

Running head: **Extracellular proteins from pea root tips**

**Martha C. Hawes**

Division of Plant Pathology and Microbiology, 204 Forbes Hall, University of Arizona, Tucson  
AZ 85721 520-621-5490; FAX: 520-621-9290. e-mail mhawes@u.arizona.edu.

Journal research area most appropriate for the paper: Plants Interacting with Other Organisms.

# **Extracellular Proteins in *Pisum sativum* L. Root Tip and Border Cell Exudates**

**Fushi Wen<sup>1</sup>, Hans D. VanEtten<sup>1</sup>, George Tsaprailis<sup>2</sup> and Martha C. Hawes<sup>1\*</sup>**

<sup>1</sup>Department of Plant Sciences, Division of Plant Pathology and Microbiology, University of Arizona, Tucson AZ 85721, <sup>2</sup>Center for Toxicology, Pharmacy 205, University of Arizona, Tucson AZ 85721

Newly generated plant tissue is inherently sensitive to infection. Yet, when *Pisum sativum* roots are inoculated with the pea pathogen, *Nectria haematococca*, most newly generated root tips remain uninfected even though most roots develop lesions just behind the tip in the region of elongation. The resistance mechanism is unknown but is correlated spatially with the presence of border cells on the cap periphery. Previously, an array of >100 extracellular proteins was found to be released while border cell separation proceeds. Here we report that protein secretion from pea root caps is induced in correlation with border cell separation. When this 'root cap secretome' was proteolytically degraded during inoculation of pea roots with *N. haematococca*, the percentage of infected root tips increased from  $4 \pm 3\%$  to 100%. In control experiments, protease treatment of conidia or roots had no effect on growth and development of the fungus or the plant. A complex of >100 extracellular proteins was confirmed, by multidimensional protein identification technology (MudPIT), to comprise the root cap secretome. In addition to defense related and signalling enzymes known to be present in the plant 'apoplast' were ribosomal proteins, 14-3-3 proteins and others typically associated with intracellular localization but recently shown to be extracellular components of microbial biofilms. We conclude that the root cap, long known to release a high molecular weight polysaccharide mucilage and thousands of living cells into the incipient rhizosphere, also secretes a complex mixture of proteins that appear to function in protection of the root tip from infection.

Plant roots detect water and nutrients by the sensing capacity of the root cap, a small organ housed within the root apex just below the apical meristem (Baluška et al., 1996). The apical meristem within the root apex adds new cells to the growing root but it is the root cap that senses water, gravity, touch, and other signals and controls the direction of root growth toward positive stimuli such as nutrients and away from deleterious stimuli such as toxins (Aiken and Smucker, 1996; Darwin, 1886; Feldman, 1984). Newly synthesized tissue such as that found in the root tip normally is inherently sensitive to physical and biotic injury. Indeed, the newly synthesized tissue in the region of elongation just behind the root tip is the primary site where infection by nematodes, fungi, and bacteria is initiated (Bauer, 1982; Curl and Truelove, 1986). Yet, surprisingly, the 1-2 mm apical region housing the root cap and root meristem in plants grown in soil, hydroponics, or laboratory conditions is largely resistant to microbial infection (Bruehl, 1986; Olivain and Alabouvette, 1999; Turlier et al., 1994). The mechanism by which the root tip is protected from infection has received little attention. However, the resistant region is correlated closely with the presence of root border cells on the cap periphery (reviewed in Hawes et al., 2003). Several lines of evidence suggest that these cells, which in most species are programmed to separate from the cap as a metabolically active population of cells into the rhizosphere, may play a role (reviewed in Hawes et al., 1998; 2000).

The infection of pea roots by the soilborne pea pathogen *Nectria haematococca* was used to test predictions of this hypothesis (Gunawardena et al., 2002). Even under conditions highly conducive to infection--including direct inoculation of roots with a suspension of fungal spores, followed by incubation in warm, moist conditions--most root tips escaped infection. Surprisingly, considering the absence of infection within root tips, was the discovery that when viewed with a microscope, a conspicuous 'mantle' of fungal hyphae was present on the cap periphery of most inoculated roots (Gunawardena et al., 2002; Hawes et al., 1998). Such hyphae, whose identity and pathogenicity were established as the inoculating strain of *N. haematococca* by expression of specific marker genes, spontaneously detached from the tip along with border cells when the root was immersed in water. After detachment of the mantle, the root tip was confirmed to be free of infection by plating onto culture medium. Border cells remained in the rhizosphere in association with detached mantles, left behind as the root tip moved downward. When root tips occasionally developed visible lesions, root cap turnover was induced and the effect was to jettison the invading fungus (Hawes et al., 1998; Gunawardena et

al., 2005). A similar mechanism of 'parasite expulsion' has been reported to occur in mammalian intestinal cells induced to undergo turnover in response to microbial infection (Cliffe et al., 2005).

In the pea-*N. haematococca* interaction, removal of border cells from roots prior to inoculation with *N. haematococca* spores resulted in a significant increase in frequency of root tip infection (Gunawardena et al., 2002). These observations supported the hypothesis that border cells play a role in protecting the root tip. Moreover, when border cells were co-cultivated with *N. haematococca* independently of the root, a complete suppression of fungal growth occurred within 48 h (Gunawardena et al., 2005). Detailed examination of hundreds of border cells growing together with *N. haematococca*, however, did not reveal hyphal penetration of any border cells. This result suggested that a product or products released into the extracellular environment might be responsible for growth inhibition and, perhaps, for preventing invasion of root tips surrounded by mantles of actively growing fungal hyphae.

In a previous study, two-dimensional gel electrophoresis was used to demonstrate that profiles of proteins synthesized by border cells are distinct from those of progenitor cells in the root cap of pea (Brigham et al., 1995). To our surprise, an array of proteins was found to be synthesized and secreted into the extracellular environment from the root cap and/or border cells as border cells undergo the process of cell wall solubilization and separate from the cap periphery. These proteins can be collected without tissue injury or cell death, because they disperse spontaneously into solution when root tips of intact seedlings are immersed in water. In the current study, the amount of extracellular protein released from pea root tips--termed the 'root cap secretome'--was quantified and found to be correlated with number of border cells present on the cap periphery. The possible impact of the secretome in protecting the root tip from infection was tested directly by measuring the ability of *N. haematococca* to infect root tips, with and without treatment with a broad spectrum protease to destroy the secretome at the time of inoculation. To follow up on previous observations using two-dimensional gel electrophoresis to document the presence of extracellular proteins released from the root tip (Brigham et al., 1995), we used multidimensional protein identification technology (MudPIT) to profile the nature of the secreted proteins. MudPIT allows analysis of complex mixtures of proteins by combining two-dimensional LC directly with MS/MS and therefore is ideally suited for profiling the root cap secretome directly as it is released into the rhizosphere (Washburn et al., 2001).

## RESULTS

### Dynamics of Extracellular Protein Delivery in Root Tip Exudates

In previous studies, pea root tips with a full set of ~4000 border cells present on the root cap periphery were exposed for 1 h to  $^{35}\text{S}$  methionine on filter paper (Brigham et al., 1995). The root tips (2-3 mm from the apex) of intact seedlings then were immersed into water to disperse border cells into suspension, which was centrifuged to pellet the border cells. Labelled proteins from excised root caps (1 mm from the apex) and the border cell pellet then were extracted and analysed by two-dimensional electrophoresis (Brigham et al., 1995). The supernatant from the washed border cells, included as a control, unexpectedly was found to contain an array of >100 proteins whose functions were unknown. Cell viability was measured based on uptake of the vital stain fluorescein diacetate and the observation of cytoplasmic streaming, as described previously (Hawes and Wheeler, 1982). Viability of the population of washed, detached border cells remained at  $90 \pm 5\%$  from the beginning to the end of the test period, so the presence of extracellular proteins could not be attributed to leakage from dead cells.

In the current study, the extracellular proteins of the root cap secretome--defined here as all the proteins released into the medium by border cells and progenitor root cap cells during the process of border cell separation--were quantified using Bradford's assay (Bradford, 1976). Exudates from each root tip of pea, collected when roots were >25 mm in length, yielded  $1.25 \pm 0.15 \mu\text{g}$  of soluble protein. Removing border cells induces renewed production of border cells by the root cap (Brigham et al., 1998; Hawes and Lin, 1990; Wen et al., 1999). Within 5-10 min after existing cells are removed, new cells can be collected from the tip and after ca 20 h a full set of ca 4000 new cells is present on the cap periphery, at which time cap turnover and border cell separation cease. Inducing root cap turnover by this method was found to induce renewed production of extracellular protein in correlation with number of border cells present (Table I). Once a full set of border cells was present, the level of protein remained stable at ca  $1.3 \mu\text{g}$  of soluble protein, per root tip.

Immersing the entire root (25 mm) into water immediately after removal of border cells and associated exudates from the tip, did not yield significant levels of extracellular protein from other parts of the root ( $0.001 \mu\text{g}$ ).

## Dynamics of Extracellular Protein Delivery in Border Cell Exudates

To determine whether populations of detached border cells continue to secrete protein after separation from the cap, protein in the supernatant of washed border cells was measured. Residual protein in the supernatant was reduced to 0.0001  $\mu\text{g}/4000$  border cells by washing, at time 0 (Table I). When the border cell pellet was immersed in fresh water, significant levels of renewed extracellular protein were detected within 15 min, and levels increased for 60 min. At 15, 30 and 60 min, respectively,  $0.08 \pm 0.04$ ,  $0.13 \pm 0.06$ , and  $0.26 \pm 0.09$   $\mu\text{g}/4000$  cells was detected. The level of extracellular protein at 2, 4, and 6 h was  $0.28 \pm 0.04$ ,  $0.27 \pm 0.03$  and  $0.27 \pm 0.06$  and was not significantly higher than at 1 h, suggesting that a plateau in protein release occurs after border cells are detached from the root cap. This plateau was not correlated with an increase in number of dead cells, whose viability remained at  $90 \pm 4\%$  for  $>6$  h. Indeed, deliberately killing border cells (by freezing or high speed centrifugation) did not result in significant release of extracellular protein. Border cell walls presumably provide a significant barrier to passive diffusion of proteins even after death; cell walls of actively growing suspension cultured cells have a pore size that would prevent diffusion of most proteins (Fleisher et al., 1999). Border cells have walls that are  $>1$   $\mu\text{m}$  in diameter and undergo secondary wall development (Hawes and Wheeler, 1982). These results suggested that viable cells capable of active secretion are required for release of proteins into the extracellular environment. When roots or border cells were treated with the secretion inhibitor Brefeldin A (Nebenfuhr et al., 2002) after removing border cells, no additional protein was detected in the extracellular milieu over a 24 h period ( $<0.001$   $\mu\text{g}/\text{root}$ ).

## Functional Impact of the Root Cap Secretome on Root Tip Infection

Most root tips escape infection when whole pea roots are inoculated with spores of the root rot pathogen, *N. haematocca* (Gunawardena et al., 2002). Because meristems are

uninvaded, growth is indistinguishable from that of uninoculated controls (Fig. 1A). Yet most root tips are enshrouded by fungal hyphae, which grow among border cell populations at the tip to develop into a 'mantle' (Fig. 1B). In response to immersion in water, mantles detach physically from the root tip, leaving the root tip uninvaded. The fact that invasion is somehow prevented while mantles of pathogenic hyphae are appressed to the cap periphery suggested the presence of a physical or chemical barrier that somehow blocks penetration into the cap tissue. The hypothesis that the proteins of the root cap secretome play a role in providing such a barrier was explored by using global destruction of the root cap secretome during inoculation with *N. haematococca*.

Roots were inoculated with fungal spores in combination with proteinase K, and frequency and severity of root tip infection were measured over the course of 3 days. The frequency of root tip infection increased from  $4 \pm 3\%$  to 100% of inoculated roots (Fig. 1C). There were no escapes; when proteins were destroyed proteolytically, every root tip developed visible necrosis and growth ceased. Microscopic examination revealed that in addition to the development of a visible lesion, there was a dramatic overgrowth of fungus (Fig. 1D, arrows); each underlying root tip developed a black rot (Fig. 1D, triangle). In control experiments, in which denatured proteinase or the digested proteins from the root tip secretome were added to inoculated roots instead of active enzyme, no increase in frequency or degree of infection occurred. This suggested that proteolytic generation of amino acid and peptide breakdown products that might act as nutrients for the pathogen, could not account for the stimulation of infection by protease treatment. However, an increase in infection did occur when washed pea roots tips were treated with Brefeldin A to prevent secretion of the extracellular proteins (as in Fig. 1B,D). Protease treatment of spores, alone, had no effect on infection or on fungal growth; and protease treatment of roots, alone, did not affect border cell viability, root growth, or gravity sensing of the root tip (not shown).

## Identification of Proteins of the Root Cap Secretome

Border cells from pea were collected from the root tip and centrifuged, as described above, and proteins in the supernatant were analysed using MudPIT. Like previous studies using

two dimensional gel electrophoresis (Brigham et al., 1995), this analysis revealed that a discrete set of >100 plant proteins is released into solution when roots with border cells are immersed into water (Table II). Some proteins represented carbohydrate metabolizing and cell wall proteins known to be components of cell walls (e.g. Albersheim et al., 1969). Such proteins, which in this study included extensin, hydroxyproline-rich glycoprotein, endoxyloglucan transferase, cell wall invertase, beta-galactosidase, cellulase, alpha-mannosidase, and oligosaccharyl transferase, have been confirmed to be present in cell walls of Arabidopsis and maize recently (Bayer et al., 2006; Zhu et al., 2006). The presence of a rhizosphere beta-galactosidase was previously identified (Celoy, 2004; Price, 2002). Calmodulin, proteases and other signal pathway associated proteins including ATPase also were found. The largest class of proteins was comprised of stress and defense related enzymes and proteins including histone H4 (Patat et al., 2004), chitinase, heat shock and dehydration responsive proteins, and reactive oxygen related enzymes such as lipoxygenase, peroxidase, and superoxide dismutase (Kawano, 2003). Also present were such 'cytoplasmic markers' as 14-3-3 proteins (Stankovic et al., 1995), ribosomal proteins, and a cytochrome P450. Only twenty proteins, including the cytochrome P450, matched proteins with predicted signal peptides (Table II).

To evaluate the contribution of detached border cells to the secretome, washed border cells were incubated for 1 h in water and the extracellular material was collected as described above and subjected to MudPIT analysis. The results revealed that proteins matching 32 of the root cap secretome species, including 14-3-3 proteins, were released from border cells within that period (Table II, asterisks). Immunolocalization was used to visualize the extracellular 14-3-3 proteins on individual border cells of pea and corn (Fig. 1).

### **Functional Impact of 14-3-3 Proteins on Fungal Infection of the Root Tip**

The presence of 14-3-3 proteins has been reported in the extracellular matrix of *Chlamydomonas* and these proteins also are thought to be secreted from human fibroblasts (Ghahary et al., 2005; Voigt and Frank, 2003). The expression of 14-3-3 proteins in plant tissues is induced in response to pathogens (Brandt et al., 1992). Because 14-3-3 proteins coordinate activity of diverse proteins through direct protein-protein interactions, their presence

in the secretome could yield clues to how so many proteins can function outside the plant cell (Ferl, 2004; Roberts, 2003). Thus, 14-3-3 proteins could function in transport, localization, stability and/or activity of the diverse proteins and enzymes secreted into the rhizosphere environment. If so, then inhibiting the function of the 14-3-3 proteins would be predicted to inhibit the ability of the secretome to function in protecting the root tip from infection. This prediction was tested using R18, a high-affinity peptide antagonist of 14-3-3 proteins (Wang et al., 1999). When roots were inoculated with R18 together with fungal spores, frequency of root tip infection increased significantly ( $p < 0.05$ ) from  $6 \pm 4\%$  to  $40 \pm 5\%$  of inoculated roots. These results are consistent with the hypothesis that 14-3-3 proteins influence root tip resistance to *N. haematococca*. However, the possibility that intracellular effects of R18 could be involved in altered root tip infection cannot be ruled out.

## DISCUSSION

The balance of microbial populations in the rhizosphere, stimulated by exudates from plant roots, has long been known to play a critical role in plant health and crop productivity (Atkinson et al., 1975; Hiltner, 1904; Hirsch et al., 2003). The components of root exudates, including those from the root cap, have been analysed and found to contain diverse products including sugars, amino acids, carbohydrates and secondary metabolites (Bais et al., 2006; Knee et al., 2000; Teplitski et al., 2000). Several investigators noted enzyme activities in the rhizosphere (e.g. Knudson, 1919, 1920; Rogers et al., 1942), but confirming the presence of proteins was difficult because most experimental systems include contributing microbial contaminants that remain difficult to control (Curl and Truelove, 1986). Antibiotics used to inhibit bacterial colonization are toxic to roots and create additional artifacts of interpretation. Indeed, pea remains our primary experimental model for border cell function because it is the only species among many tested (e.g. Hawes and Pueppke, 1986) in which we can reliably obtain roots whose root caps are free of culturable microbial species. The release of an array of newly synthesized proteins from living cells, free of microbial contamination, confirmed the secretion of proteins from the pea root cap during border cell separation (Brigham et al., 1995). In recent years the secretion of several specific endogenous proteins from the root cap, including a glycine rich glycoprotein and ribosome

inactivating proteins, has been documented (Matsuyama et al., 1999; Park et al., 2002). Basu et al., (2006) used MudPIT and LC-MS/MS to document that 52 extracytosolic proteins are released from roots of *Arabidopsis* grown in liquid shake culture for 11 days. There was little overlap among the proteins identified in that study and those in the pea root cap secretome (Table II). This may reflect the divergence in species, collection methods and seedling age, the possible presence of microbial activities, or the fact that root caps of Brassicaceae species do not produce populations of living border cells (Hawes et al., 1997; Hawes et al., 1998).

Sharp and coworkers (2006) have summarized limitations of current protein identification technology, given existing limitations in understanding plant proteome function. Large gene families, of which most pea root cap secretome proteins are members (Table II), are especially problematical with methods like MudPIT which may not distinguish among closely related protein sequences. Additional problems are inherent in the use of databases for plant species like pea whose genome sequences are not complete, and therefore extensive comparative analysis of 'probable' function based on predicted function is not warranted. In general, however, the identification of the root cap secretome proteins has revealed that the profile of the secreted proteins is, in some respects, similar to apoplast proteins extracted from other cells of the root. Thus, the 'cell wall proteome,' in the maize primary root elongation zone and root tip also includes energy related, carbohydrate processing, and defense-related proteins (Zhu et al., 2006, 2006a). Many of the secreted proteins found within the root cap secretome, including the 'pathogenesis related' chitinase and glucanase, have been known to be present within the plant apoplast for many years (Bol et al., 1990). Carbohydrate processing enzymes as well as peroxidases and other components of reactive oxygen pathways have been known to be an integral component of cell wall associated defense responses (Albersheim et al., 1969; Brisson et al., 1994; Cassab, 1998). Proteases also have been implicated in peptide based extracellular signalling responses, and the presence of ATP in the rhizosphere has recently been reported (Bergey et al., 1996; Stacey G, personal communication). That the root cap secretome is not strictly synonymous with the cell wall proteome was indicated by the observation that two cell wall enzymes known to be present in peripheral cells of the pea root cap were not represented (Hawes and Lin, 1990; Wen et al., 1999). Thus, a polygalacturonase and a pectinmethylesterase can be extracted from cell walls by treatment with NaCl but neither activity was detected in the water soluble materials collected from pea root tips (Table II). Conversely, a beta-galactosidase

which is secreted from pea border cells is not detectable within root cap tissue (Celoy, 2004; Price, 2002).

Actin, 14-3-3 proteins, glyceralde 3 phosphate dehydrogenase, heat shock proteins, and histones, generally viewed as cytoplasmic markers, increasingly have been implicated in extracellular functions (Chivasa et al., 2005). Actin was reported to be associated with plasmodesmata within the cell wall by White et al., (1994) who speculated that it might play a role in cell-cell communication. Secreted 14-3-3 proteins appear to play a role in cross-linking of hydroxyproline rich glycoproteins in the *Chlamydomonas* cell wall (Voigt and Frank, 2003) and also are associated with human fibroblasts (Ghahary et al., 2005). Secretion of proteins such as ribosomal proteins and cytochrome P450s has not been reported from plant cells and their presence might represent leakage that occurs during the cell separation process. However, the periwinkle cytochrome P450 related to a pea root cap secretome protein is among the minority predicted to have a signal peptide (Table II). Moreover, recent studies have revealed that such 'intracellular' components are found outside the cells of gram-positive bacteria (Bendtsen et al., 2005). Thus, ribosomal proteins, enolase, elongation factor, and chaperonins are components of extracellular matrices produced by on-filter growth and analysed by LC-MS/MS analysis (Gallaher et al., 2006). Histones are a component of the extracellular defense system in some vertebrates, and an extracellular complex of proteins including ribosomal proteins and histones is a key component in the innate immunity of Atlantic cod (*Gadus morhua*) (Bergsson et al., 2005; Patat et al., 2004).

Current understanding of how proteins are secreted in diverse organisms is evolving rapidly, and even in bacteria, whose secretion machinery has been studied extensively, there continue to be surprises emerging from secretome studies. For example, analysis of the complete *Bacillus subtilis* genome revealed that 300 proteins have structural motifs that predict they would be exported from the cytoplasm (Tjalsma et al., 2004). However, direct analysis under a wide range of conditions yielded only 90 that were exported and among those, nearly 50% would not have been predicted to be exported based on sequence. The export of proteins from the plant cytoplasm past the plasma membrane into the cell wall and apoplast is inherent in plant function, but little is known about the detailed mechanisms for secretion into the membrane or the cell wall, let alone into the extracellular spaces beyond the wall (reviewed in Bais et al., 2006). Secretion from plant cells can occur by a default pathway when proteins are targeted to the

endoplasmic reticulum by signal-peptide mediated translocation, but may also occur by nonspecific migration through exocytosis (Denecke et al., 1990). The root cap has long been known to be a secretory organ whose active secretion of a high molecular weight polysaccharide-based mucilage made it a favored model for studies of Golgi function (e.g. Feldman, 1984; Pickett-Heaps, 1967). Our data suggest that its role in the secretion of soluble proteins into the rhizosphere may have been underestimated by those of us working in the field of root-rhizosphere interactions.

Understanding the relative contribution of the root cap vs border cells to the secretome will require additional research. Newly synthesized proteins from these two cell types have very different protein profiles in two dimensional gel electrophoresis, but spots common to both can be found in extracellular proteins (Brigham et al., 1995). In the current study, border cells were found to continue to secrete at least a subset of the secretome, after they are dispersed into suspension. It will be of interest to define the ways this process may be influenced by challenges such as pathogenic bacteria and aluminum, which induce a marked increase in the dimensions of the extracellular mucilage layer around individual border cells (Hawes et al., 1998; Miyasaka and Hawes, 2000). By virtue of the fact that border cells can be isolated into suspension without osmotica, hormones, or other artifactual conditions, these cell populations may provide a convenient system to explore the ways diverse proteins are delivered to the extracellular environment of plant cells. Alternatively, this process may constitute a unique system that is not representative of normal secretion mechanisms. In any case, the proteins we identified can be collected simply by transient immersion of the root tip into water, an action that closely duplicates conditions normally encountered by roots in diverse growing conditions. Therefore, it is reasonable to conclude that the proteins identified here are an integral component of the complex of materials delivered into the rhizosphere of plants under natural conditions. Our results support the hypothesis that proteins released along with the root cap mucilage during the process of border cell separation play a vital role in the plant's system of innate immunity, by protecting the vulnerable root tip as it moves through the soil environment.

## MATERIALS AND METHODS

### Plant Material

Seeds of *Pisum sativum* L. cv Little Marvel (Meyer Seed Company, Baltimore, MD) and *Zea mays* L. cv Golden Bantam (Vegetable Seed Warehouse, Graniteville, SC) were germinated as described (Brigham et al., 1995). This protocol includes incubation for 15 min in 95% ethanol followed by 1 h in full strength commercial bleach (12.5% NaOH), and imbibition with repeated rinses in sterile water for 6 h during which seeds that float are discarded leaving only those with an intact seed coat. In pea, this yields roots from which no microbial colonies emerge when root tips are immersed into water which is then plated onto rich media such as Luria broth or potato dextrose agar (e.g. Gunawardena et al., 2005). In *Zea mays*, *Arabidopsis thaliana*, *Medicago truncatula* and numerous other species examined (e.g. Hawes and Pueppke, 1986), significant residual microbial contamination remains on root tips of aseptically grown seedlings, and antibiotic treatment sufficient to eliminate the contamination results in browning and root growth inhibition. This confounds interpretation of the components of extracellular material from the root tip. Therefore the proteomic analysis presented here is limited to pea, and immunolocalization using a specific antibody was used to confirm the presence of 14-3-3 proteins in the maize border cell extracellular matrix (below).

Root tips (ca. 2-3 mm of the root apex including the root cap and apical meristem) of intact two-day-old seedlings with a radicle length of 2.5 cm were immersed in 1 ml of water and washed by gently pipetting with water, as described (Brigham et al., 1998; Zhu et al., 1997). Border cells were removed from the collected exudates by centrifugation at 3500 g for 1 minute, and washed twice to remove all extracellular material. "Root tip exudate" refers to the supernatant component obtained after this centrifugation step, and "root cap secretome" refers to proteins within root tip exudate which includes material from root cap and from border cells accumulated during the process of border cell separation. "Border cell exudate" refers to material collected exclusively from border cells over time, after washing to remove all residual extracellular material at the time of border cell collection.

## Global Destruction of Root Cap Secretome: Impact on Root Tip Resistance to Infection

*N. haematococca* MPVI strains were maintained, collected, and quantified as described previously (Gunawardena et al., 2002; Wassman and VanEtten, 1996). Conidia of isolate 77-13-4 ( $10^5$  or  $10^6$ /ml) were added, with and without proteinase K (0.5 mg/ml) to 200  $\mu$ l pipette tips. Roots were placed horizontally into cellophane growth pouches (Mega International, St. Paul, MN) with root tips inserted into the the pipette tip containing conidia in water for 24 h, to retain contact among the root tip, spores, and proteinase K (Sigma Aldrich). Then, the pipette tips were removed and growth pouches containing the seedlings were placed upright. Root tip infection was evaluated by direct observation of lesion development and measurement of root growth, as described (Gunawardena et al., 2002). Results are based on at least 7 independent experiments with at least 10 replicate seedings for each treatment.

### Protein Sample Isolation and Preparation

Protein was isolated as described (Brigham et al., 1995), and quantified by the method of Bradford (1976). Absorption of the supernatant was measured at 595 nm wavelength ( $A_{595}$ ), which was plotted on the standard curve to find the protein concentration. To collect proteins in border cell exudate, the border cell pellet was washed once and fresh water was added to the cells which were incubated at room temperature for 1-2 h. Protein from the extracellular medium was harvested by centrifuging the cells and collecting the supernatant. For each root tip exudate sample, protein (ca 50  $\mu$ g) was collected in 2 ml water from tips of 50 pea roots. For border cell samples, protein (ca 20  $\mu$ g) was collected from washed border cells from 300 roots. Samples that yielded microbial colonies when plated onto nutrient media were discarded. Protein samples were collected four times from independently grown batches of pea roots.

Protein analysis was carried out essentially as described (Andon et al., 2003; Eng et al., 1994; Washburn et al., 2001; Wilkins et al., 2006; Yates et al., 1995). Ammonium bicarbonate (AMBIC) was added to a concentration of 0.1 M, and 40  $\mu$ L of 10 mM DTT was added before reducing at 56°C for 45 min (Washburn et al., 2001). Reduced cysteines were alkylated by addition of 40  $\mu$ L of 55 mM iodoacetamide (10 mM final concentration) and incubation for 30 min at room temperature. Proteolysis was initiated with a 1:50 ratio (by weight) of sequencing grade modified trypsin (Promega, Madison, WI) and allowed to proceed for 2 h at 37°C. A

second aliquot of 1:50 trypsin was added and the digestion continued overnight at 37°C. The digest was stored at -20°C prior to analysis.

### **Protein Separation and Identification by MudPIT**

Mass spectra of the four independent samples each were acquired in the AZCC/SWEHSC Proteomics Core, University of Arizona, Tucson AZ. For the nanoLCLC-MS/MS used to fractionate peptides from the digest, a microbore HPLC system (Paradigm MS4, Michrom, Auburn, CA) was used with two separate SCX and RP columns: A 100 µm I.D. capillary packed with 10 cm of 5 µm Vydac C18 reversed phase resin and a separate 250 µm I.D. capillary packed with 8cm of 5 µm Partisphere strong cation exchanger resin (Whatman, Clifton, NJ). The sample (23 µg) was acidified using TFA and manually injected onto the SCX column, the effluent from the column being fed through RP column.

A representative twelve step MudPIT analysis would be as follows. Solutions used are 10% methanol/0.1% formic acid, 0.01% TFA (buffer A), 95% methanol/0.1% formic acid, 0.01% TFA (buffer B), 10% methanol/0.1% formic acid, 0.01% TFA (buffer C) and 500 mM ammonium acetate/10% methanol/0.1% formic acid, 0.01% TFA (buffer D). Step 1 consists of a 5 min equilibration step at 100% buffer A, followed by another equilibration step for 5 min at 25% buffer B (75% buffer A), followed by a 40 min gradient from 25% buffer B to 65% buffer B, followed by a 10 min 65% buffer B and 10 min of 100% buffer A. Chromatography steps 2 to 12 follow the same pattern: 15 min of the appropriate % of buffers C & D followed by a 2 min 100% buffer C wash, a 5 min wash with 100% A, equilibration with 25% buffer B for 5 min, followed by a gradient from 25% buffer B to 65% buffer B in 40 min, followed by a 10 min 65% buffer B and 10 min of 100% buffer A. The buffer C/D percentages used were 95/5%, 90/10%, 85/15%, 80/20%, 70/30%, 60/40%, 40/60%, 20/80%, 0/100%, 0/100%, 0/100% respectively, for the 11 salt steps. The flow rate is approximately 350 nl/min, with elution directly into the electrospray ionization source of a ThermoFinnigan LCQ-Deca XP Plus ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Eluting peptides were electrosprayed into the mass spectrometer with a distally applied liquid junction spray voltage of 1.6 kV. Spectra are scanned over the range 380-2000 mass units. Automated peak recognition, dynamic exclusion,

and daughter ion scanning of the most intense ion was performed using the Xcalibur software as described previously (Andon et al., 2002, 2003).

MS/MS data were analyzed using SEQUEST (Eng et al., 1994, Yates et al., 1995). The criteria for a positive peptide identification for a doubly-charged peptide are a correlation factor (Xcorr) greater than 2.0, a delta cross-correlation factor ( $\Delta$ Xcorr) greater than 0.08, a minimum of one tryptic peptide terminus, and a high preliminary scoring. For triply- and singly-charged peptides the correlation factor threshold is set at 3.5 and 1.5, respectively. All spectra were searched against the non-redundant (nr) database from NCBI. Of proteins listed, 5 were detected in all 4 samples, 73 were detected in 3 samples, 27 in 2 samples, and 16 were present in a single sample. A survey for the presence of signal peptides was predicted using Signal-P V3 program (Bendtsen et al., 2004) ([www.cbs.dtu.dk/services/TargetP](http://www.cbs.dtu.dk/services/TargetP)).

### **In situ Immunolocalization of Extracellular 14-3-3 Protein**

Washed border cells were immersed in phosphate buffer saline (PBS) for 5 min, then treated with 3% blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN) in PBS for one hour, and washed twice in PBS, then incubated 2 h with a 1:1000 dilution of *Arabidopsis thaliana* 14-3-3 monoclonal antibody GF14-omega raised in mouse (a gift from Robert J. Ferl, University of Florida, Gainesville FL)([www.hos.ufl.edu/ferllab/14-3-3's/index.htm](http://www.hos.ufl.edu/ferllab/14-3-3's/index.htm)), followed by two 10-min rinses in PBS. The second antibody, Hilyte Fluor<sup>TM</sup>488-labeled goat anti-mouse IgG (1:1000 in PBS) (AnaSpec, San Jose, CA) was incubated with border cells for 1 h, followed by 30 sec rinses in PBS and water, respectively. P88C, the virus peptide induced antiserum from mouse (a gift from Dr. Z. Xiong, University of Arizona) was used as negative control antibody. Specimens were examined under an Olympus fluorescence microscope under ultraviolet radiation (480-500 nm filter). The images were captured with an Olympus digital camera using Micfire<sup>TM</sup> software.

### **Effects of R18, an Oligopeptide Conserved in the 14-3-3 Protein Binding Sites, on Root Tip Resistance to Infection**

The effect of R18 was tested by adding to roots at a concentration of 4  $\mu$ g/ml (Wang et al., 1999), as described above. *N. haematococca* spores were added, and infection was measured

after 24, 48, and 72 h (Gunawardena et al., 2002; 2005). Three independent experiments, with ten replicate seedlings in each experiment, were carried out to test the R18 effect.

## LITERATURE CITED

- Aiken RM and Smucker AJM** (1996) Root system regulation of whole plant growth. *Ann Rev Phytopathol* **34**: 325-346
- Albersheim P, Jones TM, English PD** (1969) Biochemistry of cell wall in relation to infective processes--involvement of cell walls in disease process. *Ann Rev Phytopathol* **7**: 171-179
- Alvarez S, Goodger JQD, Marsh EL, Chen S, Asirvatham VC, Schachtman DP** (2006) Characterization of the maize xylem sap proteome. *J Proteome Res* **5**: 963-972
- Andon NL, Eckert D, Yates JR, Haynes PA** (2003) High-throughput functional affinity purification of mannose binding proteins from *Oryza sativa*. *Proteomics* **3**: 1270-1278
- Atkinson TG, Neal JL, Larson RI** (1975) Genetic control of the rhizosphere microflora of wheat. In GW Bruehl, ed, *Biology and Control of Soil-borne Plant Pathogens*. American Phytopathological Society, St. Paul MN, pp 116-122
- Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM** (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol* **57**: 233-266
- Baluška F, Volkmann D, Barlow PW** (1996) Specialized zones of development in roots: View from the cellular level. *Plant Physiol* **112**: 3-4
- Bauer WD** (1981) Infection of legumes by rhizobia. *Ann Rev Plant Physiol* **32**: 407-49
- Basu U, Francis JL, Whittal RM, Stephens JL, Wang Y, Zaiane OR, Goebel R, Muench DG, Good AG, Taylor GJ** (2006) Extracellular proteomes of *Arabidopsis thaliana* and *Brassica napus* roots: analysis and comparison by MudPIT and LC-MS/MS. *Plant Soil* **286**: 357-376
- Bayer EM, Bottrill AR, Walshaw J, Vigouroux M, Naldrett MJ, Thomas CL, Maule AJ** (2006) Arabidopsis cell wall proteome defined using multidimensional protein identification technology. *Proteomics* **6**: 301-311

- Bendtsen JD, Nielsen H, von Heijne G, Brunak S** (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **3**: 783–795
- Bendtsen JD, Kiemer L, Fausboll A, Brunak S** (2005) Non-classical protein secretion in bacteria. *BMC Microbiol* **5**: 58-62
- Berg D, Holzmann C, Riess O** (2003) 14-3-3 proteins in the nervous system. *Nature Reviews Neuro* **4**: 752-762
- Bergey DR, Howe GA, Ryan CA** (1996) Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. *PNAS* **93**: 12053-12058
- Bol JF, Linthorst HJM, Cornelissen BJC** (1990) Plant pathogenesis related proteins induced by virus infection. *Ann Rev Phytopathol* **28**: 113-138
- Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254
- Brandt J, Thordal-Christensen H, Vad K, Gregersen P, Collinge DB** (1992) A pathogen induced gene of barley encodes a protein showing high similarity to a protein kinase regulator. *Plant J* **2**: 815-820
- Brigham LA, Woo HH, Hawes MC** (1995) Differential expression of proteins and mRNAs from border cells and root tips of pea. *Plant Physiol* **109**: 457-463
- Brigham LA, Woo HH, Wen F, Hawes MC** (1998) Meristem specific suppression of mitosis and a global switch in gene expression in the root cap of pea by endogenous signals. *Plant Physiol* **118**: 1223-1231
- Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinraugh Y, Zychlinsky** (2004) Neutrophil extracellular traps kill bacteria. *Science* **303**: 1532-1535
- Brisson LF, Tehnaken R, Lamb C** (1994) Function of oxidative cross linking of cell wall structural proteins in plant disease resistance. *Plant Cell* **6**: 1703-1712
- Bruehl GW** (1986) *Soilborne Plant Pathogens*. Macmillan Publishing Company, New York
- Cassab GI** (1998) Plant cell wall proteins. *Ann Rev Plant Physiol Plant Mol Biol* **49**: 281-309
- Celoy R** (2004) Identification and characterization of a galactosidase secreted from border cells of *Pisum sativum*. Master's thesis, University of Arizona, Tucson AZ
- Chivasa S, Simon WJ, Yu X, Yalpani N, Slabas AR** (2005) Pathogen elicitor-induced changes in the maize extracellular matrix proteome. *Proteomics* **5**: 4894-4904

- Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grecis RK** (2005) Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* **308**: 1463-1465
- Curl EA, Truelove E** (1986) *The Rhizosphere*, Springer-Verlag, Berlin, Heidelberg, New York
- Darwin C** (1896) *The Power of Movement in Plants*, AMS Press, New York
- Denecke J, Botterman J, Deblaere R** (1990) Protein secretion in plant cells can occur via a default pathway. *Plant Cell* **2**: 51-59
- Eng JK, McCormack AL, Yates JR III** (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Amer Mass Spectrom* **5**: 976-989
- Feldman LJ** (1984) Development and dynamics of the root apical meristem. *Am J Bot* **7**: 1308-1314
- Ferl RJ** (2004) 14-3-3 proteins: regulation of signal-induced events. *Physiol Plant* **120**: 173-178
- Fleischer A, O'Neill MA, Ehwald R** (1999) The pore size of non-graminaceous plant cell walls is rapidly decreased by borate ester cross linking of the pectic polysaccharide RG-II. *Plant Physiol* **121**: 829-838
- Gallaher TK, Wu S, Webster P, Aguilera R** (2006) Identification of biofilm proteins in non-typeable *Haemophilus influenzae*. *BMC Microbiology* **6**: 65-74
- Ghahary A, Ghahary A, Marcoux Y, Karimi-Busheri F, Li Y, Tredget EE, Kilani RT, Lam E, Weinfeld M** (2005) Differentiated keratinocyte-releasable stratifin (14-3-3 sigma) stimulates MMP-1 expression in dermal fibroblasts. *J Investigative Dermatol* **124**: 170-177
- Gunawardena U, Hawes MC** (2002) Tissue specific localization of root infection by fungal pathogens: role of root border cells. *Mol Plant-Microbe Int* **15**: 1128-1136
- Gunawardena U, Rodriguez M, Straney D, VanEtten HD, Hawes MC** (2005) Tissue-specific localization of root infection by *Nectria haematococca*: Mechanisms & Consequences. *Plant Physiol* **137**: 1363-1374
- Hawes MC, Bengough GA, Cassab G, Ponce G** (2003) Root caps and rhizosphere. *J Plant Growth Regul* **21**: 352-367
- Hawes MC, Brigham LA, Woo HH, Zhu Y, Wen F** (1998) Function of root border cells in plant health: Pioneers in the rhizosphere. *Ann Rev Phytopathol* **36**: 311-327

- Hawes MC, Brigham LA, Woo HH, Zhu Y, Wen F** (1997) Root border cells: Phenomenology of signal exchange. pp. 210-218. In *Radical Biology: Advances and Perspectives on the Function of Plant Roots*. HE Flores, JP Lynch, D. Eissenstat eds. American Society of Plant Physiologists.
- Hawes MC, Gunawardena U, Miyasaka S, Zhao X** (2000) The role of root border cells in plant defense. *Trends Plant Sci* **5**: 128-133
- Hawes MC, Lin H-J** (1990) Correlation of pectolytic enzyme activity with the programmed release of cells from root caps of pea. *Plant Physiol* **94**: 1855-1859
- Hawes MC, Pueppke SG** (1986) Sloughed peripheral root cap cells: yield from different species and callus formation from single cells. *Am J Bot* **73**: 1466-1473
- Hawes MC, Wheeler HE** (1982) Factors affecting victorin-induced cell death: temperature and plasmolysis. *Physiol Plant Pathol* **20**: 137-144
- Hiltner L** (1904) *Über neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Grundungung und Brache*. *Arb Dtsch Landwirt Ges* **98**: 59-78
- Hirsch AM, Bauer WD, Bird DM** (2003) Molecular signals and receptors: Controlling rhizosphere interactions between plants and other organisms. *Ecol* **84**: 858-868
- Kawano T** (2003) Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. *Plant Cell Rep* **21**: 829-837
- Knee EM, Gong FC, Gao MS, Teplitski M, Jones AR, Foxworth A, Mort AJ, Bauer WD** (2001) Root mucilage from pea and its utilization by rhizosphere bacteria as a sole carbon source. *Mol Plant-Microbe Int* **14**: 775-784
- Knudson L** (1920) The secretion of invertase by plant roots. *Am J Bot* **7**: 371-379
- Knudson L, Smith RS** (1919) Secretion of amylase by plant roots. *Bot Gaz* **68**: 460-466
- Matsuyama T, Satoh H, Yamada Y, Hashimoto T** (1999) A maize glycine rich protein is synthesized in the lateral root cap and accumulates in the mucilage. *Plant Physiol* **120**: 665-674
- Miyasaka S, Hawes MC** (2001) Possible role of root border cells in detection and avoidance of aluminum toxicity. *Plant Physiol* **125**: 1978-87
- Nebenfuhr A, Ritzenthaler C, Robinson DG** (2002) Brefeldin A: deciphering an enigmatic inhibitor of secretion. *Plant Physiol*. **130**: 1102-1108

- Olivain C, Alabouvette C** (1999) Process of tomato root colonization by a pathogenic strain of *Fusarium oxysporum* f. sp. *lycopersici* in comparison with a non-pathogenic strain. *New Phytol* **141**: 497-510
- Park SW, Lawrence CB, Linden JC, Vivanco JM** (2002) Isolation and characterization of a novel ribosome inactivating protein from root cultures of pokeweed and its mechanism of secretion from roots. *Plant Physiol* **130**: 164-178
- Patat SA, Carnegie RB, Kingsbury C, Gross PS, Chapman R, Schey KL** (2004) Antimicrobial activity of histones from hemocytes of the Pacific white shrimp. *Eur J Biochem* **271**: 4825-4833
- Pickett-Heaps J** (1967) The use of radioautography for investigating wall secretion in plant cells. *Protoplasma* **64**: 49-66
- Price I** (2002) Characterization and cloning of a pea beta-galactosidase. Master's Thesis, University of Arizona.
- Roberts MR** (2000) Regulatory 14-3-3 protein-protein interactions in plant cells. *Curr Opin Plant Biol* **3**: 400-405
- Rogers HT, Pearson RW, Pierre WH** (1942) The source and phosphate activity of exoenzyme systems of corn and tomato roots. *Soil Sci* **54**: 353-366
- Segarra CI, Casalongue CA, Pinedo ML, Ronchi VP, Conde RD** (2003) A germin-like protein of wheat leaf apoplast inhibits serine proteases. *J Exp Bot* **10**: 2013-2020
- Stankovic B, Garic-Stankovic A, Smith CM, Davies E** (1995) Isolation, sequencing, and analysis of a 14-3-3 brain protein homolog from pea. *Plant Physiol* **107**: 1481-1482
- Teplitski M, Robinson JB, Bauer WD** (2000) Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Mol Plant-Microbe Int* **13**: 637-648
- Tjalsma H, Antelmann H, Jongbloed DH, Braun PG, Darmon E, Dorenbos R, Dubois YF, Westers H, Zanen G, Quax WJ et al.** (2004) Proteomics of protein secretion by *Bacillus subtilis*: Separating the 'secrets' of the secretome. *Microbiol Mol Biol Reviews* **68**: 207-233
- Turlier M, Eparvier A, Alabouvette C** (1994) Early dynamic interactions between *Fusarium oxysporum* f. sp. *lini* and the roots of *Linum usitatissimum* as revealed by transgenic GUS-marked hyphae. *Can J Bot* **72**: 1605-1612

- Voigt J, Frank R** (2003) 14-3-3 proteins are constituents of the insoluble glycoprotein framework of the *Chlamydomonas* cell wall. *Plant Cell* **15**: 1399-1413
- Wang B, Hongzhu Y, Liu Y, Jelinek T, Zhang L, Ruislahti E, Fu H** (1999) Isolation of high affinity peptide antagonists of 14-3-3 proteins by phage display. *Biochem* **38**: 12499 -12504.
- Washburn MP, Wolters D, Yates JR** (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nature Biotech* **19**: 242-247
- Wassmann CC, Van Etten HD** (1996) Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decrease the virulence of *Nectria haematococca* on pea. *Mol Plant Microbe Int* **9**: 793-803
- Wen F, Zhu Y, Hawes MC** (1999) Effect of pectin methylesterase gene expression on pea root development. *Plant Cell* **11**: 1129-1140
- White RG, Badelt K, Overall RL, Vesik M** (1994) Actin associated with plasmodesmata. *Protoplasma* **180**: 169-184
- Wilkins MR, Appel RD, Eyk JEV, Chung MCM, Görg A, Hecker M, Huber LA, Langen H, Link AJ, Paik YK, Patterson SD, Pennington SR, Rabilloud T, Simpson RJ, Weiss W, Dunn MJ** (2006) Guidelines for the next 10 years of proteomics. *Proteomics* **6**: 4-8
- Yates, JR, III, Eng J, McCormack AL, Schieltz D** (1995) Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal Chem* **67**: 1426
- Zhu Y, Pierson LS III, Hawes MC** (1997) Release of nodulation gene inducing chemicals from root border cells. *Plant Physiol* **115**: 1691-1698
- Zhu J, Chen S, Alvarez S, Asirvatham VS, Schachtman DP, Wu Y, Sharp RE** (2006) Cell wall proteome in the maize primary root elongation zone. I Extraction and identification of water-soluble and lightly ionically bound proteins. *Plant Physiol* **140**: 311-325
- Zhu J, Chen S, Alvarez S, Asirvatham VS, Schachtman DP, Wu Y, Sharp RE** (2006a) Proceedings, Root Biology Symposium, Columbia Missouri, May 23-27, 2006

**ACKNOWLEDGEMENTS:** We thank Robert J. Ferl, University of Florida, for GF14-omega antibody for these experiments. Mass spectra were acquired in the AZCC/SWEHSC Proteomics Core supported by NIEHS grant ES06694 and NIH/NCI grant CA023074-26. This study was supported by grant# DE-FG02-04ER15551 from the Department of Energy, Division of Energy Biosciences, and the University of Arizona College of Agriculture and Life Sciences.

**Table I.** *Dynamics of protein secretion during induced border cell separation*

<u>Time (hours)</u>	<u>Number border cells</u>	<u>Extracellular protein (<math>\mu\text{g}/\text{root}</math>)</u>
0	0	0.0001
2	430 $\pm$ 57	0.16 $\pm$ 0.02
5	1000 $\pm$ 94	0.35 $\pm$ 0.05
10	2120 $\pm$ 350	0.62 $\pm$ 0.15
15	3140 $\pm$ 380	0.84 $\pm$ 0.06
20	3650 $\pm$ 420	1.0 $\pm$ 0.08
24	4190 $\pm$ 450	1.3 $\pm$ 0.05
36	4285 $\pm$ 395	1.3 $\pm$ 0.09

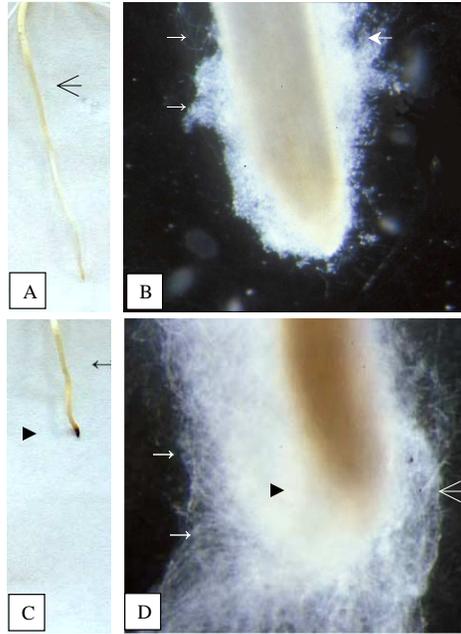
To collect proteins in border cell exudate, the border cell pellet was washed once and fresh water was added to the cells which were incubated at room temperature. Protein from the extracellular medium was harvested by centrifuging the cells and collecting the supernatant, and protein was quantified by the method of Bradford (1976). Each values represents the mean and standard error from at least 3 independent experiments, with at least 3 replicates per sample.

## Figure legends.

Fig. 1. Proteolytic destruction of the root cap secretome resulted in increased root tip infection (A,C) and increased fungal growth on root tips (B,D). (A) Most pea roots ( $94 \pm 4\%$ ) inoculated with  $10^5$  spores of *N. haematococca* show no obvious signs of infection 3 days later and growth proceeded at ca 2.5 cm/day (black arrow denotes the site of the root tip at the time of inoculation); (B) At 10X magnification, the presence of a 'mantle' of fungal hyphae (white arrows) growing among border cells was evident. No necrosis at the root tip was visible; (C) When roots were treated with proteinase K at the time of inoculation with *N. haematococca* spores, root tip infection was apparent within 2-3 days; a brown-black necrosis encompassed the root apex (arrowhead) of every inoculated root, and growth ceased within 24-48 h after inoculation (black arrow denotes the site of the root apex at the time of inoculation); (D) Root tip necrosis (arrowhead) and cessation of root growth occurred in correlation with a marked increase in hyphal growth (white arrows). Each experiment was repeated at least 7 times, with 10 replicate seedlings for each experiment, and photos (C,D) show results representative of all replicate roots; there were no escapes.

Fig. 2. Localization of extracellular 14-3-3 proteins. Immunolocalization with GF14-omega 14-3-3 antibody (Ferl, 2004) highlighted a sheath of material surrounding border cells from corn (arrows) and pea (inset). No intracellular penetration of the antibodies was detected.





**Table II. Proteins of the pea root cap secretome**

NCBI Accession #	Identification	SignalP <sup>a</sup>	Coverage <sup>b</sup> %	MW(D)	PI	Unique Peptides <sup>c</sup>	Best Xcorr <sup>d</sup>	Organism matched
33504428	9/13 hydroperoxide lyase	-	2.3	53933	6.6	1	4.2	<i>Medicago truncatula</i>
*695767	14-3-3 protein homolog	-	7.3	29524	4.9	1	3.9	<i>Vicia faba</i>
*13928452	14-3-3 protein	-	6.6	29203	4.8	1	4.1	<i>Vigna angularis</i>
*15778154	14-3-3 protein	-	7.7	29479	4.9	1	4.7	<i>Nicotiana tabacum</i>
*4850247	14-3-3-like protein	-	6.5	29359	4.8	4	4.1	<i>Pisum sativum</i>
*4775555	14-3-3-like protein	-	6.5	29434	4.8	2	4.9	<i>Pisum sativum</i>
11138320	14-3-3c protein	-	6.5	29683	4.9	1	4.9	<i>Vicia faba</i>
47027073	2-cys peroxiredoxin-like protein	-	7.1	21856	5.0	1	3.1	<i>Hyacinthus orientalis</i>
23397065	40S ribosomal protein S15A	-	10.8	14804	9.9	1	3.5	<i>Arabidopsis thaliana</i>
3413170	40S ribosomal protein S6	-	7.5	24007	11.0	1	4.4	<i>Cicer arietinum</i>
25294932	60S ribosomal protein L10A	-	6.3	25100	9.9	1	3.7	<i>Arabidopsis thaliana</i>
*47027024	60S ribosomal RL5	-	7.4	21752	10.1	1	4.6	<i>Medicago sativa</i>
12054507	ribosomal protein L2	-	4.6	28013	10.5	2	4.9	<i>Glycine max</i>
6688812	ribosomal protein L3	-	2.6	44433	10.1	1	2.2	<i>Medicago sativa</i>
*396639	ribosomal protein S13	-	11.3	17116	10.4	1	3.2	<i>Pisum sativum</i>
2565340	ribosomal protein S14	-	12.0	16292	10.9	1	4.0	<i>Lupinus luteus</i>
44662864	ribosomal protein S6	-	6.1	28112	10.7	1	4.4	<i>Glycine max</i>
33318760	ribosomal protein S7	-	5.0	29743	11.4	1	2.7	<i>Podocarpus chinensis</i>
56462228	ribosomal protein S18.A	-	6.9	13669	10.3	1	2.8	<i>A. thaliana</i>
*20633	ABA-responsive protein	-	7.6	17023	5.4	1	3.3	<i>Pisum sativum</i>
1498338	actin	-	5.4	37250	6.2	1	5.0	<i>Glycine max</i>
30685069	actin 2 (ACT2)	-	4.9	41232	5.7	1	3.7	<i>A. thaliana</i>
29691168	adenosylhomocysteinase	-	4.7	53197	6.0	2	3.6	<i>Medicago truncatula</i>
58038194	alpha-dioxygenase	-	2.8	73297	6.8	1	3.9	<i>Pisum sativum</i>
11602477	apyrase	+	16.3	13710	6.3	1	4.3	<i>Pisum sativum</i>
*32879783	ascorbate peroxidase	+	4.2	51188	5.5	1	5.3	<i>Oryza sativa</i>
525291	ATP synthase beta subunit	+	2.7	59249	5.9	1	3.2	<i>Triticum aestivum</i>
7436097	F1 ATPase	+	1.6	60151	7.1	2	2.8	<i>Pisum sativum</i>
510147	Fo ATPase subunit 6	+	3.2	55140	8.1	1	2.1	<i>Beta vulgaris</i>
3169287	vacuolar H <sup>+</sup> -ATPase catalytic subunit	-	2.9	68534	5.5	1	4.3	<i>Gossypium hirsutum</i>
12585490	H <sup>+</sup> -ATPase subunit	-	2.9	68681	5.4	1	4.3	<i>Citrus unshiu</i>
902938	beta-amylase	-	2.8	56072	5.5	1	5.1	<i>Glycine max</i>
7939621	beta-galactosidase	+	2.8	97047	8.1	1	5.1	<i>Lycopersicon esculentum</i>
*3201554	beta-D-glucosidase	+	2.6	71671	7.8	1	4.5	<i>Tropaeolum majus</i>
47028285	calmodulin	-	11.0	17363	4.2	1	3.3	<i>Bigeloviella natans</i>
*21913287	calmodulin	-	22.1	16862	4.3	2	5.0	<i>Medicago truncatula</i>
*6969639	calmodulin-like protein	-	17.6	20973	4.8	2	5.0	<i>Oryza sativa</i>
*3702620	calnexin	+	2.9	62532	4.9	3	4.5	<i>Pisum sativum</i>
3288109	calreticulin	-	4.1	48136	4.6	1	4.2	<i>Beta vulgaris</i>
*861157	cell wall invertase II	+	2.8	65967	8.0	1	4.0	<i>Vicia faba</i>
50355740	putative cell wall protein	-	1.2	121397	8.5	1	2.2	<i>Oryza sativa</i>
*7488790	cellulase precursor	+	2.5	53990	8.5	1	3.9	<i>Pisum sativum</i>
33357717	chain D, pea lectin-sucrose complex	-	43.8	5230	4.7	1	4.9	<i>Pisum sativum</i>
388361	chitinase	+	6.9	34951	8.2	1	4.6	<i>Ulmus americana</i>
*30526289	chitinase-like thermal hysteresis protein	-	8.2	28845	8.5	1	5.3	<i>Solanum dulcamara</i>
1335862	clathrin heavy chain	-	0.7	193354	5.5	1	3.2	<i>soybean</i>

Accession #	Identification	SignalP <sup>a</sup>	Coverage <sup>b</sup> %	MW(D)	PI	Unique Peptides <sup>c</sup>	Best Xcorr <sup>d</sup>	Organism matched
829119	cyclophilin	-	7.0	18160	8.2	1	2.5	<i>Phaseolus vulgaris</i>
395328	cytochrome P450	+	25.0	6492	9.8	1	4.7	<i>Madagascar periwinkle</i>
3819164	cytosolic chaperonin, delta-subunit	-	2.8	57574	7.3	1	4.7	<i>Glycine max</i>
9955324	decarboxylase	-	4.3	49744	6.5	1	4.0	<i>Pisum sativum</i>
21928175	dehydration-responsive protein	+	1.9	93049	5.2	1	3.4	<i>Arabidopsis thaliana</i>
*436313	disease resistance response protein	-	17.7	16747	5.1	2	4.2	<i>Pisum sativum</i>
*118934	disease resistance response protein	-	9.5	16791	4.9	3	5.5	<i>Pisum sativum</i>
29367381	elongation factor 1 gamma-like protein	-	2.7	47075	6.5	2	3.3	<i>Oryza sativa</i>
18958499	elongation factor eEF-1B gamma	-	2.6	47711	6.7	1	3.3	<i>Glycine max</i>
*99902	translation elongation factor eEF-1a	-	2.7	49366	9.1	1	3.4	<i>Glycine max</i>
*42521309	enolase	-	3.4	47719	5.5	1	1.7	<i>Glycine max</i>
1015937	extensin class 1 protein	+	3.3	53896	9.5	1	3.6	<i>Vigna unguiculata</i>
*927505	fructose-1,6-bisphosphate aldolase	-	11.4	38491	7.3	5	5.2	<i>Pisum sativum</i>
10645188	fructose-1,6-bisphosphate aldolase	-	3.9	38515	7.3	1	3.7	<i>Pisum sativum</i>
3021338	fructose-1,6-bisphosphate aldolase	-	4.5	38491	6.7	5	5.2	<i>Cicer arietinum</i>
1370285	glutathione reductase	-	3.2	53897	7.0	1	3.2	<i>Pisum sativum</i>
11385441	glutathione S-transferase GST 13	-	6.8	25198	5.7	1	3.8	<i>Glycine max</i>
*30315017	glutathione-S-transferase	-	8.3	15654	8.7	1	2.8	<i>Vitis vinifera</i>
16226117	glyceraldehyde 3-phosphate dehydrogenase	-	15.0	14107	8.3	1	5.4	<i>Fragaria x ananassa</i>
*3831571	glyceraldehyde-3-phosphate dehydrogenase 1	-	21.3	10153	4.9	1	4.5	<i>Glycine max</i>
1842232	heat shock protein	-	2.5	70332	5.4	1	3.9	<i>Babesia rodhaini</i>
886740	histone H4	-	9.7	11552	11.7	2	3.5	<i>Zea mays</i>
1199967	histone H4	-	17.2	6634	9.9	1	4.1	<i>Allium cepa</i>
123593	HSP70	-	2.5	70605	5.3	2	4.5	<i>Maize</i>
22652	heat shock protein 90 homologue	+	1.5	92917	4.9	1	3.5	<i>Hordeum vulgare</i>
*21592544	hydroxymethyltransferase	-	3.4	51706	7.0	1	4.7	<i>Arabidopsis thaliana</i>
21592483	late embryogenesis abundant proteins	-	3.4	35907	4.9	1	3.4	<i>Arabidopsis thaliana</i>
2459611	lipoxygenase	-	2.0	97418	6.5	3	3.9	<i>Pisum sativum</i>
9909849	lipoxygenase	-	3.8	97199	7.2	2	4.5	<i>Pisum sativum</i>
*541746	lipoxygenase	-	1.8	97113	6.8	2	4.7	<i>Pisum sativum</i>
*469156	lipoxygenase	-	3.8	97629	6.5	8	5.0	<i>Pisum sativum</i>
4165128	lipoxygenase LoxN3	-	3.5	55817	6.3	5	5.4	<i>Pisum sativum</i>
27463709	metallo-exopeptidase	+	2.6	60281	7.8	1	4.5	<i>L. esculentum</i>
7434000	methionine adenosyltransferase	-	3.8	42842	5.8	1	3.9	<i>Hordeum vulgare</i>
609223	methionine adenosyltransferase	-	4.6	39940	6.9	3	4.3	<i>Pisum sativum</i>
*51860738	monodehydroascorbate reductase I	+	3.5	47351	6.1	1	4.3	<i>Pisum sativum</i>

Accession #	Identification	SignalP <sup>a</sup>	Coverage <sup>b</sup> %	MW(D)	PI	Unique Peptides <sup>c</sup>	Best Xcorr <sup>d</sup>	Organism matched
56550701	no apical meristem (NAM) family protein	-	3.6	40781	6.8	1	2.0	<i>Arabidopsis thaliana</i>
*1236951	nucleoside diphosphate kinase	-	11.4	16443	6.3	2	4.5	<i>Glycine max</i>
*1389640	nucleoside-diphosphate kinase	-	10.7	16462	6.8	4	4.9	<i>Pisum sativum</i>
1362008	polyubiquitin	-	4.2	42403	6.1	2	3.1	<i>Arabidopsis thaliana</i>
1684857	polyubiquitin	-	4.2	24089	6.4	1	3.4	<i>Phaseolus vulgaris</i>
396819	porin	-	7.6	29597	9.1	1	4.4	<i>Pisum sativum</i>
20269067	protease inhibitor	+	6.0	23553	6.2	1	4.2	<i>Sesbania rostrata</i>
871515	Psst70 (stress 70 protein)	-	3.2	64901	5.0	1	4.8	<i>Pisum sativum</i>
53749369	putative 1,4-benzoquinone reductase	-	7.3	21630	6.8	1	3.8	<i>Oryza sativa</i>
31430108	putative alpha-mannosidase	+	2.3	71468	8.3	1	4.5	<i>Oryza sativa</i>
17065616	putative ATP citrate lyase	-	5.4	65828	7.6	2	4.7	<i>Arabidopsis thaliana</i>
31430108	putative alpha-mannosidase	+	2.3	71468	8.3	1	4.6	<i>Oryza sativa</i>
31431675	putative calmodulin-binding protein	-	0.7	115753	6.1	1	1.5	<i>Oryza sativa</i>
50915968	putative fibrillarlin	-	5.6	32403	10.2	1	3.6	<i>Oryza sativa</i>
52353701	putative oligosaccharyl transferase STT3	+	1.3	87014	9.0	1	2.0	<i>Oryza sativa</i>
50915890	putative potassium channel beta subunit	-	3.4	36449	7.4	1	2.8	<i>Oryza sativa</i>
*21068664	putative quinone oxidoreductase	-	7.4	21722	7.1	1	3.9	<i>Cicer arietinum</i>
5139543	putative tonoplast intrinsic protein	-	6.8	25375	6.4	1	4.8	<i>Pisum sativum</i>
14532836	putative UDP-glucose pyrophosphorylase	-	3.2	51920	6.0	1	2.3	<i>Arabidopsis thaliana</i>
17065642	putative serine-type carboxypeptidase II	+	2.3	53608	7.9	1	3.4	<i>Arabidopsis thaliana</i>
58500257	quinone reductase 2	-	7.4	21750	6.4	1	3.5	<i>Triticum monococcum</i>
34582497	reversibly glycosylatable polypeptide	-	3.3	41573	6.1	1	3.5	<i>Pisum sativum</i>
38194918	reversibly glycosylated protein	-	3.4	40193	6.2	2	5.9	<i>Phaseolus vulgaris</i>
*807089	root hair protein RH2	-	9.7	16504	4.9	2	3.5	<i>Pisum sativum</i>
18157331	S-adenosyl-L-methionine synthetase	-	5.4	43060	5.9	3	4.1	<i>Pisum sativum</i>
*726030	S-adenosylmethionine synthetase	-	9.2	42519	6.8	2	4.2	<i>Actinidia chinensis</i>
21593291	S-adenosylmethionine synthetase	-	9.2	43158	5.8	4	4.5	<i>Arabidopsis thaliana</i>
14600068	S-adenosylmethionine synthetase	-	3.6	43141	6.2	4	4.6	<i>Brassica juncea</i>
37051117	S-adenosylmethionine synthetase-2	-	9.2	37634	6.8	2	5.4	<i>Pisum sativum</i>
21536731	semialdehyde dehydrogenase family protein	-	5.3	40746	7.0	1	3.8	<i>Arabidopsis thaliana</i>
14596185	similar to dihydroflavonol reductase	-	4.1	43694	5.9	1	4.0	<i>Arabidopsis thaliana</i>
21555840	succinate dehydrogenase iron-protein subunit	-	3.9	31270	8.5	1	2.7	<i>Arabidopsis thaliana</i>
20902	superoxide dismutase	-	6.7	26637	7.7	1	2.8	<i>Pisum sativum</i>
*37727999	superoxide dismutase	-	17.6	7698	5.6	1	4.2	<i>Pisum sativum</i>
*20900	Cu/Zn superoxide dismutase II	-	6.4	20610	6.4	1	4.7	<i>Pisum sativum</i>

Accession #	Identification	SignalP <sup>a</sup>	Coverage <sup>b</sup> %	MW(D)	PI	Unique Peptides <sup>c</sup>	Best Xcorr <sup>d</sup>	Organism matched
38326766	tasselseed2 protein	-	4.9	27546	8.1	1	2.1	<i>Bouteloua hirsuta</i>
*3980198	thiolprotease	+	3.4	51250	6.4	2	4.8	<i>Pisum sativum</i>
48478827	UDP-glucose:protein transglucosylase-like protein	-	5.0	41192	6.2	2	5.9	<i>Lycopersicon esculentum</i>
21553496	unknown	-	9.0	15458	5.0	1	1.6	<i>Arabidopsis thaliana</i>
25345736	unknown protein	-	3.1	52066	8.6	1	2.8	<i>Arabidopsis thaliana</i>
9294394	unnamed protein product	+	1.2	72711	6.3	1	1.6	<i>Arabidopsis thaliana</i>
*3986172	xyloglucan endotransglucosylase	+	4.8	34096	8.4	1	3.4	<i>Pisum sativum</i>

\* Proteins also secreted by border cells. a. SignalP: Proteins matched contain signal peptide. b. Sum of peptide(s) amino acid residues over the total amino acids residue count of the corresponding protein. c. Unique peptide number identified by SEQUEST. d. Xcorr measures how closely the measured spectrum fits to the ideal spectrum. Xcorr values above 2.0 indicate a good correlation for doubly charged peptides (Eng et al., 1994, Yates et al., 1995). Of proteins listed, 5 were detected in all 4 samples, 73 were detected in 3 samples, 27 in 2 samples, and 16 were present in a single sample.