Meloidogyne javanica Chorismate Mutase 1 Alters Plant Cell Development

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Root-knot nematodes are obligate plant parasites that alter plant cell growth and development by inducing the formation of giant cells for feeding. Nematodes inject secretions from their esophageal glands through their stylet and into plant cells to induce giant cell formation. *Meloidogyne javanica* chorismate mutase 1 (MjCM-1) is one such esophageal gland protein likely to be secreted from the nematode as giant cells form. MjCM-1 has two domains, an N-terminal chorismate mutase (CM) domain and a C-terminal region of unknown function. It is the N-terminal CM domain of the protein that is the predominant form produced in root-knot nematodes. Transgenic expression of MjCM-1 in soybean hairy roots results in a phenotype of reduced and aborted lateral roots. Histological studies demonstrate the absence of vascular tissue in hairy roots expressing MjCM-1. The phenotype of MjCM-1 expressed at low levels can be rescued by the addition of indole-3-acetic acid (IAA), indicating MjCM-1 overexpression reduces IAA biosynthesis. We propose MjCM-1 lowers IAA by causing a competition for chorismate, resulting in an alteration of chorismate-derived metabolites and, ultimately, in plant cell development. Therefore, we hypothesize that MjCM-1 is involved in allowing nematodes to establish a parasitic relationship with the host plant.

Additional keywords: auxin, hormones, pathogen, phenolic, salicylic acid, shikimate pathway.

Root-knot nematodes (*Meloidogyne* spp.) are obligate plant parasites that infect most cultivated plant species (Sasser 1980). The second-stage juvenile (J2), the infectious stage of the root-knot nematode, migrates through the soil and penetrates the host plant root in the zone of elongation. J2s move between plant cells until the developing vascular tissue is reached (Paulson and Webster 1970). The J2s then inject secretions through their stylets into the symplast or apoplast of approximately six developing xylem parenchyma cells (Hussey and Grundler 1998). The affected plant cells undergo a dramatic transformation, becoming giant multinucleate cells on which the nematode nondestructively feeds throughout the rest of its life cycle (Huang 1985; Williamson and Hussey 1996).

Secretions originating from the phytoparasitic nematode esophageal glands are thought to play key roles in host penetration and giant cell formation (Hussey 1989; Davis et al. 2000). Root-knot nematodes have three esophageal gland cells, one dorsal and two subventral gland cells. Each gland is a single enlarged cell that is specialized to export secretions into the nematode's esophageal lumen (Hussey and

Grundler 1998). Once in the lumen, secretions from the glands are actively exported out of the nematode via the stylet. The subventral glands have long been thought to produce pectinases and cellulases that assist the nematode in penetrating the host root (Bird et al. 1974; Dropkin 1963). Recently, this hypothesis has been confirmed by the identification of several genes encoding cellulases and pectate lyases that are expressed in the subventral esophageal glands of cyst and root-knot nematodes (De Boer et al. 2002; Doyle and Lambert 2002; Gao et al. 2002; Goellner et al. 2000; Popeijus et al. 2000a; Rosso et al. 1999; Smant et al. 1998). The dorsal esophageal gland is thought to be the source of nematode factors responsible for the maintenance of giant cells. Both types of glands may play a role in the initiation of giant cells (Hussey 1989; Davis et al. 2000). While the number of esophageal gland genes that have been cloned is increasing (Davis et al. 2000; Ding et al. 1998; Gao et al. 2001; Popeijus et al. 2000a, 2000b; Wang et al. 2001), much remains to be learned about the function of many of the nematode proteins involved in parasitism. While many of the genes identified to date are involved in penetration of the host root by the nematode, little is known about the proteins that act inside the plant cell to aid the nematode in the initiation of giant cell formation (Davis et al. 2000). One such protein believed to be involved in giant cell initiation is the nematode chorismate mutase (CM) (EC 5.4.99.5). The root-knot nematode CM was cloned from a cDNA library constructed from the esophageal gland region of *Meloidogyne javanica* (MjCM-1; Lambert et al. 1999a). MjCM-1 is about 20 kDa in size, with an N-terminal domain displaying significant homology to CMs from gram-negative bacteria (Lambert et al. 1999a). The C-terminal domain of MjCM-1 displays no homologies to CMs or to other proteins. Additional nematode CMs have also recently been cloned. These include CMs from the potato cyst nematode (Popeijus et al. 2000b) and the soybean cyst nematode (Bekal et al. 2002).

CMs are part of the shikimate pathway, a primary metabolic route in plants and microorganisms. The end-product of the common shikimate pathway is chorismate, a branch-point metabolite that is the precursor for the three aromatic amino acids phenylalanine, tyrosine, and tryptophan. In plants, chorismate-derived compounds (CDCs) are converted into a vast array of secondary metabolites that either are derived directly from chorismate or are formed from an aromatic amino acid (Strack 1997). These CDCs play critical roles in plant growth, in development and defense, and in interactions with other organisms (Schmid and Amrhein 1995; Weaver and Herrman 1997). For example, indole-3-acetic acid (IAA), an auxin, is derived either directly from chorismate or indirectly from chorismate via the amino acid tryptophan (Bartel 1997). Other important CDCs include salicylic acid (SA), a key sec-

ond messenger in plant defense (Dong 2001; Ryals et al. 1994). Other defense compounds, including phytoalexins, are also derived from phenylalanine via the phenylpropanoid pathway (Dixon et al. 1983).

CM catalyzes the conversion of chorismate to prephenate, providing the precursors for the synthesis of phenylalanine and tyrosine. This enzymatic reaction is unique, since it is the only example in primary metabolism of a pericyclic Claisen-rearrangement (Romero et al. 1995). Because CM is an unusual and essential enzyme, it has been studied extensively in many organisms; it is found in bacteria, fungi, plants, and protists, but generally not in animals (Roberts et al. 1998; Romero et al. 1995; Stryer 1988). It is commonly believed that animals do not have the shikimate pathway or CMs because they obtain their essential amino acids in their diet (Schmid and Amrhein 1995). While this is true for most animals, CMs might still be useful to animals, like phytoparasitic nematodes, that have a close association with plants.

In this paper, we further characterize the nematode enzyme MjCM-1. By expressing MjCM-1 transgenically in soybean hairy roots, we can begin to assess the function of the protein in nematode parasitism. Expression of MjCM-1 in soybean hairy roots leads to alterations in plant cell development, suggesting that MjCM-1 is manipulating the host cell metabolism for the benefit of the nematode.

RESULTS

Expression and secretion of MjCM-1 in the root-knot nematode.

An antibody was prepared to a peptide derived from the CM domain of MjCM-1. To evaluate the quality of this antibody, 4-day tomato seedlings were infected with root-knot nematode J2s. After inoculation (2 days), the infected and uninfected seedlings were harvested, the proteins were extracted, and protein gel blotting was conducted using the anti-MjCM-1 antibody. Recombinant MjCM-1 produced in *Escherichia coli* was included as a positive control. As illustrated in Figure 1, the peptide antibody detects three proteins in *E. coli*, the full size MjCM-1 and two smaller putative degradation products approximately 15 and 12.5 kDa in size. However, in seedlings infected with J2s, only one protein was detected; this protein is approximately 12.5 kDa in size. This smaller-sized protein is the same size as the predicted CM domain of MjCM-1. Since no additional proteins were detected in infected seedlings, this

is the mature size of MjCM-1. In mock-inoculated tomato seedlings, no proteins were detected, confirming the specificity of the antibody.

The anti-MjCM-1 antibody was also used for immunolocalization studies to determine if the protein is secreted from the nematode. Figure 2B demonstrates that MjCM-1 protein is produced as early as one day or less after infection; however, the signal is localized to two gland basal cells, indicating that the protein is not secreted at this early stage. When the nematodes have been in the tomato roots three days or less, MjCM-1 can be detected in the gland extensions and in the esophageal lumen up to the base of the stylet (Fig. 2D). Detection of MjCM-1 on the anterior end of the nematode also indicates the protein is secreted (Fig. 2D). Two parallel fluorescent signals can be observed in the nematode in Figure 2D. The lower signal is from the dorsal gland with the signal ending at the base of the nematode's stylet. The second signal is from a subventral esophageal gland; the signal initiates at the basal gland cell, intensifies at what appears to be the subventral gland ampulla, and then continues through the esophageal lumen to the base of the stylet. The resolution of the immunolocalizations is too low to determine if MjCM-1 is injected into the plant cell.

Characterization of transgenic roots expressing MjCM-1.

To determine the effect of MjCM-1 within the cytoplasm of developing plant cells, we heterologously expressed the protein in tomato and soybean hairy roots via *Agrobacterium rhizogenes*-mediated transformation. Attempts to drive expression of the *MjCM-1* gene using a 35S promoter were unsuccessful; no transgenic roots were generated in tomato or in soybean (data not shown). Therefore, a glucocorticoid-mediated transcriptional induction system (Aoyama and Chua 1997) was employed so that expression of *MjCM-1* could be regulated by the addition of dexamethasone (DEX). Despite having expression of *MjCM-1* under the control of a DEX-inducible promoter, transgenic roots could only be generated in soybean. Prior to analysis, the transgenic hairy roots were transferred several times on media containing high concentrations of carbenicillin to assure the root lines were free of *Agrobacterium rhizogenes*.

Polymerase chain reaction (PCR) with primers specific to the transformation vector was used to confirm the presence of the *MjCM-1* transgene (data not shown). To confirm the correct gene product was being expressed, reverse transcriptase (RT)-PCR was conducted, and the resulting PCR product was cloned and sequenced. The sequence was identical to the published sequence of *MjCM-1* (Lambert et al. 1999a), indicating the correct product was being expressed.

Two transgenic soybean root lines were chosen for further analysis: lines 1-7 and 2-1. Figure 3 illustrates the expression pattern of the *MjCM-1* mRNA in these two lines. Total RNA was prepared and reverse-transcribed using oligo(dT) as a primer. *MjCM-1*-specific primers were then used to PCR-amplify the *MjCM-1* gene product, while soybean cyclophilin primers were used as a control to demonstrate the viability of cDNA in all samples. When *MjCM-1*-specific primers are utilized, two PCR products are observed (Fig. 3). The larger PCR product corresponds to the *MjCM-1* cDNA. The smaller PCR product is most likely due to nonspecific priming. Figure 3A demonstrates the inducible expression of the *MjCM-1* transgene in the presence of DEX, while very little *MjCM-1* mRNA is detected in the absence of DEX. Control RNA that was not reverse transcribed is also included as a control for the presence of genomic DNA and did not indicate any contaminating DNA. While the *MjCM-1* transcript is DEX-inducible in line 1-7, the *MjCM-1* transcript is detected in line 2-1, both in the absence of and the presence of DEX (Fig. 3A). Again, control RNA that was not reverse transcribed is also

Fig. 1. Protein gel blot of MjCM-1 (*Meloidogyne javanica* chorismate mutase 1) in tomato seedlings infected with *Meloidogyne javanica* second-stage juveniles. The first lane is a protein extract from infected seedlings (I), while the second lane is a protein extract from mock-inoculated seedlings (U). Seedlings were harvested 2 days postinoculation. The third panel is a protein extract from recombinant *E. coli* expressing MjCM-1. Sizes of molecular weight markers are listed to the left.

included and indicates there was no contaminating genomic DNA. Figure 3B shows the PCR of a soybean cyclophilin-like expressed sequence tag (EST) from the same cDNA pools, indicating that the cDNA is of good quality in all samples.

In addition to RNA expression data, attempts were also made to detect MjCM-1 protein by protein gel blotting. However, despite extensive experimentation, no MjCM-1 protein could be detected, suggesting the protein accumulates at a very low level (data not shown).

Phenotypes of transgenic roots.

Transgenic root line 1-7 was grown on media in the presence of and the absence of the inducer, DEX. In the presence of DEX, MjCM-1 is expressed, and it was observed that the hairy roots grew much more slowly. It was also observed that in the presence of DEX, fewer lateral roots formed. Those lateral roots that did initiate often failed to lengthen (Fig. 4A). This phenotype can be observed in closer detail in Figure 4B. Again, fewer lateral roots were formed in the presence of DEX, and those that do form are thin and abnormal. DEX had no effect on hairy roots not containing the *MjCM-1* transgene, indicating the phenotype was not due to the DEX itself (data not shown).

To further investigate this abortive lateral root phenotype, histological studies were performed on induced and uninduced transgenic roots. The developing roots were excised, fixed and embedded, and stained using Johansen's safranin and fast green procedure (Ruzin 1999). Lateral roots were observed in uninduced and induced tissue from line 1-7. In induced roots, many of the developing lateral root buds had no vascular tissue (Fig. 4C and D). The lateral roots in induced tissue were much less structured and regular and the overall cell size was larger.

Uninduced roots were also stained as controls. In uninduced roots, vascular tissue was observed in the developing lateral roots of the same approximate size (Fig. 4G, H, and I). The developing roots were also more organized and the overall cell size appeared smaller.

A second transgenic root line was also studied in more detail. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis suggested line 2-1 constitutively expressed *MjCM-1* at a low level. Likewise, the root line formed fewer lateral roots, even in the absence of inducer (Fig. 5A). Many of the lateral roots that did form failed to elongate, making this line somewhat difficult to propagate. These transgenic roots were also fixed, embedded and stained using Johansen's safranin and fast green procedure. As illustrated in Figure 5B, the lateral roots in line 2-1 also often lacked vascular tissue. Like the induced roots in line 1-7, these developing lateral roots were less organized, and the overall cell size was increased. Again, this phenotype correlated with the expression of *MjCM-1* (Fig. 3).

The suppression of vascular tissue formation and lateral root development observed constitutively with line 2-1 and in the presence of inducer with line 1-7 suggested an auxin effect (Aloni 1995; Lomax et al. 1995). Therefore, attempts were made to restore the wild-type phenotype using exogenously applied IAA. Line 2-1, which constitutively expressed a low level of *MjCM-1* even in the absence of the inducer, was chosen for analysis, because it displayed the most extreme phenotype.

When line 2-1 was grown on media containing 1.0 to 2.5 μ M IAA, normal lateral root formation and a normal rate of root growth were observed (Fig. 5C). In the presence of IAA, increased numbers of lateral roots formed, and the roots were able to elongate. Tissue grown on IAA was also fixed, embed-

Fig. 2. Immunofluorescence of MjCM-1 (*Meloidogyne javanica* chorismate mutase 1) in parasitic second-stage juveniles. **A**, Bright field view. **B**, Fluorescent view of the same nematode section incubated with anti-MjCM-1 antibody. Fluorescent signal appears in two gland basal cells (G), but no signal is seen in the gland extensions. This nematode was in the root for 1 day or less, demonstrating that MjCM-1 is expressed early in nematode infection of the root and that the protein is not immediately mobilized for secretion. **C**, and **D**, show nematodes that have been in the root for 3 days or less, incubated with anti-MjCM-1 antibody. **C** is a bright field view and **D** is the fluorescent view of the same section. **D** shows two parallel fluorescent signals inside the nematode. The lower signal is from the dorsal gland (DG), with the signal ending at the base of the stylet (S). The second signal is from a subventral esophageal gland (SVG), starting at the basal gland cell, intensifying at what appears to be the subventral gland ampulla (marked with an arrow), and then continuing through the esophageal lumen to the base of the stylet. The signal resumes outside of the nematode in the surrounding root tissue, indicating MjCM-1 is secreted.

ded, sectioned, and stained using Johansen's procedure. Figure 5D illustrates that in the presence of exogenous IAA, the lateral roots become much more organized and the overall cell size is reduced, and vascular tissue is also present.

DISCUSSION

Our data demonstrate that MjCM-1 is secreted from the J2 nematode at approximately three days after infection. MjCM-1

is processed by the nematode prior to secretion, resulting in a 12.5-kDa protein equivalent in size to the CM domain. While the processed size is small, this is not inconsistent with bacterial CMs. Several bacterial CMs are also approximately 10 to 13 kDa in size; these include AroQ_F from *Methanococcus jannaschii* (MacBeath et al. 1998) and the CM domain from AroQ_B in *E. coli* (Stewart et al. 1990). MjCM-1 itself has homology to these small bacterial CMs (Lambert et al. 1999a).

MjCM-1 is secreted by the nematode at the precise time that plant cells are being modified for parasitism and is able to alter plant cell development, suggesting that MjCM-1 itself is an important protein in the establishment of parasitism. Assuming that MjCM-1 is secreted in planta, it is our hypothesis that this nematode CM is active in the cytoplasm, where it alters the shikimate pathway to suppress auxin formation and plant cell vascularization in pregiant cells.

We propose that MjCM-1 injected into the plant cell would most likely reside in the cytoplasm, since the MjCM-1 protein does not have a chloroplast transit peptide. Because MjCM-1 is not feedback-inhibited by any of the three aromatic amino acids (Lambert et al. 1999a), it probably lacks a regulatory mechanism in the plant. Therefore, it is likely that MjCM-1 increases the flow of carbon through the cytoplasmic branch of the shikimate pathway. There are plastid and cytoplasmic forms of CM in plants, but the complete shikimate pathway has been experimentally demonstrated to exist only in plastids. This results in a competition between the plastid and cytoplasmic CM isozymes for the plastid-produced chorismate (Eberhard et al. 1996; Sommer and Heide 1998). If the nematode introduces an additional CM into the cytosol, this compartment may out-compete the plastid for chorismate. Different subcellular compartments are thought to funnel their aromatic amino acids towards specific secondary metabolites (Hrazdina and Jensen 1992); therefore, a change in the net production of one compartment could lead to dramatic changes in the production of

Fig. 3. Reverse transcriptase-polymerase chain reaction (RT-PCR) of transgenic soybean roots expressing *MjCM-1* (*Meloidogyne javanica* chorismate mutase 1). Total RNA was isolated from induced and uninduced transgenic roots. The total RNA was reverse-transcribed using oligo(dT), and the resulting cDNA was used as template in PCR reactions. **A**, PCR reactions using *MjCM-1*-specific primers on cDNA from lines 1-7 and 2-1. **B**, PCR using cyclophilin-specific primers on cDNA from lines 1-7 and 2-1. +RT indicates reactions with reverse transcriptase, and -RT indicates reactions without reverse transcriptase.

Fig. 4. Phenotype of soybean hairy roots expressing *MjCM-1* (*Meloidogyne javanica* chorismate mutase 1). Transgenic soybean hairy roots with the *MjCM-1* gene driven by a dexamethasone-inducible promoter were grown with or without dexamethasone (DEX) for 1 to 3 weeks. **A**, through **D**, Hairy roots from line 1-7 grown with DEX. **A** shows roots grown for 22 days on DEX, while the roots in **B** were grown on DEX for 9 days. **C** and **D** show root tips that were grown on DEX, were harvested, fixed and sectioned, and then, were stained using Johansen's procedure for safranin and fast green. Note the absence of vascular tissue in the lateral root buds in **C** and **D**. **E**, through **I**, Transgenic roots from line 1-7 grown on media without DEX. Note the dramatic increase in the number of lateral roots in the absence of DEX, as well as the presence of vascular tissue in the lateral root buds in **G** through **I**. Arrows indicate lateral root buds.

specific phenolic compounds elsewhere in the cell. Consequently, we propose a “chorismate competition model” for how a nematode CM could redirect the production of specific phenolic compounds (Fig. 6) and, thus, alter plant cell development as observed in our transgenic soybean roots expressing *MjCM-1*.

The observation that *MjCM-1* can alter plant cell development is exciting for several reasons. First, *MjCM-1* is causing a phenotype consistent with some changes that occur during giant cell formation. Second, the overall phenotype of suppression of lateral root formation and vascular tissue formation is consistent with a plant that is deficient in IAA or auxin (Aloni 1995; Lomax et al. 1995). If one considers how a root-knot nematode establishes a feeding site, it becomes clear that the nematode must reprogram the developmental fate of the plant cells upon which it feeds. J2s initiate feeding on developing vascular parenchyma cells; in a normal root, these cells are destined to form the cells of the xylem, the phloem, and their associated cells. However, when a root-knot nematode feeds upon these developing vascular parenchyma cells, the plant cells are reprogrammed to form giant feeding cells (Williamson and Hussey 1996). This nematode-induced reprogramming of cellular fate is one of the first events that must occur in feeding cell formation; otherwise the prefeeding cells would lignify, die, or eventually function as vascular cells. The suppression of vascularization and lateral root initiation induced by *MjCM-1* in soybean hairy roots is the first example of an esophageal gland-specific enzyme from a nematode actually altering plant development.

At this point, we know the enzymatic function of *MjCM-1*, and we have a phenotype caused by expression of *MjCM-1* in plants. The next step is to determine which one or more plant metabolites are altered, resulting in our plant phenotype. This is another reason why the suppression of vascular tissue formation and lateral root initiation is exciting; both of these processes are controlled by the plant hormone IAA or auxin (Aloni 1995; Baima et al. 2001; Casimiro et al. 2001; Malamy and Benfey 1997). In our hairy root system, it is known that the roots synthesize extra IAA, because the T-DNA in the Ri plasmid of *Agrobacterium rhizogenes* contains several genes involved in plant hormone synthesis and metabolism. Present are two genes for enzymes that synthesize IAA, three genes for enzymes that alter plant hormone metabolism, and one gene that encodes an enzyme that synthesizes cytokinin (Buchmann et al. 1985; Gaudin et al. 1993; Nilsson and Olsson 1997). The fact that hairy roots actively manufacture endogenous IAA gives *MjCM-1* a potential site of action in the hairy roots. In normal plants, IAA is produced in root tips and, to a lesser extent, in vascular cells (Bartel and Fink 1994; Ljung et al. 2001; Müller et al. 1998), making it possible that nematodes could be altering IAA biosynthesis in normal root systems as well. IAA, a CDC, is reported to be synthesized only in the plastid (Zhao and Last 1995). According to our model, IAA levels should decrease upon increased flow of chorismate to the plant cytosol. With this information in mind, it seems plausible that, in giant cells, *MjCM-1* could be lowering IAA levels to cause changes in plant cell development. Consistent with the model, the observed phenotypes in line 2-1 could, in fact, be rescued by the addition of exogenous IAA. Recent studies on nematode-infected roots expressing an auxin sensitive reporter gene also support our hypothesis. In this experiment, the auxin-responsive promoter GH3 was fused to the *gusA* reporter gene and transformed into white clover. *GusA* activity was then detected in plant cells with significant auxin levels. In this study, the reporter gene indicated auxin accumulation in giant cell precursors but no auxin accumulation in giant cells at 96 h (Hutangura et al. 1999). Given the long half-life of the *gusA* protein, it is likely that

auxin was eliminated in the giant cells sooner than 96 h. This result is consistent with our hypothesis that *MjCM-1* lowers auxin levels in newly formed giant cells. While our chorismate competition model explains our results in transgenic soybean roots, the situation in the whole plant could be more complex. For example, polar auxin transport, which has been demonstrated to be involved in the induction and morphogenesis of cyst nematode feeding sites (Goverse et al. 2000), may be involved in feeding cell development. A balance between auxin transport and synthesis may be critical in nematode feeding-cell formation and development.

While auxin is best known for regulating plant developmental processes, it also has been implicated in plant defense. In 1994, Bartel and Fink published their analysis of *Arabidopsis* nitrilase, an enzyme that catalyzes the last step in the biosynthesis of IAA. In their analysis, they noted that one nitrilase isozyme was pathogen-inducible. Later, Xu and associates (1998) discovered that ethylene-response-element binding proteins (EREBPs), proteins that are involved in the signal transduction of plant defense responses, specifically bind to nitrilase. They speculate that when activated, the nitrilase may produce auxin, which, along with ethylene, may amplify the defense response in the plant. While the role of auxin in plant defense remains unclear, it is still tempting to speculate that nematode suppression of IAA production may somehow interfere with plant defense mechanisms. Along these lines, Mazarei and associates (2002) have shown that an EREBP from *Glycine max* is down-regulated in the resistance response to invading soybean cyst nematodes, suggesting an ethylene-auxin link in soybean cyst nematode resistance.

Fig. 5. Recovery of wild-type root growth phenotype when *MjCM-1* (*Meloidogyne javanica* chorismate mutase 1)-expressing roots are grown with exogenous indole-3-acetic acid (IAA). **A**, Transgenic soybean roots (line 2-1) expressing *MjCM-1* grown on MXB media (MS [Murashige and Skoog 1962] basal nutrient salts, B5 [Gamborg et al. 1968] vitamins, and 3% sucrose [pH 5.7]). **B**, Transgenic soybean roots (line 2-1) expressing *MjCM-1* were fixed, embedded, and stained using Johansen's procedure for safranin and fast green. **C**, Transgenic soybean roots (line 2-1) grown on MXB media supplemented with 1.0 μ M IAA. **D**, Transgenic soybean roots (line 2-1) grown on media supplemented with 1.0 μ M IAA were fixed, embedded, and stained using Johansen's procedure. Arrows indicate lateral root buds.

One key point of our model should be reemphasized; we predict that all plastid-produced CDCs should be lowered by the overexpression of cytoplasmic MjCM-1 (Fig. 6). Potentially lowered CDCs would include flavonoids, important molecules in biotic interactions and plant defense (Gheysen and Fenoll 2002; Hutangura et al. 1999) and endogenous regulators of auxin transport (Hutangura et al. 1999; Mathesius et al. 1998). Another key plastid CDC is SA, which plays roles in plant development and in defense against plant pathogens (Wildermuth et al. 2001; Rate and Greenberg 2001). In order for a nematode to cause the formation of a feeding cell, it must be able to suppress host plant defenses or avoid detection. A plausible method for suppressing host plant defense would therefore be to suppress SA levels. Along the same theme, the reduction of phytoalexins derived from plastid CDCs could also reduce the general toxicity of the plant cell to the feeding nematode. Thus MjCM-1 has the potential to be a multifunctional enzyme for promoting nematode pathogenicity.

Overall MjCM-1 has the potential to play two main roles in nematode parasitism of plants. MjCM-1 can assist in the developmental reprogramming of prefeeding cells, or it could be involved in suppressing plant defense compound formation. Either one of these roles would make MjCM-1 an important focus of study for understanding plant-nematode parasitism. In the future, metabolic analysis will help determine the full spectrum of chorismate-derived compounds altered by MjCM-1, furthering our understanding of this important nematode enzyme.

MATERIALS AND METHODS

Oligonucleotides.

Oligonucleotides were prepared by Genemed Synthesis (NCMSP, NCMXH, NCM3, NCM4; South San Francisco, CA, U.S.A.) or by Integrated DNA Technologies (Cyc1F, Cyc1R; Coralville, IA, U.S.A.). NCMSP: 5'-GTACTAGTTAATTATGTTTACATGCTGGGCAGAG-3', NCMXH: 5'-GACTCGAGATGGATACCGATACTAATGCTGATATT-3', NCM-3: 5'-AGTCCATGGGTGATACCGATACTAATGCTGATATT-3', NCM-4: 5'-CATCCATGGTTAATTATGTTTACATGCTGGGCAGAG-3', Cyc1F: 5'-GCTTCTCTCTCTCTGCAGCAA-3', Cyc1R: 5'-CCTTCGATGACCTGTCCG-3'

Fig. 6. Chorismate competition model for chorismate partitioning. Diagram of a plant cell depicting the subcellular location of the shikimate pathway and phenylalanine and tyrosine biosynthesis. Erythrose 4-phosphate (E4P), phosphoenolpyruvate (PEP), and chorismate mutase (CM). The hollow arrows indicate the flow of metabolites through the shikimate pathway when MjCM-1 (*Meloidogyne javanica* chorismate mutase 1) is overexpressed in the plant cell cytoplasm.

Nematode infections.

Parasitic J2 nematodes were collected from hydroponic nematode cultures (Lambert et al. 1992). Plant debris was removed by allowing the nematodes to crawl through six layers of Kimwipes as previously described (Ho et al. 1992). Tomato seeds (*Lycopersicon esculentum* Mill. UC82) were surface sterilized in 10% bleach and germinated for four days as described in Ho and associates (1992). The germinated tomato seedlings were then transferred to infection boxes and inoculated with 300 to 600 nematodes, as previously described (Lambert et al. 1999b). Control roots were mock inoculated with 0.5% carboxymethylcellulose (Sigma Chemical Co, St. Louis). After two days, infected and mock-inoculated root tips were excised and were frozen in liquid nitrogen.

Protein gel blotting.

The frozen, infected and uninfected root tips were then broken into small pieces using a tissue pulverizer (Fisher Scientific, Pittsburgh). The frozen tissue fragments were then mixed with 0.5 ml of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.125 M Tris-HCl, pH 6.8, 2% 2-mercaptoethanol, 4% SDS, 20% glycerol, 0.001% bromophenol blue) and vortexed to extract the proteins. Infected root extract (5 µl each), mock-inoculated root extract, and MjCM-1-expressing *E. coli* extract were subjected to SDS-PAGE, using standard techniques (Ausubel et al. 1992). The protein gel was then transferred overnight at 10 volts to PVDF membrane (Millipore, Bedford, MA, U.S.A.) in 10% ethanol, 10 mM CAPS, pH 11.0 (United States Biochemical, Cleveland). The membrane was blocked in 0.1% bovine serum albumin (BSA) and then incubated with a commercially prepared mouse anti-MjCM-1 peptide antibody (diluted 1:10,000) that was prepared to the MjCM-1 peptide sequence: N'-CGGRLTSLDYVALYKIVNNQSIDPKREEKL-C' (Covance Research Products, Richmond, CA, U.S.A.). The anti-mouse-horseradish peroxidase conjugated secondary antibody (Pierce Biotechnology Inc., Rockford, IL, U.S.A.) was diluted 1:750,000. The membranes were developed using SuperSignal West Femto Chemiluminescent Substrate (Pierce Biotechnology) according to the manufacturer's instruction.

Immunolocalizations.

To prepare for immunolocalizations, excised nematode-infected tomato root tips were fixed in AA (50% ethanol, 5% glacial acetic acid), were paraffin embedded, and were sectioned as described by Schichnes and associates (1999). The slides were treated with xylene to remove the paraffin, followed by a 1-min incubation in cold acetone. The tissue was then rehydrated as previously described (Ruzin 1999). After treating with 4 µg of Proteinase K per ml for 45 min, the tissue was treated with 100 µl of PNGase F per slide (25 µl denaturing buffer, 25 µl G7 buffer, 25 µl 10% NP-40, 22.5 µl dH2O, 2.5 µl PNGase F) (New England Biolabs, Beverly, MA, U.S.A.) and was incubated for 2 h at 37°C. Next, the tissue was fixed in 4% formalin for 10 min, was washed twice in PBS, and was blocked in 1% goat serum in phosphate-buffered saline (PBS) for 1 h. Next, the slides were incubated at room temperature for 3 h with mouse anti-MjCM-1 peptide antibody diluted 250-fold in PBS with 0.1% BSA. After washing three times in PBS with 0.1% BSA, the slides were incubated for three h at room temperature with Oregon Green 488 goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, U.S.A.) diluted 1:250 in PBS with 0.1% BSA. The slides were washed three times in PBS with 0.1% BSA and, then, were permanently mounted using the ProLong antifading kit (Molecular Probes). Labeled cells were examined with a Zeiss Axiomscope 2 microscope with a fluorescent light source, and images were captured with a Zeiss Axiocam.

Subcloning of *MjCM-1*.

Plasmid pTA7002 was a gift from N.-H. Chua (Rockefeller University, New York). The *MjCM-1* gene was amplified from the original *MjCM-1* cDNA clone with Pfu DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) using primers NCMSP and NCMXH. The resulting PCR product was subcloned into the pCR2-TOPO vector (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.). Plasmids containing *MjCM-1* were sequenced and a plasmid containing *MjCM-1* was then digested with *Xho*I and *Spe*I restriction enzymes. The digested *MjCM-1* was gel-purified and ligated into pTA7002 also digested with *Xho*I and *Spe*I producing the resulting product, pTA7002:*MjCM-1*.

Hairy root transformation.

Plasmid pTA7002:*MjCM-1* was transformed into *Agrobacterium rhizogenes* K599 cucumopine strain (Savka et al. 1990) using electroporation (Cho et al. 1998, 2000). Soybean seeds, genotype Williams 82, were sterilized and germinated as previously described (Cho et al. 2000). After incubation at 28°C for 36 h, colonies were picked, and plasmid DNA was extracted. The resulting plasmid DNA was analyzed using PCR and restriction enzyme analysis to confirm that no rearrangements had occurred.

Plant inoculation was conducted according to Cho and associates (2000), modified as follows. Cotyledons from 7- to 9-day-old seedlings were harvested and wounded on the abaxial surface with a scalpel previously dipped into an overnight culture of *A. rhizogenes* K599/pTA7002:*MjCM-1*. The wounded cotyledons were incubated abaxial side up on moistened filter paper for 3 days. Three days postinoculation, the cotyledons were transferred and were cultured abaxial side up on MXB medium (MS [Murashige and Skoog 1962] basal nutrient salts, B5 [Gamborg et al. 1968] vitamins, and 3% sucrose [pH 5.7]), solidified with 3 g of Gelrite (PhytoTechnology Laboratories, Shawnee Mission, KS, U.S.A.) per liter. Carbenicillin (500 µg/l; Fisher Scientific) was added to inhibit the growth of *A. rhizogenes* and hygromycin B (25 µg/l; Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) was added to select for transformed roots. Approximately 10 to 14 days after root emergence, 1- to 2-cm-long root tips were transferred and were freed from bacteria by four passages on the same media at 25°C. The established cultures were then transferred every 2 to 4 weeks on MXB medium with 50 µg of carbenicillin and 2.5 µg of hygromycin B per l. To induce expression of the *MjCM-1* transgene, DEX (Sigma Chemical) was dissolved in ethanol and then added to MXB media to a final concentration of 10 µM. To evaluate the ability of IAA to rescue the *MjCM-1* phenotype, IAA (Sigma Chemical) was added to MXB to a final concentration of 1.0 to 2.5 µM.

Analysis of transgene expression.

To evaluate transgene expression, RNA was extracted from DEX-induced and uninduced tissue, according to the procedure of McCarty (1986). The extracted RNA was treated with DNase I, according to the manufacturer's instructions for severely contaminated RNA (DNA-free, Ambion Inc., Austin, TX, U.S.A.). RNA (1 µg) was then reverse-transcribed using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen Life Technologies), according to the manufacturer's instructions for oligo(dT) priming. The resulting cDNA was then used as template for PCR with *MjCM-1*-specific primers (NCM3 and NCM4). Primers (Cyc1F, Cyc1R) to a cyclophilin-like EST from soybean (GenBank accession number BI699846) were used as a control. PCR product (3 to 10 µl) was electrophoresed on an agarose gel using standard techniques.

Histology.

DEX-induced, uninduced, and IAA-rescued root tips were excised, fixed, and embedded as previously described (Schichnes et al. 1999), with the substitution of 2.5% paraformaldehyde as a fixative. The tissue was sectioned to 10-µm sections on a rotary microtome and was stained using the alternative microwave protocol for Johansen's safranin and fast green method (Ruzin 1999). Stained root sections were examined with a Zeiss Axioskop 2 microscope, and images were captured with a Zeiss AxioCam.

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