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Resistance of Coat Protein Transgenic Papaya and Development of Homozygous Transgenic Papaya Line 116/5 Resistant to *Papaya ringspot virus* (PRSV) under Screenhouse Conditions in Thailand

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ABSTRACT

Transgenic papaya, cv. 'Khak Nual', line 116/5 R_0 was regenerated via particle bombardment-mediated transformation with coat protein (*CP*) gene of *Papaya ringspot virus* (PRSV), Chiang Mai isolate (PRSV-CM). This line showed high resistance to PRSV-CM and contained 4 copies of the transgene. Homozygous lines of transgenic papaya line 116/5 were developed by self-pollination. Progenies were tested for resistance to PRSV by mechanical inoculation under screenhouse conditions. PRSV-*CP* transgene was detected by PCR, dot blot and Southern blot analysis. The results showed that R_1 progenies contained 2-4 copies and 20% were resistant to PRSV-CM. We selected resistant lines that contained only 2 copies of transgene for self-pollination to produce R_2, R_3 and R_4 progenies. The level of resistance in the R_2, R_3 and R_4 were 34, 71 and 95-100%, respectively. All 426 plants of the R_4 generation contained the PRSV-*CP* transgene. Sixty R_4 resistant plants were randomly selected for the determination of *CP* transgene copy. The results showed that all plants contained 2 copies of the transgene. Stability of R_5 homozygosity and biosafety assessment are being investigated.

Keywords: *Carica papaya* L., coat protein gene, pathogen-derived resistance, kanamycin resistance

INTRODUCTION

Papaya (*Carica papaya* L.) is an important economic fruit crop grown widely in tropical and subtropical areas. Ripe papaya is consumed as a dessert and is processed as canned fruit products. Green papaya is used as a salad; latex from green fruit is used in the wool and silk industry, beer and breweries, as a meat tenderizing agent, in cosmetic and pharmaceutical applications, and also in medicine. Further, aqueous extracts of papaya seeds have been tested in male rabbits for contraceptive and toxicological effects (Lohiya *et al.* 2000) and have been used as an anthelmintic in dogs in Trinidad and Tobago (Lans *et al.* 2000).

Nowadays, 'Khak Dum' and 'Khak Nual' are two major of papaya cultivars grown commercially in Thailand (Kumcha *et al.* 2008). The volume of papaya produced in 2006 was 131,000 tonnes with exported quantity 1,600 tonnes (FAO 2009). Although, Thailand is one of the largest papaya production countries in Asia, almost all productions are usually commercial for the local market as well. However, the production of papaya in many areas is limited by a destructive disease caused by *Papaya ringspot virus* (PRSV), a *Potyvirus* that is rapidly transmitted by numerous species of aphids in a non-persistent manner (Ferreira *et al.* 2002; Fermin and Gonsalves 2003). Therefore, spread of the viral disease is difficult to control by conventional methods, such as rouging, aphid control and cross protection with a mild strain of PRSV (Yeh *et al.* 1988; Tennant *et al.* 1995). Nowadays, the effort to develop virus resistant papaya is focused on genetic engineering involving the insertion a part of viral genome into the nucleus of papaya cells. This technique was derived from the concept of pathogen-derived resistance (Sanford and Johnson 1985; Powell-Abel *et al.* 1986; Luis *et al.* 1997; Fermin and Gonsalves 2003). PRSV genes that have been used for papaya transformation include the translatable and un-translatable coat protein (*CP*) gene, replicase (*NIb*) gene and *CP* linked with inverted repeat of *CP* gene (Chen *et al.* 2001; Davis and Ying 2004; Kertbundit *et al.* 2007). Using the *CP* transgene, Chowpongpang *et al.* (2002) successfully transformed a Thai papaya cultivar, 'Khak Nual', via microprojectile bombardment and subsequently obtained transgenic papaya with *CP* gene of PRSV-CM isolate as well as the *npt*II gene for selection. The resistant line 116/5 was completely resistant to PRSV-CM and contained 2-4 copies of transgene in R_1 plants under screenhouse condition (Warin *et al.* 2003). However the transgenic plants do not produce CP protein which may result from post-transcriptional gene silencing (PTGS) (Chowpongpang *et al.* 2002; Ruanjan *et al.* 2007).

The homozygous progenies were selectively screened through self-pollination of a hermaphrodite tree under screenhouse condition. The stable lines obtained from the breeding regime depended upon the insertion copy of the target gene, parental genotype, and morphology; the lower copy number of insertion tends to be easy for breeding into homozygous line (Phironrit *et al.* 2007). This breeding regime was employed in order to achieve stable homozygous PRSV resistance and preferred sex type (Phironrit *et al.* 2004). In this study, the copy number of PRSV-*CP* transgene determined for development of homozygous transgenic papaya line 116/5 resistant to PRSV under screenhouse condition.

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Original Research Paper

MATERIALS AND METHODS

Screening for PRSV resistant papaya

Transgenic and non-transformed papayas were inoculated with PRSV-CM isolate. The inoculum was prepared by grinding PRSV infected leaves with 0.1 M phosphate buffer (0.1 KH_2PO_4, 0.1 M Na_2HPO_4), pH 7.0 containing 0.5% w/v of celite®545 (Fluka, Switzerland) for wounding. The ratio of leaf to buffer was 1:10 (1 g infected leaf to 9 ml buffer). The plants were inoculated by rubbing the viral inoculum onto 3 leaves of the papaya plants very gently. After inoculation, celite®545 (Fluka) was rinsed off with water to prevent shading. The inoculated plants were kept in a screenhouse for symptom observation. After 14 days of inoculation, non-infected plants were repeated and determined the CP of PRSV by ELISA with polyclonal antibody against PRSV-*CP* for confirmation of PRSV symptom.

Detection of PRSV-CP transgene by PCR

Genomic DNA was extracted by CTAB standard method (Rogers *et al.* 1996). A 20 μl PCR reaction mixture consisted of 1x PCR buffer, 1.5 mM $MgCl_2$, 100 μM each dNTPs, 0.5 μM each primers; CP1 (5′-ATT GCG CAT ACC TAG GAG AGA GTG-3′), CP2 (5′-AAA GTG GTA TGA GGG AGT GAG GAA-3′), 1 U *Tag* DNA polymerase and 1 μl genomic DNA (~200 ng). PCR cycles for PRSV-*CP* are as follow; one cycle of 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final cycle of 72°C for 10 min. The 501 bp products size were analyzed by gel electrophoresis.

PRSV-CP transgene analysis in transgenic papaya by DNA dot blot and Southern blot analysis

Ten micrograms of genomic DNA were denatured in denaturation solution (0.125 N NaOH and 0.125X SSC) and doted on positively charged nylon membrane (Roche, Germany), fixed with UV 260 nm for 3 min. The membrane was hybridized with DIG non-radioactive PRSV-*CP* probe then followed the procedures as suggested by the manufacturer manual (Roche). The PRSV-*CP* insertion number was analyzed by Southern blot hybridization. Approximately 20 μg of genomic DNA was digested with *Eco*RI restriction endonuclease, and separated on 1% agarose gel with TAE buffer system; transferred to membrane, and then hybridized with DIG non-radioactive PRSV-*CP* probe with PCR-DIG labeling mix, DIG labeling probe as describe by DIG Application for Filter Hybridization (Roche). Detection procedure was similar to the DNA dot blot hybridization.

Screening for transgenic papaya line 116/5 R_4 resistant to kanamycin

Seeds were collected in bulk from transgenic papaya line 116/5 R_3 for self-pollination (and generation of R_4 progenies) and non-transgenic papaya for control. For kanamycin selection, seedlings at the stage of first pair of true leaves were sprayed 3 times with 2 mg/ml kanamycin in 0.1**%** dimethy sulfoxide (DMSO) solution using a spray bottle (Xiang *et al.* 1999; Raweerotwiboon and Chunwongse 2007). The sprayed plants were kept in glasshouse at 25°C for symptom observation. After 21 days the chlorotic symptoms. The results were compared to those obtained by the PCR amplification of the transgene using specific primers.

RESULTS AND DISCUSSION

A number of resistant transgenic papaya line 116/5 which shown high resistant level to PRSV in R_1 generation and have good performance the same as parental was identified. Progenies were tested for PRSV resistance by mechanical inoculation under the screenhouse conditions. PRSV-*CP* transgene was detected by PCR, dot blot and Southern blot analysis. Non-transformed papaya was used as positive control for symptom observation in every inoculation. After 14 days of inoculation, non-transformed papaya developed severe systemic symptoms while transgenic plants were resistant and did not exhibit any symptom development (**Fig. 1**). In addition, *CP* gene of PRSV was not detected by ELISA in these plants after 1 month of inoculation. Interestingly, the results show that R_1 progenies contained 2-4 copies (**Fig. 2**) and had 20% resistance to PRSV-CM. We selected resistant lines that contained 2 copies of transgene for self-pollination to produce R_2, R_3 and R_4 progenies. We found that the level of resistance in R_2, R_3 and R_4 were 34, 71 and 97-100%, respectively (**Table 1**). Plants with high resistant level against PRSV-CM were obtained the R_4 plants of each 116/5 transgenic lines such as R_4 - 116/5-200-17-1, R_4 - 116/5-200-4-1, R_4 - 116/5-200-13-4 and R_4 - 116/5-200-20-2. Progenies from these lines show 97-100**%** resistance to PRSV (**Table 2**). All progenies of R_4 contained the PRSV-*CP* transgene (**Figs 3, 4**) Sixty R_4 progenies randomly selected showed that all of them contained 2 copies of transgene (**Fig. 5**). The differences in protection observed

Fig. 1 PRSV-CM challenge inoculation of R_4 plants of the transgenic papaya line 116/5 inoculated with PRSV-CM for 14 days. (**A**) Non-transgenic papaya seedlings showing systemic infections after 14 days. (**B**) PRSV resistant, asymptomatic transgenic progenies after 14 days.

Table 1 Screening for PRSV resistance in R_1, R_2, R_3 and R_4 progenies of the transgenic papaya line 116/5.

Papaya regeneration[1/]	Progeny	% PRSV resistance	Copy number of PRSV-*CP* gene
p116/5 R_1	150	20.66 (31/150)	2-4
p116/5 R_2	1,850	34.05 (630/1,850)	2
p116/5 R_3	2,573	70.08 (1,829/2,573)	2
p116/5 R_4	426	95.05 (405/426)	2
Non-transformed papaya	80	0 (0/80)	NT

[1/] Plant of each regeneration
NT= not tested

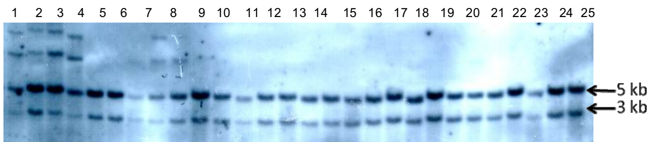

Fig. 2 A PRSV-*CP* gene insertion copy in R_1 plants of the transgenic papaya 116/5. Genomic DNA was digested with *Eco*RI restriction endonuclease. DNA fragments were separated on 1% agarose gel electrophoresis, transferred to positive charged nylon membrane and hybridized with DIG-PRSV-*CP* probe (Roche, Germany).

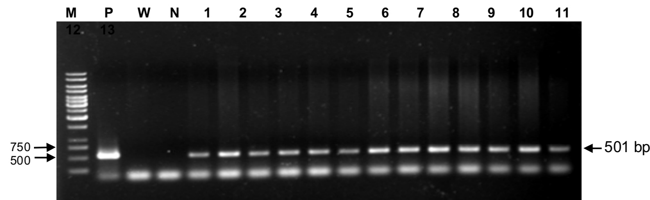

Fig. 3 PRSV-*CP* PCR product in 116/5 R_4 transgenic papaya was analyzed by gel electrophoresis. A 501 bp DNA fragment was observed in all lines. M=DNA Molecular weight marker, P = plasmid p2CMCP, W = water, N = non-transformed papaya, 1-13 = transgenic papaya.

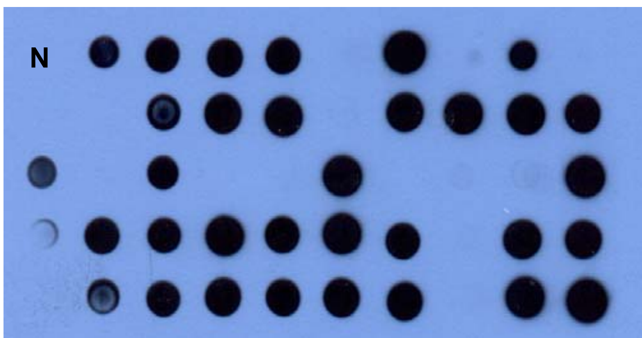

Fig. 4 DNA dot blot hybridization for PRSV-*CP* specific transgene in transgenic papaya line 116/5 R_4 progenies. Genomic DNA was immobilized on positively charged nylon membrane and hybridized with DIG PRSV-*CP* specific probe. N = non-transformed papaya, ● = PRSV-*CP* positive transgenic lines.

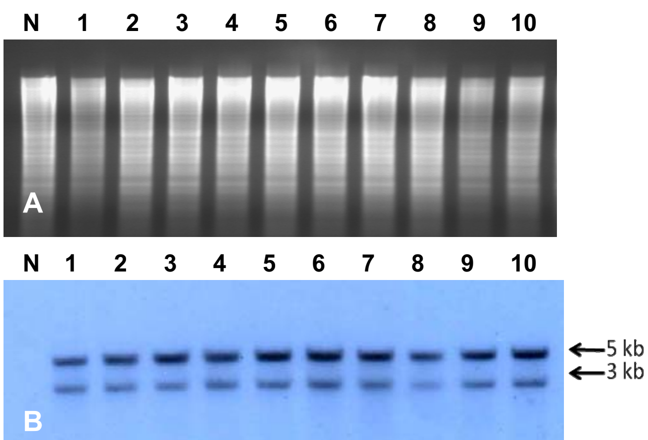

Fig. 5 A PRSV-*CP* insertion copy in R_4 plants of the transgenic papaya line 116/5. Genomic DNA was digested with *Eco*RI restriction endonuclease. DNA fragments were separated on 1% agarose gel electrophoresis (A) and transferred to positively charged nylon membrane and hybridized with DIG PRSV-*CP* probe (B). N = non-transformed papaya.

in our transgenic plants when analyzed by generation may be due to transgene dosage, which plays a fundamental role in the phenotypic manifestation of resistance in transgenic papaya. We expect to identify a homozygous line in the fifth generation that we are currently analyzing.

Fig. 6 Seedlings at the stage of their first of true leaves were sprayed 3 times with 2 mg/ml kanamycin in 0.1% DMSO solution using a spray bottle. Comparison between transgenic papaya line 116/5 R_4 (A) and non-transformed papaya (B) after 21 days post spraying.

Table 2 PRSV-CM resistant, PRSV-*CP* gene and copy number of transgene of R_4 plants of the transgenic papaya line 116/5.

Papaya line[1/]	% PRSV[2/] resistance	PRSV-*CP* gene[3/]	Copy number of *PRSV-CP* gene
R_4-116/5(200-17-1)	100[a]	+40/40	2
R_4-116/5(200-4-1)	100[a]	+40/40	2
R_4-116/5(200-13-4)	97.90[a]	+40/40	2
R_4-116/5(200-20-2)	100[a]	+40/40	2
Non-transformed papayas	0[b]	NT	NT

[1/] R_4 plants of each 116/5 transgenic lines
[2/] Mean followed by a common letter are significantly different at the 5% level by Duncan's Multiple Range Test
[3/] Plants tested by PCR for presence of PRSV-*CP* gene
NT= not tested

In addition, the spraying of kanamycin on young transgenic papaya and non-transgenic papaya seedlings was extremely effective in differentiating transgenic and non-transgenic seedlings. Chlorotic symptom development on leaves of the untransformed seedling and non-chlorotic symptom on leaves of the transformed plants were observed 21 days following application (**Fig. 6**). All 160 of the tested plants were positive with the PRSV-*CP* gene by PCR technique (**Table 3**). This reaction occurred regardless of age of tested plants, and the method has proven to be simple, fast, non-destructive, relatively inexpensive, and reliable (Weide

Table 3 Screening for transgenic papaya line 116/5 R_4 resistance to kanamycin.

Papaya lines[1/]	No. of progeny	% Kanamycin resistance[2/]	% of PRSV resistance[3/]	PRSV-*CP* gene[4/]
R4 (200-17-1)	40	100[a]	100[a]	+40/40
R4 (200-4-4)	40	100[a]	100[a]	+40/40
R4 (200-4-1)	40	100[a]	100[a]	+40/40
R4 (200-20-2)	40	100[a]	100[a]	+40/40
Non-transgenic papaya	80	0[b]	0[b]	NT

[1/] R_4 plants of each 116/5 transgenic lines
[2,3/] Mean followed by a common letter are significantly different at the 5% level by Duncan's Multiple Range Test
[4/] Plants tested by PCR for presence of PRSV-*CP* gene
NT= not tested

et al. 1989; Xiang *et al.* 1999; Ilardi and Barba 2001; Hadi *et al.* 2002; Freitas-Astua *et al.* 2003; Raweerotwiboon and Chunwongse 2007).

Although the selection of stable resistant transgenic lines require labor and time, the procedure is necessary to obtain lines with desirable traits. Various research groups are also attempting the development of transgenic lines using these conventional procedures (Chen *et al.* 2001; Davis and Ying 2004; Tennant *et al.* 2001). Prior to commercialization of virus resistant transgenic plants, it is necessary to know as much as possible about the gene that has been incorporated into the plant. Additional information will serve to expand our information about the properties of newly generated transgenic plants. This information is of special interest to breeders and researchers assessing the risks associated with introducing transgenic plants into agriculture (Kaniewski and Thomas 1999).

For future plan, these transgenic lines must be tested in the restricted field plot in different locations of Thailand to evaluate their performance against infection from different PRSV isolates. And also next generations of R_n will be generated for homozygous genotype that processes stable agronomical characteristics and PRSV traits.

FUTURE PLANS

We will continue with the selection and the development of homozygous stable lines. Biosafety assessments under screenhouse condition are ongoing. These preliminary results envisage the potential benefits to the farmers for papaya production and future papaya breeding program must incorporate for sustainable papaya production in Thailand.

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REFERENCES

Chen G, Ye CM, Huang JC, Yu M, Li BJ (2001) Cloning of the *Papaya ringspot virus* (PRSV) replicase gene and generation of PRSV-resistant papayas through the introduction of the PRSV replicase gene. *Plant Cell Reports* **20**, 272-277

Chowpongpang S, Warin N, Bhunchoth A, Romayanon K, Rodaree R, Kositratana W, Attathom S (2002) Thai transgenic papaya protects PRSV infection to different geographical isolates. *The Proceeding of the First International Conference on Tropical and Subtropical Plant Disease.* November 5-8, 2002. The Imperial Mae Ping Hotel Chiang Mai, Thailand, p 131

Davis MJ, Ying Z (2004) Development of papaya breeding lines with transgenic resistance to *Papaya ringspot virus*. *Plant Disease* **88**, 352-358

FAO (2009) FAOSTAT Database. http://faostat.fao.org

Fermin G, Gonsalves D (2003) Papaya: Engineering resistance against *Papaya ringspot virus* by native, chimeric and synthetic transgenes. In: Loebenstein G, Thottapilly G (Eds) *Virus and Virus-like Diseases of Major Crops in Developing Countries*, Kluwer Academic Publishers, Dordrecht, pp 497-518

Ferreira SA, Pitz KY, Manshard R, Zee F, Fitch M (2002) Virus coat protein transgenic papaya provides practical control of *Papaya ringsport virus* in Hawaii. *Plant Disease* **86**, 101-105

Freitas-Astua J, Astua-Monge G, Polston JE, Hiebert E (2003) A simples and reliable method for the screening of transgenic tobacco plants. *Pesquisa Agropecuaria Brasileira* **38 (7)**, 893-896

Hadi MZ, Kemper E, Wendeler E, Reiss B (2002) Simple and versatile selection of *Arabidopsis* transformation. *Plant Cell Reports* **21**, 130-135

Ilardi V, Barba M (2001) Assessment of functional transgene flow in tomato fields. *Molecular Breeding* **8**, 311-315

Kaniewski WK, Thomas PE (1999) Field testing for virus resistance and agronomic performance in transgenic plants. *Molecular Biotechnology* **12**, 101-115

Kertbundit S, Pongtanom N, Ruanjan P, Chantasingh D, Tanwanchai A, Panyim S, Juricek M (2007) Resistance of transgenic papaya plants to *Papaya ringspot virus*, Thai isolate. *Biologia Plantarum* **51 (3)**, 333-339

Kumcha U, Chaikiattiyos S, Prasatsri V (2008) Varietal improvement of papaya (*Carica papaya* L.) for fresh consumption. *Acta Horticulturae (ISHS)* **787**, 147-150

Lohiya NK, Pathak N, Mishra PK, Manivannan B (2000) Contraceptive evaluation and toxicological study of aqueous extract of the seeds of *Carica papaya* in male rabbits. *Journal of Ethnopharmacology* **70**, 17-27

Luis S, Manshardt RM, Fitch MM, Slightom JL, Sanford JC, Gonsalves D (1997) Pathogenderived resistance provides papaya with effective protection against *Papaya ringspot virus*. *Molecular Breeding* **3**, 161-168

Lans C, Harper T, Georges K, Bridgewater E (2000) Medicinal plants used for dogs in Trinidad and Tobago. *Preventive Veterinary Medicine* **45**, 201-220

Phironrit N, Bhunchoth A, Warin N, Attathom S, Chowpongpang S (2004) Reliable sex determination in papaya (*Carica papaya* L.) Thai cultivars. *Agricultural Science Journal (Supplement)* **35 (5-6)**, 379-438

Phironrit N, Chowpongpang S, Warin N, Bhunchoth A, Attathom S (2007) Small scale field testing of PRSV resistance in transgenic papaya line KN116/5. *Acta Horticulturae (ISHS)* **740**, 169-176

Powell-Abel P, Nelson RR, Hoffman N, Rogers, S Fraley R, Beachy R (1986) Delay of disease development in transgenic plants that express the *Tobacco mosaic virus* coat protein gene. *Science* **232**, 738-743

Raweerotwiboon A, Chunwongse J (2007) Screening of *nptII* in transgenic 'Seedathip3' tomato by spraying with kanamycin solution. *Agricultural Science Journal* **38**, 15-24

Rogers HJ, Burns NA, Parkes H (1996) Comparison of small-scale methods for the rapid extraction of plant DNA suitable for PCR analysis. *Plant Molecular Biology Reporter* **14**, 170-184

Ruanjan P, Kertbundit S, Juricek M (2007) Post-transcriptional gene silencing is involved in resistance of transgenic papayas to *Papaya ringspot virus*. *Biologia Plantarum* **51 (3)**, 517-520

Sanford JC, Johnsonston SA (1985) The concept of pathogen-derived resistance-deriving resistance genes from the parasite's own genome. *Journal of Theoretical Biology* **113**, 295-405

Tennant P, Fitch MM, Lius S, Gonsalves C, Manshardt RM, Sanford J, Slightom JL, Gonsalves D (1995) Coat protein-mediated protection and classical cross protection to control *Papaya ringspot virus* in papaya. *Acta Horticulturae (ISHS)* **370**, 7-11

Tennant P, Fermin G, Fitch MM, Manshardt RM, Slightom JL, Gonsalves D (2001) *Papaya ringspot virus* resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development and coat protein homology. *European Journal of Plant Pathology* **107**, 645-653

Warin N, Chowpongpang S, Bhunchoth A, Romyanon K, Rodaree R, Kositratana W, Attathom S (2003) New papaya cultivars for *Papaya ringspot virus* resistance. *Proceedings of the 41st Kasetsart University Annual Conference*, 3-7 February, 2003, pp 539-546

Weide R, Koornneef M, Zabel P (1989) A simple, nondestructive spraying assay for the detection of an active kanamycin resistance gene in transgenic tomato plants. *Theoretical and Applied Genetics* **78**, 169-172

Xiang C, Han P, Oliver DJ (1999) *In solium* selection for *Arabidopsis* transformants resistant to kanamycin. *Plant Molecular Biology Reporter* **17**, 59-65

Yeh SD, Gonsalves D, Wang HL, Namba R, Chui RJ (1988) Control of *Papaya ringspot virus* by cross protection. *Plant Disease* **72**, 375-380