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GENOME-WIDE IDENTIFICATION, MOLECULAR CHARACTERIZATION AND EXPRESSION ANALYSIS OF THE ROP GTPase FAMILY IN PEPPER (*CAPSICUM ANNUUM*)

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Abstract

ROP/RAC GTPases is a plant-specific subfamily of Rho GTPases that plays a versatile role in the regulation of plant growth, development, in hormone signal transduction and response to the environment. Prior to the present study, only one *Rop* gene in pepper has been described. However, with the recent release of the draft genome sequence of pepper allowes us to conduct a genome wide search to identify how many *Rop* family members existed in pepper genome. We carried out bioinformatics analysis to establish the conserved as well as divergent regions on the protein sequences, phylogenetically analysis and the corresponding result shows that, CaROPs could be distributed into four groups as described in the literature for their homologs in *Arabidopsis*. To understand the function of nine *Rop* genes in pepper, we accordingly studied the tissue, fruit development and ripening expression patterns of *CaRop* genes by obtained RNA-seq data from public database. From our analysis, we realized that the expression of *CaRop* genes shows no total tissue or developmental specific expression. Furthermore, gene expression profiles of *CaRop* in response to environment stresses and hormone treatment, such as inoculated with *Ralstonia solanacearum*, by heat stress as well as treated with four phytohormones respectively and evaluated with real time RT-PCR. The potential involvement of specific *CaRop* genes in growth, fruit development, ripening, environment stresses as well as hormone responses discussed and may lay the foundation for future functional analysis to unravel their biological roles.

Key words: ROP GTPase, Pepper, Phylogenetic analysis, Phytohormones and *Ralstonia solanacearum*.

Introduction

Rho-family small GTPases are monomeric guanine nucleotide-binding proteins that act as versatile molecular switches in the regulation of many important cellular processes. Plants possess a single subfamily of Rho GTPases termed ROPs (Rho-related GTPase from plants), and their amino acid sequence are most similar to RACs, thus have also been referred to as RACs (Yang, 2002). ROP/RAC is the solo subfamily of signaling small GTPases in plants. The solitude of ROP in small GTPase signaling in plants could explain why ROPs have emerged as signal integrator and coordinators of a wide range of signaling pathways that regulate fundamental developmental processes, responses to pathogens and environmental stresses (Fu *et al*., 2008). Moreover, there is strong evidence that ROPs participate in regulating plant responses to several hormones such as abscisic acid, auxin and brassinosteroids (Fischer *et al*., 2006; Fujita *et al*., 2006; Li *et al*., 2005; Xin *et al*., 2005).

Since the first plant homolog of Rho-related GTPases was discovered in the garden pea, other members of the ROP family have been identified widely from a number of plant species such as *Arabidopsis* (Hai *et al*., 1998), barley (Schultheiss *et al*., 2003), rapeseed (Chan & Pauls, 2007), grapevine (Abbal *et al*., 2007), peach (Falchi *et al*., 2010), rice (Christensen *et al*., 2003), and maize (Hassanain *et al*., 2000). Genome sequencing reveals *Arabidopsis* contains 11 *Rop* genes: three closely related *Rops*, *Rop1*, *Rop3* and *Rop5* are expressed in pollen and functionally redundant in the control of pollen tube tip growth (Fu *et al*., 2008; Vernoud *et al*., 2003). However, *Rop2*, *Rop4* and *Rop6* mediated auxin regulation of jigsaw puzzle appearance in pavement cells also participated in root hair elongation (Fu *et al*., 2005; Jones, 2002; Jones *et al*., 2007; Xin *et al*., 2005; Xu *et al*., 2010). Immune responses mediated by *Rop* genes have been studied in rice and showed that a series of immune responses induced by CA-OsRAC1 leads to enhance disease resistance to virulent race of *Magnaporthe grisea* and *Xanthomonas oryzae* (Ono *et al*., 2001). In tobacco, expression of DN-OsRAC1 delays lesion formation when inoculated tobacco mosaic virus, which is correlated with reduced ROS production, altered PR gene expression and reduction of salicylic acid accumulation (Moeder *et al*., 2005).

Pepper, the world's most widely grown spice crop is not only satisfies people's spicy taste as a major ingredient in cuisines, but also as a model crop systems for research in the mechanisms of resistance against biotic and abiotic stresses (Dang *et al*., 2013; Kim *et al*., 2014). Recently, the genome of *C. annuum* cv. CM334 and Zunla-1 were sequenced and released. These new reference genome sheds light on the biology of the pepper's hallmark pungency or spiciness, its fruit-ripening and disease-resistance mechanisms (Kim *et al*., 2014; Qin *et al*., 2014). Compared to the extensive studies of ROP GTPases in *Arabidopsis* and rice, no research has been reported in pepper so far. Considering the economic importance of this crop as well as the ROP as significant signal integrator and coordinators in plant developmental processes and stress responses, it was of interest for us to characterize the ROP family in pepper. In this study, we performed a genome-wide analysis and identified nine ROP GTPases coding genes distributed in pepper. Then, we analyzed the expression profiles of these *CaRop* genes in pepper across different tissues, fruit development and ripening by obtained from public RNA-seq databases.

Additionally, real time RT-PCR was employed to detect the transcript abundance of *CaRop* genes under *Ralstonia solanacearum* challenge, heat stress as well as four phytohormones treatment, in order to gain further insights into their roles in pepper defense responses.

Materials and Methods

Sequence database searches: *Arabidopsis* ROP protein sequences were obtained from TAIR (http://www.Arabidopsis.org), 11 AtROP proteins as query sequences and carried out BLASTP searches against the annotated CM334 pepper genome platform (http://peppergenome.snu.ac.kr/) and Zunla-1 genome database (http://peppersequence.genomics.cn), respectively. Candidate proteins were selected and further checked whether it represented or not a complete CDS. After that, the ROP-like sequences were reconfirmed by in turn used reiteratively to search the CM334 annotated proteins and CDS until no new sequences were found. Files of CM334 annotation CDS (v1.5) and Zunla-1 CDS (v2.0) were download from the pepper genome platform and Zunla-1 pepper genome respectively.

Multiple sequence alignment and phylogenetic analysis: The amino acid sequence of nine CaROP proteins and the selected AtROP9 protein (At4g28950) was used to create multiple protein sequence alignments using DNAMAN software and default setting was applied for the alignment in Fig. 1. For phylogenetic analysis ,amino acid sequence alignments and comparision of the nine CaROP proteins and eleven AtROP proteins were performed with ClustalW (Thompson *et al*., 1994) and MEGA5.05 program (Tamura *et al*., 2011) using the neighbor-joining method.

RNA-seq data extract and cluster analysis: The RNA-seq data for various tissues, pericarp and placenta development and ripening stages were obtained from public database (Kim *et al*., 2014), and the corresponding *CaRop* genes expression value were extracted (listed in Table 2) and imported into Genesis program (v1.75) (Sturn *et al*., 2002) for cluster analysis. Hierarchical clustering was performed based on Pearson coefficients with average linkage rule.

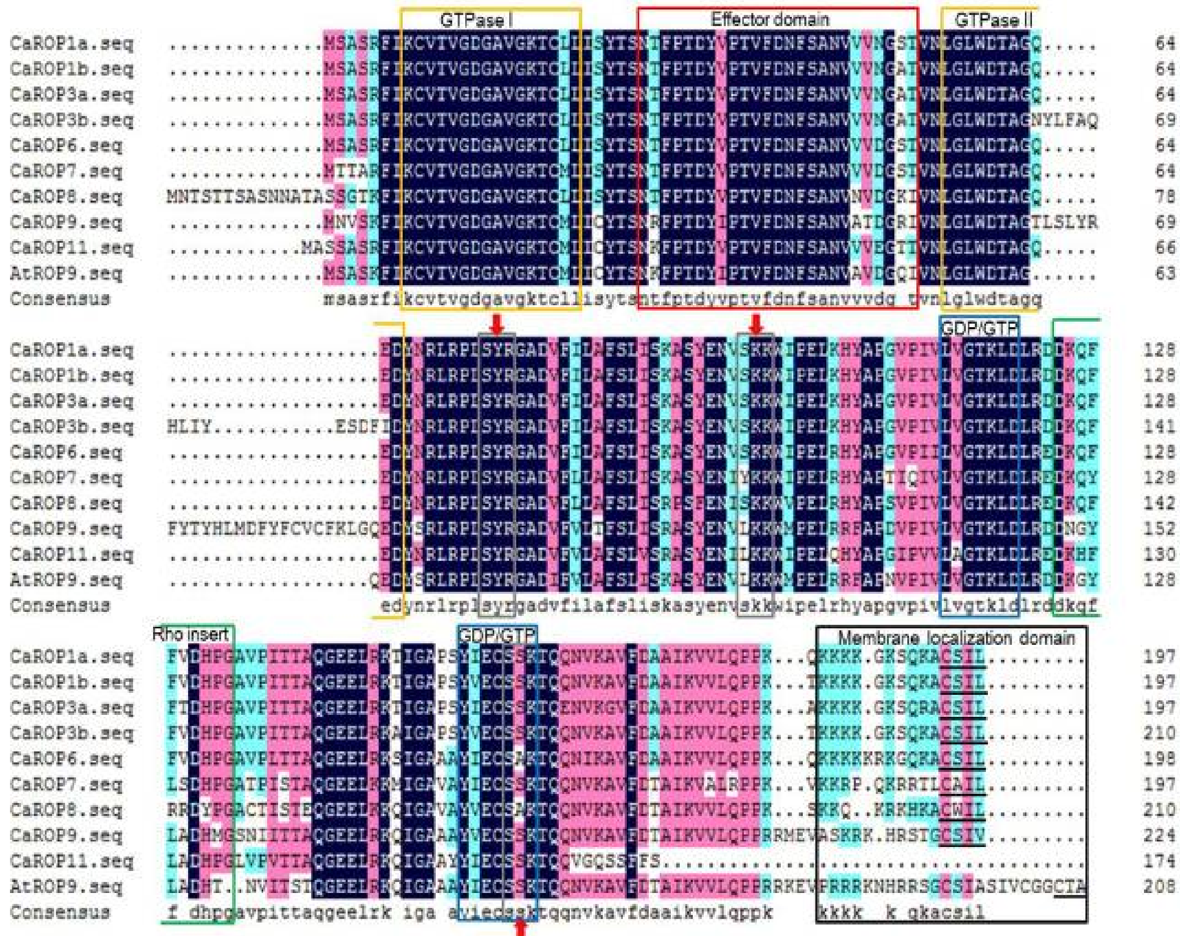

Fig. 1. Alignment of the nine *C. annuum* ROP deduced amino acid sequences with ROP9 of *Arabidopsis*. Identical amino acid residues are highlighted by black backgrounds. Disctinct functional domains are assigned according to Zheng & Yang (2000): the N-terminal two GTPase domains (GTPase I and GTPase II), an effector domain (Effector domain), two domains responsible for GDP/GTP-binding (GDP/GTP), the RHO insert region (Rho insert), and the Membrane localization domain. The three motifs are shown in grey boxes and highlighted by red arrowhead are putative serine/threonine-dependent phosphorylation sites.

RNA-seq data extract and cluster analysis: The RNA-seq data for various tissues, pericarp and placenta development and ripening stages were obtained from public database (Kim *et al*., 2014), and the corresponding *CaRop* genes expression value were extracted (listed in Table 2) and imported into Genesis program (v1.75) (Sturn *et al*., 2002) for cluster analysis. Hierarchical clustering was performed based on Pearson coefficients with average linkage rule.

Plant materials and treatments: Pepper (*C. annuum*, 68-2#, a cultivar provided by pepper breeding group in Fujian Agriculture and Forestry University) seeds were sown in a soil mix [peat moss: perlite (2/1)] in plastic pots, and grown for additional 3-4 weeks. Pepper plants were grown in a greenhouse with 16/8 h photoperiod at 25±2°C, 60-70μmol photons m^{-2} s^{-1}, and a relative humidity of 70%. For hormone treatment, pepper plants at the four-leaf stage were sprayed with 10 μM 1-Naphthaleneacetic acid (thereafter with NAA), 10 μM 24-epibrassinolide (thereafter with EBR) (both dissolved in 1% ethanol). Mock plants were sprayed with 1% ethanol. One month old pepper plants were sprayed with 100 μM abscisic acid (thereafter with ABA) and 10 mM ethephon (thereafter with ET) in ddH_2O and mock plants were sprayed with ddH_2O. For *R. solanacearum* inoculation and heat stress treatment were performed as described in (Dang *et al*., 2013). Samples collected at various hour post treatments or inoculation (thereafter with hpt or hpi), were immediately frozen in liquid nitrogen and stored at -80°C before use. To ensure that hormone treatments were eliciting expected responses in our research, we first examined the responses of ABA and ET responsive genes as described in (Dang *et al*., 2013). Also examined a few genes that are proposed to be orthologs of *Arabidopsis* genes reported specifically respond to NAA and BR treatment according to (Nemhauser *et al*., 2006). Results shown that expression of these marker genes was induced when treated by respective hormones, verified the efficiency of these hormone applications (data not shown).

RNA extract and real-time RT-PCR: Total RNA was isolated from reserved samples using Trizol reagent (Invitrogen), according to the manufacture's instruction. The RNA samples were reverse-transcribed using the PrimeScript RT-PCR kit (TaKaRa, China). A 10-fold dilution of the resulting cDNA was amplified employing SYBR® Premix ExTaq™ II using BIO-RAD CFX96 Real-time PCR system (Foster City, CA, USA) in standard volume with the following program: 95°C for 30 s; 40 cycles of 95°C for 5 s, 60°C for 34 s, with a final melt gradient starting from 60°C and heating to 95°C at a rate of each 1°C increment. The amplification of the target genes was investigated (see Table 3 for gene-specific primers) and the primer specificity was reconfirmed by analysing dissociation curves of the PCR amplification products. The Ct (threshold cycle), defined as the real-time RT-PCR cycle at which a statistically significant increase of reporter fluorescence was first detected, used as a measure for the starting copy numbers of the target genes. Three replicates of each experiment were performed. Data were analyzed by the Livak method and expressed as a normalized relative expression level ($2^{-\Delta\Delta CT}$) of respective genes (Livak & Schmittgen, 2001). The relative transcript levels of the *CaRop* genes were normalized to the transcript levels of *CaEF-1a* and the default value for each gene was set as 1 at 0 h point. In each case, three technical replicates were performed for each of at least three independent biological replicates, each value represents mean ± standard error of three replicates. Single or double asterisks indicating a significant difference of $p<0.05$ or $p<0.01$ between controls and treatments respectively.

Results

Identification of genes encoding ROP GTPases in *Capsicum annuum*: To identify ROP GTPases coding genes in the pepper genome, we used eleven *Arabidopsis* ROP proteins as query sequences, and then carried out BLASTP searches against a total of 34903 predicated pepper gene coding proteins from the *C. annuum cv.* CM334 pepper genome platform (Kim *et al*., 2014). The sequences were selected as candidate proteins if the Limite Expect Value was ≤ -50, then each candidate protein sequences were further checked whether it represented or not a complete sequence. After that, the ROP-like sequences were reconfirmed by in turn used reiteratively to search the CM334 pepper annotated proteins and CDS until no new sequences were found. In this manner, we identified nine ROP GTPase protein coding genes in the CM334 pepper genome. By the same manner, also nine *Rop* genes were identified in genome of Zunla-1(data not show). The *CaRop* genes were named according to their clear sequences similarity to *Rops* from *A.thaliana* and showed below (Table 1) (Abbal *et al*., 2007).

Sequences alignment and structure analysis of the nine *CaRop* genes: As expected, Sequence identity among the open reading frames of nine *CaRops* ranged from 55.2% to 90.5% at the nucleotide level, and 65.57% to 98.5% similarities at the amino acid sequences. This range of sequences conservation is similar in *Arabidopsis* ROPs. The *CaRops* encoded proteins with 197-224 residues, with predicated molecular masses about 21 kDa, and theoretical isoelectric points from 9.1 to 9.3. Except the *CaRop11*, due to missing the C terminal region results a lower molecular mass and theoretical isoelectric. The amino acid sequences of all CaROPs contain the common characteristics of other plant ROPs, which typically possess the seven functional domains according to previously researches (Zheng & Yang, 2000).

Table 1. Characteristics of the deduced ROP GTPases from *C. annuum*, accession number, genome location, the theoretical pI, molecular mass and their closest *Arabidopsis* homologues.

Gene	Accession number[1]	The genome location	Number of residues (aa)	Pmm[2] (kDa)	Theoretical pI	*Arabidopsis* closest homologue	*Arabidopsis* AGI gene
CaRop1a	CA00g55680	PGAv.1.5.scaffold1274	197	21.5	9.3	*AtROP1*	AT3G51300
CaRop1b	CA00g84620	PGAv.1.5.scaffold1817	197	21.4	9.3	*AtROP1*	AT3G51300
CaRop3a	CA02g05500	PGAv.1.5.scaffold225	197	21.4	9.2	*AtROP3*	AT2G17800
CaRop3b	CA04g03710	PGAv.1.5.scaffold354	210	23.0	9.1	*AtROP3*	AT2G17800
CaRop6	CA00g09420	PGAv.1.5.scaffold588	198	21.7	9.4	*AtROP6*	AT4G35020
CaRop7	CA02g04310	PGAv.1.5.scaffold79	197	21.9	9.3	*AtROP7*	AT5G45970
CaRop8	CA00g20730	PGAv.1.5.scaffold756	210	23.2	9.3	*AtROP8*	AT2G44690
CaRop9	CA00g82910	PGAv.1.5.scaffold1774	224	25.2	9.0	*AtROP9*	AT4G28950
CaRop11	CA03g24880	PGAv.1.5.scaffold781	174	19.1	6.2	*AtROP11*	AT5G62880

The theoretical pI and molecular mass analysis was performed using compute pI/Mw tool on (http://web.expasy.org/compute_pi/).
1 Indicated the corresponding accession numbers derived from the pepper CM334 annotation CDS (v1.5).
2 Indicated "Predicated molecular mass".

Table 2. RNA-seq data for genes expression on each tissue and several development stages, which were deposited on (Kim *et al.*, 2014). We extracted the corresponding *CaRop* genes expression value to construct heatmap.

Gene	Leaf	Root	Stem	PC-6DPA	PC-16DPA	PC-25DPA	PC-MG	PC-B	PC-B5	PC-B10	PL-6DPA	PL-16DPA	PL-25DPA	PL-MG	PL-B	PL-B5	PL-B10
CaRop1a	20.0	56.2	52.7	62.5	36.2	40.9	25.1	20.2	19.4	24.2	46.9	45.5	40.8	32.1	30.4	34.4	29.3
CaRop1b	26.0	69.1	58.8	42.3	40.9	28.1	25.5	18.8	21.4	19.0	62.2	50.9	36.5	30.4	27.4	33.0	23.4
CaRop3a	3.3	9.1	13.5	15.0	6.7	6.2	3.3	1.4	1.2	2.3	14.8	2.9	3.9	2.8	3.8	5.2	3.4
CaRop3b	6.7	14.8	15.6	10.1	7.8	7.0	4.8	3.2	4.1	5.0	18.9	18.6	8.6	7.7	7.9	10.0	7.2
CaRop6	35.6	73.2	72.0	171.1	230.2	193.9	116.2	74.5	72.0	95.7	68.7	82.8	70.0	50.1	55.2	100.2	90.5
CaRop7	3.2	45.8	135.9	0.85	3.1	1.1	0.89	0.14	0.0	0.0	6.1	9.7	2.0	0.9	0.2	0.0	0.2
CaRop8	3.1	16.8	29.7	3.7	1.9	2.8	1.0	0.6	0.5	0.1	14.4	8.2	4.1	1.7	1.2	0.5	0.0
CaRop9	7.7	43.9	131.8	19.6	8.6	6.5	3.1	1.8	1.6	0.9	90.5	11.1	2.8	3.8	1.3	0.6	0.0
CaRop11	13.7	32.5	38.3	126.8	122.8	101.7	74.3	41.2	38.2	34.5	27.4	16.4	37.6	21.0	28.6	25.2	19.8

Table 3. Gene specific primers used for real-time quantitative PCR experiments.

Genes	Forward primer sequence	Reverse primer sequence	Size (bp)
CaRop1a	5'-GGATGATAAGCAGTTCTTCGT	5'-TGGAGCACGACCTTGATAGCC	167
CaRop1b	5'-CTGCTGGACAGGAGGACTACAAT	5'-CTATTGGGACACCAGGAGCATAG	150
CaRop3a	5'-CGAGATGATAAGCAATTCTTCAC	5'-GACTTTCCTTTCTTTTTCTTTGC	202
*CaRop3b**	5'-TTGCTGCACTTACATCTTTGATGG	5'-TTGAAGCATTGTAATTCGAGCCAA	108
CaRop6	5'-GACCATCCAGGTGCCGTTCCGC	5'-CAAAAACAGCCTTAATATTCTGT	120
CaRop7	5'-TAAGTGATCATCCAGGGGCTACT	5'-CACCTTAGGGGGTCGCAATGCT	142
CaRop8	5'-GAGAGGACAAGCAGTTTAGAAGG	5'-GTGTTTTCTTTTCTGCTTTTTGGA	192
CaRop9	5'-GCTGATCATATGGGGTCAAATATCA	5'-TCTTACTTGCAACTTCCATTCTCCG	180
CaRop11	5'-TAGCATTGTCCTTGGTTAGTCGTG	5'-GTGCAGTGGTGACAGGAACTAAT	177
CaEF1a	5'-CTCCAGGCTGATTGTGCT	5'-GAAGGGCTTGTCTGATGG	340

*: Indicate the primer pair target sequence located on 5'-UTR of *CaRop3b*

A comparison between the amino acid sequences of nine CaROPs reveals that the N-terminal of two GTPase domains (GTPase I and GTPase II), two GDP/GTP binding domain and the effector domain are almost absolutely identical (Fig. 1) which domains corresponding to the effector loop that is thought to be responsible for interaction with GTPase activating proteins. However, most striking differences are the Rho insert region and the C-terminal hypervariable region (or Membrane localization domain). The Rho insert region is unique to ROP subclass and consists of 9-10 amino acid residues that is necessary for interaction with other downstream signaling proteins such as RICs (Rho-interacting CRIB domian containing proteins). CaROPs could be divided into three groups according to the hypervariable region: seven of them contained the geranylgeranylation motif CAAL (C, cysteine; L, leucine; A, aliphatic amino acid), while the others showed a C-terminal region with the CAAX (the terminal X can be any amino acids except for Leucine) farnesylation motif (CaROP9) or with a mutated C-terminal resulted from deletion of the hypervariable region (CaROP11). In addition, all CaROPs contained three putative serine/threonine phosphorylation sites (SSK, SKK and SYR), that have been suggested to interact with receptor like kinases (Trotochaud *et al*., 1999).

Phylogenetic relationships analysis of ROP GTPases in *Capsicum annuum*: An analysis of phylogenetic relationships among the nine CaROP and the AtROP family was performed, that was used as a boostrap analysis to construct an unrooted consensus phylogenetic tree (Fig. 2). The resulting tree showed that the nine CaROPs could be classified into four groups corresponding to group , , and in *Arabidopsis* (Zheng & Yang, 2000). Results shows CaROP8 assigned to group with AtROP8, CaROP7 distributed to group with AtROP7. It is worth noting that CaROP9 and CaROP11 group together with AtROP9, AtROP10 and AtROP11 corresponding to group with different C-terminal sequences. As expected that CaROP1a, CaROP1b, CaROP3a, CaROP3b and CaROP6 contains almost the same C-terminal QKACSIL sequence with AtROP1, AtROP3, AtROP5 and AtROP6 were assigned to the group .

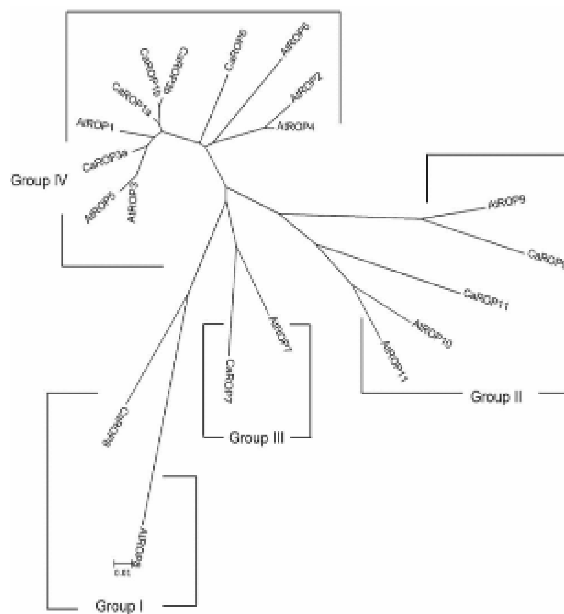

Fig. 2. Phylogenetic relationships analysis of the nine *C. annuum* ROP GTPase and eleven *A.thaliana* ROP GTPase. The four groups identified previously in *Arabidopsis* ROP sequences are shown(Zheng & Yang, 2000).

Expression profiles of *CaRop* genes across different tissues, fruit development and ripening processes: To assess how widespread ROP signaling occurs in pepper growth and fruit development, we first investigated the expression patterns of *CaRop* genes in different vegetative tissues and at seven crucial stages of pepper fruit development, by reanalyzing the RNA-seq data on each tissue and several development stages, which were deposited on (Kim *et al*., 2014). Result shows the nine *CaRop* genes were expressed in all vegetative tissues investigated and displayed preferentially somewhat higher expression level in stem and root than in leaf (Fig. 3). Among them, *CaRop7* and *CaRop9* were exhibited a higher expression level in stem and root, suggesting a high probability for it to participate the stem and root development processes.

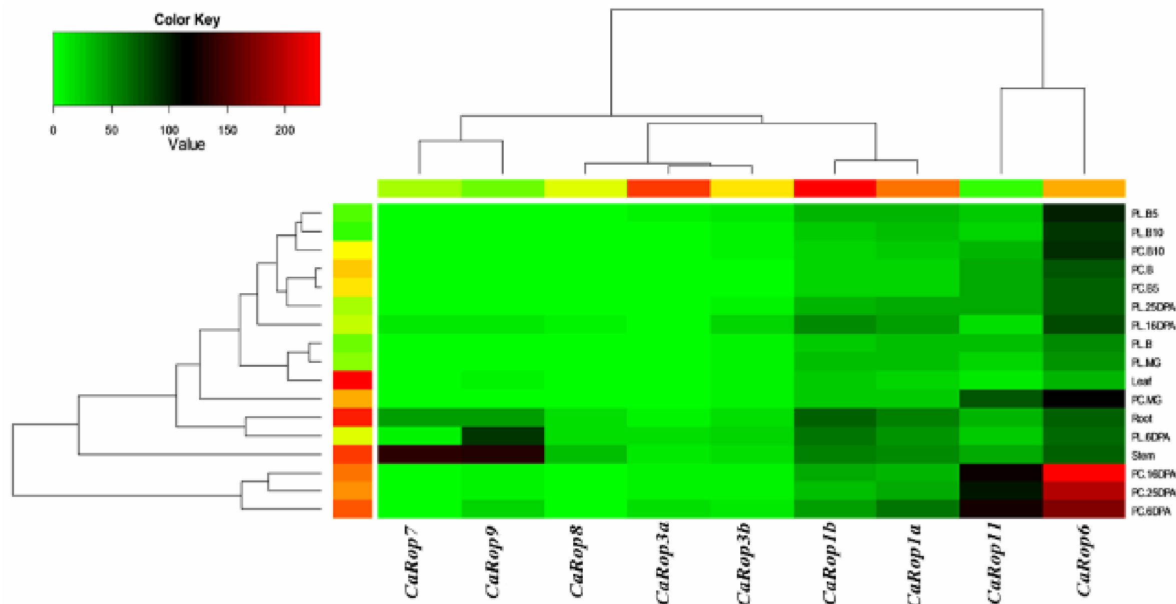

Fig. 3. Expression profiles of *C. annuum Rop* genes across different tissues, fruit development and ripening visualized as heatmap. PC: pericarp, PL: placenta, green fruit stages (6, 16 and 25 DPA), fruit ripening stages (B, B5 and B10), MG: mature green, DPA: day post anthesis, B: breaker.

It is very interesting to determine whether the widespread ROP signaling occurs in pepper fruit development, and if so, whether the different *CaRop* genes were expressed in distinct patterns. As shown in Fig. 3, all *CaRop* genes were expressed in pericarp at most stages of fruit development and displayed a similar developmentally regulated pattern of expression. It is interesting to note that *CaRop6* and *CaRop11* showed a remarkably high expression level at green fruit stages (at 6DPA, 16DPA, 25DPA and MG), and decreased progressively towards fruit ripening (at B, B5 and B10).

To gain deeper insights into whether the ROP signaling occurs in capsaicinoids biosynthesis on the placenta, in which the primary capsaicinoids are produced exclusively in glands on it. As shown in Fig. 3, all *CaRop* genes transcripts on planceta displayed a developmentally regulated expression profile as in pericarp. It is interesting to note that *CaRop9* with a high expression level at 6 DPA, whereas it sharply down-regulated to rarely expressed in subsequent development and ripening stages. Compared with others, *CaRop6* exhibited a higher expression level at green fruit stages (at 6DPA, 16DPA and 25DPA) and further enhanced toward subsequent ripening stages (at B5 and B10), that appears coincide with peaks of capsaicinoids biosynthesis speed and accumulation (B.Estrada *et al.,* 1999).

Expression analysis of *CaRop* genes response to *Ralstonia solanacearum* infection: Plant-specific ROP small GTPases functions as molecular switches in defense signal transduction (Thao *et al*., 2007). *R. solanacearum,* a Gram-negative necrotrophic pathogen, is classified as one of the most important pepper phytopathogenic bacteria due to its lethality and caused great yields lost (Salanoubat *et al*., 2002). To test whether *Rop* genes in pepper is involved in the compatible interaction with *R. solanacearum*, we employed real time RT-PCR to investigate the responses of *Rop* genes after inoculated with *R. solanacearum*. As shown in Fig. 4, compared with the mock plants, *R.solanacearum* infection caused upregulation of only *CaRop3b* (with mild enhancement) while downregulation of other eight *CaRop* genes. Among them, the transcripts for *CaRop1a, CaRop1b, CaRop3a, CaRop6, CaRop7* and *CaRop9* were slightly repressed after *R. solanacearum* infection being 3-9 folds lower at 6 and 12 hpi, then enhanced gradually to their ground states. However, the transcripts for *CaRop8* and *CaRop11* were significantly repressed with 3-40 folds lower between 6 and 48 hpi.

Expression of *CaRop* genes under heat stress conditions: Higher temperature stress is a real problem and becoming one of the major concerns for plant scientists worldwide due to the human activities are substantially adding the existing concentrations of greenhouse gases. In general, an elevation in temperature, usually 10-15°C above ambient, is considered heat stress, which often occurred during July and August in much of the country. Heat stress has devastating effects on plant growth and metabolism, as these processes have their optimum temperature limits in every plant species (Wahid *et al*., 2007). However, plants have evolved a variety of responses to heat stress in order to minimize damages and maintaining cellular homeostasis. So it was very interesting to determine whether the versatile ROP signaling pathway involved in pepper response to heat stress. As shown in Fig. 5, all *CaRops* expression were modulated when response to heat stress (at 42°C), caused upregulation of six *CaRop* genes and downregulation of three *CaRop* genes. It is interesting to note that *CaRop3b, CaRop6* and *CaRop7* were strongly up-regulated and displayed a similar expression pattern, especially *CaRop3b* showing a 30 fold induction at 12 hpt. However, the transcript for *CaRop9* was repressed, being nearly 5 fold lower at 24 hpt when compared with the control plants.

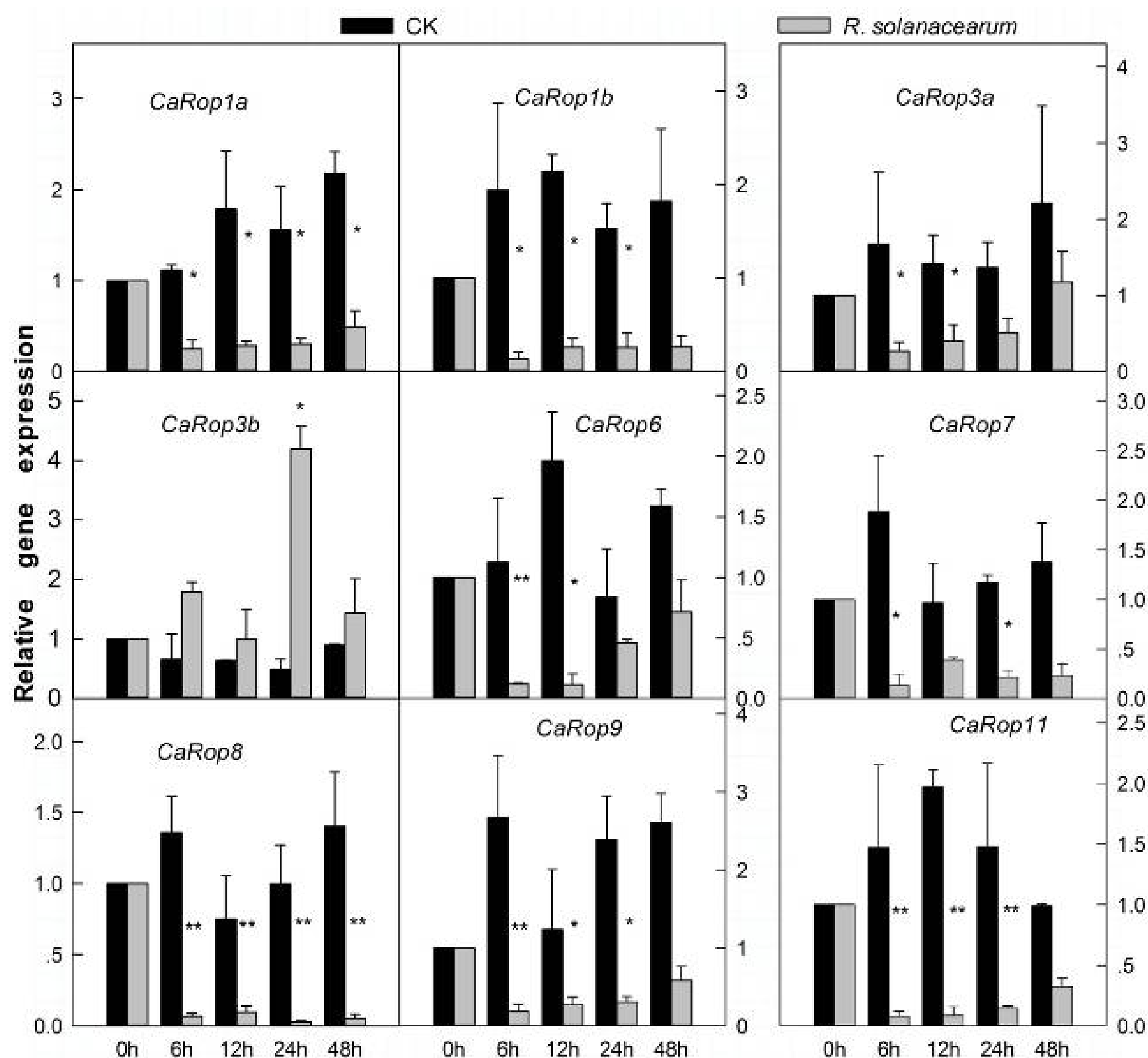

Fig. 4. Time-course analysis the transcript profiles of *CaRop* genes when respond to *R.solanacearum* challenge.

Expression analysis of *CaRop* genes response to hormone treatments: To investigate the possible involvement of *CaRop* genes in signaling pathways utilized by the phytohormones or hormonal controled mechanisms underlying the nine *CaRop* genes expression. We treated pepper seedlings with four phytohormones ABA, NAA, BR and ET, then monitored the transcript abundance of these *CaRop* genes by real time RT-PCR.

To test if *CaRop* genes were involved in ABA signaling pathway, which serve as important signaling molecules and play crucial roles in controlling the expression of downstream defense genes and physiological reactions against various abiotic stresses (Fujita *et al*., 2006). Results shown in Fig. 6, the amplitude of transcripts modulation was substantially weaker after ABA treatment, five *CaRop* genes transcript abundance were enhanced, on the contrary expressions of four *CaRop* genes were repressed. Among them, *CaRop1b, CaRop3b* and *CaRop7* displayed a similar expression pattern and peaked at 12 hpt with about 3-8 fold enhancement respectively. However, *CaRop9* displayed significantly repressed with 3-5 fold lower between 6 and 48 hpt.

When responded to exogenous application of ET, transcripts of four *CaRop* genes were enhanced while five *CaRop* genes were repressed as shown in Fig. 7. The transcripts of *CaRop3b* was enhanced notably and peaked at 24 hpt with 16 fold induction. Whereas, the transcripts of *CaRop7, CaRop8* and *CaRop9* were downregulated significantly and shared a similar expression pattern with 4-55 fold lower between 3 and 48 hpt when compared with the control plants.

To further confirm the *CaRop* genes expression when response to auxin treatment, we treated pepper seedlings with NAA and investigated their expression modulation. Results showed in Fig. 8, only *CaRop3b* was repressed, while transcripts of other eight *CaRop* genes were upregulated and exhibited mainly similar expression patterns between 3 and 48 hpt. The eight *CaRop* genes transcripts were slightly upregulated after NAA treatment and being peaked at 6 or 12 hpt with 3-5 fold enhancements, after which returned to their ground states.

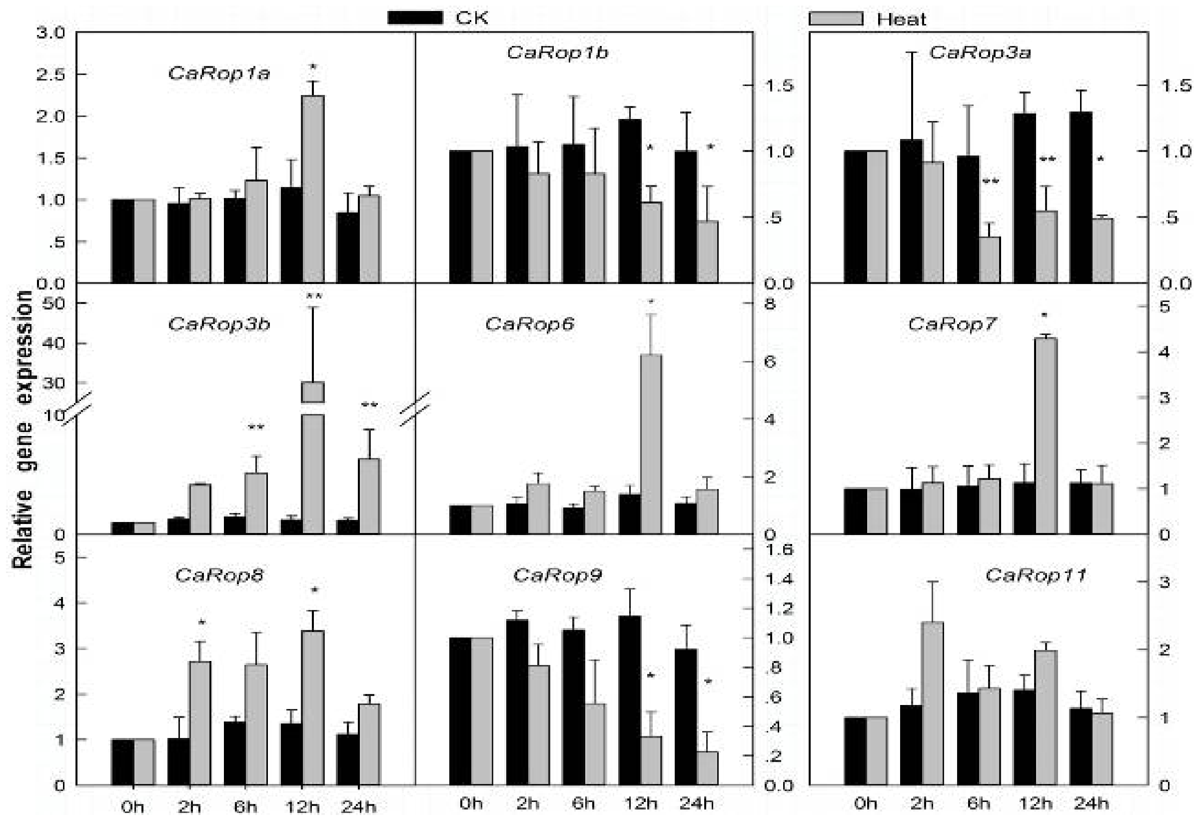

Fig. 5. Time-course analysis the transcript profiles of *CaRop* genes treated by 42°C heat stress.

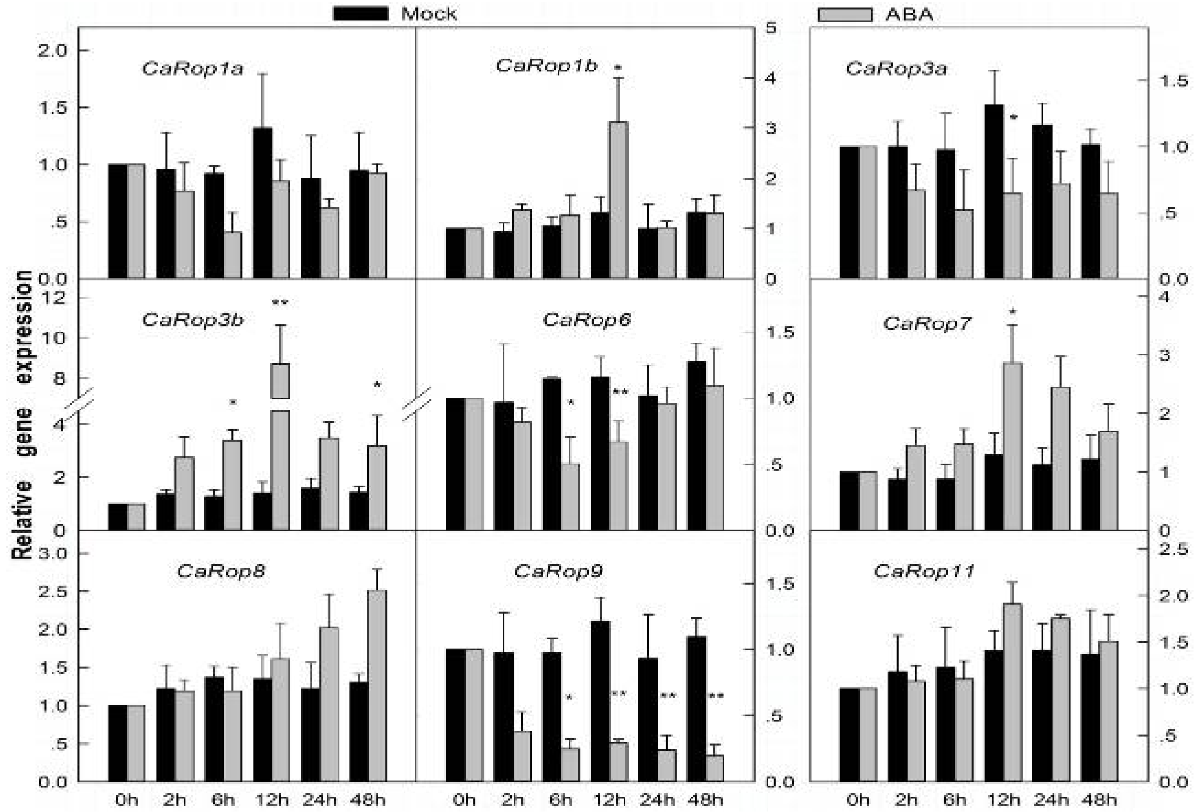

Fig. 6. Time-course analysis the transcript profiles of *CaRop* genes when respond to ABA treatment.

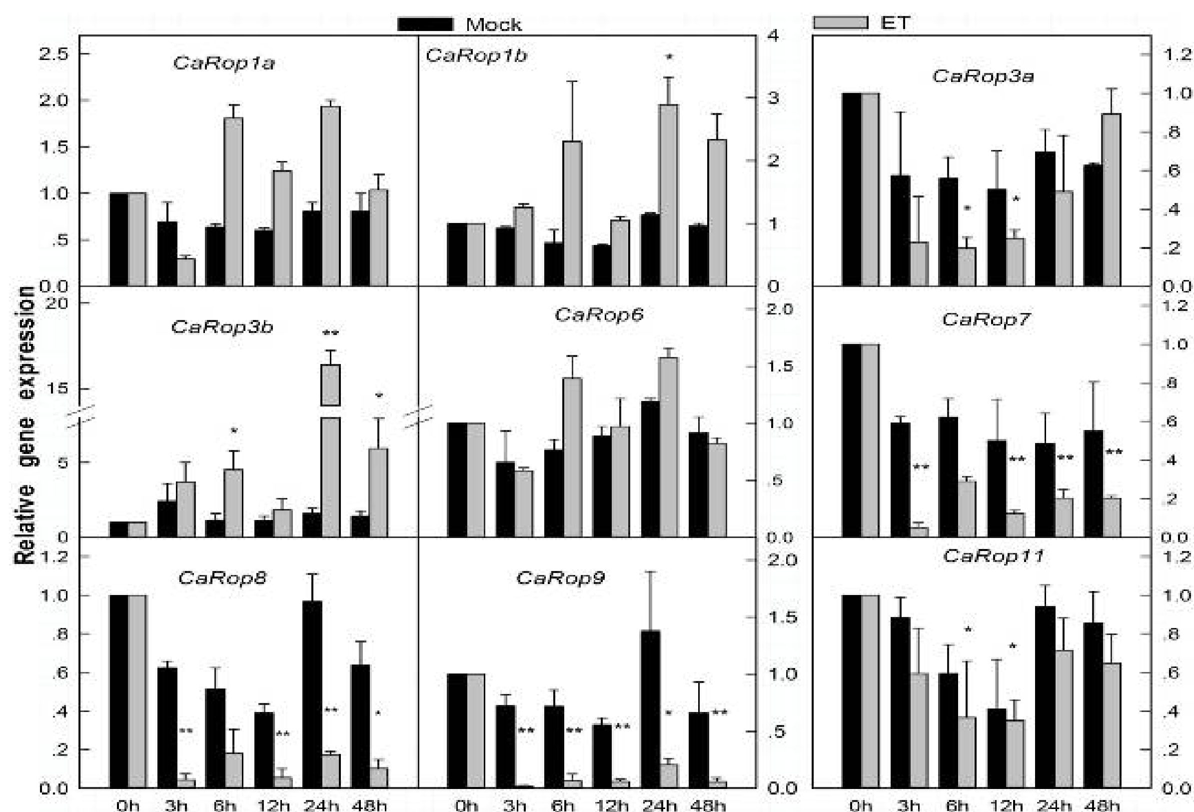

Fig. 7. Time-course analysis the expression patterns of *CaRop* genes when sprayed with ethephon.

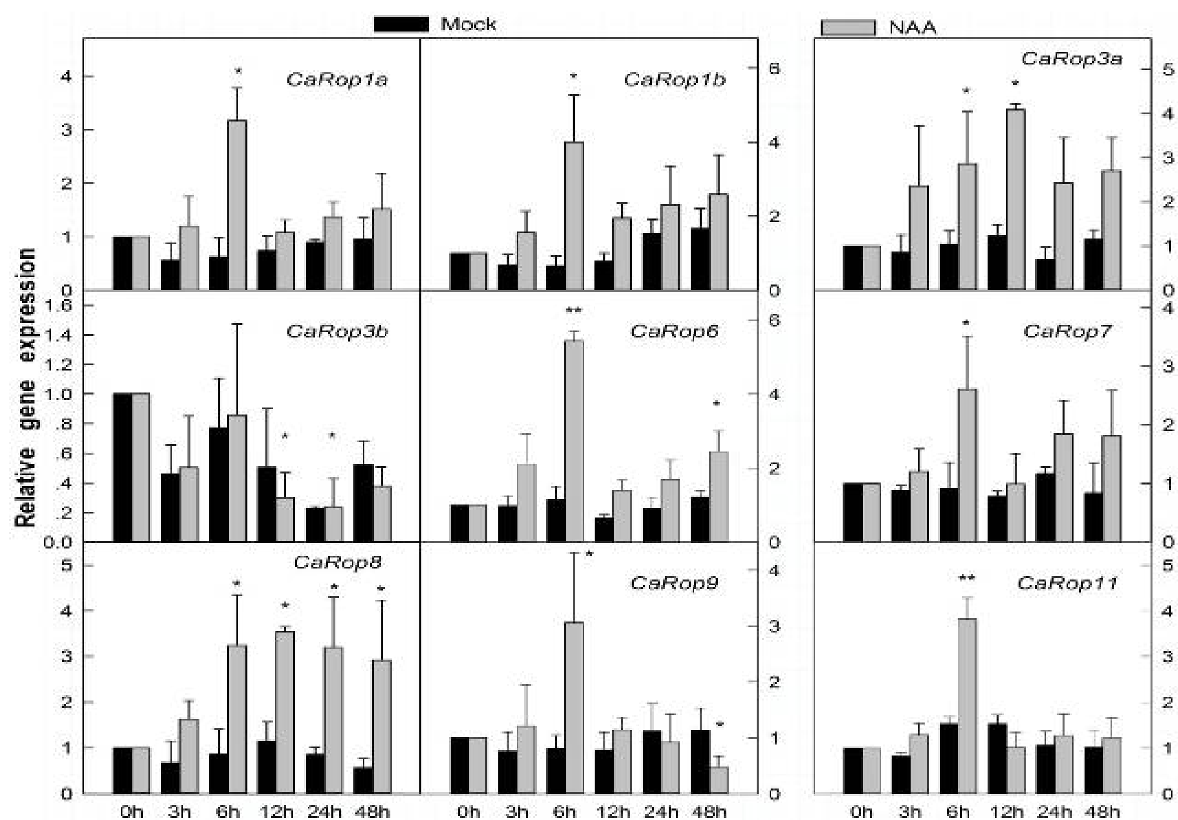

Fig. 8. Time-course analysis the transcript profiles of *CaRop* genes respond to NAA treatment.

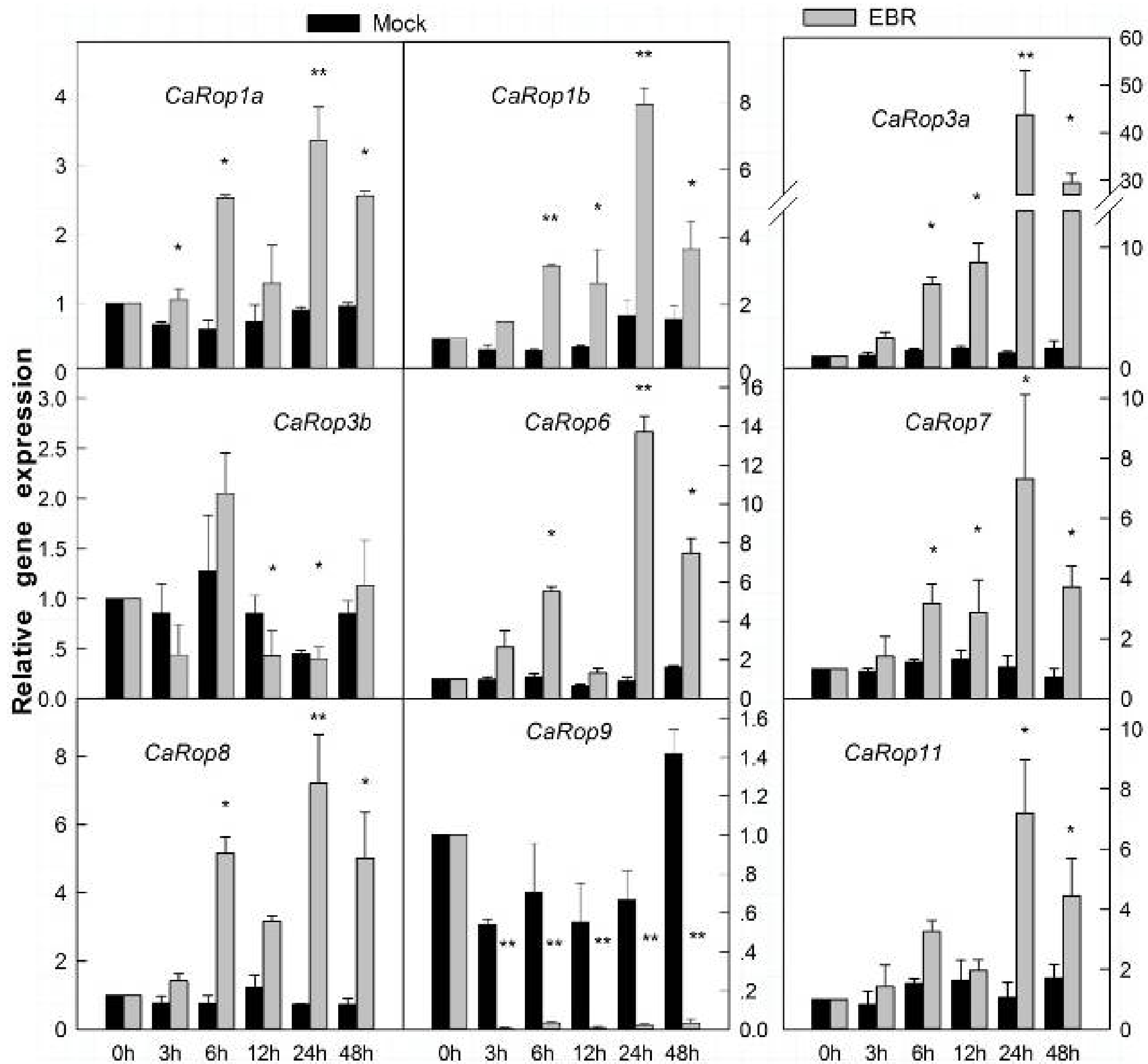

Fig. 9. Time-course analysis the expression patterns of *CaRop* genes when treated with EBR.

Expression of *CaRop* genes was also investigated after treatment with 24-epibrassinolide (EBR), a BR. As shown in Fig. 9, two *CaRop* genes (*CaRop3b* and *CaRop9*) were downregulated, while other seven *CaRop* genes were upregulated and yielded mainly similar expression profiles. The transcripts of seven *CaRop* genes were gradually increased and reached their highest expression level at 24 hpt. It is interesting to note that the expressions of *CaRop3a* and *CaRop6* with 14 or 45 fold induction at 24 hpt respectively. However, the transcripts of *CaRop9* was significantly repressed by BR treatment, being 30-170 fold lower between 3 and 48 hpt when compared with the mock plants.

Discussion

The complete genome sequence of pepper recently released shows that, CM334 cultivar has a total of 34 903 protein-coding genes, 337 328 contigs and 37 989 scaffolds whilst, Zunla-1 cultivar contains a total of 35 336 predicated protein-coding genes. Our investigation shows that both CM334 and Zunla-1 contains nine ROP GTPase protein coding genes according to our bioinformatic analysis of genome sequence obtained from respective database. The number of ROP GTPase protein coding genes in pepper genome corresponds with the number of Rops found in other plant species (Chen *et al*., 2010; Zheng & Yang, 2000).

Further, phylogenetic assessment reveals that, CaROPs could be categorized into four different phylogenetic groups and were accordingly numbered a manner coinciding with their similarities with *Arabidopsis* ROPs. Subsequent comparative sequence analysis showed that CaROPs are highly conserved and contained seven typical functional domains identified in other plants ROPs (Falchi *et al*., 2010; Zheng & Yang, 2000). While five out of the seven domains are perfectly conserved, there is however a variation within the Rho insertion region and the C-terminal hypervariable region of CaROPs in group and group .More so, group and group CaROPs exhibites 100% identity with each other and adequately suggests that

these variable domains may be exerting functions different from other CaROPs groups and validates previous knowledge that, the hypervariable region at the C-terminus is required for directing the proteins to specific membrane (Lavy *et al*., 2002). Our aligments result shows that in exception of group , all CaROPs are terminated with the geranylgeranylation motif CAAL, the target for geranylgeranyl transferase and hence, suggesting that these ROPs are recruited to specific plasma membrane through geranylgeranylation modification. As a result, CaROP11 mutant generated by deletion the hypervariable region of the C-terminal resulted in partial loss of affinity to plasma membrane. It is however, interesting to know that CaROP9 terminated with the CAAX motif, which implicated as acceptor for farnesylation, exert different functions from others.

Possible functions of CaROPs were investigated by studying the expression patterns in different tissues and fruit development processes as carried out for some plant species in others studies (Abbal *et al*., 2007). Our results showed that, all *CaRop* genes are expressed but their expression patterns are varied with stem and root exhibiting the highest expression pattern. Individual evaluation of *CaRop* from gene expression trials shows that, *CaRop7* and *CaRop9* were most abundant and evidently implicating their possible involvement in the development of stems and roots. During pericarp development, the expression of *CaRop6* and *CaRop11* were remarkably higher at green stages (at 6DPA, 16DPA, 25DPA and MG), but were downregulated in correlation with maturity and ripening (at B, B5 and B10) and suggests the possible functions of *CaRop6* and *CaRop11* in pepper fruit development in accordance with similar function of *VvRop* genes in grape berry development (Abbal *et al*., 2007). Further more, we investigated the link between expressions of *CaRop* genes and capsaicinoid biosynthesis during the placenta development and ripening, findings from this investigation showed that, *CaRop6* was most abundant during placenta development and was upregulated constitutively towards placenta ripening and also coincides with peaks of capsaicinoids biosynthesisas well as it accumulation. So, we speculated that the higher expression of *CaRop6* in placenta could be contributing to capsaicinoid biosynthesis or accumulation. Taken together, these results indicates that the expression of *CaRop6*, *CaRop7*, *CaRop9* and *CaRop11* or it's involved signaling pathways may execute important roles in pepper plant growth, fruit development and capsaicinoid biosynthesis.

Previous reports indicate that *Rop* genes have general roles in disease responses in plants (Moeder *et al*., 2005; Ono *et al*., 2001). Furthermore, mild heat stress will promote cultured *B. napus* microspores shift toward embryo production which accompanied with highly expression of some *BnRop* (Chan & Pauls, 2007). In this study, *R.solanacearum* infection caused upregulation of only *CaRop3b* and downregulated the other eight *CaRop* genes, consistent with the *Rop* family members in rice which are known to play multiple roles and has both positive and negative functions in rice blast resistance (Chen *et al*., 2010). However, heat stress induced upregulation of six *CaRop* genes while repressing the other three *CaRop* genes. It is interesting to note that *CaRop3b* was upregulated by both treatments, implicating that it play a positive role in response to *R. solanacearum* infection and heat stress. However, the expression of *CaRop1b, CaRop3a* and *CaRop9* were repressed by both treatments and seems to portray it as a negative regulator of resistance to *R. solanacearum* and heat stress. Taken together, we tentatively proposed that different *CaRop* genes could function as positive or negative regulators and may function synergistically or antagonistically in *R. solanacearum* during infection and heat stress, as an adaptive mechanism to fine-tune the host responses in plants.

Several observations suggest that ROP GTPases play a pivotal role in phytohormone signaling, and thus help to coordinate plant growth, development and physiology (Berken, 2006). In this study, expression of *CaRop* genes was affected by hormonal treatments, which is in accordance with regulatory role of ROP GTPases in the regulation of hormones responses and developmental processes (Fu *et al*., 2008). In this study, treatment with ABA enhanced the abundance of five *CaRop* genes, while four *CaRop* genes expression were weakly repressed except the *CaRop9*, which displayed 3-5 folds lower abundance between 6 and 48 hpt. As regards the response of *CaRop9* to ABA, this expression profile, as well as their C-terminal region contain the CAAX farnesylation motif, suggests that CaROP9 is functionally analogous to the AtROP10 which is implicated as a negative regulator of ABA responses in *Arabidopsis* (Zheng *et al*., 2002). When exogenous application of ET increased the transcripts of *CaRop3b* and achieved 16 folds abundance at 24 hpt, while transcripts of *CaRop7, CaRop8* and *CaRop9* were dramatically repressed with 4-55 folds lower between 3 and 48 hpt. It should be note that both ABA and ET treatment significantly enhanced the abundance of *CaRop3b*, whereas pronouncedly repressed the expression level of *CaRop9*. These data suggested an involvement in ABA and ET signaling cascade, especially *CaRop3b* and *CaRop9* have a higher probability be common downstream integrators and may play important roles in both ABA and ET signaling participated cellular responses. Compared with ABA and ET treatment, *CaRop* genes were commonly upregulated by NAA and BR, except *CaRop3b* slightly repressed by NAA treatment and BR treatment downregulate the expression of *CaRop3b* and *CaRop9*. Marked modulation in expression of *CaRop* genes after NAA and BR treatment, suggest that these CaROPs participate in cellular processes triggered by auxin and brassinosteriod as reported in other plants (Li *et al*., 2001; Tao *et al*., 2002).

In conclusions, the present results are consistent with a regulation the expression of *CaRop* genes by developmental and hormonal signals, as well as by boitic and abiotic stresses. However, *CaRop* genes probably respond to distinct signalings payhways as inferred from their various responses to phytohormones and biotic stress, and some of those may make contributions to

pepper fruit development, ripening and capsaicinoid biosynthesis. Based on all the results in this study, we speculated that the functional versatile of CaROP proteins has played a critical role in the development of pepper throughout the entire life cycle of the plant and also contributes to pepper plant response to various stresses.

Conclusion

We identified nine ROP GTPases protein coding genes in the pepper genome, and analyzed their expression patterns for tissues, fruit development and ripening processes. In addition, these genes expression profiles in response to pathogen inoculation and various hormone treatments, as well as heat stress was obtained by real time RT-PCR. To our knowledge, this is the first research showing Rop genes respond to heat stress and ethylene treatment in plants. Also we first described a CaRop seem to be associated with capsaicinoid biosynthesis or accumulation in placenta. These results may lay the foundation for future research to unraveling the widespread ROP signaling and their biological roles.

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