Isolation and Characterization of a *Chlamydomonas reinhardtii* Mutant Resistant to Photobleaching Herbicides

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A group of highly active N-phenylimide photobleaching herbicides have been synthesized. These N-phenylimide herbicides as well as diphenyl ether herbicides induce protoporphyrin IX accumulation and inhibit protoporphyrinogen oxidase activity at extremely low concentrations in higher plants. The binding of a ^{14}C-labeled N-phenylimide herbicide S-23121 [N-[4-chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl]-3,4,5,6-tetrahydrophthalimide] to the solubilized plastid fractions of greening corn seedlings is competed by the diphenyl ether herbicide acifluorfen-ethyl, but not by diuron, an inhibitor of photosynthetic electron transport. These results indicate a similar mode of action for both N-phenylimide and diphenyl ether herbicides.

In order to investigate the mechanism of photobleaching herbicides at the molecular level, a strain of *Chlamydomonas reinhardtii* RS-3 resistant to N-phenylimide S-23142 [N-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide] was isolated by mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine. The 90% inhibition concentration of N-phenylimide S-23142 for growth of RS-3 was 100 times higher than that for wild type. Maximum accumulation of protoporphyrin IX was reached at 0.03 μM of S-23142 for the wild type and 3 μM for RS-3. RS-3 was resistant to oxadiazon, oxyfluorfen and acifluorfen-ethyl which had been shown to have the same mechanism of action as N-phenylimide herbicides, but not to paraquat, diuron or fluridone. Genetic analysis of RS-3 strain showed that the resistance results from a dominant mutation *(rs-3)* in the nuclear genome. The magnesium protoporphyrin IX synthesizing activity from 5-aminolevulinic acid in chloroplast fragments isolated from RS-3 was less sensitive to S-23142 than that from wild type (CC-407). Protoporphyrinogen oxidase activity in Percoll™-purified chloroplasts from RS-3 was also less sensitive to S-23142 than that from wild type. These results indicate that the resistance of RS-3 is specific for photobleaching herbicides, and that the mutation is related to protoporphyrinogen oxidase, the primary site of the photobleaching herbicide action.

Introduction

Photobleaching herbicides, for example *ortho*-substituted diphenyl ether (DPE) herbicides such as acifluorfen-ethyl (AFE), cause rapid bleaching of the treated foliar region of higher plants. The mode of action of photobleaching herbicides has been studied extensively, and recently these herbicides have been shown to inhibit protoporphyrinogen oxidase (PPO), which oxidizes protoporphyrinogen IX to protoporphyrin IX (Proto) [1–3]. It is generally accepted that the inhibition of PPO and autoxidation of protoporphyrinogen IX lead to the accumulation of a strong phytotoxic photosensitizer Proto, which causes peroxidation and consequently membrane destruction. Recently, plasma membrane fractions have been shown to contain a PPO activity which is not inhibited by a photobleaching herbicide and this activity leads to an abnormal accumulation of tetrapyrroles that are thought to work as phytotoxic photosensitizers [4].

We have synthesized two novel, highly active N-phenylimide photobleaching herbicides, designated S-23121 [N-[4-chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl]-3,4,5,6-tetrahydrophthalimide] and S-23142 [N-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide] [8, 9]. Despite a completely different chemical structure from DPEs, they show a similar mode of action. While extensive studies have attempted to

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clarify the mode of action of photobleaching herbicides, a precise characterization of their target site at the molecular level has yet to be made. Thus investigation of the target site of new, chemically distinct photobleaching herbicides is of great interest.

One of the strategies to investigate the site of herbicide action involves the use of mutants of genetically well-characterized and normally sensitive organisms which show resistance to herbicides. The unicellular green alga *Chlamydomonas* species has been used to isolate many mutants for cellular and molecular biological studies, including the mode of action of herbicides [5, 6]. In this short review, we describe the mode of action of N-phenylimide herbicides in comparison with that of DPEs and the isolation and characterization of a mutant *Chlamydomonas reinhardtii* stain resistant to N-phenylimide herbicides.

Discovery of novel photobleaching herbicides

We have recently discovered several highly active N-phenylimide herbicides. These include experimental herbicides S-23031 [pentyl 2-chloro-4-fluoro-5-[(3,4,5,6-tetrahydro)phthalimido]-phenoxyacetate], S-23121, S-23142 and S-53482 [7-fluoro-6-[(3,4,5,6-tetrahydro)phthalimido]-4-(2-propynyl)-1,4-benzoxazin-3(2H)-one] which have a number of potential uses (Fig. 1) and show strong herbicidal activity against a wide variety of weeds by both pre- and post-emergence application [7–10]. Susceptible weeds are controlled at extremely low dosages in the range of about 10 to 100 g/ha. Post-emergence foliar application of these herbicide causes rapid bleaching, wilting, desiccation and necrosis in the presence of light and oxygen [9, 11–13]. These symptoms are characteristic of photobleaching herbicides such as *ortho*-substituted diphenyl ethers.

Mode of action of N-phenylimide photobleaching herbicides

The mode of action of N-phenylimide herbicides has been studied using S-23121 and S-23142. At extremely low concentrations, S-23142 induced massive Proto accumulation (Fig. 2) followed by evolution of lipid peroxidation products, such as ethane, in cucumber cotyledons [13]. This result led us to examine the effect of N-phenylimide herbicides on porphyrin synthesis. Plastids were isolated from greening corn leaves and Proto synthesizing activity from 5-aminolevulinic acid was solubilized by *n*-dodecyl β-D-maltoside, which had been found to be effective in solubilizing a polypeptide to which Proto binds specifically [14, 15]. Both S-23121 and S-23142 strongly inhibited Proto synthesis from 5-aminolevulinic acid in solubilized enzymes ([15], Fig. 3). Next, inhibition of

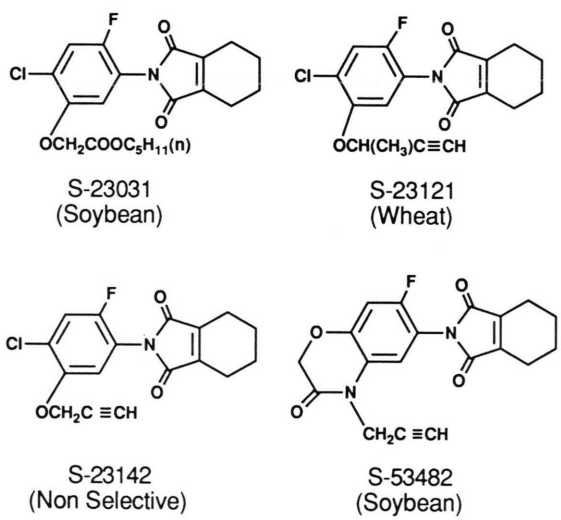

Fig. 1. Chemical structures of N-phenylimide photobleaching herbicides. The main use of each compound is shown in parentheses.

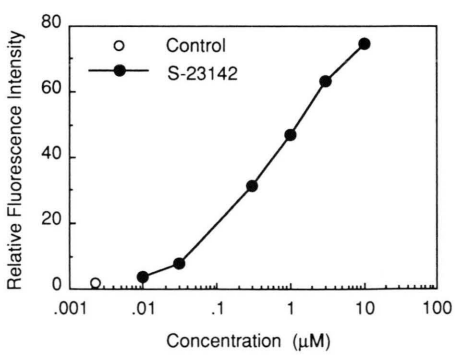

Fig. 2. Effect of S-23142 on porphyrin accumulation in cucumber cotyledons. Cucumber cotyledons treated with appropriate concentrations of S-23142 for 6 h under fluorescent light (11 W/m^2) were homogenized in buffer (10 mM sodium pyrophosphate, 0.3 M sucrose), and filtered with 6 layers gauze. The filtered solution was centrifuged at 3000 × *g* for 10 min. Fluorescence of the supernatant was measured at excitation of 410 nm and emission of 635 nm wavelength.

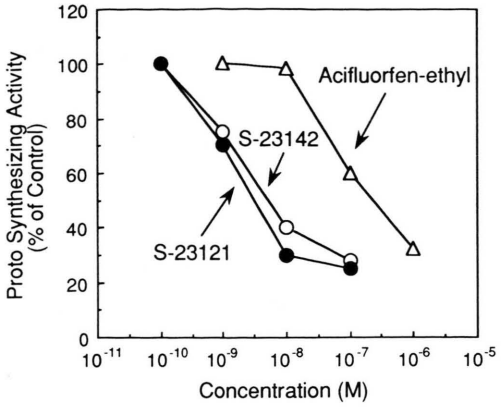

Fig. 3. Dose-dependent inhibition of solubilized Proto synthesizing activity by photobleaching herbicides. Proto synthesizing activity of corn plastid solubilized by *n*-dodecyl β-D-maltoside was measured using 5-aminolevulinic acid as the substrate. The mixture was incubated for 2 h in darkness, and the reaction was stopped by adding 90% acetone containing 0.1 N NH_4OH. After centrifugation at 10,000 × *g* for 5 min, the Proto synthesized was measured using HPLC. Proto detection was performed using a fluorescence monitor with excitation and emission wavelengths at 420 nm and 630 nm, respectively (from [15]).

PPO, which has been identified as the target enzyme of some photobleaching herbicides [1–3], was evaluated in the solubilized plastid enzyme fraction, which was isolated from greening radish cotyledons by the method described [15]. S-23142 inhibited PPO activity more strongly than AFE (Fig. 4).

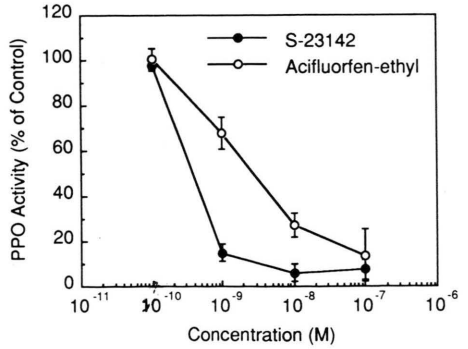

Fig. 4. Effect of photobleaching herbicides on PPO activity in radish plastid enzymes solubilized by *n*-dodecyl β-D-maltoside. PPO activity was determined by measuring fluorescence intensity at 630 nm of the Proto formed from protoporphyrinogen IX at 30 °C for 10 min as described [1, 2]. Bars are standard errors.

In order to examine the interaction of N-phenylimide herbicides with their target sites, binding of [^{14}C]S-23121 to the solubilized corn plastid fraction was investigated using the polyethyleneimine filtration method as described [16]. The binding of [^{14}C]S-23121 to the solubilized plastid fraction was displaced by AFE and S-23142, but not by the photosystem II electron transport inhibitor diuron (DCMU) ([15], Fig. 5). These results indicate that N-phenylimide herbicides have a very similar mode of action to DPEs and that both share a common binding site.

Isolation of Chlamydomonas reinhardtii mutants resistant to N-phenylimide herbicides

One of the most useful methods for analyzing the mode of action of herbicides involves the use of mutants that show resistance to the herbicides, preferably in an organism in which genetic and molecular biological analysis could be applied [17]. Investigation of the biochemical characteristics of such mutants, as well as isolation of the resistance

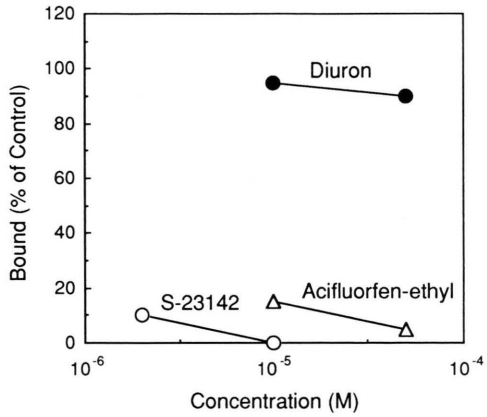

Fig. 5. Displacement of [^{14}C]S-23121 binding to a solubilized corn plastid fraction by photobleaching herbicides. The binding mixtures were incubated at 30 °C for 1 h, placed on ice, and filtered through a glass fiber filter coated with polyethyleneimine. After filtration, the filters were washed three times with 5 ml of ice-cold 10 mM Tris-HCl buffer (pH 7.6). Specific binding was defined as the radioactivity difference between the incubation with and without unlabeled S-23121. The amount of [^{14}C]S-23121 bound was corrected for non-specific binding in the presence of 10 μM unlabeled S-23121 and plotted as a percentage of the amount obtained without competition. Total and specific binding of control were 7.6–8.2 and 3.6–4.1 pmol/mg protein, respectively (from [15]).

gene, permits a great increase in our understanding of herbicide action. For this purpose, we attempted to isolate mutants of the unicellular green alga *Chlamydomonas reinhardtii* resistant to N-phenylimide herbicides.

Wild type *C. reinhardtii* (strain CC-407) is normally highly sensitive to N-phenylimide photobleaching herbicides; 0.006 μM S-23142 inhibited cell growth of CC-407 by 90% over 48 h in cell suspension culture [20]. Three cell lines showing growth on agar plates containing 0.3 μM S-23142 were isolated following mutagen treatment [18]. Since one of the three resistant cell lines, RS-3, showed the highest resistance to S-23142, we chose it for further characterization. The S-23142 concentration inhibiting RS-3 growth for 48 h in liquid culture by 90% was 0.6 μM, which was 100 times higher than that for wild type. RS-3 was also found to be resistant to the photobleaching herbicides oxadiazon, oxyfluorfen and AFE, which are considered to have the same action mechanism as S-23142, but not to other herbicides with different modes of action such as paraquat, diuron (DCMU) and fluridone ([20], Table I). This suggests that the mutation affects a common site of action for photobleaching herbicides.

Characterization of the Chlamydomonas reinhardtii RS-3 mutant strain

Like susceptible higher plants, CC-407 cells treated with 0.3 μM S-23142 accumulate fluorescent pigment(s) having spectral characteristics similar to Proto [18]. Maximum porphyrin accumulation in cell suspensions of CC-407 and RS-3 cultured for 24 h was reached at 0.03 μM and 3 μM respectively [18, 20], thus demonstrating the same 100-fold resistance for porphyrin accumulation in RS-3 as was seen for cell growth.

Then, the effect of S-23142 and AFE on protoporphyrin synthesis in chloroplast fragments isolated from CC-407 and RS-3 was examined. Cells were broken in 50 mM Tris-HCl, 0.5 M sucrose, 20 mM $MgSO_4$, 0.25% (w/v) bovine serum albumin, 2 mM 2-mercaptoethanol by sonication and the homogenate was centrifuged for 1.5 min at 500 × *g*. Chloroplast fragments were prepared by centrifugation of the supernatant for 5 min at 1900 × *g*, resuspended and precipitated by the same centrifugation twice. Mg-Proto synthesizing activity from 5-aminolevulinic acid in chloroplast fragments isolated from RS-3 was less sensitive to S-23142 and AFE than that from wild type CC-407 (Fig. 6). These results indicated that the mode of action of S-23142 in *Chlamydomonas* is essentially the same as in higher plants and strongly suggest that the mutation in the RS-3 occurs at the site of action of S-23142 in the porphyrin synthesis pathway.

Genetic analysis has shown that the herbicide resistance phenotype in the RS-3 strain results from a single, dominant mutation *(rs-3)* in the nuclear genome [20]. These results are particularly useful for developing strategies to clone the *rs-3* gene.

To determine where the *rs-3* mutation acts in porphyrin biosynthesis, PPO activity in Percoll™-purified chloroplast fragments from *Chlamydomonas* cells was measured [20]. PPO activity of untreated RS-3 and CC-407 was 0.22 ± 0.01 and

Table I. Effect of various types of herbicides on the growth of *Chlamydomonas reinhardtii* in liquid culture (from ref. [20]).

Compound	I_{90} (μM) wt (CC-407)	*rs-3*	Ratio *rs-3*/wt	Mode of action
S-23142	0.006	0.6	100	PPO inhibition
Oxadiazon	1.3	107	82	PPO inhibition
Acifluorfen-ethyl	1.4	20	14	PPO inhibition
Oxyfluorfen	0.03	0.34	11	PPO inhibition
Paraquat	1.8	1.8	1.0	radical formation
Diuron	1.2	1.8	1.5	PS II inhibition
Fluridone	0.46	0.76	1.7	inhibition of carotene synthesis
Butachlor	0.048	0.045	0.9	inhibition of cell division

I_{90} = Herbicide concentration which inhibits cell growth by 90%.

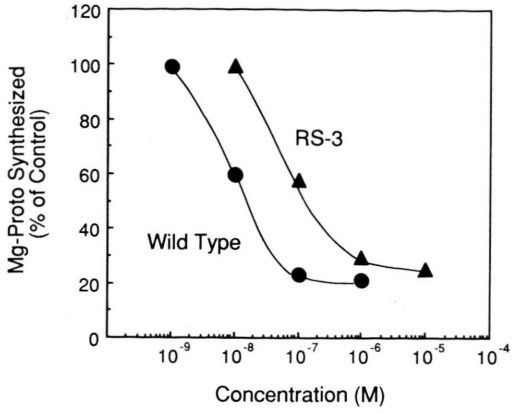

Fig. 6. Effect of S-23142 on Mg-Proto synthesizing activity in chloroplast fragments of *C. reinhardtii*. Mg-Proto synthesizing activity in chloroplast fragments was measured using 5-aminolevulinic acid as the substrate as described [1, 2]. The mixture was incubated for 2 h at 30 °C in darkness, and the reaction was stopped by adding acetone. After centrifugation at 12,000 × *g* for 5 min, the Mg-Proto synthesized was measured using HPLC. Mg-Proto detection was performed using a fluorescence monitor with excitation and emission wavelength at 410 nm and 594 nm, respectively.

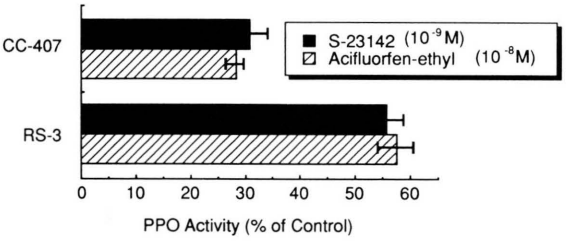

Fig. 7. Effect of photobleaching herbicides on PPO activity in chloroplast fragments from *rs-3* and wild type of *C. reinhardtii*. PPO activity was determined by measuring fluorescence intensity at 630 nm of the Proto formed from protoporphyrinogen IX by isolated chloroplast fragments at 30 °C for 10 min as described [1, 2]. Chloroplast fragments were prepared by sonication and purified by Percoll™ linear density gradient (10 ~ 80%, v/v) centrifugation by modifying the method described [21]. The reaction mixture contained 75 µg protein/ml of chloroplast fragments. Data are shown with standard errors (from [20]).

0.23 ± 0.01 pmol/µg protein/min respectively, suggesting that the catalytic site of PPO is not affected by this mutation. However, PPO activity in Percoll™-purified chloroplast fragments isolated from RS-3 was significantly less inhibited by S-23142 and AFE than that from wild type CC-407 (Fig. 7), suggesting that the *rs-3* mutation affects the possible herbicide binding site of PPO [20].

Conclusions

In this study, we demonstrate that N-phenylimide and DPE herbicides have similar mechanisms and share the same binding site. Results obtained using the herbicide-resistant *Chlamydomonas reinhardtii* strain RS-3 support this idea. Once the molecular alteration present in the *rs-3* mutation is known, it will be a very useful tool for future elucidation of the mechanism of action common to N-phenylimide and DPE herbicides, since the *rs-3* mutation is thought to affect the interaction between PPO and the photobleaching herbicides. Analyses of this interaction at the molecular level by utilizing the resistant gene reported here will be very useful in developing "biorational" approaches for the synthesis of new photobleaching herbicides, creation of crops resistant to photobleaching herbicides and understanding of the chlorophyll synthesis pathway. Further genetic analysis and molecular cloning of the resistant gene are in progress.

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