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# Global fluorescence lifetime distribution analyses of PSII energy dissipation mechanisms at low physiological temperatures: xanthophyll photoprotection, photochemistry and photoinhibition

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## Introduction

One of the main problems associated with measuring photosynthetic activity at low physiological temperatures using chlorophyll (chl *a*) fluorescence is that photosynthetic, photoprotective and photoinhibitory damage associated processes all tend to reduce the PSII fluorescence yield concomitantly. It is therefore difficult to distinguish the relative influence of each process especially with relative or ratiometric intensity yields of the chl *a* fluorescence, as obtained with conventional pulse-amplitude modulation (PAM) chl fluorometers (Schreiber et al 1986). Conventional PAM instruments do not provide the necessary direct information concerning the excited state lifetimes or energy levels of the chl fluorescence emission. This paper illustrates how measuring both wavelength- and time-dependent changes in the chl fluorescence and subsequent global analysis of the data facilitate resolution and quantification of the main processes competing for excitation in PSII under low physiological temperature conditions *in vivo*.

This study first shows how recent breakthroughs in molecular engineering have allowed us to systematically control and study the key photoprotective energy dissipation mechanism and thereby both photochemical and photoinhibitory damage related processes influencing PSII when plants are exposed to excess light. Key photoprotection mutants include those influencing various aspects of the xanthophyll cycle-dependent energy dissipation process including PsbS protein accumulation (Li et al 2000), the xanthophyll cycle deepoxidation reaction (Niyogi et al 1998, Gilmore 2001) and lutein biosynthesis (Pogson et al 1998, Niyogi et al 2001, Gilmore 2001). Further data is presented on the use of global analysis of fluorescence lifetime data to elucidate processes associated with low physiological temperatures and dark-sustained energy dissipation mechanisms *in vivo*. Two scenarios are studied: scenario 1) involves the low-temperature enhancement of lumen acidification and xanthophyll cycle- and PsbS-dependent energy dissipation, whereas scenario 2) involves more complex changes that are independent of lumen acidification and associated with both altered protein organization and composition and sustained deepoxidation of xanthophyll cycle components during winter acclimation.

## Materials and methods

*Plant materials*

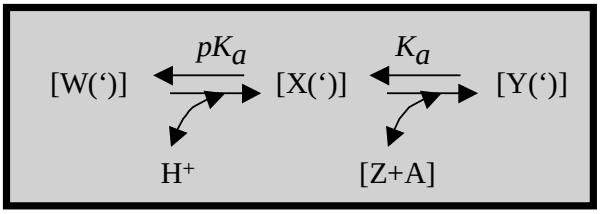
Three main types of plant material were analyzed, namely, single and double nuclear gene mutants of *Arabidopsis thaliana*, winter acclimated seedlings of *Eucalyptus pauciflora* and cells of the marine prasinophyte alga *Mantoniella squamata*. There were three Arabidopsis mutant strains: 1) *npq4-1* which inhibits the PsbS protein accumulation, 2) the F1 progeny of the WT and *npq4-1* that accumulates approximately 65% of the WT PsbS titre (Li et al 2000)

and 3) *lut2-npq1*, where *lut2* inhibits the lycopene epsilon cyclase reaction and *npq1* inhibits the violoaxanthin deepoxidase (Pogson et al 1998, Niyogi et al 1998, 2001, Gilmore 2001). The basic growth and experimental conditions for the winter acclimated *E. pauciflora* seedlings were described by Gilmore and Ball (2000). The growth and experimental conditions for the *M. squamata* cells were described by Gilmore and Yamamoto (2001).

*Steady state fluorescence*
PSII chl *a* fluorescence kinetics at room temperature were determined with a PAM chl fluorometer (PAM 101-103, ED-101BL, Heinz-Walz, Effeltrich Germany). All fluorescence, temperature and light-intensity protocols were described earlier (Gilmore et al 1998, Gilmore and Ball 2000, Gilmore and Yamamoto 2001, Gilmore 2001).

*Time- and wavelength-resolved fluorescence and global analysis*
PSII chl *a* fluorescence lifetime data were obtained with a K2-004 multifrequency phase and modulation fluorometer (ISS Inc., Urbana , IL USA) and globally analyzed as described earlier (Gilmore and Ball 2000, Gilmore and Yamamoto 2001, Gilmore 2001)

**Results and discussion**
*Model interactions between nonphotochemical and photochemical quenching in PSII*
With respect to xanthophyll cycle and lumen pH-dependent photoprotection under conditions where PSII photochemistry is inhibited with dcmu, Gilmore et al. (1998, 2001) identified three PSII chlorophyll fluorescence lifetime states: 1) W, a PSII unit containing an unprotonated PsbS protein inactive with respect to binding either a zeaxanthin, Z, or antheraxanthin, A, molecule, 2) X, a PSII unit containing the protonated form of the PsbS potentially active for binding a Z or A, molecule and finally 3) Y, a PSII unit with a protonated PsbS protein actively binding a Z or A molecule and with an increased rate constant of heat dissipation, $k_h$. The protonation of PsbS is defined with a simple Henderson-Hasselbach titration (defined by a $pK_a$ value). The concentration dependence of the binding of Z and A to the protonated PsbS associated site is defined with an equilibrium-association constant $K_a$. The Gilmore et al (1998) model is herein expanded to six states to include the influence of populations of PSII with either oxidized (or reduced) primary quinone electron acceptors, Qa (or $Qa^-$), respectively. The influence of the xanthophyll and PsbS concentrations on PSII is outlined as follows:




The six PSII states are normalized and defined as [W(‘)]+[X(‘)]+[Y(‘)]=1, herein the (‘) notation denotes PSII units in the $Q_a^-$ state, the $pK_a$ = pH – log[W]/[X] and $K_a$ = [Y]/[X]·[Z+A]. It is considered unlikely that the $Q_a$ redox state would influence either the $pK_a$ or $K_a$ in the above scheme. It is expected, on the other hand, that the increased $k_h$ should directly decrease the probability of $Q_a$ reduction and therefore increase, on average, the probability the unit will exhibit an increased rate constant of PSII photochemistry ($k_p$). The following scheme depicts the same model simplified to 4 states for PSII, the W(‘) and

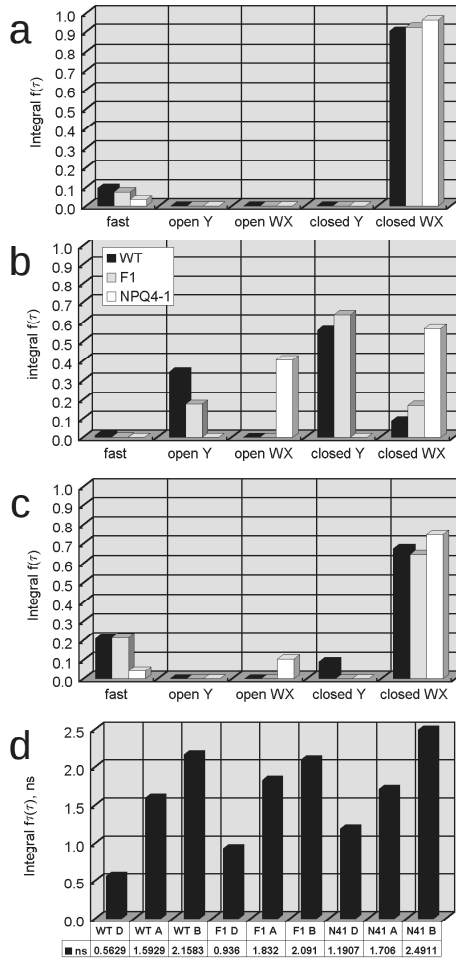
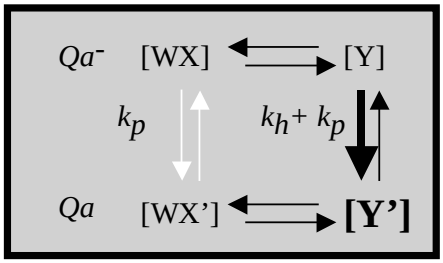



**Fig. 1**. PSII Chl fluorescence lifetime fractional distributions (a-c) and integral lifetimes (d) in leaves of *npq4-1*, WT and F1. Conditions (at 685 nm) represent before illumination (a), during illumination (b) and after illumination (c) see text for details. The global reduced $X^2$ value was 1.68 for 9 files fit to the model described in the text.

X(‘) states are summed together because their fluorescence lifetimes differ usually by little more than 20%. The scheme shown here,




represents the empirical view that for any steady state condition that is subsaturating for yielding $Q_a^-$, increasing the fraction of centers in the [Y(‘)] state should directly increase the fraction [Y`] , representing PSII traps with increased $k_h$ and oxidized $Q_a$.

The above model was tested by global analysis in the leaf experiments shown in Figure 1 using the *npq4-1* mutant lacking PsbS, the WT and their F1 generation progeny exhibiting around 65% of the WT PsbS levels. Figure 1a shows the fractional distribution of the four PSII states in dark-adapted plants under dcmu treated conditions to reduce $Q_a$ and to prevent photoinhibition and pH-gradient formation. It is clear that all three materials show the major fraction of PSII is in the closed or [WX] state plus a minor fraction of rapid decays attributed to rapid exciton equilibration, transfer and PSI emission processes. Figure 1b compares PSII fractional distribution of the 3 materials following induction of saturating levels of [Z+A] and lumen acidification followed by a chilling treatment (5º C) to maintain the lumen acidity (Gilmore and Björkman 1995) but still maintained under actinic illumination subsaturating (approximately 40%) for $Q_a$ reduction (around 100 μE $m^{-2}$ $s^{-1}$). The *npq4-1*, lacking PsbS, exhibited only two PSII fractions namely open and closed or [WX] and [WX’] in 1b. As predicted, the WT exhibited the lowest [WX(‘)] and largest fraction of [Y’] and the F1 mutant exhibited an intermediate levels of [WX(‘)] and [Y’] indicating the PsbS and xanthophylls increased both $k_h$ and $k_p$. Figure 1c shows the fractional distribution of PSII returns to close to the same levels as in Fig. 1a after warming, dark-adapting and DCMU infiltration of the leaves to both reverse the pH-gradient and reduce $Q_a$, respectively (see Fig. 2 below). Figure 1c illustrates the integral lifetimes of the three materials before (B), during (D) and after (A) the illumination treatments. The WT exhibits the fastest decay time during the illumination, compared to the F1 and *npq4-1*. All three materials exhibited similar lifetime distributions (2.1-2.4 ns) under the before conditions. The *npq4-1* exhibited the largest difference in the before and after

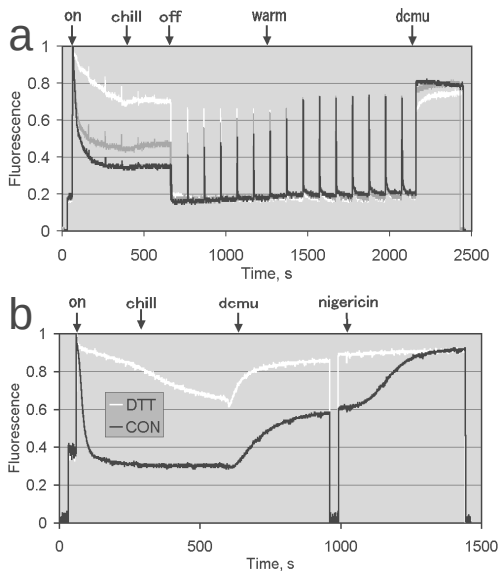



**Fig. 2.** PAM chl fluorometer traces of experiments concerning light-induced and dark-sustained fluorescence decreases at chilling temperatures in *npq4-1*, WT and F1 leaves (a) and cells of *Mantoniella squamata* (b).

treatments which was attributed to higher levels of damage due to the inhibited photoprotection without PsbS. There was no evidence of any reaction center type of pH-dependent energy dissipation mechanism in these materials (Mohanty and Yamamoto 1996).

*Dark-sustained energy dissipation in Arabidopsis leaves and Mantoniella cells at chilling temperatures*

Gilmore and Björkman (1995) showed that following light induction of [Z+A] and lumen acidification with a decrease in the temperature can cause a sustained level of energy dissipation in the dark in various plant materials. It has been questioned whether 1) the sustained energy dissipation observed *in vivo* is actually dependent on a trans-thylakoid pH-gradient (as opposed to a sustained pH-independent conformational change) and 2) how the component is physically and chemically related to the pH-dependent NPQ component at room temperature. Both questions were answered in experiments with Arabidopsis mutant leaves and cells of *Mantoniella squamata* as shown in Figure 2. Figure 2a illustrates PAM traces from experiments with intact leaves of the same *npq4-1* (white), WT (black) and F1 (gray) materials described in Fig. 1. The protocol involved inducing maximal levels of [Z+A] and lumen acidification at room temperature with a saturating actinic light intensity (on), followed by chilling (chill) then turning off the actinic light (off) and monitoring the sustained depression of the fluorescence yields with a train of saturating pulses and the low intensity measuring beam. The leaf samples were later warmed to 25º C (warm) and then vacuum infiltrated with dcmu (dcmu) to ensure full pH-gradient reversal and $Q_a$ reduction, respectively. The WT exhibited the largest decrease in the fluorescence yield in the light and sustained decrease of the fluorescence yields (between pulses) and maximal levels (during pulses) in the dark. The F1 exhibited an intermediate level of energy dissipation, while the *npq4-1* showed no light/dark or temperature reversible changes in the fluorescence yields. Thus the dark sustained energy dissipation in leaves is simply an extension of the PsbS-dependent energy dissipation induced in the light. Similar complete inhibition of the dark-sustained energy dissipation is also observed in the *lut2-npq1* mutant (data not shown), that lacks lutein and violaxanthin deepoxidation (Niyogi et al. 2001, Gilmore 2001). Figure 2b shows a similar protocol using cells of *Mantoniella squamata* except herein all the energy dissipation in the control (CON) sample depends on antheraxanthin (Goss et al.1998, Gilmore and Yamamoto 2001) and the dark sustained energy dissipation is rapidly reversed by nigericin indicating dependence on a pH-gradient. The cells treated with dithiothreitol (DTT) to inhibit antheraxanthin formation exhibited no dark-sustained energy dissipation. Thus we conclude the dark sustained energy dissipation *in vivo* depends on the PsbS protein, deepoxidized xanthophylls (Z, A and possibly lutein) and a pH-gradient. The pH-gradient is likely maintained by chloroplast ATPase activity and or chlororespiratory proton translocation (Gilmore and Björkman 1995, Gilmore and Yamamoto 2001). Global analysis

of fluorescence lifetime distribution data under the dark-sustained, chilling conditions for the Arabidopsis *npq4-1* mutants (not shown) and the *M. squamata* cells (Gilmore and Yamamoto 2001) indicate similar patterns of change associated with lumen acidification and deepoxidation of violaxanthin, as shown by Gilmore et al. (1998).

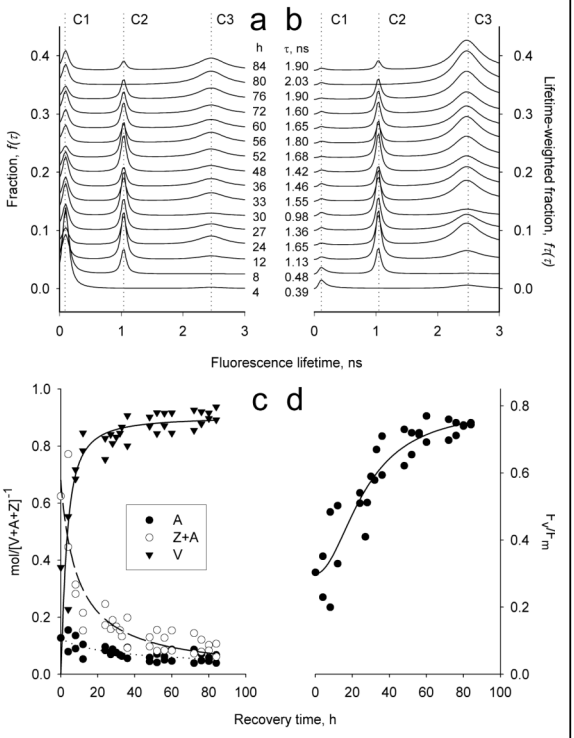



**Fig. 3**. Changes in the room temperature PSII chl *a* fluorescence lifetime distributions (a and b), xanthophyll cycle components (c) and maximal PSII quantum efficiency (d) at room temperature during winter deacclimation. The global reduced $X^2$ for the three component fit and 16 data files was 1.42.

*PSII fluorescence lifetime distributions during winter deacclimation in Eucalyptus pauciflora*

Winter-acclimated leaves of the snow gum (*E. pauciflora*) exhibit large decreases in the room temperature chl *a* fluorescence yield that are insensitive to warm temperatures and nigericin (Gilmore and Ball 2000). Figure 3 shows the distributed fluorescence lifetime fractional intensity (a) and lifetime-weighted fractional intensity profiles (b) from PSII for 4 days of winter deacclimation under room temperature and in low-light. Leaves were warmed (25° C) and treated with dcmu and or nigericin to reduce $Q_a$ and inhibit the pH-gradient. The recovery time in hours is right of Panel a and the integrated fluorescence lifetime is left of Panel b. At time = 4 h (lowest traces in a and b) the fractional intensity was dominated by a component (C1) centered at less than 0.2 ns; due to the short fluorescence lifetime of this component it was most clearly observed in the plots of fractional intensity in Fig. 3a compared to the lifetime-weighted fractional intensity in 3b. Later, the fractional intensity profiles exhibited an intermediate component (C2) centered around 1 ns, that formed at the expense of C1. Subsequently, the 2.5 ns distribution component (C3) emerged as the predominating component at the expense of both C1 and C2. The rapid decrease in the C1 component in the first 24 h correlated most strongly with the decrease in [Z+A] in Panel c; however, detectable amplitudes of C1 and [Z+A] persisted throughout the recovery period. Figure 3d shows that the PSII quantum efficiency, $F_V/F_m$, also exhibited the most rapid excursions in the first 24 hours. Thus we conclude the largest factor influencing the PSII efficiency is the xanthophyll deepoxidation associated component.

The energy dissipation processes responsible for the more rapid ~1 ns C2 mode compared to the 2.5 ns mode (C3) may be more directly due to aerobic photoinhibition related chlorophyll and or protein oxidation (Gilmore et al 1996a) than to xanthophyll cycle deepoxidation, which is more strongly correlated with the C1 component. The 77K spectral changes shown by Gilmore and Ball (2000) indicate that the core antenna structure of PSII recovers relatively slowly to the fully functional form over the 4 day deacclimation;

consistent with the slower C2 conversion to C3 in Panels a and b. The slower changes of C2 to C3 probably require protein synthesis, including D1, D2 and perhaps other components, and reassembly of functional PSII units (Ottander et al 1995). The initial fast 0-24 h decrease in deepoxidation and C1 may be related to the phosphorylation state of key PSII proteins as suggested by Ebert et al (2001) and or changes associated with the PsbS and its interactions with other proteins and xanthophylls as suggested by Ottander et al (1995).

**Concluding remarks**

It has been possible with the aid of key photoprotection mutants of Arabidopsis to perform experiments and targeted global analyses that simultaneously, and unequivocally, resolve fluorescence changes attributed to PSII photochemistry, photoprotective heat dissipation and photoinhibitory damage to PSII. The data herein clearly indicate that no lumen-pH-dependent, xanthophyll cycle-independent (or PsbS-independent) mechanisms were observed to decrease the PSII fluorescence yields *in vivo*. The exclusive PsbS-dependence of the NPQ mechanism is consistent with the model proposed by Gilmore et al (1998) and recent data in this volume and elsewhere indicating NPQ levels are largely independent of the levels of most peripheral PSII antennae proteins including LHCIIb, CP24, CP26 and CP29 (Gilmore et al 1996b, 1998, Andersson et al. 2001, Li et al 2000). It is also clear that protein-related changes in PSII must somehow, independent of lumen acidification, allow the xanthophylls Z and A to remain engaged in energy dissipation during winter acclimation conditions (Ottander et al 1995, Gilmore and Ball 2000, Ebert et al 2001).

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