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Biosynthesis of Wound Ethylene in Morning-Glory Flower Tissue[1]

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ABSTRACT

Production of wound ethylene was investigated in rib segments excised from flower buds of morning-glory (*Ipomoea tricolor*). Segments of the ribs were cut from buds 2 days before flower opening, floated overnight on 5 mM KCl solution, and transferred to agar the following morning. These immature segments evolved only a small quantity of ethylene during incubation on agar, with most of the production occurring in the morning. When such segments were wounded mechanically early in the afternoon, the rate of ethylene production rose more than 10-fold within 1 hour and returned to a low rate after about 3 hours.

Production of ethylene by both untreated and wounded rib segments was inhibited more than 95% by overnight pretreatment with the ethoxy analog of rhizobitoxine (3×10^{-5} and 10^{-4} M). After overnight exposure of segments to 9 μM L-methionine-U-^{14}C, the specific radioactivity of the ethylene evolved by untreated and wounded tissue was determined and compared to the specific radioactivities of carbon atoms 3 plus 4 of methionine and S-methylmethionine (SMM) extracted from the segments. The specific radioactivity of methionine was about one-half that of SMM; neither value was significantly affected by wounding. The specific radioactivity of ethylene evolved by untreated tissue was close to that of SMM. In wounded tissue the specific radioactivity of the ethylene evolved was lower, but still above that of methionine. These results are consistent with the interpretations that wound ethylene is synthesized from carbon atoms 3 plus 4 of either SMM or methionine. On the basis of earlier experiments with senescing rib segments, it is suggested that methionine serves as the precursor of the wound ethylene.

Production of ethylene by many plant tissues increases in response to stresses of various types, including mechanical stimuli, ionizing radiation, application of toxic chemicals, extremes of temperature and drought (for a review see ref. 2). Of these responses to stress, the evolution of ethylene by plants subjected to mechanical stress (wound ethylene) has been the most widely studied. When tissues of a number of species are cut, bruised, or pressed, the rate of ethylene production begins to rise immediately after injury, increases 10- to 100-fold in 1 to 10 hr, and then declines. These characteristics of production of wound ethylene have been observed in vegetative tissues such as bean petioles (7), potato tubers (11), and sweet potato roots (6), in unripe fruit tissues including cantaloupe (12), banana (11), sycomore fig (17), and tomato (5, 9), and in juvenile flower tissue (8, 15). Except for potato and sweet potato, all of these tissues can also produce large amounts of ethylene, without the stimulus of wounding, during ripening or senescence.

Although carbon atoms 3 and 4 of methionine are known to give rise to the ethylene evolved during senescence of several fruits (for a review see ref. 16), the role of the methionine pathway in stress-induced ethylene synthesis has not been extensively studied, and the data presently available are conflicting. Abeles and Abeles (1) have shown that the ethylene produced by chemically injured bean and tobacco leaves was derived at least in part from ^{14}C-methionine supplied to the leaves via the transpiration stream. In these experiments the conversion efficiency for the transfer of carbon atoms 3 plus 4 into ethylene (specific radioactivity of ^{14}C-ethylene evolved/calculated specific radioactivity of C3 + C4 of methionine supplied) was below 1% for unstressed samples and lower still for stressed samples. If all of the ethylene evolved was derived from methionine, an extensive dilution of labeled methionine by endogenous methionine must have occurred; direct evidence of such a dilution was not provided. Experiments with tomato fruits suggest that wound ethylene produced by unripe fruit and ethylene produced during ripening may have different biochemical origins. Fruits of the *rin* mutant ripen slowly and abnormally, with very little production of ethylene, but evolve the same amount of ethylene in response to slicing as do normal fruits (5).

We have shown previously that ethylene evolved during the senescence of rib segments excised from morning-glory flowers is synthesized from methionine (4). We have also observed that wounding of immature rib segments leads to a transient burst of ethylene production (8). In this communication, we report on the contribution of the methionine pathway to wound ethylene production in immature flower tissue of morning-glory.

MATERIALS AND METHODS

Plant Material. Morning-glory plants (*Ipomoea tricolor* Cav. cv. Heavenly Blue) were grown in an environmental chamber with a 16-hr photoperiod as described previously (3). Buds were harvested two days before flower opening (day −2) between 5:00 and 7:00 PM and were used for preparation of 10-mm rib segments (8). Batches of 15 to 60 segments were floated on 2 to 5 ml of 5 mM KCl in 6-cm Petri dishes and incubated overnight in the environmental chamber in which the morning-glory plants were kept. Between 8:00 and 9:30 AM on the following day (day −1) the segments were blotted dry and transferred gently to 25 or 50-ml Erlenmeyer flasks containing 5 or 10 ml (respectively) of 5 mM KCl solidified with 1% (w/v) agar. The flasks were closed with serum caps and incubated in darkness at 27 C. Production of ethylene was monitored by gas chromatography (8). In some experiments, measurements were made of the rolling up of the segments (3).

In most experiments, rib segments to be wounded were removed from the incubation flask, placed flat on a single layer of tissue paper spread over a tile, and pressed firmly with a lightweight file (Dr. Scholl's callus and corn remover). This treatment

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resulted in a regular pattern of small perforations about 1.4 mm apart. Other methods of wounding are described in the text. The wounded segments were then returned to incubation flasks. The flasks were flushed with ethylene-free air, resealed, and returned to darkness at 27 C. In control treatments, the incubation flasks were opened, flushed with ethylene-free air, and resealed without disturbing the segments.

Pretreatments with Rhizobitoxine Analog. L-2-Amino-4(2'-amino ethoxy)-*trans*-3-butenoic acid (the ethoxy analog of rhizobitoxine) was a gift of Dr. M. Lieberman, Agricultural Research Center, Beltsville, Md. In some experiments, this analog was included at a concentration of 3×10^{-5} or 10^{-4} M in the 5 mM KCl solution on which the segments were floated overnight. The inhibitor had no effect on the appearance of the segments at either concentration.

Experiments with ^{14}C-Methionine. The incubation conditions used have already been described in detail (4). Two batches of 60 to 100 segments were cut on day −2 and incubated overnight on L-methionine-U-^{14}C (9 μM, 260 mCi/mmole). On the morning of day −1, the segments were washed thoroughly to remove unabsorbed methionine, and a sample of segments was taken for extraction. The remaining 50 to 75 segments of each batch were transferred to two 50-ml flasks containing agar. Filter-paper wicks soaked in CO_2-trapping solution (4) were placed in these flasks, which were then closed with serum caps and incubated in darkness at 27 C. At about 2:00 PM, samples of ethylene were withdrawn from each flask for determination of specific radioactivity, the flasks were opened, and a second sample of segments was taken for extraction. The segments were removed from one flask, wounded as described above, and replaced. The second (control) flask was flushed with ethylene-free air and resealed immediately. The ethylene evolved by both control and wounded tissues was collected after about 4 hr of further incubation, and the segments were extracted.

The specific radioactivities of the ethylene evolved by the segments and those of carbon atoms 3 + 4 of methionine and SMM[2] extracted from the tissue were determined using the methods described previously (4). Thin layer chromatography and radiochromatogram scanning were also performed as already described (4).

RESULTS

Ethylene Production by Untreated and Wounded Rib Segments. Segments cut on day −2 and incubated overnight on 5 mM KCl produced 1.5 to 5 nl ethylene/25 segments during incubation on agar on day −1, mainly during the morning hours (control segments of Fig. 1, and ref. 8). Such immature segments showed no tendency to roll up. The higher rate of production of ethylene in the morning was not due to slight wounding of the strips during transfer from KCl solution to agar, because it was also observed when segments were incubated overnight on agar and were not disturbed before measurement of ethylene production (8). Furthermore, it is improbable that the increased rate of production of ethylene in the morning was a residual effect of the stress from the original excision of the segments, because production of wound ethylene usually subsided after about 4 hr (Fig. 1).

The segments were wounded in the afternoon, when the rates of ethylene evolution in untreated (control) segments had declined to a low level. When segments were wounded, the rate of ethylene evolution rose within 1 hr, reached a maximum equal to about 10 times the control rate after an additional 1 to 2 hr, and then declined (Fig. 1). The ethylene produced by wounded tissue did not accumulate in the incubation flask to a concentration sufficiently high to provoke the rolling up response during the experiment. The nature of the mechanical injury was not critical, since results similar to those of Figure 1 were obtained when segments were wounded by multiple perforations, by cutting them into five pieces or by crushing them gently with a glass rod.

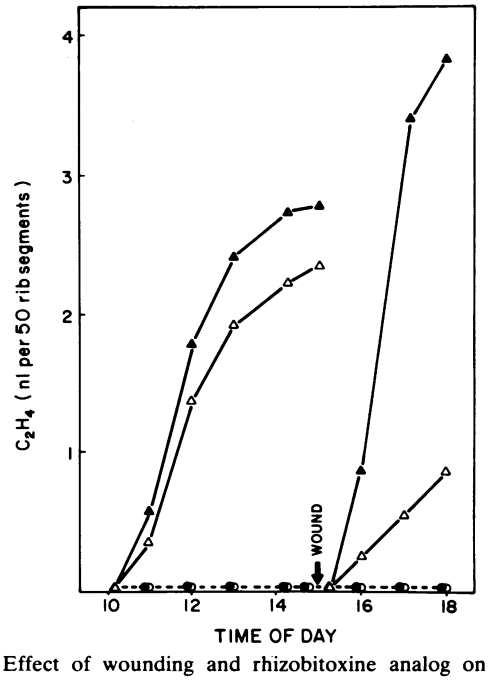

FIG. 1. Effect of wounding and rhizobitoxine analog on ethylene evolution by immature rib segments. Four batches of segments were cut on day −2, incubated overnight on 5 mM KCl (control) or on 5 mM KCl containing 10^{-4} M rhizobitoxine analog, and transferred to agar on the morning of day −1. One control batch and one inhibitor-treated batch were wounded by crushing with a glass rod (arrow). △——△: ethylene evolution by control, unwounded segements; ▲——▲: ethylene evolution by control, wounded segments; O-----O: ethylene evolution by inhibitor-treated, unwounded segments; ●-----●: ethylene evolution by inhibitor-treated, wounded segments. The total production of ethylene by unwounded and wounded segments treated with inhibitor was less than 0.1 nl/15 segments.

Effect of Rhizobitoxine Analog. Figure 1 demonstrates that pretreatment of segments with 10^{-4} M rhizobitoxine analog during the night between day −2 and day −1 almost completely abolished ethylene production on day −1 in both untreated and wounded segments. When 3×10^{-5} M inhibitor was used, results were similar to those of Figure 1, with the synthesis of ethylene reduced more than 95% in both control and wounded tissues.

^{14}C-Methionine Metabolism and ^{14}C-Ethylene Evolution. We have previously shown that, in immature rib segments, the major metabolite formed from ^{14}C- and ^{35}S-labeled methionine is SMM, which is present at an endogenous level greater than that of free methionine (4). For these reasons, the metabolism and specific radioactivities of both SMM and methionine were investigated in relation to production of wound ethylene.

The distribution of radioactivity in ^{14}C-methionine and ^{14}C-SMM before and after wounding was assessed by radioscanning of TLC plates, and the specific radioactivities of C3 + C4 of both compounds were determined for comparison with the specific radioactivity of the ^{14}C-ethylene evolved by the tissue. No effect of wounding on the metabolism of SMM and methionine was apparent from TLC. In both untreated and wounded segments, SMM was the major metabolite formed from ^{14}C-labeled methionine, and accounted for about 40% of the ethanol-soluble ^{14}C. Only 10 to 20% of the ethanol-soluble ^{14}C was present as free methionine. Figure 2 shows that wounding did not affect the specific radioactivities of C3 + C4 of either SMM or methionine. In both untreated and wounded segments, the specific radioac-

[2] Abbreviation: SMM: S-methylmethionine.

tivities of SMM and methionine fell following the overnight exposure to ^{14}C-methionine, with that of SMM being always about double that of methionine. The decreases in the specific radioactivities of SMM and methionine were due to increases in the chemical concentrations of both substances that occurred during day −1 (results not shown).

The specific radioactivity of ethylene evolved by segments before wounding was well above that of extracted methionine and was close to that of SMM. The small amount of ethylene produced between about 3 and 6 PM by untreated segments (Fig. 2A) also showed a specific radioactivity close to that of SMM. Wounded segments produced about seven times more ethylene than did control segments during the afternoon. Although the specific radioactivity of the wound ethylene was lower than that of ethylene evolved by untreated segments, it was above that of methionine (Fig. 2B).

That overnight exposure to ^{14}C-methionine did not significantly expand the endogenous methionine pool was demonstrated by comparing the specific radioactivity for C3 + C4 of extracted methionine (about 1 nCi/nmole) with that for the methionine fed (determined to be 66.6 nCi/nmole). The methionine content of the segments was thus increased only by about 2%.

DISCUSSION

On the morning of day −1, the immature rib segments showed a fairly high rate of ethylene production that was probably related to the growth of these segments; such relatively high rates of ethylene evolution have been frequently observed in juvenile flower and fruit tissues (14). During the afternoon of day −1, control segments produced little ethylene. The transient rise in the rate of ethylene evolution by segments subjected to mechanical injury was typical of the synthesis of wound ethylene.

Ethylene evolution by unwounded and wounded segments was almost completely abolished by pretreatment with rhizobitoxine analog, implicating methionine as the only precursor of ethylene in both cases (10, 13). We have reported previously that the ethoxy analog of rhizobitoxine is also a potent inhibitor of ethylene production in senescing rib segments (8).

The ^{14}C-ethylene evolved by unwounded segments had a specific radioactivity well above that of carbon atoms 3 + 4 of extracted methionine and close to that of SMM. These results would arise if SMM, rather than methionine, were the immediate precursor of ethylene. Our data on the metabolism of SMM in immature tissue suggest an alternative explanation (4). In immature segments, SMM acts as a methyl donor; transfer of one methyl group to an acceptor molecule results in the formation of methionine, which is then remethylated to SMM. The specific radioactivity of C3 + C4 of this actively metabolized methionine would be very close to that of C3 + C4 of SMM. If this methionine were localized in the same compartment as the ethylene-generating system, and thus constituted the only precursor available for production of ethylene, the ethylene evolved would show the observed specific radioactivity. This explanation is consistent with the relationship between ethylene synthesis and SMM metabolism in senescing segments (4). In these segments, the methionine available for ethylene production appears to be derived principally from SMM, but has a lower specific radioactivity than SMM because the major methyl-accepting species is unlabeled homocysteine. Methionine, the only product of this transmethylation reaction, has a mean specific radioactivity half that of SMM.

The specific radioactivity of the ethylene evolved by wounded segments was lower than that of C3 + C4 in SMM but still above the specific radioactivity of C3 + C4 of methionine. It is possible that wounding causes a temporary breakdown in compartmentation between the higher specific radioactivity pool of methionine which serves as a precursor of ethylene and a pool of methionine with a lower specific radioactivity. As a consequence, the mean specific radioactivity of the methionine available for ethylene synthesis would fall; this temporary increase in methionine con-

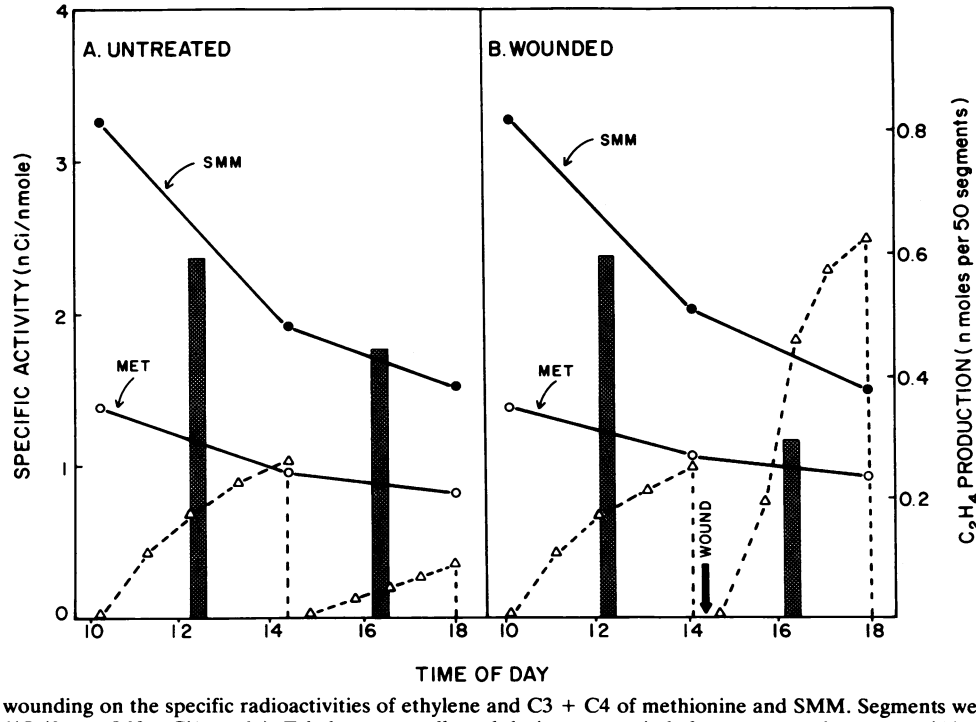

FIG. 2. Effect of wounding on the specific radioactivities of ethylene and C3 + C4 of methionine and SMM. Segments were incubated overnight on L-methionine-U-^{14}C (9 μM, 260 mCi/mmole). Ethylene was collected during two periods from untreated segments (A) and from segments that were wounded by multiple perforations (B). The specific radioactivities of the ethylene evolved during the two collection periods are shown as bars. △-----△: ethylene evolved; ○——○: specific activity of C3 + C4 of methionine; ●——●: specific radioactivity of C3 + C4 of SMM. This experiment was repeated, with almost identical results.

centration at the site of ethylene generation could cause the transient rise in the rate of ethylene production.

Our present data and those reported previously (4) indicate that the pathway of synthesis of wound ethylene in juvenile flower tissue of morning-glory is closely related, if not identical, to the pathways of ethylene synthesis in unwounded juvenile tissue and in senescing tissue. All three pathways are inhibited by the ethoxy analog of rhizobitoxine and appear to utilize methionine as substrate.

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