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

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**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
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 Nb 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 °C, 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 Nb 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-C-T-T-3’, BamHI 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 Nb and CP genes, together with a degenerate primer which anneals at the Nb gene (5’-G-C-G-G-G-G-G-T-C-C-G-T-G-T-G-T-G-T-G-G-G-G-C-G-G-G-C-G-G-3’, BglII site underlined), as described (Braz, 1999). After digestion with BamHI, 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/1 BAP, 100 mg/l vitamin B5 complex, 50 mg/l myo-inositol, 0.26% phytigel, pH 5.8) and kept in darkness for 48 h. Transgenic plants were regenerated in selective medium containing kanamycin (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% phytigel, 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 nptII gene (5’-C-C-G-G-G-G-G-C-G-C-G-C-A-C-T-C-C-C-C-G-G-C-C-C-C-C-G-G-C-G-G-3’ and 5’-T-C-A-C-C-C-C-C-G-G-C-G-G-C-G-G-C-G-G-C-C-T-G-G-T-3’) which allow the amplification of a DNA fragment of 800 nt.

**Resistance to CABMV in R0 plants**

The transgenic plants were vegetatively propagated by removing cuttings of approximately 20 cm from the R0 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.
Resistance to CABMV in R₀ plants

In order to determine whether the transgenic lines were resistant to infection by CABMV, the R₀ 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 R₀ 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 R₀ plant TE5-10 is resistant to this isolate, the same used for transformation. Plants obtained from the remaining R₀ transformants developed mosaic and leaf deformation symptoms upon inoculation with both isolates (data not shown). Results of indirect Enzyme 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 R₀ 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 R₀ transformant but inoculated with isolate PE1 had absorbance values above the threshold value (Figure 3).

Accumulation of transgenic and viral RNA in R₀ 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 R₀ transformant TE5-4 (Figure 4, lane 1), but was not detected in non-inoculated plants derived from the resistant R₀ 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 \textit{nptII} gene. Total DNA extracted from transformed yellow passionfruit (\textit{Passiflora edulis} f. \textit{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 \textit{et al.}, submitted). While this level of identity is sufficient to classify these isolates as members of the same viral species (Van Regenmortel \textit{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 \textit{Papaya ringspot virus} (PRSV), family \textit{Potyviridae}, genus \textit{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 \textit{et al.}, 1997; Tennant \textit{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 \textit{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
Our results demonstrate that CABMV-resistant transgenic passionfruit plants have the potential to provide adequate control of passionfruit woodiness disease. The Rₚ plants are currently being self-crossed in order to generate homozygous Rₛ lines. If these Rₛ 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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