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Root cap specific expression of an endo-β-1,4-D-glucanase (cellulase): a new marker to study root development in *Arabidopsis*

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Abstract

The sloughing of root cap cells from the root tip is important because it assists the growing root in penetrating the soil. Using a promoter–reporter (GUS) and RT-PCR analysis, we identified an endo-β-1,4-glucanase (*AtCel5*) of *Arabidopsis thaliana* that is expressed exclusively in root cap cells of both primary and secondary roots. Expression is inhibited by high concentrations of IAA, both exogenous and internal, as well as by ABA. *AtCel5* expression begins once the mature tissue pattern is established and continues for 3 weeks. GUS staining is observed in both root cap cells that are still attached and cells that have already been shed. Using *AtCel5-GUS* as a marker, we observed that the root cap cells begin to separate at the sides of the tip while the cells of the central region of the tip separate last. Separation involves sequential tiers of intact cells that separate from the periphery of the root tip. A homozygous T-DNA insertion mutant that does not express *AtCel5* forms the root cap and sheds root cap cells but sloughing is less efficient compared to wild type. The reduction in sloughing in the mutant does not affect the overall growth performance of the plant in loose media. The modest effect of abolishing *AtCel5* expression suggests that there are multiple redundant genes regulating the process of sloughing of the root cap, including *AtCel3*/At1g71380, the paralog of the *AtCel5* gene that is also expressed in the root cap cells. Thus, these two endo-1,4-β-D-glucanases may have a role in the sloughing of border cells from the root tip. We propose that *AtCel5*, provides a new molecular marker to further analyze the process of root cap cell separation and a root cap specific promoter for targeting to the environment genes with beneficial properties for plant growth.

Abbreviations: ABA, abscissic acid; ACC, 1 aminocyclo-propane-1 carboxylic acid; CM, carboxymethyl; GUS, β-glucuronidase; MUG, 4-methylumbelliferyl-β-D-glucuronide; X-Glu, 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside; NPA, N-1-naphthylphthalamic acid ; SEM, scanning electron microscopy

Introduction

Endo-1,4-β-D-glucanases (EC 3.2.1.4) are a widespread group of enzymes that hydrolyze the β-1,4-glucosidic bond between two glucose moieties. These genes are thought to be important to basic plant development and are also referred to as cellulases. In *Arabidopsis*, the endo-1,4-β-D-glucanase family is composed of 25 members, of which approximately half are attributable to tandem duplication of genes and duplication of genome DNA segments. This relatively large family is comprised of many proteins with a putative signal peptide at the amino terminus (predicted to be

secreted) and a few proteins with a membrane spanning domain (predicted to be non-secreted) (del Campillo, 1999). Both secreted and non-secreted forms share the same catalytic amino acid signature toward the carboxy terminal end. This signature places all 25 genes into the Glycosyl Hydrolase family 9 (GH9), formerly known as cellulase family E (Henrissat, 1991; Henrissat and Bairoch, 1993; 1996). Thus, while the protein function of all members of this family is known (endo-1,4-β-D-glucanases), for most members, the role they play in plant development is unknown. In many plants, the secreted endo-1,4-β-D-glucanases (cellulases) have been correlated with processes that require progressive disassembly and breakdown of the cell wall, including fruit ripening, (Lashbrook *et al*., 1994), anther dehiscence (del Campillo and Lewis, 1992), vascular tissue differentiation (Milioni *et al*., 2001, 2002) and abscission of plant organs, (Tucker *et al*., 1988; del Campillo and Bennett, 1996; Gonzalez-Bosch *et al*., 1997).

In this study, we have focused on At1g22880, an *Arabidopsis* endo-1,4-β-D-glucanase that is predicted to be soluble and secreted. We initially isolated this gene as a genomic clone that cross-hybridized with the tomato abscission cellulase *Cel5* (del Campillo and Bennett, 1996; Kalaitzis *et al*., 1999), and thereby it was designated *AtCel5*. We analyzed the spatial and temporal expression of *AtCel5* using a promoter–GUS reporter approach and RT-PCR. Although we initially speculated that this gene would play a role in *Arabidopsis* flower abscission, we show here that it is expressed exclusively in root cap cells. *AtCel5-GUS* expression is distinct from other known promoter–reporter constructs that are specific to the root tip and thus provides a new molecular tool for studying root cap development and root cap cell–cell separation. We also identified a homozygous, T-DNA knockout of At1g22880, *cel5,* from the Salk collection, (Alonso *et al*., 2003) and we show that this mutant does not express *AtCel5*. Morphological characterization of wild type and mutant *cel5* failed to display distinct phenotypic differences at the whole plant level. However, a close examination of the root tip revealed that the mutant displays an increase in the retention of the root cap compared to wild type in response to friction and handling. Analysis of the duplicated segments of chromosome 1 of *Arabidopsis* revealed that the *AtCel5* gene is positioned in a segment of the upper arm, which is duplicated in the lower arm. The *AtCel5* duplicon (*AtCel3*/At1g71380) shares not only 81.9% sequence identity over the coding region but also the identity extends to the non-coding promoter region as well. Therefore, in addition to the characterization of *AtCel5*, we analyzed the expression of the *AtCel3*. We conclude that these two genes have redundant functions.

Materials and methods

Plant material and growth conditions

Arabidopsis ecotype Columbia was used in all the experiments described here. Seeds were surface-sterilized using 0.3% sodium hypochlorite for 5 min, rinsed in sterile water five times and plated on medium consisting of half-strength MS (Murashige and Skoog, 1962) basal salts (Sigma), 0.5 g/l 2-[N-Morpholino] ethanesulfonic acid (Sigma, St. Louis, MO, USA), pH 5.7, supplemented with 1× Gamborg's vitamins and solidified with 1% (w/v) plant tissue culture agar (Type E, Sigma). Additional filter-sterilized stock chemicals (1000×) were added to the warm agar mixture after autoclaving. Plated seeds were cold treated for 4 days at 4 °C and then plates were placed vertically in a growth chamber at constant 20 °C under 16-h light/8-h-dark regime. For the root cap retention assay, plates were sliced in the middle and one half of the agar media was removed. Seeds were plated on the ledge of the agar so that the roots were forced to grow vertically through the agar. For germination in dark conditions, plates were set in the same incubator and covered with two layers of aluminum foil. Seeds were also planted and grown to maturity on soil (Metro-Mix 300, Scotts Company) in a growth chamber at 20 °C under 16-h light/8-h-dark cycle.

Hormone experiments

To investigate the influence of indole-3-acetic acid (IAA; Sigma) plants were grown under liquid conditions as well as vertical plates. In all experiments the roots were separated from the shoots, frozen in liquid Nitrogen, and stored at −80 °C prior to analysis. Seedlings expressing a promoter–

reporter (*AtCel5-GUS*) were grown for 2 weeks in flasks containing 50 ml of half-strength MS media supplemented with 50 μM IAA (50 μl of a 50 mM solution in DMSO), with control consisting of the same volume of liquid media containing a comparable amount of DMSO. Seeds were cold treated for 4 days at 4 °C to promote even germination. Liquid cultures were placed on a rotary shaker at 120 rpm and grown under 24 h of fluorescent light at room temperature.

To determine the effects of auxin, and the IAA transport inhibitor, *N*-1-naphthylphthalamic acid (NPA; Pfaltz and Bauer, Inc.), seeds expressing *AtCel5-GUS* were grown in half-strength MS media solidified with agar and supplemented either with, 10 μM IAA, 1 μM NPA or a comparable amount of DMSO solvent (control). Plates were transferred to the light incubator for 10 days and set in a vertical position. The inhibitory effect of NPA on root gravity perception was examined by observing the direction of root growth in plates set in a horizontal position.

For analysis of ethylene regulation, seeds expressing *AtCel5-GUS* were plated on agar containing half-strength MS medium supplemented with 10 μM of 1 aminocyclo-propane-1 carboxylic acid (ACC; Sigma), made from a stock (1000×) prepared in water. After cold treatment for 4 days at 4 °C, plates were transferred to the light incubator for 7 days and set in a vertical position.

For analysis of the effects of ABA, a 100 mM ABA stock solution was prepared in 100% ethanol (13.22 mg ABA/0.5 ml) and then diluted 1000× in water. Seeds expressing *AtCel5-GUS* were plated on agar basal medium and grown for 7 and 10 days. Under sterile conditions, plates with seedlings were opened and 10 ml of 100 μM ABA was added to bathe the seedlings. Plates were then set horizontally in the light incubator for 24 h.

Promoter–reporter construction

To characterize the *AtCel5* promoter, a chimeric construct was generated by fusing a fragment of the putative promoter in frame with the GUS gene. The promoter was derived from an *Arabidopsis* 5 kb genomic clone that contained the gene. This clone was first restricted with *Xba*I/*Nco*l to generate a 3.2 kb fragment, containing the promoter plus the first exon of the *AtCel5* gene. The 3.2 kb fragment was then restricted with *Xmn*I in order to separate the first exon from the promoter. The fragment flanked by *Xmn*I restriction sites that contained 1400-bp of the putative promoter plus the 5′ UTR and the ATG translation start of ATCel5 was cloned upstream of the *GUS* gene in the pBI101 plant transformation vector that was opened at the *Sma*I site. A plasmid containing the promoter fragment in the sense orientation with respect to the *GUS* gene was selected based on endonuclease restriction digestion and confirmed by DNA sequence. The construct also contains the *NPT*II gene which confers kanamycin resistance and was delivered to wild-type plants *via Agrobacterium* transformation by the floral dip method (Bent, 2000). Seeds derived from transformed plants were selected in germination media containing kanamycin.

SEM microscopy

Two to 14 day-old seedlings were collected on ice and fixed in 4% (w/v) glutaraldehyde in 50 mM potassium phosphate buffer, pH 7.2 under vacuum overnight at 4 °C. After fixation, tissues were rinsed with buffer, dehydrated in an ethanol series, and dried in a critical point dryer in liquid carbon dioxide at the SEM facility (University of Wisconsin, Madison, WI, USA). Tissues were then mounted on scanning electron microscope stubs, coated with gold palladium, and examined using a scanning electron microscope (Hitachi S-570; Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV. Fifteen to 20 samples of each plant line were photographed using Gatan Digital capture system.

Histochemical GUS analysis

Plant tissues were collected in 90% acetone and incubated on ice for at least 10 min. Tissues were rinsed with 50 mM $NaPO_4$ pH 7.2, 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$ and then placed in staining solution (2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside [X-Glu; Sigma] in rinse solution), vacuum infiltrated for 10 min and finally incubated at 37 °C for 2 h or overnight. Seedlings were examined using a microscope and photographed with a Digital Nikon 990 Camera.

Fluorometric GUS analysis

Quantification of GUS activity was performed according to the method of Jefferson *et al.* (1987)

using the fluorogenic substrate MUG (4-methylumbelliferyl-β-D-glucuronide). Only root tissue was used to analyze GUS activity. Tissues were ground in MUG extraction buffer and centrifuged for 5 min, at maximal speed in a microfuge, to clear the supernatant. An aliquot of crude extract containing the same number of root tips per treatment was mixed with the MUG reaction buffer containing 2 mM MUG and incubated at 37 °C. Five or six aliquots were taken from the enzyme reaction at 60 min intervals. At each time point, the reaction was stopped with 0.2 M Na_2CO_3. Fluorescence was determined using a one-channel fluorometer (Turner Designs Picofluor) with an excitation range of 365–395 nm and emission wavelengths of greater than 430 nm. The fitted linear slope ($\pm$standard error) of fluorescence vs. incubation time was used as a relative measure of GUS content for comparison of controls and treatments.

RNA isolation and RT-PCR analysis

For tissue specificity studies, total RNA was isolated from 200 mg of different plant tissues (buds, green siliques, rosette leaves, stems and roots) as described in the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA). For each sample, a one step RT-PCR (Qiagen kit) was performed with 1 μg of total RNA for a total of 35 cycles following the recommendations of the manufacture. The primers (AtCel5 set I) used for these PCR reactions, Fw-5′-GATGCTG-GGGACAATGTGAA-3′, Rv-5′-ACGGCTCGGCTCGGGAGAGAGGAA-3′, were derived from the first exon and last exon, respectively. Thus, there was an approximate 400 bp size difference between the PCR product derived from the reverse transcribed mRNA and that derived from traces of genomic DNA present in the sample. Products were run on 1% agarose gels. PCR cycles included 1 min denaturation at 94 °C, followed by 1 min annealing at 48 °C and a final 2 min extension at 72 °C.

For comparative RT-PCR analysis of roots exposed to various treatments, or the analysis of expression of the *AtCel5* duplicon, a two-step RT-PCR was performed using the Retroscript kit (Ambion, Austin, Texas). Total root RNA was pretreated with DNAse prior to reverse transcription (Ambion DNA-*free* kit). The first strand cDNA was prepared with oligo dT primers and used as template for PCR reactions. RT-PCR was normalized using *Actin*-11 or *KOR* as internal standards. The primers used were: for *AtCel5 (set II)*, Fw-5′-AAGATCCTTCCAAATTCTCCATCC-TCGTCA, Rv-5′AAGAGCCAAAGATGGGCGTTTCTA-3′; for *KOR*, Fw-5′GGAAGGACGAGGAGAGGGAGATATAGTGCAGGCACTG-3′, Rv-5′GGATCTAGCAAAGTCACGTA GCACACT-TGTCGAATAG-3′; For *Actin*-11, Fw-5′-ATGGCAGATGGTGAAGACATTCA G-3′; Rv-5′-GAAGCACTTCCTGTG GACTATT GA-3′. For *AtCel3*, primers were Fw-5′-GATTCT CCTTCTT-CCTCTACCCAA-3′, Rv-5′-GTAATG ATGATGGTTAGAGTTAAATA-3′. Each PCR reaction was run for 25 cycles. Each cycle included a denaturation and an extension step at 94 °C for 45 s, and at 72 °C for 70 s, respectively. The annealing step was carried out for 30 s at the temperature optimal for each primer pair: *AtCel5 (set II)*, 58 °C; *KOR*, 62 °C; *Actin*-11, 60 °C; *AtCel3*, 65 °C.

Root length measurements

Approximately 20 seedlings were grown on vertical plates for 10 days in conditions as previously described. Seedlings and a ruler held adjacent to the seedlings were digitally photographed. Root length calculations were performed on the digital images using the NIH ImageJ software.

Results

*Cloning and basic At*Cel5 *information*

We initially isolated a lambda clone containing the *AtCel5* gene from an *Arabidopsis* genomic library that was probed with the tomato cellulase, *Cel5*. The tomato *Cel5* gene is expressed in tomato flower abscission (del Campillo and Bennett, 1996; Kalaitzis *et al.*, 1999). When the *Arabidopsis* clone was partially sequenced, we found that it matched a putative endo-1,4-β-D-glucanase gene in the BAC clone F19G10. The predicted amino acid sequence of the *AtCel5* gene is 484 aa long, with a molecular weight 54 kDa, pI 9.53 (GenBank Accession number AY075630, MIPS At1g22880; BAC F19G10.16). The gene is located on the upper arm of chromosome 1, positions 8,095,768 to 8,097,537 bp. The open reading frame of *AtCel5*

is composed of five exons interrupted by four short introns. The protein has a predicted 22 aa signal peptide indicating that the nascent polypeptide is imported into the endoplasmic reticulum and then secreted outside the cell. In addition, this protein appears not to be glycosylated as indicated by the absence of predicted ASN N-glycosylation sites (PS00001).

We also analyzed the 5′ flanking region upstream from the translation start site. This segment is 1400 nucleotides long, AT-rich (69% A + T; 31% C + G) and contains the motif TAACAA/GA and the CAAT boxes common to many actively transcribed plant genes. Based on the Plant *cis*-acting regulatory DNA elements (PLACE) signal database (Higo *et al*., 1999), the root motif ATATT (S000098) is repeated 13 times through the 1400 bp sequence. Moreover, in the 5′ upstream sequence there are two consensus sequences, GAGAGA and GAAAAAG, which have been found in genes up-regulated 30 min after gravistimulation (Moseyko *et al*., 2002) and two TACGTG elements that relate to drought and ABA response (Iwasaki *et al*., 1995). We also found the cis-acting regulatory elements CAGGTG and CACTTGT, which have been described as binding sites for basic helix-loop-helix transcription factors that regulate gene expression in drought and ABA responsiveness in *Arabidopsis* (Jaglo-Ottosen *et al*., 1998).

Tissue specificity and developmental regulation

Although the tomato *Cel5* gene is expressed in floral abscission zones, we found no expression of *AtCel5* in aerial plant tissues of *Arabidopsis* by northern blot analysis (data not shown). A search of EST databases revealed expression of this gene in a root cDNA library (GenBank Accession number AV540005). The specificity of gene expression to the root was confirmed by RT-PCR analysis (Figure 1). Only root RNA showed a PCR product of the expected size and the sequence of *AtCel5* cDNA.

To further analyze tissue and cell specific expression of *AtCel5*, a promoter–reporter fusion (*AtCel5-GUS*) was prepared between an upstream DNA segment (1400 bp) of the *AtCel5* gene and the *E. coli* β-glucuronidase (*GUS*) gene (Jefferson, 1987). The putative promoter fragment included also the 5′ UTR and the ATG translation start of

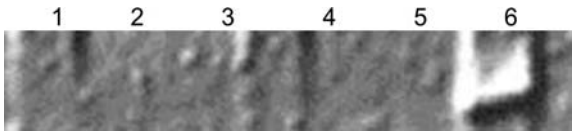

Figure 1. Detection of *AtCel5* mRNA in wild-type plants by RT-PCR using as template 1 μg of total RNA prepared from the following: flower buds (lane 1), green siliques (lane 2), leaves (lane 3), stem (lane 4), shoots (lane 5) and roots (lane 6). The forward and reverse primers were based on the sequence of the *AtCel5* first and last exons, respectively (Set I).

the *AtCel5* gene. Five independent lines resistant to kanamycin were selected and all displayed root-specific *GUS* expression. The expression was localized to the primary root apex (Figure 2A, B) and to the lateral root tips of young seedlings grown on agar plates (Figure 2C). Staining was seen throughout the outermost layer of the root tip (Figure 2B). GUS activity was also observed in root tips of plants grown in soil (data not shown).

To determine the onset of *AtCel5* expression during seedling development, GUS staining was monitored daily on seedlings growing in agar plates. There was no detectable *AtCel5-GUS* expression during the initial emergence of the primary root (Figure 2, E and F) suggesting that this gene is not linked to seed germination. We determined that *AtCel5-GUS* expression begins in the primary root tip around 30–48 h post-germination and continues for at least three weeks. Similarly, in lateral roots, *AtCel5-GUS* expression was not detected at emergence (Figure 2G) and only began once the roots were approximately 2 mm in length (Figure 2H), after the mature tissue pattern was established (Laskowski *et al*., 1995). Expression was initially localized to the cells in the center of the outermost layer of the root tip (Figure 2H). It is important to note that staining was not detected at initiation or elongation of root hairs (data not shown).

The expression of *AtCel5-GUS* is specific to the root cap (Figure 2I), in contrast with the expression of other genes that are specifically expressed in the root tip, such as DR-5 (Ulmasov *et al*., 1997) (Figure 2J) and mitotic cyclin CYCB1 (DiDonato *et al*., 2004) (Figure 2K). By comparison, DR-5 is expressed throughout the root tip (Figure 2J), whereas mitotic cyclin B1 expression is specific to the meristematic cells positioned behind the root cap (Figure 2K).

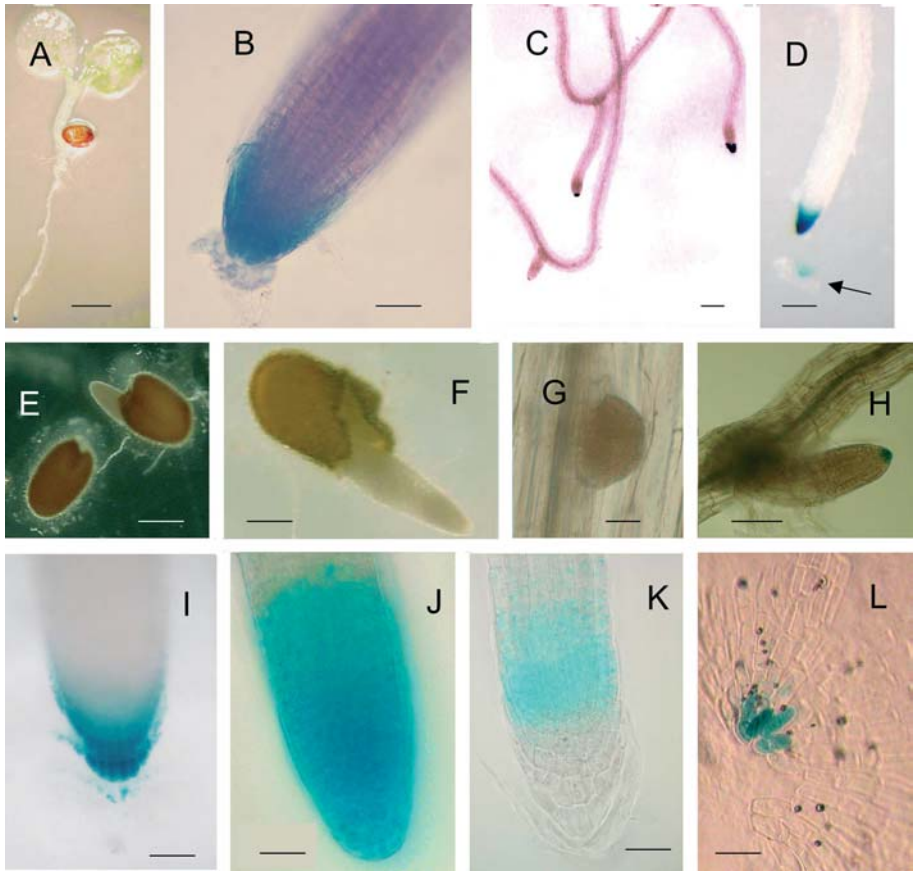

Figure 2. Transgenic *AtCel5-GUS* seedlings stained for GUS activity. (A) 2 day-old seedling. (B) Root tip of 10 day-old seedling. (C) 3-week-old seedling with abundant secondary roots. (D) Root from a 10 day-old seedling that was stained for GUS without removing the seedling from the plate. (E) Seeds 10–12 h post germination. (F) 24 h post germination. (G) Emerging secondary root. (H) Young secondary root around 2 mm in length. (I) Root of 5 day-old seedling with *AtCel5*-GUS transgene. (J) Root of 5 day-old seedling with *DR-5-GUS* transgene. (K) Root of 5 day-old seedling with *CYCB1-GUS* transgene. (L) Cells of a root cap layer recovered from the surface of the agar plates. Bars represent 1 mm in A, C–D , H and 50 μm in B, I, J–L.

When the GUS assay was performed on seedlings growing on agar plates without removing the roots from the media, stained material was detected on the surface of the agar (Figure 2D, black arrow). A microscopic examination of this material shows to be cells shed from the root cap (Figure 2L). When seedlings were grown in the dark, at 20 °C, *AtCel5-GUS* expression was detected in the root tip approximately 48 h post-germination, but was not present after 1 week. In the dark, root elongation is inhibited and we detected no change in root length between days 2 and 7. Nevertheless, when the etiolated seedlings were returned to the light, the seedlings started to green, elongation of the roots resumed and *AtCel5-GUS* expression at the root tips was restored (data not shown). Lastly, GUS staining was not detected in any aerial tissue of seedlings or mature flowering plants (data not shown). These data suggest that *AtCel5* is expressed exclusively in root cap cells, that expression is active as long as the root tips are growing and that *AtCel5* appears to be associated with the process of root cap separation, usually referred to as sloughing. The sloughing of root cap is a process of programmed cell–cell separation and hydrolase genes such as *AtCel5* are likely to be involved.

Characterization of Arabidopsis *root cap using* AtCel5 *expression*

Given the unique pattern of expression, we used *AtCel5-GUS* expression to examine root cap sloughing in *Arabidopsis*. Microscopic examination

of a shedding root tip revealed that separation involved several tiers of cells that remained intact as they separated from the root tip (Figure 3A). In Figure 3B, we show a close-up of the stained root tip after we attempted to detach a stained root cap manually with a thin brush. The root cap remained mostly intact and still attached to the center of the root tip, while the cells at the side of the root cap were detached. This would suggest that the side of the root cap loosens and separates first while the center of the root cap separates last. Note also that GUS staining was considerably less in the loosely attached root cap cells (Figure 3A) than in the cells still firmly adhered to the root (Figure 3B).

Root cap sloughing has been linked to the ability of the root to penetrate through the soil (Hawes, 1990). To investigate the effect of physical impediment on *AtCel5* expression, we grew seeds expressing *AtCel5-GUS* under conditions of increased resistance during growth by varying the agar content in the MS media. The plates were set at an angle that forced the roots to penetrate the agar. After 10 days of growth, RT-PCR failed to detect any significant changes in *AtCel5* transcript accumulation in total root RNA from seedlings growing in different concentrations of agar (Figure 3C). Yet, when *AtCel5-GUS* staining was performed in the agar plates, without removing the roots from the media, we detected many instances of root cap sloughing in the plates with the maximum percent agar (Figure 3D). We also detected carboxymethyl (CM)-cellulase activity in the agar plates where seedlings were growing. Seedlings were grown for 10 days in a media that was supplemented with 0.5% soluble CM-cellulose. Before performing the activity assay, seedlings were removed from the plates, and the imprinted agar was stained for CM-cellulose (0.2% Congo Red). CM-cellulase activity was distinguished as clear patches in the positions where seedlings had been growing (Figure 3E). While it is not clear which cellulase is producing this activity in the plates, this is consistent with our data above suggesting that *AtCel5* could be released to the media as cells are shed from the tip.

Expression of AtCel5-GUS *with exogenous auxin and* NPA

GUS activity was greatly reduced compared to controls (Figure 4A) when seedlings expressing

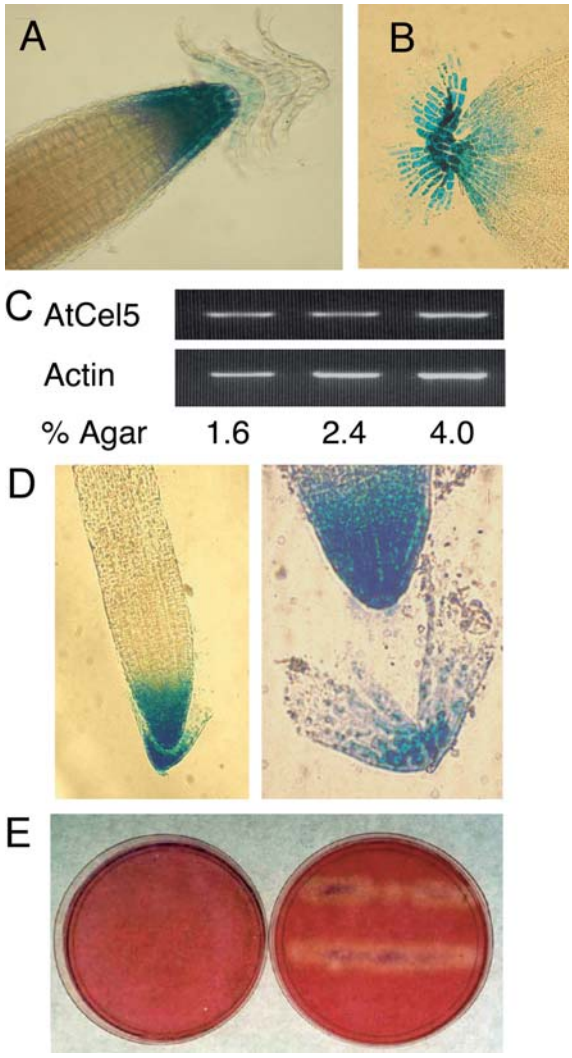

Figure 3. GUS activity staining performed on roots of transgenic *AtCel5*-GUS seedlings growing for 10 days on agar plates. (A) Root tip removed from the growth plate and stained showing sloughing of a root cap. (B) Root tip that was not removed from the growth plate prior to staining. The root cap was partly detached from the tip manually using a thin brush. (C) RTPCR analysis of *AtCel5* message in root RNA derived from wild type seedlings growing in half-strength MS plates containing increasing amounts of agar and oriented at an angle that forced the roots to penetrate the agar, top, *AtCel5*, bottom, *Actin-11* as internal reference. (D) Root tips of *AtCel5*-GUS seedlings growing in plates solidified with 4% agar showing examples of root cap cells at different stages of separation. (E) CM-cellulase activity detected with Congo Red in plates containing the basal MS agar supplemented with 0.5% soluble CM-cellulose. A 0.2% Congo Red solution was added to the media before *Arabidopsis* seeds were plated, control, (left plate) and after seeds germinated and grew for one week in light (right plate). Seedlings were removed prior to addition of Congo Red.

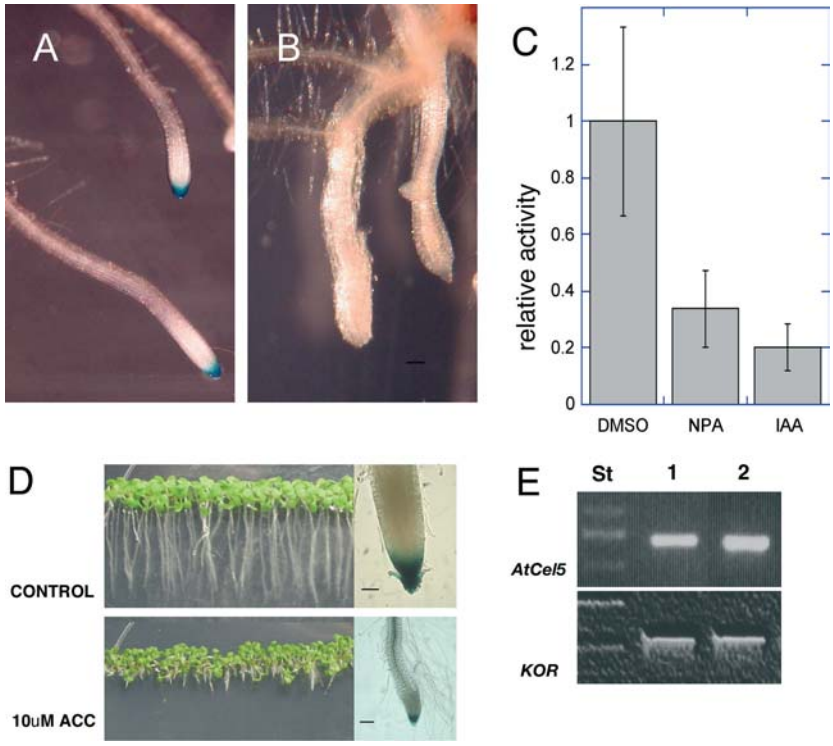

Figure 4. Auxin regulates negatively *AtCel5* expression and is independent of ethylene. (A) Transgenic *AtCel5-GUS* plants growing on liquid basal media supplemented with DMSO, or (B) basal media supplemented with 50 μM IAA dissolved in DMSO, (C) fluorometric analysis of GUS activity in roots of transgenic *AtCel5-GUS* plants grown in plates set horizontally, containing basal media supplemented with DMSO (Control), 1 μM NPA dissolved in DMSO (NPA), and 10 μM IAA dissolved in DMSO (IAA). Vertical bars represent the standard error of the fitted linear slope of fluorescence vs. incubation time (D) transgenic *AtCel5-GUS* seedlings growing in half-strength MS basal medium, control, or medium supplemented with 10 μM ACC, and stained for GUS activity (right panels), control (top) vs. 10 μM ACC (bottom). Horizontal bars represent 50 μm in control and 100 μm in ACC. (E), RT-PCR of RNA extracted from the roots of seedlings growing in control plates (lane 1) and plates with 10 μM ACC, (lane 2) that was amplified with *AtCel5* gene specific primers (top gel) or *KOR* gene specific primers (bottom gel).

AtCel5-GUS were grown on liquid MS containing IAA (50 μM). Moreover, the roots in treated plants were deformed, bulging and seemed to lack a root cap (Figure 4B). When seedlings were removed from the high auxin media and transferred to an auxin-free media, *AtCel5-GUS* expression was again detected in the tips after a few days (data not shown).

To determine if *AtCel5* expression was related to the internal IAA concentration, we tested the effect of NPA, a compound that generates an increase in the level of endogenous IAA at the tip by inhibiting the basipetal transport of IAA (Casimiro *et al*., 2001) and also abolishes the root gravitropic response (Jensen *et al*., 1998). Thus, when growing on horizontal plates the primary root of control seedlings penetrated the agar while the roots of seedlings growing in plates supplemented with 1 μM NPA grew along the surface of the agar (data not shown). Quantitative determination of GUS activity showed a large decrease in expression in the roots of seedlings grown in agar plates supplemented with 1 μM NPA or 10 μM IAA compared to controls (Figure 4C). Thus, high internal concentrations of IAA caused by the inhibition of basipetal auxin transport by NPA and exposure to high exogenous IAA concentrations negatively affect *AtCel5* expression.

AtCel5 *is ethylene independent*

To determine if *AtCel5* expression is affected by ethylene, seedlings were grown for 10 days on MS plates containing 10 μM ACC (which is constitutively converted to ethylene). In the presence of this high concentration of ACC, roots were

significantly shorter, more branched and had a considerable increase in root hair density compared to seedlings grown in control plates (Figure 4D). Despite these pronounced morphological changes, GUS staining was still observed in the root cap in both cases (Figure 4D). Similarly, RT-PCR showed no significant changes in *AtCel5* transcript between roots of control and ACC treated seedlings (Figure 4E) when normalized to the transcript level of *KOR,* a membrane endo-1,4-*β*-D-glucanase that is highly expressed in roots and not regulated by ethylene (Nicol *et al.*, 1998). After 10 days on ACC plates, growth was stalled and *AtCel5-GUS* expression disappeared, however, growth resumed and staining reappeared at the tips when seedlings were transferred to fresh media without ACC (data not shown).

AtCel5 *is down-regulated by exogenous ABA*

Previous studies by (Brigham *et al.*, 1998) have shown the essential role of water in border cell separation; and thus, we examined the effect of water stress on root cap sloughing. During water stress the internal content of ABA increases (Zeevaart and Creelman, 1988), and thus it is possible to mimic aspects of the response to water stress by exposing plant tissues to high exogenous ABA concentrations. Table 1 includes the results of two independent experiments where GUS activity was quantified fluorometrically and compared between control and ABA treated seedlings. In both experiments, *AtCel5* expression was reduced by almost half after 24 h ABA treatment.

Table 1. AtCel5-GUS expression is modulated by ABA.

	Relative activity[1]	
	Experiment number 1	Experiment number 2
Control roots	1.00 ± 0.06	1.00 ± 0.04
ABA roots	0.53 ± 0.04	0.42 ± 0.06

[1]Transgenic *AtCel5-GUS* seedlings at 7 days post-germination (experiment #1) and 10 days post germination (experiment #2) were overlaid for 24 h with either 10 ml of sterile water (control) or 10 ml of 100 μM ABA solution as described in Materials and methods. Roots were collected and analyzed fluorometrically for GUS activity. The activity (fitted slope ± standard error) in roots of ABA treated seedlings is expressed relative to the activity in controls.

T-DNA knockout mutant for AtCel5

A T-DNA insertion mutant line for the *AtCel5* gene was obtained from the Salk collection (Alonso *et al.*, 2003) http://signal.salk.edu/cgi-bin/tdnaexpress. In this line, the T-DNA is inserted in the *AtCel5* coding region, 160 bases downstream from the last exon (Figure 5A). Since this is the exon that bears the catalytic signature (position 210 bp), it was expected that the insertion would either completely abolish *AtCel5* gene expression, or at least, interrupt gene expression to generate a shorter transcript and an inactive enzyme. Using a gene-specific primer and a T-DNA border primer, a homozygous *cel5* mutant was identified. Total RNA from *cel5* and wild-type roots, (10 days post-germination) were used as templates for RT-PCR. No *AtCel5* transcript was detected in *cel5* (Figure 5B). Nevertheless, when grown in soil, *cel5* displayed no observable differences with wild-type plants in terms of overall plant height, size, seed production and response to gravity. We also measured the root length of wild-type and mutant seedlings (7 days post-germination) growing in MS agar media (control) or media supplemented with 4% mannitol or 100 mM NaCl which negatively affects root growth. Both wild type and mutant responded to the treatment but showed no significant differences in their responses (data not shown).

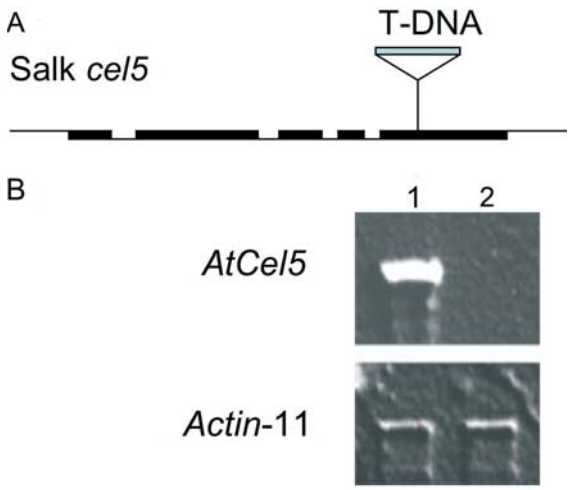

Figure 5. (A) Diagram of SALK-079921 illustrating the T-DNA insertion in the last exon. (B) RT- PCR from total RNA derived from roots of wild type (lane 1) and homozygous *cel5* mutant (lane 2), using *AtCel5* gene specific primers (top panel) and *Actin*-11 gene specific primers (bottom panel).

AtCel5 *and* AtCel3 *are paralogs*

A large segment of the upper arm of chromosome 1 of the *Arabidopsis* genome, which includes the *AtCel5* gene, is duplicated in the lower arm of the same chromosome (http://wolfe.gen.tcd.ie/athal/dup). The paralog of the *AtCel5* gene in the duplicated region was identified as *AtCel3*/At1g71380, GenBank Accession number U17888, BAC F26A9-24. The amino acid alignment (Corpet, 1988) of both genes showed 87.4% identity and after removing the predicted signal peptide the identity increased to 89.1% (Figure 6A).

To detect *AtCel3* expression by RT-PCR, gene specific primers were designed and tested for specificity using *AtCel5* cDNA (AV540005) and genomic DNA as templates. The *AtCel3* primers amplified the expected size fragment on genomic DNA template (Figure 6B, lane 1) but amplification on *AtCel5* cDNA template failed (Figure 6B, lane 2). In contrast, *AtCel5* primers amplified the expected fragments in both templates (Figure 6B, lanes 3 and 4). Once the specificity was confirmed, *AtCel3* expression was analyzed in wild-type *Arabidopsis* roots by RT-PCR. Results indicated that *AtCel3* is also expressed in roots (Figure 6C, lane 2), but at a slightly lower level of transcript accumulation than *AtCel5* (Figure 6C, lane 1).

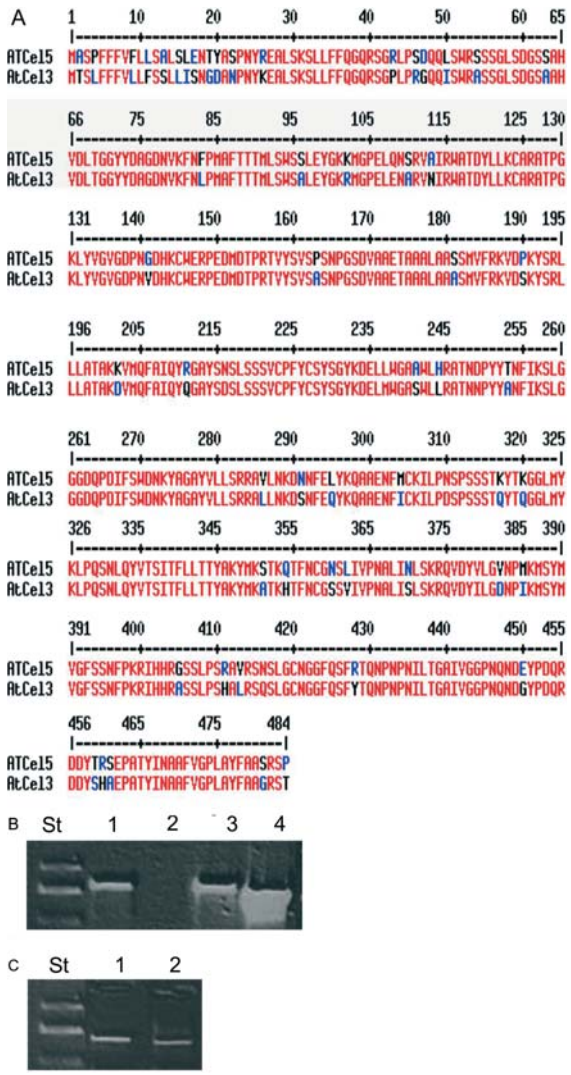

Figure 6. (A) Alignment of the amino acid sequence of *AtCel5* and its duplicon *AtCel3* (Corpet, 1988) showing the strong sequence identity shared between them. High consensus color is red, low consensus is blue and no consensus is black. (B) Analysis of PCR products using as template wild type genomic DNA (lane 1 and 3), and *AtCel5* cDNA (AV54005) (lane 2 and 4). The primers used for PCR were *AtCel3* gene specific (lane 1 and 2) and *AtCel5* gene specific (lane 3 and 4). (C) RT-PCR analysis of total RNA from wild-type roots that was reverse transcribed and then amplified by PCR with *AtCel3* gene specific primers (lane 1) and *AtCel5* gene specific primers (lane 2).

Root cap retention assay

Since expression of *AtCel5-GUS* is specific to the root cap, we compared the root cap cells of mutant and wild-type plants. After microscopic examination of many root tips from mutant and wild-type plants, we noticed that the root of the mutant forms a root cap and sheds root cap cells just like the root of wild type but sloughing appeared to be less efficient in the mutant. Therefore, we analyzed the retention of the root cap cells after removal of roots from the agar media. Seedlings were grown for 10 days on agar blocks oriented so that the roots were forced to penetrate the agar. At this point the root had grown more than 1 cm into the agar. After 10 days, the seedlings were pulled straight up from the agar and the root tips were examined microscopically. In wild-type root tips, the stress resulting from the removal from the agar usually detached the root cap (66% of cases) or left few tiers of cells loosely attached at the center of the tip (33% of cases). In contrast, the root cap was usually still attached to the root tip of the mutant (69% of cases). Similar results were obtained using a slightly different approach where seedlings of wild type and mutant were lifted from a paper substrate. Seedlings were grown using a

modification of the vertical mesh technique (VMT) described by Murphy and Taiz (1995). The modified VMT consisted of a paper (GB002, Schleicher and Schuell)-glass plate assembly, without the nylon membrane, mounted vertically within a Magenta jar containing 50 ml of liquid growth medium. Sterile seeds were set on a row 1 cm below the top of the wet paper and germinated under the same light conditions as the agar plates. After 10 days, the paper-glass plate assembly was set horizontally on the bench, seedlings were lifted from the paper and the root tips were examined microscopically. Using this technique, which resulted in a more gentle pulling, we observed that the wild type had only a few tiers of root cap cells loosely attached at the center of the tip and the sides of the cap were flaring out (Figure 7A), whereas the mutant retained the root cap in most cases (Figure 7B).

In addition, we analyzed the root tips of both control and mutant by scanning electron microscopy, which required sequential ethanol washes and high pressure critical drying prior to the observation. The high-pressure treatment usually resulted in complete removal of the root cap in the wild type (Figure 7C), but some material was often observed (> 50% cases) in SEMs of the *cel5* mutant root tips (Figure 7D). These results indicate that there is more sloughing in the wild type compared to *cel5* mutant (Figure 7A and B).

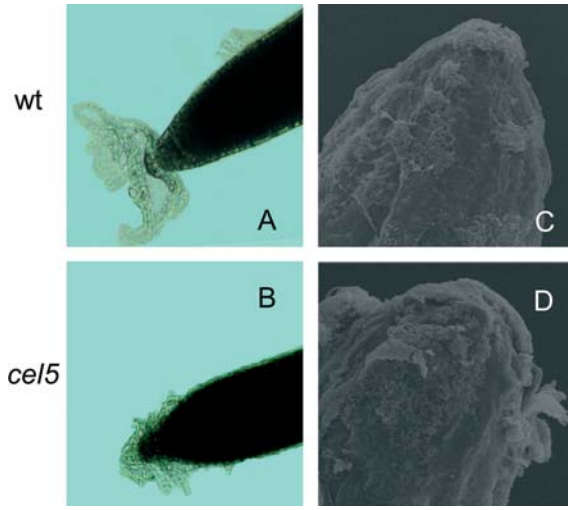

Figure 7. Comparison of the retention of root cap layers in wild type (wt) and *cel5* mutant. Root tips of (A) wild type 10 day-old seedlings lifted from VMT plates and observed on transmission microscopy. (B) As in A except *cel5* mutant. (C) root tip of a wild type 10 day-old seedling as observed by SEM. (D) root tip of a *cel5* 10 day-old seedling as observed by SEM.

Discussion

The family 9 of glycosyl hydrolases (endo-1,4-β-D-glucanases) constitutes a group of enzymes that can hydrolyze internal linkages in 1,4-β-glucan substrates. The most conspicuous β-1,4 glucan present in plants is cellulose, which is the most important structural component of the cell wall. In addition, other more complex glucan polymers bearing β-1,4 linkages are also found in the cell wall. These polymers are secreted outside the plasma membrane and organized into a complex cell wall matrix.

Most plants contain in their genome multiple sequences coding for the family 9 glycosyl hydrolases (GH9). In *Arabidopsis*, this family consists of 25 members and comprises a few membrane proteins that could be anchored at the plasma membrane and a large number of proteins with a predicted amino acid signal in the N terminus that would direct secretion to the cell wall. Although the specific glucans that this family acts on in the plant cell wall are not known, it is generally believed that the secreted members are important in processes that entail cell wall disassembly.

Here, we analyzed the expression of one member of the *Arabidopsis* GH9 family. This putative hydrolase, predicted to be soluble and secreted, was initially isolated from an *Arabidopsis* genomic library that was probed with the tomato cellulase, *Cel5*. Since the tomato *Cel5* gene is expressed in tomato flower abscission (del Campillo and Bennett, 1996; Kalaitzis *et al*., 1999), we initially expected the *Arabidopsis AtCel5* gene to be expressed in *Arabidopsis* flower abscission. What we discovered was quite different and surprising. There was no *AtCel5* expression in abscission zones or any other aerial tissue of mature plants. Instead, expression was strong and exclusive to the root cap cells. Consistent with these results, a search of EST databases revealed expression of this gene only in root cDNA libraries (http://mpss.udel.edu/at/). Moreover, our analysis of the *Cel5* promoter region indicated the presence of cis-motifs common to root specific genes. *AtCel5* expression starts at the very tip of the root cap cells approximately 30–48 h post-germination and

continues for at least 3 weeks. Expression was not linked to growth processes such as the emergence of roots from seeds, the initiation of lateral roots or root hairs, or the elongation of roots.

Root cap cells are ultimately shed from the plant tips and GUS activity was detected not only in the root cap cells that were still attached to the root, but also in cells that had already been shed. Thus, this transgenic *Arabidopsis*, with GUS expression exclusively in the root cap cells, provides a new molecular marker to further analyze the process of root cap cell separation. A xylogalacturonan (XGA) epitope that is specific to detaching cells (Willats *et al*., 2004) has already been identified in several plant species. The use of these markers should stimulate research to specifically address root cap cell separation in *Arabidopsis*.

The sloughing of root tips is primarily a process of cell–cell separation that results in the shedding of living cells with modified cell walls. These cells then degenerate to contribute the mucilaginous material around the root tip (Hawes, 1990). The whole process is likely to require an ensemble of hydrolytic enzymes. Recent evidence indicates that in pea roots (Wen *et al*., 1999), a pectin methyl esterase gene is important for root cap cell separation. The results from this work suggest that *AtCel5* is also involved in this process, either during cell–cell separation or cell–wall breakdown after shedding to provide polysaccharide precursors for mucilage production or both.

Our microscopic observations revealed that in *Arabidopsis*, the root cap cells begin to separate at the sides of the tip while the cells of the central region of the tip separate last. Separation involves sequential tiers of intact cells that separate from the periphery of the root tip. Since *AtCel5* begins to accumulate in the central region of the root tip, this would suggest that the root cap separation process involves more than the expression of *AtCel5*. Nonetheless, an additional factor to consider is that as the root meristem develops and the root extends and expands, the shearing forces experienced by the peripheral cells might be greater than the cells positioned directly at the tip. These forces may thereby accelerate separation in the peripheral cells.

Our description is consistent with observations of bean root border cells that also separate as intact, metabolically active root cells, and eventually degenerate to contribute the mucilaginous material at the root tip (Hawes, 1990; Hawes *et al*., 1998; Miyasaka and Hawes, 2001). To our knowledge, this is one of the first detailed descriptions of the root cap separation process in *Arabidopsis*. Root cap sloughing has been linked to the ability of the root to penetrate through the soil (Bengough and Mckenzie, 1997; Iijima *et al*., 2003). Consistent with this, we detected more stained root cap cells in the process of separation from the root tip on plates containing 4% agar that imposed more physical impediment as well as osmotic stress. Although this observation suggests there was more root cap separation, RT-PCR did not detect an increase in the *AtCel5* transcript levels as the percent agar increased in the medium. It is possible that the RT-PCR was not sensitive enough to detect these differences. An alternative interpretation could be that the increase in stained root cap cells was the result of greater retention or appression of the root cap cells around the root tip periphery.

We also found that high auxin and ABA concentration both negatively regulate *AtCel5* expression. High exogenous or internal auxin had an inhibitory effect on root elongation and ultimately arrested root growth. Similarly, the addition of ABA, which mimics some aspects of the water stress responses, inhibited *AtCel5-GUS* expression. Moreover, sequence analysis of the *AtCel5* promoter region identified several *cis*-acting elements involved in regulating gene expression in response to ABA, drought and dehydration. These experiments showed that when root elongation stopped, *AtCel5-GUS* expression ceased, as was also observed in seedlings growing in high external ACC concentration or etiolated seedlings kept for 1 week in the dark. These observations suggest that *AtCel5* is associated with active root cap growth, more specifically, with processes such as the initial loosening and ultimate sloughing of cells of the root cap.

In addition to the above, we identified a homozygous *cel5* knockout mutant plant and confirmed by RT-PCR that the *AtCel5* mRNA was not expressed. Although the *cel5* mutant appeared phenotypically the same as wild type under normal growth conditions, a more detailed comparison of root cap tissue indicated that there is more root cap sloughing in the wild type compared to *cel5* mutant. The reduction in sloughing

in the mutant apparently did not affect the overall growth performance of the plant in loose media. This would account for the lack of obvious phenotypic differences. The modest effect of abolishing *AtCel5* expression suggests that there are multiple redundant genes regulating the process of sloughing of the root cap, or that overall growth performance is only significantly affected by the rate of sloughing for plants cultivated on compacted soil (cf. Iijima *et al.*, 2003). With regard to the former, we identified in the *Arabidopsis* genome a duplicon of *AtCel5*, which is referred to as *AtCel3*/At1g71380. A cDNA for this paralog was initially isolated from *Arabidopsis* cells growing in suspension cultures and referred to as a β-glucanase (Lu and Ferl, 1995). A proteomic analysis of cell wall proteins in *Arabidopsis* confirmed that this gene is abundantly expressed in cell suspension cultures (Borderies *et al.*, 2003). A recent conference report described expression of *AtCel3* in abscission zones of *Arabidopsis* flowers (Thoma *et al.*, 2003) and thus it's role in the root could be overlapping with *AtCel5,* while distinct in abscission zones.

The *AtCel3* and *AtCel5* genes have the same number of amino acids (484 aa), same gene organization (five exons and four introns in the same relative position), and share a 89.1% amino acid identity. At the nucleotide level, both CDSs have 81.9% identity and the whole unspliced sequence shares 75.5% identity. Moreover, the sequence identity between *AtCel5* and *AtCel3* extends 1000 bp upstream from the ATG. Consistent with such promoter similarities, we found that the *AtCel5* duplicon is also expressed in roots. In a recent work that mapped the expression of genes in distinct root cell types in *Arabidopsis* by microarray (Birnbaum *et al.*, 2003), both genes were shown to be expressed in the epidermal cells of the root cap region (LED 5, stage 1). Consistent also with our data, Birnbaum *et al.*, demonstrated that the expression of both genes drops precipitously in stages 2 and 3 (Birnbaum *et al.*, 2003 supplemental material). Therefore, we believe that both genes *AtCel5* and *AtCel3* are expressed in root cap cells and both are involved in root cap sloughing. In addition, we propose that the significant levels of CM-cellulase activity that was detected in the media in which *Arabidopsis* seedlings were growing is most likely resulting from *AtCEL5* and *AtCEL3* activity in the root cap. Our data support the model that *Arabidopsis* root elongation and root cap cell separation are concomitant processes and that separation requires *AtCel5* and *AtCel3* expression. In order to establish the role of each gene in root cap separation, it will be necessary to inhibit both *AtCel5* and *AtCel3* and these efforts are currently underway.

We believe that more detailed analyses may demonstrate that the plant's ability to shed cells at the root cap is necessary to survive certain environmental conditions. The function of shedding the root cap is unknown; however, it has been suggested that the root cap could provide a selective advantage to the plant by releasing specific chemicals that regulate root-associated microorganisms (Hawes, 1990; Hawes *et al.*, 1998). Future studies of root cap development will be aided by using *AtCel5-GUS* expression as a specific marker of root cap cells. In addition, the root cap specificity of the *AtCel5* promoter provides a new tool for targeting to the environment genes with beneficial properties for plant growth.

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