Competitive action between Brassinosteroid and tracheary element differentiation inhibitory factor in controlling xylem cell differentiation

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Received October 8, 2021; accepted November 9, 2021 (Edited by A. Iwase)

Abstract For permanent secondary growth in plants, cell proliferation and differentiation should be strictly controlled in the vascular meristem consisting of (pro)cambial cells. A peptide hormone tracheary element differentiation inhibitory factor (TDIF) functions to inhibit xylem differentiation, while a plant hormone brassinosteroid (BR) promotes xylem differentiation in (pro)cambial cells. However, it remains unclear how TDIF and BR cooperate to regulate xylem differentiation for the proper maintenance of the vascular meristem. In this study, I developed an easy evaluation method for xylem differentiation frequency in a vascular induction system Vascular cell Induction culture System Using Arabidopsis Leaves (VISUAL) by utilizing a xylem-specific luciferase reporter line. In this quantitative system, TDIF suppressed and BR promoted xylem differentiation in a dose-dependent manner, respectively. Moreover, simultaneous treatment of TDIF and BR with (pro)cambial cells revealed that they can cancel each other's effect on xylem differentiation, suggesting a competitive relationship between TDIF and BR. Thus, mutual inhibition of “ON” and “OFF” signal enables the fine-tuned regulation of xylem differentiation in the vascular meristem.

Key words: BR, TDIF, vasculature, VISUAL, xylem.

Introduction Continuous radial growth is brought by vascular secondary development. In this process, (pro)cambial cells (PCs) undergo self-renewal and give rise to xylem or phloem cells to the opposite side (De Rybel et al. 2016; Shi et al. 2017). Previous genetic analysis revealed that tracheary element differentiation inhibitory factor (TDIF) peptide signaling is required for the proper maintenance of the vascular meristem in Arabidopsis thaliana (Etchells et al. 2016; Hirakawa et al. 2010, 2008; Ito et al. 2006; Kondo and Fukuda 2015; Kondo et al. 2014). TDIF secreted from the phloem tissue is perceived by a membrane-penetrated receptor PHLOEM INTERCALATED WITH XYLEM (PXY) / TDIF RECEPTOR (TDR) in PCs (Etchells and Turner 2010; Fisher and Turner 2007; Hirakawa et al. 2008). Then, the signal is transduced from intracellular kinase domain of TDR to GSK3-like kinases including BRASSINOSTEROID INSENSITIVE 2 (BIN2) (Kondo et al. 2014). Ultimately, transcription factors BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE RESISTANT 1 (BZR1) are inactivated via direct phosphorylation by GSK3-like kinases to inhibit the differentiation into xylem cells (XYs) (Furuya et al. 2021; He et al. 2002; Saito et al. 2018; Yin et al. 2002). On the other hand, brassinosteroid (BR) promotes XY differentiation in Zinnia tracheary element culture (Yamamoto et al. 2001, 2007). In addition, BR is known to facilitate XY differentiation in tomato (Lee et al. 2019). Similarly to TDIF, BR intracellular signaling is also mediated by GSK3-like kinases and BES/BZR transcription factors (Li and Nam 2002; Yan et al. 2009). However, it remains unknown whether BR and TDIF signal is integrated to control XY differentiation.

For the investigation of vascular cell differentiation, a tissue culture system named Vascular cell Induction culture System Using Arabidopsis Leaves (VISUAL) has been widely used (Kondo 2018). VISUAL can efficiently induce PCs and subsequent XYs with the addition of...
auxin, cytokinin, and a GSK3 inhibitor bikinin (Kondo et al. 2015, 2014, 2016). Indeed, the inhibitory effect of TDIF on xylem differentiation was examined in VISUAL using true leaves (Kondo et al. 2015). In VISUAL, co-treatment of bikinin completely blocks the inhibitory effect of TDIF on XY differentiation (Kondo et al. 2015), because GSK3s are known to act downstream of TDIF signaling (Kondo et al. 2014). Furthermore, genetic analysis with VISUAL revealed that BES1 is critical for promoting XY differentiation (Saito et al. 2018), indicating that TDIF inhibits XY differentiation via GSK3-like kinases and BES1 transcription factor in VISUAL. In this study, I examined the effects of TDIF and BR on XY differentiation in VISUAL by employing luciferase reporter system for quantifying XY differentiation rates. Quantitative measurement of XY-specific luciferase reporter confirmed that TDIF suppresses and BR promotes XY differentiation. Dual treatment assay revealed their mutual inhibitory effects, indicating an antagonistic relationship between BR and TDIF in the regulation of XY differentiation.

Materials and methods

Plant materials

All Arabidopsis seeds used in this study are Col-0 background. tdr-1 mutant (SALK_002910) was reported in Hirakawa et al. 2008. To generate the constructs for pUBQUITIN 14 (UBQ14):EMERALD LUCIFERASE (ELUC) and pIRREGULAR XYLEM 3 (IRX3):ELUC, an approximately 1–2 kb DNA fragment upstream of the predicted start codon of each gene were cloned and then fused with ELUC (TOYOBO) in pGWB1 vector by In-Fusion (Takara-Bio). These constructs were introduced into the WT and/or the tdr-1 mutant via Agrobacterium (Rhizobium radiobactor) strain GV3101.

VISUAL assay

VISUAL experiment was performed according to the previous method (Kondo et al. 2016). For the treatment assay for brassinolide (BL) and TDIF, the cotyledon samples were transferred to the medium following the wash with hormone-free medium. For calculating XY differentiation rates from images, VISUAL-induced samples were fixed with ethanol: acetic acid (3:1, v/v) and mounted onto microscope slides with a clearing solution (chloral hydrate: glycerol: water=8:1:2 [w/w/v]). Autofluorescent images illuminated by UV were obtained using a fluorescent microscope (BX51, Olympus). Autofluorescent area per whole cotyledon area was calculated for XY differentiation rate (%).

RNA extraction and realtime PCR

Total RNA was extracted from cotyledons cultured with VISUAL medium for 72h with RNeasy Plant Mini Kit (QIAGEN). After reverse transcription, quantitative PCR was performed using LightCycler (Roche Diagnostics). Relative expression of IRX3 was normalized by the expression of UBQ14 as an internal control. Three biological replicates were done for statistical analysis.

LUC measurement

VISUAL induction was conducted according to the previous protocol (Kondo et al. 2016). Cotyledons were cut out from shoots subjected to VISUAL induction for 72h and then treated with 5 µM D-luciferin solution (Wako) at 1h before LUC measurement. A cotyledon was put into a well on white 96-well-plate containing 5 µM D-luciferin solution. LUC intensity of the samples was measured by luminometer (TriStar2 LB942, Berthold).

Results

A new quantitative method for XY differentiation rates in VISUAL

VISUAL is known as a useful tool for the studies on vascular cell differentiation. In VISUAL, mesophyll cells (MSs) initially transdifferentiate into PCs and subsequently differentiate into XYs by culturing Arabidopsis cotyledons with auxin, cytokinin, and a GSK3 inhibitor bikinin (Kondo et al. 2015, 2014) (Figure 1A). While VISUAL enables the easy XY induction in various genetic backgrounds (Figure 1B, C), it was troublesome to calculate the ectopic XY area from obtained fluorescent images for quantitative analyses (Figure 1B, C, D). Usually, the WT cotyledons exposed to VISUAL induction for 4 days showed approximately 60% XY differentiation rates in the presence of bikinin (Figure 1D). Similarly, expression of IRX3, which encodes a synthetic enzyme for secondary cell wall during XY differentiation (Taylor et al. 1999), was highly upregulated by the culture with bikinin for 3 days (Figure 1E). Therefore, here I tried to measure the promoter activity of IRX3 with the use of ELUC reporter for the easier evaluation of XY differentiation rates. As a control, the promoter activity of one of house-keeping genes UBQ14 was not altered by the addition of bikinin during VISUAL (Figure 1F). By contrast, the promoter activity of IRX3 was dramatically increased upon bikinin treatment (Figure 1F). Thus, pIRX3:ELUC intensity can be used as an indicator of XY differentiation rates for further studies.

Quantitative analysis of the effects of BR and TDIF on XY differentiation

For examination of TDIF and BR effect on XY differentiation at PCs, here I set the experimental procedure in which cotyledons harboring pIRX3:ELUC were cultured with the VISUAL induction medium for 30h and then transferred to a new medium containing TDIF or an active BR, BL (Figure 2A), according to the previous method (Kondo et al. 2015). First, I examined
the effect of bikinin on XY differentiation in VISUAL. *pIRX3:ELUC* cotyledons were transferred to a medium containing bikinin at various concentrations following the culture with 10 µM bikinin for 30 h. As the bikinin concentration increases, LUC activity of *pIRX3:ELUC* at total 72 h after induction was gradually strengthened (Figure 2B), indicating that XY differentiation rates highly depend on GSK3 activity. BR is known to inhibit GSK3 activity through dephosphorylation by BRI1 SUPPRESSORs (BSUs) (Kim et al. 2009). In the same transfer assay, BL application increased the LUC activity in a dose-dependent manner (Figure 2C). In contrast to BL, TDIF activates GSK3 (Cho et al. 2014; Kondo et al. 2014). Indeed, TDIF application decreased the LUC activity in a dose-dependent manner (Figure 2D). These results coincided with the previous notion that BR promotes XY differentiation and TDIF inhibits XY differentiation. Quantitative analyses revealed that these promotive and suppressive effects are dependent on the concentrations.
Competitive relationship between BR and TDIF in the regulation of XY differentiation

Next to examine the relationship between BR and TDIF in the regulation of XY differentiation, BL and TDIF were simultaneously applied to the culture medium in the transfer experiment at various combinations. Two-way ANOVA revealed that BL tends to increase the LUC activity of pIRX3:ELUC even in the presence of TDIF (Figure 3). Vice versa, TDIF tended to suppress the LUC activity regardless of BR concentrations (Figure 3). Moreover, there was no interaction effect between BL and TDIF (Figure 3C), suggesting that their main effects do not depend on with each other. Next, I statistically examined the effect of 1,000 nM BL and 100 nM TDIF in the WT and the mutant defective in the TDIF receptor (tdr-1) in the VISUAL transfer assay. In the WT background, simultaneous treatment caused intermediated LUC intensity, suggesting that BL and TDIF mutually inhibits the effects with each other (Figure 4A). By contrast, in the TDIF insensitive mutant tdr-1, the inhibitory effect of TDIF on the LUC activity was totally abolished regardless of the absence or presence of BL (Figure 4B), suggesting that the TDIF suppresses BR signaling via the receptor TDR. To further confirm the competitive relationship, XY differentiation rates were calculated from auto-fluorescent images of XY secondary cell wall. Indeed, BL increased and TDIF decreased XY differentiation rates in the transfer assay (Figure 4C, D, E). In addition, simultaneous application of BL and TDIF resulted in intermediated levels of XY differentiation rates (Figure 4F, G), which corresponded to the data from the LUC assay. All these data indicate a competitive relationship between BR and TDIF in controlling XY differentiation from PCs (Figure 4H).

Discussion

In this study, I showed that BR and TDIF competes to control XY differentiation with the use of tissue culture VISUAL (Figures 3, 4). TDIF-TDR signaling inhibits XY differentiation via activating GSK3-like kinases (Kondo...
et al. 2014). GSK3-like kinases also mediate BR and other signaling to control plant growth and development as signal hubs (Li et al. 2021). Previous studies have shown that bikinin completely attenuates TDIF signaling (Kondo et al. 2015), while BL had a competitive function against TDIF in this study (Figure 3). Taken together, it is reasonable to expect that BR and TDIF signaling is integrated by GSK3-like kinases. TDIF induces the dissociation of GSK3-like kinases from the cytoplasmic kinase domain of TDR, which allows GSK3-like kinases to contact with BES1 and BZR1 (Kondo et al. 2014). On the other hand, BR induces the dephosphorylation of GSK3-like kinases by BSUs (Kim et al. 2009). Therefore, it would be important to show how opposing two signals are integrated by GSK3-like kinases at the molecular level. Moreover, the competitive relationship was only examined with the in vitro culture VISUAL. It will be important to validate the cross-talk from in vivo genetic experiments. Signals integrated by GSK3-like kinases are finally conveyed to the transcription factors BES1 and BZR1, which directly down-regulates BR biosynthetic genes such as DWARF 4 (DWF4) and CONSTITUTIVE PHOTOMORPHOGENIC DWARF (CPD) (Tanaka et al. 2005). Such a feedback mechanism would enable balancing BR and TDIF signaling for robust control of XY differentiation in PCs.

To date, several molecular mechanisms behind strict control of XY differentiation have been reported. BIL1, one of GSK3-like kinases, can bind to the TDR, but cannot dissociate from TDR. Therefore, BIL1 has an opposite behavior to other GSK3-like kinases to control the vascular meristem maintenance (Han et al. 2018; Kondo et al. 2014). As another case, the competitive action among BES/BZR family members contributes to fine-tuned regulation of the vascular meristem. BES1 HOMOLOG 3 (BEH3), which possesses much weaker transcriptional repressor activity, competes with other BES1 family proteins via competitive binding to the BRRE cis element (Furuya et al. 2021). Here we found the competitive relationship of TDIF and BR upstream of GSK3-like kinases and BES/BZR transcription factors. Thus, multi-layered competitive interplay between TDIF and BR signaling cascade would fulfill a more robust regulation of the vascular meristem maintenance.

Acknowledgements

I thank Yasuko Ozawa for technical supports, and Tomoyuki Furuya and Hiroo Fukuda for discussion. This work was supported partly by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Scientific Research on Priority Areas and Scientific Research on Innovative Areas) (17H06476 and 20H05407), from the Japan Society for the Promotion of Science (20K15815).

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