Pak. J. Bot., 50(3): 1013-1022, 2018.

A COMPREHENSIVE VIEW OF EXPRESSION PROFILES DYNAMICS OF CAPSAICINOID BIOSYNTHESIS-RELATED GENES DURING PEPPER FRUIT DEVELOPMENT AND UNDER MEJA TREATMENT

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Abstract

Capsaicinoids are a group of secondary plant metabolites which are synthesized and accumulated only in the fruits of peppers (*Capsicum annuum* L.). In this paper, the fruits of nadao chili peppers were used as experiment materials and the mechanism of capsaicinoid biosynthesis was studied. HPLC studies revealed that capsaicinoid accumulation in the developing fruits initially occurred at 24 days after pollination (DAP), was increasing at 36 DAP, and peaked at 48 DAP. Eleven genes that encoded enzymes involved in capsaicinoid biosynthesis were isolated and characterized. Gene expression with quantitative reverse-transcription polymerase chain reaction analysis demonstrated that capsaicin synthase (*CaCS*) was expressed only in the placenta of the fruit, while the other ten genes were expressed in all tissues tested, with nine of the eleven genes (with the exception of cinnamic acid-4-hydroxylase [*CaCa4H*] and *p*-coumaric acid-3-hydroxylase [*CaCa3H*]) being strongly expressed in placenta tissue. Spatial expression analysis demonstrated that the 11 genes could be grouped into four categories, based on the patterns of relative expression of the genes during fruit development. Category I contained two genes, which displayed a bell-shaped expression pattern, with peak expression at 24 DAP. Category II contained five genes, the expression of which increased steadily from 0 to 36 DAP, peaking at 36 DAP. Category III comprises two genes, expression of which peaked at 48 DAP. Category IV consists of two genes, which were not expressed from 0 to 12 DAP, but then showed a high level of expression at 36 and 48 DAP. Treatment of the developing fruit with methyl jasmonate (MeJA) resulted in upregulation of the expression of each of the 11 genes. These results provide the first information on capsaicinoid biosynthesis and regulation during pepper fruit development.

Key words: *Capsicum annuum* L., Capsaicinoid synthesis, Gene expression, Fruit development, MeJA.

Introduction

Chili peppers (*Capsicum annuum* L.) are a major vegetable crop worldwide (Zou, 2002). The high demand for chili pepper fruits is due to their pungent taste, which is caused by a group of alkaloid compounds known as capsaicinoids (Bosland *et al*., 2012). These compounds are exclusively synthesized and accumulated in the fruit of the pepper (Kehie *et al*., 2015). Capsaicinoids have been widely investigated and explored for their use in the food, medical, and pharmaceutical industries (Cuevas-Glory *et al*., 2015; Ludy *et al*., 2012; Luo *et al*., 2011; Korkutata & Kavaz, 2015; Lau *et al*., 2014; McCormack, 2010).

There are more than ten natural capsaicinoids (Blum *et al*., 2013). Among them, capsaicin and dihydrocapsaicin are the two predominant ones, making up 90% of the total capsaicinoid content in pepper fruits (Luo *et al*., 2011). The biosynthesis of these metabolites occurs in the epidermal cells of the fruit placenta, and they accumulate within blisters located on the surface of the placenta. The accumulation of capsaicinoids in chili pepper fruits starts at approximately 20 DAP, with the maximum level reached at 40–50 DAP (Barbero *et al*., 2014; Iwai *et al*., 1979; Salgado-Garciglia & Ochoa-Alejo, 1990).

The capsaicinoid biosynthetic pathway is outlined in Fig. 1 (Curry *et al*., 1999; Islam *et al*., 2015). The pathway is complex and uses intermediates from two other independent pathways as precursors (Aza-González *et al*., 2011; Keyhaninejad *et al*., 2014; Kim *et al*., 2014; Liu *et al*., 2013; Mazourek *et al*., 2009). One is the phenylpropanoid pathway, in which the intermediate L-phenylalanine is used as the precursor to produce cinnamic acid, *p*-coumaric acid, caffeoyl-shikimate and feruloyl-CoA and subsequently vanillin and vanillylamine, by the action of the enzymes CaPAL, CaCa4H, CaCa3H, CaCOMT and CapAMT in a series of reactions (Kobata *et al*., 2013; Phimchan *et al*., 2014). The second pathway is the branched-chain fatty acid pathway, in which either leucine or valine is the precursor for the synthesis of α-isovaletate, 8-methylnonenoic acid and 8-methyl-6-nonenoyl-CoA, catalyzed by the enzymes CaBCAT, CaKAS, CaACL, CaFAT and CaACYase (Aluru *et al*., 2003). Finally, capsaicin is synthesized by condensation of vanillylamine and 8-methyl-6-nonenoyl-CoA with the catalytic action of the capsaicin synthase (CaCS) enzyme (Han *et al*., 2013).

The enzymes in the phenylpropanoid pathway were established by several authors (Phimchan *et al*., 2014). Curry *et al*., (1999) isolated cDNAs belonging to *PAL*, *C4H*, *pAMT* and *Comt* genes from a *Capsicum chinense* cv. Habanero cDNA library and reported that the transcripts of *PAL*, *Ca4H*, *pAMT* and *Comt* genes accumulated in mature fruit placentas of Habanero chili peppers. Stewart *et al*., (2005) and Mazourek *et al*., (2009) identified some of the enzymes involved in capsaicinoid biosynthesis based on different experimental sources in the phenylpropanoid pathway.

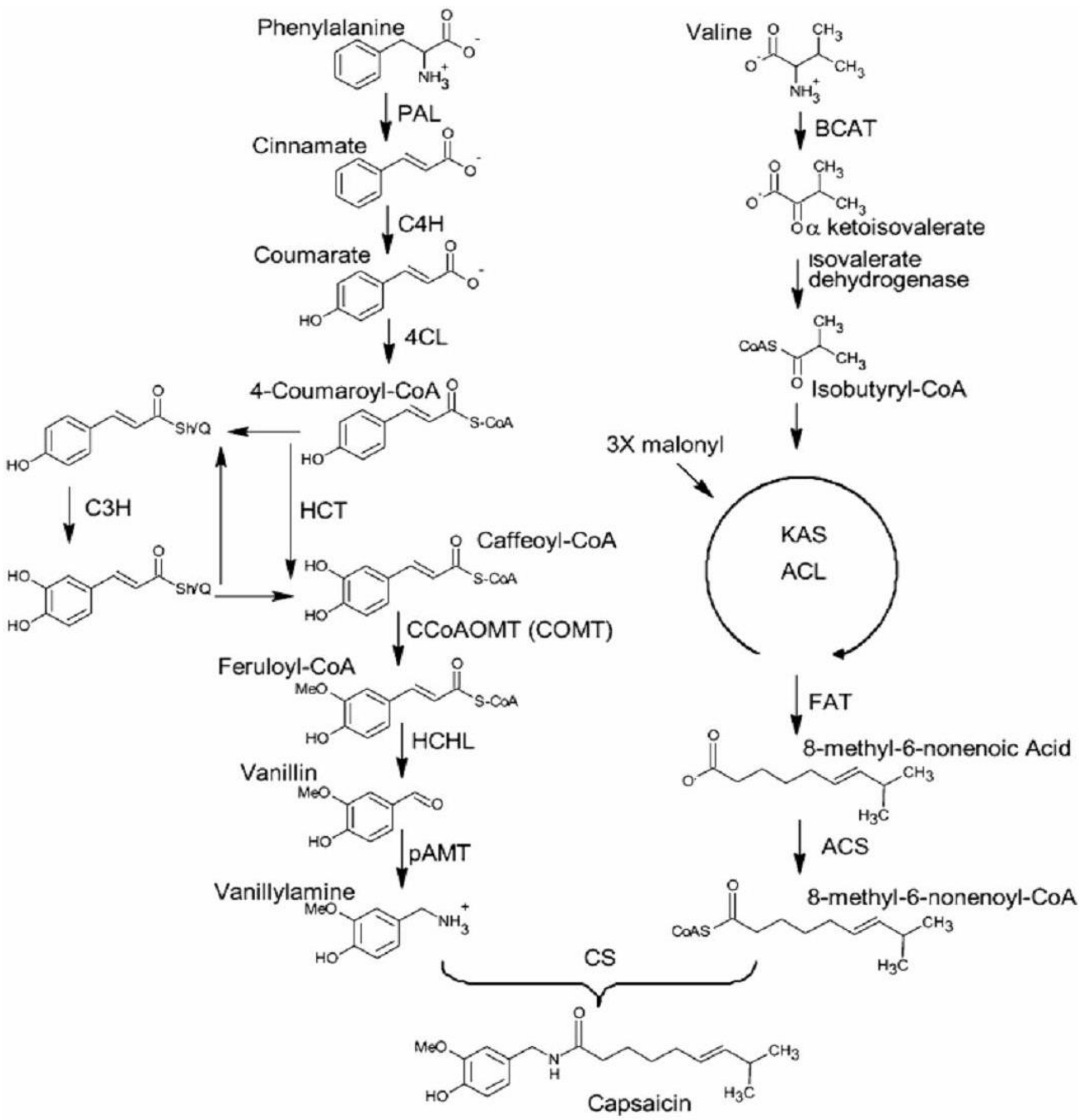

Fig. 1. Capsaicinoid biosynthetic pathway.

The activities of enzymes from the branched-chain fatty acid pathway in *Capsicum* fruits have been quantified and the corresponding genes identified (Aluru *et al*., 2003; Weber *et al*., 2014). Differential expression analysis of fatty acid synthesis (FAS) genes *Acl1*, *FatA* and *Kas* in *Capsicum* fruits was investigated and a positive relationship between the degree of pungency and transcript levels of these genes in placental tissue was established (Aluru *et al*., 2003).

Employing virus-induced gene silencing, del Rosario Abraham-Juáre *et al*., (2008) observed that the virus-infected plants with undetectable levels of transcripts had correspondingly undetectable capsaicinoid levels. In this study, the role of genes *Comt*, *pAmt* and *Kas* in capsaicinoid biosynthesis was confirmed.

The key regulatory enzyme of capsaicinoid biosynthetic pathway is capsaicin synthase (CS) (Ogawa *et al*., 2015; Phimchan *et al*., 2014; Stewart *et al*., 2007). CS catalyzes the final condensation of vanillylamine and 8-methyl-6-nonenoyl-CoA from the branched-chain fatty acid pathway (Wyatt *et al*., 2012). Recent studies identified the *Pun1* locus corresponding to the presence of capsaicinoid in chili pepper fruits, while a novel gene *AT3* which co-localized with this locus was isolated and characterized (Arce-González & Ochoa-Alejo, 2015). In addition, Prasad *et al*., (2006, 2013) reported a 35-kDa CS able to catalyze the *in vitro* synthesis of capsaicin from vanillylamine and 8- methylnonenoic acid. Expression of the candidate gene *Pun1* in *C. annuum* L. has been positively correlated with the accumulation of capsaicinoids (Reddy *et al*., 2014).

In recent years, numerous studies have demonstrated that both biotic and abiotic elicitors such as Ag^{+}, Co^{2+}, β-aminobutyric acid (BABA), MeJA, and salicylic acid (SA) can stimulate biosynthesis of bioactive secondary metabolites in plants (Altuzar-Molina *et al*., 2011; Ancona-Escalante *et al*., 2013; Gutierrez-Carabajal *et al*., 2010; Kehie *et al*., 2014, 2016; Prasad *et al*., 2006). Several studies have confirmed that methyl jasmonate (MeJA) can upregulate the activities of CaPAL and CaCOMT and enhance capsaicin production in cell suspension cultures of *Capsicum* (Prasad *et al*., 2006; Gutiérrez-Carbajal *et al*., 2010).

In order to achieve a better understanding of the biosynthetic pathway of capsaicinoids, we described the sequence homology, spatial and temporal expression, and post-MeJA expression changes of selected genes from this pathway. The results could provide a more comprehensive understanding of the role of the individual genes of the capsaicinoid biosynthetic pathway.

Materials and Methods

Plant material: Nadao pepper (*C. annuum* var. nadao), a local very hot pepper variety in Yunnan Province, was grown in experimental fields of Yunnan Agricultural University. In summer, different tissues (roots, stems, leaves, flowers, pericarps, placentas and seeds) were obtained for analysis. The developing fruits were harvested at different interval time after pollination (0, 12, 24, 36, 48, 60DAP). The fruit samples were carefully separated into pericarp and placenta tissue. Placentas were cut into small pieces which were instantly frozen in liquid nitrogen and stored at −80°C until further processing.

Separation and quantification of capsaicinoid: The isolation and quanlification of capsaicinoid were performed according to Deng *et al*., (2009). Peak areas of capsaicin and dihydrocapsaicin were converted to ppm as described by Collins *et al*., (1995).

MeJA treatment: MeJA, the key signaling molecule, modulate various physiological events during plant growth and development (Avanci *et al*., 2010). Fruit of 36 DAP were treated with 150 μM MeJA by continually spraying for 5 min in the greenhouse. The fruits were collected at 0, 2, 4, 8, 12, 18, 24 and 36 h after treatment. As a control, fruits were sampled immediately before treatment.

RNA isolation and First-strand cDNA synthesis: The total RNA was isolated from treated and untreated experiments according to (Deng *et al*., 2012). The first-strand cDNA was also synthesis as per instruction and phylogenetic analysis.

cDNA cloning: The RT-PCR products were used as template for cloning of the full lenghs of *CaPAL*, *CaACL*, *CaACYase*, *CaBCAT*, *CaCa3H*, *CaCa4H*, *CaCOMT*, *CaFAT*, *CaKAS*, *CapAMT* and *CaCS* genes. The primers design, PCR amplification reaction, the PCR products detection and sequencing of products were performed as described earlier (Deng *et al*., 2012). All primers used in this research are listed in Table 1. The phylogenetic analysis was also carried out using same strategies as described earlier (Deng *et al*., 2012).

Table 1. Primer pairs for RT-PCR and qRT-PCR.

Gene		Fwd 5′--3′	Rev 5′--3′
CaPAL	RT-PCR	TCATGGCATCAACAATTGCAC	CCGCCTAACAGATTGGAAGGGGAG
	qRT-PCR	TTTGCCTATGCTGATGATACCTG	GCTGTTCACATTCTTCTCGCTTT
CaCa4H	RT-PCR	CCCTAAAAGAAAACTCAT	TCAGATAGGCAGAACTTAC
	qRT-PCR	TCAGATTCCTTCCATTCGGT	CTTTCTCCGTGGTGTCGAG
CaCa3H	RT-PCR	ACCATGGCAA TTCCCTTAGC	CTAACAAGAGTAGTACATGC
	qRT-PCR	AGTAGAGATGGAGCGGATCTGAT	AGCCTTGTTATGTTGTTGAAGGA
CaCOMT	RT-PCR	TCTTCTACTCTAGAATTTCCGAA	GGTTTTCTCAATAAATACAAGGA
	qRT-PCR	AAACAAGCCATAGCCTAACTCAAAC	AAGTAGCAAGAAGCCTAAACATTCG
CapAMT	RT-PCR	AGAAATCTTGAAGGAATG	ATAGCACAAAGAGGAAAT
	qRT-PCR	TTTCATTGCCGAACCAGTC	GTCCCAAGTCTTCCAAATCCA
CaBCAT	RT-PCR	CCTCTACCTAATCTGTTGCTTGC	GTAAAATAACTTTAAGACGATTCA
	qRT-PCR	AAAGCGTTTAGAAGAGAGGATGG	GACAAGGAATGTGTACTCAGGTG
CaKAS	RT-PCR	TGAGAAGATGAGTAGTATTA	AGAAATTATGAGCTTGTGTT
	qRT-PCR	ATGAGTTTGGTAGATGCGGGA	CGGTGTCAATTGTAACCTGAGG
CaACL	RT-PCR	ATCAATGGCTTCTATTACTG	AATACGACGAGTCTTACAG
	qRT-PCR	ATCTCTTCCTTCAAGCACAACCA	TCCTCAAGTCCCATGACAATCTC
CaFAT	RT-PCR	ATGTTGTCTCGGGGGAGTTTT	CGCTAGTACTTAGGCAACAATGAA
	qRT-PCR	ACCTCGTAACACCTAACAATAAACTTT	AGAGAGAGTAAGAGTAAGCAGCAAGT
CaACYase	RT-PCR	ATGGAAATCATTATTCTCTC	CATCTTTTTATGACTATTGC
	qRT-PCR	CCAAACCAACACCTCCAAAC	CCAGCAAGCGGATAGAACA
CaCS	RT-PCR	GGAGGGTGTTAGGTGTATT	GACCGTAAACTTCCGTTG
	qRT-PCR	CGCACAAGATTGGTGATGG	TTCTGTACGCACTCGTTGAGAT
ACTIN	RT-PCR	TGCAGGAATCCACGAGACTAC	TACCACCACTGAGCACAATGTT
	qRT-PCR	TGCAGGAATCCACGAGACTAC	TACCACCACTGAGCACAATGTT

Quantitative Real-time PCR (qRT-PCR) analysis: The expression profiles of 11 genes were monitored by qRT-PCR analysis with protocols as described earlier (Deng *et al*., 2012). All primers used for qRT-PCR in this research are listed in Table 1. β-ACTIN gene was used as control. For the tissue specific expression assay, the expression of each gene was normalized to its expression in the placenta. For the developing fruit expression assay, the expression of each gene was normalized to its expression in the 24 DAP. For the MeJA elicitation experiments, the gene expression was normalized to that of the uninduced sample. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Three repeated experiments, including internal controls and negative controls, were conducted.

Results

Temporal accumulation of total capsaicinoid content: This study showed that capsaicinoids were undetectable in the first 12 DAP of development of the pepper fruit. Total capsaicinoid content increased slowly from 24 (1.84 mg/g) to 36 DAP (3.29 mg/g). There was a marked accumulation of total capsaicinoids from 36 to 48 DAP, with capsaicinoid content reaching the maximum level on 48 DAP (10.06 mg/g). After 48 DAP, there was a clear reduction (29.5% decrease) in total capsaicinoid content in the fruit.

Isolation and characterization of 11 full-length cDNA sequences: The five selected genes of the phenylpropanoid pathway were cloned and analyzed. The results showed that genes *CaPAL*, *CaCa4H*, *CaCa3H*, *CaCOMT* and *CapAMT* contained a 2154, 1518, 1536, 1086 and 1380 bp open reading frame (ORF), respectively, encoding proteins containing 717, 505, 511, 361 and 459 amino acid residues, respectively. The MW of the corresponding proteins were 77.912, 58.020, 57.981, 39.621, 50.929 kDa, while the corresponding pI values were 6.31, 9.25, 8.40, 5.30, 6.04, respectively.

The five selected genes from the branched-chain fatty acid biosynthesis pathway were cloned and analyzed. The results showed that *CaBCAT*, *CaKAS*, *CaFATA*, *CaACL* and *CaACYase* contained a 1158, 1467, 1116, 399 bp ORF, respectively, and the predicted translation products of the coding region contained 385, 488, 371, 132 and 456 amino acid residues, respectively. The MW and pI of the proteins encoded by the five genes were 42.459, 52.405, 41.942, 14.000, 51.089 kDa and the pI of the proteins were 8.34, 7.98, 6.48, 5.16, 5.52, respectively.

The *CaCS* gene was also cloned, and analysis showed that *CaCS* contained a 1323 bp ORF encoding 440 aa residues. The MW and pI of corresponding protein were 49.296 kDa and 6.52, respectively.

Sequence homology and phylogenetic analysis: Each of the eleven proteins lacked an N-terminal signal peptide and so were non-secretory proteins. Four proteins (CaPAL, CaCOMT, CapAMT, CaKAS) were probably located in the plasma membrane with up to more than 70% probability, and two (CaCa4H and CaCa3H) were probably located in the endoplasmic reticulum with up to 68.5% and 82% probability, respectively. The predicted CaCa4H and CaCa3H proteins possessed one transmembrane helix at positions 5-24 aa and 2-24 aa, respectively. The other nine proteins were not potential membrane proteins.

The conserved domains of the proteins encoded by the eleven genes were identified, and analysis showed that CaPAL, CaCOMT, CapAMT, CaKAS belong to the Lyase-class I-like superfamily, the Dimerization superfamily, the AdoMet-dependent MTase superfamily, the AAT_I superfamily and the cond_enzymes superfamily, respectively. Some of the other bioinformatics data, such as the base composition of the eleven genes, the number of each of the different amino acids in each protein, etc., were also predicted (Tables 2-5).

Analysis of sequence identity and evolutionary relationships: Similarity comparison and phylogenetic tree analysis of the 5 proteins in the phenylpropanoid pathway (CaPAL, CaCa4H, CaCa3H, CaCOMT and CapAMT) showed great similarity with the corresponding proteins from other plants, exhibiting 94%, 95%, 95%, 85% and 86% identity to *Solanum tuberosum* (AGT63063), *S. tuberosum* (ABC69046), *Withania somnifera* (ADM47799), *Solanum lycopersicum* (XP_004235028) and *S. lycopersicum* (XP_004244777), respectively.

The results also indicated that the five proteins in the branched-chain fatty acid biosynthesis pathway (CaBCAT, CaKAS, CaFAT, CaACL and CaACYase) shared over 91%, 87%, 90%, 90% and 74% sequence identity with the BCAT of *S. tuberosum* (XP_006356815), KAS of *S. tuberosum* (XP_006343829), FAT of *S. lycopersicum* (XP_004242408), ACL of *S. lycopersicum* (XP_004229194) and ACYase of *S. tuberosum* (XP_006345840), respectively.

Table 2. The base composition of 11 genes.

Gene	A	G	T	C
CaPAL	28.92% (623)	24.74% (533)	26.32% (567)	20.01% (431)
CaCa4H	27.73% (421)	25.36% (385)	28.26% (429)	18.64% (283)
CaCa3H	25.65% (394)	25.85% (397)	24.87% (382)	23.63% (363)
CaCOMT	27.26% (296)	23.48% (255)	29.01% (315)	20.26% (220)
CapAMT	30.07% (415)	22.25% (307)	29.35% (405)	18.23% (253)
CaBCAT	30.57% (354)	23.14% (268)	28.84% (334)	17.44% (202)
CaKAS	28.77% (422)	25.09% (368)	27.33% (401)	18.81% (276)
CaFATA	29.39% (328)	25.72% (287)	28.23% (315)	16.67% (186)
CaACL	26.82% (107)	23.56% (94)	27.32% (109)	22.31% (89)
CaACYase	32.31% (443)	20.42% (280)	29.39% (403)	17.87% (245)
CaCS	30.16% (399)	19.88% (263)	30.23% (400)	19.73% (261)

Table 3. The alpha helix, extended strand, beta turn and random coil of 11 protein.

Protein	Alpha helix	Extended strand	Beta turn	Random coil
CaPAL	360	94	66	197
CaCa4H	208	80	41	176
CaCa3H	226	87	41	157
CaCOMT	137	71	37	116
CapAMT	177	91	41	150
CaBCAT	119	107	47	112
CaKAS	137	123	54	174
CaFATA	116	84	23	148
CaACL	58	21	6	47
CaACYase	181	98	39	138
CaCS	185	67	43	145

No proteins in taxa outside the *Capsicum* genus shared high homology with the predicted CaCS protein, but the CaCS protein exhibited 99% identity with the acyltransferase proteins of *Capsicum frutescens* (tabasco pepper) (AAV66308) and *Capsicum chinense* (Scotch bonnet pepper) (AAV66309).

Spatial expression of capsaicinoid biosynthetic pathway genes in different tissues: The genes *CapAMT*, *CaKAS* and *CaCYase* were highly expressed in the placenta, but only weakly expressed in the other six tissues tested. *CaPAL*, *CaCOMT*, *CaFAT*, *CaACL*, *CaBCAT* were strongly expressed in the placenta as well as in the seed, but moderately or weakly expressed in the other five selected tissues. *CaCa4H* and *CaCa3H* were expressed only in the placenta, and then only weakly, while *CaCS* was expressed only in the placenta, albeit at very high levels (Fig. 2). In conclusion, nine of the eleven capsaicinoid biosynthesis genes (with the exception of genes *CaCa4H* and *CaCa3H*) were strongly expressed in the placenta, indicating their active participation in capsaicinoid biosynthesis (Fig. 2).

Table 4. The number of twenty kinds of amino acids, total number of negatively charged residues (Asp + Glu) and total number of positively charged residues (Arg + Lys) of the eleven proteins.

Protein	CaPAL	CaCa4H	CaCa3H	CaCOMT	CapAMT	CaBCAT	CaKAS	CaFATA	CaACL	CaACYase	CaCS
Ala (A)	67	25	47	30	41	31	46	19	15	24	25
Arg (R)	32	33	34	10	11	20	24	27	3	15	18
Asn (N)	44	26	19	14	22	13	23	17	4	40	23
Asp (D)	29	25	27	22	18	20	28	22	7	22	22
Cys(C)	13	3	5	10	6	6	9	8	4	6	13
Gln (Q)	23	18	15	5	12	15	11	11	4	12	14
Glu (E)	50	35	31	21	32	22	21	29	10	30	28
Gly (G)	54	30	31	26	33	34	49	22	7	24	20
His (H)	17	13	17	10	11	1	10	8	1	8	9
Ile (I)	40	32	25	18	25	27	39	21	11	34	21
Leu (L)	77	55	53	33	45	30	32	32	11	55	49
Lys (K)	41	37	27	21	33	25	27	22	11	28	30
Met (M)	20	13	16	17	11	8	13	8	3	14	12
Phe (F)	21	29	23	16	23	15	16	9	5	25	22
Pro (P)	30	30	32	20	25	17	17	12	5	23	22
Ser (S)	54	22	23	24	32	29	41	34	14	33	45
Thr (T)	39	20	22	21	27	20	26	7	7	21	20
Trp (W)	4	8	11	5	6	3	3	10	0	4	3
Tyr (Y)	15	10	17	10	19	21	17	29	0	8	13
Val (V)	47	41	36	28	27	28	36	0	10	30	31
(Asp + Glu)	79	60	58	43	50	42	49	51	17	52	50
(Arg + Lys)	73	70	61	31	44	45	51	49	14	43	48

Table 5. The formula, Ext. coefficient, the instability index (II), aliphatic index, Grand average of hydropathicity of 11 induced protein.

Protein	Formula	Ext. coefficient	the instability index (II)	aliphatic index	Grand average of hydropathicity
CaPAL	$C_{3418}H_{5509}N_{959}O_{1051}S_{33}$	45100	33.60 S	91.99	-0.157
CaCa4H	$C_{2638}H_{4167}N_{719}O_{722}S_{16}$	59025	48.29 U	95.68	-0.251
CaCa3H	$C_{2626}H_{4081}N_{719}O_{724}S_{21}$	86080	33.94 S	89.16	-0.210
CaCOMT	$C_{1768}H_{2760}N_{456}O_{522}S_{27}$	43025	28.58 S	85.90	0.029
CapAMT	$C_{2300}H_{3557}N_{587}O_{672}S_{17}$	61685	47.71 U	85.47	-0.140
CaBCAT	$C_{1903}H_{2997}N_{503}O_{568}S_{14}$	48165	46.19 U	86.88	-0.163
CaKAS	$C_{2306}H_{3674}N_{644}O_{705}S_{22}$	42330	34.66 S	87.56	-0.072
CaFATA	$C_{1832}H_{2929}N_{525}O_{570}S_{16}$	53900	44.18 U	83.50	-0.453
CaACL	$C_{612}H_{1012}N_{162}O_{196}S_{7}$	250	41.18 U	98.33	0.121
CaACYase	$C_{2295}H_{3637}N_{601}O_{675}S_{20}$	34295	44.73 U	100.46	-0.055
CaCS	$C_{2194}H_{3468}N_{582}O_{656}S_{25}$	36620	37.46 S	88.16	-0.162

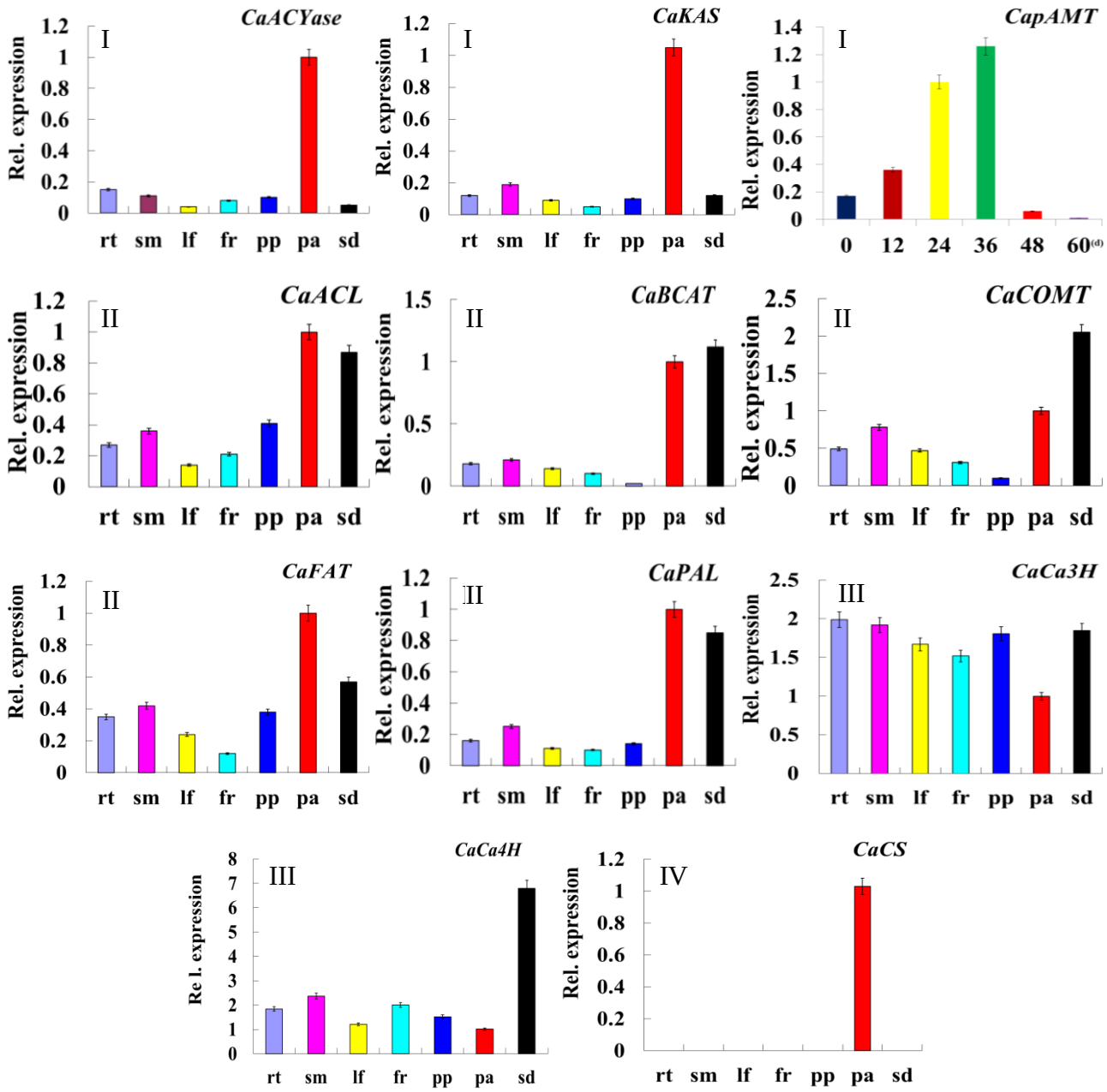

Fig. 2. Expression pattern of capsaicinoid biosynthesis genes by qRT-PCR method in different tissues (each data point represents the mean ± SD of three replicates; values in graph indicate relative expression fold; rt denotes root; sm denotes stem; lf denotes leaf; fr denotes flower; pp denotes pericarp; pa denotes placenta; sd denotes seed).

Transcription profile at pepper fruit development: Based on the patterns of relative expression of the different genes during fruit development, the eleven genes could be divided into four categories (Fig. 3). Category I contained two genes (*CaPAL* and *CaCa4H*), which displayed a bell-shaped pattern of expression, with a steady increase in gene expression from 0 to 36 DAP, peaking at 24 DAP, and then falling to a low level of expression at 60 DAP (Fig. 3-I). Category II consisted of five genes (*CaCOMT*, *CapAMT*, *CaKAS*, *CaFAT* and *CaACL*), which showed a marked increase in gene expression from 0 to 36 DAP, peaking at 36 DAP (Fig. 3-II). Category III contained the *CaCa3H* and *CaBCAT* genes, and both genes exhibited maximum expression at 48 DAP (Fig. 3-III). Finally, category IV consisted of two genes, *CaCS* and *CaACYase*, with both of them displaying a high level of expression at 36 and 48 DAP, but no expression at either 0 or 12 DAP (Fig.3-IV).

Gene expression profile in response to MeJA treatment: The expression pattern of genes involved in the capsaicinoid biosynthetic pathway in fruits following exposure to MeJA are shown in Fig. 4. MeJA caused upregulation of relative expression of genes *CaPAL*, *CapAMT* and *CaBCAT*, reaching maximum expression at 24 h after treatment. The relative expression of *CaCa4H*, *CaCa3H* and *CaACL* increased slightly at 2 and 4 h after treatment, increased markedly at 12 h, before peaking at 18 h, then decreasing. The relative expression of genes *CaCOMT*, *CaKAS*, *CaFAT* and *CaCS* was upregulated in response to MeJA treatment and reached the highest level at 12 h, before decreasing. A low level of upregulation of expression following MeJA treatment was observed for *CaACYase*, reaching its highest level at 8 h before decreasing.

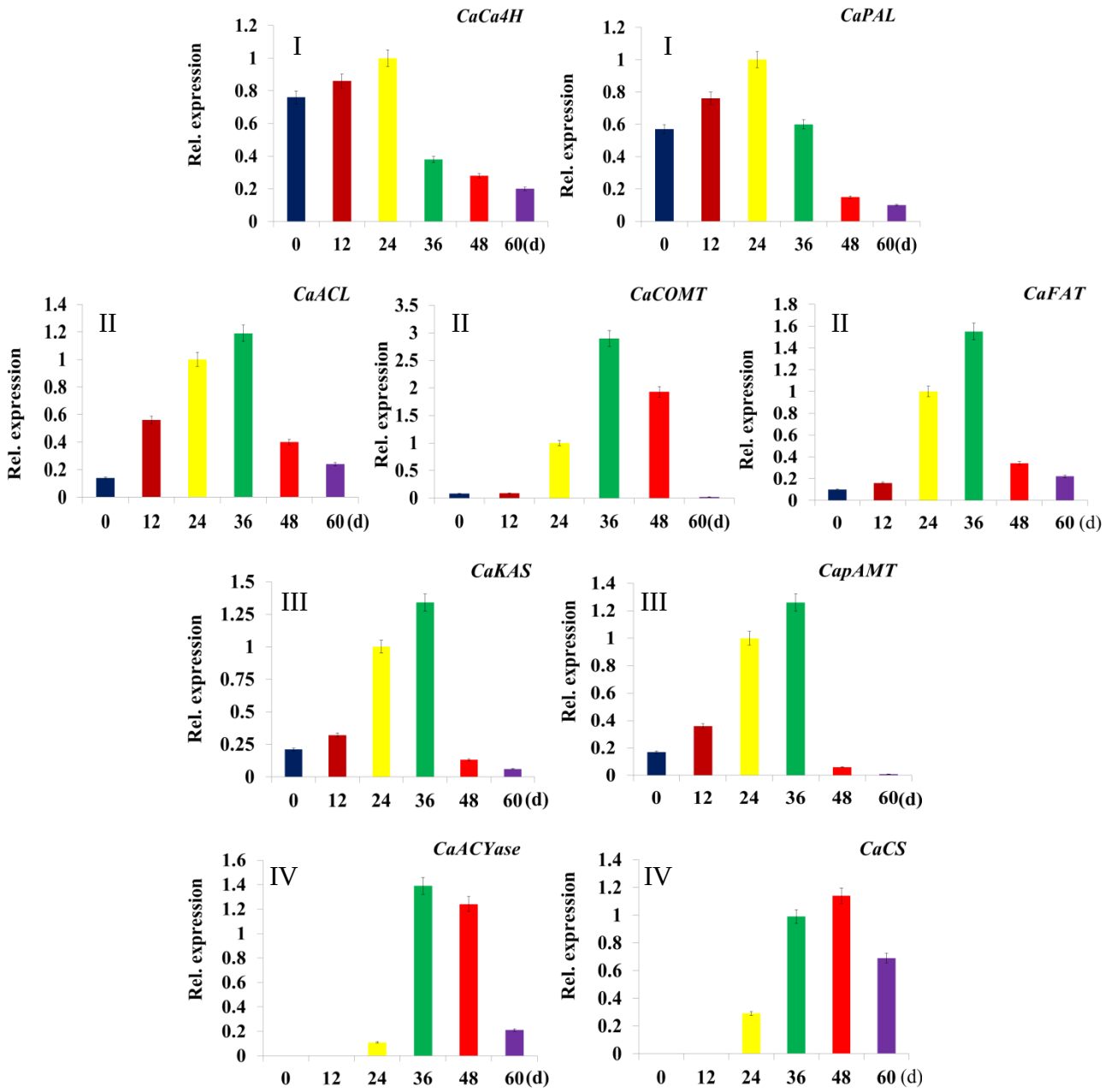

Fig. 3. Expression pattern of capsaicinoid biosynthesis genes by qRT-PCR method in different stage of development fruit.

Discussion

Capsaicinoids are well-known secondary metabolites in chili pepper (Barbero *et al*., 2014). A time-course study was set up to study capsaicinoid accumulation during pepper fruit development. During the first 12 DAP, capsaicinoids were undetectable. From 24 DAP to 48 DAP, the capsaicinoids were biosynthesised and accumulated. The capsaicinoid content declined slightly during the natural senescence of the fruit. It had previously been reported that capsaicinoids start to be synthesized and to accumulate in the fruits 20 DAP, and reach the maximum level at 40-50 DAP (Salgado-Garciglia and Ochoa-Alejo, 1990). We confirmed the general observation that biosynthesis of capsaicinoid usually occurred during the middle stage of fruit development (Barbero *et al*., 2014; Iwai *et al*., 1979; Keyhaninejad *et al*., 2014).

Investigation during fruit development of the expression profiles of genes associated with capsaicinoid biosynthesis has contributed to our understanding of the molecular basis of capsaicinoid accumulation (Kehie *et al*., 2015). In the studies, it is unsurprising that 10 of the 11 genes studied (with *CaCS* being the exception, the expression of which was placenta-specific; Fig. 2) were expressed within both fruit and non-fruit tissues in chili pepper (Fig. 2), since the phenylpropanoid and branched-chain fatty acid pathways function and are expressed in all tissues in plants. Eight of these 10 tissue non-specific genes (except *CaCa3H* and *CaCa4H*) were highly expressed in the placenta tissue and weakly expressed in the root, stem, leaf, flower and pericarp (Fig. 2), indicating their involvement in capsaicinoid biosynthesis. Capsaicinoids were synthesized and accumulated only in the placenta tissue of pepper fruit (Stewart *et al*., 2007). Our results supported the previously proposed capsaicinoid biosynthetic pathway, from which the genes studied were selected (Kehie *et al*., 2015; Keyhaninejad *et al*., 2014).

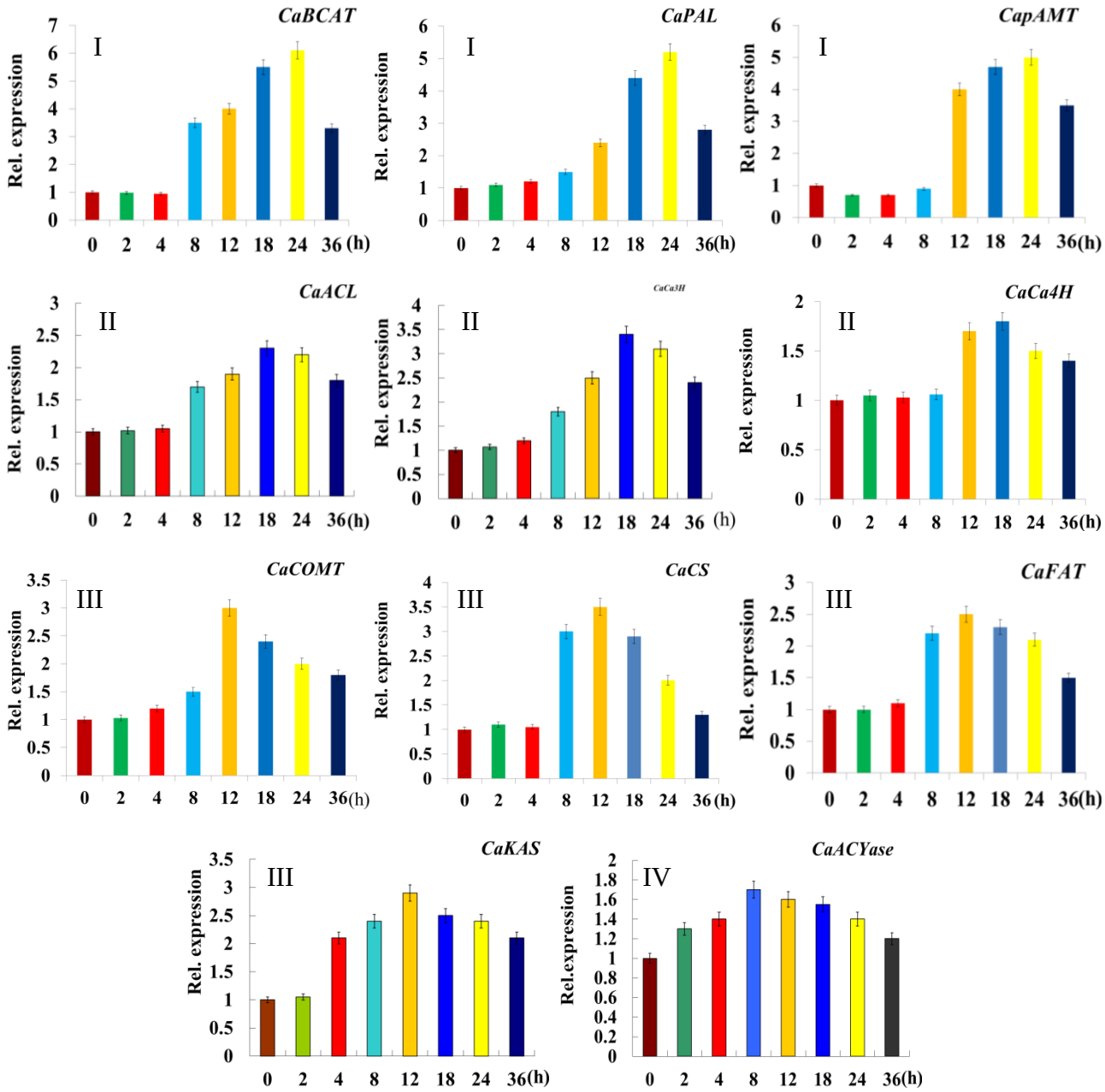

Fig. 4. Expression pattern of capsaicinoid biosynthesis genes under MeJA treatment by qRT-PCR method.

Many studies have reported that the various capsaicinoid biosynthesis genes exhibit different temporal expression patterns as the chili pepper fruit develops (Aza-González *et al*., 2011; Barbero *et al*., 2014; Kehie *et al*., 2015; Keyhaninejad *et al*., 2014). In our research, the 11 genes studied exhibited various temporal expression patterns (Fig. 3). All the 11 genes were highly expressed at some stage as the fruit developed, and expression displayed an S-shaped pattern. This result indicated that the expression of these genes was closely correlated with capsaicinoid accumulation. Capsaicinoid accumulation peaked at 48 DAP, before declining as the fruit started to senesce, with expression of nine of the 11 genes peaking at 36 or 48 DAP.

Six genes (*CaCOMT*, *CapAMT*, *CaKAS*, *CaACL*, *CaFAT* and *CaACYase*) were highly expressed in the middle period of chili pepper fruit development, peaking at the 4th stage (36 DAP), earlier than the maximum stage of capsaicinoid accumulation (48 DAP). The possible reasons for this discrepancy were that these genes are located in the upstream part of the capsaicinoid biosynthesis pathway. *CaPAL* and *CaCa4H* are the first two genes in the phenylpropanoid pathway; they exhibited rapidly increasing levels of expression at the three earliest stages of pepper fruit development (0-24 DAP), showing a downregulated expression pattern at the last three stages (36-60 DAP). It has been speculated that capsaicinoids might downregulate the capsaicinoid biosynthetic pathway as a feedback inhibitor (Aza-González *et al*., 2011). Post-transcriptional regulation of gene expression might be the reason of the lack of a correlation between maximal expression level of *CaPAL* and *CaCa4H* and capsaicinoid concentrations.

MeJA has been shown to play an important role inducing the accumulation of a wide range of plant secondary metabolites (Kehie *et al*., 2014, 2015). A higher level of capsaicinoid production in cell suspension after

treatment with MeJA has also been observed (Prasad *et al*., 2006; Gutiérrez-Carbajal *et al*., 2010).

The expression of all 11 genes was upregulated within 24 h of treatment of the fruit with MeJA. Our studies showed that expression of genes *CaPAL*, *CapAMT* and *CaBCAT* was upregulated after MeJA treatment, achieving the highest level of expression at 24 h after treatment. Our results showed that transcription of genes *CaCa4H*, *CaCa3H* and *CaACL* also began to increase after MeJA elicitation, peaking at 18 h. Expression levels of genes *CaCOMT*, *CaKAS*, *CaFAT* and *CaCS* were induced after elicitation with MeJA and peaked at 12 h. Transcript levels of *CaACYase* increased after MeJA treatment, with the highest level being achieved at 8 h. The upregulated expression of these genes would result in increased capsaicinoid accumulation by MeJA treatment, as has been reported in cell suspension culture.

Numerous studies have reported that the *CaCS* gene exhibited tissue-specific expression in the placenta tissue (Han *et al*., 2013; Ogawa *et al*., 2015). In the current study, the expression of the *CaCS* gene could not be detected in the early fruit development stages, but it was highly expressed at the color-red turn stage of the pepper fruit (Barbero *et al*., 2014; Prasad *et al*., 2006; Wyatt *et al*., 2012). The *CaCS* expression pattern was similar to that of capsaicinoid accumulation during pepper fruit development. *CaCS* expression increased 6-fold after exposure to MeJA, compared to a 6.15-fold increase in capsaicinoid content triggered by MeJA treatment (Prasad *et al*., 2006). Our results here supported earlier reports (Barbero *et al*., 2014; Han *et al*., 2013; Ogawa *et al*., 2015; Prasad *et al*., 2006; Wyatt *et al*., 2012), suggesting that CaCS catalyzes a limiting step in the capsaicinoid biosynthetic pathway.

CaPAL is an important target for metabolic regulation of capsaicinoid biosynthesis (Phimchan *et al*., 2014). Some researchers have demonstrated that *CaPAL* played an important role in capsaicinoid synthesis. Our work here also showed that *CaPAL* was highly expressed in the placenta, the expression pattern was approximated to that of the capsaicinoid content, and *CaPAL* expression was highly upregulated by MeJA treatment. Hence, *CaPAL* could be considered as one of the key genes in capsaicinoid biosynthetic pathway.

Acknowledgements

This study was supported by National Natural Science Foundation of China (Grant No. 31160394, 31560556).

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(Received for publication 18 January 2017)