Phytochemistry, 1980, Vol. 19, pp. 1075–1079. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0601-1075 $02.00/0

METHIONINE METABOLISM AND ETHYLENE BIOSYNTHESIS IN SENESCING PETALS OF *TRADESCANTIA**

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(*Received* 23 *July* 1979)

Key Word Index—*Tradescantia*; Commelinaceae; ethylene biosynthesis; methionine metabolism; senescence; 1-aminocyclopropane-1-carboxylic acid.

Abstract—The endogenous content of methionine in isolated petals of *Tradescantia* was found to increase during petal senescence while the levels of *S*-methylmethionine and protein were found to decline. The increase in free methionine was, at least in part, the result of protein degradation. Methionine and homocysteine were shown to be intermediates in ethylene biosynthesis while *S*-methylmethionine was not involved. Application of 1-aminocyclopropane-1-carboxylic acid (ACC) to all floral tissues resulted in large stimulations of ethylene production. ACC was shown to be an endogenous amino acid the internal levels of which correlated positively with the rate of ethylene production. Application of L-methionine-[U-^{14}C] led to a rapid appearance of radioactivity in both ethylene and ACC. The specific radioactivity of C-2 and C-3 of ACC and that of ethylene were found to be nearly identical which indicated that ACC was the immediate precursor of ethylene in senescing petals of *Tradescantia*.

INTRODUCTION

Methionine has been shown to be the *in vivo* precursor of ethylene in all plant tissues that have been examined thus far [1]. Investigations on the metabolism of methionine in senescing flower tissue of *Ipomoea tricolor* [2] have shown that methionine, in addition to being converted to ethylene, is also converted to *S*-methylmethionine (SMM). In this tissue SMM serves as a reserve form of methionine which is converted back to methionine during senescence.

Evidence based on labelling data has been presented which indicates that *S*-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) are intermediates in the conversion of methionine to ethylene in apple tissue [3, 4]. Subsequent work (using an *in vitro* enzyme system from ripening tomato fruit) has confirmed the validity of the above hypothesis [5]. Thus, the biosynthetic pathway of ethylene formation is thought to proceed as follows: Methionine $\rightarrow\rightarrow\rightarrow$ SAM $\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow$ ACC $\rightarrow\rightarrow\rightarrow\rightarrow$ ethylene. The well-known inhibitor of ethylene biosynthesis, aminoethoxyvinylglycine (AVG) has been shown to inhibit the enzyme that catalyzes the conversion of SAM to ACC [5].

In this communication we describe our studies on the metabolism of methionine in relation to ethylene biosynthesis in senescing petals of the ephemeral flower of *Tradescantis*.

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RESULTS

Endogenous levels of amino acids and protein during petal senescence

An analysis of the endogenous levels of several amino acids and protein is presented in Table 1. The level of free methionine increased over 2-fold during petal senescence reaching a final value of 14.8 nmol/3 petals. Similarly, the level of free leucine also increased as the petals senesced. The level of free SMM declined as the petals senesced from an initial value of 7.4 nmol/3 petals to a final value of 2.6 nmol/3 petals. As in other senescing tissues, the protein content of these petals was also found to decline during petal senescence. From the data presented in Table 1, it was calculated that less than 1% of the free methionine present in the petals on the morning of senescence would be needed as a substrate to account for the total amount of ethylene produced by these petals as they senesced.

Methionine metabolism during petal senescence

In order to investigate the metabolic fate of methionine during petal senescence, petals excised from flower buds on the afternoon before the day of opening were incubated overnight on a 10 μM solution of methionine which contained 4 μCi of L-methionine-[U-^{14}C]. On the day of senescence, the petals were rinsed and extracted at various times during the course of senescence. Throughout the day, radioactivity was found in the following fractions: ethanol-soluble, ethanol-insoluble and carbon dioxide. Acid hydrolysis of the ethanol-insoluble fraction followed by TLC of the hydrolysate demonstrated that

Table 1. Levels of endogenous amino acids and protein in senescing petals of *Tradescantia*

Compound	Time of day (hr) 09:00	15:00	21:00
Methionine	6.80	10.23	14.77
Leucine	44.20	67.73	74.17
S-Methylmethionine	7.39	4.27	2.63
Protein	225.00	160.00	175.00

Amino acid levels are expressed as nmol/3 petals. Protein is expressed as μg BSA equivalents/3 petals. A total of 0.04 nmol of ethylene was produced during the experiment.

the radioactivity in this fraction was associated with methionine originally bound in protein. TLC of the ethanol-soluble fraction, followed by scanning of the TLC plate for radioactivity, showed three major zones of radioactivity. Using a variety of criteria (see Experimental and [2]), these zones were shown to be *S*-methylmethionine, methionine, and methionine sulfoxide. Methionine sulfoxide was considered to have arisen as an artifact of extraction and its occurrence was not investigated further.

The results of a time-course study of methionine metabolism during petal senescence in *Tradescantia* petals are shown in Table 2. During petal senescence, we observed a large increase in radioactivity associated with free methionine, a small decline in the radioactivity associated with SMM and a much larger decrease in the radioactivity associated with protein. These results indicated that the increase in free methionine observed during petal senescence in *Tradescantia* was, at least in part, the result of protein degradation rather than the result of the conversion of SMM to methionine.

Table 2. Metabolic fate of L-methionine-[U-^{14}C] in senescing petals of *Tradescantia*

Time of day extracted (hr)	Radioactivity (nCi/petal) Methionine	*S*-Methylmethionine	Protein
09:15	5.10	0.30	12.31
12:15	6.18	0.46	11.64
16:20	7.38	0.32	8.01
21:00	7.88	0.26	5.50

Petals were floated overnight on a 10 μM solution of L-methionine which contained 4 μCi of L-methionine-[U-^{14}C] (256 mCi/mmol) and were extracted at various times on the following day.

Methionine metabolism in relation to ethylene biosynthesis during petal senescence

Since radioactivity was found associated with two soluble compounds, SMM and methionine, it was of interest to determine which of these two compounds was the closer precursor of ethylene in petals of *Tradescantia*. In order to differentiate between these two possibilities, the ability of SMM, methionine, and homocysteine to reduce the specific radioactivity of ethylene in petals which had been previously incubated on L-methionine-[U-^{14}C] was tested. The results of one such experiment are shown in Table 3. Methionine was the best dilutant of the specific radioactivity of ethylene. Homocysteine was also effective as a dilutant. SMM, on the other hand, exhibited only marginal ability to reduce the specific radioactivity of ethylene. These results indicated that methionine was a closer precursor of ethylene than was SMM.

Effect of ACC on ethylene production

ACC has been proposed as the immediate precursor of ethylene in ripening apples [4]. Therefore, it was of interest to determine the effect of this compound on ethylene biosynthesis in petals of *Tradescantia*. The results of one such experiment are shown in Fig. 1. Ethylene production could be detected in untreated petals by 14:00 hr, and by the end of the experiment, these petals had produced 1.93 nl of ethylene. Petals exposed to ACC produced large amounts of ethylene, and the onset of ethylene production could be detected within 15 min of ACC application. By the end of the experiment, these petals had produced 42.7 nl of ethylene. Aminoethoxyvinylglycine (AVG) was found to have little effect on either the initiation of ethylene production or on the final amount of ethylene

Table 3. Reduction of the specific radioactivity of ethylene produced by senescing petals of *Tradescantia* by unlabelled amino acids

Collection period (min)	Dilutant	Ethylene formed (nmol)	Specific activity of ethylene nCi/nmol	% Dilution
0–75	Water	0.04	5.72	0
	Methionine	0.05	2.61	54
	SMM	0.04	4.93	14
	Homocysteine	0.05	3.80	34
75–170	Water	0.02	5.35	0
	Methionine	0.03	2.34	56
	SMM	0.02	4.71	12
	Homocysteine	0.03	3.68	31

Petals were incubated overnight on 15 μCi of L-methionine-[U-^{14}C] (256 mCi/mmol) and were transferred to flasks the next morning. When ethylene production commenced, water or a solution of an unlabelled amino acid was introduced into each flask. Concentration of unlabelled amino acids: L-methionine and L-homocysteine-thiolactone: 1mM; DL-*S*-methylmethionine: 2 mM.

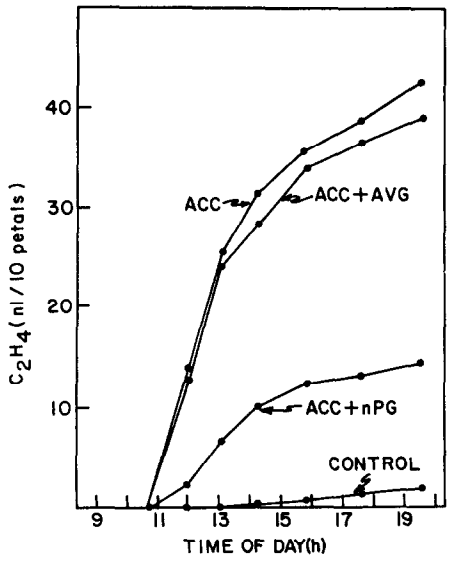

Fig. 1. The effect of ACC on ethylene production by mature petals of *Tradescantia* in the presence and absence of inhibitors of ethylene biosynthesis. Concentration of ACC: 0.5 mM; aminoethoxyvinylglycine (AVG): 50 μM; *N*-propyl gallate (nPG): 0.5 mM. Petals were excised at 09:00 hr and treatments with inhibitor solutions were initiated immediately. Petals were placed on ACC solutions in the presence and absence of inhibitors at 10:30 hr.

produced by these petals in response to ACC. On the other hand, *n*-propyl gallate inhibited the rate of ethylene production in ACC-treated petals (Fig. 1).

We have previously shown [6] that petals of *Tradescantia* develop the ability to produce ethylene as they mature, with only the mature petals being capable of 'autocatalytic' ethylene production. It was of interest to determine if the ability of ACC to stimulate ethylene production in flower tissues paralleled the tissue's ability to produce ethylene in the absence of ACC. As can be seen from Table 4, application of ACC to petals and sepals resulted in the production of large amounts of ethylene. Interestingly, immature petals, which normally produce only small amounts of ethylene, produced the greatest amount of ethylene in response to ACC. Thus, the ability of ACC to stimulate ethylene production in flower tissues of *Tradescantia* in no way correlated with the level of ethylene production in the absence of ACC.

Table 4. Effect of ACC on ethylene production in sepals and petals of different ages isolated from flowers of *Tradescantia*

Tissue	Age	Treatment	Ethylene produced (nl)
Sepals	−2	Water	0.31
Sepals	−2	ACC	5.71
Petals	−2	Water	0.31
Petals	−2	ACC	29.95
Sepals	−1	Water	0.23
Sepals	−1	ACC	6.24
Petals	−1	Water	0.20
Petals	−1	ACC	36.33
Sepals	0	Water	0.23
Sepals	0	ACC	11.85
Petals	0	Water	0.95
Petals	0	ACC	14.26

Nine petals or sepals were placed in 25 ml flasks to which was added 1 ml of water or 0.1 mM ACC. Tissue ages: −2, tissue isolated 2 days prior to flower opening; −1, tissue isolated 1 day prior to flower opening; 0, tissue isolated the day of flower opening.

Endogenous levels of ACC in relation to ethylene production

Experiments were conducted to determine if the endogenous levels of ACC correlated with the rate of ethylene production in mature tissues. The results of a time-course study comparing the endogenous content of ACC with ethylene synthesis is shown in Fig. 2. The endogenous content of ACC remained low in control petals until the onset of ethylene production when it began to rise. Thereafter, the content of ACC continued to increase as did the rate of ethylene production. Pretreatment of the petals with ethylene hastened the initiation and increased the subsequent rates of ethylene production and it also exerted a similar effect on the endogenous content of ACC. Petals pretreated with ethylene in the presence of cycloheximide produced no ethylene and showed no increase in the endogenous levels of ACC.

Comparison of the specific radioactivities of ethylene and ACC

In order to establish a precursor–product relationship between ACC and ethylene, a comparison was made between the specific radioactivities of ethylene and ACC at various times following application of L-methionine-[U-^{14}C]. The results of one such comparison are shown in Table 5. The specific radioactivity of ethylene increased with the incubation time on labelled methionine. Radioactivity was found in ACC at

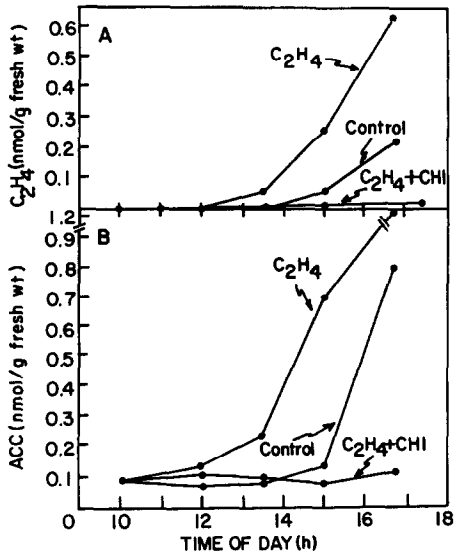

Fig. 2. Ethylene production (A) and endogenous levels of ACC (B) in mature petals of *Tradescantia*. Cycloheximide (CHI) treatment was initiated immediately after excision (09:00 hr). Ethylene pretreatment was begun 60 min following excision and lasted for 60 min (10:00–11:00 hr).

Table 5. Specific radioactivities of ethylene and carbon atoms 2 + 3 of ACC at various times following the addition of L-methionine-[U-^{14}C] to mature petals of *Tradescantia*

Trapping period (min)	Specific radioactivity (nCi/nmol) Ethylene	ACC
0– 40	0.268	0.309
40– 80	0.667	0.556
80–120	0.960	0.895

6.3 μCi of L-methionine-[U-^{14}C] (256 mCi/mmol) was added at zero time to mature petals actively producing ethylene.

each determination which demonstrated that it was derived from methionine. Moreover, the specific radioactivity of both ACC and ethylene closely paralled each other throughout the experiment. This agreement indicated that these two compounds were metabolically related and that ACC was the *in vivo* precursor of ethylene mature petals of *Tradescantia*.

DISCUSSION

The results presented in Table 1 indicate that the availability of methionine does not limit ethylene production in mature petals of *Tradescantia*. Consistent with this hypothesis is the observation that application of 1 mM L-methionine has no effect on either the initiation or the total amount of ethylene produced by these petals [7]. This situation is similar to that found in senescing flower tissue of *Ipomoea tricolor* [2], but differs from that in ripening apple tissue [8].

The results presented in Tables 1 and 2 demonstrate that SMM is a naturally occurring amino acid in petals of *Tradescantia* and that it is a principal metabolite of applied methionine. The results presented in Table 2 indicate that the increase in free methionine which occurs during petal senescence in *Tradescantia* cannot be accounted for by the conversion of SMM to methionine. Rather, these results indicate that the increase in free methionine observed during petal senescence is, at least in part, the result of protein degradation. This situation differs from that found in senescing flower tissue of *Ipomoea tricolor* where methionine is synthesized through a transfer of a methyl group from SMM to homocysteine [2].

The results of the dilution experiment indicate that SMM is not directly involved in ethylene biosynthesis (Table 3). The fact that methionine reduces the specific radioactivity of ethylene more efficiently than SMM is most easily explained by the hypothesis that methionine is a closer precursor of ethylene than is SMM. The ability of homocysteine to reduce the specific radioactivity of ethylene is consistent with the fact that it is an immediate precursor of methionine in plant tissues. In other studies [7], it was found that SMM is readily taken up by isolated petals of *Tradescantia;* therefore, the inability of SMM to reduce the specific radioactivity of ethylene reflects its lack of involvement in ethylene biosynthesis rather then a lack of uptake.

In order to demonstrate that ACC is involved in ethylene biosynthesis, three criteria must be met: (a) ACC must be converted to ethylene, (b) ACC must be an endogenous amino acid, and (c) ACC must be formed from methionine. The results presented in Fig. 1 and Table 4 leave no doubt that ACC is readily converted to ethylene in petals of *Tradescantia*. The copious amounts of ethylene produced in response to ACC application make it seem unlikely that ACC stimulates ethylene production by a mechanism other than its being a substrate. The results presented in Fig. 2 demonstrate that ACC is an endogenous amino acid in petals of *Tradescantia* the levels of which correlate with the amount of ethylene being produced. Finally, the results presented in Table 5 show that ACC is derived from methionine and the close agreement between the specific radioactivities of ACC and ethylene confirms a precursor–product relationship, as was first suggested by Adams and Yang [4].

The ability of ACC to stimulate ethylene production regardless of age in all flower tissues tested indicates that the formation of ACC is the rate-limiting step in ethylene biosynthesis in petals of *Tradescantia*. The inability of AVG to inhibit the conversion of ACC to ethylene (Fig. 1) is consistent with the hypothesis [4, 5] that this compound inhibits the formation rather than the utilization of ACC. The ability of *n*-propyl gallate to inhibit the conversion of ACC to ethylene suggests a site of action for this inhibitor. However, in other studies (not shown) application of *n*-propyl gallate to petals of *Tradescantia* did not lead to an accumulation of ACC as would be expected if only its utilization was impaired. In fact, the ACC-forming enzyme from tomato [5] and a cell-free, ACC-dependent ethylene-forming system from peas [10] were both inhibited by *n*-propyl gallate.

EXPERIMENTAL

Plant culture and ethylene analysis. Clone 02 of *Tradescantia* was grown as previously described [6]. Ethylene was analysed in 1 ml gas samples by GLC as previously described [2].

Amino acid and protein analysis. Petals were excised from open flowers between 08:00 and 08:30 hr on the day of senescence and groups of 6 petals (*ca* 0.1 g) were transferred to 25 ml flasks. The flasks were flushed with ethylene-free air and were sealed. At the times noted, the petals were extracted with 2 ml 80% EtOH containing 5mM β-mercaptoethanol (extraction medium). The extracts were centrifuged at 13 000 g for 10 min, and the supernatant was decanted. The pellet was re-extracted with 1 ml of extraction medium and was recentrifuged. The combined supernatants were evaporated at 40° in N_2. They were redissolved in 0.1 N HCl, and amino acid levels were determined in aliquots of this solution as previously described [2, 9]. Protein was determined in the washed, ethanol-insoluble pellet by the method of Lowry *et al.* [11].

Methionine metabolism. Petals were excised from unopened buds on the evening prior to flower opening and were floated overnight on a 2 ml solution of 10 μM L-methionine containing 4 μCi of L-methionine-[U-^{14}C] (256 mCi/mmol, New England Nuclear). The next morning, the petals were rinsed with distilled H_2O for 20 min and groups of 12 petals were transferred to 25 ml flasks which were then sealed. At the times noted, a group of petals was removed from a flask and was extracted as above. The dried EtOH-soluble fraction was redissolved in 80% EtOH and aliquots of this were subjected to TLC on cellulose plates developed with *n*-BuOH–Me_2CO–$HNEt_2$–H_2O (30:30:6:15). Following

TLC, the plates were scanned for radioactivity, and the radioactive zones were scraped from the plate. For quantitation, these zones were combusted in a sample oxidizer, and the radioactivity was determined by liquid scintillation counting. Identification of the EtOH-soluble, radioactive zones was accomplished as described previously [2]. The EtOH-insoluble fraction was subjected to acid hydrolysis in concentrated HCl at 110° under N_2.

Dilution experiments. Petals were excised from unopened buds and were floated overnight on 3 ml dist. H_2O containing 7 μCi L-methionine-[U-^{14}C]. The next morning, the petals were rinsed with distilled H_2O, and groups of 24 petals were transferred to 25 ml flasks. The flasks were flushed with ethylene-free air and were sealed. When petals began producing ethylene, the flasks were opened and 1.25 ml H_2O or an unlabelled amino-acid solution was added to each flask. Concentrations of unlabelled amino acids were L-methionine: 1 mM; L-homocysteine-thiolactone: 1 mM; and D,L-*S*-methylmethionine: 2 mM. Flasks were flushed with ethylene-free air and were sealed with a CO_2 trap inside. The CO_2 trap consisted of a strip of fluted filter paper wetted with a solution of N NaOH saturated with $Ba(OH)_2$ which was suspended over the petals in a vial. After 75 min, a 50 ml gas sample was removed from the flask and the specific radioactivity of ethylene was determined as previously described [2]. Flasks were then opened, flushed with ethylene-free air, and resealed. 95 min later the trapping procedure was repeated.

Effects of ACC on ethylene production. Petals were isolated from open flowers in the morning. Petals to be treated with ACC in the presence of either AVG or *n*-Propyl gallate were initially floated for 90 min on 5 ml of the following inhibitor solutions: 0.1 mM AVG or 1.0 mM *n*-propyl gallate. After this pretreatment, the petals were transferred to 50 ml flasks containing 1.5 ml of a soln of 0.5 mM ACC plus the inhibitor solution at one-half of the concn employed during the pretreatment. Petals to be exposed to ACC alone were initially floated on distilled water prior to transfer to the incubation flasks. Control petals were maintained on distilled water throughout.

Endogenous content of ACC. Petals were isolated from open flowers. Groups of 15 petals were transferred to 25 ml flasks which were flushed with ethylene-free air and sealed. Petals pretreated with ethylene were exposed to 10 μl/l. of ethylene for 1 hr prior to transfer to the incubation flasks. Cycloheximide treatment was initiated 60 min prior to the pretreatment with ethylene and was maintained throughout the experiment. At the appropriate times, the ethylene content in the headspace above the petals was determined, and the petals were extracted with 80% EtOH as before. Aliquots of the EtOH-soluble fraction were applied to cellulose TLC plates and the plates were developed as before. Authentic standards of ACC were run in parallel with each extract. Following development, the zone corresponding to the position of the standard was scraped from the plate. ACC was eluted from the cellulose powder with EtOH and was evapd. Residue was redissolved in 1 ml 0.1 M Na-Pi buffer (pH 11.5). One half of this was used directly in the ACC assay [5]. To the remaining half 10 nmol of authentic ACC were added prior to the assay in order to determine the efficiency of the test.

Determination of the specific radioactivity of ethylene and ACC. Petals were excised from open flowers and were placed in 50 ml flasks. Ethylene production was monitored, and when the petals began producing ethylene, 2.5 ml of an aq. soln containing 6.3 μCi of L-methionine-[U-^{14}C] were added to each flask. Flasks were sealed with CO_2 trap inside. After 40 min, an aliquot of the gas phase was removed from one flask for the determination of the specific radioactivity of ethylene. Petals were then removed from that flask, homogenized in 80% EtOH, and ACC was isolated as before. At this time, a second flask was opened, flushed with ethylene-free air and resealed. After 40 min, the ethylene trapping and homogenization were performed with the second sample. At this time the third flask was opened and flushed with ethylene-free air and resealed. After 40 min, the trapping and homogenization were repeated for a third time. ACC was converted to ethylene by the method of Boller *et al.* [5]. The specific radioactivity of the ACC-derived ethylene was then determined as before [2].

Acknowledgements—This research was supported by the U.S. Department of Energy under Contract EY-76-C-02-1338.

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