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THE SEGREGATION PATTERN OF INSECT RESISTANCE GENES IN THE PROGENIES AND CROSSES OF TRANSGENIC ROJOLELE RICE

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ABSTRACT

Successful application of genetic transformation technique, especially in developing rice variety resistant to brown plant hopper and stem borer, will depend on transgene being expressed and the gene inherited in a stable and predictable manner. This study aimed to analyse transgene segregation pattern of the progenies and the crosses of transgenic rice cv. Rojolele harboring *cry*1Ab and *gna* genes. The third generation (T_2) of five transgenic Rojolele events containing *gna* and/or *cry*1Ab were evaluated for two generations to identify the homozygous lines and to study their inheritance. The homozygous lines were selected based on the result of PCR technique. The segregation patterns of *gna* and *cry*1Ab were studied in eight F_2 populations derived from Rojolele x transgenic Rojolele homozygous for *cry*1Ab and or *gna* and their reciprocal crosses. Data resulted from PCR of F_2 population were analysed using a Chi Square test. The study obtained six homozygous lines for *gna*, namely A22-1-32, A22-1-37, C72-1-9, F11-1-48, K21-1-39, K21-1-48, and two homozygous lines for *cry*1Ab, namely K21-1-39 and K21-1-48. Both *cry*1Ab and *gna* transgenes had been inherited through selfing and crossing with their wild type as indicated from the F_1 containing *gna* and *cry*1Ab as many as 48.4% and 47.4%, respectively. In six of the eight crosses, *gna* was inherited in a 3:1 ratio consistent with Mendelian inheritance of a single dominant locus, while in the remaining two crosses, *gna* was segregated in a 1:1 ratio. The presence of *cry*1Ab in F_2 populations also showed a 3:1 segregation ratio in all crosses. In the F_2 population derived from F_1 plant containing *cry*1Ab and *gna*, both transgenes segregated in a 9:3:3:1 dihybrid segregation ratio. This study will add to the diversity of genetic sources for insect resistance and allow further use of these transgenic lines for pyramiding resistance to brown plant hopper and stem borer or separately in rice breeding programs whenever the efficacy tests and biosafety requirements have been completed.

[***Keywords:*** *Oryza sativa*, transgenic plant, genetic resistance, segregation, genetic inheritance]

INTRODUCTION

Brown plant hopper and stem borer are the major insects causing rice production loss in Indonesia. The progress of classical rice breeding for brown plant hopper and stem borer resistance is hampered due to the quick formation of the new biotype of brown plant hopper causing break of resistance even in the elite breeding lines, while for stem borer the major problem is the lack of resistant genes available in the respective species and their wild relatives (Rao and Padhi 1988). The International Rice Research Institute (IRRI) has screened more than 1500 entries for yellow rice stem borer and 6000 entries for stripe rice stem borer resistance (Jackson 1995), resulted some lines which have low to moderate resistance.

Plant genetic engineering is one of the complimentary tools to the conventional breeding for the development of new varieties. This technique has some advantages such as the wider germplasm options, reduction of the number of backcrossing needed to eliminate one of the parental genetic backgrounds and the precision of transferring only the target gene without any other unknown genes introgressed (Conner 1997). A number of transgenic food crops have been released globally using this techniques such as maize, soybean, and rice (www.agbios.com). In this reported work, the parental material harbors *gna* gene encoding for snowdrop (*Galanthus nivalis*) agglutinin and synthetic *cry*1Ab gene encoding crystal protein from *Bacillus thuringiensis* (Sardana *et al*. 1996). Genetic transformation using *gna* gene in tobacco for resistance to aphid was first reported by Hilder *et al*. (1995), followed by the work on rice targeted to brown plant hopper (Rao *et al*. 1998; Sudhakar *et al*. 1998), while *cry* gene has been extensively used in maize for resistance to stem borer (Amstrong *et al*. 1995; Jansens *et al*. 1997) and also in rice (Ghareyazie *et al*. 1997; Breitler *et al*. 2000). Other biotic stress resistance genes introduced to rice were among others the *wasabi defensin* gene for resistance to blast diseases (Kanzaki *et al*. 2002), *potato proteinase inhibitor* II gene (Duan *et al*. 1996), and *CpTi* gene (Xu *et al*. 1996).

Plants have mechanisms for defending themselves from microorganisms and insects. Plant produces

lectins as one of their defense strategies against insects (Carlini *et al*. 2002). GNA that binds specifically to a sugar called mannose is rather toxic to certain kinds of insect pests of important crops, including rice (Fitches *et al*. 2001). Pusztai studies showed that purified GNA was not toxic to rats (Pusztai *et al*. 1990). The gene coding for GNA was an attractive choice for increasing insect resistance of crops and at that time the gene was introduced into a number of crops, including potatoes and rice and proved to increase their resistance to some important insect pests (Rao *et al.* 1998; Foissac *et al.* 2000).

Bacillus thuringiensis (Bt) crystal proteins are protoxins that emerge when expose to alkaline medium. The protoxins are proteolitically cleaved into smaller active form (molecular weight 60-70 kDA) derived from the N-terminal half of the protein. Although the mode of toxin is largely unknown, it is predicted to bind to receptor proteins of the insect gut causing pore formation (Hoffman *et al*. 1988). Bt insecticide is commonly used in organic farming.

Transformation of Indonesian cultivar Rojolele rice by particle bombardment (Slamet-Loedin *et al.* 1998) and *Agrobacterium* has been developed in Indonesia using a number of genes (Slamet-Loedin *et al*. 1997; Mulyaningsih *et al*. 2004; Rahmawati and Slamet-Loedin 2006). In this reported work, the inheritance study was carried out on five different independent transgenic lines harboring target genes the *cry*1Ab and snowdrop lectin genes obtained through co-transformation by particle bombardment techniques (Slamet-Loedin *et al.* 1998). For further use in the breeding programs, it is important to ensure that the genes are dominant genes and segregate following Mendelian segregation. The study aimed to analyse the segregation pattern of *cry*1Ab and *gna* genes in F_2 populations derived from F_1 progenies of crosses between a transgenic Rojolele rice containing *cry*1Ab and/or *gna* genes, and their wild types as well as their reciprocal crosses.

MATERIALS AND METHODS

This study was carried out from June 2000 to July 2002 at the laboratories and the transgenic biosafety containtment glass house of the Research Center for Biotechnology, Indonesian Institute of Science, Bogor, Indonesia.

Plant Materials

All breeding lines in this reported study were the progenies of Rojolele, a local long duration variety from javanica subgroup (150-160 days) that has premium grain quality, few number of tillers, good grain filling, low productivity, and sensitive to brown plant hopper and stem borer. Rojolele was chosen because it has good cell regeneration ability and high economic value, but it is susceptible to brown plant hopper and stem borer.

The plant materials consisted of progenies of the third generations (T_2) of five transgenic events of Rojolele resulted from the earlier experiments (Slamet-Loedin *et al.* 1998), namely line A22-1, C72-1, E65-1, F11-1, and K21-1. The lines were obtained through co-transformation of three different plasmids. The first plasmid was p-Ubi-Cry IAb containing *cry*1Ab gene coding for δ endotoxin from *B. thuringiensis*, for resistance to stem borer. The second plasmid was p-Ubi-Gna containing *gna* gene coding for lectin from snowdrop for resistance to brown plant hopper and both genes were controlled by ubiquitine promoter. The third plasmid was pWRG 1515, containing *hpt* gene coding for *hygromycine phosphotransferase* and *gus A* gene coding β-*glucuronidase* controlled by CaMV35S promoter. *Hpt* gene was used to recognize the transformant among the bombarded cells in the early transformation process, while *gus A* gene was used to detect whether targeted gene has already been inserted into plant genome. All lines at the stage of the primary transgenic (T_0) have been analysed by molecular and biochemical techniques to consist of the two genes of interest (*gna* and *cry*1Ab) or individually apart from the marker genes (*hpt* and *gus-A*). The material used in this study were the segregated T_2 populations. The lines were selected based on the presence of the genes on T_1 generation. Transgenic lines A22-1 and C72-1 harbor only *gna* (lectin from snowdrop coding for resistance to brown plant hopper). Line E65-1 harbors only *cry*1Ab gene from *B. thuringiensis* (coding for δ endotoxin responsible for resistance to stem borer), whereas F11-1 and K21-1 contain both *gna* and *cry*1Ab genes.

PCR Analysis

The presence of inserted genes was analysed using PCR techniques. Specific primer sets were used to detect the presence of the codon optimized synthetic *cry*1Ab gene (Sardana *et al*. 1996) and the *gna* gene (Hilder *et al*. 1995). Primer sets used were as follows: forward (5'-CATTGTGTCTCTCTTCCC-3') and reverse (5'-CCGTTAGAGAAGTTGAAAGG-3') for *cry*1Ab and forward (5'-ATGGCTAAGGCAAGTC TCCTC-3') and reverse (5'-TCATTACTTGCCGTCAC AA G-3') for *gna*. As an internal control to ensure the plant

DNA was present in the reaction in most PCR reactions, a multiplex PCR was carried out with *gos-5* internal rice gene (Mayer *et al.* 1992) using the following primer set: forward (5'-CGACCTCGAGGAC ATCGGC AACAC-3') and reverse (5'-GCCGAGCAGC AGGAACTTGAGCAG G-3'). Expected band gene fragment sizes for *cry*1Ab, *gna* and internal control *gos-5* are 1012, 400 and 231 bp, respectively.

Seeds from the progenies of the five transgenic lines were germinated for 2 weeks. Five centimeters of the first leaf were harvested and stored in the liquid nitrogen. Genomic DNA isolation was carried out following the method of Zheng *et al*. (1995).

Individual genomic DNA samples were subjected for PCR. The PCR reaction contained 14 µl mix buffer (1x PCR buffer, dNTP 0,05 mM, primer forward 2,50 ng μl^{-1} and primer reverse 2,50 ng μl^{-1} and Taq polymerase 0,05 µl μl^{-1}) were put into PCR tube then added 1 µl DNA sample. This mixture was put into thermocycler *Gene Amp* (PCR system 2400, Perkin Elmer) and amplified for 40 cycles. PCR conditions for *gna* gene were 1 cycle 95°C 1 minute, 40 cycles with temperature of 95°C, 65°C, and 72° C, 1 minute each, 1 cycle 72°C 2 minutes and storage under 4°C. For *cry* gene, the PCR conditions were 1 cycle 95°C 1 minute, 40 cycles with temperature of 95°C, 55°C, and 72°C, 1 minute each, 1 cycle 72°C 2 minutes and storage under 4°C. Amplified DNA separation was done by electrophoresis in the 1.2% gel agarose and the result was documented using Bio Rad Gel Doc 1000.

Determination of Homozygosity of the Transgenic Lines

T_2 generation lines were selected from the T_1 parental which positively contained both *gna* and/or *cry*1Ab genes and having more than 30 seeds each. From the T_2 generation of rice lines A22-1, C72-1, E65-1, F11-1, and K21-1, eight progeny lines were selected. Fifty seeds of each selected progeny lines were grown and analysed for their inheritance. Selection of homozygous lines was done based on the gene occurrence identified from PCR result. Eleven individual plants were then selected to fullfill testing requirement number for each gene study. Eleven is the minimum number of plants to show at least one unexpected phenotype appeared under 95% probability for character controlled by a recessive allele (Sedcole 1977). However, to reduce the number of PCR reactions needed and cost, instead of carrying out directly 11 reactions for each line, initially the test was carried out on five individual plant samples from each line. Since if from five samples (five PCR reactions) there was already sample showing no amplicon (negative for the presence of the gene), the parental must be heterozygous, therefore addition of six other sample assay would not be needed. When the five initial samples gave positive results for the presence of the expected band, six additional samples were added to obtain 11 sample number, the minimum set of number to ensure the homozygosity at the level of confidence 95%.

The Segregation Pattern of *cry*1Ab and *gna* Genes in the Transgenic Rice

The segregation pattern of both *cry*1Ab and *gna* genes in transgenic rice was studied based on the PCR positive result on F_2 population derived from both Rojolele wild type x transgenic Rojolele and their reciprocal crosses. The crossing was made between transgenic Rojolele and its wild type to ensure the success rate, since inter-varietal group crossing such as to the Ciherang will have to overcome biological incompatibility if exists. In the first season, crossing between Rojolele and transgenic Rojolele homozygous for *gna* and *cry*1Ab and their respective reciprocal crosses were made. In the second season, F_1 seeds from the crossing were planted. The presence of both *cry*1Ab and *gna* genes in each individual F_1 plant was identified using PCR. The F_1 and its reciprocal crosses positively containing *cry*1Ab and *gna* genes were then selfed to produce F_2 seeds. In the third season, the F_2 seeds resulted from the F_1 and its reciprocal cross showing positive *cry*1Ab and or *gna* were planted and analysed their gene inheritance. The segregation pattern of both genes was analysed using a Chi Square test.

RESULTS AND DISCUSSION

Determination of Homozygousity of the Transgenic Lines Based on the Molecular Analysis

Plant materials used in this experiment were five transgenic progeny lines resulted from genome modification of Rojolele variety. The lines was obtained from co-transformation of *gna* and *cry*1Ab genes located at different plasmids, therefore some lines have two genes and some lines only have one of the two genes (Slamet-Loedin *et al*. 1998). In this study further analysis was carried out to identify the homozygousity status of the parents for further reciprocal analysis. In the case of transgenic application, the inserted gene will be integrated in one of

the chromosome pairs. Therefore if the gene is a dominant gene and inserted in one locus, it will follow a 3:1 Mendelian segregation pattern for a single dominant gene in the subsequent generation since rice is primarily self-pollinated plant.

As previously described in the protocol, PCR analysis using specific primer for the *gna* gene was carried out on the two steps to reduce the cost. At the step 1 at screening, five PCR reactions of each germinated seed from the same line were carried out. When all five samples showed the expected bands, then additional six samples are added to obtain 11 positive progenies. The PCR analysis results of the two-step analyses are summarized in Table 1 and 2. Based on the presence of the PCR amplicon using primers specific for *gna* on the first five samples, in total there were 10 transgenic lines harbour *gna* gene (A22-1-27, A22-1-31, A22-1-32, A22-1-37, C72-1-9, F11-1-31, F11-1-48, K21-1-46, K21-1-39, and K21-1-48), thus we continued with additional samples to reach 11 samples. The result showed that six lines (A22-1-32, A22-1-37, C72-1-9, F11-1-48, K21-1-39, and K21-1-48) derived from five transgenic events were homozygous for *gna* within 95% confidence interval (Table 1). As mentioned above, Sedcole (1977) explained statistically that 11 is the minimum number of plants must be tested to show at least one unexpected phenotype appeared under 95% probability for a character controlled by a recessive allele. If from the 11 individual plants there is one phenotype from unexpected recessive allele, the plant population must have originated from heterozygous parent plant. On the contrary, if all of the 11 individual plants showed phenotype from expected dominant allele, it means that the population was originated from homozygous parent plant for the respective gene. PCR analysis results of K21-1-48 and A22-1-32 lines are presented in Figure 1 and 2, respectively.

Table 1. Transgenic lines of rice harboring *gna* gene based on PCR amplicon presence.

Transgenic event	Target genes presence in T_1	Lines showing *gna* amplicon on first five samples	Lines showing *gna* amplicon on 11 samples
A22-1	*gna*	A22-1-27	A22-1-32
		A22-1-31	A22-1-37
		A22-1-32	
		A22-1-37	
C72-1	*gna*	C72-1-9	C72-1-9
E65-1	*cry*1Ab	-	-
F11-1	*cry*1Ab and *gna*	F11-1-31	F11-1-48
		F11-1-48	
K21-1	*cry*1Ab and *gna*	K21-1-46	K21-1-39
		K21-1-39	K21-1-48
		K21-1-48	

Table 2. Transgenic lines of rice harboring *cry*1Ab gene based on PCR amplicon presence.

Transgenic lines	Target genes presence in T_1	Lines showing *cry*1Ab on first five samples	Lines showing *cry*1Ab amplicon on 11 samples
E65-1	*cry*1Ab	-	-
F11-1	*cry*1Ab and *gna*	-	-
K21-1	*cry*1Ab and *gna*	K21-1-39	K21-1-39
		K21-1-44	K21-1-48
		K21-1-46	
		K21-1-48	

Step 1 PCR analysis for *cry*1Ab gene on five DNA samples of four lines (K21-1-39, K21-1-44, K21-1-46, and K21-1-48) harbored the *cry*1Ab gene (Table 2), thus six additional samples were analysed. Figure 3 showed the gel electrophoresis of K21-1-48 showing amplicon band of *cry*1Ab. Furher analysis using 11 DNA samples showed only two lines namely K21-39 and K21-48 that possibly homozygous for *cry*1Ab gene within confidence interval of 95% (Fig. 4).

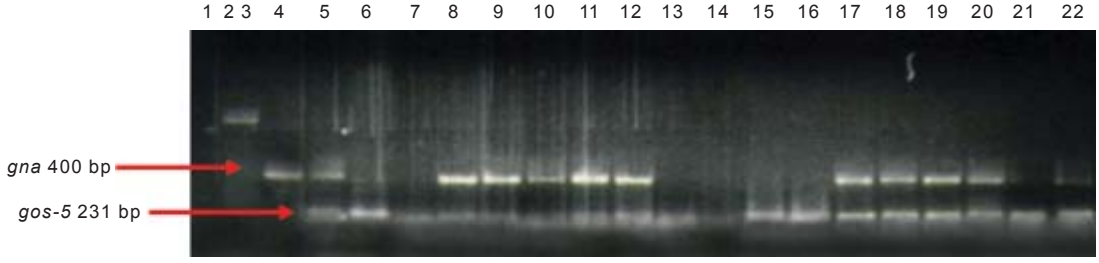

Fig. 1. PCR result of five samples for *gna* gene in the second generation of transgenic rice of K21-1-48 line, 1 = λ, 2 = -, 3 = fragment *cry*, 4 = fragment *gna*, 5 = control (+), 6 = control (-), 7 = water, 8-22 = five DNA samples of transgenic line K21-1-48.

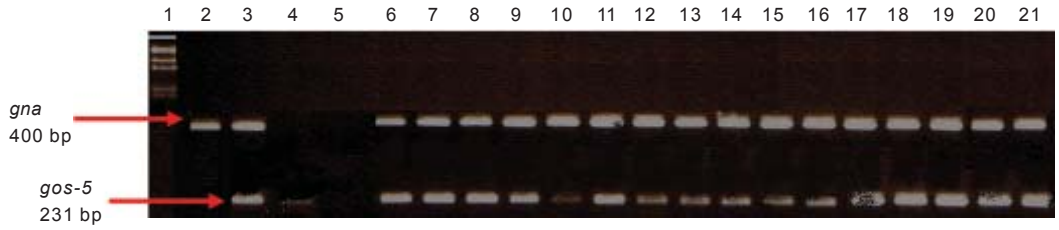

Fig. 2. PCR result of transgenic rice of A22-1-32 line homozygous for *gna*; 1 = λ *Hind*III, 2 = fragment *gna*, 3 = control (+), 4 = control (-), 5 = water, 6-16 = DNA samples of transgenic rice A22-1-32.

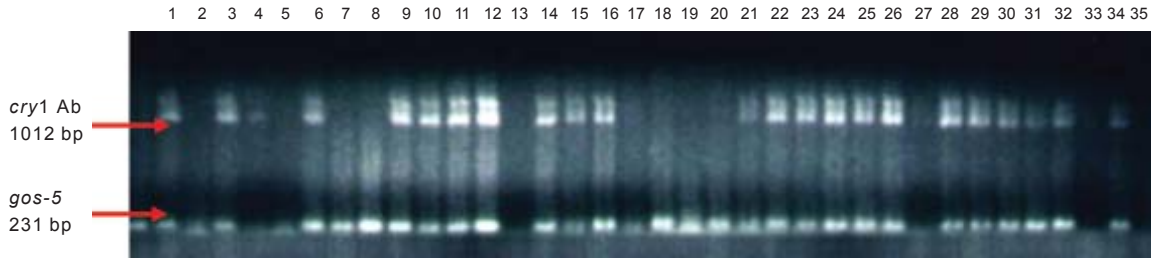

Fig. 3. PCR result of five samples for *cry*1Ab gene in the transgenic rice of T2 generation, 1 = λ *Hind*III, 2 = water, 3 = fragment *cry*, 4 = control (+), 5 = control (-), 6-35 = DNA samples of transgenic lines (27-31 = five DNA samples for K21-1-48). Positive control plant is the plant DNA sample of earlier generation shown to have *cry*1Ab gene by southern and PCR analysis. Negative control was DNA sample of the wild type nontransgenic Rojolele.

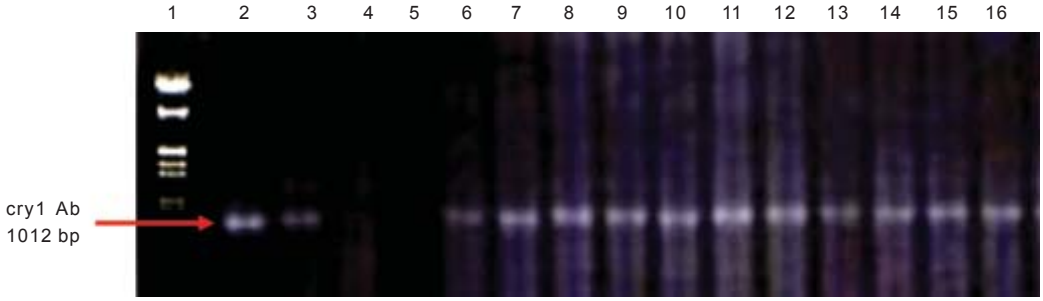

Fig. 4. PCR result of homozygous transgenic rice of K21-1-48 line containing *cry*1Ab gene; 1 = λ *Hind*III, 2 = fragment *cry*, 3 = control (+), 4 = control (-), 5 = water, 6-16 = DNA samples of the homozygous transgenic line K21-1-48.

The Segregation Pattern of *Cry*1Ab and *gna* Genes in the Transgenic Rice

All lines identified to be homozygous for the transgenes were crossed with the Rojolele wild type; reciprocal crosses were also carried out. It was observed that eventhough the genetic background of parental plants was the same, some of transgenic lines flowered earlier than their wild type, and therefore, the number of plants suitable for crossing was limited. In general, there was no difficulty to cross Rojolele wild type to the homozygous transgenic lines.

Rojolele wild type x transgenic Rojolele crosses produced F_1 seeds with seed set varied from 16% (Rojolele x K21-1-39) to 63% (C72-1-9 x Rojolele) (Table 3). Rojolele line K21-1-39 had the lowest percentage of seed set because it produced less pollen.

PCR of the F_1 plants showed that 31 out of 64 plants (48%) contained *gna* gene, while for *cry*1Ab, 9 out of 19 plants (47%) contained the gene (Table 4). PCR analyses of F_1 plants of transgenic lines homozygous for *gna* and *cry*1Ab with Rojolele were presented Figure 5 and 6, respectively.

Some of the F_1 plants that have neither *gna* nor *cry*1Ab genes may be because the crossing was

failed to take place, therefore the parent (Rojolele) was self-polinated. Another possibility was that the parent of the expected homozygous plant may actually be a heterozygous plant due to the stringency of our identification test that was at 95% level. Similar result was reported by Datta *et al*. (2002) in the crosses between two homozygous transgenic lines, TT-103 (homozygous for *Xa*21 gene) and TT-9 (homozygous for *RC*7 and *Bt* genes) resulted some F_1 that have no *RC*7 gene. Another cross (TT-9 X TT-103) also produced some F_1 plants having no *Xa*21 gene. The inheritance of transgenes through various crossing experiments performed in the present study indicates that the transgenes have already integrated into the plant genome and will be useful in rice breeding as the lines can be used as donor parent to improve the resistance of rice varieties to stem borer.

Results of the PCR analyses of samples taken from the F_2 population derived from the F_1 plants showed that there were three genotypes observed, those are genotypes containing *gna* or *cry*1Ab, or both genes (*gna* and *cry*1Ab), and those neither have any of the gene. This result indicates that both *gna* and *cry*1Ab were integrated independently in the rice genome. The recent report of Pusztai indicated the possibility that lectin from snowdrop gene may have not passed

Table 3. Number of F_1 seeds resulted from crosses between Rojolele wild type and homozygous transgenic rice lines.

Crosses	Number of crosses	Number of F_1 seeds resulted from each cross	Seed set (%)
Rojolele x A22-1-32	13	476	61
Rojolele x A22-1-37	12	443	62
Rojolele x C72-1-9	22	755	57
Rojolele x K21-1-39	5	47	16
Rojolele x K21-1-48	17	545	53
A22-1-32 x Rojolele	19	677	59
A22-1-37 x Rojolele	14	323	39
C72-1-9 x Rojolele	18	678	63
K21-1-39 x Rojolele	18	232	22
K21-1-48 x Rojolele	16	452	47

Table 4. Number of F_1 rice plants positively harbor *gna* or *cry*1Ab gene.

Crosses	Number of plants used in PCR analysis for *cry*1Ab gene	Number of plants positively harbor *cry*1Ab gene	Number of plants used in PCR analysis for *gna* gene	Number of plants positively harbor *gna* gene
Rojolele x A22-1-32			14	3
A22-1-32 x Rojolele			4	4
Rojolele x A22-1-37			7	3
A22-1-37 x Rojolele			3	3
Rojolele x C72-1-9			10	7
C72-1-9 x Rojolele			12	4
Rojolele x K21-1-48	9	5	7	4
K21-1-48 x Rojolele	10	4	7	3
Total	19	9	64	31

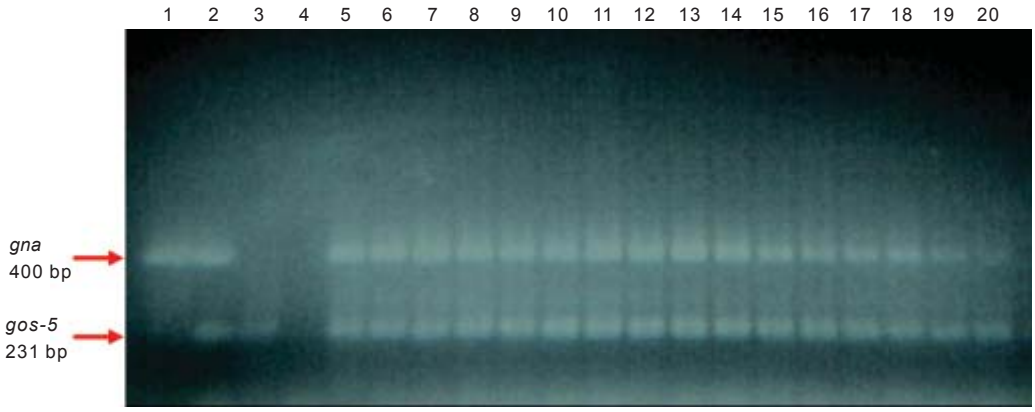

Fig. 5. PCR for *gna* gene in F_1 rice plants; 1 = *gna* gene fragment, 2 = control (+), 3 = control (-), 4 = water, 5,6,7 = samples of C72-1-9 x Rojolele, 8,9,10 = samples of A22-1-37 x Rojolele, 11,12,13,14 = samples of A22-1-32 x Rojolele, 15,16,17 = samples of Rojolele x A22-1-37, and 18,19,20 = samples of Rojolele x A22-1-32.

the food safety test for release yet, therefore, in our subsequent works the *gna* gene study has been discontinued to avoid possible food safety issues. This results negate the hypothesis suggested that transgenes tend to cluster in one locus even they are located in different plasmids (Kohli *et al*. 1999). This result also suggests that co-transformation is a possible approach when a gene is intended to be out-segregated in the final product, such as the selectable antibiotic marker gene required for transformation initially but preferred not to be presence in the final product.

The amplification result of F_2 population for segregation pattern of either *gna* or *cry*1Ab gene, and segregation of both genes was presented in Table 5, 6, and 7. In general F_1 plants performed better than their parents particularly for plant height and percentage of unfilled grains. The height of the F_1 plants was normally higher than that of both parents suggesting that the *cry*1Ab and *gna* transgenes have been inherited through selfing and crossing. When crossed with the wild type, 48.4% and 47,4% of the F_1 population contained *gna* and *cry*1Ab, respectively. In six of the eight crosses, *gna* was inherited in a 3:1 segregation ratio consistent with Mendelian inheritance of a single dominant locus. In the remaining two crosses, Rojolele x C72-1-9 and C72-1-9 x Rojolele, *gna* was segregated in 1:1 ratio. Similar result was

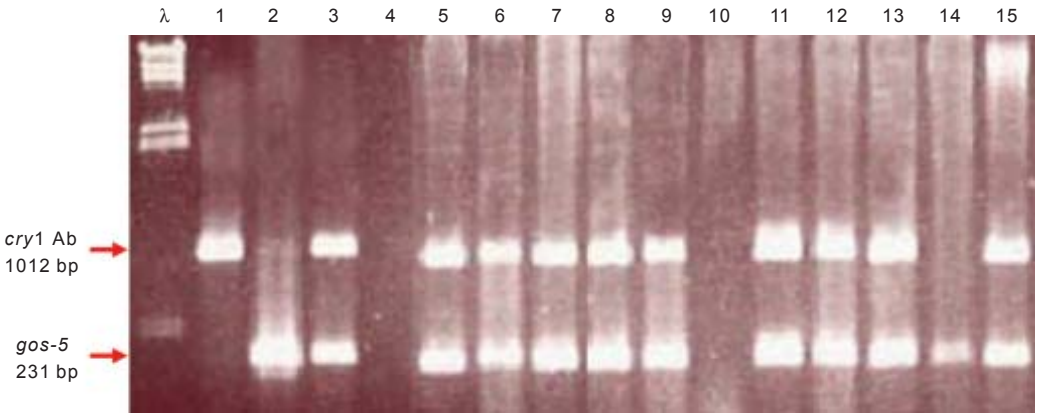

Fig. 6. PCR for *cry*1Ab gene in the F_1 rice plants. λ = lambda DNA cut by *Eco*R1, 1 = *cry*1Ab gene fragment, 2 = control (-), 3 = control (+), 4 = water, 5-9 = Rojolele x K21-1-48, 11-15 = K21-1-48 x Rojolele.

Table 5. Segegration of *gna* gene in F_2 rice populations derived from F_1 and their respective reciprocal crosses.

Crosses	n	Expected ratio[1)]		Observed ratio		X^2	df = 1; α = 0.05; X^2 = 3.84
		(+)	(-)	(+)	(-)		
Rojolele x A22-1-32	27	20.25	6.75	20	7	0.012	Ratio 3:1
A22-1-32 x Rojolele	27	20.25	6.75	16	11	3.560	Ratio 3:1
Rojolele x A22-1-37	26	20.50	6.50	19	7	0.150	Ratio 3:1
A22-1-37 x Rojolele	30	22.50	7.50	26	4	2.170	Ratio 3:1
Rojolele x C72-1-9	58	29.00	29.00	27	31	0.280	Ratio 1:1
C72-1-9 x Rojolele	51	25.50	25.50	26	23	0.250	Ratio 1:1
Rojolele x K21-1-48	59	44.25	14.75	48	11	1.270	Ratio 3:1
K21-1-48 x Rojolele	60	45.00	15.00	48	12	0.800	Ratio 3:1

[1)](+) = present and (-) = absent of *gna* gene in the F_2, progeny of the respective crosses.

Table 6. Segregation of *cry*1Ab gene in F_2 rice population derived from F_1 and its reciprocal cross.

Crosses	n	Expected ratio[1)]		Observed ratio		X^2	df = 1; α = 0.05; X^2 = 3.84
		(+)	(-)	(+)	(-)		
Rojolele x K21-1-48	59	44.25	14.75	49	10	2.04	Ratio 3:1
K21-1-48 x Rojolele	60	45.00	15.00	47	13	0.6	Ratio 3:1

[1)](+) = present and (-) = absent of *cry*1Ab gene in the F_2, progeny of the respective crosses.

Table 7. Segregation of *cry*1Ab and *gna* genes in F_2 rice populations derived from F_1 plant and its reciprocal cross.

Crosses	n	Expected ratio				Observed ratio				X^2	df = 3; α = 0.05; X^2 = 7.82
		cry/*gna*	*cry*/-	-/*gna*	-/-	*cry*/*gna*	*cry*/-	-/*gna*	-/-		
Rojolele x K21-1-48	29	16.4	5.4	5.4	1.8	19	4	5	1	1.16	Ratio 9:3:3:1
K21-1-48 x Rojolele	30	16.9	5.6	5.6	1.9	23	2	4	1	5.39	Ratio 9:3:3:1

reported by Christou *et al.* (1996) for *bar* gene which means that this aberrant segregation can be explained by the passage of the transgene exclusively through one gamete.

The presence of *cry*1Ab in F_2 population also showed a 3:1 segregation ratio in all crosses. In the F_2 population derived from F_1 plants containing *cry*1Ab and *gna*, both transgenes segregated in a 9:3:3:1 dihybrid segregation ratio.

CONCLUSION

Both *cry*1Ab and *gna* transgenes had been inherited through selfing and crossing with their wild type (48.4% of F_1 containing *gna* and 47.4% of F_1 containing *cry*1Ab). In six of the eight crosses, *gna* was inherited in a 3:1 ratio consistent with Mendelian inheritance of a single dominant locus. In the remaining two crosses, Rojolele x C72-1-9 and C72-1-9 x Rojolele, the *gna* gene was segregated in 1:1 ratio. Progeny of Rojolele wild type x Rojolele transgenic and its respective reciprocal crosses showed the same segregation pattern indicating that both *gna* and *cry*1Ab genes have been integrated in the rice genome.

Both *gna* and *cry*1Ab genes, which originally derived from different plasmids, were independently inherited and segregated in a 9:3:3:1 ratio indicating that both genes were located in two nonhomologous chromosomes; thus negated the other reports suggesting transgenes introduce by particle bombardment tend to integrate in the same loci even they were located at different plasmids originally.

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