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Aminoethoxyvinylglycine (AVG) reduces ethylene and protein biosynthesis in excised discs of mature-green tomato pericarp tissue

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Abstract

Application of an aqueous solution of aminoethoxyvinylglycine (AVG) ([S]-*trans*-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride) to the locular surface of excised 1 cm diameter × 4 mm thick pericarp discs of pre-climacteric, mature-green tomato (*Lycopersicon esculentum* Mill., cv. Castlemart) fruit significantly reduced both ethylene and protein biosynthesis in a log-linear fashion. Exposure to 1.0 μL L^{-1} 1-MCP (SmartFresh™) increased ethylene production by about 30% at each AVG concentration. Incorporation of H^3-leucine into protein in tomato pericarp discs was reduced 65%, 76%, and 93% by the application of 20 μL of 0.1, 3.0, and 10 mM AVG, respectively. In comparison, ethylene production was reduced 57%, 73%, and 89% by 20 μL 0.1, 3.0, and 10 mM AVG, respectively. Application of similar AVG concentrations had no significant effect on CO_2 production by the tissue. A tissue concentration of 6 μM AVG (16-fold dilution of the 0.1 mM applied concentration: 20 μL in 0.3 g of tissue) significantly reduced both ethylene and protein biosynthesis. The ability of AVG to reduce ethylene production was highly correlated (R^2 = 0.98) to its ability to reduce protein synthesis in both air and 1-MCP treated pre-climacteric tomato fruit tissue. Some of the physiological effect of AVG may be dependent on it ability to alter protein synthesis.
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1. Introduction

The mode of action by which an inhibitor of ethylene synthesis (e.g., AVG; [S]-*trans*-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride) reduces the ripening of climacteric fruit is readily apparent since elevated internal levels of ethylene, produced by autocatalytic ethylene synthesis, are necessary for ripening of these fruit (Burg et al., 1971; Abeles et al., 1992; Saltveit, 1999). However, its mode of action in reducing the rate of ripening and maintaining the quality of vegetative and non-climacteric fruit tissue is less obvious. While ethylene production in climac-

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teric fruit is promoted by the endogenous concentration of ethylene through positive feedback and increases dramatically during ripening, the feedback of ethylene on ethylene production in vegetative, non-climacteric, and pre-climacteric fruit tissue is negative (Abeles et al., 1992; Saltveit et al., 1998). In these tissues, ethylene actually inhibits ethylene synthesis. Apart from transient increases in ethylene production associated with the traumas of harvesting or processing for fresh-cut, endogenous ethylene levels are maintained at low levels by negative feedback, and endogenously produced ethylene probably has minimal effect on the postharvest quality of vegetables and non-climacteric fruit.

Exogenously applied ethylene has a significant effect on reducing the postharvest life of both climacteric and non-climacteric commodities (Abeles et al., 1992; Saltveit, 1999). The postharvest life of a number of climacteric and non-climacteric fruits and vegetables was prolonged by ventilating them with air containing $<0.005\ \mu L\ L^{-1}$ ethylene compared to air containing $0.1\ \mu L\ L^{-1}$ ethylene (Wills et al., 1999). Inhibiting ethylene perception with 1-methylcyclopropane (1-MCP), reduced ethylene induced browning in lettuce exposed to $0.1\ \mu L\ L^{-1}$ ethylene in air (Wills et al., 2002), but it did not reduce wound-induced browning (Saltveit, in press).

AVG is frequently used as a specific inhibitor of ethylene biosynthesis to determine the affects of ethylene on plant growth, development, and response to stress (Abeles et al., 1992). AVG has been used to study the participation of ethylene synthesis in bud break (Pereira-Netto, 2001), dry matter partitioning in rice (Mohapatra et al., 2000), fruit ripening (Clayton et al., 2000; Wang and Dilley, 2001), fungal pathogenesis (Robison et al., 2001), nodulation in legumes (Mann et al., 2001; Spronsen et al., 2001) and response to chilling stress (Hong and Gross, 2000). Effective tissue concentrations of AVG are hard to determine from published studies since the methods of application include foliar sprays ($124\ g\ ha^{-1}$), soil drenches (5–20 μM), and sprays or dips of excised tissue ($1\ g\ l^{-1}$).

Like many other inhibitors, AVG may affect more metabolic pathways than those attributed to its mode of action (i.e., inhibition of ethylene biosynthesis). For example, stress-induced ethylene production is involved in petiole epinasty of potted poinsettia plants (Saltveit et al., 1979; Saltveit and Larson, 1981). Foliar application of silver, an inhibitor of ethylene action, reduced epinasty while doubling the rate of ethylene production. Application of foliar sprays of AVG (10 mM) also reduced stress-induced petiole epinasty and ethylene production to control levels (Saltveit and Larson, 1981). Although foliar sprays of cycloheximide (CHX; 20 μM), an inhibitor of protein synthesis, promoted ethylene production, they also reduced epinasty and protein synthesis (Saltveit and Larson, 1983). Likewise, application of 100 mM AVG not only reduced ethylene production and epinasty to control levels, but also reduced protein synthesis to the level in CHX-treated plants.

One possible mode of action for AVG could be through its effect on the synthesis of functional proteins. Many proteins are synthesized during the ripening of non-climacteric fruit and during the senescence of vegetables (Grierson, 1984). If the synthesis of a significant portion of these proteins is altered, the rate of ripening and senescence could be significantly affected.

Ethylene perception and action are reduced by exposure to 1-MCP (Sisler and Lallu, 1994; Serek et al., 1995; Sisler and Serek, 1997). Development of the ethylene-induced disorder, russet spotting, was delayed in iceberg lettuce leaves and the storage life of shredded iceberg lettuce was increased by exposure to 1-MCP (Fan and Mattheis, 2000; Wills et al., 2002). Ripening of tomato fruit is controlled by an increase in ethylene production at the onset of ripening (Lelièvre et al., 1997). Exposure to 1-MCP delayed color development, softening, and the ethylene climacteric in fruit harvested at the mature-green and orange stage of ripeness (Sisler and Blankenship, 1993; Sisler and Lallu, 1994; Hoeberichts et al., 2002).

Research reported in this paper was undertaken to investigate the effect of AVG on protein and ethylene biosynthesis and on the rate of CO_2 production by pre-climacteric, mature-green tomato fruit tissue. Wounding, as well as ripening, induces protein synthesis, and some wound responses appear to act through wound-induced ethylene synthesis. Ethylene action was inhibited with 1-MCP so that the effect of AVG on protein synthesis could be separated from the effect of AVG on ethylene synthesis, and thereby on the synthesis of proteins induced by wound- and ripening-induced ethylene synthesis.

2. Materials and methods

2.1. Plant material

Mature-green tomato (*Lycopersicon esculentum* Mill., cv. Castlemart) fruit were hand-harvested from plots grown under standard cultural practices at the Department of Vegetable Crops Farm Facility, University of California, Davis (UCD). The fruit were quickly transported to the Mann Laboratory at UCD where they were washed in a 1:20 dilution of commercial bleach, and air-dried in a laminar transfer hood. Using a cork borer, 1 cm diameter discs of pericarp tissue were excised from the equatorial portion of the fruit and trimmed of adhering locular material to produce 4 mm thick disks. Each disc weighed 0.40 ± 0.02 g. Fruit were visually inspected at this point to ascertain if they were at the MG-1 stage of maturity that precedes the onset of the climacteric in respiration and ethylene production. Only pericarp discs from MG-1 fruit were used. The discs were washed twice in sterile, de-ionized water, blotted dry and eight discs were put epidermis surface down into disposable 100 mm × 20 mm plastic Petri dishes. The dishes were placed in plastic tubs lined with moist paper towels, loosely covered with aluminum foil and held overnight (ca. 16 h) in a humid, ethylene-free atmosphere at 12 °C.

2.2. Application of treatments

Uncovered tubs containing dishes of excised pericarp discs were put into a 117 L opaque plastic container. A concentration of 0.0 or 1.0 μL L^{-1} 1-MCP (SmartFresh™) was established in the container following instruction for use of the SmartFresh™ tablets provided by AgroFresh Inc. (Spring House, PA, USA). The treatment was continued overnight (ca. 16 h) in a humid, ethylene-free atmosphere at 20 °C.

The day after excision, 20 μL of an aqueous AVG solution was applied to the cut, locular surface of each of the pericarp discs per dish, and the discs held for an additional 12 h under the same condition as before. Then, 10 μL of uniformly labeled H^3-leucine was applied to four discs in each dish, and the dishes held under the same conditions for four additional hours.

2.3. Carbon dioxide and ethylene production

Carbon dioxide and C_2H_4 production was measured using four tomato discs. The tissue was enclosed in a 10 mL glass syringe set to 6 mL and closed with a rubber stopper. After 30 min, 1 mL gas samples were withdrawn with a 1 mL plastic syringe. Some of the samples were injected into an infrared gas analyzer and the concentration of CO_2 calculated by comparing the peak height of the sample with that produced by injection of a 0.5% CO_2 gas standard (Saltveit and Strike, 1989). The other gas samples were injected into a gas chromatograph equipped with a flame-ionization detector (Saltveit and Yang, 1987). Ethylene concentration was calculated with a peak integrator calibrated to a 1.1 μL L^{-1} ethylene in air standard.

2.4. Protein determination

The four tissue discs per treatment were ground in 2.0 mL *tris*-Mes buffer (pH 7.5), centrifuged for 5 min at 12,000 × *g* and the clear supernatant collected. The pellet was washed twice with another 0.5 mL of buffer at 20 °C and the supernatants combined. Then 50 μL of bovine serum albumin was added to 150 μL of the supernatant, the tube vigorously shaken, and 0.8 mL of acetone (0 °C) was added to precipitate the proteins. After being held overnight at −20 °C to permit complete precipitation of the proteins, the solution was centrifuged at 12,000 × *g* in an Eppendorf table top centrifuge for 5 min and the pellet collected. The pellet was dissolved in 0.5 mL of 0.1N NaOH and transferred to a scintillation vial. A xylene-surfactant liquid scintillation cocktail was added to the vials and the radioactivity measured by liquid scintillation spectroscopy.

2.5. Statistical analysis

Each experiment had at least three replicates of each treatment and all experiments were run at least twice with similar results. Measurements from all the replicates were combined and subjected to statistical analysis.

3. Results and discussion

3.1. Carbon dioxide and ethylene production

Application of aqueous solutions of AVG to the locular surface of excised tomato pericarp discs slightly stimulated the rate of CO_2 production at the higher

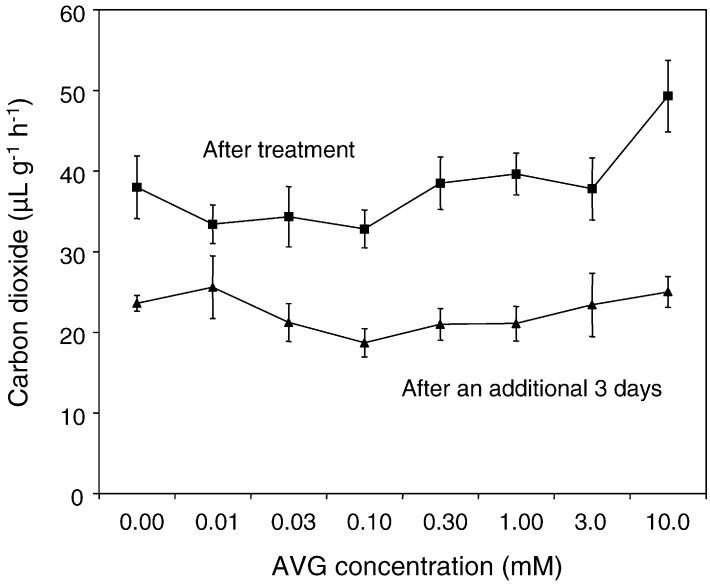

Fig. 1. Carbon dioxide production by 1 cm diameter × 4 mm thick discs of pre-climacteric mature-green tomato fruit pericarp tissue. The discs were treated with 20 μL of an aqueous AVG solution 16 h after excision, and CO_2 production was measured after an additional 12 h and after three additional days at 20 °C. Vertical bars at each data point represent the standard deviation about that mean.

AVG concentrations (Fig. 1). However, variability among the replicates and among the responses of the tissue to the various AVG concentrations rendered differences among the concentrations non-significant. Production of CO_2 was significantly higher when measured 12 h after AVG application than when measured after an additional 3 days at 20 °C for all concentrations.

In contrast to its effect on CO_2 production, application of AVG had a significant and concentration-dependent effect on ethylene evolution by pericarp discs (Fig. 2). The significant effect of AVG on ethylene production was expected since AVG is known to inhibit ethylene synthesis in other plant tissues (Halder-Doll and Bangerth, 1987; Abeles et al., 1992). The relation between AVG concentration and ethylene production in air had an R^2 of 0.95. Application of 20 μL of 0.01, 0.1, 1.0, and 10.0 mM AVG solutions reduced ethylene production by 14%, 42%, 73%, and 85%, respectively.

Exposure to 1-MCP increased ethylene production by about 30% at every AVG concentration (Fig. 2). The relation between AVG concentration and ethylene production in tissue treated with 1-MCP had an R^2 of 0.98. Application of 20 μL of 0.01, 0.1, 1.0, and 10.0 mM AVG solutions to the 1-MCP treated tissue reduced ethylene production by 17%, 56%, 71%, and 83%, respectively.

The regression lines for ethylene production versus AVG concentration for tissue exposed to either air or to 1-MCP were of the same form and roughly parallel (Fig. 2). The increase in ethylene production from 1-MCP treated pre-climacteric tissue is consistent with a negative feedback of ethylene on ethylene synthesis in non-climacteric tissue (Abeles et al., 1992). While AVG reduced the capacity of treated tissue to synthesize ethylene, it apparently did not alter the tissue's feedback control over ethylene synthesis.

If we assume that the applied AVG was equally distributed throughout the pericarp discs, the 0.3 mL volume of the discs would have diluted the AVG about 16-fold and produced concentrations of 6, 62 and 625 μM for the applied 0.1, 1.0 and 10 mM solutions, respectively. Applications of AVG that would result in these low concentrations in whole tomato fruit tissue should significantly reduce ethylene production and the rate of ripening. However, since tomato fruit are enclosed in a very impermeable cuticle and epidermis,

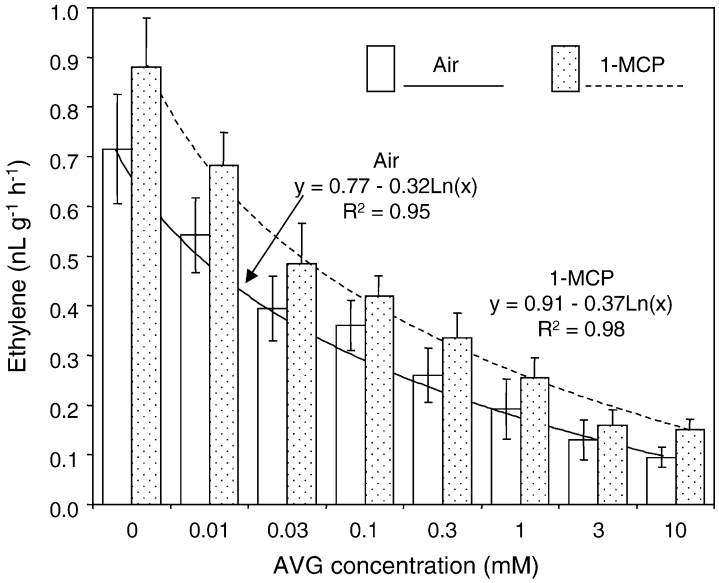

Fig. 2. Ethylene production by 1 cm diameter × 4 mm thick discs of pre-climacteric mature-green tomato fruit pericarp tissue. Discs were exposed to 0.0 or 1.0 μL L^{-1} 1-MCP (SmartFresh™) during holding at 20 °C for 16 h. Discs were then treated with 20 μL of an aqueous AVG solution (0–10 mM), and C_2H_4 production measured after an additional 12 h at 20 °C. The vertical line atop each bar represents the standard deviation about that mean.

it may be difficult to get enough AVG into the fruit to have a significant effect. Spray applications of 10 mM aqueous AVG solutions to leaves and petioles were successful in reducing petiole epinasty in poinsettia plants (Saltveit and Larson, 1983). However, absorption and translocation from the leaf blade to the petiole may be easier than from leaves to ripening tomato fruit.

3.2. Protein synthesis

The incorporation of the radio-labeled amino acid leucine into protein was significantly reduced as the concentration of AVG applied to the excised discs of MG-1 pericarp tissue increased (Fig. 3). Similar rates of incorporation occurred with discs exposed to air or 1-MCP at each AVG concentration. The relation between AVG concentration and incorporation of label in discs exposed to either air or 1-MCP had an R^2 of 0.98 (Fig. 3). Application of 20 μL of 0.01, 0.1, 1.0, and 10.0 mM AVG solutions produced an inhibition of 45%, 64%, 81%, and 90%, respectively, while it was 35%, 64%, 85%, and 85%, respectively, for tissue exposed to 1-MCP. The degree of protein synthesis inhibition was roughly the same whether the tissue was exposed to air or 1-MCP. The degree of inhibition was also similar to the inhibition of ethylene synthesis in tissue treated with 0.1–10.0 mM concentrations of AVG (Fig. 2).

Ripening of mature-green tomato fruit is commercially stimulated by exogenously applied ethylene, while inhibiting either ethylene biosynthesis or action delays tomato fruit ripening (Burg and Burg, 1965; Saltveit et al., 1978; Abeles et al., 1992). Application of AVG to excised tomato tissue inhibited ethylene production (Hong and Gross, 2000) and delayed the increase in lycopene biosynthesis by 6 days (Edwards et al., 1983). In discs treated with 20 μL of an aqueous 10 mM AVG solution, the application of 10 μL L^{-1} ethylene promoted lycopene synthesis, but did not stimulate ethylene synthesis (Edwards et al., 1983). Although lycopene synthesis was stimulated, lycopene levels in the AVG-treated discs never attained the levels found in controls. Similar effects have been reported on the ripening of 'Barlett' pear fruit with applied AVG and its reversal with applied ethylene (Ness and Romani, 1980).

In both tomato and pear fruit it appears that the principle action of AVG on reducing ripening is on reducing

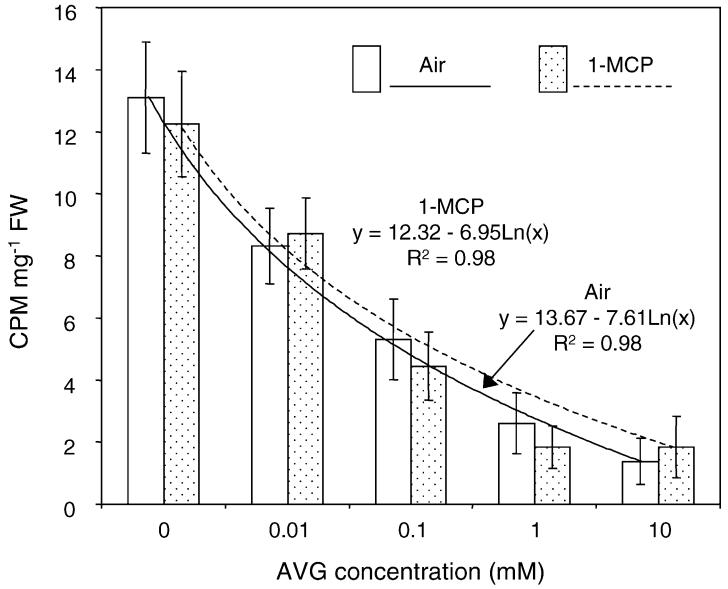

Fig. 3. Incorporation of radio-labeled amino acid leucine into protein by excised 1 cm diameter × 4 mm thick discs of pre-climacteric mature-green tomato fruit pericarp tissue. Discs were exposed to 0.0 or 1.0 μL L^{-1} 1-MCP (SmartFresh™) during holding at 20 °C for 16 h. Discs were then treated with 20 μL of an aqueous AVG solution (0–10 mM), 16 h after excision, held for 12 h, treated with 10 μL of H^3-leucine, and proteins extracted after 4 h at 20 °C. The vertical line atop each bar represents the standard deviation about that mean.

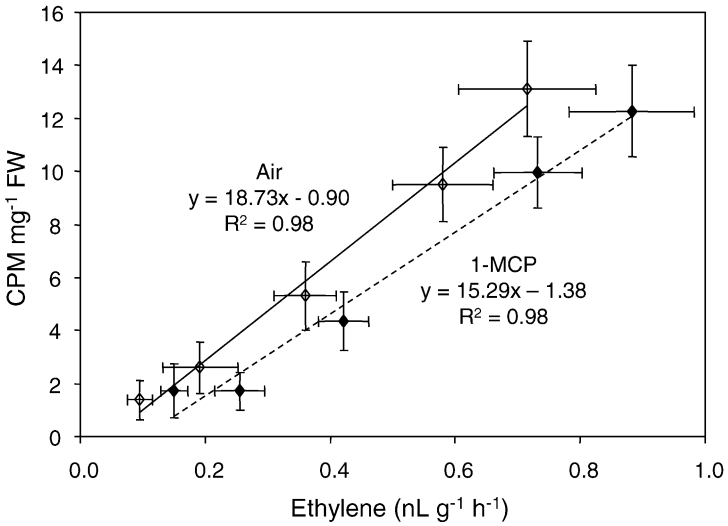

Fig. 4. Relationship between ethylene production and incorporation of radio-labeled amino acid leucine into protein by excised 1 cm diameter × 4 mm thick discs of pre-climacteric mature-green tomato fruit pericarp tissue. Discs were exposed to 0.0 or 1.0 μL L^{-1} 1-MCP (SmartFresh™) during holding at 20 °C for 16 h. Discs were then treated with 20 μL of an aqueous AVG solution (0–10 mM). Ethylene production was measured after an additional 12 h at 20 °C, or, treated with 10 μL of H^3-leucine for 12 h, and proteins extracted after 4 h at 20 °C. The vertical and horizontal lines represent the standard deviation about that mean.

ethylene synthesis. However, that part of the inhibition by AVG that was not overcome by exogenously applied ethylene could have resulted from reduced protein synthesis. In non-climacteric tissues and pre-climacteric tissues that are not producing physiologically active levels of ethylene, the ability of AVG to reduce protein synthesis may be a significant factor in its mode of action.

The ability of AVG to reduce ethylene production was highly correlated ($R^2 = 0.98$) to its ability to reduce protein synthesis in both air and 1-MCP treated pre-climacteric tissue (Fig. 4). When ethylene production was compared to protein synthesis at each AVG concentration, the logarithmic association between AVG concentrations and either ethylene production or protein synthesis was replaced by a linear relation between ethylene production and protein synthesis. For air or 1-MCP treated tissue, these relationships had an R^2 of 0.98 (Fig. 4). Enhanced ethylene production by 1-MCP treated tissue accounts for the comparatively uniform displacement of each 1-MCP data point from that of its comparable air data point at each AVG concentration. The standard deviation of most of the air and 1-MCP pairs of data points overlap.

4. Conclusion

Results from this series of experiments with excised pericarp discs of pre-climacteric, MG-1 tomato fruit showed that a tissue concentration as low as 5 μM AVG was active in reducing both ethylene and protein biosynthesis. Part of the physiological effect of AVG, especially in vegetative, non-climacteric, and pre-climacteric plant tissue may be dependent on its ability to reduce protein synthesis.

Some stress responses of plants that require protein synthesis to produce their characteristic symptoms (e.g., wound-induced browning of lightly processed lettuce) could be significantly altered by application of an AVG solution at the time of wounding. Application of 1-MCP to tissue prior to AVG application should permit the study of AVG on protein synthesis unencumbered by it effect on ethylene synthesis. Care should be exercised when using AVG to make sure the effect it is having is the one under investigation; i.e., inhibition of ethylene synthesis, rather than its effect on protein synthesis.

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