Broomrape Can Acquire Viruses from Its Hosts

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ABSTRACT

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Broomrapes (*Phelipanche*, formerly *Orobanchae*) are parasitic plants that physically connect with the vascular systems of their hosts through haustorial structures. In this study, we found that *Cucumber mosaic virus* (CMV), *Tomato mosaic virus* (ToMV), *Potato virus Y* (PVY), and *Tomato yellow leaf curl virus* (TYLCV) translocate from infected host plants to *Phelipanche aegyptiaca*. In order to examine whether these viruses, and specifically CMV, replicate in the parasite, we tested several replication parameters. We detected accumulation of both plus and minus strands of CMV genomic RNA and CMV-derived siRNAs in the shoots of *Pheli-*

panche grown on CMV-infected tobacco and tomato plants. We purified CMV particles from *Phelipanche* grown on CMV-infected plants. These particles were present in amounts comparable to those found in the hosts' leaves. These data indicate that CMV replicates in *Phelipanche* tissues. In addition, viable ToMV and PVY were observed, and the plus and minus strand RNAs of ToMV were detected in *Phelipanche* shoots grown on infected hosts. However, we found only low levels of ToMV coat protein and did not detect any PVY coat protein. We also detected genomic TYLCV DNA in shoots of *Phelipanche* grown on TYLCV-infected tomato. Thus, for the first time, we demonstrate that broomrape is a host for at least one plant virus CMV, and possibly various other viruses.

Additional keywords: parasitic weed.

Parasitic plants display a wide range of parasitic strategies, from obligate holoparasitism to facultative hemiparasitism (2,24). These plants and their hosts are physically linked by haustorial structures (26), which connect the parasite with the host's vascular system. Several parasitic weeds form connections with their hosts that contain both xylem and phloem bridges (15). The translocation and uptake of molecules and macromolecules from host plants by the parasites, especially *Cuscuta* sp., has been well documented. The movement of nucleic acids from host to parasite (12) or from parasite to host (35) is facilitated by direct contact between the host and the parasite. Physiological studies have documented the movement of radiolabeled sugar (1), herbicides (25,36,46), and proteins between hosts and various parasitic plant species (4,18,19). Additionally, specific tomato mRNA transcripts have been shown to move through the phloem of a host plant into *Cuscuta* sp. (44), and a silencing signal generated in transgenic host (siRNA) has been shown to silence exogenous transgene GUS in the *Orobanchaceae* parasite *Triphysaria* (48). Recently, we demonstrated the silencing of the parasitic M6PR endogenous gene as a result of the movement of a silencing signal from transgenic tomato roots into *Phelipanche aegyptiaca* Pers. (3).

Until recently, very little was known about the translocation of viruses and macromolecules such as nucleic acids or proteins from host plants to *Phelipanche* sp. (genus formerly named *Orobanchae*). An early study (5–7) showed that viruses could be transmitted from an infected plant to a healthy plant through a dodder (*Cuscuta* spp.) plant that parasitized both the infected and the

healthy host plants, forming a bridge between them. Hosford (23) demonstrated the transmission of viruses from various taxonomic groups to different hosts by *Cuscuta* sp. However, dodder is a shoot parasite whose anatomy and haustorial connections are different from those of root-parasitic plants and the acquisition and transmission of viruses among different host plants has not been reported for root parasites such as *Phelipanche*.

Plant viruses move from cell to cell via cellular connections (plasmodesmata) in an active process that is mediated by specialized virus-encoded movement proteins (8,21,32). Some viruses (i.e., *Tobacco mosaic virus* [TMV]) can move as ribonucleoprotein complexes (20). Other viruses (e.g., potyviruses, cucumovirus, and geminiviruses) probably move as assembled virions, in which the coat protein is essential for the virus's long-distance movement.

In this study, we used three positive single-stranded (ss) RNA viruses and one ssDNA virus, representing four distinct genera: *Cucumovirus*, *Tobamovirus*, *Potyvirus*, and *Geminivirus*.

Cucumber mosaic virus (CMV) has a broad host range, which includes more than 800 plant species, and is transmitted by aphids in a nonpersistent manner. CMV virions are isometric particles 30 nm in diameter, and the CMV genome is organized into three single-stranded genomic RNAs (RNAs 1, 2, and 3) and two major subgenomic RNAs (RNAs 4 and 4A). RNAs 1 and 2 code for components of the replicase complex. RNA 2 also codes for the 2b protein (4A), which is involved in the suppression of gene silencing. RNA 3 encodes the 3a protein, which is essential for virus movement, and the coat protein (24 kDa), which is expressed from subgenomic RNA 4 (39).

Tomato mosaic virus (ToMV) is mechanically transmitted and infects tobacco, tomato, and other Solanaceae. The rod-shaped virus (300 nm in length) consists of a single-stranded, messenger-sense RNA of about 6,400 nucleotides, which is encapsidated by a

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single coat protein (CP). The RNA is capped at the 5' end and a tRNA-like structure exists at the 3' end. The RNA encodes two replicase proteins, 126K and its read-through 183K. In addition to replication, 126K is involved in cell-to-cell movement (30) and silencing suppression (14). In the 3' region, a movement protein and a 17.5-kDa coat protein are encoded. The replicase is translated from the genomic RNA that enters a cell, whereas the movement protein (MP) and CP are expressed from subgenomic RNAs that are produced during virus replication (20).

Potato virus Y (PVY) is a flexuous filamentous virus that mostly infects Solanaceae species and is transmitted by aphids in a nonpersistent manner. The 9.6-kb RNA genome is covalently linked to a viral-encoded protein (the VPg) at the 5' end and has a 3' poly-A tail. Translation of this RNA results in a polypeptide that is processed into 9 to 10 mature proteins (see review by Riechmann et al. [42]).

Tomato yellow leaf curl virus (TYLCV), a monopartite begomovirus, has a 20 × 28 nm twinned isometric particle containing a circular single-stranded DNA molecule of 2,787 nucleotides. This virus, which infects tomato (*Solanum lycopersicum* Mill.) and a few other plant species, is transmitted by whitefly (*Bemisia tabaci*) in a persistent and circular manner. The virus's ssDNA genome is replicated in phloem cell nuclei via a rolling circle mechanism. The TYLCV genome encodes two genes on the viral strand (the coat and movement proteins) and four genes on the complementary strand (see review by Czosnek [10]).

In this study, we demonstrate, for the first time, the movement of three RNA viruses (CMV, ToMV, and PVY) and an ssDNA geminivirus (TYLCV) into the root-parasitizing plant *P. aegyptiaca* from its hosts. We also present data supporting the replication of CMV in *P. aegyptiaca*. We conclude that parasitic weeds, such as *Phelipanche* species, are virus hosts, like many other plant species.

MATERIALS AND METHODS

Parasite, host plants, and material. *P. aegyptiaca* seeds were collected from an infested tomato field in the Golan Heights, Israel (16) and used to infect tomato and tobacco host plants. Tomato (*Solanum lycopersicum* (Mill.) cv. Marmande) and tobacco (*Nicotiana tabacum* L. cvs. Samsun NN, Xanthi, and Samsun) plants that served as host plants were maintained in a temperature-controlled greenhouse at 25°C with supplementary lighting. Tobacco (*N. tabacum* L. cvs. Xanthi, Samsun, and Samsun NN) and tomato (*S. lycopersicum* cv. Marmande) seedlings were used to maintain cultures of PVY, CMV, ToMV, and TYLCV, respectively, which were used as inocula.

Virus strains. Four viruses were tested: PVY strain-H (PVY^H accession no. M95491), which is closely related to a Hungarian strain (47); CMV (Fny-CMV) (43); ToMV (an Israeli strain closely related to ToMV1-2, accession no. DQ873692); and TYLCV (accession no. X15656) (37).

Field screening for virus-infected *Phelipanche*. During 2007 and 2008, a survey was conducted in a tomato field located in Lefkosia (Nicosia district), Cyprus, in order to identify TYLCV in *Phelipanche* species. *P. aegyptiaca* and *P. ramosa* were observed parasitizing tomato plants in this field. *Phelipanche* shoots (28 samples) were randomly collected from a TYLCV-infected tomato field in Cyprus (70 to 80%) and were screened for TYLCV infection in the lab. DNA was extracted from the shoots using a commercial kit (Qiagen Plant DNeasy Kit) and polymerase chain reaction (PCR) was performed according to the protocol described by Martinez-Culebras et al. [33]).

Parasite and virus inoculations. Tomato and tobacco seedlings (four to five leaves) were transplanted into 0.5-liter pots filled with soil (light to medium clay with 63% sand, 12% silt, and 22% clay) containing *P. aegyptiaca* seeds (20 mg seeds/liter soil, approximately 3,200 seeds/liter). A week after transplanting

to soil containing *P. aegyptiaca* seeds, plants were mechanically infected with CMV, ToMV, and PVY. Tomato seedlings (three to four leaves) were infected by TYLCV 2 weeks prior to being transplanted into soil containing *P. aegyptiaca* seeds. The inoculated plants were grown for 2 months in a greenhouse under natural light, with an average 14 h of daylight and an average temperature of 20 ± 6°C. Mechanical inoculation with CMV, ToMV, and PVY was performed by rubbing leaves after dusting with carborundum. Inocula were prepared by grinding young leaves from tobacco source plants in distilled water (1:5 wt/wt, leaves/water). TYLCV inoculation was performed by adult whiteflies after acquisition on TYLCV-infected tomato source plants for 48 h, as described by Lapidot et al. (29). Plants were watered and fertilized as necessary.

Host plants. To determine whether viable virus existed in *Phelipanche* tissues, back-inoculation was performed with *Phelipanche* extracts on susceptible test plants. Pools of three *Phelipanche* shoots (5 to 15 cm) grown on two to three host plants were ground (ca. 1:5 tissue/water), and the extracts were used for mechanical inoculation of systemic and local lesion host plants (with three to four leaves). CMV infectivity was evaluated in *Nicotiana glutinosa*, *N. tabacum* L. cv. Samsun NN, and *Chenopodium quinoa*. ToMV infectivity was evaluated in *N. tabacum* L. cv. Samsun, *N. benthamiana*, *N. glutinosa*, *C. quinoa*, and *C. amaranticolor*. PVY infectivity was evaluated in *N. glutinosa* and *N. tabacum* L. cv. Samsun NN. The appearance of symptoms on the treated plants was evaluated during the 2 weeks following the inoculations.

RNA isolation and reverse transcription (RT)-PCR analysis. Total RNA was extracted from four leaf disks (9 mm diameter) collected from tobacco and tomato leaves (leaves three and four from the top), as well as from *P. aegyptiaca* tubercles, shoots, and flower organs (ca. 100 mg fresh weight). *P. aegyptiaca* samples were pooled from three *Phelipanche* shoots grown on two to three different plants. RNA was isolated using the TRI-REAGENT kit (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's instructions. RNA concentrations were measured using a spectrophotometer (Nanodrop ND-1000, Nanodrop, Wilmington, DE) and comparable amounts of the different RNAs were subjected to RT-PCR analysis to detect the different viruses. RT-PCR analysis was used to examine the accumulation of CMV, ToMV, and PVY in host plants and *P. aegyptiaca*. The first-strand cDNA was synthesized using 1 µg of total RNA extract and complementary primers specific to each virus using Verso cDNA Kit (ABgene, Epsom, UK) according to the manufacturer's instructions. Two microliters of the resulting cDNA was used as a template for a standard PCR. cDNA was denatured at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 35 s, and a final 5 min at 72°C. For the PCR amplification of the CMV cDNA, we raised the annealing temperature to 62°C. The primers used to detect CMV, ToMV, and PVY were as follows. Forward primer 5'-YASY-TTTDRGGTTCAATTCC-3' and reverse primer 5'-GACTGAC-CATTTTAGCCG-3' were used for CMV RNA3 (935 nucleotide product). Forward primer 5'-ATGTCTTACAGTATCACTACTC-CA-3' and reverse primer 5'-GTTGCAGGACCAGAGGTCCAA-ACC-3' were used for ToMV coat protein gene (475 nucleotide product). And, forward primer 5'-GTGTATCCCTGTTGTTGAC-3' and reverse primer 5'-CCACCAACTCTATAGTGCTTAATG-3' were used for PVY HC-Pro (511 nucleotide product).

DNA extraction, PCR, and rolling circle amplification analysis. PCR and rolling circle amplification (RCA) were used to detect TYLCV in *Phelipanche*. Total DNA was extracted from TYLCV-infected tomato (four leaf disks, each 9 mm in diameter, ca. 150 mg) and from *Phelipanche* shoots (ca. 200 mg) according to the protocol described by Delaporta et al. (13). The extracted DNA (1 µl) was diluted in 25 µl of a PCR mixture containing the primer pair TYC1F (5'-GGGCCTAGAGACCTGGCCCCAC-3')

and TYC1R (5'-CCGGTAATATTATACGGATGGC-3'), as used by Lapidot et al. (29).

The PCR conditions were as follows: one cycle of 4 min at 94°C, followed by 30 cycles at 94, 58, and 72°C, each of 30 s, and 5 min at 72°C. We used one unit of Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) per reaction for the amplification of TYLCV.

The circular genomic DNA of TYLCV was amplified by RCA (17) using the TempliPhi amplification kit (GE Healthcare, Chalfont St. Giles, UK) as follows. One microliter of TYLCV-infected plant total DNA preparation was added to 5 µl of the sample buffer, the mixture was heated to 95°C for 3 min to denature the DNA, and then snap-chilled on ice and combined with 5 µl of reaction buffer plus 0.2 µl of enzyme mix (containing Phi29 DNA polymerase and random hexamers in 50% glycerol). The reaction mixture was then incubated for 18 h at 30°C, followed by inactivation of the enzyme at 65°C for 10 min. To detect TYLCV, the products of the RCA reaction (1 µl) were digested using the *ApaI* restriction enzyme. The following bands were expected from the TYLCV digestion using the *ApaI* restriction enzyme: 1,300, 900, and 600 bp. The TYLCV digestion pattern was confirmed by agarose gel analysis.

CMV purification. CMV was purified from tobacco (*N. tabacum* cv. Xanthi NN) leaves 2 months after inoculation (100 g) and from pools of *Phelipanche* shoots (ca. 88 g) grown on three infected tobacco plants, as previously described by Palukaitis et al. (40). CMV particles were observed using EM Tecnai G² Spirit TEM (FEI Company, Philips, Netherlands).

Detection of viral coat protein by western blot analysis. At 65 days postinfection, protein samples were collected from the virus-infected host plants (tobacco or tomato) and from *Phelipanche* grown on those plants. Total protein was extracted by grinding pooled samples of six leaf disks (9 mm diameter; ca. 70 mg total fresh weight) taken from the young tobacco and tomato leaves, as well as pooled samples from *Phelipanche* shoots (70 mg fresh weight). The protein samples were extracted in 150 µl of ESB buffer (75 mM Tris/HCl, pH 6.8; 9 M urea; 4.5% [vol/vol] sodium dodecyl sulfate [SDS]; and 7.5% [vol/vol] β-mercaptoethanol). The samples were boiled for 5 min and were centrifuged for 10 min at 10,000 × *g*. Then, a 1:1 mixture of the supernatant was mixed with 2× SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer and 10 µl of supernatant from each sample was fractionated by SDS-PAGE on a 15% polyacrylamide gel. The fractionated proteins were electroblotted onto nitrocellulose membranes (Amersham Biosciences, Sweden), blocked with 1% nonfat dry milk for 1 h and probed with rabbit antiserum specific to either LS-CMV CP (1:2,000 dilution) or ToMV CP (1:5,000 dilution) overnight at 4°C. The membranes were washed three times with Tris-buffered saline–Tween and then incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:4,000 dilution). The viral capsid proteins were visualized by incubating the membranes with the chromophore substrates NBT and BCIP (Promega, WI) according to the manufacturer's instructions.

Detecting the accumulation of CMV siRNA in the host and parasite. To test for the presence of CMV siRNA in *Phelipanche*, total RNA was extracted from *Phelipanche* shoots that were grown on three plants and then ground to a fine powder with liquid nitrogen. RNA samples (10 µg) were separated on 15% PAGE containing 7 M urea in a 0.5× Tris-borate EDTA as described by Shiboleth et al. (45). The separated RNAs were transferred to a Hybond NX membrane (Amersham Biosciences) and cross-linked to the membrane with highly sensitive EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), as described by Pall et al. (38). The blot was hybridized with a ³²P-labeled transcript. Sense transcripts were produced using an AmpliScib T7-FlashTH Transcription Kit (EPICENTER, WI) on a linear template of a pGEM-T plasmid harboring part of RNA3 of the CMV

fragment (935 bp). Prehybridization and hybridizations were performed as described previously (45). The membranes were exposed to a phosphorimager screen (Fuji) for about 3 h.

RESULTS AND DISCUSSION

Phelipanche spp. (broomrape) is a parasitic plant that subsists on the roots of host plants, to which it connects via direct bridges of xylem and phloem tissue (15). These bridges allow the parasite to acquire water, minerals, carbohydrates, proteins, and probably nucleic acids (4,19,22,44) as well. To date, viral infection has not been reported in this parasitic organism, even though plant RNA viruses such as CMV have been shown to multiply in parasitic *Cuscuta* (dodder) (7,9). Accordingly, our study concentrated on the acquisition of CMV by *P. aegyptiaca* from infected hosts.

Tobacco and tomato plants infected with Fny-CMV (subgroup I) showed typical leaf mosaic symptoms when grown in soil containing *P. aegyptiaca* seeds (Fig. 1A and B). Tobacco leaves infected with CMV showed a variety of symptoms. Some infected leaves showed typical mosaic symptoms, while others did not display any visible symptoms. Tomato plants infected with CMV showed severe symptoms (leaf deformation with mosaic symptoms and chlorosis were observed; Fig. 1A). No differences were observed in the symptoms of CMV-infected tomato and tobacco plants grown with or without the parasite *P. aegyptiaca* (data not shown). In addition, the height of CMV-infected tobacco plants was not affected by the presence of the parasitic plant (Table 1). However, the number of *Phelipanche* shoots per plant was significantly higher in uninfected tobacco than in CMV-infected plants 45 days postinoculation (dpi). However, at 65 dpi more parasitic shoots were observed in CMV-infected tobacco and the total number of *Phelipanche* shoots per plant was similar to that of the control, uninfected tobacco plants (Table 1), indicating a delay in the appearance of *P. aegyptiaca* on CMV-infected tobacco plants.

CMV infection has been shown to reduce the accumulation of carotenoids (41). Thus, it is possible that the delay in the appearance of shoots of *Phelipanche* grown on CMV-infected tobacco is due to an effect of CMV on secondary metabolite stimulators such as strigolactones, which are produced via the carotenoid biosynthesis pathway (31). In addition, some inflorescences of *Phelipanche* grown on CMV-infected tobacco developed abnormally (deformed shoots and short nodes). In comparison, these abnormalities were not observed in the *Phelipanche* inflorescences on healthy host plants (Fig. 1D). As mentioned, these abnormalities appeared only in some of the inflorescences. Similarly, CMV-infected dodder grown on tobacco showed a range of growth abnormalities (9).

To confirm the presence of infectious CMV in *P. aegyptiaca* tissues, we back-inoculated both systemic (*N. tabacum* and *N. glutinosa*) and local lesion (*C. quinoa*) host plants with extract from *P. aegyptiaca*. Back-inoculation of *N. tabacum* with sap extract from parasitic shoots grown on tobacco and tomato plants resulted in systemic infectivity rates of 100 and 60%, respectively (Table 2, CMV). These infectivity rates were similar to those observed for CMV-infected tobacco and tomato leaves (Table 2, CMV). Back-inoculation of *C. quinoa* led to the formation of typical local lesions, similar to those obtained when the plants were inoculated with extract from CMV-infected tobacco. These data indicate that the *P. aegyptiaca* grown on tobacco and tomato infected with CMV acquired the virus from its host and that viral infectivity was maintained. Similarly, in an earlier study, the accumulation of viable CMV in dodder stems was observed through the back-inoculation of *N. tabacum* and *N. glutinosa* (7,9).

Additionally, we were able to detect CMV RNA in *Phelipanche* shoots grown on tobacco and tomato plants infected with CMV, using RT-PCR with specific primers corresponding to CMV

RNA3 (Fig. 2). To test the distribution of CMV in the parasite's organs, total RNA from the tubercles, shoots, and flowers (stamen and stigma) of *P. aegyptiaca* grown on infected tobacco was extracted and subjected to RT-PCR (Fig. 2A). CMV RNA was detected in all of the examined *P. aegyptiaca* organs (Fig. 2A), indicating long-distance movement of the virus acquired from the host.

To examine CMV replication in *Phelipanche* cells, we tested the accumulation of CMV minus strand (Fig. 2C) using the same primers used for the analysis of CMV RNA3, in which the forward primer was used for first-strand synthesis. We were able to detect the minus strand of CMV RNA3 in all of the samples of *Phelipanche* shoots grown on CMV-infected tobacco (Fig. 2C) and tomato (data not shown). This result may indicate that CMV replicates in the parasite's cells. Interestingly, an indication of the multiplication of CMV in dodder was reported in earlier studies involving transfer inoculation and mechanical inoculation (7,9).

The existence of viral small RNA (vsiRNA) in plant cells is additional, indirect evidence for virus replication, since vsiRNA is processed from dsRNA by the DCLs, which are part of the host's gene-silencing machinery (14). The accumulation of CMV siRNA has been demonstrated in several hosts of CMV (14). In our study, CMV-specific siRNA was detected in CMV-infected host plants and in shoots of *P. aegyptiaca* grown on tomato (Fig. 2D) and

tobacco (data not shown). The detection of the minus strand of CMV siRNA using plus strand-labeled RNA transcript as a probe (Fig. 2D) provides additional evidence for the accumulation of minus-strand CMV RNA in the parasite's tissue. Our observation

TABLE 1. Effect of *Cucumber mosaic virus* (CMV)-infected tobacco on the appearance of *Phelipanche aegyptiaca* shoots

	Tobacco height (cm)	Number of shoots	
		45 dpi ^a	65 dpi
Virus-free			
1 ^b	104	28	28
2	106	37	37
3	75	26	26
4	77	20	20
5	85	36	36
CMV-infected			
1	120	6	28
2	130	6	47
3	86	0	14
4	110	10	25
5	95	0	23

^a Days after inoculation with *P. aegyptiaca*.

^b Plant replicates.

Fig. 1. Effect of *Cucumber mosaic virus* (CMV)-infected tobacco and tomato plants on *Phelipanche aegyptiaca* development. **A**, *P. aegyptiaca* growing on infected tomato (CMV) and uninfected (healthy) tomato. **B**, *P. aegyptiaca* growing on tobacco infected with CMV and mosaic symptoms on secondary tobacco shoots. **C and D**, Representative inflorescences of *P. aegyptiaca* grown on tobacco infected with CMV and control healthy tobacco. Pictures were taken 65 days after inoculation with *Phelipanche* seeds. **E**, Close-up picture of *Phelipanche* spp. growing on a Tomato yellow leaf curl virus-infected tomato plant in the field.

of the accumulation of tasi mir390, which is processed by DCL4 in the cytosol from a nontranslated mRNA (27), in the parasite shoot (3) provides additional support for the existence of silencing machinery in *Phelipanche*.

Western blot analysis of total protein extracted from *P. aegyptiaca* shoots grown on tobacco plants infected with CMV detected the accumulation of CMV CP in *Phelipanche* (Fig. 3). We observed differences in the accumulation of CMV CP in two

TABLE 2. Infectivity rates of viruses from the shoots of *Phelipanche aegyptiaca* grown on plants infected with *Cucumber mosaic virus* (CMV), *Tomato mosaic virus* (ToMV), and *Potato virus Y* (PVY)

Virus	Source of inocula	Back-inoculation on a susceptible host ^a				
		<i>Nicotiana tabacum</i>	<i>N. benthamiana</i>	<i>N. glutinosa</i>	<i>Chenopodium quinoa</i>	<i>C. amaranticolor</i>
CMV	Tomato	6/9		3/5	+	
	<i>Phelipanche</i>	18/29		2/5	+	
	Tobacco	5/5		n.t.	+	
	<i>Phelipanche</i>	5/5		n.t.	+	
ToMV	Tomato	8/8	4/4	4/4	n.t.	n.t.
	<i>Phelipanche</i>	11/11	7/7	4/4	n.t.	n.t.
	Tobacco	12/12	4/4	n.t.	+	+
	<i>Phelipanche</i>	14/17	2/4	n.t.	+	+
PVY	Tobacco	4/4		4/4		
	<i>Phelipanche</i>	1/12		1/10		

^a n.t. = not tested. + = local lesions were observed but not counted.

Fig. 2. Accumulation of *Cucumber mosaic virus* (CMV) RNA in *Phelipanche aegyptiaca* grown on CMV-infected host plants. Total RNA was extracted from host plants and *Phelipanche* sp. The presence of CMV RNA was determined by reverse transcription-polymerase chain reaction analysis using one set of primers for the detection of plus and minus strands of RNA3 of CMV. **A**, Detection of CMV RNA3 in tubercles, shoots, and stamen and stigma (Stam+Stig) of *Phelipanche* sp. grown on CMV-infected (+) and virus-free tobacco plants (-). Detection of **B**, plus strand and **C**, minus strand CMV RNA3 in *Phelipanche* (Ph) grown on tomato (Tom) infected with CMV (+) and uninfected tomato (-). Arrows indicate the size of the expected cDNA fragment. **D**, CMV siRNA accumulated in *P. aegyptiaca* shoots grown on tomato and tobacco plants infected with CMV. Total RNA was extracted from CMV-infected tomato (Tom) and tobacco (Tob), and from *Phelipanche* shoots (Ph). Ten-microgram aliquots of RNA from each sample were separated with urea-polyacrylamide gel electrophoresis gel. The blotted membrane was hybridized with CMV RNA3 ³²P-labeled transcript. (The arrow indicates the 21 nucleotide siRNA size marker, which was stained with ethidium bromide prior to the hybridization.)

tobacco plants (Tob1 and Tob2 Fig. 3A). This could be explained by the slight difference in the quantities loaded in the gel and/or by differences in the accumulation of the virus in different tobacco leaves. However, the difference in the accumulation of CP in the different samples of tobacco leaves was less pronounced than the difference in the accumulation of CP in different *Phelipanche* shoots (Ph 1 and Ph 2, Fig. 3A).

The detection of CMV RNAs and CP in *P. aegyptiaca* led us to verify the accumulation of CMV particles in *Phelipanche* organs. CMV particles were isolated from tobacco leaves and from aerial *Phelipanche* organs (shoots and flowers) 55 days after the plants were inoculated with CMV, using common purification methods. Purified CMV virions were successfully extracted from *Phelipanche* organs, and the concentration of 16 mg/ml was equivalent to 170 mg/kg from parasitic tissues compared with 20 mg/ml (220 mg/kg) from tobacco leaves. The comparable levels of virus accumulation in the host and the parasite may indicate similar replication rates in the different plants or similar stability in the cells of tobacco and *Phelipanche*. Transmission electron microscopy confirmed the presence of typical isometric CMV particles

in the examined aerial organs of *Phelipanche* (Fig. 4), which were comparable to particles isolated from tobacco leaves.

These results demonstrate, for the first time, that the parasite *P. aegyptiaca* is a host for CMV. Therefore, we decided to examine the susceptibility of *Phelipanche* to additional viruses belonging to different groups, such as ToMV (type member of the tobamoviruses), PVY (type member of the potyviruses), and TYLCV (a geminivirus). *Phelipanche* grown on ToMV-infected tomato and tobacco did not show any visible developmental abnormalities, as were observed in parasite tissue infected with CMV. In this case, the parasitic *Phelipanche* was not altered by its association with ToMV-infected tomato or tobacco plants (data not shown).

Back-inoculation of test plants with extracts from *Phelipanche* shoots grown on ToMV-infected tomato and tobacco demonstrated systemic infectivity in *N. tabacum* and *N. glutinosa* and induced the formation of local lesions in *C. quinoa* and *C. amaranticolor* (Table 2, ToMV). RT-PCR analysis detected the presence of plus and minus strands of ToMV RNA in *Phelipanche* grown on tomato infected with ToMV (Fig. 5). ToMV coat protein could barely be detected in *Phelipanche* shoots grown on ToMV-

Fig. 3. Cucumber mosaic virus (CMV) coat protein (CP) accumulated in *Phelipanche aegyptiaca* grown on CMV-infected tobacco. **A**, Immunoblotting of CMV CP in tobacco leaves (Tob) and *Phelipanche* shoots (Ph) 65 days after inoculation with the parasite seeds. Total protein was extracted from CMV-infected and uninfected tobacco leaves (200 mg from two plants, Tob1 and Tob2) and from *Phelipanche* shoots (200 mg from Ph1 and Ph2) grown on those tobacco plants. Leaf extracts from virus-free tobacco (Healthy) and *Phelipanche* that grew on this plant served as controls. Fifteen microliters from each sample were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the blotted membrane was immunoblotted with CMV antibodies (anti-CP) at a 1:2,000 dilution. **B**, Ponceau staining demonstrated the amount of total protein loaded. Purified CMV virus (Vir) served as a positive control. Arrows indicate the sizes of the nearest protein marker (M) bands.

Fig. 4. Cucumber mosaic virus (CMV) particles accumulated in *Phelipanche aegyptiaca* shoots. Electron micrographs of CMV particles from purified preparations of CMV-infected tobacco and *Phelipanche* shoots that grew on those tobacco plants. Magnification factor: $\times 135,000$. Bars represent 100 nm.

infected tomato (Fig. 5C). Very low accumulation of coat protein was observed in *Phelipanche* shoots grown on one out of two tomato plants, as compared to the high level of coat protein accumulation observed in host tissue infected with ToMV (Fig. 5C). A small amount of ToMV coat protein was found in *Phelipanche*, and the fact that we could not observe virus particles in the dip preparation examined under the transmission electron microscope may indicate a very low rate of replication of ToMV in the parasite. Alternatively, ToMV may not replicate in the parasite's cells and the detection of ToMV plus and minus strands may have been the result of the movement of nonencapsidated RNA from the ToMV-infected host into *Phelipanche*.

TMV RNA can move as a ribonucleoprotein complex (20). Therefore, it is possible that the movement of ToMV from the host to the parasite *Phelipanche* is based on a mechanism similar to that reported for the movement of host-mobile mRNA transcripts into dodder (11,44). Interestingly, Costa (9) demonstrated a similar result. That study showed that TMV does not persist in dodder, suggesting the passive movement of TMV with the reversals of phloem nutrient flow, allowing virus transmission between plants.

Tobacco plants infected with PVY^H showed severe developmental defects, such as stunting and leaf necrosis around the veins. *Phelipanche* shoots grown on PVY-infected tobacco appeared late, as observed with CMV. Moreover, the number of shoots per host plant was about one-third that of *Phelipanche* grown on virus-free tobacco 60 dpi (data not shown). The pres-

ence of PVY in *Phelipanche* shoots was determined by back-inoculation and by RT-PCR, as with ToMV and CMV. Extracts from *Phelipanche* shoots grown on PVY-infected tomato were not infectious on tobacco test plants, and a low rate of infectivity (2:22) was observed for extracts from *Phelipanche* grown on PVY-infected tobacco (Table 2). However, back-inoculation with an extract from host plant leaves infected with PVY exhibited 100% infectivity on test plants. In addition, RT-PCR analysis detected PVY RNA in *Phelipanche* grown on tobacco plants infected with PVY, but not in *Phelipanche* grown on tomato plants infected with PVY (Fig. 6A). The weaker cDNA band of PVY obtained from the RT-PCR analysis of *Phelipanche* grown on tobacco plants (Fig. 6A) is representative of similar results obtained in three biological repeats. These results may indicate a low level of PVY in *Phelipanche* grown on PVY-infected host plants. The existence of PVY in parasitic plants has not been described previously, although the transmission of a potyvirus by dodder has been reported (23). The above-mentioned RNA viruses (CMV, ToMV, and PVY) replicate and accumulate in the cytosol of plant cells (i.e., mesophyll). Therefore, we decided to examine additional virus such as geminivirus, which are restricted to phloem cells.

Phelipanche physically attaches to hosts by haustorial structures, through which nutrients from the host xylem and phloem enter the parasite. Such a connection is predicted to allow the trafficking of small geminivirus particles especially TYLCV which are found in roots (34).

Fig. 5. Plus and minus strands of *Tomato mosaic virus* (ToMV) RNA accumulated in *Phelipanche aegyptiaca* grown on ToMV-infected tomato. **A**, Plus and **B**, minus strands of ToMV RNA were detected in *Phelipanche* (Ph) and tomato (Tom) when reverse transcription-polymerase chain reaction (RT-PCR) was performed with primers specific for ToMV coat protein. Total RNA and total protein were extracted from ToMV-infected tomato leaves (100 mg) and the shoots of virus-free tomato (healthy) and *Phelipanche* (100 mg) grown on ToMV-infected and uninfected tomato plants. RT-PCR products were separated on 1% agarose gel (**A** and **B**). **C**, The presence of ToMV coat protein was determined by western blotting. Total proteins (10 μ l from 100 mg of extract) were separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred to nitrocellulose membrane, and immunoblotted with ToMV antibodies (anti-CP) at a 1:5,000 dilution (**C**). Arrows indicate the sizes of the amplified ToMV fragment (nucleotides) and the nearest protein marker bands.

TYLCV-infected tomato plants showed symptoms typical of viral infection, such as curled leaves and stunted growth. The shoots of *Phelipanche* grown on infected tomato appeared later than those of *Phelipanche* grown on virus-free tomato, and the number of shoots per plant was reduced. The presence of TYLCV in *Phelipanche* was determined by PCR and by RCA. Total DNA was extracted from *Phelipanche* shoots grown on four tomato plants. Using TYLCV specific primers, a predicted fragment was PCR-amplified from the four TYLCV-infected tomato plants, but not from the virus-free tomato plants (Fig. 6B). In four biological replicates, the intensities of the PCR-amplified fragment from *Phelipanche* shoots were lower than the intensities of those from infected tomato leaves after 30 cycles (Fig. 6B). We do not have an explanation for the lower bands that appeared in the third sample (Or3), although similar faint PCR bands can be seen in tomato leaves from plant No. 3 (Fig. 6B, tom3). Using RCA amplification technology, the intact circular ssDNA of TYLCV was identified in *Phelipanche* shoots. The amplified ssDNA obtained from *Phelipanche* shoots and tomato leaves was digested by *Apa*I. Fragments of a similar size were obtained from TYLCV-infected tomato leaves and *Phelipanche* shoots, and these fragments provide further evidence for the movement of circular TYLCV ssDNA molecules from host to parasite (Fig. 6C); however replication of TYLCV in the parasite needs to be confirmed.

These data were strengthened by our finding of *Phelipanche* plants that had been found to harbor TYLCV in the field. In collaborative work carried out during 2007 and 2008, a field survey

was conducted to identify weed hosts of *Tomato yellow leaf curl virus* (TYLCV) in Cyprus. In the Kokkinotrimithia area, which is located in the Nicosia district, we observed that *Phelipanche* species (*P. ramosa* or *P. aegyptiaca*) were parasitizing tomato plants that were infected with TYLCV. Typical TYLCV symptoms (leaf curling, yellowing and stunting) were observed in a tomato field that had a 70 to 80% infection incidence (Fig. 1E). However, *Phelipanche* weeds growing on those TYLCV-infected tomato plants did not show significant symptoms (Fig. 1E). PCR analysis indicated that 12 of 28 *Phelipanche* samples collected in the field survey were infected with TYLCV. This is the first documented case of virus-infected *Phelipanche* in a field. Similarly, TYLCV was also detected in *Cuscuta* in a field survey in Spain (28).

Plasmodesmata have been shown to exist in *Phelipanche* (24). Therefore, we assume that cell-to-cell movement of plant viruses in *Phelipanche* is probably similar to the movement of viruses in photosynthetic plants. Viruses' penetration of host cells via haustorial connections has not been reported. This penetration probably involves symplasmic connections, such as the interspecific plasmodesmata observed between host and parasite phloem (15). Additionally, we observed that, unlike dodder, *Phelipanche* is not a host to all viruses. This is normal and characteristic of photosynthetic host plants. The accumulation of viruses in the seeds of a parasitic plant and transmission of viruses via these seeds has been observed in *Cuscuta campestris* (6) and should be studied further in *Phelipanche* species.

Fig. 6. *Potato virus Y* (PVY) RNA and *Tomato yellow leaf curl virus* (TYLCV) DNA accumulated in *Phelipanche aegyptiaca* grown on PVY-infected tobacco and TYLCV-infected tomato. **A**, Detection of PVY RNA by reverse transcription-polymerase chain reaction (RT-PCR) in *Phelipanche* (Ph) grown on tobacco plants (Tob). Total RNA (100 mg) was extracted from PVY-infected tobacco leaves (+) from virus-free plants (-) and from *Phelipanche* shoots (Ph) grown on PVY-infected (+) and uninfected (-) host plants. **B**, Detection of TYLCV DNA by PCR from four tomato plants and the *Phelipanche* shoots that grew on those plants (Or1-Or4). Total DNA (100 mg) was extracted from TYLCV-infected tomato leaves (+), virus-free plants (-), and *Phelipanche* shoots (Ph) grown on PVY- or TYLCV-infected (+) and uninfected (-) host plants. **C**, Samples of TYLCV ssDNA from infected tomato plants (Tom1) and the *Phelipanche* shoots that grew on these plants (Ph1) were assayed using the rolling cycle amplification method. Arrows indicate DNA nucleotide size markers (M).

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