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Resistance of non-transgenic papaya plants to papaya ringspot virus (PRSV) mediated by intron-containing hairpin dsRNAs expressed in bacteria

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Summary. – RNA-mediated virus resistance based on natural antiviral RNA silencing has been exploited as a powerful tool for engineering virus resistance in plants. In this study, a conserved 3'-region (positions 9839–10117, 279 nt) of the capsid protein (CP) gene of papaya ringspot virus (PRSV), designated *CP279*, was used to generate an intron-containing hairpin RNA (ihpRNA) construct by one-step, zero-background ligation-independent cloning (OZ-LIC). The RNaseIII-deficient *Escherichia coli* strain M-JM109lacY was identified as the best choice for producing large quantities of specific ihpRNA-CP279. Resistance analyses and ELISA data verified that most papaya plants mechanically co-inoculated with TRIzol-extracted ihpRNA-CP279 and PRSV were resistant to PRSV, and resistance was maintained throughout the test period (>2 months post-inoculation). In contrast, a 1–2 day interval between sequential inoculation of PRSV and ihpRNA-CP279 did not result in complete protection against PRSV infection, but delayed the appearance of viral symptoms by 3 to 4 days. These findings indicate that direct mechanical inoculation of papaya plants with bacterially-expressed ihpRNA-CP279 targeting the PRSV *CP* gene can interfere with virus infection. This work lays a foundation for developing a non-transgenic approach to control PRSV by directly spraying plants with ihpRNA or crude bacterial extract preparations.

Keywords: papaya ringspot virus; RNA silencing; RNA-mediated virus resistance; hairpin RNA; RNaseIII

Introduction

Papaya ringspot virus (PRSV; the genus *Potyvirus*, the family *Potyviridae*) is a highly destructive virus that affects production in almost all papaya plantation areas of the world. The PRSV-P type, which affects papayas (the W type affects cucurbits but not papaya), causes mosaic, yellow mottling and distortion of the leaves, vein clearing, classic "ringspot" and streaking on fruits, and water-soaked streaks on stems and petioles, resulting in the decline in fruit production (Gonsalves, 1998; Gonsalves *et al*., 2008; Tripathi *et al*., 2008). Disease prevention is achieved best with transgenic resistance mediated by viral protein or RNA silencing (Ratcliff *et al*., 1999; Prins *et al*., 2008; Wang *et al*., 2012). The coat protein (CP) and replicase (NIb) genes and their fragments have been amplified from different geographical PRSV isolates – including China, Thailand, Australia, and Brazil – for development of region-specific transgenic varieties with resistance to PRSV (Tripathi *et al*., 2008; Ye and Li, 2010; Tecson Mendoza *et al*., 2008). PRSV-resistant transgenic papayas engineered with the *CP* or *NIb* gene have been commercialized in Hawaii and China, respectively, representing the first commercialized transgenic fruit crop (Gonsalves, 1998; Gonsalves *et al*., 2008; Ye and Li, 2010). However, these commercialized transgenic papayas resistant to PRSV, like

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Abbreviations: CP = capsid protein; dsRNA = double-stranded RNA; ihpRNA = intron-containing hairpin RNA; PRSV = papaya ringspot virus; dpi = day post inoculation

other genetically modified organisms, are disputed regarding their potential risks to human health and the environment, although their safety has been assessed (Fermín *et al.*, 2011; Lin *et al.*, 2013; Tecson Mendoza, *et al.*, 2008).

PRSV resistance in commercialized transgenic papaya in Hawaii relies on RNA-mediated mechanisms or post-transcriptional gene silencing (PTGS), not CP protein-mediated resistance (Tennant *et al.*, 2001; Souza *et al.*, 2005; Tripathi *et al.*, 2008). RNA-mediated virus resistance based on natural antiviral RNA silencing has been exploited widely to engineer virus-resistant plants (Tecson Mendoza *et al.*, 2008; Mitter and Dietzgen, 2012; Lin, *et al.*, 2013). Double-stranded (ds) RNA derived from viral sequences as the key trigger of RNA silencing-mediated resistance causes specific degradation of the viral RNA and resultant protection against virus infections. Besides the antiviral strategies based on transgenesis, directly delivering exogenous dsRNA or hairpinRNA (hpRNA) to plants through either mechanical or spray inoculation has been used to protect plants against virus infections (Tenllado and Díaz-Ruíz, 2001; Tenllado *et al.*, 2003a, 2004; Simón-Mateo and García, 2011; Robinson *et al.,* 2014).

Both *in vitro* and *in vivo* expression systems are available for the production of dsRNA (Robinson *et al.*, 2014). Since the yield of dsRNA *in vitro* expression systems is limited, an *in vivo* expression system based on dsRNA-specific endonuclease (RNAse III)-deficient *Escherichia coli* strains such as HT115, M-Jm109LacY, and M-HMS174 is often used to produce large amounts of dsRNAs for silencing studies (Yin *et al.*, 2009; Robinson *et al.*, 2014). Many studies have demonstrated that dsRNAs or hpRNAs expressed in bacteria or the crude bacterial extracts effectively protect plants from diverse RNA plant viral infections such as pepper mild mottle virus (PMMoV) (Tenllado *et al.*, 2003b), plum pox virus (PPV) (Tenllado *et al.*, 2003b), cucumber mosaic virus (CMV) (Zhang *et al.*, 2008), tobacco mosaic virus (TMV) (Yin *et al.*, 2009; Sun *et al.*, 2010a), sugarcane mosaic virus (SCMV) (Gan *et al.*, 2010), and potato virus Y (PVY) (Sun *et al.*, 2010a, 2010b). Compared with the transgenic method for antiviral resistance, this approach is simpler, safer, environmentally friendly, and relatively inexpensive.

In this study, a large number of intron-containing hairpin RNAs (ihpRNAs) derived from a 279-bp fragment of the PRSV *CP* gene were produced in bacteria. The purified ihpRNAs were applied to papaya plants to confer resistance to PRSV infection. The mechanical application of ihpRNA-CP279 on plants conferred specific protection against PRSV, thus providing an alternative strategy to transgenesis to provide PRSV resistance in papaya.

Materials and Methods

Construction of plasmids expressing ihpRNAs. One-step, zero-background ligation-independent cloning (OZ-LIC) was used to generate an ihpRNA construct based on the pRNAi-LIC vector (Xu *et al.*, 2010). The sense and antisense orientation fragments from a conserved 3'-region (positions 9839–10117, 279 nt) of PRSV *CP* were amplified by PCR from full-length cDNA of PRSV-P isolate HN (GenBank ID: EF183499) using specific primers (Wei *et al.*, 2007; Lu *et al.*, 2008) (Fig. 1) (Table 1). Amplified fragments were subcloned into the *Sma* I-linearized pRNAi-LIC vector (Xu *et al.*, 2010) using T4 DNA polymerase, and the vector was transformed into *E. coli* DH5α, resulting in the clone pRNAi-CP279. The positive colonies were screened by sequence analysis for insertion.

For the construction of the ihpRNA bacterial prokaryotic expression vector, pRNAi-CP279 was cleaved with *Xho*I and *Cla*I to

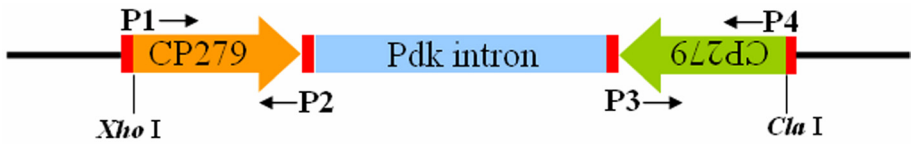

Fig. 1
ihpRNA-CP279 construct design based on pRNAi-LIC vector (Xu *et al.*, 2010)
P1-2 primers were used to produce the PCR product of sense orientation; P3-4 primers were used to produce the PCR product of antisense orientation; Pdk, pyruvate orthophosphate dikinase intron.

Table 1. Primer sequences used to produce the ihpRNA-CP279 construct

Primer	Sequence (5′– 3′)*	Description
P1	CGACGACAAGACCC*TCTCGAG***AATGCAACTGAGAGGTACAT**	For PCR product of sense orientation
P2	GAGGAGAAGAGCCC*T***TCAATTGCGCATACCCAG**	
P3	CCAGCACGGAACCC*T***TCAATTGCGCATACCCAG**	For PCR product of antisense orientation
P4	AGAGCACACGACCC*TATCGAT***AATGCAACTGAGAGGTACAT**	

*The underlined sequences are four adaptors in pRNAi-LIC vector; *Xho*I and *Cla*I sites are respectively marked in the italic; the sequences of *CP* gene are shown in bold.

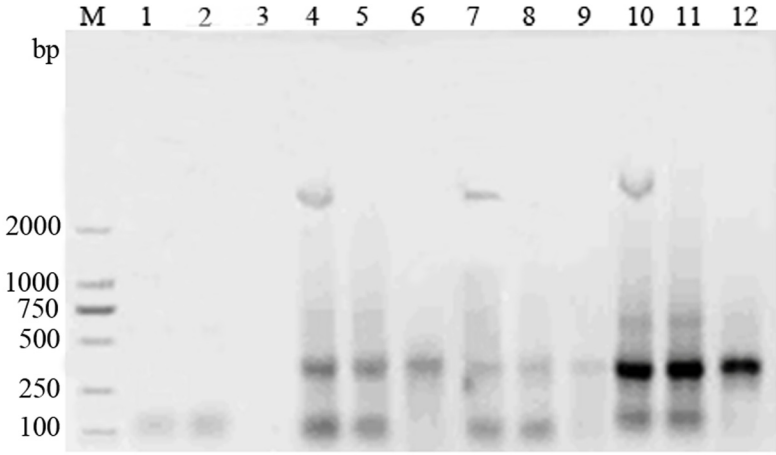

Fig. 2
Agarose gel electrophoresis of TRIzol-extracted ihpRNA-CP279 expressed in HT115, M-HMS174, and M-Jm109LacY bacterial strains
Lane M: DL 2000 DNA Marker; Lanes 1-3: total RNA extracted from the HT115/pSP73; Lanes 4-6: total RNA extracted from the HT115/pSP-CP279; Lanes 7–9: total RNA extracted from the M-HMS174/pSP-CP279; Lanes 10–12: total RNA extracted from the M-Jm109LacY/pSP-CP279; Lanes 1,4,7,10: total RNA; Lanes 2,5,8,11: total RNA+DNase I; Lanes 3,6,9,12: total RNA+DNase I+RNase A.

produce the ihpRNA-CP279 cassette, and this was inserted into an *Xho*I-*Cla*I-digested pSP73 vector (Promega, USA) to be controlled under a T7 RNA polymerase promoter. The resultant plasmid construct, pSP-CP279, was verified by restriction analysis and DNA sequencing. To measure the ihpRNA-CP279 expression level in different RNaseIII-deficient *E. coli* strains, pSP-CP279 was transformed into HT115, M-Jm109LacY, and M-HMS174 strains.

Expression and extraction of ihpRNAs. Single positive colonies of HT115, M-Jm109LacY, or M-HMS174 bacteria containing pSP-CP279 plasmids were grown for 16 hr in LB with 100 μg/ml ampicillin at 37°C. The HT115 (DE3) strain was supplemented with 12.5 μg/ml tetracycline; M-Jm109LacY and M-HMS174 were supplemented with 50 μg/ml kanamycin. The cultures were diluted 1/50 in LB broth supplemented with antibiotics and cultured at 37°C to A_{600} = 0.5. ihpRNAs were induced with 0.4 mmol/l IPTG at 37°C with shaking until A_{600} reached 1.5. Total RNA was extracted from a 2 ml bacterial culture using TRIzol (Invitrogen, USA), and the nucleic acid pellet was resuspended in 20 μl of TE buffer (pH7.5). The ihpRNAs of pSP-CP279 expressed in bacteria were confirmed by agarose gel electrophoresis after they were digested with 0.4 U/ml DNase I (TaKaRa, Japan) and100 μg/ml RNase A (TaKaRa) under high-salt conditions (0.3 mol/l NaCl, 0.03 mol/l sodium citrate) for 37°C. The dsRNA quantities were estimated by comparing band fluorescence intensity with that of DNA marker (TaKaRa) bands of known concentration.

PRSV and ihpRNAs inoculation. PRSV was purified from papaya plants as described previously (Gonsalves and Ishii, 1980). The method used to evaluate the capability of the purified ihpRNA-

Table 2. ELISA detection of PRSV resistance mediated by bacterially expressed ihpRNA-CP279

Experiments[a]	Phenotype[b]	Mean ELISA values(A_{405}) ± standard derivation (n = 3)		
		10 dpi	20 dpi	30 dpi
Non-inoculated negative controls	R 20/20	0.013±0.004	0.016±0.003	0.014±0.002
TE (negative controls)	R 20/20	0.015±0.003	0.016±0.005	0.015±0.006
PRSV (positive controls)	S 20/20	0.314±0.013	0.457±0.011	0.533±0.016
PRSV +ihpRNA-CP279	R 13/20	0.018±0.005	0.019±0.007	0.019±0.005
	S 7/20	0.127±0.006	0.212±0.012	0.378±0.019
ihpRNA-CP279+PRSV (1 day)	S 20/20	0.193±0.005	0.237±0.009	0.433±0.016
ihpRNA-CP279+PRSV (2 days)	S 20/20	0.113±0.008	0.357±0.005	0.473±0.004
ihpRNA-CP279+PRSV (3 days)	S 20/20	0.298±0.012	0.409±0.007	0.517±0.013
ihpRNA-CP279+PRSV (5 days)	S 20/20	0.325±0.004	0.428±0.012	0.543±0.009

[a]PRSV sap was mechanically inoculated on papaya seedlings simultaneously or 1, 2, 3 or 5 days after inoculating with an equal volume of TRIzol-extracted ihpRNA-CP279 preparation in the same leaves. The positive control was inoculated with PRSV sap; TE buffer (pH 7.5) inoculation and uninoculated plants were used as the negative controls. [b]The number of plants showing resistant (R) or susceptible (S) phenotypes to PRSV infection is indicated.

CP279 from bacteria to interfere with PRSV infection was based on previous reports (Tenllado and Díaz-Ruíz, 2001; Yin *et al.*, 2009; Sun *et al.*, 2010a,b). Briefly, 3 μg of PRSV sap was mechanically inoculated on six- to eight-week-old papaya seedlings simultaneously or 1, 2, 3, or 5 days after inoculating with an equal volume of TRIzol-extracted ihpRNA-CP279 (100 μg) preparation in the same leaves. In these experiments, each treatment included 20 papaya plants. The inoculated plants were grown in a controlled environment with a cycle of 16 hrs of light at 28°C and 8 hrs of dark at 25°C. The positive controls were inoculated with PRSV sap only; negative controls were inoculated with TE buffer or were uninoculated. After the papaya leaves are mechanically inoculated with PRSV, the appearance of symptoms (mosaic, ring spots and distortion on leaves, and water soaking streaks on petioles) on the leaves is usually shown 10–12 days post inoculation (dpi). However, in this study the PRSV infection symptoms were observed 2 months post-inoculation. The virus level in plants inoculated with ihpRNA-CP279 was detected in upper leaves by PRSV DAS-ELISA kit (Agdi, USA) 10, 20, and 30 dpi.

Results

Quality and yields of ihpRNA-CP279

To compare the expression level of ihpRNA-CP279 in different *E. coli* strains defective for RNase III, recombinant pRNAi-CP279 plasmids containing ihpRNA constructs were transformed into HT115, M-HMS174, and M-Jm109LacY strains. Expression was induced with 0.4 mmol/l IPTG before total bacterial nucleic acids were purified. IhpRNA-CP279 was observed as a clear band around the expected size (279 bp) for all transformants from three different *E. coli* strains by agarose gel electrophoresis, but the M-JM109lacY strain expressed significantly higher levels of ihpRNA-CP279 (Fig. 2). When extracted ihpRNA-CP279 was treated with DNase and RNaseA, gel electrophoresis revealed degradation of the DNA and the single-stranded RNA loop (Fig. 2). Based on dsRNA band intensities in agarose gels relative to that of the DNA marker bands, each A_{600} = 1 of ihpRNA-CP279/M-JM109lacY culture was estimated to have yielded about 30 μg of ihpRNA-CP279.

ihpRNA-CP279 inhibits PRSV infection

To assess the capability of ihpRNA-CP279 to inhibit systemic PRSV infection in papaya plants, papaya plants were co-inoculated with ihpRNA-CP279 and PRSV. This protocol resulted in 65% of papaya plants being free of symptoms and appearing resistant to PRSV. ELISA results (10, 20, 30 dpi) indicated that PRSV accumulated at similar rates across PRSV-inoculated ihpRNA-CP279 plants up to 30 dpi (Table 2). Interestingly, when plants were challenged with virus in 1–2-day intervals post ihpRNA-CP279 application, the appearance of viral symptoms was delayed by 3to 4 days compared to the positive control plants, and the virus concentration in the susceptible plants was lower than in the positive control, but higher than in the negative controls (Table 2). However, the virus concentration in the susceptible plants is similar to that in the negative controls and there was no observation of PRSV resistance when plants were challenged with virus at more than 2-day intervals (3- and 5-day) post ihpRNA-CP279 application.

Discussion

Although the commercialized PRSV-resistant transgenic papaya generated by expression of the *CP* gene revived the papaya industry in Hawaii, those plants remain susceptible to PRSV isolates outside of Hawaii and can be overcome by PRSV with geographically-distant homology to the transgene, or by helper component-proteinase (HC-Pro) of PRSV, a potyvirus RNA silencing suppressor (Tripathi *et al.*, 2008). Therefore, to overcome this limitation and make papaya with broad-spectrum resistance to different PRSV strains in China, in this study, the conserved 3'-region (positions 9839–10117, 279 nt) of PRSV *CP* was used to generate an ihpRNA construct based on the alignment of *CP* gene sequences of seven PRSV isolates originating from different regions of papaya production in China (Wei *et al.*, 2007). A previous study confirmed that transgenic papaya plants transformed with an ihpRNA-CP279 construct can interfere with PRSV infection (Wei *et al.*, 2008), but this ihpRNA was constructed by the traditional ligase-based vector pHANNIBAL (Wesley *et al.*, 2001) which requires several rounds of restriction and ligation, and is therefore, tedious and time-consuming. In this work, OZ-LIC method was successfully applied to rapidly generate ihpRNA-CP279 constructs without considering traditional restriction site limitation and ligase use (Xu *et al.*, 2010). In addition, the yields of ihpRNA-CP279 differed among bacterial strains HT115, M-HMS174, and M-Jm109LacY, with M-JM109lacY serving as the best choice for producing large quantities of specific ihpRNA-CP279 (30 μg /each A_{600} = 1). This finding is consistent with the result of Yin *et al.* (2009) because the *endA* and *recA1* mutation in M-JM109lacY will make the exogenous plasmids or the transcribed dsRNA more stable.

In this study, direct mechanical inoculation of nontransgenic papaya plants with bacterially expressed ihpRNA-CP279 targeting the PRSV *CP* gene interfered with virus infection. Most papaya plants co-inoculated with ihpRNA-CP279 and PRSV were resistant to PRSV, and the resistance was maintained throughout the test period (beyond 2 months post-inoculation). Similar resistance was found

when plants were co-inoculated with dsRNA and PMMoV, TMV, or PVY (Tenllado and Díaz-Ruíz, 2001; Tenllado *et al.*, 2003b; Yin *et al.*, 2009; Robinson *et al.,* 2014). Additionally, a protective function of the construct was found when plants were challenged with virus in 1–2-day intervals post dsRNA application: symptom onset was delayed by 3–4 days and virus concentration in the susceptible plants was lower than in the positive control, but higher than in the negative control. This finding suggests that a weak systemic protective effect had been achieved, but PRSV was able to overcome the RNA silencing mediated by ihpRNA-CP279. Previous studies showed that dsRNA transcribed *in vitro* failed to protect against PMMoV in *Nicotiana benthamiana* plants when plants were challenged with virus at 24 hr intervals post dsRNA application (Tenllado and Díaz-Ruíz, 2001). Those findings were believed to be attributable to the labile nature of dsRNA, which provides only a short-lived opportunity post application in which the plant may be protected against virus infection (Robinson *et al.,* 2014). However, a remarkable report showed that a delay of up to 5 days between spraying with crude extracts of bacterially expressed dsRNA and virus inoculation can protect plants against PMMoV or PPV until their life cycles are completed (Tenllado *et al.*, 2003b). Similar results were obtained when experiments were conducted with SCMV in maize plants (Gan *et al.*, 2010). The RNA-mediated antiviral protection induced by the crude extracts of bacteria expressing dsRNAs or ihpRNAs is a more simple, rapid, and inexpensive method than the direct mechanical inoculation of the purified dsRNA. Therefore, based on these results and our study, the development of a novel technology by spraying plants with crude preparations containing effector dsRNAs or ihpRNAs to control PRSV is feasible. To make it practical for field application, further studies on the relationship between RNA silencing and the dsRNA sequence length, dose dependency, and persistence on the leaves are needed.

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