한국식물병리학회지 11(2) : 173-178(1995)
Korean J. Plant Pathol. 11(2) : 173-178(1995)

Double-Stranded RNA-Dependent Protein Kinase Gene Expression in Tobacco Plant

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연초식물체에서의 dsRNA 의존성 인산화 효소 유전자 발현

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ABSTRACT : Double-stranded (ds) RNA-dependent protein kinase (PKR) participates in an antiviral mechanism by regulating cellular protein synthesis in the mammalian host system; however, it has been little characterized in plants. To elucidate their roles in plant sysem, the human PKR gene has been constructed into the plant expression vector and established transgenic tobacco plants. A 1.8 kb *Hin*dIII/*Pst*I fragment of phPKR containing complete open reading frame (550 amino acids) of PKR cDNA franked with untranslated regions at both ends was cloned into the site of *Sac*I/*Sma*I in GUS region-deleted pBI121 and designated as p12168 which was verified by restriction enzyme digestion. PKR transgenic tobacco plants were obtained by *Agrobacterium*-mediated transformation. Transformed tobacco plants selected based on kanamycin (100 μg/ml) resistance were regenerated in Murashige and Skoog (MS) agar media supplemented with 2 mg/l benzyladenine (BA), 0.5 mg/l naphtaleneacetic acid (NAA) and 500 μg/ml carbenicillin. Integration of PKR cDNA in plant genomic DNA was confirmed by polymerase chain reaction (PCR). A 1.4 kb DNA fragment produced by reverse transcription PCR (RT-PCR) suggested the expression of CaMV 35S promoter-driven 1.8 kb-PKR mRNA.

***Key words* : dsRNA-dependent protein kinase gene, transgenic tobacco plant, mRNA expression, PCR, RT-PCR.**

The double-stranded (ds) RNA-activated protein kinase (PKR) in human, a member of serine/threonine kinases, is induced by interferon treatment and becomes autophosphorylated on its serine/threonine residues by the presence of dsRNA (11, 21, 25). The initiation factor eIF2-a, which is required as a component of the translation initiation ternary complex, is phosphorylated by the activated PKR, sequesters the GTP/GDP exchange factor (eIF-2B), and inhibits the initiation of protein synthesis (16). The activation of PKR is triggered by dsRNA molecules during the viral infection or by single-stranded RNA containing stem region in its secondary structure. The interferon-induced PKR, therefore, has been considered to participate in an antiviral system as well as cell proliferation in mammals (5, 24).

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The PKR-mediated antiviral system in plants, however, is in question even though it has been reported that a protein of 68 kDa in tomato was immunologically reacted to human PKR and was differentially phosphorylated by potato spindle viroid (PSTV) (17), and that tobacco mosaic virus (TMV) or synthetic dsRNA could phosphorylate the 68 kDa protein from tobacco plant (18). It is only suggestive that these 68 kDa phosphoproteins are related to the antiviral action in plants. It is interesting to understand whether or how the kinase-regulated protein synthesis plays a role as a part of the antiviral mechanism in plants.

In this study, we have established transgenic tobacco plants expressing the PKR gene in an effort to initiate molecular study of PKR. The construction of CaMV 35S promoter-linked PKR gene, transgenesis of tobacco plants by *Agrobacterium*-mediated transfor-

mation, and the analysis of PKR gene expression from the transgenic tobacco plants were performed.

MATERIALS AND METHODS

Plants. Seeds of tobacco (*Nicotiana tabacum* cv. Xanthi-nc) were surface-sterilized in 70% ethanol and 5% sodium hypochlorite solution, and grown on Murashige-Skoog (MS) agar medium (26) without phytohormone. Tobacco leaves were used for *Agrobacterium*-mediated transformation (1).

Plasmid. Double-stranded RNA-dependent protein kinase (PKR) gene inserted in pBluescript SK+ plasmid vector (phPKR) was obtained from Dr. Hovanessian (23). The phPKR plasmid was amplified in *Escherichia coli* DH5 by transformation and plasmid purification method (29). The 1.8 kb-*Hin*dIII/*Pst*I fragment from the phPKR plasmid was separated in 1% agarose gel electrophoresis and purified by the Gene Clean method (Bio101 Inc., CA) for its cloning in plant expression vector pBI121.

Transgenic plant. Tobacco leaf discs (about 5 mm^2) were immersed for 10 min in the culture of *A. tumefaciens* LBA4404 harboring recombinant plasmid grown in Luria-Bertani (LB) liquid medium containing streptomycin (50 μg/ml) (1), and placed on MS shooting agar medium containing phytohormones (benzyladenine 2 mg/L, naphtaleneacetic acid 0.5 mg/L) without antibiotics for two days. Samples were transferred to the new plate of MS shooting agar medium containing 100 μg/ml kanamycin and 500 μg/ml carbenicillin for eradication of the bacteria. After 2 to 3 weeks, shoots were transferred for rooting to phytohormone-free MS agar medium with antibiotics. Transgenic tobacco plants were transferred to pots and maintained in a greenhouse.

Polymerase chain reaction (PCR) for detection of DNA integration. Leaves from plant transformants were ground in liquid nitrogen to fine powder, and genomic DNA was isolated by the CTAB-based method (31). PKR DNA integration in plant genomic DNA was identified by PCR. For PCR, 100 ng of genomic DNA was mixed with two synthesized primers 5'-TTTCCAGAAGGTGAAGGT-3' (primer 1) (10, 30) and 5'-TGTGTTAGGTCGATCCTC-3' (primer 2). DNA fragments were amplified by 40 thermocyclings (95°C, 30 sec; 53°C, 45 sec; 72°C, 80 sec). The reaction products were chloroform-extracted, followed by the precipitation step with 0.1 vol. of 7 M ammonium acetate and 2 vol. of absolute ethanol (20). The PCR products were analyzed in 1% agarose gel electrophoresis.

Reverse transcription (RT)-PCR. Total RNA was extracted from leaves of either nontransformed or transformed tobacco plants. Samples ground in liquid nitrogen to fine powder were suspended in the denaturing solution containing guanidinium isothiocyanate (29). Total RNA was monitored either by spectrophotometer for its quantitation or in 1 % agarose gel in TAE buffer to confirm intact RNAs. Using 1 μg of total RNA, first strand cDNA was synthesized at 42°C for 1 hour using AMV reverse transcriptase in the presence of primer 2 according to the manual from Promega Inc. (Madison, WI). The first strand cDNA was amplified with primers 1 and 2 by PCR in 40 thermocyclings of 95°C, 30 sec / 54°C, 30 sec / 72°C, 90 sec followed by 5 min of the postreaction period. The reaction products were analysed in 1% agarose gel electrophoresis.

RESULTS

Construction of PKR in plant expression vector. To know how PKR gene from human origin may play a certain role in respect of the regulation of protein synthesis and of antiviral system in plant cells, we first constructed PKR cDNA in the plant expression vector pBI121 as shown in Fig. 1. PKR cDNA maintained in pBluescript SK+ was digested with *Hin*dIII and *Pst*I restriction enzymes to obtain 1.8 kb fragment containing open reading frame franked with 5' untranslated 24 nucleotides with the conserved Kozak box (GAAGAAATGG) (19) and with 3' untranslated region. The *Hin*dIII/*Pst*I fragment was re-introduced to pBluescript SK+ by which additional cloning sites could be available for the following manipulation. The *Hin*dII/*Sac*I fragment was ligated to the plant expression vector pBI121 cut with *Sac*I and *Sma*I restriction enzymes to produce p12168.

The construction of p12168 was identified by restriction enzyme digestion. Restriction fragments were separated on 1% agarose gel and the results are shown in Fig. 2. Intact pBI121 (Fig. 2, Lane 1) or p12168 (Lane 2) was linearized by digestion with *Hin*dIII (Lane 6) or *Sac*I (Lane 3), respectively. The 1.8 kb PKR DNA insert was produced by digesting p12168 with *Sac*I and *Hin*dIII (Lane 4) where the 0.8 kb CaMV promoter region was also shown as seen from *Hin*dIII-digested p12168 (Lane 5). After confirmation

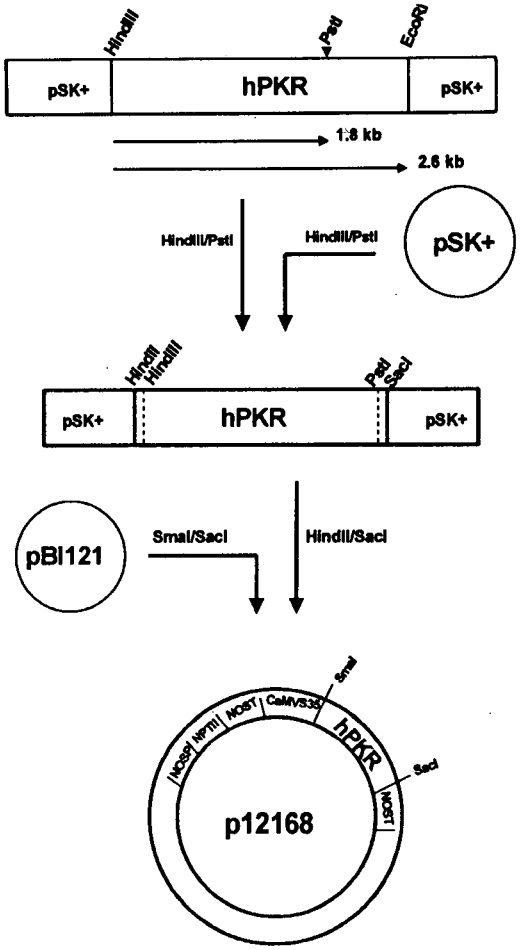

Fig. 1. The construction of hPKR in plant expression vector.

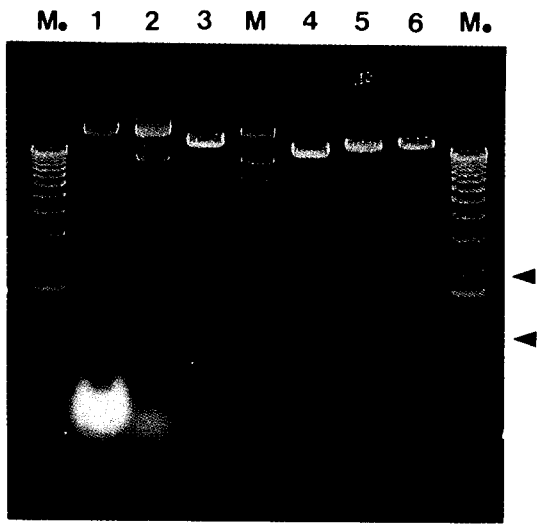

Fig. 2. Agarose gel electrophoresis of recombinant plasmid DNA. Lane M (left) : 1 kb ladder DNA marker, Lane M (right) : lambda DNA/*Hin*dIII DNA marker; Lane 1 : pBI121; Lane 2 : p12168; Lane 3 : p12168 *Sac*I cut; Lane 4 : p12168 *Sac*I/*Hin*dIII cut; Lane 5 : p12168 *Hin*dIII cut; Lane 6 : pBI121 *Hin*dIII cut. Upper and lower arrows indicate the inserted PKR DNA of 1.8 kb and the 0.8 kb CaMV 35S promoter region, respectively.

of the orientation and site of PKR cDNA by restriction enzymes digestion, p12168 was introduced into *A. tumefaciens* LBA4404. Bacterial transformants selected on LB agar medium containing kanamycin (50 μg/ml) and streptomycin (50 μg/ml) were used to transform tobacco, *Nicotiana tabacum* cv. Xanthi-nc.

Transgenic tobacco plants and its expression of PKR gene. p12168 harbored in *A. tumefaciens* LBA 4404 was transferred to tobacco leaf discs by incubating them with overnight-grown *Agrobacterium* transformants. The selected transgenic tobacco plants were subject to analysis of PKR DNA insertion or their mRNA expression. Genomic DNAs were purified from normal, pBI121 vector- or p12168-transformed tobacco plants. Fig. 3-A (Lane D) shows genomic DNA band migrating with 23 kb DNA fragment of the *Hin*dIII-digested λ DNA marker. hPKR DNA insertion was examined by PCR method. Expected product from PCR is 1.4 kb in length due to use of internal primers, primer 1 partly representing the site of RNA binding domain of N-terminus and primer 2 at kinase domain of C-terminus. Fig. 3-B shows that normal (Lane 1) or pBI121-transgenic tobacco plant (Lane 2) has no PCR products, while many of transformed plants tested in this experiment (Lane 3~7) showed 1.4 kb DNA fragment in varying amounts, suggesting stable integration of PKR DNA in plant genome. Other types of DNA fragments did not appear probably due to low homology of tobacco DNA to hPKR gene under our experimental conditions.

To analyze PKR gene expression in transformed tobacco plants, reverse transcription PCR (RT-PCR) was performed. Although this technique is not precisely quantitative as northern hybridization technique (29), it determines certain level of mRNA expression in a short period of time with ease. Total RNAs isolated from transformed plants analyzed by PCR (Fig. 3-B, Lane 3, 4, 6 and 7) were monitored for their degradation by 1% agarose gel electrophoresis in non-

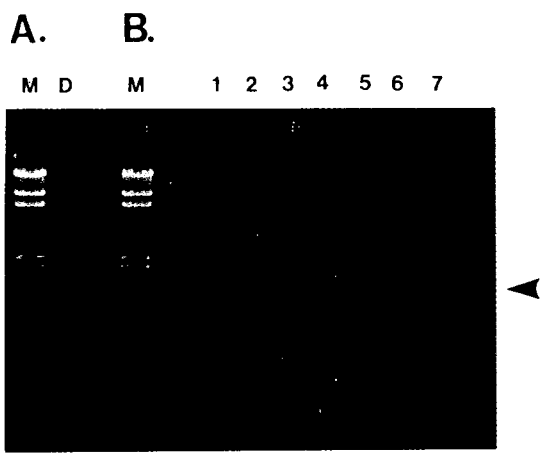

Fig. 3. hPKR DNA in transgenic plants. A) Lane M represents lambda/*Hin*dIII DNA marker. Lane D is genomic DNA extracted from transgenic plants. B) Lane M is lambda/*Hin*dIII DNA marker. Lane 1~7 : PCR products in 1% agarose gel electrophoresis (Lane 1 : normal; Lane 2 : pBI121 transformed; Lane 3~7 : hPKR transformed tobacco plants). Arrow indicates the 1.4 kb DNA fragments produced by PCR.

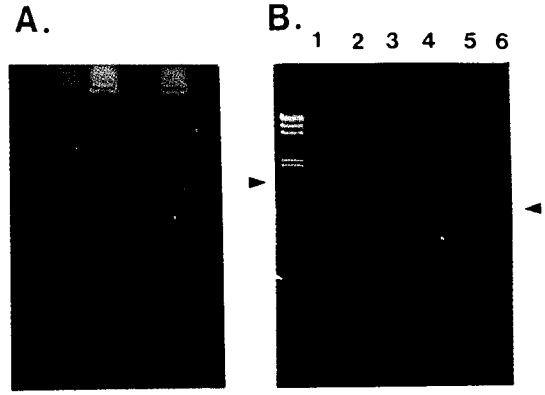

Fig. 4. hPKR gene expression in transgenic plants. A) Electrophoresis of RNA in 1% agarose gel in TAE buffer. B) Lane 1~6 : RT-PCR products of total RNA isolated from transgenic plants(Lane 1~4 and 6) or from normal plant (Lane 5). Left and right arrows indicate the 1.4 kb DNA fragments produced by RT-PCR and about 1.0 kb RT-PCR product respectively.

denaturing conditions. Two discrete bands of RNA (28S and 18S rRNA) from intact RNA of transgenic plants are shown in Fig. 4-A. One microgram of total RNA was used for reverse transcription and the following first strand cDNA was PCR-amplified. Although the level of cDNA synthesis was quite low and slightly variable, transgenic plants produced PKR mRNA expressed as 1.4 kb DNA fragment in Fig. 4-B (Lane 1~4). Normal plant (Lane 5) did not show any cDNA fragments. cDNA in short length (about 1 kb) was produced when RNA sample for Lane 1 was amplified in the presence of primer 1 and a primer representing upstream of primer 2 by RT-PCR (Lane 6).

DISCUSSION

As an effort to genetically fortify self-defense system in plants against viral infection, genes from virus sources have been introduced into plant genome. For example, viral coat protein gene product has been reported to confer a molecular cross protection from infection of viruses such as TMV (27), potato virus X (15), and alfalfa mosaic virus (32) on its transgenic plant. Antisense viral RNA produced in transgenic plants mediates resistance by competition with challenging viral sense RNA (7, 28). In contrast to viral gene-aided plant protecion, plants themselves in nature show hypersensitive response as a way of resistance to virus infection (2). For example, induction of pathogenesis-related (PR) proteins during necrosis-causing viral infection has been recognized as a defense reaction (3, 4).

Double-stranded RNA-dependent protein kinase (PKR) as well as 2'-5' oligoadenylate synthase activated by dsRNA and induced by interferon treatment have been described as agents involved in the inhibition of the virus proliferation in mammalian systems (11, 14). PKR in the presence of dsRNA is autophosphorylated to phosphorylate-subunit of the protein initiation factor, IF2, mediating the inhibition of protein synthesis and virus multiplication as a consequence. In plants, 68 kDa protein has been reported to be immunologically crossreactive to human PKR antibody and activated in the presence of dsRNA (17). Therefore, it is possible to propose that the putative PKR in plants may be a novel subject in the light of setting up defense strategy at molecular level.

Based on the assumption of analogy in plants of mammalian cellular protein synthesis machinery, and of antiviral PKR activity, we manipulated human PKR cDNA so as to be expressed in transgenic tobacco, with a purpose to monitor any sign of inhibition of viral multiplication ultimately. In this study, we described PKR transgenesis in tobacco plants by demonstrating its stable integration in plant genome by

PCR and expression by RT-PCR. Recombinant PKR protein produced in bacterial system shows inhibitory effect on bacterial growth due to its unknown toxicity (personal communication). PKR in plant, however, should be seemed to have stimulating effects on either plant regeneration or growth. In overall observation, transgenic plant physiology was quite normal when compared to normal plants during our experiment (data not shown). Although we need to analyze PKR protein synthesis in correlation with mRNA expression, PKR transgenic plant may be utilized as a material for elucidation of PKR activity in relation to viral multiplication and protein synthesis in plant system as well. Regulation of protein synthesis by kinase activity in plants will add an understanding how plant virus multiplication is regulated in plants or how plant virus escapes from PKR-mediated antiviral system as demonstrated in animal virus; for example, small RNA inhibitor of PKR activation (Adenovirus VAI RNA), dsRNA binding viral proteins that compete with PKR (Vaccinia virus E3L protein), proteins that serve as surrogate substrate for phosphorylation by PKR (Vaccinia virus K3L as a homolog eIF2), virus-induced host cellular protein inhibitor of PKR, virus-induced proteolysis of PKR, and down regulation of interferon regulated proteins by virus coded proteins (adenovirus E 1A) (8, 9, 13, 22, 25).

요 약

동물계에서 항바이러스와 관련된 dsRNA 의존성 인산화 효소(PKR)의 유전자를 식물체에서 발현시킬 경우 PKR에 의한 단백질합성 및 식물바이러스의 증식 조절 가능성에 대한 기초자료를 확보하기 위하여 사람에서 분리된 PKR cDNA를 *Agrobacterium* 방법에 의하여 연초식물체(*Nicotiana tabacum* cv. Xanthi-nc)로 형질전환시켰다. *Hin*dIII/*Pst*I 처리에 의해 얻어지는 약 1.8 kb의 phPKR cDNA 절편을 일련의 유전자 조작 방법을 통하여 식물발현벡터인 pBI121에 도입하여, p12168을 재조합하였다. 이를 *A. tumefaciens* LBA 4404에 형질전환시켜 연초식물체형질 전환에 이용하였다. 2 mg/l BA와 0.5 mg/l NAA가 포함되고 100 μg/ml의 kanamycin이 첨가된 MS배지에서 shooting시킨 후 phytohormone이 첨가되지 않은 MS배지상에서 rooting을 시켜 형질전환 연초식물체를 얻었으며, 형질전환식물체는 정상식물체와 유사한 생육양상을 나타내었다. 형질전환식물체의 유전자도입은 hPKR cDNA의 전사여부는 RT-PCR 방법에 의하여 확인되었다.

ACKNOWLEDGEMENT

The present study was supported by the Basic Science Research Institute Program, Ministry of Education (BSRI-'95-4403) and by the research fund of Taegu Hyosung Catholic University (1994).

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