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Research article

Stage- and tissue-expression of genes involved in the biosynthesis and signalling of ethylene in reproductive organs of damson plum (*Prunus domestica* L. subsp. *insititia*)

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Abstract

In this work, four cDNA clones (*Pd-ACS1*, AJ890088; *Pd-ETR1* and Pd-ERS1, AJ890092, AJ890091; and Pd-CTR1, AJ890089) encoding an ACC-synthase, two putative ethylene (ET) receptors, and a putative MAPKKK, respectively, were isolated and phylogenetically characterized in *Prunus domestica* L. subsp. *insititia*. Their expression was studied by real-time PCR during flower (closed, open and senescent) and fruit (*early green*, *late green*, *maturation* and *ripening)* development of damson plum, which is climateric. While two peaks of ET production were quantified at *early green* and *ripening* stages in whole fruits, the seed was not able to produce it during *maturation* and *ripening* stages. All studied genes were differentially expressed during flower and fruit development. In general, the level of transcripts of *Pd-ACS1* was higher in fruits than in flowers. However, it was noteworthy that: (1) *Pd-ACS1* expression was hardly detected in closed flowers and at low levels during *early green* stage; and fruit development provoked a notable differential expression in seeds, and pericarp; (2) the results of *Pd-ACS1* expression during fruit development suggest a preponderant role of this gene from *late green* stage onward. The stamen was the only floral organ in which expression of both *Pd-ETR1* and *Pd-ERS1* receptor genes was not significantly altered during development; however, their expression decreased concomitantly with development of pistil (only floral organ to register a net ET production when fertilized) and during first days of ovary development (the highest ET production during all fruit development). Contrary to *Pd-ERS1*, the level of *Pd-ETR1* mRNA was temporally quite similar in the seed. With regard *Pd-ETR1*, even its expression was very scarce during *maturation* of mesocarp, was stimulated during *ripening*. In the epicarp, *Pd-ERS1* and *Pd-ETR1* were low expressed during pit hardening increasing onward and decreasing during *ripening*. *Pd-CTR1* expression was in the seed > mesocarp > > epicarp. Spatial and temporal levels of *Pd-ACS1*, *Pd-ETR1*, *Pd-ERS1* and *Pd-CTR1* mRNAs described in this work demonstrate that the expression of these genes is not always constitutive and that control of its transcription may play an important role in regulating the development of reproductive organs of damson plum.

Keywords: ACS1; CTR1; Epicarp; ERS1; Ethylene signalling; ETR1; Fleshy fruit; Mesocarp; Pericarp; Pistil; Damson plum; Seed

1. Introduction

The hormone ethylene (ET) regulates a wide range of developmental processes in higher plants [1]. The responses to ET are mediated by a family of receptors that show a structure similar to bacterial two-component regulators [2–4]. The first ET receptor (*ETR1*) was identified in *A. thaliana* by map-based cloning and its recombinant protein expression in yeast confirmed its capability to bind ET *in vivo* [2,5,6]. Subsequently, it has been confirmed that ET–ETR1 complex is located in the endoplasmic reticulum membrane [7]. The family of ET receptors can be divided into two categories according to structural differences of their deduced amino acid sequences: *ETR1*-like subfamily, formed by ETR1 and ERS1, which presents 3 hydrophobic subdomains at the NH_2-terminal, has the

Abbreviations: ACO, ACC-oxidase; ACS, ACC-synthase; DAP, days after pollination; ET, ethylene; HK, His-kinase.

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highest conservation of the His kinase (HK) elements [4] and shows HK activity *in vitro* [8,9], although HK activity does not appear to be required for signalling [10–12]; and *ETR2*-like subfamily, consisting of ETR2, ERS2 and EIN4, presents 4 hydrophobic subdomains, contain degenerate HK domains [8] and has Ser/Thr kinase activity *in vitro* [9]. Family members can also be classified as to whether they contain a receiver domain at the C-terminus (ETR1, ETR2, and EIN4), or lack such a domain (ERS1 and ERS2) (for review, see [13,14]). Receiver domains play a role in the recovery from growth inhibition. Thus, receptor isoforms with receiver domain have a more important function in growth recovery from ET than those without this domain [11].

A considerable body of evidence indicates that the ET receptors identified in *Arabidopsis* are conserved in agronomically important dicots and monocots ([15] and references therein), although some differences are observed [16,17]. These orthologues show typical traits highlighted by researches on *Arabidopsis* ET receptors; e.g. negative regulation of downstream responses and redundancy (six members in tomato; [13]). Beside ET receptors isolated from *Arabidopsis* and tomato, putative ET receptor orthologues have been cloned and characterized in many others plant species, including *Rumex* [18], melon [19], carnation [20], peach [21], and rice [22]. All of these receptors have the greatest level of amino acid conservation in the ET-binding domain (EBD). Recently, the EBD of the *Arabidopsis* ETR1 receptor was studied. The results yielded a notable insight into the structure and function of the EBD and suggested a conserved role of the EBD as a negative regulator of the signal transmitter domain [23].

The use of biochemical and molecular genetic approaches has led to the identification of the many components responsible for sensing and responding to ET (for recent reviews, see [14,15,24–26]). ET signal transduction begins with ET binding to and inactivating a family of ET receptors. In the absence of ET, these receptors activate CTR1, a mitogen-activating protein kinase (MAPKKK) that negatively regulates the pathway [27,28]. After CTR1 inactivation, EIN2, a positive regulator of the signalling cascade, that shares homology with the N-Ramp family of metal transporters [29], promotes ET responses via the downstream transcription factor EIN3 and most likely also via EIN3-like1 (EIL1), EIN5, EIN6, EIN7 and others components. For instance, the identity of EIN5/EIN7 has now been shown to be XRN4, an exoribonuclease component of the ET response pathway in Arabidopsis [30,31]. The ET receptors prevent EIN3 degradation and leads to an increase in EIN3 protein levels [14,24], whereas in the absence of ET, EIN3 levels are reduced by proteasome-mediated degradation (Ub/26S) involving EBF1/EBF2 (E3 complex) [32–35]. However, in spite the increase of molecular information on ET signalling in higher plants, the contribution of each receptor to the perception and signalling of ET remains unknown since it is difficult to assign specific functions to each of them, considering the demonstrated existence in *A. thaliana* and tomato of a compensation mechanism between receptors [3,36,37]. In contrast, it was recently demonstrated in Arabidopsis that the ETR1 receptor has a unique and non-overlapping function in ET-stimulated mutations [38], and a strong correlation between receptor transcript level and ET binding capability in *Arabidopsis* [37].

Damson plum (*Prunus domestica* L. subsp. *insititia* cv. Syriaca) fruit is a climacteric drupe [39,40]. Due to its perishable nature, marketing of the fresh fruit is very limited. Therefore, the lengthening of the ripening period would increase its commercial and socio-economical values, apart from constituting a useful experimental tool in fruit physiology since no study exists about this subject in plums. Previously, we have demonstrated that *Pd-ACO1* and *Pd-ACO2* genes had differential expression during flower and fruit development and that the seed actively participates in the *Pd-ACO1* expression, synthesis of both ET and its precursors, and polyamines during *early green* and *late green* stages [39,40]. To gain insight in the regulation by ET of this plum, in this study, four cDNAs (*Pd-ACS1, Pd-ETR1*, *Pd-ERS1* and *Pd-CTR1*), have been isolated, characterized and studied by means of its spatial and temporal expression throughout flower and fruit development by real-time PCR.

2. Materials and methods

2.1. Plant material

Damson plum (*Prunus domestica* L. subsp. *insititia* cv. Syriaca) trees were grown in the experimental farm of the O'Areeiro Phytopathology Centre (Pontevedra, Spain). Closed, open and open-pollinated flowers (with advanced symptoms of senescence) were collected in 2005. Growth and development of whole fruit (divided in epicarp, mesocarp and seed from 28 days after pollination, DAP) and seeds, were monitored from fertilized ovaries (zero DAP) to senescent fruit (104 DAP) [39]. Until 96 DAP, fruits were harvested at 7-day intervals, while close to the ET burst (climacteric phase), fruits were collected every 2 days. All samples were immediately used for ET determination or frozen with liquid N_2 and stored at −80 °C. The fruit development stages studied here were: *early green* (from zero until 35 DAP), *late green* (from 35 until 70 DAP, pit hardening), *maturation* (from 70 until 98 DAP) and *ripening* (from 98 until 104 DAP).

2.2. ET determination

Samples of plant material (3–4 replicates at least of whole fruits and individual seeds) were transferred to appropriate flasks containing filter paper wetted with sterile distilled water. The flasks were hermetically sealed and incubated in darkness at 30 °C. After a 60 min, 1 ml of sample of internal atmosphere was sampled for ET determination as described [40].

2.3. DNA extraction and Southern analysis

Genomic DNA was extracted as described by [41] from 0.5 g of young fully expanded leaves. For Southern blot analysis, DNA (10 μg) was digested with *Eco*RI (E), *Hin*dIII (H) and *Xho*I (X), separated in agarose gels (1%), and then transferred to Hybond-N^+ membranes (Amersham, Buckinghamshire,

UK) according to the manufacturer's instructions. Hybridizations were carried out using as probes *Pd-ACS1*, *Pd-ETR1*, *Pd-ERS1* and *Pd-CTR1* cDNA labelled with ^{32}P following standard procedures [42].

2.4. RNA isolation, cDNA synthesis and transcript analysis by real-time PCR

Total RNA was isolated from appropriate organs basically as described by [43]. To separate the RNA from the large amount of carbohydrate, a precipitation in 2-butoxyethanol was included prior to LiCl precipitation [44]. The cDNA was synthesized from 0.8 μg of total RNA DNA-free using the Super Script™ First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) primed with oligo-dT_{12-18} following the manufacturer's instructions.

Real-time semi-quantitative PCR analysis was performed with cDNA from different stages of fruit and flower development, using appropriate oligonucleotides (Table 1). Actin and 18S-RNA were examined as possible controls (data not shown); actin was found to be expressed at constant levels through the development and was used as control for the gene studied. The cDNA was amplified using IQTM SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). PCR reaction mixes included the following components: water (9.5 μl), SYBR-Green® Supermix (12.5 μl), 12 μM each primer (1 μl) and cDNA template (1 μl). PCR conditions were as follows: 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C; and a final elongation step of 7 min at 72 °C. The melting curve was designed to increase 0.5 °C every 10 s from 67 °C, for 66 cycles. Amplification experiments were conducted in triplicate. PCR products were examined for the correct size and homogeneity after agarose electrophoresis, and sequenced once for identity confirmation. All the amplifications included cDNA obtained from epicarp (32 DAP), used as a reference for calculations. Quantification was based on analysis of threshold cycle (Ct) value as described by [45].

Table 1
Primers used for amplification and sequencing of *PdACS1*, *PdETR1*, *PdERS1* and *PdCTR1*

Primer	Sequence	Gene
ACS1 Fw	5′-CAGATGGGGTTGGCGGAGAA-3′	*PdACS1*
ACS1 Rv	5′-GCAATGGAAAGAACCTG-3′	*PdACS1*
ETR1 5′Fw	5′-GAAGCATGCAATTGTATTGAG-3′	*PdETR1*
ETR1 5′Rv	5′-CTAAATTTAATGAAACCGAT-3′	*PdETR1*
ETR1 M Fw	5′-AGGTAGAACTTTGGCATTGG-3′	*PdETR1*
ETR1 M Rv	5′-ATCCATGACAAGGACTTT-3′	*PdETR1*
ETR1 3′Fw	5′-TTTAACCTCCACTCTG-3′	*PdETR1*
ETR1 3′Rv	5′-CATAGCCTCAAATAAAACTCG-3′	*PdETR1*
ERS1 5′Fw	5′-ATGGATTCCTGTGATTGCAT-3′	*PdERS1*
ERS1 5′Rv	5′-CAGCATGTGAAAGAGCAACAG-3′	*PdERS1*
ERS1 M Fw	5′-TGCCAATAAACCTTCCTATA-3′	*PdERS1*
ERS1 M Rv	5′-ATAAAATTCAGGTGGTCGCCA-3′	*PdERS1*
ERS1 3′Fw	5′-CTTAATGTTGCTGGTAATGC-3′	*PdERS1*
ERS1 3′Rv	5′-AATGCTTCTTTGATAGCGGG-3′	*PdERS1*
CTR1 Fw	5′-CAAATCACGTGGAATCTCAAG-3′	*PdCTR1*
CTR1 Rv	5′-GGMATGGMWCWGAKGTTGCTG-3′	*PdCTR1*

2.5. Sequence analysis of Pd-ACS1, Pd-ETR1, Pd-ERS1 and Pd-CTR1

In order to obtain sequence data, cDNA was used as template for PCR reactions using the appropriate oligonucleotides (Table 2). The PCR conditions were 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s for 35 cycles. The PCR products were cloned into the pGEM®-T easy vector (Promega, Madison, WI, USA) using as host DH5α *Escherichia coli* cells. The nucleotide sequencing was performed using the Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Amino-acid sequence analysis, multiple alignments, and the phylogenetic tree were conducted by Clustal-X [46].

3. Results

3.1. Characterization of cDNA clones for Pd-ACS1, Pd-ETR1, Pd-ERS1 and Pd-CTR1

In the present work we isolated four cDNAs, named *Pd-ACS1* (AJ890088), *Pd-ETR1* (AJ890092), *Pd-ERS1* (AJ890091) *and Pd-CTR1* (AJ890089). The deduced protein Pd-ACS1 (AJ890088) is comprised of 356 amino acids with a molecular mass of 39.8 kDa and a p*I* of 7.0. Sequence analysis revealed that this fragment encoded a partial damson plum ACS. The hydropathic profile analysis supports its high solubility (data not shown). With the exception of an ACS from the apple fruit [47], all the ACS described were soluble. Phylogenetic analysis showed that *Pd-ACS1* clustered with ACSs isolated from genus *Prunus* (e.g. *Pa-ACS3*, Q9FVH7), *Pyrus* (e.g. *Pp-ACS2*, AB007639; *Pc-ACS3*, AY442145; *Pc-ACS2*, AF386519) and *Malus* (e.g. *Md-ACS3*, AY821542) showing the highest homology with *P. persica* (*Pp-ACSS*, AF239663, 97%). Southern blot analysis (data not shown) suggested the existence in *P. domestica* of an ACS multigene family, as reported for other species.

The deduced Aa sequence of Pd-ETR1 presents 70.7% identity with respect to Pd-ERS1 (Fig. 1A). Pd-ETR1 and Pd-ERS1 clustered with ET receptors identified in *P. persica* (accession numbers AF12452, AY061640), and are also closely related to ETR and ERS from other fruits (Fig. 1B). The hydropathic profile of both Pd-ETR1 and Pd-ERS1 sequences showed three main hydrophobic regions on the

Table 2
Primers used for transcript analysis by real-time PCR

Primer	Sequence	Gene
rtACS1 Fw	5′-TGGGACAAATCAGAGGAG-3′	*PdACS1*
rtACS1 Rv	5′-CCTGGATAGTATGGGGTT-3′	*PdACS1*
rtETR1 Fw	5′-GGATGTGTGCATGCCTGG-3′	*PdETR1*
rtETR1 Rv	5′-CCAACCCTCATGCAGTTCTCC-3′	*PdETR1*
rtERS1 Fw	5′-GCCCAGAAGTGGATCCAA-3′	*PdERS1*
rtERS1 Rv	5′-CACAAGGATTGTCGCATATCCC-3′	*PdERS1*
rtCTR1 Fw	5′-GCTGCGGCATCCAAATATAG-3′	*PdCTR1*
rtCTR1 Rv	5′-CGCCTCTCATCCAATGCC-3′	*PdCTR1*
rtACTIN Fw	5′-CCATCACCAGAATCCAGCAC-3′	*Actin*
rtACTIN Rv	5′-GTATGTTGCCATTCAGGCTG-3′	*Actin*

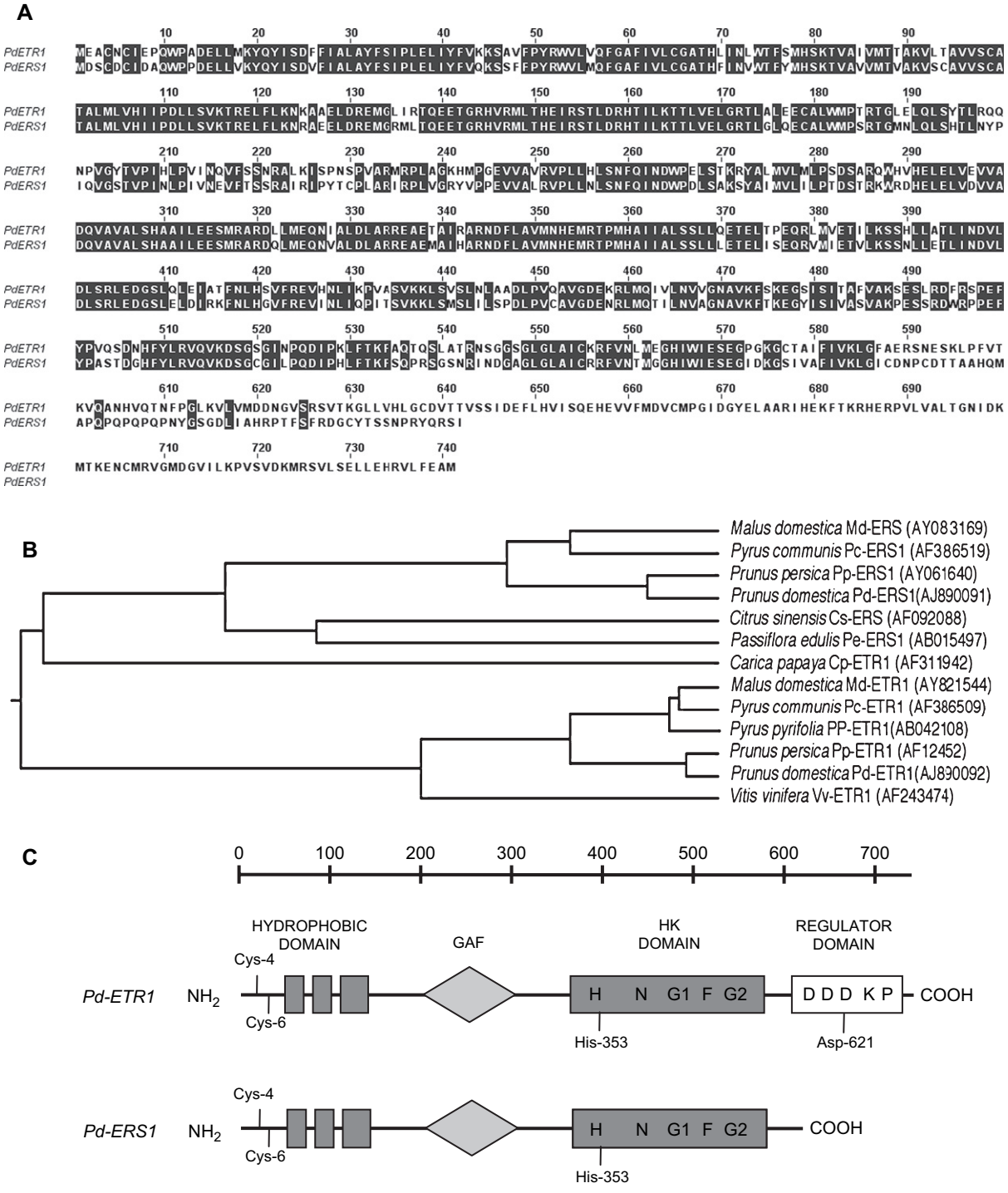

Fig. 1. Analysis of *Pd-ERS1* and *Pd-ETR1* sequence data. (A) Alignment of the amino-acidic sequence of Pd-ETR1 and Pd-ERS1 receptors from *P. domestica*. (B) Phylogenetic tree including PdETR1 and PdERS1; accession numbers are given in parentheses. (C) Possible structure of Pd-ETR1 and Pd-ERS1 receptors from *P. domestica*.

N-terminal end and subsequently an alternation of hydrophilic and hydrophobic regions (data not shown). Therefore, Pd-ETR1 and Pd-ERS1 belonged to the subfamily called ETR1-like, whose members are characterized by the presence of three highly conserved hydrophobic segments in the sensor domain [48] (Fig. 1C). Also, both receptors of *P. domestica* bear the HK domain, characterized by the presence of 5 boxes necessary for the HK activity in the ETR1-like subfamily [49], and the F-box required for binding to ATP [50]. Likewise, as with previously reported for other homolog receptors [3,51–53], the regulator domain appears in Pd-ETR1 but not in Pd-ERS1 (Fig. 1C). Finally, the Pd-CTR1 isolated in this work was homologous to other MAPKKK previously described (e.g. *Lycopersicon esculentum*, Q5YKK5 90%;

Arabidopsis thaliana, Q05609; 89%; *Cucumis sativus*, Q7XAV0 88%; *L. esculentum* Q5YKK6, 87%; *Pyrus communis*, Q8W231 77%).

3.2. Temporal and spatial distribution of ET production in flowers and fruits

The fertilized pistil was the only floral organ to register a detectable ethylene production (0.4 ± 0.1 nmol g^{-1} FW^{-1} h^{-1}) while in petals of pollinated flowers the hormone remained at a basal level (50 ± 10 μmol g^{-1} FW^{-1} h^{-1}). ET production was not detected in sepals, petals and stamens of both closed and open-not pollinated flowers (data not shown). Fruit development of damson plum displays a typical double sigmoidal curve (*early green* and *maturation* stages displayed an exponential growth, while *late green* and *ripening* ones are characterized by a slower growth rate) [39]. Two peaks of ET production from whole fruit were detected at 14 DAP (*early green*) and at 117 DAP (*ripening*) (Fig. 2). Seed growth from *early green* and *maturation* stages also undergoes exponential growth similar to that of the whole fruit [36]. The isolated seed reached two peaks of ET production at the end of *early green* phase (42 DAP) and at the beginning of the *late green* phase (pit hardening; 63 DAP) (Fig. 2); the seed was not capable of producing ET at *maturation* and *ripening* stages. The ACC content paralleled the ET evolution both in fruit and seed [39].

3.3. Stage- and tissue-expression of Pd-ACS1, Pd-ETR1, Pd-ERS1 and Pd-CTR1 genes

In order to understand the possible roles played by these genes in damson plum, real-time semi-quantitative PCR analysis was performed with cDNA from different stages and tissues of flower and fruit development. *Pd-ACS1* expression was not detected in any studied part of closed flower (Fig. 3). In contrast, its expression was induced in all flower organs by the anthesis process, stamen and petals being more affected. However, *Pd-ACS1* expression in ovary did not change after fecundation. In this work we show that the spatial and temporal *Pd-ACS1* expression was higher in fruits than in flowers (Figs. 3 and 5). The separation of the fruit in seed, mesocarp and epicarp was not possible in the *early green* phase due to the size of the fruit. The amount of *Pd-ACS1* transcripts in seed rose from *late green* phase until beginning of *ripening*, diminishing onward. The profiles of *Pd-ACS1* expression in mesocarp and seed were similar, being in mesocarp quantitatively higher. On the other hand, the levels of *Pd-ACS1*-mRNA linearly augmented in epicarp during fruit development, reaching in soft fruits the greatest level of this study (Fig. 5).

Pd-ETR1 and *Pd-ERS1* genes are expressed during the entire floral development, the transcriptional activity of both receptors being spatial and temporally similar (Fig. 3). It is striking that: (a) in closed flowers, the maximum transcription of both receptors took place in the sepals and pistil, clearly declining with the floral-opening process; (b) in open flowers, the greatest accumulation of these mRNAs was detected in the pistil and floral envelops; and (c) fecundation was accompanied by a decrease in the expression of both genes in the pistil. Similar to *Pd-ETR1* and *Pd-ERS1*, *Pd-CTR1* expression in

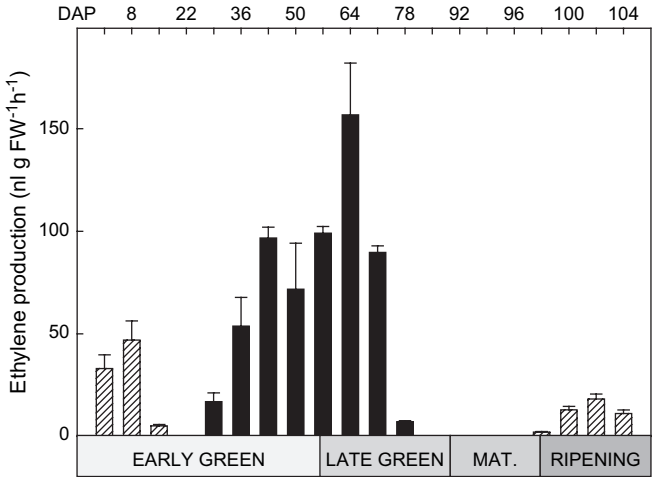

Fig. 2. Alterations in ethylene production from whole fruits (hatched bars) and seeds (solid bars) of *Prunus domestica* L. subsp. *insititia*. Data represent the mean of 4–5 measurements ± SD.

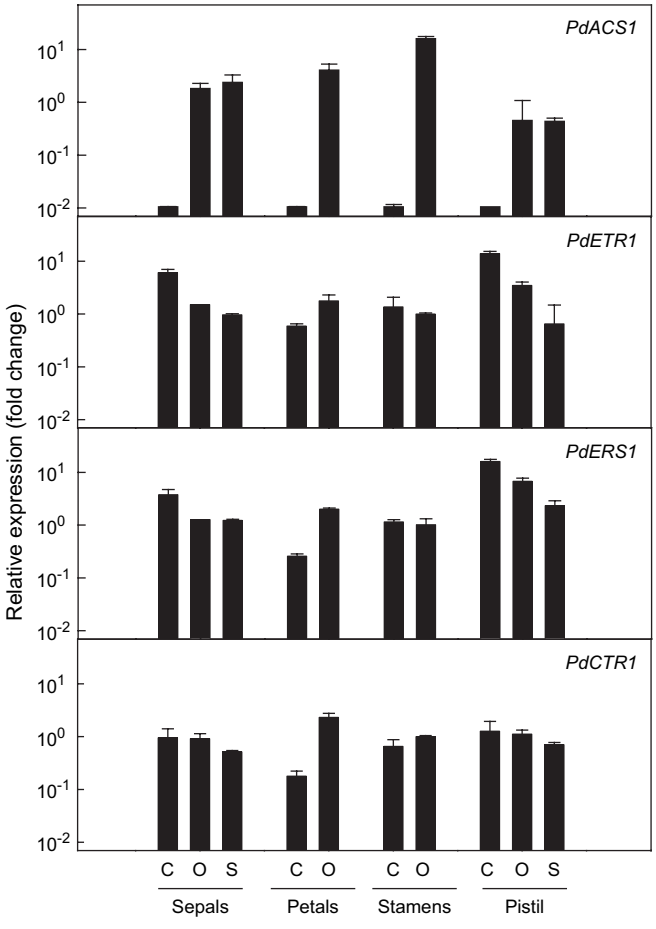

Fig. 3. Expression of *Pd-ACS1*, *Pd-ETR1*, *PdERS1* and *Pd-CTR1* genes during floral development of *Prunus domestica* L. subsp. *insititia*. C, closed; O, open: S, senescent.

closed petals was the lowest during flower development, strongly increasing with the floral-opening process (Fig. 3). The sepals and pistil development correlated with a decrease in expression of *Pd-CTR1*.

The spatial and temporal expression of *Pd-ETR1* and *Pd-ERS1* in *P. domestica* fruits has these characteristics: (a) in *early green* fruits the expression of both receptors decreased during the first 28 DAP to rise again at the end of this stage (Figs. 4, 6, 7); (b) in the seed, the transcription of *Pd-ETR1* was quite similar over time and appears constitutive (Fig. 6); in contrast, *Pd-ERS1* expression increased up to the beginning of the climacteric phase (Fig. 7); (c) in the mesocarp, *Pd-ETR1* expression was similar in the *late green* phase, strongly decreased during *maturation* and increased during *ripening* (Fig. 6); *Pd-ERS1* expression remained low during the *late green* phase (pit hardening) compared to the other phases (Fig. 7); and (d) in the epicarp, *Pd-ERS1* and *Pd-ETR1* had low expression during pit hardening, increased afterwards until *ripening* when levels of both decreased again (Figs. 6, 7). On the other hand, *Pd-CTR1* expression was low in the early whole fruit (Fig. 4) and peaked in the seed, mesocarp and epicarp early in the *maturation* phase. Expression of Pd-*CTR1* decreased in all three during *maturation* but rose again during ripening (Fig. 8).

4. Discussion

4.1. Gene expression involved in ET synthesis and signalling during flower development

The role of ET during flower development is not known in detail since ET production and sensitivity to it varies widely among higher plants. However, it has been demonstrated that pollination is accompanied by changes in ACS and ACO gene expression [17,54–56]. In order to increase our knowledge on ET production in flowers of damson plum, the temporal and spatial expression of *Pd-ACS1* was studied. *Pd-ACS1*-mRNA was hardly detected in pre-anthesis; but anthesis triggered an accumulation of this transcript in all studied floral organs. Taking into account that in pre-anthesis and anthesis ET synthesis was not detected, *Pd-ACS1*, *Pd-ACO1* and *Pd-ACO2* should be post-transcriptionally regulated since both ACO genes were also sharply expressed [40]. On the other hand, because the pollinated pistil was the only organ capable of producing ET it seems evident to conclude that the gene products of *Pd-ACS1*, *Pd-ACO1* and *Pd-ACO2* should be implicated in its synthesis. Moreover, as stated in this work *Pd-ACS1* was greatly expressed in the stamens of open not-pollinated flowers, as well as *Pd-ACO1* and *Pd-ACO2* [40]. This fact suggests that the anthers and more specifically the housed pollen grains may be implied into this stamen expression. One consequence of this should be the synthesis of the ACC necessary for the flower senescence and other post-pollination processes that require the presence of ET [17]. In pollinated flowers of petunia, the first ET burst evolves from the stigma and can be attributed mainly to oxidation of pollen-borne ACC which was synthesized by a pollen-specific ACS [57].

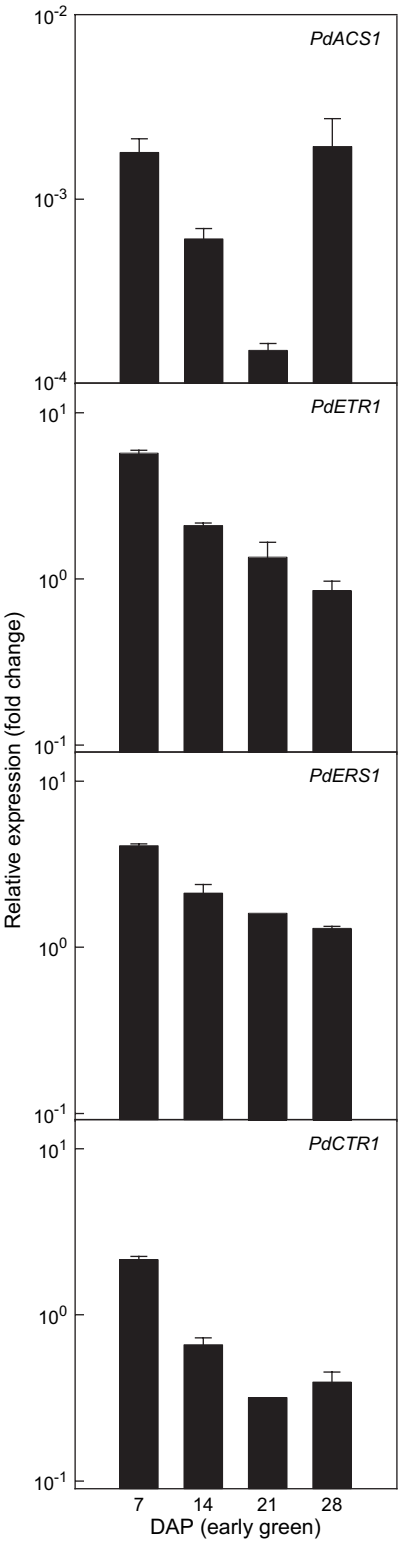

Fig. 4. Expression of *Pd-ACS1*, *Pd-ETR1*, *PdERS1* and *Pd-CTR1* genes in whole fruits of *Prunus domestica* L. subsp. *insititia*. during the first 28 DAP.

In this work, two putative ET receptor genes, *Pd-ETR1* and *Pd-ERS1* were isolated from *P. domestica*. Both exhibit sequence similarity to ET receptors from various plant species such as tomato [58], Arabidopsis [48], muskmelon [19], peach

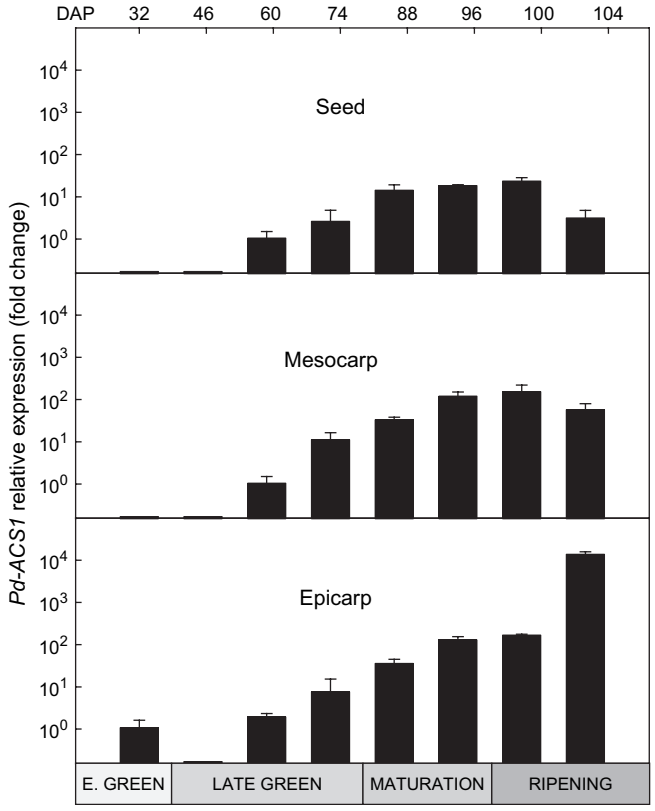

Fig. 5. Alterations in the expression of *Pd-ACS1* gene during development of seed, mesocarp and epicarp of *Prunus domestica* L. subsp. *insititia* fruits.

[21,43], pear [53], and rice [22]. The expression of both receptors demonstrated their tissue and developmental stage specificity. The model proposing that in the absence of ET the receptors are active repressors of the hormonal response has an important physiological consequence because the variations in the levels of the receptors would alter the sensitivity to ET [3,48,59,60]. *Pd-ETR1* and *Pd-ERS1* receptor genes were expressed during all of floral development with similar alterations in transcript levels for both genes over time. The fecundation process was accompanied by a decrease in the expression of *Pd-ETR1* and *Pd-ERS1* genes in the sepals and gyneceum. In damson plum, the fecundated pistil was the only floral organ to produce ET [39]. A major accumulation of *Cs-ETR2* and *Cs-ERS* mRNAs was observed in the gyneceum of cucumber, due probably to the higher level of endogenous ET [61]. In rice, ET treatment appeared to stimulate the accumulation of *Os-ETR2* mRNA and inhibit those of *Os-ERS2*. Moreover, the expression pattern of *Os-ETR2* was parallel with those of ET production and the abundance of *Os-ETR2* mRNA was increased [22]. Likewise, the expression of *Rh-CTR1* increased during floral senescence, coinciding with the increase in ET production [62,63]. However, in *Passiflora edulis*, the level of transcripts of *Pe-ERS1* and *Pe-ERS2* increased during floral senescence; but ET production did not rise [64]. A similar phenomenon has also been observed in carnation [20]. On the other hand, the profile of *Pd-CTR1* gene expression in flowers was temporal and spatially similar to that of *Pd-ETR1* and *Pd-ERS*. In the mutant Nr of tomato, the induction of *Le-CTR1* was associated with the increase of ET during petal senescence [65]. In petals of *Rosa hybrida*, the abundance of *Rh-CTR1* transcript was triggered by senescence, *Rh-CTR2* was constitutively expressed, and both *Rh-CTR1* and *Rh-CTR1* mRNAs levels rose in the presence of ET [62]. Contrary to *Rosa hybrida*, the great *Pd-CTR1* mRNA increase in petals of damson plum took place in the absence of detectable ET.

4.2. Expression of genes involved in ET synthesis and signalling in edible and non-edible parts of damson plum fruit

We have demonstrated here that *Pd-ACS1* expression was very low in whole fruits (*early green*). Moreover, during these early stages of development there was a notable accumulation of *Pd-ACO1* transcripts coinciding with peak of ET production which is higher than ET climateric [40]. ET production in *early green* phase is perhaps needed for regulating abundant cell division existing in fertilized ovary. Initial ET synthesis has also been reported in fruits of species phylogenetically close to *P. domestica*, such as *P. persica* [66,67], and both *Pp-ACO1* and *Pp-ACO2* genes, on the contrary to damson plum, were expressed [43]. ET production in the *early green* phase of damson plum must involve the gene *Pd-ACS1*. However, due to scarce *Pd-ACS1* transcription perhaps another

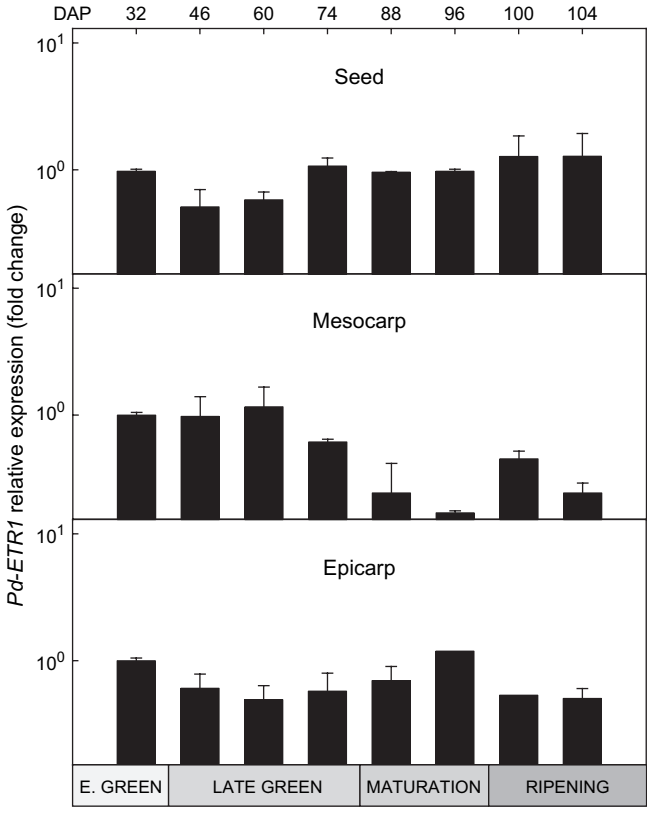

Fig. 6. Alterations in the expression of *Pd-ETR1* gene during development of seed, mesocarp and epicarp of *Prunus domestica* L. subsp. *insititia* fruits.

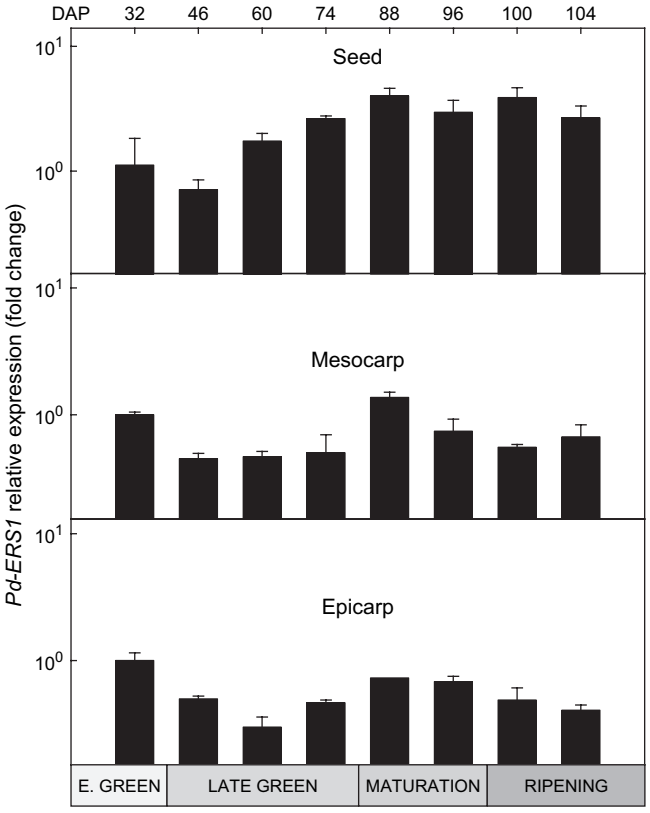

Fig. 7. Alterations in the expression of *Pd-ERS1* gene during development of seed, mesocarp and epicarp of *Prunus domestica* L. subsp. *insititia* fruits.

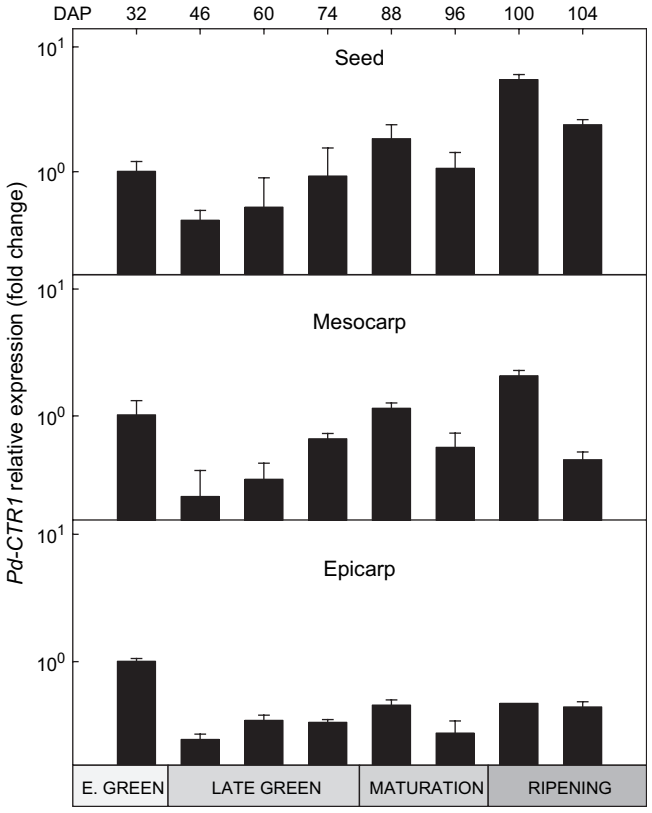

Fig. 8. Alterations in the expression of *Pd-CTR1* gene during development of seed, mesocarp and epicarp of *Prunus domestica* L. subsp. *insititia* fruits.

member of the Pd-ACS multigene family (data not shown) could be also implicated. The results of this work also demonstrated that ET production from early whole fruit corresponded with high levels of *Pd-ETR1* and *Pd-ERS1* mRNAs.

On analysing the kinetics of *Pd-ACS1* expression during development of mesocarp and epicarp of *P. domestica*, and taking into account the high transcriptional activity of *Pd-ACO1* [40], we suggest that *Pd-ACS1* gene has participation in ET production during the climateric period. However, as reported for several fruits, the involvement of other ACS and ACO enzymes cannot be ruled out [68,69]. The expression patterns of *Pd-ETR1*, *Pd-ERS1* and *Pd-CTR1* were different in the plum mesocarp. Thus, *Pd-ERS1* transcript barely changed during the cell expansion stage (*late green*) and *ripening*. In contrast, *Pd-ETR1* expression declined throughout mesocarp development until the late *maturation* phase. After this, it peaked in the early stage of *ripening*. ET could induce the accumulation of mRNA levels of *Pe-ERS2* and *Pp-ERS1* in arils of passion fruits and peach fruits, respectively [52,64]. As a whole, we speculate that Pd-ETR1 and Pd-CTR1 actively participates in the ripening signalling of plum. *Le-ETR1* and *Os-ERS1* were expressed constitutively in all tissues examined of tomato and rice [22,70], whereas *Le-ETR3*(NR) increased during tomato ripening [70]. In fully enlarged fruit of melon *Cm-ETR1* mRNA was very high in seeds, and *Cm-ERS1* mRNA was low in all tissues. However, during ripening *Cm-ERS1* mRNA increased slightly in the pericarp before the marked increase of *Cm-ETR1* mRNA paralleled climateric ET production [19]. Epicarp development of *P. domestica* was not correlated with notable alterations of *Pd-ETR1*, *Pd-ERS1* and *Pd-CTR1* mRNAs during *maturation* and *ripening* stages.

Finally, data on ET signalling in seeds of fleshy fruits are very scant [26]. In tomato, *Le-ETR2* was up-regulated in seeds before germination [70], whereas *Cm-ETR1* was the only studied melon ET receptor to show a high expression in fully enlarged fruit [19]. In damson plum, even though the seed was not capable of producing ET during maturation and ripening stages, perhaps because *Pd-ACO1* and *Pd-ACO2* genes were transcriptionally inactive [40], the levels of *Pd-ACS1*, *Pd-ERS1* and *Pd-CTR1*-mRNAs were very high and those of *Pd-ETR1* possibly constitutive. The existence of a raised accumulation of these transcripts concomitant with the maturation and desiccation of damson plum seed is, at present, not easy to explain.

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