한국농화학회지 제 39권 제 2호(1996)
Agricultural Chemistry and Biotechnology
Vol. 39, No. 2, pp. 112~117(1996)

Molecular Cloning and Nucleotide Sequencing of a DNA Clone Encoding Arginine Decarboxylase in Rice (*Oryza sativa* L.)

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Abstract : Arginine decarboxylase (ADC) is the first enzyme in one of the two pathways of diamine putrescine biosynthesis in plants. The genes encoding ADC have previously been cloned from *Escherichia coli*, oat and tomato genome. Two degenerate oligonucleotides (17-mer) corresponding to two conserved regions of ADC were used as primers in polymerase chain reaction of rice (*Oryza sativa* L.) genomic DNA, and an approximately 1.0 kbp fragment was obtained. This amplified PCR product showed an open reading frame which contains 1,022 bp of nucleotide sequences. This PCR product was cloned into pGEM-originated T vector and the short 500 bp *Pst*I digested fragment was subcloned into pGEM-3zf(+/−) vectors to facilitate sequencing. The nucleotide sequence of this PCR product showed about 74% and 70% identity with the same regions of the oat and tomato ADC cDNA sequences, respectively. The predicted amino acid sequence exhibited 45% and 62% identity with oat and tomato ADC polypeptide fragments, respectively. The sequence similarities of 34%, 47% and 38% were previously reported in oat and *E. coli*, tomato and oat, and tomato and *E. coli* ADC amino acids, respectively. Therefore, similarities and identities between rice and oat or tomato are remarkably higher than those others of the previous reports. In the highly conserved regions in both the amino acid sequence and spacing regions among the sequences of these three, rice ADC open reading frame also has the exactly same regions with the striking similarity. RNA blot analysis showed that ADC is expressed as a transcript of approximately 2.5 kbp in the rice seedling leaf tissues(Received January 8, 1996; accepted March 18, 1996).

Introduction

The diamine putrescine and the polyamine spermidine and spermine are naturally occurring compounds found in all living organisms. It seems clear that polyamines influence many aspects of cellular metabolism, such as nucleic acid and protein synthesis, cell division, embryogenesis, fruit development, senescence, flowering, and response to environmental stress,[6,19] because of their polycationic interaction with nucleic acids and other anionic macromolecules. Polyamines are found not only in the form of free but also in the forms of conjugates in many species of higher plants, such as glutathionylspermidine, putreamine and bleomycins. In plant, hydroxycinnamic acid amides formed from binding of one or two coumaric, caffeic and ferulic acid are general polyamine conjugates, and the polyamine conjugates appear to play an important role as a storage form of polyamines in cell growth.[20]

The biosynthetic pathways for polyamines have been well documented.[9] Putrescine synthesis arises via either of two alternate decarboxylation pathways directly from ornithine via ornithine decarboxylase (ODC : EC 4.1.1.17) and from arginine via arginine decarboxylase (ADC : EC 4.1.1.19) through intermediate agmatine. Besides, interconversion of arginine and ornithine is carried out by enzymes of the ornithine cycle. The ADC pathway seems to be present exclusively in plant and some bacteria, including *Escherichia coli*, while in animal and fungi tissues the ODC pathway predominates.[13,19] In plant system, polyamine metabolism has been studied in angiosperm, but not in gymnosperm. The synthesized putrescine is converted to spermidine and spermine by the consecutive addition of propylamino residues, which is originated from decarboxylated S-adenosyl-methionine generated via the enzyme S-adenosyl-methionine decarboxylase (SAMDC : EC 4.1.1.50).[4]

Recently, molecular analysis of polyamine biosynthesis in plants has been initiated. Cloning and sequence analysis of the speA gene encoding arginine decarboxylase in *E. coli* was reported by Moore and Boyle (1990).[11] Hammil *et al.* (1990)[8] produced transgenic tobacco roots which overexpressed yeast ODC gene. Bell and Malmberg (1990)[1] reported the cloning of oat ADC cDNA.

Key words : arginine decarboxylase(ADC), putrescine, degenerate oligonucleotides, polymerase chain reaction(PCR)
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More recently, a partial cDNA clone, isolated from a tomato meristem library and showing homology to the oat ADC, was reported to be expressed in the meristem in a tissue-specific manner.[7] It was also reported that the fruit ripening and storage of tomato is closely related to polyamine metabolism, and that it appears to be due to an increase in the activity of ADC.[14,15] Tomato ADC cDNA was cloned and the predicted amino acid sequence exhibited 47% and 38% identity with oat ADC and *E. coli* ADC, respectively.[16] Molecular approaches including cloning of genes for polyamine biosynthetic enzymes should allow a better understanding of the function of polyamine in the plant growth and development.

In this study, to characterize rice ADC DNA clone which has not yet been identified, we have cloned the PCR product of rice genomic DNA amplified with sense and antisense degenerate primers corresponding to highly conserved regions of oat, *E. coli* and tomato ADCs, and analyzed the sequence by comparison with the same regions of oat and tomato ADC sequences.

Materials and Methods

Plant Materials

The plants of rice (*Oryza sativa* L. cv. Jin-mi) were grown as plant materials at the pots in greenhouse. The day and night temperature was 25℃ and 17℃, respectively. After 2 weeks of cultivation, seedling leaf samples were collected for isolation of genomic DNA for PCR application and Southern blot analysis. These leaf samples were also used for isolation of mRNA for Northern blot analysis and cDNA library construction.

Extraction of Genomic DNA

Genomic DNA was isolated using a modified version of both CTAB method[12] and microextraction method.[2,5] Leaf tissue (100 mg) was ground in a 1.5 m*l* microcentrifuge tube with a pestle with the addition of 30 μ*l* extraction buffer [50 mM Tris-HCl pH 8.0, 0.25 mM EDTA, 0.35 M sorbitol, 0.1% β-mercaptoethanol]. After grinding, 236 μ*l* of extraction buffer and 36 μ*l* of 5% sarcosyl were added individually to the homogenate, and the tube was placed at room temperature for 5 min. Then, 46 μ*l* of 5 M NaCl and 37 μ*l* of CTAB buffer [8.6% CTAB in 0.7 mM NaCl] were added separately, and incubated at 65℃ for 15 min. Two microliter of RNase (1 mg/m*l*) were added and incubated at 37℃ for 20 min to remove RNAs in the sample. The mixture was extracted with phenol/chloroform. The DNA was precipitated with 0.5 volume of 7.5 M NH_4OAc and 2 volumes of ethanol at −70℃ for 30 min, and resuspended in TE buffer [10 mM Tris pH 8.0, 0.1 mM EDTA].[17]

Primer synthesis

Two degenerate oligonucleotides were designed as PCR primers. These correspond to two conserved regions in the oat and *E. coli* ADC proteins. Sense primer corresponds to the amino acid residues 152 to 157 of the oat ADC and 171 to 176 of the *E. coli* ADC. Antisense primer corresponds to the amino acid residues 474 to 479 of the oat ADC and 527 to 532 of the *E. coli* ADC (Fig. 1). These primers were synthesized by Korea Biotech Co.

PCR Amplification of Genomic DNA and Cloning

Genomic DNA (100 ng) was used in a 50 μ*l* PCR amplification sample containing 150 pmole of each primer (sense and antisense), 1X buffer [10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100], 200 μM each deoxyribonucleotide triphosphates, and 1 unit of DynaZyme (*Thermus brockianus*, Finezymes, Finland), overlayed with mineral oil. The PCR amplification conditions were 94℃ for 1.5 min for denaturation, 55℃ for 2 min for primer annealing, and 72℃ for 2 min for extension, for a total of 45 cycles, using a water-bath thermal cycler (Fine PCR, Korea). Samples were predenatured for 4 min at 94℃ and final extension was for 7 min at 72℃. Amplification fragments generated were separated by electrophoresis on a 1.0% agarose gel and cloned into pGEM-originated T vector (Promega, U.S.A.).

Southern Blot Analysis

The PCR products were separated by electrophoresis on a 1.0% agarose gel and transferred onto a nylon membrane with 0.4 N NaOH. Membranes were prehybridized at 65℃ in the hybridization buffer [1 mM EDTA, 7% SDS, 0.25 M disodium phosphate] for 1 hour and then hybridized with alkaline phosphatase labeled probes prepared by primer extension at 65℃ overnight in the hybridization buffer according to manufacturer's instruction (Southern Light Chemiluminescent Detection System, Tropix, U.S.A.). Filters were washed at room temperature for 20 min each with 2X SSC/1% SDS and 1X SSC and then at 65℃ for 40 min in 0.1X SSC/1% SDS. Filters were covered with plastic wrap, placed under a sheet of X-ray film in an exposure cassette, and film exposure was performed for 40 min at room temperature.

DNA Sequencing

The PCR products were sequenced using the dideoxy sequencing[18] method with Sequenase version 2.0 (US Biochemical, U.S.A.) or silver staining method (Promega, U.S.A.). Plasmid DNAs (1~2 pmole) were mixed in a total 16 μ*l* of reaction solution containing 5 pmole of

each primer, 1X sequencing buffer [50 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$], and 5 units of sequencing grade *Taq* polymerase (Promega, U.S.A.). The mixture of four microliters was added to each 2 μl d/ddNTP mixture and was overlayed with mineral oil. PCR amplification conditions were 95℃ for 1 min for denaturation, 42℃ for 1 min for primer annealing, 70℃ for 2 min for extension, for a total 60 cycles using a water-bath thermal cycler, and then 3 μl of stop solution [10 mM NaCl, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol] was added to each tube. The mixture was heated at 70℃ for 2 min immediately before loading on a 6% polyacrylamide (19 : 1 acrylamide : bis-acrylamide) sequencing gel with 0.4 mm spacers and was separated by electrophoresis (80 V/cm).

The plate was fixed for 20 min in 10% glacial acetic acid, stained for 30 min in the staining solution [0.1% AgNO3, 0.056% formaldehyde] and developed in the developing solution [3% Na_2CO_3, 0.056% formaldehyde, 2 mg/L $Na_2S_2O_3 \cdot 5H_2O$] until the bands were visible. To terminate the developing reaction and fix the gel, 10% glacial acetic acid was added directly to the developing solution. The plate was dried at room temperature and exposed with EDF film (Kodak, U.S.A.). All DNA and the deduced amino acid sequence anaylses were performed using the DNASIS (Hitachi, Japan) and PCGENE software (Intelligenetics, Switzerland).

RNA extraction and Northern Blot Anaylsis

Total RNA was extracted from leaf tissue using a modified version of the method described by Chomczynski and Sacchi (1987).[3] Leaf tissue (5 g) was powdered in liquid nitrogen and mixed with 30 ml of extraction buffer [5 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 2 mM EDTA, 100 mM β-mercaptoethanol]. The mixture was extracted once with 1 volume of 25 : 24 : 1 phenol : chloroform : isoamyl alcohol, and once with the same volume of chloroform. The nucleic acids were precipitated with 1/10 volume of 3 M sodium acetate, pH 5.2 and 2.5 volumes of ethanol at −70℃ for 30 min. The pellet was resuspended in 0.9 ml of DEPC (Diethylpyrocarbonate)-treated water and incubated with 0.3 ml of 8 M LiCl overnight at the 4℃. The mixture was centrifuged, resuspended in 350 μl of DEPC-treated water and centrifuged again. The supernatant was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol at −70℃ for 30 min. The total RNA was dried at room temperature, resuspended in DEPC-treated water, and fractionated through 1.0% agarose-formaldehyde gel. The RNA quality was checked by UV spectrophotometer ($OD_{260/280}$=1.7~2.0) and gel analysis (rRNA bands should be obvious and distinct).

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                    C   N   G   Y   K   D
Sense primer    : 5'- TGT AAT GGT TAT AAA GA - 3'
                      C   C   A   C   G

                    G   D   S   D   C   T
Antisense primer : 5'- CC ATC ACT ATC ACA AGT - 3'
                      G   G   G   G   G
                                      T
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Fig. 1. Primers used for PCR DNA amplification of rice ADC genomic fragment. The corresponding amino acids and degenerate sequences are shown above and under each oligonucleotide sequence, respectively. Sense primer corresponds to amino acid residues 152 to 157 of the oat ADC and 171 to 176 of the *E. coli* ADC. Antisense primer corresponds to amino acid residues 474 to 479 of the oat ADC and 527 to 532 of the *E. coli* ADC.

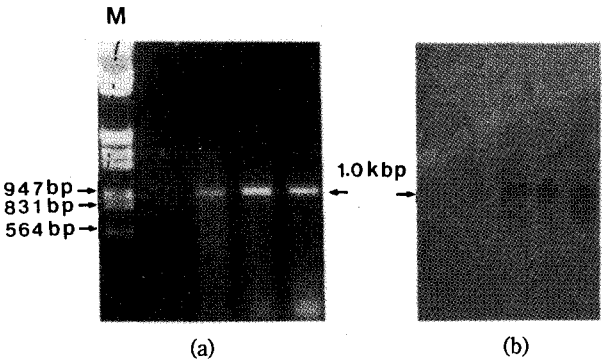

Fig. 2. Gel electrophoresis and Southern blot analysis of PCR product of rice genomic DNA using the highly degenerated primers. (a) 1.0% agarose gel stained with ethidium bromide, (b) Southern blot probed with oat ADC clone. M: lambda *Eco*RI-*Hin*dIII molecular weight marker. Arrows indicate the molecular weight of DNA fragments.

Poly(A)$^+$ mRNA was fractionated through oligo-dT cellulose column. Poly(A)$^+$ mRNA samples were separated by electrophoresis on a 1.0% agarose-formaldehyde gel and transferred to a nylon membrane. Hybridization conditions were the same as used for the Southern blot. The probe was the 1.02 kbp rice PCR product, oat and tomato ADC cDNAs.

Results and Discussion

PCR Amplification and Southern Analysis

Two degenerate oligonucleotides (Fig. 1), corresponding to conserved regions in the oat, tomato and *E. coli* ADC proteins, were used for PCR amplification of rice genomic DNA. To amplify rice genomic DNA, each 37℃, 40℃ and 55℃ of annealing temperature was tried sepa-

rately and sense primer was added up to three times as much as antisense primer. A conserved single band of about 1.0 kbp was generated by the PCR at 55℃ of annealing temperature (Fig. 2a). In rice genome, the specific band could not be produced at 40℃ which is the temperature recommended in the tomato genome,[16] but it was validated that the degenerate primer in rice genome would be reacted more specifically at 55℃, probably due to its dissimilar genome size with tomato. Amplified PCR product was analyzed in Southern blot with oat cDNA probe under the high stringence hybridization conditions, and gave a positive signal (Fig. 2b). The molecular weight of amplified positive band was similar to the size of conserved sequences of tomato and oat ADC gene corresponding to 1.05 kbp and 0.98 kbp, respectively.

Therefore, this rice PCR product was confirmed as an amplified genomic fragment representing rice ADC sequence. Even though bands of 0.7 kbp and 0.5 kbp size besides 1.0 kbp size band were obtained in PCR, those were revealed as negative bands using by Sourthern blot analysis. The sequence analysis certified that only antisense primer was used in those negative bands to be amplified (data not shown). This phenomenon might be caused by the Tm value of antisense primer which is relatively higher than that of sense primer due to its higher GC content. The problem for negative band should be eliminated when the positive band was uniquely selected under the high stringent condition in Southern blot analysis.

Cloning and Sequence Analysis of PCR product

Amplified PCR products were eluted from 1 % agarose gel with Gene Clean II Kit (BIO 101, U.S.A.) and cloned into pGEM-originated T vector (Promega, U.S.A.). The middle portion of PCR product digested with Pst I was subcloned into pGEM-3zf(+/−) vectors for sequence analysis (Fig. 3).

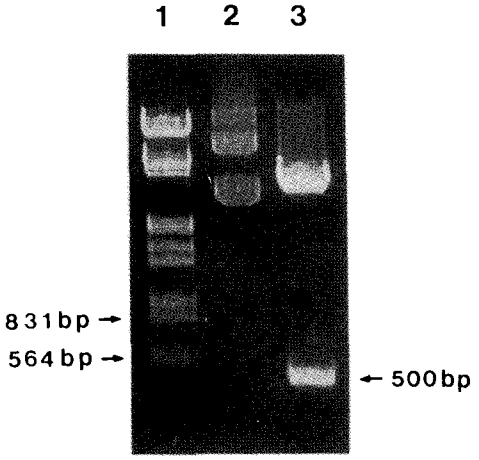

Fig. 3. Restriction digestion of inserted rice PCR product. PstI was subjected to digest the cloning sites. Lane 1 : lambda *Eco*RI-*Hin*dIII molecular weight marker, Lane 2 : pGEM-derived T cloning vector control, Lane 3 : *Pst*I digested rice PCR product clone. Arrows indicate the molecular weight of DNA fragments.

The PCR products were sequenced using dideoxy sequencing method with Sequenase version 2.0 or silver staining method to confirm the sequence identity with the previously published data. The sense and antisense primer were existed on both ends of PCR product. The nucleotide length of rice PCR product was 1,022 bp, and revealed that this fragment contains an open reading frame encoding a polypeptide of 340 amino acids, which showed strong similarity to the conserved regions of oat with 74% identity (Fig. 4), and of tomato ADC cDNA with 70% (Data not shown). It is likely that rice is more closely related to monocotyledonous *Gramineae* oat than to dicotyledonous tomato. The most noticeable feature

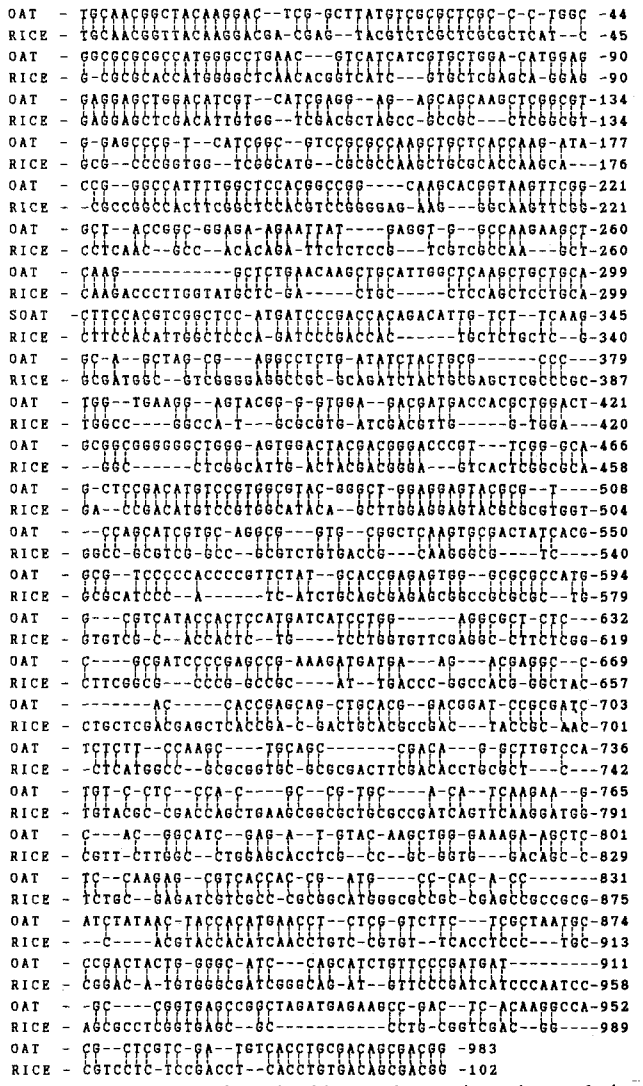

Fig. 4. Comparison of nucleotide maximum homology of rice PCR product and oat ADC cDNA sequences (GenBank accession No. X56802).

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TOMATO- CNGFKDTEYISLALVARKLLLNSVIVLEQEEELDLVIDISRKMSVWPVIG -50
RICE  - CNGYKDDEYVSLALIARTMGLNTVIVLEQEEELDIVVDASRRLGVRPVVG -50

TOMATO- LRAKLTTKHSGHFGSTSGEKGKFGLTTTQILRVVKKLDESGMLDCLQLLH -100
RICE  - MRAKLRTKHAGHFGSTSGEKGKFGLNATQILSVVAKLKTLGMLDCLQLLH -100

TOMATO- FHIGSQIPTTELLADGVGEATQIYSELVRLGAGMKFIDIGGGLGIDYDGS -150
RICE  - FHIGSQIPTTALLGDGVGEAAQIYCELARW-PAMRVIDVGGGLGIDYDGS -149

TOMATO- KSSNSDVSVCYSIEEYASAVVQAVLYVCDRKGGKHPVICSESGRAIVSHH -200
RICE  - HSAQTDMSVAYSLEEYARGGRVGR--VCDRKGVAHPIICSESGRALVSHH -197

TOMATO- SILIFEAVSASTSHVSTQPSSGGLQSLVETLNEEARADYENLSAAAVR-- -248
RICE  - SVLVFEAFSAS----------------------------------APGRID -214

TOMATO- ---------------------------GEYDTCLIYSDQLKQRCVEQFKDG -272
RICE  - PATGYLLDELTDDCTPTTATSWPRGARDFDTCALYADQLKWRCADQFKDG -264

TOMATO- SLDIEQLAAVDSICDWVSKAIGVADPVRTYHVNLSVFTSIPDFWGFSQLF -322
RICE  - VLGLEHLAAVDSLCEIVARGMGAAEPPRTYHINLSVFTSLPDMWAIGQMF -314

TOMATO- PIVPIHRLDEKPTMRGILLDLTCDSD -348
RICE  - PIIPIQRLGERPAVDGVLSDLTCDSD -340
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Fig. 5. Comparison of the derived amino acid sequences of rice PCR product and tomato ADC cDNA. The character to show that two aligned residues are identical is;' |', similar is; '.', and that amino acids said to be 'similar' are: A,S,T; D,E; N,Q; I,L,M,V; F,Y, W.

in the case of the pairwise sequence comparisons of rice PCR product with oat, tomato and *E. coli* ADC cDNAs is that there are lots of corresponded homologous sequences that are located within the regions of the conserved amino acid sequences of them. Therefore, the nucleotide sequence analysis reconfirmed us that this amplified genomic fragment indeed represented the rice ADC sequence. Among the four sequences, there are several regions of striking similarity that are completely conserved both in the amino acid sequence and spacing. Unexpectedly, however, comparison of the predicted amino acid sequence of rice PCR product revealed similarities to the oat and tomato ADC cDNAs with 45 and 62% identity, respectively (Fig. 5). Therefore, at the amino acid level, 62% amino acid identity between rice PCR product and tomato ADC cDNA was more similar to each other with comparison of 47% amino acid identity between tomato and oat ADC cDNAs, or of 38% amino acid identity between tomato and *E. coli* ADC cDNAs, or of 45% identity between oat ADC cDNA and rice PCR product. It appears that duplication and divergence of the ancestral ADC gene preceded the divergence of monocotyledons and dicotyledons, followed by translational and post-translational modification of the ADC gene. In animal systems, the regulation of ODC and SAMDC expression at translational and post-translational level has been well known. In oat, it has been shown that the ADC polypeptide is post-translationally processed,[10] but whether this processing is related to

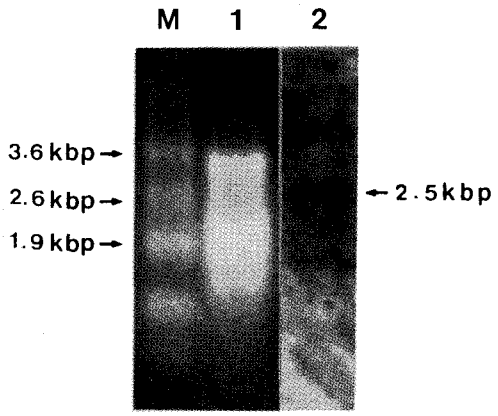

Fig. 6. RNA blot analysis of ADC expression in seedling leaf of rice. Poly(A)$^+$ mRNA (3 μg) was loaded in the lane. M: lambda *Eco*RI-*Hin*dIII molecular weight marker, Lane 1 : 1.0% agarose-formamide gel stained with ethidium bromide, Lane 2 : Northern blot probed with 1.02 kbp ADC PCR product. Arrow indicates approximate 2.5 kbp of rice ADC mRNA.

ADC gene modification is not clear. This question may be resolved if the entire sequence analysis of rice ADC cDNA is completed and if further work is done. We hope that this approach will help explain some of biological roles of polyamine in plant growth and development.

RNA Blot Analysis

To determine the size of ADC mRNA in rice, Northern blot analysis of mRNA using this 1,022 bp PCR product from rice was conducted. The rice ADC was expressed as an approximately 2.5 kbp transcript as shown by Northern blot analysis (Fig. 6), and the gel blotting showed that rice ADC in seedling leaf tissue is encoded by a single gene. This is in an agreement with the size of the ADC transcript detected in other crops. Rastogi *et al.*[16] reported that the correlation between ADC activity and ADC mRNA levels was lack in tomato genome. Further work is needed to understand whether this kind of gene expression is also existed in rice genome.

Acknowledgments

This study was supported by a grant for Genetic Engineering Research Fund from the Ministry of Education (1994).

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벼의 arginine decarboxylase DNA clone의 재조합 및 염기서열 분석

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초록 : ADC는 diamine인 putrescine 생합성의 두가지 경로중에서 식물계에서 특히 중요한 효소이며, ADC 유전자는 *E. coli*, 귀리, 토마토 genome에서 이미 cloning된 바 있다. 벼 (*Oryza sativa* L.) 게놈 DNA의 PCR 증폭을 위해서 토마토와 *E. coli*의 ADC cDNA의 보존된 부분과 일치하는 두개의 degenerate oligonucleotides (17 mer)를 인위 합성하였으며, 증폭의 결과 약 1 kbp 크기의 DNA가 관찰되었다. 증폭된 DNA 절편은 1,022 bp 염기서열을 포함하고 있는 ORF (open reading frame)으로 확인되었다. 이 PCR product는 pGEM-originated T vector에 재조합하였으며, *Pst*I 제한효소로 약 500bp 크기로 절단하여 pGEM-3Zf(+/−) vector에 subcloning하였다. 벼 ADC clone의 염기서열은 귀리와 토마토 ADC cDNA 서열의 같은 부분과 각각 74%와 70%의 동질성을 갖는 것으로 나타났으며, 예상되는 아미노산 서열은 귀리와 토마토 ADC 단백질과 각각 45%와 62%의 동질성이 관찰되었다. 귀리와 *E. coli*, 토마토와 귀리 그리고 토마토와 *E. coli* ADC 아미노산 서열에서 각각 34%, 47% 그리고 38%의 유사성 정도가 보고된 것을 비교하여 볼 때, 벼와 귀리 및 토마토 사이의 유사성 정도는 다른 비교 보다도 월등히 높았다. 벼 유묘기 잎조직에서 추출한 RNA를 이용한 Northern blot 분석에서 ADC는 약 2.5 kbp의 전사체로 발현됨이 확인되었다.

*연락저자