An organ-specific role for ethylene in rose petal expansion during dehydration and rehydration

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Received 27 September 2012; Revised 22 February 2013; Accepted 5 March 2013

Abstract

Dehydration is a major factor resulting in huge loss from cut flowers during transportation. In the present study, dehydration inhibited petal cell expansion and resulted in irregular flowers in cut roses, mimicking ethylene-treated flowers. Among the five floral organs, dehydration substantially elevated ethylene production in the sepals, whilst rehydration caused rapid and elevated ethylene levels in the gynoecia and sepals. Among the five ethylene biosynthetic enzyme genes (RhACS1–5), expression of RhACS1 and RhACS2 was induced by dehydration and rehydration in the two floral organs. Silencing both RhACS1 and RhACS2 significantly suppressed dehydration- and rehydration-induced ethylene in the sepals and gynoecia. This weakened the inhibitory effect of dehydration on petal cell expansion. β-glucuronidase activity driven by both the RhACS1 and RhACS2 promoters was dramatically induced in the sepals, pistil, and stamens, but not in the petals of transgenic Arabidopsis. This further supports the organ-specific induction of these two genes. Among the five rose ethylene receptor genes (RhETR1−5), expression of RhETR3 was predominantly induced by dehydration and rehydration in the petals. RhETR3 silencing clearly aggravated the inhibitory effect of dehydration on petal cell expansion. However, no significant difference in the effect between RhETR3-silenced flowers and RhETR-genes-silenced flowers was observed. Furthermore, RhETR-genes silencing extensively altered the expression of 21 cell expansion-related downstream genes in response to ethylene. These results suggest that induction of ethylene biosynthesis by dehydration proceeds in an organ-specific manner, indicating that ethylene can function as a mediator in dehydration-caused inhibition of cell expansion in rose petals.

Key words: cut roses, dehydration, ethylene biosynthesis, ethylene perception, petal expansion, rehydration.

Introduction

Dehydration typically causes numerous morphological and developmental changes in plants, such as a reduced life cycle, inhibition of shoot and leaf growth, promotion of root growth, and early flowering (Xiong and Zhu, 2002). In the past two decades, the dehydration signalling pathway has been thoroughly studied (Seki et al., 2007). Although abscisic acid is generally regarded as the major hormonal signalling molecule for plants in response to dehydration (Seki et al., 2007), previous reports have demonstrated that ethylene can also play an important role in this biological process (Wilkinson and Davies, 2010).
The role of ethylene appears to be highly species and/or organ specific. Dehydration cannot induce ethylene production in an organ attached to a plant in many plant species, including wheat, beans, cotton, and miniature rose (Morgan et al., 1990; Narayana et al., 1991). However, dehydration can result in ethylene production in detached plant organs, such as cotton bolls and petioles (McMichael et al., 1972; Guinn, 1976), wheat leaves (McKeon et al., 1982), carnation and valencia orange flowers (Ben-Yehoshua and Aloni, 1974; Yakimova and Woltering, 1997), and avocado and persimmon fruits (Adato and Gazit, 1974; Nakano et al., 2003). In addition, ethylene has also been reported to function in rehydration, which usually occurs after dehydration during post-harvest handling. In Cleopatra mandarin seedlings, a rapid and substantial increase in ethylene production is observed in the leaves of water-stressed plants after rehydration, resulting in leaf abscission (Tudela and Primo-Millo, 1992). In wheat ears, rehydration of plants at full turgor after desiccation cause a high level of ethylene production (Beltrano et al., 1997).

As elevated ethylene production is reported in both dehydration and rehydration, it is of interest to study how the regulation of ethylene biosynthesis is involved in a plant’s response to dehydration and rehydration. Over the past three decades, the ethylene biosynthesis mechanism has been well documented in higher plants. The rate-limiting step of ethylene biosynthesis is conversion of AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). ACS is then oxidized to ethylene by ACC oxidase (Yang and Hoffman, 1984). Ethylene is perceived by a family of endoplasmic reticulum-localized receptors (ETR1, ERS1, ETR2, EIN4, and ERS2) that are similar to the bacterial two-component system and function as negative regulators of the ethylene response (Chen et al., 2005).

ACS plays a key role in the rate-limiting step in ethylene biosynthesis, even though ACC oxidase can control ethylene production in some cases (Shi et al., 2006). ACS is encoded by a divergent multigene family and is regulated at both the transcriptional and post-transcriptional levels by various internal and external factors (Argueso et al., 2007). Expression of ACS genes exhibits spatial- and temporal-specific patterns in post-pollinated orchid flowers: PhalACS2 and PhalACS3 correlate with higher ACS activity in the stigma and ovary. A sequential increase in ACS activity in the labellum is attributed to the increased expression of ethylene-inducible PhalACS1 (Bui and O’Neill, 1998). In carnations, DCACS2 and DCACS3 are preferentially expressed in the styles, whereas DCACS1 mRNA is most abundant in the petals (Jones and Woodson, 1999). In detached persimmon fruits, water-loss-induced expression of DkACS2, a wound-induced ACS gene, in the calyx, caused large amounts of ethylene production. This triggers expression of DkACS1 and DkACS2, which leads to ethylene production in other tissues, such as the pulp, peel, and core (Nakano et al., 2003).

The rose is thought of as one of the most beautiful flowers in the world, with many romantic and sentimental associations. Cut roses account for approximately 31 and 21% of all cut-flower trade business in European and Chinese markets, respectively (Heinrichs, 2008). As a fresh product, rose flowers are particularly susceptible to dehydration-induced stress, which usually results in damage, such as the failure of buds to expand, wilted flowers, and bent necks (Jin et al., 2006). In cut roses, after ethylene treatment, the rapid and substantial increase in ethylene production in the gynoecia is associated with a rapid and enhanced expression of RhACS2 and RhACS3 (Xue et al., 2008). Expression of two receptor genes, RhETR1 and RhETR3, is enhanced by ethylene (Ma et al., 2006), and RhETR3 has been proven to be expressed in an organ-specific manner (Xue et al., 2008).

Currently, there is no direct evidence showing that members of the ACS gene family correspond to dehydration- and rehydration-induced ethylene biosynthesis in detached plant organs. It is also unclear which receptor gene plays a crucial role in the perception of ethylene during dehydration and rehydration.

In the work presented here, the temporal and spatial expression of ACS genes in rose floral organs was measured during dehydration and rehydration. Furthermore, the role of members of the ACS gene family in rose petal expansion in response to dehydration and rehydration was investigated using a virus-induced gene silencing (VIGS) approach. The perception of ethylene and the effect of the key receptor genes on the expression of potential ethylene-downstream genes related to cell expansion in rose petals were also investigated. The results suggested that induction of ethylene biosynthesis during dehydration proceeds in a tissue-specific manner and allows ethylene to function as a mediator in inhibition of cell expansion of rose petals caused by dehydration.

Materials and methods

Plant materials
Cut rose (Rosa hybrida) cv. Samantha was produced in a local solar greenhouse using standard commercial practices in Beijing, PR China. The flowers were harvested at flower opening stage 2 (completely open bud) (Ma et al., 2005) and placed immediately in water. The flowers were delivered to the laboratory within 1 h of harvesting and the stems were re-cut to 30 cm in length under water and placed in deionized water (DW) until further processing.

Dehydration treatment and phenotype observations

For the dehydration treatment, the flowers were placed horizontally on test beds and exposed to air for 6, 12, 18, and 24 h. The environmental conditions were: 25 °C, 40–50% relative humidity, and 150 μmol m⁻² s⁻¹. After dehydration treatment, the bottom stems of the flowers were re-cut under water, removing approximately 1 cm from the 30 cm total length, and were then placed in DW for rehydration and further evaluation of their opening. To investigate the role of ethylene in dehydration, the rose flowers were sealed in a 64 l chamber with 2 ppm of 1-methylcyclopropene (1-MCP) for 12 h and then with 10 ppm of ethylene for 24 h at 25 °C, 40–50% relative humidity. The flowers exposed to ordinary atmospheric air served as the control group. NaOH (1 M) was used to prevent the accumulation of CO₂.

For observations of the phenotype and cell counting, one petal was chosen randomly from the second layer of each flower on the third day after dehydration. Photos of petals were taken using a USB scanner (Microtek Scanmaker 8700), and petal areas were measured independently using Adobe Photoshop 7.0 software. Abaxial subepidermis (AbsE) cell photography and cell counting were performed as described by Ma et al. (2008). A tissue sample (0.5 × 0.4 cm) was excised at 25% of the petal length from the petal tip. The tissue slices
were fixed in formaldehyde and cleared using ethanol. The number of AbsE cells was counted using ImageJ software in a visual field of 1360 × 1024 μm².

Ethylene production of whole flowers and different floral tissues, including sepals, petals, androecia, gynoecia, and the receptacle, was determined as described by Xue et al. (2008). Based on previous work, the floral organs can produce wound ethylene when the incubation time is longer than a particular time. To avoid contamination of wound-induced ethylene, the gas chromatography vials were capped and incubated at 25 °C for 1 h for sepals, petals, androecia, and receptacle, and for 40 min for gynoecia (Xue et al., 2008).

**RNA extraction and semi-quantitative RT-PCR analysis**

For RNA extraction, one flower was defined as an independent sample. All sepals and gynoecia from the flower were collected. Petals from the second and third whorl were taken from the flower. Total RNA from the sepals and petals was extracted using the hot borate method described by Wan and Wilkins (1994). Total RNA from the gynoecia was extracted using the hot phenol method (Ma et al., 2005).

For semi-quantitative RT-PCR analysis, cDNAs were synthesized from 1 μg of total RNA using Moloney marine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). The rose ubiquitin1 gene (Ubi1, JK622648) was used as the internal control. The primer sequences are listed in Table S1 at JXB online. The specificity of each primer pair was checked by sequencing of the PCR products. PCRs were carried out with 31 cycles for RhACS1–5, 27 cycles for Ubi1, and 27 cycles for RhETR1–5. The linearity of response with these cycle numbers was tested using cDNA dilutions. To control for background DNA contamination, a reaction using each gene primers but no reverse transcriptase, was performed. The relative transcript levels were determined by the densitometry of the signals using AlphaImagerTM2200 software (Alpha Innotech, USA), and a statistical analysis was performed using Duncan’s multiple range tests (P < 0.05).

Silencing of RhACS and RhETR genes in rose flowers by VIGS

A tobacco rattle virus (TRV)-based vector, including pTRV1 and pTRV2 VIGS vectors (Liu et al., 2002), used in this work were graciously provided by Dr Yule Liu (Tsinghua University, PR China). The gene silencing in rose flowers by VIGS was performed according to the procedures described by Ma et al. (2008) with some modifications. VIGS experiments were performed a minimum of five times.

For ACS gene silencing, a 360 bp gene-specific fragment at the 3′ end of RhACS1 and a 325 bp fragment at the 3′ end of RhACS2 were amplified using cDNA as template. For RhACS1/2 silencing, the fragments of RhACS1 and RhACS2 were fused by overlapping PCR. The resulting products were inserted into pGEM-T Easy vector and subjected to sequencing. The vector was digested to produce fragments of 360 bp for RhACS1, 325 bp for RhACS2, and 685 bp for RhACS1/2, which were then inserted into TRV2 plasmids. For ethylene receptor gene silencing, a 410 bp gene-specific fragment at the 3′ end of RhETR3 and a 852 bp fragment possessing a conserved domain of the ethylene receptor gene was used to construct the pTRV2-RhETR3 and pTRV2-RhETRs vectors, respectively, as described above (Table S2 at JXB online).

The constructs were transformed into Agrobacterium GV3101 by electroporation. Agrobacterium containing pTRV1, pTRV2, pTRV2-RhACS1, pTRV2-RhACS2, pTRV2-RhACS1/2, pTRV2-RhETR3, or pTRV2-RhETRs were grown at 28 °C in Luria–Bertani medium supplemented with 10 mM MES, 20 mM acetosyringone, and 50 mg L⁻¹ of kanamycin for approximately 24 h. Agrobacterium cells were harvested and suspended in the infiltration buffer (10 mM MgCl₂, 200 mM acetosyringone, and 10 mM MES, pH 5.6) to a final optical density at 600 nm of 1.5. A mixture of Agrobacterium cultures containing pTRV1 and pTRV2 or its derivatives (pTRV2-RhACS1, -RhACS2, -RhACS1/2, -RhETR3, or -RhETRs) at a ratio of 1:1 (v/v) were placed at room temperature for 4 h before vacuum infiltration.

For vacuum infiltration, flower stems were placed upside down in an 81.64 l container, with the whole flower immersed in the bacterial suspension solution. They were then infiltrated by vacuum at 30 mmHg for 2 min and allowed to slowly recover. The rose flowers were washed with DW and kept in DW for 3 d at 8 °C before the dehydration treatment.

A preliminary experiment observing the phenotype showed that it was difficult to see the difference in petal areas between TRV control and VIGS-silenced petals from the outer layers, because the petals of the outer layers partially extended during virus infection for 3 d at 8 °C before dehydration treatment. Therefore, petals were chosen randomly from the fourth layer of each flower on the second day after the treatment for measurements. Petal areas and cell numbers were determined according to the methods described above.

**Results**

Induction of ethylene production by dehydration in rose floral parts

After 24 h of dehydration, flowers lost 22.8% of their initial weight and the flower water potential decreased to −3.2 MPa from −0.5 MPa (Supplementary Fig. S1 at JXB online). The flowers were capable of recovery with full opening once they had been rehydrated in water. However, after 36 h dehydration, flower opening was severely impeded resulting in buds that would not open in over 50% of flowers during rehydration (data not shown). Therefore, in the following experiments, the dehydration treatment was only performed for a measurement of 24 h.

Compared with the control, flowers subjected to dehydration developed irregular shapes including uneven unfurling of the outer layer petals and curly edges (Fig. 1A). Dehydration also resulted in vertically compressed flowers with a decreased flower height-to-diameter ratio (Fig. 1B). This phenomenon was similar to those that were treated with ethylene. Pre-treatment with 1-MCP, an ethylene action inhibitor, clearly weakened the dehydration-induced negative effects on flower opening (Fig. 1A, B). Dehydration significantly decreased the petal area of the second layer in comparison with control flowers, similar to the observations on ethylene-treated flowers. As expected, 1-MCP pre-treatment effectively prevented petals from all negative influence caused by dehydration (Fig. 1C).

The cell size of the petals was also measured to determine whether or not dehydration decreased petal area by inhibiting cell expansion. As shown in Fig. 1C, dehydration significantly increased cell density by 18.1% compared with the control. This indicated that dehydration caused a decrease in the cell size of the petals. Moreover, dehydration also resulted in a severely interlocking AbsE cell shape that mimicked the ethylene-treated petal cells. As expected, 1-MCP pre-treatment weakened the influence of dehydration on AbsE cell size and shape, indicating that ethylene may mediate the influence of dehydration on petal expansion of rose flowers.

Ethylene production was determined in different floral parts during dehydration and rehydration. For the whole flower, ethylene production levels were low in untreated controls in a vase (Supplementary Fig. S2 at JXB online). It substantially increased from 18 to 24 h in the control during
Surprisingly, 1-MCP pre-treatment promoted ethylene production during dehydration (Figs 2 and S2). Among the five floral organs, sepal-produced ethylene exhibited a clear increase from 12 to 24 h, accounting for 44.3 and 65.4% of ethylene production in whole flowers at 18 and 24 h (Table S4 at JXB online). Additionally, 1-MCP pre-treatment showed a significantly elevated effect on ethylene production in sepal organs compared to the other floral organs (Fig. 2).

During rehydration for the whole flower, ethylene slightly and transiently decreased at 1 h and then sharply increased at 3 h, followed by a gradual decline until 24 h. Additionally, ethylene production of flowers was further promoted by 1-MCP pre-treatment during rehydration (Figs 2 and S2). In the gynoecia, ethylene production increased dramatically and attained a peak value, accounting for 20-fold that before rehydration, at 1 h. In the flowers, rehydration-induced ethylene production decreased at 1 h and then recovered to a level close to that before rehydration. After recovery, it then decreased gradually. Ethylene production in the androecia and the receptacle maintained a constant level after slightly increasing at 3 h. During the entire rehydration period, ethylene production in petals remained at a low level. 1-MCP pre-treatment further elevated ethylene production in all five tissues at an earlier time during rehydration.

**Dehydration-induced expression of RhACS genes in the sepals and gynoecia**

As ACS is the rate-limiting enzyme in ethylene biosynthesis, the mRNA levels of five known ACS genes in rose were determined during dehydration and rehydration. As dehydration-induced ethylene production is organ specific, more focus was given to the sepals and gynoecia, which are more responsible for ethylene production, during gene expression. **RhACS1** and **RhACS2** displayed substantially increasing levels of expression throughout the dehydration period (Fig. 3). **RhACS3** mRNA levels maintained a constant level, whereas the mRNA levels of the **RhACS4** and **RhACS5** genes were hardly detectable. During dehydration, in the sepals, expression of **RhACS1** was slightly enhanced at 12 h and then increased linearly. Expression of **RhACS2** was increased at 18 h. In the sepals, there was a significant difference in gene expression at late times of dehydration for **RhACS2** but not for **RhACS1** between the air control and 1-MCP pre-treatment.

In the gynoecia, the **RhACS1** mRNA levels strongly increased at 1 h during rehydration and remained at a high level.
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level until 3 h, before gradually decreasing. Meanwhile, *RhACS2* expression increased slightly at 0.5 h and attained a peak value at 1 h, before dramatically decreasing during rehydration. In the sepals, expression of *RhACS1* exhibited a fluctuating pattern, and the expression decreased within 1 h, began to increase at 3 h, and then decreased gradually during rehydration. Expression of *RhACS2* clearly increased at 0.5 h and then declined (Fig. 3).

Effect of *RhACS1/2* silencing on petal expansion of rose flowers during dehydration

In order to understand the contribution of *RhACS1* and *RhACS2* in the dehydration-caused ethylene production, *RhACS1*, *RhACS2* or *RhACS1/2* were silenced in rose flowers using a VIGS approach (Liu et al., 2002; Ma et al., 2008) (Supplementary Fig. S3 at JXB online).

As described above, increased levels of ethylene were observed in the sepals from 12 to 24 h in the dehydration treatment. In the gynoecia and sepals, elevated ethylene levels occurred within 6 h of rehydration. Therefore, ethylene production was determined at 18 h of dehydration and 3 h of rehydration in the sepals and gynoecia of gene-silenced flowers.

At 18 h of dehydration, when compared with the TRV control, ethylene production was significantly reduced in *RhACS1-* or *RhACS1/2*-silenced sepals (Fig. 4A). *RhACS1/2*-silenced sepals had a greater reduction (79%) in ethylene production when compared with *RhACS1*- or *RhACS2*-silenced petals. At 3 h of rehydration, in contrast to TRV, silencing of *RhACS1*, *RhACS2*, or *RhACS1/2* significantly reduced ethylene production in the sepals and gynoecia (Fig. 4B).

Flower opening was then observed in the *RhACS1/2*-silenced flowers. Consistent with ethylene production, the ratio of flower height to diameter was increased significantly.

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**Fig. 2.** Ethylene production in whole flowers and five floral organs of cut roses treated by dehydration. Dark grey shading indicates 1-MCP pre-treatment duration; light grey shading indicates the duration of dehydration, and a white background indicates the rehydration duration. Results are shown as means ± standard error (SE) (n=30).
in RhACS1-, RhACS2-, and RhACS1/2-silenced flowers after 24 h of dehydration (Supplementary Fig. S4 at JXB online). This was in stark contrast to the TRV control. Again using TRV as a comparison, petal areas in the fourth layer of flowers significantly increased in RhACS1-, RhACS2-, and RhACS1/2-silenced flowers (Fig. 4C). Furthermore, the cell density of AbsE cells decreased significantly in RhACS1-, RhACS2-, and RhACS1/2-silenced petals (Fig. 4C).

**Induction of the RhACS1/2 promoter activities in Arabidopsis floral parts by dehydration**

To test whether or not dehydration directly influenced the expression of RhACS1 and RhACS2, the 2080 bp 5'-upstream sequence of RhACS1 and 1584 bp 5'-upstream sequence of RhACS2 were isolated from rose flowers. A number of cis-elements, including abscisic acid, dehydration-responsive, and tissue-specific elements, were identified in the two promoters (Supplementary Fig. S5 at JXB online) using the PLACE program (Higo et al., 1999). The RhACS1 promoter contained eight putative MYC (CANNTG) and two MYB (WAACCA) motifs, and the RhACS2 promoter possessed six putative MYC motifs and one MYB motif. Putative GATA boxes and OSE were also found in both promoters, which are considered to be involved in tissue-specific expression (Aird et al., 1994; Vieweg et al., 2004). A promoter assay using β-glucuronidase as reporter showed that the expression of
As ethylene may mediate the effect of dehydration on petal expansion, tests were performed to determine whether petals perceived the signal of ethylene induced by dehydration in the sepal and gynoecia. mRNA levels of \textit{RhETR3} and \textit{RhETR4} were undetectable, whilst \textit{RhETR5} expression was constant. Similarly, \textit{RhETR1} expression was not induced by dehydration. In contrast, \textit{RhETR3} expression was induced gradually in petals during the 24 h dehydration treatment. During rehydration, \textit{RhETR3} expression maintained high levels until 1 h of rehydration and then visibly decreased at 3 h, and maintained an almost constant level within 24 h of determination.

1-MCP pre-treatment strongly inhibited the expression of \textit{RhETR3} during dehydration and rehydration (Fig. 5).

In addressing the role of \textit{RhETR3} in petal expansion during dehydration, the effect of \textit{RhETR3} or \textit{RhETR}-genes silencing on petal expansion was explored in rose flowers treated by dehydration using a VIGS approach (Liu \textit{et al.}, 2002; Ma \textit{et al.}, 2008) (Supplementary Fig. S8 at \textit{JXB} online). Following infiltration treatment for the whole flower, \textit{RhETR3}- and \textit{RhETR}-genes-silenced flowers were successfully obtained, although the degree of silencing varied among the three \textit{RhETR} members (Fig. 6A).

On the second day after dehydration treatment, dehydration-caused flower-opening phenotypes, such as irregular shape, were further aggravated in \textit{RhETR3}-silenced flowers compared with TRV (Supplementary Fig. S9 at \textit{JXB} online). Dehydration-caused inhibition of petal expansion and cell expansion was also significantly aggravated in \textit{RhETR3}-silenced rose flowers (Fig. 6B).

In addition, the changes in petal cell expansion contrasted between \textit{RhETR3} and \textit{RhETR}-silenced flowers. The decrease in petal area in \textit{RhETR3}-silenced petals (11.3%) was equivalent to 85.0% of that in \textit{RhETR}-silenced petals (13.3%). The increased in cell density accounted for 73.7% of that in \textit{RhETR} silenced petals (Fig. 6B). These findings illustrated that \textit{RhETR3} plays an important role in perception of ethylene production induced by dehydration.

The expression of relevant ethylene-downstream genes was also determined in \textit{RhETR3}-silenced flowers on the third day after 24 h of dehydration. The results showed that, among the 32 genes, \textit{RhETR3} silencing substantially upregulated ten genes, including endoxyloglucan transferase, xyloglucan endotransglycosylase-related protein, \(\beta\)-1,3-glucanase-like protein, two of the sugar transporters and one of the putative microtubule and kinesin proteins. This was in contrast to the TRV controls. Moreover, \textit{RhETRs} silencing also substantially downregulated 11 genes including expansin and expansin, cellulose synthase-like protein, cellulase, sugar transporter, actin depolymerizing-factor, putative microtubule proteins, kinesin, and gibberellin 20-oxidase (Fig. 7).

These results indicated that the inhibitory effect of dehydration-caused ethylene on petal expansion may occur partially through regulating the expression of relevant downstream genes related to cell expansion.
Decades ago, several reports indicated that detached vegetative organs usually produce an ethylene burst in response to dehydration. Detached wheat and orange leaves produce large amounts of ethylene under dehydration (Ben-Yehoshua and Aloni, 1974; McKeon et al., 1982). In cotton, bracts as green organs, lose much more water and produce greater amounts of ethylene than the rest of the boll during dehydration (Guinn, 1976). For detached reproductive organs, ethylene has been reported to be induced rapidly and dramatically in the calyx in detached persimmon fruit under dehydration (Nakano et al., 2003). Here, we found that ethylene was primarily produced in the sepals among the five floral organs of detached rose flowers during dehydration treatment. Interestingly, the response of the calyx in persimmon suggests that different plants respond similarly to dehydration stress.

Surprisingly, we found that ethylene production appeared to be highly induced in the gynoecia during the initial period of rehydration, and in the sepals (Fig. 2); it is suggested here that gynoecia are more sensitive than the other floral organs in the production of ethylene as a response to rehydration. Interestingly, the response of the calyx in persimmon suggests that different plants respond similarly to dehydration stress.

**Discussion**

**Spatial-specific induction of ethylene biosynthesis by dehydration in rose flowers**

Decades ago, several reports indicated that detached vegetative organs usually produce an ethylene burst in response to dehydration. Detached wheat and orange leaves produce large amounts of ethylene under dehydration (Ben-Yehoshua and Aloni, 1974; McKeon et al., 1982). In cotton, bracts as green organs, lose much more water and produce greater amounts of ethylene than the rest of the boll during dehydration (Guinn, 1976). For detached reproductive organs, ethylene has been reported to be induced rapidly and dramatically in the calyx in detached persimmon fruit under dehydration (Nakano et al., 2003). Here, we found that ethylene was primarily produced in the sepals among the five floral organs of detached rose flowers during dehydration treatment. Interestingly, the response of the calyx in persimmon suggests that different plants respond similarly to dehydration stress.

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In most reported plants, tissue-specific ethylene induction is attributed to the temporal and spatial regulation of ACS genes (Lin et al., 2009). In post-pollinated orchid flowers, field of 1360 × 1024 μm². Different letters indicate significant differences between different treatments according to Duncan’s multiple range tests (P < 0.05).
Dehydration influences petal expansion in rose flowers.

Elevated expression of PhalACS2 and PhalACS3 in stigma and ovary subsequently resulted in increased expression of PhalACS1 in the labellum (Bui and O’Neill, 1998). In carnation, ethylene treatment affects the induction of ethylene biosynthetic genes with different kinetics in all flower organs. Expression of DCACS2 and DCACS3 in styles causes enhanced ethylene production, which is able to induce DCACS1 expression in petals (Jones and Woodson, 1999). In cut roses, after ethylene treatment, the rapid and substantial increase in ethylene production in the gynoecia is attributed to rapid and enhanced expression of RhACS2 and RhACS3 (Xue et al., 2008).

Here, we found that expression of RhACS1, a wounding-inducible gene, and RhACS2, a senescence-inducible gene (Ma et al., 2005), exhibited spatial and temporal specificity during dehydration and rehydration in detached rose flowers.
(Fig. 3) in a pattern consistent with the ethylene production in the two organs (Fig. 2). Gene silencing by VIGS further confirmed the role of the RhACS1 and RhACS2 genes in producing ethylene. This ethylene resulted in sequential phenotypic changes in response to dehydration and rehydration (Fig. 4).

Similarly, in detached persimmon fruit, water loss also induced expression of a wound-induced ACS gene, DkACS2, in the calyx and caused large amounts of ethylene production.

Together, these findings indicate that, in detached rose flowers during dehydration and rehydration, the sepals and gynoecia are the main organs of ethylene induction, and RhACS1 and RhACS2 primarily contribute to ethylene induction at the transcriptional level.

Regulation of ETR genes in response to dehydration and rehydration

It has been well documented that the ethylene receptors act as negative regulators of ethylene responses. This means that reducing the levels of receptor increases signal output, and vice versa (Hua and Meyerowitz, 1998). Ethylene receptor genes exhibit higher expression levels in reproductive tissues, such as the androecia and gynoecia of Arabidopsis (Sakai et al., 1998), anthers of rice (Yau et al., 2004), pollen and embryo of tobacco (Zhang et al., 2001), flowers and fruits of tomato (Tieman and Klee, 1999), and gynoecia of rose flowers (Xue et al., 2008). Here, among the five rose ETR genes, RhETR1 and RhETR3 were upregulated during dehydration and rehydration in petals (Fig. 5).

As the ethylene receptors are negative regulators of ethylene signalling, silencing of the ethylene receptor might increase ethylene sensitivity in plants. A double mutant of subfamily I receptor, etr1ers1, exhibited hypersensitivity to ethylene, whereas single- and double-gene knockouts of the other subfamily did not clearly display different phenotypes. This observation indicates that the subfamily I receptors are more important than the subfamily II receptors in determining competency in response to ethylene in Arabidopsis (Wang et al., 2003). In tomato, however, silencing of the LeETR4 or LeETR6 gene, both subfamily II members, displays exaggerated ethylene response phenotypes, including epinastic growth, premature flower senescence, and early fruit ripening (Tieman et al., 2000). In the present study using the VIGS approach, it was confirmed that silencing of RhETR3, a subfamily II receptor, significantly aggravated dehydration-caused inhibition of petal expansion in rose flowers (Fig. 6), indicating that RhETR is a negative regulator for ethylene signalling in rose flowers. Therefore, ethylene perception and signalling might vary in different plant species.

A dehydration-caused abnormal phenotype, such as inhibition of petal expansion, might result from inappropriate cell expansion. Generally, cell expansion is thought to depend on degradation and resynthesis of cell-wall substrates, changes in cell turgor, and remodelling of the cytoskeleton. In the present work, it was found that, in RhETR-genes-silenced petals, there were 21 substantially altered genes among 32 rose genes that were related to cell expansion.

Of the genes related to the cell wall, RU00040 and RU10722 encode xyloglucan endotransglycosylase-related protein, which is a key enzyme in catalysing the biosynthesis of major structural polysaccharides for primary cell walls (Hayashi and Kaida, 2011). RU06454, RU00964, and RU20002 encode endo-xyloglucan transferase, which can catalyse the cleavage and molecular grafting of xyloglucan polymers. A gene encoding endo-xyloglucan transferase has been suggested as one of the most likely agents responsible for cell-wall loosening (Nishitani, 1997).

Of the genes related to cell turgor, RU04803, RU04906, RU00922, and RU08526 encode sugar-transport proteins, which play a crucial role in long-distance and cell-to-cell distribution of sugars. These are key signalling molecules that can potentially regulate cell growth throughout the plant (Williams et al., 2000). In diffusely growing cells, microtubules provide tracks for the movements of cellulose synthases and hence provide directional deposition of cellulose, the major factor controlling cell expansion. The binding of microtubules and microfilaments in plant cells appears to regulate cytoskeleton bundling (Petrásek and Schwarzerová, 2009).

Taken together, these results indicated that RhETR-genes silencing leads to substantial expression changes in genes associated with the cell wall, cytoskeleton, and cell turgor, which regulate cell expansion.

Conclusions

Based on the present findings, a working model of the role of ethylene under dehydration in rose petal expansion is proposed. As shown in Supplementary Fig. S10 at JXB online, for ethylene biosynthesis, dehydration- and rehydration-caused ethylene primarily contributes to RhACS1 and RhACS2 gene expression in the sepals and gynoecia, and the perception of ethylene is chiefly attributable to RhETR3 in petals. The perception of ethylene by RhETR3 further influences the expression of genes related to cell expansion and finally influences petal cell expansion.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Changes in fresh weight loss rate during dehydration.

Supplementary Fig. S2. Ethylene production in whole flowers.

Supplementary Fig. S3. Alignment of the cDNA sequence of RhACS1–5 genes and the specific fragments used in VIGS.

Supplementary Fig. S4. Phenotype of RhACS1-, RhACS2-, and RhACS1/2-silenced flowers (upper) and ratio of height to diameter (lower).

Supplementary Fig. S5. The promoters of RhACS1 and RhACS2.

Supplementary Fig. S6. Induction of RhACS1 promoter activity by dehydration in flowers of transgenic Arabidopsis.

Supplementary Fig. S7. Induction of RhACS2 promoter activity by dehydration in flowers of transgenic Arabidopsis.

Supplementary Fig. S8. Alignment of the cDNA sequence with RhETR1–5 genes and the specific fragments used in VIGS.
Supplementary Fig. S9. The phenotype of RhETR3- and RhETR-genes-silenced flowers (upper) and the ratio of height to flower diameter (lower).

Supplementary Fig. S10. Proposed model of dehydration-affected flower opening mediated by ethylene in rose flowers.

Supplementary Table S1. Primer sequences of the various genes in RT-PCR analysis.

Supplementary Table S2. Primer sequences of the various genes for construction of VIGS vectors.

Supplementary Table S3. Primer sequences of the various genes in RT-PCR analysis for gene silencing.

Supplementary Table S4. Proportion of each floral tissue contributing to increased ethylene production.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (grant nos 31130048 and 30671480) and the ‘948’ project (grant no. 2011-G17) from the Ministry of Agriculture. We thank Mr Gabriel M. Garcia (University of New Mexico, USA) for careful proofreading of our manuscript.

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