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Silencing of ORFs C2 and C4 of Tomato Yellow Leaf Curl Virus Engenders Resistant or Tolerant Plants

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Abstract: The IL-60 system is a transient universal vector system for expression and silencing in plants [1]. This vector has been derived from Tomato yellow leaf curl virus (TYLCV). The viral intergenic region (IR) is a non-coding short (314 b) sequence separating the viral sense-oriented genes from the complementary-oriented genes. IR carries the viral origin of replication as well as a promoter at each end. Placing a gene segment between two IRs at opposite orientations followed by trans-activation of the construct by the plasmid IL-60-BS, caused silencing of the pertinent gene as indicated by the silencing of the endogenous gene *PDS*.. The viral genes *C2* and *C4* are implicated as having a role in viral-directed silencing suppression. The silencing *of C2* and *C4* intervened with the virus ability to counter-react to viral silencing by the host plant, thus engendering resistance or tolerance.

Keywords: TYLCV, silencing, silencing suppressors, resistance.

INTRODUCTION

The Tomato yellow leaf curl virus (TYLCV) genome is made up of a single-stranded (ss) DNA of approximately 3000 bases (depending on the strain; the Israeli strain which was disarmed and developed into IL-60 is 2787 bases long). The ssDNA is encapsidated, and the virus is transmissible by whiteflies (*Bemisia tabaci*). The coat protein (CP) directs the viral DNA to the nucleus, where the ssDNA is converted to double-stranded (ds) DNA and viral RNAs are transcribed. The genome carries six overlapping open reading frames (ORFs). Two ORFs, V1 (CP) and V2, are transcribed to RNA in sense orientation, and four ORFs, C1 to C4, are transcribed to RNA in the complementary orientation. Rolling circle replication is initiated by the viral replicase-associated protein (REP, the product of ORF C1), producing progeny ssDNAs. The sense-oriented and complementary-oriented ORFs are separated by a short (314-bases long) intergenic region (IR). The IR carries the viral origin of replication and REP-binding motifs. Strong promoters are situated at both ends of the IR, directing transcription of the sense- and complementary-oriented viral RNAs. TYLCV DNA assembles into minichromosomes, but is accessable to plant factors at the origin of replication and the promoter regions [2]. Structure-function relationships in geminiviruses have been described [3-6].

The IL-60 system [1] comprises a series of plasmids derived from a modified disarmed form of TYLCV DNA. Briefly, a deletion of 60 bp in the coat protein ORF of a clone of the Israeli isolate of TYLCV rendered the construct non-symptomatic. In addition, The C1 ORF was interrupted by insertion of a bacterial plasmid (usually BlueScript, ca. 3 Kbp in length). Since no functional C1 can be expressed, the only mode of DNA replication is dsDNA to dsDNA in a REP-independent manner, presumably recombination-depended replication [7]. Various combinations of this system have allowed for replication, movement and expression (when applicable) of foreign sequences in every plant tested to date. The vectors and the genes they carry do not integrate into the plant's genome and are not heritable. Therefore, although active throughout the plant's life span, expression or silencing derived from IL-60 components are not transgenic. When a construct carrying the same sequence in opposite orientations was transcribed, RNA silencing of the pertinent sequence was obtained due to presumed backfolding of the transcript. Thus the IL-60 system provides a universal expression/silencing system in plants. Any gene fused to the IR (IR-X) is stabilized in plants. Challenge infection of these plants with a native TYLCV initiates mobilization, replication and expression of IR-X. Hence, IR-X is a TYLCV-dependent satellite. The disarmed form of TYLCV dsDNA (IL-60-BS) can replace TYLCV as the driving force for movement, replication and expression of IR-X without causing disease [1].

Even though TYLCV is a DNA virus, the host plant reacts to infection by promoting a virus-specific RNA-silencing mechanism, probably induced by a segment of overlapping sense- and complementary-oriented RNAs forming a segment of dsRNA [7-9]. The virus counter-reacts by producing silencing-suppressor proteins. The product of ORF C2 (and its analogs in other geminiviruses) is a transcription activator [10, 11] and has also been implicated in silencing suppression [12-16]. Silencing suppression by C2 appears to be a nuclear process [15]. Since C2 is a transcription activator, it is not surprising that it contributes to silencing suppression indirectly, by upregulating gene expression of the host, leading, among other things, to

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the intervention of native plant proteins (such as WEL1) in silencing pathways [10]. Adenosine kinase has been implicated in the methylation cycle of silencing [17, 18], and inhibition of its expression by C2 and its analogs in other geminiviruses brings about silencing suppression, although this might be a case of transcriptional gene silencing rather than a post-transcriptional event [18]. C4 and its analogs in other geminiviruses have also been pinpointed as silencing suppressors. C4 inhibits accumulation of siRNA, and therefore interferes with the guiding of RISC to its target, resulting in elevated RNA accumulation [19, 20]. Transgenic plants expressing C4 exhibit virus-like symptoms interpreted as possible intervention in normal developmental processes, in which siRNAs have been demonstrated to participate in several organisms [19-25]. More recently, after the presently reported work was already completed, the product of ORF V2 was also convincingly demonstrated to be a silencing suppressor [26, 27]. Here we report that placing a gene segment between two opposing IRs brings about silencing of the inserted gene, and that silencing of C2 and C4 leads to the production of TYLCV-resistant and/or tolerant plants.

MATERIALS AND METHODS

Clones and Constructs

A PCR-amplified segment of TYLCV containing the IR and 159 bp of ORF V2 (bases 61-473, GenBank accession no. X15656) was cloned into the T/A cloning vector pDRIVE. This construct carries the ribosomal binding site of *V2*. The above IR segment was amplified with primers carrying restriction sites at their 5' ends. A forward IR was cloned into pDRIVE with *Kpn*I and *Pst*I, and the IR in opposite orientation was cloned into the same plasmid with *Sal*I and *Hind*III. The opposing IRs flanked the T/A cloning site of pDRIVE, enabling the insertion of any PCR product between the two opposing IR promoters. A segment of tomato *PDS* (bases 943 to 1135, GenBank accession no. M88683) and the entire ORFs of TYLCV *C2* and TYLCV *C4* (bases 1398 to 1809 and 2343 to 2647, respectively, GenBank accession no. X15656) were inserted between the opposing IRs. The resultant constructs (IR-PDS-RI, IR-C2-RI and IR-C4-RI, respectively) are illustrated in Fig. (**1**). IRs in all constructs (including constructs carrying opposing IRs) were positioned so that the transcripts would be regulated by the promoter controlling *C1* to *C4* transcription. The only viral component in the aforementioned constructs was the IR, and they were therefore considered viral satellites. Constructs were injected into plants along with IL-60-BS [4] which promotes replication, movement and transcription of the satellites.

Propagation of Satellites and their Administration into Plants

Escherichia coli cells were transformed with the pertinent satellite construct, propagated under ampicillin selection, and the construct was extracted by standard procedures [28]. A stem or leaf petiole of a recipient plant was punctured with a hypodermic needle. A capillary tube was inserted into the resultant hole, and approximately 2 µg of DNA (in 100 µl H_2O) was pipetted into the capillary tube until fully absorbed by the plant. Only about 50% of the plants injected with the various satellite forms demonstrated successful administration, as indicated by PCR. Therefore, each experiment was carried out with at least 20 plants, and only plants found by PCR to carry the inserted construct were further analyzed. Each reported experiment was repeated at least twice (more in most cases).

Molecular Analyses

Southern, PCR and semi-quantitative PCR analyses were carried out according to standard procedures [28]. Unless otherwise stated, 40 cycles were run for each PCR assay. Probes for Southern analyses were labeled by the PCR-DIG procedure (Roche Molecular Biochemicals). Probes were prepared according to the manufacturers' protocols.

Semi-quantitative PCR was carried out by removing aliquots from an ongoing PCR of a target gene (or cDNA) at different cycles and determining the threshold of band appearance. A similar assay, with the same templates, was carried out with primers for a constitutive gene, and the threshold of its band appearance was determined and served as a standardizing parameter for possible quantitative deviations.

Unlike the case of real-time PCR, the exact threshold in a semi-quantitative analysis is only an estimate. Nevertheless we used the following calculation (developed for real-time PCR) to compare quantities between samples. Each treatment threshold was given an arbitrary quantitative value according to the formula $\Delta ct = 2^{-(ct\ target\ gene\ -\ ct\ constitutive\ gene)}$, *ct* being the cycle threshold. The relative quantitative increase/decrease in templates between control and treated plants was estimated from the ratio of their respective Δ*ct*s.

RESULTS

The Artificial Satellites

Construction of the IR-carrying artificial satellites is described in detail in Materials and Methods. In general, the TYLCV IR was cloned into a bacterial plasmid. In satellites designed for silencing, a segment of the gene to be silenced was inserted between two opposing IRs (IR-X-RI), generating transcripts in both orientations and providing dsRNA which, in turn, brought about silencing. The stronger IR promoter, transcribing the viral antisense genes, was oriented towards the inserts. In these constructs, we placed a segment of the endogenous phytoene desaturase gene (*PDS*, as a positive control for silencing) or the complete *C2* and *C4* ORFs of TYLCV. The bacterial plasmid component for all of the IR-carrying constructs was pDRIVE (Qiagen), but other plasmids, such as pBluescript (Stratagene), have also been used successfully. Replication, expression and movement of the satellites were promoted by IL-60-BS [4]. The various types of IR-carrying satellites are illustrated in Fig. (**1**). Note that the only TYLCV-derived sequence in these satellites is the IR.

Satellite-Mediated Silencing of an Endogenous Gene

The first step was to determine whether a sequence placed between two opposing IRs would bring about silencing of the pertinent gene following TYLCV infection. The gene for PDS was selected for this assay. PDS is a key enzyme in the plant's carotenoid-biosynthesis pathway, leading to the production of zeaxanthin, which protects chlorophyll from excessive light bleaching [29-31]. *PDS*-

silencing results in chlorophyll bleaching under strong light and provides a distinctive, easily recognizable phenotype.

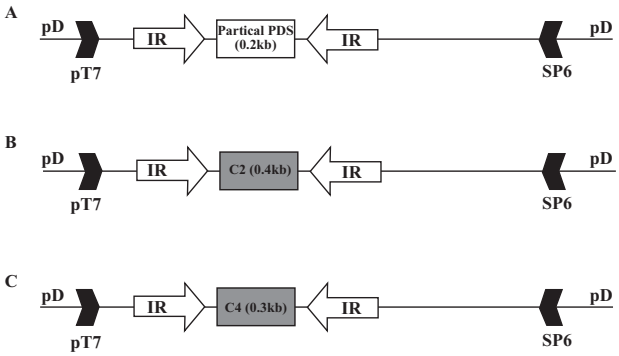

Fig. (1). Illustrations (not to scale) of the various IR-X-RI satellites. **A**. IR-PDS-RI. **B**. IR-C2-RI. **C**. IR-C4 -RI. pD denotes pDRIVE plasmid sequences. pT7 and SP6 denote the positions of the respective promoters.

A segment of the tomato *PDS* gene was inserted into a satellite between two opposing IRs as described in Materials and Methods and illustrated in Fig. (**1A**). We expected that transcription would result in *PDS*-related RNA in both orientations and that the resultant dsRNA would engender RNA silencing of *PDS*. The construct (IR-PDS-RI) was injected into tomato plants and the plants were whitefly-inoculated with TYLCV 7 days post-injection. Bleaching along the veins appeared about a month after TYLCV inoculation and then progressed rapidly to all parts of the plant (Fig. **2A**). Bleaching symptoms are distinguishable from TYLCV-induced symptoms, and to the naked eye, *PDS*-silenced plants either did not exhibit TYLCV-derived symptoms or developed only very mild ones. Silencing of PDS was corroborated by the appearance in treated plants of siRNAs corresponding to the PDS sequence (Fig. **2B**).

Engendering of TYLCV Resistance/Tolerance/Immunity by Satellite-Mediated Silencing of Putative Viral-Silencing Suppressor

The IL-60 system has also been developed as an inductive system comprising of two elements: The expressing/silencing element (the target element) carries a sequence to be expressed/silenced under the control of IR. The driver element provides all necessary factors for replication, expression and movement. TYLCV itself is a driver element, and in order to avoide disease IL-60-BS has been developed as a driver [4]. We have demonstrated the ability of the IL-60 system to silence the aforementioned endogenous gene (*PDS*) by the opposing promoters approach driven by IL-60-BS, and a stem-loop folding structure for the silencing of a viral gene (p10 of GV; 4) we attempted to find out whether TYLCV itself can activate a IR-X-RI construct, where X is a viral gene, and thus promote silencing of its own transcripts.

Post-transcriptional gene silencing has been reported to play a role in the plant's reaction to infection. In the case of TYLCV, the products of ORFs C2 and C4 have been reported to be involved in the suppression of this viral silencing (summarized in 6). We therefore attempted to engender non-transgenic resistance by silencing *C2* or *C4*, thereby arresting the virus's ability to exercise counter-silencing measures.

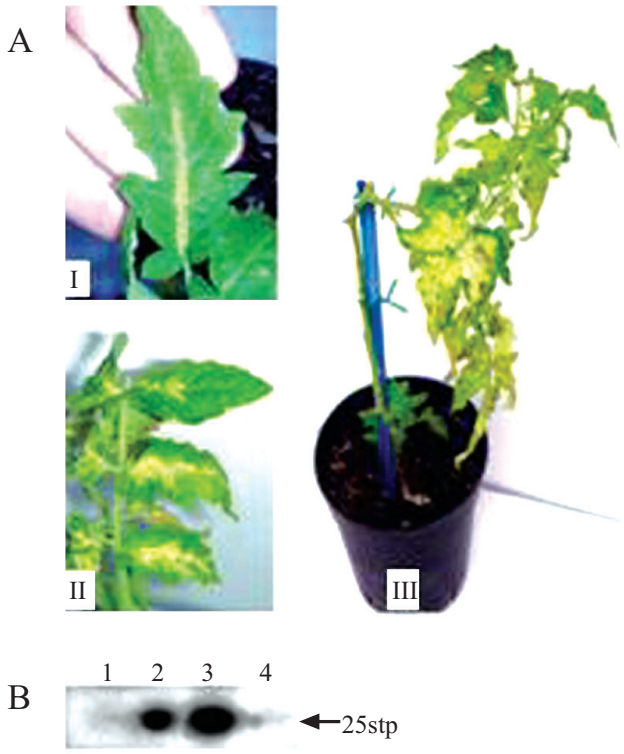

Fig. (2). Silencing of *PDS* following injection with the artificial satellite IR-PDS-RI. **A**. Photographs of chlorophyll bleaching following *PDS* silencing. Initially, major veins were bleached (AI), followed by its spread into neighboring tissue (AII) and finally, to all parts of the plant (AIII). **B**. Appearance of siRNAs of PDS sequences following treatment with IR-PDS-RI, Lane 1: siRNA analysis of an untreated plant. Lane 2: siRNA analysis of a plant treated with IR-PDS-RI. Lane 3: A 25-base-long size marker. Lane 4: Negative control (sample buffer only).

The *C4* ORF was placed between two opposing IR promoters (IR-C4-RI), as described in Materials and Methods and illustrated in Fig. (**1C**). The construct was injected into tomato plants. As already stated, this construct cannot replicate in the plant, but by virtue of its inherent IR, is stable and can be induced by the invading virus to replicate, move and be expressed. Transcription from IR-C4-RI was expected to provide RNAs in both orientations, with the resultant dsRNA initiating silencing of *C4*-carrying transcripts. In the absence (or presence of reduced levels) of C4, the virus can no longer effectively suppress its own silencing by the host plant, and resistance (or tolerance) may thus be engendered. In contrast to other cases (such as the P10 in 4) the resistance machinery was not pre-introduced, and the virus does not find a protection mechanism "waiting" for it. Here, the virus itself activates resistance. In effect, the system directs the invading virus to commit suicide.

Tomato seedlings were injected with IR-C4-RI. Seven days later, these plants, as well as their control, non-injected counterparts, were whitefly-inoculated with TYLCV (30 viruliferous insects per plant). Sixty days post-inoculation,

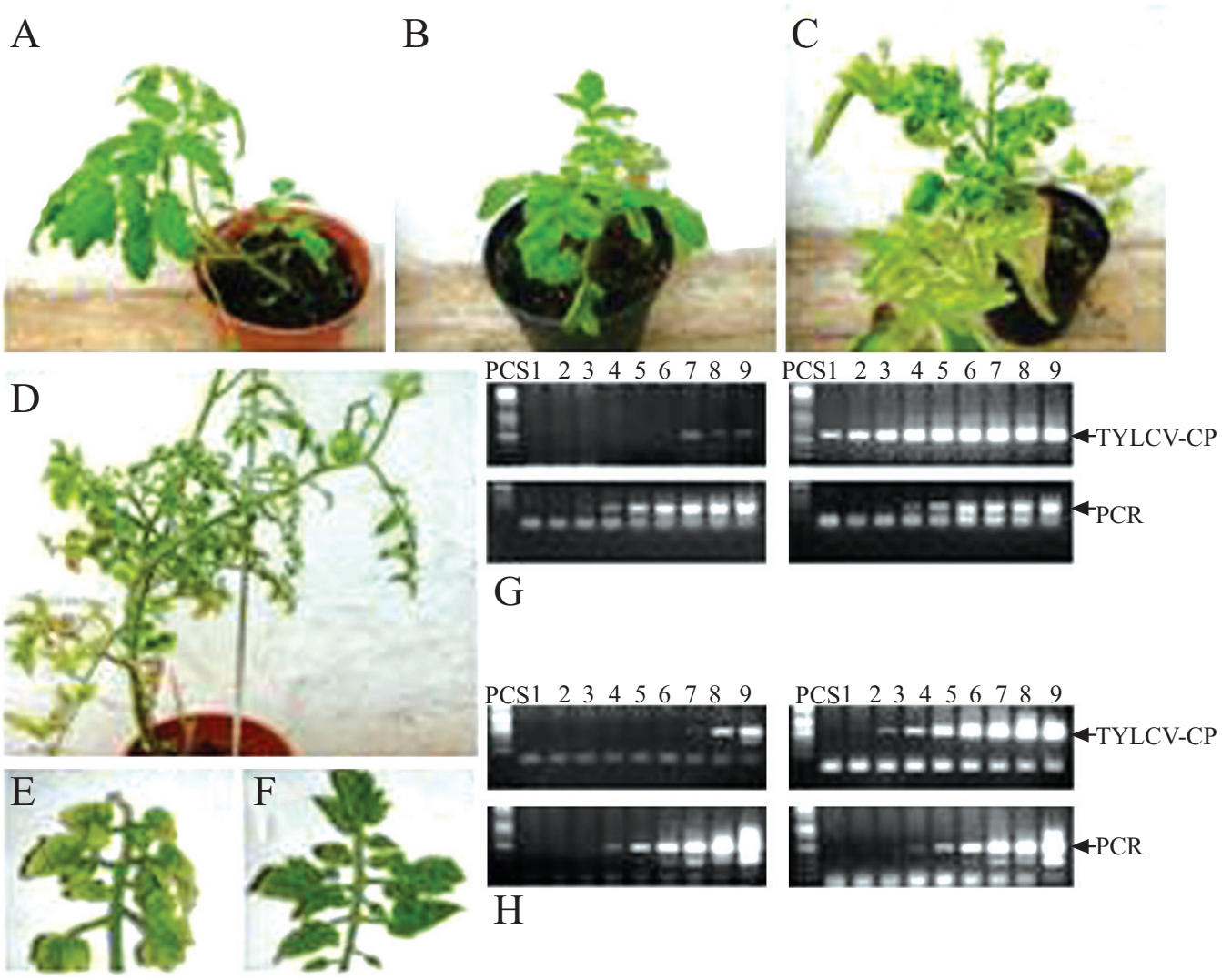

Fig. (3). Examples of TYLCV resistance/tolerance obtained by C4 silencing. Frames **A**, **B** and **C** exemplify the engendering of resistance/tolerance. Plants shown in frames **A** and **B** were injected with pIR-C4-RI 7 days prior to inoculation. The plant in frame C is a TYLCV-infected untreated control. Pictures were taken 30 days post-inoculation. Plant **A** is a one of the symptomless resistant plants, and plant B has only mild symptoms. Frames **D**, **E**, and **F** show an example of recovery. The plant shown in frame **D** was injected with IR-C4-RI 3 months after inoculation. The new growth of the heavily infected plant was symptomless; the plant overcame stunting, and produced flowers and normal-looking fruit. Frame E exhibits symptom-laden leaves of the lower part of the plant. Frame F shows recovered leaves of the upper part of the plant. Frames **G** and **H** demonstrate the reduction in virus titer in resistant and recovered plants by semi-quantitative PCR with TYLCV-CP primers. Frame **G** shows PCR products with DNA from Plant **A** (upper left panel) and plant **C** (upper right panel) following 18 to 34 PCR cycles (lanes 1-9). The lower panels show the results obtained with the same DNA similarly amplified with primers for the constitutive gene *PDS*. Frame H shows the results of semi-quantitative PCR obtained from DNA extracted from the recovered upper leaves of plant **D** (Upper left panel) versus that of the recovered upper leaves (upper right panel). Internal controls with the constitutive gene *PDS* is shown in the respective lower panels.

all of the control plants exhibited severe symptoms, while none of the IR-C4-RI-treated plants showed any. By 90 days post-infection, about 60% of the IR-C4-RI-treated plants had developed very mild symptoms. However, the deleterious effects of the disease were not observed: these plants (symptomless or showing mild symptoms) looked normal in size and set flowers and fruits. The experiment was repeated twice with 30 treated plants in each experiment. An estimation of the virus titer by semi-quantitative PCR indicated an over 10 million-fold reduction in viral DNA in the C4-silenced, non-symptomatic plants. Development of resistance was detected, by semi-quantitative PCR and Southern-blot analysis, as early as 1 week post-inoculation (data not shown). Furthermore, recovery from infection was obtained following injection of IR-C4-RI into plants which were already heavily infected (inoculated in the laboratory or collected in the field). The new growth was asymptomatic, and the plants recovered from stunting and developed

flowers and fruits. In this case, semi-quantitative PCR indicated an over 100,000-fold reduction in TYLCV titer in leaves above the site of injection relative to leaves below this site in the same treated plant, the latter retaining disease symptoms. The results of the engendered resistance/tolerance and recovery are shown in Figs. (**3**, **4**).

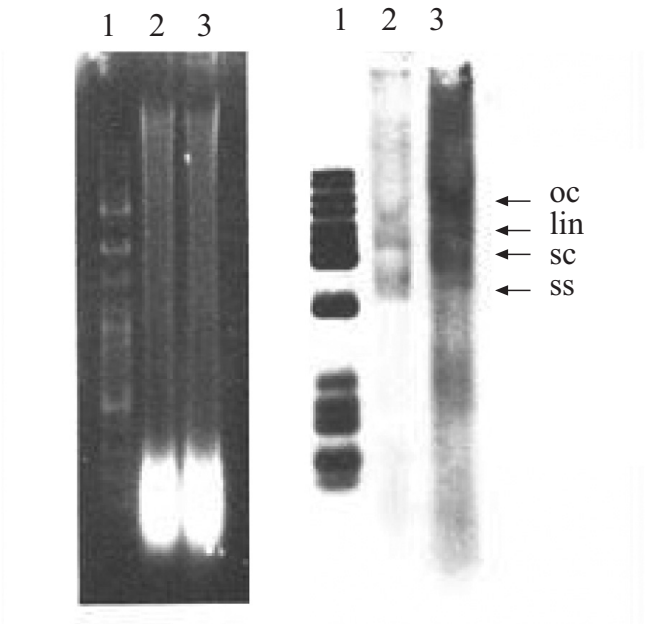

Fig. (4). A Southern blot for TYLCV. DNA was extracted from the upper leaves of the plant shown in Fig. (**3D**, lane 2) and from the symptom-laden lower leaves of the same plant (lane 3). Size markers are shown in lane 1. Left panel, EtBr-stained gel before transferring to the membrane (loading control).

The viral *C2* gene was also placed between two opposing IRs as described in Materials and Methods (Fig. **1B**). IR-C2-RI was injected into plants which were later whitefly-inoculated with TYLCV as described for *C4*. The experiment consisted of 40 treated plants and was repeated twice. Symptoms appeared following inoculation but all new growth was symptomless. The plants grew to normal size and (except for some symptoms on their lower part) were indistinguishable from healthy plants (Fig. **5A**). Semi-quantitative RT-PCR could not detect any TYLCV at all (Fig. **5B**). Total suppression of infection was also indicated by comparison of TYLCV titers in symptomatic versus asymptomatic leaves of the very same plant (Fig. **5C**). C2-siRNA was detected in IR-C2-RI-tretaed plants before and after TYLCV inoculation (data not shown). It appears that the new growth had become immune to TYLCV. Silencing-suppressor-derived resistance/tolerance/immunity is discussed further on.

DISCUSSION

This paper describes a rapidly responding platform for silencing in plants. The IR-carrying segment of TYLCV fused to a foreign gene and introduced into plants is stable, possibly due to its inherent IR sequence. A helper geminivirus may be introduced at a later time, contributing trans-activating factors and engendering the spread of, and expression from the IR-carrying segment [4]. By expressing constructs leading to the formation of dsRNA, the system can also engender silencing of a target gene.

We demonstrated inducible silencing of an endogenous gene (*PDS*). We proceeded to construct a system in which virus infection stimulates the silencing of one of its own genes, bringing about resistance/tolerance. The treated plants became resistant/tolerant within a few days of injection, as compared to conventional breeding which, after many years of development, has been only partially successful. After the completion of this work, a new candidate gene for TYLCV silencing suppression (*V2*) was reported [26, 27]. The IR constructs employed in this study carried 159 bp segment of *V2* harboring the gene's ribosomal binding site. The *V2* segment was also bidirectionally transcribed which conceivably could have resulted in *V2* silencing as well. The presence of three genes for silencing suppression in a virus carrying only six genes is quite peculiar and may suggest that TYLCV is particularly prone to silencing. Therefore, the interplay between the plant's silencing of TYLCV infection and the viral counter-activity in suppression of its own silencing is a major factor in infectivity. The mere fact that TYLCV infection may be counteracted by disarming the virus from its silencing-suppression capability indicates that an innate host immune response is a considerable factor in the plant's survival following infection. The IL-60 platform offers some advantages in plant manipulation. It is non-species-specific, it can be inducible, and can be used for both expression as well as silencing. It is stable compared to the PVX system and, in contrast to the TRV system, is applicable to many plant species (including monocots and woody plant) A disadvantage is the need for injection (which is not always successful) in order to introduce the plasmids into the plant.

Silencing of viral genes coupled with the development of resistance or tolerance has been demonstrated. Since X silencing engendered by IR-X-RI develops into a phenotype, it is apparent that the satellite continues to transcribe and is not turned off due to self silencing. This can be explained by the unique features of the IRs at both ends of the insert. The IR carries strong signals for RNA-polymerase II termination [14]: A C:G rich stem-loop structure starting at position 147, a polyadenylation signal (AATCAAA) at position 234-240 followed by a downstream AGTGTTCA. Therefore, it is conceivable that transcription stops at both ends of X, and the formed dsRNA, leading to silencing, represent only sequences of X (which is silenced) and not of the entire plasmid (which is not affected).

In addition, involvement of transcription gene silencing (TGS) can not be ruled out. Once siRNAs are formed they may affect chromatin remodeling [32], TYLCV-dsDNAs appears as histone-coated minichromosomes [33, 34] with exposed active sites. The IR may be the origin of assembly of minichromosomes. Such remodeling may turn promoter regions inaccessible, shutting down transcription from those sites. siRNA may also cause TGS by engendering DNA methylation.

In conclusion, a plasmid of the type IR-X-RI brings about X silencing by engendering the production of dsRNA which is processed to siRNA and presumably resulting in post-transcriptional gene silencing. However, TGS is not ruled out. The *in-trans* down regulation of TYLCV may be

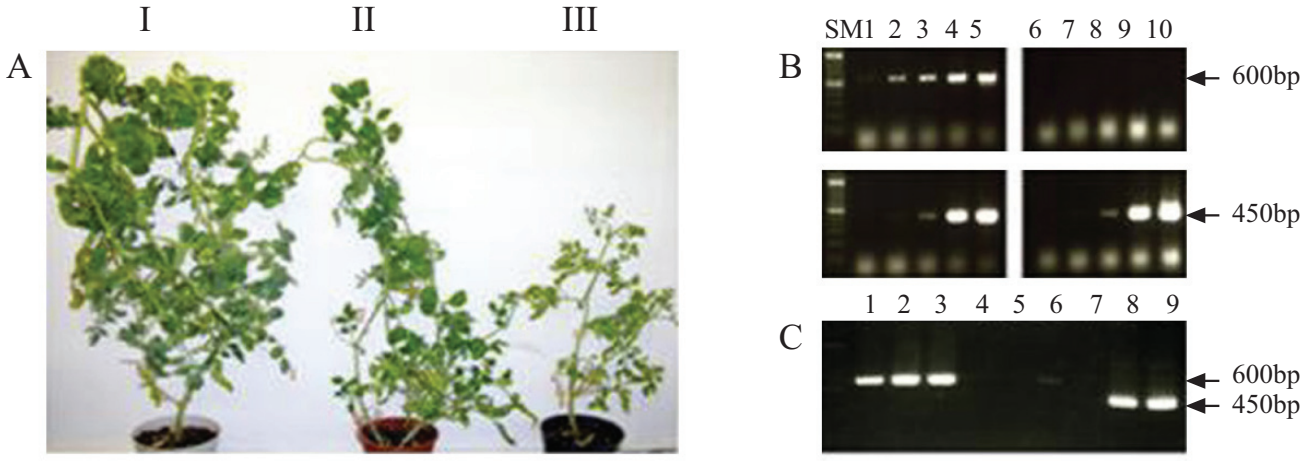

Fig. (5). Examples of TYLCV immunity following C2 silencing. Frame **A** shows TYLCV-inoculated plants. Plant I: Treated with RI-C2-RI, 3 months post-TYLCV-inoculation. Plant II: Treated with RI-C2-RI, 2 months post-inoculation. Plant III: Untreated plant, 3 months post-inoculation. Frame **B**: Semi-quantitative PCR. Samples were drawn every three cycles from cycle 18 to 30. Upper frames: PCR with primers for TYLCV CP (product size: 600 bp). Lower frames: PCR with primers for 18S rDNA (product size: 450 bp). SM: Size markers. Lanes 1-5: Samples from a TYLCV-infected plant (plant III in **A**). Lanes 6-10: Samples from an IR-C2-RI-injected, TYLCV-infected plant (plant I in **A**). No TYLCV was detected in the IR-C2-RI-treated plant. Frame **C**: Comparison of TYLCV titer in symptom-carrying and asymptomatic leaves of the same plant (plant II in **A**). Lanes 1-3: PCR (35 cycles) of symptom-showing leaves. Lanes 4-6: As in lanes 1-3 but from asymptomatic leaves. Lane 7: Negative control, PCR was carried out without template. Lane 8: Internal control; rDNA level from a symptomatic leaf. Lane 9: Internal control; rDNA level from an asymptomatic leaf.

due to siRNA- depended silencing as well as to competition between an efficient satellite and the virus.

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