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Differential Expression of Proteins and mRNAs from Border Cells and Root Tips of Pea[1]

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Many plants release large numbers of metabolically active root border cells into the rhizosphere. We have proposed that border cells, cells produced by the root cap meristem that separate from the rest of the root upon reaching the periphery of the cap, are a singularly differentiated part of the root system that modulates the environment of the plant root by producing specific substances to be released into the rhizosphere. Proteins synthesized in border cells exhibit profiles that are very distinct from those of the root tip (root cap, root meristem, and adjacent cells). In vivo-labeling experiments demonstrate that 13% of the proteins that are abundant in preparations from border cells are undetectable in root tip preparations. Twenty-five percent of the proteins synthesized by border cells in a 1-h period are rapidly excreted into the incubation medium. Quantitative variation in levels of specific marker proteins, including glutamine synthetase, heat-shock protein 70, and isoflavone reductase, also occurs between border cells and cells in the root tip. mRNA differential-display assays demonstrate that these large qualitative and quantitative differences in protein expression are correlated with similarly distinct patterns of gene expression. These observations are consistent with the hypothesis that a major switch in gene expression accompanies differentiation into root border cells, as expected for cells with specialized functions in plant development.

Many plants can produce large numbers of metabolically active root "border" cells, which are programmed to separate from each other and be released from the root cap periphery into the external environment (Hawes, 1990; Hawes and Lin, 1990; reviewed by Hawes and Brigham, 1992). Experimentally, border cells are defined as those cells that can be released into suspension by a brief immersion of the root tip into water (Hawes and Brigham, 1992). In the absence of free water, the separated cells remain adhered to the root tip. Border cell separation is a self-delimited process that is regulated in response to developmental and environmental signals (Hawes and Lin, 1990). The number of cells produced by a single root can vary by orders of magnitude among different species, but cell number is conserved at the family level (Hawes and Pueppke, 1986). The function of border cells (previously referred to as "sloughed root cap cells") and their impact on plant growth and development are unknown (Hawes and Brigham, 1992).

Most literature on root function in the rhizosphere attributes three functions to the root cap: protection of the root meristem, sensing of gravity, and lubrication of the root through the soil by way of mucilage production and "sloughing" cells (Sievers and Hensel, 1991). More than 50 years ago, Rogers et al. (1942) pointed out that data supporting a role for sloughed cells in lubrication are lacking. This remains true (Hawes and Brigham, 1992). Rogers advanced the alternative hypothesis that such cells constitute an "extraroot" digestive system (Rogers et al., 1942) that functions as an exoenzyme system releasing substances such as phosphatases into the rhizosphere.

Several properties of border cells are consistent with the hypothesis that they have the capacity to protect plant health by conditioning the environment of the growing root tip (reviewed by Hawes and Brigham, 1992). Depending on the genotypes of the plant and microorganism, border cells can specifically attract or repel bacteria (Hawes and Pueppke, 1989) or produce papillae in response to infection by pathogenic fungi (Sherwood, 1987). Of particular interest to the current study are observations of border-cell-specific properties. Border cells appear to lack several phenotypes exhibited by cells of the root proper. For instance, certain enzyme activities present at high levels in the root cap are undetectable upon differentiation of progenitor root cap cells into border cells (Hawes and Lin, 1990; Stephenson and Hawes, 1994). In other cases, border cells exhibit phenotypes that are not shared by the rest of the root. Zoospores of several plant pathogenic fungi are specifically attracted to border cells (Goldberg et al., 1989) but not to the root per se, suggesting that border cells are a source of biologically active chemicals not found in other cells of the root. One explanation for such observations is that border cells constitute a specialized component of the root system that carries out functions distinct from those of other root tissues.

A prediction of the hypothesis that border cells become uniquely differentiated when they separate from the root cap is that proteins and mRNAs made by border cells will be distinct from those made by progenitor cells in the root cap. The objectives of this study were to compare the gene products made by pea (*Pisum sativum* L.) root border cells

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Abbreviations: GS, glutamine synthetase; HSP70, heat-shock protein 70; IFR, isoflavone reductase.

with those of the intact root tip; to compare the levels of three marker proteins that are found in roots—GS, HSP70, and IFR—using immunoblot analysis; and to relate the differences in protein profiles by comparing differences in gene expression using mRNA differential display.

MATERIALS AND METHODS

Plant Material

Seeds of *Pisum sativum* L. cv Little Marvel (Royal Seed Company, Kansas City, MO) were surface sterilized by immersion in 95% (v/v) ethanol for 10 min, then in 5.25% sodium hypochlorite (full-strength commercial bleach) for 30 min. During five rinses in sterile, distilled water, contaminated seeds (those that floated) were discarded, and the remaining seeds were placed on 1.2% water agar overlaid with sterile germination paper (Anchor Paper Co., Hudson, WI) in plastic Petri dishes and incubated in the dark at 24°C.

Release of Border Cells

Border cells were isolated from the root tips of seedlings when the radicle was 2.5 cm long. The root tip was immersed in 2 to 5 mL of sterile, distilled water, which was agitated to release the cells into suspension (Hawes and Pueppke, 1989). Border-cell preparations were assayed for microbial contamination by plating samples onto plates with solidified Luria broth (10% [w/v] tryptone, 5% [w/v] yeast extract, 5% [w/v] NaCl); any samples that developed bacterial or fungal colonies were discarded.

Protein Extraction

All protein extractions were performed on tissue taken from seedlings whose roots were 2.5 cm in length. Border cells were collected as described above. Root tips were excised 1 mm from the apex. All tissue was homogenized in microfuge grinder tubes (Kontes, Vineland, NJ) in SDS extraction buffer (4% SDS, 5% 2-mercaptoethanol, 5% Suc) as described by Colas des Francs et al. (1985). Samples were centrifuged for 20 min at 10,000*g*. Proteins were precipitated from the supernatant with acetone at −20°C overnight and then resuspended in sample buffer (60 mM Tris-HCl, pH 8.8, 60 mM DTT, 2% SDS, 15% Suc, 5 mM amino-*N*-caproic acid, 1 mM benzamidine, 0.01% bromphenol blue) and stored at −20°C. Protein content was measured according to the Coomassie blue dye-binding procedure combined with scanning densitometry (Ghosh et al., 1988).

In Vivo Protein Labeling

Radioactive labeling of proteins was carried out by incubating intact seedlings, with border cells still adhering to the tip, on moist filter paper saturated with 500 to 600 mCi of [^{35}S]Trans-Label (85% Met, 15% Cys; >1000 TBq/mmol; ICN Biomedicals, Costa Mesa, CA) for 1 h. Border cells were then released into sterile, distilled water and proteins were extracted from the cells and from the root tips as described above. Alternatively, border cells and root tip proteins were labeled separately as follows. Border cells from 60 root tips were released in 0.5 mL of 1% Suc to which 500 to 600 mCi of [^{35}S]Trans-Label was added. The apical 0.5 cm of 20 intact roots from which the border cells had been removed were immersed in 100 μL of 1% Suc to which 80 to 90 mCi of [^{35}S]Trans-Label was added. Both border cells and roots were incubated at room temperature for 1 h and extracted as described above. Incorporation of radioactivity into protein during in vivo labeling was measured by the method of Mans and Novelli (1961). The eukaryotic translational inhibitor cycloheximide was added at a concentration of 50 μg/mL to a set of controls.

Protein Secretion

Incubation medium from the in vivo-labeling experiments was retained. Proteins were precipitated from the medium with acetone at −20°C overnight, resuspended in sample buffer, and subjected to electrophoresis under the same conditions as the SDS-soluble proteins.

Gel Electrophoresis

One-dimensional SDS-PAGE was carried out essentially according to Laemmli (1970). Protein samples suspended in sample buffer were separated in 12.5% acrylamide gels or on a linear gradient gel of 5 to 20%. Two-dimensional PAGE of the protein samples was carried out according to O'Farrell (1975). Proteins were resolved by IEF in the first dimension. Electrophoresis was carried out at 400 V for 16 h and 800 V for 2 h. The IEF tube gels were equilibrated in SDS sample buffer and stored at −20°C. Second-dimension SDS-PAGE was performed as above. Proteins were stained using the Silver Stain Plus kit (Bio-Rad). After fluorography, radioactive gels were exposed to XAR-5 film (Eastman-Kodak, Rochester, NY).

Immunoblot Analysis

Immunoblotting of gel-separated proteins was performed at 30 V for 16 h at 4°C. The polyclonal antisera to bean GS, pea HSP70, and pea IFR were gifts of Malcom Bennett (University of Warwick, Coventry, UK), Elizabeth Vierling (University of Arizona, Tucson, AZ), and Geza Hrazdina (New York State Agricultural Experiment Station, Cornell University, Geneva, NY), respectively. Antiserum to the different proteins was used at 1:500 dilutions. Immune complexes were detected by a colorimetric assay using the Immune-Blot Assay Kit (Bio-Rad).

mRNA Differential Display

mRNA differential display (Liang and Pardee, 1992) was used to compare mRNA patterns of root border cells with those of cells in the root tip. First-strand cDNA was synthesized from either 100 ng of poly(A)$^+$ mRNA or 200 ng of total RNA by SuperScript reverse transcriptase (GIBCO-BRL). Total RNA was treated with RNase-free DNase I (Ambion Inc., Austin, TX) to remove chromosomal DNA contamination. Poly(A)$^+$ mRNA was isolated using the

PolyATtract mRNA isolation system (Promega). First-strand cDNA synthesis was primed by one of the $T_{12}MN$ primers (M stands for G, C, or A but not T, and N is one of the four deoxynucleoside triphosphates). A portion of this first-strand reaction was used for PCR amplification with sets of the arbitrary 10-mer primer for the 5′ end and the same $T_{12}MN$ primer for the 3′ end. PCR was performed with *Taq* DNA polymerase (exonuclease-free) (Boehringer Mannheim) and [α-^{35}S]dATP for 40 cycles at 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s and a 5-min extension at 72°C. The amplified PCR products were size-fractionated on a 6% denaturing PAGE gel for 4 h. After drying, the gel was exposed to XAR-5 film. Each set of experiments was repeated three times with different batches of RNA samples.

RESULTS

Proteins from Pea Root Tips and Pea Root Border Cells

Each root tip yielded approximately 3 mg of tissue (fresh weight) and 131 mg of SDS-soluble protein. Border cells from a single root (2500–3500 cells) weighed 0.5 mg (fresh weight) and yielded 1.26 mg of SDS-soluble protein (Table I). To determine if significant differences in protein populations from pea root tips and root border cells could be detected, several approaches were used: SDS-soluble proteins were separated on one- and two-dimensional PAGE; newly synthesized proteins were compared using one- and two-dimensional PAGE; and levels of specific marker proteins were compared using immunoblot analysis.

Total Proteins in Border Cells and Root Tips

The protein profiles of root tips and border cells were very different, both qualitatively and quantitatively, when analyzed by SDS-PAGE based on size (Fig. 1A). The total number of bands was greater in the root tips, and there were distinct differences in the band patterns between the two cell populations. At least 10 bands were apparent in root tips that were not present in border cells (two examples are shown in Fig. 1A, arrows labeled "t"). On the other hand, at least three bands seen in the border-cell preparations were not apparent in the root-tip preparations (Fig. 1A, arrows labeled "b"). When SDS-soluble proteins were resolved in two dimensions (data not shown), the differences were even more apparent. Approximately 300 silver-stained root-tip proteins and 200 border-cell proteins were resolved by two-dimensional PAGE.

Newly Synthesized Proteins in Root Tips and Border Cells

To determine the differences among proteins synthesized at a particular time by the two tissues, the incorporation of [^{35}S]Met and [^{35}S]Cys was assayed in root tips and in border cells after their removal from the root tips. After 1 h of incubation with label, root tip proteins incorporated 640,000 cpm per root tip (4,872 cpm/μg protein). Border-cell proteins incorporated 16,000 cpm per border cell in a single root tip (12,730 cpm/μg protein) (Table I). On a total protein basis, the border cells incorporated more than 2.5 times the amount of label into new proteins as the root tips. As was found with the silver-stained proteins, newly synthesized proteins in the root tip and border cells were very different when separated by SDS-PAGE (Fig. 1B). At least

Table I. *Physical and biochemical characteristics of pea root tips and pea border cells*

Data are from comparisons of silver stained proteins, in vivo-labeled proteins, and mRNA species.

Characteristic	Border Cells/Root Tip	Root Tip
Amount of tissue	2,500–3,500 cells/tip	1 mm of apex
Tissue weight	0.5 mg	2–4 mg
SDS-soluble protein	1.26 ± 0.67 mg	131 ± 49.61 mg
(after cycloheximide)	1.25 ± 0.42 mg	150 ± 35.36 mg
Protein separation—silver stained SDS-PAGE		
Total bands	approx. 35	approx. 50
Unique bands	approx. 3	approx. 10
IEF/SDS-PAGE		
Total polypeptides	approx. 200	approx. 300
Unique polypeptides	approx. 5	ND[a]
Labeled proteins		
Incorporation of label (in 1 h of incubation)	16,000 ± 5,000 cpm	640,000 ± 300,000 cpm
(after cycloheximide)	2,805 ± 1,587 cpm	3,851 ± 1,295 cpm
SDS-PAGE		
Total bands	approx. 39	approx. 44
Unique bands	approx. 7	ND
IEF/SDS-PAGE		
Total polypeptides	approx. 150	approx. 200
Molecular mass range	20–100 kD	20–190 kD
Majority molecular mass	20–43 kD	30–68 kD
Majority pI	6.5–7.5	6.5–7.5
Unique polypeptides	approx. 20	ND

[a] ND, Not determined.

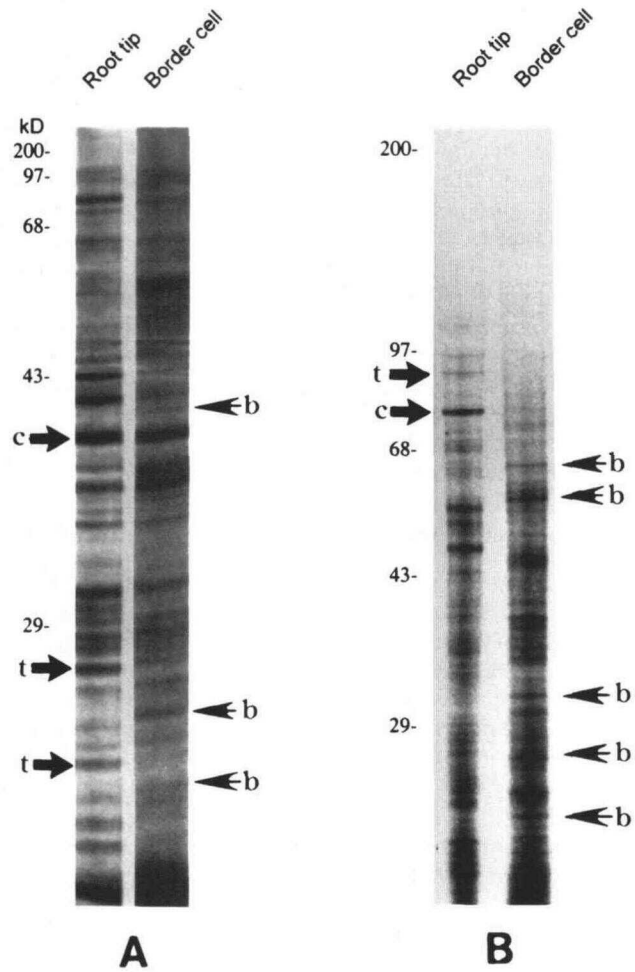

Figure 1. Gel electrophoresis of SDS-soluble proteins from pea root tips and pea root border cells. A, Ten micrograms of protein separated by 12.5% SDS-PAGE and silver stained. B, In vivo [^{35}S]Met-labeled proteins separated by 5 to 20% SDS-PAGE. b, Bands unique to border cells; t, bands unique to root tips; c, bands common to both.

five polypeptides present in the border-cell preparations were not detectable in preparations from root tips (Fig. 1B, arrows labeled "b").

When two-dimensional SDS-PAGE was used to compare proteins synthesized by border cells and root tips within 1 h after release, a marked difference in profile between the two populations was observed (Fig. 2). Differences in numbers of proteins, size range, and pI have been tabulated (Table I). As expected, many proteins were common to both root tips and border cells (see, for example, Fig. 2, A and B, arrows) However, at least 20 proteins (13%) can be distinguished only in border-cell preparations (three examples are shown by solid triangles in Fig. 2B). In addition, many proteins seen in root tips were not visible in border cells (see, for example, Fig. 2A, open triangles)

This distinct border cell profile was detectable even when border cells continued to adhere to the root tip, before they were released into suspension (data not shown). When border cells were released and allowed to incubate in water for several hours, the border cell profile was maintained (data not shown). Throughout the time course, up to 6 h, the profile of the proteins from root tips remained constant, and the border-cell protein profile remained distinct from it. Viability of the border cells was 90 ± 5% immediately after collection. Viability remained at this level for at least 16 h, indicating that, although dead cells existed at the start of the experiments, there was not a measurable amount of additional cell death for the duration of the experiments.

Cycloheximide treatment at the time of incubation in [^{35}S]Met and [^{35}S]Cys resulted in no significant change in total protein amount, which remained at 1.25 mg per border cell from a single tip and 150 mg for 1 mm of root tip. Incorporation of label, however, was decreased by 99% in root tips and by 83% in border cells (Table I).

Protein Secretion

Root tips are known to contain secretory cells. To determine which, if any, proteins were secreted from the root border cells, the incubation medium was analyzed by two-dimensional PAGE. Many peptides identifiable in border-cell profiles are also present in the extracellular medium (Fig. 2C, solid triangles). Of the total proteins synthesized in 1 h by border cells, 25% were found extracellularly.

Expression of Marker Proteins

To determine the ability of border cells to express specific root proteins and to determine if the levels of expression differed from those in the root tips, expression of known proteins in root-tip and border-cell samples were compared by immunoblot analysis (Fig. 3). GS, one of the enzymes involved in assimilation of ammonia-nitrogen by plants and found in root plastids (Bennett and Cullimore, 1989), was found in the SDS-soluble proteins of both the pea-root-tip and pea-border cells. On a protein weight basis (μg SDS-soluble protein) the root tip had substantially more GS. HSP70, a protein expressed constitutively and in response to heat shock, was found at a much higher level in root tips than in border cells (Fig. 3, arrow). It is interesting that the HSP70 antibody cross-reacted with an unidentified protein in the border-cell samples that was not in the root-tip samples. IFR (Sun et al., 1991), an enzyme involved in plant stress responses, was present in equal amounts (per μg of protein) in both root tips and border cells.

mRNA Comparison by Differential Display

mRNA differential display (Liang and Pardee, 1992) was used to test whether a change in transcripts produced by border cells corresponds to the observed change in proteins. This method uses distinct PCR primers to divide total mRNA populations into subsets small enough that individual mRNAs can be distinguished from each other when separated on PAGE. mRNAs expressed in two or more tissues are compared by running their reverse-transcribed PCR products on PAGE in adjacent lanes. This assay was

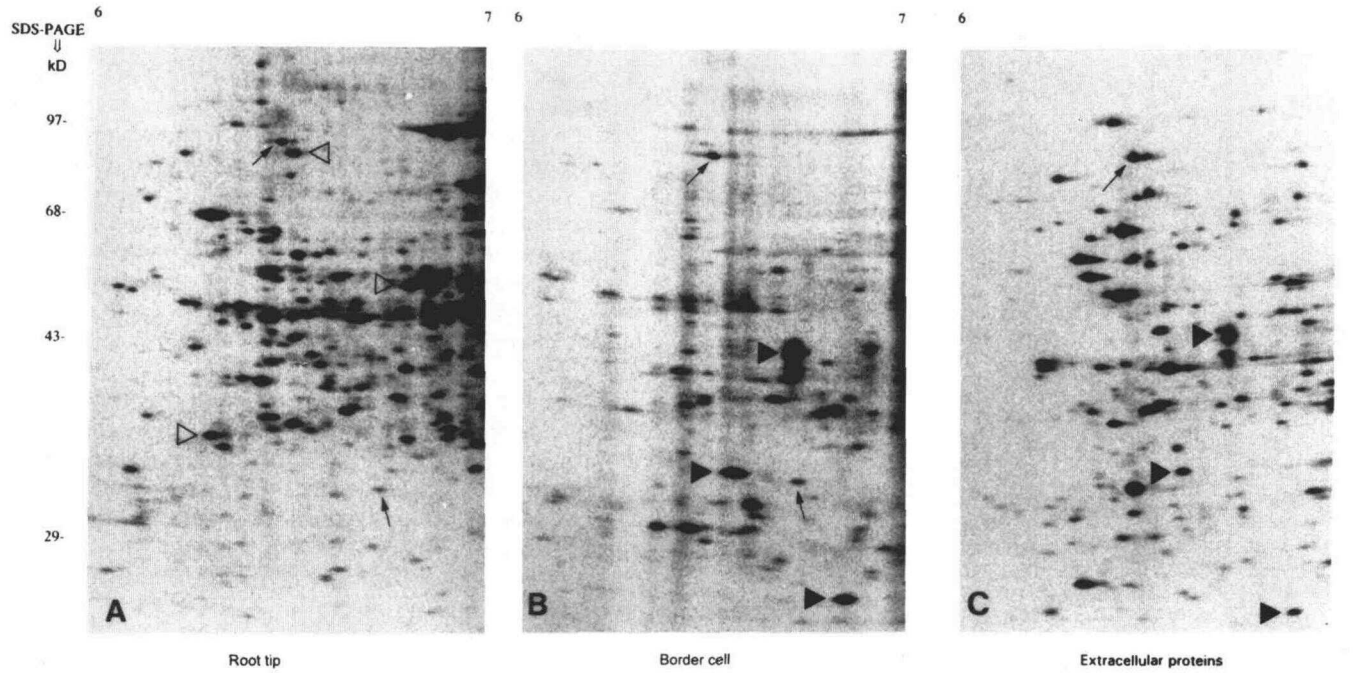

Figure 2. Fluorographs of in vivo [^{35}S]Met-labeled proteins separated by two-dimensional gel electrophoresis employing IEF in the first dimension and 5 to 20% gradient SDS-PAGE in the second dimension. A, Proteins from root tips; B, proteins from border cells; C, extracellular proteins synthesized by border cells. Open triangles indicate polypeptides seen only in root-tip samples; closed triangles indicate polypeptides seen only in root border-cell samples. Arrows indicate two polypeptides observed in both samples.

used to compare profiles of mRNAs from leaves, stems, whole roots, root caps, and root border cells (Fig. 4). Twenty primer sets were used, and 50 to 100 mRNA species were detected with each set of PCR amplification. Profiles of mRNAs from leaves, stems, and roots exhibited small differences, but overall profiles were very similar to each other (Fig. 4A). To assess how accurately differential display patterns reflect tissue-specific expression of the mRNA, several bands, representing different expression patterns, were cloned, sequenced, and analyzed by northern blot and RNase protection. One of the bands appeared in all tissue at an equal band intensity (Fig. 4B, solid arrowhead). The cDNA was found to have >80% sequence homology to tomato and Arabidopsis ubiquitin-conjugating enzyme (Woo et al., 1994). Results of northern analysis and RNase protection showed the message to be equally expressed in all tissue, consistent with the results from mRNA differential display gels. Comparable results were obtained for a differentially expressed band, H1 histone (Woo et al., 1995). Using the differential display cDNA clone as a probe, two H1 histones were identified, one with 100% homology to a previously identified pea H1 histone, PsH1b (Gantt and Key, 1987), and one to a new pea H1 histone sequence that has 59.1% homology to PsH1b (Woo et al., 1995). Both were shown by RNase protection to be differentially expressed in several pea tissues in a manner comparable to their deduced expression in the mRNA differential display gels. In contrast to the uniformity of different tissues, border cells were markedly different from root tips by mRNA differential-display banding patterns; four examples of border-cell-specific bands are illustrated with arrows in Figure 4, B and C. At least 1 to 2 mRNA changes were observed in border-cell RNAs from each set of PCR primers. Because 50 to 100 bands were visible from each set of PCR primers, this suggests that a change of at

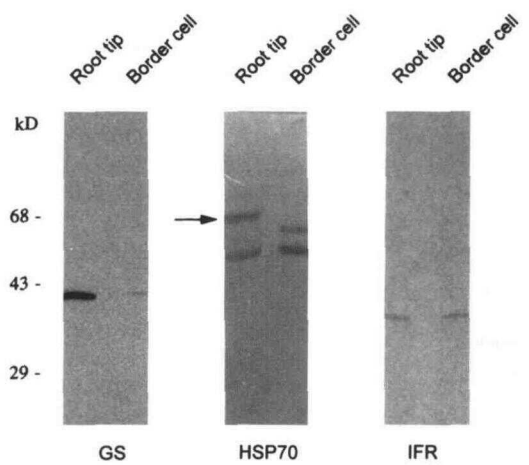

Figure 3. Immunoblot analysis of GS, HSP70, and IFR in pea-root tips and pea-root-border cells. Ten micrograms of total protein were prepared as described in "Materials and Methods" and subjected to SDS-PAGE and immunoblot analysis. The arrow indicates the position of the HSP70 band. Two other bands show cross-reaction with the polyclonal antiserum.

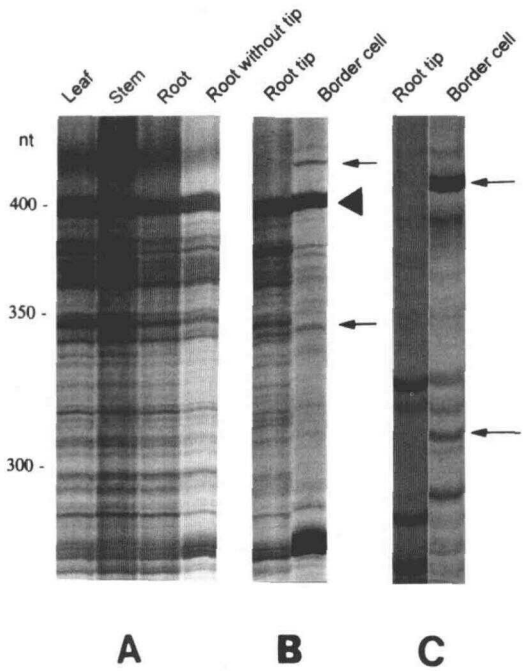

Figure 4. mRNA differential display showing results using one set of PCR primers. Single-strand cDNAs were synthesized from 200 μg of total RNA, PCR amplified, and size-fractionated on 6% urea denaturing PAGE as described in "Materials and Methods." Expression patterns of mRNAs in leaf, stem, root, and root without the tip are quite similar (A). However, comparison of root-tip and root-border cells show many qualitative and quantitative differences in the band patterns using two different sets of primers (B and C).

least 1 to 2% of the poly(A)$^+$ mRNA populations occurs during border-cell development.

DISCUSSION

The root cap of plants is a dynamic, multifunctional tissue. Cells of the cap originate by mitosis within the root-cap meristem; the cells then progress through a number of distinct developmental stages in which they participate in processes including starch synthesis, gravity sensing, and mucilage secretion (Rougier, 1981). These stages are distinguishable both morphologically and physiologically (Moore and McClelen, 1983). Such changes in function are likely to be associated with substantial changes in gene expression, but little is understood about the molecular events underlying cap differentiation. Separation of root border cells from the periphery of the cap constitutes the final stage of root-cap differentiation. The data presented here reveal that the unique morphology of border cells—a population of single cells external to the root—is associated with a marked switch in protein synthesis. Upon differentiation from root cap cells into border cells, the cells cease making one population of proteins and begin synthesizing another. The magnitude of the change is more pronounced than those that occur with other known major switches in cell function. For example, during a 24-h period in which pea lateral buds switch from a state of dormancy to active growth, approximately 0.7% of the proteins are detected uniquely in the active state (Stafstrom and Sussex, 1988). After the switch from root cap to border cells, which is complete within 24 h, at least 13% of the proteins made by border cells are detectable only in border cells. In contrast, protein changes that occur in different stages of root development are most often quantitative (Allan and Trewavas, 1989). The simplest explanation for the observed changes in protein profiles is that, upon differentiation into border cells, a signal is perceived that results in a large change in gene expression. The observation that changes in protein profiles are correlated with similarly holistic changes in mRNA profiles is consistent with this hypothesis.

How the functions of border cell proteins differ from those made by the root cap is unknown, although overall profiles vary based on protein number, size, and charge. The most striking change between the two cell populations is that many of the proteins synthesized by border cells appear so rapidly in the external medium. This is especially noteworthy, given that cells of the root cap themselves are known to secrete large amounts of material, including proteins (reviewed by Curl and Truelove, 1986; Narváez-Vásquez et al., 1993). Whereas 2% of the newly synthesized protein in the cap appears extracellular during the 1-h test period, 25% of the new border-cell proteins are released into the external medium. One explanation for such differences is that the detached border cells in suspension are more prone to nonspecific cell leakage due to stress or general loss in viability. Given that border-cell viability remains unaltered for many hours after the test period, this explanation does not seem likely. Indeed, the health of the border cells is best illustrated by the fact that they incorporate labeled amino acids into protein 2.6-fold more efficiently than do the cells of the root cap.

That the root cap of some species produces cells that can live apart from the root for extended periods has been known for many years (Knudson, 1919), and a possible role for the cells in extracellular enzyme production was proposed 50 years ago (Rogers et al., 1942). The results of this study reveal that differentiation into border cells is associated with large changes in protein expression, which is controlled at least in part at the level of transcription. Correlated with changes in gene expression are an increase in efficiency of protein synthesis and a high rate of extracellular protein extrusion. The results are consistent with the hypothesis that border cells constitute a uniquely specialized tissue of the root system whose function is to modulate properties of the growing root tip by releasing proteins and other special chemicals into the external environment.

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