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Role of Peroxidase During Ethylene-Induced Chlorophyll Breakdown in *Cucumis sativus* Cotyledons

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Abstract. Yellowing is a visible result of ethylene-enhanced senescence. In certain plants, such as *Cucumis sativus*, an increase in peroxidase levels occurs during this period. Experiments described here were designed to test the hypothesis that peroxidase levels induced during senescence play a role in chloroplast degradation. Inhibitors of heme synthesis and protein glycosylation, which had no effect on chlorophyll degradation, reduced the synthesis of pI 9 peroxidase. Decapitation of seedlings, which enhanced greening of cotyledons, increased levels of peroxidase. These observations are consistent with the view that while the role of aging- or ethylene-induced peroxidases are not known, they are not involved in chlorophyll degradation.

The general pattern of senescence of cucumber cotyledons follows that observed in other plants. Butler (1967), studying ultrastructural changes in cucumber cotyledons during 50 days of development and senescence, observed that the degradation of chloroplasts was initiated by 25 days, ribosomes by 26 days, mitochondria and tonoplasts by 31 days, and plasma membrane and nuclei by 36 days. Lewington et al. (1967), who also examined cucumber cotyledon senescence, observed that peroxidase levels increased 10-fold during the first 40 days of development and remained high for 65 days. At the same time total protein, chlorophyll, RNA, but not DNA, were degraded. Both structural and DNA content data suggest that the nucleus remains intact until the cell dies. These observations suggest that peroxidases are involved in some aspect of either degradation, mobilization, or export of cellular constituents. The synthesis, location, and other possible functions of plant peroxidases, such as lignification, has been reviewed (Van Huystee 1987).

The experiments reported in this article reexamine the role of peroxidase in

chlorophyll degradation. Earlier work by Abeles et al. (1989) evaluated the suggestion by Matile (1980) that peroxidase plays a role in chlorophyll degradation. This hypothesis is supported by the evidence that peroxidases destroy chlorophyll in vitro (Matile 1980), peroxidase activity increases in senescing cucumber cotyledons (Abeles et al. 1988; Lewington et al. 1967), and that pI 9 peroxidase is localized next to starch grains inside the chloroplast (Abeles et al. 1989). On the other hand, we observed that there was no direct correlation between chlorophyll content and pI 9 peroxidase when cucumber seedlings were treated with other compounds that retard or promote senescence (Abeles et al. 1989). Previously, Ford and Simon (1972) showed that both chlorophyll and peroxidase levels were increased in the cotyledons of decapitated cucumber seedlings. This observation suggests that peroxidase is associated with some other aspect of aging in these decapitated plants.

The chlorophyll catabolism hypothesis was evaluated here by treating cotyledons with compounds that are known to inhibit protein synthesis, protein glycosylation, or heme synthesis. Since all three processes are essential for peroxidase synthesis, these experiments can test the idea that it is possible to differentially reduce peroxidase levels without effecting chlorosis. Elimination of chlorophyll degradation as a role for peroxidase would enable us to focus our attention on other possible functions for peroxidase in aging leaf or fruit (Rothan and Nicolas 1989) tissue.

Materials and Methods

Plant Material

Cucumis sativus cv. Poinsett 76 seeds were purchased from W. Atlee Burpee Co. (Warminster, PA, USA). Plants were grown in the greenhouse or in the dark in 57 × 27 × 7 cm trays or 10 × 10 cm pots filled with vermiculite and watered with 1 g L^{-1} Peters soluble 20-20-20 fertilizer (W. R. Grace & Co., Allentown, PA, USA).

Ethylene Treatment

For the experiments described in Tables 1–3, cotyledons were cut from 2-week-old seedlings, submersed for 30 min in the indicated solutions containing 0.05% Triton X-100, and then placed on moist Whatman No. 3 filter paper in 9-cm Petri plates. The plates were stored in 4-L plastic containers fitted with rubber vaccine stoppers and stored in the dark at 37°C. Ethylene was injected into the containers with a syringe.

In the decapitation experiment shown in Table 4, 2-week-old seedlings were decapitated by excising the tissue above the cotyledons and were then grown for an additional 2 weeks in the greenhouse.

Peroxidase Assay

The pI 9 and pI 4 peroxidases were purified, and antibodies to these proteins were raised in rabbits (Abeles et al. 1988). Peroxidase was isolated from acetone powders of 10 cucumber cotyledons (~2 g fresh weight) extracted with 2 ml of 0.1 M potassium phosphate buffer (pH 6.8) and measured using a radial immunodiffusion assay (Abeles et al. 1989). Only the pI 9 form of the peroxidase was assayed for most of the experiments described herein because antibodies to the pI 4 isozyme were not available until most of the experiments were complete. However, as shown in Table 5, both the acidic and basic peroxidase isoenzymes, normally increased at the same time. The minimum level of peroxidase detectable with the radial diffusion assay was 4 μg/ml.

Chlorophyll Assay

Chlorophyll was extracted, from a piece of tissue cut from the upper 1.5 cm of a cotyledon, in 2 ml of 80% ethanol heated to 80°C for 10 min, and the absorbance at 652 nm measured. Five cotyledons from etiolated seedlings (Table 5) were used, and the heating period was extended to 1 h. Data shown are the average of five samples.

Inhibitors

Gabaculin was purchased from Fluka Chemical Corp. (Ronkonkoma, NY, USA). The GABA (γ-aminobutyric acid) analogs, 4-amino-4-hexynoic acid (acetylenic-GABA, MDL 71,645) and 4-amino-5-fluoropentanoic acid (fluoromethyl-GABA, MDL 71,109) were gifts of Dr. Ekkehard H. W. Bohme (Merrell Dow Research Institute, Merrell Dow Pharmaceuticals Inc., Cincinnati, OH, USA). The action of gabaculin and fluoromethyl-GABA on chlorophyll synthesis was tested to verify that physiologically appropriate concentrations were employed in these experiments. Dark-grown, 5-day-old cotyledons were soaked in these inhibitors for 10 min before exposing them to light. Chlorophyll synthesis was inhibited 62% by 1 mM gabaculin and 73% by 1 mM fluoromethyl-GABA compared to untreated controls.

MDMP [2-(4-methyl-1,6-dinitroanilino)-*N*-methyl propionamide], (racemic mixture) was a gift of Dr. R. Romani (University of California, Davis, CA, USA).

Effect of Ethylene on Chlorophyll Synthesis

Cotyledons from 1-week-old etiolated plants were excised in dim light and preincubated with air or 100 μl/L ethylene in the dark for 18 h. Samples from each group were then placed in the light for an additional 6 h in the presence or absence of ethylene.

Table 1. Effect of MDMP and cycloheximide on chlorophyll and pI 9 peroxidase levels in ethylene-treated cucumber cotyledons.

Treatment	Air		Ethylene (100 μl/L)	
	Chlorophyll (mg/g fw)	Peroxidase (μg/g fw)	Chlorophyll (mg/g fw)	Peroxidase (μg/g fw)
Initial (0 day)	0.80a	<0.8d*		
Control (2 days)	0.36cd	6.6b	0.25e	36.0a
5 μM MDMP	0.53b	3.6c	0.32de	3.8c
10 μM MDMP	0.48b	<0.8d	0.44bc	<0.8d
Initial (0 day)	0.76a	<0.8c		
Control (2 days)	0.47cd	3.8b	0.38de	12.0a
100 μM cycloheximide	0.47cd	3.0b	0.33e	3.4b
500 μM cycloheximide	0.54bc	<0.8c	0.58bc	<0.8c

fw, fresh weight.
The two experiments shown were performed at separate times. Similar letters within a column indicate the means were identical at the 5% level.
* The minimum sensitivity of the peroxidase assay was 0.8 μg/g fresh weight.

Statistics

Data were compared by Duncan's multiple range test. Different letters in the tables indicate that the means were different at the 5% level.

Results and Discussion

Two inhibitors of protein synthesis often used in plant studies are D-MDMP (Weeks and Baxter 1972) and cycloheximide (Frenkel et al. 1968). As shown in Table 1, it was not possible to inhibit peroxidase synthesis without blocking the loss of chlorophyll. As previously observed by de Laat et al. (1981), MDMP was more effective than cycloheximide as a protein synthesis inhibitor. Since it is assumed that chlorophyll degradation requires protein synthesis (Thomas 1976), the effect described in Table 1 suggests that application of these inhibitors causes a simultaneous inhibition of chlorophyll-degrading enzymes as well as peroxidase.

Evidence suggesting that peroxidase synthesis was not needed for chlorophyll breakdown was obtained with the antibiotic tunicamycin. Tunicamycin inhibits protein glycosylation and has been shown to inhibit tomato ripening (Handa et al. 1985). The data in Table 2 indicated that tunicamycin did not reduce chlorophyll breakdown but did inhibit both endogenous and ethylene-induced peroxidase synthesis.

The transaminase inhibitors gabaculin and fluoromethyl-GABA block the synthesis of the precursor porphyrin, 5-aminolevulinic acid and have been used to inhibit the synthesis of chlorophyll and phytochrome (Gardner et al. 1988). Fluoromethyl-GABA and acetylenic-GABA are irreversible inactivators of GABA transaminase (Silverman and Levy 1980). As shown in Table 3, ga-

Table 2. Effect of tunicamycin on the induction of pI 9 peroxidase and chlorophyll levels in cucumber cotyledons.

Treatment	Air		Ethylene (100 μl/L)	
	Chlorophyll (mg/g fw)	Peroxidase (μg/g fw)	Chlorophyll (mg/g fw)	Peroxidase (μg/g fw)
Initial (0 day)	0.65b	<0.8d		
Control (2 days)	0.44cd	5.2b	0.35d	20.8a
0.03 μg/ml tunicamycin	0.74a	2.4c	0.44cd	6.0b
0.11 μg/ml tunicamycin	0.48c	<0.8d	0.36d	4.2b

fw, fresh weight.
Similar letters within a column indicate the means were identical at the 5% level.

Table 3. Effect of transaminase inhibitors on chlorophyll levels and the induction of pI 9 peroxidase in cucumber cotyledons.

Treatment	Air		Ethylene (100 μl/L)	
	Chlorophyll (mg/g fw)	Peroxidase (μg/g fw)	Chlorophyll (mg/g fw)	Peroxidase (μg/g fw)
Initial (0 day)	0.62a	<0.8c		
Control (2 days)	0.53abc	<0.8c	0.24f	37.4a
Gabaculin	0.44bcd	<0.8c	0.31f	19.2b
Actylenic-GABA	0.54ab	<0.8c	0.24f	17.6b
Fluoromethyl-GABA	0.40de	<0.8c	0.21f	14.5b

fw, fresh weight.
Similar letters within a column indicate the means were identical at the 5% level.
Gabaculin, 4-amino-4-hexynoic acid (acetylenic-GABA), and 4-amino-5-fluoropentanoic acid (fluoromethyl-GABA) were used at a concentration of 5 mM.

baculin and the GABA analogs did not reduce chlorophyll breakdown, but did inhibit ethylene-induced peroxidase synthesis.

The data in Table 4 confirm earlier observations by Ford and Simon (1972) that decapitation of cucumber seedlings results in an increase in both chlorophyll and peroxidase levels. While the mechanism for enhanced greening of tissue normally destined to senescence is not known, it is possible that cytokinins produced by the roots are involved in the process (Dei 1978).

The tacit assumption in the above experiments is that ethylene promotes yellowing by initiating the synthesis of chlorophyll-degrading enzymes. However, it is also possible that pigment synthesis is also important in determining chlorophyll levels, and that ethylene action may be due to an inhibition of chlorophyll synthesis. The data in Table 5 indicate that ethylene reduced chlorophyll synthesis if it was applied to etiolated cotyledons in both the dark and light phase of a chlorophyll synthesis experiment. On the other hand, ethylene had no effect if present in only the light or dark phase. A simple interpretation of these results is not possible, and it appears that chlorophyll synthesis is reduced if events initiated by ethylene in the dark are allowed to continue in

Table 4. Effects of decapitation on chlorophyll and peroxidase levels in cucumber cotyledons.

Treatment	Chlorophyll (mg/g fw)	Peroxidase (μg/g fw)	
		pI 9	pI 4
Initial (2 weeks)	0.65b	<0.8d	3.6c
Intact (4 weeks)	0.67b	<0.8d	5.8b
Decapitated (4 weeks)	1.20a	4.8b	16.6a

fw, fresh weight.
Similar letters within a column indicate the means were identical at the 5% level.
Initial seedlings were 2-weeks-old. The levels of chlorophyll and peroxidase of cotyledons were measured in both intact and decapitated seedlings after an additional 2-week growth period.

Table 5. Effect of ethylene (100 μl/L) on chlorophyll synthesis in 1-week-old etiolated cucumber cotyledons.

Dark period (18 h)	Light period (6 h)	Chlorophyll (μg/cotyledon)
Air	Air	4.80a
Air	Ethylene	4.68a
Ethylene	Air	4.44a
Ethylene	Ethylene	3.36b

Similar letters within a column indicate the means were identical at the 5% level.
Initial level of chlorophyll was 0.36 μg/cotyledon.

the light. Earlier work by Alscher and Castelfranco (1972) with cucumber cotyledons and Buhler et al. (1978) with *Sinapis alba* L. (mustard) indicated that treating seedlings or cotyledons with ethylene in the dark before exposing them to light caused an increase in chlorophyll synthesis. However, Alscher and Castelfranco (1972) also observed that inhibition of chlorophyll synthesis was observed if cotyledons were exposed to a 50-s light flash before the dark incubation. In the experiments reported here, the cotyledons were isolated in dim room light of sufficient intensity to permit assembling the experiment and then incubated in the dark during the 18-h ethylene treatment. It is likely that our dim room light and Alscher and Castelfranco's 50-s light flash had a similar physiological effect, facilitating the inhibition of chlorophyll synthesis by ethylene.

In conclusion, the observations, that it was possible to decrease peroxidase synthesis without decreasing chlorophyll degradation and that decapitation led to simultaneous increase in both peroxidase and chlorophyll content, are consistent with the idea that peroxidase does not mediate chlorophyll degradation.

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