



Analysis of capsaicinoid biosynthesis pathway genes expression in callus cultures of *Capsicum chinense* Jacq. cv. ‘Umorok’

Khaidem Chanu Kabita¹ · Susheel Kumar Sharma² · K. Sanatombi¹

Received: 7 January 2019 / Accepted: 26 February 2019 / Published online: 2 March 2019
© Springer Nature B.V. 2019

Abstract

The placental tissue of the highly pungent chilli cultivar, *Capsicum chinense* Jacq. cv. ‘Umorok’, is used as explants for callus induction. Callus cultures were subcultured after every 32 days and growth curves for a period of six consecutive growth cycles were studied till a stable capsaicinoids producing callus cultures were obtained. The capsaicinoids content in placental tissue explants decreased gradually during the first 2 months of culture as the explants dedifferentiated to form friable callus while the biomass and capsaicinoid content did not show much change in the subsequent growth cycles. The maximum callus biomass of 7.8 g freshweight (FW) or 0.56 g dry weight (DW) per culture were obtained on the 24th day of every growth cycle and the maximum average capsaicinoids content (1.6 mg g⁻¹ FW capsaicin and 0.78 mg g⁻¹ FW dihydrocapsaicin) were obtained on the 20th day of every growth cycle. To investigate the underlying dynamics for capsaicinoid biosynthesis during callus formation, comparative gene expression analysis of the genes involved in capsaicinoid biosynthesis pathway were also studied by qRT-PCR analysis. When compared with placental tissue, all the studied genes showed reduced expression during callus formation, especially putative aminotransferase (pAMT) and pungent gene 1 (Pun1), which were extensively down regulated from the 3rd month onwards in the callus cultures. Therefore, the present study revealed that the down-regulated expression of mainly two putative genes in capsaicinoid biosynthetic pathway (pAMT and Pun1) resulted in lower accumulation of capsaicinoids in callus cultures compared to placental tissues of fruits.

Key message

The present study reports the gene expression analysis of the genes involved in capsaicinoid biosynthesis pathway during callus formation from placental tissue explants of *Capsicum chinense* Jacq.

Keywords Placental tissue explants · Callus · Capsaicinoid · Gene expression · qPCR

Introduction

Capsaicinoids are compounds that produce pungency in chillies and capsaicin and dihydrocapsaicin are the main pungent principles that account for most of the pungency in chillies. The site for capsaicinoid synthesis is mainly in the placenta and interocular septum, where they are produced by

epidermis cells and are secreted through glandular vesicles called “blisters” located on the surface of placental tissue (Iwai et al. 1979; Rowland et al. 1983). Some amount of capsaicinoids has also been shown to accumulate in the pericarp tissue of the fruits (Tanaka et al. 2016; Sugiyama 2017; Park et al. 2019). Capsaicin has many pharmacological and physiological properties and is commonly used for its analgesic, anticancer, anti-inflammation, anti-obesity and antioxidant effects (Deal et al. 1999; Moore and Moore 2003; Kempaiah et al. 2005; Sawynok 2005). Since capsaicinoids are also used in food and other industries, the demand for capsaicinoids is increasing and cell and tissue cultures of highly pungent chili cultivars may serve as an alternative potential source of capsaicinoids. To sustain a stable production of capsaicinoids, a number of earlier studies have reported the production of capsaicinoids from cell suspension cultures

Communicated by Jose M. Segui-Simarro.

✉ K. Sanatombi
ksanatombi@rediffmail.com

¹ Department of Biotechnology, Manipur University, Canchipur, Imphal 795003, India

² ICAR Research Complex for NEH Region, Manipur Centre, Imphal 795004, India

established from hypocotyl explants obtained from seedlings germinated in vitro or immobilized placental tissues of different *Capsicum* cultivars (Lindsey 1985; Johnson et al. 1990, 1996; Kehie et al. 2014). The capsaicin content in cell cultures established from callus obtained from the hypocotyl explants were low (Holden et al. 1987) and the immobilized placenta cultures of different *Capsicum* species, on the other hand, showed higher capsaicin accumulation using various enhancement strategies (Johnson et al. 1990; Johnson and Ravishankar 1996; Aldana-Iuit et al. 2015). However, since the immobilized placenta does not provide a consistent source and capsaicinoids biosynthesis occurs mainly in the placental tissue of fruits, the use of placental tissue as explants for establishing in vitro cultures are expected to accumulate higher amount of capsaicinoids and may provide an alternative source for production of capsaicinoids (Aza-Gonzalez et al. 2011; Ancona-Escalante et al. 2013). Callus induction and assessment of capsaicinoids production potential of callus derived from placental tissues of *Capsicum annuum* have been reported only by Umamaheswari and Lalitha (2007). Since callus derived from placental tissue explant showed higher capsaicinoid accumulation than other explants of *Capsicum annuum* L. (Umamaheswari and Lalitha 2007) and the average capsaicinoid content of *Capsicum chinense* Jacq cv. ‘Umorok’ (synonym-Naga jolokia, Naga King Chilli, Bhut jolokia) fruits is high (Sanatombi and Sharma 2008), the cultures derived from placental explants of this highly pungent cultivar may have the potential for higher capsaicinoid production if the descendant cells retain their potential.

Further, capsaicinoid biosynthesis involves two converging pathways, phenylpropanoid pathway for synthesis of vanillylamine and the pathway for synthesis of fatty acids (Fujiwake et al. 1980). Numerous enzymes are involved in capsaicinoid biosynthesis pathway and phenylalanine ammonia-lyase (PAL) is the first enzyme in the phenylpropanoid pathway (Perucka and Materska 2001) followed by 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), which catalyses the formation of vanillylamine. In the branched-fatty-acid synthesis pathway, 8-methyl-6-nonenoyl-CoA is formed (Suzuki et al. 1981), which finally undergoes condensation reaction with vanillylamine from the phenylpropanoid pathway to form capsaicinoids catalysed by acyl-transferase encoded by Pun1 (Stewart et al. 2005). Most of the structural genes of capsaicinoid biosynthesis pathway have been identified and their expression pattern in different chilli cultivars has been determined (Curry et al. 1999; Liu et al. 2012; Tanaka et al. 2016; Zhang et al. 2016) and non-pungent cultivars have been shown either to accumulate very few capsaicinoids or to accumulate another

group of compounds called capsinoids (Yazawa et al. 1989; Kobata et al. 1998). Although several studies have been conducted to assess the capsaicinoid production potential of tissue cultures obtained from different explants and the studies reported lower production of capsaicinoids in cell cultures compared to chilli fruits, the genes involved in capsaicinoid synthesis responsible for lower capsaicinoid accumulation in cultures have not been studied. Therefore, the present study aimed at investigating the capsaicinoids production potential of callus cultures established using placental tissue explants of the highly pungent chilli cultivar and to investigate the change in expression patterns of genes involved in capsaicinoid synthesis during callus formation so that the down-regulated genes may be identified for future metabolic engineering considerations.

Materials and methods

Callus culture induction from placental tissue explants

Fresh and healthy fruits of *Capsicum chinense* Jacq cv. ‘Umorok’ at matured green stage were washed with running tap water and then treated with 0.1% Carbendazim for about 10 min and then rinsed with distilled water for 3 times. The fruits were then surface sterilized by flaming for 2–3 s after dipping in 70% ethanol under aseptic condition followed by treatment with 0.5% HgCl₂ solution for 5 min. After 5 min, the fruits were washed for about 5 times using autoclaved distilled water. The fruits were then cut open with a sterile surgical blade; the placental tissues were taken out and trimmed to about 0.5 cm long pieces. These explants were then inoculated on MS medium supplemented with 0.5 mg L⁻¹ Kinetin (Kin) along with 1, 2 or 3 mg L⁻¹ 2,4-Dichlorophenoxyacetic acid (2,4-D); 0.5 mg L⁻¹ Kin along with 2 mg L⁻¹ indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or α -naphthalene acetic acid (NAA); 0.5 mg L⁻¹ Benzylaminopurine (BAP) alone or 2 mg L⁻¹ 2,4-D alone. The culture tubes were incubated in dark condition at 25 \pm 2 °C. The biomass growth and capsaicinoid production of the placental callus cultures were studied for a period of six consecutive growth cycles, each comprising of 32 days. The cultures were subcultured by transferring about a gram of callus in 25 mL of medium after every 32 days. The growth curve for the callus was established by measuring FW, DW and capsaicinoids (viz. capsaicin and dihydrocapsaicin) content at 4 days interval. The experiments were repeated thrice with ten replicates each.

Extraction and quantification of capsaicinoids

Capsaicinoids were extracted from callus and placental tissue by following the method of Collins et al. (1995). The capsaicinoid content of the extracts were measured by UV/Vis Spectrophotometer at 296 nm by following the methods of Salgado-Roman et al. (2008). Capsaicinoid content was further confirmed by high performance liquid chromatography (HPLC) 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 compare the expression levels of genes involved in capsaicinoid biosynthetic pathway during callus formation, qRT-PCR analysis was performed. The placental tissue of mature dark green fruits of *C. chinense* Jacq cv. ‘Umorok’, callus cultures from the first six months and 1 year-old callus tissue collected on the day with maximum capsaicinoids content of every growth cycle studied were taken for RNA extraction. Total RNA was extracted using ZR Plant RNA MiniPrep (Zymo Research, Germany) and the RNeasy Mini spin column (Qiagen). The RNA

extracted from the samples used for RT-PCR were treated with DNase I prior to cDNA synthesis to remove DNA contamination. Four hundred nanograms of RNA were converted into cDNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (BIO-RAD). Quantitative PCR was performed using the SYBR^R Green JumpStart™ Taq Ready Mix™ (Sigma–Aldrich, USA) on the QuantStudio™ 5 System according to the manufacturer’s instructions. The thermal cycle used was 94 °C for 2 min followed by 39 cycles of 94 °C for 15 s, 60 °C for 1 min and a melting curve analysis was performed at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 1 s. The dissociation temperature range extends from 65 to 95 °C. Actin gene was used as the reference gene for normalization and the Livak ($\Delta\Delta C_T$) 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. The primer sequences used for qRT-PCR are listed in Table 1.

Statistical analysis

All the tissue culture experiments were repeated thrice, each consisting of three technical replicates and data were analyzed using one-way analysis of variance (ANOVA, $p < 0.05$). The significant differences among the means were determined by Duncan’s multiple range tests.

Table 1 Nucleotide sequences of primers used for qRT-PCR

Gene	Gene ID	Primer sequence (50–30)	References
PAL	CA05g20790	R: AACTGCTCAACAACAACATTACC F: CATCCAACACTTCTCCGTTAGG	Zhang et al. (2016)
NADH-GOGAT	CA03g19580	R:TCCAGTCAGGTTGAAGAAGAGAAG F: CAGATTGTGTCGTGAGGAGAGG	Zhang et al. (2016)
C4H	CA06g25940	R: ACGGTGAGCATTGGAGGAAGATG F: CCATTCGTGCGAGATTCAGGATTC	Zhang et al. (2016)
HCT	CA03g30250	R: ACTTGATGGCACGAGGACTAGC F: GCAACAGGAGTGGCAGTGAATATC	Zhang et al. (2016)
C3H	CA08g09680	R: GGCAATGGCTGAGGTGATC F: GTTGGCGTTGGCTCTGTG	Zhang et al. (2016)
CCoAOMT	CA02g14470	R: ATCGGGGAGAACTATGAGATTGGTC F: TTGTCCTTGTCAGCGTCCAC	Zhang et al. (2016)
GST	CA02g04610 G	R: ACTTTGGTGGTGATGACAATTTGG F: GTGAGGATGAGGAAGGGAGTTTG	Zhang et al. (2016)
pAMT	AF085149	F: ATACTCAAAGAGGGGCTGAAACAG R: TTCCAAATCCACATACCACCTCATC	Tanaka et al. (2016)
Pun1	AY819027	F: ATCTCAACGAGTGCCTACAGAAAAGACT R: GTGAACCAACTTTGATGGTAGCATTGAT	Tanaka et al. (2016)
Actin	CA04g06670	F: TGTTGGACTCTGGTGATGGTGTG R: CCGTTCAGCCGTGGTGGTG	Zhang et al. (2016)

Results and discussion

Callus culture induction

The placental segment explants cultured on callus induction medium showed increase in size accompanied by swelling and bulging with slight callusing on the margins in the first month of culture. When these explants were subcultured after 32 days, the explants cultured on callus induction medium containing 2 mg L⁻¹ 2, 4- D and 0.5 mg L⁻¹ K in produced friable callus after about 15 days. The effect of different auxins and cytokinins on callus induction from placental tissue explants at the end of the second month is shown in Table 2. Among the different combinations and concentrations of auxins with cytokinins used, MS medium containing 2 mg L⁻¹ 2, 4- D and 0.5 mg L⁻¹ Kin was found to be the most effective for callus induction producing rapidly growing friable calli (Fig. 1a–d) while the explants cultured on the media containing other combinations and concentrations of auxins with cytokinins produced compact calli with low proliferation rate. Similar effectiveness of the use of 2 mg L⁻¹ 2, 4-D and 0.5 mg L⁻¹ Kin together in callus induction from placenta explants of *Capsicum annum* L. was reported by Umamaheswari and Lalitha (2007). When the friable callus obtained from placental tissue explants cultured on medium containing 2 mg L⁻¹ 2,4-D with 0.5 mg L⁻¹ Kin were subcultured on fresh medium supplemented with the same combination of growth regulators after every 32 day, the callus tissue proliferated rapidly on the medium showing a growth curve characteristic of cultured plant cells. From the third month onwards, the biomass growth of the callus cultures were maintained in a similar manner till the sixth month of cultures studied (Fig. 2a). The growth curve of the callus cultures consisted of an initial slow lag phase comprising of the first 4 days followed by an exponential growth phase ending at day 15. The cultures then entered a

linear growth phase lasting from day 16 to day 23 followed by a stationary phase of growth from day 24 onwards. The maximum increase in callus biomass were obtained on day 24 of every cycle reaching up to 7.8 g FW culture⁻¹ and 0.56 g DW culture⁻¹ on the 4th month which was about 16-fold over the initial explant FW. During the first growth cycle comprising of 32 days, the amount of capsaicinoids in the placental tissue gradually decreased from 19 mg g⁻¹ FW capsaicin and 8.8 mg g⁻¹ FW dihydrocapsaicin in fresh placental tissue explants to 12 mg g⁻¹ FW capsaicin and 5.3 mg g⁻¹ FW dihydrocapsaicin respectively in the callus cultures (Fig. 2b, c). The decrease in capsaicin and dihydrocapsaicin content further continued in the second month showing maximum content of 6.3 mg g⁻¹ FW and 2.8 mg g⁻¹ FW at day 20 as the placental cells undergo dedifferentiation to form callus tissue. The optimum capsaicinoid accumulation were obtained on 20th day of every growth cycle and in the third growth cycle, the amount of capsaicin was 1.9 mg g⁻¹ FW and the amount of dihydrocapsaicin was 0.9 mg g⁻¹ FW, which further decreased a little to 1.65 mg g⁻¹ FW and 0.8 mg g⁻¹ FW in the fourth month. However, from the fourth month onwards, the capsaicin and dihydrocapsaicin content of the callus on 20th day of every growth cycle remained stable up to 6 months at an average of 1.6 mg g⁻¹ FW (equivalent to 12.5 mg culture⁻¹) and 0.78 mg g⁻¹ FW, respectively (Fig. 2b, c). The average content of capsaicinoid is maintained in the 12 months-old cell line maintained for further studies. In vitro capsaicinoid production from different explants of *Capsicum* species using various enhancement strategies have been reported earlier by several workers as reviewed by Kehie et al. (2014). However, the earlier reported capsaicin content in suspension cultures after enhancement using different strategies were in the range of 0.0307 mg culture⁻¹ in *Capsicum frutescens* Mill (Sudha and Ravishankar 2003) to 3.83 mg culture⁻¹ in *Capsicum frutescens* Mill (Rao and Ravishankar 2000), which is lower than the content (12.5 mg culture⁻¹) reported in the present

Table 2 Effects of plant growth regulators in callus induction from the placental tissue explants of *Capsicum chinense* Jacq. cv. ‘Umorok’

	Plant growth regulators (mg L ⁻¹)						Callus morphology		Response (%)
	2,4-D	IBA	IAA	NAA	BAP	Kin	Proliferation	Texture	
1						0.5	Low	Less friable	30
2						0.5	High	Friable	100
3						0.5	Low	Compact	50
		2				0.5	Low	Compact	40
				2		0.5	Very low	Compact	0
			2			0.5	Medium	Compact	70
2							Low	Compact	70
1					0.5		Low	Compact	70
2					0.5		Medium	Less Friable	60
3					0.5		Low	Compact	40

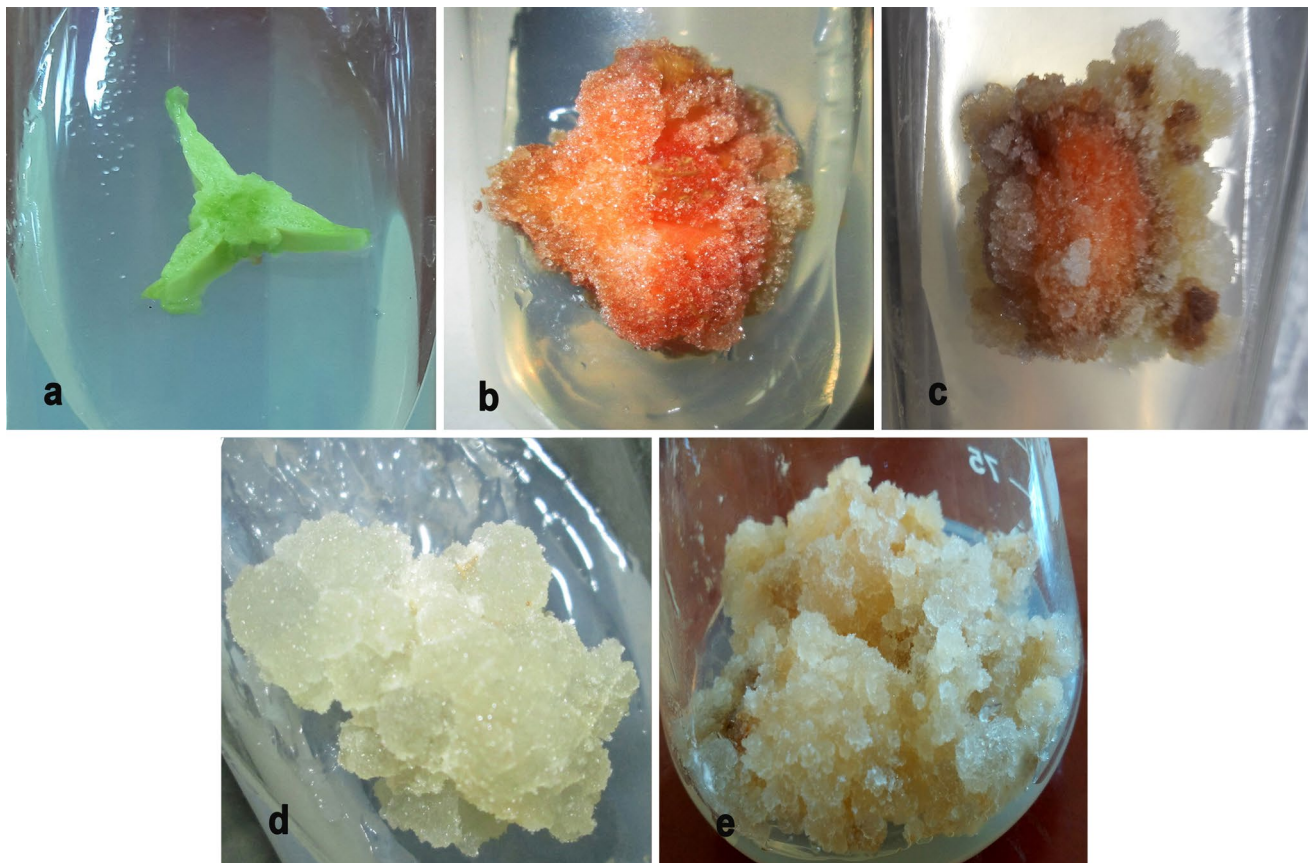


Fig. 1 Induction of callus cultures from placental tissue explants of *Capsicum chinense* Jacq. cv. 'Umorok' on MS medium containing 2 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} Kin: **a** placental explant inoculated

on MS medium; **b** compact callus at 1st month; **c** semi friable callus at 2nd month; **d** friable callus at 3rd month and **e** fully grown friable callus at 4th month culture

study. Similarly, suspension cultures established from callus derived from seedling explants of *Capsicum chinense* Jacq. 'Umorok' syn. 'Naga King Chilli' were shown to accumulate maximum of 1.6 mg g^{-1} FW capsaicin after enhancement, (Kehie et al. 2012) which is comparable to the capsaicin content in the callus (1.6 mg g^{-1} FW) without enhancement being reported in the present study. Earlier, several studies have shown the use of immobilized placental culture to accumulate higher amount of capsaicin when treated with elicitors or precursors. Capsaicin production was increased to 0.593 mg g^{-1} FW after enhancement in immobilized cell culture derived from seedlings of *Capsicum annuum* by Ravishankar et al. (1988) while Johnson and Ravishankar (1996) reported the accumulation of 5.78 mg g^{-1} DW capsaicin from cells of placenta of *Capsicum frutescens* Mill following immobilization and enhancement treatment. Similarly, in case of *Capsicum chinense* Jacq. (Habanero peppers), Aldana-Iuit et al. (2015) reported maximum capsaicinoid production from immobilized placental tissue of 1.56 mg g^{-1} DW. Thus, the capsaicinoids content in the present stable callus cultures reported in this study is higher than the content in most

of the earlier reports of capsaicin production by cultures derived from seedling explants of different *Capsicum* species. Although the content in immobilized placental tissues of different *Capsicum* species after enhancement is comparable to the content in the callus cultures obtained in the present study, the callus cultures have the advantage of rapid proliferation compared to the immobilized tissues. Thus, the present study reports the production of high capsaicinoid producing callus from the placental tissue explants of the highly pungent chilli cultivar *C. chinense* Jacq cv. 'Umorok' that have the potential to serve as an alternative source of capsaicinoids.

Expression patterns of capsaicinoid biosynthesis genes in callus cultures

To understand the gene expression patterns during callus formation from the placental tissue explants, the expression levels of genes involved in capsaicinoids biosynthetic pathway were determined by RT-qPCR analysis. Overall, all the candidate genes are down-regulated in callus cultures compared with the placental tissue. Nine structural

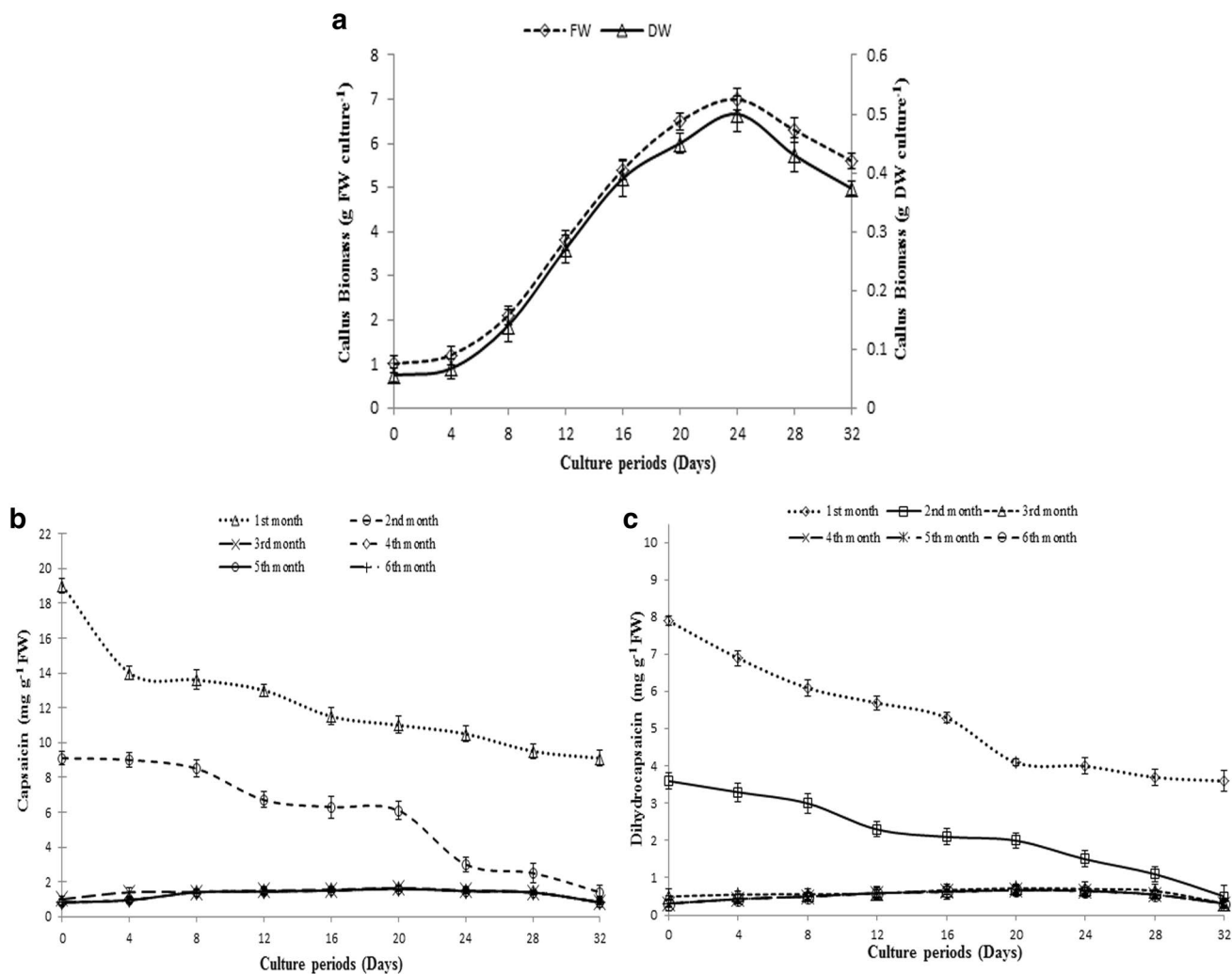


Fig. 2 Growth cycles for placental callus of *Capsicum chinense* Jacq. cv. ‘Umorok’: **a** biomass growth; **b** capsaicin and **c** dihydrocapsaicin accumulation during the growth cycles for 6 months after subsequent subcultures at 32nd day. Error bars shows standard errors

genes involved in capsaicinoids biosynthetic pathways, including Phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), hydroxycinnamoyl transferase (HCT), coumarate 3-hydroxylase (C3H), caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT), NADH-Glutamine oxoglutarate aminotransferase (NADH-GOGAT), glutathione S-transferase (GST), pAMT, and Pun1 were studied for comparative gene expression analysis and the expression pattern of each candidate gene is shown in Fig. 3. Among the nine candidate genes analysed, Pun1 and pAMT showed decreased expression during the first two months of culture compared to the placental tissue and were highly down regulated in the further growth cycles of callus cultures (Fig. 3h, i). Pun1 encodes an acyltransferase enzyme, which is believed to catalyze the last step in the capsaicinoid biosynthetic pathway (Stewart et al. 2005; Lang et al. 2006) and loss-of-function in Pun1 gene results in non-pungency (Stewart et al. 2005).

The expression pattern of Pun1 gene is consistent with capsaicinoid accumulation in callus cultures during the growth cycles. The expression of Pun1 gene was 3 and 4-fold lower respectively than the placental tissue in the first and second callus growth cycles and the expression was greatly reduced in the further growth cycles (Fig. 3h). A similarity was also observed for pAMT gene expression pattern, which showed approximately 9- and 13-fold lower expression in the first two months and the expression level was almost negligible from the third month onwards (Fig. 3i). These expression patterns in callus cultures correspond with the capsaicinoid accumulation during the growth cycles. pAMT gene catalyses the conversion of vanillylamine from vanillin in phenylpropanoid pathway, which is an important step in capsaicinoid biosynthesis (Lang et al. 2009; Tanaka et al. 2015). Earlier, pAMT and Pun1 genes were reported to play crucial roles in capsaicinoid accumulation in chilli peppers

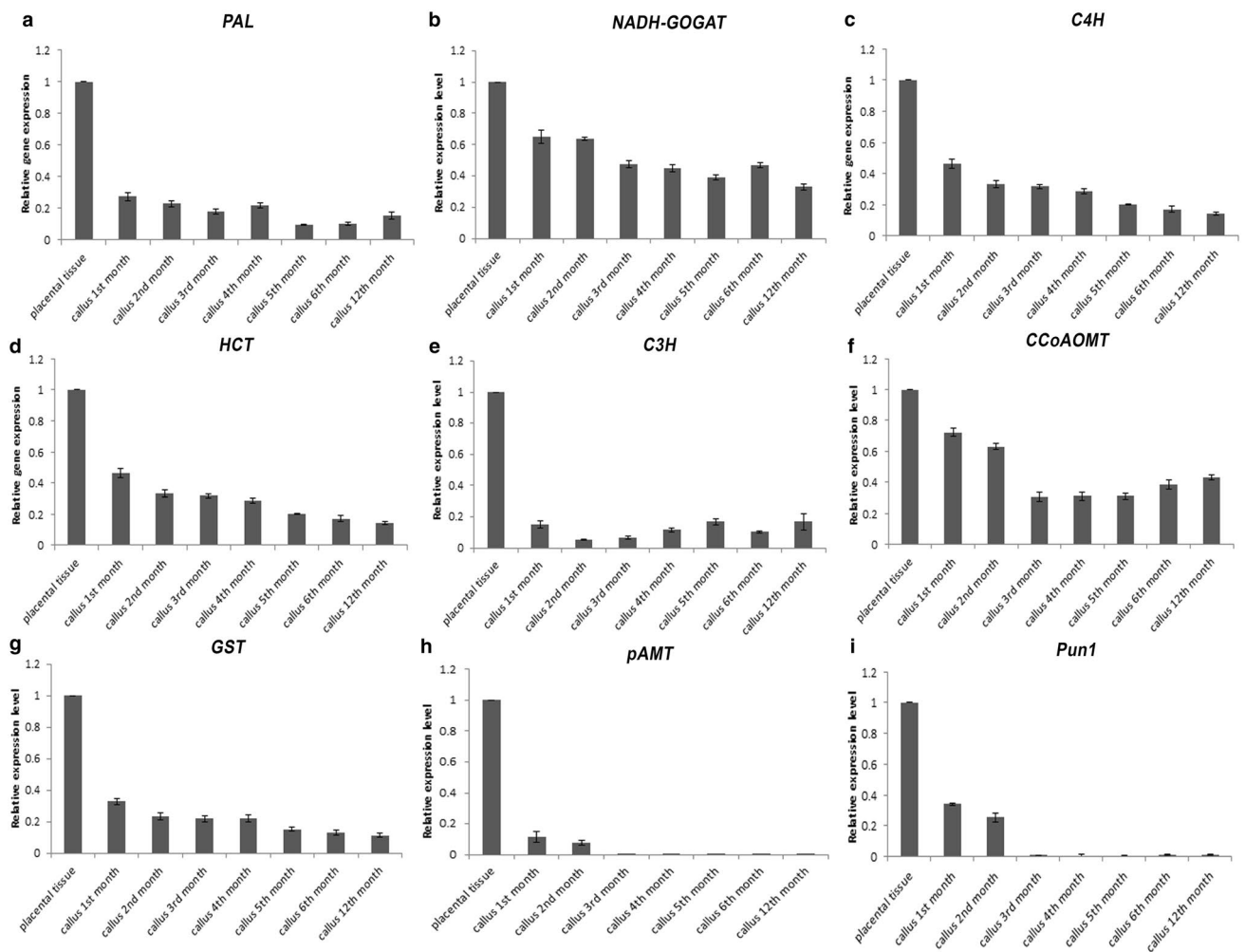


Fig. 3 Differential gene expression levels of capsaisinoid biosynthesis genes during callus formation. The relative expression levels of 9 structural genes were determined by comparative qRT-PCR analysis in the placental tissue and 1st–6th and 12th months-old cal-

lus cultures: **a** PAL, **b** NADH-GOGAT, **c** C4H, **d** HCT, **e** C3H, **f** CCoAOMT, **g** GST, **h** pAMT and **i** Pun1. All data are presented as means of three repeats. Error bars shows standard errors

including *C. Chinense* (Stewart et al. 2005; Ogawa et al. 2015; Sarpras et al. 2016), and silencing of the pAMT gene expression or loss-of-function in a particular mutant (CH-19 Sweet) has been shown to result in significant reduction in capsaicin production in placental tissues (Lang et al. 2009). Similarly, in the present study, the expression pattern of these genes are observed to correspond with capsaisinoid accumulation in callus cultures and down-regulation of these two putative genes (pAMT and Pun1 gene) in the friable callus cultures may be responsible for the lower capsaisinoid accumulation in callus cultures compared to placental tissues. For the other candidate genes (PAL, C4H, HCT, C3H, CCoAOMT, NADH-GOGAT and GST), the expression patterns were not consistent with the capsaisinoid accumulation during the growth cycles (Fig. 3a–g). The reason of this inconsistency might be that they are involved in early stages of the capsaisinoid biosynthesis pathway and also participate

in the production of other secondary metabolites such as flavonoids, coumarins and lignins (Vogt 2010; Fraser and Chapple 2011). Thus, the present study shows, for the first time, the regulation of capsaisinoid accumulation during callus formation from placental tissue explants of *C. chinense* Jacq. and observed marked down-regulation in the expression of two candidate genes (pAMT, and Pun1) in the callus cultures and these two genes may be considered as potential targets for enhancement of in vitro capsaisinoid production using different strategies including metabolic engineering. Studying the mechanism underlying the down-regulation of these capsaisinoid biosynthesis pathway genes in cells cultures will be useful in controlling the in vitro capsaisinoid production potential of cell cultures.

Acknowledgements The authors are thankful to Department of Biotechnology, Government of India, for financial support.

Author Contributions KCK: planned the experiments and Carried out the experiment, analysed the data, wrote the manuscript with the help of KS. SKS: helped in performing the experiment, supervised the experiments. KS: conceived the idea, planned the experiments, supervised the experiments, revised and review the data and manuscript.

Funding This study was funded by the Department of Biotechnology, Government of India (Grant No. BT/PR6387/AGII/106/876/2012).

Compliance with ethical standards

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

References

- Aldana-Iuit JG, Sauri-Duch E, Miranda-Ham ML, Castro-Concha LA, Cuevas-Glory LF, Vázquez-Flota FA (2015) Nitrate promotes capsaicin accumulation in *Capsicum chinense* immobilized placentas. *BioMed Res Int*. <https://doi.org/10.1155/2015/794084>
- Ancona-Escalante WR, Baas-Espinola FM, Castro-Concha LA, Vázquez-Flota FA, Zamudio-Maya M, Miranda-Ham ML (2013) Induction of capsaicinoid accumulation in placental tissues of *Capsicum chinense* Jacq. requires primary ammonia assimilation. *Plant Cell Tissue Organ Cult* 113:565–570
- Aza-Gonzalez C, Nunez-Palenius HG, Ochoa-Alejo N (2011) Molecular biology of capsaicinoid biosynthesis in chili pepper (*Capsicum* spp.). *Plant Cell Rep* 30(5):695–706
- Collins MD, Wasmund LM, Bosland PW (1995) Improved method for quantifying capsaicinoids in *Capsicum* using high performance liquid chromatography. *Hort Sci* 30(1):137–139
- Curry J, Aluru M, Mendoza M, Nevarez J, Melendrez M, O'Connell MA (1999) Transcripts for possible capsaicinoid biosynthetic genes are differentially accumulated in pungent and non-pungent *Capsicum* spp. *Plant Sci* 148:47–57
- Deal CL, Schnitzer TJ, Lipstein E, Seibold JR, Stevens RM, Levy MD, Albert D, Renold F (1999) Treatment of arthritis with topical capsaicin: A double-blind trial. *Clin Ther* 13:383–395
- Fraser CM, Chapple C (2011) The phenylpropanoid pathway in Arabidopsis, *The Arabidopsis book*. American Society of Plant Biologist Doi: 1199/tab.0152
- Fujiwaka H, Suzuki T, Iwai K (1980) Intracellular localization of capsaicin and its analogues in *Capsicum* fruit. II. The vacuole as the intracellular accumulation site of capsaicinoids in the protoplast of *Capsicum* fruit. *Plant Cell Physiol* 21:1023–1030
- Holden MA, Hall RD, Lindsey K, Yeoman MM (1987) Capsaicin biosynthesis in cell cultures of *Capsicum frutescens*. In: Webb C, Mavituna F, Foria JJ (eds) Process possibilities for plant and animal cell cultures. Harwood, Chichester, pp 45–63
- Iwai K, Suzuki T, Fujiwaka H (1979) Formation and accumulation of pungent principle of hot pepper fruits, capsaicin and its analogues, in *Capsicum annum* var. annum cv. karayatsubusa at different growth stages after flowering. *Agric Biol Chem* 43(12):2493–2498. <https://doi.org/10.1080/00021369.1979.10863843>
- Johnson TS, Ravishankar GA (1996) Precursor biotransformation in immobilized placental tissues of *Capsicum frutescens* Mill. I. influence of feeding intermediate metabolites of the capsaicinoid pathway on capsaicin and dihydrocapsaicin accumulation. *J Plant Physiol* 147:481–485
- Johnson TS, Ravishankar GA, Venkataraman LV (1990) In vitro capsaicin production by immobilized cells and placental tissues of *Capsicum annum* L. grown in liquid medium. *Plant Sci* 70(2):223–229
- Johnson TS, Ravishankar GA, Venkataraman LV (1996) Biotransformation of ferulic acid and vanillylamine to capsaicin and vanillin in immobilized cell cultures of *Capsicum frutescens*. *Plant Cell Tissue Organ Cult* 44:117. <https://doi.org/10.1007/BF00048188>
- Kehie M, Kumaria S, Tandon P (2012) Osmotic stress induced-capsaicin production in suspension cultures of *Capsicum chinense* Jacq. cv. Naga King Chili. *Acta Physiol Plant* 34:2039–2044. <https://doi.org/10.1007/s11738-012-0991-1>
- Kehie M, Kumaria S, Tandon P, Ramchiary N (2014) Biotechnological advances on in vitro capsaicinoids biosynthesis in capsicum: a review. *Phytochem Rev*. <https://doi.org/10.1007/s11101-014-9344-6>
- Kempaiiah RK, Manjunatha H, Srinivasan K (2005) Protective effect of dietary capsaicin on induced oxidation of low-density lipoprotein in rats. *Mol Cell Biochem* 275:7–13
- Kobata K, Todo T, Yazawa S, Iwai K, Watanabe T (1998) Novel capsaicinoid-like substances, capsate and dihydrocapsate, from the fruits of a nonpungent cultivar, CH-19 Sweet, of pepper (*Capsicum annum* L.). *J Agric Food Chem* 46(5):1695–1697. <https://doi.org/10.1021/jf980135c>
- Lang YQ, Yanagawa S, Sasanuma T, Sasakuma T (2006) A gene encoding a putative acyl-transferase involved in pungency of *Capsicum*. *Breed Sci* 56(1):55–62
- Lang YQ, Kisaka H, Sugiyama R, Nomura K, Morita A, Watanabe T, Tanaka Y, Yazawa S, Miwa T (2009) Functional loss of pAMT results in biosynthesis of capsinoids, capsaicinoid analogs, in *Capsicum annum* cv. CH-19 sweet. *Plant J* 59:953–961
- Lindsey K (1985) Manipulation by nutrient limitation, of the biosynthetic activity of immobilized cells of *Capsicum frutescens* Mill. cv. annum. *Planta* 165:126–133
- Liu S, Chen C, Chen G, Cao B, Chen Q, Lei J (2012) RNA-sequencing tag profiling of the placenta and pericarp of pungent pepper provides robust candidates contributing to capsaicinoid biosynthesis. *Plant Cell Tissue Organ Cult* 110:111–121. <https://doi.org/10.1007/s11240-012-0135-8>
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* 25(4):402–408
- Moore DJ, Moore DM (2003) Synergistic Capsicum-tea mixtures with anticancer activity. *J Pharm Pharmacol* 55:987–994
- Ogawa K, Murota K, Shimura H, Furuya M, Togawa Y, Matsumura T (2015) Evidence of capsaicin synthase activity of the Pun1-encoded protein and its role as a determinant of capsaicinoid accumulation in pepper. *BMC Plant Biol* 15:93. <https://doi.org/10.1186/s12870-015-0476-7>
- Park M, Lee J, Koeun Han K, Jang S, Han J, Lim J, Jung J, Kang B (2019) A major QTL and candidate genes for capsaicinoid biosynthesis in the pericarp of *Capsicum chinense* revealed using QTLseq and RNAsEq. *Theor Appl Genet* 132:515–529. <https://doi.org/10.1007/s00122-018-3238-8>
- Perucka I, Materska M (2001) Phenylalanine ammonia-lyase and antioxidant activities of lipophilic fraction of fresh pepper fruits *Capsicum annum* L. *Innov Food Sci Emerg Technol* 2:189–192
- Rao SR, Ravishankar GA (2000) Biotransformation of protocatechuic aldehyde and caffeic acid to vanillin and capsaicin in freely suspended and immobilized cell cultures of *Capsicum frutescens*. *J Biotechnol* 76:137–146
- Ravishankar GA, Sarma KS, Venkataraman LV, Kadyan AK (1988) Effect of nutritional stress on capsaicin production in immobilized cell cultures of *Capsicum annum*. *Curr Sci* 57:381–383
- Rowland BJ, Villalon B, Burns EE (1983) Capsaicin production in sweet bell and pungent jalapeno peppers. *J Agric Food Chem* 31(3):484–487. <https://doi.org/10.1021/jf00117a005>
- Salgado-Roman M, Botello-Alvarez E, Rico-Martínez R, Jiménez-Islas H, Cárdenas-Manríquez M, Navarrete-Bolaños JL (2008) Enzymatic treatment to improve extraction of capsaicinoids and

- carotenoids from chili (*Capsicum annuum*) fruits. *Agric Food Chem* 56(21):10012–10018. <https://doi.org/10.1021/jf801823m>
- Sanatombi K, Sharma GJ (2008) Capsaicin content and pungency of different *Capsicum* spp. cultivars. *Not Bot Horti Agrobot Cluj-Napoca* 36(2):89–90
- Sarpras M, Gaur R, Sharma V, Chhapekar SS, Das J, Kumar A et al (2016) Comparative analysis of fruit metabolites and pungency candidate genes expression between bhut jolokia and other *Capsicum* species. *PLoS ONE* 11(12):e0167791. <https://doi.org/10.1371/journal.pone.0167791>
- Sawynok J (2005) Topical analgesics in neuropathic pain. *Curr Pharma Design* 11:2995–3004
- Stewart C, Kang BC, Liu K, Mazourek M, Moore SL, Yoo EY, Kim BD, Paran I, Jahn MM (2005) The Pun1 gene for pungency in pepper encodes a putative acyltransferase. *Plant J* 42:675–688
- Sudha G, Ravishankar GA (2003) Influence of methyl jasmonate and salicylic acid in the enhancement of capsaicin production in cell suspension cultures of *Capsicum frutescens* Mill. *Curr Sci* 85:8–25
- Sugiyama R (2017) Capsaicinoids production and accumulation in epidermal cells on the internal side of the fruit pericarp in ‘Bhut Jolokia’ (*Capsicum chinense*). *Cytologia* 82(3):303–306. <https://doi.org/10.1508/cytologia.82.303>
- Suzuki T, Kawada T, Lwai K (1981) Biosynthesis of acyl moieties of capsaicin and its analogues from valine and leucine in *Capsicum* fruits. *Plant Cell Physiol* 22:23–32
- Tanaka Y, Sonoyama T, Muraga Y, Koeda S, Goto T, Yoshida Y, Yasuba K (2015) Multiple loss-of-function putative aminotransferase alleles contribute to low pungency and capsinoid biosynthesis in *Capsicum chinense*. *Mol Breed* 35:142
- Tanaka Y, Nakashima F, Kirii E, Goto T, Yoshida Y, Yasuba K (2016) Difference in capsaicinoid biosynthesis gene expression in the pericarp reveals elevation of capsaicinoid contents in chili peppers (*Capsicum chinense*). *Plant Cell Rep*. <https://doi.org/10.1007/s00299-016-2078-8>
- Umamaheswari A, Lalitha V (2007) In vitro effect of various growth hormones in *Capsicum annuum* L. on the callus induction and production of capsaicin. *J Plant Sci* 2(5):545–551
- Vogt T (2010) Phenylpropanoid biosynthesis. *Mol Plant* 3:2–20
- Yazawa S, Suetomi N, Okamoto K, Namiki T (1989) Content of Capsaicinoids and Capsaicinoid-like substances in fruit of pepper (*Capsicum annuum* L.) hybrids made with ‘CH-19 Sweet’ as a parent. *J Japan Soc Hort Sci* 58(3):601–607
- Zhang Z, Zhao S, Liu G, Huang Z, Cao Z, Cheng S, Lin S (2016) Discovery of putative capsaicin biosynthetic genes by RNA-Seq and digital gene expression analysis of pepper. *Sci Rep* 6:34121. <https://doi.org/10.1038/srep34121>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.