Alternaria alternata Crofton-Weed Toxin: a Natural Inhibitor of Photosystem II in *Chlamydomonas reinhardtii* Thylakoids

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The action site of *Alternaria alternata* Crofton-weed toxin (AAC-toxin), isolated first from *Alternaria alternata* (Fr.) Keissler, was investigated in *Chlamydomonas reinhardtii* thylakoids. The results revealed that AAC-toxin inhibited photophosphorylation in a concentration-dependent pattern. Similarly, toxin inhibited uncoupled, basal electron flow and photosystem II (PSII) electron transport as well. However, toxin did not affect photosystem I (PSI) activity or the partial reaction of electron transport from H_2O to silicomolybdic acid (SiMo). Therefore, the action site of toxin was located at Q_B level. In addition, the toxin may behave as an energy-transfer inhibitor at high concentrations by inhibiting phosphorylating electron transport and Mg^{2+}ATPase activity. Chlorophyll *a* fluorescence induction and JIP test corroborated the inhibition at Q_B level. Through observations of the different sensitivity of toxin on D1 mutants of *C. reinhardtii*, evidence further confirmed that AAC-toxin inhibited electron transport by displacing the Q_B on the D1 protein, and the mode of action was similar to phenol-type PSII inhibitors.

INTRODUCTION

Crofton weed (*Eupatorium adenophorum* Spreng) is a troublesome invasive weed in China. *Alternaria alternata* (Fr.) Keissler is a natural pathogenetic fungus isolated from Crofton weed for the first time, which caused a brown diseased leaf spot and produced a secondary metabolite, *Alternaria alternata* Crofton-weed toxin (AAC-toxin) (*1*). The chemical structure of AAC-toxin is shown as **Figure 1**. The capability of AAC-toxin to kill the seedlings of many mono- and dicotyledonous weeds even at low concentrations (*2*) makes it a possible candidate for the development of a biological herbicide. However, not much is known about the mechanism of its action. Primary work has indicated that the action of AAC-toxin was likely to inhibit electron transport of PSII (*3*). But a more detailed and systemic study is needed in order to understand precisely the target and mode of toxin action. In addition, the influences of toxin on photophosphorylation, noncyclic electron transport, and ATPase activity have not yet been investigated.

The eukaryotic unicellular green alga *Chlamydomonas reinhardtii* resembles higher plant cells (*4*) and constitutes a powerful experimental model system for study of the photosynthetic machinery. It can be used to isolate numerous photosynthetic mutants, which have helped to examine the function of the photosynthetic apparatus (*5*). It is well-known that the herbicide binding protein is the D1 protein of the RC of PSII, but the X-ray structure of PSII at high resolution is not
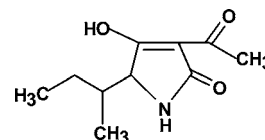

Figure 1. Chemical structure of AAC-toxin.

yet available. So, herbicide-resistant or -supersensitive mutant is used to identify amino acids in the target that were involved in herbicide binding (*6*). By analyzing D1 mutants of *C. reinhardtii* for their sensitivity toward different inhibitors, we can gain useful insight into their inhibitor binding modes (*7*). Therefore, in this paper, we investigate in detail the action site and mode of AAC-toxin by measuring ATP synthesis, O_2 evolution or uptake, Chl *a* fluorescence induction transient, and the resistance or sensitivity of D1 mutants of *C. reinhardtii*.

MATERIALS AND METHODS

Chemicals and Reagents. All reagents and solvents were analytical grade. DCMU (D-7763), DCIP (D-1878), DBMIB (271993), SiMo (383368), MV (856177, 98%), sodium ascorbate (B13011), and PMS (P-9625) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (J0014), HEPES (B2012), and Tricine (D20031) were obtained from Ameresco (Solon, OH). NaN_3 was purchased from Chemical Reagent Co. (Shanghai, China). Luciferin/luciferase (dry powder) kit was from Chinese Academic of Science (Shanghai, China). ADP and Na_2HPO_4 were purchased from Fluka (Steinheim, Germany). Other reagents, including methanol, ethanol, sucrose, $MgCl_2$, EDTA, glycerol, NaCl, FeCy, KH_2PO_4, NH_4Cl, PD, trichloroacetic acid,

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sorbitol, KCl, MnCl$_2$, and Na$_2$EDTA, were prepared from Shanghai Chemical Co. Ltd. (Shanghai, China).

AAC-Toxin Purification and Treatment Procedures. AAC-toxin was isolated and purified from the culture of *A. alternata* isolate 501 by the procedures previously reported (*8, 2*), and dissolved in methanol. In order to avoid solvent-induced damage to photosynthetic membranes, the final concentration of methanol in the thylakoid suspension was kept well below 1% (v/v) in all measurements, and the results showed the effect of methanol was negligible (data not shown). Thylakoids treated with different concentrations of AAC-toxin were first preincubated for 10 min in the dark and then were added to reaction medium to make a final Chl concentration of 20 μg/mL. The treatment for Chl *a* fluorescence induction is described below.

Cultivation of *C. reinhardtii* Cells. *C. reinhardtii* cells were obtained from the Chlamydomonas Genetic Center (Department of Botany, Duke University, Durham, NC). The *C. reinhardtii* wild type (CC-124) and five mutants of D1 protein (D1-V219I, D1-F255Y, D1-S264A, D1-L275Y, and D1-G256D) were cultivated mixtrophically in liquid TAP medium at 25 °C, with a light/dark cycle of 12/12 h and constant shaking. The growth rate of wild type and the mutant was determined by measuring the optical density of the cells in the TAP culture medium at 750 nm (*5*). The cells in the second hour of the light phase during the log phase of their optimal growth conditions were harvested and used for subsequent measurements.

Thylakoid Preparation and Chlorophyll Determination. The thylakoid preparation was as described earlier (*9*) with some modifications. The log-phase cells were centrifuged at 2000*g* for 4 min at 4 °C. The pellet was washed twice with a buffer containing 350 mM sucrose, 20 mM HEPES (pH 7.5), and 2.0 mM MgCl$_2$. The cells were resuspended in the above buffer to ~0.5 mg of Chl/mL and were sonicated slightly. The broken cells (thylakoids) were centrifuged at 100000*g* for 20 min at 4 °C. The pellet was resuspended in a buffer containing 400 mM sucrose, 20 mM HEPES (pH 7.5), 5.0 mM MgCl$_2$, 5.0 mM EDTA, 1.0 mg/mL bovine serum albumin, and 20% (v/v) glycerol. It was briefly centrifuged at 1000*g* for 10 s to remove the unbroken cells. The supernatant containing the thylakoids was recentrifuged at 14000*g* for 1 min. The pellet was resuspended in the above-described buffer to a Chl concentration of ~1 mg/mL. The aliquots of resuspension were quickly frozen in liquid nitrogen until use. Chl determinations were performed according to Arnon's method (*10*).

Photophosphorylation Determination. Photophosphorylation of thylakoids was measured as described by Wei et al. (*11*). The reaction medium with a final volume of 1 mL contained 50 mM Tricine-NaOH (pH 8.0), 5 mM NaCl, 5 mM MgCl$_2$, 1 mM FeCy for noncyclic photophosphorylation or 50 μM PMS for cyclic photophosphorylation, and thylakoids. After 1 min of preincubation, the reaction was initiated by addition of 1 mM ADP and 2 mM Na$_2$HPO$_4$. ATP content was measured by the luciferin/luciferase luminescence assay (*12*).

Photosynthetic Electron Transport Measurement. Photosynthetic noncyclic electron flow from water to MV was determined with a Clark-type electrode (Chlorolab2, Hansatech, U.K.) at a light intensity of 500 μE m^{-2} s^{-1} and temperature of 25 °C (*13, 14*). The basal electron transport reaction medium contained 50 mM HEPES (pH 7.6), 50 μM MV, 2 mM NaN$_3$, 5 mM MgCl$_2$, and 10 mM NaCl. Both phosphorylating and uncoupled electron transport were the same as basal electron transport except that 1 mM ADP plus 3 mM KH$_2$PO$_4$ or 5 mM NH$_4$Cl was added, respectively.

The measurement of PSI and PSII electron transport was similar to that of noncyclic electron flow. For uncoupled PSI electron flow, the reaction medium contained 50 mM HEPES (pH 7.6), 5 mM MgCl$_2$, 10 mM NaCl, 50 μM DCMU, 0.2 mM DCIP, 2 mM sodium ascorbate, 2 mM NaN$_3$, 50 μM MV, and 5 mM NH$_4$Cl. For uncoupled PSII electron flow, the reaction medium contained 50 mM HEPES (pH 7.6), 5 mM MgCl$_2$, 10 mM NaCl, 1 μM DBMIB, 5 mM NH$_4$Cl, 1 mM PD, and 4 mM FeCy. For the partial reaction of uncoupled electron transport from H$_2$O to SiMo, the reaction medium was the same as uncoupled PSII electron flow in the absence of PD and FeCy, except that 200 μM SiMo and 10 μM DCMU were added.

Mg^{2+}ATPase Activity Assays. Thylakoids were preincubated in 25 mM Tricine/NaOH (pH 8.0), 5 mM MgCl$_2$, and 25% ethanol at 37 °C. Then the reaction was started by the addition of 5 mM ATP

Table 1. Effect of AAC-Toxin on Photophosphorylation of *C. reinhardtii* Thylakoids[a]

concn (μg/mL)	activity (%)	
	noncyclic photophosphorylation, water to FeCy	cyclic photophosphorylation, water to PMS
0	100 ± 0.00 Aa	100 ± 0.00 Aa
10	87.4 ± 4.40 ABb	ND
40	76.3 ± 2.47 BCc	90.4 ± 3.52 Bb
80	65.3 ± 2.97 Cd	78.2 ± 1.09 Cc
120	27.2 ± 1.11 De	68.4 ± 2.08 Dd
160	11.5 ± 0.03 Ef	37.5 ± 1.40 Ee

[a] Other conditions are described under Materials and Methods. Control values for noncyclic and cyclic photophosphorylation are 27.19 ± 0.76 and 55.39 ± 1.12 mmol of ATP (mg of Chl)$^{-1}$ h^{-1}, respectively. Data are expressed as mean ± SE for 3–5 replications. Values followed by different capital letters are significantly different at $P < 0.01$, while those followed by different lowercase letters are different at $P < 0.05$ (Tukey's HSD test). ND, not determined.

and was stopped by the addition of 3% trichloroacetic acid (*15*). Inorganic phosphate content was determined according to Taussky and Shorr (*16*).

Chl *a* Fluorescence Induction. Chl *a* fluorescence induction transients were registered by using a plant efficiency analyzer (Handy PEA, Hansatech Instruments, U.K.) at room temperature. Experiments were performed in 1-cm diameter vials containing 0.5 mL of reaction medium [0.1 M sorbitol, 20 mM HEPES (pH 7.0), 10 mM MgCl$_2$, 10 mM KCl, and 0.1 mM NH$_4$Cl], which contained thylakoids at a Chl concentration of 5 μg/mL (*9*). The reaction medium was dark-adapted for 10 min; at the end of the period, different concentrations of AAC-toxin were added in the dark, followed by gentle shaking. After a 10 min dark incubation, induction kinetic curves were recorded for a period of 1 s at actinic irradiance of 3000 μmol m^{-2} s^{-1}. Each fluorescence induction curve was a mean of 6−8 repetitions.

Determination of AAC-Toxin Resistance or Sensitivity. The resistance or sensitivity was determined as described by De Prado et al. (*17*) with a few modifications. The reaction medium contained 25 mM Tricine-NaOH (pH 7.6), 1 mM MgCl$_2$, 1 mM MnCl$_2$, 2 mM Na$_2$EDTA, 0.4 M sorbitol, 3 mM FeCy, and 2.5 mM NH$_4$Cl. EC$_{50}$ value (the concentration that inhibits the Hill reaction by 50%) was determined. The resistance factor is defined as R/S (R$_{EC50}$/S$_{EC50}$). In this paper, R represents the D1 protein mutants of *C. reinhardtii*, and S is the wild-type *C. reinhardtii*.

Data Analysis. EC$_{50}$ values were calculated on the basis of logistic regression with SAS software (version 8.0, *18*). Tukey's HSD test was performed with SPSS statistical software, version 13.0, to identify differences among different concentrations.

RESULTS AND DISCUSSION

Inhibition of AAC-Toxin upon Photophosphorylation. Photosynthetic phosphorylation encompasses both cyclic photophosphorylation, which is concerned only with PSI, and noncyclic photophosphorylation, which is concerned with both PSI and PSII. Phosphorylation rates of *C. reinhardtii* were lower than those of higher plants (*19*), which was confirmed in our result. As shown in **Table 1**, both noncyclic photophosphorylation from water to FeCy and cyclic photophosphorylation from water to PMS in thylakoids of *C. reinhardtii* were inhibited in a concentration-dependent manner ($P < 0.01$). Moreover, higher inhibition of noncyclic photophosphorylation was observed at the same time (EC$_{50}$ values were 85.02 and 142.5 μg/mL for noncyclic and cyclic photophosphorylation, respectively).

Inhibition upon Photosynthetic Electron Transport. It is well-known that ATP synthesis is coupled to electron flow. The inhibition of ATP synthesis could be due to uncoupling ATP synthesis from electron transport, blocking electron transport, or blocking the phosphorylation reaction itself (*20*). In order to

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Table 2. Effects of AAC-Toxin on Noncyclic Electron Transport of *C. reinhardtii* Thylakoids from Water to MV[a]

concn (μg/mL)	activity (%)		
	uncoupled	basal	phosphorylating
0	100 ± 0.00 Aa	100 ± 0.00 Aa	100 ± 0.00 Aa
5	95.3 ± 0.42 Bb	96.8 ± 0.80 AaBb	99.1 ± 0.82 Aa
10	92.8 ± 0.85 Cc	95.2 ± 2.95 ABb	98.5 ± 1.42 Aa
20	89.8 ± 1.50 Dd	93.8 ± 0.22 BbCc	98.0 ± 1.81 Aa
40	85.4 ± 1.77 Ee	88.8 ± 1.63 Cd	94.9 ± 1.56 ABb
100	41.8 ± 1.60 Ff	71.6 ± 2.56 De	76.5 ± 1.36 Cc
120	26.5 ± 1.25 Gg	62.8 ± 0.69 Ef	54.6 ± 0.16 Dd
200	0.0 ± 0.00 Ff	47.4 ± 0.91 Fg	13.5 ± 1.00 Ee

[a] Other conditions are described under Materials and Methods. Control values of basal, phosphorylating, and uncoupled electron transport are 23.94 ± 0.51, 28.2 ± 0.46, and 30.35 ± 0.65 mmol of O_2 (mg of Chl)$^{-1}$ h^{-1}, respectively. Data are expressed as mean ± SE for 3–5 replications. Values followed by different capital letters are significantly different at $P < 0.01$, while those followed by different lowercase letters are different at $P < 0.05$ (Tukey's HSD test).

Table 3. Effects of AAC-Toxin on Uncoupled PSII and PSI Electron Transport and Partial Reaction of Uncoupled PSII[a]

concn (μg/mL)	activity (%)		
	uncoupled PSII electron transport		uncoupled PSI electron transport
	H_2O to PD/FeCy	H_2O to SiMo[b]	DCIP$_{red}$ to MV
0	100 ± 0.00 Aa	100 ± 0.0 Aa	100 ± 0.00 Aa
5	94.2 ± 1.79 Bb	ND	100 ± 2.07 Aa
10	90.5 ± 1.52 Cc	98.4 ± 3.20 Aa	99.5 ± 0.86 Aa
20	86.1 ± 1.61 Dd	ND	98.8 ± 2.10 Aa
40	71.1 ± 1.08 Ee	97.7 ± 2.64 Aa	99.2 ± 1.61 Aa
100	28.4 ± 1.02 Ff	99.2 ± 1.71 Aa	86.7 ± 1.17 Bb

[a] Other conditions are described under Materials and Methods. Control values for uncoupled PSII, uncoupled PSI, and partial reaction are 52.9 ± 0.86, 48.2 ± 0.12, and 20.2 ± 0.62 mmol of O_2 (mg of Chl)$^{-1}$ h^{-1}, respectively. Data are expressed as mean ± SE for 3–5 replications. Values followed by different capital letters are significantly different at $P < 0.01$, while those followed by different lowercase letters are different at $P < 0.05$ (Tukey's HSD test). ND, not determined. [b] Partial reaction of uncoupled PSII.

study the inhibition of photophosphorylation by AAC-toxin, we determined the effect of toxin on basal, phosphorylating, and uncoupled electron transport from water to MV. The one-way ANOVA analysis (Tukey's HSD) showed that both uncoupled and basal electron flow were inhibited as concentrations of toxin increased ($P < 0.01$). However, phosphorylating electron transport was not affected until the concentrations of toxin reached 40 μg/mL ($P > 0.05$, **Table 2**). In addition, the inhibitory effect on uncoupled electron transport was greater than on basal and phosphorylating electron transport (EC$_{50}$ values were 81.8, 188.5, and 127.8 μg/mL, respectively). Electron transport activity decreased to 26.5%, 62.8%, and 54.6% for uncoupled, basal, and phosphorylating electron transport, respectively, at the concentration of 120 μg/mL compared to the control. The results suggested that the action site of AAC-toxin was exposed when the thylakoid was unenergized (uncoupled state) but was partially buried during the energization of the membrane (phosphorylating and basal state). At high concentrations, greater inhibition of phosphorylating electron transport was observed compared to basal electron transport. So, toxin might affect the phosphorylation reaction itself. By comparison of uncoupled and basal electron flow, it was thought that the toxin has slight or even no effect on uncoupling ATP synthesis from electron transport. Altogether, inhibition of ATP synthesis was mainly induced by the block of electron transport.

Subsequently, we measured the effect of AAC-toxin on PSII and PSI electron transport. The results showed that the AAC-toxin inhibited uncoupled PSII electron transport from H_2O to PD/FeCy in a concentration-dependent pattern, and significant differences were detected among concentrations ($P < 0.01$). The activity of electron transport decreased to 28.4% at the highest concentration of 100 μg/mL compared to the control (**Table 3**). However, uncoupled PSI electron transport from DCIP$_{red}$ to MV was not affected, although slight inhibition was detected at 100 μg/mL ($P < 0.01$), as described previously by others (*21*). At the same time, the partial reaction of uncoupled PSII electron transport from H_2O to SiMo was not inhibited ($P > 0.05$). It is known the partial reaction from H_2O to SiMo measures H_2O to Q_A electron flow. Therefore, the action site of AAC-toxin was located at the Q_B level of PSII electron transport.
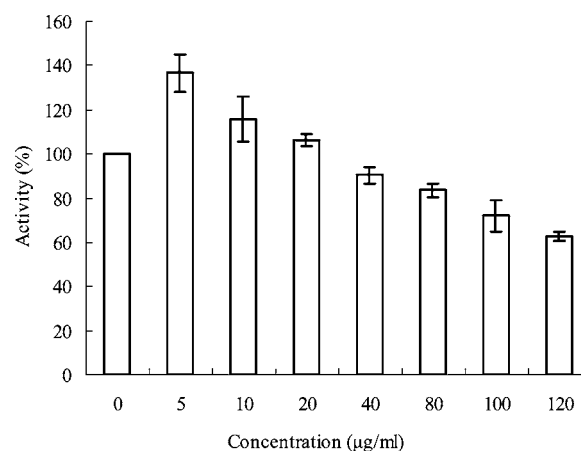
Influence on Mg^{2+}ATPase Activity. As shown in **Figure 2**, toxin tended to mildly stimulate the ATPase activity at concentrations lower than 20 μg/mL, with enzyme activity

Figure 2. Effect of AAC-toxin on Mg^{2+}ATPase activity of *C. reinhardtii* thylakoid in the presence of 25% ethanol. Control value is 170.22 ± 2.40 μmol of P$_i$ (mg of Chl)$^{-1}$ h^{-1}. Other conditions are described under Materials and Methods. Data are expressed as mean ± SE for 3–5 replications.

increased 37% at 5 μg/mL. This suggested that the effect of toxin on uncoupling property was slight and could be related to the light perturbation of thylakoid membranes caused by the lipophilic character of toxin (*14, 22, 23*). However, the toxin inhibited ATPase activity at high concentrations, which corresponded with the inhibition of phosphorylating electron transport. The inhibitors blocking the phosphorylation reaction are frequently referred to as energy transfer inhibitors, which blocked the Mg^{2+}ATPase (*20*). So, AAC-toxin could behave as an energy transfer inhibitor at high concentrations. Accordingly, the AAC-toxin might have inhibited Mg^{2+}ATPase at high concentrations, but further work will be needed to clarify it.

Effects on Fast Chl *a* Fluorescence Kinetics. Fast Chl *a* fluorescence transients of thylakoids from *C. reinhardtii* incubated with different concentrations of AAC-toxin for 10 min were measured (**Figure 3**). The control curve showed two steps J (about 2 ms) and I (about 30 ms), between O and P, with J more clearly observed than I at the light intensity used. The toxin significantly affected the Chl *a* fluorescence induction in a concentration-dependent manner. First, the increase of the J level was observed. Second, although all treatments were kept in complete darkness, after the addition of AAC-toxin, the initial fluorescence (F_0) of toxin-treated sample was higher than that

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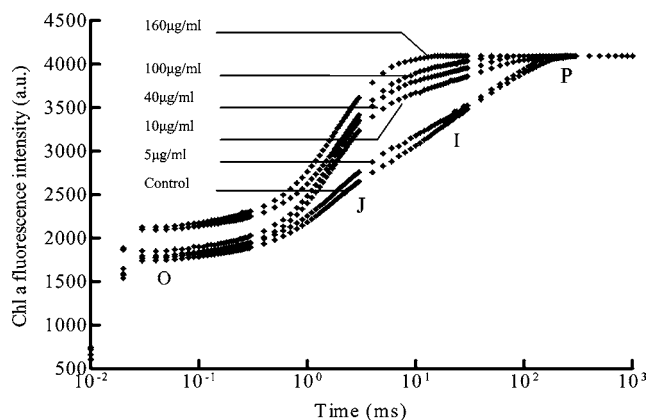

Figure 3. Effects of increased concentrations of AAC-toxin on Chl *a* fluorescence transients of *C. reinhardtii* thylakoids. Different concentrations are shown in the figure. Others conditions are described in Materials and Methods.

Table 4. R/S Values of AAC-Toxin in D1 Mutants of *C. Reinhardtii*

strains	R/S value	resistance or sensitivity
Val219Ile	1.87	R
Gly256Asp	1.67	R
Leu275Phe	2.13	R
Ser264Ala	0.88	S
Phe255Tyr	0.81	S

of the control. Finally, no K peak (300 μs) was observed. As shown in **Figure 3**, with the increase of concentrations, AAC-toxin tended to elevate the amplitude of J-step fluorescence progressively. Thereafter, the magnitude of J-step fluorescence yield was readily saturated at the highest concentration (160 μg/mL), thus transforming the OJIP transient into an OJ sequence.

Strasser and Govindjee (*24*) had suggested that the O step was minimal Chl *a* fluorescence yield (highest yield of photochemistry); the J step reflected an accumulation of $Q_A^- Q_B$ form; the I step was an accumulation of $Q_A^- Q_B^-$ form; and the P step was an accumulation of $Q_A^- Q_B^{2-}$ form. Therefore, upon treatment with AAC-toxin, the increased J level accounted for the lower rate of electron flow between Q_A and Q_B (*25*). As for the rise of F_0 level, it had been observed earlier (*26*). The explanation was that when Q_A was fully oxidized in dark-adapted control thylakoids, F_0 was attained. After addition of the toxin in the dark, Q_B was replaced and an electron was transferred to Q_A from the reaction centers in S_0, thus leading to increased fluorescence (*27*). In addition, Briantais et al. (*28*) thought that a decreased quantum efficiency of PSII photochemistry and a disconnection of a minor antenna complex (containing a small percentage of total PSII chlorophyll), not the LHCII *b*, could also increase the F_0 level. The JIP test allows it (unpublished data). The K peak was attributed to inactivation of the OEC (*29*); no K peak in the induction curves indicated that AAC-toxin did not affect the donor side of PSII (data not shown). On the basis of the results, it can be concluded that AAC-toxin mainly inhibited electron transport from Q_A to Q_B on the acceptor side of PSII, as demonstrated by the JIP test (unpublished data). Afterward, the action mode of toxin was discussed in detail.

Studies with D1 Mutants of *C. reinhardtii*. The D1 protein is the herbicide binding protein. Different amino acid substitutions that confer resistance to many herbicides are clustered in the Q_B binding region between helices IV and V from residue 211 to 275 of the D1 subunit of PSII (*6*). The five D1 protein mutants of *C. reinhardtii* in our paper were resistant to many common herbicides. So, we examined the sensitivity profile of AAC-toxin on the Hill reaction rate for five D1 mutants and wild type. In the case of resistance, the R/S value (resistance factor) is always >1. In the case of supersensitivity or negative cross-resistance, the R/S value is <1 (*6*). The results are shown in **Table 4**. Mutants Ser264Ala and Phe255Tyr were not tolerant against AAC-toxin (R/S values 0.88 and 0.81, respectively);

however, mutants Val219Ile, Gly256Asp, and Leu275Phe showed slight resistance (R/S values were 1.87, 1.67, and 2.13, respectively). It was suggested that the amino acid sites 275, 219, and 256 of D1 protein were likely to participate in toxin binding. Furthermore, these results indicated that toxin surely displaced the Q_B on the D1 protein. The principal difference between the phenols and classical herbicides rests on the fact that the Ser264Ala mutant is not tolerant to phenol-type herbicides, in contrast to the classical ones (*30*). Accordingly, the mode of action of AAC-toxin was similar to that of phenol-type herbicides.

Many natural products and derivatives, which inhibit photosynthesis, have been studied widely in order to develop new biodegradable and environmentally safe herbicides. And the results indicated that these inhibitors have multiple actions in thylakoid membranes. The target can be located at different levels in the electron transport chain: (1) the OEC complex (*31–33, 22*); (2) the span of P_{680} to Q_A (*32, 33*); (3) the Q_B level (*33, 34*); (4) the plastoquinone pool (*35*); or (5) the Cytb6f-PC level (*14*). Otherwise, these inhibitors can behave as uncouplers (*31*) or energy transfer inhibitors (*36*) or as both uncoupler and energy transfer inhibitor at the same time (*14*). AAC-toxin was a natural product isolated from *A. alternata*. According to our studies with *C. reinhardtii* thylakoids, toxin was a photosynthetic electron transport inhibitor, and the target was located at the Q_B level. In addition, toxin may behave as an energy transfer inhibitor, which needs further study.

PSII inhibitors, including numerous compounds and many herbicides, inhibited the electron transport at the acceptor side of PSII by competing with the native plastoquinone Q_B. It can be grouped two families, that is, the classical triazine-urea herbicides and the phenol-type group, by the different modes of action in the Q_B binding site (*30*). The classical herbicides orient themselves preferentially toward Ser 264 of the D1 protein, whereas the binding of phenolic herbicides is toward His 215 (*6*). That is, the classical diuron-type herbicides mostly interacted with Ser264. When Ser264 was substituted by another amino acid (Ala), mutants became tolerant to the classical herbicides but were sensitive to phenol-type herbicides. AAC-toxin, a natural PSII inhibitor, was sensitive to Ser264Ala. So, its mode of action was similar to that of phenol-type PSII inhibitors. However, the resistance to mutants Val219Ile, Gly256Asp, and Leu275Phe suggested that binding of toxin in the Q_B niche has a close correlation with amino acids 275, 219, and 256 of D1 protein, and it displaced Q_B indeed. Next, we will focus on computer models, with the aim to further specify the correct orientation of toxin toward a particular amino acid residue in the binding niche of the Q_B site. On the other hand, the competitive binding method can be generally used for the identification of a common binding site and mechanism of two different PSII inhibitors (*37*). Therefore, we will also employ radioactive labeling methods to study the competing binding of the toxin, so as to understand the mode of toxin more clearly. In conclusion, our aims were to study the mechanism of action of AAC-toxin completely and, furthermore, to synthesize more potent analogues to generate commercially viable herbicides.

ABBREVIATIONS

PSI, photosystem I; PSII, photosystem II; Chl, chlorophyll; Q_A, primary bound plastoquinone; Q_B, secondary bound plastoquinone; TAP, Tris−acetate−phosphate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichloroindophenol; DBMIB, 2,5-dibromo-3-methyi-6-isopropyl-*p*-benzoquinone; FeCy, $K_3Fe(CN)_6$; PD, phenylenediamine; SiMo, silicomolybdic acid; RC, reaction center; MV, methyl viologen; PMS, phenazine methosulfate; OEC, oxygen evolution complex; LHCII, light-harvesting complex II; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ANOVA, analysis of variance.

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