Thermal energy dissipation in reaction centres and in the antenna of photosystem II protects desiccated poikilohydric mosses against photo-oxidation

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Abstract
Seasonal differences have been observed in the ability of desiccated mosses to dissipate absorbed light energy harmlessly into heat. During the dry summer season desiccation-tolerant mosses were more protected against photo-oxidative damage in the dry state than during the more humid winter season. Investigation of the differences revealed that phototolerance could be acquired or lost even under laboratory conditions. When a desiccated poikilohydric moss such as Rhytidiadelphus squarrosus is in the photosensitive state, the primary quinone, QA, in the reaction centre of photosystem II is readily reduced even by low intensity illumination as indicated by reversibly increased chlorophyll fluorescence. No such reduction is observed even under strong illumination in desiccated mosses after phototolerance has been acquired. In this state, reductive charge stabilization is replaced by energy dissipation. As a consequence, chlorophyll fluorescence is quenched. Different mechanisms are responsible for quenching. One is based on the presence of zeaxanthin provided drying occurs in the light. This mechanism is known to be controlled by a protonation reaction which is based on proton-coupled electron transport while the moss is still hydrated. Another mechanism which also requires light for activation, but no protonation, is activated during desiccation. While water is slowly lost, fluorescence is quenched. In this situation, an absorption band formed at 800 nm in the light is stabilized. It loses reversibility on darkening. Comparable kinetics of fluorescence quenching and 800 nm signals as well as the linear relationship between non-photochemical fluorescence quenching (NPQ) and loss of stable charge separation in photosystem II reaction centres suggested that desiccation-induced quenching is a property of photosystem II reaction centres. During desiccation, quenchers accumulate which are stable in the absence of water but revert to non-quenching molecular species on hydration. Together with zeaxanthin-dependent energy dissipation, desiccation-induced thermal energy dissipation protects desiccated poikilohydric mosses against photo-oxidation, ensuring survival during drought periods.

Key words: Chlorophyll fluorescence, energy dissipation, mosses, photoprotection, photosystem II, reaction centre, zeaxanthin.

Introduction
Whereas vegetative organs of most higher plants do not survive the complete loss of water, many lower plants are...
desiccation-tolerant. This property enables mosses and lichens to colonize habitats which are inaccessible for higher plants. However, recovery of life functions after prolonged desiccation under irradiation requires more than desiccation tolerance. Sunlight is absorbed by the pigments of the photosynthetic apparatus whether water is present or not. During photosynthesis of hydrated plants, light energy is used to produce an oxidant in the reaction centre of PSII, P680\(^{+}\), which is strong enough to oxidize water. Oxygen is evolved and liberated electrons are used to reduce CO\(_2\). However, P680\(^{+}\) threatens to oxidize components of the photosynthetic apparatus when water is absent. Even if reaction centres were inactive in the absence of water, light exciting chlorophyll to its short-lived singlet state would give rise to long-lived triplet chlorophyll by intersystem crossing (1Chl\(^{*}\) \rightarrow 3Chl\(^{*}\)). When deactivated in the presence of triplet oxygen (3O\(_2\)), 3Chl\(^{*}\) produces highly oxidative singlet oxygen (1O\(^{2}\)). Singlet oxygen production in photosynthesis has recently been discussed by Krieger-Liszkay (2005). Owing to the reactivity of this oxygen species, damage to the photosynthetic apparatus is unavoidable if 1Chl\(^{*}\) is not deactivated before significant formation of 3Chl\(^{*}\) or the accumulation of P680\(^{+}\) can take place. Mechanisms of dissipation of excess excitation energy are known to protect hydrated photosynthetic organisms against photo-oxidative damage during photosynthesis. They are activated when more light is available than needed to sustain photo-oxidative damage during photosynthesis. They are known to protect hydrated photosynthetic organisms against excessive light and darkness before drying them slowly in the dark. For *Rhytidiaedelphus squarrosus*, prolonged darkening was occasionally effective in removing zeaxanthin completely (Bukhov et al., 2001). Drying was done at room temperature in air of a relative humidity below 60% or a water potential below \(-70 \text{ MPa}\). Alternatively, dark-adapted hydrated mosses were immersed for 1 h in 20 \(\mu\text{M}\) or 50 \(\mu\text{M}\) DCMU to block electron flow between QA and QB in the reaction centre of PSII (Trebst, 1981), or in 5 \(\mu\text{M}\) nigericin to facilitate fast K\(^{+}\)/H\(^{+}\) exchange across thylakoid membranes, or in 1 mM or 5 mM dithiothreitol to inhibit the formation of zeaxanthin from violaxanthin (Yamamoto and Kamite, 1972). Slow drying was then done either under illumination (PPFDs from 2.5 up to 500 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)), or in darkness, or in near darkness (PPFD 0.07 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)) in order to facilitate the recording of fluorescence during drying. Effectiveness of DCMU-inhibition of electron flow and of nigericin-induced absence of pH control of electron flow was checked by measuring the response to low light of chlorophyll fluorescence. Absence of zeaxanthin-dependent energy dissipation under the conditions of the experiments was controlled by making sure that quenching of basal or F\(_{\text{c}}\) chlorophyll fluorescence was absent immediately after illumination with strong light pulses (Katona et al., 1992; see also Fig. 1B). Active zeaxanthin-dependent energy dissipation was indicated by post-illumination F\(_{\text{o}}\) quenching (Koppecky et al., 2005; see also Figs 1A, 2, 3). The measuring beam was reduced in control experiments to a PPFD <0.1 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) to reduce fluorescence of hydrated mosses reliably to the F\(_{\text{c}}\) level. It should be noted that it is difficult at higher PPFDs to distinguish between post-illumination F\(_{\text{o}}\) quenching and post-illumination oxidation of reduced QA (Fig. 2). Modulated chlorophyll fluorescence was monitored as fluorescence emission beyond 700 nm (using the far-red transmitting filter RG 9 of Schott, Mainz, Germany) by the pulse amplitude modulation fluorometer 101 (PAM) of Walz, Effeltrich, Germany (Schreiber et al., 1986). The detector was protected against strong actinic light by a combination of the filters Calflex c and DT-Cyan from Balzers (Liechtenstein). A red measuring beam of very low PPFD produced modulated fluorescence. Short pulses (usually 1 s) of white light (filters: Calflex c and DT-Cyan of Balzers, Liechtenstein) from a halogen lamp (KL 1500 electronic of Schott, Mainz, Germany) were brought to the cuvette by fibre optics. The PPFD of the light pulses was usually 12 000 \(\mu\text{mol m}^{-2}\text{s}^{-1}\). Whenever necessary, the temperature of the samples was monitored by a thermocouple. Light-dependent absorption changes at 800 nm were measured in reflection using thermal energy dissipation contributes to photoprotection of organisms such as poikilohydric mosses and lichens. It is thought to be located in the reaction centre of PSII and is activated when water is lost during desiccation.

### Materials and methods

The poikilohydric shade-tolerant moss *Rhytidiaedelphus squarrosus* (L. Ap Hedw.) Warmst. (family Rhytidaceae) was collected under trees in the botanical garden of the University of Wuerzburg. The moss *Hylodium splendens* (Hedw.) Br. Eur. (family Hylodiumaceae) was from a forest floor under pine near Leinach, 25 km from Wuerzburg. Both mosses are moderately homochlorophyllous (Proctor and Tuba, 2002), but lose chlorophyll very slowly owing to photo-oxidation when exposed for several days to full sunlight in the desiccated state. Both are desiccation-tolerant in the sense that they tolerate full desiccation after slow drying in air and subsequent storage in a desiccator over silica gel.

Dark adaptation of hydrated mosses (intended to minimize or eliminate zeaxanthin by its conversion to violaxanthin) was achieved by exposing the mosses for prolonged times (several hours to a few days) to dim light and darkness before drying them slowly in the dark. For *Rhytidiaedelphus squarrosus*, prolonged darkening was occasionally effective in removing zeaxanthin completely (Bukhov et al., 2001). Drying was done at room temperature in air of a relative humidity below 60% or a water potential below \(-70 \text{ MPa}\). Alternatively, dark-adapted hydrated mosses were immersed for 1 h in 20 \(\mu\text{M}\) or 50 \(\mu\text{M}\) DCMU to block electron flow between QA and QB in the reaction centre of PSII (Trebst, 1981), or in 5 \(\mu\text{M}\) nigericin to facilitate fast K\(^{+}\)/H\(^{+}\) exchange across thylakoid membranes, or in 1 mM or 5 mM dithiothreitol to inhibit the formation of zeaxanthin from violaxanthin (Yamamoto and Kamite, 1972). Slow drying was then done either under illumination (PPFDs from 2.5 up to 500 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)), or in darkness, or in near darkness (PPFD 0.07 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)) in order to facilitate the recording of fluorescence during drying. Effectiveness of DCMU-inhibition of electron flow and of nigericin-induced absence of pH control of electron flow was checked by measuring the response to low light of chlorophyll fluorescence. Absence of zeaxanthin-dependent energy dissipation under the conditions of the experiments was controlled by making sure that quenching of basal or F\(_{\text{c}}\) chlorophyll fluorescence was absent immediately after illumination with strong light pulses (Katona et al., 1992; see also Fig. 1B). Active zeaxanthin-dependent energy dissipation was indicated by post-illumination F\(_{\text{o}}\) quenching (Koppecky et al., 2005; see also Figs 1A, 2, 3). The measuring beam was reduced in control experiments to a PPFD <0.1 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) to reduce fluorescence of hydrated mosses reliably to the F\(_{\text{c}}\) level. It should be noted that it is difficult at higher PPFDs to distinguish between post-illumination F\(_{\text{o}}\) quenching and post-illumination oxidation of reduced QA (Fig. 2). Modulated chlorophyll fluorescence was monitored as fluorescence emission beyond 700 nm (using the far-red transmitting filter RG 9 of Schott, Mainz, Germany) by the pulse amplitude modulation fluorometer 101 (PAM) of Walz, Effeltrich, Germany (Schreiber et al., 1986). The detector was protected against strong actinic light by a combination of the filters Calflex c and DT-Cyan from Balzers (Liechtenstein). A red measuring beam of very low PPFD produced modulated fluorescence. Short pulses (usually 1 s) of white light (filters: Calflex c and DT-Cyan of Balzers, Liechtenstein) from a halogen lamp (KL 1500 electronic of Schott, Mainz, Germany) were brought to the cuvette by fibre optics. The PPFD of the light pulses was usually 12 000 \(\mu\text{mol m}^{-2}\text{s}^{-1}\). Whenever necessary, the temperature of the samples was monitored by a thermocouple. Light-dependent absorption changes at 800 nm were measured in reflection using
the PAM instrument in combination with the ED800 T emitter/detector unit of Walz. This attachment was modified for the measurement of absorption changes at 950 nm by replacing the original LED of the emitter/detector unit with a LED with peak emission at 950 nm. For carotenoid determinations, desiccated mosses were stored in a freezer at \(-20^\circ\text{C}\). Before extraction, they were rewetted in darkness for 1 min and were then ground in liquid nitrogen in a mortar before 80% acetone was added. After centrifugation, the pellet was extracted twice with 100% acetone. Combined extracts were analysed for carotenoids and chlorophyll \(a\) and \(b\) using an Agilent 1100 HPLC system with diode array detection at 445 nm and 407 nm following the method published in Niinemets et al. (1998). For measuring fluorescence emission spectra, the spectrofluorometer SPF 500 of Aminco Instruments (Silver Spring, Maryland, USA.) was used. 440 nm light served for fluorescence excitation.

Results and discussion

Field observations

Light, when absorbed by the pigment system of photosynthesis, has only three possible fates which exclude one another. Photochemical use of light energy, thermal energy

Fig. 1. Modulated chlorophyll fluorescence of the moss *Rhytidiadelphus squarrosus* before and after hydration by water. (A) The moss was collected in the sun-dried state. Zeaxanthin was present. (B) The moss was collected wet and dried in the dark. Zeaxanthin-dependent energy dissipation was absent. Very strong light pulses (1 s; photon flux 12 000 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\), or about seven times sunlight) given every 200 s increased fluorescence transiently only after hydration in (A), but before and after hydration in (B). \(F_o\) denotes basal fluorescence when \(Q_A\) is largely oxidized, \(F_m\) maximum fluorescence when \(Q_A\) is reduced. \(F_m\) was fully quenched by desiccation in (A) and only partially in (B). Importantly, the very low background photon flux of 2.5 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\) was sufficient to increase fluorescence of the desiccated moss substantially beyond \(F_o\) in (B). It was ineffective in (A).

Fig. 2. Modulated chlorophyll fluorescence of hydrated *Rhytidiadelphus squarrosus* as affected by very strong light pulses of 1 s duration (PPFD=12 000 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\)) which were given once every minute. Background light for fluorescence excitation was extremely low (PPFD=0.08 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\)). It was increased to 3.1 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\) for only about 4 min after the first six light pulses and then returned to 0.08 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\). Actinic light of PPFD=1000 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\) was given for 2 min as indicated to activate zeaxanthin-dependent energy dissipation. For explanation, see text.
dissipation, and re-emission of light as fluorescence compete with one another. Therefore, measurements of fluorescence are a convenient and reliable means to follow changes in thermal energy dissipation as long as photochemistry can be suppressed or neglected. This is the case, when photochemistry is either saturated or negligible in extent. In this way, changes in chlorophyll fluorescence can be used to indicate changes in thermal energy dissipation. Increased quenching of modulated fluorescence shows increased thermal energy dissipation and vice versa (Schreiber et al., 1986; Krause and Weis, 1991; Krause and Jahn, 2004). In fact, mosses, when collected desiccated during late spring or summer, exhibit very low modulated chlorophyll fluorescence. Hydration results in increased \( F_o \) fluorescence indicating a decrease of thermal energy dissipation (Heber et al., 2001). In addition, PSII is activated. This is exemplified in Fig. 1A for the shade-tolerant moss *Rhytidiadelphus squarrosus* which was collected desiccated during a sunny summer day. Strong light pulses were given every 200 s to probe for photochemical activity. Pulse-induced increases in fluorescence are known to indicate charge separation in RCs of PSII and reduction of the primary quinone acceptor QA. No response was seen in the dried moss, but reversible reduction of QA was indicated by large fluorescence responses to short light pulses soon after hydration had resulted in increased fluorescence, i.e. decreased energy dissipation.

However, when the moss was collected in the late autumn or winter under humid conditions, drying under darkness did not quench \( F_o \) fluorescence (Heber and Shuvalov, 2005). This observation is important. It shows that desiccation by itself does not necessarily result in strongly increased thermal energy dissipation, a prerequisite of photoprotection. Rather, very sensitive responses of fluorescence even to PPFDs as low as 2.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) showed that transfer of excitation energy from the light-harvesting pigments to reaction centres is not interrupted by desiccation (Fig. 1B). Very similar observations were made with another shade-tolerant poikilohydric moss, *Hylocomium splendens* (not shown), or when leaves of higher plants were dried (Shuvalov and Heber, 2003; Kopecky et al., 2005). They simultaneously indicated functional energy transfer to the RC of PSII and reductive RC activity even in the absence of water, although this activity was reduced in extent in dried leaves and in the desiccated moss (Fig. 1B). Hydration, instead of increasing fluorescence, rather decreased basal fluorescence in contrast to what was observed after hydration of desiccated mosses in spring and summer.

**Laboratory analysis**

**Experimental manipulation of fluorescence characteristics:**

These seasonal differences in fluorescence responses which suggested seasonal differences in thermal energy dissipation proved to be accessible to experimental manipulation. It appeared that some ‘summer’ mosses could be converted into ‘winter’ mosses and vice versa. Requirements for the conversion of a ‘summer’ moss into the ‘winter’ state were prolonged exposure of a hydrated moss to dim light and darkness. Thus, when the moss *Rhytidiadelphus squarrosus* was collected in the summer and incubated in the hydrated state for one or two days under PPFD=2 or 3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), followed by another day in darkness, it exhibited after slow drying in the dark fluorescence responses similar to those shown in Fig. 1B. Conversely, drying of hydrated ‘winter’ mosses under actinic illumination was sufficient for producing ‘summer’ characteristics of fluorescence (Fig. 1A). This was observed with several shade-tolerant moss species, particularly *Rhytidiadelphus squarrosus* and *Hylocomium splendens*.

**Post-illumination quenching of \( F_o \) fluorescence as indicator of active zeaxanthin-dependent energy dissipation in hydrated mosses:**

The requirement of light for the conversion of a ‘winter’ moss to a ‘summer’ moss suggested a prominent role of zeaxanthin in desiccation-induced fluorescence quenching because zeaxanthin formation from violaxanthin is known to require light (Sapozhnikov et al., 1966; Hager, 1969; Pfündel and Dilley, 1993). In addition, by coupling transthylakoid proton transport to electron transport, light lowers the intrathylakoid pH, a prerequisite not only for zeaxanthin synthesis but also for the protonation of the PsbS protein which is needed to activate zeaxanthin-dependent energy dissipation when zeaxanthin is already present (Graßes et al., 2002; Ruban et al., 2005). Energy dissipation is accompanied, and indeed indicated, by fluorescence quenching if other quenching processes such as chemical quenching can be excluded. In the experiment of Fig. 2, modulated chlorophyll fluorescence is recorded of hydrated *Rhytidiadelphus squarrosus* which had been exposed for 2 d to dim light (PPFD=3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). In higher plants, this is known to result in the conversion of zeaxanthin to violaxanthin. Fluorescence was excited by a measuring beam of the very low PPFD of 0.08 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (0.00005% of full sunlight). It increased fluorescence to the \( F_o \) level at which QA in the reaction centre (RC) of PSII is known to be oxidized. Saturating light pulses of 1 s duration (PPFD=12 000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), i.e. seven times full sunlight) increased fluorescence transiently to its maximum, the \( F_m \) level, at which QA is fully reduced. Fluorescence relaxed completely after the light pulses reflecting the slow oxidation of reduced QA. Importantly, transient post-illumination \( F_o \) quenching was not observed. Its absence indicated that zeaxanthin-dependent energy dissipation was not active (Katona et al., 1992; Kopecky et al., 2005). Either zeaxanthin had been absent, or the light pulse had failed to produce the protonation of the PsbS protein which activates thermal energy dissipation when zeaxanthin is available. In fact, HPLC analysis revealed the
presence not only of appreciable levels of zeaxanthin (Z) but also of antheraxanthin (A). Z was 16±3 moles/1000 moles of chlorophyll (n=6), A was 7.7±1 moles/1000 moles of chlorophyll (n=6). The ratio (antheraxanthin plus zeaxanthin)/(antheraxanthin plus zeaxanthin plus violaxanthin)=\((A+Z)/(A+Z+V)\) was 0.31±0.03 (n=6) indicating considerable de-epoxidation of violaxanthin.

Increasing the measuring beam after the first six light pulses to PPFD=3.1 \(\text{mol m}^{-2} \text{s}^{-1}\) increased fluorescence by reducing \(Q_A\) partially (Fig. 2). Now, fluorescence excited by the strong light pulses relaxed rapidly even below the steady-state fluorescence level which had been raised by increasing the light intensity. However, the post-illumination fluorescence quenching observed in this situation is unrelated to thermal energy dissipation. Rather, it reflects chemical fluorescence quenching because cytochrome \(f\), which had been oxidized during the light pulse, accepted electrons from reduced \(Q_A\) faster than electrons could be re-supplied by photosystem II in low light. This shows that fluorescence is an ambiguous signal as long as it cannot be correctly interpreted by carefully defining conditions of the measurements. After the initial situation was restored by reducing the intensity of the measuring beam, fluorescence relaxed to the \(F_o\) level. Strong light was then given for 2 min to activate zeaxanthin-dependent energy dissipation by protonation (for \(Rhytidiadelphus squarrosus\), see Bukhov et al., 2001). After the actinic light was turned off, strong light pulses were followed by post-illumination fluorescence quenching even below the initial \(F_0\) level. At this level, PSII RCs are open and \(Q_A\) is oxidized. Quenching even below the \(F_0\) level is caused by effective thermal energy dissipation. It is accompanied by an absorbance change at 535 nm (Heber, 1969; Katona et al., 1992) which has been shown to shift zeaxanthin absorption from about 500 to 535 nm (Li et al., 2000; Ruban et al., 2002). Z was increased to 25.8±4.9 moles/1000 moles of chlorophyll (n=6) and A to 8.2±3 moles/1000 moles of chlorophyll (n=6). \((A+Z)/(A+Z+V)\) had increased during illumination to 0.51±0.0, indicating increased conversion of violaxanthin to antheraxanthin and zeaxanthin.

It is important to note that post-illumination \(F_o\) quenching demonstrates that thermal energy dissipation competed successfully with energy conservation in functional PSII RCs. As the latter initiates charge separation within about 3 ps (Zinth and Kaiser, 1993), zeaxanthin-dependent energy dissipation is faster than the time needed for excitation energy to reach open reaction centres, i.e. faster than the travelling time of excitation energy (about 60 ps). Nevertheless, part of the excitation energy still reached the reaction centres as shown by transient light-induced fluorescence peaks.

In the first part of the experiment of Fig. 2, short light pulses did not result in post-illumination \(F_o\) quenching even though zeaxanthin had been present. This shows that zeaxanthin had not been able to initiate energy dissipation. Apparently, the strong light pulses had been ineffective to protonate the PsbS protein. By using \(CO_2\) as a weak acid for protonation instead of illumination, it is possible to find out whether zeaxanthin is absent, or whether it is present, but inactive. In \(Rhytidiadelphus\), \(CO_2\) is incapable of quenching fluorescence below the \(F_o\) level after zeaxanthin had been fully converted to violaxanthin. This has been shown in a preceding publication (Bukhov et al., 2001).

Increasing the measuring beam after the first six light pulses to PPFD=3.1 \(\text{mol m}^{-2} \text{s}^{-1}\) for 2 d and was then darkened for 2 d, post-illumination \(F_o\) quenching was initially absent after strong light pulses, but addition of 20\% \(CO_2\) quenched both \(F_m\) and \(F_o\) fluorescence reversibly, showing that zeaxanthin had actually been present, but was inactive in the absence of 20\% \(CO_2\). It was activated by \(CO_2\). In fact, HPLC analysis revealed the presence of low levels of zeaxanthin and antheraxanthin. \(Z\) was 6.06±0.72 moles/1000 moles of chlorophyll (n=5) and A 5.1±0.82 moles/1000 moles of chlorophyll (n=5). \((A+Z)/(A+Z+V)\) had the low value of 0.106±0.01 showing that prolonged darkening had resulted in the conversion of most zeaxanthin to violaxanthin.

\(F_o\) quenching by \(CO_2\) was reduced after incubation of the moss with 1 mM dithiothreitol (DTT) for 2 h (Fig. 3B). Thus, DTT, a known inhibitor of zeaxanthin formation from violaxanthin (Yamamoto and Kamite, 1972), also inhibited \(F_o\) quenching by \(CO_2\) (Bukhov et al., 2001). In fact, \(Z\) was reduced by about 20\% during incubation to 4.73±0.87 moles/1000 moles of chlorophyll (n=5). \(A\) was reduced by about 35\% to 3.3±0.31 moles/1000 moles of chlorophyll (n=5). \((A+Z)/(A+Z+V)\) was reduced to 0.076±0.009. Apparently, some loss of zeaxanthin had occurred during the 2 h period of incubation with 1 mM DTT. It is still not clear whether this small loss accounts for the decreased \(CO_2\)-dependent quenching of chlorophyll fluorescence shown in Fig. 3B. It is also possible that DTT not only inhibits zeaxanthin formation but also interferes with the binding of zeaxanthin to the protonated PsbS-protein, thereby inhibiting zeaxanthin-dependent thermal energy dissipation even when zeaxanthin is present.

The experiments of Figs 2 and 3 show that the effects of high concentrations of \(CO_2\) on \(F_o\) and \(F_m\) fluorescence reveal, in the absence of actinic illumination, whether zeaxanthin is present or not in \(Rhytidiadelphus squarrosus\) and other mosses and lichens (Bukhov et al., 2001). Presence or absence of post-illumination \(F_o\) quenching after brief strong light pulses gives information on whether zeaxanthin-dependent energy dissipation is active or not when zeaxanthin is present.

Desiccation-induced fluorescence quenching; effects of nigericin, DCMU, and DTT: There is the question whether the combined requirement for desiccation and light for the increased photoprotection in the desiccated state (see
section ‘Field observations’) is attributable fully or partially or not at all to zeaxanthin-dependent energy dissipation. It can be answered by preventing either the formation of zeaxanthin or the protonation of the PsbS-protein in the light or both during desiccation.

DCMU, by inhibiting electron transport, inhibits the light-dependent protonation of the PsbS protein, a prerequisite of the activation of zeaxanthin-dependent energy dissipation when zeaxanthin is present. Nigericin is an antibiotic known to prevent the intrathylakoid acidification which is necessary for zeaxanthin synthesis and, in addition, for activating zeaxanthin-dependent energy dissipation. It facilitates H+/K+ exchange across the thylakoid membrane. Dithiothreitol is an inhibitor of zeaxanthin synthesis. All three agents are expected to interfere with zeaxanthin-dependent energy dissipation. Moreover, the dissipation reaction is not active in hydrated photosynthetic organisms under very low light intensities when energy conservation is the dominant photoreaction (Fig. 2).

Figure 4 shows representative examples of three different experiments performed with hydrated Rhytidiadelphus squarrosus. The moss had been kept for 2 d in low light (PPFD=3 μmol m$^{-2}$ s$^{-1}$) and thereafter for an additional 2 d in darkness in order to deplete zeaxanthin (Bukhov et al., 2001). In the experiment of Fig. 4A, an untreated moss sample was used as a control. In Fig. 4B, the moss had been incubated in 5 μM nigericin (1 h) and in Fig. 4C in 50 μM DCMU (1 h). After turning a low modulated measuring beam on to activate fluorescence (averaged PPFD=0.07 μmol m$^{-2}$ s$^{-1}$), a very strong light pulse (1 s, PPFD=12 000 μmol m$^{-2}$ s$^{-1}$) was given to raise fluorescence to the $F_m$ level which indicates full reduction of the primary quinone acceptor QA in the RC of PSII. Subsequently, 1 s light pulses of PPFD=3.5 μmol m$^{-2}$ s$^{-1}$ were given every 500 s to probe for the extent of transient QA reduction. In the presence of nigericin (Fig. 4B) or DCMU (Fig. 4C) transient fluorescence responses were larger than in Fig. 4A. They also increased fluorescence more than in Fig. 4A indicating increased reduction of QA compared with the control. The low PPFD of 3.5 μmol m$^{-2}$ s$^{-1}$ was almost sufficient in Fig. 4B and C to reduce QA completely during the short light pulses. Pulse-induced fluorescence spikes decreased in all experiments as desiccation progressed. However, they were not completely
suppressed, particularly in the control experiment of Fig. 4A even after equilibrium between dry air and the moss had been achieved at water potential below $-60$ MPa. Loss of pulse-induced fluorescence spikes during drying was accompanied by fluorescence quenching. A very strong light pulse (PPFD=12 000 μmol m$^{-2}$ s$^{-1}$) given after desiccation was complete increased fluorescence to a lowered maximum level $F_m$ in Fig. 4A. In remarkable contrast to this,
fluorescence was not increased but quenched by the single strong light pulse in Fig. 4B and C, although the quenching effect was small. Apparently, a quencher was produced by the strong light pulse in the experiments with nigericin and DCMU. Non-photochemical fluorescence quenching \( NPQ = (F_o/F_m - 1) \) was 1.32 in the first part of the experiment of Fig. 4A, 1.27 in Fig. 4B, and 1.38 in Fig. 4C. There was some variability in these NPQ values. For instance, the average NPQ of the first part of the experiment of Fig. 4A was 1.35 ± 0.28 (n = 8). Similar variability was observed in the other experiments.

Hydration by a few drops of water reversed desiccation-induced quenching of \( F_m \). After four very strong light pulses (PPFD = 12 000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) had been given, background illumination was increased from 0.07 to 3.5 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). The mosses were then permitted to desiccate slowly in the presence of the increased photon flux. Light pulses given every 500 s now had a PPFD of 12 000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). Fluorescence quenching during desiccation always increased beyond the quenching observed under a background illumination of only PPFD = 0.07 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (see first part of the experiments of Fig. 4). Thus, increased photon flux during desiccation increased fluorescence quenching. It is particularly important to note that \( F_o \) decreased to \( F_m \) during desiccation in the presence of PPFD = 3.5 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) below the level observed in the hydrated mosses. Whereas desiccation under PPFD = 0.07 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) had failed to suppress positive pulse-induced fluorescence responses particularly in the control experiment of Fig. 4A, negative fluorescence responses were usually observed after desiccation under PPFD = 3.5 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). Strong light pulses now produced quenchers in the desiccated mosses. NPQ was, after desiccation in the presence of PPFD = 3.5 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), 3.3 in Fig. 4A, 2.1 in Fig. 4B, and 2 in Fig. 4C. When, after desiccation under PPFD = 3.5 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), water was again added to the dry mosses, quenching not only of \( F_m \) but also of \( F_o \) was reversed. This is shown for the experiment of Fig. 4A in the inset of Fig. 4A.

All three experiments of Fig. 4 were designed to avoid the activation of zeaxanthin-dependent energy dissipation. Nevertheless, desiccation caused fluorescence quenching in all three experiments which increased when the photon flux present during slow drying was increased (second part of the experiments of Fig. 4). Whereas post-illumination \( F_o \) quenching in the experiment of Fig. 1A after hydration had suggested that zeaxanthin-dependent energy dissipation might have been responsible for lowered fluorescence in desiccated phototolerant mosses, the experiments of Fig. 4 show that desiccation causes strong fluorescence quenching even under conditions in which the activation of zeaxanthin-dependent energy dissipation by protonation of the PsbS protein has not been possible. This eliminates zeaxanthin as the sole requirement for desiccation-induced fluorescence quenching.

![Fig. 5. Relaxation of thermal energy dissipation (measured as non-photochemical fluorescence quenching NPQ; left ordinate, solid line) and recovery of reductive charge separation (measured as \( \Delta F/F_m \); right ordinate, dashed line) during hydration of desiccated Rhytidiadelphus squarrosus and reversal of these effects during subsequent slow dehydration. Photon flux throughout the experiment was 2.5 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). Before the experiment, the moss had been incubated with 1 mM DTT and was then slowly dried under illumination with a photon flux of 500 \( \mu \)mol m\(^{-2}\) s\(^{-1}\).](image)

![Fig. 6. Relationship between inhibition of charge separation in PSII RCs (as indicated by loss of \( F_o/F_m \)) and non-photochemical fluorescence quenching NPQ during slow desiccation of Rhytidiadelphus squarrosus, or during activation of zeaxanthin-dependent energy dissipation by protonation. For the desiccation experiments, hydrated Rhytidiadelphus had been incubated with DCMU (x), nigericin (filled diamonds) or DTT (●) to prevent activation of zeaxanthin-dependent energy dissipation. Background illumination during desiccation was 0.07 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (DCMU and nigericin) or 500 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (DTT). Effectiveness of zeaxanthin-dependent energy dissipation (▲) was modulated by CO\(_2\).](image)
fluorescence quenching during desiccation is localized in the RCs of PSII.

In the experiment of Fig. 5, changes in non-photochemical fluorescence quenching NPQ and in stable charge separation in PSII reaction centres (as expressed by $\Delta F/F_m$) are compared during hydration of a desiccated moss in which DTT had been used to suppress zeaxanthin synthesis before the moss had been dried. As in the case of the experiments of Fig. 4, the moss had been photosensitive after zeaxanthin-depletion in darkness. After incubation with 1 mM DTT, the moss was dried slowly under illumination with the photon flux of 500 $\mu$mol m$^{-2}$ s$^{-1}$ in order to ensure considerable QA reduction in the PSII RCs. As in the experiments of Fig. 4, drying in the light had quenched fluorescence. Hydration reversed quenching and decreased NPQ rapidly (Fig. 5). With a slight delay, charge separation in PSII RCs recovered. As the experiment was continued, loss of water by evaporation resulted in a reversal of these changes. NPQ increased and charge separation was simultaneously lost.

**Relationship between energy dissipation in the reaction centre and zeaxanthin-dependent energy dissipation:** During slow drying of untreated ‘winter’ moss in the presence of PPFD=500 $\mu$mol m$^{-2}$ s$^{-1}$, which permits zeaxanthin synthesis and protonation of the PsbS protein, NPQ increased from initially zero in near darkness to values of more than 5. These values exceed the NPQ values (usually <3.5) obtained when inhibitors such as DTT, nigericin, or DCMU prevented activation of zeaxanthin-dependent energy dissipation during slow drying under illumination (Fig. 4). Increased NPQ values indicate that the complex formed in the light between the protonated PsbS protein and zeaxanthin during hydration survives desiccation and contributes to photoprotection in the desiccated state (see Eickmeier et al., 1993; Deltoro et al., 1998; Kopecky et al., 2005, for desiccated lichens).

However, there is an important difference between zeaxanthin-dependent energy dissipation and desiccation-induced energy dissipation. Zeaxanthin-dependent energy dissipation is protective only by draining excitation energy from the reaction centres, but does not alter RC function. It does not protect RCs directly from photo-oxidation. As a consequence, dissipating centres and RCs compete for excitation energy (Fig. 6). By contrast, during desiccation, PSII RCs completely lose their energy-conserving function which makes them sensitive to photo-oxidation. Simultaneously, NPQ increases depending on how much light is present during desiccation (Fig. 6). As will be shown below, energy conservation is replaced by energy dissipation. The relationship between non-photochemical fluorescence quenching NPQ and the loss of stable charge separation of PSII RCs as shown by decreasing $\Delta F/F_m$ ($=F_s/F_m$) is linear during desiccation of moss which had been treated with DTT to eliminate a contribution of zeaxanthin-dependent energy dissipation (Fig. 6). Stable charge separation was progressively inhibited during desiccation while NPQ increased. At the maximum of NPQ, reversible charge separation was lost. Similar observations were made after electron transport was inhibited by DCMU, or after protonation was suppressed by nigericin. In these cases, both maximum $F_s/F_m$ and NPQ values were lower than in the DTT experiment because the measurements had to be made in near darkness in order to avoid excessive QA reduction by the measuring light. Nevertheless, $F_s/F_m$ decreased to zero while NPQ increased. In remarkable contrast to this, $F_s/F_m$ was only partially inhibited when zeaxanthin-dependent energy dissipation was changed towards the same NPQ values which resulted in the complete loss of $F_s/F_m$ in the desiccation experiments (Fig. 6). The differences result from different localization of zeaxanthin-dependent energy dissipation and of desiccation-induced energy dissipation in the thylakoid membrane system. The former dissipates energy within the antenna system of PSII, the latter within PSII RCs.

**Reaction centre activities in the absence of water:** Whereas sunlight failed either to increase or decrease the low modulated fluorescence of phototolerant desiccated mosses (Fig. 1A), small reversible fluorescence increases were observed in desiccated photosensitive mosses after slow drying under darkness or very low light (Figs 1B, 4A). These responses revealed not only effective transfer of excitation energy from the antenna of PSII to the RCs but also reduction of QA in the RCs. There was some heterogeneity of responses. In part of the RCs, QA was reduced in the light, accumulated in the reduced form. In other RCs, QA reduced during a short light pulse was still capable of reoxidizing via recombination reactions (see small reversible pulse-induced fluorescence changes in desiccated mosses as shown in Figs 1B, 4A). Similar observations were also made with dried leaves of higher plants (Kopecky et al., 2005; Heber and Shuvalov, 2005).

![Fig. 7. Increased absorption at 800 nm by hydrated DCMU-treated Rhytidiadelphus squarrosus as a function of PPFDs (white light). P700 was kept oxidized by strong far-red background light. At saturation, the ratio $\Delta A_{\text{white}}/\Delta A_{\text{far-red}}$ was 2.65.](image-url)
Once QA is reduced in the absence of water (as it is at the fluorescence level $F_0$ in the experiments of Fig. 4), other redox reactions can be observed. In dried leaves, chloroplasts and subchloroplast particles, transmission spectroscopy revealed loss of absorption bands at 500 nm and 430 nm and the appearance of a band at 450 nm during illumination. This was interpreted to show the reversible photo-oxidation of β-carotene and the reversible reduction of a chlorophyll in PSII RCs (Shuvalov and Heber, 2003). Observed photoreactions were not restricted to dried leaves. Bleaching at 500 nm (Heber and Shuvalov, 2005) and band formation at 950 nm (data not shown) indicated reversible photo-oxidation of β-carotene also in desiccated photosensitive Rhytidiadelphus. As the quantum efficiency of these photoreactions was very low, they are unlikely to contribute appreciably to photoprotection.

### Relationship between an 800 nm absorption band and fluorescence quenching during desiccation

Cationic chlorophyll radicals such as P700$^+$ in the RC of PSI are known to increase absorption around 800 nm. The same is true for anionic radicals of chlorophyll or pheophytin (Fujita et al., 1978). Such radicals are known to be effective fluorescence quenchers (Klimov et al., 1977, 1985). It has previously been shown that formation of an 800 nm absorption band was kinetically correlated to fluorescence quenching in desiccated spinach leaves (Heber and Shuvalov, 2005). PSII RCs contain four chlorophylls and two pheophytins. Whereas PPFDs approaching sunlight caused little changes in 800 nm absorption in hydrated mosses except for those changes which could be readily explained resulting from redox changes of P700$^+$ in PSII RCs, an 800 nm absorption band appeared under very strong illumination while P700 was kept oxidized by strong background illumination with far-red light. Figure 7 shows a sigmoidal relationship between 800 nm absorption of hydrated Rhytidiadelphus squarrosus and photon flux. As long as the moss was fully hydrated, the reaction was fully reversible. However, it became partially irreversible as dehydration progressed (Fig. 8). Simultaneously, fluorescence quenching increased. The relationship between

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**Fig. 8.** Kinetics of the light-dependent increase in 800 nm absorption by hydrated DCMU-treated Rhytidiadelphus squarrosus (A) and during slow desiccation (B, C). Measurements in (B) were taken after 40 min and in (C) after 280 min compared with (A). Note complete relaxation of the reaction in (A) and incomplete relaxation in (B) and (C). Illumination for 5 s with PPFD=12 000 μmol m$^{-2}$ s$^{-1}$ on a background of strong far-red light as indicated by arrows.

**Fig. 9.** Kinetics of the light-dependent increase in 800 nm absorption by partially dehydrated DCMU-treated Rhytidiadelphus squarrosus (A) and of the quenching of chlorophyll fluorescence (B). Illumination for 5 s with PPFD=12 000 μmol m$^{-2}$ s$^{-1}$ on a background of strong far-red light as indicated by arrows.
the increase in 800 nm absorption and quenching of chlorophyll fluorescence after partial dehydration is shown in Fig. 9. While 800 nm absorption increased, chlorophyll fluorescence was quenched. As 800 nm absorption relaxed after darkening, chlorophyll fluorescence increased. Importantly, quenching persisted after darkening under the same conditions which prevented complete relaxation of the 800 nm change. The kinetics of the 800 nm reaction and of fluorescence quenching were virtually identical (Fig. 9). Apparently, increased 800 nm absorption represents formation of a quencher. Irreversible accumulation of the quencher during desiccation is held responsible for fluorescence quenching. It accounts for the progressive loss of the reversible part of the 800 nm reaction during desiccation (Fig. 8). The quencher is stabilized in the absence of water. It reverts to its non-quenching state on hydration (data not shown). Simultaneously, fluorescence quenching is reversed and stable charge separation in PSII reaction centres is restored (Fig. 5). From the relationship between non-photochemical fluorescence quenching NPQ and the loss of stable charge separation in PSII RCs during desiccation (Fig. 6) it is concluded that desiccation-induced fluorescence quenching is a property of PSII RCs. This conclusion is strengthened by the observation that very short strong light pulses given to DCMU-poisoned desiccated Rhytidio delphus produced more transient 800 nm absorption (∆A=1.49±0.25×10^{-3}; n=5) than in unpoisoned controls (∆A=1.02±0.13×10^{-3}; n=3). Thus the quencher represented by the 800 nm absorption change is a component of PSII RCs. Its molecular nature is not yet known. However, a comparison between the extent of the 800 nm reaction and the oxidation of P700 in PSI RCs which also results in increased 800 nm absorption is informative. ∆A_{white}/∆A_{far-red}, the ratio of the maximal 800 nm absorption change measured under saturation illumination with white light in the presence of strong far-red background illumination (to exclude P700 oxidation) to the 800 nm absorption change which is attributable to P700 oxidation, was 2.42±0.97 (n=9). The ratio PSII RCs to PSI RCs is not known for Rhytidio delphus squarro sus. For different photosynthetic organisms, it varies with growth conditions (Buchanan et al., 2000) and may, under sunlight, approach 2 in higher plants. It can also be lower than 2. As the ratio of extinction coefficients for P700+ and for cationic and anionic radicals of Chl (or of Pheo) at 800 nm is close to unity, ∆A_{white}/∆A_{far-red} ratios above 2 may mean than more than one quencher can accumulate per PSII RC during desiccation. However, in different 800 nm experiments with Rhytidio delphus from different locations and seasons, ∆A_{white}/∆A_{far-red} ratios lower than 2 (occasionally approaching unity) were also frequently observed. This testifies to the efficiency of RC quenching which does not always require the full conversion of functional energy-conserving to energy-dissipating centres during desiccation (see also residual reversible QA reduction after desiccation in the first part of the experiment of Fig. 4A).

Spectral observations: The main feature of fluorescence emission spectra of Rhytidio delphus squarro sus. taken at room temperature (where photosystem I is known to be essentially non-fluorescent) is the presence of two emission bands at 685 nm and 730 nm. Both are related to PSII emission (Heber and Shuvalov, 2005). The main emission at 685 nm was quenched more during desiccation than a secondary band with main emission around 730 nm. This is true whether or not agents such as DCMU were used to suppress the contribution of zeaxanthin-dependent energy dissipation. Figure 10 shows fluorescence emission of DCMU-treated Rhytidio delphus. In the lower spectrum, emission from dry moss is shown after slow desiccation in the presence of PPFD=2.5 μmol m^{-2} s^{-1}. Desiccation-induced energy dissipation caused strong fluorescence quenching both at 685 nm and at 730 nm compared with fluorescence emission after hydration which resulted in strongly increased fluorescence (upper spectrum). Very similar observations were made with nigericin- or DTT-treated mosses (not shown).

Conclusions

Increased thermal energy dissipation as expressed by increased fluorescence quenching forms the basis of increased photoprotection. It has been observed that light is required for increased fluorescence quenching while shade-tolerant poikilohydric mosses such as Rhytidio delphus squarro sus are slowly desiccated. Reversible charge separation in PSII RCs can no longer be observed after desiccation in the light because QA in PSII RCs accumulates in the reduced form. Suppression of the activation of zeaxanthin-dependent thermal energy dissipation by inhibitors of electron transport (such as DCMU), of protonation (such as nigericin) and of zeaxanthin synthesis (such as DTT) revealed the presence of another...
mechanism of thermal energy dissipation which requires light for activation during desiccation.

Recently, it has been proposed that PSII RCs can be converted under desiccation from energy-conserving to energy-dissipating units (Heber et al., 2006). The basis of the proposal was that light–dark difference spectra had revealed photo-oxidation of β-carotene (Car) and reduction of a chlorophyll, presumably ChlP1 or ChlD2 next to the dimeric chlorophyll of P680 in the reaction centre of PSII (Shuvalov and Heber, 2003). Similar, but less detailed, spectra were also obtained with dried photosensitive Rhytidiadelphus squarrosus (Heber and Shuvalov, 2005). Importantly, no photoaccumulation of reduced pheophytin was observed. This was attributed to a desiccation-induced shift of the redox potential of Pheo to more negative values which prevents secondary charge stabilization by Chl− + Pheo −→ Chl+Pheo . Alternatively, it is possible that in the absence of water Pheo is stabilized in the reduced form, which is known to be an effective quencher (Klimov et al., 1977). Most Car5 relaxed to Car within 750 ms in spinach, but faster (less than 250 ms) in Rhytidiadelphus as shown in the moss by the relaxation of an absorption increase at 950 nm where Car5 absorbs (Tracewell et al., 2001). Relaxation times are far too slow to attribute a significant contribution of the relaxation of (Chl− Car+) to thermal energy dissipation. Next neighbour analysis revealed a distance between the chlorophyll dimer of P680 and the chlorophyll ChlD1 of less than 5 Å (Kamiya and Shen, 2003). Primary charge separation in the reaction centre in the dried state is represented by P680−Chl− rather than by P680−Pheo− (Shuvalov and Heber, 2003). Secondary charge stabilization in the reaction P680+Car−→P680+Car+ occurred with very low quantum efficiency. The distance between Car and P680 is about 20 Å. Accordingly, charge recombination P680−Chl−→P680Chl becomes highly probable. This fast competitive reaction could actually explain the low quantum efficiency of the photoaccumulation of Car5 and Chl−. Also, a fast recombination P680−Chl−→P680Chl could represent an effective photoprotective reaction similar to the recombination reaction recently proposed by Holt et al. (2005) for zeaxanthin-dependent energy dissipation.

However, the findings of the present work suggest an additional, or an alternative, mechanism of photoprotection. In fully hydrated Rhytidiadelphus, the 800 nm reaction which leads to the formation of a quencher (Fig. 8) has a low quantum efficiency because the electron transport chain is largely oxidized. However, very low light intensities were sufficient for considerable QA reduction and for fluorescence quenching during desiccation (Fig. 4). In this situation, the light curve for the formation of the 800 nm quencher is shifted to lower intensities because an increasing number of RCs contain QA rather than QA. As dehydration progresses, more quencher is stabilized in the RCs contributing to fluorescence quenching. If Pheo− should really prove to be the quencher, energy dissipation in PSII RCs is very effective, because all excited states of RC pigments are transferred to the 800-nm band of the radical which converts excitation energy into heat in the absence of light emission.

By preventing photo-oxidation in the desiccated state, increased thermal dissipation of absorbed light energy permits survival of large groups of photosynthetic organisms under the most extreme environmental conditions. It also permits photosynthetic productivity in arctic zones and in deserts as soon as water becomes available either in the form of rain, dew or high relative humidity in the atmosphere (Green and Lange, 1994).

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References


Protection of desiccated mosses against photo-oxidation


