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Agrobacterium-Mediated Transformation of a Plant with *Saccharomyces cerevisiae* Acid Phosphatase Gene(*PH05*)

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*Agrobacterium*을 이용한 *Saccharomyces cerevisiae* Acid Phosphatase 유전자(*PH05*)의 식물체로의 도입

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摘 要

Agrobacterium tumefaciens(strain LBA4404)를 媒介로 한 외래유전자의 식물체로의 도입에 관한 실험을 하여 아래와 같은 결과를 얻었다.

모델 植物로는 담배(*Nicotiana tabacum* c.v. *samsun*)를 사용하였으며, 외래 유전자는 효모 유래의 *Acid phosphatase* 유전자인 *PH05*를 사용하였다. pVC727G에서 *PH05*를 잘라내어 電氣泳動 및 graphical estimation法으로 確認한 結果, *PH05*의 크기는 1.5kb였다. pVC727G와 廣域 plasmid인 pBI121을 利用하여 植物體 形質轉換을 爲한 vector인 pBKJ I을 만들었다. pBKJ I을 *Agrobacterium* LBA4404에 subcloning 한후 담배의 leaf disc를 *Agrobacterium* LBA4404과 共培養하여 형질전환을 유도하였다. kanamycin을 添加한 MS-n/B培地에서 形質轉換된 shoots를 얻었으며, 이들의 再分化를 實施하여 形質轉換된 식물체를 얻었다. 形質轉換된 식물체의 genomic DNA를 *PH05*로서 southern hybridization하여 形質轉換을 確認하였으며, 식물체의 잎, 줄기 및 뿌리의 Apase(*PH05*)活性을 測定하여 導入한 *PH05*가 發現됨을 確認하였다.

I. INTRODUCTION

Not only in plants but also in all organisms, phosphrous is an essential element and normal growth of plants is not expected without it. In soils, however, phosphorus is always deficient for plant growth and the concentration is not more than 10 μM even in fertile soils(Arnon, 1953, Fried and Brosehart, 1967). The phosphorus in the soil solution is in the form of either $H_2PO_4^-$ in acidic soils or HPO_4^{2-} in basic soils(Hagen and Hopkins, 1955). In acidic soils, $H_2PO_4^-$ is readily reacted with aluminum or iron ions to form insoluble salts as varisite or strengite, respectively and they phosphate deficient acidic soil environments because they were equipped with adaptation system genetically to phosphorus deficiency. The plants perceive phosphorus deficiency when soil available phosphorus concentration is lowered, and synthesize acid phosphatase(EC 3.1. 3.2. denoted Apase) under acidic soil conditions. The plants exude the synthesized Apase out of cells to the soil solution, and it resolves phosphate ester molecules contained abundantly in the soil solution. Then plants absorb ortho-phosphate and consequently be adapted to the phosporus deficient soils. In some plants, on the other hand, they can not survive in Pi deficient soils because either they may not be equipped with Apase genes at all or may have genes with very low expression activity. Plants without Apase genes or with gene of low Apase

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expression activity can be cured by introduction of Apase genes from other genetical sources. In this expreiment, we tried to transform tobacco as a model plant with *PH05*, an Apase gene derived from *Saccaromyces cereviciae* by binary vector system of *Agrobacterium tumefaciens* to confirm the possibility to cure Apase gene abnormality.

Ⅱ. MATERIALS AND METHODS

1. microorganisms, plasmids and plants

The yeast strain, *Saccharomyces cerevisiae* NA 87-11A and *Escherichia coli* strains of HB101, JM8 3, JM105 and JM109 were used for cloning and manipulation of plasmids. Plages of $M13_{mp18}$ and $M13_{mp19}$ were used for the sequencing of 5' and 3' end of *PH05* fragment. *Agrobacterium tumefaciens* LBA4404 was used for transformation of plant tissures. Plasmid pVC727G from professor Yongil Hwang of Kyungnam University was used as the source of acid phosphatase gene, *PH05* and pBI 121 as the binary vector for plant transformation. *Nicotiana tabacum* cv. Samsun was used as a model plant in the transformation experiments. Plants were grown for 20 days for the genomic DNA isolation and for tissue culture.

2. Media

E. coli strains were grown at 37℃ in LB medium with vigorus shaking. YEP medium was used for the growth of *A. tumefaciens*. YPD and YM medium were used for the growth of Yeast strains. Ampicillin(50 μg/ml), kanamycin(50 μg/ml) and rifampicin(100 μg/ml) were added, if necessary. Transgenic plants were induced in the MS medium.

3. Staining of Acid Phosphatase activity

Acid phosphatase activity was assayed by Tohe and Oshima method(1974). Color development was observed after overlaying following solutions of 0.1M Na-acetate buffer(pH 4.0), α-naphthyl phosphate and Fast Blue Salt B onto the Apase sources.

4. Preparation of plasmid DNA

Devenish and Newlon method(1982) was used for the preparation of plasmid DNA from *S. cerevisiae*. Plasmid DNA in *E. coli* was isolated by the methods of Birnboim and Doly(1979) and Ish-Horowitz and Burke(1981). All plasmids isolated from Yeasts or *E. coli* were stored at −20℃ with small aliquots. Plasmid DNAs and DNA fragments digested with restriction enzymes were observed after electrophoresis on the 0.8% agarose gel.

5. Subcloning of *E. coli* and *A. tumefaciens*

Preparation of competent cells and subcloning of *E. coli* were conformed with Dower et al.(19 88) and Taketo(1988) methods. Plasmid DNA was transfered into *A. tumefaciens* as described by Holsters et al.(1978: freeze thaw method).

6. Sequencing and hybridization

The sequencing of 5' and 3'end of *PH05* was performed by dideoxy chain termination method (Sanger et al.(1977). $M13_{mp18}$ and $M13_{mp19}$ with *PH 05* were infected to *E. coli* strain JM105. After obtaining ssDNA, primers were annealed at 65℃ water bath for 30min. Then Klenow fragment and deoxynucleotides(dATP, dTTP, dGTP and dCTP) were added for DNA synthesis. After chain termination reaction, various sizes of DNA fragments synthesized were electrophoresed and autoradiographed for 20 hours. Genomic DNA isolated was treated with several restriction enzymes and hybridized with *PH05* as a probe for confirmation of plant transformation with Southern's method (1975). Colony hybridization was performed by Hanahan and Meselson method(1983) for screening of modified plasmid.

7. Construction of pBKJ I

For transfering *PH05* into tobacco plants, the pBKJ I was constructed. Firstly pVC727-1 was constructed from pVC727G by removing 0.9kb GAP promoter region pBI 121 was double-digested with

Sst I and Sma I to remove GUS fragment and end-filled for blunt end ligation to construct pBKS 1, pBKS1 was modified by adding termination codon to make pBKS1-1, Finally the binary vector, pBKJ I for the plant transformation was constructed by ligation pBKS1-1 and 1.5kb *PH05* fragment from pVCT727-1.

8. Transformation of plant tissues

Transformation of plant tissues was conformed as described by Horsch et al.(1985) and An et al. (1986). Sterilized tobacco leaves were sliced into 1 cm sections and infected with *Agrobacterium* cells. After co-cultivation for 2 days, *Agrobacterium* cells were washed out with sterilized water. The transformed tobacco shoots were selected with kanamycin resistance and were rooted on MS medium. Finally the transgenic plants were transplanted to soil medium. For another confirmation of transformation, genomic DNA was isolated as described by Murray and Thompson method(1980) for southern hybridization.

Ⅲ. RESULTS AND DISCUSSION

1. Isolation and confirmation of *PH05* from pVC 727G

Apase activity of S. cerevisiae NA87-11A with plasmid pVC727G was remarkably shown up as deep red color while the yeast without pVC727G did not show Apase activity when assayed by Tohe and Oshima method (1974). So the pVC727G was isolated from S. cerevisiae NA87-11A and transfered to *E. coli* strain HB101 as the pVC727G had been designed to have co-host(Hwang et al., 1989). The pVC727G isolated from *E. coli* was digested with several restriction enzymes to confirm the *PH05* fragment(Fig. 1).

When pVC727G was digested with EcoR I /Hind Ⅲ, the resulted fragments were 3.8, 1.5, 0.8 and 0.6 kbs as expected from the restriction map of pVC727G(no shown) among which the 1.5 kb fragment was thought to be the *PH05*. Both ends of 5' and 3' of *PH05* were sequenced using dideoxy chain termination method. The result was well coincided with the full length nucleotide sequences of *PH05* fragment(Arima et al., 1983) elucidated already.

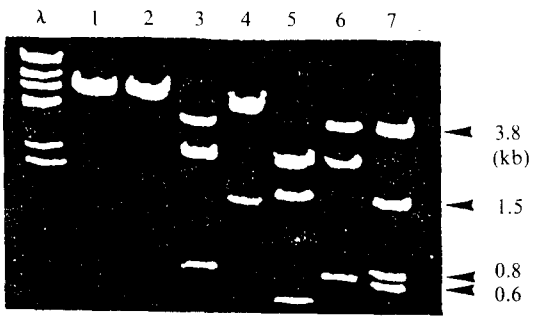

Fig. 1. Restriction enzyme fragments of pVC727G. λ: Hind Ⅲ digested λ DNA Marker, Lane 1: BamH I digestion, Lane 2: EcoR I digestion, Lane 3: Hind Ⅲ digestion, Lane 4: Sal I digestion, Lane 5: Pst I digestion; Lane 6: BamH I /Hind Ⅲ digestion, Lane 7: EcoR I /Hind Ⅲ digestion.

2. Construction of binary vector pBKJ I

The GAP promoter with the size of 0.9 kb was removed from pVC727G by BamH I /EcoR I double digestion and the resulted plasmid with 5.8 kb was end-filled and designated to pVC727-1. The pVC 727-1 was confirmed b electrophoresis for the fragments formed by BamH I, Hind Ⅲ and Kpn I digestion. The 0.9 kb GAP fragment was not found but the 1.5 kb *PH05* fragment was only found at the fragments of pVC727-1 digestion while the 2.4 kb fragment of *PH05* plus GAP was detected at those of pVC727G digestion(Fig. 2).

The *PH05* fragment of 1.5 kb was obtained from pVC727-1 by digestion with Hind Ⅲ followed by end filling and Xba I digestion again. The resulted fragment was with Xba I site at 5' end and blunted at 3' end.

The plasmid pBKS-1(12 kb) with 35S promoter which can be expressed in plants was double-digested with Xba I /Sma I and *PH05* fragment was inserted

to it. Thus formed plasmid was designated to pBKJ I (Fig. 3).

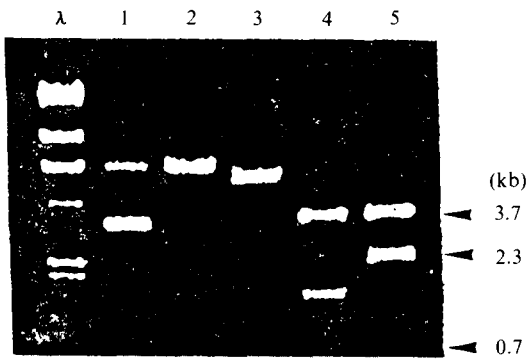

Fig. 2. Restriction patterns of pVC727-1 and pVC 727G.
λ: Hind Ⅲ digested λ DNA Marker, Lane 1, 3 and 4; digestion of pVC 727-1 with BamH I, Kpn I, and Hind Ⅲ, respectively, Lane 2 and 5; digestion of pVC727G with BamH I, and Hind Ⅲ, respectively.

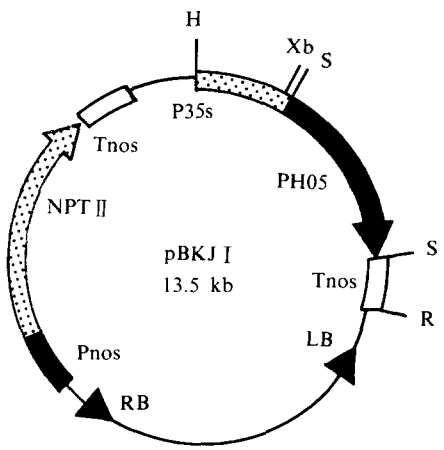

Fig. 3. Restriction map of pBKJ I.
P35S: 35S promotor of CaMV, Tnos: Terminator of nopaline synthase, Pnos, Promoter of nopaline synthase: NPT Ⅱ, Neomycin phosphotransferase Type Ⅱ: *PH05, Saccharomyces cerevisiae* APase gene: LB, Left boarder: RB, Right boarder; H, Hind Ⅲ: S, Sal I: R, EcoR I: Xb, Xba I.

E. coli strain JM83 was transformed with pBKJ I and was grown at the LB medium with kanamycin(50 μ/ml). Transformed colonies were screened by colony hybridization with the α-^{32}P labeled *PH05* s probe. Average 2-4 colonies were screened from one of total 10 petri dishes.

The correct positioning of *PH05* in pBKJ I between CaMV(Cauliflower Mosaic Virus) 35S promoter and NOS(Nopaline Synthase) terminator was assayed with restriction pattern after electrophoresis. When pBKJ I was digested with Sal I, about a 12 kb band and a 1.4kb band was observed as was expected because *PH05* has two Sal I sites on both 5' and 3' ends(Fig. 4 lane 6). Without *PH05* clone that has only one Sal I site showed a single 20 kb band(Fig. 4 lane 6).

The restriction pattern of pBKJ I with Xba I / EcoR I double digestion revealed a 2 kb fragment which was thought to be that of *PH05* plus Nos terminator(Fig. 4 lane 4). The southern hybridization result also convinced the existence of *PH05* in pBKJ I (data not shown).

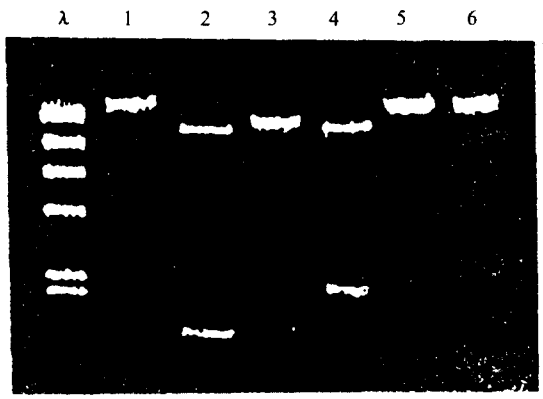

Fig. 4. Restriction pattern of pBKJ I and pBKS1.
λ: Hind Ⅲ digested λ DNA Marker, Lane 1: intact pBKJ I, Lane 2, 3 and 4: digestion of pBKJ I with Sal I, Xba I and Xba I/EcoR I, respectively, Lane 5: intact pBKS1, Lane 6: Sal I digested pBKS1-1.

3. Transformation of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens LBA4404 was transformed with the constructed pBKJ I. The transformed colonies were selected on YEP medium containing 50 μg/ml kanamycin and 100 μg/ml rifampicin. For the confirmation of transformed

Agrobacterium tumefaciens, plasmid DNA was isolated from the selected *Agrobacterium* and electrophoresed for the restriction pattern analysis(data not shown).

4. Transformation of tobacco plants

Tobacco leaf discs were cocultivated with the transformed *Agrobacterium tumefaciens* suspension (10^8 cell/ml) for 24 hours and transformed shoots were selected on kanamycin-containing MS-n/B medium. In all 20 treated plates, shoots were induced vigorously while in the untreated plates, leaf discs did not formed shoots in the presence of 50 μg/ml kanamycin. Shoots and roots were induced about 20 and 15 days after inoculation, respectively. Plantlets were transplanted to soil medium for sampling for the confirmation of transformation.

5. Genomic DNA southern hybridization

About 1.5mg of genomic DNA was extracted from 5g of transgenic tobacco plants. As a control, genomic DNA of nontransgenic tobacco plants was also isolated. Both DNAs were full-digested with Sal I and agarose gel electrophoresed for southern hybridization. Capillary transfered DNA to nylon membrane was hybridized with α-^{32}P labelled *PH 05* as a probe. Two bands were observed in transgenic plant(lane 1 of Fig. 5) while only a single band was observed in nontransgenic plant(lane 2 of Fig. 5).

The bands appeared in both transgenic and nontransgeic plants were thought to be the plant origin Apase genes but another band found in transgenic plant was inferred to *PH05*.

6. Expression of *PH05* gene in transgenic tobacco plants

As explained in the previous chapter, pBKJ I was constructed to have 35S promoter that can express in dicotyledonous plants, the transformed plants should have not only *PH05* itself but also show Apase activity. Therefore, the Apase activity was compared between the transgenic and nontransgenic plants.

Roots of plants were severed longitudinally for the extrusion of hidden root cells and color was developed with the addition of α-naphthyl phosphate as a substrate of Apase and Fast Blue Salt B. Five minutes later, deep red color was clearly developed in the roots of transgenic plant but in those of nontransgenic plant, no color was developed at all (Fig. 6). Leaf and stem tissues also showed the same result but deep dark color was developed instead of red color because of chlorophyll. In nontransgenic plants, original Apase activity was ignorable, which can be inferred that this plants will be suffered from inorganic phosphate deficiency under acidic soil conditions.

Under ordinary plant growing conditions, phosphate ions are absorbed only from roots not from leaves or stems. Therefore, the expression of Apase in leaves and stems is extravagance of energy for plants. In this experiment, however, transgenic plants expressed *PH05* in all tissues, leaves and stems as well as roots. Therefore, plasmids which have T-DNA containing *PH05* should be constructed for the root specific expression.

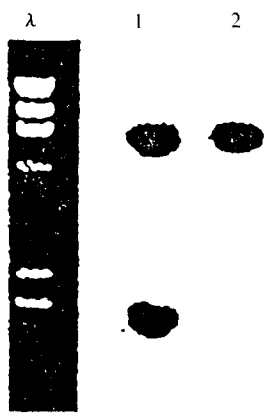

Fig. 5. Southern Hybridization of Genomic DNA from transgenic and nontransgenic plants
λ: Hind III digested λ DNA Marker, Lane 1: Sal I digested genomic DNA from transgenic plants, Lane 2: Sal I digested genomic DNA from nontransgenic plants.

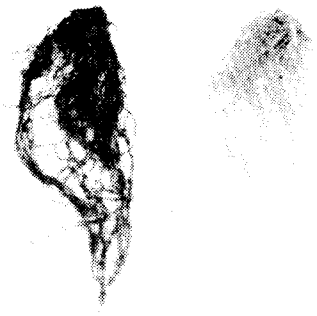

Fig. 6. Comparison of apase activity between transgenic (left) and nontransgenic(right) plant roots.
* Color was developed with application of α-naphyl phosphate and Fast blue B salt to severed plant roots. Picture was taken after 5 minutes incubation with color developing reagents.

Ⅳ. SUMMARY

This study was conducted to obtain the transformed tobacco plants with *S. cerevisiae* Acid phosphatase gene(*PH05*) using *Agrobacterium tumefaciens* and to confirm plant transformation and gene expression. The results obtained were summarized as follows: APase activity of *Saccharomyces cerevisiae* NA 87-11A was remarkably showed up as deep red color when assayed by Tohe and Oshima(1974). *PH05* fragment, Apase gene, was obtained from pVC727G and the graphically estimated size was about 1.5 kb by agarose gel electrophoresis. The sequencing results of 5'end and 3'end of *PH05* using dideoxy chain termination method were coinsided with the full length nucleotide sequence of *PH05* fragment having elucidated already.

pBKJ Ⅰ vector was constructed by isolation of *PH05* fragment from pVC727-1 and pBKS Ⅰ-1 digested with Sma Ⅰ and Xba Ⅰ. Isolated plasmid from transformed *A. tumefaciens* with constructed pBKJ Ⅰ when it was electrophoresed with agarose gel. The disc of tobacco leaf was cocultivated with transformed *Agrobacterium tumefaciens*. Transformed shoots were selected on kanamycin-containing MS-n/B medium and they were regenerated. The transgenic tobacco plants were elucidated by isolation of genomic DNA and genomic southern hybridization using α-^{32}P labelled *PH05* fragments. The *PH 05* in transformed tobacco plants was expressed in leaf, stem and root, and its APase activity was estimated as deep red color by Tohe method.

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