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Molecular Transfer of Nematode Resistance Genes[1]

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Abstract: Recombinant DNA techniques have been used to introduce agronomically valuable traits, including resistance to viruses, herbicides, and insects, into crop plants. Introduction of these genes into plants frequently involves *Agrobacterium*-mediated gene transfer. The potential exists for applying this technology to nematode control by introducing genes conferring resistance to nematodes. Transferred genes could include those encoding products detrimental to nematode development or reproduction as well as cloned host resistance genes. Host genes that confer resistance to cyst or root-knot nematode species have been identified in many plants. The best characterized is *Mi*, a gene that confers resistance to root-knot nematodes in tomato. A map-based cloning approach is being used to isolate the gene. For development of a detailed map of the region of the genome surrounding *Mi*, DNA markers genetically linked to *Mi* have been identified and analyzed in tomato lines that have undergone a recombination event near *Mi*. The molecular map will be used to identify DNA corresponding to *Mi*. We estimate that a clone of *Mi* will be obtained in 2–5 years. An exciting prospect is that introduction of this gene will confer resistance in plant species without currently available sources of resistance.

Key words: Agrobacterium tumefaciens, DNA, *Lycopersicon esculentum*, *Meloidogyne* spp., *Mi*, nematode, resistance, tomato.

The ability to genetically engineer plants provides a major opportunity for modification and improvement of crops. There has been considerable progress in the development of systems for transferring genes into plants (reviewed by Gasser and Fraley, [14]). The most widely used method involves DNA transfer mediated by the plant-pathogenic bacterium, *Agrobacterium tumefaciens* (9,17,45). In nature, *A. tumefaciens* causes tumors on plants by inserting into the plant genome a defined region of its own DNA, the T-DNA (for transferred DNA), which is carried on a large plasmid, the Ti (or tumor-inducing) plasmid (45). The T-DNA encodes several genes, including those that cause the formation of the tumorous galls. This natural gene transfer system has been modified into a genetic engineering tool by deleting the tumor-forming genes from the T-DNA and replacing the deleted genes with the gene to be introduced and a selectable marker, usually an antibiotic resistance marker. The selectable marker allows detection of plant cells that have incorporated the T-DNA. Figure 1 contains a generalized scheme for *A. tumefaciens*-mediated DNA transfer of a resistance gene (R-gene).

Other plant transformation techniques: Although numerous transgenic plant species have been produced with *A. tumefacies* (14), the method has not been useful for some important crop plants such as rice and maize. Other techniques have transferred DNA into plant protoplasts; however, difficulties have occurred in regenerating fertile, intact plants for maize and many other species. Consequently, molecular transformation of these crops has lagged behind more easily engineered species. Microprojectile bombardment has recently been used for stable transformation of maize suspension culture cells from which fertile, transgenic plants have been regenerated (15). Through improvements in this and other transformation techniques, most agriculturally important crops should become accessible to molecular gene transfer techniques in the near future. The next crucial step is to identify and design appropriate genes to transfer to crop plants. We shall discuss two categories of genes that may impact nematode control: those encoding defined products that are detri-

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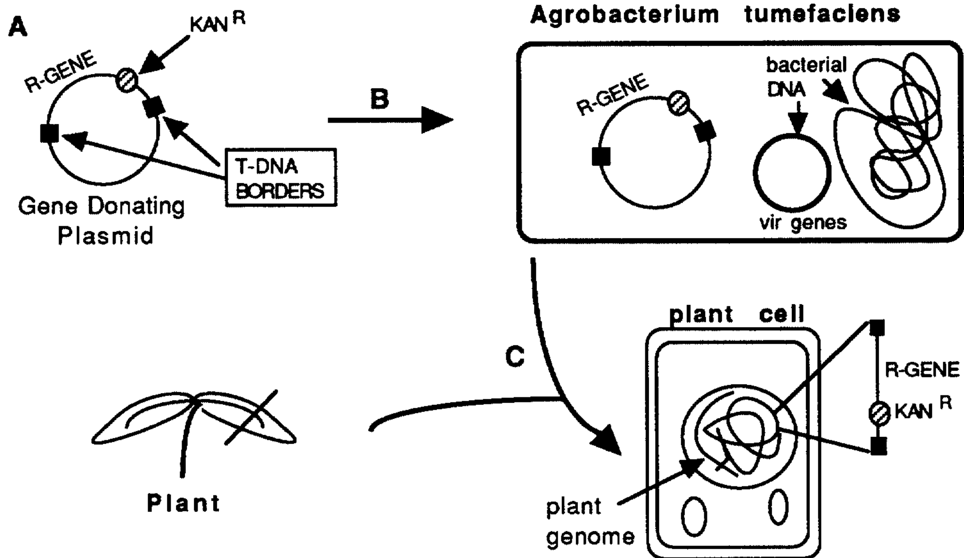

FIG. 1. Molecular transfer of genes mediated by *Agrobacterium tumefaciens.* A) The gene to be transferred, the R-gene in this example, is introduced between the T-DNA borders of the Gene-Donating Plasmid. The T-DNA borders are short DNA sequences that are required for DNA transfer and that delimit the transferred region. The plasmid also contains a plant selectable marker, in this case a gene conferring resistance to kanamycin (KAN^R), and sequences that allow the plasmid to replicate in both *Escherichia coli* and *A. tumefaciens.* For convenience, the plasmid is proliferated in an *E. coli* host. B) The Gene-Donating Plasmid is transferred to *A. tumefaciens.* This bacterium carries virulence or *vir* genes required for pathogenesis and DNA transfer. C) A fragment of plant tissue, e.g., a piece of cotyledon, is dipped into a solution carrying the engineered *A. tumefaciens* that transfers the T-DNA to the plant cell, where it is incorporated into the genome. Plant cells with the newly incorporated DNA are selected by their ability to grow on kanamycin-containing medium.

mental to nematodes and natural plant resistance genes whose products are unknown.

ENGINEERING RESISTANCE

Recombinant DNA techniques have been used to transfer into crop plants agronomically useful traits, such as herbicide, insect, and virus resistance (14). One of the first such applications was the transfer of a gene encoding a glyphosate-tolerant form of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (7). Transgenic plants display glyphosate tolerance. More recently, genes conferring resistance to several other herbicides have been transferred to plants (reviewed in Gasser and Fraley [14]). Resistance to tobacco mosaic virus (27) or other viruses (3) has been achieved by expressing the corresponding virus coat protein genes in transgenic plants. Regarding insects, plants engineered to produce an insecticidal protein from *Bacillus thuringiensis* are resistant to certain Lepidoptera (26). Increased expression of protease inhibitor genes in transgenic plants produces partial resistance to several insect species by inhibiting their digestive enzymes (32).

Applications of plant biotechnology to nematode control have thus far not been developed, and one can only hypothesize about potential control strategies. One strategy might be to transform plants with a gene encoding a product known to be detrimental to nematodes or to inhibit expression of plant genes required for host–parasite interactions. An example of the former would be transformation of a plant to produce collagenase in response to nematode invasion, as collagenase adversely affects *M. javanica* juveniles (12). Regulation of collagenase gene expression would require attaching a control region or promoter that would turn on the gene in response to nematode attack. A gene that is specifically expressed in potato at or near the syncytium induced by *Globodera*

rostochiensis has recently been identified (16), and its control sequences may be able to induce expression of the collagenase gene at the site of nematode infection.

Host Resistance Genes

A second approach to engineering nematode-resistant plants is to identify and clone natural plant resistance genes and then introduce the cloned genes into susceptible plants. Although resistance genes are available in many plant species, undesirable traits are often genetically linked to these genes due to their origin in wild or agronomically primitive plant material. Transfer of a cloned resistance gene could reduce by many years the conventional breeding required to introduce resistance into commercially acceptable cultivars. In addition, a cloned gene conferring resistance to a nematode that has a broad host range, such as *Meloidogyne incognita,* could be transferred to many plant species with no curently available sources of resistance.

Cloning genes with unknown products: Programs to isolate plant genes conferring resistance to diverse pathogens are underway in several different laboratories but have had limited success (23). Although cloning genes that encode known proteins is relatively straightforward (1,41), the lack of knowledge of the products of nematode resistance genes, as well as other plant disease resistance genes, severely limits the available cloning strategies. One basic strategy for cloning genes with unknown products is map-based cloning or chromosome "walking." The approach starts with a DNA clone, usually identified as a restriction fragment length polymorphism (RFLP) that co-segregates in genetic crosses with the resistance gene. The process proceeds with the identification of DNA fragments progressively closer to the gene of interest by sequentially obtaining clones from a DNA "library," a collection of DNA clones representing the entire genome of the organism. The isolation of the human disease genes responsible for cystic fibrosis and neurofibromatosis are excellent examples of the power of the technology (31,39). Pulsed field gel electrophoresis (PFGE), a recently developed technique to fractionate very large DNA molecules such as intact DNA from entire yeast chromosomes, greatly facilitates map-based cloning (5,33). Another tool, yeast artificial chromosome (YAC) vectors, has been useful for cloning very long fragments of DNA (6). DNA libraries representing most of the DNA of the nematode *Caenorhabditis elegans,* the small plant *Arabidopsis thaliana,* and *Homo sapiens* have been created using YAC vectors (8,18,40).

Transposon tagging: An alternative approach for cloning genes with unknown products, such as resistance genes, utilizes transposon mutagenesis. Transposable elements are pieces of a genome that move around and cause mutations, often by inserting into and thus disrupting genes. Consequently, the inserted element tags the targeted gene sequences, which are now immediately adjacent to the introduced transposon. A DNA clone corresponding to the transposon can then be used as a probe to identify a fragment of DNA corresponding to the transposon and adjacent target sequence. This strategy has been successful in maize, which has several well-characterized transposable elements (11). A similar strategy, using the *A. tumefaciens* T-DNA as a tag, has been used to clone genes in *Arabidopsis* (42).

Selection of Candidate Genes

Nature of resistance: Cloning a resistance gene is a major undertaking, requiring an extensive commitment of resources. Genes that confer effective field resistance to a broad group of nematodes or to nematodes that have a broad host range may be more appropriate choices than those conferring resistance to only one nematode race. In addition, initial attempts should concentrate on resistance conferred by a single gene rather than multigenic resistance because of the relative simplicity of cloning and transferring a single gene. An excellent candidate for cloning is *Mi,* a tomato gene that confers effective laboratory

and field resistance to *Meloidogyne incognita, M. javanica,* and *M. arenaria* (2,29).

Stability: Another consideration for selecting genes for cloning arises when resistance genes have been characterized but traditional breeding has not produced stably transmitted resistance in an acceptable cultivar. An example is resistance to *Heterodera schachtii.* Although resistance has been transferred to sugarbeet, *Beta vulgaris,* as an intact chromosome or a chromosome fragment from other *Beta* species, it is unstable and has not been incorporated into useful cultivars despite extensive efforts (19). An alternative approach would be to isolate the corresponding genes so that resistance can be directly transferred by recombinant DNA techniques (19,25).

Progress Toward Cloning *Mi*

The tomato plant: One reason for our choice of *Mi* as a cloning target is that tomato is an excellent molecular and genetic model organism. Genetic analysis in tomato is straightforward, many morphological and DNA markers have already been mapped (35), and techniques for isolation and analysis of long fragments of DNA from tomato are available (13,38). Tomato has a modest genome size, and DNA transformation by *Agrobacterium*-based vectors is relatively efficient. The large number of workers pursuing aspects of tomato biotechnology increases the likelihood of cooperation on labor-intensive techniques, such as construction of YAC libraries.

The Mi gene: Mi was introduced into cultivated tomato (*Lycopersicon esculentum*) from a wild tomato species (*L. peruvianum*) by using tissue-culture techniques to rescue a hybrid embryo (34). A single F_1 plant was the source of nematode resistance in all currently available fresh-market and processing tomato cultivars (21). The early material has been backcrossed extensively to *L. esculentum.* The observation (28) of tight genetic linkage between *Mi* and *Aps-1,* a gene-encoding acid phosphatase-1, greatly facilitated the introduction of the resistance gene into commercial cultivars. Many nematode-resistant tomato lines contain a variant allele, *Aps-1*^{1}, that originated from *L. peruvianum,* whereas susceptible lines of *L. esculentum* contain the typical *Aps-1*$^{+}$ allele. The products of the two *Aps-1* alleles can easily be distinguished by starch gel or cellulose acetate electrophoresis (4,24). Early speculation that the *Aps-1*^{1} allele was responsible for nematode resistance has not held up, as at least three genetic recombinants between these two loci have been identified.

Chromosome walking: Aps-1 maps very closely to *Mi* (less than 0.89 recombination units away); thus a clone of this gene is a logical starting point for chromosome walking to *Mi. Aps-1* has been cloned from nematode-resistant tomato plants (1,41). On the average, there are about 600 kilobases (kb; 600,000 nucleotides of DNA) for each recombination unit in tomato. If recombination were constant throughout the tomato genome, *Mi* would be <534 kb from *Aps-1.* This is a feasible distance to cover by modern chromosome walking techniques. However, the relationship between genetic and physical distance is far from linear in plants (10), and the distance between these markers could be ten or even a hundred-fold greater.

The alien origin of *Mi* and the surrounding DNA sequences has been useful for obtaining additional DNA markers linked to *Mi.* DNA sequence differences, observed as RFLPs, between cultivars of the domestic tomato are uncommon. However, between different *Lycopersicon* species, such polymorphisms are abundant (22). Thus, RFLPs between inbred nematode-resistant and susceptible cultivars are likely be located in the region of DNA containing *Aps-1* and *Mi,* as this region is derived from *L. peruvianum.* Such an RFLP, obtained by probing a Southern blot with a labeled DNA clone (*CD67*) that maps near *Aps-1* (36), clearly differentiates tomato lines with different *Aps-1* isozymes (Fig. 2). In comparison, another clone (CD29) that maps further away from *Mi* but is still on the same chromosome displays no poly-

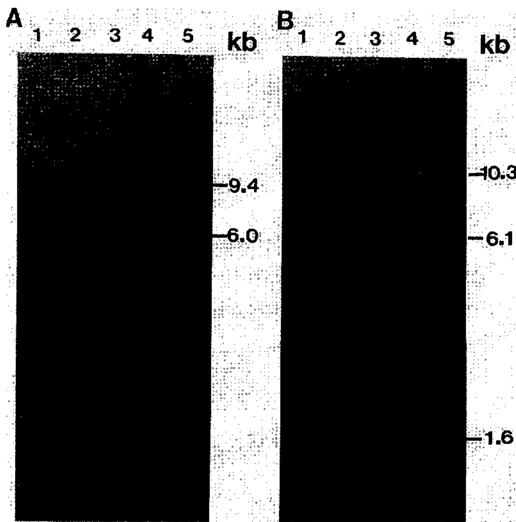

FIG. 2. RFLP analysis of resistant and susceptible tomato (*Lycopersicon esculentum*). A Southern blot of tomato DNA digested with Hind III was probed with cDNA probes CD29 (A) or CD67 (B). DNA in lane 1 is from *L. esculentum* cv. Rossol (*Mi Aps-1*$^{+}$); lane 2, cv. VFNT cherry (*Mi Aps-1*^{1}); lane 3, line Sun 6082 (*Mi Aps-1*^{1}); lane 4, cv. Castlerock II (*mi Aps-1*$^{+}$), lane 5, cv. Roma (*mi Aps-1*$^{+}$). Approximate sizes of bands in kilobases (kb) are indicated to the right. The less intense of the two bands hybridizing to CD29 is linked to *Mi*. Probes CD29 and CD67 were obtained from S. Tanksley, Cornell University.

morphism (Fig. 2). Because a polymorphism was observed between *L. esculentum* and *L. peruvianum* with CD29 as a probe (not shown), it was concluded that this locus is outside the introgressed area in the nematode-resistant tomato lines. The potato RFLP marker GP79 and the tomato genomic DNA clone H6A2c2 also display polymorphisms and map near *Mi* (20). Additional RFLP markers (LC75, LC216, LC379, and LC471) that are linked to *Mi* have been obtained by using random clones from a tomato cDNA library as probes for polymorphisms correlated with the *Mi* or *Aps-1* allele (J. Y. Ho and V. M. Williamson, unpubl.).

To determine the relative locations of the linked markers in the tomato genome and the extent of the introgressed region, we have analyzed DNA from several different tomato lines and cultivars. The root-knot nematode-resistant cultivar Anahu, developed in the Hawaiian breeding program, was particularly important in the analyses. This line does not carry the *Aps-1*^{1} allele characteristic of *L. peruvianum,* presumably due to a recombination event between *Aps-1* and *Mi* (21). The tomato line N118, identified in a processing tomato breeding program at the University of California, Davis, represents a second, independent recombination event between *Mi* and *Aps-1*. N118 carries the *Aps-1*^{1} allele but is susceptible to root-knot nematodes. Comparison of Southern blots of DNA from these and other tomato lines using DNA probes linked to *Mi* allowed us to establish the partial order of DNA markers and to obtain an estimate of the relative extent of introgressed DNA (Fig. 3). Our results suggest that the lines derived from the Hawaiian breeding program (cultivars Anahu and Rossol) have retained substantially less *L. peruvianum* DNA than those with the *Aps-1*^{1} allele (cultivar VFNT cherry and line Sun 6082). Analysis of recombination events in segregating tomato populations supports these results. Additional recombinants and a long-range physical map of existing recombinants will be required to determine the order of all the markers presented. PFGE is being used to develop a physical map of the region of the genome corresponding to *Mi*. The physical map will facilitate identification of the DNA fragment corresponding to *Mi*. Identity of the fragment will be confirmed by transformation of susceptible tomato with the *Agrobacterium* transformation system outlined in Figure 1.

Transposon tagging: The map-based cloning approach has been used successfully to clone a number of animal genes but, as yet, no plant genes. Transposon tagging is an alternative approach for cloning *Mi*. Although no endogenous transposable elements have been characterized in tomato, the maize transposable element activator (*Ac*) is capable of transposition in tomato (43). A multistep strategy, taking advantage of the propensity of *Ac* to move to nearby chromosomal locations, is in progress to tag *Mi*. A plant has already

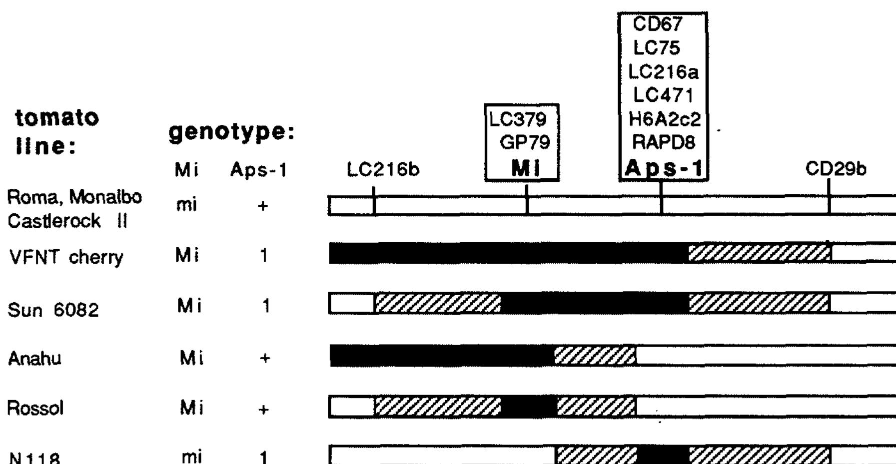

FIG. 3. Genetic map of the *Mi* region. The map of chromosome 6 contains over 100 recombination units, but *Mi* and *Aps-1* are separated by <0.9 recombination units; therefore, this map represents only a small portion of the chromosome. Roma, Monalbo, and Castlerock II are susceptible tomato cultivars. VFNT cherry, Sun 6082, Anahu, and Rossol are resistant to nematodes and differ in the amount of remaining *L. peruvianum* DNA surrounding *Mi*. Line N118 carries the *Aps-1*[1] allele but is susceptible to nematodes. White horizontal bars represent regions of *L. esculentum* origin, black bars represent regions derived from *L. peruvianum*, and cross-hatched areas contain the junction between these species.

been identified in which *Ac* is inserted on chromosome 6, the location of *Mi* (B. Baker, Plant Gene Expression Center, Albany, CA, pers. comm.). We will screen progeny of this plant to identify individuals with *Ac* located very close to *Mi*. Plants in which *Ac* has moved again will be screened for loss of nematode resistance resulting from insertion of *Ac* into *Mi*.

EXPECTATIONS

Less than 10 years have passed since production of the first genetically engineered plants (9,17). Engineered plants now have increased herbicide, insect, or virus resistance in the field. Gasser and Fraley (14) predict that the first transgenic varieties will be released between 1995 and 1997. Field testing of transgenic plants containing novel nematode resistance genes is likely to occur within 5–10 years. We predict that within the next 5 years, the *Mi* gene will be cloned. Transfer of *Mi* directly into susceptible tomato varieties with other agronomically desirable traits could save years of breeding. An exciting experiment will be to transfer *Mi* to plant species where root-knot nematodes are a problem and in which no effective resistance genes are available. If resistance is conferred, the *Mi* clone would have an important impact on agriculture, particularly in light of use restrictions and withdrawal of nematicides. It may be possible to modify *Mi* and alter its properties to allow recognition of additional nematode species. Also, if *Mi* is homologous to other resistance genes, it will be useful as a probe to identify such genes.

Plants protected by engineered resistance will need to be incorporated into an integrated control strategy if their utility is to be preserved. The potential for proliferation of resistance-breaking pathotypes that can infect plants with engineered resistance is a serious concern (44). There have been several reports of the appearance of nematode populations that can infect tomato plants with *Mi* (30,37). Careful monitoring, rotations, and integrated pest-control strategies will be necessary or the engineered resistance will be short-lived. Plants engineered to carry more than one type of resistance gene may also reduce the risk of proliferation of resistance-breaking nematode populations.

Public concerns over possible ramifications of the use of recombinant plants must be resolved. For example, the introduction of herbicide resistance into plants

has raised concern that such plants will increase herbicide use. Concern also exists about possible dangers from spread of transgenes to other plant species in the environment. On the positive side, heightened interest in reducing use of chemical pesticides has increased acceptance of new approaches. Barriers to the commercialization of resistance genes include the complex regulations for the release of recombinant plants and concern for proprietary protection for industry investment in producing transgenic products. The first group of transgenic plant products are currently undergoing evaluation and scrutiny and will pave the way for applications of recombinant DNA technology to problems in nematode control.

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