Brassinosteroids promote parenchyma cell and secondary xylem development in sugar beet (Beta vulgaris L.) root

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Abstract
Increasing crop yield has always been an important goal in agriculture. Brassinosteroids (BRs) are growth-promoting steroid hormones with vital roles in many root developmental processes. Sugar beet (Beta vulgaris L.) is a root crop with a tertiary root structure. The differentiation of vascular bundles and the division of cambial cells increase root diameter. However, little is known about how BRs regulate the transverse growth of beetroot. Therefore, sugar beet with eight leaves was grown in medium containing epibrassinolide or brassinazole, an inhibitor of BR biosynthesis. BRs increased the spacing between the cambial rings by increasing the size of parenchyma cells between the rings and ultimately increasing root diameter. BRs also promoted secondary xylem differentiation. Moreover, the gene expression analysis of BvXTH33, BvSHV3, BvCESA6, BvPARVUS, and BvCEL1, which were related to the cell wall biosynthesis, indicated that BR could promote the growth of cell wall. These findings showed that BRs function in transverse development in beetroot.

KEYWORDS
brassinosteroid, parenchyma cell, sugar beet, xylem

1 INTRODUCTION

Brassinosteroids (BRs) are ubiquitous plant hormones that have been used widely in agriculture since the 1970s (Divi & Krishna, 2009). Recent analyses have revealed that BRs are involved in many aspects of root development, including cell elongation, maintenance of meristem size, root hair formation, lateral root initiation, and the gravitropic response (Wei & Li, 2016). In Arabidopsis, a low BR concentration stimulated root elongation in wild-type plants by up to 50% and by up to 150% in BR-deficient mutants, such as dwf1-6. The root growth-promoting effect of BRs appears to be largely independent of auxin and gibberellin (Müssig et al., 2003). In roots, cell proliferation and postmitotic cell enlargement form a developmental gradient along the apical–basal axis that eventually determines their length (Petricka et al., 2012). Moreover, cell enlargement is regulated by the extensibility of the surrounding wall (Cosgrove, 2005; Fujita et al., 2011; Wolf et al., 2012). Interestingly, recent chromatin-immunoprecipitation microarray (CHIP-chip) experiments identified several BR-regulated BZR1 target genes that play vital roles in cell wall biosynthesis, such as cellulose synthase 6 (CESA6), xyloglucan endotransglycosylase/hydrolases (XTH), and pectinesterases, which ultimately promote cell elongation (Sun et al., 2010).

In recent years, growing sugar beet (Beta vulgaris L.), one of the important sugar crops, has been the pillar industry in Northern China. Especially in remote areas, it has become the main source of farmers’ income. Taproot is the main product organ that could be used to extract sugar. Based on the changes of material metabolism and the transfer of substance distribution center, the vegetative growth process of sugar beet in the first year could be divided into four stages: seedling stage, rapid growth stage of leaf clusters, growth stage of...
taproot and sugar content, and sugar accumulation stage. During the first year of the biennial life cycle of the sugar beet, root swelling and sucrose accumulation are the ultimate goals (Elliott & Weston, 1993). The beetroot is a crucial organ in sugar beet and accounts for 30% of the global sucrose output (Zhang et al., 2017). When a seedling becomes established, the plant enters a period of leaf initiation with very little root growth. At the 8–10 leaf stage, the leaves and root grow simultaneously; eventually, roots comprise the major proportion of the total plant dry weight (Bellin et al., 2007; Elliott & Weston, 1993). The increase in girth of the tap root results from the activity of the cambia. The innermost cambium is produced between the primary xylem and phloem. Subsequent cambia are initiated centrifugally in the outer portion of the previous ring (Milford, 1973). Although 12–15 cambial rings can form, the greatest contributions to root development are from rings 1 and 2, whereas rings 3–8 show progressively less activity. Rings 1–6 make up approximately 75% of the storage root (Elliott & Weston, 1993). Therefore, the rings that contribute most to the final yield of the root were already present when the plants had produced 12–13 leaves. The ringed structure of the beetroot in transverse section results from the development of parenchymatous zones and vascular bundles that contain xylem toward the inside and phloem toward the outside. However, few studies have examined whether BR promotes transverse growth by alternating the size or number of parenchyma cells in sugar beet.

By observing the microscopic structure of the taproot cross section, this study aims to preliminarily clarify the regulation mechanism of exogenous BR on the enlargement of taproot from the anatomical point of view and fill in the gap in the regulation of BR in the development of plant root with a tertiary root structure. In addition, the expression level of genes which related to cell wall biosynthesis after treated with exogenous BR was determined to verify that BR could promote cell expansion by regulating the expression of these genes and finally, increase the root diameter. This study offers a theoretical basis for further work on the molecular mechanisms of beetroot growth.

2 | RESULTS

2.1 | BR increased the root diameter

Figure 1a shows sugar beets treated with BR, Brz, or water (control group) for 10 days. Compared with the controls, BR-treated plants had a significant (P < .01) increase in root diameter, whereas Brz-treated plants were not significantly altered (Figure 1b,c). Therefore, BR increased the root diameter.

2.2 | BR increased the spacing between cambial rings

To assess how root structure influences root diameter, we examined cross sections of root apex treated with BR, Brz, or water (Figures 2 and 3). After 10 days, all the experimental groups had four cambial rings. The second and third cambial rings, which

**Figure 1** The effects of exogenous BR and Brz on sugar beet. There were biological replicates done for the test. (a) Morphological phenotype of sugar beet irrigated with BR, water, or Brz. From left to right, BR (0.1 mg/L)-treated, control and Brz (20 μmol/L)-treated plants are shown. Sugar beets were grown for 10 days. Scale bar equal to 3 cm. (b) Root diameter treated with BR, water, or Brz in 10 days. Values not connected by the same uppercase letter are significantly different (Student’s t test, P < .01). (c) Morphological phenotype of taproot irrigated with BR, water or Brz. From left to right, BR (0.1 mg/L)-treated, control, and Brz (20 μmol/L)-treated plants are shown. Sugar beets were grown for 10 days. Scale bar equal to 3 cm.
contain the most vigorous cambial cells, were examined to assess the role of BR. Compared with the controls, BR-treated plants had a significant \( (P < .05) \) 37.5% increase in spacing between the first and second cambial rings (Figure 4). The spacings between the second and third rings in BR-treated plants were increased \( (P < .01) \) by 8%, compared with the control plants (Figure 4). Therefore, BR increases the spacing between cambial rings.

**FIGURE 2** The effect of exogenous BR on the spacing between the first and second cambial rings in beetroot. Root cross sections were stained with Safranin and Astra Blue. The distance between the two arrows indicates the spacing between cambial rings. pc, primary cambium; sx, secondary xylem; sc, secondary cambium; sp, secondary phloem. (a) Root cross section of control before the treatment. (b) Root cross section of BR (0.1 mg/L)-treated sugar beet grown for 10 days. (c) Root cross section of control sugar beet grown for 10 days. (d) Root cross section of Brz (20 \( \mu \)mol/L)-treated sugar beet grown for 10 days. Bar = 100 \( \mu \)m
2.3  |  BR increased the size of parenchyma cells

Figure 5 shows the parenchyma cells between the first and second cambial rings in plants treated with BR, Brz, or water. In BR-treated plants, the parenchyma cells between the first and second rings were significantly larger (23.8%). In comparison, parenchyma cells between the first and second rings in Brz-treated plants were 19.9% smaller ($P > .05$) (Figure 5a–d). Compared with the controls, the layers of parenchyma cells between the first and second rings were significantly larger in BR-treated plants, but not Brz-treated plants (Figure 5a–c, e).

Whereas BR treatment increased the size of parenchyma cells between the second and third cambial rings by 6.7% compared with the controls, Brz treatment decreased the size by 6.5% (both $P < .01$; Figure 6a–d). The number of layers of parenchyma cells did not differ among the BR-treated, Brz-treated, and control groups (Figure 6a–c, e). Accordingly, BR increases the size of parenchyma cells.

2.4  |  BR promoted the development of secondary xylem

The areas of secondary xylem in the second and third cambial rings were calculated using CaseViewer. In BR-treated plants, these areas increased significantly (15.58% and 53.15%, respectively; $P < .01$)
compared with the controls (Figure 7). Hence, BR promoted development of the secondary xylem.

2.5 | Cell wall biosynthesis genes expression in response to BR

BR-related BZR1 target genes were reported to function in cell wall biosynthesis, such as XYLOGLUCAN:XYLOGLUCOSYL TRANSFERASE 33 (XTH33), CELLULOSE1 (CEL1), GLYCEROPHOSPHODIESTER PHOSPHODIESTERASE (GDPD) LIKE 3 (SHV3), CELLULOSE SYNTHASE 6 (CESA6), and GALACTURONOSYLTRANSFERASE-LIKE 1 (PARVUS) (Sun et al., 2010). To investigate whether the expression levels of genes related to cell wall biosynthesis are regulated by exogenous BR in sugar beet, the beetroots were sprayed with BR, Brz, or water (control group) for 4 h. As shown in Figure 8, compared with the control group, the expression level of BvXTH33,
BvSHV3, BvCESA6, and BvPARVUS increased after spraying with BR, whereas decreased after spraying with Brz. The expression level of BvCEL1 gene decreased after BR treatment and increased after Brz treatment, suggesting that BR could promote the biosynthesis of cell wall.

3 | DISCUSSION

There have been many recent reports on how BRs influence plant root development. At certain concentrations, BRs decrease the root length by inhibiting apical meristem growth; for example, in wheat, 0.1 and 1 nmol/L of 24-eBL promoted root growth, whereas 10 nmol/L of 24-eBL inhibited root growth. However, the research on the effects of BRs on plant roots has focused on longitudinal growth, whereas transverse growth is rarely studied. In sugar beet, studying root swelling is important. Comparing the three treatments, BR promoted root swelling by increasing the spacing between the cambial rings, which depends on the number and size of parenchyma cells between the rings. BR produced larger parenchyma cells, but had no significant effect on the number of parenchyma cells, which is consistent with the findings that BR mediates root cell elongation (Wei & Li, 2016). BR-related BZR1 target genes were reported to function in multiple cellular processes such as cellular transport and cell wall synthesis (Sun et al., 2010). The expression level of XTH, CEL1, SHV3, CESA6, PARVUS, and ATCWINV1 that related to cell wall synthesis upregulated in response to BR, whereas suppressed when responding to Brz. So BR could stimulate the cell elongation by regulating the synthesis of cell wall and finally accelerate root swelling. These findings validate the previous histological results.

Vascular bundles are necessary for the growth and development of higher plants, as they transport plant materials. BR affects xylem development. When some BR synthesis genes were mutated, the xylem decreased and phloem increased in vascular tissue (Choe et al., 1999). Brz treatment inhibited the secondary xylem in cress (Nagata et al., 2001). Researcher found that the TCP1 gene, which regulates BR synthesis, is involved in the differentiation and formation of vascular epigenetic xylem in Arabidopsis. We found that the area of secondary xylem in beetroot increased with BR treatment, but decreased with Brz treatment. The area of xylem in the third cambial ring of beetroot treated with BR increased by 53.15% compared with the controls. Therefore, BR promotes beetroot xylem.
**FIGURE 7** The effect of exogenous BR on secondary xylem in beetroot. Root cross sections were stained with Safranin and Astra Blue. sx, secondary xylem. (a) Root cross section of BR (0.1 mg/L)-treated sugar beet grown for 10 days. (b) Root cross section of control sugar beet grown for 10 days. (c) Root cross section of Brz (20 μmol/L)-treated sugar beet grown for 10 days. (d) The area of the secondary xylem at the second cambial ring after treatments for 10 days. (e) The area of the secondary xylem at the third cambial ring after treatments for 10 days. Values not connected by the same uppercase letter are significantly different (Student’s t test, \( P < .01 \)). Bar = 1000 μm in (a, b, & c).

**FIGURE 8** The expression level of cell wall biosynthesis-related genes after BR treatment. The beetroots were sprayed with BR, Brz, or water (control group) for 4 h to investigate whether the expression levels of genes related to cell wall biosynthesis are regulated by exogenous BR in sugar beet. Values not connected by the same lowercase letter are significantly different (Student’s t test, \( P < .05 \)).
4  | CONCLUSION

On treating sugar beet at the eight-leaf stage with BR, Brz, or water, BR increased the beetroot diameter. Histological analyses revealed that BR increases the spacing between the cambial rings by increasing the size of parenchyma cells between the rings. BR also promotes the differentiation of xylem.

5  | MATERIAL AND METHODS

5.1  | Plant materials and growth conditions

The BS02 sugar beet cultivar used in this study was bred in our laboratory. Seeds were cultivated in the Inner Mongolia Agricultural University phytotron (Hohhot, Inner Mongolia, China) and sown in vermiculite with one seedling per 8 × 8 × 8 cm³ float tray. Hoagland solution was added every 14 days, and the plants were grown at 22°C and under 16-h light/8-h dark conditions.

5.2  | BR and Brz treatments

The eight-leaf plants were treated with 0.1 mg/L of epibrassinolide (epiBL; Sigma, USA) or 20 μmol/L of Brz (Sigma). The epiBL and Brz solutions were prepared by dissolving the solute in DMSO and diluting with distilled water. The control group was treated with solutions prepared by dissolving the solute in DMSO and diluting with distilled water. The control group was treated with water. On Day 10, the morphological and physiological parameters of all plants were measured, and the root tissues were harvested for histological analyses. Roots were collected from three biological replicates.

5.3  | Histological parameters

Samples were collected from the root 1 cm from the root apex. All specimens were fixed in 70% formaldehyde acetic acid for 24 h and dehydrated in an increasing ethanol series. Transverse 12-μm-thick sections were obtained with a rotary microtome, stained with Safranin and Astra Blue, and mounted on paraffin (Maia et al., 2018). The slides were observed under a microscope (Pannoramic DESK, Hungary) and photomicrographed. The images were analyzed with CaseViewer. The following histological parameters were evaluated: size of the xylem and parenchyma cells and spacing between the cambial rings. CaseViewer was used to randomly select 10 adjacent cells for the total area, and then the average area was calculated. Five groups of data were randomly selected for analysis. CaseViewer was also used to calculate areas of individual xylems and the total area of xylems in the cross section. The distance between the outmost xylem of two vascular cambia is defined as spacing between cambial rings. Five groups of data were randomly selected for analysis. All experiments had three biological replicates.

5.4  | Data analysis

The differences between the mean values were assessed at 5% and 1% probability levels using SPSS (ver. 18.0). The figures were made with GraphPad Prism (ver. 8.0).

5.5  | Real-time quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from each sample using Trizol, under the operation instruction of TaKaRa RNAiso Plus (Code No.9109). Complementary DNA (cDNA) was diluted 16 times before use as the template. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA) in a 20-μl reaction containing 10 μl of iTaq Universal SYBR Green Supermix (Bio-Rad), 0.5 μl of each primer (10 μM), 2 μl of cDNA template, and 7 μl of double-distilled H₂O. The PCR program was as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 30 s. After every reaction, a melting curve analysis was conducted to confirm that only one product had been amplified and could be detected. The cycle threshold (Ct) values were used to calculate fold-change differences. Relative expression levels were determined using the 2^(-ΔΔCt) method (Livak & Schmittgen, 2001). ΔCt = Ct[control] - Ct[experimental]: ΔΔCt = ΔCt[experimental group] - ΔCt[control group]. The 2^(-ΔΔCt) method was used to calculate the multiple relationships between the experimental group and control group. All qRT-PCR experiments included three biological replicates, each with three technical replicates. The primer sequences were in Table S1.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

W.W. conceived the original screening and research plans; Y.Q.S. and S.Y.Z. supervised the experiments; G.L.L. sowed the sugar beet in vermiculite and W.W. performed the anatomical map using the paraffin method; S.Y.Z. provided technical assistance to W.W.; Y.Q.S. designed the experiments, and G.L.L. analyzed the data; W.W. conceived the project and wrote the article with contributions of all the authors; Y.Q.S. supervised and complete the writing. S.Y.Z. agrees to serve as the author responsible for contact and ensures communication.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.
CONSENT FOR PUBLICATION
Not applicable.

CONFLICT OF INTEREST
The Authors did not report any conflict of interest.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this published article and its supporting information. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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