Magnetic field protects plants against high light by slowing down production of singlet oxygen

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Abstract

Recombination of the primary radical pair of Photosystem II (PSII) of photosynthesis may produce the triplet state of the primary donor of PSII. Triplet formation is potentially harmful because chlorophyll triplets can react with molecular oxygen to produce the reactive singlet oxygen ($^1O_2$). The yield of $^1O_2$ is expected to be directly proportional to the triplet yield, and the triplet yield of charge recombination can be lowered with a magnetic field of 100-300 mT. In the present study, we illuminated intact pumpkin leaves with strong light in the presence and absence of a magnetic field and found that the magnetic field protects against photoinhibition of PSII. The result suggests that radical pair recombination is responsible for significant part of singlet oxygen production in the chloroplast. The magnetic field effect vanished if leaves were illuminated in the presence of lincomycin, an inhibitor of chloroplast protein synthesis, or if isolated thylakoid membranes were exposed to light. These data, in turn, indicate that $^1O_2$ produced by the recombination of the primary charge pair is not directly involved in photoinactivation of PSII but instead damages PSII by inhibiting the repair of photoinhibited PSII. We also found that an *Arabidopsis thaliana* mutant lacking α-tocopherol, a scavenger of $^1O_2$, is more sensitive to photoinhibition than the wild type in the absence but not in the presence of lincomycin, confirming that the target of $^1O_2$ is the repair mechanism.

Abbreviations – F_V/F_M, ratio of variable to maximum chlorophyll *a* fluorescence; $^1O_2$, singlet oxygen; P_{680}, $^3P_{680}$, the ground state and the triplet excited state of the primary donor of PSII; PPFD, photosynthetic photon flux density; PSII, Photosystem II.

1. Introduction

In Photosystem II (PSII), the primary radical pair is formed by charge separation after excitation of the reaction center chlorophyll (Chl) P_{680}. Recombination of the primary pair may produce the triplet state of the primary donor ($^3P_{680}$) if the radical pair is in a virtual triplet state at the moment of recombination. This virtual triplet state is formed by spin dephasing in ~10 ns after charge separation (Hoff 1981). In open PSII reaction centers, the triplet yield is close to zero because the reduction of the quinone acceptor Q_A occurs in approximately 0.3 ns (Renger and Holzwarth 2005). However, in a closed reaction center the primary radical pair lives longer and recombination may therefore produce $^3P_{680}$ (van Mieghem et al. 1995).

Production of $^3P_{680}$ is potentially harmful because triplet Chl like $^3P_{680}$ reacts with molecular oxygen (O_2), producing singlet oxygen ($^1O_2$) (for reviews, see Halliwell and Gutteridge 1998, Schweitzer and Schmidt 2003, Tyystjärvi 2004). $^1O_2$ is a short-lived, highly reactive molecule capable of oxidizing proteins, lipids and nucleic acids. Plants, algae and cyanobacteria produce $^1O_2$ during illumination (Barta et al. 2004, Flors et al. 2006, Fufezan et al. 2007, Hideg et al. 1998, 2001, 2002), and *in vitro* experiments suggest that thylakoid membranes of chloroplasts are the source of $^1O_2$ (Hideg et al. 1994a, 1994b, Jung and Kim 1990, Telfer et al. 1994). Carotenoids and tocopherols of thylakoid membranes quench and deactivate $^1O_2$ (Fahrenholtz et al. 1974, Kruk and Strzalka 1995, Munné-Bosch and Alegre 2002).

External magnetic fields affect the reactions of radical pairs, including the primary pair of PSII (see reviews by Hoff 1981, Katz et al. 1978, Rodgers and Hore 2009). In an external magnetic field, only the middle triplet energy level becomes populated during the short lifetime of a

photosynthetic radical pair, and therefore a magnetic field lowers the triplet yield of charge recombination in photosynthetic reaction centers (Hoff 1981). Thus, a magnetic field is also expected to lower the amount of $^1O_2$ produced by the charge recombination mechanism. In reaction centers of a carotenoidless strain of *Rhodobacter sphaeroides*, an external magnetic field of ~8-100 mT has been shown to lower the production of $^1O_2$ in the light by up to 50 % (Liu et al. 2005). Earlier experiments have also shown that delayed light emission from PSII is enhanced by magnetic fields of 100-300 mT, apparently because a magnetic field lowers the $^3P_{680}$ yield (Sonneveld et al. 1980). Recent experiments with higher plants, in turn, have shown that magnetic fields (125-250 mT) have significant positive effects on growth of seedlings of rice, pea, lentil and maize (Flórez et al. 2004, 2007, Martínez et al. 2009). However, connection of the growth enhancement to protection against singlet oxygen production has not been considered.

It has been shown that $^1O_2$ can cause decrease in PSII activity (Hideg et al. 2007, Knox and Dodge 1985, Nishiyama et al. 2004, Trebst et al. 2002), but controversial results have been reported about the primary target of the damage caused by chemically induced $^1O_2$ (Hideg et al. 2007, Nishiyama et al. 2004). Production of $^1O_2$ has often been linked to photoinhibition of PSII, a reaction in which light causes loss of the electron transfer activity of the PSII reaction center (for reviews, see Tyystjärvi 2008, Vass and Aro 2008). According to the acceptor-side photoinhibition hypothesis (Vass et al. 1992), $^1O_2$ is produced due to recombination of the primary radical pair in already photoinhibited PSII centers in which the lifetime of the radical pair is exceptionally long (van Mieghem et al. 1995, Vass et al. 1992, 1993). This view is supported by the finding that the production of $^1O_2$ increases with decreasing PSII activity (Hideg et al. 1994a, 1998). A recent hypothesis suggests that photoinhibition under strong light is caused by $^1O_2$ produced due to recombination of the primary radical pair in closed PSII centers (Vass

and Aro 2008, Vass and Cser 2009), but the hypothesis has not yet been experimentally tested. Under low light or under illumination with short flashes, photoinhibition has been suggested to be mediated by $^1O_2$ produced via the slow recombination reactions between the quinone electron acceptors and the manganese cluster (Keren et al. 1997, 2000, Szilard et al. 2005). In addition to recombination reactions in the reaction center of PSII, intersystem crossing may produce Chl triplets and $^1O_2$ in the light-harvesting antennae (Katz et al. 1978, Santabarbara et al. 2002). Also energetically uncoupled Chls (Santabarbara et al. 2001), or iron-sulfur centers and cytochromes (Jung and Kim 1990) have been suggested to act as $^1O_2$ sensitizers in chloroplasts.

Two hypotheses explain photoinactivation of PSII without direct involvement of $^1O_2$. The donor-side hypothesis (Anderson et al. 1998) assumes that PSII is damaged by inappropriate electron transfer to long-lived P_{680}^+ when electron donation from the manganese cluster does not function. The manganese hypothesis (Hakala et al. 2005, Ohnishi et al. 2005), in turn, explains photoinhibition by direct light-induced damage to the manganese cluster. Experimental evidence in support of the manganese mechanism is accumulating (Antal et al. 2009a, Hakala et al. 2006, Sarvikas et al. 2006, Tyystjärvi et al. 2002) but it has also become obvious that an independent Chl-dependent mechanism functions in parallel with the manganese mechanism (Sarvikas et al. 2010a).

When an intact photosynthetic organism is exposed to light, light-induced damage to PSII is continuously repaired in a complicated pathway consisting of degradation and *de novo* synthesis of the PSII reaction center protein D1 and activation of the repaired reaction center (for reviews, see Nishiyama et al. 2005, Nixon et al. 2010, Vass and Aro 2008). Reactive oxygen species,

including $^1O_2$, may inhibit the concurrent repair of photoinhibited PSII by interfering with protein synthesis (Nishiyama et al. 2001, 2004).

In the present study, we utilized the unique capacity of a magnetic field to specifically lower the $^1O_2$ yield of charge recombination reactions. Intact leaves and isolated thylakoid membranes were illuminated in the presence and absence of a strong magnetic field, and the photoinhibitory loss of PSII activity was measured. The results show that recombination reactions cause production of $^1O_2$ and the produced $^1O_2$ causes damage in the chloroplast.

2. Materials and Methods

2.1. Biological material

Pumpkin (*Cucurbita maxima*) and Arabidopsis (*Arabidopsis thaliana*) leaves and pumpkin thylakoids were used in photoinhibition experiments. Pumpkin plants were grown at the photosynthetic photon flux density (PPFD) of 150 µmol m^{-2} s^{-1} in a 16 h day, 8 h night rhythm, and fully expanded leaves of 4-5 week old plants were used in the *in vivo* experiments. Arabidopsis plants were grown at the PPFD of 100 µmol m^{-2} s^{-1} in 8 h day, 16 h night rhythm, and fully expanded leaves of 7-8 week old plants of the wild type or the tocopherol cyclase knock-out strain *vte1* (Porfirova et al. 2002) were used in experiments.

2.2. Photoinhibition treatments in vitro *and* in vivo

Thylakoid membranes for *in vitro* experiments were isolated according to Hakala et al. (2005). Isolated thylakoids (50 µg Chl ml^{-1}) were illuminated with white light, PPFD 900 or 1800 µmol m^{-2} s^{-1}, as indicated, in the presence or absence of an external magnetic field. The light source

was a 300 W high-pressure ozone-free Xenon lamp (Oriel Instruments, Stratford, CT, USA) equipped with an ultraviolet blocking filter (Schott GG400). In another set of experiments, thylakoid membranes were illuminated with laser pulses (532 nm, pulse width 5-ns, energy density 170 mJ cm^{-2}), fired at 10 s pulse-to-pulse intervals in the presence and absence of a magnetic field.

Magnetic fields from 2 to 160 mT were obtained with an electrical magnet and a 350 mT field was obtained by placing the sample between a pair of strong permanent magnets. We also tested the effect of compensating for the Earth's magnetic field (~0.03 mT) by placing the sample in the middle of three electrical magnets and adjusting the strength of the field at all three axes to zero.

For *in vivo* photoinhibition, both pumpkin and Arabidopsis leaves were pretreated by immersing the cut petiole either in 2.3 mM lincomycin or in water, and incubating the leaf for 16 h in dim light before the illumination treatment. Leaves were illuminated in a temperature controlled incubator at 23 °C on a wet paper towel with white light, PPFD 1500 µmol m^{-2} s^{-1} for 1.5 to 6 hours. Pumpkin leaves were illuminated either on top of a 170 mT permanent magnet or on a similar piece of non-magnetic metal.

2.3. Measurements of oxygen evolution and Chl fluorescence

Prior to fluorescence measurements, unilluminated and illuminated leaves were dark-incubated for 20 min. The ratio of variable to maximum Chl *a* fluorescence (F_V/F_M) was measured from intact leaves with the FluorPen fluorometer (PSI, Brno, Czech Republic). After the fluorescence measurement, thylakoids were isolated (Hakala et al. 2005) from the same leaf disks, and the light-saturated rate of oxygen evolution was measured from the isolated thylakoids (10 µg Chl

ml^{-1}) with an oxygen electrode (Hansatech, King's Lynn, UK), using 125 µM 2,6-dichlorobenzoquinone as electron acceptor. The rate constant of photoinhibition (k_{PI}) was obtained by fitting the photoinhibitory loss of PSII activity to a first-order reaction equation.

2.4. Determination of the D1 protein

Thylakoids (2 µg Chl) were solubilized for 5 min at 65 °C, and the proteins were separated with SDS-polyacrylamide gel electrophoresis using a 13 % NextGel™ (Amresco Inc., Solon, OH, USA). A protein standard (Dual color, Bio-Rad, Hercules, CA, USA) was included. After electrophoresis, the separated proteins were electroblotted onto Immobilon P membrane (Millipore, Billerica, MA, USA) and stained with Ponceau S solution (Sigma-Aldrich, Wilmington, DE, USA) to check even loading and even transfer of proteins. The D1 protein was detected with the CDP-Star™ chemiluminescence kit (Sigma-Aldrich) using the AS06124A D1 antibody (Agrisera, Vännäs, Sweden). Quantification of D1 protein was carried out with FluorChem™ 8000 image analyzer (Alpha Innotech, San Leandro, CA, USA).

3. Results

3.1. Magnetic field protects against loss of PSII activity in intact leaves

Intact, detached pumpkin leaves were illuminated with strong light (PPFD 1500 µmol $m^{-2}s^{-1}$) in the presence and absence of a magnetic field of 170 mT. The treatment led to gradual loss of PSII activity (Fig. 1). After 6 h illumination in a magnetic field, 40 % of oxygen evolution activity remained, while in the absence of a magnetic field, only only 25 % of original PSII activity was retained (Fig. 1A). A similar protective effect of the magnetic field was seen also when photoinhibition was measured as decrease in F_V/F_M (Fig. 1B). The D1 protein content of the

leaves decreased by ~20 % during the 6-h treatments irrespective of the presence or absence of a magnetic field (data not shown).

3.2. Magnetic field does not affect the rate of photoinactivation

Repair of photoinhibited PSII occurs concurrently with photoinactivation, and therefore data in Fig. 1 do not tell whether the magnetic field protected PSII by slowing down photoinactivation or by enhancing repair. To solve this question, we illuminated pumpkin leaves that had been pre-treated with lincomycin, an inhibitor of chloroplast protein synthesis. In the presence of lincomycin, the light-saturated rate of oxygen evolution, measured from thylakoids isolated from treated leaves, decreased to 18 % of the control value in 6 h at the PPFD of 1500 µmol $m^{-2}s^{-1}$ both in the absence and presence of an external magnetic field (Fig. 2). Also loss of the D1 protein occurred similarly in the absence and presence of a magnetic field (Fig. 2). The disappearance of the clear protective effect of a magnetic field in the presence of lincomycin indicates that the magnetic field protects PSII by enhancing the repair of photoinhibited PSII, not by directly slowing down photoinactivation of PSII.

To confirm that the magnetic field does not directly affect the photoinactivation reaction, we used isolated pumpkin thylakoids and varied the magnetic field strength between zero and 350 mT. In these experiments, PPFD was either 900 or 1800 µmol $m^{-2}s^{-1}$. The results showed that photoinactivation occurred with similar kinetics irrespective of the strength of the magnetic field (Fig. 3). Also photoinhibition in zero magnetic field, obtained by compensating for Earth's magnetic field, occurred with a similar rate as photoinhibition in the higher magnetic fields (Fig. 3). These data confirm the conclusion that magnetic fields protect the repair reactions, as repair of photoinhibited PSII does not occur in isolated membranes.

Photoinhibition can be induced with short flashes, instead of using continuous light. The effect of magnetic field on flash-induced photoinhibition was of interest because $^1O_2$, produced as a side product of recombination reactions in PSII, has been suggested to be the causal agent of photodamage in flash-induced photoinhibition (Keren et al. 1997, 2000). We illuminated isolated thylakoids with 25 laser pulses at 10 s intervals (532 nm, 5-ns, 170 mJ cm^{-2}) in the presence and absence of an external magnetic field of 350 mT. In accordance with our data from thylakoids illuminated with strong continuous light, no magnetic field effect was observed, as the treatment led to 50 % inhibition of oxygen evolution both in the presence and absence of the magnetic field (Table 1).

3.3. Repair of photoinhibited PSII is impaired in a tocopherol-deficient mutant vte1

Magnetic fields are not expected to affect $^1O_2$ production by antenna Chls or other non-radical-pair sensitizers. To test the general importance of $^1O_2$ in photoinhibition, we measured photoinhibition in the tocopherol-deficient *vte1* mutant of Arabidopsis (Porfirova et al. 2002). Tocopherols are efficient $^1O_2$ scavengers in plants and algae, and the *vte1* mutant does not accumulate tocopherols under any conditions. When leaves of the *vte1* strain were illuminated at the PPFD of 1300 µmol $m^{-2}s^{-1}$, PSII activity was found to decrease more rapidly in *vte1* than in the wild type (Fig. 4A). However, when the illumination was done in the presence of lincomycin, photoinhibition proceeded similarly in the *vte1* mutant and in the wild type (Fig. 4B), further strengthening the conclusion that $^1O_2$ produced in the light interferes with repair of photoinhibited PSII but does not affect the rate of photoinactivation.

4. Discussion

Mechanisms of production and action of different reactive oxygen species often overlap, which makes it difficult to assign a specific cellular function to a particular molecule (Halliwell and Gutteridge 1998). Furthermore, piperidine derivatives used for detection of $^1O_2$ have serious side effects on PSII (Hakala-Yatkin and Tyystjärvi 2011). As radical pairs are the only plant constituents that respond to external magnetic fields, a magnetic field is an exceptionally specific tool to study the importance of the reactions of the photosynthetic radical pairs.

Participation of recombination reactions and $^1O_2$ in photoinactivation of PSII has earlier been suggested on the basis of indirect evidence (Fufezan et al. 2007, Hideg et al. 1994a, 1998, 2007, Keren et al. 1997, Vass and Aro 2008, Vass et al. 1992). The effect of a magnetic field that lowers the triplet yield of charge recombination is a crucial test for the importance of the recombination of the primary radical pair in photoinhibition. Our magnetic field data suggest that a significant part of $^1O_2$ produced by the photosynthetic machinery (Hideg et al. 1994a, 1998, 2001) originates in PSII recombination reactions. However, the lack of a protective effect of a magnetic field against photoinactivation *in vitro* (Fig. 3) and in the presence of lincomycin *in vivo* (Fig. 2) indicates that $^1O_2$ produced by the recombination reactions does not directly damage PSII. Therefore, photoinhibition mechanisms in which $^1O_2$ produced by the recombination reactions is the main cause of damage to PSII, need to be reconsidered. Photoinhibition mechanisms in which $^1O_2$ does not have an explicit inhibitory role (Anderson et al. 1998, Hakala et al. 2005, Ohnishi et al. 2005) are favored by the present data.

Adverse effects of reactive oxygen species can also be alleviated by compounds that scavenge or quench the already produced harmful molecule. α-tocopherol is an important $^1O_2$ scavenger (Fahrenholtz et al. 1974, Munné-Bosch and Alegre 2002), and experiments with the green alga

Chlamydomonas reinhardtii have shown that rapid loss of the D1 protein occurs during illumination when tocopherol biosynthesis is inhibited (Kruk et al. 2005, Trebst et al. 2002). Under severe conditions, also tocopherol-deficient Arabidopsis mutants have been found to be more sensitive to light stress than the wild type (Havaux et al. 2005, Porfirova et al. 2002). These results were originally interpreted to indicate that tocopherols slow down photoinactivation by scavenging $^1O_2$. However, in the cited experiments, the PSII repair cycle was allowed to run, and therefore it remained unclear whether the effect of tocopherol was to protect the repair of PSII or whether tocopherol slowed down the photoinactivation reaction. The repair cycle of PSII is highly sensitive to reactive oxygen species (see reviews by Allakhverdiev and Murata 2004, Murata et al. 2007, Nishiyama et al. 2005). Our results from the *vte1* mutant (Fig. 4) indicate that in Arabidopsis, α-tocopherol protects against the harmful effect of $^1O_2$ on the repair of photoinhibited PSII but does not affect photoinactivation of PSII. A similar result was recently obtained with a tocopherol-deficient strain of the cyanobacterium *Synechocystis* sp. PCC 6803 (Inoue et al. 2011). The adverse effect of $^1O_2$ on the repair of PSII in chloroplasts may be based on inhibition of translation elongation, as shown for cyanobacteria (Kojima et al. 2007, Nishiyama et al. 2001, 2004).

Recombination reactions occur inevitably during illumination in PSII. The triplet yield of the recombination of pairs $S_{2/3}Q_{A/B}^-$ and $P_{680}^+Q_A^-$ is relatively high because the spins of the recombination partners are not correlated. $^3P_{680}$ is frequently produced in continuous light because the $P_{680}^+Q_A^-$ recombines during the misses of the oxygen-evolving complex (Renger and Holzwarth 2005) and the miss probability in pumpkin thylakoids is approximately 9 % (Antal et al. 2009b). During illumination with single turnover flashes, $^3P_{680}$ is also produced by recombination of the pair $S_2Q_B^-$ with a half-time of 20-30 s (Keren et al. 1997). Data showing

that $^1O_2$ produced by continuous light does not directly cause photoinactivation of PSII may suggest that $^1O_2$, produced due to the rare $S_2Q_B^-$ recombination events, does not mediate inhibition of PSII during illumination by single turnover flashes (Table 1). The similarity of the light intensity response of photoinhibition induced with saturating Xenon flashes and with continuous light *in vitro* (Hakala et al. 2005) and *in vivo* (Sarvikas et al. 2010b) supports the conclusion that the slow recombination reactions do not cause photoinactivation of PSII.

Metabolic energy is required to counteract the adverse effects of $^1O_2$ in the chloroplast. A magnetic field is expected to lower these metabolic costs by lowering $^1O_2$ production. It is likely that the recently discovered stimulation of plant growth by magnetic fields (Flórez et al. 2004, 2007, Martínez et al. 2009) can be explained by decrease in the production of $^1O_2$ in chloroplasts. However, alternative explanations of the growth stimulation cannot be fully excluded, as magnetic field of ~0.5 mT has recently been shown to inhibit hypocotyl growth in Arabidopsis (Ahmad et al. 2007) due to an effect on a radical pair located in cryptochrome. Interestingly, migratory birds sense Earth's magnetic field with a radical pair mechanism in which cryptochrome is the sensor molecule (Rodgers and Hore 2009). Anyway, magnetic field effects in plants seem to require stronger fields than found in nature, suggesting that these phenomena are side effects of radical pair reactions (Hoff 1981, Katz et al. 1978, Rodgers and Hore 2009) rather than features that give a fitness advantage.

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Figure legends

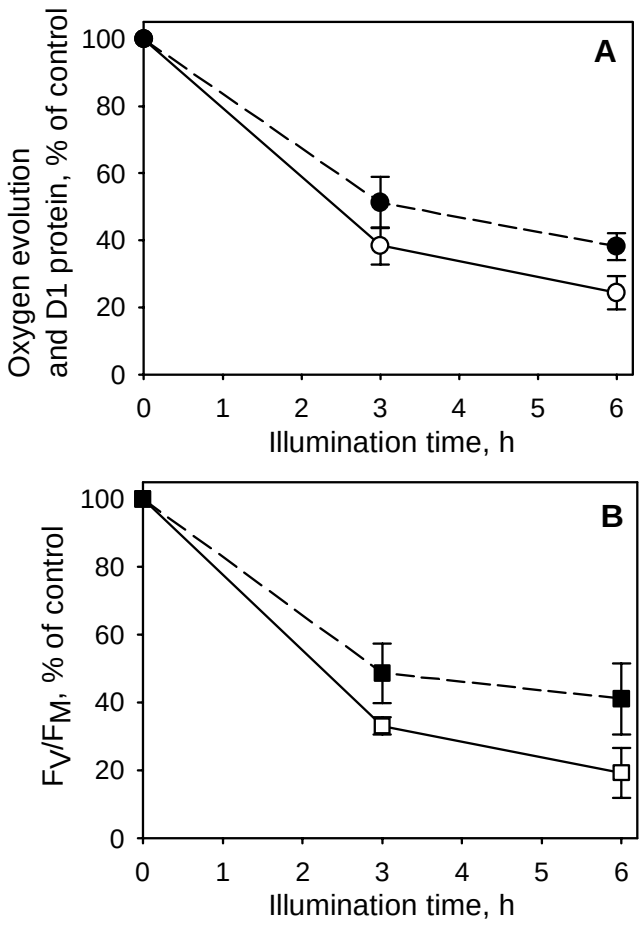
Fig. 1. Loss of oxygen evolution (A) and decrease in F_V/F_M (B) during illumination of intact pumpkin leaves at the PPFD of 1500 µmol m^{-2} s^{-1} in the absence (open symbols) and presence (solid symbols) of a magnetic field of 170 mT. The light-saturated rate of oxygen evolution (H_2O to 2,6-dichlorobenzoquinone) was measured from thylakoids isolated from control and treated leaves, and F_V/F_M was measured from the leaves. Each symbol represents an average of three independent experiments and the error bars denote SD. The control rate of oxygen evolution was 345 ± 19 µmol O_2 (mg Chl x h)$^{-1}$ and the F_V/F_M of the control leaves was 0.84 ± 0.01.

Fig. 2. Loss of oxygen evolution (circles) and D1 protein (triangles) during illumination of lincomycin-treated pumpkin leaves at PPFD 1500 µmol m^{-2} s^{-1} in the absence (open symbols, solid line) and presence (solid symbols, hatched line) of a magnetic field of 170 mT. Both oxygen evolution and D1 protein were measured from thylakoids isolated from control and treated leaves. For oxygen evolution, the lines represent the best fit to the first-order reaction equation. Each symbol represents the average of three independent experiments and the error bars denote SD.

Fig. 3. The rate constant of *in vitro* photoinhibition of isolated pumpkin thylakoids at the PPFD of 900 (hatched bars) or 1800 µmol m^{-2} s^{-1} (open bars) in different strengths of an external magnetic field. The zero field was obtained by compensating for the Earth's magnetic field. The rate constant k_{PI} was measured by fitting the loss of the light-saturated rate of oxygen evolution to the first-order reaction equation. Each bar represents an average of at least three independent experiments and the error bars denote SD. The control rate of oxygen evolution was 279 ± 17

μmol O_2 (mg Chl x h)$^{-1}$. Pairwise T-tests showed no significant difference between the k_{PI} values measured at the same PPFD in the presence or absence of an external magnetic field.

Fig. 4. Loss of oxygen evolution during illumination of leaves of wild type Arabidopsis (open circles, solid line) and leaves of the *vte1* mutant (solid symbols, hatched line) at the PPFD of 1300 μmol m^{-2} s^{-1} in the absence (A) and presence (B) of lincomycin. Oxygen evolution was measured from thylakoids isolated from control and treated leaves. The lines in (B) represent the best fit to the first-order reaction equation. Each symbol represents an average of at least three independent experiments and the bars, shown if larger than the symbol, denote SD. The control rate of oxygen evolution was 143 ± 14 μmol O_2 (mg Chl x h)$^{-1}$.


A
Oxygen evolution
and D1 protein, % of control
100
80
60
40
20
0
0
1
2
3
4
5
6
Illumination time, h
B
F_V/F_M, % of control
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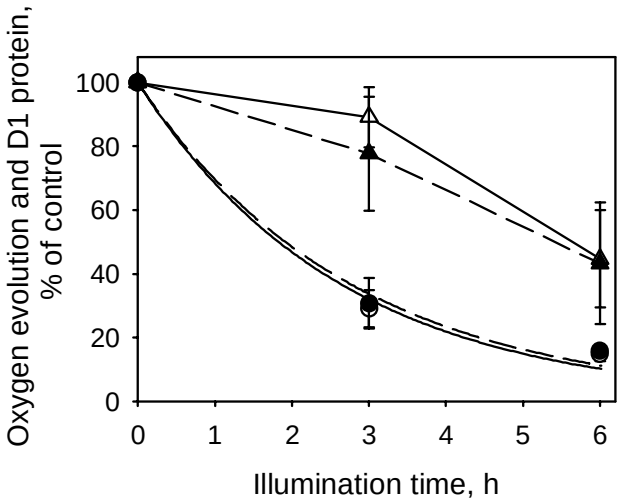
Oxygen evolution and D1 protein, % of control
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20
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0
1
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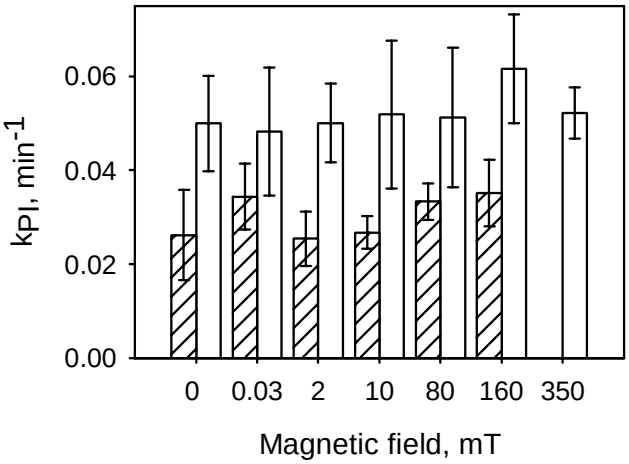
6
Illumination time, h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0.03
2
10
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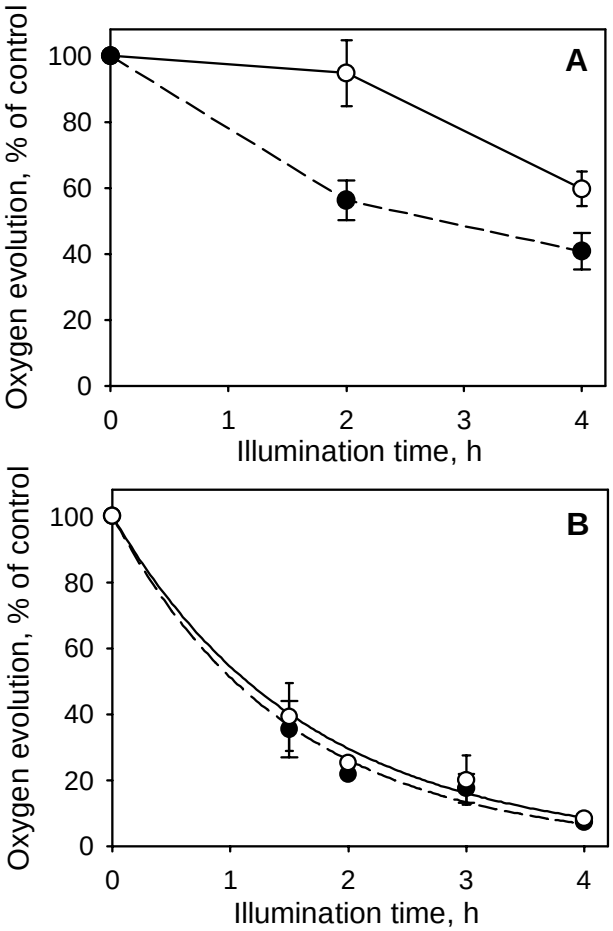
350
Magnetic field, mT


A
B
Oxygen evolution, % of control
Illumination time, h
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20
40
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80
100
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1
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4