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Isolation and Characterization of a Jasmonic Acid Carboxyl Methyltransferase Gene from Hot Pepper (*Capsicum annuum* L.)

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Methyl jasmonate, the methyl ester of jasmonic acid, is a volatile plant hormone that acts as an important cellular regulator, mediating diverse developmental processes and defense responses. Methyl jasmonate is synthesized by methylation of jasmonic acid; this reaction is catalyzed by jasmonic acid carboxyl methyltransferase (JMT). Although JMT cDNA had previously been described only for *Arabidopsis thaliana*, here we used PCR to isolate it from *Capsicum annuum* L. The 389-amino-acid sequence deduced for the *JMT* gene showed 92% identity to that from *A. thaliana*. Southern blot analysis revealed that *JMT* is present in the genome as two copies. Our preliminary northern blot detected no JMT transcript, but, through RT-PCR and subsequent Southern blot analysis of products using gene-specific probes, we found that transcript levels increased after leaf-wounding. Likewise, 10 μM methyl jasmonate induced *JMT* gene expression in leaves. Transcription levels began to increase 10 min after wounding, and were maintained for 1 to 4 h. Moreover, expression of the *CaJMT* and *PIN2* genes was increased by both wounding and MeJA applications, but was not enhanced by treatment with H_2O_2.

Keywords: hot pepper, hydrogen peroxide, jasmonic acid carboxyl methyltransferase, methyl jasmonate, wounding

Methyl esters of secondary metabolites are important components of plant volatiles. For example, methyl jasmonate (MeJA) is a fragrant compound initially identified from the flowers of *Jasminum grandiflorum* (Arimura et al., 2000). The term 'jasmonate' includes the biologically active intermediates in the pathway for jasmonic acid biosynthesis, as well as the biologically active derivatives of jasmonic acid. These widely distributed compounds affect a variety of processes (Creelman and Mullet, 1997), including fruit ripening, production of viable pollen, root growth, tendril coiling, responses to wounding and abiotic stress, and defense against insects and pathogens. Jasmonates are synthesized in plants via the octadecanoid pathway (Creelman and Mullet, 1997; Beale and Ward, 1998), then further catabolized to form a volatile counterpart, MeJA, and numerous conjugates. Jasmonic acid carboxyl methyltransferase (JMT) is a key enzyme for jasmonate-regulated plant responses. MeJA can act as an intra-cellular regulator, a diffusible intercellular signal transducer, or an airborne signal, mediating intra- and inter-plant communications (Beale and Ward, 1998). JMT cDNA had previously been isolated only from *Arabidopsis thaliana* (Seo et al., 2001). Here, we report its characterization from *Capsicum annuum* L., as well as our analyses of the functioning, biochemical activity, and signal regulation of the enzyme encoded by this gene.

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MATERIALS AND METHODS

Plant Material and Treatments

Young plants of hot pepper (*C. annuum* L. cv. Bugang) were reared in a growth chamber or greenhouse at 25°C, under a 16-h photoperiod. Healthy and well-expanded leaves from 2-month-old plants were used for mechanical wounding and chemical treatments. All samples were floated on 20 mM sodium phosphate buffer. For wounding, the tissues were cut with a cork borer. Chemical treatments involved the application of 1 to 100 μM MeJA or 0.1 to 100.0 mM H_2O_2. During most experiments, the leaves were kept in an open area, but the MeJA-treated leaves were enclosed in a jar to separate them from the others. Afterward, all samples were harvested, frozen immediately in liquid nitrogen, and stored at –80°C prior to the RNA or DNA extractions.

Cloning of Hot Pepper *JMT* (*CaJMT*)

To clone the hot pepper *JMT* genes, first-strand

cDNA was prepared from treated leaves. *Arabidopsis* JMT-specific primers (forward, 5´-ATGGAGGTAAT-GCGAGTTCT-3´ and reverse, 5´-TCAACCGGTTC-TAACGAGCG-3´ encoding the amino acids MEVMRI and LVRTG, respectively) were used in the amplification reaction. PCR fragments were cloned into pGEM T-Easy (Promega, USA) and sequenced via the dideoxy chain-termination method (Sanger et al., 1977), on an ABI373 automated DNA sequencer (Perkin-Elmer, USA).

Northern and Southern Blot Analyses and RT-PCR

For our northern blot analysis, 20 μg of total RNA, isolated from the leaves, stems, or roots, was separated on a formaldehyde agarose gel (Sambrook et al., 1989). In addition, 2 μg of total RNA was amplified by RT-PCR (10, 20, or 30 cycles), and the products were analyzed by Southern blotting. Hot pepper PIN2 and actin served as quantity controls. Serial 10-fold dilutions of the total RNA were prepared in

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1 ATG GAG GTA ATG CGA GTT CTT CAC ATG AAC AAA GGA AAC GGT GAA ACG AGT TAT GCC AAG
  M  E  V  M  R  V  L  H  M  N  K  G  N  G  E  T  S  Y  A  K    20

61 AAC TCC ACC GCT CAG AGT AAC ATA ATA TCT CTA GGC AGA AGA GTA ATG GAC GAG GCC TTG
  N  S  T  A  Q  S  N  I  I  S  L  G  R  R  V  M  D  E  A  L    40

121 AAG AAG TTA ATG ATG AGC AAT TCA GAG ATT TCG AGC ATT GGA ATC GCC GAC TTA GGC TGC
  K  K  L  M  M  S  N  S  E  I  S  S  I  G  I  A  D  L  G  C    60

181 TCC TCC GGT CCG AAC AGT CTC TTG TCC ATC TCC AAC ATA GTT GAC ACG ATC CAC AAC TTG
  S  S  G  P  N  S  L  L  S  I  S  N  I  V  D  T  I  H  N  L    80

241 TGT CCT GAC CTC GAC CGT CCT GTC CCT GAG CTC AGA GTC TCT CTG AAC GAC CTC CCT AGC
  C  P  D  L  D  R  P  V  P  E  L  R  V  S  L  N  D  L  P  S   100

301 AAT GAC TTC AAC TAC ATA TGT GCT TCT TTG CCA GAG TTT TAC GAC CGG GTT AAT AAT AAC
  N  D  F  N  Y  I  C  A  S  L  P  E  F  Y  D  R  V  N  N  N   120

361 AAG GAG GGT TTA GGG TTC GGT CGT GGA GGA GGA GAA TCG TGT TTT GTG TCG GCC GTC CCA
  K  E  G  L  G  F  G  R  G  G  G  E  S  C  F  V  S  A  V  P   140

421 AGT TCG TTC TAC GGA CGT TTG TTT CCT CGC CGG AGC CTT CAC TCT GTG CAT TCT TCT TCT
  S  S  F  Y  G  R  L  F  P  R  R  S  L  H  S  V  H  S  S  S   160

481 AGT TTA CAT TGG TTA TCT CAA GTT CCA TGT CGT GAG GCG GAG AAG GAA GAC AGG ACA ATA
  S  L  H  W  L  S  Q  V  P  C  R  E  A  E  K  E  D  R  T  I   180

541 ACA GCT GAT TTA GAA AAC ATG GGG AAA ATA TAC ATA TCA AAG ACA AGT CCT AAG AGT GCA
  T  A  D  L  E  N  M  G  K  I  Y  I  S  K  T  S  P  K  S  A   200

601 CAT AAA GCT TAT GCT CTT CAA TTC CAA ACT GAT TTC TTG GGT TTT TTG AGA TCG CGA TCT
  H  K  A  Y  A  L  Q  F  Q  T  D  F  L  G  F  L  R  S  R  S   220

661 GAG AAG TTG GTC CCG GAA GGC CGA ATG GTT TTA TCG TTC CTT GGT AAA AGA TCA CTG GAT
  E  K  L  V  P  E  G  R  M  V  L  S  F  L  G  K  R  S  L  D   240

721 CCC ACA ACC GAA GAG AGT TGC TAT CAA TGG GAA CTC CTA GCT CAA GCT CTT ATG TCC ATG
  P  T  T  E  E  S  C  Y  Q  W  E  L  L  A  Q  A  L  M  S  M   260

781 GCC AAA GAG GGT ATC ATC GAG GAA GAG AAG ATC GAT GCT TTC AAC GCT CCT TAC TAT GCT
  A  K  E  G  I  I  E  E  E  K  I  D  A  F  N  A  P  Y  Y  A   280

841 GCG AGC TCC GAA GAG TTG AAA ATG GTG ATA GAG AAA GAA GGG TCA TTT TCG ATC GAT AGG
  A  S  S  E  E  L  K  M  V  I  E  K  E  G  S  F  S  I  D  R   300

901 CTT GAG ATA AGT CCG ATT GAT TGG GAA GGT GGG AGT ATC AGT GAG GAG AGT TAT GAC CTT
  L  E  I  S  P  I  D  W  E  G  G  S  I  S  E  E  S  Y  D  L   320

961 GTA ATA AGG TCC AAA CCC GAA GCC CTA GCT AGT GGC CGA AGA GTG TCT AAT ACC ATA AGA
  V  I  R  S  K  P  E  A  L  A  S  G  R  R  V  S  N  T  I  R   340

1021 GCT GTG GTC GAG CCG ATG CTA GAA CCT ACT TTC GGT GAA AAT GTG ATG GAC GAG CTT TTT
  A  V  V  E  P  M  L  E  P  T  F  G  E  N  V  M  D  E  L  F   360

1081 GAA AGG TAT GCA AAG ATC GTG GGA GAG TAC TTC TAT GTA AGC TCG CCA CGA TAC GCT ATT
  E  R  Y  A  K  I  V  G  E  Y  F  Y  V  S  S  P  R  Y  A  I   380

1141 GTT ATT CTT TCG CTC GTT AGA ACC GGT TGA
  V  I  L  S  L  V  R  T  G  *                                  390
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Figure 1. Nucleotide sequence and deduced amino acid sequence of *CaJMT* cDNA. Amino acid sequence represent full-length gene product. A total of 1170 nucleotides are presented and numbered in 5´→ 3´ direction.

nuclease-free water, and the RT-PCR product complementary to the actin gene was amplified using the following gene-specific primers: for PIN2 (forward, 5′-CCTTGCTTCCCTACTTGTACTTGG-3′ and reverse, 5′-CATATAGACGCCCTAGCGTATTACG-3′) and actin (forward, 5′-GAGCACCCTGTCCTGCTCACTG-AA-3′ and reverse, 5′-ATGCTGCTGGGAGCCAAAG-CAGT-3′). ^{32}P-radio-labeled probes for *CaJMT*, *PIN2*, and actin were hybridized at 42°C, washed, and exposed to a phosphor-image plate. These probes were prepared with the Klenow enzyme.

RESULTS AND DISCUSSION

Nucleotide Sequence and Deduced Amino Acid Sequence of the *CaJMT* Gene

To obtain the *JMT* gene in hot pepper, first-strand cDNA was prepared from extracted poly(A)$^+$ RNA (Chomczynski and Sacchi, 1987). *JMT* cDNA was isolated by PCR using heterologous *Arabidopsis*-specific primers. The full-length PCR product, *CaJMT* cDNA, contained a 1170-b open reading frame (ORF). Its sequence revealed an ORF encoding 390 amino acids (Fig. 1). The CaJMT protein sequence was compared through multiple alignments with other available JMT sequences (NCBI protein Blast) using global comparison methods (ClustalW v. 1.8). CaJMT resembled five other related proteins (Fig. 2), showing the following identities: 92% to JMT from *Arabidopsis* (AY008434), 78% to NTR1 (floral nectary-specific protein) from *Brassica campestris* (AF179222), 41% to salicylic acid methyl transferase (SAMT) from *Stephanotis floribunda* (AJ308570), 38% to SAMT from *Atropa belladonna* (AB049752), and 38% to SAMT from *Clarkia breweri* (AF133053) (Ross et al., 1999; Dudareva et al., 2000; Murfitt et al., 2000; Song et al., 2000; Seo et al., 2001). *JMT* was first reported for *Arabidopsis*, where it was described as a single-copy gene. In contrast, here we detected more than two hybridizing bands in all lanes containing the restricted hot pepper genomic DNA (Fig. 3A). Therefore, we are convinced that this gene is *JMT* of hot pepper (*CaJMT*). The two hybridizing bands in Figure 3B were caused by internal cutting of the probe.

The recently obtained crystal structure for *C. breweri* SAMT has led to the identification of specific res-

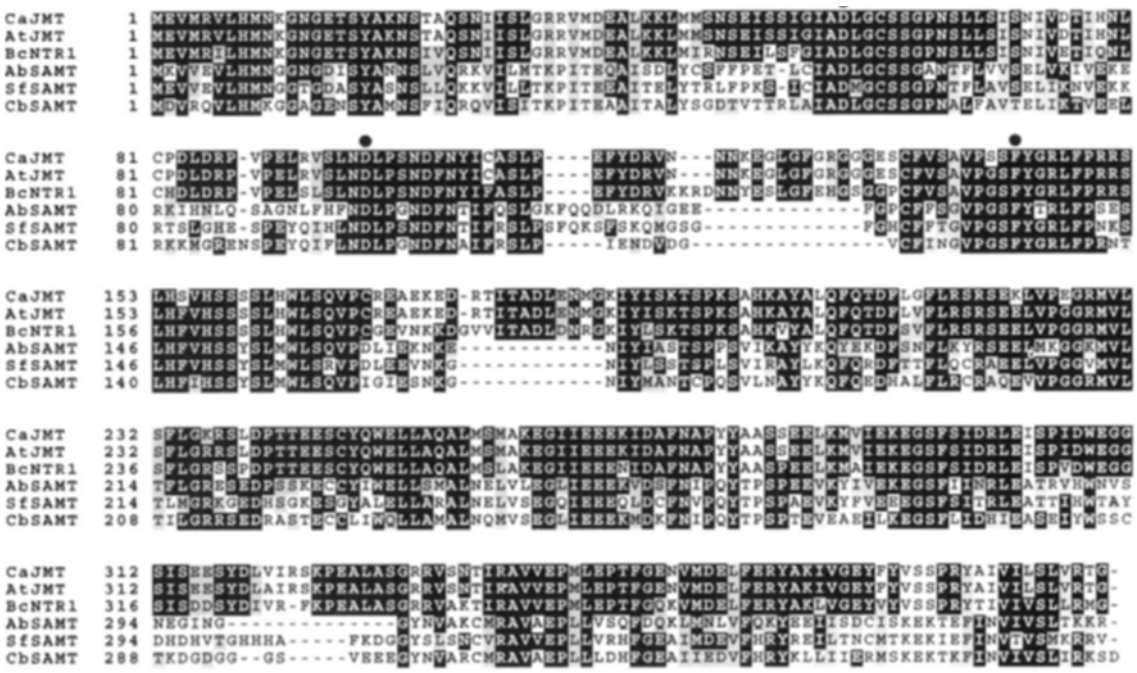

Figure 2. Comparison of predicted amino acid sequences of hot pepper JMT and related proteins. Hot pepper JMT (CaJMT) sequence was aligned via ClustalW with JMT from *Arabidopsis* (AtJMT; AY008434), NTR1 from *B. campestris* (BcNTR1; AF179222), SAMT from *S. floribunda* (SfSAMT; AJ308570), SAMT from *A. belladonna* (AbSAMT; AB049752), and SAMT from *C. breweri* (CbSAMT; AF133053). Alignment was shaded using Boxshade 3.21 software program (ch.EMBnet.org) to show conserved amino acid residues in black and similar residues in gray. Dashes indicate gaps inserted for optimal alignment.

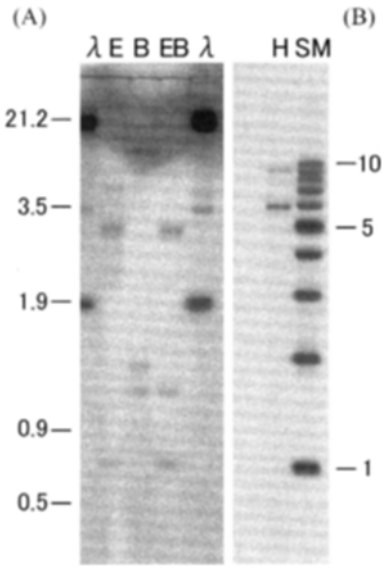

Figure 3. Genomic Southern blot analysis of *CaJMT*. Blots were hybridized with full-length *Arabidopsis* cDNA probe **(A)** and *CaJMT*-specific probe **(B)**. **(A)** Twenty-μg sample of hot pepper genomic DNA isolated from wild-type leaves was digested with *Eco*RI (E), *Bam*HI (B), and *Eco*RI+*Bam*HI (EB) and electrophoresed. **(B)** Twenty-μg sample of *Arabidopsis* genomic DNA isolated from wild-type leaves was digested with *Hin*dIII (H) and electrophoresed. Marker sizes (in kbp) are indicated on left- or right-hand side of gel. λ, lambda DNA/*Eco*RI+*Hin*dIII markers; SM, 1-kbp DNA ladder.

idues responsible for substrate binding in carboxyl methyltransferase (Zubieta et al., 2003). Here, we found that Asp-57, Asp-97, and Phe-143 (labeled in Fig. 2 with dots) are involved in *S*-adenosyl-L-methionine (SAM) binding. While those amino acid residues are conserved in the aligned sequences, there are some variations in the residues involved in the acceptor molecule site. These variations most likely determine the substrate specificity of carboxyl methyltransferases (Negre et al., 2002).

Expression Patterns of *CaJMT*

Transcripts of *CaJMT* accumulated in the leaves and flowers, but were barely detectable in the roots and stems (Fig. 4). The band associated with the "flower" lane may be the floral nectary-specific NTR1 because *Arabidopsis* JMT and NTR1 (Song et al., 2000) are orthologues (Seo et al., 2001). Those two enzymes seem to catalyze MeJA production in the cytoplasm, because the lack of these proteins apparently organ-specific transit signals peptide and hydrophobic regions long enough to be integrated membranes

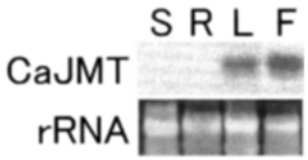

Figure 4. Accumulation of *CaJMT* transcripts in different organs. Samples of total RNA (2 μg) isolated from stems (S), roots (R), flowers (F), and leaves (L) were amplified by RT-PCR; products were hybridized using *CaJMT*-specific probe. rRNA in ethidium bromide-stained gels served as loading control.

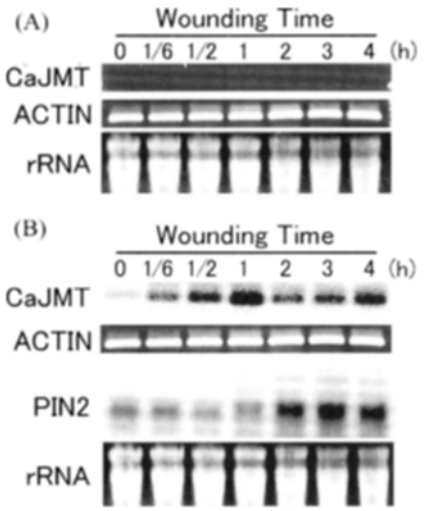

Figure 5. Effect of wounding on *CaJMT* and *PIN2* transcription levels in hot pepper leaves. **(A)** Time course of accumulation of *CaJMT* transcripts detected after wounding. Total RNA was extracted from leaves at 0, 10, and 30 min, and 1, 2, 3, and 4 h after wounding. A 2-μg sample of isolated total RNA was amplified by RT-PCR. **(B)** Expression patterns for pepper *PIN2* and *CaJMT* genes after wounding. PCR products were hybridized using *CaJMT*-specific probe. Actin PCR products are shown as quantitative loading control. Total RNA was prepared from leaves at various times after wounding. rRNA in ethidium bromide-stained gels served as loading control.

(Seo et al., 2001).

Because their levels were very low, *CaJMT* transcripts were undetectable by RNA-blot hybridization in the floral and leaf tissues. However, we did perform Southern blotting to analyze the quantity of *CaJMT* gene expression after RT-PCR, using *CaJMT*-specific primers (Fig. 5A and B). The detection sensitivity of the RT-PCR dictated the number of amplification cycles and the quantity of template. *CaJMT* transcript levels started to rise 10 min after wounding, although the PIN2 transcription level increased only after 2 h (Fig. 5B).

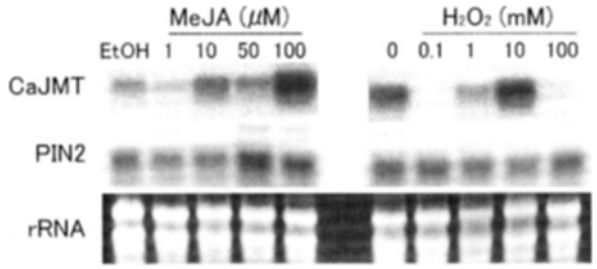

Figure 6. Expression pattern for *CaJMT* after MeJA and H_2O_2 treatments. Detached pepper leaves were treated with indicated concentrations for 24 h. MeJA treatment was carried out in a jar; H_2O_2 treatment, in an open area. For 0 μM MeJA, ethanol was used. *PIN2* gene served as positive control; rRNA in EtBr-stained gels is shown as loading control.

JMT in Hot Pepper Is an "early gene"

H_2O_2 directly regulates the expression of numerous genes, some of which are involved in plant defenses and the hypersensitive response (Levine et al., 1994; Korsmeyer et al., 1995; Desikan et al., 1996; Alvarez et al., 1998; Kovtun et al., 2000). Jasmonic acid activates the signal pathway genes (early genes) in the vascular bundles, whereas H_2O_2, produced by cell wall-derived oligogalacturonides released by polygalacturonides, is a second messenger that activates defense genes (late genes) in the mesophyll cells (Orozco-Cardenas et al., 2001). In our study, the expression of *CaJMT* and *PIN2* genes was increased by wounding and MeJA treatments, but not by hydrogen peroxide (Fig. 6). This suggests that *CaJMT* is an "early gene" in the signaling cascade, because HO is a second messenger that activates late genes.

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LITERATURE CITED

Alvarez ME, Penell RI, Meijer PJ, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92: 773-784

Arimura G, Ozawa R, Shimoda T, Nishioka T, Boland W, Takabayashi J (2000) Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature 406: 512-525

Beale MH, Ward JL (1998) Jasmonates: Key players in the plant defence. Nat Prod Rep 15: 533-548

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-189

Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonates in plants. Annu Rev Plant Physiol Plant Mol Biol 48: 355-381

Desikan R, Hancock JT, Coffey MJ, Neill SJ (1996) Generation of active oxygen species in elicited cells of *Arabidopsis thaliana* is mediated by NADPH oxidase-like enzyme. FEBS Lett 382: 213-217

Dudareva N, Murfitt LM, Mann CJ, Gorenstein N, Kolosova N, Kish CM, Bonham C, Wood K (2000) Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. Plant Cell 12: 949-961

Korsmeyer SJ, Yin XM, Oltuai SN, Veis-Novack DJ, Linette GP (1995) Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. Biochim Biophys Acta 1271: 63-66

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci USA 97: 2940-2945

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583-593

Murfitt LM, Kolosova N, Mann CJ, Dudareva N (2000) Purification and characterization of S-adenosyl-L-methionine: benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methyl benzoate in flowers of *Antirrhinum majus*. Arch Biochem Biophys 382: 145-151

Negre F, Kolosova N, Knoll J, Kish CM, Dudareva N (2002) Novel S-adenosyl-L methionine: salicylic acid carboxyl methyltransferase, an enzyme responsible for biosynthesis of methyl salicylate and methyl benzoate, is not involved in floral scent production in snapdragon flowers. Arch Biochem Biophys 406: 261-270

Orozco-Cardenas ML, Narvaez-Vasquez J, Ryan CA (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell 13: 179-191

Ross JR, Nam KH, D'Auria JC, Pichersky E (1999) S-Adenosyl-L methionine: salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. Arch Biochem Biophys 367: 9-16

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463

Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD (2001) Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant

responses. Proc Natl Acad Sci USA 97: 4788-4793

Song JT, Seo HS, Song SL, Lee JS, Choi YD (2000) NTR1 encodes a floral nectary specific gene in *Brassica campestris* L. ssp. *pekinensis*. Plant Mol Biol 42: 647-655

Zubieta C, Ross JR, Koscheski P, Yang Y, Pichersky E, Noel JP (2003) Structural basis for substrate recognition in the salicylic acid carboxyl methyltransferase family. Plant Cell 15: 1704-1716