Rh-PIP2;1, a Rose Aquaporin Gene, Is Involved in Ethylene-Regulated Petal Expansion

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Aquaporins are water channel proteins that facilitate the passage of water through biological membranes and play a crucial role in plant growth. We show that ethylene treatment significantly reduced petal size, inhibited expansion of petal abaxial subepidermal cells, and decreased petal water content in rose (Rosa hybrida ‘Samantha’). Here, we report the isolation of a plasma membrane aquaporin (PIP) gene, Rh-PIP2;1, and characterized its potential role in ethylene-inhibited petal expansion. Rh-PIP2;1 is mainly localized on the plasma membrane and belongs to the class 2 subfamily of PIP proteins. We show that Rh-PIP2;1 is an active water channel. The transcripts of Rh-PIP2;1 are highly abundant in petal epidermal cells, especially in the abaxial subepidermal cells. The expression of Rh-PIP2;1 is highly correlated with petal expansion and tightly down-regulated by ethylene. Furthermore, we demonstrate that in Rh-PIP2;1-silenced flowers, petal expansion was greatly inhibited and anatomical features of the petals were similar to those of ethylene-treated flowers. We argue that Rh-PIP2;1 plays an important role in petal cell expansion and that ethylene inhibits petal expansion of roses at least partially by suppressing Rh-PIP2;1 expression.

The gaseous phytohormone ethylene modulates various plant developmental processes, including organ growth, flowering, fruit ripening, leaf senescence, and abscission (Abeles et al., 1992; Wang et al., 2002). Particularly, the inhibitory role of ethylene in organ growth has been well documented (Pierik et al., 2006). In Arabidopsis (Arabidopsis thaliana), reduction of hypocotyl elongation in dark-grown seedlings occurred merely 15 min after the plant was subjected to ethylene treatment (Binder et al., 2004). Ethylene-inhibited growth was also found in stems and petioles of Arabidopsis (Smalle and Van der Straeten, 1997), roots of Rumex and cucumber (Cucumis sativus; Visser et al., 1997; Pierik et al., 1999), and leaves of Poa species (Fiorani et al., 2002). In flower development, ethylene has been reported to be mainly involved in sexual determination (Yamasaki et al., 2003) and petal senescence (Woltering and van Doorn, 1988; van Doorn and van Meereren, 2003; van Doorn and Woltering, 2008).

Especially, several reports proved that ethylene also played an important role in floral organ abscission (Patterson and Bleecker, 2004; Butenko et al., 2006). In roses (Rosa spp.), Reid et al. (1989a, 1998b) showed that ethylene could promote or inhibit petal growth in a cultivar-dependent manner. However, little is known concerning the regulatory mechanisms of ethylene in petal development during the process of flower opening.

It has been reported that ethylene inhibits plant organ growth by regulating cell elongation and expansion processes. Ethylene impedes cell expansion in roots along the direction of the long axis but enhances cell radial expansion (Chadwick and Burg, 1967). Ethylene induces the formation of apical hypocotyl hooks by regulating asymmetrical cell elongation on the adaxial and abaxial sides of the apical hook region in Arabidopsis and pea (Pisum sativum; Lehman et al., 1996). Moreover, Arabidopsis ethylene constitutive response mutant plants (ctr1) are tiny and their leaf cell sizes are also much smaller (Kieber et al., 1993), while the ethylene-insensitive mutants (e.g. ers1 and etr1-1) are larger and their larger leaf area is thought to be caused by increased cell size (Bleecker et al., 1988; Hua et al., 1995). Cell expansion requires the synergistic action of cell wall loosening, deposition of cell wall materials, and water absorption. In Rumex, ethylene can influence petiole elongation by regulating expression of the genes related to cell expansion (Vreeburg et al., 2005). However, little is known about the mechanism of ethylene-regulated cell expansion.

Aquaporins (AQPs) are the primary channels of water transport across biological membranes. AQPs...
are a class of small (24–34 kD) transmembrane proteins and are localized on various cellular membranes (Maurel and Chrispeels, 2001). Plasma membrane intrinsic proteins (PIPs), which are plasma membrane-associated AQPs, can be classified into two major subgroups, PIP1 and PIP2. PIP2 has a shorter N terminus and a longer C terminus than PIP1 (Johansson et al., 2000). PIP2 has a shorter N terminus and a longer C terminus than PIP1 (Daniels et al., 1994; Yamada et al., 1995; Moshelion et al., 2002; Fetter et al., 2004).

AQPs have been implicated in many developmental processes in plants, including cell expansion, organ movement, and elongation (for review, see Tyerman et al., 2002). In Arabidopsis, reduction of AtPIP1;2 (previously named AtPIP1b) mRNA levels decreased cellular permeability and increased root mass (Kaldenhoff et al., 1998). Overexpression of AtPIP1;2 in tobacco (Nicotiana tabacum) significantly increased plant growth rate and resulted in larger plants (Aharon et al., 2003). The transcriptional activity of a tobacco AQP, NtAQP1, was highly associated with cell expansion regions (e.g. young petioles and corolla; Siefritz et al., 2004). In tobacco, PIP1 and PIP2 showed distinct expression patterns during stigma and anther development, and they might contribute to the development of these organs in different ways (Bots et al., 2005a, 2005b). In Arabidopsis, eight PIP genes were detectable in flower buds, suggesting the involvement of PIPs in flower development (Quigley et al., 2002). In tulip (Tulipa spp.), temperature-dependent changes of a putative plasma membrane-associated AQP activity were associated with petal opening and closing (Azad et al., 2004). However, very limited direct evidence is available to support the role of PIPs in petal expansion.

Roses have been one of the most important ornamental crops in the floriculture industry for centuries, and flowering is one of the most important traits. Generally, flower opening requires programmatic expansion of petals. Considering that postmitotic development of petals mainly depends on water uptake-driven cell expansion, it is highly possible that AQPs play an important role in petal expansion.

Our previous studies showed that ethylene could accelerate the flower-opening process in roses and could result in irregular petal shape (Ma et al., 2006; Xue et al., 2008). In this study, we found that ethylene significantly reduced petal size and water content in roses and inhibited petal expansion by inhibiting cell expansion. Furthermore, we identified a rose AQP gene from the public EST collection and demonstrated that this gene was tightly down-regulated by ethylene and was involved in the regulation of rose petal expansion.

RESULTS

Ethylene Inhibits Petal Expansion

We have reported that ethylene plays a crucial role in the flower-opening process of roses (Ma et al., 2006; Xue et al., 2008). Here, we further investigated the effects of ethylene on petal expansion during flower opening. Our results showed that ethylene treatment resulted in vertically compressed flowers with decreased flower height-to-flower diameter ratio in rose (Rosa hybridra ‘Samantha’). An ethylene action inhibitor, 1-MCP (for 1-methylcyclopropene), was used to inhibit the ethylene effects because of its high specificity of action and lack of deleterious side effects (Serek et al., 1995; Sisler et al., 1996; Sisler and Serek, 1997; Cho and Cosgrove, 2002; Tanaka et al., 2005; Iwai et al., 2006). Expectably, 1-MCP increased the ratio significantly and resulted in vertically stretched flowers (Fig. 1, A and B). In addition, ethylene significantly decreased both petal length and width, while 1-MCP increased petal length significantly (Fig. 1C).

During normal flower opening, although the petals expanded significantly, their thickness significantly decreased, especially in the upper and middle regions (25% and 50% of the petal length from the top, respectively). Ethylene treatment significantly increased petal thickness in the upper and middle regions, while 1-MCP significantly decreased petal thickness in the upper region (Table I).

Ethylene Inhibits Cell Expansion of Petals

In general, petal cells cease division right before anthesis (Drews et al., 1992; Martin and Gerats, 1993; Guterman et al., 2002; Dafny-Yelin et al., 2005). Consequently, during flower opening, petal growth is driven mainly by cell expansion. Here, we determined the effects of ethylene on petal cell expansion.

Scanning electron microscopy showed that the petal could be roughly divided into five sections anatomically: adaxial epidermis, parenchyma cell, vascular bundle, abaxial subepidermis (AbsE), and abaxial epidermis (Fig. 2A). Among the five sections, AbsE cells appeared to have a regular shape and to form a flat layer. The expansion of AbsE cells was tightly related to petal expansion (Supplemental Figs. S1 and S2). Therefore, we examined the size of AbsE cells by counting the number of cells in a 1,360 × 1,024-µm² microscope visual area. We found that ethylene treatment significantly increased cell numbers per visual area, indicating a significant decrease in cell size, while 1-MCP significantly reduced cell numbers, especially in the upper region of petals, indicating a significant increase in cell size (Fig. 2B). Interestingly, ethylene treatment also resulted in interlocking AbsE cells of irregular shape, suggesting that normal cell polar expansion might be impeded by ethylene (Fig. 2B). These results indicated that ethylene inhibited petal expansion, at least partially, through the inhibition of AbsE cell expansion.

Ethylene Reduces Water Content of Petals

As indicated above, petal growth during flower opening is driven mainly by cell expansion, a process of rapid water uptake. In this study, we investigated

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petal water content during flower opening and upon ethylene or 1-MCP treatment. The fresh weight of petals in control flowers increased rapidly during the first 4 d of flower opening and then started to decrease, while the dry weight stayed at a steady level during the first 7 d of the flower-opening process (Fig. 1D). During flower opening, ethylene treatment substantially decreased the petal fresh weight, while 1-MCP significantly increased the fresh weight. However, no significant differences in dry weight were observed between the control flowers and those treated with ethylene or 1-MCP (Fig. 1D). This indicated that ethylene caused significant petal water loss while 1-MCP increased petal water content during flower opening.

Cloning and Characterization of Rh-PIP2;1

Since AQPs play important roles in regulating the water intake of plant cells and ethylene reduces the water content of rose petals, it is highly possible that ethylene impedes petal expansion of roses by regulating certain AQPs. To identify potential AQPs involved in petal expansion, we first searched the public rose EST database for sequences with similarity to known AQPs. Among the identified rose AQP-like ESTs, one (GenBank accession no. BQ104371) showed approximately 20-fold increase in expression in rose petals during the early period of flower opening, according to a microarray analysis (Guterman et al., 2002). Therefore, we characterized this EST for its functional role in rose petal expansion.

The full-length cDNA of the EST was isolated via RACE. The full transcript is 1,145 bp in length and contains an 846-bp open reading frame, a 91-bp 5'-untranslated region (UTR), and a 208-bp 3'-UTR. The deduced amino acid sequence contains the signature motifs of plasma membrane-associated AQPs (GGGANXXXXGY and TNPARSL/FGAAI/VI/VF/YN) and shares significant homology with plasma membrane AQPs from Arabidopsis, grape (Vitis vinifera), Brassica spp., and poplar (Populus spp.; Fig. 3). Based on the length of its C and N termini, this gene was classified as a class 2 subfamily PIP protein. Therefore, we named it Rh-PIP2;1.

We then determined the localization of Rh-PIP2;1 by expressing a 35S::GFP-Rh-PIP2;1 fusion gene in Arabidopsis thaliana (Fig. 1A). The 10-micron fluorescence signal showed that Rh-PIP2;1 was preferentially expressed in the petals of the flowers. Double asterisks indicate significant differences in length or width between untreated control and ethylene- or 1-MCP-treated flower petals (n = 15, P < 0.01). D, Changes of petal fresh weight (top blue lines) and dry weight (bottom red lines) after ethylene and 1-MCP treatments. Each data point represents the mean ± se (n = 15). Asterisks indicate significant differences (Student's t test; P < 0.05) between untreated control and ethylene- or 1-MCP-treated flower petals.

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Figure 1. Morphological changes of cut rose flowers in response to exogenous ethylene. A, Vertical (left) and side (right) views of flowers after ethylene and 1-MCP treatments. Flowers were exposed to air (control), 10 μL L⁻¹ ethylene, or 2 μL L⁻¹ 1-MCP for 24 to 72 h, and the photographs were taken at the end of individual treatment times. Twenty-five flowers were used in each treatment, and representative results are shown. B, The ratio of flower height to flower diameter. Each data point represents the mean ± se (n = 15). Asterisks indicate significant differences (Student’s t test; P < 0.05) between untreated control and ethylene- or 1-MCP-treated flowers. C, The size of petals after ethylene and 1-MCP treatments. The length and width of petals were measured on the 2nd d after 24-h treatments. One petal was chosen randomly from the second layer of each flower. Double asterisks indicate significant differences in length or width between untreated control and ethylene- or 1-MCP-treated flower petals (n = 15, P < 0.01). D, Changes of petal fresh weight (top blue lines) and dry weight (bottom red lines) after ethylene and 1-MCP treatments. Each data point represents the mean ± se (n = 15). Asterisks indicate significant differences (Student’s t test; P < 0.05) between untreated control and ethylene- or 1-MCP-treated flower petals.
dopsis. To determine a more detailed localization, we isolated protoplasts from the transgenic Arabidopsis harboring the 35S::GFP-Rh-PIP2;1 cassette. We found that the green fluorescence was primarily distributed on the plasma membrane while also distributed on some internal membranes (Fig. 4A). Thus, we conducted colocalization of GFP-Rh-PIP2;1 with a mCherry (a red fluorescent protein mutant derived from mRFP1 that is a monomeric mutant of DsRed [Shaner et al., 2004])-labeled plasma membrane marker (PM-rk; The Arabidopsis Information Resource stock no. CD3-1007) and an endoplasmic reticulum marker (ER-rk; The Arabidopsis Information Resource stock no. CD3-959; Nelson et al., 2007), respectively. The results indicated that Rh-PIP2;1 was mainly distributed on the plasma membrane and much less on the endoplasmic reticulum (Fig. 4B).

We also examined the spatial expression pattern of Rh-PIP2;1 in petals through in situ hybridization. A 216-bp fragment in the 3′ end of Rh-PIP2;1 was used to generate digoxigenin (DIG)-labeled RNA antisense and sense probes. In situ hybridization of Rh-PIP2;1 transcripts in petals at the early stages of flower opening was performed as described previously (Nelson et al., 2007). The results showed that Rh-PIP2;1 transcripts were expressed in the periphery region of petals, especially at the abaxial epidermis, as well as in the vascular bundles (Fig. 4B). The same expression pattern was observed when petals were treated with ethylene or 1-MCP on the first day of flower opening. However, the expression level of Rh-PIP2;1 was significantly reduced when petals were treated with ethylene or 1-MCP on the third day of flower opening (Fig. 4B).

Table 1. Effect of ethylene on petal thickness (µm)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Upper (25%)</th>
<th>Middle (50%)</th>
<th>Bottom (75%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (day 0)</td>
<td>459.2 ± 10.3 a</td>
<td>574.6 ± 21.4 a</td>
<td>820.2 ± 56.2 a</td>
</tr>
<tr>
<td>Control (day 3)</td>
<td>183.7 ± 26.5 b</td>
<td>446.8 ± 15.1 b</td>
<td>844.1 ± 65.8 a</td>
</tr>
<tr>
<td>Ethylene (day 3)</td>
<td>358.2 ± 38.2 a</td>
<td>567.2 ± 14.3 a</td>
<td>886.4 ± 35.0 a</td>
</tr>
<tr>
<td>1-MCP (day 3)</td>
<td>113.9 ± 34.3 c</td>
<td>461.4 ± 10.8 b</td>
<td>854.1 ± 37.6 a</td>
</tr>
</tbody>
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**Figure 2.** Inhibition of the cell elongation of AbsE in petals by ethylene. A. The typical anatomic structure of a petal at flower full opening stage. Left, A scanning electron microscope image. Bar = 50 µm. Right, A light microscope image with semithin section. Bar = 200 µm. AbsE, Abaxial epidermis; AdE, adaxial epidermis; PC, parenchyma cells; VB, vascular bundle. B. Traces of outlines of AbsE cells (top) and cell numbers (bottom) in different regions of petals. Bars = 200 µm. AbsE cells of petals were photographed using a Nikon IX-71 microscope. Petal samples were taken as a 0.5-cm × 0.4-cm slice at 25%, 50%, and 75% of the petal length from petal top on the 2nd day after 24-h treatments. The slices were fixed in formaldehyde and then cleared in ethanol. The traces were drawn using Photoshop 7.0 software. Fifteen flowers were used in each treatment, and representative results are shown. Cell numbers were counted using ImageJ software in a visual field of 1,360 × 1,024 µm². Each data point represents the mean ± SE (n = 15). Different letters in the same row indicate significant differences between different treatments according to Duncan’s multiple range test (P < 0.05).
opening showed that the expression of Rh-PIP2;1 was much stronger in AbsE than in adaxial epidermis, and the expression was also detected in parenchyma cells but at a relatively lower level (Fig. 4C).

Water Channel Activity of Rh-PIP2;1

Plant PIP2 proteins usually have high water channel activities, whereas PIP1 proteins are often inactive or have low activities (Kaldenhoff and Fischer, 2006). Here, we tested the water channel activity of Rh-PIP2;1 by comparing osmotic water permeability ($P_f$) of leaf protoplasts between Arabidopsis wild-type (ecotype Columbia [Col]) plants and transgenic plants harboring the 35S::Rh-PIP2;1 cassette. The number of leaf protoplasts with high $P_f$ was significantly higher in transgenic plants (Fig. 5A). As shown in Figure 5B, on average, the $P_f$ value of leaf protoplasts in transgenic plants was significantly higher than that in the wild type (14.4 and 6.6, respectively; $P < 0.01$). This indicates that Rh-PIP2;1 is an active water channel.

Regulation of Rh-PIP2;1 Expression by Ethylene

We determined the tissue-specific expression pattern of Rh-PIP2;1 by RNA gel-blot analysis. As shown in Figure 6A, Rh-PIP2;1 was mainly expressed in petals. We then determined its expression levels in petals over the course of flower opening. Rh-PIP2;1 was highly expressed within the first 2 d of flower opening, when petal expansion was the most active. The expression of Rh-PIP2;1 decreased dramatically
after 3 d of flower opening, when the petal expansion process was almost completed (Fig. 6B). These results indicated that Rh-PIP2;1 expression was highly correlated with the petal expansion process.

We further investigated the effects of ethylene and its action inhibitor, 1-MCP, on Rh-PIP2;1 expression in petals during the first 72 h of flower opening. Ethylene treatment significantly inhibited the expression of Rh-PIP2;1, while 1-MCP maintained its expression at a higher level for a longer time compared with the controls (Fig. 6C).

We then examined Rh-PIP2;1 expression in petals on a narrower time scale during the first 24 h of ethylene or 1-MCP treatment in order to understand how quickly Rh-PIP2;1 responds to ethylene. The results showed that the expression of Rh-PIP2;1 changed within 0.5 to 1 h upon ethylene treatment (Fig. 6D), suggesting a quick response of Rh-PIP2;1 expression to ethylene.

Characterization of Rh-PIP2;1-Silenced Flowers

We functionally characterized the role of Rh-PIP2;1 in petal expansion by silencing Rh-PIP2;1 in rose flowers using a virus-induced gene silencing (VIGS) approach. The 290-bp fragment of Rh-PIP2;1 3’ end was used to construct the TRV-Rh-PIP2;1 silencing vector. We successfully silenced Rh-PIP2;1 in rose petals (Fig. 7A), although the efficiency of silencing (about 9:300) was much less than that reported in tobacco and tomato (Solanum lycopersicum; Liu et al., 2002; Fu et al., 2005). Flowers with silenced Rh-PIP2;1 had petals with significantly decreased length and width as well as reduced water content (Fig. 7B), a phenotype similar to that of ethylene-treated flowers. Rh-PIP2;1 silencing also resulted in significantly increased cell numbers of AbsE per microscope visual area in the upper region of petals, indicating that the silencing reduced the cell volume; however, the cell shapes of AbsE were more regular than those of ethylene-treated petals (Fig. 8).

In addition, we also obtained 12 partially Rh-PIP2;1-silenced flowers, and their phenotypes were more similar to those of the wild-type flowers (Figs. 7 and 8).

DISCUSSION

Ethylene Influences Petal Expansion by Modulating AbsE Cells in Roses

In plant development, proliferative growth and postmitotic expansion are coordinated and modulated...
by phytohormones like auxin, brassinosteroids, GA, and ethylene (Mizukami, 2001; Ingram and Waites, 2006). In Arabidopsis, auxin, abscisic acid, GA, and brassinosteroids can enhance longitudinal expansion of many organs, such as leaves and roots, while ethylene increases lateral expansion of hypocotyls and roots (Saab et al., 1990, 1992; Spollen et al., 2000; for review, see Mizukami, 2001). Compared with wild-type plants, ethylene overexpression (eto) and constitutive response (ctr1) mutants have smaller organs, while ethylene-insensitive (ctr) mutant plants appear to have larger leaves and floral organs (Ecker, 1995). Ethylene causes asymmetric cell elongation and results in formation of the apical hooks of pea (Peck et al., 1998). Treatment with ethylene or its precursor, 1-aminocyclopropane-1-carboxylic acid, greatly inhibits cell elongation in Arabidopsis roots (Le et al., 2001; De Cnodder et al., 2005). Thus, ethylene can play an important role in suppressing organ expansion in plants. Ethylene promotes flower opening in carnation (Dianthus caryophyllus; Jones and Woodson, 1997), orchid (Phalaenopsis spp.; Bui and O’Neill, 1998), and Petunia (Tang and Woodson, 1996). In rose, ethylene may promote or inhibit flower opening in a cultivar-dependent manner (Reid et al., 1989a, 1989b). In our previous work, we observed that ethylene promoted flower opening in Samantha rose (Cai et al., 2002; Ma et al., 2005, 2006; Xue et al., 2008). Despite these reports describing the effects of ethylene on ornamental plant flower opening, little is known about the effect of ethylene on petal expansion.

In this study, we show that ethylene treatment significantly reduced petal size (Fig. 1C) and resulted in vertically compressed flowers (Fig. 1, A and B). In addition, ethylene suppressed the expansion of AbsE cells and resulted in cells with irregular shapes (Fig. 2B). These findings strongly suggest that inhibited expansion of AbsE cells may contribute to the inhibition of petal expansion by ethylene.

**Rh-PIP2;1 Is Involved in Petal Expansion of Rose**

PIPs belong to a multigene family in higher plants, and 13 PIPs have been identified in both Arabidopsis (Johanson et al., 2001) and maize (Zea mays; Chaumont et al., 2001). It has been reported that high levels of PIP expression are usually associated with developmental...
processes requiring substantial water intake, including root elongation and expansion (Javot et al., 2003; Ranathunge et al., 2004), anther dehiscence (Bots et al., 2005a, 2005b), and petiole movement (Moshelion et al., 2002; Siefritz et al., 2004). During flower opening, petal expansion is mainly driven by cell expansion, a process of rapid water uptake (Drews et al., 1992; Martin and Gerats, 1993; Guterman et al., 2002). In this study, we show that petal fresh weight increased quickly and dry weight stayed at a steady level during petal expansion in rose, indicating that petal expansion should be primarily caused by water uptake-driven

Figure 7. Silencing of the Rh-PIP2;1 gene in rose petals by VIGS. Rose branches were infiltrated with Agrobacterium containing TRV alone (TRV, pTRV1 + pTRV2) or TRV carrying a fragment of Rh-PIP2;1 (TRV-Rh-PIP2;1, pTRV1 + pTRV2-Rh-PIP2;1). A. RT-PCR analysis of Rh-PIP2;1 in the Rh-PIP2;1-silencing flower petals. The first-strand cDNA was generated from 1 μg of total RNA and was used to amplify Rh-PIP2;1 and the 18S rRNA gene using gene-specific primers. The PCR cycles was 27 and 18 for Rh-PIP2;1 and 18S rRNA, respectively. Completely, Completely silenced flowers; partially, partially silenced flowers; non, no silenced flowers. B. The phenotype of Rh-PIP2;1-silencing flowers (top) and petal sizes and water content (bottom). The photographs were taken on the 7th d after infiltration. The petal size and water content were determined on the 7th d after infiltration as described in Figure 1. Each data point represents the mean ± se (n = 15 for control, TRV-Rh-PIP2;1 [non], and TRV; n = 9 for TRV-Rh-PIP2;1 [completely]; n = 12 for TRV-Rh-PIP2;1 [partially]).

Figure 8. Silencing of Rh-PIP2;1 inhibits the cell elongation of the AbsE in petals. Petal samples were taken as a 0.5-cm × 0.4-cm slice at 25% of the length from petal top on the 7th d after infiltration and treated as described in Figure 2B. Bars = 200 μm. Cell numbers were counted as described in Figure 2C. Completely, Completely silenced flowers; partially, partially silenced flowers; non, no silenced flowers. Each data point represents the mean ± se (n = 15 for control, TRV-Rh-PIP2;1 [non], TRV, and ethylene treatment; n = 9 for TRV-Rh-PIP2;1 [completely]; n = 12 for TRV-Rh-PIP2;1 [partially]). Different letters indicate significant differences between different treatments according to Duncan’s multiple range test (P < 0.05).
cell expansion in rose (Fig. 1D). In addition, we also showed that ethylene significantly inhibited petal expansion and reduced petal water content. Thus, we speculated that PIPs could be involved in the process of ethylene-inhibited petal expansion. Guterman et al. (2002) reported an AQP-like EST (BQ104371) that exhibited a 20-fold increase of mRNA concentration in petals of opened flowers compared with unopened buds, suggesting that it has a potential role in rose petal expansion. In this study, we further functionally characterized this EST, which we named Rh-PIP2;1, by investigating its role in ethylene-inhibited petal expansion.

The deduced amino acid sequence of Rh-PIP2;1 shares high homology with members of the PIP2 subfamily from other plant species (Fig. 3). Rh-PIP2;1 was expressed predominantly in petals, and its transcript abundance was tightly correlated with the flower-opening process (Fig. 6, A and B). In addition, among petal tissues, Rh-PIP2;1 expression was stronger in AbsE, whose expansion is tightly related to the increase of petal size during flower opening (Fig. 4C). These results strongly suggest that Rh-PIP2;1 is involved in petal expansion.

In general, PIPs are localized on the plasma membrane. However, Siefritz et al. (2001) reported that a tobacco PIP2 protein, NtAQP1, was also localized on some endomembranes in protoplasts. We also found that Rh-PIP2;1 was mainly plasma membrane localized, but its presence was also observed in some internal membrane compartments. Furthermore, using a set of subcellular fluorescence-labeled markers (Nelson et al., 2007), we proved that the internal membrane where Rh-PIP2;1 was localized was the endoplasmic reticulum (Fig. 4, A and B). Mammalian AQPs and plant TIPs (for tonoplast intrinsic proteins) can shuttle between subcellular compartments in response to various stimuli (reviewed by Luu and Maurel, 2005). However, little is known concerning the dynamic localizations of plant PIPs. Recently, Zelazny et al. (2007) reported that PIP interaction could regulate their subcellular localization. Thus, further analysis would be necessary to understand the mechanisms of Rh-PIP2;1 dynamic localization.

Ethylene Regulates Rh-PIP2;1 Expression in Rose Petals

PIPs are likely to play divergent and unique roles in plants with fewer functional redundancies (Luu and Maurel, 2005). The expression of PIP genes can be regulated by developmental and environmental cues at both the transcriptional and posttranscriptional levels (Kjellbom et al., 1999; Hachez et al., 2006). At the transcriptional level, PIP gene expression is regulated by various environmental factors, including light (Cochard et al., 2007), drought (Aroca et al., 2006; Galmés et al., 2007), cold (Jang et al., 2004), salt (Jang et al., 2004; Bourissac et al., 2005), and phytohormones, including gibberellic acid (Phillips and Huttly, 1994; Suga et al., 2002), abscisic acid (Kaldenhoff et al., 1993; Zhu et al., 2005; Aroca et al., 2006), and brassinolide (Morillon et al., 2001).

At the posttranscriptional level, AQP channel activities are modulated by several mechanisms, including protein phosphorylation, pH, protein trafficking, and protein oligomerization (Luu and Maurel, 2005; Hachez et al., 2006). Phosphorylation at certain residues of some AQPs is thought to be an important modification mechanism for channel activity. For instance, the channel activities of an Arabidopsis α-TIP gene, AtTIP3;1, the spinach (Spinacia oleracea) SoPIP2;1 (PM28A), and the soybean (Glycine max) nodulin 26 are all regulated through protein phosphorylation (Maurel et al., 1995; Johansson et al., 1996; Guenther et al., 2003). Recently, Tornroth-Horsefield et al. (2006) reported that phosphorylation-induced structural remodeling led to channel gating of SoPIP2;1.

The involvement of ethylene in the modification of AQP channel activity has been suggested in a few reports. A general inhibitor of AQP activities, HgCl₂, decreased ethylene-enhanced water flow in aspen (Populus spp.) roots (Kamaluddin and Zwiazek, 2002). A protein kinase inhibitor, okadaic acid, could inhibit the ethylene-induced phosphorylation in mung bean (Vigna radiata) hypocotyls (Kim et al., 1997), orchid petals (Wang et al., 2001), and pea (Berry et al., 1996; Kwak and Lee, 1997), and the inhibitor also inhibited the phosphorylation of AQP PM28A in spinach leaves (Johansson et al., 1996). Since the phosphorylation of AQPs is considered to be an important regulatory mechanism controlling channel activity (Tornroth-Horsefield et al., 2006), it is reasonably speculated that ethylene may be involved in the regulation of water channel activities via the protein phosphorylation/dephosphorylation pathway.

However, little direct evidence has been obtained at either the transcriptional or posttranscriptional level to support the role of AQPs in ethylene-regulated water transport.

In this work, we found that ethylene substantially decreased the transcript levels of Rh-PIP2;1 (Fig. 6, C and D). The suppressing effect of ethylene on Rh-PIP2;1 expression was observed within only 0.5 to 1 h of the treatment (Fig. 6D), suggesting that Rh-PIP2;1 expression is likely regulated directly by ethylene. Additionally, the Rh-PIP2;1-silenced flowers exhibited similar morphological and anatomical phenotypes to flowers treated by ethylene (Figs. 7 and 8), further supporting the notion that ethylene inhibits petal expansion of rose by suppressing Rh-PIP2;1 expression. Taken together, we argue that Rh-PIP2;1 is able to respond to ethylene promptly at the transcriptional level and is involved in ethylene-regulated petal expansion. However, we cannot rule out the possibility that ethylene also contributes to regulating petal water uptake and petal expansion by influencing the preexisting Rh-PIP2;1 protein activity. However, further work is required to clarify this possibility.

In this study, we also found that although the Rh-PIP2;1-silenced and ethylene-treated flowers exhibited...
similar morphological and anatomical phenotypes, overall the ethylene-treated flowers showed a more severe phenotype regarding the inhibited petal expansion. This indicates that some other ethylene-regulated genes might also be involved in ethylene-inhibited petal expansion.

On the other hand, it is possible that the cross talk between different hormones is involved in the regulation of plant organ expansion. In maintaining leaf expansion of Arabidopsis and tomato, and primary root elongation of Arabidopsis, endogenous abscisic acid was thought to play an important role by restricting ethylene production (Sharp et al., 2000; Spollen et al., 2000; LeNoble et al., 2004).

In Arabidopsis, the inhibitory effect of ethylene on GA-induced root elongation was accomplished through enhancing the stability of the conserved repressors of GA signaling, GAI (GA insensitive) and RGA (repressor of ga1-3; Achard et al., 2003), suggesting that there may be cross talk in influencing root elongation between ethylene and GA.

Taken together, ethylene, abscisic acid, and GA may synergistically influence petal expansion by regulating relevant genes, like AQP, at the transcriptional and/or the posttranscriptional level. However, further studies are required to understand the possible mechanisms.

MATERIALS AND METHODS

Plant Materials

Cut roses (Rosa hybrida ‘Samantha’) were harvested at stage 2 of flower opening from a local commercial grower and placed in water immediately.

Flower-opening stages were as defined by Wang et al. (2004) and Ma et al. (2005): stage 0, unopened bud; stage 1, partially opened bud; stage 2, completely opened bud; stages 3 and 4, partially opened flower; stage 5, fully opened flower with anther appearance (yellow); stage 6, fully opened flower (2005): stage 0, unopened bud; stage 1, partially opened bud; stage 2, completely opened bud; stages 3 and 4, partially opened flower; stage 5, fully opened flower (Wang et al., 2004 and Ma et al., 2005). Flower-opening stages were as defined by Wang et al. (2004) and Ma et al. (2005).

Based on our previous work (Ma et al., 2006), we used 10 μL L−1 ethylene or 2 μL L−1 1-MCP for treatments. The flowers were sealed in a 64-L chamber with ethylene, 1-MCP, or regular air as the control at 25°C for different periods of time. NaOH (1 mol L−1) was put into the chamber to prevent the accumulation of CO2. After treatment, flowers were placed in a vase with deionized water, which was refreshed daily. For all analyses in this study, samples were collected from outermost ray petals of the flowers.

Treatment of Flowers with Ethylene and 1-MCP

Based on our previous work (Ma et al., 2006), we used 10 μL L−1 ethylene or 2 μL L−1 1-MCP for treatments. The flowers were sealed in a 64-L chamber with ethylene, 1-MCP, or regular air as the control at 25°C for different periods of time. NaOH (1 mol L−1) was put into the chamber to prevent the accumulation of CO2. After treatment, flowers were placed in a vase with deionized water, which was refreshed daily. For all analyses in this study, samples were collected from outermost ray petals of the flowers.

Microscopic Examination and Cell Counting

Petal samples were taken as 0.5-cm × 0.4-cm slices from regions at 25%, 50%, and 75% of the petal length from the top. For scanning electron microscopy, slices of the petal middle region (50% of the length) of fully opened flowers were selected and then fixed and processed according to a standard protocol (Bowman et al., 1989). The slices were flat mounted on an agar block and fractured to reveal internal anatomy transverse to the petal longitudinal axis. Scanning electron microscopy was performed using a Philips 53400 apparatus.

Abscission cell photography and cell counting were performed as described by Devitt et al. (2007). In brief, rose flowers were treated at the 1st d (day 0) of opening for 24 h, and petal samples were taken on the 2nd d after the respective treatments. Petal samples were taken as 0.5-cm × 0.4-cm slices at 25%, 50%, and 75% of the petal length from the petal top. The slices were fixed in formaldehyde and then cleared in ethanol. Abscission cells of the slices were photographed using a Nikon IX-71 camera. The traces were drawn using Photoshop 7.0 software. Fifteen flowers were used in each treatment.

Numbers of Abscission cells were counted using ImageJ software in a visual field of 1,360 × 1,024 μm².

Rh-PIP2;1 Subcellular Localization

Rh-PIP2;1 cDNA was amplified with primers Rh-PIP2;1_up (5′-GGAGCG-ACTGGTCACAAGATGAAAGAAGTGC-3′) and Rh-PIP2;1_down (5′-GGCG-TGATCTCCTTTGAGGAGGTGTCTCCC-3′) for cDNA was amplified with primers gfp_up (5′-ATCCCTGATGTGACGCAAGGGCGAGAGCT-3′) and gfp_down (5′-CACCTCTTCTGCACTGTAGCCTGCC-3′). The resulting fragments were used to generate the GFP-Rh-PIP2;1 fusion gene by overlapping PCR. The fused segment was inserted into pGEM T-Easy vector (Promega) and subjected to sequencing for verification. After Ncol and BamHI digestion, the cassette was inserted into pRTLTR2 to construct the pRTL-GFP-Rh-PIP2;1 transient expression vector. Transient expression was conducted according to Kowtan et al. (2000). Arabidopsis (Arabidopsis thaliana) leaf mesophyll protoplasts were isolated from well expanded leaves of 3- to 4-week-old plants. Leaf sections of roughly 1 g were cut into 0.5- to 1-mm strips with a razor blade. The strips were placed into 15 mL of preheated enzyme solution (1% cellulase R10, 0.25% macerozyme R10, 20 mL KCl, and 20 mL MES, pH 5.7) and gently shaken at 23°C in darkness for 3 h. Then, the protoplasts were filtered and spun at 1,000 rpm for 1 min. The resulting pellets were washed once with cold WS solution (154 mL NaCl, 125 mL CaCl2, 5 mL KCl, 5 mL Glc, and 2 mL MES, pH 5.7). Then, the protoplasts were centrifuged at 1,000 rpm for 1 min, and then resuspended in MMg solution (0.4 mL mannitol, 15 mL MgCl2, and 4 mL MES, pH 5.7). Around 15 μL of plasmid DNA was mixed with 5 × 106 protoplasts in 0.1 mL of MMg solution. An equal volume of 40% (w/v) PEG4000 (Fluka) in 0.1 mL Ca(NO3)2 and 0.4 mL mannitol solution of pH 10 was added and gently mixed, and then the mixture was incubated at 23°C for 25 min. After incubation, a 4-fold volume WS solution was added and mixed gently, and the mixture was centrifuged at 1,000 rpm for 2 min. The pellets were resuspended gently in 1 mL of WS solution and then the mixture was incubated at 23°C for 20 to 22 h in darkness. All transformation was performed three times, and assays were repeated on at least three separate occasions.

pRTL-GFP-Rh-PIP2;1 was digested by HindIII, and the 350-bp GFP-Rh-PIP2;1Nos cassette was inserted into the binary vector pGreen 0229 and transformed into Agrobacterium tumefaciens GV3101. Arabidopsis plants were infiltrated with Agrobacterium using the floral dip method (Clough and Bent, 1998). The GFP fluorescence was visualized using a confocal microscopy (Nikon). Colocalization experiments were performed using the mCherry-labeled plasma membrane marker (PM-rk; CD3-1007) and the endoplasmic reticulum marker (ER-rk; CD3-959) purchased from the Arabidopsis Biological Resource Center (Nelson et al., 2007). The plasmids of CD3-1007 and CD3-959 were separately transformed into protoplasts of Arabidopsis transgenic plants overexpressing GFP-PPIP2;1. Imaging of protoplasts was conducted with a laser scanning confocal microscope (Nikon C1). The excitation wavelengths for GFP and mCherry were 488 and 543 nm, respectively, and the emission filter wavelengths were 505 to 530 nm for GFP and 560 to 615 nm for mCherry.

In Situ Hybridization

In situ hybridization was as described by Xu et al. (2005) with minor modifications. A 216-bp fragment at the 3′ end of Rh-PIP2;1 was amplified using the following primer pairs: 5′-CGCGGCTCAACTAAUGGAGAAGACG3′-3′-GCTAATGGCATCTACATAGGCGAAAACACCCTACTCTATTCGAC3′ for the antisense probe and 5′-GCTAATGCCTACTACTAGGGCCCTCAACTAAAGGAAAGAAGC-3′ and 5′-CAACCAACACTACTCTATTCGAC3′ for the sense probe. The PCR products were purified (Axyscreen DNA gel extraction kit; Axyscreen) and used as the template to generate the antisense or sense probe via in vitro transcription using the DIG RNA labeling mix (Roche).

Petal samples were fixed in cold phosphate-buffered saline (pH 7.0) containing 4% (w/v) paraformaldehyde overnight at 4°C. After being washed twice with cold phosphate-buffered saline, the samples were dehydrated with an ethanol series and then embedded in Paraplast plus (Sigma). Subsequently,
the samples were cut into 7-µm sections and mounted on slides pretreated with 0.01% poly-L-lys (Sigma). The hybridization was performed at 43°C overnight with a 0.1 to 0.2 ng µL⁻¹ RNA probe concentration. After washing and digestion by RNase A, the slides were incubated with Anti-Digoxigenin AP-Conjugate (Roche). Hybridizations were visualized using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate stock solution (Roche) and photographed with an Olympus BX51 microscope.

**Measurement of Pᵢ on Protoplasts**

The Pᵢ of Rh-PIP2;1 in leaf protoplasts of Arabidopsis wild-type and transgenic plants was measured according to Ramahaleo et al. (1999), Mosher and et al. (2004), and Cabalero et al. (2006). In brief, leaves of 20- to 22-d-old plants were harvested, and three different preparations of protoplasts were generated. Fifteen-microliter protoplast suspensions were dispensed on a microscope slide and, the same amount of water was added to decrease the osmotic potential of the medium to half. Cell volumes were captured with an inverted Nikon microscope (IX-71), and images were acquired at 3-s intervals for a total of 90 s using a CCD camera (DP 70; Nikon). Protoplast swelling was analyzed using the ImageJ program (http://rsb.info.nih.gov/ij/). Pᵢ was calculated according to the equation from Zhang and Verkman (1991): $Pᵢ = \left( \frac{Vᵢ}{Sᵢ} - \frac{Vₑ}{Sₑ} \right) \left( \frac{Osmₑ}{Osmᵢ} - 1 \right)$, where $Vᵢ$ is the initial cell volume, $Vₑ$ is the initial cell surface area, $Sᵢ$ is the initial cell surface area, $Sₑ$ is the molar volume of water (18 cm³ mol⁻¹), k is the fitted exponential rate constant of the initial phase of the swelling, and osmolₒ and osmolᵢ are the internal and external osmoralties, respectively. Measurements of osmolarity (mosmol kg⁻¹) were carried out using an osmometer (Fiske ONE-TENTM Osmometer).

For each line, the Pᵢ of 75 protoplasts was measured, and the initial diameter of the protoplasts was about 60 µm.

**RNA Extraction, Northern-Blot Analysis, and Reverse Transcription-PCR**

The full-length cDNA of Rh-PIP2;1 was obtained by RACE (SMART RACE cDNA Amplification; Clontech) and deposited into GenBank under accession number EU727217. Procedures for total RNA extraction and RNA gel-blot analysis were reported by Ma et al. (2006). Petals were ground with liquid nitrogen and homogenized with the preheated extraction buffer (200 mM sodium tetraborate decahydrate, 30 mM EGTA, 1% deoxycholic acid sodium salt, 10 mM dithiothreitol, 2% polyvinylpyrrolidone 40, and 1% Nonidet P-40). RNA was first precipitated overnight with 2× LiCl, then washed by 2× LiCl and redissolved in 1× Tris-Cl (pH 7.5). Subsequently, the RNA was precipitated with 100% ethanol at −80°C for 2 h. For RNA gel-blot analysis, 10 µg of total RNA was fractionated on 1.2% agarose gels containing 2.5% (v/v) formaldehyde. The 3′-UTR region of Rh-PIP2;1 was used as the probe. DIG-labeled probes were generated by PCR using a PCR DIG probe synthesis kit (Boehringer Mannheim). Hybridization was carried out overnight at 46°C. The membranes were washed twice in 2× SSC at 37°C and twice in 0.1× SSC at 59°C, with 30 min for each wash, and then were incubated with Anti-Digoxigenin AP-Conjugate (Roche). The membranes were then subjected to a chemiluminescence reaction with CDP-Star according to the manufacturer's protocol (Roche) and exposed to Fuji Medical X-Ray Film (Fuji Photo Film). All of the hybridizations were performed with at least three biological replicates.

For reverse transcription (RT)-PCR, first-strand cDNA was synthesized from 1 µg of total RNA using SuperScript Moloney murine leukemia virus reverse transcriptase (Promega). Semiquantitative RT-PCR was performed as described by Fu et al. (2005). The primers used for RT-PCR were 5′-TTC-AAAAATGACGAAGGAAGTGAGCGA-3′ and 5′-CCGGATCAGAGAGACCTTACGAG-3′, which were outside the region used for VIGS. This ensured that only the endogenous Rh-PIP2;1 transcripts were examined in VIGS plants. The 18S RNA gene was used as an internal control for RNA quantity. The PCR product amplified from a reaction without reverse transcription was used as the negative control. The PCR products were analyzed on 1% agarose gels and imaged by Alphalmager 2200 (Alpha Innotech).

**Silencing of Rh-PIP2;1 in Rose Flowers by VIGS**

The pTRV1 and pTRV2 VIGS vectors (described by Liu et al., 2002) were kindly provided by Dr. S.P. Dinesh-Kumar at Yale University. The Rh-PIP2;1 silencing was performed as described by Fu et al. (2005). A 302-bp fragment at the 3′ end of Rh-PIP2;1 was amplified using primers 5′-CGTCTTACAGTG- CACGCCGGTACCAACGACG-3′ with an XhoI restriction site and 5′-CATCTC- GAGGGTTGCAAAACACACTA-3′ with an XhoI restriction site. The resulting product was cloned into pTRV2 to form the pTRV2-Rh-PIP2;1 construct. pTRV2-Rh-PIP2;1, as well as the pTRV1 and pTRV2 vectors, were transformed into Agrobacterium GV3101 by electroporation. The Agrobacterium strain GV3101 containing pTRV1, pTRV2, or pTRV2-Rh-PIP2;1 was grown at 28°C in Luria-Bertani medium supplemented with 10 µM MES, 20 µM acetylcytosingone, and 50 mg L⁻¹ kanamycin for about 24 h. Agrobacterium cells were harvested and suspended in the infiltration buffer (10 mM MgCl₂, 150 mM acetylcytosingone, and 10 µM MES; pH 5.6) to a final optical density at 600 nm of around 1.0. A mixture of Agrobacterium cultures containing pTRV1 and pTRV2-Rh-PIP2;1 in a ratio of 1:1 (v/v), as well as a mixture containing pTRV1 and pTRV2 in a 1:1 ratio (which served as the negative control), were shaken at room temperature for 4 h before vacuum infiltration.

For vacuum infiltration, rose flower branches at flower stage 1 were placed into the bacterial suspension solution and infiltrated by vacuum at 30 mmHg for 30 s. After release of the vacuum, the flower branch base was washed by deionized water and kept in deionized water for about 10 d at 18°C. The petals were collected when flowers fully opened, approximately at the 7th d after the infiltration.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU572717.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Anatomic structure of a petal by scanning electron microscopy.

**Supplemental Figure S2.** Petal AbsE cell expansion during flower opening.

**Supplemental Figure S3.** The quantification of the in situ signals of Figure 4C.

**Supplemental Figure S4.** The quantification of the northern hybridization signals in Figure 6, C (left) and D (right).

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