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ORNITHINE DECARBOXYLASE ACTIVITY AND THE HYPERSENSITIVE REACTION TO TOBACCO MOSAIC VIRUS IN *NICOTIANA TABACUM*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco mosaic virus; hypersensitivity; ornithine decarboxylase; tyrosine decarboxylase; hydroxycinnamic acid amides.

Abstract—The activity of ornithine decarboxylase (ODC) is increased 20 fold in leaves of *Nicotiana tabacum* cv. Xanthi n.c. following infection with tobacco mosaic virus at 20°. The activity reaches its maximum when localized necrotic lesions appear. There is little or no increase in plants kept at 32° when infection is systemic. However, if the infected plants are transferred to 20°, a marked and rapid increase in ODC activity occurs in the upper leaves, which collapse seven to nine hours after the transfer. ODC activity therefore parallels the activity of phenylalanine ammonia lyase during the hypersensitive reaction. Tyrosine decarboxylase was found to be activated in the same conditions. By contrast no increase in arginine decarboxylase activity could be detected. Temperature has a much greater effect on the polyamine and tyramine content of Xanthi n.c. leaves than does infection with TMV.

INTRODUCTION

Infection of different cultivars of tobacco plants by tobacco mosaic virus (TMV) leads to a generalized infection (Samsun nn) or to the hypersensitive reaction (Xanthi n.c. or Samsun NN) in which virus multiplication is limited to the vicinity of local necrotic lesions. It is well established that a strong stimulation of the phenylpropanoïd pathway occurs during the hypersensitive reaction. The activity of phenylalanine ammonia lyase (PAL) and of the other enzymes of the pathway is increased [1–3]. This activation leads to an accumulation of phenolics derived from phenylalanine [4, 5].

The occurrence of hydroxycinnamic acid amides of putrescine (feruloylputrescine, *p*-coumaroylputrescine) in TMV infected leaves of *Nicotiana tabacum* L. cv. Xanthi n.c. or Samsun NN [6] led us to investigate putrescine metabolism during the hypersensitive reaction. The amides accumulate mainly in the living cells surrounding the necrotic lesions [6]. Acetyl derivatives of the di- and polyamines are known to occur in animals and microorganisms and have been postulated as intermediates in polyamines catabolism [7]. However, in higher plants, the great diversity of the hydroxycinnamic acid amides identified so far, and the diverse physiological conditions in which they have been found to accumulate [6, 8, 9–12] make any interpretation of their function in polyamine metabolism difficult.

In contrast with mammalian cells, in which putrescine is synthesized by decarboxylation of ornithine, in higher plants putrescine was first found to be formed mainly via agmatine through the action of arginine decarboxylase (ADC; EC 4.1.1.19) [13]. The occurrence of ornithine decarboxylase (EC 4.1.1.17) however has now been demonstrated in several plants [14], especially those within the Solanaceae. In tobacco, putrescine is known to be formed from ornithine or arginine [15, 16]. We, therefore, followed both ODC and ADC activities during the hypersensitive reaction at 20°. When Xanthi n.c. or Samsun NN plants are grown at temperatures higher than 29°, TMV can multiply and the hypersensitive reaction does not occur [17]. Transfer of a plant, 72 hr after inoculation from 32° to 20° results in the collapse of the part of the plant where the virus is multiplying [18], i.e. the upper leaves and the inoculated leaf. This property is used to study the early events taking place before the cell collapse which leads to the death of most of the infected cells.

The changes of ODC and ADC activities are also correlated with changes in polyamine content during the hypersensitive reaction. Evidence for the activation of tyrosine decarboxylase (EC 4.1.1.25) is also presented.

RESULTS

Amine levels in healthy and TMV infected Xanthi n.c. leaves

The formation of local necrotic lesions on Xanthi n.c. leaves inoculated by TMV at 20° does not strongly affect free amine levels in comparison with the water inoculated control. Putrescine and tyramine levels however are slightly increased (Table 1). The effect of a temperature rise from 20° to 32° on amine concentration in both the healthy or TMV inoculated leaves is much more drastic. The ratio spermine/putrescine rises *ca* 140 fold in the healthy leaf and *ca* 200 fold in the inoculated one in which TMV multiplication is systemic.

Optimal requirements for ODC

These were determined with the crude enzyme extract obtained after elution from a Sephadex G25 column. Enzyme activity was maximal in Tris–HCl buffer pH 7 (30°) with 50% activity at pH 6 and 7.5. A saturating concentration of ornithine (25 mM) was used in the

Table 1. Polyamine and tyramine content of healthy or TMV inoculated leaves of Xanthi n.c. grown at 20° or 32°

	20°		32°	
	Healthy	TMV	Healthy	TMV
Putrescine	96	120	6	6
Spermidine	163	143	99	109
Spermine	5	5	33	63
Tyramine	70	125	40	53

Results are expressed as nmol/g fr. wt. Mature leaves were taken 3 days after inoculation with water (control) or TMV using carborundum as an abrasive.

standard incubation mixture. The apparent K_m for ornithine was 1 mM. Less than 5% activity was found in the absence of pyridoxal phosphate.

ODC and ADC activities in TMV inoculated or healthy leaves of Xanthi n.c. at 20°

Figure 1 shows the time course of changes in ODC activity during the hypersensitive reaction to TMV. At 20°, a 20 fold increase in ODC activity can be detected during this period. However, this activation is transient and the activity declines rapidly during the following days. In contrast, we could not detect any activation of ADC. In mature leaves ADC activity remained very low and nearly undetectable using the standard assay (see Experimental).

ODC activity in the healthy or 72 hr infected apical leaves transferred from 32° to 20°

Figure 2 shows the effect of a transfer from 32° to 20° on ODC in the apical leaves of various tobacco plants. ODC activity remains unchanged and very low when the plant is healthy or when the infected plant is not hypersensitive to TMV (Samsun nn). In contrast, a marked and rapid activation is detectable 4–5 hr after the transfer to 20° in the hypersensitive varieties Samsun NN or Xanthi n.c. Between the seventh and the ninth hour, the infected tissues begin to collapse and die very quickly afterwards. The specific activity of ODC reaches 30 pkat/mg protein in the infected cells before their death.

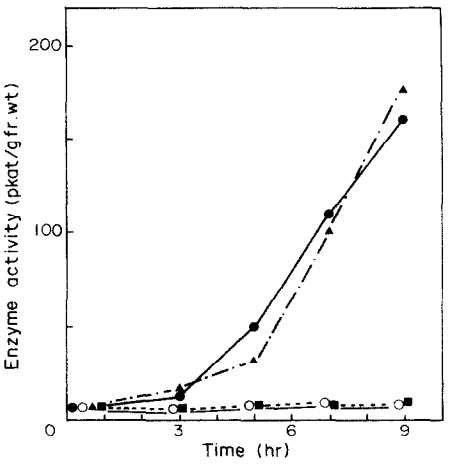

Fig. 2. ODC activity in the apical part of tobacco plants transferred from 32° to 20° three days after inoculation with TMV. Xanthi n.c. (●—●), Samsun NN (▲—▲), Samsun nn (■—■), water inoculated controls (○ - - ○).

Tyrosine decarboxylase activity

The hypersensitive reaction also triggered an increase of TDC activity. It is shown on Fig. 3 for infected plants transferred from 32° to 20°. At 20° the time course of changes was very similar to the one observed for ODC, with a maximum 48 hr after inoculation (data not shown). However, TDC remained 3–5 fold less active than ODC.

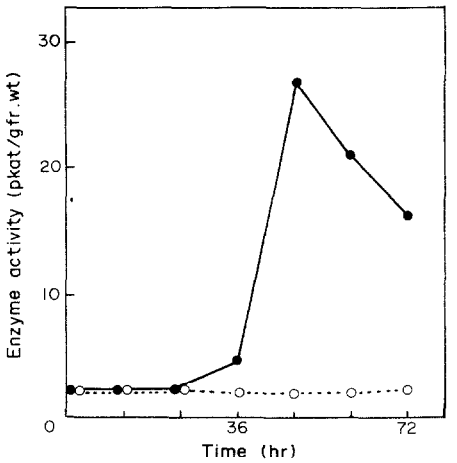

Fig. 1. Effect of TMV inoculation on ODC activity in Xanthi n.c. leaves at 20°. TMV (●—●), water inoculated control (○ - - ○). Necrotic local lesions appeared 36–40 hr after inoculation.

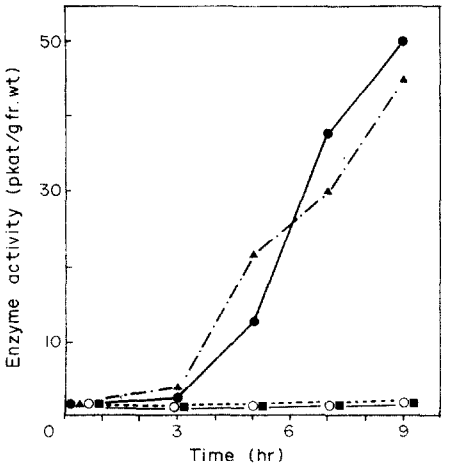

Fig. 3. TDC activity in the apical part of tobacco plants transferred from 32° to 20° three days after inoculation with TMV. Xanthi n.c. (●—●), Samsun NN (▲—▲), Samsun nn (■—■) water inoculated controls (○ - - ○).

Effect of tobacco necrosis virus (TNV) inoculation

A 20 fold increase in ODC and TDC activities could also be induced in Xanthi n.c. leaves 48 hr after inoculation by TNV at 20°.

Feeding of L-[U-^{14}C]ornithine or L-[U-^{14}C]tyrosine

When L-[U-^{14}C]ornithine was fed *in vivo* for 1 hr to infected or healthy apical leaves of Xanthi n.c. plants transferred from 32° to 20°, no qualitative change was observed in the distribution of radioactivity among the different metabolites (Table 2). Putrescine was quickly labelled but the 50% difference between the inoculated and healthy leaves was small compared with the observed difference in enzymic activity. This is probably due to the rapid metabolism of ornithine and especially the incorporation of arginine into proteins. When L-[U-^{14}C]ornithine was fed for 2 hr, some radioactivity was incorporated into spermidine. No label could be detected in *p*-coumaroylputrescine or feruloylputrescine. Putrescine could also be labelled after 1 hr from L-[U-^{14}C]arginine (data not shown). However, no radioactivity was found in agmatine, suggesting that putrescine had been mainly formed through the action of ODC. The activation of TDC was also confirmed by feeding L-[U-^{14}C]tyrosine in the conditions used to feed ornithine (Table 2).

Influence of high temperature (32°) *on the activation of ODC*

When 72 hr infected Xanthi n.c. plants are transferred from 32° to 20° for 5 or 2 hr only, before taking them back to 32°, the increase of ODC is either reduced or transient (Fig. 4). In the first case (5 hr at 20°), necrosis occurs. In the second case (2 hr at 20°) the cell collapse does not take place and virus multiplication goes on. The lag time between the temperature change (32° to 20° or 20° to 32°) and its measurable effect on ODC activity is between 4 and 5 hr.

DISCUSSION

The formation of putrescine is the first step in polyamine biosynthesis. In higher plants, as in microorganisms, putrescine synthesis is achieved through the action of ADC and ODC [14]. These biosynthetic enzymes have been extensively studied in microorganisms [19]. All mammalian cells lack ADC and much data is also available on the occurrence and properties of ODC extracted from mammalian tissues or cell cultures. ODC activity is very low in quiescent cells but it can increase many fold within a few hours, in regenerating tissues or following treatment with hormones and growth factors [20].

Although in higher plants, the occurrence of amino acid decarboxylases have often been linked with the synthesis of alkaloids, it appears that polyamine biosynthesis is also associated with cell division [21]. In this respect, plants do not differ from mammals or bacteria. Polyamine biosynthesis has been found to be enhanced in rapidly proliferating cells: seedlings [22], plant tumours [23], cell cultures in exponential phase, ovaries after pollination [24]. Growth factors can also induce an activation of putrescine biosynthesis when they are applied to receptive tissues [25].

We present evidence of a 20 fold increase in ODC activity during the hypersensitive reaction of tobacco plants to TMV. This activation is very strong in the infected cells and precedes their death. This is confirmed by temperature shift experiments where the apical part of the plant, in which virus multiplication is systemic, is used to monitor ODC activity. The systemic multiplication of TMV in Xanthi n.c. leaves at 32° does not induce a detectable activation of ODC. TMV has been found to contain only traces of polyamines [26] and from this point of view, the effect of TMV multiplication on polyamine synthesis is simpler to study than it is with viruses like turnip yellow mosaic virus (TYMV), in which spermidine is known to be an intrinsic constituent of the viral particule [26].

In mammals, ODC is known to have a very short half life [27] and it is likely that this is also true for the plant ODC although information is lacking on this subject. In this respect, the control by temperature of the increase of ODC activity in the TMV infected apical leaves (Fig. 4) could be a good model to study the mechanism of activation of ODC, its biosynthesis and the possible occurrence of macromolecular effectors [28]. Radioimmunoassay and titration of ODC with the irreversible inhibitor α-DFMO should prove useful in this study [29]. Furthermore, the high specific activity of ODC after

Table 2. Metabolism of L-[U-^{14}C]ornithine or L-[U-^{14}C]tyrosine *in vivo* during the hypersensitive reaction

		Radioactivity (% dose)				
Substrate		Putrescine	Ornithine	Arginine	Citrulline	Proteins
L-[U-^{14}C]Ornithine	Healthy	10.1	19.2	8.6	10.1	52
	TMV	15.8	33.7	7	6.2	37.3
		Tyramine		Tyrosine		Proteins
L-[U-^{14}C]Tyrosine	Healthy	7.6		11.4		81
	TMV	17.2		14.8		68

Apical parts (2–2.5 g fr. wt) of healthy or TMV inoculated Xanthi n.c. transferred from 32° to 20° after 3 days at 32° were left on a solution of either L-[U-^{14}C]ornithine (0.5 ml, 2 μCi/ml) or L-[U-^{14}C]tyrosine (0.5 ml, 2 μCi/ml) for 1 hr at 20°, between the sixth and the seventh hour after the transfer.

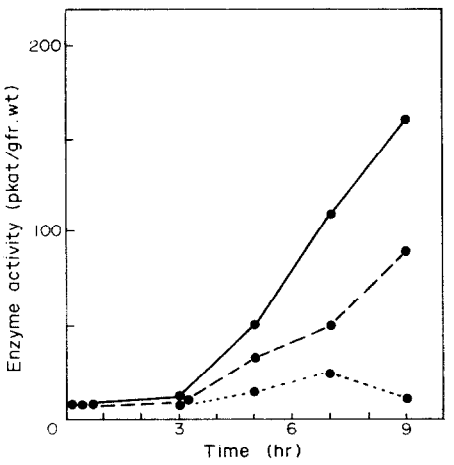

Fig. 4. Effect of temperature on the activity of ODC in Xanthi n.c. apical leaves. 72 hr after inoculation, plants were transferred to 20° (zero time) for 2 (●- - -●) or 5 hr (●— —●), and then transferred back to 20°. Other plants were kept at 20° for the duration of the experiment (●——●).

extraction from inoculated hypersensitive tobacco plants should simplify its purification.

The activation of ODC in infected cells prior to their death is paradoxical. These cells are not undergoing very active divisions and the rapid TMV multiplication at 32° does not induce the activation of ODC, although it appears to intensify the temperature induced spermine biosynthesis. The fact that temperature has such a drastic effect on the di- and polyamine content in tobacco is another possible indication of their involvement in growth processes and certainly deserves a detailed study. In this context, the fact that a decrease in temperature (from 32° to 20°) results in a reduction of the growth rate of the healthy plant and in an activation of ODC in the infected tissues of the inoculated one seems contradictory. One must keep in mind however the abnormal metabolism of infected hypersensitive cells. Moreover, the formation of local necrotic lesions in tobacco is known to be accompanied by the production of the growth inhibitors ethylene [30, 31] and abscisic acid [32]. Putrescine biosynthesis is known to be activated in response to an osmotic shock [33] and by mineral deficiency [34], which can also lead to the formation of local necrotic lesions. High putrescine concentration has also been reported in TYMV infected Chineese cabbage leaves just prior to the death of the plant [26].

During the hypersensitive reaction, the increase of ODC activity parallels the activation of PAL [1, 2]. The fact that it is an early event preceding any visible symptoms in the hypersensitive reaction illustrates the importance of both enzymes in the reguiation of plant cell metabolism. Very little is known on the occurrence of TDC [35] and the function of tyramine in plants, although aromatic amines are widespread in higher plants [36]. The simultaneous activation of TDC, ODC and PAL certainly indicates that tyramine metabolism deserves further investigation.

Microscopic studies are in agreement with the observed metabolic activation during the hypersensitive reaction. The mesophyll cells surrounding the necrotic local lesions exhibit structural features suggestive of metabolically active tissues. Endoplasmic reticulum with associated ribosomes is especially abundant [37]. The fact that hydroxycinnamic acid amides of putrescine have been found to accumulate in the living cells surrounding the local necrotic lesions and are detectable 3 days after inoculation, i.e. after a strong activation of ODC, is an indication that hydroxycinnamic acid amides synthesis could play a part in polyamine catabolism in plants. From this point of view, it now seems important to study the biosynthesis of these amides and to characterize and study the properties of the postulated cinnamoyl CoA putrescine transferase.

EXPERIMENTAL

Plant material. Nicotiana tabacum cv Xanthi n.c., Samsun nn or NN were grown in a greenhouse for 7 weeks and then transferred 3 days before inoculation to a controlled growth room (16 hr photoperiod; 16000 lux; 70% relative humidity at 20°, 50% at 32°).

Fully expanded leaves were inoculated with a purified TMV soln (10 μg/ml) to give 4–5 lesions per cm^2 using carborundum as an abrasive. Crude TNV solutions were used. A TNV infected Xanthi n.c. leaf was ground in 0.01 M NaPi buffer (3 vols), pH 7. The homogenate was then centrifuged (10000 *g* for 10 min) and the supernatant used immediately as inoculum. Plants inoculated with H_2O were used as blanks.

Polyamines and tyramine were extracted according to the method of ref. [38].

Amines were analysed by ion exchange chromatography [39] and detected using *o*-phthalaldehyde as reagent [40].

Protein was determined by the method of ref. [41].

Enzyme preparation. Mature leaves or apical parts, including only very small leaves, were extracted in a mortar with 0.1 M Tris–HCl buffer (3 vols), pH 7.5, containing 10 mM mercaptoethanol, 1 mM EDTA, 0.5% (w/v) ascorbic acid and 2% (v/v) polyethylene glycol 600. After centrifugation for 10 min at 10000 *g*, the volume of supernatant corresponding to 1.5 g fr. wt was layered on a Sephadex G25 column (2.5 × 15 cm) buffered with 0.01 M Tris–HCl buffer, pH 7.5 (ME 10 mM, EDTA 1 mM). The enzyme was eluted with the protein peak in 10 ml.

The incubation mixture for ODC contained 1 ml enzyme extract and 1 ml 0.1 M Tris–HCl buffer, pH 7 (30°) containing 50 μmol ornithine and 0.1 μmol pyridoxal phosphate. The mixture was maintained at 30° for 1 hr and stopped with 50 μl 17 M HOAc. Boiled extracts were used as blanks. After precipitation and centrifugation of the proteins, the putrescine formed during the reaction was measured in 50–200 μl aliquots using ion exchange chromatography and *o*-phthalaldehyde reaction in an amino acid analyser.

ADC was assayed in the same conditions using the same concentration (25 mM) of arginine at pH 7. No or very low activity could be detected in the pH range 6–8.

The incubation mixture for TDC contained 2 ml enzyme extract and 10 ml 0.1 M NaPi buffer, pH 7.5, containing 20 μmol tyrosine, 0.2 μmol pyridoxal phosphate and 100 μmol ME (optimal requirements for TDC). The mixture was maintained at 30° for 2 hr and the reaction stopped with 0.5 ml 12 M HCl. After removal of the proteins by centrifugation, and evaporation *in vacuo*, the residue was dissolved in 1 ml 0.1 M HCl. Aliquots (50–200 μl) were analysed for tyramine by ion exchange chromatography. Due to the concentration of the incubation mixture, endogenous polyamines extracted with the protein fraction [42] could be detected. Endogenous tyramine however was removed on the Sephadex column.

Feeding experiments. The apical part of the plant (*ca* 2–2.5 g fr. wt) was left in a small beaker containing L-[U-^{14}C]ornithine (210 mCi/mmol) or L-[U-^{14}C]tyrosine (388 mCi/mmol) for specified periods, the radioactive soln being absorbed through the stem. After washing with H_2O and grinding in EtOH (4 vols) containing 0.1% 12 M HCl, the extract was centrifuged (10 000 *g*, 10 min) and the pellet washed (× 2) with 80% EtOH. The pellet was then hydrolysed in 6 M HCl (110°, 16 hr). The supernatants were collected in a separating funnel, H_2O was added and the EtOH–H_2O phase extracted with $CHCl_3$ (1 vol).

The EtOH phase was taken to dryness (< 40) *in vacuo* and redissolved in 0.01 M HCl (1 ml/g fr. wt). Aliquots were then analysed by ion exchange chromatography and by 2D chromato-electrophoresis on cellulose plates (Merck Avicel). Electrophoresis was performed in HOAc–pyridine–H_2O (5:1:94) for 1 hr at 300 V; mobility relative to a putrescine standard (= 1): spermidine 0.9; ornithine 0.63; tyramine 0.63; arginine 0.54; tyrosine 0.40; citrulline 0.38.

TLC was performed in *n*-BuOH–HOAc–pyridine–H_2O (4:1:1:2). R_f values: tyramine 0.56, tyrosine 0.34, putrescine 0.15, arginine 0.12, citrulline 0.12, ornithine 0.09, spermidine 0.09.

Radioactivity was detected and quantified using an anthracene cell linked to the ion exchange column. Radioactivity on cellulose plates was detected by autoradiography and counted on the plates using a Geiger counter.

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