

Expression of Phenylalanine Ammonia Lyase Genes in Maize Lines Differing in Susceptibility to *Meloidogyne incognita*

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Abstract: Maize is a well-known host for *Meloidogyne incognita*, and there is substantial variation in host status among maize genotypes. In previous work it was observed that nematode reproduction increased in the moderately susceptible maize inbred line B73 when the *ZmLOX3* gene from oxylipid metabolism was knocked out. Additionally, in this mutant line, use of a nonspecific primer for phenyl alanine ammonia lyase (PAL) genes indicated that expression of these genes was reduced in the mutant maize plants whereas expression of several other defense related genes was increased. In this study, we used more specific gene primers to examine the expression of six PAL genes in three maize genotypes that were good, moderate, and poor hosts for *M. incognita*, respectively. Of the six PAL genes interrogated, two (*ZmPAL3* and *ZmPAL6*) were not expressed in either *M. incognita*-infected or noninfected roots. Three genes (*ZmPAL1*, *ZmPAL2*, and *ZmPAL5*) were strongly expressed in all three maize lines, in both nematode-infected and noninfected roots, between 2 and 16 d after inoculation (DAI). In contrast, *ZmPAL4* was most strongly expressed in the most-resistant maize line W438, was not detected in the most-susceptible maize line CML, and was detected only at 8 DAI in the maize line B73 that supported intermediate levels of reproduction by *M. incognita*. These observations are consistent with at least one PAL gene playing a role in modulating host status of maize toward *M. incognita* and suggest a need for additional research to further elucidate this association.

Key words: genetics, host susceptibility, maize, *Meloidogyne incognita*, phenylalanine ammonia lyase, root-knot nematode, *Zea mays*.

Maize (*Zea mays* L.) has long been recognized as a host for the root-knot nematode *Meloidogyne incognita* (Windham, 1998). Further, it is known that maize genotypes (inbred lines and hybrids) vary greatly in their susceptibility to *M. incognita* (Baldwin and Barker, 1970; Windham and Williams, 1988). The variation in reproduction of the nematode on different maize genotypes appears to be a continuum from high to low levels of reproduction with no known reports of distinct resistant genotypes that are based on major genes for resistance. To date, few studies have examined the basis for the observed variation in host status of maize toward *M. incognita*.

Lipoxygenases are a class of enzymes that catalyze the oxidation of fatty acids. The direct products of these reactions or their derivatives are signaling molecules that play important roles in plant responses to abiotic and biotic stresses (Howe and Schimmler, 2002; Porta and Rocha-Sosa, 2002). One of the key lipoxygenases in maize, especially with regard to maize interactions with several plant-pathogenic fungi, is *ZmLOX3*. When the maize *ZmLOX3* gene was knocked out, the mutant was more resistant to several foliar pathogens and to fumonisin accumulation when infected by *Fusarium verticillioides* (Gao et al., 2007). Interestingly, knockout disruption of *ZmLOX3* in the inbred line B73 resulted in increased susceptibility and attractiveness to *M. incognita* (Gao et al., 2008). With this increased susceptibility to *M. incognita*, the mutants unexpectedly displayed increased and constitutive expression of several defense-related genes including the proteinase inhibitor MPI, the pathogenesis related protein PR1, and several jasmonic

acid biosynthesis genes. These same genes were induced by nematode infection in the near-isogenic wild-type roots. These genes were not expressed in untreated *ZmLOX3* mutant maize roots but were induced by nematode infection in the near-isogenic wild-type roots. An exception to this trend of increased expression of defense-related genes was phenylalanine ammonia lyase (PAL), the expression of which was suppressed in the mutants prompting the hypothesis that the lack of PAL gene response in the mutant plays a key role in the increased susceptibility of the *ZmLOX3* mutant to the root-knot nematode (Gao et al., 2008).

PAL is a key enzyme in phenylpropanoid metabolism in plants and is involved in plant response to biotic and abiotic stresses. The work of Gao et al. (2008) did not determine the number of PAL genes in maize or which of these genes were active in response to infection by *M. incognita* and might therefore play a role in modulating the host status of different maize inbred lines to the nematode. The objectives of this study were to (i) characterize the host status of a group of maize inbred lines to *M. incognita*, and (ii) characterize the activity of several individual PAL genes in maize inbred lines that differed in susceptibility to the nematode.

MATERIALS AND METHODS

M. incognita (isolate 98-1) was maintained on susceptible tomato, *Solanum lycopersicum* ‘Rutgers’. Eggs were collected from infected plants using 0.6% NaOCl (Hussey and Barker, 1973). To test the host status of maize inbred lines, a collection of 14 lines was obtained from the Texas AgriLife Research germplasm collection and from the maize collection at the USDA North Central Regional Plant Introduction Station at Ames, IA. Except for B73, which was known to be a host (Gao et al., 2008), the host status of the inbred lines relative to *M. incognita* was unknown. In each experiment, three

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seeds of each line were planted separately in 15-cm-diam. pots containing a steam pasteurized soil mix of coarse sand and peat (6:1, v/v). Plants were thinned to 1/pot 1 wk after emergence. Immediately thereafter the soil was infested with a suspension of 10,000 eggs of *M. incognita*. Inoculated plants were maintained in a greenhouse where temperatures ranged from 25°C to 35°C and watered as needed. The experiments were terminated approximately 8 wk after inoculation. Nematode reproduction was measured by extraction of eggs from a 5-g sample of the roots of each plant using NaOCl. Egg count data were subjected to analysis of variance using SPSS, Inc. (Armonk, NY) with mean separations by Tukey's honest significance difference (HSD) test. This experiment was a completely randomized design and was conducted three times with eight single-plant replications of each inbred line.

Three inbred lines that consistently supported respectively high, moderate, and low levels of reproduction by *M. incognita* were selected for analysis of nematode development and PAL activity. Seeds of the selected lines were planted as above but in these experiments each seedling was inoculated with 5,000 freshly hatched second-stage juveniles (J2) collected as described by Vrain (1977). Plants were then harvested at 0, 2, 4, 8, and 16 d after inoculation (DAI). There were six replications of each treatment-inbred line combination for each sample time. At each harvest time the roots were gently washed with tap water, blotted dry with paper towels, frozen in liquid N₂, and stored at -80°C (Gao et al., 2008). Samples for extraction of RNA consisted of roots from three inoculated plants or three non-inoculated plants. Total root RNA was isolated with TRI Reagent[®] (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. After treatment with RNase-free DNase (Thermo Fisher Scientific Inc., Waltham, MA) at 37°C for 30 min, 5 µg of RNA was reverse transcribed into cDNA using the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol. For the determination of transcript quantities, the first-strand cDNA was amplified in a semi-quantitative rtPCR reaction using specific primers for six PAL genes (Table 1) and glyceraldehyde-3-phosphate-dehydrogenase (*GAPc*), which was used as the internal control. The first strand of each sample was diluted to

obtain equal amounts of template for quantification of each gene. To define the optimal number of polymerase chain reaction (PCR) cycles for linear amplification of each gene a range of PCR amplifications was performed. Template cDNA was dissociated at 94°C for 10 min, followed by PCR cycling in a GeneAmp PTC-100 thermocycler. PCR conditions for the *GAPc* gene were 23 cycles at 30 sec at 94°C, 45 sec at 58°C, and 1 min at 72°C. Amplified DNA fragments were separated on 1.5% agarose gels and recorded under ultraviolet light after staining with ethidium bromide. This experiment was conducted twice.

To measure the rate of nematode development in the three inbred lines, three additional root samples of each inoculated inbred line were collected at the same time as the root samples for RNA extraction and were processed as described by Thies et al. (2002) to stain the developing nematodes in the roots. The stained root samples were examined with a compound light microscope and observed stages of nematode development were recorded.

RESULTS AND DISCUSSION

The inbred maize lines tested differed in susceptibility to *M. incognita* ($P \leq 0.05$) with CML176 consistently supporting the highest level of nematode reproduction, W438 supporting the lowest level of reproduction, and B73 being intermediate (Fig. 1). Nematode reproduction on CML176 was fivefold greater than reproduction on W438. Rates of development of *M. incognita* on these three inbred lines were consistent with the observed differences in total reproduction supported by the inbred lines. Mature females were observed in the roots of CML176 at 16 DAI whereas the nematodes in W438 were only slightly swollen at that time (Fig. 2). Nematode development on B73 appeared to be intermediate between CML176 and W438; the juveniles were greatly swollen but had not yet molted to the adult stage. Because of the consistent differences in susceptibility to *M. incognita*, these three inbred lines were selected for comparison of expression of PAL genes in response to nematode infection.

The initial evaluation of the maize genome data base revealed the presence of six PAL genes (Maize B73 RefGen_v2; <http://gbrowse.maizegdb.org/gb2/gbrowse/>

TABLE 1. Genes encoding phenylalanine ammonia lyases in *Zea mays* and primer sequences for each gene.

Gene name	GenBank accession	Forward primer (5'-3')	Reverse primer (5'-3')
<i>ZmPAL1</i>	L77912	CGAGGTCAACTCCGTGAACG	GCTCTGCACGTGGTTGGTGA
<i>ZmPAL2</i>	BT069509	CCGGTGCCAAGAGGCAAGAC	CACGATCACTGCTACAAGCTCC
<i>ZmPAL3</i>	BT041432	GTGAAGAAGTCCGTGACGCAG	CGACGGTAGATATAGAGAGCATCTTC
<i>ZmPAL4</i>	EU955384	GAGGTGGACAAGGTGTTTCGT	TTGCCACAACCCACAACATAA
<i>ZmPAL5</i>	EU957015	TAAGCCGGAGTACACCAACC	ATCTCGATGGCTFCAGCAGT
<i>ZmPAL6</i>	EU970905	CATCGACATCCTGAAGCTGA	TGAAACGCTCTGTTTTGTGC

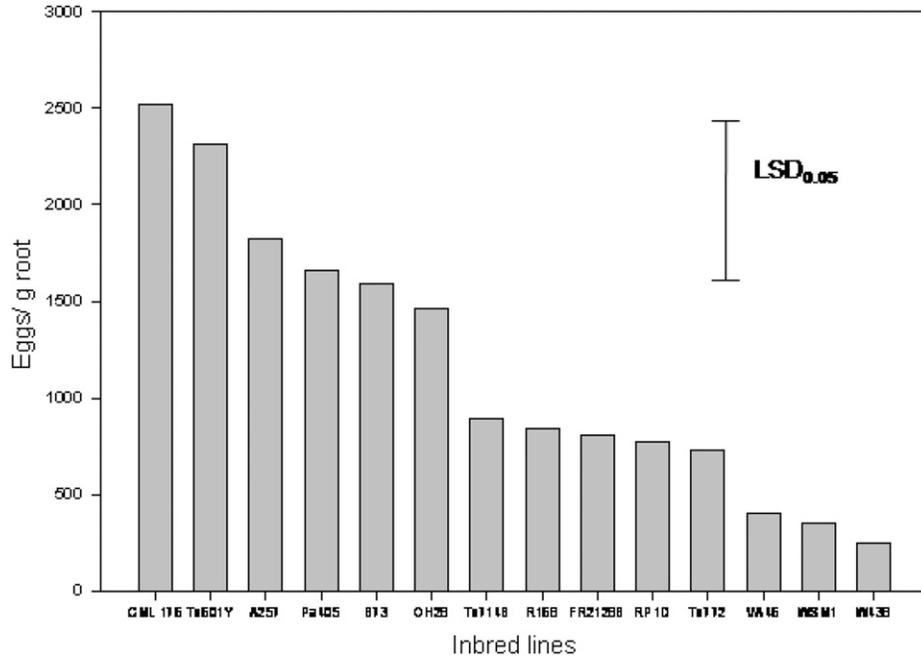


FIG. 1. Variation in reproduction of *Meloidogyne incognita* on maize inbred lines measured as number of eggs per g root tissue at 8 wk after inoculation.

maize_v2/). Gene-specific primers were designed based on the sequences and used for semi-quantitative PCR analysis (Table 1). The genes *ZmPAL1*, *ZmPAL2*, *ZmPAL4*, and *ZmPAL5* were expressed in the roots of infected and noninfected maize at each sample time (0 to 16 DAI). *ZmPAL3* was not detected in the roots and *ZmPAL6* was found expressed in the leaves but not in the roots (data not shown).

ZmPAL1, *ZmPAL2*, and *ZmPAL5* were strongly expressed in all root samples, inoculated and non-inoculated, at all sample times (Fig. 3). Interestingly, *ZmPAL4* transcripts were not observed in the highly susceptible CML176. In the intermediately resistant B73 line, relatively low expression of *ZmPAL4* was observed at most time points, except at 8 DAI, when *ZmPAL4* was expressed at a greater level in inoculated than in noninoculated roots. In contrast with CML176 and B73, in the nematode-resistant W438 line, *ZmPAL4*

was strongly expressed in all root samples at all sample times (Fig. 3). Similar results were obtained from each independent experiment. The *ZmPAL4* gene was identified from genomic DNA of all three maize inbred lines by PCR using gene-specific primers (data not shown).

These data confirm previous reports concerning the variability among maize inbred lines with respect to host status toward *M. incognita* (Baldwin and Barker, 1970; Windham and Williams, 1988). Additionally, our observation that expression of the PAL gene *ZmPAL4* is increased in response to nematode infection in B73 confirms the observation of Gao et al. (2008). The observation that the most resistant maize inbred W438 had the highest level of expression of *ZmPAL4* whereas the most susceptible inbred had no detectable expression is consistent with the hypothesis that this PAL gene may play a role in modulating susceptibility in maize to *M. incognita*. Unfortunately, no PAL mutants have been



FIG. 2. Development of *Meloidogyne incognita* in maize inbred lines B73 (left), CML 176 (center), and W438 (right) at 16 DAI. Note the greater development as indicated by greater body diameters in B73 and CML176 than in the more resistant W438.

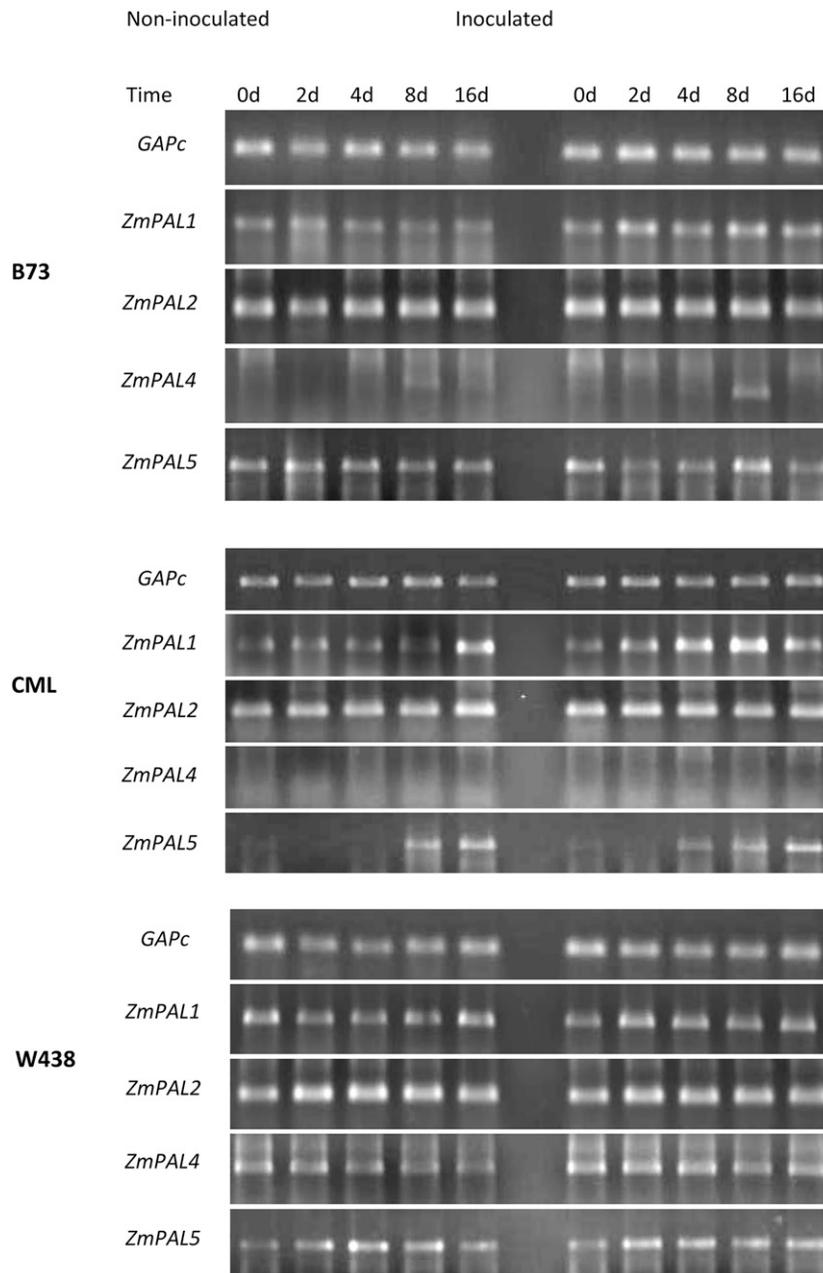


FIG. 3. Expression of PAL genes in three maize inbred lines. Roots of three inbred lines, B73, CML176, and W438, were infected with root-knot nematode *Meloidogyne incognita* and root total RNA was extracted at the time points indicated. Expression of selected PAL genes was measured by semi-quantitative RT-PCR. Expression of glyceraldehyde-3-phosphate-dehydrogenase (*GAPc*) was used as a quantitative standard. Two independent experiments were conducted and similar results were obtained.

identified among the available collection of 48,000 Mu-insertion mutations (May et al., 2003), thus we have not been able to further test this hypothesis. The latest release of the maize B73 genome sequence (Maize B73 RefGen_v2; http://gbrowse.maizgdb.org/gb2/gbrowse/maize_v2/) contains three additional PAL genes, *ZmPAL7*, *ZmPAL8*, and *ZmPAL9* not found initially. The expression patterns of these genes in maize roots needs to be examined to further explore the role of PAL in modulating maize host status to *M. incognita*.

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