EXPRESSION OF KOREAN MISTLETOE LECTIN A-CHAIN GENE IN GENETICALLY MODIFIED *NICOTIANA TABACUM*

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ABSTRACT

Genetically modified (GM) tobacco (*Nicotiana tabacum*) harboring *KMLA* has been constructed to develop a plant expression system for the protein, KMLA. The 747 bp-long of the *KMLA* gene was ligated into *BamHI/SacI*- cut pCAMBIA3300 to obtain a recombinant plasmid, *pCAMBIA3300-kmla*. It was then transformed into *Agrobacterium tumefaciens* LBA4404 by liquid nitrogen method and the *A. tumefaciens* LBA4404/ pCAMBIA3300-*kmla* was inoculated into leaf discs of tobacco (20 days old). After co-cultivation, numerous calli were developed from transformed tobacco leaf explants which were cultured on MS basal medium containing kanamycin (Kan) (50 mg/ml), and then there were three GM tobacco plants generated from these calli. Transgenicity of the tobacco plants was confirmed by PCR screening and the *KMLA* gene expression was observed by Western blot

Keywords: *Agrobacterium tumefaciens, Lectin A-subunit from Korean Mistletoe, transformation, pCAMBIA3300-kmla, PCR, Western blot.*

INTRODUCTION

Plant molecular breeding is a rapidly developing scientific issue in global agriculture owing to *in vitro* gene manipulation along with *A. tumefaciens*-mediated transformation (Gelvin 2003, McCullen&Binns 2006, Bedo *et al.* 2009). Thus, there are numerous documents of GM plants, especially economically important crops such as wheat, barley, oats, rice, corn, soybean, tobacco and so on (Craig *et al.*, 2008). Manufacturing pharmaceuticals, therapeutic proteins and vaccines from GM plants has become more and more attractive over the past few years because of its cost-effectiveness (Ma *et al.* 2005, Crowell *et al.* 2008, Luisa *et al.* 2009, Cardi *et al.* 2010, Masoumiasl *et al.* 2010).

Indigenous mistletoes in Korea belongs to *Viscumalbum* subsp. *coloratum* Kom (Long 1982, Lee 2001). It is a hemi-parasitic shrub, which grows on the stems of other trees, such as some species of *Acer, Carpinus, Juglans, Sorbus, etc.* (Long 1982). It has stems 30–100 centimeters (12–39 in) long with dichotomous branching.

From ancient time, plant extract has been used in Asian and European traditional medicine such as spasmolytic, sedative and anti-cancer (Maekelae 1957). It is now known that the major ingredient of the extract is lectin. A lectin is a sugar-binding protein of non-immune origin that agglutinates cells or precipitates glycoconjugates (Paszuti 1991) Korean mistletoe lectins (KMLs) are classified into two group (Paszuti 1991), which react with *N*-acetyl-D–galactosamine and/or D–galactose. The biological activity of the KMLs has been recognized as Type II ribosome inactivating proteins (RIPs) (Barbieri *et al.* 1993). The RIPs consists of two chains with subunits of A-chain and B-chain. Two chains are linked with a disulfide bond. The A-chain disrupts the selective hydrolysis of the *N*-glycosidic bond at the adenine-4324 in the eukaryotic 28S ribosomal RNA (Paszuti 2004). The properties of *lectin* gene have well been elucidated by using molecular cloning methods. There were numbers of cloned gene of *Korean misletoe lectins (KMLs)* and their nucleotide sequences have been submitted to GenBank (GenBank accession number AF508915, AF508918, A58597). The objective of this research is to

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investigate whether the *kmla* gene, can be expressed in a photoautotroph using a well-studied plant material, tobacco (*Nicotiana tabacum* cv. Petit Havana).

MATERIALS AND METHODS

Materials

In our experiments, reagents and other materials were used as follows: bacterium culture medium (Difco, U.S.A.); gene transfection reagents (Stratagene, U.S.A.); acrylamide and agarose (Sigma or Bio-Rad, U.S.A); PCR and immunoblotting reagents (Sigma, USA); organic solvents (Merk, Germany). Oligonucleotides were synthesized by Cosmo Genetech, Korea. The plant binary vector pCAMBIA3300 was from Clontech (California, USA). The strain was cultured in Luria-Bertani (LB) (Sambrook *et al.* 1989). Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) (Suwon, Korea) were mediated by *Agrobacterium tumefaciens* LBA4404 (*Ach5*, *pTiAch5*, Sm*R*, Sp*R*). Oligonucleotide primers were used in PCR to amplify fragment A-chain gene from transgenic tobacco plants are: *kml-F:*

5' TACGAGAGGCTAAGACTCAGAGTT 3'

kml-R:

5' GTCCTCGCATACAAACAACATGAT 3'

Methods

Agrobacterium - mediated transformation and leaf explant culture

DNA fragments spanning entire region of open reading frame (ORF, 747 bp) of the *kmla* gene was obtained from pGEM-T-*kmla* restricted by *Bam*HI/*Sac*I (Kong 2005). It was inserted into pCAMBIA3300 binary vector restricted with *Bam*HI/*Sac*I to generate a recombinant plasmid pCAMBIA3300-*kmla*. All the tissue culture procedures described below, were followed in previous publications (Sambrook *et al.*1989, Gelvin 2003). The pCAMBIA3300-*kmla* was transfected into *A. tumefaciens* LBA4404 by liquid nitrogen method. The selected *A. tumefaciens* LBA4404/ pCAMBIA3300-*kmla* were further screened by plasmid mini-preparation and sizing on agarose gel (0.8 *per cent*) Tobacco leaf discs (1.0 cm × 1.0 cm) were infected with the *A. tumefaciens* LBA4404/ pCAMBIA3300-*kmla* ($A600$ = 0.5–0.7) for 30 min. After two days in the darkness, the leaf explants were transferred onto regeneration medium supplemented with Kan (50 $\mu g/\mu\ell$) and cefotaxime (200 $\mu g/\mu\ell$) to select GM tobacco cells harboring the gene *kmla* in a growth chamber set at 4,000 lux for 16 h per day at 24°C. After 3 – 4 weeks, the GM tobacco cells could produce calli. When the size of calli is larger than 5 mm, they were transferred into a new regenerating medium for the root induction. Transgenic tobacco explants were shooted after 3-4 weeks later and then transferred to root medium containing 4.4g/L MS mixture, 250mg/L cefotaxime sodium and 8g/L plant agar. Transgenic tobacco explants were rooted after 2 weeks later and transferred into pots containing sterilized commercial soil.

Identification of *kmla* transgene in GM tobacco plants

Kmla transgene in transgenic tobacco plants was identified by PCR with primers *kml-F/kml-R*. PCR conditions for *kmla* gene: one cycle at 94°C for 5 min, following 30 cycles (1 min of denaturation at 94°C; 1 min of annealing at 56°C; and 1 min 30 sec of extension at 72°C, final hold at 4 °C). The PCR products were analyzed by electrophoresis in agarose gel 0.8% followed by ethidium bromide staining. The molecular size of PCR products is expected to be 747 bp.

Detection of KMLA protein in GM-tobacco by Western blot

KMLA protein in GM-tobacco was detected by Western blot (Kim *et al.* 2006) and procedures were followed by the directions of manufacture in Bio-Rad immune blot™ (GAR-HRP) Kit procedure.

RESULT AND DISCUSSION

Generations of pCAMBIA3300-*kmla*, *A. tumefaciens* LBA4404 /pCAMBIA3300-*kmla* and transgenic tobacco plants

Kmla gene was subcloned into a plant transformation vector. Only the ORF of the 747 bp DNA was ligated into *Bam*HI/*Sac*I-cut pCAMBIA3300 to construct the recombinant clone of pCAMBIA3300-*kmla* (Fig.1). The ligation was introduced into the host cells, *A. tumefaciens* LBA4404, to increase the number of putative transformants, *A. tumefaciens* LBA4404/ pCAMBIA3300-*kmla*. After that, some colonies harboring the T-DNA with *kmla* were selected on the solid agar plate supplemented with the antibiotic (Kan) as described in the method. Therefore, it is safe to use pCAMBIA3300-*kmla* constructed as an *Agrobacterium*–based transformation vector.

Agrobacterium-mediated transformation of pCAMBIA3300-*kmla* construct via leaf infection and regeneration of tobacco plants (Fig.2). Some Kan-resistant calli were selected from tobacco leaf explants inoculated with *A. tumefaciens* LBA4404/ pCAMBIA3300-*kmla* on MS basal medium plus Kan (50 μg/μℓ). After 3 – 4 weeks, GM-tobacco roots were produced from the calli (Fig. 2A, 2B, 2C). Subsequently, soot systems were generated after 1-3 months (Fig.2D). Three transgenic plantlets were grown on the agar medium for additional 2-weeks, and then transferred into pots containing sterilized commercial soil in GM-room (Fig. 2E, 2F).

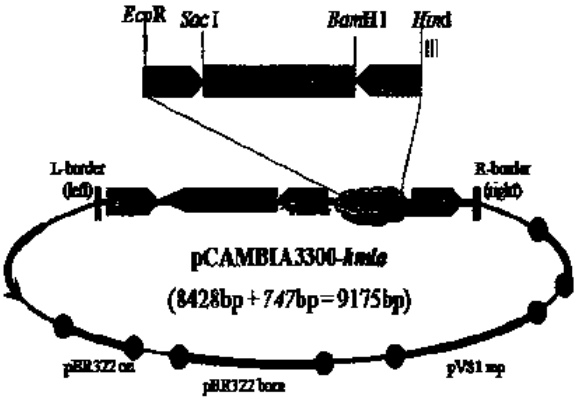

Fig. 1. *Structure of pCAMBIA3300-kmla, 9175bp*

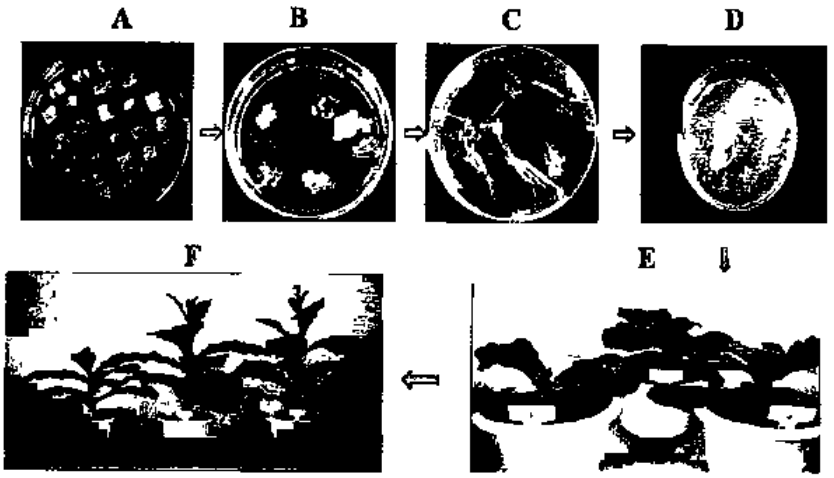

Fig. 2. *Agrobacterim-mediated transformation and regeneration of GM tobacco. Pannels: (A) Leaf explants inoculated A. tumefaciens LBA4404-kmla, (B) Induced calli ,(C) Rooting, (D) Shooting, (E) Growing in soil, (F) Flowering*

Identification of GM-tobacco harboring *kmla* gene

Transformic tobacco plants from the Kan-selected calli were examined at the DNA-level. Total DNA was extracted from two GM tobacco plants and non-transgenic tobacco plants were then amplified by PCR. Some 747 bp-long DNA fragments being equal to the size of the inserted *kmla* gene has been identified in the gel, which belongs to GM-tobacco plants. In contrast, the DNA band has not been recognized in the control plant (Fig. 3), because it has been expected that the control plant does not have *kmla* gene.

Analysis of the expression of *kmla* gene in GM tobacco

To examine the expression of *kmla* gene, total protein from the GM tobacco were analyzed by Western blot. The protein extracts were fractionated on a denatured gel, and then they were electrically blotted on nitro cellulose membrane. A unique blot from was seen in 30.3 kDa size at the position of two transgenic tobacco plants, which was expected molecular weight of kmla protein (Fig. 4). The novel band could not be detected in wild type tobacco.

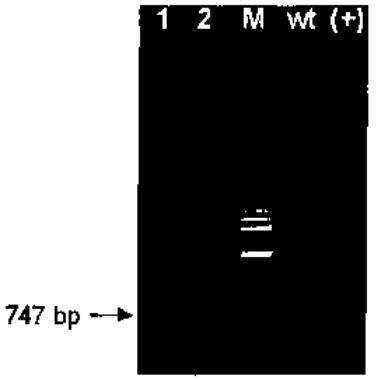

Fig. 3. *PCR amplification of kmla in GM tobaccos. M: 1Kb DNA marker, 1, 2: two GM tobacco plants; wt: Non- GM tobacco plant; (+): Positive control*

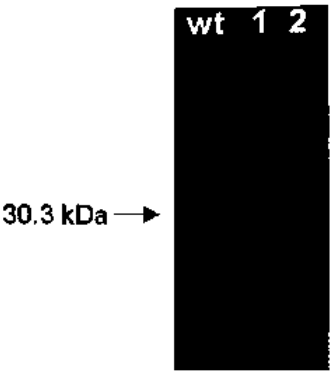

Fig. 4. *Western blot analysis of kmla expression in GM tobacco plants.* wt: *non-transgenic tobacco plants;* 1, 2: *transgenic tobacco plants*

Agrobacterium mediated transformation is definitely a useful way to manage basic plant research and a valuable technology to generate economically important crops (Bedo *et al.* 2009). Nowadays it is not a problem to transfer gene into plant cells, but rather to maintain the genetic homeostasis of the integrated gene in the transformants. GM plants are routinely cultivated to produce various non-self proteins, especially medicinal agents (Craig *et al.* 2008) for a successful achievement of the genetic homeostasis in a target GM plant: stable / reliable integration and expression of integrated gene (s), susceptibility of the recombinant protein(s), and GM plants tolerance to biotic and/or abiotic stresses. (Vasil IK, 2007). According to Pusztai (1991) lectins are hardy proteins that do not break down easily. They are resistant to stomach acid and digestive enzymes. It would mean that lectins could be produced in a transgenic plant as a food.

Ever since Murashige and Skoog (1962) set up tobacco transformation system, it became a model for plant gene manipulation (Craig *et al.* 2008, Masoumiasl *et al.* 2010). The expression of kmla protein has been successfully done in this research. It will be basic to provide a safe and economically kmla source for human's need.

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TÓM TẮT
BIỂU HIỆN GEN *LECTIN A* CỦA CÂY TÀM GỬI HÀN QUỐC TRÊN CÂY THUỐC LÁ BIẾN ĐỔI GEN

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Thuốc lá (*Nicotiana tabacum)* đã được thiết kế biến đổi gen để biểu hiện protein kmla. Gen *kmla* có kích thước 747 bp được gắn vào vector pCAMBIA3300 Bam HI/SacI-cut tạo plasmid tái tổ hợp là *pCAMBIA3300-kmla*. Sau đó vector *pCAMBIA3300-kmla* được biến nạp vào vi khuẩn *A. tumefaciens* để tạo *A. tumefaciens* LBA4404/Pcamia3300-kmla tái tổ hợp. *A. tumefaciens* LBA4404/Pcambia3300-kmla được lây nhiễm vào lá thuốc lá và được nuôi cấy trên môi trường MS cơ bản có chứa Kan (50 mg/ml). Kết quả sau khi tái sinh đã tạo được hai cây thuốc lá chuyển gen *kmla*, điều này đã được khẳng định bằng kết quả PCR. Gen *kmla* đã biểu hiện thành protein tái tổ hợp và được chứng minh bằng phân tích Western blot

Từ khóa: *Agrobacterium tumefaciens, Lectin tiểu đơn vị A từ cây tầm gửi Hàn Quốc, biến nạp, pCAMBIA3300-kmla, PCR, Western blot.*

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