Transgenic Passionfruit Expressing RNA Derived from *Cowpea aphid-borne mosaic virus* Is Resistant to Passionfruit Woodiness Disease

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ABSTRACT

Sixteen transgenic yellow passionfruit (*Passiflora* spp.) plants (R_0) were obtained which express a non-translatable transgenic RNA corresponding to the 3' region of the *NIb* gene and the 5' region of the *CP* gene, derived from the genome of a Brazilian isolate of *Cowpea aphid-borne mosaic virus* (CABMV). The transgenic plants were propagated by stem cuttings and challenged by sap inoculation with isolates CABMV-MG1 and CABMV-PE1. One transgenic plant (TE5-10) was resistant to the isolate CABMV-MG1, but susceptible to CABMV-PE1. The remaining transgenic plants developed systemic symptoms, equal to non-transformed plants, when inoculated with either isolate. The absence of virus in TE5-10 plants was confirmed by indirect ELISA. Transcription analysis of the transgene demonstrated that the TE5-10 plant did not accumulate transgenic mRNA, even before inoculation. After inoculation, viral RNA was only detected in plants inoculated with CABMV-PE1. These results confirm that the transgenic plant TE5-10 is resistant to isolate CABMV-MG1, and suggest that the resistance mechanism is post-transcriptional gene silencing, which is already activated in the transgenic plants before virus inoculation.

Additional keywords: *Passionfruit woodiness virus*, PWV, potyvirus, resistance.

RESUMO

Plantas transgênicas de maracujá-amarelo expressando um RNA derivado do genoma do *Cowpea aphid-borne mosaic virus* são resistentes ao endurecimento dos frutos

Dezesseis plantas transgênicas (R_0) de maracujá-amarelo (*Passiflora* spp.) foram obtidas expressando um RNA não-traduzível correspondente à região 3' do gene *NIb* e 5' do gene *CP* de um isolado brasileiro do CABMV. As plantas R_0 foram propagadas vegetativamente por estaquia e inoculadas com os isolados CABMV-MG1 e CABMV-PE1. Uma das plantas (TE5-10) foi resistente ao isolado CABMV-MG1, porém suscetível ao isolado CABMV-PE1. As demais plantas transgênicas foram suscetíveis a ambos os isolados, apresentando sintomas sistêmicos semelhantes ao de plantas não-transformadas. A ausência de replicação viral nas plantas TE5-10 foi confirmada por ELISA indireto. A análise da transcrição do transgene nas plantas TE5-10 demonstrou que estas não acumulam o mRNA transgênico, mesmo antes da inoculação com o vírus. Após a inoculação, o RNA viral foi detectado apenas em plantas inoculadas com o isolado CABMV-PE1. Esses resultados confirmam que a planta TE5-10 é resistente ao isolado CABMV-MG1, e sugerem que o mecanismo da resistência é baseado em silenciamento gênico pós-transcricional, o qual já se encontra ativado nas plantas antes da inoculação com o vírus.

Palavras-chave adicionais: *Passionfruit woodiness virus*, PWV, potyvirus, resistência, maracujá amarelo.

INTRODUCTION

Passionfruit woodiness is one of the most important diseases of the passionfruit (*Passiflora* spp.) (Kitajima & Chagas, 1984; Chagas, 1991). The causative agent was described and named *Passionfruit woodiness virus* (PWV) by Cobb, in Australia, in 1901 (cited by Shukla *et al.*, 1988). Shukla *et al.* (1988) determined the amino acid sequence of the capsid protein (CP) of three PWV isolates of Australasian origin. Later, McKern *et al.* (1994) showed that an isolate from South Africa classified as PWV was actually a member of a distinct species, designated South African passiflora virus (SAPV). Additional studies identified SAPV as a strain of *Cowpea aphid-borne mosaic virus* (CABMV) (Sithole Niang *et al.*, 1996). Therefore, it is currently acknowledged that passionfruit woodiness disease can be caused by PWV or CABMV, family *Potyviridae*, genus *Potyvirus* (Van Regenmortel *et al.*, 2000). Phylogenetic analysis based on the capsid

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protein amino acid sequence grouped a number of Brazilian isolates causing passionfruit woodiness disease together with CABMV, distant from PWV isolates, indicating that they comprise a strain of CABMV (Nascimento *et al.*, 2004).

The control of passionfruit woodiness disease is difficult and often impossible, mostly due to the non-circulative mode of virus transmission by aphid vectors and to the fact that both CABMV and PWV have several wild hosts that can serve as virus reservoirs (Taylor & Greber, 1973; Bock & Conti, 1974). Cross-protection would be an alternative control method. However, reports of a synergistic effect between PWV and *Cucumber mosaic virus* (CMV), family *Bromoviridae*, genus *Cucumovirus*, causing tip necrosis (Pares *et al.*, 1985) have hindered the use of cross-protection. Breeding for resistance is complicated by self-incompatibility and inter-specific incompatibility in *Passiflora* species (Martin & Nakasone, 1970; Lopes, 1994; Bruckner *et al.*, 2002). An interesting alternative control method would be the use of transgenic plants with pathogen-derived resistance (PDR).

Transgenic plants expressing genes or partial viral sequences can be resistant to viral infection (Goldbach *et al.*, 2003). This form of resistance is known as PDR (Sanford & Johnston, 1985). The PDR can be manifested at the protein or RNA level. Resistance due to protein expression is, in most cases, directly correlated with protein accumulation. On the other hand, RNA-mediated resistance is typically associated with transgenic plants which accumulate the transgenic mRNA at low or non-detectable levels. This kind of resistance is known as homology-dependent resistance (Baulcombe, 1996) and is associated with the process of post-transcriptional gene silencing (PTGS) (Baulcombe, 2002; Goldbach *et al.*, 2003), which leads to the highly sequence-specific degradation of a homologous RNA population (Baulcombe, 2002). This phenomenon was first observed in transgenic plants when the introduction of a second copy of an endogenous gene led to the inactivation (silencing) of the transgene itself and the homologous endogene (Napoli *et al.*, 1990; Smith *et al.*, 1990; Van Der Krol *et al.*, 1990).

In this study, we showed that transgenic yellow passionfruit (*Passiflora edulis* f. *flavicarpa*) plants expressing an untranslatable RNA encompassing the 3'-terminal region of the *NIb* gene and the 5'-terminal region of the *CP* gene from isolate CABMV-MG1 are resistant to the virus. Transcription analysis of the transgene indicates that the resistance mechanism is PTGS, which is already active even in the absence of viral replication.

MATERIALS AND METHODS

Viral isolates

The viral isolates used in this study were obtained by Costa (1996) in the states of Pernambuco (isolate PE1) and Minas Gerais (isolate MG1). The isolates were stored at –20 ºC, in the form of dried leaf material of common bean (*Phaseolus vulgaris* L.) cv. Preto 153. After reactivation by sap-inoculation onto 'Preto 153' bean and yellow passionfruit plants, the isolates were maintained in plants of these species under greenhouse conditions. Inoculations were carried out mechanically using potassium phosphate buffer 0.05 M, pH 7.2, with 0.1% (p/v) sodium sulfite, and carborundum as an abrasive.

Cloning of *NIb* and *CP* genes

Concentrated viral preparations of isolate CABMV-MG1 were obtained from infected leaves of 'Preto 153' bean according to Lane (1992). Viral RNA was extracted as described by Krause-Sakate *et al.* (2001). An oligo-dT (5'-G-A-C-T-G-G-A-T-C-C-T_{14}-3', *Bam*H I site underlined), which is supposed to anneal at the poly-A sequence at the 3'-end of the viral genome, was used for cDNA synthesis. The same primer was used for amplification of the *NIb* and *CP* genes, together with a degenerate primer which anneals at the *NIb* gene (5'-G-C-G-G-G-A-T-C-C-G-T-N-T-G-Y-G-T-N-G-A-Y-G-A-Y-T-T-T-Y-A-A-Y-A-A-3', *Bam*H I site underlined), as described (Braz, 1999). After digestion with *Bam*H I, amplified fragments were ligated to the pBluescript KS+ vector, previously digested with the same enzyme. The fragment was subcloned into the expression cassette of the binary vector pBI121 and transferred to the *Agrobacterium tumefaciens* strain LBA 4404.

Plant transformation

Transformation of leaf disks of yellow passionfruit was mediated by *A. tumefaciens*. The explants were immersed in a suspension of *A. tumefaciens* (OD_{600} 0.4) for 15 to 20 min, transferred to non-selective MS medium (3% sucrose, 0.1 mg/l BAP, 100 mg/l vitamin B5 complex, 50 mg/l myo-inositol, 0.26% phytagel, pH 5.8) and kept in darkness for 48 h. Transgenic plants were regenerated in selective medium containing kanamicin (150 mg/l) and cefotaxim (500 mg/l). After stem elongation, these were transferred to rooting medium (1/2 MS, 2% sucrose, 100 mg/l vitamin B5 complex, 50 mg/l myo-inositol, 500 mg/l cefotaxim, 0.88 mg/l IAA, 0.26% phytagel, pH 5.8). Rooted plants were transferred to substrate and, after acclimation, maintained in a greenhouse. The presence of the T-DNA was determined by polymerase chain reaction (PCR) based on genomic DNA extracted from the regenerated plants as described by Gama (1998) using specific primers for the *npt*II gene (5'-C-C-G-G-T-C-A-G-C-C-C-A-T-T-C-G-C-C-G-C-C-3' and 5'-T-C-A-G-C-G-C-A-G-G-G-G-C-G-C-C-C-G-G-T-T-3') which allow the amplification of a DNA fragment of 800 nt.

Resistance to CABMV in R_0 plants

The transgenic plants were vegetatively propagated by removing cuttings of approximately 20 cm from the R_0 plants. About four centimeters of the base of each cutting was immersed for 5 min in 200 mg/l IBA, and transferred to plastic trays containing sterile sand (the treated end into the sand bed), and kept for the next 20 days under greenhouse conditions. The cuttings were then transferred to pots and maintained in the greenhouse until they developed four-six leaves. At this point, cuttings were sap-inoculated as described

above. Five plants per transformant were inoculated with CABMV isolate MG1, five with isolate PE1, and three were mock-inoculated with buffer. After three days, all plants were reinoculated to avoid escapes. Plants were maintained under greenhouse conditions and visually evaluated for the incidence of symptoms until 45 days after inoculation. The experiment was repeated four times, for a total of 20 inoculated plants derived from each R_0 transformant. Virus identification in inoculated plants was carried out by indirect ELISA using a polyclonal antiserum raised against isolate MG1 (Costa, 1996).

Analysis of transgene transcription

Total RNA was extracted from transgenic and non-transgenic plants as described by Mallory *et al.* (2001). Twenty micrograms of total RNA were separated on a denaturing agarose gel (1.2%) and capillary-transferred to a nylon membrane (Hybond-N+, Amersham Biosciences). The clone with the viral fragment in pBluescript KS+ was used as a probe. Approximately 50 ng of DNA were labeled with $[\alpha^{32}P]$-dCTP by random priming and hybridization was carried out at high stringency, according to standard techniques (Sambrook *et al.*, 1989).

RESULTS

Transformation of yellow passionfruit

Yellow passionfruit plants were transformed with the 3'-terminal region of the *NIb* gene and the 5'-terminal region of the *CP* gene from isolate CABMV-MG1. To determine which R_0 plants contained at least one copy of the transgene, we carried out PCR reactions using total DNA from these plants with primers which anneal specifically to the *npt*II gene. The primer pair allows the amplification of a fragment with approximately 800 nt when DNA from the pBI121 vector, which contains the *npt*II gene, is used as a template (Figure 1, lane 1). Amplification of a fragment of the same size was observed for 15 out of 16 R_0 plants (Figure 1, lanes 3 to 18). No amplification was observed when the total DNA of non-transformed plants was used as a template (Figure 1, lane 2), thus confirming the specificity of the primers. Amplification was not observed with the TE5-2 plant (Figure 1, lane 4), which suggests that this plant was not transformed, or that the T-DNA insertion was not complete.

Resistance to CABMV in R_0 plants

In order to determine whether the transgenic lines were resistant to infection by CABMV, the R_0 plants were vegetatively propagated by cuttings, and plants developed from the cuttings were sap-inoculated with CABMV isolates MG1 and PE1. Non-transformed plants developed evident mosaic and leaf deformation symptoms upon inoculation with both isolates (Figure 2A, B). Plants obtained from the R_0 transformant TE5-10 developed the same symptoms upon inoculation with isolate PE1 (Figure 2C), but did not show any symptoms upon inoculation with isolate MG1 (Figure 2D). This result indicates that the R_0 plant TE5-10 is resistant

to this isolate, the same used for transformation. Plants obtained from the remaining R_0 transformants developed mosaic and leaf deformation symptoms upon inoculation with both isolates (data not shown). Results of indirect Enzime Linked Immunosorbent Assay (ELISA), done in all 20 plants propagated from transformant TE5-10 and in 20 non-transformed control plants, were in agreement with the visual observations. Non-transformed plants inoculated with isolates MG1 and PE1 had absorbance values clearly above the positive/negative threshold (twice the absorbance of healthy plants) (Figure 3). Plants obtained from the R_0 transformant TE5-10 and inoculated with isolate MG1 had absorbance values below the threshold, at the same level as the control plants (Figure 3), demonstrating that these plants were actually virus-free. Plants obtained from the same R_0 transformant but inoculated with isolate PE1 had absorbance values above the threshold value (Figure 3).

Accumulation of transgenic and viral RNA in R_0 plants

Northern blot analysis was carried out to determine whether the plants obtained from the TE5-10 transformant displayed the resistance phenotype due to PTGS.

The transgenic mRNA was detected in a non-inoculated plant derived from the susceptible R_0 transformant TE5-4 (Figure 4, lane 1), but was not detected in non-inoculated plants derived from the resistant R_0 transformant TE5-10 (Figure 4, lane 2). This result indicates that the PTGS mechanism was already activated in TE5-10-derived plants before inoculation with the viral isolates. The fact that transgenic mRNA was not detected in non-transformed plants (Figure 4, lane 3) confirmed the specificity of the probe.

Accumulation of viral RNA was detected in TE5-10-derived plants after inoculation with CABMV-PE1 (Figure 4, lane 7), evidence of viral replication in these plants. However, it was not detected in TE5-10-derived plants after inoculation with CABMV-MG1 (Figure 4, lane 6), further evidence that viral replication did not take place in these plants. Viral RNA accumulation was also detected in non-transformed plants inoculated with both isolates (Figure 4, lanes 4 and 5), which confirmed that the probe could detect both isolates.

DISCUSSION

Results obtained in this study demonstrate that the construct used to transform yellow passionfruit was efficient to confer resistance to isolate CABMV-MG1, and suggest that the resistance mechanism is PTGS.

Resistance in the TE5-10 transgenic plant was specific against isolate MG1. Plants derived from this transformant were susceptible to infection by isolate PE1. The resistance against isolate MG1 can be explained based on the mechanism of PTGS (Waterhouse *et al.*, 2001), since the sequence used for transformation is derived from the genome of this isolate. Sequence comparisons between these two isolates indicate an identity of 92% for the amino acid sequence of the capsid protein and of 94% for the nucleotide sequence of the 3'NTR
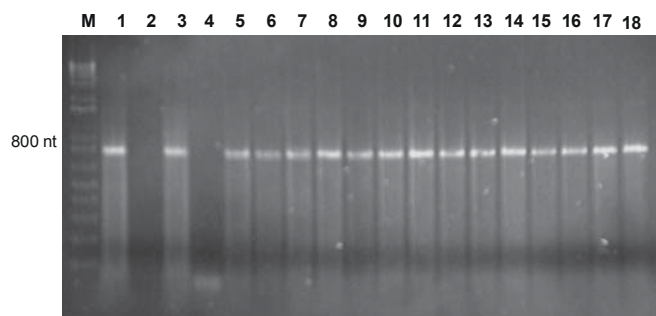
FIG. 1 - Polymerase chain reaction (PCR) - amplification products of the *npt*II gene. Total DNA extracted from transformed yellow passionfruit (*Passiflora edulis* f. *flavicarpa*) plants (lanes 3 to 18, corresponding to the R_0 plants TE5-1 to TE5-16, respectively), non-transformed plant (lane 2) and plasmid DNA of pBI121 (lane 1). M, size marker ("1 kb Plus DNA ladder"). The size of the amplified fragment is indicated at the left.

(Nascimento *et al.*, *submitted*). While this level of identity is sufficient to classify these isolates as members of the same viral species (Van Regenmortel *et al.*, 2000), there could be enough nucleotide differences between MG1 and PE1 to prevent recognition of the PE1 RNA by the silencing machinery

activated in the TE5-10 plants. Also, CABMV-PE1 could present differences in HC-Pro, which may determine that the HC-Pro of this isolate is a more efficient silencing suppressor than the HC-Pro of isolate MG1.

It has been demonstrated that in papaya plants transformed with the capsid protein gene of *Papaya ringspot virus* (PRSV), family *Potyviridae*, genus *Potyvirus,* resistance against a wide range of isolates is dependent on gene dosage, that is, plants that are homozygous for the transgene are resistant to several isolates of the virus (Lius *et al.*, 1997; Tennant *et al.*, 2001). Since the R_0 plants that we tested are hemizygous, it is possible that R_1 plants will be resistant to other isolates when in homozygosis for the transgene.

The fact that *A. tumefaciens*-mediated genetic transformation is a random process may explain why only one R_0 plant was resistant. Inverted repeat copies might have been inserted into the TE5-10 plant, which would facilitate the production of double stranded RNA required to trigger the silencing mechanism. Still, Southern blot and PCR analyses are required to determine the copy number and orientation of transgene insertions into these plants. Another factor that must be taken into consideration is the occurrence of methylation in the coding region of the transgene, since it has been proposed that methylation may lead to the production
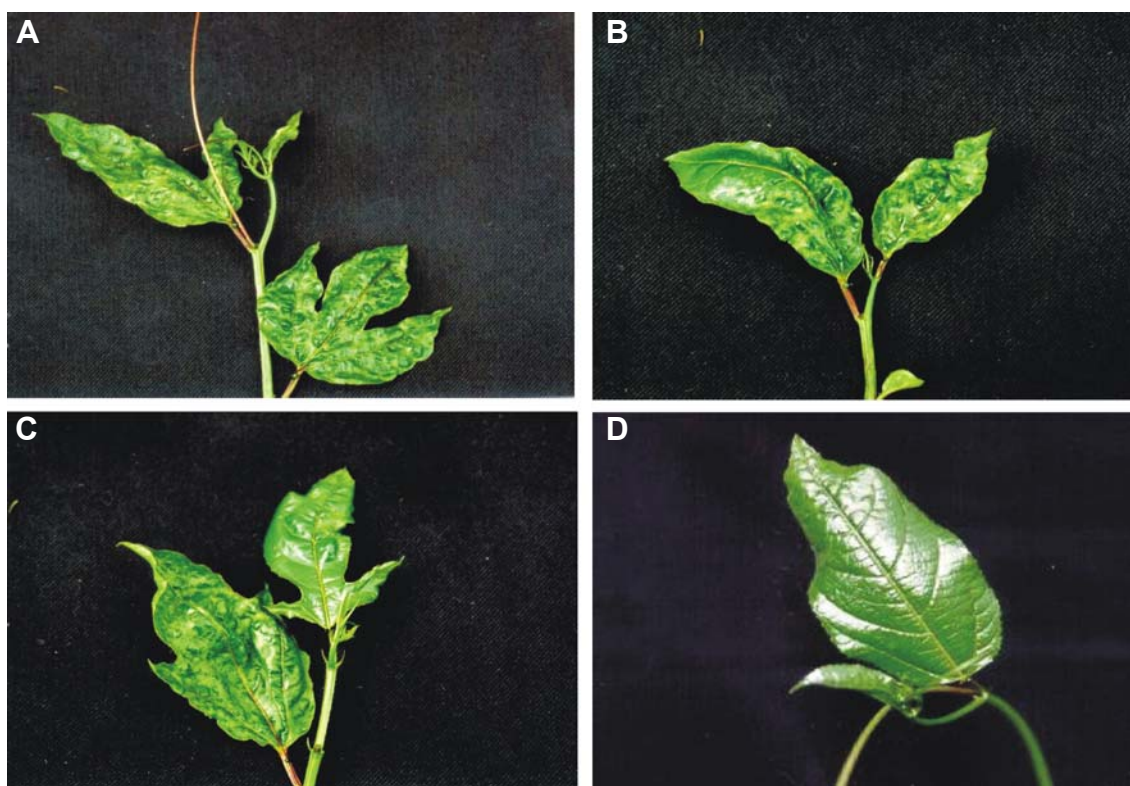

FIG. 2 - Symptoms observed in inoculated yellow passionfruit (*Passiflora edulis* f. *flavicarpa*) plants. A, B - Non-transformed plant inoculated with isolates *Cowpea aphid-borne mosaic virus* (CABMV) -PE1 and -MG1, respectively, showing mosaic and leaf deformation symptoms at 14 days post-inoculation (dpi). C - Transformed plant derived from the TE5-10 plant and inoculated with isolate CABMV-PE1, showing the same symptoms as the non-transformed control, at 14 dpi. D - TE5-10-derived plant inoculated with CABMV-MG1, showing no symptoms at 35 dpi.
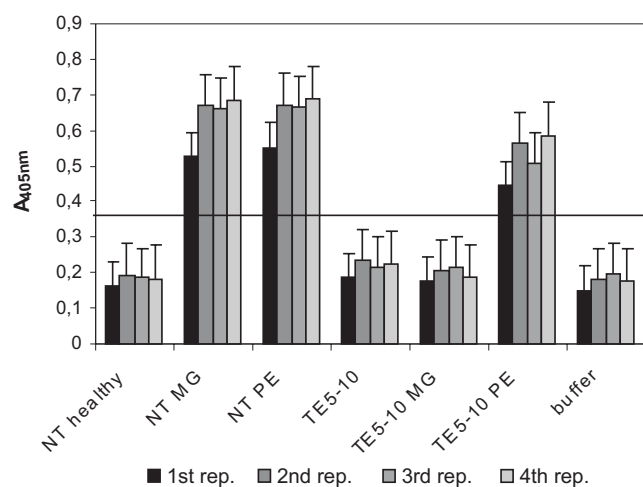
FIG. 3 - Virus detection via indirect ELISA in transgenic yellow passionfruit (*Passiflora edulis* f. *flavicarpa*) plants inoculated with isolates *Cowpea aphid-borne mosaic virus* (CABMV) -MG1 and -PE1; **NT healthy**, Non-transformed, non-inoculated plant. **NT MG**, Non-transformed plants inoculated with CABMV-MG1; **NT PE**, Non-transformed plants inoculated with CABMV-PE1; **TE5-10**, Non-inoculated plants derived from the TE5-10 transformant; **TE5-10 MG**, TE5-10-derived plants inoculated with CABMV-MG1; **TE5-10 PE**, TE5-10-derived plants inoculated with CABMV-PE1. Buffer, grinding buffer for I-ELISA. Each vertical bar represents the average absorbance value of five plants, for a total of 20 plants tested for each treatment. The horizontal line represents the positive/negative threshold, which corresponds to twice the absorbance value for healthy, non-inoculated plants.
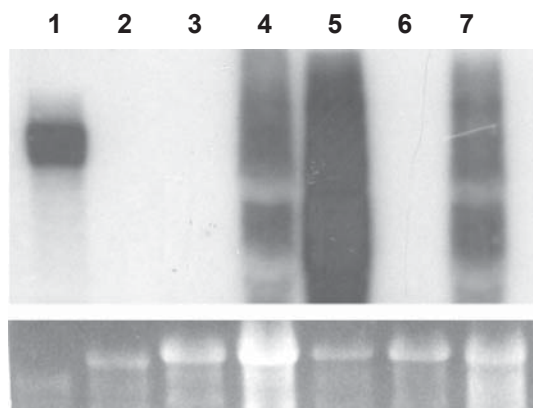

FIG. 4 - Expression analysis of the transgene in R_0 yellow passionfruit (*Passiflora edulis* f. *flavicarpa*) plants resistant or susceptible to *Cowpea aphid-borne mosaic virus* (CABMV). Total RNA was extracted from inoculated or non-inoculated plants derived from the R_0 plants TE5-4 (susceptible) and TE5-10 (resistant) and hybridized with a specific probe for the *NIb* and *CP* genes of CABMV. 1, TE5-4-derived plant, non-inoculated. 2, TE5-10-derived plant, non-inoculated. 3, Non-transformed, non-inoculated plant. 4, 5, Non-transformed plants inoculated with CABMV-MG1 and CABMV-PE1, respectively. 6, 7, TE5-10-derived plants inoculated with CABMV-MG1 and CABMV-PE1, respectively. Ethidium bromide-stained gel prior to transfer in the region corresponding to the 25S rRNA is shown at the bottom.

of aberrant mRNAs, which in turn could trigger the PTGS mechanism (Wassenegger, 2000).

Our results demonstrate that CABMV-resistant transgenic passionfruit plants have the potential to provide adequate control of passionfruit woodiness disease. The R_0 plants are currently being self-crossed in order to generate homozygous R_1 lines. If these R_1 lines display a broader spectrum of resistance, field trials would be the next logical step towards the commercial application of transgenic passionfruit.

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