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Ethylene and Indole-3-Acetic Acid Participate in the In-Rolling and Opening of Carnation Petal Segments

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We have examined the inward-rolling and outward-opening of petals from 90° stage carnation flowers (*Dianthus charyophyllus* L. cv. Pink Donor). Ethylene released from 2-chloroethylphosphonic acid (CEPA) induced in-rolling in the lower portions of the petals while that action was suppressed by an inhibitor of auxin transport. Another plant hormone, indole-3-acetic acid (IAA), intensified this ethylene-induced in-rolling. In contrast, when ethylene was not applied, the same IAA concentration promoted the opening of petal segments. Our data suggest that a low level of ethylene acts on IAA-induced opening. Likewise, we can speculate that endogenous concentrations of ethylene could be an important determinant of petal responses that involve interactions between ethylene and IAA.

Keywords: carnation ethylene, IAA, in-rolling, opening, petal

In cut carnations, corolla senescence is intimately associated with climacteric ethylene production in the flower (Strydom and Whitehead, 1990; Whitehead and Vasiljevic, 1993). Cultivars such as 'Shinkibo' (Kim et al., 1998), 'Yellow Liberty', and 'Pink Donor' show a typical response pattern during the senescence process. We previously demonstrated (Kim et al., 1998) that the lower portion of a petal from 'Shinkibo', i.e., from its outermost whorl, is an excellent model system for examining the biosynthesis of ethylene and its possible role in corolla senescence. By studying petal segments, we have determined that their inward-rolling phenomenon can be induced artificially by treatment with exogenous ethylene before the acceleration of corolla decline. Recently, we found that the expression of genes participating in auxin-transport is up- or down-regulated in petal segments exposed to ethylene (unpublished data). Based on these results, we postulate that auxin, or at least auxin transport, participates in this in-rolling process from ethylene-treated segments. In contrast, applications of IAA alone might induce the outward expansion of petal segments, similar to that observed when carnation flowers bloom. However, ethylene is required for this IAA-associated opening of petals. As examples of this interaction between ethylene and auxin, these two growth regulators contribute to the regulation of ovary and ovule development in orchid (Zhang and O'Neill, 1993), while, in *Arabidopsis*, both function in the shading response to low-light intensity (Vandenbussche et al., 2003). The formation and growth of *Arabidopsis* root hairs also is affected by auxins and ethylene (Rahman et al., 2002).

The mimicked floral responses in carnation petal segments could reflect the role of both hormones for in-rolling related to corolla senescence and the opening of blossoms, especially with climacteric cultivars. Here, we investigated the involvement and interaction of ethylene and IAA in these two phenomena in carnation cv. Pink Donor.

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MATERIALS AND METHODS

Plant Material

Flowers of cut carnation (*Dianthus charyophyllus* L. cv. Pink Donor) at the paint brush stage were purchased from a local market in Seoul, Korea. Their stems were placed in a solution composed of 0.2% sucrose and 0.1% 8-hydroxyquinoline, and were kept in a chamber maintained at 25°C under constant cool-white florescent light. To eliminate the effects of ethylene during this period, a plastic box containing 40 mM $KMnO_4$ was placed in this chamber. The stages of petals in the outermost whorl were defined by their degree of reflected angle with respect to the axis of the pedicle. After those petals were detached at the 90° stage, they were divided into upper and lower portions, according to the method of Mor et al. (1985).

Treatment Chemicals for Petal Segments

The lower portion of our petal segments were placed in gas-tight Petri dishes containing 15 mL of 10 mM MES-Tris buffer (pH 7.2) with 50 mg L^{-1} chloramphenicol, and were incubated at 27°C for 8 to 12 h. To explore the effects of chemicals on these segments, agar blocks (2 × 5 × 2 mm) were prepared with various compounds that were added by using the incubation buffer as solvent. These were loaded onto either the left or right side of the adaxial or abaxial epidermis. For pre-treatment with 1-methylcyclopropane (1-MCP), carnation flowers at the 90° stage were placed in a gas-tight chamber and exposed to 10 nL L^{-1} of 1-MCP for 12 h (In, 2001). Chemicals except 1-MCP were purchased from Sigma (USA). 1-MCP was donated from Rohm an d Haas Korea.

Analysis of the In-Rolling and Opening of Petal Segments

Following incubation, the cut surfaces of transverse sections from our petal segments were photographed with a CCD camera. The effect of hormones on in-rolling or

opening was then analyzed by determining the ratio of adaxial/abaxial epidermis lengths. These adaxial and the abaxial sides were magnified on a 17-inch monitor and marked by a mouse pen. Then the length of each side was transformed to pixel units using KS300 software (Karl Zeiss, Germany). Ratios for the chemicals applied per half side were determined according to the particular concentration contained within each agar block. An SSPE package (Microsoft, USA) was used for our statistical analyses.

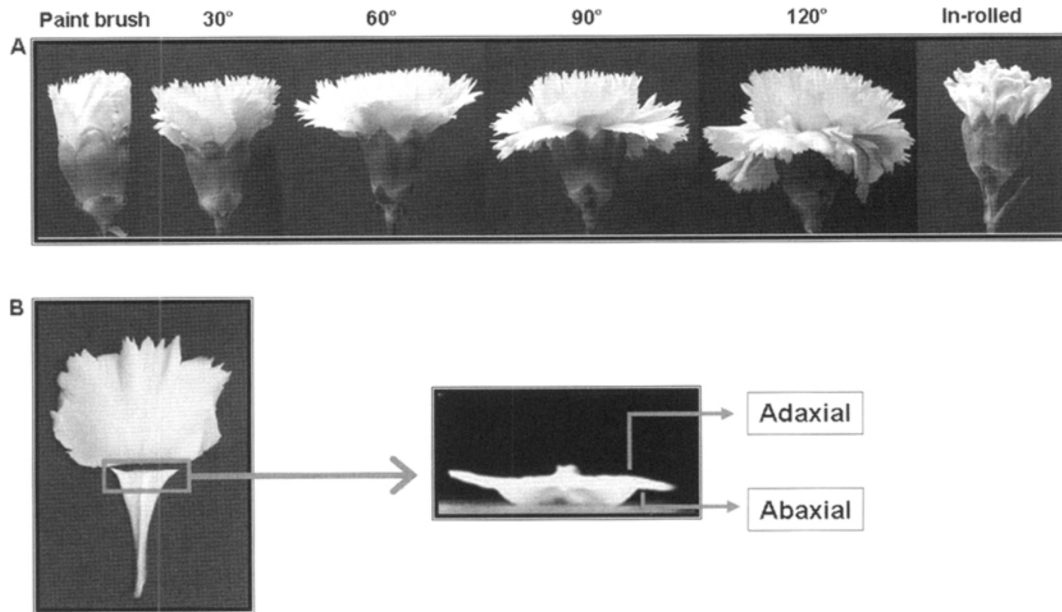

Figure 1. (**A**) Stages of carnation flower. (**B**) Lower portion of petal, from outermost whorl at 90° stage (left), and front view of transverse-cut surface (in box) of petal segment (right).

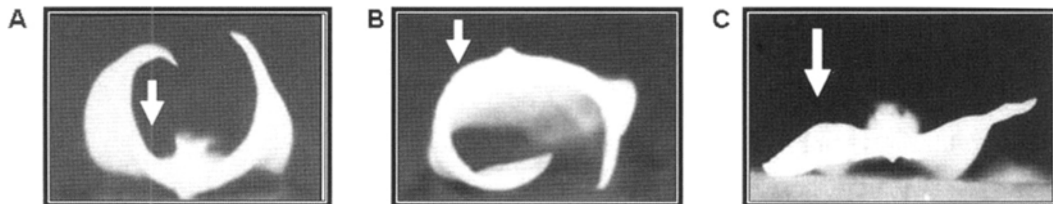

Figure 2. (**A**) Agar block containing 100 μM IAA was loaded on left side of adaxial epidermis from petal segment, then incubated in medium with 5 μM 2-chloroethylphosphonic acid (CEPA). (**B**) Block containing 100 μM IAA was loaded on left side of abaxial epidermis and incubated in medium with 5 μM CEPA. (**C**) Block containing 100 μM IAA was loaded on left side of adaxial epidermis and incubated in medium without CEPA. For all trials, arrows indicate loading side for chemical. Agar block without chemical was loaded on other, untreated, side of petal. All segments were incubated for 8 or 12 h.

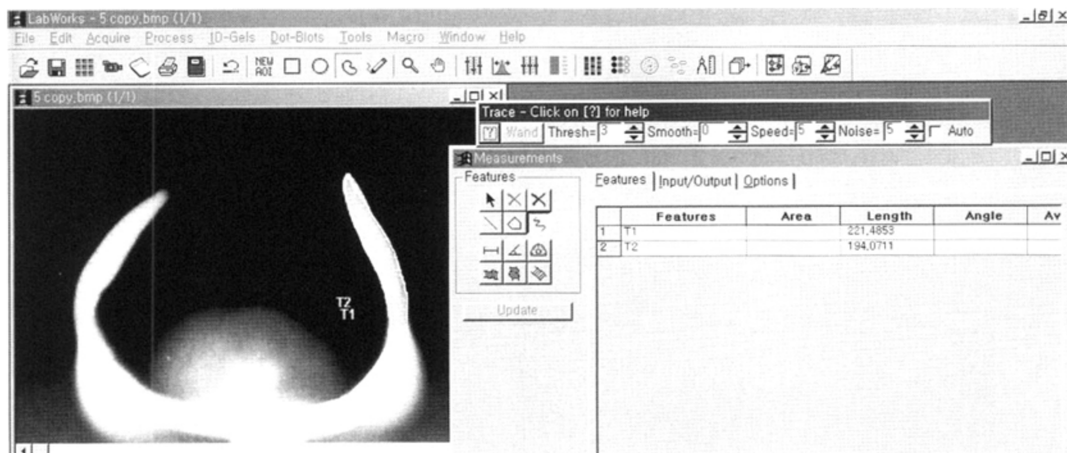

Figure 3. Analytical format, using KS300 software, for determining ratio of adaxial/abaxial lengths of segment cut surfaces, for which transverse sections were photographed (after chemical treatment and incubation) with CCD camera and zoom lens. Lengths of both adaxial and abaxial epidermal layers were measured with mouse pen coupled to software program.

RESULTS

Ethylene-Induced In-Rolling of Carnation Petal Segments

In-rolling by the segments of 'Pink Donor' carnation petals was examined in response to hormonal treatments. This phenomenon was quantified as a ratio of the lengths of the adaxial and abaxial epidermal layers. A decrease in ratio meant that the petal segments were rolling inwardly (Fig. 2A, B, 3).

Segments rolled inwardly after treatment with 2-chloroethylphosphonic acid (CEPA), an ethylene-releasing agent (Fig. 4). When segments were incubated for 8 h in a medium lacking CEPA, the ratio of adaxial/abaxial lengths approximated 1.0 compared with a decrease in this ratio (from 1.0 to 0.8) when petals were treated with 5 μM CEPA. Kim et al. (1998) have reported a similar response to exogenous ethylene by petals of carnation cv. Shinkibo, in which ethylene is produced climacterically. To clarify whether this CEPA-induced in-rolling resulted from an unknown by-product(s), we introduced 1-methylcyclopropane (1-MCP), an inhibitor of ethylene activity. Pre-treatment with 1-MCP completely suppressed CEPA-induced in-rolling. In a previous study using 'Shinkibo', Kim et al. (1998) reported that an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), induced similar in-rolling, thus implying a role for ethylene in this event.

Requirements for Auxin and/or Auxin Transport in Ethylene-Induced Petal In-Rolling

Using DD (differential display) - RT (reverse transcription)- PCR, we have isolated genes whose expressions varied during ethylene-induced petal in-rolling. Interestingly, some of these are supposed participants in auxin transport. Because ethylene is known to re-distribute auxin in some plant tissues (Vandenbussche et al., 2003), we speculated that auxin, or at least its transport, was active when this process was induced by exogenous ethylene. Therefore, we tested the effect of exogenous IAA, using a concentration that, based on previous tests in the range of 10 μM to 1 mM, causes minimal damage or a change in tissue color (Ishiki et al., 2000; Taguchi et al., 2001). For local treatment of our petal segments, we placed an agar block containing 100 μM IAA on the left side of either the adaxial or abaxial

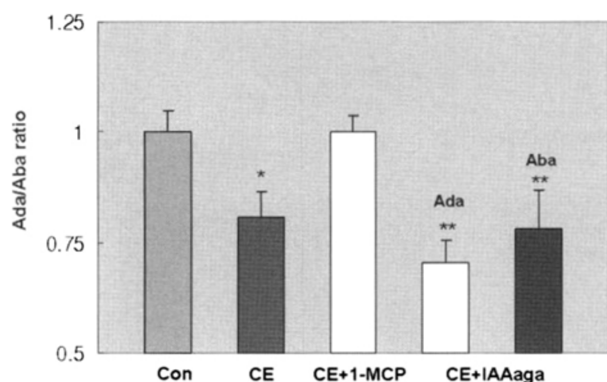

Figure 4. In-rolling of petal segments induced by ethylene that was released from 2-chloro-ethylphosphonic acid (CEPA), and effects of IAA within agar block on ethylene-induced in-rolling. Segments were incubated for 8 h in medium with 5 μM CEPA (CE) or without (Con). Segments treated for 12 h with 10 nL L^{-1} 1-methylcyclopropane (1-MCP) were incubated in medium with 5 μM CEPA (1-MCP + CE). Agar block containing 100 μM IAA (IAAaga) was loaded on one side of adaxial (Ada) or abaxial (Aba) epidermal layer. (*, $p < 0.05$ compared with control and 1-MCP; **, $p < 0.05$ compared with CEPA treatment).

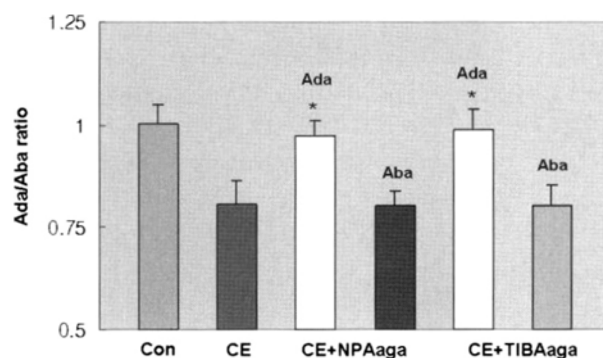

Figure 5. Effects of auxin-transport inhibitors on ethylene-induced in-rolling of petal segments from 90° stage carnation. NPAaga, an agar block containing 100 μM N-1-naphthylthalmic acid (NPA), was loaded on one side of adaxial or abaxial epidermal layer; TIBAaga, an agar block containing 100 μM 2, 3, 5-triiodobenzoic acid (TIBA), was loaded on one side of adaxial or abaxial epidermis. Segments were incubated for 12 h in medium containing 5 μM CEPA (CE). Ada, adaxial epidermis; Aba, abaxial epidermis. (*, $p < 0.05$ compared with CEPA treatment).

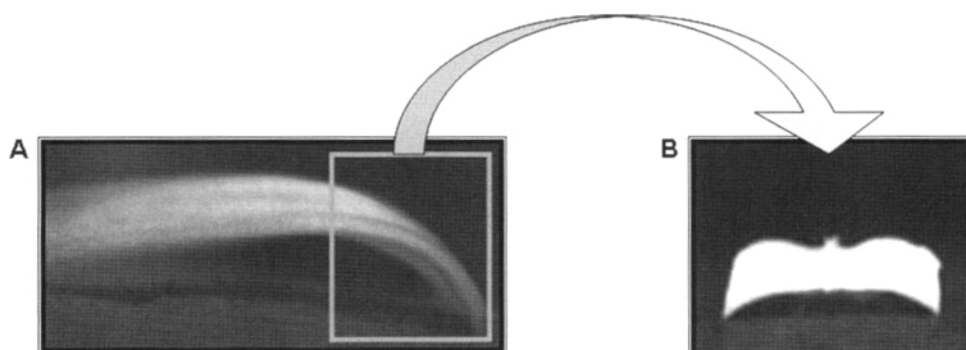

Figure 6. Outward expansion (opening) of petal segment following chemical treatment. (**A**) Side view of lower portion. (**B**) Front view of cut surface from transverse section of petal.

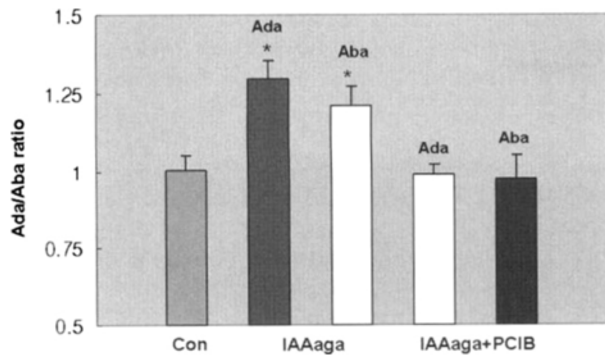

Figure 7. Auxin-induced opening of petal segments. Agar block containing 100 μM IAA was loaded on one side of adaxial (Ada) or abaxial (Aba) epidermis, and incubated in medium for 12 h. Agar block containing 100 μM IAA (IAAaga) was loaded on one side of adaxial or abaxial epidermis, and incubated in medium containing 100 μM 2-(*p*-chlorophenoxy)-2-methylpropionic acid ethylester (PCIB). (*, $p < 0.05$ compared with each control and IAAaga+PCIB).

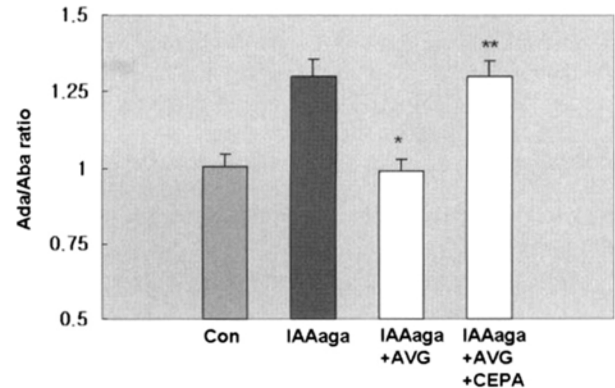

Figure 8. Requirement of ethylene for IAA-induced opening. Con, petals pre-treated with 1-methylcyclopropene (1-MCP) as in Figure 6; IAAaga + AVG, an agar block containing 100 μM IAA was loaded on one side of tissue pre-treated with 1-MCP. Segments were incubated in medium with 10 μM aminoethoxy-vinylglycine, (AVG) for 12 h; IAAaga + AVG + CEPA, an agar block containing 100 μM IAA was loaded on one side of tissue pre-treated with 1-MCP. Segments were incubated in 10 μM AVG and 5 μM CEPA for 12 h. (*, $p < 0.05$ compares with IAAaga; **, $p < 0.05$ compares with IAAaga + AVG).

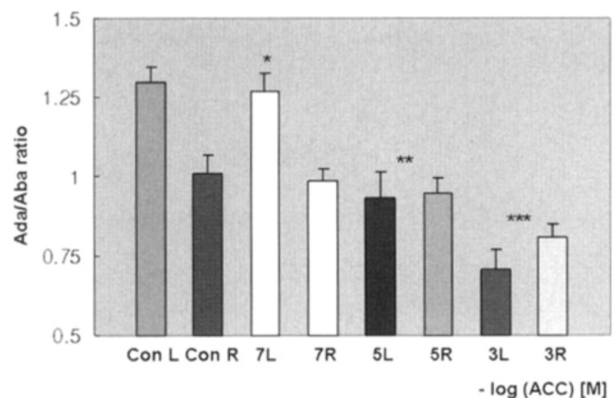

Figure 9. Effect of various concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC) on petal segments in presence or absence of IAA. Agar block containing 100 μM IAA was loaded on left side (L) and block without IAA was loaded on right side (R) of epidermis. Segments were incubated in medium with various concentrations of ACC (100 nM to 1 mM). Con, incubation of petal segments in medium without ACC. (*, $p < 0.05$ compared with control R; **, $p < 0.05$ compared with ACC 10^{-7}; ***, $p < 0.05$ compared with control)

epidermis (Fig. 2A, B). Such application intensified the ethylene-induced in-rolling effect (Fig. 4), although that response was apparent only on the side contacted to the IAA agar block. Furthermore, this effect was slightly different for each site: i.e., when exogenous IAA was applied to the adaxial epidermis, the ratio of adaxial/abaxial length decreased from 0.81 to 0.72, whereas this ratio was 0.76 when loading occurred on the abaxial epidermis.

We also tested the influence of auxin-transport inhibitors on ethylene-induced in-rolling. In contrast to IAA, compounds such as 100 μM N-1-naphtylphthalmic acid (NPA) or 2, 3, 5-triiodobenzoic acid (TIBA) completely suppressed that response (Fig. 5). Specific concentrations of both inhibitors used here were based on previous experiments of side effects associated with a range of 10 μM to 1 mM (Plieth and Trewavas, 2002; Oono et al., 2003; Poupart et al., 2005). In our study, this inhibition was observed only when the compounds were loaded onto the adaxial epidermis.

IAA-Induced Opening of Petal Segments

When 100 μM IAA was loaded onto petal segments treated without CEPA, the outward expansion of the adaxial epidermis, i.e., petal-opening, was induced (Fig. 6). This phenomenon resembled that of a carnation flower as it blooms from the paint brush to the 120° stage. Treatment with exogenous IAA alone increased the ratio of adaxial/abaxial lengths from 1.00 (original state) to values of 1.30 and 1.23 when the hormone was loaded on the adaxial and abaxial epidermis, respectively (Fig. 7). However, this IAA-induced petal-opening was entirely suppressed when we also applied 100 μM 2-(*p*-chlorophenoxy)-2-methylpropionic acid ethylester (PCIB), an auxin action inhibitor. The particular concentration of inhibitor used here was selected based on reports from previous experiments with 10 to 100 μM PCIB (Taguchi et al., 2001; Oono et al., 2003; Takahashi et al., 2003).

Requirement of Ethylene for IAA-Induced Opening

The wounding that results when one prepares experimental tissue segments or treats them with exogenous IAA are well-known factors for increased ethylene production, particularly through the activation of ACC synthase (Abeles et al., 1992; Nam et al., 2007). Therefore, we chose to block endogenous ethylene formation and, tested for the effect of 1-MCP on IAA-induced opening. Localized auxin application also promoted petal-opening when segments were pre-treated with 1-MCP for 12 h (Fig. 8). However, we could not exclude the possibility that the method used for this pre-treatment was not sufficient to block all ethylene receptors in the petals. Therefore, we next attempted to suppress endogenous ethylene production by treating segments with 10 μM aminoethoxyvinylglycine (AVG), an inhibitor of ACC synthase. Here, AVG profoundly suppressed the IAA-induced opening of segments pre-treated with 1-MCP (Fig.

8). Likewise, this inhibitory property of AVG was mostly recovered when we used CEPA. These data led us to speculate that a low amount of ethylene is required for IAA-induced petal-opening. Therefore, we propose, based on these observations, that both in-rolling and petal-opening result from an interaction between ethylene and IAA.

Factors That Determine the Responses of Petal Segments

To identify the factor responsible for this response of petal segments to ethylene and auxin, we tested different amounts of ethylene in the presence or absence of IAA, using various concentrations of ACC instead of CEPA. IAA induced petal-opening when segments were incubated in media with and without 100 nM ACC (Fig. 9), the latter inducing a level of ethylene that was too low to determine. In contrast, higher ACC concentrations, e.g., 10 μM or 1 mM, were associated with in-rolling as well as the production of 28 $\mu L\ g^{-1}$ and 480 $\mu L\ g^{-1}$ ethylene, respectively, after 18 h of incubation. The IAA contained an agar block also intensified the degree of ethylene-induced in-rolling (Fig. 9). Therefore, based on these data, we propose that the endogenous level of plant ethylene could be a determinant of the carnation petal response to interactions between ethylene and IAA.

DISCUSSION

Ethylene-Induced In-Rolling of Petal Segments

Kinetic analysis has revealed variations in the length of the epidermis from carnation petals when segments are treated with ethylene. That of the adaxial layer tends to decrease while the abaxial layer shows steady elongation (Kim et al., 1998). Such a response may result from the accompanying selective reduction in turgor pressure in adaxial epidermal cells as they decrease in length. Here, however, water content did not change after flowers were exposed to ethylene for 12 h (data not shown). We also examined post-treatment alterations in the volume and shape of epidermal cells from both layers. While abaxial cells steadily enlarged, those of the adaxial epidermis showed no significant change in volume (data not shown). In contrast, the shapes of those adaxial cells were extremely modified, with their ratio of cell height to width increasing from 1.7 to 2.8. This was especially true for cells near the petal edge. In comparison, the ratio for cells of the abaxial epidermis changed less so (from 1.4 to 1.5; unpublished data).

These results lead us to speculate that the longitudinal elongation of cells by ethylene could be a basis for petal in-rolling. One possible explanation for the observed changes in height/width ratios is a variation in the direction of cell expansion. Taiz (1984) has suggested that ethylene and auxin might control this direction by effecting the orientation of cell wall microfibrils. Such an association has also been reported, based on the alignment of cellular microtubules, in the cortical array (Fisher and Cyr, 1998; Baskin et al., 2004), so that when those microtubules and co-aligned microfibrils are arranged transversely around the cell, turgor pressure drives elongation. Wounding can also prompt a short-term re-orientation of microtubules, from transverse to vertical, in pea stem epidermal cells (Yuan et al., 1994). Therefore, we believe that ethylene alone or its interaction with auxin can induce selective re-orientation of microtubules in adaxial epidermal cells, thereby causing in-rolling.

As illustrated in Figure 4, auxin transport in petal segments participates in the process of ethylene-induced in-rolling. A prerequisite for auxin transport has been proposed, and is based on the isolation of genes regulated through exogenous ethylene in carnation petal segments. Auxin is imported into cells by auxin-import proteins e.g., AUX1 (Yang et al., 2006) and the P-glycoprotein PGP4 (Terasaka et al., 2005), and is exported via auxin-efflux carrier complexes such as auxin efflux proteins, PIN and multi-drug resistance proteins (Benjamins et al., 2005). Using DD - RT - PCR, we have found that the expression of genes for auxin-transport proteins (multi-drug-resistant P-glycoprotein) changes during ethylene-induced in-rolling (unpublished data). This has been confirmed by blocking auxin-transport with inhibitors such as NPA and TIBA. In the current study, these inhibitors appeared only when the chemicals were applied to the adaxial epidermis (Fig. 5). Such a site-dependent influence is concurrent with observations that PCIB, an IAA-action inhibitor, suppresses this type of in-rolling when applied to the abaxial epidermis (data not shown). Therefore, we suggest that a redistribution of auxin to the abaxial epidermis by ethylene could participate in this ethylene-induced process.

As indicated by Figure 4, local treatment with auxin intensified ethylene-induced in-rolling. By adding 100 μM IAA to the incubation medium and not via agar block, ethylene was produced (2 $\mu L\ g^{-1}$) in petal segments within 10 h (unpublished data). Although this amount of ethylene was relatively minor compared with the 150 $\mu L\ g^{-1}$ ethylene that resulted from treatment with 5 μM CEPA, nonetheless IAA in an agar block was capable of locally inducing ethylene production at the side on which loading occurred, thereby intensifying the role of ethylene in targeted petal in-rolling. This indirect effect of IAA is consistent with our results that demonstrated how elevated amounts of ACC could also enhance in-rolling (Fig. 9). Although the discrepancy in IAA effect between adaxial and abaxial layers was relative small, we cannot exclude the possibility that locally applied IAA could play a direct role, such as by driving cell elongation.

IAA-Induced Opening of Petal Segments

Agar blocks containing IAA induced the outward opening or expansion of the adaxial epidermis in carnation petals (Fig. 7). When segments were incubated in a medium supplemented with 100 μM IAA but not with CEPA, a similar response was observed (data not shown). Interestingly, this effect of IAA appeared to be more significant on the adaxial than on the abaxial side when an IAA-laden agar block was loaded locally. However, this reaction was entirely suppressed when an auxin-action inhibitor, PCIB, was present in the incubation medium (Fig. 7).

Epinasty results from differential cell growth in the petioles (Ursin and Bradford, 1989). Gravitropic and phototropic

responses as well as the formation of an apical hook in dark-grown seedlings are other consequences of differential growth rates. For example, in *Arabidopsis*, an asymmetric distribution of auxin causes both gravitropic and phototropic responses (Friml et al., 2002). Unequal auxin dispersion in hypocotyls may also be a causal factor for the formation of apical hooks (Lehman et al., 1996). Because ethylene induces the redistribution of auxin (Vandenbussche et al., 2003), we might suggest, based on our current data (Fig. 8, 9) that a lower level of ethylene might participate in IAA-induced opening by redistributing exogenous IAA, and that this asymmetry of IAA concentrations between the adaxial and abaxial epidermal layers may cause differential growth rates, leading to petal-opening. In this context, such discrepancies that depend upon which side of the epidermis is being treated (Fig. 7) indicate that more auxin is being distributed to the adaxial epidermis.

Ethylene Is the Determinant for the Responses of Petal Segments, both In-Rolling and Opening

In conjunction with the data related to *de novo* synthesis of ethylene in the petals of cv. Shinkibo, e.g., amount of endogenous ethylene production, ACC content, and enzyme activities during biosynthesis, Kim et al. (1998) have reported that a transient minimal production peak of ethylene is detected before the larger main peak appears. Furthermore, in previous study by this lab (data not shown), we have analyzed variations in the lengths of adaxial and abaxial epidermis layers of 'Shinkibo' petals, and observed transient petal-opening at the beginning of the period for ethylene treatment released from CEPA. These past results are consistent with data for 'Pink Donor' presented in Figure 9. Here, 100 μM IAA, the maximum concentration linked with this opening, was associated with the production of 2 $\mu L\ g^{-1}$ of ethylene over a 10-h span. In contrast, 28 $\mu L\ g^{-1}$ of ethylene was induced by ACC treatment, a level that was the minimum required to induce in-rolling. This amount of ethylene also was released from segments incubated in CEPA for 2 to 3 h. Therefore, we conclude that the critical endogenous level of ethylene for determining petal response ranges from 2 to 28 $\mu L\ g^{-1}$.

At present, we are unable to satisfactorily explain how ethylene determines the local response of petal segments as a function of quantity. For example, we must still investigate whether the arrangement of microtubules dictates the direction for expansion of cells in a manner different from the level of ethylene. As mentioned previously, auxin can be transported from the adaxial to the abaxial epidermis or *vice versa* and may participate in petal in-rolling and -opening through the induction of specific cellular growth that depends on ethylene content.

A connection is likely to exist between the activity of ethylene and the redistribution of auxin. Therefore, further examination is required into the individual roles of ethylene and auxin during in-rolling and petal-opening. Additional study is also needed to determine if cross-talk between plant hormones participates in the overall regulation of plant dynamics within the entire carnation flower, rather than merely being limited to the petal segments.

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