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# Stability and inheritance analysis of transgenic Tobacco expressing Hepatitis B surface antigen

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**Abstract**

Plants provide ample advantages over other expression systems for the production of recombinant proteins efficiently at low cost. The current focus of plant transformation is towards stable expression of a transgene. However, some unintended transgene instability has occurred in the expression of recombinant proteins. To study the genetic stability of a transgene, we transformed leaf explants of tobacco with the hepatitis B surface antigen (HBsAg) gene along with *npt-II* as an antibiotic selection marker using *Agrobacterium* mediated transformation. The presence of the HBsAg gene in putative transformants was confirmed by polymerase chain reaction (PCR) analysis. The crude protein obtained from the transformed tobacco plants was tested by western-blot analysis and Enzyme linked immunosorbant assay (ELISA) to prove the antigen specificity and immunogenic nature of the hepatitis B surface antigen. The $T_1$ to $T_4$ generation seeds obtained from the transgenic tobacco plants were tested after germination in the presence of kanamycin and Mendelian segregation was confirmed. This phenotypic and molecular characterization strengthens the genetic stability and inheritance of the HBsAg gene in different generations of transgenic tobacco plants.

**Keywords:** Agrobacterium; Genetic stability; Kanamycin; Mendelian inheritance; Transgenic plants.
**Abbreviations:** CTAB-N,N,N,N,-Cetyl trimethyl ammonium bromide; ELISA-Enzyme linked immunosorbant assay; HBsAg-Hepatitis B surface antigen; kDa-Kilo Dalton; *npt* II-Neomycin phosphotransferace.

## Introduction

Advances in genetic engineering over the last two decades have enabled alterations of plants for production of pharmaceutical proteins. Plant expression systems are economical, rapid methods for producing vaccines (Langer, 2011). More recently, plants are being engineered as bioreactors to produce new and modified proteins, some of which are already in clinical and industrial trials. This has been possible due to their post-translational modifications that enable these recombinant proteins to maintain their structure and functional integrity (Obembe et al., 2011).

Hepatitis B virus infection causes more than one million deaths per annum and 360 million people are persistently infected throughout the world (Ott et al., 2012). Hepatitis B virus is a 42 nm coated DNA virus of the hepadnaviridae family, with a circular partially double-stranded 3.2 kb genome, that preferentially infects hepatocytes. The complete virion consists of an inner core (nucleocapsid or hepatitis core antigen, HBcAg) surrounded by an outer protein coat or envelope (the hepatitis B surface antigen, HBsAg). The hepatitis B virus vaccine has been recommended as a routine infant vaccination worldwide since 1991 and as a routine adolescent vaccination since 1995. The vaccine is delivered in a series of three intramuscular injections over a six-month period. It requires refrigeration and injections must be administered by a medical professional, with the total cost ranging between $100 and $150 per person. Hence, plant-based production of vaccine for hepatitis B may be an economically feasible alternative (Sharma et al., 1999). HBsAg expression has been reported in transgenic tobacco plants (*Nicotiana tabacum* cv. 'Samsun') (Mason et al., 1992), lupin (*Lupinus albus*) and lettuce (*Lactuca sativa*) (Kapusta et al., 1999), potato (*Solanum tuberosum*) (Richter et al., 2000), carrot (*Daucus carota*) (Imani et al., 2002), tobacco cell lines (*N. tabacum* NT-1) (Sunil Kumar et al., 2003), banana (Sunil Kumar et al., 2005; Elkholy et al., 2009), tomato (Salyaev et al., 2007; Srinivas et al., 2008), cherry tomato (Guan et al., 2012) and maize (Hayden et al., 2012). However, variation in transgene expression is not unusual in primary transformants in plants and is undesirable, as it requires screening a large number of transformants in order to select transgenic lines with acceptable levels of stable transgene expression. Therefore, the current focus of plant transformation is toward fine tuning transgene expression and stability in the transgenic plants. Although a number of studies have reported relatively stable transgene expression for several target traits, including herbicide resistance, insect resistance, and lignin modification, some unintended transgene instability in expression of recombinant proteins has also been noted (Doran, 2006). In this study, we transferred a recombinant HBsAg gene into tobacco plants using *Agrobacterium* mediated transformation and studied the genetic stability and inheritance pattern of the HBsAg gene in the $T_1$ to $T_4$ generations of the tobacco plants by phenotypic and molecular characterization.

## Results

The use of transgenic plants as a source of an edible vaccine is a tantalizing possibility that has been pursued with varying degrees of success for half a decade now. In the last several years, a novel approach for developing improved edible vaccines has emerged by exploiting the use of genetically

**Table 1.** Comparison of growth parameters of control tobacco with different generations ($T_1$ - $T_4$) of transgenic tobacco with Hepatitis B Surface Antigen 30 and 60 days after sowing.

| | Plant height (cm) | | | Leaf area ($cm^2$) | | | Number of leaves | | |
|---|---|---|---|---|---|---|---|---|---|
| | 30 days | 60 days | Least Square Mean | 30 days | 60 days | Least Square Mean | 30 days | 60 days | Least Square Mean |
| Control | 3.62 | 67.82 | 35.72[a] | 7.07 | 196.18 | 101.62[a] | 4.57 | 18.38 | 11.48[c] |
| T1 | 3.68 | 70.53 | 37.11[a] | 5.86 | 198.92 | 102.39[a] | 4.72 | 18.62 | 11.67[cb] |
| T2 | 3.71 | 70.51 | 37.11[a] | 6.84 | 195.06 | 100.95[a] | 4.51 | 20.15 | 12.33[ab] |
| T3 | 3.64 | 70.68 | 37.16[a] | 5.88 | 198.00 | 101.94[a] | 4.67 | 19.87 | 12.27[ab] |
| T4 | 3.77 | 70.51 | 37.14[a] | 5.85 | 201.20 | 103.52[a] | 4.60 | 21.18 | 12.89[a] |
| Mean | 3.68 | 70.01 | | 6.30 | 197.87 | | 4.62 | 19.64 | |
| | LSD | | | LSD | | | LSD | | |
| Days | 13.99 | | | 14.66 | | | 3.10 | | |
| Treatment | 8.85 | | | 9.27 | | | 1.96 | | |
| Days * Treatment | 19.79 | | | 20.73 | | | 4.38 | | |

[a] Means with the same letter are not significantly different. [abc] Means with different letters are significantly different.

**a. Transgenic Plants** **b. Non-Transgenic Plants**

**Fig 1.** Overview of transgenic and control tobacco plants grown in greenhouse.

modified plants against infectious diseases including hepatitis B. We transferred the HBsAg gene into tobacco plants by *Agrobacterium* mediated transformation and studied the phenotypic and molecular characterization of the developed transgenic tobacco plants for genetic stability and inheritance from generation to generation ($T_1$-$T_4$).

***Phenotypic characterization***

Different generations of transgenic tobacco plants along with untransformed plants were grown in a greenhouse. An overview of the transgenic tobacco progeny and control tobacco plants are illustrated in Fig 1 (a, b). To investigate correlations between phenotypic variations among different generations of transgenic progeny ($T_1$- $T_4$) and control tobacco plants, data on plant height, leaf area and number of leaves per plant were recorded and subjected to ANOVA analysis using the SAS GLM procedure (Table 1). No significant variation occurred in the plant height or leaf area between different generations of transgenic plants and control plants, whereas significant variation was observed in the number of leaves per plant. The heights of the $T_4$ (3.77 cm) and $T_3$ (70.68 cm) transgenic plants were higher than those of the corresponding control plants (3.62 cm and 67.82 cm) on the $30^{th}$ and $60^{th}$ days, respectively. However, leaf area was greater in control (7.07 $cm^2$) compared to transgenic plants on day 30, and the leaf area of the $T_4$ transgenics (201.20 $cm^2$) was greater on the $60^{th}$ day comparable to control plants; whereas significant variation was observed in the numbers of leaves on control and transgenic plants between the $T_1$-$T_2$, $T_1$-$T_4$, $T_1$-$T_3$, $T_2$-$T_4$ and $T_3$-$T_4$ generations.

***Molecular characterization***

When genomic DNA from transgenic and control tobacco plants from generations $T_1$ to $T_4$ and pHB118 plasmid DNA from *Agrobacterium* were subjected to PCR analysis, gene-specific primers showed the presence of a 900 bp amplified HBsAg gene fragment in transgenics that corresponded to the amplified band of plasmid DNA. But it was absent in the control tobacco when run on a 1.2% agarose gel (Fig 2). The protein product of the HBsAg gene was detected on western blots using a monoclonal antibody specific for the hepatitis B virus. Successful expression of the HBsAg gene in the transgenic tobacco plants was confirmed by positive results in a 24 kDa HBsAg antigen test, which were negative in non-transformed control plant samples (Fig 3). This indicates that the transgenic tobacco plants definitely contained the immunogenic HBsAg. ELISA is a rapid and sensitive technique by which an immunogen can be screened for in a large number of samples. Enriched mouse anti-hepatitis B surface antigen was used as the primary antibody. The absorbance of transgenic samples from $T_1$ to $T_4$ was 0.160, 0.198, 0.183 and 0.164, respectively, significantly higher than the control plant absorbance value of 0.012, whereas yeast derived HBsAg protein recorded an absorbance of 0.325 (Fig 4). The higher absorbance values of the transgenic plant samples indicated the immunogenic nature of the samples, confirming the presence of the recombinant HBsAg. This validated the earlier Western blot analysis results.

*Table 2.* Kanamycin assay to study transgene expression in transgenic ($T_1$ to $T_4$) and control tobacco seeds by germination on 100 ppm kanamycin.

| | No. of seeds sown | Kanamycin (100ppm) | |
|---|---|---|---|
| | | Germinated | Non-Germinated |
| Non-Transgenic | 100 | 0 | 100 |
| T1 | 100 | 76 | 24 |
| T2 | 100 | 70 | 30 |
| T3 | 100 | 74 | 26 |
| T4 | 100 | 80 | 20 |
| chi square ($\chi$2)test | | | |
| | Observed | Expected | chi square ($\chi$2) value |
| Transgenic seeds | | | |
| Germinated | 300 | 300 | 0.0 |
| Non-Germinated | 100 | 100 | 0.0 |
| Non-Transgenic | | | |
| Germinated | 0 | 0 | 0.0 |
| Non-Germinated | 100 | 100 | 0.0 |

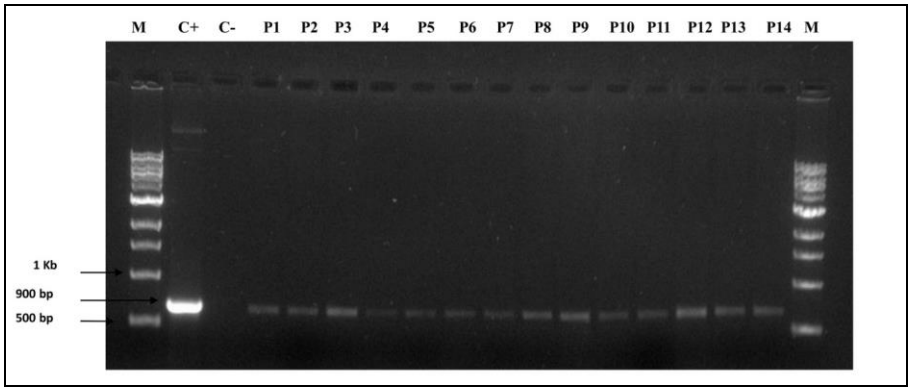


**Fig 2.** PCR confirmation of Hepatitis B surface antigen in different generations of transgenic tobacco plants using HBsAg gene-specific primers. M, 1 Kb DNA markers (Chromus Biotech Pvt. Ltd. Bangalore, India); C+, Plasmid DNA; C-, control tobacco plant; P1- P14, Transgenic plants.

***Inheritance analysis of HBsAg gene in seeds obtained from transgenic tobacco***

The seeds obtained from transgenic tobacco plants with the HBsAg gene were tested for their germination in the presence of 100 ppm kanamycin. The seeds from the transgenic tobacco plants showed a segregation ratio for hepatitis B surface antigen of 3:1, as expected, confirming Mendelian inheritance, when they were germinated in the presence of 100 ppm kanamycin (Table 2). A chi-square test indicated that the observed data/ratio fit with Mendelian segregation. We conclude there was no significant difference between the observed and expected ratio and the null hypothesis was accepted.

**Discussion**

Tobacco is readily amenable to genetic transformation. Hence, most researchers use tobacco for expression of recombinant proteins. Another advantage is it is a short duration crop producing enormous biomass. In addition, transgenic tobacco is not a food crop; hence, there is no chance of it entering the food chain. Even though tobacco contains nicotine, a process can be employed to remove the nicotine content from tobacco extracts so that a recombinant protein can be used for therapies. The yield of extractable tobacco protein varied between 155 and 228 kg $ha^{-1}$ (Woodleif et al., 1981). Mason et al. (1992) reported expression of HBsAg in tobacco (*Nicotiana tobaccum* cv. 'Samsun'). The expression levels were as high as 66 ng $mg^{-1}$ of the total soluble protein in the leaves. To study stable expression of hepatitis B surface antigen, we employed *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tobaccum* cv. 'Kanchun') with pHB118 and compared four generations of transgenic tobacco progeny ($T_1$ to $T_4$) for variations in morphological characters. Variations in the transgenic plants might result independently from one or more of the following sources (a) Tissue culture derived somaclonal variations (Larkin and Scowcroft, 1981) (b) Breakdown of plant genes caused by transgene insertion (Lijsebetens et al., 1991) (c) Pleiotropy, whereby one or more transgenes may have multiple effects on apparently unrelated genes or the products of the transgenes may have effects on plant growth (d) Transgene-induced endogene silencing (Matzke et al., 2000). According to many reports, variations in transgenic plants would seem to be the main source of the greater morphological variations in $T_1$ plants. However, morphologically there were no significant variations between transgenic and control plants for plant height and leaf area, except for the number of leaves per plant in all the generations. Even though expression of the HBsAg gene did not cause any abnormal phenotypic variations, contrary to this, Nagesha and Ramanjini Gowda (2011) reported that expression of rabies glycoprotein in generations $R_1$ to $R_4$ of tobacco variety FCV (Flue Cured Virginia) caused significant variations in plant height and leaf area in all generations of the plants; though, no significant differences were observed in the number of leaves per plant in any generation. The presence of the transgene in the transgenic plants was evident by PCR analysis in generations $T_1$ to $T_4$. The transgenic lines yielded a 0.9 kb band, the same as the pHB118 plasmid; the PCR product was absent in non-transgenic plants. The results

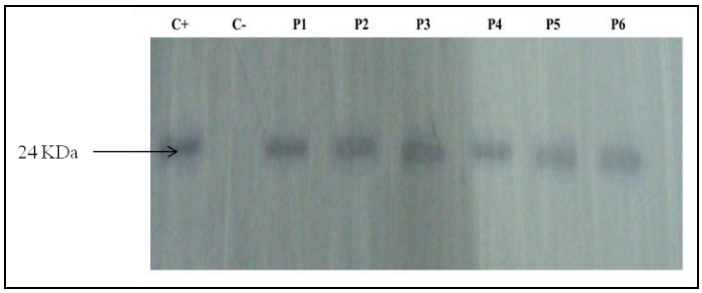


**Fig 3.** Western blot confirmation of HBsAg expression in different generations of transgenic tobacco. C+, Commercial vaccine (Engerix$^{TM}$ B); P1-P6, crude protein from transgenic tobacco plants; C, crude protein from control tobacco.

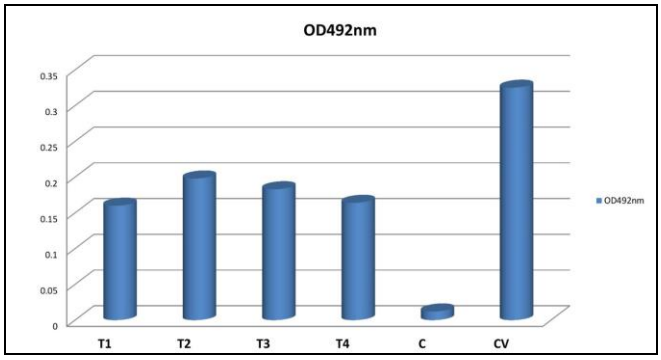


**Fig 4.** ELISA absorbance values detected at 492 nm for transgenic (T1-T4) and control tobacco leaf protein (crude) samples. T1-T4, Transgenic tobacco leaf protein samples; C, Control tobacco leaf protein sample; CV, Commercial vaccine (Engerix$^{TM}$ B)

confirm those of Yogendra et al. (2009), who reported the presence of the HBsAg gene in *Nicotiana tobaccum*, Zhong et al. (2005) transformed *Nicotiana benthamina* plants with pHB117 and pMHB plasmid constructs and Guan et al. (2012) transformed 681 bp recombinant hepatitis B surface antigen of the adr serotype (*HBsAg*/adr) gene containing pBI121/HB to a cherry tomato. Recombinant pHB118 protein was extracted from mature tobacco leaves by ammonium sulphate precipitation. Western blotting was performed to confirm the expression of the HBsAg protein in the transgenic plants. A 24 kDa yeast-derived HBsAg protein was also compared with the recombinant protein produced in transgenic tobacco plants. ELISA was conducted using transgenic and control tobacco plant proteins. The higher absorbance values of the transgenic plant samples indicated the presence of the immunogen in the sample, confirming the presence of recombinant HBsAg. This result is consistent with those obtained by Yogendra et al. (2009), who confirmed the presence of the 24k Da recombinant hepatitis B protein in *Nicotiana tobaccum*. Similar results were obtained by Ganapathi et al. (2007) in soybean cell culture suspension, Shekhawat et al. (2007) in potato and Guan et al. (2012) in cherry tomato. Seeds obtained from the $T_1$ to $T_4$ generations of the transgenic tobacco plants showed the hepatitis B surface antigen segregated in a 3: 1 ratio, as expected, confirming Mendelian inheritance, when they were germinated in the presence of 100 ppm kanamycin. This result is in agreement with the findings of Vasil et al. (1993), who transferred the gene coding for phosphinothricin acetyl transferase (PAT) into wheat plants; PAT activity was detected in a 3: 1 ratio in $R_1$ generation plants following cross or self-pollination. Both male and female transmission of the PAT gene and its segregation as a dominant Mendelian ratio in $R_1$ and $R_2$ plants were demonstrated. Contrary to this, Becker et al. (1994) showed histochemically stained pollen grains of *GUS* gene-transformed wheat plants showed a 1:1 segregation of the *uid A* gene in all plants tested. A 3:1 segregation of the introduced gene was demonstrated by an enzyme sensitivity test and southern blot analysis of $R_1$ generation plants.

## Material and methods

### *Plasmid constructs*

The plant binary vector pHB118 contained the complete unmodified hepatitis B surface antigen from hepatitis B virus strain *npt*-II as a selectable marker gene, the nos (nopaline synthase) terminator gene and a modified CaMV 35S promoter. The plasmid pHB118 was mobilized into an *Agrobacterium* strain LBA4404 (obtained from the National Centre for Biological Sciences, Bangalore, India) by heat shock transformation. During this process *Agrobacterium* competent cells were prepared using 0.1 M $CaCl_2$ and the construct was transferred to competent cells by freezing the cells in liquid nitrogen for 5 min and immediately incubating at 37°C for 5 min. This construct was provided by Dr. Hugh S. Mason, Arizona Biodesign Institute, USA.

### *Transgenic plants*

Tobacco plant variety kanchan (*N. tabacum* cv. 'Kanchun') procured from the Central Tobacco Research Institute, Rajamundry, India was used as the experimental material. The tobacco leaf explants were transferred using *Agrobacterium tumefaciens* and in-vitro regeneration was conducted on kanamycin (50 mg $l^{-1}$) selective media supplemented with 2 mg $l^{-1}$ BAP (6-benzyl amminopurine) and 0.2 mg $l^{-1}$ NAA (naphthalene acetic acid). Cefotaxime (500 mg $l^{-1}$) was also used in this medium to inhibit over-growth of the *Agrobacterium*. Rooted $T_0$ tobacco plants were hardened in a 3:1 (v/v) peat: sand mix, transferred to pots containing soil, and maintained in a controlled environment growth chamber for 15 d before transfer to a greenhouse. The plants were allowed to grow to maturity, seeds were collected and germinated in soil, and 80 transgenic plants from the $T_1$ to $T_4$ lines of tobacco along with 20 control tobacco plants were evaluated for the presence of the HBsAg gene. For each progeny, 20 plants were planted along with the same number of controls in the greenhouse. The transgenic and control tobacco plant progenies were then evaluated for plant growth parameters *viz.*, plant height (cm), number of leaves per plant and leaf area ($cm^2$). The observations were recorded on the $30^{th}$ and $60^{th}$ days after sowing. The data were analyzed using SAS 9.3 with the PROC GLM procedure (SAS Institute, 2011). Treatment means were separated with Fisher's protected least significant difference (LSD) test ($p = 0.05$).

### *PCR analysis*

Genomic DNA was isolated from the second and third leaves from the top of transgenic tobacco plants by the CTAB method (2% CTAB, 1.4 M NaCl, 0.02 M EDTA, 0.2% β-mercaptoethanol, 0.1 M Tris-HCl and 1% PVP). The genomic DNA was then subjected to PCR analysis. Each PCR mix contained 10 pMol of each primer, 1 U of *Taq* DNA polymerase, 2.5 µM of each dNTP, 1X Taq buffer (Bangalore Genei) and 0.2 µg of genomic DNA as template

for a total volume of 25µL. The PCR conditions were 94°C for 2 min of initial denaturation followed by 30 cycles of amplification, with each cycle consisting of the following steps: 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, with a final extension at $72^0$C for 5 min and a final hold at 4°C (Nagaraju et al., 1998). The amplified products were separated on 1.2% agarose (Sigma-Aldrich, Bangalore, India) gels. The primer sequences used to amplify a 900 bp fragment of the HBsAg gene are: Forward primer (5′-CATTCTACTTCTATTGCAGC-3′) and Reverse primer (5′-ACGTGGTAACTTAGATGTACACCCAAAG-3′) (Zhong et al., 2005).

***Western blot analysis***

Total protein was extracted from transgenic and control tobacco plants using a protein extraction buffer (pH 8.0, 0.1 M Tris-Hcl, 0.001 M PMSF, β-mercaptoethanol, sodium sulphate, 2% PVP) (Nagesha et al., 2006). These crude proteins were subjected to ammonium sulphate precipitation and concentrated by dialysis. The concentrated protein samples were then run on 12% SDS-PAGE and transferred to a PVDF membrane (Whatman, India) by electroblotting. This was carried out using a Mini trans-blot electrophoretic transfer cell (Bio-Rad, India) by applying 60 V for 2 h and then transferring to a blocking solution containing skimmed milk powder [5% (w/v)] in 1X phosphate buffered saline (PBS). This was followed by incubation in primary antibody (mouse anti-hepatitis B surface antigen, Invitrogen) and secondary antibody (goat anti-mouse IgG horseradish peroxidase conjugate, Invitrogen) for 1 h each at room temperature. The signal was detected using DAB (Diamino benzidine) substrate (Bangalore Genei, India).

***Enzyme linked immunosorbant assay (ELISA)***

The crude protein samples of control and transgenic tobacco leaves were also used as an antigen source for ELISA. They were incubated in blocking solution for 1 h at 37°C, followed by incubation with the primary and secondary antibodies for 1 h each at 37°C. Detection involved 0.4 mg $mL^{-1}$ of chromogen orthophenylene diamine (Sigma-Aldrich, India) with 1 µL $mL^{-1}$ of hydrogen peroxide as substrate. Absorbance was recorded at 492 nm. The protein extracted from non-transgenic tobacco plants was used as a negative control, and yeast derived HBsAg protein was used as a positive control.

***Inheritance of the hepatitis B surface antigen by kanamycin assay***

Seeds obtained from putative transgenic tobacco plants with hepatitis B surface antigen gene and non-transgenic plants were tested for their germination in the presence of 100 ppm kanamycin (Sigma-Aldrich, India) (non-transgenic plants cannot survive at this concentration). The *npt*II gene was used as a selectable marker gene in transforming tobacco with the HBsAg gene. Expression of the *npt*II gene in transgenic plants confers resistance to kanamycin and aids in the selection of putative transgenics in tissue culture. This trait can also be used to study stable gene expression in transgenics over generations. Observations were made after 10 days of incubation by counting the number of germinated seeds. The ratios of germinated and non-germinated seeds were recorded.

***Statistical analysis***

The goodness of fit of the observed segregation ratio for the transgene was tested against the Mendelian segregation ratio (3: 1) using the chi-square ($\chi^2$) test. The $\chi^2$ values were calculated using the formula (Greenwood et al., 1996):

$$\chi^2=\sum \frac{(\text{Observed frequencies} - \text{Expected frequencies})^2}{\text{Expected frequencies}}$$

**Conclusion**

The Results of the present study reveal that the transgene can be efficiently inherited from generation to generation and maintains stable transgene expression. The expression of 24 kDa hepatitis B surface antigen in transgenic tobacco plants was confirmed by western blotting and ELISA. In conclusion, we report that no morphological variations occurred in different generations of tobacco plants and the hepatitis B surface antigen was stably inherited in all the generations.

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