Ethylene Production in Spinach Leaves during Floral Induction

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ABSTRACT

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Spinach plants were induced to flower by transferring them from short days to continuous light. Their leaf laminae released more ethylene than those from vegetative plants. These leaves also exhibited a greater capacity to convert exogenous ACC into ethylene. Cell wall preparations from the leaves of continuously illuminated plants also converted exogenous ACC into ethylene more readily than extracts from short day plants. These effects and also those previously reported for peroxidases appear very similar to these brought about by various environmental stresses such as pollution and mechanical irritation.

Key words—Ethylene, floral induction, stress, photoperiod, spinach.

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INTRODUCTION

'There is little question that ethylene can play a significant role in flower initiation . . . Studies of SAM (*S*-adenosylmethionine) or ACC (1-aminocyclopropane-1-carboxylic acid) biosynthesis and transport in relation to environmental and chemical controls of floral initiation should provide important clues as to how ethylene itself may participate in induction and perhaps evocation . . .' (Bernier, Kinet, and Sachs, 1981). There are indeed few studies on ethylene metabolism and production in relation to the induction of flowering. Suge (1977) was probably the first to follow endogenous ethylene in relation to flowering. He found that ethylene production increased gradually and then declined in both radish and pea exposed to vernalizing temperature. After return to normal growing temperatures ethylene production was slower in the vernalized than in the non-vernalized plants. These results may help to explain why the absolute requirement of vernalization for the flowering ability of buds of *Cichorium intybus* can be replaced by an ethylene treatment at room temperature (Joseph, Billot, Soudain, and Come, 1985). It also may explain why ethylene

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; CL, continuous light; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid; MES, 2-(*N*-morpholino)-ethane sulphonic acid; SAM, *S*-adenosylmethionine; SD, short days; TRIS, α,α,α-Tris-(hydroxymethyl)-methylamine.

treatment of tobacco and *Pharbitis* immediately after an exposure to a floral inductive environment favoured vegetative growth and retarded flowering (Suge, 1974; Kasperbauer and Hamilton, 1978). More recently, De Greef, van Dijck, de Proft, and Mekers (1983) have shown that *Aechmea* plants can be induced to flower either by ethylene or ACC application but only when such plants reached 'flowering maturity'. Flowering maturity of these plants was precisely correlated with their capacity to convert large amounts of ACC into ethylene. It was also shown that flowering induction of some *Bromeliaceae* could be retarded by AVG (aminoethoxyvinylglycine), an inhibitor of ethylene biosynthesis (Mekers, de Proft, and Jacobs, 1983).

In order to elucidate further the possible role of ethylene synthesis in the flowering induction process, the extent of ethylene production and the conversion of ACC to ethylene was examined in the leaves of the long-day plant *Spinacia oleracea* currently used in our laboratory for the study of flowering (Penel and Greppin, 1974, 1975; Karege, Penel, and Greppin, 1982). From this work it seems that the earliest steps of this process involve fast biophysical and biochemical modifications of cell structures that support vegetative growth (Greppin, Auderset, Bonzon, and Penel, 1978).

MATERIALS AND METHODS

Plant material

Spinach plants (*Spinacia oleracea* L. cv. Nobel) were grown in a phytotron under standard conditions of illumination (100 μE m^{-2} s^{-1} provided by Sylvania fluorescent lamps 'daylight', 40 W, Sylvania Electric products, Danvers, Mass., U.S.A.), temperature (21 °C) and humidity (70% during the dark, 50% during the light period).

Photoperiodic treatments

Three different light treatments were used in order to characterize: (i) the vegetative state (5 weeks in short days of 8 h light), (ii) the transition to the floral state (5 weeks in short days, then transfer in continuous light for 24 h), and (iii) the effect of a transfer as in (ii), but with plants already induced by 24 h continuous illumination but transferred back to short days for 5 d. This treatment is called 'mimicked inductive treatment'.

Sampling

The two youngest leaves (3 and 4) fully expanding at the time of analysis were excised and divided into laminae and petioles.

Ethylene measurements

Two leaf laminae were dipped by their bases in a 2×10^{-6} m^3 glass test tube containing 0.7×10^{-6} m^3 H_2O or ACC (10 mol m^{-3}) fixed at the bottom of a 60 cm^3 glass bottle hermetically closed with a rubber plug. Petiole segments 20 mm long were set on a filter paper sheet imbibed either with H_2O or ACC (10 mol m^{-3}) in 35×10^{-6} m^3 glass bottles similarly closed. Whole plants with their four leaves and with their roots (previously rinsed with tap water) were similarly enclosed in 150×10^{-6} m^3 bottles (1 plant per bottle) in the presence of H_2O or ACC. Ethylene production was quantified after 24 h at room temperature by G.C. according to the method used by Boyer, Desbiez, Hofinger, and Gaspar (1983).

Fractionation

Samples of leaves weighing 10 g were ground in a precooled mortar in 25 cm^3 m^{-3} MES buffer containing 500 mol m^{-3} sucrose, and adjusted to pH 7·2 with TRIS buffer. After filtration on a nylon cloth, the extract was centrifuged at 3 000 g for 10 min. The supernatant was centrifuged at 6 000 g for 10 min, then at 20 000 g for 30 min. The resulting supernatant and the pellet constituted the soluble and the microsomal fractions respectively. The pellet obtained after the first centrifugation was resuspended in 25 mol m^{-3} MES buffer (pH 7·2) and centrifuged at 1 000 g for 10 min. This yielded a wall pellet. Both the microsomal and the wall pellets were resuspended in 10 mol m^{-3} HEPES buffer, pH 8·2. Aliquots of the fractions corresponding to 200 μg proteins were absorbed onto a filter paper sheet in 35×10^{-6} m^3

glass bottles in the presence of ACC (1.0 mol m^{-3}) and MnCl$_2$ (0.5 mol m^{-3}); ethylene production was measured after 24 h at room temperature.

ACC content

Samples (1.0 g) of leaf lamina or petiole were extracted with 80% ethanol and centrifuged at $5\,000\,g$ for 10 min. The supernatant was evaporated to dryness at 37 °C and the residue was resuspended in 1×10^{-6} m^3 distilled water. Free ACC content was then determined according to Lizada and Yang (1979) from the ethylene evolved after 2 h from 1.0 cm^3 solution containing: 0.8×10^{-6} m^3 aqueous extract, sodium hypochlorite 0·44% (wt./vol.), HgCl$_2$ 1.0 mol m^{-3} and 3·3% (v/v) of a NaOH saturated solution. This was enclosed in a sealed 35 cm^3 glass bottle.

Freshly prepared standards were run routinely. Each experiment was repeated at least four times with different batches of plants.

RESULTS

Ethylene production by excised laminae of leaves 3 and 4 from vegetative plants (grown under SD) measured at the end of a darkness period was 52 μm^3 g^{-1} over 24 h (Table 1). Leaf laminae from plants that have been induced to flower (by a 24 h period of continuous light) produced twice as much ethylene. Comparable results were obtained using whole plants instead of excised leaf laminae (Table 1). Further analyses indicated that this increase already was measurable during the first hours following the transfer of SD plants to continuous light. Ethylene production by excised petioles of the same leaves 3 and 4 was quite the same for vegetative and induced plants (Table 1).

When ACC was applied to leaf laminae and to petioles, ethylene production from both vegetative plants and plants induced to flower was increased severalfold (Table 1). The ability of laminae from induced plants to convert exogenous ACC to ethylene was three times greater than that from vegetative plants (Table 1); that of petioles was twice as high (Table 1).

Table 2 indicates that ACC in the laminae was more concentrated in vegetative plants than that in florally induced plants. However, the ACC content in the petioles was similar in both types of plants.

Laminae of plants which had been induced by 24 h continuous light and transferred back to short days for 5 d returned to a rate of ethylene production and ability to convert ACC to ethylene typical of non-induced plants (Table 3). A second inductive treatment of 24 h continuous illumination at the end of these 5 short days again resulted in the changes mentioned before (Table 3).

TABLE 1. *Ethylene production (μm^3 g^{-1} over 24 h) by excised leaf laminae, petioles and whole vegetative and induced spinach plants in the absence and in the presence of ACC (10 mol m^{-3}). Means of four replicates and standard errors*

	Vegatative plants (SD)	Induced plants (SD + 24 h CL)
Leaf laminae		
−ACC	52·44 ± 4·32	113·86 ± 8·43
+ACC	1217 ± 204	3646 ± 450
Whole plants		
−ACC	13·50 ± 4·35	20·32 ± 4·88
+ACC	82·73 ± 12·39	148·97 ± 21·46
Leaf petioles		
−ACC	256·64 ± 22·57	252·22 ± 34
+ACC	1198 ± 72·92	2598 ± 280

TABLE 2. *ACC content (10^{-9} mol g^{-1} fr. wt.) in leaf laminae and petioles from vegetative and induced plants*

ACC content in	Vegetative plants (SD)	Induced plants (SD + 24 h CL)
Leaf laminae	$2·36 \pm 0·20$	$1·50 \pm 0·19$
Petioles	$1·12 \pm 0·17$	$1·13 \pm 0·18$

Means of four replicates and standard errors.

TABLE 3. *Ethylene production (μm^3 g^{-1} over 24 h) by excised leaf laminae from plants subjected to various photoperiod sequences*

Photoperiodic treatments	Leaf laminae + H_2O	Leaf laminae + ACC (10 mol m^{-3})
37 SD	$50 \pm 10·78$	1287 ± 170
36 SD + 24 h CL	$94 \pm 12·58$	4505 ± 380
31 SD + 24 h CL + 5 SD	$54 \pm 5·13$	1399 ± 192
30 SD + 24 h CL + 5 SD + 24 h CL	$137 \pm 16·93$	5714 ± 152

Means of four replicates and standard errors.

TABLE 4. *Ethylene production (μm^3 mg^{-1} protein over 24 h) by microsomal, soluble and cell wall fractions from leaf laminae of vegetative and induced spinach plants*

Fraction	Components added to the fraction	Vegetative plants	Induced plants
Microsomal	Buffer	17	13
	Buffer + ACC mol m^{-3}	178	185
	Buffer + ACC mol m^{-3} + $MnCl_2$ 0·5 mol m^{-3}	277	292
Soluble	Buffer	6	4
	Buffer + ACC mol m^{-3}	28	19
	Buffer + ACC mol m^{-3} + $MnCl_2$ 0·5 mol m^{-3}	104	112
Cell wall	Buffer	19	20
	Buffer + ACC mol m^{-3}	177	246
	Buffer + ACC mol m^{-3} + $MnCl_2$ 0·5 mol m^{-3}	338	428

As expected from literature data (Mayak, Legge, and Thompson, 1981; McRae, Baker, and Thompson, 1982; Lynch, Sridhara, and Thompson, 1985; Kevers and Gaspar, 1985), a microsomal fraction from spinach leaves converted ACC to ethylene *in vitro* in the presence of manganese (Table 4). Calcium ions up to 1·0 mol m^{-3} appeared uneffective (data not shown) but no difference could be seen in the rates of ACC conversion to ethylene by microsomes between vegetative and induced plants (Table 4). Soluble fractions from both types of plants showed very low and similar capabilities for such a conversion. The cell wall

fractions from both induced and vegetative plants were seen to convert ACC to ethylene, with a higher activity for those prepared from induced plants. Activity of this fraction was maintained after both a washing in a sucrose gradient and a treatment with 1000 mol m^{-3} NaCl.

DISCUSSION

For many years, ethylene or ethylene-releasing compounds such as 2-chloroethane-phosphonic acid are known to promote flowering of pineapple (Cooke and Randall, 1968; Adriansen, 1976; Zeevaart, 1978). Application of exogenous ethylene is now standard commercial practice for the controlled flowering of pineapple and also of other bromeliads. Besides those in the Bromeliaceae, a few other species have also been found to respond positively to ethylene applications. Ethylene may thus play a role in flowering induction. There are, however, few studies of endogenous ethylene in relation to flowering (Suge, 1977; De Greef *et al.*, 1983). We show here that the transfer of spinach plants from non-inductive to inductive conditions rapidly increases the rate of ethylene production by leaf laminae and also their capacity to convert ACC to ethylene. ACC measurements in laminae indicate a lower concentration within induced plants. This probably corresponds to a faster conversion to ethylene. However, the transfer to continuous light of previously induced plants (mimicked induction) indicates that the changes in ethylene release and ACC–ethylene conversion may not be caused by the onset of flowering since they can be reproduced twice on the same plants. They may be considered rather as a more direct response to photoperiod.

Two cell fractions are able to convert ACC into ethylene in spinach leaves i.e. microsomal and cell wall. Activity of the microsomal fraction is in good agreement with studies indicating that ACC conversion to ethylene is associated with membrane systems (Imaseki and Watanabe, 1978; Mayak *et al.*, 1981; McRae *et al.*, 1982; Lynch *et al.*, 1985). As far as we know, conversion of ACC to ethylene by a cell wall fraction has not been reported until now. However, studies have shown that cell wall digesting enzymes and their cell wall products are capable of stimulating ethylene biosynthesis, and suggest that certain cell wall fragments produced as a consequence of stress may serve as signals eliciting the synthesis of ethylene (Anderson, Matoo, and Lieberman, 1982; Vandermolen, Labavitch, Strand, and DeVay, 1983; Anderson, Chalutz, and Matoo, 1985). This suggests a role for the cell wall in ethylene metabolism. We show that increased capacity of the leaves from induced plants to convert ACC to ethylene is related to an increased capacity of the cell wall fraction.

Although the enzyme responsible for ACC–ethylene conversion has not been isolated, some studies indicate that peroxidases may be considered as a part of the enzyme complex catalysing this step of ethylene metabolism (Yang, Ku, and Pratt, 1967; Rohwer and Mäder, 1981; Vioque, Albi, and Vioque, 1981; Boyer and De Jaegher, 1986). That peroxidases may play a role in ethylene synthesis was suggested by data indicating a relation between ethylene production and membrane binding of peroxidases in vitrifying tissues (Kevers and Gaspar, 1985) and in tissues subjected to stresses, for instance mechanical stress (Boyer *et al.*, 1983). In spinach, it was previously shown that flowering induction is characterized by an increase of the total peroxidase activity and of the acid/basic ratio (Penel and Greppin, 1975). These changes in peroxidases and those reported here for ethylene are very similar to those described in various plant tissues in response to different types of stresses, for instance, mechanical perturbation (Boyer and De Jaegher, 1986) indicating relations between changes in ethylene and in cellular distribution of peroxidases (Gaspar, Penel, Castillo, and Greppin, 1985). This leads us to propose that flowering induction could be considered as a stress response of the plants to a change in their light regime. Results of the mimicked induction are

in good agreement with this view. Further studies are now needed to determine whether the changes in ethylene biosynthesis described here in spinach plants transferred to continuous light can be related to biochemical (sugar composition, enzyme association) modifications at the cell wall level.

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