J. Plant Biol. 41 (4) : 304~311 (1998)

The Inrolling Phenomena of Petals During Senescence in Cut Carnations (*Dianthus caryophyllus* L. cv. Shinkibo)

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The inward rolling of the petals is one of typical symptoms observed in the process of climacteric corolla senescence. Inrolling was mimicked by treating the lower part of the petal instead of the whole petal of cut carnations (cv. Shinkibo) with exogenous ethylene. In these petal segments, the climacteric ethylene burst occurred right at the inrolling stage, indicating that these petal segments may be an excellent model system for examining corolla senescence. According to kinetic analysis, an asymmetry in the lengths of the adaxial and abaxial sides of petal segments appeared to be the direct cause of the inward rolling. While the length of the abaxial side of the transverse section of petal segments increased during the analysis, the ultimate length of the adaxial side was shrunken by the same ethylene action. Interestingly, the kinetics curve of the adaxial side consisted of two distinct phases. The rate of expansion/shrink of either side of the petal and the slope of each phase varied with the chemicals affected in the rolling process of the petal segments: e.g., *n*-octanoic acid, polyamines, and inhibitors of the Ca^{2+}-channel blocker.

Keywords: Ca^{2+}, carnation petals, ethylene, polyamine, verapamile

INTRODUCTION

The wilting of petals in carnations is a typical symptom observed in the later stage of corolla senescence. Before wilting, inrolling of the petals that expand outwardly from the paint brush stage to the anthesis occurs promptly at a critical point of senescence. In cut carnations, corolla senescence is well known to be intimately associated with a climacteric pattern in ethylene production (Strydom and Whitehead, 1990; Whitehead and Vasiljevic, 1993).

Ethylene production during the process of senescence in cut carnations cv. Shinkibo cultured in the southwest province of Korea showed a typical climacteric pattern. Particularly, the lower part of the petals located in the outmost whorl was sensible to ethylene and rolled inwardly in response to treatment with exogenous ethylene. Therefore, these petal segments provided us with a chance to artificially mimic the typical inrolling phenomena of carnation petals by exogenous ethylene and to elucidate the mechanisms of the inward rolling of the petals. In addition, the effects of some chemicals on the inrolling phenomenon could be tested with this model system; *n*-octanoic acid, known as 'the sensitivity factor' (Whitehead and Vasiljevic, 1993), and polyamines have shown their anti-senescence effects during the senescence of plant tissues (Kaur-Sawhney and Galston, 1991).

Recently, the requirement of Ca^{2+} was elucidated in ethylene-induced actions such as the shortening of pea epicotyls (Berry *et al.*, 1996) and the induction of pathogenesis related genes (Raz and Fluhr, 1992, 1993). Therefore, this model system makes possible to test whether the increase of Ca^{2+} in cytosol is indispensable in the process of the rolling of petal segments.

In this work, we elucidated the mechanism of the inrolling of petal segments by elongation kinetics analysis. Also, we determined how the petal segments respond to ethylene when n-octanoic acid and/or polyamines are treated to the petal segments, and furthermore, how the rolling of petals was altered when the influx of Ca^{2+} into cytosol was artificially inhibited.

MATERIAL AND METHODS

Material

Carnations (*Dianthus caryophyllus* L. cv. Shinkibo) at the paint brush state were harvested from a

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commercial greenhouse located in the Kimhae area in Korea. The carnations were further incubated in a solution containing 0.2% sucrose and 0.1% 8-hydroxyquinoline and placed in a chamber maintained at 20°C under constant cool-white florescence light. For the elimination of ethylene effects during incubation, water containers containing 4×10^{-5} M $KMnO_4$ were placed in the growth chamber. The stages of the petals in the outmost whorl were defined depending on the degree of reflected angles with respect to the axis of the pedicle as the paint brush (almost 0°), 60°, 90° and 120° stages. When completely expanded petals (ca. 120°) were rolled inwardly, this stage was defined as the inrolled phase. After detachment of petals at each stage, they were divided into upper and lower parts according to the methods of Mor *et al.* (1985).

Determination of Ethylene

Ethylene production was determined with a gas chromatography (Shimazu GC-RIA, 3 m stainless column; air, 0.4 kg/cm^2; carrier N_2, 50 mL/min; H_2, 0.6 kg/cm^2) equipped with an active alumina column (80/100 mesh). Petal segments were incubated in a 10 mL gas tight vial containing 10 mM Mes-Tris buffer and 1 mM aminooxyacetic acid (AOA), an inhibitor of ACC-synthase, for 3 h at 27°C under dark conditions. One ml of the gas sample containing ethylene converted from endogenous ACC during incubation was removed and used for the determination of ethylene.

Determination of ACC Content

The method of Lizada and Yang (1979) was modified to determine the ACC (1-aminocyclopropane-1-carboxylic acid) content in petal segments. One g of the petal segments was powdered by liquid nitrogen and extracted with 3 mL of 80% ethanol.

Assay of Enzyme Activities

ACC-oxidase activity was assayed according to the a modification of method of Hoffman and Yang (1982). Petal segments were incubated in a 20 mL vial containing 2 mM ACC and 1 mM AOA for 30 min at 27°C under dark conditions. ACC-synthase activity was determined by the modified method of Woodson *et al.* (1992). In this case, Sephadex G-50 was used and equilibrated by elution of a buffer including 10 mM Hepes-KOH (pH 8.0), 4 mM DTT, 2 M pyridoxal phosphate and 10% (v/v) glycerol.

Biotest of the Rolling of Petal Segments

The lower part of the petal segments were incubated in a petridish containing 15 mL of 10 mM MES-Tris buffer (pH 7.2) and chloroamphenicol (50 mg/L) and sealed by parafilm at 27°C under dark condition for 8 h or 18 h. After incubation, the petal segments were photographed.

Elongation Kinetics of Each Side of the Petal Segment's Cut Surface

Petal segments in the 90° stage were placed on a sponge and incubated in a gas tight glass jar for plant tissue culture (Sigma, USA) containing 15 mL of 10 mM MES-Tris buffer (pH 7.2) at 27°C. For maintenance of incubation temperature, the glass jar was placed in a water-bath maintained at 42°C (Fig. 1A). Cut surfaces of the transverse sections of the petal's lower parts were magnified and photographed at every 10 min by a CCD camera equipped with a zoom lens (Fig. 1A). The adaxial and the abaxial sides of the cut surface, magnified further on a 17-inch monitor (Fig. 1B), were marked by a mouse pen, and the kinetics curve of the length of each side was analyzed by the KS300 software (Karl Zeiss, Germany).

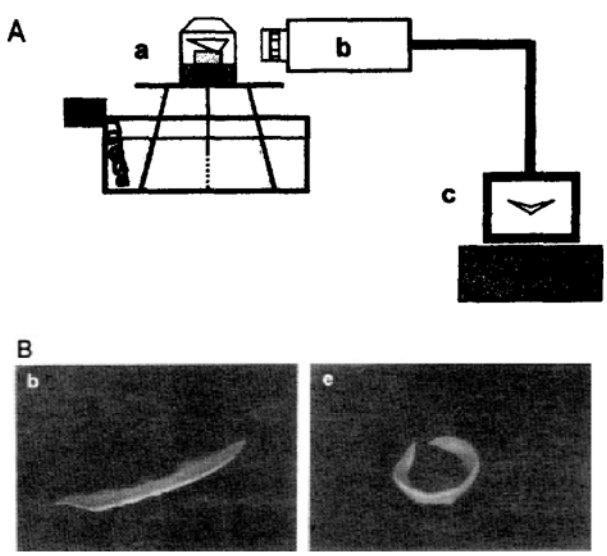

Fig. 1. A, Assembly of the system for the analysis of the inrolling of petal segments. a, A petal segment laid on the sponge floating on the incubation solution. Glass jar containing petal segments located in the water-bath; b, CCD-camera equipped with zoom lens; c, a computer equipped with analyzing software. B, The shape of cut surface of a petal segment at the beginning (b) and at the end (e) of the 8 h incubation.

RESULTS

Ethylene-induced Rolling of Carnation Petals Inwardly

We examined, first of all, whether exogenous ethylene mimics the inrolling of petals as observed under natural conditions in corolla senescence. We also determined whether the lower part of carnation petals is suitable as a model system for our purpose. As shown in Fig. 2A, the lower part of carnation petals was rolled inward by the treatment of 5×10^{-6} M CEPA (2-chloroethylphosphonic acid), an ethylene-releasing agent, during 8 h incubation. This effect of CEPA was more remarkable in the 90° stage of petal segments than in the 60° or 120° stages. Further, the effect of 10^{-3} M ACC, an ethylene precursor, on the rolling petal segments was examined. Particularly, the 90° stage petal segments were more prominently inrolled than petal segments of any other stage (Fig. 2B). To confirm whether this phenomenon is due to by-products of CEPA, such as chloride and phosphate, or not, we examined the effect of STS (silver thisulfate), an ethylene action inhibitor, on the rolling of the petals. As shown in Fig. 2C, the inrolling of petals with treatment of CEPA or ACC was considerably inhibited by the addition of 10^{-4} M STS.

The inrolling of petals was profoundly inhibited by the treatment of 10^{-4} M polyamines (e. g., putresine, spermidine, and spermine) that have anti-senescent effects (Fig. 3). However, there were no differences in the effects between the polyamines.

To elucidate that the increase of cytosolic Ca^{2+} is intimately associated with the process of the rolling,

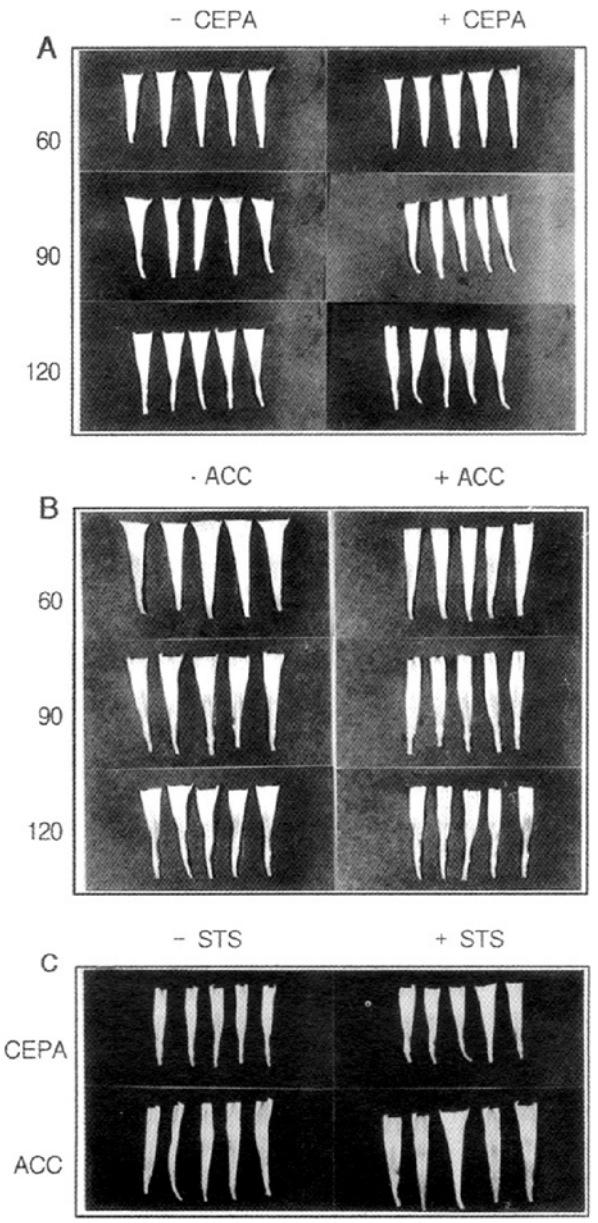

Fig. 2. Bioassay for the determination of chemicals effects on the inrolling of petal segments in the 60°, 90°, and 120° stages. A, The effect of 5×10^{-6} M CEPA on the inrolling of petal segments; B, The effect of 10^{-3} M ACC on the inrolling of petal segments. 60, 90, and 120 mean an each defined stage of petal segments; C, The effect of 10^{-4} M STS on the inrolling of the 90° stage of petal segments.

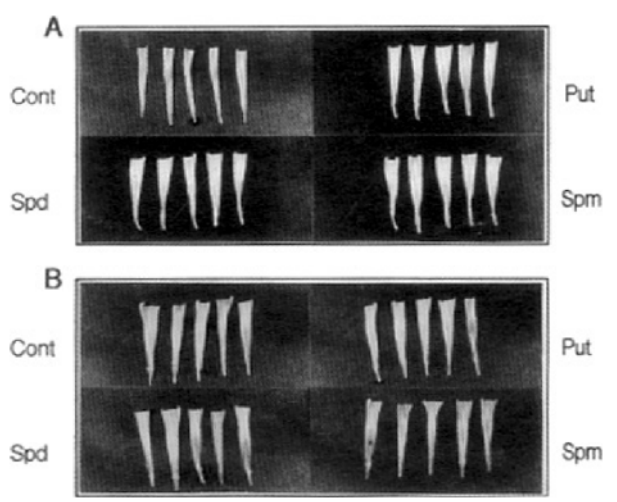

Fig. 3. Bioassay for the determination of polyamines effects on the inrolling of petal segments. A, The effect of 10^{-4} M polyamines on the inrolling of petal segments induced by 5×10^{-6} M CEPA during 8 h incubation; B, The effect of 10^{-4} M polyamines on the inrolling of petal segments induced by 10^{-3} M ACC during 18 h incubation. Put, putresine; Spd, spermidine; Spm, spermine.

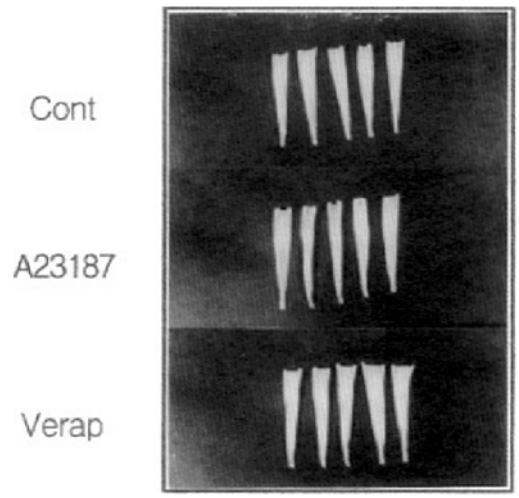

Fig. 4. Bioassay for the determination of Ca^{2+} effect on the inrolling of petal segments. A, The effect of 10^{-7} M A 23187, a specific Ca^{2+}-ionophore on the inrolling of petal segments induced by 5×10^{-6} M CEPA during 8 h incubation; B, The effect of 10^{-4} M verapamile, a Ca^{2+}-channel blocker on the inrolling of petal segments induced by 5×10^{-6} M CEPA during 8 h incubation.

we artificially altered the level of cytosolic Ca^{2+} and compared the degree of the inrolling of petals. As shown in Fig. 4, it was found that Ca^{2+}-ionophore, 10^{-7} M A23187, slightly stimulated the rolling of petals, whereas a Ca^{2+}-channel blocker, 10^{-4} M verapamil, inhibited the rolling of petals.

Endogenous Ethylene Production in Petals

We tested further whether the ethylene that induced the inrolling originated from the *de novo* synthesis in petals. For the purpose of elucidation of this question, some parameters indicating the *de novo* synthesis of ethylene such as the amount of endogenous ethylene production, ACC content, and a couple of enzyme activities that participate in biosynthesis were determined. As shown in Fig. 5A, the amount of endogenous ethylene in petals increased explosively in the inrolled stage in a climacteric manner. Unexpectedly, transient slight ethylene production occurred in the 60° stage. The content of ACC (Fig. 5B) and both the activities of ACC-syn-

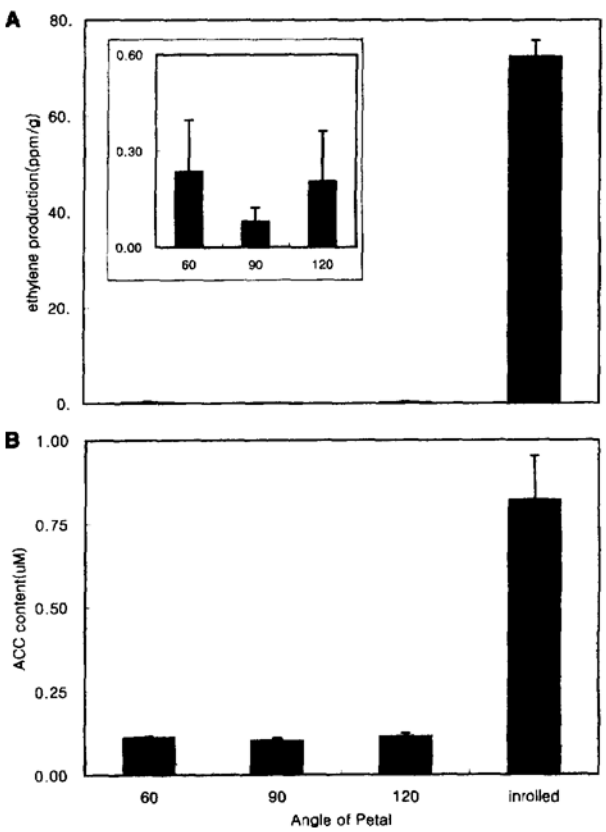

Fig. 5. The endogenous ethylene production (A) and the content of ACC (B) in the 60°, 90°, 120°, and inrolled stage of petal segments. Inset: Etylene production in the 60°, 90°, and 120° stage of petal segments is enlarged 65 times.

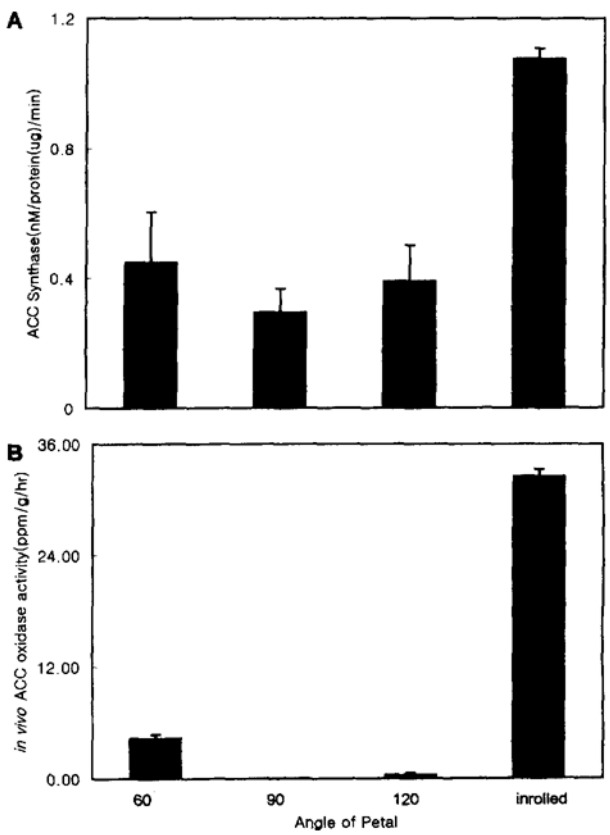

Fig. 6. Determination of the activities of ACC-synthase (A) and ACC-oxidase (B) in the 60°, 90°, 120°, and inrolled stage of petal segments.

thase (Fig. 6A) and ACC-oxidase (Fig. 6B) were promoted prominently in the inrolled stage. Even though low, the activities of both ACC-synthase and ACC-oxidase were also detected in the 60° stage. Therefore, the above data suggests that both the transient peak in the 60° stage and the burst of ethylene in the inrolled stage could resulted from *de novo* synthesis in petals.

Kinetics of Length of Adaxial and Abaxial Sides of Petal

Finally, we tried to address the mechanism of the petal rolling by ethylene. We analyzed the variation of length of each side of the petal segment's cut surface. Before the kinetics studies, we tested whether petal segments set on a sponge in a glass jar (Fig.

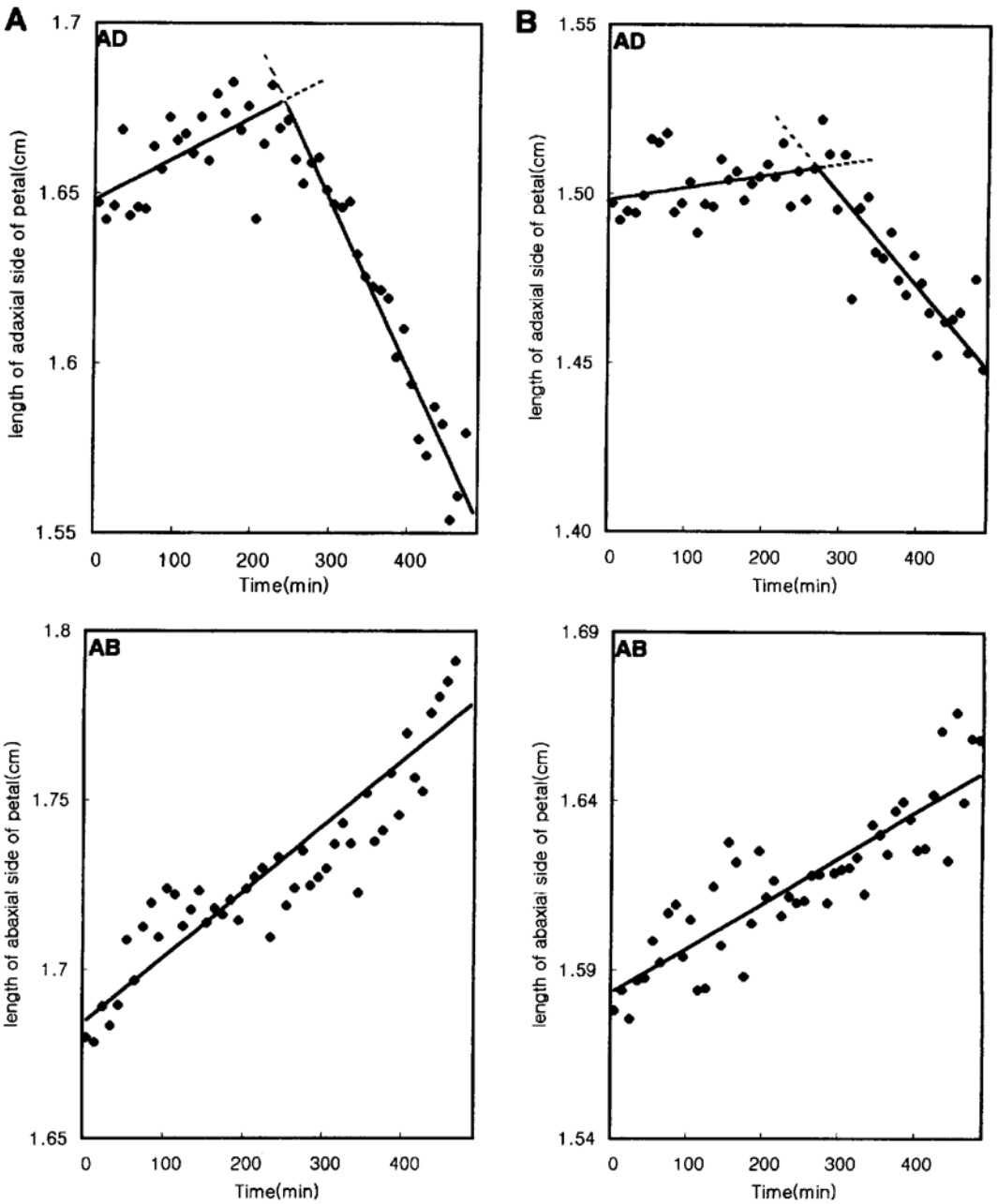

Fig. 7. The length kinetics curve of the adaxial (AD) and the abaxial side (AB) of petal segment's cut surface in 90° stage. A, The petal segment was treated by 5×10^{-6} M CEPA; B, The petal segment was treated by 5×10^{-6} M CEPA and 10^{-4} M spermidine at the same time.

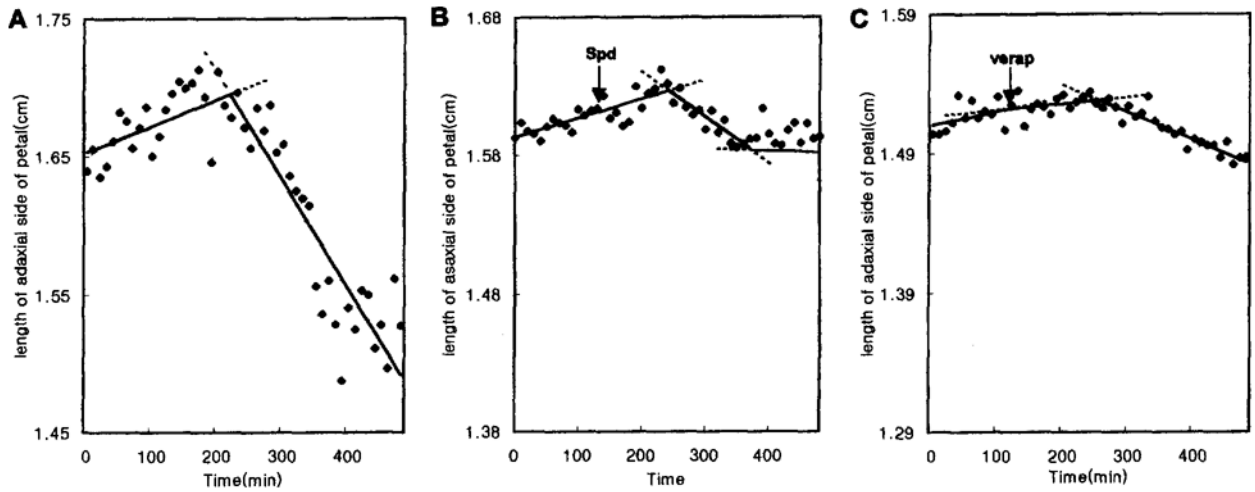

Fig. 8. The length kinetics curve of the adaxial side of petal segment's cut surface in 90° stage pretreated by 5×10^{-7} M n-octanoic acid for 5 h. A, control; B, treatment of 10^{-4} M spermidine at 150 min after incubation indicated as arrow; C, treatment of 10^{-4} M verapamile at 150 min after incubation indicated as arrow.

1) rolled due to the decrease of turgor pressure according to water deficiency during the incubation time or not. However, the inrolling was not detected during 13 h long incubation without the treatment of CEPA (data not shown). According to the data shown in Fig. 7A-AD, the kinetics curve consisted of two different phases. The length of the adaxial side of the cut surface increased at the rate of 120×10^{-6} cm/min in the first phase, which extended up to near 250 min, and then shrunk rapidly at the rate of -497×10^{-6} cm/min in the second phase by 5×10^{-6} M CEPA. Ultimately, total length of the adaxial side of petals shrunk during incubation as compared to that at the beginning stage. In contrast, the length of the abaxial side of petals expanded steadily at the rate of 194×10^{-6} cm/min (Fig. 7A-AB). Therefore, the expansion in abaxial side and the shrinkage in adaxial side could cause petals to roll inwardly physically. Further, we examined how polyamines inhibit the rolling of petals as shown in Fig. 3A. The detected kinetics curve consisting of two distinct phases was altered by the treatment of 10^{-4} M spermidine, one of the polyamines (Fig. 7B). In this cas, the rate of the expansion of abaxial side was altered slightly to at the rate of 133×10^{-6} cm/min as shown in Fig. 7A-AB. However, the rate of expansion (36×10^{-6} cm/min) and shrinkage (-27×10^{-6} cm/min) in each phase composing the kinetics curve of the adaxial side was prominently reduced (Fig. 7B). Therefore, polyamine caused only a slight difference in length between both sides of the petal's cut surface and ultimately inhibited the rolling of petals. The effect of short-chain saturated fatty acids known to increase ethylene sensitivity (Whitehead and Vasiljevic, 1993) on the rolling of petals was also examined (Fig. 8A). After incubation of 5×10^{-7} M *n*-octanoic acid for 5 h, the expansion rate in the adaxial side detected in the first phase increased to 192×10^{-6} cm/min. The rate of shrinkage in the same side detected in the second phase was increased to -780×10^{-6} cm/min. In this case, the rate of expansion in the abaxial side was also not significantly changed (data not shown). Therefore, we focused on investigating the alteration of the kinetics curve only in the adaxial side of the petals. While the rate of expansion in the first phase was slightly reduced to 138×10^{-6} cm/min by the treatment of polyamine as shown in Fig. 8B, the later part of second phase was altered extremely. In this case, the first phase of the kinetics curve continued until almost 250 min, as shown in Fig. 7. In contrast, the blocking effect of Ca^{2+}-influx into cytosol by 10^{-4} M verapamile appeared quickly and altered the kinetic curve of the later part of the first phase extremely (Fig. 8C). Though the pattern of the first part of the kinetic curve was altered, the transit point to enter the second phase of the kinetic curve was nearly 250 min, and the rate of shrinkage that occurred in the second phase was also reduced to -200×10^{-6} cm/min.

DISCCUSION

We elucidated in this work that the inrolling of petals of cut carnations of cv. Shinkibo could be ultimately

caused by asymmetric responses during the growth and consecutive senescence process of the abaxial and adaxial side of petals (Fig. 2 and Fig. 7).

In cut carnations of cv. White Shim, short-chain saturated fatty acids in chain lengths ranging from C 7 to C10 (e. g. *n*-octanoic acid used in this work) were supposed as 'the sensitivity factor' (Whitehead and Vasiljevic, 1993). These are known to be responsible for the increase of tissue sensitivity to ethylene during senescence via increasing the ability of the tissue to bind ethylene (Whitehead and Vasiljevic, 1993). As shown in Fig. 7 and Fig. 8, while the kinetics curve of the adaxial side of cut surface became steeper by *n*-octanoic acid, the slope of the abaxial side was not significantly altered (data not shown), as like the result by polyamine (Fig. 7). So, we suppose that the adaxial side of petals could be more sensitive to ethylene than the abaxial side. This asymmetry of tissue sensitivity to ethylene in the same petals could be attributed to the difference in the amount of ethylene receptors in each side that trigger the signal transduction pathway.

Unfortunately, how the adaxial side of carnation petals in the second phase of kinetic curve (Fig. 8A) was shrunken by ethylene is not yet understood. However, there seems to be a clue to explain this phenomenon. A loss of membrane integrity is a well known characteristic observed during the senescence of plant tissues (Thompson, 1988), resulting from the enhancement of lipid peroxidation (Dhinsda *et al.*, 1982). When an abundant amount of ethylene is treated continually to the adaxial side of petals having higher ethylene-sensitivity, ethylene could trigger lipid peroxidation and cause the loss of membrane integrity. Then, the turgor pressure of cells located on the adaxial side could be reduced and subsequently result in the shrinkage of the cells. But, it remains to be explained how two distinct phases of growth kinetics occurred in the same tissues by the same ethylene action. Further research is needed to explain how the duration of the first phase continued to nearly 250 min (Fig. 7 and Fig. 8). In addition to an increase in the sensitivity to ethylene, a climacteric rise in ethylene is concomitant with senescence as described elsewhere in the introduction. According to the data shown in Fig. 5 and Fig. 6, climacteric ethylene production in unpollinated petals of carnations of cv. Shinkibo could be a result of *de novo* biosynthesis in petal segments. Particularly, it could be logically postulated that the existence of the first transient peak in ethylene production in petals suggest the possibilities of *de novo* biosynthesis in the 60° stage on the basis of the data shown in Fig. 5 and Fig. 6. Interestingly, recent data acquired in the co-author's laboratory confirmed this postulation by the fact that the transient ethylene production in cut roses is a prerequisite to the expansion of petals to anthesis (data not shown). From our data, we strongly suggest that a transient ethylene peak in the petal segments plays an important role in the outward expansion of the petals. This outward expansion phenomenon to anthesis could be explained by the first phase of the kinetics curve of adaxial side maintained until almost 250 min shown in Fig. 7. In carnations of cv. White Sim, the initial burst of ethylene production in styles has been determined to catalyze the later ethylene production in styles responsible for pollination-induced corolla senescence and ovary development (Jones and Woodson, 1997). However, it is not clear whether the observed burst of ethylene production in the in-rolled petals of carnations of cv. Shinkibo is caused by autocatalytic biosynthesis via preceding transient ethylene production in the same petals or via ethylene originated from other organs.

As shown in Fig. 2A, Fig. 3A, and Fig. 3B, the petal segment in the 90° stage, in which the ethylene production was at the null level, rolled inwardly more prominently than at any other stage of the petals. These phenomena could be caused by factors such as the physical condition of petals, i. e., degree of outward expansion or inward rolling, and the differences in tissue sensitivity to ethylene and/or in ethylene production according to the stage of the petals.

Polyamine and verapamile has different effects on the rolling of petal segments, especially, depending upon the specific phase of the growth kinetics curve of the adaxial side. While the effect of the inhibition of Ca^{2+}-influx into cytosol by verapamile appeared promptly, that of polyamine had a lag period of about 100 min (Fig. 8B and 8C). This differing effect of polyamines could be produced with a decrease in tissue sensitivity to bind ethylene via alteration of membrane structure. In addition, the possibility of the inhibition of lipid peroxidation by polyamine in the later phase of the growth kinetics curve as observed in oat leaves (Borrell *et al.*, 1997) is not exclusive. However, further study is needed to reveal whether the effect of spermidine was due only to its cationic nature, namely non-specific interaction with plasma membrane or plant cell wall at a physiological pH range, or not.

ACKNOWLEDGEMENT

This work was supported by a grant from KOSEF (No. 95-0401-05-01-3) to SEO.

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Received September 30, 1998
Accepted November 7, 1998