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Research Article

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A Novel Tool for Genetic Transformation in Trees

Rita Mozes-Koch*, Edna Tanne-Sela and Ilan Sela

Abstract

Genetic manipulation of trees is difficult. Using the universal vector IL-60 derived from Tomato Yellow Leaf Curl Virus (TYLCV) and auxiliary constructs, we introduced and expressed genes in grapevine, and orange trees. The genes were expressed in the trees after the constructs were introduced by direct injection into the phloem, root uptake or grafting, or in cuttings from intact plants that had been expressing the introduced genes for years. Moreover, because the disarmed virus does not support rolling-circle replication, viral progeny single-stranded DNA is not produced, IL-60 is not integrated into the plant's genome, and the construct, including the expressed gene, is not heritable. Moreover, IL-60 is not transmitted by natural insect vectors of TYLCV. The introduction of these constructs into trees, seeds and cuttings or grafts provides an easy, inexpensive and rapid way of introducing genes into trees and vegetatively propagated plants such as grapevine.

Keywords

Genetic transformation in trees; Grafting; Non-transgenic; IL-60; Root uptake; TYLCV

Abbreviations

TYLCV: Tomato Yellow Leaf Curl Virus; PRN: Pyrrolnitrin; GVA: Grapevine Virus A

Introduction

Tomato Yellow Leaf Curl Virus (TYLCV) belongs to the Geminiviruses, a major class of single-stranded DNA viruses. Its DNA genome is 2.7 kb in length and it encodes six genes with partially overlapping open reading frames (ORFs) in a bidirectional organization for viral replication and spread [1,2]. Two of these ORFs (V1 and V2) are present in the virion sense orientation and four of them (C1–C4) are in the antisense orientation, separated by a 314-nucleotide (nt) intergenic region (IR). The IR contains the key elements responsible for replication and transcription of the viral genome. V1 encodes the viral coat protein (CP, 30.3 kDa), which is responsible for encapsidation of the genome and is involved in virus movement and vector recognition [3]. V2 (13.5 kDa) encodes the pre-coat protein, which is involved in viral spread from cell to cell [2]. The replication-associated protein (Rep, 41 kDa) is encoded by C1. C2 (15.6 kDa) encodes a transcriptional activator protein that is involved in the activation of transcription. C3 (15.9 kDa) and C4 (10.9 kDa) encode proteins involved in viral DNA accumulation [4].

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Geminivirus-derived vectors can be classified into expression vectors and virus-induced gene silencing (VIGS) vectors.

New approaches and techniques for expanding the genetic boundaries are constantly being developed and applied to achieve, for example, tolerance to biotic and abiotic stresses, as well as improved nutritional quality and crop productivity [5-8].

The first step in genetic transformation is DNA delivery to the cells or tissues. Microprojectile bombardment and *Agrobacterium*-mediated gene transfer are the most widely used methods. This is followed by tissue culture and selection to permit only those tissues and cells that have the marker gene (for instance, a herbicide- or antibiotic-resistance gene) to proliferate. Plants are then grown from the surviving cells.

Transgenesis is a major way of expanding the genetic pool beyond the species/genus reservoir. The advanced technologies for stable and heritable transformation, however, are expensive, labor-intensive, and time-consuming [9-12]. Therefore, the rapidly multiplying DNA viruses are ideal vectors for plant transformation because they produce high copy numbers of the genes of interest [13].

Viral expression systems are considered transient expression systems because the viral vectors are not integrated into the host genome. However, viral vectors have many limitations. They can cause disease in their plant hosts, they can spread naturally among plants in the field, and in some cases, they can be spread through pollen or seeds to the next generation. Viral vectors are also limited with respect to their systemic spread in the plant, expression stability, and the size of the insert that can be tolerated. Finally, like transgenic plants, modified viruses are classified as genetically modified organisms (GMO) and are thus subject to regulatory constraints.

In this study, we chose the TYLCV-CP clone IL-60, which replicates and spreads in plants, which nevertheless remain symptomless for the duration of observation (months or years). Thus, IL-60 is a symptomless clone with a 60-nt deletion (position 552–612) in the TYLCV-DNA.

Materials and Methods

Artificial satellites require a helper virus for replication, movement, and expression. A plasmid was inserted into the *rep* gene of TYLCV, disabling rolling-circle replication. Replication from double-stranded DNA to double-stranded DNA, which is directed solely by host factors, was retained. The short (314 bp) viral noncoding IR functioned as the origin of replication and carried a promoter on each end. IR alone has been previously shown to direct the replication of IL-60, as well as of foreign genes the expression of which it directed [1,14,15]. Any DNA placed downstream of the viral IR will replicate in the cells to which it has been delivered. However, it requires a helper virus to spread to other cells. In our case, the helper virus was IL-60-BS, which promoted movement throughout the plant without causing disease. All viral functions (replication, expression and movement) were determined by the IR and the sense-oriented genes. The IL-60 plasmid is a bi-component system consisting of a "driver" (IL-60) and an "expresser" (IR-X). The following reporter genes were cloned downstream of the TYLCV- IR into the plasmid

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pD-IR: *GUS* (GenBank accession No. M14641), *GFP* (GenBank accession No. U87974), *PRN* operon of *Pseudomonas fluorescens* Pf-5 (GenBank accession No. CP000076.1; bases 4157074–4162815) and *P10* ,the entire ORF5 of Grapevine virus A (GVA) coding for P10. The resulting constructs were termed IR-GUS, IR-GFP, IR-PRN and IR-P10, respectively. The IR segment of TYLCV was PCR-amplified and cloned into the T/A cloning vector pDRIVE (Figure 1).

Escherichia coli cells were transformed with the IL-60 construct and propagated under ampicillin selection. The IL-60 construct was extracted by standard procedures [16]. IL-60 and the various constructs employed in this study were administered to the plants in different ways:

a. injection with a syringe directly into the phloem [1].

b. root uptake by seedlings and cuttings introduced into a solution of 2 µg/ml IL-60 and 2 µg/ml of the expressers (IR-GUS, IR-GFP, IR-PRN or IR-P10) for 3 days at room temperature.

c. soaking seeds in the solutions listed in (b).

d. grafting cuttings from successfully treated plants and seedlings.

Replication of the constructs was monitored by PCR using the reporter genes' primers. Positive reactions with DNA templates in leaves above the point of injection were observed as early as 3 to 4 weeks after inoculation.

PRN {(3-chloro-4-)2'-nitro-3-chlorophenyl-pyrrole} was extracted as detailed in Mozes-Koch et al. (2012).

GUS activity was detected by staining [17]. The staining solution, composed of 1 mM EDTA (pH 8.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 100 mM sodium phosphate (pH 7.0), and 1% Triton X-100, was vacuum-infiltrated into the leaves. The plants were then stored at 37°C for 24 to 48 h.

GFP activity was detected by fluorescence [18]. For confocal imaging, leaves were manually excised under a binocular, sectioned longitudinally or transversely, and then immediately mounted on slides and immersed in one drop of Fluorogel Tris buffer. Observations were performed in a Zeiss 100M confocal microscope with excitation at 488 nm emitted by a krypton-argon laser equipped with a 505 nm-550 nm emission filter [19]. Chlorophyll autofluorescence was detected at wavelengths longer than 560 nm. Data were processed using the built-in LSM 51 software (Zeiss).

Results

Positive reactions with DNA templates further up the leaves from the point of injection were observed as early as 3 to 4 weeks after inoculation of TYLCV, which is a phloem-limited virus. Indeed, gene expression was initially observed in the vascular system of the plants (Figures 2 and 3), and then the product was found in the whole plant. Plants carrying IL-60+IR-GUS (β-glucuronidase) carried and expressed GUS for years. The same result was obtained with plants carrying IL-60+IR-GFP (green fluorescent protein), IL-60+IR-PRN (pyrrolnitrin) or IL-60+IR-P10 (the entire ORF5 of GVA the movement protein gene of Grapevine Virus A. The treated plants were not transgenic, since the viral DNA did not integrate with their DNA, and the inserted constructs replicated for years in the plants (Figures 4-10) (Tables 1 and 2).

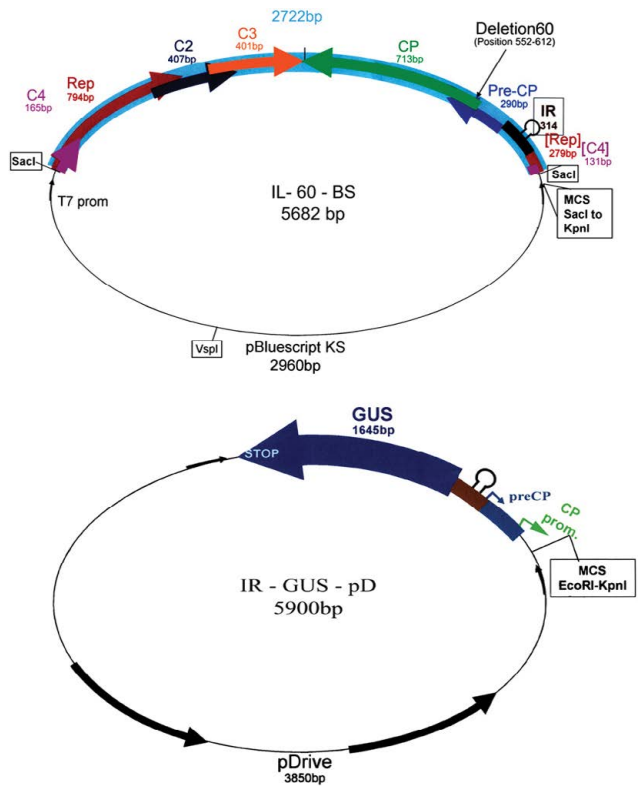

Figure 1: Top: IL-60-BS. The arrows represent viral genes and their orientation. The thin line represents the inserted plasmid pBluescript II KS1. The stem–loop structure represents the intergenic region (IR). The location of the 60-bp deletion is marked.

Bottom: IR-GUS as an example of a construct that is stimulated by TYLCV infection or IL-60-BS. The thick rectangles represent TYLCV sequences. The thick arrow represents a reporter gene. The bold arrows represent the inserted plasmid pDrive. Thin arrows represent pT7 and SP6 promoters.

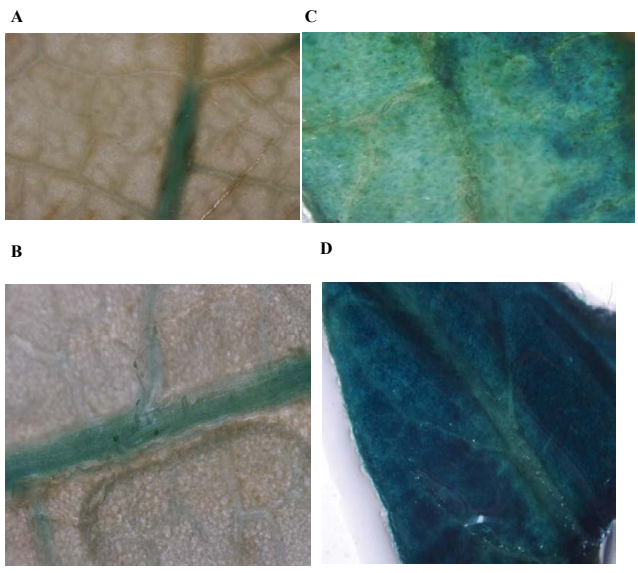

Figure 2: GUS-stained leaves of citrus (A,C) and grapevine (B,D) inoculated with IR-GUS 4 weeks (A,B) and 3 years (C,D) after treatment.

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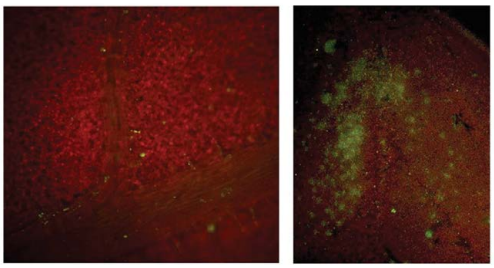

Figure 3: Citrus plants express GFP 3 weeks (a) and 6 months (b) after treatment with IR -GFP.

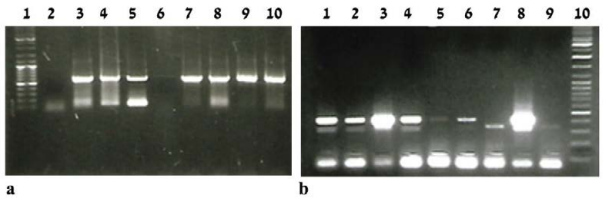

Figure 4: Grapevine plants were treated with IR-GUS and IL-60 plasmid. DNA was extracted from leaves of the same plant 1 month (a) and 3 years (b) after plasmid introduction. (a) Lane 1, size markers; lane 2, negative plant control; lanes 3 to 10, DNA extracted from treated plants. (b) Lanes 1 to 7, DNA extracted from treated plants; lane 8, positive control; lane 9, negative control; lane 10, size markers.

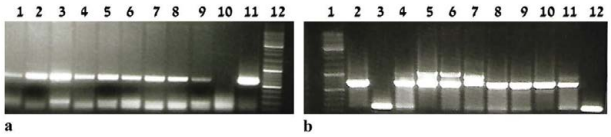

Figure 5: Grapevine plants were treated with IR-P10 of GVA and IL-60 plasmid. DNA was extracted from plants 2 years (a) and 4 years (b) after the treatment. (a) Lanes 1–9, DNA from the treated plants; lane 10, DNA from a negative control plant; lane 11, plasmid DNA (positive control); lane 12, size markers. (b) Lane 1, size markers; lane 2, positive control; lane 3, negative control; lanes 4–12, treated plants.

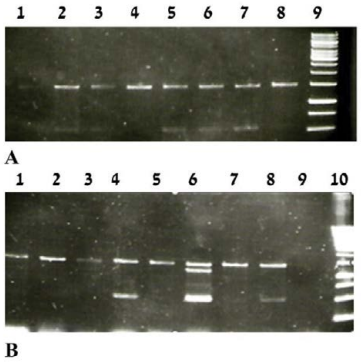

Figure 6: Grapevine plants treated with IR-PRN and IL-60 plasmid, (A) 1 year and (B) 3 years after the treatment. (A) Lane 1, negative control; lanes 2-9, DNA from treated plants; lane 10, size markers. (B) Lanes1-8, DNA from treated plants; lane 9, DNA from untreated plant; lane 10, size markers.

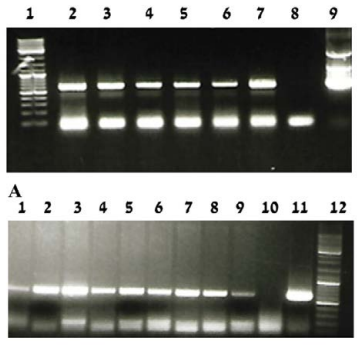

Figure 7: Citrus trees treated with IR-GUS and IL-60 plasmid. (A) Citrus trees 1 year after introduction of the plasmids. Lane 1, size markers; lanes 2-8, DNA from treated plants; lane 9, negative control; lane 10, positive control. (B) Grafted citrus trees 3 years after grafting. Lanes 1-9, DNA from grafted plants; lane 10, negative control; lane 11, positive control; lane 12, size markers.

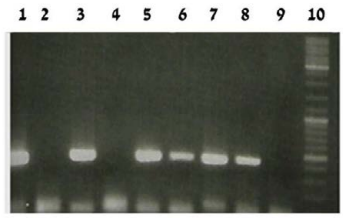

Figure 8: Citrus trees 2 years after introduction of IR-GFP and IL-60 plasmid. Lane 1, positive control; lanes 2-8, DNA from treated plants; lane 9, negative control; lane10, size markers.

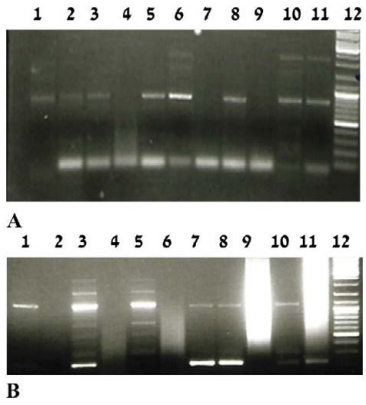

Figure 9: Citrus trees 1 year after introduction of IR-PRN and IL-60 plasmid (A) and 1 year after grafting (B). (A) Lanes 1-11, DNA from treated plants; lane 12, size markers. (B) Lanes 1-8 and 10, DNA from treated plants; lanes 9 and 11, overloaded plasmids; lane 12, size markers.

Discussion

In most woody fruit species, transformation and regeneration are difficult to perform, their efficiency is low, and they are limited to a few genotypes or to seed-derived tissues [20]. We used the plasmid IL-60, derived from TYLCV, as a platform for the constructs. The IL-60 system has been shown to provide a universal expression or silencing for all tested plants [1].

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Table 1: Details of primers used in the various PCR assays.

Primer Designation	Sequence 5' —+3'	Description
966 (reverse)	attggecipttttccataperc	Bases 928 to 908 of IL-60
977 (forward)	gaaggctpaacttcgacag	Bases 530 to 548 of IL-60
939 (forward)	agagacaccgaticatttcaac	Bases 1 to 21 of IL 60 BS
940 (reverse)	gcpgataacaatttcacacag	Bases 826 to 845 of BS
167 (reverse)	Cagcgtaapggtaatacpag	Bases 2,468 to 2,449 of GenBank acc. M14641
408 (forward)	gaacaacpaactpaactpecapac	Bases 1,867 to 1,890 of GenBank acc. M14641
345 (reverse)	tpiptppacagptaaipe	Bases 694 to 669 of GenBank acc. U87974
895 (forward)	geccpaattcaptaaappapaap	Bases 77 to 99 of GenBank acc. U87974
PDS (forward)	cagccectitgatttctce	Bases 934 to 953 of GenBank acc. M88683
PDS (reverse)	cacaccttgetttctcatcc	Bases 1,133 to 1,114 of GenBank acc. M88683
18S-rRNA (forward)	agpaattgacppaagpecac	Bases 1,142 to 1,446 of GenBank acc. AJ236016
18S rRNA (reverse)	gtgcencccagaacatctaag	Bases 1,466 to 1,446 of GenBank acc. AJ236016

Table 2: List of pyrrolnitrin (PRN) primers.

Primer Designation	Sequence 5'3'	Amplified Sequence
#100 forward	ATGGATCCATGAACAAGCCAATCAAGATATCGTCA	Part of *prn*A
#101 reverse	GGCCCCAGCATCGGAATCTT	838bp
#102 forward	GCGAACGAACACGATAGCAA	Parts of *prn*B and
#103 reverse	CGTCAATGAGGGCGTGAAT	*prn*C. 1461 bp
#104 forward	ATGAACAACTTCAATTG	The entire prnD
# 105 reverse	ATTCTAGACACGAGTTGCAACAGCCAGATG	1091 bp
# 106 forward	TATCATATGGAACCTTTCGAGCTGCGTC(with Nde1 site)	The coding region of GFP

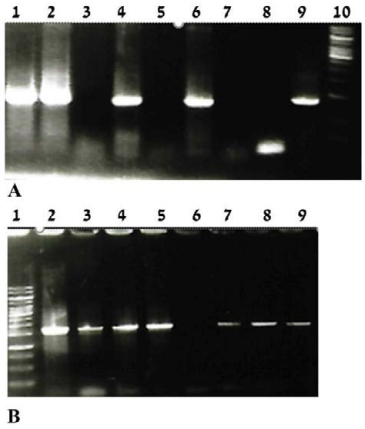

Figure 10: Citrus trees 6 years after treatment with IR-GUS (A) and IR-PRN (B). DNA was extracted from plants 6 years after treatment. (A) Lanes 1- 9, DNA from treated plants; lane 10, size markers. (B) Lane 1, size markers; lane 2, DNA from the positive sample; lanes 3-9, DNA from treated plants.

The DNA components inserted into IL-60 replicate in plant cells only as double-stranded DNA [1,14]. Here, the universal DNA plant vector system IL-60 mediated the introduction and expression of a number of genes (*PRN*, *GFP*, *GUS* and GVA *P10*) in orange trees and grapevine. We transformed whole seedlings, plants and trees, in contrast to most previous work where transformation was performed in the meristematic tissue [20,21]. In the last 30 years, many geminivirus-based vectors have been used for coding and non-coding-gene expression, gene silencing, vaccine production, microRNA expression, silencing suppression, CRISPR/Cas9 system construction, and VIGS for functional genomics analyses. Vaccines using geminivirus-based vectors are already available and in clinical trials, and some are even licensed for medical production [3,13]. The advantages of infection that does not involve genome insertion and high-copy multiplication make geminiviruses important vectors for genomic modifications. Transgenic plums expressing resistance to viral, nematode, bacterial, and fungal diseases have been obtained, as well as those showing resistance to abiotic stresses, and altered fruit ripening [21,22]. In this case, transformation was performed in meristematic tissues and the hypocotyl.

In recent years, site-directed nucleases (SDNs) such as CRISPR/Cas9 became an attractive technology for the production of mutated crops/cultivars [23,24]. Successful applications of the CRISPR/Cas9 system to modify gene expression in several species, including perennial plants, have been reviewed by [25,26].

We demonstrated the possibility of transforming trees' DNA with IR-X (X designating the gene of interest) using an easy technique. The viral DNA spreads, multiplies and is expressed for years after its introduction. We further developed a system that can propagate the modification from a single positive plant into a high number of seedlings and cuttings, to be rooted or grafted on untreated plants, respectively. These plants can be used as nuclear stock or as a mother plantation. The advantage of our system is that it allows generating, easily, rapidly and inexpensively, large quantities of transformed orange trees and grapevines.

In memoriam

The late Professor Ilan Sela was a well-known and respected scientist in the fields of Plant Virology and Molecular Biology. He was regarded as a pioneer plant virologist in Israel. He was an innovative, groundbreaking scientist carrying out basic science and implementing it to solve real world problems. He published more than 100 papers and numerous chapters in books. He was an enthusiastic, inspiring teacher for many generations of students. He supervised more than hundred Ph.D. and M.Sc. students. He continued working till his last days. Ilan is greatly missed.

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