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Endogenous Levels of Abscisic Acid in Aging Carnation Flower Parts

Kathleen M. Hanley[1] and William J. Bramlage

Department of Plant and Soil Sciences, University of Massachusetts, Amherst, Massachusetts 01003, USA

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Abstract. Aging carnation flower parts were used to determine whether or not any correlation existed between the concentration of abscisic acid (ABA) and a predisposition of the tissue for ethylene synthesis. Levels of ABA were measured using an enzyme-linked immunosorbent assay (ELISA) following purification steps including prepacked silica gel columns. Increased ABA levels paralleled the increase of ethylene and the onset of irreversible wilting in the carnation petals. Neither the green tissue nor the receptacle showed any sign of wilting with the remainder of the flower parts, but increased ABA was detected in both tissues subsequent to, or coincident with, the ethylene climacteric peak in the senescing petals. An increase of ABA in both the styles and the ovary was detected in the preclimacteric flower, and did not appear to be triggered by the production of ethylene. Increased ABA in the gynoecium also did not result in the onset of ethylene production in the preclimacteric flower.

Accelerated ethylene evolution is involved in the senescence of carnation flower petals; its deleterious effects are related to an alteration of cell membrane composition and integrity (Thompson et al. 1982). However, the event(s) that triggers the onset of increased ethylene production is still unknown. Abscisic acid (ABA), as well as ethylene, is involved in promoting the physiological events of senescence (Mayak and Halevy 1981); however, the relationship between these two hormones in promoting flower senescence is not well understood. When exogenous ABA was administered to cut carnation flowers,

[1] Present address: University of Kentucky, Department of Agronomy, Lexington, Kentucky 40546, USA.

the ethylene climacteric rise and subsequent petal wilting were both advanced by two days (Ronen and Mayak 1981, Mayak and Dilley 1976). This response was prevented by treatment with aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis (Ronen and Mayak 1981), or by hypobaric ventilation of the ethylene produced (Mayak and Dilley 1976). Thus, it was concluded that exogenous ABA was stimulating the production of ethylene. In detached carnation petals an increase of endogenous ABA paralleled the ethylene climacteric rise and consequent petal wilting (Eze et al. 1986). Nowak and Veen (1982) detected a large increase of ABA in the carnation pistil and a small increase in the petals two days prior to the ethylene surge. When they pretreated these same flowers with silver thiosulfate complex (STS), an inhibitor of ethylene action (Veen 1979), the increase of ABA was completely prevented. Other studies have reported an inhibition of both cytokinin accumulation (Van Staden and Dimalla 1980) and gynoecium enlargement (Dimalla and Van Staden 1980) in senescing carnations pretreated with STS.

Immunological analysis is now a recommended method in the quantification of ABA and related compounds (Hirai 1986). The enzyme-linked immunosorbent assay (ELISA) is based on the competition between free ABA in the sample and an alkaline-phosphatase-labeled ABA tracer for a fixed number of high-affinity antibody binding sites (Weiler 1984).

In this study we attempted to determine whether or not any correlation exists between the concentration of ABA in the plant tissue and the ontogeny of the individual flower parts. Through the use of a sensitive monoclonal antibody (McAb), quantitative ABA measurements could be made on small tissue samples. In this way, we anticipated elucidation of a possible predisposition of tissues for ethylene synthesis.

Materials and Methods

Greenhouse-grown carnation flowers (*Dianthus caryophyllus* L. cv Scanea 3C), including 30 cm of stem, were harvested when outer petals first started to form a right angle to the stem and kept in continuous light at 22°C for 0–9 days. At each day, petals (five from the outer whorl) and green tissue (including the calyx, leaves, and 12 cm of stem) were excised, and fresh weights and ethylene production (petals) were recorded. Subsequently, the petals and green tissue were frozen at −20°C, lyophilized, and saved for ABA analysis. To evaluate the endogenous ABA levels in the remaining flower parts, each day the ovary, receptacle, and styles (including the stigmatic surface) were excised, fresh weights were recorded, and flower parts were frozen at −20°C.

Each sampling day excised petals were placed in 25 ml flasks that were sealed with rubber septa. After incubation in the light for one hour, ethylene was determined from a 1-ml gas sample withdrawn from the headspace using a 3-ml disposable syringe. Gas samples were injected into a Shimadzu GC-8A gas chromatograph equipped with an activated alumina column and flame ionization detector. Data were compiled using a Shimadzu GC integrator. Immediately after ethylene measurements, petals were frozen, lyophilized, and ground using a Wiley mill (20 mesh screen).

Generally, a preliminary purification of the tissue sample is advised as a precaution against substances in the crude extract interfering with the activity of the antibody (Pengelly 1977). However, in other studies the use of this antibody precluded the need for prior purification of the crude extract, and very similar levels of sensitivity were observed between the immunoassay technique and physicochemical methods of detection (Mertens et al. 1983, Walker-Simmons 1987). A modified version of Subbiah and Powell's (1987) method for ABA extraction and purification was utilized. At each sampling day frozen styles, ovaries, and receptacles were homogenized twice in 5 ml methanol:acetic acid:water (80:19:1, vol:vol) (including 20 mg/l BHT as an anti-oxidant), using a hand-held homogenizer. Lyophilized, 50 mg samples of petal and green tissue were extracted twice in 10 ml of extracting solvent at 4°C for 24 h. Homogenates and tissue extracts were centrifuged at 10,000 *g;* supernatants were combined and placed in 25-ml beakers. Crude extracts of petals, styles, and green tissue were each administered 25 μl (0.02 μCi/μl) [2-^{14}C]ABA (SA = 8 μCi/μM) (Amersham, Arlington Hts., IL, USA) for estimating recovery at each purification step. Methanol was evaporated from the supernatants in the dark under the strong draft of a hood. Aqueous extracts were placed in 20-ml plastic scintillation vials; pH was adjusted to 3.0 with 0.5 M acetic acid, and the extracts were partitioned three times against diethyl ether. Preliminary examination revealed the recovery of varying quantities of radiolabeled ABA tracer in the emulsion formed between the aqueous and organic fractions after partitioning. To dissipate this emulsion, after each partitioning step the vials were capped and centrifuged at 5000 *g* for 10 min. The organic fractions were collected and made to a final volume of 1:1, ether:hexane (vol/vol) (ET:HX). Disposable, prepacked, Baker-SPE 3 ml silica gel columns were preconditioned by first aspirating through 2 column volumes of methanol, followed by 2 column volumes of ET:HX. The ET:HX samples were loaded onto and aspirated through wetted, preconditioned columns, and were washed twice with 3 ml ET:HX. The ABA fraction was eluted four times with 0.5 ml methanol:acetonitrile (1:3, vol/vol) and the eluate was saved for quantification using ELISA. All solvents used in extraction and purification were HPLC grade.

The ABA was quantified using the Phytodetek-ABA monoclonal antibody kits (Idetek, San Bruno, CA, USA) following the general procedure of Weiler et al. (1981). The antibody was raised against S-(+)ABA attached to the carrier protein, bovine serum albumin (BSA) at the ketone group, resulting in an antibody that is highly specific for the free, physiologically active ABA (Quarrie et al. 1988). A stock solution of 1 mM (+/−)ABA (Sigma, St. Louis, MO, USA) standard was prepared in 100% methanol and diluted in Tris buffer, pH 7.5. Concentrations ranging from 0–2 pmol/100 μl and 100 pmol/100 μl (nonspecific binding) were used to form the standard curve. Tissue sample eluates were serial-diluted in Tris buffer pH 7.5, to 1/1000. Strips of flat-bottomed reaction wells coated with ABA McAB were placed in strip holder plates. To each reaction well, 100 μl of either diluted tissue sample or ABA standard was added. Following addition of the standards or samples, 100 μl ABA-alkaline phosphatase conjugate in Tris-buffered saline was added to each well. The holder plates were sealed and placed in the dark at 4°C for 3 h. After

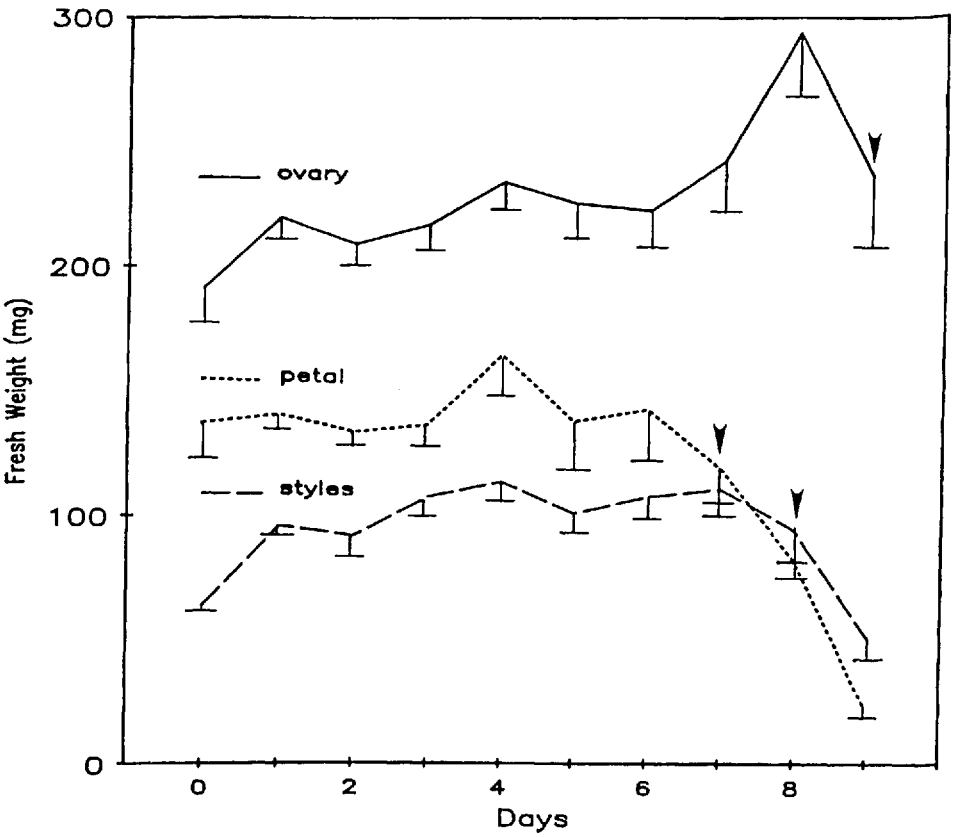

Fig. 1. Fresh weights of aging carnation flower parts. Arrows indicate first visible sign of wilting in each flower organ. Each value represents the mean (N = 3). SE bars are indicated.

incubation, solutions were decanted and wells were washed three times with a tween–saline solution. After washing, enzyme tracer activity was assayed by the addition of *p*-nitrophenyl phosphate substrate (200 µl) to each well. Substrate, wash solution, and enzyme tracer were each added using a four-channel micropipettor (Titertek, Flow Labs, Mclean, VA, USA). Holder plates were subsequently incubated at 37°C in a forced-air microtitration plate oven (Titertek) for 60 min. At the end of the incubation period, strips were removed and 1 drop of 1 N NaOH was immediately added to each well to stop the reaction. Activity in each well was read using a Titertek miniskan vertical path spectophotometer at 405 nm. All standards and samples were replicated three times.

Absorbance readings for the (+/−)ABA standards were converted to percent binding (B/B_0 %) and then logit transformed (Weiler et al. 1981), resulting in a linear standard curve. The racemic ABA used to form the standard curve contains 50% of each of the enantiomorphs, R and S-ABA. The monoclonal antibody is raised against S-(+)ABA, and does not bind to R-(−)ABA, allowing the use of the racemic ABA with no distortion of the standard curve (Mertens et al. 1983). All sample absorbance readings were converted to

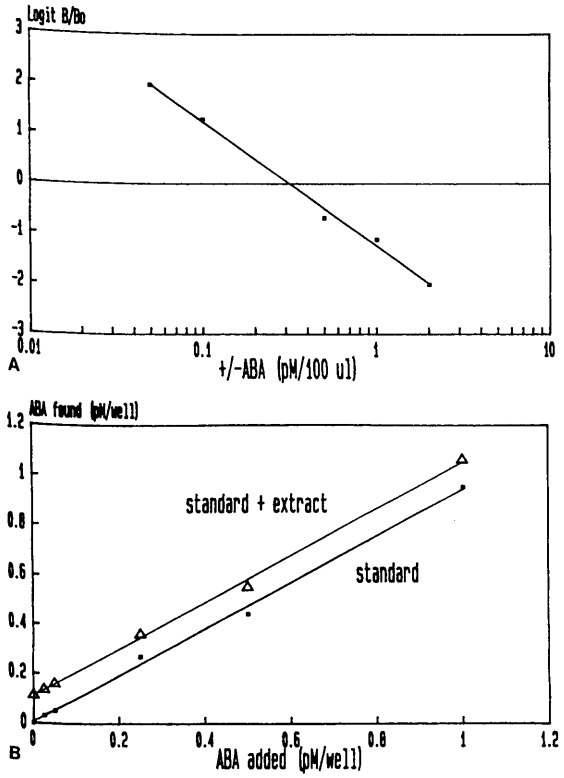

Fig. 2. ABA enzyme immunoassay (**A**) standard curve and (**B**) validation using purified petal extracts. Data values are the mean of two to three replications. Linear regression lines for data are indicated.

B/B_0 %, and logit transformed; ABA concentration was extrapolated from the linear regression line of the (+/−)ABA standard curve, and amounts were halved to equal only quantities of physiologically active S-(+)ABA. To determine whether there was any interference in the assay from the sample extracts, replicate samples of petal extracts were added to increasing concentrations of ABA standards (Walker-Simmons 1987). ABA standards alone and ABA standards plus extract were assayed for ABA content as outlined above, and contents plotted as ABA added versus ABA found. Any interfering substances in the petal extract should change the slope of the curve (Pengelly 1977). The degree of parallelism between lines was evaluated.

Results

At 22°C the fresh weight of the petals remained fairly constant through day 6 of postharvest, after which it decreased rapidly throughout the senescing period (Fig. 1). Petal wilting was recognizable at day 7. The fresh weight of the styles increased from days 0 to 4, remained fairly constant from days 4 to 7, and then decreased. Visible loss of turgidity was observed at day 8. Fresh weight of the ovary increased from days 6 to 8, and then decreased at day 9 as it lost turgidity. Apparently, neither the receptacle nor the green tissue senesced with the remainder of the flower, there being no significant decrease of fresh weight

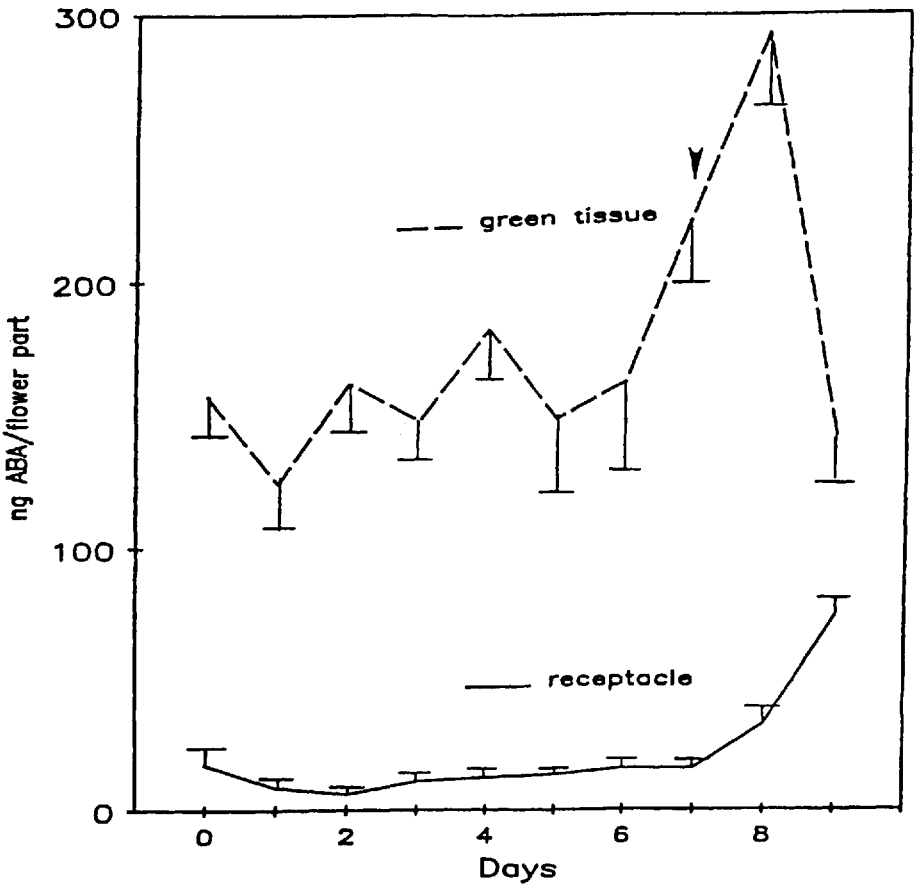

Fig. 3. ABA content of aging receptacle and green tissue parts. Arrow indicates the ethylene climacteric peak in the petals. Each value represents the mean (N = 3). SE bars are indicated.

or visible loss of turgidity of either part as the flower aged and senesced (data not shown).

When radiolabeled ABA was added to samples of styles, ovaries, and green tissue as an internal standard, there was no significant difference in percent recovery between any of the three plant parts. After partitioning, 89–91% of the label was recovered; and after silica gel chromatography 55–77% was recovered. At both steps, the differences among tissues were within the standard errors (N = 3).

A linear standard curve was constructed from data points for 0.05–2.0 pM per assay (+/−)ABA (Fig. 2A). The sensitivity of the assay is optimum between 0.02 and 5.0 pM (Phytodetek, personal communication). The addition of purified petal extract did not interfere with the activity of the immunoassay procedure. Plotting the ABA added vs. ABA found for both ABA standard and ABA standard + extract resulted in parallelism between lines (Fig. 2B).

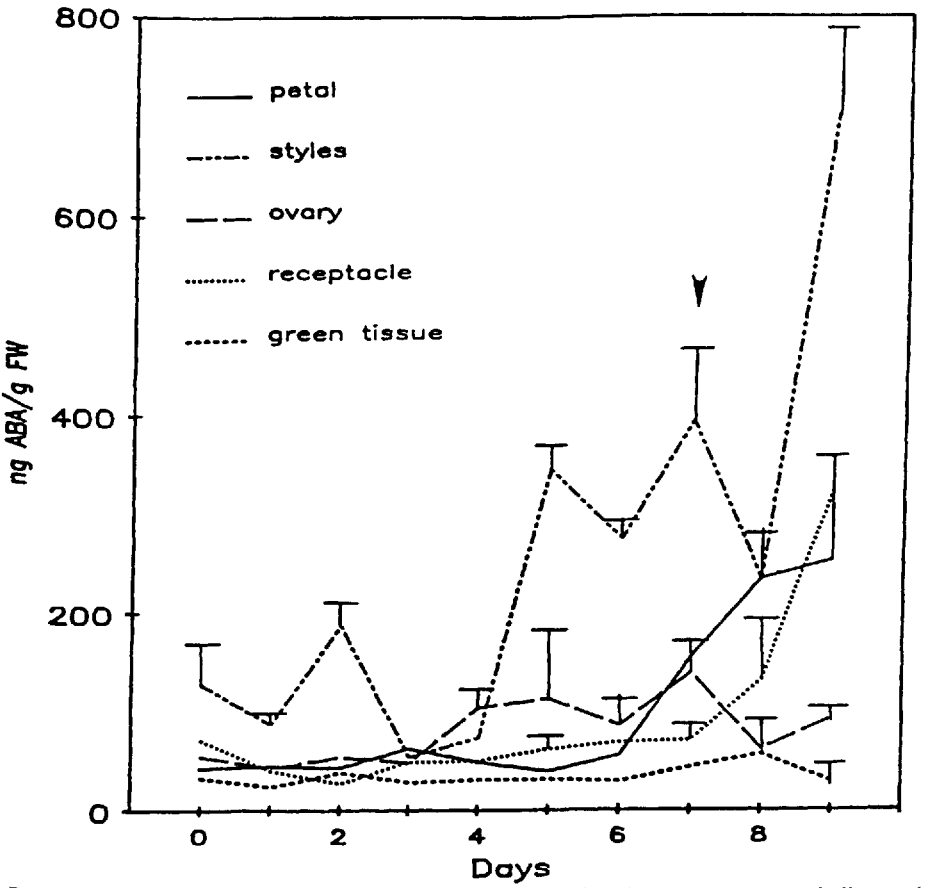

Fig. 4. ABA content per gram fresh weight on aging carnation flower parts. Arrow indicates the ethylene climacteric peak in the petals. Each value represents the mean (N = 3). SE bars are indicated.

A significant increase in the total activity of ABA per flower part in the green tissue and receptacle was detected after days 6 and 7, respectively (Fig. 3). Levels decreased substantially in the green tissue on day 9, while in the receptacle ABA continued to accumulate on day 9. When levels of ABA were calculated on a fresh weight basis, there was almost a sevenfold increase during days 0 to 9 in the receptacle, but mostly during days 7 to 9, versus less than a twofold increase in the green tissue during days 0 to 8 (Fig. 4). In the petals a small, transient increase of ABA was observed at days 3 and 4, followed by a large increase after day 5 (Fig. 5). The ethylene climacteric in the petals corresponded with the ABA increase after day 5 (Fig. 5). By day 9 both ABA levels and ethylene production had returned to presenescent levels.

A modest peak of ABA activity was detected in developing styles at day 2, with a decrease at day 3, and a subsequent increase after day 4 (Fig. 6). Levels of ABA in the ovary started increasing after day 3. Beginning at day 5, and

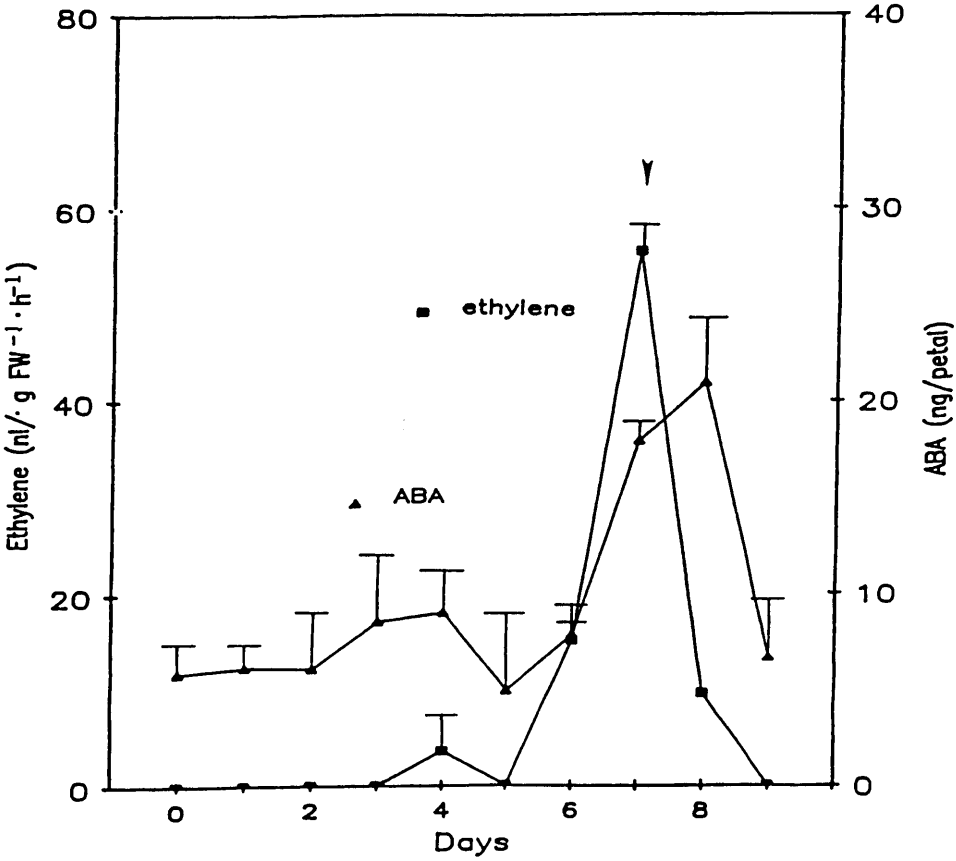

Fig. 5. The ethylene production and ABA content of aging carnation flower petals. Arrow indicates first visible sign of wilting. Each value represents the mean (N = 3). SE bars are indicated.

continuing through day 9, the levels of ABA in both the styles and the ovary paralleled each other, with large peaks observed at day 7 and high levels remaining at day 9. The styles contained the highest amount of ABA of any flower part when calculated on a fresh weight basis, at approximately 700 ng/g fr wt (Fig. 4).

Discussion

From the time a carnation flower first opens to when it ultimately senesces, many morphological and biochemical changes occur. The morphological changes include an elongation of the styles, enlargement of the petals (Manning 1981), and swelling of the ovary (Mor et al. 1980). Without fertilization these organs will die, leaving behind the chlorophyllous parts of the flower

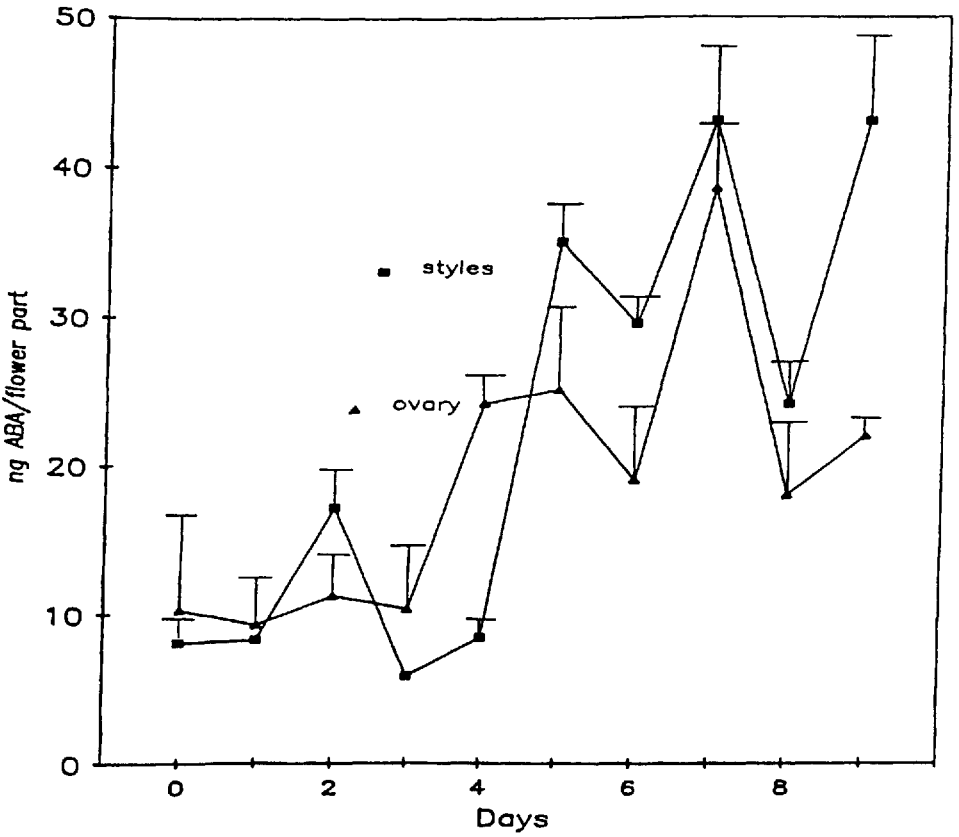

Fig. 6. The ABA content of aging carnation flower styles and ovaries. Each value represents the mean (N = 3). SE bars are indicated.

seemingly unaffected. Carnation petal wilting, coincident with the ethylene climacteric, is very distinct and reproducible.

Abscisic acid is thought to play a major role in the regulation of flower senescence (Mayak and Halevy 1981). Previous studies have tried to correlate the endogenous levels of ABA and the increase of ethylene production (Eze et al. 1986, Harris and Dugger 1986, Nowak and Veen 1982). The synchronous behavior of these two hormones in senescing tissue has made it very difficult to assess their roles with the physiological event. In this study we used an enzyme immunoassay that enabled us to quantify ABA levels in small amounts of tissue. In previous studies ABA in aging carnation flowers was quantified using electron capture-gas liquid chromatography (EC-GLC) following methylation (Eze et al. 1986, Nowak and Veen 1982). In this investigation a small, temporary increase of ABA was noted in the preclimacteric petals (Fig. 5). Eze et al. (1986) did not detect an increase of ABA in aging carnation petals until the onset of petal wilting, when large amounts of ABA were being produced. It appears that the immunoassay may be a more appropriate means of quantification due the level of sensitivity of the assay.

In this investigation, levels of ABA in both the petals and the green tissue paralleled the ethylene climacteric rise in the petals (Figs. 3 and 5). A similar coupling of ABA and ethylene levels is evident in some ripening fruit, and it is thought that increased ABA content may be involved in the ripening process once ripening is initiated (Rhodes 1981). This involvement may be related to the de novo synthesis of degradative enzymes induced by ABA (Ho 1983). A similar mechanism may be involved in flower senescence. The synthesis of degradative enzymes, including ribonuclease, deoxynuclease, and ATPase, has been detected in various flower types with the onset of senescence (Adam et al. 1983, Matile and Winkenbach 1971).

The ABA content in the receptacle also increased with petal wilting, but not until after the rise in ethylene (Figs. 3 and 5). An increase in ABA content is frequently associated with water stress in the tissue (Davies and Mansfield 1983). Although neither the green tissue nor the receptacle seemed to be under water stress, they both appeared to be influenced by wilting in the petals, which began at day 7. The styles and the ovary began wilting at days 8 and 9, respectively (Fig. 1). Given the anatomical proximity of the receptacle to the other parts of the flower, perhaps it continues to accumulate ABA longer than the green tissue in response to wilting in the styles and ovary, as well as the petals.

If ABA content is to be used as an indicator of ontogeny of carnation flower parts, it is difficult to speculate what role increased levels are having in the presenescent flower. In both the styles and the ovary an initial peak of ABA was observed in the young, developing flower, with a subsequent peak occurring coincident with petal wilting (Figs. 5 and 6). A similar increase of ABA was observed in both preclimacteric carnation flower pistils (Nowak and Veen 1982) and developing citrus flower styles (Goldschmidt 1980). Although ABA is frequently regarded as a deleterious plant hormone, it is also involved in many physiologically promotive events (Ho 1983). The involvement of ABA in regulating assimilate partitioning (Tietz et al. 1981) has been proposed as a possible explanation for increased ABA in the developing gynoecium (Nowak and Veen 1982). In our investigation, however, the increase of fresh weight in the styles occurred prior to the ABA increase (Figs. 1 and 6). In the ovary, an increase of fresh weight was not apparent until several days subsequent to the initial ABA increase (Figs. 1 and 6). The ABA produced in the ovary with the onset of senescence may be involved in assimilate partitioning, as a substantial increase of fresh weight was observed at day 8 (Fig. 1).

In this investigation ethylene production in the petals peaked at seven days after opening (Fig. 5). Previous studies have shown synchronous ethylene climacteric peaks in all the individual parts of the carnation flower (Nichols 1977). The increase of ABA in the styles and ovary, prior to any increase of ethylene, indicates a separation of the relationship between these two hormones. This separation may be related to the pattern of senescence in pollinated and unpollinated carnation flowers. Unpollinated, fully open carnation flowers begin to senesce five to seven days after harvest (Nichols 1977), while wilting in pollinated flowers commences in two to three days (Nichols 1977; Nichols et al. 1983). Pollination-induced senescence is mediated by an increase in ethylene production at this time. Manning (1981) found substantial ethylene-

forming enzyme activity in young, developing carnation flower styles. It has been suggested that the style may be the triggering organ for pollination-induced flower senescence (Goldschmidt 1980). Given the relationship between ABA and ethylene in senescing flower tissue (Mayak and Halevy 1981), the increased production of ABA found in the developing gynoecium may be a necessary component for the onset of pollination-induced senescence.

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