Down-regulation of the *IbEXP1* gene enhanced storage root development in sweetpotato

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Abstract

The role of an expansin gene (*IbEXP1*) in the formation of the storage root (SR) was investigated by expression pattern analysis and characterization of *IbEXP1*-antisense sweetpotato (*Ipomoea batatas* cv. Yulmi) plants in an attempt to elucidate the molecular mechanism underlying SR development in sweetpotato. The transcript level of *IbEXP1* was high in the fibrous root (FR) and petiole at the FR stage, but decreased significantly at the young storage root (YSR) stage. *IbEXP1*-antisense plants cultured in vitro produced FRs which were both thicker and shorter than those of wild-type (WT) plants. Elongation growth of the epidermal cells was significantly reduced, and metaxylem and cambium cell proliferation was markedly enhanced in the FRs of *IbEXP1*-antisense plants, resulting in an earlier thickening growth in these plants relative to WT plants. There was a marked reduction in the lignification of the central stele of the FRs of the *IbEXP1*-antisense plants, suggesting that the FRs of the mutant plants possessed a higher potential than those of WT plants to develop into SRs. *IbEXP1*-antisense plants cultured in soil produced a larger number of SRs and, consequently, total SR weight per *IbEXP1*-antisense plant was greater than that per WT plant. These results demonstrate that SR development was accelerated in *IbEXP1*-antisense plants and suggest that *IbEXP1* plays a negative role in the formation of SR by suppressing the proliferation of metaxylem and cambium cells to inhibit the initial thickening growth of SRs. *IbEXP1* is the first sweetpotato gene whose role in SR development has been directly identified in soil-grown transgenic sweetpotato plants.

Key words: Development, expansin, storage root, sweetpotato, thickening growth.

Introduction

The storage roots (SRs) of sweetpotato (*Ipomoea batatas*) provide high levels of digestible nutrients and fibres. The sweetpotato plant initially produces colourless fibrous roots (FRs), with some of these subsequently acquiring pigmentation and undergoing ‘thickening’ growth to form thick roots (TRs) that ultimately develop into SRs. Three distinct types of sweetpotato roots have been identified on the basis of anatomical studies: the FR, TR, and SR (*Kokubu*, 1973; *Wilson and Lowe*, 1973; *Nakatani and Komeichi*, 1991; *Noh et al.*, 2010). The characteristic features of the TR are active cambium differentiation to form a circular primary cambium around the central metaxylem and anomalous proliferation of the metaxylem cells at the centre part of the stele, while the SR is characterized by anomalous secondary cambial activity inside of a primary cambium.

Many authors have proposed that growth hormones, such as cytokinins, auxins [indole-3-acetic acid (IAA)], and abscisic
acid (ABA), play various roles in the formation and thickening growth of SRs (Akita et al., 1962; Matsuo, 1983; Nakatani and Komeichi, 1992; Wang et al., 2006; Eguchi and Yoshida, 2008; Noh et al., 2010). Cytokinin and auxin levels are high during the early stage of SR formation (Akita et al., 1962; Matsuo, 1983; Nakatani and Komeichi, 1992; Noh et al., 2010). Alternatively, secondary thickening growth of SRs is positively correlated with concentrations of ABA and cytokinin—and not with IAA levels (Wang et al., 2006), which actually show a gradual decrease with secondary thickening growth (Akita et al., 1962; Matsuo, 1983; Nakatani and Komeichi, 1992). The results of a recent transcriptome analysis of the SR of sweetpotato suggest a possible involvement of jasmonic acid (JA) in SR development (McGregor, 2006). Furthermore, stele lignification in FRs and TRs may prevent SR development (Togari, 1950). Wilson and Lowe (1973) and Belehu et al. (2004) observed an association between stele lignification and the inability of FRs and TRs to develop into SRs (Firon et al., 2009). In addition, the growth and/or yield of SRs have been shown to be affected by several environmental factors, including soil temperature, humidity, light, photoperiod, carbon dioxide, and drought (Loretan et al., 1994; Hill et al., 1996; Mortley et al., 1996; Eguchi et al., 1998; Pardales et al., 1999; Kano and Ming, 2000; van Heerden and Laurie, 2008).

The molecular mechanism underlying SR development is unknown. Recent advances in various molecular approaches have enabled the mining of genes possibly involved in SR development in sweetpotato, resulting in the identification of a number of genes that are differentially expressed in the developing SR (You et al., 2003; Tanaka et al., 2005). Tanaka et al. (2008) isolated three class I knotted1-like homeobox (KNOX) genes from sweetpotato and, based on the results of their subsequent comparison of the distribution of the expression of these KNOX genes and that of endogenous trans-zeatin riboside (t-ZR) in sweetpotato roots, suggested that these genes could be possible regulators of cytokinin levels in the SR. Ku et al. (2008) isolated IbMADS1 from sweetpotato and analysed its functional role in SR development using IbMADS1-overexpressing (ox) potato (Solanum tuberosum) plants grown in vitro. Very recently, another MADS-box gene, SRD1, from sweetpotato was identified and it was demonstrated that it had 99% nucleotide sequence identity with IbMADS1. Characterization of its role in SRD1-ox sweetpotato plants grown in vitro revealed that SRD1 is involved in the auxin-mediated initial thickening growth of the SR by enhancing proliferation activity in metaxylem and cambium cells (Noh et al., 2010). Further elucidation of the molecular mechanism underlying SR development will require identification of the full set of genes involved in SR development and direct characterization of their functional roles in soil-grown sweetpotato plants. Within this framework, Wang et al. (2010) and Xie et al. (2012) performed large-scale transcriptome sequencing of the SRs of sweetpotato using the Illumina paired-end sequencing technology and characterized the sweetpotato root transcriptome. However, functional characterization of the transcriptome in SR development has not yet been reported.

Expansins have been characterized as cell wall-loosening proteins (McQueen-Mason et al., 1992; McQueen-Mason and Cosgrove, 1995; Li et al., 2003). They are believed to be important regulators of wall extension during plant cell growth (Lee et al., 2001; Cosgrove et al., 2002; Li et al., 2003), and the expression levels of expansin genes have been found to be strongly correlated with elongation growth in the root, internode, flower, and leaf (Wu et al., 1996; Cho and Cosgrove, 2000; Lee and Kende, 2002; Choi et al., 2003; Gookin et al., 2003; Lee et al., 2003; Zenoni et al., 2004; Sloan et al., 2009; Harada et al., 2011), with expansion growth of grain (Lizana et al., 2010), and with fruit ripening (Rose et al., 1997; Brumme et al., 1999; Hayama et al., 2006; Mbéguié-A-Mbéguié et al., 2009). Expansin activities have also been found to be influenced by various abiotic stresses, such as drought (Wu et al., 2001; Jones and McQueen-Mason, 2004; Li et al., 2011; Zhao et al., 2011), flooding (Cho and Kende, 1997; Huang et al., 2000; Kim et al., 2000; Vriezen et al., 2000; Colmer et al., 2004), and salt and osmotic stress (Buchanan et al., 2005; Geiffers et al., 2010). Very recently, using gain- and/or loss-of-function approaches, a number of researchers have demonstrated that expansins are involved in the elongation growth of root hairs (Lin et al., 2011; ZhiMing et al., 2011) and stomatal opening (Wei et al., 2011; Zhang et al., 2011). Expansins are encoded by multifamily genes consisting of four gene subfamilies: α-expansin, β-expansin, expansin-like A, and expansin-like B (Sampedro and Cosgrove, 2005). These genes are similar in size, share a number of conserved motifs, and express similar wall-loosening activities (Cosgrove et al., 1997; Sampedro and Cosgrove, 2005). Expansin-like genes have been identified in tomato (Solanum lycopersicum) (Keller and Cosgrove, 1995), rice (Oryza sativa) (Lee and Kende, 2002), and Dicotyledon discoreaeum (Darley et al., 2003; Ogasawara et al., 2009), but the roles of expansin genes in SR development have not yet been investigated.

In an earlier study, cDNAs of three expansin genes (IbEXP1, IbEXP2, and IbEXPL1) from the young storage root (YSR) cDNA library of sweetpotato (I. batatas cv. Jinhongmi) were identified (You et al., 2003) and the transcriptional regulation of these three genes in response to chilling temperature was investigated (12–28 °C; Noh et al., 2009). In the study reported here, the functional role of IbEXP1 in SR development was characterized using IbEXP1-antisense sweetpotato plants. The results demonstrate that the initial thickening growth of the SR was enhanced in IbEXP1-antisense plants by an increased multiplication of metaxylem and cambium cells, which consequently promoted SR development, resulting in an increased production of SRs. These finding provide experimental evidence that the functional role of IbEXP1 involves blocking the initial thickening growth of the SR by suppressing metaxylem and cambium cell proliferation in the FR.

Materials and methods

Plant materials and growth conditions

Sweetpotato (I. batatas cv. Yulmi) plants were propagated by cutting and planting apical stems bearing 2–3 leaves in large [27 × 27 × 24 (height) cm] pots containing commercial horticultural potting soil (Baroker; Seoul Bio, Chungcheongbuk-do, Korea) in the greenhouse at 25–30 °C under a long-day photoperiod (16/8h, light/dark). No additional fertilizer was added.
RNA gel blot analysis
Total RNA was extracted from various tissues at three different developmental stages (FR (diameter <0.2 cm), YSR (diameter 0.5–1.0 cm)), and mature storage root (MSR; diameter >5.0 cm) stages] using the modified method with guanidinium–SDS lysis buffer and the CsCl gradient method as described in You et al. (2003). Total RNA (25 μg) was denatured, electrophoresed, and then transferred onto nylon membranes (Tropilon-Plus, Tropix) using the downward alkaline capillary method. A biotin-labeled probe was prepared by PCR amplification of the full-length IbEXP1 cDNA with T3 and T7 primers. The PCR cycling conditions consisted of pre-denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s at 95 °C, 20 s at 58 °C, and 30 s at 72 °C using dNTP mixed with biotin-labeled dCTP (Invitrogen). The labelled probe was purified using a PCR purification kit (Qiagen) according to the manufacturer’s instructions. Hybridization, washing, and detection were performed as described previously (You et al., 2003).

Generation of IbEXP1-antisense sweetpotato plants
Embryogenic calli were induced from L. batatas (L.) Lam. cv. ‘Yulmi’ shoot apical meristems cultured on MS medium (Murashige and Skoog, 1962) supplemented with 1 mg l−1 2,4-dichlorophenoxy acetic acid (2,4-D), 3% sucrose, and 0.4% geltie (MSID), kept at 25 °C in the dark, and proliferated by subculture at 4 week intervals on the same fresh medium. The full-size IbEXP1 cDNA was amplified with IbEXP1-specific primers (Supplementary Fig. S1 available at JXB online) (forward: 5'-gatggattcccATTCCTCTACCAATTCACTGAA-3’; reverse: 5'-gatggctactacctctcactcagctcag-3’). Kpnl and BamHI restriction sites were introduced at the ends of the forward and reverse primers, respectively, in order to facilitate subcloning. The PCR products were then subcloned into the pGEM-T Easy vector (Promega), digested with BamHI and Kpnl restriction enzymes, and fused to the Cauliflower mosaic virus (CaMV) 35S promoter in an antisense orientation by insertion of the IbEXP1 fragments at the BamHI and Kpnl sites of the pMBP1 binary vector. The resulting construct was used to transform sweetpotato plants by particle bombardment. DNA-coated microparticles were prepared using the CaCl2/spermide method as described by Kikkert (1993). A 1 μg aliquot of the plasmid DNA was mixed with 500 μg of gold particles (1.0 Micron Gold, Bio-Rad) in the presence of 1M spermide and 16mM CaCl2. A biolistic gun device (PDS-1000/He, Bio-Rad) was used to deliver the plasmid-coated gold particles (500 ng per bombardment) at the following parameters: the stopping disk was positioned 6 cm below the target calli were positioned 6 cm below the stopping screen; helium pressure was 1100 psi. The bombarded embryogenic calli were ruptured disk; target calli were incubated in flasks containing distilled water for 3 weeks. After FRs had developed from the distal end of the petiole, the single-leaf plantlets were incubated in various concentrations of IAA, JA, and 6-benzylaminopurine (BA) at 25 °C in the dark for 3 h. After the hormone treatment, total RNA was extracted from the FRs using the RNaseasy Plant Mini Kit (Qiagen) and used for real-time PCR.

qRT-PCR
The first-strand cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer’s instructions. The primers for real-time PCR were as follows: SRD1-specific primers (5'-AGAGGAGAAAAATGTTGTGTATGA-3’; 5’-GTGACCAGGAACCTCCCTTCT-3’); IbEXP1-specific primers used in the RT-PCR, and CAD primers (5’-ATTGTGATCATGACGTTGGCACA-3’; 5’-AGGACATTACATACACTCATTATATTG-3’). The real-time PCR analysis was performed using the LightCycler® 480 quantification system (Roche Diagnostics) as described in Noh et al. (2010). Expression levels were normalized with β-tubulin expression amplified with the β-tubulin-specific primers used in the RT-PCR. The real-time PCR mixture was prepared with 1× KAPA SYBR® Fast Master mix (KAPABIOSYSTEMS) in a final reaction volume of 16 μl. For the SRD1, IbEXP1, and CAD genes, the PCR was performed by subjecting the samples to an initial denaturation at 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s. The melting curve and cooling were performed under the same conditions as those described by Noh et al. (2010).

Results
Transcript level of IbEXP1 was down-regulated during SR development
With the aim of elucidating the functional roles of expansin genes in SR development, IbEXP1 was further characterized.

Microscopic observation
Apical meristems of sweetpotato plants bearing 2–3 leaves were grown on MS medium (Murashige and Skoog, 1962) at 25 °C for 10 d under long-day conditions (16/8h, light/dark). The thickest primary FR on each plant was collected, and transverse and longitudinal sections of the root were prepared from samples obtained at 5 mm from the root tip. The samples were first fixed in 2% (w/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1M sodium phosphate buffer, pH 7.0, at 4 °C for 10 d. After fixation, the tissues were washed twice with 0.1M sodium phosphate buffer (43.2 mM Na2HPO4 and 57.7 mM NaH2PO4) for 5 min, dehydrated stepwise for 30 min in a graded series of ethanol (50, 60, 70, 80, 90, 95, and 100%), embedded in acrylic resin (LR White; London Resin Company) for 5 d, and cut into 1.0 μm thick sections on an ultramicrotome (BROMMA 2088; LKB).

For the histochemical analysis of lignin, the maturation zone prepared from the thickest FR of sweetpotato plants cultured in vitro for 3 weeks was used. Hand-prepared sections of fresh samples were treated with 0.01% (w/v) phloroglucinol in 95% ethanol for 10 s, washed with 50% (v/v) HCl for 10 s, and mounted in 5 N HCl. The stained sections were observed by bright-field microscopy (BX51; OLYMPUS).

Hormone treatment
Sweetpotato plantlets bearing a single leaf and petiole (single-leaf plantlets) were collected from sweetpotato plants and incubated in flasks containing distilled water for 5 weeks. After FRs had developed from the distal end of the petiole, the single-leaf plantlets were incubated in various concentrations of IAA, JA, and 6-benzylaminopurine (BA) at 25 °C in the dark for 3 h. After the hormone treatment, total RNA was extracted from the FRs using the RNaseasy Plant Mini Kit (Qiagen) and used for real-time PCR.

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20 μl. PCR amplification was performed with an initial denaturation for 5 min at 95 °C, followed by 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C, and terminated with a 5 min final extension at 72 °C. The numbers of cycles used for each amplification were: β-tubulin, 24 cycles; IbEXP1, 27 cycles; IbEXP2, 35 cycles; and IbEXP1L, 25 cycles. The amplified products were electrophoresed on a 1.2% agarose gel.

For the histochemical analysis of lignin, the maturation zone prepared from the thickest FR of sweetpotato plants cultured in vitro for 3 weeks was used. Hand-prepared sections of fresh samples were treated with 0.01% (w/v) phloroglucinol in 95% ethanol for 10 s, washed with 50% (v/v) HCl for 10 s, and mounted in 5 N HCl. The stained sections were observed by bright-field microscopy (BX51; OLYMPUS).
Transcriptional regulation of *IbEXP1* during SR development was investigated in detail by an RNA gel blot hybridization analysis of RNAs extracted from various tissues (root, stem, leaf, and petiole) at three different root developmental stages, namely, the FR, YSR, and MSR stage, respectively (Fig. 1). Due to relatively low nucleotide sequence identity between *IbEXP1* and two other sweetpotato expansin genes (~46% with *IbEXP2* and 33% with *IbEXPL1*) (Noh et al., 2009), the full-length cDNA fragment (1213 bp) was used as a probe. At the FR stage, high-level expression of the *IbEXP1* transcript was found in the FR and petiole, and relatively low-level expression was found in the stem and leaf. In comparison, at the YSR stage, the expression level of the *IbEXP1* transcript had clearly decreased in the YSR, stem, leaf, and petiole, with the extent of the decrease being remarkable in the YSR and petiole. *IbEXP1* mRNA was not detected at all in the FR at the MSR stage. These results indicate that the expression of *IbEXP1* is transcriptionally down-regulated during SR development.

Transcriptional regulation of *IbEXP1* in response to exogenous hormones

Various authors have suggested that growth hormones, including auxins, JA, and cytokinins, play a role in the formation and thickening growth of SRs (Akita et al., 1962; Matsuo, 1983; Nakatani and Komeichi, 1992; Koda, 1997; McGreger, 2006). To test this notion, the possible transcriptional regulation of *IbEXP1* in response to exogenous IAA, JA, and BA was investigated by incubating sweetpotato plantlets bearing a single leaf and petiole (single-leaf plantlets) for 3 h in a solution containing various concentrations (0, 50, 100, 200, 500, and 1000 µM) of IAA, JA, or BA (Fig. 2). Total RNAs were then extracted from the FRs of the hormone-treated plants and subjected to analysis by real-time RT-PCR using *IbEXP1*-specific primers. *SRD1* was found to be involved in the initial thickening growth of the SR, and its transcript level increased following treatment with exogenous and endogenous IAA (Noh et al., 2010). The transcriptional regulation of *IbEXP1* by IAA, JA, and BA, respectively, was compared with that of *SRD1* by evaluating the transcriptional regulation of *SRD1* in response to these three hormones using *SRD1*-specific primers. The transcript level of *IbEXP1* remained almost unchanged in response to exogenously applied IAA, although the mRNA level of *SRD1* increased remarkably at 500 µM IAA (~300-fold). The transcript levels of *IbEXP1* increased gradually...
in response to increasing concentrations of JA, reaching a maximum transcript level (~10-fold) at 200 µM JA, but the extent of the increase in *IbEXP1* transcript level was relatively low compared with the robust increase in *SRD1* transcript level (~40- to 50-fold) at 200 µM and 500 µM JA. The transcript levels of both *SRD1* and *IbEXP1* increased in response to BA treatment, but the sensitivity of these two genes to BA treatment was clearly different in that the maximum increase in their respective transcript levels was detected at different concentrations of BA, with *IbEXP1* transcript levels reaching a maximum at 100 µM BA and those of *SRD1* at 500 µM BA. These results indicate that *IbEXP1* expression is nearly insensitive to IAA treatment and relatively weakly regulated by JA. Thus, transcriptional regulation of *IbEXP1* in response to IAA and JA differed from that of *SRD1*, suggesting that the role of *IbEXP1* in SR development is different from that of *SRD1*.

**IbEXP1-antisense sweetpotato plants showed an earlier thickening growth in FRs**

To ascertain the effect of *IbEXP1* down-regulation on SR development, *IbEXP1*-antisense sweetpotato plants with a full-length *IbEXP1* fragment under the control of the CaMV35S promoter were generated. Three independent *IbEXP1*-antisense transgenic lines (#1, #2, and #3) were obtained by *in vitro* culture. To identify the stable phenotype and expression of *IbEXP1* in these transgenic lines, the regenerated *IbEXP1*-antisense plants of each of these lines were propagated by cutting and planting three times *in vitro* prior to characterization. Quantitative RT-PCR analysis using *IbEXP1*-specific primers revealed that *IbEXP1* transcript levels in the FRs of *IbEXP1*-antisense plants of lines #1, #3, and #2 decreased to 35 ± 12, 52 ± 18, and 71 ± 1%, respectively, of that of the wild-type (WT) plants (Fig. 3A). These three *IbEXP1*-antisense lines were therefore used in subsequent analyses.

Cuttings of *IbEXP1*-antisense plants #1, #2, and #3 bearing the apical meristem and 2–3 developing leaves were planted on hormone-free MS medium. After 10 d of growth, the plantlets were examined for any alteration in root morphogenesis. No change in phenotype was observed in the aerial parts of the *IbEXP1*-antisense plants, but the FRs were shorter and thicker than those of the WT plants (Fig. 3B). Specifically, in the three *IbEXP1*-antisense lines, the length of the FRs was significantly reduced (Fig. 3C), and the diameter of the FRs had increased markedly in #1 and #3, and moderately in #2 (Fig. 3D). The extent of the reduction in root length and of the increase in root diameter was more severe in *IbEXP1*-antisense plants #1 and #3 than plants #2, suggesting that the observed root phenotype was attributable to a decrease in *IbEXP1* transcript levels.

Three expansin genes (*IbEXP1*, *IbEXP2*, and *IbEXPL1*) are expressed in the YSR of sweetpotato (*You et al.*, 2003; *Noh et al.*, 2009). To verify that the root phenotype of *IbEXP1*-antisense plants was caused by the observed decrease in *IbEXP1* transcript level—and not due to changes in the transcript levels of the other two expansin genes (*IbEXP2* and *IbEXPL1*)—the transcript levels of *IbEXP2* and *IbEXPL1* in *IbEXP1*-antisense plants were determined using RNAs extracted from the FRs of *IbEXP1*-antisense plants #1, #2, and #3 and *IbEXP2*- and *IbEXPL1*-specific primers. The results of the RT-PCR analysis on the extracted RNAs

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**Fig. 3.** Characterization of *IbEXP1*-antisense sweetpotato plants. (A) Transcript levels of *IbEXP1* in *IbEXP1*-antisense plants. Real-time RT-PCR analysis was carried out with total RNAs extracted from fibrous roots grown *in vitro*. Data were normalized to those for the endogenous *β-tubulin* gene. Error bars indicate the standard deviation (SD) between three technical replicates measured on fibrous roots collected from at least three different sweetpotato plantlets. (B) Morphology of the *IbEXP1*-antisense plants. Pictures were taken at 10 d after planting. (C) Root length of *IbEXP1*-antisense plants. Root length was measured with the three longest roots of each plant. (D) Root diameter of *IbEXP1*-antisense plants. Root diameter was measured with a dial caliper by measuring the thickest root of each plant. (C and D) Data were collected from sweetpotato plants cultured *in vitro* for 10 d after planting and are the means ±SD from three separate measurements of three individual plants. Different letters above the bars indicate significantly different means (*P* < 0.05) as analysed by Duncan’s multiple range test using the SAS statistical program (SAS Institute, Cary, NC, USA). (A–D) Numbers #1–#3 represent *IbEXP1*-antisense sweetpotato lines #1–#3, respectively. WT, wild type. (This figure is available in colour at *JXB* online.)
demonstrated that the transcript levels of *IbEXP2* and *IbEXPL1* had remained unchanged even though the *IbEXP1* mRNA level was significantly reduced in *IbEXP1*-antisense plants #1, #2, and #3 (Supplementary Fig. S2 at JXB online). This result suggests that mRNAs of antisense *IbEXP1* specifically reduced the transcript level of *IbEXP1* but did not affect the transcript levels of *IbEXP2* and *IbEXPL1*.

**Cell proliferation was enhanced in FRs of *IbEXP1*-antisense sweetpotato plants**

In order to study the root morphology phenotype at the cellular level, longitudinal and transverse sections of the maturation zone (collected at 5 mm from the root tip) were prepared from the thickest FR of the WT and *IbEXP1*-antisense plants cultured *in vitro*. Cells from *IbEXP1*-antisense lines #1 and #2, representing the strong and moderate root phenotype, respectively, were then characterized phenotypically. Elongation growth of the epidermal cells was distinctly different between the FRs of WT and *IbEXP1*-antisense plants (Fig. 4A), with the length of the epidermal cells in the FRs of *IbEXP1*-antisense plants of lines #1 and #2 being 38% and 47%, respectively, of that of WT plants (Fig. 4B). The extent of this reduction almost accounts for the decreased length of FRs from *IbEXP1*-antisense plants (32% and 61% of that of WT plants in lines #1 and #2, respectively), suggesting that the decreased length of FRs in *IbEXP1*-antisense plants was attributable to the reduced elongation growth of the epidermal cells. There was no significant difference in the width of the epidermal cells of WT and *IbEXP1*-antisense plants (Fig. 4C).

As vigorous proliferation of cambium and metaxylem cells has been shown to be a morphological marker of the initial thickening growth process leading to SR development in sweetpotato (Noh *et al.*, 2010), cell proliferation in the stele was examined in more detail. The cellular structure of the FR of *IbEXP1*-antisense plants was compared with that of WT plants (Fig. 5). WT plants showed the typical anatomy of FRs, with a relatively small root stele in which metaxylem and metaphloem had recently differentiated. Cell proliferation was, however, significantly enhanced in the *IbEXP1*-antisense plants, resulting in an increased number of cells in the stele compared with the WT (Table 1). Both metaxylem and cambium cell proliferation were markedly elevated in *IbEXP1*-antisense plants, with a 2.0- to 2.4-fold increase in the number of metaxylem cells and a 1.4- to 2.0-fold increase in the number of cambium cells relative to WT plants. These results suggest that the enhanced proliferation activity in metaxylem and cambium cells resulted in an earlier thickening growth in the FRs of *IbEXP1*-antisense plants. In addition, based on differences in the numbers of metaxylem and cambium cells, proliferation activity was higher in *IbEXP1*-antisense plants #1 than #2, indicating that the proliferation activity was positively correlated with the extent of reduction in *IbEXP1* mRNA levels. The increased proliferation activity was therefore attributed to the reduction in *IbEXP1* transcripts.

**Lignification was reduced in the FRs of *IbEXP1*-antisense sweetpotato plants**

It has been suggested that lignification in the steles of FRs and TRs prevents SR development (Togari, 1950; Wilson and Lowe, 1973; Firon *et al.*, 2009). In addition, Belehu *et al.* (2004) suggested that the presence of lignified central metaxylem cells is a typical structure of FRs and TRs that lack the potential to develop into SRs. The degree of lignification in the stele of FRs from WT and *IbEXP1*-antisense sweetpotato plants was therefore examined. Lignification in the FRs was detected by preparing transverse sections of the maturation zone from the thickest FR of WT and *IbEXP1*-antisense plants cultured *in vitro* for 3 weeks, staining these with phloroglucinol, and observing the staining intensity microscopically (Fig. 6A). The central metaxylem cells were deeply stained in the FR of WT plants but remained unstained in the hexarch stele of the FR from *IbEXP1*-antisense plants of line #1. The central metaxylem cells in the stele of FRs from *IbEXP1*-antisense plants of line #2 were faintly stained, but the lignification level of the metaxylem cells from this line was much lower than that of WT plants. These results indicate that stele lignification was significantly reduced in the FRs of *IbEXP1*-antisense plants.
Cinnamyl alcohol dehydrogenase (CAD) is a key enzyme in the biosynthesis of lignin (Walter et al., 1988; Goujon et al., 2003). Quantitative RT-PCR analysis performed to determine the transcript level of the CAD gene in these transgenic mutants revealed that the transcript levels of CAD were moderately decreased in *IbEXP1*-antisense plants of line #2 and markedly decreased in those of line #1, as compared with WT plants (Fig. 6B), suggesting that down-regulation of *IbEXP1* expression led to a decrease in the transcript abundance of the *CAD* gene and, consequently, to a reduction in the extent of lignification. This reduced level of stele lignification in the *IbEXP1*-antisense plants suggests that the FRs of *IbEXP1*-antisense plants have a higher potential to develop into SRs than those of WT plants. The reduction in stele lignification and CAD transcript abundance was more severe in *IbEXP1*-antisense plants of line #1 than in those of line #2, indicating that the reduction level was positively correlated with the extent of reduction in *IbEXP1* mRNA levels and suggesting that the reduced lignification in these mutants is attributable to the decreased levels of *IbEXP1* mRNA accumulation.

**IbEXP1-antisense sweetpotato plants produced a higher number of SRs**

*IbEXP1*-antisense plants were transferred to the soil, propagated by cutting and planting three times in the greenhouse, and then examined for growth and phenotypic characteristics. At 6 weeks after planting, compared with WT plants, the aerial parts of *IbEXP1*-antisense plants grew less vigorously, their stems, petioles, and internodes were shorter, and their leaves were smaller (Supplementary Fig. S3A at *JXB* online). There were also distinct differences between the WT and the *IbEXP1*-antisense plants at 6 weeks of growth in terms of root morphology (Supplementary Fig. S3B). The most prominent phenotypic difference was that a higher number of primary roots had already begun thickening growth in the *IbEXP1*-antisense plants, whereas most of the primary roots in the WT plants had not yet initiated the thickening growth process, resulting in a higher ratio of TRs to total primary roots (TR/PR) in *IbEXP1*-antisense plants than in the WT (Supplementary Fig. S3C). These TRs in the *IbEXP1*-antisense plants were observed to develop into SRs at 8 weeks after planting (Supplementary Fig. S3B). These results indicate that the initiation of thickening growth was accelerated in FRs from *IbEXP1*-antisense plants.

At 10 weeks after planting, the initial growth retardation observed in the leaf and petiole had almost disappeared, but the elongation growth of the stems differed markedly between the WT and *IbEXP1*-antisense plants (Supplementary Fig. S3D, E at *JXB* online). In WT plants, the primary stem showed vigorous elongation, but the growth of additional stems was not

### Table 1. Effect of IbEXP1 down-regulation on cell proliferation activities in the fibrous root

Data are means ±SD from three separate measurements of three sections from three individual plants and are collected from the in vitro cultured sweetpotato plants at 10 d after planting.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Wild type</th>
<th><em>IbEXP1</em>-antisense #1</th>
<th><em>IbEXP1</em>-antisense #2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cells</td>
<td>Rate (fold)</td>
<td>No. of cells</td>
</tr>
<tr>
<td>Cambium</td>
<td>46 ± 6</td>
<td>(2.0)</td>
<td>64 ± 7</td>
</tr>
<tr>
<td>Metaxylem</td>
<td>30 ± 4</td>
<td>(2.4)</td>
<td>60 ± 1</td>
</tr>
</tbody>
</table>

*a* Counted on transverse sections of fibrous roots.

*b* Proliferation rate relative to that of wild-type plants.

*c* Numbers of metaxylem cells, including mature and immature metaxylem cells.
observed. In contrast, elongation growth of the primary stem was reduced in *IbEXP1*-antisense plants, resulting in a much shorter primary stem than that formed in WT plants, as well as the production of multiple stems showing similar levels of elongation growth. Consequently, the WT produced a long vine-like stem, while *IbEXP1*-antisense plants acquired a bush form with multiple stems.

After 5 months of cultivation, WT plants and *IbEXP1*-antisense plants #1, #2, and #3 were harvested and the growth of both the SR and shoot was examined (Fig. 7, Table 2; Supplementary Fig. S4 at *JXB* online). Relative to the WT plants, *IbEXP1*-antisense plants produced a higher number of stems and delayed leaf senescence. Leaf yellowing, which is due to chlorophyll loss, and subsequent leaf loss are typical symptoms of age-dependent senescence. At 5 months post-planting, most of the leaves of the WT plants had fallen off the plants, while those of the *IbEXP1*-antisense plants were still attached to the stems and had remained green (Supplementary Fig. S4). Consequently, the total shoot weight per *IbEXP1*-antisense plant was greater than that per WT plant (Table 2). SR development was also altered in *IbEXP1*-antisense plants, with *IbEXP1*-antisense plants #1, #3, and #2 producing 7.3 ± 1.5, 7.0 ± 0.6, and 6.0 ± 0.6 SRs, respectively, in comparison with the 4.5 ± 2.4 SRs produced by the WT plants (Fig. 7, Table 2). This elevated number of SRs in each *IbEXP1*-antisense plant resulted in a significant increase in the total weight of SRs in comparison with that of each WT plant, namely, 393.8 ± 35 g (*IbEXP1*-antisense plant #1), 359.7 ± 44.2 g (*IbEXP1*-antisense plant #3), and 299.8 ± 42.4 g (*IbEXP1*-antisense plant #2) versus 226.1 ± 19.1 g (WT plant) (Table 2). The extent of the increase in SR number and total SR weight was greater in *IbEXP1*-antisense plants #1 and #3 than in *IbEXP1*-antisense plants #2, suggesting that the observed phenotype in SR development was attributable to a decrease in *IbEXP1* transcript level. Another noteworthy phenotype was the difference in level of uniformity in the size of the SRs between WT and *IbEXP1*-antisense plants. SRs from *IbEXP1*-antisense plants...
were relatively uniform in size, while those of WT plants varied in size. These results indicate that although the shoot growth of \( \text{IbEXP1} \)-antisense plants was retarded during the early developmental stage, the final yield in terms of both SRs and shoots was elevated in \( \text{IbEXP1} \)-antisense plants.

**SR development was accelerated in \( \text{IbEXP1} \)-antisense sweetpotato plants**

Sweetpotato plants initially produce FRs from underground stems. These FRs undergo elongation growth, followed by thickening growth at certain positions that leads to the development of SRs. Thus, in the FR, there is always a certain distance between the proximal end of the SR and the underground stem.

The length of the FRSS [fibrous root between storage root and underground stem (red arrows in Fig. 8A)] decreased markedly in \( \text{IbEXP1} \)-antisense plants in comparison with WT plants, being <1.5 cm in the former and >5.0 cm in the latter (Fig. 8B). Consequently, in contrast to the random positions of SRs on FRs in WT plants, all of the SRs in \( \text{IbEXP1} \)-antisense plants were almost directly attached to the underground stem, indicating that the formation of SRs took place at identical sites, namely the proximal end of the FR. This result indicates that the thickening growth of the SRs commenced right from the initial stage of FR growth in \( \text{IbEXP1} \)-antisense sweetpotato plants, further suggesting that SR development was accelerated in \( \text{IbEXP1} \)-antisense sweetpotato plants.

### Discussion

The expression levels of expansin genes have been found to be strongly correlated with elongation growth in various plant organs (Wu et al., 1996; Lee and Kende, 2002; Choi et al., 2003; Gookin et al., 2003; Lee et al., 2003; Zenoni et al., 2004). Similar to expansin genes in other plants, in the present study, the \( \text{IbEXP1} \) transcript level was also found to be high in actively growing tissues, such as the FR and petiole tissues at the FR stage; however, this high transcript level decreased markedly at the YSR stage (Fig. 1). This type of expression pattern suggests the possibility that \( \text{IbEXP1} \) plays a negative role in SR development.

It has been suggested that cytokinin and auxin (IAA) have some function during the onset and subsequent primary thickening growth of SRs (Akita et al., 1962; McDavid and Alam, 1980; Matsuo, 1983; Nakatani and Komeichi, 1992; McGregor, 2006). This hypothesis is supported by recent work in our laboratory demonstrating that \( \text{SRD1} \) activates the initial thickening growth of the SR and that its transcript level increases in response to exogenous and endogenous treatment with IAA.
JA has been shown to inhibit root elongation (Staswick et al., 1992; Feys et al., 1994). In sweetpotato, the initiation of SR formation is accompanied by the cessation of root elongation (Kim et al., 2002), suggesting the possible involvement of JA in SR formation (Firon et al., 2009). Based on the transcriptome analysis of the sweetpotato SR, McGregor et al. (2006) also suggested a possible involvement of JA in SR development. In the study reported here, it was found (i) that IAA had almost no effect on regulating the transcript level of \(IbEXP1\) although it did markedly increase that of \(SRD1\); and (ii) that transcriptional induction of \(IbEXP1\) by JA was much weaker than that of \(SRD1\) (Fig. 2). These results suggest a possible negative role for \(IbEXP1\) in SR formation.

\(IbEXP1\)-antisense sweetpotato plants cultured in vitro produced shorter FRs than WT plants (Fig. 3), and microscopic observation revealed that the reduced length of FRs could be attributed to the limited elongation growth of epidermal cells (Fig. 4). This observation implies that \(IbEXP1\) plays a role in the elongation growth of epidermal cells in the sweetpotato FR, a role that is supported by the expression patterns of other expansin genes strongly correlating with elongation growth in the root, internode, flower, and leaf (Wu et al., 1996; Cho and Cosgrove, 2000; Lee and Kende, 2002; Choi et al., 2003; Gookin et al., 2003; Lee et al., 2003; Zenoni et al., 2004; Sloan et al., 2009; Harada et al., 2011), and also consistent with the roles of other expansin genes involved in the elongation growth of root hairs (Lin et al., 2011; ZhiMing et al., 2011). In addition, the FRs of \(IbEXP1\)-antisense sweetpotato plants cultured in vitro were thicker than those of the WT plants (Fig. 3), and there were significantly more metaxylem and cambium cells in \(IbEXP1\)-antisense plants than in WT plants (Fig. 5, Table 1), leading to an earlier thickening growth in the FR. The roots of sweetpotato have been classified into three distinct root types according to their diameters: FR (diameter <2 mm), TR (diameter 2–5 mm), and SR (diameter >5 mm) (Kokubu, 1973; Wilson and Lowe, 1973). The process of SR formation in sweetpotato begins with the formation of colourless FRs, followed by the pigmentation and primary thickening growth of some of these FRs, ultimately leading to the development of SRs by secondary thickening growth. Based on this classification by root type, the formation of the TR from the FR is a prerequisite in the process of SR development, and the proliferation of cambium and metaxylem cells has been determined to be a morphological characteristic of the TR and SR (Noh et al., 2010). Thus, the earlier thickening growth shown by the \(IbEXP1\)-antisense plants implies that the potential to develop SRs is enhanced in the FRs of \(IbEXP1\)-antisense plants. Moreover, earlier studies have shown that stele lignification in the FRs and TRs in sweetpotato inhibit the thickening growth into SRs (Togari, 1950; Wilson and Lowe, 1973; Belehu et al., 2004). Morphological and anatomical studies of sweetpotato roots have demonstrated that those roots without lignified central metaxylem cells have the potential to develop into SRs, whereas those with lignified central metaxylem cells do not (Wilson and Lowe, 1973; Belehu et al., 2004). A significant reduction was observed in the lignification of the stele, especially that of the central metaxylem cells, of FRs from \(IbEXP1\)-antisense plants and a corresponding decrease in the transcript level of the lignin biosynthesis-related gene (\(CAD\) gene) (Fig. 6). Therefore, these results also suggest that the potential to develop SRs is enhanced in the FRs of \(IbEXP1\)-antisense plants. A correlation between lignification level and expansin gene expression has also been observed in loquat fruit (Yang et al., 2008) and water bamboo shoot (Song et al., 2011). Specifically, Yang et al. (2008) reported that the low expression of \(EjEXP1\) and \(EjEXP4\) is associated with the low level of chilling-induced lignification in loquat fruit (cv. Baisha), and Song et al. (2011) demonstrated that the suppression of lignification by 1-methylcyclopropane (an inhibitor of lignification) treatment is accompanied by a decrease in the expression of expansin (\(ZeExp\)) in peeled water bamboo shoot (\(Zizania caduciflora\) L.).

In this study, the earlier thickening growth in the FRs of \(IbEXP1\)-antisense plants cultured in vitro was verified by the observation of the earlier thickening growth in the FRs from soil-grown \(IbEXP1\)-antisense plants, leading to an increased ratio of TRs to total primary roots (TR/PR) in the \(IbEXP1\)-antisense plants relative to WT plants (Supplementary Fig. S3 at JXB online). Ultimately, \(IbEXP1\)-antisense plants produced a higher number of SRs than WT plants (Fig. 7, Table 2), resulting in an elevation in the total weight of SRs per \(IbEXP1\)-antisense plant. The most striking phenotype in \(IbEXP1\)-antisense plants was a remarkable decrease in the length of the FRSS (Fig. 8). In WT sweetpotato plants, SR development took place at various positions along the FR at different time points, but in \(IbEXP1\)-antisense plants most of the SRs developed simultaneously at the proximal ends of the FRs. Therefore, it seems likely that, in contrast to the situation in WT plants in which the elongation growth phase of FRs precedes the thickening growth of SRs, in \(IbEXP1\)-antisense plants, thickening growth and elongation growth of FRs proceeded simultaneously at the proximal ends of the FR. This result indicates that the thickening growth of FRs was initiated earlier in \(IbEXP1\)-antisense plants than in those of the WT and that, consequently, SR development was accelerated in \(IbEXP1\)-antisense sweetpotato plants. Taken together, these results demonstrate that down-regulation of the \(IbEXP1\) gene caused an acceleration of SR development through an enhancement of thickening growth in FRs, suggesting that \(IbEXP1\) plays a negative role in the formation of SRs by inhibiting the initial thickening growth of SRs.

Not only the SR of \(IbEXP1\)-antisense plants exhibited significant phenotypic alterations, but also the shoot. Although \(IbEXP1\)-antisense plants did show shoot growth retardation and restricted elongation growth in the primary stem during the early stage of development (Supplementary Fig. S3 at JXB online), they eventually produced higher number of stems than WT plants (Fig. 7, Table 2). This increased number of stems may be attributable to reduced apical dominance in \(IbEXP1\)-antisense plants. Sweetpotato normally produces a single primary stem in the early stage of development that grows vigorously to form a long vein-like stem. The apical dominance of this primary stem prevents additional stems from growing. In \(IbEXP1\)-antisense plants, however, the elongation growth of the primary stem was significantly reduced, possibly weakening the apical dominance of the primary stem to such a degree that it was unable to inhibit the growth of other stems. Consequently, \(IbEXP1\)-antisense plants produced more stems than WT plants. Another distinctive phenotype associated with the shoots was the delayed leaf
senescence in \textit{ibEXP1}-antisense plants compared with that of WT plants (Fig. 7; Supplementary Fig. S4). This resulted in an increase in total shoot weight per \textit{ibEXP1}-antisense plant at harvest (Table 2). With this increased shoot weight, \textit{ibEXP1}-antisense plants may be able to supply much more photosynthetic energy needed for thickening growth of SRs. However, it was observed that during the early developmental stage \textit{ibEXP1}-antisense plants exhibited an earlier and enhanced formation of SRs relative to WT plants, although they did show retarded shoot growth (Supplementary Fig. S3B, C). This observation strongly suggests that unlike the increased total SR weight, the enhanced SR formation in \textit{ibEXP1}-antisense plants is not affected by the increased shoot weight.

To date, little progress has been made in elucidating the molecular mechanism underlying SR development in sweetpotato. Although expression analysis studies have identified a number of genes possibly involved in SR development (You \textit{et al.}, 2003; Tanaka \textit{et al.}, 2005; 2008), further functional characterization of these genes has been hindered due to a difficulty in generating transgenic sweetpotato plants. Ku \textit{et al.} (2008) employed an alternative approach to characterize the role of \textit{IbMADS1} in SR development by generating \textit{IbMADS1}-ox potato plants. These authors observed that the FRs of \textit{IbMADS1}-ox potato plants cultured \textit{in vitro} were partially swollen and that the metaxylem cells had undergone enhanced multiplication. However, this phenotype was not confirmed in sweetpotato. Very recently, \textit{SRD1}-ox transgenic sweetpotato plants were successfully generated and it was demonstrated that \textit{SRD1} plays a role in the formation of SRs by activating the proliferation of cambium and metaxylem cells to induce the initial thickening growth of SRs in an auxin-dependent manner (Noh \textit{et al.}, 2010). This gain-of-function study, however, was performed with FRs from \textit{SRD1}-ox sweetpotato plants cultured \textit{in vitro}, but the proposed role of \textit{SRD1} was not verified in mature \textit{SRD1}-ox sweetpotato plants grown in soil. To date, researchers have been unable to verify whether a sweetpotato gene is directly involved in regulating SR development in soil-grown sweetpotato plants. As such, \textit{IbEXP1} is the first sweetpotato gene whose role in SR development has been directly identified in soil-grown transgenic sweetpotato. Not only can \textit{ibEXP1}-antisense plants be effectively used for elucidating the molecular mechanism involved in SR development, but, as a promising commercial cultivar, they have an increased productivity in terms of both the SR and shoot.

\section*{Supplementary data}

Supplementary data are available at JXB online.

- Figure S1. Nucleotide positions of \textit{ibEXP1}, \textit{ibEXP2}, and \textit{IbEXPL1}-specific primers.
- Figure S2. Transcript levels of \textit{IbEXP}2 and \textit{IbEXPL1} in \textit{IbEXP1}.
- Figure S3. Growth of \textit{ibEXP1}-antisense sweetpotato plants grown in soil.

\section*{Figure S4.} Phenotypic alteration in \textit{ibEXP1}-antisense sweetpotato plants grown in soil.

\section*{Acknowledgements}

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\section*{References}


