Correlation of Pectolytic Enzyme Activity with the Programmed Release of Cells from Root Caps of Pea (Pisum sativum)¹

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ABSTRACT

In many plant species, the daily release of hundreds to thousands of healthy cells from the root cap into the soil is a normal process, whose function is unknown. We studied the separation of the cells in pea (Pisum sativum) using an aeroponic system in which separated cells were retained on the root until they were washed off for counting. We found that cell separation is a developmentally regulated, temperature-sensitive process that appears to be regulated independently of root growth. Cells were released from very young roots. When plants were grown aeroponically, cell numbers increased with increasing root length to a mean of 3400 cells per root, at which point the release of new cells ceased. The process could be reset and synchronized by washing the root in water to remove shed cells. Cell separation from the root cap was correlated with pectolytic enzyme activity in root cap tissue. Because these cells that separate from the root cap ensheathe the root as it grows and thus provide a cellular interface between the root surface and the soil, we propose to call the cells “root border cells.”

Root caps of many plant species shed cells that become part of a mucilaginous sheath that surrounds the growing root (24). These shed cells, thought to be a by-product of root cap turnover, have traditionally been referred to as “sloughed” root cap cells because of the belief that the cells were dead (21, 24, 28). To the contrary, populations of shed root cap cells from more than 30 species in 10 families examined, including cereals and legumes, exhibit 90 to 100% viability (12). The cells are capable of surviving independently of the root, under field conditions (27) as well as in culture (9, 12, 16), and they can be induced to divide and grow into callus (12). In some species, up to 10,000 cells can be released from the root cap daily (8, 12). Because of the ability of these shed cells to ensheathe the root as it grows, we will refer to the cells as “root border cells (BRD).” BRD cells have been excluded from most analyses of the root cap itself (for example, refs. 18, 19), presumably because the cells are easily detached from the root tip; any abrasion of the root or contact with liquid medium during germination or processing causes the loss of nearly all BRD cells from the root (12). Indeed, BRD cells can be experimentally defined as those which can be nondestructively dissociated from each other and from the root by gentle agitation in water (11, 16).

The function of the release of BRD cells in plant development is not known. Rogers et al. (23) pointed out nearly 50 years ago that “early recognition of the sloughing away of certain root tissues as a normal process during root growth led to little more than mere speculation as to what the role of such a process might be. The process was assigned, quite summarily it seems, the function of lubricating or protecting the advancing root tip as it forced its way into the soil.” Information about BRD cells and their properties remains limited, but recent studies have suggested that BRD cells can influence the levels of microbes that predominate in the immediate vicinity of roots (for review, see ref. 11). BRD cells exhibit specificity in their interactions with pathogens, and recognition of the cells can be important in pathogenesis (13, 14, 16, 26). Mutants of Agrobacterium tumefaciens that are deficient in chemotactic recognition of BRD cells are avirulent on pea plants grown in soil (14, 15). In addition, zoospores of the soilborne fungus Pythium dissotocum are attracted specifically to BRD cells associated with cotton root caps (8). After attraction to the cells, the zoospores germinate, penetrate the BRD cells and grow into the root, killing the plant within 1 d. BRD cells washed from the root are attractive to zoospores, which penetrate and kill the cells within minutes. However, attraction to the washed root is almost completely eliminated in the absence of BRD cells.

BRD cells are the product of a root cap meristematic region distal to and separate from the apical root meristem. The root cap has been used to study Golgi function, cell turnover, and mitosis, and as a result there are many papers describing the physiological dynamics of this tissue (reviewed in 1, 6, 7, 17, 24). However, nothing is known about processes controlling the separation of BRD cells from each other and from the organized tissue of the root cap itself. We report here that the differentiation of peripheral root cap tissue into a population of discrete BRD cells is a developmentally programmed process; that the process can be readily reinitiated and synchronized; and that the time course for cell separation is correlated with pectolytic enzyme activity.

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²Abbreviations: BRD cells; root border cells; PGA, polygalacturonide acid;
MATERIALS AND METHODS

Plant Material

Pea seeds (Pisum sativum L., cv "Little Marvel", Royal Seeds, Kansas City, MO) were sterilized by immersing for 10 min in 95% ethanol, followed by 40 min in full strength commercial bleach and 20 min in 50% bleach. Discolored or swollen seeds were discarded, and the remaining seeds were rinsed and imbibed in sterile water for 12 h, and then germinated on water agar until the radicle emerged to a length of 1 to 5 mm. The seedlings were then placed in a root growth chamber consisting of a 500-mL beaker containing 250 mL of water and covered by a sheet of aluminum foil. The roots of the seedlings were suspended through holes in the foil into water saturated air, and the chamber was sealed with plastic wrap secured with a rubber band. Plants were grown in incubators with 12 h day/12 h night cycles. BRD cells produced by a single root were collected at intervals by removing the seedling from the root growth chamber, and dipping the root into 100 μL of water and agitating gently (12, 16). The total number of cells in 10 μL was counted directly (<100 magnification). Alternatively, suspensions were diluted to approximately 100 cells per 10 μL, to facilitate rapid counting. The seedling was returned to the chamber.

Cell Separation during Development

Seedlings were transferred to the root growth chamber and were maintained aeroponically at 25°C. At hourly intervals, 30 seedlings were removed and root length was measured, and BRD cells were collected and counted. To photograph roots releasing BRD cells, the tip of a root was immersed in a 100-μL droplet of water on a microscope slide or the bottom of a plastic Petri dish, covered with a cover slip, and observed with a Zeiss STEMI SV8 dissecting microscope equipped with an automatic camera. Photographs were taken after hydration of the root cap mucilage encasing the BRD cells (about 30 s) and before cells began to dissociate from the root and become dispersed into the water. Dispersal of the cells occurred within 2 to 3 min even when care was taken not to disturb the mount.

Temperature Effects on BRD Cell Separation

Seeds were germinated on water agar at 25°C, and transferred to the root growth chamber when roots were 1 mm long. The chambers were maintained at 20, 25, 30, or 35°C, and root length and numbers of BRD cells released were measured at 2-h intervals. BRD cells were harvested into a 50-μL droplet of water and the number of cells in triplicate 10-μL samples was counted directly. At least 20 seedlings were included in each treatment.

Gravity Sensing by Roots during Development

The ability of roots to sense gravity was detected as follows: seedlings were removed from the beaker apparatus at different stages of development, and the roots were washed to remove BRD cells. The seedlings were then placed into growth pouches (Northrup King, Minnesota), oriented horizontally, and observed after 5 h. The gravitropic responses of at least 10 seedlings for each treatment were evaluated by measuring the degree of curvature of the root tip.

Pectolytic Enzyme Assays

All assays were carried out aseptically, and replicate samples were plated onto Luria broth agar to ensure that microbial contaminants were not responsible for enzyme activity. Induced root caps (1-1.5 mm segment of the root tip) harvested 1 h after BRD cell removal, and uninduced root caps (not washed) were ground in 50 μL of sodium acetate buffer (50 mM, pH 5.5) in a conical Belco tissue grinder. Replicate unfraccionated samples (25 μL) were placed in the center of a Petri dish on a thin (about 2 mm) overlay containing 0.5% PGA solidified with agarose (22). After 16 h, the plates were flooded with 1% ruthenium red or cetyl trimethyl ammonium bromide to precipitate undigested PGA; pectolytic enzyme activity was detected by the presence of cleared regions where the substrate had been degraded.

Nelson's reducing sugar assay (20) was used to measure enzyme activity during the course of BRD cell release. Seedlings were grown aeroponically in the root growth chamber until roots were 25 mm long, and were then removed and the root caps washed to induce cell release. Root cap tissue was extracted at 0 and 60 min, 15 h, 20 h, and 25 h after induction. For each treatment, 15 root caps were ground in 50 μL buffer, centrifuged to remove insoluble material, and the supernatant was frozen at -20°C. For the assay, 60 μL of tissue extract were mixed with 40 μL PGA (40% w/v), and incubated for 0, 30, and 60 min at 30°C. The enzymatic degradation of the substrate was measured with Nelson's assay for reducing sugars (20). Controls included tissue extract without substrate, boiled extract with substrate, and substrate alone.

RESULTS

Roots of seedlings in the root growth chamber developed normally and at a rate of 1 mm/h at 25°C, the same rate as those grown on agar or in growth pouches. Growth of the roots suspended in air allowed BRD cells to remain associated with roots as they grew, without loss or redistribution of the cells due to abrasion or contact with water. Thus, at any given time the total number of cells released from the root cap of an individual plant could be collected and counted. The gravitropic responsiveness of the seedlings was normal.

Separation of BRD Cells during Root Development

From the time of germination until the root was 5 mm long, no cells could be separated from the tip, even when the root caps were agitated vigorously using a Vortex mixer, or when roots were grown in solid medium which mechanically abraded the root cap (Fig. 1). The number of BRD cells per root increased with increasing root length until the root was about 25 mm long (Fig. 1), at which point cell production ceased and the number remained at 3400 ± 500 (Fig. 1B). In roots that were 10 mm or less in length, cell release was confined to the root cap, which is approximately 0.5 to 0.8 mm in length (Fig. 1A). After the point of maximum cell
Release, the cells were distributed several mm behind the cap itself (Fig. 1A), even in the absence of mechanical forces for distributing the cells.

Removal of BRD cells by washing had no effect on gravitropic responses: 5 h after BRD cell release, before new BRD cells could be detached by washing, the degree of curvature in roots without BRD cells was identical to that of those with a full complement of associated cells (data not shown). Ces- sation of BRD cell release from root caps of seedlings longer than 25 mm had no apparent effect on ability of the root tips to respond to gravity (data not shown).

Temperature Sensitivity of BRD Cell Separation

We tested the effects of temperature to determine if the process of root growth could be separated from BRD cell production. We found that while both processes were temperature sensitive, the reduction in the rate of root growth at high temperatures was not correlated with the reduction in the rate of cell release. At 25°C, the roots grew at a rate of 1 mm/h, and the root caps released an average of 175 cells per h. At 35°C the mean rate of cell release was reduced to 7 cells per h, and 25 mm roots had a total of fewer than 500 cells (Fig. 2). The rate of root growth at 35°C was reduced to 0.42 mm/h at 25°C.

Synchronization of BRD Cell Separation

Five hours after washing all BRD cells from root tips, new cells could be harvested (Fig. 3). Root caps washed to remove BRD cells and to initiate separation of new cells are referred to as "induced" caps. The separation of new cells paralleled the process that occurred during development. No cells were released for 5 h (at 25°C), or the first 5 mm of growth. After 25 h, the mean number of cells leveled off at 3400 per root. In contrast to the process in newly germinated seedlings, however, the separation process following induction was syn- chronized, with very little variation among individual seed-lings (Fig. 3). The process was repeated with individual seed-lings through three cycles, with no apparent changes in the kinetics of cell release.

BRD Cell Separation is Preceded by Activity of Pectin-Degrading Enzymes

In the overlay assay, pectolytic enzyme activity was detectable within 2 to 8 h as a clear halo around extract from induced root caps (data not shown). No clearing was observed around extract from noninduced root caps, or from BRD cells washed from the root caps. No pectolytic enzyme activity was detected around induced root caps that were not crushed prior to the assay. In the reducing sugar assay, measurements from induced root caps harvested at 0, 5, 10, and 15 min after washing did not differ from controls. However, degra- dation of substrate was detectable after 60 min, and the pectolytic enzyme activity remained high after 15 h (Fig. 4). Activity in induced root caps returned to control values within 20 h after the roots were washed. No pectolytic enzyme activity was detected in uninduced root cap tissue, or in BRD cell extracts.

All Resources for BRD Cell Separation are Contained in the Root Tip

To determine whether the entire root or whole plant participates in BRD cell separation, roots were cut at random distances from the root tip, and the cap was washed to remove BRD cells. The cut end was inserted into solidified medium in a Petri plate and the plate was inverted over a 200-mL beaker containing 150 mL water, such that the roots were suspended in saturated air. After 24 h, the roots were washed again to harvest new cells. Although the resources of the
excised cap were apparently depleted by the production of one set of viable cells, the cell release process in excised caps as well as whole roots was repeatedly reinitiated in response to cell removal. There was no difference in the induction of cell release by roots of intact plants and by excised root tips: all that was required to begin to release cells within 5 h was a 1 to 2 mm segment containing the root cap itself. The number of cells was reduced to a mean of 2800 per root, but viability was 90%. When the segment was again washed to induce cell release, the mean number harvested after 24 h was reduced to 1200, and cell viability was reduced from 90% to 1%. When excised root tips were induced a third time, only 300 cells were released within 24 h.

DISCUSSION

We have shown that the separation of root border cells from the root cap varies during the course of development. No cells were released when roots first emerged from the seed, under any of the conditions we used. In addition, when roots were grown under conditions such that the BRD cells remained associated with the root, the machinery for releasing cells from the cap apparently received a signal to stop cell release when 3400 cells were present. As the number of cells associated with the root tip did not increase beyond this point, the processes leading to cell release appear to have been turned off in response to this signal. In roots longer than 25 mm, root growth continued normally in the absence of BRD cell turnover, suggesting that the root apical meristem and the root cap meristem may be regulated independently. The fact that root growth continued at 35°C, a temperature that inhibited BRD cell release almost completely, is also consistent with the possibility that activity of the meristems that control the processes are independent of each other, although we cannot rule out the possibility that growth occurred as a result of cell elongation rather cell division. In either case, the data indicate that pea roots have regulatory mechanisms that allow them to turn the process of cell release from root caps off and on in response to environmental or developmental signals. The discovery that cell separation is not necessarily active continually may help to explain the source of a long-standing controversy. Estimates of the time required for the turnover of cap cells vary from 24 h to 9 d (1-4, 6, 7). Clowes (3, 4) used a variety of methods to calculate that the entire maize root cap containing 10,000 cells turns over within 24 h at 23°C, and theorized that 7,000 to 10,000 cells sloughed from the cap daily. In contrast, Barlow (2) reported that at 23°C the maize root cap turns over only once in 7 d. Based on our results, we suggest that the discrepancy between the estimates of Clowes and Barlow results from differences in their experimental protocols. Clowes (3, 4) grew his experimental roots in aerated water, such that BRD cells would be continually released, perhaps keeping the meristem in a constant state of activity. Barlow (2) maintained the roots on damp moss, which may not have removed BRD cells efficiently as the roots grew, so meristemic cells did not receive a signal to undergo mitosis consistently.

We have defined a simple means to synchronously reset the BRD cell separation mechanism in pea. The release of BRD cells that occurs during germination and early development (root length between 5 and 20 mm) is not uniform among individual seedlings: standard deviations in mean numbers of cells harvested from several roots of the same length were up to 100% of the mean. However, removing BRD cells from root tips that have reached the maximum of 3400 cells resets the cell separation mechanism, with minimal variation among individual roots. The availability of this simple technique for turning the process on and off provides a tool for studying the biochemical and molecular events leading to the release of BRD cells from the root cap. We used this method to demonstrate that pectolytic enzyme activity in excised root caps was correlated with the release of BRD cells. Activity was detectable several hours before the first cells could be dissociated from the root by washing and remained high until after 20 h, when cell release began to level off. The pectolytic enzyme activity appeared to be localized within the root cap tissue and could not be detected in

![Figure 3](image-url) Synchronization of BRD cell release. After seedlings were 25 mm in length, the roots were washed to remove BRD cells, then returned to the root growth chamber. Cells were harvested at indicated intervals. Values are means from at least 30 seedlings; standard deviations were less than 10% of the mean.

![Figure 4](image-url) Correlation of pectolytic enzyme activity (circles) with BRD cell release (triangles). Enzyme activity is expressed as units, where 1 unit is the amount of enzyme required to release 1 μM sugar/h.
suspensions of BRD cells or in the medium of intact root caps. The observed correlation between pectolytic enzyme activity and cell release is consistent with the hypothesis that a root cap localized pectin-degrading enzyme is involved in the systematic separation of BRD cells from each other and from root tissue.

The large numbers of BRD cells that are released from the roots of some species undoubtedly contribute much of the nutrient-rich exudate found in the immediate vicinity of the root. Such root exudates have long been known to exert a profound influence on the levels and the composition of microbial populations in the soil (5, 25, 29). However, virtually nothing is known about plant genetic or metabolic factors that influence the release of such exudates from roots. The packaging of exudates in living BRD cells that act as an interface between the root and the soil could provide an important mechanism for regulating levels of biologically active molecules that function in plant-microbe recognition.

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LITERATURE CITED