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Resistance gene *Sw-5* of tomato confers resistance to TCSV in *Solanum melongena*

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Summary: Eggplants transformed with *Sw-5* gene, regenerated by organogenesis and somatic embryogenesis, were resistant to the *Tomato chlorotic spot virus*, while wild plants did present systemic infection. T0 plants were selfed and the segregation analysis of T1 and T2 generation indicated the existence of one or more insertion sites. Southern blot analysis confirmed one or two independent insertions in T2 plants. Different lesions associated with the insertion number were observed in T1 and T2 plants. T2 plants with two copies displayed faster hypersensitive reactions and characteristic necrotic lesions that contrasted with slower responses and necrotic ring lesions in plants with one copy. These results suggest that the *Sw-5* confers resistance to tospovirus in transgenic eggplants and that the resistant phenotype depends on the number of transgene copies.

Key words: *Tospovirus*, hypersensitive response, genetic transformation, transgene expression

Introduction

Eggplant (*Solanum melongena* L.) is a non-tuberous solanaceous crop that has increasing commercial importance in Brazil and worldwide. It is very appreciated in North America, Asian, European, North Africa and Mediterranean countries where its fruits, which vary greatly in color and shape, are consumed *in natura* or as spices. Breeding for disease resistance in this crop has been limited due to the lack of desirable traits in the eggplant genome and sexual incompatibility with related species containing resistance genes (*Rotino* et al., 1997; *Collonnier* et al., 2001).

Eggplant regeneration, either by organogenesis or somatic embryogenesis, and transformation protocols are available and can be easily accomplished (*Collonnier* et al., 2001). Albeit being considered an amenable species to *in vitro* studies, most studies have dealt with the optimization of genetic transformation protocols (*Filippone & Lurquin*, 1989; *Rotino & Gleddie*, 1990; *Iannacone* et al., 1995; *Fári* et al., 1995; *Picoli* et al., 2002) and transformation for insect resistance (*Rotino & Gleddie*, 1990; *Chen* et al., 1995; *Iannacone* et al., 1995; *Arpaia* et al., 1997; *Jelenkovic* et al., 1998), while virus, fungi and bacterial resistance have been neglected.

Tospovirus is a genus of the *Bunyaviridae* family with a wide host range and transmitted in a propagative manner by thrips (Thysanoptera: *Thripidae*) (*Pozzer* et al., 1996). Amongst the tospoviruses, *Tomato spotted wilt virus* (TSWV) ranks among the top ten economically most important plant viruses worldwide (*German* et al., 1992; *Goldbach & Peters*, 1994). *Jordá* (1992), cited by *Roselló* et al. (1996), and *Parisi* et al. (1998) observed TSWV-infected eggplant crops in Spain and Italy, respectively. Although there are few studies on eggplant viruses in Brazil and a lack of data on tospovirus susceptibility under field conditions, systemic infection under greenhouse conditions was observed (*Lima* et al., 2002). Brazilian eggplant varieties displayed susceptibility to TSWV and to TCSV, which an isolate (V1-3) caused more aggressive symptoms, and Embú was one of the most susceptible cultivars (*Lima* et al., 2002). Based on these results, cultivar Embú and V1-3 isolate were used in the present study.

The *Sw-5* gene from tomato was introgressed from an unknown *Lycopersicon peruvianum* accession and confers resistance to tospoviruses (*Boiteux* et al., 1993). Originally, *Sw*-5 was considered to confer incompletely dominant resistance, which was characterized by the absence of symptoms or the appearance of necrotic lesions in the

inoculated leaves (*Stevens* et al., 1992). This gene conferred tospovirus resistance not only to tomato species and cultivars but also to others solanaceous species such as *Nicotiana tabacum* and *N. benthamiana* (*Lau*, 2001; *Lau* et al., 2005).

The deduced protein encoded by the *Sw-5* gene contains a putative NBS domain composed of kinase 1a, kinase 2a and kinase 3a motifs and the C-terminal region is composed of 14 imperfect LRRs (*Brommonschenkel* et al., 2000). These structural features are similarly found in other resistance genes such as *Mi*, *I2*, *Prf* (tomato), *RPM1* (*Arabidopsis*) and *Rx* (potato), already characterized (*Martin* et al., 2003). Considering the resistant phenotype and the nature of the SW-5 protein, it is thought that resistance depends on pathogen recognition, which triggers a series of responses involving a hypersensitive response (HR) and the contention of the pathogen near the primary infection site.

The cloning of the *Sw-5* gene made its insertion possible in species that do not have efficient tospovirus resistance in its genetic pool. The purpose of this work was to transfer the *Sw-5* gene into the eggplant and to evaluate its functionality in this heterologous system. This study intended to provide data concerning the signal transduction pathways in solanaceous species, and also to generate tospovirus-resistant eggplant lines, which can be incorporated into a breeding program.

Material and methods

Eggplant genetic transformation

Eggplant (*S. melongena* L cv. Embú) seeds were surface-sterilized and germinated as described in *Picoli* et al. (2002). Cotyledons and hypocotyls of 16 to 20 day old seedlings were aseptically removed and used as explants. Cotyledons were cut longitudinally into two segments and placed with the abaxial in contact with the embryogenesis induction medium. Hypocotyl segments varying from 6 to 10 mm were used as explants for organogenesis induction. Eight cotyledonary explants and twelve hypocotyl segments were placed in each Petri dish (90 x 15 mm) with 25 ml of medium. Organogenesis and somatic embryogenesis induction media were prepared according to *Sharma & Rajam* (1995) and *Picoli* et al. (2000), respectively. Cultures were maintained under a 16 h light regime and 24 mol $m^{-2}s^{-1}$ radiation, provided by two fluorescent tubes (20 W Daylight, Osram, Brazil). The growth chamber temperature was set at 26° ± 2 °C.

The cosmid pCLDO04541, harboring a 19 kb genomic region introgressed from *L. peruvianum* (TC134) spanning the *Sw-5 Tospovirus* resistance gene was used for transformation (*Figure 1*). The construct was electroporated into the non-oncogenic *Agrobacterium tumefaciens* strain LBA4404. Bacterial suspensions were grown in 50 ml Rhizo medium (*Tepfer & Cassel-Delbart*, 1987) plus 3 mg l^{-1} tetracycline and 50 mg l^{-1} streptomycin (Sigma Chem. Co., USA), for 24 hours in a rotatory shaker (200 rpm) at 28 °C. They were then centrifuged at 3,500 rpm for 5 minutes at 22 °C. The pellet was diluted in MS0 liquid medium plus 2% sucrose (*Frary & Earle*, 1996), and the optical density was adjusted to 0.4 (λ= 600 nm). The explants soaked in this solution for 5 minutes, and then transferred to non-selective medium. Pre- and co-culture periods of two days were adopted. Selective medium contained 300 mg l^{-1} timentin (Smithkline Beecham, Brazil) and 100 mg l^{-1} kanamycin (Sigma Chem. Co., USA).

Regenerated shoots were cultivated in TMR medium (*Szász* et al., 1998) containing 0.5 mg l^{-1} IAA, and embryos were matured in MR medium supplemented with 150 nM GA3, 1.5% sucrose and 1% Phytagel (Sigma Chem. Co., USA). *In vitro*-regenerated putative transformants had their roots washed and were transferred to plastic cups containing distilled water and covered with plastic bags. Approximately a week later, they were transferred to organic substrate (Plantagro, Brazil) and grown in standard greenhouse conditions under 50% shading.

Transgenesis confirmation and evaluation of tospovirus resistance

Description of transgenic plant generations was according to *Chaleff* (1981). DNA samples were collected (*Fulton et al.*, 1995) and TC134 specific sequences were PCR-amplified with oligonucleotides COS134FR and COS134RF2 (0.8 kb) and 3331 and 19 PST (2.7 kb) (*Figure 1*). Transgene insertion in T0 plants was confirmed based on the amplification of the 0.8 kb fragment.

T0 plants were selfed and the T1 plants were screened to determine the number of *Sw-5* insertions. Deduction of the number of transgene insertion sites was carried out with the

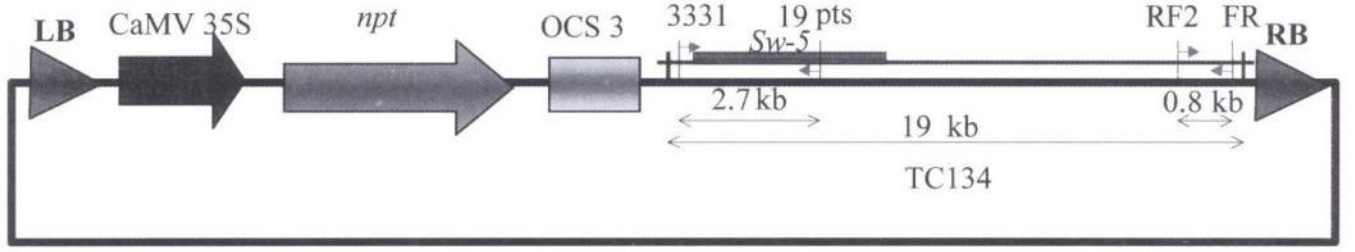

Figure 1. Cosmid pCLDO04541 containing the TC134 clone with the *Sw-5* gene. RB: right border; CaMV: 35S RNA CaMV promoter; OCS3: *ocs3* gene termination sign; *npt*: neomycin phosphotransferase; LB: left border, and annealing sites of the primers 3331 and 19pst (inside the *Sw-5* gene) and COS134FR (FR) and COS134RF2 (RF2) (inside the TC134 fragment and outside the *Sw-5* gene)

chi-square test based on the presence or absence of the 0.8 and 2.7 kb specific bands. T1 plants were selfed and the T2 were used to evaluate the resistance to tospovirus infection. *N. tabacum* cv. 'Havana 425' was used to multiply the *Tomato chlorotic spot virus* (TCSV) V1-3 isolate, avoiding *Tomato mosaic virus* (ToMV) co-infection. Inoculum was prepared from one gram of systemically infected leaves ground in 10 ml of 0.1 M sodium phosphate pH 7.0 with 0.01 M Na_2SO_3, and kept in ice. Carborundum 600 mesh was added, homogenized and spread on the leaf surface with gauze. Mechanical inoculation was performed in plants with 2 to 3 fully expanded leaves. Untransformed plants were used as controls for virus inoculation. Visual evaluation of disease symptoms was monitored for 6 to 8 weeks, and DAS-ELISA (*Clark & Adams*, 1977) was performed with nucleocapsid (N) protein antiserum to confirm virus infection.

Disease progression was evaluated in T2 plants observing the period necessary for hypersensitive response to appear. T2 seeds, from T1 plants 19, 20 and 14, all derived from one transformation event in T0, were chosen based on T1 leaf lesions attributed to 0, 1 or 2 insertion sites, respectively. Inoculation was performed as described above. The number of plants displaying HR was scored on the inoculation day and on the fourth, eighth, twelfth and sixteenth day after inoculation. In this disease progression approach there were surveyed 14, 26 and 38 individuals for plants 19, 20 and 14, respectively.

Molecular analyses

Standard molecular biology techniques were performed according to *Sambrook* et al. (1989). For Southern blot analysis, total DNA extraction from *S. melongena* cv. Embú wild type and *Sw-5*-transformed was performed as previously described (*Fulton* et al., 1995). Nylon membranes (Hybond N^+/Amersham) containing immobilized electrophoresed DNA from T2 plants displaying characteristic HR lesions were used. These individuals were selected from T1 plants used for the evaluation of the resistance to tospovirus infection. The DNA was digested with *Bam*HI (which cuts in the *Sw-5* gene once) and were hybridized with a ^{32}P-labeled probe corresponding to a 1 kb 5'-end *Sw-5* fragment, at 65 °C for 16 hr. The probe was obtained from the amplification of the 1 kb fragment from the cosmid pCLDO04541. Membranes were washed three times for 1 hr at 65 °C in 0.5 × SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS).

Results and discussion

Agrobacterium tumefaciens-mediated genetic transformation

Transgenic plants were regenerated by somatic embryogenesis and organogenesis. Little callusing was observed in hypocotyl segments, while cotyledonary explants displayed necrosis in most of their area. Some sectors developed friable calli, from which embryos differentiated. The regeneration frequency of putative transformants was 4.16% and 3.7% for embryogenesis and organogenesis, respectively. Two out of three plants regenerated by organogenesis were actually transformed, while both somatic embryo-derived plants had the transgene. Transgenic insertion was confirmed by PCR amplification product (0.8 Kb) using COS134FR/COS134FR2 primer pair (data not shown). A third embryo-derived plant, thus not screened, and one of the transformed plants were lost in the acclimatization process.

Although the transformation efficiency obtained in this work was low, as compared to 20.8% (*Billings et al.*, 1997), 6.9% (*Filippone & Lurquin*, 1989) and 7.6% (*Rotino & Gleddie*, 1990), we were successful in obtaining regenerants both from organogenesis and somatic embryogenesis protocols. It is possible that the cultivar Embú does not have a genetic background as favorable as other cultivars in terms of *in vitro* regeneration.

Number of Sw-5 inserted copies

The number of *Sw-5* inserted copies in the three transformation events (T0 generation) and in three T1 plants, inferred on the basis of the number of insertion sites, was estimated by Mendelian segregation analysis. Tissue samples from T1 and T2 plants were collected and DNA extracted. PCR amplification of a specific sequence of the TC134 cosmid was expected in the *Sw-5* containing plants with the

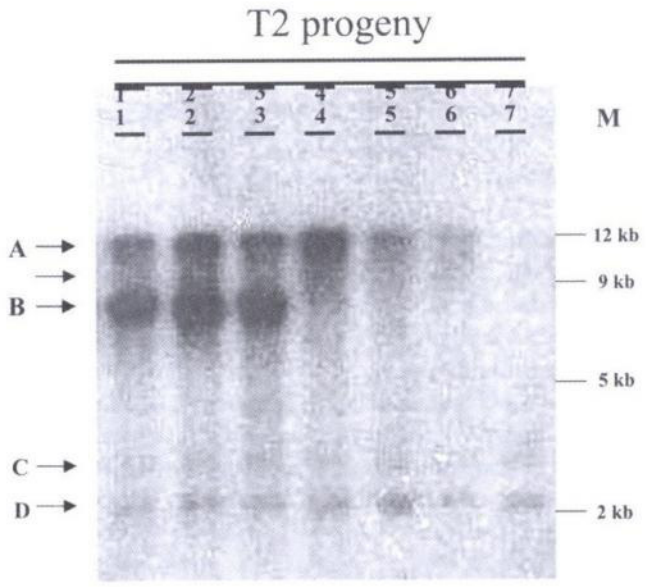

Figure 2. Southern blot analysis of transgenic eggplant genomic DNA digested with *Bam*HI and probed with a 1 kb 5'-end fragment of the *Sw-5* gene to reveal the number of T-DNA insertions. 1–3: T2 progeny with two *Sw-5* insertion sites; 4–6: T2 progeny with one *Sw-5* insertion site; 7: T2 plant with no *Sw-5* insertion. Plants corresponding to lanes 1–3 (Pl 1–14 progeny), 4–6 (Pl 1–20 progeny), and 7 (Pl 1–19 progeny), displayed necrotic lesions, ring necrotic lesions, and mosaic, respectively. Arrows A and B indicate transgene-containing hybridization fragments with approximately 8 and 12 kb, respectively. Arrows C and D indicate hybridization with eggplant *Sw-5* homologous sequences. M, size marker

Table 1. Estimated number of *Sw-5* inserted copies (insertion sites) in eggplant T0 transformants by Mendelian segregation analysis the T1 generation, and in T1 transgenic eggplants estimated by analysis of T2 generation.

Plants (T0)									
Expected segregation		Observed		Expected		DAS-ELISA		Chi-square (χ^2)	Significance (%)
		P	A	P	A	Neg.	Pos.		
3:1	1	67	2	51.75	17.25	66	3	16.81	0.0041
	2	30	0	22.5	7.5	30	0	8.71	0.3164
	3	37	2	29.25	9.75	37	2	8.77	0.3062
15:1	1	67	2	64.8475	4.3125	66	3	2.85	9.1373
	2	30	0	28.125	1.875	30	0	1.07	30.0945
	3	37	2	36.5625	2.4375	37	2	0.36	54.8506
Plants (T1)									
Expected segregation		Observed		Expected		DAS-ELISA		Chi-square ($\lambda 2$)	Significance (%)
		P	A	P	A	Neg.	Pos.		
		P	A	P	A	–	–		
3:1	1–14	44	3	35.25	11.75	–	–	11.20	8.1797
	1–20	30	17	35.25	11.75	–	–	2.85	9.1373
15:1	1–14	44	3	44.0625	2.9375	–	–	0.06	79.2798
	1–20	30	17	44.0625	2.9375	–	–	67.43	1.4x10-10
	1–19	–	12	–	–	–	–	–	–

T1 plant 1–19: non-transformed plant.
P: transgene presence; A: transgene absence; Neg.: DAS-ELISA negative, Pos.: DAS-ELISA positive.
$\chi^2 = \sum [|(\text{observed} - \text{expected})| - 1/2 \, ^{\S}]^2 / (\text{expected})$.
§ Yates correction factor

use of COS134FR/COS134FR2 primers (*Figure 1*). Given the occurrence of plants displaying the resistance phenotype (hypersensitive response or necrotic lesions) but not amplifying the expected 0.8 kb PCR product, a second primer combination was used, 3331 and 19 PST (*Figure 1*), which anneal to sequences internal to the *Sw-5* ORF to amplify a 2.7 kb fragment.

The chi-square test was performed based on the presence or absence of the expected amplified sequences. All tested events fit better to the hypothesis of more than one insertion (*Table 1*). We hypothesized that the observation of tospovirus resistant plants presenting the 2.7 kb PCR product, but not the 0.8 kb, was due to a possible deletion on the inserted T-DNA. Southern blot (*Figure 2*) and PCR analysis (*Table 1*) in T2 plants confirmed the insertion of two independent *Sw*-5 copies.

Tospovirus infection and resistance analysis

Two types of lesions were recognized in transformed eggplants (PCR positive), which were associated with one or two insertions sites of the transgene in the T1 generation and inoculated with the *Tomato chlorotic spot virus* (TCSV) V1-3 isolate (*Figures 3A, 3B*). Southern blot analysis with the respective T2 generation (*Figure 2*) further confirmed this hypothesis. Considering the *Sw*-5 dosage effect observed for *Lycopersicon* sp. (*Stevens* et al., 1992), a more pronounced effect for some tospovirus isolates (*Lau*, 2001) and the present data, it is possible that different phenotypes might be detected depending on the time necessary to elicit hypersensitive responses.

The observation of two different types of local lesions in the segregating T1 generation suggests that the number of transgene insertion sites may determine how fast the defense response evolves. A lower copy number would lead to a delayed defense response generating ring necrotic lesions, while a higher copy number leading to quicker and/or stronger response would generate necrotic lesions. This was also supported by Southern-blot and segregation analyses (*Figure 2*), as well as the infection kinetics with T2 plants (*Figure 4*). It must be highlighted that this copy number effect is only manifested against tospovirus isolates that probably have a weaker interaction between the viral elicitor protein and the *Sw-5* gene product, as observed for tomato and transgenic tobacco. On the other hand, some isolates, which lead easily to HR, induced necrotic lesions irrespective to the number of the *Sw*-5 copies (*Lau*, 2001).

Most of transformed T2 plants displayed hypersensitive response (HR) in the form of necrotic lesions, five days after inoculation, while some of them showed chlorosis. Plants with two insertion sites exhibited necrotic lesions, and single insertion plants presented ring necrotic lesions (*Figures 3A, 3B*). Faster HR elicitation in plants with two transgene copies (insertion sites), compared to events with one copy, reinforces the dosage effect hypothesis in the kinetics of HR

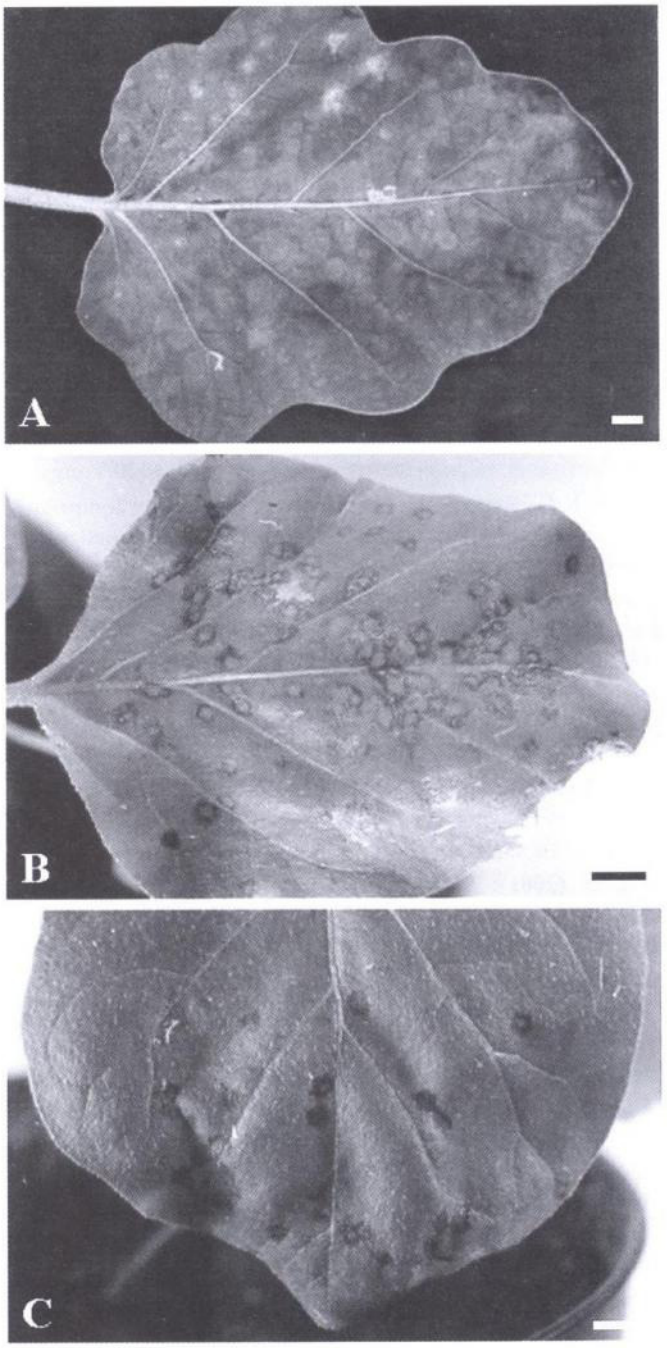

Figure 3. TCSV V1-3 hypersensitivity responce and susceptibility response one week after inoculation of transgenic eggplant. A: Local lesion (necrotic rings) attributed to the presence of one *Sw-5* copy (insertion site); B: Local lesion attributed to the presence of two *Sw-5* copies (insertion sites); C: Mosaic and chlorosis in a susceptible leaf. Bar = 1 cm.

response (*Figure 4*). Both types of lesions in transgenic eggplant appeared more rapidly than the chlorotic lesions observed in non-transformed plants. Non-transformed and control plants did not exhibit HR, although mosaic, ring-spots and chlorotic lesions were observed (*Figure 3C*). Ring necrotic lesions displayed a green core akin to non-inoculated tissue. *Lima et al.* (2002) observed that, in non-transformed plants, the reaction to tospovirus infection on inoculated leaves varied with the eggplant genotype and virus isolate.

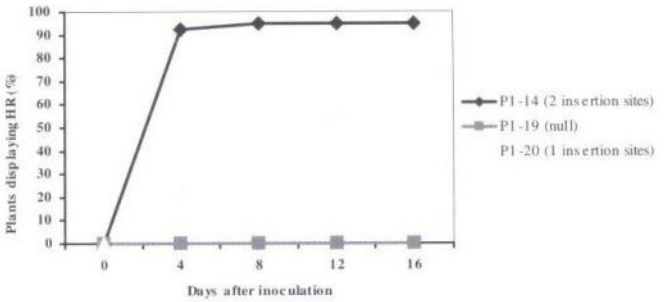

Figure 4. Infection kinetics in T2 plants: percentage of T2 plants displaying hypersensitivity response (HR) after inoculation with the TCSV V1-3 isolate. P1-14, P1-19 and P1-20: selected plants of the first transformation event with two, zero and one *Sw-5* copies, respectively.

The absence of systemic infection in T1 was confirmed by DAS-ELISA. Except for two plants, all transgenic plants were ELISA negative, what suggests that the virus was confined to the lesion area. The TCSV V1-3 isolate was able to systemically infect *Sw-5*-transgenic tomatoes, although this was dependent on the number of transgene copies (*Lau*, 2001). *Stevens* et al. (1992) had already observed 98.7% penetrance in a TSWV resistance inheritance study using *Sw-5* transgenic tomatoes. These responses may be a consequence of heterologous expression of the *Sw-5* gene. Also, the lesion reddish shadowing suggests that some sort of defense response might still be occurring in this region. This is supported by the fact that isolates that are considered efficient HR elicitors on *Sw-5* plants (tobacco and tomato) (*Lau*, 2001) always generate lesions with a necrotic core.

Two additional hybridization bands (2.2 and 3.3 kb), common to all individuals screened in the Southern blot analysis of T2 plants, could be observed (*Figure 2*). It should be noted that *S. melongena* cv. Embú naturally displays a level of resistance to some tospovirus isolates (*Lima* et al., 2002). Thus, the observed cross hybridization pattern in the Southern blot analysis suggests the presence of *Sw-5* homologs in the *S. melongena* genome.

Grube et al. (2000) reported the sinteny and conservation of phenotypically defined disease resistance genes (R genes) and R gene homologues in solanaceous crops. An analysis of the available data for solanaceous R genes suggests that, while the taxonomic specificity of host R genes may be evolving rapidly, general functions of R alleles may be conserved at homologous loci in related plant genera. On support of this hypothesis, *Tai* et al. (1999) verified functional activity of the pepper *Bs2* gene, which confers resistance to *Xanthomonas campestris*, in tomato and *N. benthamiana*. *Grube* et al. (2000) observed hybridization bands, attributed to R gene homologs, in the pepper genome, when tomato probes were used. Similarly, pepper *Sw-5* homologs were found in two positions. Considering gene mapping position and similarity across genera, these results suggest that these alleles may maintain similar function, and probably, similar signal transduction pathways. *Van der Hoorn* et al. (2000) reinforced this hypothesis, observing that tomato *Cf-9* and *Cf-4* resistance genes to *Cladosporium fulvum* were functional in *Nicotiana* spp. and *Petunia hybrida,*. Nonetheless, this signal transduction

pathway seems to be conserved only in solanaceous species, as heterologous expression approaches in species belonging to other families did not result in resistant phenotypes (*Tai* et al., 1999; *van der Hoorn* et al., 2000).

Our results suggest that the *Sw-5* gene is functional in eggplant and is efficiently incorporated into the signal transduction pathway that leads to tospovirus resistance, or to the activation of other virus resistance genes. *Lau* (2001) verified that F1 tomato plants, from a cross between a tospovirus resistant (*Sw-5*) and a susceptible (*sw-5*) tomato plant, still allowed systemic infection of the V1-3 isolate. An efficient signal transduction pathway or synergistic effect with other genes associated to tospovirus resistance must be occurring, as HR was observed in eggplant similarly as in tomato. Tospovirus control is difficult for several reasons (*Boiteux* et al., 1993), and the use of resistant plants is one of the few efficient means of containing the pathogen (*Roselló* et al., 1996). The present report is an example where transgenesis, associated to traditional breeding, may provide the conditions for the production of eggplant cultivars with superior agronomical traits and tospovirus resistance.

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