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Short communication

Cloning of genes whose expression is correlated with mitosis and localized in dividing cells in root caps of *Pisum sativum* L.

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Abstract

Removal of border cells from pea roots synchronizes and induces root cap cell division, wall biogenesis and differentiation. Three messages which are expressed differentially in such induced root caps have been cloned. Sequence analyses showed that the *PsHRGP1*-encoded protein has high homology with a hydroxyproline-rich glycoprotein. The *PsCaP23*-encoded protein has high homology with an alfalfa callus protein or translationally controlled human or mouse tumor protein P23. The *PsRbL41*-encoded protein has high homology with a highly basic 60S ribosomal protein L41. *In situ* hybridization showed that *PsHRGP1*, *PsCaP23* and *PsRbL41* messages are localized within dividing cells of the root cap. *PsHRGP1* is highly expressed in uninduced root caps, but its message is repressed by 10–11 times as soon as cell division and differentiation begin. Expression of *PsHRGP1* recovers to higher than (180%) its initial level in 30 min. *PsHRGP1* is root-specific. *PsCaP23* and *PsRbL41* messages increase ca. 3-fold within 15 min after root cap induction. All three genes represent small families of 3–5 closely related genes in the pea genome.

Introduction

Root caps have long been recognized as an excellent system to study cell structure and function, because their final differentiated state is well defined and easily accessible [23]. Root caps, attached to the apices of roots, protect the apical meristem, serve as gravisensing tissue, and have been proposed to open a passage for the growing roots by producing root cap mucilage [23]. Gravity sensing statocytes renewed by meristematic cell divisions develop and are transformed into secretory cells, which produce mucilage before they finally reach the periphery of root cap. The terminal step in root cap development is the complete separation of peripheral root cap cells as they differentiate into border cells [3, 4]. The time required for a single cell to proceed through the root cap depends on the size of the root cap, the species, and undefined environmental variables [2, 4, 12].

Border cell production is elicited by an unknown signals(s), which sequentially induces transcription of genes involved in root cap cell differentiation [3]. The process of border cell production is tightly regulated, such that when a species specific maximum number of cells accumulates outside the root cap periphery, cell production ceases [12]. In *Pisum sativum*, this set number of border cells is ca. 3500 [4, 12]. The process can be experimentally induced and synchronized from plant to plant by removing the existing border cells, either by immersing the root tip in water or by gently wiping the cells from the root tip surface [3, 12]. This results in a ca. 400% increase in mitosis in cells of the root cap meristem within 30 min; after 2 h, presum-

The GenBank, EMBL and DDBJ accession numbers for *PsHRGP1*, *PsCaP23* and *PsRbL41* messages and deduced amino acid sequences are U78952, L47968 and L47967, respectively.

ably when ca. 3500 new cells have been made, mitosis returns to preinduction levels. Within 1 h new border cells can be collected from the root tip [3]. Concomitant with induction of renewed border cell production is a switch in gene expression that can be detected, using mRNA differential display, within 15 min after removing border cells; mRNA profiles continue to change sequentially for at least 60 min after border cells have been removed [3]. We exploited this system to test the hypothesis that a change in expression of specific genes occurs in parallel with cell division and/or wall biogenesis in root caps. The experimental approach was to screen a cDNA library of 'induced root caps', excized from the root during the period of maximum cell division. Genes induced or repressed at this early stage of root cap cell differentiation have been cloned and their expression patterns confirmed by *in situ* hybridization and northern blot analysis. Sequence analyses showed that they encode a hydroxyproline-rich glycoprotein, a callus protein P23, and a highly basic 60S ribosomal protein L41, and that their expression is localized within dividing cells of the root cup [25].

Cloning of messages whose expression is correlated with induction of cell division, wall biogenesis and differentiation in root caps

Removal of border cells in pea root tips initiates a new round of mitosis, cytokinesis, wall biogenesis, differentiation, and border cell production until a full complement of border cells is restored after 20–24 h [3, 4, 25]. Based on Barlow's model for root cap development [2], together with observed molecular responses to its experimental induction [3], we predicted that there was a high probability that changes in expression of genes required for cell division and/or cell wall biogenesis would occur as mitosis peaks at 30 min to 1 h after induction. A cDNA library of mRNAs from root tips induced in 30–60 min was made and screened to isolate differentially expressed messages. After screening 2×10^5 pfu of an unamplified cDNA library, three messages which showed differential expression within 30 min, *PsHRGP1*, *PsCaP23* and PsRbL41, were cloned [25].

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           VPHPVYSPPK KPYKYS          16
SPPPPVPTPY VPHPVYSPPK KPYKYS          42
SPPPPVPTPY VPHPVYSPPK KPYKYS          68
SPPPPVPTPY VPHPVYSPPR KPYKYS          94
SPPPPVPSPY IPHPVYSPPK KPYKYS         120

SPETLEKMPF KRFVEIGRVA LINYGKDYGR     150
LVVIVDVIDQ TRALVDAPDM ERSPINFKRL     180
SLTDLKIDIK RVPKKKDLIK ALEAADVKNK     210
WAKSSWGRKL IVKKTRAALN DFDRFKIMLA     240
KIKRAAGVRQ ELAKLKKTAA *              260
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Figure 1. Repeating motifs of *PsHRGP1*-encoded protein.

Primary structures of PsHRGP1*,* PsCaP23 *and* PsRbL41

PsHRGP1*: hydroxyproline-rich glycoprotein*
PsHRGP1 cDNA has 55–60% homology with a hydroxyproline-rich glycoprotein gene of *Phaseolus vulgaris* [22] and soybean [13]. At the amino acid level, the *PsHRGP1*-encoded protein has 40–50% homology with extensins of carrot [5], or tobacco [16, 18]. In *PsHRGP1* cDNA, the termination codon occurs at the position +1172 to +1174, followed by a 3′-untranslated sequence consisting of about 250 nucleotides. A consensus recognition sequence [8] for addition of the poly(A) tail is missing. The poly(A) tail was also found at the 3′ end of the cloned cDNA. RNA blot analysis of the total RNA from pea root tips leads us to estimate the length of the *PsUDPGT1* mRNA to be ca. 2 kb. The partial sequence of *PsHRGP1*-encoded protein has four repeating motifs of 'S-$(Hyp)_4$'. However, it also has five repeating motifs of 'VPTPYVPHPVYSPPKKPYKYS' after every 'S-$(Hyp)_4$' (Figure 1). According to Kieliszewski and Lamport's classification [14], these repeating motifs belong to the dicot P1 type. Hydropathy shows that the encoded protein is hydrophilic (not shown). Efforts to get the full length sequence of *PsHRGP1* were not successful. Rescreenings of a cDNA library and 5′-RACE (rapid amplification of cDNA end) were not successful in obtaining the full-length sequence of *PsHRGP1*.

PsCaP23*: callus protein*
PsCaP23 mRNA has 75–85% homology with a gene encoding alfalfa callus protein [19], or translationally controlled human or mouse tumor protein P23 [6, 11]. The *PsCaP23* mRNA has 70 nucleotides of untranslated region at the 5′ end before the translation initiation codon. The translation initiation codon starts at the position +71. The termination codon occurs at the position +632 to +634, followed by a 3′-untranslated sequence consisting of about 60 nucleotides. A con-

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         10        20
MRAKWKKKRMRRLKRKRRKMRQRSK      PsRbL41
|||||||||||||||||||||||||
MRAKWKKKRMRRLKRKRRKMRQRSK      Cotton RL41
|||||:|||||||||||||||||||
MRAKWRKKRMRRLKRKRRKMRQRSK      Human HG12
|||||:||| |||||||||:|:|||
MRAKWRKKRTRRLKRKRRKVRARSK      Yeast YL41
         10        20
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Figure 2. Comparison of ribosomal protein L41 amino acid sequences between *PsRbL41*, cotton RL41, human HG12, and yeast YL41. A bar (|) represents absolute matches of each sequences. A colon (:) represents conservative subsitutions between these sequences and *PsRbL41*.

sensus recognition sequence [8] for addition of the poly(A) is missing. The poly(A) tail was also found at the 3′ end of the cloned cDNA. RNA blot analysis of the total RNA from pea root tips leads us to estimate the length of the *PsCaP23* mRNA to be ca. 0.9 kb. The length of the cDNA clone here appears to represent a full-length copy of the mRNA. Its encoded polypeptide has 187 amino acids with a relative molecular weight of 21 314 and isoelectric point of 4.59. *Ps*CaP23 polypeptide has two highly conserved consensus sequences which were also found in alfalfa callus protein [19] or translationally controlled human tumor protein P23 [6, 11]. These two consensus sequences are; I-G-A-N-P-S-A-E-G-G-X-D-X-X-X-D-D at residues between +45 and +61, and L-F-X-G-E-X-M-X-D-X-G-X-L at residues between +129 and +149. It has also a putative *O*-glycosylation site (N-X-S) on amino acid residue 48. Hydropathy shows that the encoded protein is hydrophilic with a strong hydrophobic region at the C-terminal end (not shown).

PsRbL41*: 60S ribosomal protein L41*

PsRbL41 has high homology with a gene encoding a highly basic 60S ribosomal protein HG12 of human [15] and 60S ribosomal protein YL41 of yeast [17]. Its encoded polypeptide has 25 amino acids with a relative molecular weight of 3428 and is a highly hydrophilic protein (not shown). It is rich in lysine (32% mol/mol) and arginine (36% mol/mol). The *PsRbL41*-encoded protein has 100% identity with 60S ribosomal protein L41 of cotton [24]. Out of 25 residues there is only one amino acid difference between *PsRbL41*-encoded protein and human 60S ribosomal protein HG12 (Figure 2). However, if conservative substitutions [7] are included, they have 100% similarity. The *PsRbL41* mRNA has a 54 nucleotides of untranslated region at the 5′ end before the translation initiation codon. The translation initiation codon starts at the position +55. The termination codon occurs at position +130 to +132, followed by a 3′-untranslated sequence consisting of about 400 nucleotides. A consensus recognition sequence [8] for addition of the poly(A) is missing. The poly(A) tail was also found at the 3′ end of the cloned cDNA. RNA blot analysis of the total RNA from pea root tips leads us to estimate the length of the *PsRbL41* mRNA to be ca. 0.5 kb. The length of the cDNA clone here appears to represent a full-length copy of the mRNA.

Expression of PsHRGP1*,* PsCaP23 *and* PsRbL41 *mRNAs is localized within the same region of the root cap*

Whole mount *in situ* hybridization showed that *PsHRGP1*, *PsCaP23* and *PsRbL41* messages were all highly expressed only in cells of the columella, within the root cap transverse meristem [2], in patterns that were virtually superimposable (Figure 3A, B and C). No detectable expression occurred in columellar cells distal to the transverse meristem, or within peripheral layers of the cap.

Expression of PsHRGP1*,* PsCaP2 *and* PsRbL41 *changes dramatically within minutes after inducing root cap cell differentiation by removing border cells*

Northern analysis showed that all three messages exhibit differential expression in response to signals controlling root cap differentiation leading to border cell production; within minutes, *PsHRGP1* was repressed by an order of magnitude, whereas *PsCaP23* and *PsRbL41* were induced by several-fold. *PsHRGP1* message was repressed 10–11 times in 5 min, and recovered to higher (180%) than the initial expression level in 30 min (Figure 4A and C). The levels of *PsCaP23* and *PsRbL41* messages were increased as early as in 5 min after induction of cell differentiation in root caps (Figure 4A and C) and reached maximum level (ie. ca. 3 times higher than its initial expression in uninduced root caps) within 15 min after induction. The differences were large enough to be readily detectable by *in situ* hybridization analysis (Figure 3A, B and C). Expression of all three messages had returned to the same levels found in uninduced root caps by 30 min after removing border cells (Figure 4A and C).

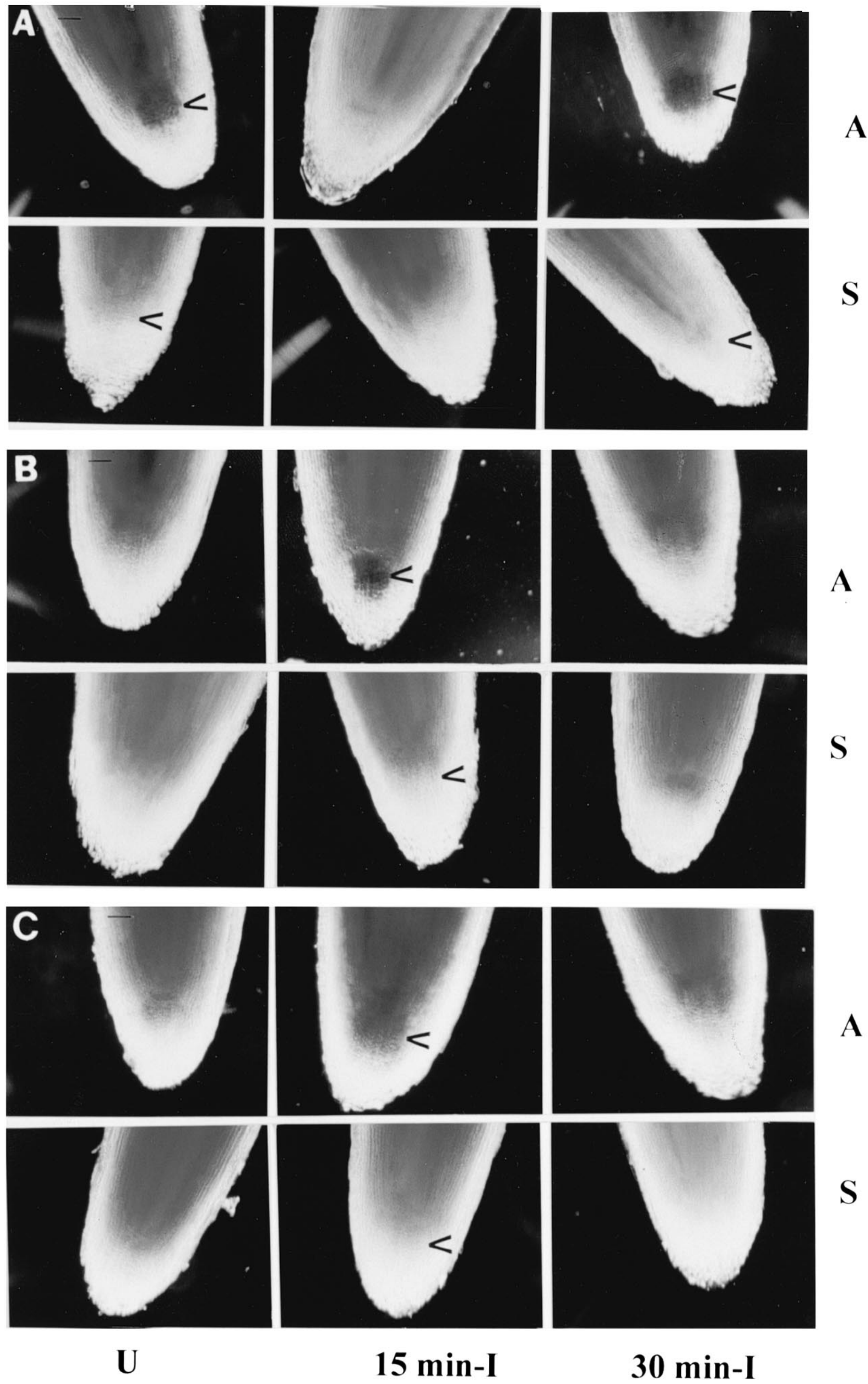

Figure 3. Localized expression of (A) *PsCaP23* and (C) *PsRbL41* in root caps of *Pisum sativum* L. Whole mount *in situ* hybridization was performed as described in Almeida Engler *et al.* [1] and Brigham *et al.* [4]. Digoxigenin-labelled antisense (upper row) and sense (lower row) mRNAs of (A) *PsHRGP1*, (B) *PsCaP23* and (C) *PsRbL41* were hybridized to localize the messages in root caps. Arrow (<) indicates where messages are localized using digoxigenin-labelled antisense mRNAs.

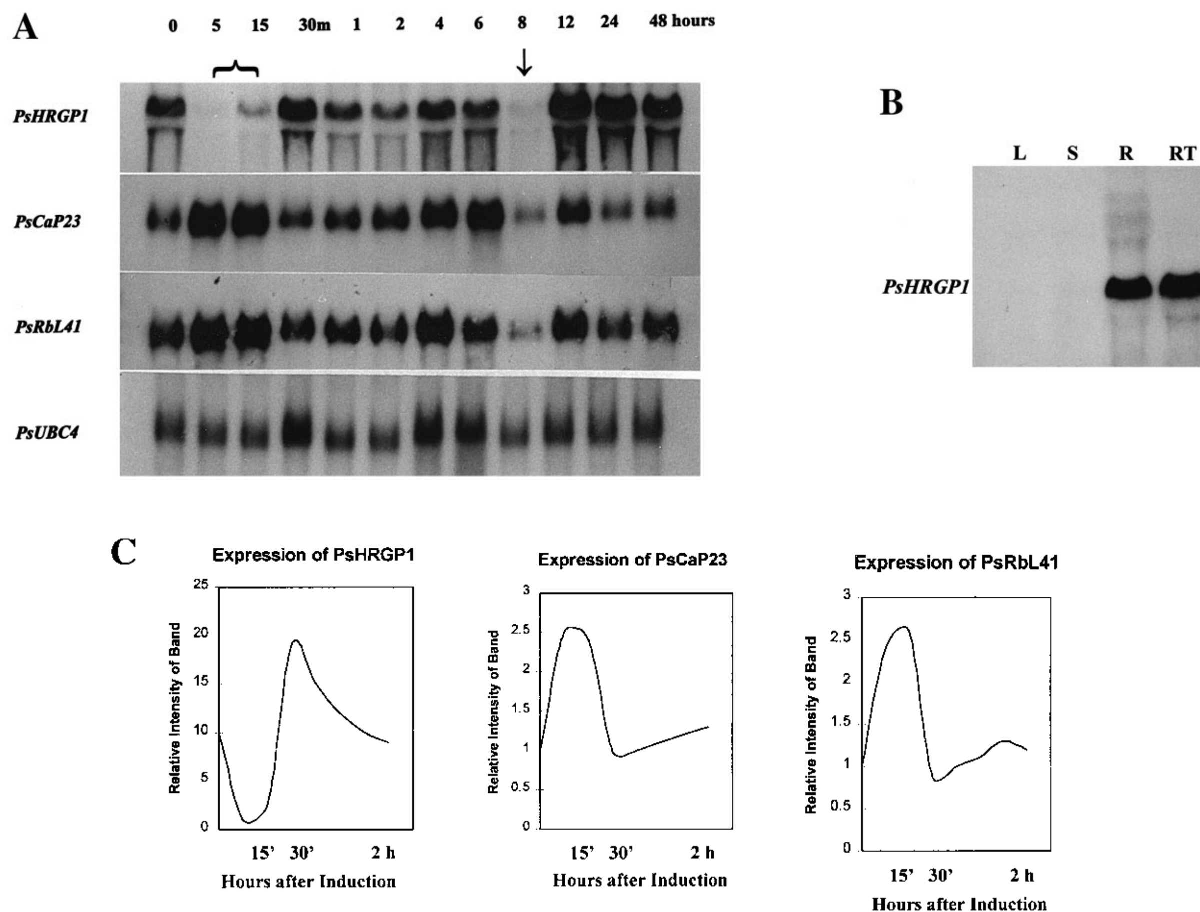

Figure 4. A (top). Developmentally regulated expression of *PsHRGP1*, *PsCaP23* and *PsRbL41* during the induced root cap cell differentiation. B (right). Root tissue-specific expression of *PsHRGP1*. Total RNA was isolated from root tips of 1 mm in length at different time point after induction for A, or from leaves, stems, roots, and root tips for B. cDNAs of *PsHRGP1*, *PsCaP23* and *PsRbL41* were ^{32}P-labelled and used for hybridization. *PsUBC4* [26] was used to demonstrate equal amount of RNA in different lanes. C. (left). To check the expression level corresponding bands from membrane were uniformly excised and measured the radioactivity by liquid scintilation counter (Beckman LS 6000SC).

Fluctuation of expression of PsHRGP1*,* PsCaP2 *and* PsRbL41 *mRNAs continues in parallel throughout border cell development*

Once initiated, border cell development is complete within 24 h. At most time points measured during this period, subsequent to the initial 30 min period of induction, expression of *PsHRGP1*, *PsCaP23* and *PsRbL41* mRNAs remained at constant pre-induction levels. A marked exception occurred at 8 h, when measurable changes in the levels of all three transcripts occurred (Figure 4A, arrow).

Small families of PsHRGP1*,* PsCaP23 *and* PsRbL41 *genes in* Pisum sativum *L.*

Genes encoding *PsHRGP1*, *PsCaP23* and *PsRbL41* in the pea genome were characterized by Southern DNA hybridization analyses using three different restriction enzymes at moderate and at high-stringency conditions. Hybridization with *PsHRGP1* cDNA sequence revealed one strong band and 3–5 minor bands at moderate stringency, and 1 strong bands at high-stringency (Figure 5A), suggesting that there are 3–5 closely related sequences of *PsHRGP1* gene in the pea genome. Hybridization with *PsCaP23* cDNA revealed 1 or 2 strong bands and 2–3 minor bands at moderate stringency, and 1–2 strong bands at high stringency (Fig-

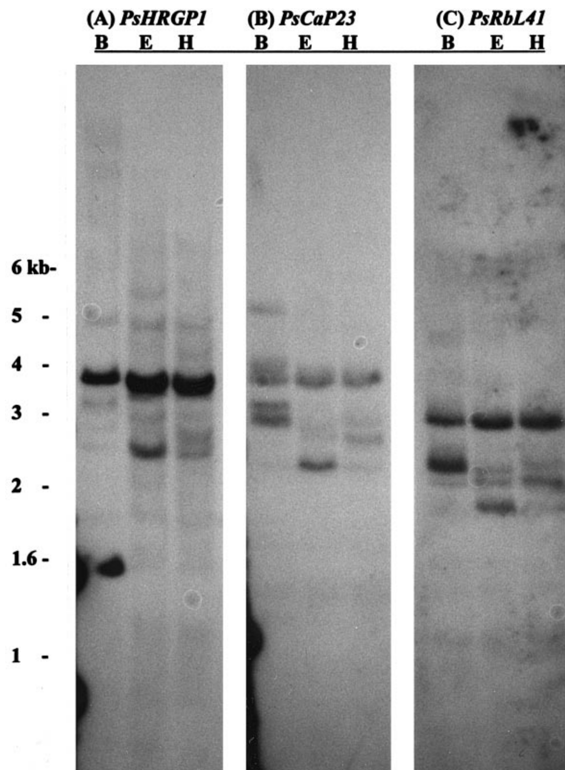

Figure 5. Gene families of (A) *PsHRGP1*, (B) *PsCaP23* and (C) *PsRbL41* in the genome of *Pisum sativum* L. Pea genomic DNA (20 μg) was digested with *Bam*HI (B), *Eco*RI (E) and *Hin*dIII (H). After blotting, the digested DNA was probed with ^{32}P-labelled cDNAs of *PsHRGP1*, *PsCaP23* and *PsRbL41*. Positions of DNA molecular length markers are indicated at left in kilobases.

ure 5B), suggesting that there are 3–5 closely related sequences of *PsCaP23* in the pea genome. Hybridization with *PsRbL41* cDNA sequence revealed one strong band and 2–3 minor bands both at moderate and high stringencies (Figure 5C), suggesting that there are 3–4 closely related sequences of *PsRbL41* gene in the pea genome.

Expression of PsHRGP1 *is root-specific*

PsHRGP1 was highly expressed in roots as well as in root tips, but its message was undetectable in leaves and stems in northern blot analysis under standard conditions (Figure 4B). When exposure times were increased from 1 h to longer than 7 days, several very faint bands were detected between 1 and 5 kb (not shown).

All three genes undergo transient changes in expression that are temporally and spatially correlated with a transient induction of mitosis at the earliest stages of root cap development when a defined set of new cells is produced. Whether or how the genes may function in the creation of new cells is unknown, but a role for an induced ribosomal protein and an HRGP can easily be envisioned. Although the function of the *PsCaP23*-encoded protein is not known, the expression of *PsCaP23* homologous sequences in other proliferating cells (such as, alfalfa callus tissues [19], human or mouse tumor cells [6, 11]) suggests that *PsCaP23*-encoded protein may be involved in cell proliferation. All three genes undergo a marked change in expression simultaneously at 8 h in addition to the initial shift between 5 and 15 min. The significance of this change is unknown, but endomitotic cycles have been observed to occur within cells of the root cap during development, and the 8 h sample could have encompassed such an event.

The root cap provides a convenient system to study molecular events underlying development because the cytology of the region has been mapped, layer by layer [2, 9, 10, 21]. Within the columella or central core of the cap, is a region called the transverse meristem [20]. Mitotic events within the transverse meristem, which give rise to cells of the root cap, are clearly delineated from those of the root apical meristem. In maize, mitosis in the columella is confined to the first 5 cell layers [2]. Though not as precisely measured as in maize with respect to the specific layer when mitosis ends, a similar localization of cell division has been observed in pea [3, 20].

The production and release of border cells from the periphery of the root cap into the external environment is a tightly regulated process controlled by developmental and environmental signals. Border cell development is functionally dependent on, and synonymous with, the sequential differentiation of all cells within the root cap. Our ability to manipulate the kinetics of gene expression associated with border cell production has made it possible to clone specific messages related to root cap development. The results suggest that the system can provide a valuable tool to define molecular processes underlying cell division and differentiation in higher plants, within a dynamic but well defined biological context.

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