Supplementary Information for

Specific thylakoid protein phosphorylations are prerequisites for overwintering of Norway spruce (Picea abies) photosynthesis

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This PDF file includes:

- Supplementary text
- Figures S1 to S16
- Legends for Datasets S1 to S3
- SI References

Other supplementary materials for this manuscript include the following:

- Datasets S1 to S3
Weather data
Temperature and irradiance (global radiation) data were collected from the Finnish Meteorological Institute (https://ilmatieteenlaitos.fi/). Temperature data was recorded from the observation station Turku Artukainen, while the irradiance was calculated as average of two closest observation stations (Parainen Utö and Jokioinen Ilmala).

*In vivo* chlorophyll fluorescence and P700 difference absorption

Simultaneous *in vivo* chlorophyll fluorescence and P700 difference absorption measurements of spruce needles were performed after 1 h dark acclimation at room temperature needles. A homemade adapter was used to ensure parallel alignment and minimize gaps in-between the needles. Initial Fv/Fm and Pm determination was followed by 3 min intervals of increasing actinic light intensities in ten steps from 0 - 2000 μmol photons m$^{-2}$ s$^{-1}$ and for Mar 2018 samples in six steps from 0 - 1200 μmol photons m$^{-2}$ s$^{-1}$. Fluorescence measuring light intensity was <1.0 μmol photons m$^{-2}$ s$^{-1}$ and saturating pulse (SP) intensity was 6000 μmol photons m$^{-2}$ s$^{-1}$ with a pulse width of 700 ms. F0' was determined with a short far-red pulse after each SP (F0' subroutine in the Dual-PAM 100 software). Pm was determined with a SP after 10 s of far-red pre-illumination (720 nm, 130 μmol photons m$^{-2}$ s$^{-1}$) with default settings for delay time of 5 ms and delay width of 30 ms. Light curve experiments were performed after 1 h dark acclimation at room temperature with initial Fv/Fm and Pm determination, followed by 3 min intervals of increasing actinic light intensities in ten steps from 0 - 2000 μmol photons m$^{-2}$ s$^{-1}$ and for Mar 2018 samples in six steps from 0 - 1200 μmol photons m$^{-2}$ s$^{-1}$. PSII and PSI yield parameters were calculated according to (1, 2). The NPQ parameter to assess regulatory non-photochemical quenching (NPQ = (Fm-Fm')/Fm') was not used for comparison of seasonal samples, as their quenching characteristics in the dark-adapted state (Fv/Fm) differed from each other (3).

Artificial recovery experiments
For recovery experiments mature needles collected from branches of three individual trees in sun and shade habitats during March 2018 and June 2018 were used. In contrast to other sampling points, needles of branches were not pooled. After transference to the laboratory, branches were kept in darkness at +7ºC and probed with *in vivo* light curve measurements after 0, 1, 24, 48, 72 and 96h. Thylakoid isolations were performed after 0, 24 and 96h. Simultaneous in-vivo chlorophyll fluorescence and P700 difference absorption light curve experiments were performed from recovery samples with identical Dual-PAM-100 settings as described above. However, a shorter light curve protocol with only 3 min intervals of increasing actinic light intensities in six steps from 0 - 1200 μmol photons m$^{-2}$ s$^{-1}$ was used, to prevent any premature relaxation of sustained NPQ. Despite shorter measuring time, sustained NPQ samples showed substantial fluorescence drift during the measurements at time point 0h, which decreased after 1h dark acclimation at +7 ºC. Since steady-state fluorescence drifted above Fm values, thus rendering calculation of fluorescence parameters not useful, we report in Fig. 1C-E and Fig. 3C only the Fv/Fm values for time point 0h and only one representative biological replicate with the highest Fv/Fm (least severe sustained NPQ) after 1h of recovery, which did not show substantial fluorescence drift.
Artificial induction experiments

Spruce branches for artificial induction experiments were collected at 26th of March 2020 at noon from three individual sun-exposed trees. After transference to the laboratory, branches were subjected to artificial day/night cycle (12h/12h) and freezing temperature treatment in modified freezer unit for four consecutive days. The white light followed stepwise increase and decrease to mimic a natural day: 15 μmol photons m\(^{-2}\) s\(^{-1}\) (6:30 – 7:30; 17:30 – 18:30), 25 μmol photons m\(^{-2}\) s\(^{-1}\) (7:30 – 8:30; 16:30 – 17:30), 70 μmol photons m\(^{-2}\) s\(^{-1}\) (8:30 – 9:30; 15:30 – 16:30), 290 μmol photons m\(^{-2}\) s\(^{-1}\) (9:30 – 10:30; 14:30 – 15:30), 900 μmol photons m\(^{-2}\) s\(^{-1}\) (11:30 – 14:30) and darkness (18:30 – 6:30) and was provided with combination of multicolored LEDs (DYNA, Heliospectra AB, Sweden). Temperature in first 24h was slowly lowered and then kept stable between -18 to -22°C (SI Appendix, fig. S16). Control needles were only subjected to temperature treatment and kept in darkness. Fv/Fm measurements were taken at noon at time points 0, 24, 48 and 96h with Dual-PAM-100 settings as described above. However, prior to Fv/Fm measurements spruce needles were dark acclimated for 1h on ice. Additionally to induction, recovery of Fv/Fm was followed in darkness for 24h and 96h. Needles (2g fresh weight) for thylakoid isolation were taken at noon at time points 0, 24, 48 and 96h and frozen in liquid nitrogen. Otherwise thylakoid isolations were performed as described in main text.

77K chlorophyll fluorescence

77K Fluorescence emission spectra of isolated thylakoids were recorded using Ocean Optics spectrophotometer (S2000) and excitation at 440 nm. Thylakoid samples were diluted with storage buffer (50 mM Hepes-KOH, pH 7.5; 5 mM MgCl\(_2\); 100 mM sorbitol; 10 mM NaF) to concentration of 10 μg Chl mL\(^{-1}\). Fluorescence spectra were background corrected and normalized to PSII fluorescence peak at 686 nm.

Gel electrophoresis, protein staining and immunoblotting

Isolated thylakoids were loaded on equal chlorophyll bases in the wells of both mono-dimensional 12% acrylamide (w/v) 6 M urea SDS-PAGE and in IpBN-PAGE. For 2D IpBN / SDS-PAGE, isolated thylakoids were first solubilized with 2% (w/v) n-dodecyl β-D-maltoside (β-DM), then subjected to IpBN-PAGE for separation of the protein complexes, and subsequently the protein subunits of individual photosynthetic complexes were separated in the second dimension SDS-PAGE as described in (4). ProQ Diamond phosphoprotein and SYPRO Ruby total protein staining of gels were performed according to manufacturer instructions (Invitrogen/Molecular Probes). Silver staining for MS/MS identification of proteins was carried out according to (5). For immunoblotting analysis, gels were electroblotted to a PVDF membrane according to (4). Protein phosphorylation was assessed by probing the membranes with phospho-threonine (p-Thr) antibody (New England Biolabs), PsαB antibody (Agrisera, Vännäs, Sweden, 1:3000 dilution), PsbS antibody (1:5000 dilution) and D1 (D-E loop, dilution 1:8000) antibody (both gifts from Prof. Roberto Barbato).

MS/MS identification

The identification of 3p-LHCII was first carried out by excising the gel pieces from the spots marked with red arrows in the gel in Fig 2A (sustained NPQ side, on the left) and in the bands as indicated in Fig S8D and S12. In the second approach, the sustained NPQ sample, the winter non-quenched sample and the summer sample were subjected to IpBN-PAGE, the LHCII trimers were excised from respective gel strips and subjected to the second dimension 12% acrylamide (w/v) 6 M urea.
SDS-PAGE to obtain a good separation of the three canonical LHCII bands 1, 2 and 3 (indicated in Fig 2C) and, in the winter samples, additionally the 3p-LHCII doublet. In the case of the spots (Fig 2A) and the trimer bands (similar to those visible in Fig. S12), the upper faint band and lower strong band of the 3p-LHCII doublet in sustained NPQ samples have been cut and identified by MS/MS separately. In the proteomics dataset PXD018941 deposited to the ProteomeXchange Consortium via the PRIDE partner repository (6), the files referring to the upper faint band of the doublet are 3p_LHCII_sustainedNPQ_band1 and 3p_LHCII_sustainedNPQ_spot1, while the files referring to the lower strong band of the doublet are 3p-LHCII_sustainedNPQ_band and 3p_LHCII_sustainedNPQ_spot. The identification of p-PSBS was carried out in the spots marked with red arrows in Fig 2A (both sustained NPQ sample and summer sample) and in the band identified with ProQ staining visible in mono-dimensional SDS-PAGE (indicated in Fig 2D).

Before excising the gel pieces, the gels were silver stained. All gel samples were cut in about 1 mm³ pieces and shrunk with 100% acetonitrile (ACN) in a clean Eppendorf tube. Next, the cysteine residues were reduced with 20 mM dithiothreitol (DTT) in 40 mM NH₄HCO₃ (ABC buffer) at 56°C for 20 min and, after shrinking again the gel pieces in 100% ACN, they were alkylated by incubation with iodoacetamide (IAA) at room temperature for 30 min. After two cycles of washes alternating 40 mM ABC and 100% ACN, the gel pieces were finally subjected to in-gel trypsin digestion for up to 14 h in 40 mM ABC, 10% (v/v) ACN and 0.3 µg of sequencing grade modified trypsin, (Promega V5111) at 0.02 µg/µl. The tryptic peptides were extracted by adding an equal volume of 100% ACN to the mixture and incubating for 15 min. After collecting the liquid in a separate Eppendorf, the tryptic peptides were extracted two more times by adding 75 µl of a buffer composed by 50% (v/v) ACN and 5% (v/v) formic acid (FA) and incubating for 15 min each time. The three peptides mixtures were pooled and vacuum dried in a vacuum centrifuge (SpeedVac) for 3 hours.

The dried peptides were re-suspended in 2% (v/v) FA and loaded on a nanoflow HPLC system (EasyNanoLC 1000, Thermo Fisher Scientific) equipped with a 20 X 0.1 mm (inner diameter) precolumn in conjunction with a 150 mm X 75 µm (inner diameter) analytical column, both packed with 5 µm Reprosil C₁₈-bonded silica (Dr Maisch GmbH). The peptides were separated by a two-step gradient elution at a flow rate of 300 nLmin⁻¹, from 3% to 43% solvent B (ACN/water (80:20 [v/v]) with 0.2% (v/v) FA) over 13 min, then from 35% to 100% over 2 min, and subsequently at 100% solvent B for 10 min, while solvent A was water/ACN (98:2 [v/v]) with 0.2% (v/v) FA. The nHPLC was coupled to a Q-Exactive (Q-Ex) or Q-Ex HF electrospray ionization-hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific), run in positive mode in data-dependent acquisition (DDA). The type of fragmentation used was higher-energy collisional dissociation (HCD). The scan range was set from 300 to 18000 m/z in MS1 with resolution at 140000 (120000 in Q-Ex HF). Up to 15 data-dependent MS/MS spectra were acquired in each scan with dynamic exclusion set for 30 s (10 s in Q-Ex HF), and the scan range in MS2 was set to 200 to 2000 m/z with resolution at 17500 (15000 in Q-Ex HF).

To identify the residues phosphorylated in the SSTT sequence of the N-Terminal peptide of the LHCB1_A in the 3p-LHCII band, the same sample was subjected to MS analysis with an Orbitrap Fusion Lumos (Thermo Fisher Scientific) equipped with the same nHPLC as described above. In this case, the gradient was 8% to 39%. The type of fragmentation used was EThcD, with resolution in MS1 at 120000, scan range 300-1750 m/z, and resolution in MS2 at 30000, scan range 120 to 2000 m/z.

All acquired spectra were analyzed against a custom Picea abies database (7) using Proteome Discoverer (version 2.2) Software (Thermo Fisher Scientific) with an in-house installation of the Mascot (version 2.7; Matrix Science) search engine, with the following search parameters:
monoisotopic mass, two missed cleavages allowed, precursor mass tolerance 10 ppm, fragment mass tolerance 0.02 D, m/z > or = 2+. Carbamidomethylation of Cys (mono Δ = 57.021464 Da) was set as static modification, while oxidation of Met (mono Δ = 15.9949 Da), phosphorylation of Ser, Thr, and Tyr (mono Δ = 79.9663 Da), and protein N-term acetylation (mono Δ = 42.01057 Da) were set as variable modifications. The identifications of phosphopeptides were validated through a PhosphoRS filter (version 3.1; (8)) and through a decoy database search for peptides (Percolator filter), with target false discovery rates of <0.01 (strict) and <0.05 (relaxed). For phosphopeptides the confidence threshold was set to P< 0.05. For the phosphorylations indicated in Fig. 2C and D and Fig. S5, only the phosphopeptides with Mascot score above identity relaxed threshold have been used. All proteins and PSMs identified in representative LHCII and PSBS bands and 2D spots are presented in Dataset S1.

Multiple sequence alignments

LHCB1, LHCB2 and PSBS sequences for different species were collected as described in (7). Multiple sequence alignments were performed in MEGA 7 (9) using the MUSCLE algorithm (10) and visualized with BioEdit v7.0.5.

Statistics analysis

Statistical analysis of Fv/Fm measurements were performed with SPSS (v26.0) and summarized in SI Appendix, Dataset S3.
Figure S1: PSII yield parameters of sun and shade needles
PSII yield parameters from light curve experiments of sun and shade needles in 2017 (A, D, G, J, M, P), 2018 (B, E, H, K, N, Q) and 2019 (C, F, I, O, R). (A-C) Effective quantum yield PSII photochemistry, \( Y_{II} \), from sun needles. (D-F) Quantum yield of regulatory non-photochemical quenching, \( Y_{NPQ} \), from sun needles. (G-I) Quantum yield of non-regulatory energy dissipation, \( Y_{NO} \) from sun needles. (J-L) Effective quantum yield PSII photochemistry, \( Y_{II} \), from shade needles. (M-O) Quantum yield of regulatory non-photochemical quenching, \( Y_{NPQ} \), from shade needles. (P-R) Quantum yield of non-regulatory energy dissipation, \( Y_{NO} \) from shade needles. Average of 3 biological replicates with standard deviations are shown, except for sustained NPQ samples from Mar 2018 (red). Here only the least quenched (highest \( F_{v}/F_{m} \)) biological replicate after 1h recovery is shown (see SI material and methods).
Figure S2: PSI yield parameters of sun and shade needles

PSI yield parameters from light curve experiments of sun and shade needles in 2017 (A, D, G, J, M, P), 2018 (B, E, H, K, N, Q) and 2019 (C, F, I, L, O, R). (A-C) Effective quantum yield PSI photochemistry, Y(I), from sun needles. (D-F) Quantum yield of oxidized P700 (Y(ND), PSI donor side limitation) from sun needles. (G-I) Quantum yield of reduced P700 (Y(NA), PSI acceptor side limitation) from sun needles. (M-O) Quantum yield of oxidized P700 (Y(ND), PSI donor side limitation) from shade needles. (P-R) Quantum yield of reduced P700 (Y(NA), PSI acceptor side limitation) from shade needles. Average of 3 biological replicates with standard deviations are shown, except for sustained NPQ samples from Mar 2018 (red). Here only the least quenched (highest Fv/Fm) biological after 1h recovery is shown (see SI material and methods).
Figure S3: IpBN of all samples from 2016 (shade and sun)
IpBN-PAGE from (A) shade and (B) sun samples of 2016. Isolated thylakoids were solubilized with 2% (w/v) n-dodecyl β-D-maltoside (β-DM). Gel was loaded on equal chlorophyll basis (8µg). sc – supercomplex, dm – dimer, mm – monomer.
Figure S4: 2D lpBN/SDS-PAGE of sustained NPQ sample vs summer sample, and phosphorylation comparison of the subunits of individual PSII and LHCII complexes in winter and summer.

(A) and (B) Full molecular mass range of the 2D lpBN/SDS-PAGE stained with Sypro and ProQ presented in Fig 2A and B. (C) lpBN-PAGE from Jan 2018 and Jun 2018 samples. Isolated thylakoids were solubilized with 2% (w/v) n-dodecyl β-D-maltoside (β-DM). Gel was loaded on equal chlorophyll basis (8µg). C2S1 and C2S2 indicate PSII-LHCII supercomplexes comprised of a PSII dimer (C2) and 1 or 2 strongly bound LHCII trimers (S), respectively. (D) Phospho-threonine (p-Thr) immuno-blot of the indicated photosynthetic complexes excised from the gel in (A) and run in 2d SDS-PAGE as separate bands. A lane with the corresponding thylakoids before solublization with β-DM (total thyl.) was loaded as control (1 µg Chl). tri- trimer, dm – dimer, mm – monomer.
Figure S5: Sequence comparison of LHCB1, 2 and PSBS in selected species
Multiple sequence alignment of LHCB1 and LHCB2 (A) and PSBS (B) from different angiosperms and gymnosperms. Arth – Arabidopsis thaliana, Spol – Spinacia oleracea, Zema – Zea mays, Piab – Picea abies, Pita – Pinus taeda, Gibi – Ginkgo biloba. Secondary structures indicated according to S. oleracea 3d structures in protein data bank for LHCII trimer (PDB: 1RWT) and PSBS (PDB: 4RI2). Transmembrane helices (TM) are indicated in dark grey. Alpha helices (H) and beta strands (β) indicated in light grey. Start of protein sequence marked by black arrow according to A. thaliana (LHCB1.2: AT1G29910, PSBS: AT1G44575). Conserved Glu residues of PSBS highlighted in orange. Phosphosites highlighted in red in A. thaliana and S. oleracea according to (11) and in P. abies according to MS/MS results. Conserved residues are indicated by (*). It should be noted, that LHCB1_A and LHCB1_B isoform nomenclature used here differs from original nomenclature in Pinus sylvestris (12).
**Figure S6:** Representative MS/MS spectrum (HCD) of N-terminal LHCB1_A triply phosphorylation

Representative MS/MS spectrum, generated by HCD type of fragmentation, of the N-terminal LHCB1_A triply phosphorylated peptide "SSpTPKKVPSSASAPSPWYGPRD". The identified y ions (in blue) and the b ions (in red) matching the theoretical fragments are reported in the table at the bottom. –P indicates ions with phosphorylation losses, while –H₂O and –NH₂ indicate ions with neutral losses.

| b⁺  | b⁻P  | b⁻²P | r1  | Seq | r2  | y⁺  | y⁻P  | y⁻H₂O | y⁻H₂O²⁻ | y⁻NH₂⁺ | y⁻NH₂²⁻ |
|-----|-------|-------|-----|-----|-----|-----|-------|--------|----------|---------|---------|
| 88  | 88    |       | 18  |     |     |     |       |        |          |         |         |
| 175 | 174   |       | 17  |     |     |     |       |        |          |         |         |
| 356 | 355   |       | 16  |     |     |     |       |        |          |         |         |
| 537 | 536   |       | 15  |     |     |     |       |        |          |         |         |
| 665 | 664   |       | 14  |     |     |     |       |        |          |         |         |
| 793 | 792   |       | 13  |     |     |     |       |        |          |         |         |
| 892 | 891   |       | 12  |     |     |     |       |        |          |         |         |
| 1059| 1058  |       | 11  |     |     |     |       |        |          |         |         |
| 1130| 1129  |       | 10  |     |     |     |       |        |          |         |         |
| 1217| 1216  |       | 9   |     |     |     |       |        |          |         |         |
| 1268| 1267  |       | 8   |     |     |     |       |        |          |         |         |
| 1375| 1374  |       | 7   |     |     |     |       |        |          |         |         |
| 1472| 1471  |       | 6   |     |     |     |       |        |          |         |         |
| 1559| 1558  |       | 5   |     |     |     |       |        |          |         |         |
| 1656| 1655  |       | 4   |     |     |     |       |        |          |         |         |
| 1842| 1841  |       | 3   |     |     |     |       |        |          |         |         |
| 2005| 2004  |       | 2   |     |     |     |       |        |          |         |         |

Table: Representative MS/MS spectrum (HCD) of N-terminal LHCB1_A triply phosphorylation -P indicates ions with phosphorylation losses, while –H₂O and –NH₂ indicate ions with neutral losses.
Representative MS/MS spectrum (EThcD) of N-terminal LHCB1_A triple phosphorylation

This type of fragmentation allows, in a multiply phosphorylated peptide, the identification of the correct phosphorylated residues with a higher degree of confidence. The identified y and z ions (in blue) and the b and c ions (in red) matching the theoretical fragments are indicated in the table at the bottom. The –NH  indicate ions with neutral losses, and –NH3–P indicates multiple neutral losses. The masses of identified precursors in the spectrum are also indicated in the table at the bottom.

Figure S7: Representative MS/MS spectrum (EThcD) of N-terminal LHCB1_A triple phosphorylation

Representative MS/MS spectrum, generated by EThcD type of fragmentation, of the N-terminal LHCB1_A triply phosphorylated peptide "SSpTtKvPSaSAssPwYyGyDPr". This type of fragmentation allows, in a multiply phosphorylated peptide, the identification of the correct phosphorylated residues with a higher degree of confidence. The identified y and z ions (in blue) and the b and c ions (in red) matching the theoretical fragments are reported in the table in the middle. –P indicates ions with phosphorylation losses, –H2O and –NH2 indicate ions with neutral losses, and –NH3–P indicates multiple neutral losses. The masses of identified precursors in the spectrum are also indicated in the table at the bottom.
Figure S8: Analysis of protein phosphorylation in recovery samples

(A) Comparison of total protein stain (Sypro), phosphoprotein stain (ProQ) and anti-phospho-threonine (p-Thr) immunoblot of isolated thylakoids separated by SDS-PAGE. The relationship between the phosphobands observed with ProQ and with p-Thr antibody in non-quenched (Feb 2018) and sustained NPQ (Mar 2018) samples is shown. (B) p-Thr immunoblot of thylakoid isolated from shade needles after 0h, 24h and 96h of artificial recovery (darkness and 7°C) from Mar 2018 (sustained NPQ) and Jul 2018 (summer control). The molecular mass region of p-PSBS in the p-Thr immunoblot is shown with longer exposure time. The 10%, 25% and 50% loading scale refer to the 0h Mar 2018 sample. Gel was loaded on equal chlorophyll basis (1µg).

(C) IpBN-PAGE from Mar 2018 and Jun 2018 spruce recovery shade samples. One lane was loaded with A. thaliana thylakoids (as described in (4)) as a control. Thylakoids were solubilized with 2% (w/v) (for spruce) and 1% (w/v) (for A. thaliana) n-dodecyl β-D-maltoside (β-DM). Gel was loaded on equal chlorophyll basis (5µg Chl) sc – supercomplex, dm – dimer, mm – monomer. (D) 2d SDS-PAGE stained with phospho protein (ProQ) and total protein stain (Sypro) of the LHCII trimer bands excised from the gel in (B) and run as separated bands. (E-F) SDS-PAGE of total thylakoids (4µg Chl) from sun and shade recovery samples stained with (E) total protein stain (Sypro) and (F) phosphoprotein stain (ProQ).
Figure S9: PSII+PSI parameters of sun and shade needles during recovery from sustained NPQ. PSII and PSI yield parameters from Mar 2018 recovery samples after 0, 1, 24, 48, 72, 96h and Jul 2018 0h summer control samples. (A-F) sun and (G-L) shade needles. (A, G) Effective quantum yield PSII photochemistry, Y(II). (B, H) quantum yield of regulatory non-photochemical quenching, Y(NPQ). (C-I) quantum yield of non-regulatory energy dissipation, Y(NO). (D, J) Effective quantum yield PSI photochemistry, Y(I). (E, K) quantum yield of oxidized P700 (Y(ND), PSI donor side limitation). (F, L) quantum yield of reduced P700 (Y(NA), PSI acceptor side limitation) from sun needles. Average of 3 biological replicates with standard deviations are shown, except for 0h (orange) and 1h (red) recovery samples of Mar 2018. For 0h only initial Fv/Fm measurement (0 μmol photons m⁻² s⁻¹) and for 1h only the least quenched (highest Fv/Fm) biological replicate is shown (see SI material and methods).
Figure S10: PSII+PSI parameters of sun and shade needles of control recovery from summer PSII and PSI yield parameters from Jul 2018 recovery samples after 0h, 24, 48, 72, and 96h. (A-F) sun and (G-L) shade needles. (A, G) Effective quantum yield PSII photochemistry, Y(II). (B, H) quantum yield of regulatory non-photochemical quenching, Y(NPQ). (C-I) quantum yield of non-regulatory energy dissipation, Y(NO). (D, J) Effective quantum yield PSI photochemistry, Y(I). (E, K) quantum yield of oxidized P700 (Y(ND), PSI donor side limitation). (F, L) quantum yield of reduced P700 (Y(NA), PSI acceptor side limitation) from sun needles. Average of 3 biological replicates with standard deviations are shown.
Figure S11: 77K fluorescence of isolated thylakoids during recovery from sustained NPQ
77K chlorophyll fluorescence spectra of isolated thylakoids (ex. 480 nm) of shade (A, C) and sun (B, D) recovery samples. Comparison of Mar 2018 samples after 0h, 24h and 96h recovery with 0h Jul 2018 of shade (A) and sun (B) needles. Comparison of 0h Mar 2018 samples with Jul 2018 after 0h, 24h and 96h recovery of shade (C) and sun (D) needles. Spectra were normalized to 686 nm.
Figure S12: Analysis of the LHCII trimers in 2016 samples
2d SDS-PAGE from LHCII trimer bands excised from the IpBN of 2016 (A) shade and (B) sun samples shown in Figure S3. Gels were stained with phospho protein stain (ProQ) and total protein stain (silver nitrate).
**Figure S13:** 2d lpBN/SDS-PAGE from 2016 winter and summer samples

2d lpBN/SDS-PAGE from sun (A-B) and shade (C-D) samples of Jan 2016 and Jun 2016 samples. The lpBN was loaded on equal chlorophyll basis (8µg). Gels were stained with (A, C) total protein stain (Sypro) and (B, D) phospho protein stain (ProQ). sc – supercomplex, dm – dimer, mm – monomer.
Figure S14: 2d lpBN/SDS-PAGE from 2017 winter and summer samples
2d lpBN/SDS-PAGE from sun (A-B) and shade (C-D) samples of Feb 2017 and Jun 2017 samples. The lpBN was loaded on equal chlorophyll basis (8µg). Gels were stained with (A, C) total protein stain (Sypro) and (B, D) phospho protein stain (ProQ). sc – supercomplex, dm – dimer, mm – monomer.
Figure S15: 2d lpBN/SDS-PAGE from 2018 winter and summer samples
2d lpBN/SDS-PAGE from sun (A-B) and shade (C-D) samples of Jan 2018 and Jun 2018 samples. The lpBN was loaded on equal chlorophyll basis (8µg). Gels were stained with (A, C) total protein stain (Sypro) and (B, D) phospho protein stain (ProQ). sc – supercomplex, dm – dimer, mm – monomer.
Freshly harvested sun-exposed spruce branches were subjected to artificial induction of sustained NPQ on 26th March 2020 13:00 (0h) in a modified freezer unit until 30th March 13:00 (96h). The white light (multicolored LEDs, DYNA, Heliospectra AB, Sweden) followed stepwise increase and decrease during day/night cycle (12h/12h) to mimic a natural day: 15 μmol photons m⁻² s⁻¹ (6:30 – 7:30; 17:30 – 18:30), 25 μmol photons m⁻² s⁻¹ (7:30 – 8:30; 16:30 – 17:30), 70 μmol photons m⁻² s⁻¹ (8:30 – 9:30; 15:30 – 16:30), 290 μmol photons m⁻² s⁻¹ (9:30 – 10:30; 14:30 – 15:30), 900 μmol photons m⁻² s⁻¹ (11:30 – 14:30) and darkness (18:30 – 6:30). Temperature in first 24h was slowly lowered and then kept stable between -18 to -22°C. The start and the end of the experiment are indicated by arrows.
**SI Datasets**

**Dataset S1:** List of the proteins identified in DDA in representative LHCII and PSBS bands and 2D spots

Each tab shows the list of identified proteins in representative samples of the LHCII trimer in a sustained NPQ sample, in a winter sample where 3p-LHCII doublet was visible, and in a summer sample. The name of the LHCII bands is in accordance to Figure 2C. For 3p-LHCII doublet, also the identifications from the upper and lower spot indicated in the 2D lpBN/SDS-PAGE in Figure 2A and B are listed. The result of the re-analysis of the winter 3p-LHCII lower band by ETHcD type of fragmentation is also listed (3p_{LHCII_winter_band_ETHcD}). For p-PSBS, each tab shows the identifications obtained in the 3 spots in the 2D in Figure 2A and B (2 from sustained NPQ sample and 1 from summer sample), and in the band from mono-dimensional SDS-PAGE of the sustained NPQ sample indicated in Figure 2D. In each tab, for each protein a second expandable layer lists the corresponding peptides spectrum matches (PSMs) identified.

The header of the proteins layer indicate: Protein FDR Confidence; accession; protein description; percentage of coverage of the whole sequence (including transit peptide, when present); number of identified peptides (# Peptides); number of peptides spectrum matches (# PSMs); number of unique peptides (# Unique Peptides); number of protein groups the protein belongs to (# Protein Groups); length of the protein sequence as number of amino acids (#AA); molecular weight of the protein; (MW [kDa]); Mascot protein score, which represents the sum of the scores of all peptides identified for the protein (Score Mascot); the post-translational modification found (protein N-terminal acetylation, Thr, Ser or Tyr phosphorylation), where the number following the AA (e.g. T10) refers to the position of the phosphorylation/acetylation in the full protein sequence, followed by the percentage of probability that the identified residue is the correct site according to ptmRS (in brackets).

In the PSMs layer, the headers indicate: the identification confidence based on Percolator FDR (high= FDR<0.01 ; medium= FDR <0.05) (Confidence) ; the level of ambiguity of the spectrum-peptide match (PSM Ambiguity); the annotated AA sequence where the modified AA is shown in lower case (Annotated Sequence); the Mascot ion score of the MS/MS match (Ions Score); Mascot Expectation Value for strict significance (significance <0.05)( Expectation Value); all protein accessions the peptide is contained in (Protein Accessions); rank in the list of peptide matches as assigned by the search engine (Mascot) (Search Engine Rank); the number of cleavage sites within the peptide that were missed by the digestion process (# Missed Cleavages); Charge of the detected peptide (m/z) (Charge); number of protein groups and number of proteins the peptide identified is contained in (# Protein Groups and # Proteins); the normalized score difference between the top two scores for the peptides identified by that spectrum (Delta Score); rank in the list of peptide matches to the same spectrum according to the main score (Rank); mass on charge (m/z [Da]); observed MH+ [Da]; the theoretical MH+ mass in Dalton, calculated from the amino acid sequence, including the modifications (Theo. MH+ [Da]); the relative difference between observed and measured mass (Delta M [ppm]); the absolute difference between observed and measured mass (Delta m/z [Da]); the type of fragmentation used to obtained the spectrum (Activation Type); the percentage of interference by co-isolated ions within the precursor isolation window (Isolation interference (%)); the time (in ms) used to accumulate ions in the mass spectrometer to reach their target value before they are scanned out (ion injection time (in ms)); the retention time when the peptide was observed (in min) (RT (min)); the number of the first scan used to identify the peptide (First Scan); Mascot identity threshold for strict significance (Identity Strict); Mascot identity threshold for relaxed significance (Identity Relaxed); Percolator Q-value; percolator PEP; ptmRS score of the modified peptide (ptmRS: Binomial Peptide Score); estimate of the probability (0-100%) that the site is truly modified (ptmRS: Best Site Probabilities); sequence of the peptide in the protein (Sequences in Protein); the position of the peptide in the protein (Positions in Protein); the position of the modified AA in the sequence and the percentage of confidence for the assigned phosphosite according to ptmRS (PSM Modification Positions in Protein).
Dataset S2: Temperature and irradiance data for individual sampling days with cumulative moving averages for 1, 3 and 5 days prior to sampling.

Dataset S3: Statistical summary of FvFm values with pre-tests and multiple pairwise comparisons.
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