RESEARCH PAPER

Cytokinin-dependent secondary growth determines root biomass in radish (Raphanus sativus L.)

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Abstract

The root serves as an essential organ in plant growth by taking up nutrients and water from the soil and supporting the rest of the plant body. Some plant species utilize roots as storage organs. Sweet potatoes (Ipomoea batatas), cassava (Manihot esculenta), and radish (Raphanus sativus), for example, are important root crops. However, how their root growth is regulated remains unknown. In this study, we characterized the relationship between cambium and radial root growth in radish. Through a comparative analysis with Arabidopsis root expression data, we identified putative cambium-enriched transcription factors in radish and analysed their expression in representative inbred lines featuring distinctive radial growth. We found that cell proliferation activities in the cambium positively correlated with radial growth and final yields of radish roots. Expression analysis of candidate transcription factor genes revealed that some genes are differentially expressed between inbred lines and that the difference is due to the distinct cytokinin response. Taken together, we have demonstrated for the first time, to the best of our knowledge, that cytokinin-dependent radial growth plays a key role in the yields of root crops.

Key words: Cambium, cytokinin, Raphanus sativus, root biomass, secondary growth.

Introduction

Vascular plants are characterized by their dynamic and indeterminate growth in both apical and radial directions. Apical (primary) and lateral (secondary) meristems are responsible for such development. Cambium, a secondary meristem, drives the radial growth in stems and roots. Undifferentiated cells in the cambium undergo asymmetric cell divisions in anticlinal or periclinal directions to generate daughter cells that become a part of the xylem or phloem tissues. Several environmental components such as temperature, photoperiod, and precipitation affect the secondary growth driven by the cambium (Antonova and Stasova, 1997; Begum et al., 2013).

Secondary growth is important for biomass production. Therefore, there has been a growing interest in understanding the physiological regulation underlying secondary growth (Miyashima et al., 2013; Zhang et al., 2014). Auxin and cytokinin are key regulators in this. Auxin is distributed differentially across the cambial regions in hybrid aspen; it reaches a maximum level in cambium regions and then decreases in regions forming tracheids (Uggla et al., 1996; Uggla et al., 1998). Changes in auxin distribution seem to affect the orientation of cambial initials in response to wounding (Kramer et al., 2008). However, the initiation of latewood formation and the cessation of cambial cell division are not caused by changes in auxin concentration in the cambium of Scots pine (Uggla et al., 2001), indicating the involvement of other plant hormones in these processes. Recently, cytokinins...
were shown to regulate the cambium activity and secondary growth. When four genes encoding isopentenyl transferase (IPT1, -3, -5, and -7) were disrupted in *Arabidopsis*, cambium formation became defective and root thickening was reduced (Matsumoto-Kitano et al., 2008). A study by Niemenen et al. (2008) also demonstrated the essential role of cytokinin in *Populus* cambium. They showed that cytokinin receptor genes are preferentially expressed in the dividing cambial cells in the *Populus* stem. Overexpression of a gene encoding CYTOKININ OXIDASE (CKX), a cytokinin-degrading enzyme, resulted in the suppression of secondary growth (Niemenen et al., 2008).

Gene regulatory networks governed by transcription factors are essential for every aspect of plant development. High-resolution transcript profiling has revealed genes that are expressed in a cambium-enriched manner in *Populus* (Schrader et al., 2004). However, only a small number of transcription factors have been identified as regulators of secondary growth so far. These include *Arabidopsis thaliana* Homeo-Box8 (ATHB8), *Populus* REVOLUTA, High Cambial Activity2 (HCA2), *Populus* LATERAL ORGAN BOUNDARIES DOMAIN 1 (PtaLBD1), and Ethylene Response Factor 109 (ERF109) and ERF18 (Baima et al., 2001; Guo et al., 2009; Yordanov et al., 2010; Robischon et al., 2011; Etchells et al., 2012).

Radial growth occurs not only in stems but also in roots. Root radial growth is particularly noticeable in storage roots, many of which serve as important sources of food and energy. Anatomical studies have shown that storage roots form cambium as well as anomalous meristemetic tissues, and their organization varies depending on plant species (Esau, 1977). However, the more detailed nature of storage root growth at structural and molecular levels remains to be elucidated. Radish (*Raphanus sativus*) belonging to the Brassicaceae is an economically important root crop in Eastern Asia. It has advantages over other root crops for the study of radial root growth for following reasons. First, radish root growth is very rapid, completing its major growth within 9 weeks. Secondly, radish is accessible for tissue-specific gene expression analysis because of its thick root morphology. Lastly, self-pollinating F2s between two cultivars, ‘Kwan-dong summer’ and ‘Pyeong-ji summer’, for 10 generations. Phenotypic analysis was carried out for the radish inbred lines grown in the field at NIHHS, Suwon (127°01′E/37°16′N), Korea. For growing radish in a growth room, seeds were germinated in pots (20 × 20 × 20 cm) filled with soil and grown at 22 °C, under a 16 h light/8 h dark photoperiod. Root circumference was measured at the thickest part of a root. Photoshop and Image J were used for processing radish images and measuring roots and shoots.

**Materials and methods**

**Plant materials, growth, and phenotypic analysis**

The radish inbred lines used in this study were obtained from National Institute of Horticultural and Herbal Science (NIHHS) of the Republic of Korea. Each inbred line was produced by manually self-pollinating F2s between two cultivars, ‘Kwan-dong summer’ and ‘Pyeong-ji summer’, for 10 generations. Phenotypic analysis was carried out for the radish inbred lines grown in the field at NIHHS, Suwon (127°01′E/37°16′N), Korea. For growing radish in a growth room, seeds were germinated in pots (20 × 20 × 20 cm) filled with soil and grown at 22 °C, under a 16 h light/8 h dark photoperiod. Root circumference was measured at the thickest part of a root. Photoshop and Image J were used for processing radish images and measuring roots and shoots.

**Embedding, sectioning, and staining**

For transverse sectioning of radish roots, specimens (0.5 × 0.5 × 0.5 cm) collected from the thickest parts of radish roots were fixed overnight in 4% paraformaldehyde dissolved in PBS (pH 7.4). After washing with PBS, fixed samples were dehydrated in an increasing concentration of ethanol (25, 50, 75, and 100%) in PBS and then in a series of Neo-Clear mixed with 100% ethanol (25, 50, 75, and 100%). The dehydrated samples were sequentially incubated in an increasing series of paraﬃn concentrations [25, 50, 75, and 100%, v/v, in Neo-Clear (Merck)] for 1 d each. Samples were then incubated in 100% paraﬃn for 2 d and then placed in moulds. Solidified samples were sectioned at a thickness of 8–10 μm with a RM 2145 microtome (Leica). Deparaﬃnized and hydrated sections were stained with 0.05% toluidine blue (pH 4.4). Images were captured with an axiоimager M1 (Zeiss) and IX70 (Olympus) light microscope system.

**Immunolocalization assay**

To visually analyse cell division activity in cambium tissues, immunolocalization of radish roots was carried out with proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology). The thickest part of the root was transversely sectioned with a razor blade and fixed overnight in 4% paraformaldehyde dissolved in PBS at 4 °C. The root sections were then washed with PBS buffer six times for 10 min each. They were pre-incubated with 2% BSA in PBS for 30 min and then with PCNA antibody diluted at a ratio of 1:100, for 1.5 h at 37 °C and again washed with PBS, six times for 10 min each. The root sections were incubated with Alexa Fluor 488-conjugated anti-goat IgG (Invitrogen), diluted 200-fold in PBS for 1 h at room temperature and washed six times in PBS for 10 min each, and then stained with propidium iodide and mounted on a slide glass with citifluor (Electron Microscopy). The fluorescence signals from the Alexa Fluor 488 were detected using an LSM700 confocal microscope (Zeiss). The excitation/emission wavelength was 488 nm and 505–530 nm for Alexa Fluor 488 and 561 and 591–635 nm for propidium iodide.

**Identification of cambium-enriched transcription factor orthologues in radish and Arabidopsis**

Transcription factors that are expressed in a cambium-enriched manner were identified by analysing the data, which combined the
root expression map (Brady et al., 2007) and the new expression data generated for early procambium/cambium. Specifically, Arabidopsis plants expressing ARR15::erfp [containing endoplasmic reticulum-targeted green fluorescent protein (erfp)], which indicates early cambium, and plants expressing ARR5::erfp, which indicates procambium and root cap were developed. Expression profiles for these cell types were generated using a method used for other root expression data based on protoplast sorting-microarray technology (Birnbaum et al., 2005; Lee, J.-Y., Nieminen, K., Elo, A., Weissmann, S. and Helariutta, Y., unpublished data). A manuscript about more details of these data is in preparation. In the combined root expression data, approximately 170 transcription factors were identified to be enriched in the early cambium from the search for co-expressed gene groups (modules) (Segal et al. 2003). For this study, 11 transcription factors were selected based on their enriched expression in the early cambium and procambium as well as their potential importance as meristem regulators, such as ANT and KNAT1.

The radish coding sequence (CDS) and EST sequence data were collected from RGD (ftp://ftp.kazusa.or.jp/pub/radish/) and RadishBase (http://bioinfo.bti.cornell.edu/cgi-bin/radish/index.cgi). Orthologous genes were searched for based on the reciprocal BLAST analysis (cut-off value of 1e-5). The top hit for both blasts (Arabidopsis CDS against radish CDS/EST and radish CDS/EST against Arabidopsis CDS) were selected and considered as putative orthologous gene pairs.

BLASTP analysis for the Arabidopsis CDS and its homologues as well as radish orthologue candidates was carried out. Alignments of assembled nucleotide sequences were conducted using TranslatorX, an online tool that aligns nucleotide sequences based on the translated amino acid sequences (Abascal et al., 2010). Poorly aligned regions were removed from tree analysis either manually or using default parameters in TranslatorX. The phylogenetic bootstrap analyses were conducted for the nucleotide sequence data of each gene-family data using maximum parsimony in the program PAUP* version 4.0b10 (Swoford, 2003). Bootstrap analysis (Felsenstein 1985) with 500 pseudoreplicates was conducted. For options of maximum parsimony analysis, we used simple sequence addition and tree bisection and reconnection branch swapping to find the best tree in each replication.

Cytokinin treatment

To analyse the short-term effect of cytokinin on line 216 and 218 root cambia, root cubes (0.5 × 0.5 × 0.5 cm) were collected from the thickest part of line 216 and 218 root grown in growth room conditions for 5 weeks, treated with MS liquid medium containing 20 μM 6-benzylaminopurine (BAP), and then incubated for 3h at room temperature. For gene expression analysis in response to cytokinin treatment, total RNAs were extracted from cambium regions isolated from these root cubes. To analyse the long-term effect of cytokinin on the root growth, 2 l of water containing 20 or 200 μM of BAP was fed to 3-week-old radish plants every alternative day for a week.

Quantitative reverse transcription PCR (qRT-PCR) analysis

To analyse temporal and spatial expression patterns of candidate genes, qRT-PCR analyses were carried out using total RNAs extracted from root tissues, which were collected at various developmental stages. Cambial zone and inner parenchyma regions 1 and 2, which are located at one-third and two-thirds of the distance from the cambium zone to the root centre, were collected. Cambial zones can be visually distinguished from neighbouring tissues because of their high cell density. These regions were carefully thin sectioned and collected. Total RNA extraction was performed with an RNeasy Plant Mini-prep kit according to the manufacturer's instructions (Qiagen). The reverse transcription reaction (20 μl) was performed for the first cDNA strand synthesis using 1 μg of total RNA and Superscript III reverse transcriptase (Invitrogen). After completion of the reverse transcription reaction, the cDNA template was diluted 5-fold by adding 80 μl of ddH2O, and 1 μl of cDNA template was used for the quantitative PCR in a total volume of 10 μl. For quantitative PCR, a master mix was prepared using an iQTM SYBR Green supermix (Bio-Rad) and PCR was carried out according to the manufacturer's instructions (initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 10 s and extension at 72 °C for 30 s). PCR and fluorescence detection were performed using a CFX96 Real-Time PCR machine (Bio-Rad), and three technical replicates of the qRT-PCRs were performed using two biological replicates. Primer information is available in Supplementary Table S1, available at JXB online. RsActin2/7 was used as an internal control gene for the analysis of candidate gene expression, and the stability of RsActin2/7 as a reference gene was tested by another internal control gene, Radish Translation elongation factor 2 (RsTEF2) (Xu et al., 2012). Expression levels obtained from real-time RT-PCR were calculated by subtracting the cycle threshold (Ct) values of each target gene from the Ct values of RsActin2/7. Expression values obtained for each tissue at different time points or in different root regions were normalized by row and then displayed as heat maps using the Multi-Experiment Viewer (MeV), which is part of the TM4 microarray software suite (Saeed et al., 2006).

Results

Root yield depends on the radial root growth in radish

As the first step towards understanding the radial root growth in radish, we analysed relationships between radial root growth and other growth parameters. We measured root fresh weight, root circumference, shoot fresh weight, and shoot length in 28 radish inbred lines grown in the field for 9 weeks (Fig. 1A, B, and Supplementary Fig. S1, available at JXB online). When average root circumferences for inbred lines were plotted against average root weights, a strong positive correlation (R²=0.70) was found between root weights and root circumferences (Fig. 1A). This indicates that radial root growth is an important factor determining root yield in radish. However, radish root growth was not tightly linked to the shoot growth. When average shoot weights or shoot lengths were plotted against root weights, the correlation was much weaker (R² =0.16 for shoot weight and 0.07 for shoot length) than the correlation between root weight and root circumference (Fig. 1B and Supplementary Fig. S1A). The poor correlation between root and shoot growth was also found when shoot weights and lengths were plotted against root circumferences (R² =0.1 for shoot weight and 0.03 for shoot length) (Supplementary Fig. S1B, C).

To further investigate the radial root growth in radish, we chose four inbred lines (lines 212, 214, 216, and 218) and characterized their radial root growth patterns over time (Fig. 1C–E, and Supplementary Fig. S2, available at JXB online). Lines 214 and 216 were selected because their radial growth in 5 weeks was superior to the growth of other inbred lines tested. Lines 212 and 218 were selected as representatives of poor radial growth. During the growth period from 5 to 9 weeks after seed planting, lines 214 and 216 showed consistently active radial growth, resulting in higher root biomass than lines 212 and 218. Even though line 212 was selected because of its poor radial growth over 5 weeks, it showed...
accelerated growth between 6 and 8 weeks. By contrast, the radial root growth of line 218 was very slow throughout the entire growth period, resulting in the lowest root biomass. Despite its poor radial root growth, the shoot growth of line 218 plants was similar to that of line 214 at all developmental stages. The poor correlation between root and shoot growth was also observed in the comparison between line 212 and other lines (Supplementary Fig. S2). Taken together, these results suggested that root-specific developmental mechanisms control radial root growth and final root yields.

**Radial root growth accompanies dynamic changes in the cambial zone**

Cambium is the stem-cell population that drives radial growth. Asymmetric cell division in the cambial zone generates cells for xylem and phloem tissues in the stem (Nieminen et al., 2008; Zhang et al., 2011). In *Populus*, the number of thin cell layers in a stem cambial zone increases during growth seasons and then decreases in winter. It is known that such seasonal changes in the size of the stem cambial zone are due to changes in the relative rates between cell division and cell differentiation.

Similar to the radial growth in the stem, the cambium might function as a key location that drives radial growth in the root. In this case, we would expect that the cambial zones would show structural differences in radish inbred lines that have varied growth dynamics. To test our hypothesis, we searched for cambium-like cell populations in radish roots that would be organized similar to the stem cambium. Time-course images obtained by free-hand transverse sectioning showed the presence of opaque areas with high cell densities that lined up parallel to the epidermis (Supplementary Fig. S3, available at *JXB* online). To analyse these areas in detail, we generated thin sections from paraffin-embedded root blocks and stained them with toluidine blue. The stained sections revealed that opaque regions are composed of layers of thin cells that are stacked in varying numbers (Fig. 2). These cellular organizations are typical of cambium zones in the stem. Inbred lines showing active radial growth such as lines 214 and 216 displayed dynamic changes in cambial zones over time (Fig. 2). In lines 214 and 216, the cambial zones of 5- and 7-week-old roots expanded to around 10 cell layers, while those in 9-week-old roots shrank to about two cell layers. In contrast to lines 214 and 216, dynamic changes in the cambial zone were less discernible in line 218 whose cambium had only two to five thin cells at all developmental stages. The size of the cambial zones in line 212 was between that seen in lines 214 and 216 and in line 218.

More detailed analyses of the cambial cell layers were performed for lines 216 and 218, which showed contrasting radial growth. This time we grew the plants in a growth room. Radish root growth in growth room conditions was almost identical to that in the field, indicating that our growth room conditions could successfully replicate the field conditions (Supplementary Fig. S4, available at *JXB* online). First, we imaged thin cross-sections of roots from 1-, 2-, and 3-week-old plants (Fig. 3A). At 1 week after germination, lines 216 and 218 had already started establishing cambium tissue, as indicated by the dense cell layers formed between the xylem and ground tissues. At 2 weeks after germination, we observed a further increase in the number of xylem vessels and neighbouring parenchyma cells. Up until this stage, no noticeable difference in cellular organization was found between lines 216 and 218. However, in 3-week-old plants, a dramatic increase in cell numbers inside the cambial layer was observed.
Cytokinin-dependent secondary growth determines in line 216. By contrast, line 218 showed only a slight increase. These results suggested that the difference in radial growth of lines 216 and 218 was from differences in the cell proliferation activities in the cambium. Next, we counted the cambial cell layers in various regions of cross-sections and compared their distributions (Fig. 3A, B) in 5-, 7-, and 9-week-old roots. At 5 and 7 weeks, when the radial growth was active for line 216, significantly more cambial cell layers were present in line 216 than in line 218 (Supplementary Table S2, available at JXB online). However, such differences between lines 216 and 218 diminished in 9-week-old plants.

It has been reported that ectopic meristems develop and contribute to the growth of root crops (Esau, 1977). We analysed this aspect further by imaging cellular organization in the cambium region, inner parenchyma region 1, inner parenchyma region 2, and root centre of line 216 roots grown for 7 weeks (Fig. 4A and Supplementary Fig. S5, available at JXB online). As reported previously, several regions filled with small cells were found, which indicated the presence of ectopic meristems. The density of ectopic meristems was slightly higher in the root centre than in the regions closer to the cambium. In cambial zones of actively growing stems and roots, cells emerging from the cambium by asymmetric cell division gradually increase their sizes to differentiate. By contrast, cells adjacent to putative ectopic meristems were fully expanded, indicating that these cells were not recent...
descendants of ectopic meristems. These results further suggested that the cambium might be primarily responsible for the radial growth of radish roots.

Cell division activities in the cambium drive radial root growth

To understand the relationships between dynamic changes in the cambial zone and cell division activity we performed immunolocalization assays in the cambial zones using an antibody against PCNA. PCNA is an evolutionarily conserved protein that is required for DNA replication. Its expression is highly upregulated in actively dividing cells (Dimova et al., 2003; Shimizu and Mori, 1998a, b). Our test experiment using 7-week-old root sections of lines 212 and 214 with or without treatment with PCNA antibody confirmed that the dividing cell-specific fluorescence signals came only from antibody-treated sections (Fig. 5A). High expression of PCNA in root cambium was also confirmed in actively growing scarlet globe radish roots (Supplementary Fig. S6, available at JXB online). We then carried out immunolocalization analyses using the roots of lines 212, 214, 216, and 218 collected at five different developmental stages (Fig. 5B). Line 214 and 216 plants showed a high level of PCNA in the cambium zone between 5 and 7 weeks, and then a very low level at 8 and 9 weeks. Line 212 plants did not show PCNA expression in 5-week-old roots but showed increasing PCNA levels in the cambium zone of 6-week-old roots. PCNA expression reached its highest level in 7-week-old root and then disappeared abruptly in line 212. In contrast to lines 212, 214, and 216, PCNA expression was always below the detection level in line 218. This indicated that the cambial cell division activity of line 218 plants was very low compared with the other inbred lines. Such developmental stage-dependent changes in cambial cell division together with dynamic changes in cambium size and cell organization in inbred lines suggest that cell division activities in the cambium play a critical role in the radial growth of radish roots.

Cross-species comparison identifies cambium-enriched transcription factors in radish

Cambium structure and its physiological regulation have been characterized in stems of woody eudicots (Savidge, 1996; Antonova and Stasova, 1997; Lachaud et al., 1999). However, underlying genetic components and molecular mechanisms are still largely unknown. Regulatory networks by transcription factors are crucial for every aspect in plant development, and cambium development and growth driven by cambial activities are no exception to this.

To understand transcriptional regulation in the radish root cambium, we searched radish ESTs and predicted CDSs that are putatively orthologous to cambium-enriched transcription factors in Arabidopsis roots. Genes for cambium-enriched transcription factors in Arabidopsis roots were identified from previous root cell-type-specific transcript profiling data combined with expression profiles in the early stage of root cambium formation (Birnbaum et al., 2003; Navy et al., 2005; Lee et al., 2006; Levesque et al., 2006; Brady et al., 2007; Carlsbecker et al., 2010) (Supplementary Fig. S7, available at JXB online). These include SHORT VEGETATIVE PHASE (SVP, AT2G22540), KNOTTED-LIKE FROM ARABIDOPSIS THALIANA (KNAT1, AT4G08150), and AINTEGUMENTA (ANT) (AT4G37750). SVP, a flowering time regulator like SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) and FRUITFULL (FUL), showed strong enriched expression in the early cambium. SOC1 and FUL have been shown to
Cytokinin-dependent secondary growth determines meristem determinacy, thereby affecting secondary growth (Melzer et al., 2008). KNAT1 and ANT (AINTEGUMENTA), key regulators in the shoot apical meristem, also show strong enrichment in the early cambium. KNAT1 was recently shown to affect the xylem cell fate in the cambium (Liebsch et al., 2014). Through reciprocal BLAST analyses between Arabidopsis and radish, we identified 12 putative orthologues from radish (Table 1). Orthologous relationships between Arabidopsis and radish gene pairs were further confirmed by phylogenetic tree analyses (Supplementary Fig. S8, available at JXB online).

To analyse the candidate gene expression patterns during radial root growth, we extracted total RNA from the cambium region and inner parenchyma regions 1 and 2 in 5-, 7- and 9-week-old roots from line 216 and analysed changes in gene expression by tracing their relative expression against RsActin2/7, a radish reference gene for qRT-PCR (Fig. 4A, B). To validate RsActin2/7 stability as a reference, we tested the relative expression of another radish reference gene, RsTEF2, against RsActin2/7 (Xu et al., 2012). The relative expression ratios between RsTEF2 and RsActin2/7 were very stable in all tissues of 5- and 7-week-old line 216 roots; however, some fluctuation in RsTEF2 was found in 9-week-old roots (Supplementary Fig. S9, available at JXB online). Thus, we chose RsActin2/7 as our primary reference gene. Among the 12 selected genes, seven exhibited a tendency to be expressed in the cambium region more highly than in other two regions (Fig. 4B), while the other five genes showed barely any enriched expression in the cambium (Supplementary Fig. S10, available at JXB online).

Thus, we further analysed the seven cambium-enriched genes in more detail, and detected a dynamic nature in their expression patterns. For example, cambium-enriched expression of RsKNAT1 was consistently detected at all developmental stages. However, the other genes showed developmental stage-dependent regulation. Expression of RsSVP in 5-week-old root cambium was similar to or slightly lower than that in the inner parenchyma tissues, but it became higher in the cambium than in the inner parenchyma tissues.

**Table 1.** Putative cambium-enriched transcription factors in radish, selected based on Arabidopsis root expression map

| Arabidopsis gene name | TF motif | Gene name of radish orthologue | Radish EST | Putative radish CDS |
|-----------------------|----------|-------------------------------|------------|--------------------|
| At2g22540 (SVP)       | MADS     | RsSVP                         | FY439133   | Rs1.0_01930.1_g00004.1 |
| At2g31180 (MYB14)     | MYB      | RsMYB14                       | EY908024   | Rs1.0_00496.1_g00007.1, Rs1.0_03050.1_g00002.1 |
| At4g08150 (KNAT1)     | Homeobox | RsKNAT1                       | EV532705   | Rs1.0_00263.1_g00002.1 |
| At4g37750 (ANT)       | AP2      | RsANT                         | EY917101   | Rs1.0_00792.1_g00008.1 |
| At5g61590 (DEWAX)     | ERF      | RsDEWAX-1                     | EV545694   | –                  |
| At1g16530 (ASL9)      | ASL      | RsASL9                        | EV528492   | Rs1.0_00056.1_g00002.1 |
| At3g23250 (MYB15)     | MYB      | RsMYB15                       | FY440652   | Rs1.0_01399.1_g00001.1, Rs1.0_02960.1_g00001.1 |
| At2g38470 (WRKY33)    | WRKY     | RsWRKY33                      | FY448049   | Rs1.0_03437.1_g00004.1 |
| At5g51190 (ERF105)    | ERF      | RsERF105                      | FY434566   | Rs1.0_07250.1_g00002.1 |
| At1g17380 (JAZ5)      | JAZ      | RsJAZ5                        | FD981520   | Rs1.0_00153.1_g000014.1 |
| At2g47260 (WRKY23)    | WRKY     | RsWRKY23                      | FY443167   | Rs1.0_01888.1_g00008.1 |

Fig. 5. Dynamic changes in the cambial activity of radish roots. (A) Immunolocalization of PCNA in radish roots. PCNA expression was analysed in 7-week-old roots of lines 212 and 214 by immunolocalization assays, with (+) and () indicate PCNA antibody-treated and untreated conditions, respectively. (B) Developmental stage-dependent regulation of cambial activity. The cambial activity of the indicated inbred lines was analysed by immunolocalization of PCNA over time. c, Cortex; p, parenchyma; arrowhead, cambium. Bars, 200 μm.
in 7- and 9-week-old roots. RsMYB14 and RsANT showed much stronger enrichment in 5-week-old root cambium than in 7- and 9-week-old root cambium. In contrast to these results, RsDEWAX1, RsDEWAX2, and RsASL9 reached their maximum expression in 7-week-old roots.

To further understand the expression patterns of the cambium-enriched genes, we extracted total RNA from the cambium region and inner parenchyma regions 1 and 2 at three different longitudinal axes of 7-week-old roots of line 216 and analysed gene expression (Fig. 4C, D). As expected, all the candidates were expressed in the cambium region more highly than in the other two tissues. Among these, expression of RsSVP, RsKNAT1, RsANT, and RsASL9 in the cambium changed dynamically along the longitudinal axis. Taking these data together, we concluded that expression of transcription factors in the cambium is regulated in a very dynamic manner over space and time.

**Differential expression of cambium-enriched transcription factors in inbred lines with distinctive radial root growth**

Line 218 plants showed suppressed radial root growth compared with line 216 plants. This suggested that expression patterns of the cambium-enriched genes might be different in these two. To test this idea, we analysed the mRNA levels of the aforementioned cambium-enriched genes in 216 and 218 roots. qRT-PCR was performed using total RNA extracted from the cambium zone and inner parenchyma region 2 of lines 216 and 218 roots grown in growth room conditions for 5, 7, and 9 weeks (Fig. 6 and Supplementary Fig. S11, available at JXB online). Interestingly, the expression pattern of RsSVP in line 216 roots was different from those in line 218. As mentioned in the previous section, line 216 plants showed dynamic regulation of RsSVP in cambium regions along root development stages. By contrast, the stage-dependent enrichment of RsSVP in the cambium was not observed in line 218 roots. Expression patterns of RsDEWAX1 and RsDEWAX2 also showed striking differences between line 216 and 218 plants. RsDEWAX1, RsDEWAX2, and RsASL9 did not show strong cambium-enriched expression in 5-week-old root cambium of line 216, although their expression increased dramatically in 7-week-old roots. By contrast, line 218 displayed very strong cambium-enriched expression of RsDEWAX1, RsDEWAX2, and RsASL9 in 5-week-old roots. Expression patterns of other genes such as RsMYB14, RsKNAT2, and RsANT were similar between line 216 and 218 roots (Supplementary Fig. S11). Despite some differences in expression levels (Fig. 6B), expression of these genes was highly enriched in the root cambium throughout all stages in both lines. These results suggested that RsSVP, RsDEWAX1, RsDEWAX2, and RsASL9 are involved in the cambium-mediated radial growth.

**Differences in radial growth caused by cytokinin responsiveness in radish**

Previously, it was shown that the expression patterns of SVP, DEWAX, and ASL9 are regulated by cytokinin (Naito et al., 2007; Mantiri et al., 2008; Bhargava et al., 2013). Cytokinin is a plant hormone essential in maintaining cell division activity and secondary growth. Because RsSVP, RsDEWAX1, RsDEWAX2, and RsASL9 showed distinctive expression patterns in lines 216 and 218, we asked whether cytokinin...
status was different between these two lines. To address this, we traced changes in cytokinin responses in line 216 and 218 root cambium by analysing the expression levels of RsRR15 and RsRR7, radish orthologues of Arabidopsis Response Regulator 15 (ARR15) and ARR7 (Fig. 7A). In line 216 roots, the expression levels of RsRR15 and RsRR7 were always higher in the cambium than in the inner parenchyma. In contrast to line 216, cambium enrichment of RsRR15 and RsRR7 was weak in line 218 roots. Furthermore, developmental stage-dependent changes in RsRR15 and RsRR7 expression in the root cambium were not observed in line 218. These results indicated that the response of line 218 to cytokinin in the root cambium might be weaker than the response of line 216. To further understand the cytokinin response in line 216 and 218 root cambium, we compared cytokinin responses induced by exogenous cytokinin treatment in these two inbred lines (Fig. 7B). Cytokinin treatment of line 216 roots induced the expression of RsRR15 and RsRR7 in the root cambium by 2- and 2.7-fold, respectively, in comparison with untreated root cambium. By contrast, RsRR15 and RsRR7 in the root cambium of line 218 were induced only slightly by cytokinin. Other genes known to be affected by cytokinin also showed expression changes in line 216 but not in line 218. RsSVP and RsASL9 were induced in line 216 but not in line 218, and RsDEWAX1 and RsDEWAX2 were significantly repressed by cytokinin in line 216 but not in line 218. These results suggested that the weak cytokinin response of line 218 cambium might be caused by defects in the cytokinin signalling and not by cytokinin biosynthesis.

If line 218 plants were defective in cytokinin signalling, their radial root growth would not be affected by cytokinin treatment. To test this hypothesis, we analysed the effect of
exogenous cytokinin on root growth in lines 216 and 218 (Fig. 8A, B). When 3-week-old plants in line 216 were grown in the cytokinin-treated condition for 1 week, their roots became noticeably thicker than untreated roots. In addition, their root growth showed dependence on cytokinin dosage; 20 and 200 nM BAP induced an increase in root circumferences of 50 and 75%, respectively, compared with the mock treatment. In contrast to line 216, line 218 plants did not display cytokinin dosage-dependent activation of radial root growth; 20 nM BAP induced only a slight increase in root circumference, while 200 nM BAP resulted in roots very similar to untreated roots. We then observed cambial cell division activity and cambium structure in response to cytokinin (Fig. 8C, D). When cell division activities in the cambium were monitored by immunolocalization of PCNA, line 216 roots grown without exogenous cytokinin did not show active cell division in the cambial area. By contrast, those treated with 20 and 200 nM BAP showed a dramatic increase in cell division activity in the cambium zone in a dosage-dependent manner. Consistent with the change in cell division activities, cell organization in the cambium of line 216 was also affected by cytokinin. Cytokinin treatment induced an increase in the cambium size. In contrast to line 216 plants, line 218 plants did not respond to cytokinin. Cambial cell division activity and the cambium structure of line 218 roots treated with cytokinin were almost identical to the mock-treated control plant. These results demonstrated that the suppressed radial growth of line 218 roots was caused by defects in cytokinin signalling. Therefore, cytokinin signalling and its downstream transcriptional regulation play an essential role in the radial growth of radish roots.

**Discussion**

The root is an essential organ required for nutrient and water uptake from soil. A growing number of studies show that plant growth and yields are affected by root development, suggesting that modulation and optimization of root development are crucial for the next green revolution (Villordon et al., 2014). In radish, secondary growth seems to be directly linked to root yield. Previous structural analyses showed the formation of both cambium and internal anomalous meristematic tissues during radial root growth in radish (Esau, 1977). However, it has been unclear to what extent the cambium and anomalous meristems contribute to the growth. In this study, we found that the cambial activity is strongly correlated with radial root growth and biomass in radish, not with anomalous meristems, indicating the essential role of cambium in radial root growth.
Cambial activity is sensitively modulated by environmental conditions, but its molecular mechanisms are still largely unknown. Cytokinin has been considered an important signalling molecule for this process because it controls cell division activity. Cytokinin levels are also sensitively regulated by environmental signals: they decrease in water-deficit conditions but increase in nutrient-rich conditions (Samuelson et al., 1992; Takei et al., 2001; Yang et al., 2001). Recent studies have demonstrated that cytokinin signalling is tightly linked to the regulation of secondary growth in the stem. Both quadruple mutant plants that lack AtIPT1, 3, -5, and -7 in Arabidopsis and transgenic plants overexpressing cytokinin oxidase in Populus showed suppressed secondary growth (Matsumoto-Kitano et al., 2008; Nieminen et al., 2008). The proliferative role of cytokinin has also been suggested based on the studies of root meristem in Arabidopsis (Mähönen et al., 2000; Dello Ioio et al., 2007). In mutants defective in cytokinin signalling such as wooden leg (wol) and ahk2 ahk3 ahk4 triple-mutant plants, the cell division that increases the number of procambial cell files is suppressed, resulting in the reduction of cell files that constitute primary vascular tissues (Scheres et al., 1995; Mähönen et al., 2006).

Previous studies by Radin and Loomis (1971) and Webster and Radin (1972) suggested the possible involvement of cytokinin in the secondary growth of radish roots. In these studies, they showed that cytokinin levels increased in radish roots with the onset of active secondary growth, and that cytokinin treatment triggered an increase in root diameter (Radin and Loomis, 1971; Webster and Radin, 1972). However, how cytokinin regulates this process at the molecular level has never been demonstrated. Here, we showed that cytokinin functions as a key modulator driving secondary growth and that cytokinin controls the secondary growth by regulating cell proliferation in the root cambium. In the inbred line with active secondary growth, the cytokinin response was stronger in the root cambium than in other neighbouring tissues, and was dynamically regulated along developmental stages. By contrast, the inbred line with suppressed secondary growth exhibited a very weak cytokinin response in the root cambium. Consistently, we could not find developmental stage-dependent changes in the cytokinin response in this line. Furthermore, exogenous cytokinin treatment could not activate cell division in the cambial zone and the secondary root growth in the inbred lines with suppressed growth. These results collectively indicate that the suppression of radial root growth in this inbred line is caused by defects in cytokinin signalling. However, we do not rule out the possibility that a key regulator of periclinal cell division is missing or defective in this line. Although further investigation needs to clarify these aspects, our investigation suggests that cytokinin function in secondary root growth resembles its function in secondary stem growth and that the proliferative role of cytokinin is essential for the secondary growth in radish roots.

Transcription factors are involved in every aspect of plant development by governing gene regulatory networks. Through a comparative analysis approach using Arabidopsis root expression data, we successfully identified cambium-enriched transcription factors in radish. Expression of RsKNAT1 was consistently enriched in the cambium regardless of root development stage. On the other hand, genes such as RsSVP, RsDEWAX1, RsDEWAX2, and RsASL9 showed developmental stage-dependent enrichment in the cambium. More importantly, the expression patterns of these genes were very different in two inbred lines with distinctive secondary root growth. Cambium-enriched expression of RsSVP was observed only in the inbred lines with active secondary growth, while RsDEWAX1 and RsDEWAX2 were expressed very strongly only in the cambium of the inbred line with suppressed secondary growth. These results showed the potential involvement of RsSVP, RsDEWAX1, RsDEWAX2, and RsASL9 in secondary growth of radish roots. Previous microarray approaches using Arabidopsis seedlings showed that these genes are regulated by cytokinin (Bhargava et al., 2013); however, their developmental functions in the cambium have not been reported. Our results uncover previously unidentified roles of RsSVP, RsDEWAX1, RsDEWAX2, and RsASL9, which are to regulate secondary root growth by controlling cytokinin-dependent cambium activity in an evolutionarily conserved manner.

A draft genome sequence of radish has become available (Kitashiba et al., 2014). More than 60 000 genes are predicted, even though the assembled genome sequence is estimated to cover only 75% of the whole radish genome. Therefore, it is possible that many of radial growth regulators have evolved specifically in the radish lineage, despite the key regulators conserved to operate in both radish and Arabidopsis. This study shows that such evolutionarily conserved radial growth regulators can be found efficiently by comparison of radish and Arabidopsis data. With technical advances in genomics tool, more thorough genome-wide investigations of root development in radish will help to deepen our understandings of molecular mechanisms underlying secondary growth and to apply them to the breeding programmes of economically important root crops.

**Supplementary data**

Supplementary data are available at JXB online.

- **Supplementary Table S1.** Primers used in this study.
- **Supplementary Fig. S1.** Relationships between radish growth factors.
- **Supplementary Fig. S2.** Shoot growth pattern in selected radish inbred lines.
- **Supplementary Fig. S3.** Images of root cross-sections in selected radish inbred lines.
- **Supplementary Fig. S4.** Radish root development in growth room and field conditions.
- **Supplementary Fig. S5.** Internal anatomy of radish roots.
- **Supplementary Fig. S6.** Cell division activities in root cambia.
- **Supplementary Fig. S7.** Cambium-enriched transcription factor genes in Arabidopsis.
- **Supplementary Fig. S8.** Phylogenetic analyses of cambium-enriched transcription factor genes in Arabidopsis and their putative radish orthologues.
Supplementary Fig. S9. Validation of Actin217 expression as a reference gene for qRT-PCR analysis in radish.

Supplementary Fig. S10. Expression patterns of the radish candidate genes.

Supplementary Fig. S11. Distinct expression pattern of the radish candidate genes between lines 216 and 218.

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