

Extracellular DNA Is Required for Root Tip Resistance to Fungal Infection^{1[W][OA]}

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Plant defense involves a complex array of biochemical interactions, many of which occur in the extracellular environment. The apical 1- to 2-mm root tip housing apical and root cap meristems is resistant to infection by most pathogens, so growth and gravity sensing often proceed normally even when other sites on the root are invaded. The mechanism of this resistance is unknown but appears to involve a mucilaginous matrix or “slime” composed of proteins, polysaccharides, and detached living cells called “border cells.” Here, we report that extracellular DNA (exDNA) is a component of root cap slime and that exDNA degradation during inoculation by a fungal pathogen results in loss of root tip resistance to infection. Most root tips (>95%) escape infection even when immersed in inoculum from the root-rotting pathogen *Nectria haematococca*. By contrast, 100% of inoculated root tips treated with DNase I developed necrosis. Treatment with BAL31, an exonuclease that digests DNA more slowly than DNase I, also resulted in increased root tip infection, but the onset of infection was delayed. Control root tips or fungal spores treated with nuclease alone exhibited normal morphology and growth. Pea (*Pisum sativum*) root tips incubated with [³²P]dCTP during a 1-h period when no cell death occurs yielded root cap slime containing ³²P-labeled exDNA. Our results suggest that exDNA is a previously unrecognized component of plant defense, an observation that is in accordance with the recent discovery that exDNA from white blood cells plays a key role in the vertebrate immune response against microbial pathogens.

Root diseases caused by soil-borne plant pathogens are a perennial source of crop loss worldwide (Bruehl, 1986; Curl and Truelove, 1986). These diseases are of increasing concern, as pesticides like methyl bromide are removed from the market due to environmental concerns (Gilreath et al., 2005). One possible alternative means of crop protection is to exploit natural mechanisms of root disease resistance (Nelson, 1990; Goswami and Punja, 2008; Shittu et al., 2009). Direct observation of root systems under diverse conditions has revealed that root tips, in general, are resistant to infection even when lesions are initiated elsewhere on the same plant root (Foster et al., 1983; Bruehl, 1986; Curl and Truelove, 1986; Smith et al., 1992; Gunawardena et al., 2005; Wen et al., 2007). This form of disease resistance is important for crop production because root growth and its directional movement in response to gravity, water, and other signals can proceed normally as long as the root tip is not invaded. The 1- to 2-mm apical

region of roots houses the root meristems required for root growth and cap development, and when infection does occur, root development ceases irreversibly within a few hours even in the absence of severe necrosis (Gunawardena and Hawes, 2002). Mechanisms underlying root tip resistance to infection are unclear, but the phenomenon appears to involve root cap “slime,” a mucilaginous matrix produced by the root cap (Morré et al., 1967; Rougier et al., 1979; Foster, 1982; Chaboud, 1983; Guinel and McCully, 1986; Moody et al., 1988; Knee et al., 2001; Barlow, 2003; Iijima et al., 2008). Within the root cap slime of cereals, legumes, and most other crop species are specialized populations of living cells called root “border cells” (Supplemental Fig. S1; Hawes et al., 2000). Border cell numbers increase in response to pathogens and toxins such as aluminum, and the cell populations maintain a high rate of metabolic activity even after detachment from the root cap periphery (Brigham et al., 1995; Miyasaka and Hawes, 2000).

As border cells detach from roots of cereals and legumes, a complex of more than 100 proteins, termed the root cap secretome, is synthesized and exported from living cells into the matrix ensheathing the root tip (Brigham et al., 1995). The profile of secreted proteins changes in response to challenge with soil-borne bacteria (De-la-Peña et al., 2008). In pea (*Pisum sativum*), root tip resistance to infection is abolished in response to proteolytic degradation of the root cap secretome (Wen et al., 2007). In addition to an array of antimicrobial enzymes and other proteins known to be components of the extracellular matrix and apoplast of

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higher plants, the DNA-binding protein histone H4 unexpectedly was found to be present among the secreted proteins (Wen et al., 2007). One explanation for the presence of histone is global leakage of material from disrupted nuclei in dead cells, but no cell death occurs during delivery of the secretome (Brigham et al., 1995; Wen et al., 2007). An alternative explanation for the presence of a secreted DNA-binding protein is that extracellular DNA (exDNA) also is present in root cap slime.

exDNA has long been known to be a component of slimy biological matrices ranging from purulent localized human infections to bacterial capsules, biofilms, and snail exudate (Sherry and Goeller, 1950; Leuchtenberger and Schrader, 1952; Braun and Whallon, 1954; Smithies and Gibbons, 1955; Catlin, 1956; Fahy et al., 1993; Allesen-Holm et al., 2006; Spoering and Gilmore, 2006; Qin et al., 2007; Izano et al., 2008). Specialized white blood cells in humans and other species including fish recently have been shown to deploy a complex neutrophil extracellular "trap" (NET), composed of DNA and a collection of enzymes, in response to infection (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2007; Palić et al., 2007; Wartha et al., 2007; Yousefi et al., 2008). NETs appear to kill bacterial, fungal, and protozoan pathogens by localizing them within a matrix of antimicrobial peptides and proteins (Urban et al., 2006; Wartha et al., 2007; Guimaraes-Costa et al., 2009). Several extracellular peptides and proteins implicated in neutrophil function, including histone, also are present within the pea root cap secretome (Wen et al., 2007). exDNA linked with extracellular histone is a structural component of NETs, and treatment with DNase destroys NET integrity and function (Wartha et al., 2007). Moreover, human pathogens including group A *Streptococcus* and *Streptococcus pneumoniae* release extracellular DNase (Sherry and Goeller, 1950). When these activities are eliminated by mutagenesis of the encoding genes, bacteria lose their normal ability to escape the NET and multiply at the site of infection (Sumby et al., 2005; Buchanan et al., 2006). Here, we report that, in addition to histone and other secretome proteins, exDNA also is a component of root cap slime. When this exDNA is digested enzymatically, root tip resistance to infection is abolished.

RESULTS

Loss of Root Tip Resistance to Infection in Response to in Situ DNase Treatment of Root Cap Slime

Deoxyribose previously was detected in root exudates of young monocot seedlings by chromatographic analysis (Vancura, 1964). If DNA is present within the slime surrounding the root cap, it also should be detectable using stains known to bind to DNA. Extracellular material was imprinted by touching the root tip of aseptically germinated seedlings to the surface

of a microscope slide, and a coverslip was added. Preliminary cytological analysis using the double-stranded DNA stain 4',6-diamidino-2-phenylindole (DAPI; Kubista et al., 1987) revealed positively staining fibrillar material within the cell-free slime (Fig. 1A). Results with the nucleic acid stain SYTOX green (Jones and Singer, 2001) were similar (Fig. 1B). This experiment was repeated more than 10 times for each stain, with the same positive results revealing the presence of positively staining fibrous material of variable shapes and sizes. These data are consistent with the possibility that exDNA is a component of root cap slime, but the possibility of nonspecific binding of histological stains cannot be ruled out.

Brinkmann et al. (2004) directly established evidence for a functional role of neutrophil exDNA by showing that the structural integrity of NETs is lost when the exDNA is degraded enzymatically. We used the same approach by proposing that, if presumptive exDNA visualized by histological staining (Fig. 1) plays a role in the defense response of plant roots, as it does in mammalian immunity, then degrades during the infection process, it would be predicted to result in increased susceptibility of root tips to fungal invasion. This hypothesis was tested directly by treating root tips with the endonuclease DNase I in the presence of spores of the pea root-rotting pathogen, *Nectria haematococca* (Fig. 2). When roots are uniformly inoculated with spores, *N. haematococca* infects most roots (>90%) in the region of elongation behind the root tip, but the tip remains uninvaded and growth proceeds normally (Gunawardena and Hawes, 2002; Wen et al., 2007). The presence of the fungus does not trigger expression of defense responses within the root tip unless there is a visible necrosis, and lateral roots that emerge during the course of the infection assay resist infection as does the primary root tip, despite growing among fungal hyphae (Gunawardena et al., 2005). In approximately 3% of inoculated roots, fungal hyphae can be seen to invade the inoculated root tip, which

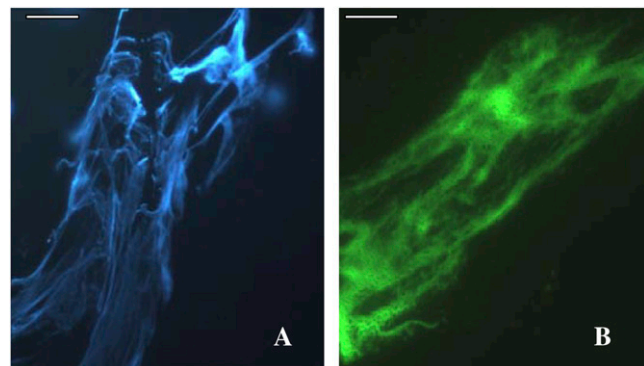


Figure 1. Light microscopic visualization of the fibrillar nature of root cap slime. A, Staining of cell-free material with DAPI reveals strands of DAPI-positive material. B, Similar structures were visible in response to staining with SYTOX green (Jones and Singer, 2001). Bars = 0.1 mm.

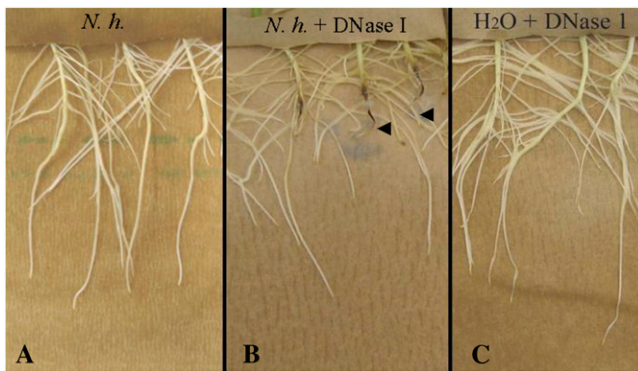


Figure 2. Loss of root tip resistance to infection in pea roots treated with DNase I at the time of inoculation with *N. haematococca* (*N. h.*). A, Most inoculated root tips resist infection, as described (Gunawardena and Hawes, 2002; Gunawardena et al., 2005; Wen et al., 2007): no lesions form at the root tip, which remains white and continues to grow in a manner indistinguishable from that of uninoculated controls. B, When coinoculated with DNase I (1.2 units) and fungal spores, 100% of inoculated root tips developed necrosis within 48 to 72 h. Root growth ceased, and fungal growth proliferated (arrowheads). C, Control roots treated with DNase I alone did not develop necrosis, and there was no significant difference in mean growth of control roots, with or without DNase I treatment.

then expresses defense genes, develops necrosis, and ceases growth within 24 h. In this study, the incidence of infection increased from $3\% \pm 3\%$ in inoculated root tips without DNase I (Fig. 2A) to 100% in inoculated root tips treated with DNase I (Fig. 2B). These differences were statistically distinct at $P < 0.0001$. Infections that developed in the presence of DNase I were marked by severe necrosis, total cessation of growth, and proliferation of hyphal growth surrounding the root tip regions (Fig. 2B, arrowheads).

One explanation for these results is that nuclease activity and/or the degradation of exDNA somehow results in plant cell death, allowing increased tissue ingress for the fungus. However, control experiments using DNase I treatment alone did not support this explanation: treatment with DNase I caused no root tip necrosis among treated roots (Fig. 2C; $n > 100$). At 48 h after treatment, the mean increase in root length, with and without DNase treatment of root tips, was 36 ± 8 mm ($n = 18$) and 34 ± 9 mm ($n = 18$), respectively. Border cell viability in uninoculated control roots was $95\% \pm 5\%$, with or without DNase treatment. Similar results were obtained when salmon sperm DNA or pea genomic DNA was added to roots and incubated with DNase I. These data suggested that it is the loss of the root cap slime exDNA, per se, that is responsible for loss of root tip resistance to infection when root tips are treated with DNase I.

An alternative explanation for increased fungal infection of root tips in the presence of DNase I is that breakdown of exDNA within the surrounding root cap slime provides an enhanced nutrient resource for the fungus, fostering more rapid growth and development

during early stages of the interaction. However, control experiments in which root mucilage collected from nuclease-treated roots was added to spores did not support this explanation: no increase in the rate of spore germination or hyphal growth was detected. Within 3 h of culturing spores with root exudates with and without DNase I treatment, spore germination rates were $78\% \pm 7\%$ ($n = 792$) and $80\% \pm 4\%$ ($n = 853$), respectively. During the 16-h course of incubation of spores with DNase I, measurements for hyphal growth in root exudates with and without DNase I treatment were $A_{620} = 0.027 \pm 0.016$ ($n = 20$) and $A_{620} = 0.028 \pm 0.012$ ($n = 20$), respectively, and were not statistically distinct.

The same results observed in growth pouch experiments (Fig. 2) occurred when the experiment was carried out under alternative conditions, with excised root tips immersed directly within the treatment solutions in wells of a microtiter plate (Fig. 3; Supplemental Fig. S2). A reporter strain of *N. haematococca* expressing GFP was used to visualize early stages of infection (Supplemental Fig. S2). At 24 h after inoculation of root tips with fungal spores alone, hyphal growth was minimal (Supplemental Fig. S2A). At 48 h, hyphal growth remained dispersed away from the root tip and no strands of GFP-expressing hyphae could be seen in contact with root tissues (Supplemental Fig. S2C). In root tips coinoculated with *N. haematococca* and DNase I (Supplemental Fig. S2, B and D), proliferation of GFP-expressing hyphae (black arrows) on the surface of and penetrating within root tissue was evident within 24 h. As in the growth pouch assay, the incidence of root tip infection, manifested as cessation of growth and development of root tip necrosis, increased within 72 h from $3\% \pm 4\%$ in the absence of DNase I to 100% of roots inoculated in the presence of DNase I ($P < 0.0001$). As in the growth pouch assay, adding salmon sperm DNA or pea genomic DNA did not alter the frequency of root tip infection, and no necrosis occurred on control roots treated with DNase I alone. Confocal microscopy revealed that in inoculated roots without DNase I treatment, growth of individual hyphae within 36 h was unbranched and limited to less than $100 \mu\text{m}$ in length (Fig. 3A), whereas growth in DNase I-treated samples hyphae was branched and more than $200 \mu\text{m}$ long (Fig. 3B). In DNase I-treated root tips, direct contact between proliferating fungal hyphae and the plant cell surface was evident (Fig. 3B). A profusion of border cells (Fig. 3B, white arrow) was apparent, and some fungal strands were seen to have penetrated the root epidermis (Fig. 3B, black arrows).

Slower DNA Digestion Is Correlated with Delayed Onset of Infection

The process of pea root infection by *N. haematococca* is a time-dependent process (Gunawardena and Hawes, 2002). If exDNA within root cap slime is necessary for preventing root tip infection, then alter-

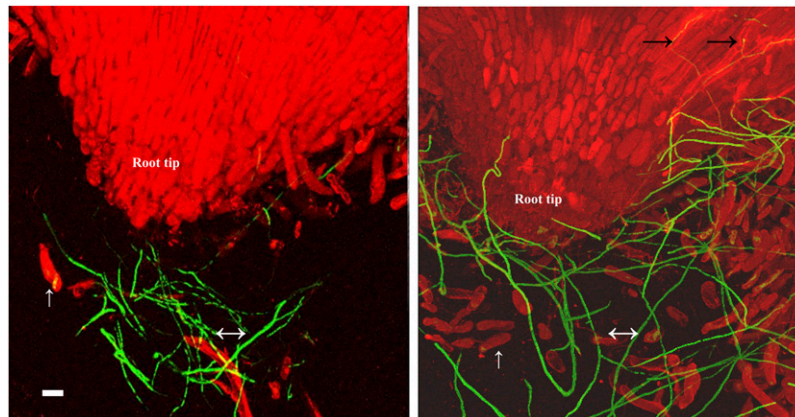


Figure 3. Early changes in *N. haematococca* (green)-root tip (red) interactions in response to DNase I treatment using a GFP reporter strain and viewed by confocal laser scanning microscopy. A, Limited growth of fungal hyphae (double white arrow) is evident at a distance from root tips (red). Detached border cells (red) are indicated with the white arrow. B, In DNase I-treated roots, proliferating fungal hyphae (double white arrow) can be seen among border cells (white arrow) and penetrating the root tip epidermis (black arrows). Photographs were taken at 36 h after inoculation, before visible necrosis had developed. GFP green fungal hyphae and red root autofluorescence images were obtained by dual excitation at 490 and 568 nm, respectively. Bar = 20 μ m.

ing the time at which loss of DNA integrity occurs might be predicted to influence the rate of disease development. This prediction was tested by comparing infection after treatment with a nuclease that requires a longer period of time to digest the exDNA.

First, BAL31, an exonuclease, was shown to require a significantly longer period of time to degrade pea DNA polymers than is required for DNase I under the conditions of our pea pathogenicity assay (Fig. 4). When a sample of pea genomic DNA was treated with DNase I under the conditions used in the root tip infection assay, as described above, the entire sample was degraded to fragments smaller than 250 bp within 2 h. By contrast, after 24 h of treatment with BAL31, the size range of pea genomic DNA remained within 250 bp to 6 kb.

If exDNA is required for root tip resistance to infection and its degradation underlies the observed changes in DNase I-treated root tip infection (Figs. 2 and 3; Supplemental Fig. S2), then the reduced rate of exDNA digestion seen with BAL31 (Fig. 4) would be predicted to result in delayed and/or reduced infection. We tested this prediction by treating inoculated roots with BAL31 instead of DNase I and found that this change was correlated with a 24-h delay in onset of necrosis and root tip destruction, compared with the response to DNase I. Most roots inoculated with *N. haematococca* (97% \pm 3%) escaped necrosis, and roots with uninvaded root tips continued to grow in a manner indistinguishable from that of uninoculated control roots (Fig. 5A). Root tips treated with BAL31 developed a tan discoloration within 72 h (Fig. 5B) but growth continued, and the degree of infection in BAL31-treated root tips was noticeably less severe than in root tips treated with DNase I (Fig. 5C). Only after 96 h did infection in roots treated with BAL31 and

N. haematococca progress to blackened necrosis and cessation of growth. This would be the predicted result if intact DNA polymers within root cap slime are required for its observed effect on the root tip resistance.

DNA in Root Cap Slime

The observation that DNase added to root tips eliminates resistance to infection lends support to the hypothesis that DNA is present in the extracellular matrix and that its presence and structural integrity are required for root tip defense. The possibility that exDNA is a component of root cap slime was confirmed by electrophoretic display of exDNA extracted from pea root cap slime (Fig. 6). In this study, the presence of DNA in the cell-free supernatant was

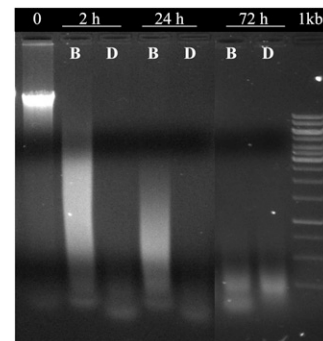


Figure 4. Delayed degradation of DNA by the exonuclease BAL31 compared with DNase I. Electrophoresis of pea genomic DNA treated with BAL31 (B) or DNase I (D), respectively, for 2, 24, or 72 h.

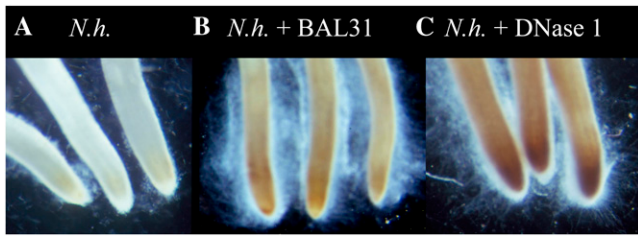


Figure 5. Delayed onset of symptoms in inoculated root tips treated with BAL31 compared with DNase I. A, Root tips 72 h after inoculation with *N. haematococca* (*N. h.*) are free of necrosis despite being surrounded by hyphal growth on surrounding border cells, as described (Gunawardena and Hawes, 2002; Gunawardena et al., 2005; Wen et al. 2007). B, Root tips 72 h after inoculation with *N. haematococca* in the presence of BAL31 exhibit tan discoloration at the root tips, but roots continue to grow. C, Root tips 72 h after inoculation with *N. haematococca* in the presence of DNase I exhibit dark brown to blackened necrosis. Root tips were treated with 1.2 units of BAL31 or DNase I.

estimated based on A_{260} and then was confirmed by analyzing a sample obtained using a standard phenol-chloroform protocol for DNA isolation (Sambrook et al., 1989). Each root yielded approximately 10 ng of exDNA by this method. DNA outside the root tip could have several sources, including plastid or nuclear DNA from the plant or nonculturable microorganisms that were undetected using growth on culture medium as an assay. Sequence analysis was used to evaluate the source of the exDNA in root cap slime. Electrophoretic display confirmed the presence of DNA, with a smear of DNA ranging in size from 150 bp to approximately 5 kb (Fig. 6A, lane 1). Its identity was confirmed based on its complete digestion by treatment with DNase I (Fig. 6A, lane 2). The slime exDNA was cloned, and 94 clones were sequenced. All were found to be of plant origin, and most were known sequences from pea or closely related legume species. Among these sequences, 25 are of unknown function. The remaining sequences are related to retroelements or repetitive DNAs, genomic sequences, pea chloroplast genes, 18S and 25S rRNAs, pea gene encoding gibberellin c20-oxidase, and pea gene encoding root-expressed trypsin inhibitor (Table I). The large proportion of repetitive DNA elements in the exDNA (approximately 70%) is representative of the pea genome, which contains an estimated 35% to 48% repetitive elements (Macas et al., 2007).

One possible source of exDNA is dead, dying, or lysed border cells that are released into slime upon immersion of root tips into water but were not detected by the methods used to control for such events. We estimated the number of cells that would be required to account for our results. In pea (cv Little Marvel), approximately 150 to 175 (5%) of the $3,500 \pm 500$ cells produced per root tip daily are nonviable when the radicle is 25 to 35 mm in length (Brigham et al., 1995). Given a genome size of approximately

4,000 Mb per haploid genome, 100% of the total nuclear DNA for the 3,500 diploid pea border cells delivered into the extracellular matrix would yield approximately 28 ng of exDNA. The 150 to 175 dead cells, assuming 100% of nuclear DNA content for every cell was extruded from all the cells and successfully collected from a fraction of the supernatant (as described above), would yield 1.4 ng, approximately 14% of the actual yield obtained when root cap slime was harvested. Moreover, this yield would be the expected result only if killed cells release exDNA through their cell walls after death. This possibility was examined. In control samples of slime from border cells that were washed to remove existing exDNA and then killed by freezing at -80°C , as described previously for secreted proteins (Wen et al., 2007), no additional exDNA could be collected from the supernatant.

Collection of Newly Synthesized exDNA from Root Cap Slime during a 1-h Period When There Is No Loss of Cell Viability

Previously, we showed that proteins of the root cap secretome are synthesized and secreted by living cells (Brigham et al., 1995; Wen et al., 2007). This was accomplished by profiling labeled extracellular proteins synthesized during a 1-h period in which root tips, with a full set of border cells present on the cap periphery, were incubated with [^{35}S]Met (Brigham et al., 1995). Labeled proteins released immediately into the supernatant when root tips were immersed

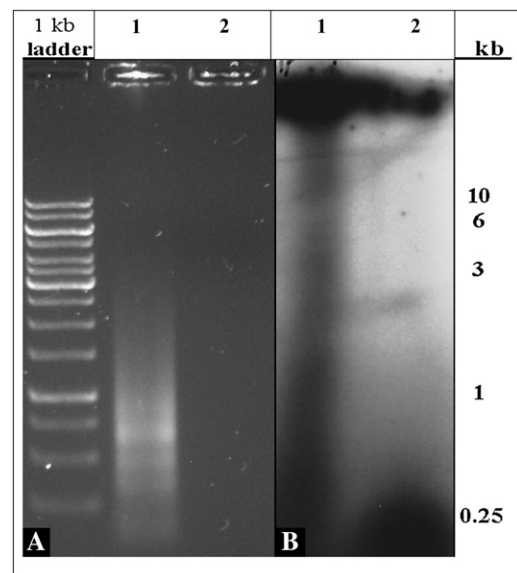


Figure 6. exDNA in root cap slime. A, exDNA isolated from root cap slime (lane 1) and after digestion by treatment with DNase I (lane 2). B, Autoradiograph of exDNA collected from root tips fed with [^{32}P]dCTP for 1 h (lane 1) and cell-free root mucilage collected, mixed with [^{32}P]dCTP for 1 h, and subjected to electrophoresis (lane 2).

Table 1. *exDNA sequence identification*

National Center for Biotechnology Information Identifier	Identity	Percent Identity	Alignment Length	Organism
AJ841794.1	mRNA for putative His-containing phosphotransfer protein 2	100.00	105	<i>Populus x canadensis</i>
X52575.1	rDNA for 18S (partial) and 25S (partial) rRNAs	100.00	63	<i>P. sativum</i>
AP006404.1	Genomic DNA, chromosome 5, clone:LjT43G19, TM0290	100.00	31	<i>Lotus japonicus</i>
AY143471.1	18S ribosomal RNA gene	99.80	489	<i>P. sativum</i>
X05423.1	Chloroplast psaA1 and psaA2 genes	98.99	597	<i>P. sativum</i>
AF300840.1	Clone PisTR-A/3 repeat region	97.50	80	<i>P. sativum</i>
AY299398.1	Clone Ps-cos16 LTR and Ogre retrotransposons	96.30	108	<i>P. sativum</i>
AY299394.1	Clone Ps-phage13 Ogre retrotransposon	95.24	273	<i>P. sativum</i>
AY299398.1	Clone Ps-cos16 LTR and Ogre retrotransposons	95.21	146	<i>P. sativum</i>
EF483939.1	Chloroplast thioredoxin <i>m</i> gene	95.12	328	<i>P. sativum</i>
EF483939.1	Chloroplast thioredoxin <i>m</i> gene	95.09	163	<i>P. sativum</i>
AY299397.1	Ps-cos14 Ogre retrotransposon	94.72	360	<i>P. sativum</i>
AY299398.1	Ps-cos16 LTR and Ogre retrotransposons	94.63	335	<i>P. sativum</i>
AY299397.1	Clone Ps-cos14 Ogre retrotransposon	94.63	242	<i>P. sativum</i>
AF155746.1	Clone Psat6 repetitive sequence	94.43	323	<i>P. sativum</i>
AJ965568.1	PDR1 retrotransposon partial right terminal repeat	94.10	305	<i>P. sativum</i>
AY299397.1	Clone Ps-cos14 Ogre retrotransposon	93.51	231	<i>P. sativum</i>
AC161106.13	Clone mth2-168f23	93.47	352	<i>Medicago truncatula</i>
AY299397.1	Clone Ps-cos14 Ogre retrotransposon	93.09	246	<i>P. sativum</i>
AF155759.1	Clone Psat22 repetitive sequence	92.86	42	<i>P. sativum</i>
AF155741.1	Psat1-1 repetitive sequence	92.74	468	<i>P. sativum</i>
AY299394.1	Clone Ps-phage13 Ogre retrotransposon	92.31	104	<i>P. sativum</i>
AY319329.1	Leb10_1394 genomic sequence	92.28	583	<i>P. sativum</i>
AY299396.1	Clone Ps-phage22 Ogre retrotransposon	91.67	204	<i>P. sativum</i>
EF483939.1	Chloroplast thioredoxin <i>m</i> gene	91.46	246	<i>P. sativum</i>
AY299394.1	Clone Ps-phage13 Ogre retrotransposon	91.39	395	<i>P. sativum</i>
AF155744.1	Clone Psat4 repetitive sequence	91.38	58	<i>P. sativum</i>
AF083074.1	Peabody/Ty3-type retrotransposon gag-pol precursor pseudogene	90.96	332	<i>P. sativum</i>
AY319329.1	Clone Leb10_1394 genomic sequence	90.91	374	<i>P. sativum</i>
AY299397.1	Clone Ps-cos14 Ogre retrotransposon	90.91	77	<i>P. sativum</i>
AF155768.1	Clone Psat30-31 repetitive sequence	90.91	66	<i>P. sativum</i>
AF138704.1	Gibberellin c20-oxidase gene, complete cds	90.91	55	<i>P. sativum</i>
AF083074.1	Peabody/Ty3-type retrotransposon gag-pol precursor pseudogene	90.08	242	<i>P. sativum</i>
AF155761.1	Clone Psat24 repetitive sequence	89.89	89	<i>P. sativum</i>
AY299398.1	Clone Ps-cos16 LTR and Ogre retrotransposons	89.68	310	<i>P. sativum</i>
AY299398.1	Clone Ps-cos16 LTR and Ogre retrotransposons	89.47	171	<i>P. sativum</i>
AJ965681.1	PDR1 retrotransposon partial right terminal repeat	89.41	85	<i>P. sativum</i>
DQ189096.1	RAPD amplicon Y15_1050 genomic sequence	89.39	66	<i>P. sativum</i>
AY299398.1	Clone Ps-cos16 LTR and Ogre retrotransposons	89.32	103	<i>P. sativum</i>
AC154867.1	Chromosome 2 clone mte1-6j20	88.65	141	<i>M. truncatula</i>
AY299398.1	Clone Ps-cos16 LTR and Ogre retrotransposons	88.35	395	<i>P. sativum</i>
AC146789.30	Clone mth2-71h24	88.27	162	<i>M. truncatula</i>
AF155749.1	Clone Psat1-9 repetitive sequence	88.26	264	<i>P. sativum</i>
AY299397.1	Clone Ps-cos14 Ogre retrotransposon	87.73	220	<i>P. sativum</i>
AF138704.1	Gibberellin c20-oxidase gene, complete cds	87.21	86	<i>P. sativum</i>
AJ277287.1	Partial enod18 gene, exon 1 and 5' UTR	86.93	153	<i>Vicia faba</i>
AY299398.1	Clone Ps-cos16 LTR and Ogre retrotransposons	86.77	189	<i>P. sativum</i>
AY299397.1	Clone Ps-cos14 Ogre retrotransposon	86.29	197	<i>P. sativum</i>
AF155759.1	Clone Psat22 repetitive sequence	86.08	79	<i>P. sativum</i>
AC124217.21	Clone mth2-36d22	86.02	415	<i>M. truncatula</i>
AY299397.1	Clone Ps-cos14 Ogre retrotransposon	85.95	555	<i>P. sativum</i>
AY303677.1	Clone E16_837 genomic sequence	85.57	194	<i>P. sativum</i>
AC137079.9	Clone mth2-27d17	85.37	82	<i>M. truncatula</i>
AF155751.1	Clone Psat12 repetitive sequence	85.33	300	<i>P. sativum</i>
AY299397.1	Clone Ps-cos14 Ogre retrotransposon	84.88	86	<i>P. sativum</i>
AC140031.22	Clone mth2-24d16	84.67	137	<i>M. truncatula</i>
CU013530.14	DNA sequence from clone MTH2-115K7 on chromosome 3	84.66	502	<i>M. truncatula</i>
AF300835.1	Clone PisTR-B/6 repeat region	84.58	201	<i>P. sativum</i>

(Table continues on following page.)

Table I. (Continued from previous page.)

National Center for Biotechnology Information Identifier	Identity	Percent Identity	Alignment Length	Organism
AF083074.1	Peabody/Ty3-type retrotransposon gag-pol precursor pseudogene	84.30	121	<i>P. sativum</i>
AJ291493.1	Ti9 gene for root-expressed trypsin inhibitor	84.00	150	<i>P. sativum</i>
AF083074.1	Peabody/Ty3-type retrotransposon gag-pol precursor pseudogene	83.04	112	<i>P. sativum</i>
AY299397.1	Clone Ps-cos14 Ogre retrotransposon	81.86	204	<i>P. sativum</i>
AY205612.1	SIRE1-13 retroelement	81.59	239	<i>Glycine max</i>
AP006690.1	Genomic DNA, chromosome 3, clone:LjT14J20, TM0388a	81.29	342	<i>L. japonicus</i>
AJ276592.1	lip1 mutant cop1 gene for constitutively photomorphogenic 1 protein, exons 1–19	80.95	189	<i>P. sativum</i>
AF279585.2	Retrotransposon TLC1.1	72.00	180	<i>Solanum chilense</i>

into water were separated electrophoretically, and their identities were confirmed by multidimensional protein identification technology (Wen et al., 2007). An analogous approach was used to examine the hypothesis that de novo synthesis by living cells can result in production of exDNA collected from root cap slime (Clark, 1988; Janska et al., 1998). Pea root tips were immersed into water containing 3,000 Ci mmol⁻¹ ³²P-labeled dCTP, a radiation dosage that does not cause significant cell death in mammalian cells even after a 5-h exposure under parallel conditions (Cooper and Burgess, 1985). At the end of a 1-h incubation, root cap slime was collected, as described above. This procedure resulted in the isolation of radioactively labeled exDNA ranging in size from 0.25 to greater than 10 kb (Fig. 6B, lane 1) in the absence of a change in cell viability, which remained at 95% ± 5%. In control experiments, root cap slime was first collected and then mixed with ³²P-labeled dCTP for 1 h to control for nonspecific labeling; no labeled DNA resulted from this procedure (Fig. 6B, lane 2).

DISCUSSION

A hallmark of root tip resistance to invasion by fungal pathogens is a phenomenon in which the pathogen appears to be prevented from forming intimate contact with the root surface (Hawes et al., 1998; Gunawardena and Hawes, 2002). Instead, there is formation of a “mantle” of germinating hyphae and border cells that detaches, leaving the root cap uninvaded. The mechanism underlying the surprising observation that a pathogenic fungus can germinate and grow within the immediate vicinity of newly emergent root tips without invading the cells or triggering a defense response has remained unclear (Gunawardena et al., 2005). Striking parallels appear to exist between the defense response in vertebrate white blood cells and in legume root tips. In response to microbial invasion in humans and other animal species, neutrophils enter the bloodstream and form NETs, extracellular structures that aggregate and kill pathogens by localizing them within a matrix of anti-

microbial peptides and proteins (Brinkmann et al., 2004; Medina, 2009). These extracellular traps also are produced by mast cells, which are present at the surface of tissues exposed to the environment and microorganisms (von Köckritz-Blickwede et al., 2008). exDNA linked with extracellular histone is a structural component of NETs, and treatment with DNase destroys NET integrity and function (Wartha et al., 2007). Perhaps the most compelling information regarding the importance of NETs is the discovery that pathogens like group A *Streptococcus* require extracellular DNases for virulence (Sumbly et al., 2005). In plants, a population of detached cells ensheathed within root cap slime is released into the environment to form a mantle that localizes and inhibits growth of pathogenic fungi (Gunawardena et al., 2005; Wen et al., 2007). When the root tip is treated with DNase, this NET-like mantle is destroyed and the normal resistance to infection is abolished. Like human pathogens that produce extracellular DNase activities now known to be involved in virulence (Sherry and Goeller, 1950; Sumbly et al., 2005; Buchanan et al., 2006), root-associated bacteria and fungi also produce extracellular DNase activities (Klosterman et al., 2001; Tavares and Sellstedt, 2001; Balestrazzi et al., 2007). Klosterman et al. (2001) have proposed that such enzymes in pathogenic fungi function by entering the nuclei of plant cells and causing DNA damage that triggers defense responses in nonhost tissues. It will be of interest in future studies to examine the alternative hypothesis that, as with human pathogens, these enzymes play a role in plant cell exDNA degradation as part of the infection process.

How exDNA might be delivered to the extracellular matrix of plants is unknown. Root caps are secretory organs, actively exporting the component polymers that make up root cap slime (Morré et al., 1967; Rougier et al., 1979; Foster, 1982; Chaboud, 1983; Guinel and McCully, 1986; Moody et al., 1988; Roy and Vian, 1991; Lynch and Staehelin, 1992; Carrolla et al., 1998; Knee et al., 2001; Barlow, 2003; Iijima et al., 2008). Delivery of the proteins of the root cap secretome also is energy dependent and requires living cells (Brigham et al., 1995). Our data from feeding root tips

with ^{32}P -labeled dCTP and collecting ^{32}P -labeled exDNA from root slime 1 h later, in the absence of measurable cell death, suggest that exDNA in pea is actively synthesized and exported by living cells. DNA previously was shown to be actively synthesized in the root cap periphery, the site of synthesis and export of root cap slime, but its significance was unclear (Phillips and Torrey, 1971; Jones, 1977). Of particular interest is a series of studies by Van't Hof and colleagues, who identified and characterized a distinct class of "extrachromosomal" genomic DNA replicated within cells of the pea root tip (Van't Hof and Bjerknes, 1982; Krimer and Van't Hof, 1983; Kraszewska et al., 1985). Their data suggested that after excision from chromosomes, this DNA can replicate autonomously and remain stable for several days, but its function was unclear. The extrachromosomal DNA was present among samples of DNA replicated in cells of the root tip, which is resistant to infection, but not in cells of the elongation zone, which is the primary site of infection by most soil-borne pathogens and symbionts (Curl and Truelove, 1986). These studies may provide tools to address whether exDNA is distinct to certain cell types like the root cap or is a common component of plant cell defense. As Kwon et al. (2008) have pointed out, defining the mechanistic compartmentalization of biosynthesis and delivery of cell walls and other components of the extracellular matrix as they relate to parasitic invasion remains a challenge for the future.

Our study indicates that, in plants as well as animals, DNA can serve in critical biological roles other than as the language of inheritance.

MATERIALS AND METHODS

Plant Material

Sterilization and germination of seeds of pea (*Pisum sativum* 'Little Marvel'; Meyer Seed), border cell collection, and viability measurement were as described (Brigham et al., 1995).

Histochemical Staining of Cell-Free Root Cap Slime

Border cells or root tips of intact seedlings were stained by DNA stain DAPI (Invitrogen) or SYTOX Green (Invitrogen) according to the manufacturer's instructions. Each treatment was replicated at least 10 times. Samples were viewed using an Olympus fluorescence microscope equipped with U-MWU2 (for DAPI excitation). The images were captured with an Olympus digital camera using MicroFire software.

Fungal Pathogenicity Assays

Nectria haematococca culture, collection of spores, root tip inoculation, and evaluation of infection using a growth pouch assay (Caetano-Anollés et al., 1992) were as described previously (Gunawardena and Hawes, 2002; Gunawardena et al., 2005; Wen et al., 2007). Root tips of radicles, prior to emergence of lateral roots, were used in all experiments. For nuclease treatments, DNase I or BAL31 (1.2 units) was added to *N. haematococca* spores ($60\ \mu\text{L}$ at 10^7 spores mL^{-1}) immediately before inoculation. Root tips treated with DNase I in water without fungal spores served as controls. The effect of DNase I on root development was examined by comparing growth

(millimeters of root length increase after time 0) of DNase I-treated control roots with water-treated control roots in three independent experiments with at least six replicate plants per experiment. For the microtiter plate assay, the apical 10 mm of root tips (15 mm in length) was inserted into wells of a 24-well microtiter plate and incubated at 24°C in the presence of $500\ \mu\text{L}$ of spore suspension (10^5 spores mL^{-1}). Values for pathogenicity assays reflect means and \pm SE from 10 to 20 independent experiments with at least six replicate plants per experiment.

Effect of DNase I-Treated Root Exudates on Fungal Spore Germination and Fungal Growth

Root exudates and border cells were collected separately from root tips incubated in water and in DNase I in microtiter plate wells. Root exudates were separated from border cells by a pulse spin of the mixture at the lowest speed in a microcentrifuge. Both exudate samples were heated at 75°C for 10 min to inactivate DNase I activity. Subsequently, fungal spores (10^4 spores mL^{-1}) were added to replicate exudate samples (at $200\ \mu\text{L}$) in independent microtiter plate wells. Five independent experiments, each with at least three replicate samples per treatment, were performed to examine the frequency of spore germination and fungal growth. Spore germination assays were assessed by direct counts of germinating spores, and fungal growth was assessed by measuring A_{620} using a microtiter plate scanner as described previously (Gunawardena et al., 2005). Nine independent tests were carried out, with 47 to 187 spores per replicate sample for each treatment.

For confocal microscopy analysis of interactions between root tips and *N. haematococca* fungal hyphae, root tips were incubated with fungal spores in microtiter plate wells with or without DNase I. Treated root tips were then observed with a confocal laser scanning microscope (Leica SP5). GFP green fungal hyphae and red root autofluorescence images were obtained by dual excitation at 490 and 568 nm, respectively. One optical section ($5\ \mu\text{m}$) is shown in Figure 3.

exDNA Extraction, Digestion, and Identification

Root tip mucilage was collected from the supernatant of washed border cells (Brigham et al., 1995; Wen et al., 2007). The slime was collected using nondestructive procedures that do not cause cellular injury or death, as described by Brigham et al. (1995). Root tips are immersed into water for 1 to 2 min and then gently agitated to disperse border cells, which are treated by a pulse of low-speed centrifugation to yield a pellet of living border cells and a supernatant that comprises "root cap slime" in a total volume of 1.5 mL. Border cells are readily visible as an approximately 50- to $100\text{-}\mu\text{L}$ pellet, and the supernatant is taken from the upper 1 mL volume, to prevent any uptake of cells at the pellet-supernatant interface. The absence of cells in a control sample of the supernatant is confirmed microscopically after high-speed centrifugation, and the presence of microbial contamination is surveyed by plating samples onto nutrient medium. Cell viability is measured at the beginning and end of the treatment using the vital stain fluorescein diacetate, which has a greater than 99% accuracy in measuring viability based on the presence of cytoplasmic streaming as the measure of viability (Hawes and Wheeler, 1982; Supplemental Fig. S1). A standard phenol-chloroform protocol for DNA extraction (Sambrook et al., 1989) was used to extract and purify exDNA from root mucilage. For DNA digestion experiments, genomic DNA was digested with 1 unit of DNase I or 1 unit of BAL31 separately in water at 24°C . Samples were removed at the indicated intervals, and the progress of DNA digestion was visualized by electrophoresis on agarose gels. Each treatment was replicated at least three times.

For sequence analysis, exDNA (200 ng) was subjected to A-tailing using RedMix Taq DNA polymerase (Sigma-Aldrich) at 72°C for 20 min and then cloned into pGEM-T Easy vector (Promega) through direct cloning as described (Janska et al., 1998). Random clones were sequenced at the University of Arizona Genomic Analysis and Technology Core Facility (<http://gatc.arizona.edu/>). Individual sequences were identified by batch BLAST search against the National Center for Biotechnology Information nonredundant nucleotide database.

Transformation of *N. haematococca*

Protoplasts of *N. haematococca* were prepared as described by Miao et al. (1991) with the exceptions that Kitalase ($6\ \text{mg}\ \text{mL}^{-1}$; Wako Pure Chemical

Industries) and Driselase (5 mg mL⁻¹; InterSpex Products) were used for cell wall digestion and the protoplasts were washed twice in 0.7 M NaCl, once in SuTC (20% Suc, 50 mM Tris-HCl, pH 8.0, and 50 mM CaCl₂), and resuspended in SuTC to obtain a concentration of 1 × 10⁷ protoplasts mL⁻¹. Plasmid pAM1292 DNA (a gift from Dr. Lei Li), containing the *Aspergillus nidulans* *grg1*-GFP fusion with a *grg1* promoter, was used for transformation of *N. haematococca* protoplasts. Transformation was performed as described by Sweigard et al. (1992) with the exceptions that the polyethylene glycol solution contained 60% polyethylene glycol 3500, 20% Suc, 50 mM Tris, pH 8.0, and 50 mM CaCl₂ and the final concentration of hygromycin B in the selection medium was 65 μg mL⁻¹. Transformants were picked after 3 to 7 d, examined microscopically for expression of GFP, and then stored as glycerol stock cultures at -80°C. Infection assays were as described above and in previous studies (Gunawardena and Hawes, 2002; Gunawardena et al., 2005; Wen et al., 2007). Care was taken to ensure at the time of collection that roots were not immersed in free water, in which case mantles will detach spontaneously, or dehydrated, in which case mantles may adhere to the surface and tear away from the root. Roots were lifted from the growth pouch or microtiter well and then placed onto a microscope slide for analysis. Each experiment was repeated at least three times, with four replicate plants per treatment.

In Vivo Labeling of Newly Synthesized exDNA

Ten seedlings, with border cells attached, were arranged radially in a sterile petri dish as described in previous assays for in vivo labeling of newly synthesized proteins (Brigham et al., 1995). Excised root tips (approximately 1 mm) were immersed in a central 100-μL droplet of water containing 150 μCi of ³²P (3,000 Ci mmol⁻¹)-labeled dCTP. After 1 h at 24°C, the mixture of root tip mucilage, border cells, and the radioactive dCTP was collected in a 1.5-mL tube. After centrifugation, border cells were visible in an approximately 30-μL pellet; of the remaining 80 μL of supernatant, a 5-μL aliquot was removed from the upper layer of fluid and subjected to electrophoresis on a 1-mm agarose gel. After removal of the aliquot, a sample of cells removed from the pellet was examined for viability. The gel was exposed directly to x-ray film and photographed. In the control sample, a 100-μL sample of mucilage was collected from 10 root tips and then mixed with 150 μCi of ³²P-labeled dCTP at 24°C. After 1 h, a 5-μL sample was processed as described above. This procedure was repeated three times, and Figure 6 is a representative example.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Root cap slime and border cells.

Supplemental Figure S2. Altered root tip colonization in response to DNase I treatment.

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