The Plant Cell, Vol. 11, 1129–1140, June 1999, www.plantcell.org

Effect of Pectin Methylesterase Gene Expression on Pea Root Development

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Expression of an inducible gene with sequences common to genes encoding pectin methylesterase (PME) was found to be tightly correlated, both spatially and temporally, with border cell separation in pea root caps. Partial inhibition of the gene's expression by antisense mRNA in transgenic pea hairy roots prevented the normal separation of root border cells from the root tip into the external environment. This phenotype was correlated with an increase in extracellular pH, reduced root elongation, and altered cellular morphology. The translation product of the gene exhibited PME activity in vitro. These results are consistent with the long-standing hypothesis that the demethylation of pectin by PME plays a key role in cell wall metabolism.

INTRODUCTION

Between the plant cytoplasm and its external environment lies a complex carbohydrate–based cell wall, which is a dynamic interface that participates directly in cellular responses to exogenous stimuli (reviewed in Albersheim et al., 1994; de Lorenzo et al., 1994). In addition to a direct role in perceiving and responding to incoming signals, the cell wall is a repository of oligosaccharides whose activity can alter the metabolism of the plant cell it encloses as well as that of other organisms that find their way into proximity with the cell. These sugar-based signal molecules are released from cell wall polymers by the action of enzymes that can come from fungi, bacteria, or other organisms in the environment, or from the plant itself. The role of specific plant cell wall–degrading enzymes in cell wall metabolism during growth and development remains unclear (reviewed in Carpita et al., 1996).

Plant enzymes that degrade pectin, or methylated polygalacturonic acid, are of special interest because this polymer is a major constituent of cell walls and because such pectolytic enzymes can solubilize cell walls (Collmer and Keen, 1986; Koutojansky, 1987). For example, genes encoding certain polygalacturonases (PGs) or pectate lyases (PLs) individually allow soft rot pathogens to macerate potato tuber tissue and to infect plants systemically (Collmer and Keen, 1986). Pectin methylesterase (PME), although it does not by itself solubilize cell walls, has been postulated to regulate cell wall degradation by several mechanisms (e.g., Goldberg et al., 1992). The action of PME reduces pH by the release of a proton when methoxyl groups of pectin are converted to carboxyl groups. This change in pH has been proposed to control the activity of other cell wall–degrading enzymes that are optimally active at low pH and thereby to facilitate cell expansion and growth (Nari et al., 1986) and/or cell separation (Koutojansky, 1987).

Demethylation by PME can alter sensitivity of polymers to the action of hydrolases (e.g., Fischer and Bennett, 1991; Liu and Berry, 1991) and expansins (Carpita et al., 1996). Small pectic fragments released by the action of such hydrolases act as signals to induce expression of other pectolytic enzymes, and the degree of methylation of such fragments, dictated by PME activity, may affect their specificity in inducing expression of genes encoding distinct pectic isozymes (McMillan et al., 1994). By its action, then, PME may regulate which enzymes are synthesized within a particular cellular environment. Finally, the generation of fixed COO^- charges accessible to neutralization by Ca^{2+} results in one of the major consequences of PME action on plant cell wall structure. The formation of Ca^{2+} bridges is responsible for the "gelling" action that probably plays a crucial role in the normal structural properties of the cell wall and middle lamella.

PME is an enzyme that is present in all plant tissues and in all species that have been examined to date (Rombouts and Pilnik, 1980). As predicted, the gene encoding PME plays a key multidimensional role in cell wall metabolism, and PME genes have been identified in several plant species (Albani et al., 1991; Hall et al., 1994; Mu et al., 1994; Qiu and Erickson, 1995; Bordenave et al., 1996; Glover et al., 1996; Recourt, 1996; Richard et al., 1996; Gaffe et al., 1997). Surprisingly,

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however, plants whose PME activities have been inhibited using antisense mRNA exhibit relatively subtle changes in phenotype (Tieman et al., 1992; Hall et al., 1993; Gaffe et al., 1997). For example, inhibition of fruit-specific PME expression affects fruit tissue integrity during senescence but does not affect growth and development of the plant or of tomato fruit (Tieman and Handa, 1994).

Root border cells provide a convenient model system in which to examine the role of cell wall–degrading enzymes in cell function and development (Stephenson and Hawes, 1994; Brigham et al., 1995a; Hawes et al., 1998). Each day, plants of many species release thousands of healthy somatic cells, with unique patterns of protein and gene expression, from the root tip into the external environment (Brigham et al., 1995b). We refer to these cells, formerly called sloughed root cap cells, as root border cells to emphasize that they are not part of the root cap and that as a population, they form a physical and biological interface or "border" between the root and the soil (Hawes and Brigham, 1992).

Border cells of pea, our primary model system, begin to separate from the root tip when emerging roots are 5 mm long, and cell number increases until the root is ~25 mm long and ~4000 cells have accumulated at the root periphery (Hawes and Lin, 1990). At this point, cell separation and root cap turnover cease as long as the existing cells are not removed. When the accumulated cells are removed by gentle agitation of root tips in water, renewed border cell separation is induced. Roots so treated are referred to herein as "induced" roots. Within 1 hr, new cells can be collected from the tips of such induced roots, and a complete new set of ~4000 cells separates from the cap within 24 hr of removing the original set of border cells (Hawes and Lin, 1990).

We have exploited this system to identify a gene with "signature" sequences common to PME-encoding genes and whose expression in peripheral cells of the root cap is correlated with border cell separation. In this study, we report the isolation of a PME-encoding gene and demonstrate that its expression is required for three phenotypes: maintenance of extracellular pH, elongation of cells within the root tip, and cell wall degradation leading to border cell separation.

RESULTS

An Inducible Root Cap cDNA Clone Has Features Common to PME Genes

A full-length cDNA clone was isolated from an induced root cap cDNA library by using a partial cDNA from the conserved 3′ half of a PME-encoding gene from French bean as a probe. The sequence of the 1799-bp insert (*rcpme1*) in pRCPME1 contained a 20-bp putative 5′ untranslated leader sequence followed by an open reading frame of 1665 bp that could encode a 555–amino acid polypeptide with a molecular mass of 61 kD (Figure 1). The proposed *rcpme1* translation initiation site (ATC AGTATGGCT) matches well with the consensus (underlined) translation initiation sequence (TAACAATGGCT) for plant genes (Joshi, 1987). The 214-bp 3′ untranslated region contains two potential polyadenylation sites (AATAAA) and a poly(A) tail (Figure 1) (Murphy and Thompson, 1988). The deduced amino acid sequence of *rcpme1* contains the signature PME motif I (xGxYxEx, where x stands for any amino acid) and motif II (GxxDFIFG) (Figure 1) (Markovic and Jornvall,

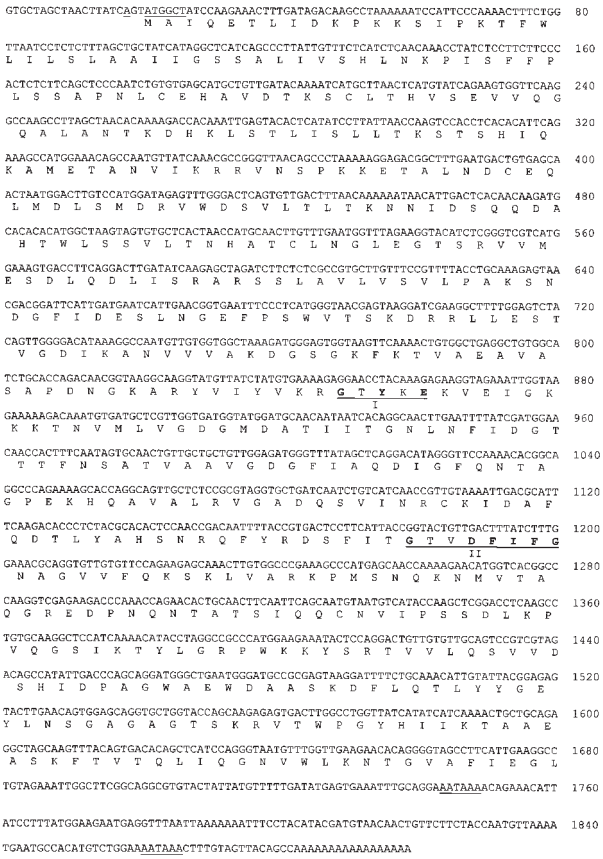

Figure 1. Structural Analysis of *rcpme1*.

Nucleotide and deduced amino acid sequences of *rcpme1* (GenBank accession number AF056493) isolated from induced pea root tips. Nucleotides are numbered from the first base after cloning site EcoRI on pBluescript SK−. The deduced amino acid sequence of *rcpme1* is below the nucleotide sequence in single-letter code. The translation initiation site and potential polyadenylation signals are underlined. The PME signature motifs (I and II) are underlined and indicated in boldface.

1992). The conserved tyrosine in motif I may play a role in the catalytic mechanism. Motif II corresponds to the best conserved region, an octapeptide located in the central part of these enzymes (Markovic and Jornvall, 1992).

These properties are consistent with the hypothesis that the cDNA encodes a root cap–expressed PME, which we therefore have designated *rcpme1* (GenBank accession number AF056493). The deduced amino acid sequence of a partial cDNA, *PsPE1*, representing the 3′ half of *rcpme1*, exhibits 80% homology with the deduced amino acid sequence of the conserved 3′ half of genes encoding PMEs from tomato and other organisms (Figure 2A). *PsPE1* was used to detect homologous sequences in Arabidopsis, maize, and alfalfa (Figure 2B).

The predicted amino acid sequence of the 5′ half of *rcpme1* shares little homology with other PME gene products (Figure 2A). A cDNA, *PsPE2*, representing the 5′ half of *rcpme1*, therefore can be used to detect only the smaller subfamily of pea PMEs represented by *rcpme1.* At high stringency, DNA gel blot analysis of pea genomic DNA using *PsPE2* as a probe revealed fewer bands than were recognized by *PsPE1* (Figure 2C).

Expression of *rcpme1* in the Root Cap Is Correlated with Border Cell Separation

Steady state levels of *rcpme1* transcript are tightly correlated with border cell separation during two distinct phases of border cell development. The first phase is germination. The transcription of *rcpme1* was high as the root emerged and border cell separation was initiated, and then it declined gradually as cell separation proceeded. Once the maximum number of border cells had separated in roots ⩾25 mm in length, *rcpme1* mRNA levels were barely detectable by RNA gel blot analysis (Figure 3). The second phase is induced border cell separation. Within 5 min of inducing renewed border cell separation by removing existing cells, an increase in *rcpme1* mRNA was detectable, and levels increased to a maximum within 2 hr (Figure 4A). Twenty-four hours after induction, when the maximum number of border cells had separated (Figure 4B), *rcpme1* transcription decreased to a low constitutive level. The same pattern of inducible expression was detected whether *PsPE1* or *PsPE2* (data not shown) was used as a probe.

rcpme1 Expression Is Localized in Peripheral Cells of the Root Cap

In situ tissue print RNA blot analysis was used to localize expression of *rcpme1* within the root tip (Figure 5). No reaction was detectable in uninduced root tips (Figure 5A), but a positive reaction was detected in the peripheral cells of induced root tips, along the peripheral surface expanse from which border cells are released (Figure 5B). A similar pattern of expression was detected (Brigham et al., 1998) using whole-mount in situ hybridization.

Expression of β-Glucuronidase in Root Caps of Pea Hairy Roots under the Control of the Cauliflower Mosaic Virus 35S Promoter Is Transitory

Transgenic pea hairy roots were used to analyze the function of *rcpme1* in root development and border cell separation. This was accomplished by expressing 1744 bp of *rcpme1* antisense or sense mRNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter in hairy roots and then examining the morphology of the root tip during development. Pea is highly susceptible to transformation with *Agrobacterium rhizogenes* (Hawes et al., 1989; Robbs et al., 1991), and border cell development and expression of reporter genes in hairy roots are indistinguishable from that which occurs in whole plants (Nicoll et al., 1995). The expression of *uidA*, the *Escherichia coli* gene encoding β-glucuronidase, was used as a reporter gene to characterize the spatial and temporal pattern of expression of the CaMV 35S promoter in pea hairy roots. The results revealed that CaMV 35S–*uidA* expression occurs in emerging root caps of hairy roots (Figure 6A) but that expression within the root cap is greatly reduced later in development. Two or more weeks after the emergence of a given root, strong expression continued to be detected throughout most of the root (Figure 6B, arrow) but not in the root cap. This pattern remained stable for at least 8 months in culture.

Inhibition of *rcpme1* Expression in Pea Hairy Roots by Antisense mRNA under the Control of the CaMV 35S Promoter Is Also Transitory

When *rcpme1* antisense mRNA was expressed under the control of the CaMV 35S promoter, inhibition of *rcpme1* expression in hairy roots was confined to the same early developmental window as CaMV 35S–*uidA.* For the first week to 10 days in culture, expression of *rcpme1* was reduced by >80% compared with control hairy roots (Figure 6C). A similar reduction in *rcpme1* expression occurred in response to sense mRNA expression, presumably as a result of cosuppression (Jorgensen, 1995). After 2 weeks in culture, however, *rcpme1* mRNA expression in hairy roots expressing *rcpme1* sense or antisense mRNA was indistinguishable from that which occurred in controls (Figure 6C). This transient inhibition of mRNA expression coincided with the transient expression of CaMV 35S–*uidA* during development. At the same time that CaMV 35S–*uidA* expression in the cap ceased to be detectable by histochemical assays, CaMV 35S–*rcpme1* antisense mRNA no longer inhibited endogenous *rcpme1* expression.

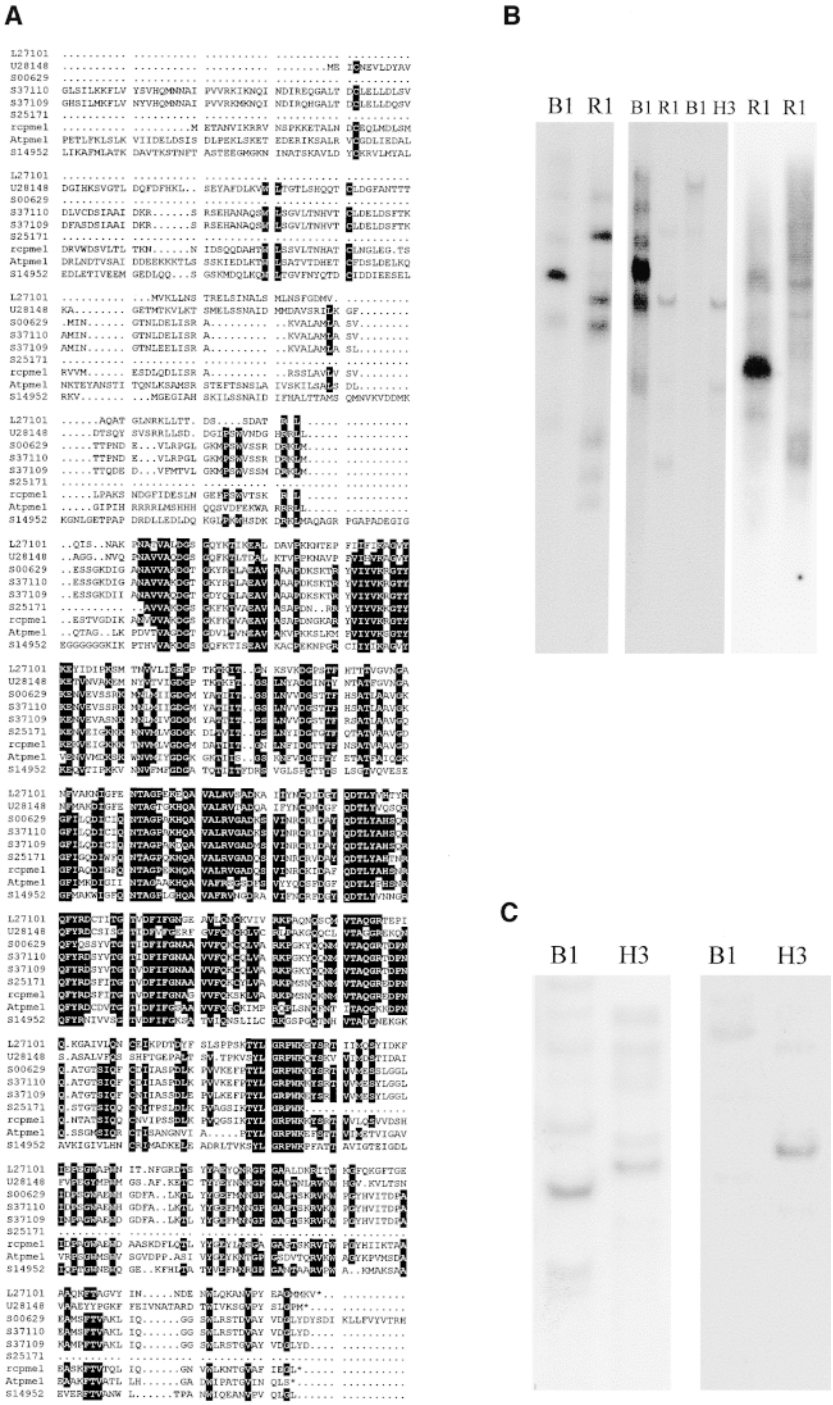

Figure 2. Homology Analysis of PME Genes.

(A) Comparison of predicted amino acid sequence of *rcpme1* with PMEs from other plants, including L27101 (petunia), U28148 (alfalfa), S00629 (tomato), S37110 (tomato), S37109 (tomato), S25171 (bean), *Atpme1* (Arabidopsis), and S14952 (rape). Sequences were aligned using the Pileup protein comparison program in the University of Wisconsin GCG sequence analysis software package (Devereux et al., 1984). Dots represent gaps introduced to optimize the alignment. Amino acids identical in six or more sequences are boxed in black.

(B) Genomic DNA gel blot analysis of sequences related to *rcpme1* in alfalfa, Arabidopsis, and maize. Genomic DNA from alfalfa (left), Arabidopsis (center), and maize (right) were digested with EcoRI (R1), BamHI (B1), or HindIII (H3) and probed with ^{32}P-labeled *PsPE1* at 65°C. The first lane in each gel is pea genomic DNA.

Inhibition of *rcpme1* Expression in Peripheral Root Cap Cells Is Correlated with an Increase in Extracellular pH

In previous studies, an assay based on fluorescein uptake was used to demonstrate that cell wall–bound PME enzyme activity is correlated with changes in extracellular pH in root cap cells of whole plants (Stephenson and Hawes, 1994). Fluorescein uptake into root cells occurs when extracellular pH is <5.5. Once inside the cell, the molecule is chemically modified, which results in a bright yellow fluorescence. Fluorescein is not taken into cells when extracellular pH is >6.0, so roots remain dark green (Dorhout and Kollöffel, 1992). During germination, extracellular pH in caps of emerging roots is >6.0, and PME activity is high. As PME activity continues, a gradual decrease in pH occurs. Once roots reach 25 mm in length 2 to 3 days after emergence and have a full complement of border cells, the extracellular pH in root caps is reduced to <5.5 and remains at this level as long as border cells are not removed.

If *rcpme1* plays a role in this change in extracellular pH, which occurs normally during border cell development, then inhibition of PME expression in transgenic hairy roots would be predicted to result in root caps whose extracellular pH does not fluctuate during border cell development but instead remains at a higher level. The fluorescein uptake assay was used to test the possibility that extracellular pH in roots expressing *rcpme1* antisense mRNA is constitutively higher than that of control hairy roots. In control hairy roots with a full set of border cells, extracellular pH was <5.5: treatment with fluorescein resulted in a bright yellow fluorescence throughout the root cap and extending upward into peripheral cells where *rcpme1* expression occurs (Figure 6D). In contrast, hairy roots expressing *rcpme1* antisense mRNA remained dark green, indicating that the extracellular pH throughout the root tip was >6.0 (Figure 6E). Efforts to reverse the pH effects by applying buffers were unsuccessful because hairy root growth was inhibited by gross changes in the pH of the growth medium (data not shown).

Root Growth Is Stunted and Cell Shape Is Altered in Roots Expressing *rcpme1* Antisense mRNA

Growth of emerging hairy roots expressing *rcpme1* antisense mRNA was stunted by >50% 1 week after subculture (60 ± 18 mm in length versus 145 ± 34 mm for control roots) (Figures 6F and 6G). This stunting occurred mainly in the region in which elongation normally occurs, between the root cap and the zone of root hair emergence (Figures 6H and 6I, area between the arrowheads) and was associated with deformities in cell shape. Whereas most cells within control root tips were square or rectangular (Figure 6J), many cells in roots expressing *rcpme1* antisense mRNA exhibited a bulging or rounded shape (Figure 6K). This area of cellular deformity corresponded closely with the region encompassed by altered uptake of fluorescein (Figures 6D and 6E).

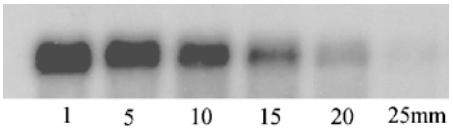

Figure 3. Expression of *rcpme1* during Emergence of the Root.

RNA gel blot analysis of *rcpme1* expression during early development of the root. *PsPE1* was used to probe an RNA gel blot containing mRNA samples isolated from roots 1, 5, 10, 15, 20, and 25 mm in length. Expression was high during emergence, when border cell separation was initiated, and gradually subsided as the number of border cells leveled off, with ~4000 cells being detected when the root was 25 mm long (Stephenson and Hawes, 1994). Results illustrate a pattern that was detected in three independently replicated experiments.

Border Cell Separation Is Inhibited in Roots Whose *rcpme1* Expression Is Inhibited by Antisense mRNA

In roots expressing *rcpme1* antisense mRNA, border cells were made, but instead of dispersing into suspension when roots were immersed in water, as do control roots (Figure 6H, arrow), they accumulated in a ball at the root tip (Figure 6I, arrow). When this ball was mechanically teased from the root cap, it became a cohesive detached clump (data not shown), and the root cap had apparently normal contours (as in Figure 6E).

When a normal root tip is sectioned for microscopy, border cells dissociate readily from the root in response to processing and handling, leaving the root cap periphery smooth and free of border cells (Figure 6J). In contrast, the tips of roots expressing antisense mRNA exhibited a ragged boundary resulting from the presence of border cells that remained associated with the root periphery (Figure 6K). Like other cells within the root tip expressing *rcpme1* antisense mRNA, border cells in the same root were deformed compared with control cells (Figure 6K, arrow).

Figure 2. (continued).

(C) Genomic DNA gel blot analysis of pea using probes *PsPE1* (left) or *PsPE2* (right), cDNA sequences representing the conserved 3′ half of *rcpme1* or its unique 5′ half, respectively. Genomic DNA was digested with BamHI (B1) or HindIII (H3).

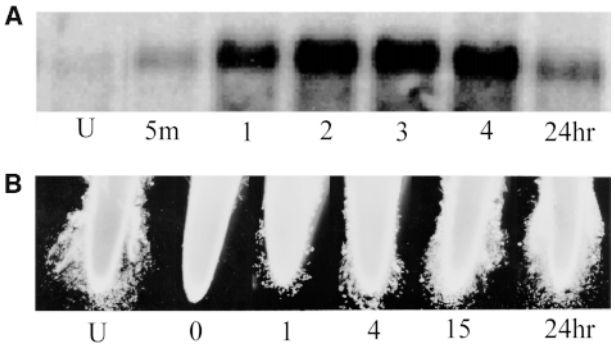

Figure 4. Expression of *rcpme1* after Experimental Induction of Border Cell Development.

(A) RNA gel blot analysis of *rcpme1* expression in uninduced 25-mm roots (U) and at 5 min (5m), 1, 2, 3, 4, and 24 hr after induction. The same results occurred in two independently replicated experiments.
(B) Border cell production after experimental induction by removal of existing border cells from uninduced (U) roots. The appearance of the root tip, as border cell number increased, is shown at time 0 and after 1, 4, 15, or 24 hr.

Changes in Root Tip Extracellular pH, Elongation, Cell Shape, and Border Cell Separation Are Transitory and Reversible within Antisense Roots

The observed changes in extracellular pH, cell morphology, root growth, and border cell separation that occurred in transgenic hairy roots were reversible. After roots were 2 weeks old, at the time when CaMV 35S antisense mRNA expression within the root tip becomes undetectable by reporter gene or RNA gel blot analysis, a normal appearance and function were recovered. Fluorescein uptake, cell shape, root elongation, and border cell development in root tips of roots expressing *rcpme1* antisense mRNA were indistinguishable from those of control roots.

rcpme1 Encodes a PME

In vitro translation of *rcpme1* yielded a protein of ~61 kD, the predicted size based on the gene sequence (Figure 1). When assayed using standard procedures, a positive dosage-dependent reaction for pectin demethylation was detected within 5 min (data not shown).

DISCUSSION

The controlled breakdown of polymers within the wall by endogenous cell wall–degrading enzymes has been proposed to play a role in ripening, abscission, cell division, growth, respiration, signal transduction, and pollen development (reviewed in Fischer and Bennett, 1991; Carpita et al., 1996). In the best-studied system, the inhibition of expression of PGs and PMEs in tomato causes predictable effects on the chemistry of cell wall polymers and can slow senescence but has little or no impact on growth and development (Tieman et al., 1992; Tieman and Handa, 1994). We report the cloning and functional analysis of an inducible root cap gene whose expression appears to be critical for root development and whose deduced amino acid sequence contains signature sequences common to PMEs from bacteria, fungi, and other plants. Based on its sequence and the tight correlation of its expression with PME enzyme activity and border cell separation in the root cap during development, we designated the gene *rcpme1*, confirmed that its product exhibits PME activity in vitro, and examined predictions of the hypothesis that it plays a role in solubilization of the cell wall.

Effect of *rcpme1* Expression on Root Development and Border Cell Separation

Our data are consistent with the hypothesis that expression of *rcpme1* in pea root caps influences cell shape, root growth, and border cell separation. The transitory expression of *rcpme1* expression driven by the CaMV 35S promoter in the root tip region offered an unusual opportunity to examine the impact of this gene on cellular development. The CaMV 35S promoter is expressed within the root cap

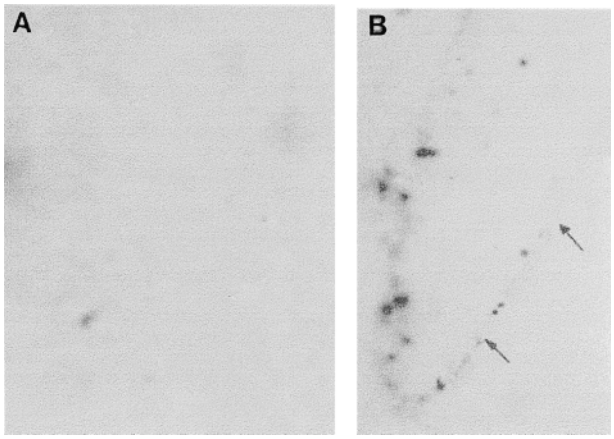

Figure 5. Localized Expression of *rcpme1* in Peripheral Cells of the Root Cap.

(A) An uninduced root tip hybridized with a *PsPE1* probe showed no reaction, and tissue prints were invisible.
(B) Tissue print of an induced root hybridized with a *PsPE1* probe. A positive reaction is detected as a dark border along the periphery of the root tip, as indicated by arrows. The same results were obtained in five independent tests.

during the first 2 weeks of development. At this point, CaMV 35S promoter expression becomes undetectable in the root cap, even though its expression remains high in the rest of the root. This made it possible to examine the impact of root cap–localized expression of *rcpme1* on cellular development for that 2-week period during which its expression was inhibited by CaMV 35S antisense mRNA. We could then compare these effects by using the same tissues in the same roots after *rcpme1* expression had returned to normal levels. As long as *rcpme1* antisense mRNA was expressed in the root cap, *rcpme1* expression in the root cap was reduced, and root growth, cell shape, and border cell separation were all visibly affected. Once the CaMV 35S promoter–driven expression of antisense mRNA in the cap ceased, normal *rcpme1* expression resumed. This provided very strong internal controls to allow interpretation of the ways *rcpme1* expression in peripheral cells of the root cap can affect cellular development, morphology, and function as well as cell wall degradation leading to cell separation.

Effect of *rcpme1* Expression on Extracellular pH

Changes in cellular development and cell separation, which occurred when *rcpme1* expression was inhibited, were correlated with a change in extracellular pH large enough to detect using an assay based on fluorescein uptake. These observations support a conceptually simple, long-standing experimental model for cell wall function—that PME activity within the cell wall generates an extracellular pH gradient that exerts a multitiered influence on the cell's biology (Collmer and Keen, 1986; Gorshkova et al., 1997). Such a gradient could account for all of the three phenotypes—changes in cell shape, root elongation, and cell separation—observed in transgenic roots whose *rcpme1* activity was inhibited by antisense mRNA expression. The acid growth hypothesis predicts that low pH at the cell wall is required for normal cellular elongation; therefore, distorted cell shape and reduced elongation are predictable effects of increased extracellular pH during critical phases of cell development (Cleland and Rayle, 1978). Cell wall solubilization leading to border cell separation would be expected to require the activity of pectin-degrading enzymes, such as PGs (Hawes and Lin, 1990) with acidic pH optima (reviewed in Collmer and Keen, 1986). In the absence of PME expression, the pH of the cell wall milieu at the root cap periphery may never reach levels appropriate for enzymatic solubilization of carbohydrate polymers that must precede border cell separation. As a result, border cell separation is inhibited in transgenic roots whose extracellular pH remains >6.0.

The increased extracellular pH, as measured by uptake of fluorescein into cells, extended well beyond the peripheral cell layers where *rcpme1* expression was detected. One explanation for this observation is that as PME deesterifies pectin in walls of peripheral root cap cells, depolymerization by enzymes, such as PGs (Hawes and Lin, 1990) and PLs (Twell et al., 1991), ensues. As a result, small acid-generating molecules may be released extracellularly, where they disperse away from the cell of origin via the root cap apoplast, which provides a continuous pathway allowing rapid movement (1 mm per min) of molecules up to a formular weight of 600 (Bayliss et al., 1996). Alternative hypotheses include the possibility that PME activity results indirectly in the solubilization of oligosaccharides that act as short-range signals to activate chemical changes throughout the cap, or that *rcpme1* expression occurs within the interior of the cap under developmental or environmental conditions that were not detected by our assays.

Irrespective of the mechanism, a PME-generated pH gradient encompassing the entire root cap and the apical meristem could affect cell surface charge, electrolyte balance, secretion, nutrient uptake, tolerance to minerals and toxins, and sensing of gravity and other stimuli. Such a gradient also could play a role in the switch in gene expression within the cap that occurs in response to the experimental removal of border cells (Brigham et al., 1998). In *Dictyostelium discoideum*, reduced extracellular pH causes a developmental shift from spore to stalk formation and is associated with the selective activation of the expression of some genes but not others (Town et al., 1987).

Our study provides evidence that, as proposed (e.g., Moustacas et al., 1986; Charney et al., 1992), endogenous PME activity in plant cell walls plays a crucial role in plant growth and development. The fact that even partial inhibition of *rcpme1* expression can cause such effects highlights the importance of this gene in cellular metabolism in plants.

METHODS

Plant Material

Pea (*Pisum sativum* cv Little Marvel; Royal Seeds, Kansas City, MO) seeds were surface sterilized as described previously (Stephenson and Hawes, 1994). Roots of varying lengths were selected by direct measurements. In certain experiments, border cells were removed to induce pectin methylesterase (PME) activity, as described previously (Hawes and Lin, 1990; Stephenson and Hawes, 1994).

Induction of Border Cell Separation

Border cells were collected from root tips during germination, beginning when roots were 5 mm in length, according to Hawes and Lin (1990). Border cell number increases with increasing root length for $\sim$24 hr, until the root is $\sim$25 mm long and $\sim$4000 cells are present in a sheath around the root cap. At this stage in development, border cell separation ceases, such that the number of cells per root remains constant as root growth proceeds. The process can be induced and synchronized by removing the existing border cells by gentle agitation in water (Stephenson and Hawes, 1994). Root caps so treated are referred to here as "induced root tips," and "uninduced root tips" are those with a full complement of border cells.

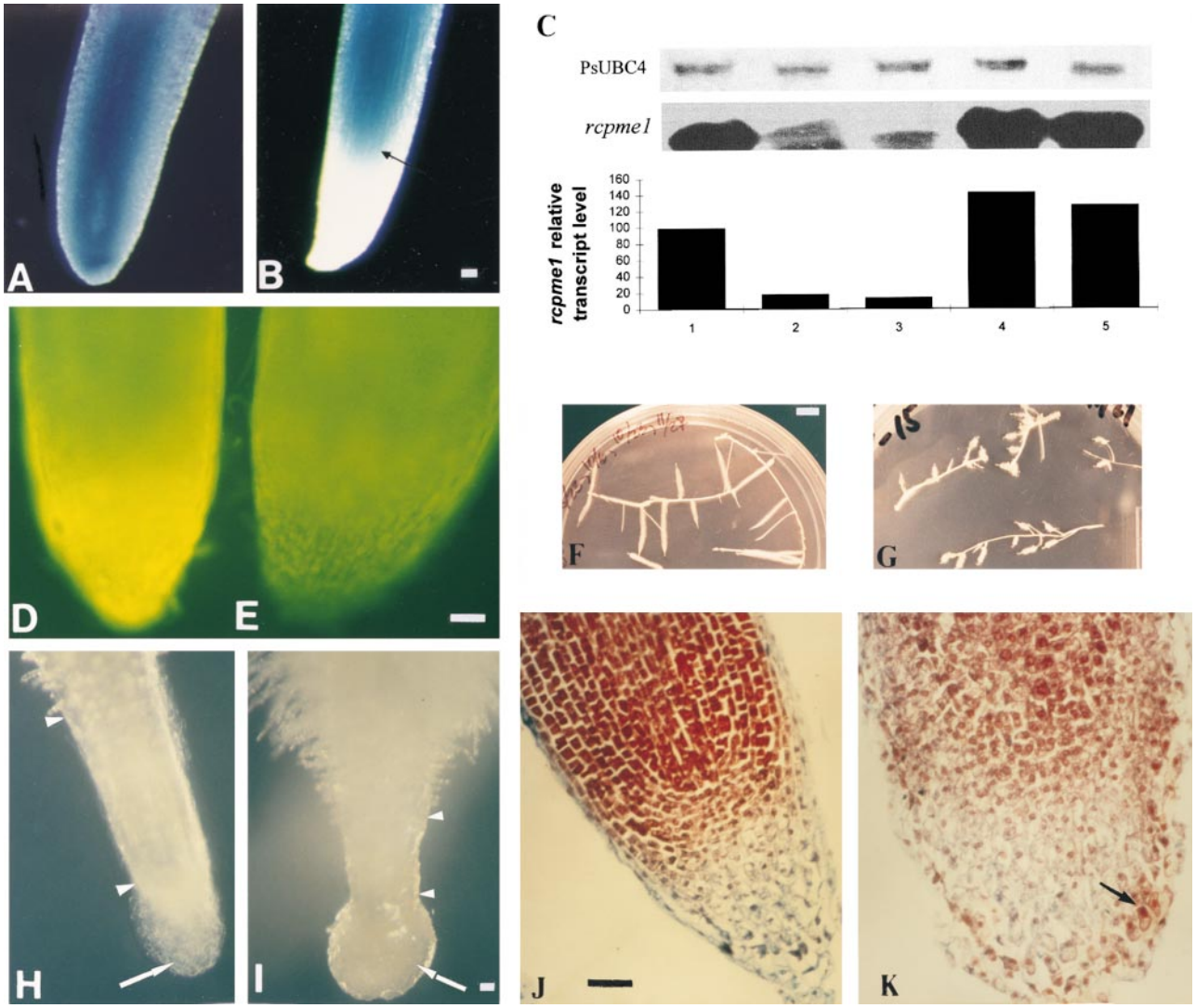

Figure 6. Effect of *rcpme1* Antisense mRNA Expression on Root Development.

(A) and **(B)** Use of CaMV 35S–*uidA* as a reporter to determine expression in hairy roots of pea. In emerging hairy roots, expression in the root cap is detectable as a blue stain **(A)**. In hairy roots that have been in culture for >2 weeks, expression is no longer detectable in root caps, but a strong positive reaction is evident above the root cap (arrow) **(B)**. Dozens of roots among 15 independently generated replicate clones were evaluated over the course of 3 years, and representative samples are shown. Bar = 100 μm for **(A)** and **(B)**.

(C) Transitory inhibition of *rcpme1* expression in hairy roots expressing *rcpme1* antisense mRNA. Expression of *rcpme1* in different roots was determined by RNA gel blot analysis using a ^{32}P-labeled single-strand *rcpme1* transcript as probe in two independently replicated experiments. PsUBC4, a gene encoding pea ubiquitin conjugating enzyme (Woo et al., 1994), showed equal expression. Values represent relative intensity of RNA gel blot samples of (bar 1) vector-only control hairy roots after 1 week in culture; (bar 2) transgenic hairy roots expressing *rcpme1* antisense mRNA after 1 week in culture; (bar 3) transgenic hairy roots expressing *rcpme1* sense mRNA after 1 week in culture; (bar 4) transgenic hairy roots expressing *rcpme1* antisense mRNA after 2 weeks in culture; (bar 5) root tips of induced 25-mm pea roots (3 days after emergence).

(D) and **(E)** Altered extracellular pH in root tips of transgenic hairy roots. Control hairy roots **(D)** exhibit an ability to take up fluorescein throughout the root cap, indicating that the extracellular pH is <5.5. In contrast, hairy roots at the same developmental stage expressing *rcpme1* antisense mRNA **(E)** do not take up fluorescein and remain dark, indicating that the extracellular pH is >6.0 (Dorhout and Kollöffel, 1992). Fifty control and 50 antisense mRNA roots were compared. A few roots exhibited patterns that were distinct from the majority or were inconclusive, but the photographs represent a pattern that is representative of at least 95% of the samples. Bar = 100 μm for **(D)** and **(E)**.

(F) and **(G)** Stunting of root growth in roots expressing *rcpme1* antisense mRNA. After 1 week in culture, control hairy roots **(F)** are >100 mm in length, whereas antisense roots **(G)** are reduced by >50%. Results of **(F)** to **(K)** represent root clones from >12 independent transformations conducted over an 18-month period, with dozens of replicate plate cultures and hundreds of individual roots. Bar = 10 mm for **(F)** and **(G)**.

cDNA Library Construction and Isolation of PME cDNA Clones *PsPE1*, *PsPE2*, and *rcpme1*

PME activity was induced by removing border cells from root caps of 25-mm long roots (Stephenson and Hawes, 1994). After incubation at 24°C for 2 hr, induced root tips (2 to 3 mm) were excised, and total RNA was extracted (Carrington and Morris, 1984). Poly(A)$^+$ RNA was extracted using Poly-A-Tract mRNA isolation systems (Promega). The cDNA library was constructed using 2 μg of poly(A)$^+$ RNA from induced root tips, as described in the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). The amplified library was screened with a ^{32}P-labeled French bean PME cDNA, *PvVPE3* (GenBank accession number X85216). After three rounds of screening, a single, isolated, positive plaque was chosen for in vivo excision of pBluescript SK− from UNI-ZAP XR, as instructed by the manufacturer (Stratagene). This cDNA clone was named *PsPE1*. RNA gel blot analysis revealed that *PsPE1* is not a full-length cDNA clone but instead represents the 3′ half of the PME mRNA.

To obtain a cDNA clone representing the 5′ half of the PME mRNA, *PsPE2*, we synthesized PME-enriched cDNAs by using poly(A)$^+$ RNA from induced root tips as template and a 34-bp oligonucleotide containing a XhoI site and corresponding to the sequence of *PsPE1* 60 bp from the 5′ end as a primer. A PME-enriched cDNA library was constructed as described in the ZAP-cDNA synthesis kit. ^{32}P-labeled *PsPE1* was used as a probe to screen this library.

To obtain the full-length PME cDNA clone *rcpme1*, we used ^{32}P-labeled *PsPE1* as a probe to screen a cDNA library synthesized from induced root tips.

DNA Sequencing

Representative clones *PsPE1*, *PsPE2*, and the full-length cDNA clone *rcpme1* were subjected to DNA sequence analysis. Plasmid DNA was purified using the Plasmid Midi kit (Qiagen, Chatsworth, CA) and then sequenced automatically using vector primer M13-20 and the reverse primer at the Biotechnology Center at the University of Arizona. Oligonucleotides were synthesized according to the sequence information obtained and were used directly as primers for further sequencing. Manual dideoxynucleotide sequencing was conducted according to the instructions accompanying the Sequenase version 2.0 kit (U.S. Biochemical).

Sequence alignment and comparison with PME sequences from other organisms were performed using the Genetics Computer Group (Madison, WI) software and the GENEMBL data library (Devereux et al., 1984).

RNA Gel Blot Analysis of PME Expression

Poly(A)$^+$ mRNA was extracted from the tips (2 to 3 mm) of roots with varying lengths during development. Alternatively, poly(A)$^+$ mRNA was extracted from uninduced or induced root tips at different times after removal of border cells. Stem or leaf tissue was collected from plants grown for 60 days. One microgram of poly(A)$^+$ mRNA from each sample was denatured with formaldehyde and separated by electrophoresis on a 1% agarose gel under denaturing conditions. Gels were blotted to a Hybond N membrane (Amersham) with 10 × SSC (1 × SSC is 0.15 M NaCL and 0.015 M sodium citrate) and hybridized under stringent conditions in 50% formamide, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), and 1% SDS at 42°C overnight with ^{32}P-labeled *PsPE1* or *PsPE2*. After hybridization, membranes were washed at room temperature three times for 20 min each in 1 × SSC and 0.1% SDS, followed by one wash in 0.2 × SSC and 0.1% SDS at 65°C for 15 min before x-ray film was exposed to them.

Tissue Print RNA Blot Analysis

The tips (10 mm) of induced roots were excised and split longitudinally into two equal halves. The tissue printing of these freshly split roots was performed as described by Cassab and Varner (1987) using Hybond N$^+$ membranes (Amersham). The riboprobe of *PsPE1* was labeled using digoxigenin. Tissue print RNA blot hybridization was performed as described previously (Tire et al., 1993). Controls included uninduced root tips subjected to the same treatments.

Construction of Transformation Vectors and Trangenes

A 1744-bp fragment of *rcpme1* was polymerase chain reaction amplified with primer 1 (5′-ATCAGGAGCTCAGCCCTTATTGTTTCTCATC-3′) containing a created Sst1 site and primer 2 (5′-AGTTCGGATCCTCCAGACATGTGGCATTCAT-3′) containing a created BamHI site (positions 116 and 1860 in the *rcpme1* sequence, respectively). This polymerase chain reaction–amplified fragment was digested by BamHI and SstI simultaneously and then inserted in both sense (*rcpme1*S) and antisense (*rcpme1*A) orientations under the

Figure 6. (continued).

(H) and **(I)** Inhibition of root elongation and border cell separation in hairy roots expressing *rcpme1* antisense mRNA. In control hairy roots **(H)**, the region of elongation (designated by arrowheads) is several millimeters in length, compared with that (indicated between the two arrowheads) in roots expressing antisense mRNA **(I)**. In control hairy roots **(I)**, border cells disperse into suspension upon contact with water (arrow in **[H]**), but in antisense mRNA roots, border cells accumulate in a ball that does not separate from the root upon immersion in water (arrow in **[I]**). Bar = 100 μm for **(H)** and **(I)**.

(J) and **(K)** Distortion of cell shape and structure in hairy roots expressing *rcpme1* antisense mRNA. In tips of control hairy roots **(J)**, cell lineages are sharply defined, most cells are elongated or square, and the root periphery is smooth because border cells disperse during the process of sectioning for microscopy. In contrast, cells within roots expressing *rcpme1* antisense mRNA **(K)** are rounded, and a ragged boundary of still-attached border cells (arrow) is present on the cap periphery. Bar = 100 μm for **(J)** and **(K)**.

control of the cauliflower mosaic virus (CaMV) 35S promoter in vector pBI121 whose *uidA* gene was removed by digestion with BamHI and SstI. The resulting constructs pBI*rcpme1*S and pBI*rcpme1*A were mobilized into *Agrobacterium rhizogenes* R1000 through triparental mating using pRK2013 as helper strain and kanamycin as selectable markers (Ditta et al., 1980; Tieman et al., 1992). R1000/pBI121 (CaMV35S–*uidA*) was used to characterize the CaMV 35S promoter expression in root caps of pea hairy roots.

Transformation

pBI*rcpme1*S and pBI*rcpme1*A were transformed into pea stems by using *A. rhizogenes* R1000 containing a kanamycin resistance gene as a selectable marker. Pea seeds were sterilized as described above. Sterilized seeds were germinated on 1% water agar in magenta vessels at 24°C in the dark until hypocotyls reached ~1 cm in length. Subsequently, seedlings were incubated at 24°C with a 16-hr light period. Sterile stem segments (1.5 to 2 cm long) were transferred aseptically in an inverted position to TM-1 solid medium (Shahin, 1985) containing 500 mg/L carbenicillin. A 3-μl drop of bacterial suspension (10^8 cells mL^{-1}) was then placed on the upper surface of the stem section. The plates were incubated at 24°C, with a 16-hr photoperiod, and 2 μE m^{-2} sec^{-1} light intensity. Ten to 15 days after inoculation, hairy roots emerged from the upper surface of the inoculated stem (Nicoll et al., 1995).

One to 2 weeks after the emergence, the primary hairy roots induced on pea stems were excised and cultured on hormone-free Gamborg's B5 medium (Sigma), pH 5.8, with 1% Difco agar, 100 mg of kanamycin, 500 mg of carbenicillin, and 20 g of sucrose per L. Putative positive hairy roots (selected on kanamycin) were subcultured once a month on the same medium without kanamycin. Two to 4 weeks after subculture, sufficient material was available for RNA gel blot analysis. For confirmation of transformation, genomic DNA from independent transformants was digested with BamHI and analyzed by DNA gel blotting using a ^{32}P-labeled CaMV 35S promoter fragment as a probe. The frequency of transformed stems that gave rise to hairy roots was ~85%. Among pBI*rcpme1*A and pBI*rcpme1*S transformed hairy roots, 80% were kanamycin resistant. Results reported here represent 10 independent transformations conducted over an 18-month period, with dozens of replicate plate cultures and hundreds of roots.

β-Glucuronidase Assay

Histochemical localization of β-glucuronidase activity in hairy root tissues was performed by incubating tissues at room temperature in 50 mM sodium phosphate, pH 7.0, containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (1 mM), by standard procedures (Liang et al., 1989; Schmid et al., 1990). Hairy roots were examined microscopically.

Fluorescein Assay for Extracellular pH

Hairy roots induced by wild-type *A. rhizogenes* R1000 and pBI*rcpme1*A, respectively, were incubated in 0.5% fluorescein for 15 min, washed three times in water for 20 min, and immersed in water for 14 to 18 hr to remove excess dye. Fluorescein uptake was evaluated by direct observation using a microscope (model D-7082; Carl Zeiss, Oberkochen, Germany) outfitted with an ultraviolet radiation source (Dorhout and Kollöffel, 1992).

Histology

Hairy root tips induced by R1000 and pBI*rcpme1*A, respectively, were excised 1 cm from the apex into HC tissue fixative MB (Amresco, Solon, OH), dehydrated in an ethanol and butanol series, embedded in Paraplast (Sigma), sectioned in 10-μm sections, dried on slides, and stained with 2% aqueous safranin O and 0.5% Fast Green in 95% ethanol. Sections through the transverse meristem (Popham, 1955) were used for analysis.

Riboprobe for RNA Hybridization and Extraction of Genomic DNA for DNA Gel Blot Analysis

A single-strand RNA probe (riboprobe) was synthesized according to MAXIscript in vitro transcription kits (Ambion, Austin, TX). *rcpme1* mRNA levels in transgenic hairy roots were detected by RNA gel blot analysis using ^{32}P-labeled single-strand *rcpme1* transcript as probe. Quantification of *rcpme1* level was conducted with a Macintosh computer using the public domain National Institutes of Health Image program. Genomic DNA from pea leaf and stem and from maize leaves was extracted according to the modified CTAB (hexadecyltrimethylammonium bromide) method of Murray and Thompson (1980). DNA from alfalfa and Arabidopsis leaves was extracted according to Saghai-Maroof et al. (1984). DNA from different species was digested (10 μg each) for 6 hr at 37°C with different restriction enzymes and separated on a 0.8% agarose gel. The DNA was transferred to Hybond N^+ membrane, according to the instructions of the manufacturer. Hybridizations with the ^{32}P-labeled *PsPE1* and *PsPE2*, respectively, were performed overnight at 55°C. After hybridization, the membranes were washed twice in 2 × SSC and 0.5% SDS (65°C for 20 min) before autoradiography.

In Vitro Translation of *rcpme1* and Enzyme Activity of the Product

In vitro translation of *rcpme1* was performed in a coupled transcription/translation system (TNT coupled reticulocyte lysate system; Promega), in the presence of Transcend tRNA (Promega), to produce labeled protein. The protein was electrophoresed on an SDS–polyacrylamide gel, blotted onto a nitrocellulose membrane, and then visualized by binding streptavidin–alkaline phosphatase followed by colorimetric detection. Luciferase DNA was used as a positive control, and a no-DNA template was used as a negative control.

To detect enzyme activity of the *rcpme1* translation product, we pooled 300 μL of the translation mixture from the above-mentioned reaction, and the hemoglobin was removed by acid precipitation to facilitate visual detection of the reaction (Thomas et al., 1984). The reaction mixture was diluted serially into assay buffer containing citrus pectin (Sigma), bromothymol blue, and water, pH 7.4, in replicate wells of a microtiter plate (Hagerman and Austin, 1986). Negative controls included buffer only or buffer containing boiled enzyme. Commercial PME (Sigma) was used as a positive control. A positive reaction was detected within 5 min by a concentration-dependent color change from blue to yellow.

ACKNOWLEDGMENTS

We thank Karen Oishi for help and Kees Recourt for the partial bean PME cDNA clone. We thank Martha B. Stephenson for assistance in RNA gel blot analysis. This work was supported by Grant No. DE-FG03-94ER20164 from the U.S. Department of Energy.

Received December 23, 1998; accepted March 28, 1999.

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