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# Recovery of ethylene sensitivity and responses in carnation petals post-treatment with 1-methylcyclopropene

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ABSTRACT

In ethylene-sensitive plants, such as carnation, ethylene perception is considered an indispensable requirement to initiate and perpetuate the ethylene-mediated senescence program. Ethylene binding antagonists, such as 1-methylcyclopropene (1-MCP) compete for ethylene binding and block the hormone signaling. Despite its antagonistic propensity, plants treated with 1-MCP often recover sensitivity to ethylene post-treatment. Here we demonstrate that increases in the transcript level of the ethylene receptor *DcETR1* results in the recovery of ethylene sensitivity in carnation petals treated with 1-MCP. This study reveals that the ethylene-induced reduction in mRNA levels of *DcETR1* and *DcCTR1* is completely suppressed by 1-MCP, and that the transcripts fluctuate periodically in association with petal senescence and ethylene biosynthesis. The results suggest that the periodic increase in receptor transcript may represent the appearance of new active receptors leading to renewed sensitivity to ethylene after treatment with 1-MCP. While ethylene sensitivity is temporarily blocked by 1-MCP, ethylene binding to new receptors is completely prevented by successive treatment of 1-MCP prior to recovery of ethylene-sensitivity, resulting in repression of petal senescence.

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## 1. Introduction

The plant hormone ethylene regulates multiple aspects of plant growth and development including germination, stress responses, senescence and abscission of plant organs, and fruit ripening (Abeles et al., 1992; Reid and Wu, 1992). Flower senescence is the final event in floral development and onset of senescence is associated with a climacteric rise in ethylene biosynthesis in ethylene-sensitive plants (Halevy and Mayak, 1981; Yang and Hoffman, 1984). Ethylene perception by ethylene receptors is an essential requirement in the initiation and maintenance of the ethylene-mediated senescence program (Borochov and Woodson, 1989). When an ethylene molecule binds to ethylene receptor isoforms, a signal is sent through a sequence of biochemical events that regulate the transcript of ethylene-responsive genes leading to the senescence process. The ability to perceive or respond to ethylene is most likely mediated by changes in ethylene signal transduction during plant development (Abeles et al., 1992; Reid and Wu, 1992).

Genetic studies conducted on *Arabidopsis* have identified a family of five receptors (*ETR1*, *ETR2*, *ERS1*, *ERS2* and *EIN4*) as well as a number of components (*CTR1, EIN2, EIN3*, *EIL1*, and *ERF1*) that act downstream in the ethylene signaling pathway (Chang et al., 1993; Hua and Meyerowitz, 1998; Hua et al., 1998; Kieber et al., 1993; Sakai et al., 1998; Schaller, 2012). Ethylene perception by the receptors inactivates the antagonist CTR1, a Raf-like MAPK kinase kinase (MAPKKK), thereby suppressing the negative regulation of the receptors at the cell membrane (Clark et al., 1998; Hua and Meyerowitz, 1998; Hua et al., 1998; Kieber et al., 1993).

Ethylene perception and action can be inhibited by the potent binding inhibitor 1-MCP (Hall et al., 2000; Sisler et al., 1990; Sisler et al., 1986). 1-MCP binds competitively to ethylene receptors with greater affinities for the receptors than that of ethylene. Specifically, the attachment of 1-MCP to ethylene receptors is irreversible in plants; and thus, 1-MCP treatment renders plants insensitive to ethylene (Binder et al., 2004a; Serek et al., 1994; Sisler, 2006; Sisler et al., 1996; Sisler and Serek, 2003). Consequently, 1-MCP works quite effectively in various ethylene-sensitive horticultural crops to extend their postharvest longevity (Blankenship and Dole, 2003; Ichimura et al., 2002; Jiang et al., 1999; Schotsmans et al., 2009; Serek et al., 1994; Serek et al., 1995; Sisler et al., 1996; Watkins et al., 2000). Despite the

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irreversible binding of 1-MCP to ethylene receptors, plants treated with 1-MCP ultimately regain sensitivity to ethylene (i.e., recovery) post-1-MCP treatment (Cameron and Reid, 2001; Ekman et al., 2004; Serek et al., 1995). And thus, this transient characteristic of 1-MCP effects can limit its practical application for prolonging the postharvest longevity of many flower species.

Based on the irreversible binding model for 1-MCP, it has been proposed that regaining ethylene sensitivity in plants after 1-MCP treatment is the consequence of synthesis of new ethylene binding sites during plant development (Binder and Bleecker, 2003; Cameron and Reid, 2001; Schotsmans et al., 2009; van Doorn and Woltering, 2008). However, to our knowledge, no data directly supports this hypothesis and the nature of changes in ethylene sensitivity post-treatment with 1-MCP has not been characterized. Previously, we demonstrated that recovery of ethylene sensitivity is completely prevented by successive treatments with 1-MCP in carnation flowers (In et al., 2013b). In this study, to address the question of how 1-MCP-treated plants regain sensitivity to ethylene, we determined expression patterns of ethylene receptor genes in carnation (*Dianthus caryophyllus* L.) petals in response to ethylene and 1-MCP. The relationship between the changes in ethylene sensitivity and senescence of petals was also characterized by monitoring petal inrolling and expression patterns of ethylene biosynthesis genes to better understand the nature of flower senescence.

## 2. Materials and methods

### 2.1. Plant materials

Rooted cuttings of carnation plants (*Dianthus caryophyllus* L. 'Glacier') were planted in growing medium (Metro-Mix Special Blend, SUNGRO Horticulture Distribution Inc., Bellevue, WA) into 15 cm-diameter plastic pots. Plants were grown in a greenhouse at 22/16 °C day/night temperatures and drip-irrigated every other day with half strength Hoagland nutrient solution (Hoagland and Arnon, 1950). Supplementary lighting (220 $\mu mol\, m^{-2}\, s^{-1}$ PPFD at plant level) was provided by high-pressure sodium lamps (Philips 600 W Master GreenPower) to assure a photoperiod of 16 h. These lamps were turned on automatically when the intensity of natural lighting was less than 350 $\mu mol\, m^{-2}\, s^{-1}$. Plants were pinched 4 weeks after planting and transplanted into 30 cm plastic pots. After harvest, carnation flowers were immediately placed in tap water and transferred to the laboratory for all experiments.

### 2.2. Kinetic analysis for petal inrolling

The individual petals of carnations have been shown as a useful system to study the physiology of senescence and ethylene action due to their high sensitivity and rapid response to ethylene (Kim et al., 1998; Reid and Çelikel, 2008; Wulster et al., 1982).

The outer whorl of petals was detached from fully open flowers ("Corolla cylindrical" according to Camprubi and Nichols (1978)) and the individual petals were immediately placed into 1.5 ml microfuge tubes containing distilled water. The petals in the tubes were incubated in a transparent plastic chamber (25 L) with 10 $\mu L\, L^{-1}$ ethylene and provided air circulation. They were photographed with a digital camera at 30 min intervals for 24 h during the incubation. The petal width was analyzed from the images using ImageJ (http://rsb.info.nih.gov/ij).

### 2.3. Ethylene treatment and measurement of petal width

The petals in the tubes with distilled water were enclosed in a plastic chamber (117 L) with air circulation provided by a small fan at 21 °C under dark conditions. Ethylene gas drawn by syringe from a 10% ethylene cylinder was injected into the chambers to give a final concentration of 10 $\mu L\, L^{-1}$. This is the optimum dose to elicit ethylene responses in carnation petals (In et al., 2013a). The petals were incubated for 12 h. For the experiments on the effects of ethylene treatment time on petal inrolling, petals were incubated in the treatment chambers with 10 $\mu L\, L^{-1}$ of ethylene for various durations (4 h, 8 h, 12 h, and 24 h) based on our previous study (In et al., 2013a). Untreated petals were incubated in the same chambers with normal air and used as the control. Ethylene treatment was terminated by transferring the petals from the treatment chambers to normal atmospheric air. After the treatments, the petals were held in the laboratory environment at 21 ± 2 °C, 30 ± 10% relative humidity and ambient laboratory fluorescent lighting at about 10 $\mu mol\, m^{-2}\, s^{-1}$ until the petals were completely wilted and petal width measurements using digital calipers were made. These petal samples were frozen in liquid nitrogen approximately 20 min after the initial removal from ethylene and stored at −80 °C for RNA isolation.

### 2.4. Treatment with 1-methylcyclopropene (1-MCP)

To explore the optimum concentration of 1-MCP to inhibit ethylene responses, carnation petals were treated with 0.1, 0.2 and 0.5 $\mu L\, L^{-1}$ 1-MCP for 10 h at day 0 and subsequently exposed to 10 $\mu L\, L^{-1}$ ethylene for 12 h, which is the threshold time for ethylene treatment to elicit the sustained progression of petal senescence in carnation 'Glacier' (In et al., 2013a). Treatment with 1-MCP was performed using SmartFresh (AgroFresh Inc., Philadelphia, PA) tablets as described previously (In et al., 2013a). To determine whether recovery of ethylene sensitivity can be suppressed by the ethylene binding antagonist 1-MCP, petals were treated with 0.2 $\mu L\, L^{-1}$ 1-MCP once (+MCP) at day 0, two times (+2MCP) at day 0 and 3, and four times (+4MCP) at day 0, 3, 6 and 9 for 10 h as shown in Fig. 4A. The petals were exposed daily to 10 $\mu L\, L^{-1}$ ethylene for 10 h.

### 2.5. Ethylene measurements

Individual petals were enclosed in 25 mL glass vials for 1 h at 21 °C. 1 mL gas samples were collected with a gas-tight hypodermic syringe through a rubber septum and analyzed for ethylene by gas chromatography (Model 8500, Perkin Elmer Corp., Norwalk, CT, USA) equipped with an alumina column and flame ionization detector as described previously (In et al., 2013a). Ethylene measurements were performed until petals inrolled completely.

### 2.6. cDNA synthesis

After measurement of petal width, individual petals were immediately frozen in liquid $N_2$ and stored at −80 °C until RNA isolation. Total RNA extraction was performed using Trizol (Invitrogen, Carlsbad, CA) as described previously (In et al., 2013a). RNA samples were treated with RNase-free DNase prior to RT-PCR and first-strand cDNA was synthesized from 2 μg of total RNA with 1 μg of oligo $(dT)_{18}$ primer, dNTPs, RNA inhibitor, buffer, and M-MLV reverse transcriptase in a final volume of 25 μl according to the manufacturer's instruction (Promega, WI). The reverse transcription was performed in a PTC-200 PCR machine (MJ Research Inc., MA) with the following temperature parameters: 15 min at 70 °C followed by 1 h at 42 °C.

### 2.7. Semi-quantitative RT-PCR

Gene-specific primers were designed for the ethylene biosynthesis genes (D*cACS1 and DcACO1*) and ethylene signaling genes (*DcETR1, DcERS1, DcERS2,* and *DcCTR1*) and synthesized by