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Transcription analysis of the ethylene receptor and CTR genes in tomato: The effects of on and off-vine ripening and 1-MCP

Clara I. Mata[a], Bram Van de Poel[b], Maarten L.A.T.M. Hertog[a,*], Dinh Tran[c], Bart M. Nicolai[a,d]

[a] KU Leuven, Division of Mechatronics, Biostatistics and Sensors (MeBioS), Department of Biosystems (BIOSYST), Willem de Croylaan 42, 3001 Leuven, Belgium
[b] KU Leuven, Division of Crop Biotechnics, Department of Biosystems (BIOSYST), Willem de Croylaan 42, 3001 Leuven, Belgium
[c] Vietnam National University of Agriculture, Faculty of Food Science and Technology, Hanoi, Vietnam
[d] Flanders Centre of Postharvest Technology (VCBT), Willem de Croylaan 42, 3001 Leuven, Belgium

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ABSTRACT

Ethylene, the main hormone regulating ripening in climacteric fruit, is perceived by the ethylene receptors starting a cascade of reactions of the ethylene signal transduction. In order to gain insight in the perception of ethylene during the ripening process, this work presents the transcriptional characterisation of the ethylene receptor (ETR) and CTR (CONSTITUTIVE TRIPPLE RESPONSE) genes of tomato fruit at different developmental stages and postharvest ripening conditions. Our results show that both ETR and CTR genes were differentially expressed when comparing on the vine ripening with off the vine (postharvest ripening) and in response to 1-MCP. Expression of the six ethylene receptors revealed *SlETR3* and *SlETR4* as the most expressed genes and together with *SlETR6* to be the most ethylene-responsive, as they were the fastest to react to changes in conditions such as the 1-MCP application in detached fruit ripening off the vine. A climacteric pattern of expression similar to that of ethylene production itself was observed for most of the receptors and CTR genes during ripening, most strongly during postharvest ripening. All together our results suggest a dedicated regulation of the expression of *ETRs* and *CTRs* that allows the plant to precisely control the timing of ripening.

1. Introduction

The plant hormone ethylene regulates many physiological processes such as germination, abscission, senescence and responses to biotic and abiotic stresses (Grierson, 2012). Furthermore, it controls ripening of climacteric fruits, which makes it one of the most studied phytohormones (Golden et al., 2014). To be perceived, ethylene needs to diffuse to the endoplasmic reticulum where it can bind to dedicated ethylene receptors (Chen et al., 2002). Here it can trigger a cascade of reactions of the ethylene signal transduction pathway (Klee, 2004). The ethylene receptors can be, based on the conserved residues of their histidine kinase, divided into two subfamilies (Gamble et al., 1998; Kevany et al., 2007). In tomato, three receptors, SlETR1, SlETR2 and SlETR3 (Never Ripe; NR) are part of subfamily I, with a well conserved histidine kinase, and receptors SlETR4, SlETR5 and SlETR6 belong to subfamily II, which lack some of the necessary residues of the histidine kinase and probably act as Ser/Thr protein kinases (Klee, 2002; Moussatche and Klee, 2004). In the absence of ethylene, the receptors remain in their phosphorylated state, physically interacting with the Raf-like kinases CTR proteins (SlCTR1-SlCTR4) (Zhong et al., 2008), which are negative regulators of the ethylene response (Adams-Phillips et al., 2004b). In *Arabidopsis* only one CTR is present, which interacts with and phosphorylates ETHYLENE INSENSITIVE 2 (EIN2) to inhibit its action (Ju et al., 2012). Initially, EIN2 was believed to interact only with CTR1, but later it was found that EIN2 also interacts with all the ethylene receptor proteins in *Arabidopsis* (Bisson and Groth, 2010). The current ethylene signalling model proposed explains that upon binding of ethylene to the receptors, their autokinase activity is inhibited, reducing its phosphorylated state, being afterwards tagged for degradation, likely through the 26S proteasome dependent pathway (Chen et al., 2007; Kevany et al., 2007). This causes the release or the conformational shift of CTR1, failing to phosphorylate EIN2. This is correlated with the cleavage and translocation of the EIN2 C-terminal part to the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012) where it activates EIN3 (ETHYLENE INSENSITIVE 3) and EIN3-like proteins (EILs) (Chao et al., 1997; Roman et al., 1995). These are transcription factors which promote the transcription of the Ethylene Response Factor family (ERFs), inducing a large range of ethylene responses (Fujimoto et al., 2000; Ju et al., 2012; Qiao et al., 2012; Tieman et al., 2001; Tournier et al., 2003). The ethylene receptors have also been characterised as negative regulators of the ethylene response, because transgenic plants with reduced receptor gene expression

* Corresponding author.
E-mail address: maarten.hertog@kuleuven.be (M.L.A.T.M. Hertog).

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showed an increased ethylene sensitivity (Hua and Meyerowitz, 1998; Tieman et al., 2000). While in *Arabidopsis* multiple ethylene receptor genes need to be inactivated to produce the constitutive ethylene response, in tomato, only the inactivation of *SlETR4* or *SlETR6* is enough to produce this phenotype (Kevany et al., 2008, 2007; Tieman et al., 2000). The ethylene signalling process can be reversed through degradation of activated receptors or synthesis of new receptors, which afterwards will repress the ethylene response (Klee, 2002). Recent discoveries on the ethylene signal transduction reveal a more complex regulation of the pathway including extra transcriptional networks and mRNA turnover regulatory modules (Liu et al., 2016; Merchante et al., 2015).

Individual analysis of the gene expression levels of ethylene receptors in tomato fruit revealed *SlETR3* and *SlETR4* as the main receptor genes expressed during ripening (Kevany et al., 2007; Liu et al., 2015). Their expression, together with *SlETR6* is highly induced during the climacteric ripening phase, while *SlETR1*, *SlETR2* and *SlETR5* maintain more constant expression levels (Kevany et al., 2007; Lashbrook et al., 1998). The expression of *SlCTR1* has been shown to be low in mature green fruit and to significantly increase during the onset of ripening, as well as upon ethylene treatment (Leclercq, 2002; Zegzouti et al., 1999). The low expression of *SlCTR2*, *SlCTR3* and *SlCTR4* did not show significant changes during ripening and did not change upon ethylene application either (Adams-Phillips et al., 2004a; Lin et al., 2008).

1-methylcyclopropene (1-MCP) is an ethylene antagonist, which represses ethylene responses through its permanent binding to the receptors with an affinity higher than that of ethylene (Jiang et al., 1999; Sisler and Serek, 1997). It has been shown that a 1-MCP treatment of turning tomatoes decreases the climacteric ethylene production and the expression of *SlETR3* and *SlETR4* (Yan et al., 2013).

The act of harvesting could be considered an abiotic stress and previous studies have shown that ethylene receptors are differentially expressed in tomato fruit during several stress conditions. For instance, root hypoxia produced an increased expression of *SlETR3* and *SlETR4* in tomato fruit, while cold storage of breaker tomatoes displayed a reduced expression of these two receptors and increased *SlETR1* and *SlCTR1* mRNA levels (Horchani and Aschi-Smiti, 2010; Rugkong et al., 2011).

While a lot of progress has been made in understanding the regulation of the ethylene signalling pathway, an extensive profiling of the gene expression of all the ethylene receptors and CTR-like genes is still missing. Therefore, the present work presents a detailed characterisation of the gene expression levels of the six ETR and four CTR genes in tomato to reveal and compare the transcriptional changes during ripening. This gene expression profiling has been performed on a high resolution set of samples, covering multiple developmental and ripening stages both on and off the vine. We also used 1-MCP to inhibit the action of the ethylene receptors and to study its effect on the feedback expression of ETR and CTR genes. This work allowed us to better characterise the individual behaviour and regulation of the two negative regulators of the ethylene signalling pathway during different ripening conditions.

2. Materials and methods

2.1. Plant material

Tomato plants (*Solanum lycopersicum* L. cv. Bonaparte) were grown in a greenhouse at the Research Station for Vegetable Production of Hoogstraten (Belgium). Plants were cultivated hydroponically on rockwool substrate under natural daylight with controlled humidity (70%) and temperature (23/21 °C day/night).

2.1.1. Fruit development and ripening

Six independent fruit of ten different developmental and ripening stages (from immature small green fruit to red ripe fruit) were harvested. Additionally, harvested red ripe fruit were used during a postharvest storage experiment for 12 d at shelf life conditions (18 °C and 80% RH). During this postharvest period 6 fruit were sampled after 3,5, 7, 10 and 12 days of storage. Pericarp tissue of the individual fruit was flash frozen in liquid nitrogen, crushed with a grindomixer (Retsch, Haan, Germany) and stored at −80 °C. Sampling procedures were identical to Van de Poel et al. (2012).

2.1.2. 1-MCP treatment

Mature green fruit, each originating from a different plant, were harvested and transported to the laboratory. One batch was treated with 5 μL L^{-1} 1-MCP (SmartFreshTM, AgroFresh Inc., Philadelphia, PA) for 24 h at 18 °C in an airtight container. The control batch was exposed to atmospheric air for the same period in a similar container. Subsequently, fruit were removed from the container and stored at shelf life conditions (18 °C and 80% relative humidity) in air. At certain intervals corresponding to the major ripening stages of the control fruit (breaker, orange and red) and some postclimacteric stages (red + 4, 8 and 12 d) 6 individual fruit were sampled per treatment. The 1-MCP-treated fruit were sampled at the same time as the control fruit regardless their actual ripening stage. A scheme of the storage experiment is presented in Fig. S1. Pericarp tissue of individual fruit was flash frozen in liquid nitrogen, crushed with a grindomixer (Retsch, Haan, Germany) and stored at −80 °C. Sampling procedures were identical to Van de Poel et al. (2013).

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from tomato fruit pericarp samples. Ground tissue samples (500 mg) were homogenised in 800 μL of extraction buffer containing cetyltrimethylammonium bromide, following Gasic et al. (2004). The mixture was incubated at 65 °C for 10 min in a thermomixer (Eppendorf, Hamburg, Germany) while shaking vigorously. Chloroform (800 μL) was added and mixed by inversion, and the mixture was centrifuged at 21,000 g for 10 min at room temperature. The supernatant was transferred to a gDNA eliminator spin column (Plant RNeasy Extraction Kit, Qiagen) and centrifuged at 8000 g for 2 min at room temperature. Half a volume of ethanol was added to the effluent, then the mixture was loaded and washed through the RNeasy mini column (Plant RNeasy Extraction Kit, Qiagen) and finally the RNA was eluted with RNAse free water. The amount of total RNA extracted was measured by spectrophotometric analysis using the NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and its purity determined by the 260/280 nm or 260/230 nm ratio. Electrophoresis in a 1% agarose gel and ethidium bromide staining was used to check the integrity of the RNA. 1 μg of purified RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) in a total volume of 20 μl following manufacturer's protocol.

2.3. Gene expression analysis by reverse transcription-qPCR

Gene expression studies were performed following MIQE (Minimum Information for publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009). Real Time PCR was carried out with SYBR® Green PCR Master Mix (Applied Biosystems) on a Rotor Gene Q (Qiagen GmbH, Hilden, Germany). The selected primers, designed with the Primer3 web tool (http://bioinfo.ut.ee/primer3/), are listed in Table S1. All RT-qPCR reactions contained 1 μL of cDNA template (50 ng/μl), 7.5 μL of Absolute QRT-PCR SYBR Green Mix (ABgene1 Ltd., Epsom, UK), and 1 μL of 0.375 μM primer pairs, in a final volume of 15 μL. The cycling conditions were as follow: denaturation step at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 63 °C for 20 s, and extension at 72 °C for 20 s. A melting curve analysis was performed for every run to confirm the specificity of the primer pairs on the amplification product, going