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AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF THE *GMAP2* GENE CONSTRUCT INTO TOBACCO

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ABSTRACT

Background: Soybean (*Glycine max*) is high-value grain crops recognized for their nutritional benefits and soil-improving capacity. However, its tolerance to drought and salinity is relatively low. Within the soybean genome, the *AP2* gene family has been identified as playing a key role in activating the transcription of stress-responsive genes. **Objectives:** The aim was to evaluate the gene's function and establish a foundation for developing transgenic soybean lines with enhanced tolerance to drought and salinity. **Methods:** In this study, a vector construct carrying the *GmAP2* gene, which encodes a transcription factor protein of the AP2 family, was successfully introduced into Nicotiana tabacum cultivar K326 via *Agrobacterium tumefacien*s-mediated transformation. Transformed samples were regenerated *in vitro*, selected using antibiotic resistance, and cultured to produce complete plants. **Results:** Results showed that 62 leaf explants produced multiple shoots, forming 145 shoot clusters. Among these, 227 shoots were selected and elongated, with 112 successfully rooted. A total of 65 transgenic plants were acclimatized and transferred to the substrate; 29 plants survived and grew under greenhouse conditions. **Conclusions:** The pBI121-*GmAP2* construct was

successfully introduced into the K326 tobacco cultivar via *Agrobacterium tumefaciens*. Transformed explants were regenerated *in vitro*, selected using antibiotic screening, and subsequently developed into transgenic tobacco plants.
Keywords: Genetic transformation; *GmAP2*; Abiotic stress; Transcription factor; Tobacco

BIẾN NẠP DI TRUYỀN CẤU TRÚC MANG GENE *GMAP2* THÔNG QUA *AGROBACTERIUM TUMEFACIENS* VÀO THUỐC LÁ

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TÓM TẮT
Đặt vấn đề: Đậu tương (*Glycine max*) là cây trồng thu hạt có giá trị kinh tế cao, giàu dinh dưỡng và góp phần cải tạo đất. Tuy nhiên, đậu tương lại có khả năng chịu hạn và mặn kém. Trong hệ gene của đậu tương, các gene thuộc họ AP2 đã được xác định có vai trò kích hoạt phiên mã các gene liên quan đến khả năng chống chịu khi cây gặp stress. **Mục tiêu:** Nhằm đánh giá chức năng của gene *GmAP2* và tạo nền tảng cho việc phát triển các dòng đậu tương biến đổi gene có khả năng chịu hạn, chịu mặn. Các mẫu sau biến nạp được tái sinh *in vitro*, chọn lọc bằng kháng sinh và tiếp tục nuôi cấy để tạo cây hoàn chỉnh. **Phương pháp:** Trong nghiên cứu này, cấu trúc vector mang gene *GmAP2*, mã hóa protein thuộc phân họ nhân tố phiên mã AP2 của đậu tương, thông qua phương pháp biến nạp trung gian bởi *Agrobacterium tumefaciens*

(A. tumefaciens) đã được chuyển thành công vào giống thuốc lá K326. **Kết quả:** Ghi nhận 62 mảnh lá hình thành đa chồi, tạo 145 cụm chồi, trong đó 227 chồi được chọn lọc và kéo dài, với 112 chồi ra rễ thành công. Tổng cộng 65 cây được trồng trên giá thể, trong đó 29 cây sống sót và phát triển trong điều kiện nhà lưới. **Kết luận:** Cấu trúc pBI121-*GmAP2* đã được biến nạp thành công vào giống thuốc lá K326 thông qua chủng *A. tumefaciens*. Các mẫu biến nạp được tái sinh *in vitro*, chọn lọc bằng kháng sinh và tạo cây thuốc lá chuyển gene.
Từ khóa: Biến nạp di truyền; *GmAP2*; Stress phi sinh học; Nhân tố phiên mã, Thuốc lá

INTRODUCTION

Soybean (*Glycine max (L.) Merrill*) is among the world's most significant food crops, including in Vietnam, due to its high nutritional value and its role in improving soil fertility. Increasing demand for soybean-based products is driven by their health benefits, including prevention of chronic diseases such as cancer, diabetes, and obesity, as well as their contributions to lowering cholesterol and protecting kidney function. Soybeans provide 32 - 52% protein and 12 - 25% lipid content and are rich in essential vitamins, amino acids, fiber, and secondary metabolites beneficial to human health [1,2]. However, abiotic stresses—particularly drought and salinity—can cause substantial yield losses, sometimes up to 40% [3]. As climate change intensifies, with prolonged droughts and increased saline intrusion, it becomes increasingly challenging to cultivate soybean under conventional conditions. Developing stress-tolerant

varieties is thus critical. Modern plant biotechnology, particularly gene transformation, offers promising tools for enhancing stress resistance [4]. Among these, transcription factors (TFs)—proteins that bind to promoter regions and regulate gene expression—play a pivotal role. The AP2 (APETALA2) transcription factor family has been shown to mediate responses to drought and salinity by activating specific stress-responsive genes [5]. The *GmAP2* gene from soybean encodes such a TF. Tobacco (*Nicotiana tabacum)* is a widely used model plant in genetic transformation due to its ease of *in vitro* regeneration, rapid life cycle, and well-established transformation protocols [6]. This study aims to introduce the *GmAP2* gene into tobacco K326 via *Agrobacterium tumefaciens*-mediated transformation to explore its potential role in stress tolerance and lay the groundwork for future soybean transformation.

METHODS

Research Materials, Time and Location

Materials: Seeds of Nicotiana tabacum K326 were provided by the Plant Cell Technology Department, Institute of Biotechnology. The *Agrobacterium tumefaciens* strain harboring the pBI121_AP2 expression vector was maintained at the Plant Cell Technology Laboratory, Faculty of Biology, Thai Nguyen University of Education.

Research Location: The study was conducted at the Department of Biology, Thai Nguyen University of Medicine and Pharmacy, and the Plant Cell Technology Laboratory, Faculty of Biology, Thai Nguyen University of Education.

Timeline: January 2025 to June 2025.

Sampling Method: Seeds were selected based on morphological characteristics, with preference given to clean, mold-free, well-preserved seeds demonstrating good germination potential.

Transformation and Regeneration Procedure

The method for introducing the *GmAP2* gene construct into tobacco was carried out following the protocol described by Topping (1998), with the following key steps:

(1) Preparation of transformation material: Tobacco seeds were surface-sterilized by immersion in 70% ethanol for approximately 20 seconds, followed by treatment with a sterilizing solution containing 30% Javel and 0.05% Tween-20 for 20 minutes. The sterilizing solution was then discarded, and the seeds were rinsed five times with sterile distilled water.

(2) Preparation of the infectious bacterial suspension: *Agrobacterium tumefaciens* harboring the transformation construct was cultured in 15 mL of liquid LB medium supplemented with kanamycin (50 mg/L) and rifamycin (50 mg/L) at 28oC, shaking at 200 rpm for 48 hours. Subsequently, 10 mL of the bacterial suspension was transferred to 50 mL of antibiotic-free LB medium and incubated under the same conditions until the optical density at OD600nm reached 0.8, which was considered the optimal cell density for transformation. The bacterial culture was then centrifuged at 5000 rpm for 15 minutes at 4oC. The resulting pellet was resuspended in 40 mL of chilled 1/2 MS solution supplemented with 50 µL of acetosyringone (AS), and kept on ice.

(3) Induction of multiple shoot regeneration: Following co-cultivation, the leaf explants were washed by immersion in 1/2MS medium supplemented with cefotaxime (400 mg/L) for 10 minutes, blotted dry, and transferred to MS medium containing kanamycin (50 mg/L) and cefotaxime (400 mg/L) for selection and shoot regeneration.

(4) Shoot selection and elongation: After 3 - 4 weeks, the formed shoot clusters were dissected into individual shoots and transferred to elongation medium (SIM), which consisted of MS medium supplemented with BAP (1 mg/L), kanamycin (50 mg/L), and cefotaxime (400 mg/L).

(5) Root induction: After 4 - 5 weeks, shoots that had elongated to a height of 2 - 3 cm were excised and transferred to rooting medium (RM), composed of MS medium supplemented with IBA (0.2 mg/L), kanamycin (50 mg/L), and cefotaxime (400 mg/L).

(6) Acclimatization and greenhouse transfer: After an additional 3 - 4 weeks, rooted plantlets with 3 - 4 true leaves were transplanted into pots containing a 1:1 mixture of husk and sand. Once the plants developed 4 - 5 true leaves, they were transferred to greenhouse conditions for further growth.

Data processing and calculations were carried out using Excel software.

RESULTS

Preparation of transformation material and co-cultivation

After surface sterilization, tobacco seeds were germinated on MS medium. The resulting seedlings were allowed to grow for approximately 3 - 5 weeks

until they developed 3 - 4 true leaves, at which point they were used for transformation experiments (Figure 1A). Leaves from these plants were excised into segments approximately 1 cm^2 in size and mechanically wounded. The wounded leaf segments were then immersed in a suspension of *Agrobacterium tumefaciens* containing the plasmid construct with the *GmAP2* gene for 10 minutes (Figure 1B). Following inoculation, the leaf segments were blotted dry on sterile filter paper and transferred to co-cultivation medium on Petri dishes. The co-cultivation medium consisted of MS supplemented with BAP (1 mg/L), kanamycin, and cefotaxime, and was incubated at 25°C (Figure 1C).

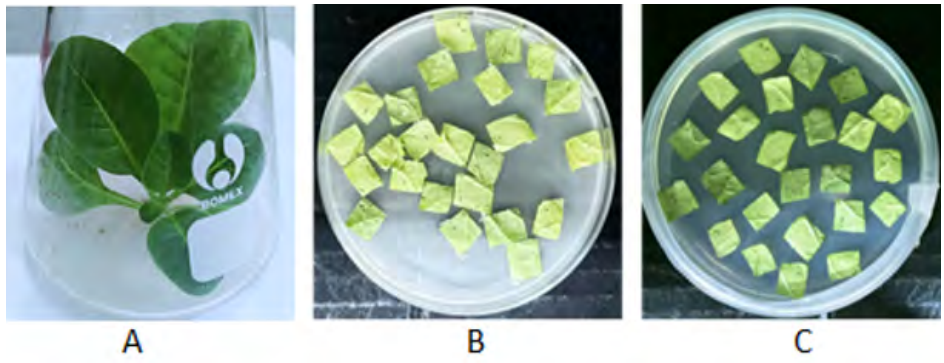

Figure 1. Genetic Transformation Process

** **A:** In vitro tobacco K326 plantlet; **B:** Leaf segment from in vitro tobacco immersed in recombinant A. tumefaciens suspension containing the gene construct; **C:** Co-cultivation on selective medium.*

The results of multiple shoot induction are presented in Figure 2. After co-cultivation in darkness for two days, the leaf explants were washed with cefotaxime (400 mg/L) and transferred to shoot induction medium (SIM) supplemented with BAP (1 mg/L) and kanamycin (Figure 2A). After two weeks of culture,

small shoot clusters began to emerge from the leaf tissues (Figure 2B). When the shoots became more distinct (after 3 - 4 weeks), the clusters were dissected into individual shoots and transferred to a specialized medium for shoot elongation (Figure 2C).

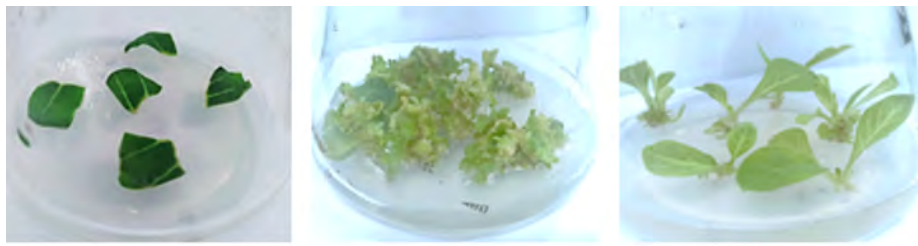

Figure 2. Multiple shoot regeneration and development of transgenic tobacco plants

** **A:** Tobacco leaf explant on SIM medium; **B:** Leaf explant forming multiple shoots on SIM after 2 weeks; **C:** Shoots growing on shoot elongation medium.*

Results of transgenic plant generation

The transformation experiments using *Agrobacterium tumefaciens* to introduce the *GmAP2* gene construct were conducted in three independent replicates. The results of the transformation trials, along with the corresponding control treatments, are presented in Table 1.

Table 1. Results of genetic transformation of the construct into tobacco

Transformation and control trials		Number of explants	Number of shoot clusters	Number of longated shoots	Number of rooted shoots	Number of plants transferred to substrate	Number of surviving plants on substrate
Experiment	1	30	45	71	42	20	9
	2	30	52	76	40	20	10
	3	30	48	80	60	25	10
	Total	90	145	227	112	65	29

C0*	30	0	0	0	0	0
C1*	30	50	100	60	40	20

: **C0: Non-transformed tobacco leaf explants cultured on regeneration medium supplemented with selective antibiotics; **C1:** Non-transformed tobacco leaf explants cultured on regeneration medium without selective antibiotics.*

Figure 3. Rooting stage and transplanting of transgenic plants to substrate

** **A:** Rooted shoots on rooting medium; **B:** Transgenic tobacco plants grown on substrate under greenhouse conditions.*

The results presented in Table 1 show that, after three transformation trials involving a total of 90 leaf explants from tobacco cultivar K326, 62 explants survived and regenerated into 145 shoot clusters. These clusters were transferred to shoot elongation medium supplemented with selective antibiotics, from which 227 individual shoots developed. Among these, 112 shoots successfully formed roots and were transferred to the substrate stage. A total of 65 plants survived the *in vitro* phase, and ultimately, 29 plants exhibited healthy growth under greenhouse conditions. Alongside the transformation experiments using recombinant *A. tumefaciens* carrying the *GmAP2* gene construct, two control groups (C0* and C1*)—each consisting of 30 explants—were cultured through the same stages of shoot induction, elongation, and rooting. In the C0* group, non-transformed tobacco leaf explants were

cultured on regeneration medium containing selective antibiotics. As a result, all explants were eliminated by the antibiotics, with no shoot formation observed, confirming the effectiveness of antibiotic selection in eliminating non-transformed tissues. In contrast, the C1* group, in which non-transformed explants were cultured on regeneration medium without selective antibiotics, yielded 50 shoot clusters, 100 elongated shoots, and 40 rooted shoots. These were subsequently transferred to substrate, with 20 plants surviving under greenhouse conditions. These results demonstrate that tobacco tissue exhibits strong regenerative capacity in the absence of selection pressure.

DISCUSSION

In recent years, tobacco has been widely utilized as a model plant system in genetic transformation studies due to its notable advantages, including ease of *in vitro* culture, high regeneration efficiency, robust capacity for multiple shoot formation, and particularly, a high transformation rate. These characteristics make tobacco an ideal platform for evaluating gene function and optimizing transformation systems prior to application in target crop species [6]. Several studies have leveraged these advantages to investigate the expression of soybean-derived genes using the tobacco model. Specifically, genes such as GmP5C [8], GmEXP1 [9], RNAi constructs [10], HA1 [11], and GmDREB [12,13] have been successfully introduced into tobacco to assess their biological roles in conferring tolerance to abiotic stress conditions. Rachmat et al. (2021) also employed *Agrobacterium*-mediated transformation to introduce the OsNAC6

gene into tobacco, reporting the highest transformation efficiency of approximately 17% when using stem explants. In comparison, leaf and callus explants yielded lower transformation rates of 10% and 15% [14], respectively. In our study, using 90 transformed leaf explants, we obtained 29 surviving plants on substrate, corresponding to a transformation success rate of approximately 25% based on the number of initial shoots. This efficiency is considered favorable. The differences in transformation efficiency observed across studies may be attributed to variations in culture conditions, bacterial strains employed, or the specific target gene used in the transformation process.

CONCLUSION

The pBI121-*GmAP2* construct was successfully introduced into tobacco cultivar K326 via *Agrobacterium tumefaciens*-mediated transformation. The transformed samples were regenerated *in vitro*, selected using antibiotics, and developed into transgenic tobacco plants. A total of 62 leaf explants produced multiple shoots, resulting in 145 shoot clusters. From these, 227 individual shoots were selected on elongation medium, and 112 shoots successfully developed roots. Ultimately, 65 transgenic plants were transferred to substrate, with 29 plants surviving and growing under greenhouse conditions.

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