Isolation and Characterization of *o*-Diphenol-*O*-Methyltransferase cDNA Clone in Hot Pepper (*Capsicum annuum* L.)

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A cDNA clone, CaOMT1 encoding an o-diphenol-O-methyltransferase (OMT), which is involved in capsaicin biosynthesis, was isolated by screening of a cDNA library prepared from the mRNA of pepper (Capsicum annuum L.) pericarp. Nucleotide sequence analysis of CaOMT1 revealed that it had an open reading frame of 1080 bp which encodes a polypeptide with a predicted molecular weight of 39,430 D, corresponding well with the size of the known OMT's of tobacco, poplar, aspen, alfalfa, and cabbage. It also had five conserved boxes which appear in all known OMT's. The nucleotide sequence of CaOMTI had 89~74% identity with the OMT cDNA's of tobacco, aspen, alfalfa, and poplar, but a relatively lower identity of 59% with the OMT cDNA of maize. Amino acid sequence analysis also revealed that CaOMT1 has high identity with the known OMT's which have a substrate of o-diphenolic compounds, especially 5-hydroxyferulic acid and caffeic acid. It supports CaOMT1 which encodes an OMT. Southern blot analysis suggested that CaOMT1 might exist in the form of multiple copies in the pepper genome. CaOMT1 is expressed preferentially in pepper fruit and its expression levels increased during pepper fruit development, but decreased during fruit ripening, suggesting that the CaOMT1 gene is fruit development-related. CaOMT1 is the first reported cDNA clone for enzymes related to the phenlypropanoid pathway in pepper.

Keywords: hot pepper (Capsicum annuum L.), o-diphenol-O-methyltransferase, capsaicin, fruit development, phenylpropanoid pathway

Capsaicin (8-methyl-N-vanillyl-6-nonenamide; Bennett and Kirby, 1968) is the major pungent component of hot pepper fruit (Capsicum annuum L.) and is used as a spice for hot taste. Capsaicin is synthesized mainly in the placenta of the pepper fruit (Iwai et al., 1979; Suzuki et al., 1981; Fujiwake et al., 1982) and accumulates in the vacuoles of epidermal cells of the placenta (Fujiwake et al., 1980; Suzuki et al., 1980). The accumulation of capsaicin is known to occur over a relatively short period during the latter stages of fruit development (Iwai et al., 1979). Capsaicin is an amide derivative of vanillylamine and trans-8-methyl-6-nonenoic acid (Leete and Louden, 1968; Bennett and Kirby, 1968), but the biosynthesis is not fully elucidated. As far as is known, capsaicin-intermediates are made through the phenylpropanoid pathway (Fujiwake et al., 1980). The intermediates can also be used for biosynthesis of monolignols, precursors of lignins, therefore biosynthesis of capsaicin and that of lignin share a common biosynthetic pathway (Sukrasno and Yeoman, 1993). Phenlyalanine ammonia-lyase (PAL), the enzyme of the first step for capsaicin biosynthesis, catalyzes L-phenylalanine into cinnamic acid, which is subsequently converted into p-coumaric acid, caffeic acid, and ferulic acid through actions of cinnamic acid-4-hydroxylase (C4H), p-coumaric acid-3-hydroxylase (C3H), and o-diphenol-O-methyltransferase (OMT; EC 2.1.1.6), respectively. The enzymes which are involved in the transformation of ferulic acid into vanillin and vanillin into vanillylamine are unknown. The final step is completed by capsaicinoid synthetase through the enzymatic condensation of vanillylamine and trans-8-methyl-6nonenoic acid.

Generally, the activities of the enzymes, PAL, C 4H, C3H, OMT, and CS, participating in capsaicin biosynthesis increase along with the development of the fruit and the growth of callus derived from the hypocotyl of hot pepper (Ochoa-Alejo and Gómez-Peralta, 1993). As for OMT and CS, the activities in

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callus are much lower than those in the fruit. This phenomenon is thought to be the result of the lower expression of OMT and CS in callus (Ochoa-Alejo and Gómez-Peralta, 1993). Therefore, OMT and CS might be the key enzymes for regulation of capsaicin biosynthesis in pepper fruit.

OMT methylates *o*-diphenol compounds such as caffeic acid and 5-hydroferulic acid using S-adenosyl-L-methionine as a methyl donor (Collendavelloo *et al.*, 1981). While in gymnosperms, caffeic acid is a main substrate of OMT, in angiosperms, OMT is a bispecific catalyst having substrates of caffeic acid and 5-hydroxyferulic acid, which are converted into ferulic acid and sinapic acid through O-methylation by OMT, respectively (Higuchi, 1990). Ferulic acid and sinapic acid are phenolic derivatives for lignin biosynthesis, and ferulic acid may also follow the capsaicin biosynthesis pathway.

Recently, several OMT cDNAs were isolated from aspen (Bugos et al., 1991), alfalfa (Gowri et al., 1991), tobacco (Jaeck et al., 1993; Pellegrini et al., 1993), poplar (Dumas et al., 1992), cider tree (Poeydomenge et al., 1994), Zinnia elegans (Ye and Varner, 1995), and OMT genomic genes were isolated from Zea mays (Collazo et al., 1992) and poplar (Tsai et al., 1995). However, neither proteins nor the cDNA of CS has been isolated yet.

In this study, we present the basis of molecular studies on the regulation of capsaicin biosynthesis and its pathway.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR) and Partial cDNA Cloning

PCR was carried out in a total volume of 50 µL containing 1×10^8 phage particles of hot pepper (Capsicum annuum L.) pericarp cDNA library, 50 pmole of OMT-1 primer [ATCGAATTCA(A/G) GTICTIATGGA(A/G)AG(T/C)TGG], 50 pmole of OMT-2 primer [ACTGAATTCTIATIAC(C/T)] TTICC(A/G)TTIGCIGG], 2 mM MgCl₂, 0.25 mM dNTP, 2.5 units of Taq polymerase (Takara), and a reaction buffer supplied by the manufacturer. PCR was performed in a thermal cycler (Pharmacia) for 35 cycles with each cycle consisting of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The amplified products were digested with EcoRI, and then electrophoresed in 0.8% agarose gel. The PCR product corresponding to approximately 600 bp was eluted with a JETSORB Kit (GENOMED), and then ligated

into pGEM-7Zf(+). The resulting plasmid was designated as pOMT600.

Screening of cDNA Library

A total of 5×10^5 plaques of the hot pepper pericarp cDNA library were plated at a density of $1 \times$ 10^s plagues per 150 mm petridish and transferred to Hybond-N+ filters (Amersham) and screened using pOMT600 as a probe. The probe was labeled with $\left[\alpha^{-32}P\right]dCTP$ (3000 Ci/mM, Amersham) by the Primea-Gene system (Promega). The filters were prehybridized for 3 h at 60° C in $6 \times$ SSC, $5 \times$ Denhardt's solution, 0.5% SDS, and 100 µg/mL salmon sperm DNA. Hybridization was performed for 16~20 h at 60° C in prehybridization solution with 3×10^{6} cpm/ mL labeled probes. The filters were washed once in $2 \times SSC$, 0.1% SDS for 10 min, once in $1 \times SSC$, 0.1% SDS for 10 min, once in $0.5 \times SSC$, 0.1% SDS for 10 min, and once in $0.2 \times$ SSC, 0.1% SDS for 10 min. at 60°C.

Sequence Analysis

cDNA inserts of the isolated phage clones were recovered by *in vivo* excision from the Uni-ZAPTM XR vector following the manufacturer's instructions. The double-strands of the cDNA insert were sequenced according to the manufacturer's instructions (USB Sequenase ver. 2.0 Kit). For generation of smaller DNA fragments for sequencing, a deletion kit for kilosequencing (Takara) was used. DNASIS and PROSIS programs (Hitachi), and the Clustal multiple alignment program (Higgins *et al.*, 1992) were used in nucleotide and amino acid sequence analyses. Nucleotide and amino acid sequence similarities were analyzed through the BLAST Network service of the National Center for Biotechnology Information.

Southern Blot Analysis

Total DNA was prepared from hot pepper leaves according to the protocol of Doyle and Doyle (1990) with a minor modification. Thirteen μ g samples of DNA were digested with *Eco*RI, *Eco*RV, *Hind*III, and *Xba*I, respectively, subjected to electrophoresis in 1% agarose gel and then blotted onto a Hybond-N+ filter (Amersham). The total insert of *CaOMT1* was radioactively labeled with $[\alpha$ -³²P]dCTP (3000 Ci/mM, Amersham) by random priming (Promega) and used as a probe. The filter was prehybridized, hybridized, and washed in the same method used in the screening of the cDNA library.

Northern Blot Analysis

Total RNA was extracted from pericarps and leaves of hot pepper, according to the guanidinium thiocyanate method (Sambrook *et al.*, 1989). Eleven μ g of total RNA was run on 1% formaldehyde agarose gel, blotted onto a Hybond-N+ filter (Amersham), prehybridized at 55°C and hybridized at 55°C with ³²P-labeled probes of which templates were approximately a 500 bp fragment digested by *Bam*HI and a 430 bp fragment double-digested by *Xba*I and *Hind*III, which were prepared from *CaOMT1*. For washing the filter, the same method in the screening of the cDNA library was used except for the temperature (55°C).

RESULTS AND DISCUSSION

Isolation of Full Length cDNA Clone

Two degenerated primers, OMT1 and OMT2, of which sequences were based on conserved regions of known OMT's of tobacco (Jaeck et al., 1993), poplar (Dumas et al., 1992), alfalfa (Gowri et al., 1991), and maize (Collazo et al., 1992), were made and used for cloning of partial cDNA in the PCR. After electrophoresing the PCR product, we observed three bands of approximately 500 bp, 600 bp, and 1000 bp on the gel. Since the DNA of 600 bp showed a high similarity to the known OMT cDNA of poplar and tobacco, we used the pOMT600 as a probe for isolating a full length cDNA clone. Among the positive clones selected after the first and second screenings, three clones were thought to have the correct size, approximately 1.4 kb for the full length which was based on the sizes of the known OMT cDNA's-tobacco, 1431 bp; poplar, 1368 bp; cider tree, 1452 bp (Poeydomenge et al., 1994); alfalfa, 1368 bp; aspen, 1503 bp (Bugos et al., 1991). As results of sequence analyses and homology searches, one of them was identified as a full length OMT cDNA and designated CaOMT1.

Sequence Analyses of CaOMT1 and CaOMT1

The cDNA of *CaOMT1* is 1377 bp in length, which consists of a 5'-leader sequence (58 bp), an open reading frame (1080 bp), and a 3'-untranslated region (239 bp) with a polyA tail (18 bp) (Fig. 1).

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221 The Lys Gly The Asn Phe Asp Leu Ser His Val The Glu Asp Ala Pro Ala Tyr Pro Gly 244 779 GTC GAA CAT GTT GOT OGG GAC ATG TTT GTT AGT GTA CCG AAA GCA GAT GCC ATT TTC ATG 833 241 Val Glu His Val Gly Arg Asp Met Phe Val Ser Val Pro Lys Ala Asp Ala The Phe Met 266 839 ANG TGG ATT TGT CAT GAT TGG AGC GAT GAG CAT TGC TTA AAA TTC TTG AAG AAC TGT TAC 899 261 Lys Trp Tile Cys His Asp Trp Ser Asp Glu His Cys Leu Lys Phe Leu Lys Asn Cys Tyr 288 840 GCA CTT CCT GCA AAT GGG AAT GCA GTA GAT GCT GAT ACT TCCA GAG ACC TCA GAG GAC ACT GCT GCA AAT GGG AAT GCA GTA GTT GAT ATG GTA AGG ACC CCA 959 959 GAA CAC TCA GCT GCC ACA AMG AAT GCA GTA CAT GTT GAT ATT GTT ATG TTA GCA CAT AAC 101 301 Asp Thr Ser Ala Ala Thr Lys Asn Ala Val His Val Asp The Val Met Leu Ala His Asn 32 959 GAC ACA TCA GCT GCC ACA AMG GAT GAG GAA ATTI GAA CTT GG GTA GGG GCT GCA TTT 107 301 Asp Thr Ser Ala Ala Thr Lys Asn Ala Val His Val Asp The Val Met Leu Ala His Asn 32 1019 CCA GGA GGC AAG GAA AGG ACT GAG GAA ACT THE GAA ACT TGG GTA GGA ATT GAG ATT GCA THE GDL Hys GIU Asg Thr Glu Lys GIU Phe Glu Ala Leu Ala Lys GIU Phe Bil Val GI Phe 113 311 The Gly Phe Arg Arg Ala Cys Cys Ala Tyr Gln Thr Try Val Met Glu Phe His Lys **** 36 1129 ATTACCAATTCCTTGGAAGGCTTGACTGCGATGTA			718 220
241 Val Slu His Val Gly Arg Asp Het Phe Val Ser Val Pro Lys Ala Asp Ala Ile Phe Het 26 283 ANG TGG ATT TGT CAT GAT TGG AGC GAT GAG CAT TGC TTA AAA TTC TTG AAG AAC TGT TAC 89 261 Lys Trp Ile Cys His Asp Trp Ser Asp Glu His Cys Leu Lys Phe Leu Lys Asn Cys Tyr 28 899 GAA GCA CTT CCT GCA AAT GGG AAA GTT TTA GTT GCA GAG TGC ATA CTT CCA GAG ACC CCA 96 996 GAA CCA CTT CCT GCA AAT GGG AAA GTT TTA GTT GCA GAG TGC ATA CTT CCA GAG ACC CCA 96 959 GAA ACA TCA GCT OCC ACA AAG AAT GCA GTA CAT GTT GAT ATT GTT ATG TTA GCA CAT AACC 95 956 GAC ACA TCA GCT OCC ACA AAG AAT GCA GTA CAT GTT GAT ATT GTT ATG TTA GCA CAT AACC 101 301 Asp Thr Ser Ala Ala The Lys Asn Ala Val His Val Asp Ile Val Het Leu Ala His Kan 32 1019 CCA GGA GGC AAA GAA ACG ATT GAG AAA GAA TTT GAA GAT TTT GAT GTT GGT GAG GGT GCA CTT TTT 107 321 Pro Gly Gly Lys Glu Arg Thr Glu Lys Glu Phe Glu Ala Leu Ala Lys Gly Ala Gly Phe 34 1029 ACT GGA TTC COC AGA GCT TGC TST GCT TAC CAA ACT TGG GTC AGG GAA TTC CAC AAG TGA 133 341 The Gly Phe Arg Arg Ala Cys Cys Ala Tyr Gln Thr Try Val Het Glu Phe His Lys **** 36 1139 ATTACCAATTCCTTTGAAGGGCTTGACTGACGATGTAGTAGTAGTAGTAGTAGTAATAAAAGAAAATTAGCTTTACATTG 129 34 1218 TTACTTTIAC			778 240
261 Lys Trp Ile Cys His Asp Trp Ser Asp Glu His Cys Leu Lys Phe Leu Lys Asn Cys Tyr 28 899 GAA GCA CTT CCT GCA ANT GGG AAA GTT TTA GTT GCA GAG TGC ATA CTT CCA GAG ACC CCA 95 810 Ala Leu Pro Ala Asn Gly Lys Val Leu Val Ala Glu Cys Ile Leu Pro Glu Thr Pro 30 959 GAC ACA TCA GCT OCC ACA ANG AAT GCA GTA CAT GTT GAT ATT GTT ATG TTA GCA CAT AACC 101 301 Asp Thr Ser Ala Ala Thr Lys Asn Ala Val His Val Asp Ile Val Net Leu Ala His Asn 32 1019 CCA GGA GGC AAA GAA ACG ATT GAG AAA GAA TTT GAA GAT TTT GAT ATG GTT AGG GTO GCA TTT 107 321 Pro Gly Gly Lys Glu Arg Thr Glu Lys Glu Phe Glu Ala Leu Ala Lys Gly Ala Gly Phe 34 1029 ACT GGA TTC COC AGA GCT TGC TST GCT TAC CAA ACT TGG GT CAG GAA TTC CAC ANG TGA 113 341 Thr GGA TTC COC AGA GCT TGC TST GCT TAC CAA ACT TGG GT CAG GAA TTC CAC ANG TGA 136 341 Thr GJ Phe Arg Arg Ala Cys Cys Ala Tyr Gln Thr Trp Val Net Glu Phe His Lys **** 36 1139 ATTATCCAATTCCTTTGAAGATTCACTGCGATGTAGTAGTAGTAGTAGTAGTAGTAGTAGTAGAAAAAA			838 260
281 Glu Ala Leu Pro Ala Asn Gly Lys Val Leu Val Ala Glu Cys Ile Leu Pro Glu Thr Pro 30 959 GAC ACA TCA GCT GCC ACA AMG ANT GCA GTA CAT GTT GAT ATT GTT ATG TTA GCA CAT AMC 101 301 Asp Thr Ser Ala Ala Thr Lys Asn Ala Val His Val Asp Ile Val Net Leu Ala His Asn 32 1019 CCA GGA GGC AMA GAA AGG ACT GAG GAA GAA TTT GAA GCT TTG GCT AMG GGT GCA GTT 107 321 Pro Gly Gly Lys Glu Arg Thr Glu Lys Glu Phe Glu Ala Leu Ala Lys Gly Ala Gly Phe 34 1019 ACT GGA TTC COC AGA GCT TGC TST GCT TAC CAA ACT TGG GTC ATG GAA TTC CAC AMG TGA 113 341 Thr GOA TTC COC AGA GCT TGC TST GCT TAC CAA ACT TGG GTC ATG GAA TTC CAC AMG TGA 133 341 Thr GJy Phe Arg Arg Ala Cys Cys Ala Tyr GIn Thr Try Val Net Glu Phe His Lys *** 36 343 TTACCAATTCCTTTGAAGGATTCAAAACAAAAATGTATTACCATTGGTGAAATAAAAGAAAATTACCATTGGGGATT 121 1218 TTACCTTTIGAAGGACTGACGAAGTAGTAGTAGTAGTAGTGTTGAATATAAAGAAAAATAAAGAAAATTAGCTTTACAATTGGTCAATTAGTGTCAATATAGAAGTAAAAAGAAAAATTAGCTTTACAATTGGTCAATATGTGTCAATATAGAGTCAAAAAAAA			898 280
301 Asp Thr Ser Ala Ala Thr Lys Asm Ala Val His Val Asp Ile Val Net Leu Ala His Asm 32 1019 CCA GGA GGC AAA GAA KGS ACT GAG AAA GAA TTT GAA OCT TTG GCT AAG GGT OCT GGA TTT 107 321 Pro Gly Gly Lys Glu Arg Thr Glu Lys Glu Phe Glu Ala Leu Ala Lys Gly Ala Gly Phe 107 341 Thr GCA TTC CCC AGA GCT TGC TGT GCT TAC CAA ACT TGG GTC ATG GAA TTC CAC ANG TGA 113 341 Thr Gly Phe Arg Arg Ala Cys Cys Ala Tyr Gln Thr Trp Val Net Glu Phe His Lys *** 36 1139 ATTATCCAATTCCTTTGAAGGATTCAAAACAAAATIGTTTACCCCTATATATATAGTGTCATTTGCAATTGGGGATT 121 1218 TTACTTTTACTTGAAGGATTGACTGCGATGTAGTAGTAGTAGTGTGTGAATATAAAGAAAAATTAGTTTACCATTG 129 ATGAGATTTATGTCACTATGAAATAATTGCTCTTGGAAATGGATGTGTTGAATATAAAGTGAAAAAAAA			958 300
321 Pro Gly Gly Lys Glu Arg Thr Glu Lys Glu Phe Glu Ala Leu Ala Lys Gly Ala Gly Phe 34 1079 ACT GOA TIC COC ARA GCT TOC TST GCT TAC CAA ACT TOG GTC ATG GAA TTC CAC ANG TSA 113 341 Thr Gly Phe Arg Arg Ala Cys Cys Ala Tyr Gln Thr Trp Val Het Glu Phe His Lys *** 36 1139 ATTATCCAATTCCTTTGAAGGATTCAAACAAAATGTTTACCCCTATATATA			1018 320
341 Thr Gly Phe Arg Arg Ala Cys Cys Ala Tyr Gln Thr Trp Val Het Glu Phe His Lys ···· 36 36 1139 ATTATCCAATTCCTTTGAAGGATTCAAAACAAAAATTGTTTACCCCTATATATA			1078 340
1218 TTACTITINCTCTGRATGOCITIGACTOCGATGIAGTAGTAGTAGTACACTGTGAAAATAAAASAAAATAACCTTTINCATTG 129 1295 ATGAGATTTATGTTCACTATGAAATAATTGCTCTTAGAAAAAGAAAATGGTTCAATATAAGTTCAAAAAGAAAATAAAAAGAAAATAGTTCAATATAGTTCAATATGGTTCAATATAAGTTCAAAAAAGAAAATAGTTCAATATGGTTCAATATAGTTCAATATGGTTCAATATGGTTCAATATAGAATGAAATAGTTCAATATGGTTCAATATAGTTCAATATGGTTCAATATAGAATGAAT			1138 360
1295 ATGAGATTTATGTCACTATGAAATAATTGCTCTTAGAAAAAGTGTTCAATATAAGTTCAATAATAAAGTTCAATAAAGTTCAATAATAAAGTTCAATAAAGTTCAATAAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAAGTTCAATAAAGTTCAATAAAGTTCAATAATAAGTTCAATAATAAAGTTCAATAATAAGTTCAATAATAAAGTTCAATAATAAGTTCAATAATAAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAATAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAAGTTCAATAATAATAAGTTCAATAATAAGTTCAATAATAATAATAAGTTCAATAATAAGTTCAATAATAATAATAATAATAATAAGTTCAATAATAATAATAATAATAATAATAATAATAATAATA	1139		1217
-m		1. Nucleotide sequence and the deduced amino	

Fig. 1. Nucleotide sequence and the deduced amino acid sequence of the *CaOMT1* cDNA clone. The terminal codon is indicated by asterisks (***). Residues homologous to the conserved flanking sequence of ATG are underlined. Putative glycosylation sites and polyadenylation signals are shadowed and boldfaced, respectively. The GenBank accession number of this sequence is U83789.

Its [G+C] content is 40.5%.

In the 58 bp of the 5'-leader sequence, [A+T] content is 69%, which corresponds to analyses of 79 plant genes, in which 53% of the plant genes had 40~50 bp of the 5'-leader sequence and 92% of them had over 50% of [A+T] content in them (Joshi, 1987a). The translation start region shows a high similarity of 87.5% to the plant consensus initiation context, AACAATGG and corresponded to purine at 3 bp upstream of ATG (Guerineau, 1992). Moreover, it corresponded to the report that at 3 bp upstream of ATG in dicots adenine appears 2~4 times more frequently than in monocots (Cavener *et al.*, 1991). In the 3'-untranslated region, there are three sequences (CACTG, AATAAA, CATTG) which seem to be polyadenylation signals and are located at 100 bp, 92 bp, and 70 bp upward from polyA tail, respectively (Joshi, 1987b).

The 1080 bp open reading frame encodes a polypeptide of 359 amino acids, whose calculated molecular weight is 39,430 D, similar to that of the known OMT's - e.g. 38.5 kD~43 kD of tobacco (Hermann et al., 1987); 38 kD of poplar (Doorsselaerc et al., 1993); 40 kD of aspen (Bugos et al., 1991); 41 kD of alfalfa (Vance and Bryan, 1981); 42 ± 2 kD of cabbage (De Carolis and Ibrahim, 1989). It also has five conserved boxes, which appear in all known OMT's, consisting of S-adenosyl-L-methionine binding motifs (Boxes I, III, and IV) (Ingrosso et al., 1989), Box 1 - extended, conserved motif (Box II), and conserved hydrophilic motif (Box V) (Fig. 2).

As a result of the NCBI BLAST sequence similarity search, the nucleotide sequence of *CaOMT1* had 89~74% identity with the OMT cDNA of tobacco, aspen, alfalfa, and poplar, but a lower identity of 59% with the OMT cDNA of maize, a monocot. Amino acid sequence analysis also revealed that CaOMT1 has high identity with the known OMT's which have a substrate of *o*-diphenolic compounds, especially 5-hydroxyferulic acid and caffeic acid. This supports the idea that *CaOMT1* encodes an OMT. In order to confirm the activity of CaOMT1, we are trying to express CaOMT1.

Based on the result of the NCBI Entrez search, CaOMT1 is the first reported cDNA clone for enzymes related to the phenylpropanoid pathway in hot pepper. We think *CaOMT1* will be helpful to study not only as regards capsaicin biosythesis and its regulation, but also in phenylpropanoid pathway related phenomena-pathogenic response (Jaeck *et al.*, 1993), stress response (Gowri *et al.*, 1991), and lignin biosynthesis (Grisebach, 1981).

Southern Blot Analysis and Northern Blot Analysis

We could observe several hybridizing signals in the Southern gel blot (Fig. 3). It suggested that the CaOMT1 gene might exist in the form of multiple copies in the hot pepper genome. However, the overall intensity of the hybridizing signals was not even suggesting that besides the genomic sequence

0.00	
CaOMT1	MDSTNONLTQTEDEAFLFAMQLASASVLPMVLKSALELDLLEIMAK
Tobacco	MGSTSQSQSKSLTHTEDEAFLFAMQLASASVLPMVLKSALELDLLELMAK
Poplar	MGSTGETQMTPTQVSDEEAHLFAMQLASASVLPMILKTAIELDLLEIMAK
Alfalfa	MGSTGETQITPTHISDEEANLFAMQLASASVLPMILKSALELDLLEIIAK
Maize	MGSTAGDVAAVVDEEACMYAMQLASSSILPMTLKNAIELGLLEVLQK
	*.**
CaOMT1	AGPGAAISPSELAAQLPTKNPEAPVMLDRMLRLLATYSVLNCTLR
Tobacco	AGPGAAISPSELAAQLSTQNPEAPVILDRMLRLLATYSVLNCTLR
Poplar	AGPGAFLSTSEIASHLPTKNPDAPVMLDRILRLLASYSILTCSLK
Alfalfa	AGPGAQISPIEIASQLPTTNPDAPVMLDRMLRLLACYIILTCSVR
Maize	EAGGGKAALAPEEVVARMPAAPSDPAAAAAMVDRMLRLLASYDVVRCOME
	. • • • •
CaOMT1	TLPDGRVERLYSLAPVCKLLTKNADGVSVAPLLLMNQDKVLMESWYHLTD
Tobacco	TLSDGSVERLYSLAPVCKFLTKNADGVSVAPLLLMNQDKVLMESWYHLKD
Poplar	DLPDGKVERLYGLAPVCKFLTKNEDGVSVSPLCLMNQDKVLMESWYYLKD
Alfalfa	TOODGKVORLYGLATVAKYLVKNEDGVSISALNLMNODKVLMESWYHLKD
Maize	D-RDGRYERRYSAAPVCKWLTPNEDGVSMAALALMNQDKVLMESWYYLKD
CaOMT1	AVLDGGVPFNKAYGMTAFEYHGTDPRFNKVFNRGMSDHSTMTMKKILEDY
Tobacco	AVLDGGIPFNKAYGMTAFEYHGTDPRFNKVFNRGMSDHSTMSMKKILEDY
Poplar	AILDGGIPFNKAYGMTAFEYHGTDPRFNKVFNKGMSDHSTITMKKILETY
Alfalfa	AVLDGGIPFNKAYGMTAFEYHGTDPRFNKVFNKGMSDHSTITMKKILETY
Maize	AVLDGGIPFNKAYGMTAFEYHGTDARFNRVFNEGMKNHSVIITKKLLDFY
	[]] J[]] J
CaOMT1	[]][]] KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV
CaOMT1 Tobacco	
	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV
Tobacco	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKHPSIKGINFDLPHVIGDAPAYPGV
Tobacco Poplar	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKHPSIKGINFDLPHVIGDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV
Tobacco Poplar Alfalfa	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKHPSIKGINFDLPHVIGDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV
Tobacco Poplar Alfalfa	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKHPSIKGINFDLPHVIGDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV
Tobacco Poplar Alfalfa	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKHPSIKGINFDLPHVIGDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV
Tobacco Poplar Alfalfa	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLPHVIGDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGLVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV
Tobacco Poplar Alfalfa Maize	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLPHVIGDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV
Tobacco Poplar Alfalfa Maize CaOMT1	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLPHVIEDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV [[]]] []V EHVGRDMFVSVPKADAIFMKWICHDWSDEHCLKFLKNCYEALPANGKVLV
Tobacco Poplar Alfalfa Maize CaOMT1 Tobacco	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKHPSIKGINFDLPHVIGDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEBAPSYPGV [[]]] []V EHVGRDMFVSVPKADAIFMKWICHDWSDEHCLKFLKNCYEALPANGKVLV EHVGGDMFASVPKADAIFMKWICHDWSDEHCLKFLKNCYEALPANGKVIV
Tobacco Poplar Alfalfa Maize CaOMT1 Tobacco Poplar	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV ELVGRDMFVSVPKADAIFMKWICHDWSDEHCLKFLKNCYEALPANGKVLV EHVGGDMFVSVPKADAIFMKWICHDWSDEHCLKFLKNCYEALPANGKVII EHVGGDMFVSVPKADAVFMKWICHDWSDAHCLKFLKNCYDALPENGKVIL
Tobacco Poplar Alfalfa Maize CaOMT1 Tobacco Poplar Alfalfa	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIGDAPAYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPSIKGINFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVISGAPFPGV EHVGGDMFVSVPKADAIFMKWICHDWSDEHCLKFLKNCYEALPANGKVLV EHVGGDMFVSVPKADAVFMKWICHDWSDAHCLKFLKNCYEALPANGKVIV
Tobacco Poplar Alfalfa Maize CaOMT1 Tobacco Poplar Alfalfa	KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLNSIVDVGGGTGATVNMIVSKYPSIKGINFDLSHVIEDAPAYPGV KGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIGDAPSYPGV TGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVIEDAPSYPGV TGFEGVSTLVDVGGGVGATLHAITSRHPHISGVNFDLPHVISEAPPFPGV EHVGGDMFVSVPKADAIFMKWICHDWSDEHCLKFLKNCYEALPANGKVIV EHVGGDMFVSVPKADAVFMKWICHDWSDAHCLKFLKNCYEALPANGKVIV RHVGGDMFASVPAGDAILMKWILHDWSDAHCATLLKNCYDALPENGKVIV
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Tobacco Poplar Alfalfa Maize CaOMT1 Tobacco Poplar Alfalfa Maize CaOMT1 Tobacco Poplar Alfalfa Maize CaOMT1 Tobacco Poplar Alfalfa Maize	KGFEGLNSI VDVGGGTGAT VNMI VSKYPSI KGI NFDLSHVI EDAPAYPGV KGFEGLNSI VDVGGGTGAT VNMI VSKYPSI KGI NFDLPHVI GDAPAYPGV KGFEGLTSL VDVGGGTGAV INTI VSKYPSI KGI NFDLPHVI EDAPSYPGV TGFEGLKSL VDVGGGTGAV INTI VSKYPSI KGI NFDLPHVI EDAPSYPGV TGFEGVSTI VDVGGGTGAVI NTI VSKYPTI KGI NFDLPHVI EDAPSYPGV TGFEGVSTI VDVGGTGAVI NTI VSKYPTI KGI NFDLPHVI SEAPPFPGV TGFEGVSTI VDVGGTGAVI NTI VSKYPTI KGI NFDLPHVI SEAPPFPGV TGFEGVSTI VDVGGTGAVI NTI VSKYPTI KGI NFDLPHVI SEAPPFPGV TGFEGVGTI VDVGGTGAVI NTI VSKYPTI KGI NFDLPHVI SEAPPFPGV TGFEGVGTI VSVPKADA I FMKWI CHDMSDEHCLKFLKNCYEALPANGKVI V EHVGGDMFVS I PKADAVFMKWI CHDMSDEHCLKFLKNCYEALPANGKVI V RHVGGDMFVS I PKADAVFMKWI CHDMSDAHCATLLKNCYDALPENGKVI V RHVGGDMFASVPAGDAI LMKWI LHDMSDAHCATLLKNCYDALPENGKVI V THVGGDMFASVPAGDAI LMKWI LHDMSDAHCATLLKNCYDALPENGKVI V NTO TA TAKAVHVDI VMLAHNPGGKERTEKEFEALAKGAGFT AECI LPETPDTSAATKNAVHVDI VMLAHNPGGKERTEKEFEALAKGAGFT VECI LPVAPDTSLATKGVVHDV VI MLAHNPGGKERTQKEFEDLAKGAGFG AECI LPVAPDSSLATKGVVHDV MI LAHNPGGKERTQKEFEDLAKGAGFG VECVLPVNTEATPKAQGVFHVDMI MLAHNPGGKERTQKEFEDLAKGAGFG VECVLPVNTEATPKAQGVFHVDMI MLAHNPGGKERTGKEFERLAKGAGFS TO TAKAGAGTI VMEFHK GFARLVALTILGSWNST-NN GFEVMCCAFNTHVI EFRKKA GFKVHCNAFNTY IMEFLKKV

Fig. 2. Alignment of the deduced amino acid sequences of CaOMT1 and OMT of tobacco, poplar, alfalfa, and maize. The amino acids identical in four proteins are marked with asterisks (*) and the amino acids similar in four proteins are marked with dots (\cdot). The five conserved boxes in OMT are shadowed.



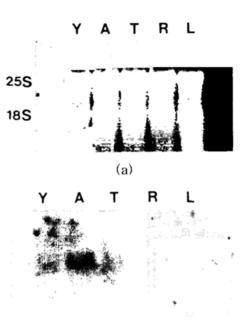


Fig. 3. DNA gel blot analysis of the gene for CaOMT1 in *Capsicum annuum*. Each 13 μ g of *Capsicum annuum* total DNA digested with *Eco*R1 (EI), *Eco*RV (EV), *Hind*III (H), and *Xba*I (X) was separated on a 1.0% agarose gel, transferred to a Hybond-N+ filter and probed with the *CaOMT1* cDNA clone.

of *CaOMT1* itself, other hybridizing sequences were distantly related. Jaeck *et al.* (1993) showed that the tobacco genome has two OMT genes as a result of Southern blot. Besides, it was reported that there are two or three OMT genes in the genomes of alfalfa (Gowri *et al.*, 1991) and aspen (Bugos *et al.*, 1991). Therefore our result agrees with these previous studies.

In order to study the *CaOMT1* mRNA level during fruit growth, the fruit growth period was divided into four stages based on the length and color of the fruit (Hubbard and Pharr, 1992; Ochoa-Alejo and G mez-Peralta, 1993) - young stage, fully developed stage, red-turning stage, and fully ripened stage.

The two former stages were considered as developmental, and the latter two as ripening. Total RNA was extracted from the pericarp of each stage and a leaf. The result of Northern blot analysis showed that *CaOMT1* mRNA expression level increased during pepper fruit development, but decreased during ripening (Fig. 4). The result agrees with the report that the activity of OMT in pepper increases during development and decreases 30 days after flowering (Ochoa-Alejo and Gómez-Peralta, 1993). This means the *CaOMT1* gene is fruit development-related, not ripening-related like geranylgeranyl

(b)

Fig. 4. RNA gel blot analysis of total RNA from hot pepper pericarp. Each 11 μ g of total RNA extracted from the pericarp of young stage (Y), fully developed adult stage (A), red-turning stage (T), fully ripened stage (R), and a leaf (L) was separated on a 1.0% formaldehyde agarose gel. (a), ethidium bromide staining; (b), autoradiogram hybridized with radiolabeled *CaOMT1*.

pyrophosphate synthase (Kuntz et al., 1992), cysteine synthase (O-acetylserine sulfhydrylase) (Romer et al., 1992), or ascorbate peroxidase (Schantz et al., 1995) which are known to increase their activities and expressions during ripening of pepper. It was also observed that CaOMT1 mRNA was not detected in the total RNA of hot pepper leaf suggesting that CaOMT1 is expressed preferentially in fruit organ. The facts that callus derived from the hypocotyl of pepper had low OMT activity (Ochoa-Alejo and Gómez-Peralta, 1993), that xylems of poplar (Doorsselaere et al., 1993), aspen (Bugos et al., 1991), and tobacco (Jaeck et al., 1992) had tissue-specifically high OMT mRNA levels, and that high OMT mRNA levels were detected in roots and stems of alfalfa (Gowri et al., 1991) support our speculation.

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