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FATTY ACID HYDROPEROXIDE LYASE IN TOBACCO CELLS CULTURED *IN VITRO*

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Key Word Index—*Nicotiana tabacum*, Solanaceae, cultured tobacco cells, green cells, fatty acid hydroperoxide lyase, linoleic acid, linolenic acid, C_6-aldehydes

Abstract—Fatty acid hydroperoxide lyase (HPO lyase) was found in green and non-green tobacco cells cultured *in vitro* The HPO lyase activity in non-green cells was $\frac{1}{3}$–$\frac{1}{2}$ of that in green cells When the cells were transferred from the light to dark conditions or *vice versa*, cells turned non-green or green according to the light conditions The HPO lyase activity also changed according to the light conditions, but the changes in HPO lyase activities were not proportional to the changes in chlorophyll contents These results suggest that at least two types of HPO lyases are present in the green cells One type of HPO lyase is perhaps common both to the green and non-green cells, another one is chloroplastic The fatty acid compositions of cells and substrate specificities of HPO lyase differed between green and non-green cells.

INTRODUCTION

Many higher plants have the ability to produce volatile C_6-aldehydes, hexanal, *cis*-3-hexenal and *trans*-2-hexenal etc, which with their corresponding alcohols are largely responsible for characteristic odour of green leaves [1, 2] The major biosynthetic pathway for these C_6-aldehydes consists of three sequential enzymic reactions, acyl hydrolysis of lipids, peroxidation of linoleic and linolenic acids, and cleavage of the fatty acid hydroperoxides [1] The enzymes involved in the above three stepwise biosynthetic reactions are lipolytic acyl hydrolase [3, 4], lipoxygenase [5, 6] and HPO lyase* [5, 6], respectively Volatile C_9-aldehydes and their alcohols are formed by a similar pathway [5, 7] From 13-hydroperoxides of the fatty acids, 12-oxo-*cis*-9-dodecenoic acid also was formed with its derivatives as the counterparts of C_6-aldehydes by the action of HPO lyase [6, 8] This C_{12}-oxo acid is presumed to be a precursor of traumatin, a wound hormone [9, 10]

HPO lyase was identified first in non-green tissues, watermelon seedlings [6] and cucumber fruits [5, 11] Subsequently, the occurrence of this enzyme was reported also in green tissues, such as peels of cucumber fruits [12], leaves of kidney bean [13, 14] and tea [15], and other plant leaves [16], as well as in non-green tissues like etiolated seedlings [17] and fruits [18–20] Wardale *et al* [12] suggested that HPO lyase occurs in multiple forms We also reported the possible presence of two types of HPO lyases [16] However, little is known about the multiple forms of HPO lyase and their properties

The present paper describes the occurrence of HPO lyase in cultured tobacco cells which turn green and non-green according to the light conditions The results indicate the presence of two types of HPO lyases in green cells and one type in non-green cells Some properties of HPO lyase present in green and non-green cells are also discussed

RESULTS

Cell growth and HPO lyase activity in green cells cultured in light

The cell line used in this experiment grew well in the light maximum content of chlorophyll was *ca* 200 μg/g fr wt Cells started growing in the presence of light without a significant lag phase after inoculation, and reached stationary phase after 3 weeks (Fig 1) The chlorophyll content of cells, however, decreased in the early log phase to 50% of that of the maximum value, but

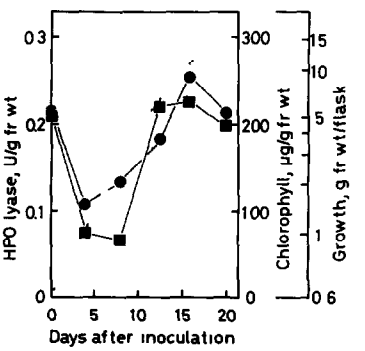

Fig 1 Cell growth, chlorophyll content and HPO lyase activity of cells cultured in light The green cells cultured in light were inoculated in a fresh medium and cultured for 20 days in light (LL) Cell growth, ( ), chlorophyll content, (●), HPO lyase activity, (■)

*Abbreviations used BSA, bovine serum albumin, 13-HPOLA, 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid, 13-HPOLNA, 13-L-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid, 9-HPOLA, 9-D-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid, 13-HPOLMe, methyl ester of 13-HPOLA, 13-HPOLNMe, methyl ester of 13-HPOLNA, 13-HOLA, 13-L-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid, HPO lyase, fatty acid hydroperoxide lyase

it reached a maximum in the late log phase The chlorophyll content of 200 μg/g fr wt was maintained during the late log and early stationary phase (Fig 1) HPO lyase activity in the cells was *ca* 0 2 U/g fr wt immediately after the inoculation and in the stationary phase The activity in the early log phase cells, however, declined to 0 07–0 10 U/g fr wt, $\frac{1}{3}$–$\frac{1}{2}$ of the activity noted in the freshly inoculated cells This initial low activity of HPO lyase was restored in the late log phase (Fig 1) The changes in the HPO lyase activity have been found to be proportional to the changes in chlorophyll content These changes in cell growth, chlorophyll content and HPO lyase activity recurred during the successive passages of subcultures

Cell growth and HPO lyase activity in non-green cells cultured in dark

When the green cells cultured in light were transferred to dark conditions and subcultured continuously in the dark, the cells turned non-green As such the cells did not contain an appreciable amount of chlorophyll These cells grew well even in the dark (Fig 2), but the growth rate was slower than that of the green cells in the light The cell growth reached stationary phase 25–30 days after the inoculation HPO lyase activity in the cells cultured in the dark was 0 06–0 10 U/g fr wt during the whole course of the culture (Fig 2) This value remained constant in successive passages during subcultures

Effect of transfer from light to dark conditions on HPO lyase activity

The green cells cultured continuously in the light were inoculated to a fresh medium, and cultured in the dark A slight decrease in growth rate of the cells was found after the transfer to the dark (Fig 3) Chlorophyll content decreased rapidly after the transfer; *ca* 30 μg/g fr wt at the end of the first passage The cells turned completely non-green at the end of the second passage However, the pattern of HPO lyase activity in the first passage was similar to that of the green cells (Fig 3) In the second passage, the HPO lyase activity decreased and reached that of non-green cells at the end of the second passage The rate of decrease in enzyme activity was not parallel to the decrease in chlorophyll

Effect of transfer from dark to light conditions on HPO lyase activity

When the non-green cells which had been cultured continuously in the dark were transferred to a fresh medium and subcultured in light, the cells grew rapidly even in the first passage after the transfer (Fig 4) Chlorophyll content increased from a negligible amount to 100 μg/g fr wt in the first passage and, in the third passage, reached the same value (*ca* 200 μg/g fr wt) as that of the green cells

In the first passage after transfer to the light, HPO lyase had a low activity, similar to that of the non-green cells, although the chlorophyll content increased (Fig 4) In the late log phase of the second passage, HPO lyase activity increased to the level between those of cells in light and dark, and the activity, along with chlorophyll content, reached a level similar to that of green cells in the third passage During further subcultures, the green cell type of

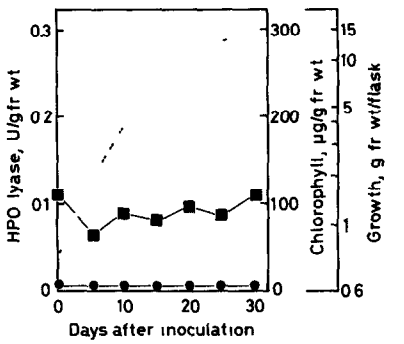

Fig 2 Cell growth, chlorophyll content and HPO lyase activity of cells cultured in dark The non-green cells cultured in dark were inoculated in a fresh medium and cultured for 30 days in dark (DD) Symbols are as in Fig 1

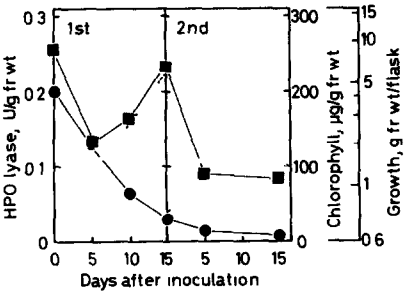

Fig 3 Effect of transfer from the light to the dark on the chlorophyll content and HPO lyase activity The green cells cultured in the light were inoculated in a fresh medium and cultured in the dark (1st passage) Then, the cells were again inoculated and cultured in the dark (2nd passage) Symbols are as in Fig 1

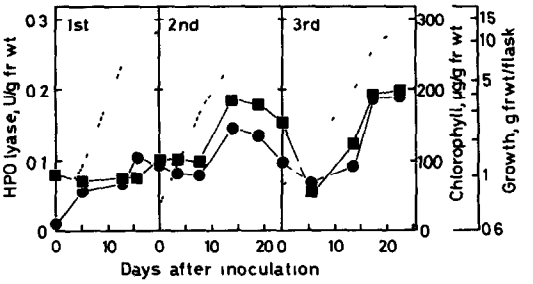

Fig 4 Effect of transfer from the dark to the light on chlorophyll content and HPO lyase activity The non-green cells cultured in dark were inoculated in a fresh medium and cultured in the light (1st passage) Then, the cells were again inoculated and cultured in the light (2nd and 3rd passages) Symbols are as in Fig 1

HPO lyase activity and the high chlorophyll content were maintained

Thus, when the non-green cells cultured in the dark were transferred to the light condition, both HPO lyase activity and chlorophyll content finally shifted to those of the green cells

Substrate specificity of HPO lyase

When 13-HPOLA was used as the substrate, hexanal was produced, the ratio of the activity of the green cells

(LL) to that of non-green cells (DD) was *ca* 3 (Table 1) 13-HPOLA was the best substrate among the compounds tested HPO lyase of the green cells (LL) utilized 13-HPOLNA as the substrate better than that of the non-green cells (DD) The reaction ratios with 13-HPOLNA/13-HPOLA were 0 5 for the green cells (LL) and 0 23 for the non-green cells (DD) (Table 1) HPO lyases from other types of cells such as LL_5, LD and DL showed $\frac{1}{3}$–$\frac{1}{2}$ of the green cell enzyme activity with 13-HPOLA (Table 1) In these cases, the ratios of the enzyme activities with 13-HPOLNA/13-HPOLA were 0 21–0 26, comparable to non-green cells (DD) 13-HPOLMe and 13-HPOLNMe were poor substrates 9-HPOLA which is a precursor for C_9-aldehydes, and 13-HOLA did not act as the substrates for HPO lyase of cultured tobacco cells

Fatty acid composition

When the cells were transferred from the light to dark conditions or *vice versa*, HPO lyase activity changed according to the light conditions Table 2 shows the fatty acid compositions of the cells cultured under the different culture conditions The major fatty acids were palmitic, linoleic and linolenic acids The result obtained from the green cells (LL) was consistent with that in ref [21] Of the total fatty acids, palmitic acid accounted for 21–24%, while linoleic and linolenic acids together constituted 70–74% The ratios of linoleic acid to linolenic acid varied according to the light condition In the green cells (LL) cultured for 20 days in the light, linolenic acid was the predominant species (40% of the total fatty acids), which was followed by linoleic (34%) and palmitic acids (21%) In case of dark cultured non-green cells (DD), linoleic acid was predominant (56%), followed by palmitic (23%) and linolenic acids (15%) This high content of linolenic acid in green cells may arise from the development of the chloroplast thylakoid [22]

When the green cells were transferred to the dark (LD), linolenic acid decreased to 20% and linoleic acid increased to 52%, the fatty acid profile registered a shift toward that of the non-green cells On the other hand, when the non-green cells were transferred to the light (DL), linoleic acid decreased to 35% and linolenic acid increased to 37% The fatty acid profiles of these cells (LD and DL) were intermediate between those of the green (LL) and non-green cells (DD) The fatty acid, *trans*-3-hexadecenoic acid, specific to chloroplast thylakoid [22] was found in the green cells (LL) and the cells transferred from the dark to light condition (DL), although in small amounts The fatty acid compositions of cells were not proportional to the HPO lyase activities, but could be correlated with chlorophyll contents

The green cells (LL_5) harvested at the fifth day had the intermediate type of the fatty acid composition (Table 2) This is probably due to delayed development of the chloroplast

In tobacco green leaves, *cis*-7,*cis*-10,*cis*-13-hexadecatrienoic acid was involved in monogalactosyldiglyceride [23] However, no appreciable amount of this fatty acid was found in tobacco green cells cultured in light [21]

Some properties of HPO lyase in green and non-green cells

Optimal pH of HPO lyase was 7 for the green cells and 7–8 for the non-green cells (Fig 5) K_m values were 1 72 mM with HPO lyase from the green cells (LL) and 0 12 mM with that from the non-green cells (DD), when 13-HPOLA was used as the substrate in the concentration range of 0 1–0 6 mM

HPO lyase activity was found in the chloroplast fraction of green cells, when chloroplasts were isolated (data not shown) This chloroplast fraction showed nitrite reductase activity but not activities of either cytochrome *c* oxidase or glyoxylate reductase

Table 1 Substrate specificities of HPO lyase

Cells inoculated	Culture condition		Cell type	HPO lyase activity (U/g fr wt)		
	Light or dark	Day		with 13-HPOLA (A)	with 13-HPOLNA (B)	B/A
Green cells	Light	20	LL	0 235	0 117	0 50
Green cells	Light	5	LL_5	0 069	0 018	0 26
Green cells	Dark	20	LD	0 112	0 023	0 21
Non-green cells	Light	20	DL	0 110	0 029	0 26
Non-green cells	Dark	30	DD	0 073	0 017	0 23

Table 2 Fatty acid compositions of cells cultured under the different conditions

Cell type*	Protein (mg/g fr wt)	Chlorophyll (μg/g fr wt)	Fatty acid composition (mol%)					
			Palmitic	Hexadecenoic†	Stearic	Oleic	Linoleic	Linolenic
LL	8 4	214	21	1	2	2	34	40
LL_5	10 8	98	24	trace‡	2	4	46	24
LD	11 1	20	23	0	2	3	52	20
DL	10 5	141	22	1	3	2	35	37
DD	10 8	0	23	0	2	4	56	15

*See Table 1

†*trans*-3-Hexadecenoic acid

‡Detectable but less than 0 5%

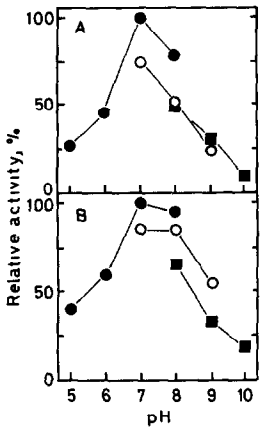

Fig 5 Effect of pH on HPO lyase activity A, green cells (LL), B, non-green cells (DD) The buffers used were citric acid–phosphate buffer (●), 50 mM pyrophosphate buffer (○) and 50 mM borate buffer (■)

DISCUSSION

The findings presented here indicate that the plant tissues have two types of HPO lyases, chloroplastic and non-chloroplastic, non-green tissue has a non-chloroplastic type of HPO lyase, and green tissue both non-chloroplastic and chloroplastic HPO lyases *Ca* 0 07 U/g fr wt of HPO lyase activity, independent of chlorophyll content, was found in non-green cells (DD) cultured continuously in the dark In the green cells (LL) cultured continuously in the light, *ca* 0 2 U/g fr wt of HPO lyase activity was found initially, but it eventually declined to 0 1 U/g fr wt, proportional to the decrease in chlorophyll contents, in the early log phase after the inoculation to a fresh medium These changes suggest that $\frac{1}{3}$ of HPO lyase activity (*ca* 0 2 U/g fr wt) in green cells may originate from non-photosynthetic compartment(s), probably the same one(s) as found in non-green cells Another $\frac{1}{2}$–$\frac{2}{3}$ of HPO lyase activity in green cells might have originated from chloroplasts Our data corroborate the results obtained with cucumber fruits [12] In the peel tissue of cucumber fruit, high activity of HPO lyase was found in the chloroplast fraction associated with chlorophyll and chloroplast marker enzymes, in the inner part of the flesh tissue, however, the activity of HPO lyase was found in the fractions of plasma and Golgi membranes, and endoplasmic reticulum [12] The fact that HPO lyase is present even in etiolated seedlings in addition to the green photosynthetic tissues also supports the conclusion drawn here

Cultured tobacco green cells (LL) have chloroplastic and non-chloroplastic HPO lyase, which indicates that the development of chloroplasts is required for full expression of HPO lyase activity The transfer of cells from the light to the dark or *vice versa* caused the changes in chloroplastic HPO lyase activities However, the fatty acid compositions and chlorophyll contents of the cells suggest that the development of chloroplastic HPO lyase activity was not proportional to the changes in chlorophyll contents and fatty acid compositions (Table 2)

It appears that there are two types of response to light with regard to chloroplastic HPO lyase In green cells (LL), where the HPO lyase synthesis has already been activated and the rate of synthesis may perhaps be proportional to chloroplast development and proliferation, as indicated by increased chlorophyll content When the cells are transferred from the dark to the light condition or *vice versa*, the chloroplast development would be switched on or off and synthesis or degradation of chlorophyll and lipids follows immediately as indicated by changes in chlorophyll contents and fatty acid compositions The rate of synthesis or degradation of chloroplastic HPO lyase was slower than those of synthesis or degradation of chlorophyll and fatty acids A similar phenomenon was observed in alfalfa callus tissue [24] HPO lyase activity decreased temporarily after the transfer from light to dark conditions but afterward it was restored to the initial activity, even in the dark Chlorophyll content and lipoxygenase activity decreased in the dark, but recovered only when the callus was transferred to the light Thus, changes in HPO lyase activity in alfalfa callus were not proportional to the changes in chlorophyll content

The results on substrate specificities of HPO lyases in the green (LL) and non-green cells (DD) revealed that the green cell type of HPO lyase catalysed hexenal formation from 13-HPOLNA at the rate of 50% of hexanal formation from 13-HPOLA However, HPO lyase from non-green cells catalysed hexenal formation from 13-HPOLNA at a lower rate than HPO lyase in the green cells If we assume the ratio of chloroplastic/non-chloroplastic HPO lyase activity is 2, the chloroplastic HPO lyase will catalyse hexenal formation with 13-HPOLNA at the rate of 60% of hexanal formation with 13-HPOLA A similar relationship was observed in green leaves and etiolated hypocotyls of cucumber plants the ratios of HPO lyase activity with 13-HPOLNA to that with 13-HPOLA were 0 83 for green leaves and 0 41 for etiolated hypocotyls (data not shown) Therefore, chloroplastic HPO lyase seems to utilize 13-HPOLNA better than non-chloroplastic Most lipoxygenases are capable of catalysing peroxidation of both linoleic and linolenic acids However, one of the cowpea lipoxygenase isoenzymes is reported to peroxidize only linoleic acid [25] Thus, some enzymes concerning lipid metabolism can distinguish linolenic acid from linoleic acid These may result in the flavour formation characteristic in the tissues The substrate specificities and the K_m values also support the presence of at least two types of HPO lyases in tobacco cells

EXPERIMENTAL

Cell culture Tobacco cells (*Nicotiana tabacum* L cv Samsun) [26] were cultured on a rotatory shaker (120 cycle/min) at 25° in the medium of ref [27] supplemented with 10 μM 1-naphthaleneacetic acid and 1 μM benzyladenine The photomixotrophic green cells were cultured for 20 days in the light (4000 lux) provided with fluorescent tubes The non-green cells were cultured for 30 days in the dark Cell suspension (5 ml) containing 0 6–0 7 g fr wt was inoculated to a fresh medium (95 ml) in 500-ml conical flasks No appreciable activity of lipoxygenase was found in cultured tobacco cells

HPO lyase assay by headspace method Cells were harvested on cellulose filter paper and washed with H_2O The washed cells were ground with a chilled mortar in 2 vols of 50 mM NaPi buffer, pH 7 The homogenate thus obtained was used as the enzyme source HPO lyase was assayed by the headspace method [28] Amounts of hexanal from 13-HPOLA and *cis*-3-hexenal and *trans*-2-hexenal from 13-HPOLNA were measured by GLC

The sum of both hexenals was signified as hexenals formed from 13-HPOLNA One enzyme unit (U) was defined as the amount of enzyme catalysing the formation of 1 μmol of net hexanal per min

Preparation of hydroperoxides and their derivatives 13-HPOLA and 13-HPOLNA were prepared from linoleic and linolenic acid, using soybean lipoxygenase [29] 9-HPOLA was prepared from linoleic acid using partially purified potato lipoxygenase [30] 13-HPOLMe and 13-HPOLNMe were derived from 13-HPOLA and 13-HPOLNA, respectively, by methylation with CH_2N_2 13-HOLA was prepared from 13-HPOLA by reduction with $NaBH_4$ Each hydroperoxide derivative represented a single spot on silica gel TLC Typical composition of 13-HPOLA was in ref [31] Concns of the hydroperoxide derivatives were determined by the *A* at 234 nm ($\varepsilon = 27000$)

Fatty acid composition of lipids in cultured cells The lipids extracted from cultured cells were analysed for their fatty acid compositions by the method of ref [32] The cells (5 g) were homogenized in a mixture of $CHCl_3$–MeOH (1 2) and lipids were extracted The fatty acids in lipids were hydrolysed and methylated with 5% H_2SO_4 in MeOH at 40° for 16 hr Me esters of the fatty acids thus obtained were extracted with hexane An aliquot of the concd hexane extract was analysed with FID-GC mounting a glass column (3 mm × 2 m) packed with 15% DEGS on Chromosorb WAW The column temp was maintained at 190° and the flow rate of N_2 was 50 ml/min Arachidic acid was added to the lipids before methylation as the internal standard

Isolation of chloroplasts Chloroplasts were isolated by the method of ref [33] with necessary modifications The green cells were homogenized gently in a chilled glass-Teflon homogenizer with 50 mM Tricine–KOH buffer (pH 7 8) containing 0 33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$ and 0 05% BSA The homogenate was filtered through four layers of gauze, then the filtrate was layered on 40% (v/v) Percoll medium containing 0 33 M sorbitol, 0 05% BSA and 50 mM Tricine–KOH buffer (pH 7 8) The chloroplasts were pelleted by centrifugation at 4000 *g* for 3 min The green chloroplast fraction was collected and layered on the Percoll density gradients consisting of three steps 80% Percoll, 6 ml, 40%, 9 ml, and 20%, 9 ml in a 30-ml tube The tube was centrifuged at 4000 *g* for 3 min The green chloroplast band was collected and the activities of enzymes, glyoxylate reductase [34], cytochrome *c* oxidase [35] and nitrate reductase [36] were determined

Determination of chlorophyll and protein Chlorophyll was determined by the method of ref [37] and protein by the method of ref [38] using BSA as the standard

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