JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Valine Pathway Is More Crucial than Phenyl Propanoid Pathway in Regulating Capsaicin Biosynthesis in *Capsicum frutescens* Mill.

BELLUR CHAYAPATHY NARASIMHA PRASAD, HARISHCHANDRA BHASKAR GURURAJ, VINOD KUMAR, PARVATAM GIRIDHAR, AND GOKARE ASHWATHNARAYANA RAVISHANKAR*

Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore 570020, India

Capsaicin, a pungency factor alkaloid of *Capsicum* fruits, is biosynthesized by enzymatic condensation of vanillylamine, a phenyl propanoid intermediate, and 8-methyl-nonenoic acid, a fatty acid derivative from the leucine/valine pathway by capsaicin synthase. Biotic elicitors, such as aqueous mycelial extracts of *Rhizopus oligosporus* and *Aspergillus niger*, and abiotic elicitors, such as salicylic acid and methyl jasmonate, were incorporated to cell-suspension cultures of *Capsicum frutescens.* The maximum elicitation of capsaicinoids to the extent of 6-folds was noticed when mycelial extracts of *R. oligosporus* were incorporated to cell-suspension cultures. The phenyl propanoid intermediates were elicited to the extent of 3−4-fold, whereas the levels of 8-methyl-nonanoic acid increased 6-fold in *R. oligosporus* mycelial-extract-treated cultures. The enzymatic assays of caffeic acid *O*-methyl transferases, keto acyl synthase, and capsaicin synthase were performed in elicited and nonelicited cell-suspension cultures. These enzymes were overexpressed in *R. oligosporus* mycelial-extract-treated suspension cultures, which was also confirmed by reverse transcriptase polymerase chain reaction studies. This is the first paper on the overexpression of 8-methyl-nonanoic acid leading to capsaicin biosynthesis using elicitors.

KEYWORDS: Capsaicin synthase; capsaicin; keto acyl synthase; 8-methyl-nonanoic acid; *Rhizopus oligosporus*; RT-PCR; elicitation

INTRODUCTION

Capsaicinoids are the major pungency imparting secondary metabolites in *Capsicum* spp. Capsaicin, a major alkaloid among capsaicinoids, produced only in *Capsicum* fruits, has wide applications in food, medicine, and pharmacy (*1*). Capsaicin synthase (CS) is reported to be responsible for the condensation reaction between vanillylamine, a phenyl propanoid pathway intermediate, and 8-methyl-nonenoic acid, a derivative of the valine pathway, leading to capsaicin production (**Figure 1**) (*2*). Ferulic acid, one of the important phenyl propanoid intermediates is biosynthesized by the enzymatic activity of caffeic acid *O*-methyl transferase (CoMT) (*3*, *4*), but there are no reports on enzymatic studies leading to the formation of vanillin from ferulic acid in the capsaicin biosynthetic pathway. Similarly, biochemical studies on enzymatic formation (amino transferase) of vanillylamine from vanillin (PAMT) have not been reported (*5*, *6*). The enzymatic synthesis of 8-methyl-nonanoic acid is catalyzed by keto acyl synthase (KAS) (*7*). Finally, the enzymatic condensation of vanillylamine and 8-methyl-nonenoic acid to yield capsaicin is presumed to be brought about by CS. Hence, CoMT, KAS, and CS appear to be the key enzymes in capsaicin biosynthesis.

Among the strategies employed to enhance the productivity of secondary metabolites, the outstanding one is the use of biotic and abiotic elicitors (*8*). Elicitors have been effective in inducing the synthesis of secondary metabolites in cell and organ cultures of different species such as berberine in *Thalictrum rugosum* (*9*), thiophene in *Tagetus patula* (*10*), indole alkaloids in *Catharanthus roseus* (*11*), and taxol as well as related taxanes in *Taxus* spp. (*12*). In our previous studies, we demonstrated the influence of 8-methyl nonenoic acid on the biosynthesis of capsaicin through substrate inhibitor studies, precursor biotransformation, enzymatic analysis, and reverse transcriptase polymerase chain reaction (RT-PCR) studies during the ontogeny of the fruit (*13*). The objectives of the present study were to investigate elicitor-mediated enhancement of capsaicinoids, in relation to the enzyme activities of CoMT, KAS, and CS in elicited and nonelicited cell-suspension cultures of *Capsicum frutescens*, and also to study mRNA transcript abundance of

* To whom correspondence should be addressed. Telephone: +91-0821-2516501. Fax: +91-0821-2517233. E-mail: pcbt@cftri.res.in.

10.1021/jf061040a CCC: $33.50
Published on Web 08/04/2006

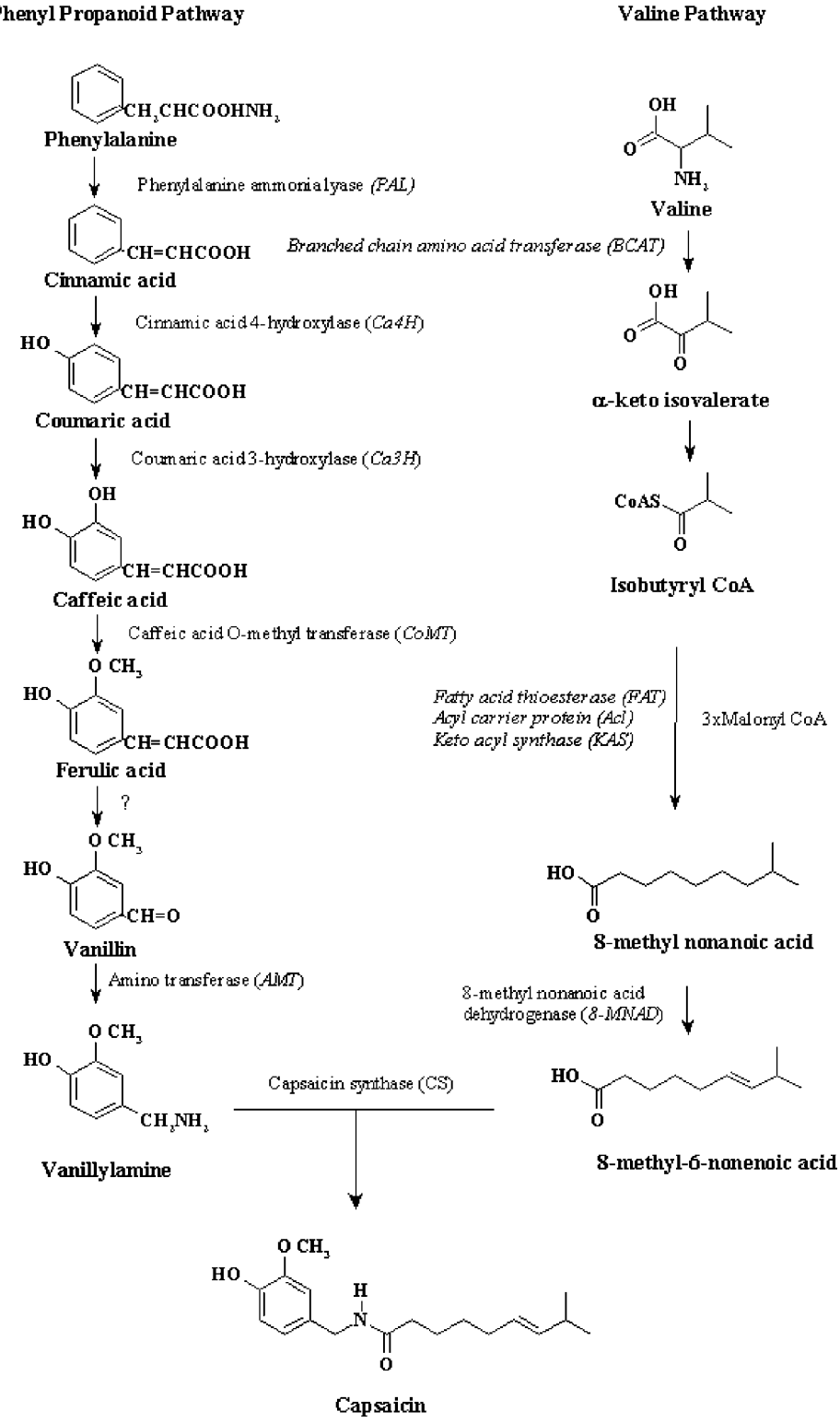

Figure 1. Pathway of capsaicin biosynthesis.

CoMT, KAS, and CS under the influence of different elicitors for utilization of this system to identify the regulatory step in capsaicin biosynthesis.

MATERIALS AND METHODS

Chemicals. Capsaicin, dihydrocapsaicin, phenylalanine, cinnamic acid, coumaric acid, ferulic acid, 8-methyl-nonanoic acid, boron trifluoride, ascorbic acid, β-mercaptoethanol, adenosine triphosphate (ATP), acetyl coenzyme A (CoA), oligo-dT (18-mer) primer, ethidium bromide, Tris, kinetin, and diethyl pyrocarbonate (DEPC) were purchased from Sigma Co., St. Louis, MO, whereas vanillylamine were purchased from Aldrich Co., St. Louis, MO. The RNA extraction kit, RNase Zap, M-MuLV reverse transcriptase, and DNase were purchased from Ambion, Austin, TX. *Taq* DNA polymerase was purchased from MBI Fermentas, Lithuania. All of the solvents used for high-performance liquid chromatography (HPLC) and gas chromatography (GC) were purchased from Merck, Germany. Salicylic acid and methyl jasmonate were purchased from Duchefa Pvt. Ltd., The Netherlands.

Callus and Cell-Suspension Cultures. *In vitro* seedlings of *C. frutescens* were raised in half-strength Murashige and Skoog (MS) medium (*14*). Callus cultures were raised in full-strength MS solid

medium supplemented with 2,4-dichlorophenoxy acetic acid (2 mg/L), kinetin (0.5 mg/L), and 3% sucrose. The cultures were regularly subcultured and transferred to 150 mL conical flasks containing 40 mL of MS liquid medium supplemented with 2,4-dichlorophenoxy acetic acid (2 mg/L), kinetin (0.5 mg/L), and 3% sucrose. Cultures were grown on a rotary shaker at 100 rpm with 12 h photoperiods at 25 ± 2 °C. The media pH was adjusted to 5.7 before autoclaving. Cells were harvested by filtration. Control and elicitor-treated cell-suspension cultures of *C. frutescens* were harvested and dried at 60 °C until they attained constant weight. Fresh and dry weights of cells were determined and recorded.

Elicitor Preparation and Treatment of Cell Cultures. For the preparation of fungal elicitors, *Rhizopus oligosporus* and *Aspergillus niger* (obtained from the Food Microbiology Department, CFTRI, Mysore, India) were grown on 250 mL of potato dextrose broth for 15 days at room temperature (25 ± 2 °C). Fully grown mycelia with spores were collected by filtration and were homogenized in a pestle and mortar using acid-washed neutralized sand. All of these operations were done under a fume hood. The homogenized suspension was centrifuged at 8000*g*, and the supernatant was used as elicitors after autoclaving for 20 min at 121 °C. The stock solution was prepared at a 10 g/mL concentration and applied to cell cultures at the concentrations of 0.5, 1, and 2% (w/v).

Stock solutions (1 mM) of abiotic elicitors, salicylic acid, and methyl jasmonate were prepared. They were administered to cell-suspension cultures at 1, 2.5, and 5 μM concentrations after filter sterilization. The cell-suspension cultures without the incorporation of biotic and abiotic elicitors were considered as controls. The suspension cells were harvested at 5, 10, and 15 days after elicitor application. Cells from each culture were separated from media, and the fresh and dry weights were recorded. The experiment was carried out with a minimum of three replications.

Quantification of Phenyl Propanoid Intermediates and Capsaicinoids in Cultured Cells. Dried cells (1 g) were homogenized in a mortar containing quartz sand with acetonitrile (1:10, w/v). The extract was centrifuged at 10000*g* at 4 °C for 15 min, and the pellet was discarded. The aliquots were evaporated to dryness *in vacuo*, and extracts were resuspended with 1.0 mL of HPLC-grade methanol. The samples were centrifuged at 6000*g* for 15 min before injecting to HPLC. Capsaicinoids and intermediates of the phenyl propanoid pathway were quantified by HPLC (*15*). The mobile phase consisted of a linear gradient of 0−100% acetonitrile in water with pH 3.0 (made with acetic acid) for 35 min then maintained at 100% for 2 min. Detection was at 236 nm, and the flow rate was maintained at 1 mL/min. A C-18 column of 250 × 4.6 mm i.d., 5 μm, was used. The reagents used were of HPLC grade. Samples and standards (5 and 10 μL) were injected in triplicate, and the mean area was calculated.

Quantification of 8-Methyl-nonanoic Acid. Portions (1 g) of dried fruits/suspension cells were homogenized in a mortar containing quartz sand with petroleum ether (1:20, w/v). The extracts were concentrated *in vacuo* at 40 °C to dryness. Oils were saponified with (0.5 N) NaOH, and free acids were converted to methyl esters with boron trifluoride and heated for 5 min at 65−70 °C. Saturated NaCl (0.5 mL) was added followed by 3 mL of hexane. The hexane fraction was used for GC and GC−mass spectroscopy (MS). A 30 m × 0.25 mm i.d., 0.25 μm film depth, DB-1 column (J & W Scientific Co. Folson, CA) was used in the study. The initial temperature of the capillary column (DB-1) was maintained at 80 °C for 5 min and increased up to 200 °C at 5 °C/min. The helium flow rate was 1 mL/min. Spiking was carried out for further corroboration.

Enzyme Assays: CoMT Assay. The activity of CoMT was assayed by the method of Shimada et al. (*16*). A total of 1 g each of fresh cells (from elicitor treated and control) was homogenized in 12 mL of 100 mM Tris-HCl at pH 7.5. The homogenate was centrifuged at 6000 rpm for 20 min at 4 °C. The supernatant was used as an enzyme extract. The reaction mixture contained 0.2 mL of 1 M Tris-HCl at pH 8.0, 10 mM caffeic acid, 40 mM $MgCl_2$, 5 mM *S*-adenosyl-L-methionine (SAM), and 1 mL of enzyme extract. The reaction mixture was incubated for 30 min at 30 °C and terminated by 0.1 N HCl. The mixture was extracted with 5 mL of diethyl ether followed by in vacuo evaporation of the solvent at 22 °C. The residue was dissolved in 0.5 mL of ethanol, and the samples were chromatographed on silica gel GF 254, developed with benzene and acetic acid as the solvent (4:1). Ferulic acid was detected in ultraviolet (UV) light; the fluorescent spot was recovered with 4 mL of methanol; and absorbance was measured at 343 nm in a spectrophotometer for quantification. The specific activity of CoMT was expressed in units of ferulic acid produced per milligram of protein per hour.

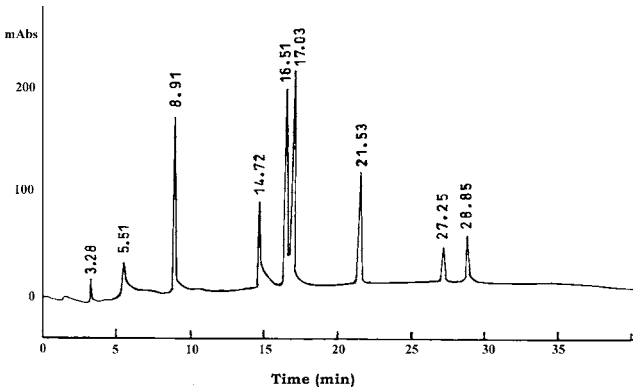

Figure 2. HPLC chromatogram depicting the intermediates of the phenyl propanoid pathway and capsaicinoids. Vanillylamine (3.28 min), phenylalanine (5.51 min), caffeic acid (14.72 min), coumaric acid (16.51 min), ferulic acid (17.03 min), cinnamic acid (21.53 min), capsaicin (27.26 min), and dihydrocapsaicin (28.85 min).

KAS Assay. The activity of KAS was measured according to Shimakata and Stumpf (*17*). The assay solution contained 0.1 M potassium phosphate buffer at pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 40 μM of malonyl-CoA, and 50 mg of protein extract. The reaction was started by the addition of malonyl-CoA, and the activity was calculated from the difference at 340 nm between the initial absorbance and that after 10 min. The activity of KAS was expressed in terms of units of 8-methyl-nonanoic acid produced per milligram of protein per hour.

CS Assay. The activity of CS was assayed according to Iwai et al. (*18*). A total of 1 g each of fresh callus (from elicitor treated and control) was homogenized in 10 mL of 0.1 M potassium phosphate buffer (pH 6.8), with 100 mg of ascorbic acid and 5 mM β-mercaptoethanol. The homogenate was centrifuged at 6000 rpm for 30 min at 4 °C. The supernatant was used as an enzyme extract. The reaction mixture contained 0.5 M potassium phosphate buffer at pH 6.8, 1 μM $MgCl_2$ and ATP, 5 μM each of vanillylamine, 8-methyl-nonanoic acid, and CoA, and 0.1 mL of enzyme extract. The reaction mixture was incubated for 2 h at 37 °C and terminated by 0.5 N HCl. The reaction mixture was taken into chloroform and later evaporated and resuspended in 100 μL of methanol. The capsaicin levels in the methanol fraction were determined by HPLC. The activity of CS was expressed in terms of units of capsaicin produced per milligram of protein per hour.

Protein Estimation. Protein extracted from cell-suspension cultures of *C. frutescens* were estimated according to the Bradford method (*19*).

Expression Analysis of CoMT, KAS, and CS by RT-PCR. To study mRNA transcripts of CoMT, KAS, and CS genes, total RNA was extracted using the total RNA extraction kit. To avoid possible RNase contamination, all plasticware was treated with 0.1% diethyl pyrocarbonate and the working area, electrophoresis tank, and other required materials were treated with RNase Zap. RNA was extracted from control and elicitor-treated *Capsicum* cell-suspension cultures. The quality and concentration of RNA were checked on denaturing agarose gel and by absorbance measurements at 230, 260, and 280 nm in a UV spectrophotometer. All of the RNA samples were subjected to DNase treatment to avoid possible artifact amplifications from contaminant genomic DNA.

The CoMT, KAS, and CS gene-specific primers were designed using Primer3 software (*20*). A control PCR was run on extracted RNA samples to check for the absence of genomic DNA. First-strand cDNAs were synthesized from 2 μg of total RNA in 20 μL of final volume, using M-MuLV reverse transcriptase and oligo-dT (18-mer) primer following the instructions of the manufacturer. The RT-PCR reaction

Table 1. Effect of Different Elicitors on the Enhancement of Phenyl Propanoid Pathway Intermediates, Capsaicinoids, and 8-Methyl-nonanoic Acid in in Vitro Cell-Suspension Cultures of *C. frutescens*[a]

		secondary metabolites (nM/g)				
elicitors	concentrations	ferulic acid	vanillylamine	capsaicin	dihydrocapsaicin	8-methyl-nonanoic acid
control		25.0 [a]	27.6 [a]	12.7 [a]	3.8 [a]	4.2 [a]
aqueous mycelial	0.5	39.3 [c]	49.6 [c]	37.0 [b]	5.4 [c]	6.4 [a]
extracts of *R. oligosporus*	1.0	57.0 [c]	77.0 [c]	77.0 [c]	7.6 [c]	26.0 [c]
(% w/v)	2.0	45.0 [c]	56.6 [c]	54.6 [c]	6.5 [c]	7.5 [b]
aqueous mycelial	0.5	31.6 [a]	39.6 [a]	25.6 [a]	4.1 [a]	5.26 [a]
extracts of *A. niger*	1.0	44.6 [c]	48.6 [c]	52.3 [c]	5.2 [c]	13.2 [c]
(% w/v)	2.0	40.0 [c]	44.6 [c]	48.3 [c]	4.8 [b]	6.4 [a]
methyl jasmonate	1.0	28.0 [a]	35.0 [a]	20.3 [a]	4.0 [a]	4.4 [a]
(μM)	2.5	34.6 [b]	40.6 [b]	36.3 [b]	4.4 [a]	6.2 [a]
	5.0	29.3 [a]	35.0 [a]	36.0 [b]	4.1 [a]	5.4 [a]
salicylic acid	1.0	25.6 [a]	29.0 [a]	16.0 [a]	4.0 [a]	4.5 [a]
(μM)	2.5	28.0 [a]	33.6 [a]	29.0 [a]	4.2 [a]	4.9 [a]
	5.0	26.3 [a]	30.0 [a]	27.3 [a]	3.9 [a]	4.4 [a]
SE		1.277	1.884	1.829	0.131	0.446[b]

[a] The data are significant at $p > 0.05$. [b] The data are significant at $p > 0.01$. [c] The data are nonsignificant.

was stopped in the early exponential phase (20th cycle) to maintain initial differences in target transcript quantities (exponential phase of amplification). PCR reactions were subjected to 28 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s with *Taq* DNA polymerase. The primers for KAS were forward 5′-GCTTGTGCAACTGCCAATAT-3′ and reverse 5′-GGATGAAGCCATCCTGTTGT-3′; the primers for CoMT were forward 5′−TTCAACCGTGGAATGTCTGA-3′ and reverse 5′-CGAAGGCCAACAATTGAACT-3′; and the primers for CS were forward 5′-TCTAGGAAATGGTCCCTCCA-3′ and reverse 5′-TTGACCGTAAACTTCCGTTG-3′. An aliquot of 10 μL from each PCR reaction was fractionated on a 1.5% (w/v) agarose gel in Tris-acetate EDTA buffer. Ethidium bromide (0.5 μg/L) stained gels were photographed with a Digital Imaging System (HeroLab, Germany). The intensity of the DNA bands was estimated by intensity histogram. α-Tubulin, used as an internal constitutive control, was amplified using primers 5′-CTGTCAACGACCCCTTCATC-3′ and 5′-CCTGTTGTCGC-CAACGAAGTC-3′. The transcript abundance of CoMT, KAS, and CS were quantified using the intensity histogram.

Statistical Analysis. All of the statistical analyses were done using the GENRES program and Tukey's procedure (*21*).

RESULTS

Separation of Phenyl Propanoid Intermediates, 8-Methyl-nonanoic Acid, and Capsaicinoids in Cell-Suspension Cultures of *C. frutescens*. HPLC conditions gave very good separation of phenyl propanoid intermediates from capsaicinoids **(Figure 2)** (*15*), and GC conditions assisted in the estimation of 8-methyl-nonanoic acid. The 8-methyl-nonanoic acid was detected at 9.39 min. This was confirmed by GC−MS also.

Effect of Elicitors on Phenyl Propanoid Intermediates, 8-Methyl-nonanoic Acid, and Capsaicinoids in Cell-Suspension Cultures of *C .frutescens*. There was a significant difference among the different elicitors on the enhancement of secondary metabolites **(Table 1)**. Biotic elicitors, aqueous mycelial extracts of *R. oligosporus* and *A. niger*, significantly enhanced ferulic acid, vanillylamine, capsaicinoids, and 8-methyl-nonanoic acid production as compared to abiotic elicitors. The maximum enhancement of ferulic acid (57 nM/g) was observed in aqueous mycelial extracts of *R. oligosporus* (1%

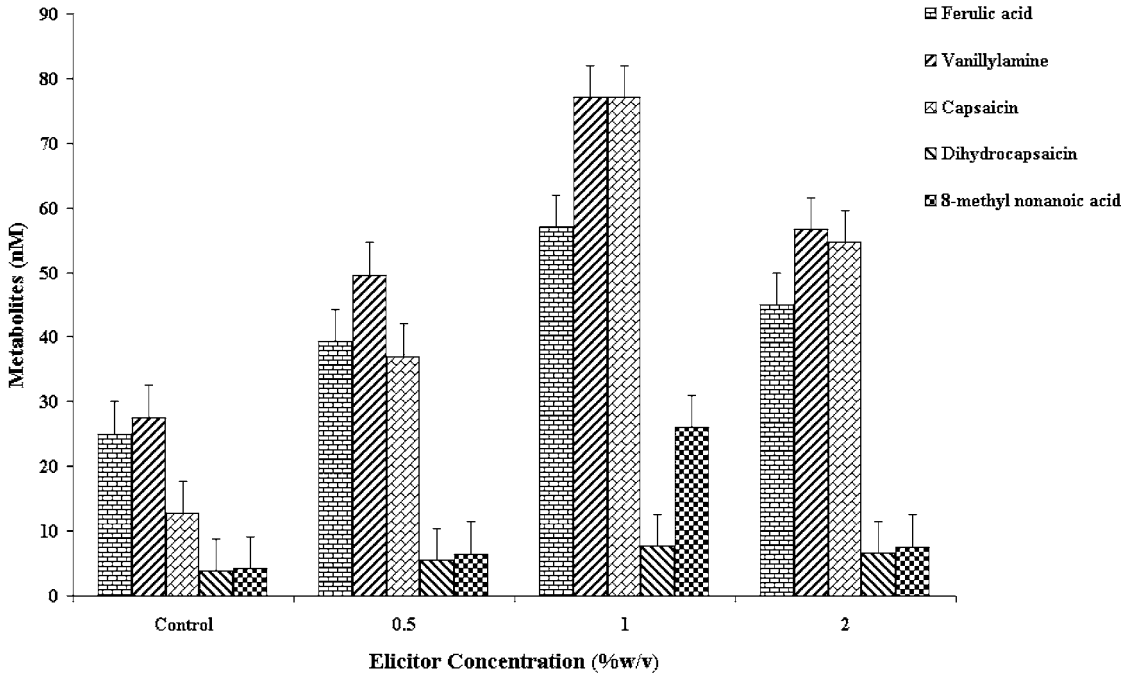

Figure 3. Elicitation of phenyl propanoid pathway intermediates, 8-methyl-nonanoic acid, and capsaicinoids in 15-day-old cell-suspension cultures of *C. frutescens* by *R. oligosporus*.

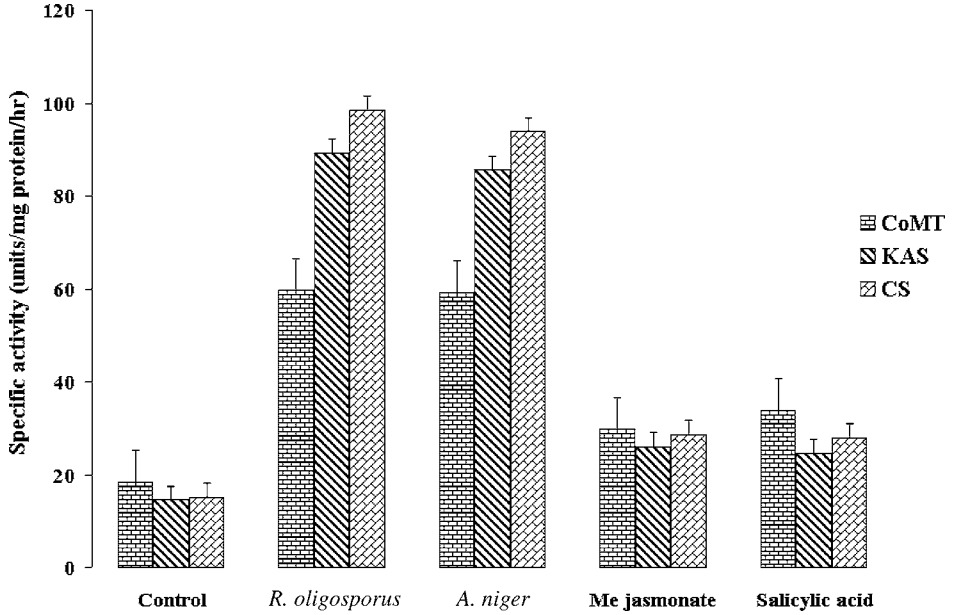

Figure 4. Activity of CoMT, KAS, and CS in elicited cell-suspension cultures of *C. frutescens*.

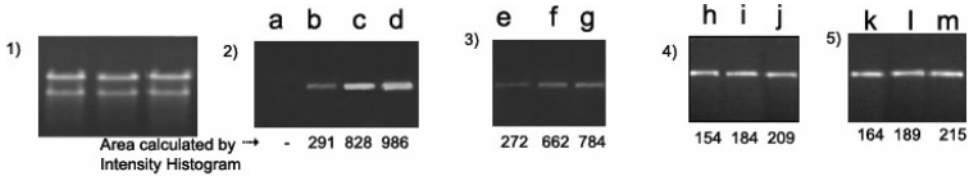

Figure 5. Expression of CoMT, KAS, and CS in fruits of *C. frutescens* under the influence of different elicitors by RT-PCR. (1) Total RNA. (2) mRNA transcript levels as influenced by the optimum concentration of *R. oligosporus*: (a) control, (b) transcript levels of CoMT, (c) transcript levels of KAS, and (d) transcript levels of CS. (3) mRNA transcript levels as influenced by the optimum concentration of *A. niger*. (e) transcript levels of CoMT, (f) transcript levels of KAS, and (g) transcript levels of CS. (4) mRNA transcript levels as influenced by the optimum concentration of methyl jasmonate: (h) transcript levels of CoMT, (i) transcript levels of KAS, and (j) transcript levels of CS. (5) mRNA transcript levels as influenced by the optimum concentration of salicylic acid: (k) transcript levels of CoMT, (l) transcript levels of KAS, and (m) transcript levels of CS.

w/v) treated cultures. Similarly, levels of vanillylamine were also enhanced to 77 nM/g by *R. oligosporus* (1% w/v), and the levels of capsaicin and dihydrocapsaicin increased to 77 and 7.6 nM/g, respectively. Moreover, 8-methyl-nonanoic acid also increased to 26 nM/g (**Figure 3**).

In terms of enhancement of secondary metabolites, capsaicin was enhanced by 6.15-fold followed by 8-methyl-nonanoic acid (6.08 fold) and vanillylamine (2.7-fold) in *R. oligosporus* treated cultures. Interestingly, the enhancement of capsaicin was positively correlated with the levels of 8-methyl-nonanoic acid.

Overexpression of CoMT, KAS, and CS in *R. oligosporus* Treated Cell-Suspension Cultures. There was linearity in the enzyme assays of CoMT, KAS, and CS. When enzymatic studies were done in elicited and nonelicited conditions, CoMT, which is responsible for ferulic acid biosynthesis, utilizing caffeic acid as a substrate, was overexpressed to the extent of 3-fold. KAS, responsible for the synthesis of 8-methyl-nonanoic acid from malonyl-CoA as a substrate, was overexpressed to the extent of 6-fold. Similarly, CS, the key regulatory enzyme, was overexpressed by 6-fold, corresponding to enhanced levels of capsaicin (6.15-fold) (**Figure 4**).

Transcript Analysis of CoMT, KAS, and CS under the Influence of Elicitors. When the elicitor-mediated regulation of the capsaicin biosynthetic pathway was further corroborated at the molecular level, RT-PCR was performed on cell-suspension cultures of *C. frutescens* treated with optimum elicitor concentrations. There was a significant difference with respect to the banding pattern for CoMT, KAS, and CS transcript levels under the influence of *R. oligosporus* and *A. niger* (each at 1% w/v). However, transcripts under abiotic elicitors, methyl jasmonate, and salicylic acid did not vary with respect to the intensity of the bands.

DISCUSSION

In the presence of a pathogen, plants develop a vast array of metabolic defense responses sequentially activated in a complex multicomponent network that may be local and/or systemic (*22*). Defense responses to pathogen infection include the production of several secondary metabolites (*23*, *24*). In our previous studies (*13*), we had demonstrated the importance of 8-methyl-nonenoic acid using biotransformation and inhibitors as potential tools to study the regulation of capsaicin biosynthesis. In the present study, we have used biotic and abiotic elicitors to study the regulation of capsaicin biosynthetic pathway and their influence on key enzymes involved in the capsaicin biosynthetic pathway.

In cell-suspension cultures of *C. frutescens*, ferulic acid, vanillylamine, capsaicin, and dihydrocapsaicin were the secondary metabolites detected by HPLC. Interestingly, vanillin was not detected in the cell-suspension cultures, which may be due to the fast metabolic conversion of vanillin to vanillylamine. Because vanillin is very reactive, it exhibits a toxic effect to most microorganisms (*25*, *26*). In most cases, vanillin is converted to its derivatives, such as vanillic acid and protocat-

echuic acid, in microbial systems (*27*, *28*), whereas the β-D-glucoside moiety is attached to vanillin in *Vanilla planifolia* (*29*). In *Capsicum* spp., vanillin is converted to vanillylamine catalyzed by aminotransferase (*3*, *5*). Hence, in the present study, it is proven that the production of vanillylamine is the reason why vanillin is not detected. Moreover, to the best of our knowledge, vanillylamine is one of the secondary metabolites whose biosynthesis is restricted to *Capsicum* spp. This could be one of the reasons for the uniqueness of capsaicin production in *Capsicum* spp. (*30*, *31*).

Interestingly, the enhancement of capsaicin by *R. oligosporus* was positively correlated with the levels of 8-methyl-nonanoic acid (**Figure 3**), the precursor of 8-methyl-nonenoic acid, which undergoes condensation with vanillylamine to form capsaicin by CS. In the present study, concomitant overexpression of KAS (involved in KAS biosynthesis) and CS (involved in capsaicin biosynthesis) by *R. oligosporus* (**Figure 4**) and overexpression of its mRNA transcripts gene (**Figure 5**) have been shown.

Methyl jasmonate and salicylic acid have been shown to induce the accumulation of a wide range of plant secondary metabolites, including isoprenoids (*32*), alkaloids (*33*), and also phenyl propanoid intermediates, which are common in plant systems (*34*), but they may not induce 8-methyl-nonanoic acid, which is a key regulatory step in capsaicin biosynthesis (*13*). On the other hand, biotic elicitors, which could induce 8-methyl-nonanoic acid, would effectively enhance capsaicinoid levels. On the basis of the enhancement of capsaicin biosynthesis intermediates by *R. oligosporus* and, concomitantly, with the overexpression of corresponding enzymes and on the basis of transcript studies, it can be concluded that the 8-methyl-nonanoic acid is a very crucial step. It may be noted that the elevated levels of 8-methyl-nonanoic acid were almost equal to number of fold enhancements of capsaicin. The enzyme activity of CoMT was not equivalent to the extent of enhanced levels of capsaicin biosynthesis, but the activity of KAS positively correlated with the activity of CS and capsaicin production. This study provides evidence that capsaicin biosynthesis depends upon the pools of 8-methyl-nonanoic acid. Hence, from earlier studies and the present study, we conclude that the valine pathway is more crucial than the phenyl propanoid pathway in regulating capsaicin biosynthesis in *Capsicum* spp. This study has a practical implication for the enhancement of capsaicinoids under field conditions (*35*), which is of agricultural importance.

LITERATURE CITED

(1) Prasad, N. B. C.; Shrivastava, R.; Ravishankar, G. A. Capsaicin as multifaceted drug from *Capsicum* spp. *Evidence-Based Int. Med.* **2005**, *2*, 147−166.

(2) Bennet, D. J.; Kirby, G. W. Constitution and biosynthesis of capsaicin. *J. Chem. Soc. C* **1968**, 442.

(3) Sukrasno, N.; Yeoman, M. M. Phenyl propanoid pathway metabolism during growth and development of *Capsicum frutescens* fruits. *Phytochemistry* **1993**, *32*, 839−844.

(4) Ochoa-Alejo, N.; Gomez-Peralta, E. J. Activity of enzymes of involved in capsaicin biosynthesis in callus tissue and fruits of Chilli pepper (*Capsicum annuum* L.). *J. Plant. Physiol.* **1993**, *141*, 147−152.

(5) Curry, J.; Aluru, M. R.; Mendoza, M.; Nevarez, J.; Melenderz, M.; O'Connell, M. A. Transcripts for possible capsaicinoid biosynthesis genes are differentially accumulated in pungent and non-pungent *Capsicum* spp. *Plant Sci.* **1999**, *148*, 47−57.

(6) Kim, M.; Kim, S.; Kim, S.; Kim, B. D. Isolation of cDNA clones differentially accumulated in pungent peppers by suppression subtractive hybridization. *Mol. Cells* **2001**, *11*, 213−219.

(7) Aluru, M. R.; Mazourek, M.; Landry, L. G.; Curry, J.; Jahn, M.; O'Connell, M. A. Differential expression of fatty acid synthesis genes, *Acl*, *Fat* and *Kas* in *Capsicum* fruit. *J. Exp. Bot.* **2003**, *54*, 1655−1664.

(8) Pitta-Alverez, S. I.; Spollanksy, T. C.; Giuletti, A. M. The influence of abiotic and biotic elicitors on the production of and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. *Enzyme Microb. Technol.* **2000**, *26*, 252−258.

(9) Brodelis, P.; Collinge, M. A.; Funk; G.; Marques, I. Studies on alkaloid formation in plant cell cultures after treatment with yeast elicitor. In *Primary and Secondary Metabolism of Plant Cell Cultures*; Kurz, W. G. W., Ed.; Springer-Verlag: Berlin, Germany, 1989; pp 190−199.

(10) Buitelar, R. M.; Cesario, M. T.; Tamper, J. Elicitation of thiopene production by hairy root cultures of *Tagetus patula*. *Enzyme Microb. Technol.* **1992**, *14*, 2−7.

(11) Vazquenz-Flota, F.; Moreno-Valenzuela, O.; Miranda-Ham, M. L.; Coello-Coello, J.; Loyala-Vargas, V. M. Catharanthine and ajmalicine synthesis in *Catharanthus roseus* hairy root cultures, Medium optimization and elicitation. *Plant Cell, Tissue Organ Cult.* **1994**, *38*, 273−279.

(12) Ciddi, V.; Srinivasan, V.; Shuler, M. L. Elicitation of *Taxus* sp. cell for production of taxol. *Biotechnol. Lett.* **1995**, *17*, 1343−1346.

(13) Prasad, N. B. C.; Gururaj, H. B.; Kumar, V.; Giridhar, P; Parimalan, R.; Sharma, A.; Ravishankar, G. A. The influence of 8-methyl-nonenoic acid on capsaicin biosynthesis in *in vivo* and *in vitro* cell cultures of *Capsicum* spp. *J. Agric. Food Chem.* **2006**, *54*, 1854−1859.

(14) Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **1962**, *15*, 473−497.

(15) Johnson, T. S.; Ravishankar, G. A.; Venkataraman, L. Separation of capsaicin from phenyl propanoid compounds by high performance liquid chromatography to determine the biosynthetic status of cells and tissues of *Capsicum frutescens* Mill. in *in vivo* and *in vitro*. *J. Agric. Food Chem.* **1992**, *40*, 2461−2463.

(16) Shimada, M.; Ohasi, H.; Higuchi, T. *O*-Methyltranseferases involved in the biosynthesis of lignins. *Phytochemistry* **1970**, *9*, 2463−2470.

(17) Shimakata, T.; Stumf, P. K. Purification and characterization of keto acyl ACP synthatases from *Spinacia oleracea* leaves. *Arch. Biochem. Biophys.* **1983**, *220*, 39−45.

(18) Iwai, K.; Suzuki, T.; Lee, K.; Kobashi, M.; Oka, S. *In vivo* and *in vitro* formation of dihydrocapsaicin in sweet pepper fruits, *Capsicum annuum* L. var. *grossum*. *Agric. Biol. Chem.* **1977**, *41*, 1877−1882.

(19) Bradford, M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(20) Rozen, S.; Skaletsky, H. J. Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols, Methods in Molecular Biology*; Krawetz, S. M. S., Ed.; Humana Press: Totowa, NJ, 2000; pp 365−386.

(21) Tukey, J. W. Some selected quick and easy methods of statistical analysis. *Trans. N.Y. Acad. Sci., Ser. II* **1953**, *16*, 88−97.

(22) Hahlbrock, K.; Bednarek, P.; Ciolkowski, I.; Hamberger, B.; Heise, A.; Liedgens, H.; Logemann, E.; Nürnberger, T.; Somssich, I. E.; Tan, J. Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *25* (supplement 2), 14569−14576.

(23) Dixon, R. A. Natural products and plant disease resistance. *Nature* **2001**, *411*, 843−847.

(24) Tan, J.; Schneider, B.; Svatos, A.; Bednarek, P.; Liu, J.; Hahlbrock, K. Universally occurring phenyl propanoid and species-specific indolic metabolites in infected and uninfected *Arabidopsis thaliana* roots and leaves. *Phytochemistry* **2004**, *65*, 691−699.

(25) Matamoros, L.; Argaiz, A.; Lopez, M. A. Individual and combined effects of vanillin and potassium sorbate on *Penicillium digitatum* and *P. glabrum* growth. *J. Food Prot.* **1999**, *62*, 540−542.
(26) Zaldivar, J.; Martinez, A.; Ingram, L. O. Effect of selected aldehydes on the growth and fermentation of ethanologenic *E. coli*. *Biotechnol. Bioeng.* **1999**, *65*, 24−33.
(27) Overhage, J.; Kresse, A. U.; Priefert, H.; Sommer, H.; Krammer, G.; Rabenhorst, J.; Steinbuchel, A. Molecular characterization of the genes pcaG and pcaH encoding protocatechuate 3,4-dioxygenase which are essential for vanillin catabolism in *Pseudomonas* spp. strain HR199. *Appl. Environ. Microbiol.* **1999**, *65*, 951−960.
(28) Priefert, H.; Rabenhorst, J.; Steinbuchel, A. Biotechnological production of vanillin. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 296−314.
(29) Valton, N. J.; Mayer, M. J.; Narbad, A. Molecules of interest—Vanillin. *Phytochemistry* **2003**, *63*, 505−515.
(30) Walsh, B. M.; Hoot, S. B. Phylogenetic relationships of *Capsicum* (Solanaceae) using DNA sequences from two noncoding regions: The chloroplast atpB−rbcL spacer region and nuclear waxy introns. *Int. J. Plant Sci.* **2001**, *162*, 1409−1418.
(31) Andrews, J. *Peppers: The Domesticated Capsicums*; University of Texas Press: Austin, TX, 1984.
(32) Choi, D.; Bostock, R. M.; Avdiushko, S.; Hildebrand, D. F. Lipid derived signals that discriminate wound and pathogen responsive isoprenoid pathway in plants. Methyl jasmonate and fungal elicitor arachidonic acid induce different 3-hydroxy-3-methyl glutaryl CoA reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *41*, 2329−2333.
(33) Menke, F. L. H.; Parchmann, S.; Mueller, M. J.; Kijne, J. W.; Memelink, J. Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor induced expression of terpenoid indole alkaloid biosynthesis genes in *Catharanthus roseus*. *Plant Physiol.* **1999**, *119*, 1289−1296.
(34) Ellard-Ivey, M.; Douglas, C. J. Role of jasmonates in the elicitor and wound inducible expression of defense genes in parsley and transgenic tobacco. *Plant Physiol.* **1996**, *112*, 183−192.
(35) Prasad, N. B. C.; Giridhar, P.; Kumar, V.; Ravishankar, G. A. A spray formulation for pungency enhancement in *Capsicum* fruits. Patent Pending U.S. application number 10/955786, PCT application number PCT/IB04/03197, 2003.

Received for review April 13, 2006. Revised manuscript received June 21, 2006. Accepted June 22, 2006. This work was supported by a research grant from the Department of Biotechnology, Government of India. B.C.N.P., V.K., and H.B.G. are thankful to CSIR for research fellowships. The institutional support of Dr. V. Prakash, Director, CFTRI, is gratefully acknowledged.

JF061040A