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Expression in Tobacco of a Functional Monoclonal Antibody Specific to Stylet Secretions of the Root-Knot Nematode

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Genes for the heavy and light protein chains of a monoclonal antibody ($6D_4$) specific to stylet secretions of *Meloidogyne incognita* were cloned as cDNAs containing a native leader sequence. Heavy and light chain constructs under control of the cauliflower mosaic virus 35S promoter were transformed independently into Xanthi tobacco. Transgenic plants producing either heavy or light chain protein were crossed, yielding progeny that produced assembled and functional $6D_4$ immunoglobulins (plantibodies). Specificity of plantibodies was identical to $6D_4$ derived from the hybridoma cell line, i.e., both bound to the root-knot nematode esophageal glands and stylet secretions. Based on tissue print analysis, plantibodies were expressed in leaves, stems, roots, and galls. However, plantibody expression had no influence on root-knot nematode parasitism of transgenic plants. Plantibodies possibly accumulate apoplastically whereas nematode stylet secretions might be injected into the cytoplasm of the parasitized cell, precluding plantibody interference with secretion function in parasitism.

Additional keywords: giant-cells, pathogen, resistance, transformation.

Plant defense against invading organisms relies on physical barriers, chemical compounds, or other cellular reactions like the hypersensitive response. Unlike vertebrate organisms, plants do not possess an antibody-mediated immune system, nor do they possess the related physiological functions and cell types involved in inactivating and killing of invading pathogens and parasites. However, engineering a plant with the ability to produce an antibody, or antibody fragments, to specifically bind and inactivate a biologically active molecule has promise to open new avenues for novel plant resistances against pests (Schots et al. 1992; Tavladoraki et al. 1993; Voss et al. 1995).

Cloning of antibody genes from hybridoma cell lines has enabled researchers to express monoclonal antibodies in non-vertebrate organisms including insect cell culture (Pulitz et al. 1990), yeast (Carlson 1988; Horwitz et al. 1988; Wood et al. 1985), and plants (Düring et al. 1990; Hein et al. 1991; Hiatt et al. 1989; Hiatt and Ma 1992; Owen et al. 1992; Ma et al. 1994; Ma et al. 1995; Van Engelen et al. 1994; Voss et al. 1995). The multimeric structure of immunoglobulins, however, poses a challenge in generating functional antibodies. The typical IgG antibody molecule, the type of antibody frequently used in molecular and biochemical studies, is made up of two identical sets of heavy and light chains. These subunits are covalently cross-linked by disulfide bonds. The IgM antibody molecule is comprised of five covalently cross-linked IgG-like molecules plus a joining protein, the J chain. In the native animal cell, individual antibody chains are targeted into the endoplasmic reticulum (ER) by leader peptides. There, leaders are proteolytically cleaved and antibody chains are assembled and cross-linked.

Expression in plants of full-size functional antibodies, termed plantibodies, has not been achieved in the cytoplasm, probably due to prevalent reducing conditions breaking down disulfide bonds or due to a lack of chaperone proteins instrumental to assembling and cross-linking of antibody chains. Successful production of full-size plantibodies has relied on the presence of peptide leader sequences to ensure proper assembly inside the ER. The site of accumulation of assembled antibodies in plant tissue has not been fully resolved. Although secretion into the apoplast seems most logical (Hiatt and Mostov 1993; Ma et al. 1995; Van Engelen et al. 1994), one immunohistochemical study of plantibody-expressing tobacco plants revealed immunoglobulins in chloroplasts (Düring et al. 1990).

Root-knot nematodes (RKN), plant parasites in the genus *Meloidogyne*, are present in most agricultural regions throughout the world. These nematodes are among the world's most damaging agricultural pests, attacking nearly all crops grown (Sasser 1980). Besides their tremendous economic importance, root-knot nematodes exhibit a complex life cycle and interaction with their host plants (Eisenback and Triantaphyllou 1991). Infective second-stage juveniles (J2) hatch from eggs in the soil and penetrate plant roots near the root tip. After migrating to the vascular tissue, they inject secretions into five to seven cells in the vascular cylinder. In roots of susceptible plants, the recipient cells presumably respond to the nematode secretions by undergoing karyokinesis uncou-

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pled from cytokinesis, ultimately leading to highly multinucleate cells. Furthermore, cell size increases dramatically, walls are remodeled by formation of ingrowths, and the cytoplasm becomes dense with an increase in cell organelles. Fully developed feeding cells, called giant-cells, are morphologically similar to transfer cells and have a high rate of metabolism (Jones 1981). With nourishment from the giant-cells, the root-knot nematode completes its life cycle. Another more conspicuous host response associated with root-knot nematodes parasitism is the galling of infected root tissue.

Secretions originating in three esophageal glands are considered to be the chemical signals with which root-knot nematodes alter plant cell cycle and cell differentiation (Hussey 1989a; Hussey et al. 1994). These nematode compounds are in all probability secreted through the nematode's stylet into root cells. However, it is not clear whether the secretions are deposited between the cell wall and plasma membrane or injected directly into the cytoplasm of the host cell. At either site, putative receptors, membrane-bound or cytoplasmic, may be activated to trigger signal transduction cascades leading to giant-cell formation.

Inactivation of giant-cell-eliciting nematode secretions in the plant tissue through binding by plantibodies has promise to interfere with compatible nematode-plant interactions. In this paper, we report expression in tobacco of the monoclonal antibody $6D_4$ (Hussey et al. 1990) under control of the cauliflower mosaic virus 35S promoter. This antibody binds to the nematode dorsal and subventral esophageal glands and to stylet secretions of infective juveniles and adult females. Although $6D_4$ is an IgM antibody, only the heavy and light chain genes were used in this study, allowing assembly into the IgG-like IgM subunits. Production of plantibodies was demonstrated in noninfected and nematode-infected transgenic plants, and the effect of the plantibody on nematode reproduction was assessed.

RESULTS

Transformation and recovery of plants.

Independent *Agrobacterium*-mediated transformation of Xanthi tobacco with cDNA constructs of the full-length $6D_4$ heavy and light chain genes, including a native murine leader sequence, generated transgenic plants producing either light or heavy chain protein as revealed by ELISA analyses. These plants were crossed and their progenies were analyzed by ELISA for production and assembly of both antibody chains. Positive plants were identified, and self progeny of one such plant was used for the remainder of this study. ELISA analyses of leaf samples revealed that positive self progeny plants accumulated immunoglobulin protein to similar levels.

Western blot analyses under reducing conditions of leaf extracts from plantibody-producing and wild-type plants showed that heavy (50 kDa) and light chain protein (30 kDa) accumulated in the transgenic plant tissue (Fig. 1). The heavy chains of the plantibodies or the hybridoma-derived antibody appeared to consistently form stable dimers (97 kDa) which were not separated under the reducing conditions used in this study (Fig. 1). The 97-kDa bands did not contain light chains as demonstrated by the lack of binding of light chain-specific antibodies in Western blots (Fig. 1). Interestingly, similarly prepared Western blots of a hybridoma-derived and plant-produced IgG antibody (data not shown) did not display the observed high molecular weight band. Plant-produced heavy chains apparently were also subject to degradation as shown by their smaller size (50 kDa) when compared to those from hybridoma culture filtrate (68 kDa). When present in the putative dimer (97 kDa), plantibody heavy chains appeared to be protected from proteolytic attack because heavy chain dimers were of equal size in the hybridoma culture filtrate and the plantibody preparation. The discrepancy between the added molecular weight of two heavy chains and the observed molecular weight of the suspected dimer is probably due to the structural conformation of the dimer. Incomplete denaturing, i.e., dimer formation, will interfere with electrophoretic separation strictly proportional to molecular weight.

Western blot analysis with antibodies to light chains visualized two bands in extracts from transgenic plants (Fig. 1). The top band, being slightly larger than the hybridoma light chain, very likely represents nonprocessed light chains still containing the 16 amino acid leader peptide.

Plantibody activity and specificity.

Leaf and root extracts from plantibody-producing plants were assayed for binding activity in ELISA tests using concentrated nematode stylet secretions as antigen. Both showed positive reactions when compared to identically prepared wild-type plant extracts. Conservative calculations using ELISA data estimated total functional antibody in leaves and roots at approximately 0.01% and 0.003% of total protein, respectively. These values were derived by estimating an average $6D_4$ concentration in hybridoma culture filtrates of 10 mg/liter and an average total protein content of leaves and roots of 1% their fresh weight. However, the hybridoma-produced antibody is present as full-size pentameric IgM molecules, whereas the plantibody only assembles into the IgG-like monomers. The IgM pentamer has the ability to bind more strongly to its antigen

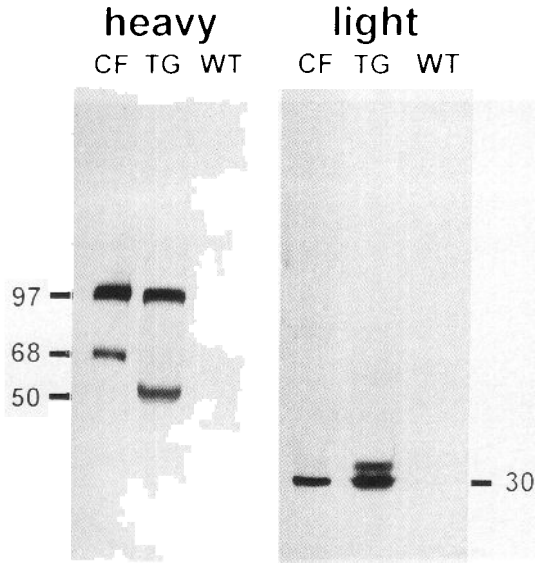

Fig. 1. Western blot of a polyacrylamide gel after electrophoresis under reducing conditions of $6D_4$ hybridoma culture filtrate (CF), leaf extracts of a transgenic plant expressing $6D_4$ heavy and light chain protein (TG), and leaf extracts of wild-type tobacco (WT). Protein sizes are given as kDa. Blots were probed with antibodies specific to either heavy (heavy) or light chains (light) of mouse IgM.

than a monomer alone. This leads to a higher proportion of antigen-antibody complexes and therefore to higher ELISA readings when comparing equal amounts of plant-derived and hybridoma-produced antibody. As a consequence, the concentration of functional plantibody will be underestimated.

To verify the plantibody binding activity and specificity, leaf extracts were used in indirect immunofluorescence analyses with J2 and adult female nematode sections. In all cases, $6D_4$ plantibody (Fig. 2A) had the same specificity in sections of J2 and adult females as did the hybridoma-derived monoclonal $6D_4$ when used directly as hybridoma culture filtrate or when added to wild-type tobacco extracts (Fig. 2B). In no instance was any labeling observable with wild-type tobacco extracts (Fig. 2C).

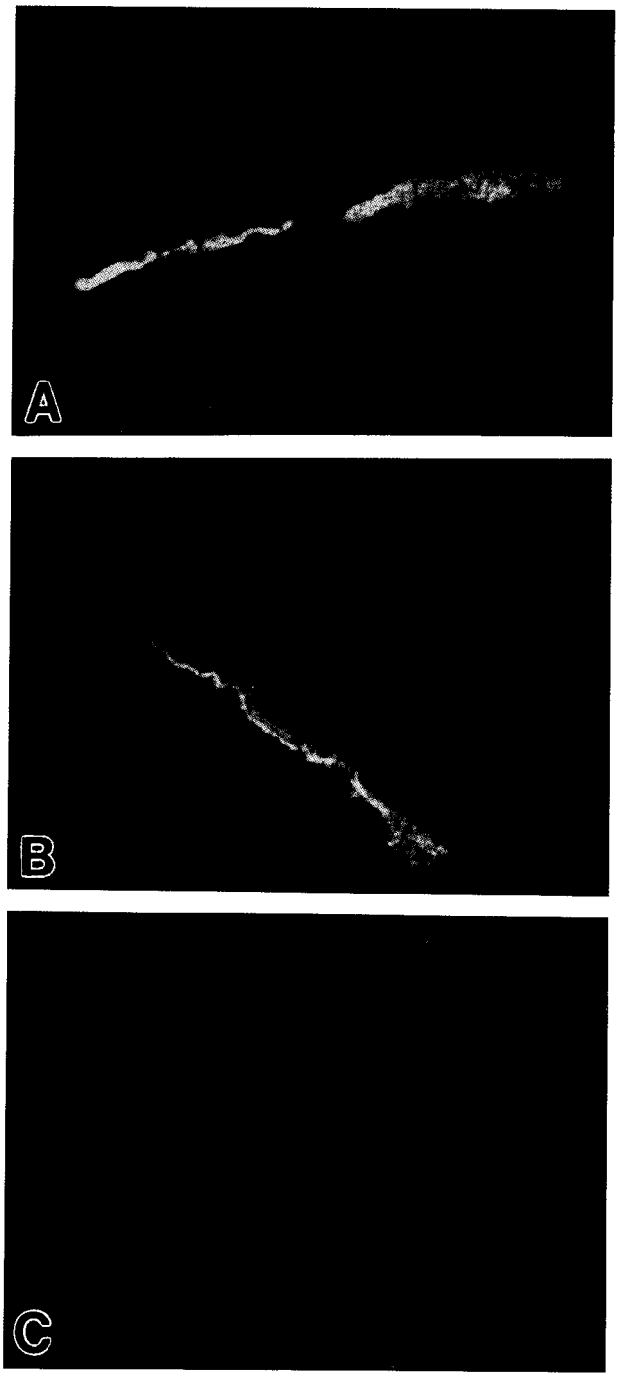

Fig. 2. Indirect immunofluorescence (FITC) microscopy of sections of *Meloidogyne incognita* second-stage juveniles using leaf extracts of a transgenic plant expressing $6D_4$ heavy and light chain protein (**A**), wild-type tobacco spiked with $6D_4$ culture filtrate before grinding (**B**), and wild type tobacco (**C**), and fluorescin-conjugated second antibody. Esophageal glands of the juveniles are labeled with FITC.

Tissue printing.

Tissue print analyses provided an understanding of the spatial accumulation of plantibody in transgenic plants. This technique identified antibody expression over the entire cross sections of stems, leaves (laminae and mid ribs), and roots (Fig. 3), as well as flowers and developing seed pods (data not shown). Much stronger signals were obtained with aboveground plant parts than with roots. Tissue printing of galled roots of nematode-infected plantibody-producing tobacco proved that immunoglobulins were also present in infected root tissues for at least the first 15 days after nematode infection. Staining was observed throughout entire cross sections of galls containing giant-cells (Fig. 3). However, whether plantibodies were present in the giant-cells could not consistently be resolved with tissue printing.

Effect on root-knot nematode parasitism.

To determine whether plantibody production in transgenic plants had an effect on nematode parasitism, plantibody-producing and control plants were inoculated with *M. incognita* in two separate greenhouse experiments. Nematode reproduction was assessed as number of eggs produced per gram of root fresh weight. Plantibody production did not affect nematode reproduction in any treatment (one experiment shown in Fig. 4).

DISCUSSION

In our experiments, we were successful in expressing a murine antibody in tobacco plants. The $6D_4$ plantibody displayed the same specificity as the hybridoma-produced monoclonal antibody, which is to say, they both bound to nematode esophageal glands and stylet secretions. As in previous work (Hiatt et al. 1989; Ma et al. 1994, 1995; Van Engelen et al. 1994), the assembled full-size antibody accumulated to easily detectable amounts in plant tissues. These results confirm that the plant protein synthesis and processing machinery can recognize molecular animal signals for transport into the ER, cleavage of leader peptides, and correct assembly.

Another interesting aspect is that the original $6D_4$ hybridoma cell line produces an IgM isotype antibody. Expression of the IgM heavy and light chains alone without the J chain was sufficient to obtain strong antigen binding activity. Similar findings were made also by Düring et al. (1990).

The antibody gene constructs used for tobacco transformation contained the full-length cDNA coding regions of heavy or light chains plus a murine leader sequence required for uptake into the ER for proper assembly. Proteins in the ER are targeted for secretion and, therefore, full-size plantibodies should accumulate mainly in the apoplast (Hiatt and Mostov 1993; Van Engelen et al. 1994). Due to the size of the multimeric immunoglobulin, passage through the cell wall can be expected to be limited because of the small size of cell wall

pores, which have an estimated size exclusion limit of approximately 20 kDa (Carpita et al. 1979). Hence, the site of highest plantibody concentration should be the space between plasma membrane and the cell wall. Van Engelen et al. (1994) reported a retardation of full-size plantibodies by the cell wall, whereas a smaller antibody fragment was able to freely traverse this barrier. Light microscopic immunolocalization of plantibodies by Ma et al. (1995) identified immunoglobulins as not penetrating cell walls. Our tissue print analyses did not further elucidate this aspect.

Plantibody production had no effect on nematode parasitism and reproduction. Six hypotheses may explain this observation. (i) The nematode secretion recognized by $6D_4$ ($6D_4$ antigen) might not be important for nematode parasitism. However, the presence of the $6D_4$ antigen in stylet secretions suggests that the protein has a role in parasitism. (ii) Binding of the plantibody to the nematode secretion does not interfere with the function of the secretion, i. e., binding is away from the effector site. Unfortunately, in vitro analyses of function and biological activity of nematode secretions have not been successful to this date. (iii) The nematode secretes the $6D_4$ antigen directly into the cytoplasm of the recipient cell and hence the antigen is never exposed to the plantibody, which probably accumulates in the ER and the apoplastic space. (iv) The amount of plantibody may have been too low. However, as mentioned above, the level of antibody accumulation is very likely considerably higher than our estimates indicate. Furthermore, immunofluorescence analyses have shown that $6D_4$ gave strong and specific labeling of its antigen in much lower concentrations than encountered in our plants. It seems likely that, if nematode secretions and plantibodies in fact do meet, even very low concentrations could allow the proposed inactivation. To use the higher plantibody expression in aboveground plant parts, we injected *Meloidogyne* juveniles into the midribs of leaves. All wild-type and transgenic plants allowed equal infection and reproduction (data not shown). (v) The 35S promoter might not direct gene expression in the specific cell type the nematode injects the secretions into (usually phloem parenchyma). Studies of 35S activity (Battraw and Hall 1990; Jefferson et al. 1987; Nagy et al. 1985) do not specifically indicate whether 35S activity occurs in this particular cell type. (vi) The 35S promoter might have been silenced under nematode attack, as reported by Goddijn et al. (1993). However, our tissue prints showed that $6D_4$ plantibody was present in galled tissue. Furthermore, the presence of the $6D_4$ antigen in J2 secretions indicates a role in the very early phases of parasitism, probably prior to nematode-induced changes in gene expression. Therefore, at the time of initial secretion, $6D_4$ plantibody, if present, should be available to neutralize the nematode antigen.

ELISA and tissue print analyses revealed that levels of antibody accumulation were considerably lower in root tissues of all ages when compared to equal amounts of leaf tissue, even though the 35S promoter has been shown to be very active in root tissue (Battraw and Hall 1990; Jefferson et al. 1987; Van Engelen et al. 1994). More detailed studies are needed to give an explanation for this observation.

We have not been able to use immunocytochemical analysis of nematode-infected roots using the $6D_4$ antibody to resolve the actual site of deposition of the $6D_4$ antigen (R. S. Hussey, unpublished). If nematode secretions are indeed injected directly into the cytoplasm, their neutralization by plantibodies mandates cytoplasmic accumulation of plantibodies. Cytoplasmic accumulation might be achieved by expression of only the variable regions of both the heavy and light chains joined by a linker peptide forming single-chain variable fragment constructs (scFv) (Owen et al. 1992). Expression of such constructs from an antibody specific to the coat protein of the artichoke mottled crinkle virus has revealed antigen-binding activity in the cytoplasm, which attenuated virus infection of transgenic tobacco (Tavladoraki et al. 1993). Currently we are transforming tobacco plants with scFv constructs of the $6D_4$ antibody. Future experiments will determine if the $6D_4$ antigen can be neutralized by cytoplasmic $6D_4$ scFv plantibodies.

In conclusion, a monoclonal antibody specific to root-knot nematode stylet secretions was expressed in tobacco. Planti-

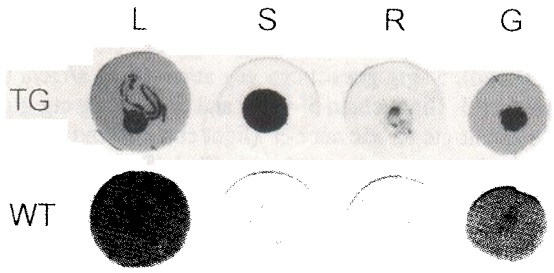

Fig. 3. Tissue print analysis of cross sections of leaves (L), stems (S), roots (R), and *Meloidgyne incognita*-infected root galls (G) of transgenic tobacco expressing $6D_4$ heavy and light chain protein (TG) and wild-type tobacco (WT). Cross sections of plant tissues were printed onto nitrocellulose membrane and probed as described in the text. Round pieces of membrane containing the tissue prints were cut out and photographed. Imprints of wild-type tissues are weakly visible due to plant pigments and are not due to alkaline phosphatase conjugated secondary antibodies.

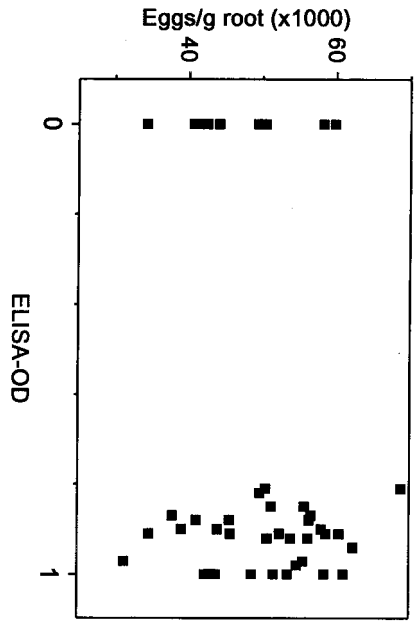

Fig. 4. Reproduction data of *Meloidogyne incognita* (eggs per gram of root) on tobacco expressing assembled $6D_4$ heavy and light chain protein (ELISA-OD around 1) and tobacco expressing either heavy or light chain protein of $6D_4$ (ELISA-OD of 0) measured in leaf tissue.

bodies and hybridoma-derived monoclonal antibodies showed identical specificity. Although tobacco plants produced functional immunoglobulins, nematode parasitism was not affected. The nematode secretion in question is either not important for parasitism or not neutralized by the $6D_4$ plantibody expressed under control of the 35S promoter and with native leader peptides.

MATERIALS AND METHODS

Monoclonal antibody generation and cDNA cloning.

The $6D_4$ antibody was generated by intraperitoneal injection of homogenized J2 *M. incognita* (Hussey 1989b; Hussey et al. 1990). Coding regions containing all domains of the full-length heavy and light chain proteins were amplified with the polymerase chain reaction as described in Ma et al. (1994). Primer sequences for individual genes were: 5′-tgg tac ctc gag cca ggt g/ca/ca a/gct gca gg/ca gtc a/tg-3′ (heavy chain 5′ end), 5′-atc tcg aga att ctc aat agc agg tgc ctc c-3′ (heavy chain 3′ end), 5′-gtg gta cct cga gcg at/ca tt/cc/g a/tgc/a tg/ca ccc aa/gt ct-3′ (light chain 5′ end), and 5′-ggg gag ctg gtg aat tcg tcg acc ttt gtc tct aac act c-3′ (light chain 3′ end). Amplification products were digested with *Xho*I (5′ end) and *Eco*RI (3′ end) and ligated into pMON530 (Rogers et al. 1987) containing a murine immunoglobulin leader sequence (MGWSWIFLFLLSGGTS) and the cauliflower mosaic virus 35S promoter (Ma et al. 1994).

Transformation and recovery of plants.

Plasmids containing the coding regions of the heavy and light chains of $6D_4$ were transformed directly into *Agrobacterium tumefaciens* strain EHA101 by the freeze-thaw method of An et al. (1988). Transformation of Xanthi tobacco and recovery of transgenic plants was as described in Hein et al. (1991).

ELISA for production of heavy or light chain protein.

Multiwell plates (Falcon, Becton Dickinson, Oxnard, CA) were coated overnight at 4°C with goat antibodies to either mu or kappa immunoglobulin chains (Fisher Biotech, Pittsburgh, PA) diluted 1:500 in PBS (136 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4). Antibodies were discarded and plates were blocked with 1% nonfat dry milk and 1% BSA (Fisher Biotech) in PBS for 1 h at 37°C. During blocking, plant samples (approximately 70 mg) were harvested into 1.5-ml microcentrifuge tubes, chilled on ice, and ground in 200 µl of PBST (PBS plus 0.1% Tween 20) supplemented with a proteinase inhibitor cocktail to the following final concentrations: 0.5 mg of leupeptin per liter; 1 mM EDTA-Na_2; 0.7 mg of pepstatin per liter; 0.2 mM PMSF; 0.2 mM iodoacetamide. Grinding was achieved in the microcentrifuge tubes with reusable plastic pestles driven by an electric motor. Homogenates were centrifuged for 10 min at 16,000 × *g* in a microcentrifuge, and 150 µl of the supernatants were transferred into blocked wells of the test plates. Plates were incubated for 1.5 h at 37°C and then rinsed twice with PBST. The same antibodies used for coating of plates were used as alkaline phosphatase (AP)-conjugates (anti kappa: Fisher Biotech; anti mu: Bio-Rad Laboratories, Hercules, CA) diluted to 1:500 in PBST to label the captured antigen for 1 h at 37°C. Plates were then washed once in PBST, in PBS, and in AP reagent buffer (10% diethanolamine, 1 mM $MgCl_2$, pH 9.8) for 5 min each. AP substrate (Sigma 104 phosphatase substrate tablets, Sigma Diagnostics, St. Louis, MO) was added and color development was measured.

ELISA for production of assembled heavy and light chain antibody.

This procedure was essentially identical to ELISA method described above, except that plates were always coated with the antibodies to the kappa chain and the antibodies to the mu chain were always used as the AP-conjugated secondary antibody.

Western blot analysis of leaf tissue.

Two leaf disks were clipped from young leaves of test plants with the lids of 1.5-ml microcentrifuge tubes as tools. The microcentrifuge tubes containing the leaf material were immediately immersed in liquid nitrogen. The tubes were removed individually from liquid nitrogen, 100 µl of extraction buffer (75 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol) was added, and the material was ground for approximately 1 min as described above. Homogenates were boiled for 10 min and left to cool to room temperature. All samples were centrifuged as described above to remove debris, and 70 µl of the supernatants was transferred to new microcentrifuge tubes. Ten microliters of loading dye (80% glycerol, 0.01% bromophenol blue) was added, and the desired amount of homogenate (5 to 30 µl) was electrophoresed in precast 4 to 15% gradient gels (Bio-Rad) at 180 V, until the bromophenol blue dye front reached the bottom of the gel. Gels were then soaked in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 10 min and blotted in a MiniTransBlot apparatus (Bio-Rad) onto 0.45 µm-pore size nitrocellulose (Schleicher & Schuell, Keene, NH) for 1 h at 100 V. Nitrocellulose membranes were then rinsed twice for 5 min in PBST and blocked in 5% nonfat dry milk in PBS for 0.5 h. After blocking, membranes were incubated for 1.5 h in AP-conjugated goat antibodies to mouse kappa chains (Southern Biotechnology Associates, Inc., Birmingham, AL) or to mouse mu chains (Bio-Rad) diluted 1:1,000 in PBST, followed by two rinses in PBST and one in AP reagent buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5). AP chromogenic substrate (BCIP and NBT, Gibco BRL, Life Technologies, Inc., Gaithersburg, MD) was added and color development was monitored.

Tissue printing.

Tissue printing followed the procedure of Cassab and Varner (1987). Printed nitrocellulose membranes were treated and developed as described for Western blots. AP-conjugated goat antibodies to kappa and mu chains, mixed in equal amounts, were used as secondary antibodies.

Assay for functionality by ELISA.

Multiwell plates were coated overnight at 4°C with 5 to 10 µl of concentrated *M. incognita* stylet secretions (Davis et al. 1992) in 50 µl of BBS (per liter: 6.2 g of boric acid; 9.5 g of $Na_2B_4O_7$ 10 H_2O; 4.4 g of NaCl; pH 8.5). After two rinses with PBST, plates were blocked and subsequently filled with 100 µl of leaf or root extracts prepared as described for ELISAs. Plantibody bound to its secretory antigen was labeled

with AP-conjugated goat antibodies to mouse mu chains and detected as for ELISAs.

Assay for functionality by indirect immunofluorescence.

Two leaf disks were harvested and ground as described for ELISA. Blocked sections of *M. incognita* J2 and adult females were incubated overnight at room temperature in leaf extracts. Preparation of nematode sections and further processing and immunofluorescence analysis are described by Hussey (1989b).

Inoculation with root-knot nematodes and reproduction assessment.

In two separate experiments, a total of 93 4- to 5-week-old wild-type or transgenic plants producing assembled and functional plantibody or producing only one plantibody chain were grown in 10-cm-diameter styrofoam cups in the greenhouse. Plants were inoculated with 10,000 eggs of *M. incognita* (Hussey and Barker 1973) and maintained in the greenhouse. Forty-five days after inoculation, roots were washed free of soil and root fresh weights were determined. Eggs were collected (Hussey and Barker 1973) and counted to determine the number of eggs per gram of root fresh weight.

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