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Transformation of *Brassica napus* with Acid Phosphatase Gene

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Acid Phosphatase 유전자 도입에 의한 유채(油菜)의 형질전환

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摘 要

담배(*Nicotiana tabacum* L. cv. Samsun)로부터 acid phosphatase 유전자를 분리하여 *Agrobacterium tumefaciens*를 매개로 한 유채(*Brassica napus* L. cv. Chungpoong)로의 형질 전환에 관한 실험을 하여 다음과 같은 결과를 얻었다.

효모의 repressible acid phosphatase 유전자인 *pho5* DNA를 probe로 사용하여 담배의 genomic DNA로부터 Southern blot analysis를 실시한 결과, 담배의 genome상에서 Apase 유전자의 존재를 확인하였다. Apase 유전자를 분리하기 위하여, 인산결핍 처리된 담배의 뿌리로부터 mRNA를 분리 정제한 다음, cDNA library를 구축하였으며, 이 library의 파지형 성능을 검정한 결과 2.58×10^5 pfu/mℓ를 나타내어 담배에서 발현되는 모든 mRNA를 포함하는 것으로 판단되었다. 작성된 cDNA library로부터 *pho5* DNA를 probe로 사용하여 screening을 실시하여 4개의 positive clones을 선발하였으며, 제한 효소 지도의 작성 및 Southern blot analysis를 통하여 이들은 동일한 clone으로, 3.6kb의 cDNA를 포함하는 것으로 나타났다. 분리한 담배의 Apase 유전자를 유채로 형질 전환하기 위하여 발현벡터인 plasmid pGA695-tcAP1을 구축하였으며, *Agrobacterium* EHA101에 도입한 후, 유채의 자엽과 공배양하여 형질 전환을 유도하였다. Kanamycin이 첨가된 선발 배지에서 재분화된 식물체를 얻은 다음 Southern blot analysis를 통하여 담배의 Apase 유전자가 유채의 genome상에 안정적으로 도입되었음을 확인하였다.

I. INTRODUCTION

It is well knwon that plants have evolved starvation rescue systems composed of psi gene systems which effectively act to increase the concentration of exogenous Pi(Reid and Bieleski, 1970; Goldstein et al., 1987). These coordinately expressed operons, collectively called a psi regulon, have been studied extensively in both bacteria and yeast(Torriani and Ludtke, 1985: Yoshida et al., 1987). In plants, as a component of psi system, acid phosphatase (orthophosphoric-monoester phosphohydrolyase; EC3.1.3.2; Apase) catalyzes the hydrolysis of organic phosphates of the acidic soil conditions(Boutin and Roux, 1974). The enzyme has been purified and characterized from a variety of plant species and the

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properties such as molecular weight, subcellular localization, different isozymes and optimum pH have been reported(Sommer and Blum, 1965; Laemmli, 1970; Reid and Bieleski, 1970; Zink and Veliky, 1979; McCain and Davies, 1984; Kummerrova, 1986; Kevin et al., 1994). It has also been demonstrated about the enhanced activity in response to Pi starvation. Ukei and Sato(1971) showed that synthesis of the enzyme was induced by Pi-depletion and Reid and Bieleski(1970) reported that Pi-depletion caused an increase in the intracellular level of acid phosphatase. Moreover, Goldstein et al.(1988) reported that the enhancement of Apase activity occurred primarily in root tissues during the early stages of Pi starvation in the whole plant study, and the induction of the psi response occurs within 24 h of transfer to the Pi-depleted medium. There may be a wide spectrum of plants to respond to Pi depletion. Some plants can survive without Pi by induction of Apase to use organic phosphates under acidic soil conditions while other plants will die off because they have not developed the Apase system genetically. If the plants with poorly developed Apase gene are transformed with Apase gene they can survive under Pi depleted conditions.

We have cloned Apase gene from the tobacco plant and have made some transgenic canolas(*Brassica napus*) by the binary vector system of *Agrobacterium tumefaciens*.

II. MATERIALS AND METHODS

1. Plant materials, DNA probe preparation, and standard DNA techniques

Nicotiana tabacum L. cv. Samsun and *Brassica napus* L. cv. Chungpoong were cultivated in a greenhouse under a regime of 16 h light(28℃) and 8 h dark(20℃).

Plasmid pVC727G containing a repressible acid phosphatase gene of yeast, *pho5*, was from professor Yongil Hwang of Kyungnam University. It was used as a probe for screening of Apase cDNA from a tobacco cDNA library.

DNA probes for hybridizations were labeled with [α-^{32}P] dCTP(Amersham) by means of the random primer labeling procedure of Feinberg and Vogelstein (1984) using a kit(Amersham), and purified using a Sephadex G-50 Quick Spin Column(Boehringer Mannheim). Preparation of phage and plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis and bacterial transformation were carried out with standard procedures(Sambrook et al., 1989).

2. Southern blot analysis

Genomic DNA was isolated from leaves of tobacco according to the method described by Murray and Thompson(1980). Genomic DNA was digested with restriction enzymes and 10 μg/lane was separated on 0.8% agarose gel, then transferred to Hybond-N$^+$ nylon membrane(Amersham).

The membrane was prehybridized for 3 h at 42℃ in a buffer containing 50% formamide, 5 × SSC, 5 × Denhardt's solution, 50mM Na-Pi(pH 6.5), 0.1% SDS and 100mg/mℓ denatured salmon sperm DNA. Hybridization was performed for 18 h at 42℃ in the same solution supplemented with α-^{32}P labeled probe DNA. The membrane was washed twice in 2 × SSC-0.1%(w/v) SDS for 30 min and once in 0.1 × SSC-0.1% SDS for 30 min at 42℃, and exposed to an X-ray film with an intensifying screen at −70℃ for 3 days.

3. cDNA library construction and screening

In order to induce the expression of psi Apase gene, 4-week old tobacco plants were water-cultured in Pi-free MS medium(pH 5.2) for 3 days.

Total RNA was isolated from tobacco roots,

extracted with 4 M guanidine isothiocyanate and ultracentrifuged in a 5.7 M CsCl solution. Poly(A)$^+$ RNA was purified from the total RNA by oligo(dT)-cellulose column chromatography.

Double stranded cDNAs were synthesized from 1.32 μg of Poly(A)$^+$ RNA using reverse transcriptase, cloned into the EcoR I site of the λ gt 10 vectors, and they were packaged *in vitro* according to the manufacturer's protocols(Amersham).

The tobacco cDNA library was plated on 90 mm petridish at a density of 1.5×10^4 plaques per plate. The plaques were transferred onto a nylon membrane. Prehybridization, hybridization, and membrane washing were performed as described in Southern blot analysis.

4. Construction of expression vector and transformation of plant tissues

For transferring tobacco Apase gene into *Brassica napus*, the 3.6 kb tobacco Apase cDNA was inserted into Hind III site of pGA695. The constructed expression vector, pGA695-tcAP1 had 35S promoter to be expressed constitutively in plants and NPT II gene for kanamycin selection. *Agrobacterium tumefaciens* EHA101 was used for transformation of plant tissues.

Transformation of plant tissues was performed as described by Son et al. (1994) and An et al.(1986). The sterilized *Brassica napus* cotyledonary petioles were infected with *Agrobaterium* cells harboring pGA695-tcAP1. After co-cultivation for 3 days, *Agrobacterium* cells were washed out with sterilized MS medium and placed on shoot induction medium containing kanamycin(30mg/ℓ). The transformed *Brassica napus* shoots were selected with kanamycin resistance and transferred to root induction medium containing kanamycin(30mg/ℓ). Finally, the transgenic plants were transplanted to soil medium. For another confirmation of transformation, the Southern blot analysis was also conducted.

III. RESULTS AND DISCUSSION

1. Preparation of probe DNA and confirmation of tobacco Apase gene in the genome

A repressible acid phosphatase gene of *Saccharomyces cerevisiae, pho5*, was used for screening of tobacco Apase cDNA. Activity staining of Apase of *S. cerevisiae* NA87-11A with plasmid pVC727G and isolation of *pho5* DNA from pVC727G were described previously by Kim et al.(1993).

In order to identify Apase gene in tobacco genome, Southern blot analysis was performed(Fig. 1). Genomic DNA was digested to completion with BamH I, EcoR I or HindIII and probed with labeled 1.5 kb *pho5*.

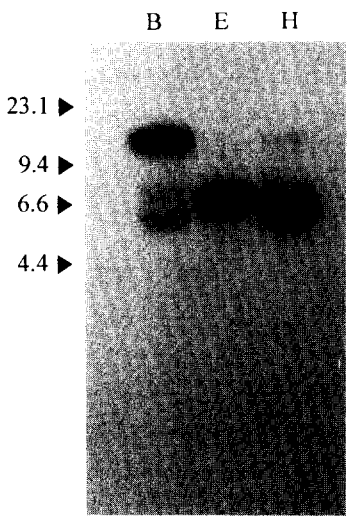

Fig. 1. Southern blot analysis of *Nicotiana tabacum* L. genomic DNA. Ten μg of tobacco genomic DNA was digested with BamH I (B), EcoR I (E) or Hind III(H). The blot was hybridized with the $\alpha - {}^{32}$P labeled *pho5* DNA. Numbers on the left-hand side are the length in kb of DNA size markers.

The probe hybridized strongly to the 6.5 kb band in EcoR I or Hind III-cut DNA(Fig. 1. lane E and H), and to the 10.5 kb and 5.5 kb bands in BamH I -cut genomic DNA(Fig. 1. laneB). These results suggest that the Apase gene may be present as a single copy, or at most two or three copies, in tobacco genome. Since the size of each distinct band in lane B, E and H is about 5.5 ~ 10.5 kb, there may be a chance of having two or three copies of a related gene that are closely linked in the tobacco genomic DNA fragment.

2. cDNA library construction and screening

Preparation of nucleic acids

A number of studies demonstrated that the intracellular and/or extracellular Apase activity were enhanced in response to Pi depletion(Reid and Bieleski, 1970; Ukei and Sato, 1971). Moreover, Goldstein et al. (1988) reported that the enhancement of Apase activity occured primarily in root tissues during the early stages of Pi starvation in the whole plant study, and the induction of the psi response occurred within 24 h of transfer to the Pi-depleted medium. In this experiment, for inducing phosphate-starvation inducible acid phosphatase mRNAs, 4 weeks old tobacco plants were water-cultured in Pi-free MS medium(pH 5.2) for 3 days. 4.1mg of total RNA was isolated from 20g of -Pi treated tobacco roots. A_{260}/A_{280} of total RNA was 1.91 and A_{260}/A_{230} was 1.95. Accoding to this data, isolated total RNA was pure without proteins and/or polysaccarides. Poly(A)$^+$ RNA was purified from total RNA by two cycles of chromatography on oligo(dT)-cellulose and it was scanned from 200nm to 300nm with a spectrophotometer. Using oligo(dT) cellulose column chromatography, 82.8 μg of poly(A)$^+$ RNA was obtained from 4.1mg of total RNA and its A_{260}/A_{280} was 1.95.

Construction of cDNA library

The amount of synthesized cDNA was determined using liquid scintillation counter. It was revealed that 42% of mRNA template was converted to first strand cDNA and 88.9% of the first strand cDNA to second strand cDNA. Obtained cDNAs were attached with EcoR I adaptors, kinased to EcoR I ends, ligated with λ gt 10 vector arm DNA, and then they were *in vitro* packaged into λ phages. The plaque forming unit of the library was 2.8×10^5 pfu/mℓ which might be enough to cover all the expressed mRNAs.

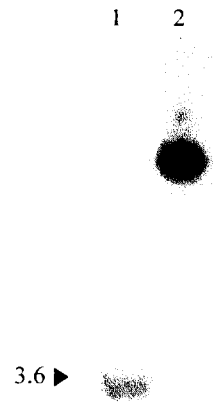

Fig. 2. Southern blot analysis of tcAP-1 DNA. tcAP-1 DNA was digested with EcoR I (1), or undigested(2). The blot was hybridized with the $\alpha - {}^{32}P$ labeled *pho5* DNA. Arrow on the left-hand side showed 3.6 kb Apase gene.

Screening of cDNA library

Using *pho5* as a probe, out of approximately 150,000 phages in the cDNA library, 21 positive plaques were retrieved. Among 21 positive plaques, four plaques hybridized strongly with the probes were used for further study. Restriction mapping and Southern blot analysis of cDNA inserts revealed that four clones were all identical(tcAP-1). In agarose gel

electrophoresis of EcoR I digested tcAP-1 DNA, we observed about a 3.6 kb cDNA band(Fig. 2A) which hybridized strongly with the *pho5* probe labeled with a random primer(Fig. 2B). These results suggested that the 3.6 kb clone contained the tobacco acid phosphatase cDNA.

3. Construction of expression vector and transformation of plant tissues

Construction of plasmid pGA695-tcAP1

Plasmid pGA695-tcAP1 was constructed by subcloning tobacco Apase cDNA into the Hind III site next to 35S promoter in pGA695, and it could be expressed constitutively in plants(Fig. 3).

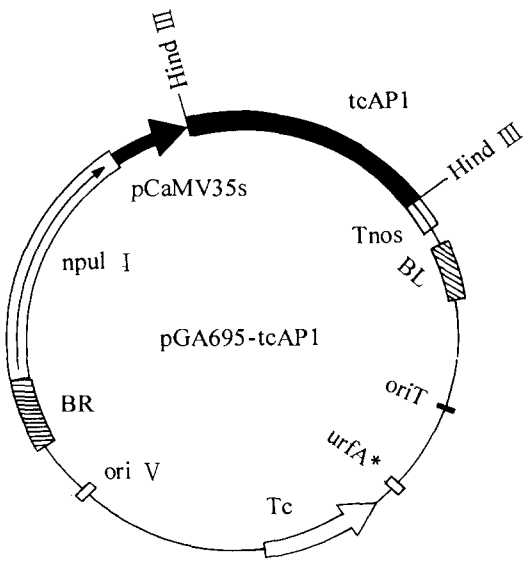

Fig. 3. The structure of expression vector, pGA695-tcAP1. The 3.6 kb Apase cDNA fragment of tobacco was inserted into HindIII site of pGA695. nptII, neomycin phosphotransferase typeII gene; Tc, tetracycline resistance gene; Tnos, nopaline synthase terminator; BR, T-DNA right border; BL, T-DNA left border.

E. coli strain XL1-blue was transformed with pGA695-tcAP1 and was grown at the LB medium containing 50 μg/mℓ of kanamycin and 10 μg/mℓ of tetracycline. Transformed colonies were selected and the correct positioning of tobacco Apase cDNA in pGA695-tcAP1 between CaMV(Cauliflower Mosaic Virus) 35S promoter and NOS(Nopaline Synthase) terminator was confirmed with restriction mapping. When pGA695-tcAP1 was digested with Hind III, about a 12 kb band and a 3.6 kb band were observed as expected. This constructed plasmid pGA695-tcAP1 was used for making transgenic *Brassica napus* plants. *A. tumefaciens* EHA101 was transformed with the constructed pGA695-tcAP1 by two cycles of freeze-thaw method. The transformed colonies were selected on YEP medium containing 50 μg/mℓ of kanamycin and 100 μg/mℓ of rifampicin.

Transformation of Brassica napus plants

The *Brassica napus* cotyledonary petioles were cocultivated with the transformed *Agrobacterium* suspension(10^8 cells/mℓ) for 72 h and were transferred to MS medium with 0.5mg/ℓ NAA, 2.0mg/ℓ BA, 30mg/ℓ kanamycin, 100mg/ℓ cefotaxime, 30g/ℓ sucrose, 3mg/ℓ $AgNO_3$ and 2g/ℓ gelrite. The cotyledonary petioles transferred to the selection medium formed green shoots(Fig. 4A). Transformed shoots were induced vigorously in the cocultivated cotyledonary petioles while shoots were not formed in the uncocultivated cotyledonary petioles. The transformed and regenerated plants(Fig. 4B) were transplanted to soil medium for sampling for the confirmation of transformation.

Confirmation of transgenic Brassica napus plants

Genomic DNA was extracted from both the transgenic and nontransgenic *Brassica napus* plant leaves. Both DNAs were full-digested with Hind III and agarose gel electrophoresed for Southern blot

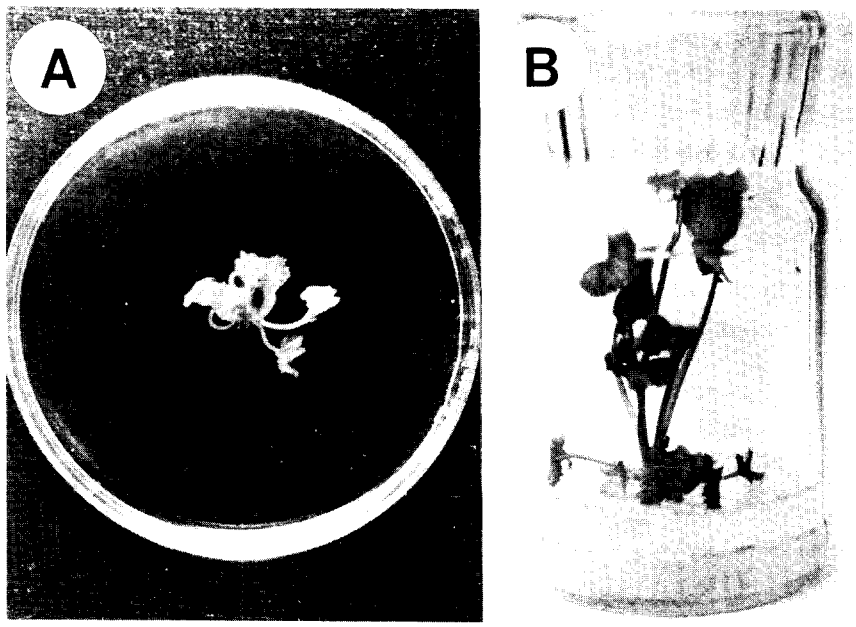

Fig. 4. Production of transgenic *Brassica. napus* plants.
A. Shoot formation from cotyledonary petioles on selection medium.
B. Normal green plant selected from the medium with kanamycin.

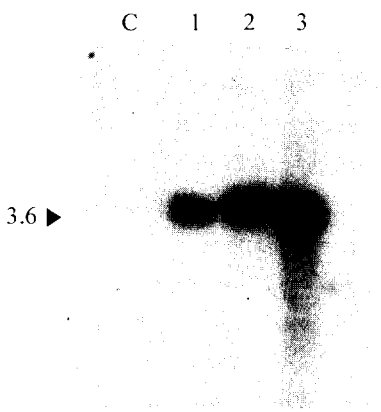

Fig. 5. Southern blot analysis of transformed *B. napus* plants. Ten μg of *B. napus* genomic DNA was digested with HindIII. The blot was hybridized with the $\alpha - {}^{32}P$ labeled tobacco Apase gene. Lane C: wild type plant. Lane 1 – 3: transgenic plants. In lane 1, 2 and 3, a band of 3.6 kb showed tobacco Apase gene.

analysis. Capillary transfered DNA to nylon membrane was hybridized with α-^{32}P labelled tobacco Apase cDNA as a probe. One band was observed at about 3.6 kb range in transgenic plants while no band was shown in nontransgenic control plant(Fig. 5). These results indicated that a foregin gene was stably integrated in genome of *B. napus* plants.

Ⅳ. SUMMARY

This study was conducted to obtain the transgenic *Brassica napus* plants with tobacco Apase gene using the binary vector system of *Agrobacterium tumefaciens*. The results obtained were summarized as follows: A repressible acid phosphatase gene of *Saccharomyces cerevisiae, pho5* was used for screening of tobacco Apase cDNA. In order to identify Apase gene in tobacco genome, Southern blot analysis was performed and the Apase gene may be present as

a single copy, or at most two or three copies, in tobacco genome. To isolate the tobacco Apase gene, tobacco cDNA library was constructed using purifed mRNA from -Pi treated tobacco root and the plaque forming unit of the library was 2.8×10^5 pfu/mℓ, therefore the library might cover all expressed mRNAs. Using *pho5* as a probe, tobacco Apase cDNA was cloned, and restriction mapping and Southern blot analysis of cDNA insert were revealed that the 3.6 kb cDNA contained tobacco acid phosphatase cDNA. Plasmid pGA695-tcAP1 was constructed by subcloning tobacco Apase cDNA into the Hind Ⅲ site of pGA695 with 35S promoter which can be expressed constitutively in plants. The *Brassica napus* cotyledonary petioles were cocultivated with the *Agrobacterium* and transferred to the selection medium. The transformed and regenerated plants were transplanted to soil medium. Southern blot analysis was done on the transformed plants, and it was confirmed that a foregin gene was stably integrated into the genomes of *B. napus* plants.

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