ROLE OF PECTOLYTIC ENZYMES IN THE PROGRAMMED SEPARATION OF CELLS FROM THE ROOT CAP OF HIGHER PLANTS

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Martha C. Hawes

FINAL ~~PROGRESS~~ REPORT (1991-199~~3~~)

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Note: Internal reviewers of the Renewal Application and Progress Report have recommended that reviewers first read the Background and Significance (Section I, Renewal Application), and then read the Progress Report before proceeding to Proposed Experiments in the Renewal Application.

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PROGRESS REPORT (1991-1993)

SUMMARY: The following objectives have been accomplished during the funding period *(reviewed in Hawes & Brigham 1992, Hawes et al below)*: 1. The use of transgenic hairy roots to study border cell separation has been optimized for pea *(Nicoll et al below)*; 2. A cDNA encoding a root cap pectinmethylesterase (PME) has been cloned *(Wen et al below)*; 3. PME and polygalacturonase activities in cell walls of the root cap have been characterized and shown to be correlated with border cell separation *(Stephenson & Hawes below)*.

Results *(summarized in Fig. 1)* are consistent with a model in which pectolytic enzymes play a role in border cell separation, and simultaneously alter the physiology of the root cap: PME and PG activities are high during cell separation, and are low after cell separation ceases. Immunoreactivity with antibodies specific to esterified or deesterified pectin revealed that the following changes are correlated with PME and PG activities during border cell separation: 1. Pectic compounds in the root cap become increasingly deesterified to polygalacturonic acid (PGA) as border cells separate; 2. The deesterified PGA changes from being cell wall bound to being water soluble; and 3. Virtually all of the increase in deesterified soluble PGA is associated with border cells as they separate from the root cap. An assay was developed to test the hypothesis that release of soluble PGA results in changes in extracellular pH in border cell progenitor cells in the root cap. Results indicate that as PGA levels increase, a parallel decrease in cell wall/apoplastic pH occurs.

Efforts to develop molecular tools that can be used to test the model in future experiments *(described in Renewal Application)* are underway. A heterologous probe was used to clone a root cap expressed cDNA (PsPE1) that encodes an amino acid sequence 87% identical to known PME genes. A gene encoding a putative pectate lyase from tomato has been shown to be expressed as it is in tomato, in peripheral cells of pea root caps, an expression pattern that is expected of genes involved in border cell separation. Attempts to clone a root cap PG have not yet been successful: Heterologous probes using full length cDNA from tomato fruit PG do not react with pea genomic DNA or with root cap mRNA.

PUBLICATIONS (1992-1993):

Hawes MC, Brigham LA (1992) Impact of root border cells on microbial populations in the rhizosphere. Advances in Plant Pathology 8: 119-149 (reprint attached)

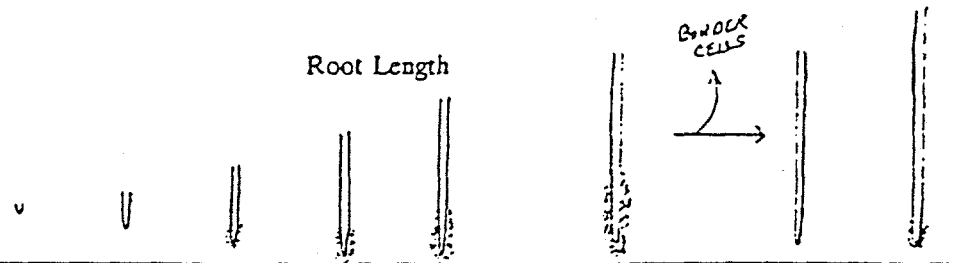
Hawes MC, Brigham LA, Nicoll SM, Stephenson MB (1994) Plant genes controlling the release of root exudates. Biotechnology and Plant Protection 4 (in press) (preprint attached)

Nicoll SM, Brigham LA, Wen F, Hawes MC Expression of transferred genes in transgenic hairy roots of pea (submitted; manuscript attached)

Stephenson MB, Hawes MC Pectinmethylesterase in root caps of pea correlated with border cell separation (Ms in preparation--results are detailed in this Progress Report). (Attached)

Wen F, Oishi KK, Hawes MC Cloning and sequence analysis of a root cap pectinmethylesterase cDNA from *Pisum sativum*. (Ms in preparation; results are detailed in this Progress Report)

Fig. 1. Summary of changes in pectolytic enzyme activity, localization of polygalacturonic acid (0-50% esterified), and in cell wall pH* during border cell development (see text for details).



PROPERTIES	1 mm	5 mm	10 mm	15 mm	20 mm	25 mm	25 mm	30 mm
Number of border cells	0	50	1000	2000	3000	3400	0	800
PME (percent highest activity)	100	100	70	30	15	15	15	50
PG (percent highest activity)	0	100	100	100	100	0	0	100
PGA in root cap								
cell wall bound	±	++	++	+	+	+	±	
water soluble	±	+	++	+++	++++	+++++	±	
Cell wall/apoplastic pH*	> 6.0	int.	int.	int.	int.	< 5.6	int.	

pH of border cell progenitor cells of the root cap. Limits of detection of assay are 5.6 and 6.0; "int." means that levels are intermediate between 5.6 and 6.0 (see text for details).

ROLE OF PECTOLYTIC ENZYMES IN THE PROGRAMMED SEPARATION OF CELLS FROM THE ROOT CAP OF HIGHER PLANTS

OBJECTIVE: To develop a model system to study border cell separation in transgenic pea roots; and to test the hypothesis that genes encoding pectolytic enzymes in the root cap play a role in the programmed separation of root border cells from the root tip.

Note: The primary focus of the original grant was to test the role of an endopolygalacturonase (endoPG) in cell separation. The rationale for this emphasis was that such enzymes that degrade pectic compounds internally are the only ones which alone can cause the separation of plant cells from each other (Collmer & Keen 1986), and therefore were most likely to have a major impact on border cell development. I broadened the focus for two reasons: 1. Enzymes like PME and exo-PGs play crucial roles in cell separation during pathogenesis by soft rot bacteria. Even though such enzymes alone do not cause cell separation, their activity is necessary for other enzymes to solubilize cell walls. 2. Reviewers of my original grant felt that including other enzymes would be much more informative about the overall process of cell separation, and I agree.

I. DEVELOPMENT OF A MODEL SYSTEM TO STUDY BORDER CELLS IN TRANSGENIC PEA ROOTS.

Our primary model plant is *Pisum sativum*, a well characterized diploid for which information is available about root development, root cap cell biology, rhizosphere biology and root exudates, and root gene expression. Pea has large root caps amenable to biochemical characterization, which has been useful in the characterization of root cap localized enzymes *(Hawes & Lin 1990, Hawes & Stephenson 1991, Stephenson & Hawes, submitted, Zhu & Hawes, unpublished)*. Like other legumes *(Hawes & Pueppke 1986)*, pea produces thousands of border cells daily, which has facilitated physiological studies and molecular work like the construction of border cell cDNA libraries *(Brigham and Hawes 1991, Brigham et al unpublished, Wen et al unpublished)*. Methods for regenerating pea from tissue culture have been established for years *(Rubluo et al 1984, Mroginski & Kartha 1981, Kysely et al 1987, Malmberg 1979, Griga et al 1986)*, and transgenic pea recently has been produced by this method *(Schroeder et al 1993)*.

Pea is highly susceptible to transformation with *Agrobacterium*, including *A. rhizogenes (Hawes et al 1991, Robbs et al 1990)*. An important advantage of using hairy roots is that hundreds of replicate genetic clones of a specific transgenic root can be obtained for use in quantitative and qualitative effects of a transferred on root development. These clones can be obtained within a few weeks, compared with months required for regeneration of whole plants. A unique benefit of using hairy roots is that manipulation of genes putatively involved in border cell development can be restricted to the root, reducing the chances that transgenes will cause pleiotropic effects on whole plant development.

The hairy root system for use with transgenic pea roots was optimized *(Nicoll et al 1991, Nicoll et al submitted* (attached). Our objectives were to characterize border cell development in hairy roots; and to assess expression of marker genes in hairy roots, especially in root border cells. We used the "defense" genes phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) from bean as markers for gene expression in hairy roots. These were chosen because their expression in normal and transgenic roots is well described. Expression of bean PAL and CHS during hairy root development was very similar to that which has been described in bean roots and in roots of transgenic tobacco *(Nicoll et al)*. PAL and CHS are not expressed constitutively in border cells of hairy roots, but are expressed in border cells under conditions of stress. Border cell development in hairy roots is comparable to that of normal roots.

Transgenic hairy roots and whole plants will be used in complementary studies of effects of genes putatively involved in border cell separation. *In vitro* studies of effects of the gene on numbers of border cells, pH effects, growth rate, etc. will be carried out using hairy roots, because of the rapid availability of multiple clones for quantitative studies. Plant regeneration will be carried out simultaneously, and transgenic plants will be used to carry out detailed studies of the impact of the gene in whole plant development and such functions as gravitropism, nutrient uptake, ability to penetrate soil, and susceptibility to soil borne pathogens and symbionts. In these studies, we can use pea as well as amplify the work to include other species like alfalfa. Given that border cell development is highly conserved at the family level *(Hawes & Pueppke 1986)*, it would be surprising to find that heterologous genes would not function appropriately at least in related genera.

II. PECTOLYTIC ENZYMES IN THE ROOT CAP.

Pectic compounds are complex polymers associated with 1,4-linked α-D-galactosyluronic acid residues *(see Table 1, Renewal Application)*. Two types of pectolytic enzyme activity--PME and PG--can be measured in extracts of pea root caps, and a gene with homology to PLs is expressed in root caps of tomato (Table 1). The status of our understanding of PME, PG and PL in pea root caps, and their relationship with border cell separation, are summarized below.

A. PECTINMETHYLESTERASE (PME). We focused first on PME, because of its pivotal role in initiating pectin degradation *in vivo* and *in vitro (Table 1 Renewal Application)*. PME is required to demethylate pectin, which yields polygalacturonic acid, a substrate that can be degraded by PG and PL.

1. ENZYMOLOGY.

a. High levels of PME activity can be detected in pea root caps. PME activity can be detected by the ability to induce the formation of a gel in the presence of pectin *(Yamaoka et al 1983)*. A crude extract of 4 root caps of pea in 100 µl pectin (1% w/v) resulted in the formation of a gel within 60 min. Boiling or treating the extract with protease eliminated

Table 1. Pectolytic enzymes in the root cap of *Pisum sativum*

Enzyme type	
Pectin methylesterase (PME) *(Stephenson & Hawes, unpublished)*	
Enzyme activity correlated with separation:	Yes
Cell wall bound	Yes
Detectable in border cells:	No
Extracellular:	No
Number of isoenzymes:	Not known
pH optimum:	Higher than 7.0
Gene expressed in peripheral cap cells:	Not known
Polygalacturonase (PG) *(Hawes & Lin 1990)*	
Enzyme activity correlated with cell separation:	Yes
Cell wall bound:	Yes
Detectable in border cells:	No
Extracellular:	No
Attacks internally or externally:	Not known
Number of isoenzymes:	Not known
pH optimum:	Lower than 6.0
Gene expressed in peripheral cap cells:	Not known
Pectate lyase (PL) (?) *(Twell et al 1990)*	
Enzyme activity	Not detected
Gene (LAT59) expressed in peripheral cells:	Yes

gel inducing activity. PME activity also can be measured by a change in pH which occurs when methoxy groups are cleaved from esterified pectin *(Moustacas et al 1986)*. One unit is defined as that amount needed to catalyze the release of 1 μm of carboxyl groups in 1 min. In the titration assay, a single root cap contained up to 0.08 units of PME, enough to lower the pH of 3 ml of pectin by an order of magnitude within 5 minutes. The titration assay is used routinely because it is more sensitive and quantitative than the gel assay.

b. Most PME is cell wall bound. PME activity is present in root caps, but not in soluble root exudates or in root border cells. Most activity (80±4%) is cell wall bound, and is only extractable in salt; 20% of the activity is water soluble.

c. Enzyme purification. A 40 to 50 fold increase in specific activity of the PME from pea root caps was achieved (Table 2). An increase in total units of activity occurred frequently (36 of 56 experiments) as a result of fractionation, suggesting that an inhibitor of PME activity may be present under some conditions. A glycoprotein inhibitor of PME activity has been identified in other species *(Balestrieri et al 1990)*, and may be one regulator of PME activity during border cell development. A single broad peak of PME activity occurred on DEAE Sephacel columns. When fractions from the gel filtration column with PE activity were subjected to SDS PAGE, fewer than ten proteins could be detected. Attempts to detect PME in non denaturing gels were not successful.

Activity of root cap PME was stable at -20 C for more than one year.

d. PME activity is optimal at pH higher than 7.0. Activity occurred over a broad pH range from from 5.1 to 7.4, but was 10 fold higher at 7.4 than at 5.1 (Table 3). Alkaline deesterification of pectin occurs at pH higher than 7.6, so activity cannot be measured accurately above 7.5.

2. Biological context

a. PME activity is correlated with border cell separation during development. Enzyme activity is high during emergence of the radicle, and remains high until the roots are 15 mm in length, at which time PME levels drop and remain low (Figure 2a,b). This corresponds to the time when most border cells have separated from the root and from each other *(Hawes & Lin 1990)*. In pea, border cell number reaches a maximum at 25 mm, and then remains largely stable at 3400 cells per root, if the cells are not removed. If cells are removed from the root tip, regeneration of border cells is reinitiated; within 5 hours new cells can be collected *(Hawes & Lin 1990)*. PME activity begins to increase again within 2.5 hours after border cells are removed (Figure 2b).

b. PME activity is present in root caps of other species that release large numbers of border cells. PME activity levels in root caps of alfalfa, sunflower, corn, and *Arabidopsis thaliana* were tested. The only species in which PME was not detected was *A. thaliana*, which is the only one of the four which does not release border cells during early development, under

TABLE 2. PARTIAL PURIFICATION OF PE FROM PEA ROOT CAPS

Treatment	Protein (µg)	Units PE	Units/mg	Increase in purification	% Recovery
4% NaCl	3320	7205	2.2	1.0	100%
30% NH_4SO_4	778	7360	9.5	4.3	102%
70% NH_4SO_4	570	14,000	24.6	11.2	190%
Sephacel	8.2	800	97.6	44.4	11%

Table 3. Effect of pH on root cap PME activity

pH	Units/ul
4.35	0
4.6	0
5.1	0.64
5.6	0.83
6.1	1.1
6.5	1.4
7.0	3.7
7.5	5.1

Table 4. PME activity in root caps of other species

Species	#Border cells/root	Enzyme activity
Maize	3000	+
Alfalfa	2800	+
Sunflower	2900	+
Arabidopsis	0	–

Figure. 2 Changes in pectolytic enzyme activity, esterification and localization of polygalacturonic acid, and cell wall pH during border cell development.

2a. **Border cell number during root development.** No cells are present at 5 mm, after which cell number increases until the root is 25 mm long and 3400 border cells surround the root. At this point the process of border cell separation ceases. If border cells are removed by washing the root tip in water, cell separation is reinitiated, and the process is synchronized. After 24 hours, 3400 border cells have separated, and cell separation again ceases. Under the conditions of the experiments, roots grow at a rate of 1 mm per hour.

2b. **Correlation of pectolytic enzyme--PME and PG--activity with border cell separation.** Values represent the percent of highest levels of enzyme activity. (Highest PME activity is 0.04 units per root cap (see text for explanation of units); highest PG activity is 1 unit per ml *(Hawes & Lin 1990.)*

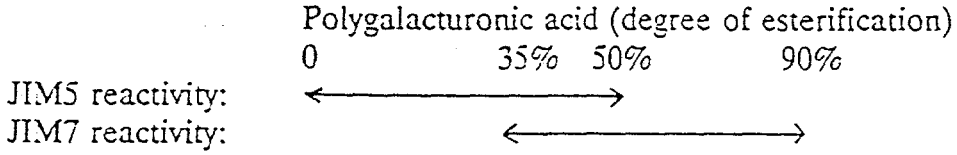
2c. **Changes in level and localization of deesterified PGA in root caps detected by immunoreactivity with JIM5** *(Knox et al 1990)* (see text). Root caps (25 @ 1 mm) were extracted into 100 µl water and centrifuged; the supernatant is the WATER SOLUBLE fraction. The pellet was mixed with 100 µl EDTA (0.1M) and 4% NaCL (100 C) and extracted at room temperature 30 min, and centrifuged; the supernatant is the CELL WALL fraction. Samples (1 µl) of each were dotted onto nitrocellulose and processed with Bio-Rad Immuno-Blot assay. Primary antibody dilution (JIM5) was 1:25 and secondary anti-rat IgG was 1:3000. Controls included BSA or extraction buffer.

2d. **Change in root cap cell wall/apoplastic pH correlated with border cell separation.** Values represent the number of fluorescent border cells obtained from roots 6 h after incubation of the root cap in fluorescein (see Fig. 3). When the cell wall pH of progenitor root cap cells was higher than 6.0 at the time of incubation in fluorescein, none of the newly separated border cells are stained; when the cell wall pH of progenitor cells was less than 5.6 at the time of incubation, 100% of border cells are stained.

the conditions used in this study (Table 4).

c. PME activity does not change in response to several other environmental factors. Several variables were tested for an effect on levels of enzyme activity *(data not shown)*. Levels of PME were not significantly affected by genotype, by circadian rhythms, light, or germination temperatures ranging from 10 C to 37 C.

d. Changes in degree of esterification, and in cellular localization, of root cap pectin during development are correlated with PME activity during border cell development. Monoclonal antibodies JIM5 and JIM 7 (a gift from Keith Roberts, John Innes Institute) were used to demonstrate that changes in degree of esterification of PGA are correlated with PME activity (Fig. 2c). JIM5 reacts immunologically to deesterified pectin and to pectin containing up to 50% esterification *(Knox et al 1990)* (see below). The JIM5 reaction to pectin decreases in intensity as degree of esterification increases. In contrast, JIM7 does not react with deesterified pectin but is equally reactive with pectin containing 35% to 90% esterification.



JIM5 has been shown to react strongly with middle lamellae of cells from roots of 3 to 4 week old peas *(VandenBosch et al 1989)*, and throughout the cell walls of oat root cap cells *(Knox et al 1990)*. As in other root tissues, JIM7 reacted at a moderate level with substrate in root caps of pea roots (1 through 25 mm in length), with or without border cells *(data not shown)*. This result suggests that PGA with greater than 35% esterification is present in the root cap throughout this period. In contrast, reactivity with JIM5 changed markedly during the same period; in 25 mm roots, reaction with JIM5 depended on whether or not border cells were present:

Water soluble PGA with 0 to 50% esterification: In 1 mm roots, there is little or no substrate in root caps that reacts with JIM5 (Fig. 2c). In roots 5 mm or longer, after border cells begin to separate from the cap, soluble JIM5 reactive substrate increases incrementally through 25 mm. Virtually all of the reactive substrate is eliminated when border cells are washed from the root prior to extraction in water.

Cell wall bound pectin with 0 to 50% esterification: Little or no cell wall bound material present in 1 mm root caps reacts with JIM5 (Fig 2C). The strongest reaction occurs in roots that are 5 to 10 mm in length. This corresponds to the time just before and after visible border cell separation can be detected. The level of cell wall bound JIM5 reactive material is constant at a moderate level at 15 through 25 mm. Washing border cells from the root tip prior to assay does not cause a significant change in the intensity of the reaction.

These results are consistent with the hypothesis that PME activity in the root cap results in

an increase in the amount of PGA with less than 50% esterification. The first effect is on cell wall bound PGA, at 5 to 10 mm. As more and more border cells separate, most of the PGA becomes water soluble, and is associated with border cells, not the root cap. The results indicate that deesterified PGA is largely confined to material that is removed by washing the root in water, when border cells are removed. Cell walls in the root cap itself primarily contain PGA which is more than 35% esterified.

d. A decrease in apoplastic pH of peripheral root cap cells during development is correlated with PME activity. High PME activity at the cell wall is thought to be capable of generating a pH gradient as a result of the deesterification of pectin to yield polygalacturonic acid *(Balestrieri et al 1990, Chamey et al 1992, Goldberg et al 1992)*. It has been speculated that the resulting low pH could inhibit PME, which is optimal at high pH, and simultaneously activate PGs and other enzymes with low pH optima, which could be an important mode of regulation for cell wall degrading enzymes *(Moustacas et al 1986)*.

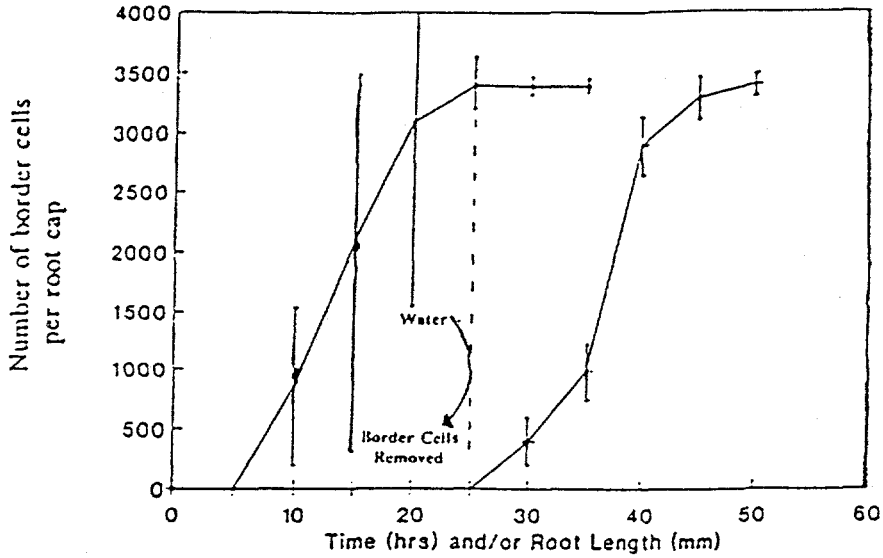
A unique quantitative assay based on uptake of fluorescein into border cell progenitor cells in the periphery of the root cap was developed to test the hypothesis that changes in apoplastic pH (cell wall and apoplastic pH are continuous and are used interchangeably) occur in root caps during border cell development (Fig. 3). Fluorescein is a weak acid which accumulates in cells with a pH higher than that of the apoplast, such that accumulation increases sharply as the pH of the apoplast is lowered. In pea root apices, fluorescein does not accumulate in cells when extracellular pH is higher than 6.0, but readily accumulates when the apoplastic pH is 5.6 or lower *(Dorhout and Kolloffe! 1992)*. Staining is irreversible, and cells which take up the dye remain fluorescent for days. Therefore the apoplastic pH of the peripheral root cap cells can be measured by simply counting the proportion of fluorescent border cells which develop several hours later.

The assay revealed that the apoplastic pH of peripheral cells of the emerging radicle is higher than 6.0, such that fluorescein uptake is minimal (Fig. 2D). The pH apparently increases during the separation of border cells, so that uptake into progenitor cells in the cap increases. The fact that some cells take up the stain and others do not may be because the pH is at a threshold level for uptake, or it may be because the apoplastic pH is not uniform, so some cells have higher external pH than others. In caps of roots more than 25 mm long, virtually every peripheral cell takes up fluorescein, indicating that the apoplastic pH within those cell layers is uniformly lower than 5.6.

If the low pH is due to the presence of soluble PGA associated with border cells, then washing border cells from roots should allow the pH to increase, which would result in fewer cells taking up fluorescein. Washing border cells from the root by brief agitation in water causes no change. This would be expected if the soluble PGA which bathes the root tip diffuses into intercellular spaces, but it should be eliminated by more extensive washing *(for instance, in order to eliminate fluorescein from intercellular spaces, roots must be washed for an hour--see Fig. 3)*. When roots were washed in water for one hour, the number of cells stained with fluorescein decreased from 100% to 50%. This is consistent with the hypothesis

Fig. 2. Changes in pectolytic enzyme activity, localization of deesterified polygalacturonic acid, and cell wall pH correlated with border cell separation.

A



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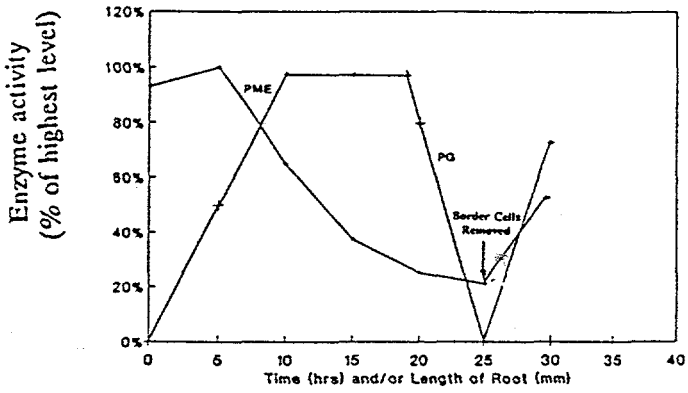

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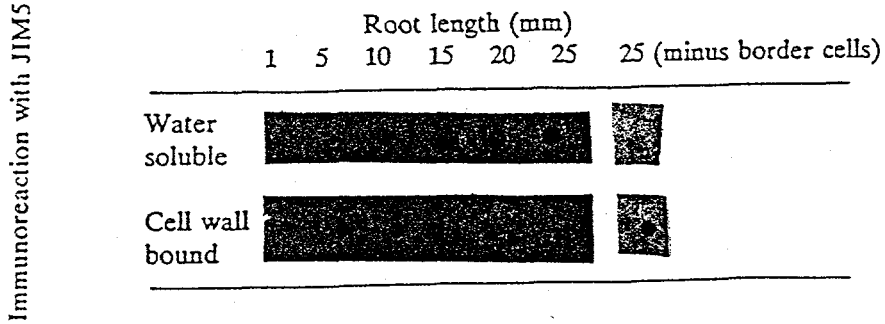

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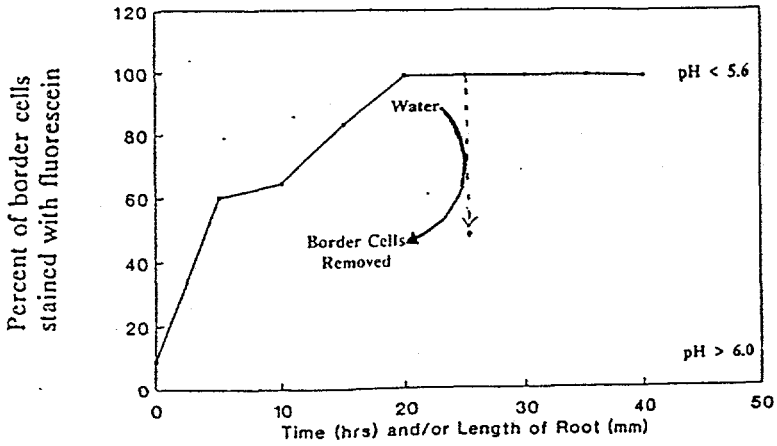
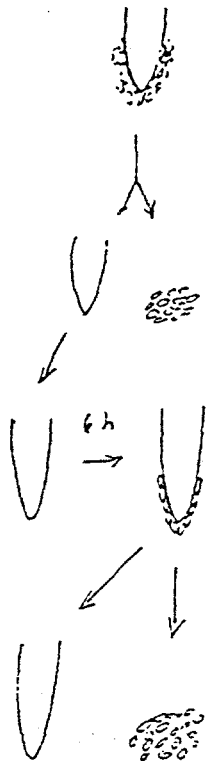


Fig. 3. QUANTITATIVE ASSAY FOR APOPLASTIC PH CHANGES IN PERIPHERAL ROOT CAP CELLS



1. Place seedlings (root lengths from 1 to 35 mm) into fluorescein pH 7.9. 15 min.

   When pH of the apoplast is less than 5.6, the dye is taken up irreversibly, resulting in fluorescent cells within the tissue.

2. Wash roots in water for one h. Collect original border cells; wash by repeated centrifugation 1 h, and count cells that fluoresce.

3. Place washed seedlings, free of border cells, onto filter paper 6 h to allow regeneration of new border cells (Hawes & Lin 1990).

4. Remove newly formed border cells, and count the number of cells which are fluorescent. Those which are derived from peripheral root cap cells that were stained in step 1 (apoplastic pH was less than 5.6) will be fluorescent. Those derived from root cap cells which were not stained in step 1 (apoplastic pH greater than 6.0) will not fluoresce.

that border cell separation is associated with the presence of soluble compounds that result in a low pH in peripheral cells of the root cap. We confirmed this by direct measurements: the pH of the supernatant from border cells is a surprisingly low 5.0.

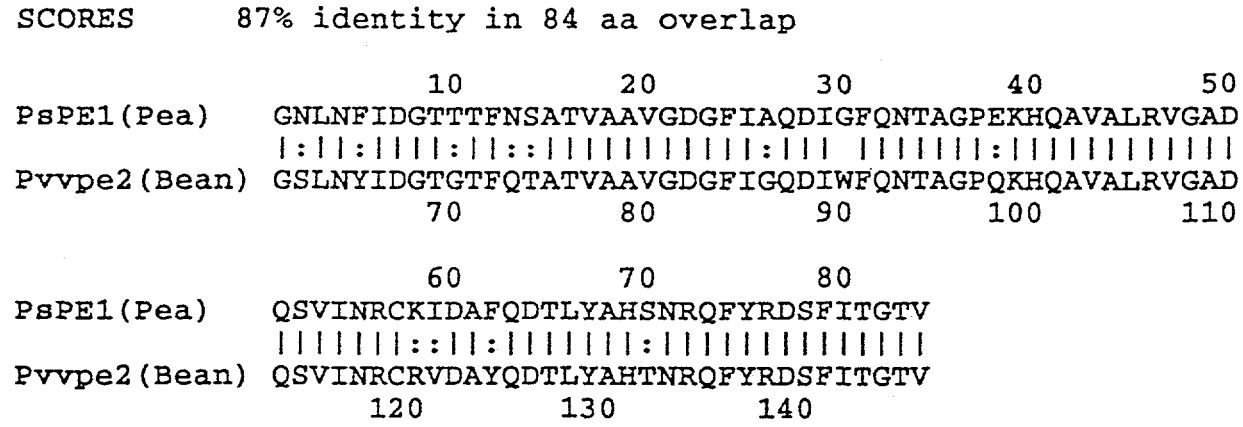
This is a very important finding. These results are consistent with the possibility that as PME hydrolyzes methoxy groups from pectin, yielding increasing amounts of deesterified PGA, the pH of the apoplast drops in response. Such changes in pH have the potential to have a drastic effect on the physiology and the biology of the root cap. This is extremely important, because abrupt changes in pH would affect virtually all aspects of the function and development of root tips, including environmental responses, enzyme reactions, solute uptake and cell growth, as well as susceptibility to fungal, bacterial, and nematode pathogens.

The border cell system provides a unique opportunity not only to dissect fundamental aspects of root development and rhizosphere biology, but also to understand ways in which plant cells regulate pH and control the cellular and molecular reactions which depend on this parameter. In the past year efforts have focused on developing cDNA libraries and isolating clones needed to manipulate activity of the enzyme in transgenic roots, so we can determine the effects of root cap pectolytic enzyme activity on growth and development. The results of this work are summarized below.

3. GENE(S) ENCODING THE ROOT CAP PME

a. A root cap-expressed cDNA with homology to PMEs has been cloned. A root cap cDNA library was constructed using 10 ng cDNA synthesized from 2 ug mRNA from root caps induced for PME activity by removing border cells and incubating for 2 h. The cDNA was annealed into EcoRI and XhoI sites of lambda ZAP containing pBluescript and transferred into E. coli SURE to yield 3.3 X 10^4 recombinants. A PME encoding cDNA clone from *Phaseolus vulgaris* pods, PvPE3, has been shown to be expressed in bean roots *(Recourt et al 1988)*. Southern hybridization was used to demonstrate that several restriction bands within pea genomic DNA react with PvPE3 *(not shown)*. PvPE3 was used as a heterologous probe to screen the PME induced root cap cDNA library amplified to 5 X 10^5 clones. After a tertiary screen, 7 cDNA clones were selected and one of them, PsPE1, was further analysed. Sequence analysis of PsPE1 shows 67% to 75% identify with PEs from tomato and bean. Its partial sequence revealed a predicted amino acid region with 73% identity in an 83 amino acid overlap with tomato PME, and 87% identity in an 84 amino acid overlap with bean PME *(Recourt et al 1992)* (Fig. 4). The data are consistent with the hypothesis that a PME encoding gene is expressed in the root cap of pea during developmental stages when PME activity is detectable.

b. A small gene family in pea reacts with PsPE1. Used as a probe in Southern analysis, PsPE1 clone reacts with 5 to 6 bands ranging in size from ca 5 to 15 kb (Fig. 5a-c). The probe reacts with the same two bands in a Bgl II digest of bean genomic DNA which react with a probe from PvPE3 (Fig. 5d). The data are consistent with the possibility that a small family of PME genes is present in the pea genome.



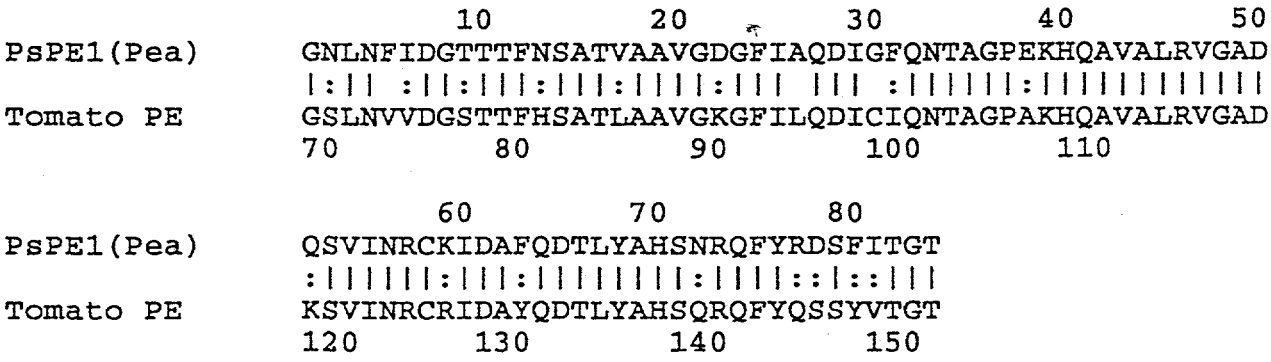


Fig. 4. Deduced amino acid sequence of 250 bp region of PsPE1, and comparison with overlapping sequences from PME genes from bean pods (top) and tomato fruit (bottom).

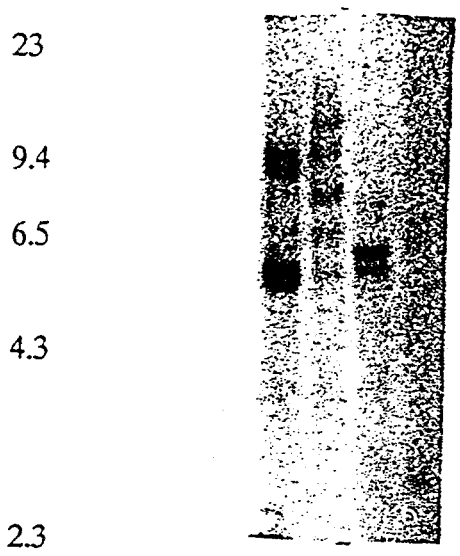


Fig. 5. Southern blot of pea genomic DNA probed with PsPE1. Pea genomic DNA was cut with restriction enzymes (A) EcoRV; (B) Bgl II; (C) Xba I. Lane D is bean genomic DNA cut with Bgl II. PsPE1 was isolated by restriction digest with EcoRI and XhoI. The fragment was isolated on a 1.4% agarose gel in TAE. The Prime It II kit (Stratagene) was used in random primer labelling the PsPE1 fragment. Hybridization conditions were 65C for 48 hours. Wash conditions were 65 C in 0.5X SSPE, 0.1% SDS.

B. POLYGALACTURONASE (PG) An assay to detect reducing sugars was used previously to demonstrate that pectin degrading activity in the root cap is correlated with border cell separation (Fig. 2a, *Hawes & Lin 1990*). In the current funding period, the PG was further characterized (Table 1): Properties of the enzyme are consistent with those of a PG, with respect to pH optimum (lower than 6.0, with high activity at 4.0 and little or none at pH higher than 7.0) and substrate preference (PGA) (*Hawes, unpublished*). Most enzyme activity is ionically bound to the cell wall of the root cap; activity is detectable when roots are extracted into water *(Hawes & Lin 1990)*, but at least 70% of the activity is extracted only with salt. PG is undetectable in border cells or root exudates. The activity is unstable upon purification: Every attempt made to fractionate extracts resulted in complete loss of the activity. This could be due to the presence of inhibitors (*O'Neill et al 1990*), or to intrinsic properties of the enzyme.

Initial attempts to identify a pea root cap expressed PG gene using a tomato PG as a probe yielded negative results: In these experiments, the full length cDNA encoding tomato fruit PG was used as a probe of pea genomic DNA and of root cap mRNA; related sequences were not detected. Additionally, antibody to the tomato fruit PG did not react with extracts of pea root caps with PME activity.

C. PECTATE LYASE (PL) A tomato gene expressed during pollen development (LAT59) is expressed in peripheral cells of the root cap of tomato *(Twell et al 1990)*. This gene is of interest to us for two reasons: 1. IDENTITY AS A PL. The gene has been proposed to encode a PL based on strong homology to bacterial PLs. Although the identity of the gene has not been confirmed *(Yoder et al 1993)*, and PL activities have not been detected in plants, including root caps (*Hawes, unpublished and Sheila McCormick, personal communication*), its identity as a PL has not been ruled out. If it is, it could be an important component of the array of cell wall degrading enzymes potentially involved in border cell separation. 2. CELL SPECIFIC EXPRESSION. Even if LAT59 does not encode a PL, its pattern of expression, confined to peripheral cells of the root cap, makes it extremely useful to us, because the promoter can be used to direct expression of antisense and other constructs in border cell progenitor cells. This will minimize the chances that inhibition of the gene will cause pleiotropic or lethal effects on development.

Southern and Northern blot analyses indicate that a gene with homology to LAT59 exists in the pea genome, and that it is expressed in pea root caps *(unpublished)*. We have constructed a fusion gene using the LAT59 promoter and *uid*A, and inserted it into *A. rhizogenes*, and have transformed it into pea hairy roots. The results indicate that the gene is expressed in peripheral cells of the pea root cap, as it is in tomato *(unpublished)*.

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