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Arabitol Provided by Lichenous Fungi Enhances Ability to Dissipate Excess Light Energy in a Symbiotic Green Alga under Desiccation

Makiko Kosugi[1,4], Hirohisa Miyake[2], Hisanori Yamakawa[2], Yutaka Shibata[2,5], Atsuo Miyazawa[1], Takashi Sugimura[3], Kazuhiko Satoh[1], Shigeru Itoh[2,6] and Yasuhiro Kashino[1,*]

[1]Department of Life Science, University of Hyogo, 3-2-1 Kohto, Kamigohri, Ako-gun, Hyogo, 678-1297 Japan
[2]Division of Material Science (Physics), Graduate School of Science, Nagoya University, Nagoya, 464-8602 Japan
[3]Department of Material Science, University of Hyogo, 3-2-1 Kohto, Kamigohri, Ako-gun, Hyogo, 678-1297 Japan
[4]Present address: National Institute of Polar Research, 10-3 Midori-cho, Tachikawa, Tokyo, 190-8518 Japan.
[5]Present address: Department of Chemistry, Graduate School of Science, Tohoku University, Aramaki aza Aoba, Aoba-ku, Sendai, 980-8578 Japan.
[6]Present address: Center for Gene Research, Nagoya University, Chikusa, Nagoya, 464-8602 Japan.
*Corresponding author: E-mail, kashino@sci.u-hyogo.ac.jp; Fax, +81-791-58-0185.
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Lichens are drought-resistant symbiotic organisms of mycobiont fungi and photobiont green algae or cyanobacteria, and have an efficient mechanism to dissipate excess captured light energy into heat in a picosecond time range to avoid photoinhibition. This mechanism can be assessed as drought-induced non-photochemical quenching (d-NPQ) using time-resolved fluorescence spectroscopy. A green alga *Trebouxia* sp., which lives within a lichen *Ramalina yasudae*, is one of the most common green algal photobionts. This alga showed very efficient d-NPQ under desiccation within the lichen thallus, whereas it lost d-NPQ ability when isolated from *R. yasudae*, indicating the importance of the interaction with the mycobiont for d-NPQ ability. We analyzed the water extracts from lichen thalli that enhanced d-NPQ in *Trebouxia*. Of several sugar compounds identified in the water extracts by nuclear magnetic resonance (NMR), mass spectrometry (MS) and gas chromatography (GC) analyses, only D-arabitol recovered d-NPQ in isolated *Trebouxia* to a level similar to that detected for *R. yasudae* thallus. Other sugar compounds did not help the expression of d-NPQ at the same concentrations. Thus, arabitol is essential for the expression of d-NPQ to dissipate excess captured light energy into heat, protecting the photobiont from photoinhibition. The relationship between mycobionts and photobionts is, therefore, not commensalism, but mutualism with each other, as shown by d-NPQ expression.

Keywords: Chl fluorescence • Drought tolerance • Lichen • Non-photochemical quenching • NPQ • Photoinhibition.

Abbreviations: DAS, decay-associated spectrum; ESI-TOF, electrospray ionization-time of flight; d-NPQ, drought-induced non-photochemical quenching; GC, gas chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; NPQ, non-photochemical quenching; PAM, pulse amplitude modulation fluorometry; TFA, trifluoroacetyl; WE, water-soluble extract from lichen thalli.

Introduction

Lichens are symbiotic organisms consisting of fungi (the mycobiont) and photosynthetic partners (the photobiont) that are either green algae or cyanobacteria (Ahmadjian 1988, Ahmadjian 1993). Their extremely high drought tolerance is one of their remarkable characteristics. They are still biologically active even after severe drought stress when they have lost almost all of the water from their thalli (Oliver and Bewley 1997). Drought conditions are frequently coupled with high light conditions, which easily damage the photosynthetic machinery and cause irreversible photoinhibition (Powles 1984, Nishiyama et al. 2006) by inducing triplet state of Chls which produces harmful reactive oxygen species (Krieger-Liszkay 2005). Photosynthetic organisms have developed various protection mechanisms to avoid photoinhibition. A major mechanism that occurs in hydrated chloroplasts is the dissipation of excess light energy into heat by the xanthophyll cycle triggered by illumination. The dissipation results in shorter lifetimes of the excited states of Chls as detected by the measurements of the lifetime and intensity of fluorescence, known as non-photochemical fluorescence quenching (NPQ) (Smirnoff 1993, Heber et al. 2006a, Heber et al. 2006b).

The xanthophyll cycle operates only in wet cells under physiological conditions (Demmig-Adams and Adams 1996).

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However, a very high drought-induced non-photochemical fluorescence quenching [designated as d-NPQ hereafter (Komura et al. 2010)] has been observed in some drought-resistant species of cyanobacteria (Satoh et al. 2002), green algae (Kosugi et al. 2009, Kosugi et al. 2010b), lichens (Heber et al. 2006b, Kosugi et al. 2010b) and bryophytes (Heber et al. 2006a, Nabe et al. 2007). Although it is not yet clear whether the unknown mechanism of this d-NPQ is shared between different groups of photosynthetic organisms, the extent of d-NPQ can be a valuable parameter to assess the capacity of the energy dissipation process.

In desiccated lichens, ultra-fast decays in fluorescence at around 740 nm (F740) with 10 and 40 ps time constants of fluorescence were detected at 290 K and 4 K, respectively, by time-resolved fluorescence analyses (Komura et al. 2010), confirming the result at 77 K (Veerman et al. 2007). The decay time at 680 nm is extremely short compared with that in a normal PSII which gave a decay time of $\geq$200 ps (Komura et al. 2010). The decay time became significantly longer after a few minutes of rewetting lichen. These results indicated that some unknown quencher of fluorescence appeared upon the dehydration of the lichen thallus. Although the molecular identity of the quencher is unknown as yet, it is reasonable to assume it is closely related to d-NPQ (Komura et al. 2010).

Recently, we reported that the cells of the photobiont *Trebouxia* became sensitive to high light with less d-NPQ activity under dehydration conditions after their isolation from *Ramalina yasudae* thalli (Kosugi et al. 2009). Kranner et al. (2005) also described that both the mycobiont and the photobiont suffered oxidative damage during desiccation if they were separated, but, in the form of lichen, each up-regulated the protective system of the other. They showed that the up-regulation was modulated by the water-soluble antioxidant glutathione. However, there have been no reports on the lichenous substance that supports the ultra-fast thermal dissipation of excitation energy of photobionts under dehydration. Most lichenous fungi synthesize metabolites such as sugars, amino acids and lichen acids in their symbiosis with photobionts (Huneck and Yoshimura 1996, Nash 1996). Therefore, we assumed that the sensitivity of isolated *Trebouxia* to high light and the resulting low d-NPQ (Kosugi et al. 2009) were due to the removal of water-soluble substances around the cells of *Trebouxia*. Such substances seem to affect the component(s) that dissipates excess light energy into heat. We identified the substance using the lichen *R. yasudae*.

Results

Arabitol is the major component of water-soluble extracts of *Ramalina yasudae*

To identify candidate components in the lichen that may be responsible for enhancing d-NPQ in the photobiont *Trebouxia*, we extracted water-soluble components from *R. yasudae* thalli, and analyzed them by nuclear magnetic resonance (NMR), electrospay ionization-time of flight mass spectrometry (ESI-TOF MS) and gas chromatography (GC).

The ^{1}H-NMR spectrum of the water-soluble extract (WE) from *R. yasudae* dissolved in D_2O showed typical signals of sugar compounds, but no α-hydrogen signals derived from reducing sugars (**Fig. 1**). After the acetylation process, the WE was subjected to ESI-MS analyses. A mother peak of the acetylated compound in the WE appeared at 385.04 m/z (100%) in the positive ion mode, indicating that the molecular weight was 362.05 after subtracting the mass of Na^+ ($[M + Na]^+ - [Na]^+ = 385.04 - 22.99$). This peak corresponded to a fully acetylated pentitol [$C_5H_7(OCOCH_3)_5$]. There were also small ion peaks assigned to tetraacetyl-pentitol ($m/z = 343.05$, 4.0%) and a deacetoxy fragment ($m/z = 325.03$, 23.1%), and peaks of fully acetylated hexitol ($m/z = 457.08$, 16.0%) and pentaacetylated hexitol ($m/z = 413.23$, 1.3%). Considering the results obtained in ESI-MS analyses, the ^{1}H-NMR spectrum of the WE was identical to that of D-arabitol [(2*R*,4*R*)-pentane-1,2,3,4,5-pentol], whose chemical shifts were 3.77 (ddd, 1H), 3.67 (dd, 1H), 3.59 (ddd, 1H), 3.50 (m, 3H) and 3.50 (dd, 1H) (**Fig. 1**).

We further analyzed the stereochemical features and contents of sugar compounds in the WE by GC analysis, comparing them with acetylated hexitols, sorbitol and mannitol. However, the retention times of the structural isomers were too close to be distinguished. To overcome this difficulty, acetyl groups were replaced by trifluoroacetyl (TFA) groups and the GC analysis conditions were tuned to separate all peaks corresponding to TFA-esters of ribitol, arabitol, xylitol, mannitol and sorbitol. We identified ribitol, arabitol and mannitol in the WE at the retention times of 11.35, 12.5 and 17.40 min, respectively, while xylitol and sorbitol were beneath the level of detection. Judging from the signals shown in **Fig. 1**, there may be other small compounds present in the WE. The proportions of arabitol : ribitol : mannitol were 76.4 : 10.4 : 13.2 by molar ratio, and 74.4 : 15.4 : 10.3 by weight ratio. Thus, the content of arabitol in the lichen thallus was calculated as 0.070 g g^{-1} air-dried weight, or 0.074 g g^{-1} oven-dried weight (120°C, 1 h). This indicated that photobionts in *R. yasudae* would be in an environment with high concentrations of arabitol under drought conditions. Therefore, we examined the effects of arabitol on the fluorescence profile and photosynthetic activities of isolated *Trebouxia* cells.

Effect of arabitol on time-integrated fluorescence spectra of isolated photobiont

The concentration of arabitol in lichen was estimated to be 0.48 M under fully wet conditions based on its yield from lichen thalli, since the wet weight is approximately twice the oven-dried weight. Therefore, the effect of arabitol on the fluorescence was assessed at 0.5 M (but see below). Fluorescence from PSII peaking at 685 and 695 nm (van Dorssen et al. 1987) was very low in dehydrated *R. yasudae* thalli (**Fig. 2A**, red line) and recovered to the high level after rehydration

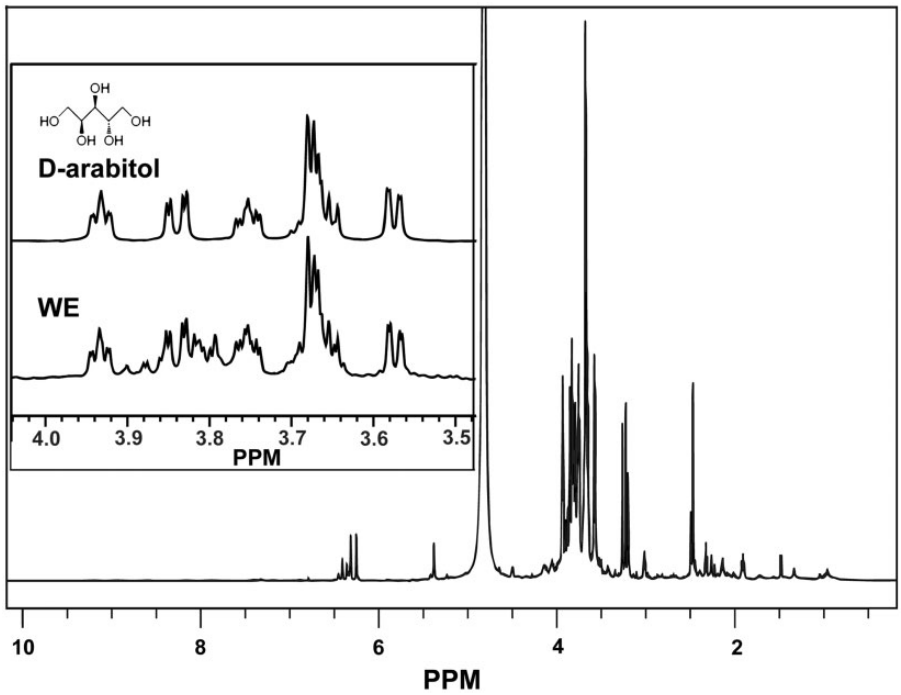

Fig. 1 ^{1}H-NMR spectrum of water-soluble extracts (WE) of *Ramalina yasudae* dissolved in D_2O. The inset shows an enlarged spectrum between 3.44 and 4.02 p.p.m. in which ^{1}H signals from D-arabitol [δ 3.93 (ddd, 1H), 3.84 (dd, 1H), 3.75 (ddd, 1H), 3.67 (m, 3H), 3.57 (dd, 1H)] and other sugar compounds were detected.

(**Fig. 2A**, black line). Desiccation induced only a low level of fluorescence quenching in the isolated *Trebouxia* cells in the absence of arabitol (**Fig. 2B**, red line), and the fluorescence intensity did not recover to its original level after rehydration (**Fig. 2B**, black and blue lines). However, *Trebouxia* cells that were desiccated in the presence of arabitol showed extremely high quenching of PSII fluorescence (**Fig. 2C**, red line). Upon rehydration, the fluorescence intensity almost fully recovered its hydrated level again (**Fig. 2C**, blue line).

Effect of arabitol on time-resolved fluorescence spectra of the isolated photobiont

The effect of arabitol on the decay kinetics of fluorescence at 5 K was assessed using *R. yasudae* (**Fig. 3A, D**) and isolated *Trebouxia* cells that were desiccated in the absence (**Fig. 3B, E**) or presence (**Fig. 3C, F**) of arabitol. The spectral bandwidths of fluorescence (shown in green in **Fig. 3**) were similar among all the cells of *Trebouxia* in different conditions, indicating almost the same conditions for the fluorescence-emitting Chl molecules. However, the decay kinetics of fluorescence varied markedly, indicating the modifications of excitation energy transfer processes. The fluorescence at 680–700 nm originating from PSII (van Dorssen et al. 1987) in *R. yasudae* decayed much faster under dehydrated conditions (**Fig. 3A**) than under wet conditions (**Fig. 3D**), showing the action of d-NPQ. Acceleration of fluorescence decay was also observed in isolated *Trebouxia* cells that were dehydrated in arabitol solution (**Fig. 3C** vs. **F**). However, in the absence of arabitol, PSII fluorescence in isolated *Trebouxia* cells decayed slowly at almost the same rate under both wet and dry conditions (**Fig. 3B, E**), indicating no or a low level of d-NPQ (see also **Supplementary Fig. S1A–C**). The acceleration of fluorescence decay in the presence of arabitol was only observed for PSII fluorescence, not for PSI fluorescence which peaks at around 720 nm (Itoh and Sugiura 2004) (**Fig. 3C, F**; **Supplementary Fig. S1C, F**).

The fluorescence kinetics shown in **Fig. 3** were further analyzed by calculating the decay-associated spectrum (DAS) (**Fig. 4**) to evaluate precisely the effect of arabitol on energy flow among pigments. Wet *R. yasudae* thalli (**Fig. 4D**) showed three DAS components with time constants of 35.4, 117 and 863 ps in PSII Chl fluorescence at 680–700 nm (van Dorssen et al. 1987, Itoh and Sugiura 2004). The slow DAS component with a time constant of 2.79 ns with a positive broad peak at 720 nm can be attributed to the radiative decay of PSI Chl fluorescence. Upon desiccation of *R. yasudae*, all the PSII fluorescence peaks decayed faster (**Fig. 4A**). DAS with a 22 ps time constant showed high positive peaks at around 680 and 740 nm, indicating the fast decay of the 680–690 nm PSII fluorescence band. The band at 720–740 nm that became detectable only after the desiccation seems to indicate the occurrence of d-NPQ, in which the excitation energy on PSII Chls is rapidly transferred to the 740 nm band to be dissipated in turn into heat in less than the detection time of this experiment (see Miyake et al. 2011). The PSI fluorescence component at 720 nm became smaller with only a small change in the time constant (**Fig. 4A, D**).

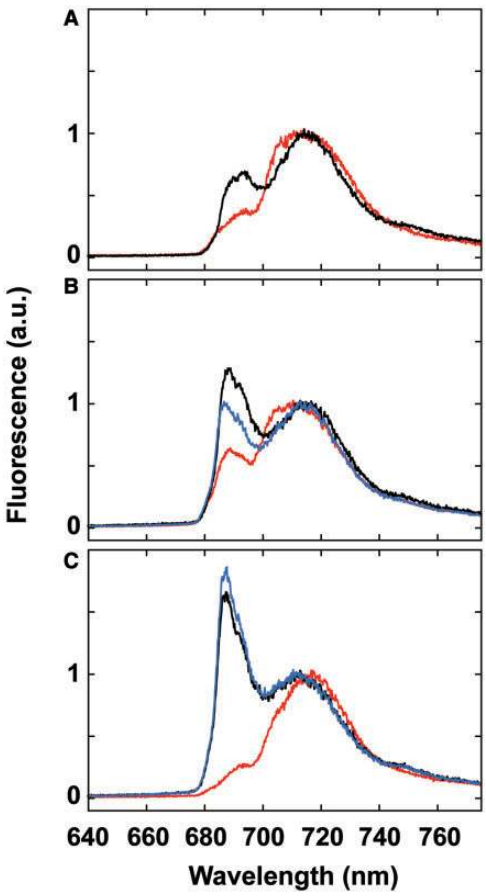

Fig. 2 Effects of desiccation on the time-integrated fluorescence spectra of *Trebouxia* cells and lichen (*Ramalina yasudae*) thallus measured at low temperature (5 K). (A) Lichen thallus of *R. yasudae*. (B) *Trebouxia* cells immersed in solution without arabitol after isolation from *R. yasudae*. (C) *Trebouxia* cells immersed in 0.5 M arabitol solution immediately after isolation from *R. yasudae* thallus. Black, red and blue lines represent fluorescence spectra measured under wet, desiccated and rehydrared conditions, respectively. The excitation wavelength was 430 nm. Spectra were normalized to the fluorescence peak of PSI at 715 nm. The rehydrated condition was considered to be the same as the wet condition for lichen thallus (A) since naturally grown lichen thallus frequently experiences a desiccation–rehydration cycle.

Wet isolated *Trebouxia* cells showed almost the same DAS characteristics as wet *R. yasudae* thalli, regardless of the presence of arabitol (**Fig. 4E, D**). When dried without arabitol (**Fig. 4B**), the DAS characteristics were almost the same as those of the wet thalli shown in **Fig. 4E** with no indication of the 740 nm fast decay band. When dried in the presence of arabitol (**Fig. 4C**), fast DAS components with a time constant of 31.4 ps and positive peaks at 680 and 740 nm became prominent, as seen in natural *R. yasudae* thalli with functional d-NPQ (**Fig. 4A, D**). The relative amplitude and time constant of the PSI fluorescence component at 720 nm, however, remained almost the same when cells were dried both in the presence and in the absence of arabitol (**Fig. 4C, F**).

Effect of concentration of arabitol

Fluorescence decay of cells dried in the presence of different concentrations of arabitol was measured. The PSII and PSI fluorescence band at 77 K at 685 and 720 nm decayed slightly faster when cells were dried with 0.2 M arabitol (**Fig. 5A, B**). The fluorescence decay at 740 nm at 0.2 M arabitol was as fast as that detected at 0.5 M (**Fig. 5C**), indicating that F740 can be induced even at arabitol concentrations <0.2 M, although no effect was observed with 0.5 M ribitol and mannitol (see below).

Effect of other sugar alcohols on d-NPQ

The effects on the fluorescence decay were compared for three sugar alcohols (arabitol, ribitol and mannitol) that were found in the WE (**Fig. 6**). The measurement was performed at the same 0.5 M concentration by assuming their activity coefficients to be similar to each other. Desiccation with arabitol remarkably accelerated the fluorescence decay at 77 K (**Fig. 6**, red line), but not with ribitol (**Fig. 6**, green line) or with mannitol (**Fig. 6**, blue line).

Effect of arabitol on light-induced NPQ

The effects of arabitol as well as ribitol and mannitol on the light-induced NPQ of cells were studied. NPQ, which occurs only in wet cells, is mainly attributable to the activity of the xanthophyll cycle (**Table 1**). The fluorescence yield is already low due to the loss of the F_v component of fluorescence, so that the NPQ is rather difficult to evaluate. However, under the wet and rehydrated conditions, NPQ values showed slightly higher values at any concentration of sugar alcohols than without them. This small effect might be caused by the higher osmotic pressure caused by a high concentration of sugar alcohols. The small NPQ values cannot be attributed to the xanthophyll cycle that should be inactive under dry conditions at which most of the metabolic processes are stopped. Nevertheless, there was no remarkable effect of sugar alcohols on the NPQ values.

Discussion

Arabitol is the major water-soluble component in the mycobiont of *Ramalina yasudae*

Trebouxia and some other green-algal photobionts inside lichen thalli synthesize ribitol [(2*R*,3*s*,4*S*)-pentane-1,2,3,4,5-pentol]. Host fungi then convert ribitol into other sugar compounds such as arabitol and mannitol (Richardson and Smith 1968, Komiya and Shibata 1971). In the present study, the ribitol content was less than a quarter of the arabitol content in the WE (the proportions of arabitol : ribitol : mannitol were 74.4 : 15.4 : 10.3 by weight). This was probably because the ribitol was continuously converted to other sugars, including the most abundant sugar compound, arabitol. Thus, arabitol was the most probable candidate of the substance promoting d-NPQ. As is described above, the concentration of arabitol

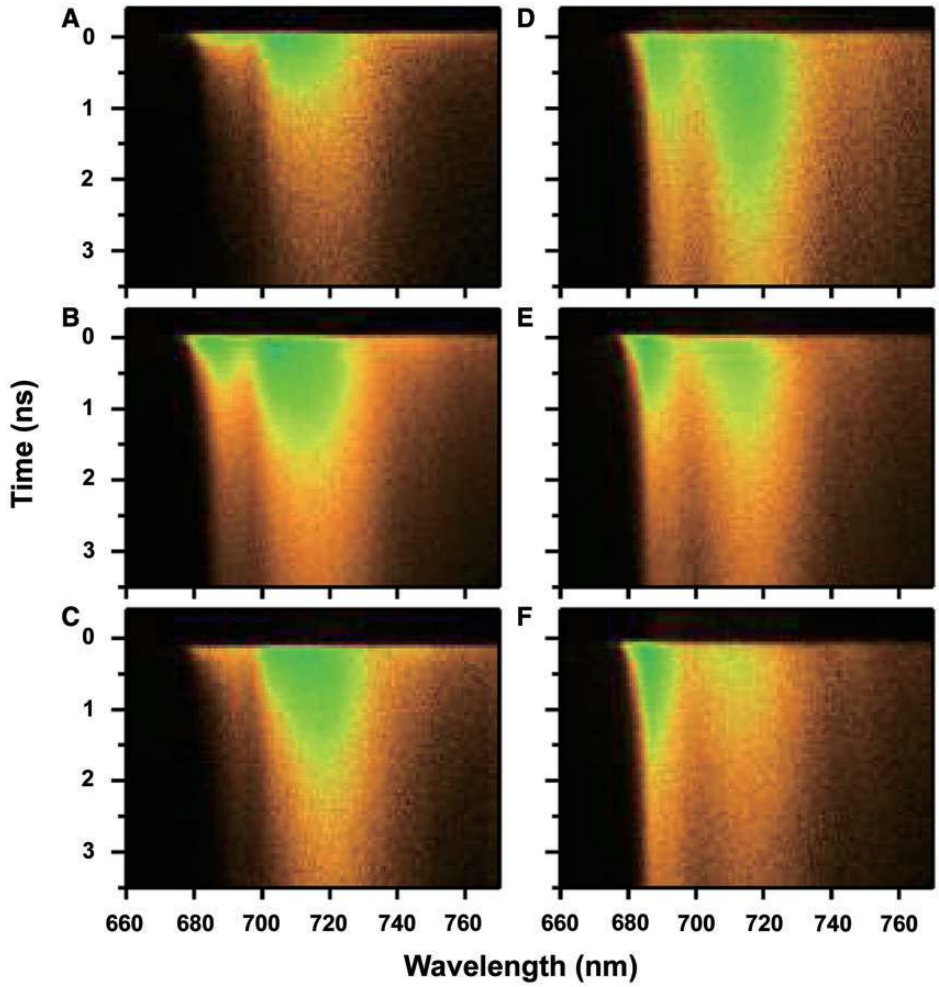

Fig. 3 Effects of desiccation on fluorescence decay measured at 5 K in lichen (*Ramalina yasudae*) thallus (upper) and isolated *Trebouxia* cells in the absence (middle) or presence of 0.5 M arabitol (lower). Fluorescence decay is shown as time–wavelength 2D images. Left panels (A, B and C), desiccated conditions; right panels (D, E and F), rehydrated conditions.

in lichen under fully wet conditions was estimated to be 0.48 M. Under air-dried conditions, the arabitol concentration was estimated to be 7.6 M, if we assume that water fills the total volume of the dried lichen thallus. Our results therefore show that *R. yasudae* contains a very large amount of arabitol in the thallus. We conducted the measurement at 0.5 M arabitol in most cases. Almost the same effect on F740 was observed at a lower concentration (0.2 M) (**Fig. 5**). Therefore, the action of arabitol on d-NPQ could be highly effective.

Lichenous component responsible for d-NPQ

In this work, the effects of sugar alcohols were assessed using the freshly isolated algal photobiont, *Trebouxia*. Although algal photobionts naturally grow free from the lichen body, and isolated algal photobionts can be cultivated in laboratories, the cells would lose the capability to cope with environmental stress after long-term cultivation. Therefore, we improved the method to isolate the algal photobiont freshly from the lichen body while retaining the original high photosynthetic capability and intactness using Percoll (Kosugi et al. 2009, Kosugi et al. 2010a). The method consists of a fast and simple procedure removing the mycobiont and damaged photobiont cells. Although the freshly isolated *Trebouxia* was sensitive to high light under either wet or dry conditions, dehydration in the dark did not affect the photosynthetic characteristics (Kosugi et al. 2009, Kosugi et al. 2010a). A similar method using Percoll was successfully applied to the physiological analysis of the photobiont *Trebouxia* by another group (Gasulla et al. 2010).

Arabitol, ribitol and mannitol were detectable compounds in the WE. Ribitol could be excluded as the candidate responsible for d-NPQ because it is synthesized in the algal photobiont, and should also be present in freshly isolated *Trebouxia* that showed d-NPQ capability when dried with arabitol but not with ribitol (**Figs. 2**, **3**, **4**, **6**). Mannitol did not affect the d-NPQ (**Fig. 6**). Some other compounds present in the WE at levels beneath the detection limits in our analyses could assist d-NPQ. However, arabitol was the most probable compound to enhance d-NPQ for the dissipation of excess light energy in

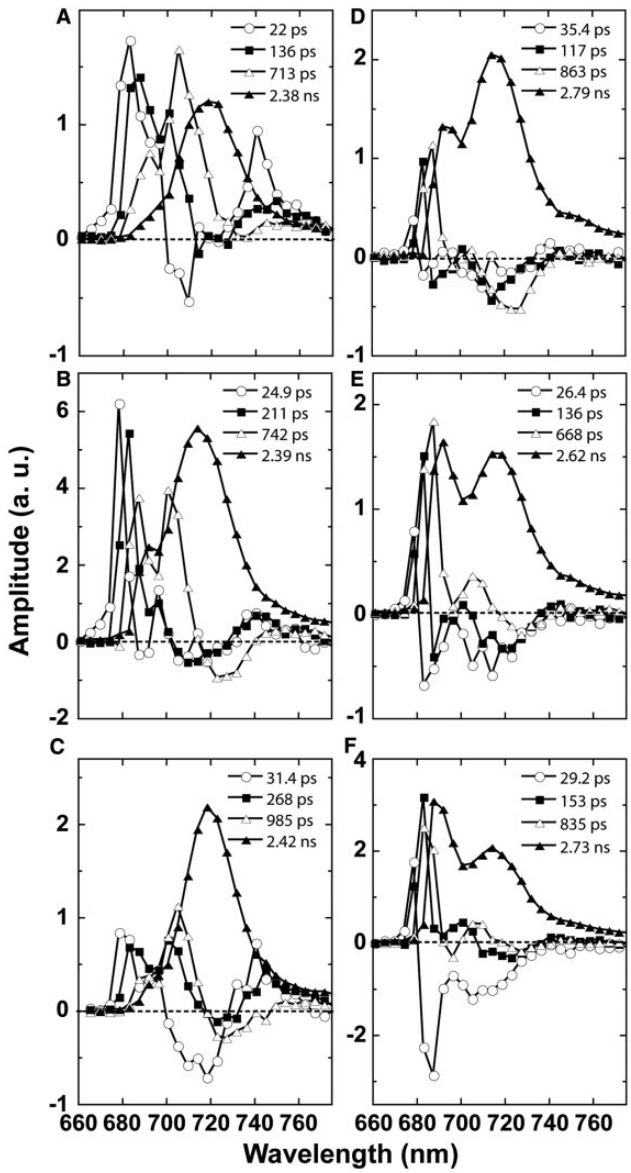

Fig. 4 Effects of desiccation on decay-associated spectra (DASs) of lichen (*Ramalina yasudae*) thallus (top) and isolated *Trebouxia* cells in the absence (middle) or presence of 0.5 M arabitol (bottom). Left (A, B and C) and right (D, E and F) panels show DAS in desiccated and re-hydrated conditions, respectively. Each DAS was calculated from the data shown in **Fig. 3**.

this lichen. In fact, the decay of fluorescence from PSII was greatly accelerated in *Trebouxia* cells that had been dried in the presence of arabitol, but not in the absence of arabitol (**Figs. 2–4**). Even though ribitol and mannitol were assessed at the same concentration as arabitol (the same activity assuming a similar activity coefficient), they did not show any effects on the fluorescence decay kinetics from PSII (685 nm) (**Fig. 6**). Since the content of these two compounds in the WE, and consequently in the thallus body, was lower than that of arabitol, it is not probable that ribitol and mannitol are the compounds responsible for the d-NPQ observed in lichen

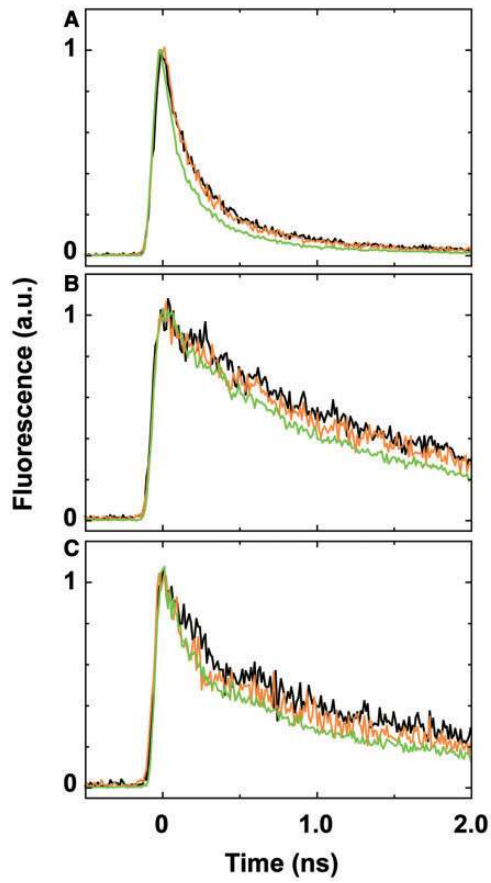

Fig. 5 Effects of arabitol concentration on the decay kinetics of fluorescence in isolated *Trebouxia* cells. Black, brown and green lines represent fluorescence decay measured in the absence of arabitol, or in the presence of 0.2 and 0.5 M arabitol, respectively. The fluorescence decay kinetics were extracted from the time–wavelength 2D images measured as shown in **Fig. 3** but at 77 K at wavelengths 685 ± 0.5 (A), 720 ± 0.5 (B) and 740 ± 0.5 nm (C).

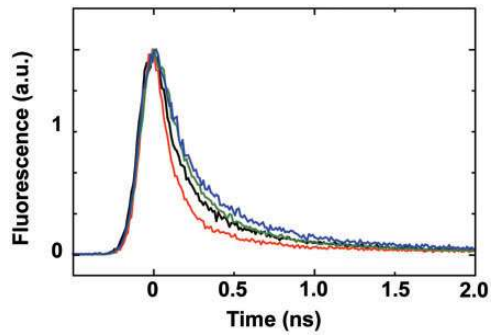

Fig. 6 Time courses of fluorescence decay at 685 nm measured at 77 K in isolated *Trebouxia* cells. Cells were desiccated in the absence (black line) or presence of 0.5 M arabitol (red line), 0.5 M ribitol (green line) or 0.5 M mannitol (blue line). Each decay curve was obtained from picosecond time-resolved fluorescence spectra measured with a streak camera spectrophotometer system at 77 K and was normalized by peak height to unity. The excitation wavelength was 430 nm.

Table 1 Effect of type and concentration of sugar alcohols on the light-induced non-photochemical quenching (NPQ)

		NPQ		
		Wet	**Dry**	**Rehydrated**
No addition		1.04 ± 0.03	0.0541 ± 0.0056	0.377 ± 0.064
Arabitol	0.5 M	1.16 ± 0.03	0.00887 ± 0.00850	0.602 ± 0.134
	1.0 M	1.12 ± 0.20	0.0572 ± 0.0643	0.679 ± 0.073
	2.0 M	1.14 ± 0.11	0.150 ± 0.011	0.452 ± 0.110
	3.0 M	1.34 ± 0.08	0.125 ± 0.040	0.595 ± 0.182
Ribitol	0.5 M	1.11 ± 0.07	0.0500 ± 0.0246	0.524 ± 0.036
	1.0 M	1.18 ± 0.19	0.127 ± 0.015	0.627 ± 0.041
	2.0 M	1.40 ± 0.14	0.0931 ± 0.043	0.562 ± 0.128
	3.0 M	1.32 ± 0.10	0.118 ± 0.007	1.07 ± 0.42
Mannitol	0.5 M	0.991 ± 0.086	0.0243 ± 0.0015	0.190 ± 0.044
	1.0 M	1.19 ± 0.06	0.0450 ± 0.0357	0.254 ± 0.018

Mannitol was assayed only at 0.5 and 1.0 M since its solubility is slightly higher than 1.0 M. ($n = 3$).

thallus (**Figs. 2–4**) although their ability to induce d-NPQ at extremely high concentration could not be completely disproved.

Mechanism by which arabitol accelerates dissipation of excess light energy into heat in the photobiont

Desiccation of lichen thalli induces a long wavelength fluorescence band, F740, which is assumed to accept excitation energy rapidly from shorter wavelength Chls in PSII and dissipate the energy as heat. This process is related to d-NPQ. Although its exact molecular mechanism is unclear as yet (Veerman et al. 2007, Komura et al. 2010, Miyake et al. 2011), it is considered that its dissipation process is quite different from that in the xanthophyll cycle (Komura et al. 2010, Miyake et al. 2011). Since the level of fluorescence quenching is remarkable in PSII rather than PSI (**Fig. 1**), and F740 appears with PSII- and light-harvesting complex II (LHCII)-related fluorescence components (**Fig. 4**), the origin of F740 could reside within PSII or LHCII. This issue was discussed in detail previously (Komura et al. 2010, Miyake et al. 2011). We detected the fast 30 ps decay component attributable to F740 in desiccated *R. yasudae* and in isolated *Trebouxia* cells desiccated in the presence of arabitol (**Fig. 4**; **Supplementary Fig. S1**) and not in the absence of arabitol (**Figs. 3**, **4**). Considering the physicochemical characteristics of arabitol, it is impossible that it functions as a quencher that accepts energy from PSII. Arabitol should affect d-NPQ indirectly (**Fig. 7**). One possible mechanism for the quenching is that arabitol modulates expression of genes related to d-NPQ. Heber et al. (2007) reported that d-NPQ decreased markedly in rapidly dried lichen, which is consistent with the assumption that de novo gene expression plays a role in this process. Sugar compounds regulate intracellular osmolality (Brown and Simpson 1972) to create high osmotic pressure inside the cells and to acquire water from ambient air or from the lichen matrix, since their hydroxyl groups form

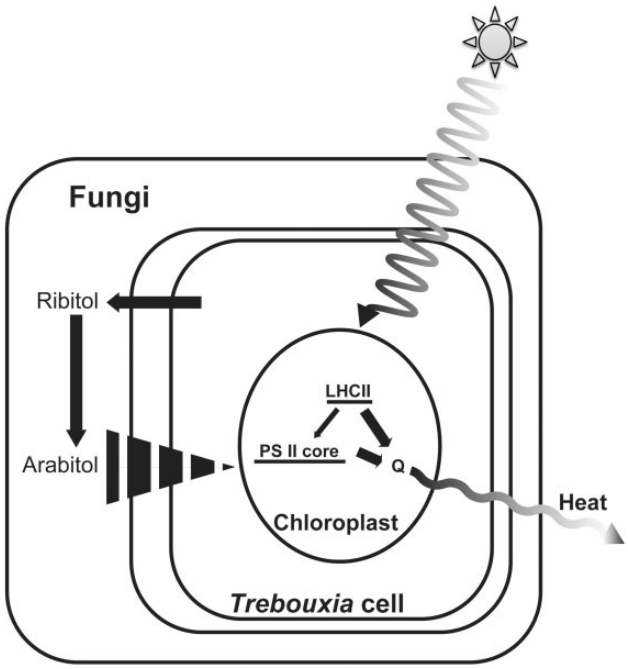

Fig. 7 Model of action of arabitol in lichen under dry conditions. Arrows and horizontal bars in *Trebouxia* represent the flow of light energy and schematic energy levels of photosynthetic pigments in pigment-binding proteins. See text for details.

hydrogen bonds with water. Within the thallus, *Trebouxia* cells might be slowly desiccated in the presence of high concentrations of arabitol during the dehydration process. If the drought-induced thermal dissipation mechanism requires gene expression and protein synthesis, arabitol would slow down the dehydration rate of the cells, allowing such gene expression and protein synthesis to occur. However, this scenario is less probable since mannitol did not affect d-NPQ capability (**Fig. 6**). Similarly, a simple osmotic effect to that shown for xanthophyll cycle activity (Azzabi et al. 2012) could be excluded because ribitol and sorbitol did not show the capability to induce d-NPQ at the same concentration as arabitol (**Fig. 6**).

Another possible role for arabitol in supporting quenching is that it may change protein conformation during dehydration. De-epoxydation of xanthophyll cycle pigments is known to be induced by the formation of a transmembrane pH gradient that also induces protonation of PsbS (Li et al. 2002). Instead of PsbS, in a green alga *Chlamydomonas reinhardtii*, LHCSR (formerly called LI818) is thought to play a critical role in qE capacity, which leads to NPQ (Peers et al. 2009). However, d-NPQ is not related to these mechanisms (Heber et al. 2006a), and the mechanisms to dissipate excess light energy are different between d-NPQ and the xanthophyll cycle (**Table 1**), as was discussed previously (Miyake et al. 2011). Furthermore, it is known that many sugar compounds with hydroxyl groups stabilize proteins (Smirnoff 1998). Those molecules take the place of water molecules that bind to the proteins during the desiccation process (Smirnoff 1998) and may protect proteins from

complete dehydration. Until now, there have been no reports showing that sugar compounds are translocated from the mycobiont to the photobiont. Therefore, we analyzed sugar compounds in *Trebouxia* cells immediately after isolation from the lichen thalli by GC analysis. Ribitol was the major component (18.6 mg g^{-1} dried cells) of isolated *Trebouxia*, while the levels of arabitol and other sugar compounds were below the detection limit. It is very unlikely that arabitol within the cells was lost during the process of isolating *Trebouxia* from lichen, leaving a high amount of ribitol within the cell. Accordingly, arabitol should accumulate in the thallus matrix. Since algal symbionts occupy approximately 10% of the thallus weight, the amount of ribitol in the photobiont cells within a lichen thallus would be approximately 13% of the total ribitol in the thallus body based on the chemical analysis described above. Although most ribitol exists in the thallus matrix or within the mycobiont body, it should not be the representative to induce d-NPQ as described above.

Concluding remarks

Photosynthetic organisms should possess several pathways to dissipate excess light energy as heat. Those pathways may function cooperatively depending on the environments. Under wet and high light conditions, mainly xanthophyll cycle activity, which can be measured by PAM (pulse amplitude modulation fluorometry) as NPQ, might drain excess light energy. The drought-induced excess light energy dissipation mechanism in PSII is important for photosynthetic organisms to survive under extreme desiccation and strong light conditions. NPQ is often used to refer to a heat dissipation mechanism activated by the lumen acidification in plants and algae (the qE component of NPQ). However, d-NPQ is stably induced by drought and does not depend on strong illumination. Both qE and d-NPQ are observed as NPQ, but their mechanisms are likely to be different. Our results showed that the mycobiont of *R. yasudae* provides an environment for the photobiont *Trebouxia* sp. to induce d-NPQ, and that this process relies on arabitol provided by the mycobiont (**Fig. 7**). Based on our results, the relationship between lichenous fungi and photobionts is not the classical view of commensalism, but mutualism (Kranner et al. 2005). *Trebouxia* strengthen the protection mechanism against high light under dehydration by their symbiosis with the mycobiont.

Materials and Methods

Materials

Ramalina yasudae was collected from the Harima Campus for Science of the University of Hyogo, Hyogo Prefecture, Japan (35°55′N, 134°26′E). The thalli were washed with water and air-dried at 60% relative humidity for 1 d in the dark. The dehydrated thalli were stored at −15°C until use.

Trebouxia sp. cells were isolated from *R. yasudae* thalli rehydrated in buffer A containing 25 mM MES-NaOH (pH 5.5) according to Millbank and Kershaw (1969) with some modifications as described in Kosugi et al. (2010a).

To extract water-soluble substances from *R. yasudae*, the thalli were rehydrated in pure water and then ground with a mortar and pestle on ice. After the removal of debris using a nylon mesh (pore size, 150 μm), the filtrate was centrifuged at 7,000×*g* for 12 min at 4°C. The resulting supernatant was passed through filter paper (No. 2, Advantec). The filtrate was freeze-dried and stored at −15°C. The yield of this water-soluble extract (designated as WE) was 9.0% (w/w) of dried thalli.

To extract the intracellular sugar compounds, *Trebouxia* cells were extracted three times with 80% ethanol solution at 80°C for 1 h. The extract was filtered through filter paper (No. 2, Advantec), and then dried by evaporation. The DW of the extract was 9.3 mg from 0.5 g DW of *Trebouxia* cells isolated from 7.7 g DW of *R. yasudae*.

Dehydration and rehydration of materials

The lichen thalli and isolated *Trebouxia* cells were dried at 25°C in the dark at 60% relative humidity for 1 d. *Trebouxia* cells were air-dried on clear plastic film so that fluorescence measurements could be conducted in situ. To rehydrate the materials, thalli or cells that were air-dried in the dark were immersed in distilled water for 1 h in the dark. To assess the effect of arabitol, the wet and rehydrated cells were incubated in 0.5 M arabitol solution in the dark until the electron transport activities assessed by PAM (Walz) reached a steady-state level (>2 h). The effect of arabitol under the desiccated condition was assessed using the isolated *Trebouxia* cells incubated in the 0.5 M arabitol solution for 2 h at 25°C in the dark followed by desiccation on a clear plastic film in the presence of arabitol solution. The dried cells were rehydrated by adding distilled water so that the arabitol concentration recovered the level of before dehydration.

Analysis of major water-soluble components of lichen

NMR measurements of the WE and sugar compounds were performed with an ECA-600 NMR spectrometer (JEOL) after the extracts were dissolved in D_2O.

MS analyses were conducted with a high resolution ESI-TOF mass spectrometer (AccuTOF, JMS-T100LC; JEOL) after acetylation of substances in the WE. Acetylation followed the standard method: a mixture of 0.0202 g of WE, 0.2 ml of acetic anhydride and 0.0114 g of anhydrous sodium acetate in a recovery flask fitted with a Liebig condenser under nitrogen was heated at 100°C for 5 h, and then extracted with methylene chloride. Intracellular sugar compounds extracted from *Trebouxia* were acetylated by the same process.

GC analyses were performed with a GC-17A (Shimadzu) instrument with a capillary column (TC-1, 30 m, i.d. 0.25 mm; GL Science Inc.) at the linear speed of 27.5 cm s^{-1}. The temperature of the injection block and detector was 230°C. The column temperature was kept at 80°C for 20 min, and then

raised to 180°C (5°C min^{-1}) to elute all substances from the column. Because acetylation structural isomers of hexane-1,2,3,4,5,6-hexol (hexitol) could not be separated by GC, fluorinated esters were used. The acylation reaction was carried out by heating 1 mg of WE and 0.1 ml of trifluoroacetic anhydride (TFAA) in 0.5 ml ethyl acetate at 50°C for 1 h in a 1 ml vial. After dilution with ethyl acetate, the mixture was injected into the gas chromatograph. Peak area calibration and peak assignments were conducted for authentic sugar compounds (arabitol, ribitol, xylitol, mannitol and sorbitol). The amounts of sugar compounds in the WE were determined by analyzing a mixture of 0.23 mg of ribitol and 1 mg of WE. When determining the amount of ribitol in the WE, a mixture of 0.5 mg of arabitol and 1 mg of WE was used.

Analysis of fluorescence kinetics

Picosecond time-resolved fluorescence spectra were measured with a streak camera spectrophotometer system as described in Komura et al. (2010) at 5 K or 77 K. DASs were obtained by a global multiexponential fitting analysis (Komura et al. 2010) of the fluorescence kinetics.

Measurement of light-induced non-photochemical quenching

Light-induced NPQ was measured using PAM as described by Kosugi et al. (2009). The parameter NPQ was calculated by the following equation;

$$\mathrm{NPQ} = \left[F_\mathrm{m} - F'_\mathrm{m}\right]/F'_\mathrm{m}$$

Freshly isolated *Trebouxia* cells were incubated for 2 h with sugar alcohol solution at 0, 0.5, 1.0, 2.0 and 3.0 M. Dehydration was performed as described above. Dried cells were re-hydrated by adding distilled water for 2 h prior to the measurements. NPQ was determined after 5 min illumination under a halogen light of 210 µmol photons m^{-2} s^{-1}, which did not induce photoinhibion in the absence of sugar alcohols.

Supplementary data

Supplementary data are available at PCP online.

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