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INDUCTION OF ETHYLENE BIOSYNTHESIS IN TOBACCO LEAF DISCS BY CELL WALL DIGESTING ENZYMES[1]

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SUMMARY: Cellulysin induces ethylene production in tobacco leaf discs by initiating the formation of 1-aminocyclopropane-1-carboxylic acid. Induction occurred within 30 to 60 min of incubation and was inhibited by aminoethoxyvinylglycine, and the antiproteases, PMSF and soybean trypsin inhibitor. Cycloheximide (CHI) at 2.8 µg/ml and chloramphenicol (CAP) at 100 µg/ml did not inhibit this induction although incorporation of the label from (3,4-^{14}C)methionine into the acid-insoluble fraction was inhibited by 57%. At 14 µg/ml CHI, and CAP, ethylene production was inhibited by 25% while protein synthesis was inhibited by 75%. We suggest that either the low amounts of protein synthesis that appear to be insensitive to CHI is sufficient to induce ethylene biosynthesis or that Cellulysin activates a preexisting but inactive form of ACC synthase to promote ethylene biosynthesis. Also, induction of ethylene production by microbial enzymes that digests plant cell walls may be an initial protective response of plants that serves to combat microbial infection.

Ethylene, the gaseous plant hormone, is produced, to varying degrees, by most higher plants and by microorganisms (1). Auxin treatment as well as mechanical or chemical wounding of higher plants lead to a considerable enhancement in the rate of ethylene production, particularly in those plant tissues that normally produce very little of this hormone (1-4). This type of stimulation of ethylene biosynthesis in higher plants has been related to the induction of 1-aminocyclopropane-1-carboxylic acid (ACC) accumulation as a result of enhanced conversion of S-adenosylmethionine (SAM) to ACC in the following metabolic sequence: methionine $\longrightarrow$ SAM $\longrightarrow$ ACC $\longrightarrow$ C_2H_4
The mechanism of this stimulation remains obscure, although it is apparently related to de novo synthesis of ACC synthase (3).

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In contrast to our knowledge of hormone- and wound-induced ethylene production in higher plants, little is known about the enhanced ethylene biosynthesis common to most host-parasite interactions during disease development. In the latter, the actual contribution of the host, or the pathogen, or both in stimulating ethylene biosynthesis is not yet established. However, one of the first events during the contact of a pathogen with the host plant involves secretion by the pathogen of enzymes that degrade plant cell walls (5). Fungi secrete a variety of extracellular plant cell wall digesting enzymes that are routinely used to obtain protoplasts from plant cells (6). In this context, we found it interesting that the fungal, cell wall degrading enzyme mixture, Cellulysin, markedly induced the production of ethylene in tobacco leaf discs. We report here that Cellulysin-induced ethylene biosynthesis is rapid, related to a considerable increase in the synthesis of ACC, and insensitive to low concentrations of the protein synthesis inhibitors, chloramphenicol and cycloheximide.

MATERIALS & METHODS

Tobacco plants (Nicotiana tobacum L. cv. Xanthi) were grown in a greenhouse under natural light at temperatures ranging between 20 and 30°C for 1-2 months. Leaves were washed with distilled water, surface-sterilized by soaking for 10 sec in 70% ethanol, and rinsed several times in sterile distilled water. Sterile technique was used in all subsequent handling of the tissue and glassware.

Discs (1 cm in diameter) were cut from leaf blades of fully expanded, deep-green leaves and were floated abaxially down under cool-white fluorescent light (1750 lux) for about 1 h in petri dishes containing, unless otherwise indicated, filter-sterilized basal medium containing 700 mM sorbitol, 10mM MES(pH 6.0), 10 mM $CaCl_2$, 50 µg/ml streptomycin sulfate and 50 units/ml penicillin G in the absence and presence of Cellulysin (Calbiochem). Six discs (70-100 mg fresh weight) were floated on 1 ml of filter-sterilized test solutions in 25-ml Erlenmeyer flasks. The flasks were sealed with rubber serum stoppers and incubated at 25°C in darkness.

Ethylene was allowed to accumulate for 1 to 2 h and quantified by gas chromatography (7). Between each sampling, the flasks were flushed with sterile fresh air. Radioactive ethylene produced by leaf discs incubated with (3,4-^{14}C)methionine was absorbed in 1 ml of ice-cold, 100 mM mercuric acetate (in methanol) and assayed in a liquid scintillation counter.

For ACC determination, leaf discs were homogenized in 70% ethanol, and the homogenate was centrifuged at 10,000 x g for 20 min. The supernatant was evaporated to dryness under vacuum at 40°C and the residue suspended in 1 ml of water. The solution was used directly or passed through a cation-exchange column (Dowex 50, H^+). In the latter case, ACC was then eluted with 2N NH_4OH and concentrated as before. ACC was then degraded to ethylene by the method of Lizada and Yang (8).

With a few exceptions, three replicates were used routinely. Experiments were repeated at least twice and gave reproducible results. Representative experiments are presented.

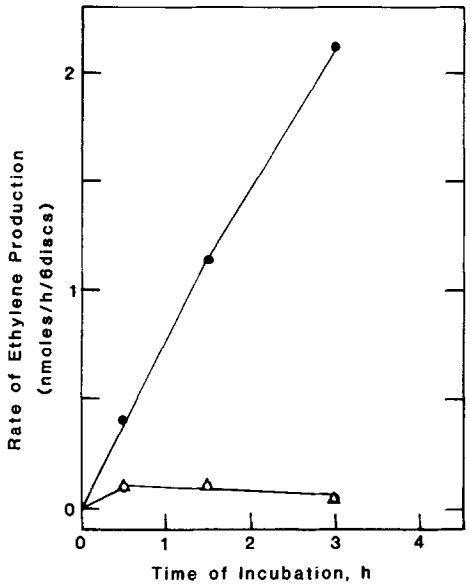

Fig. 1. Induction by Cellulysin of ethylene production in tobacco leaf discs. Leaf discs (6 per flask) prepared as described in Materials and Methods were floated on 1 ml of basal medium containing 600 mM sorbitol, 10mM $CaCl_2$, 10 mM MES, pH 6.0, 50µg/ml streptomycin sulfate and 50 U/ml penicillin G in the absence (○) and presence of either 1.3% Cellulysin (●) or 0.05mM IAA (△). Rate of ethylene production is shown in relation to the time of incubation.

RESULTS

Previously Aharoni and Lieberman (9) reported that the rate of endogenous ethylene production by tobacco leaf discs, floating on water in the dark, was very low, taking as many as 7-8 days to reach 40-80 pmoles/h/g. A rapid induction of ethylene production was observed when tobacco leaf discs were treated with Cellulysin (Fig. 1). The Cellulysin-dependent ethylene production occurred in less than 1 h and rates (2120 pmoles/h/6 discs) which were over 17-fold greater than the control were reached within 3 h(Fig. 1). In the same time period, boiling Cellulysin or supplementing IAA in the absence of Cellulysin were ineffective in stimulating ethylene production (Fig. 1). In some experiments, a lag of less than 0.5 h was observed in Cellulysin-induced ethylene production. The induction of ethylene production occurred prior to the detection of protoplasts in the medium, using a light microscope. Over the 2 years during which this investigation was carried out variation was found in the rate of ethylene production induced by Cellulysin. Much of this variability was related to the age of the leaf, from which discs were prepared.

Table 1. Inhibition of Cellulysin-induced ACC formation and ethylene production by aminoethoxyvinylglycine(AVG) in tobacco leaf discs

Additions to the incubation medium*	C_2H_4 nmoles/h/g fr wt.	ACC nmoles/h/g fr wt.
None	0.25	0.12
Cellulysin	3.03	1.83
Cellulysin + 0.1 mM AVG	0.20	0.07

*The concentration of Cellulysin was 2%. Ethylene production was measured between 0-2h. ACC was isolated from the leaf discs after a 6 h incubation period and the concentration determined as described in Materials and Methods.

The marked increase in ethylene production by tobacco leaf discs in contact with Cellulysin was accompanied with an increase in the formation of ACC (Table 1), suggesting that the conversion of SAM to ACC is the target of Cellulysin action. This conclusion is supported by the inhibition of both the Cellulysin-induced ethylene production and ACC formation by aminoethoxyvinylglycine (AVG) (Table 1), a potent inhibitor (10,11) of ACC synthase which catalyzes the SAM → ACC reaction.

Cycloheximide(CHI) (10 μM), an inhibitor of cytoplasmic protein synthesis, did not block Cellulysin-mediated induction in ethylene biosynthesis (Table 2), shedding doubts on the involvement of de novo protein synthesis in this induction. This was further tested in experiments using (3,4-^{14}C)methionine supplemented with 1mM ^{12}C-methionine (to adjust for pool saturation) as a substrate for ethylene biosynthesis as well as protein synthesis.

Cellulysin induced the conversion of (3,4-^{14}C)methionine into $^{14}C_2H_4$, which was comparable to the rise in total ethylene production over the controls (Table 2). Under these conditions, Cellulysin caused 22% inhibition in the uptake of radioactive methionine and 42% inhibition in the incorporation of radioactivity into acid-insoluble material. Inclusion of CHI (10μM) and chloramphenicol(CAP) (100μg/ml) along with Cellulysin in the incubation medium further inhibited uptake of radioactive methionine by 14% and its incorporation into acid-insoluble (protein) radioactivity by 57%, but neither total ethylene production (+10%) nor ^{14}C-methionine incorporation

Table 2. Effect of chloramphenicol(CAP), cycloheximide(CHI), isopropanol and PMSF on Cellulysin-induced ethylene production and on protein synthesis*

Addition to the basal medium	Total C_2H_4 nmoles	$^{14}C_2H_4$	Protein (PCA-insoluble)	^{14}C-met Uptake	Ratio $^{14}C_2H_4$/ ^{14}C-protein
		----------nCi----------			
None	0.11	0.04	3.84	93.0	0.01
Cellulysin	1.43	0.66	2.21	72.6	0.30
Cellulysin + CAP + CHI (10μM)	1.57	0.60	0.96	69.9	0.63
Cellulysin + CAP + CHI (50μM)	1.07	0.26	0.56	65.3	0.46
Cellulysin + isopropanol	1.18	0.43	1.79	82.4	0.24
Cellulysin + PMSF	0.56	0.22	1.50	76.2	0.15

*Each data point is the mean of 2, 6 leaf disc replicates, prepared as described in the "Materials & Methods". Discs were preincubated in 1 ml of filter-sterilized basal medium containing 1μCi L-(3,4-^{14}C)methionine (50μCi/μmol),1μmole ^{12}C-methionine and additions listed in the table. After 3 hr, total C_2H_4 and $^{14}C_2H_4$ were determined. Discs were then rinsed with basal medium and stored at -20°C until extraction with perchloric acid. The concentrations of various additives were: Cellulysin, 1.2%; CAP, 100 μg/ml; CHI, as indicated; PMSF in isopropanol, 1mM; isopropanol control for PMSF, 10μl.

into $^{14}C_2H_4$ (-8%) were affected (Table 2). Only at much higher concentrations (50μM) was CHI effective in inhibiting both the total ethylene production (by 25%) and radioactive ethylene (by 61%), when 75% of radioactivity into acid-insoluble material was inhibited. Therefore, under conditions when 57% of protein synthesis is inhibited neither total ethylene nor radio-labeled ethylene formation were affected. Indeed, the ratio of $^{14}C_2H_4/^{14}C$-protein in Cellulysin-treated tissue, which was 0.3, increased to 0.63 in the presence of 10μM CHI and CAP, and to 0.46 when 50μM CHI was used (Table 2).

It was suspected that Cellulysin-induced ethylene production may involve a modification of the already present but inactive ACC synthase, e.g. by a protease-mediated activation phenomenon observed elsewhere (12,13)in other systems. Therefore, ethylene production and ACC levels were analyzed in tobacco leaf discs after incubation with Cellulysin in the absence and presence of known protease inhibitors. The results (Table 3) show that

Table 3. Effect of protease inhibitors on Cellulysin-mediated increase in ACC level and rate of ethylene production*

Addition to the basal medium	Ethylene Production (nmoles/h/g fr. wt.)	ACC (nmoles/h/g fr. wt.)
None	0.25	0.11
Cellulysin	3.10	1.84
Cellulysin + PMSF	0.40	0.05
Cellulysin + trypsin inhibitor(1 mg)	1.83	0.50
Cellulysin + Pepstatin A (0.1 mg)	3.10	1.39

*Details are given in the text and in the legend to Table 1.

phenylmethanesulfonic acid (PMSF) and soybean trypsin inhibitor, but not Pepstatin A (an inhibitor of carboxyl proteases), markedly inhibit ethylene production induced by Cellulysin and that the inhibition parallels a corresponding decrease in the level of ACC. There was some inhibition of ACC formation but no inhibition of ethylene production by Pepstatin A (Table 3). This experiment, however, did not rule out the possibility that the serine-type protease inhibitors could also prevent any contaminating protease present in Cellulysin from acting on the leaf discs, if, in fact, that was the mechanism of induction of ethylene production by Cellulysin. To test this, Cellulysin was preincubated with PMSF for 30 min to inactivate any serine-type protease or esterase. The precipitated protein was centrifuged down and supernatant gel-filtered through a column of Sephadex G-25 (30 ml bed volume) to remove any free PMSF present and tested for ethylene-producing activity in tobacco leaf discs. PMSF-treated Cellulysin was as effective in stimulating ethylene biosynthesis as the untreated enzyme mixture (Table 4), while the addition of PMSF to the incubation medium inhibited Cellulysin-induced ethylene production. PMSF inhibited production of both the total and radioactive ethylene from (3,4-^{14}C)methionine in the presence of Cellulysin (Table 2) confirming effective inhibition of Cellulysin-induced ethylene production by PMSF. During the short incubation periods used, CHI, PMSF and

Table 4. Pretreatment of Cellulysin with PMSF does not prevent Cellulysin-induced ethylene production*

Addition to the basal medium	Ethylene production (nmoles/2h/6 discs)
None	0.08
Cellulysin	1.64
Cellulysin + isopropanol	1.37
Cellulysin + PMSF	0.83
Isopropanol-treated Cellulysin after gel filtration	1.67
PMSF-treated Cellulysin after gel filtration	1.54

*For details see text and the legend to Table 1. Incubation period was 2 hr.

Pepstatin A did not affect the conversion of exogenously applied ACC to ethylene, while trypsin inhibitor slightly stimulated this conversion (data not shown).

DISCUSSION

We have demonstrated a rapid induction of ethylene biosynthesis in tobacco leaf discs by Cellulysin treatment. This induction appears to be related to a tremendous increase in the activity of ACC synthase as judged by the formation of ACC and inhibition by AVG. In this respect, these results are similar to stress- (3,14) and auxin-induced (4) ethylene production where SAM→ACC conversion has been invoked as the rate-limiting step. Such an increase in the activity of ACC synthase has been attributed to de novo synthesis because of its inhibition by relatively high concentrations of CHI (3). However, doubts about this conclusion have been expressed (14,21) and no direct proof of actual de novo synthesis of ACC synthase has been reported.

In the phenomenon described here, either the low amount of protein synthesis that occurs in the presence of CHI is sufficient to induce ethylene biosynthesis at the level of ACC synthase activity or, alternatively, in certain situations, the increase in ACC levels and ACC synthase can occur by a

post-translational modification, involving modification of a pre-existing, enzymatically inactive protein. The absence of lag, specificity of inhibition of both Cellulysin-mediated ethylene production and ACC formation by a serine protease inhibitor, PMSF(since the carboxyl protease inhibitor, Pepstatin A, was not inhibitory), and ineffectiveness of CHI to inhibit this process support our contention that a post-translational modification/activation of an "inactive" ACC synthase may be involved. Also, we have observed (15) that PMSF inhibits the increase but not the activity of ACC synthase in wounded tomato fruit slices. However, elucidation of the activation mechanism alluded to above awaits preparing antisera to pure ACC synthase and analyzing the content of this protein in tissues unactivated and activated for ethylene production.

Cellulysin-mediated increase in ethylene biosynthesis demonstrated here has other implications. For example, the marked increase in ethylene production during host-parasite interactions (16,17) may be caused by an interaction between cell wall degrading enzyme(s) secreted by the pathogen and the host plant tissue in the same manner as reported here. Such a rapid induction of ethylene production by a pathogenic organism could be a defense mechanism involving a hypersensitive reaction (18) by which plants eliminate infected areas and/or possibly a mechanism which enhances the pathogencity or virulence of the parasite.

Finally, ethylene is known to cause abscission of leaves and fruits by inducing cellulase and polygalacturonase activity in the abscission zone (19,20). It remains to be understood whether this induction of cellulase and polygalacturonase activities is a mechanism by which the initial trigger caused by ethylene leads to continued, self-activation of ethylene production via the stimulation of ACC synthase by cellulase and/or polygalacturonase. This phenomenon would generate a cyclic process until the leaf or fruit abscisses.

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