Incompatibility conditioned by the *Mla* gene in powdery mildew of barley: timing of the effect of cordycepin on hypersensitive cell death

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The effect of cordycepin, an inhibitor of mRNA synthesis, on the hypersensitive host cell death response (HR), was tested with epidermal tissues from barley coleoptiles containing the *Mla* gene for resistance, inoculated with incompatible race 3 of *Erysiphe graminis* f.sp. *hordei*. In untreated control tissues, HR occurred 18–30 h after inoculation in 61–91% of cells with haustoria. The percentage of HR was strongly reduced by cordycepin at 10–31 μM but only if applied 4 h after inoculation or before; not if applied 8 h or later. Effective cordycepin treatments had no inhibitory effect on the development of germ tubes or appressoria and usually increased the development of haustoria and hyphae. The results indicate that mRNAs required for the HR are synthesized in the host within the first 0–8 h after inoculation and that mRNA synthesis is not required thereafter. The timing of protein synthesis required for the HR in the host could not be determined because the inhibitors of protein synthesis used, blasticidin S and cycloheximide, inhibited parasite development at concentrations required to inhibit the HR.

INTRODUCTION

Several lines of evidence indicate increased gene expression in cereal leaf tissue in response to attack by powdery mildew parasites. Synthesis of several enzymes is enhanced, including β-1,3 glucanase [*23*], chitinase [*28*], peroxidase [*25*] and phenylalanine ammonium lyase [*19*, *40*]. All are thought to be involved in defence against pathogens. Synthesis of several other polypeptides is also induced as detected by two-dimensional gel electrophoresis [*35*] and also by *in vitro* translation of mRNA isolated from infected tissue [*5*, *18*]. Furthermore, several cDNA clones of host response genes have been obtained by differential screening of infected *v.* uninfected leaves.

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Abbreviations used in text: AlgR, Algerian/4*(F14)Man R; AlgS, Algerian/4*(F14)Man S; HR, hypersensitive host cell death response; ^{3}H-ATP, tritiated adenosine triphosphate.

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These clones, in turn, have been shown by dot blot or Northern blot analyses to represent genes whose expression is enhanced or newly initiated in response to powdery mildew parasites [*3*, *11*, *12*, *13*, *37*, *39*, *47*, *50*]. Gene expression in these investigations was consistently enhanced 24 h after inoculation and in several cases as early as 3–6 h. However, several of these genes are expressed in about the same amount in both resistant and susceptible leaves in the first 15 h after inoculation [*11*] and a role in resistance has not been established for any of the genes activated in response to powdery mildew parasites.

We have used an inhibitor of mRNA synthesis, cordycepin, to determine when after inoculation mRNA synthesis is required for the development of the hypersensitive cell death response (HR) in barley with the *Mla* gene for resistance. Cordycepin (3′deoxyadenosine), which inhibits polyadenylation of mRNA [*17*, *33*], has been used to inhibit resistance responses in crown rust of oats [*42*, *52*]. Cordycepin has been used widely in other investigations of gene expression in plants [*4*, *14*, *20*, *32*, *45*] including barley [*41*]. In our experiments, cordycepin solutions were applied directly to single cell layers of epidermal tissues partially isolated from coleoptiles, so that movement of cordycepin through other tissues of the plant was not a factor. HR and fungal development could be assessed by light microscopy. Comparable experiments were performed with two inhibitors of protein synthesis, blasticidin S and cycloheximide. However, these experiments were less successful.

MATERIALS AND METHODS

Culture CR3, race 3, of *Erysiphe graminis* D.C. Merat f.sp. *hordei* Em. Marchal was used with two near-isogenic barley (*Hordeum vulgare* L.) lines: Algerian/4*(F14)Man R (AlgR), containing the *Mla* gene for resistance (incompatible with race 3), and Algerian/4*(F14)Man S (AlgS) with the *mla* gene for susceptibility (compatible with race 3) [*36*].

Conidiospore inoculum was produced on AlgS in a plant growth chamber as described earlier [*6*]. To inoculate tissues, spores were blown into a settling tower directly from individual leaves that had been inoculated 7 days earlier.

Epidermal tissues were partially dissected from 7-day-old barley coleoptiles, generally following the mounting procedures of Bushnell *et al.* [*7*]. A 1·6-mm diameter experimental zone was exposed on the outer (cuticularized) epidermal surface for inoculation and microscope observation. The inner (non-cuticularized) surface was exposed directly to test solutions contained in a 2 ml reservoir beneath the tissue.

An average of 33 spores mm^{-2} was applied to the experimental zone; the conidia produced an average of nine haustoria mm^{-2}. Inoculated tissue mounts were placed in boxes containing saturated $ZnSO_4$ solution to give 95 % RH and incubated in darkness at 19 ± 1 °C. Mounts were usually prepared in sets of 20, with two to four mounts per treatment. Where data for more than four mounts per treatment are reported, data were combined from two to three experimental sets.

All test solutions contained 0·01 M CaMOPS buffer [0·209 g 3-(N-morpholino)-propane sulphonic acid (MOPS); 0·015 g $Ca(OH)_2$; 0·188 g $Ca(NO_3)_2$/100 ml, pH 6·5]. Cordycepin, MOPS and cycloheximide were obtained from Sigma Chemical Co., St Louis and blasticidin S was kindly provided by S. Ouchi, Kinki University,

Japan. Solutions in reservoirs were changed with Pasteur pipettes, by rinsing the tissue twice with the new solution before the final volume was introduced.

The total number of spores, germinated spores, appressoria, papillae, haustoria and dead cells associated with haustoria were recorded at 24 h and usually at 43–48 h after inoculation for each mount. In addition, the number of haustoria in dead cells (usually) and in living cells, and the number of dead and living cells with haustoria were recorded. Finally, the number of haustoria in each of six developmental index stages [see Fig. 1(b)] was recorded. The total number of spores in the three to five mounts usually used for a given treatment was 190–360; the total number of haustoria, 50–100. In some experiments, the lengths of a selected number of hyphae (growing from appressoria) were measured with an ocular micrometer. The number of hyphae used to determine hyphal length is given for individual experiments. From these primary data, the following values were calculated: spore germination rate (as the percentage of spores with visible germ tubes, whether primary or appressorial); appressoria (as the percentage of germinated spores); haustoria (as the percentage of spores with appressoria); average haustorial developmental index; hyphae (as the percentage of appressoria with haustoria); HR (dead cells as the percentage of host cells with haustoria); and average hyphal length (per appressorium that produced one or more hyphae). Mean values were calculated for individual mounts and these, in turn, were averaged for the overall means reported here.

Significance of differences between individual treatments and untreated controls was determined using Students *t*-test based on variation among mounts. The arcsine transformation was used for percentage data before the *t*-test was applied.

In some experiments, the haustorial developmental index and dead cells were recorded in a series of observations at 2 h intervals. For a given set of experimental mounts, each time of observation required an interval of 30–60 min. The average time of observation was calculated in these cases.

To assess the effect of cordycepin on mRNA synthesis, the amount of label incorporated into poly(A)$^+$RNA from tritiated adenosine triphosphate (^{3}H-ATP, Amersham) was determined. Uninfected coleoptiles were partially dissected so that each had a 2–3 × 5–7 mm area of inner epidermis in a monolayer. The coleoptile pieces were mounted flat on a glass slide with two cover slips, one covering 2 mm of the basal end, the other covering 2 mm of the tip. CaMOPS buffer was allowed to flow under the coleoptile piece until all the undersurface was wetted. As required, solutions were withdrawn from under the tissue with filter paper touched to the solution edge. The CaMOPS buffer was replaced with CaMOPS buffer with or without cordycepin at 31 μM, containing ^{3}H-ATP at a concentration sufficient to supply 5–10 μCi per coleoptile piece. The slides with tissues were incubated in a moist chamber overnight and then dissected to remove the vascular bundles bordering the epidermal monolayer [7]. The epidermal monolayer was then rinsed in water five times, combined with other pieces from a given treatment, ground in liquid nitrogen, and then homogenized with a Polytron (Brinkmann) homogenizer. Total RNA was extracted by the guanidium thiocyanate method [*10*]. Poly(A)$^+$RNA was isolated using oligo d(T) cellulose columns according to Maniatis *et al.* [*34*]. The resulting poly(A)$^+$RNA was quantified by spectrophotometry. Equal fractions of poly(A)$^+$RNA were then subjected to scintillation counting to determine the extent of labelling.

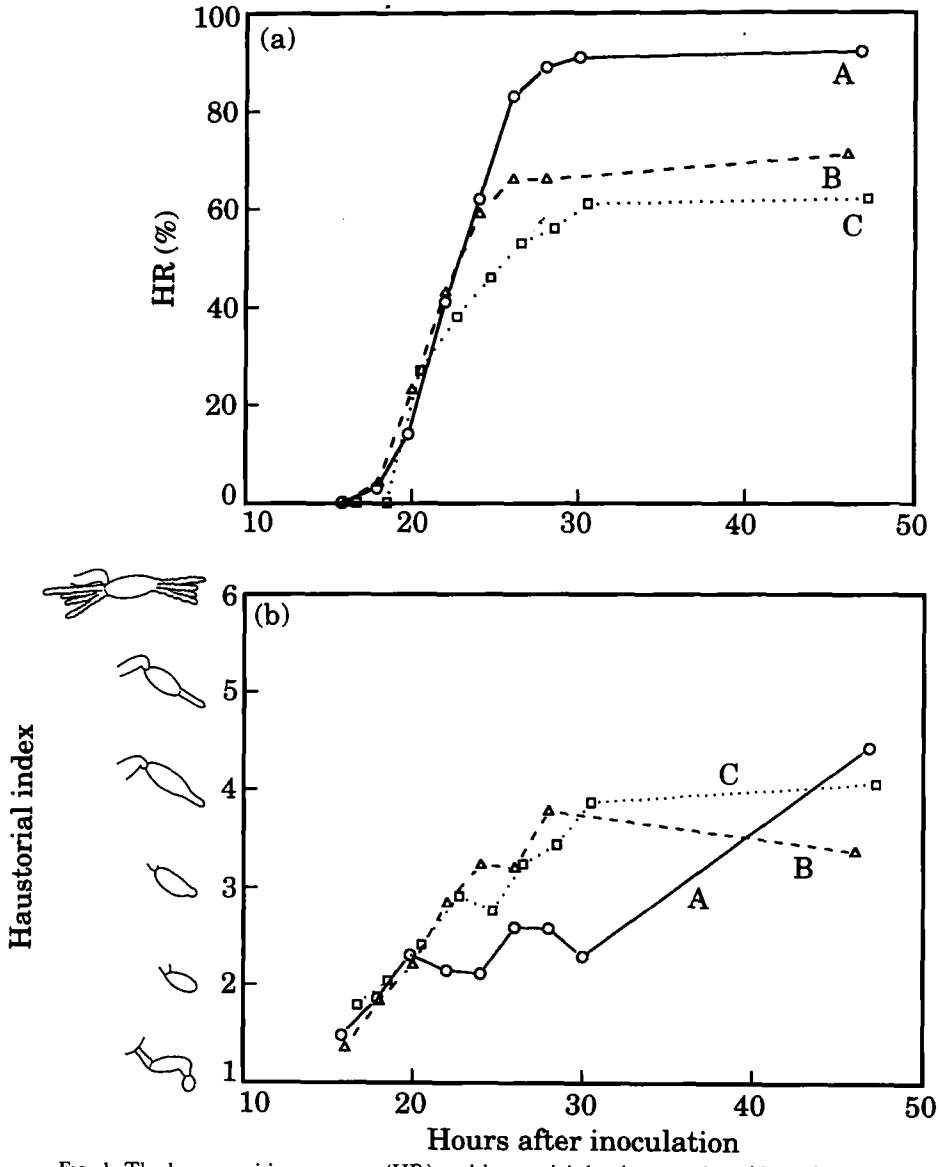

FIG. 1. The hypersensitive response (HR) and haustorial development in epidermal tissues of Algerian/4*(F14)Man R (resistant) barley, inoculated with *Erysiphe graminis* f.sp. *hordei* in three typical experiments, A, B and C. (a) HR (dead host cells as percentage of cells containing haustoria). (b) Haustorial development (haustorial index of Hazen & Bushnell [*21*] illustrated on left).

RESULTS

HR

HR began 18 h after inoculation of AlgR and usually reached maximum percentages (of haustorium-containing cells) by 28–30 h [Fig. 1(a)]. This pattern was consistent among experiments, but the final percentage (at 43–48 h) varied from 61–91%,

averaging 75% for all experiments. HR occurred when the average developmental index indicated haustorial fingers were beginning to develop [Compare Fig. 1(a) and (b)].

Effects of cordycepin

In preliminary trials, cordycepin applied at 10 μM had no effect on HR when applied at 12 or 16 h after inoculation but was highly inhibitory when applied at the time of inoculation. Subsequently, cordycepin at 10 or 31 μM reduced the percentage of haustorium-containing cells that underwent HR if treatment started at 0 or 4 h after inoculation, but not if started at 8 or 12 h (Table 1). Cordycepin had no effect on fungal germination, appressorium formation or haustorium formation in the first experiment (Table 1) or in all subsequent experiments (data not shown). On the other hand, the haustorial developmental index increased (Table 1) as the rate of HR was reduced; i.e., more haustoria continued to elongate because fewer host cells died. Likewise, hyphae (as a percentage of appressoria with haustoria) increased when cordycepin treatments reduced HR (Table 1).

TABLE 1

The effect of cordycepin treatments starting 0, 4, 8 and 12 h after inoculation on the hypersensitive cell death response (HR) and fungus development in Algerian/4(F14)Man R (resistant) barley inoculated with* Erysiphe graminis *f.sp.* hordei. *Data were obtained 45 h after inoculation*

		10 μM Cordycepin Initiation of treatment (h)[b]				31 μM Cordycepin Initiation of treatment (h)[b]			
	Control[a]	0	4	8	12	0	4	8	12
HR (%)[c]	91	30*[d]	51	83	73	6	36	72	94
Fungus development									
Germination (%)[e]	76	64	70	70	69	76	75	73	73
Appressoria (%)[f]	66	68	58	72	51	63	64	65	61
Haustoria (%)[g]	60	48	56	71	41	52	42	68	52
Haustorial index[h]	2·7	4·7*	3·9*	3·5	3·9	4·9*	4·6*	3·4	3·2
Hyphae (%)[i]	11	55*	46*	6	5	42[j]	23*	21	5

[a]No cordycepin.
[b]Hours after inoculation. Treated until 45 h after inoculation.
[c]Dead host cells as percentage of cells with haustoria.
[d]*Indicates value is significantly different from value for untreated control tissue by Students *t*-test, $P < 0{\cdot}05$.
[e]As percentage of spores.
[f]As percentage of germinated spores.
[g]As percentage of appressoria.
[h]Index of haustorial development; see Fig. 1(b)
[i]As percentage of haustoria.
[j]Not significant because of unusually large variation among mounts.

To provide further evidence that cordycepin was ineffective when applied later, we treated tissues 12 and 16 h after inoculation with cordycepin at high concentration (Table 2). At 1000 μM, cordycepin was toxic to some mounts 24 h after inoculation, so the experiment was terminated when the percentage of HR in untreated tissues was

only 49%. HR was not reduced with cordycepin concentrations of 31, 100, or 1000 μM applied at 12 or 16 h after inoculation (Table 2). The rate of cell death scored as HR actually increased, either because the toxic effects of cordycepin added to the number of dead cells or possibly because of a synergistic effect of cordycepin and the fungus in inducing HR. In any case, high concentrations of cordycepin did not inhibit HR when applied at 12 or 16 h after inoculation.

TABLE 2

The effect of high concentrations of cordycepin on the hypersensitive cell death response (HR) and haustorium development in Algerian/4(F14)Man R (resistant) barley inoculated with* Erysiphe graminis *f.sp.* hordei. *Data were obtained 24 h after inoculation*

		31 μM Cordycepin Initiation of treatment (h)[b]		100 μM Cordycepin Initiation of treatment (h)[b]		1000 μM Cordycepin Initiation of treatment (h)[b]	
	Control[a]	12	16	12	16	12	16
HR (%)[c]	49	63	93	75	70	93*[d] (toxic)	90* (toxic)
Haustorial index[e]	3·4	3·9	2·4	3·1	2·9	1·8*	2·1*

[a]No cordycepin.
[b]Hours after inoculation. Treated until 24 h after inoculation.
[c]Dead host cells as percentage of cells with haustoria.
[d]*Indicates value is significantly different from value for untreated control tissue by Students *t*-test, $P < 0{\cdot}05$.
[e]Index of haustorial development; see Fig. 1(b).

Cordycepin at 31 μM applied at inoculation inhibited HR almost completely whether removed 4, 8 or 12 h after inoculation (Table 3). As before, the haustorial index and percentage of hyphae increased (Table 3). However, hyphal length was not increased in this experiment.

To further delimit the time that treatments were effective, a set of 4 h cordycepin treatments was applied, starting 12, 8 or 4 h before inoculation (Table 4). At 10 μM, cordycepin effectively suppressed HR when applied during the −4 to 0 h treatment period, but not with earlier 4 h treatments (Table 4), indicating that the effect of cordycepin at this concentration lasted no more than 4 h after cordycepin was removed. At 31 μM, cordycepin effectively suppressed HR with treatments of 4 h duration ending 8 or 4 h before inoculation, indicating the effect lasted for at least 8 h (Table 4). Neither the haustorial index, the percentage of hyphae, nor hyphal length was increased significantly in this experiment (Table 3).

To obtain a clearer picture of the effect of cordycepin on fungal development, we compared the effects of cordycepin (at 31 μM) on fungal development in both AlgR (resistant) and AlgS (susceptible) barley lines (Table 5). Whether applied for 0–4 h or 0–45 h, cordycepin again inhibited HR in AlgR. (The rate of HR was only 1–2% in AlgS, regardless of treatment.) The haustorial index was not increased significantly in AlgR, but the percentage of hyphae was increased about four-fold and hyphal length increased two-fold. In AlgS the 0–4 h treatment had no effect on fungal development,

TABLE 3

The effect of cordycepin (31 μM) treatments terminated at various times after inoculation on the hypersensitive cell death response (HR) and fungus development in Algerian/4(F14)Man R (resistant) barley inoculated with* Erysiphe graminis *f.sp.* hordei. *Data were obtained 48 h after inoculation*

		Termination of treatment (h)[b]			
	Control[a]	4	8	12	48
HR (%)[c]	69	5*[d]	3*	2*	2*
Fungus development					
Haustorial index[e]	3·7	5·9*	5·5*	5·8*	5·1*
Hyphae (%)[f]	19	88*	69*	76*	49
Hyphal length (μm)[g]	72	94	38	39	56

[a]No cordycepin.
[b]Hours after inoculation. Treatments started at the time of inoculation.
[c]Dead host cells as percentage of cells with haustoria.
[d]*Indicates value is significantly different from value for untreated control tissue by Students *t*-test, $P < 0{\cdot}05$.
[e]Index of haustorial development; see Fig. 1(b).
[f]As percentage of appressoria.
[g]*Length of hyphae per colony. Means from each of four mounts were averaged. Total of 17–32 colonies per treatment.

TABLE 4

The effect of cordycepin treatments of 4 h duration at various times before inoculation on the hypersensitive cell death response (HR) and fungus development in Algerian/4(F14)Man R (resistant) barley inoculated with* Erysiphe graminis *f.sp.* hordei. *Data were obtained 43 h after inoculation*

		10 μM Cordycepin Duration of treatment (h)[b]			31 μM Cordycepin Duration of treatment (h)[b]		
	Control[a]	−12 to −8	−8 to −4	−4 to 0	−12 to −8	−8 to −4	−4 to 0
HR (%)[c]	68	—	62	20*[d]	3*	16*	18*
Fungus development							
Haustorial index[e]	4·2	—	3·9	5·3	5·9	5·5	5·6
Hyphae (%)[f]	47	—	41	69	91	66	78
Hyphal length (μm)[g]	97	—	40	56	96	120	55

[a]No cordycepin.
[b]Hours from inoculation.
[c]Dead host cells as percentage of cells with haustoria.
[d]*Indicates value is significantly different from value for untreated control tissue by Students *t*-test, $P < 0{\cdot}05$.
[e]Index of haustorial development; see Fig. 1(b).
[f]As percentage of haustoria.
[g]Length of hyphae per colony; mean of eight colonies per treatment.

but the 0–45 h treatment reduced the haustorial index, as well as the percentage and length of hyphae. Thus, in AlgS, 31 μM cordycepin was somewhat inhibitory to haustorial and hyphal development if left in mounts for 45 h after inoculation, but not if left for only 4 h—a treatment sufficient to inhibit HR in AlgR.

TABLE 5

The effect of cordycepin (31 μM) treatment, at 0–4 h and 0–45 h after inoculation on the hypersensitive cell death response (HR) and fungal development in Algerian/4(F14)Man R (AlgR) (resistant) and Algerian/4*(F14)Man S (AlgS) (susceptible) barley inoculated with* Erysiphe graminis *f.sp.* hordei. *Data were obtained 45 h after inoculation*

	AlgS			AlgR		
		Termination of treatment (h)[b]			Termination of treatment (h)[b]	
	Control[a]	4	45	Control[a]	4	45
HR (%)[c]	2	2	1	84	7*[d]	9*
Fungus development						
Haustorial index[e]	5·9	4·9	4·1*	3·0	5·0	5·3
Hyphae (%)[f]	91	64	8*	13	57*	62*
Hyphal length (μm)[g]	146	94	15*	29	56*	47*

[a]No cordycepin.

[b]Hours after inoculation. Treatments started at the time of inoculation.

[c]Dead host cells as percentage of cells with haustoria.

[d]*Indicates value is significantly different from value for untreated control tissue of corresponding host as determined by Students *t*-test, $P < 0{\cdot}05$.

[e]Index of haustorial development; see Fig. 1(b).

[f]As percentage of haustoria.

[g]Length of hyphae per colony; mean for six mounts, all colonies up to eight mounts were included; total of 15–48 colonies per treatment.

The effect of cordycepin on synthesis of mRNA in uninoculated coleoptile epidermis was estimated from its effect on the rate of incorporation of label from ^{3}H-ATP into mRNA. In two experiments (Table 6), cordycepin at 31 μM reduced incorporation to 48–77 % of amounts in untreated tissues.

As an alternative to cordycepin, another inhibitor of mRNA synthesis, actinomycin, was tested for effect on HR. In several experiments, actinomycin applied 0 or 16 h after inoculation at concentrations of 0·1, 1·0, 10 and 100 μg ml^{-1} had no effect on rates of HR. At 100 μg ml^{-1}, the actinomycin was toxic to coleoptile tissue in some experiments. The reason for the ineffectiveness of actinomycin in reducing rates of HR was not investigated.

TABLE 6

The effect of cordycepin (31 μM) on the amount of radioactivity from tritiated adenosine triphosphate (^{3}H-ATP) incorporated into mRNA in barley coleoptile tissues

	CPM[a]	
Experiment	Untreated control	Cordycepin
1	304	234
2	317	152

[a]mRNA was extracted 24 h after ^{3}H-ATP was applied to tissues. Thirty uninoculated coleoptile-pieces per treatment.

Effects of blasticidin S and cycloheximide

By using the inhibitors of protein synthesis, blasticidin S and cycloheximide, we attempted to determine when host protein synthesis was required for HR. A range of concentrations and treatment times was tested in unsuccessful efforts to inhibit HR and enhance fungal growth. Haustorium formation was strongly inhibited if treatments were applied at inoculation with concentrations of 0·05 μg ml^{-1} for blasticidin S or 0·03 μg ml^{-1} for cycloheximide. Lower concentrations applied at inoculation had no effect on HR. With treatments started at 14–18 h after inoculation, HR was inhibited, but, again, fungal development was also inhibited. Thus, with cycloheximide (data not shown), fungal development was always reduced at the minimum concentration (0·1 μg ml^{-1}) required to inhibit HR. With blasticidin S (Table 7), a concentration of 0·5 μg ml^{-1} inhibited HR and fungal development regardless of the time treatments started. A concentration of 0·1 μg ml^{-1} was ineffective when treatment started at 16 or 18 h, whereas HR was partially inhibited when treatment started at 14 h. Although fungal development was not reduced in this case, haustoria and hyphae developed no more than in untreated controls.

TABLE 7

The effect of blasticidin S treatments starting 14, 16 and 18 h after inoculation on the hypersensitive cell death response (HR) and fungus development in Algerian/4(F14)Man R inoculated with* Erysiphe graminis *f.sp.* hordei. *Data were obtained 47 h after inoculation*

	0·1 μg ml^{-1}				0·5 μg ml^{-1}			
		Initiation of treatment (h)[b]				Initiation of treatment (h)[b]		
	Control[a]	14	16	18	Control[a]	14	16	18
HR (%)[c]	74–79	31*[d]	74	67	63–74	1*	15*	26*
Fungus development								
Haustorial index[e]	5·4–5·9	5·1	4·7	5·4	5·4–6·0	1·4*	2·5*	3·6*
Hyphae (%)[f]	17–27	34	12	16	27–38	0*	2*	6*

[a]No blasticidin S.
[b]Hours after inoculation. Treated until 47 h after inoculation.
[c]Dead host cells as percentage of cells with haustoria.
[d]*Indicates value is significantly different from value for untreated control tissue by Students *t*-test, $P < 0·05$.
[e]Index of haustorial development; see Fig. 1(b).
[f]As percentage of haustoria.

With both cycloheximide (data not shown) and blasticidin S (Table 7), the amount of HR inhibition was greater the earlier treatments were started. Thus, at 0·1 μg ml^{-1}, blasticidin S reduced HR rates to 41% of control rates when applied at 14 h, but insignificantly when applied at 16 h or later (Table 7). Generally, the final percentage of HR equalled values reached in untreated tissues 4–6 h after treatments were started (data not shown), indicating that development of HR was not stopped immediately by the inhibitors.

DISCUSSION

The results of experiments in which cordycepin was applied at various times before and after inoculation indicate that host mRNAs produced during the first 8 h after inoculation are required for the HR. To be effective, treatments had to start no later than 4 h after inoculation, even though HR occurred much later (18–30 h after inoculation). Results with treatments given before inoculation (Table 4) indicate that the effect of cordycepin at 10 μg ml^{-1} persisted no more than 4 h after the end of treatment. Thus, 4 h treatments at this concentration ending 4 h before inoculation were ineffective. It follows that cordycepin applied at 10 μg ml^{-1} in the 4-h period immediately before inoculation probably acted no later than 4 h after inoculation. Treatments applied in the 4-h period immediately after inoculation probably acted no later than 8 h after inoculation. This strongly suggests that mRNA synthesis in the first 8 h is essential for processes leading to the HR. Conversely, mRNA synthesis at 8 h or later following inoculation is apparently not required, even though the HR did not occur until 18–30 h.

During the period in which cordycepin treatments were effective, the powdery mildew spore produces a short, terminal germ tube, usually 3–6 h after inoculation. The host cell wall is at least partially penetrated beneath the germ tube tip, so that contact is established for water and small molecules to move between host and parasite [*9, 29–31*]. Host cytoplasm aggregates temporarily beneath the germ tube tip and deposits a small papilla. Furthermore, these early physiological responses are associated with enhanced or newly initiated expression of several host response genes. This has been shown by Northern or dot blot hybridization, using cDNA clones isolated from infected resistant plants, as probes [*3, 5, 11, 39, 47, 50*]. These investigations consistently show that mRNA transcripts accumulate 3–6 h after inoculation. Thus, a number of host response genes are activated in the first few hours after inoculation. Our experiments with cordycepin suggest that one or more of these early response genes is required for the HR. Alternatively, cordycepin may have inhibited a constitutively expressed gene whose expression in the first 8 h after inoculation is required for the HR.

The need to start treatments no later than 4 h after inoculation in order to inhibit the HR was probably not a consequence of slow uptake of cordycepin by plant cells. Concentrations much higher than were effective at 4 h after inoculation were ineffective when applied later (Table 2). Furthermore, cordycepin solutions were applied directly to the non-cuticularized surface of the experimental epidermal tissues. Given the general effectiveness of cordycepin in higher plants, slow uptake is unlikely to have contributed significantly to our results.

Our interpretation rests on the assumption that cordycepin inhibited the HR by inhibiting mRNA synthesis in host tissues. Support for this assumption was provided by reduced incorporation of ^{3}H-ATP into mRNA (Table 6), although the inhibition was only partial. Furthermore, the results may have been influenced by low rates of uptake of the ^{3}H-ATP into cells. Ideally, the magnitude and duration of inhibition should be measured to complement experiments on HR.

The continued, and often enhanced, development of *E. graminis* on cordycepin-treated AlgR host tissues indicated that the inhibition of the HR was through effects on the host and not on the parasite. Germination and appressorium formation were

consistently unaffected, including treatments started at or before inoculation. Average haustorium development usually increased as did rates of initiation of hyphae from appressoria (Tables 1, 3 and 5) and average hyphal lengths (Tables 3 and 4). However, hyphal growth was partially inhibited by cordycepin in susceptible AlgS (Table 5) and hyphae in treated AlgR never reached the full length of hyphae in untreated AlgS. By using the criterion of hyphal length, we could not demonstrate that resistance was completely abolished by cordycepin. This point needs to be investigated in leaves which can be maintained longer after treatment than can dissected coleoptile tissues.

The reasons for the absence of a strong inhibitory effect by cordycepin on the fungus are unknown. Cordycepin probably reached appressoria (and possibly spores) by way of the apoplast, and could have reached haustoria by way of either the apoplast or host cytoplasm [*8*]. Apparently, the fungus either failed to take up cordycepin or was insensitive to it after uptake, or (less likely) had sufficient mRNA in the spore for production of infection structures.

Although primary germ tubes were not involved, Tani & Yamamoto [*42*, *43*] obtained results with crown rust of oats that partially parallel ours with powdery mildew of barley. Resistance was expressed by retarded growth of infection hyphae from substomatal vesicles by 20 h after inoculation, before haustoria were produced. Cordycepin treatments starting 10 h after inoculation inhibited this expression of resistance. Treatments starting 12 h or later after inoculation were less effective. The interval between treatment and the effect on resistance was shorter than in our experiments, but the overall implication was the same: host gene expression required for resistance occurred early in the interaction between host and parasite, well before haustoria were produced.

In our experiments, the inhibitors of protein synthesis, cycloheximide or blasticidin S, both strongly inhibited HR, but always with concomitant inhibition of fungal development. The inhibitors stopped HR when applied as late as 18 h after inoculation; HR usually ceased within 4–6 h after treatments were started. This indicates that protein synthesis was required in the host, the parasite or possibly both, until 4–6 h before HR developed, but the experiments do not distinguish between the activities of the two organisms. Of the essential mRNAs synthesized in the host in the first 8 h, few are likely to remain until 18 h after inoculation for translation. Therefore when applied at 18 h, the effect of blasticidin S in limiting HR was more likely to be on the parasite than on the host.

Efforts elsewhere to use blasticidin S to relate host protein synthesis to HR have given mixed results. The HR has been inhibited or delayed without inhibition of the parasite in some host–parasite combinations [*24*, *51*] but not others [*16*]. Phytotoxicity by blasticidin S interfered in some cases [*24*, *26*]. In oat crown rust, blasticidin S they had no effect on the rate of hypersensitive cell death, but nevertheless increased rates of fungus development [*44*]. Overall, the relation between host protein synthesis and HR remains unclear. On the other hand, inhibitors of protein synthesis have consistently inhibited phytoalexin formation [*15*, *46*, *53*], and also have inhibited resistance that prevents penetration of host cell walls by fungal pathogens, as in papilla-associated resistance of epidermal cells [*2*, *38*, *48*, *49*], or in resistance to rust fungi expressed by failure of haustorium formation in mesophyll cells [*22*]. Papilla-associated resistance is expressed 12–15 h after inoculation in the near-isogenic isolines used in our

investigations [27]. However, this resistance is of a general type, mostly unrelated to the race-specific *Mla* resistance expressed as a HR [1]. The effect of cordycepin on HR is, therefore, probably unrelated to papilla-associated resistance. On the other hand, cordycepin has reduced the effectiveness of papilla-associated resistance in some preliminary experiments (unpublished data), implicating mRNA synthesis in this type of resistance.

A challenge for future research is to determine which genes expressed in the host in the first 8 h after inoculation are essential for HR. These might include the primary resistance gene, *Mla*, so that its product is not expressed to trigger gene-for-gene interaction in the presence of cordycepin. More likely, other genes, constitutive or inducible, must be expressed by 8 h after inoculation for the events leading to HR to be completed.

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