Genetic Transformation of Passionflower and Evaluation of R₁ and R₂ Generations for Resistance to *Cowpea aphid borne mosaic virus*

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Abstract

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We report on the production and evaluation of passionflower transgenic lines for resistance to *Cowpea aphid borne mosaic virus* (CABMV). Genetic transformation was done using *Agrobacterium tumefaciens* and transgene integration was confirmed by Southern blot analyses, resulting in nine transgenic lines for 'IAC 275' and three for 'IAC 277'. Transgenic lines were clonally propagated and evaluated for resistance to CABMV. After the third inoculation, under higher inoculum pressure, only propagated plants of the transgenic line T16 remained asymptomatic, indicating a high resistance to infection with CABMV. This transgenic line was self-pollinated and the R₁ generation was evaluated together with the R₁ generation of another resistant

transgenic line (T2) identified previously. Plants were inoculated with CABMV by means of viruliferous *Myzus nicotianae*. All 524 T2R₁ plants became infected, whereas 13 of 279 T16R₁ remained asymptomatic after four successive inoculations. A T16R₂ generation was obtained and plants were inoculated with CABMV mechanically or by aphids. After successive inoculations, 118 of 258 plants were symptomless, suggesting that the resistance to CABMV was maintained in the plant genome as the homozygous condition was achieved. Five selected resistant T16R₂ plants which contained the capsid protein gene are being crossed for further analyses.

Brazil is the world's largest producer of passion fruit, with an estimated planted area of 44,300 ha and fruit yield of 615,196 t (12). Yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) is the predominant species (98%), with the remaining area (2%) planted with sweet passion fruit (*P. alata*). However, this production is threatened by several pathogens, including the *Potyvirus* sp. *Cowpea aphid borne mosaic virus* (CABMV) (4,23,24), which was previously misidentified as *Passion fruit woodiness virus* (6,7). The disease caused by CABMV was first detected in Brazil in the late 1970s in the State of Bahia (6), and then spread throughout the country (3,7,11,16). The virus is transmitted in a nonpersistent manner by several aphid species, and also mechanically, during pruning. The disease causes severe yield losses and reduces the orchard lifespan by approximately half (13).

The control of passion fruit woodiness disease in passionflower orchards is problematic. All cultivars of yellow and sweet passion fruit are susceptible to infection, and chemical vector control is usually inefficient because of the nonpersistent manner of transmission. Cross protection with mild strains of the virus have also failed to control the disease (26). Recently, two Brazilian hybrids (BRS Ouro Vermelho and BRS Sol do Cerrado) tolerant to this disease were introduced into the market but are still being evaluated by growers (<http://www.cpac.embrapa.br/maracuja/inicio/>). In Brazil, at present, several cultural practices have been recommended to minimize the problems associated with CABMV in

passionflower orchards. These include the use of virus-free seedlings for new plantings, eradication of old and abandoned orchards before starting new crops, care during pruning operations to prevent mechanical transmission of the virus, avoidance of leguminous plants which may harbor the virus in the proximity of the orchard, and roguing of diseased plants by means of systematic inspections during the first 5 months after transplanting (11,13).

Attempts to develop CABMV-resistant transgenic passionflower which can provide a more effective and long-lasting alternative for woodiness control have been studied in Brazil for many years (1,29). Transgenic passionflower plants (R₀) containing the 3' region of the *Nib* gene and the 5' region of the capsid protein (CP) gene of Brazilian isolate CABMV-MG1 showed resistance to the transgene donor isolate but not to isolate CABMV-PE1 (1). Using a full-length CP gene from a severe CABMV isolate from the State of São Paulo, Brazil, Trevisan et al. (29) obtained seven transgenic lines showing different numbers of insertion events for the CP gene. The results of three successive mechanical inoculations of the transgenic plants using three Brazilian isolates of CABMV from different geographic origins showed that the primary transgenic line, T2, of 'IAC-277' was resistant to all isolates.

Because passionflower is a self-incompatible species, fruit yield is dependent on cross-pollination among flowers from plants with different genetic backgrounds. Therefore, the development of a commercial resistant transgenic cultivar of passionflower requires more than one transgenic line from different explant sources and genetic transformation events. In the present work, we report on the production and the analyses of 12 new transgenic lines of passionflower 'IAC-275' and IAC-277 transformed with CP gene construct obtained by Trevisan et al. (29), as well as the reaction of R₁ and R₂ generations of CABMV-resistant transgenic lines subjected to both mechanical and aphid inoculations.

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Materials and Methods

Plant material and genetic transformation. Passionflower seed of IAC-275 and IAC-277 were germinated in potting mix Plantmax (Eucatex, São Paulo, Brazil), at $27 \pm 1^\circ\text{C}$, and 50- to 75-day-old seedlings were used as explant source. The explants consisted of leaf disks (6 mm in diameter) collected from young leaves. Genetic transformation was done according to Trevisan et al. (29) with the full-length CP gene cloned from a severe CABMV isolate from the state of São Paulo, Brazil (CABMV-SP, GenBank accession number AY433950). The gene was inserted into the pCambia 2300 binary vector, which was renamed 2300-CABMV. In summary, the explants were inoculated with *Agrobacterium tumefaciens* EHA 105 containing pCambia 2300-CABMV (20 min) and co-cultivated (3 days; 24°C) in Murashige and Skoog (MS) medium (22) supplemented with thidiazuron (TDZ; 0.25 mg/liter), silver nitrate (AgNO_3 ; 4 mg/liter), and acetosyringone (100 μM /liter). After co-cultivation, the explants were transferred to a selection and regeneration medium consisting of MS supplemented with TDZ, AgNO_3 , kanamycin (100 mg/liter), and cefotaxime (500 mg/liter). Cultures were incubated in the dark for 6 to 8 weeks (27°C) and then transferred to a 16-h photoperiod (63 $\mu\text{mol}/\text{m}^2/\text{s}$) at $27 \pm 1^\circ\text{C}$. Developed shoots were transferred to Magenta boxes, covered with vented lids (Sigma-Aldrich, St. Louis) containing elongation medium (i.e., MSM medium [21] supplemented with coconut water [10%] and cefotaxime [500 mg/liter]), and incubated under a 16-h photoperiod.

Molecular analyses of transgenic plants. The presence of the CABMV-CP gene in the shoots developed in vitro was detected by polymerase chain reaction (PCR) using the CABMV-P1 and CABMV-P2 primers (formerly named PWV-P1 and PWV-P2; 30). Genomic DNA was extracted from young leaves of well-developed in vitro plantlets according to the protocol described by Doyle and Doyle (9), and PCR reactions were performed as outlined by Trevisan et al. (29). PCR-positive plants were transferred to pots containing sterile potting mix and acclimatized to greenhouse conditions.

Genetic transformation was confirmed by Southern blot analysis. Genomic DNA was isolated from fully expanded leaves of both acclimatized transgenic and nontransgenic control plants according to Doyle and Doyle (9). *Hind*III-digested DNA samples (60 μg for 16 h at 37°C) were separated on agarose gel (1%) by electrophoresis, transferred to nylon membrane (Hybond-N⁺, GE Healthcare, Buckinghamshire, UK), and subsequently fixed at 80°C . A PCR-amplified alkaline phosphatase-labeled (AlkPhos Direct Labelling and Detection System; GE Healthcare) fragment from the CABMV-CP gene coding region (850 bp) was used as a probe. Prehybridization, hybridization (60°C), washing, and detection were performed with CDP-Star Detection Reagent (GE Healthcare) following the supplier's instructions.

Evaluation of resistance to CABMV in R_0 transgenic lines. Twelve transgenic lines (R_0 generation)—namely, nine lines of IAC-275 (T9, T11, T12, T13, T14, T15, T17, T19, and T20), three lines of IAC-277 (T10, T16, and T18), and a nontransgenic control—were propagated by stem cuttings under greenhouse conditions. Propagated clones of each R_0 transgenic line and a nontransgenic control line were mechanically inoculated with CABMV Brazilian isolates from São Paulo (CABMV-SP), Rio de Janeiro (CABMV-RJ), and Ceará (CABMV-CE) States, separately. The inoculum was prepared by grinding fresh leaves from CABMV-infected plants in phosphate buffer (0.02 M; pH 7.0) containing sodium sulfite (0.02 M). For inoculation, two leaves of each plant at the four- to five-leaf development stage were dusted with Carborundum and rubbed with 1:200 (wt/vol) diluted inoculum (200 μl /plant). One month later, two newer expanded leaves of the same plants were reinoculated with 1:100 (wt/vol) diluted inoculum. A third inoculation was performed 30 days later with 1:50 (wt/vol) diluted inoculum. Disease resistance was assessed by symptom development.

Evaluation of resistance to CABMV in R_1 and R_2 generations of T2 and T16 transgenic lines. The CABMV-resistant

transgenic lines T2, reported by Trevisan et al. (29), and the transgenic line T16, identified in the present work, were self-pollinated in a greenhouse according to the biosecurity policy established in Brazil (<http://www.ctnbio.gov.br>). Seed collected from T2R₁ and T16R₁ fruit were germinated in 2-liter pots containing potting mix and plants at the three- to four-leaf development stage were analyzed for their resistance to CABMV by mechanical or aphid inoculation with the CABMV-SP. Mechanical inoculation was performed as described above, using 200 μl of inoculum, diluted 1:200, per plant. Vector inoculation was performed with the tobacco aphid (*Myzus nicotianae*). Virus-free colonies were maintained on tobacco plants (*Nicotiana tabacum*) grown in the greenhouse. For vector inoculation, groups of apterous aphids were starved in plastic boxes (30 min), and then transferred to CABMV-SP-infected leaves for an acquisition access period of 20 min. Groups of five aphids were transferred to each test plant for an inoculation access period of 1 h. Disease resistance was assessed as described above. Symptomatic plants were properly discarded and asymptomatic plants were inoculated once more. This procedure was repeated at least three times. The absence of CABMV-SP in symptomless plants was confirmed by recovery test on *Chenopodium amaranticolor*, which is a local lesion host for CABMV (4). *C. amaranticolor* was mechanically inoculated with extract from the upper leaf of each plant. The evaluation was based on the presence of characteristic local lesions on inoculated leaves.

Transgene transcription was investigated in 12 propagated clones of the T16R₁ transgenic line. Total RNA was extracted from leaves (60 mg) collected from T16R₁ and T16R₀ transgenic plants, as well as from a healthy nontransgenic line (negative control) and from a nontransgenic line inoculated with CABMV (positive control). RNA extraction was performed by means of the Trizol reagent protocol (Invitrogen, Carlsbad, CA) and genomic DNA was removed using RQ1 RNase-Free DNase (Promega Corp., Madison, WI). RNA was quantified using a Qubit fluorometer (Invitrogen). First-strand cDNA synthesis was carried out with M-MLV Reverse Transcriptase (Invitrogen) and primers oligo(DT) (Invitrogen) from 1 μg of total RNA. The PCR protocol followed the same procedure described above.

Asymptomatic T16R₁ plants (after four inoculations) were selected and self-pollinated. The R_2 seed were germinated and the plants were evaluated for resistance to the CABMV-SP isolates by mechanical or aphid inoculation as described before. Promising R_2 -resistant plants after successive inoculations with CABMV-SP were analyzed for the presence of the CP transgene. The identity of this gene was confirmed by direct nucleotide sequence of the respective amplified PCR fragments (Macrogen, Seoul, South Korea). CP expression was also verified in these plants by indirect double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using polyclonal antisera produced in rabbit and chicken, as described by Novaes and Rezende (25), and by Western blot following the protocol described by Trevisan et al. (29), using the polyclonal antiserum produced in chicken.

Reevaluation of the resistance of R_0 T2 and T16 transgenic lines through mechanical and aphid inoculation. Because of the absence of resistance to vector-transmitted CABMV-SP in the R_1 generation of the T2 transgenic line, the reaction of the R_0 transgenic lines T2 and T16 to CABMV-SP through mechanical and aphid inoculations was reevaluated. Plants were vegetatively propagated in the greenhouse and inoculated as described earlier. Seven independent inoculation experiments were done. Six nontransgenic control plants were included in each assay. Evaluations were based on symptom expression and bioassays on *C. amaranticolor*, as described before. Means were compared using a *t* test ($P < 0.05$) (SAS Institute Inc., Cary, NC).

Results

Genetic transformation and plant characterization. *A. tumefaciens*-mediated genetic transformation was successful and transgenic plants were obtained for the two passionflower cultivars studied. From 1,330 IAC-275 and 1,560 IAC-277 explants inocu-

lated with *A. tumefaciens*, 9 and 3 putative transgenic shoots, respectively, were identified. These results correspond to a genetic transformation efficiency of 0.67% for IAC-275 and 0.19% for IAC-277. The Southern blot analyses confirmed the integration of the CP gene into the genome for all plants analyzed (Fig. 1). Most of the transgenic lines had at least two integration events. The Southern blot profile indicated independent transformation events. No hybridization signal was detected with nontransgenic control plants. The transgenic lines showed normal phenotype. No abnormalities in leaf morphology or plant vegetative development could be detected in acclimatized plants.

Resistance to CABMV in R_0 transgenic lines. Thirty days after the first inoculation, mosaic symptoms could be detected in propagated plants of several transgenic lines, except for T9, T11, and T16 inoculated with CABMV-SP; T10 and T16 inoculated with CABMV-RJ; and T10, T16, and T20 inoculated with CABMV-CE (Table 1). After the second mechanical inoculation

using a double inoculum concentration, at least one propagated plant of each transgenic line became infected, except for transgenic line T16, in which all propagated plants remained asymptomatic. After the third inoculation, under higher inoculum pressure, propagated plants of transgenic line T16 remained asymptomatic, indicating a high resistance to infection against all three isolates of CABMV tested. Propagated plants from all the other transgenic lines showed virus symptoms. The incidence of nontransgenic plants infected by the different virus isolates ranged from 60 to 100%. No relation could be detected between the number of transgene insertion events and resistance to CABMV.

Resistance of the R_1 transgenic lines T2 and T16 to CABMV-SP. In all, 24 and 7 fruit, with a total of 1,998 and 810 seeds, were obtained from self-pollinated R_0 plants of transgenic lines T2 and T16, respectively.

Of the 1,373 T2 R_1 sowed seeds, 38% germinated, resulting in 524 plants for resistance evaluation. Following the first inoculation, 47% of the evaluated plants did not show CABMV-SP symptoms (Table 2). However, the percentage of asymptomatic plants decreased after three successive inoculations, resulting in all plants becoming infected. Similarly, of the 360 T16 R_1 seeds planted, 77% germinated, resulting in 279 plants for resistance evaluation. After the first inoculation, 55% of evaluated plants did not show CABMV-SP symptoms (Table 2). Although other susceptible plants were identified after each additional inoculation, 13 plants (4.6%) remained asymptomatic following the fourth inoculation with CABMV-SP using *M. nicotianae*. Efficiency of virus transmission to nontransgenic passionflower ranged from 70 to 100% (data not shown). Mechanical inoculations using leaf extracts from 13 asymptomatic T16 R_1 plants did not cause local lesions to develop on *C. amaranticolor*. PCR analysis revealed that the CP transgene was present in all asymptomatic plants (data not shown). CP transgene expression was investigated by reverse-transcription (RT)-PCR in 12 T16 R_1 transgenic plants. RT-PCR analyses showed the CP transgene mRNA in all analyzed plants, which indicated the presence of the transgene transcription (Fig. 2). The 13 T16 R_1 asymptomatic plants were self-pollinated, from which 20 fruit and 2,694 seeds were obtained. Two batches of 144 seeds were then planted, resulting in 133 and 125 plants, respectively, for resistance evaluation. After two aphid and two mechanical inoculations with CABMV-SP, 42 plants (31.6%) out of 133 T16 R_2 plants of the first sowing remained asymptomatic (Table 2). Efficiency of virus transmission to nontransgenic passionflower was 100%, except for the second vector inoculation which had 30% transmission efficiency. For the second sowing of T16 R_2 , 125 plants were evaluated by means of two successive mechanical inoculations with CABMV-SP and 76 plants (60.8%) remained asymptomatic (Table 2). All nontransgenic control plants were infected (data not shown).

The CP transgene was detected in all of 20 selected T16 R_2 resistant plants. The partial nucleotide sequence of the CP gene-ampli-

Fig. 1. Southern blot analyses of *Passiflora edulis* f. *flavicarpa* 'IAC-275' (T9, T11, T12, T13, T14, T15, T17, T19, and T20) and 'IAC-277' (T10, T16, and T18). DNA was digested with *Hind*III and hybridized with a probe corresponding to an 850-bp fragment from the *Cowpea aphid borne mosaic virus* capsid protein (CABMV-CP) gene. C+ = positive control (0.85-kb CABMV-CP gene fragment); C- = negative control (DNA from nontransgenic plant).

Table 1. Evaluation of *Cowpea aphid borne mosaic virus* (CABMV) resistance in R_0 passionflower transgenic plants carrying the CABMV capsid protein gene

	Transgenic lines ^y												C ^z
Inoculum (wt/vol)	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18	T19	T20	
CABMV-SP													
1st inoculation (1:200)	0/7	2/8	0/2	2/5	1/2	2/6	2/3	0/9	2/6	3/9	4/5	1/2	3/4
2nd inoculation (1:100)	5/7	4/8	2/2	2/5	2/2	5/6	2/3	0/9	4/6	6/9	5/5	2/2	4/4
3rd inoculation (1:50)	5/7	5/8	–	5/5	–	6/6	2/3	0/9	5/6	9/9	–	–	5/8
CABMV-RJ													
1st inoculation (1:200)	5/6	0/6	1/1	2/8	3/5	2/4	...	0/3	2/3	5/7	2/9	5/6	4/6
2nd inoculation (1:100)	6/6	2/6	1/1	8/8	5/5	4/4	...	0/3	2/3	7/7	5/9	5/6	5/5
3rd inoculation (1:50)	–	3/6	–	–	–	–	...	0/3	3/3	–	9/9	5/6	6/6
CABMV-CE													
1st inoculation (1:200)	...	0/4	2/3	2/2	2/5	1/1	2/2	0/2	3/3	1/4	–	0/3	5/5
2nd inoculation (1:100)	...	4/4	2/3	2/2	5/5	1/1	2/2	0/2	3/3	4/4	–	3/3	2/3
3rd inoculation (1:50)	...	...	2/3	–	–	–	–	0/2	–	–	–	–	3/5

^y Number of symptomatic plants/inoculated plants; — = not tested.

^z Number of nontransgenic inoculated plants.

fied fragment (835 bp) shared 98 to 99% identity with the CP gene of different isolates of CABMV (*data not shown*). The CP was not detected in all analyzed plants by Western blot and indirect DAS-ELISA (*data not shown*).

Resistance of R_0 transgenic lines T2 and T16 through mechanical and aphid inoculation. T2R₀ and T16R₀ transgenic lines were retested after the results obtained for the T2R₁ transgenic line after *M. nicotianae* inoculation. Aphid transmission studies to T2R₀ transgenic plants resulted in 96.5% of the plants displaying mosaic and leaf malformation symptoms. Conversely, T16R₀ transgenic plants showed a high level of resistance to CABMV-SP after both mechanical and aphid inoculations. Transmission efficiency for both methods of inoculation was very high for all assays, with 90% of nontransgenic control plants showing virus symptoms (Table 3). Attempts to recover CABMV from asymptomatic T2R₀ and T16R₀ plants through biological virus recovery tests on *C. amaranticolor* produced negative results.

Discussion

Genetic engineering has been presented as a powerful tool for the production of disease-resistant plants. However, most of the promising results obtained so far are related to the control of plant diseases caused by viruses (8). To the best of our knowledge, the only examples of practical application of genetic engineering for controlling plant diseases are the papaya cultivars resistant to *Papaya ringspot virus* (PRSV) (14,17), and summer squash cultivars resistant to *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus*, or *Watermelon mosaic virus* released for commercial use in the United States (15). Tomato and sweet pepper cultivars resistant to CMV and a papaya cultivar resistant to PRSV were also released commercially in the People's Republic of China (15).

For passionflower, no natural source of resistance to CABMV is available. *P. suberosa* has been reported to be immune to this virus but it presents chromosome incompatibility with the cultivated species (20). As a consequence, Brazilian researchers have concentrated efforts on using genetic engineering in order to obtain passionflowers resistant to the *Potyvirus* sp. CABMV (1,29). These results suggested that the development of a commercial transgenic passionflower resistant to CABMV may be possible.

Passionflower genetic transformation protocol via the genus *Agrobacterium* is feasible; however, genetic transformation efficiency is low. Trevisan et al. (29) reported genetic transformation efficiencies of 0.11 and 0.21% for passionflower IAC-275 and IAC-277, respectively. The main constraint to the process is related to problems in shoot elongation and plantlet development (10,28). Therefore, the use of a high number of initial explants is necessary. In spite of such difficulties, we report here on the production of 12 new independent passionflower transgenic lines containing the CABMV-CP gene (9 IAC-275 and 3 IAC-277).

The evaluation of primary transformants (R_0) for resistance to virus pathogens has been reported for a range of crop species (5,29,30). The results usually show transgenic lines with different degrees of resistance. Because only a few lines have a high degree of resistance or are immune to the virus (5,19,29,30), a high genetic transformation efficiency is necessary in order to increase the

chances to obtain highly resistant or immune transgenic lines. Also, even among cloned plants from the same transgenic line, there may be a variable response. The transgenic lines may be graded into categories from susceptible to highly resistant or immune, based on the percentage of clonally propagated individuals showing disease symptom (19,30).

In our work, this occurred while evaluating R_0 transgenic lines. All transgenic lines were propagated and challenged with three isolates of CABMV in order to identify resistant lines. Clones of few transgenic plants showed some resistance to infection when mechanically inoculated with 1:200 diluted inoculum. In order to identify highly resistant or immune lines, we persisted in inoculating the plants using a higher inoculum pressure (1:100 and 1:50). Subsequently, only plants of the T16 transgenic line (IAC-277) were highly resistant to infection with CABMV isolates, with no individual clones showing virus symptoms. Trevisan et al. (29) also found the same variability of response of transgenic passionflower when inoculated with the same virus isolates used in the present work. Out of seven transgenic lines they evaluated, only one (T2; IAC-277) was resistant to all three virus isolates, after three successive mechanical inoculations (29).

Subsequent experiments focused on advanced generations of T2 (29) and T16 transgenic lines, which had been identified as highly resistant to CABMV. After four successive inoculations with *M. nicotianae*, none of the T2R₁ plants were resistant to CABMV-SP, contrasting with the results obtained for T2R₀ mechanically inoculated plants. Variable results comparing the reaction of transgenic plants after mechanical or vector transmission with their respective viruses have been reported by others. Lawson et al. (18) showed that the transgenic potato line 303, expressing the CP genes of *Potato virus Y* (PVY) and *Potato virus X* (PVX) was resistant to PVY infection by mechanical and aphid inoculations, whereas transgenic lines 108 and 204 were only resistant to mechanical inoculation. Transgenic *N. tabacum* 'Samsun NN' expressing the CP gene of *Tobacco rattle virus* was resistant to infection by mechanical inoculation but trichodoriid nematode vectors successfully

Fig. 2. Reverse-transcription polymerase chain reaction analyses performed in T16R₁ transgenic plants. Lane + = positive control (T16R₀ transgenic line); lane Pi = nontransgenic passionflower inoculated with *Cowpea aphid borne mosaic virus* São Paulo isolate; lanes 1–17 = T16R₁ transgenic plants; – = negative control (nontransgenic noninoculated passionflower); lane Pc = negative control (water); lane M = 1-kb ladder (Invitrogen).

Table 2. Evaluation of resistance to *Cowpea aphid borne mosaic virus* in R_1 and R_2 generations of T2 and T16 passionflower transgenic lines^x

Plants ^y	T2R ₁	T16R ₁	T16R ₂ ^z	
Inoculated	524	279	133	125
Asymptomatic				
1st inoculation	245 A	154 A	122 M	110 M
2nd inoculation	103 A	111 A	112 A	76 M
3rd inoculation	22 A	50 A	87 A	nt
4th inoculation	0 A	13 A	42 M	nt

^x A = aphid inoculation, M = mechanical inoculation, and nt = not tested.

^y Inoculated = number of inoculated plants and Asymptomatic = number of asymptomatic plants after given inoculation.

^z Plants from two different sowings.

Table 3. Evaluation of resistance to *Cowpea aphid borne mosaic virus* in R_0 passionflower transgenic lines T2 and T16 by means of mechanical and *Myzus nicotianae* inoculations across seven independent experiments

Method ^y	Number of infected plants/inoculated plants ^x		
	T2	T16	C ^z
Aphid	28/29 aA	1/25 aB	19/21 aA
Mechanical	0/21 bA	4/22 aA	21/21 aB

^x Values within a column followed by the same lowercase letter were not significantly different according to a *t* test with *P* < 0.05. Values within a row followed by the same uppercase letter were not significantly different according to a *t* test with *P* < 0.05.

^y Method of inoculation.

^z Nontransgenic control.

transmitted the virus to the plant's roots and leaves (27). Transgenic *N. benthamiana* plants encoding the *Beet necrotic yellow vein virus* (BNYVV) CP were immune to foliar mechanical inoculation but susceptible to root inoculation with viruliferous zoospores of *Polymyxa betae*. Analysis of small interfering RNAs revealed that the RNA silencing-mediated resistance to BNYVV was less effective in roots than in leaves (2).

As to T16R₁, 13 transgenic plants remained symptomless after four successive inoculations with aphids and, for the advanced generation, 118 T16R₂ also remained symptomless. This suggested that resistance to CABMV was being maintained in the plant genome, together with homozygosity. The CP gene was detected in 20 selected T16R₂ resistant transgenic plants although the expression of the CP was not found in any such plants by indirect PTA-ELISA or Western blot. This suggests that the protein was not being expressed or is being synthesized at an undetectable level. Expression of the CP in transgenic passionflower is not a requirement to confer resistance to the virus, as shown by Trevisan et al. (29).

T16R₂ resistant transgenic plants containing the CP transgene are being crossed to obtain the R₃ generation. The resistance to CABMV will be evaluated under greenhouse and field conditions under official permission from Brazilian biosecurity agency.

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