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Gene Expression in Tomato for Glucosylation of Indole-3-Propionic Acid, a Selective Antibacterial Agent against a Bacterial Wilt Pathogen *Pseudomonas solanacearum*

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Abstract

To examine the mechanism for *in planta* detoxification of phytotoxicity of indole-3-propionic acid, the gene expression for glucosylation of this compound was detected in treated tomato seedlings by Northern and *in situ* hybridization with the specific probe. The probe was obtained by polymerase chain reaction of tomato chromosomal DNA using the primers designed on the basis of amino acid sequences which were highly conserved in several enzymes catalyzing glucosylation. The positive hybridization was intensively detected in stems of the treated tomato seedlings. The increase of hybridized transcript was well coincident with the accumulation of glucosyl indole-3-propionic acid analyzed by thin layer chromatography. The result suggests that the PCR clone obtained from tomato chromosome encodes partial sequences of a gene for glucosyl conjugation of the compound.

1. Introduction

Pseudomonas solanacearum is a soil-borne bacterium causing bacterial wilt in a wide range of important crop plants, such as tomato, eggplant, sweet pepper, potato, bean, tobacco, and some floral plants. However, the protection of these plants from the disease is not necessarily effective, and an efficient control of the pathogen has been an urgent matter in agriculture. In our attempt to develop chemical control agents, we found that indole-3-propionic acid (IPA) was capable of selectively suppressing the growth of *P. solanacearum* [1] and have extensively examined the relationship between the chemical structure and the antibacterial function of this compound [2]. In our practical strategy for controlling phytopathogenic microorganisms, the chemical agent was applied to hydroponically cultured tomato plants. This approach enables us to expand the chemical agent rapidly and extensively in water phase of hydroponics and therefore would be expected to be effective for suppressing the pathogens in aqueous rhizoplane of tomato plants. At the same time, it is essential to examine phytotoxicity of the treated compounds on tomato plants, especially in IPA because of its auxin activity [3]. In our previous work, we reported the capability of tomato to conjugate IPA with glucose [4]. The similar glucosyl conjugation has been recognized in indole-3-acetic acid (IAA), by which IAA is conjugated with glucose to be inactive in an auxin activity [5]. In the present study, we focus on the glucosylation of IPA by tomato for inactivation or detoxification of phytotoxicity of the antibacterial compound and clarify gene expression for IPA glucosyl conjugation in tomato plants using some molecular biological techniques such as polymerase chain reaction (PCR) and *in situ* hybridization.

2. Materials and Methods

2. 1 *Genetic marking of* P. solanacearum

The marker genes (tetracycline-resistance gene and *lux* operon)-integrated virulent strain (K-101/lux8) of *P. solanacearum* [6] was used for a rapid and effective monitoring of the pathogen. The K-101/lux8 was detected by spreading bacterial suspension onto a selective medium (10 g peptone, 1 g casamino acids, 10 g glucose, and 10 mg tetracycline in 1 liter of water, solidified by 1. 5 % agar) and then confirmed by checking the bioluminescence production derived from the expression of the *lux* operon genes. The bioluminescence was detected by exposing luminiferous bacterial

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colonies to a X ray film overnight in the dark [7].

2. 2 *Assay for phytotoxicity and antibacterial activity of IPA*

One-month-old seedlings of tomato (*Lycopersicon esculentum* Mill, cv. "Ponderosa") were hydroponically cultured using a culture solution (77. 5 mg $NH_4H_2PO_4$, 400 mg KNO_3, 356. 3 mg $Ca(NO_3)_2 \cdot 4H_2O$, 250 mg $MgSO_4 \cdot 7H_2O$, 24 mg NaFe • EDTA, 3 mg H_3BO_3 and 2 mg $MnSO_4 \cdot 4H_2O$ in 1 *l* of water) containing various concentrations (1-100 mg/*l*) of IPA in order to examine phytotoxicity of IPA. Namely, one-month-old seedlings of tomato were gently pulled out of vermiculite of pots and their roots were carefully rinsed with distilled water and cultivated in a culture solution containing various concentrations of IPA for several days at 26 °C under a continuous light illumination (70μmol/cm^2/sec.), using a Hyponica 302 (Kyowa Co. Ltd., Osaka, Japan). An effective concentration of IPA to suppress the pathogen in the hydroponic culture system was determined by dipping tomato seedlings in a IPA-containing culture solution infested with the pathogen. In this experiment, the culture solution was supplemented with different concentrations of IPA and then mixed with bacterial suspension of the K-101/lux8 so as to give a final density of 10^6 cells/m*l*. The appearance of systemic wilting in tomato plants was recorded on a daily basis.

2. 3 *TLC analysis of glucoside of indole derivative*

Tomato seedlings were harvested at the various times after the treatment with IPA and their roots, stems, and leaves were detached and powdered in liquid nitrogen with a pestle and mortar. IPA and its glucoside were extracted from powdered samples with 10 volumes of ethyl acetate according to the method described previously [4]. The extracts were reduced *in vacuo*, charged onto a silica gel plate (Merck silica gel 60 F_{254}), and developed using a solvent system of $CHCl_3$-MeOH (6: 1, v/v) for TLC analysis. These compounds were detected by staining with a Ehrlich reagent.

2. 4 *Primer construction and polymerase chain reaction*

Amino acid sequences of the genes [8-10] encoding enzymes which transfer glucose moiety of UDP-glucose were computer-analyzed by the DNA data bank of DDJB and the PCR primers were artificially synthesized with an Applied Biosystems 391 DNA Synthesizer. On the basis of codon usage frequencies of tomato, three kinds of primers were designed for each two regions of amino acid sequences conserved in the genes; the antisense primers AS1 (5′-TACGTCTCCTTCGGC-3′), AS2 (5′-TATGTTTCATTTGGA-3′), and AS3 (5′-TATGTCTCTTTCGGT-3′), and the sense primers S1 (5′-ACCTCAAGGTTACCA-3′), S2 (5′-GCCACATTACCA-3′) and S3 (5′-GCCGCACG-GATACCA-3′) for priming the 5′- and 3′-end of coding strand, respectively. The PCR of tomato chromosomal DNA was conducted in nine combinations of these sense and antisense primers.

Chromosomal DNA was extracted from detached tomato leaves by the method described previously [11] and used for PCR. Namely, purified chromosomal DNA was dissolved in 10 mM Tris-HCl (pH 8. 3) containing 50 mM KCl, 1. 5 mM $MgCl_2$, 0. 2 mM dNTPs, and 1 unit of AmpliTaq DNA polymerase (Takara Biochemical, Co. Ltd., Kyoto, Japan). The PCR was carried out with 25 cycles of denaturation (94°C for 45 sec.), annealing (42°C for 45 sec.), and extension (72°C for 2 min.). The DNA extension in the final cycle was additionally conducted at 72°C for 5 min. The temperature cycling and reaction time were controlled with Atto Zymoreactor II (Tokyo, Japan). Amplified DNAs were treated with T4 DNA polymerase and ligated into the *Sma*I site of a plasmid vector pUC19, which was used for transformation of *Escherichia coli* JM109.

2. 5 *Nucleotide sequence determination*

The plasmid was extracted from transformed *E. coli* cells, and the insert DNA was clipped out of the vector and ligated to RF DNAs of M13mp118 and M13mp119. Single stranded DNA was isolated from M13 phages by standard techniques [12] and used for determining DNA sequences. Sequencing of inserted DNAs was carried out using the dideoxy method [13] with an aid of 370A Auto-DNA Sequencer (Applied Biosystems).

2. 6 *Probe preparation and hybridization analysis*

The subcloned PCR fragment was labeled with horseradish peroxidase for Northern hybridization and with digoxigenin for *in situ* hybridization, using ECL-Gene Detection System (Amersham International, Buckinghamshire, England) and PCR Dig Labeling Kit (Boehringer Mannheim GmbH Biochemica, Mannheim, FRG), respectively. Each labeling was conducted according to the manufacturer's instructions. For Northern hybridization, total RNAs were extracted from detached tissues of IPA-treated tomato seedlings by the method of Chomczynski *et al.* [14] and electrophoresed using a 1. 16 % agarose gel containing formaldehyde and MOPS according to the standard protocols [15].

For *in situ* hybridization, stems were detached from tomato seedlings whose roots were dipped in 10μg/m*l* IPA-containing water, fixed at 4°C for 30 min. with 4 % paraformaldehyde dissolved in 0. 01 M phosphate

buffer (pH 7.0) containing 4.37 mM NaCl and 2.68 mM KCl. The fixed samples were washed with 0.1 M phosphate buffer and sliced with a microtome to prepare sections of 20μm thickness. *In situ* hybridization was conducted by the method of Hirota *et al.* [16]. The enzymatic detection of hybridized complex was conducted according to the manufacturer's instruction (Boehringer Mannheim GmbH Biochemica, Mannheim, FRG). The positive *in situ* hybridization was recognized as a brownish color derived from the enzyme substrates (330 μg nitroblue tetrazolium and 167 μg 5-bromo-4-chloro-3-indoryl phosphate dissolved in 1 m*l* of TTBS buffer).

3. Results and Discussion

In the first experiment, the annihilative densities of the pathogen to be inoculated into the hydroponic culture system of tomato were determined. The bacterial density of 10^8 cells/m*l* was high enough to cause systemic wilting in all of tested tomato plants within seven days. Secondarily, the minimal effective concentration of IPA for suppressing the pathogen inoculated into hydroponic culture solution was examined. The pathogenic bacteria were isolated from the culture solution at the various periods after transplantation and identified by their typical fluidal colony and expression of the integrated marker genes (**Fig. 1A**). In control (non-treatment with the compound), the phytopathogenic bacteria were retained at the high densities of 10^7-10^8 cells/m*l* during the incubation periods. In the IPA treatment, however, the bacterial population was decreased in proportion to concentrations of IPA and less than 10^2 cells/m*l* in the culture solution treated with more than 10μg/m*l* of IPA. In this hydroponic culture system, the appearance of disease symptom in tomato seedlings corresponded to the decrease of the pathogen (**Fig. 1B**). In tomato plants treated with less than 5μg/m*l* of IPA, the decrease of the pathogen was not sufficient and some of tomato plants wilted 4-6 days after transplantation. On the other hand, no disease symptom was observed during the entire culture periods when tomato seedlings were treated with more than 10μg/m*l* of IPA. Tomato seedlings treated with more than 25μg/m*l*, however, showed severe bending of stem, probably because of auxin activity of IPA. Judging from these results, we concluded that the amendment of the pathogen-infested hydroponic culture system could be achieved without causing any detrimental effects in tomato seedlings by the treatment with 10μg/m*l* of IPA.

The present study demonstrated that tomato plants were capable of detoxifying IPA by any means and normally growing, when the level of IPA was less than

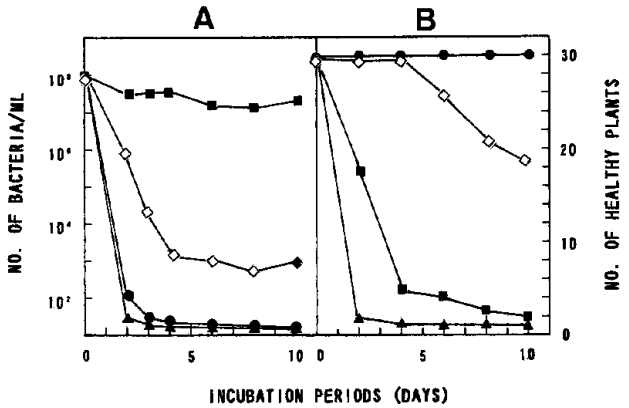

Fig. 1 Amendment of the hydroponic culture solution by IPA treatment assessed by decrease of both the pathogenic bacteria (A) and wilted tomato plants (B).
The hydroponic culture solution was inoculated with the genetically marked pathogen (K-101/lux8) so as to give a final density of 10^8 cells/m*l* and then amended with 1 (■), 5 (◇), 10 (●), and 25 μg/m*l* of IPA (▲). One-month-old seedlings of tomato were transplanted 2 days after the treatment with IPA. In the treatment with the highest concentration of IPA, the decrease of healthy plants was due to severe stem bending, but not bacterial wilting.

10μg/m*l*. In order to examine the mechanism for IPA detoxification by tomato, the TLC analysis of metabolites of IPA was carried out at the various periods after the treatment. Tomato seedlings treated with 10μg/m*l* IPA were cut into three segments (roots, stems and leaves) and each homogenate was analyzed by TLC. Consequently, the glucosylation of IPA was prominently observed in detached stems and negligibly in other segments. **Fig. 2** showed the time-course of accumulation of IPA and its glucoside in detached stems of the IPA-treated seedlings. As shown in a previous paper [4], the samples involved two Ehrlich-positive spots; one was IPA and the other 1-*O*-(3-indole-propionyl)-β-D-glucopyranoside (IPA-glu). IPA-glu was first detected 30 min. after treatment, and rapidly increased with a decrease of IPA, and reached the maximum 8 hr after the treatment. This glucoside was recovered from the TLC plate according to the method described previously [4] and examined for their phytotoxicity by dipping tomato seedlings in the solution containing various concentrations (1-50μg/m*l*) of IPA-glu for one week. The result showed that the glucoside did not cause any detrimental effect on tomato seedlings even when treated with the highest concentration of IPA-glu, indicating that IPA was detoxified by glucosylation.

It has been well recognized that the biosynthesis of glucosidic compounds was accompanied by glucosyl transfer from UDP-glucose, and therefore the

enzymes catalyzing glucosylation were considered to hold highly conserved regions in their amino acid sequences [10]. In the present study, therefore, these amino acid sequences were computer-searched among this type of genes, *bronze-1* genes of corn [8] and barley [9] and *iaglu* gene of corn [10]. On the basis of homologous amino acid sequences of these genes, the primers were synthesized and used for PCR of tomato chromosomal DNA. Some PCR products were successfully obtained and cloned into the plasmid vector. The cloned PCR products were excised from the vector, labeled, and used for the Northern hybridization of total RNA obtained from IPA-treated tomato seedlings. As a result, the insert (CIPA-02) (approximately 0.6 kilo base-pairs) which was obtained by PCR with the primers AS1 and S3 showed the positive hybridization, and the nucleotide sequence of the insert was determined [17]. **Fig. 3** shows the alignment of deduced amino acid sequence of the CIPA-02 with the *iaglu* gene product. The data revealed that the amino acid sequence deduced from the CIPA-02 possessed high homology with amino acids 282 to 287, 311 to 316, and 369 to 374 of the *iaglu* gene product. These homologous sequences were conserved among other UDP-glucose glucosylating proteins [8,9,18]. Because all of these proteins transfer glucose to specific acceptors, these sites are probably necessary for binding UDP. In addition, the highly conserved region involving the glucosylation

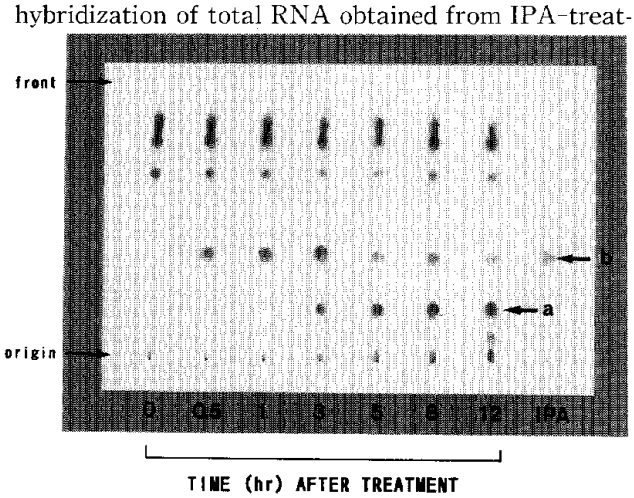

Fig. 2 TLC analysis for time-course accumulation of 1-*O*-(3-indolepropionyl)-β-D-glucopyranoside (IPA-glu) (a) from IPA (b) in detached stems of 10μg/m*l* IPA-treated tomato seedlings.

Tomato seedlings were dipped in water containing 10μg/m*l* of IPA and then detached stems were homogenized for TLC analysis at various periods after treatment. Indole derivatives were detected by staining with a Ehrlich reagent. The compound indicated by "a" was identified as IPA-glu in a previous paper [4], and IPA represents the authentic compound (indole-3-propionic acid) purchased from Sigma Chemical Co., St. Louis, MO. USA.

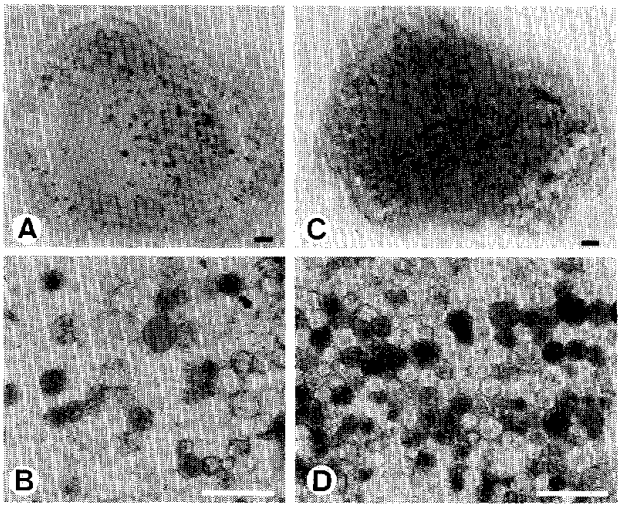

Fig. 4 *In situ* hybridization analysis of stem sections obtained from non-treated (A, B) and 10μg/m*l* IPA-treated tomato seedlings (C, D) by the DIG-labeled PCR clone CIPA-02 (3 hr after the treatment with IPA).

B and D represent enlarged pictures of A and C, respectively. Note the increased numbers of positively stained cells with intensified hybridization signal in IPA-treated tomato seedlings.

Bar represents 100μm.

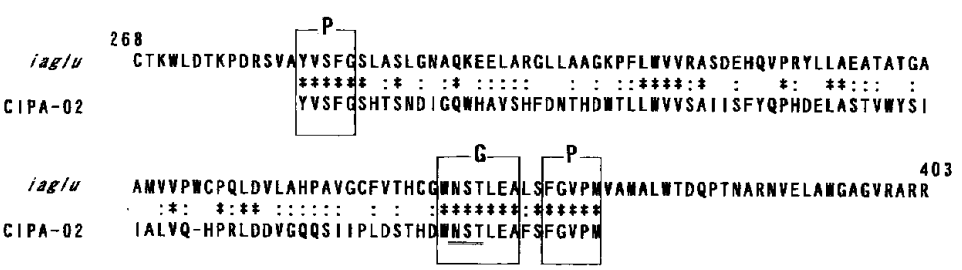

Fig. 3 Alignment of the deduced amino acid sequence of the PCR clone CIPA-02 with the product of the *iaglu* gene.

The P boxes were amino acid sequences used for synthesis of the primers and the G box represents the highly conserved regions involving the glucosylation site (doubly underlined). Asterisks indicate identical amino acids and dots indicate similar residues.

site "NST" (amino acids 363-365 of the *iaglu*) [10]

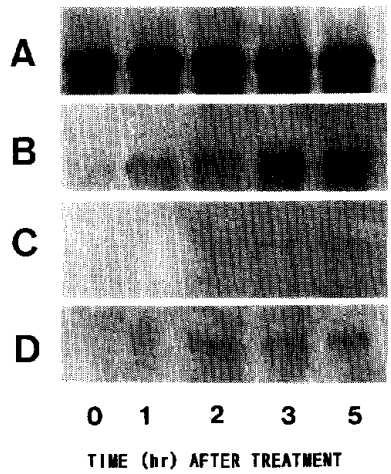

Fig. 5 Northern slot blot analysis of total RNAs obtained from detached stems (B), roots (C), and leaves (D) of 10μg/m*l* IPA-treated tomato seedlings by the labeled PCR clone CIPA-02.
Ten μg of total RNAs from detached tissues was loaded per lane. The panel A represents hybridization with a ribosomal DNA probe for load control.

was identified in the amino acid sequence of the CIPA-02. These homologies suggest that the CIPA-02 is likely to partially encode a gene for glucosylating IPA. Therefore, the CIPA-02 was used for the following Northern and *in situ* hybridization detection of specific-transcripts in IPA-treated tomato seedlings.

Fig. 4 showed the positive *in situ* hybridization with the labeled clone CIPA-02 in stem section of IPA-treated tomato seedlings. Although the hybridization was weakly detected in non-treated control (**Fig. 4A** and **B**), the signal of hybridization was conspicuously intensified in the section of the IPA-treated seedlings (**Fig. 4C** and **D**). The total RNAs were obtained from the IPA-treated seedlings and Northern-analyzed using the labeled CIPA-02 probe (**Fig. 5**). The present analysis indicated that the CIPA-02 probe hybridized the transcript with approximately 1.3 kilo bases in all of RNA samples obtained from detached roots, stems and leaves of the IPA-treated seedlings. However, the signal intensity was the highest in the detached stem sample. The hybridizable mRNA was weakly detected before the treatment, but the transcript increased rapidly and reached the maximum level 3 hr after the treatment. These results support the postulation that the CIPA-02 clone obtained in the present PCR encodes the partial sequences of a gene for glucosylation of IPA and the transcription of this

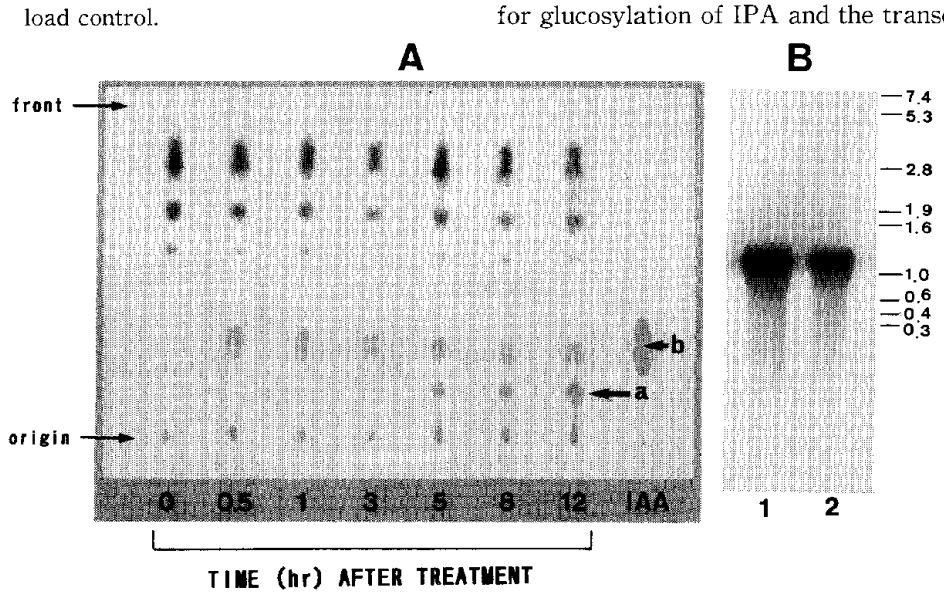

Fig. 6 TLC analysis for IAA glucoside formation (A) and Northern hybridization detection of CIPA-02-hybridizable transcripts (B) in IAA-treated tomato seedlings.
The seedlings were treated with 10μg/m*l* IAA for the definite periods and then detached stems were homogenized for TLC and Northern hybridization analyses. The compounds a and b reveal IAA-glucoside and IAA, respectively. For northern hybridization, 10 μg of total RNAs extracted from detached stems of IAA-(lane 1) and IPA-treated seedlings (lane 2) 3 hr after the treatment was loaded per lane, and the labeled CIPA-02 clone was used as probe for hybridization. The ladder indicates kilo bases of RNAs obtained from the RNA-molecular weight marker I (purchased from Boehringer Mannheim).

gene was enhanced by the treatment with IPA, especially in stems of tomato plants.

The present study suggested that the conjugation of IPA with glucose results in *in planta* detoxification of this antibacterial compound. The similar mechanism for inactivation of auxinic indole derivatives has been reported in glucose-conjugation of IAA [19]. In the present study, therefore, tomato seedlings were also treated with IAA and similarly examined for their capability to conjugate IAA with glucose by TLC analysis. The results indicated that the glucosylation of IAA was intensively detected in detached stem regions at the similar time course after the treatment with IAA (**Fig. 6A**). The Northern hybridization with the labeled CIPA-02 probe indicated that the CIPA-02 showed positive hybridization with the corresponding, similar size-transcripts acceleratedly produced in IAA-treated tomato seedlings (**Fig. 6B**). The results suggest that the genes for glucosylation of IAA and IPA may be identical or highly homologous to each other. The isolation of the cDNA with the complete length is underway.

References and Notes

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