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Resistance to Peanut Stripe Virus in Transgenic Peanuts (*Arachis hypogaea* L.) Carrying PStV *cp* Gene Was Stabile up to Seven Generations of Selfing

Resistensi Kacang Tanah Transgenik yang Membawa Gen *cp* PStV Stabil Sampai Tujuh Generasi Silang-Dalam terhadap Peanut Stripe Virus

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Abstrak

Penyakit bilur kacang tanah yang disebabkan oleh *peanut stripe virus* (PStV) merupakan salah satu penyakit utama pada pertanaman kacang tanah (*Arachis hypogaea* L.). Penggunaan varietas tahan merupakan alternatif paling efektif untuk mengatasi penyakit tersebut. Rekayasa genetika merupakan metode efektif untuk mendapatkan varietas kacang tanah yang resisten PStV. Tujuan penelitian ini adalah untuk (1) mengetahui respons tanaman kacang tanah transgenik yang membawa gen *cp* PStV terhadap infeksi PStV dan (2) menguji stabilitas transgen sampai tujuh generasi silang-dalam. Tanaman kacang tanah transgenik cv. Gajah generasi T_0, T_1, T_2, T_3, T_5, T_6, dan T_7 diinokulasi secara mekanik dengan PStV. Terdapat tiga jenis respons kacang tanah transgenik terhadap infeksi PStV, yaitu resisten, *recovery*, dan rentan. Pada tanaman resisten gejala tidak muncul. Pada tanaman *recovery*, gejala *chlorotic ring mottle* muncul pada satu daun atau lebih, selanjutnya gejala tidak tampak pada daun-daun yang tumbuh kemudian. Pada tanaman rentan, gejala *severe blotch* muncul pada suatu daun, selanjutnya gejala tersebut tetap muncul pada seluruh daun yang tumbuh kemudian. Transgen *cp* PStV tetap stabil setelah mengalami tujuh generasi silang-dalam. Sejumlah galur murni kacang tanah transgenik yang resisten PStV telah diidentifikasi.

Kata Kunci: Kacang Tanah Transgenik, PStV, gen *cp*, stabil

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Introduction

Peanut stripe disease caused by peanut stripe virus (PStV) is one of the most important diseases in peanuts (*Arachis hypogaea* L.). It could cause 30-60% decrease in production (Saleh & Baliadi 1992). This disease is rapidly spread by insect vector in a persistent manner. Therefore, control of the disease by pesticides is not effective (Saleh *et al*., 1991). Since peanut stripe disease is also seed-borne (Sudarsono *et al*., 1997), it can be found in virtually all fields of peanut in Indonesia.

The use of PStV-resistant peanut cultivars has been considered the most effective means of controlling peanut stripe disease. However, PStV-resistant peanut cultivars have not been available yet and breeding for peanut resistance to PStV through hybridization has been hampered by lack of the resistant gene in the gene pools of *Arachis hypogaea* L. Several wild types of peanut were reported to be resistant to PStV. However, the

introgression of the resistant gene into desired cultivars through hybridization is faced with the problem of incompatibility and lengthy backcrossing.

Plant genetic engineering could be used to cope with the problem. A body of evidence is accumulating that plants carrying a viral gene showed resistance to the corresponding virus. The resistance mechanism has been reported to be through post-transcriptional gene silencing (PTGS) (Smith *et al*., 1994; Mueller *et al*., 1995; Goodwin *et al*., 1996; English *et al*., 1996; Jan *et al*., 2000; Scorza *et al*., 2001; Guo *et al*., 2003; Asad *et al*., 2003). In PTGS, a gene is silenced because its transcription product is degraded. With the same mechanism, a viral gene inserted into a plant genome is silenced and the genome of the corresponding virus is degraded so that the plant becomes resistant to the virus. PTGS in transgenic virus resistance requires that RNA of the transgene has homology with that of the virus.

Transgenic peanuts containing coat protein (*cp*) gene of PStV has been produced (Higgins & Dietzgen, 2000) but the stability of the transgene from generation to generation has not been tested yet. This research aimed to determine the response of the transgenic peanut containing PStV *cp* gene to PStV infection and to test the stability of the transgene up to seven generations of selfing.

Materials and Methods

Transgenic peanuts

A transgenic peanut of Gajah variety carrying PStV gene (Figure 1) has been regenerated (Higgins & Dietzgen, 2000). This transgenic plant, which resulted from one transformation event, was obtained through transformation using particle bombardment conducted in Australia and sent to Indonesia as a plantlet.

Plantlet was vegetatively propagated in vitro through axilllary branching to ensure the genetic fidelity. Each plant was grown in soil contained in a polybag under a plastic house. The transgenic plants of T0 generation were mechanically inoculated with PStV and consecutively used to produce $T_{0:1}$, $T_{1:2}$, $T_{2:3}$, $T_{3:4}$, $T_{4:5}$, $T_{5:6}$, and $T_{6:7}$ seeds.

Nomenclature of plants used in experiment was as follows. T0 plants were designated as G. T1, T2, T3, T4, T5, T6 and T7 plants were designated as G (n), G(n.o), G(n.o.p), G(n.o.p.q), G(n.o.p.q.r), G(n.o.p.q.r.s), G(n.o.p.q.r.s.t), respectively, where n, o, p, q, r, s, and t are cardinal numbers, respectively. As an ilustration, G(1) is a T1 plant number 1, G(2) is a T1 plant number 2, G(3) is a T1 plant number 3, and so on. G(1.1) is a T2 plant number 1 derived from G(1). G(1.2) is a T2 plant number 2 derived from G(1). G(2.1) is a T2 plant number 1 derived from G(2). Arbitrarily, G(8.10.8.4.1.2) is a T6 plant number 2 derived from a T5 plant G(8.10.8.4.1).

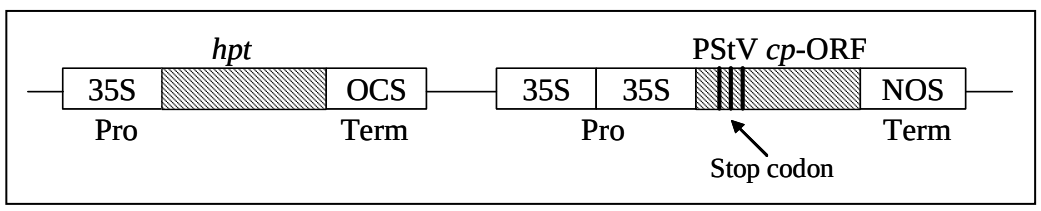

Figure 1. Gene construct used in transformation of peanut cv.Gajah through particle bombardment to obtain transgenic peanuts resistant to PStV (peanut stripe virus) (Higgins and Dietzgen, 2000). The transformation used selectable marker gene *hpt* (hygromycin phosphotransferase) driven by promoter (Pro) 35S CaMV and terminator (Term) OCS (octopine synthase) and PStV *cp* (coat protein) gene driven by double promoter 35S CaMV and terminator NOS (nopaline synthase). A stop codon was put in *cp*-ORF (open reading frame) to make the gene untranslatable.

Production of T2 to T7 plants

$T_{0:1}$ seeds were planted in a mix of soil and sand medium (2:1) contained in polybags (45 x 50 cm). T1 plants were maintained under plastic house conditions (in a screenhouse) to produce $T_{1:2}$ seeds. $T_{2:3}$, $T_{3:4}$, $T_{4:5}$, $T_{5:6}$, and $T_{6:7}$ seeds were then produced through selfing. Watering until field capacity was done every day. When needed, pest control was done using Kelthane, Confidor, and Furadan, while disease control was carried out using Dithane-M45. Plants were fertilized with 2 gram per liter of NPK fertilizer (15-15-15) at planting date and 8 weeks after sowing.

Response of transgenic plants to PStV infection

T0, T2, T3, T5, T6, and T7 plants were mechanically inoculated with PStV at least three times, i.e at 2, 4, and 6 weeks after sowing. Inoculation was continued each week for plants that did not show disease symptoms until 10 weeks after sowing to ensure that the lack of symptoms was indeed a resistance response and not a failure in doing inoculation. Non-transgenic plants were inoculated in the same way and used as a positive control.

Inoculum of PStV was maintained and propagated in peanut plants cv. Rabbits which had been inoculated with PStV isolate Bogor that caused severe blotch-stripe symptom in peanut plants cv. Landak (Akin, 1998; Avivi, 2000; Yasin, 2001). The fully open youngest leaves were sprayed with carborundum powder (600 mess) and rubbed with cutton bud previously dipped in inoculum solution. The inoculum was prepared by grinding PStV-infected leaves (0.5 cm in diameter) in 200 µl of phosphate buffer solution pH 7. Effectiveness of the inoculation was evaluated using an indicator plant, i.e *Chenopodium amaranticolor*.

Detection of PStV *cp* transgenes

Total nucleic acid was extracted using CTAB method (Murray & Thompson, 1980). Four or five leaves were frozen in liquid nitrogen and ground to powder using pestle and mortar. The powdered leaves were added with 3-4 ml of extraction buffer of 65^0C, shaken slowly, and the suspension was incubated for 1 hour in a water bath at 65^0C. The suspension was added with the same volume of chloroform and isoamylalcohol mix (24:1), slowly shaken, and centrifuged at 10,000 rpm at room temperature for 30 minutes. The liquid face on the upper part was pipetted and put into a new tube, added with 0.6 volume of isopropanol and 0.1 volume of sodium acetate 5 M, incubated for 10 minutes, and centrifuged at 10,000 rpm at room temperature for 30 minutes. The supernatant was removed and the pellet was resuspended in 70% alcohol, centrifuged at 8,000 rpm at room temperature for 20 minutes. The pellet was solubilized in 500 µl of aquadest and DNA concentration in the solution was measured with spectrophotometer at 260 nm. 2-5 µl of DNA samples were run in gel electrophoresis with agarose 1% to check the quality of the DNA.

Total nucleic acid PCR (polymerase chain reaction) analysis of T5 plants was carried out using specific primers for PStV *cp* gene to detect existence of the gene in genome. A pair of primers used was PST1 (5'-GCATGCCCTCGCCATTG CAA-3') and PST2 (5'GCACACACTTCTTG GCATGG-3') (Higgins & Dietzgen, 2000). The size of the amplified product was 234 bp. Amplification reactions were carried out in 25 µl containing 100 ng template DNA, 0,5 µl of each primer 20 µM, 0,4 µl dNTP, 0,75 µl $MgCl_2$ 50mM, 2,5 µl TRIS-HCl 10x (pH 8.3), and 3 µl Taq polymerase 10000 U/ml. Reaction conditions consisted of 3 minutes at 94^0C and 35 cycles of 30 seconds at 94^0C, 30 seconds at 55^0C, and 1 minute at 72^0C. The PCR reaction was concluded by 7-minute extension at 72^0C.

Results and Discussion

Results of the experiment showed that there were three types of response to PStV inoculation (Figure 2). Resistant plants were those that did not exhibit symptoms of PStV infection. Recovery plants were those that showed chlorotic ring mottle symptoms on one or more leaves and no disease symptoms on newly-emerging leaves. Susceptible plants were those that showed severe blotch

symptoms on one leaf and all newly-emerging leaves.

One T0 transgenic plant used in this experiment, designated as G, showed chlorotic ring mottle symptoms, while non-transgenic plants cv.Gajah showed severe blotch symptoms. Selfing T0 transgenic plants resulted in 18 $T_{0:1}$ seeds, giving rise to T1 plants and $T_{1:2}$ seeds. T1 plants were not PStV-inoculated, so their response was not known.

Response of T2 plants to PStV inoculation was presented in Table 1. Of 18 T1 lines, 6 lines produced T2 plants that were all susceptible (No.1-6), 2 lines produced recovery and susceptible T2 plants (No.7-8), 1 line produced recovery T2 plants (No.9), 3 lines produced resistant and recovery T2 plants (No.10-12), 4 lines produced resistant and susceptible T2 plants (No.13-16), and 2 lines produced resistant, recovery, and susceptible T2 plants (Table 1).

Response of T3 plants to PStV inoculation was also presented in Table 1. Of 6 resistant T2 lines, 2 lines produced only resistant T3 plants (No.19-20), 3 lines produced resistant and recovery T3 plants (No.21-23), and 1 line produced resistant, recovery, and susceptible T3 plants (No.24). Of 8 recovery T2 lines, 1 line produced resistant T3 plants (No.25), 6 lines produced resistant and recovery T3 plants (No.26-31), and 1 line produced resistant, recovery, and susceptible T3 plants (No. 32). Two other T2 lines, even though susceptible, produced resistant T3 plants in addition to susceptible ones (No.33-34). Based on the data, after three generations of selfing, the transgenic T0 plants produced resistant progenies.

All of $T_{3:4}$ seeds were planted and the T4 plants were grown to maturity, giving rise to $T_{4:5}$ seeds. T4 plants were not inoculated, so their response to PStV infection was not known. Response of T5 plants to PStV inoculation was presented in Table 2. Of 21 T4 lines evaluated, 5 lines produced only resistant T5 plants (No.1-5), 5 lines produced resistant and recovery T5 plants (No.6-10), 4 lines produced resistant and susceptible T5 plants (No.11-14), 3 lines produced resistant, recovery, and susceptible T5 plants (No.15-17), 2 lines produced recovery and susceptible T5 plants (No.18-19), and 2 lines produced only susceptible T5 plants (No.20-21). Those data showed that after five generations of selfing, most of the T4 plants evaluated produced resistant progenies and segregation in T5 plants still occurred with respect to their response to PStV inoculation.

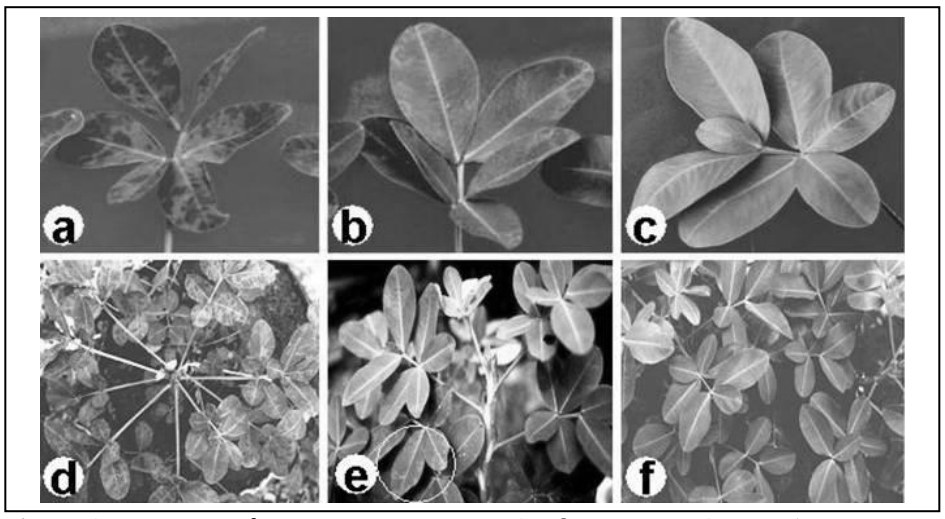

Figure 2. Response of transgenic peanuts cv.Gajah carrying PStV *cp* (peanut stripe virus coat protein) gene to PStV infection. a and d= susceptible (Sc) plants, showing severe blotch systemic symptoms on their leaves. b and e= recovery (Rc) plants, showing chlorotic ring mottle symptoms (circle mark) on one or several leaves and no symptoms on newly-emerging leaves. c and f= resistant (Rs) plants, showing no symptoms of PStV infection.

Progenies of some of the resistant, recovery, and T5 lines were then evaluated for their response to PStV inoculation (Table 2). Resistant T5 plants produced only resistant T6 plants (No.22-25), or resistant and recovery T6 plants (No.26-27). Recovery T5 lines produced only resistant T6 plants (No.28-29) or resistant and recovery T6 plants (No.30-31). A susceptible T5 line produced only susceptible T6 plants (No.32). Three resistant T6 lines, i.e. G (8.10.8.4.1.1), G (8.10.8.4.1.2), and G (8.10.8.4.1.3), which were derived from resistant T5 plants, produced only resistant T7 plants (No.33-35).

PCR analysis of T5 plants resulted in an expected amplified product of 234 bp (Fig.3), indicating that those plants contained PStV *cp* genes.

Even though PCR analysis of T0 plant was not carried out, the appearance of the signal in T5 plants was also an indication that the transgene was integrated in T0 plant genome since the transgene was still detected after five generations of selfing. That the T0 plant was transgenic was again proved by the fact that most of the T5 plants had only resistant or resistant and recovery progenies (Table 2). In addition, three resistant T6 plants produced only resistant T7 plants (Table 2).

Table 1. Response of transgenic peanuts cv. Gajah from T2 and T3 generations to PStV inoculation

No.	Peanut Lines	Response to PStV*	Number of Progenies Tested	Number of resistant, recovery, and susceptible progenies		
				Resistant (Rs)*	Recovery (Rc)*	Susceptible (Sc)*
				T2 Generations		
1	G(1)	ND**	7	0	0	7
2	G(5)	ND	6	0	0	6
3	G(10)	ND	2	0	0	2
4	G(15)	ND	2	0	0	2
5	G(17)	ND	4	0	0	4
6	G(20)	ND	32	0	0	32
7	G(3)	ND	5	0	1	4
8	G(4)	ND	4	0	1	3
9	G18)	ND	2	0	2	0
10	G(6)	ND	7	1	6	0
11	G(8)	ND	18	3	15	0
12	G(9)	ND	6	2	4	0
13	G(2)	ND	5	1	0	4
14	G(7)	ND	3	2	0	1
15	G(11)	ND	3	2	0	1
16	G(16)	ND	4	1	0	3
17	G(12)	ND	11	1	2	8
18	G(19)	ND	8	1	2	5
					T3 Generations	
19	G(8.4)	Rs	9	9	0	0
20	G(9.2)	Rs	15	15	0	0
21	G(6.1)	Rs	3	2	1	0
22	G(8.10)	Rs	15	13	2	0
23	G(9.4)	Rs	17	15	2	0
24	G(16.4)	Rs	20	10	8	2
25	G(8.15)	Rc	17	17	0	0
26	G(6.2)	Rc	20	18	2	0
27	G(8.11)	Rc	9	7	2	0
28	G(8.14)	Rc	5	3	2	0
29	G(8.17)	Rc	22	21	1	0
30	G(18.1)	Rc	25	18	4	0
31	G(18.2)	Rc	9	8	1	0
32	G(6.4)	Rc	14	5	7	2
33	G(1.5)	Sc	16	7	0	9
34	G(17.1)	Sc	17	11	0	6

*Rs = resistant; Rc = recovery; Sc = susceptible; as described in Figure 2 and in the text
** ND = not determined

The population of T0 plants employed in this experiment resulted from clonal propagation of one T0 plant in vitro. Therefore, all plants in the population were genotipically the same in term of the transgene. Phenotype of T0 plants were also the same, exhibiting symptoms of chlorotic ring mottle, which were similar to those of severe blotch shown by susceptible plants, except that the former was less severe. Therefore, plants that showed the chlorotic ring mottle was called less susceptible. This symptom was also shown by F1 plants derived from cross between PStV-resistant transgenic peanuts and PStV-susceptible non-transgenic peanuts (data not shown).

With respect to PStV *cp* transgenes, T0 plants may be hemizigous since T2 population was segregated into resistant, recovery, and susceptible plants (Table 1). The resistant and recovery plants might contain functional transgene being in homozygous condition due to inbreeding, while the susceptible plants might not contain any transgene because of the transgene being segregating out. Or, the susceptible plants might contain the transgene, but in non-functional loci.

Table 2. Response of transgenic peanuts cv. Gajah from T5, T6, and T7 generations to PStV inoculation

No.	Peanut Lines	Response to PStV*	Number of Progenies Tested	Number of resistant, recovery, and susceptible progenies		
				Resistant (Rs)*	Recovery (Rc)*	Susceptible (Sc)*
				T5 Generations		
1	G(8.4.3.1)	ND**	1	1	0	0
2	G(8.10.8.4)	ND	5	5	0	0
3	G(8.10.8.6)	ND	8	8	0	0
4	G(9.4.16.1)	ND	3	3	0	0
5	G(16.4.19.1)	ND	2	2	0	0
6	G(6.1.3.1)	ND	4	3	1	0
7	G(8.15.4.1)	ND	3	1	2	0
8	G(9.2.5.1)	ND	5	3	2	0
9	G(8.14.4.1)	ND	5	2	3	0
10	G(18.2.2.1)	ND	4	2	2	0
11	G(6.4.14.1)	ND	3	1	0	2
12	G(6.4.14.2)	ND	7	2	0	5
13	G(8.17.1.1)	ND	7	4	2	1
14	G(6.4.3.1)	ND	4	0	2	2
15	G(18.1.5.1)	ND	1	0	0	1
16	G(6.2.4.1)	ND	8	7	0	1
17	G(16.4.15.1)	ND	8	1	0	7
18	G(8.11.6.1)	ND	4	2	1	1
19	G(16.4.1.1)	ND	8	1	4	3
20	G(1.5.2.1)	ND	2	0	1	1
21	G(17.1.8.1)	ND	3	0	0	3
				T6 Generations		
22	G(8.10.8.4.1)	Rs	10	10	0	0
23	G(8.17.1.1.3)	Rs	12	12	0	0
24	G(9.2.5.1.2)	Rs	10	10	0	0
25	G(9.4.16.1.3)	Rs	6	6	0	0
26	G(8.4.3.1.1)	Rs	10	9	1	0
27	G(8.10.8.6.1)	Rs	10	8	2	0
28	G(8.17.1.1.5)	Rc	10	10	0	0
29	G(9.2.5.1.1)	Rc	10	10	0	0
30	G(6.4.3.1.1)	Rc	5	4	1	0
31	G(8.11.6.1.2)	Rc	12	3	3	6
32	G(17.1.8.1.3)	Sc	10	0	0	10
				T7 Generations		
33	G(8.10.8.4.1.1)	Rs	8	8	0	0
34	G(8.10.8.4.1.2)	Rs	7	7	0	0
35	G(8.10.8.4.1.3)	Rs	10	10	0	0

*Rs = resistant; Rc = recovery; Sc = susceptible; as described in Figure 2 and in the text
** ND = not determined

Up to seven generations of selfing, resistant individual plants produced progenies some of which were resistant and/or recovery (Table 1 and 2). Even some resistant and recovery individual plants produced only resistant and/or recovery progenies (Table 1 and 2). It was evident that up to T7 generation the *cp* PStV transgene was functionally stabile. Since gene expression was, among other things, dependent upon its location in genome, a phenomenon called positional effects (Meyer, 1995; Matzke & Matzke, 1995), the PStV *cp* transgene being functionally stabile was an indication that the transgene was also stably integrated in genome. Direct prove for this stable transgene integration would need Southern analysis of plants from different generations.

PStV *cp* gene employed in research was theoretically untranslatable because there is a stop codon in the front of its open reading frame. Use of untranslatable coat protein gene of virus to produce virus-resistant transgenic plants has been reported, for example TEV (tobacco etch virus) *cp* gene in tobacco (Goodwin *et al*., 1996), SqMV (squash mosaic virus) *cp* gene in squash (Jan *et al.*, 2000), PPV (*plum pox virus*) *cp* gene in plum (Scorza *et al*., 2001), and SrMV (*sorghum mosaic virus*) *cp* gene in sorghum (Butterfield *et al*., 2002). Their experiments showed that mechanism of resistance was through post-transcriptional gene silencing (PTGS).

A test of transgene stability from generation to generation is a requirement before a transgenic plant is used commercially or as a parent in a breeding program. To evaluate transgene stability, some researchers used 2 generations (T0 and T1) (Vain *et al*., 2002; Okada *et al*., 2002; Rooke *et al*., 2003), 3 generations (T0, T1, and T2)) (Cheng *et al*., 1997; Campbell *et al*., 2000), 4 generations (T0, T1, T2 and T3) (Webb *et al*., 1999; Gahakwa *et al*., 2000; James *et al*., 2002), 5 generations (T0, T1, T2, T3 and T4) (Satoto, 2003). Iglesias *et al*., (1997) reported that chimeric genes *hpt* and *cat* in homozygous condition were stabile up to eight generations of selfing in some independent transformants of tobacco but not stabile in other independent transformants. Those two groups of transgenic tobacco were then used to study correlation between stability of transgene and its integration sites.

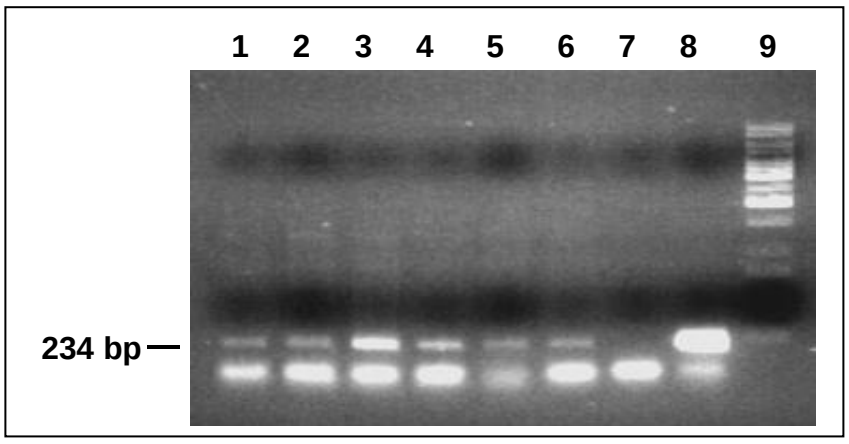

Figure 3. PCR analysis of T5 transgenic peanuts cv.Gajah to detect PStV *cp* gene. Column 1-6 were samples of DNA of G(9.2.5.1.1), G(8.4.3.1.3), G(8.15.4.1.2), G(18.1.5.1.2), G(8.15.4.1.1), and G(8.17.1.1.1), respectively. Column 7 was aquadest and column 8 was pBINRCP3, a plasmid used in transformation. Column 9 was a 1-kb ladder.

In our experiment, some pure lines of PStV-resistant transgenic peanuts have been identified; among others was G (8.10.8.4.1) which produced only resistant T6 plants (Table 4, No.22) and three of these T6 plants also produced only resistant progenies (Table 4, No.33-35), suggesting that the *cp* transgene was in homozygous condition. Other pure lines of PStV-resistant transgenic peanuts were G (8.17.1.1.3), G (9.2.5.1.2), and G (9.4.16.1.3) (Table 4, No.23-25) because they had only resistant progenies. However, since those lines were derived from one T0 transgenic plant, it is possible that at least some of those lines were genotipically the same with respect to copy number and integration sites of the PStV *cp* transgenes. Southern analysis is required to confirm this possibility.

Conclusions

The transgenic peanut plants containing PStV coat protein gene obtained in this experiment showed three types of response to PStV inoculation: resistant, recovery and susceptible. Resistant plants were those showing no symptoms of PStV infection. Recovery plants were those that showed chlorotic ring mottle symptoms on one or more leaves and no disease symptoms on newly-emerging leaves. Susceptible plants were those that showed severe blotch symptoms on one leaf and all newly-emerging leaves. The transgenic resistance character has been shown to be stable up to seven generations of selfing.

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