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New herbicide-binding site in the photosynthetic electron-transport chain

Competitive herbicide binding at the photosystem I phylloquinone-(vitamin K_1)-binding site

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The binding of herbicides to the phylloquinone-(primary electron acceptor A_1)-binding site in green plant photosystem (PS) I reaction centers is shown. Dissociation constants (K_d) of various herbicides to the phylloquinone-binding site were estimated by analyzing their competitive inhibition of the reconstitution of the phylloquinone analogue, menadione (vitamin K_3), to the phylloquinone-extracted spinach PS I particles. The phylloquinone-binding site was found to bind *o*-phenanthroline ($K_d = 1.2 \times 10^{-4}$ M), but only weak binding was observed with atrazine ($K_d > 10^{-2}$ M), although both are known to bind specifically to the quinone-(Q_B)-binding site in reaction centers of purple photosynthetic bacteria or PS II. The inhibitors of the cytochrome $b/c_1(f)$ complex, myxothiazol ($K_d = 9.5 \times 10^{-6}$ M) or antimycin A ($K_d = 2.8 \times 10^{-6}$ M), also strongly bound to the phylloquinone site. This is the first report showing that the PS I reaction center complex also has a herbicide-binding site, although the site is probably not sensitive in vivo to these herbicides due to its higher affinity for phylloquinone than herbicides. The inhibitor specificity of the PS I phylloquinone site is different from that of the other quinone-functioning sites in the photosynthetic or respiratory electron-transfer chain, suggesting it to have a unique structure.

Photosystem I; Quinone; Phylloquinone; Vitamin K-1; Herbicide; Reaction center

1. INTRODUCTION

Photochemical reaction centers of photosynthetic bacteria and plants may be grouped into two types from current knowledge of the features of constituent polypeptides and prosthetic groups. One group consists of reaction centers of purple photosynthetic bacteria [1] and photosystem II (PS II) of oxygenic photosynthetic organisms [2], where two quinones function in series as the primary (Q_A) and secondary (Q_B) stable electron acceptors. In this type of reaction center, the prosthetic groups required for charge separation are associated with two, partially homologous, polypeptides of molecular mass about 30 kDa [1–3]. The Q_B site, where Q_B is reduced to quinol, together with the quinol-binding sites in the cytochrome $b/c_1(f)$ complex, has long been a target of herbicide studies [4,5]. The other type contains the PS I reaction center from plants [3,6] and probably that of anaerobic green photosynthetic bacteria [7]. In the case of PS I, it has been proposed that the stable primary electron acceptor is a phylloquinone but that the secondary electron acceptors are iron-sulfur centers [3]. The prosthetic groups PS I are associated with two polypeptides of approx. 80 kDa which show almost no

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Abbreviations: DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; prometone, 2,4-bis(isopropylamino)-6-(methoxy)-*s*-triazine; prometryne, 2,4-bis(isopropylamino)-6-(methylthio)-*s*-triazine; PS, photosystem; Q_ϕ site, PS I phylloquinone-binding site

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homology to the polypeptides associated with the reaction centers of PS II and of purple photosynthetic bacteria [3,6]. Similar prosthetic groups (menaquinones and iron-sulfur centers) and large polypeptides are likely to constitute the reaction center of green anaerobic photosynthetic bacteria [7].

Recent studies have indicated that the PS I reaction center contains two molecules of phylloquinone (= vitamin K_1)/reaction center [8,9], one of which presumably functions as the primary electron acceptor A_1 [10–15] and mediates electron transfer between the reduced electron acceptor intermediate, chlorophyll *a* (A_0^-), and F_X the iron-sulfur secondary electron acceptor. Some arguments against the function of phylloquinone, however, still remain [16,17] and its binding site has not been well characterized.

Many herbicides are known to bind to the Q_B site in a competitive manner with in situ ubi- or plastoquinone [5,18,19]. An X-ray crystallographic study of the *Rps. viridis* reaction center [20] has clearly shown that different types of herbicides, terbutryne and *o*-phenanthroline bind to different positions in the Q_B site interacting with different amino acid residues, respectively. Mutational modifications of these residues are known to alter resistance to these herbicides [5,19].

Binding of herbicides to the PS I reaction center has not yet been reported. We have reported that PS I phylloquinone can be extracted with diethyl ether leaving the primary charge separation activity virtually intact [10]. However, this is accompanied by an enhancement of the rapid 35 ns charge recombination between the oxidized donor $P700^+$ and A_0^- [10–14] due to the loss of the mediator for the electron transfer between A_0^- and F_X. The charge recombination can be suppressed by reconstitution with phylloquinone, phylloquinone analogues such as menadione [10–14] or artificial naphtho- and anthraquinones (Iwaki, M. and Itoh, S., in preparation). In addition, normal electron transfer from P700 to iron-sulfur clusters F_A and F_B, as shown below [3], can be restored [10,12].

$$P700 \xrightarrow{\text{light}} A_0 \xrightarrow{35\ \text{ps}} A_1(\text{phylloquinone?}) \xrightarrow{<\mu s} F_X \longrightarrow F_BF_A$$
$$\uparrow\ 35\ \text{ns}$$

Here, the PS I phylloquinone-binding site (designated Q_ϕ site) was studied through the effects of herbicides on menadione reconstitution into phylloquinone-extracted PS I particles. The PS I Q_ϕ site also binds herbicides and seems to have structural characteristics in common with other quinone-binding sites in photosynthetic or respiratory electron-transfer chains.

2. MATERIALS AND METHODS

Lyophilized spinach PS I particles were extracted twice with diethyl ether containing a 50% saturated amount of water followed by washing with dry ether to remove completely phylloquinone as described [10–12]. The resultant particles retained full primary charge separation activity, with 15% of antenna chlorophylls remaining (about 20 chlorophylls/P700), but contained no phylloquinone and carotenoids [10]. The iron-sulfur centers were not destroyed [10] and were active in accepting electrons when quinones were reconstituted [12]. In the quinone reconstitution studies, ether-extracted particles, dissolved in medium containing 50 mM Tris-Cl (pH 7.5) and 0.1% Triton X-100, were diluted 50 times with the same medium containing 30% glycerol without Triton, and incubated with menadione in the presence or absence of herbicides overnight at 0°C. 10 mM ascorbate was added to the reaction mixture together with 0.1 mM dichloroindophenol to reduce P700, 1–2 h before measurement. The absorption change of P700, measured with a time response of 1 μs, was induced by excitation with a 10 ns, 532 nm flash from an Nd-YAG laser; this excited about half of the reaction centers [10,12]. When reconstitution was performed with a saturating concentration of menadione, more than 70% of the reaction centers were estimated to be reconstituted with menadione as judged from the extent of the absorption change of P700 measured at the saturated laser intensity. Concentrations of chlorophylls and P700 were determined according to [10]. All measurements were performed at 6°C (to avoid damaging samples). The atrazines used here were gifts from Dr Kimiyuki Satoh, Okayama University. Other inhibitors and menadione were purchased from Sigma or Aldrich.

3. RESULTS

Extraction of phylloquinone from PS I particles resulted in a decrease in the flash-induced absorption change of P700 in the micro- to millisecond time range [10] due to the enhanced 35 ns charge recombination between $P700^+$ and A_0^- [13,14] caused by inhibition of the normal A_0^- oxidation step by phylloquinone. Flash excitation then induces an absorption change at 695 nm composed of a small, rapid decay (80 μs half-time) of the charge-recombination-induced $P700^T$ state and a very small, slow (100 ms half-time) $P700^+$ decay which reflects reduction of $P700^+$ by added dichloroindophenol (fig.1a) as reported [12]. The

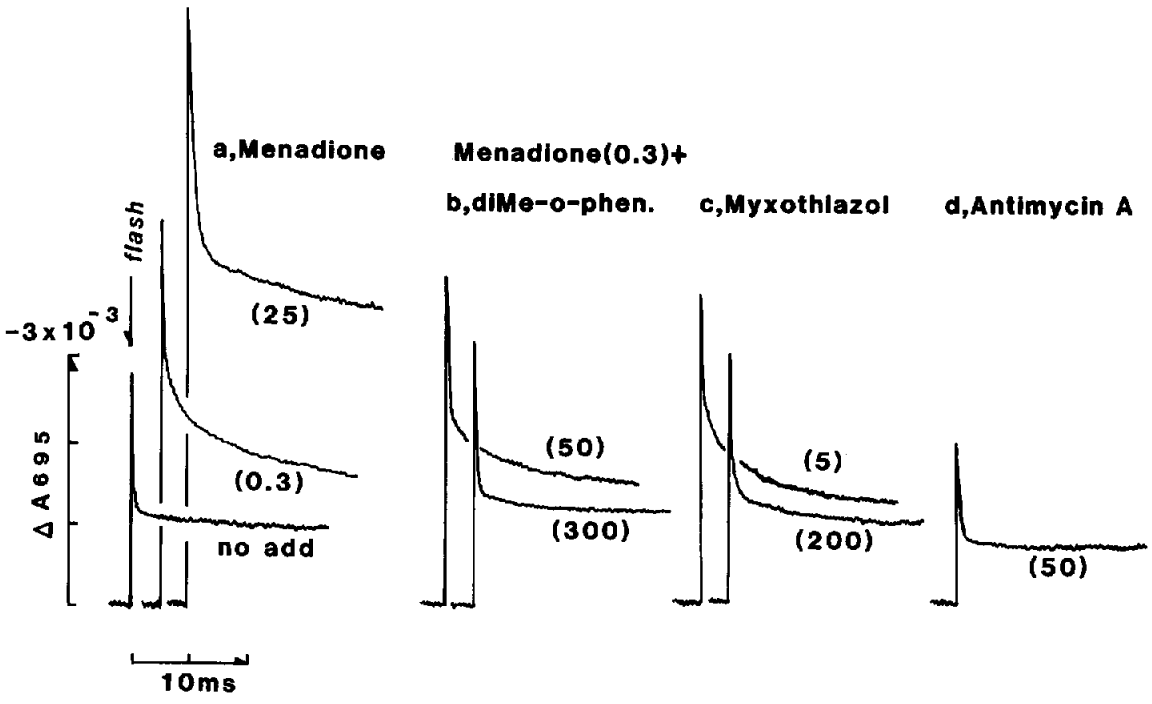

Fig.1. Flash-induced oxidation of P700 after reconstitution of menadione in the absence and presence of inhibitors in ether-extracted PS I particles. (a) Effect of concentration of menadione used for the reconstitution; (b–d) ether-extracted PS I particles incubated overnight with 0.3 μM menadione in the presence of (b) 4,7-dimethyl-*o*-phenanthroline, (c) myxothiazol and (d) antimycin A, at the indicated concentrations. The reaction mixture contained ether-extracted (20 chlorophylls/P700) PS I particles equivalent to 0.22 μM P700, 20 mM Tris-Cl buffer (pH 7.5) and 30% glycerol. Numbers in parentheses denote concentrations in μM.

latter phase reflects leakage of electrons from A_0^- to iron-sulfur centers which occurs even in the absence of phylloquinone [10]. Preincubation with menadione, which has a methylnaphthoquinone ring similar to that of phylloquinone but lacks a long phytyl chain, enhances the initial extent of $P700^+$ by suppressing the charge recombination (fig.1) with an apparent dissociation constant of 3.5×10^{-7} M (see fig.2A). This value is higher than that previously estimated for phylloquinone ($< 10^{-8}$ M) [12] and shows weaker binding of menadione. The enhanced micro/millisecond component of $P700^+$ is interpreted by the longer stay of electrons in the reducing side of PS I. Electrons in A_0^- are thought to be transferred to the iron-sulfur centers F_X, F_B and F_A mediated by menadione [10–12], and then slowly to $P700^+$. The extent of $P700^+$ in a micro/millisecond time range (fig.1a) thus represents the extent of the PS I reaction center reconstituted with menadione. Fig.1a demonstrates that 0.3 μM menadione was sufficient to induce almost half of the initial extent of $P700^+$ obtained with saturating concentration of menadione.

Traces b–d in fig.1 show the $P700^+$ decay kinetics in PS I particles reconstituted with 0.3 μM menadione in the presence of various reagents. Menadione reconstitution was suppressed in the presence of dimethyl-*o*-phenanthroline, myxothiazol or antimycin A. At higher concentrations of these reagents the effect of menadione was fully suppressed.

Fig.2A shows the dependence of the extent of $P700^+$ at 250 μs (this time was chosen to minimize the contribution of the recombination-induced P700 triplet state) on the concentration of menadione when reconstitution was performed with or without antimycin A. In the presence of the inhibitor, a higher concentration of menadione was required to attain the same extent of $P700^+$. Values of the dissociation constant (K_d) of 3.0 and 0.35 μM were calculated for menadione with and

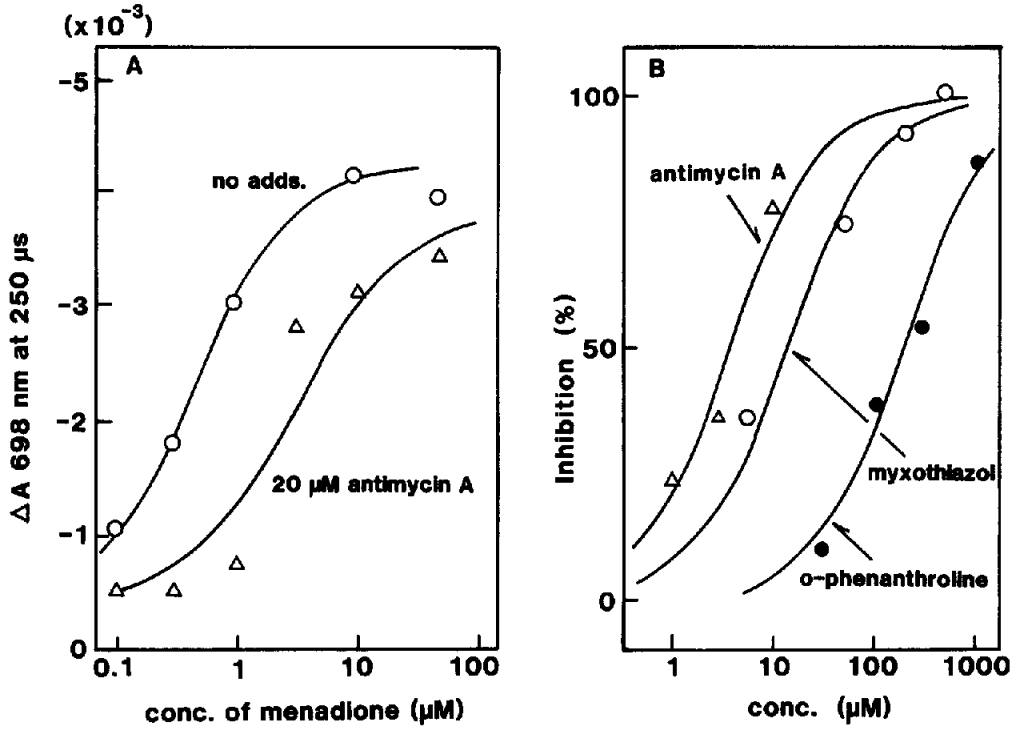

Fig.2. (A) Dependence of the extent of $P700^+$ at 250 μs after flash excitation on the concentration of menadione in the absence and presence of 20 μM antimycin A. Solid lines represent theoretical curves for the dissociation equilibrium calculated using the equation in the legend to table 1, with K_d values of 0.35 and 3.0 μM for the cases in which antimycin A was absent and present, respectively. (B) Suppression of 0.3 μM menadione-induced $P700^+$ stabilization by inhibitors. The extent of suppression was calculated from the inhibitor-induced depression of the extent of $P700^+$ measured at 250 μs after flash excitation. Other conditions similar to those in fig.1.

without 20 μM antimycin A, respectively. This reagent, thus, acts as a competitive inhibitor of menadione binding to the Q_ϕ site. From the shift of the apparent K_d value for menadione, the K_d for antimycin A was calculated to be 2.6×10^{-6} M using the equation in table 1 which assumes competitive binding of menadione and the inhibitor. Fig.2B shows the dependence of the reconstitution with 0.3 μM menadione on the concentration of *o*-phenanthroline, myxothiazol and antimycin A. The curves are interpreted as being for competitive binding of these reagents and menadione to the Q_ϕ site (see legend to table 1). The dissociation constants for antimycin A, myxothiazol and *o*-phenanthroline were calculated to be 2.8×10^{-6}, 9.5×10^{-6} and 1.2×10^{-4} M, respectively, from fig.2B. The value for antimycin A was consistent with that obtained in fig.2A.

The effects of various herbicides were estimated in a similar way and listed in table 1. Phenanthrolines, which are known to bind to the Q_A and Q_B sites of purple bacterial reaction centers or to the Q_B site of the PS II reaction center [5,18,19], showed K_d values of the order of 10^{-5}–10^{-4} M. These values are a little higher than those reported for PS II or purple bacteria [5,18,19]. Similar or lower K_d values were also estimated for HOQNO and myxothiazol, which are known to be potent inhibitors in the cytochrome b/c_1 complex [4] and in the Q_B site of purple bacterial reaction center [19]. Antimycin A, also known to be a potent inhibitor of the cytochrome b/c_1 complex [4], showed the tightest K_d value. On the other hand, atrazine and its derivatives which are known to bind specifically to the Q_B sites of the purple bacterial reaction center [5,18–20] and PS II [1,5] more effectively than do phenanthrolines, were rather ineffective in the Q_ϕ site showing K_d values greater than 10^{-3} M (fig.2A and table 1). DCMU, which is also known to inhibit strongly at the PS II Q_B site at concen-

Table 1

Effects of various herbicides on the reconstitution of menadione in ether-extracted PS I particles

Additions	Estimated K_d (M)
Phenanthrolines	
o-Phenanthroline	1.2×10^{-4}
4,7-Dimethyl-*o*-phenanthroline	2.4×10^{-5}
Benzoquinone analog	
DBMIB	5.7×10^{-3}
Atrazines and urea	
Atrazine	$>10^{-2}$
Prometone	5.7×10^{-3}
Prometryne	1.1×10^{-3}
DCMU	1.8×10^{-4}
Inhibitors of b/c_1 complex	
on the quinol-oxidase site	
Myxothiazol	9.5×10^{-6}
on the quinone-reductase site	
Antimycin A	2.8×10^{-6}
HOQNO	1.7×10^{-4}

K_d values (dissociation constant between the Q_ϕ site and herbicides) were calculated as shown in fig.2B from inhibition of 0.3 μM menadione-supported stabilization of $P700^+$ (calculated from the extent at 250 μs after laser excitation) using the equation below, which assumes competitive, one-site binding of menadione and herbicides to the Q_ϕ site:

$$R = \frac{1}{\frac{K_Q}{[Q]}\left(1 + \frac{[I]}{K_d}\right) + 1}$$

where K_Q represents the dissociation constant between the reaction center and menadione and equals 3.5×10^{-7} M under the present experimental conditions; R, the ratio of menadione-supported activity with herbicide to that without herbicide; I, the concentration of herbicide. Decrease in the concentration of free menadione due to binding was also taken into account

trations of the order of 10^{-7} M [5], was not effective in PS I. Also, the benzoquinone analogue DBMIB, a known inhibitor of both the Q_B site of PS II [5] and cytochrome b/c_1 complex [4], was not effective. However, this result is not unexpected, since it is known that DBMIB functions as an electron acceptor in the Q_B site of photosynthetic bacterial reaction centers [2].

4. DISCUSSION

The present results indicate that the PS I Q_ϕ site cannot be reconstituted with menadione in the presence of inhibitors. These inhibitors also suppressed the stabilization of $P700^+$ when added after the reconstitution of menadione or other quinones (Iwaki, M. and Itoh, S., unpublished). The Q_ϕ site, therefore, is also estimated to be a herbicide-binding site like the other quinone-functioning sites in reaction centers of PS II and purple bacteria or in cytochrome b/c_1 complex. Our finding that the quinone-site inhibitors suppress menadione-supported stabilization of $P700^+$ seems to confirm the idea that phylloquinone bound to the Q_ϕ site in the PS I reaction center functions as the primary electron acceptor A_1, as do the quinones in the Q_A site of purple bacterial or PS II reaction centers.

The Q_ϕ site may have structural features in common with the quinone-binding sites of PS II or purple bacterial reaction centers judging from the sensitivity to *o*-phenanthroline. This inhibitor is known to bind to the Q_B site by forming a hydrogen bond with a histidine residue, residue 190, of the L subunit (Hs-L190) of the reaction center polypeptides of *Rps. viridis* [20]. The histidine is coordinated to the Fe^{2+} situated between Q_A and Q_B quinones, and anchors one of the carbonyl groups of the benzoquinone ring of ubiquinone by H-bond [20]. A similar histidine residue also exists in the Q_A site [20]. In the Q_A and Q_B sites of the PS II reaction center, H-bonds between quinone carbonyls and similar histidine residues are assumed [5,20]. An amino acid residue, which can H-bond with *o*-phenanthroline, may also exist in the Q_ϕ site. However, an analogous histidine or nonhaem Fe^{2+}-containing site has not been identified in PS I [3,6]. At present, the identification of the binding site of phenanthrolines in PS I is difficult, since there are more than 50 histidine residues even in the α-helix portion of the PS I core polypeptides [22,23] in which the Q_ϕ site is likely to be embedded.

The low sensitivity to atrazines and DCMU indicates a feature of the Q_ϕ site differing from the Q_B site. Atrazines are believed to bind to the Q_B site by making an H-bond with the tryptophan residue (L223) of the purple bacterial reaction center polypeptides, which normally functions to anchor the other carbonyl group of in situ ubiquinone [20]. Similar H-bonding is also proposed in PS II from homology of the amino acid sequences with those of the purple bacterial reaction center polypeptides and the site of mutation of herbicide-tolerant mutants [5]. Such an H-bonding

site may be lacking in the Q_ϕ site of PS I reaction center. The structure of the Q_ϕ site might be more like that of the Q_A site than of the Q_B site, as judged from its function of binding the primary electron acceptor quinone.

The sensitivity to antimycin A, HOQNO and myxothiazol, which are known to be inhibitors of the cytochrome b/c_1 complex [4], may indicate that the Q_ϕ site has some common structural feature with the quinone-binding sites in the b/c_1 complex, although only low homology seems to exist between the primary amino acid sequences of PS I core proteins and subunits of the cytochrome b/c_1 complex [22,23]. It is clear, however, that the inhibitor specificity of the Q_ϕ site is somewhat different from that of the quinone/quinol-binding sites in the b/c_1 complex, since menadione binding is markedly disturbed by both antimycin A and myxothiazol, each of which acts on a different site in the b/c_1 complex, i.e. the quinone-reductase and quinol-oxidase site, respectively [4]. Elucidation of the binding characteristics of these reagents will facilitate biochemical determination of the phylloquinone-binding site in the PS I reaction center complex.

The reducing side of PS I is characterized by its extremely low redox potential. Phylloquinone is estimated to function as the primary electron acceptor A_1 which mediates electron transfer from the acceptor intermediate chlorophyll *a*-690 (A_0) to the iron-sulfur centers [3,6,12]. The Q_ϕ site appears to provide highly apolar environments which enable the phyllosemiquinone$^{\cdot -}$/phylloquinone couple to exist at an extremely low redox midpoint potential of about -900 mV [6]. With this low E_m value, phyllosemiquinone$^{\cdot -}$ may be able to reduce the low-potential iron-sulfur center F_X ($E_m = -705$ [24] or 730 mV [25]) situated inside the same reaction center protein. The estimated low E_m value of phylloquinone contrasts with those of menaquinone (-70 to -150 mV), which has the same naphthoquinone ring as phylloquinone, in the Q_A site of purple bacteria [26,27]. This clearly distinguishes the Q_ϕ site from the Q_A site.

It may be concluded that the Q_ϕ site exhibits an inhibitor sensitivity which is somewhat similar to the sensitivity observed for the other quinone/quinol-binding sites in the reaction centers of PS II or purple bacteria, or in the cytochrome b/c_1 complex. This suggests that the quinone/quinol-binding sites in the different electron-transfer complexes, even when their constituent polypeptide sequences have only low homology to each other, have a common three-dimensional structural feature which functions to bind the aromatic ring(s) and/or two carbonyl groups of quinones. The characteristics of the Q_ϕ site reported here are somewhat different from those of the other quinone-binding sites [4,5] Their elucidation will contribute to a better understanding of the structure of the PS I reaction center and the evolutionary relationship between different types of photosynthetic reaction centers, and may help in the design of new herbicides.

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