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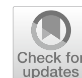

Efficient enhancement of capsaicinoids biosynthesis in cell suspension cultures of *Capsicum chinense* Jacq. cv. 'Umorok' by elicitors and differential gene expression analysis of elicited cultures

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Abstract

Capsaicinoids are responsible for pungency in chillies and have multiple health benefits and industrial applications. In vitro cell cultures of *Capsicum* with high capsaicinoids content may have the potential to serve as an alternative source for production of capsaicinoids. The present study investigated the effects of various elicitors, precursors and intermediates on the production of capsaicinoids in cell suspension cultures established from callus derived from the placental tissue explants of the highly pungent chilli cultivar, *Capsicum chinense* Jacq. cv. 'Umorok'. The study also, for the first time, reports the effect of elicitors on expression patterns of capsaicinoids biosynthesis genes in cell suspension cultures of *C. chinense* by quantitative real-time PCR. The maximum capsaicinoids content was obtained in cells elicitated with chitosan with average capsaicin and dihydrocapsaicin contents of 2.87 mg g^{-1} fresh weight (FW) and 1.03 mg g^{-1} FW, respectively. The expressions of the studied candidate genes were significantly up-regulated in response to elicitation. Enhancement of capsaicinoids biosynthesis by elicitation with chitosan was found to be associated with the up-regulation of the expression of all the studied candidate genes. The present study shows the association of different enhancement potential of different elicitors with differential expression pattern of the candidate genes of capsaicinoids biosynthesis pathways in cell suspension cultures of *C. chinense* and the information provided may help in understanding the key mechanism for enhancement of capsaicinoids biosynthesis by elicitation.

Key message

The present study reports the change in gene expression profiles of capsaicinoid biosynthesis pathway genes in response to elicitation in cell suspension cultures of *Capsicum chinense* Jacq.

Keywords Capsaicinoids · *Capsicum chinense* · Elicitor · Gene expression · Intermediate · Precursor

Introduction

Capsicum chinense Jacq. cv. 'Umorok' (syn. 'Naga jolokia' or 'Bhut jolokia'), found in the North Eastern states of India, is known for its extreme hotness and unique taste. The chilli cultivar was acknowledged as the hottest chilli in the world measuring 1,001,304 Scoville Heat Units (Bosland and Baral 2007). Capsaicinoids, mainly capsaicin and dihydrocapsaicin, account for more than 90% of the pungency in chillies and are synthesized in the placental tissue by two pathways, phenylpropanoid pathway and the pathway for synthesis of fatty acids (Fujiwake et al. 1980). Recent advances in RNA-seq technologies and comparative gene expression analysis have revealed most of the structural genes of capsaicinoids biosynthesis pathways and even elucidated their expression pattern in different chilli cultivars (Curry et al. 1999; Liu et al. 2012; Tanaka et al. 2015, 2016; Zhang et al. 2016). Phenylalanine ammonia-lyase (PAL) is the first enzyme in the phenylpropanoid pathway (Perucka

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and Materska 2001) followed by intermediate enzymes, such as cinnamate-4-hydroxylase (C4H), 4-coumarate: Coenzyme A Ligase (4CL), hydroxycinnamoyl transferase (HCT), coumarate-3-hydroxylase (C3H), caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) and a putative aminotransferase (pAMT). pAMT catalyses the formation of vanillylamine in the phenylpropanoid pathway. And, in the branched-fatty-acid synthesis pathway, an acyl-transferase encoded by Pun1 catalyzes the final condensation reaction of 8-methyl-6-nonenoyl-CoA from the phenylpropanoid pathway with vanillylamine to form capsaicinoids (Suzuki et al. 1981; Stewart et al. 2005).

Since in vitro cell cultures of *Capsicum* with high capsaicinoids content may have the potential to serve as an alternative source for production of capsaicinoids, several studies have been conducted to study in vitro capsaicinoids production in cell cultures of *Capsicum*. Kehie et al. (2014b) reviewed the production of capsaicinoids by in vitro cultured cells and tissues of different *Capsicum* species using immobilized placenta and cell cultures, as well as the application of various strategies to enhance capsaicinoids production including nutrient limitation, elicitor treatment and precursors and intermediates feeding. However, the reported capsaicinoids production potential of the cell cultures were low and optimization of effective enhancement strategies for enhancement of in vitro capsaicinoids production is essential. Addition of precursors, feeding of intermediates and elicitation has been adopted as effective strategies for improving the production of valuable secondary metabolites in plant cell cultures. In cell suspension cultures, immobilized cell cultures and in immobilized placental tissue cultures of *C. annuum* and *C. frutescens*, externally fed precursors, phenylpropanoid intermediates and valine pathway intermediate 8-methylnonenoic acid were shown to have the ability to biotransform into vanillin and capsaicin (Johnson and Ravishankar 1996; Rao and Ravishankar 2000; Nunez-Palenius and Ochoa-Alejo 2005; Prasad et al. 2006b).

Elicitation is one of the most effective methods to significantly induce secondary metabolism in plant cell cultures (Kamalipourazad et al. 2016). The plant defense signaling compounds, such as salicylic acid (SA), jasmonic acid (JA) and methyl jasmonate (MeJA) as well as other biotic elicitors, such as chitosan and other fungal elicitors have been used as potent elicitors and their ability to induce secondary metabolism in in vitro cultures is also well-documented (DiCosmo and Misawa 1985; Namdeo 2007; Kehie et al. 2014b; Shakya et al. 2019). Chitosan is an important structural component of the cell wall of several fungal plant pathogens, especially Zygomycetes (Bartnicki-Garcia 1970) and is known to elicit defense responses in plants thereby stimulating the production of secondary metabolites (Chakraborty et al. 2008; Ferri and Tassoni 2011; Sathiyabama et al. 2016). It is believed to imitate the effects of some pathogenic microorganisms in its activity to induce the synthesis of defense-related secondary metabolites in plants (Benhamou and Theriault 1992). Chitin and its derivative chitosan are two well-known biotic elicitors that induce secondary metabolite synthesis in several plant species (Baque et al. 2012; Chakraborty et al. 2008; Coqueiro et al. 2015; Ferri et al. 2008; Golkar et al. 2019; Lim et al. 2013; Malerba and Cerana 2016; Simic et al. 2015). Earlier, elicitation in cell and tissue cultures of *Capsicum* species to enhance capsaicin production have been achieved by using SA and MeJA (Sudha and Ravishankar 2003; Gutierrez-Carbajal et al. 2010; Kehie et al. 2016; Ferri et al. 2017). Recently, chitosan and other biotic elicitors, such as b-cyclodextrin and fungal elicitor extracts of *Rhizopus oligosporus* and *Aspergillus niger* were also used to enhance capsaicin content in cell suspension cultures of *C. chinense* and *C. frutescens* (Ferri et al. 2017; Rao and Ravishankar 1999; Prasad et al. 2006b).

However, detailed understanding of the gene expression changes in cell suspension cultures of *Capsicum* species in response to elicitor treatment and feeding of precursors and intermediates have not been reported. In our previous study, the expression pattern of these candidate genes involved in capsaicinoids biosynthesis was studied in in vitro callus cultures of *Capsicum chinense* and two genes (*CcpAMT* and *CcPun1*) were reported to be the main regulatory genes for capsaicinoids biosynthesis in the callus cultures (Kabita et al. 2019). However, the gene expression changes in elicitor enhanced production of capsaicinoids in cell cultures of *Capsicum* species have not been studied yet. Moreover, understanding the molecular mechanism underlying the response of plant secondary metabolism to elicitors and other enhancement strategies in cell cultures will help in enhancing capsaicinoids production in *Capsicum* cell cultures. Therefore, the present study focuses on the application of different enhancement strategies for enhancement of capsaicinoids biosynthesis in cell suspension cultures derived from the placental callus of the highly pungent cultivar *C. chinense* Jacq. cv. 'Umorok' and to gain a comprehensive knowledge of regulatory molecular mechanisms involved in enhancement of capsaicinoids biosynthesis by elicitors.

Materials and methods

Callus induction and establishment of cell suspension culture

Callus was induced from the placental tissue explants of *C. chinense* Jacq cv. 'Umorok' cultured on MS medium supplemented with 2 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg L^{-1} Kinetin (Kin) following the method described in our earlier study (Kabita et al. 2019).

The calli were subcultured by transferring about a gram of calli in 25 mL of medium after every 32 days. Cell suspension cultures were initiated from the friable callus and maintained in liquid MS medium supplemented with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kin and the cultures were kept on a rotatory shaker (125 rpm) at 25 ± 2 °C under dark condition.

Treatment with precursors, intermediates and elicitors

Cell cultures were treated with freshly prepared solutions of phenylalanine, cinnamic acid, *p*-coumaric acid, ferulic acid, vanillylamine, vanillin and valine, individually at three different doses (50, 100 and 200 µM) and also with 8-methyl-6 nonenoic acid at 2, 5 and 10 µM concentrations to study their influence on capsaicin and dihydrocapsaicin biosynthesis and the cultures were analyzed at 4-day intervals during a growth cycle of 32 days. The capsaicin content, dihydrocapsaicin content and biomass growth of cells cultured in MS medium containing 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kin and supplemented with precursors and intermediates were compared with those of control cells cultured on MS medium containing 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kin only. For elicitation treatments, *C. chinense* cells cultured in MS medium containing 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kin were treated with three different doses (100, 200, 500 µM) of MeJA, JA, Chitosan and SA on the 16th day of the growth cycle and their influences on capsaicin and dihydrocapsaicin biosynthesis were analysed at 0, 12, 24, 36, 48, 60, 72 and 84 h after treatment using cells cultured in medium without elicitors as control. All the experiments were carried out by inoculating 2 g cells (FW) from 20-day old callus cultures in 150 mL Erlenmeyer flasks containing 50 mL MS liquid medium under the same culture conditions described above.

Extraction and quantification of capsaicinoids

Capsaicinoids were extracted from the cells by following the method of Collins et al. (1995). The capsaicinoids content of the extracts were measured by UV/Vis Spectrophotometer at 296 nm following the methods of Salgado-Roman et al. (2008). Capsaicinoids content was further confirmed by High Performance Liquid Chromatography using Agilent 1260 infinity series equipped with Zorbax Eclipse Plus C18 (4.6 × 100 mm, 3.5 micron). The mobile phase consisted of a binary mixture of solvent A (methanol)- B (water) at 50%:50% ratio for 3 min followed by an increasing gradient to 100% solvent B till 10th min and further maintained at 100% solvent B for another 5 min. Detection was at 230 nm, and the flow rate was maintained at 1 mL min^{-1}.

RNA isolation for cDNA synthesis and qPCR analysis

To investigate the gene expression pattern of candidate genes involved in capsaicinoids biosynthesis in response to various enhancement strategies in *C. chinense* cell suspension cultures, qRT-PCR analysis was performed. Total RNA was extracted using ZR Plant RNA MiniPrep (Zymo Research, Germany) and the RNeasy Mini spin column (Qiagen). The extracted RNA was treated with DNase I to remove DNA contamination. For cDNA synthesis, four hundred nanograms of RNA were converted into cDNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (BIO-RAD). Quantitative PCR analysis was performed using the SYBRR Green Jump-Start™ Taq Ready Mix™ (Sigma–Aldrich, USA) on the QuantStudio™ 5 System according to the manufacturer's instructions. The dissociation temperature ranged from 65 to 95 °C and actin gene was used as the reference gene for normalization. The primer sequences used for qRT-PCR are the same as described in our earlier study (Kabita et al. 2019) and the Livak (ΔΔCT) method was used to analyse the expression levels (Livak and Schmittgen 2001). The RNA extraction was conducted with three biological repeats and each data represent the mean relative expression of three biological repetitions with standard error bars.

Statistical analysis

All experiments were performed with three biological repeats, each consisting of three replicates and data were analysed using one-way analysis of variance (ANOVA, $P < 0.05$). The statistical significance among the means for cell culture experiments were determined by Duncan's multiple range tests (MRT) and the statistical significance for gene expression levels between control and treated samples were determined by Dunnett's test.

Results and discussion

Establishment of cell suspension culture

Fine cell suspension cultures were successfully established by culturing the friable callus tissue in liquid MS medium and the cells exhibited a typical growth curve. The optimum cell biomass with an average of 6.7 g FW $culture^{-1}$ and 13% (v/v) packed cell volume (PCV) were obtained on the 24th day of the growth cycle, while the optimum capsaicin and dihydrocapsaicin contents (1.4 mg g^{-1} FW and 0.59 mg g^{-1} FW, respectively) were obtained on the 20th day of the growth cycle (Fig. 1a, b). Although, several earlier studies have reported *in vitro* capsaicinoids production from cell cultures derived from different explants of

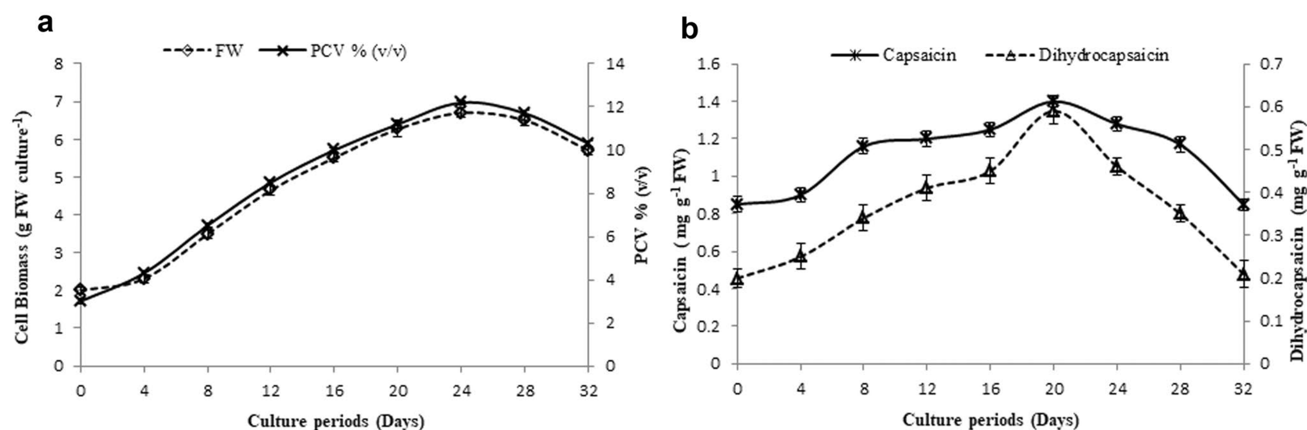

Fig. 1 Growth cycle of cell suspension cultures of *Capsicum chinense* Jacq. cv. 'Umorok': **a** cell biomass growth, and **b** capsaicin and dihydrocapsaicin accumulation. All data are presented as means of three repeats. Error bars shows standard errors

Capsicum cultivars as reviewed by Kehie et al. (2014b), the present study is the first report on the establishment of cell suspension cultures from callus derived from placental tissue explants. Earlier, we reported high capsaicinoids content (1.6 mg g^{-1} FW capsaicin and 0.78 mg g^{-1} FW dihydrocapsaicin) of callus tissue derived from the placental tissue explants (Kabita et al. 2019) and the capsaicinoids production potential is also observed to be maintained in the cell suspension cultures.

Influence of precursors, intermediates and elicitors on capsaicinoids biosynthesis

The effects of feeding precursors and intermediates of phenylpropanoid and valine pathways on capsaicin and dihydrocapsaicin biosynthesis in cell suspension cultures of *C. chinense* are shown in Table 1. Significant variation in the level of capsaicinoids biosynthesis was observed in response to the varying concentrations or the type of precursors and intermediates used. The results indicate that precursors and intermediates feeding in culture media significantly enhance both the capsaicinoids content and the cell biomass of the cell suspension cultures. Similar effectiveness of the addition of precursors and intermediates of the target metabolites to the culture medium in enhancing biosynthetic level of capsaicin in *Capsicum* cell cultures have been reported earlier (Nunez-Palenius and Ochoa-Alejo 2005; Prasad et al. 2006a; Kehie et al. 2014a; Ferri et al. 2017). The maximum capsaicinoids content and cell biomass yield were observed on the 20th and 24th day, respectively of the growth cycle. The highest capsaicin (2.75 mg g^{-1} FW) and dihydrocapsaicin (0.98 mg g^{-1} FW) contents were observed on the 20th day in the cells fed with 5 µM 8-methyl-6-nonenoic acid followed by cells treated with 100 µM vanillin (2.55 mg g^{-1} FW capsaicin and 0.91 mg g^{-1} FW dihydrocapsaicin). However, the optimal increase in cell biomass (9.63 g $culture^{-1}$) was observed in cells treated with 100 µM vanillin followed by cells treated with 5 µM 8-methyl-6-nonenoic acid (8.91 g $culture^{-1}$) on the 24th day of culture. Such effectiveness of 8-methylnonenoic acid and vanillin in enhancing capsaicin biosynthesis in cell cultures of *Capsicum* has also been reported earlier (Prasad et al. 2006a; Kehie et al. 2014a). Further, the effects of the addition of other phenylpropanoid pathway intermediates (phenylalanine, cinnamic acid, *p*-coumaric acid, ferulic acid, vanillylamine and valine) are also shown in Table 1. Cultures treated with 200 µM phenylalanine, 100 µM cinnamic acid, 100 µM *p*-coumaric acid, 200 µM ferulic acid or 100 µM valine showed significant increase in capsaicin and dihydrocapsaicin production compared to the control cultures. The level of enhancement of capsaicin and dihydrocapsaicin production by these intermediates in the cell cultures were lesser than that of the cells treated with 8-methyl-6-nonenoic acid. Thus, the addition of precursors and intermediates resulted in differential increase in capsaicinoids content, which may be due to the difference in the activity of enzymes of the capsaicinoids biosynthesis pathway.

The enhancement of capsaicin production in cell suspension and immobilized cell cultures derived from hypocotyl explants and immobilized placenta of different *Capsicum* species by using biotic and abiotic elicitors have been reported by earlier workers (Johnson et al. 1990, 1991; Rao and Ravishankar 1999; Prasad et al. 2006b; Kehie et al. 2016; Ferri et al. 2017). In the present study also, significant variation in capsaicin and dihydrocapsaicin biosynthesis were observed in cell suspension cultures of *C. chinense* in response to different biotic and abiotic elicitor concentrations and at different hours of treatment (Tables 2, 3). Elicitation of cell cultures with 200 µM chitosan for 72 h resulted in the maximum enhancement of capsaicin and

Table 1 Effect of precursors and intermediates on capsaicin, dihydrocapsaicin and cell biomass production in cell suspension cultures of *Capsicum chinense* Jacq. cv. 'Umorok'

Treatment	20th day		24th day
	Capsaicin (mg g^{-1} FW)	Dihydrocapsaicin (mg g^{-1} FW)	Cell biomass (g FW $culture^{-1}$)
Control	1.40 ± 0.03^{e}	0.59 ± 0.03^{f}	6.70 ± 0.12^{e}
50 µM vanillin	1.42 ± 0.04^{e}	0.60 ± 0.03^{f}	7.55 ± 0.12^{cd}
100 µM vanillin	2.55 ± 0.03^{b}	0.91 ± 0.02^{b}	9.63 ± 0.12^{a}
200 µM vanillin	1.31 ± 0.03^{f}	0.55 ± 0.02^{f}	7.62 ± 0.15^{cd}
50 µM vanillylamine	1.40 ± 0.03^{e}	0.58 ± 0.02^{f}	6.75 ± 0.12^{e}
100 µM vanillylamine	2.44 ± 0.03^{c}	0.83 ± 0.03^{c}	7.81 ± 0.16^{c}
200 µM vanillylamine	1.42 ± 0.04^{e}	0.59 ± 0.01^{f}	7.34 ± 0.17^{d}
50 µM cinnamic acid	1.40 ± 0.03^{e}	0.58 ± 0.02^{f}	6.72 ± 0.12^{e}
100 µM cinnamic acid	1.87 ± 0.09^{d}	0.68 ± 0.02^{e}	7.57 ± 0.13^{cd}
200 µM cinnamic acid	1.17 ± 0.02^{g}	0.34 ± 0.03^{h}	7.31 ± 0.14^{d}
50 µM phenylalanine	1.31 ± 0.03^{f}	0.48 ± 0.02^{g}	6.76 ± 0.16^{e}
100 µM phenylalanine	1.46 ± 0.05^{e}	0.59 ± 0.02^{f}	7.27 ± 0.19^{d}
200 µM phenylalanine	1.97 ± 0.04^{d}	0.76 ± 0.02^{d}	7.29 ± 0.15^{d}
50 µM valine	1.31 ± 0.03^{f}	0.43 ± 0.04^{g}	6.76 ± 0.12^{e}
100 µM valine	1.97 ± 0.03^{d}	0.76 ± 0.02^{d}	7.60 ± 0.16^{cd}
200 µM valine	0.99 ± 0.04^{h}	0.56 ± 0.02^{f}	7.55 ± 0.13^{cd}
50 µM ferulic acid	1.31 ± 0.02^{f}	0.44 ± 0.04^{g}	6.76 ± 0.13^{e}
100 µM ferulic acid	1.48 ± 0.07^{e}	0.57 ± 0.02^{f}	7.28 ± 0.12^{d}
200 µM ferulic acid	1.90 ± 0.05^{d}	0.74 ± 0.04^{de}	7.35 ± 0.13^{d}
50 µM *p*-coumaric acid	1.49 ± 0.07^{e}	0.57 ± 0.02^{f}	6.73 ± 0.14^{e}
100 µM *p*-coumaric acid	1.97 ± 0.03^{d}	0.75 ± 0.02^{d}	7.55 ± 0.13^{cd}
200 µM *p*-coumaric acid	1.45 ± 0.04^{e}	0.56 ± 0.03^{f}	6.75 ± 0.17^{e}
2 µM 8-methyl-6-nanonoic acid	1.48 ± 0.06^{e}	0.57 ± 0.02^{f}	7.50 ± 0.11^{cd}
5 µM 8-methyl-6-nanonoic acid	2.75 ± 0.04^{a}	0.98 ± 0.02^{a}	8.91 ± 0.16^{b}
10 µM 8-methyl-6-nanonoic acid	2.00 ± 0.05^{d}	0.75 ± 0.02^{d}	7.55 ± 0.13^{cd}

Data scored at 4-day interval during the growth cycle of culture, mean ± SD. Duncan's comparisons are significant when letters are different within columns (ANOVA, $P < 0.05$)

Table 2 Effect of elicitors on capsaicin production in cell suspension cultures of *Capsicum chinense* Jacq. cv. 'Umorok'

Treatment	Capsaicin production (mg g^{-1} FW) at different time intervals							
	0 h	12 h	24 h	36 h	48 h	60 h	72 h	84 h
Control	1.25 ± 0.02^{a}	1.26 ± 0.03^{d}	1.28 ± 0.03^{d}	1.32 ± 0.03^{d}	1.35 ± 0.04^{c}	1.36 ± 0.02^{d}	1.37 ± 0.06^{d}	1.40 ± 0.04^{d}
100 µM SA	1.25 ± 0.02^{a}	1.25 ± 0.03^{d}	1.29 ± 0.03^{d}	1.35 ± 0.02^{d}	1.35 ± 0.05^{c}	1.37 ± 0.03^{d}	1.37 ± 0.05^{d}	1.37 ± 0.03^{d}
200 µM SA	1.25 ± 0.03^{a}	1.52 ± 0.03^{b}	1.55 ± 0.03^{b}	2.30 ± 0.05^{a}	1.88 ± 0.04^{a}	1.84 ± 0.02^{b}	1.83 ± 0.03^{c}	1.81 ± 0.04^{b}
500 µM SA	1.25 ± 0.02^{a}	1.27 ± 0.04^{d}	1.30 ± 0.02^{d}	1.33 ± 0.03^{d}	1.37 ± 0.04^{c}	1.39 ± 0.04^{d}	1.37 ± 0.05^{d}	1.37 ± 0.04^{d}
100 µM MeJA	1.25 ± 0.03^{a}	1.43 ± 0.03^{c}	1.46 ± 0.02^{c}	1.49 ± 0.03^{c}	1.50 ± 0.03^{b}	1.65 ± 0.03^{c}	1.93 ± 0.03^{b}	1.82 ± 0.02^{b}
200 µM MeJA	1.25 ± 0.02^{a}	1.10 ± 0.05^{e}	1.10 ± 0.03^{e}	1.10 ± 0.03^{e}	1.09 ± 0.04^{f}	1.09 ± 0.03^{f}	1.08 ± 0.03^{f}	1.05 ± 0.03^{e}
500 µM MeJA	1.25 ± 0.03^{a}	1.00 ± 0.05^{e}	1.09 ± 0.04^{e}	1.07 ± 0.05^{e}	0.95 ± 0.02^{g}	0.93 ± 0.03^{g}	0.93 ± 0.03^{g}	0.90 ± 0.02^{f}
100 µM JA	1.25 ± 0.02^{a}	1.42 ± 0.04^{c}	1.44 ± 0.02^{c}	1.43 ± 0.04^{c}	1.51 ± 0.04^{b}	1.94 ± 0.05^{a}	1.86 ± 0.05^{b}	1.70 ± 0.03^{c}
200 µM JA	1.25 ± 0.03^{a}	1.24 ± 0.03^{d}	1.23 ± 0.05^{d}	1.27 ± 0.05^{d}	1.29 ± 0.05^{c}	1.24 ± 0.02^{e}	1.21 ± 0.02^{e}	1.09 ± 0.04^{e}
500 µM JA	1.25 ± 0.02^{a}	1.23 ± 0.02^{d}	1.23 ± 0.04^{d}	1.27 ± 0.03^{d}	1.28 ± 0.03^{c}	1.21 ± 0.02^{e}	1.19 ± 0.03^{e}	1.02 ± 0.04^{e}
100 µM chitosan	1.25 ± 0.03^{a}	1.27 ± 0.02^{d}	1.30 ± 0.03^{d}	1.31 ± 0.03^{d}	1.31 ± 0.03^{c}	1.33 ± 0.03^{d}	1.34 ± 0.05^{d}	1.35 ± 0.04^{d}
200 µM chitosan	1.25 ± 0.03^{a}	1.81 ± 0.04^{a}	1.89 ± 0.03^{a}	1.89 ± 0.04^{b}	1.91 ± 0.03^{a}	1.95 ± 0.03^{a}	2.87 ± 0.08^{a}	2.71 ± 0.02^{a}
500 µM chitosan	1.25 ± 0.03^{a}	1.26 ± 0.02^{d}	1.31 ± 0.04^{d}	1.32 ± 0.02^{d}	1.33 ± 0.02^{c}	1.35 ± 0.02^{d}	1.36 ± 0.05^{d}	1.36 ± 0.03^{d}

Data represents mean ± SD. Duncan's comparisons are significant when letters are different within columns (ANOVA, $P < 0.05$)

SA salicylic acid, *MeJA* methyl jasmonate, *JA* jasmonic acid

Table 3 Effect of elicitors on dihydrocapsaicin production in cell suspension cultures of *Capsicum chinense* Jacq. cv. 'Umorok'

Treatment	Dihydrocapsaicin production (mg g^{-1} FW) at different time intervals							
	0 h	12 h	24 h	36 h	48 h	60 h	72 h	84 h
Control	0.45 ± 0.02[a]	0.45 ± 0.02[d]	0.46 ± 0.03[d]	0.49 ± 0.02[c]	0.52 ± 0.04[c]	0.55 ± 0.02[c]	0.57 ± 0.03[c]	0.59 ± 0.02[c]
100 μM SA	0.45 ± 0.02[a]	0.45 ± 0.02[d]	0.46 ± 0.04[d]	0.48 ± 0.03[c]	0.50 ± 0.02[c]	0.53 ± 0.02[c]	0.55 ± 0.02[c]	0.55 ± 0.03[cd]
200 μM SA	0.45 ± 0.03[a]	0.63 ± 0.02[b]	0.67 ± 0.03[b]	0.85 ± 0.03[a]	0.73 ± 0.05[a]	0.75 ± 0.04[a]	0.75 ± 0.03[b]	0.68 ± 0.03[b]
500 μM SA	0.45 ± 0.01[a]	0.45 ± 0.02[d]	0.46 ± 0.03[d]	0.47 ± 0.02[c]	0.51 ± 0.04[c]	0.54 ± 0.01[c]	0.55 ± 0.03[c]	0.56 ± 0.01[cd]
100 μM MeJA	0.45 ± 0.02[a]	0.54 ± 0.02[c]	0.57 ± 0.03[c]	0.61 ± 0.04[b]	0.61 ± 0.02[b]	0.63 ± 0.02[b]	0.79 ± 0.02[b]	0.69 ± 0.03[b]
200 μM MeJA	0.45 ± 0.02[a]	0.39 ± 0.01[d]	0.40 ± 0.03[de]	0.35 ± 0.03[d]	0.34 ± 0.02[e]	0.34 ± 0.02[e]	0.33 ± 0.02[e]	0.31 ± 0.03[e]
500 μM MeJA	0.45 ± 0.03[a]	0.39 ± 0.02[d]	0.35 ± 0.03[e]	0.33 ± 0.04[d]	0.29 ± 0.04[e]	0.25 ± 0.03[f]	0.25 ± 0.03[f]	0.21 ± 0.03[f]
100 μM JA	0.45 ± 0.03[a]	0.54 ± 0.02[c]	0.56 ± 0.02[c]	0.57 ± 0.03[b]	0.62 ± 0.03[b]	0.80 ± 0.03[a]	0.76 ± 0.05[b]	0.65 ± 0.02[b]
200 μM JA	0.45 ± 0.02[a]	0.40 ± 0.03[d]	0.43 ± 0.04[d]	0.45 ± 0.03[c]	0.44 ± 0.04[c]	0.43 ± 0.03[d]	0.42 ± 0.03[d]	0.35 ± 0.02[e]
500 μM JA	0.45 ± 0.03[a]	0.41 ± 0.02[d]	0.43 ± 0.03[d]	0.45 ± 0.03[c]	0.43 ± 0.05[c]	0.42 ± 0.02[d]	0.41 ± 0.04[d]	0.27 ± 0.01[de]
100 μM chitosan	0.45 ± 0.02[a]	0.45 ± 0.03[d]	0.46 ± 0.04[d]	0.46 ± 0.03[c]	0.47 ± 0.04[c]	0.48 ± 0.05[cd]	0.49 ± 0.05[cd]	0.51 ± 0.03[d]
200 μM chitosan	0.45 ± 0.02[a]	0.75 ± 0.03[a]	0.78 ± 0.02[a]	0.78 ± 0.05[a]	0.79 ± 0.04[a]	0.81 ± 0.03[a]	1.03 ± 0.03[a]	0.80 ± 0.02[a]
500 μM chitosan	0.45 ± 0.03[a]	0.44 ± 0.03[d]	0.46 ± 0.04[d]	0.47 ± 0.02[c]	0.48 ± 0.03[c]	0.49 ± 0.04[c]	0.50 ± 0.03[c]	0.51 ± 0.02[d]

Data represents mean ± SD. Duncan's comparisons are significant when letters are different within columns (ANOVA, $P < 0.05$)

SA salicylic acid, *MeJA* methyl jasmonate, *JA* jasmonic acid

dihydrocapsaicin contents (2.87 mg g^{-1} FW and 1.03 mg g^{-1} FW, respectively) followed by treatment with 200 μM SA for 36 h (2.3 mg g^{-1} FW capsaicin and 0.95 mg g^{-1} FW dihydrocapsaicin). In a similar study, Ferri et al. (2017) reported the enhancement of capsaicinoids biosynthesis (17.5 μg g^{-1} FW) in cell cultures of *C. chinense* derived from hypocotyl explants by elicitation with 50 μg mL^{-1} chitosan. In addition to elicited capsaicin synthesis, chitosan has been reported to enhance the permeability of capsaicin allowing its secretion from the cells to the exterior (Johnson et al. 1991). Other elicitors, such as SA, MeJA and JA, were also used earlier to enhance capsaicin biosynthesis in *Capsicum* cell cultures (Kehie et al. 2016; Prasad et al. 2006b). However, in the present study, although SA, MeJA and JA increased the production of capsaicinoids in the cell cultures, the enhancements were lesser when compared to the enhancement by chitosan.

Gene expression analysis in response to elicitation in cell suspension cultures

To investigate the underlying mechanism of capsaicinoids enhancement in response to elicitation, the expression levels of genes involved in capsaicinoids biosynthesis were determined by RT-qPCR analysis. The elicitor concentrations and the exposure time of cells cultures resulting in optimum capsaicinoids accumulation (cultures treated with 200 μM SA for 36 h, 100 μM MeJA for 72 h, 100 μM JA for 60 h and 200 μM chitosan for 72 h) were selected and the corresponding cultures were used for comparative gene expression analysis. Nine structural genes involved in capsaicinoids biosynthetic pathways, including phenylalanine ammonia-lyase *Solanum lycopersicum* (*SlPAL*), cinnamatc 4-hydroxylase *Capsicum annuum* (*CaC4H*), hydroxycinnamoyl transferase (*CaHCT*), coumarate3-hydroxylase (*CaC3H*), caffeoyl-CoA 3-O-methyltransferase (*CaCCoAOMT*), NADH-Glutamine oxoglutarate aminotransferase (*SlNADH-GOGAT*), glutathione S-transferase (*CaGST*), *CcpAMT*, and *CcPun1* were studied for comparative gene expression analysis and the expression patterns of each candidate gene are shown in Fig. 2. Although all the elicitors up-regulated the candidate genes involved in capsaicinoids biosynthesis pathways, the nine candidate genes were differentially up-regulated by the different elicitors. Cell cultures elicited with 200 μM chitosan for 72 h, which shows maximum enhancement of capsaicinoids biosynthesis, significantly up-regulated all the candidate genes involved in capsaicinoids biosynthesis pathway, including up to 3.5 and 4.5 folds relative increase in expression of the two key regulating genes, *CcPun1* and *CcpAMT*, respectively (Fig. 2h, i). The two genes *CcPun1* and *CcpAMT* were reported to be significantly down-regulated in the callus cultures with reduced capsaicinoids content compared to placental tissues of *C. chinense* (Kabita et al. 2019). Therefore, although all the studied genes were up-regulated in response to elicitation with chitosan, the up-regulation of these two candidate genes (*CcPun1* and *CcpAMT)* may be primarily responsible for the significant enhancement of capsaicinoids biosynthesis in the cell suspension cultures of *C. chinense*. Earlier, chitosan treatment was shown to enhance the synthesis and release of several metabolites by regulating proteomic expression profile in *Vitis vinifera* cell suspensions (Ferri et al. 2008). It has also been shown to up-regulate the genes of the ROS pathway enhancing the antioxidant potential of grape tissues (Singh et al. 2019)

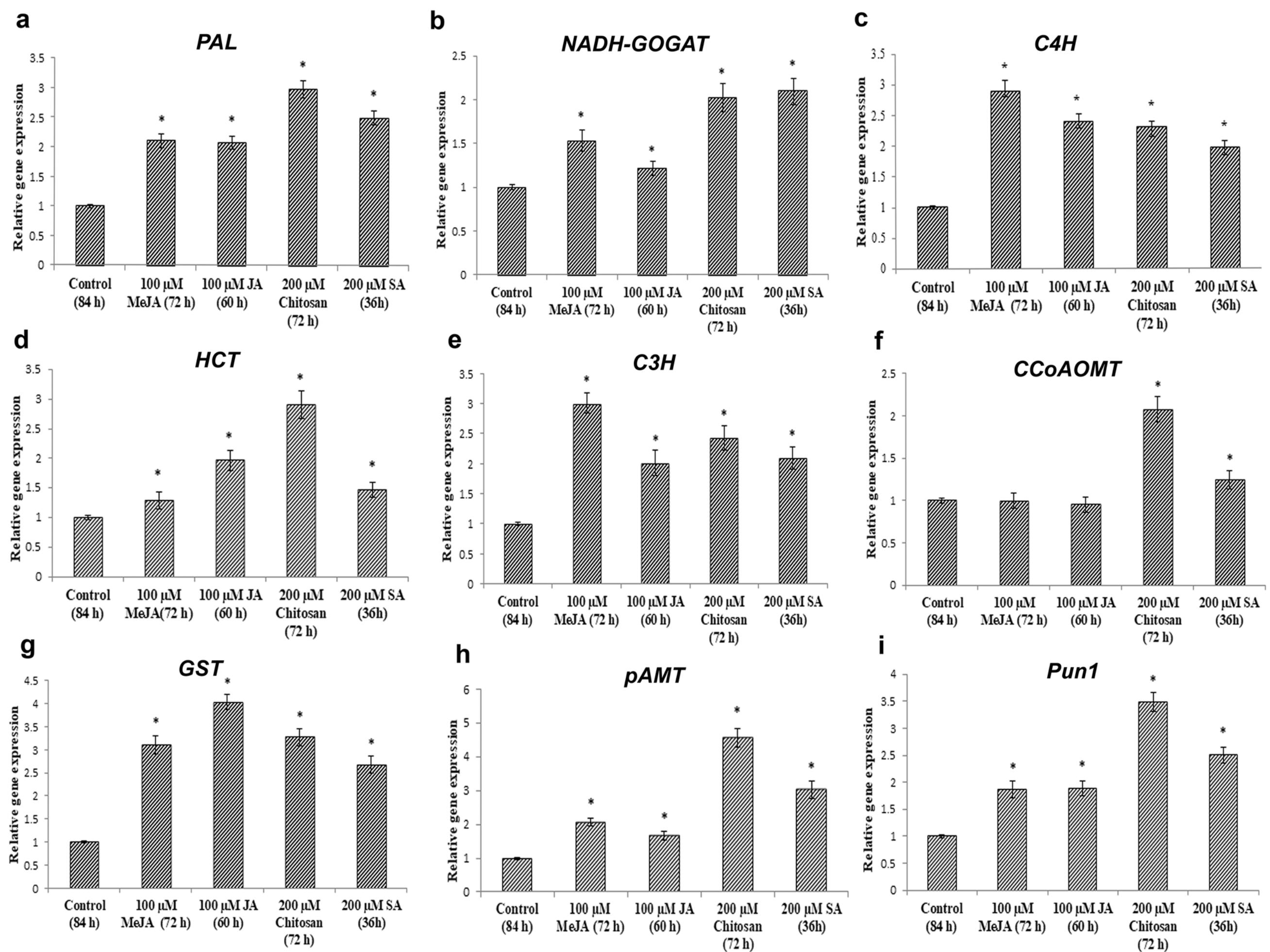

Fig. 2 Differential gene expression levels of capsaicinoids biosynthesis genes in response to elicitation in cell suspension cultures of *C. chinense*. The relative expression levels of 9 structural genes were determined by comparative qRT-PCR analysis in cell cultures treated with methyl jasmonate (MeJa), jasmonic acid (JA), chitosan and salicylic (SA): **a** *SlPAL*, **b** *SlNADH-GOGAT*, **c** *CaC4H*, **d** *CaHCT*, **e** *CaC3H*, **f** *CaCCoAOMT*, **g** *CaGST*, **h** *CcpAMT*, and **i** *CcPun1*. Asterisks indicates significant difference (Dunnett's test, ANOVA, $P < 0.05$). All data are presented as means of three repeats. Error bars shows standard errors

and the genes of the phenylpropanoids pathway in avocado fruit epicarp (Xoca-Orozco et al. 2018). Chakraborty et al. (2008) also reported chitosan-induced elicitation enhancing accumulation of phenylpropanoid derivatives and increased activities of the early phenylpropanoid pathway enzymes, such as phenylalanine ammonia lyase (PAL), *p*-coumaroyl-CoA ligase (4CL) and *p*-hydroxybenzaldehyde dehydrogenase (HBD) in cell suspension cultures of *Cocos nucifera*. Similarly, the up-regulation of *PAL* gene in cell cultures of *Scrophularia striata* Boiss increasing the production of phenylpropanoid compounds (Kamalipourazad et al. 2016) and the up-regulation of *PAL*, *C4H* and other associated genes involved in flavonoid biosynthesis pathway in hairy root cultures of *Isatis tinctoria* L. in response to chitosan elicitation (Jiao et al. 2018) further demonstrated the role of chitosan in up-regulating the genes involved in the production of phenylpropanoid compounds, which are also precursors and intermediates of capsaicinoids biosynthesis. Therefore, the up-regulation of genes involved in capsaicinoids biosynthesis in response to chitosan observed in the present study and its potential to up-regulate other genes of phenylpropanoid pathway enzymes might be responsible for the increased capsaicinoids contents.

In case of cells treated with 100 µM MeJA for 60 h, the genes *CaC3H* and *CaC4H* were significantly up-regulated indicating that these two genes might possibly be playing a role in induction of secondary metabolite synthesis in response to MeJA elicitation. Earlier, SA has been shown to induce capsaicin biosynthesis in *Capsicum* cell cultures (Sudha and Ravishankar 2003; Prasad et al. 2006b; Gutierrez-Carbajal et al. 2010; Kehie et al. 2016) and in the present study also, elicitation with 200 µM SA for 36 h enhanced capsaicinoids biosynthesis although the effect was lesser when compared to that of chitosan. Campos et al.

(2003) suggested that SA might be stimulating the activities of enzymes involved in secondary metabolites pathways. In the present study, elicitation with 200 µM SA for 36 h resulted in significant up-regulation of all the studied genes, except the genes *CaHCT* and *CaCCoAOMT*. It is possible that the inability of SA to up-regulate the genes *CaHCT* and *CaCCoAOMT*, which were up-regulated by chitosan, may be responsible for the lower enhancement of capsaicinoids biosynthesis by SA.

Earlier, genome wide transcript profiling studies have revealed that elicitation by JA and MeJA induces extensive transcriptional reprogramming (De Geyter et al. 2012; Rahnamaie-Tajadod et al. 2017) leading to the activation of several metabolic pathways including the pathways for the synthesis of terpenoids (Misra et al. 2014), phenylpropanoids (Cocetta et al. 2015) and alkaloids (Kang et al. 2004). In the present study, although cell cultures treated with 100 µM MeJA for 72 h or 100 µM JA for 60 h enhanced capsaicinoids biosynthesis compared to the control cultures, the rate of enhancement was lower compared to that of cells treated with 200 µM chitosan for 72 h or 200 µM SA for 36 h. It was also observed from the expression analysis of the candidate genes that the cultures treated with MeJA or JA significantly up-regulated the other candidate genes for capsaicinoids synthesis pathways but the genes *CaCCoAOMT*, *CaHCT* and *SlNADH-GOGAT* were not up-regulated. Hence, the failure to up-regulate higher number of genes by MeJA and JA might be responsible for their lesser efficiency to enhance capsaicinoids biosynthesis. Thus, the present study revealed that the optimum concentration of the most efficient elicitor, chitosan, up-regulated the activities of all the studied genes for capsaicinoids biosynthesis pathways and the less effective elicitors (SA, MeJA and JA) could up-regulate only some genes of the pathways.

Conclusion

In the present report, cell suspension cultures of *Capsicum chinense* Jacq. were successfully established for the first time using callus derived from placental tissue explants. For the enhancement of capsaicinoids biosynthesis, the effect of elicitation and feeding of precursors and intermediates were studied. The exogenous application of phenylpropanoid intermediate vanillin, valine pathway intermediate 8-methyl-6-nonenoic acid or the elicitor chitosan significantly enhanced capsaicinoids biosynthesis well as cell biomass growth in the cultures. To study the molecular mechanism in response to elicitation, gene expression analysis was carried out. Chitosan was found to considerably increase the expression of all the studied candidate genes of capsaicinoids biosynthesis pathways compared to other elicitors, which failed to up-regulate some of the studied genes. Thus, the present study reports the effective capsaicinoids enhancement strategy for efficient improvement of capsaicinoids biosynthesis in cell suspension cultures of *Capsicum chinense*. The study also reports, for the first time, the analysis of capsaicinoids biosynthesis gene expression in the cell suspension cultures of *Capsicum* in response to elicitors in an attempt to understand the molecular mechanism of enhancement of capsaicinoids biosynthesis by elicitation.

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Author Contributions KCK planned the experiments, carried out the experiments, analysed the data, and wrote the manuscript. KS conceived the idea, planned the experiments, supervised the experiments, revised and reviewed the data and manuscript. SKS helped in performing the experiments and supervised the experiments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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