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Identification of tenuazonic acid as a novel type of natural photosystem II inhibitor binding in Q_B-site of *Chlamydomonas reinhardtii*

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Abstract

Tenuazonic acid (TeA) is a natural phytotoxin produced by *Alternaria alternata*, the causal agent of brown leaf spot disease of *Eupatorium adenophorum*. Results from chlorophyll fluorescence revealed TeA can block electron flow from Q_A to Q_B at photosystem II acceptor side. Based on studies with D1-mutants of *Chlamydomonas reinhardtii*, the No. 256 amino acid plays a key role in TeA binding to the Q_B-niche. The results of competitive replacement with [^{14}C]atrazine combined with JIP-test and D1-mutant showed that TeA should be considered as a new type of photosystem II inhibitor because it has a different binding behavior within Q_B-niche from other known photosystem II inhibitors. Bioassay of TeA and its analogues indicated 3-acyl-5-alkyltetramic and even tetramic acid compounds may represent a new structural framework for photosynthetic inhibitors.

Keywords: Tenuazonic acid; Photosystem II inhibitor; JIP-test; Binding niche; *psb*A mutant; Resistance

1. Introduction

Tenuazonic acid (TeA) is a phytotoxin produced by some phytopathogenic fungi. It has been found in many plant materials such as olives, sunflower seeds, mandarins, peppers, sorghum, tobacco, rice, melons and linseed [1–3]. Consequently, most of the research on TeA has focused on concerns with toxicity. Davies et al. [4] reported that TeA was toxic to chicken embryos and could cause haemorrhage and death in mice. Conversely, TeA also exhibits desirable bioactivity, such as antitumor, antiviral and antibacterial activity [5].

However, only a few studies have addressed the bioactivity of TeA to plants, of which include cause of brown leaf spot in leaves of rice, tobacco, tomato and jimsonweed and weak inhibition of seed germination of rice, lettuce, wheat, tomato, rye, carrot and *Striga hermonthica* [6–9]. Recently, Qiang et al. found a phytotoxin produced by an *Alternaria alternata* isolated from *Eupatorium adenophorum* (Asteraceae), which causes a brown leaf spot disease in many weeds [10–12]. The phytotoxin was later shown to be tenuazonic acid [13,14]. Prior studies on the mechanism of action of TeA seems to support that TeA inhibits protein synthesis in eukaryotic cells [15], and moreover, has weak activity on the inhibition of HPPD (*p*-Hydroxyphenylpyruvate dioxygenase) [16], and reduces root and shoot length of seedlings [17]. In our laboratory, bioassay results showed that TeA is phytotoxic to a wide range of plants, from weeds to crops [11], and then quickly kills the seedlings of mono- and dicotyledonous weeds[13,14]. Our preliminary studies indicated that TeA greatly inhibited photosynthesis by blocking photosystem II electron flow from Q_A to Q_B, but did not affect the level and activity of RuBP carboxylases, photosynthetic pigment content, thylakoid membrane protein and donor side of photosystem II [18,19]. Consequently, it is

Abbreviations: DCMU, (3-(3, 4-dichlorophenyl)-1,1-dimethylurea); F_o, F_M, initial and maximal fluorescence; $F_{300\ \mu s}$, fluorescence at 300 μs; F_J, fluorescence at the J-step; V_J, relative variable fluorescence at the J-step; tF_M, time to reach maximal fluorescence F_M; M_o, approximated initial slope of the fluorescence transient $V=f(t)$; S_m, normalized total complementary area above the O–J–I–P transient; Q_A, Q_B, primary and secondary quinone acceptor; N, number of Q_A reduction events between time o and tF_M; ψ_o, probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-; φ_{Eo}, quantum yield for electron transport (at $t=0$); RC, reaction center; ET, energy flux for electron transport; PDB, Protein Data Bank

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possible that TeA may represent a new type of photosystem II inhibitor.

It is well known that photosystem II is a large protein complex that is located on the thylakoid membranes of higher plants, algae and cyanobacteria, which contains light-harvesting complex, reaction center [20,21]. The reaction center consists of D1, D2, cytochrome b-559 protein and gene products of *psb*I and *psb*W [22,23], Q_B binding-site locates in the D–E region of D1 protein, which is the part between the fourth and fifth transmembrane helices. After it accepted two electrons and is reduced, Q_B is released off Q_B-niche and replaced by a new plastoquinone from pool or a molecule of photosystem II inhibitors [21]. It has been reported that Photosystem II inhibitors work at the acceptor side of photosystem II where they compete with Q_B for the binding niche, hence, interrupt photosystem II electron transfer from Q_A to Q_B [24]. It has been documented that known photosystem II inhibitors are grouped into two families according to chemical specifications and inhibitory patterns, a ureas/triazine and a phenol family [25]. The first family inhibitors have the common structure group N–C═X, where X signifies N or O not S. The second family contains the aromatic hydroxyl group bearing nitro and/or halogen and /or nitrile substituent [26]. Many compounds from this family of inhibitors have been successfully developed as commercial herbicides: diuron, atrazine, bromanil, and phenols to cite a few [27]. Therefore, knowledge of the herbicide and Q_B binding site of the photosystem II reaction center is important for the design of new herbicides and for the generation of herbicide resistant plants [28]. A number of mutations in the gene *psb*A, which encodes D1 Protein, have been found to confer herbicide-resistance. In algae mutants, mutations at positions 219, 251, 252, 255, 256, 264 and 275 are most common and result in resistance to several classes of herbicides [29]. All these *psb*A mutations are located on helices IV and V helices and their connecting loop in D1 protein, which is a specific region of the polypeptide between amino acid residues 211 and 275 [30].

TeA has an N–C═O moiety that is characteristic of compound in the first family of photosystem II inhibitors. As a new photosynthesis inhibitor, more studies are required to elucidate where TeA binds and how it works. In this paper, we describe here a series of physiological and chemical experiments conducted to determine the binding site of TeA in the photosystem II electron transport chain, and thus accurately define its cause of inhibition in photosynthesis.

2. Materials and methods

2.1. Chemical extraction and purification

Tenuazonic acid was isolated and purified from a culture of *A. alternata* isolate 501[10–12,31]. Fungal spores were inoculate into a 1000 ml flask with 400 ml sterile potato, sugar and potassium dihydrogen phosphate liquid medium (PSK), and cultured for 6 days on an orbit shaker (Model 3527X Orbit Environ-Shaker; Lab-Line Instruments, Inc., Ill. USA) at 110 rpm in the dark at 25 °C. The filtrate was passed through a column of macroporous resin DA201, and the toxin eluted with alcohol. The alcohol-diluted extraction was concentrated by rotary evaporation under regular pressure at 80 °C. The concentrate was extracted with an equal volume of ethyl acetate three times. The extract was concentrated into the crude toxin by rotary evaporation under regular pressure at 70 °C. The toxin was further purified by thee rounds of column chromatography on Si gel, and the fractions were prepared by silica TLC until the purified toxin was obtained. The purity was determined by TLC and HPLC (Waters, US) to be higher than 98%. For all experiments, TeA stock solution (100 mM) was dissolved in 50% methanol, further diluted in 50% methanol. DCMU and HEPES were purchased from SIGMA. Other common chemical reagents used in this work were obtained from Amresco.

2.2. Chlamydomonas reinhardtii strains and culture of cells

Chlamydomonas reinhardtii wild-type strain CC124 and chloroplast *psb*A D1 mutant strains, CC1403 *dr-u-2mt*$^+$ (DCMU resistant, *psb*A Val219Ile), CC1847 *Ar207 mt*$^+$ (Atrazine resistant, *psb*A Phe255Tyr), CC2059 *Ar204 mt*$^+$ (Atrazine resistant, *psb*A Gly256Asp), CC2857 DCMU4 *er-u-1a mt*$^-$ (DCMU and Atrazine cross resistant, *psb*A Ser264Ala), CC1844 *Br202 mt*$^+$ (Bromacil resistant, *psb*A Leu275Tyr) were obtained from the *Chlamydomonas* Center (Duke University, USA). Cells were grown at 25 °C in liquid Tris–acetate–phosphate (TAP) medium under approximate 100 μmol m^{-2} s^{-1} white light (day/night, 12 h/12 h). The cell culture reaching late logarithmic phase (approximately 4 or 5 days) was harvested and used for latter determinations and preparations for thylakoid membranes. The chlorophyll concentration was analyzed as described by Porro et al. [32]. For chlorophyll determinations from intact cells, the cells were suspended in 80% acetone at 40 °C for 20 min. The samples were centrifuged at 10,000×*g* for 5 min, and the pellet was removed. The absorbance of the supernatant was determined at 663.6 and 664.6 nm using a UV754 spectrophotometer. Chlorophyll concentration was calculated according to the following equations: $[\text{Chl } a+b]=17.76\text{OD}^{646.6}+7.34\text{OD}^{663.6}$ (μg ml^{-1}).

2.3. Preparation of thylakoid membranes of C. reinhardtii

The thylakoids were prepared by the method described by Xiong et al. [33] with slight modification. The late log-phase cells were centrifuged at 2000×*g* (Avanti J-25, Beckman Coulter) for 2 min at 4 °C. The pellet was washed twice with buffer A containing 20 mM HEPES–KOH (pH 7.5), 350 mM sucrose and 2.0 mM $MgCl_2$. The cells were resuspended with buffer A to 100 mg Chl ml^{-1} and broken for 3 min with ultrasonic cell crusher (VCX-750, USA). The broken cells were centrifuged at 100,000×*g* for 20 min at 4 °C (Optimal-80xp, Beckman Coulter). After discarding the supernatant and washing the pellet twice with buffer A, the pellet was resuspended in buffer B containing 20 mM HEPES–KOH (pH 7.5), 40 mM sucrose, 5.0 mM MaCl2, 5.0 mM EDTA, 1.0 mM bovine serum albumin, and 20% (v/v) glycerol. The suspensions were further homogenized with a tissue grinder and briefly centrifuged at 1000×*g* (Allegra™ 64R, Beckman Coulter) for 30 s at 4 °C to remove the unbroken cells. The supernatant was re-centrifuged at 14,000×*g* for 15 min at 4 °C (Allegra™ 64R, Beckman Coulter). The pellet was resuspended with buffer B to a concentration of about 1 mg ml^{-1} in 1.5 ml Eppendorf tubes. The aliquots of thylakoid resuspensions were quickly frozen in liquid nitrogen and stored for later use. The chlorophyll concentration measurements followed the method of Porro et al. [32].

2.4. Photosystem II electron transfer rate

Measurement of electron transfer rate of photosystem II was performed using a Clark type oxygen electrode (Hansatech, the U.K.) according to the method of Coombs et al. [34]. TeA was added to 0.5 ml thylakoid suspensions (100 μg Chl ml^{-1}) to make final concentrations of 0, 5, 10, 50, 100, 500, 1000, 2000 μM and then, the thylakoids were incubated 15 min in dark at 4 °C before measurements began. The photosystem II reaction medium (2.0 ml) contained 50 mM HEPES–KOH buffer (7.6), 4 mM $K_3Fe(CN)_6$, 5 mM NH_4Cl, 1 mM p-phenylenediamine and thylakoids of 40 μg chlorophyll. After the reaction mixtures were illuminated with 400 μmol photons m^{-2} s^{-1} red light, the rate of O_2 evolution was recorded. The experiment was repeated for 3 times. The I_{50} (the concentration producing 50% inhibition) value was determined from plots of photosystem II activity in the presence of various concentrations TeA.

2.5. The chlorophyll a fluorescence induction kinetics and JIP-test

Chlorophyll *a* polyphasic fluorescence rise OJIP curves were measured at room temperature with a plant efficiency analyzer (Handy PEA fluorometer, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK) as described as Strasser and Govindjee [30]. Before the measurements, the cells (the late log-phase) were resuspended in a buffer containing 20 mM HEPES (pH 7.5), 350 mM sucrose, 2.0 mM $MgCl_2$ with a Chl concentration of 1 μg ml^{-1}; the thylakoids were resuspended in the reaction medium of 20 mM MES–NaOH (pH 6.5), 5 mM NaCl, 5 mM $CaCl_2$, 2 mM $NaHCO_3$ at a Chl ($a+b$) concentration of 10 μg ml^{-1}. Intact cells and thylakoid membranes were performed in 1-cm diameter channel cuvette containing 250 μl of suspension and dark adapted for 15 min before measurement and then, in darkness, TeA or DCMU were added to cells or thylakoids suspension to give final concentrations of 0, 5, 10, 50, 100, 500 μM TeA and 1 μM DCMU. The samples were incubated for different time, as described in the legends of correlative experiments, before JIP-test determinations began. Chlorophyll fluorescence was excited with a 1-s pulse of continuous red light (650 nm wavelength) at 3000 μmol photons m^{-2} s^{-1}. Fluorescence data were recorded with a variable sampling rate from 10 μs to 100 ms per point during 1 s. The experimental traces represent the averages of three to four samples each illuminated a single time. The fluorescence signal at 20 μs, 2 ms, 30 ms were considered as F_o (initial fluorescence), F_J, F_I (fluorescence at the I-step), respectively. The data were analyzed with Handy PEA Software V1.3 and BiolyzerHP3.

In this paper the following original data were utilized: the initial fluorescence F_o was measured at 20 μs at this time all reaction centers (RCs) are open; the fluorescence intensity at 300 μs was denoted as $F_{300\ \mu s}$; the fluorescence intensity F_J is at 2 ms (J-step); the maximal fluorescence intensity F_M is equal to F_p since all RCs are closed after illumination of high enough excitation light intensity. For dark-adaptation samples, based on data before some parameters for quantification of photosystem II behavior were calculated as the following formulae [35–37]: (a) the relative variable fluorescence at J step denoted as V_J,

$$V_J = (F_J - F_o)/(F_M - F_o) \quad (1)$$

reflecting the accumulation of Q_A^- and rate of Q_A^- reoxidation (b) dV/dto is the slope at the origin of the relative variable fluorescence and indicates the net rate of the RCs's closure;, which is expressed according to formula

$$M_o = dV/dto = 4(F_{300\mu s} - F_o)/(F_M - F_o) \quad (2)$$

(c) the probability Ψ_o that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- is given as

$$\Psi_o = 1 - V_J \quad (3)$$

(d) φ_{Eo}, the maximum yield of electron transport, was given the following expression:

$$\varphi_{Eo} = (1 - F_o/F_M).(1 - V_J) \quad (4)$$

(e) the maximum rate of electron transport per reaction center (ET_0/RC) can be calculated as

$$ET_0/RC = (M_o/V_J)\Psi_o \quad (5)$$

(f) so-called turnover number N, which expresses how many times Q_A has been reduced in the time interval from 0 to tF_M, is defined as

$$N = S_m.M_o/V_J \text{ with } S_m \equiv \text{Area}/(F_M - F_o) \quad (6)$$

where Area is the parameter of total complementary area between fluorescence induction curve and $F=F_M$.

2.6. Competitive experiments of [^{14}C]atrazine bound to C. reinhardtii thylakoids

Displacement experiments were carried out using the method of Xiong et al. [33] with minor modifications. [^{14}C]Atrazine binding was performed in 1.5 ml Eppendorf tube. The reaction mixture of 1 ml final volume contained 20 mM HEPES (pH 7.0), 100 mM sorbitol, 10 mM KCl, 10 mM $MgCl_2$, 0.1 mM NH_4Cl, and 0.08 μM to 0.32 μM [^{14}C]atrazine (equivalent to 23 to 92 Bq) (Moravek Biochemicals Inc., 9.3 mCi/mmol). [^{14}C]Atrazine was dissolved in methanol. Then thylakoids of *C. reinhardtii* were added to the reaction mixture with a chlorophyll concentration of 50 μg ml^{-1}. After incubating 25 °C in darkness for 5 min, TeA and DCMU was further added to the reaction mixture at final concentration 0, 1, 5, 10, 50, 100, 500 μM TeA and 1 μM DCMU, and the mixture was incubated in the dark for 15 min at 25 °C with gentle shaking (50 rpm on a platform shaker). Total volume of reaction mixture was 1 ml. The thylakoids were then centrifuged for 10 min at 10,000×*g*, and 0.5 ml of the supernatant and 4.5 ml of scintillator fluid (contained 2 mM PPO, 0.1 mM POPOP were dissolved in toluene: Triton X-100 (2:1,v/v)) were measured by liquid scintillation spectrometry (LS6500 Multi-purpose Scintillation Counter, Beckman Coulter). The amount of bound [^{14}C]atrazine was calculated from the total radioactivity added to the thylakoid suspension and the amount of free [^{14}C]atrazine in the supernatant after centrifugation.

2.7. Electrophoresis

Thylakoid membranes proteins were separated by gel electrophoresis in a modified Jursinic et al. [38]. A total of 12.5 μg Chl was loaded per lane for electrophoresis. SDS-PAGE containing 6 M urea was used with a slab gel containing 8% (stacking) and 13.75% (resolving) acrylamide. Molecular mass standards were purchased from Bio-Rad. After electrophoresis, gels were dried onto glass paper at room temperature (about 2 days) and exposed to X-ray film at −40 °C for 9 weeks.

2.8. Analysis for TeA and its analogues of growth rate and ETR

TeA and its analogues were synthesized from natural amino acid by five step reactions including neutralization, esterification, acylation, ring formation and acidification. 20 ml ethanol was taken as solvent into 100 ml flask, 0.02 mol natural amino acid such as isoleucine, valine, alanine, glycine, phenylalanine, tyrosine and *aspartic acid* corresponding to different analogues respectively was added, and 0.022 mol dry chlorine hydride was submitted in, the mixture was heated at 60 °C with stirring for 4 h, then the 0.022 mol sodium ethoxide was added to the reaction system with continuous stirring for 0.5 h, and 0.022 mol ketene was slowly added with stirring for 1 h. After which, 15 ml benzene and another 0.022 mol sodium ethoxide were added. The reaction mixture was then refluxed at 120 °C for 5 h. After which 50 ml water was added and the solution was acidified with sulphuric acid and extracted with ethyl acetate and aim compound was recrystallized or separated by chromatography. The final yield was about 45.1–67.2%. Cells of *C. reinhardtii* wild type at logarithmic phase (O.D. 750 nm=0.05 to 0.5) were diluted to an O.D. 750 nm=0.08 using TAP medium. Aliquots (250 μl) of cell suspension were added to the sterile 96 well cell plate and then various concentration of TeA and its analogues were added to make final concentrations of 0, 10, 20, 50, 100, 200, 400 μg ml^{-1}. Cells were grown at 25 °C with approximate 100 μmol m^{-2} s^{-1} white light (day/night, 12 h/12 h). The growth rates were determined by measuring the optical density of the cells at 750 nm (O.D. 750) [33] at 48 h using an auto multi-functional microplate reader (TECAN Infinite™ M200, Switzerland).

ETR (electron transfer rate) was determined using a commercial pulse-modulated PAM2000 fluorometer (Heinz Walz, Germany) at room temperature [39,40]. Measuring and actinic light were provided by red-light-emitting diodes. The intensity of the measuring light was 0.7 μmol m^{-2} s^{-1} and the intensity of the actinic light was 590 μmol m^{-2} s^{-1}. Before the measurements, cells of *C. reinhardtii* wild type at late log-phase were resuspended in a buffer containing 20 mM HEPES (pH 7.5), 350 mM sucrose, 2.0 mM $MgCl_2$ with a Chl concentration of 1 μg ml^{-1}. Cell suspension were performed in 2-cm diameter channel cuvette containing 250 μl of suspension and 0, 10, 20, 50, 100, 200, 400 μM TeA or its analogues and then were incubated in darkness for 15 min before measurement. The inhibition I_{50} value was determined to be various concentrations TeA and its analogues compared to control (2% methanol).

For every experiment the final concentration of methanol in the cells or thylakoids never exceeded 1% (v/v).

3. Result

3.1. Effect of TeA on O_2 evolution rate of photosystem II of thylakoids

By using different electron transfer inhibitors, electron donors, electron acceptors, O_2 evolution rate of photosystem II can be measured for analyzing action mechanism of TeA in photosystem II. Results show that the O_2 evolution rate of photosystem II was reduced in a concentration-dependent relationship after treatment with TeA (see Fig. 1). However, with an I_{50} of 261 μM, TeA is a weak photosystem II inhibitor compared to the specific photosystem II inhibitor DCMU [3-(3, 4-dichlorophenyl)-1,1-dimethylurea].

3.2. Effect of TeA on chlorophyll a fluorescence induction rise kinetics OJIP of C. reinhardtii

In recent years, chlorophyll *a* fluorescence induction kinetics has become a useful tool to investigate the conditions of the structure, conformation and function of the overall photosystem II since chlorophyll *a* fluorescence is assumed to be emitted from photosystem II complex only [41].

To assess the effect of TeA on photosystem II acceptor side, chlorophyll fluorescence rise transient kinetics OJIP curves of intact cells of wild-type *C. reinhardtii* treated with 500 μM of TeA for different time were determined. The fluorescence rise transients obtained from control cells (at time 0) show a clear OJIP shape, with a sharp increase in the J level with increasing treatment time of TeA (Fig. 2A). After cells were incubated for 10 min or longer, the level of step-J was much higher compared to that of 0 and 5 min, especially at 30 min fluorescence rises quickly of OJ phase so as to F_J (fluorescence at the J-step) being close to F_M (maximal fluorescence), showing distinct time-dependent. The increase of step-J implies that TeA affected electron flow from Q_A to Q_B or Q_B^-, which resulted in the fast accumulation of Q_A^-.

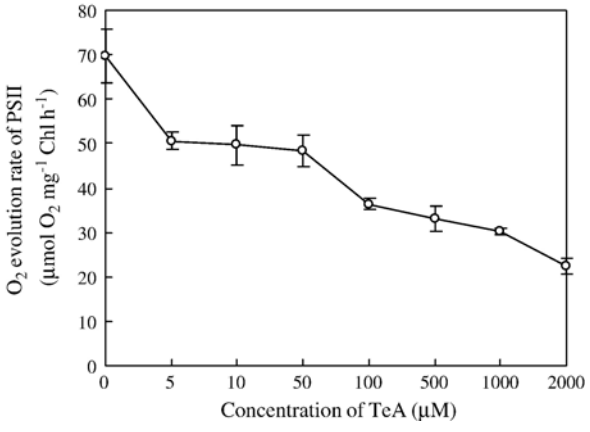

Fig. 1. Effect of TeA at various concentrations on O_2 evolution rate of thylakoids of wild-type *C. reinhardtii*. H_2O and p-Phenylenediamine was the electron donor and acceptor, respectively. O_2 evolution rate of PSII was measured after samples were treated for 30 min in dark at 4 °C. The concentration of half inhibition (I_{50}) is 261 μM. Data shown were mean values±SE of 3 times experiments.

To further check the effect of TeA on the acceptor side of photosystem II, chlorophyll fluorescence rise transients of thylakoids incubated with various concentration of TeA and 1 μM DCMU (as special control) were measured 15 min after treatment (Fig. 2B). Fluorescence transients of the control thylakoids measured under enough high excitation light represent also visible J and I step between O and P. The fluorescence rise transients in the presence of 1 μM DCMU (top curve of Fig. 2B), resulted in an OJ shape with $F_J=F_M$ due to Q_A being reduced entirely and all RCs (reaction centers) closed. It is well known that DCMU is an excellent photosystem II inhibitor, which can interrupt the electron transfer from Q_A to Q_B by occupying the Q_B site at quite low concentrations. TeA can also accelerate the rise of thyalkoids fluorescence similar to DCMU, such that the J step lift is more obvious, showing a trend of concentration-dependence. After thylakoids were incubated with 100 μM or higher TeA, F_J was so close to its maximum value of F_M that the OJ part has become a mainly part of OJIP transient and the step-I disappeared, which should be due to fast reduction of Q_A. The results indicate that the TeA action site is possibly quite similar to DCMU, blocking photosystem II electron transport from Q_A to Q_B by occupying Q_B-niche.

3.3. JIP-test: effect of TeA on activity of photosystem II reaction center

Each transient can be analyzed according to the JIP-test [42], from which different parameters yield information on the kinetics of electron flow reactions on the acceptor as well as the donor side of photosystem II. In order to further demonstrate the effect of TeA on the acceptor side of photosystem II, some functional parameters to quantitate photosystem II behavior and activity were applied (as Figs. 2C and D). V_J (relative variable fluorescence at the J-step) corresponding to the relative variable fluorescence at J-step showed a distinctly uptake after cells were incubated for 5 min. At 30 min, the V_J value increased to 0.65, which was over twice that of control (0.30). With uptake incubation time, ETo/RC (the maximum rate of electron transport per reaction center) and φ_{Eo} (the maximum yield of electron transport) decreased pronouncedly and the value of Ψ_o (the probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-) was reduced significantly. In addition, the turnover number N declined sharply, which expresses how many times Q_A has been reduced in the time interval from 0 to tF_M (time to reach maximal fluorescence F_M).

Thylakoids treated for 15 min by various concentration of TeA had a remarkable increase in V_J as well as a significant decrease in the value of ETo/RC, φ_{Eo}, Ψ_o and N in Fig. 2D. Moreover, this trend would get clearer and clearer while increasing of concentration of TeA treatment (Fig. 2D). On the other hand, the change of these parameters under TeA treatment was slower than that of 1 μM DCMU treatment. It is obvious that the five parameters from JIP-test for TeA treatment show a significant time-concentration-dependence. The above results further prove that TeA affects the rate of Q_A^- reducing reoxidation by interrupting the electron flow from Q_A to Q_B.

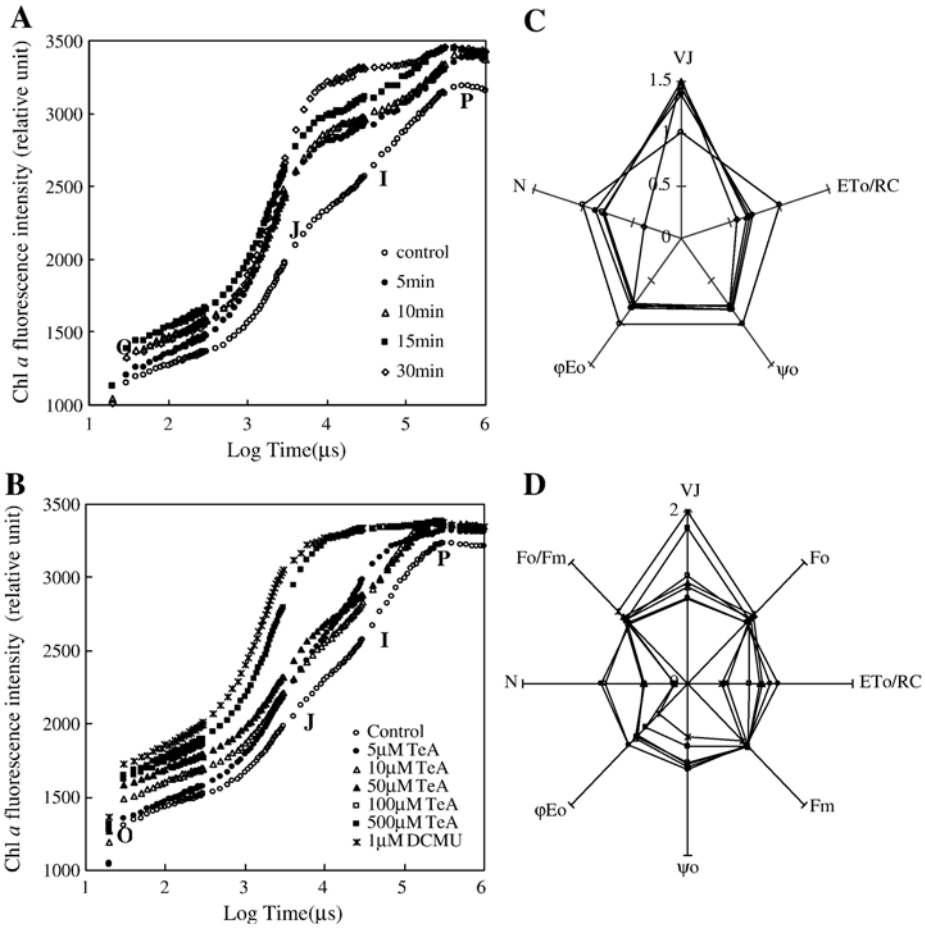

Fig. 2. Effect of TeA on chlorophyll *a* fluorescence induction kinetics of *C. reinhardtii* wild type. (A) OJIP curves of intact cells incubated with TeA for various treatment time 0 (control), 5, 10, 15, 30 min. (B) OJIP curvers of thylakoids incubated for 15 min in the present of 1 μM DCMU or various concentrations of TeA. (C) Spider plot showing selected JIP-test parameters from A (V_J, ET_0/RC, ψ_0, φE_0, and N) quantifying the behaviour of PSII of intact cells treated by TeA for different time. (D) Spider plot presentation of a constellation of selected parameters from B (F_o, F_m, F_o/F_m, V_J, ET_0/RC, ψ_0, φE_0, and N)) quantifying the behaviour of PSII of thylakoids incubated with various concentration of TeA and 1 μM DCMU. Results are averages of at 5 repetitions. Each experiment result was repeated at least 3 times. See Materials and methods for further details.

3.4. Competitive binding between TeA and atrazine assay

Although TeA is similar to diuron in blocking photosystem II electron transport, having the same effect on electron transport does not necessarily mean that the two inhibitors act at same binding site [43]. According to current reports, it is not doubtful that the action site of some excellent photosystem II inhibitors, such as diuron, atrazine, etc., are located at the Q_B-niche. In order to study the binding properties of TeA within the Q_B binding niche, competition binding experiments between non-labeled-TeA and [^{14}C]atrazine were determined (no radioactively labeled TeA is available to conduct the displacement of TeA by atrizine). The amount of free [^{14}C]atrazine in the reaction mixture of the competitive experiments increased with the addition of non-labeled TeA (Fig. 3B). Thus, TeA prevented the binding of atrazine. However, the double-reciprocal plots of the amount of free [^{14}C]atrazine (1/μM) versus the amount of bound [^{14}C]atrazine (mg Chl/nM) reveals a non-competitive displacement (see Fig. 3A), which is attributed to an identical abscissa intercept in the control and at three various concentrations of TeA. The displacement between DCMU and [^{14}C]atrazine proceeds competitively since their regression lines lies on the *y*-axis (Fig. 3A) [44]. Based on these results, TeA can displace atrazine-like inhibitors in a non-competitive manner.

3.5. Effect of TeA on photoaffinity labeling protein of atrazine

The fluorograph obtained from the gel of the polypeptides of [^{14}C]atrazine-labeled thylakoid membranes from *C. reinhardtii* wild type in the presence of various concentration TeA and 1 μM DCMU revealed that in each thylakoid sample a single band which is 32–34 kDa protein was labeled with [^{14}C] atrazine (Fig. 3C). It is well known that 32–34 kDa protein (D1 protein) is the binding polypeptide of atrazine and DCMU [38,45,46]. Competitive binding between TeA and atrazine with increasing concentration of TeA caused a decrease in the amount of bound [^{14}C]atrazine to 32–34 kDa protein, which is reflected in a weaker radioactive band (Fig. 3C, lanes 1–8). Additionally, a high concentration of TeA (500 μM) (lane 7) and DCMU (1 μM) resulted in a remarkable decrease of the atrazine bound to D1 protein (Fig. 3C, lane 8). Therefore, we conclude

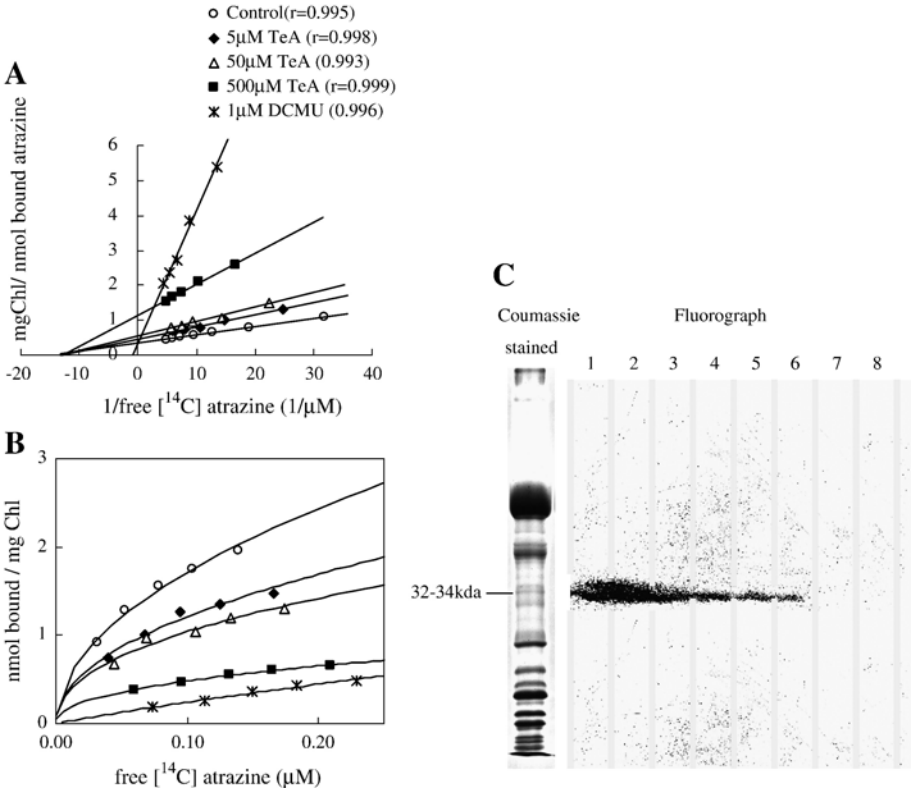

Fig. 3. Competitive experiments of [^{14}C]atrazine bound to thylakoid membranes of *C. reinhardtii* wild type by non-labeled TeA and DCMU. (A) Double-reciprocal plot of the concentration of free [^{14}C]atrazine vs. the amount of bound [^{14}C]atrazine. (B) [^{14}C]atrazine binding curves. (C) Effect of TeA on photoaffinity labelling of thylakoid membranes of *C. reinhardtii* wild type with 0.24 [^{14}C]atrazine. Seeing from left to right, A–H, a 9-week fluorography under different concentration TeA and 1 μM DCMU treatment: Control (1); 1 μM (2); 5 μM (3); 10 μM (4); 50 μM (5); 100 μM (6); 500 μM (7) TeA; 1 μM DCMU (8).

that occupation of the binding site of the D1 protein by atrazine can be affected by the binding of TeA.

3.6. Thylakoids of wild-type and mutants D1 of C. reinhardtii sensitivity to TeA assay

As demonstrated above, TeA differs in the binding niche from atrazine although both compounds have the common action target as Q_B-niche. To further corroborate amino acid residues involved TeA binding, the pI_{50}-values of TeA in five different D1 mutants of *C. reinhardtii* were to that of wild type *C. reinhardtii*. The D1-Phe255Tyr, D1-Gly256Asp and D1-Ser264Ala mutants were resistant against TeA, with *R/S*-values of 2.45, 37.39 and 7.99, respectively (Table 1). The D1-Val219Ile and D1-Leu275Tyr mutants were supersensitive to TeA, with *R/S*-values <1 (Table 1). It was reported that D1-Phe255Tyr and D1-Gly256Asp mutants are against atrazine, cyanoacrylate and bromacil as well as D1-Ser264Ala mutant has high cross-resistance to diuron (*R/S*-values of 200), bromacil (*R/S*-values of 106), metribuzin (*R/S*-values of 5000–10000) and atrazine (*R/S*-values of 125–500) in *C. reinhardtii*. Resistance towards metribuzin, ioxynil and metamitron is also observed in mutants D1-Val219Ile and D1-Leu275Phe [29]. Our results showed that a mutational change at amino acid position 256 confers a more resistance to TeA than at position 264. Conversely, *C. reinhardtii* mutants with an amino acid alteration at position 264 are more

Table 1
pI_{50} Values of TeA for wild type and mutants D1 of *C. reinhardtii*

Strain	Codon change	Position	pI_{50}	Resistance R/S	Corresponding equation	
CC-124 wild type mt-137c	–	–	3.58		$y=-15.182x+104.41$	$R^2=0.91$
CC-1403 dr-u-2 mt+	Val→Ile	219	3.66	0.85 (<1)	$y=-18.17x+116.43$	$R^2=0.93$
CC-1847 Ar207 mt+	Phe→Tyr	255	3.19	2.45	$y=-24.509x+128.28$	$R^2=0.90$
CC-2059 Ar204 mt+	Gly→Asp	256	2.01	37.39	$y=-17.161x+84.50$	$R^2=0.99$
CC-2857 DCMU4 er-u-1a mt-	Ser→Ala	264	2.68	7.99	$y=-21.218x+106.88$	$R^2=0.89$
CC-1844 Br202 mt+	Leu→Tyr	275	3.82	0.58 (<1)	$y=-32.681x+174.81$	$R^2=0.97$

PS II electron transfer activity from H_2O to p-phenylenediamine was determined in isolated thylakoid membranes. pI_{50} values are the negative logarithm of concentration of TeA that inhibits the electron by 50% (I_{50}). The *R/S*-value is the ratio of the I_{50}-value in electron transport in thyalkoids isolated from resistant versus susceptible *C. reinhardtii.* In case of resistance, the *R/S*-value is always >1. In equation, y=% inhibition, $x=-\lg$[TeA].

resistant than mutants with an amino acid alteration at position 256 to other known photosystem II inhibitors. Therefore, the requirement for binding at the Q_B-niche is different for TeA than for other inhibitors. While both D1-Gly256Asp and D1-Ser264Ala mutants show resistance to TeA, the former mutant exhibited higher resistance compared to the latter. Therefore, we conclude that the amino acid at position 256 plays a key role in the binding of TeA to the Q_B-niche.

3.7. Effect of TeA on some parameters from JIP-test of wild-type and mutant D1-Gly256Asp of C. reinhardtii

To further confirm the D1-Gly256Asp mutant's resistance to TeA, chlorophyll fluorescence transients of wild-type and Gly256Asp mutant whole-cells and thylakoids were investigated. Five parameters from JIP-test (OJIP-curves not shown), which express precisely on electron transfer from Q_A to Q_B in photosystem II acceptor side, were analyzed to confirm the effect degree of amino alteration at position 256 on competition for Q_B-niche between TeA and Q_B. With an increase of incubation concentration of TeA, the V_J and M_o (the slope at the origin of the relative variable fluorescence) began to increase, while the other three parameters, φ_{Eo}, Ψ_o and N, showed a declining trend (Fig. 4). The JIP-test parameters of the D1-Gly256Asp mutant changed little, while these parameters of the wild-type had a pronounced change (Fig. 4). Most importantly, among the five JIP-test parameters, N was most sensitive to TeA. It is well known that once electron transport

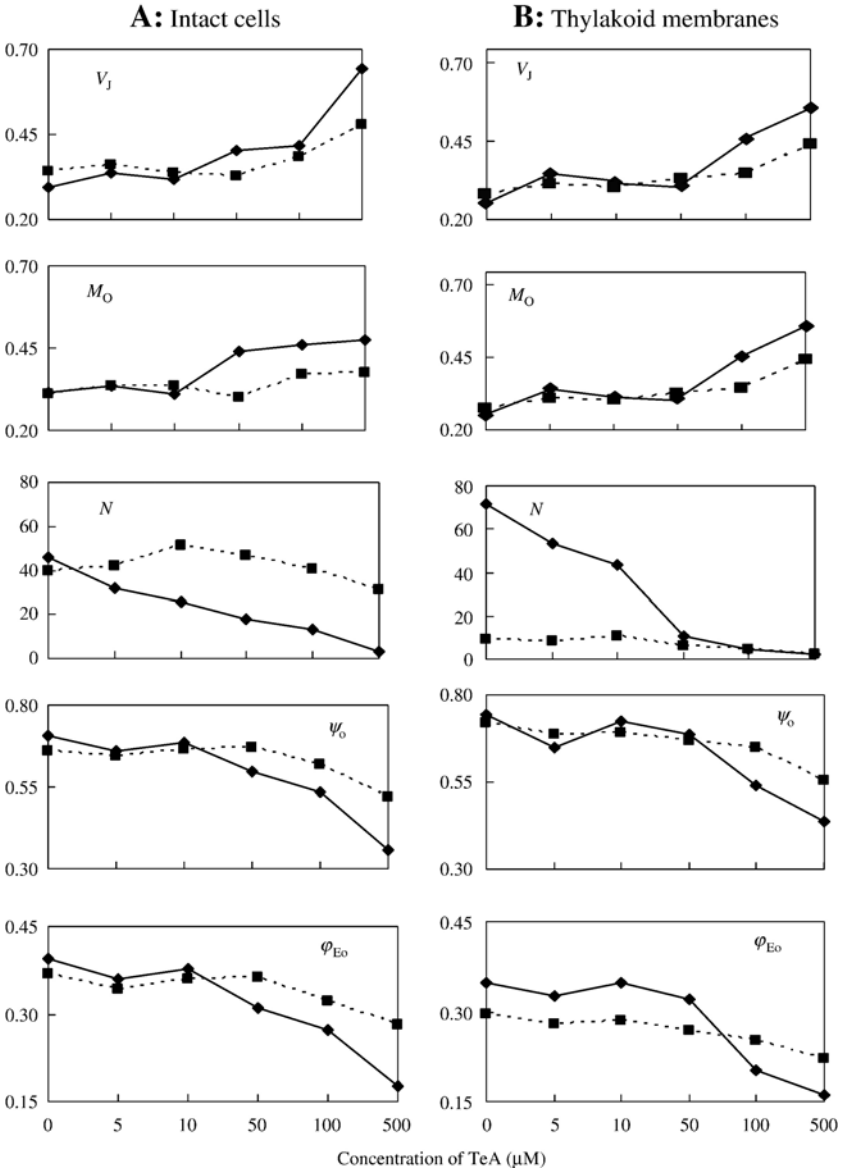

Fig. 4. Effect of various concentration of TeA on different parameters from JIP-test of wild type (solid lines) and D1-Gly256Asp mutant (broken lines) (A) In intact cell determinations, the concentration of Chlorophyll ($a+b$) was about 1 μg ml^{-1} and the excitation intensity was 3000 μmol photons m^{-2} s^{-1}; (B) in thylakoids determinations, a chlorophyll ($a+b$) concentration of 10 μg ml^{-1} and 3000 μmol photons m^{-2} s^{-1} excitation intensity were used. Cells and thylakoids were incubated with TeA for 30, 15 min before determinations, respectively. The results in the figure are the mean of 4 times experiments.

from Q_A to Q_B and Q_B^- is blocked by a photosystem II inhibitor, which results in a quick increase of the various fluorescence at J-step (V_J), the net rate of the RCs's closure (M_o), and a rapid decrease of yield for Q_A^- reoxidation (Ψ_o), yield for electron transport (φ_{Eo}) and times of Q_A are reduced in the time interval from 0 to tF_M (N). Moreover, there is a positive correlation between amplitude change of the parameters and the inhibition degree of electron transport of on the acceptor side of photosystem II. Our results indicate that the effect of TeA on electron transfer at the acceptor side of photosystem II of the D1-Gly256Asp mutant is much less than that of the wild-type. These results further demonstrate that an alteration at amino position 256 in the D1 protein yields resistance to TeA, corresponding to *R/S*-value of approximately 37).

3.8. Competitive binding of TeA by thylakoids of wild type and mutant D1-Gly256Asp of C. reinhardtii

In order to obtain more insight into the TeA resistance in the D1-Gly256Asp mutant, competition binding experiments between non-labeled TeA and [^{14}C]atrazine were performed in thylakoids of the wild-type and D1-Gly256Asp mutant. With increasing amounts of TeA, reduction in the amount of [^{14}C]atrazine binding to thylakoids of D1-Gly256Asp mutant was much slower than of the wild-type (Fig. 5). The effect of TeA on the atrazine bound to thylakoids of the D1-Gly256Asp mutant was less than that of wild-type. The inhibition constant, Ki, values calculated by the slope of the regression lines of double-reciprocal plots (curve not shown) for the wild-type and D1-Gly256Asp mutant were about 95 and 271 μM, respectively. These results further demonstrate that the amino acid at position 256 is a very important in the binding of TeA to the Q_B-niche.

3.9. Proposed modeling: TeA binding sites

Based on the available crystal structure information of photosystem II from *Cyanobacterium* [47] and some herbicides binding in the photosynthetic bacterial reaction center [48–51] as well as the previous model of [25,52] for the herbicide-binding environment, a proposed model of TeA binding sites is presented (in Fig. 6). The TeA molecule binds to the Q_B binding pocket formed predominantly by D1 residues that fall between the helices D and E of the D1 protein (Fig. 6A). Additionally, there are also a number of D2 protein residues in the Q_B pocket. The main residues coordinates TeA binding is Gly256, which is located between the two transmembrane helices D and E of D1 protein, as shown in Fig. 6B. This requirement is different from other photosystem II inhibitors.

3.10. Analysis of biology activity of TeA and its analogues

To further clarify the mechanism of TeA binding to Q_B-niche, a series of TeA analogues were synthesized using natural amino acid as former body, which differ only in side chain at the

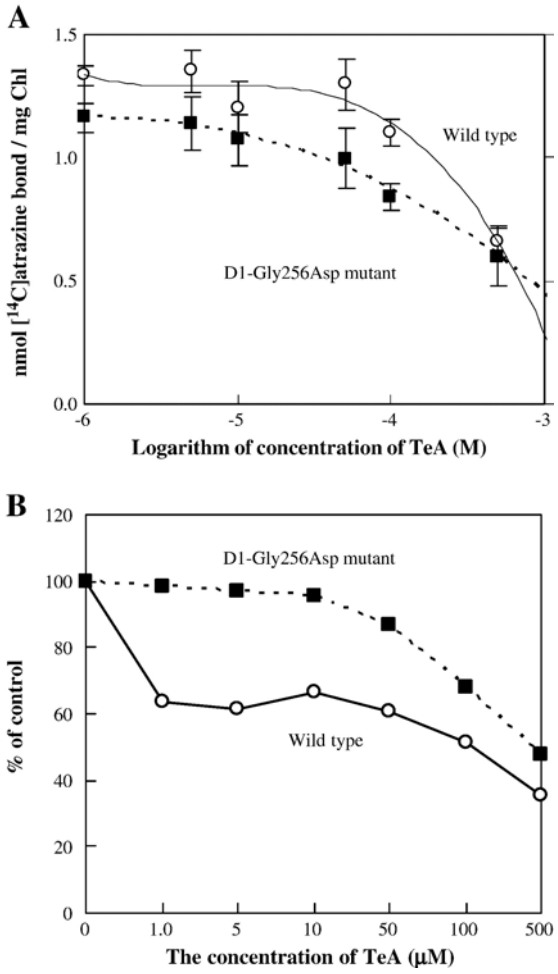

Fig. 5. Effect of various concentrations of TeA on atrazine binding of thylakoids of *C. reinhardtii* wild type (solid line) and D1-Gly256Asp mutant (broken line). (a) The details of [^{14}C]atrazine binding in present of TeA were shown. (b) The percentages of [^{14}C]atrazine binding in the present of various concentrations of TeA (100% of control (1% methanol) binding). The figure indicated 0.24 μM [^{14}C]atrazine binding.

5-position (Fig. 7). The I_{50} value for TeA and its analogues in growth rate and photosystem II electron transfer rate (*ETR*) of whole cells of *C. reinhardtii* wild type are presented in Table 2. Compounds TeA and **I** have the alkyl side chain with more carbons in the 5-position, showing higher activity while compound **II** with one carbon alkyl side chain exhibited much lower activity. Interestingly, compounds **III** and **VI** with phenyl side chain or hydrogen-group exhibited only slight activity or were completely inactive in inhibiting *ETR*. Compound **IV** having a hydrophilic group showed no inhibiting activity in growth but slight activity in *ETR*. Similar to compound **III**, compound **V** with a larger hydrophilic group (4-hydroxy benzyl, $-CH_2$ $(C_6H_4)OH$) in position 5 had no inhibiting activity in growth or *ETR*. The results demonstrate that, there is an important relationship between the biology activity and the character of the side chain in the 5-position of the TeA analogs. Furthermore, the presence of the hydrophobic group (alkyl side chain) in 5-position for TeA and its analogues

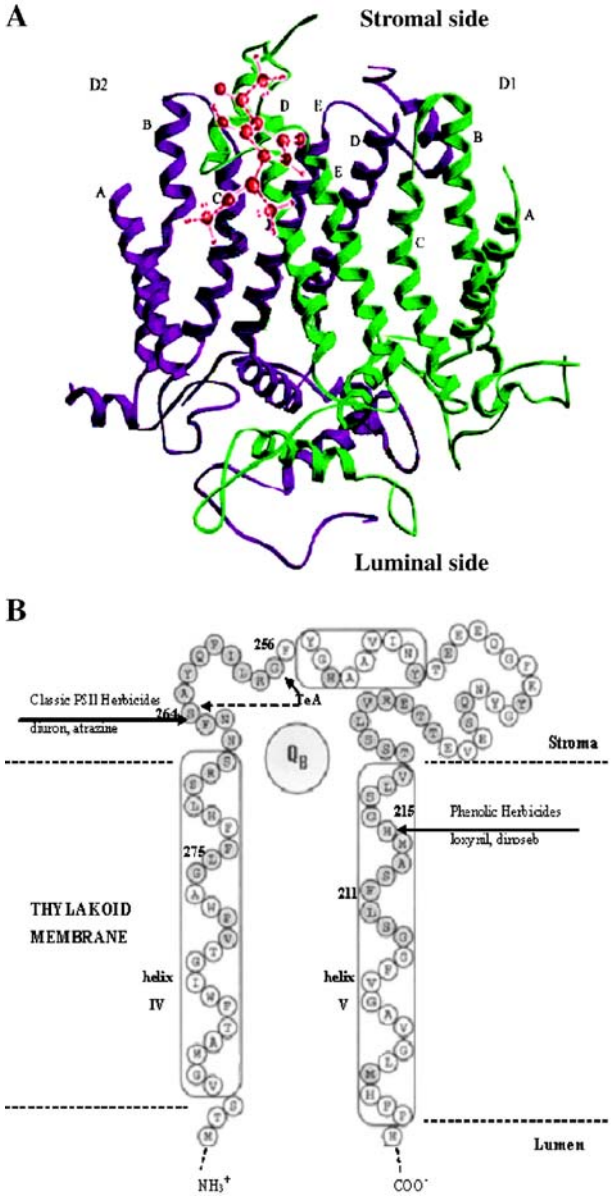

Fig. 6. Simplified model of TeA binding in D1 protein. (A) The three dimensional model of TeA-binding niche in the D1 and D2 protein of the PSII reaction center according to Kamiya and Shen [47]. The ribbon form indicates the *a*-helix structure. A–E represent helix numbers of D1 and D2. The D1 protein is shown in purple ribbon and D2 is shown in green ribbon and TeA molecular is shown in red. (B) Scheme based upon the model of Trebst [25,52] for the herbicide-binding environment in D1 protein. Predicted *a*-helical residues are boxed. Only that region of D1 is shown which covers the segment from the beginning of the 4th (D) membrane-spanning helix to the end of 5th (E) membrane-spanning helix. Arrows denote amino acid residues involved in fixation of 'Classic' PSII herbicides (diuron, atrazine) (264), phenolic herbicides (ioxynil, dinoseb) (215) and TeA (256).

is important for their high inhibitory potency. Moreover, inhibitory potency increases with increasing length of the side chain. However, moieties, such as a phenyl-group, in 5-position can reduce the inhibitory activity of TeA analogs. On the contrary, the substitution of a hydrophobic group with a hydrophilic group at position 5 leads to a great decrease or loss of inhibiting activity.

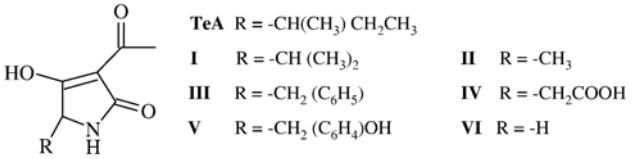

Fig. 7. Structure formula of TeA and its analogues.

4. Discussion

The above results first time proved that TeA worked as a novel photosystem II inhibitor to inhibit plant activity. In our lab we have studied TeA as a potential herbicide and tried to search for action mode killing weeds since it was found that it is one main metabolite of *A. alternata* with phytotoxic activity isolated from *E. adenophorum* [10–14,18,19,31]. Blocked photosystem II electron transport activity (Fig. 1) and then inhibited photosynthesis, which is possibly the major action mechanism of TeA. According to the theory of chlorophyll fluorescence transient kinetics, chlorophyll fluorescence induction rise reveals a characteristic O–J–I–P polyphasic transient at room temperature [53]. If the reoxidation of Q_A^- reducing is inhibited by any stress, such as the presence of inhibitors, fluorescence transient rise from O (origin point) to P (fluorescence peak) is markedly faster. During this O to J phase mainly single turn over events with respect to Q_A reduction are occurring [54]. Q_A is reduced quickly once electron transport from Q_A to Q_B is blocked in the presence of photosystem II inhibitors, leading to a strong increase of the level of step J. A fast increase of the fluorescence yield of step-J suggests that there is a large accumulation of Q_A^- due to a blockage in electron transfer between Q_A and Q_B [30]. From the chlorophyll fluorescence rise of intact cells and thylakoids of *C. reinhardtii*, it is obviously observed that TeA treatment resulted in enhanced F_J values. Moreover, the F_J value approached its maximum value F_M with increased TeA treatment time and concentration (see Fig. 2A and B). Increase of the V_J values indicated TeA blocked transport of electron from Q_A^- to Q_B and leaded to an increase in reduced Q_A. Consequently, in the presence of TeA, the electron transport per reaction center (ET_0/RC), the quantum yield of

Table 2
Half-inhibition concentrations of TeA and its analogs in growth rate and electron transfer rate (ETR) of whole cells of *C. reinhardtii* wild type

No.	*R* group	Growth rates I_{50} (μg ml^{-1})	ETR I_{50} (μg ml^{-1})
TeA	–CH(CH$_3$) CH$_2$CH$_3$	192.94	41.53
I	–CH(CH$_3$)$_2$	294.71	110.98
II	–CH$_3$	>1000	305.49
III	–CH$_2$(C$_6$H$_5$)	–	–
IV	–CH$_2$COOH	–	850.94
V	–CH$_2$(C$_6$H$_4$)OH	–	–
VI	–H	–	–

Growth rates were expressed by OD750 at 48 h (control rates are 0.39±0.06). Electron transfer rate (in μmol electron m^{-2} s^{-1}) of control is 11.05±1.59.

electron transport (φ_{Eo}), and yield of Q_A^- reoxidation (Ψ_o) decrease sharply, resulting in a decrease in number of Q_A reduction (N) (Fig. 2C and D). The above results demonstrate that similar to other photosystem II inhibitors, TeA interrupts electron flow from Q_A to Q_B.

It is generally accepted that many photosystem II inhibitors inhibit electron transport from Q_A to Q_B by competing for the Q_B binding niche in D1 protein [29,55,56]. Competition experiments between non-labeled TeA and [^{14}C]atrazine showed TeA has a similar action to atrazine, which binds to the Q_B-site since atrazine binding to Q_B-site could be prevented by TeA (Fig. 3B and C). Double-reciprocal plots of the binding of [^{14}C]atrazine in the presence of various concentration of TeA (Fig. 3A), indicated that binding behavior of TeA exhibited different characteristics from that of atrazine-like inhibitors. From these results it was concluded TeA has the same action target as other photosystem II inhibitors herbicides (e.g. DCMU and atrazine), i.e., binding to the Q_B-niche. However, the binding of TeA to the Q_B-niche is dependent on different amino acid residues than those identified for other photosystem II inhibitors.

The D1-protein well known in higher plants, called L-protein in the photosynthetic bacteria, is encoded by the chloroplasts gene *psb*A [57]. In bacterial reaction center, the herbicide binding site is located on the L subunit in the connecting loop between the fourth (D) and the fifth (E) transmembrane helices [23]. For significant sequence and functional homology is known to exist between Dl proteins and the L subunits, a great deal of the knowledge about the structure and function of photosystem II and the mode of action of herbicides therein is based on the X-ray structures of the reaction centers of photosynthetic bacteria [28,29,58,59]. According to photosynthetic bacteria data and the up-to-the-minute crystal structure of photosystem II from *Cyanobacterium*, it is well known that D1 protein contains five trans-membrane α-helices and several short nonmembrane helices between the transmembrane helices on either the luminal side or the stromal side [20,23,47,60,61]. The Q_B binding niche falls between the helices IV and V (or D and E), which is also the site of photosystem II inhibitor herbicide binding [23]. The specific region of D1 protein starts at Phe211 and ends at Leu275, where any amino acid change may lead to reduce herbicide binding and consequently increased resistance [30,62,63]. This assumption can be proven best by the use of X-ray structure analysis of the herbicide bound to the Q_B site. However, in the absence of high resolution crystal structure for the photosystem II reaction centers of higher plants, molecular models, based upon the bacterial data and mutant studies of the Q_B-binding domain, provide a useful alternative technique for investigating potential interactions between herbicides and the Q_B binding site [28,29,46,58]. At present, it has been well established by X-ray crystallography that the binding site of herbicides stigmatellin (PDB entry 4PRC) [49], atrazine (PDB entry 5PRC) as well as triazine-a (PDB entry 6PRC) and triazine-b (PDB entry 7PRC) [50], terbutryn [48,51], capsaicin [64] and their model of interaction with Q_B^-site of photosynthetic bacteria. Significantly, the amino acids HisL190, SerL223, GluL212, PheL216 involved in these inhibitors binding in the bacterial reaction center are found to be conserved in photosystem II D1-His215, D1-Ser264, D1-Ala251, D1-Phe255 [23,29,48–51,64]. Furthermore, mutants of photosynthetic bacteria have been isolated and characterized which are resistant against certain inhibitors and commercial herbicides. These mutants include positions GlyL192, GluL212, PheL216, TyrL222, SerL223, ThrL226, GlyL228, LeuL229 and GluM234 corresponding to D1-Ser217, D1-Ala251, D1-Phe255, D1-Tyr262, D1-Ser264, D1-Ser268, D1-Ser270, D1-Leu271 and D2-Ala234 respectively [23,29,65,66]. Our experimental results with D1-mutants of *C. reinhardtii* show that a change of amino acid at 256 position yielded about 37 fold resistance to TeA, while D1-Ser264Ala and D1-Phe255Tyr mutants had approximately 8 and 2 fold resistance of TeA, respectively. In contrast, D1-Val219Ile and D1-Leu275Tyr mutants showed TeA-supersensitive (see Table 1). The D1-Gly256Asp mutant exhibits a significant resistance to TeA. This conclusion was supported strongly by the information from JIP-test and competition experiments of the wild type and D1-Gly256Asp mutant. In the presence of TeA, it is observed from Fig. 4 that change trend of D1-Gly256Asp mutant's JIP-test parameters, V_J, M_o, φ_{Eo}, Ψ_o, N, which reflected the electron transport state of photosystem II acceptor side, was slower than that of the wild type, whether intact cells or thylakoids of *C. reinhardtii* were used for the assay, indicating that electron flow between Q_A and Q_B of D1-Gly256Asp is significantly less affected by TeA than the wild type. On the other hand, the ability of competition of [^{14}C]atrazine for TeA was weaker in thylakoids of D1-Gly256Asp mutant than wild type since mutant showed a slower decrease of the amount of bound [^{14}C] atrazine and a litter inhibitor constant Ki (see Fig. 5). These results indicate that amino acid at position 256 plays an important role in the process of TeA binding to Q_B-niche. Govindjee et al. [67] had suggested that both D1-G256 and D1-264 play important roles in electron transfer from Q_A to Q_B and in the Q_B binding. Furthermore, the major effect of Gly256Asp and Ser264Ala mutants may be to alter the distribution pattern of the binding of plastoquinone at the Q_B binding site. Although D1-G256 and D1-S264 residues participate in the herbicide binding environment, the role of D1-G256 needs to be further examination [23,67].

Based on previous studies [23,25,28,47,52,58,59] and our experimental results, a simplified model of TeA binding to Q_B-niche is given in Fig. 6. As shown in Fig. 6A, TeA is located in Q_B binding niche which is a hydrophobic pocket formed by D1 residues from Phe211 to Leu275 and D2 residues from Glu219 to Ala260 [23,60]. The TeA head group (pyrrolidone ring) binds in the pocket and the butyl side chain at 5-position is fixed in the lumen hydrophobic environment under the pocket. Obviously, a compound with a hydrophobic side chain group is optimally suited for tight binding in the Q_B niche and has high inhibitory potency. So, it is understandable that compounds TeA, **I** and **II** with an alkyl side chain have higher inhibiting activity compared to compounds **III**, **IV**, **V** and **VI** with a phenyl, or a hydrophilic side chain, or hydrogen-group (see Fig. 7 and Table 2). Phenyl in compound **III** may be too big to fit the pocket of D1. Liu et al. [68] believed that a hydrophobic group for photosystem II inhibitor herbicides (such as urea and triazine)

may be necessary as well as an alkyl chain length of 1 to 4 carbon atom may be the best choice in binding to Q_B niche. Moreover, the inhibiting activity may be enhanced with the increase in alkyl chain length ($-CH_3 < -CH_2CH_3 < -CH_2CH_3CH_3 < -CH(CH_3)_2 < -CH_2CH(CH_3)_2 < -CH_2CH_3CH_3CH_3 < -CH(CH_3)CH_2CH_3 < -C(CH_3)_3$).

All inhibitors bind in the Q_B niche, although they do not necessarily bind in the same region of the Q_B niche. According to the pattern of hydrogen bonding geometry for the RC–stigmatellin complex (4PRC), one hydrogen bond could be donated by the stigmatellin carbonyl oxygen O4 to the HisL190 N atom, the other by the hydroxyl O8 to the O of SerL223. In addition, a bifurcated hydrogen bond is between the hydroxyl O8 and the backbone amides of IleL224 and GlyL225 [29,49]. For atrazine or two its derivatives, the triazine molecule is apparently bound to the Q_B-site directly by three hydrogen bonds on the distal side, and only indirectly via water molecules on the proximal side. The three hydrogen bonds from the backbone carbonyl oxygen of TyrL222 to the triazine cyanobutylamino nitrogen and from the backbone amide of IleL224 to the N5 of the triazine ring and from the aminoethyl NH to SerL223 O had been inferred. HisL190 and GluL212 are involved in the indirect hydrogen-bonding. Further contacts are formed with ValL220, IleL229 and PheL216 [50]. Taken from the model of terbutryn binding to RC from *Rhodopseudomonas viridis* wild type [29,48] and mutant (PDB entry 1DXR) [51], the interaction mechanism of RC-terbutryn complex is very similar to that reported earlier for the binding of atrazine [51]. The X-ray structure of capsaicin binding in the bacteria reaction center shows that capsaicin forms two hydrogen bonds with Q_B site: one is between the capsaicin hydroxyl group and the side chain oxygen of SerL223 and the other between the capsaicin methoxy oxygen and the peptide amino of HisL190 [64]. As templates as the high resolution crystal structures of herbicide-binding niche models in the bacteria reaction center, several attempts at modeling the interaction of herbicides with Q_B site in the D1 protein have been constructed by various groups [23,25,28,52,58,59]. According to these modeling, the protein binding environment for photosystem II inhibitor herbicides (e.g., DCMU) is founded to be overlapping with that for Q_B. D1-H215 is likely to provide a weak hydrogen bond to the carbonyl group of DCMU and D1-S264 may provide another hydrogen bond with the amide group of DCMU [23,54]. Additionally, from the inhibitory activity of herbicides in herbicide resistant mutants of cyanobacteria, alga and higher plants, it has concluded that classical photosystem II inhibitors herbicides (like diuron, atrazine, terbutryn) orient themselves preferentially towards Ser264 of the D1 protein, and the binding of phenolic herbicides (e.g. ioxynil, dinoseb et al.) occurs via His215 [27] (see Fig. 6B). Whereas, our research shows that it is Gly256 not Ser264 residue that represents a direct and important role in TeA bound to Q_B-site because D1-Ser264Ala mutant has a weak resistance. It maybe attribute to the difference of chemical structure between TeA and other photosystem II inhibitor herbicides. Previous studies of different classes of herbicides indicated that the degree of resistance was related to chemical structure [43]. As a new type of photosystem II inhibitor, the structure of TeA is different obviously from the "classic" photosystem II inhibitors despite they share a common group N–C═X (where X signifies N or O). So, the pyrrolenode ring compounds like TeA and its above analogues, even tetramic acid may be a novel structural framework of a potential inhibitor to photosynthesis because of having a common group N–C═X (where X signifies N or O). Thus, the conclusion was drawn that in the process of TeA and its analogues interacting with Q_B-site D1-G256 residue may provide a hydrogen bond with the carboxyl in 2-position of TeA and its analogues and plays important role as well as D1-S264 and D1-F255 residues may be of marginal importance, and then D1-V219 and D1-L275 residues are not necessary (see Fig. 6B). In consideration of significant sequence and functional homology of photosystem II reaction center between higher plant and algae, this conclusion can also be applied in higher plants. Thus, the different binding behavior in Q_B-niche resulting in the different mechanism of killing weed may be a reasonable explanation that TeA has similar or even higher activity of killing weeds despite that it has lower inhibitor activity compared with commercial atrazine and DCMU herbicides [13,14].

In conclusion, TeA, as a new type of photosystem II inhibitor, is a phytotoxin from fungal source of *A. alternata*. Interestingly, why does *A. alternata* only synthesize TeA as phytotoxin against the resistance of host plant during its infection? The fungi easily use all 20 kinds of natural amino acid available as raw materials to synthesize 3-acyl-5-alkyltetramic acid as phytotoxin. *A. alternata* selectively use only isoleucine, having butyl side chain, to synthesize TeA with long side chain at 5-position best to keep high biological activity which possibly is an important factor that *A. alternata* isolated from *E. adenophorum* produces TeA not other phytotoxin. The novel finding may contribute to the expansion of our knowledge on the microbe–plant interaction. The characteristics of inhibition of TeA and its analogues to plants, which makes us deduce 3-acyl-5-alkyltetramic acid compound and even tetramic acid having same bioactivity, perhaps provides a new idea and approach in search for new herbicides through mimic synthesis as molecular structure model.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2007.02.007.

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