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MOLECULAR BIOLOGY AND PHYSIOLOGY

Comparison of *MIC-3* Protein Accumulation in Response to Root-knot Nematode Infection in Cotton Lines Displaying a Range of Resistance Levels

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ABSTRACT

The *MIC-3 (Meloidogyne Induced Cotton protein)* cDNA, corresponding to a 14 kDa protein whose expression is induced by infection with root-knot nematode (RKN) in a RKN resistant (R) cotton breeding line, was previously cloned and sequenced. In this study, a polyclonal antibody to the *MIC-3* protein was developed through transformation and over-expression of a *MIC-3* fusion protein in an *E. coli* system. The antibody was then used as a probe to further verify the relationship between the *MIC-3* cDNA and the 14 kDa protein and to assess levels of 14 kDa (*MIC*) protein accumulation in a larger group of RKN resistant breeding lines and susceptible cultivars of cotton. The *MIC* antiserum recognized the 14 kDa protein and revealed a negative correlation between the amount of MIC protein in root tissues and root galling index.

Host plant resistance for the control of nematode pathogens, such as *Meloidogyne incognita* (RKN), has long been a desirable goal; however, for both economical and practical reasons, available resistant (R) breeding lines of cotton have not been developed into commercial cultivars. A primary obstacle to the use of R germplasm has been the labor intensive and prolonged time necessary to breed resistance into desirable cultivars, due in part to a lack of available molecular markers linked to the trait and the absence of cloned resistance genes from cotton. To overcome these limitations, research on many fronts is focused on understanding the molecular interaction between nematodes and the plant root cell with regard to factors and/or signals that allow feeding site formation, as well as resistance mechanisms that restrict or prevent development and reproduction of nematodes (Davis et al., 2000; Gheysen and Fenoll, 2002; Abad et al., 2003).

A ~14-kDa protein was previously identified that was induced 8 to 10 d after inoculation (8-10 DAI) in the immature root galls of an RKN resistant cotton isoline (Callahan et al., 1997). Based on a limited internal amino acid sequence for the 14-kDa protein, a degenerate primer was designed to amplify a sequence from a cotton λZAPII cDNA library constructed from mRNA from excised, immature galls of resistant cotton (M-249) roots at 10 DAI with RKN. A full-length cDNA, *MIC-3 (Meloidogyne Induced Cotton)* protein, was cloned and sequenced (Zhang et al., 2002). The putative open reading frame of *MIC-3* encoded a protein of 141 amino acids with a calculated molecular mass of 15.3 kDa. Southern blot analysis of the novel *MIC-3* gene indicated it belonged to a multi-gene family of at least six members. RNA blot analysis with a *MIC-3* cDNA probe was in agreement with earlier protein work regarding root localization and apparent up-regulation of the *MIC* gene in the inoculated R line, which indicated that *MIC-3* may be involved in the resistance response to root-knot nematode. To our knowledge, characterization of *MIC-3* was the first report of a root-specific gene with increased expression in nematode resistant plants following nematode infection.

A concern of the previous work was that it was limited to a single resistant breeding line (M249) and its recurrent susceptible parent. In this study, a polyclonal antibody to the *MIC-3* protein was developed through transformation and over-expression of a *MIC-3* fusion protein in an *E. coli* system. The antibody was then used as a probe to verify the relationship between the *MIC-3* cDNA and the 14

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kDa protein and to measure accumulation of the 14 kDa (*MIC*) protein in a larger group of RKN resistant breeding lines and susceptible cultivars of cotton.

MATERIALS AND METHODS

Plant material. Lines of *Gossypium hirsutum* L. that had different levels of resistance to RKN were grown in the greenhouse and inoculated with RKN. RKN eggs were collected from tomato and cotton roots using 1.05 % sodium hypochlorite (Shepherd, 1983). Eggs were then suspended in an aqueous solution and calibrated to 5000 eggs/mL. The cultivars, DES 119, DPL 90, DPL 50, DPL 61, Suregrow 125, (Delta Pine and Land; Scott, MS), Stoneville La887 (Stoneville Pedigreed Seeds; Memphis, TN), and NemX (California Planting Cotton Seed Distributors, Shafter, CA); and the RKN resistant breeding lines, M-92, M-315, M-272, M-155, M-331, M-120, M-240, and the susceptible check M-8 (USDA-ARS, Mississippi State, MS) were planted in plastic 7.6-cm pots that contained screened Tallassee soil (a fine, loamy sand) fumigated with methyl-bromide. There were five pots per plot with one plant per pot. The experimental design was a randomized complete block with a split-plot arrangement of treatments and two replications. Seeds were planted into 1.5 cm by 2.0 cm holes made in the soil in the pots. Whole plots were inoculated and non-inoculated plants, and split plots were cotton lines. For the inoculated plots, RKN eggs were administered directly onto the seed in 2 mL aliquots, and the holes were covered with moistened soil. The beds were covered with a layer of black plastic followed by aluminum foil for 3 d. Plants were grown for up to 44 d following emergence at 27 ºC/24 ºC (+/- 3 ºC) day/night with 14-h day lengths. The stems were cut 5 cm above the soil and the roots were removed from the pots. Roots were placed in wire baskets and rinsed free of soil using tap water. For protein analysis, roots were collected at ~10 d post-emergence, when roots of RKN inoculated plants just began to display immature galls along the root system (Callahan et al., 1997). A root gall index was conducted on plants at 44 d post-emergence. For root galling determinations, bare roots were maintained in plastic cups filled with water until evaluations were completed. The galling index was based on ratings described by Shepherd (1983), where 1 = no galling, 2 = very light galling, 3 = moderate galling, 4 = heavy galling, 5 = severe galling.

Expression of *MIC-3* fusion protein in *E. coli*. The *MIC-3* cDNA was cloned into an expression vector pET-32b(+) (Novagen; Madison, WI) and expressed as a recombinant *MIC-3* protein in *E. coli* cells. Two PCR primers, MIC-EXP-01-A (5'-CGGAATTCAAAAATGGCTTGTCCTC-CAACTCATAAAGT-3') and MIC-EXP-02-B (5'-GGCAAGCTTATTGCAACCGCTCCACAT-GATTTGTGCT-3'), were used to amplify the cDNA sequence of *MIC-3* from a plasmid template, pGEM-TEasy-*MIC-3* (Zhang et al., 2002), with *Pfu* DNA polymerase (Stratagene; LaJolla, CA). An *Eco*RI (GAATTC) or *Hin*dIII (AAGCTT) restriction site was engineered at the 5' end of the primers. The amplified *MIC-3* cDNA was digested with *Eco*RI and *Hin*dIII and cloned into the pET-32b(+) expression vector. The recombinant plasmid, pET-32b(+)-*MIC-3*, was initially transformed into *E. coli* XL1-Blue cells, and the transformants were selected on LB plates containing 50 µg/mL ampicillin. In this construct, the *MIC-3* protein was fused with a Trx-tag (thioredoxin protein) at the N-terminus and with His-tags at both N- and C-termini. In order to increase antigenicity toward the *MIC* protein sequence, the Trx-tag (109 aa) was removed by digestion of pET-32b(+)-*MIC-3* DNA with *Nde*I. The re-ligated recombinant pET-32b(+)-*MIC-3*/NdeI DNA without the Trx-tag was then transformed into *E. coli* XL1-Blue cells, and the transformants were selected on LB plates containing 50 µg/mL ampicillin. After PCR and DNA sequencing to confirm that the *MIC-3* protein was correctly fused to both N- and C-terminal His-tags, the pET-32b(+)-*MIC-3*/NdeI recombinant DNA was transformed into *E. coli* AD494 host cells and selected on LB plates containing 50 µg/mL ampicillin. *E. coli* AD494 host cells carrying pET-32b(+)-*MIC-3*/NdeI were grown in LB medium containing 50 µg/mL ampicillin and 30 µg/mL kanamycin at 37 ºC to an optical density of 0.6 at a wavelength of 600 nm. Expression of recombinant MIC fusion protein was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 1 mM and continued incubation at 37 ºC for 3 h. Cells were chilled on ice and collected by centrifugation at 3000 x g for 10 min. Preliminary experiments showed that the protein was largely expressed in the soluble fraction, so inclusion bodies were not used. The soluble protein fraction was extracted from the induced cells using B-PER Bacterial Protein Extraction Reagent (Pierce Biotechnology Inc.; Rockford, IL) with addition of 100 µg/mL of phenylmethylsulfonylfluoride, and the

recombinant *MIC* fusion protein was purified by affinity (His-tag) chromatography on nickel-chelated columns (Pierce Biotechnology Inc.) according to manufacturer's protocols. Aliquots of cell extracts and protein fractions obtained during column purification were analyzed by SDS-PAGE and immunoblotting with mouse monoclonal anti-HIS antibody (1/5000, Invitrogen Life Technology; Carlsbad, CA) to confirm the expected size (~25 kDa) of the *MIC*-3 fusion protein. Secondary antibody in this case was goat anti-mouse IgG alkaline phosphatase conjugate (1/7500, Promega Corp.; Madison, WI).

Production of polyclonal antibodies. The purified, recombinant *MIC-3* fusion protein fraction was resolved on preparative SDS-PAGE gels and reversibly stained with GelCode E-Zinc Stain Kit (Pierce Biotechnology Inc.). Six gel slices (~38 mm x 4 mm x 1.5 mm) containing 150 µg purified protein each were used to immunize rabbits at Antibody Solutions (Palo Alto, CA).

Cotton root protein extraction. Roots from the noninoculated control and from RKN-inoculated cotton seedlings at 10 d post-emergence were washed free of soil, gently blotted dry on paper towels, quick frozen in liquid nitrogen, and stored at -80 ºC in sealed freezer bags. Total root mass from each plant minus the tap root was ground in a mortar in 400 µL of 3X SDS sample buffer (Laemmli, 1970) immediately upon removal from the freezer. Homogenate was transferred to 2.0 mL centrifuge tubes and briefly spun in a microcentrifuge to pellet cellular debris. Supernatants were heated at 95 ºC for 5 min to denature the protein, cooled on ice, and then centrifuged at 12,000 X g for 10 min at 4 ºC to pellet any remaining solids. Final supernatants representing total protein extracts from whole root tissues were transferred to new tubes, protein concentrations were estimated (Marder et al., 1986), and samples were stored at -80˚C prior to SDS-PAGE/immunoblotting.

Immunoblot analysis. Root total protein extracts (~40 µg protein/lane) were resolved by SDS-PAGE on gels consisting of 4.5%(w/v) stacking and 15% (w/v) resolving gel acrylamide concentrations using the buffer system of Laemmli (1970) in a Hoefer SE600 electrophoresis apparatus (Hoefer Inc.; San Francisco, CA). Gels were run in duplicate for total protein staining with SYPRO Ruby gel stain (Molecular Probes; Eugene, OR) and electrotransfer to nitrocellulose. Prestained molecular weight markers (Bio-Rad; Hercules, CA) were electrophoresed for subsequent alignment of stained gels and immunoblots. Proteins were transferred electrophoretically onto nitrocellulose (0.1 µm, Schleicher and Schuell; Keene, NH) using a Hoefer TE 42 Transphore Unit (Hoefer, Inc.). Transfer buffer consisted of 25 mM Tris, 0.192 M glycine, 0.02% SDS, and 20% methanol. Transfer voltage (40 V) was maintained for ~12 h at 20 ºC. Following transfer, the membranes were blocked for 2 h in TBS (25 mM Tris, 0.15 M NaCl, pH 7.2) containing 1% milk powder (Carnation; Los Angeles, CA) and 0.5% bovine serum albumin (BSA), then washed twice in TBST (TBS plus 0.05% Tween 20) for 5 min each. Blots were then incubated for 2h with *MIC* antisera at 1/4000 dilution in TBS containing 0.1% milk powder and 0.05% BSA at room temperature. Following four washes in TBST as described above, the blots were incubated for 1 h with goat-anti-rabbit IgG-alkaline phosphatase conjugate (1/7500, Promega; Madison, WI) in TBS containing 0.1% milk powder and 0.05% BSA. Blots were washed 5 times for 5 min each in TBST before development with Western Blue Substrate (Promega; Madison, WI). Developed blots were rinsed in 0.1% SDS followed by deionized water and air dried before quantitation of antigen levels with a Molecular Dynamics Densitometer SI (Sunnyvale, CA). The densitometer provided an image of the blot with pixel values linearly proportional to the optical density. ImageQuant 5.0 software (Molecular Dynamics; Sunnyvale, CA) was used to calculate peak areas corresponding to the MIC protein band on the immunoblots.

RESULTS

The identification of a 14 kDa protein and its correlation with RKN resistance (Callahan et al, 1997) and subsequent cloning of a full length cDNA (*MIC-3*, Zhang, et al., 2002) have been previously reported. These earlier studies were limited to the RKN-resistant breeding line M-249 and its recurrent susceptible parent, Stoneville 213. To assess accumulation levels of the *MIC-3* protein in a larger number of cotton breeding lines and cultivars and to evaluate its association with RKN resistance response, a polyclonal antibody to specifically label the protein was developed. The *MIC-3* cDNA was cloned into expression vector pET-32b(+) and expressed in *E. coli* as a fusion protein to produce the protein antigen for production of antibodies in rabbits. The resulting serum (*MIC* antisera) was initially tested on immunoblots of protein extracts from the transformed

bacterial cells harboring the plasmid for the 25 kDa *MIC* fusion protein. Immune serum, but not preimmune serum, recognized the 25 kDa fusion protein from IPTG induced cells, and optimal dilutions of the antiserum for use on western blots were determined (data not shown).

As a preliminary step to this study, *MIC* antiserum was tested to determine its ability to recognize the 14 kDa protein from cotton roots in susceptible and resistant lines. The most RKN resistant (R) breeding line, M315, was compared with a susceptible (S) line, M8, with and without inoculation with RKN. Because earlier studies indicated that the 14 kDa protein was largely expressed in the developing, immature galls of infected resistant plants (Callahan et al., 1997), protein extracts were prepared separately from regions of the root displaying small immature galls versus symptom-less regions within a single root system of inoculated S and R plants. Root proteins were resolved by SDS-PAGE and either stained for total protein or transferred to nitrocellulose and reacted with the *MIC* antiserum (total protein vs. anti-*MIC*, Fig.1). The *MIC* antiserum recognized a 14 kDa protein, and in agreement with previous work, revealed higher accumulation of the 14 kDa protein in the galled areas of the inoculated R line (Fig. 1, lane 4) as compared with inoculated S line (Fig. 1, Lane 2). Labeling was not detected in non-inoculated controls (Fig. 1, Lane 1 and 3). There was also weaker labeling of the 14 kDa protein in extracts from symptom-less regions of the root of the inoculated R line (Fig. 1, Lane 6) that was absent in equivalent tissue from the inoculated S line (Fig.1, Lane 5). This result may represent an effect of infective juveniles within the sampled root sections but where feeding sites had not yet developed into visible young galls. In addition, the root specificity of 14 kDa accumulation was verified using the *MIC* antiserum on immunoblots of protein extracts from various cotton plant tissues (data not shown) in agreement with previous Northern blot analysis (Zhang et al., 2002).

The high specificity of the *MIC* antiserum for reaction with the 14 kDa protein made it possible to use the antibody to probe the accumulation of the protein in response to RKN infection in a larger subset of cultivars and breeding lines. Figure 2 summarizes *MIC* (14 kDa protein) levels obtained from densitometric scans of immunoblots of total protein extracts from whole roots of RKN-inoculated cotton lines displaying a range of resistance levels. MIC

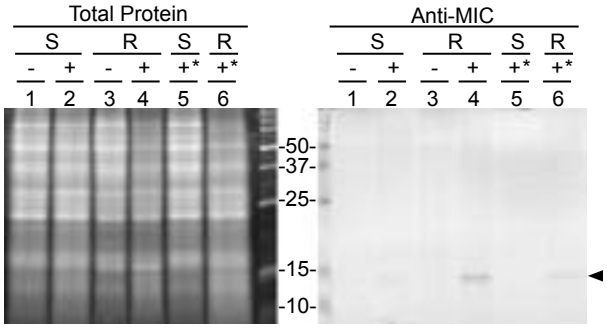

Figure 1. Immunological detection of the 14 kDa cotton root protein with the rabbit antiserum raised against the *MIC-3* fusion protein. Root protein extracts from control and RKN inoculated seedlings (10 d after emergence) were resolved by SDS-PAGE and either stained with SYPRO Ruby (Total Protein) or electrotransferred to nitrocellulose for immunodetection with the rabbit antiserum (Anti-*MIC*). Lanes 1 and 2 are the RKN susceptible (S) line M8 without(-) and with(+) inoculation, respectively; Lanes 3 and 4 are the RKN resistant (R) line M315 without (-) and with (+) inoculation, respectively. The root protein extracts of lanes 2 and 4 (inoculated S and R plants, respectively) were from pooled dissected sections of the individual root system showing visible signs of RKN infection in the form of young immature galls (Fig. 1, Callahan et al., 1997). Lanes 5 and 6, inoculated S (M8) and R (M315), respectively, were root protein extracts from pooled regions of the individual root system lacking visible young galls (+*). Molecular weight markers are indicated for alignment of the stained gel and immunoblot.

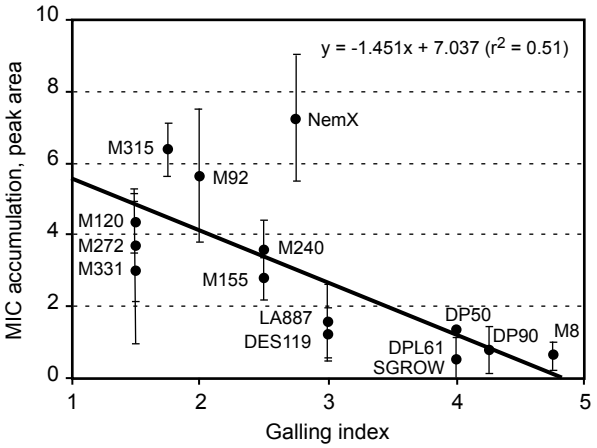

Figure 2. Correlation of 14 kDa (*MIC*) protein levels with root gall indices from a group of cotton lines representing a range of resistance levels. Whole root protein extracts from inoculated seedlings at ~10 d post-emergence were resolved by SDS-PAGE and electrotransferred to nitrocellulose for immunodetection with anti-*MIC* antiserum. Blots were scanned with a Molecular Dynamics densitometer to quantify the immunoreactive 14 kDa (*MIC*) protein. Values for peak area of the MIC band represent the average from three independent plants within the experiment. Regression equations were calculated for MIC accumulation levels (peak area) versus galling index (1 to 5), the latter estimated from inoculated plants at 44 d post-emergence. Accumulation of MIC protein was undetected in non-inoculated controls.

protein was not detected in non-inoculated controls. Regression equations were calculated for MIC accumulation levels compared with the galling index of the various cotton lines tested. Predicted values based on the regression equation are plotted in Figure 2. Although the R^2 value was rather low (0.51), the trend shows that susceptibility to RKN increases as MIC protein levels decrease.

DISCUSSION

Analysis of MIC protein accumulation in this expanded group of breeding lines and susceptible cultivars confirmed our previous findings that *MIC* protein levels are generally higher in the resistant lines following infection by RKN. Dissection and immunoblot analysis of galled versus symptomless regions of the roots of resistant M315 substantiated previous work (Callahan et al., 1997) that indicated the accumulation of the protein is in the vicinity of the developing feeding site of the nematode. Given that further development of feeding sites and reproduction of RKN is halted soon after this initial interaction in the resistant lines (Creech et al., 1995; Jenkins et al., 1995), it is tempting to speculate that the *MIC* gene is involved in the resistance response to RKN. Given the lack of homology of the *MIC-3* gene to other genes of known or unknown function in the major gene databases and the lack of any clues from its structure (i.e. no DNA or protein binding domains are apparent), we can still only speculate as to its function or specific role in the resistance mechanism. We do note that the size of the *MIC* protein (14 kDa) is compatible with passing through RKN feeding tubes within feeding cells, which are thought to exclude compounds larger than 40 kDa (Urwin et. al., 1998; Davis et. al., 2000). Full characterization and sequencing of putative homologues of the *MIC* gene family in susceptible and resistant cotton backgrounds and creation of molecular markers for the genes will help to clarify the relationship between increased accumulation of the *MIC-3* protein(s) in response to RKN infection.

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DISCLAIMER

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REFERENCES

Abad, P., B. Favery, M.-N. Rosso, and P. Castagnone-Sereno. 2003. Root-knot nematode parasitism and host response: molecular basis of a sophisticated interaction. Mol. Plant Pathol. 4:217-224.

Callahan, F.E., J.N. Jenkins, R.G. Creech, and G.W. Lawrence. 1997. Changes in cotton root proteins correlated with resistance to root knot nematode development [Online]. J. Cotton Sci. 1:38-47. Available at http://www.cotton.org/journal/1997-01/1/38.cfm

Creech, R.G., J.N. Jenkins, B. Tang, G.W. Lawrence, and J.C. McCarty. 1995. Cotton resistance to root-knot nematode: I. Penetration and reproduction. Crop Sci. 35:365-368.

Davis, E.L., R.S. Hussey, T.J. Baum, J. Bakker, A. Schots, M.-N. Rosso, and P. Abad. 2000. Nematode parasitism genes. Annu. Rev. Phytopathol. 38:365-396.

Gheysen, G., and C. Fenoll. 2002. Gene expression in nematode feeding sites. Annu. Rev. Phytopathol. 40:191-219.

Jenkins, J.N., R.G. Creech, B. Tang, G.W. Lawrence, and J.C. McCarty. 1995. Cotton resistance to root-knot nematode: II. Post-penetration development. Crop Sci. 35:369-373.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Marder, J.B., A.K. Mattoo, and M. Edelman. 1986. Identification and characterization of the *psbA* gene product: the 32 kDa chloroplast membrane protein. Methods Enzymol. 118:384-396.

Shepherd, R. L. 1983. A quantitative technique for evaluating cotton for root-knot nematode resistance. *In* Host Plant Resistance Research Methods for Insects, Diseases, Nematodes and Spider Mites in Cotton, Southern Cooperative Series, Bulletin 280. Mississippi Agricultural and Forestry Experiment Station, Mississippi State University, Mississippi State, MS.

Urwin, P.E., M.J. McPherson, and H.J. Atkinson. 1998. Enhanced transgenic plant resistance to nematodes by dual proteinase inhibitor constructs. Planta 204:472-479.

Zhang, X.-D., F.E. Callahan, J.N. Jenkins, D.-P. Ma, M. Karaca, S. Saha, and R.G. Creech. 2002. A novel root-specific gene, *MIC-3*, with increased expression in nematode-resistant cotton (*Gossypium hirsutum* L.) after root-knot nematode infection. Biochem. Biophys. Acta 1576:214-218.