Plant Biotechnology 29, 339–349 (2012)
DOI: 10.5511/plantbiotechnology.12.0215b

Original Paper

Evaluation of selected transgenic papaya (*Carica papaya* L.) lines for inheritance of resistance to papaya ringspot virus and horticultural traits

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Received December 5, 2011; accepted February 15, 2012 (Edited by G.-T. Kim)

Abstract Three selected transgenic papaya lines 124-3, 132-2 and 142-3 across the T_1 and T_2 generations were preliminarily evaluated under Biological Containment Level 2 (BL2) greenhouse approved by the National Committee on Biosafety of the Philippines (NCBP) to determine the partial stability of expression of resistance to papaya ringspot virus (PRSV) and selected phenotypic traits. The PRSV reaction of the three transgenic lines using mechanical inoculation test and enzyme-linked immunosorbent assay was assessed. The qualitative and quantitative horticultural traits at flowering and ripe fruit stages of the transgenic lines were also evaluated. Based from preliminary results, the percentages of resistant progenies to PRSV in the T_1 generation were 83% for 124-3, 74% for 132-2, and 70% for 142-3. However, in the same generation, no difference between the transgenic and non-transgenic were observed for stem, leaf and petiole color, fruit shape, peel, flesh and seed color, stem diameter, number of days to first flower, and fruit edible portion, indicating that the transgenic is seemingly stable. The percentage of resistant progenies to PRSV in the T_1 generation decreased in the T_2 generation, indicating that the inherited resistance of the three transgenic lines was unstable, and a continuous evaluation of the transgene could be done in later generations. In contrast, the light green (RHS 141B) stem color, dark green (RHS 141A) leaves and petioles and creamy white flowers (RHS 155A) were stably expressed in both the T_1 and T_2 generations. The plant height for lines 124-3 and 142-3, internode length for line 142-3 and number of nodes to first flower for lines 132-2 and 142-3 were also stable in both T_1 and T_2 generations. In addition, the pyriform fruit shape for hermaphrodite sex form and rounded fruit shape for the female sex form, light yellow orange (RHS 17A) peel color, bright yellow orange (RHS 23A) flesh color and black (RHS 202A) seeds of the three transgenic lines were stably expressed in both generations. The fruit weight, total soluble solids and fruit edible portion for lines 124-3 and 142-3 was stably expressed in both generations.

Key words: *Carica papaya* L., coat protein gene, horticultural traits, inheritance, papaya ringspot virus, resistance, enzyme-linked immunosorbent assay

The papaya (*Carica papaya* L.), a member of the family *Caricaceae* is a single-stemmed semi-woody herbaceous plant which is widely grown in tropical and subtropical regions of the world. The melon-like fruits are edible and highly nutritious because they are rich in vitamins, minerals and fiber. Its flesh when ripe may be golden yellow like 'Kapoho', and 'Waimanalo' Solo and 'Cavite Special' or red like 'Sunrise' and 'Sunset' Solo and 'Red Lady'. The unripe fruits are rich sources of papain, an enzyme used as a component of meat tenderizers and also used the in the manufacture of pharmaceutical and cosmetic products.

Papaya plantations often range from small to large-scale commercial farms. The area planted to papaya in the Philippines is 9,280 ha with a production volume of 182,908 metric tons (DA-BAS, 2009). The production of papaya especially in the Southern Tagalog region is a lucrative business until the outbreak of the deadly papaya ringspot virus (PRSV) that devastated the papaya industry in this region. The disease has been spreading like a wild-fire because it is being transmitted by aphids in a non-persistent manner (Magdalita et al. 1989). The virus can infect papaya plants at any stage of growth. To date, there is no known ultimate solution for this disease. However, conventional breeding efforts led to the development of moderately tolerant varieties like the 'Sinta' hybrid. Intergeneric hybridization between papaya and its wild relatives produced hybrids but hybrid breakdown prevented further backcrossing to recover a resistant variety (Magdalita et al. 1996, 1997). Hence, the

This article can be found at http://www.jspcmb.jp/
Published online September 9, 2012

use of genetic engineering to develop resistance offers an alternative solution to solve the problem.

In agriculture, genetic engineering is utilized mostly for the development of resistant crops to pests and diseases, tolerance to abiotic stresses and in the elucidation of gene functions and gene products via plant transformation. The most effective method of controlling plant viruses is through building up resistance (Fuchs and Gonsalves 2007). For instance, the PRSV-resistant Hawaiian transgenic papaya variety 'SunUp' was developed through transformation of somatic embryos with the coat protein gene of the Hawaiian PRSV mutated strain (Fitch et al. 1992). The transgenic plants are therefore protected from the infecting virulent Hawaiian PRSV strain only.

In the development of transgenic crops resistant to viruses, it is important to assess fully the resistance and its inheritance pattern. In addition, the phenotypic characteristics need to be tested whether they are similar to the non-transgenic counterpart, which is important for regulatory purposes and variety release. In other transgenic crops agronomic traits and yield performance has been evaluated for instance, in transgenic squash that contained single or multiple virus coat protein gene (Tricoli et al. 1995), burley tobacco that expresses tobacco vein mottling virus or alfalfa mosaic virus coat protein genes (Xu et al. 1999), peanut resistant to tomato spotted wilt virus (Yang and Ozias-Akins 2004), tomato hybrids resistant to tomato spotted wilt virus (Accotto et al. 2005) and papaya resistant to papaya ringspot virus Hawaiian isolate (Bau et al. 2004; Ferreira et al. 2002; Phironrit et al. 2007; Tennant et al. 2005). There were either no or few differences in agronomic traits of the transgenic crops in comparison to their non-transgenic counterparts. In most cases, the transgenic crops had better yield performance than their non-transgenic counterparts.

Finally, the test for a successful plant transformation especially for resistance to viral pathogens is an assessment of the stability of incorporated transgenes through characterization of resistance from one generation to the next. Hence, the main objective of this study is to: i) assess the resistance to PRSV of the selected transgenic lines and stability of resistance trait across two generations and, ii) evaluate the selected horticultural traits of the three transgenic lines in T_1 and T_2 generations.

Materials and methods

The materials used in this study were obtained from a project, "Development and Commercialization of GM Papaya for Fresh Fruit and Papain Production", under the leadership of Dr. Pablito M. Magdalita and being conducted at the Institute of Plant Breeding, Crop Science Cluster, College of Agriculture, University of the Philippines Los Baños, College, Laguna. The project was approved by the National Biosafety Committee of the Philippines (NCBP) dated 28 December 1999. The screening for PRSV resistance and phenotypic characterization of T_1 and T_2 transgenic lines begun on 2004 and all experiments were terminated on 2006.

Plant materials

The yellow 'Solo' papaya was used in this study. Somatic embryos were induced from immature zygotic embryos isolated from immature green papaya. The somatic embryos were transformed via *Agrobacterium*-mediated transformation system using a binary vector containing the coat protein (CP) gene from a Philippine PRSV isolate to develop the T_0 generation plants. The plant expression vector used contains an enhanced 35S promoter, a GMhsp 17.9 leader sequence from soybean, a 35S 3′UTR, *npt II* selectable marker gene. The transformation work was conducted at the Malaysian Agricultural Research and Development Institute (MARDI) in Malaysia. Proliferating somatic embryos and calli were co-cultivated with the activated bacterial culture (OD_{600}=0.2–0.5) for 2 h at 120 rpm at 28°C. After co-cultivation, the embryos were transferred to solid co-cultivation medium and incubated for 3 days. After this period, the embryo clusters were washed, blotted dry and incubated in somatic embryo induction medium plus carbenecillin (500 mg l^{-1}) for 2 weeks and subcultured in a selection medium with kanamycin (150 mg l^{-1}) until plantlets developed. The T_0 plantlets in tissue culture were brought to the Philippines in January 2002 with permission from the Plant Quarantine Service-Bureau of Plant Industry (PQS-BPI). They were self-pollinated and grown to maturity to generate the T_1 progeny lines.

Seedlings of the three selected T_1 progeny lines were subjected to virus challenge and only seedlings that showed resistance were selected. The resistant T_1 progeny plants from each of the three selected lines were transplanted in the BL2 greenhouse. The three selected transgenic lines namely: 124-3, 132-2 and 142-3 and the number of plants transplanted in soil in the BL2 greenhouse which were used as sources of progeny plants used in this study are shown in Table 1. The three T_1 lines came from the 11 lines that showed a Mendelian segregation for PRSV resistance.

Screening for PRSV resistance

Screening for PRSV resistance was done inside the BL2 greenhouse at IPB. Thirty to forty seedlings per transgenic line at the T_1 and T_2 generation and the non-transgenic control were raised. The seedlings were mechanically inoculated with the sap of PRSV-infected leaves by gently rubbing the virus inoculum onto the upper surface of the three uppermost expanded leaves previously dusted with carborundum. After inoculation, the leaves were rinsed briefly with distilled water. Three weeks after inoculation, the seedlings were assessed on a weekly basis for the presence or absence of the virus. Likewise, the symptom/s that developed on each test seedling was noted. The plants

Table 1. The number of transgenic plants from T_1 and T_2 generation of selected resistant lines 124-3, 132-2 and 142-3 and the non-transgenic control plants transplanted in soil inside the BL2 greenhouse.

Transgenic line	Number of plants transplanted in soil	
	T_1	T_2
Control	6	8
124-3	16	7
132-2	15	17
142-3	12	3

which appeared symptomless after two to three weeks from inoculation were re-inoculated to make sure that the resistance observed is not an escape from the virus. All inoculated and re-inoculated test seedlings were observed for their reaction to PRSV. A visual rating score of one (1) was given to test seedlings that showed PRSV symptoms, while a zero (0) score was given to the symptomless test seedlings. The visual rating for resistance of each test seedling was validated using the enzyme-linked immunosorbent assay (ELISA, Hull 2002, Nelson and Cox 2005). Each of the 30–40 test plants for each line, 2 leaf samples were used and each sample was replicated 2 times in the ELISA plate. All ELISA tests were repeated 2 times. Leaf sample was collected from the youngest fully expanded leaf of each test plant. The tissue was homogenized using mortar and pestle in 0.05 M Carbonate Buffer, pH 9.6 at 1 : 10 dilution. About 100 μl of the sap was loaded onto each well of the ELISA microtiter plate. The plates were incubated at 4°C overnight and then washed 3 times at 5 min interval using a washing buffer 1× phosphate buffer saline (PBS) plus 0.05% Tween 20, then 200 μl of the freshly prepared blocking solution (PBS and BSA) was loaded onto each well, incubated under room temperature for 1 h and the plates were washed following the procedure described above. This was followed with the addition of 100 μl of the captured antibody at 1 : 200 in the antibody buffer was loaded onto each well of the ELISA plate. Cross absorption was done by dissolving the capture antibody using antibody buffer to which sap from healthy papaya leaf was added at dilution of 1 g tissue per 30 ml buffer. The plates were incubated at room temperature for 2–3 h and then washed following the procedures mentioned above. Then 100 μl of the secondary antibody enzyme conjugate, Goat AntiRabbit Alkaline Phosphatase, at 1 : 1000 dilution was added to each well of the ELISA plate and then incubated at room temperature for 2–3 h and the plates were washed. Each well was then loaded with 100 μl of *p*-nitrophenyl phosphate substrate (PNP) (1PNP tablet per 5 ml diethanolamine buffer pH 9.6) and incubated at room temperature. The wells were observed for the development of yellow color reaction indicating the presence of the virus in the sample and assessed based on the absorbance value measured in an ELISA plate reader.

The resistant transgenic seedlings were selected and transferred to soil placed in bigger plastic bags (6×8) and grown inside the BL2 greenhouse. The percent resistance was computed by dividing the number of resistant plants by the total number of inoculated plants, multiplied by 100%.

The selected resistant plants from each line at the T_1 and T_2 generation were subsequently transplanted directly in soil inside the BL2 greenhouse extension under the supervision of the representatives from the regulatory agencies including the University of the Philippines Los Banos-Institutional Biosafety Committee (UPLB-IBC), Bureau of Plant Industry-Plant Quarantine Service (BPI- PQS) and the NCBP.

Horticultural evaluation and line purification

The selected resistant plants from each of the three T_1 lines were transplanted directly in soil inside the BL2 extension greenhouse. Due to space limitations, only the best plants in each line were planted accordingly as follows: 16 from 25 resistant plants of T_1 line 124-3, 15 from 19 resistant plants of T_1 line 132-2, 12 from 17 resistant plants of T_1 line 142-3 and six non-transgenic control plants but only two reached maturity.

Three months after transplanting directly in soil and when the first flower developed, phenotypic evaluation of each plant was done. Selected horticultural traits at flowering stage such as plant height, stem diameter, length of internodes and days to first flower were assessed. In addition, the ripe fruits were evaluated for selected fruit characters such as fruit weight, total soluble solids (TSS) and edible portion (EP). Evaluation of the selected characters was based on the descriptor criteria set for evaluating papaya by the International Board for Plant Genetic Resources (IBPGR 1988). The color of the stem, petiole, leaf, flower, fruit peel, flesh and seed was determined based from the Colour Chart of the Royal Horticultural Society (RHS) of London (RHS 1966).

The three selected transgenic lines (T_0) were either self- or sib-pollinated to produce the T_1 and T_2 generation progenies. Hermaphrodite flowers at the full-balloon stage produced by the transgenic lines were enclosed in glassine bags and were secured with a paper clip to allow self-pollination. The self-pollinated flowers were properly labeled. On the other hand, the female flowers were sib-pollinated artificially by pollens taken from a sibling hermaphrodite line and enclosed with glassine bag. The artificially pollinated flowers were monitored until fruits developed. The fruits that developed were properly labeled. The seeds extracted from self-pollination of T_0 composed the T_1 lines while self-pollination of T_1 lines gave rise to the T_2 progeny lines.

Statistical analysis

A standard Student *t*-test using the SAS program version 9.1 (SAS System 1985) was performed to determine any differences in the selected horticultural traits between the transgenic lines and the non-transgenic control both at the T_1 and T_2 generations.

Table 2. Reaction to PRSV based on visual rating and ELISA reaction of inoculated seedlings of transgenic T_1 lines 124-3, 132-2 and 142-3 and the non-transgenic control plants screened for PRSV resistance two months after mechanical inoculation.

Transgenic lines	No. of inoculated plants	Reaction to PRSV (R:S)	Resistant plants (%)	ELISA reaction (−:+)
Control	10	0:10	0	0:10
124-3	30	25:5	83	25:5
132-2	27	19:8	70	19:8
142-3	23	17:6	74	17:6

Results and discussion

Evaluation of T_1 generation

Screening for PRSV resistance

Initially, 77 T_1 lines were screened for PRSV resistance and showed varying reaction to PRSV. Eight weeks from inoculation, the test seedlings of the T_1 lines showed different reactions to the virus. Twenty-four T_1 lines were resistant to PRSV while the remaining 53 lines were susceptible. The resistance reaction of the lines as analyzed by Chi-square test showed that 11 out of 77 T_1 lines showed a segregation pattern similar to Mendelian segregation ratio of 3:1 (Magdalita et al. 2004). Out of the 11 T_1 lines almost having the Mendelian segregation, three lines including 124-3, 132-2 and 142-3 were selected.

The PRSV reaction and the pattern of segregation (resistant:susceptible) of test plants of the selected T_1 lines 124-3, 132-2 and 142-3 two months after inoculation are presented in Table 2. While all three T_1 lines showed relatively high resistance to PRSV, notably, T_1 line 124-3 had the highest percentage of resistant plants of 83.0% out of 30 inoculated seedlings. The segregation of resistant to susceptible plants based on mechanical inoculation and ELISA tests of T_1 line 124-3 is very similar to 3 : 1 Mendelian segregation. This suggests that a single gene controls resistance in this line. However, since the results are preliminary, further evaluation of the transgene expression could be done in later generations. Conversely, T_1 lines 142-3 and 132-2 had only 74.0% and 70.0% resistant plants respectively based on both mechanical inoculation and ELISA tests. All non-transgenic control plants were susceptible. The occurrence of resistant plants in the 3 T_1 lines and all susceptible plants in the non-transgenic control spells the difference between the transgenic and non-transgenic plants.

Symptoms of PRSV such as severe leaf deformation, mottling, mosaic, shoe-stringing, vein banding, chlorotic spots and water-soaked streaks on the stems of the inoculated non-transgenic control seedlings developed as early as two weeks post-inoculation. The PRSV symptoms on the susceptible T_1 seedlings also developed two weeks post-inoculation same as in the control. The results of the ELISA obtained for each test plant for all three T_1 transgenic lines and non-transgenic control plants were consistent with the visual rating and confirmed the presence (or absence) of PRSV in the inoculated T_1 seedlings.

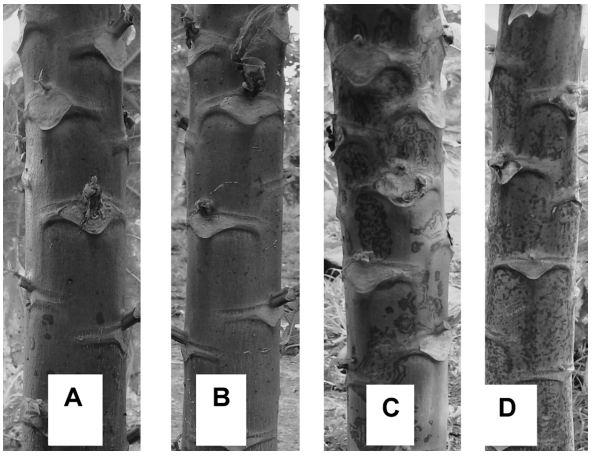

Figure 1. The light green (RHS 141 B) stem color of the T_1 transgenic line 132-2 (A), 124-3 (B) and 142-3 (C) is not different to the non-transgenic control (D).

Horticultural evaluation

The plants of T_1 lines 124-3, 132-2 and 142-3 were morphologically similar to the non-transgenic control. Phenotypically, the T_1 transgenic lines have typical papaya characteristics similar to the non-transgenic control. Both the T_1 transgenic lines and the non-transgenic control had the same light green (RHS 141 B) stem color (Figure 1). Moreover, the leaf shape (palmate), leaf color (dark green, RHS 141 A), number of main veins (7) and petiole length of the T_1 transgenic lines were also similar to the non-transgenic control (Figure 2), indicating that the transgenic are comparable to the non-transgenic. These observations corroborated with the results on evaluation of transgenic papaya in Thailand (Burns et al. 2003) and in Indonesia (Damayanti et al. 2005).

On the other hand, there was a difference between the transgenic T_1 lines and the non-transgenic control in terms of plant height, and internode length (Table 3). Transgenic T_1 lines 124-3, 132-2 and 142-3 were significantly shorter than the non-transgenic control. The T_0 plants from where these T_1 lines were derived were short-statured, one of the criteria being considered in line selection and purification, hence the T_1 progenies

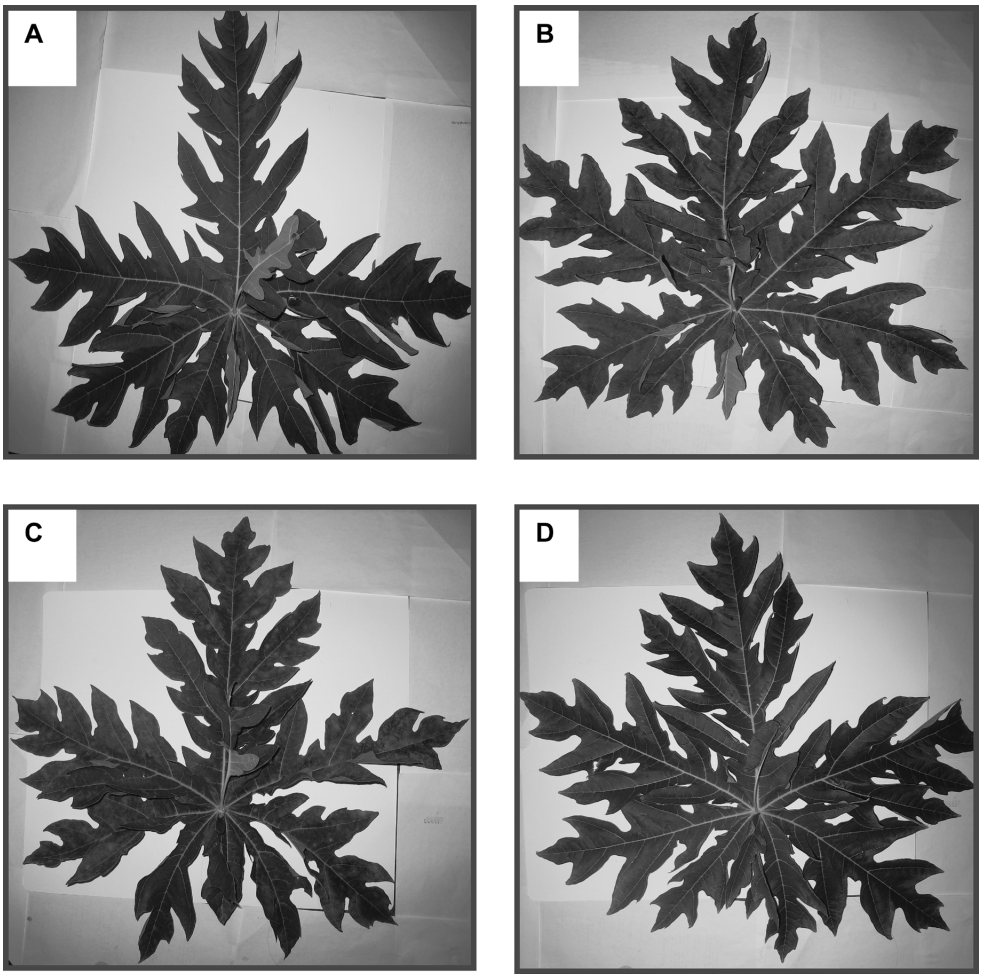

Figure 2. The palmate leaf shape, dark green (RHS 141 A) leaf color and, number (7) of main veins of the transgenic T_1 lines 124-3 (A), 132-2 (B) and 142-3 (C) are not different to the non-transgenic control (D).

were also short. This difference in height and internode length shows a difference between the transgenic and non-transgenic control. In the Philippines, papaya growers prefer relatively short-statured papaya plants than taller ones because of convenience in harvesting the fruits. Furthermore, being a country often visited by typhoons or tropical storms, shorter trees are preferred because taller ones are more prone to damage by strong winds.

The stem diameter of the T_1 transgenic lines was thinner than the non-transgenic control. In Malaysia, transgenic 'Eksotika' papayas had similarly thinner stem than the non-transgenic control (Ravindranathan et al. 2002).

In the present study, the T_1 transgenic lines had shorter internodes than the non-transgenic control. However, the T_1 lines 132-2 and 142-3 had significantly shorter internodes than the non-transgenic control and line 124-3. The internode length was directly correlated to the plant height of the transgenic and the non-transgenic control. In the case of the T_1 transgenic lines, their shorter internodes contributed to their plant

Table 3. Selected horticultural traits at flowering stage of the three transgenic T_1 lines and the non-transgenic control.

Transgenic lines	Horticultural traits[1]				
	Plant height (cm)	Stem diameter (cm)	Length of internodes (cm)	No. of days to first flower	No. of nodes to first flower
Control	121.5	6.48	4.25	81	33
124-3	86.3*	5.11	2.41	67	37
132-2	86.1*	5.45	2.26*	64	35
142-3	72.4*	5.24	2.08*	66	31

[1] Mean values with asterisk (*) are significantly different from the control at 5% level of significance by *t*-test.

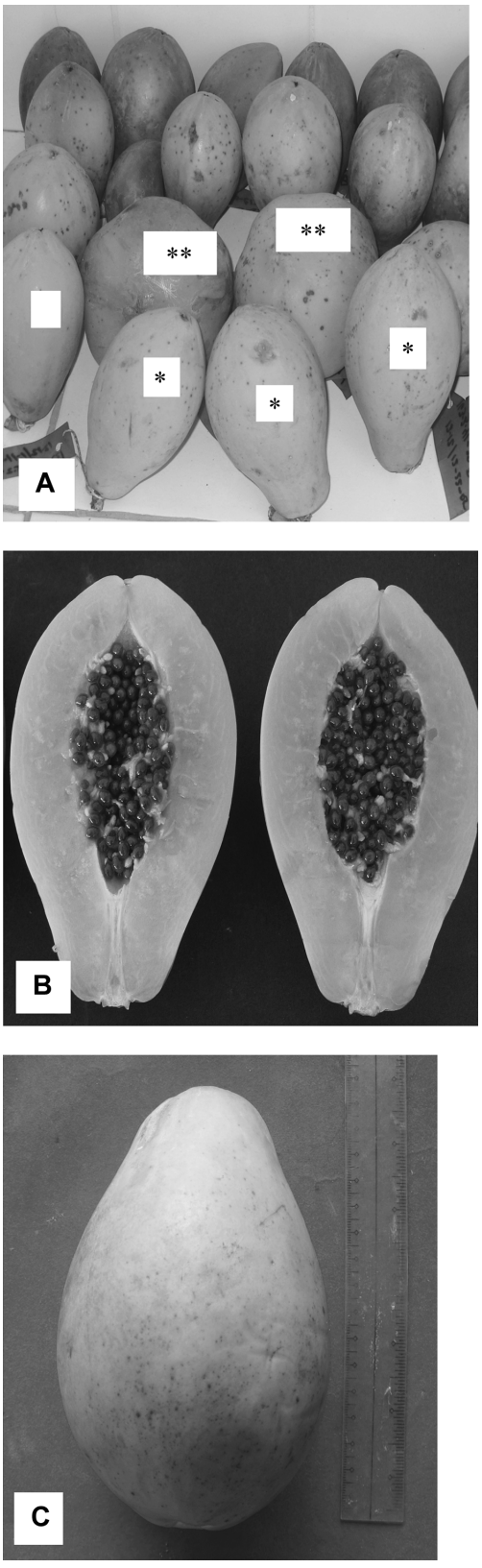

Figure 3. The hermaphrodite fruit form that is pyriform in shape* (female is rounded**), light yellow orange (RHS 17A) peel color (A), bright yellow orange (RHS 23A) flesh color and black (RHS 202A) seeds of the transgenic T_1 lines (B) is not different to the hermaphrodite pyriform fruit shape (the female is rounded) of the non-transgenic control (C).

Table 4. Selected fruit characters evaluated in the three T_1 transgenic lines and the non-transgenic control.

Transgenic lines	Fruit characters[1]		
	Fruit weight (g)	Total soluble solids (°B)	Edible portion (%)
Control (3)	567.3	12.2	75.3
124-3 (76)	508.7	10.5*	71.5
132-2 (131)	460.0*	10.4*	72.2
142-3 (62)	483.7	10.0*	70.8

[1] Mean values with asterisk (*) are significantly different from the control at 5% level of significance by *t*-test. Numbers in parenthesis ( ) refer to the total number of fruits evaluated in each line.

stature. It has been reported that internode length affects the plant height of 'Solo' papaya in Hawaii prior to flowering (Storey 1986).

The number of days to first flower of the T_1 transgenic lines was not significantly different from the non-transgenic control. However, the three T_1 transgenic lines produced the first flower much earlier than the non-transgenic control. Probably, the transgenic lines are more genetically precocious than the non-transgenic control. A genetically determined number of nodes that developed following a short period of juvenility have been reported to induce flowering in 'Solo' papaya (Storey 1986). Precocious flowering in papaya is a desirable trait that promotes an earlier harvesting season for ripe fruits. Furthermore, the number of nodes to first flower of the three T_1 transgenic lines was also not significantly different with the non-transgenic control.

Generally, the fruit gross morphology, light yellow orange (RHS 17A) peel and bright yellow orange (RHS 23A) flesh color of the three transgenic lines are no different to the non-transgenic control phenotypically (Figure 3). Both the transgenic T_1 lines and the non-transgenic control having hermaphrodite sex form, they borne bulb-shaped (pyriform) fruits, a typical shape of 'Solo' papaya. On the other hand, both the female transgenic lines and the non-transgenic control have rounded fruits. The two T_1 transgenic lines 124-3 and 142-3 had fruit weight not significantly different with the non-transgenic control. However, transgenic line 132-2 had fruit weight that is significantly different from the control.

All T_1 transgenic lines had total soluble solids values that were significantly different from the non-transgenic control. Contrastingly, it has been reported that there is no significant difference in the fruits' total mass sucrose (°Brix) between transgenic and non-transgenic 'Khak Nual' papaya variety in Thailand (Chowpongpang et al. 2003).

Furthermore, there was no significant difference in the fruit's edible portion between the T_1 transgenic lines and the non-transgenic control, suggesting that they are not different from each other. The fruits of both the T_1

transgenic lines and the non-transgenic control have many black (RHS 202A) seeds, indicating that both of them are highly fertile, indicating that indeed the transgenic lines are not different to the non-transgenic control.

Evaluation of T_2 generation

Screening for PRSV resistance

Among the T_2 plants that showed resistance to PRSV four months after inoculation under BL2 greenhouse conditions, six representative test plants of the three T_2 transgenic lines (3 from 124-3, 2 from 132-2 and 1 from 142-3) that are free from PRSV symptoms were selected (Table 5). Based from the ELISA assay, the values obtained were similar to the negative control, but these values are several folds lower than the positive control. This result confirms the absence of the virus in the six symptomless transgenic T_2 representative test plants. This may suggest inheritance of the transgene from the T_1 generation.

However, because there are susceptible test plants in the three T_2 transgenic lines, this indicate instability of the resistance. Similar observation was made among transgenic papaya transformed with PRSV coat gene via microprojectile bombardment (Magdalita et al. 2011).

Expectedly, all the inoculated non-transgenic control test plants were susceptible to PRSV and had an ELISA value similar to the positive control and several folds higher than the negative control. The PRSV symptoms that developed on the susceptible T_2 plants were similar to the symptoms of the inoculated control non-transgenic control.

Horticultural evaluation

The six selected resistant test plants in the three T_2 transgenic lines had gross morphological characteristics not different to the non-transgenic control (Table 6). The same observation was reported for transgenic papaya containing the PRSV coat protein gene that was developed in Thailand (Chowpongpang et al. 2003).

The plant height of the three T_2 transgenic lines is significantly different from the non-transgenic control (Table 6). This observation was consistent with the result obtained in the T_1 generation which is attributable to the selection for short statured progenies implemented in the T_0 generation. In addition, the internode length of the three lines is significantly different from the non-transgenic control. Like the plant height, the intenode length of the transgenic lines is significantly shorter than the non-transgenic control. This shorter internode length is a function of the similarly shorter plant height. In addition, the number of nodes to first flower of the three transgenic T_2 lines is significantly different from the non-transgenic control. The number of nodes to first flower of the transgenic T_2 is significantly greater than the non-transgenic control. While the transgenic T_2 lines have greater number of nodes to first flower, they are shorter than the non-transgenic control, hence the shorter plant height of the transgenic T_2 lines. It has been reported that flowering in papaya is induced after

Table 5. Reaction to PRSV using mechanical inoculation and to ELISA of representative test plants of the three selected transgenic T_2 lines and non-transgenic control four weeks after inoculation.

T_2 transgenic line	PRSV symptoms*	ELISA reading for the test plants (OD_{405})*	ELISA reading for the control (OD_{405})	
			−	+
124-3.9-12.4	−	0.191±0.05 (−)	0.174±0.07	0.911±0.11
124-3.9-21.17	−	0.109±0.08 (−)	0.174±0.09	0.911±0.09
124-3.9-21.30	−	0.131±0.04 (−)	0.174±0.12	0.911±0.06
132-2.9-13.27	−	0.236±0.06 (−)	0.230±0.13	0.735±0.12
132-2.17-2.37	−	0.208±0.10 (−)	0.230±0.11	0.735±0.11
142-3.13-16.7	−	0.222±0.13 (−)	0.230±0.07	0.735±0.13
Non-transgenic	+	0.890±0.12 (+)	0.230±0.06	0.735±0.12

* − indicates negative symptom of PRSV and negative reaction to ELISA.

Table 6. Selected horticultural traits of the three transgenic T_2 lines and the non-transgenic control at flowering stage.

Transgenic lines	Horticultural traits[1]				
	Plant height (cm)	Stem diameter (cm)	Length of internodes (cm)	No. of days to first flower	No. of nodes to first flower
Control	114.9	4.29	3.13	134	32
124-3 (7)	81.3*	4.00	1.60*	142	47*
132-2 (17)	78.0*	3.72	1.61*	115	39*
142-3 (3)	76.8*	3.27	1.80*	126	53*

[1] Mean values with asterisk (*) are significantly different from the control at 5% level of significance by *t*-test. Numbers inside parenthesis ( ) represent the number of plants per line.

a genetically determined number of nodes ranging from the 24th node from the ground level in a short statured variety and at the 48th node from the ground in a tall statured variety provided that the plants are growing under favorable environmental conditions (Storey 1986).

In contrast, the stem diameter of the transgenic T_2 lines is not significantly different with the non-transgenic control, suggesting that the transgenic T_2 lines and the non-transgenic control are no different to each other in terms of stem diameter. Among the three T_2 lines, line 124-3 had the stoutest stem diameter or girth of 4.00 cm, while the transgenic T_2 line 142-3 had the thinnest stem diameter of 3.27 cm. Likewise, the number of days to first flower of the transgenic T_2 lines is not different to the non-transgenic control. This result also suggests that the three transgenic T_2 lines are no different to the transgenic control, in terms of the number of days to first flower.

In terms of gross fruit morphology, fruit shape, light yellow orange (RHS 17A), peel color, and bright yellow-orange (RHS 23A) flesh color of ripe fruits of the transgenic T_2 lines and the non-transgenic control are the same, suggesting that the T_2 transgenic lines and the non-transgenic control are indeed not different. However, the fruit weight of the T_2 transgenic lines was significantly different to the non-transgenic control plants (Table 7). In addition, the TSS and EP of the T_2 transgenic lines were significantly different to the non-transgenic control. While the fruit weight, TSS and EP of the three transgenic T_2 lines are significantly different to the non-transgenic control, this does not imply that the transgenic lines are no longer equivalent to the non-transgenic control, because phenotypically the gross morphological characteristics of the plant and fruit are still falling within the characteristic range of the non-transgenic 'Solo' papaya.

Comparison between T_1 and T_2 generations

Resistance to PRSV

In both T_1 and T_2 generations, the selected plant progenies that were transplanted directly in soil were resistant to PRSV until the flowering stage but towards the end of the first fruiting cycle, they were infected. However, during the early screening stage for resistance to PRSV of the seedling progenies, the percentage of the PRSV resistant progenies differ in both T_1 and T_2 generations among the three transgenic lines.

In the T_1 generation, line 124-3 had the highest percentage of resistance having 83% out of the 30 inoculated seedlings, 132-2 had 70% out of the 27 inoculated seedlings and line 142-3 had 74% out of the 23 inoculated seedlings (Table 8). However, the non-transgenic control had no resistance.

In comparison to the T_1 generation, the T_2 generation of the three transgenic lines had lower percentages of resistance than in T_1 generation in all the three T_2 transgenic lines, possibly because coat protein accumulation could be lesser in the T_2 generation than in the T_1 generation, hence the coat protein offers a lesser degree of protection against the severe invading virus. This result indicates that the resistance of the three transgenic lines is unstable. In coat protein mediated protection against tobacco mosaic virus (TMV), it has been reported that the accumulation of coat protein in the transgenic tobacco plants is indispensable for protection against the virus and it appeared that the coat protein expressed in the transgenic plants interferes in the disassembly of the TMV particles, hence diminishing the protection rendered by the transgenic (Osbourn et. al. 1989; Register III and Beachy 1988).

In addition, the unstability of the resistance reaction observed in the three transgenic lines could be attributed to the segregation of the coat protein transgene, hence further evaluation of transgene expression could be done in later generations. It has been reported that the variation in the PRSV resistance of the R_2 seedling progenies of the transgenic KN49 papaya variety in Thailand's is due to the segregation of the coat protein transgene (Chowpongpang et al. 2005). Similarly, in Malaysia, it has been observed that the resistance is also unstable, as the number of resistant progenies (119) in the R_0 (original transgenic plants) regeneration decreases to 13 in the R_1 generation and no resistant progenies were observed in the R_2 generation (Habibuddin et al. 2007).

Table 7. Selected fruit characters at ripe stage of the transgenic T_2 lines and the non-transgenic control.

Transgenic lines	Fruit characters[1]		
	Fruit weight (g)	TSS (°B)	EP (%)
Control (20)	475.8	12.8	69.2
124-3 (10)	552.2*	10.5*	72.0*
132-2 (57)	587.8*	10.4*	71.9*
142-3 (10)	580.5*	10.3*	71.0*

[1] Mean values with asterisk (*) are significantly different from the control at 5% level of significance by *t*-test. Numbers inside parenthesis ( ) represent the total number of fruits evaluated in each line.

Table 8. Percentage resistance to PRSV of the three transgenic lines in T_1 and T_2 generations. Reaction to PRSV of the test plants was assessed 60 days after inoculation.

Transgenic lines	Percent Resistance (%)	
	T_1	T_2
124-3	83	41
132-2	70	7
142-3	74	8
Control	0	0

Horticultural evaluation

The plant height of transgenic lines 124-3 and 142-3 in the T_1 and T_2 generation were not significantly different (Table 9). This result suggests that these two lines are stable in terms of plant height. The same trait in the T_1 and T_2 generations for transgenic line 132-2 was significantly different but it is still within the range of the height of 'Solo' papaya. In terms of internode length, there was no significant difference in the T_1 and T_2 generations for transgenic line 142-3, also suggesting stability of this transgenic line. On the other hand, the same trait for lines 124-3 and 132-2 in the two generations was significantly different, but the values for internode length still fall within the range of 'Solo' papaya.

On the overall, there is a fluctuation from T_1 to T_2 in terms of plant height (except for 142-3), stem diameter and length of internodes for all the 3 lines. This decrease may be due to the higher temperature (30°C) that prevailed when the T_2 are grown compared to the temperature (28°C) when the T_1 were grown, suggesting that less soil moisture is available for plant growth and development. In addition, rainfall is lesser during the growing season of the T_2 (148.0 mm) than during the growing season of the T_1 (137.0 mm). This further possibly explains why plant height, stem diameter and length of internode decreased.

In terms of number of nodes, there was no significant difference in the T_1 and T_2 generations for lines 132-2 and 142-3, but this trait was significantly different for line 124-3. However, in terms of stem diameter and number of days to flower, significant differences between the T_1 and T_2 generations for three transgenic lines were detected. It is interesting to note that generally, the number of days to flower of the three transgenic lines was much higher in the T_2 where the plants were grown from August 2005 until March 2007, than in the T_1 generation where the plants were grown from March 2004 to March 2005. Varying environmental conditions such as light duration, temperature and frequency of rainfall during the two different growing periods may have contributed to the difference in the number of days to flower by the three transgenic lines.

The qualitative traits at flowering stage such as light green (RHS 141B) stem color, dark green (RHS 141B) leaves and petiole, and creamy white (RHS 155A) flowers, are not different in both T_1 and T_2 generations for the three transgenic lines. Furthermore, a comparison of the selected fruit characters of the transgenic lines in the T_1 and T_2 generations was shown in Table 10. The fruit weight in the T_1 and T_2 generations of transgenic lines 124-3 and 142-3 was not significantly different, suggesting that these lines are relatively stable for fruit weight. However, in the same fruit character, for line 132-2, there is was a significant difference in the two generations. In terms of total soluble solids, there was no significant difference in the T_1 and T_2 generations among the three transgenic lines. In addition, there was no significant difference in terms of fruit edible portion of the T_1 and T_2 generations for three transgenic lines. The results suggest that edible portion and total soluble solids were stable for T_1 and T_2 generations for lines 124-3 and 132-2.

Moreover, the fruit shape, light yellow orange (RHS

Table 9. Comparison of the selected horticultural traits at flowering stage of the three transgenic lines in the T_1 and T_2 generations.

Horticultural traits[1]	Transgenic line/Generation					
	124-3		132-2		142-3	
	T_1	T_2	T_1	T_2	T_1	T_2
Plant height (cm)	86.3	81.3	86.7	78.0*	72.4	76.8
Stem diameter (cm)	5.11	4.00*	5.45	3.72*	5.24	3.27*
Length of internode (cm)	2.41	1.60*	2.26	1.60*	2.08	1.80
No. of days to first flower	67	142*	64	115*	66	126*
No. of nodes to first flower	37	47*	35	39	31	53

[1] Mean values with asterisk (*) are significantly different to T_1 at 5% level of significance.

Table 10. Comparison of selected fruit characters at the ripe stage of the transgenic lines in the T_1 and T_2 generations.

Fruit characters[1]	Transgenic line/Generation					
	124-3		132-2		142-3	
	T_1	T_2	T_1	T_2	T_1	T_2
Fruit weight (g)	508.7	552.2	460.0	587.6*	509.0	515.8
TSS (°Brix)	10.5	10.5	10.7	10.4	10.2	10.4
Edible portion (%)	71.5	72.0	72.2	71.9	72.8	72.0

[1] Mean values with asterisk (*) are significantly different to T_1 at 5% level of significance using *t*-test.

17A) peel and bright yellow-orange (RHS 23A) flesh color of the three transgenic lines were not different in the T_1 and T_2 generations. The fruit shape of the three transgenic lines was consistently pyriform or bulb-shape for the hermaphrodite form and rounded for the female form in both generations, which is typical of 'Solo' fruits. The fruits of the transgenic lines also consistently produced a lot of black (RHS 202A) seeds in both T_1 and T_2 generations, indicating that they were fertile.

Summary and conclusion

A preliminary study aimed to determine the partial stability of the expression of resistance to PRSV and horticultural characters of transgenic lines previously transformed using the coat protein gene of the papaya ringspot virus by the *Agrobacterium*-mediated system was conducted under BL2 greenhouse conditions approved by the NCBP. Three selected transgenic lines namely: 124-3, 132-2 and 142-3 in the T_1 and T_2 generations were assessed for resistance to PRSV using mechanical inoculation test and ELISA assay. Evaluation of the horticultural traits at flowering and ripe fruit stages was also done.

Based from preliminary results, the percentage of resistant seedling progenies in the T_1 generation decreased in the T_2 generation, an indication that the inherited resistance of the three transgenic lines was unstable, hence further evaluation of the transgene expression could be done. In contrast, several horticultural traits of the transgenic lines were stable in both the T_1 and T_2 generations. Specifically, the light green (RHS 141B) stem color, dark green (RHS 141B) leaves and petiole, and creamy white (RHS 155A) flowers were stably expressed in the flowering stage by the three transgenic lines in both the T_1 and T_2 generations, indicating that these traits are seemingly stable and are comparable to the non-transgenic control. The plant height for lines 124-3 and 142-3, internode length for line 142-3 and number of nodes to first flower for lines 132-2 and 142-3 were also stable in both T_1 and T_2 generations.

Further, the qualitative ripe fruit characters such as the pyriform fruit shape for hermaphrodite sex form and rounded fruit shape for the female sex form, light yellow orange (RHS 17A) peel color, bright yellow-orange (RHS 23A) flesh color and black (RHS 202A) seeds of the three transgenic lines were stably expressed in both T_1 and T_2 generations. The fruit weight for lines 124-3 and 142-3 was stably expressed in both generations including the total soluble solids and fruit edible portion.

Acknowledgments

This study was part of a project, "Development and Commercialization of GM Papaya for Fresh Fruit and Papain Production", implemented at the Crop Science Cluster-Institute of Plant Breeding, College of Agriculture, University of the Philippines Los Baños led by Dr. Pablito M. Magdalita. This project was funded by the Department of Science and Technology-Philippine Council for Agriculture, Aquatic, Forestry and Natural Resources Research and Development (DOST-PCAARRD) and supported by the International Service for the Acquisition of Agri-biotech Applications (ISAAA), Agricultural Biotechnology Support Project II (ABSP II) and Economic Modernization through Efficient Reforms and Governance (EMERGE). The authors acknowledged the contributions of Dr. Vermando M. Aquino, Ms. Lolita M. Dolores, Mr. Pierriden Perez, Mr. Marcelino T. Gregorio and Ms. Teresa de Leon. The suggestions of the anonymous reviewers were also acknowledged.

References

Accotto GP, Nervo G, Acciarri N, Tavella L, Vecchiati M, Schiavi M, Mason G, Vaira AM (2005) Field evaluation of tomato hybrids engineered with Tomato Spotted Wilt virus sequences for virus resistance, agronomic performance, and pollen-mediated transgene flow. *Phytopathology* 95: 800–807

Bau H-J, Cheng Y-H, Yu T-A, Yang J-S, Yeh S-D (2003) Broad-spectrum resistance to different geographic strains of *Papaya ringspot virus* in coat protein gene transgenic papaya. *Phytopathology* 93: 112–120

Bau H-J, Cheng Y-H, Yu T-A, Yang J-S, Liou P-C, Hsiao C-H, Lin C-Y, Yeh S-D (2004) Field evaluation of transgenic papaya lines carrying the coat protein gene of Papaya Ringspot Virus in Taiwan. *Plant Dis* 88: 594–599

Bregitzer P, Tonks D (2003) Inheritance and expression of transgenes in barley. *Crop Sci* 43: 4–12

Burns P, Kumdee O, Bandee S (2003) Progress report of papaya delayed ripening project in Thailand for 2003. Unpublished country progress report presented at the Papaya Biotechnology Network of Southeast Asia Technical and Coordination Meeting held in Kamphaengsaen, Nakhom Pathom and Bangkok, Thailand, December 15–16, 2003

Chowpongpang S, Warin N, Bhunchotch A, Rodaree R, Pironrit N, Attathom S (2003) PRSV resistance and yield of transgenic papaya in the small scale field trial. Unpublished country progress report presented at the Papaya Biotechnology Network of Southeast Asia Technical and Coordination Meeting. Held in Kamphaengsaen, Nakhom Pathom and Bangkok, Thailand, December 15–16, 2003

Department of Agriculture-Bureau of Agricultural Statistics (2010) Crop production statistics of the Philippines. Diliman, Quezon City, 96

Damayanti D, Mariska I, Herman M (2005) Delayed ripening of papaya: current status and future activity in Indonesia. Unpublished country progress report presented at Papaya Biotechnology Network of Southeast Asia. Technical and Coordination Meeting held in the First World Hotel, Genting Highlands, Kuala Lumpur, Malaysia, November 20–21, 2005

Ferreira SA, Pitz KY, Manshardt R, Zee F, Fitch MMM, Gonsalves D (2002) Virus coat protein transgenic papaya provides practical control of Papaya Ringspot Virus in Hawaii. *Plant Dis* 86: 101–105

Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1992) Virus resistant papaya derived from tissues bombarded with the coat protein gene of Papaya Ringspot Virus. *Bio/Tech* 10: 1466–1472

Fuchs M, Gonsalves D (2007) Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. *Annu Rev Phytopathol* 45: 173–202

Habibuddin H, Abubakar UK, Tan CS, Ong CA, Pillai V, NoorSuhana A (2007) Evaluation of the transgenic Eksotika papaya lines for resistance against the Papaya Ringspot Virus in Malaysia. Unpublished country progress report presented at Papaya Biotechnology Network of Southeast Asia Technical and Coordination Meeting held in Makati City and Los Baños, Laguna Philippines, June 14–15, 2007

Hull R (2002) Matthew's Plant Virology. 4th ed. Elsevier Academic Press, San Diego, California, USA. 1001 p

International Board for Plant Genetic Resources (IBPGR) (1988) Descriptors for Papaya. IBPGR, Rome, 33 p

Magdalita PM, Opina OS, Espino RRC, Villegas VN (1989) Epidemiology of Papaya Ringspot in the Philippines. *Phil Phytopath* 25: 1–11

Magdalita PM, Adkins SW, Godwin ID, Drew RA (1996) An improved embryo-rescue protocol for a *Carica* interspecific hybrid. *Aust J Bot* 44: 343–353

Magdalita PM, Parsley DM, Godwin ID, Drew RA, Adkins SW (1997) Screening *Carica papaya*×*C. cauliflora* hybrids for resistance to Papaya Ringspot Virus-type P. *Plant Pathol* 46: 837–841

Magdalita PM, Aquino VM, Dolores LM, Galvez HF (2004) *Agrobacterium*-mediated transformation of Philippine papaya for PRSV resistance. Unpublished terminal report May 1999 to April 2004 submitted to the Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD)

Magdalita PM, Valencia LD, Villegas VN (2011) Cloning of papaya ringspot virus coat protein gene and transformation of 'Solo' papaya through microprojectile bombardment. *Philipp J Crop Sci* 36: 45–56

Nelson DL, Cox MM (2005) Lehninger Principles of Biochemistry. 4th ed. Prentice Hall, New Jersey, USA. 1119 p

Osbourn JK, Watts JW, Beachy RN, Wilson TAM (1989) Evidence that nucleocapsid disassembly and a later step in virus replication are inhibited in transgenic tobacco plants expressing the coat protein gene of Tobacco Mosaic Virus. *Virol* 158: 126–132

Phironrit N, Chowpongpang S, Warin N, Bhunchoth A, Attathom S (2007) Small scale field testing of PRSV resistance in transgenic papaya line KN116/5. (Abstr). *Acta Hortic* : 740

Presting GG, Smith OP, Brown CR (1995) Resistance to Potato Leafroll Virus in potato plants transformed with the coat protein gene or with vector control constructs. *Phytopathology* 85: 469–442

Ravindranathan P, Muda P, Ying Kwok C, Abu Bakar UK, Pillai V, Peng Fatt L, Mat Daud H2002. Contained field evaluation of delayed ripening transgenic 'Eksotika' papaya. Unpublished country progress report presented at Papaya Biotechnology Network of Southeast Asia Technical and Coordination Meeting held in. Port Dickson, Malaysia, October 7–9, 2002

Register JC 3rd, Beachy RN (1988) Resistance to TMV in transgenic plants results from interference with an early event in infection. *Virology* 166: 524–532

Royal Horticultural Society (RHS) of London and Flower Council of Holland (1966) RHS Colour Chart. Leiden, Holland

Statistical Analysis System Institute, Inc. (1985) SAS/STAT Guide for personal computers. 6th ed. Cary, N.C.: SAS Institute, Inc. 378 p

Storey WB (1986) *Carica papaya*. In: Halvey AH, (ed.). CRC Handbook of Flowering Vol. 2, Boca Rotan, Florida: CRC Press, Inc. pp 147–154

Tennant P, Ahmad MH, Gonsalves D (2005) Field resistance of coat protein transgenic papaya to Papaya Ringspot Virus in Jamaica. *Plant Dis* 89: 841–847

Tricoli DM, Carney KJ, Russell PF, McMaster JR, Groff DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML, Quemada HD (1995) Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus 2 and Zucchini Yellow Mosaic Virus. *Bio/Tech* 13: 1458–1465

Xu D, Collins GB, Hunt AG, Nielsen MT (1999) Agronomic performance of transgenic burley tobaccos expressing TVMV or AMV coat protein genes with and without virus challenges. *Crop Sci* 39: 1195–1202

Yang H, Ozias-Akins P, Culbreath AK, Gorbet DW, Weeks JR, Mandal B, Pappu HR (2004) Field evaluation of Tomato Spotted Wilt Virus resistance in transgenic peanut (*Arachis hypogaea*). *Plant Dis* 88: 259–264