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Visualising the mobility and distribution of chlorophyll-proteins in higher plant thylakoid membranes: effects of photoinhibition and protein phosphorylation

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SUMMARY

Diffusion of proteins in chloroplast thylakoid membranes is believed to be important for processes including the Photosystem II repair cycle and regulation of light harvesting. However, to date there is very little direct information on the mobility of thylakoid proteins. We have used Fluorescence Recovery after Photobleaching in a laser-scanning confocal microscope to visualize in real time the exchange of chlorophyll-proteins between grana in intact spinach and *Arabidopsis* chloroplasts. Most chlorophyll-proteins in the grana appear immobile on the 10-minute timescale of our measurements. However, a limited population of chlorophyll proteins (accounting for around 15% of chlorophyll fluorescence) can exchange between grana on this timescale. In intact, wild-type chloroplasts this mobile population increases significantly after photoinhibition, consistent with a role for protein diffusion in the Photosystem II repair cycle. No such increase in mobility is seen in isolated grana membranes, or in the *Arabidopsis stn8* and *stn7 stn8* mutants, which lack protein kinases required for phosphorylation of Photosystem II core proteins and light-harvesting complexes. Furthermore, mobility under low-light conditions is significantly lower in *stn8* and *stn7 stn8* than in wild-type *Arabidopsis.* The changes in protein mobility correlate with changes in the packing density and size of thylakoid protein complexes as observed by freeze-fracture electron microscopy. We conclude that protein phosphorylation switches the membrane system to a more fluid state, thus facilitating the Photosystem II repair cycle.

INTRODUCTION

Thylakoid membranes are densely packed with proteins. For example, the protein complexes occupy about 80% of the membrane area of grana thylakoid membranes

(Kirchhoff et al., 2002; 2008b). This dense macromolecular crowding may cause problems. Protein diffusion is crucial for the function of most biological membranes, but the dense packing of thylakoids must lead to a severe reduction in the mobility of protein complexes (Kirchhoff et al., 2004). Nevertheless it is clear that some proteins in the grana must be mobile under some conditions. Two well-characterised examples are the redistribution of LHCII light-harvesting complexes during state transitions (Allen and Forsberg, 2001), and migration of Photosystem II (PSII) reaction centres as part of the PSII repair cycle (Tikkanen et al., 2008). In both cases, protein phosphorylation has been implicated in triggering the redistribution of complexes (Allen and Forsberg, 2001; Tikkanen et al., 2008). Fractionation of thylakoid membranes into grana and stroma lamellae shows redistribution of complexes between the two fractions, over a timescale of a few minutes (Drepper et al., 1993). However, this biochemical approach does not show how far the complexes migrate. Some authors have argued that LHCII migrates only a short distance during state transitions, between the grana and the "grana margins", without ever moving into the stroma lamellae (Allen and Forsberg, 2001). The biochemical approach also gives only a crude indication of the timescale on which protein movements occur, and it does not distinguish between two possibilities for the relationship between grana structure and protein movement:

a. A sub-population of proteins can diffuse in and out of the grana, which are relatively stable structures.
b. Protein escape from the grana membranes requires the partial or complete disassembly of a proportion of the grana.

Freeze-fracture electron microscopy has revealed considerable detail of the distribution of chlorophyll-protein complexes in thylakoid membranes (reviewed by Staehelin, 2003) and shown quantitative differences in the supramolecular organisation of chlorophyll-protein complexes resulting from state transitions (Staehelin and Arntzen, 1983). Electron tomography also suggests large-scale structural changes in grana during state transitions (Chuartzmann et al., 2008). However, electron microscopic techniques can only give part of the story, since the preparation required (fixing and/or freezing) obviously prevents the dynamic tracking of protein movements and membrane re-arrangement. Techniques based on fluorescence microscopy offer the best hope of resolving these issues by tracking the movement of protein complexes in real time, albeit with much lower spattial resolution than that achieved with electron microscopy. Fluorescence microscopy in thylakoid membranes is facilitated by the natural fluorescence of the chlorophylls, which allows the distribution of some protein complexes to be visualised without the need for artificial fluorescent tagging (Mullineaux and Sarcina, 2002). Fluorescent tagging with Green Fluorescent Protein (GFP) or fluorescent antibodies is likely to perturb the system, particularly in the grana where the tight appression of the membranes will almost certainly exclude any bulky fluorescent tags (Consoli et al., 2005).

Fluorescence Recovery after Photobleaching (FRAP) has been used to probe the mobility of protein complexes in some photosynthetic membranes (Mullineaux et al., 1997; Mullineaux and Sarcina, 2002). In favourable cases, FRAP is fully quantitative and can be used to measure the diffusion coefficients of photosynthetic complexes. However, this requires a simple, predictable membrane topography, and a membrane that can be assumed

to be homogeneous over a distance of a micron or more. This is the case with the thylakoid membranes of some cyanobacteria (Mullineaux et al., 1997; Mullineaux and Sarcina, 2002). Green plant thylakoid membranes are much more difficult systems for FRAP due to their lateral heterogeneity and their complex three-dimensional architecture, which is still not fully-understood (Garab and Manella, 2008). Kirchhoff et al. recently used FRAP to measure the mobility of chlorophyll-protein complexes in isolated grana membranes from spinach, taking advantage of the tendency of these membranes to fuse laterally into larger membrane patches *in vitro* (Kirchhoff et al., 2008a). There most of the chlorophyll-proteins are completely immobile, at least on timescales of a few minutes. However, a sub-population accounting for about 25% of chlorophyll fluorescence is able to diffuse surprisingly fast (diffusion coefficient D ~ 0.005 $\mu m^2 s^{-1}$). It was suggested that this mobile sub-population might be able to exchange rapidly between the grana and stroma lamellae *in vivo* (Kirchhoff et al., 2008a).

Here we report the extension of the FRAP approach to probe the mobility of chlorophyll proteins in a more physiologically-relevant system: the thylakoid membranes of intact spinach and *Arabidopsis* chloroplasts. Due to the complex membrane architecture, we cannot measure diffusion coefficients in this system. However, we can accurately measure the "mobile fraction": in this case the proportion of chlorophyll fluorescence that can exchange *between* grana, due to chlorophyll-proteins escaping from one granum and diffusing through the stromal lamellae to another granum. This leads to recovery of fluorescence in a bleached granum. We use a range of control measurements to show that this fluorescence recovery is due to protein diffusion. Our technique provides a new tool for the direct measurement of the dynamics of plant thylakoid membranes. We show that

photoinhibition increases the mobility of chlorophyll proteins, suggesting that mobilisation of these proteins is important for the repair cycle. We use *Arabidopsis* mutants lacking the STN7 and STN8 protein kinases (Bonardi et al., 2005) to show that mobilisation requires these proteins. Freeze-fracture electron micrographs indicate that changes in protein mobility within the thylakoid system correlate with changes in the packing density and size of the complexes.

RESULTS

Visualization of chloroplast intactness

We set out to measure the mobility of chlorophyll-proteins in chloroplasts with the outer envelope intact, in order to ensure that our results were as physiologically relevant as possible. We tried several methods for isolating intact chloroplasts, including the use of centrifugation on a Percoll cushion to separate intact and broken organelles (Napier and Barnes, 1995). However, we could never obtain 100% intactness in the preparation. Since our confocal FRAP measurements are done on individual chloroplasts it is important to assess whether the chloroplast under examination is broken or intact. We found that this could be achieved by staining the preparation with the green lipophilic fluorophore BOPIDY FL C_{12}, previously used to stain membranes in the cells of cyanobacteria (Sarcina et al., 2003). In intact chloroplasts the dye stains only the chloroplast envelope and does not enter the chloroplast interior: it is visualized as a continuous halo of green fluorescence surrounding the red chlorophyll fluorescence from the thylakoid membranes (Fig. 1). In broken chloroplasts the distribution of green fluorescence appears very different: there is only fragmentary staining outside the thylakoid membranes and considerable staining of the

thylakoid membranes themselves (Fig. 1). We used this method to select intact chloroplasts for our FRAP experiments.

FRAP measurements in isolated intact chloroplasts: optimisation and controls

For FRAP it is important to immobilise the sample to ensure that any fluorescence recovery is due to diffusion within the sample rather than movement of the sample as a whole. Slides coated with polylysine are often used to immobilise bacterial cells (e.g. Lenn et al., 2008) and we found this method to be effective with intact chloroplasts. Most chloroplasts adhered to the polylysine-coated slide and remained immobile during the experiment. Presumably the negatively-charged outer membrane (Stocking and Francheschi, 1982) interacts electrostatically with the positively-charged polylysine film. In this system there is no direct interaction between the thylakoid membranes and the support, and thus no danger that such interactions might perturb membrane conformation and the mobility of membrane proteins.

For FRAP measurements we first selected intact, immobilized chloroplasts as described above, and then visualised chlorophyll fluorescence by scanning in two dimensions (see Experimental Procedures). At the imaging laser intensity, chlorophyll fluorescence was stable: repeated scans across the same chloroplast did not lead to detectable photobleaching (not shown). The grana within the chloroplasts appeared as bright fluorescent spots (dark spots in the inverted images shown in Figs. 2-4). For our measurements it was important to resolve the fluorescence from individual grana as cleanly as possible. Because the grana are tightly packed together, this is problematic at optical

resolution. We used a routine deconvolution procedure taking into account the measured point-spread function (see Experimental Procedures) to improve the resolution of individual grana. To photobleach a region of the chloroplast we increased the laser power by a factor of 32 and then scanned the confocal laser spot repeatedly in one dimension across the sample for 5-7 s. This bleached a line across the chloroplast which could be visualised by subsequent imaging at lower laser power (Figs 2-4). Under some conditions we could then observe a partial recovery of fluorescence in the bleached zone over a timescale of a few minutes (Fig. 4).

We needed to establish whether or not this fluorescence recovery was due to the diffusion of chlorophyll protein complexes into the bleached area. The other possibility would be some form of reversible fluorescence quenching allowing fluorescence recovery in the bleached area without any movement of complexes. In geometrically simpler systems (for example isolated, laterally fused grana membranes and the quasi-cylindrical thylakoid membranes of some cyanobacteria) fluorescence redistribution can clearly be visualised. Recovery of fluorescence in the bleached area is matched by loss of fluorescence in the neighbouring regions of the membrane, leading to a characteristic "blurring" of the bleached line which is a clear indication of diffusion (Mullineaux et al. 1997; Kirchhoff et al., 2008a). However, the situation is more complex in intact chloroplasts due to the lateral heterogeneity and intricate geometry of the membrane, combined with the small extent of fluorescence recovery. To test the possibility of reversible fluorescence quenching we bleached entire chloroplasts, rather than just a line across the chloroplast (Fig. 2a). If recovery were due to reversible fluorescence quenching,

we would still expect to observe it in this experiment. If recovery were to diffusion we would expect not to see it, since bleaching the entire chloroplast removes the pool of unbleached complexes whose diffusion into the bleached area causes the recovery. Fluorescence recovery was very slight in this experiment (Fig. 2a). This is strong evidence against any significant reversible fluorescence quenching under our conditions. As a second control we used treatment with glutaraldehyde, a very effective protein cross-linker (Habeeb and Hiramoto, 1968). If fluorescence recovery were due to protein diffusion we would expect it to be strongly inhibited by glutaraldehyde treatment, and indeed we observed very little fluorescence recovery in glutaraldehyde-treated chloroplasts (Fig. 2b). Both the controls shown in Fig. 2 suggest that the fluorescence recovery we observe is due to protein diffusion, as it is in isolated grana membranes (Kirchhoff et al., 2008a).

For our purposes it is also important to assess the effect of the bleach on the grana membrane structure. To do this we carried out FRAP measurements on broken chloroplasts in which the thylakoid membrane system was stained with BODIPY FL C_{12} as in Figure 1. Following the bleach, fluorescence recovery was observed simultaneously in the red channel (monitoring chlorophyll fluorescence) and the green channel (monitoring BODIPY fluorescence) (Fig. 3). Recovery of chlorophyll fluorescence in the bleached granum is slow and incomplete, however there is complete recovery of BODIPY fluorescence on a timescale of a few minutes (Fig. 3). This indicates relatively rapid lipid mobility within the thylakoid membrane system, and it indicates that the basic structure of the granum is not destroyed by photobleaching.

Mobility of chlorophyll-proteins in intact spinach chloroplasts

Fig. 4a shows an example of a FRAP measurement in an intact spinach chloroplast. After 8 minutes, the bleached line remains clearly visible, however there is a partial recovery of fluorescence which we attribute to long-range diffusion of chlorophyll proteins within the thylakoid membrane system. The fluorescence recovery curve shown in Fig. 4c is obtained by selecting a region of interest in the images corresponding roughly to one individual granum whose fluorescence was strongly decreased during the bleach. This particular granum shows about 17% fluorescence recovery over about 10 minutes (Fig. 4c). Thus the mobile fraction of chlorophyll fluorescence is small, but measurable and fairly consistent under these conditions. There is some variation between measurements, but the mean mobile fraction from measurements on 10 individual chloroplasts is about 13% (Table 1). The controls described above and shown in Figs. 2 and 3 indicate that this partial fluorescence recovery is due to protein diffusion. We considered the possibility that this diffusion might be "vertical" movement between the different membrane layers of the granum, which may be interconnected (Shimoni et al., 2005). However, the limited resolution of our measurements in the X,Y and Z directions (see Experimental Procedures) will ensure that the full depth of the granum is bleached. It also means that any "vertical" movement within the granum will not change the fluorescence signal observed. This means that our measurements do not report on "vertical" diffusion within a granum: we are not able to assess whether such protein movements occur. The diffusion we observe must be due to exchange between grana, with complexes escaping from one granum, diffusing through the stroma lamellae and entering another granum. Our results indicate that a limited pool of chlorophyll-protein complexes is able to exchange between grana on a timescale of a few minutes.

Effect of photoinhibition on the mobility of chlorophyll-proteins in spinach chloroplasts

Most models for the PSII repair cycle involve the migration of photodamaged PSII reaction centres from the grana to the stroma lamellae for repair (Aro et al., 1993; Tikkanen et al., 2008). Therefore photoinhibition might be expected to result in an increase in the mobility of chlorophyll-protein complexes within the thylakoid membrane system. To test this possibility we carried out FRAP measurements on photoinhibited spinach chloroplasts (Fig. 4b). Intact chloroplasts were photoinhibited in suspension by illuminating for 10-15 minutes with actinic light at ~ 3000 µmol photons m^{-2} s^{-1}. During photoinhibition chlorophyll fluorescence was monitored with a PAM fluorometer (Fig. 5). The treatment resulted in a decrease of about 50% in the maximal yield of fluorescence with closed PSII reaction centres (F_m) which was not reversed even after 2 hours (Figure S1). Photoinhibited chloroplasts were immediately adhered to polylysine-coated slides and FRAP measurements carried out as described above. Chlorophyll fluorescence recovery was faster and more complete in photoinhibited chloroplasts (Fig. 4b,d) as compared to chloroplasts that had not been photoinhibited (Fig. 4 a,c). The mean mobile fraction of chlorophyll fluorescence in photoinhibited chloroplasts was significantly increased, as compared to chloroplasts that had not been photoinhibited (Table 1).

FRAP measurements in intact spinach chloroplasts in the presence of an uncoupler

As an additional control for our FRAP measurements we repeated the measurements in the presence of nigericin, an uncoupler which effectively prevents the formation of a

transmembrane pH difference (ΔpH). ΔpH is an indispensable trigger for the induction of qE, a reversible quenching mechanism that converts excess excitation energy to heat (Horton et al., 1996; Ruban et al. 2007). PAM fluorometer measurements (not shown) confirmed the absence of qE in the presence of nigericin. Mobile fractions calculated from this control did not differ significantly when compared to the corresponding measurements on chloroplasts where nigericin was not added (Table 1). As with chloroplasts in the absence of nigericin (Fig. 4, Table 1) we observed a significant increase in the mobile fraction in chloroplasts photoinhibited in the presence of nigericin (Table 1). These measurements confirm that changes in the extent of qE are not involved in the fluorescence bleaching and recovery that we observe in our FRAP measurements. They also show that the increase in mobility of chlorophyll-proteins that we observe following photoinhibition (Fig. 4, Table 1) is not dependent on the induction of qE.

Effect of photoinhibition on mobility of chlorophyll-proteins in isolated grana membranes

For comparison with our results on intact chloroplasts we examined the effect of photoinhibition on the mobility of chlorophyll-proteins in isolated grana membranes from spinach. These experiments used the system previously described in which isolated grana membranes are adsorbed onto an artificial lipid bilayer support. Membrane fragments tend to fuse together forming patches large enough for quantitative FRAP measurements of mobile fraction and diffusion coefficient (Kirchhoff et al., 2008a). As previously reported (Kirchhoff et al., 2008a), grana membranes show a mobile fraction of chlorophyll fluorescence of 28% ± 3% (SE), much higher than we observe in intact chloroplasts.

Photoinhibition of the grana membranes was carried out in the same way as described above for intact chloroplasts, with similar effects on chlorophyll fluorescence as monitored by pulsed-amplitude modulation (PAM) fluorometry (not shown). However, in contrast to the result in intact chloroplasts, we could detect no significant increase in the mobility of chlorophyll-proteins induced by photoinhibition in isolated grana membranes (Table 1).

Mobilization of chlorophyll proteins under photoinhibitory conditions requires protein kinases

Upon photoinhibition, the PSII core proteins, in particular the D1 polypeptide, undergo a rapid light-induced phosphorylation cycle which is connected to the regulation of PSII protein turnover and the repair of the damaged proteins (Rintamäki et al., 1996). The *Arabidopsis thaliana stn7* and *stn8* mutants lack protein kinases required for the phosphorylation of thylakoid membrane proteins (Bonardi et al. 2005). The STN8 protein kinase appears to be primarily responsible for phosphorylation of PSII core proteins (Bonardi et al., 2005) however the *stn7 stn8* double mutant shows a more complete loss of capacity for PSII phosphorylation at high light intensities (Tikkanen et al., 2008). As a test for the involvement of PSII phosphorylation in the mobilization of chlorophyll-proteins after photoinhibition, we carried out FRAP measurements on intact chloroplasts from *Arabidopsis* wild-type (Col-0) , *stn8* and *stn7 stn8* mutants. Results obtained for wild-type *Arabidopsis* chloroplasts were comparable to those from spinach. Photoinhibition resulted in a small but significant increase in the mobile fraction of chlorophyll fluorescence (Fig. 6). However, in the *stn8* and *stn7 stn8* mutants, mobility of chlorophyll proteins was

significantly lower than in the wild-type and there was no increase in the mobile fraction following photoinhibition (Fig. 6).

Correlation of protein mobility with supramolecular organisation

We obtained freeze-fracture electron micrographs from intact spinach chloroplasts either dark-adapted or photoinhibited prior to freezing (Fig. 7). There were no dramatic changes in PSII organisation as a result of photoinhibition (Fig. 7) but quantitative analysis of the images indicates that photoinhibition results in a significant decrease in the density of PSII particles in the granal regions, with a concomitant increase in the mean distance between particles (Fig. 8). Photoinhibition also induced a small but significant decrease in the mean size of granal PSII particles. Mean PSII dimensions in dark-adapted samples were (16.0 ± 2.4) x (10.8 ± 2.0) nm, decreasing to (14.2 ± 2.6) x (9.2 ± 1.8) nm in photoinhibited samples (± SD, $P \leq 0.0002$ from a T-test).

DISCUSSION

Here we show that confocal microscopy and FRAP can be used to probe the mobility of chlorophyll-protein complexes in higher plant thylakoid membranes. We visualise the proteins using the native fluorescence from the chlorophylls. This has the advantage that we are not perturbing the membrane structure. It may be the only way that we can track proteins through the grana, where the tight appression of the membranes (Dekker and Boekema, 2005) is likely to exclude extrinsic fluorescent tags such as GFP and antibody-linked fluorophores. At the same time, the approach brings some difficulties:

a. We have no direct way to distinguish the different chlorophyll-protein complexes (PSII, LHCII etc) which puts obvious limits on the information we can obtain.

b. Chlorophyll fluorescence yield is influenced by a complex set of quenching mechanisms including, for example, photochemical quenching by the reaction centres and various mechanisms that dissipate excitation energy as heat (Maxwell and Johnson, 2000). We have to control carefully to make sure that any fluorescence recovery we observe is genuinely the result of protein diffusion rather than recovery from some reversible quenching process.

c. Problems are caused by the complex three-dimensional structure and lateral heterogeneity of higher-plant thylakoid membranes. This prevents us from quantifying diffusion coefficients as can be done in cyanobacteria (Mullineaux et al., 1997; Mullineaux and Sarcina, 2002) and in isolated, laterally fused grana membranes (Kirchhoff et al., 2008a). It also makes it harder to check that fluorescence changes are due to diffusion. In a simple, homogeneous membrane, diffusion is easily recognisable because of the characteristic redistribution of fluorescence. This is much harder to check in laterally-segregated thylakoid membranes, where the fluorescence distribution is very inhomogeneous.

Despite these problems, we are confident that our FRAP measurements do reveal protein movements within the thylakoid membrane system, since the controls shown in Figs 2 and 3 and Table 1 eliminate the other possibilities.

Obviously a FRAP measurement is somewhat disruptive. Bleaching out fluorescence in a region of the membrane will significantly perturb the processes occurring there. This is particularly true of a photosynthetic membrane, where bleaching chlorophyll fluorescence means destroying the function of photosynthetic proteins. However it must be remembered that the bleach is very localised. Although we damage function in the bleached region, we do not do any damage in the remainder of the chloroplast. The line bleach used in our experiments generally bleaches out a single granum, while leaving neighbouring grana unaffected. If we see fluorescence recovery in the bleached area, it indicates that chlorophyll-proteins must be able to diffuse within the neighbouring, undamaged regions of the membrane, and escape from unbleached grana.

Our results on intact spinach chloroplasts show a partial fluorescence recovery in bleached grana. Although we cannot quantify the diffusion coefficients within this complex system, we can quantify the mobile fraction. There is variation from chloroplast to chloroplast, but on average about 13% of chlorophyll fluorescence is mobile (Table 1). The simplest explanation for our observation is that a relatively small proportion of granal chlorophyll-proteins are mobile to the extent that they are not only able to diffuse *within* the appressed membrane region of a single granum, but they can also escape from the granum, diffuse through the connecting stroma lamellae, and enter the appressed membranes of

another granum. Thus, a limited fraction of chlorophyll proteins are relatively loosely associated with the grana, and are able to exchange between grana on a timescale of a few minutes. This result can be compared to our previous result in isolated spinach grana membranes, where we showed that a fraction of chlorophyll-proteins accounting for about 25% of chlorophyll fluorescence is mobile within the grana membrane (Kirchhoff et al., 2008a). The remainder of the chlorophyll-proteins appear completely immobile. Our current results extend this finding to a much more intact and physiologically-relevant system, suggesting that some of the chlorophyll-proteins that are mobile within the grana can also readily migrate into the stroma lamellae. It is clear that the grana and the stroma lamellae are part of a continuous membrane system, although the precise three-dimensional architecture of the system and the nature of the connections between the granal membranes and the stroma lamellae remain controversial (Shimoni et al., 2005; Mustárdy et al., 2008; Garab and Manella, 2008). Our results confirm that protein diffusion through these connections is possible. Once in the stroma lamellae diffusion is likely to be relatively rapid. There is one report using single-particle tracking for direct visualization of antibody-labelled LHCII (Consoli et al., 2005). Given the large size of the fluorescent tag used, the LHCII visualized would have been excluded from appressed grana membranes, and was probably in the stroma lamellae. The tagged LHCII exhibited a random walk confined to a limited membrane area, with a mean diffusion coefficient of about 0.008 $\mu m^2 s^{-1}$, rising to about 0.027 $\mu m^2 s^{-1}$ for phospho-LHCII (Consoli et al., 2005). Our results confirm that there is at least some long-range diffusion within the thylakoid membrane system. Thus models for state transitions and PSII repair which involve the migration of LHCII and PSII

core complexes out of the grana and into the bulk phase of the stroma lamellae are plausible.

Our experiments indicate that grana are relatively stable structures *in vivo*. Even after considerable photodamage (photobleaching an entire granum) some chlorophyll-proteins diffuse back into the same region of the sample, and there is rapid and complete diffusion of a lipophilic fluorophore back into the granum (Fig. 3). This indicates that the location of the granum does not change. High-resolution studies using cryo-electron tomography suggest considerable effects of illumination and adaptation on grana structure (Chuartzmann et al, 2008). Such changes would probably not be detectable at optical resolution. However, our studies indicate that grana remain in place - they do not totally disintegrate or reform even after drastic light exposure.

To further investigate the mobility of chlorophyll-proteins in intact chloroplasts, we measured the effect of a pre-illumination to induce photoinhibition. Such a treatment will initiate the PSII repair cycle, whose operation is essential for maintaining efficient photosynthesis under most conditions (Long et al., 1994). Most models for the PSII repair cycle involve the migration of photodamaged PSII complexes out of the grana and into the stroma lamellae for repair (Aro et al., 1993; Baena-Gonzalez and Aro, 2002). Thus we might expect a photoinhibitory pre-treatment to mobilize the thylakoid membrane system, causing an increase in the mobile fraction in our measurements. We found this to be the case: a photoinhibitory pre-illumination significantly increases the mean mobile fraction from about 13% to about 18%. This suggests that additional population of chlorophyll-

proteins is able to escape from the grana under these conditions. A simple interpretation would be that the mobile fraction under low light conditions consists of a sub-population of LHCII (Drepper et al., 1993; Allen and Forsberg, 2001). After photoinhibition, a proportion of PSII complexes may become mobile as well. The result may be compared to previous findings in a cyanobacterium, where pre-illumination with bright red light results in the mobilization of up to about 50% of chlorophyll fluorescence (Sarcina et al., 2006). In the cyanobacterium we can be confident that the mobilized fraction does consist of PSII core complexes, since these contribute most of the chlorophyll fluorescence (Sarcina et al., 2006).

In contrast to our finding with intact chloroplasts, we found that photoinhibitory pre-illumination has no effect on the mobility of chlorophyll-proteins in isolated grana membranes (Table 1). There are several possible explanations for this discrepancy. Mobilisation after photoinhibition may require some stromal factor which is absent from the isolated grana membrane preparation. Alternatively, mobilisation may only be possible if there is adjacent membrane space available in the stroma lamellae. This idea would envisage a progressive increase in diffusion space in the grana as complexes escape into the stroma lamellae, starting with the complexes closest to the grana-stroma lamellae connections. Obviously this would not occur in the isolated grana membranes. Finally, there might be a diffusion barrier or "gatekeeper" structure at the grana-stroma lamellae junctions. In this case, movement of proteins between grana and the stroma lamellae, and exchange between grana, would not directly related to mobility *within* the appressed grana membrane. Again, this idea would explain why we saw no photoinhibition-induced

mobilisation of complexes in isolated grana membranes. The mobile fraction of chlorophyll fluorescence *within* isolated grana membranes is considerably higher than the fraction that diffuses *between* grana in intact thylakoids (Table 1). This might suggest a partial barrier to exchange between the grana and the stroma lamellae, however it must be considered that the forces acting on complexes in isolated grana membranes could be different from those in the intact system. Freeze-fracture electron micrographs (Fig. 7) provide some clues to the reasons for the increased protein mobility in photoinhibited thylakoids. We could detect no drastic changes in PSII organisation within the grana, suggesting that PSII mobilisation is not a consequence of changes in the large-scale supramolecular interactions. Photoinhibition causes a small but significant decrease in the mean size of PSII complexes in the grana (perhaps due to loss of part of the light-harvesting antenna) and it significantly increases the mean distance between complexes. This suggests loss of PSII complexes from the grana (presumably due to escape to the stroma lamellae). Progressive loss of PSII complexes from the grana would make the system more fluid: studies on isolated grana membranes show that protein mobility is increased in a more "dilute" system (Kirchhoff et al., 2008a).

We used *Arabidopsis* mutants to further explore the factors required for mobilization of chlorophyll-protein complexes after photoinhibition. *Arabidopsis* mutant studies have shown that STN7 and STN8 protein kinases are required for phosphorylation of PSII components (PSII core proteins and LHCII) (Bonardi et al., 2005). Protein phosphorylation by STN7 and STN8 is not absolutely required for the PSII repair cycle (Bonardi et al., 2005). However the dynamics of the repair cycle are impaired in the

absence of these proteins and this effect seems to be related to impairment of the disassembly of PSII supercomplexes (Tikkanen et al., 2008). Therefore it was suggested that PSII phosphorylation enables the disassembly of PSII supercomplexes, facilitating the migration of damaged PSII complexes into the stroma lamellae for repair (Tikkanen et al., 2008). Fristedt et al. (2009) further propose that protein migration in the *stn7stn8* mutant is impaired by an increase in the diameter of the grana discs. Our results on the *Arabidopsis stn8* and *stn7 stn8* mutants (Fig. 6) are consistent with these models. Firstly, we find that the mobility of chlorophyll-proteins in intact chloroplasts is lower in the *stn8* and *stn7 stn8* mutants than in the wild-type. In both mutants, the mobile fraction of chlorophyll fluorescence is significantly lower than in the wild-type (Fig. 6). Secondly, we find that in *stn8* and *stn7 stn8* there is no increase in the mobile fraction following photoinhibition (Fig. 6), in contrast to the effect seen in wild-type *Arabidopsis* (Fig. 6) and spinach (Fig. 4, Table 1). This provides direct evidence that PSII phosphorylation facilitates the exchange of chlorophyll-proteins between the grana and the stroma lamellae. Phosphorylation switches the thylakoid membrane system to a more fluid state.

EXPERIMENTAL PROCEDURES

Plant material

Spinach leaves (*Spinacia oleracea*) (L.) were purchased fresh from a local supermarket and kept overnight at 4 °C in the dark prior to use. WT *Arabidopsis thaliana* (L.) ecotype Columbia (Col-0), the *stn8* and the *stn7 stn8* mutant plants were grown in a Conviron plant growth room with an 8-h photoperiod at a light intensity of 200 μmol photons m^{-2} s^{-1} and a

day/night temperature of 22/18 °C, respectively. Mature rosette leaves from 10 – 12 weeks old plants were dark adapted for 30 min prior to use for experiments.

Isolation of intact chloroplasts and grana membranes

Intact chloroplasts were isolated from spinach and *Arabidopsis* leaves using a modification of the procedure described by Crouchman et al. (2006). Fresh, dark-adapted leaves were homogenized in ice-cold grinding buffer (450mM sorbitol, 20 mM Tricine, 10mM EDTA, 10 mM $NaHCO_3$, 5 mM $MgCl_2$ and 0.1% BSA at pH 8.4) with a Polytron (Kinematica GmbH). The homogenate was then filtered through four layers of muslin followed by four layers of muslin and one layer of cotton wool. The filtrate was centrifuged for 30 s at 4000 × g and 4 °C. The chloroplast-enriched pellet was then washed twice and finally resuspended with a small volume of the buffer containing 300 mM sorbitol, 20 mM Tricine, 5 mM $MgCl_2$ and 2.5 mM EDTA, pH 7.6 and put on ice until use. The washing step was carried out with the resuspension medium. Chlorophyll concentration was determined according to Porra et al. (1989). Isolated grana membranes were prepared from spinach leaves following the procedure described previously (Kirchhoff et al., 2008a).

Control experiments with glutaraldehyde and nigericin

For the glutaraldehyde control, chloroplasts were resuspended in resuspending buffer containing 2% glutaraldehyde (Agar Scientific Ltd.) and incubated for 30 min at 4 °C in the dark followed by centrifugation (60 s at 5000 g). The clean pellet was then resuspended in the resuspending buffer and chlorophyll content was measured. For the nigericin control, 4

μM nigericin (Sigma) was added to the chloroplast suspension at a chlorophyll concentration of 10 μg.ml^{-1} prior to FRAP measurements.

Photoinhibitory treatment of intact chloroplasts and isolated grana membranes

Photoinhibition in intact chloroplasts and grana membranes was induced by high light exposure and monitored by PAM fluorescence measurements (Walz-101 PAM fluorometer) as presented in Fig 5. In brief, the chloroplast suspension or grana membranes (at a chlorophyll concentration of 10 μg ml^{-1} and 40 μg ml^{-1}, respectively) was illuminated with a high light (3000 μmol photons m^{-2} s^{-1}) for approximately 10 – 15 min at room temperature following saturating light pulses at 30-s intervals for about 5 min. Prior to illumination, 25 μM (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (DCMU) (Sigma) was added to eliminate the photochemical contribution to fluorescence quenching. The decrease in F_v/F_m after photoinhibitory treatment was more than 50% and did not recover significantly even after two hours (Figure S1). Photoinhibited chloroplasts and grana membranes were immediately used for FRAP measurements.

Sample preparation for FRAP

Prior to experiments, chloroplast suspensions were diluted in resuspension buffer containing 5 μM 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (BODIPY FL C_{12}; Invitrogen) to a final chlorophyll concentration of 10 μg ml^{-1}. A glass slide was sealed with a cover-slip using vacuum grease so as to form a flow chamber. 60 μl of a 0.5% aqueous solution of polylysine (Sigma) were applied to the chamber, washed with the resuspension buffer followed by application of 60 μl of the chloroplast

suspension. After 5 min incubation the chloroplasts that were not immobilised were washed out with resuspension buffer. Isolated grana membranes were immobilised by adsorption onto an artificial lipid bilayer as described previously (Kirchhoff et al., 2008a).

FRAP measurements

FRAP measurements were carried out with a Nikon PCM2000 laser-scanning confocal microscope as previously described (Kirchhoff et al., 2008a) using a 60x oil-immersion objective (numerical aperture 1.4). Images were recorded with pixel dimensions of 28 nm. The 488 nm line of a 100 mW Argon laser (Spectra-Physics) was used for exciting both chlorophyll and BODIPY fluorescence. BODIPY fluorescence was selected with a 505 nm dichroic mirror and an interference filter with a transmission range of 500 – 527 nm. Chlorophyll fluorescence was selected with a Schott RG665 red-glass filter transmitting above about 665 nm. Chloroplasts were visualised using a 20-µm confocal pinhole giving a point-spread in the Z-direction of about 1.3 µm (full width at half maximum). For FRAP, a line was bleached across sample by withdrawing neutral density filters to increase laser power by a factor of 32. The laser was then scanned repeatedly in one dimension for 5-7 s. Laser power was then reduced again and 10 post-bleach images were recorded at 60-s intervals. For the total bleach control, the entire sample was bleached out by increasing laser power and scanning across the entire field of view in XY mode for 15-20 s.

Image processing and FRAP data analysis

In the intact chloroplast measurements, confocal images were converted to grey-scale and deconvolved using the DeconvolutionJ plugin of the public domain NIH ImageJ software

(http://rsb.info.nih.gov/ij/) using 2D deconvolution based on the Wiener filter. The regularisation parameter (gamma) was 0.0001. Point spread function was determined by visualisation of 0.175 µm fluorescence microspheres (PS-Speck Microscope Point Source Kit, Invitrogen, Molecular Probes) with the same microscope set-up. The point-spread function in the XY plane was 0.76 µm (full width at half-maximum), reduced to 0.68 µm after deconvolution. The images were aligned to correct for slight drift with time during the FRAP series using ImagePro Plus software (Media Cybernetics, USA) and analysed with ImageJ. An individual granum was selected as a region of interest and the fluorescence intensity of that region was measured in pre- and post-bleach images. Simultaneously, the fluorescence in unbleached regions in post-bleach images was normalized to the same total fluorescence as in pre-bleach images. Mobile fractions were determined by fluorescence recovery curves as presented in Figs. 3 and 4 according to the following equation (Reits and Neefjes, 2001):

$$R = (F_\infty - F_0)/(F_i - F_0)$$

where R – mobile fraction, F_∞ - fluorescence intensity in the bleached region after full recovery, F_0 – fluorescence intensity just after bleaching (time 0), F_i – fluorescence intensity in the pre-bleach image. F_0 values were normalized to 0 in all measurements and an exponential curve was plotted to the experimental points with the Origin software (OriginLab Co., USA). Mathematical analysis and calculations of diffusion coefficients for BBY membranes were done as described previously (Kirchhoff el al., 2008a).

Freeze-fracture electron microscopy

Freshly prepared spinach chloroplasts suspensions were concentrated and rapidly frozen as a thin film by rapid immersion in slushy liquid nitrogen (-210°C) using Bal-Tech double replicas carriers and were then fractured at -150°C in a Polaron E7000 freeze-fracture device. Replicas were prepared by shadowing with platinum and carbon, cleaned with bleach and examined with a FEI Tecnai T12 electron microscope at ×120000 magnification. PSII particles average density and distance measurements (n ≈ 2000) were conducted using the Pixcavator IA 4.2 (Intelligent Perception Co., USA) and the Delaunay Voronoi plugin of the Image J software. Measurements of PSII particles sizes were carried out with ImagePro Plus software.

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SHORT LEGENDS FOR SUPPORTING INFORMATION

Figure S1

PAM fluorescence measurement on intact spinach chloroplasts subject to high-light illumination (3000 μE m^{-2} s^{-1}) in the presence of 25 μM DCMU. As in Fig. 5, but with extended timescale.

TABLE 1

Effect of photoinhibition on the mobility of chlorophyll-proteins in intact spinach chloroplasts and isolated grana membranes

Means (± SE) from 10 FRAP measurements on individual chloroplasts or 16-18 measurements on isolated grana (BBY) membranes. In intact chloroplasts the mobile fraction indicates the proportion of chlorophyll-proteins able to exchange between grana, whereas in isolated grana membranes it indicates the proportion of chlorophyll proteins that is mobile within the granal membrane. P values are from T-tests for significance of the difference between the mobile fractions in dark-adapted and photoinhibited samples.

Sample	Mobile fraction (%)		P
	Dark-adapted	Photoinhibited	
Intact chloroplast	13.3 ± 1.1	18.0 ± 1.3	0.014
Intact chloroplast + nigericin	12.3 ± 1.5	16.4 ± 1.0	0.04
Isolated grana membrane	28.0 ± 3.0	27.0 ± 5.0	0.86

LEGENDS TO FIGURES

Figure 1

Confocal fluorescence images of intact (Panel I) and broken (Panel II) chloroplasts from spinach. Scale-bars 2 μm.

a. Green fluorescence from the BODIPY FL C_{12} stain.

b. Chlorophyll fluorescence.

c. Merged pseudocolor images (chlorophyll fluorescence shown in magenta, BODIPY FL C_{12} fluorescence shown in green).

Figure 2

Control FRAP experiments on intact spinach chloroplasts. Fluorescence images are shown in inverted grey-scale. Scale-bars 3 μm.

a. "Total bleach" experiment bleaching out the entire thylakoid membrane area. Note the lack of fluorescence recovery.

b. Bleaching a line across a chloroplast fixed with glutaraldehyde. The circle in the prebleach image shows the region of interest selected for quantitative analysis and the panel below shows enlarged and contrast-enhanced images of the bleached area (position of granum indicated by the arrow). Fluorescence recovery is minimal.

c. Mobile fractions in experiments of the type shown in (a) and (b), compared to experiments of the type shown in Fig. 4. Means of 10 experiments ± S.E. Asterisks indicate values significantly different from the control (one-way ANOVA with Tukey post-hoc test, $P<0.05$).

Figure 3

Control FRAP experiment on broken spinach chloroplast. Fluorescence images are shown in inverted grey-scale. Scale-bars 3 μm. The chloroplast was stained with the green lipophilic fluorophore BODIPY FL C_{12} . Following photobleaching, fluorescence was monitored simultaneously in (a.) the red channel (chlorophyll fluorescence) and (b.) the green channel (BODIPY fluorescence). The arrowed granum shows only partial chlorophyll fluorescence recovery, but complete BODIPY fluorescence recovery.

Figure 4

FRAP measurements on intact spinach chloroplasts.

a. Chlorophyll fluorescence image sequence (inverted grey-scale) for a dark-adapted chloroplast. The circle indicates the position of an individual granum (used as the region of interest for quantitative data analysis). Scale-bar 2 μm. Lower panel shows enlarged and contrast-enhanced images of the bleached area (granum indicated by the arrow).

b. Similar image sequence for a photoinhibited chloroplast.

c. Fluorescence recovery curve for the bleached granum in (a.).

d. Fluorescence recovery curve for the bleached granum in (b.)

Note the greater mobile fraction and faster recovery of fluorescence in the photoinhibited chloroplast.

Figure 5

Photoinhibition of intact spinach chloroplasts monitored by pulsed-amplitude modulation (PAM) fluorometry. A sample was withdrawn and used for FRAP measurements (Fig. 4) at the end of the actinic illumination.

Figure 6

Chlorophyll-protein mobility in intact *Arabidopsis* chloroplasts, with and without photoinihibition. Bars represent mean mobile fractions (± S.E.) from 10 measurements. P-values are from unpaired T-tests.

a. Wild-type (Col-0). Photoinhibition induces a significant increase in the mobile fraction (P = 0.013).

b. *stn8* mutant. Photoinhibition does not increase the mobile fraction.

c. *stn7 stn8* mutant. Photoinhibition does not increase the mobile fraction, and mobility in non-photoinhibited chloroplasts is significantly lower than in the wild-type (P = 0.0001).

Figure 7

Freeze-fracture electron micrographs from intact spinach chloroplasts (scale-bars 100 nm).

a.) Dark-adapted sample. The thylakoid fracture faces EFs, EFu, PFs and PFu have been labelled according to the nomenclature of Branton et al. (1975).

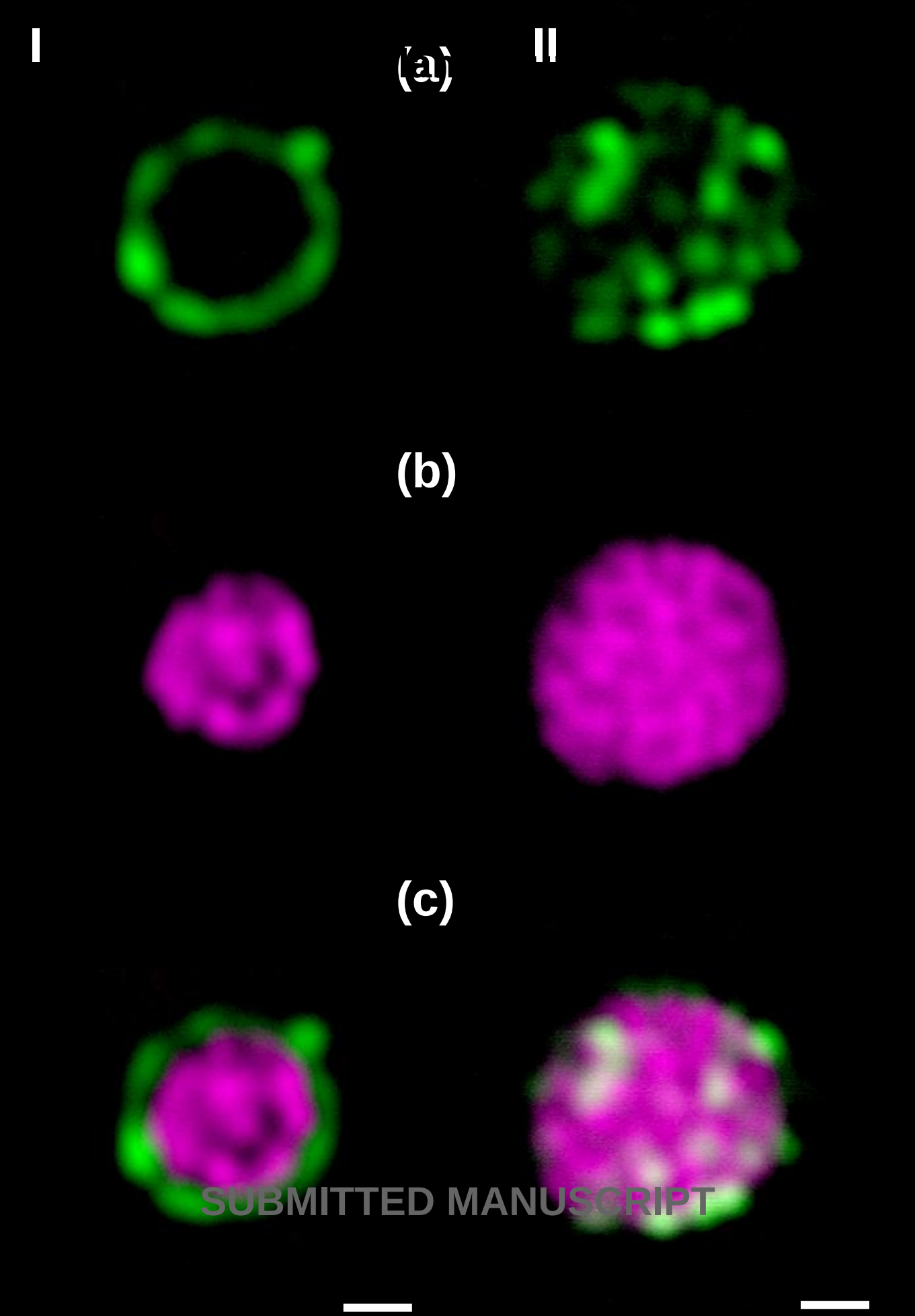
b.) Photoinhibited sample.

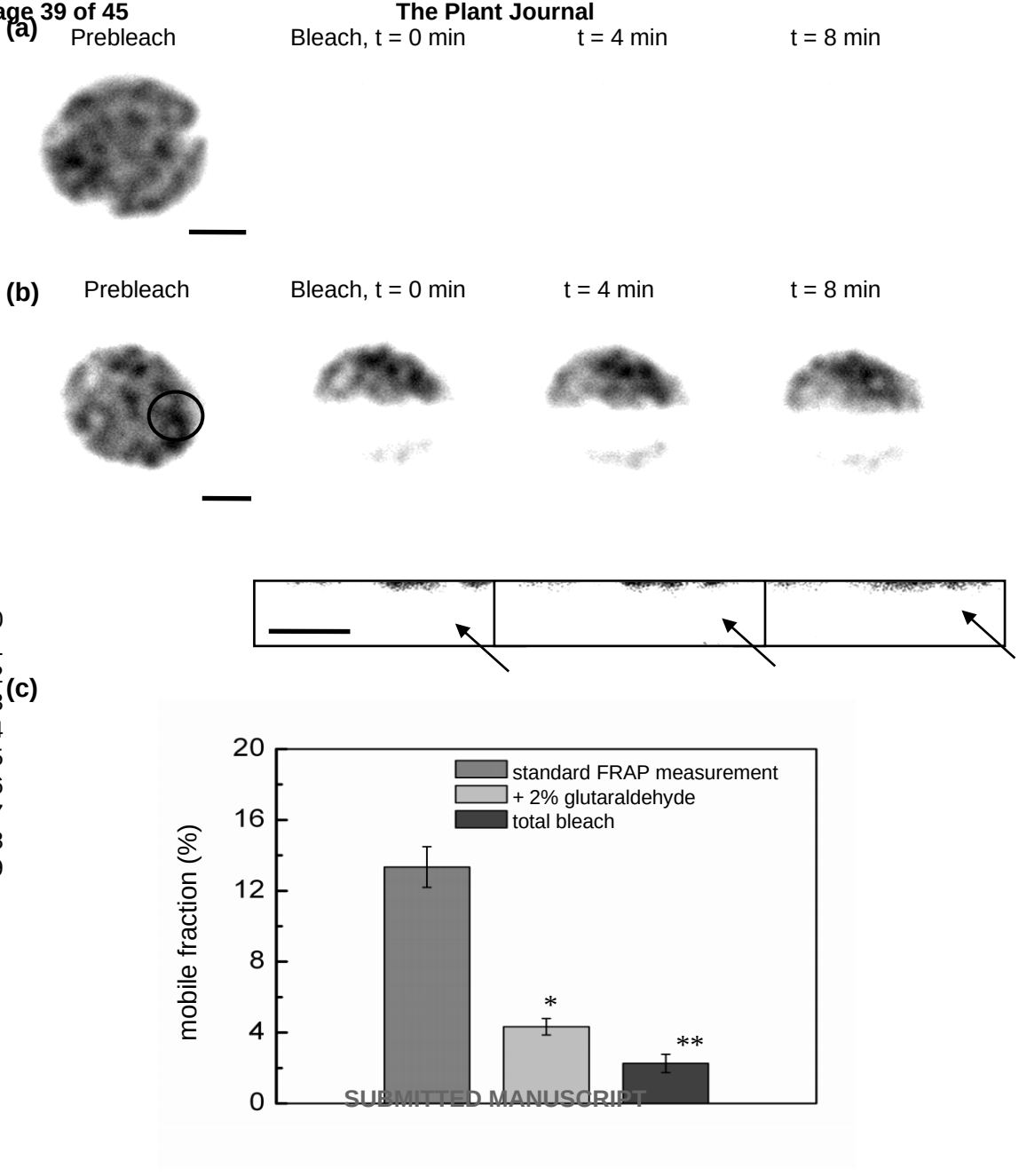
Figure 8

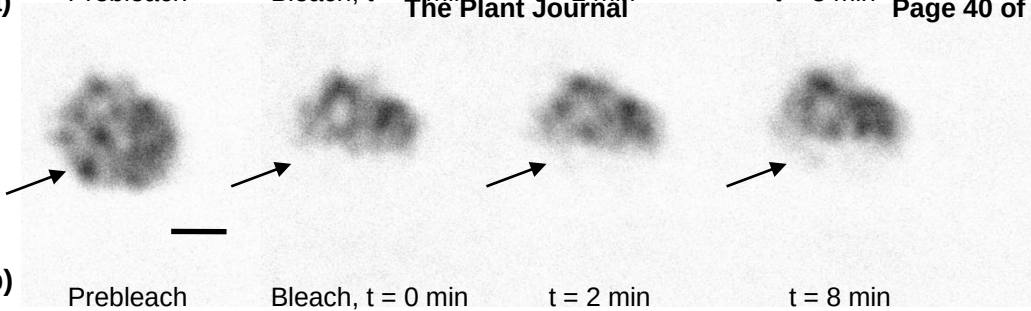
Differences in PSII density in the grana regions of dark-adapted and photoinhibited spinach chloroplasts revealed in the EFs faces of freeze-fracture electron micrographs such as those shown in Fig. 7.

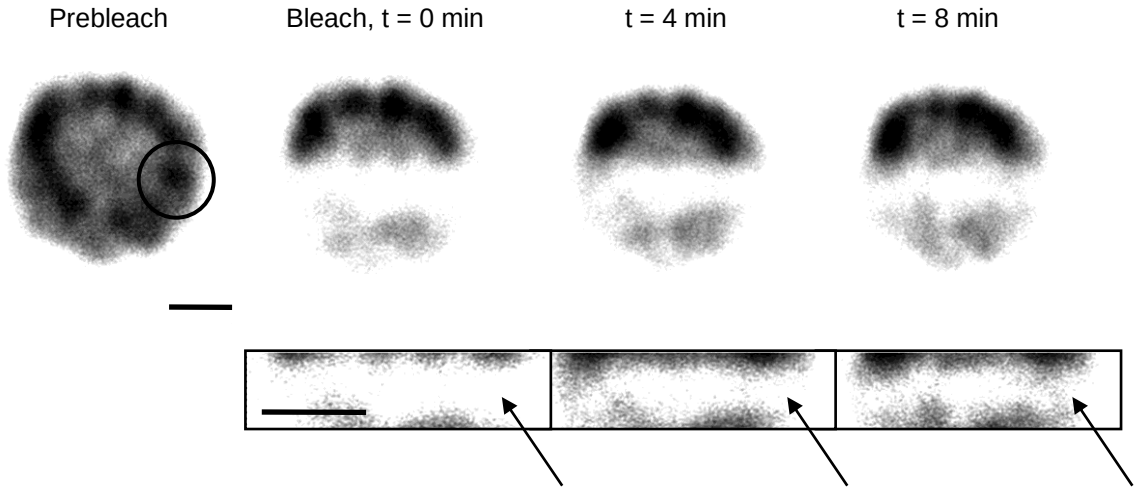
a.) Mean PSII density in μm^{-2}. Mean ± SE (n=30) is shown, the difference is significant (T-test, $P < 0.001$).

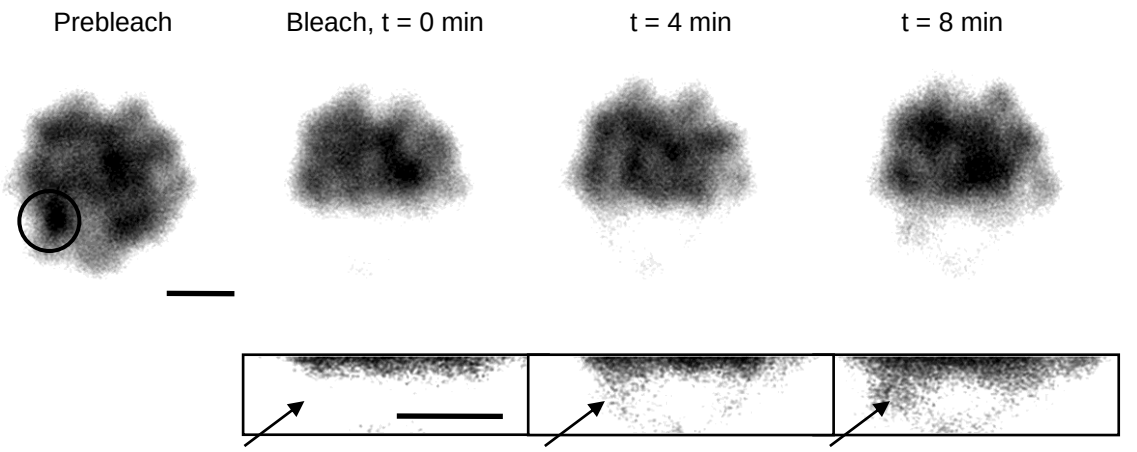
b.) Nearest-neighbour distances between PSII particles in photoinhibited (PI) and dark (D) states. The lines show the smoothed distributions of nearest-neighbour distances, and the histogram shows the difference between the distributions (PI-D). Note the shift towards greater nearest-neighbour distance in the photoinhibited state.








(a)
Prebleach
Bleach, t = 0 min
t = 4 min
t = 8 min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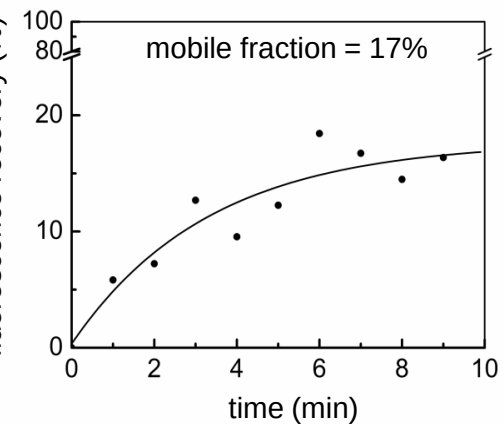
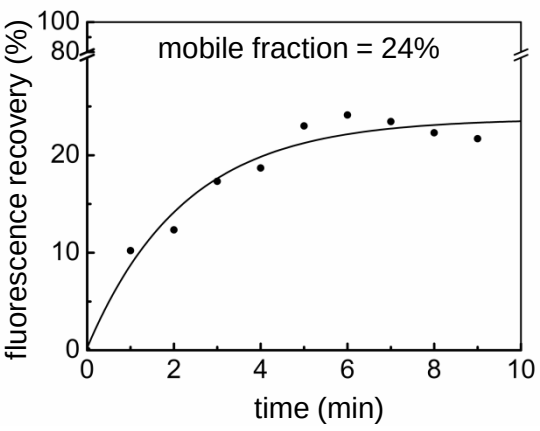
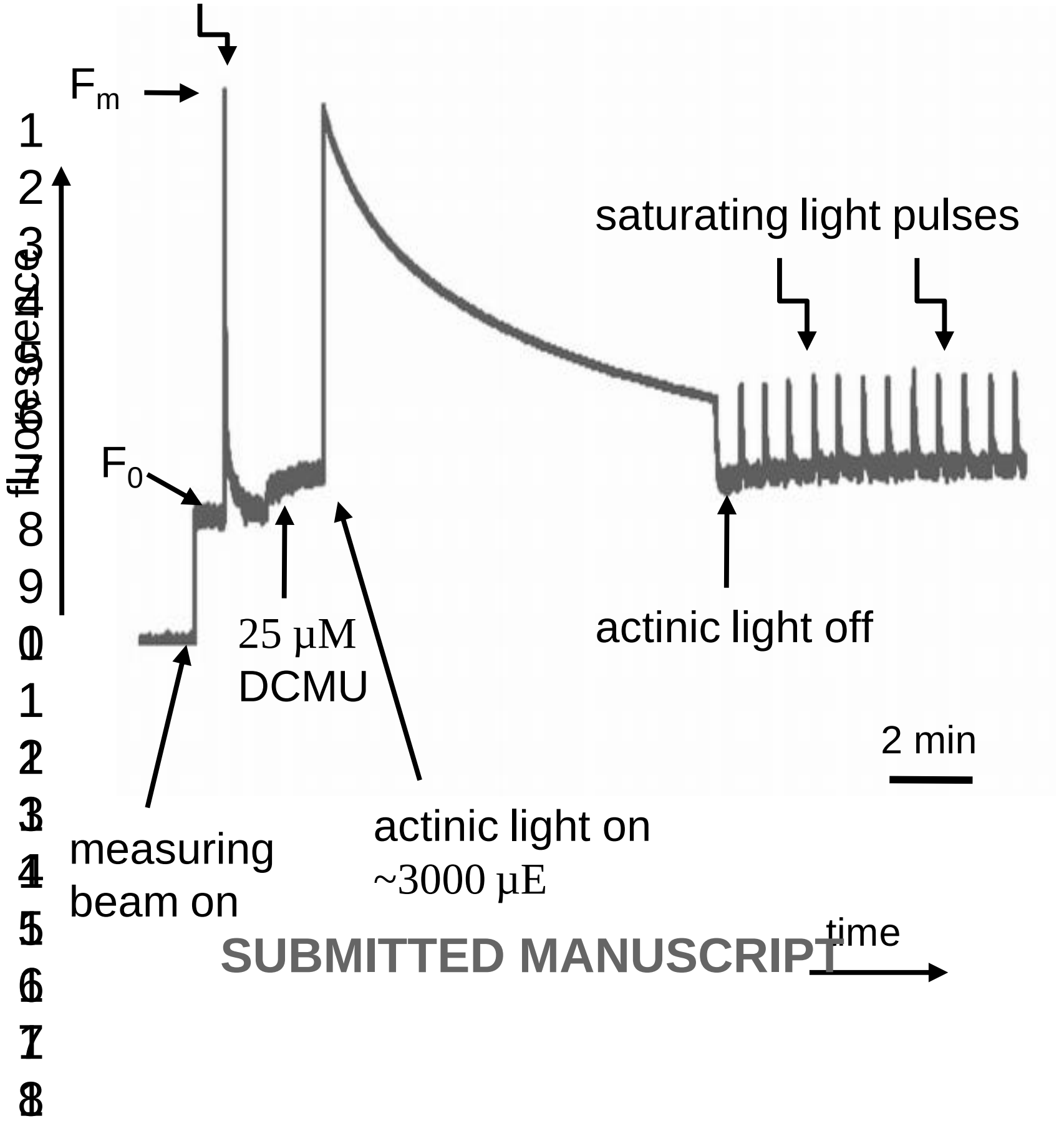
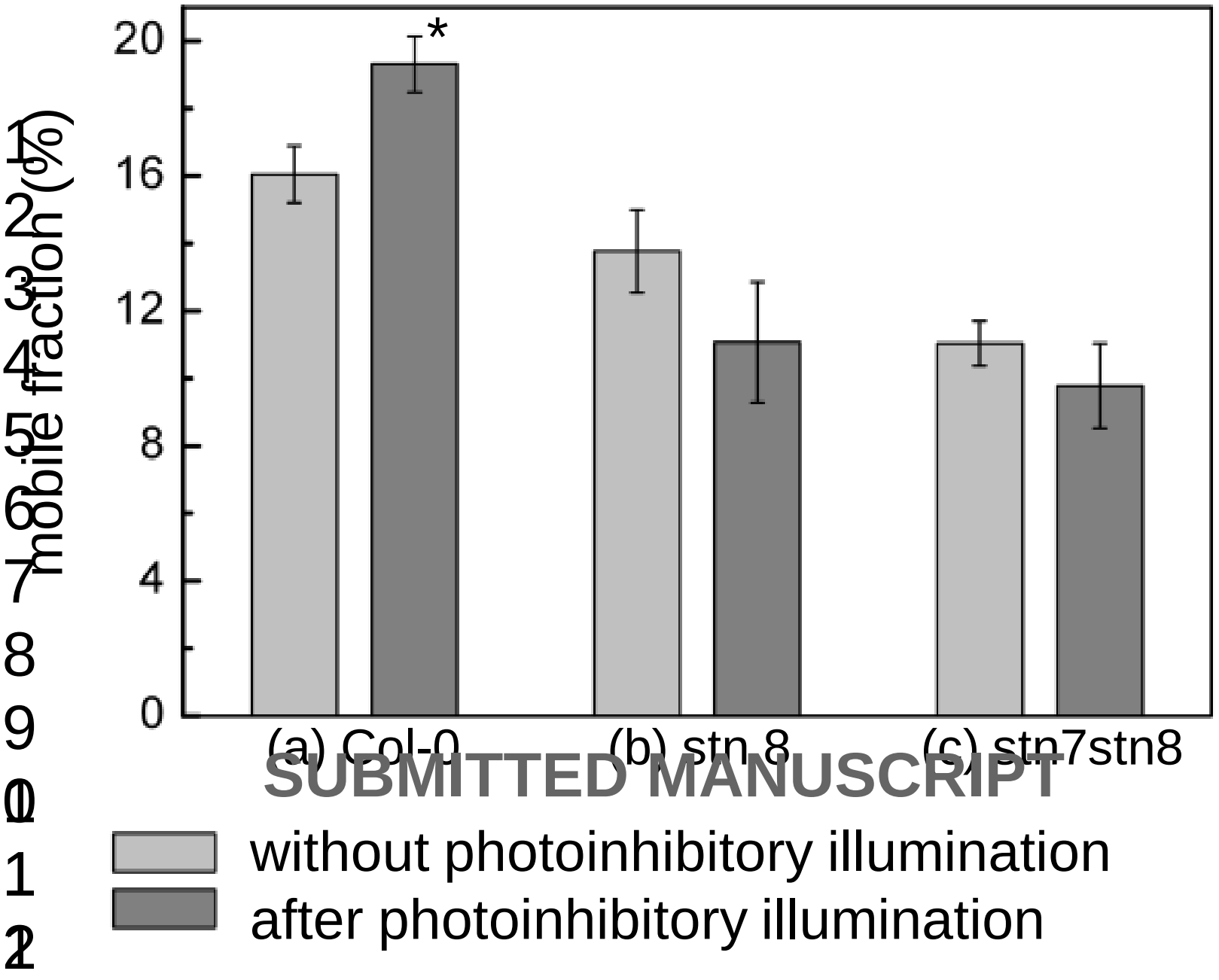
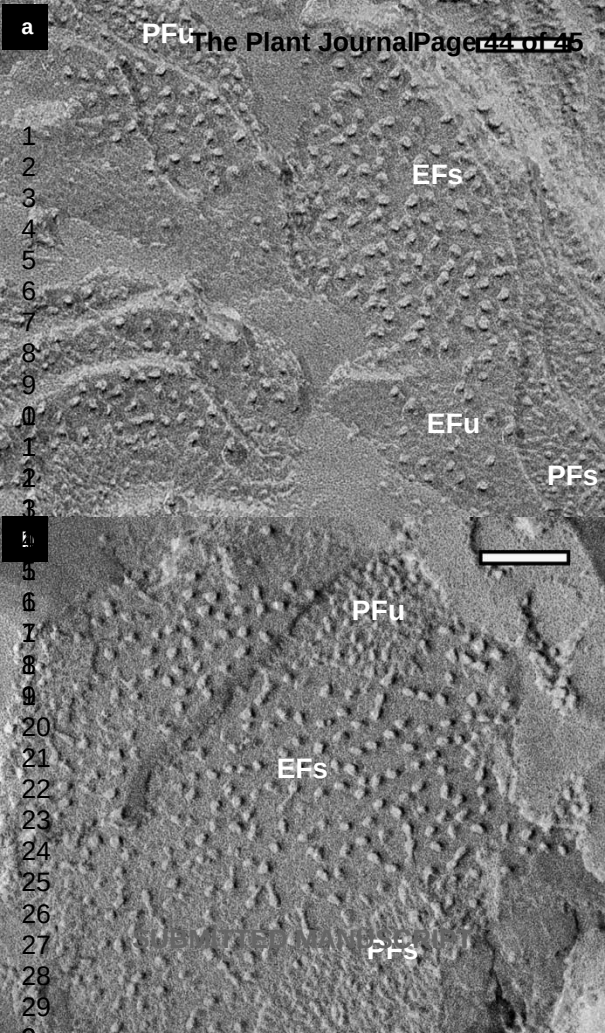

(c)
mobile fraction = 17%
fluorescence recovery (%)
time (min)

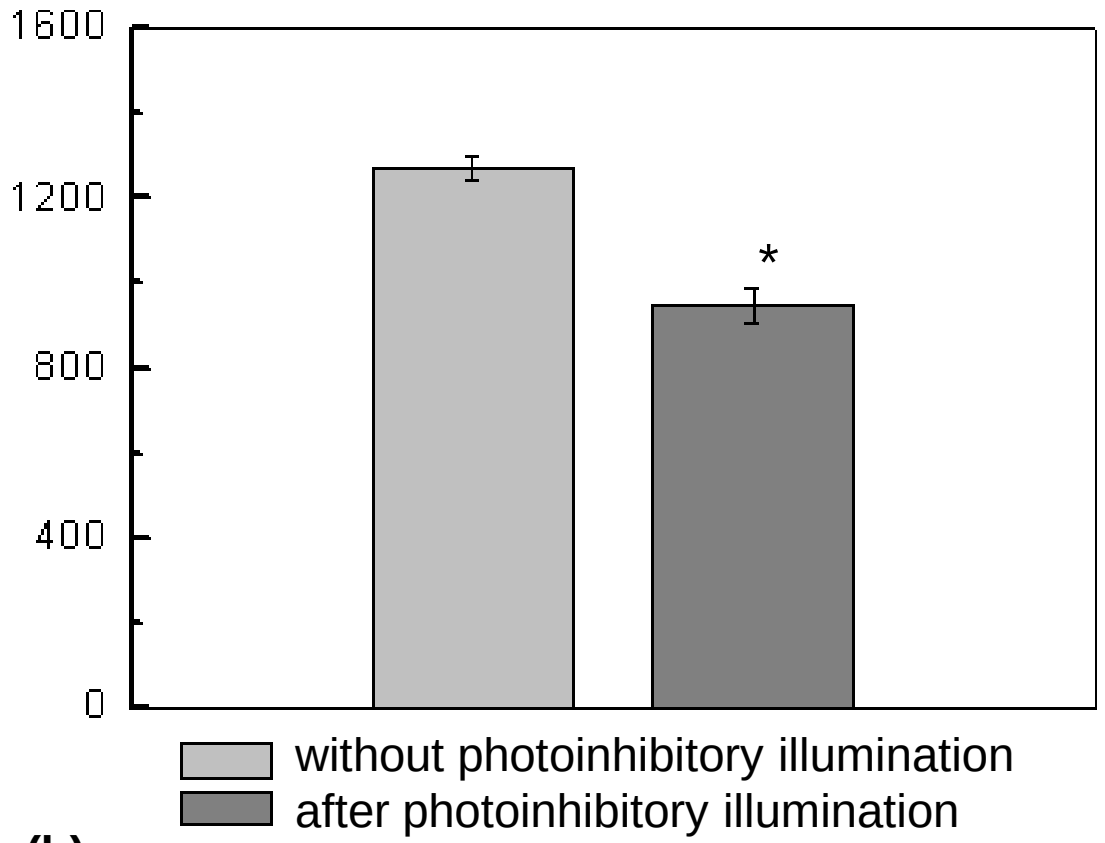

(d)
mobile fraction = 24%
fluorescence recovery (%)
time (min)



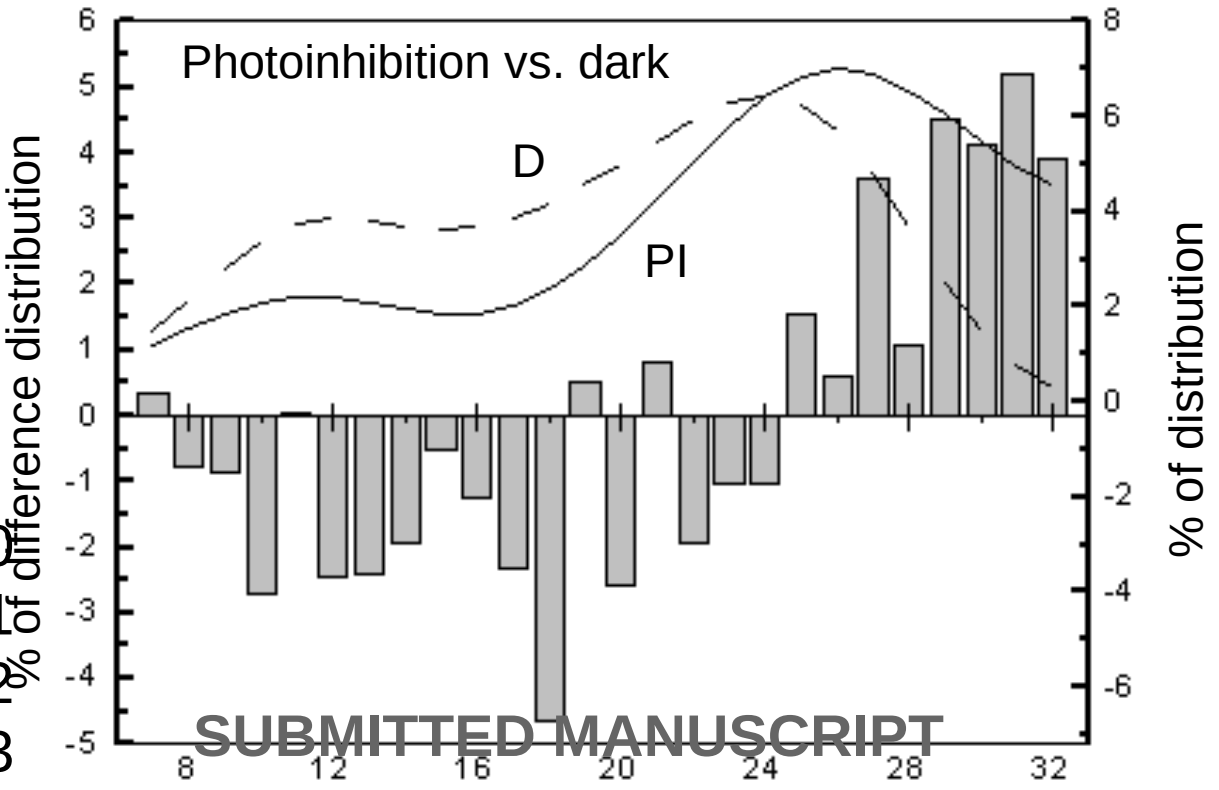



a
PFu
EFs
EFu
PFs
b
PFu
EFs
PFs

(a)



(b)



Nearest neighbour distance between PSII particles (nm)