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Expression of Low Temperature Regulated Gene H28 in *Solanum tuberosum* L.

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감자식물체에서 H28 저온조절유전자의 발현

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ABSTRACT

Leaf disc explants of *Solanum tuberosum* cultivar, Desiree and Atlantic, were infected with a *Agrobacterium* MP90 strain containing chimeric gene construct, consisting of antibiotic and low temperature regulated gene (H28) for transformation. Regenerated multiple shoots were selected on a medium containing kanamycin and carbenicillin after exposure to *Agrobacterium*. Both PCR analysis of NPT II, H28 genes and northern blot analysis indicated that the genes coding for the enzyme were successfully integrated into the potato genome and could be expressed in potato plants.

Key words: *Agrobacterium*, chimeric gene, northern blot analysis, PCR

INTRODUCTION

Plants from temperate regions generally can acclimate to cold temperature and survive in this stress, which is called cold acclimation. In 1970, Weiser(1970) first suggested that cold acclimation may involve changes in gene expression. Since then, considerable efforts have been achieved for determining the molecular basis for this enhancement of freezing tolerance. Indeed, there is evidence that altered gene expression occurs during cold acclimation in a wide range of plant species(Gilmour et al., 1988; Guy et al., 1985). During the past 5 years, cold induced genes have been cloned from several plants, including alfalfa(Mohapatra et al., 1988), *Arabidopsis*(Hajela et al., 1990), barley(Dunn et al., 1990), wheat(Houde et al., 1992) and *Brassica*1(Weretilnyk et al., 1993). A cDNA clone, pBN H28, encoding a low temperature regulated transcript in winter *Brassica napus* has been isolated(Weretilnyk et al., 1993).

Potato(*Solanum tuberosum* L.) is a major food source in many parts of the world. In Korea, potato is the major horticultural crop, however its production faces several problems due to diseases, pests and chilling injury. In early spring, young potato seedlings are frequently damaged by frost, so not only production has decreased but also quality has deteriorated. For this reason much efforts have been devoted to the breeding of cold resistant potato cultivar. However, potato cultivars, in general, are highly heterozygotes which show significant difficulties to breeding programs. Recently, the introduction of foreign genes into potato by genetic engineering methods such as *Agrobacterium* mediated gene transfer has become possible.

The aim of our study was to develop antifreeze potato cultivar by using gene manipulation technology. Therefore, we have tried to introduce cold regulated gene into the potato genome by using leaf disc transformation mediated by *A. tumefaciens* MP90. PCR analysis of both NPT II and H28 gene, and northern blot analysis was carried out to confirm successful integration and expression of the gene in transgenic potato plants.

MATERIALS AND METHODS

Plant material: Growing apices and axillary buds from in vitro cultivated plants of the cultivars, Atlantic and Desiree, were subcultured every two weeks in a MS solidified medium without growth regulators. These plants were grown at 22±2℃ and exposed 16 h to 3000 lux illumination. After 14 days under these conditions, leaf discs were excised by trimming their borders.

Bacterial growth: *Agrobacterium tumefaciens* MP90 carrying the plasmid pBN::H28 was used to infect potato explants. This plasmid carries two genes that could be expressed in plant cells, such as low temperature regulated gene(H28), and neomycin phosphotransferase(NPT Ⅱ) which confers kanamycin resistance. *Agrobacterium* cultures were maintained on AB medium(An, 1987) containing the appropriate antibiotics : 25 ㎍/mL kanamycin and 25 ㎍/mL gentamycin.

Transformation and selection: Leaf discs were cocultivated with *Agrobacterium* cells of exponential phase. Co-cultivation was carried out in a AB liquid medium containing antibiotics in Petri dishes for 10 minutes. Leaf discs were then dried on sterile filter paper to remove bacterial culture medium excess. For an effective transformation, the explants were placed in the co-culture medium containing 2 mg/L of 2,4-D. Two days later, the explants were transferred to the regeneration medium(MS medium, supplemented with 2.0 mg/L Zeatin, 0.01 mg/L NAA and 0.1 mg/L GA_3, 3% sucrose at pH 6.0, solidified with 0.8% agar) supplemented with 100 ㎍/mL kanamycin for transformant selection and 500 ㎍/mL carbenicillin for *Agrobacterium* elimination. The explants were transferred to the regeneration medium every two weeks. Induced shoots were then transferred to MS medium supplemented with 0.25 mg/L BA and 0.1 mg/L GA3. Antibiotics were added as above.

Polymerase Chain Reaction for NPT II and H28 genes: Genomic DNA from 200 mg control plants and from all putative kanamycin resistant plants were extracted from leaves as described by Edwards et al.(1991) We have utilized the sequence information derived from pBN::H28 in *Brassica napus*, and designed degenerated oligonucleotides of corresponding genes from *S. tuberosum* genomic DNA for PCR amplification. The oligonucleotide 5′ -CAAGGTCAAGGATGCTGCTA -3′ was used as the sense primer and the oligonucleotide 5′ -AGCTTAA-ATCAGATGTTCAT-3′ as the antisense primer. The primer for the NPT II gene fragment, 5′ - GAGGCTATTC-GGCTATGACTG-3′ as the sense primer and 5′ - ATC-GGGAGCGGCGATACCGTA-3′ as the antisense primer were used for PCR screening of kanamycin resistant plants. Gene Amp PCR system 9600(Perkin Elimer Cetus, Norwalk, USA) was used in a reaction mixture of 20㎕ containing 0.1-0.5 ㎍ of genomic potato DNA using a Pre-mix Top(Bioneer). Mineral oil were overlaid with the mixture, and heated to 94℃ for 5 min. The PCR was performed with 35 cycles, each consisting of 1 min at 94℃, 1 min at 60℃ and 1 min 30 s at 72℃. PCR products were electrophoresised in 1% agarose gel and detected by ultraviolet light.

Northern blot analysis: Total RNA was extracted by the Ultraspec™-II RNA isolation system(Bio Tecx). Total RNA(30 ㎍) was fractionated by electrophoresis in 1.0% agarose gels at 4 V/cm in the presence of 2.2 M formaldehyde as a denaturant. After electrophoresis, the RNA was transferred to nylon membranes(Schleicher and Schuell), and fixed by cross-linking in a UV Spectrolinker(Spectronics Corporation). The RNA gel-blot filter was added to the hybridization buffer and the hybridization reaction was carried out at 42℃. RNA dot blot was performed

with the DIG labeled insert of H28.

RESULTS AND DISCUSSION

Transformation, regeneration and selection : Two cultivars of *in vitro* potato leaf discs were trimmed and co-cultivated with *A. tumefaciens* MP90 containing the binary vector for 10 min without agitation. Leaf discs were transferred to the selection medium(Fig. 1-A). After one week of culture, explants were enlarged twice. Calli formed on the cut edges of the explants were observed in the presence of 100 ㎍/mL kanamycin after three weeks of cocultivation(Fig. 1-B). Control leaf discs, that is, discs not infected with *Agrobacteria*, became chlorotic and did not produce any calli on medium containing 100 ㎍/mL kanamycin. Within 6 weeks after placing these calli on fresh selection medium, many of them formed shoots(Fig. 1-C). Putatively transformed shoots were excised when they were about 1 ㎝ tall and transferred to shoot elongation medium containing carbenicillin and kanamycin(Fig. 1-D). Thirty five shoots of cultivar Desiree and eleven shoots of cultivar Atlantic were selected in the presence of 100 ㎍/mL kanamycin. After this first selection, a stem and leaf fragments from each excised shoot were taken for identification of transgenic plants. After one to two weeks, rooted transformants were transferred to soil in a greenhouse(Fig. 1-E).

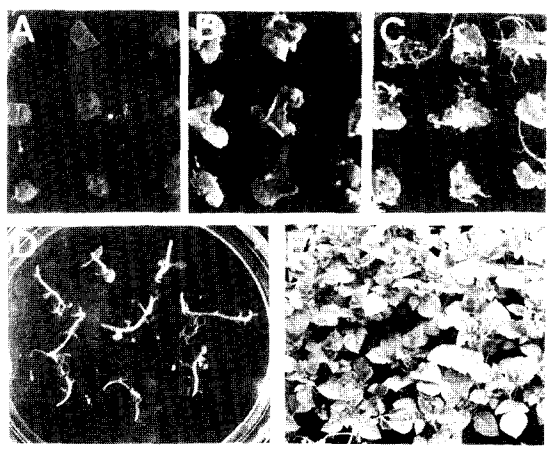

Fig. 1. Shoot regeneration from *Solanum tuberosum* leaf discs A) Leaf discs were cultured on MS selection medium after transformation with *Agrobacterium tumefaciens*. B) Kanamycin resistant calli developed on leaf discs. C) Numerous shoots formed on the explant. D) Transformed plantlets transfer to medium containing 100 ㎍/mL kanamycin, 500 ㎍/mL carbenicillin, 0.25 mg/L BA and 0.1 mg/L GA. E) Mature transformants in a greenhouse. The plants are vigorous and phenotypically normal.

Polymerase chain reaction analysis for selection of transgenic plants: DNA amplification of all 46 plants regenerated in the presence of kanamycin was analysed by PCR for the co-integration of the NPT II(Fig. 2) or cold regulated gene(Fig. 3). NPT II primers amplified a 700 bp fragment. Three of thirty five kmr Desiree transformants and three of eleven kmr Atlantic showed NPT II integration. To show the presence of the cold regulated gene(H28) from the T-DNA in the potato genome, PCR analyses were performed for the genomic DNA isolated from in vitro grown shoots. By using internal H28 primers for DNA amplification, the presence of the 328 bp H28 fragment was demonstrated in DNA from three lines of Desiree and Atlantic(Fig. 3), indicating that the H28 gene was incorporated into the genomic DNA of the transformants. No such fragment was detected in DNA from untransformed potato.

Northern blot analysis: Transgenic potatoes produced H28 mRNA transcripts that were detected by northern

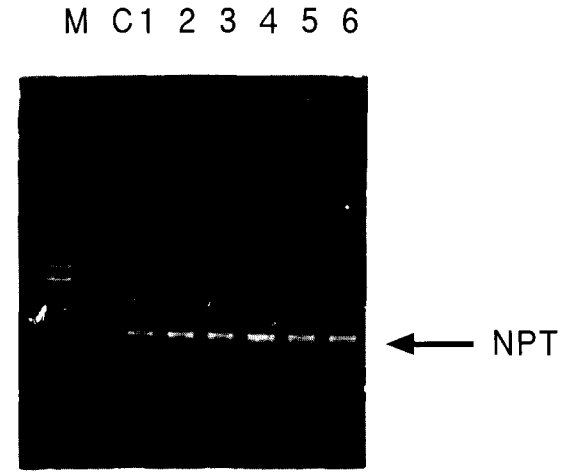

Fig. 2. PCR analysis of NPT II gene from genomic DNA of transgenic and control potato plants. M: 1Kb size marker, C: Control potato plant, lanes 1-6:Transgenic plants.

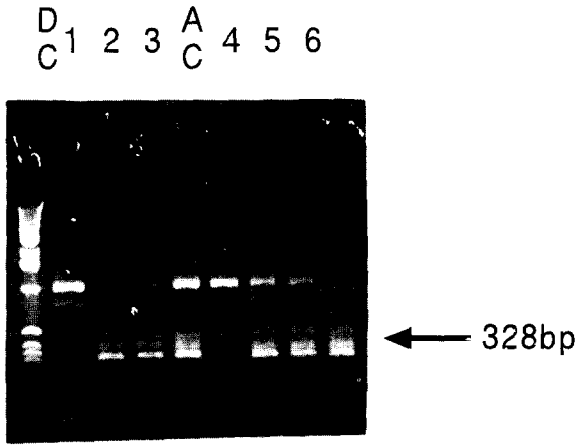

Fig. 3. PCR analysis of H28 gene from genomic DNA of NPT II positive transgenic and nontransgenic potato plants. PCR products obtained about 328 bp bands after amplification of the insert cold regulated gene(H28) in transgenic potatos. M: 1Kb size marker, DC:Control pototo plant(cv. Desiree), lanes 1-3: Transgenic plants(cv. Desiree), AC:Control pototo plant(cv. Atlantic), lanes 4-6: Transgenic plants(cv. Atlantic).

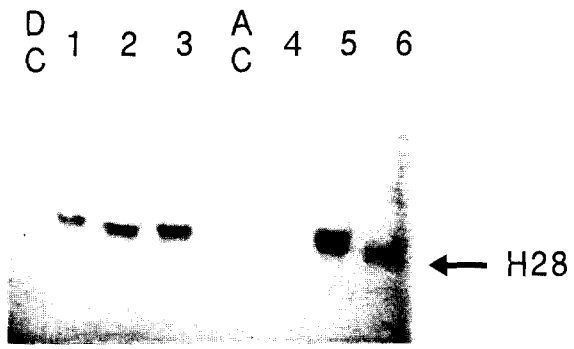

Fig. 4. Northern hybridization of total RNA isolated from transgenic and normal potato plants. Thirty ㎍ total RNA was electrophoresed, blotted and then hybridized with DIG labelled H28 as a probe. Lanes DC and AC, control potato cv. Desiree and cv. Atlantic; lanes 1-3, transgenic plants(cv. Desiree); lanes 4-6, transgenic plants (cv. Atlantic).

blot analysis(Fig. 4). No signal could be detected in the control potatoes(lanes DC and AC). In the cultivar of Desiree, similar levels of transcripts were observed in all three lanes(lanes 1,2 and 3). Transcripts hybridizing to H28 were observed in all three lines of cultivar Atlantic(lanes 4, 5 and 6) whereas the transcript could be detected in the lane 4, although level was much lower than others.

Shoot regeneration from transformed cells has been a major problem in transformation of potato. Tuber discs(Sheerman and Bevan, 1988; Stiekema et al., 1988), leaf discs(De Blok, 1988; Ooms et al., 1987), stem segments of explants(Twell and Ooms, 1987) were used in the transformation. In our experments, leaf discs gave the most uniform and rapid response and produced the largest number of transgenic shoots compared to tuber discs and stem explants. This result agree with Horsch et al.(1984). Therefore, the leaf discs have been successfully utilized to regenerated transgenic plants from potato. There is a difference in shoot regeneration ability between different cultivars. Over 80% discs of the Desiree produced multiple shoots, while 20% of leaf discs from cultivar Atlantic was regenerated. Desiree was more efficient than Atlantic. For Atlantic cultivar, it may be necessary to use several protocols to increase transformation efficiency. When subjected to selection, transformed leaves were able to produce some calli along the wound tissues, while untransformed leaves became progressively chlorotic in the presence of 100 ㎍/mL kamamycin. We obtained 6 independently transformed lines that contained cold regulated gene of the H28. A strong hybridizing signal was observed in lane 5. Such data suggest that lane 5 may have the most cold resistance activity among the 6 transgenic potatoes.

Cold acclimation in higher plants is a complex process that results in increased freezing tolerance. Numerous changes have been associated with cold acclimation, for example, alterations in lipid composition, increased levels of soluble proteins and sugars, changes in gene expression, and the appearance of new isozymes(Steponkus and Lynch, 1989). However, the critical role that a given change has in the freezing tolerance is uncertain. Therefore, further analysis is necessary to evaluate numerous changes and the cold resistance in field environment. Experiments are now in progress to assay for the tolerance of transgenic potatoes to cold exposure.

적 요

저온관련 유전자인 H28 유전자를 갖고 있는 *Agrobacterium* MP90균주를 Desiree와 Atlantic 감자 품종의 잎절편과 공동배양한 후 약 6-8주 후에 항생제가 첨가된 선발배지에서 multiple shoots를 얻을 수 있었다. 선발된 형질전환체를 대상으로 PCR 방법을 이용한 NPT II 유전자와 H28를 확인한 결과 내한성유전자가 식물체의 genome에 안정적으로 삽입되었음을 확인하였고 northern blot 확인에 의하여 내한성 유전자가 발현됨을 확인하였다.

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