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Restoration of Fertility by Suppression of Male Sterility-Induced Gene Using an Antisense Construct

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ABSTRACT This study was carried out to restore the fertility by suppression of male sterility-induced gene using an antisense construct. Tobacco (cv. Petit Havana SR1) was transformed with the binary vector containing a *GBAN215-6* promoter, an antisense diphtheria toxin (*DTx-A*) gene (*pKDA215b*) and a hygromycin resistant gene. Seventy-six confirmed transgenic plants regenerated from leaf disks were designated as the R_0 generation and selfed to produce the R_1 generation. From the inheritance study, five R_1 lines with multiple copies of the antisense construct were selected and selfed to identify homozygosity for the antisense construct. In order to restore fertility and finally to select restore lines, five R_2 lines with multiple copies of the antisense construct were crossed with male sterile plants. From these crosses, three different phenotypes have been observed: completely restored, partially restored, and not restored pollens, and otherwise tobacco plants were phenotypically same as normal plants. These plants were scored for the degree of restoration and selected for further study.

Additional key words: diphtheria toxin (*DTx-A*) gene, tobacco, transformation

Introduction

Hybrid seeds which show heterosis effect play an important role in production of horticultural crops. However many crops have both male and female reproductive organs and give the problems retarding the production of hybrid seeds. In order to prevent self-pollination, artificial emasculation is required. But it raises costs and labor expenses of seed production. To overcome these problems, the utilization of male sterility is one of the most efficient approaches.

Homology-dependent gene silencing can be observed when multiple copies of a transgene or a transgene with homology to an endogenous plant gene are present in a plant genome. Two general types of silencing, involving either transcriptional inactivation or a post-transcriptional process, have been identified. The former has been associated with homology in promoter regions, increased methylation, and meiotically heritable reductions in gene activity, while the latter requires homology in the protein coding region and does not usually lead to heritable alterations in either methylation or gene expression (Matzke et al., 1994; Park et al., 1996).

To obtain sterility in plants, several approaches using molecular biological techniques such as homologous recombination, transposon tagging or antisense RNA have been used. Among them, expression of antisense RNA in transgenic plants, which has lead post-transcriptional inactivation, has been shown to be more powerful.

This study was carried out to restore the fertility by suppression of a male sterility-inducing gene (Kim et al., 1996) using the antisense construct.

Materials and Methods

Construction of antisense *GBAN215-6/DTx-A*

The 428 bp of *DTx-A* gene (Yamazumi et al., 1978) was excised from *pBPE5* construct (Kim et al., 1997) by digestion with *Sac*I and inserted into a pUC119. After cleavage with *Sac*I and *Kpn*I from pUC119, the fragment was cloned in antisense orientation into the binary vector pBI101. The resulting chimeric construct was designated as *pKDA215b* and transferred into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method.

Tobacco transformation

Male sterile plants containing the *DTx-A* gene were obtained from Cytogenic Division, National Agricultural Science and Technology Institute, RDA. To obtain transgenic plants containing *pKDA215b*, leaf disk of *Nicotiana tabacuum* L. (cv. petit havana SR1), prepared from plants ca. 10cm in height and grown on Murashige and Skoog (MS) medium in sterile glass jars, were transformed with *Agrobacterium tumefaciens* as described by An (1987). Direct regeneration of transformed shoots were induced on solid MS medium supplemented with 1.5 mg/L 6-benzylamino purine (BAP), 50 mg/L hygromycin and 200 mg/L cefatoxime. After root induction, plants were transferred to soil and grown to flower in greenhouse.

Outcrossing

Outcrossing progenies were obtained by crossing male sterile plants with transgenic plant containing the *pKDA215b* construct. Crosses were performed by applying pollen from the desired flower using a cotton-tipped swab after hand-emasculating before flower opened. Sterilized seeds from progenies of crosses were placed on solid MS medium, containing 40 mg/L hygromycin and 100 mg/L kanamycin. The progenies containing *pBPE5* and *pKDA215b* constructs were selected and grown to flower in greenhouse.

PCR analysis

PCR amplification were performed with two synthetic primers, 5'-AACTTTCTTCG TACCAC-3' and 5'-GCTTTCGCCTGTTCC CAG-3' from the *DTx-A* gene. Thirty PCR cycles were performed: the denaturation step was at 95℃ for 1 min, the annealing step at 60℃ for 1 min., and the polymerization reaction was performed at 72℃ for 1 min.

Results and Discussion

A recombinant plasmid, *pBPE5* containing the pollen specific promoter *GBAN215-6* fused to the diphtheria toxin A (*DTx-A*) gene was constructed and introduced into tobacco to induce male sterility (Kim et al., 1996). Transformed tobacco plants by *pBPE5* were completely male sterile. Progenies of trangenic male sterile tobacco plant containing *GBAN215-6* promoter and *DTx-A* gene were analyzed by kanamycin screening, pollen observation, and PCR.

Tobacco was also transformed with the construct of *GBAN215-6* promoter and antisense *DTx-A* gene (*pKDA215b*). Transgenic tobacco plants with the antisense construct were selected on the medium containing hygromycin. To confirm the hygromycin resistance gene in transgenic

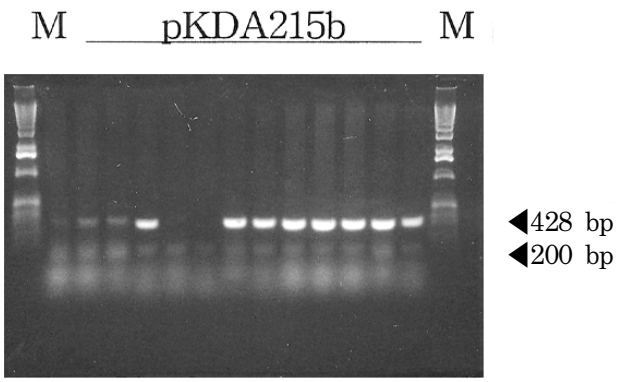

Fig. 1. Detection of hygromycin resistant (Hyg^R) gene in transgenic tobacco plants with the antisense construct by PCR. Two synthetic primers, 5'-CCGACCTGACCTGATGCAGCTCTCGGAGG-3' and 5'-GGCCGTGGTTGGCTTGTATGGAGC-3', from the sequence of Hyg^R gene were used for PCR. Intergenic primers (5'-TACTA TGATGGCAGATACTGGACCATGTGGAAG-3', 5'-TTAGTAGGCC TTCTGGCTTGTAGGCGATGAAACT-3') from the ribulose bisphosphate carboxylase/oxygenase (rubisco) small subunit gene (*rbc*S) were also used for PCR as the control. The expected sizes a of Hyg^R (663 bp) and *rbc*S(200 bp) of PCR products are shown.

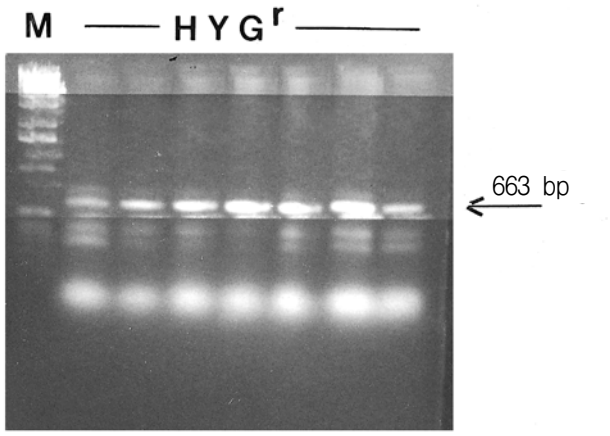

Fig. 2. Detection of the antisense *DTx-A* gene in transgenic tobacco plants by PCR. Two synthetic primers, 5'-AACTTT CTTCGTAC CAC-3' and 5'-GCTTTC GCCTGTTCCCAG-3' from the sequence of DTx-A gene were used for PCR. Intergenic primers (same as Fig. 1) from the ribulose bisphosphate carboxylase (rubisco) small subunit gene (*rbc*S) were also used for PCR as the control. The expected sizes of *DTx-A* (428 bp) and *rbcS* (200 bp) of PCR products are shown.

plants, PCR was performed by two synthetic primers, 5'-GGCCGTGGTTGGCTTGAT GGAGC-3' and 5'-CCGACCTGATGCAGCT CTGGAGG-3' from the sequence of hygromycin (Fig. 1). Also the antisense *DTx-A* gene was confirmed by PCR using two synthetic primers, 5'-AACTTTCTTCGTACC AC-3' and 5'-GCTTTCGCCTGTTCCCAG-3' from the sequence of *DTx-A* gene. The fragment of 428 bp was appeared for antisense *DTx-A* gene (Fig. 2). For these two PCR analyses, fragment of 200 bp from the ribulose bisphosphate carboxylase/oxygenase (rubisco) small subunit gene was also used as an internal control. Seventy-six confirmed transgenic plants with antisense construct were named the R_0 generation.

Previous studies reported copy number and silencing ability. The connection between copy number and silencing ability arises from the fact that multiple closely linked copies of transgenes (Linn et al., 1990; Scheid et al., 1991), or transgene constructs containing repeated elements arranged *in cis* (Assaad et al., 1993), tend to spontaneously inactive and become methylated *de novo*. Other reports suggest that multicopy inserts are more effective silencers of unlinked, homologous transgens (Matzke and Matzke, 1993; Hobbs et al., 1993). In this study, copy number of transgens in individual lines were investigated to increase the silencing ability so that increase efficiency of restoration of the fertility. Seventy-six lines with *pKDA 215b* were selfed and produced the R_1 generation. Sterilized seeds from the R_1 generation were placed on solid MS medium, contatining 40 mg/L hygromycin. After 6~8 weeks, seedlings were scored for the resistance to hygromycin. Resistant seedlings were green and sensitive seedlings never formed roots and turned white shortly after germination. From the inheritance study, five R_1 lines (21505, 21507, 21511, 21522, 21525) with multiple copies of the antisense construct, which showed higher hygromycin resistant ratio than lines with single copy, were selected and selfed to obtain homozygosity for the antisense construct. Homozygous lines had all resistant seedlings on MS medium with 40 mg/L hygromycin and these lines were used to suppress the *DTx-A* gene and finally to restore fertility.

In order to restore fertility and finally to select restore line, five R_2 lines with multiple copies of the antisense construct were crossed with five male sterile plants which had different number of *DTx-A* gene, respectively (Fig. 3). The progenies containing *pBPE5* and *pKDA215b* constructs from progenies of crosses were selected on solid MS medium, containing 40 mg/L hygromycin and 100 mg/L kanamycin. Seedlings with two constructs, which showed green

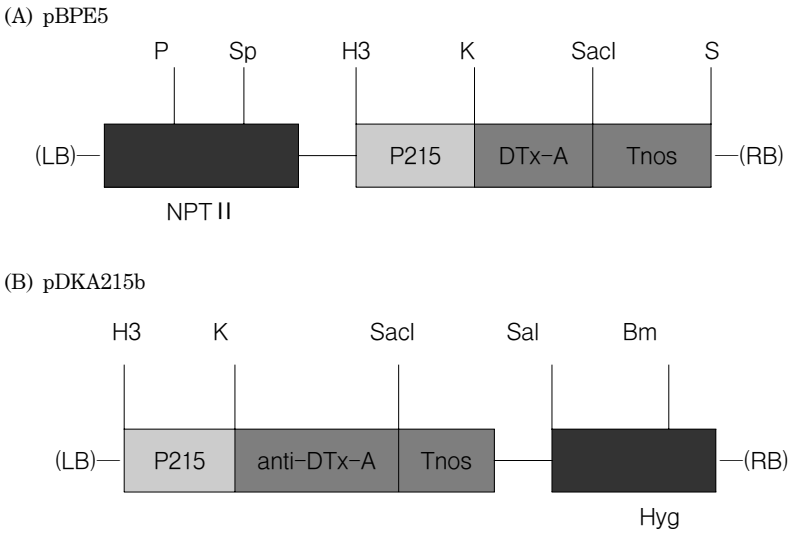

Fig. 3. Maps of binary vectors used to induce male sterility and to restore fertility. Cross fertilization of male sterile plants with the *pKDA215b* (Hyg^R-P215/anti-sense DTx-A/T-nos) gene for restoration of fertility. Construct of *pBPE* (A: sense direction of *DTx-A* gene) and *pKDA215b* (B: antisense direction of *DTx-A* gene) used for a cross fertilization.

shoot and vigorous roots on the medium containing antibiotics, were easily distinguishable from sensitive seedlings, which never formed roots and turned white shortly after germination. Rooted plants were transferred to soil and grown to flower in the greenhouse to observe the restoration.

The observation showed three different types: completely restored, partially restored, and not restored pollens, respectively (Fig. 4). Except pollen sterility other phenotypes were same as normal plants for all three types. These plants were scored and selected for further study. Coordinate co-suppression was described for transgenic petunia, in which both the transgene and a homologous endogenous gene were inactivated (Napoli et al., 1990). In the present study, expressions of *DTx-A* gene were reduced by varying degrees.

Although the mechanism of suppression remains unknown, the presence of sense and antisense oriented transgene with homology to *DTx-A* sequence appeared to perfectly or partially suppress each other and finally restored fertility. This means that the degree of suppression was different for the two genes of the constructs. Greater understanding of this phenomenon will be required in order to design the production system of hybrid seed to incorporate several economically useful genes.

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A B C

Fig. 4. Observation of restoration of male fertility to male sterile tobacco transformants by a cross fertilization using the antisense construct (Hyg^R-P215/antisense *DTx*-A/Tnos) gene. Completely restored (A), partially restored (B), and not restored pollens (C), are shown.

웅성불임 유전자의 발현억제를 이용한 임성회복

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초 록

본 연구는 antisense 유전자를 이용하여 웅성불임 식물체를 유기하는 웅성불임 유기 유전자의 발현을 억제함으로써 임성을 회복하기 위하여 실시하였다. 약특이 promoter(*GBAN215-6* promoter)와 antisense 방향의 diphtheria toxin(*DTx-A*) 유전자로 제작된 *pKDA215b*로 담배(cv. petit Havana SR1)를 형질전환시키고 형질전환이 확인된 76개의 R_0 세대를 자가수분하여 R_1 세대를 획득하였다. R_1 세대의 유전분석후 antisene 유전자가 복수로 존재하는 5개의 R_1 계통 (21505, 21507, 21511, 21522, 21525)을 선발하고 동형접합체를 획득하기 위해 자가수분을 하였다. 임성회복을 유도하고 회복친을 선발하기 위하여 antisense 유전자를 가진 R_2 계통과 웅성불임 식물체를 교배하였다. 그 결과 꽃가루가 완전이 회복된 개체, 부분적으로 회복된 개체, 회복되지 않은 개체 등 3종류의 식물체를 획득하였으며 이들 식물체의 화분 이외의 표현형은 정상식물체와 같았고 그 기작 구명을 위해 선발하고 계속 연구가 진행되고 있다.

추가 주요어 : diphtheria toxin (*DTx-A*) 유전자, 담배, 형질전환