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Identification of genes related to root shape in radish (*Raphanus sativus*) using suppression subtractive hybridization

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Radish is an important vegetable, and displays a wide variation in root shape. To understand the mechanisms controlling development and/or inheritance of different root shapes, we attempted to identify the genes expressed differentially in roots of two cultivars with different root shapes; long and thick type (Lt) cv. Taibyousoubutori and skinny type (St) cv. Kosena. Morphological studies revealed that significant differences in thickness of roots began at 4 weeks after sowing. By suppression subtractive hybridization using roots at this stage, 140 and 70 non-redundant ESTs were identified from subtraction of St (tester) × Lt (driver) and reverse subtraction, respectively. Of these ESTs, 102 and 52 showed high similarity to previously identified genes, and the remaining 38 and 18 showed no matches to known genes. Quantitative RT-PCR analysis for selected eleven genes demonstrated that they are differentially expressed in roots of two radish cultivars according to the direction of subtraction. These results will contribute to a better understanding of the mechanisms and inheritance of radish root shape.

Key Words: radish (*Raphanus sativus*), root thickness or shape, ESTs, suppression subtractive hybridization, real-time RT-PCR.

Introduction

Radish (*Raphanus sativus* L.) is an important crop that is cultivated all over the world. The root axis of radish is composed of two anatomically distinct parts. The upper part originates from the hypocotyls, and lateral roots are not present on this part. The lower part consists of true root tissue, and lateral roots are developed in this part. These two parts of the radish root can thicken and form succulent tissue, which is used as a vegetable (Tsuro *et al.* 2008).

Radish was domesticated in the eastern Mediterranean area and subsequently spread to China and Japan. Extensive breeding has produced a large number of varieties with different root shapes (Kitamura 1958). In the West, the radish is commonly perceived as a small-rooted, short-season vegetable normally consumed in salads. However, in the Far East a variety of large-rooted radishes are grown. The roots exhibit great variation in skin color, flesh color, and shape. The roots are eaten raw, cooked, or preserved by storage, pickling, or drying. Radish is not only a vegetable crop but also an important source of medicinal compounds described by Watt and Breyer-Brandwijk (1962). East Asian countries are currently the biggest producers of radish, and the large-rooted varieties are produced much more widely than the small rooted varieties (Crisp 1995).

The molecular basis and inheritance of storage root development in radish are not well understood (Iwata *et al.* 2004). Storage root development begins with a thickening of the hypocotyl (Ting and Wren 1980) and is controlled by complex interactions among genetic, environmental and physiological factors. It is reported that the initiation of storage root development depends on a supply of sucrose and growth regulators from the shoots in root crops (Gupta *et al.* 2001, Rouhier and Usuda 2001, Lu and Zhang 2004).

The shapes and sizes of the storage roots change during the course of vegetative growth. Information about these processes is essential for optimization at the time of harvest, because shape and size are important traits that influences the efficiency of transportation and processing as well as consumer appeal. Hence, the root shape is directly related to the market price of the product (Reid and English 2000). Although several studies have been conducted on the morphological development of the roots of root vegetables (Rosenfeld 1998, Soujala 1999, Rosenfeld *et al.* 2002), little attention has been paid to genetic mechanisms that affect changes in shape during growth.

Radish root shape varies among varieties, and it can be treated as a quantitative trait (Tsuro *et al.* 2008). Some quantitative trait loci (QTLs) controlling root-related traits have been identified in rice (Price *et al.* 2000), bean (Beebe *et al.* 2006), and lettuce (Johnson *et al.* 2000). Lu *et al.* (2008) detected 18 QTLs affecting root morphological traits in *Brassica rapa*. These included 7 QTLs for taproot thickness, 5 QTLs for taproot length, and 6 QTLs for taproot weight. Tsuro *et al.* (2008) reported three QTLs affecting root shape and two QTLs affecting root thickening in radish. However,

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detailed analyses of differential gene expression in radish cultivars with different root shapes are lacking. Among the various methods available for identifying differentially expressed genes, suppression subtractive hybridization (SSH) is an efficient technique for isolating novel genes when access to whole genome information and expression analysis tools such as microarrays is not available.

In the present study, we identified genes that are differentially expressed during root formation or development in two radish cultivars with different root shapes using SSH. Two hundred ten non-redundant genes were isolated and classified by their molecular functions. In addition, root specific expressions of isolated ESTs were confirmed by quantitative RT-PCR.

Materials and Methods

Plant materials and root morphological studies

Two radish cultivars having different root shapes; a long type with thick roots cv. 'Taibyousoubutori' (Lt type) (Takii Seed Co. Ltd., Kyoto, Japan) and a skinny type cv. 'Kosena' (St type) (Watanabe Seed & Co. Ltd., Miyagi, Japan) were used in this study. Seeds were germinated in commercial soil (Gattirikun N-120, Tokita Seed Co., Saitama, Japan) in pots at 25/15°C day/night temperatures under natural light conditions for 6 weeks. In order to investigate the changes in root shape during the 6-week period, root morphological characters were examined each week. The shoot and root of each plant were separated, and the root was divided into three sections, top, middle and bottom, by cutting the root transversely at two sites of equal distance along the vertical length. The diameter was measured at the center of each section (I, II and III) (Fig. 1A). This experiment was independently repeated three times with six replicates.

Poly (A)$^+$ RNA extraction and SSH library construction

Plants were harvested from the Lt and St cultivars at four weeks after sowing, and the middle sections of the roots were excised for use in the SSH experiments. The root sections were smashed thoroughly to remove water and kept at –80°C. Poly (A)$^+$ RNA was isolated from the roots using a micro FastTrack 2.0 mRNA isolation kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. SSH was carried out using the PCR-select subtractive hybridization kit (Clontech, Palo Alto, CA, USA). cDNAs were synthesized from 2 µg of poly (A)$^+$ RNA isolated from each sample. The cDNAs generated from the two cultivars were used for subtraction in two directions: St (tester) × Lt (driver) and Lt (tester) × St (driver). cDNA digestion, adaptor ligation, hybridization, and PCR amplification were carried out as described by Diachenko *et al.* (1999).

Cloning and sequence analysis

The subtracted cDNAs (secondary PCR products) were cloned into the TA cloning vector pCR2.1 (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions and transformed into *E. coli* DH5α. Over 1000 positive clones were obtained. A total of 415 clones, which consisted of 226 clones in St (tester) × Lt (driver) and 189 ones in Lt (tester) × St (driver), were selected and sequenced with a DNA sequencing kit and an ABI 310 DNA sequencer (Applied Biosystems, Lincoln Centre Drive, Foster City, CA, USA). The sequences were used in a homology search of the NCBI (http://www.ncbi.nlm.nih.gov) and TAIR (http://www.arabidopsis.org) databases using the BLAST program. Sequences that showed 95% identity or more over at least 100 bp were grouped together. The grouped ESTs were used to search the Arabidopsis database at the TAIR website to identify genes with known molecular functions.

Expression analysis of isolated genes by RT-PCR and real-time quantitative RT-PCR analysis

Total RNAs were isolated from three sections (top, middle, bottom) of roots and leaves using RNAiso (Takara). cDNAs were synthesized from 1 µg of total RNA in a total volume of 20 µl containing 1 µl of oligo (dT) primer (0.5 µg µl^{-1}), 4 µl of first-strand buffer (5 × concentrated), 2 µl of dithiothreitol (100 mM), 2 µl of dNTPs (10 mM) and 0.2 µl Superscript II (300 unit). The reaction was carried out at 30°C for 10 min, 42°C for 50 min, and 70°C for 10 min. To evaluate the differential expression of the subtracted genes in roots of two radish cultivars with different root shapes, RT-PCR was carried out using gene-specific primers (ESM 1). Conditions for the thermal cycling were 94°C for 2 min; 28 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 40 s; and finally 72°C for 2 min. The reproducibility of the results was confirmed using samples from three independently grown plants.

The detail expression profiles of the subtracted genes on different sections and developmental stages of roots were analyzed by real-time quantitative RT-PCR. Real-time RT-PCR reactions were performed using a LightCycler system (Roche, Mannheim, Germany). The reaction volume was 20 µl containing 10 µl SYBR Premix Ex Taq II (Takara), 6.4 µl H_2O, 2.0 µl cDNA, and 0.8 µl of each 10 µM primer solution. The primer sequences used in this study are presented in ESM 2. These primer positions were predicted using Primer Express version 2.0 software (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Standard cycling conditions (30 s at 95°C followed by 40 cycles of 5 s at 95°C and 20–25 s at 60–63°C) were used for product formation. The real-time PCR products were analyzed using LightCycler software version 3.5.3 (Roche, Basel, Switzerland), and quantitative data for each gene are presented as ratios compared with actin gene expression. Each gene was analyzed at least two times.

Results

Root morphological studies

The two radish cultivars with different root shapes used in this study are shown in Fig. 1B–C. Both cultivars were

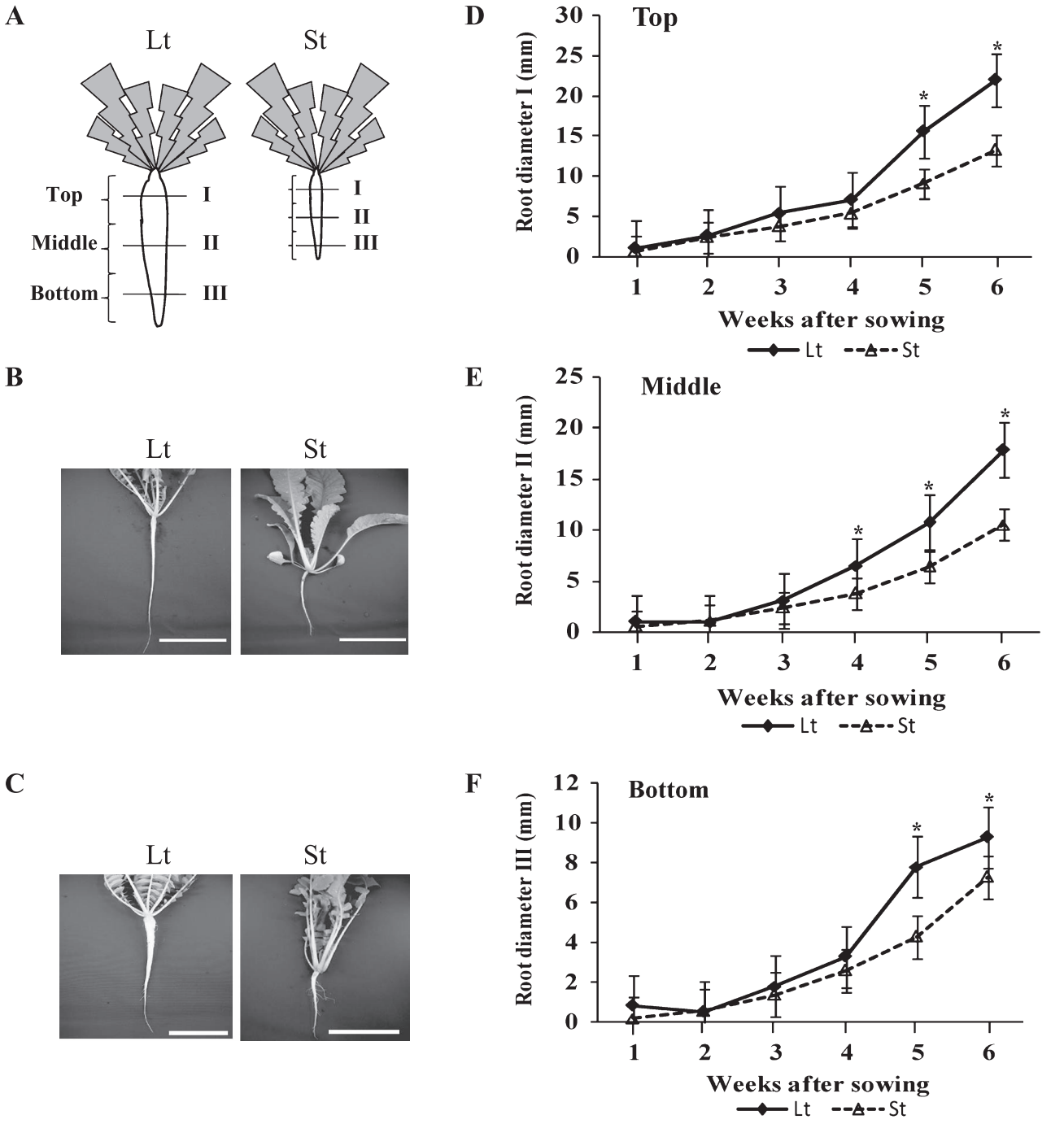

Fig. 1. Morphological analysis of radish root development using two cultivars with different root shape. Lt: long and thick type cv. 'Taibyou-soubutori', St: skinny type cv. 'Kosena'. (A) The roots were separated into three sections (top, middle, and bottom) by cutting at two sites at equal distances along the vertical lengths. The diameters were measured in each section (I, II, and III). (B) Four-week-old plants. (C) Six-week-old plants. (D), (E), (F) Developmental changes in top, middle and bottom parts of root, respectively. Measurement was repeated independently three times with six replicates, and values represent means ± SD. *: Significance at 5% level. Bars = 3 cm

grown for six weeks, and every week plants were harvested and the root diameter was measured. By the fifth week, significant differences between the cultivars were observed in the diameters of all three root sections (top, middle, bottom) (Fig. 1D–F). At first, thickening of the hypocotyl/root axis was similar in both cultivars, beginning in the mid-hypocotyl region and extending to both the top and bottom sections, so that a significant different was first observed in the middle section at four weeks (Fig. 1E). The upper part of the taproot continued to thicken very slowly in the St cultivar, but growth was much greater in the Lt cultivar, and by six weeks the roots were very different in shape between the two cultivars (Fig. 1C). At the same time, the Lt taproot was heavier and longer than that of the St (data not shown).

Based on these results, we selected the middle sections of four-week-old roots, which consisted of the lower part of the hypocotyl and the upper part of the taproot, to use in the SSH analyses.

Identification of radish root shape genes by differential screening of SSH library

Subtracted libraries were constructed to clone and identify genes showing differential expression in roots between the Lt and St cultivars. The middle sections of roots collected from three independent four-week-old plants in each cultivar were pooled for use in SSH library preparation. One subtraction was done by using St as a tester and Lt as a driver, and the other by using Lt as a tester and St as a driver. Two hundred and twenty-six clones from the St (tester) × Lt (driver) subtraction and 189 clones from the Lt (tester) × St (driver) subtraction were selected for sequencing. BLAST searches of the NCBI and TAIR databases were done to identify the putative functions of the DNA sequences. Of 226 clones identified from St × Lt, 114 were singletons and 112 were assembled into 26 groups with their common genes ranging from 2 to 10 (ESM 3). Of 189 clones identified from Lt × St, 47 were singletons and 142 were assembled into 23 groups with their common genes ranging from 2 to 14 (ESM 3). From the result of this grouping, 140 and 70 non-redundant sequences were identified in the St × Lt and Lt × St subtraction, respectively. These 140 and 70 EST groups were assigned to functional groups according to their homologies with sequences in the Arabidopsis database at the TAIR website. Of the 140 and 70 EST groups, 102 and 52 showed high similarity to previously identified sequences (Fig. 2), and the remaining 38 and 18 showed no matches to known genes in St × Lt and Lt × St, respectively. Of the 102 EST groups identified from St × Lt subtractions, other enzyme activity (14.2%), protein binding (13.5%), transferase activity (10.6%) and hydrolase activity (10.6%) were highly represented classes of genes with known potential function (Fig. 2A). Of the 52 ESTs from Lt × St, other enzyme activity (17.9%), protein binding (10.7%) and nucleotide binding (10.7%) were highly represented classes of genes with known potential function, and a high portion (17.9%) of ESTs represented genes with unknown molecular functions (Fig. 2B).

Expression analysis of isolated genes by RT-PCR and real-time quantitative RT-PCR analysis

To evaluate the differential expression of the subtracted genes in roots of two cultivars, eleven selected genes, i.e., seven genes from St × Lt (*RsSt-9*, *10*, *14*, *15*, *16*, *27*, *28*) and four genes from Lt × ST (*RsLt-2*, *7*, *13*, *44*), which are composed of genes previously isolated from *R. sativus* and genes related to cell development, transcription factor and signal transduction, were analyzed by RT-PCR. The former 7 genes showed higher expression in the St cultivar than in the Lt cultivar, and the latter 4 genes showed higher expression in the Lt cultivar (Fig. 3).

Real-time RT-PCR was used to investigate the expression of the same eleven genes in the top, middle and bottom sections of roots and leaves of the four-week-old plants (Fig. 4). In general, the relative levels of expression of these genes were higher in root parts than in leaves. The seven genes isolated from St × Lt were divided into two groups based on their levels of expression. The first group, *RsSt-9*, *14*, *15* and *28*, showed much higher levels of expression in the St than in the Lt cultivars (Fig. 4A). The second group, *RsSt-10*, *16* and *27*, was expressed abundantly in both St and Lt cultivars, although the levels were still higher in the St than in the Lt cultivars (Fig. 4B). The four genes isolated from Lt × St (*RsLt-2*, *7*, *13* and *44*) exhibited higher levels of expression in the Lt than in the St plants (Fig. 4C). In particular, all four genes exhibited higher expression in middle sections of the Lt roots, which were the parts used to create the subtraction libraries.

We also investigated the developmental changes in expression levels of the eleven genes in the middle section of roots. In general, the expressions of these genes were developmentally regulated in the roots of the two cultivars, and various changes in expression levels were observed. The seven genes isolated from St × Lt were divided into two groups according to their levels of expression. *RsSt-9*, *14*, *15*, *16* and *28* were developmentally up-regulated at later stages of development (4–6 weeks) in the St cultivar (Fig. 5A). On the other hand, *RsSt-10* and *27* showed high expression levels from one to six weeks in both cultivars, with general increases in expression levels over time (Fig. 5B). The four genes isolated from Lt × St were up-regulated specifically in the Lt cultivar and showed higher expression in the earlier stages from one to four weeks, with their highest expression at four weeks (Fig. 5C). It was notable that the expression levels of these eleven genes tended to change at around four weeks from sowing, when the two cultivars diverged in root shape and thickness.

Discussion

Various differences in size and shape of the storage organ occur in many root crops, and developmental changes in several species have been described (Rosenfeld 1998, Soujala 1999, Rosenfeld *et al.* 2002, Malamy 2005). However, little is known about the underlying molecular mechanisms and inheritance of traits that determine the size and shape of the mature storage organ (Iwata *et al.* 2004). In this study, we attempted to identify genes involved in root shape and size of radish by SSH. A morphological study using two cultivars, one a long and thick type and the other a skinny type, revealed that significant differences in thickness started in the middle sections of the roots at four weeks after sowing. From these results, isolation of genes was carried out using the tissue of this section.

A total of 210 non-redundant ESTs, which were composed of 140 from subtraction of St × Lt and 70 ESTs from reverse subtraction, were isolated. It is considered that the genes isolated from the former subtraction are related to the

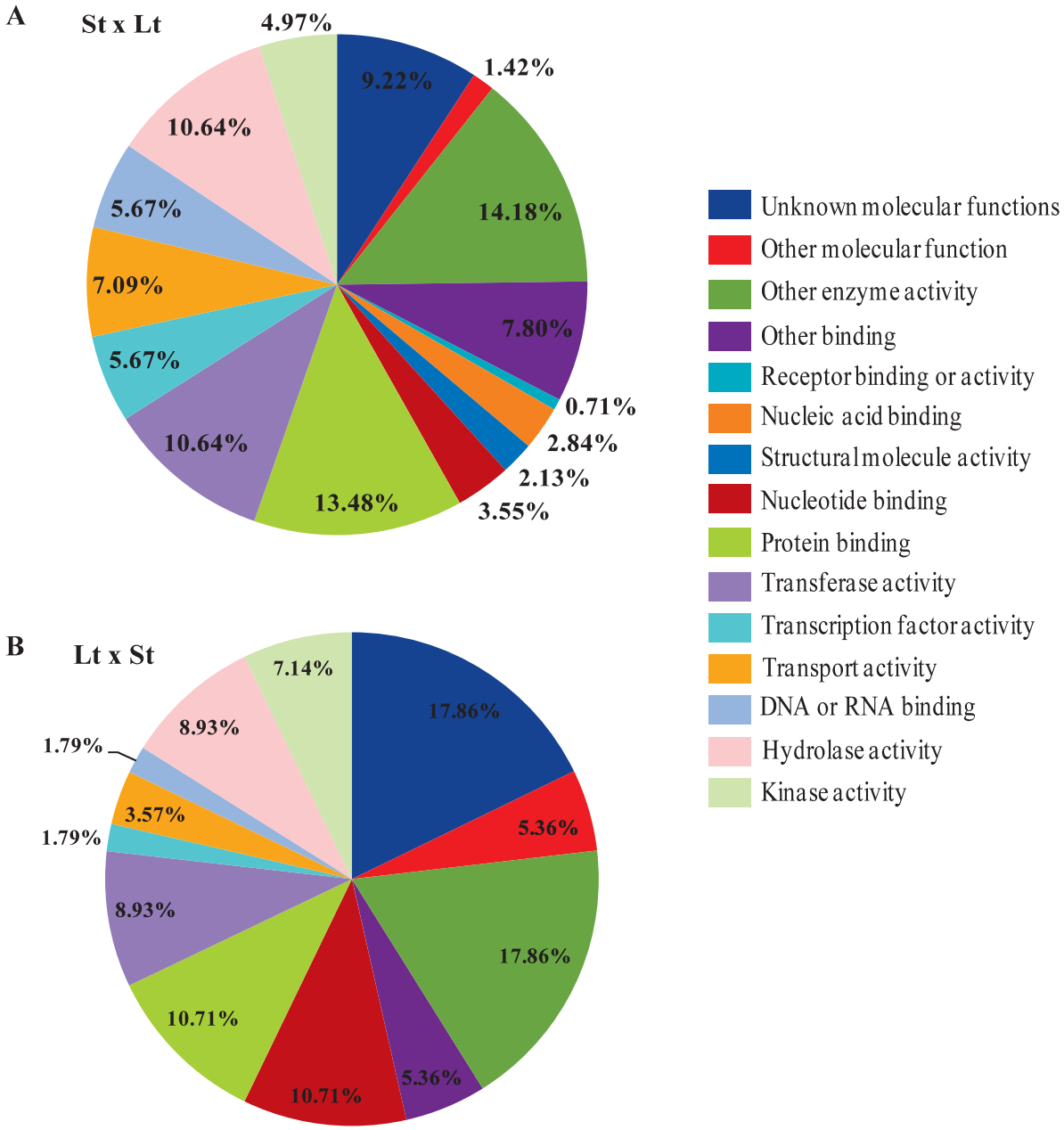

Fig. 2. Functional classifications of the isolated EST groups. The putative functions of identified 154 ESTs were determined by sequence comparisons using the BLAST program at the NCBI (http://www.ncbi.nlm.nih.gov) and TAIR (http://www.arabidopsis.org) databases. (A) Subtraction between St (tester) and Lt (driver). (B) Subtraction between Lt (tester) and St (driver). All ESTs (102 ESTs in A and 52 ESTs in B) were functionally classified based on sequences at the TAIR website.

genes repressing root enlargement and/or maintaining root development of the skinny type, in contrast, the genes isolated from the latter subtraction are related to the genes promoting root enlargement and/or repressing the genes characteristic of skinny type development. In general, root thickening is brought about by a combination of cell division in the cambium and cell enlargement in the differentiating xylem and phloem (Ting and Wren 1980), and variations in cell number and/or cell size account for wide differences in root shape (Dolan *et al.* 1993). Many ESTs identified in this study were homologous to genes reported to be involved in root development, cell number, and/or cell size. The differential expression of selected eleven genes in roots of two cultivars confirmed the success of the subtraction. The relationships between the function of these genes and their expression are discussed below.

The deduced product of *RsSt-9* shows high similarity to PAL (phenylalanine ammonia-lyase), which commits the flux of the biosynthesis of phenylpropanoids. Phenylpropanoids perform a variety of functions in plant development and in the interactions of plants with their environments (Shufflebottom *et al.* 1993), and they are also the precursors

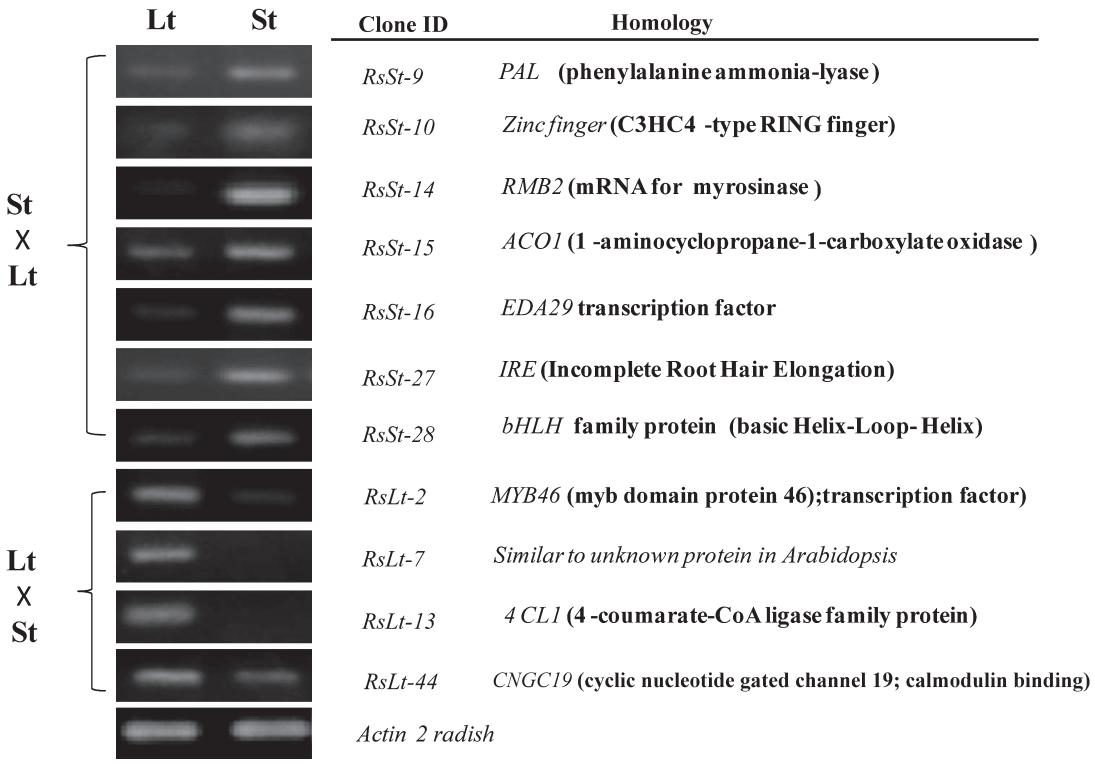

Fig. 3. Expression analysis of selected ESTs by RT-PCR. St × Lt: St (tester) × Lt (driver), Lt × St: Lt (tester) × St (driver).

in lignin synthesis. Higher expression of *RsSt-9* was found in the roots of St plants compared with those of Lt plants. On the other hand, *RsLt-13*, which has a higher expression in Lt type, has high similarity to *4CL1* (4-coumaroyl-CoA ligase). 4CL1 is one of the key enzymes in phenylpropanoid metabolism (Lee *et al.* 1995), and it is associated with the biosynthesis of lignin. Despite the possibility that *RsSt-9* and *RsLt-13* may be involved in lignin biosynthesis, they show different patterns of expression in Lt and St radish roots. Although their specific roles are unclear, our results suggest that lignin may play some important roles in determining root shape.

RsSt-14 is homologous to *RMB2* (radish B type mRNA myrosinase). Myrosinase is known as a myrosinase-glucosinolate defense system in Brassicaceae species (Bennet and Wallsgrove 1994). Myrosinase is a cytosolic plant enzyme present in radish roots, and it hydrolyzes 4-methylthio-3-butenyl glucosinolate into the pungent agent 4-methylthio-3-butenyl isothiocynate (Nakamura *et al.* 2008). Nakamura *et al.* (2008) reported that heirloom varieties produced higher levels of myrosinase than cv. Aokubi (=cv. Taibyosoubutori, Lt type). Their results agree with our results indicating that *RsSt-14* showed higher expression in St roots than in Lt ones.

RsSt-15, which showed higher expression in all sections of the roots of St than of Lt, has high similarity to *ACO1* (1-aminocyclopropane-1-carboxylate oxidase gene family). ACO plays an important role in the ethylene production. ACO catalyzes the conversion of ACC (1-aminocyclopropane-1-carboxylate) to ethylene. Interestingly, ethylene can promote and inhibit growth depending on the cell type and plant species. In Arabidopsis, it inhibits cell expansion and elongation throughout development in most tissues (Kieber *et al.* 1993, Guzman and Ecker 1990). Although the role of ACO in radish root development is largely unknown, these results might explain why *RsSt-15* showed higher expression in all sections of the roots of St. The putative product of *RsSt-27* shows homology to the IRE protein, which is strongly expressed in elongating root hair cells of Arabidopsis, suggesting a cell autonomous function of IRE in root hairs (Oyama *et al.* 2002). We observed that the radish St cultivar produced more lateral roots than Lt did, though did not examine root hairs. *RsSt-27* showed higher expression in the St plants, suggesting that there may be a relationship between root shape and the development of the root hairs.

Real-time RT-PCR analysis for several genes (*RsSt-10*, *16*, *28*, *RsLt-2*, *7*, *44*), which is related to the transcription factor and/or signal transduction (*Zinc finger*, *EDA29/BLH1*, *bHLH*, *MYB-46* etc.) and to unknown genes, demonstrated that they are differentially expressed according to the direction of subtraction. Although it is reported that these genes are related to cell division, cell expansion and/or cell fate (Laity *et al.* 2001, Birnbau and Benfey 2004, Zhong *et al.* 2007), the relationship of these genes with root shape is not well understood.

In this study, we have succeeded in identifying a large number of genes whose expression levels correlate with variations in root shape in radish. These results will contribute to

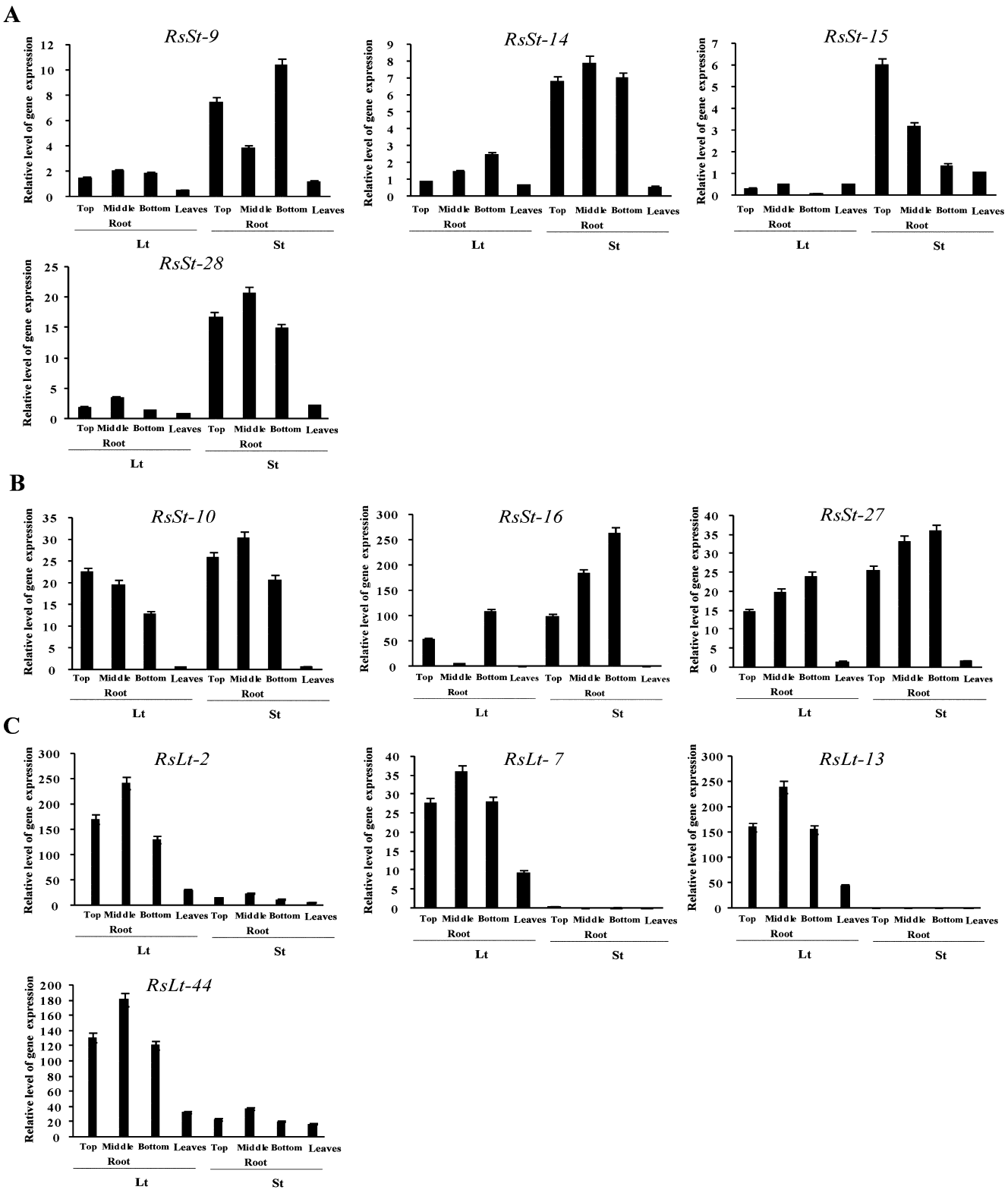

Fig. 4. Tissue specific expression analysis of selected ESTs by real-time RT-PCR. Analyzed ESTs are divided to three groups based on their expression pattern: (A) *RsSt-9*, *14*, *15* and *28*; (B) *RsSt-10*, *16* and *27*; (C) *RsLt-2*, *7*, *13* and *44*. The reproducibility of the results was confirmed using samples from three independently grown plants. Data presented are the means of two technical replicates ± SD.

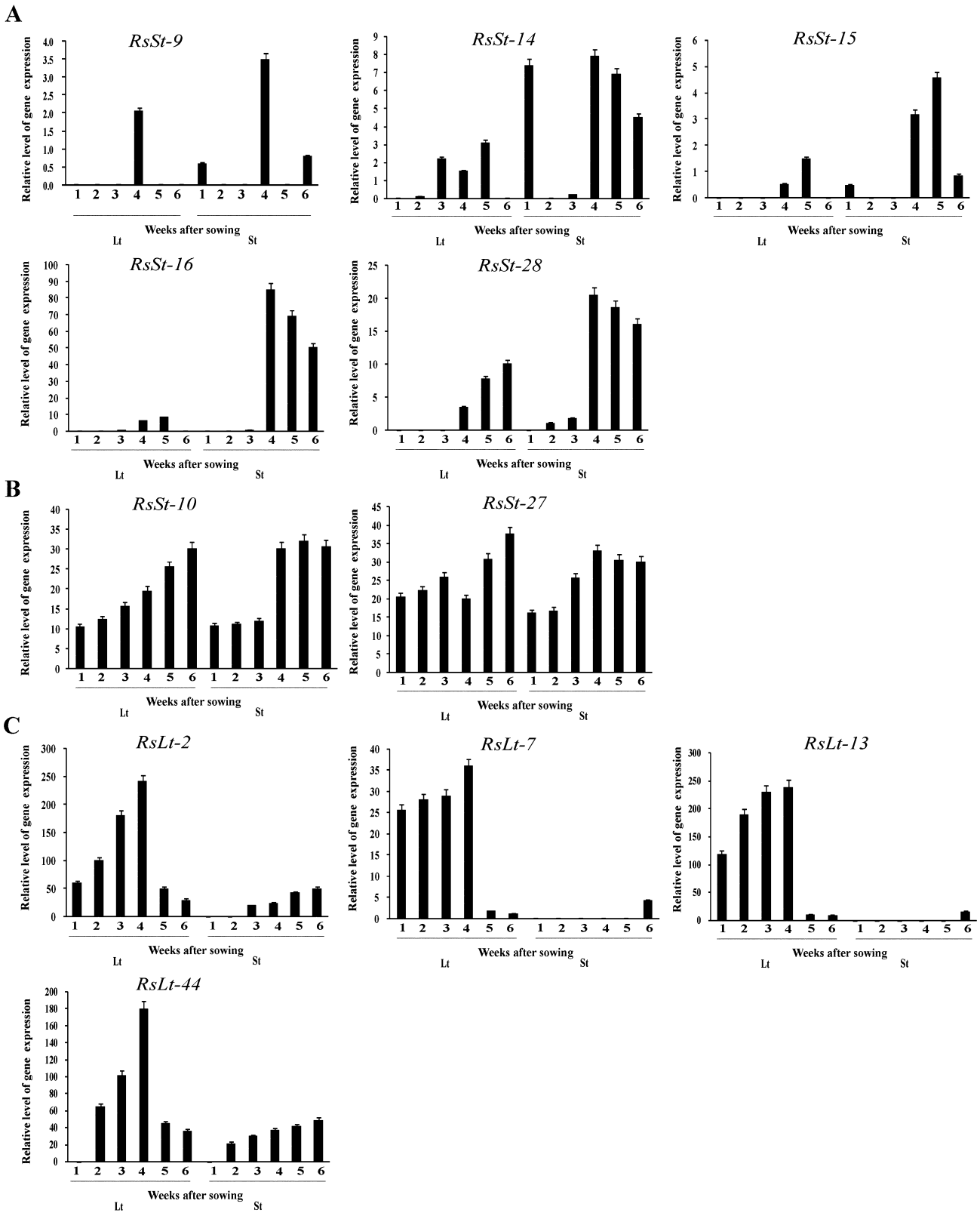

Fig. 5. Expression analysis of selected ESTs during root development by real-time RT-PCR. ESTs are divided to three groups based on their expression patterns: (A) *RsSt-9*, *14*, *15*, *16* and *28*; (B) *RsSt-10* and *27*; (C) *RsLt-2*, *7*, *13* and *44*. Data presented are the means of two technical replicates ± SD.

a better understanding of the mechanisms and inheritance of root shape. Further analysis of the individual genes is needed to elucidate their specific roles in determining root shape. We are currently carrying out function analysis of individual genes using homologous genes in Arabidopsis.

Acknowledgments

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