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Application of heterogeneous RNA probes labeled immuno -fluorescent agent DIG for the screening a noble gene in cucumber

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ABSTRACT

We tried to isolating a noble gene from cucumber library with heterogeneouse RNA probe labeled DIG of *Arabidopsis* PIN3 gene. Two kinds of RNA probes which had no significant homology each others, were designed from the 5'- and 3'-prime nucleotides of the AtPIN3 gene. In the first and second screenings of the cDNA library of cucumber with the probes, two positive clones were identified with specific duplicate signals. However, we isolated cDNA fragments homologous with putative nucleases from *Nicotiana, Arabidopsis, Cordialis*, and *Oryza sativa*, there was no significant homology with any other PIN family genes.

Key words : DIG, AtPIN3, *Arabidopsis*, Cucumber, Nuclease

INTRODUCTION

At though protocols of cloning a new gene were invented more than ten years ago, applications of the probes of DNA, RNA, and protein like antibodies including labeling isotopes and immunofluorescents like the DIG and T-DNA tagging procedure (Sambrook et al. 1989, Bennett et al. 1996), and so on, are for the most part still unclear and imperfect.

In some organisms with completed genome sequences, such as yeast, *Caenorhabdtis elegans*, and *Arabidopsis thaliana*, it is possible to profile their gene expression at the transcription level (De Risi et al. 1997, Hill et al. 2000, Moseyko et al. 2002). Microarray technology with cDNA and oligonucleotide probe allow the accurate measurement of mRNA transcript for hundreds thousands of genes in parallel (Schena et al. 1995, Schena et al. 1996, Chee et al. 1996, Lipshutz et al. 1999). To screen a new gene, the application of a DNA probe increases the possibility of taking a partial clone that includes partial sequences from the library. On the other hand, RNA probe has one of merit to take full sequence because it is impossible for the probe to hybridize on any plaque if it did not express. The

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technique for cloning new genes is generally related to a homogeneous nucleotide probe (Müller et al. 1998, Friml et al. 2002a, 2002b, Choo et al. 2001). The application of a homogeneous probe is adopted to design degenerated primer and to isolate high homology EST clones including domain (Fujii et al. 2000). In labeling probes, the use of isotope is one of the effective methods in screening target genes but radioactive permits possible dangers to humans and environments. Though a DIG-labeled heterogeneous probe is hard to hybridize, the application of heterogeneous probe does not need a degenerated primer and an EST clone, and thus can save time and costs.

In our work, we try to the possibility of screening a noble gene from the cucumber cDNA library using heterogeneouse DIG labeled RNA probe from *Arabidopsis* PIN3 (AtPIN3).

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* Heynh., ecotype Columbia were surface sterilized with 1.0% NaOCl and rinsed several times in sterile distilled water. The sterilized seeds were placed on MS-agar media in disposable plastic petri-dishes and incubated at 24℃ for 16 days with the vertical position.

Isolation of total RNA

Total RNA of *Arabidopsis* was isolated from the roots of 16 days old seedling using the TRI-reagent (Sigma T-9424). The isolation procedures were followed by the descriptions as Chomczynski and Mackey (1995).

Purification of cDNA fragments with RT-PCR

For the cDNA synthesis, the RT-PCR analysis was performed using MMLV RTase (MMLV Reverse Transcriptase, GenHunter Co.) with 0.5 ug of total RNA. The PCR reaction were performed on a PCR System 2700 (Applied Biosystems) under following condition; 1 min at 95℃, followed by 30 sec at 94℃, 30 sec at 55℃, and 1 min 72℃ for 35 cycle, and 5 min at 72℃ for a final extension. To amplify AtPIN3RN (5'-stream region in AtPIN3) and AtPIN3RC (3'-stream region in AtPIN3) regions, two types of oligonucleotide sets were designed from the *Arabidopsis* cDNA (accession no. AF087818). AtPIN3RN and AtPIN3RC fragments were amplified using two sets of oligonucleotides, 5'-ATGGCACGACCTCTACACGG-3' (AtPIN3RNF)/ 5'-GCTCCGG TGAGATTTGAC GG-3' (AtPIN3RNR), and 5'-GCGCTACAATCC AAGAC AGG-3' (AtPIN3RCF)/ 5'-AACCA GCGTGATCGGAAGCG-3' (AtPIN3RCR). PCR products were size-fractionated by electrophoresis in 1.0% agarose gel, and then, recovered from the gel with a Gel Extraction Kit Millipore (QIAEX II, QIAGEN).

Cloning and sequencing of AtPIN3RN and AtPIN3RC

Amplified DNA fragments, AtPIN3RN and AtPIN3RC, were ligated into a pGEM-T easy vector (Promega) overnight at 16℃ and then the resultant plasmids were transformed into the E. *coli* DH5*α*. The plasmid DNAs were prepared by the alkaline lysis method and purified by equilibrium centrifugation in CsCl/EtBr gradients (Sambrook et al. 1989). The sequence analysis of AtPIN3RN and AtPIN3RC fragments were performed with the BigDye terminator V3.0 cycle sequencing ready reaction kit (ABIprism 4390242) and ABI sequencer (Model 310, Perkin-Elmer Japan Co.). The sequence data were analyzed using DNASIS 3.7.

Preparation of AtPIN3RN and AtPIN3RC RNA probes

The plasmid DNAs containing AtPIN3RN and AtPIN3RC fragments were linearized by restriction

enzyme SpeI and purified by a phenol/chloroform extraction and ethanol precipitation. RNA probes were synthesized with T7 RNA polymerase and a DIG labeling mixture (Boehringer Mannheim 1277 073).

Screening of a gene from cDNA library

Two kinds of cDNA libraries were constructed using total RNA which isolated from the auxin treated hypocotyls or peg region (transition zone) of cucumber (Fujii et al. 2000). For the screening of positive plaques, plaque-lift was carried out using nylon membrane (the first screening, Schleicher & Schuell 10416196; the second screening, Roche 1 699 075) and hybridized overnight at 58℃ with the RNA probes after prehybridizing in a standard buffer by 2 hour at 58℃ according to the manufacturer's manual (Boehringer Mannheim). Positive signal plaques were automatically subcloned with *in vivo* excision according to the manufacturer's protocol (Stratagene). The nucleotide sequences of isolated clones were determined using the BigDye terminator V3.0 cycle sequencing ready reaction kit and ABI sequencer.

RESULTS AND DISCUSSION

The 5'- and 3'- prime cDNA fragments of AtPIN3 were amplified with the oligonucleotide sets designed from a highly conserved region with *Arabidopsis* PIN1, PIN2, PIN4, PIN6, PIN7, and cucumber PIN1 from the cDNA of *Arabidopsis* seedlings (Fig. 1A). The sequences of the 5'- and 3'- prime DNA fragments were compared with that of AtPIN3. AtPIN3RN sequence is different from AtPIN3 sequence in 3'-stream region. AtPIN3RN sequence is different from that of AtPIN3 sequence in 3 bases. In the 3'- prime DNA fragment, AtPIN3RC sequence is identical with that of AtPIN3 sequence. The sizes of AtPIN3RN and AtPIN3RC are 684bp and 537bp, respectively. The RNA probes were made from AtPIN3RN and AtPIN3RC sequence for screening cDNA using two kinds of cucumber libraries (Fig. 1B). Two duplicate-signal plaques were obtained from the auxin treated hypocotyls library using AtPIN3RN and AtPIN3RC probes in the first screening. Forty eight single-signal plaques were performed for the second screening together (Fig 1C). As expected, we obtained a duplicate-signal from plaque number 6 with the AtPIN3RN probe and a duplicate-signal from plaque number 49 with an AtPIN3RC probe in the second screening (Fig. 1C). The cDNA inserts of positive clones were sequenced from three positive clones (red cycle, a, b, c in Fig. 1C) per plate and then *in vivo* excision was accomplished.

In study of screening the cDNA fragments with RNA probes, two clones were hybridized with the two kinds of probes encoded identical sequences (Fig. 2). The cDNA is judged to be at full-length. The 176 amino acids in 5'- prime were similar, from 57 to 68%, to the gene product from *Nicotiana tabacum, Arabidopsis thaliana, Corydalis sempervirens*, and *Oryza sativa* in the cDNA from cucumber by BlastX at DDBJ (Table 1, DNA Data Bank of Japan; http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). Although a well-characterized extra-cellular nuclease had been identified from *Staphylococcus aureus* (Anfinsen et al. 1971, Shortle 1983), only limited information about nuclease was reported as a functionally uncharacterized protein from *Corydalis sempervirens* (Schaller et al. 1992), *Oryza sativa* (Sasaki et al. 2002), and a nucleotide sequence from *Arabidopsis* as an unknown protein (DDBJ) in higher plants. A few characteristic functions from *Arabidopsis* have recently been revealed in cDNA encoded a noble Ca^{2+}-dependent nuclease of *Arabidopsis* (Isono et al. 2000). However, the biological mechanism of the nuclease family gene in plant still remains unclear. Nevertheless, it is an urgent issue and no evidence that the nuclease gene related to the PIN gene because the PIN gene is products in cytoplasm (Müller et al. 1998, Friml et al. 2002, Guan et al. 1998).

In the plaque hybridization, one of the possibilities of positive hybridization is the homology of pGEM-T easy vector. There is no significant homology with the DNA sequence of the pGEM-T easy vector and nucleotide of the two probes. No possibilities are inferred from the two occasions of hybridizations with two kinds of DIG labeling RNA probes on the pGEM-T easy vector (the first and second screening) which showed specific duplicate signals distinctly on the membrane (Fig. 1C). We propose that heterogeneous RNA probe labeled with DIG is possible to isolate a candidate gene like PIN in cucumber. CsPIN2 is homologous to AtPIN3RN and AtPIN3RC as the heterogeneouse probes. With screening a cucumber cDNA library using homogeneous RNA probe base on the conserved amino acid sequence among AtPIN3, AtPIN4 and AtPIN7, we also isolated cDNA of CsPIN2 and the amino acid sequence showed 71% identities with AtPIN3 (Hotta, T. et al. 2003).

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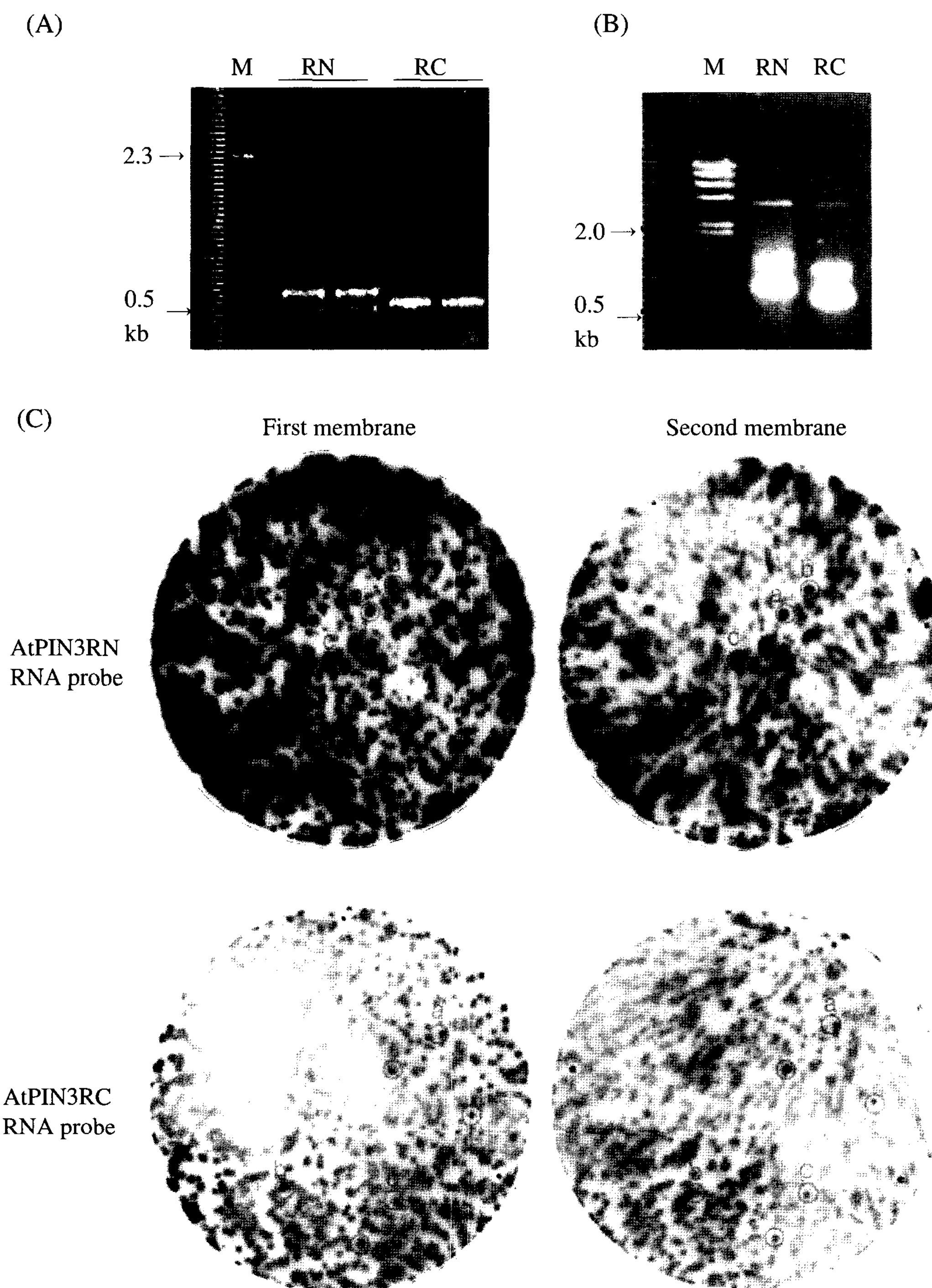

Fig. 1. Screening a cucumber cDNA library using Dig labeled heterologous RNA probe of *Arabidopsis*. (A), RT-PCR with two types of oligonucleotide sets to isolate DNA probe in AtPIN3 region. (B), The RNA probes of AtPIN3RN and AtPIN3RC synthesized with T7 RNA polymerase just after digesting restriction enzyme, *SpeI*. (C), Duplicated signals hybridizing with the probes showed on the membrane of the second screening. Above lines of membrane to 6 plaques were hybridized with AtPIN3RN RNA probe. Below lines of membrane to plaques were hybridized with AtPIN3RC RNA probe. Red cycles mean duplicated signal.

A

CAAAAAAATG	GGAAACGCAC	TCAGGTTCCT	CTGCGGCCAC	TTCTCCCGTC	50
CCACCTCCGA	TTCCTTCCCT	CCTCACGCCG	CCGTATCTTC	CTCCACTGCC	100
GGCGTCTCCG	CTCTCCCTCA	CGATCTCTTC	GAATTCGAGA	TCACATCACA	150
GGTTCCTCCA	GGGCTTAGTA	AGCATGTGGT	TTCATCGAAG	AAGGCACAGG	200
CGAATTGGTA	TGCAAAATTG	GCTGAGGCTT	GGAAGACGGA	GAAACCAACT	250
CCACGAACTC	CTGAAGAAGC	TTCTAGACTT	GTGATTCAGA	CTCTCAAAAG	300
ACACCAAAAG	AAAGATGTCG	AGGGACTATT	GACATTCTAT	GGTCTTCCTC	350
TTCCTCATAC	TCTCGTCAAA	CCTTCTGCTC	CGGTTCCAAC	TGCCGTCTCG	400
ATACCTGACG	GAGTCAAGTT	TGAGCTGCAG	ACGCTGCCAG	TTGATGCCAA	450
AGCTGTAGCA	GATGGTGATA	CAGTGACAGT	GTATGTGAGC	ACATCACACC	500
CGAGAGAGTC	CTCTTGTGTT	CCAAAAGAGG	TTAGA		

B

TTTTTTTTTT	TTTTTGATTT	TTTTAATATT	ATTACGAATA	TAACAATGTG	50
TTCCTTTTCA	TTTCTCTTCA	GTTACAAATT	TACAAATTAC	CAGCAATGTT	100
GAAGATGTTG	ATTAATAAAT	ACTTTAAAGA	CAGAAGAGAC	CTTTTTGGGT	150
TGATTTTAGG	GTTCCCTCTT	CTGCTTCTTG	TCGTTGGTAG	ACATGAAATG	200
GATGAACGTC	CTTCATATAT	AAATTATAAG	TGTTGTGTAT	TGAAACAGCA	250
ATAGCAACAA	GCAGCCATTA	TTATTTCCCC	TCACGCTTTC	CCTTTCTCCA	300
TTCCCATGGC	TGCTCTGGGT	TTGAAGAAGC	CCACAACCCA	GCCCTTTTAG	350
CTCGAGCCTC	GTTTTCCCAC	TTTGACAGCT	CTGGACGTTT	GTCATATGCT	400
GTGTAATGCC	AAGCAAACCC	CTTTTTCAGC	ATTGCTTCCT	GAATAAACTT	450
TCCATTACAA	TACAAGTCAC	CCACACATCG	GATTGTACCG	ATCTTCACCA	500
TACACATGTA	CTCTCAAACA	CTTGCCTTCA	ACAAGCCTTT	TCAGTTCTTC	550
TTTTGCCTCT	TTCCCATAA				569

Fig. 2. The front and rear nucleotide sequences of number 6 and 49 clones sequenced with primer. (A), sequence with T3 primer. (B), sequence with T7 primer.

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Table 1. The list of significant gene products aligned with the N-terminal sequence (nucleotide 1-528) of the cDNA from cucumber by BlastX at DDBJ (DNA Data Bank of Japan; http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html)

Sequences producing significant alignments	Score (bits)	E Value	Identities (%)
dad|AY029749-1|AAK52082.1| 330|Nicotiana tabacum nuclease prot...	220	4e-56	68
dad|AY128927-1|AAM91327.1|226|Arabidopsis thaliana unknown pr...	211	2e-53	63
dad|AY093027-1|AAM13026.1|226|Arabidopsis thaliana unknown pr...	211	2e-53	63
dad|X63595-1|CAA45139.1|338|Corydalis sempervirens protein of...	205	2e-51	61
sp|Q39635|Y38K_CORSE Hypothetical 38.1 kDa protein.	205	2e-51	61
pir|S24930|S24930 hypothetical protein - pink corydalis	205	2e-51	61
prf|1906382A|pCSC71 protein	205	2e-51	61
dad|AC007020-20|AAD25675.1|288|Arabidopsis thaliana hypotheti...	203	6e-51	63
pir|B84829|B84829 hypothetical protein At2g40410 [imported] - A...	203	6e-51	63
dad|AP003301-16|BAB64802.1|331|Oryza sativa (japonica cultiva...	201	1e-50	59
dad|D84226-1|BAA95210.1|323|Arabidopsis thaliana Ca(2+)-depen...	186	5e-46	57
pir|T52640|T52640 nuclease (EC 3.1.-.-), Ca(2+)-dependent [vali...	186	5e-46	57
prf|2612258A|Ca-dependent nuclease	186	5e-46	57
dad|AL163763-13|CAB87416.1|276|Arabidopsis thaliana putative ...	186	5e-46	57
pir|T47734|T47734 hypothetical protein F18O21.130 - Arabidopsis...	186	5e-46	57

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