1982 Engineering durable root-knot nematode resistance in crops by RNAi silencing of a root-knot nematode parasitism gene

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Secreted proteins coded by parasitism genes expressed in esophageal gland cells mediate infection and parasitism of plants by root-knot nematodes. An essential parasitism gene, designated as *16D10*, encodes a conserved root-knot nematode secretory peptide that stimulates root growth and functions as a ligand for a plant transcription factor. Plants were engineered to silence this parasitism gene in root-knot nematodes using RNA interference (RNAi). *In planta* expression of *16D10* dsRNA in *Arabidopsis* resulted in resistance against the four common root-knot nematode species (Meloidogyne spp.). No known natural resistance gene elicits this wide and effective range of root-knot nematode resistance. Genetically modifying crop cultivars to express dsRNA that silence essential nematode parasitism genes represents a viable and flexible means to develop novel durable broad-spectrum root-knot nematode resistant crops. This strategy could also be used to develop root-knot nematode resistant crops for which natural resistance genes have not been found.

Root-knot nematodes (*Meloidogyne* spp) are among nature's most successful parasites. They parasitize more than 2,000 plant species from diverse plant families and represent a tremendous threat to crop production worldwide (Sasser 1980). Four common species, *M. incognita, M. javanica, M. arenaria,* and *M. hapla*, account for 95% of all root knot nematode infestations in agricultural land with *M. incognita* being a major parasite of cotton. These biotrophic pathogens have evolved highly specialized (and complex) feeding relationships with their hosts. Successful nematode-host interactions require molecular signals from the parasite to modify the host plant's root cells into elaborate multi-nucleate feeding structures, called giant-cells, which are the sole site of direct acquisition of nutrients that provide for nematode development and reproduction. Giant-cell formation represents one of the most complex responses elicited in plant tissue by any parasite or pathogen (Hussey, 1985). Infective root-knot nematode second-stage juveniles penetrate near the root tip and migrate intercellularly to a site near the differentiating vascular tissue. Secretory parasitism proteins injected through the nematode's hollow, protrusible stylet (oral feeding spear) transform five to seven root vascular cells into the metabolically active feeding cells by inducing repeated nuclear divisions uncoupled from cytokinesis (Hussey and Grundler, 1998). The parasitism proteins are encoded by parasitism genes expressed in the

root-knot nematode's three large and complex esophageal gland cells, a single dorsal (DG) and two subventral (SvG) (Hussey 1989; Davis et al. 2004). These secreted parasitism proteins mediate the dynamic interaction of the root-knot nematode with its plant hosts during migration through roots, establishment of giant-cells, and feeding.

Parasitism Genes

A powerful experimental approach to elucidate the parasitism genes of root-knot nematodes was developed by combining microaspiration methods with molecular tools (Gao et al., 2001; Karrer et al., 1995). A microaspiration technique was used to collect the cytoplasm of the nematode esophageal gland cells where the parasitism genes are expressed. cDNAs libraries were generated from extracts of the cytoplasm using RT-PCR (Huang et al., 2003). The mRNA isolated from transcriptionally-active gland cells of a range of root-knot nematode parasitic stages was pooled and used to generate esophageal gland cell-specific cDNA libraries that provided a comprehensive profile of nematode esophageal gland genes expressed in *M. incognita* during plant parasitism. Parasitism gene candidates were identified among the numerous gland cell-specific cDNA clones by detecting the presence of a putative secretion signal peptide on the encoded proteins. The presence of the signal peptide suggested that the nematode gene product could be actively secreted into plant tissues to mediate the parasitic interaction. In an expressed sequence tag (EST) analyses of parasitism genes from a gland-cell specific cDNA library in root-knot nematodes, 37 unique clones were confirmed by *in situ* mRNA hybridization to be specifically expressed within the subventral (13 clones) or dorsal (24 clones) esophageal gland cells of *M. incognita* (Huang et al., 2003). Developmental expression patterns of the parasitism genes vary greatly throughout the *M. incognita* life cycle. In BLASTP analyses, the majority (73%) of the predicted proteins were novel proteins and those with similarities to known proteins included a pectate lyase, an acid phosphatase, as well as hypothetically identified proteins from other organisms. EST analysis of a second gland-cell specific cDNA library from *M. incognita* (Huang et al., 2004) identified 11 additional parasitism genes of which six had similarities to known proteins that included a pectate lyase, beta-1,4-endoglucanases, and chorismate mutase.

Analysis of parasitism gene *16D10*

Functional analysis of the secreted products of root-knot nematode parasitism genes is critical for understanding the molecular basis of nematode pathogenesis. Functional analysis of a parasitism gene designated as *16D10*, which is expressed in the subventral esophageal gland cells, has revealed that the secreted product of this gene appears to mediate an early signaling event in root-knot nematode-host interactions (Huang et al., 2003; Huang et al., 2006a).

Parasitism gene *16D10* encodes a deduced protein of 43 amino acid including a 30 amino acid N-terminal hydrophobic signal peptide. The peptide is secreted by root-knot nematode infective second-stage juveniles at the point of initiation of the specialized feeding cells, suggesting the peptide has a biological function in parasitism. The mature 13 amino acid 16D10 peptide (GKKPSGPNPGGNN*,* M_r 1,223) has no significant BLASTP similarity, suggesting it is unique to root-knot nematodes (Huang et al., 2006a). A DNA blot containing genomic DNA from *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* hybridized with a *16D10* cDNA probe showed that *16D10* was conserved in each of the four agriculturally important *Meloidogyne* species with 3-4 copies or homologues.

Overexpression of *16D10* without the signal peptide under the control of the CaMV 35S promoter in *Arabidopsis* significantly accelerates root growth, giving rise to a much-enlarged root system without affecting shoot growth. In the root-growth assay, significant differences in root length between *35S::16D10* transformants and control lines were observed at 12 days after germination, with the length of primary root in *35S::16D10* transformants was increased by 85% (Huang et al., 2006a).

Yeast-two-hybrid analysis (Fields ad Song, 1989) identified tomato proteins that directly interact with the root-knot nematode parasitism protein (Huang et al., 2006a). The yeast-two-hybrid analysis led to the identification of three identical tomato cDNA clones that encoded partial proteins with 30-78% identity to the C-termini of plant SCARECROW-like (SCL) transcription regulators, which control plant growth and development (Bolle 2004). For confirmation that 16D10 interacted with a plant SCL protein, 12 *Arabidopsis* SCL genes homologous to the tomato SCL gene were cloned from *Arabidopsis* and expressed as fusion proteins in a yeast-two-hybrid assay. Two of the *Arabidopsis* SCL proteins, AtSCL6 and AtSCL21, interacted with 16D10 in yeast. Domain analysis revealed the specific interaction of 16D10 with the SAW domain of AtSCL6 and AtSCL21, and no interaction of 16D10 with the rest of the domains of the SCL proteins, indicating the two SCL transcription factors were putative targets of the secreted 16D10 during root-knot nematode parasitism of plants. *In vitro* and *in planta* interaction of 16D10 with AtSCL6 and AtSCL21 was verified by co-immunoprecipitation assays (Huang et al., 2006a).

These data suggest that the peptide 16D10 secreted from the subventral esophageal gland cells of root-knot nematodes specifically induces host root growth by directly interacting with a host intracellular SCL transcription regulator. Furthermore, since *16D10* is conserved in root-knot nematode species, it can be inferred that 16D10 is a fundamental signal for regulating root-knot nematode-host interactions by activating SCL-mediated signal-transduction mechanism in parasitized root cells. In host roots, vascular parenchyma cells near the primary xylem in the zone of elongation are preferred for root-knot nematode feeding cell initiation. The transition from parenchyma cell to a fully differentiated giant-cell occurs early in the parasitic association, indicating that secreted signaling molecules from the early parasitic second-stage juveniles mediate giant-cell development (Hussey 1989; Williamson and Hussey 1996). In root-knot nematode infected roots, cell cycle genes are up-regulated in the parasitized cells within the first hours of parasitism (Niebel et al. 1996; De Almeida Engler et al. 1999). Because the conserved root-knot nematode secreted signaling peptide is strongly expressed in the subventral gland cells of second-stage juveniles at the time when the giant-cells are being developed and functions as a ligand for specific plant transcription factor proteins, *16D10* has been postulated to have a role in the reprogramming of gene expression required for giant-cell formation (Huang et al., 2006a).

Engineering root-knot nematode resistant crops by RNAi silencing of parasitism genes

The most cost-effective and environmentally sustainable method for reducing root-knot nematode damage to food and fiber crops is to develop resistant plants which suppress nematode development and reproduction (Hussey and Janssen, 2002). However, use of host plant resistance is limited for several reasons the most fundamental of which is that only a small number of plant species are resistant to root-knot nematodes and there are many crops for which appropriate resistance loci have not been identified (Roberts, 2002; Williamson and Kumar, 2006). As with other plant resistance genes, the function of available root-knot nematode resistance genes involves recognition of specific root-knot nematode biotypes. For many of these the resistance can be overcome by virulent biotypes

(Roberts, 1995). Consequently, there is a need for new approaches for developing nematode resistant plants, and bioengineering provides a strategy to design effective and durable forms of root-knot nematode resistant crops.

One strategy that shows tremendous potential for bioengineering plant resistance to parasitic nematodes is RNA interference (RNAi) technology. RNAi, first demonstrated in *Caenorhabditis elegans* (Fire et al., 1998), has evolved into a powerful gene silencing tool for analysis of gene function in a wide variety of organisms (Hannon, 2002). In plants and nematodes, introducing or expressing double-stranded RNA (dsRNA) triggers the target gene-specific RNAi pathway (Novina and Sharp, 2004) including RNAi of target genes distal to the location of dsRNA that is ingested by nematodes (Timmons and Fire, 1998). Vectors designed to direct the expression of hairpin dsRNA within host plants (Wesley et al., 2001) may be used to promote the *in vivo* ingestion of the dsRNA by the nematode and consequent silencing of target nematode genes. Such an effect may be used to functionally analyze nematode parasitism genes and potentially to create novel transgenic crops that are resistant to nematodes (Davis, et al. 2004).

RNAi silencing of parasitism gene *16D10* was tested by engineering *Arabidopsis* to produce truncated or full-length *16D10* dsRNA molecules driven by the CaMV 35S promoter using the pHANNIBAL vector (Wesley et al., 2001). The transgenic lines had no observed morphological differences when compared to wild-type *Arabidopsis* suggesting there were no off-target effects from expression of the *16D10* dsRNA (Huang et al., 2006b). The *16D10* dsRNAs were transcribed in the transgenic lines and processed by *Arabidopsis* cells to approximately 21-bp small interfering (si) RNA. This processing of constitutively expressed *16D10* dsRNA in transgenic *Arabidopsis* provides *16D10* siRNA molecules for ingestion by parasitic stages of root-knot nematode and subsequent RNAi silencing of *16D10* in the subventral esophageal glands of the nematode.

The transgenic *Arabidopsis* lines were inoculated with each of the four major root-knot nematode species, *M. incognita, M. javanica, M. arenaria,* or *M. hapla*, to determine the effects of the host-generated *16D10* dsRNA molecules on root-knot nematode parasitism. At four weeks after inoculation, numerous large galls were present on the control transgenic plants while the *16D10* dsRNA transgenic lines showed a significant reduction (63-90%) in the number of galls as well as an overall decrease in gall size, compared to the vector-transformed line (Huang et al., 2006b). Significantly (69-93%) fewer root-knot nematode eggs per gram root were present on the *16D10* dsRNA transgenic lines when compared to the infected control plants. This silencing of *16D10* by RNAi demonstrates that a) parasitism gene *16D10* is essential for successful root-knot nematode infection of *Arabidopsis* and b) that *in planta* delivery of RNAi of *16D10* in root-knot nematode can provide broad-spectrum root-knot nematode resistance.

Additional evidence supporting the RNAi silencing of *16D10* was obtained by RNAi silencing of *16D10* in *Arabidopsis* by crossing the transgenic *Arabidopsis* line overexpressing *16D10* with the *16D10* dsRNA transgenic line to generate F1 hybrid lines. Root growth on *Arabidopsis* is strongly stimulated by overexpression of *16D10* (Huang et al., 2006a) whereas root growth on the root-knot nematode resistant *16D10* dsRNA transgenic *Arabidopsis* line is comparable to root growth on wild-type plants. *16D10* mRNA, which is present in the maternal *16D10*-overespressing transgenic line, was not detected in the F1 hybrid line, but a higher level of *16D10* siRNA was present in the hybrid line when compared to the paternal *16D10* dsRNA transgenic line (Huang et al., 2006b). Furthermore, the RNAi silencing of *16D10* expression in the F1 hybrid line restored the *16D10* stimulated root growth phenotype of the maternal *16D10*-overespressing transgenic line to a wild-type

normal root growth phenotype. This *in planta* silencing of overexpressed 16D10 confirms that host-generated 16D10 dsRNA can trigger RNAi of *16D10* to subsequently impede the function of the root-knot nematode 16D10 parasitism peptide in plants.

Future prospects for engineering root-knot nematode resistant crops by RNAi

Targeting the root-knot nematode parasitism gene *16D10* for silencing by expressing dsRNA in transgenic *Arabidopsis* enabled the development of transgenic plants that were resistant to multiple root-knot nematode species. Because no known resistance gene has this wide effective range of root-knot nematode resistance, bioengineering crops expressing dsRNA that silence target nematode parasitism genes to disrupt the parasitic process represents a viable and flexible means to develop novel durable nematode resistant crops and could provide crops with unprecedented broad-spectrum resistance to root-knot nematodes. Indeed *in planta* RNAi silencing of *16D10* in root-knot nematode could provide a strategy for developing root-knot nematode-resistant crops for which natural resistance genes do not exist.

Bioengineering root-knot nematode resistant crops by expressing dsRNA that silence root-knot nematode parasitism genes has several advantages. In addition to achieving broad-spectrum root-knot resistance, this type of resistance has the potential to be more durable. The specificity of RNAi-mediated resistance is based on RNA hybridization rather than receptor-ligand binding interactions characteristic of traditional plant resistance genes, which should make the resistance highly durable (Escobar et al. 2001). Durable resistance may also be achieved by co-expressing dsRNA constructs targeting more than one parasitism gene.

Root-knot nematode is a major pathogen of cotton and commonly forms a disease complex with Fusarium wilt where the severity of Fusarium wilt is significantly increased in the presence of *M. incognita* (Starr and Page, 1990). The root-knot nematode-Fusarium wilt disease complex in cotton is effectively controlled by root-knot resistance for races 1 and 2 of *Fusarium oxysporum f. sp. vasinfectum,* the most commonly occurring races in the U. S. (Wang and Roberts, 2006). There is need for novel genes conditioning root-knot resistance in cotton and expressing dsRNA that silences a root-knot nematode parasitism gene has the potential to provide a new source of root-knot resistance for cotton with the advantage that the dsRNA resistance gene can be directly incorporated into elite cotton cultivars

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