Tenuazonic Acid, a Novel Natural PSII Inhibitor, Impacts on Photosynthetic Activity by Occupying the Q_B-Binding Site and Inhibiting Forward Electron Flow

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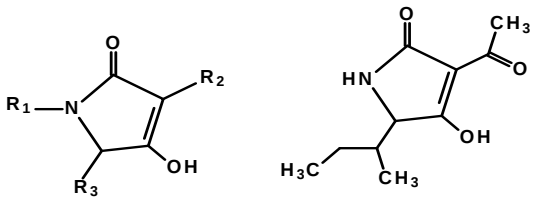
Abstract: Tenuazonic acid (TeA), a member of representative natural tetramic acids, is a phytotoxin produced by the fungus *Alternaria alternata* isolated from diseased Croftonweed (*Eupatorium adenophorum*). TeA strongly inhibits photosynthesis, especially photosystem II (PSII) activity. Evidence from fast chlorophyll fluorescence induction transients of host plant shows that the most important action site of TeA is that it interrupts electron transport beyond Q_A, on the acceptor side of PSII, and this is due to its binding at the Q_B-site. On the basis of competition experiments with [^{14}C]atrazine, it is further confirmed that TeA does not share the same binding environment as atrazine, despite their common action target: the Q_B-site.

Keywords: Tenuazonic acid; Chl *a* fluorescence induction transients; JIP-test; PSII inhibitor; Reaction center

Introduction

Tetramic acid (pyrrolidine-2,4-diones ring system) is a recurrent motif among natural products originating from a variety of marine and terrestrial species. Tenuazonic acid (TeA) is a member of representative natural tetramic acids.



1 **2**

Fig. 1 The structure of tetramic acid **1**, tenuazonic acid **2**.

Based on evidence from the model organism *Chlamydomonas reinhardtii*, TeA is a novel photosynthesis inhibitor, which mainly interrupts forward electron transport beyond Q_A by competing with Q_B for the Q_B-niche of the D1 protein (Chen *et al*., 2007). However, for a group of new potential herbicidal natural chemicals, understanding its mechanism of action is critical and this includes not just identifying the target site in an isolated model system but knowing all aspects of interaction with the whole plant, especially the host plant and the target weed. Fast Chl *a* fluorescence transient kinetics, a non-invasive spectroscopic technique, has become an excellent tool to probe the mode of action of photosynthetic inhibitors. Combined with photoaffinity labeling with radioactive technique, we have focused here on the effect of TeA on PSII activity by determining Chl *a* fluorescence transients of the host plant.

Materials and Methods

Plant materials and chemicals

Croftonweed (*E. adenophorum*) was grown for 3 months by rooting in soil at 20–25 ºC under approximately 300 μmol photons m^{-2} s^{-1} of white light (day/night, 12/12 h) and relative humidity (about 70%) in a glasshouse.

TeA was isolated and purified from a culture of *A.*

alternata isolate 501 (Chen *et al*., 2007). [^{14}C]Atrazine was purchased from Moravek Biochemicals Inc. (Specific activity is 9.3 mCi/mmol).

Chl a fluorescence transients and the JIP-test

Croftonweed plants were placed in darkness for about 3 h before TeA treatment at room temperature (25 ^{o}C), and then pairs of the second top leaves (without detaching them from the plant) were put in trays filled with 10 mL of TeA solution (1mmol TeA, distilled water). After treatment for the indicated time, leaves (still not detached and in darkness) were removed from the TeA solution and wiped to avoid possible effects of anaerobiosis.

Detached Croftonweed leaves (the second top leaf pair) were arranged in Petri dishes between two layers of filter paper with the lower surface upwards. TeA solution or water was added to cover the paper. The samples were left for incubation for a period of 12 h in complete darkness at 25 ^{o}C.

Chl *a* fluorescence transients were measured at room temperature with a fluorometer (Handy-PEA, Hansatech Instruments Ltd., UK). Raw fluorescence OJIP transients were analysed with the JIP-test (Strasser *et al*., 2004). The initial fluorescence F_O is measured at 20 μs —at this time all reaction centers (RCs) are open; fluorescence intensity at 300 μs (K-step), 2 ms (J-step), 30ms (I-step) is denoted as F_K, F_J and F_I, respectively. The maximal fluorescence intensity F_M is equal to F_P.

The probability ψ_{Eo} that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- is given as: $\psi_{Eo} = PSI_0 = ET_0/TR_0 = 1–V_J$. The maximum quantum yield of primary photochemistry, φ_{Po}, is defined as: $\varphi_{Po} = PHI(Po) = TRo/ABS = 1–F_O/F_M$. The maximum yield of electron transport (φ_{Eo}) has the following expression: $\varphi_{Eo} = PHI(Eo) = ET_0/ABS = (1–F_O/F_M)(1–V_J)$.

The amount of Q_A reducing centers = $(RC/RC_{ref.})(ABS/ABS_{ref.}) = [(RC/CS)_{treated}/(RC/CS)_{control}][(ABS/CS)_{treated}/(ABS/CS)_{control}]$. Non-$Q_A$ reducing centers (%) = 100–Q_A reducing centers.

Q_B reducing centers was calculated according to the protocol used for the so called double hit experiments (Appenroth *et al*., 2001). Dark adapted leaves were exposed twice for 1 s with saturating light at an interval of 10 s dark. The fraction of Q_B reducing centers can be calculated as follows: Q_B reducing centers = $(1–F_O/F_M)_{(second\ exposure)}/(1–F_O/F_M)_{(first\ exposure)}$. Non-$Q_B$ reducing centers = ΔV_0 = 100%–Q_B reducing centers.

An estimate of Oxygen Evolving Complexes (OEC) can be made by utilizing the value of V_K (= $(F_K–F_O)/(F_M–F_O)$) and V_J (= $(F_J–F_O)/(F_M–F_O)$). The fraction of OEC is calculated in comparison with the control sample as: Fraction of OEC = $[1–(V_K/V_J)]_{treated}/[1–(V_K/V_J)]_{control}$.

The ratio S_m/t_{Fmax} expresses the average fraction of open RCs: $S_m/t_{Fmax} = [RC_{open}/(RC_{close}+RC_{open}]av$, where S_m =(Area/$(F_M–F_O)$) is the working integral of the energy needed to close all reaction centers.

Competitive experiments of [^{14}C]atrazine bound to D1 protein

Displacement experiments were carried out using the method of Chen *et al*. (2007). The amount of bound [^{14}C]atrazine to Q_B-site was calculated from the total radioactivity added to the thylakoid suspension and the amount of free [^{14}C]atrazine in the supernatant after centrifugation.

Results and Discussion

TeA blocked PSII electron flow beyond Q_A

The following is observed from fluorescence transient OJIP curves of leaves in vivo (Figs. 2A and 2B): (1) the fluorescence rise transients, obtained from control leaves, show a typical OJIP shape; (2) with an

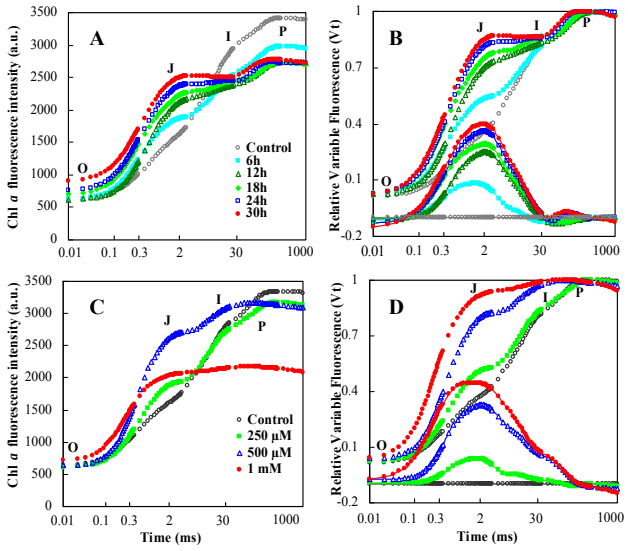


Fig. 2 Effect of TeA on Chl *a* fluorescence transients plotted on logarithmic time scale of Croftonweed leaves in vivo (A, B) or detached-intact leaves (C, D). Figs. A and C show raw curves without any normalization; in Figs. B and D the top figures show curves normalized by F_O and F_M, the bottom figures shows delta V (gain 1) full symbol curves minus control.

increase of the treatment time with TeA, the major changes are treatment-time dependent increase in the J level. Figs. 2C and 2D show that fluorescence transients OJIP of various concentrations of TeA treated detached-intact leaves. A clear rise in the J-step is observed with the penetration of TeA into the leaf. Moreover, with increasing of concentration of TeA, the J level became closer to the P level and then the IP phase began to disappear. An increase of the J step is usually interpreted as evidence for a large accumulation of Q_A^- due to a slowdown of electron transport beyond Q_A (Strasser and Govindjee, 1992).

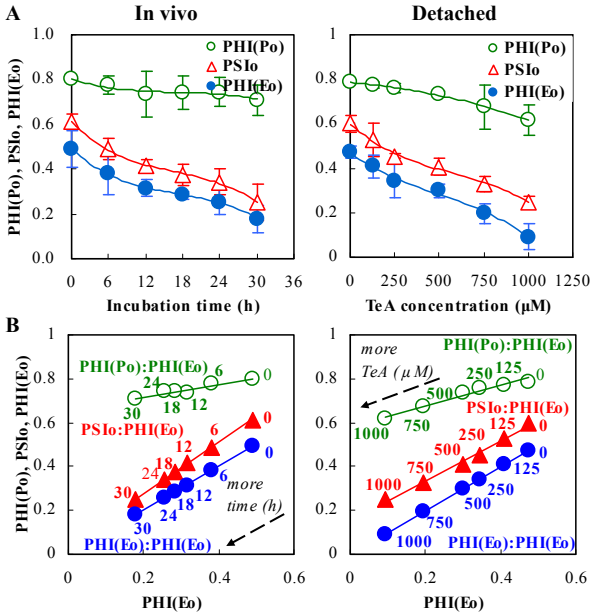


Fig. 3 Panel A: Effect of TeA on the maximum quantum yield of primary photochemistry (PHI(P_0)), the probability that an electron is going further than Q_A into the electron transport chain (PSI_0), the maximum quantum yield of electron transport (PHI(E_0)). Panel B: Analysis of the correlation for PHI(P_0), PSI_0 and PHI(E_0) versus PHI(E_0) of Croftonweed leaves treated with TeA.

In order to further demonstrate the effect of TeA on PSII, some functional parameters were used to quantify the PSII behavior and its activity. As shown in Fig. 3A, the maximum yield of primary photochemical (PHI(Po) = φ_{Po}) values do not change much. They remain in a narrow range. In contrast to φ_{Po}, the probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A ($PSI_0 = \psi_0$) and the quantum yield for electron transport (PHI(Eo) = φ_{Eo}) decreased strongly as a function of the incubation time or concentration with TeA. The three parameters can be plotted as φ_{Eo}: φ_{Po}, ψ_0 and φ_{Eo} versus φ_{Eo} (Fig. 3B). By increasing the time and concentration of TeA treatment, the values of ψ_0 and φ_{Eo} declined approximately linearly with φ_{Eo}, with φ_{Po} being inactive kept constant. These results indicate that TeA reacts like DCMU: it does not inhibit the primary light reaction but the redox reaction after Q_A due to interruption of electron flow beyond Q_A.

TeA resulted in inactive of PSII reaction centers

A large accumulation of Q_A^- must lead to inactive PSII RCs. With increased time and concentration of TeA treatment, the ratio S_m/t_{Fmax} values decreased further (Fig. 4A), which means that TeA caused severe closure of PSII RCs. In order to further check the influence of TeA on PSII RCs, three types of PSII RCs, non-Q_A reducing RCs, Q_A reducing RCs including non-Q_B reducing RCs and Q_B reducing RCs, were calculated by fluorescence transients (Figs. 4B and 4C). During TeA treatment, an approximately linearly sharp increase of non-Q_A reducing RCs was observed. In contrast, amount of Q_A reducing RCs showed an approximately linear decrease. Amount of Q_B binding centers containing Q_B reducing RCs (active) and non-Q_B

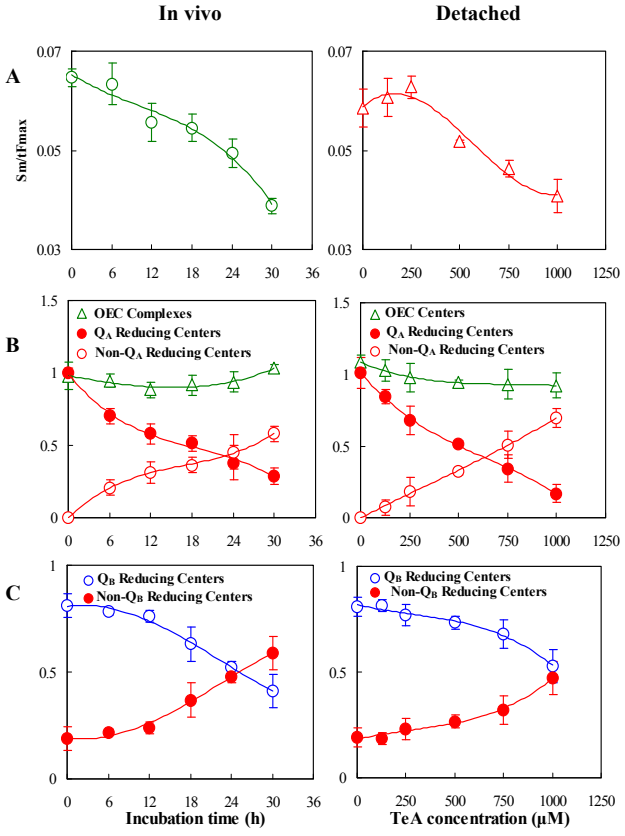


Fig. 4 Time and concentration dependent effect of TeA on (A) average fraction of open RCs (Sm/t_{Fmax}) in the time interval from 0 to t_{Fmax}; (B) fraction of Oxygen Evolving Centers (OEC) centers, Q_A-reducing RCs and non-Q_A-reducing RCs; (C) fraction of Q_B-reducing RCs and non-Q_B reducing RCs.

reducing RCs (inactive) is presented in Fig. 4C. Approximately 19% Q_B binding centers were inactive in control samples, which is equal to 19% non-Q_B reducing RCs. However, TeA treatment inactivated further the number of Q_B binding centers, which would lead to a significant increase in the amount of non-Q_B reducing RCs and a clear decrease of fraction of Q_B reducing RCs. After 30 h of 1 mmol TeA incubation of leaves in vivo, there were about 59% non-Q_B reducing RCs and 41% Q_B reducing RCs. Detached-intact leaves showed a similar change in amount of Q_B reducing RCs. However, TeA treatment had no distinct effect on OEC centers. Thus, it is concluded that TeA causes inactivity of PSII RCs by blocking electron transport of PSII acceptor side due to an increase of non-Q_B reducing RCs attributed to the binding of TeA to the Q_B site.

TeA bound to the Q_B-site

Although TeA is similar to the classical PSII herbicides (*e.g.* DCMU and atrazine) in binding to Q_B reaction centers, which does not necessarily mean that they act at the same binding site. As can be seen from the Fig. 5A, the amount of free [^{14}C]atrazine in the reaction mixture of the competition experiment increased in proportion to the addition of non-labeled TeA. This means that [^{14}C]atrazine binding to thylakoids of Croftonweed was affected by the presence of non-labeled TeA. Double-reciprocal plots of 1/μM free atrazine versus mg Chl/nmol bound atrazine were made for control thylakoids (no TeA) and in the presence of six concentrations of TeA (Fig. 5B),

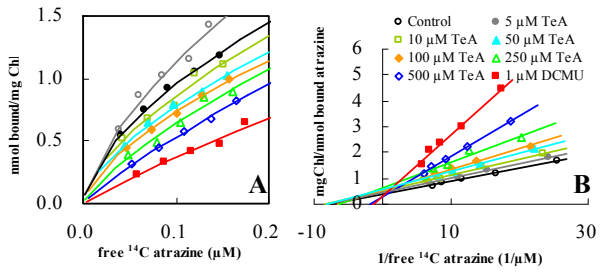


Fig. 5 Competitive experiments of [^{14}C]atrazine bound to thylakoid membranes of Crofton weed by non-labeled TeA and DCMU. (A) [^{14}C]atrazine binding curves. (B) Double-reciprocal plot of the concentration of free [^{14}C]atrazine vs. the amount of bound [^{14}C]atrazine.

which reveal a non-competitive displacement because there is not an identical ordinate intersect in various TeA concentrations. This demonstrates that TeA displaces atrazine-like inhibitors in a non-competitive manner. Hence, TeA has a different binding behavior within the Q_B-niche than by other PSII inhibitors.

Acknowledgements

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