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BRIEF COMMUNICATION

Post-transcriptional gene silencing is involved in resistance of transgenic papayas to papaya ringspot virus

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Abstract

Transgenic papayas carrying the papaya ringspot virus coat protein gene were inoculated with *Papaya ringspot virus*. Infection was monitored by evaluating symptoms and by reverse transcription polymerase chain reaction (RT-PCR). Among eight tested transgenic lines, clone G2 was found highly resistant to virus infection during 3 years of testing. Further analysis of this clone revealed complex multicopy transgene insertion with aberrant copies. The suspected post transcriptional gene silencing was confirmed by siRNA detection. While the R0 generation of G2 transgenic papaya was found to be fully resistant to the infection, *Papaya ringspot virus* was able to break this resistance in subsequent generations by suppressing post-transcriptional gene silencing (PTGS).

Additional key words: *Carica papaya*, reverse transcription PCR.

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Papaya ringspot virus (PRSV), a species of *Potyviridae*, is the etiological agent of one of the most important disease in papaya (*Carica papaya* L.), which occurs wherever papaya is grown. While the use of resistant germplasm for developing virus resistant cultivars is a preferred method of control, no source of such resistance was previously found in papaya. Thus, the only successful control of this disease so far can be achieved through genetic engineering (Ling *et al.* 1991, Lius *et al.* 1997). We used the pathogen-derived approach (Sanford and Johnson 1985) to generate transgenic papaya resistant against PRSV.

Eight transgenic lines, bearing PRSV coat protein gene, were previously identified under kanamycin selection out of 1980 bombarded calli of papaya somatic embryos. One line, labeled G2, was found to be highly resistant to this virus and the nature of this resistance was found to be RNA mediated. Because of a low steady state level of PRSV CP mRNA and the presence of truncated and rearranged transgene, the involvement of post transcriptional gene silencing (PTGS) was suggested (Kertbundit *et al.* 2006).

With a discovery that some geographically distant PRSV viruses are able to overcome the coat protein mediated resistance in papaya, it was originally suggested, that the resistance to the PRSV is strain specific and it must therefore be targeted to strains from the same geographical region (Tennant *et al.* 1994). More recent reports have shown however, that the sequence divergence of the virus from the transgene does not always correlate with the ability to overcome PRSV resistance (Chiang *et al.* 2001). An involvement of silencing suppressor seems to be more likely the main factor involved in breaking of PRSV transgenic resistance (Tripathi *et al.* 2004). It has been demonstrated that silenced plants infected with a virus encoding a suppressor of gene silencing resulted in reversal of the silencing (Anandalakshmi *et al.* 1998, Marathe *et al.* 2000). In the potyvirus group, a viral protein called helper component proteinase (HC-Pro) was clearly shown to

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Abbreviations: CP - coat protein; HC-Pro - helper component protein; PRSV - *Papaya ringspot virus*; PTGS - post-transcriptional gene silencing.
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mediate such suppression. Despite years of study however, the detailed mechanisms plant resistance based on PTGS is still not well understood. In this paper, we report on the mechanisms of PRSV resistance in transgenic papaya in R0 and R1 generations.

Transgenic papaya (*Carica papaya* L.) lines G1, G2, T1 - T7 used in this study were previously described (Kertbundit *et al.* 2007). Transformed plants originated from a single somatic embryo were transferred to soil in greenhouse and considered as R0 generation. R1 subsequent transgenic papaya lines were backcrossed R0 with non transgenic cv. Kaek-dum. The inheritance of the transgene was analyzed by Southern blot analysis using PRSV CP as a probe and resistance was tested by mechanical inoculation with Thai isolate of PRSV. For this testing, transgenic papaya lines were grown in the greenhouse to height of about 6 - 8 cm. The plants were then dusted with 600-mesh carborundum powder on the third and the fourth youngest leaves and rubbed with 0.2 cm^3 inoculum extracted from PRSV infected papaya tissue. Non-transformed papaya plants were used as a positive control. Symptoms development was monitored daily for at least two months by visual evaluation and by PCR detection of PRSV HC-Pro and NiaB fragments.

Genomic DNA from papaya for Southern blot analysis was isolated by homogenizing 2 g of young leaf tissue as described by Rogers and Bendich (1994). 20 µg DNA from each line was then digested overnight with *Hind*III restriction endonuclease, separated on a 0.8 % agarose gel and blotted onto *GeneScreenPlus*® membrane (*Perkin Elmer Life Sciences*, USA). Transferred DNA was hybridized overnight at 42 ºC with the full length PRSV CP probe labeled with the gene images random prime labeling kit (*Amersham Biosciences*, UK). Gene images *CDP-Star* (*Amersham Biosciences*) was used for detection.

The total RNA extract was used for high and low molecular mass RNA gel blot analysis according to the procedure described by Hamilton and Baulcombe (1999) and Szittya *et al.* (2002) with slight modification. The high molecular RNAs were precipitated out and the lower molecular mass RNAs fractions were recovered from the supernatant and separated on 15 % polyacrylamide gel and transferred to *Hybond* N^+ membrane (*Amersham Biosciences*) by electroblotting with *Trans-Blot*® *SD* semi-dry transfer cell (*Bio-Rad*, Hercules, USA). DNA oligomers were loaded on the same gels for size and polarity controls. For generating an RNA probe, the full-length PRSV CP sequence or 400 bp of the PRSV HC-Pro sequence was inserted into pGEM-3zf(+) plasmid (*Promega*, Madison, USA) in the *Sma*I site. Clones with inserts in sense or antisenese orientation relative to the T7 promoter were linearized with *Bam*HI and purified. After *in vitro* transcription in the presence of [α^{32}P]UTP, the template was digested with RNase-free DNase and hydrolyzed in carbonate buffer to an average length of 40 nucleotides. After neutralization with 3 M sodium acetate, pH 5.0, the probe was added to the prehybridization buffer. Hybridization and washes were performed as described Hamilton and Baulcombe (1999) and Szittya *et al.* (2002) at 30 ºC. All experiments were done at least in triplicate.

Once the papaya response to transgenic infection was determined, the total DNA prepared from infected leaves was used in Southern hybridization. *Hind*III restriction enzyme, which cuts out the CP expression cassette, was used to analyze the transgene structure in all both resistant and non resistant transgenic plants. Assuming the full integration of both cassettes, the *Hind*III digestion would yield single band of 1.6 kb in size. This expected banding pattern was observed only in the transgenic lines G1 and T2 (Fig. 1). The remaining transgenic papaya showed various degree of rearrangement depending on each line. All the nonresistant lines revealed at least one intact 1.6 kb CP expression cassette. The G2 resistant line contained greatest number of bands of all transformed lines. The expression cassette, however, is about 500 bp larger than the expected size despite the fact that the CP within this cassette is 166 bp shorter (Kertbundit *et al.* 2007). This result indicates, that not only separate insertions of duplicated fragments have occurred but also a rearrangement of the transgene itself took place. The larger size of other fragments may also result from mutation of the restriction site or its deletion. Only the G1 and T2 transgenic lines bearing a single copy integration of the CP transgene were previously tested positive to CP expression. None of the remaining transgenic lines with multiple copy insertion showed CP protein expression. The association of virus resistance with the copy number of integrated CP gene was already reported (Pang *et al.* 1997) but there was no correlation found between the copy number and resistance, similarly to this study. The important condition for the RNA-

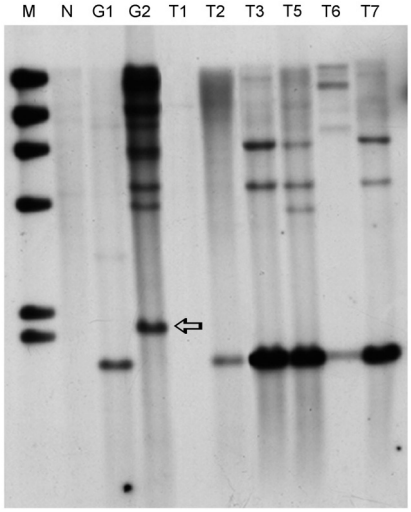

Fig. 1. Southern blot analysis of transgenic papaya DNA. Total plant genomic DNA from each transgenic line was digested with restriction enzyme *Hind*III which flanks the 1.6 kb CP expression cassette in the binary vector and probed with CP. The *arrow* points to the rearranged 2.1 kb cassette in G2 transgenic plant. M - *Hind*III λ-DNA marker, N - non-transgenic line, G1, G2, T1-T7 - transgenic lines.

mediated resistance is generation of dsRNA from transgenes which is then cleaved to siRNAs. If this assumption is true, only G2 transgenic papaya line would exhibit the presence of siRNA.

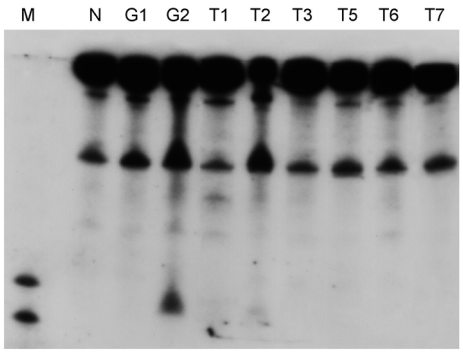

Fig. 2. RNA gel blot analysis of siRNA in transgenic papaya lines. Low molecular mass RNA was obtained from untransformed line (N) and from transgenic lines G1, G2, T1-T7. siRNAs were detected by hybridization with hydrolyzed single stranded ^{32}P-labelled PRSV CP probe. 21 and 25 nt long oligonucleotides were used as molecular standards. An equal amount of RNA (10 μg) was loaded per lane. M - 21 and 25 nt long oligonucleotides as size markers, N - non-transgenic line, G1, G2, T1-T7 - transgenic lines.

The presence of siRNA is now considered the most important characteristic of PTGS or RNA interference in global. Therefore, siRNAs were analyzed by Northern blot analysis of low molecular mass RNA, which was prepared form young fully expanded leaves of non infected plants. As expected, a distinct hybridization signal between the size of 21 and 25 oligonucleotide standards, homologous to the CP transcript, was detected in the resistant line G2 (Fig. 2). No such a signal was observed in non resistant transgenic papaya lines. The resistance to PRSV in the G2 lines can be therefore attributed to PTGS. Its origin was not investigated but it probably comes from the presence of transgene rearrangement and/or inverted repeat within the transgene. The resistance of the R0 G2 line to PRSV was very stable, we did not observe any resistance breakage during 3 years of testing with different isolates from various geographical regions of Thailand. This is not surprising because the sequence similarity of all these isolates was high, over 95 %. It is generally considered, that transgene similarity greater than 90 % to the virus is sufficient to induce durable resistance (Moreno *et al.* 1998), although only as 84 % identity was sufficient to induce plum pox resistant plum trees (Guo *et al.* 1998). In the case of transgenic papaya in Taiwan, Bau *et al.* (2003) recorded resistance towards PRSV bearing 89.7 % transgene similarity. According to this work, the crucial CP part for the resistance against PRSV is the 100 bp region in the 3' part including the 3'NTR. Among our tested isolates these regions were almost 100 % similar. A different situation was observed in R1 and subsequent generations.

Mendelian inheritance of the resistance over successive generations together with Southern blot analyses has shown that the transgene integration in G2 is homozygous (data not shown). Resistance screening over sufficient number of generation would be therefore a proper mechanism to generate line with stable and durable resistance to PRSV. Unfortunately, already in R1 and subsequent lines, various isolates of PRSV were able to break this engineered resistance. Although all transgenic R0 resistant plants were fully imune to virus infection during several years of testing and majority of R1 lines remained immune during the whole period of testing (up to 6 months), some R1 and subsequent lines showed only a delayed resistance. In these plants, the PRSV virus was able to infect after certain time post inoculation. This delayed resistance in R1 did not correlate with the virus RNA similarity to the transgene and the average time for resistance breakage was 4 to 6 months. We therefore suspected a suppression of PTGS in these lines as it is illustrated in R1 line G2-18-2 (Fig. 3*A*). While the parental R0 line always exhibited a stable resistance and steady state of siRNA presence, the in one daughter line the siRNA started to slowly decline after 90 day post inoculation (dpi) and it become undetectable around 150 dpi. Consequently, the first symptoms of PRSV infection start to appear after 120 dpi and around 150 dpi the symptoms of PRSV infection were clearly visible and the virus was detected by PCR. The

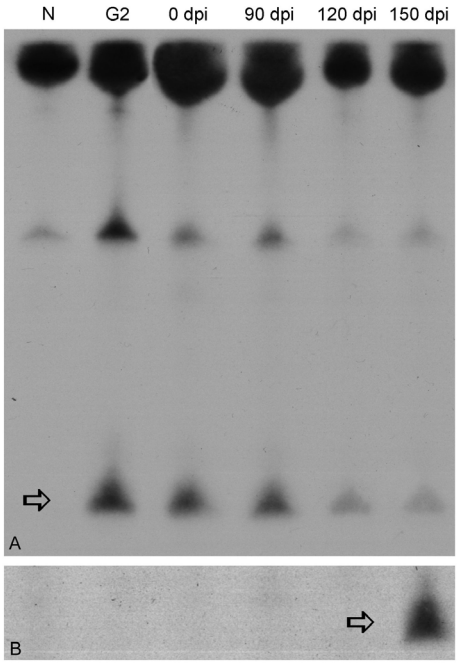

Fig. 3. RNA gel blot analysis of siRNA of G2 transgenic papaya lines. Low molecular mass RNA was obtained from non-transformed line (N) and from transgenic lines G2 in R0 and in R1 generation at 0, 90, 120 and 150 d post inoculation. An equal amount of 50 μg low molecular mass RNA was loaded on each well and the siRNAs were detected by hybridization with hydrolyzed single stranded ^{32}P-labelled PRSV CP probe (*A*) or PRSV HC-Pro probe (*B*). The *arrows* points to the transgene siRNA band (*A*) and the degradation product of infecting PRSV (*B*). N - non-transgenic line, G2 - R0 siRNA at 150 dpi as a control, 0 - 150 dpi siRNA of R1 generation.

transgene siRNA was either very low level or it was not present at all. The siRNA band at 150 dpi (Fig. 3*A*) comes from virus degradation as it was confirmed by re-probing the membrane with another probe – HC-Pro (Fig. 3*B*) that does not hybridize with transgene or plant RNA and thus the signal may come only from the infecting virus.

This resistance breakage seems to work in the opposite way to the well known recovery phenotype (Lindbo *et al.* 1993, Tanzer *et al.* 1997). Recovery plants are initially susceptible to the virus, accumulating full-length transgene mRNA, but eventually they develop resistance, at which point there is little transgene mRNA (Lindbo *et al.* 1993, Dougherty *et al.* 1994). The resistance induced in the recovery phenotype is reacquired after pathogen infection in the next generation. Thus meiotic resetting (Dehio and Schell 1994) plays an important role in this process to triger PTGS. The above described manifestation of resistance breakage on the other hand requires meiotic resetting for the infecting virus to be able to suppress PTGS and overcome the transgene engineered resistance. The mechanisms is unknown, perhaps changes in transgene methylation pattern in daughter lines may play the key role in this process. In any case, this resistance breakage poses an obstacle in breeding of transgenic papaya for durable resistance. For the present time, only R0 PRSV resistant papaya plants may be suitable for commercial growing. These findings generally confirm the commercial strategy of growing PRSV resistant transgenic papaya in Hawaii, where only R0 transgenic PRSV line is used.

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