Carbohydrates Stimulate Ethylene Production in Tobacco Leaf Discs

II. SITES OF STIMULATION IN THE ETHYLENE BIOSYNTHESIS PATHWAY

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ABSTRACT

Galactose, sucrose, and glucose (50 millimolar) applied to tobacco leaf discs (Nicotiana tabacum L. cv 'Xanthi') during a prolonged incubation (5-6 d) markedly stimulated ethylene production which, in turn, could be inhibited by aminoethoxyvinylglycine (2-amino-4-(2'-aminoethoxy)-trans-3-butenolic acid) (AVG) or Co2+ ions. These three tested sugars also stimulated the conversion of L-[3,4,14C]methionine to [14C]1-amino-cyclopropane-1-carboxylic acid (ACC) and to [14C]ethylene, thus indicating that the carbohydrates-stimulated ethylene production proceeds from methionine via the ACC pathway. Sucrose concentrations above 25 mM considerably enhanced ACC-dependent ethylene production, and this enhancement was related to the increased respiratory carbon dioxide. However, sucrose by itself could directly promote the step of ACC conversion to ethylene, since low sucrose concentrations (1-25 mM) enhanced ACC-dependent ethylene production also in the presence of 15% CO2.

The data suggest that the stimulation of ethylene production by sugars in tobacco leaf discs results from enhancement of ACC formation as well as from the conversion of ACC to ethylene, when both steps could be involved in regulation of ethylene biosynthesis.

Ethylene plays a considerable role in leaf senescence (3-5), as well as in many other aspects of plant growth and development (1, 12). Unlike fruits (1), intact leaves (3-5, 18) and other vegetative organs (8, 23) produce very small amounts of ethylene. Ethylene production rates could be increased in vegetative tissues by employing various carbohydrates (6, 9, 16, 18), by various stresses (1, 10, 12), and by the addition of growth regulators (1, 3, 12, 20, 21). D-Galactose promotes IAA-dependent ethylene evolution in mung bean hypocotyls, and methionine appears to be the substrate of this galactose-stimulated ethylene (9). Recently, Rivov and Yang (18) demonstrated that wound ethylene formation, induced in citrus leaf discs by excision, was stimulated considerably by mannotol treatment, and this was ascribed to a chemical stress. On the other hand, our study (16) with tobacco leaves, showed that the carbohydrates-stimulating effect (except that of galactose) is a physiological rather than stress, toxic, or osmotic effect. Among the 14 carbohydrates tested, galactose and sucrose were the most active in stimulating ethylene production, while mannitol had no effect (16). We also found that all sugars tested acted synergistically with IAA in respect to ethylene production, as reported previously for galactose in mung bean hypocotyls (9).

Adams and Yang (2) established the biosynthesis pathway of ethylene in apple as follows: methionine → SAM → ACC → C2H4. The validity of this sequence has since been confirmed in other systems, including IAA-induced ethylene production in vegetative tissues (20, 21, 23, 24). The present study was conducted to determine at which step in the aforementioned ethylene biosynthesis pathway the sugars exert their promotive effect. For this purpose, long term experiments (5-6 d) with tobacco leaf discs as a model system were performed.

MATERIALS AND METHODS

Plant Material and Treatments. Experiments were performed with fully expanded mature leaves of tobacco plants (Nicotiana tabacum L. cv 'Xanthi'), grown in a greenhouse under LD conditions (18 h light) at temperatures between 20° and 30°C. Leaves were washed and sterilized as described previously (16). All subsequent handling of the tissue involved sterile techniques. Discs, 1 cm in diameter, were excised, treated, and incubated as described previously (5, 16), in 2 ml of 50 mM Na-phosphate buffer (pH 6.1) containing 50 μg/ml chloramphenicol. Each flask contained a sample of eight leaf discs, weighing about 90 mg. Where indicated, 50 mM of sugars (d-glucose, d-galactose, or sucrose), 0.1 mM IAA, 0.1 mM AVG, 0.1 mM ACC, 0.5 mM Co2+ or L-[3,4,14C]methionine was included. In one experiment, a 15% CO2 atmosphere was provided by injecting 10 ml of 100% CO2 into each sealed flask. Ethylene and CO2 evolved were absorbed, respectively, by 0.25 M Hg(ClO4)2 (22) and 10% KOH solutions in two plastic center wells as detailed previously (16). The flasks, sealed with rubber serum caps, were incubated in darkness at 30°C, and ethylene production or ACC content was assayed periodically.

Chemicals. All chemicals used were of analytical grade and were purchased from commercial sources. IAA and ACC were purchased from Sigma; L-[3,4,14C]methionine was from SEA, France; and AVG, d-glucose, d-galactose, and sucrose were from BDH Chemicals Ltd. AVG was kindly supplied by Dr. S. F. Yang, Davis, CA (Hoffmann-La Roche).

Determination of Ethylene. The ethylene absorbed during the


2 Abbreviations: SAM, S-adenosylmethionine; ACC, 1-amino-cyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine (2-amino-4-(2'-aminoethoxy)-trans-3-butenolic acid); EFE, ethylene-forming enzyme which catalyzes the conversion of ACC to ethylene.
indicated incubation periods was released from the Hg(ClO₄)₂ complex by injecting 0.2 ml saturated LiCl (22) as described previously (16), and then assayed by a Packard gas chromatograph equipped with an activated alumina column and a flame ionization detector.

Determination of CO₂. The CO₂ trapped with 10% KOH during the indicated incubation periods was released by injecting 0.2 ml of 25% lactic acid. After 2 h of incubation, a 5-ml gas sample was withdrawn from each flask and CO₂ concentration was analyzed with a Packard gas chromatograph equipped with a Porapack Q column and a thermal conductivity detector.

ACC Extraction and Assay. Eight leaf discs (90 mg), incubated under the conditions described above, in the presence or absence of 0.5 mm Ca²⁺ and the different sugars, were removed periodically and homogenized in 2 ml of cold 80% ethanol. The extraction procedure was performed according to Riviè and Yang (17), except that the ACC extract was suspended in 1 ml of H₂O. ACC content in the extract was assayed by the method of Lizada and Yang (13); the efficiency of the conversion of ACC to C₂H₄ as determined with authentic ACC was usually between 70% and 85%.

Radioactivity Measurements. For determination of radioactive C₂H₄ and ACC, 1 μCi of L-[3,4-¹⁴C]methionine (57 mCi/mmol) was added to the incubation medium. To measure the uptake of L-[3,4-¹⁴C]methionine by the leaf discs, a sample of 25 μl was withdrawn from the incubation medium to a 5-ml scintillation solution (toluene-Triton X-100), and the radioactivity was assayed by a Kontron liquid scintillation counter. The counting efficiency was between 80% and 90%. Each center well with the ¹⁴C₂H₄ trapped by the Hg(ClO₄)₂ solution was transferred to a new 50-ml Erlenmeyer flask with another center well containing 0.5 ml of 0.1 M mercury acetate solution, and sealed with a rubber serum cap. The ¹⁴C₂H₄ was released from the Hg(ClO₄)₂ complex by injecting 0.2 ml of saturated LiCl (22) and then reabsorbed by the mercury acetate solution during 2 h of incubation (3). The center wells with the ¹⁴C₂H₄ thus reabsorbed were placed each in a 10-ml scintillation solution for radioactivity assay, as described above. Two flasks from each treatment were sampled for [¹⁴C]ACC extraction and then both Hg(ClO₄)₂ and KOH solutions were renewed in the rest of the flasks for further incubation.

[¹⁴C]ACC content was assayed as described above, with aliquots of 0.25 ml withdrawn from the radioactive extract. An empty plastic center well was hung in each 14-ml test tube, which was sealed with a rubber serum cap. After conversion of ACC to ethylene by NaOCl reagent (13), a 0.5 ml gas sample was withdrawn from the test tube for ethylene determination by GC. Immediately afterwards, 0.1 ml of Hg(ClO₄)₂ reagent was injected to the empty center well for absorption of the remaining ethylene (22). The radioactive ethylene thus absorbed was assayed by liquid scintillation as described above. The radioactive ACC content (nCi) in the sample was calculated as the quotient of ¹⁴C₂H₄ evolved and the conversion efficiency.

All experiments were performed at least twice, with similar results. Treatments within each experiment were tested in duplicate or triplicate flasks and the data presented are averages of the measurements. Standard deviations of the mean are indicated.

RESULTS

Stimulation of Ethylene Production Rates. The effect of different carbohydrates on rates of ethylene production in the course of prolonged incubation was examined with tobacco leaf discs held in darkness at 30°C. As reported previously (4–6, 16), freshly excised leaf discs incubated in a sugar-free medium, produced very small amounts of ethylene during incubation (Fig. 1). Application of 50 mm sugars (glucose, galactose, or sucrose) stimulated ethylene production during incubation (Fig. 1) after a lag period of at least 8 h (see Ref. 16).

From the data illustrated in Figure 1, it can be seen that the pattern of the stimulatory curve of ethylene production during incubation depends on the type of sugar tested. Thus, in the presence of galactose, ethylene production rapidly increased, peaking at about 40 h and then sharply declining, whereas in glucose-treated discs ethylene production gradually increased followed by a slow decrease after reaching the peak at about 70 h. In sucrose-treated discs, the stimulatory curve of ethylene production during the first 40 h was similar to that of glucose-treated discs, except that the increase lasted for more than 100 h of incubation. The maximal stimulation extent of the sugars-enhanced ethylene production, as compared with buffer-treated discs, was about 50-fold by galactose, 40-fold by glucose, and 20-fold by sucrose (Fig. 1).

Stimulation of Ethylene Biosynthesis via Methionine. Carbons 3 and 4 of methionine are specifically converted into the carbon skeleton of ethylene in most of the higher plants tested (12). Hence, the stimulatory effect of carbohydrates on the conversion of L-[3,4-¹⁴C]methionine into ¹⁴C₂H₄ was studied. Figure 2 demonstrates that sucrose stimulated the conversion of labeled methionine into ¹⁴C₂H₄ and a greater enhancement of ethylene production was obtained with a simultaneous treatment of sucrose and IAA. This points to a synergistic effect between these two agents, as demonstrated previously for endogenous ethylene production (16). It is noteworthy that the different extents of ethylene stimulation observed in sucrose- and IAA-treated discs, as compared with buffer, could not be due to different uptake rates because a similar uptake of L-[3,4-¹⁴C]methionine was observed with all treatments (not shown).

Sites of Stimulation. Recent studies on mannitol-stimulated ethylene production suggest that either ACC formation from SAM or ACC conversion to ethylene (or both) might become rate limiting (18). Accordingly, the effects of sugars on ACC level and on ACC-dependent ethylene formation were studied. The effect of sucrose on the endogenous ACC level in leaf discs at various periods after excision is illustrated in Figure 3. In untreated freshly excised discs, ACC content was very low (0.4 nmol/g) and it increased insignificantly during incubation. When incubated with sucrose, ACC content of the leaf discs was markedly stimulated at 70 h after excision.

These data represent the effect of sugars on the pattern of ACC production.
treated leaf discs

Fig. 2. Effect of sucrose or sucrose-plus-IAA pretreatment on the conversion of L-[3,4-\textsuperscript{14}C]methionine into \textsuperscript{14}C\textsubscript{2}H\textsubscript{4} by tobacco leaf discs. Samples of eight discs were preincubated for 24 h with 50 mM sucrose in the presence or absence of 0.1 mM IAA for inducing ethylene production, and thereafter 1 \mu Ci of L-[3,4-\textsuperscript{14}C]methionine was added to the medium.

Fig. 3. Effect of sucrose on the endogenous ACC level in tobacco leaf discs during long term incubation. Fifty mM of sucrose was used. The bars indicate mean ± SD. Inset. Sucrose stimulation of the endogenous ACC level in the presence of 0.5 mM Co\textsuperscript{2+}. Content in the tissue when the whole metabolic pathway of ethylene is active. To determine the effect of sugars on the specific step of ACC formation by itself, Co\textsuperscript{2+} was employed as an inhibitor of ACC conversion to ethylene (24). The inset of Figure 3 demonstrates that with Co\textsuperscript{2+}, ACC level increased during incubation in both buffer and sucrose-treated discs, when the sucrose treatment markedly stimulated (6-fold) this accumulation of ACC as compared with buffer. Rate of inhibition of ACC-dependent ethylene production by 0.5 mM Co\textsuperscript{2+} in the sucrose-treated leaf discs was about 90% during the first 24 h of incubation, and then gradually decreased to around 70% at the 4th d. The data illustrated in Figure 4 show that, as expected, sugars also stimulated the conversion of L-[3,4-\textsuperscript{14}C]methionine into \textsuperscript{14}C\textsubscript{2}H\textsubscript{4}. Thus, in the sugar-treated discs there was an increase in the incorporation of label into ACC, which was more pronounced when Co\textsuperscript{2+} was present (Fig. 4).

These results indicate, as reported previously (18), that a part of ACC synthesized does not accumulate in the leaves but is converted to ethylene. We, therefore, examined the effect of carbohydrates on this last metabolic step. The in vivo conversion of ACC to ethylene is determined by employing AVG to block endogenous ACC synthesis (2, 24) in the ACC-treated leaf tissue and measuring the resulting ethylene production. The data presented in Figure 5 show that AVG almost completely inhibited endogenous ethylene production, both in buffer and in sucrose-treated discs. Application of exogenous ACC to buffer-treated discs caused a 4-fold stimulation of the ACC-dependent ethylene production (Fig. 5A), and a 12-fold increase in sucrose-treated discs (Fig. 5B). The enhancement effect of sucrose on the ACC-dependent ethylene production, as compared with buffer, was 6-fold (Fig. 5, A and B).

We have considered the possibility that carbohydrates may enhance ACC conversion to ethylene by increasing the respiratory CO\textsubscript{2} (Fig. 6A), which in turn was found to enhance this step and to regulate EF-E activity in vegetative tissues (11). Indeed, exogenously supplied CO\textsubscript{2} (15%) increased ACC-dependent ethylene production in untreated discs by about 23-fold (Fig. 6, A and B), whereas 200 mM sucrose enhanced it by only 16-fold.
FIG. 5. Effect of sucrose on basal and ACC-dependent ethylene production in tobacco leaf discs during long-term incubation. Discs were incubated with or without 50 mM sucrose, 0.1 mM AVG, and 0.1 mM ACC, where indicated.

(Fig. 6A). The saturating level for the CO2 action was 15% (data not shown). Rates of respiratory CO2 and ACC-dependent ethylene production of the leaf discs were greater as sucrose concentrations increased (Fig. 6A). Sucrose between 1 and 10 mM caused little increase in ACC-dependent ethylene production, but a sharp increase at, and above, 25 mM. The data in Figure 6A indicate that the increasing respiratory CO2 in the presence of 1 to 200 mM sucrose are still below saturation. Thus, the drastic increase in ethylene production observed above 25 mM sucrose (Fig. 6A) is probably due to the increased CO2 evolved. This is supported by the data of Figure 6B showing that a saturating level of 15% CO2 caused a relatively moderate change in ethylene production in discs treated with more than 25 mM sucrose. However, the 3-fold increase in ACC-dependent ethylene production obtained between 1 and 50 mM sucrose in the presence of saturating 15% CO2 (Fig. 6B) indicates that sucrose by itself can also stimulate ACC conversion to ethylene in addition to its CO2-mediated effect (Fig. 6A). The synergistic effect between CO2 and sucrose became evident mainly with the low sucrose concentrations between 1 and 100 mM. Thus, at 1 mM sucrose, 15% CO2 enhanced ACC-dependent ethylene production by 50-fold, whereas at 200 mM sucrose the CO2-enhancing effect was only 4-fold (Fig. 6, A and B).

DISCUSSION

Our preceding report (16) demonstrated that various sugars stimulated ethylene production significantly in tobacco leaf discs, acting synergistically with IAA. One possible explanation for this synergism is that the carbohydrates may be acting to maintain a sufficient level of free IAA to elicit ethylene biosynthesis (6, 9, 16). The present study has further established this unique effect of sugars by demonstrating that the biosynthesis of the carbohydrates-stimulated ethylene proceeds via the ordinary methionine-ACC pathway, as was observed in many other plant tissues. This conclusion was based on the following observations: (a) galactose, glucose, or sucrose stimulated conversion of added l-[3,4-14C]methionine to [14C]ACC (Fig. 4) and to 14CO2 (Fig. 2); (b) sucrose stimulated both ACC formation (Fig. 3) and conversion of ACC to ethylene (Fig. 5); and (c) AVG (Fig. 5) or Co2+, inhibitors of ethylene biosynthesis in these aforementioned steps, almost completely abolished sucrose-stimulated ethylene production. Thus, promotive effect of the tested sugars resulted from enhancement of the final two steps in the ethylene biosynthesis sequence, namely ACC formation from SAM and conversion of ACC to ethylene. These results are similar to those of Riov and Yang (18) obtained with maninitol in citrus leaf discs, except that their absolute rates of ethylene production were six times higher than ours. This is probably due to the fact that, unlike those of Riov and Yang (18), our experiments were carried out in a CO2-free atmosphere, which might account for the lower rates of ethylene production (11).

Fruits and vegetative tissues share a common pathway for ethylene biosynthesis although the mechanism of regulation is different (21). It has been established in vegetative tissues that in general the only rate-controlling step in the ethylene pathway is the reaction converting SAM to ACC (21). However, some variations to that view have been advanced recently (17, 18), suggesting that in some circumstances or tissues, the conversion
of ACC to ethylene might also participate in the regulation of ethylene biosynthesis. Our data support the latter possibility, showing that tobacco leaf discs, which normally produce very small amounts of ethylene (3–5), gave relatively high rates of ACC-dependent ethylene production (Fig. 5A), and sucrose treatment further increased it 6-fold (Fig. 5B). However, it is important to note the following facts: (a) the sugars stimulatory effect has a lag time of several hours (9, 16), which may be due to the unavailability of ACC in freshly excised leaves (Fig. 3); (b) the sugars act synergistically with IAA which is known to exert its effect only on the step converting SAM to ACC (21, 23); and (c) untreated leaves had a relatively moderate ethylene production in response to CO₂ (3), whereas the response of ACC-treated leaves to CO₂ was very remarkable (Fig. 6). These observations suggest that the availability of ACC during the first 24 h is the limiting factor regulating ethylene production. It seems, therefore, that the stimulatory effect of sugars on the endogenous ethylene production (which is believed to represent an actual physiological situation in the leaf) occurs mainly through stimulation of ACC formation. This step is the rate-limiting one, at least during the initial period after excision (16). However, once the leaf becomes older and ACC is formed, its conversion to ethylene may become rate-limiting too, depending on the type of sugar present in the tissue.

The mechanism by which the carbohydrates stimulate ethylene production is not yet clarified. In view of our studies (6, 16), it seems most plausible that sugars in concert with IAA, as suggested previously for galactose in mung bean hypocotyls (9), promoted ethylene production by stimulating the step of ACC formation. Our results imply that the sugars may act by maintaining the endogenous levels of free IAA (16), thereby inducing increased ACC and ethylene production.

The present study suggests an additional mechanism, involving interaction between CO₂ and carbohydrates in stimulating the conversion of ACC to ethylene. Fifteen % CO₂ increased ACC-dependent ethylene production in untreated leaf discs by 23-fold (Fig. 6). Increasing the sucrose concentrations up to 50 mm, in the presence of a saturating level of 15% CO₂, caused a 3-fold increase in ACC-dependent ethylene production (Fig. 6B). This shows that sucrose by itself can directly promote the conversion of ACC to ethylene. However, the results shown in Figure 6A imply that most of the sucrose stimulation in this step can be attributed to an interaction between sucrose and the increased respiratory CO₂. The synergistic effect between sucrose and CO₂ becomes evident at very low sucrose concentrations, which may represent the physiological situation in leaves held in darkness. Thus, simultaneous application of only 1 mm sucrose with 15% CO₂ to the leaf discs, increased the CO₂-promotive effect on ethylene production from 23- to 50-fold (Fig. 6B). Regarding the CO₂-mediated effect of the sucrose, it seems that between 1 and 100 mm sucrose concentrations the system is not yet saturated by internal CO₂. Based on the ratio of 1:10 found between internal ethylene gas and the rate of its emanation from tobacco leaves (Fig. 1 of Ref. 5), the evolution rate of 45 ml CO₂/45 h obtained for tobacco leaf discs treated with 200 mm sucrose (Fig. 6A), corresponds with a level of about 10% endogenous CO₂. Indeed, this CO₂ level was found to saturate our system of tobacco leaf discs (unpublished data) and, similarly, 200 mm sucrose saturated both ACC-dependent (Fig. 6A) and endogenous ethylene production (Fig. 1 of Ref. 16). The discrepancy between our 10% CO₂ saturating level and the 1.5% CO₂ level obtained by Kao and Yang (11) can be ascribed to differences in the experimental conditions (darkness versus light), as well as in the tobacco species employed. The synergistic effect between CO₂ and sucrose in ACC-dependent ethylene production might suggest a different mode of action of these two agents in the mechanism of ethylene formation from ACC.

Besides involvement of IAA and CO₂ as mediators of the sugars-stimulating effect, results from the first two steps of the ethylene pathway, we may consider the possibility that the sugars can serve as energy suppliers of the ethylene system, probably via ATP. Accumulated data imply that increased ethylene production may be accompanied by increased new ATP synthesis (14) or higher ATP levels (15, 19) in the tissue.

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