Arch Virol (2007) 152: 1323–1339
DOI 10.1007/s00705-007-0942-0
Printed in The Netherlands

Archives of Virology

Making a friend from a foe: expressing a GroEL gene from the whitefly *Bemisia tabaci* in the phloem of tomato plants confers resistance to tomato yellow leaf curl virus

F. Akad[1], **A. Eybishtz**[1], **D. Edelbaum**[1], **R. Gorovits**[1], **O. Dar-Issa**[2], **N. Iraki**[2], and **H. Czosnek**[1]

[1] The Otto Warburg Minerva Center for Agricultural Biotechnology & The Robert H. Smith Institute for Plant Science and Genetics in Agriculture, The Hebrew University of Jerusalem, Rehovot, Israel
[2] UNESCO Biotechnology Center, Bethlehem University, Bethlehem, West Bank, Palestinian Authority

Received October 24, 2006; accepted January 22, 2007; published online March 5, 2007

Summary

Some (perhaps all) plant viruses transmitted in a circulative manner by their insect vectors avoid destruction in the haemolymph by interacting with GroEL homologues, ensuring transmission. We have previously shown that the phloem-limited begomovirus tomato yellow leaf curl virus (TYLCV) interacts *in vivo* and *in vitro* with GroEL produced by the whitefly vector *Bemisia tabaci*. In this study, we have exploited this phenomenon to generate transgenic tomato plants expressing the whitefly GroEL in their phloem. We postulated that following inoculation, TYLCV particles will be trapped by GroEL in the plant phloem, thereby inhibiting virus replication and movement, thereby rendering the plants resistant. A whitefly GroEL gene was cloned in an *Agrobacterium* vector under the control of an *Arabidopsis* phloem-specific promoter, which was used to transform two tomato genotypes. During three consecutive generations, plants expressing GroEL exhibited mild or no disease symptoms upon whitefly-mediated inoculation of TYLCV. *In vitro* assays indicated that the sap of resistant plants contained GroEL-TYLCV complexes. Infected resistant plants served as virus source for whitefly-mediated transmission as effectively as infected non-transgenic tomato. Non-transgenic susceptible tomato plants grafted on resistant GroEL-transgenic scions remained susceptible, although GroEL translocated into the grafted plant and GroEL-TYLCV complexes were detected in the grafted tissues.

Introduction

GroELs are well-characterized proteins belonging to the chaperonin family. They are involved in many important biological processes such as protein post-translational folding and subunit assembly. In addition, chaperonins play a role in the infection cycle of some plant viruses [19]. Once ingested by their insect vector, these viruses are not immediately available for infection. They need to translocate from the digestive tract to the salivary glands, from

Author's address: Dr. Henryk Czosnek, The Otto Warburg Minerva Center for Agricultural Biotechnology & The Robert H. Smith Institute for Plant Science and Genetics in Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel. e-mail: czosnek@agri.huji.ac.il

which they are excreted with the saliva during feeding [7]. To avoid destruction in the haemolymph of their vector, these circulative viruses interact with GroEL homologues produced by insect endosymbiotic bacteria. First shown for the potato leafroll virus (PLRV), a circulative virus transmitted by the aphid *Myzus persicae*, the GroEL homologue produced by the insect primary endosymbiotic bacteria exhibited affinity for the virus. Interference in this interaction resulted in reduced capsid integrity and loss of infectivity [33]. We have shown that tomato yellow leaf curl virus (TYLCV), a geminivirus transmitted by the whitefly *Bemisia tabaci*, interacts *in vivo* with a GroEL homologue produced by insect endosymbiotic bacteria. Disturbing this interaction *in vivo* resulted in a dramatic reduction in TYLCV transmission [21]. We have also shown that the coat protein (CP) of TYLCV binds to *B. tabaci* GroEL in the yeast two-hybrid system [22]. We have exploited these phenomena to devise tools allowing trapping *in vitro* of plant viruses by either GroEL purified from the whitefly *Bemisia tabaci* or by whitefly GroEL over-expressed in *E. coli* [2]. PCR tubes or 96-well plates coated with a GroEL preparation were incubated with cleared sap of virus-infected plant leaves or insect vectors. GroEL-bound viruses were then identified by PCR (DNA viruses) or RT-PCR (RNA viruses) using virus-specific primers or by ELISA with virus specific antibodies. In this way, tomato yellow leaf curl virus (TYLCV) – a whitefly-transmitted geminivirus – was detected in plant sap, in extracts of leaf squashes and in homogenates of individual viruliferous whiteflies. Anti-GroEL antibody prevented TYLCV binding to GroEL. The viruses able to bind GroEL chaperonins, in general, have a CP with a basic isoelectric point, have a marked positive charge, are rich in arginine residues and have a globular (or geminate) shape. Every virus we have tested followed these predictions [2]. For example, in addition to several geminiviruses, GroEL was able to bind to a variety of RNA viruses such as cucumber mosaic virus (CMV), prune dwarf virus (PDV) and tomato spotted wilt virus (TSWV), but not to potato virus X and potato virus Y (PVX and PVY), grapevine leafroll virus (GLRV) and tobacco mosaic virus (TMV).

Since binding to insect vector endosymbiotic GroEL homologues could be a mechanism shared by plant circulative viruses to avoid destruction in the haemolymph, we wondered whether this phenomenon could be exploited to generate transgenic tomato plants expressing the whitefly GroEL in their phloem. We expect that once inoculated by their vector, phloem-limited circulative viruses will be trapped in the plant phloem. As a result proper invasion of phloem-associated cells and long-distance movement may be significantly inhibited, rendering the plants resistant to the virus. This approach was attempted by infecting GroEL-expressing transgenic tomato plants with TYLCV.

Material and methods

Sources of virus, insects, plants and antibodies

Bemisia tabaci of the B biotype [10] was reared on cotton plants (*Gossypium hirusutum* cv. Akala) grown in insect-proof wooden cages at 24–27 °C, as previously described [38]. An isolate of TYLCV from Israel [27] was maintained in tomato plants (*Solanum esculentum* cv. Daniella FA144) by whitefly-mediated transmission. The tomato line MP1 [5] and an interspecific hybrid of *S. pennellii* × *S. esculentum* [15], named in short F1, were used for transformation. Grafts were done in a specialized commercial nursery (Hishtil, Ashkelon, Israel). Grafting was performed just below the cotyledons; hence scions did not include leaves. Antibodies to native *Buchnera* GroEL from *Myzus persicae* raised in rabbits were a gift of Dr. JFJM van den Heuvel [33].

Cloning of the GroEL gene from B. tabaci under the promoter of the phloem-specific sucrose transporter gene SUC2 from Arabidopsis thaliana

The gene encoding the GroEL homologue from secondary endosymbiotic bacteria of *B. tabaci* (accession number AF130421) was cloned under the control of the promoter of the *Arabidopsis thaliana SUC2* sucrose-H+ transporter gene, *At*SUC2 (accession number X79702), a phloem-specific gene [32]. The insect full-length GroEL gene [22] was isolated by PCR using the forward primer: 5′-TTTCATGACAGCTAAAGACTTAAAATTTGG-3′, which contains the AUG translation initiation codon as part of an added *Rca*I site (changes to obtain the *Rca*I site are underlined), and the reverse complement primer: 5′-TGAGCTC TTACATCATACCATTCATTCCGCC-3′, which contains an added *Sac*I site (the added *Sac*I is underlined). The PCR cycling consisted of a 4 min initial denaturation at 94 °C, annealing of 2 min at 54 °C, and elongation of 3 min at 72 °C, followed by 25 cycles of 94 °C for 30 s, 54 °C for

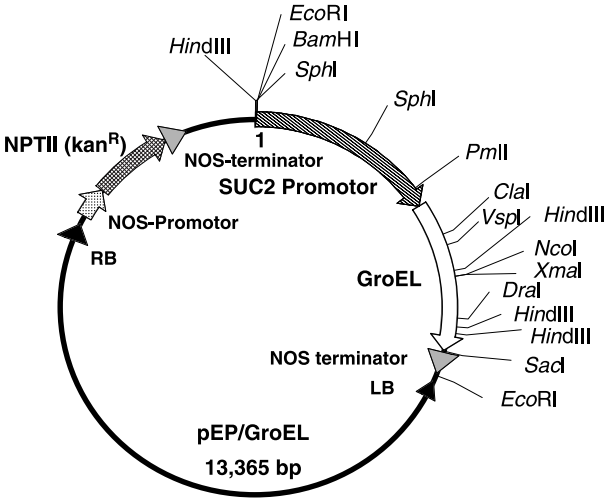


Fig. 1. *Agrobacterium tumefaciens* binary vector pEP/GroEL used to transform tomato plants with the GroEL gene from the whitefly *Bemisia tabaci*

1 min, 72 °C for 2 min, and a final step at 72 °C for 15 min. The amplified 1665-bp GroEL gene was initially cloned into pGEM-T Easy (Promega, Madison, USA) following *Rca*I/*Sac*I cleavage. The *Rca*I/*Sac*I fragment was further sub-cloned into the corresponding restriction sites of plasmid pUC19-AtSUC-mpCMV-GFP [31], which contains the movement protein gene of cucumber mosaic virus fused to GFP (mpCMV-GFP) under the control of the *Arabidopsis* phloem-specific promoter of the *SUC2* gene (a gift of Dr. S. Wolf). As a result, GroEL which replaced the mpCMV-GFP, was under the control of the *SUC2* promoter. The cassette containing the GroEL gene and the *SUC2* promoter was cleaved by *Pst*I/*Sac*I and inserted into the *Sda*I/*Sac*I sites (*Pst*I and *Sda*I have compatible ends) of the *Agrobacterium tumefaciens* binary vector pBI121 (accession number AF485783), replacing the 35S CaMV promoter and the GUS gene (Fig. 1). The binary plasmid (named pEP/GroEL) was mobilized into *Agrobacterium* LBA4404 by electroporation.

Transformation and regeneration of tomato plants

Surface-sterilized tomato seeds (MP1 and F1) were germinated on germination Nitsch medium [25]. Cotyledon and hypocotyls were excised from 10-day-old seedlings, discarding both extremities (leaving approximately 2/3 of the tissues), and cultured for 48 h on MS agar medium [23] in 9-cm-diameter Petri dishes. *Agrobacterium* containing the binary vector pEp/GroEL was grown for 16 h in YT medium containing 50 mg/l rifampicin and 50 mg/l kanamycin. The culture was diluted with MS medium to O.D.600 nm = 0.3; acetosyringone was added to a final concentration of 100 mM. Cotyledons and hypocotyls were submerged in the bacteria suspension for 2 h. The bacteria were removed and the explants were cultivated on MS agar plates for 48 h in the dark at 24 °C. The explants were then transferred to selective regeneration medium (solidified MS salts with Nitsch vitamins [25], containing 400 mg/l carbenicillin, 70 mg/l kanamycin and 1 mg/l zeatin). Regenerated explants were transferred to fresh medium biweekly. Green shoots, 1–3 cm high, were separated from the original explants and transferred to Nitsch medium containing 150 mg/l carbenicillin, 50 mg/l kanamycin and 1 mg/l indolebutyric acid (IBA) for rooting. Rooted plants were transplanted to soil in 1-liter pots and kept in the greenhouse in controlled conditions fitting the regulations of the Israel Plant Protection Authorities.

PCR-detection of GroEL and NPTII DNA in tomato plants

Plants were assayed for the presence of the GroEL gene by PCR using the sense primer GroEL-P751 5′-GCAGAA GATGTTGAAGGTGAAGC-3′ (starting 751 nucleotides from the initiation codon), and the reverse complementary primer GroEL-P1229 5′-CCTTCTTCTACTGCTGCTTCT TGT-3′ (nucleotides 1229–1206). PCR was performed as described above, amplifying a 478-bp GroEL DNA fragment. Plants were also analyzed by PCR for the presence of the *NPTII* gene using primers NptII-F (nt 2874 to 2895: 5′-GCCGCTTGGGTGGAGAGGCTAT-3′) and NptII-R (nt 2550 to 2539: 5′-GAGGAAGCGGTCAGCCCATTCG-3′) designed from the sequence of the gene (GenBank accession number AF485783).

Detection of GroEL DNA by Southern blot hybridization

Thirty micro gram of tomato total genomic DNA extracted according to [6] were digested to completion with *Eco*RI. The DNA fragments were subjected to 0.8% agarose gel electrophoresis and transferred to Hybond N+ membranes (Amersham, UK). GroEL-specific probes were prepared by random primer labeling with α^{32}P-dCTP. Hybridization was carried out at 65 °C for 18 h. The blot was washed with 0.1 × SSC at 65 °C and exposed for 72 h at −70 °C using an intensifying screen and Kodak Biomax film.

Screening for TYLCV resistance

Transgenic and wild-type plants were kept in an insect-proof greenhouse at 19–25 °C. Viruliferous whiteflies (following 24 h of acquisition access on TYLCV-infected tomatoes) were used to inoculate tomato plants [38]. The insects were caged with plants (30–50 insects/plant) for a 48-h inoculation access feeding period. The whiteflies were eliminated by spraying with imidacloprid. The plants were transferred to an insect-proof greenhouse to monitor appearance of symptoms. Severity of symptoms was monitored using the following disease severity index (DSI) [17]: DSI = 0: no visible symp-

toms; DSI = 1: very slight yellowing of leaflet margin on apical leaf; DSI = 2: some yellowing and minor curling of leaflet ends; DSI = 3: leaf yellowing, curling and cupping, plant has reduced size but continues to develop; DSI = 4: very severe stunting, yellowing, cupping and curling, plant ceases to grow.

Semi-quantitative PCR analysis of TYLCV DNA

A 100-µl reaction mixture was prepared, containing 100 ng of tomato total genomic DNA, 1 µl of a 25 mM mixture of the four dNTPs, 10 µl Taq polymerase buffer (×10), 1 unit of Taq DNA polymerase, 2.5 pmoles of two TYLCV-specific primers (accession number X15656): virion strand (position 61–80) 5′-ATACTTGGACACCTAATGGC-3′ and complementary strand (position 473–457) 5′-AGTCACGGGCC CTTACA-3′. Tomato β-actin served as positive control; 2.5 pmoles of two β-actin-specific primers were used (accession number BT013524): sense (position 772–791) 5′-GGAAAAGCTTGCCTATGTGG-3′, and complementary sense (position 951–932) 5′-CCTGCAGCTTCCATACC AAT-3′. The mixture was aliquoted in 10 tubes and subjected to PCR. Cycling was as follows: initial denaturation at 95 °C for 3 min followed by cycles of 30 sec at 95 °C, 30 sec at 55 °C and 1 min at 72 °C. The reaction was stopped after various numbers of cycles, and 5 µl of the PCR products was subjected to electrophoresis in a 1% agarose gel in Tris–phosphate-EDTA buffer (TAE) and stained with ethidium bromide (0.5 µg/ml).

Western blot immunodetection of GroEL

Leaf protein extracts were prepared in sample buffer [16] and subjected to 10% SDS-PAGE. After electrophoresis, proteins were electroblotted onto a Hybond-C Extra membrane (Amersham) at 4 °C for 2 h at 110 V, using transfer buffer (25 mM Tris–HCl, pH 8.3–192 mM glycine) supplemented with 10% (v/v) methanol. The membranes were blocked for 1 h at 22–25 °C with 2% bovine serum albumin (BSA) in 10 mM Tris–HCl, pH 7.5–150 mM NaCl, containing 0.1% Tween 20 (TBS-T). GroEL was immunodetected as described [2]. Briefly, membranes were incubated for 18 h at 4 °C with the GroEL antiserum (diluted 1:1000). All subsequent steps were done at 22–25 °C. Following five washes of 10 min each in TBS-T, membranes were incubated for 1 h with horseradish-peroxidase-linked anti-rabbit IgG. After intensive washes with TBS-T, immobilized conjugates were visualized by enhanced chemiluminescence (ECL, Amersham Life Science), followed by exposure to X-ray film.

Immunodetection of GroEL in tissue prints

Tomato plant stems were cut, and sections were stamped on Hybond-C Extra membranes (Amersham Life Science). Detached leaves were laid on membrane wetted with TBS-T and subjected to vacuum at ambient temperature for 1 h. The tissue prints [27] were incubated with 2% BSA blocking solution for 1 h. GroEL was detected using the GroEL antibody as described above.

Capture of TYLCV using anti-GroEL-coated tubes and detection by PCR

PCR tubes were filled with anti-GroEL antibody diluted (1:1000) in ELISA coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 0.02% NaN_3, pH 9.6), incubated for 3 h at 37 °C, followed by incubation with 2% BSA in TBST for 1 h at 37 °C, and washed with TBST. One hundred micro liter of homogenates of cleared sap from tomato stems was added to the anti-GroEL antibody-coated support, incubated for 1 h at 37 °C, and washed four times with TBST. PCR reagents were added directly to the tube with the TYLCV-specific primers, and the reaction was carried out as described above. The PCR products were subjected to electrophoresis in a 1% agarose gel in TAE buffer and stained with ethidium bromide (0.5 µg/ml) before photography. The same procedure was used with non-coated tubes. A mixture 1:1 of sap from two plants was incubated together for 1 h at 37 °C prior to binding to the GroEL-coated tube binding tests.

Results

Transformation and regeneration of tomato plants ("T_0" generation)

Two different tomato lines, F1 and MP1, were chosen for this study to avoid genotype-related artifacts. These two genotypes are easy to transform and have high regeneration competence. A gene encoding a GroEL homologue from the whitefly *B. tabaci* endosymbiotic bacteria was cloned into an *Agrobacterium* binary vector, under the control of the promoter from the *Arabidopsis* phloem-specific sucrose transporter gene *SUC2* (Fig. 1). The vector was introduced into *Agrobacterium*. Tomato cotyledons and hypocotyls were co-cultivated with the cells. Eighty-nine independent tomato plants were regenerated from cotyledons and hypocotyls: 40 from F1 hypocotyls, 15 from F1 cotyledons, 27 from MP1 hypocotyls and 7 from MP1 cotyledons.

DNA extracted from each plant was subjected to PCR analysis using GroEL-specific primers. Transgenic plants yielded the expected 500-bp product. Of the 89 regenerated plants, a GroEL-specific PCR product was obtained from 24 plants only: 10 from F1 hypocotyls (plant numbers 1, 2, 4, 10, 14, 22, 34,

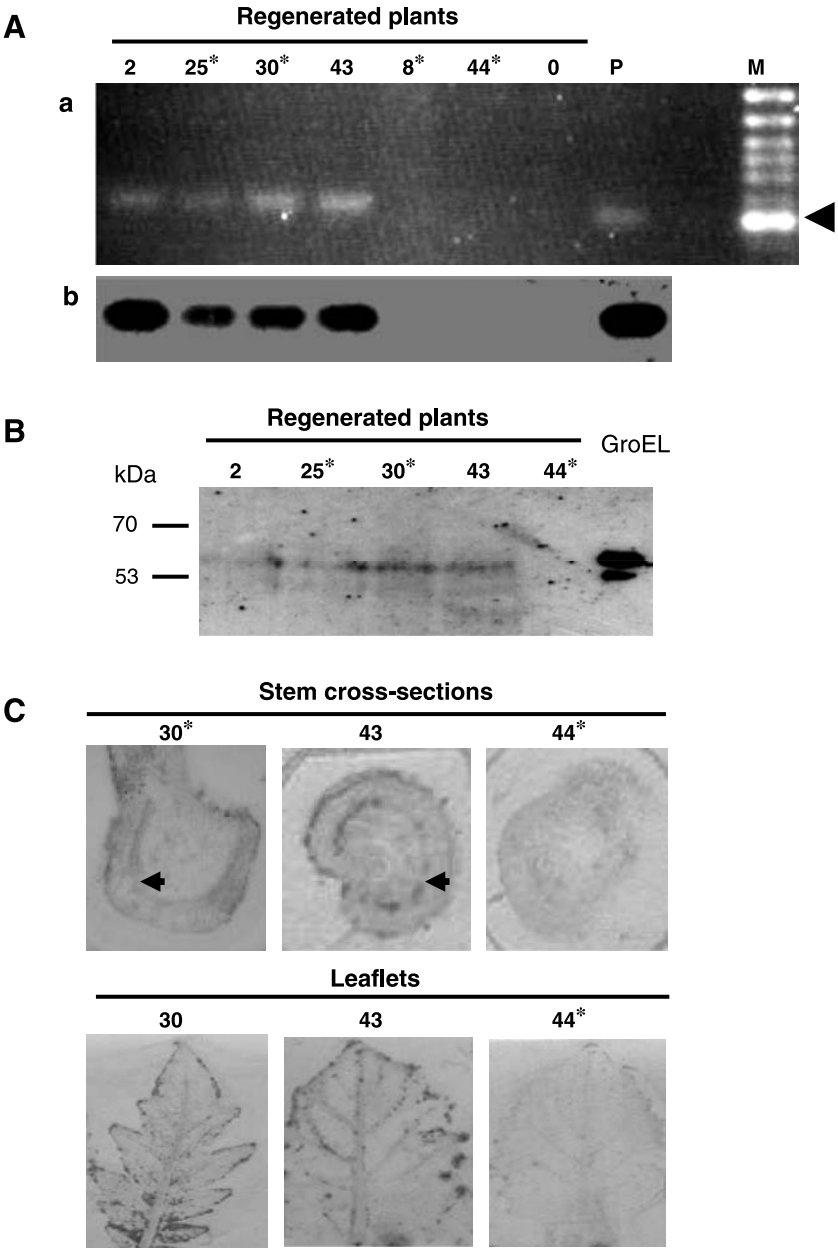
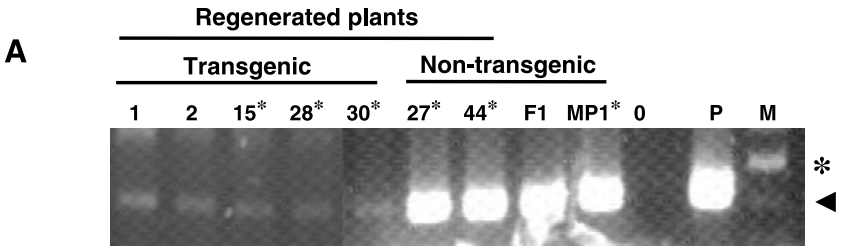


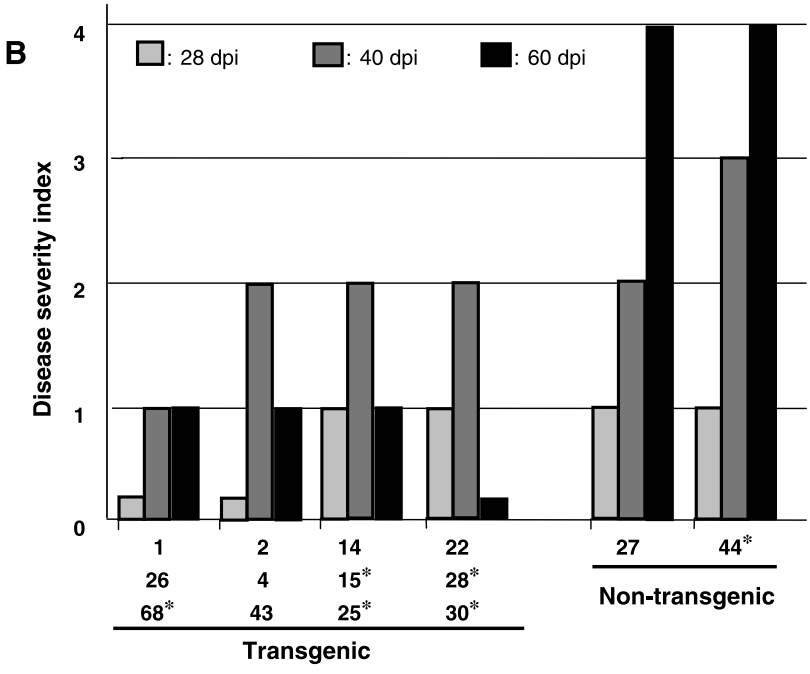
Fig. 2. Screening of tomato plants that regenerated following co-cultivation of explants with *Agrobacterium* containing the binary vector pEP/GroEL ("T_0" generation): presence of GroEL DNA (**A**) and GroEL protein (**B**, **C**). **A** DNA of selected plants was subjected to PCR using GroEL-specific primers; transgenic plants yielded the expected ~500-bp product. **a** Agarose gel showing PCR products obtained with a selected number of plants; *0* MP1 plant from seed, *P* plasmid containing the GroEL clone, *M* 100-bp ladder, arrow head: 500 bp. **b** The gel was blotted on a membrane and subjected to hybridization with a GroEL-specific probe. **B** Western blot analysis of selected regenerated plants using a GroEL-specific antibody; *GroEL*: protein over-expressed in *E. coli*. **C** Immunolocalization of GroEL in tissue prints; arrow head points to label in the inner phloem. Note that the non-transgenic regenerated plant 44 is not expressing GroEL. * Regenerated plants of the MP1 genotype

69, 71, 86), 5 from F1 cotyledons (plant numbers 16, 26, 29, 35, 43), 7 from MP1 hypocotyls (plant numbers 15, 28, 30, 45, 76, 80, 82) and 2 from MP1 cotyledons (plant numbers 25, 68). The nature of the PCR product was confirmed by hybridization with a GroEL-specific probe (Fig. 2A). The ratio of regenerated plants to transgenic plants was low because of the necessity to remove kanamycin selection during explant rooting, a fact that lead to a large number of escapes.

Selection of primary transformants ("T_0" generation) expressing GroEL and symptomless upon whitefly-mediated TYLCV inoculation

In order to select those transgenic plants that express GroEL, protein extracts from the leaves and the stems of plants of the "T_0" generation, were subjected to Western blot analysis. Figure 2B shows the results for a selected number of plants, regenerated from cotyledons and hypocotyls of the F1 and MP1





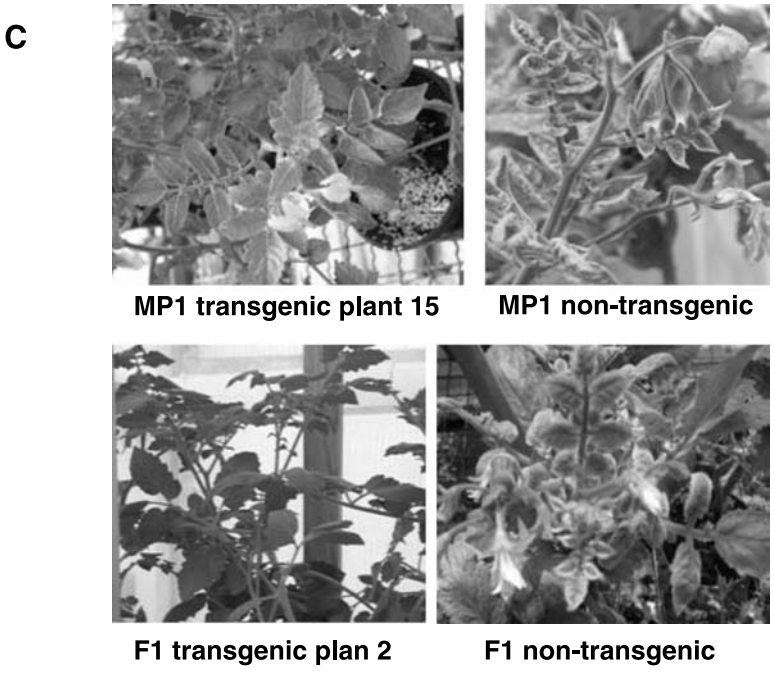


Fig. 3. Resistance of "T_0" plants to whitefly-mediated TYLCV infection**.** **A** PCR amplification of TYLCV DNA from selected regenerated transgenic and non-transgenic tomato plants, two weeks after whitefly-mediated inoculation; 10 ng DNA from a young leaf of infected and uninfected plants were used as template, and the products were analyzed after 30 cycles; F1 and MP1: inoculated plants issued from seeds, *0* non-infected MP1 plant, *P* plasmid pTYJ20.4 containing the cloned full-length TYLCV genome, *M* 100-bp molecular weight ladder; 500 bp is indicated by a star. **B** Following inoculation, the severity of symptoms (disease severity index, DSI) was scored 28, 40 and 60 days post inoculation (dpi); note that regenerated plants 27 and 44 were not transgenic. * Plants of the MP1 genotype. **C** Absence of disease symptoms at 90 dpi in infected transgenic plants from MP1 and F1 genotype; compare with strong disease symptoms presented by infected non-transgenic plants

genotypes. Transgenic plants 2, 25, 30 and 43 expressed the GroEL protein while the regenerated non-transgenic plant 44 did not. The GroEL protein was immunolocalized in tissue prints of stem sections and of young leaves, using an anti-GroEL antibody. Examination of the prints (Fig. 2C) indicated that GroEL was localized mainly in the inner phloem tissue of the GroEL PCR-positive transgenic tomato plants 30 and 43 but was not detected in non-transgenic tomato plant 44 issued from the same transformation events. The results from the tissue prints and of the Western blots were totally compatible. All twenty-four of the regenerated plants that contained PCR-amplifiable GroEL DNA expressed the GroEL protein.

The twenty-four "T_0" transgenic plants (from MP1 and F1) were challenged with TYLCV by caging plants with viruliferous whiteflies. Two weeks thereafter, DNA was extracted from a young leaflet sampled from each plant and was assayed for the presence of TYLCV DNA by PCR. The results shown in Fig. 3A indicate that at this early stage of infection the transgenic plants contained far less viral DNA than infected non-transgenic plants issued from tissue culture or from seeds. Severity of symptoms was scored 28, 40 and 60 days post-inoculation (dpi): symptomless plants were given a disease severity index (DSI) of 0 (as non-inoculated plants), and fully symptomatic plants were scored as 4 (as inoculated non-transgenic plants). A graphic representation of the behavior of a randomly selected number of plants upon TYLCV inoculation is shown in Fig. 3B. Three of the 24 "T_0" transgenic plants (1, 26, 68) remained almost symptomless, even at 60 dpi, with a DSI of 0–1. Twenty-one plants showed no or slight symptoms at 40 dpi (DSI of 1–2); the symptoms tended to improve or to disappear altogether thereafter (DSI of 0–1). Non-transgenic regenerated plants (e.g. 27 and 44) issued from the same transformation event presented typical TYLCV disease symptoms with a DSI of 4, as did susceptible control plants issued from seeds. At 90 dpi, all the transgenic plants contained PCR-amplifiable viral DNA in similar amounts, excluding the possibility of infection escapes (not shown). An example of the appearance of the symptomless inoculated transgenic plants and of the symptomatic non-transgenic plants at 60 dpi is shown in Fig. 3C. In the greenhouse, infected symptomless transgenic tomato plants yielded fruits as did uninfected non-transgenic plants from the same genotype. Infected non-transgenic plants did not yield at all. Symptomless, or nearly-symptomless, infected tomato plants (DSI from 0 to 1) were considered resistant, although they contained significant amounts of viral DNA.

Production of the "T_1" transgenic generation: integrity of the GroEL gene construct, expression of the GroEL protein and resistance to TYLCV

The 24 primary transformed "T_0" plants were self-pollinated. Twenty seeds from nine randomly selected "T_0" plants were germinated in soil. All 20 of the "T_1" progenies (named 'A' to 'T') from each of the nine primary "T_0" transformants were analyzed for the presence of the GroEL gene by PCR. Approximately 65–70% of the plants, regardless of the identity of the mother plant, contained amplifiable GroEL DNA (not shown), indicating that this generation segregated for the GroEL gene. Those plants that contained GroEL DNA were further investigated.

The integrity of the GroEL gene, flanked by the phloem-specific promoter and the NOS terminator, in the PCR-positive GroEL transgenic plants was confirmed by Southern blot hybridization of *Eco*RI-digested plant DNA (two *Eco*RI restriction sites encompass the *SUC2*-GroEL-NOS DNA, see Fig. 1). Figure 4A shows that a unique DNA fragment of about 4200 bp hybridized with a probe consisting of the radiolabeled full-length GroEL gene. All plants that tested positive for GroEL DNA by PCR displayed the GroEL-containing 4200-bp DNA fragment. This fragment was absent in non-transgenic plants.

Presence of the GroEL protein was assayed by Western blot using the GroEL-specific antibody. Figure 4B shows the analysis of a number of selected plants. GroEL was detected in the all the transgenic plants assayed, but not in progeny of non-transgenic plants (e.g. plant 44D).

The transgenic plants from the "T_1" generation were tested for resistance to whitefly-mediated in-

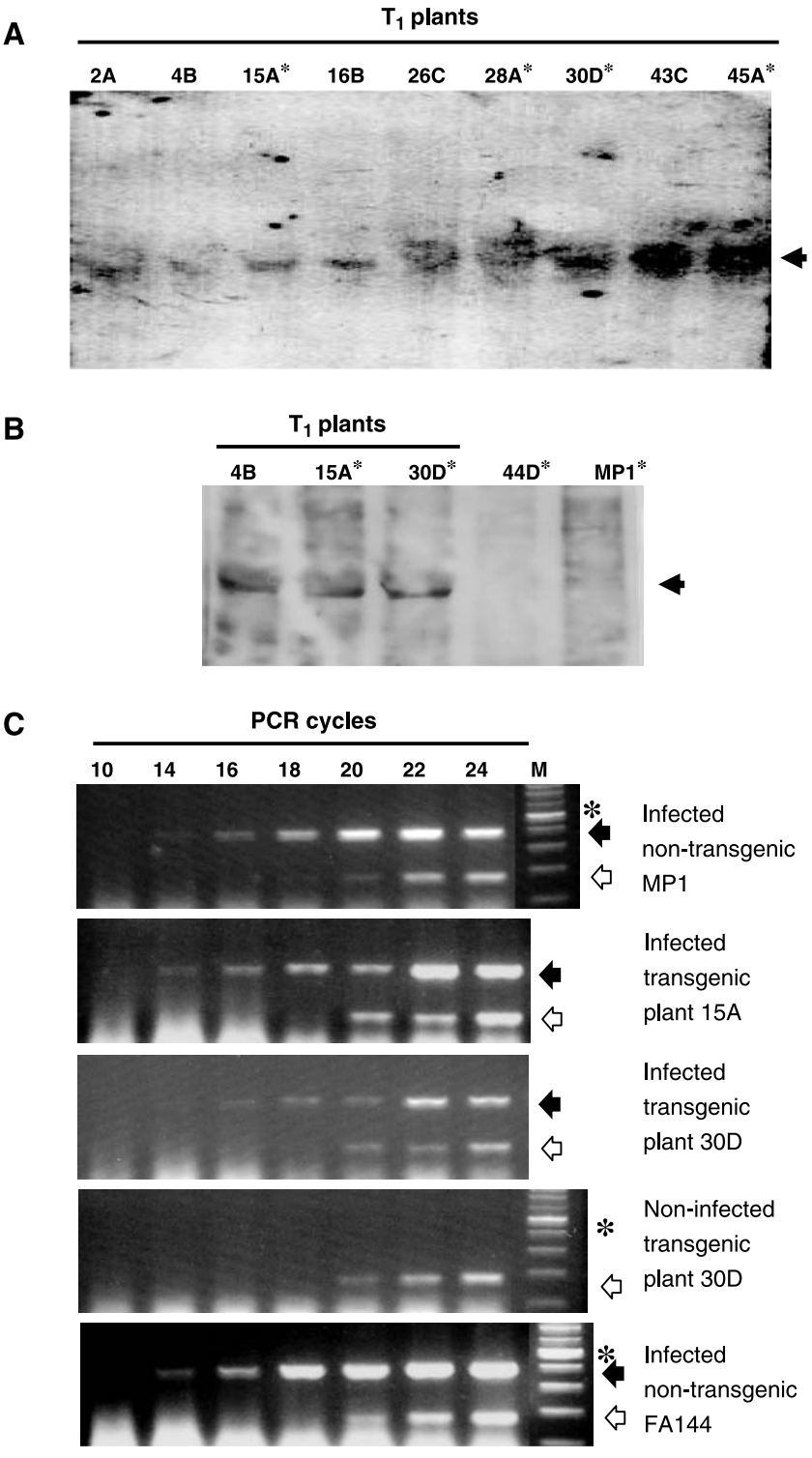


Fig. 4. Whitefly GroEL DNA and protein in transgenic plants from the "T_1" generation; viral DNA amounts upon inoculation. **A** Southern blot hybridization of *Eco*RI-digested plant DNA with radiolabeled GroEL gene; arrow head: 4200-bp DNA fragment containing the GroEL gene flanked by *SUC2* promoter and the *NOS* terminator. **B** Presence of the GroEL protein as assayed by Western blot in selected plants using the GroEL-specific antibody; note that plant 44 is not transgenic. * Plants of the MP1 genotype. **C** Comparison of amounts of viral DNA at 60 dpi in infected transgenic T_1 plants 15A and 30D and in infected non-transgenic MP1 and F1 plants by semi-quantitative PCR analysis of viral DNA (full arrow); β-actin served as internal standard (empty arrow); 50 ng plant DNA was used as template; the reactions were sampled after the number of cycles indicated and the products subjected to agarose gel electrophoresis. *M* 100-bp ladder, star: 500 bp

oculation of TYLCV. As a first approach, 3–5 plants, progeny from each of the nine selected transgenic "T_0" parents that expressed the GroEL protein, were challenged with TYLCV as described above. Following whitefly-mediated inoculation, each plant was scored for symptoms, and the DSI

was noted at 30, 60 and 90 dpi. The progeny of the nine "T_0" transgenic parents showed very mild symptoms (DSI of 1–2) and either maintained this level of symptoms or the symptoms disappeared. The DSI within each group of plants was homogenous and varied only by one point, if at all (not shown). These results showed that all the transgenic plants that were resistant to TYLCV inoculation at the "T_0" generation (Fig. 3B) engendered resistant "T_1" progeny plants.

The amounts of viral DNA in resistant transgenic plants of the "T_1" generation were compared to those of non-transgenic tomato plants, two months after inoculation (at 60 dpi). Semi-quantitative PCR was used, with β-actin as internal standard. Figure 4C shows the results obtained with GroEL-expressing transgenic plants 15A and 30D, regenerated from MP1 cotyledons, which remained nearly symptomless upon inoculation. In plants 15A and 30D, as well as in the non-transgenic MP1 and FA144 varieties, the 410-bp TYLCV amplicons were conspicuous after 14–16 cycles, while the 195-bp actin amplicon was detected after 18–20 cycles. These results indicated that infected transgenic and non-transgenic plants contained about the same amount of viral DNA at 60 dpi, although the transgenic plants were virtually symptomless and the non-transgenic plants exhibited strong disease symptoms.

Virus-GroEL complexes in the phloem of "T_1" transgenic tomato plants

At the beginning of this study, we postulated that GroEL expressed in the phloem of tomato plants would be able to trap virions, thereby preventing virus spread and infection. However, similar amounts of viral DNA were present in symptomless transgenic plants and in symptomatic non-transgenic

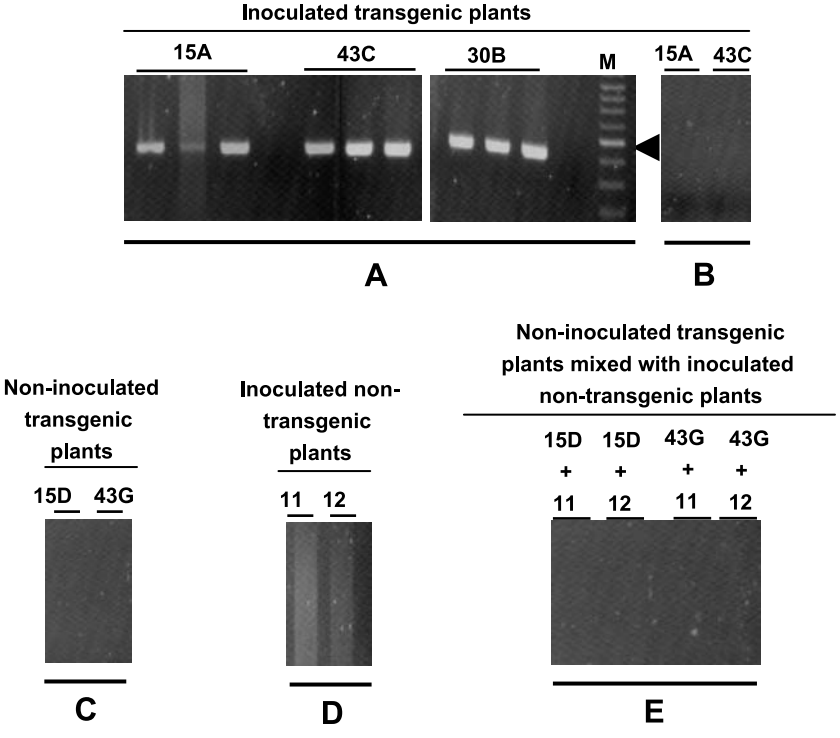


Fig. 5. Detection of virus bound to GroEL in the sap of "T_1" transgenic plants. Sap of infected transgenic plants 15A, 43C and 30B (at 60 dpi) was added to PCR tubes coated with anti-GroEL antibody (**A**) and with uncoated tubes (**B**); similarly, extracts of non-inoculated transgenic plants 15D and 43G (**C**), extracts of infected non-transgenic plants 11 and 12 (**D**), and mixtures of non-infected transgenic plants 15D and 43G with infected non-transgenic plants 11 and 12 (**E**), were incubated in PCR tubes coated with anti-GroEL antibody. After thorough washes, a PCR mix containing TYLCV-specific primers was added to the tube and the PCR products were analyzed by agarose gel electrophoresis. *M* 100-bp ladder, arrow head: 500 bp

tomato (Fig. 4C). Such a result could be explained by the fact that in the resistant plants the virus was trapped by GroEL, all or part of it, and could not interact with plant factors to induce symptoms. Using a previously described test [2], we investigated whether GroEL-TYLCV complexes could be identified in infected transgenic tomato plants or whether GroEL and TYLCV were not physically linked (Fig. 5). In these tests, a TYLCV-specific PCR product was detectable only if a pre-existing TYLCV-GroEL complex would bind to anti-GroEL-coated tubes (case A). No PCR product would be detected if a pre-existing TYLCV-GroEL complex was incubated with non-coated tubes (case B). Similarly, no PCR product would be detected with sap from either non-infected transgenic plants (case C), infected non-transgenic plants (case D), and mixtures of sap from non-infected transgenic plants and sap from infected non-transgenic plants (case E).

PCR tubes were coated with anti-GroEL antibody; stem sap of infected (at 60 dpi) transgenic "T_1" plants 15A, 30B and 43C (with DSI close to 1) were added to the coated tube, as described before. After thorough washes, a PCR mix containing TYLCV-specific primers was added to the tube, and the PCR products were analyzed. A virus-specific PCR product was detected, indicating that pre-existing GroEL-TYLCV complexes did bind to the anti-GroEL-coated tubes (Fig. 5, case A). No PCR product was obtained when sap of plants 15A and 43C was added to uncoated tubes (case B). Similarly, no product was obtained when homogenates of non-inoculated transgenic plants 15D and 43G (case C), infected non-transgenic plants 11 and 12 (case D), or 1:1 mixtures of saps of non-infected transgenic plants 15D and 43G and infected non-transgenic plants 11 and 12 were incubated with the anti-GroEL-coated tubes (case E).

These results indicated that GroEL expressed in the phloem of transgenic plants was able to complex virions, and that these complexes could be trapped by an anti-GroEL antibody. GroEL-TYLC complexes were not formed simply by mixing and incubating saps of non-infected transgenic plants and infected non-transgenic plants, possibly because of the low concentration of chaperon and virions in the sap of the respective plants and in the sap mixture. Hence we believe that such complexes need to be formed *in vivo*. The formation of the GroEL-TYLCV complex in the plant phloem might be the cause of the observed resistance observed in progeny of transformed plants 2, 4, 15, 30 and 43.

Production of the "T_2" transgenic generation: homozygocity for the GroEL gene and expression of the GroEL protein

Two GroEL-expressing TYLCV-resistant transgenic plants of the "T_1" generation, 15A and 30D (Fig. 4), were self-pollinated, and 70 seeds from each plant were germinated to give the "T_2" generation. Fifty-six plants that were progeny of 15A, and sixty-five plants that were progeny of 30D were tested by PCR for the presence of GroEL DNA as well as for DNA from the NPTII selective marker. All of the plants tested were positive for the two genes (not shown), indicating that the 15A and 30D parents, and their progeny, were homozygous for GroEL and NPTII.

Infected TYLCV-resistant plants may serve as inoculum source for whitefly-mediated transmission of virus to test tomato plants

We have tested whether whitefly-mediated transmission of virus from the infected transgenic plants was impacted by GroEL. To find out whether or not resistant transgenic plants can serve as a virus inoculum for whiteflies, insects were caged for a 72-h acquisition access period with: 1) two infected symptomless "T_2" tomatoes that were progeny of 15A (plants 3 and 7); 2) one infected symptomless "T_2" tomato progeny of 30D (plant 62); 3) one non-infected "T_2" tomato progeny of 30D (plant 68); 4) one infected non-transgenic susceptible tomato (cv. Daniella, FA144). PCR indicated that the infected transgenic and non-transgenic plants contained viral DNA, while the non-infected transgenic control plant did not (Fig. 6A). The insects were collected separately from each plant source, and each group was caged with ten non-transgenic, non-infected susceptible test plants FA144. Following a 72-h inoculation access period, ten insects

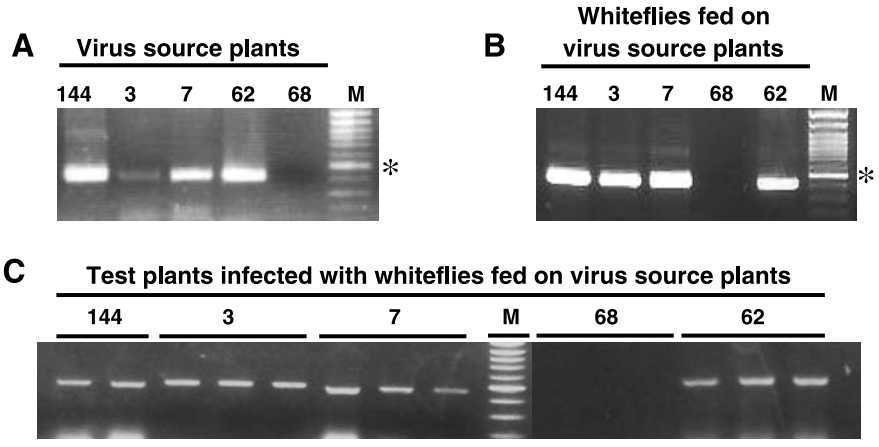


Fig. 6. Acquisition of TYLCV from resistant "T_2" plants by whiteflies and transmission to tomato test plants. **A** PCR detection of viral DNA in infected "T_2" plants 3, 7 and 62 and in uninfected "T_2" plant 68; 144 is infected non-transgenic FA144 cultivar. **B** PCR detection of viral DNA in whiteflies that fed on plants in **A**. **C** PCR detection of viral DNA in test plants inoculated by whiteflies fed on resistant "T_2" plants in **A** (3 target test plants per source plant, except for 144). *M* 100-bp ladder, star: 500 bp

were collected from each plant group; PCR analyses indicated that these insects had acquired virus from the infected plants, whether they were transgenic or not (Fig. 6B). The plants were then treated with insecticide. Four weeks thereafter, all of the test plants inoculated by insects fed on infected plants presented typical disease symptoms. PCR analyses indicated that the inoculated plants contained viral DNA (Fig. 6C). There was no difference in inoculation efficiency when either transgenic or non-transgenic tomato source plants served as virus source. Therefore, infected TYLCV-resistant transgenic plants may constitute a source of virus for whitefly-mediated transmission that is as effective as non-transgenic plants.

Non-transgenic susceptible tomato plants grafted on resistant GroEL-transgenic scions remain susceptible to TYLCV infection

We have investigated the question of whether GroEL can move long-distance from TYLCV-resistant transgenic scion to TYLCV-susceptible non-transgenic graft, thus conferring resistance to the non-transgenic tissues. Progeny plants of 15A and 30D (6 weeks after sowing) were used as scions on which susceptible FA144 plants were grafted. One week before grafting, the presence of GroEL was confirmed in the would be scions (Fig. 7A). We have also grafted GroEL-expressing progeny of plants 15A and 30D on FA144 susceptible scions. As controls, we used FA144 plants and progeny of 15A and 30D plants grafted on themselves, and non-grafted resistant and susceptible plants. Two weeks after grafting, the plants were inoculated with viruliferous whiteflies as described above. DSI was recorded 20, 40, 60 and 80 days after inoculation. The results graphically summarized in Fig. 7B showed that the FA144 plants grafted on resistant 15A and 30D progeny plants were as susceptible as FA144 plants grafted on themselves or on non-grafted FA144 plants (Fig. 7C). In comparison, 14A and 30D progeny plants grafted on FA144 retained their resistance to the virus and in this respect behaved similarly to 14A and 30D grafted on themselves or to non-grafted 15A and 30D progeny plants. PCR analyses performed one week after inoculation (not shown) indicated that all the plants contained TYLCV DNA (scions and grafts, or whole plants) and that there was no escape from inoculation.

In the light of these results, we have investigated whether the symptomatic FA144 grafts contained GroEL-TYLCV complexes 60 days after inoculation. Western blot analysis showed that GroEL did translocate from scions of GroEL-expressing plants

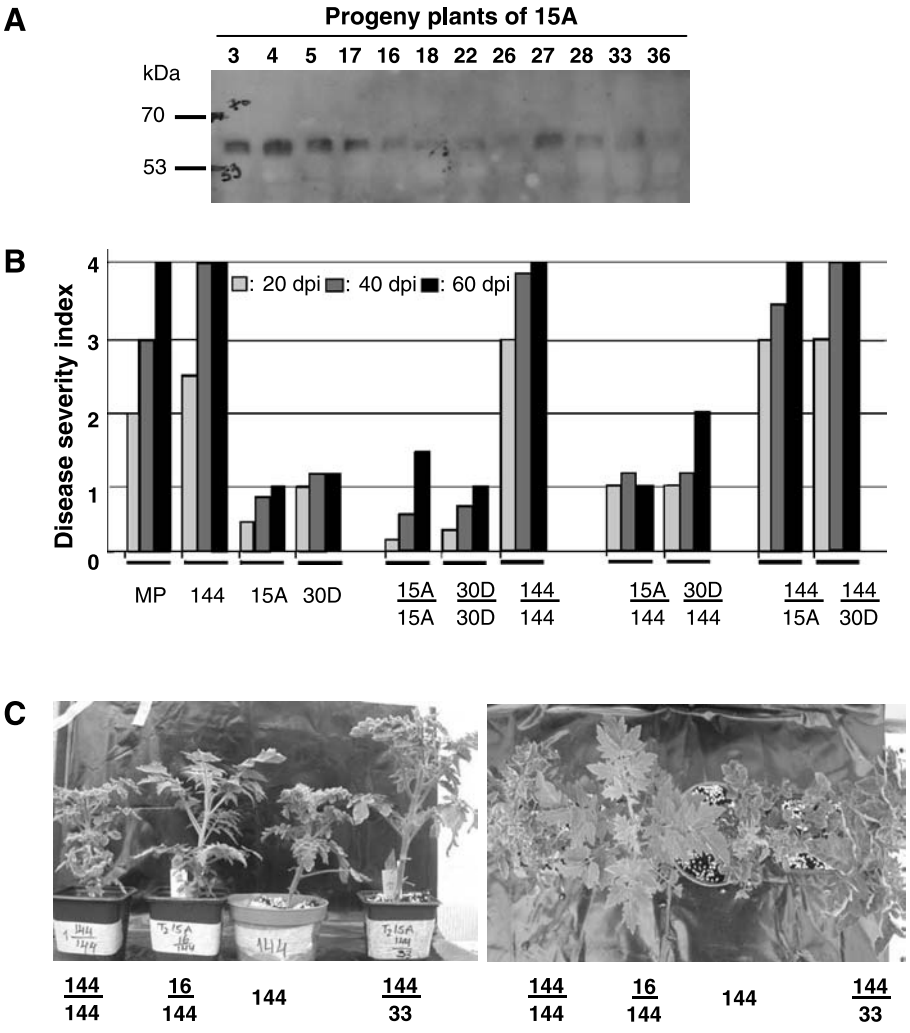


Fig. 7. Behavior of non-transgenic susceptible tomato plants FA144 grafted on resistant GroEL-transgenic "T_2" scions. Specification of graft and scion is graft/scion. **A** Presence of GroEL in tomato plants serving as scions, detected by Western blotting using a GroEL antibody. **B** Disease severity index of grafts 20, 40 and 60 days after whitefly-mediated inoculation; infected non-transgenic MP (MP1) and FA144 (144) plants served as susceptible controls; infected 15A and 30D "T_1" resistant plants served as resistant controls. **C** Presence of severe disease symptoms presented by FA144 plant grafted on GroEL-producing resistant "T_2" plant 33 (144/ 33) at 60 dpi; note the very mild symptoms presented by resistant "T_2" plant 16 grafted on susceptible non-transgenic FA144 scion (16/144)

into susceptible grafts. Figure 8A shows that GroEL present in the nearly symptomless (DSI of about 1) plants 16 (progeny of 15A) and 43 (progeny of 30D) was conspicuous in the symptomatic FA144 plants grafted on the 16 and 43 scions. The presence of virions bound to GroEL was investigated as described above. Plant sap was incubated in PCR tubes coated with anti-GroEL antibody. After washing, a PCR mix containing TYLCV-specific primers was added and the PCR products were analyzed. Figure 8B shows that a virus-specific PCR product was detected in the FA144 plants grafted on transgenic plants 16, 28 and 33 (progeny of 15A) and 43 (progeny of 30D), indicating that GroEL-TYLCV complexes were present in the sap of the infected symptomatic FA144 grafts. All the FA144 plants grafted on GroEL-transgenic scions gave identical results. No PCR product was obtained when the

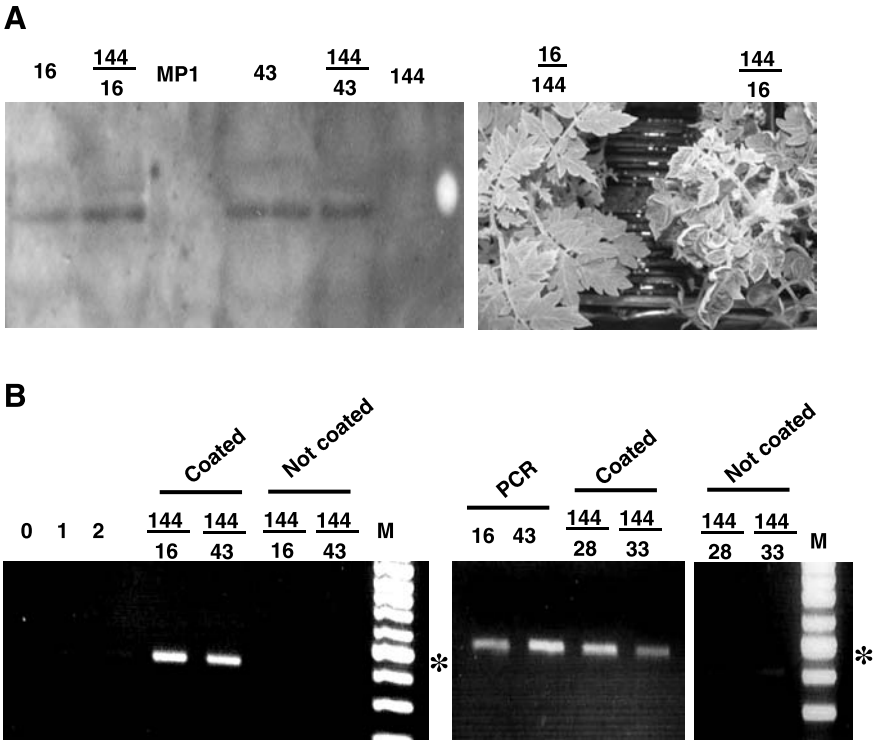


Fig. 8. Translocation of GroEL from transgenic scions to non-transgenic grafts and detection of GroEL-TYLCV complexes in the grafts. **A** Left panel: immunodetection of GroEL in FA144 plants grafted on scions of GroEL-expressing "T_2" plants 16 (144/16) and 43 (144/43); right panel: appearance of severe symptoms in FA144 plants grafted on plant 16, and of very mild symptoms on plant 16 grafted on 144. **B** Detection of GroEL-TYLCV complexes in inoculated FA144 grafts (see also Fig. 5); sap from FA144 grafts (grafted on plants 16, 43, 28 and 33: 144/16, 144/43, 144/28, 144/33) was incubated in PCR tubes coated or not coated with anti-GroEL antibody; after washing, a PCR mix containing TYLCV-specific primers was added and the PCR products were analyzed; *0* no template, *1,2* coated tubes without sap, *16* and *43* infected non-grafted plants, *M* 100-bp molecular marker, star: 500 bp

sap of the FA144 scions was incubated in tubes not coated with GroEL antibody. Hence, despite the fact that GroEL translocated from the transgenic scion into the non-transgenic grafts and was able to bind virions, the FA144 grafts were symptomatic, as were infected FA144 plants.

Discussion

Breeding for virus resistance in transgenic crop plants has been reviewed recently [26]. A variety of strategies have been devised based on the pathogen-derived resistance concept [29], which involves the expression of functional as well as dysfunctional pathogen genes such as coat protein, replicase, and movement protein [35]. RNA-mediated virus resistance proved to be more efficient than protein-mediated resistance but was shown to be highly sequence-dependent and therefore with a narrower-range potential. It was shown to trigger existing plant antiviral mechanisms leading to the degradation of viral RNA [4], and was collectively named RNA-mediated gene silencing. It was found that some viruses establish themselves by expressing viral proteins that interfere with plant host silencing mechanisms [34]. Transgenic plants have been developed which exploited the mechanism of silencing via double-stranded RNA sequences [30]. However, transgenic RNA-mediated resistance can be overcome by strong silencing suppressors of unrelated viruses in mixed infections [20]. Recent strategies have targeted the formation of infectious virions by expressing peptides in transgenic plants which interfere with homo-multimerization of the

coat protein [28]. Some of these strategies have been used to obtain TYLCV-resistant tomato plants [1, 3, 8, 15, 37].

The strategy used in the present research is based on a totally new concept. It takes advantage of the fact that some, and perhaps all, plant viruses transmitted by their insect vectors in a circulative manner interact in the insect haemolymph with GroEL homologues produced by the vector endosymbiotic bacteria. Binding between GroEL and virions has been shown to occur *in vivo* and *in vitro* [2, 21, 22, 33]. It has been suggested that GroEL-virus interaction could be a mechanism shared by plant circulative viruses to avoid destruction in the haemolymph [12]. In this study, we wondered whether this phenomenon could be exploited to generate transgenic tomato plants expressing the whitefly GroEL in their phloem. We expected that once inoculated by their vector, phloem-limited circulative viruses would be trapped by GroEL in the plant phloem. As a result, invasion of phloem-associated cells and long-distance movement could be significantly inhibited, rendering the plants resistant to the virus.

This approach was implemented by expressing a gene encoding a GroEL homologue from the endosymbiotic bacteria of the whitefly *B. tabaci* in transgenic tomato plants and testing these plants following whitefly-mediated inoculation of TYLCV. A whitefly GroEL gene was cloned in an *Agrobacterium* binary vector under the control of an *Arabidopsis* phloem-specific promoter (Fig. 1). In *Arabidopsis* leaves, this promoter regulates the expression of the companion cell-specific *AtSUC2* Suc-H^+ symporter gene [32]. The tissue-specificity of the promoter has been demonstrated in tobacco [36] but not in tomato. Immunodetection of GroEL indicated that the *Arabidopsis* promoter drove the expression of this protein in the tomato phloem (Fig. 2). The GroEL gene is of prokaryote origin, and the question of its efficient expression in a eukaryotic environment has been raised. Examination of codon usage (Graphic Codon Usage Analyser http://www.gcua.de) indicated that the codon usage of the GroEL gene from *B. tabaci* is fit for expression in tomato plants. Indeed, GroEL was detected by western blot analysis and was immunolocalized in the vascular system of the transgenic plants. The plants expressing the insect GroEL did not show any obvious phenotype or malformation.

GroEL was expressed in the phloem of transgenic tomato plants issued from two different genotypes (Fig. 2), which presented excellent transformation and regeneration rates: an interspecific hybrid of *S. pennellii* × *S .esculentum* [15] and the tomato line MP1 [5]. Results obtained with the hybrid and with MP1 were similar. MP1 was described as tolerant to TYLCV [5]; however, in our hands, and under our harsh inoculation conditions, this line was as susceptible cultivars such as FA144 (cv. Daniella). The GroEL-transgenic tomato plants exhibited good levels of resistance to whitefly-mediated inoculation of TYLCV, exhibiting very mild or no symptoms (Fig. 3). The transgenic progeny of the resistant plants were as resistant as their parents.

In "T_1" and "T_2" resistant plants, *in vitro* assays indicated that viral particles were bound to GroEL in the plant sap (Figs. 5 and 8). In all plants tested, there was a strict correlation between the detection of GroEL-TYLCV complexes and the symptomless phenotype presented by the transgenic plants upon inoculation. These complexes were pre-formed; GroEL-TYLCV complexes were not detected when mixes of saps of uninfected transgenic plants and infected non-transgenic plants were incubated together in anti-GroEL-coated tubes, probably because of the low concentration of free GroEL and free virus in the plant saps.

Our investigation was based on the paradigm that interaction between GroEL and TYLCV in the phloem may prevent invasion of phloem-associated cells, a first step necessary for the virus to replicate. PCR analyses showed that, 2–3 weeks after inoculation, the resistant transgenic tomatoes contained less viral DNA than susceptible non-transgenic plants; however, 6 weeks later, the viral DNA amounts were similar in the symptomless transgenic plants and in the symptomatic non-transgenic plants (Figs. 3 and 4). The pattern of viral DNA accumulation patterns in the GroEL-expressing plants suggests that the virus replicates in these plants. It also implies that not all particles are immediately trapped by the chaperonins upon in-

oculation. Some virions seem to escape and invade companion cells, where they replicate. It is also possible that the virus replicates after GroEL-virion complexes have penetrated these cells and have dissociated (it is unlikely that complexes penetrate the cell nucleus). The fact that strong symptoms do not develop in infected transgenic plants indicates that the accumulation of virions is slow enough not to interfere with the growth of the plant. It is also possible that GroEL disturbs interactions between viral and plant proteins at one or more of the steps of the virus cycle: entry into the cell nucleus, exit into the cytoplasm, cell-to-cell and long-distance movement (see reviews in [11, 13, 14]).

In the field, infected resistant tomato plants (resistance obtained by classical breeding) may serve as virus inoculum as do infected susceptible plants. Efficacy and rates of whitefly-mediated transmission of the TYLCV disease to susceptible plants is similar whether the virus source is from susceptible plants or from resistant ones, especially in the later stages of infection [18]. We have tested whether whitefly-mediated transmission of virus from the infected transgenic plants was impacted by GroEL. We found that infected GroEL-expressing resistant plants were as good a source of virus for whitefly-mediated transmission as were infected non-transgenic plants (Fig. 7). We do not know whether whiteflies selectively ingurgitate free particles from the transgenic tomatoes or ingurgitate GroEL-virus complexes as well. There might be enough free virions in the transgenic plant sap to be picked up by the insect stylets and transmitted to test plants but not enough to induce a systemic infection. Alternatively, GroEL-virus complexes may be acquired during feeding and dissociate at some point in the insect digestive tract.

We have hypothesized that by grafting susceptible non-transgenic tomato on scions from GroEL-expressing resistant plants, GroEL would systemically move into the graft, conferring resistance to the grafted plant upon whitefly-mediated inoculation by virtue of binding TYLCV particles. Although we found that GroEL indeed translocated into the grafted tissues, and that GroEL-TYLCV complexes could be detected upon inoculation, the susceptible grafts remained susceptible, exhibiting strong disease symptoms (Fig. 8). In comparison, GroEL-expressing transgenic resistant plants grafted on susceptible scions retained their resistance and presented very mild or no symptoms (Fig. 8). It is possible that the transgenic scions did not contain enough GroEL-producing tissues in order to complex sufficient amounts of virions to prevent the development of disease symptoms. Indeed, the scions consisted only of the stem without any leaf, with the part from the roots up to the cotyledons excluded.

As is the case with all antiviral strategies, the GroEL approach has its own limits. Resistance provided by expression of viral proteins, antisense RNA and siRNA in transgenic plants is usually virus-sequence-specific [1, 3, 37]. Similarly, the GroEL approach may work only for those viruses which are transmitted in a circulative manner by their insect vector [2]. We have investigated whether or not GroEL expressed in transgenic *Nicotiana benthamiana* provides resistance to infection by tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV). CMV is transmitted in a circulative manner by aphids, while TMV is not [9]. CMV binds to GroEL *in vitro*, while TMV does not [2]. The results indicated that GroEL-producing *N. benthamiana* was resistant to CMV, but not to TMV (in preparation).

Acknowledgements

This research was supported by Grant No. M21-037 funded by the U.S.-Israel Cooperative Development Research Program, Bureau for Economic Growth, Agriculture, and Trade, U.S. Agency for International Development, Middle East Research and Cooperation (MERC) Program.

References

1. Abhary MK, Anfoka GH, Nakhla MK, Maxwell DP (2006) Post-transcriptional gene silencing in controlling viruses of the Tomato yellow leaf curl virus complex. Arch Virol 148: 405–421
2. Akad F, Dotan N, Czosnek H (2004) Trapping of *Tomato yellow leaf curl virus* (TYLCV) and other plant viruses with a GroEL homologue from the whitefly *Bemisia tabaci*. Arch Virol 149: 1481–1497
3. Antignus Y, Vunsh R, Lachman O, Pearsman M, Maslenin L, Hananya U, Rosner A (2004) Truncated

Rep gene originated from *Tomato yellow leaf curl virus-Israel* [Mild] confers strain-specific resistance in transgenic tomato. Ann Appl Biol 144: 39–44
4. Baulcombe DC (1996) Mechanisms of pathogen-derived resistance to viruses in transgenic plants. Plant Cell 8: 1833–1844
5. Barg R, Pilowsky M, Shabtai S, Carmi N, Szechtman AD, Dedicova B, Salts Y (1997) The TYLCV-tolerant tomato line MP-1 is characterized by superior transformation competence. J Exp Botany 48: 1919–1923
6. Bernatzky R, Tanksley SD (1986) Majority of random cDNA clones correspond to single loci in the tomato genome. Mol Gen Gene 203: 8–14
7. Brown JK, Czosnek H (2002) Whitefly transmission of plant viruses. In: Plumb RT (ed), Advances in botanical research. Plant virus vector interactions, Vol. 36. Academic Press, New York, pp. 65–100
8. Brunetti A, Tavazza M, Noris E, Tavazza R, Caciagli P, Ancora G, Crespi S, Accotto GP (1997) High expression of truncated viral rep protein confers resistance to tomato yellow leaf curl virus in transgenic tomato plants. Mol Plant-Microbe Interact 10: 571–579
9. Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ, Watson L, Zurcher EJ (eds) (1996) Plant viruses online: descriptions and lists from the VIDE database. Version: 20th August 1996. URL http://image.fs.uidaho.edu/vide/refs.htm
10. Cohen S (1993) Sweet potato whitefly biotypes and their connection with squash silver leaf. Phytoparasitica 21: 174
11. Gafni Y, Epel B (2002) The role of host and viral proteins in intra- and inter-cellular trafficking of geminiviruses. Physiol Mol Plant Pathol 60: 231–241
12. Gibbs M (1999) Chaperonin camouflage. Nature 399: 415
13. Hanley-Bowdoin L, Settlage SB, Robertson D (2004) Reprogramming plant gene expression – a prerequisite to geminivirus DNA replication. Mol Plant Pathol 5: 149–156
14. Hehnle S, Wege C, Jeske H (2004) Interaction of DNA with the movement proteins of geminiviruses revisited. J Virol 78: 7698–7706
15. Kunik T, Salomon R, Navot N, Zeidan M, Michelson I, Zamir D, Gafni Y, Czosnek H (1994) Transgenic tomato plants expressing the tomato yellow leaf curl virus capsid protein are resistant to the virus. BioTechnology 12: 500–504
16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
17. Lapidot M, Friedmann M (2002) Breeding for resistance to whitefly-transmitted geminiviruses. Ann Appl Biol 140: 109–127
18. Lapidot M, Friedmann M, Pilowsky M, Ben-Joseph R, Cohen S (2001) Effect of host plant resistance to *Tomato yellow leaf curl virus* (TYLCV) on virus acquisition and transmission by its whitefly vector. Phytopathology 91: 209–1213
19. Mayer MP (2005) Recruitment of Hsp70 chaperones: a crucial part of viral survival strategies. Rev Physiol Biochem Pharmacol 153: 1–46
20. Mitter N, Sulistyowati E, Graham MW, Dietzgen RG (2001) Suppression of gene silencing, a threat to virus-resistant transgenic plants. Trends Plant Sci 6: 246–247
21. Morin S, Ghanim M, Zeidan M, Czosnek H, Verbeek M, van den Heuvel JFJM (1999) A GroEL homologue from endosymbiotic bacteria of the whitefly *Bemisia tabaci* is implicated in the circulative transmission of *Tomato yellow leaf curl virus*. Virology 256: 75–84
22. Morin S, Ghanim M, Sobol I, Czosnek H (2000) The GroEL protein of the whitefly *Bemisia tabaci* interacts with the coat protein of transmissible and non-transmissible begomoviruses in the yeast two-hybrid system. Virology 276: 404–416
23. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15: 473–479
24. Navot N, Pichersky E, Zeidan M, Zamir D, Czosnek H (1991) *Tomato yellow leaf curl virus*, a whitefly-transmitted geminivirus with a single genomic component. Virology 185: 151–161
25. Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163: 85–87
26. Prins M (2003) Broad virus resistance in transgenic plants. Trends Biotech 21: 373–375
27. Reid PD, del Campillo E, Lewis LN (1990) Anatomical changes and immunolocalization of cellulase during abscission as observed on nitrocellulose tissue prints. Plant Physiol 93: 160–165
28. Rudolph C, Schreier PH, Uhrig JF (2003) Peptide-mediated broad-spectrum plant resistance to tospoviruses. Proc Natl Acad Sci USA 100: 4429–4434
29. Sanford JC, Johnston SA (1985) The concept of parasite-derived resistance – deriving resistance genes from the parasite's own genome. J Theor Biol 113: 395–405
30. Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. Nature 407: 319–320
31. Stadler R, Wright KM, Lauterbach C, Amon G, Gahrtz M, Feuerstein A, Oparka KJ, Sauer N (2005) Expression of GFP-fusions in *Arabidopsis* companion cells reveals non-specific protein trafficking into sieve elements and identifies a novel post-phloem domain in roots. Plant J 41: 319–331
32. Truernit E, Sauer N (1995) The promoter of the *Arabidopsis thaliana SUC2* sucrose-H^+ symporter gene directs expression of β-glucuronidase to the phloem: evidence for phloem loading and unloading by *SUC2*. Planta 196: 564–570

33. van den Heuvel JFJM, Verbeek M, van der Wilk F (1994) Endosymbiotic bacteria associated with circulative transmission of *Potato leafroll virus* by *Myzus persicae*. J Gen Virol 75: 2559–2565
34. Voinnet O, Pinto YM, Baulcombe DC (1999) Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc Natl Acad Sci USA 96: 14147–14152
35. Wilson TMA (1993) Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. Proc Natl Acad Sci USA 90: 3134–3141
36. Wright KM, Roberts AG, Martens HJ, Sauer N, Oparka KJ (2003) Structural and functional vein maturation in developing tobacco leaves in relation to *AtSUC2* promoter activity. Plant Physiol 131: 1555–1565
37. Yang Y, Sherwood TA, Patte CP, Hiebert E, Polston JE (2004) Use of tomato yellow leaf curl virus (TYLCV) Rep gene sequences to engineer TYLCV resistance in tomato. Phytopathology 94: 490–496
38. Zeidan M, Czosnek H (1991) Acquisition of *Tomato yellow leaf curl virus* by the whitefly *Bemisia tabaci*. J Gen Virol 72: 2607–2614