



## Cloning and expression of ethylene receptor ERS1 at various developmental and ripening stages of mango fruit

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**ABSTRACT.** Ethylene induces characteristic ripening reactions in climacteric fruits through its binding to histidine-kinase (HK) receptors, activating the expression of ripening genes. Ethylene receptors have been found in *Arabidopsis thaliana* (Brassicaceae) and some fruits; number and expression patterns differ among species. In mango, only ethylene receptor ETR1 was known. We cloned *ERS1* cDNA from mango, and evaluated the expression of Mi-*ERS1* and Mi-*ETR1* by qPCR in developmental and ripening stages of this fruit. The Mi-*ERS1* coding sequence is 1890 bp long and encodes 629 amino acids, similar to ERS1 from other fruits. Also, the amino acid sequence of ERS1 C-terminal HK domain shows the cognate fold after molecular modeling. Mi-*ERS1* expression levels increased as mangoes ripened, showing the highest

levels at the climacteric stage, while Mi-*ETR1* levels did not change during development and ripening. We conclude that the patterns of expression of Mi-*ERS1* and Mi-*ETR1* differ in mango fruit.

**Key words:** Ethylene receptor; *Mangifera indica* L.; Expression; Ripening; Molecular modeling

## INTRODUCTION

Ethylene is involved in many physiological and developmental events in plants, playing an important role in ripening in all climacteric fruits, including mango (*Mangifera indica* L.) (Bleecker and Kende, 2000). Ethylene induces the characteristic reactions for fruit ripening, including the synthesis of enzymes involved in softening (cellular wall degradation) via depolymerization of matrix glycans (Brummell et al., 2004), the release of volatile compounds, and pigment production. These reactions occur in response to the binding of ethylene to receptors located in the membrane of the endoplasmic reticulum, which in turn initiates the multistep pathway involved in ethylene perception and signal transduction events (Giovannoni, 2001; Guo and Ecker, 2004).

Ethylene receptors (ETRs) are encoded by a small gene family of catalytic receptors found in bacteria and some eukaryotic systems known as 2-component regulators, and most of these are transmembrane sensor proteins (Marina et al., 2005). These systems, first described in bacteria, consist of 2 proteins - a sensor and a response regulator - that can modulate responses to a great variety of environmental stimuli (Grefen and Harter, 2004). The number of ETR isoforms varies from species to species, but all of the receptors share similar structures and contain an ethylene binding domain, a cGMP-specific phosphodiesterase, adenylyl cyclase and Fh1A (GAF) domain of unknown function, and a histidine kinase (HK) domain. Five ETRs were initially identified in the model plant *Arabidopsis thaliana* [ETR1, ETR2, ethylene response sensor (ERS) 1, ERS2, and ethylene insensitive (EIN) 4] (Bleecker et al., 1998) and 6 in tomato (*Lycopersicon esculentum*) (Lashbrook et al., 1998; Tieman and Klee, 1999).

Based on ethylene binding domain sequence comparisons, various ETR isoforms in plants can be separated into 2 subfamilies: subfamily I members contain 3 transmembrane segments at their N-terminal hydrophobic domain (ETR1 and ERS1), and 4 of these segments occur in subfamily II (ETR2, ERS2, and EIN4) (Bleecker et al., 1998). The ETRs contain a receiver domain at the C-terminus in addition to these domains. The 2 type of receptors function as homodimers linked by a disulfide bond at their N-terminals (Hall et al., 2000). In the absence of ethylene, the HK domain of the constitutive triple response 1 domain (CTR1), a negative regulator of the pathway, represses ethylene response; ethylene binding by the receptor induces a CTR1 conformational change, reducing its kinase activity and thereby activating the ethylene response pathway (Gao et al., 2003; Huang et al., 2003).

Despite the growing knowledge of the characterization of these receptors, little is known about the expression of ETR and ERS coding genes in fruits that are not model systems. Currently, 5 ETRs have been identified in apple (Tatsuki et al., 2007; Wiersma et al., 2007), 4 in pear (El-Sharkawy et al., 2003), 3 in peach (Rasori et al., 2002; Trainotti et al., 2005), 3 in persimmon (Pang et al., 2007), 2 in melon (Sato-Nara et al., 1999), and 1 in mango (Martínez et al., 2001). Expression studies of ETR genes have revealed a differential expression pattern related to the type of tissue, developmental stage, and exogenous application of ethylene. In *A. thaliana*, the

expression of *At-ETR1* is ubiquitous and ethylene independent, whereas that of *At-ERS1* is tissue specific, ethylene dependent, and developmentally regulated (Hua et al., 1998). In peach (*Prunus persica*), *Pp-ETR1* appears to be constitutively expressed and ethylene independent, whereas the *Pp-ERS1* transcription rate dramatically increases in activated leaf and fruitlet abscission zones as well as in ripening fruit (Rasori et al., 2002). In melon fruit, *ERS1* accumulates during enlargement on both the transcript and protein levels (Ma et al., 2006a).

In this study, the nucleotide sequence of *ERS1* complementary DNA (cDNA) was obtained and its expression was evaluated in mango fruit at various developmental and ripening stages. We also examined some structural features of *ERS1* based on the molecular modeling of its HK domain, which is characteristic for this receptor type.

## MATERIAL AND METHODS

### Plant material

*M. indica* L. fruits from the cultivar 'Kent' were harvested by hand at the research station Valle del Mayo (27°0437.96 N and 109°2705.56 W) of the National Institute of Forestry, Agricultural and Veterinary Research in Navojoa, Sonora. Fruits were picked based on days after flowering (DAF) at developmental stages S1 (106 DAF) and S2 (122 DAF) and at physiological maturity or S3 (189 DAF). To obtain samples of stage S4 (commercial maturity), fruits were ripened under laboratory conditions by storing them at 20°C with 65% humidity.

### Respiration

CO<sub>2</sub> production was measured using gas chromatography. The measurement was made with a gas chromatograph Varian Star 3400 (Varian, Walnut Creek, CA, USA) equipped with flame ionization detectors and thermal conductivity and a 2-m metal column filled with Haysep N 800/100 (Varian) (Watada and Massie, 1981). Mango fruits selected and harvested in each developmental stage were measured 3 times. The fruits were placed in sealed plastic containers for 1 h at 20°C, and CO<sub>2</sub> headspace concentration was analyzed by withdrawing a 1-mL sample from the container and injecting it into the gas chromatograph. A standard of 5% was used, and CO<sub>2</sub> concentration was obtained using the following formula:

$$\text{mL CO}_2 \cdot \text{kg}^{-1} \cdot \text{h}^{-1} = \frac{(\text{sample area}) (\text{standard concentration}) (\text{head space area})}{(\text{incubation time}) (\text{standard area}) (\text{sample weight})}$$

### RNA isolation and cDNA synthesis

Total RNA was isolated and purified from mango seeds for cloning purposes and from mesocarp tissue for expression analysis using a Qiagen RNeasy Plant Mini Kit<sup>®</sup> (Qiagen, Mississauga, Ontario, Canada) and the supplied RLT extraction buffer according to manufacturer instructions. RNA quantity was estimated at 260 nm using a NanoDrop ND-1000 ultraviolet-visible spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). RNA integrity was detected via agarose gel electrophoresis. The cDNA synthesis from RNA obtained from both tissues was performed via reverse transcription of 1.0 µg RNA using a Creator<sup>™</sup> SMART<sup>™</sup> cDNA Library Construction Kit (Clontech Laboratories, Palo Alto, CA, USA).

## Cloning of *ERS1* cDNA

Mi-*ERS1* cDNA was obtained using degenerated primers ReEt Fw (5'-GAITGTGCI TTGTGGATGCC-3') and ReEt Rw (5'-ATIGCAGCATGIGAIAGAGC-3'). Reactions were performed on a Peltier thermal cycler (DNA Engine, BioRad, Hercules, CA, USA) using Platinum PCR Super Mix (Invitrogen) and 100 ng cDNA as a template under the following conditions: 5 min at 94°C, followed by 30 cycles of 45 s at 94°C, 90 s at 50°C, 1 min at 72°C, and 5 min at 72°C as a final extension. The polymerase chain reaction (PCR) products were thoroughly sequenced at the University of Arizona Genetics Core (Tucson, AZ, USA) and analyzed using the Basic Local Alignment Search Tool algorithm (Altschul et al., 1990) against the GenBank database. Specific primers were designed to walk over the cDNA and were used to obtain the 5'- and 3'-ends in accordance with manufacturer instructions for a DNA Walking kit (SeeGene, USA). The following primers were used to walk over the 5'-end: Mi-ERS4, 5'-GAGACAGTTCTAAAGAGTAG-3'; Mi-ERS2, 5'-GCCACTTGCAGATTGAAC-3', and Mi-ERS1, 5'-GTGAAGATTGAATGGTCC-3'. The following primers were used for the 3'-end: Mi-ERS5, 5'-GACTTGCCATTAGCAGAAGG-3'; Mi-ERS1F, 5'-CTTGAGTGTTCGCT TGTGGATG-3', and Mi-ERS3F, 5'-GCGCTGTAATGAACCATG-3'. Contiguous sequences were assembled using the DNA sequence assembly program CAP3 from non-ambiguous sequences of both strands to assemble the Mi-*ERS1* cDNA sequence.

## Mi-*ERS1* and Mi-*ETRI* expression analysis

Quantitative reverse transcription coupled with quantitative PCR (qRT-PCR) was carried out using the Full Velocity™ SYBR® Green qRT-PCR Master Mix (Stratagene, La Jolla, CA, USA). Specific primers for Mi-*ERS1* (sense, 5'-CTTTGATAAAGCCAATCGCA-3'; antisense, 5'-GTAAATTTACAGCGTTGCC-3'), Mi-*ETRI* (sense, 5'-AATATATGCCTGGCGA GGTG-3'; antisense, 5'-TGCACTATCTGAGGGAAGCA-3'), and mango glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) (sense, 5'-GTGGCTGTTAACGATCCCTT-3'; antisense, 5'-GTGACTGGCTTCTCATCGAA-3') were designed to amplify products of 150 bp. Three replications were carried out for each sample in reactions with a final volume of 25 µL containing 100 ng total RNA, 75-125 nM gene-specific primers, 0.0625 µL Stratascript RT/RNase block (Stratagene), and Full Velocity™ SYBR® Green qRT-PCR Master Mix. The PCR products were amplified in a SmartCycler (Cepheid, Sunnyvale, CA, USA) using the following conditions: 50°C for 30 min (cDNA synthesis), 95°C for 5 min, and 50 cycles of 95°C for 10 s and 60°C for 30 s. To calculate the expression of Mi-*ERS1* and Mi-*ETRI* receptors, we used the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). Data were analyzed based on the  $C_T$  value of each sample during PCR amplification, where  $-\Delta\Delta CT$  was  $-(C_T \text{ target} - C_T \text{ GAPDH}) - (C_T \text{ Avg target} - C_T \text{ Avg GAPDH})$ , and *Avg* corresponded to the averaged  $C_T$ s from runs of developmental stage S1. The results are reported as relative mRNA steady-state levels of the target gene and normalized to *GAPDH* expression levels. Statistical analysis was carried out with one-way analysis of variance, and for *post hoc* analysis, we used the Tukey test. Statistical significance was considered when the P value was 0.05, and analyses were performed using the NCSS (2001) software (Kaysville, UT, USA).

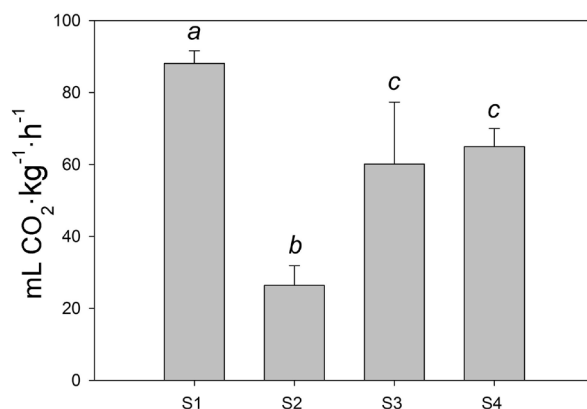
## Molecular modeling of ERS1

The molecular structure of the cytoplasmic portion of Mi-ERS1 was predicted through homology modeling with the HK topology of bacteria (Marina et al., 2005; Casino et al., 2009). The theoretical structure was obtained with the Molecular Operating Environment software version 2010.10 (Chemical Computing Group, Montreal, Canada). A multiple sequence alignment was used to improve the homology model using the sequences of the ETR for other plants. The coordinates used for molecular model building were those for the HK of *Thermotoga maritima* bacteria from 2 different crystal structures deposited in the Protein Data Bank (codes 3DGE and 2C2A). A total of 50 intermediate models were generated from the alignment for the cytoplasmic region of Mi-ETR under the CHARMM27 force field. The final model was further refined through an energy minimization scheme in Molecular Operating Environment starting from the best of the intermediate models according to the protein geometry utility of the software.

## RESULTS

### CO<sub>2</sub> production

Mangos in 2 stages of development (S1 and S2) were selected based on 106 and 122 DAF, respectively; stage S3 was obtained at 189 DAF (physiological maturity), and the final stage (S4, commercial maturity) was obtained through storage at 20°C with a 60% relative humidity for 10 days. The respiratory rates determined with gas chromatography appear in Figure 1. The CO<sub>2</sub> production trend is typical for climacteric fruits. The first stage corresponded to S1, and the respiratory rate yielded a CO<sub>2</sub> concentration of 87 mL·kg<sup>-1</sup>·h<sup>-1</sup>. At the S2 stage, metabolism slowed during development, and CO<sub>2</sub> production diminished until respiration reached 25 mL·kg<sup>-1</sup>·h<sup>-1</sup>. An increase in respiration with CO<sub>2</sub> levels between 60 and 65 mL·kg<sup>-1</sup>·h<sup>-1</sup> occurred in the next 2 stages because the fruit returns to an accelerated metabolism, synthesizing enzymes for metabolic processes that result in the organoleptic characteristics of the mature fruit; these processes are reflected by changes in fruit color, texture, and aroma.



**Figure 1.** Levels of CO<sub>2</sub> produced by mango fruits in two stages of development (S1 and S2) and two ripening stages (S3 and S4). Data are means of at least three determinations. Mean values in each bar followed by a different letter at each stage of development are significantly different ( $P < 0.05$ ).

### cDNA and deduced amino acid sequence of Mi-ERS1

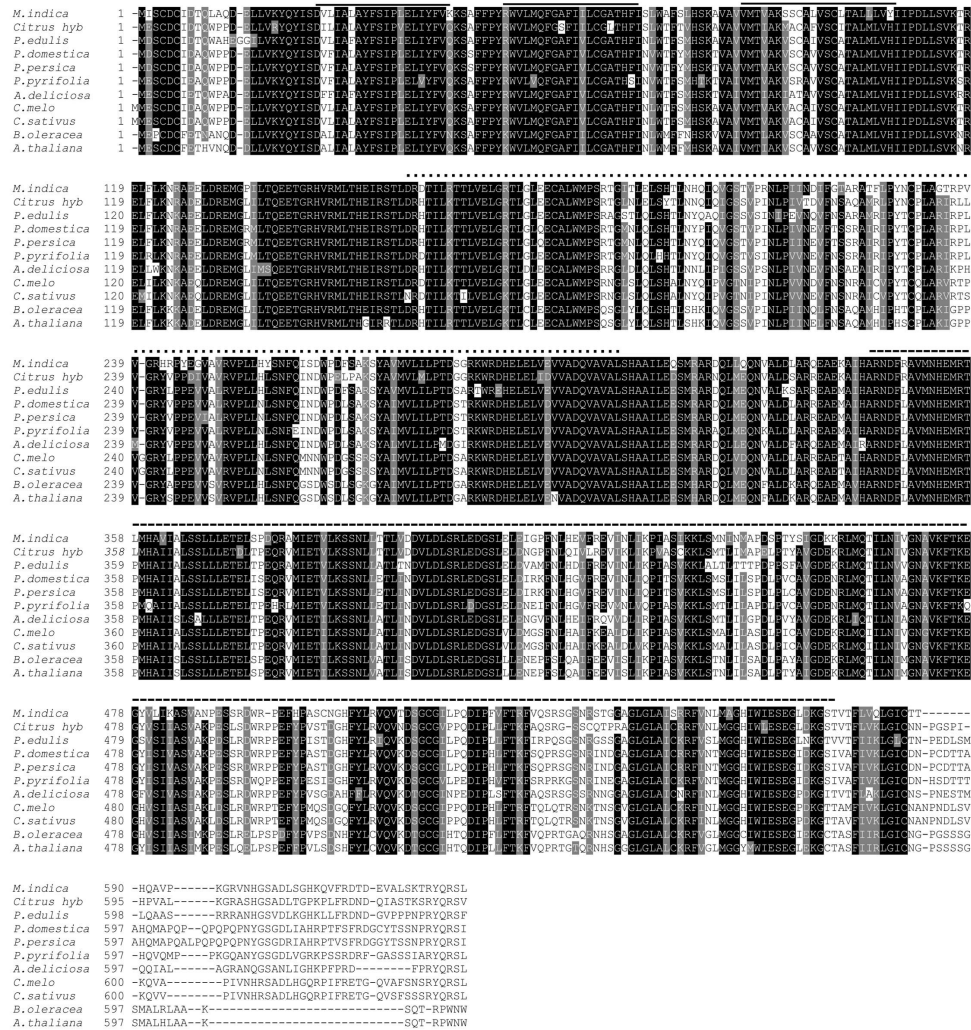
The complete sequence of the Mi-ERS1 cDNA (GenBank JN851132) is 1908 bp with an 18-bp 5'-untranslated region and an 1890-bp open reading frame including the initial methionine and the stop codon (Figure 2). The 1890-bp open reading frame encodes a protein of 629 amino acid residues with a theoretical isoelectric point of 6.86 and a molecular weight of 70.3 kDa, which is similar to that of other ETRs from fruits. Multiple amino acid sequence alignment with other species showed that Mi-ERS1 is highly conserved between climacteric and non-climacteric fruits (Figure 3). The deduced protein was 78% identical to ERS1 from a citrus hybrid cultivar (ADB25216.1) and 76% to ERS1 from passion fruit (*Passiflora edulis*, BAA37137.1), plum (*Prunus domestica*, CAI64504.1), and peach (*P. persica*, AAM89517.1). The Mi-ERS1 sequence contained the 3 characteristic domains for ETR subfamily I members: an ethylene binding domain containing 3 membrane-spanning motifs (residues 25-119), a GAF domain (159-307), and an HK domain (344-577).

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1       M I S C D C I D T Q L A Q D E L L V K Y Q Y I S D V L I A L A Y F S
1     CCATAAGGCAATTGTCATGATATCTTGATTGCATTGATACACAATTGGCCAGGATGAACCTCGTGAATATCAGTATATCTCAGATGATTGATGCCCTGCGTATTCTC
35  I P L E L I Y F V K K S A F F P Y R W V L M Q F G A F I I L C G A T H F I S L W
121 ATTCCCTTGGAGCTTATATATTTCGTGAAGAAGTCAGCATTCTTCCATATAGATGGGTCTAATGCAGTTCGGTGCTTTTATTATTCTTTGGAGCAACCCACTTCATAAGCTTGGG
75  A F S L H S K A V A V V M T V A K S S C A L V S C L T A L L L V Y I I P D L L S
241 GCATTCTCTGCACCTCCAAAGCGTCGCTGTGTGATGACTGTGCAAAAGAGTCTCTGTGCTAGTGTCACTACTGCTTTGCTACTTGTTCATCATCTCTGATCTTTTGAGT
115 V K T R E L F L K N R A E E L D R E M G P I L T Q E E T G R H V R M L T H E I R
361 GTCAAAACCTCGGAATTATCTCTGAAGAACAGGGCTGAAGAGCTTGACCGGGAGATGGCCCTATCTTACTCAAGAAAGAACTGGAAGGCATGTTAGAATGCTGACTCATGAATAGG
155 S T L D R D T I L R T T L V E L G R T L G L E E C A L W M P S R T G I T L E L S
481 AGCACACTCGATAGAGATACAATTTAAGAACCACCTCTGTTGAGCTAGTAGGACCTTAGGCTGGAGGAATGTGATGTGGATGCCTCAAGAACGGTATCACTTAGAACCTTCT
195 H T L N H Q I Q V G S T V P R N L P I I N D I F G T A R A T F L P Y N C P L A G
601 CACACTCTGAACCATCAAATACAAGTTGGATCTACCGTCTCCTAGAAAACCTTCCATAAATAATGACATCTTTGGCACTGCTCGAGCAACCTTTTACCCATAACTGCCACTGGCAGGG
235 T R P V V G R H R P Y E G V A V R V P L L H Y S N F Q I S D W P D F S A K S Y A
721 ACCAGCTCTGTGGGAAGACATAGGCCCTTATGAGGTTGCGCTGTTGGGTACTCTTTTACATTACTCAAATTTCCAAATCAGCGATTGGCCTGACTCTCTGCAAAAAGCTATGCA
275 V M V L I L P T D S G R K W R D H E L E L V E V V A D Q V A V A L S H A A I L E
841 GTCATGGTCTGATCCTCCCTGACAGTGGTAGAAAATGGAGAGATCATGAGTTGGAAGTTGTGAGCAGCCAGGTTGCTTGTCTTTCTCATGCTGCAATCTGGAA
315 Q S M R A R D Q L L Q Q N V A L D L A R Q E A E K A I H A R N D F R A V M N H E
961 CAATCTATCGGGCTCGTATCAGCTTTCACAACAGATGTTGCTTTAGATTGGCTGACAAAGGAGGCAAGGCAATCCATGCTCGAAATGATTTCCCGCGTGAATGAACATGAA
355 M R T L M H A V I A L S S L L L E T E L S P D Q R A M I E T V L K S S N L L T T
1081 ATGAGGACTCTGATGATGATGATTTGATTTGCTTCTCTCTGGAAGACAGAATTAAGTCCAGATCAAAGGCAATGATAGAGACAGTCTTAAAGATGATGAACCTCTGACAA
395 L V D D V L D L S R L E D G S L E L E I G P F N L H E V F R E V I N L I K P I A
1201 CTTGTTGATGATGTTTTGGATCTTCCCGACTTGAAGATGGTAGTCTAGAAATAGAAAATGGACCATCAATCTTCCGAAAGTTTTCAGAGAGTCAATTAATTTGATAAAAACATATTGCA
435 S I K K L S M N I N M A P D S P T Y S I G D K K R L M Q T I L N I V G N A V K F
1321 TCTATAAAGAGTTATCCATGAATATAAATATGGCCGAGATTCCACCAACATACTCAATTTGGCCGACAAAGAAAGACTGATGCAAACTATTTTGAACATTGGGGTAAATGCTGTAAGTTT
475 T K E G Y V L I K A S V A N P E S S R D W R P E F H P A S C N G H F Y L R V Q V
1441 ACAAGGAGGTTATGTTTTGATTAAAGCTTCTGTGCAAAATCCAGAGTCTTCAAGAGACTGGCGACCTGAAATTCACCCCTGCGTCATGCAATGGCCACTTCACTTCGAGTACAGGTT
515 T D S G C G I L P Q D I P F V F T R F V Q S R S G S N R S T G G A G L G L A I S
1561 ACTGATTCTGGCTGGTATTCTGCCACAAGACATTCATTGTCCTTACCAGATTTGTTCAATCTCGAAGTGGCTCCAACAGAAGTACAGGTGGAGCAGGACTGGACTTGCATTAGC
555 R R F V N L M A G H I W I E S E G L D K G S T V T F L V Q L G I C T T H Q A V P
1681 AGAAGGTTTGTCAATCTCCGACGGGCACATTTGGATTGAGAGTGAAGTCTTGAACAAGTACACAGTACACATTTCTGTCCAATTTGGGATATGACACCACACACCAAGCTGTACCC
595 K G R V N H G S A D L S G H K Q V F R D T D E V A L S K T R Y Q R S L *
1801 AAAGTCCGATAAATCATGGAAGTGCAGATCTCTCTGGACATAAACAAGTATTTAGAGACACCGATGAGGTAGCTTTATCCAAAACACCATACCAAGAAGGCTTTAGTGCACAGTGACA
1921 GCAGATCGACATTACGGACCAGCTTATTCAGGATATGATTTTCTCATATTCAGTAAAAGCTGAAGGCCGAAATCCCGGTGTACATTTATACATAG

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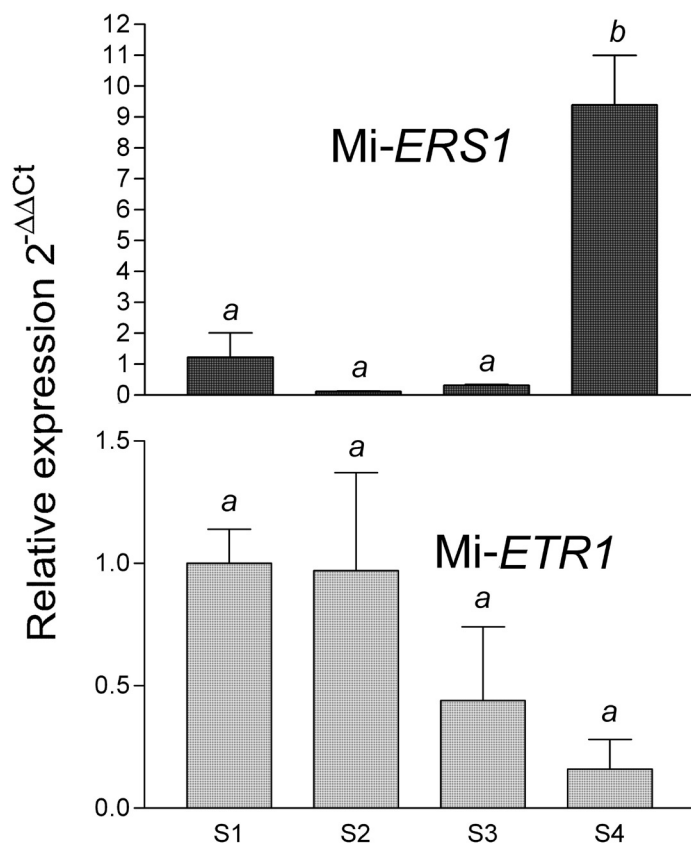
**Figure 2.** Nucleotide and deduced amino acid sequence for mango ERS1. The stop codon is labeled with an asterisk. In boldface, Cys 4 and Cys 6 involved in dimerization and phosphorylation site His 353. Subdomains N, G, F, and G present in the histidine kinase domain of subfamily I ethylene receptors are in boxes.



**Figure 3.** Multiple amino acid sequence alignment of *Mangifera indica* L. ERS1 with other plant ERS. *Citrus* hybrid cultivar (ADB25216.1), passion fruit (*Passiflora edulis* BAA37137.1), Damson plum (*Prunus domestica* subsp. *insititia*, CAI64504.1), peach (*Prunus persica*, AAM89517.1), Nashi pear (*Pyrus pyrifolia*, BAB13735.1), kiwi (*Actinidia deliciosa*, ABY28263.1), Cantaloup melon (*Cucumis melo* var. *Cantalupensis*, BAB13735.1), cucumber (*Cucumis sativus*, BAA85818.1), cabbage (*Brassica oleracea*, AAC31157.1), and *Arabidopsis thaliana* (AAB86454.1). The three  $\alpha$ -helix domains spanning the membrane are marked with a continuous line, the GAF domain is marked with a pointed line and the histidine kinase domain in dashed line. Identical amino acids are indicated in black regions, and conservative replacements in gray regions.

### Expression of Mi-ERS1 and Mi-ETR1 during development and ripening

To explore Mi-ERS1 and Mi-ETR1 expression during fruit development, we analyzed their transcripts with quantitative PCR (Figure 4). Mi-ERS1 mRNA levels remained constant during stages S1 to S3, then showed an 8-fold increase in expression at stage S4 (mature mango). The Mi-ETR1 mRNA levels did not change significantly in fruits during the S1 developmental stage or throughout the processes that followed until the commercial ripening stage (S4).



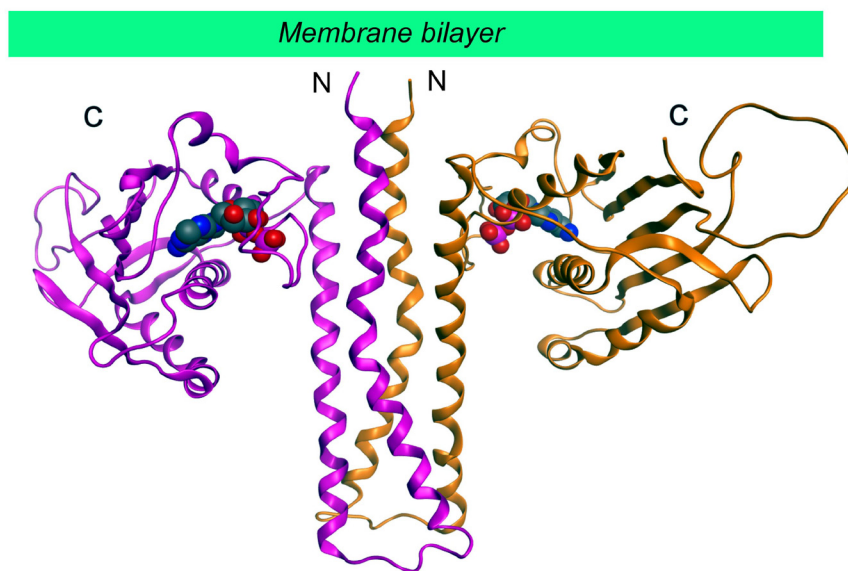
**Figure 4.** Relative expression of Mi-ERS1 and Mi-ETR1 at developmental stages S1, S2, and ripening fruits at S3 and S4 stages. Different letters show statistical difference ( $P < 0.05$ ) between stages of development. S1 = 106 days after flowering, S2 = 122 days after flowering, S3 = physiological maturity, and S4 = commercial maturity. Data was normalized with the *GAPDH* constitutive gene.

### Molecular modeling of ERS1 cytoplasmic domain

The deduced protein sequence of Mi-ERS1 displayed the N-terminal sensor domain and the C-terminal HK domain, connected through the GAF domain (Ma et al., 2006b). A multiple sequence alignment of ERS1 to other homologs from plants showed a high degree of con-



ervation for the 3 domains (Figure 5). The N-terminus showed a high potential for forming transmembrane  $\alpha$ -helices, which span the membrane of the endoplasmic reticulum, as seen in a protease protection assay (Ma et al., 2006b). The C-terminal HK portion of ERS1 (residues 344-577) was modeled, adopting the topology for this receptor type, which is a dimer with an interface of 4  $\alpha$ -helices. The HK portion of ERS1 had 31% sequence identity with the bacterial HK that was used as a template for homology modeling.



**Figure 5.** Molecular modeling of mango ERS1 cytoplasmic domain. Ribbon representation of ERS1 dimer based on homology modeling, including ADP molecule shown in sphere representation. The N- and C-terminal positions are labeled in each subunit, A (pink) and B (golden). The membrane would be located on the top of N-terminal residues.

## DISCUSSION

Harvested fruits are classified as climacteric or non-climacteric based on their respiratory pattern and ethylene biosynthesis during ripening. Climacteric fruits harvested at full maturity can be ripened out of the parent plant. The respiration rate and ethylene formation, although minimal at maturity, rise dramatically to a climacteric peak at the onset of ripening, after which they decline (Prasanna et al., 2007).

Respiration rate, measured as  $\text{CO}_2$  production, is a strong indicator of maturity in climacteric fruit. Akamine and Goo (1973) have reported 1-day difference between  $\text{CO}_2$  level and ethylene production peaks in the mango ‘Kensington Pride’ at ripening. Studies carried out by Lalel et al. (2003) to study the respiration process in mango fruit development have shown a low  $\text{CO}_2$  level in early developmental stages and later an increment of  $\text{CO}_2$  production at ripening. This result is in accordance with our data, which revealed a decrease in  $\text{CO}_2$  level at the S2 stage followed by a 2.4-fold increase at the S3 and a 2.6-fold increase at the S4 stages compared with that at S2.

Ethylene in minute amounts can trigger many cellular metabolic events, including rip-

ening initiation and senescence, particularly in climacteric fruit. Ethylene is synthesized auto-catalytically at levels as low as 0.0016 nM in the 'Kent' mango (Kader, 2002), triggering the ETR response leading to the ripening process. The expression of Mi-*ERS1* and Mi-*ETR1* was evaluated in this study using qRT-PCR of Mi-*ERS1* and Mi-*ETR1*. During mango development, Mi-*ERS1* transcripts remained unchanged and dramatically increased at the commercial ripening stage (S4) as a primary response to ethylene. Rasori et al. (2002) have reported that Pp-ERS genes are upregulated with auto-catalytically synthesized ethylene.

In contrast, Mi-*ETR1* expression levels were similar during the 4 stages of mango fruit development, whereas Mi-*ERS1* levels were higher than those of Mi-*ETR1* and increased during ripening. This expression behavior for *ETR1* has been reported for other climacteric fruits, such as the passion fruit (Mita et al., 1998), peach (Bassett et al., 2002; Rasori et al., 2002), and kiwi (Yin et al., 2008), in which *ETR1* expression is constant throughout the various developmental stages. As shown in this study (Figure 4), the expression of *ETR1* in mango is constitutive during fruit development and ripening, unlike that of *ERS1*. This difference can be explained by the important structural differences between ERS1 and ETR1 proteins already known in the ETRs of other fruits. Both proteins, as ETR subfamily I members, have an overall similar sequence and share all the residues known to be essential for ethylene binding, although ERS1 lacks the response regulator domain found in ETR1 (Bleecker, 1999).

The ERS1 deduced amino acid sequence had 66% similarity to a reported Mi-ETR1 (Martínez et al., 2001) in accordance with the 63% similarity reported between Pp-ETRs (Rasori et al., 2002). The low identity between Mi-ETR1 and Mi-ERS1 is mainly due to the lack of the response regulator domain in the latter. The response regulator domain is involved in phosphate transfer within the signaling pathways. Moreover, the ethylene response is regulated by its interaction with the CTR1 protein (Yoo et al., 2009). Huang et al. (2003) have reported an analysis of loss of CTR1 function in *A. thaliana*, in which the interaction of the N-terminal domain of CTR1 with ETR1 is required for the negative regulation of ethylene signaling. Thus, the authors have suggested that CTR1 kinase activity is directly activated by ETR1 in the absence of ethylene, and that the active CTR1 phosphorylates the downstream targets, turning off the ethylene response pathway.

Although ETR1 and ERS1 have important structural differences that may lead to quantitative differences in signaling, they share similar biochemical properties for ethylene sensing and phosphorylating activity in the HK domain. A highly conserved HK motif is shared by the deduced protein of Mi-ERS1 and a previously reported Mi-ETR1 protein (Martínez et al., 2001). This HK domain adopted the topology for the 2-component system for signal transduction in prokaryotes, fungi, and plants. This domain contains an HATPase-like region that is critical in phosphorylation and ATP binding. Until now, no ERS protein structure has been reported, and only 2 crystal structures exist for the HK domain from a single bacteria with homology to HK domains from plants (Marina et al., 2005). These crystallographic structures were used to obtain the HK cytoplasmic domain structure of Mi-ERS1. The HK domain is characterized by the presence of His (autophosphorylation site), Asn, Gly, Phe, and Gly (NGFG) residues, as reported for subfamily I receptors of *A. thaliana* (Hall et al., 2007) and *L. esculentum* (Klee and Tieman, 2002), and they are also present in Mi-ERS1.

Furthermore, Mi-ERS1 has the N-terminal hydrophobic domain containing 3 transmembrane regions capable of forming  $\alpha$ -helices, which is characteristic for members of subfamily I. The N-terminal domain is located in the endoplasmic reticulum lumen, at which ethylene is sensed, and it forms homodimeric links via a disulfide bond between Cys5 and

Cys7 residues in *Cucumis melo* ERS1 (Ma et al., 2006a). These residues are present in the Mi-ERS1 deduced polypeptide, corresponding to Cys4 and Cys6. Although ETRs function as homodimers, the GAF domain has recently been reported to be capable of mediating heteromeric receptor interactions (Gao et al., 2008) as well as cyclic guanosine monophosphate binding and light regulation functions in some proteins.

A number of ETR subfamily I- and II-associated genes have been identified in fruits. The biochemical functions and interactions of those genes are still being studied because of the signaling and response complexity in the ripening process. An overlapping expression in plant tissues is exhibited by the ethylene response system receptors, but it may maintain distinct functional identity, responding differentially to developmental challenges that may vary among species. More detailed molecular information is required to reach a more comprehensive understanding of the ethylene hormone signaling pathway in climacteric fruits and determine how the expression of these genes may coordinate ethylene perception in fruits during the development process.

Mi-ERS1 was differentially expressed during mango fruit ripening, whereas Mi-ETR1 levels remained largely unchanged. Future experiments should aim to clone the components of the ethylene signal transduction pathway in mango fruits and to evaluate the expression of these genes. An understanding of these processes could be helpful in the development of transgenic mango plants with retarded ripening.

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