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Induction of Microbial Genes for Pathogenesis and Symbiosis by Chemicals from Root Border Cells[1]

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Reporter strains of soil-borne bacteria were used to test the hypothesis that chemicals released by root border cells can influence the expression of bacterial genes required for the establishment of plant-microbe associations. Promoters from genes known to be activated by plant factors included *virE*, required for *Agrobacterium tumefaciens* pathogenesis, and common *nod* genes from *Rhizobium leguminosarum* bv *viciae* and *Rhizobium meliloti*, required for nodulation of pea (*Pisum sativum*) and alfalfa (*Medicago sativum*), respectively. Also included was *phzB*, an autoinducible gene encoding the biosynthesis of antibiotics by *Pseudomonas aureofaciens*. The *virE* and *nod* genes were activated to different degrees, depending on the source of border cells, whereas *phzB* activity remained unaffected. The homologous interaction between *R. leguminosarum* bv *viciae* and its host, pea, was examined in detail. *Nod* gene induction by border cells was dosage dependent and responsive to environmental signals. The highest levels of gene induction by pea (but not alfalfa) border cells occurred at low temperatures, when little or no bacterial growth was detected. Detached border cells cultured in distilled water exhibited increased nod gene induction (*ini*) in response to signals from *R. leguminosarum* bv *viciae*.

The balance of beneficial and detrimental microorganisms that inhabit root systems is crucial to plant health. Plant genotype can control the ability of certain microorganisms to colonize plant roots, but the mechanism of such effects is not understood (Atkinson et al., 1975; Hawes et al., 1994). One way plants influence microbial growth and gene expression is by the programmed release of root "border" cells from their root tips (Hawes and Lin, 1990). Plants of many species produce thousands of these healthy somatic cells, which are released daily into the rhizosphere in response to endogenous and environmental signals (Hawes and Pueppke, 1986). Release of the cells occurs because the intercellular connections among cells at the root cap periphery are broken, yielding populations of separated cells with intact cell walls. We refer to these cells as root border cells to emphasize that under natural conditions, they create a physical and biological interface between the root surface and the soil environment (Hawes and Lin, 1990).

Border cells, which were called "sloughed root cap cells" before their distinctive properties were recognized, can survive in hydroponic culture (Knudson, 1919) or under field conditions (Vermeer and McCully, 1982) and can be induced to grow into organized tissue in culture (Hawes and Pueppke, 1986; Hawes et al., 1991). By definition, border cells are those cells that become dispersed into suspension in response to gentle agitation of the root tip in water (Hawes and Lin, 1990). As such, they provide a convenient in vitro system for studying cellular interactions of soil-borne microorganisms with root cells. The ability to culture border cells in water or in other simple media has been exploited to measure their ability to attract, repel, and support the growth of numerous fungal and bacterial pathogens (Sherwood, 1987; for review, see Hawes and Brigham, 1992; Brigham et al., 1995a).

Based on their selective interactions with soil-borne microorganisms, we have proposed that border cells control the ecology of the rhizosphere by the programmed release of biologically active chemicals that regulate growth and gene expression in microbial populations (Hawes, 1990; Hawes and Brigham, 1992; Hawes et al., 1996). It has been known for nearly a century that root exudates—the chemicals released from root systems into the soil—have profound effects on microorganisms in the rhizosphere (for review, see Curl and Truelove, 1986; Rovira, 1991). In recent years, specific sugars and phenolic compounds from plant exudates have been shown to induce microbial genes required for pathogenesis and symbiosis (Rossen et al., 1985; Stachel et al., 1985; Shearman et al., 1986; Peters and Long, 1988), and the cellular sources of such chemicals are now being examined (Maxwell and Phillips, 1990; Graham, 1991; Oommen et al., 1994; McKhann et al., 1997).

Several groups have used reporter genes to demonstrate that the region surrounding the root tip is a primary source of chemicals that affect the expression of the nodulation (*nod*) gene of *Rhizobium* spp. (Firmin et al., 1986; Redmond et al., 1986; Djordjevic et al., 1987; Peters and Long, 1988). In those studies a plate assay in which host plants were cocultivated overnight at 28°C with *Rhizobium* strains was used (Djordjevic et al., 1987; Peters and Long, 1988). The region of intense reporter gene induction at the root tip, distal to the region of root hair emergence, corresponds to the area where border cells are released (Hawes and Brigham, 1992). Within this region, during the 24-h period after emergence of the radicle, extracellular chemicals are released from at least three sources: (a) secretory cells

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Abbreviation: MS, Murashige-Skoog.

within the root tip, which synthesize and export a high-molecular-weight mucilage (Rougier, 1981); (b) root border cells, which synthesize and export an array of low-molecular-weight proteins (Brigham et al., 1995b); and (c) cell wall polysaccharides and other structural components that are degraded during border cell separation.

The objective of this paper was to use reporter genes to test the hypothesis that chemicals from border cells can selectively influence gene expression in soil-borne microorganisms. Instead of whole roots, bacteria were cocultivated with washed border cells, based on the premise that any reporter gene activity detected is in response to signals released from border cells during the cocultivation period. We compared the ability of border cells of pea (*Pisum sativum*), corn (*Zea mays*), and alfalfa (*Medicago sativa*) to induce genes known to play important roles in the establishment of plant-microbe relationships. The *lacZ* gene from *Escherichia coli* was expressed under the control of promoters from the virulence gene of *Agrobacterium tumefaciens* (*vir*E), the nodulation gene of the *Rhizobium leguminosarum* bv *viciae* operon (*nod*ABCIJ), the nodulation gene from *Rhizobium meliloti* (*nod*C), and the phenazine antibiotic synthesis gene from *Pseudomonas aureofaciens* (*phz*B).

*vir*E is one of the inducible genes of the *A. tumefaciens vir* regulon that is regulated in response to sugars and simple phenolic compounds (Stachel et al., 1985; Cangelosi et al., 1990). The common *nod* genes, which encode enzymes involved in the biosynthesis of signal molecules required for nodulation, are induced in response to specific flavonoid molecules from plant roots (Peters and Long, 1988; Lerouge et al., 1990; van Brussel et al., 1990). *phz*B is a gene involved in the biosynthesis of phenazine antibiotics that play a role in biological control of *Gaeumannomyces graminis* var *tritici* in wheat. The expression of *phz*B is not known to be affected directly by signals from the roots. Instead, expression is induced when conditions allow population growth to a critical threshold density in response to the accumulation of a diffusible signal from *P. aureofaciens* (Pierson et al., 1994). Conditions affecting the interaction between pea, our primary model system for examining border cell biology and function, and *R. leguminosarum* bv *viciae*, a strain that nodulates pea, were studied in detail.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Pea (*Pisum sativum* L. cv Little Marvel [Royal Seeds, Kansas City, MO]) seeds were surface sterilized by immersion for 10 min in 95% ethanol, followed by 30 min in full-strength commercial bleach. Seeds then were rinsed in distilled water and allowed to soak for 30 min. After swollen or discolored seeds were removed, seeds were placed on Petri plates containing 15 mL of solidified water agar (1.2%, w/v) overlaid with germination paper (Anchor Paper, Hudson, WI) and incubated at 24°C for 3 to 4 d in darkness. Corn (*Zea mays* cv Funk F) and alfalfa (*Medicago sativa* cv CUF-101) seeds were treated by the same procedure except that exposure to ethanol and bleach was limited to 5 min. Except in the experiments designed to test the effect of border cell age on *nod* gene induction, border cells were collected from seedlings with radicles 20 to 25 mm in length approximately 24 h after the radicle began to emerge from the seed.

Root border cells were collected as described previously (Hawes and Lin, 1990). Root tips were immersed in 100 μL of water or MS basal salts (Sigma) medium adjusted to pH 7.0 with 1 N NaOH. After 1 to 2 min, the root tips were agitated gently using a Pasteur pipette to disperse border cells. Suspensions of border cells were washed by low-speed centrifugation and then resuspended in fresh MS salts or water. Washing the cells twice was found to be sufficient to remove all residual extracellular gene-inducing activity. This was done to ensure that all gene induction that occurred during cocultivation was exclusively in response to signals from border cells. The concentration of border cells was determined by direct counts. The viability of border cells was measured by microscopic observation of cytoplasmic streaming or by staining with fluorescein diacetate (Hawes and Wheeler, 1982).

Duplicate experiments were carried out using water or MS salts for cocultivation, with no difference in results with respect to plant cell viability, bacterial cell growth, or levels of reporter gene expression. All values reported herein represent results obtained using water.

"Root tip exudate" is defined here as all material that could be washed from the tips of roots 25 mm in length, excluding border cells, after germination of seeds on 1.2% water agar overlaid with germination paper. Root tip exudate is a cell-free supernatant obtained by agitating root tips only (0.5–1 cm) in liquid, using a stream of water from a Pasteur pipette to remove all extracellular material from the root, and then centrifuging to pellet border cells. Care was taken to ensure that no part of the seed touched the liquid. Excluding chemicals that may have diffused into the germination paper and/or the water agar during this period, root tip exudate includes all material secreted by cells of the root tip and by border cells and all chemicals from cell walls released as border cells separate from the root during germination.

In most experiments the effects of border cell exudate were determined by measuring reporter gene expression in bacteria after a period of cocultivation with washed border cells. In the experiment measuring the *ini* response of border cells to *Rhizobium leguminosarum* bv *viciae*, washed border cells were incubated in water, with or without cocultivation with bacteria, for various intervals. The sample was centrifuged to pellet border cells and bacteria. The cell-free supernatant, which included all chemicals that had been released from the border cells into the external medium during cocultivation, was then incubated with fresh bacteria, and reporter gene expression was measured by standard procedures, as described below.

Bacterial Strains and Growth Conditions

Agrobacterium tumefaciens A723 pSM358, containing a *vir*E-*lacZ* fusion, was grown in MG/L liquid medium supplemented with kanamycin and carbenicillin (100 μg/mL; Stachel et al., 1985; Cangelosi et al., 1990). Strain RBL5560 pMP154, a derivative of *R. leguminosarum* bv *viciae*

LPR5045, contains the *sym* plasmid of *R. leguminosarum* bv *viciae* 248 and also pMP154 (derived from IncQ expression vector pMP190, Sm^r, Cm^r), a fusion gene consisting of *lacZ* expressed under the control of the promoter controlling expression of genes in the *nod*ABCIJ operon of *R. leguminosarum* bv *viciae*. The *Rhizobium* strain was grown in liquid yeast extract-mannitol (YEM) medium supplemented with chloramphenicol (10 $\mu g\ mL^{-1}$; Spaink et al., 1987; van Brussel et al., 1990). *Rhizobium meliloti* 1021(pRmM57), containing the *R. meliloti nodC-lacZ* fusion, was grown on TY medium supplemented with tetracycline (10 $\mu g\ mL^{-1}$) and spectinomycin (50 $\mu g\ mL^{-1}$; Yelton et al., 1987). *Pseudomonas aureofaciens* strain 30–84Z, which contains a chromosomal fusion between the phenazine biosynthetic gene *phz*B and *lacZ*, was grown in Luria Bertani or AB minimal medium (Pierson et al., 1994). Cultures of all four strains were prepared by inoculating a single colony to 100 mL of liquid medium and grown to logarithmic phase at 28°C for 18 to 20 h. Bacterial cells were collected by centrifugation and washed twice with sterile, distilled water to remove residual medium and then resuspended in water or MS salts adjusted to pH 7.0. Cell concentration was estimated by measuring the A_{600} (and confirmed by testing samples with direct plate counts) and then adjusted to 10^8 cells mL^{-1} in assays.

Growth of Bacteria on Root Tip or Border Cell Exudates

Growth of bacteria was determined by measuring bacterial numbers before and after cocultivation with border cells or incubation in root tip exudate. Border cells and root tip exudates were collected as described below.

Quantitative Assay of Reporter Gene Activity

Preparation of Bacteria and Border Cells

Bacteria were harvested after overnight growth to the logarithmic phase and were washed twice in water to remove the culture medium. Washed bacteria were diluted to a concentration of 2×10^8 cells mL^{-1} by turbidimetric estimation at A_{600} and mixed 1:1 with plant cells, for a final standard concentration of 10^8 cells mL^{-1}. Border cells were collected from root tips and washed twice in water (a treatment found sufficient to remove extracellular inducing activity), and the numbers were adjusted by direct counts. Unless otherwise indicated, plant cell number was adjusted to 6.0×10^4 mL^{-1} (approximately the number of cells from 20 pea root tips and 25–30 alfalfa root tips) and diluted 1:1 for cocultivation, for a final concentration of 3.0×10^4 border cells mL^{-1}.

Cocultivation of Bacteria and Border Cells

Unless otherwise indicated, bacteria and plant cells were mixed 1:1 and incubated for 16 h. Cocultivation was carried out in the dark, without shaking, with a total volume of 1.0 mL.

Collection of Border Cell Exudates

In one experiment in which the *ini* activity (van Brussel et al., 1990) from border cells was measured in response to bacteria, inducing activity was measured after bacteria were incubated for 16 h with border cell exudates rather than with border cells per se. The exudates were collected from border cells after 12 h at 10°C, with or without cocultivation with *R. leguminosarum* bv *viciae*. Cells were pelleted by centrifugation, and the cell-free supernatant was mixed 1:1 with bacteria and incubated for 16 h prior to enzyme assay.

β-Galactosidase Assay

In all experiments values representing β-galactosidase activity levels accumulated during the cocultivation period. The β-galactosidase assay was carried out according to standard conditions at pH 7.0 and 28°C. Enzyme activity is expressed as Miller units (Miller, 1972). At the end of cocultivation, the mixture of plant and bacterial cells was pelleted and resuspended in 0.5 mL of Z buffer and the A_{600} was measured. After the sample was vortexed with 20 μL of chloroform and 20 μL of 0.1% SDS, the enzyme assay was started by adding 100 μL of *O*-nitrophenyl-β-D-galactoside (Sigma) at 4 mg mL^{-1}. The assay was allowed to proceed for 5 min for *R. leguminosarum* bv *viciae* and *P. aureofaciens* and 30 min for *R. meliloti* and *A. tumefaciens*. The reaction was stopped by adding 250 μL of 1 M Na_2CO_3.

After cell debris was pelleted, the A_{420} was measured and Miller units were calculated by the following formula: $(A_{420} \times 1000)/(A_{600} \times \text{time of reaction in min})$. Controls for each experiment included bacteria without border cells and border cells without bacteria. Border cells never contributed significantly to the absorbance and never exhibited measurable β-galactosidase activity under the conditions tested. Control values for bacteria without border cells constitute the background level of β-galactosidase activity; these values were 79 ± 5, 295 ± 14, 250 ± 85, and 27 ± 6, respectively, for *A. tumefaciens*, *P. aureofaciens*, *R. leguminosarum* bv *viciae*, and *R. meliloti*. Each sample was tested in duplicate, and all experiments were repeated at least twice.

RESULTS

Species-Dependent Variation in Induction of Reporter Gene Expression by Chemicals from Border Cells of Corn, Pea, and Alfalfa

Conditions of the plate assay used in previous studies (Firmin et al., 1986; Redmond et al., 1986; Djordjevic et al., 1987; Peters and Long, 1988)—incubation of plant roots with bacteria overnight at 28°C—were duplicated, but in the current study the only source of chemicals was border cells. A quantitative assay was used to measure enzyme activity reflecting the level of the reporter gene product present at the end of the cocultivation period. Under these conditions, little or no correlation between gene induction and host range was observed. A very small increase in the expression of *A. tumefaciens virE* was detected in response to border cells from pea, the roots of which are highly susceptible to infection by *A. tumefaciens* (Robbs et al., 1991).

A similar increase in expression occurred in response to border cells from corn, a nonhost species. Inducing activity was not significantly different between the two plant species (Fig. 1A). Border cells of corn, the roots of which are also refractory to infection by rhizobia, nevertheless caused a slight increase in *R. leguminosarum* bv *viciae nod* gene expression (Fig. 1B). The same reporter gene was induced 2-fold by border cells from its host, pea, and was induced 5-fold by border cells from alfalfa, a nonhost species (Fig. 1B). In fact, at 28°C the only response that was directly correlated with host range was the expression of the *R. meliloti nod* gene, the activity of which was more than 2-fold higher in response to cocultivation with border cells from its host, alfalfa, than in response to nonhost pea border cells (Fig. 1C). *P. aureofaciens* was grown to a cell density at which gene expression is initiated linearly in response to density-dependent autoregulation (Pierson et al., 1994). Cocultivation with border cells did not stimulate any increase in gene expression beyond this background level (Fig. 1D).

Increase in Bacterial Numbers in Response to Border Cell Exudate

Differences in reporter gene induction were not correlated with growth of the bacteria. Border cells stimulated similar small increases in numbers of all of the test strains from 25 to 43% (Table I); the values were not significantly different from each other. Responses of bacteria to incubation in root tip exudate were similar; all four bacterial species increased by 18 to 40% (Table I). No increase in the number of cells occurred when bacteria were incubated in water or MS salts alone (data not shown).

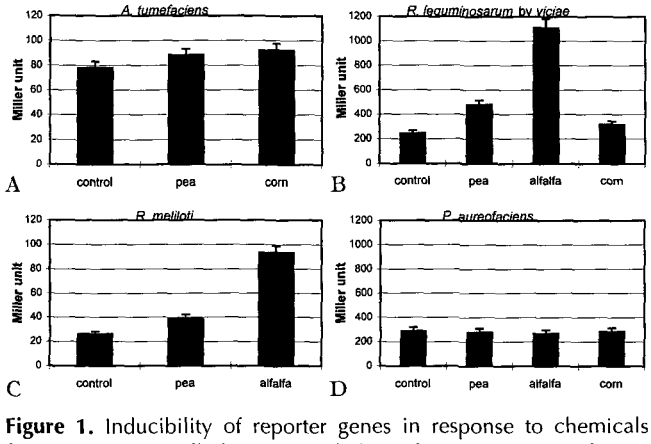

Figure 1. Inducibility of reporter genes in response to chemicals from root border cells from pea, alfalfa, and corn. A, *A. tumefaciens virE* gene. B, *R. leguminosarum* bv *viciae nod*ABCIJ. C, *R. meliloti nod*C. D, *phz*B gene from *P. aureofaciens*. Border cells (30,000 mL^{-1}) were cocultivated at 28°C with bacteria (10^8 mL^{-1}) for 16 h. Enzyme activity in bacteria was then assayed at 28°C as described in "Materials and Methods." Values are means and SEs from duplicate samples in at least two independent experiments.

Developmental and Environmental Factors Affecting *R. leguminosarum* bv *viciae Nod* Gene Expression during Cocultivation with Border Cells of Pea

Root Tip Exudate versus Border Cell Exudate

The gene-inducing activities resulting from incubation with root tip exudate, included as a control in all experiments, or with border cells were virtually the same. In three representative samples (containing material from 10 pea root tips in 1 mL), enzyme activities were 760, 814, and 918 units for root tip exudates and 754, 800, and 897 units, respectively, for border cell exudate.

Time Course

Experiments were conducted to determine how quickly border cells in isolation can release measurable amounts of extracellular *nod*-inducing chemicals. When washed border cells were cocultivated with *R. leguminosarum* bv *viciae*, induction of *nod* gene expression was detected within 4 h, and activity increased over a 24-h period (Table II). Higher levels of activity occurred with larger numbers of border cells.

Age of Border Cells

Each pea root is programmed to produce a set number of border cells by the time it is about 25 mm in length. Cells begin to separate when the root is 5 mm in length (5 h after emergence) and continue to increase in numbers for about 25 h. Cell separation then ceases and the total number of border cells per root remains at this level unless the border cells are removed (Hawes and Lin, 1990; Stephenson and Hawes, 1994; Brigham et al., 1995a). Thus, the set of border cells that is made within 24 h remains on the root indefinitely under conditions in which the cells are not removed.

To determine whether the age of border cells during a 48-h period after separation affects their ability to release chemicals that induce *R. leguminosarum* bv *viciae nod* genes, seedlings were maintained on water agar overlaid with germination paper for several days, and border cells were collected from roots 1 to 2, 2 to 3, and 3 to 4 cm in length. This corresponds to 24, 40, and 48 h after emergence of the radicle, with populations in which individual border cells range in age from about 20 to 43 h. The border cells were cocultivated with bacteria for 12 h, and units of reporter gene expression were determined. The values for the different samples, 450 ± 40, 450 ± 55, and 448 ± 37 Miller units, respectively, did not differ significantly from one another. The results indicate that short-term differences in the age of the border cells did not influence their ability to release *nod* gene-inducing chemicals.

Temperature

Expression of the *R. leguminosarum* bv *viciae nod* gene in the presence of pea border cells was detected at temperatures from 4 to 37°C (Fig. 2A). Nearly 4-fold more activity was present after cocultivation at 16 than at 28°C. The

Table I. *Growth of bacteria in response to root tip or border cell exudates as the sole nutrient source*

Root tip exudates were obtained as described in "Materials and Methods." Bacteria were cocultivated with border cells or root tip exudates. At the end of cocultivation, the concentration was estimated by turbidimetric measurements, which were confirmed by direct counts. Values are means ± SDs from duplicate samples in at least two independent experiments.

Strain	Increase in Bacterial Nos.			
	Root tip exudate		Border cell exudate	
	0 h	16 h	0 h	16 h
P. aureofaciens	1×10^8	$1.19 \times 10^8 \pm 0.13$	1×10^8	$1.31 \times 10^8 \pm 0.06$
R. leguminosarum bv *viciae*	1×10^8	$1.40 \times 10^8 \pm 0.24$	1×10^8	$1.43 \times 10^8 \pm 0.26$
A. tumefaciens	1×10^8	$1.26 \times 10^8 \pm 0.15$	1×10^8	$1.34 \times 10^8 \pm 0.06$
R. meliloti	1×10^8	$1.18 \times 10^8 \pm 0.14$	1×10^8	$1.25 \times 10^8 \pm 0.18$

effect of temperature on *nod* gene expression was not correlated with effects of temperature on viability of border cells and on the growth of *R. leguminosarum* bv *viciae* under the test conditions. Survival of border cells was inversely proportional to temperature (Fig. 2B), whereas the number of bacteria present after cocultivation was directly proportional to temperature (Fig. 2C). Thus, at 4 and 10°C, no loss in border cell viability occurred during the test period (Fig. 2B), but all cells died when incubated at 37°C. Little or no increase in bacterial numbers occurred at 10 and 16°C, when *nod* gene expression was highest, but bacterial numbers more than doubled when cocultivation was at 28 or 37°C, when *nod* gene expression was very low. Subsequent experiments with pea border cells were carried out at 10°C, which yielded the highest *nod* gene induction, with no loss of border cell viability and no increase in bacterial numbers.

The effect of temperature on the ability of border cells from two different legume species, pea and alfalfa, to induce *R. leguminosarum* bv *viciae nod* gene expression was compared (Fig. 3). The release of *nod* gene inducers from border cells of both species was significantly but inversely affected by temperature. Thus, at 28°C, border-cell-inducing activity was 1.55-fold higher in alfalfa than at 10°C, whereas activity from pea border cells was 2.18-fold higher at 10 than at 28°C. At 28°C, inducing activity was not correlated with host range. Border cells from the non-host species, alfalfa, caused a 2.8-fold higher induction of *nod* gene expression than those of the host species, pea. At 10°C, in contrast, the situation was reversed: *R. leguminosarum* bv *viciae nod* gene expression in response to pea border cells was 1.24-fold higher than that of alfalfa.

Table II. *R. leguminosarum bv viciae nod gene expression during cocultivation with pea border cells*

Pea border cells were washed twice and then cocultivated at 28°C with bacteria (10^8 mL^{-1}) for the indicated times. The level of reporter gene expression in the bacteria was then measured at 28°C for 5 min as described in "Materials and Methods." Values are means ± SDs from duplicate samples in four independent experiments.

No. of Border Cells per Milliliter	Cocultivation Time			
	0 h	4 h	16 h	24 h
	units of activity			
0	286 ± 21	292 ± 38	274 ± 18	279 ± 36
15,000	289 ± 60	342 ± 16	464 ± 22	504 ± 23
30,000	297 ± 10	369 ± 21	579 ± 87	624 ± 87

Dosage Response

In response to increasing numbers of border cells, higher levels of reporter gene activity occurred (Fig. 4). When border cells were diluted to fewer than 2000 mL^{-1} (less than half the number produced by a single root), no significant β-galactosidase activity was detected (Fig. 4), suggesting that a threshold concentration of border cells may be required for the production and/or release of *nod* gene-inducing chemicals.

ini Activity in Response to Cocultivation with *R. leguminosarum* bv *viciae*

Whole roots of host legumes incubated with *R. leguminosarum* bv *viciae* exhibit *ini* activity as a result of stimulated

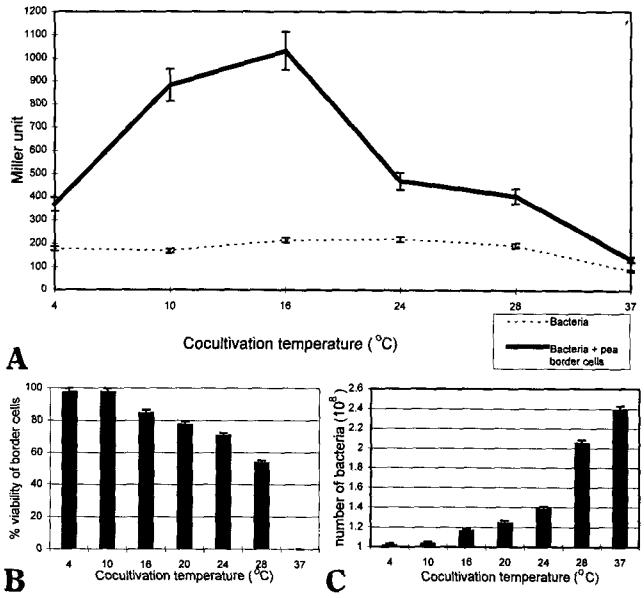

Figure 2. A, The effect of cocultivation temperature on the amount of reporter gene expression by *R. leguminosarum* bv *viciae* in response to border cells. B, The effect of cocultivation temperature on border cell viability. C, The effect of cocultivation temperature on bacterial numbers. Border cells (30,000 mL^{-1}) were cocultivated at the indicated temperatures with bacteria (10^8 mL^{-1}) for 16 h. Enzyme activity in bacteria was then assayed for 5 min at 28°C, as described in "Materials and Methods." Values are means and SEs from duplicate samples in four independent experiments.

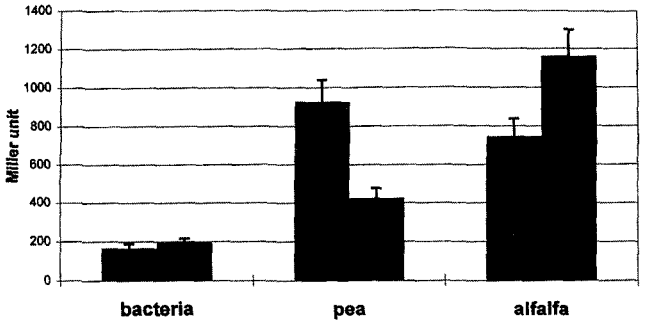

Figure 3. The effect of cocultivation temperature on *R. leguminosarum* bv *viciae nod* gene expression in response to border cells from pea and alfalfa. Border cells from both species were adjusted to 30,000 mL^{-1} and cocultivated for 16 h with bacteria (10^8 mL^{-1}) at 10°C (gray bars) and 28°C (black bars). Reporter gene expression in washed bacteria was measured as described in "Materials and Methods." Values are means and SEs from duplicate samples in two independent experiments.

Table III. *ini by border cells in response to cocultivation with R. leguminosarum bv viciae*

Border cells (15,000 mL^{-1}) were incubated in water at 10°C, with or without *R. leguminosarum* bv *viciae* (10^8 mL^{-1}), for 12 h and then centrifuged. The cell-free supernatant was then added to fresh *R. leguminosarum* bv *viciae* (10^8 mL^{-1}), incubated at 10°C for 16 h, and then assayed as described in "Materials and Methods." Cell-free supernatant from *R. leguminosarum* bv *viciae* was obtained from overnight cultivation in water without border cells as a control. Values are means ± SDs from duplicate samples in two independent experiments.

Supernatant Source	Units of Activity	Border Cell Viability
R. leguminosarum bv *viciae*	250 ± 36	NA[a]
Border cells	336 ± 42	95 ± 4%
Border cells cocultivated with *R. leguminosarum* bv *viciae*	580 ± 80	95 ± 4%

[a] NA, Not applicable.

production of *nod* gene-activating flavanones and chalcones (van Brussel et al., 1990; Recourt et al., 1991), and this response is correlated with the induction of plant genes required for their production (McKhann et al., 1997). To determine whether border cells exhibit a similar response to *R. leguminosarum* bv *viciae*, border cells were incubated for 12 h with or without bacteria. Exudate from border cells incubated in the absence of bacteria yielded significantly less *nod* gene-inducing activity than exudate from border cells cocultivated with bacteria (Table III). This increase in *nod* gene expression occurred under conditions in which no increase in bacterial numbers and no loss in viability of border cells was detected.

DISCUSSION

Root border cells constitute a uniquely differentiated and largely ignored part of the root system of many higher plants (Hawes et al., 1996). It is now clear that border cells survive and function independently of the root and that they exhibit phenotypes that are distinct even from their immediate progenitor cells at the periphery of the root cap (for review, see Hawes and Brigham, 1992). Border cells exhibit properties that potentially allow them to exert large and rapid effects on rhizosphere populations (Hawes et al., 1996). In vitro, for example, border cells can drastically and specifically alter the distribution of fungal zoospores on the root within minutes (Goldberg et al., 1989). Such effects presumably are conditioned by specific gene expression patterns now known to occur in border cells (Brigham et al., 1995b). As soon as root cap cells differentiate into border cells, a massive switch in transcription occurs, followed by the synthesis of an array of low-molecular-weight proteins that are exported almost immediately into the external environment. The functions of these extracellular border cell proteins remain to be determined.

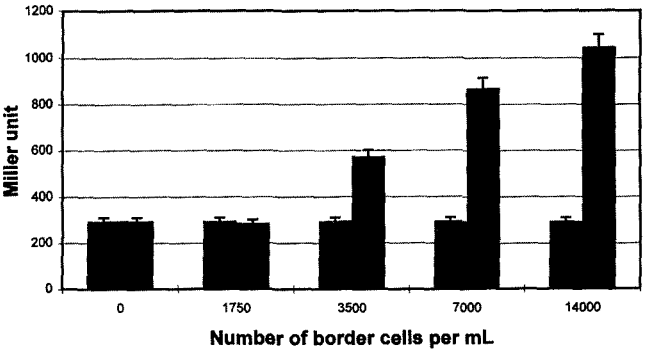

Figure 4. Dosage effect of border cells on *nod* gene expression in *R. leguminosarum* bv *viciae*. Washed border cells from 10 roots were serially diluted and cocultivated at 10°C with bacteria (10^8 mL^{-1}) for 16 h. Values are means and SEs from duplicate samples in at least two independent experiments. Gray bars, Bacteria; black bars, bacteria and pea.

In this study we report that border cells cultured in water release extracellular signals that induce microbial gene expression and that environmental conditions influence the levels of such activity. The approach was to use as markers microbial reporter genes with expression in response to specific signals that have already been shown to play important roles in plant-microbe recognition. Although root exudates are known to contain metabolites that influence the expression of microbial genes, the cellular sources of such signals have not been delineated.

The term "root exudates" can refer to anything that can be washed from roots, and terms and conditions have seldom been standardized or described sufficiently to allow comparisons among studies (Rovira, 1991). One observation that has been consistent among independent laboratories is that the root tip is rich in chemicals that specifically influence reporter gene expression (Firmin et al., 1986; Redmond et al., 1986; Djordjevic et al., 1987; Peters and Long, 1988). Because this region of intense activity corresponds with some precision to the region where border cells are released, it was of interest to test the hypothesis that border cells constitute one cellular source of signals that induce microbial gene expression. The biol-

ogy of border cells, which by definition are populations of detached, developmentally uniform cells, provides a unique opportunity to measure the contribution of one root "tissue" to gene expression in root-associated microorganisms.

The ability of border cells to induce gene expression was selective. Little or no *vir* gene or *phz* gene induction occurred in response to cocultivation with border cells of corn, pea, or alfalfa. In contrast, border cells of both legumes caused a substantial increase in the expression of *nod* genes of *R. meliloti* as well as *R. leguminosarum* bv *viciae*. The fact that border cells can influence expression of some genes but not others highlights the selective impact that the cells potentially can exert in fostering colonization by diverse microorganisms.

The release of *nod* gene-activating signals by legume border cells was responsive to environmental stimuli. Perhaps most interesting were the distinct effects of temperature on the release of *nod* gene-inducing signals from border cells of pea and alfalfa. At 28°C, the level of extracellular *nod* gene inducers released from border cells of alfalfa was 1.55-fold higher than at 10°C, whereas in pea the effect was reversed: 2.18-fold more extracellular activity occurred at 10 than at 28°C. Whether the levels of gene induction were correlated with host range therefore depended on temperature of cocultivation. Thus, at 10°C *R. leguminosarum* bv *viciae nod* gene expression was higher in response to border cells of its host, pea, than in response to border cells of the nonhost species, alfalfa, but at 28°C induction by alfalfa was higher.

The induction of *R. meliloti nod* gene expression in response to cocultivation at 28°C with border cells of its host, alfalfa, was also higher than induction by border cells of the nonhost species, pea. The divergent effects of temperature on the release of *nod* gene inducers from border cells of pea and alfalfa may correlate with the growth habits of the two species. Alfalfa (CUF101) grows in the desert southwest, where soil temperatures routinely exceed 30°C, whereas pea is a cool-weather species and its roots undergo heat shock at 28°C (Vierling, 1991). Little is understood about the actual habitats of soil-borne bacteria such as rhizobia under natural conditions, and assays are frequently designed based on laboratory conditions most conducive to rapid growth in pure culture.

In the current study, *nod* gene activation during cocultivation was independent of large increases in bacterial growth. In fact, the highest inducing activity from pea border cells occurred under low-temperature conditions, when no bacterial growth occurred at all, and the lowest levels occurred at 28 and 37°C, when bacterial growth was highest. The results indicate that the characteristics of the plant species should be considered in designing assays to examine *nod* gene expression in response to plant signals and that host range correlations obtained using in vitro assays at a single temperature should be interpreted with caution.

Flavonoid signals that induce *nod* genes are synthesized in plants via the phenylpropanoid pathway. Although rapid progress is being made in characterizing the genes involved in their synthesis (Dixon and Paiva, 1995; McKhann et al., 1997), the mechanisms by which such signals are released extracellularly to influence microbial gene expression are not known. Rhizobia are one source of signals that stimulate increased levels of extracellular *nod* gene-inducing signals from plant roots in a phenomenon described as the *ini* response (van Brussel et al., 1990). The *ini* response is correlated with an increased expression of flavonoid biosynthetic genes in specific root tissues (McKhann et al., 1997) and may occur in response to specific signals from *nod* genes (van Brussel et al., 1990; Recourt et al., 1991). Our results reveal that detached border cells, in the absence of exogenous nutrients, constitute one defined cell type that can respond to signals from rhizobia by an increase in extracellular chemicals that stimulate *nod* gene induction.

At this time, the absolute level of gene-inducing chemicals required to initiate a pathogenic or symbiotic relationship under natural conditions is unknown. Therefore, it is not possible to judge how significant the relative contribution of border cells to microbial gene induction may be based on the values observed in this study. Under soil conditions in which free water can vary drastically millimeter by millimeter, the actual concentration of chemicals could be much higher or lower than that released by 30,000 border cells into 1 mL of water. Under the conditions used, however, the levels of inducing activity from border cells during a 16-h period of cocultivation were very similar to values obtained in other studies in which whole-root exudates were used (Rossen et al., 1985; Shearman et al., 1986; Coronado et al., 1995; McKhann et al., 1997).

In addition, the amount of activity from pea border cells was virtually identical to the total inducing activity released from whole pea root tips during a 36- to 48-h period of germination. Even allowing for loss of some root tip exudate by diffusion into the agar or absorption into paper, the amount from border cells alone could easily account for a significant part of the intense reporter gene activity that occurs at root tips of legumes (Firmin et al., 1986; Redmond et al., 1986; Djordjevic et al., 1987; Peters and Long, 1988).

The fact that the age of border cells during a 2-d period had no measurable impact on their ability to induce *nod* gene expression means that a given population of border cells can influence microbial gene expression selectively for a day or more after they separate from the root tip. Because extracellular chemicals that induce *nod* genes may be important limiting factors in nodulation (Kapulnik et al., 1987), the ability of border cells to release such chemicals into the rhizosphere makes them a potentially important partner in the *Rhizobium*-legume interaction. In future studies, this hypothesis can be tested directly using transgenic roots in which the production of border cells has been genetically altered (Hawes et al., 1996).

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