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Influence of Herbicide Binding on the Redox Potential of the Quinone Acceptor in Photosystem II: Relevance to Photodamage and Phytotoxicity†

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ABSTRACT: Here we show that herbicide binding influences the redox potential (Em) of the plastoquinone Q_A/Q_A^- redox couple in Photosystem II (PSII). Phenolic herbicides lower the Em by approximately 45 mV, while DCMU raises the Em by 50 mV. These shifts are reflected in changes in the peak temperature of thermoluminescence bands arising from the recombination of charge pairs involving Q_A^-. The herbicide-induced changes in the Em of Q_A/Q_A^- correlate with earlier work showing that phenolic herbicides increase the sensitivity of PSII to light, while DCMU protects against photodamage. This correlation is explained in terms of the following hypothesis which is based on reactions occurring in the bacterial reaction center. The back-reaction pathway for $P_{680}^+Q_A^-$ is assumed to be modulated by the free-energy gap between the $P_{680}^+Q_A^-$ and the $P_{680}^+Ph^-$ radical pairs. When this gap is small (i.e., when the Em of Q_A/Q_A^- is lowered), a true back-reaction is favored in which $P_{680}^+Ph^-$ is formed, a state which decays forming a significant yield of P_{680} triplet. This triplet state of chlorophyll reacts with oxygen, forming singlet oxygen, a species likely to be responsible for photodamage. When the free-energy gap is increased (i.e., when the Em of Q_A/Q_A^- is raised), the yield of the $P_{680}^+Ph^-$ is diminished and a greater proportion of the $P_{680}^+Q_A^-$ radical pair decays by an alternative, less damaging, route. We propose that at least some of the phytotoxic properties of phenolic herbicides may be explained by the fact that they render PSII ultrasensitive to light due to this mechanism.

Photosystem II (PSII)[1] is the membrane−protein complex in thylakoid membranes which catalyzes the oxidation of water and the reduction of plastoquinone (*1*, *2*). PSII is similar in many ways to the simpler photosynthetic reaction center of purple bacteria. Given the extremely detailed understanding of both its structure and its function (*3*, *4*), the purple bacterial reaction center has served as an extremely useful model on which to base research on PSII (*1*, *5*, *6*).

Certain commercial herbicides (urea, triazines, and phenolic herbicides) bind to the exchangeable quinone (Q_B) site on D1, the protein subunit which constitutes half of the

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[1] Abbreviations: Chl, chlorophyll; D1, reaction center-binding protein; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Em, midpoint redox potential; P680, primary electron donor of PSII; Ph, pheophytin, primary electron acceptor in PSII; PSII, photosystem II; Q_A, primary quinone acceptor in PSII; Q_B, second quinone electron acceptor in PSII; S-states, oxidation states of the O_2-evolving enzyme; TL, thermoluminescence.

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heterodimeric reaction center of PSII (*7*, *8*). In this way, these herbicides block photosynthetic electron flow after the one-electron reduction of the bound quinone, Q_A (*7*, *8*). It is known however that the inhibition of electron transfer is not the direct cause of herbicide-induced plant death. Instead, pigment-mediated photodamage is supposed to be responsible. The mechanism of this process is poorly understood (*7*, *8*). Phenolic herbicides are known to act additionally as uncouplers of the bioenergetic membrane and to accelerate the decay of the S-states of the O_2-evolving complex (ADRY reagents), but these effects are not thought to be their main phytotoxic mode of action (*7*, *8*).

Under normal conditions of plant growth, the D1 protein is turned over extremely rapidly compared to all of the other proteins in the plant cell (*9*, see refs *10*, *11*). This is usually assumed to be a result of photodamage mediated by the cofactors unique to D1. These include Q_B, the photooxidizable chlorophyll, P_{680}, the pheophytin electron acceptor, Ph, the kinetically competent oxidizable tyrosine Tyr_Z, and the Mn cluster that provides the catalytic site for oxygen evolution. When exposed to excess strong light, electron transfer is inhibited and D1 is degraded faster than it is replaced (*10*, *11*). Several different photoinduced reactions potentially contribute to the photodamage depending on the conditions (*10*, *11*).

Binding of herbicides to the Q_B site of the D1 protein can influence photodamage in PSII in two ways: first they determine what photochemical reactions can occur, and second, they bind to the D1 protein and effect protein degradation. The urea herbicide, DCMU, has been reported to protect against both photodamage (*12*−*14*) and degradation of D1 (*9*, *12*, *13*, *15*−*20*). The origins of these effects are poorly understood, and both binding (*19*, *21*) and photochemical effects (*14*) are ascribed to conformational effects for want of any other explanation. In contrast to DCMU and related herbicides, the phenolic herbicides which bind in the same site as DCMU have the opposite effect on the stability of PSII to photodamage, leading to greatly increased sensitivity to light (*15*, *22*). In addition, unlike DCMU, some phenolic herbicides do not inhibit the degradation of D1 (*17*, *19*).

In this work we show that DCMU and phenolic herbicides have opposite effects on the Em of the Q_A/Q_A^- couple and we propose a thermodynamic mechanism to explain the contrasting effects of these herbicides on the photosensitivity of PSII. We suggest that this mechanism may contribute to the phytotoxic properties of phenolic herbicides.

MATERIALS AND METHODS

PSII-enriched membrane fragments from spinach were prepared essentially as described in ref *23* with modifications as described in ref *24*. PSII-enriched membrane fragments without Q_B were obtained by incubating thylkaoid membranes at a higher Triton X-100 concentration (5% Triton for 30 min (final Chl content: 2 mg/mL)). The activity of both types of samples was about 500 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. The presence and absence of Q_B in these different preparations were verified by thermoluminescence.

Ca^{2+} depletion was performed by incubation of PSII samples at room temperature for 5 min in room light (10−12 μmol of quanta m^{-2} s^{-1}) in a buffer containing 300 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, and 25 mM succinic acid (pH 4.5). Following this, the same medium was added but with 80 mM Mes instead of succinic acid in order to readjust the pH to 6.5. The residual activity after this treatment was very low (20−50 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}) but could be restored by the addition of $CaCl_2$.

Mn depletion by NH_2OH washing was carried out by incubating PSII samples (0.5 mg of Chl mL^{-1}) in a buffer containing 5 mM NH_2OH, 400 mM sucrose, 15 mM NaCl, and 50 mM Mes (pH 6.5) for 1 h in the dark on ice. This treatment was followed by two washes in the same buffer without NH_2OH. The residual activity after NH_2OH treatment was low (less than 5% of the activity prior to the treatment).

Mn depletion by Tris washing was carried out by incubating PSII samples (0.1 mg of Chl mL^{-1}) in 0.8 M Tris-HCl for 10 min in room light on ice. This treatment was followed by two washes in 400 mM sucrose, 15 mM NaCl, and 50 mM Mes (pH 6.5). The residual activity after Tris treatment was low (less than 5% of the activity prior to the treatment).

The level of variable fluorescence was used as a measurement for the reduction state of Q_A; the measurements were performed without redox mediators essentially as described in ref *25*. In PSII mediators have been shown to perturb the measurements of the Em Q_A/Q_A^- particularly in centers capable of O_2 evolution (*25*). Reversible (and therefore valid) redox titrations are obtained in the absence of mediators when dilute samples are used. Reductive titrations were performed by gradual addition of sodium dithionite (in 0.5 M Mes), oxidative titrations by the addition of potassium ferricyanide. Chlorophyll fluorescence was measured with a PAM 101 fluorimeter (Walz, Effeltrich, Germany). Fluorescence was measured using the weak measuring light of the PAM fluorimeter set to 1.6 kHz, as described previously (*25*).

Thermoluminescence was measured with a home-built apparatus as described in ref *26*. Samples were illuminated with a single-turnover flash at −20 °C to measure the Q-band ($S_2Q_A^-$ recombination) and C-band (probably $Tyr_D^+Q_A^-$ recombination). For measuring the A_T-band (probably $Tyr_Z^+Q_A^-$ recombination), samples were illuminated with 10 single-turnover flashes at −13 °C, then cooled to −20 °C. The samples were heated with a rate of 0.4 °C/s.

RESULTS

Figure 1 shows redox titrations of the Q_A/Q_A^- couple in active PSII, measured as the change in chlorophyll fluorescence which is associated with this redox change. In samples without the addition of herbicides, the Em was found to be −80 mV, a value which agrees well with our earlier measurements (*25*). The presence of DCMU leads to an upshift in the Em potential of Q_A/Q_A^- to −28 mV (Figure 1A), while the presence of bromoxynil, a phenolic herbicide, shifts the potential down to −125 mV (Figure 1B).

We showed previously that the removal of the Mn cluster resulted in an increase in the Em of Q_A/Q_A^- by approximately 150 mV and designated this as the high-potential form of Q_A (*27*, *28*). Figure 2 shows titrations of chlorophyll fluorescence, reflecting the redox state of Q_A/Q_A^-, done in Mn-depleted PSII in the presence and absence of the herbicides. Herbicide binding has the same effect on the

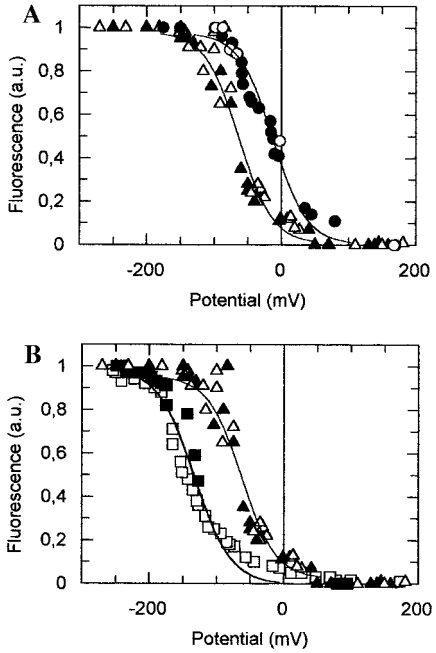

FIGURE 1: Redox titration of the yield of chlorophyll fluorescence in active PSII membranes: filled symbols, reductive titration; open symbols, oxidative titration. The curves shown represent one-electron Nernst curves. (A) triangles, no addition; circles, addition of DCMU. The midpoint potentials of these specific curves are −70 mV in the absence of herbicides and −30 mV in the presence of 10 μM DCMU. (B) triangles, no addition; squares, addition of 100 μM bromoxynil. In the presence of bromoxynil, the midpoint potential of the curve is −130 mV.

high-potential form of Q_A as seen for active samples (Figure 1). Without herbicides the midpoint potential is about 70 mV, the presence of DCMU leads to an upshift to about 125 mV (Figure 2A), and the presence of bromoxynil leads to a downshift to about 30 mV (Figure 2B).

The data from a series of titrations with DCMU and bromoxynil and another phenolic herbicide, ioxynil, are shown in Table 1. The size of the potential shifts induced by DCMU and bromoxynil are comparable for active and Mn-depleted PSII. In addition it is shown that ioxynil influences the Em of Q_A/Q_A^- in a similar way to bromoxynil in active PSII.

Thermoluminescence (TL) from PSII reflects charge recombination reactions occurring within the reaction center, and the peak temperatures of the thermoluminescence bands reflect the stability of the charge pairs involved. Changes in the Em of Q_A/Q_A^- should directly affect the thermoluminescence bands arising from charge pairs involving Q_A^-. When herbicides are present, the recombination luminescence occurs from the $S_2Q_A^-$ charge pair (*29*). Binding of phenolic herbicides results in $S_2Q_A^-$ recombination occurring at a lower temperature than when DCMU and related herbicides are bound, that is, −9 °C in the presence of bromoxynil compared to 7 °C in the presence of DCMU (*30*, *31*). Table 2 presents data which verify this effect; in addition we compare with $S_2Q_A^-$ recombination occurring without herbicides. This was done using a membrane preparation in which Q_B was removed by incubation with detergent. The present results show that phenolic herbicides destabilize the $S_2Q_A^-$ charge pair and that DCMU stabilizes the $S_2Q_A^-$ charge pair relative to that without herbicide binding. In PSII preparations which contained Q_B prior to the herbicide addition, the peak temperatures in the presence of DCMU and bromoxynil were the same as those given in Table 2 (not shown).

Ca^{2+}-depleted PSII shows a high-temperature band (around 45 °C, C-band) which seems to arise from $Tyr_D^+Q_A^-$ (*24*, but see ref *32*). Also in the absence of the Mn cluster charge recombination can still take place giving rise to a weak luminescence band at temperatures lower than 0 °C (A_T-band), probably arising from $Tyr_Z^+Q_A^-$ (*33*, but see ref *34*). Although these bands are less well-characterized than the $S_2Q_A^-$ recombination band and although there remains some doubt over the attribution of the charge pairs involved, the doubts mainly concern the origin of the donor side radical rather than that on the acceptor side (*24*, *32*−*34*). As can be seen in Table 2, for all measured TL bands, DCMU leads to a rise of the peak temperature while bromoxynil lowers the peak temperature. These results are in good qualitative agreement with the measurements of the Em of Q_A/Q_A^-. In the presence of DCMU, the rise of the peak temperature of the A_T-band is smaller than that of the Q-band and the C-band. This might be due to less accurate determinations of the peak position for the A_T-band due to its smaller amplitude.

DISCUSSION

Here we show that the binding of DCMU and phenolic herbicides to PSII shifts the Em of Q_A/Q_A^- in opposite directions: DCMU raises the potential while phenolic herbicides lower it. In addition we show that this corresponds to shifts in the temperature maxima of the TL bands arising from charge recombination reactions involving Q_A^-. A comparison of the redox titration data and the TL data arising from several different charge pairs indicates that the herbicide-induced shifts in the TL positions are due to changes in the Em of $Q_AQ_A^-$.

A wide range of herbicides have been screened by TL (*30*, *31*). The herbicides were classified into two groups: those like DCMU (the urea/triazine herbicides) and those like bromoxynil (the phenolic herbicides). It seems likely that the upshift in the Em of Q_A seen for DCMU and the downshift in the Em of Q_A seen for bromoxynil and ioxynil are representative characteristics of the other herbicides in their respective classes.

There are at least two earlier publications in which thermoluminescence arising from $S_2Q_A^-$ recombination has been reported in the absence of a herbicide. First, in the work of Rutherford et al. (*29*) the PQ pool and Q_B were reduced chemically allowing $S_2Q_A^-$ recombination to take place. The TL peak occurred at a slightly lower temperature than that in the presence of DCMU. It was suggested at that time that DCMU binding could result in an upshift in the Em of Q_A. The present work confirms that suggestion. Second, Wydrzynski and Inoue (*35*) extracted the PQ pool and Q_B with organic solvents from freeze-dried thylakoid membranes and generated a $S_2Q_A^-$ recombination TL band in rehydrated samples. In this case no shift of the peak temperature was reported. The discrepancy between this result and the present work may be attributed to differences induced by the pretreatment. In our case, the removal of Q_B by detergent incubation does not perturb the Em of Q_A/Q_A^- measured directly in redox titrations (data not shown). In fact the TL

Table 1: Effect of PSII Herbicides on the Midpoint Potential of the Q_A/Q_A^- Redox Couple[a]

active PSII	active PSII + DCMU	active PSII + bromoxynil	active PSII + ioxynil	Mn-depleted PSII	Mn-depleted PSII + DCMU	Mn-depleted PSII + bromoxynil
-80 ± 16 mV	-28 ± 18 mV $\Delta E = 52$ mV	-125 ± 16 mV $\Delta E = -45$ mV	-113 mV $\Delta E = -33$ mV	73 ± 17 mV	123 ± 5 mV $\Delta E = 50$ mV	24 ± 14 mV $\Delta E = -49$ mV

[a] The midpoint potential was measured using room-temperature chlorophyll fluorescence in active and NH_2OH-washed (Mn-depleted) PSII membranes. The concentrations of the herbicides were 10 μM DCMU or 100 μM bromoxynil or ioxynil. Standard deviations for six (active samples) and three (inactive samples) independent measurements are given.

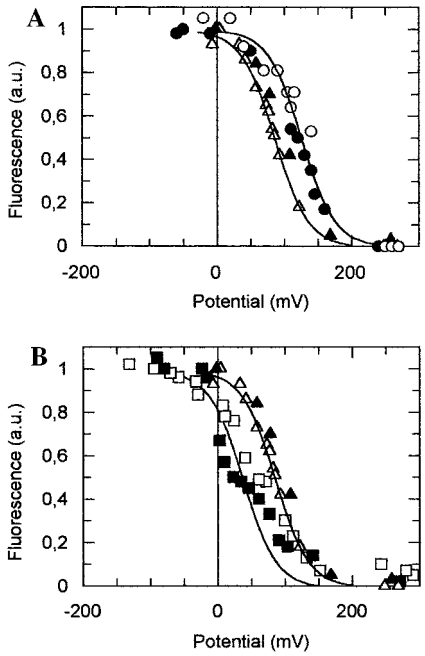

FIGURE 2: Redox titration of the yield of chlorophyll fluorescence in NH_2OH-washed PSII membranes: filled symbols, reductive titration; open symbols, oxidative titration. The curves shown represent one-electron Nernst curves. (A) triangles, no addition; circles, addition of 10 μM DCMU. The midpoint potentials of these specific curves are +70 mV in the absence of herbicides and +125 mV in the presence of DCMU. (B) triangles, no addition; squares, addition of 100 μM bromoxynil. In the presence of bromoxynil, the midpoint potential of the curve is +30 mV.

Table 2: Influence of Herbicides on the Maximal Emission Temperature of TL-Bands[a]

	no addition	+ DCMU	+ bromoxynil
Q-band	−2 °C	8 °C	−10 °C
A_T-band	−8 °C	−5 °C	−14 °C
C-band	46 °C	55 °C	41 °C

[a] The Q-band was measured in Q_B-depleted PSII membranes, the other bands in PSII membranes which contained Q_B. The Q-band and C-band were excited by one single-turnover flash at −20 °C, and the A_T-band was excited by 10 single-turnover flashes and subsequently cooled to −20 °C. The heating rate was 0.4 °C/s for all samples.

bands in the presence of the herbicides have the same peak temperature irrespective of the pretreatment.

It has been reported that DCMU and other urea/triazine herbicides protect against photodamage of PSII activity (*12*−*15*) while phenolic herbicides render PSII more sensitive to light (*15*, *22*). The present results, showing opposite effects of DCMU and bromoxynil on the Em of Q_A/Q_A^-, provide the basis for explaining the opposing effects of these herbicides on photosensitivity. This explanation relies on well-established photochemical reactions occurring in the purple bacterial reaction center.

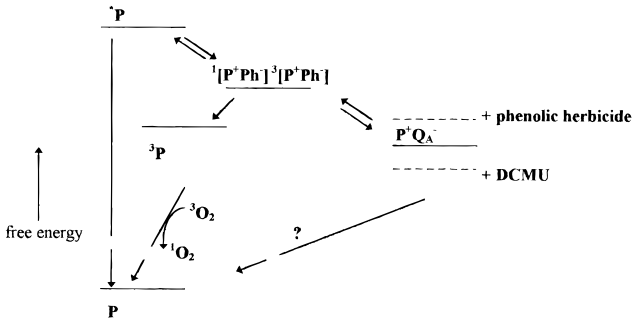

FIGURE 3: A schematic diagram of the free-energy levels of the states involved in recombination of the $P^+Q_A^-$ radical pair in PSII showing the influence of the herbicide binding on the Em of Q_A/Q_A^-. When phenolic herbicides are bound, the back-reaction via the $P680^+Ph^-$ radical pair is favored. When $P680^+Ph^-$ is formed in the back-reaction, both singlet and triplet forms of the radical pair are present. The latter can decay forming ^{3}P680 and $^1O_2$. When DCMU is bound, this back-reaction is disfavored and direct recombination or an alternative route to the ground state may occur.

In bacterial reaction centers, it has been demonstrated that the free-energy gap between the $P^+Q_A^-$ radical pair and the P^+BPh^- radical pair has a major influence on the back-reaction pathway (*36*−*39*). When the gap is smaller than 400 meV, the back reaction via the P^+BPh^- radical dominates, while under conditions where the gap is greater than this value, a direct recombination pathway dominates involving nuclear tunneling reactions (*36*−*39*). In PSII (Figure 3), it seems likely that the modulation of the Em of Q_A/Q_A^- by herbicides will also influence the free-energy gap between $P_{680}^+Q_A^-$ and $P_{680}^+Ph^-$. With DCMU, it is predicted that the increase in the Em should increase the energy gap and thereby diminish the yield of the true back-reaction via the $P_{680}^+Ph^-$ radical pair. By analogy to the bacterial reaction center a direct recombination reaction between $P_{680}^+Q_A^-$ may take place which acts as a nondamaging pathway for the decay of the radical pair $P680^+Q_A$. Alternatively some kind of cyclic electron-transfer pathway may be favored.

On the other hand, the decreased Em of Q_A/Q_A^- induced by phenolic herbicides should make the energy gap smaller. It is expected that the back-reaction via the $P680^+Ph^-$ radical pair will result in a significant fraction of charge recombination to form the P680 triplet (*40*, see ref *13*). The P680 triplet is expected to have a relatively long lifetime under these conditions (*41*, *42*) and will therefore have a significant chance of encountering O_2, especially since O_2 is generated by the enzyme itself. The O_2 that reacts with P680 triplet will be converted to singlet oxygen which is then likely to cause damage to the protein and the cofactors in its environment (*43*−*45*).

This mechanism provides then an explanation for the observations in the literature that the binding of phenolic herbicides leads to greater sensitivity to photodamage while DCMU and related herbicides protect against photodamage

(*15*). Other mechanisms may also play a role; indeed the present work does not bear on the influences of the herbicides on D1 degradation (e.g., refs *17*, *19*) and we do not rule out that the special binding properties of the different classes of herbicide could contribute additional stabilization and destabilization effects as suggested earlier (*14*, *15*).

For phenolic herbicides, it seems reasonable to suggest that the mechanism of photodamage proposed here contributes to the phytotoxicity of the herbicide. A stimulation of chlorophyll bleaching has been observed in leaves in the presence of phenolic herbicides compared to the presence of urea herbicides (*22*).[2]

In the present study we have no information on how herbicide binding in the Q_B site results in an Em shift of Q_A. Of possible relevance is the observation in bacterial reaction center mutants, in which the absence of Glu L212 in the Q_B site was compensated for in a suppressor mutant by an additonal Phen (Leu L227 to Phen) (*46*). It was suggested (*46*) that an arginine residue, also present in the Q_B site, might be partially neutralized by the π electron system on the aromatic residue (see ref *47*). Since it has been shown that charged groups in the Q_B site have an influence on the free-energy level of Q_A (*48*), the partial neutralization of a positive charge in the Q_B site of PSII by a phenolic herbicide may contribute to the Em shift of Q_A. Of course, several other modes of action can be imagined, particularly herbicide-induced conformational changes. Future spectroscopic studies may help elucidate the structural basis of the herbicide effects.

It is of note that the inhibitor, *o*-phenanthroline, which also binds in the Q_B site of bacterial reaction centers, also shifts the potential of Q_A/Q_A^- (*49*, *50*). In the bacterial reaction center, the binding of *o*-phenanthroline has been modeled by X-ray crystallography (*51*). It would be particularly interesting to study the influence of DCMU and ioxynil on the kinetics and thermodynamics of the back-reactions occurring in the bacterial reaction center where crystallographic models are already or potentially available. This would involve the terbutryn-resistant mutant, T4, which is the only bacterial reaction center studied so far in which these plant herbicides bind (*52*).

Finally, the modulation of the back-reaction pathway through the Em of Q_A/Q_A^-, as suggested here for herbicide binding and earlier during photoactivation (*28*), may be relevant to PSII under any conditions where the Em of Q_A/Q_A^- is modified. We should now consider a wide range of possible cases where the electrostatic environment of Q_A could be affected: for example, in the presence of membrane potentials, in certain herbicide-resistant mutants (*7*), in the presence of carboxylic acids (such as bicarbonate, glycolate, etc.) which bind to the iron (*53*), in PSII when the phosphorylation occurs in the vicinity of the Q_A site (*54*), under conditions where Ca^{2+} could be lost (*55*), or in mutants lacking the 23 kDa protein (*56*). In all of these cases it may be worth looking for correlations between the Em of Q_A/Q_A^- and the sensitivity of PSII to light. If the charge recombination pathway is modulated by the Em of Q_A as proposed here, such correlations would be predicted.

[2] It has been shown that DCMU protects PSII from photodamage in vitro. Yet in vivo it seems to be clear that phytotoxicity of this herbicide is still due to light-driven processes presumed to be Chl triplet-mediated $^1O_2$ damage. There are several ways by which the binding of the herbicide could trigger such a mechanism. For example, the perturbation of PSII reaction center assembly due to binding of DCMU in the quinone site could leave the partially assembled chlorophyll-bearing proteins, both reaction center and antenna, in a state where the excited Chl cannot be quenched before Chl triplets are formed. Herbicide-induced perturbations of the reaction center disassembly mechanism, as suggested by Trebst (personal communication), could have a similar effect. Another possibility is that a DCMU-induced blockage of the photoactivation of the oxygen-evolving enzyme results in an increase in photoinhibition (*57*). Specific experiments are required in order to determine which of the potential mechanisms is actually the dominant one for the phytotoxicity of DCMU. Given the similarities in the binding site of phenolic herbicides, the mechanisms by which DCMU could trigger Chl triplet-mediated $^1O_2$ production are potentially applicable to phenolic herbicides also. However, the specific effect of phenolic herbicides on the Em of Q_A represents an additional, highly efficient reaction for the generation of Chl triplet which is unique to this class of herbicides.

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