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Biotechnological Reduction of Tobacco (*Nicotiana Tabacum* L.) Toxicity

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ABSTRACT

Background: *Nicotiana tobacco* contains large amounts of alkaloid nicotine. Tobacco plant is used for smoking and causes many health problems since it is high in nicotine which is one of the widely-recognized toxic compounds with serious side effects for different body organs. Reducing nicotine content of this plant is a way to reduce its health hazards in cigarette smokers. Utilizing the new methods of genetic engineering can modify nicotine levels in the plant. In this study, through transferring the blocking gene, the pathway of nicotine biosynthesis was blocked to produce transgenic tobacco with low levels of nicotine.

Methods: Transgenic plants carrying T DNA, and non-transgenic plants were grown on MS medium. Then their leaves were dried and powdered. The plants were extracted with alkali solution. Eventually, the nicotine content of the extract were analyzed using GC.

Results: The analysis of extracts showed a reduction in the nicotine content of the transgenic plant (contain T-DNA) in comparison with non-transgenic plants.

Conclusion: Tobacco with lower nicotine reduction can reduce the toxic effects of smoking on smokers and can facilitate withdrawal from it.

Keywords: Nicotine, Non-Transgenic Tobacco, Tobacco, Toxicity, Transgenic.

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INTRODUCTION

Extensive studies have been done on the toxic properties of nicotine and its physiological impacts on human. In fact, nowadays, nicotine is introduced as one of the most harmful toxins affecting human health with great certainty. It has been demonstrated that upon entering human body, nicotine induces severe physiologic disorders and its high doses can lead to death (1). Several reports have shown that despite improved public awareness of the toxic effects of nicotine and smoking, the smoking rate of the produced cigarettes throughout the world has been more than doubled in 1967-92 years (from 2.8 to 5.7 trillion); nearly 25% of the produced cigarettes were smoked in this period.

The consequences of this increase can now be seen in developed countries where smoking was quite prevalent in the 1950s and 1970s. Nearly 20% of all deaths in developed countries in 1990 were due to using tobacco products. In the 35-69 age group, around 35% of deaths in men and 15% in women have been reported to be due to smoking (2).

According to WHO projection, in 2020-30, 10 million deaths will occur per annum (of these, 70% in developing countries) (3). Estimations have shown that only in the United States, more than 76 billion dollars of health care costs are currently spent on dealing with the adverse effects of smoking and even a greater sum of money is needed for providing the costs

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of disabilities in smokers (4). It has also been reported that 70% of smokers try to quit smoking; however, due to serious physiologic dependency on nicotine, only 5% of them succeed (5, 6). A variety of ways , such as producing light cigarettes or using filter, have been suggested for reducing nicotine intake but research has indicated their inefficiency (7,8,9).

One of the most important guidelines for minimizing health hazards associated with nicotine is to reduce the amount of nicotine in tobacco before use. This can be achieved through inhibiting or lowering the expression of key enzymes of the nicotine biosynthesis pathway. Therefore, by using the gene transfer system through agrobacterium, foreign genes can be transferred to the plant and alter the production of secondary metabolites such as nicotine (10, 11, 12).

Noticing the importance of social health and the effects of nicotine, this study was conducted to link T-DNA containing Kanamycin resistant genes and β-glucuronidase, as selective genes, and reporting genes to the cells of tobacco plant and reproducing transgenic plants for investigating nicotine level alterations in them.

MATERIALS AND METHODS

Reproduction of the transgenic plant

Using *Agrobacterium tumefaciens* containing Pzm1047 plasmid with T-DNA, which carries the Kanamycin (NPTII) resistance gene, and β-glucuronidase and its concomitant culture with tobacco plant leaves in suspension environment, the genes were transferred. Then the leaves of the transgenic plants were transferred to the solid culture media. Through creating a callus and reproducing the plant, the transgenic tobacco was obtained. The transgenic test of the reproduced plant was done through PCR by using NPTII primer (13).

For proliferation of the transgenic plant containing T-DNA and non-transgenic plants, basic culture media (Murashing and Skoog) was used. In order to provide this culture media, 1000 ml of double-distilled water was poured into the Erlenmeyer and a magnet was placed inside it which was placed on a shaker. First, 30g of Sucrose was added to the mixture and when it was completely dissolved and the temperature of the flask reached 60-70 °C, 4.5g of the MS culture media powder was gradually added so that it could spread well. When the temperature of the content of the flask reached 80-90 °C, agar was gradually added. By using NaoH and HCL, The pH of the solution was maintained at 5.6-5.8 (14).

Nicotine extraction

After reproducing the transgenic and non-transgenic plants, the leaves were carefully collected and dried in a 50 °C degree oven for 10 hours. After complete drying, the leaves were milled and powdered. One of the powdered plants (each plant separately) was carefully weighed and mixed with 40ml of hexane containing 0.05% heptadecane, as the internal standard, 20 ml of distilled water, and 10 ml of normal sodium hydroxide, 8% (w/v) aqueous solution, for one hour. Finally, the hexanic phase was separated and it was analyzed by GC (15).

For GC analysis, nicotine stock was first prepared by dissolving 0.24g of standard nicotine in 50ml of the distilled water that 100ml of hexane containing 0.05% heptadecane, as the internal standard, was added to it. Then 25ml of the normal sodium hydroxide, 8% (w/v) aqueous solution, was added to this solution and it was kept in 4-8°C. Next, 1.25, 2.5, 3.75, 6.25, and 10 ml of the hexanic phase which contained nicotine were transferred to different 25ml volumetric flasks and with hexane containing 0.05% heptadecane it reached the required volume. This way, 0.22, 0.45, 0.675, 1.125, and 1.8 mg/l concentrations of the standard nicotine were provided (16).

After that, 0.5 µl of the standard nicotine concentration of each one was separately injected into the GC three times and according to the area under the nicotine curve and internal standard of nicotine, the standard nicotine curve was drawn. Finally, by adding 0.5 µl of the extract obtained from transgenic and non-transgenic and based on the low area under the curve and the internal standard in the extract, the concentration of the nicotine present in the extract was measured by standard nicotine curve equation (15).

The specifications of the GC device used in this study were as follows:

Column	OV1-capillary column
Type of detector	FID
Temperature of (FID) detectors	280°C
Ramp rate	15°C/minute
Temperature of the oven	60-275°C
Injector temperature	275°C

RESULTS

Nicotine analysis

In nicotine analysis, based on this method, the standard samples and plant extracts were evaluated. The results for nicotine standards and the samples of the plant extract with the peak nicotine level with the retention time of 8.2 minutes and 12.36 minutes for heptadecane were obtained.

For each one of the standard samples and plant extracts, three repetitions of the analysis were done and their mean was measured. Then a graph was drawn based on the mean of area under the curve to the internal standard in proportion to the nicotine concentration and the equation of the line was obtained.

By using this equation and the mean of the area under the curve for nicotine to the internal standard for alcoholic extracts, nicotine concentration was measured for each plant. The results of this analysis are shown in Figure 1. As it can be observed, the amount of nicotine in the transgenic plant containing T-DNA compared to the non-transgenic plant showed a significant difference.

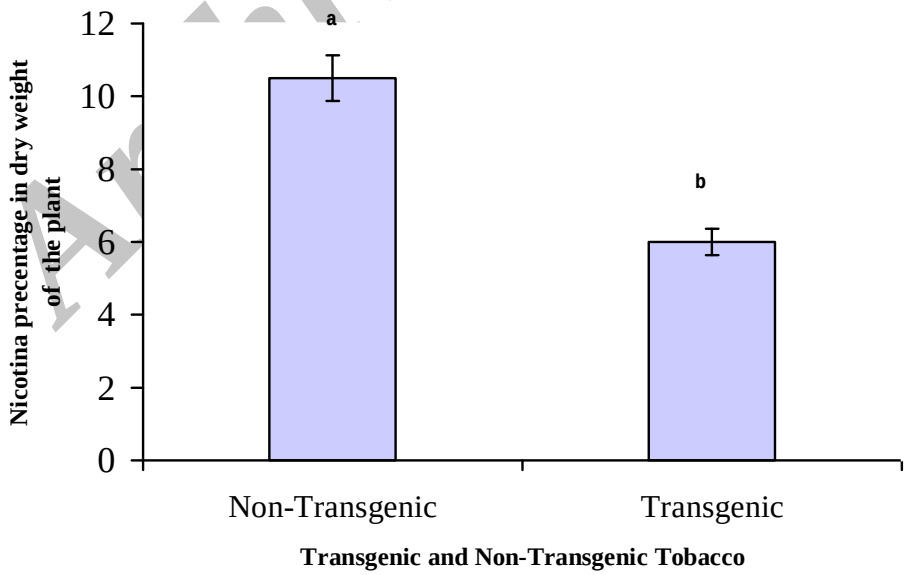

Figure 1. The amounts of nicotine alkaloids in transgenic plants containing T-DNA and the non-transgenic plants according to GC analysis (the letters which are not common between the two groups indicate the significance of the results based on Tukey test, P<0.05)

DISCUSSION

In this study, the change in the amount of nicotine alkaloid in transgenic and non-transgenic plants was evaluated. The results of the quantitative analyses of alkaloids indicated a significant difference in nicotine concentrations in aerial part of the plant. Transgenic plants showed a significant decrease in the amount of nicotine in comparison with the non-transgenic plants. From this decrease of nicotine in transgenic plants, it can be inferred that T-DNA transfer to plant cells has probably affected the production of these products by affecting the genes synthesizing nicotine and, as a result, the expression of the enzymes biosynthesizing alkaloids.

Type, intensity, and amount of this effect depend on many factors such as the number of T-DNA copies merged inside the plant cells genome and the merging location of these copies. At this level, it can be hypothesized that T-DNA transfer to the genome of tobacco plant has occurred through shutting down the transcription pathway of key enzymes genes of the biosynthesis route of alkaloids in the transgenic plant.

Overall, noticing the presence of little information on the effects of transferring T-DNA carrying Gus gene and NPTII on the amount of the alkaloids of this plant, the findings of this study can probably be used as primary data for explaining how this transformation affects the biosynthesis of nicotine in transgenic and non-transgenic plants used in future research.

The difference in nicotine values in the aerial part of the plant might be associated with the disruption of nicotine transfer from the roots to the air segment of the transgenic plant under study since lack of biosynthesis and transfer to the air organs can both justify the reduction in the amount of nicotine.

In general, every mechanism leading to nicotine decrease in tobacco can result in the reduction of tobacco toxicity and consequently less harm to cigarette smokers. Several attempts have been made in this regard (17).

CONCLUSION

However, research has shown that even cigarettes with lower nicotine levels due to the presence of other chemical compounds can induce health problems. Higher levels of nicotine result in cardiovascular diseases and other chemicals present in cigarettes are associated with cancer, yet what causes addiction to cigarettes and makes withdrawal from it difficult is nicotine. Reduction in tobacco facilitates its withdrawal and allows educational programs to have a greater chance of success for having a cigarette-free society. This way, not only are cigarette smokers freed from the dangers of this toxin, but also passive smokers in contact with them who inhale the smoke are protected since, in the United States alone, nearly 3000 patients with lung cancer and 35000 (18) of those with coronary heart diseases are individuals who are condemned to live with smokers.

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