Europ.J.Hort.Sci., **78** (6). S. 241–248, 2013, ISSN 1611-4426. © Verlag Eugen Ulmer KG, Stuttgart

Characterization of *Pp-CTR1* Gene Expression during Fruit Development and Ripening in Peach (*Prunus persica*)

C. P. Zhao[1)], J. H. Zhi[1)], M. Y. Han[1)], H. K. Liu[1)], N. An[2)] and A. Z. Wang[1)]
([1)]College of Horticulture, Northwest A & F University, Yangling, Shaanxi, China and [2)]College of Life Sciences, Northwest A & F University, Yangling, Shaanxi, China)

Summary

Ethylene is an important factor for the development and maturation of fruits. The cDNA full sequence of *Pp-CTR1* involving in ethylene signalling was cloned from *Prunus persica* 'Rui pan 4' and its expression in the fruits and seeds were analyzed. The main results were as follows. The cDNA of *Pp-CTR1* (FJ982370) with 3124 bp full-length and 2532 bp ORF encoded a polypeptide of 843 amino acid. *Pp-CTR1* had the conserved serine/threonine kinase domain, including ATP-binding site, and serine/threonine kinase catalytic site. *Pp-CTR1* expression patterns in fruits and seeds were significantly different with developmental stages. During the first exponential growth phase of peach fruit (S1), *Pp-CTR1* expression in the young fruits enhanced rapidly. At the slower growth stage of peach fruit (S2), opposite to its expression with quick decrease in young fruits, the expression of *Pp-CTR1* in seeds increased obviously. In the third stage with fruits growing rapidly again (S3), with the *Pp-CTR1* expression in fruits raising again, its expression in seeds reduced. These results suggested that *Pp-CTR1* probably played an important role in regulating peach fruit and seed development. During the ripening and softening of peach fruit, *Pp-CTR1* expression in fruit kept a lower level with a slight change. An opposite tendency was observed between *Pp-CTR1* expression pattern and ethylene evolution. However, the expression level of *Pp-CTR1* in seeds was higher than that of in fruit, and showed significant increase at the late stage of storage.

Key words. stone fruit – *Pp-CTR1* – cloning – expression pattern – postharvest

Introduction

Fruit development follows fertilization, and occurs simultaneously with seed development and maturation in plants (White 2002). Fruit ripening is accompanied by dramatic changes in fruit appearance, texture, flavour, and aroma, such as colour, hardness, fragrance, taste, nutritional quality, and so on. Fruit development and maturation involve the expressions and regulations of a series of developing and ripening genes (Giovannoni 2004; Yokotani et al. 2009). In the recent years, great progresses have been achieved in understanding in fruit development and maturation regulation by ethylene signal action, in many cases by research results on model plants such as Arabidopsis and tomato (Kendrick and Chang 2008; Rohrmann et al. 2011). Research showed that ethylene mediated regulation of ripening at various levels, which includes ethylene biosynthesis, its perception, signal transduction cascade, and finally regulation of target gene expression. These target genes can control fruit firmness, taste, colour and aroma directly (Barry and Giovannoni 2007; Bapat et al. 2010). Ethylene signalling plays a crucial role in regulating fruit developing and ripening, the different members in the ethylene signal transduction path were isolated from different plants, such as, ethylene receptor-coding genes (ETR) (Rasori et al. 2002; Pang et al. 2007), constitutive triple response 1 (CTR1) (Adams-Phillips et al. 2004; Manzano et al. 2010), ethylene insensitive 2 (EIN2) (Wang et al. 2007b), ethylene insensitive 3 (EIN3) (Tieman et al. 2001; Yokotani et al. 2003), ethylene response factors (ERF) (Tournier et al. 2003; Wang et al. 2007a).

CTR1 is a negative regulator in the downstream of ethylene receptors and actively represses the ethylene response pathway in the absence of the hormone. In tomato, the *CTR* gene existed as a gene family which including four *CTR* like genes (*LeCTR1*, *LeCTR2*, *LeCTR3* and *LeCTR4*) (Leclercq et al. 2002; Adams-Phillips et al. 2004; Lin et al. 2008). Adams-Phillips et al. (2004) proved that the tomato *LeCTR1* transcript increased markedly coincident with the onset of ripening, and *LeCTR1* induction was associated with tissues at development stages of re-

lated to the increase of ethylene (fruit ripending, pedicel abscission, petal senescence). Two *CTR-like* genes in *Rosa hybrida* and *Cucurbita pepo* had also been identified, and the regulation of their expression happened during the flower development and senescence (Müller et al. 2002; Ma et al. 2006; Manzano et al. 2010). In some plants, such as apple, plum, peach and delphinium, the *CTR1* gene appears to be unique as in *Arabidopsis* (Yin et al. 2009; Kieber et al. 1993).

Peach, as a stone fruit, exhibits a typical double sigmoid growth pattern during fruit development, with distinct and recognizable growth stages (S1–S4: The first stage (S1), which is the first exponential growth phase, is characterized by a rapid increase in cell division and elongation. During the second stage (S2), the endocarp hardens to form the pit hardening. In the third stage (S3), known as the second exponential growth phase, a rapid increase in fruit size takes place. In the final stage (S4), the fruit reaches the final full size and enters the fruit ripening stage) (Tonutti et al. 1997; El-Sharkawy et al. 2007). Furthermore, a peach is a typical climacteric fruit, which undergo a program of enhanced ethylene production and an associated increase in respiration rate at the onset of ripening (Barry and Giovannoni 2007). Therefore, softening and senescence occurred rapidly after peach fruits were harvest, and the difficulty of storing and transporting for peaches restricts its production. A better understanding of the physiological basis of the peach fruit developing and ripening process should make it possible to develop further strategies to regulate developing and ripening. *CTR1* genes were related to plant development, maturation and senescence in many crops, while different results were found among these studies (Adams-Phillips et al. 2004; Cin et al. 2006; El-Sharkawy et al. 2007; Fernández-Otero et al. 2007; Wiersma et al. 2007; Begheldo et al. 2008; Yin et al. 2008; Wang et al. 2010). In this study, we cloned the full length of *CTR1* cDNA from peach fruit, and then determined the expression patterns of the *CTR1* gene and its possible role in the developing and ripening of peach fruit.

Materials and Methods

Plant material

The fruits from three peach trees [*Prunus persica* 'Rui pan 4'] growing in the Experimental Station of College of Horticulture's Northwest A & F University in Yangling, Shaanxi, China, were used for this study.

For a fruit development series, young fruit samples were hand-picked from 14 days after full bloom (DAFB) and seed samples were from 42 DAFB. Fruit and seed samples were collected every two weeks until the fruits reached their commercial maturity (the criterion for the commercial maturity were as follows: the ground colour of peel showed light green, partly red colour, and the firmness value was about 80N). At least 20 fruits at each developmental stage were collected for measuring fruit diameter and sampling of flesh and seed.

Fruits for analysis the further ripening and softening were hand-picked at commercial maturity, and then were stored one by one in plastic bag at 25 ± 1 °C. During fruit shelf life, fruit and seed samples were taken each two day, until the flesh was too soft and firmness could not be measured any more. All the samples were frozen with liquid nitrogen and stored at −80 °C in refrigerator for future analysis.

Peach fruit ethylene evolution and flesh firmness measurement

When peach fruits reached their commercial maturity, the ethylene evolution was measured on 5 individual fruits from 3 trees using a gas chromatograph (Trace GC Ultra, Thermo Fisher, Italy) as described by Liguori et al. (2004), with 3 replications. Flesh firmness was measured using 5 fruits (one fruit for each replication) after removing a small disc of skin from each side of the fruit, using a penetrometer (GY-I, MMI, China) with a 4 mm probe, and depth of penetration was 1 cm.

RNA extraction and reverse transcription

The total RNA was isolated from flesh and seed sample using the protocol described by Zhang et al. (2003). Five micrograms of total RNA was pretreated with RQ1 DNase I (Promega), using RNase Inhibitor (TAKARA) as a protective measure, to remove any contaminating genomic DNA. RNA yield and purity were measured using a spectrophotometer, while RNA integrity was checked using electrophoresis in agarose gels. First-strand cDNA was synthesized using 1.0 μg of treated total RNA, Primescripe (TAKARA), and oligo $d(T)_{20}$ to a total volume of 10 μl. The cDNA was diluted 1:5 with water.

Full-length cDNA cloning of Pp-CTR1

According to the EST (Expressed Sequence Tag) (serial number: AY945799) of CTR1 from Gene bank, the gene-specific RACE primers of the target gene were designed with Primer Premier 5.0 and Oligo 6.0 as follows: GSP1: 5'-TGTGAAAATTCTTATGGAGCAAGACT-3', GSP2: 5'-GAC GCTGTTTCGCATCGGTTTTGGGT-3'. The 5' and 3' Smart-RACE was performed according to SMARTTM cDNA Amplification kit (Clonetech). The amplification fragments were inserted into the pMD18-T vector (TAKARA) and sequenced.

Bioinformatic analysis of Pp-CTR1

The sequencing results were analyzed with the following applications: The ORF sequence was checked and translated by http://us.expasy.org/tools/protparam.html. The

sequence homology was measured using the BLAST program of the Genbank. The first order structures of amino acids, including isoelectric points and molecular weights, were estimated by Computer pI/MW. The amino acid phylogenetic tree was analyzed using MAGA 4.0.

Quantitative PCR

The real time PCR was conducted on IQ5 fluorescence Quantitative PCR Meter (Biorad, USA) using the primers showed in Table 1. The qPCR reactions were carried out with 10 μL Premix EX Taq™, 0.5 μL Primer F(20 μM), 0.5 μL Primer R(20 μM), 0.8 μL fluorescence probe solution, 1.0 μL cDNA template, and 7.2 μL ddH_2O. The qPCR amplification program included a preliminary step at 95 °C for 30 s, followed by 40 three-step cycles of template denaturation at 95 °C for 5 s, primer annealing at 57 °C for 20 s, and an extension at 72 °C for 30 s. Peach 18 s RNA was used as the reference gene (Cin et al. 2006).

Fluorescence data were collected and relative gene expression was calculated using the comparative 2-ΔΔ Ct method (Livak and Schmittgen 2001). Each assay was run in triplicate and repeated three times using different samples.

Results

Cloning of Pp-CTR1 cDNA

The electrophoresis results showed that the lengths of amplification product of 3'-RACE and 5'-RACE of *Pp-CTR1* were 2.5 kb and 1.2 kb respectively (Fig. 1a and 1b), and the full length cDNA sequences of the *Pp-CTR1* gene with 3124 bp (GenBank accession no. FJ982370) was obtained by sequence-joining, containing a open reading frame (ORF) with 2532 bp (146-2 677 bp).

Table 1. The Primers of *Pp-CTR1* for the real-time Fluorescence quantitative PCR

Gene	Primer
CTR1F	5'-CGATGGCGGGTTTAAGAGTTTC-3'
CTR1R	5'-TTGTTGTTCTTGCTCTCCGAATC-3'
CTR1P	5'-(FAM) CGCCGCCGTCTGGTCGTCG (Eclipse)-3'
R18 s F	5'- GTTACTTTTAGGACTCCGCCA-3'
R18 s R	5'- ATTCCTTTAAGTTTCAGCCTTG-3'
R18 s P	5'-(FAM) CCCCCGGAACCCAAAGACT (Eclipse)-3'

Bioinformatic analysis of Pp-CTR1 gene

The open reading frame of *Pp-CTR1* encoded a 843 amino acid polypeptide. The molecular weight of protein was 93.2 kD and the isoelectric point was 5.84. Homology comparison showed that *Pp-CTR1* shared the highest identity with the partial fragment of *Prunus domestica Pd CTR1* (AJ890089) and *Prunus salicina Ps CTR1* (EF585298) genes, with the homology of 99 %, 97 % in nucleotide and 95 %, 97 % in amino acid, respectively. The *Pp-CTR1* also showed 85, 85, 75 and 74 % homology in nucleotide and 87, 83, 68 and 69 % homoglogy in amino acid with those of *Malus* × *domestica* (DQ847148), *Rosa hybrid* (AY032953), *Arabidopsis thaliana* (NM_80429.3) and *Cucumis melo* (HQ848663). In addition, a ~62–69 % amino acid sequence identity was observed between *Pp-CTR1* and *CTR-like* regulators in tomato.

According to the deduced amino acid sequences from different plants *CTR1* published in GenBank, the phylogenic trees of CTR1 constructed by MEGA 4 indicated that *Prunus persica* Pp-CTR1 was genetically most close to *Prunus salicina* Ps-CTR1, both of which belong to the stone fruit group, followed by *Malus* × *domestica* Md-CTR1 and *Rosa hybrid* Rh-CTR1. Pp-CTR1 did not cluster together with CTR1-like kinases in tomato and European pear (Fig. 2).

The molecular structure of CTR1 proteins were largely conserved in different plant species. The C-terminal end of Pp-CTR1 had highly conserved Ser/Thr kinase domains (Fig. 3), as well as the consensus sequences for the ATP-binding site GXGXXGXV and Ser/Thr protein kinase active site signature (IVHRDLKSPNLLV) (Fig. 3).

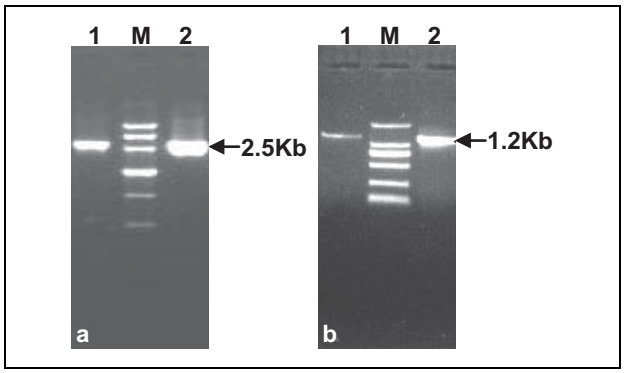


Fig. 1. RT-PCR amplification of *CTR1* genes from peach by agarose gel-electrophoresis. a: 1. Amplication result of CTR1 with 5'-RACE; M: DL5000 Maker; 2. PCR amplification result of the bacterium solution after CTR1 recombination and transformation; b: 1. Amplication result of CTR1 with 3'-RACE; M: DL2000 Maker; 2. PCR amplification result of the bacterium solution after CTR1recombiantion and transformation.

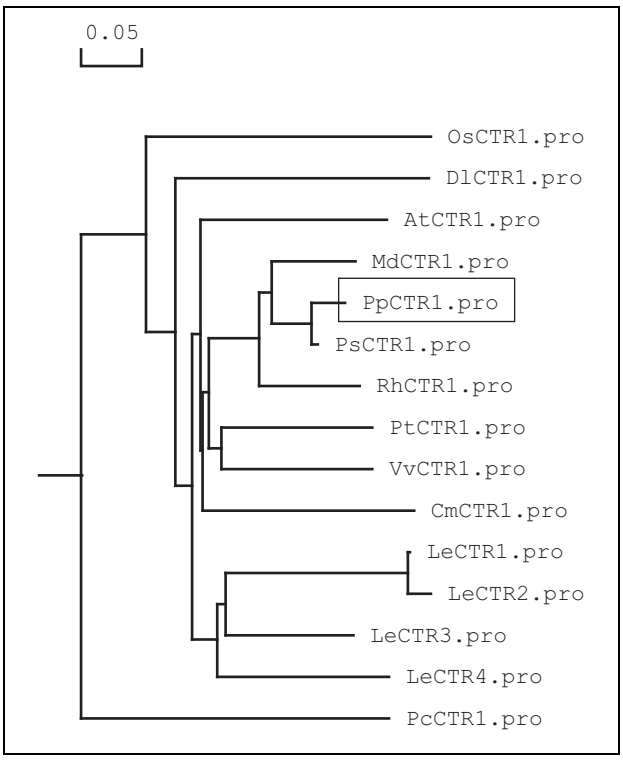


Fig. 2. Phylogenetic tree was established by the amino acid sequences of known plant CTR1. Accession numbers of sequences used to build the tree were as follows. *Prunus persica*: *Pp-CTR1* (FJ982370); *Malus × domestica*: *Md-CTR1* (DQ847148); *Rosa hybrid*: *Rh-CTR1* (AY032953); *Arabidopsis thaliana*: *At-CTR1* (NM_120454.3); *Prunus salicina*: *Ps-CTR1* (EF585298); *Pyrus communis*: *Pc-CTR1* (AF386508); *Cucumis melo*: *Cm-CTR1* (HQ848663); *Solanum lycopersicum*: *Le-CTR1* (AY079048), *Le-CTR2* (AF110519), *Le-CTR3*(AY382676), *Le-CTR4* (AY382677); *Populus trichocarpa*: *Pt-CTR1*(XM_002326209); *Vitis vinifera*: *Vv-CTR1*(XM_002277324); *Oryza sativa*: *Os-CTR1* (AP004777.3); *Delphinium*: *Dl-CTR1* (AB100686).

Pp-CTR1 expression during the developmental process of young fruit and seed

To understand the possible roles of *Pp-CTR1* gene during fruit development, the expression changes of *Pp-CTR1* in the mesocarp and seed of 'Rui pan 4' were examined using real-time quantitative PCR technique. The results showed that *Pp-CTR1* expression in the mesocarp enhanced rapidly from 14 to 43 DAFB (this stage was just the first period of peach fruit exponential growth, S1 stage, Fig. 4a, b), and decreased quickly from 43 to 88 DAFB (in this stage, most of dry matter was employed in pit hardening and seed and embryo growth, S2 stage, Fig. 4a, b), then increased from 88 to 101 DAFB (in this stage, fruits began to grow rapidly once again, S3 stage, Fig. 4a, b), and after that always kept lower level until fruit maturation, thought slightly increase of *Pp-CTR1* expression was observed from 129 to 141 DAFB (Fig. 4b).

Six weeks after full bloom, young seeds of 'Rui pan 4' were separated from fruit, and then the *Pp-CTR1* expression in the seed was assessed. The assessment results showed that *Pp-CTR1* expression enhanced stably in S2 stage (from 43 to 88 DAFB), and decreased from 88 to 101 DAFB (S3 stage), then always kept lower level until fruit ripening (Fig. 4a, c).

Pp-CTR1 expression in fruit and seed during storage and relation to the change in ethylene production and firmness

When the fruits of 'Rui pan 4' reached their commercial maturity, the fruit were hand-picked and stored, and the firmness, ethylene production and *Pp-CTR1* expression were analyzed during the fruit shelf life. In the first 2 days after harvest, the fruit firmness was stable, while from 12 August, fruit firmness began to decline rapidly to 18N (Fig. 5a).

The expression of *Pp-CTR1* in fruit decreased during the first 3 days after harvest and reached its minimum level in the 13 August, and then tended to increase (Fig. 5c). However, the production rate of endogenous ethylene in fruit was characterized by opposite extremes and showed the significant increase during this period, and then obvious decrease (Fig. 5b).

The expression level and pattern of *Pp-CTR1* in seed was different with that of in fruit during the storage. The expression level of *Pp-CTR1* in seed was higher than that of in fruit, and tended to increase slowly during the first 7 days after harvest (from 10 August to 17 August), and enhanced nearly fourfold from the 17 August to 19 August, and then declined rapidly (Fig. 5d).

Discussion

Phylogenetic analysis and deduced protein characteristic of Pp-CTR1 genes

In this study, the full-length cDNA of *CTR1* in peach was cloned. Homology comparison showed that compared to other plants, *Pp-CTR1* shared higher identity with those of *Rosaceae* plants, such as plum, apple and rose, which suggested that the function of *CTR1* was similar in those plants. While *Pp-CTR1* did not cluster together with *CTR1-like* kinases in tomato, which is a model plant studying the ethylene signal transduction mechanism. In addition, *Pp-CTR1* encoded protein also had all the features of a serine/threonine specific protein kinase domain of CTR with the ATP-binding site motif, GAGSFGTV, and the consensus sequence for serine/threonine kinase, IVHRDLKSPNLLV (Kieber et al. 1993). These analyses showed that we got the right ethylene signalling regulator gene in peach, *Pp-CTR1*.

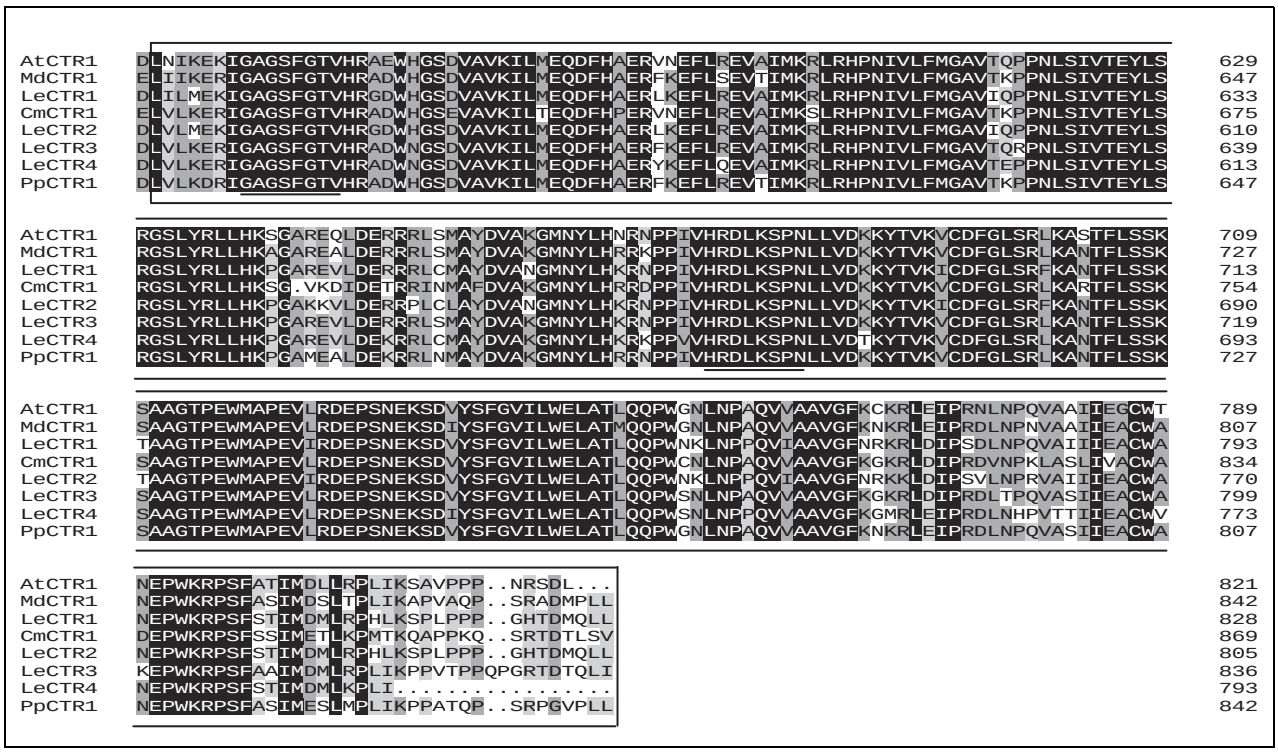


Fig. 3. Amino acid sequence alignments of the conserved C-terminal Ser/Thr kinase domain in the CTR1-like proteins of several plant species. The boxed sequence in the C-terminal region indicates the Ser/Thr kinase domain. The ATP-binding site motif (GAGSFGTV) and Ser/Thr kinase active site consensus sequence (HRDLKSPN) were underlined in PpCTR1. All accession numbers of sequences were the same as for Fig. 2.

Possible effect of Pp-CTR1 on the development of peach fruit and seed

Ethylene signals regulate many aspects of plant growth and development, ranging from seed germination to senescence processes, including abscission of flowers, fruits and leaves (Lin et al. 2009). However, the molecular basis of this regulation is not completely understood in some plants, and especially the studies on regulation of fruit and seed development were very little. Stone fruits, including peach, exhibit a typical double sigmoid growth pattern during fruit development and ripening (Tonutti et al. 1997; Layne and Bassi 2008). The results of this studies showed that in the first period of peach fruit exponential growth (S1 stage, from 14 DAFB to 43 DAFB, Fig. 4a), *Pp-CTR1* expression in young fruit enhanced rapidly, and with a peak at 43 DAFB (Fig. 4b). In the following slower growth stage of fruits (S2 stage, from 43 DAFB to 88 DAFB), *Pp-CTR1* expression of the young fruits decreased quickly (Fig. 4b), however *Pp-CTR1* expression of seed increased obviously (Fig. 4c). After S2 stage, fruits began to grow rapidly once again (Fig. 4a) because cell enlargement and elongation (S3 stage), and correspondingly, fruit *Pp-CTR1* expression went up again, while seed *Pp-CTR1* expression reduced (Fig. 4b, c). The seed and fruit compete strongly for assimilation product throughout fruit development, and the high nutrient demand are created in the pericarp at S1 and S3 stage and in the seed at S2 stage, which result in the fruit growth rapidly at S1 and S3 stages and seed growth at S2 stage (Chalmers and van Ende 1977).

All of the above studies indicated that the *Pp-CTR1* expression were concordant with the developmental period of young fruits and seeds. Two homologous genes of kiwifruit *AdCTR1* and *AdCTR2* also presented strong expressions in the initial fruit development period (Yin et al. 2008). Damson plum *PdCTR1* also showed a higher expression in the early fruit development (Fernández-Otero et al. 2007). The study of El-Sharkawy et al. (2007) showed that the accumulation of Japanese plum Ps-CTR1 mRNAs increased during fruit second exponential growth phase in an ethylene-independent manner. So, it could be suggested that *Pp-CTR1* played an important role in regulating peach fruit and seed development.

Possible role of Pp-CTR1 gene in the process of fruit ripening

Studies of *CTR1* expression pattern during fruit ripening and softening had been reported in some plants, while different results were found among these studies. Tomato *LeCTR1* transcript markedly increased at the onset of ripening (Adams-Phillips et al. 2004). The expression of

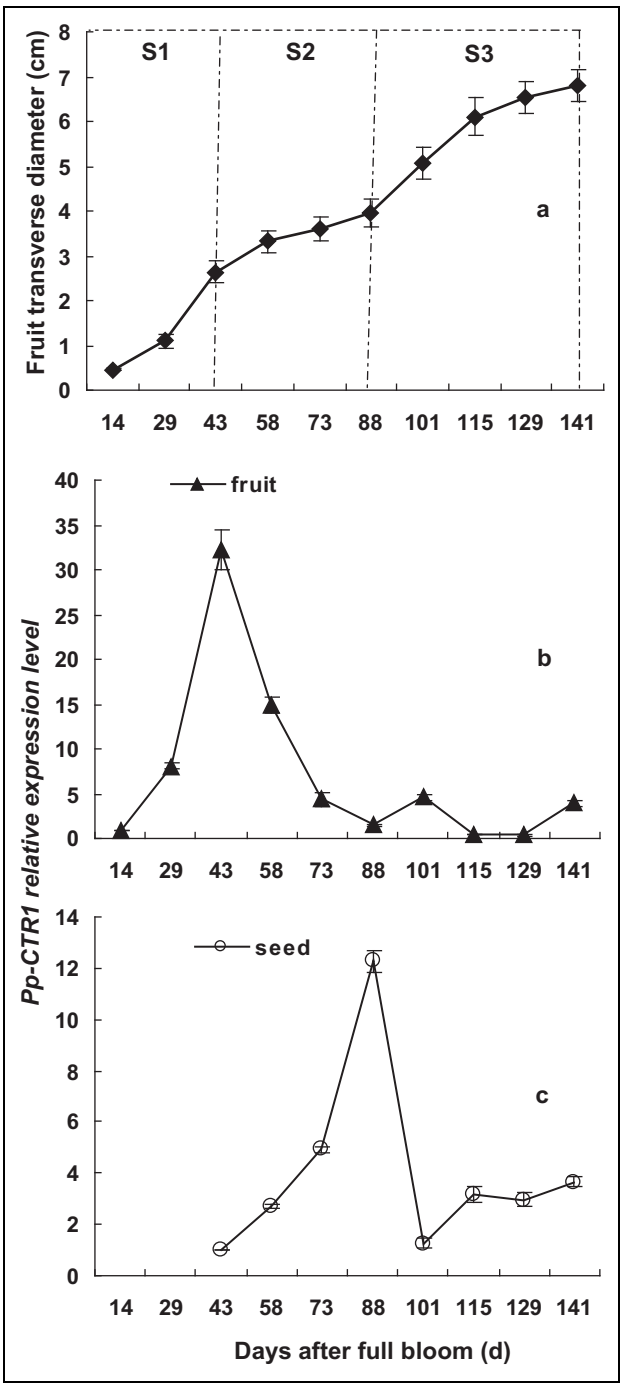


Fig. 4. *Pp-CTR1* expressions during the developmental process of young fruit and seed. a: Peach fruit growth curve; b, c: *Pp-CTR1* expressions in fruit and seed were evaluted by real time PCR, and peach 18 s RNA was used as the reference gene. Bars indicate SD.

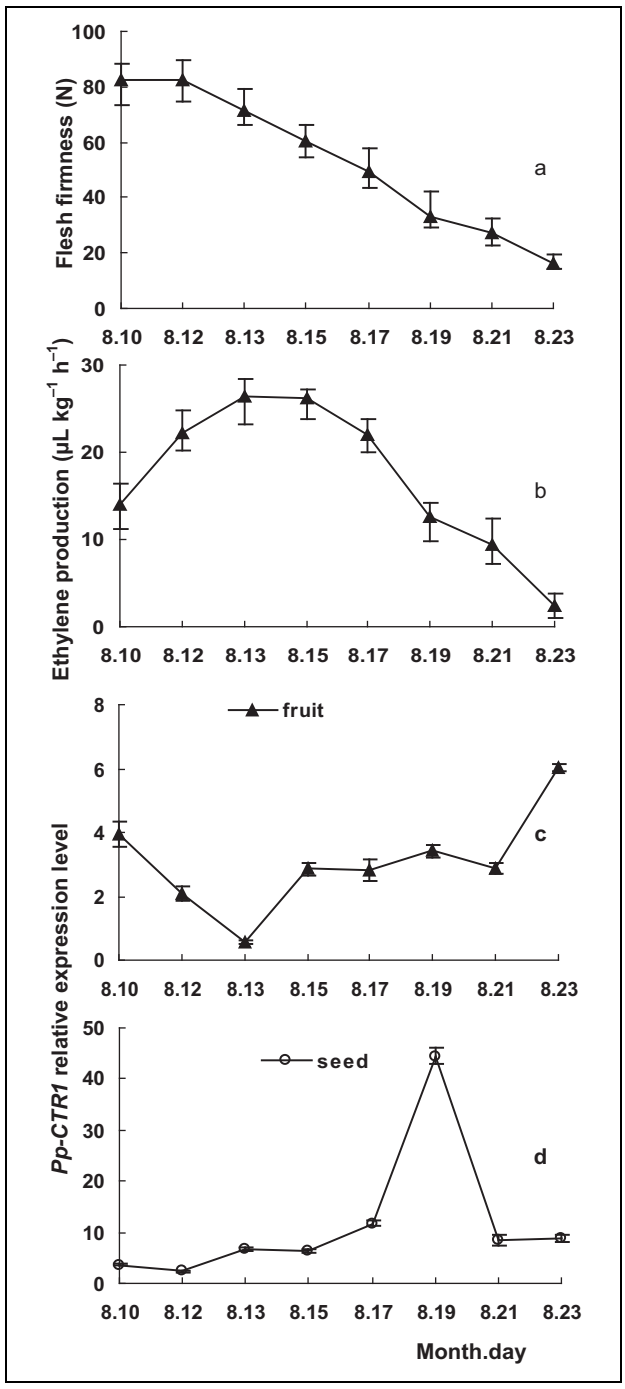


Fig. 5. Flesh firmness, ethylene production and Pp-CTR1 expression levels during peach fruit storage at 25 °C. *Pp-CTR1* expressions in fruit and seed were evaluted by real time PCR, and peach 18 s RNA was used as the reference gene. Bars indicate SD.

the genes *PcCTR1*, *PsCTR1* and *PdCTR1* in pear, plum and damson plum respectively, also increased during fruit ripening and after ethylene treatment (El-Sharkawy et al. 2003, 2007; Fernández-Otero et al. 2007). The highest accumulation level of peach *PpCTR1* occurred at the onset of ethylene climacteric (Begheldo et al. 2008). In ripening kiwifruit flesh, the transcript abundance of *AdCTR1* increased with the ethylene climacteric, but was unresponsive to the external ethylene treatment. While, *AdCTR2* presented a constitutive expression during fruit ripening, showing no response to exogenous ethylene and 1-MCP treatments (Yin et al. 2008).

However, *MdCTR1* and *PpCTR1* showed little change in expression during apple and peach fruit ripening, and *PpCTR1* showed no response to 1-MCP, and *MdCTR1* was suppressed at a late stage in ripening (Cin et al. 2006). Wang et al. (2010) also found that transcript level of Loquat *EjCTR1* in fruit during post-harvest storage displayed no significant changes, but expression was strongly induced by low temperature. In addition, Wiersma et al. (2007) found that apple *MdCTR1* expression decreased predominantly at commercial harvest. Our study indicated that *PpCTR1* expression in fruit kept a lower level with a slight decrease first and then increase during the ripening and softening of peach fruit. An opposite tendency was showed between *PpCTR1* expression pattern and ethylene evolution after the onset of ethylene climacteric when *Pp-CTR1* expression level decreased to the minimum value (Fig. 5b, c). *CTR1* is a negative regulator of ethylene responses, so the decrease in expression during ripening could correspond to an increase in sensitivity to ethylene. Our result was similar to those from Cin et al. (2006), Wang et al. (2010) and Wiersma et al. (2007). Therefore, *CTR1* probably played a complex role in the process of fruit ripening.

Ethylene signal transduction in seeds of fleshy fruit was rarely studied. The research of Bonghi et al. (1998) indicated that at ripening of peach fruit, ethylene evolution was greatly enhanced in epicarp, while strongly inhibited in seed. However, our results showed that during the fruit shelf life, the expression level of *Pp-CTR1* in seed was higher than that in fruit, and significantly increased at the late stage of ripening. Fernández-Otero et al. (2007) got the similar results in damson plum. Until now, it is not clear why *CTR1* expression increases in seed at maturation stage of fruit, whether seed further development, such as after-ripening process, is accompanied with fruit ripening and softening.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31101514), and the Breeding Project by Tangzhouying Fund.

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Received 09/09/2012 / Accepted 10/21/2013

Addresses of authors: C. P. Zhao, J. H. Zhi, M. Y. Han (corresponding author), H. K. Liu, A. Z. Wang, College of Horticulture, Northwest A & F University, Yangling, Shaanxi, China, and N. An, College of Life Sciences, Northwest A & F University, Yangling, Shaanxi, China, e-mail (corresponding author): hanmy@nwsuaf.edu.cn.