ELSEVIER

Identification and characterization of ethylene receptor homologs expressed during fruit development and ripening in persimmon (*Diospyros kaki* Thumb.)

Jin Huan Pang [a,1], Biao Ma [a,1], Hyeon-Jin Sun [a], Guinevere I. Ortiz [a],
Shunsuke Imanishi [b], Sumiko Sugaya [a], Hiroshi Gemma [a], Hiroshi Ezura [a,*]

[a] *Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8572, Japan*
[b] *National Institute of Vegetable and Tea Science, National Agricultural and Bio-oriented Research Organization (NARO) Ano, Mie 514-2392, Japan*

Abstract

Persimmon (*Diospyros kaki* Thumb.) is an atypical climacteric fruit that produces a small amount of ethylene during ripening, whereas it exhibits a climacteric-like increase in ethylene production in detached young fruit. To better understand the regulatory role of ethylene in fruit ripening, we isolated three full-length persimmon cDNAs homologous to *Arabidopsis* ethylene receptor genes *ERS1*, *ETR1*, and *ETR2*, designated as *DkERS1*, *DkETR1*, and *DkETR2*, respectively, and examined their expression during fruit development and ripening. *DkETR1* mRNA expression remained at a basal level throughout all stages examined and was not affected by ethylene treatment. In contrast, expression of *DkERS1* and *DkETR2* mRNAs was correlated with ethylene production during fruit development and ripening and was enhanced after ethylene treatment. Because the abundance of the *DkERS1* transcript was far higher than those of the other two genes, we further examined DkERS1 expression at the protein level. Western blot analysis using anti-DkERS1 antibody showed that expression of DkERS1 protein decreased gradually towards maturation and reached the lowest level at the ripening stage. Possible roles of the ethylene receptors in regulating fruit development and ripening are discussed.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Ethylene receptor; Fruit ripening; Gene cloning; Persimmon

1. Introduction

The gaseous hormone ethylene regulates a number of plant growth and developmental processes, including fruit ripening (Abeles et al., 1992; Lelièvre et al., 1997; Giovannoni, 2004). Fruit have been classified as climacteric and non-climacteric based on the respiration pattern and ethylene production during ripening. In climacteric fruit, onset of ripening is accompanied by a sharp increase in respiration and ethylene production. The climacteric ethylene is thought to regulate fruit ripening by inducing the expression of many ripening-related genes responsible for autocatalytic ethylene production, cell wall metabolism, chlorophyll degradation, synthesis of carotenoids and volatiles, and conversion of starch to sugar (Gray et al., 1992; Theologis,

1993; Alexander and Grierson, 2002). In non-climacteric fruit, there is no dramatic change in the rate of respiration, and ethylene production remains at a very low level. However, in some plant species, some aspects of ripening, such as chlorophyll degradation and fruit softening, are controlled or at least partially controlled by ethylene (Goldschmidt et al., 1993; Wills and Kim, 1995). Ethylene is biosynthesized from methionine via a well-defined pathway in which 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO) function as key enzymes. Considerable progress has been made in the characterization of ACS and ACO genes, and this has allowed the generation of anti-sense plants with reduced ethylene production and delayed fruit ripening (Lelièvre et al., 1997).

Ethylene is perceived by a family of receptors that are similar to bacterial two-component histidine kinase (Bleecker et al., 1998). Apart from ethylene production, ethylene perception also plays an important role in regulating fruit ripening, as indicated by the identification of the *Never ripe* (*Nr*) tomato mutant as an ethylene receptor mutant (Lanahan et al., 1994; Wilkinson et al., 1995). These findings provide an alternative to control

fruit ripening by manipulation of the sensitivity of fruit tissue to ethylene. In this regard, ethylene receptors have been identified in many important fruit species, such as tomato (Tieman and Klee, 1999), melon (Sato-Nara et al., 1999; Takahashi et al., 2002), apple (Lee et al., 1998), banana (Wu et al., 1999), mango (Martinez et al., 2001), peach (Bassett et al., 2002), passion fruit (Mita et al., 2002), and pear (El-Sharkawy et al., 2003).

Ethylene receptors are encoded by a small gene family. In *Arabidopsis*, five members have been identified, including *ETR1*, *ETR2*, *ERS1*, *ERS2*, and *EIN4* (Bleecker et al., 1998). All receptor proteins have a similar structural organization: an N-terminal hydrophobic domain that contains three transmembrane segments in subfamily-I (*ETR1* and *ERS1*) and four in subfamily-II (*ETR2*, *ERS2*, and *EIN4*), a GAF domain in the middle portion, followed by a histidine kinase (HisKA) domain. In addition to these domains, ETR-type receptors contain a receiver domain at the C-terminus. Genetic and biochemical studies suggest that ethylene receptors function as negative regulators of ethylene responses and ethylene binding is thought to inactivate the receptors (Hua and Meyerowitz, 1998). Ethylene signaling involves a multi-step pathway in which ethylene receptors act at the first cascade and play a critical role in regulating ethylene responses (Guo and Ecker, 2004).

Persimmon is an important fruit in Japan. Along with grape, persimmon ranks fourth in production, just behind citrus and apple (Yonemori, 1997). Persimmon fruit often exhibits rapid softening during postharvest distribution, after deastringency, or sometimes on the trees. This is a major problem in the marketing of persimmon. Fruit softening is one of the ripening processes that is most sensitive to ethylene (Lelièvre et al., 1997). Persimmon fruit is classified as climacteric because it produces a small but significant amount of ethylene during ripening and is sensitive to exogenously applied ethylene (Abeles et al., 1992). The close relationship between ethylene production and fruit softening has been clearly demonstrated (Takata, 1983; Itamura et al., 1991). All established methods to extend the shelf life of persimmon fruit are based on the control of ethylene production and action. Recently, the molecular mechanisms of ethylene biosynthesis during fruit softening have been studied in persimmon (Nakano et al., 2002, 2003). However, the mechanism of ethylene perception involved in these physiological processes is still unknown.

Here, we describe the isolation and characterization of ethylene receptor genes expressed in persimmon fruit. Three putative ethylene receptor cDNAs, *DkERS1*, *DkETR1*, and *DkETR2*, were isolated. We examined their expression at the mRNA and protein levels (for *DkERS1*) throughout fruit development and ripening and after ethylene treatment. Based on the expression patterns, we discuss their possible roles in fruit development and ripening in persimmon.

2. Materials and methods

2.1. Plant material and treatments

Japanese persimmon (*Diospyros kaki* Thumb.) cv. Hiratanenashi was grown at the orchard of the University of Tsukuba (Tsukuba, Ibaraki, Japan). Persimmon fruit were harvested at stage I (July to early August), stage II (September 2), stage III (September 23), and the mature stage (October 24). Ethylene production in ten individual fruit per stage was measured as described previously (Ortiz et al., 2005). For postharvest ripening, 10 fruit were harvested at the mature stage and kept at $20\,^{\circ}C$ for about 1 month until ripening-associated ethylene was produced. For ethylene treatment, fruit (four individuals) harvested at the mature stage were placed separately in airtight jars and treated with $10\,\mu L\,L^{-1}$ ethylene at $25\,^{\circ}C$ for 12 h. Fruit tissues were frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until use. These experiments were repeated twice during two seasons (2004–2005).

2.2. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from pulp tissue using the hot borate method (Wan and Wilkins, 1994). RNA samples ($5\,\mu g$) isolated from young or ripening fruit were reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Life Technologies) following the manufacturer's instructions. The synthesized cDNAs were used as a template for RT-PCR using degenerate oligonucleotide primers that corresponded to conserved regions of ethylene receptor genes. The primers that gave positive results were as follows: 5′-AGR ATG YTN CAN CAT GAR AT-3′ (sense) and 5′-TTT GWR AAY TTH ACA GCA TT-3′ (anti-sense) for *DkERS1*, and 5′-GAG ACT GGN AGA GCA CTG GNC AGA GAT G-3′ (sense) and 5′-CAT CGG GNG TCT CGT CAT CTT CGA TGG ATT C-3′ (anti-sense) for *DkETR1* and *DkETR2*. Reactions were carried out under the following conditions: initial denaturation at $94\,^{\circ}C$ for 5 min, then 40 cycles consisting of 1 min at $94\,^{\circ}C$, 1 min at $45\,^{\circ}C$ and 1 min at $72\,^{\circ}C$, and final extension at $72\,^{\circ}C$ for 5 min. The amplified cDNA fragments with the proper size were cloned into the pGEM-T vector (Promega) by TA cloning and sequenced (BigDye Terminator on an ABI 310 sequencer; Applied Biosystems) using either the T7 or SP6 primer.

2.3. Rapid amplification of cDNA ends (RACE)-PCR

Full-length cDNA sequences of putative ethylene receptors were determined using RACE-PCR. The 5′ and 3′ RACE-PCR were performed using a Marathon cDNA amplification kit (Clontech, Palo Alto, CA) following the manufacturer's instructions. Gene-specific primers were derived from the partial cDNA sequences determined above. For 5′-end amplification, the specific primers 5′-GCT ACC TGG TCA GCA ACA ACT TCC ACG AG-3′, 5′-GTG CAC TAG CAT AAG AGC TGT TAC AC-3′, and 5′-GAA ACC AGA GCC GTG AGG AAC TTG AA-3′ were used for *DkERS1*, *DkETR1*, and *DkETR2*, respectively. For 3′-end amplification, the specific primers 5′-GCA GAG ACA GCC ATC CGT GCT CGC AAT G-3′, 5′-GAA GGC TAG AGA TCT TCT CAT GGT GCA-3′, and 5′-CAG CTG GTT GAG CGA AAT CGA GTG TTG C-3′ were used for *DkERS1*, *DkETR1*, and *DkETR2*, respectively. Reactions were carried out at the following conditions: initial denatura-