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RESEARCH ARTICLE

Development of a Sri Lankan rice variety Bg 94-1 harbouring *cry2A* gene of *Bacillus thuringiensis* resistant to rice leaffolder [*Cnaphalocrocis medinalis* (Guenée)]

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Abstract: The rice leaffolder (RLF) [*Cnaphalocrocis medinalis* (Guenée)] is a major lepidopteran insect pest of rice in Sri Lanka and the larvae cause significant yield losses annually. Insecticidal crystal protein gene, *cry2A* driven by the 35S cauliflower mosaic virus (CaMV) promoter with a double enhancer sequence was transformed into scutellum derived embryogenic calli of Bg 94-1 *via Agrobacterium tumefaciens* mediated transformation procedure. Transformation vector pCAMBIA 1305.1 containing the transgene construct CaMV 35S d-cry2A-NOS, hygromycin phosphotransferase and β-glucuronidase (GUS)-Plus was employed in this study. Transformed calli were subjected to 4 – 5 rounds of hygromycin selection. Molecular analysis of the generated plants together with an insect bioassay using RLF larvae confirmed successful transformation and expression of this transgene in T_0 generation of rice plants. A total of 19 putative transgenic plants could be confirmed from a total of 60 T_0 generation plants examined. Insect feeding bioassay demonstrated that T_0 transgenic rice plants are effective against RLF larvae, and showed approximately 89 % larval mortality after 3 days and 100 % mortality after 5 days of feeding. Further molecular analysis of T_1 generation of the transgenic lines confirmed the stable integration of *cry2A* transgene into the Bg 94-1 genome. Findings of this study demonstrated that the *cry2A* gene of *Bacillus thuringiensis* (*Bt*) was successfully transformed into Bg 94-1 *via Agrobacterium* mediated transformation and have developed resistance against the RLF larvae. Thus, this study highlights the importance of developing transgenic rice, which can provide insect resistance against a major lepidopteran insect pest of rice in Sri Lanka.

Keywords: *Agrobacterium tumefaciens*, *Bacillus thuringiensis*, *cry2A*, rice, rice leaffolder, transformation.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important food crops and is the staple food for more than half of the global population (Khush & Brar, 2002). In Sri Lanka, rice is the predominant crop both in terms of land use and dietary importance. The total area where rice is cultivated at present is about 708,000 ha, which amounts to about 34 % of the total cultivated land area in Sri Lanka (*www.statistics.gov.lk*).

Rice productivity is greatly affected by both biotic and abiotic stresses. Insect pest attacks and damage is considered as one of the major causes of rice yield losses throughout the rice producing countries of the world; it is estimated to cost at least several billion dollars (Heinrichs *et al.*, 1985). Insect pests not only reduce the quantity, but also the quality of rice grain. Rice leaffolder (RLF) (*Cnaphalocrocis medinalis*) is one of the main rice pests in Asia and especially in Sri Lanka. It has been reported that approximately 20 % of the annual rice growing area of Sri Lanka is affected by RLFs and 30 % of the insecticides that is used in rice cultivation is targeted against RLF infestations (Nugaliyadde *et al.*, 1997). RLF was previously considered as a minor pest, however it has become a major pest in many parts of rice growing regions world over due to the use of high yielding rice varieties with little resistance to leaffolder, the changes in cultural practices of rice farmers and climate change (Bautista *et al.*, 1984; Heinrichs *et al.*, 1985; Kandibane *et al.*, 2010; Gangwar, 2015).

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When rice plants are in the early phase of growth, the larvae of RLF spin the leaves longitudinally into a roll by stitching together the opposite rims of the leaf. The larvae feed by scraping the green tissues inside the rolled leaf, which causes the leaves to become membraneous, whitish in colour and withered. The presence of a large number of leaf folds in rice fields is the characteristic symptom of RLF damage. The reduction in effective leaf area for photosynthesis in damaged leaves causes lowering in photosynthesis leading to retarded plant growth, and reduced yield (Rao *et al.*, 2010). Moreover, the infected plants are prone to bacterial and fungal infection (Bashir *et al.*, 2004).

Chemical insecticides are used to protect rice against RLF infestations. However, excess spraying of these chemicals increases the production cost, threaten human health and pollutes not only ground water but also the environment away from the fields (Chen *et al.*, 2009; Li *et al.*, 2014). In addition, these insecticides pose a real potential risk for rice consumers in terms of food safety as many insecticides are not degraded before rice is consumed (High *et al.*, 2004; Dors *et al.*, 2011).

The development of rice lines with resistance to RLF *via* conventional breeding is the most preferable approach to counteract RLF insect attacks. However, the genetic basis of RLF resistance in rice germplasm collections is not well understood (Selvaraju *et al.*, 2007; Rao *et al.*, 2010); thus, the development of RLF resistant rice lines through conventional breeding remains a challenge. Therefore, introduction of genes expressing *Bacillus thuringiensis* (*Bt*) insecticidal crystal protein into rice *via* transgenic technology is recognised as the most effective and suitable alternative to conventional breeding methods to introduce insect resistance into rice varieties (High *et al.*, 2004; Chen *et al.*, 2009; Li *et al.*, 2015).

Transgenic technology is considered as one of the most powerful 21st century technologies, which provides the driving force for 'gene revolution' in agriculture. Scientists have deployed several strategies using the transgenic approach to confer insect resistance in crop plants. One of the most successful strategies that is deployed is the introduction of *Bt* delta endotoxin crystal insecticidal protein genes (c*ry* genes). There is a wide range of *Bt* genes that have been isolated and identified from different strains of *Bt* (Chen *et al.*, 2009). The *Bt* proteins are highly toxic to lepidopteran, dipteran and coleopteran insect pests (Cheng *et al.*, 1998). The primary action of *Bt* Cry protein is reported to be *via* formation of pores caused by binding of *Bt* toxin to membranes of epithelial cells in the mid gut of target pests, which causes lysis of cells leading to death (Gill *et al.*, 1992). The Cry proteins are highly specific in their action, and therefore are not toxic to other insects including beneficial insects, birds, and mammals (Vaeck *et al.*, 1987; Stewart Jr. *et al.*, 1996).

The stable inheritance and expression of *Bt* genes has been deployed to obtain resistance specifically against lepidopteran insects of rice, namely, yellow stem borer (*Scirpophaga incertulas*), striped stem borer (*Chilo suppressalis*), and RLF (Fujimoto *et al.*, 1993; Datta *et al.*, 1998; Maqbool *et al.*, 1998; High *et al.*, 2004). Since the first *Bt* rice was developed in 1989 by a group of Chinese scientists (Yang *et al.*, 1989), several insect-resistant *Bt* rice lines have been developed over the past two to three decades in China (Chen *et al.*, 2011; Li *et al.*, 2014). Field trials have confirmed that these *Bt* rice lines can provide effective control over lepidopteran insects and significantly reduce the use of chemical insecticides. Extensive safety assessment studies on these *Bt* rice have shown that *Bt* rice poses a negligible risk to the environment and have a lesser impact on the environment when compared with present day practices (Huang *et al.*, 2005; Li *et al.*, 2014). Comprehensive assessment of food safety issues such as toxicity, allergenicity, digestibility and nutritional value has revealed that *Bt* rice and their products are as safe as non-*Bt* rice (He *et al.*, 2008; Xiao *et al.*, 2012). Furthermore, evaluation of the consequences of growing *Bt* rice lines have demonstrated an increase in the yield and profitability (High *et al.*, 2004). In addition, the adoption of *Bt* rice has demonstrated a significant improvement in farmers' health as a result of reduced insecticide use (Huang *et al.*, 2005; Huang *et al.*, 2015). Therefore, the development of insect-resistant genetically modified rice lines expressing *Bt* insecticidal proteins appears to be an attractive alternative for methods predominantly based on chemical insecticides. However, despite the evidence gathered and documented from recent research on environmental biosafety, food safety impacts as well as socioeconomic issues, and benefits of growing *Bt* rice, these transgenic rice lines have not been commercially planted to date (Li *et al.*, 2015). It is interesting to note that unlike *Bt* rice, *Bt* maize and *Bt* cotton, which also contain introduced genes encoding insecticidal *Bt* proteins have been cultivated extensively since 1996. The land area under transgenic crops has increased rapidly worldwide. It has been reported that in 2013 transgenic crops were grown on 175 million ha, of which 43 % were crop plants that expressed insect-resistant genes (James, 2013).

The transgenic approach of plant genetic engineering provides access to an unlimited gene pool for the transformation of desirable genes between any two species of interest, irrespective of their evolutionary or

taxonomic relationship. The important prerequisites for generation of transgenic plants are, positive response of cells in the target tissue for transformation and the capacity of those cells to regenerate into an intact plant (Hansen & Wright, 1999). The first reliable *Agrobacterium tumefaciens* mediated transformation and regeneration of transgenic rice was reported in 1994 for *japonica* rice (Hiei *et al.*, 1994). However, it is reported that *indica* rice varieties are often sensitive to tissue culture conditions and poorly responsive to transformation mediated by *A. tumefaciens* or other transformation methods (Zhang *et al.*, 1997; Zaidi *et al.*, 2006). Improvements to the transformation protocols and tissue culture techniques such as callus induction, somatic embryogenesis and regeneration have made *indica* rice amenable to *A. tumefaciens* mediated transformation, regeneration and recovery of intact transgenic rice plants (Aldemita & Hodges, 1996; Rashid *et al.*, 1996; Kumar *et al.*, 2005).

The research reported in this paper reveals the successful deployment of *A. tumefaciens* mediated transformation technology and *in vitro* tissue culture techniques for generating transgenic rice from a local rice variety Bg 94-1. Efficient and reproducible *Agrobacterium* mediated transformation and regeneration procedure developed in this study permitted the genetic transformation of Bg 94-1 with *cry2A* gene of *B. thuringiensis,* which conferred resistance against RLF larvae as demonstrated by the insect feeding assay of T_0 leaf tissues that revealed very high mortality within 3 – 5 days. Furthermore, the findings of this study also indicates the usefulness of *A. tumefaciens* mediated transformation technology as an effective tool to improve local rice with important traits like insect resistance.

METHODOLOGY

Agrobacterium tumefaciens strain and transformation vector

In this study, *A. tumefaciens* strain GV3101 harbouring the binary vector pCAMBIA 1305.1 was used to introduce insect resistance into the local rice variety, Bg 94-1. The transformation vector pCAMBIA 1305.1 containing the CaMV 35S d–cry2A–NOS construct was obtained from Prof. Illimar Altosaar, Department of Biochemistry, Faculty of Medicine, University of Ottawa, Ontario, Canada. The transformation vector pCAMBIA 1305.1 in its T-DNA region contained the modified *cry2A* gene (1.902 kb) coding sequence, which encodes the insecticidal protein of *Bt*. This particular *cry2A* gene has been modified to remove cryptic polyadenylation sites, aberrant splicing sequences and RNA instability motifs as well as to change in codon usage to fit plant codon preference (Cao *et al.*, 1999). This *cry2A* gene was placed under the control of double enhancer sequence of 35S cauliflower mosaic virus promoter (CaMV 35S d) and Nopaline synthase (NOS) termination sequence (Figure 1). The CaMV 35S d promoter is reported to cause significant increase in the transcriptional activity compared to CaMV 35S normal promoter (Kay *et al.*, 1987). A translation initiation context sequence (38 bp) of alfalfa mosaic virus (AMV), which is known to enhance translation of genes (Datla *et al.*, 1993) has been incorporated at the 5′ position of the *cry2A* gene (Figure 1).

In addition, pCAMBIA 1305.1 in its T-DNA region contained hygromycin phosphotransferase II (*HptII*),

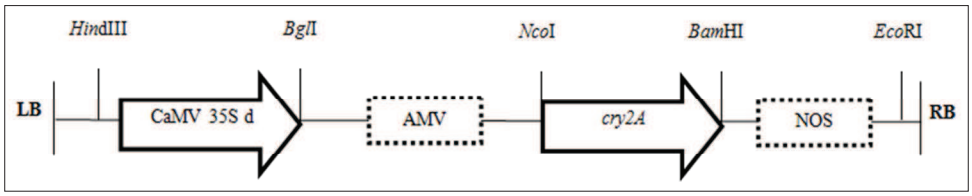

Figure 1: Schematic representation of the expression cassette. *cry2A* gene is driven by CaMV 35S d promoter and terminated by NOS terminator; translation initiation sequence, AMV placed between the promoter and *cry2A*. This expression cassette was cloned into *Hin*dIII and *Eco*RI sites of the transformation vector pCAMBIA 1305.1. LB and RB are left and right border of T-DNA.

and is used as the plant selectable marker gene, which was driven by CaMV 35S promoter and 35S terminator. GUSPlus (GUS: β-Glucuronidase) was used as the reporter gene, which was driven by CaMV 35S promoter and NOS terminator (Figure 2). The first intron of the castor bean *catalase* gene interrupts the *GUS*-coding sequence to ensure expression only after transfer into the plant cell.

The pCAMBIA 1305.1 transformation vector carrying the above mentioned *cry2A* transgene construct was transformed into *A. tumefaciens* strain GV3101 by

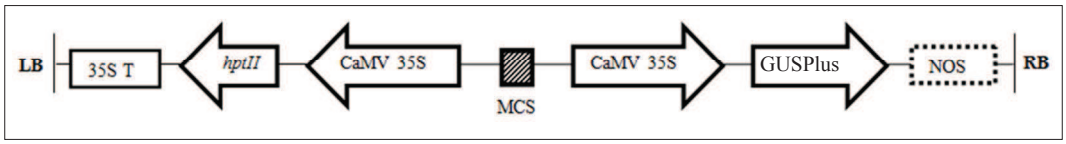

Figure 2: Schematic representation of the T-DNA region in pCAMBIA 1305.1. The selectable marker gene, *hptII* is driven by CaMV 35S promoter and 35S CaMV terminator (35S T). The reporter gene, GUSPlus is under the control of CaMV 35S promoter and NOS terminator. MCS: multiple cloning site; LB and RB are left and right border of T-DNA.

Table 1: Primer sequences used for amplification of *cry2A*, CaMV 35S d promoter and NOS terminator sequences

	Primer sequence (5´ -> 3´)	Expected fragment size (bp)	References
cry2A	F1: TCCCTCGACACCATCCAGAAG R1: GAGTAGAGGAACGGCCAGTTC	806	Zaidi *et al.*, 2005
CaMV 35S d	F1: CCACGTCTTCAAAGCAAGTGG R1: TCCTCTCCAAATGAAATGAAGTTC	123	Lipp *et al*., 2001
NOS	F1: GCATGACGTTATTTATGAGATGGG R1: GACACCGCGCGCGATAATTTATCC	119	Lipp *et al*., 2001

F1: forward primer; R1: reverse primer

freeze thaw method (Weigel & Glazebrook, 2006) and was selected on LB medium (10 g/L bactotryptone, 5 g/L bacto yeast, 10 g/L NaCl; pH 7.0) containing 25 µg/mL rifampicin and 50 mg/L kanamycin antibiotics. The presence of transformation vector carrying the *cry2A* transgene construct in *A. tumefaciens* strain GV3101 was confirmed by molecular analysis of isolated pCAMBIA 1305.1 DNA. This was achieved by performing *Hin*dIII and *Eco*R1 restriction enzyme digestion along with diagnostic PCR amplification using specific primers for *cry2A* transgene construct (Table 1).

*Agrobacterium tumefacien*s mediated transformation of rice embryogenic calli

Mature seeds of *O. sativa* L. subspecies *indica* variety Bg 94-1 were obtained from the Rice Research and Development Institute (RRDI), Bathalagoda, Ibbagamuwa, Sri Lanka. Seeds were dehusked manually, washed with water for several times and surface sterilised with 70 % (v/v) ethanol for 3 min, and 50 % sodium hypochlorite [Clorox™ (v/v)] with 2 – 3 drops of Tween-20 for 30 min, followed by several washes with sterile distilled water.

Embryogenic calli were initiated from the scutellum of mature rice seeds in the presence of 3 different plant hormone combinations in solid callus induction medium [CI/NB medium: N6 major and minor salts, B5 vitamins, 30 g/L maltose, 2 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D), 1 mg/L 6-benzylaminopurine (BAP), 1 mg/L 1-naphthaleneacetic acid (NAA), 4 g/L phytagel, 2 g/L agar; pH 5.8)]. Following surface sterilisation, seeds were transferred to the CI/NB medium and the cultures were incubated at 25 ºC in the dark. After 3 wks of callus growth, the proliferating calli were sub-cultured (the plumule, radical and the intact endosperm were excised from the seed) onto the same medium and were incubated for another 2 ds at 25 °C in the dark. Actively growing white harder calli appeared at this stage, and those calli were used for *Agrobacterium* infection. *A. tumefaciens* containing pCAMBIA 1305.1 transformation vector carrying *cry2A* transgene construct was grown in LB medium supplemented with 50 mg/L kanamycin and 25 µg/mL rifampicin as the selective antibiotics with continuous shaking for 48 h at 28 °C. A single well grown colony of *A. tumefaciens* strain GV3101 harbouring the *cry2A* transgene construct was inoculated onto 5 mL of LB medium supplemented with appropriate antibiotics and continuously shaken overnight on low speed (55 rpm) at 28 °C. Subsequently, the above cultures were inoculated into 50 mL pre-induction medium containing antibiotics and 100 µm acetosyringone. The cultures were incubated overnight with 55 rpm shaking at 28 °C, and used for transformation of embryogenic calli. *Agrobacterium* was grown to an optical density (OD) of 1.0 at 550 nm in liquid

pre-induction medium containing 10 g/L bactotryptone, 5 g/L bacto yeast, 10 g/L NaCl, appropriate antibiotics and 100 µM acetosyringone. Thereafter, the culture was spun at 3220 g for 10 min at 4 ºC and the pellet was re-suspended in 15 – 20 mL of liquid re-suspension medium (NB liquid medium containing 20 g/L sucrose, 10 g/L glucose and 100 µM acetosyringone; pH 5.6). The temperature in all bacterial cultures was maintained at 28 ºC in order to eliminate plasmid curing. The calli were immersed in bacterial suspension for 3 min and the excess bacterial suspension was removed by blotting on sterile tissue papers. The infected calli were transferred onto NB co-cultivation medium (CI/NB medium with plant hormones, 30 g/L maltose, 10 g/L glucose, 3 g/L phytagel, 100 µM acetosyringone; pH 5.6) and incubated at 25 °C for 48 h in dark. After co-cultivation, the infected calli were washed 4 – 5 times with sterile distilled water and finally with a dilution series of the antibiotic cefotaxime (1 g/L, 750 mg/L and 500 mg/L) in order to remove any traces of *Agrobacterium*. Then, the calli were blotted dry on sterile filter papers and transferred to callus induction medium devoid of hygromycin (NB callus induction medium containing 600 mg/L cefotaxime) and grown for 14 ds at 25 ºC in the dark imposing a resting period. Actively growing calli were observed at the end of the resting period. Scutellum derived embryogenic calli of mature Bg 94-1 seeds (untransformed) were used as the control throughout this experiment.

Selection and regeneration

Actively proliferating calli, which appeared creamy white in colour and hard in nature at the end of the 2 wks resting period were transferred to callus induction medium with selection (30 mg/L hygromycin) and grown for 1 – 5 wks at 25 °C. After the 1st wk of selection, brown necrotic areas on calli were removed and healthy calli segments (2 – 3 mm) were sub-cultured onto the same medium and grown in dark at 25 °C in the presence of hygromycin (30 mg/L). During 2nd to 5th rounds of selection with hygromycin, sub-culturing was done at weekly intervals in order to remove any necrotic areas on the calli. At the end of the 3rd round of selection, sub-culturing was done with the exposure of calli to continuous illumination. By the end of the 4th round of selection (28 – 35 ds) with hygromycin, the appearance of yellowish green regions was observed in some parts of the calli. Towards the end of the 5th round of selection (5th wk of incubation) green shoot primordia development was visible in the areas, which were in yellowish green colour. During the 5th round of selection the concentration of cefotaxime in the culture medium was lowered to 300 mg/L.

After the selection with hygromycin, actively growing calli segments with shoot primordia were transferred to shoot regeneration NB medium (CI/NB medium, 200 mg/L ascorbic acid, 100 mg/L sorbitol, 100 mg/L adenine sulfate, 100 mg/L cysteine, 30 g/L maltose, 500 mg/L cefotaxime; pH 5.8 – 6.0) containing 3 mg/L BAP and 1.5 mg/L NAA, and grown at 25 ºC under continuous illumination. Once well-developed shoots appeared, the intact shoots were transferred to fresh shoot regeneration medium with lower concentrations of cefotaxime (250 mg/L), and the cultures were incubated for shoot elongation. With the development of numerous shoots, shoot systems were sub-cultured onto the same fresh shoot regeneration NB medium in order to get single shoot systems, and further grown under continuous illumination. The concentration of cefotaxime in shoot regeneration media was lowered at each sub-culture step to eliminate negative effects on shoot regeneration. The regenerated shoots were transferred to MS rooting medium (MS major salts, MS minor salts, MS vitamins, 1 g/L ascorbic acid, 1.3 g/L $FeSO_4.7H_2O$, 1.68 g/L $EDTA.2H_2O$, 30 g/L sucrose, 8 g/L agar; pH 5.8) without plant hormones and cefotaxime, and grown until well developed roots appeared.

Acclimatisation of putatively transformed plants

With the development of roots, the plantlets were transferred to pots containing sterilised soil. The roots were washed in a fungicide solution (2.5 g/L 'shoot' carbendazim 50 % w/w) for a few minutes and transferred to pots with sterilised soil. The plants were grown under controlled environmental conditions. After 4 – 5 wks they were transferred to the greenhouse and grown until maturity. *In vitro* grown plants regenerated from untransformed scutellum derived calli of mature Bg 94-1 seeds were used as control plants in subsequent experiments.

Molecular analysis of putative primary transformants

Genomic DNA was isolated from putative primary transformants (T_0 generation) and untransformed control plants according to the method described by Dellaporta *et al.* (1983). Specific primer sequences were used to amplify a part of the gene cassette to screen the putative transformant lines (Table 1). The PCR reactions were performed as follows: hot start step at 94 ºC for 3 min followed by 35 cycles at 94 ºC for 1 min, 59 ºC for 1 min for *cry2A* (at 50 ºC for 30 s for CaMV 35S d and NOS), 72 ºC for 2 min and final extension at 72 ºC for 10 min. Fragment sizes of the amplified products were

confirmed using agarose gel electrophoresis. In these experiments, the DNA isolated from untransformed rice plants and pCAMBIA 1305.1 binary plasmid containing the transgene construct was used as the negative and positive controls, respectively.

GUS expression analysis

The expression of GUSPlus gene and the resulting GUS enzyme activity in embryogenic calli transformed with *cry2A* undergoing the 3rd round of selection were assayed following the GUS staining histochemical method described by Jefferson (1987). The GUSPlus gene contained a *catalase* intron, preventing expression in *A. tumefaciens* cells. The transformed calli were transferred to microfuge tubes (one callus per tube) containing 500 µL of fixative solution [0.3 % formaldehyde, 10 mM MES (2-*N*-morpholino ethanesulfonic acid) (pH 5.6), 0.3 M mannitol]. These calli were incubated at room temperature for 3 min. Following incubation, the fixative was removed and 500 µL of 50 mM Na_3PO_4 at pH 7.0 was added. The content was vacuum infiltered for 1 min, followed by removal of the phosphate buffer. Subsequently, the calli were washed thrice with 50 mM Na_3PO_4 at pH 7.0. After the removal of phosphate buffer, 200 µL of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-Glucuronide) was added and vacuum infiltered for 1 – 2 min. Afterwards, the tubes were incubated at 37 °C in dark for 3 ds. Following incubation, tissues were de-stained with 70 % ethanol and observed under the microscope. Untransformed calli were used as the negative control.

Analysis of T_1 transgenic lines

Selfed seeds from the primary transformants (PCR positive for the presence of *cry2A*) were collected to obtain T_1 generation of seeds. These seeds were dehusked and surface sterilised as mentioned earlier. The seeds were cultured on MS medium supplemented with 30 mg/L hygromycin and grown at 25 °C under continuous illumination until well-developed shoot and root systems appeared. The plants that were hygromycin resistant were selected, and potted in soil and transferred to greenhouse conditions. Plantlets were maintained under greenhouse conditions until maturity. To further confirm transgene integration, genomic DNA was isolated according to the method mentioned by Dellaporta *et al.* (1983) and PCR reactions were performed using primers specific for *cry2A*, CaMV 35S d promoter and NOS terminator (Table 1). The procedure adopted for PCR amplification was the same as the procedure used for T_0 generation transgenic rice plants described earlier.

Sequence analysis

To further validate the identity of the *cry2A* gene in transformant lines, amplified PCR products of *cry2A* were sequenced by an ABI cyclic sequencer. The resulted DNA sequences were searched against the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST), supported through the National Centre for Biotechnology Information (NCBI) (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*).

Insect bioassay

The insecticidal activity of 19 *cry2A* containing transgenic rice plants of T_0 generation towards the RLF larvae was assayed following a modified method described by Maqbool *et al.* (1998). In brief, 3 freshly cut leaf pieces (average size: 5 cm × 1.5 cm) from each 35 d old transgenic rice plants were placed on moist filter papers in a Petri plate. Similarly, as the negative control, 3 freshly cut leaf pieces of rice plants regenerated *in vitro* (without transformation) was used. Two leaf folder larvae in the 2nd instar stage were placed in each plate. The plates were incubated at 28 °C in light with 70 % relative humidity for 5 ds. At regular intervals up to the 5th day, the larvae were fed with leaves of both transgenic as well as control plants, and examined to determine the larval mortality and to evaluate the larval characteristics in terms of morphology and condition. Additionally, leaf segments were examined for any visible tissue damage.

Biosafety management

All the experiments with transgenic rice lines containing *cry2A* gene were carried out under contained environmental conditions in the laboratory and the greenhouse by following the guidelines as given in "Guidelines for the Safe use of Recombinant DNA Technology in the Laboratory" prepared by the National Science Foundation (2005). In order to prevent pollen transfer and insect attacks, rice plants were grown in an insect proof cage constructed using insect proof mesh (dimensions: 18 × 16 steel mesh/inch on its four sides). This cage was kept in the greenhouse. Plants were grown under greenhouse conditions until maturity.

RESULTS

Agrobacterium tumefaciens mediated transformation, selection and regeneration of primary transformants

DNA isolated from pCAMBIA 1305.1 binary plasmids containing *cry2A* transgene construct, which was transformed into *A. tumefaciens* strain GV3101 were

subjected to molecular analysis by diagnostic *Hin*dIII and *Eco*RI restriction digestion. This showed a 2810 bp band for *cry2A* gene cassette and a 11849 bp band for the pCAMBIA 1305.1 backbone (data not shown). Additionally, PCR analysis using *cry2A* gene specific primers resulted in the expected 806 bp amplified band (data not shown). This analysis confirmed the presence of pCAMBIA 1305.1 transformation vectors in *A. tumefaciens* strain GV3101, which were used in subsequent transformation studies.

The establishment of embryogenic calli is a prerequisite for successful transformation and subsequent regeneration of intact transgenic rice plants. Embryogenic calli derived from the scutellum of mature Bg 94-1 seeds were employed as explants tissue (Figure 3a). A total of 512 calli segments were transformed with *Agrobacterium* harbouring pCAMBIA 1305.1 binary plasmid. Following transformation, the calli were incubated in callus induction medium in the dark for 2 weeks without hygromycin selection (Figure 3b). After 2 weeks of resting period, transformed calli were allowed to grow and subjected to selection in callus induction medium containing 30 mg/L hygromycin for 4 – 5 weeks along with 4 to 5 rounds of selection. The necrotic regions of calli, which appeared in dark brown colour (Figure 3c) were removed while preserving the proliferating live calli. These proliferating calli appeared in creamy white colour. At the end of the 3rd round of selection when the calli were exposed to continuous illumination, green regions were visible in some parts of the calli (Figure 3c) from which shoot primordia emerged during the 4th and 5th rounds of selection (Figure 3d). Intact shoots were regenerated when the calli were transferred to shoot regeneration medium, followed by root development (Figure 3e). The rooted plants were transferred to pots containing sterilised soil, and were acclimatised and established under greenhouse conditions (Figure 3f).

Following the tissue culture manipulations and *in vitro* regeneration techniques described in this study, a total of 78 hygromycin resistant calli were selected from a total of 512 scutellum derived callus segments subjected for transformation. It was observed that these selected calli have been developed from different callus segments or well separated regions of individual callus. These 78 callus lines, therefore, could be considered most likely to be the result of independent *A. tumefaciens* mediated transformation events. These selected actively growing callus lines, when further cultured, started to develop shoot primordia subsequently giving rise to developed shoot systems as described above.

A total of 110 putative transformed plants were regenerated and recovered from the 78 hygromycin

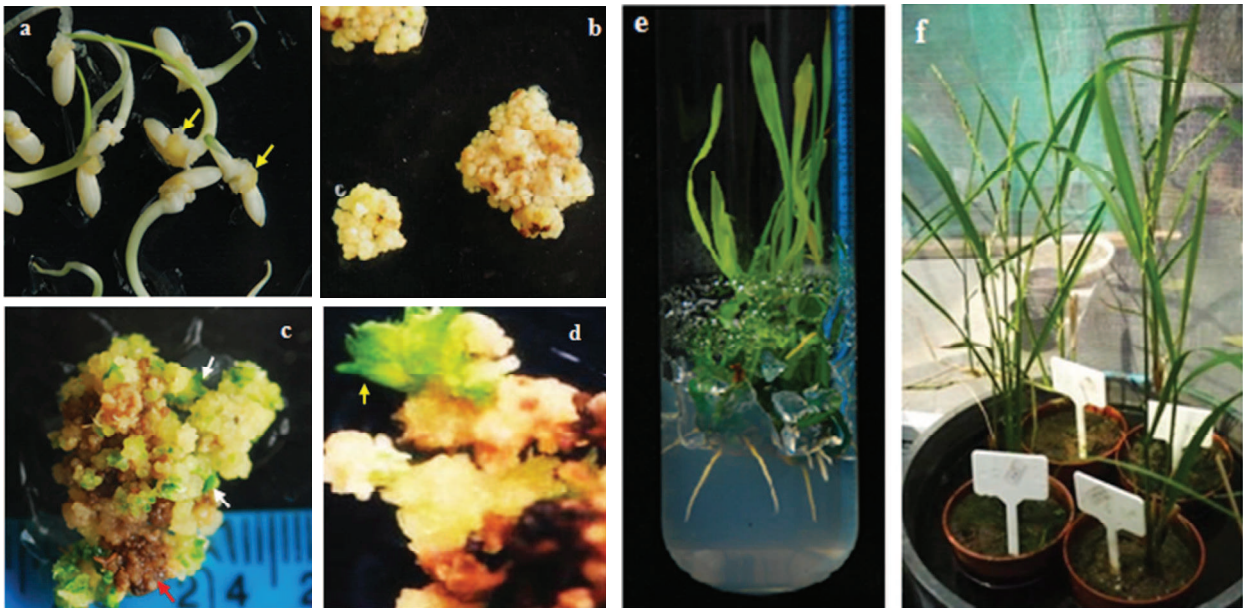

Figure 3: *A. tumefaciens* mediated transformation of Bg 94-1. a - scutellum derived embryogenic calli (yellow arrows); b - callus proliferation after transformation (without hygromycin selection); c - hygromycin (30 mg/L) resistant calli. Necrotic and green regions are indicated by red and white coloured arrows, respectively; d - development of shoot primordia from transformed calli (yellow arrow); e - transformed plants in rooting medium; f - regenerated plantlets under greenhouse conditions

resistant calli lines of Bg 94-1 transformed with CaMV 35S d–cry2A transgene construct. On an average 1.4 putative transformed plants were regenerated from each callus line. However, it was observed that there was notable difference in the regeneration capacity among different callus lines.

Molecular analysis of putative primary transformants (T_0 generation lines) for transgene integration of *cry2A* gene

Transgene integration of *cry2A* into the Bg 94-1 genome and subsequent identification of primary transformants was performed by PCR analysis of DNA isolated from putative transformants lines, employing specific primers for CaMV 35S promoter, *cry2A* gene and NOS terminator (Table 1). The amplified products obtained for primary transformant plants transformed with *cry2A* transgene construct is shown in Figure 4. In all PCR analyses, pCAMBIA 1305.1 plasmid DNA of *A. tumefaciens* with *cry2A* transgene construct was used as the positive control and this resulted in an 806 bp amplified product for *cry2A* (data not shown). The genomic DNA of non-transgenic untransformed Bg 94-1 rice plants regenerated *in vitro* was used as the negative control.

Sixty (60) putative transformed plants (out of 110) regenerated from 60 different callus lines were selected for further molecular analysis. The expected amplified band of 806 bp for *cry2A* (Figure 4) was detected in 19 out of 60 putative transformed plants. However, no such bands were observed for the untransformed control plants and water control under identical conditions. Furthermore, PCR analysis revealed the expected amplified bands of 123 bp for CaMV 35S promoter region and 119 bp for NOS terminator for all 19 *cry2A* positive primary transformants (Figure 4). This further increased the validity of the results obtained.

Histochemical analysis of GUS activity in putatively transformed callus tissues

The pCAMBIA 1305.1 transformation vector as shown in Figure 2 contains GUSPlus reporter gene, in which the *GUS* coding sequence is interrupted by the *catalase* intron. The expression of intron containing *GUS* gene (intron-GUS) in plant cells results in GUS enzyme activity, which can be assayed by a histochemical staining assay. In this analysis, calli obtained from the 3rd round of hygromycin selection showed blue spots in some sectors of the transformed calli when assayed for GUS activity during incubation with GUS substrate for 3 days. The calli at this stage of selection as mentioned earlier consisted of actively proliferating sectors, which appeared in creamy white colour and necrotic browning sectors. On close examination it was revealed that actively proliferating calli were stained with blue colour (Figure 5a), indicating possible intron-GUS gene transfer in those calli cells and subsequent GUS enzyme activity. In contrast, the untransformed calli sectors which appeared in brown colour did not get stained to give the characteristic blue colour. Additionally, untransformed control calli which were subjected to the exact same

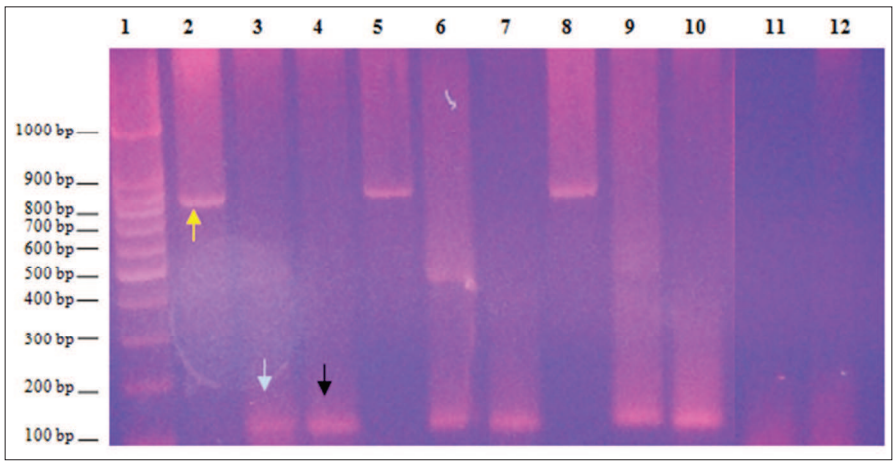

Figure 4: PCR analysis of putative primary transformants of Bg 94 - 1. Lane 1: 100 bp ladder; Lane 2 - 4: putative transgenic line 1; Lane 5 - 7: putative transgenic line 2; Lane 8 - 10: putative transgenic line 3; Lane 11: untransformed control plant; Lane 12: negative control (water). Yellow, blue and black arrows denote amplified products of *cry2A* (≈ 806 bp), CaMV 35S d promoter (≈ 123 bp) and NOS terminator (≈ 119 bp), respectively.

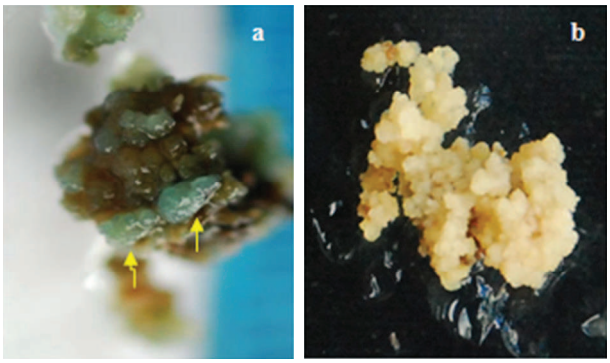

Figure 5: *GUS* expression in transformed calli segments. a - histochemical staining of transformed calli (yellow arrows); b - untransformed calli

treatment during GUS staining did not show any blue colour (Figure 5b).

Inheritance of *cry2A* transgene into the T_1 generation of rice plants

In order to analyse the inheritance of *cry2A* transgene, selfed seeds collected from T_0 transgenic plants were germinated in the presence of hygromycin (30 mg/L). The resulting hygromycin resistant plant DNA was tested through PCR to confirm the presence of *cry2A* transgene using the primers designed for CaMV 35S, NOS terminator, and *cry2A* (Figures 6a and 6b). Hygromycin resistance and PCR analysis of T_1 plants indicated the stable integration of hygromycin resistance gene (*HptII*) and *cry2A* transgene into the Bg 94-1 genome.

To establish the definitive transgenic status of *cry2A* transformed rice plants, genomic DNA of T_1 generation lines was subjected to PCR amplification using *cry2A* gene specific primers, and the resulted amplified product was sequenced using the ABI cyclic sequencer. The 806 bp long DNA sequence obtained was further analysed through the NCBI BLAST search, which revealed 100 % similarity with the reference sequence, EU109565.1-Synthetic construct insecticidal *cry2Aa* delta-endotoxin gene, complete cds 9 (data not shown).

Determination of insecticidal activity in transgenic rice plant tissues *via* an insect bioassay

Results of the insect bioassay revealed that there was less damage caused by the RLF larvae feeding on leaf pieces taken from all nineteen (19) *cry2A* transgenic plants, compared to leaf pieces taken from the control plants. In the untransformed control plants, larvae fed extensively, were healthy and developed normally causing severe damage to leaf tissues during the bioassay period (Figures 7a – 7c). The leaf pieces demonstrated the typical symptoms of leaf folder feeding such as membraneous, whitish and withered regions in the leaf blade. It was noted that the appearance of these detached leaves of the control plants are quite similar to the symptoms demonstrated by leaf folder larvae infested rice plants under field conditions.

On transgenic plants, there were only a very few surviving larvae after 3 days. The larvae that survived up to 3 days appeared to have undergone deformation and showed symptoms such as browning (Figure 7d). After

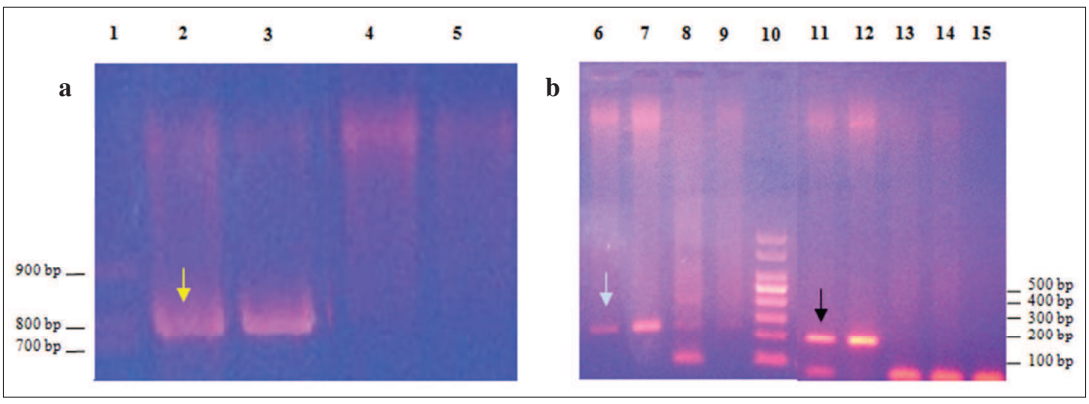

Figure 6: PCR analysis of T_1 generation transgenic rice lines. (a) - amplification of *cry2A*; (b) - amplification of CaMV 35S d promoter and NOS terminator regions. Lane 1 and 10: 100 bp ladder; Lane 2, 6 and 11: T_1 transgenic line 1; Lane 3, 7 and 12: T_1 transgenic line 2; Lane 4, 8, 9, 13 and 14: negative control (untransformed control plant DNA); Lane 6 and 15: negative control (water). Yellow, blue and black arrows denote amplified products of *cry2A* (≈806 bp), CaMV 35S d promoter (≈123 bp) and NOS terminator (≈119 bp), respectively.

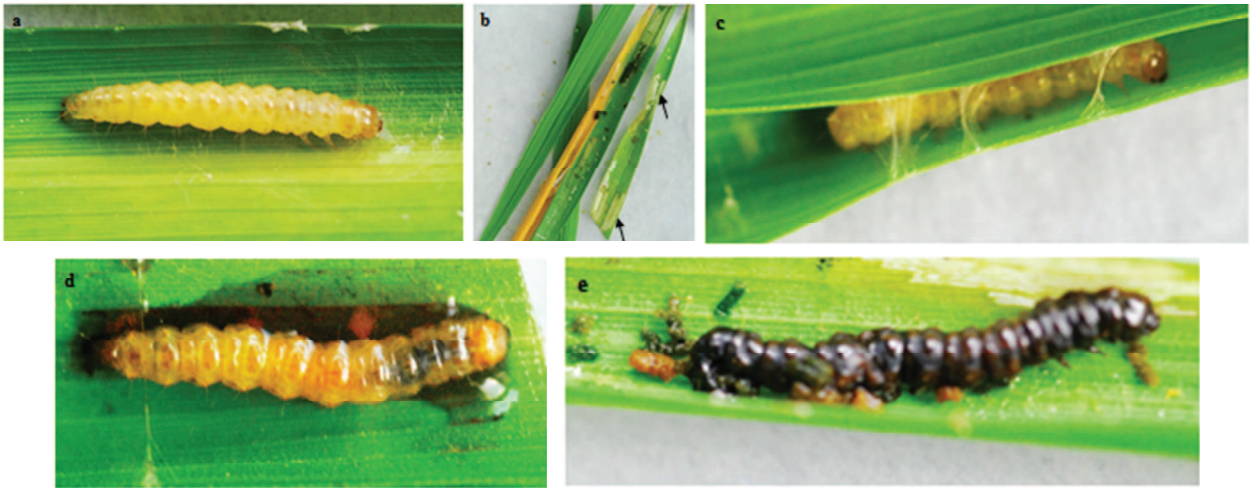

Figure 7: Insect feeding bioassay for T_0 generation transgenic rice lines expressing *cry2A*. a - RLF larva; b - untransformed control plant leaves with typical RLF infestation symptoms [membraneous and whitish leaf blades (black arrows)]; c - folded leaf blade (control leaves); d - RLF larva after 3 days of feeding on transgenic plant material; e - dead RLF larva on leaves expressing *cry2A*

Table 2: Mortality of RLF larvae feeding on leaf pieces of T_0 generation transgenic plants transformed with *cry2A* gene

Rice line	Average larval mortality after 3 days of feeding (%)	Average larval mortality after 5 days of feeding (%)
Control (untransformed)	0	0
cry2A lines: 1-2, 4-7, 9-11, 13-15, 17-19	100	100
cry2A lines: 3, 8, 12, 16	50	100

3 days of feeding the average larval mortality was 89 % and by day 5 the larval mortality reached 100 % (Table 2; Figure 7e). However, on leaves taken from control plants all the larvae were alive resulting in 0 % mortality.

DISCUSSION

The local rice variety, Bg 94-1 used in the present study is a very popular cultivar grown in many parts of the country particularly in the North Western region of Sri Lanka. This variety, most likely due to the lack of natural resistance is found to be susceptible to RLF outbreaks at all stages of plant growth (Dhanapala & Claridge, 1989). Bg 94-1, therefore was selected and successfully transformed with *cry2A* gene of *Bt* in order to confer resistance against the RLF. Transgenic Bg 94-1 lines expressing *cry2A* gene were generated by employing *A. tumefaciens* mediated transformation protocol and *in vitro* tissue culture techniques developed and optimised in our laboratory.

Scutellum derived embryogenic calli initiated from the mature seeds of *japonica* and *indica* rice are considered as excellent source of starting material for *A. tumefaciens* mediated transformation (Hiei *et al.*, 1994; Rashid *et al.*, 1996). Therefore in the present study, three-week old embryogenic calli derived from the scutellum of mature rice seeds of Bg 94-1 were used to introduce *cry2A* gene driven by CaMV 35S d promoter into Bg 94-1 rice variety through an optimised *A. tumefaciens* mediated transformation method. A total of 512 individual segments of calli were transformed with *A. tumefaciens* strain GV3101 carrying pCAMBIA 1305.1, which contained the CaMV 35S d-cry2A transgene construct in its T-DNA region. In this study, 78 hygromycin resistant calli were selected, that developed from different regions of individual calli segments or from well separated regions of the same calli. Therefore, these calli lines are considered to be the result of independent *A. tumefaciens* mediated transformation events. Furthermore, a total of 110 intact putative transformants of rice (T_0 generation

of plants) were generated from these 78 hygromycin resistant calli lines by following *in vitro* regeneration techniques described in this paper.

Successful amplification of different regions of the *cry2A* transgene construct of transformed plants indicates that when transferred, it is very likely that the transgene construct remained intact when integrated to the genome of Bg 94-1 rice plants. This analysis also revealed a transformation efficiency of 32 % for *cry2A* transformed Bg 94-1 plants. The results obtained in the present study demonstrate clearly the feasibility and efficiency of *A. tumefaciens* mediated transformation and *in vitro* regeneration techniques employed in producing transgenic Bg 94-1 rice carrying *cry2A*. Moreover, the results obtained are comparable to the results reported on development of insect resistant transgenic rice (Cheng *et al.*, 1998; Ramesh *et al.*, 2004).

The presence of blue colour observed during GUS staining in large sectors of transformed calli undergoing the third round of selection suggests that the T-DNA in the PCAMBIA 1305.1 has been transferred to many calli cells during *A. tumefaciens* infection and co-cultivation. The incorporation of 100 µM acetosyringone in induction and co-cultivation medium as the *vir* inducer is likely to promote the initiation of transfer of T-DNA to many calli cells during the early part of co-cultivation as reported in other studies (Hiei *et al.*, 1994; Ramesh *et al.*, 2004). The intron-GUS gene that was used in this study is considered as an excellent reporter gene since it is expressed only when it is transferred to plant cells but not in *A. tumefaciens* that attach to the callus tissue (Roberts *et al.*, 1998). *GUS* expression analysis confirms the observation that embryogenic calli cells are good target cells for *A. tumefaciens* meditated transformation of rice.

In order to establish the definitive transgenic status of the primary transformants of Bg 94-1 containing *cry2A*, the stable inheritance of transgene through seeds was examined in T_1 individuals. The seeds collected from T_0 plants were germinated in the presence of hygromycin and initially screened for hygromycin resistance. T_1 individuals showing resistance to hygromycin when analysed for the presence of *cry2A* gene by PCR analysis revealed expected amplified fragments of *cry2A*, CaMV 35S d promoter and NOS terminator. The results obtained for T_0 plants on the presence of transgenes, taken together with the results obtained for T_1 plants clearly demonstrated stable integration of transgenes into Bg 94-1 genome of T_0 plants and subsequent inheritance of transgenes in T_1 generation plants. In addition, nucleotide sequencing of the introduced *cry2A* coding region of Bg 94-1 belonging to T_1 generation individual plant, and the sequence similarity observed when compared with the respective reference sequence confirmed and validated the identity of stably integrated *cry2A* gene in Bg 94-1 genome clearly establishing its transgenic nature. When all the results are considered together, it demonstrated agreement with the criteria suggested as proof of stable transformation mediated by *A. tumefaciens* (Potrykus, 1991; Langridge *et al.*, 1992).

The insecticidal activity of transgenic Bg 94-1 rice plants expressing *cry2A* gene was determined by an insect feeding bioassay using detached rice leaves and RLF larvae on the PCR positive T_0 plants and control plants. It was clearly observed that the RLF larvae fed on the leaves of transgenic rice lines caused limited damage to tissues. These larvae did not grow and suffered from severe deleterious effects showing typical symptoms of browning, and began to die 2 days after feeding, reaching 100 % mortality after 5 days. In comparison, the RLF larvae fed on untransformed tissues or control plants caused extensive damage to leaf tissues and grew well; there was no larval mortality. These larvae exhibited typical RLF feeding habits similar to what was observed in RLF infested rice fields. In addition, the larvae feeding on untransformed leaves spun the rice leaves longitudinally into a roll by stitching together the opposite edges of the rice leaf to give the characteristic leaf roll. Although these initial observations certainly require further investigation, findings of this study clearly suggest that the transgenic Bg 94-1 plants possess a high degree of resistance to RLF larvae. This observed resistance to RLF larvae is likely a resultant of enhanced expression of *cry2A* in transgenic Bg 94-1 plants, which could be attributed to several modifications devised into the transgene construct. These modifications include the use of double enhancer sequence of 35S CaMV promoter for increased transcription (Kay *et al.*, 1987), improvements done to enhance translation efficiency, which comprise incorporation of AMV RNA4 (AMV leader) to act as a *cis*-active translational activator (Datla *et al.*, 1993), and modification of codon usage of *cry2A* to match the codon preference in plants (Perlak *et al.*, 1991; Fujimoto *et al.*, 1993; Cao *et al.*, 1999).

With the experience gathered since 1996 from successful commercialisation of *Bt* maize (Koziel *et al.*, 1993) and *Bt* cotton (Perlak *et al.*, 1991), the development of insect resistance in rice by expression of insecticidal *cry* genes was considered to be immensely important for establishing a suitable insect management strategy for rice (Chen *et al.*, 2011).

Several transgenic rice lines expressing *Bt* genes and displaying high resistance against lepidopteran pests have been developed over the past 15 years by Chinese scientists (Chen *et al.*, 2011; Li *et al.*, 2015). These *Bt* rice lines have been subjected to extensive laboratory and field tests for assessing their environmental and food safety impacts, and evaluating their socio-economic consequences. Analysis of the results of these studies very clearly highlights two salient features; planting of *Bt* rice can reduce application of insecticides by 50 – 60 % compared to non-*Bt* rice, and *Bt* rice could increase rice yield by 60 – 65 % compared with non-*Bt* rice when no insecticide was applied (Huang *et al.*, 2015; Li *et al.*, 2015).

Paddy farmers in Asia including Sri Lanka spray highly toxic insecticides such as pyrethroid, organophosphate, carbamates and neonicotinoid in early season as a response to initial visual symptoms of leaf folder damage (Heong & Schoenly, 1998; Escalada *et al.*, 1999). This traditional management of RLF pests by spraying highly toxic insecticides done in the first 40 days is reported to cause reduction in the population of natural enemies of the planthopper (Matteson, 2000; Lou *et al.*, 2013), resulting in an increase in vulnerability of paddy crop by 10-fold to invading planthoppers especially brown planthoppers [*Nilaparvata lugens* (Sta l)], the main sap-sucking insect pest of rice, which causes significant annual reduction in the rice yield (Heinrichs, 1979; Sogawa *et al.*, 2003).

Recently, Han *et al.* (2014) have reported an important finding revealing that *Bt* rice expressing *cry2A* while exhibiting high resistance against leaffolders, does not cause any detrimental effects to the non-target organism *Cyrtorhinus lividipennis*, the major predator of eggs and young nymphs of planthoppers. Therefore, *C. lividipennis* is permitted to control the population density of brown planthoppers in rice fields. This highlights the possibility that *Bt* rice could provide an effective and environmentally friendly alternative method to reduce the use of toxic insecticides and control lepidopteran pests, while protecting the natural enemy of brown planthoppers in rice fields. This strategy highlights the fact that adoption of *Bt* rice can effectively bring about decrease in insecticide use while promoting biocontrol services of natural enemies of pests.

The overall results clearly demonstrate the efficacy of *A. tumefaciens* mediated transformation method and *in vitro* tissue culture techniques developed and optimised in this study for generating transgenic rice from a local rice variety Bg 94-1. In addition, the results also showed that transgenic Bg 94-1 containing *cry2A* insecticidal gene displayed sufficient resistance to RLF larvae, which warrants further investigation. These results also highlight the possibility that *Bt* rice could provide an effective, efficient and environmentally friendly alternative method to reduce the use of toxic insecticides and control RLF.

CONCLUSION

The local rice variety, Bg 94-1 was transformed with *cry2A* gene *via Agrobacterium* mediated genetic transformation. The findings of molecular screening and insect bioassay experiments provide evidence that the genetically transformed Bg 94-1 with *cry2A* gene of *Bt* has an enhanced resistance to RLF. In conclusion, this study provides a significant step in the development of transgenic rice lines effective against RLF in Sri Lanka.

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