Toward Stacked Transgenic Virus Resistance In Tomato.

J.K. Brown[1] (jbrown@ag.arizona.edu), K. Mills-Lujan[2] (millsk@uga.edu), A.M. Idris[1] (idrissud@ag.arizona.edu), Maria Mercedes Roca[3] (mmroca@zamorano.edu), Dale T. Krigsvold[4] (dkrigsvold@fhia.org.hn), C. Michael Deom[2] (deom@uga.edu). [1]Plant Sciences Department, The University of Arizona, Tucson AZ USA; [2]Department of Plant Pathology, University of Georgia, Athens GA USA; [3]Escuela Agricola Panamericana, Honduras; [4]Fundación Hondureña de Investigación Agrícola, Honduras.

Abstract

Tomato mosaic virus (ToMV) and *Pepper golden mosaic virus* (PepGMV) are major deterrents to tomato production in Honduras. To counter crop losses, transgenic tomato lines have been produced with the long-term goal of 'stacked' transgenic resistance to both ToMV and PepGMV. Tomato lines were transformed either with viral coat protein (CP) constructs (ToMV), or with inverted repeat constructs of viral gene sequences (ToMV, PepGMV). Inverted repeat constructs are expected to induce an RNAi response (gene silencing), which results in virus resistance. RNAi is now the most common approach for inducing high levels of transgenic resistance in plants. Approximately 70-80 transgenic open pollinated tomato (cv 'Moneymaker') plants were obtained carrying sequences from each virus. Transgenic plants are being evaluated to identify lines having the most robust resistance to ToMV and PepGMV. The transgenic plants will be used as a source of resistance for crossing into locally adapted varieties and to 'stack' resistance, followed by further selection toward high-level resistance in tomato for the American Tropics.

Introduction

Tomato mosaic virus (ToMV, Fig. 2B) and *Pepper golden mosaic virus* (PepGMV) (Fig. 2C) were selected as target viruses for this project because the viruses were found to be two of the most important viral pathogens of tomato in Central America. PepGMV is a single-stranded DNA *Begamovirus* composed of two components, DNA A and DNA B. *PepGMV* causes mosaic and leaf curl symptoms and yield losses in pepper (Fig. 1A) and tomato (Fig. 1B). Clones of the DNA components were used to determine the experimental host range of the virus and to complete Koch's Postulates (i.e. demonstrate causality) (1). Similarly, CP gene sequences from three field isolates of ToMV, a *Tobamovirus* having a single-stranded RNA genome, were obtained from Honduras and cloned and sequenced. All coat protein sequences were identical. One of the CP clones was used in generating trangenic tomato plants.

Objective

To genetically engineer disease resistance in tomato to ToMV and PepGMV.

a. Clone and sequence Rep (Replication-associated protein) and BV1 genes from PepGMV, and CP gene from ToMV.
b. Clone viral genes into transformation vector and transform into *Agrobacterium*
c. Transform tomato independently with PepGMV (Rep, BV1), and ToMV (CP) transgenes.
d. Screen T0/T1 lines for extent of homologous virus protection.

Transgenic tomato strategies

Our primary approach to developing transgene-mediated virus resistance in tomato is to induce the host cell's RNAi response (2, 4, 5) by expressing inverted repeats of viral sequences, that form double-stranded RNA (dsRNA) following transcription. The formation of double-stranded RNA sequences induce Dicer, a cellular dsRNA endonuclease, to cleave the viral dsRNA into siRNA (small interfering RNAs). The siRNAs associate with RISC (RNA-inducing silencing complex) causing the siRNA to unwind. A single-stranded siRNA coupled to RISC targets complementary RNA (i.e. ToMV genomic and subgenomic RNA and PepGMV mRNA) in a sequence specific manner. The target RNA is cleaved by Slicer, a component of RISC. The cleaved RNA is recognized as aberrant in the host cell and destroyed. Begomoviral replication requires expression of Rep (encoded by the AC1 gene) and down-regulation of its expression would interfere with, and possibly eliminate completely, the synthesis of Rep protein, thereby blocking viral replication. Similarly PepGMV movement depends on the BV1 gene. Down-regulation of BV1 would interfere with virus movement and block infection. Likewise, targeted degradation of the ToMV genome and CP mRNA would block virus infection.

In addition to the RNAi approach for developing resistance to ToMV, a full-length translatable CP gene from ToMV, was introduced into transgenic tomato to induce CP-mediated protection, a mechanism that is widely used for transgenic resistance for tobamoviruses (3). The presence of coat protein in transgenic plants inhibits uncoating of the viral genome following entry, therefore inhibiting infection.

Figure 1. Pepper (A) and tomato (B) plants showing typical *Pepper golden mosaic virus* symptoms. The virus causes a golden mosaic in pepper and golden leaf curl and stunting in tomato plants. PepGMV is transmitted in nature by the whitefly vector *Bemisia tabaci* (center).

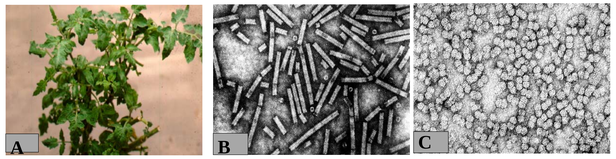

Figure 2. Symptoms in tomato (A) caused by *Tomato mosaic virus* (rod shaped), (B) tobamovirus particles (ToMV), and (C) typical begomovirus particles (PepGMV) (geminiate).

Table 1. Number of transgenic plants obtained for each PepGMV construct, and PCR primers used for detection of viral transgenes (using PCR) and transgene expression (using RTPCR). AC1 encodes the Rep protein an BV1 encodes the nuclear shuttle protein. IR, inverted repeat.

Viral Construct Designation	# of Lines	PCR Primer Target	Frequency of Transgene Detection	Frequency of Detectable Transgene Expression	Viral Transgene
1862	10	TPV-AC1C, TPV-AC1V	10 (100%)	10 (100%)	AC1 sense
1854	12	TPV-AC1C, TPV-AC1V	12 (100%)	8 (67%)	AC1 anti-sense
1921	38	TPV-AC1C, TPV-AC1V	37 (97%)	34 (89%)	AC1 IR
1901	34	TPV-BVIC, TPV-BVIV	32 (94%)	29 (85%)	BV1 IR

Table 2. Number of transgenic plants obtained for each ToMV construct with detectable viral transgenes (using PCR). CP encodes the coat protein. IR, inverted repeat.

Viral Construct	# of Lines	Frequency of Trangene Detection	Viral Gene
CPASS	57	48/57 (84%)	CP IR
ToCP	38	32/38 (84%)	CP sense

Table 3. Transgenic plants carrying the RNAi construct for the ToMV coat protein showing tentative resistance during initial screening. VC, transgenic plants containing only the transformation vector.

Plant Designation	Frequency of ToMV Inoculated Plants Positive for ToMV	Frequency of Mock Inoculated Plants Positive for ToMV
CPASS-4	1/7	0/3
CPASS-6	4/7	0/3
CPASS-7	3/7	0/3
CPASS-8	5/7	0/3
VC-2	7/7	0/3
VC-6	7/7	0/3

Because RNAi is sequence specific, it is important that the viral gene sequences used to generate transgenic plants be obtained from the predominant ToMV and PePGMV isolates present in tomato in Honduras.

Methods

Viral gene sequences were cloned into pBI121 under the regulatory control of the 35S promoter and transgene tomato lines were generated at the Ralph M. Parsond Plant Transformation Facility, Davis, CA by *Agrobacterium*-mediated transformation. Because the majority of cultivars are hybrids, transformation was conducted in the generic variety, 'Moneymaker', which subsequently will be crossed into local varieties. Inverted repeats of the CP gene of ToMV and the Rep (replication associated protein) and BV1 (nuclear shuttle protein) gene sequences of PepGMV were transformed into tomato for induction of RNAi. For ToMV the full-length translatable coat protein gene was introduced into transgenic tomato to induce coat protein-mediated protection. 'Moneymaker' was also transformed with additional constructs form PepGMV: sense Rep (control) and anti-sense Rep (control). In addition, 'Moneymaker' was transformed with pBI121 as a vector control. Kanamycin resistant regenerated plants were analyzed by PCR for the transgenes and /or northern blotting for transgene expression. Challenge inoculations with ToMV or PePGMV are initially being done in greenhouses at UGA and UAZ, respectively.

Results and Discussion

Transgenic lines transformed with begamovirus gene sequences are being assessed by quantitative PCR for levels of transgene expression. The highest expressers will then be assessed to determine transgene copy number using restriction digestion and Southern analysis. Transformants (10+/transgene) having one or multiple copies of the transgenes will be selected for challenge-inoculation using infectious viral clones. Plants (T1) will be inoculated with infectious PepGMV clones using biolistics, to determine their level of resistance to (homologous) virus infection. Inoculated plants will be assessed for (i) mRNA transgene and (ii) RNAi expression, and (iii) presence of PepGMV ss/dsDNA by Southern analysis using viral DNA-A and –B probes. Additional promising material will be selected and analyzed for RNAi expression and monitored for symptom expression over time.

Approximately 84% of the transgenic ToMV lines are transformed with ToMV CP sequences (Table 2). T1 seedlings of transgenic lines expressing ToMV CP gene sequences are also being evaluated for virus resistance by virus inoculation. To date, 8 lines carrying the RNAi construct have been screened for resistance. Initial screening suggests that 4 of the 8 lines show a resistant phenotype (Table 3).

Seed will be produced from promising transgenic lines showing virus resistance and T2 and T3 plants will be assessed as described. Those stably inheriting the resistance will be used to transgress transgenes into tomato varieties that are compatible with the target area for field-testing and eventual release in Honduras and the Central American region. 'Stacking' resistance to both ToMV and PepGMV in cultivars with favorable agronomic traits will be a primary goal of the breeding program. These cultivars have been developed for specific agronomic traits (resistance to nonviral pathogens, fruit quality, and heat tolerance) and will be preferred by farmers and local consumers. Because of the mechanism behind RNAi, we foresee the RNAi induced response as a highly durable type of transgenic resistance that will greatly increase the economic viability of tomato production in Honduras and the American Tropics.

References

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