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Primary Site of Action of Amitrole in *Arabidopsis thaliana* Involves Inhibition of Root Elongation but Not of Histidine or Pigment Biosynthesis

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ABSTRACT

Interference with histidine metabolism, inhibition of pigment biosynthesis, or both have been the principal candidates for the primary site of action of 3-amino 1,2,4-triazole (amitrole). *Arabidopsis thaliana* is sensitive to 1,2,4-triazole-3-alanine, a feedback inhibitor of histidine biosynthesis, and this effect is reversed by histidine. The combination of triazolealanine and histidine, however, does not reverse the herbicidal effect of amitrole. This indicates that amitrole toxicity is not caused by histidine starvation, nor is it caused by the accumulation of a toxic intermediate of the histidine pathway. Amitrole inhibits root elongation at lower concentrations than it causes pigment bleaching in the leaves. In contrast, fluridone, a known inhibitor of the carotenoid biosynthetic pathway does not block root elongation. Fluridone also inhibits carotenoid accumulation in etiolated seedlings in the dark, but amitrole does not. Last, gabaculine and acifluorfen, but not amitrole, prevent chlorophyll accumulation in greening etiolated seedlings of *Arabidopsis*. These experiments cast doubt on pigment biosynthesis as the primary site of action of amitrole.

Amitrole (3-amino 1,2,4-triazole) is a herbicidal compound whose mode of action at the molecular level has not been satisfactorily elucidated. It has been claimed that this compound is an inhibitor of the enzymes phytoene desaturase (8), lycopene cyclase (24), and imidazoleglycerol phosphate dehydratase (13) as well as catalase (22).

Amitrole has been shown to be an inhibitor of the enzyme imidazoleglycerol phosphate dehydratase, a part of the histidine biosynthetic pathway, in *Escherichia coli* (7), *Salmonella typhimurium* (13), and *Saccharomyces cerevisiae* (12). Interestingly, amitrole does not have a lethal effect on these organisms. Amitrole has been shown to inhibit this enzyme in higher plants (9), however, consistent reversal by histidine of the effect of amitrole, has not been reported (3).

The appearance of albino tissue after treatment with amitrole is one of the most striking and commonly observed effects of its toxicity (3). Pigment bleaching may be the most important factor in the toxicity of this compound in the field (16). This has led to studies identifying various enzymes involved in pigment biosynthesis (8, 24) as the primary target of this herbicide. Pigment bleaching, however, is a common secondary effect of herbicides (19, 20), and thus conclusions about whether it is the primary site of action of a herbicide should be treated with caution.

We have reexamined the mode of action of amitrole using *Arabidopsis thaliana* as our test system in the belief that a body of data based on a single species with a homogeneous genetic background would be more informative than trying to correlate effects using different species of varying genetic homogeneity.

The results presented here eliminate the possibility that the herbicidal site of action is in the histidine biosynthetic pathway. These results also strongly indicate that neither the carotenoid nor the tetrapyrrole biosynthetic pathways are likely to be the site of action of amitrole. Root elongation in *Arabidopsis* is more sensitive to the effect of amitrole than is pigment bleaching. The inhibition of root growth, unlike pigment bleaching, is light independent. This inhibition indicates that amitrole has an effect on cell division and/or cell elongation which may be indicative of the primary block caused by this herbicide.

METHODS AND MATERIALS

Plant Materials

Our seed stock of *Arabidopsis thaliana* var Columbia is of clonal origin, descendants of a single seed from a stock which was the kind gift of Dr. C. Somerville, Michigan State University. All of the experiments described below were conducted with third generation seed, G3 (where the original seed is designated the zero generation, G0). The composition of the nutrient medium was as described in Somerville and Ogren (25).

Chemicals

DMSO was from EM Science. Amitrole was from Alfa Products 1,2,4-Triazole-3-alanine (triazolealanine), histidine, 3-amino 2,3-dihydrobenzoic acid (gabaculine), and δ-aminolevulinic acid were from Sigma Chemical Co. 5-[2-Chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate, sodium salt (acifluorfen) was from Chem Service Inc. 1-Methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1H)-pyridinone (fluridone) was the kind gift of Dr. R. Abdulla (Lilly Research Laboratories). Agarose was from Fisher.

Plate Tests

Thirty mL of sterile nutrient media (25), supplemented with 0.5% sucrose, was solidified with 0.8% agarose and

poured into standard 100 × 15 mm sterile Petri dishes (Falcon). The herbicides were added to the sterile media as concentrated solutions in DMSO. The final concentration of DMSO was 0.025%. Before pouring the plate, 5 mL of the media containing the herbicide were withdrawn for use as top agar. Seeds, sterilized as in Somerville and Ogren (25), were suspended in the top agar and evenly distributed on top of the solidified agar plate. The plates were sealed with Parafilm and incubated for 10 d at 150 μE m^{-2} s^{-1} at 23°C under continuous fluorescent lighting for experiments involving high light conditions. These were also our standard growth conditions. For the experiments under low light conditions, the plates were incubated at 50 μE m^{-2} s^{-1} under continuous fluorescent lighting. Experiments needing intermittent lighting used the same light intensity as the low light conditions but had a 15 min light/150 min dark cycle superimposed. For experiments in total darkness, plates were wrapped in two layers of aluminum foil and incubated at room temperature. At 10 d, plants were removed for the relevant measurements.

Greening Experiments

Etiolated *Arabidopsis thaliana* seedlings were obtained by growing batches of seedlings (18 × 150 mm borosilicate test tubes), in liquid culture in a rotary drum (New Brunswick Scientific TC-7) in the dark at 27°C for 3 d. The appropriate herbicide concentration was then added to the plants in DMSO as above, and these were transferred to the light at 150 μE m^{-2} s^{-1} at 23°C in a rotary drum. After 16 h the samples were removed, and Chl measurements were taken.

Chl Measurements

The Chl extraction was done according to the method of Hiscox and Israelstam (14). For the plate assay, 10 individual plants were combined and weighed, and the Chl was extracted. The Chl concentration was measured as in Arnon (1). The results were recorded as μg Chl/mg wet weight. Each extraction was done in triplicate. For the greening experiments, the contents of an entire borosilicate test tube was weighed after blotting on filter paper as fully as possible and the Chl extracted and measured as above. Each measurement was done in triplicate.

Carotenoid Measurements

Arabidopsis seedlings were grown in liquid culture as above. The appropiate herbicide concentration was added at the same time as the seed. Carotenoids were extracted in DMSO as above and quantified at 485 nm (18). The concentration of carotenoids was expressed as OD_{485}/mg wet weight and the I_{50} determined from the appropriate graph.

Root Measurements

Root of *A. thaliana*, when grown in agarose, characteristically elongate as a single tap root with little or no branching except at high light intensities. Therefore, root measurements were done by carefully extracting individual plants from the agarose and measuring the size of the tap root. Each reported value is the average of 10 such measurements. Since the radicle is of a finite size, even totally inhibited samples had a root length which was arbitrarily set at 1 mm. This was the smallest size which could be measured accurately. All values were rounded to the nearest millimeter. All reported values are shown as percent of control at the same conditions to eliminate any possible variability due to differing growth conditions.

RESULTS

Effect of Triazolealanine on *Arabidopsis thaliana* and Its Reversal by Histidine

Triazolealanine is a feedback inhibitor of the enzyme ATP phosphoribosyl transferase, the first committed step of the histidine biosynthetic pathway (23). This compound is herbicidal to *A. thaliana*, causing a concentration-dependent decrease both in Chl and in root length (Fig. 1, A and B). This effect is reversed by the addition of histidine to the media (Fig. 1, A and B). These experiments allow us to conclude that inhibition of carbon flow through the histidine pathway is lethal, and that this lethality can be reversed by the addition of exogenous histidine.

Neither Histidine nor Triazolealanine Reverse Amitrole Toxicity

The above experiments allowed us to ask if the histidine pathway was the primary site of action of amitrole. As can be seen in Figure 2, A and B, neither histidine alone nor histidine in combination with triazolealanine had any effect on amitrole toxicity either in terms of pigment accumulation or root elongation. This provides a powerful argument against any major involvement of this pathway as the primary site of action of amitrole. Since histidine does not reverse the effects of amitrole, the toxic effects of this compound cannot solely be due to starvation of the plant for histidine. Since the triazolealanine plus histidine combination also had no effect, it cannot be argued that the toxicity of amitrole is due to a buildup of a toxic intermediate of the metabolic pathway.

Effect of Fluridone and Amitrole on Etiolated Seedlings in the Dark

If the primary site of action of amitrole is on carotenoid biosynthesis, one would expect amitrole to prevent synthesis of carotenoids in the dark as well as in the light. Fluridone, a known carotenoid biosynthesis inhibitor (6), was able to inhibit the accumulation of carotenoids into etiolated seedlings in the dark, with an I_{50} of 10 nM. In contrast amitrole did not reduce the concentration of carotenoids when expressed on a per weight basis, even at 1 mM. This is an indication that inhibition of carotenoid biosynthesis is not the primary site of action of amitrole. In contrast, at 10^{-4} M amitrole completely stopped elongation of the root in this system. Fluridone had no effect on root elongation (data not shown).

Effect of Light Intensity on the Decrease in Pigment Accumulation and Root Elongation Caused by Amitrole

It has been reported (11) that amitrole-induced bleaching of leaves is light mediated. This suggests that this process

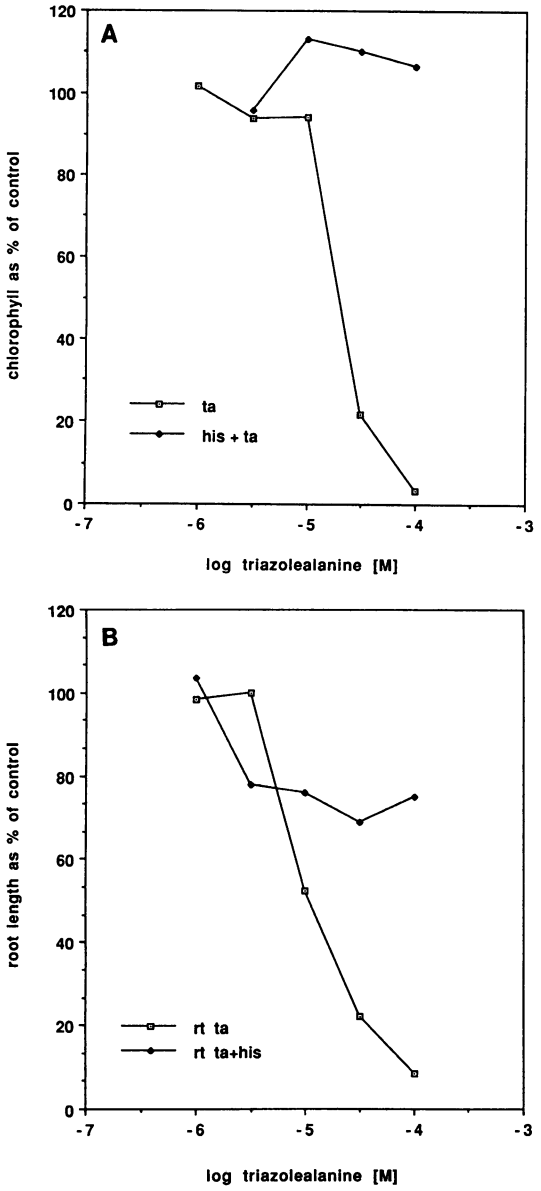

Figure 1. A, Open squares show the effect of increasing concentrations of triazolealanine on the Chl content of *A. thaliana*, closed diamonds show the reversal of this effect by 1 mM histidine; B, open squares show the effect of triazole alanine on root elongation in *A. thaliana*, closed diamonds show the reversal of this effect by 1 mM histidine.

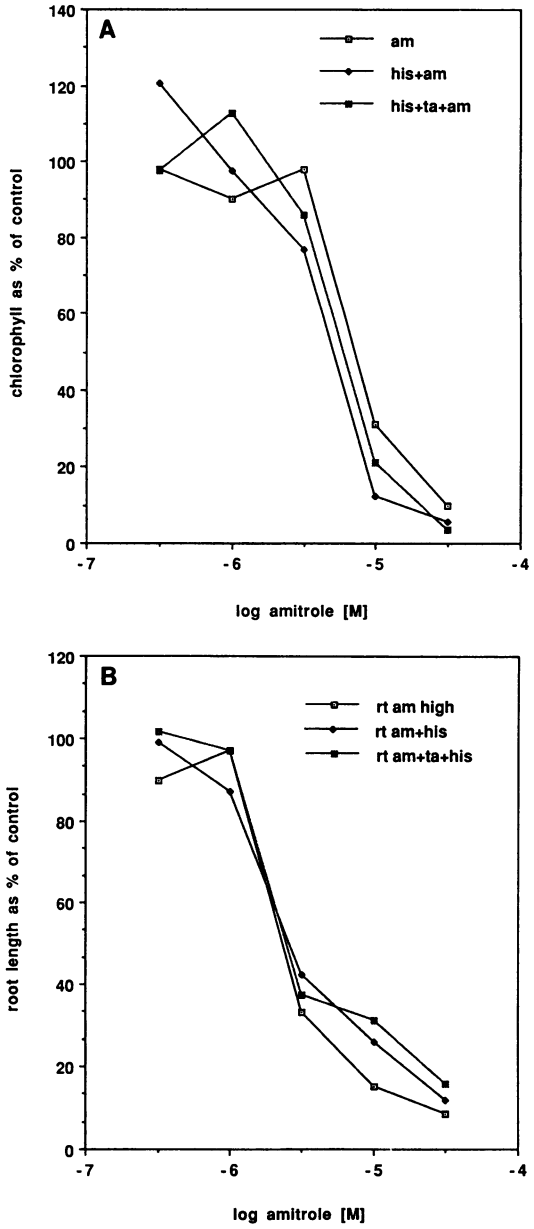

Figure 2. A, Open squares show the effect of increasing concentrations of amitrole on the Chl content of *A. thaliana*, closed diamonds shows the effect of 1 mM histidine on this aspect of amitrole toxicity, closed squares show the combined effect of 0.1 mM triazolealanine and 1 mM histidine on this aspect of amitrole toxicity; B, open squares, closed diamonds, and closed squares as above but showing the effect on root elongation.

involves degradation rather than blockage of pigment biosynthesis and is thus a secondary phenomenon. It has also been reported (2) that amitrole has an effect on root elongation. We thought that it would be informative to compare in *Arabidopsis* the sensitivities of both of these processes to amitrole as well as the modulation of light on these processes. As can be seen in Figure 3A, the bleaching effect of amitrole is light dependent since it is responsive not only to light intensity but also to the total flux of light. Although we only quantitated the Chl concentration, the same can be said for the carotenoids; the affected plant had no visible carotenoids.

In contrast, the amitrole effect on the root system is nearly light independent as can be seen in Figure 3B. Indeed, this inhibition occurs in total darkness (data not shown). Root elongation was five times more sensitive to amitrole than was the inhibition of pigment accumulation. This result was surprising, since the literature on amitrole (3) tends to stress its bleaching effect.

These data lead us to postulate that bleaching of the leaves, although a highly visible phenomenon is, in fact, a secondary

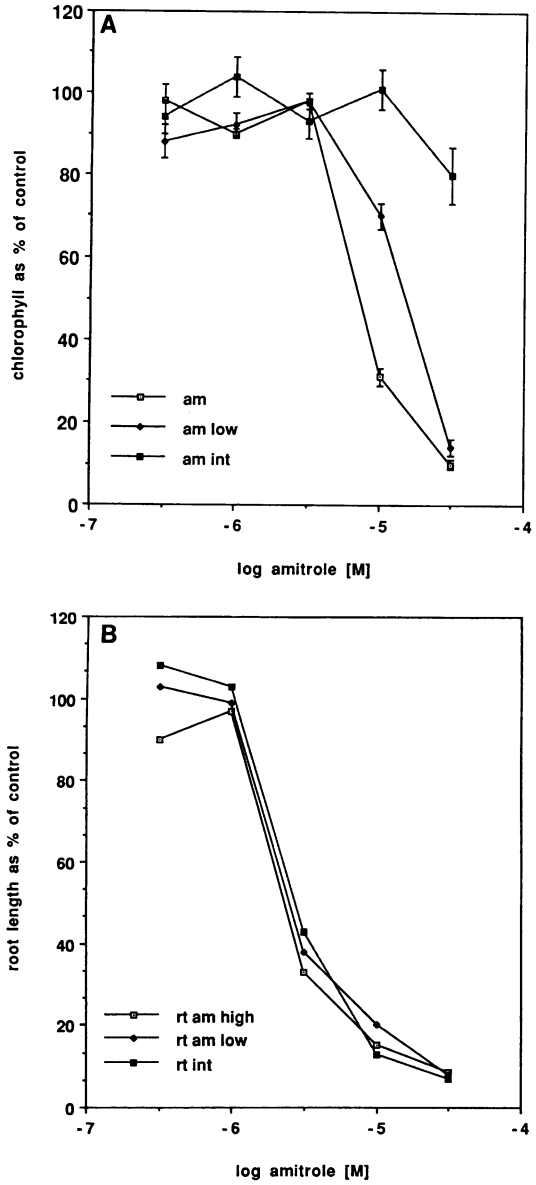

Figure 3. A, Open squares show the effect of increasing concentrations of amitrole on the Chl content of *A. thaliana*, under high light conditions, closed diamonds are as above but under low light conditions, closed squares are as above but under intermittent light conditions; B, open squares, closed diamonds, and closed squares as above but showing the effect on root elongation. Error bars represent the standard error of the mean.

effect and that the primary site of amitrole is more likely to be one which would lead to the inhibition of root elongation, which is more sensitive to amitrole and is light independent.

Comparison of Amitrole with Known Carotenoid and Tetrapyrrole Inhibitors

If our above conclusion is correct, one would expect to see major differences between the toxic effects of true pigment inhibitors and that of amitrole. Therefore, we set out to compare the effects of fluridone (a carotenoid inhibitor) and gabaculine and acifluorfen (both tetrapyrrole inhibitors) to those of amitrole.

As can be seen in Table I, amitrole is a very poor inhibitor of Chl biosynthesis, with an I_{50} of 8 mM in greening, etiolated *Arabidopsis* seedlings. As a comparison, amitrole is more than 50 times less active than gabaculine, itself a very poor inhibitor, and 5 orders of magnitude less active than acifluorfen. Acifluorfen also causes photobleaching (19, 20) and some of the above effect could be due to secondary destruction of Chl. This should be a minor effect since the seeds germinated in total darkness would contain little starting Chl and also would be photosynthetically incompetent. It can be concluded that amitrole does not interfere with the Chl biosynthetic pathway as its primary mode of action.

I_{50} for inhibition of Chl accumulation by amitrole in this test is 500 times that needed for a similar inhibition of Chl in long-term experiments (see Table II). In contrast, both gabaculine and acifluorfen have similar inhibition constants for both long- and short-term inhibition. This can be easily seen by comparing the inhibition constants in Table I with those in Table II.

We also compared, as shown in Table II, the ratios for inhibition of root elongation and Chl bleaching for the above herbicides and amitrole. As has already been stated, root elongation is more sensitive to amitrole than Chl bleaching. This is clearly not the case with fluridone where bleaching is 500 times more sensitive than is root elongation. In fact, we have not been able to measure an effect of fluridone on root elongation.

This makes it highly unlikely that amitrole has any direct, significant effect on the carotenoid biosynthetic pathway after phytoene desaturase, the enzyme affected by fluridone. This, together with the inability of amitrole to block carotenogenesis in the dark, indicates that carotenoid biosynthesis is unlikely to be the primary site of action, although the failure to

Table I. *Effect of Different Herbicides on the Chl Content of Greening, Etiolated Seedlings of A. thaliana*

Herbicide	Chl Content	
	I_{50}	Arbitrary units
Amitrole	8.0×10^{-3}	108,696
Gabaculine	4.2×10^{-6}	56
Acifluorfen	7.4×10^{-8}	1

Table II. *Effects of Different Herbicides on the Chl Accumulation and Root Elongation of A. thaliana*

Herbicide	I_{50}		
	Root length	Chl	Ratio
		M	
Fluridone	$>10^{-6}$	5.2×10^{-9}	>520
Acifluorfen	1.1×10^{-5}[7]	1.1×10^{-8}	10
Gabaculine	2.4×10^{-6}	3.8×10^{-6}	0.63
Gabaculine+ala[a]	4.5×10^{-5}	6.0×10^{-6}	7.5
Amitrole	2.6×10^{-6}	1.5×10^{-5}	0.17
Amitrole+ala[a]	1.6×10^{-6}	5.3×10^{-6}	0.30

[a] 50 μM δ-aminolevulinic acid.

accumulate carotenoids may be one of the most important herbicidal effects of this compound.

Gabaculine, like amitrole, affects root elongation more than leaf bleaching; however, this effect is reversed by the addition of the product of the enzyme inhibited by gabaculine, δ-aminolevulinic acid. Amitrole toxicity is not affected by the addition of this compound. It might be argued that there could be a block upstream in the porphyrin biosynthetic pathway. However, acifluorfen, which inhibits the enzyme protoporphorinogen oxidase (19, 20), which is upstream in the pathway, has a more pronounced effect on the leaves than on the roots. This is in keeping with the mode of action of acifluorfen (19, 20), which has a light-mediated component. Although the biosynthetic block could theoretically be even further upstream in the pathway, these data and the data on greening seedlings indicate that amitrole does not affect the Chl biosynthetic pathway directly.

DISCUSSION

Amitrole is a herbicide that, in spite of an enormous literature, has not yielded a clear answer about its primary site of action. Amitrole is a known inhibitor of the enzyme imidazoleglycerol phosphate dehydratase (13) and thus this would seem to be a prime candidate for its site of action. Indeed, amitrole is routinely mentioned as an amino acid inhibitor in reviews of the literature on herbicides (17).

Amitrole also affects chloroplast function and structure (4, 5). Furthermore, the most striking visible effect of amitrole is the albino appearance of new foliar tissue (3). It is not surprising, therefore, that pigment biosynthesis has also been sited as a primary site of action of amitrole.

Unfortunately, the literature on amitrole encompasses many vastly different species. Therefore, it is difficult to extrapolate an effect from one plant to another, especially since plants vary in their sensitivity to amitrole and such a large variation in rates have been used. It was our hope that by doing an extensive series of experiments using a single species with a uniform genotype, and a uniform protocol, we would be better able to dissect primary from secondary effects. The weakness of this approach, of course, is that the data collected might not extrapolate to other plants besides the experimental material due to idiosyncrasies of the test system.

We first sought to either confirm or rule out the two main contenders for the primary site of action of amitrole. We have shown that inhibition of histidine biosynthesis cannot account for the herbicidal activity of amitrole. If the plant was starved for histidine, it would be rescued by histidine. If it was poisoned by a toxic intermediate, it would be rescued by triazolealanine and histidine combined. This does not mean that amitrole is incapable of inhibiting this enzyme. It does mean that this inhibition is neither sufficient nor necessary for the mode of action of amitrole. In fact, from Figures 1 and 2, we can conclude that little, if any, of the herbicidal activity of amitrole can be accounted for by a block in histidine metabolism.

It could be argued that amitrole has multiple sites of action and that the failure of histidine to reverse amitrole toxicity was expected. Therefore, the failure by histidine to reverse amitrole toxicity would have no bearing on whether amitrole blocks this pathway. This is a flawed argument. If amitrole has an effect at more than one site, it can be expected that each of the sites would be associated with a specific set of toxic responses. Relieving the effects of one of the sites of action should eliminate the effects associated with that particular block. In fact, plants treated with amitrole and histidine simultaneously are indistinguishable from plants treated with amitrole alone.

We also tested the hypothesis that an enzyme of the carotenoid biosynthetic pathway is the primary site of action of amitrole since these have been implicated in its mode of action (8, 24). The hypothesis that there might be a block in Chl biosynthesis was tested since known inhibitors of this pathway are bleaching agents which are light dependent (19, 20). The appearance of unusual porphyrins has been reported after treatment with amitrole (15), as well as the of excretion of porphyrins after treatment with amitrole (10). This might imply a block in tetrapyrrole biosynthesis.

The results in Tables I and II show that it is highly unlikely that amitrole acts via either of these two mechanisms. Amitrole is inefficient at blocking Chl or carotenoid biosynthesis in greening, etiolated seedlings of *Arabidopsis*. Acifluorfen is 5 orders of magnitude better than amitrole in the inhibition of Chl accumulation. Furthermore, we have shown that *Arabidopsis* seedlings grown in the dark in the presence of fluridone are lacking in carotenoids while those grown in amitrole contain carotenoids.

Amitrole is effective at blocking root elongation. A known carotenoid inhibitor, fluridone, is incapable of blocking root elongation indicating that blockage of the pathway at phytoene desaturase does not affect this function. It is possible that amitrole blocks previous to this step. This could not be tested due to the lack of inhibitors which affect *Arabidopsis*. Even though acifluorfen and gabaculine block root elongation, both of these herbicides can also be distinguished from amitrole. Acifluorfen can be distinguished because the ratio of root elongation to pigment bleaching is 50 times that of amitrole, and gabaculine can be distinguished because it can be reversed by δ-aminolevulinic acid, which does not reverse amitrole.

Last, we have shown that root elongation is a more sensitive phenomenon than pigment accumulation, and that, in addition, unlike the block in pigment accumulation, it is a light-insensitive process. This indicates that this inhibition is a more fundamental, if less spectacular effect, of amitrole. Furthermore, amitrole causes albinism only in new tissue; it can neither prevent the synthesis of Chl in preexisting but etiolated tissue nor appreciably lead to photooxidation of preexisting Chl.

These facts have led us to postulate that amitrole is involved in the inhibition of a stable or abundant cell component. In rapidly growing tissue, this component is rapidly diluted, allowing us to see the effect of amitrole. In preexisting tissue the factor is already present and its stability or abundance allows it to perform its function unaffected by the presence of amitrole. It is interesting that thiamine-deficient mutants of *Nicotiana* (21) have recently been described whose foliar phenotype, albinism in the absence of exogenous thiamine, is strikingly similar to amitrole. Although thiamine does not

reverse amitrole toxicity (DR Heim, IM Larrinua, personal observation), another such cofactor of cell component might.

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