Two dominant boreal conifers use contrasting mechanisms to reactivate photosynthesis in the spring

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Boreal forests are dominated by evergreen conifers that show strongly regulated seasonal photosynthetic activity. Understanding the mechanisms behind seasonal modulation of photosynthesis is crucial for predicting how these forests will respond to changes in seasonal patterns and how this will affect their role in the terrestrial carbon cycle. We demonstrate that the two co-occurring dominant boreal conifers, Scots pine (Pinus sylvestris L.) and Norway spruce (Picea abies), use contrasting mechanisms to reactivate photosynthesis in the spring. Scots pine downregulates its capacity for CO2 assimilation during winter and activates alternative electron sinks through accumulation of PGR5 and PGRL1 during early spring until the capacity for CO2 assimilation is recovered. In contrast, Norway spruce lacks this ability to actively switch between different electron sinks over the year and as a consequence suffers severe photooxidative damage during the critical spring period.
Oreal forests are dominated by evergreen conifers, and the boreal climate presents a particular seasonality characterized by periods of active growth interspersed with periods of seasonal dormancy to minimize damage from severe cold. The direct consequence of this lifestyle is an environmentally regulated seasonal photosynthetic activity that at larger scales affects the global CO2 budget1-3. The CO2 photosynthetically fixed by boreal forests represents ~22% of global CO2 storage by established forests, which in turn account for ~30% of the global C uptake4. Thus, the boreal forests are key players in balancing the global carbon cycle to reduce the impact of greenhouse emissions on future global climate. The spring recovery of photosynthesis in evergreen conifers is a crucial process for boreal forests, and proper timing of this event is a trade-off between maximizing the full growing season and minimizing damage from exposure to the combined stresses of cold temperatures and high irradiance5. To maintain a balance between the light energy captured to drive photosynthesis and the metabolic demand through these dynamic seasonal growth cycles, cold-tolerant plants deploy two main strategies to cope with low temperature: (i) upregulation of metabolic sink capacity3,5 and/or (ii) downregulation of photosynthetic responses in Scots pine and Norway spruce over the entire year. We found profound differences between the two species. Pine demonstrated a clear modulation of electron sink capacity over the year where the capacity for CO2 assimilation was downregulated during winter and then gradually upregulated during spring in response to warming. To compensate for the reduced CO2 assimilation capacity during the critical late winter–early spring months pine increased alternative electron sinks to protect the photosystems from photodamage. In contrast, Norway spruce lacks this mechanism and as a consequence suffered more severe photooxidative damage during late winter and early spring as shown by larger fluctuations in Fv/Fm and increased thylakoid lipid peroxidation. Our results demonstrate that the two co-occurring dominant boreal conifers use contrasting mechanisms to reactivate photosynthesis in the spring.

### Results

#### Seasonal changes in photosynthetic performance

To investigate the seasonal photosynthetic performance of the two boreal key species, Scots pine and Norway spruce, the photosynthetic capacity and functionality was investigated over a full year from mature (80 + year old) trees growing together in a mixed coniferous forest in northern Sweden (64° 00′ 21.24″N, 19° 54′ 00.24″E) (Fig. 1). Ambient temperature during this time period varied by 50 °C (Fig. 1a). Measurements of chlorophyll fluorescence demonstrated a clear seasonal pattern of photosynthetic performance where both species maintained a fully functional PETC, with a maximum quantum yield of PSII (Fv/Fm) > 0.8, for only 5 months (June–October) of the year (Fig. 1b). During the winter period, PSII is inactivated with both species showing a decline in Fv/Fm beginning in late October, reaching low values of Fv/Fm of 0.38 through to the end of March (Fig. 1b). During spring recovery (April–May), Fv/Fm increased in both species but, especially in Norway spruce, large variations in the Fv/Fm values were observed where high values were followed by a sharp drop, indicative of photodamage associated with frost events. Scots pine demonstrated a slower and less volatile recovery of Fv/Fm, which in turn supported higher rates of electron transport of PSII (ETR(I)), and in particular by very high rates of electron transport by PSI (ETR(I)) during the critical March to May period of spring reactivation (Fig. 1c, d). The large variation in PSI activity over the year observed in Scots pine was not shown in Norway spruce (Fig. 1c, d).

### Norway spruce chloroplasts are more sensitive to photodamage

Analysis of the chloroplast ultrastructure during the winter-to-spring transition showed that between winter (February) and early spring (March), chloroplasts showed an almost complete loss of grana structures (Fig. 2a; Supplementary Fig. 1). Quantification of the TEM images revealed that during spring recovery (March), Norway spruce underwent more extensive remodeling of the thylakoid membranes compared with Scots pine, possessing fewer grana stacks per plastid (19 ± 4 (mean ± SD, n = 6) than in Scots pine (31 ± 9) and reduced effective surface per granum (2.4 ± 0.3 and 3.2 ± 0.4 thylakoid membranes/granum for Norway spruce and Scots pine, respectively) (Fig. 2a). In addition, Norway spruce demonstrate larger numbers of plastoglobuli (Fig. 2b, c) and higher levels of malondialdehyde (MDA) (Fig. 2d), a by-product of lipid peroxidation, compared with Scots pine. Taken together, the data suggest Norway spruce suffers greater oxidative stress during the spring recovery phase compared with Scots pine, as also indicated by the large volatility in Fv/Fm observed in Norway spruce during the spring period (Fig. 1b).

#### Seasonal and temperature responses of CO2 assimilation

Scots pine demonstrated variable rates of ETR(I) over the year, with particularly high rates during the critical spring reactivation period (Fig. 1d). Scots pine also showed a large variation in photosynthetic CO2 assimilation capacity under saturating conditions over the year. The light- and CO2-saturated CO2 uptake4. Thus, the boreal forests are key players in balancing the global carbon cycle to reduce the impact of greenhouse emissions on future global climate. The spring recovery of photosynthesis in evergreen conifers is a crucial process for boreal forests, and proper timing of this event is a trade-off between maximizing the full growing season and minimizing damage from exposure to the combined stresses of cold temperatures and high irradiance5. To maintain a balance between the light energy captured to drive photosynthesis and the metabolic demand through these dynamic seasonal growth cycles, cold-tolerant plants deploy two main strategies to cope with low temperature: (i) upregulation of metabolic sink capacity3,5 and/or (ii) downregulation of photosynthetic responses in Scots pine and Norway spruce over the entire year. We found profound differences between the two species. Pine demonstrated a clear modulation of electron sink capacity over the year where the capacity for CO2 assimilation was downregulated during winter and then gradually upregulated during spring in response to warming. To compensate for the reduced CO2 assimilation capacity during the critical late winter–early spring months pine increased alternative electron sinks to protect the photosystems from photodamage. In contrast, Norway spruce lacks this mechanism and as a consequence suffered more severe photooxidative damage during late winter and early spring as shown by larger fluctuations in Fv/Fm and increased thylakoid lipid peroxidation. Our results demonstrate that the two co-occurring dominant boreal conifers use contrasting mechanisms to reactivate photosynthesis in the spring.

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assimilation rate was low in needles collected during the winter and spring months, and then increased dramatically toward the end of May once $T_{\text{min}}$ rose above 0 °C in the field (Fig. 1), to remain high during the summer months, and then decline in October to again reach low flux capacities in early winter (Fig. 3a). Similar trends were also observed for the CO$_2$ assimilation rates at ambient CO$_2$ concentrations (Supplementary Fig. 2). This response pattern in Scots pine is similar to what has been reported from field measurements and indicates that the low fluxes measured during autumn and winter are not only a result of the inhibition of photosynthesis by low temperature but are also due to a downregulation of CO$_2$ assimilation capacity during the autumn and winter months. In contrast to Scots pine, no seasonal variation in the maximal capacity for CO$_2$ assimilation was observed in Norway spruce (Fig. 3a), although field measurements show seasonal variation in CO$_2$ assimilation reflecting low-temperature inhibition of CO$_2$ assimilation.

 PSI activity is essential during the winter–spring transition. During exposure to high levels of excitation energy at low-temperatures plants are prone to PSI acceptor-side limitation, which eventually leads to PSI photodamage from which plants recover more slowly compared with recovery from photodamage to PSII. Furthermore, PSI photoinhibition is believed to
have more severe consequences for plant metabolism compared with PSII photoinhibition, making the avoidance of damage to PSI particularly important. The maintenance of an increased ETR(I) activity in Scots pine during the spring recovery phase (Fig. 1) suggests winter acclimation has led to some change in the redox poise of the PETC in Scots pine, but not in Norway spruce.

Alternative electron flows (AEF) around PSI have been proposed as alternative electron pathways that can function to minimize the risk of overreduction of the PETC and damage to PSI. The relative quantum yield of AEF (Y(AEF)) was calculated during the spring period, representing the Δ flow between PSI and PSII contributed by CET and pseudo-CET (Fig. 4a). It is clear that in Scots pine Y(AEF) is significantly elevated during the critical spring period and then reduced during the summer months. Norway spruce, on the other hand, showed very little variation in Y(AEF) between the spring and summer months (Fig. 4a). In the controlled recovery experiment with field samples collected in April, a reduction in Y(AEF) was observed following 24 h in warm temperatures in Scots pine (Fig. 4b; Supplementary Fig. 5). No difference was observed in Norway spruce (Fig. 4b). The reduced Y(AEF) following warm exposure in Scots pine correlated with the recovery of the CO₂ assimilation capacity observed in the warming-recovered samples (Fig. 3b).

To test if AEF could protect PSI from photoinhibitory spring conditions in conifers, we used a dynamic saturating pulse protocol including periods of high-light (Supplementary Fig. 6) designed to mimic natural fluctuating light (FL) conditions that are the main cause of PSI photoinhibition. We evaluated samples collected from the field in early spring (April) and from greenhouse-grown plants (control). The greenhouse grown control seedlings presented similar Y(AEF) values to their summer field samples (Fig. 4a; Supplementary Fig. 7). During the high-light treatment, the field samples collected from Scots pine showed the highest Y(I), and Norway spruce the lowest Y(I), of all four sample types measured (Fig. 4d). This high-flux capacity of PSI shown in the April samples from Scots pine was supported by the ETR(I) data (Fig. 4g). Consistent with the data from the fluctuating light experiment, ETR(I) values under saturating constant irradiances were also higher in the early spring Scots pine samples from the field compared with greenhouse-grown Scots pine samples, whereas the early spring Norway spruce samples from the field had lower rates of ETR(I) compared with their greenhouse controls (Supplementary Fig. 7). Furthermore, in Scots pine, there was no sign of an acceptor-side limitation during the high-light treatment, indicating that PSI in the Scots pine spring needles was almost fully oxidized.
Fig. 3 The capacity for CO₂ assimilation is downregulated in winter and reactivated with warm temperature in Scots pine. a Seasonal photosynthetic CO₂ assimilation ($A_N$) was measured under saturated conditions (light intensity, 1200 μmol photons m⁻² s⁻¹ and CO₂ concentration, 800 μmol mol⁻¹, 23 °C). Needles of Scots pine (open circle) and Norway spruce (closed triangle) were collected from March 2017 through January 2018. The dates of budburst in Scots Pine and Norway spruce are indicated with open and closed arrows, respectively. b Response of assimilation ($A_N$) to the internal CO₂ concentration ($C_i$) in a controlled recovery experiment. Samples of Scots pine (closed circle) and Norway spruce (closed triangle) were collected from the field in April. After the initial measurements, the Scots pine (open circle) and Norway spruce (open triangle) samples were recovered in room temperature for 24 h, and measured again. c Response of assimilation ($A_N$) to the internal CO₂ concentration ($C_i$) in seedlings grown in climate chambers. The $A_N/C_i$ curves were measured in 1-year-old cold-acclimated Scots pine (open circle) and Norway spruce (open triangle) seedlings. Seedlings were transferred either to warm (22 °C, indicated in black) or cold (5 °C, indicated in gray) chambers for 4 weeks, and $A_N/C_i$ curves were determined again. d rbcL expression during the spring recovery phase in Scots pine (open circle) and Norway spruce (closed triangle). Relative expression values were normalized against the reference gene PP2A and related to the amount present in the February samples. Each data point represents the mean (± SE) of at least three replicates. The daily mean of air temperature (gray line) for the period February to May 2017 is shown in light gray. e Photosynthetic parameters calculated from the $A_N/C_i$ curve are shown in b and c. $A_N$ rate of CO₂ assimilation, $V_{\text{cmax}}$ maximum rate of carboxylation, $J_{\text{max}}$ maximum rate of electron transport, $g_s$ stomatal conductance. Units are μmol m⁻² s⁻¹. Asterisks indicate the significant difference between values calculated from treatments and control (one-way ANOVA, $P < 0.01$). Each data represent mean of 4–6 replicates (mean ± SE, $n = 4$–6).
throughout the entire experiment (Fig. 4f). This is despite the reduced CO₂ assimilation capacity in the Scots pine spring needles (Fig. 3), suggesting AEF might have been activated as an alternative electron sink.

The role of AEF and PGR5/PGRL1 during critical spring months. The photosynthetic parameters indicate that AEF could contribute to photoprotection of PSI during the spring recovery phase, especially in Scots pine. The abundance of PGR5, PGRL1, and FLVB were determined by western blot analysis in Scots pine and Norway spruce during the winter–summer transition period (Fig. 5a). Scots pine showed increasing amounts for both PGR5 and PGRL1 proteins in spring with the highest amounts present in the needles in March and April (Fig. 5a, b). In contrast, Norway spruce showed no change in PGR5 and PGRL1 protein levels in spring compared with winter or summer (Fig. 5a, b). The increase in PGR5 protein in Scots pine during the spring months correlated with an increase in PGR5 expression (Supplementary Fig. 8). As shown previously, the PGRL1 protein exists in a
Fig. 4 Large electron sinks downstream of PSI protect Scots pine and Norway spruce under illumination mimicking fluctuating growth light condition. a, b The relative yield of AEF (Y(AEF)), representing the Δ flow in conifers between PSI and PSII mainly contributed by CET and pseudo-CET. Y(AEF) is calculated as Y(AEF) = Y(I) - Y(II). The yield of PSII and PSI were measured simultaneously with rapid light curves, and the values under moderate high light (536 μmol photons m<sup>-2</sup> s<sup>-1</sup>) were presented here. Needles of Scots pine (open circle) and Norway spruce (closed triangle) were collected from the pine (open circle) and Norway spruce (open triangle) grown in growth chamber (22 °C, 150 μmol photons m<sup>-2</sup> s<sup>-1</sup>) as controls. Significance of differences of AEF were indicated in Supplementary Fig. 9. c-h Photophysical fluorescence measurement of Scots pine and Norway spruce under illumination mimicking fluctuating growth light condition. In vivo fluorescence and P700 signals monitored under 2 min dark, followed by four cycles of 5 min low light (58 μmol photons m<sup>-2</sup> s<sup>-1</sup>, gray bar) and 1 min high light (1599 μmol photons m<sup>-2</sup> s<sup>-1</sup>, yellow bar). Fluorescence parameters: c Y(II), operating efficiency of PSI; d Y(I), operating efficiency of PSI; e Y(ND), quantum yield of non-photochemical energy dissipation in PSI reaction centers that are limited due to a shortage of electrons (donor-side limitation); f Y(NA), quantum yield of non-photochemical energy dissipation in PSI reaction centers that are limited due to shortage of electron acceptors (acceptor-side limitation); g ETR(I)/ETR(II), the ratio of the electron transport rate of PSI to PSII; h ETR(I), the electron transport rate of PSI. Sun-acclimated needles from Scots pine (closed circle) and Norway spruce (closed triangle) were collected from the field in April. Seedlings from Scots pine (open circle) and Norway spruce (open triangle) grown in growth chamber (22 °C, 150 μmol photons m<sup>-2</sup> s<sup>-1</sup> and 8/16 h light/dark cycle) were used as controls. Significant differences are indicated with different letters above the bars (one-way ANOVA, P < 0.05). Each data point represents the mean of 4-6 biological replicates (mean ± SE, n = 4-6).

Discussion

The boreal biome is characterized by periods of active growth interspersed with periods of dormancy, resulting in strongly regulated seasonal photosynthetic activity. Our results demonstrate that the two dominant tree species of the boreal biome (Scots pine and Norway spruce) do not regulate their seasonal photosynthesis in the same way. Scots pine downregulates its capacity for CO<sub>2</sub> assimilation during winter, induces very high rates of ETR(I) and accumulates PGR5 and PGRL1 during late winter and early spring as a temporary alternative electron sink (Fig. 6). In the late spring and early summer when temperatures rise, Scots pine recovers its capacity for CO<sub>2</sub> assimilation and is coordinated with a re-poising of the rates of ETR(II) (Supplementary Fig. 10) and ETR(I) and reduced amounts of PGR5 (Fig. 6). However, in Norway spruce the capacity for AEF and CO<sub>2</sub> assimilation is constant throughout the year and when the photosynthetic apparatus is challenged during the late winter–early spring months, when cold temperatures are experienced in combination with high irradiance, Norway spruce suffers severe photooxidative damage as shown by large fluctuations in F<sub>i</sub>/F<sub>o</sub> and increased thylakoid lipid peroxidation (Figs. 1, 2).

Evergreen species such as Scots pine and Norway spruce maintain their foliage year-round and they therefore must possess efficient photoprotective mechanisms to cope with excess energy absorbed by a functional PETC during winter and early spring, when general metabolism (including CO<sub>2</sub> assimilation) is strongly inhibited by cold temperatures. It has been suggested that a key factor explaining how gymnosperms outcompete angiosperms at high latitudes is their ability to maintain a highly functional PSI factor explaining how gymnosperms outcompete angiosperms at high latitudes is their ability to maintain a highly functional PSI and CO<sub>2</sub> assimilation can again act as the main electron sink, the PGR5 pathway is downregulated. Norway spruce, on the other hand, appears to lack this ability to actively switch between different electron sinks over the year.
needles (Fig. 6), results in increased tolerance to photoinhibition by minimizing acceptor-side limitations on PSI (i.e., photo-inhibitory PSI overreduction) when needles are exposed to periods of high-light irradiance at low temperatures. Our experiments conducted on field samples (Fig. 4) revealed that AEF in Scots pine needles are able to control the electron transfer to PSI (high Y(ND)) and thermally dissipate the excess excitation energy (Fig. 4d). Thus, although FLV may be the dominate component of AEF during periods of productive growth, the significant decay of PSI capacity in response to AA-inhibition in the Scots pine needles, which is lost once CO2 assimilation is recovered (Fig. 4d), suggests PGR5 plays a key role as an inducible temporary electron sink during the critical spring recovery phase in Scots pine.

The main constraint on the growth of the boreal forests is temperature-dependent season length21,36, and ongoing climate change has caused a mean annual temperature increase of 1.5 °C in high-latitude boreal forests, driving earlier bud flush37,38 at a time when plants remain at risk of exposure to freezing events39.
Recent observations made in boreal forest biomes over Fennoscandia, North America, and Russia have identified accelerated growth in response to longer growing seasons over 40–42, with the earlier arrival of spring shown to yield the greater growth benefits over 43,44. Our results show that two of the dominant conifer species utilize fundamentally different mechanisms to manage spring recovery of photosynthetic activity. This difference may in part reflect the divergent positions these two species occupy in the ecosystem, with Scots pine being an early pioneer species whereas Norway spruce is a shade tolerant late successional species that develops under a covering canopy over 20. Norway spruce and Scots pine also have different shoot and canopy structures, resulting in greater self-shading both within the shoot and within the canopy of Norway spruce plants, even in mature Norway spruce trees that have emerged from their sheltering overstory canopy. These two factors might indicate that Norway spruce has less of a need for such protective mechanisms in spring compared with Scots pine. However, we show that mature exposed Norway spruce canopies do suffer wider fluctuations in Fv/Fm during spring recovery, and therefore suffer more repeat damage to the reactivating photosynthetic apparatus than Scots pine. With climate-driven earlier bud flush and an increase in frequency of spring backlash events over 45, Norway spruce is likely to be more vulnerable to spring frost damage to their canopy in the coming years. It has also recently been shown that Scots pine is more able than Norway spruce to acclimate photosynthesis and respiration to both increased seasonal temperatures and elevated CO2 over 46. Scots pine increased growth in response to temperature increases as high as +8 °C, whereas Norway spruce showed minimal capacity to acclimate energy metabolism and suffered growth losses at elevated seasonal temperatures over 46. These findings, together with those we report here, indicate that the pioneer species Scots pine may generally have a greater capacity to cope with environmental fluctuations and challenges than the more ecologically conservative late successional species Norway spruce. Elucidating the divergent mechanisms utilized by the dominant species in these forests will be crucial for predicting how they will respond to future changes in the timing of spring arrival. The differential responses of these two dominant species need to be accounted for in the estimations of carbon sequestration by the boreal forests, particularly in continental climates.

**Methods**

**Plant material and growth conditions.** Two mature conifer trees, *Pinus sylvestris* Linn. (Scots pine) and *Picea abies* (L.) Karst. (Norway spruce), located near Vännäs, Umeå, Sweden (63° 54′ 24.34″N, 19° 45′ 25.63″E), were selected for...
analysis. Air temperature at the location was monitored every day. The dates of budburst in Scots Pine (average apical shoot length reached 20 mm) and Norway spruce (average Krutzsch index 3, which represents the budburst stage) are on June 8th and June 15th, 2017, respectively47. To characterize seasonal photosynthetic activity, needles on south-facing branches that developed in 2016 were collected during February 2017 through January 2018. Branches were also collected from the field in April 2018 and 2019, and kept at room temperature for 24 h and 6 days for controlled electrolyte stress. All experiments were performed with needle samples collected from the field unless specified. For the growth chamber experiments, 1-year old seedlings of P. sylvestris and P. abies were grown in soil in 1L pots with a photoperiod of 8 h light/16 h dark at an irradiance of 150 µmol photons m⁻² s⁻¹ under 22°C temperatures as indicated. For additional climate chamber experiments, the cold-acclimated seedlings were grown under gradually increasing temperature from 4°C to 22°C (increase 1°C per day) with 8 h light/16 h dark or under gradually increasing day length from 4 h to 22 h light (increase 1 h per 2 days) with 5°C.

In vivo chlorophyll fluorescence, P700 measurement. In vivo chlorophyll a fluorescence and signal from oxidized P700 were monitored simultaneously with a Dual PAM-100 fluorimeters (Walz, Effeltrich, Germany) at room temperature. Needles from mature P. sylvestris and P. abies trees were dark acclimated for 30 min and then bundles of needles that were aligned in parallel to form a single layer used for the measurements. A saturation Pulse (10000 µmol photons m⁻² s⁻¹ for 300 ms) was applied with a sequence of increasing actinic light intensity from 0 to 2000 µmol photons m⁻² s⁻¹. Each measurement was made with four-to-six replicates. Photosynthetic parameters were calculated as described in refs. 18,49. Y(ND), quantum yield of non-photochemical energy dissipation in PSI reaction centers that are limited due to a shortage of electrons (donor-side limitation). Y(NA), quantum yield of non-photochemical energy dissipation in PSI reaction center s that are limited by a shortage of acceptor-side (acceptor-side limitation). Relative yield of AEF (Y(AEF)), representing the ∆ flow in conserves PSI and PSII mainly contributed by CET and pseudo-CET29. Y(AEF) was calculated as Y(AEF) = Y(I) – Y(II).

For measurements mimicking fluctuating light, needles from mature P. sylvestris and P. abies trees were collected on 9th April 2018. The needles from P. sylvestris and P. abies seedlings grown under 22°C were used as controls. The chlorophyll a and P700 signal were monitored after 2 min dark, followed by four cycles of 5 min low light (58 µmol photons m⁻² s⁻¹) and 1 min high light (1599 µmol photons m⁻² s⁻¹). The measurements were made in the steady-state conditions, the light intensity was set for 5 min either at high light (1599 µmol photons m⁻² s⁻¹) or moderate light (536 µmol photons m⁻² s⁻¹). Each measurement was made with four-to-six replicates. To address the effect of inhibition of PGR5-dependent cyclic electron transport, branches from P. sylvestris and P. abies were collected on 10th of April 2019. Detached needles were soaked in distilled water or water containing 200 µM antimycin A (AA). Needles were vacuum infiltrated for 15 min, and the treatment repeated four times. Needles were sandwiched with wet tissue paper and incubated in the dark for 30 min before applying fluctuating light measurements. After the initial measurements, branches were allowed to recover in room temperature for 24 h, and measured again. Each measurement was made with three replicates.

Gas-exchange analysis. The net CO₂ assimilation rate (Aₐ) was measured with the gas-exchange system (LI6400xt, Li-Cor, Lincoln, NE, USA). Aₐ over the seasons was measured at a CO₂ concentration (Cₐ) of 800 µmol mol⁻¹ and a photon flux density of 1200 µmol photons m⁻² s⁻¹ with branches collected from the field in the morning. The cuvette temperature was set to 25°C, and the airflow was set to 500 µmol s⁻¹. Aₐ, versus the calculated intercellular CO₂ partial pressure (A/CI curve) was measured with samples collected on 19th April 2018 and 8th April 2019. The assimilation rate was assessed after 2 to 3 min of exposure to CO₂ concentrations of 400, 200, 150, 100, 50, 400, 650, 800, 1000, and 1200 µmol mol⁻¹ CO₂, based on a protocol described by Chang et al.52. All measurements were performed at 25°C and 1400 µmol photons m⁻² s⁻¹ with four-to-six biological replicates. The same set of samples was allowed to recover in room temperature for 24 h after the initial measurements, and then measured again with the same protocol. Cold-acclimated seedlings were transferred either to warm (22°C, indicated in black) or cold (5°C, indicated in gray) chambers for 4 weeks. Aₐ were determined again. The maximum carboxylation rate (Vₘₐₓ) and maximum electron transport rate (Jₘₐₓ) were estimated according to Sharkey et al.53.

Transmission electron micrographs. Samples were prepared with a modified procedure according to Jonsson et al.54. In all, 0.5 mm-long, cross-sectional needle samples were cut from the middle region of five needles and placed into tubes containing a fixation solution (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4) and kept at 4°C overnight. The samples were then rinsed two times in 0.1 M cacodylate buffer (pH 7.2–7.4) for 10 min and fixed in 1% OsO₄ dissolved in the cacodylate buffer for 2 h in darkness. After wash with MQ water × 2 × 10 min, tissue samples were dehydrated in graded ethanol series (50%, 60%, 70%, 80%, 90%, 100%) followed by propylene oxide fixation for 20 min, and then embedded in Spurr epoxy resin medium. After trimming, sections for three samples per species for imaging were cut with an EM UC7 Ultra Microtome (Leica) using diamond knife, then mounted on copper grids and stained with 5% uranyl acetate (dissolved in MQ water) and Reynolds lead citrate. Whole cells and chloroplasts were photographed with a digital camera (Gatan Orius CCD and Ceta CMOS) connected to a transmission electron microscope (JEOL 1230 and FEI Talos L120 C). The digital images were analyzed using ImageJ (version 1.51j8, National Institute of Health) and Photoshop CC (version 2017.0.1, Adobe software). The number and total area of plastoglobules per chloroplast from the transmission electron micrographs (n = 8–12) were quantified from three independent experiments using the program ImageJ software55.

Carbohydrates and lipid peroxidation analysis. Soluble sugars including sucrose, glucose, and fructose were determined in ethanol extracts as described by Stitt et al.56. The pellets of the ethanol extraction were used for starch determination with methods described by Smith and Zeeman57 with slight modification. The incubation time was increased from 4 h to 12 h. The level of general lipid peroxidation was measured using the modified thiobarbituric acid-malondialdehyde (TBA-MDA) method56. Needles were powdered in liquid nitrogen and homogenized in 5% TCA. The homogenate was centrifuged at 12,000 g for 15 min. Reaction buffer of 300 µl of 0.65% (w/v) thiobarbituric acid (TBA) containing 2% (w/v) of 2-thiobarbituric acid, and hydrogen peroxide (0.67 µl, 33.3% w/v) was added to the tube to extract lipids and proteins from the pellets. After the mixture was centrifuged at 16,000 g for 3 min, 0.4 ml phenol phase extraction was mixed with 1.6 ml methanol buffer and then centrifuged at 16,000 g, 4°C for 3 min. The pellets were washed twice with methanol and 80% acetic acid. Final lipid and protein pellets were dissolved in 0.2 ml Laemmli sample buffer. Total proteins were quantified with a Pierce BCA protein assay kit (Thermo Scientific). SDS-PAGE was performed in 12% polyacrylamide gels with 25 µg total protein loaded per well. The gels were stained with Coomassie Brilliant Blue R as a loading control. Proteins were transferred to a polyvinylidine difluoride (PVDF) membrane (GE). Immunoblot analysis were performed with antibodies raised against the PGR5 (dilution 1:1000) and PGR1 (dilution 1:1000) of Arabidopsis (Agrisera) and antibody of FLV8 (dilution 1:2000) which was provided by Dr. Shikana59. Secondary antibody was anti-rabbit (Agrisera). The protein sequences of PGR5 and PGR1 are highly conserved between Scots pine and Norway spruce (Supplementary Fig. 11), and the peptide targets for PGR5 antibody in these two species are identical. Each analysis was repeated with three biological replicates. Protein levels were quantified from three independent experiments using the program ImageJ software55.

Gene expression analysis. Needles from mature P. sylvestris and P. abies trees were collected during February 2017 to May 2017. The total RNA was isolated with the Spectrum Plant Total RNA Kit (Sigma Aldrich) following the protocols of the manufacturer. RNA was quantified using the NanoDrop spectrophotometer (Thermo Scientific). cDNA synthesis and real-time PCR performed as described by Diaz et al.38. Gene-specific primers were designed with Primer360 and listed in Supplementary Table 1. Three biological and three technical replicates were performed for each experiment. Data analysis was performed with CFX manager software (Bio-Rad). Relative expression values were normalized against the reference gene PP2A (locus name: lclPgdhPlysCys,72007 for P. sylvestris and lcl MA_10426832010000 for P. abies). All values were related to the February samples in the same species.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The underlying Figs. 1, 2b–d, 3 and 4 and Supplementary Figs. 2, 3a, 5, 7, 8, 9 and 10 are provided as a Source Data file. Any other data that support the findings of this study are available within the paper and its supplementary files or are available from the corresponding author(s) upon request.
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Author contributions

Å.S., Q.Y., N.E.B. and V.H. designed the research. Q.Y., N.E.B., C.H.-C. and N.L. performed the research. All authors contributed to data analysis, writing of the paper, and reviewed and approved the final version of the paper.

Competing interests

The authors declare no competing interests.

Additional information

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