Molecular Cloning and Sequence Analysis of Catalase cDNA from Green Pepper Seedlings Elicited with Arachidonic Acid

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We isolated a cDNA encoding catalase from green pepper seedlings elicited with arachidonic acid, based on the amino acid sequences of the purified protein. The nucleotide sequence of the isolated cDNA contained a single open reading frame predicted to encode 492 amino acid residues with a calculated molecular mass of 56439.0 daltons. The deduced amino acid sequence contained the amino acid sequences found by sequencing of the peptides. The total deduced amino acid sequence showed high similarity with those of the other plant catalases reported so far and was found to possess the peroxisomal targeting sequence conserved among plant catalases. Transcription of the catalase gene in green pepper seedlings was found to be induced by treatment with arachidonic acid.

Key words: green pepper; catalase; cDNA cloning; arachidonic acid; elicitation

Higher plants have developed broad and complex biochemical systems to defend themselves against wounding, environmental changes, absorption of xenobiotics, and attacks of pathogens and herbivores. Biosynthesis of phytoalexins is one of the important defense mechanisms in higher plants. Capsidiol is a sesquiterpenoid phytoalexin produced in green pepper and tobacco, and has been studied for its biosynthesis and induction mechanisms. In these plant species, farnesy1 pyrophosphate is cyclized to yield the intermediate 5-epi-aristolochene, which is oxidized to produce capsidiol. The biosynthesis of capsidiol was induced by treatment with arachidonic acid in green pepper seedlings and by treatment with a number of fungal elicitors in cultured tobacco cells. The farnesyl pyrophosphate cyclization is an important reaction for switching the biosynthesis pathways of terpenoids from a healthy state to a stressed state. In addition, the final oxidation from 5-epi-aristolochene to capsidiol by 5-epi-aristolochene 3-hydroxylation seemed to be an important step in the biosynthesis. Hoshino et al. reported that a 59-kDa protein partially purified from the microsomes of green pepper seedlings treated with arachidonic acid was 5-epi-aristolochene 3-hydroxylase.

In this report, we attempted to analyze amino acid sequences of the purified 59-kDa protein from the green pepper seedlings elicited with arachidonic acid for cDNA cloning. Based on the deduced amino acid sequence of the cDNA isolated, we attempted to identify the protein encoded on the cDNA.

Materials and Methods

Plant materials. Green pepper (Capsicum annuum) seeds were purchased from Sapporo Konoen Seed Co. Ltd. (Sapporo, Japan). Seeds were sterilized in 3% sodium hypochlorite solution for 10 min, and plated on an autoclaved medium prepared from 0.1% (v/v) Hypoxen and 0.7% agar, and incubated at 25°C under light and dark conditions for 12 h each. After 10 days, seedlings were collected by removing them from the agar carefully, immersed in water containing 1 mM arachidonic acid solution, and then incubated for an appropriate time at 25°C.

PCR cloning. Green pepper seedlings grown for 10 days were treated with arachidonic acid for 6, 12, or 24 h and then poly(A) RNA was prepared from them using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). First strand cDNA was synthesized from 0.1 μg of poly(A) RNA using a first strand cDNA synthesis kit (Pharmacia) and 10% of the mixture was used as a template for PCR, which was done in 100 μl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 50 pmol of degenerate primers synthesized on the base of amino acid sequences and 1.25 units of AmpliTaq DNA polymerase (Takara Shuzo Co., Ltd., Otsu, Japan). The reaction was done through 35 cycles of 1 min at 94°C, 2 min at 45°C, and 3 min at 72°C. PCR products were separated by 5% polyacrylamide gel electrophoresis. The DNA fragment was extracted and cloned into pBluescript KSII+ (Stratagene Cloning Systems, La Jolla, CA).

Construction and screening of cDNA library. A cDNA library consisting of 2×105 recombinants was

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Abbreviations: CNBr, cyanogen bromide; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; MOPS, 3-morpholinopropanesulfonic acid.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession number AB007190.
constructed from 5 μg of the poly(A)⁺ RNA using a λZAP cDNA synthesis kit (Stratagene) according to the manufacturer’s instructions. The cDNA library was screened by plaque hybridization with a random-primed ³²P-labeled PCR fragment as a probe. Hybridization was done at 42°C for 15 h in a solution containing 50% (v/v) formamide, 5× SSC (1× SSC: 15 mM sodium citrate buffer containing 150 mM NaCl), 5× Denhardt’s solution, 100 μg/ml salmon sperm DNA, 0.5% SDS, and a probe DNA (about 5×10⁶ cpm/μg of DNA). Membranes were washed in 2× SSC-0.1% SDS for 5 min at a room temperature at once, 30 min at 50°C twice, and then in 0.2× SSC-0.1% SDS for 30 min at 50°C. After autoradiography, positive plaques were isolated and converted to phagemids by in vivo excision according to the manufacturer’s instructions.

DNA sequencing and sequence analysis. Insert DNA fragments of positive clones as well as PCR fragment were sequenced with an AutoRead Sequencing Kit (Pharmacia) using a Shimadzu model DSQ1 DNA sequencer (Shimadzu Co., Ltd., Kyoto, Japan). Sequence analysis was done with a DNASIS-Mac software (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). Multiple sequence alignment was done using a BLAST computer program.⁹

Northern hybridization. Poly(A)⁺ RNA preparations were put onto 1.0% agarose gel containing 1.8% formaldehdye with running buffer of 20 mM MOPS Good’s buffer (pH 7.0) containing 5 mM EDTA and 8 mM sodium acetate. RNA bands were transferred to a Hybond N nylon membrane (Amer sham International plc., Buckinghamshire, U.K.). About a 400-bp fragment of cCT1 cDNA and 1.3-kbp fragment from the β-ATPase gene were used as probe DNA. Hybridization was done at 42°C for 15 h in a solution containing 50% (v/v) formamide, 5× SSPE (1× SSPE: sodium phosphate buffer (pH 7.4) containing 180 mM NaCl and 1 mM EDTA), 5× Denhardt’s solution, 100 μg/ml salmon sperm DNA, and 0.5% SDS. A membrane was washed in 2× SSPE-0.1% SDS for 5 min at room temperature once, 30 min at 50°C twice, and in 0.1× SSPE-0.1% SDS for 20 min at 50°C twice.

Peptide sequencing. A 59-kDa protein sample purified from green pepper seedlings treated with arachidonic acid was given to us by Dr. T. Hoshino (Hokkaido National Industrial Research Institute, Sapporo, Japan). The protein sample of 1 μg with about 90% purity were put onto 9% SDS polyacrylamide gel electrophoresis as described by Laemmli,¹¹ and then electroblotted onto a PVDF membrane.¹² Protein bands were stained with Coomassie Blue and then a major band of 59-kDa was cut out. About 100 pmol of protein on the membrane was soaked in 150 μl of 70% formic acid containing 1.0% CNBr. Cleavage reactions were done at room temperature in the dark overnight. Then peptides were eluted from the membrane with 10 mM Tris-HCl buffer (pH 6.8) containing 2% SDS by shaking for 1.5 h. Then, these peptides were separated by 15% SDS polyacrylamide gel electrophoresis and electroblotted onto a PVDF membrane. The amino acids of stained bands were sequenced using an Applied Biosystems model 470A protein sequencer (The Perkin-Elmer Co., Foster City, CA).

Results and Discussion

Peptide sequences

We attempted to sequence the amino terminal region of the 59-kDa protein, that was purified from green pepper seedlings treated with arachidonic acid. From the preliminary sequencing, the amino terminus of the purified protein seemed to be modified. Therefore the protein sample was cleaved with CNBr. As shown in Fig. 1, three major bands were obtained. The amino terminal amino acid sequences of three peptides with 31.6-kDa (P1), 23.2-kDa (P2) and 14.5-kDa (P3) were found to be Asp-Leu-Ser-Leu-Tyr-X-Pro-Ser-Ser-Ala-Tyr-Asp-Ser-Pro for P1 and P2, and X-X-Asp-Glu-Glu-Val-Asp-Tyr-Leu-Pro-Ser-Ser-Phe for P3 (X: not identified). The amino-terminal sequences of P1 and P2 were identical. These two amino terminal sequences (P1/P2 and P3) showed high similarity with those of plant catalases reported. Both P1/P2 and P3 sequences had 85.7% and 76.9% identity with those of tobacco catalase.¹³

cDNA cloning

Based on the amino acid sequences found, four degenerate oligonucleotide primers were synthesized. The

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Fig. 1. CNBr Cleavage of 59-kDa Protein Purified from the Micromolar Fraction of Green Pepper Seedlings Treated with Arachidonic Acid.

Cleaved peptides were separated by SDS-polyacrylamide gel electrophoresis (15% gel) and electroblotted onto a PVDF membrane. Lane 1, cleaved peptides. Lane 2, protein markers. 66-kDa, bovine serum albumin; 45-kDa, egg albumin; 36-kDa, glyceraldehyde-3-phosphate dehydrogenase; 29-kDa, carbonic anhydrase; 24-kDa, trypsinogen; 20-kDa, trypsin inhibitor; 14-kDa, α-lactalbumin. Main bands (P1, P2, and P3) were cut out and their amino acids were sequenced.
cDNA Cloning of Catalase from Green Pepper

(A)  

\[ \begin{align*}  
\text{C01S} & : 5'-\text{aactcag} \text{g} \text{ccc} \text{a} \text{c} \text{gc} \text{tac} \text{gac} \text{ac} \text{cc-3'} \text{XhoI} \text{tg} \text{tg} \\
\text{C02AS} & : 3'-\text{ctc} \text{ctc} \text{ca} \text{i} \text{ata} \text{a} \text{a} \text{a} \text{ggt} \text{tca} \text{gag} \text{g} \text{3'-HindIII} \\
\end{align*} \]

Fig. 2. PCR Amplification of a DNA Fragment with the First Strand cDNA from Green Pepper Seedlings Treated with Arachidonic Acid.

(A) The DNA sequences of degenerate oligonucleotides C01S and C02AS, aligned with the peptide sequences. (B) PCR products amplified with the first strand cDNA as a template (lane 1). The DNA markers were a mixture of λ HindIII and φX174 HaeIII (lane 2). The 1.2-kbp fragment was subcloned, sequenced, and used as a probe for screening a cdNA library.

sense degenerate primer C01S [5'-AATCGAGGCl (A/T) (C/G) I (A/T) (C/G) IGCITA (C/T) GA (C/T) (A/T) (C/G) ICC-3'; I=inosine] and the antisense primer C01AS [5'-AACCTCGAGGGC (A/T) (A/G) TC (A/G) TAIGCI (C/G) (A/T) 1 (C/G) (A/T) IGG-3'] were based on the peptide sequence Pro-Ser-Ser-Ala-Tyr-Asp-Ser-Pro from P1/P2 with a XhoI site at the 5' end. The sense degenerate primer C02S [5'-GAAGCTTGA (A/G) GA (A/G) GTGAC (C/T) TA (C/T) (C/T) TTTCC-3'] and antisense primer C02AS [5'-GAAAGCTTGGTG (A/G) (A/G) TA (A/G) TCIAC (C/T) (C/T) TCC-3'] were based on the peptide sequence Glu-Glu-Val-Asp-Tyr-Leu-Pro from P3 with a HindIII site at the 5' end. PCR was carried out with first strand cDNA as a template using these primers. With a set of C01S and C02AS, 1.2-kbp fragment was obtained as a major product (Fig. 2). The 1.2-kbp fragment was subcloned and sequenced. The deduced amino acid sequence contained the peptide sequences as described before, and showed high similarity with that of tobacco catalase.\(^{(13)}\)

The cloned PCR fragment was used as a probe for screening a green pepper cDNA library, which was prepared from a poly(A)\(^{+}\) RNA fraction of the seedlings treated with 1 mm arachidonic acid for 12 h. About 3.0 x 10\(^5\) plaques were screened by plaque hybridization. After screening was repeated three times, over 14 positive clones were isolated. The insert sizes of these clones were analyzed by PCR with the oligonucleotide primers corresponding to the nucleotide sequence of T3 and T7 promoter in pBluescript SKII + . The clone pCT01 contained the longest insert DNA (1.8-kbp), and its sequence was analyzed.

**Sequence analysis**

Figure 3 shows the restriction map and sequencing strategy for the 1.8-kbp insert of pCT01. The cdNA was found to contain a 1479-bp open reading frame from the first ATG codon to the TGA stop codon, a 71-bp 5'-untranslated region and a 295-bp 3'-untranslated region as well as a 18-bp poly(A) tail (Fig. 4). The 5'-untranslated region was AT-rich, as found with plant cdNA clones.\(^{(14)}\) The poly(A) additional signal, AATAAA was present 122-bp upstream of the poly(A). The sequence (ACTATGGAT) resembled the initiation start consensus sequence in plant genes.\(^{(15)}\) From the first ATG codon, the open reading frame encoded 492 amino acids. The calculated molecular weight of the polypeptide was 56439.0, and agreed well with the molecular mass of the purified protein estimated on SDS polyacrylamide gel electrophoresis. The peptide sequences of P1/P2 and P3 were also found in the deduced amino acid sequence of pCT01 from the 2nd to 15th and 387th to 397th residues, respectively, although both sequences contained one different residue of Lys at 5th position instead of Ser and Arg at 596th position instead of Ser. In addition, putative CNBr cleavage sites were found in the deduced amino acid sequence. The deduced amino acid sequence of pCT01 showed high similarity to those of

Fig. 3. Restriction Map and Sequencing Strategy for the Insert DNA of pCT01.

The rectangular box shows the cdNA insert of pCT01. The closed box presents the coding region and the open box presents the 5' - and 3' - untranslated regions. Horizontal arrows indicate the direction and extent of sequencing. Ec, EcoRI; Sc, SacI; Bm, BamHI; Cl, Clal; Hc, HindII; Ev, EcoRV; Xh, XhoI.
plant catalases reported. Figure 5 shows a comparison of the deduced amino acid sequence of pCT01 with those of three catalases. Eggplant, tobacco, 13) and bovine 16) catalases showed 97.8%, 94.1%, and 38.8% amino acid sequence identity with that of pCT01. The primary structures of plant catalases were also analyzed with sweet potato, 17) maize, 18) castor bean, 19) tobacco, 20) barley, 21) tomato, 22) and rice. 23) These sequences were found to be highly conserved. Based on the three-dimensional structure of bovine catalase, 26) the amino acid residues involved in the catalytic site (His-65, Ser-104, and Asn-138), and distal (Val-64, Arg-102, Thr-105, Phe-143, and Phe-151) and proximal (Pro-326, Arg-344, and Tyr-348) heme binding sites were also conserved in pCT01. A putative targeting sequence (Ser-Arg-Leu) to peroxisomes was also conserved in pCT01. 19) Actually, Gould et al. reported that Ser (Cys or Ala)-Lys (His or Arg)-Leu is a signal sequence for targeting to peroxisomes. 25) Therefore, the cloned cDNA pCT01 was found to encode green pepper catalase.

We could not confirm whether the green pepper catalase in this study shows 5-epi-aristolochene 3-hydroxylase activity because of the lack of availability of the substrate, 5-epi-aristolochene. However it is possible to postulate that 5-epi-aristolochene is oxidized to produce capsidiol in the presence of O2 produced by catalase, since the compound is easily autooxidized (Hoshino, personal communication).
Expression of green pepper catalase mRNA

The expression of the green pepper catalase gene was analyzed by northern blotting using pCT01 cDNA as a probe. When green pepper seedlings were treated with 1 mM arachidonic acid, the transcript reached a maximum level after 6 h. The expression level was maintained even after 48 h (Fig. 6). The 59-kDa protein was also induced by the same treatment in green pepper seedlings.7 Thus, arachidonic acid seemed to induce the catalase gene in green pepper seedlings.

Arachidonic acid, which is an unsaturated fatty acid, is not present in higher plants, but is present in lower plants and fungi. It was reported that arachidonic acid was released from the cell walls of incompatible fungi and elicited fungitoxic sesquiterpenes in the potato.26 Arachidonic acid or its hydroperoxide produced by oxygen attack may be a signal molecule that induces defense-related genes, resulting in hypersensitive responses. In plant-pathogen interactions, reactive oxygen species (ROS) are generated at the infection site.
and the produced ROS seemed to play an important role in plant defense. H₂O₂ contributes to the structural reinforcement of plant cell walls and salicylic acid (SA) accumulation, as well as alteration of redox balance in the reacting cells. However, H₂O₂ is also toxic and causes damage not only to the pathogen but also to host plants. Catalase converts H₂O₂ to H₂O and O₂. So, induction of the catalase gene may be important for protection of host plants against the damage caused by H₂O₂. In addition, catalase may be involved in the oxidation of 5-epi-aristolochene to capsidiol because 5-epi-aristolochene is oxidized immediately under aerobic conditions. Based on these results of our study, it is possible that catalase inducible by arachidonic acid may play an important role in plant defense mechanisms in green pepper.

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