Direct energy transfer from photosystem II to photosystem I is the major regulator of winter sustainability of Scots pine

Authors

Pushan Bag^{1¶}, Volha Chukhutsina^{2¶#}, Zishan Zhang^{1&}, Suman Paul^{1#}, Alexander G. Ivanov^{3,4}, Tatyana Shutova¹, Roberta Croce², Alfred R. Holzwarth^{2*}, Stefan Jansson^{1**}

Affiliations

¹Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, Umeå, Sweden.

²Department of Physics and Astronomy, Faculty of Sciences, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

³Department of Biology, University of Western Ontario, London, Ontario, Canada.

⁴Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria.

¶ P.B and V.C. contributed equally to this work

≠ New address: Department of Life Sciences, Imperial College London, United Kingdom

New address: Department of Biochemistry and Biophysics, Stockholm University, Sweden

& New address: State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, China

** **Lead corresponding author:** stefan.jansson@umu.se

* **Second corresponding author:** a.holzwarth@vu.nl

Abstract

Evergreen conifers in boreal forests can survive extremely cold (freezing) temperatures during the long dark winter and fully recover during the summer. A phenomenon called ‘sustained quenching’ putatively provides photoprotection and enables their survival, but its precise molecular and physiological mechanisms are not understood. To unveil them, we have analyzed the seasonal adaptation of the photosynthetic machinery of Scots pine (*Pinus sylvestris*) trees by monitoring multi-year changes in weather, chlorophyll fluorescence, chloroplast ultrastructure, and changes in pigment-protein composition. Recorded Photosystem II and Photosystem I performance parameters indicate that highly dynamic structural and functional seasonal rearrangements of the photosynthetic apparatus occur. Although several mechanisms might contribute to ‘sustained quenching’ of winter/early spring pine needles, time-resolved fluorescence analysis shows that extreme down-regulation of photosystem II activity along with direct energy transfer from photosystem II to photosystem I plays a major role. This mechanism is enabled by extensive thylakoid destacking allowing for mixing of PSII with PSI complexes. These two linked phenomena play crucial roles in winter acclimation and protection.

Graphical abstract

Keywords: Conifer, Seasonal adaptation, Photosystem II, Photosystem I, Quenching, Thylakoid ultrastructure, Non-photochemical quenching, Sustained quenching.

Introduction

Photosynthesis is the basis for most life on earth, and the ability to sustain growth by harvesting sunlight confers such an enormous evolutionary advantage that photosynthetic organisms have developed numerous adaptations that enable them to photosynthesize in diverse environments. These include vast boreal forests, which cover ~70% of all coniferous forest of the world (mainly in the northern hemisphere)¹, which have lower species diversity than many other terrestrial ecosystems and are often dominated by evergreen conifers, like pine and spruce². Broadleaf deciduous trees and shrubs are also present, and sometimes deciduous conifers such as Larch (*Larix*) species, but there is no doubt that evergreen conifers are particularly well adapted to harsh boreal conditions, including short growing seasons and cold, snowy winters. Thus, evergreen conifers' adaptations to boreal regions must presumably include regulatory processes that protect the photosynthetic apparatus in their needles during the harsh winter and early spring. Knowledge of photoprotective mechanisms in various kinds of photosynthetic organisms has increased considerably in recent decades. Several control systems have been identified that allow the photosynthesis machinery to harmlessly dissipate excess excitation energy³⁻⁷. In 2003, Öquist and Huner⁸ published a seminal review of photosynthesis in evergreen plants, which pointed out that overwintering conifer needles enter a state of 'sustained quenching' during winter. They found strong evidence for major alterations in the organization and composition of the photosystem II (PS II) antenna but also concluded that photosystem I (PS I) may play an important role, via nonphotochemical quenching of absorbed light or via quenching absorbed light photochemically through cyclic electron transport. Recently it was also shown that alternative electron transport might add up to this as well⁹. Up to date there exists no clear mechanism rationalizing winter quenching in conifers, although there are several proposed hypotheses^{8,10}. Several protection mechanisms

may also occur in parallel, and (if so) they are likely activated largely well before the extreme stress occurs^{11,12}. But the major conundrum arises in early spring, when temperatures are typically still low, so plants' biochemical and metabolic activities are strongly limited, but solar radiation is already high⁸. Once induced, the mechanisms need to be sustained, i.e. locked-in over the long winter season and protect the photosynthetic machinery. When conditions improve late in the spring, photosynthetic apparatus is restored and attains its active growth state in summer.

In previous explorations of these phenomena, a marked drop in steady-state fluorescence (measured as F_v/F_m) has been recorded in several overwintering conifers, and some indications of the quenching mechanisms involved have been obtained. These reportedly include destacking of thylakoid membranes and associated changes in chloroplast ultrastructure¹³, in accordance with the drop in PSII maximal fluorescence⁸. In higher plants, thylakoid stacking and heterogeneity play crucial roles in the localizations of PSII and PSI in grana (tightly appressed thylakoid layers) and stroma lamellae, respectively. In conifers, although an extreme drop in maximal fluorescence in winter/early spring has been observed, its mechanistic relationship to thylakoid structural changes has not been explained. In the study reported here, we monitored steady-state chlorophyll fluorescence, ultrafast time-resolved fluorescence, and chloroplast ultrastructure in Scots pine needles from autumn to summer in three successive years. Our data strongly indicate that chlorophyll fluorescence quenching and thylakoid destacking, are strongly linked, mutually dependent, and crucial for the survival of evergreen conifers in the extreme northern boreal winter and early spring, when temperatures are low but solar radiation levels may be high.

Results:

Seasonal changes in Photosystem II- and Photosystem I-related functional activities

To monitor photosynthetic performance, we recorded several PSII and PSI parameters during three consecutive growth seasons (2015-2016, 2016-2017, and 2017-2018) along with concomitant changes in daily air temperature and solar radiation (Fig. 1A-I, II, III). For simplicity, in the main figures we only present (here onwards) data from 2017-2018, which we divided into five distinct seasons, based on weather parameters: Summer (S, June-Aug), autumn (A, Sept-mid Nov), winter (W, mid Nov- mid Feb), early spring (ES, mid Feb-mid Apr), and late spring (LS, mid Apr-June). Data from the other two growth seasons are provided in Supplementary information 1, 2 and 3.

We found characteristic seasonal patterns in maximum PSII fluorescence (F_m) and maximum quantum efficiency of PSII (expressed as F_v/F_m), in accordance with earlier reports^{8,10}. F_v/F_m was highest in S, fell with reductions in ambient temperatures during A and W (Fig. 1B-I, II), and was lowest in ES (63% lower than in S), when low temperatures coincided with rises in solar irradiance (Fig. 1A-I-III; see also SI 2A). During LS, F_v/F_m gradually increased with increasing temperatures and peaked in S. The seasonal changes recorded in F_v/F_m were found to be mostly affected by changes in F_m rather than F_o (basic intrinsic fluorescence) (Fig. 1B-I). For deeper understanding of PSII performance, the fraction of absorbed light energy utilized by PSII photochemistry¹⁴ (Φ_{PSII}) was measured (Fig. 1B-III). During W Φ_{PSII} decreased significantly and reached minimal values (19% of S values) in ES (SI 2B).

Furthermore we tried to quantify the amount of absorbed light energy thermally dissipated by non-photochemical quenching (NPQ)³. The component of NPQ that play a crucial role under fluctuating light conditions is the fast component (ΔpH -PsbS dependent- qE^3 or zeaxanthin dependent- qZ^4), which increased with increasing light intensities in all the seasons.

In S and A, the fast component did not reach a stationary phase even at $1500 \mu\text{mol m}^{-2} \text{sec}^{-1}$ illumination, while in W and LS samples a stationary phase was reached at $500 \mu\text{mol m}^{-2} \text{sec}^{-1}$ and in ES already at $300 \mu\text{mol m}^{-2} \text{sec}^{-1}$ (Fig. 1C-I). Most importantly, in ES inducible steady-state NPQ was much smaller and overall slower due to the smaller amplitude of the fast component (~50% less than in S) in ES (Fig. 1C-II). This is due to the fact that ES needles have already developed static NPQ. Instead the quantum yield of non-regulated and/or constitutive loss (Φ_{NO}) of energy was high during ES (Fig. 1C-III) (SI 2D). This strongly suggests that this static NPQ is the fraction of absorbed light energy neither going to drive photochemistry (Φ_{PSII}) nor thermally dissipated by rapid regulated NPQ processes (qE/qZ).

To confirm that this static quenching is a manifestation of a ‘sustained mode of quenching’ we artificially relaxed the ES needles (hereafter, ESR) for 48h in low light ($80 \mu\text{mol m}^{-2} \text{sec}^{-1}$) with 18/6 h photoperiod. During this recovery we observed no significant changes in F_v/F_m for 6-8 hours (less than a 10% increase), a modest increase (30%) after 24 h (Fig. 1D-I) and almost complete (95%) recovery to S levels after 48 h. F_o did not change significantly throughout the recovery period (Fig. 1D-I), but changes in F_m followed the same recovery dynamics as F_v/F_m (Fig. 1D-I), although maximal fluorescence (F_m) was much higher in S than in 48 h ESR samples. Φ_{PSII} did not change significantly until 12 h of recovery but recovered to 50% and 90% of S levels after 24 and 48 h, respectively (Fig. 1D-II). qE/qZ -dependent fast NPQ kinetics followed similar patterns to Φ_{PSII} (Fig. 1D-III). It should be noted that light-dependent induction of the fast component of NPQ (qE/qZ) was slower in the ESR samples than in S samples, even after 48 h of recovery, which appears to be mainly due to photoinhibition.

Seasonal changes in partitioning of absorbed light energy within PSI showed that $Y(I)$ was highest in S, declined in A and W, and reached minimal values in ES (Fig. 2A-I). Although it decreased, $Y(I)$ was much less affected than the PSII activity (Φ_{PSII}) during the

cold periods (Fig. 1B-III) (SI 3A). Y(NA) followed the same seasonal pattern, with minimum values registered in ES and a steady recovery until S (Fig. 2A-II) (SI 3B). In contrast to Y(NA), Y(ND) considerably increased during the cold periods and peaked (at 69% higher than S values) in ES, then gradually declined to minimum values in S (Fig. 2A-III, SI 3C). This suggests that during early spring most of the absorbed light energy is dissipated by oxidized P700 (P700⁺) to prevent photoinhibition caused by over reduction of the acceptor side as iron sulfur clusters get damaged¹⁵. During the first 3 h of the recovery period, Φ PSI did not significantly change, but it gradually returned to S levels over 48 h (Fig. 2B-I). Y(NA) did not change much during recovery (Fig. 1E-II). Y(ND) increased slightly during the first 3 h, but then gradually decreased, and ESR samples did not quite reach S values. Based on these results, we conclude that the restoration of PSI light use efficiency takes longer than 48 hours.

Sustained non-photochemical quenching dominates in early spring needles

To elucidate the mechanism of “sustained quenching” observed in pine during the early spring periods, ultrafast time-resolved fluorescence measurements on intact pine needles were performed at a temperature of -20°C using the setup and approach described previously^{16,17}. In addition, a special chamber was designed to keep the rotational cuvette with the sample at -20°C (SI 4.1). Fluorescence decay traces of S needles in the original state (dark-adapted, Fig. 3A, Summer dark) were compared to the needles collected in ES when sustained quenching was present (Fig. 3A, Early spring) and after the quenching in the needles had been relaxed at room temperature (ESR) (Fig. 3A, E. spring recovered). To understand whether sustained quenching observed in ES occurs via a similar quenching mechanism to light-induced quenching, S needles exposed to HL for 30 min (SQ) were also measured (Fig. 3A, Summer quenched). As shown by the direct comparison of the decay curves (Fig. 3A), the fluorescence in SQ needles is much shorter-lived as compared to that of S needles. However, the fluorescence decays of

ES needles are still pronouncedly shorter-lived than that of the SQ samples, thus characterizing the “sustained quenching” in the ES samples as the most pronounced quenching at all detection wavelengths and under all conditions. In contrast, ESR and S samples showed very similar fluorescence decays, indicating that the ES sample recovered quite (although not completely, vide infra) well from sustained quenching within 48 h of recovery treatment.

Global analysis of the fluorescence kinetics of pine needles

To get the first hints on the mechanism(s) underlying sustained quenching in early spring global analysis¹⁸ was performed on all datasets: ES, S, SQ (Fig. 3 B) and ESR (SI 4.2) needles. Six Decay Associated Spectra (DAS) were required to fit the fluorescence kinetics in all four cases. Three DAS were tentatively assigned to PSI: 11-16 ps, 50-60 ps, 150-180 ps (Fig 3B, SI 4.1). Their spectra and lifetimes are reminiscent of previously reported PSI-related DAS in other plant species in vivo^{16,17,19}. The fastest component represents energy equilibration between Chl_{red} and Chl_{blue}^{20,21}. The second DAS in S needles peaks around 685-690 nm with a broad emission in the 700-720 nm region. Therefore, it mainly represents PSI core decay in combination with the 710-720 Chls of Lhcas. The third DAS (185 ps) peaks at 680 nm with some contribution at 730 nm, and therefore, it should be primarily assigned to LHCII and some Chl_{red} in PSI. In quenched states (SQ and ES, in particular) these two DAS have substantially higher contribution around 680 nm and, as a result, the reconstructed steady-state PSI spectra have higher emission around 680-685 nm as compared to the unquenched states (S and ESR see SI 4.2). It indicates that upon quenching PSII and/or LHCII kinetics also contribute to these PSI-related components. In S and ESR states, the remaining three DAS peak at 682 nm and therefore, are assigned to PSII (Fig 3B and SI 4.1). Their lifetimes are 0.5-0.6 ns, 1.5-2.0 ns and 4 ns, similar to what was resolved previously for PSII in the closed state^{16,17}. However, in ES two considerably shorter-lived PSII-related DAS were observed with lifetimes of 0.3 ns

and 0.8 ns. This demonstrates that PSII kinetics is indeed quenched strongly in ES needles. Additionally, we resolved a DAS with 8 ps lifetime in the ES sample representing excitation energy transfer (EET) from Chl a pools emitting at ~680 nm to those emitting at 700-730 nm, respectively. This indicates that there occurs efficient EET between PSII and/or LHCII and PSI in the ES needles.

In a nutshell, global analysis results show that sustained quenching in ES state involves both PSII/LHCII and PSI through a direct energy transfer. This mechanism provides much stronger quenching than the high-light-induced quenching in the summer needles (SQ).

Target modeling of the fluorescence kinetics of unquenched pine needles

To resolve the mechanism(s) responsible for the ‘sustained quenching’ in pine needles in detail we performed target analysis²² of all the kinetic data. By testing kinetic models that had been validated previously on isolated subcomplexes^{23–25} it was possible to separate the PSI from the PSII kinetics. In S needles (Fig 4C, Summer dark) the PSI kinetics was described with two emitting compartments (PSI red, Ant/core) and one non-emitting radical pair (RP, Fig 3C). The resolved rates and Species Associated Spectra (SAS) are strongly reminiscent of the ones reported for the PSI-LHCI complex²⁵. Kinetics from the PSII antenna/RC complex was described by one emitting compartment (Ant/core) and two non-emitting radical pairs (RP1 and RP2, Fig 3C) as reported previously^{16,23,24,26}. According to their SAS and rates, the emitting species represents the equilibrated excited state of the PSII-LHCII super-complex. In our analysis, to achieve a very good fit, two pools of PSII complexes with different decay rates were required (pool 1, pool 2, Fig 3C, see the detailed comparison of the residuals plots in SI 4.3) for the S sample. The two pools differ in the rate constants of charge separation in a manner that is typical for PSII particles with different antenna sizes (see ^{18,19} for discussion and explanation), which result in different average lifetimes (Table 3 SM). In the S sample, PSI

with its antenna accounts for 30% of the total absorption cross-section at the excitation wavelength (662 nm), while the two PSII pools account for 40% and 30% of the total absorption cross-section. No internal quenching related to any NPQ process of these PSII compartments was observed.

The target model for the ESR needles is rather similar to that of S needles (SI 4.2). The main difference is that the smaller PSII pool (with a very small absorption cross-section of ca. 10%) shows an unusually small charge separation rate. The most likely explanation of this phenomenon is that a small percentage of PSII RCs has been photo inhibited during the 48 h relaxation process from the sustained quenching state, which agrees with NPQ measurements.

Direct energetic transfer between PSII and PSI is the dominating component of “sustained quenching”

As for the S samples, also the description of the ES sample (Fig. 3C) required a three - compartmental target model for PSI and the presence of two PSII pools, each described by a three-compartmental model. The two PSII pools differ in the rate constants of charge separation in a similar manner as in S samples. The kinetics are also very similar to that of the S samples, however with a slightly increased charge separation rate. This can be interpreted as a consequence of partial reduction in the physical antenna size. This goes in line with an increase in the Chl a/b ratio in ES as compared to S samples (SI 6 Table 2).

The striking feature is the presence of a very strong contribution of a direct energy transfer from PSII to PSI. Without allowing for this direct energy transfer, the data could not at all be fitted adequately (SI 4.3, 4.4). This process has a transfer rate of 4.4 ns^{-1} . A small compartment representing quenched and functionally detached LHCII was also needed for a satisfactory description of the ES samples (See more details on this component in the next section). Overall,

the target analysis shows that direct energy transfer from PSII to PSI complexes provides the dominant quenching mechanism of PSII complexes in ES needles causing the pronounced “sustained quenching”.

Comparison of sustained quenching and light-induced quenching

To model the fluorescence kinetics of SQ needles (Fig 4C) again three-compartmental models for PSI and PSII were required. However, unlike in previous cases, one single PSII pool was sufficient to describe the PSII kinetics.

Besides PSII and PSI, one additional component was needed to satisfactorily describe the fluorescence kinetics in the SQ samples. The rates, spectra and average lifetime (0.4 ns, Table 3 SI) of these compartments are strongly reminiscent of what was reported previously for highly quenched LHCII aggregates²⁷, and the quenched LHCII complexes under NPQ conditions in wild type *Arabidopsis*¹⁶ and in *Arabidopsis* mutants depleted of the reaction centers²⁸. We therefore assign this highly quenched component to the quenched LHCII antenna energetically disconnected from both PSII and PSI. However, the addition of this quenched LHCII compartment was not sufficient to fully describe the fluorescence kinetics (SI 4.4). The species-associated spectra (SAS) and kinetics of PSII still contained a substantial amount of 690-700 and 730 nm emission, entirely uncharacteristic for PSII super-complexes, but characteristic of PSI complexes^{21,25}. Interestingly, also the SAS of the PSI antenna/core complex contains a 680 nm emission, which is characteristic for PSII. This strongly suggests – as can be directly deduced from the spectral properties - the presence of a direct energetic contact between PSI and PSII (Fig 3C). Introducing such a connection in the target model indeed resulted in a considerably better fit (χ^2 decreased from 1.198 to 1.093, see SI 4.3). Also, the PSII and PSI kinetics became well-separated in such a model. The resolved direct energy transfer rate from PSII to PSI is quite high (2.3 ns^{-1}), implying that a part of energy absorbed

by PSII is actually funneled to PSI. However, this rate of direct energy transfer in SQ needles is only about half of that in ES needles, suggesting, that unlike in cold-induced sustained quenching, upon light illumination, spillover is not the major process. Contribution of the quenched antenna, on the other hand plays the major role.

Efficiency of direct energy transfer quenching in different conditions

By evaluating the kinetic information provided in Fig. 3C one can get a quantitative estimation of the degrees of light use efficiencies (for photosynthesis) on the one hand, and for photoprotection/quenching on the other hand, present in both PSII and PSI in the different situations (c.f. SI 4.5, SI Table 4). In S needles 70% of the total absorbed energy drives PSII charge separation and only 30% PSI charge separation. In the SQ needles the percentage of energy flowing into the PSII reaction center drops to 7.1%, while it increases to 67% for PSI. Upon strong light illumination most of PSII reaction centers will be closed, and a fraction of that total energy flowing through the RC can produce harmful reactive species like ROS^{29,30}. Since the percentage of energy flow into PSII decreases 10 times (70% in S and 7.1% in SQ) in SQ as compared to S, this means that the potential of oxidative damage is reduced by a factor of 10 by the quenching process. This factor is increased by a further factor of approx. 2 due to the large pool of functionally detached and quenched LHCII. So, in SQ needles the overall quenching provides a protection factor of 20 for PSII compared to S needles. In the sustained quenched ES samples this effect is much more extreme: Only 1.5% of the total absorbed energy in the system flows through the PSII reaction center potentially causing oxidative damage (assuming all other factors are equal). Thus, in total, ‘sustained quenching’ in ES samples provides a protection factor of approx. 40 to PSII (double of SQ) compared to S needles. This huge protection factor explains well why “sustained quenching” is so effective enabling pine needles to survive the harsh winter and early spring conditions.

Massive destacking of grana membranes in the winter

In accordance with early reports^{31,32}, we observed strong seasonal changes in chloroplast ultrastructure, including loss of grana stacks during early spring (Fig. 4A-III). Ultrastructural changes derived from morphometric analysis of electron micrographs (Fig. 4A) indicate that average numbers of grana per chloroplast steadily decreased from autumn to winter and reached minimal values in early spring, corresponding to just 68% of S values (SI 6 table 1). More strikingly, the number of appressed thylakoid layers/granum dramatically declined from A (4.97) to ES (2.72) and rose back in S (6.50) (Table 1). These changes corroborate the shift in grana stacking illustrated in Fig 4B. In S and A, 3-6 layered grana stacks accounted for 60-70% of total stacks. In contrast in ES samples, 70-75% and 15-20% of the grana had only two and three layers, respectively (Fig. 4B-II). These changes in ES samples were accompanied by a transient doubling of the number of lipid globules (plastoglobuli) per cell (SI 6, table 1) when the chloroplast structure deviated most strongly from the S state.

To obtain deeper understanding of the thylakoid plasticity in ES needles we also subjected needles collected at specific early spring and summer dates to EM analysis after artificially induced recovery under different light conditions (Fig. 5A-I to IV). When both S and ESR samples were exposed to HL for 30 (SQ1 for S samples, ESRQ1 for ESR samples) and 60 mins (SQ2 for S samples, ESRQ2 for ESR samples) similar destacking of grana (as of ES samples) have been observed (Fig 5B). These changes in grana structures recorded in ESR and S samples (Fig 5B-II) following short term HL exposure strongly corroborate the high dynamic plasticity of the thylakoid membrane, which appears to be essential for acclimation to the harsh boreal winters.

Changes in pigment and protein composition of ES, ER, SQ and S samples

We also analyzed pigments of the pine needles used for spectroscopic measurements and found substantial differences in pigment contents between S and ES needles (SI 6 table 2). In S and

ES samples the Chl a/b ratios were 2.8 and 3.4, respectively, indicating that S needles had higher relative amounts of Lhcbs and other Chl b-binding proteins. The Chl /carotenoid ratio was also substantially lower in ES samples than in S samples, indicating that they had higher amounts of carotenoids (mainly lutein and violaxanthin/zeaxanthin). We observed no significant difference in total Chl pigment contents between S and SQ samples.

For quantifying the core and antenna proteins, isolated thylakoids were subjected to SDS-PAGE and were immunoblotted against specific antibodies (SI 5). This revealed that ES samples contain a lower abundance of core (PsaD, PsbD) and antenna (Lhcb2, Lhca4) proteins than in S, in line with earlier reports³³ (SI 5). ESR samples contained lower amounts of PsbD and Lhcb2 compared to S, which might reflect on the minor amount of photoinhibition seen in fluorescence data.

Discussion

Distinct changes in chloroplast ultrastructure, including loss of grana thylakoids and disorganization resulting in mostly single-/bi-stromal thylakoid membranes during the cold winter season, followed by recovery during the late spring/summer season have been observed in various conifers^{8,13,32}. We observed similar strong seasonal variations in the chloroplast architecture of pine chloroplasts (Fig. 4, SI 6 table 1) and together with the fluorescence data presented above we are able to decipher the major molecular mechanism that connects these changes to the pronounced ‘sustained quenching’ described in conifers in the winter.

The seasonal reductions in maximal quantum efficiency of PSII (Fig. 1B-II) and light energy utilization during the winter (Fig. 1B-III) were associated with a substantial increase in excitation pressure, indicating overall reduction of the photosynthetic electron transport chain³⁴. In early spring, conifers harvest light energy but use of the excitation energy for

photosynthesis is severely restricted, resulting in potential photoinhibition. Hence, all overwintering conifers must develop highly efficient mechanisms for photoprotection of the photosynthetic apparatus. The major photoprotective mechanism for de-excitation of excess light energy in green plants and algae is the rapidly/ reversible ΔpH - dependent non-photochemical quenching (qE) that occurs in the pigment bed of LHCII proteins^{3,16,35}. However, several alternative and/or supplementary photoprotective mechanisms for effective thermal deactivation of excess light energy have also been proposed^{5,12,13,36–38}.

At first glance, the sharp decline in $\Phi PSII$ during early spring (Fig. 1B-III), might suggest that excess irradiance is dissipated via qE, which has been established as one of the most effective mechanisms for coping with the excess energy flux³. However, fast inducible qE was extremely low (Fig 1C-II,III) in ES samples and was not restored to S levels even after 48 h of recovery treatment. (Fig 1D-III). This is not unexpected, since, in contrast to the fast energy dissipation (qE) depending on ΔpH and PsbS-protein³ the energy dissipation during winter (sustained quenching) occurs under strong down-regulation, even perhaps in the absence of ΔpH ^{8,10,39}. However, we can assign this relatively small qE contribution to quenching to the small component (accounting for 4.7% of the absorption cross-section) of detached and quenched LHCII. This fraction was much smaller than in light-quenched samples (18%), and absent in both the dark-adapted S samples and recovered ES samples. Incidentally, a so-called ‘cold hard band’ has been reported in low temperature fluorescence spectra and kinetics of cold-acclimated evergreens^{39,40}. Features of this band are reminiscent of quenched LHCII aggregates at low temperature⁴¹, hence it is probably correlated with the fraction of detached and quenched LHCII observed in ‘sustained quenching’ conditions. This led us to conclude that this quenching seen in conifers is not only ‘Sustained NPQ’ but also has a different mechanism.

Detailed quantitative energy partitioning analysis of total absorbed light energy by PSII demonstrates a strong increase (4 fold) of the fraction of constitutive thermal dissipation (Φ_{NO})^{42,43} (Fig 1C-III). Usually, this thermal dissipation (Φ_{NO}) contribution is not explained by any clear mechanisms. However, in the case of ES samples it is strongly suggested by the data that the high levels of Φ_{NO} do reflect the ΔpH -independent “sustained” energy quenching^{8,10,39}. Our lifetime data provide an explanation for the origin of this component: A very high rate of direct energy transfer from PSII to PSI, provides this delta-pH independent (non-qE) quenching of PSII. Other photo protective mechanisms proposed, such as, role of photo-inactivated PSII complexes may also effectively dissipate excess excitation energy as heat⁴⁴, and since the photoinhibitory quenching (qI), dependent on inactivation and/or degradation of D1, relaxes within hours (or longer), the process could also be considered a form of ‘sustained quenching’. Moreover, the involvement of RC quenching, based on enhanced S2QA- and S2QB- charge recombination, favoring non-radiative PSII RC dissipation of excess light, has been suggested to supplement or even replace the fast component of non-photochemical quenching during winter, and thus play a significant role in overwintering conifers^{36,37}. However, we found no indication of photo inhibited PSII centers in the unrelaxed ES samples that displayed sustained quenching. A component with these characteristics was not observed in the ES samples. However, even if it would be present it could not explain the strong quenching effect since this component has longer average lifetime and higher fluorescence yield than the intact PSII units. In addition, we found no experimental evidence for PSII RC quenching either. All rate constants obtained from target modeling of the PSII antenna/RC pools in both unquenched and quenched states are quite normal. Thus, neither photoinhibition nor RC quenching alone could explain the strong ‘sustained quenching’. Moreover, changes in Y(I) suggest a lowered activity of PSI, even though PSI activity was less affected than PSII. In the absence of carbon fixation, one would expect higher acceptor side

limitation, but contrastingly we found Y(NA) less affected, and donor side limitation [Y(ND)] was very high (Compared to S). This makes sense if PSII directly transfers energy to PSI, making PSI an energy sink for PSII by reducing linear electron flow, and in turn causing PSI donor side limitation (Oxidised P700) to increase.

The detailed structural and functional data presented here enable inference of a coherent quenching/photoprotection mechanism involving structural rearrangements of the thylakoids. The model explains Scots pine's acclimation to the combination of harsh freezing temperatures and high solar radiation that occurs during early spring in northern boreal forests (Fig. 6). Acclimation to early spring conditions (Fig 6-II) results in massive loss of grana stacks (destacking) and formation of uniform membrane structures of stromal thylakoids. This allows for a redistribution and randomization of PSII and PSI. The reduction in the spatial distance between PSII and PSI complexes increases the probability for direct energy transfer from PSII to PSI⁴⁵⁻⁴⁷. This protective mechanism severely restricts linear electron flow since it strongly quenches PSII activity. It also has strong similarities to the potent quenching observed in other extreme environmental conditions when PSII requires strong protection. Examples include drying-induced quenching in lichen⁴⁸ and heat-induced quenching in *Symbiodinium* cells of coral⁴⁹. In both cases, destacking and reorganization of the thylakoid membrane occur, resulting in proximity of PSII to PSI complexes with direct energy transfer.

In conclusion, during early spring only a very small fraction of the absorbed light energy in PSII antennae is utilized for PSII photochemistry (Φ PSII) and the fast inducible NPQ (qE) is low since it not only requires a high Δ pH^{3,6,16} but there is a strong static quenching present already. On the other hand, thylakoid rearrangement and movement of complexes are likely coupled to the high zeaxanthin content⁵⁰ (SI 6 Table 2), and probably to pronounced phosphorylation of antenna and core proteins present under these conditions⁵¹. In this situation, the excess energy is dissipated primarily through direct energy transfer from PSII to PSI, which

appears in steady-state (PAM) fluorescence measurements as a non-regulated constitutive energy quenching. An additional, albeit smaller, amount of quenching and photoprotection is provided under sustained quenching conditions by the qE mechanism in functionally detached LHCII. It is possible that this fraction is linked to large aggregates containing LHCII, PsbS and other small proteins, previously detected in cold-adapted pine needles⁵². Thus, conifers appear to have a powerful protective mechanism that resembles those found in other photosynthetic organisms of relatively harsh environments^{48,49}. This protective mechanism plays a crucial role in the survival of pine from harsh boreal winters.

Material and methods

Weather. Weather data were recorded from October 2015 to May 2016, from November 2016 to June 2017, and from October 2017 to June 2018 on an hourly basis at the weather station of the Sweden's meteorological and hydrological institute, Umeå stations. (available on <https://www.smhi.se>).

Plant material and sampling. Fully developed needles were collected frequently, from Oct 2015 to May 2016, from Nov 2016 to June 2017 and Oct 2017 to June 2018, inclusive, at midday (8.00-10.00) from fully exposed south-facing branches of five Scots pine (*Pinus sylvestris* L.) trees growing on Umeå University campus (63° 49' N, 20° 18' E). The collected needles were immediately stored in darkness, at 0-5°C, in a laboratory at Umeå Plant Science Centre, and subjected to measurements (described in the following sections) shortly after sampling to minimize any further changes in the photosynthetic machinery. For recovery treatment samples from winter-stressed branches were collected in mid-March and subjected to recovery treatment, involving exposure to very low light (80 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) at 20°C⁵².

Further details are provided (together with other details regarding the sampling dates and procedures) in SI 1.

Modulated chlorophyll fluorescence and (P700+) measurements. Chlorophyll fluorescence was measured using a Dual-PAM-100 instrument (Heinz Walz GmbH, Effeltrich, Germany) at room temperature (20°C) after 30 min dark adaptation at low temperature [below 5°C, winter samples (samples collected during November to May)] or room temperature [(20°C, summer samples (samples collected during June to October)]. Maximum photochemical efficiency of PSII was calculated as $F_v/F_m = (F_m - F_o)/F_m$. To perform all measurements, we chose to record a light response curve where we measured F_v/F_m first, followed by P_m (maximal P700 oxidation), and then changing the actinic light intensity stepwise from 33 μE to 1469 μE . During recording light response curve, samples were kept in one light intensity for 3 mins followed by a saturating pulse of 4000 μE to get the optimal values. Partitioning of absorbed light energy was estimated as $\Phi_{PSII} + \Phi_{NPQ} + \Phi_{NO} = 1$ ^{42,43}. The effective photochemical quantum yield of PSI, $Y(I)$, was calculated as $Y(I) = 1 - (Y(ND) + Y(NA))$ ⁵³. Here, $Y(I)$ is the effective quantum yield of PSI (Φ_{PSI}) when reaction centers (RCs) are open with reduced donor side (P700) and oxidized acceptor side (A); $Y(NA)$ is the energy dissipation due to acceptor side limitation, reflecting the redox state of the acceptor side when RCs are closed with P700 reduced and both acceptors reduced; and $Y(ND)$ is the energy dissipation due to donor side limitation, reflecting the redox state with both P700 and A oxidized.

Ultrafast fluorescence measurements. Intact needles were subjected to ultrafast time-correlated single photon counting measurements at -20 °C in a rotation cuvette, as previously described^{16,17}. Pine needles were immersed in 60% PEG solution (pH 7.5), which was used as an anti-freeze. Tests confirmed that this solution did not significantly affect either the F_v/F_m values or fluorescence kinetics. These conditions allowed maintenance of the physiological and quenching states for a long time during the measurements and prevention of radiation damage

or further quenching induction by the very weak measuring light. Needles sampled in summer (S needles) were measured in both unquenched and fully light-quenched states. For measurement in the unquenched state, they were dark-adapted for 30 min, then quickly frozen to -20°C in the rotation cuvette. For measurement in the light-quenched state, the needles were illuminated by red light (intensity 1000 $\mu\text{mol m}^{-2}\text{sec}^{-1}$) for 30 min. at room temperature. During this time, fluorescence intensity was monitored and after 30 min. a steady-state level was reached. The needles were then frozen quickly under illumination in the rotation cuvette to -20°C. Early spring samples (ES samples) were measured either in the original ‘sustained quenching state’ by transferring them directly at -20 °C into the cooled rotation cuvette (SI 4.1), or in a ‘recovery state’ induced by exposing them to very low light (80 $\mu\text{mol m}^{-2}\text{s}^{-1}$) at 20°C, as described earlier, then freezing them (as in the measurements of the summer needles in unquenched state). After each fluorescence lifetime measurement, the samples were fast-frozen in liquid nitrogen and stored at -80 °C for further pigment analysis. The laser settings for the measurements were 40-60 μW power, 4 MHz repetition rate, and 1 mm diameter beam size.

Pigment and protein analysis. Pigments were extracted from frozen pine needles with 80% acetone. Chl a/b and Chl/carotenoid ratios were calculated by fitting the extracts’ absorption spectra with the spectra of the individual pigments, and the relative amounts of carotenoids were determined by HPLC as previously described⁵⁴. For protein composition analysis intact thylakoids were isolated followed by Grebe et al. 2018⁵⁵, subjected to SDS PAGE separation followed by Damkjær et al. 2009⁵⁶ and immunoblotted against Anti PsbD, PsaD, Lhcb2 and Lhcb4 as per manufacturer’s instructions (Agrisera AB, Sweden).

Transmission electron microscopy. Thin slices (< 0.5 mm) from the middle region of pine needles were cut in tap water and fixed in 4% paraformaldehyde, 2.5% glutaraldehyde (TAAB Laboratories, Aldermaston, England) in 0.1M or 0.05M (May and June) sodium cacodylate

buffer, pH 7.4 (TAAB Laboratories, Aldermaston, England). Thoroughly washed samples were post-fixed in 1% osmium tetroxide (TAAB Laboratories, Aldermaston, England). The fixed material was dehydrated in ethanol series with increasing concentrations and propylene oxide and finally embedded in Spurr resin (TAAB Laboratories, Aldermaston, England). Ultrathin sections (70 nm) were post contrasted in uranyl acetate and Reynolds lead citrate and further examined with Talos 120C electron microscope (FEI, Eindhoven, The Netherlands) operating at 120kV. Micrographs were acquired with a Ceta 16M CCD camera (FEI, Eindhoven, The Netherlands) using TEM Image & Analysis software ver. 4.14 (FEI, Eindhoven, The Netherlands). The chloroplast ultrastructure was analyzed from the electron micrographs by measuring the average number of chloroplasts per cell, average number of grana per chloroplasts and average number of appressed thylakoids per grana stack (Ng)^{57,58}.

Statistical analysis. The significance of between-mean differences was assessed by t-tests and p values were recorded. One (*), two (**) and three (***) asterisks in the presented figures indicate $P \leq 95\%$, $P \leq 99\%$, and $P \leq 99.90\%$, respectively, for fluorescent and electron microscopic measurements.

Acknowledgements:

This work was supported by SE2B Horizon 2020 under grant agreement no. 675006 (SE2B) to SJ (Umeå University) and RC (Vrije Universiteit Amsterdam), the Swedish Research council (VR) and the Kempe foundation to SJ and the Netherlands Organization for Scientific Research (NWO-Vici grant to RC).

Author Contributions:

SJ and ARH conceived the idea; PB, AGI, ARH, and SJ designed the experiments; PB, ZZ, SP and TS performed the chlorophyll fluorescence and P700 experiments; PB performed TEM studies and protein quantification; PB and AGI analysed chlorophyll fluorescence data; VC performed ultrafast time-resolved fluorescence measurements; VC and ARH analyzed the time-resolved fluorescence data; PB, VC, AGI, ARH, RC and SJ analysed and discussed all results; PB, AGI, VC, ARH and SJ wrote the paper.

References

1. Gowthrop D. *Vanishing Halo*. Greystone Books; 2009.
<https://books.google.se/books?id=t-SDCgAAQBAJ>
2. Nystedt B, Street NR, Wetterbom A, et al. The Norway spruce genome sequence and conifer genome evolution. *Nature*. 2013;497(7451):579-584.
3. Li X-P, BjoÈrkman O, Shih C, et al. A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature*. 2000;403(6768):391-395.
4. Jahns P, Holzwarth AR. The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. *Biochim Biophys Acta (BBA)-Bioenergetics*. 2012;1817(1):182-193.
5. Malnoë A. Photoinhibition or photoprotection of photosynthesis? Update on the (newly termed) sustained quenching component qH. *Environ Exp Bot*. 2018;154:123-133.
6. Niyogi KK. Photoprotection revisited: genetic and molecular approaches. *Annu Rev Plant Biol*. 1999;50(1):333-359.
7. Horton P, Ruban A V, Walters RG. Regulation of light harvesting in green plants. *Annu Rev Plant Biol*. 1996;47(1):655-684.
8. Öquist G, Huner NPA. Photosynthesis of overwintering evergreen plants. *Annu Rev Plant Biol*. 2003;54(1):329-355.
9. Yang Q, Blanco NE, Hermida-Carrera C, Lehotai N, Hurry V, Strand Å. Two dominant boreal conifers use contrasting mechanisms to reactivate photosynthesis in the spring. *Nat Commun*. 2020;11(1):1-12.
10. Verhoeven A. Sustained energy dissipation in winter evergreens. *New Phytol*. 2014;201(1):57-65.
11. Harsch MA, Zhou Y, HilleRisLambers J, Kot M. Keeping pace with climate change:

- stage-structured moving-habitat models. *Am Nat.* 2014;184(1):25-37.
12. Demmig-Adams B, Adams III WW. Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. *New Phytol.* 2006;172(1):11-21.
13. Krol M, Hurry VM, Maxwell DP, Malek L, Ivanov AG, Huner NPA. Low growth temperature inhibition of photosynthesis in cotyledons of jack pine seedlings (*Pinus banksiana*) is due to impaired chloroplast development. *Can J Bot.* 2002;80(10):1042-1051.
14. Genty B, Briantais J-M, Baker NR. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim Biophys Acta (BBA)-General Subj.* 1989;990(1):87-92.
15. Tiwari A, Mamedov F, Grieco M, et al. Photodamage of iron–sulphur clusters in photosystem I induces non-photochemical energy dissipation. *Nat Plants.* 2016;2(4):1-9.
16. Holzwarth AR, Miloslavina Y, Nilkens M, Jahns P. Identification of two quenching sites active in the regulation of photosynthetic light-harvesting studied by time-resolved fluorescence. *Chem Phys Lett.* 2009;483(4-6):262-267.
17. Chukhutsina VU, Holzwarth AR, Croce R. Time-resolved fluorescence measurements on leaves: principles and recent developments. *Photosynth Res.* 2019;140(3):355-369.
18. Van Stokkum IHM, Larsen DS, Van Grondelle R. Global and target analysis of time-resolved spectra. *Biochim Biophys Acta (BBA)-Bioenergetics.* 2004;1657(2-3):82-104.
19. Chukhutsina VU, Liu Xin, Xu Pengqi CR. The major light-harvesting complex (LHCII) is an antenna of photosystem I in dark-adapted plants. *Nat Plants.* Published online 2020. doi:10.1038/s41477-020-0693-4
20. Croce R, Dorra D, Holzwarth AR, Jennings RC. Fluorescence decay and spectral evolution in intact photosystem I of higher plants. *Biochemistry.* 2000;39(21):6341-

- 613 6348.
- 614 21. Wientjes E, Van Stokkum IHM, Van Amerongen H, Croce R. Excitation-energy
615 transfer dynamics of higher plant photosystem I light-harvesting complexes. *Biophys J.*
616 2011;100(5):1372-1380.
- 617 22. Holzwarth AR. Data analysis of time-resolved measurements. In: *Biophysical*
618 *Techniques in Photosynthesis*. Springer; 1996:75-92.
- 619 23. Holzwarth AR, Müller MG, Niklas J, Lubitz W. Ultrafast transient absorption studies
620 on photosystem I reaction centers from *Chlamydomonas reinhardtii*. 2: mutations near
621 the P700 reaction center chlorophylls provide new insight into the nature of the
622 primary electron donor. *Biophys J.* 2006;90(2):552-565.
- 623 24. Miloslavina Y, Szczepaniak M, Müller MG, et al. Charge separation kinetics in intact
624 photosystem II core particles is trap-limited. A picosecond fluorescence study.
625 *Biochemistry.* 2006;45(7):2436-2442.
- 626 25. Slavov C, Ballottari M, Morosinotto T, Bassi R, Holzwarth AR. Trap-limited charge
627 separation kinetics in higher plant photosystem I complexes. *Biophys J.*
628 2008;94(9):3601-3612.
- 629 26. Szczepaniak M, Sander J, Nowaczyk M, Müller MG, Rögner M, Holzwarth AR.
630 Charge separation, stabilization, and protein relaxation in photosystem II core particles
631 with closed reaction center. *Biophys J.* 2009;96(2):621-631.
- 632 27. Miloslavina Y, Wehner A, Lambrev PH, et al. Far-red fluorescence: A direct
633 spectroscopic marker for LHClI oligomer formation in non-photochemical quenching.
634 *FEBS Lett.* 2008;582(25-26):3625-3631.
- 635 28. Ware MA, Giovagnetti V, Belgio E, Ruban A V. PsbS protein modulates non-
636 photochemical chlorophyll fluorescence quenching in membranes depleted of
637 photosystems. *J Photochem Photobiol B Biol.* 2015;152:301-307.

29. Lambrev PH, Miloslavina Y, Jahns P, Holzwarth AR. On the relationship between non-photochemical quenching and photoprotection of photosystem II. *Biochim Biophys Acta (BBA)-Bioenergetics*. 2012;1817(5):760-769.
30. Khorobrykh S, Havurinne V, Mattila H, Tyystjärvi E. Oxygen and ROS in Photosynthesis. *Plants*. 2020;9(1):91.
31. Parker J, Philpott DE. An electron microscopic study of chloroplast condition in summer and winter in *Pinus strobus*. *Protoplasma*. 1961;53(4):575-583.
32. MARTIN B, ÖQUIST G. Seasonal and experimentally induced changes in the ultrastructure of chloroplasts of *Pinus silvestris*. *Physiol Plant*. 1979;46(1):42-49.
33. Verhoeven A, Osmolak A, Morales P, Crow J. Seasonal changes in abundance and phosphorylation status of photosynthetic proteins in eastern white pine and balsam fir. *Tree Physiol*. 2009;29(3):361-374.
34. Huner N, Dahal K, Hollis L, et al. Chloroplast redox imbalance governs phenotypic plasticity: the “grand design of photosynthesis” revisited. *Front Plant Sci*. 2012;3:255.
35. Nilkens M, Kress E, Lambrev P, et al. Identification of a slowly inducible zeaxanthin-dependent component of non-photochemical quenching of chlorophyll fluorescence generated under steady-state conditions in *Arabidopsis*. *Biochim Biophys Acta (BBA)-Bioenergetics*. 2010;1797(4):466-475.
36. Ivanov A, Sane P, Zeinalov Y, Simidjiev I, Huner N, Öquist G. Seasonal responses of photosynthetic electron transport in Scots pine (*Pinus sylvestris* L.) studied by thermoluminescence. *Planta*. 2002;215(3):457-465.
37. Sveshnikov D, Ensminger I, Ivanov AG, et al. Excitation energy partitioning and quenching during cold acclimation in Scots pine. *Tree Physiol*. 2006;26(3):325-336.
38. Tikkanen M, Aro E-M. Integrative regulatory network of plant thylakoid energy transduction. *Trends Plant Sci*. 2014;19(1):10-17.

39. Gilmore AM, Ball MC. Protection and storage of chlorophyll in overwintering evergreens. *Proc Natl Acad Sci.* 2000;97(20):11098-11101.
40. Gilmore AM, Matsubara S, Ball MC, Barker DH, Itoh S. Excitation energy flow at 77 K in the photosynthetic apparatus of overwintering evergreens. *Plant Cell Environ.* 2003;26(7):1021-1034.
41. Ostroumov EE, Götze JP, Reus M, Lambrev PH, Holzwarth AR. Characterization of fluorescent chlorophyll charge-transfer states as intermediates in the excited state quenching of light-harvesting complex II. *Photosynth Res.* Published online 2020:1-23.
42. Kramer DM, Johnson G, Kiirats O, Edwards GE. New fluorescence parameters for the determination of Q A redox state and excitation energy fluxes. *Photosynth Res.* 2004;79(2):209.
43. Hendrickson L, Furbank RT, Chow WS. A simple alternative approach to assessing the fate of absorbed light energy using chlorophyll fluorescence. *Photosynth Res.* 2004;82(1):73.
44. Krause GH. Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. *Physiol Plant.* 1988;74(3):566-574.
45. Barber J. An explanation for the relationship between salt-induced thylakoid stacking and the chlorophyll fluorescence changes associated with changes in spillover of energy from photosystem II to photosystem I. *FEBS Lett.* 1980;118(1):1-10.
46. Barber J. Influence of surface charges on thylakoid structure and function. *Annu Rev Plant Physiol.* 1982;33(1):261-295.
47. Barber J. Photosynthetic energy conversion: natural and artificial. *Chem Soc Rev.* 2009;38(1):185-196. doi:10.1039/B802262N
48. Slavov C, Reus M, Holzwarth AR. Two different mechanisms cooperate in the desiccation-induced excited state quenching in *Parmelia* lichen. *J Phys Chem B.*

- 2013;117(38):11326-11336.
49. Slavov C, Schrameyer V, Reus M, et al. “Super-quenching” state protects Symbiodinium from thermal stress—implications for coral bleaching. *Biochim Biophys Acta (BBA)-Bioenergetics*. 2016;1857(6):840-847.
50. Vajravel S, Kis M, Kłodawska K, et al. Zeaxanthin and echinenone modify the structure of photosystem I trimer in *Synechocystis* sp. PCC 6803. *Biochim Biophys Acta (BBA)-Bioenergetics*. 2017;1858(7):510-518.
51. Merry R, Jerrard J, Frebault J, Verhoeven A. A comparison of pine and spruce in recovery from winter stress; changes in recovery kinetics, and the abundance and phosphorylation status of photosynthetic proteins during winter. *Tree Physiol*. 2017;37(9):1239-1250.
52. Ottander C, Campbell D, Öquist G. Seasonal changes in photosystem II organisation and pigment composition in *Pinus sylvestris*. *Planta*. 1995;197(1):176-183.
53. Schreiber U. Saturation pulse method for assessment of energy conversion in PSI. *PAM Appl Notes*. 2008;1:11-14.
54. Xu P, Tian L, Klotz M, Croce R. Molecular insights into Zeaxanthin-dependent quenching in higher plants. *Sci Rep*. 2015;5(1):1-10.
55. Grebe S, Trotta A, Bajwa AA, et al. The unique photosynthetic apparatus of Pinaceae: analysis of photosynthetic complexes in *Picea abies*. *J Exp Bot*. 2019;70(12):3211-3225.
56. Damkjær JT, Kereiche S, Johnson MP, et al. The photosystem II light-harvesting protein Lhcb3 affects the macrostructure of photosystem II and the rate of state transitions in *Arabidopsis*. *Plant Cell*. 2009;21(10):3245-3256.
57. Ivanov AG, Ignatova NS, Christov AM. Comparative ultrastructural and fluorescence studies of grapevine (*Vitis vinifera* L.) chloroplasts isolated from stem and leaf tissues.

Plant Sci. 1990;67(2):253-257.

58. Ivanov AG, Kitcheva MI, Christov AM, Popova LP. Effects of abscisic acid treatment on the thermostability of the photosynthetic apparatus in barley chloroplasts. *Plant Physiol.* 1992;98(4):1228-1232.

738 Fig 1. Bag et al 2020

A. Weather during three measuring periods

B. Fluorescence intensities of PSII

C. Regulated and non-regulated energy dissipation

D. Recovery of PSII under artificial conditions

739

740 **Figure 1. Seasonal dynamics of weather and photochemical performance of PSII measured by chlorophyll**
741 **fluorescence in Scots pine needles** A. Changes in temperature (°C) (Left Y axis) and solar radiation (watt m⁻²)
742 (Right Y axis) during 2015-2016 (I), 2016-2017 (II), 2017-2018 (III) measuring seasons. B. Seasonal dynamics
743 of PSII photochemistry: (I) Changes in maximal (Fm) and basic (Fo) fluorescence. (II) Maximal quantum
744 efficiency of PSII measured as Fv/Fm (III) Effective quantum yield of PSII (Φ(II)). C. Energy dissipation
745 measured as regulated and non-regulated non photochemical quenching. (I) Changes in NPQ with increasing PAR
746 (II) Induction of NPQ with constant actinic light, (III) Quantum yield of non-regulated non photochemical
747 quenching. D. Recovery of pine needles under artificial conditions at 80 μmol photons m⁻² s⁻¹ of light for 48h with
748 18/6 photoperiod (I) Changes in Fo, Fm and Fv/Fm, (II) Changes in Φ(II), (III) Induction of NPQ with constant
749 actinic light of 300 μmol photons m⁻² s⁻¹. All measurements were taken after 30 mins of dark adaptation at 4°C in
750 winter and room temperature in summer. All data are means ± SD (n = 3-5) and the statistically significant
751 differences are marked by asterisks: * - p ≤ 95%; ** - p ≤ 99%; *** - p ≤ 99.9%.

Fig 2. Bag et al. 2020

A. Energy partitioning in PSI

B. Recovery of PSI under ambient conditions

Figure 2. Seasonal changes of PSI photochemistry in Scots pine needles | A. Energy distribution in PSI considering $Y(I)+Y(ND)+Y(NA) = 1$, where Y(I) (I), Y(NA) (II) and Y(ND) (III) are photochemical quantum yield of PSI (when P700 is reduced and A is oxidised), energy dissipation in PSI (measure of acceptor side limitation, when P700 and A both are reduced) and energy dissipation in PSI (measure of donor side limitation, when P700 and A both are oxidised), respectively. B. Recovery of PSI photochemistry under ambient conditions: (I) Y(I), (II) Y(NA), (III) Y(ND) of PSI during the recovery period. All measurements were taken after 30 mins of dark adaptation at 4°C in winter and room temperature in summer. All data are means $\pm$ SD (n = 3-5) and the statistically significant differences are marked by asterisks: * - p ≤ 95%; ** - p ≤ 99%.

770 Fig 3. Bag et al 2020

771 **Fig 3. Time-resolved fluorescence of intact pine needles measured using TCSPC** (A) Fluorescence decay
772 traces measured at -20 °C and at four characteristic wavelengths: 686 nm (mainly PSII, LHCII contributions), 698
773 nm (PSII, PSI contributions), 723 nm (mainly PSI contribution), and 741 nm (mainly PSI contribution). (B) Global
774 analysis of pine needles in three states: Summer dark (S, dark-adapted summer needles, left row), ES (E.spring
775 needles with “sustained quenching” present, middle row), Summer quenched (SQ, right row) (C) Kinetic target
776 analysis of pine needles in the three states. The kinetic target modeling analysis (SAS top, kinetic model with rate constants
777 in ns⁻¹, bottom) shows the results of the detailed target modeling of the fluorescence kinetics of pine needles. The
778 rate constants (ns⁻¹) were determined from global target modeling analysis. Species-associated emission spectra (SAS)
779 resulted from the fit of the target kinetic model in the corresponding state. *Note* that fluorescence decay
780 measurements below 680 nm to detect the decreasing short-wavelength part of the spectra were not possible due
781 to the extremely high scattering of the pine needles. This has no effect however on the ability to distinguish the
782 various lifetime components kinetically and spectrally.

Fig 4. Bag et al 2020

A. Seasonal changes in chloroplast ultrastructure

B. Changes in thylakoid layers in grana

Figure 4. Transmission electron microphotographs depicting seasonal variations in chloroplast ultrastructure in pine needles A. Chloroplast structure in (I) Autumn (II) Winter (III) E. Spring (IV) L. Spring and (V) Summer. B. Histograms of frequency distributions of numbers of thylakoids per granum during the five distinct seasonal periods. The histograms were calculated from 80-100 electron micrographs per season, representing 2-3 chloroplasts per image (Error bars represent SD calculated from 800-1000 stacks per season).

Fig 5. Bag et al 2020

Figure 5. Artificial induction of changes in chloroplast ultrastructure of pine needles | **A.** Changes in chloroplast ultrastructure in (I) E. spring (ES), (II) E. spring samples recovered (ER) at 18°C for 48 hours with a photoperiod of 18 h at $80 \mu\text{mol m}^{-2}\text{s}^{-1}$, (III) ER samples treated with $800 \mu\text{mol m}^{-2}\text{s}^{-1}$ high light for 30 min (ERQ1), (IV) for 60 min (ERQ2). (V) Summer (S), (VI) Summer samples treated with $1200 \mu\text{mol m}^{-2}\text{s}^{-1}$ high light for 30 min (SQ1), (IV) for 60 min (SQ2). **B.** The number of grana per chloroplasts (40 images typically containing 2-3 chloroplast per image) (I); Histograms of frequency distributions of numbers of thylakoids per granum in different (II) E. spring treated (III) Summer treated samples. Error bars represent SD obtained from analysis of 460-540 grana stacks.

816 Fig 6. Bag et al 2020

817
818 **Figure 6. Molecular model for acclimation of photosynthetic machinery under changing**
819 **natural environmental conditions**, such as, in Summer unquenched (A); Winter quenched
820 (B); Summer quenched (C) in Scots pine.
821

826 **Supplementary information.**

827 **Supplementary information 1. Sampling for seasonal profiling**

828 1A. Sampling dates for Fluorescence (2015-2016, 2016-2017 and 2017-2018) and P700
829 measurements (only 2016-2017 and 2017-2018)

830 Season 2015-2016			Season 2016-2017				Season 2017-2018			
Year	Month	Week	Year	Month	Week	Date	Year	Month	Week	Date
2015	Sept	37	2016	Nov	45	7.11.16	2017	Oct	42	17.10.17
2015	Oct	40	2016	Nov	45	11.11.16	2017	Dec	51	19.12.17
2015	Oct	42	2016	Nov	46	14.11.16	2018	Mar	11	12.03.18
2015	Nov	45	2016	Nov	47	22.11.16	2018	May	18	02.05.18
2015	Nov	47	2016	Nov	47	24.11.16	2018	July	30	24.07.18
2015	Dec	50	2016	Nov	48	28.11.16				
2015	Dec	52	2016	Dec	48	2.12.16				
2016	Jan	1	2016	Dec	49	7.12.16				
2016	Jan	2	2016	Dec	50	14.12.16				
2016	Feb	6	2017	Jan	1	05.01.17				
2016	Feb	7	2017	Jan	3	16.01.17				
2016	Mar	9	2017	Jan	3	19.01.17				
2016	Mar	11	2017	Jan	4	24.01.17				
2016	Apr	14	2017	Feb	8	22.02.17				
2016	Apr	15	2017	Feb	8	24.02.17				
2016	May	19	2017	Mar	9	02.03.17				
2016	May	21	2017	Mar	10	06.03.17				
			2017	Mar	10	09.03.17				
			2017	Mar	11	17.03.17				
			2017	Mar	12	23.03.17				
			2017	Mar	13	30.03.17				
			2017	Apr	15	11.04.17				
			2017	Apr	15	14.04.17				
			2017	Apr	16	20.04.17				
			2017	Apr	17	25.04.17				
			2017	May	18	02.05.17				
			2017	May	18	04.05.17				
			2017	May	19	08.05.17				
			2017	May	20	15.05.17				
			2017	June	22	03.06.17				

Color Code	
	Summer
	Winter
	Early Spring
	Late Spring
	Summer

831

832

833

834

835 1B. Sampling for Time resolved measurements (2016-2017 and 2017-2018)

Year	Month	Week	Date
2017	Mar	9	02.03.17
2017	Mar	10	06.03.17
2017	Mar	10	09.03.17
2017	May	20	15.05.17
2017	June	22	03.06.17

2018	July	30	24.07.18
------	------	----	----------

842

843 1C. Sampling for seasonal Electron Microscopy (2016-2017 and 2017-2018)

844

Year	Month	Week	Date
2016	Nov	45	11.11.16
2016	Nov	46	14.11.16
2016	Dec	48	2.12.16
2016	Dec	50	14.12.16
2017	Jan	1	05.01.17
2017	Feb	8	24.02.17
2017	Mar	9	02.03.17
2017	Mar	10	06.03.17
2017	Mar	10	09.03.17
2017	Apr	15	14.04.17
2017	Apr	16	20.04.17
2017	Apr	17	25.04.17
2017	May	18	02.05.17
2017	May	20	15.05.17
2017	June	22	03.06.17

Year	Month	Week	Date
2017	Oct	42	17.10.17
2017	Dec	51	19.12.17
2018	Mar	11	12.03.18
2018	May	18	02.05.18
2018	July	30	24.07.18

856

857 1D. Sampling for protein quantification (2016-2017 and 2017-2018)

Year	Month	Week	Date
2017	Mar	9	02.03.17
2017	Mar	10	06.03.17
2017	Mar	10	09.03.17
2017	May	20	15.05.17
2017	June	22	03.06.17

2018	July	30	24.07.18
------	------	----	----------

864

Supplementary information 2. Seasonal performance of PSII

Supplementary information 3. Seasonal performance of PSI

Supplementary information 4. Lifetime measurements of pine needles

4.1 Measuring cuvette with pine needles inside in S state (A) or ES (B). Temperature control chamber, with the cuvette inside it during the experiment.

887 4.2 (A) and target (C) analysis of E.spring needles relaxed. (B) Reconstructed steady-state
888 PSI spectra in four measured states

889
890
891 4.3. Targeted analysis of fluorescence kinetics of pine needles without spillover mechanism
892 present

893
41

B. Target analysis: E.spring (no spillover)

4.4. Auto co-relation and residuals plots

A. Example traces showing fitting of the data

924 B. Auto correlation and residual plot (Summer and Summer quenched)

925

926

927

928 C. Auto correlation and residual plot (E.spring and E.spring recovered)

929

930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945

4.5. Time-dependent (on log time scale) populations of selected PSII and PSI compartments as calculated from the fluorescence kinetics (Fig. 3C). The dashed/dotted curves show the kinetics energy (normalized to the total absorption cross-section) flowing into PSI (purple dashed curves) and PSII (dotted black curves). The initial excitation input was taken from the excitation vectors of corresponding target analysis results (Fig 3C). Depending on the state of the respective reaction center, that energy will be either used for photochemistry or will be deactivated non-radiatively (quenching). See Table 4 SI for the percentages. Black (PSI) and green (PSII) curves show the time course of the excited state populations.

Supplementary Information 5. I. SDS_PAGE separation of thylakoid proteins loaded based on equal chlorophyll. II. Quantification protein by specific antibodies against PsbD, Lhcb2, PsaD and Lhca4.

Supplementary Information 6.

Table 1.

Parameters	Autumn	Winter	E.Spring	L.Spring	Summer
Number of Chloroplasts	15.2±3.93a	12.73±3.72c	13.46±4.05b	14.2±4.26	16.3±2.38
Number of grana per chloroplast	23.07±6.90c	18.66±9.24c	18.73±7.06c	25.38±9.88	27.47±8.62
Number of thylakoids per grana	4.97±0.27c	4.02±0.34c	2.72±0.46c	2.85±0.51c	6.50±0.33
Lipid globules per Chloroplast	27.3±19.81c	50.37±16.23c	55.7±15.07c	33.125±18.00c	15.67±6.29

Table 1. Quantitative analysis of seasonal changes in chloroplast ultrastructure as seen in Transmission electron microscopy. Statistical significance levels are referred as a, b, c denoting 99.95%, 99.99% and 99.999% level of significance.

Table 2.

	Summer	Summer Quenched	E.spring (recovered)	E. spring
Chl a/b	2.85±0.15	2.83±0.14	2.54±0.16	3.36±0.20
Chl /Car	4.90±0.48	4.67±0.34	2.72±0.10	2.98±0.45
Chl/fr w, mg/g	1.06±0.17	1.05±0.31	0.64±0.26	0.55±0.05
Carotenoids/ Chl a				
neo	0.23±0.02	0.23±0.02	0.52±0.08	0.37±0.28
vio	0.26±0.03	0.29±0.06	0.77±0.22	0.15±0.03
lut	0.83±0.21	0.96±0.27	2.61±0.14	2.15±0.43
beta	0.27±0.09	0.37±0.12	0.16±0.003	0.48±0.09
zea	n.d.	n.d	n.d	0.58±0.11

Table 2. Pigment composition analysis by HPLC. Chl, Chlorophyll; fr w, fresh weight; neo, neoxanthin; vio violaxanthin; lut, lutein; beta, beta-carotene; zea, zeaxanthin. Shown is ±SD. n=3

Table 3

< τ >, ps		Summer	Summer Quenched	E.spring (recovered)	E. spring
PSI		95	95	90	42
PSII	pool 1	988	357	820	228
	pool 2	1228		2113	2950
	total	1086		1137	273
LHCII quenched			399		420
total		779	296	572	170

Table 3. To assess differences in excited-state energy relaxation of different decaying components we calculated the average excited state relaxation time as $\langle \tau \rangle = \sum A_i \tau_i$, where A_i are the relative areas of each Decay-associated spectra (DAS). DAS were obtained from global target analysis (Fig. 3).

Table 4.

Sample condition	PSI (CS and quenched)	PSII (CS) (Both pool taken together)	Comments
Summer unquenched	50%	30%	figure shows only main pool contribution
Summer quenched	67%	7.1%	detached and quenched LHCII not considered
E.Spring relaxed	27%	55%	small amount of photoinhibited PSII pool not considered
E.Spring quenched	89%	1.5%	small unquenched PSII pool and small quenched detached LHCII component not considered.

Table 4. Percentages of total energy flow into PSII and PSI as calculated from Fig. 4.3 SI.

For S state both PSII pools were taken into calculation