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Resistance of transgenic papaya plants to *Papaya ringspot virus*

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Abstract

The coat protein gene isolated from *Papaya ringspot virus*, Thai isolate, was used to generate transgenic papayas. A binary vector containing the coat protein gene under the control of a 35S promoter, was constructed and transformed into somatic embryos of papaya cultivar Khak Dum by microprojectile bombardment. Eight transgenic lines were identified from 1980 bombarded calli of papaya somatic embryos under kanamycin selection. Integration of the transferred genes into kanamycin resistant papaya calli was verified by PCR amplification of the coat protein gene, GUS assays and Southern blot hybridization. Although the coat protein gene was detected in all transgenic lines, only line G2 was found to be highly resistant to virus. This resistant line showed high degree of rearrangement of the inserted coat protein expression cassette while the coat protein gene itself had a deletion of 166 bp on the 3' end of its sequence. Although the transcription of the coat protein gene was detected in all transgenic lines by RT-PCR, only two transgenic papayas expressed the intact coat protein. Moreover, in the resistant line G2 the amount of the truncated coat protein mRNA was significantly decreased. These results point to an RNA mediated mechanism of coat protein mediated resistance in papaya, probably based on post-transcriptional gene silencing.

Additional key words: biolistic transformation, coat protein, plant regeneration.

Introduction

Papaya (*Carica papaya* L.) is a fruit crop grown in both tropical and subtropical countries. In Thailand, papaya is one of the staple foods and is grown both in commercial plantations as well as in backyard gardens. Since 1975, however, papaya production has been severely limited by *Papaya ringspot virus* (PRSV) (Srisomchai 1975), which is now endemic in the country.

PRSV is a positive single stranded RNA virus in the potyvirus group (Purcifull *et al.* 1984.) with the virions being flexuous, filamentous particles of 780 × 12 nm. PRSV is classified into two types according to the host range, type P, which infects papaya and some cucurbits and type W, which infects only cucurbits. These two types are not distinguishable by serological methods. PRSV type P can infect papaya at any growth stage. The infected papaya plants show a range of symptoms including yellowing and vein-clearing of younger leaves, mottling, leaves distortion and narrowing, ringed spots on the fruit and dark green streaks on the petioles and stems. Infected papaya plants exhibit significant stunting and a

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Abbreviations: AMV - alfalfa mosaic virus; CP - coat protein; CPMR - coat protein mediated resistance; GUS - glucuronidase; NTR - non translated region; PCR - polymerase chain reaction; PRSV - *Papaya ringspot virus*; PTGS - post-transcriptional gene silencing; RT-PCR - reverse transcription PCR.
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reduction in quality and quantity of fruit production. Several techniques have been applied to control the disease in the past including mild strain cross protection and conventional breeding programs, however, none of these techniques proved to be successful to date.

Coat protein mediated resistance (CPMR) has been successfully used to produce transgenic plants resistant to viruses (Baulcombe 1996, Beachy 1997), including transgenic papaya resistant to PRSV from Hawaii (Fitch *et al.* 1990, 1992). Transgenic plants of papaya cultivar Sunset containing the coat protein gene of the mild strain HA 5-1 of PRSV showed resistance to the severe strain of PRSV isolated from Hawaii but not to PRSV isolated from Thailand and other countries (Tennant *et al.* 1994), leading to the suggestion that the resistance to the PRSV is strain specific and it must therefore be targeted to strains from the same geographical region.

In our previous work, we compared the sequences of the coat protein gene and the 3' non-translated region of PRSV isolates from Thailand and other countries (Kertbundit *et al.* 1998). A very high degree of similarity was found among PRSV Thai isolates (95 - 99 %) while up to 13 % variation was observed comparing PRSV CP isolates from Thailand and other countries. The high variability in the N terminus together with the absence of a lysine residue in all the Thai isolates may play an important role in overcoming transgenic resistance against Hawaiian PRSV strain HA5-1.

The CPMR resistance was originally based on a concept of pathogen derived resistance (PDR) which proposes that a pathogen trait, expressed in a host organisms, may disrupt the parasitic relationship and result in host resistance (Sanford and Johnson 1985). Recently it was discovered, however, that CPMR is based on a post-transcriptional gene silencing (PTGS), which is closely related to RNA interference, and represents an ancient eukaryotic phenomenon for adaptive protection against viruses (Lecellier and Voinnet 2004, Voinnet 2002). In transgenic plants, this kind of RNA-mediated resistance is usually a result of transgene rearrangement leading to inverted repeats of transgene, which have been shown to be the inducers of PTGS and virus resistance (Smith *et al.* 2000, Wesley *et al.* 2001, Kalantidis *et al.* 2002).

In this work, we used the coat protein gene of a PRSV Thai isolate to generate transgenic papaya plants resistant to PRSV and analyze whether this CPMR is protein or RNA-mediated.

Materials and methods

Plasmid construction: The binary vector pGV4042 was derived from pGV941 (Deblaere *et al.* 1987). The β-lactamase gene in pGV941 was inactivated by a *Pst* I/*Sca* I deletion and the *Hin*d III and *Eco*R I recognition sites, flanking the left and the right borders in pGV941 were removed by restriction enzyme digestion, blunting and ligation. Multiple cloning site (*Bgl*II, *Xba*I, *Eco*RI, *Sal*I, *Hin*dIII, *Eco*RV, *Bam*HI) was inserted between the *Hpa* I and *Bam*HI sites upstream of the chimeric *nptII* gene to facilitate cloning. The *Hind*III/*Eco*RI fragment of pBI121 (Jefferson *et al.* 1987), which contains CaMV35S promoter-*uidA* gene-3' *nos* was inserted into the polylinker of pGV4042 to generate the binary vector pSA1001.

The full-length coat protein (CP) gene was amplified from the RNA of PRSV isolated from Ratchaburi province, Thailand by RT-PCR. The 5'CP forward primer (ATCATTCCATGGCTGTGGATGCTGGTTTGAATG) was designed to contain an ATG start codon and the 3'CP reverse primer (GTCAAGCCATGGTTGCGCAGCC ACNCTGTATTCTAATG) was designed to contain a 3' stop codon for the CP gene. *Nco*I sites were introduced into both primers to facilitate further cloning. The amplified CP gene was then inserted into pPRT103 (Topfer *et al.* 1987) between the CaMV 35S promoter and CaMV polyA signal at the *Nco*I site. The non-translated region of alfalfa mosaic virus (AMV) (Gallie *et al.* 1987) as included into the *Kpn*I site between the 35S CaMV promoter region and the PRSV coat protein gene yielding a CP plant expression cassette. This expression cassette was then inserted between the *nptII* gene and the *uid*A gene binary vector pSA1006 (Fig. 1).

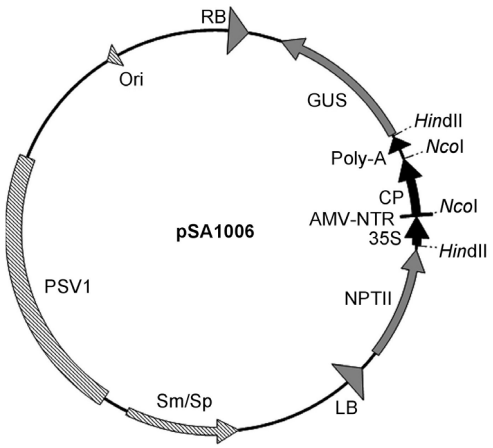

Fig. 1. Schematic map of binary vector pSA1006. The CP sequence is under control of CaMV 35S with NOS terminator and AMV NTR as enhancer. Only *Nco*I flanking the CP gene and *Hin*dIII flanking the CP expression cassette are shown.

Plants and culture: Zygotic embryos were dissected from seeds of immature fruits of the papaya (*Carica*

papaya L. cv. Khak Dum), and cultured in the dark at 26 °C on induction medium (M1 medium) which consisted of half-strength MS salts (Murashige and Skoog 1962), 50 mg dm^{-3} myo-inositol, full-strength MS vitamins, 400 mg dm^{-3} glutamine, 10 mg dm^{-3} 2,4-D, 6 % sucrose and 8 g dm^{-3} *Difco Bacto* agar, pH 5.8. After three weeks of incubation, embryogenic calli developed from zygotic embryos. Somatic embryos comprising of globular, heart and torpedo stages, developed after one to two months of maintaining the embryogenic calli in the induction medium. One week before micro projectile bombardment, the tissue containing somatic embryos were transferred to fresh M1 medium in groups of 30 clusters per Petri dish sealed with *Parafilm*.

Plant transformation: The *M10 tungsten* were coated with pSA1006 vector according to the *Bio-Rad* manual and used for bombardment of the somatic embryos clusters with *PDS1000* particle gun delivery system (*Bio-Rad*). The gap distance between the rupture disk and the macro carrier was 1.2 cm and the Petri dish was placed at the target level 2 (6.0 cm). The chamber was evacuated to 88 kPa (26 in Hg) and the helium gas pressure was 6.2 MPa (900 psi). The bombarded papaya calli were cultured in the M1 medium for 2 d and then transferred to the callus propagation medium (M1 medium supplemented with 75 mg dm^{-3} kanamycin and 500 mg dm^{-3} cefotaxime). After 14 d, papaya calli were subcultured to fresh M1 medium containing 100 mg dm^{-3} kanamycin and 250 mg dm^{-3} cefotaxime. Cultures were transferred monthly to the fresh medium until resistant calli developed (4 - 5 months).

Plant regeneration: The kanamycin resistant calli were transferred to 1 dm^3 glass jars with plastic lids containing regeneration medium (MS medium supplemented with 0.2 mg dm^{-3} BAP, 0.1 mg dm^{-3} kinetin, 3 % sucrose, 8 g dm^{-3} *Difco Bacto* agar, 100 mg dm^{-3} kanamycin, 250 mg dm^{-3} cefotaxime, pH 5.7) and incubated at 26 °C under a 12-h photoperiod by cool white *Sylvania* fluorescent lamps (irradiance of 700 μW cm^{-2}). The tissues started to regenerate after 5 - 6 months and multiple shoots were formed within 6 - 12 months. Shoots with 3 - 4 leaves and about 1.5 cm tall were sliced and transferred to the growth-regulator-free rooting medium consisting of half strength MS salts medium, half strength MS vitamins, 1.5 % sucrose, 8 g dm^{-3} *Difco Bacto* agar at pH 5.8. Rooting plants were transferred to 1:1 mixture of soil and *Vermiculite* and acclimatized under the same light and temperature conditions.

GUS fluorimetric analysis: The GUS expression in kanamycin resistance embryogenic calli was analyzed for β-glucuronidase activity by fluorimetric assay (Jefferson *et al.* 1987).

PCR analysis: Total genomic DNA was isolated from transgenic calli or leaves of regenerated plants by the CTAB method (Guillemaut and Maréchal-Drouard 1992) and used as a template. The 0.05 cm^3 of total PCR reaction was composed of 100 ng DNA template, 50 pmol of each forward and reverse primers, 200 μM of dNTPs mix, 1× PCR buffer (20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1 % Triton
X-100, and 100 μg cm^{-3} nuclease-free BSA), 0.5 mm^3 (1.5 units) *Pfu* DNA polymerase and water. The amplification was carried out in the thermal cycle 2400 (*Perkin-Elmer*, USA) under the following condition: 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min, for 35 cycles. PCR products were analyzed by agarose gel electrophoresis. 5'CP (ATCATTCCATGGCTGTGGA TGCTGGTTTGAATG) and 3'CP (GTCAAGCCATGG TTGCGCAGCCACNCTGTATTCTAATG) primers were used to amplify the coat protein gene. 5'GUS (ATGTTACGTCCTGTAGAAAC) and 3'GUS (GCGCC AGGAGAGTTGTTGAT) primers used to amplify the *gus* gene.

RT-PCR analysis: Total RNA was isolated from transgenic calli or leaves of regenerated plants using the *TRIzol* reagent kit (*Gibco BRL*, USA). The RNA was treated with DNaseI. First strand cDNA was synthesized using Superscript II™ (*Gibco BRL*) and oligo-dT primer according the manufacturer's instruction. Five to ten mm^3 of this first strand cDNA reaction were directly used as the template for PCR amplification using 5'CP and 3'CP primers for detection of the CP mRNA and 5'GUS and 3'GUS primers for detection of GUS mRNA.

Southern blot hybridization: Ten micrograms of total DNA isolated from each transgenic plant were digested with *Nco*I. The resulting fragments were subjected to electrophoresis in a 0.8 % agarose gel, transferred to *Hybond*-N^+ membrane (*Amersham Biosciences*, UK) and hybridized using the 643 bp 3' end of the amplified coat protein gene fragment as a probe. Labeling of the probe, hybridization and detection was performed using *AlkPhos* direct non-radioactive DNA labelling system (*Amersham Biosciences*) and *CDP-Star* detection kit (*Amersham Biosciences*) following manufacturer's instructions.

Western blot analysis: Thirty five micrograms of total proteins isolated from transgenic and PRSV infected papaya (Wu and Wang 1984) were separated by SDS-PAGE and transferred onto *Protran* nitrocellulose membrane (*Schleicher & Schuell*, Germany) by semi-dry method using *Trans-Blot SD* Electrophoretic Transfer Cell (*Bio-Rad*). The blotting was performed with a constant current (0.8 mA cm^{-2}) for 1.5 - 2.5 h depending on the thickness of the gel. The polyclonal antibody used for detection was prepared from intraperitoneally

immunized mice as described in Harlow and Lane (1988). The membrane was first incubated with a 1:2400 dilution of anti GST-hIre1 polyclonal antibody, which composed of anti-GST polyclonal antibody and anti-hIre1 polyclonal antibody, and then incubated with 1:5000 dilution of secondary antibody (horseradish peroxidase conjugated with anti-mouse IgG). The antibody detection was performed by ECL plus Western blotting detection reagent (*Amersham Biosciences*).

PRSV inoculation: Five regenerated plants from each transgenic line were tested for PRSV infection. The PRSV isolate Ratchaburi was propagated in normal papaya plants. The infected leaves were ground in 0.01 M phosphate buffer, pH 7.0, and this leaf extract was rubbed onto two celite-dusted upper leaves of transgenic plants. After 10 min, the inoculated leaves were rinsed with water. Plants were observed for at least 90 d for the symptoms of PRSV infection.

Results and discussion

The major result reported in this paper is a high degree of resistance to PRSV associated with the presence of an aberrant CP gene and/or rearrangement of the integrated CP cDNA in the resistant transgenic papaya.

Calli containing somatic embryos of papaya cultivar Khak Dum were used in this experiment. Transgenic calli were identified as light-green somatic embryo clusters among the brown tissue of non-transformed calli after bombardment and subculture for 5 - 6 months in selective media containing 100 mg dm^{-3} kanamycin. Eight transgenic lines (G1, G2, T1, T2, T4, T5, T6 and T7) were successfully regenerated from 1 980 calli bombarded with plasmid pSA1006. Thus, the transformation efficiency was rather low - about 0.4 %.

The coat protein gene presence in the transgenic lines was initially analyzed by PCR. The intact 964 bp fragment of the coat protein gene was detected in all transgenic lines – with an exception of line T1 (Fig. 2).

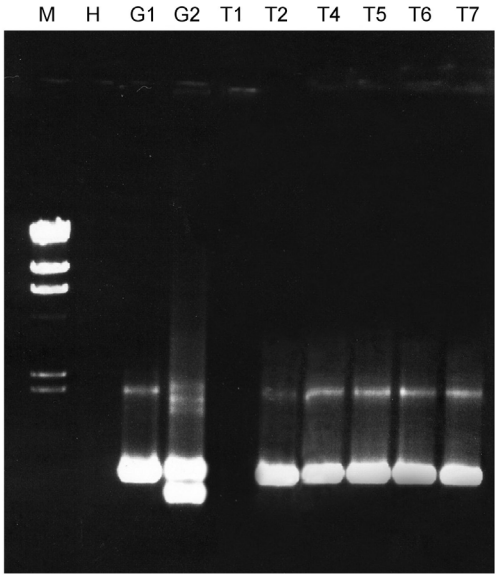

Fig. 2. PCR amplification of the coat protein gene inserted in the transgenic lines. The PCR products were analyses in 0.8 % agarose gel and stained with ethidium bromide. M - λ-DNA digested with *Hind*III, N - PCR amplification from non-transgenic papaya.

In addition, the transgenic lines G2 exhibited a truncated form of the CP gene of about 0.8 kb. This shorter form of the CP was isolated from the PCR mixture and sequenced using an ABI 377 automated sequencing system. 166 bp fragment was found missing from the 3' end of the CP gene. However, using specific PCR primers to this missing region, we were able to detected it in all transgenic plants so it seems there are both full length and shorter form of the coat protein sequence in line G2.

Southern blot analysis of *Nco*I digested total DNA with a 3' end CP probe (Fig. 3) confirmed the data obtained by PCR amplification. Transgenic line G2 showed a deletion in the CP gene, while all the other lines appear to have a full length CP gene insertion. Only lines G1 and T2 seem to have single transgene copies while the remaining lines exhibit multiple rearranged and/ or truncated transgene fragments that are however different

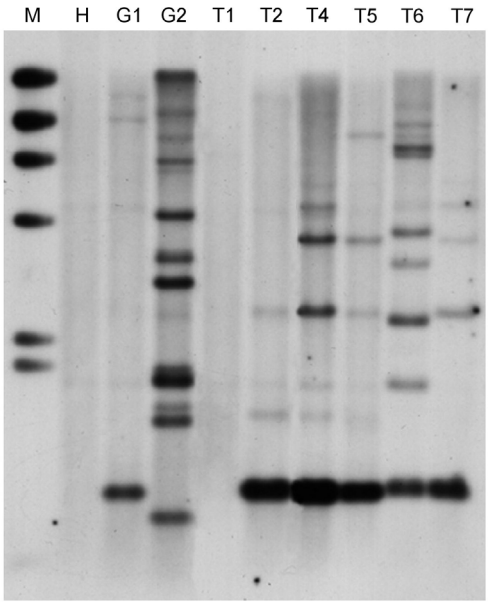

Fig. 3. Southern blot analysis of transgenic papaya DNA. Total plant genomic DNA from each transgenic line was digested with restriction enzyme *Nco*I, which flanks the PRSV coat protein and hybridized with CP probe. M - *Hind*III λ-DNA marker, N - DNA from non-transgenic papaya.

from the PRSV resistant line G2.

The transcriptional product of the PRSV coat protein gene in all eight transgenic lines was determined by RT-PCR analysis (Fig. 4). The binary vector used for biolistic transformation and integration of the CP gene into the papaya genome was originally designed as a plant expression vector (Fig. 1). We can therefore see the CP mRNA transcription. All lines with an exception of T1 showed an amplified fragment of the intact CP mRNA. Apart from the strong band of intact CP, line G2 shows a faint band of the shorter form of CP as well.

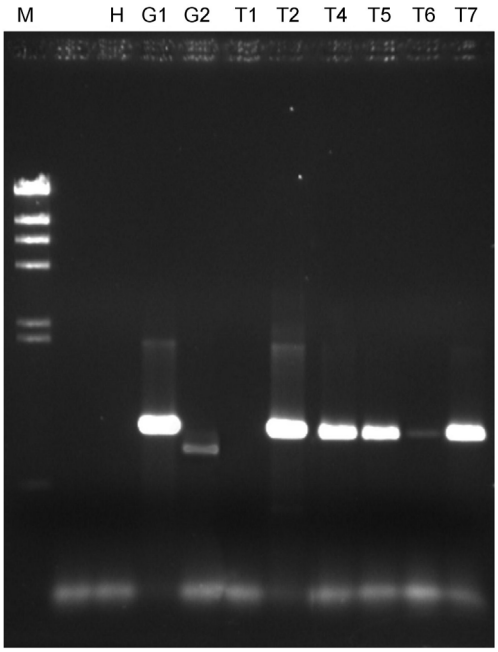

Fig. 4. RT-PCR amplification of the coat protein gene inserted in the transgenic lines. The PCR products were analyzed in 0.8 % agarose gel and stained with ethidium bromide. M - λ-DNA digested with *Hin*dIII, N1, N2 - PCR amplification from non-transgenic papaya as a negative control. P - PCR amplification from PRSV infected papaya as a positive control.

Despite the fact, that CP DNA is transcribed into mRNA in seven transgenic lines, the coat protein expression can be detected only from lines G1 and T2. SDS-PAGE gel shows a clear 35 kDa band of the PRSV coat protein when using polyclonal antibody against this CP sequence (Fig. 5). It indicates that the DNA sequence rearrangement seen from Southern blots (Fig. 3) probably caused a disruption in expression in other transgenic papaya lines (G2, T4, T5, T6 and T7). This experiment excludes protein as a causative agent of PRSV resistance in transgenic papaya.

These results clearly point to an RNA mediated resistance mechanism with involvement of post-transcriptional gene silencing. The underlying mechanism of RNA-mediated virus resistance, also referred to as homology-dependent resistance, is post-transcriptional gene silencing (Baulcombe 1996). The integration of foreign DNA into the chromosome after biolistic bombardment is a rather complex process, which is still poorly understood. Transgenic loci frequently consist of multiple copies of transgenes and with various degrees of rearrangement. These rearrangements, such as repeats, inverted repeats or gene fusions are believed to be responsible for PTGS. CPMR in trangenic plants based on PTGS mechanism and mediated by an aberrant or truncated coat protein genes were previously confirmed in with a number of viruses (Silva-Rosales *et al.* 1994,

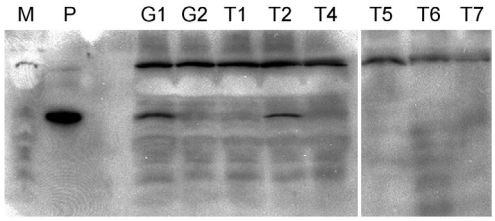

Fig. 5. Detection of CP protein in the transgenic lines by Western blot analysis. 35 μg of total protein extract from transgenic papaya leaves were transferred to a nitrocellulose membrane and treated with a 1:500 dilution of the primary antibody (anti-CP polyclonal antibody), followed by incubation with a 1:5000 dilution of the secondary antibody. M - broad range protein marker, P - 500 ng of total protein extract from PRSV infected papaya leaves.

Leclerc and Abou Haidar 1995, Pang *et al.* 1997).

Presently, we are unable to determine if the observed high resistance against PRSV in G2 lines is related to the truncated CP or to the missing 166 bp sequence. It also remains unclear why the suspected PTGS lead to the resistance only in line G2 and not in other transgenic lines - T4, T5, T6 and T7, which are showing multiple copy and/or rearranged sequences as well.

There is a discrepancy between the PCR, RT-PCR and Southern blot results in transgenic line G2. While both PCR amplification of the CP gene from genomic DNA and RT-PCR amplification of the cDNA from mRNA showed the presence of an intact 964 bp CP sequence, no such band was detected by Southern blot analysis. However the PCR results of CP DNA amplification from G2 lines were not always identical. In repeated experiments and under identical conditions we were occasionally unable to amplify the intact CP gene from some samples of the G2 line, while the truncated form of CP was always present. The truncated form of the smallest band of the CP gene in G2 line (Fig. 3) was confirmed by sequencing; the identity of the 1 kb band seen in PCR results comes probably from multicopy and/or rearranged insertions of the coat protein sequence, which are seen as larger size bands in Fig. 3.

All eight transgenic lines were tested for mechanical PRSV infection. 10 - 15 cm tall papaya plants were

Table 1. Summary of various analyses performed on the transgenic papaya lines. GUS activity [nmol mg^{-1} min^{-1}] was calculated as mean value ± standard deviation from three independent experiments. S - susceptible to PRSV infection, R - resistant to PRSV infection, NT - non transformed.

Line	GUS activity	RT-PCR of GUS gene	PCR of CP gene [kb]	RT-PCR of CP gene [kb]	Resistance to PRSV
NT	0.41 ± 0.16	–	–	–	S
G1	269.71 ± 34.10	+	1	1	S
G2	0.37 ± 0.14	–	1, 0.8	1, 0.8	R
T1	285.34 ± 4.35	+	1	1	S
T2	35.34 ± 43.48	+	1	1	S
T4	1.60 ± 0.64	+	1	1	S
T5	37.51 ± 47.76	+	1	1	S
T6	0.41 ± 0.18	–	1	1	S
T7	45.84 ± 10.51	+	1	1	S

inoculated with PRSV particles under greenhouse condition. The first symptoms appeared both in the control plants and in the susceptible transgenic plants approximately 3 weeks after inoculation. There was no delay observed. Only the G2 transgenic line showed high resistance against PRSV infection - all G2 R0 lines remain immune to the virus during repeated mechanical inoculations over 3 years of testing. No symptoms were observed on the inoculated leaves or on new emerging leaves during plant maturation and no PRSV sequence could be amplified in RT-PCR tests.

From the results summarized in Table 1 it is clear that no prediction about virus resistant transgenic papaya can be made from kanamycin resistance, from GUS expression or from PCR results. The GUS expression test did not correlate with neither the data obtained from mechanical infection of transgenic papaya plants nor with the RT-PCR experiments. The only reliable test was an actual infection of the transgenic papaya plants with the virulent papaya ringspot virus. Thus, our results are contradictory to the transgenic papayas reported by others (Cabrera-Ponce *et al.* 1995, Yang *et al.* 1996), where GUS expression is detected in all transgenic papaya plants.

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There are many books now published on the topic of plant tissue cultures. In the series "Methods in Molecular Biology™" Vol. 318 the 2nd edition of Plant Cell Culture Protocols has appeared. This 2nd edition pursues the similar goals as its predecessor – to provide an updated step-by-step guide to the most common and applicable techniques and methods for plant tissue and cell culture. Readily reproducible and extensively annotated, the methods range from general methodologies, such as culture induction, growth and viability evaluation and contamination control to such highly specialized techniques as chloroplast transformation.

The total of 30 chapters is divided in 6 sections. The first section introduces readers to *in vitro* technology with the insight into the history of plant, cell, tissue, and organ cultures. The following part "Cell Culture and Plant Regeneration: the Fundamentals" contains eight quite different chapters. They bring some basic and useful information, but when we have in mind that it is the methodical book, the continuation of follow-up articles is rather pure. Inside the third part mostly dealing with plant propagation techniques, one very important paper about use of statistics in plant biotechnology is included. The protocols of isolation, culture and plant regeneration from protoplasts we can find in the fourth part. The fifth part describes the methods for genomic manipulation – via *Agrobacterium*, particle bombardment and chloroplast transformation. The one of possibilities of commercial utilization of plant tissue cultures is the production and accumulation of pharmaceutically interesting metabolites and this is the topic of four articles in the last part of the book.

All the chapters are reviews of up-to-date literature. They start with short introduction summarizing the basic information. Further, the necessary equipment and reagents are mentioned. It is followed by step-by-step laboratory instructions. We can find there many diagrams and schemes illustrating the processes or strategies. The high formal level of this book is decreased with some photographs of pure quality and presence of only 3 colour pictures. Very beneficial is addition of two appendices with the composition of commonly used plant cultures media and the list with useful internet sites. I would like to recommend this book as a major resource of information to the all research team, which start with new plant cell culture techniques.

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