

TOBACCO (*NICOTIANA BENTHAMIANA* DOMIN) PLANT TRANSFORMATION WITH AND WITHOUT SELECTABLE MARKER GENE FOR MULTIPLE VIRUS RESISTANCE

Kassa Getu

Era Agri-Link PLC, PO Box 22927, Addis Ababa, Ethiopia. E-mail: Kassagetu@yahoo.com

ABSTRACT: Transformation of tobacco plants (*Nicotiana benthamiana* Domin), containing non-translatable coat protein (CP) gene fragments of *Potato virus Y* (PVY), *Potato leafroll virus* (PLRV) and *Potato virus X* (PVX) with and without selectable marker gene was conducted using *Agrobacterium*-mediated transformation. Preliminary identifications of the transformants were done based on the expression of the reporter gene, Beta-Glucuronidase (*GUS*) gene. *GUS* activity test was conducted in the first (T0) generation while the plants were in test tubes and after transferring to a greenhouse for marker-free and marker-aided transformations, respectively. In the transformation with marker gene, three lines, which later on (in T1 generation) became virus resistant and PCR-positive for the insert, were generated from *GUS*-negative mother plants. Although regeneration of plants from marker-free transformation was relatively easy, it was not possible to get transgenic plants using histochemical *GUS* staining selection system.

Key words/phrases: Beta-Glucuronidase gene, potato leafroll virus, potato virus X, potato virus Y, tobacco (*Nicotiana benthamiana*)

INTRODUCTION

Protection of plants from viral diseases has often been a difficult task unless there exists a source of natural resistant gene within a species to be used for gene introgression. Hence, pathogen-derived resistance (PDR), which has also been known by the names “parasite-derived resistance”, “non-conventional protection”, “transgenic resistance” and “engineering resistance”, has attracted major interest and is the main one by which transgenic protection is being produced against viruses in plants since mid-1980’s (Hull, 2002). PDR was first demonstrated on tobacco plant expressing the coat protein gene of the *Tobacco mosaic virus* (TMV), whereby the transgenic plants delayed the development of the disease (Powell-Abel *et al.*, 1986). This approach has also been tried for *Potato virus Y* (PVY) (Lindbo *et al.*, 1993; Farinelli and Malnoe, 1994; Han *et al.*, 1999), *Potato leafroll virus* (PLRV) (Herbers *et al.*, 1997; Lee *et al.*, 2002) and *Potato virus X* (PVX) (Braun and Hemenway, 1992; Angell and Baulcombe, 1997; Kobayashi *et al.*, 2001).

The two principal means by which PDR has been induced are protein and RNA mediated resistance. At first during the discovery of this

mechanism, researchers had been using full gene(s) and the resulting resistance to viruses was attributed to the expression of integrated genes. Later on, however, Lindbo and Dougherty (1992) showed that untranslatable CP fragment from *Tobacco etch virus* (TEV) conferred resistance to plants against TEV infection. Van der Vlugt *et al.* (1992) also showed sense RNA-mediated protection to PVY in tobacco plants transformed with the viral coat protein cistron.

Transformation is a very rare phenomenon, and hence, in order to increase the chance of recovering the real transformants from a large pool of untransformed cells, dominant selectable marker genes coding usually antibiotic or herbicide resistance have been used (Bevan *et al.*, 1983; de Vetten *et al.*, 2003). However, there has been a growing concern from consumers and environmentalist that the incorporation of genes coding antibiotic or herbicide resistance might have negative impacts on human or animal health and on biodiversity. Moreover, these genes generally have negative effects on proliferation and differentiation of cells. Therefore, gene transfer without the incorporation of antibiotic and/or herbicide-resistance genes should ease public concerns over the field release

of transgenic organisms expressing traits of interest (Ebinuma *et al.*, 1997; de Vetten *et al.*, 2003). Accordingly, attempts to develop marker-free plants have been employed in different crops such as rice (Lu *et al.*, 2001), tobacco (Sugita *et al.*, 2000), potato and cassava (de Vetten *et al.*, 2003).

The concerns have not been limited to the selectable marker genes but also to the gene of interest, though the putative negative impacts of such transgenic plants have not been scientifically proved. In the case of transgenic virus resistance, the current focus is, therefore, on RNA-mediated resistance, which alleviate at least some of the putative concerns associated to protein-mediated protection (Puchta, 2003). RNA-mediated resistance was our choice in this study too. The mechanism in RNA-mediated virus resistance is believed to function in post-transcriptional gene silencing (PTGS), which represents a novel cellular pathway conserved in a diverse group of organisms (Ding, 2000). According to the same author, the mechanism has been named as RNA silencing since it involves a homology-dependent RNA degradation. In plants, gene silencing refers to a natural phenomenon *i.e.*, a genetic control mechanism, which is manifested in virus resistance (Ratcliff *et al.*, 1997; Covey *et al.*, 1997). It can be induced by pathogens such as virus and hence the name virus-induced gene silencing, VIGS, (Ratcliff *et al.*, 1999). Viruses are potentially initiators and targets of gene silencing at the same time.

Crops/plants are usually infected with multiple pathogens under field conditions. Therefore, in order to increase durability of crops for production, development of varieties with multiple pathogen infections has been a wise approach. Tobacco, as a model crop and as one of the cash crops, can be infected with multiple viruses. The hypothesis in this study was that multiple virus resistance could be integrated into the test plant and hence it would be possible (a) to reduce resources and time spent in research to tackle every virus disease separately and (b) to increase field resistance. This paper describes the engineering and introduction of a construct derived from PVY, PVX and PLRV truncated coat protein gene, which was fused with *GUS* reporter gene into Tobacco plants. The objective was to

determine the outcome of the construct on enhancing resistance to viral infections (PVY, PVX and PLRV).

MATERIALS AND METHODS

Plant material

Leaf explants of a tobacco plant (*Nicotiana benthamiana* Domin) were collected from three-month-old seedlings for transformation with the DNA construct mentioned below.

Coat protein genes construction and Agrobacterium transformation

A DNA construct (Fig. 1) composed of double 35S promoter from Cauliflower mosaic virus (CaMV), β -Glucuronidase (*GUS*) gene, and fragments of coat protein genes from the three viruses and a termination sequence from pCaMV (polyadenylation signal from CaMV) gene were used as a transformant. For the transformation with selectable marker gene, the construct was inserted in a binary vector pLX_222, which has *nptII* gene in the T-DNA region for kanamycin resistance (Landsmann *et al.*, 1988). The same construct was also cloned in pGreen0000 binary vector, which is devoid of selectable marker gene (Hellens *et al.*, 2000) and was supported by a helper vector, pSoup. The two binary vectors carrying *GUS_PPX* (PVY, PLRV and PVX) construct were used to transform *Agrobacterium tumefaciens* strain LBA4404 by electroporation.

Plant transformation and regeneration

The transformed bacteria were then used to transform a tobacco plant (*Nicotiana benthamiana* Domin) using leaf-disc transformation method (Varrelmann, 1999). For the transformation with selectable marker gene, MS-medium supplemented with kanamycin and growth hormones (IAA and BAP) was used to selectively regenerate transformed plantlets. The same procedure was followed in marker-free transformation with the exception of adding the selection agent, kanamycin, in the MS-media. Regenerated plants were transferred to a greenhouse for virus resistance test and seed production.

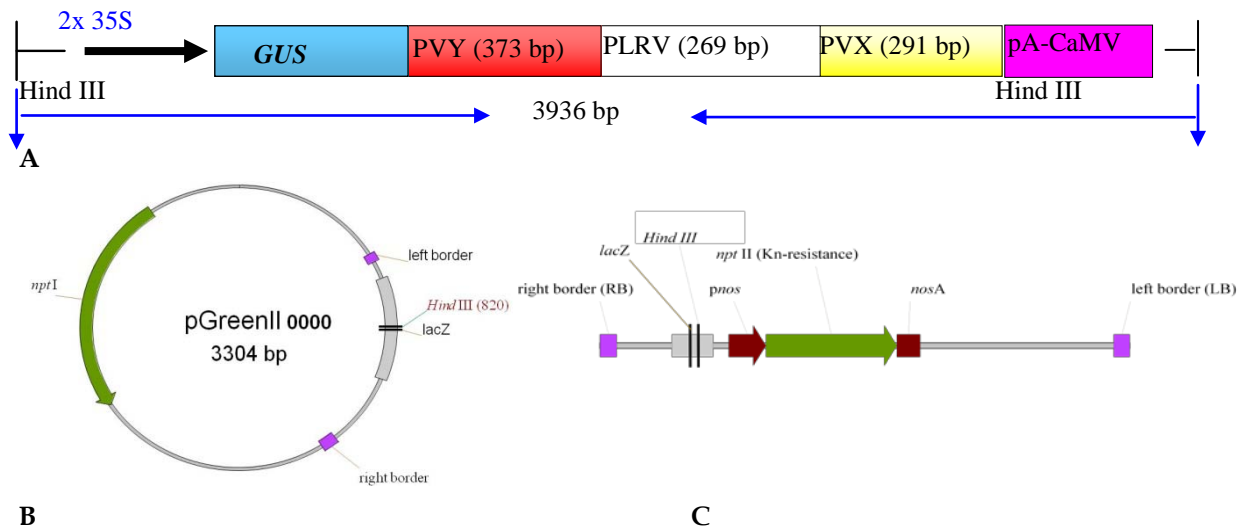


Figure 1. A) The *GUS_PPX* construct (35S= promoter from CaMV; *GUS*= β -Glucuronidase gene; *PVY*= Coat protein (CP) gene fragment from *Potato virus Y*; *PLRV*= CP gene fragment from *Potato leafroll virus*; *PVX*= CP gene fragment from *Potato virus X*, pA-CaMV= Polyadenylation signal from CaMV); B) *pGreenII0000* binary vector used for marker free transformation in combination with a helper vector, *pSoup*; C) *pLX222RB-LB* binary vector used for transformation with selectable marker gene.

Characterization of transgenic lines

In the identification of transgenic mother plants among the regenerated ones, enzymatic method *i.e.*, histochemical *GUS* staining test (Jefferson *et al.*, 1987) was employed after acclimatization in a greenhouse and while plantlets were in test tubes for transformations with and without marker gene, respectively. However, confirmation was done by polymerase chain reaction (PCR) only for transformation with marker gene. Since plants that did not express the *GUS* gene frangement-sequence, the presence of the *GUS* gene was checked by PCR. For this purpose, a pair of primers (5'ACGCGTCGACCGAG-CAACTCAATCACAGTT3' and 5' CCCTTATCTGGGAAC-TACTCACAC 3') was used in order to amplify the viral gene fragments. Another pair of primers (5'GCAAGTCAAGATGTCCA-TGGTACG 3') and anti-sense (5' GCGATGGATT-CCGGCATAGTTA 3') were used to amplify the *GUS* gene. Plant DNA samples for PCR analyses were isolated according to Edwards *et al.* (1991). In the DNA electrophoresis, lambda phage DNA digested with *Pst*I (Al Abdellah, 2002) was used as a ladder.

Virus Inoculation and plant resistant test

In the preliminary disease resistance test, three kinds of viruses; namely, *PVY* (strain *PVY* 15), *PVX* (strain *PVX* 0018) and *PLRV* (Full-length clone in bacteria, clone 28) were used for inoculation of transgenic plants. Plants at the age of 40 days

after transplanting were used for inoculation. *PVY* and *PVX* were inoculated mechanically, whereas *PLRV* inoculation was conducted by agro-infiltration using *Agrobacterium tumefaciens* strain ATHV. Evaluations of disease resistance were conducted visually, tissue print technique (Katul, 1992) and DAS-ELISA (Casper and Mayer, 1981) methods. The OD readings were analysed according to the method developed by Rek (1987). Anti- *PLRV* and *PVY* antibodies from Loewe Phytodiagnostica GmbH were used in the DAS-ELISA.

RESULTS

Transformation with marker gene (*GUS_PPX* in *pLX_222* vector)

Histochemical *GUS* staining test on mother plants (*T0*)

Sixteen plants were regenerated from transformation with *GUS_PPX* construct, which was in *pLX_222* vector. Eleven out of the sixteen mother plants were *GUS* positive except 278/11, which showed negative response in the first test. In the leaf age comparison for *GUS* activity, only 278/1 and 278/2 were negative in their lower leaves. Line 278/5 was *GUS* positive only once; all the next subsequent three tests showed that this line was *GUS* negative. In general, there were five *GUS* negative plants, which comprised 31% of the total regenerated plants (Table 1).

Table 1. Summarized results of histochemical GUS staining on mother plants of 278 construct.

Line No.	Series of GUS test					Leaf age comparison		Summary
	1*	2	3	4	5	Younger	Older	
278/1	+	+	+	+	+	-	+	+
278/2	+	+	+	+	+	-	+	+
278/3	+	+	+	+	+	+	+	+
278/4	+			+	+	+	+	+
278/5	+			-	-	-	-	-
278/6	-	-	-	-	-	-	-	-
278/7	+			+	+	+	+	+
278/8	+			+	+	+	+	+
278/9	-	-	-	-	-	-	-	-
278/10		+		+	+	+	+	+
278/11		-	+	+	+	+	+	+
278/12		-	-	-	-	-	-	-
278/13				-	-	-	-	-
278/14				+	+	+	+	+
278/15				+	+	+	+	+
278/16					+			+

Note: - and + refer to GUS negative and GUS positive, respectively; *1-5 refer to the series of tests, which had no fixed time interval.

PCR analyses on the mother plants (T₀)

PCR analysis of the two groups of plants showed that all plants contained both the viral gene fragments; the expected 1071 bp virus se

quence was amplified from all plants (Fig. 2). Thereby, it was found that all GUS negative plants possessed the gene (Fig. 3). The expected 531 bp *GUS* gene fragment was amplified from the plants.

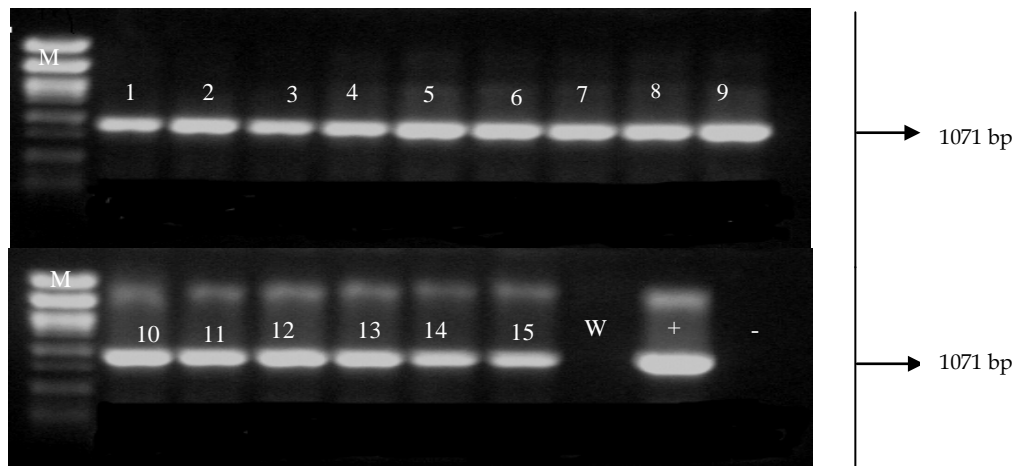


Figure 2. Virus gene fragments amplified from mother (T₀) plants. M = marker; 1-15 = transgenic plants of lines 278/1-278/15 (see Table 1); W = Wild/non transgenic plant; + = Plasmid positive control; and - = water.

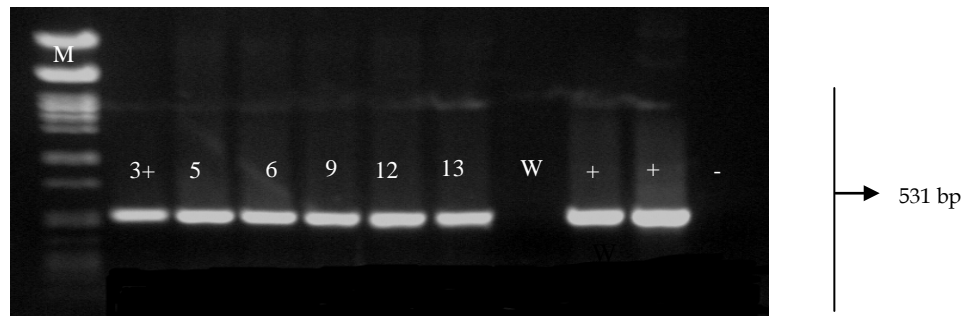


Figure 3. The GUS gene fragment amplified from GUS negative mother plants. M = marker; 3+ = plant number 3 as a positive control; 5, 6, 9, 12 & 13 = GUS negative mother plants of 278 construct (see Table 1); W = Wild/non-transgenic plant; + = Plasmid positive control; and - = water.

Disease resistance test on T1 generation

In the preliminary disease resistance test on T1 generation against the three viruses (PVY, PLRV and PVX), only nine plants were tested since there were enough number of resistant lines. Three lines *i.e.*, 278/5, 278/6 and 278/9 were immune to PVY infection (Figs 4 and 5). The same lines had shown mixed reaction for PLRV infection, which was not clear enough (Fig. 6). However, all lines were susceptible to PVX infection (Fig. 7) Surprisingly, PVY resistant plants were obtained only from lines/plants that came from previously GUS-negative mother plants and all virus resistant plants were GUS positive before virus inoculation. GUS activity in these plants disappeared two – three weeks after inoculation (Kassa Getu, 2003 and Kassa Getu *et al.*, 2004).

Results of the DAS-ELISA test conducted on the three PVY resistant lines showed that most of the

plants (73%) were highly resistant to PVY infection (Fig. 5). Since pre-selections for transgenic plants were not conducted, the above-mentioned percentage gave crude information on the mechanism of resistance. The arrow in Figure 5 indicates the mean OD reading used for a cut-off value determination after multiplying by a factor of 1.4 (Rek, 1987). There was no difference between the visual evaluation and the ELISA test for PVY inoculated plants. The results for PLRV resistance test were not as clear as PVY resistant test. From the raw data, however, there were plants, which had almost equivalent OD readings with the negative control. All the plants were positive to PLRV based on the tissue print tests (Kassa Getu, 2003). However, the data did not fit to Rek’s graphical method of cut-off point determination (Fig. 6).

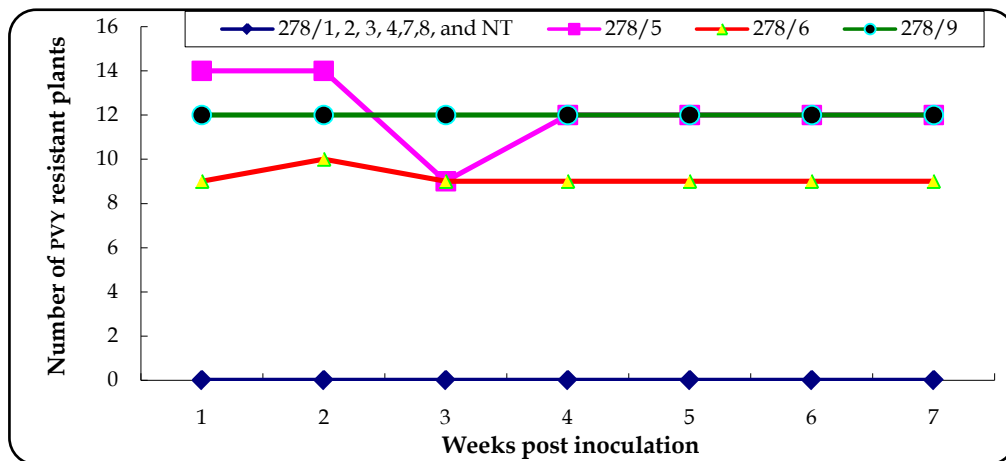


Figure 4. The number of healthy plants of the 9 lines and non-transgenic control plant after PVY infection. NT = Wild.

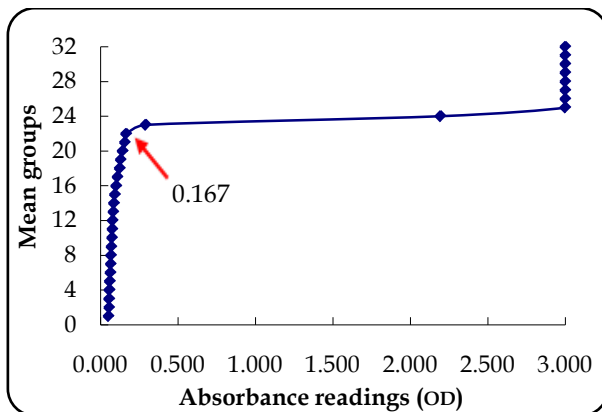


Figure 5. A curve for boundary value in the ELISA test on the three lines (278/5, 278/6 and 278/9) for their resistance to PVY infection.

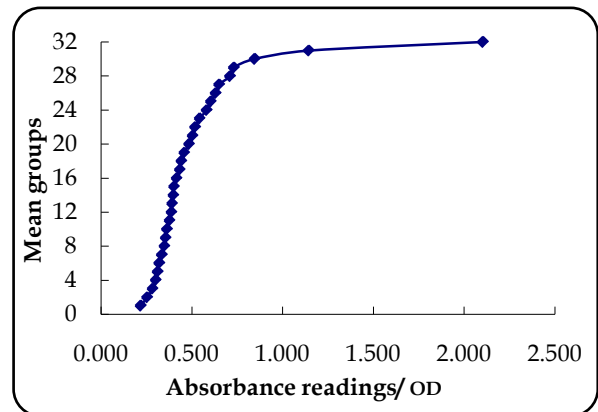


Figure 6. A trend showing the distribution of the mean OD values against the mean groups in the PLRV resistance test.



Figure 7. A) PVX (strain PVX0018) on susceptible *N. benthamiana* plants. B) Sample resistant and susceptible plants in line 278/9 (1 = susceptible and 2 = resistant plants).

GUS_PPX in pGreen vector

Histochemical GUS staining

Plants transformed with *GUS-PPX*, which was in pGreen0000 binary vector, were easily regenerated since there was no selection marker gene. Owing to the aforementioned reason, every cell has a potential to grow in the medium where a selection agent was absent. It would, therefore, be a costly task to bring all regenerated plants to the greenhouse for further analysis. As a result, *GUS* activity-test was conducted while the plants were in tubes.

All the 97 plants examined for *GUS* expression were found to be *GUS* negative, *i.e.*, it was wrongly assumed that they did not contain the insert DNA. Assuming that these plants would not contain the DNA insert, work on this construct was discontinued only because of the high cost to be incurred for analysis. The question here is whether there would be real transformants among the regenerated plants, which might have been thrown away owing to the inefficiency of our selection criteria *i.e.*, the expression of the reporter (*Gus*) gene? That was a lesson learnt after these plants were thrown and after a strange situation in plants with marker gene was found.

DISCUSSION

Transformation with marker gene (GUS_PPX in pLX_222 vector)

Currently employed transformation systems require a selectable agent in the culture media to selectively recover transformed cells among a large population of untransformed cells (de Vetten *et al.*, 2003). Hence, dominant genes encoding either antibiotics or herbicide resistance are widely used as selectable marker in plant transformation (Bevan *et al.*, 1983). The Kanamycin resistance gene used in this study yielded 100% selection efficiency, for all regenerated plants were transgenic. However, wrong selections were about to be done due to the absence of reporter gene expression in some of the transgenic plants, which later on became resistant to PVY infection. The absence of *GUS* gene expression after virus inoculation and the occurrence of PVY resistance justified RNA-mediated resistance. The first question here is why the resistance was associated only to previously *GUS* negative mother plants. The absence of *GUS* gene expression could be due to methylation of transgenes up on integration into the nuclear genome of the plant (Sijen *et al.*, 1996), which causes aRNA (aberrant RNA) that in turn mediate resistance as described by the qualitative model of Baulcombe

(1996) and Sijen *et al.* (1996). In this model, small pools of RNAs are formed even before the entrance of a virus into the cells. Another question one can raise in this case is that, if the formation of small pools of RNAs is independent from the genome of the invading virus, why the GUS gene in the transgene was not silenced before virus inoculation in T1 generation. According to the current and widely accepted model of Waterhouse *et al.* (2001), dsRNA formation precedes siRNA or crRNA (as Sijen *et al.* (1996) called it) preparation, and hence require the possible involvement of the invading viral genome. However, the question why the observed resistance is limited to lines originated from GUS negative mother plants is still unanswered, and this inspires further investigations. The probability that such results cannot happen coincidentally can be checked by testing the remaining two lines (278/12 and 278/13, shown in Table 1) which also originated from GUS negative mother plants. The presence of GUS activity in plants derived from GUS negative mother plants could be due to reactivation of the GUS gene expression in T1 generation. Guo *et al.* (1999) have reported similar findings.

The transgene sequence similarity as low as 23-nt is believed to trigger PTGS (Thomas *et al.*, 2001). The absence of disease resistance to PVX infection could, therefore, be the presence of silencing suppressor proteins and a change in the transcription of the transgene. Voinnet *et al.* (2000) and Li and Ding (2001) reported that the 25 kDa viral movement protein (p25) encoded by PVX is able to prevent or interfere with systemic silencing. According to their findings, the effect of p25 on systemic silencing could result from blocking of signal production from initial site of infection. A small change in transgene transcription can affect homology-dependent virus resistance and gene silencing (English and Baulcombe, 1997). Hence, the complete absence of resistance to PVX in our lines might have a link to a change in transcription. In principle, RNA-mediated resistance against PLRV is also possible (Barker *et al.*, 1994, Rovere *et al.*, 2000; Thomas *et al.*, 2000). The response of the lines to PLRV infection in the study was difficult to conclude due to the conflicting results obtained in the different tests. The presence of a few plants with almost equivalent OD readings with the negative control in the ELISA test could indicate the

existence of some kind of resistance, *i.e.*, intermediate type of resistance, in those plants.

GUS_PPX in pGreen vector

Marker-free plant transformation has been reported on a number of plants such as rice (Lu *et al.*, 2001), tobacco (Sugita *et al.*, 2000), and potato and cassava (de Vetten *et al.*, 2003). However, attempts to develop transgenic tobacco plants without selectable marker gene in this study had failed, for the research was discontinued based on the GUS activity test. In our marker-free transformation, absence of transformants could have two reasons. Firstly, ninety-seven plants were probably too small number for analysis in addition to the lower efficiency of the *Agrobacterium* strain, LBA4404 (de Vetten *et al.*, 2003). De Vetten *et al.* (2003) have compared transformation efficiency of two *Agrobacterium* strains using PCR-analysis on a population composed of about 8000 putative transgenic potato plants (variety Karnico). After transformation with strain LBA4404, they found that <0.2% of the harvested potato shoots were PCR-positive, whereas with *Agrobacterium* strain AGLO the average rate of transformation was 4.5%. The frequency of PCR positive transformants of their five independent transformation experiments with AGLO ranged between 1.3% and 5.6%, whereas for LBA4404 it varied from 0% to 0.8%. Secondly, in our analysis of putative transformed plants, we used the expression of GUS gene as a basis for classification. This, however, would have worsened the analysis, for it is not as sensitive as PCR and the expression of the GUS gene might have been influenced by many unforeseen phenomena in the mother plants. Such effects have been observed in one of our study, whereby we found about 31% GUS-negative and yet transgenic plants in T0 generation (Table1). Similarly, some transgenic plants might have been missed due to the inefficiency of the selection methodology employed. As a result, PCR analysis in combination with GUS activity test would have been favourable for the analysis of our transformants.

CONCLUSION AND RECOMMENDATIONS

In this study, pyramiding virus resistance for all viruses was not achieved; the GUS_PPX transgene conferred immune-type of resistance only to PVY.

The transgenic plants which were completely susceptible to PVX and had mixed reactions to PLRV need to be investigated further. In the resistant plants, complete silencing of the reporter gene on young developing leaves is exhibited three weeks after virus inoculation. The observed association between GUS negative T0 plants and PTGS/ virus resistance should be proved in future research.

The lesson learnt in the study was that selections of transformants based on the expression of reporter genes might result in loss of useful transgenics, for the expression is influenced by many genetic, epigenetic and environmental factors. Based on the findings of this study, we recommend early selection activities to depend on careful examination of both reporter gene expressing and non-expressing mother plants. If identification of transgenic plants in marker-free transformation at the early stage of seedling development is required, histochemical GUS staining method alone should not be considered as a better method of choice for selection, rather it should be coupled with PCR analysis that targets both the reporter gene and the gene of interest of the construct.

ACKNOWLEDGEMENTS

The author is grateful to Prof. Dr Edgar Maiss for his supervision when the research was conducted in his laboratory in the Institute of Plant Diseases and Plant Protection, University of Hannover, now called Leibniz University of Hannover. The author's sincere gratitude goes to Jill Engelman and Jutta Zimmermann for their unreserved technical assistance in the laboratory and greenhouse experiments. Catholic Academic Exchange Service (KAAD) deserves special credit for sponsoring my study at the University of Hannover.

REFERENCES

- Al Abdellah, Q. (2002). A plum pox virus (PPV) chimera as a model for a possible recombination event and its use for detection of homologous recombination PPV. MSc thesis. University of Hannover. p.17.
- Angell, S.M. and Baulcombe, D.C. (1997). Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *EMBO J.* **16**:3675-3684.
- Barker, H., Webster, K.D., Jolly, C.A., Reavy, B., Kumar, A. and Mayo, M.A. (1994). Enhancement of resistance to potato leafroll virus multiplication in potato by combining the effects of host genes and transgenes. *Mol. Plant Microbe. Interact* **7**:528-30.
- Baulcombe, D.C. (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* **8**:1833-1844.
- Bevan, M.W., Flavell, R.B. and Chilton, M.D. (1983). A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* **304**:184-187.
- Braun, C.J. and Hemenway, C.L. (1992). Expression of amino-terminal portions or full-length viral replicase genes in transgenic plants confers resistance to potato virus X infection. *Plant Cell* **4**:735-744.
- Casper, R. and Meyer, S. (1981). Die Anwendung des ELISA - Verfahrens zur Nachweis pflanzenpathogener Viren. *Nachrichtenbl. Deut. Pflanzenschutz* **33**:49-54.
- Covey, S.N., Al-Kaff, N.S., Langara, A. and Turner, D.S. (1997). Plants combat infection by gene silencing. *Nature (London)* **385**:781-782.
- de Vetten, N., Wolters, A.-M., Raemarkers, K., van der Meer, I., ter Stege, R., Heeres, E., Heeres, P. and Visser, R. (2003). A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. *Nature Biotechnol* **21**:439-442.
- Ding, S.W. (2000). RNA silencing. *Curr Opin in Biotechnol* **11**:152-156.
- Ebinuma, H., Sugita, K., Matsunaga, E. and Yamakado, M. (1997). Selection of marker-free transgenic plants using the isopentenyl transferase gene. *Proc. Natl. Acad. Sci. USA* **94**:2117-2121.
- Edwards, K., Johnstone, C. and Thompson, C. (1991). A simple and rapid method for preparation of plant genomic DNA for PCR analysis. *Nucl. Acids. Res.* **19**:1349.
- English, J.J. and Baulcombe, D.C. (1977). The influence of small changes in transgene transcription on homology-dependent virus resistance and gene silencing. *Plant J.* **12**:1311-1318.
- Farinelli, L. and Malnoe, P. (1993). Coat protein gene-mediated resistance to potato virus Y in tobacco: examination of the resistance mechanisms--is the transgenic coat protein required for protection. *Mol. Plant Microbe. Interact.* **6**:284-292.
- Guo, H.S., Lopez-Moya, J.J. and Garcia, J.A. (1999). Mitotic stability of infection-induced resistance to plum pox potyvirus associated with transgene silencing and DNA

- methylation. *Mol. Plant. Microbe. Interact.* **12**:103–111.
16. Han, S.J., Cho, H.S., You, J.S., Nam, Y.W., Park, E. K., Shin, J.S., Park, Y.L., Park, W.M. and Paek, K.H. (1999). Gene silencing-mediated resistance in transgenic tobacco plants carrying potato virus Y coat protein gene. *Mol. Cells* **31**:376–83.
 17. Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. and Mullineaux, P.M. (2000). pGreen: a versatile and flexible binary vector for Agrobacterium-mediated plant transformation. *Plant Mol Biol.* **42**:819–832.
 18. Herbers, K., Tacke, E., Hazirezaei, M., Krause, K. P., Melzer, M., Rohde, W. and Sonnewald, U. (1997). Expression of a luteoviral movement protein in transgenic plants leads to carbohydrate accumulation and reduced photosynthetic capacity in source leaves. *Plant J.* **5**:1045–56.
 19. Hull, R. (2002). *Matthews' Plant Virology* (4th ed.). Academic Press, London. pp. 1001.
 20. Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987). GUS fusion: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**:3901–3907.
 21. Katul, L. (1992). Serologische und molekularbiologische charakterisierung des ban leaf roll virus (BLRV) und des faba bean necrotic yellows virus (FBNYV). Dissertation zur Erlangung des Doktorgrades der Georg-August-Universitaete zur Goettingen. Fachbereich Agrarwissenschaften. P. 37.
 22. Kassa Getu (2003). Examination of Multiple Virus Resistance in Transgenic Tobacco Plants (*Nicotiana Benthamiana* Domin). MSc Thesis. University of Hannover, Germany, 151 pp.
 23. Kassa Getu, Engelman, J. and Maiss, E. (2004). Transgenic resistance to potato viruses in tobacco plants (*Nicotiana Benthamiana* Domin). Paper presented at the 12th International Biotechnology Symposium. October 17–22, 2004, Santiago, Chile.
 24. Kobayashi, K., Cabral, S., Calamante, G., Maldonado, S. and Mentaberry, A. (2001). Transgenic tobacco plants expressing the potato virus X open reading frame 3 gene develop specific resistance and necrotic ring symptoms after infection with the homologous virus. *Mol. Plant Microbe. Interact.* **14**:1274–1285.
 25. Landsmann, J., Liewellyn, D., Dennis, E.S. Peacock, W.J. (1988). Organ regulated expression of the *Parasponia andersonii* haemoglobin gene in transgenic plants. *Mol. Gen. Genet.* **214**:68–73.
 26. Lee, L., Palukaitis, P. and Gray, S.M. (2002). Host-dependent requirement for the Potato leafroll virus 17-kda protein in virus movement. *Mol. Plant Microbe. Interact.* **15**:1086–1094.
 27. Li, W.X. and Ding, S.W. (2001). Viral suppressor of RNA silencing. *Curr. Opin. Biotechnol.* **12**:150–154.
 28. Lindbo, J.A. and Dougherty, W.G. (1992). Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. *Virology.* **189**:725–33.
 29. Lindbo, J.A., Silva Rosales, L., Proebsting, W.M. and Dougherty, W.G. (1993). Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *Plant-cell.* **5**:1749–1759.
 30. Lu, H-J., Zhou, X.R., Gong, Z-X. and Upadhyaya, M. (2001). Generation of selectable marker-free transgenic rice using double right-border (DRB) binary vectors. *Aust. J. Plant Physiol.* **28**:241–248.
 31. Powell-Abel, P., Nelson, R.S., De, B., Hoffman, N., Rogers, S.G., Faley, R.T. and Beach, R.N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein. *Science* **232**:738–43.
 32. Puchta, H. (2003). Marker-free transgenic plants. *Plant Cell, Tissue and Organ Culture* **74**:123–134.
 33. Ratcliff, F.G., MacFarlane, S.A. and Baulcombe, D. C. (1999). Gene silencing without DNA: RNA-mediated cross protection between viruses. *Plant Cell* **11**:1207–1215.
 34. Ratcliff, F., Harrison, B.D., and Baulcombe, D.C. (1997). A similarity between viral defence and gene silencing in plants. *Science* **276**:1558–1560.
 35. Rek, J. (1987). Untersuchungen über die eignung des ELISA- verfahrens zum serienmässigen nachweis von viren (PLRV und PVY) bei der kartoffel unter berücksichtigung des infektionszeitpunktes der pflanzen und des physiologischen zustandes der knolle. Doktors der Technischen Wissenschaften der Eidgenössischen Technischen Hochschule Zürich. p. 37–41.
 36. Rovere, C.V., Asurmendi, S. and Hopp, H.E. (2000). Transgenic resistance in potato plants expressing potato leaf roll virus (PLRV) replicase gene sequences is RNA-mediated and suggests the involvement of post-transcriptional gene silencing. *Arch. Virol.* **146**:1337–1353.

37. Sijen, T., Wellink, J., Hiriart, J.B. and van Kammen, A. (1996). RNA-mediated virus resistance: Role of repeated transgenes and delineation of targeted regions. *Plant Cell* **8**:2277-2294.
38. Sugita, K., Kasahara, T., Matsunaga, E. and Ebinuma, H. (2000). A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. *Plant J.* **22**:461-469.
39. Thomas, C.L., Jones, L., Baulcombe, D.C. and Maule, A.J. (2001). Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. *Plant J.* **25**:417-425.
40. Thomas, P.E., Lawson, E.C., Zalewski, J.C., Reed, G.C. and Kaniewski, W.K. (2000). Extreme resistance to potato leafroll virus in potato cv. Russet Burbank mediated by the viral replicase gene. *Virus Res.* **72**:49-62.
41. van der Vlugt, R.A., Ruiter, R.K. and Goldbach, R. (1992). Evidence for sense RNA-mediated protection to PVYN in tobacco plants transformed with the viral coat protein cistron. *Plant Mol. Biol.* **20**:631-639.
42. Varrelmann, M. (1999). Begrenzung von heterologer Enkapsidierung und Rekombination bei pathogen-vermittelter Resistenz gegen das Plum Pox Virus der Pflaume (PPV). Doktors der Gartenbauwissenschaften geheimigte Dissertation. P. 27.
43. Voinnet, O., Leader, C. and Baulcombe, D.C. (2000). A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**:157-167.
44. Waterhouse P.M., Wang, M-B and Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature* **411**:834-842.