식물조직배양학회지 제25권 제3호
Korean J. Plant Tissue Culture
Vol. 25, No. 3, 147-152 (1998)

Transgenic Plants Expressing an Antisense RNA of AL1-Gene from Tomato Golden Mosaic Virus(TGMV)

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Tomato Golden Mosaic Virus(TGMV) AL1-gene의 antisense RNA 발현 형질 전환 식물체

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AL1-gene, necessary for the replication of the genome of a gemini virus TGMV, was inserted in the opposite direction to the promoter CaMV35S resulting in the construction of a plant transformation binary vector pAR35-2. The vector pAR35-2 contains the chimeric gene cassette involving the duplicated promoter CaMV35S, opposite direction of AL1-gene fusioned with hygromycin resistant gene, and the gene cassette of the neomycin phosphotransferase II gene. The plasmid was transferred to tobacco and tomato plants by leaf disk infection via Agrobacterium. The transgenic plants were selected and grown on the MS-agar medium containing kanamycin and hygromycin. The shoots induced from the calli were regenerated to the whole transgenic plants. The antisense AL1-gene was detected in the genomic DNA isolated from the leaves by using the PCR mediated Southern blot analysis. The expression of the antisense AL1-gene was also observed using the RT-PCR mediated Southern blot analysis. The observation of chloroplasts in guard cell pair indicated that the transgenic tomato plants were diploid.

Keywords:TGMV geminivirus, AL1-gene, antisense RNA, transgenic plants

Geminiviruses, a family of plant viruses characterized by their single-stranded circular DNA genomes and small, twinned isometric particle morphology (Lazarowitz et al., 1988), can be separated into two major groups by their genomic organization and insect vector. They are transmitted by whitefly to dicot plants, and by leafhoppers, predominantly to monocots. The whitefly-transmitted geminiviruses typically have bipartite genomes with each genome (DNA A and DNA B) encapsidated separately. Tomato Golden Mosaic Virus (TGMV) is a member of the bipartite genomes (Bisaro et al., 1982), whitefly-transmitted group. The genetic organizations of the two TGMV genomes (DNA A and DNA B) are similar to those of all the bipatite genome. In DNA A, AL1, AL2 and AL3 are encoded on the viral minus strand. AL1 is essential for replication of TGMV DNA in plant. AL1 mutants are completely unable to replicate (Brough et al., 1988). The function of AL2 and AL3 are unclear, but both are required for efficient replication. The coat-protein deletion analysis suggested that the product of the AL1-gene and the 200 bp region common to both DNA A and DNA B are the only essential requirements for TGMV replication. Therefore, AL1 is an ideal target for antisense RNA technology. Antisense RNA, complementary to a target RNA, has recently been exploited to artificially suppress gene expression with high specificity.

In this report, we have developed transgenic plants expressing an antisense RNA of AL1-gene. As a target for the antisense RNA, a region of the TGMV DNA A genome encompassing the entire AL1 gene ORF was chosen. For the expression, the fragment was inserted in the opposite direction to the promoter CaMV35S resulting in the construction of a plant transformation binary vector pAR35-2. The vector contained the neomycin phosphotransferase II gene (NPTII) cassette and the chimeric gene cassette involving the

duplicated promoter CaMV35S, opposite direction of AL1-gene, hygromycin and terminator sequence of the octopine synthase gene. The plasmid pAR35-2 was then transferred to tobacco and tomato plants by leaf disk infection via *Agrobacterium*. The antisense RNA was observed in the transgenic plants using the RT-PCR/Southern blot analysis. Furthermore we determined the ploidy level of the transgenic tomato plants.

MATERIALS AND METHODS

Construction of Plasmids

The antisense AL1 gene fusioned to the CaMV35S promoter and selectable marker of hygromycin resistance, about 2.9kb, was cut off from pP2AEN (Bejarano et al., 1992, friendly gift from Dr. Suh, Suk-Cheol in RDA, Korea) by cleavage with *Kpn* I and subcloned into a plant transformation vector pBinAR containing originally a chimeric gene expression cassette of NPTII within the two border sequences (Rhim et al., 1995). The recombinant plasmid was named to pAR35-2, which then transferred to *Agrobacterium tumefaciens* strain LBA 4404 by electroporation (Easy Jet System) under the conditions of 2mm cubette, 25μF and 2.5 kV in 500 ul of 10% glycerin solution.

Plant materials

Lycopersicum esculentum cv. Seokwang and *Nicotiana tabacum* cv. NC82 were used as host plants for plant transformation. At fiirst, these seeds were sterilized with 20% hypochlorine solution(content more than 4% chlorine) for 15 min. and washed 2-3 times with autoclaved distilled water. The seeds were maintained on MS-agar medium(Murashige and Skoog, 1962) and incubated at 25°C under the condition of 16h light in a day. After grown, the young leaves were used for the plant transformation.

The plant transformation and tissue culture

The wounded young leaves were cocultured with *A. tumefaciens* harboring the binary vector pAR35-2 on the MS-agar medium for two days and washed with 100 mg/L of carbenicilin. The leaves were cultured for the selection of transgenic plants on the MS-shoot inducing medium(MSSIM) containing the kanamycin(300 mg/L), hygromycin(100 mg/L), 2.5 mg/L BA and 0.2 mg/L IAA for about 4 weeks. After the induction of the shoots from the calli, the shoots were grown for about 3 weeks on the MS-root inducing medium in which BA and IAA were deleted from MSSIM. Most of them were converted to intact plantlets and grown in the incubator.

Polymerase chain reaction(PCR) mediated Southern hybridization analysis

Total plant DNA was extracted from the leaves by the method of Rech et al.,(1987). Five grams of the plants were frozen in the liquid nitrogen and powdered. The plant powder was extracted with the buffer containing 70 mM NaCl, 50 mM Tris-CL(pH8.0), 10 mM EDTA, 1% β-mercaptoethanol, 1% SDS or CTAB, which was heated to 60°C. The plant extract was mixed with phenol/chloroform/isoamylalchohol in the inverting incubator at 60°C for 10–15 min. After centrifugation, the overphase was precipitated with ethanol. The DNA was picked out and dried in the aspirator, and used for the analysis of the PCR. The two primers complementary with AL1 gene in both termini were 20-mer oligonucleotides (AL1-a:5'-GGATCCAAAATGCCATCGCA-3', AL1-b: 5'-TCTAGAGCTCTTCGTTTAGC-3'). PCR amplification was performed in 100μL of total volume. Each reaction mixture contained 1μg of genomic DNA template, 100μM each of dNTPs, 1 pmole of each primer and 1.5 units of DNA polymerase. The mixture was subjected for optimal results at 37 cycles of each consisting of 94°C for 1 min, 54°C for 2 min and 72°C for 3 min. The samples following the PCR-analysis were stored at 4°C for further experiments. The PCR products were separated on 1.5% agarose gel and blotted onto nylon membrane. The membrane was hybridized with AL1 DNA probe(1083bp fragment produced from PCR based on pAR35-2), labeled with dioxigenin(DIG)(Boehringer Mannheim) at 68°C for 16h and then washed stringently at 68°C. After the washing, the fragment of DNA was colorized using the DIG-detection kit (Boehringer Mannheim).

Reverse Transcriptase(RT)-PCR mediated Southern hybridization analysis

Total RNA was isolated from the leaves of the plants transformed with pAR35-2 by using the method of Rhim et al.(1995). Briefly, five grams of the leaves were frozen in liquid nitrogen, powderd and mixed with phenol in incubator at 50°C for 20 min. After the short centrifugation, the

overphase was mixed with 8M LiCl followed by ethanol precipitation for overnight at 20°C. The precipitate after centrifugation was washed with 70% ethanol and dissolved with autoclaved distilled water. The total RNA was analyzed on the agarose gel containing formaldehyde. The isolated RNA was subjected to RT-PCR. The reverse transcription was carried out in the reaction buffer containing 5 ㎍ RNA, 20 μL of RT buffer containing 0.5 mM each of dNTPs and 1 pmole of AL1-a primer. The reaction was performed at 70°C for 5 min with 40 units of M-MuLV reverse transcriptase(Boehringer Mannheim). After the further incubation for 60 min at 37°C, 10 μL of the RT-product was used for PCR. The product of PCR was separated on the agarose and hybridized with the AL1 DNA probe (1083bp fragment) labeled with DIG. The PCR and southern hybridization analysis were carried out as above.

Determination of ploidy level in transgenic tomato plants

The ploidy level of the transgenic tomato plants was determined by counting the number of chloroplasts per guard cell pair in leaf epidermal tissue(Koornef et al., 1989). The epidermal tissue was peeled from the lower surface of the leaves, mounted in 30mM KCl, 10mM K^{+}-Mes(pH6.0) solution on a slide glass and observed. The number of chloroplasts were counted under a microscope(Zeiss Jena, Germany) with 100 x objective lens immersed in oil.

RESULTS

Construction of transformation vector

As a target for the antisense RNA, a region of the TGMV DNA A genome encompassing the entire AL1 gene ORF was chosen. The plasmid pP2AEN was cleaved with *Kpn*I. The fragment of 2.9kb was subcloned into the *Kpn*I-site of the plant transformation binary vector pBinAR treated with phosphatase. Accordingly, the hygromycin resistant gene and the antisense AL1 were located between two CaMV35S promoters and the terminator sequence of the octopin synthase gene. The resulting recombinant plasmid was named as pAR35-2 (Figure 1). Because the vector pBinAR originally harbored a expression unit of NPTII gene under control of the promoter and terminator of the nopaline synthase gene, the plasmid pAR35-2 contained the two selection marker genes of hygromycin and kanamycin. The plasmid was

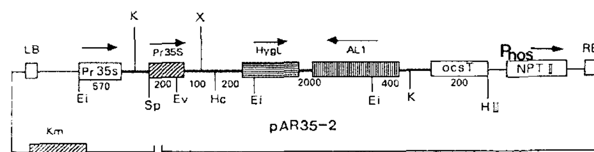

Figure 1. Gene map of the plant transformation vector pAR35-2. B: BamHI, Ei: EcoRI, Ev: EcoRV, HIII: *Hind*III, Hc: *Hinc*II, K: *Kpn*I, P: *Pst*I, Sp: *Sph*I, X: *Xba*I, AL1: AL1-gene, HygL: hygromycin resistant gene, NPTII: neomycin phosphotransferase gene II, Pr35S: CaMV35S promoter, ocsT: octopine synthase gene terminator-sequence, Pnos: nopaline synthase gene promoter, LB: left border sequence, RB: right border sequence(The arrows indicate the directions of the original transcription)

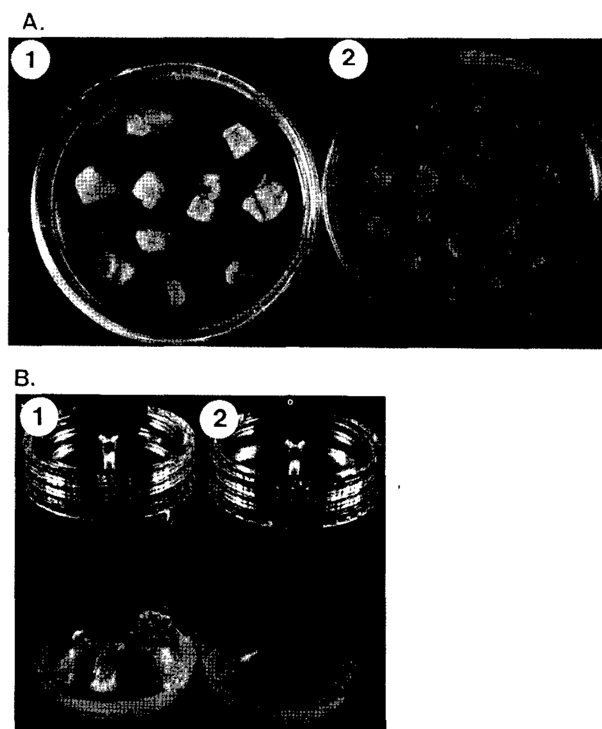

Figure 2. Selection of transgenic tobacco plants transformed with *Agrobacterium* bearing pAR35-2 on the selective and growing medium containing kanamycin and hygromycin.
A: Selection of transgenic tobacco plants
1) Leaf disks(not infected).
2) Leaf disks infected
B: Selection and growing the transgenic tobacco plants
1) plant not infected with *Agrobacterium* pAR35-2
2) plant infected with *Agrobacterium* pAR35-2

transferred to *Agrobacteium tumefaciens* strain LBA 4404 by electroporation for the transformation of tomato and tobacco plants.

Plant transformation

The leaf disks of young tobacco and tomato plants were

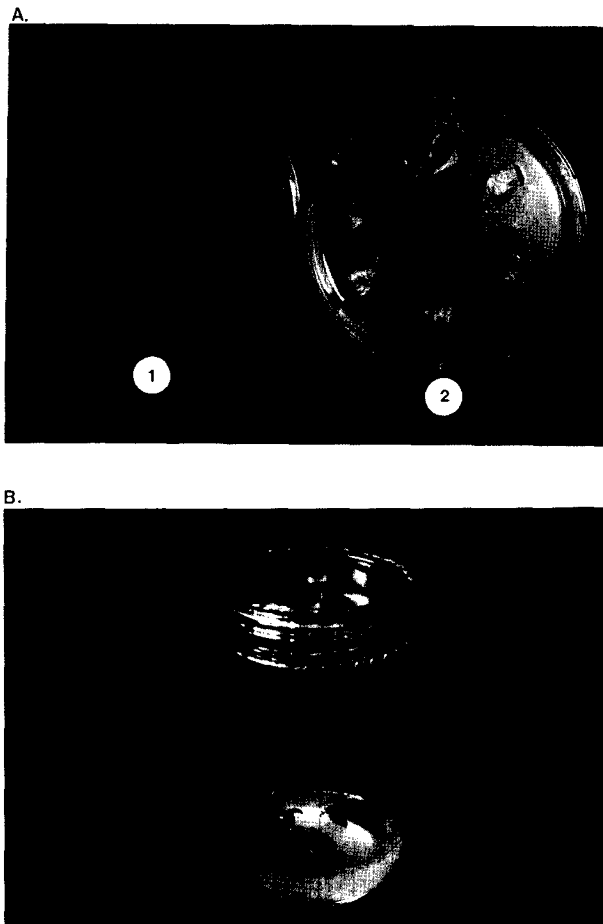

Figure 3. Selection of transgenic tomato plants on the selective and growing medium containing kanamycin and hygromycin.
A: Selection of tomato plants transformed by leaf disk infection
1) Leaf disks infected with *Agrobacterium* bearing pAR35-2
2) Leaf disks not infected
B: Selection and growing the transgenic tomato plants

cocultured with *A. tumefaciens* harboring the binary vector pAR35-2 for 1 hour and layed on the MS-agar medium for two days. After washing with carbenicilin, the leaf disks were cultured on the selection agar medium containing the kanamycin and hygromycin. After the induction of the shoots from the calli, the shoots were grown on the MS-root inducing medium. In these transformation experiments, 21 shoots were regenerated from the 81 leaf disks of tobacco plants. For the tomato plants, 5 shoots were regenerated from the 58 leaf disks infected with *Agrobacterium.*

The efficiencies of transformation were estimated as 26.3% in tobacco and 8.6% in tomato plants. Figures 2 and 3 showed an example for the selections of the transgenic tobacco and tomato plants on the selection agar medium. All of the shoots were converted to intact plantlets and cultured to grown plants in the incubator for further experiments.

Detection of antisense AL1-gene

A modified PCR method, PCR mediated southern blot hybridization, was used to detect the antisense AL1 gene in the transformed tobacco and tomato plants. The region of 1083 bp in the antisense AL1 gene was amplified from the isolated total DNA using the PCR method. The PCR products were separated on 1.5% agarose gel and blotted onto nylon membrane. The membrane was hybridized with AL1 DNA probe labeled with dioxigenin. As shown in the Figure 4 A and B, the DNA fragments of antisense AL1 gene were detected from the regenerated plants(lanes 4 and 6, respectively). However, the antisense AL1 gene was not detected from the control tomato and tobacco plants(lanes 3 and 5, respectively). The results showed that the antisense AL1-gene is located in the transgenic tomato and tobacco plants.

The further hybridization experiments detected that the fragment of antisense AL1-gene was located in all of the other regenerated plants(data not shown).

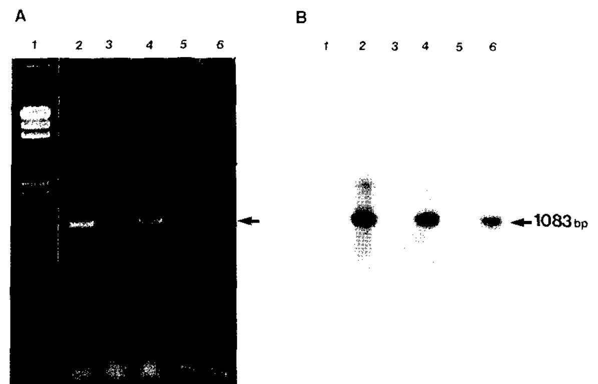

Figure 4. Detection of AL1-gene by using PCR mediated Southern blot analysis
A: Agarose gel electrophoresis of PCR products
B: Southern hybridization analysis of the PCR products to detect antisense AL1-gene
lane 1: λ-DNA digested with *Hin*dIII
lane 2: PCR-product from pAR35-2
lane 3: PCR-product from wildtype of tomato plant(control)
lane 4: PCR-product from transgenic tomato plant
lane 5: PCR-product from wildtype of tobacco plant(control)
lane 6: PCR-product from transgenic tobacco plant
(The arrows indicate the antisense AL1-gene)

Detection of the antisense RNA

The expressions of antisense AL1 gene were detected from

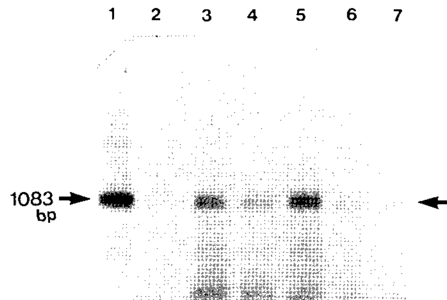

Figure 5. Detection of antisense RNA in the transgenic plants.
lane 1: PCR product of AL1-gene fragment from pAR35-2(marker)
lane 2: RT-PCR product from wild type tomato plant(control)
lanes 3-5: RT-PCR product from transgenic tomato plants
lanes 6-7: RT-PCR product from transgenic tobacco plants
(Arrow indicates the antisense RNA)

the regenerated tomato and tobacco plants using the Reverse Transcriptase(RT)-PCR mediated southern hybridization with the DIG-labeled AL1 DNA fragment(Figure 5). Total RNA isolated from the leaves of the regenerated plants was subjected to RT-PCR. After the gel analysis, the antisense RNA was detected with the hybridization with the AL1 DNA-fragment. In this experiment, it was estimated that the antisense AL1-gene was expressed at a low level in the tobacco plants comparing to that in tomato plants(Figure 5, lanes 3-7). However, the antisense RNA of AL1 gene was not detected from the control plant(lane 2). The results showed that the antisense AL1-gene may be integrated in the genomic DNA of the transgenic tomato and tobacco plants, and expressed at a low level.

Determination of ploidy level in transgenic tomato plants

The ploidy levels of the transgenic tomato plants were determined by counting the number of chloroplasts per guard cell pair in leaf epidermal tissue to identify whether the transgenic tomato plants were normally differentiated and grown. Eight chloroplasts were counted under the microscope with 100 x objective lens(Figure 6), which indicated that the plants were diploid and normally transformed. The ploidy levels of the transgenic tobacco plants were not counted, because the relationship between the ploidy level and the number of guard cells were not known so far. However, the morphological analysis in the height and shape of the plant showed that the transgenic tobacco plants could also normally be transfromed and grown.

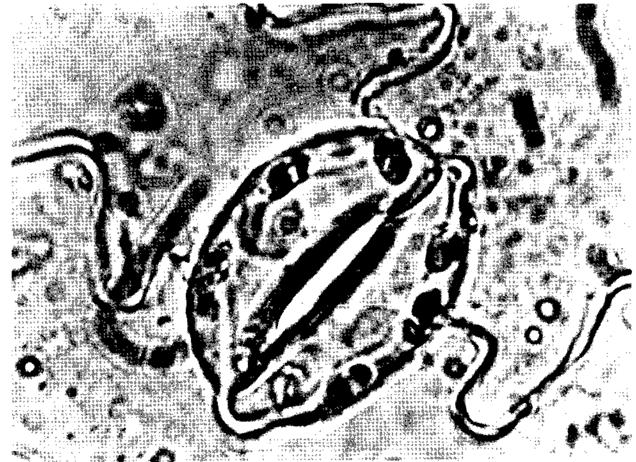

Figure 6. Determination of the ploidy level in the transgenic tomato plants.
The ploidy level was determined by measuring the number of the chloroplasts in stomata guard cell pair under light microscope(× 1,000). Eight chloroplasts indicated that the transgenic tomato plants were normal plants containing the ploidy level of diploid.

DISCUSSION

The plasmid pAR35-2 was constructed for the expression of the antisense AL1-gene fusioned to the end of the hygromycin resistant gene under the control of two CaMV35S promoters. The terminator sequence of the octopine synthase gene was attachted to the fusioned DNA fragment. The chimeric gene cassette was ligated to the chimeric gene expression cassette of NPTII within the right and left border sequences in the vector plasmid. Therefore, the transgenic plants harboring the plasmid pAR35-2 could be selected on the MS-agar medium containing kanamycin and hygromycin. It was supposed that this plasmid may be useful for selections and gene expression in plant transformation system. The results of the analyses using the methods of PCR and RT-PCR showed that the antisense AL1 gene is located and expressed in the transgenic plants. These methods of PCR and RT-PCR have, recently, been used for the detection of the DNA and the expression in various cells. However, the PCR-method only may not be enough to determine the transformation of the plants. But, the results using the method together with the RT-PCR can be accepted for the determination of the plant transformation. From this point of the view, the newly adopted method of PCR and RT-PCR mediated southern hybridization seemed to be fast and efficient, and more useful for the detection of DNA and the expression in the plants. These methods may, therefore, be used commonly as one of the methods for the detection of DNA and the expression.

The expression of the antisense AL1-gene fusioned after the hygromycin resistant gene indicated that the fusioned DNA fragment could be succesfully transcribed under the control of the two CaMV35s promoters. The expression levels were low, for which the reasons were not known. It was interested whether the expression of the antisense AL1-gene could protect the infection of the TGMV. The bioassay was proposed for the protection test against the infection of TGMV. However, the protection test was not performed, because TGMV could not be obtained in Korea. The existence of TGMV in Korea has not been known so far. Furthermore, it is not possible to obtain TGMV from foreign countries recently. The antisene RNA technology of the fusioned DNA as described above may be used or necessary for the development of the transgenic plants showing the resistant against certain virus infections.

Acknowledgments- I would like to thank Mr. Kim,W.-S. for skillfull works and Dr. Suh, Suk-Cheol for the gift of pP2AEN. This paper was suported by KOSEF(92-05-00-02-3).

적 요

AL1-gene은 TGMV의 복제에 매우 중요한 역할을 하고 있다. 이 AL1 gene의 발현을 억제하기 위해서는 식물체내에서 AL1 gene의 antisense RNA의 발현에 의한 억제가 효과적 방법 중에 하나로 알려져 있다. 이런 발현을 식물체내에서 실현 시키기 위해 hygromycin 저항성 유전자에 antisense AL1-gene을 연결 시키고, 연결된 부위를 CaMV35s-promoter와 octopine synthase gene terminator 사이에 연결 시켰다. 이 유전자 발현 단위 부분을 다시 kanamycin 저항성 유전자 발현 단위 부분을 지니고 있는 형질 전환 벡터인 pBinAR에 삽입시켜 새로운 형질 전환 벡터인 pAR35-2를 개발 하였다. 이 벡터를 *Agrobacterium tumefaciens* LBA4404에 형질 전환 시킨 다음, 토마토와 담배 잎사귀 조직에 감염시켜 식물체들을 kanamycin과 hygromycin이 함유된 배지위에서 배양하여 형질전환된 식물체들을 선발하였다. 형질 전환된 식물체들로부터 antisense AL1-gene 및 antisense RNA를 각각 PCR 및 RT-PCR를 이용한 southern hybridization 방법을 이용하여 증명하였고, 토마토 식물체의 공변세포쌍 내에 있는 엽록체 숫자가 여덟 개라는 것이 확인되어 형질 전환된 토마토 식물체가 2 배수체로서 정상적인 식물체라는 것을 증명하였다. 이러한 형질 전환 식물체는 앞으로 항 바이러스성 형질을 지니는 식물체들을 개발하는 데 많은 도움을 주리라 여겨 진다. 그리고, 본 연구에서 제조된 벡터 pAR35-2는 두 개의 항생제에서 동시 선발 할 수 있도록 되어 있고 promoter가 두 개로 되어 있어 형질 전환 식물체 선발 및 유전자 발현 연구에 효과적으로 이용되어 질 수 있으리라 여겨진다.

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(1998년 2월 3일 접수)