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Maize polyamine oxidase: primary structure from protein and cDNA sequencing

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Abstract The first complete amino acid sequence of a flavin-containing polyamine oxidase was solved by a combined approach of nucleotide and peptide sequence analysis. A cDNA of 1737 bp, isolated from maize seedlings by reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends strategies, was cloned and its sequence determined. This cDNA contains information for a polypeptide chain of 500 amino acids. Its amino-terminal sequence shows the typical features of secretion signal peptides. The primary structure of the mature protein was independently confirmed by extensive amino acid sequencing. Structural relationships with flavin-containing monoamine oxidases are also discussed.

Key words: Flavin oxidase; Hydrogen peroxide; Polyamine oxidase; Primary structure; *Zea mays* L.

1. Introduction

Despite the ubiquitous occurrence of aliphatic polyamines in prokaryotic and eukaryotic organisms as well as their implication in cell growth and developmental processes [1–3], little attention has been devoted to the study of polyamine catabolism. Only recently, the biochemical properties and the physiological modulations of the enzymes involved in this process have been studied in detail [3–6]. The oxidation of polyamines at the primary amino group is catalysed by copper-containing amine oxidases (EC 1.4.3.6), giving the corresponding aminoaldehydes, ammonia and hydrogen peroxide. Copper amine oxidases are homodimeric proteins containing at the active site a tyrosyl residue post-translationally modified to 2,4,5-trihydroxyphenylalanine quinone [3–5]. The oxidation of polyamines at the secondary amino group is catalysed by FAD-containing enzymes, known as polyamine oxidases (PAOs). These enzymes are usually classified on the basis of their biochemical properties, metabolic products, physiological roles and source [4–6]. Vertebrate PAOs (EC 1.5.3.11) efficiently transform N^1-acetyl derivatives of spermidine and spermine into putrescine and spermidine, respectively, plus acetamidopropanal and H_2O_2, and participate in the interconversion of polyamines [1,4,6]. Recent experimental data have suggested that H_2O_2 from amine oxidase-dependent catabolism of polyamines is a mediator of programmed cell death in mammals [7,8]. On the other hand, plant [4], bacterial [1] and protozoan [9] PAOs oxidise spermidine and spermine to 4-aminobutyral or 3-aminopropyl-4-aminobutyral, respectively, plus 1,3-diaminopropane and H_2O_2. As these compounds cannot be converted directly to other polyamines, this class of PAO is considered to be involved in terminal catabolism of polyamines. Growing evidence suggests that the production of H_2O_2 in the cell wall is a mediator of several physiological events such as programmed cell death, lignification, wall stiffening and cellular defense [10,11]. These metabolic functions have led to a new biotechnological interest in copper-containing amine oxidases and flavin polyamine oxidases. Plant PAOs, which apparently occur mainly in the cell wall of monocots, have been purified and partially characterised from a few species [4,5]. Maize PAO, the most studied member of this enzyme class, is a monomeric glycoprotein with a molecular mass of 53 000 Da, as determined by SDS-PAGE, containing one molecule of FAD [12,13]; the sugar content of the enzyme is 2.5%, mainly represented by arabinose [12]. In this paper we present the complete amino acid sequence determination of maize PAO, obtained by a combined approach of protein and cDNA sequencing. To our knowledge, this is the first primary structure reported for a flavin-containing polyamine oxidase.

2. Materials and methods

2.1. Molecular cloning and DNA sequence analysis

Total RNA was extracted from 5-day-old etiolated maize (*Zea mays* L.) seedlings, using Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. Poly(A)$^+$ mRNA was isolated with the Oligotex mRNA kit (Qiagen) and utilised for reverse transcription-polymerase chain reaction (RT-PCR). First-strand cDNA was synthesised using the Gibco-BRL Superscript Preamplification System and a $(dT)_n$-adaptor primer. PCR was performed in a 50 μl reaction containing 3 μl of the first-strand cDNA, 0.3 mM of each dNTP, 20 pmol of each primer (degenerate or specific, according to the experimental strategy), GeneAmp 1×PCR buffer II (Perkin-Elmer), 1.5 mM $MgCl_2$ and 2 units of AmpliTaq Gold (Perkin-Elmer). Amplification was carried out in a DNA GeneAmp PCR System 2400 (Perkin-Elmer) with the following temperature parameters: 5 min at 94°C followed by 40 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 2 min; followed by a 10 min final extension at 72°C. Rapid

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Abbreviations: dNTP, deoxynucleoside triphosphate; MAO, monoamine oxidase; MPAO, maize polyamine oxidase; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; UTR, untranslated region

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amplification of the 5′ and 3′ cDNA ends (5′ and 3′ RACE) was done essentially as reported by Frohman et al. [14]. For 5′ RACE the corresponding kit from Gibco-BRL was used following the manufacturer's recommendations. The first-strand cDNA and PCR conditions were as previously described, with the utilisation of the specific primer PaoIV. For 3′ RACE the first-strand cDNA and PCR conditions were mainly as above, with the following modifications: 10% (v/v) dimethyl sulphoxide was included in the PCR mixture, the specific primer PaoIII and the adaptor primer were used, a pre-PCR cycle (95°C, 5 min; 55°C, 2 min; 72°C, 40 min) was added. Full-length cDNA was obtained from a first-strand cDNA pool using two specific primers (PaoI and PaoVI) and following the conditions of the 3′ RACE. Oligonucleotides used were: PaoI, 5′-CACACTGCTGTGCAGAAC-3′ (nt 16–33 on the cDNA sequence, Fig. 1); PaoII, 5′-ATGTCNGGNATHTCNGCNGC-3′ (nt 193–212); PaoIII, 5′-GAGGCCGGGATAACCGACCT-3′ (nt 226–245); PaoIV, 5′-TAACTCGACGTTGATGCCGG-3′ (nt 321–302); PaoV, 5′-CCYTCNACCCARTTNGCNCC-3′ (nt 341–322); PaoVI, 5′-ATCGAACCAGCCGCTAGC-3′ (nt 1736–1719); $(dT)_n$-adaptor, 5′-CGTCTAGAGTCGACTAGTGC$(T)_{20}$-3′; adaptor, 5′-CGTCTAGAGTCGACTAGTGC-3′.

PCR products were purified using QIAquick PCR purification kit (Qiagen) and QIAquick gel extraction kit (Qiagen) and cloned in the TA cloning vectors pCR2.1 (Invitrogen) and pGEM-T (Promega). Plasmid DNAs were prepared for restriction analysis and DNA sequencing using column purification kits (Qiagen). DNA sequencing was performed on double-stranded plasmid DNA and PCR products using Amplicycle sequencing kit (Perkin-Elmer). A progressive sequencing strategy was carried out with design of further primers to complete the sequence of both strands of the cDNA inserts.

2.2. Amino acid sequence analysis

Procedures for preparation of carboxymethylated apoprotein were according to Hirs [15]. A sample of carboxymethylated maize PAO (2 mg) was suspended in 0.5 ml of 0.1 M ammonium bicarbonate and incubated at 37°C for 4 h with 40 μg trypsin (Worthington, code TRTPCK). A second aliquot (0.4 mg) was incubated in 50 mM Tris-HCl, pH 8.6, for 12 h at 37°C, with 15 μg Asp-N endoproteinase (Boehringer, sequence grade). A last aliquot (0.2 mg) of the native protein was incubated for 18 h in 0.7 ml 70% formic acid with a few crystals of CNBr. Isolation of peptides was carried out by HPLC, using a Beckman System Gold Model 126 instrument, on a macroporous reverse-phase column (C8, Aquapore RP300, 7 mm, 4.6 mm×250 mm, Brownlee Labs) with gradients of 0–70% acetonitrile in 0.2% (v/v) trifluoroacetic acid, at a flow rate of 1.0 ml/min. Elution of peptides was monitored with a diode-array detector (Beckman, Model 168). Automated Edman degradation of the intact protein and the purified peptides was performed on an Applied Biosystems model 476A gas-phase sequencer. Samples were loaded onto ProBlott membranes (Applied Biosystems) coated with polybrene, and run with a Blott cartridge according to the manufacturer's instructions. N-terminal sequence analysis of CNBr fragments was performed on samples electrotransferred on ProBlott membranes after SDS-PAGE [16].

3. Results and discussion

3.1. Primary structure of maize polyamine oxidase

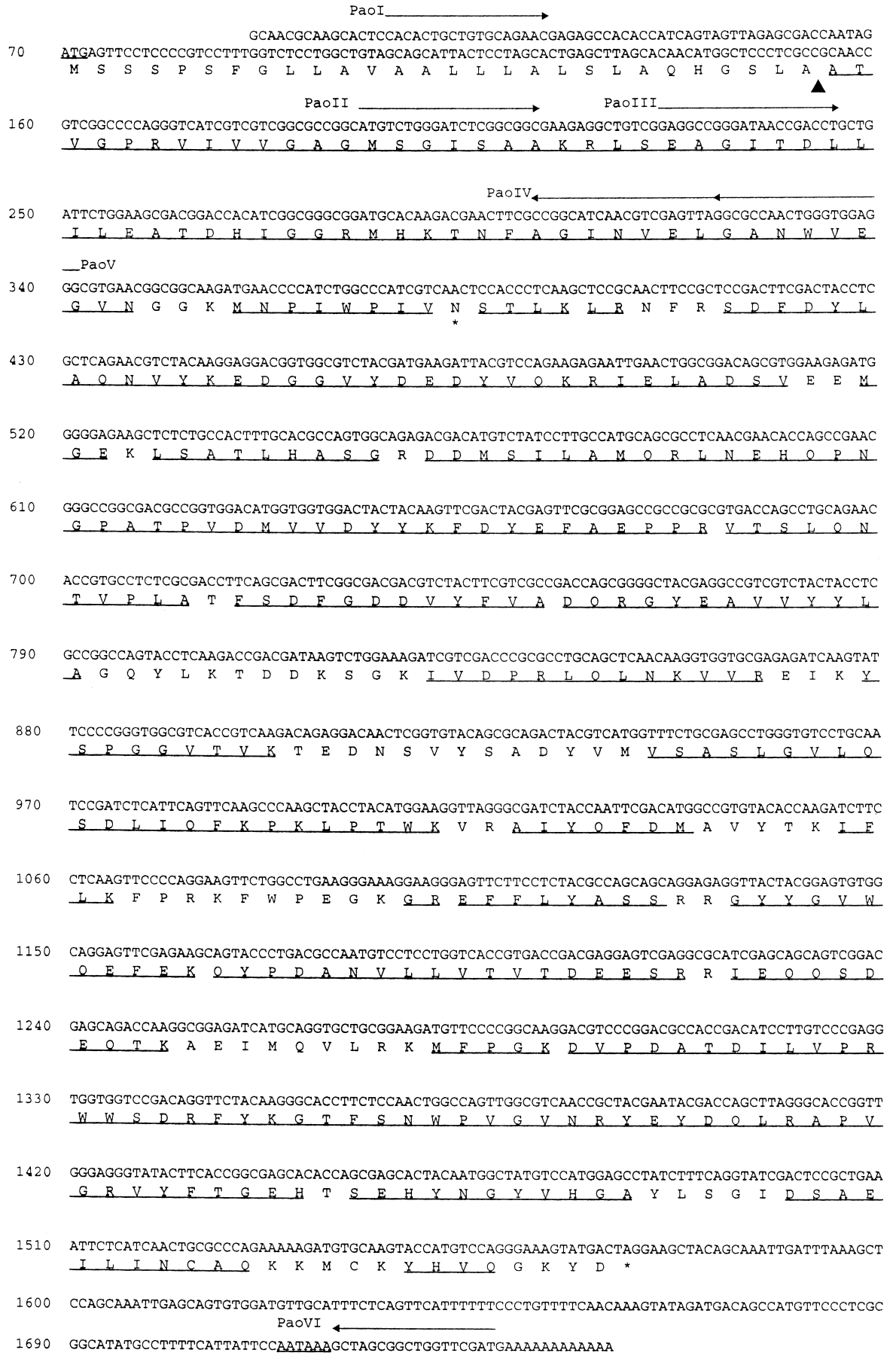
The nucleotide sequence of maize PAO (MPAO) full-length cDNA is shown in Fig. 1. This was obtained by RT-PCR and RACE strategies on the basis of amino acid sequences provided by the independent structural analysis of the purified protein (region from 29 to 91 in Fig. 1). Two degenerate primers, PaoII and PaoV, were designed as described in Section 2 and used for RT-PCR. A 150 bp product was obtained, cloned and sequenced. Its deduced amino acid sequence perfectly matched the protein sequence in region 42–91 (numbering according to Fig. 1). From the sequence of this DNA fragment, specific primers (PaoIV and PaoIII) were designed and used for 5′ and 3′ RACE, respectively. A fragment of 321 bp was obtained by 5′ RACE, whereas a fragment of 1509 bp was obtained by 3′ RACE. The full-length cDNA was synthesised by RT-PCR using the specific primers (PaoI and PaoVI), designed on the basis of the sequences of the 5′ and 3′ RACE fragments. The PCR product was directly sequenced and then cloned. The cDNA sequence is 1737 bp in length (Fig. 1), shows a single open reading frame extending from nt position 70 to 1573 and 5′- and 3′-untranslated regions (UTRs) 69 and 165 nt long, respectively. In the 3′-UTR, a canonical polyadenylation site (AATAAA) is present at position 1713. Northern analysis of poly(A)$^+$ mRNA from etiolated maize seedlings, using the MPAO cDNA as a probe, revealed a single transcript of approximately 1900 nt (data not shown).

In Fig. 1, the putative translational product deduced from the nucleotide sequence is also reported. The amino acid sequence from position 29 perfectly matches the previously reported N-terminal sequence of the protein [17]. This region does not correspond to the start of the open reading frame, because it follows a 28 amino acid sequence with the typical features of secretion signal peptides [18], i.e. a Leu-rich hydrophobic core and an Ala residue at the cleavage site. This is in agreement with the extracellular localisation of MPAO, as previously suggested on the basis of biochemical and immunocytochemical evidence [19,20].

The MPAO cDNA-deduced sequence has been confirmed by analysis of a number of tryptic, Asp-N protease and CNBr fragments derived from the protein. As indicated in Fig. 1, information from the protein sequence accounts for 400 residues, that is 84.7% of the primary structure of the mature protein. The calculated molecular mass is 53 636 Da, and supports previous determinations based on SDS-PAGE analysis [12]. Direct sequencing of the protein not only proves the identity of the cloned cDNA, but also provides information on the post-translational modification of the mature protein. During automated Edman degradation of tryptic peptides, we were unable to confidently assign a residue at the cycle corresponding to the predicted Asn-105. Since the sequence adjacent to the unidentified residue corresponds to the consensus sequence for *N*-glycosylation (Asn-Xxx-Ser/Thr), the presence of an *N*-linked carbohydrate chain has been inferred. Analytical studies are under way to definitely establish the presence of *O*-glycosylation, as strongly suggested by previous determination of sugar content [12].

3.2. Sequence comparison

The amino acid sequence reported here is the first structure available for a polyamine oxidase. In order to establish structure-function relationships among MPAO and other flavin-containing oxidases, a search of protein, nucleic acid and structural motif data bases has been carried out [21–23]. A significant structural similarity (about 20% amino acid identity) has been detected between MPAO and vertebrate flavin-containing monoamine oxidases (MAOs). These are highly conserved enzymes (70–80% identity), known to catalyse oxidative deamination of neuroactive, vasoactive and xenobiotic amines. In Fig. 2, the MPAO primary structure is compared with that of human type B MAO, taken as representative of this class of enzymes [24]. Identical residues are shown in bold. In the human enzyme, the 68 residues identical in all known MAOs (from human, ox, rat, fish and *Aspergillus niger*) are underlined. On the basis of the sequence alignment reported in Fig. 2, 28 out of 68 amino acids are conserved also in MPAO (41% identity). These residues are mainly clustered


PaoI
PaoII
PaoIII
PaoIV
PaoV
PaoVI

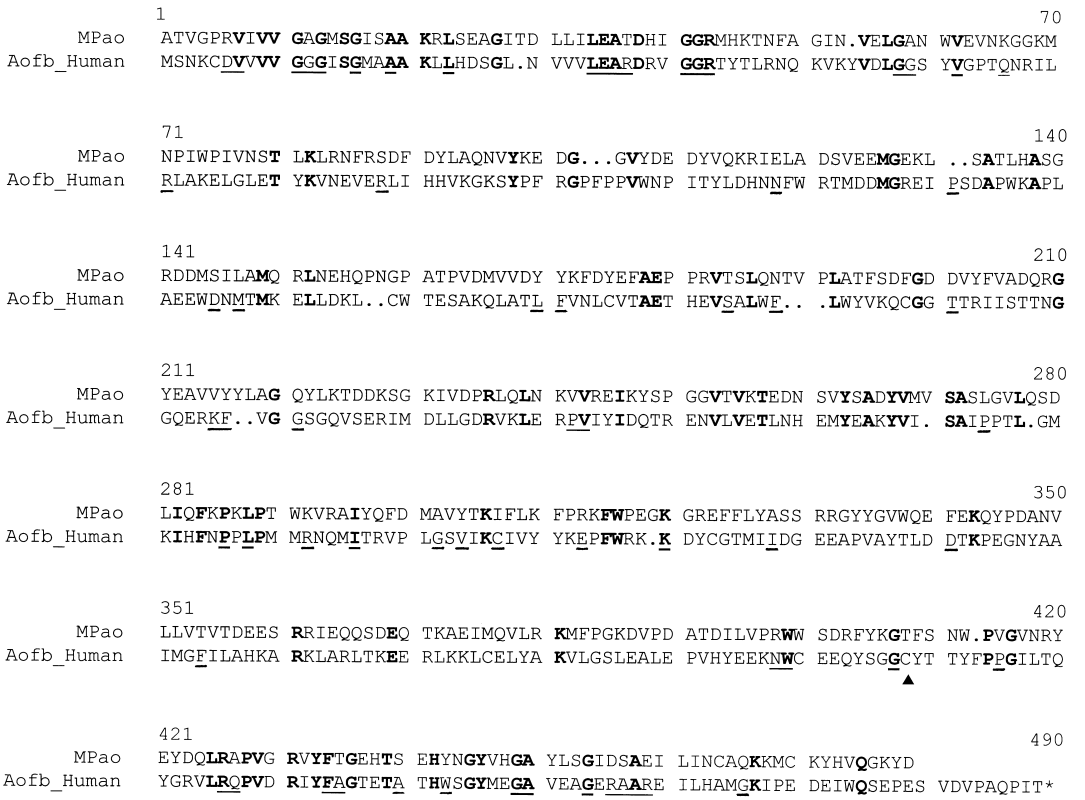


Fig. 2. Amino acid sequence comparison among maize polyamine oxidase (MPAO) and human type B monoamine oxidase (Aofb_Human [24]). Identical residues are in bold. Underlined amino acids in Aofb_Human indicate residues conserved in all flavin-containing monoamine oxidases. Dots represent insertions/deletions introduced by the multiple alignment program (Pileup from GCG package). For the sake of clarity, the C-terminal 42 residues of Aofb_Human have been deleted (*). The Cys residue covalently binding FAD in vertebrate monoamine oxidases is also indicated (▲).

in the N-terminal region, with the characteristic AMP binding motif, near residue 285, and in the C-terminal region, possibly involved in the flavin binding domain. In this respect, the absence of the Cys residue involved in the covalent binding of FAD in vertebrate MAOs (position 408) confirms a non-covalent interaction of the flavin [13], reported also for *A. niger* MAO [25].

Monoamine and polyamine oxidases, although differing in their specificity towards substrates (MAO being mainly active on primary amines and PAO on secondary ones), could be classified in the same structural and functional class of flavin-dependent oxidases. According to Ghisla and Massey [26], the plausible catalytic mechanism(s) operating in this class of enzymes should imply the interaction with 'non-activated' groups, in contrast to other flavin-containing oxidases, such as D-amino acid oxidase, in which electron-withdrawing activating groups are located next to the position of dehydrogenation [26].

The MPAO sequence reported will be of great importance in assisting the interpretation, currently under way, of the electron density map provided by X-ray crystallography. Moreover, the availability of MPAO cDNA will make it possible to perform site-directed mutagenesis experiments for a deeper insight into structure-function relationships and biotechnological applications in this class of enzymes.

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Fig. 1. Nucleotide and predicted amino acid sequences of maize flavin-containing polyamine oxidase. Nucleotides are numbered on the left and amino acids in italics on the right. Initiation ATG codon and putative polyadenylation site in the 3′-UTR are underlined; termination TAG codon is indicated by an asterisk (*). The length and the orientation of synthetic oligonucleotides used to prime the PCR are indicated (→); numbers refers to the oligonucleotides decribed in Section 2. Primers PaoII and PaoV are degenerate. The nt sequences 5′ and 3′ flanking the primers PaoI and PaoVI, respectively, are derived from RACE experiments. Underlined amino acids were identified by automated Edman degradation of peptides obtained from enzymatic and chemical cleavage. Signal sequence cleavage and potential *N*-linked glycosylation sites are indicated by arrowhead and asterisk, respectively. The DNA sequence has been deposited in the EMBL Data Bank with accession number AJ002204.

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