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INHIBITION OF CHLOROPLAST DEVELOPMENT BY TENTOXIN

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Key Word Index—*Pisum sativum*; Leguminosae; garden pea; chloroplast; RNA synthesis; protein synthesis; Fraction I protein; tentoxin; photophosphorylation.

Abstract—Light-dependent chloroplast development in detached pea shoots was measured in terms of chlorophyll synthesis and the synthesis of Fraction I protein. Both synthetic processes were inhibited more than 90% by the fungal metabolite, tentoxin (1 or 10 μg/ml). These results place *Pisum sativum* in the class of tentoxin-sensitive higher plants. Tentoxin, actinomycin D, lincomycin, D-*threo*-chloramphenicol and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were compared in their ability to inhibit RNA and protein synthesis by isolated pea chloroplasts. Energy for the synthetic reactions was supplied either by light or by added ATP. Only CCCP gave the same pattern of inhibition as tentoxin, i.e. inhibition of both RNA and protein synthesis in the light-driven system but no inhibition in the ATP-driven system. It is concluded that chloroplast developmental processes are inhibited by tentoxin through the inhibition of photophosphorylation.

INTRODUCTION

The fungal phytopathogen *Alternaria tenuis* induces chlorosis in susceptible seedlings by secreting a cyclic peptide, tentoxin [1]. The ultrastructural changes induced by tentoxin appear to be limited to the chloroplast [2], suggesting that this organelle is the site of action of the toxin. The most obvious ultrastructural effect of the toxin is to prevent formation of photosynthetic lamellae [2] and in this respect the toxin resembles lincomycin, a specific inhibitor of chloroplast ribosomal function [3]. However, if tentoxin is inhibitory to chloroplast protein synthesis, it may act only indirectly on the ribosomes. This is suggested by the work of Arntzen [4] who has recently shown that tentoxin inhibits photophosphorylation in chloroplasts isolated from untreated lettuce and corn seedlings. He proposes that chloroplast development is dependent upon photophosphorylation and that this dependence renders the developmental process sensitive to the toxin. Thus, the inhibition of lamella formation would be a consequence of a deficiency of ATP for protein synthesis.

Support for this proposal would be provided by demonstrating that tentoxin inhibited chloroplast development processes such as RNA and protein synthesis only when light was the energy source, i.e. that tentoxin did not act directly on the synthetic processes themselves. Such a demonstration is probably impossible to perform *in vivo* because of the problem of providing an alternative energy source, but it has recently become possible to perform the experiment *in vitro* following the discovery of a procedure for isolating higher plant chloroplasts that are capable *in vitro* of incorporating ^{3}H-uridine into RNA and ^{35}S-methionine into protein [5]. The important feature of this isotope incorporation system is that it may be driven either by light or by exogenous ATP in darkness. Only the former involves photophosphorylation.

In this paper I show that RNA and protein synthesis by isolated pea chloroplasts are sensitive to tentoxin when driven by light but insensitive when driven by added ATP, results which fully support Arntzen's notion that tentoxin inhibits chloroplast development through the inhibition of photophosphorylation [4].

RESULTS

The garden pea *P. sativum* has been frequently used for the isolation of chloroplasts capable of *in vitro* macromolecular synthesis [5] and has accordingly been employed in this study.

Inhibition by tentoxin of pea chloroplast development. It was firstly necessary to show that chloroplast development in the pea was sensitive to tentoxin. Development was assayed in relation to chlorophyll synthesis and the synthesis of Fraction I protein, the most abundant protein in the chloroplast. Pea shoots were detached from etiolated plants and allowed to green in the presence or absence of tentoxin. As reported in Table 1, two days of continuous illumination with white light resulted in the accumulation of chlorophyll and Fraction I protein in shoots standing in distilled water whereas shoots standing in tentoxin solution (1 or 10 μg/ml) accumulated pigment and protein at less than 10% of the rate exhibited by the illuminated controls. Attached, etiolated controls also synthesized very little chlorophyll and Fraction I protein in 48 hr, confirming the marked light-dependence of chloroplast development in this species [6]. These results establish that chloroplast development in *P. sativum* is sensitive to tentoxin.

In vitro *effects of tentoxin.* Chloroplast RNA and protein synthesis were assayed *in vitro* using rapidly isolated plastids. Usually between 50–60% of the isolated organelles were refractile under phase-contrast. This indicates that they possessed a continuous envelope and a

Table 1. Inhibition by tentoxin (TT) of chlorophyll and Fraction I protein synthesis in detached shoots of *P. sativum*

Incubation conditions	Chlorophyll (μg/bud)	Fraction I protein (μg/bud)
Dark (0 hr)	0·5	75
Dark (48 hr)	0·5	98
Light (48 hr)	11·2	503
Light (48 hr, 1 μg TT/ml)	1·4	120
Light (48 hr, 10 μg TT/ml)	1·4	110

substantial content of stroma, but it does not imply that the chloroplasts were strictly intact nor necessarily capable of high rates of CO_2 fixation. They were, however, able to incorporate ^{3}H-uridine into RNA (cold-acid-insoluble, ribonuclease-sensitive) and ^{35}S-methionine into protein (hot acid-insoluble, pronase-sensitive). Light and added ATP (2 mM) were alternative energy sources for the incorporation of the two isotopes (Table 2).

Tentoxin was found to inhibit both RNA and protein synthesis in isolated chloroplasts but only when light was used as energy source (Table 2). At a toxin concentration of 10 μg/ml, uridine incorporation was inhibited 98% while methionine incorporation was inhibited 91%. In contrast, no inhibition was observed when added ATP was energy source. These results provide strong evidence that tentoxin is not a direct inhibitor of either RNA or protein synthesis but inhibits these processes only when they are dependent upon ATP formation by photophosphorylation, as proposed by Arntzen [4].

In vitro *effects of other inhibitors.* In Table 3, the inhibitory properties of tentoxin are compared with those of four familiar inhibitors: actinomycin D, lincomycin, D-*threo*-chloramphenicol and CCCP. Uridine incorporation was sensitive to actinomycin D but not to lincomycin, whereas the reverse was true for methionine incorporation, irrespective of the energy source. D-*threo*-chloramphenicol inhibited methionine incorporation in the light and in the dark with ATP, presumably as a result of its well known ability to inhibit chloroplast protein synthesis [5]. However, it also inhibited uridine incorporation in the light, probably as a consequence of its less familiar ability to inhibit photophosphorylation at the concentration used in the experiment [7]. This is an example of a substance with two direct sites of action. Finally, CCCP, a specific inhibitor of photophosphorylation, abolished only light-dependent incorporation, to give the same pattern of inhibition as tentoxin.

DISCUSSION

In studying the mode of action of toxins, it is important to distinguish between direct and indirect effects and

Table 2. Differential effects of tentoxin (TT, 10 μg/ml) on light-driven and ATP-driven RNA and protein synthesis by isolated pea chloroplasts

Incubation conditions	Isotope incorporation (cpm/30 min/assay) ^{3}H-RNA	^{35}S-protein
No energy	559	508
Light	5926	3945
Light + TT	662	838
ATP	2810	2730
ATP + TT	3027	2946

Table 3. Percentage inhibition of RNA and protein synthesis in isolated pea chloroplasts exposed to tentoxin, actinomycin D, lincomycin, D-*threo*-chloramphenicol (CAM) and carbonyl cyanide *m*-chlorohydrazone (CCCP)

Inhibitor	Light-driven synthesis RNA	protein	ATP-driven synthesis RNA	protein
Tentoxin (10 μg/ml)	98	91	0	0
Actinomycin D (10 μg/ml)	75	5	92	0
Lincomycin (5 μg/ml)	0	76	0	63
D-*threo*-CAM (150 μg/ml)	69	80	0	78
CCCP (5 μM)	75	72	0	22

also to recognise that some toxins may have more than one direct site of action. D-*threo*-chloramphenicol has already been quoted as an example of an inhibitor with two sites of action. It may not be possible from *in vivo* studies alone to define the mode of action of a toxin and in such a case recourse must be made to appropriate *in vitro* systems. There is clearly great scope for the use of chloroplasts and other cellular components in the detection, assay and characterization of toxins associated with plant disease.

Tentoxin prevents chloroplast development in some plant species but has no effect on others [1]. Thus, chloroplast development in cabbage seedlings is insensitive to the toxin [2]. This could be due to any one of a number of factors, including: (i) the impermeability of the plasma membrane or the chloroplast envelope to the toxin, (ii) the existence of a detoxification mechanism somewhere in the plant, (iii) the insensitivity of photophosphorylation to tentoxin, and (iv) the lack of dependence of chloroplast development on photophosphorylation. Clearly, such a complex situation could not be analyzed adequately without recourse to *in vitro* systems.

EXPERIMENTAL

Imbibed pea seeds (*Pisum sativum* L. var. Feltham First) were sown in shallow trays of potting fibre and allowed to grow at 20–22° either in the dark or under a 12 hr photoperiod of 2000 lx provided by white fluorescent tubes. The effect of tentoxin on chloroplast development was examined by the use of detached pea shoots. After 7 days of growth, etiolated shoots were detached, placed in sterile H_2O containing tentoxin, and illuminated continuously for 48 hr under 14000 lx. Duplicate samples of 10 buds were then homogenised in a mortar with ice-cold buffer (2·5 mM tris, 5 mM 2-mercaptoethanol, adjusted to pH 8·5 with glycine) and the homogenates (each 10 ml final vol.) were centrifuged at 30000 *g* for 30 min. Chlorophyll was extracted from the pellets with Me_2CO–H_2O (4:1), and assayed [8]. The Fraction I protein content of the supernatants was measured by running aliquots on 5% polyacrylamide gels under non-denaturing conditions [9]. The gels were stained with Coomassie blue, the stained bands were scanned, and the amounts of protein were estimated by comparison with a range of loadings of purified pea Fraction I protein. The effects of tentoxin and certain other inhibitors on RNA and protein synthesis by isolated chloroplasts were assessed by published procedures [9,10]. Chloroplasts were prepared rapidly from 9-day-old illuminated plants (15 g of buds) and resuspended in KCl medium [9]. They were immediately assayed for their ability to incorporate ^{3}H-uridine into cold acid-insoluble material [10] and ^{35}S-methionine into hot acid-insoluble material [9]. The incubation mixtures with

a final vol. of 500 μl contained 100 μmol KCl, 33 μmol tricine–KOH (pH 8·3). 3·3 μmol $MgCl_2$, 300 μl chloroplast suspension and either 10 μCi ^{3}H-uridine (15 Ci/mmol) or 0·5 μCi ^{35}S-methionine (200 Ci/mmol). Mixtures to be incubated with ATP in the dark were supplemented with 1 μmol ATP and covered with foil. The concentrations of tentoxin and the other inhibitors used in this study are indicated in the appropriate tables. Incubations were performed at 20° for 30 min in an illuminated waterbath [9]. The post-incubation work-up to determine the amount of radioisotope incorporated into RNA and protein has been described previously [9,10].

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