Low temperature induced modulation of photosynthetic induction in non-acclimated and cold-acclimated Arabidopsis thaliana: chlorophyll a fluorescence and gas-exchange measurements

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Abstract
Cold acclimation modifies the photosynthetic machinery and enables plants to survive at sub-zero temperatures, whereas in warm habitats, many species suffer even at non-freezing temperatures. We have measured chlorophyll a fluorescence (ChlF) and CO2 assimilation to investigate the effects of cold acclimation, and of low temperatures, on a cold-sensitive Arabidopsis thaliana accession C24. Upon excitation with low intensity (40 µmol photons m−2 s−1) ~ 620 nm light, slow (minute range) ChlF transients, at ~ 22 °C, showed two waves in the SMT phase (S, semi steady-state; M, maximum; T, terminal steady-state), whereas CO2 assimilation showed a linear increase with time. Low-temperature treatment (down to −1.5 °C) strongly modulated the SMT phase and stimulated a peak in the CO2 assimilation induction curve. We show that the SMT phase, at ~ 22 °C, was abolished when measured under high actinic irradiance, or when 3-(3, 4-dichlorophenyl)-1,1- dimethylurea (DCMU, an inhibitor of electron flow) or methyl viologen (MV, a Photosystem I (PSI) electron acceptor) was added to the system. Our data suggest that stimulation of the SMT wave, at low temperatures, has multiple reasons, which may include changes in both photochemical and biochemical reactions leading to modulations in non-photochemical quenching (NPQ) of the excited state of Chl, “state transitions,” as well as changes in the rate of cyclic electron flow through PSI. Further, we suggest that cold acclimation, in accession C24, promotes “state transition” and protects photosystems by preventing high excitation pressure during low-temperature exposure.

Keywords Low-temperature effect · Cold acclimation · Chlorophyll fluorescence transients · Slow SMT fluorescence phase · Gas-exchange measurements · State transition · 3-(3, 4-dichlorophenyl)-1, 1- dimethylurea · Methyl viologen

Abbreviations

A CO2 assimilation rate
AC Cold acclimated

A gross Gross CO2 assimilation rate
A max Maximum CO2 assimilation rate under saturating light
Chl a Chlorophyll a
ChlF Chlorophyll a fluorescence
DCMU (also called diuron) 3-(3, 4-dichlorophenyl)-1, 1- dimethylurea
F m Maximum fluorescence intensity during actinic light exposure
F m′(t) Maximum fluorescence intensity during dark-relaxation
F683 Fluorescence emission band, with a maximum at 683 nm
F735 Fluorescence emission band, with a maximum at 735 nm

Five of us (KBM, AM, JK, OU, and AGH) pay tribute to three pioneers of Photosynthesis Research, Agepati S. Raghavendra (carbon reactions, specifically for C4 photosynthesis), William A. Cramer (bioenergetics, specifically for biochemistry and biophysics of cytochromes), and Govindjee (primary photochemistry and electron transport, specifically for the unique role of bicarbonate in Photosystem II; also a co-author of this manuscript), for their contributions and commendable leadership in photosynthesis research.

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Extended author information available on the last page of the article

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$F_m$ Maximum fluorescence when the (plasto) quinone $Q_A$ is fully reduced

$F_O$ Minimum fluorescence when $Q_A$ is fully oxidized

$F_P$ Fluorescence intensity at the P level

$F_{M1}$ Fluorescence intensity at peak $M_1$

$F_{M2}$ Fluorescence intensity at peak $M_2$

$F_T$ Terminal steady-state fluorescence

$F_v$ Maximum variable ChlF ($F_m - F_O$)

$I_{S60}$ Induction state of $A$ at 60 s after illumination, expressed as a percent of $A_{max}$

$IT_{50}$ Induction time required to reach 50% of $A_{max}$

$k$ ($k^{-1}$) Rate constant (inverse of rate constant) [of the P-to-S phase]

$LHCs$ Light-harvesting complexes

$M_1, M_2$ First and second maxima after peak P ($F_P$) in the SMT phase of ChlF transient

$MV$ Methyl viologen

$NAC$ Non-acclimated

$NPQ$ Non-photochemical quenching (of the excited state of Chl a)

$PQ$ Plastoquinone

$PSI$ Photosystem I

$PSII$ Photosystem II

$Q_A, Q_B$ The first and the second (plasto) quinone acceptors of electrons in the reaction center of PSII

$R_{FD}$ Fluorescence decrease ratio defined as $F_D/F_T$, where $F_D = F_P - F_T$

$RuBP$ Ribulose-1,5-bisphosphate

$SMT$ Slow phase of chlorophyll $a$ fluorescence transient (where S is semi steady-state, M is a maximum and T is terminal steady-state)

$F_{M1}$ Time required to reach $M_1$ level of ChlF transient

$F_{M2}$ Time required to reach $M_2$ level of ChlF transient

$t_{50}$ Time required for 50% decline from $P$ ($F_P$) to the S level

$ΔpH$ pH difference across the thylakoid membrane

$Φ_{PSII}$ Quantum yield of PSII photochemistry

$Φ_{NPQ}$ Quantum yield of “regulated” non-photochemical quenching

$Φ_d$ Quantum yield of photoinhibition (I) quenching of Chl fluorescence

$Φ_{qT}$ Quantum yield of state-transition (T) quenching of Chl fluorescence, during State I (high fluorescence) to State II (low fluorescence)

Introduction

Low temperature, classified as chilling (0 °C < T < 15 °C) or freezing (T < 0 °C), is a major factor in limiting growth, survival and geographical distribution of plants (Berry and Björkman 1980; Allen and Ort 2001; Hasdai et al. 2006). Plants adapted to warm habitats are highly susceptible to cold (prolonged exposure to low temperature, or a sudden temperature drop). Under such conditions, there is a decrease in the yield, or a complete failure, of important agricultural crops (Leegood and Edwards 1996). In cold habitats, however, plants overcome the effects of low temperature, and survive even at sub-zero temperatures. The gain in cold tolerance is due to a highly complex process of cold acclimation (Guy 1990; Thomashow 2010; Crosatti et al. 2013), activated by dynamic interaction between the exposure to non-freezing temperatures (0–10 °C) and light (Wanner and Junttila 1999; Smallwood and Bowles 2002; Franklin and Whitelam 2007; Catalá et al. 2011). It is well known that the capacity for cold acclimation is dependent on the latitudinal range of habitats in natural accessions of Arabidopsis thaliana (hereafter Arabidopsis) (Hannah et al. 2006; Mishra et al. 2011, 2014; Lukas et al. 2013). However,
mechanisms underlying cold acclimation as well as regulatory steps controlling re-adjustment of the physiological homeostasis at low temperatures remain poorly understood. This is particularly true for responses of photosynthetic reactions, and associated metabolic pathways, when plants experience a sudden drop in temperature. Understanding the underlying biophysical, biochemical, and physiological processes is crucial for elucidating how photosynthesis functions at these temperatures, particularly for the development of breeding strategies (Ort et al. 2015).

Plants have evolved several regulatory strategies for protecting their photosystems while optimizing efficiency under unfavorable environmental stimuli. In general, low temperature leads to a decline in membrane fluidity that inhibits electron transport by restricting mobilization of hydrophobic proteins, redox homeostasis, and the repair cycle of D1 protein (Aro et al. 1990; Allen and Ort 2001). Low temperature affects the rate of enzymatic reactions of the Calvin–Benson cycle far more than the primary physophysical and photochemical processes, involved in light absorption, excitation energy transfer, and conversion of light energy into chemical energy, and, thus, there is the possibility of imbalance between the absorbed and the used light energy (Berry and Björkman 1980; Bernacchi et al. 2002; Ensinger et al. 2006; Sage and Kubien 2007; Horton 2012; Khanal et al. 2017). The decreased biochemical consumption of the products of photochemical reactions, i.e., NADPH and ATP, in the enzymatic reactions, in turn, creates a situation as if there is high irradiance, which may cause elevated excitation pressure (Öquist et al. 1987; Huner et al. 1998) and over-reduce the system (Endo et al. 2005). This stimulates several stress responses, e.g., increases in the cyclic electron transport around photosystem (PS) I (Endo et al. 2005), in the water–water cycle (Asada 1999), and production of damaging reactive oxygen species (Triantaphylides and Havva 2009) that may further result in photo-inhibition (Allakhverdiev et al. 1997; Tyystjärvi 2013) of both PSI and PSII. The light-harvesting complexes (LHCs) of both photosystems must, therefore, dispose of potentially harmful absorbed energy by dissipating it as heat through photo-protective processes, such as non-photochemical quenching (NPQ) of the excited state of chlorophyll d (Chl d) (Demmig-Adams and Adams 2000; Müller et al. 2001; Ruban et al. 2007; Murchie and Niyogi 2011; Horton 2012; Demmig-Adams et al. 2014). Moreover, cold acclimation reduces the functional absorption cross section of PSII antenna to avoid over-excitation of the system (Huner et al. 1998; Khanal et al. 2017).

Adjustments in photosynthetic mechanisms in response to low temperatures can be probed in vivo through non-invasive and highly sensitive Chl d fluorescence (ChlF) measurements. ChlF induction kinetics, fluorescence emission spectra at room temperature and at 77 K, and their parameters have been successfully used to probe the effects of biotic as well as abiotic stress on photosynthetic reactions (Oxborough 2004; Papageorgiou and Govindjee 2004; Baker 2008; Malenovsky et al. 2009; Kalaji et al. 2012; Goltsve et al. 2016; Mishra et al. 2016a; Stirbet et al. 2018). Application of saturation pulses on ChlF transient has been used for understanding the fate of absorbed energy into highly complex photochemical and non-photochemical processes. Efforts have been undertaken by several laboratories to extend the so-called “saturation pulse methods” for energy partitioning, for the evaluation of the quantum yield of various photochemical and non-photochemical processes (Cailly et al. 1996; Hendrickson et al. 2004; Kramer et al. 2004; Ahn et al. 2009; Guadagno et al. 2010; reviewed in Lazár 2015). Information on the quantum yield of various processes is also needed for a detailed understanding of the responses of the photosynthetic apparatus to environmental factors and acclimation. Nevertheless, ChlF methods have not been fully exploited for the in vivo analysis of photosynthetic responses to cold acclimation and low temperatures. However, attempts have already been made to use ChlF parameters for the screening of cold tolerance (Baker and Rosenqvist 2004; Ehlerl and Hincha 2008; Mishra et al. 2011, 2014; Humplík et al. 2015) and for the investigation of certain metabolic effects on PSII integrity (Knaupp et al. 2011). The relative contributions of PSII and PSI, as measured through both ChlF induction and emission spectra, were found to change during cold exposure (Agati et al. 2000; Franck et al. 2002). By measurement of ChlF parameter, $F_v/F_m$, i.e., the ratio of variable to maximal fluorescence, which represents maximum quantum yield of PSII photochemistry, during cooling of detached leaves to the freezing temperature of $-20$ °C, Ehlerl and Hincha (2008) and Knaupp et al. (2011) showed that damage to PSII occurs at temperatures below those causing damage to the plasma membrane. Usually, $F_v/F_m$ is very stable at or near sub-zero temperatures (Pospíšil et al. 1998; Mishra et al. 2011), and its decline at lower temperatures might be due to freezing-induced severe dehydration stress (Hacker et al. 2008), which occurs probably by “blocking” of the reaction centers at lower temperatures (Pospíšil et al. 1998). Mishra et al. (2011) have measured ChlF transients in differentially cold-tolerant Arabidopsis accessions during progressive cooling ($-2$ °C h$^{-1}$) and reported that low-temperature treatments stimulate slow (seconds to minute range) SMT fluorescence phase (for a review of this phase, see Papageorgiou and Govindjee 2011; also see Stirbet and Govindjee 2016). Although the presence of slow SMT phase in ChlF transients has been reported in various photosynthetic organisms (Papageorgiou et al. 2007; Papageorgiou and Govindjee 2011), yet, the mechanisms involved in the formation of SMT phase and its relation to photosynthetic processes at low temperature is not at all clear.
In order to better understand how photosynthetic processes and the SMT phase of ChlF induction are influenced by low temperatures, we have measured changes in slow ChlF transients, in the fluorescence emission spectra in the 650–780 nm range, and evaluated quantum yield of photochemical and non-photochemical quenching of the excited state of Chl α, at selected temperatures during controlled cooling from 22 °C down to −1.5 °C, in non-acclimated (NAC) and cold-acclimated (AC) leaves of the cold-sensitive Arabidopsis accession C24 (Hannah et al. 2006). Further, we measured fluorescence emission spectra at 77 K, before and after low-temperature treatment at −1.5 °C to access effects on “state changes” (Kaňa et al. 2012). In order to understand how the slow SMT phase of ChlF transient is formed, we have analyzed detached leaves that were vacuum infiltrated with the electron transport inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and a PSI electron acceptor, methyl viologen (MV). In addition, we have measured the induction of CO₂ assimilation (A) in intact NAC and AC C24 plant rosettes to investigate how low temperatures affect CO₂ assimilation. Here, we present new datasets that revealed photochemical as well as biochemical effects of low temperature on the modulation of SMT fluorescence phase and the importance of cold acclimation in regulation of photosynthetic processes at low temperatures.

Materials and methods

Plant material and growth conditions

Arabidopsis C24, originating from the Iberian Peninsula (see Schmid et al. 2003; Hannah et al. 2006), was grown, for four weeks, in GS90 soil and vermiculite (1:1). Three plants per 100-mm diameter pot were placed in a growth chamber [8-h fluorescent light (120 µmol photons m⁻² s⁻¹; 22 °C)/16-h dark; 16 °C]. Then the plants were transferred to long day conditions (16-h light, 8-h dark), but kept under the same irradiance and the temperature regime. Plants were watered daily and fertilized every two weeks with standard nitrogen (N), phosphorus (P), and potassium (K) fertilizer. Six weeks after germination, half of the plants (24 in number) were transferred to a 4 °C chamber for cold acclimation (AC) for two weeks, whereas the non-acclimated (NAC) plants were kept at 22 °C (day) and 16 °C (night). All physiological measurements were made on 8-week-old NAC and AC plants. For measuring CO₂ assimilation rates of the whole rosettes, we used Arabidopsis plants as described above, but their seedlings (two weeks after germination) were transplanted in Ray Leach cone-type pots (140 mm long; 38 mm diameter; Stuewe & Sons Tangent, Oregon, USA) that were suitable to fit into a whole plant Arabidopsis chamber (6400-17; LI-COR Biosciences, USA).

Chlorophyll a fluorescence (ChlF) transients

For measuring ChlF transients, detached leaves, from both NAC and AC plants, were arranged on a black wooden plate, and their petioles were kept under drops of water. The HANDY chlorophyll fluorescence imaging system (Photon Systems Instruments, Brno, CZ) was set above the black plate with leaf samples inside a cooling device that was slowly cooled at a rate of −2 °C h⁻¹, from 10 °C to −2 °C. Cooling from 22 °C to 10 °C was accomplished within 1 h, and then the slow cooling (−2 °C h⁻¹) down to −2 °C was started. The cooling device was programmed to stop cooling at selected temperatures during the measurement. The temperatures in the vicinity of the detached leaves were measured by two sets of data loggers [DS1921 Thermocron, Maxim Integrated, San Jose (CA), USA], which monitored the temperature every five minutes. The fluorescence imaging system, used for measuring ChlF transients (Fig. 1), as described by Mishra et al. (2014), was equipped with four orange light (620 nm) emitting diode panels, programmed to generate 10-µs measuring flashes (−1 µmol photons m⁻² s⁻¹; frequency: 19.63 Hz), saturating flashes of −1800 µmol photons m⁻² s⁻¹, and actinic light of selected intensity. Flashes of the light-emitting devices (LEDs) were synchronized with the opening of the electronic shutter of the charged-coupled device (CCD) camera (512×512 pixels, 12 bits). An optical dark red glass filter (RG695) was placed in front of the CCD camera to block the light of the LEDs. ChlF transients are usually measured after 20–30 min of dark adaptation (which is long enough to have all Qₐ in the oxidized state); longer dark adaptation may inactivate several Calvin–Benson cycle enzymes; these changes also influence ChlF transients (see Mishra et al. 2016b). Thus, we programmed our imaging system to measure ChlF transients automatically at 30-min intervals during the cooling of the leaves from 22 °C to −1.5 °C.

The experimental protocol for measuring ChlF transients (Fig. 1) began with the measurement of minimum (O, F₀) and maximum (Fₘ) fluorescence levels by exposing leaves, respectively, to the measuring and the saturating light flashes. After ~60 s dark adaptation, leaves were exposed to actinic irradiance [40 µmol photons m⁻² s⁻¹] for about 8 min to measure the kinetics of the ChlF transient up to the terminal steady-state (Fₜ). At the end of the actinic illumination, a saturating flash was applied to probe the non-photochemical quenching [NPQ = (Fₘ − Fₐ)/Fₘ, where Fₘ is the maximal fluorescence in light; Maxwell and Johnson 2000] and the effective quantum yield of PSII photochemistry [ΦPSII = (Fₘ − Fₜ)/Fₘ; Genty et al. 1989] of the leaves. Further, the fluorescence decrease ratio (Rₖ/Fₜ) was calculated. After the actinic light was switched off, a saturating flash was given after ~30 s...
Photosynthesis Research

13

to measure the relaxation of maximum fluorescence in the dark, \( F''_m(t) \), for investigating the relaxation of the faster energy-dependent quenching (qE).

Quantum yield of photochemical and non-photochemical quenching processes

To estimate the quantum yields of photochemical and different types of non-photochemical processes using energy partitioning approach (Cailly et al. 1996; Hendrickson et al. 2004), ChlF transients of 20-min dark-adapted leaves of both the NAC and the AC plants were measured for about 15 min in the presence of actinic light; this was followed by the measurement of the dark-relaxation of the \( F'_m, F''_m(t) \), at several time intervals ranging from 2 to 1200 s, as used by Roháček et al. (2008). For further details on the measuring protocol, see Supplementary Fig. S1 (also see Roháček et al. 2008; Roháček 2010). We used here the equations given by Hendrickson et al. (2004) to quantify the partitioning of absorbed energy into the quantum yield of PSII photochemistry (\( \Phi_{\text{PSII}} = (F'_m - F_T)/F'_m \); Genty et al. 1989), the quantum yield of “regulated” NPQ (\( \Phi_{\text{NPQ}} = F_T/F'_m - F_T/F'_m \); Cailly et al. 1996; cf. Lazár 2015), and the “non-regulated” NPQ composed of the sum of quantum yield of fluorescence and “constitutive” thermal dissipation (\( \Phi_{\text{t,d}} = F_T/F'_m \); Cailly et al. 1996; cf. Lazár 2015). Further, we analyzed the dark-relaxation kinetics of NPQ by non-linear regression of three exponentially decay components in order to resolve the half times of the following processes: ΔpH dependent (fast), excitation energy re-distribution due to state transition (medium), and photo-inhibition (slow), as proposed by Walters and Horton (1991). We have used here methods given by Guadagno et al. (2010) for the calculation of quantum yields of the components of the regulatory NPQ, \( \Phi_{\text{NPQ},i} \), i.e., \( \Phi_{\text{qE}}, \Phi_{\text{qT}} \), and \( \Phi_{\text{qI}} \). For further detailed information, see Supplementary Fig. S1, the paper by Guadagno et al. (2010) and the review by Lazár (2015).

ChlF spectra measured during low-temperature treatment

Individual detached leaves were placed in a cooling device to measure ChlF emission spectra by FluorMax 3 spectrofluorometer (Jobin Yvon, Horiba Scientific, Japan). Leaves were illuminated with blue light (480 ± 0.5 nm, bandwidth 1 nm), obtained from a xenon lamp in the FluorMax instrument, and the steady-state ChlF emission spectra, after 5 min of illumination, were measured in the 640–780 nm range with an integration time of 0.5 s and a bandwidth of 1 nm. Both the excitation light (falling on the leaf surface)
and the fluorescence (emitted by the leaf) were remotely sensed through a fiber-optic bundle that was connected to the excitation spectrometer and the front-face detector of the FluorMax via F-3000 Fiber Optic Mount. The sample was cooled at 2 °C h⁻¹, the cooling was stopped at selected temperatures, and then the fluorescence emission spectra were measured.

Low-temperature (77 K) fluorescence spectra

In order to check if there was state change (State I has relatively higher PSII than PSI fluorescence), we measured 77 K (using liquid nitrogen) ChlF spectra of the control and low-temperature (− 1.5 °C)-treated detached leaf discs from NAC and AC Arabidopsis plants. Leaf discs were placed, in a closed translucent Dewar flask, at 77 K, on a specially designed metal probe. All the leaf discs used had equal size and their ChlF spectra were recorded by a spectrometer (SM9000, Photon Systems Instruments, Brno, CZ) following excitation with LED blue light (λ = 470 nm, Δλ = 20 nm).

Treatment with diuron (DCMU) and methyl viologen (MV)

Detached leaves of both NAC and AC Arabidopsis plants were vacuum infiltrated for 20 s, at a pressure of ~ 400 mbar, with 10 ml solutions of (i) Diuron, 3-(3,4-dichlorophenyl)-1,1- dimethylurea [DCMU, which inhibits electron transport from PSII to PSI by displacing Qb in PSII (see Govindjee and Spilotro 2002 for the procedure)] or (ii) Methyl viologen [MV, which accepts electrons from PSI (Munday and Govindjee 1969)] at room temperature. Because of the low solubility of DCMU in water, we first dissolved it in ethanol and then made stock solutions. The final concentrations of the chemicals used were: 100 μM and 200 μM DCMU, and 100 μM and 500 μM MV. To compare results between the control and chemically treated samples, all the samples had 2% ethanol in the 10 ml solvent.

Gas-exchange measurements of CO2 assimilation rate

Plants, grown in Ray Leach cone-type pots, were kept in a whole plant Arabidopsis chamber (6400-17, LI-COR Biosciences, Lincoln, NE, USA) that was air sealed; this chamber was attached to RGB (red–green–blue) light source (6400-18A, LI-COR Biosciences, USA) and a gas-exchange measuring system (LI-6400-18, LI-COR Biosciences, USA). The whole plant, fitted within its chamber, was placed in a freezer with controlled cooling (as described above), where 45-min dark-adapted rosettes were exposed to 300 μmol photons m⁻² s⁻¹ irradiance to measure CO₂ assimilation rates (A). Plant rosettes were provided with constant ambient CO₂ (385 ± 5 μmol CO₂ mol⁻¹) at 50 ± 5% air humidity. The time course of A was automatically recorded throughout the experiment, at 15-s intervals, during the dark adaptation period as well as during the light phase. The precise setting of temperature between 22 and 0 °C inside the assimilation chamber was achieved by using an integrated Peltier thermoelectric cooler of the LI-6400-18 type. The measured CO₂ assimilation rate was normalized per unit leaf area, which was estimated from the corresponding photographs of the rosettes with the help of Adobe Photoshop software (Adobe Systems Software, Dublin, Ireland). The gross CO₂ assimilation rate (A_{gross}) was estimated by adding the rate of respiration to the rate of net photosynthesis, the former measured during the dark phase of the assimilation curve. From the photosynthetic induction curves, we calculated the A after 15 min of light exposure (A_{max}), and at 60 s after illumination, expressed as a percentage of A_{max} (IS₆₀); in addition, we recorded the time required to reach 50% of A_{max} (IT₆₀). For further details, see Chazdon and Pearcy (1986) and Urban et al. (2008).

Tools for data analysis and statistical analysis

Image processing software integrated with the FluorCam (http://www.psi.cz) was used to process the “captured” fluorescence image sequences and to calculate the averaged fluorescence parameters over the whole leaf area. Specific features of Graphpad Prism Software, version 5 (GraphPad software, La Jolla, CA, USA), were used for a quantitative comparison of the area under different fluorescence phases, e.g., OPS and SMT of ChlF transients, for evaluating the parameters of ChlF decay from the peak P (Fₚ) to the semi steady-state S, e.g., t₅₀ (the time to reach 50% of the fluorescence intensity from the peak P to the S level) and the associated rate constant (k), and for calculating the applicable tests (t test, one-way ANOVA, two-way ANOVA) to evaluate statistical significance of differences between the parameters. For evaluating the areas under different phases (OPS, S₁M₁T₁, and S₂M₂T₂), F₀ was set as a baseline for each curve; lower and upper limits of points in the x-axis were manually controlled for obtaining precise and accurate integration of the area under each peak. The CO₂ induction curve was fitted using distance-weighted least-squares function, and then the IS₆₀ and IT₆₀ were calculated.

Results

Cold acclimation induced changes in ChlF transients of detached Arabidopsis leaves

ChlF transients of detached leaves of NAC and AC plants, measured at ~22 °C, upon illumination with low (40 μmol
photos $m^{-2} s^{-1}$) actinic orange (620 nm) light are shown in Fig. 2 (solid lines) (here, and in all the following figures, we do not include the relaxation of $F''_m (t)$ in the ChlF transients for better presentation of the slow fluorescence phase). The curves of ChlF transients are expressed as $F/F_m$ since $F_m$ was almost invariable in the measured temperature range.

ChlF transients of both NAC and AC leaves show a rise in fluorescence intensity from the O ($F_O$) level to the peak P ($F_P$) followed by a slow decline that includes two fluorescence waves with maxima $M_1$ (NAC $\sim 33.3 \pm 0.4$ s; AC $\sim 31.2 \pm 1.0$ s) and $M_2$ (NAC $\sim 72.6$ s; AC $\sim 66.7 \pm 0.8$ s) (Table 1), both being much lower than $F_P$ (Fig. 2). The time to reach the P and the $M_1$ levels decreased with increasing actinic irradiance, from 80 to 160 $\mu$mol photons $m^{-2} s^{-1}$, while the time to reach the $M_2$ level increased. Fluorescence intensity at $M_2$ showed a stronger decrease at 160 $\mu$mol photons $m^{-2} s^{-1}$ as compared to that at 80 $\mu$mol photons $m^{-2} s^{-1}$ (see Supplementary Figs. S2A, S3A, and Table S1). Further, at ~300 $\mu$mol photons $m^{-2} s^{-1}$, both $M_1$ and $M_2$ were absent (see Supplementary Figs. S2B, S3B, and Table S1).

A comparison of ChlF curves (Fig. 2) and the calculated parameters (Table 1) of NAC and AC leaves showed that cold acclimation significantly increased $F_O$ ($p < 0.01$) as well as the terminal steady-state fluorescence $F_T$ ($p < 0.01$), but decreased the $F_P$ ($p < 0.01$) and the $M_2$ ($p < 0.05$) levels. Cold acclimation, when measured at 22 °C, did not show any effect on $F_S/F_m$ (and, thus, $\Phi_{PSII}$), but it significantly decreased the $R_{FD}$ value (Table 1). Quantitative comparison of changes in ChlF transients from the peak P ($F_P$) to the semi steady-state S confirmed that two weeks of cold acclimation (AC) significantly increased $k^{-1}$ and $t_{50}$ by ~20%, ~4%, respectively, and decreased the value of $(F_P − F_S)/F_S$, by ~13%, as compared to their NAC counterparts (see Table 2).

Low temperature induced changes in ChlF transients in NAC and AC leaves

There was no significant difference in $F_S/F_m$ between NAC and AC leaves during low-temperature treatment from 22 °C to −1.5 °C; however, cooling down from 22 °C and 10 °C,

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**Table 1** The basic chlorophyll fluorescence parameters from detached leaves of non-acclimated (NAC) and cold-acclimated (AC) *Arabidopsis thaliana* accession C24

<table>
<thead>
<tr>
<th>ChlF parameters</th>
<th>NAC</th>
<th>AC</th>
<th>NAC (+ ethanol)</th>
<th>AC (+ ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_(F_P)$ (s)</td>
<td>3.4 ± 0</td>
<td>3.4 ± 0</td>
<td>3.4 ± 0</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>$t_(M_1)$ (s)</td>
<td>33.3 ± 0.4</td>
<td>31.2 ± 1.0 (*)</td>
<td>23.2 ± 0</td>
<td>27.2 ± 2.5</td>
</tr>
<tr>
<td>$t_(M_2)$ (s)</td>
<td>72.6 ± 0.8</td>
<td>66.7 ± 0.8 (**)</td>
<td>79.2 ± 8.8</td>
<td>73.2 ± 7.0</td>
</tr>
<tr>
<td>$F_O$</td>
<td>0.141 ± 0.001</td>
<td>0.154 ± 0.002 (**)</td>
<td>0.147 ± 0.001</td>
<td>0.158 ± 0.001 (**)</td>
</tr>
<tr>
<td>$F_P$</td>
<td>0.52 ± 0.01</td>
<td>0.46 ± 0.01 (**)</td>
<td>0.55 ± 0.03</td>
<td>0.47 ± 0.01 (*)</td>
</tr>
<tr>
<td>$F_(M_1)$</td>
<td>0.286 ± 0.003</td>
<td>0.267 ± 0.003 (**)</td>
<td>0.329 ± 0.006</td>
<td>0.289 ± 0.018</td>
</tr>
<tr>
<td>$F_(M_2)$</td>
<td>0.312 ± 0.001</td>
<td>0.280 ± 0.002 (**)</td>
<td>0.347 ± 0.005</td>
<td>0.286 ± 0.018 (*)</td>
</tr>
<tr>
<td>$F_(p)$</td>
<td>0.168 ± 0.001</td>
<td>0.182 ± 0.002 (*)</td>
<td>0.191 ± 0.006</td>
<td>0.204 ± 0.002 (*)</td>
</tr>
<tr>
<td>$F_(p)/F_m$</td>
<td>0.853 ± 0.003</td>
<td>0.842 ± 0.002</td>
<td>0.849 ± 0.002</td>
<td>0.837 ± 0.007</td>
</tr>
<tr>
<td>$\Phi_{PSII}$</td>
<td>0.790 ± 0.001</td>
<td>0.787 ± 0.004</td>
<td>0.766 ± 0.021</td>
<td>0.780 ± 0.019</td>
</tr>
<tr>
<td>$R_{FD}$</td>
<td>2.08 ± 0.04</td>
<td>1.58 ± 0.03 (**)</td>
<td>1.88 ± 0.06</td>
<td>1.33 ± 0.09 (**)</td>
</tr>
</tbody>
</table>

Mean ± SE ($n=4$ for AC/NAC). Asterisks (*) in the column of AC samples denote statistical significance of the data with respect to their NAC counterparts (*$p<0.05$, **$p<0.01$, ***$p<0.001$). See list of abbreviations for the definition of the parameters used here.

Photosynthesis Research
respectively, to −1.5 °C had a significant effect on $F_p/F_m$ in NAC leaves, but not in AC leaves (Table 2). ChlF transients of the leaves of the control NAC and AC plants, recorded at selected temperatures, during slow cooling from 22 °C to −1.5 °C, showed temperature-dependent changes in the OPS phase as well as in the SMT phase of the ChlF transients (see panels A and B in Fig. 3). Cooling of NAC leaves to 10 °C slightly increased $F_p$ (−4%), but further cooling to 4 °C, 0 °C, and −1.5 °C significantly reduced its value by ~42%, 37%, and 33%, as compared to that measured at 22 °C (Fig. 3A). We do not yet understand the reasons for this change. On the other hand, in AC leaves cooling to 10 °C and 4 °C, respectively, increased the level of $F_p$ by 19% and 28%, while further cooling to 0 °C and −1.5 °C brought back its value close to what was observed at 22 °C (Fig. 3B). For quantitative evaluation of effects of low temperature on ChlF transients, we compared the changes in the areas under the OPS (with peak $M_1$) and the SMT (with peaks $M_1$ and $M_2$) phases during cooling (see panels C and D of Fig. 3). At 22 °C, the area of the fast OPS phase was smaller (by ~8.3% in NAC and by ~16.6% in AC leaves) as compared to that under the SMT phase (with ~0.6% for $M_1$ and ~90.1% for $M_2$ in NAC; and ~2.3% for $M_1$ and ~81.1% for $M_2$ in AC). Further cooling below 10 °C led to a decrease of the OP phase, followed by an increase of the SMT phase, where the area under $M_1$ predominated over that under $M_2$ (see Fig. 3C, D).

The time to reach $F_p$ (~3.4 s) was essentially unaffected by low-temperature treatment, except in the NAC leaves when cooled to −1.5 °C; we found that, at this temperature, the position of $F_p$ was delayed by ~6 s as compared to that measured at 22 °C (Table 3). At 10 °C, 4 °C, 0 °C, and −1.5 °C, the intensity of the peak $M_1$ in the NAC samples increased by ~26%, 33%, 47%, 57%, as compared to that measured at 22 °C (Fig. 3A). However, in AC samples, cooling to the same temperatures increased the intensity of $M_1$ by ~19%, 68%, 75%, and 81%, respectively (Fig. 3B). The linear cooling caused high fluctuations in the position of peak $M_1$, but it significantly increased the time to reach $M_2$ in both the NAC and the AC leaves, as compared to that measured at 22 °C (Table 3; Fig. 3A, B). The position of $M_2$ in NAC leaves showed shifts of 124 s, 128 s, 297 s, and ~92% and ~73%, respectively, in NAC and AC leaves, as compared to its value at 22 °C (Table 2). Further, we found that cooling induced an increase in the intensity of the $M_1$ peak (~4%), but further cooling when cooled to 10 °C and 4 °C, respectively, and its intensity was also reduced. However, further cooling abolished the $M_2$ peak (Table 3; Fig. 3A). In addition, a continuous increase in $F_p$ during the low-temperature treatment was observed for both the NAC and the AC leaves (Fig. 3A, B). We note that cooling induced an increase in the intensity of $F_p$ at 10 °C in the NAC, and at 10 °C and 4 °C in the AC leaves. This was accompanied by an increase in $k^{-1}$ or $t_{50}$, but this was transient, peaking at a lower temperature in AC than in the NAC leaves (Table 2). The decrease in the fluorescence intensity from the P to the S level, as represented by the “quenching” ratio ($F_p - F_S)/F_S$, was shown by Briantais et al. (1979) to be linearly related to the transmembrane proton difference. In our experiments, low-temperature treatments decreased the P–S quenching ratio ($F_p - F_S)/F_S$, and at −1.5 °C, this ratio decreased by ~92% and ~73%, respectively, in NAC and AC leaves, as compared to its value at 22 °C (Table 2). Further, we found that the correlation between the P–S quenching ratio and temperature was stronger in NAC than in AC leaves ($R^2$ of 0.94 vs. 0.82). Comparatively higher value of quenching ratio, at −1.5 °C, and lower $R^2$ between this quenching ratio and temperature for AC leaves may indicate that cold-acclimated plants have a higher capacity to energize thylakoids by pumping comparatively more protons (H+s) into the lumen; in other words, they seem to be able to induce a steeper proton concentration difference (ΔpH) across the thylakoid membrane, which may favor cyclic electron flow through PSI for adjusting the rate of ATP production, according to the metabolic needs of the sample (see, e.g., Shikanai 2007).

### Table 2

The effect of low temperature on several parameters: maximum quantum yield of PSII photochemistry ($F_p/F_m$), rate constant ($k^{-1}$), time to reach 50% from peak P to S ($t_{50}$), and the ratio ($F_p - F_S)/F_S$ for NAC and AC plant leaves

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$F_p/F_m$</th>
<th>$k^{-1}$ (s)</th>
<th>$t_{50}$ (s)</th>
<th>($F_p - F_S)/F_S$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NAC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>22</td>
<td>0.853 ± 0.003</td>
<td>14.3 ± 0.3</td>
<td>7.23 ± 0.12</td>
</tr>
<tr>
<td>+2% ethanol</td>
<td>22</td>
<td>0.849 ± 0.002</td>
<td>16.6 ± 0.6</td>
<td>6.91 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>0.855 ± 0.002</td>
<td>38.1 ± 7.6</td>
<td>10.05 ± 0.87</td>
<td>0.45 ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.841 ± 0.006</td>
<td>–</td>
<td>6.44 ± 0.47</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>0</td>
<td>0.837 ± 0.009</td>
<td>–</td>
<td>7.12 ± 0.12</td>
<td>0.10 ± 0.07</td>
</tr>
<tr>
<td>−1.5</td>
<td>0.827 ± 0.009</td>
<td>–</td>
<td>10.32 ± 0.21</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td><strong>AC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>22</td>
<td>0.842 ± 0.002</td>
<td>17.2 ± 0.4</td>
<td>7.51 ± 0.07</td>
</tr>
<tr>
<td>+2% ethanol</td>
<td>22</td>
<td>0.837 ± 0.007</td>
<td>21.9 ± 3.9</td>
<td>7.63 ± 0.41</td>
</tr>
<tr>
<td>10</td>
<td>0.845 ± 0.011</td>
<td>30.8 ± 6.7</td>
<td>8.81 ± 1.83</td>
<td>0.59 ± 0.11</td>
</tr>
<tr>
<td>4</td>
<td>0.842 ± 0.010</td>
<td>–</td>
<td>9.57 ± 0.86</td>
<td>0.35 ± 0.10</td>
</tr>
<tr>
<td>0</td>
<td>0.839 ± 0.010</td>
<td>–</td>
<td>9.91 ± 3.15</td>
<td>0.34 ± 0.10</td>
</tr>
<tr>
<td>−1.5</td>
<td>0.830 ± 0.012</td>
<td>–</td>
<td>7.28 ± 0.46</td>
<td>0.24 ± 0.13</td>
</tr>
</tbody>
</table>

Mean ± SE (n=3/3 for control, 7/8 AC/NAC). Asterisks (*) in the column of AC samples denote statistical significance of the data with respect to their NAC counterparts (*p < 0.05, **p < 0.01, ***p < 0.001)
Fig. 3 Chlorophyll \(a\) fluorescence transients of leaves of NAC (A) and AC (B) *Arabidopsis thaliana* accession C24 at selected temperatures inside the freezer; samples were cooled at the rate of 2 °C h\(^{-1}\). Corresponding cooling induced modifications of areas under the OPS [with peak \(P (F_p)\)] phase and the SMT phase (with peaks \(M_1\) and \(M_2\)) are shown in panels C (NAC) and D (AC). In the panels A and B, the vertical lines at semi steady-states \(S_1\) & \(S_2\), and at maxima \(M_1\) & \(M_2\), are for the curves measured at 22 °C. Other experimental conditions are as described in the legends of Figs. 1 and 2.

Table 3 The effect of low temperature on time in seconds to reach \(P (t_{F_p})\), \(M_1 (t_{M1})\) and \(M_2 (t_{M2})\), for NAC and AC plant leaves. Mean ± SE (\(n = 4\) for AC and 3 for NAC)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>(t_{F_p}) (s)</th>
<th>(t_{M1}) (s)</th>
<th>(t_{M2}) (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAC</td>
<td>AC</td>
<td>NAC</td>
</tr>
<tr>
<td>+2% ethanol</td>
<td>22</td>
<td>3.4 ± 0.0</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>4.2 ± 0.0</td>
<td>3.6 ± 0.3</td>
<td>28.2 ± 1.2</td>
</tr>
<tr>
<td>4</td>
<td>3.9 ± 0.0</td>
<td>4.7 ± 0.6</td>
<td>29.5 ± 2.9</td>
</tr>
<tr>
<td>0</td>
<td>3.3 ± 0.8</td>
<td>4.5 ± 0.6</td>
<td>35.2 ± 5</td>
</tr>
<tr>
<td>−1.5</td>
<td>9.2 ± 3.0</td>
<td>3.8 ± 0.2 (*)</td>
<td>37.1 ± 7</td>
</tr>
</tbody>
</table>

For control samples, detached leaves were used after vacuum infiltration in distilled water containing 2% of ethanol. Asterisks (*) in the column of AC samples denote statistical significance of the data with respect to their NAC counterparts (* \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\)).
Low temperature induced changes in the quantum yield of photochemical and regulated and non-regulated non-photochemical quenching

Low temperatures induced changes in the quantum yield of PSII photochemistry ($\Phi_{\text{PSII}}$), of regulated NPQ ($\Phi_{\text{NPQ}}$), and of the sum of quantum yields of fluorescence and basal constitutive thermal dissipation ($\Phi_{f,d}$), as evaluated according to the energy partitioning approach (see Hendrickson et al. 2004). Our data, recorded for NAC as well as AC leaves, are shown in Fig. 4. Contribution of the regulatory quantum yield of NPQ, $\Phi_{\text{NPQ}}$, is very small (~4%) as compared to $\Phi_{f,d}$ (~18%) and $\Phi_{\text{PSII}}$ (~78%) for both NAC and AC at 22 °C. Both the $\Phi_{\text{NPQ}}$ and the $\Phi_{\text{PSII}}$ decreased by 2% with cooling until 4 °C; however, they had almost similar values for NAC and AC leaves (Fig. 4). Reductions in the $\Phi_{\text{NPQ}}$ and $\Phi_{\text{PSII}}$, while cooling down to 4 °C, were compensated by similar increases in $\Phi_{f,d}$. However, further cooling to 0 °C and −1.5 °C led to an increase in the contribution of $\Phi_{\text{NPQ}}$ to 10% and 20% in NAC, and to 7% and 15% in AC leaves, and this increase in the $\Phi_{\text{NPQ}}$ was compensated by a decrease in the $\Phi_{\text{PSII}}$. Significantly higher $\Phi_{\text{NPQ}}$ and lower $\Phi_{\text{PSII}}$ were observed in leaves of NAC (Fig. 4A) as compared to those from AC (Fig. 4B) plants at both 0 °C and −1.5 °C.

In order to further investigate non-photochemical processes being affected by low temperatures under the prevailing actinic irradiance, we evaluated components of quantum yield of NPQ, $\Phi_{\text{NPQ}}$: (1) a fast reversible energy-dependent quenching caused by $\Delta pH$ ($\Phi_{qE}$), (2) a slow reversible phosphorylation of light-harvesting complexes of PSII that stimulates state-transition quenching ($\Phi_{qT}$; State I to State II), and (3) the slowest of all, the photoinhibitory quenching ($\Phi_{qI}$), in both the NAC and the AC leaves at selected temperatures (Fig. 5). Although $\Phi_{\text{NPQ}}$ declined during the initial cooling from 22 to 4 °C, we did not find any statistically significant

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Fig. 4 Estimated quantum yields of PSII photochemistry ($\Phi_{\text{PSII}}$), of regulated non-photochemical quenching ($\Phi_{\text{NPQ}}$) and of non-regulated basal constitutive thermal dissipation and fluorescence ($\Phi_{f,d}$) for NAC (A) and AC (B) Arabidopsis thaliana accession C24, measured at selected temperatures during cooling. We have used the method given by Hendrickson et al. (2004) for estimating $\Phi_{\text{PSII}} = (F_m' - F_T)/F_m'$; $\Phi_{\text{NPQ}} = F_T/F_m' - F_T/F_m$; and $\Phi_{f,d} = F_T/F_m$. For details, see Material and Methods and Supplementary Fig. S1. Standard errors (± SE) are shown ($n = 3$ or 4).

Fig. 5 Components of quantum yield of regulated non-photochemical quenching ($\Phi_{\text{NPQ}}$) measured from the detached leaves of NAC (A) and AC (B) Arabidopsis thaliana accession C24. $\Phi_{qE}$ is the quantum yield for high-energy quenching, $\Phi_{qT}$ is the quantum yield of state change (State I to State II), and $\Phi_{qI}$ is the quantum yield of photoinhibition. For measuring components of $\Phi_{\text{NPQ}}$, chlorophyll fluorescence of the corresponding leaves were measured for 15 min under actinic light, followed by the measurement of relaxation of variable fluorescence at different time intervals for another 20 min in the dark, and their values were calculated as described by Guadagno et al. (2010). For further details, see Material and Methods and Supplementary Fig. S1. Standard errors (± SE) are for $n = 3$ or 4.
difference between the different components of $\Phi_{\text{NPO}}$ in both NAC and AC leaves (Fig. 5). A two-way ANOVA confirmed a significant increase in $\Phi_{\text{qE}}, \Phi_{\text{qT}},$ as well as in $\Phi_{\text{qI}},$ in NAC leaves (Fig. 5A), whereas in the AC leaves, the increase was significant only for $\Phi_{\text{qE}}$ and $\Phi_{\text{qT}}$ and the value of $\Phi_{\text{qI}}$ remained similar to that at the higher temperatures (Fig. 5B).

Low temperature induced changes in fluorescence spectra at room temperature

We observed that low temperatures induced a larger increase in the far-red fluorescence band (F735, due to both PSII and PSI; cf. Agati et al. 2000; Franck et al. 2002; Lamb et al. 2018) than in the red fluorescence band (F683, mostly due to PSII), and this rise was most prominent at $-1.5 \, ^\circ\text{C}$ in NAC (Fig. 6A). However, in AC (Fig. 6B) leaves, it was prominent not only at $-1.5 \, ^\circ\text{C}$ but also at $4 \, ^\circ\text{C}$ and $0 \, ^\circ\text{C}$. This caused a decrease in $F_{683}/F_{735}$ by $\sim 7\%$ for NAC at $-1.5 \, ^\circ\text{C}$ and $8\%, 19\%,$ and $23\%$ for AC at $4 \, ^\circ\text{C}, 0 \, ^\circ\text{C},$ and $-1.5 \, ^\circ\text{C},$ respectively, as compared to the values at $10 \, ^\circ\text{C},$ which were $3.02 \pm 0.08$ (for NAC) and $3.40 \pm 0.07$ (for AC). Such a change could mean that low temperatures promoted State I to State II change, but in view of the complexities involved in deciphering PSI and PSII fluorescence at room temperature (see above, and Govindjee and Yang 1966; Franck et al. 2002), this conclusion cannot be made. Thus, we measured emission spectra at $77 \, ^\circ\text{K}$ (see below).

Low temperature induced changes in fluorescence spectra at $77 \, ^\circ\text{K}$

To obtain information on low-temperature-induced state transitions (Fork and Satoh 1986; Kaňa et al. 2012), affected possibly by “migration” of LHCs between PSII and PSI, we measured $77 \, ^\circ\text{K}$ ChlF spectra of leaf discs from NAC and AC plants. The samples were either directly taken from their adaptive growth temperatures or after low-temperature treatment at $-1.5 \, ^\circ\text{C}$ (Fig. 6C). The $F_{695}$ band (at $77 \, ^\circ\text{K}$) is known to originate from Chls in CP47 and the $F_{683}$ from Chls in both CP43 and CP47 (see, e.g., Andrizhiyevskaya et al. 2005); in addition, data of Chen et al. (2015) suggest that $F_{683}$ may also include a contribution of RC Chls. Further, it is known that the $F_{735}$ band (at $77 \, ^\circ\text{K}$) originates mostly from Chl $a$ in PSI (see reviews in Govindjee et al. 1986; and Papageorgiou and Govindjee 2004). Our results show that cold acclimation led to a reduction in the intensity of the $F_{735}$ band by $\sim 22\%$ in AC as compared to that in NAC plants (Fig. 6C); $F_{683}/F_{735}$ increased from $0.226 \pm 0.009$ in NAC leaves to $0.290 \pm 0.018$ in AC leaves ($\sim 28\%, p < 0.01$), indicating that cold acclimation “shifts” LHCs towards PSII, a slightly larger State II to State I transition in AC plants. However, when treated at $-1.5 \, ^\circ\text{C},$ this band of both NAC and AC leaf samples was broadened.
but the ratio F683/F735 was significantly reduced by 13% ($p < 0.05$) only in leaves from the AC plants, indicating that $-1.5 ^\circ C$ causes State I to State II change, as implied by room temperature data (see above).

**Effects of DCMU and methyl violugen (MV) on ChlF transients of NAC and AC leaves at room temperature**

In order to investigate the impact of cold acclimation and low temperatures on specific photochemical processes, we measured ChlF transients of detached leaves treated with DCMU (that blocks electron flow between $Q_A$ and the PQ pool, and thus, from PSII to PSI; see Holub et al. 2007) and MV (that accepts electrons from PSI; see Munday and Govindjee 1969). For both AC and NAC plant leaves, 20-s vacuum infiltration (at $\sim 400$ mbar) with 2% ethanol had negligible effects on the pattern of ChlF transients, including the SMT fluorescence phase (dotted lines in Fig. 2), and on several ChlF parameters, such as the maximum and effective quantum yield of PSII photochemistry ($F_v/F_m$ reflecting $\phi_{PSII}$) and the time to reach $F_p$ ($t_Fp$) (Table 1). Although vacuum infiltration did cause an increase in $F_O$ and $F_T$, and a decrease in $R_{FP}$, in both NAC and AC samples, yet differences in these parameters between NAC and AC leaves were preserved (Table 1). Further, vacuum infiltration of detached leaves with 2% ethanol had insignificant effects on $k^{-1}$ ($t_{50}$) and ($F_p - F_S$)/$F_S$ in AC plants; however, the same treatment significantly increased $k^{-1}$ by 2.3 s (16%) and decreased ($F_p - F_S$)/$F_S$ to $\sim 25\%$ in NAC plants (Table 2).

To check if the vacuum-infiltrated chemicals indeed entered leaf discs, ChlF transients were measured at 22 °C. Clear and significant effects were observed in ChlF transients of leaves from both NAC (solid lines of Fig. 7) and AC (dotted lines see Fig. 7) plants. We conclude that higher concentrations of chemicals were needed in the leaves from NAC as compared to those from AC plants: (1) 200 µM of DCMU did not fully abolish the P to S decay of the ChlF transients in NAC leaves (blue solid line of Fig. 7), whereas 100 µM DCMU abolished both the fast P–S and slow SMT fluorescence phase in AC leaves (blue dotted line of Fig. 7); (2) increasing concentrations of MV gradually decreased the slow SMT phase, and 500 µM MV was enough to eliminate the SMT phase in NAC (solid magenta line of Fig. 7), whereas only 100 µM MV was needed to do the same in AC samples (dotted magenta line of Fig. 7).

**Low-temperature treatment modifies the slow SMT phase in DCMU and MV-infiltrated leaves**

In order to investigate effects of low temperature on ChlF transients of leaves with fully abolished SMT phase (e.g., when infiltrated with DCMU or MV), we measured ChlF transients of AC leaves treated with 100 µM DCMU (Fig. 8A), as well as with 100 µM MV (Fig. 8B), during cooling from 22 °C down to $-1.5 \ ^\circ C$. For a comparison of changes in the SMT fluorescence phase, we plotted the
data on a log scale starting at 10 s in Fig. 8. Corresponding data for NAC leaves treated with 200 µM DCMU and 500 µM MV are not shown here (however, see Supplementary Fig. S4). Our data show that in both DCMU- and MV-infiltrated leaves of NAC plants, the SMT phase was present at ~10 °C (Fig. S4), whereas in AC leaves, SMT fluorescence phase reappeared only at ~4 °C (Fig. 8). In both DCMU- and MV-infiltrated leaves, SMT fluorescence phase was prominent both at 0 and ~1.5 °C (Fig. 8).

Fig. 8 Chlorophyll a fluorescence transients, measured at different temperatures during slow cooling (2 °C h⁻¹) of DCMU-treated (A) and MV-treated (B) (see Material and Methods for details) detached leaves of AC Arabidopsis thaliana accession C24. The y-axis (on the left) is F/F₀, and the y-axis (on the right) of the upper panel (A) is for ChlF transients of control leaves at room temperature (black line); these curves have been staggered to allow easier observation of differences between the different samples. The vertical lines, at semi steady-state S₁ & S₂ and at maxima M₁ & M₂, are for the curve indicated as control in both the panels. Control leaves were vacuum infiltrated in distilled water plus 2% ethanol. The ChlF transient was measured as described in the legends of Figs. 1 and 2; all data are plotted on a log time scale.

Effects of cold acclimation and low-temperature treatment on whole plant CO₂ assimilation rate

For finding a relationship between the dynamics of the low-temperature-induced changes in ChlF transients and photosynthesis, we measured CO₂ assimilation rate (A) of NAC and AC plant rosettes at selected low temperatures during cooling down to 0 °C. A comparison of the ratio between the induction of light-induced gross CO₂ assimilation rate (A₀) and the maximal CO₂ assimilation rate at saturating light (Aₘₐₓ) of NAC and AC plant rosettes, for the first 200 s after the light was switched on, is shown in Fig. 9. For both NAC and AC plant rosettes, the induction curves of A₀ were almost linear at 10 °C. However, there is an inflection with a peak at ~50 s in the induction curve from both AC and NAC plants at temperatures below 7 °C. Lowering the temperature broadened this peak and prolonged the position of its maximum by ~10 s at 0 °C (Fig. 9); note that the CO₂ assimilation in AC plant rosettes was measured only in the range of 10 °C to 0 °C. Surprisingly, for the AC plants, the initial photosynthetic induction was slower than that for the NAC plants (Fig. 9). Nevertheless, under saturating light, the
\(A_{\text{gross}}\) was significantly higher in the AC plants, as expected (Supplementary Fig. S5A). In both groups, however, \(A_{\text{gross}}\) declined with temperature, and at 0 °C the NAC and AC leaves had approximately the same \(A_{\text{gross}}\) (Supplementary Fig. S5A). Low temperature (e.g., 0 °C) significantly \((p < 0.01)\) increased the time to reach 50% of the \(A_{\text{max}}\) (IT\(_{50}\)) in AC plants as compared to its value at 10 °C, whereas IT\(_{50}\) was almost constant below 7 °C in NAC plants. Further, at 7 °C and below, IT\(_{50}\) of AC plants was significantly higher than in the NAC plants (Supplementary Fig. S5B). For a better understanding of the low-temperature-induced peak during the initial induction of CO\(_2\) uptake (Fig. 9), the induction of CO\(_2\) assimilation rate at 60 s as a percentage of \(A_{\text{gross}}\) [IS\(_{60}(\%))\] is shown in Supplementary Fig. S5C. It is clear that this peak is much more pronounced in NAC than in AC plants, and its intensity is significantly higher below 7 °C, while for AC plants its rise is significantly lower for temperatures below 7 °C (Supplementary Fig. S5C).

## Discussion

### Effect of cold acclimation is reflected in chlorophyll fluorescence transients

Several parameters of ChlF transients are known to provide important insights into the molecular processes of photosynthesis in different photosynthetic organisms and under different biotic and abiotic stresses (Baker and Rosenqvist 2004; Baker 2008; Papageorgiou and Govindjee 2011; Gururani et al. 2015; Mishra et al. 2016a). The shape of the slow ChlF transients is affected by complex interactions within the photosynthetic machinery (this includes PSII, PSI, and associated LHCs, see Lazár 2015 for a review); ChlF transient is highly variable, and it has been used to obtain information on the qualitative effects of different experimental conditions on the photosynthetic processes (Strasser et al. 1995a, b; Govindjee 1995; Lazár 1999; Oxborough 2004; Kaňa and Govindjee 2016). Because of the observed large variations in the OPS and SMT fluorescence phases of ChlF transients, we have used specific quantitative features of ChlF transients, such as the rate constant \((k)\) and the time to reach 50% of P–S quenching \((t_{50})\), as well as the areas underneath the OPS and SMT phases, in order to understand the effects of low temperature, discovered in this work. These quantitative parameters proved to be highly valuable for the analysis of slow phase of ChlF transients (see the following sections).

We show here that although cold acclimation of C24 Arabidopsis did not change the pattern of ChlF transients (including the slow SMT phase) in NAC plants (Fig. 2), it significantly affected various parameters of these transients (Tables 1, 2). Variation in some of these parameters may be due to low-temperature-induced modification of the structural and functional properties of the chloroplasts, changed Chl content, and changes in rates of CO\(_2\) assimilation, carbon metabolism, and other photosynthetic activities (cf. Telfer et al. 1976; Martindale and Leegood 1997; Strand et al. 1999). However, an increase in the minimal fluorescence \((F_{\text{m}})\) of cold-acclimated leaves (Fig. 2) points to modulation in light-harvesting chlorophyll antenna size of one or both photosystems and their activities, whereas an increase in the time needed for the P-to-S decline in cold-acclimated leaves (Fig. 2), as shown by an increase in \(k^{-1}\) (or \(t_{50}\)) (Table 2), may indicate structural changes or changes in acidification of the thylakoid lumen (Bräntais et al. 1980; Walters et al. 1996) and/or in events related to carbon fixation (Walker 1981; Seaton and Walker 1990; Savitch et al. 1997). Further, comparatively higher terminal steady-state fluorescence \((F_{\text{p}})\), at 22 °C, in AC leaves under identical exposure of actinic irradiance, may indicate a shift of energy distribution in different pathways in order to have efficient photochemical and carbon fixation reactions of photosynthesis at low temperatures.

### Low-temperature treatment largely affects the slow phase of ChlF transient and the CO\(_2\) assimilation induction curve

We show here that lowering of temperature has a marked effect on ChlF transients in the leaves of both the NAC and the AC plants (Fig. 3A, B), and these changes are reflected in the quantitative parameters, e.g., areas underneath the OPS and the SMT fluorescence phases (Fig. 3C, D), peak positions, and the values of \(k\), \(t_{50}\), and \((F_{\text{p}} - F_{\text{m}})/F_{\text{S}}\) (Tables 2, 3). The O–P rise is mainly due to the closure of PSII reaction centers with the reduction of primary electron acceptor \(Q_{A}\), as well as electron acceptors beyond PSI (in other words, it reflects the reduction of the entire electron transport chain); in the presence of saturating light, \(Q_{A}\) is fully reduced to \(Q_{A}^{-}\), and \(F_{\text{p}}\) attains the value of \(F_{\text{m}}\) (Govindjee 1995; Baker 2008; Stirbet and Govindjee 2011, 2012). Cold acclimation essentially changes the redox state of photosynthetic electron transport components; in part, this is due to \(Q_{A}\) being kept in the oxidized state (see Huner et al. 1998) and by changing the redox potential not only of \(Q_{A}\), but also of \(Q_{B}\) (Sane et al. 2003; cf. Ensminger et al. 2006; Khanal et al. 2017). Any change in the ratio of \(Q_{A}\) to \(Q_{A}^{-}\), obviously, changes the \(F_{\text{p}}\) level, when measured under identical light (here, measured at actinic irradiance of \(~40\) μmol photons m\(^{-2}\) s\(^{-1}\)).

ChlF transients beyond \(F_{\text{p}}\) are much more complex than the fast O–P rise (see, e.g., Stirbet and Govindjee 2016; Bernát et al. 2018) because of several processes, e.g., both photochemical and non-photochemical quenching, due to changes in the acidification of the thylakoid lumen, ATP synthesis, and the activation of the Calvin–Benson cycle. All of these affect ChlF, especially beyond \(F_{\text{p}}\), and this is
reflected in changes in the parameter $t_{50}$ ($k^{-1}$). Further, the corresponding areas underneath OPS would also be modulated. In addition to that, an indirect effect on the O–P curve must be considered. It has been demonstrated that the area above O–P reflects the size of the PSII acceptor pool, i.e., plastoquinone, and the oxidized carriers between PSII and the Calvin–Benson cycle, i.e., the acceptors of PSI (see, e.g., Joliot and Joliot 2002; Tóth et al. 2007). Given that low temperature slows down the Calvin–Benson cycle, a feedback that affects the O–P curve must also be expected.

In the ChlF transients from leaves of NAC and AC plants (Fig. 3A, B), the area under the $M_1$ peak increased with decreasing temperature, and this effect was much more pronounced in AC than in the NAC leaves (Fig. 3C, D). The time to reach $M_1$ (see Table 3) was about 10–25 s ahead of the peak in the $CO_2$ assimilation curve (see Fig. 9), indicating a link between the decline in fluorescence following $M_1$ and the initiation of carbon fixation. This is supported by the observation that the low-temperature-induced shift in the position of $M_1$ (Fig. 3; Table 3) coincides with a shift in the peak of the $CO_2$ assimilation (Fig. 9). Considering that the decrease of $CO_2$ fixation following the peak in the $CO_2$ assimilation curve and its shift most likely reflect exhaustion of Calvin–Benson cycle intermediates, our results may be interpreted to indicate a transition from carboxylation to ribulose-bisphosphate (RuBP) regeneration-limited photosynthesis at low temperatures (Hikosaka et al. 2006). Low temperature may favor RuBP-limited photosynthesis when there are limitations on either (i) the rate by which the bisphosphatase, in the stroma, regenerate RuBP in the photosynthetic carbon reduction cycle, or (ii) the rate at which triose-phosphates are exported from the chloroplasts or metabolized to release inorganic phosphate (Pi) for use in subsequent photophosphorylation (Allen and Ort 2001). However, a direct effect of the electron transport processes on RuBP regeneration cannot be excluded. The time to reach 50% of $A_{max}$ (IT$_{50}$) was much longer in AC, than in NAC, leaves (Supplementary Fig. S5B), which may indicate that enzymatic reactions of the Calvin–Benson cycle may take longer time for activation. Five of the enzymes of the Calvin–Benson cycle (Fructose-bisphosphatase, Sedoheptulose-bisphosphatase, Phosphoribulokinase, Glyceraldehyde 3-phosphate dehydrogenase, and Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) via Rubisco activase) are known to be redox regulated via the thioredoxin system (Buchanan 1991). Zhang and Scheller (2004) have shown that low temperature causes a persistent reduction in PSI activity, expressed as the rate of NADPH production, which is coupled to a reduced proportion of photo-oxidizable P700. Reduced photo-oxidation of P700 would affect the reduction of ferredoxin pool, and thus the activation of Calvin–Benson cycle enzymes might be affected. This may mean that increased $F_p$ and $F_\gamma$ along with broadening of the OPS and S$_2$M$_1$T$_1$ fluorescence phases at suboptimal temperatures (at 10 °C for NAC, 10 °C and 4 °C for AC) may be induced by a slowdown in the electron transport chain, and that there exists a correspondence between further rise of the SMT (specifically with peak $M_1$) fluorescence phase (Fig. 3A, B) and the peak in the $CO_2$ assimilation curve (Fig. 9A, B). The latter seems to be due to limitations of photosynthesis by both the substrates of the Calvin–Benson cycle and/or RuBP regeneration (see Furkbank and Walker 1986). Thus, ChlF transients can indeed be used to interpret changes in low-temperature-induced “light reactions” as well as the “carbon reactions” of photosynthesis.

**Cold-acclimated plants have better modulation of quantum yield of regulated NPQ via state transition and photoinhibition at low temperatures**

We have observed a significant decrease in $F_s/F_m$ (at $−1.5$ °C) and in $F_P$ (at 4 °C and $−1.5$ °C) in NAC, but not in AC, leaves (Fig. 3A, B; Table 2). This corresponds to a higher degree of quantum yield of photoinhibition, $\Phi_{ql}$, in NAC leaves (Fig. 5A). Further, this may indicate a vulnerability of RCs of PSI in NAC leaves that may have resulted because of a block of the D1-repair cycle at low temperatures (Mohanty et al. 2007). Photoinhibition has been shown to be directly proportional to increasing light intensity (Tyystjärvi and Aro 1996), but its underlying mechanisms are complex and still not fully understood (Allakhverdiev et al. 2005, 2007; Tyystjärvi 2013; Khanal et al. 2017). Sane et al. (2003) reported, in *Arabidopsis* accession Col-0, that dark acclimation predisposes photoinhibition by facilitating radical pair backward recombination reactions of P680$^+$ Q$\Lambda^−$ that enhances the dissipation of excess light energy within the reaction center of PSI.

Temperatures below 4 °C were shown here to lead to an increase in the quantum yield of regulated NPQ, $\Phi_{NPQ}$, and a dramatic reduction in $\Phi_{PSII}$ especially in the NAC leaves (Fig. 4). Comparatively lower $\Phi_{NPQ}$ in AC leaves than that in NAC, at low temperatures, may be due to higher enzyme activity in the Calvin–Benson cycle, which is reflected in higher $A_{gross}$ that may further allow the plant to keep high photochemical quenching. A simultaneous increase in $\Phi_{NPQ}$ and decline in $\Phi_{PSII}$ is an indicator of an activation of alternative electron transport pathways, e.g., cyclic electron transport through PSI (Ort and Baker 2002). Analysis of the different components of $\Phi_{NPQ}$ suggests that state transition (qT; see, e.g., Allen 2003; Horton 2012) involved in the re-distribution of LHCs from the high fluorescent PSI to the low fluorescent PSI has a significant role at temperatures near or below the freezing point (Fig. 5). Our results on the fluorescence emission spectra, measured during low-temperature treatment (Fig. 6), confirm this hypothesis. A comparison of 77 K spectra, and of F685/F735 ratios,
for AC and NAC leaves, before and after low-temperature (−1.5 °C) exposure, further suggests that re-distribution of LHCs in favor of PSI, i.e., State I to State II transition, may indeed serve as a regulatory mechanism to protect the photosystems, and that cold acclimation may stimulate this process (Fig. 6C). Yun et al. (1997) reported destacking of inter-grana lamellae, after only 3 s of exposure to 5 °C, in the chilling-sensitive African violet (Saintpaulia ionantha). This led to homogeneous distribution of proteins within the thylakoid membrane and resulted in “spillover” of excitation energy from PSII to PSI (Agati et al. 2000). It is unlikely that destacking of grana was the reason for the decreased F685/F735 ratio in accession C24, as observed in our work, since this would have been accompanied by a large decrease in ChlF (Yun et al. 1997), which was not observed in C24 Arabidopsis plants. This points out to us that state transition must play a key role in cold-acclimated leaves, and that this process prevents high excitation pressure and damage of PSII during low-temperature exposure.

**Analysis of ChlF transients in the presence of different photosynthetic inhibitors**

When chemicals (such as DCMU and MV) that modify or interfere with the electron transport process were applied to NAC and AC leaves, it appeared that AC leaves required lower concentrations to give the same effect (Fig. 7). This might be due to lower water content of AC leaves that may interfere with the electron transport process beyond 

\[ F \]

m and inhibited ChlF quenching beyond 

\[ F \]

(Munday and Govindjee 1969; Joliot and Joliot 2006). Considering that MV effectively competes with electron transport to ferredoxin as well as to cytochrome \( b_{f} \), re-occurrence of the peak M (Fig. 8B) in MV-treated leaves at low temperatures indicates a slowdown in the electron transfer reactions competing with MV, which in turn must mean that, at low temperature, cyclic electron transport significantly contributes to the quenching of ChlF. This has been interpreted as a means to increase ATP production at low temperatures (Joliot and Johnson 2011; Kramer and Evans 2011), but it could also act as a regulatory factor in offsetting the slow turnover of NADPH in the Calvin–Benson cycle at the lower temperatures. Therefore, the re-appearance of maxima M in the ChlF transients at low temperatures might reflect a strategy for regulating photosynthetic efficiency through combination of processes that may include state transition (State II to State I transition) and cyclic electron transport (Fig. 8).

**Regulation of slow SMT fluorescence phase**

The SMT fluorescence phase has been observed in cyanobacteria (Papageorgiou and Govindjee 1968a), green algae (Papageorgiou and Govindjee 1968b; Mohanty and Govindjee 1974), red algae (Mohanty et al. 1971), lichens (Mishra et al. 2015; Marečková and Barták 2016), as well as in higher plants (Bradbury and Baker 1984; Pandey and Gopal 2012). It is highly pronounced in phycobilisome (PBS)-containing cyanobacteria; here, in contrast to other organisms, the intensity of the fluorescence maxima M is much higher than that of the peak P, \( F_{\text{p}} \) (Kaña et al. 2012). As is known in several systems (Yamagishi et al. 1978; Fork and Satoh 1986; Papageorgiou et al. 2007; Papageorgiou and Govindjee 2011), the SMT fluorescence phase has two waves in Arabidopsis accession C24. Yamagishi et al. (1978) suggested that protonation-induced structural changes in thylakoids may partially contribute to the formation of the SMT phase in isolated chloroplasts from a green alga Bryopsis maxima. Sivak et al. (1985a) compared the ChlF transients and light scattering at 535 nm, in spinach leaves, after 1 min and 4 min of dark intervals, and found simultaneous changes in scattering and the formation of the SMT phase, in 4-min, but not in 1-min, dark-adapted leaves. Sivak et al. (1985a) assumed that longer dark adaptation transiently energizes thylakoids that induce high scattering at 535 nm because of the development of transmembrane proton difference, and...
consequently, the SMT phase was formed due to low consumption of ATP in low irradiance. Sivak et al. (1985b) further interpreted the dynamics of the SMT phase as an “indicator of protonation of thylakoids” and found that the proton motive force increases and decreases slightly in advance of the rise and the fall of the CO₂ assimilation rate. Further, Bradbury and Baker (1981, 1984) have suggested that both the redox state of the PSII electron acceptor as well as an interplay between photochemical and non-photochemical quenching play a major role during the P–T decline, and the S–M rise is predominantly caused by a decrease in photochemical quenching (qP) that implies a decrease in the linear electron flow. This would agree with our interpretation of an involvement of insufficient RuBP regeneration as one of the causes for SMT development. Kaňa et al. (2012) have suggested that induction of SMT phase is a safe way of decreasing excess light in Synechococcus that lacks certain NPQ mechanisms. They have demonstrated that state transition is the major phenomenon behind the S–M rise; the S–M rise is due to transition of lower fluorescence “State II” to a higher fluorescence “State I” since a mutant blocked in state change did not show the S–M rise. It is possible that the M–T decline could be, partly, due to the “State I” to “State II” transition in cyanobacteria (Papageorgiou and Govindjee 2011; Kaňa et al. 2012; Bernát et al. 2018). A similar conclusion for S–M rise has been made for the green alga Chlamydomonas reinhardtii (Kodru et al. 2015). Further, our results show the occurrence of SMT as well NPQ at low temperatures in both the NAC and the AC leaves of Arabidopsis natural accession C24. Our data clearly show that state transition plays an important role in Arabidopsis, and that it is activated already at 4 °C in NAC plants, whereas in AC plants, it is prominent only at a lower temperature, −1.5 °C.

Conclusions

We have demonstrated that cold acclimation modulates photosynthetic activity of a cold-sensitive natural Arabidopsis accession C24 since cold acclimation improves not only the CO₂ assimilation rate at saturating light, but also the maximum quantum yield of PSII photochemistry and the regulatory non-photochemical quenching at ~0 °C. We have discovered that cold acclimation slows down quenching reactions during the fluorescence decline from the P to S level, and this may facilitate improved quenching ratio (an indicator of proton pumping capacity) at low temperatures. We speculate that improvement in both photochemical and carbon reactions of photosynthesis may have been due to a general increase in the activity of several Calvin–Benson cycle enzymes (e.g., Rubisco) and regulatory proteins (e.g., thioredoxin) that better facilitate the regulation of photosynthesis at low temperatures. Furthermore, the estimated changes in the quantum yield of regulatory NPQ suggest that cold-acclimated plants develop an ability to reduce photoinhibition. On the other hand, the slow SMT fluorescence phase (S₁M₁S₂M₂T), measured at 22 °C, seems to reflect constraints in “photochemical pathways” because it disappears when exposed to high irradiance or after application of DCMU (blocking electron transport) and MV (accelerating electron transport). The redox state of Qₐ and inhibition in kinetics of redox reactions of photosynthetic electron transport (between PSII and PSI and beyond) are expected to increase Fₚᵣ, Fₚᵣ, and the area underneath the OPS fluorescence phase at suboptimal temperatures (10 °C for NAC; 10 °C and 4 °C for AC plants). However, low temperature-induced strong modulation of the SMT fluorescence phase (in AC and NAC leaves), and re-appearance of the SMT phase in DCMU and MV treated leaves that had no SMT fluorescence phase at 22 °C, indicates a contribution of cyclic electron flow through PSI. Low-temperature-induced re-distribution of LHCs from PSII towards PSI also supports the possibility of cyclic electron transport through PSI being involved in the formation of SMT fluorescence phase. Further, we suggest that both the initiation of state transition and cyclic electron transport stimulate SMT fluorescence phase at low temperatures, in response to limitations in the Calvin–Benson cycle and/or RuBP regeneration. Thus, there must exist a dynamic regulation of photochemical and biochemical activities in response to low-temperature treatment in Arabidopsis leaves, and low-temperature-induced restrictions on biochemical reactions are not only reflected in CO₂ assimilation rates but also in ChlF transient measurements that are more sensitive, and easier to use; they provide much more information for the same phenomena. Further research involving Arabidopsis mutants will help in reaching a better understanding of cold acclimation-induced modifications in the photosynthetic machinery.

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