Journal of Phytology 2011, 3(7): 11-16
ISSN: 2075-6240
www.scholarjournals.org

JOURNAL OF PHYTOLOGY

www.journal-phytology.com

Phenotypic and Molecular Analysis of Transgenic Tobacco with Rabies Glycoprotein for Stability of Gene Expression

N. Nagesha[1*] and P.H. Ramanjini Gowda[2]

[1]*Department of Agriculture Biotechnology, College of Agriculture, University of Agricultural Sciences (Bangalore), Karekere, Hassan, Karnataka-573225*
[2]*Department of Plant Biotechnology, University of Agricultural Sciences (Bangalore), GKVK, Bangalore, Karnataka, India-560065.*

Article Info

Article History

Received : 12-02-2011
Revised : 18-03-2011
Accepted : 14-04-2011

*Corresponding Author

Tel : + 91-9448713777
Fax : +91-8172221177

Email:
nageshabt@gmail.com
ramanjini@yahoo.com

©ScholarJournals, SSR

Summary

The agronomic and morphological characteristics of *Agrobacterium* mediated transformed plants carrying the ERA strain of the rabies glycoprotein gene in different progenies of tobacco (FCV var.) were investigated. Significant variation in plant height and the leaf area was observed in all the generations of the plants. However no significant differences were observed for number of leaves per plant. Most of the variations in R_1 to R_4 plants have no significant correlation with transgene insertion and were proved heritability characters in the progenies. None of the plants in R_1 to R_4 generation showed any dwarfism or stunted growth. The molecular analysis of the transgenic tobacco progenies did not show any significant differences in the expression of rabies glycoprotein, thus indicating the stability of the gene expression.

Key Words: Transgenic Plants, Morphological characters, Rabies glycoprotein, Molecular characterization and stability

Introduction

Genetic engineering of the crop plants is becoming a reality and plant gene transfer is now a fertile field. The global commercial area of genetically modified GM crops reached 67.7 m ha in 2003 (1). Since the mid-1990's transgenic plants with enhanced tolerance to herbicides, resistance to diseases and insect pests and with improved quality have been commercially exploited. More recently, plants are being engineered as bioreactors to produce new and modified proteins and new polymeric compounds, some of which are already in clinical and industrial trails (2-3).

In the last decade, plant biotechnology has emerged as a promising strategy that combines innovations in medical science with novel protein bio-manufactures as a means to create affordable protein pharmaceuticals. Extensive research has shown that a wide range of valuable proteins can be expressed efficiently in plants, which have emerged as a convenient, safe and economically alternative to microbial and animal cell factories or transgenic animals for the production of clinically useful therapeutic proteins (4-8).

Various methods of gene transfer techniques are currently available for the genetic transformation of crops. The success of plant genetic manipulation requires not only the ability to deliver functional DNA into the cell, but also that of producing multiple transgenic plants that stably inherit and express the transgenes. Many have reported the stability of primary transformants (9-14), which revealed that the inheritance and stability of the transgene varied in the off springs.

Enterotoxigenic *Escherichia coli* (ETEC) strains are important pathogens in developing countries. Some vaccine formulations containing the heat labile toxin B subunit (LTB) have been used in clinical trials; however, the induction of neutralizing antibodies against the heat-stable toxin (ST), a poor immunogenic peptide, is necessary, as most ETEC strains can produce both toxins. A plant optimized synthetic gene encoding for the LTB-ST fusion protein has been introduced into plastids of tobacco leaf tissues, using biolistic microprojectile bombardment, in an effort to develop a single plant-based candidate vaccine against both toxins. Transplastomic tobacco plants carrying the LTB-ST transgene have been recovered (15).

Rabies virus (RV), the etiological agent of one of the oldest recognized infectious diseases, almost always causes a fatal encephalomyelitis in several species of mammals, including humans (16). RV, the prototype of the *Lyssavirus* genus of the family *Rhabdoviridae*, is an enveloped, nonsegmented, negative-stranded RNA virus. RV has a simple genome of about 12kb encoding five proteins: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), and the RNA-dependent RNA polymerase (L). The viral RNA, which is always encapsidated by N, forms the ribonucleoprotein (RNP), which is the template for viral replication and transcription (17).

Glycoprotein of rabies virus (RV) is known to play a predominant role in the pathogenesis of rabies. To further investigate the roles of these proteins in viral pathogenicity, chimeric recombinant viruses were constructed by exchanging the G and M genes of the attenuated SN strain with those of the highly pathogenic SB strain. Infection of mice with these chimeric viruses revealed a significant increase in the pathogenicity of the SN strain bearing the RV G from the pathogenic SB strain (18).

The rabies glycoprotein was first expressed in Tomato; unfortunately the tomato produced a low glycoprotein yield. So it could not be used for immunization (19). We have developed the transgenic crops viz., Tobacco, muskmelon and groundnut expressing the rabies glycoprotein. The molecular

characterization of the transgenic crops through the PCR, SDS-PAGE and western blot confirmed the integration and expression of the rabies glycoprotein (20).

Transgenic maize was developed expressing the rabies virus glycoprotein (G) of the Vnukovo strain and evaluation of its immunogenicity in mice, by the oral route. The ubiquitin maize promoter fused to the whole coding region of the rabies virus G gene, and a constitutive promoter from cauliflower mosaic virus (CaMV) were used. The presence of the G gene and its product was detected by PCR and western blot, respectively. The amount of G protein detected in the grains was approximately 1% of the total soluble plant protein. The G protein of the Vnukovo strain expressed in transgenic maize may be considered as an oral immunogen against rabies, conferring cross-protection (21).

In the present investigation, we have studied the effects on phenotypic characters of transgenic Tobacco progenies between four generations from R_1 to R_4. The molecular characterizations of the transgenic plants were carried out to study the stability of the rabies glycoprotein between four generations.

Materials and Methods

Transgenic plants

The tobacco variety FCV (Flue Cured Virginia) was used for the study of rabies glycoprotein expression. In total, the study involved evaluation of 72 transgenic plants from R_1 to R_4 lines of tobacco along with 18 control tobacco plants. In each progeny, 18 plants were planted along with same number of control in the green house. The morphological and molecular characters were recorded in these plants.

Observations on agronomic and morphological characters of Transgenic and control Tobacco Plants

Plants regenerated from transformed calli are designated as R_0 and their subsequent progenies as R_1 to R_4. The morphological traits of the Tobacco plant progenies viz., plant height, number of leaves per plant and leaf area were recorded for both control and transgenic tobacco plants. The observations were recorded on 30th Day and 60th Day.

Statistical Analysis

The data was analyzed using factorial CRD analysis with different generation transgenic and non-transgenic plants. The significance of difference between means was determined.

Molecular characterization through SDS-PAGE and Western Blot

Total protein was extracted from young leaves on 30th and 60th day. The protein samples were stored at -20^{0}C freezer for long-term storage. The protein was assayed using Lowry's method. The total protein from four-generations of transgenic and un-transformed Tobacco plants were analyzed on 10 % SDS-PAGE gel. The SDS-PAGE confirmed transgenic tobacco proteins with rabies glycoprotein were purified by using concanvalin A Agarose columns. Western blot were used to detect the expression of rabies glycoprotein in different generations. After the extraction and separation of the proteins through the SDS-PAGE gel the proteins were transformed on to PVDF membrane according to standard methods. After transfer, the membrane was processed in blocking solution followed by incubation in primary and secondary antibody. The signals were detected using horseradish peroxidase (HRP) substrate Diaminobenzidine (DAB).

Results

Transgenic plants

Different generations of transgenic Tobacco plants along with untransformed plants were grown in green house. Each generation consists of 18 plant progenies along with control plants. The overview of the seedling stage transgenic Tobacco progenies and matured Tobacco plants are illustrated in the Figure -1 (a, b).

Observations on agronomic and morphological characters of Transgenic and control Tobacco Plants

To investigate the correlation between phenotypic variations among different generations of transgenic progenies R_1, R_2, R_3, R_4 and control Tobacco plants, the data on plant height, leaf area and number of leaves per plant were recorded and subjected to the statistical analysis.

The result of correlation analysis for the four-generations of transgenic tobacco and control plants for plant height is presented in the Table-1. No significant variation occurred in the first observation on 30th day between R_1-R_2, R_1-R_3, R_1-R_4 and R_2-R_3 generation transgenic plants, but significant differences were observed between R_2-R_4 and R_3-R_4 generation transgenic plants. The significant differences were also observed between control and transgenic progenies. The maximum height of 22 cm was recorded in R_4 generation Tobacco progenies and least height of 12 cm in R_2 progenies. Similarly on 60th day of second observation, maximum and minimum height of 90 cm and 66 cm were recorded in R_4 and R_3 progenies respectively. The untransformed tobacco progenies showed slightly more height of 31 cm and 95 cm on 30th and 60th day respectively when compared to the transgenic Tobacco progenies.

The results of correlation analysis for four generations of transgenic Tobacco progenies and untransformed Tobacco plants for leaf area and number of leaves per plant are summarized in the Table 2 and 3. No significant differences were observed between the transgenic and control tobacco plant progenies for the number of leaves per plant. The average number of leaves per plant in the Tobacco progeny was 12, where as average number of leaves per plant for four generations were 11, which was recorded on 60th day, similarly the average leaf area in control Tobacco progeny was 334 cm^2 and average leaf area of all the four generations of Tobacco progenies were 458 cm^2, where no effect of transgenes on leaf area was observed.

Figure 1: a) Over view of seedling and b) Mature stages of four generation (R_1, R_2, R_3, R_4) Transgenic and Control Tobacco plants grown in green house.

Table-1: The seedling height of transgenic progenies and control tobacco at the age of 30th day and 60th day

Duration/ Generation	30th Day	60th Day	Mean
R_1	16.363	78.796	47.547
R_2	12.527	68.596	40.558
R_3	14.957	66.763	40.860
R_4	22.137	90.933	56.535
C	31.830	95.747	63.788
Mean	19.551	80.165	49.858
Comparison of Mean F	SEM ±	CD (P=0.05)	
Days	2.60	8.030	
Generation	4.11	12.695	

Table- 2: The number of leaves of the transgenic progenies and control tobacco at the age of 30th and 60th day

Duration/ Generation	30th Day	60th Day	Mean
R_1	7.551	17.33	12.432
R_2	6.587	17.167	11.877
R_3	7.320	16.267	11.793
R_4	8.033	15.70	11.867
C	9.300	15.66	12.480
Mean	7.755	16.425	12.090
Comparison of Mean F	SEM ±	CD (P=0.05)	
Days	0.3829	1.182	
Generations	0.6054	1.870	

Table -3: The leaf area (cm^2) of the transgenic progenies and control tobacco at the age of 30th and 60th day.

Duration/ Generation	30th Day	60th Day	Mean
R_1	404.480	439.613	422.047
R_2	399.907	480.677	440.292
R_3	428.937	562.243	495.590
R_4	408.95	543.423	475.940
C	274.70	395.290	334.290
Mean	383.296	484.249	433.773
Comparison of Mean F	SEM ±	CD (P=0.05)	
Days	32.9093	101.65	
Generations	52.0343	160.73	

Molecular characterization of transgenic Tobacco progenies: SDS-PAGE and Western blot

The four-generations of transgenic Tobacco progeny leaves along with the untransformed Tobacco progeny grown in the green house were harvested. The leaves were harvested twice to extract the protein for comparison. The total protein was extracted and quantified by using spectrophotometer. The crude total protein was purified through Concanvalin Agarose column affinity chromatography. The samples including both purified and unpurified proteins were analyzed on 10 % SDS-PAGE gel where expression of single band of 66 kDa was found in all the four generation transgenic Tobacco progenies (Figure-2&3). The percentage of the column purified rabies glycoprotein in the sample is summarized in the Table 4. The column purified protein samples were subjected to western blot using primary and secondary antibody specific to the rabies glycoprotein. The expression of specific 66 kDa band was found only in the transgenic Tobacco progenies (Figure-4).

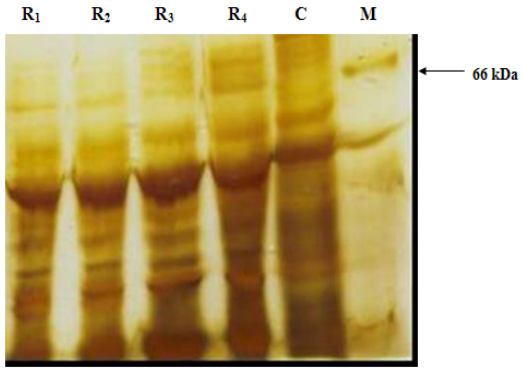

Figure 2: SDS-PAGE gel showing the expression of the 66 kDa rabies glycoprotein along with total proteins from the R_1, R_2, R_3 and R_4 generation transgenic and Control (C) Tobacco Leaf samples

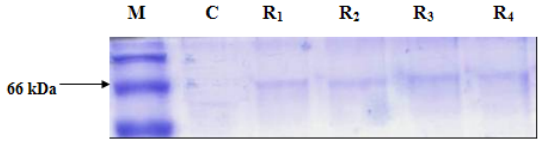

Figure 3: SDS-PAGE gel showing the expression of the 66 kDa rabies glycoprotein from the R_1, R_2, R_3, R_4 generation transgenic Tobacco and control (C) protein purified from Concanvalin Agarose column purification

Table 4: Column purified Protein percentage for the R_1, R_2, R_3 and R_4 generation Tobacco progenies and control Tobacco Progenies recorded on 30th and 60th day

Duration/ Generation	30th Day (%)	60th Day (%)	Mean (%)
R_1	1.76	1.83	1.80
R_2	1.73	1.83	1.78
R_3	1.76	1.90	1.83
R_4	2.03	2.22	2.12**
C	0.16	0.23	0.19
Mean	1.48	1.60	1.54
Comparison of Mean F	SEM ±	CD (P=0.05)	
Generation	0.021	0.061	

** = Significant

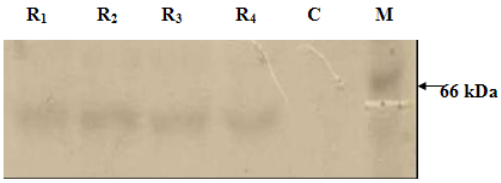

Figure 4: Western blot analysis showing the expression of the 66 kDa rabies glycoprotein from the R_1, R_2, R_3, R_4 generation transgenic Tobacco and control Tobacco protein purified from Concanavalin Agarose column purification

Discussion

Here we report the stable expression of the rabies glycoprotein gene in tobacco progenies. We have employed *Agrobacterium* mediated transformation for the transfer of gene and compared four generations of transgenic tobacco lines (R_1 to R_4) for the variations in the morphological characters. The variations in the transgenic plants might result independently from either one or more of the following sources (a) Tissue culture derived somaclonal variations (22). (b) Breakdown of plant genes caused by transgene insertion (23). (c) Pleiotropy, whereby one or more of the transgenes may have multiple effects on apparently unrelated genes or the products of the transgenes have effects on plant growth. (d) Transgene induced endogene silencing (24). According to many reports variations would seem to be the main source of the greater morphological variations in R_1 plants. We have also observed significant variations for plant height and leaf area in the transgenic plants. The control tobacco plants were recorded maximum level of plant height compared to transgenic tobacco plants. However there were no significant variations for the characters like number of leaves per plant.

Even though the expression of rabies glycoprotein did not cause any abnormal phenotypic variations, the over expression of the rabies glycoprotein might have resulted in lesser plant height in transgenic plants. The correct processing of the antigen is an important phenomenon, considering that the antigenic proteins are being synthesized in an external environment, it may not favour the correct folding in their native confirmation.

Transplastomic tobacco plants carrying the LTB-ST (heat labile toxin B subunit & heat-stable toxin) transgene have been recovered. Transgene insertion into the plastid was confirmed by both PCR and Southern blot analysis. GM1-ELISA revealed that the LTB-ST fusion protein retained its oligomeric structure, and displayed antigenic determinants for both LTB and ST. Western blot analysis, using LTB antisera, confirmed the presence of a 17-KDa protein in transplastomic lines, with the correct antigenicity of the fusion protein. Expression levels of this fusion protein in different lines reached up to 2.3% total soluble protein (15).

A synthetic gene coding for the surface glycoprotein (G protein) of rabies virus was strategically designed to achieve high-level expression in transgenic plants. The native signal peptide was replaced by that of the pathogenesis related protein, PR-S of *Nicotiana tabacum*. An endoplasmic reticulum retention signal was included at C-terminus of the G protein. Tobacco plants were genetically engineered by nuclear transformation. Selected transgenic lines expressed the chimeric G protein at 0.38% of the total soluble leaf protein. The results establish that plants can provide a safe and effective production system for the expression of immunoprotective rabies virus surface protein (25).

In the present study, the rabies glycoprotein was processed correctly in the transgenic tobacco plants expressing glycoprotein size of 66 kDa (Molecular weight of authentic rabies glycoprotein). The likely explanation is glycosylation of the plants are different from yeast and also the growing temperatures of the plants. All the four generations of tobacco (R_1 to R_4) recorded the same size of the rabies glycoprotein. It is known that plants can glycosylate heterogeneous proteins and attach a variety of carbohydrates including some which are not present in animal cells (26). These extra carbohydrates can alter the mobility of protein gels.

Conclusions

The Results of the present study reveals that the transgene can be efficiently inherited from generations to generations and maintains the stability of transgene expression. The immunization of rabies glycoprotein produced in R_1 to R_4 generations of tobacco confirmed adequate antibody production in mice. This data proves that the stability of inheritance of the rabies glycoprotein gene through $R_1$to R_4 generations. In conclusion we report that there is no morphological variations occurred in different generations of tobacco plants and the rabies glycoprotein was stably inherited in all the generations.

Acknowldgement

We are very thankful to Department of Biotechnology (DBT) and National Agricultural Technology Programme (NATP) New Delhi for providing the funds.

References

[1] Griffiths, B.S., Birch, A.N.E., Caul, S. & Thompson, J, (2004). Below ground effects of transgenic maize. Environment. 162-163

[2] Herbers, K. & U. Sonnewald, 1999, Production of new/modified proteins in transgenic plants. Current Opinion in Biotechnology. 10:163–168.

[3] Poirier, Y, (2001), Production of polyesters in transgenic plants. Adv.Biochem.Eng.Biotechnol. 71: 209–240.

[4] Koprowski, H and Yusibov, V. (2001). The green revolution: plants as heterologous expression vectors. Vaccine. 19:2735–41.

[5] Mason, H.S., Warzecha, H., Mor, T., Arntzen, C.J, (2002), Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine.Trends. Mol. Med. 8:324–329.

[6] Ma, J.K., Drake P.M and Christou, P, (2003). The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet. 4:794–805.

[7] Streatfield, S.J. and Howard, J.A. (2003), Plant-based vaccines. Int.J.Parasitol. 33:479–493.

[8] Mor, T.S., Mason, H.S., Kirk, D.D., Arntzen, C.J., Cardineau, G.A, (2004), Plants as production and delivery vehicle for orally delivered subunit vaccines. New

generation vaccines. 3rd Ed. New York-Basel: Marcel Dekker Inc: p. 305–12.

[9] Fromm, M.E., Morrish, F., Armstrong, C., Williams, R., Thomas, J & Klein T.M, (1990), Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Biotechnology. 8: 833–839.

[10] Register, J.C., Peterson, D.J., Bell, P.J., Bullock, W.P., Evans, E.J., Frame, B., Greenland, A.J., Higgs, N.S., Jepson, I., Jiao, S., Lewnau, C.J., Sillick, J.M. & Wilson, H.M, (1994), Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant.Mol.Biol.25: 951–961.

[11] Peng, J., F., Wen, R.L., Lister & Hodges, T.K, (1995), Inheritance of *gusA* and *neo* genes in transgenic rice. Plant.Mol.Biol. 27: 91–104.

[12] Duan, X.L, Li X.G., Xue, Q.Z., Abo El Saad M., Xu, D.P., Wu. R, (1996). Transgenic rice plants harboring an introduced potato Proteinase inhibitor II gene is insect resistant. *Nat* Biotechnology. 14: 494–498.

[13] Scott, A., Woodfield, D. and White D.W.R. (1998), Allelic composition and genetic background effects on transgene expression and inheritance in white clover. Mol.Breed. 4:479–490.

[14] Wu, G., Cui, H., Ye, Y. G., Xia, Sardana, R., Cheng, X., Li, Y., Altosaar, I.& Shu, Q., (2002), Inheritance and expression of the *cry1Ab* gene in Bt (*Bacillus thuringiensis*) transgenic rice. Theor.Appl.Genet. 104: 727–734.

[15] Rosales-Mendoza S, Alpuche-Solís AG, Soria-Guerra RE, Moreno-Fierros L, Martínez-González L, Herrera-Díaz A, Korban SS, (2009). Expression of an Escherichia coli antigenic fusion protein comprising the heat labile toxin B subunit and the heat stable toxin, and its assembly as a functional oligomer in transplastomic tobacco plants. Plant J. 57(1):45-54.

[16] Conzelmann, K. K. (2004). Reverse genetics of mononegavirales. Curr. Top. Microbiol. Immunol. 283:1–41.

[17] Dietzschold, B., C. E. Rupprecht, Z. F. Fu, and H. Koprowski. (1996). Rhabdoviruses 3rd ed. Lippincott-Raven, Philadelphia, PA.

[18] Pulmanausahakul R, Li J, Schnell MJ, Dietzschold B (2008), The glycoprotein and the matrix protein of rabies virus affect pathogenicity by regulating viral replication and facilitating cell-to-cell spread. J. Virol. 82 (5):52330-2338.

[19] Mc Garvey, P.B., Hammond, J., Dienelt, M.M., (1995), Expression of the rabies virus glycoprotein in transgenic tomatoes. Biotechnology. 13:1484–1487.

[20] Ramanjini Gowda, P.H., Shilpa, R., Madhusudhana, S.N., Dinesh, N., Prakash,C.S., Gowda,T.K.S., Deviah, B.N., Nagesha, N. (2002), Expression and immunogenicity of rabies glycoprotein produced in Tobacco and Muskmelon plants by Agrobacterium mediated transformation. Paper presented in the IAPTC &BC, June 23-28, 2002, Orlando, USA.

[21] Loza-Rubio E, Rojas E, Gómez L, Olivera MT, Gómez-Lim MA (2008). Development of an edible rabies vaccine in maize using the Vnukovo strain. Dev Biol (Basel). 131:477-82.

[22] Larkin, P.J and Scowcroft, W.R, (1981), Somaclonal variation, a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60: 197–214.

[23] Lijsebetens, V.M., Vanderhaeghen, R .and. Van Montagu, M, (1991), Insertional mutagenesis in *Arabidopsis thaliana:* isolation of a T-DNA-linked mutation that alters leaf morphology. Theor. Appl. Genet. 81: 277-284.

[24] Matzke, M.A., Mette, M.F. &.Matzke, A.J.M, (2000), Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. Plant Mol.Biol. 43: 401–415.

[25] Ashraf S, Singh PK, Yadav DK, Shahnawaz M, Mishra S, Sawant SV, Tuli R, (2005). High level expression of surface glycoprotein of rabies virus in tobacco leaves and its immunoprotective activity in mice. J Biotechnol. 119(1):1-14.

[26] Faye, L., Boulaflous, A., Benchabane, M., Gomord, V., and Michaud, D. (2005). Protein modifications in the plant secretary pathway: current status and practical implications in molecular pharming. Vaccine 23. 1770−1778.