Diurnal regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity and its content in Norway spruce needles

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Abstract

Diurnal changes of ribulose-1,5- bisphosphate carboxylase/oxygenase (RuBPCO) activity and its content were measured to find the mechanism of RuBPCO activity regulation in Norway spruce needles. Both initial and total RuBPCO activities as well as the activation state had a typical pattern with two peaks in the morning and afternoon, respectively, and a midday depression. On the 19 October, RuBPCO content decreased during the day from 3.1 to 1.4 g m^{-2}, while on the 20 October it was approximately constant both in the morning and in the afternoon (2.7 g m^{-2}). Neither initial nor total activity of RuBPCO copied irradiances. Relatively low morning and evening values of total activities indicate that nocturnal inhibitor CA1P is important in Norway spruce. However, the midday depression of total activity indicates that besides CA1P there function some other inhibitors of RuBPCO. In addition, the diminution of RuBPCO content during the day may indicate repression of its gene expression.

Additional key words: CA1P; daily inhibitors; midday depression; *Picea*.

Introduction

The only quantitatively significant link between the pools of inorganic and organic carbon in the biosphere is enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBPCO (EC 4.1.1.39) – the most abundant protein on the Earth. This enzyme catalyses carboxylation of D-ribulose-1,5-bisphosphate (RuBP), the first step of the Calvin cycle in competition with oxygenation of RuBP that leads to the photorespiratory pathway. Catalytic effectiveness of RuBPCO is feeble (k_{cat} = 2–12 s^{-1}) and therefore photosynthetic organisms invest up to 50 % of soluble leaf protein (Roy and Andrews 2000) in RuBPCO to support acceptable rates of photosynthesis.

RuBPCO is a key enzyme of photosynthesis regulation. For the low internal CO_2 concentrations [C_i; up to 200 μmol(CO_2) mol^{-1}] carboxylation is limited by the RuBPCO activity (Farquhar *et al.* 1980) and therefore the physiology of RuBPCO has been studied more than that of any other plant enzyme. RuBPCO must be reversibly activated with CO_2 and Mg^{2+} before catalysis can occur. At first, the CO_2 molecule reacts with ε-amino group of lysine 201 within the active site to form carbamate (process of carbamylation) and then follows rapid binding of Mg^{2+} (Lorimer *et al.* 1976). The activation *in vivo* must be facilitated by the presence of a second protein, RuBPCO activase (Portis 1990).

RuBPCO activity depends on the content of enzyme and the activation state of its active sites. For this reason there is not a simple correlation between RuBPCO content and RuBPCO activity. RuBPCO content varies over the time scale of hours and days in dependence on specific saccharide contents (*e.g.* glucose, sucrose). Increased contents of these sugars lead, *via* a hexokinase-related signal, to the repression of RuBPCO gene expression and subsequent decrease in the content of RuBPCO protein (Drake *et al.* 1997, Moore *et al.* 1999). Decrease in RuBPCO content may be also the result of non-selective decrease in leaf nitrogen content (Makino *et al.* 1997, Curtis *et al.* 2000). RuBPCO activity changes within several minutes depending on temperature, RuBP supply, irradiance, CO_2 concentration, inorganic phosphate

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Abbreviations: C_i – intercellular CO_2 partial pressure; CA1P – 2-carboxy-D-arabinol-1-phosphate; DTT – dithiothreitol; HEPES – N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PPFD – photosynthetic photon flux density; RuBP – D-ribulose-1,5-bisphosphate; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase; SDS – sodium dodecyl sulfate; T_{air} – air temperature; TCA – trichloroacetic acid; Tris – Tris[hydroxymethyl] aminomethane; VPD – vapour pressure deficit.
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content, and presence of inhibitors in the active site (Caemmerer and Quick 2000).

The effect of carbamylation on RuBPCO activity may be determined by the extent to which its activity, measured immediately in extracts prepared rapidly in the cold, is less than the activity measured following incubation with saturating concentration of CO_2 and Mg^{2+}. These measurements are called initial and total activities, respectively. The term total activity is a misnomer since it does not include active sites blocked by tight binding inhibitors, for example 2-carboxy-D-arabinol-1-phosphate (CA1P), which are not removed by incubation with CO_2 and Mg^{2+}. Consistent with this is the observation that total activity may change significantly during the day, presumably due to regulation by inhibitors (Parry *et al.* 1997). Whilst the difference between initial and total activities of RuBPCO is a measure of the regulation by carbamylation, changes in total activities can be attributed to regulation by CA1P or similar inhibitors.

In this work we measured diurnal changes of RuBPCO initial and total activities *in vitro* as well as RuBPCO content to find the mechanism of RuBPCO activity regulation in Norway spruce needles.

Materials and methods

Plants: The experiment was conducted on 19 and 20 October, 2005 on the full developed Norway spruce (*Picea abies* [L.] Karst.) shoots. Current-year needles were sampled at 06:00 (before the sunrise) and then every unpaired hour till 19:00 (after the sunset). Actual air temperature and actual photosynthetic photon flux density (PPFD) in sampling times are shown in Table 1. Quantum Sensor *LI-190* (*Li-Cor*, USA), located at the level of investigated shoot, was used for incident PPFD measurement. Needles were put into liquid nitrogen after the estimation of their fresh mass and projected area using the technique of Kalina and Slovák (2004).

Table 1. Average values of photosynthetic photon flux density (PPFD) and air temperature (T_{air}) in the course of sampling.

Time [h]	19 Oct. 2005 PPFD [μmol m^{-2} s^{-1}]	T_{air} [°C]	20 Oct. 2005 PPFD [μmol m^{-2} s^{-1}]	T_{air} [°C]
06:00	0	1	0	–2
07:00	13	2	7	–1
09:00	62	2	140	1
11:00	747	12	589	11
13:00	599	12	446	15
15:00	94	12	604	15
17:00	33	11	46	13
19:00	0	9	0	10

RuBPCO protein was extracted from the needles and assayed for its activity using the techniques described in Hrstka *et al.* (2005). Approximately 0.3 g of needle tissue was homogenized in a chilled mortar with 0.1 g inert sand, liquid nitrogen, and 10 cm^3 extraction buffer composed of: 50 mM HEPES, 5 mM Na_2EDTA, 5 mM dithiothreitol (DTT), and 1 % insoluble polyvinylpolypyrrolidone, all at pH 8.0. The homogenate was centrifuged at 10 000×*g* for 2 min and an aliquot of the supernatant was used immediately for spectrophotometric RuBPCO activity assay, based on the continuous measurement of 3-phosphoglycerate-dependent NADH oxidation in a coupled enzyme system (Lilley and Walker 1974).

The initial RuBPCO activity was determined by adding 15 mm^3 of extract to 470 mm^3 of assay solution which contained: 50 mM HEPES (pH 8.0), 25 mM $KHCO_3$, 20 mM $MgCl_2$, 5 mM Na_2EDTA, 5 mM DTT, 3.5 mM ATP, 0.35 mM NADH, 3.5 mM phosphocreatine, 34 nkat glyceraldehyde-3-phosphate dehydrogenase, 56 nkat 3-phosphoglyceric phosphokinase, and 89 nkat creatine phosphokinase. The reaction was started by the addition of 15 mm^3 of RuBP at final concentration 0.4 mM and the changes in absorbance at 340 nm were immediately measured at 25 °C using *Helios γ* (*Spectronic Unicam*, UK) spectrophotometer.

The total activity was measured after 10-min incubating 15 mm^3 of the extract with 470 mm^3 of the assay solution. The assay was again started by adding 15 mm^3 of RuBP at final concentration 0.4 mM and the changes in absorbance at 340 nm were measured. The activation state was calculated as the ratio of initial activity to total activity.

RuBPCO content was determined by trichloroacetic acid/acetone method following SDS-PAGE according Damerval *et al.* (1986) and Nie *et al.* (1995) with some adaptations by authors. About 0.3 g of frozen needles was powdered in liquid nitrogen with a mortar and a pestle. Total protein was extracted with 10 cm^3 of extraction buffer containing 62 mM Tris, 2 % (m/v) sodium dodecyl sulphate (SDS), 65 mM DTT, and 10 % (v/v) glycerol, all at pH 6.8. A 5 cm^3 of the supernatant was precipitated by 5 cm^3 of 10 % (m/v) TCA and 0.07 % (v/v) 2-mercaptoethanol in acetone for 45 min at –18 °C. Then the extract was centrifuged at 10 000×*g* for 5 min, the supernatant was discarded, and the pellet was washed three times with acetone containing 0.07 % 2-mercaptoethanol, and dried under vacuum. Dry pellet was solubilized in 1 cm^3 sample buffer composed of 3 % (m/v) Tris, 5 % 2-mercaptoethanol, 10 % (m/v) SDS, 20 % (v/v) glycerol, and 0.2 % (m/v) bromphenol blue.

RuBPCO content was determined by SDS-PAGE with a *Mini-PROTEAN 3* system (*Bio-Rad*). Resolving gels contained 10 % (m/v) acrylamide, 0.27 % (m/v) N,N´-methylene-bis-acrylamide, 0.37 M Tris-HCl (pH 8.8), 0.1 % (m/v) SDS, 0.04 % (v/v) N,N,N',N'-tetra-

methylethylenediamine (TEMED), and 0.1 % (m/v) ammonium persulphate. Stacking gels contained 5 % (m/v) acrylamide, 0.13 % (m/v) N,N´-methylene-bis-acrylamide, 0.19 M Tris, 0.02 % (v/v) TEMED, and 0.1 % (m/v) ammonium persulphate.

A 5 mm^3 of sample solution were loaded on polyacrylamide gel. Electrophoresis ran 100 min at the constant current 15 mA. The large subunit of RuBPCO was detected by staining with Coomassie brilliant blue and its identity was confirmed by co-electrophoresis of RuBPCO standard (*Sigma-Aldrich*) (Fig. 2). Quantification of individual bands was performed on *HP Scanjet 5590P* with programme Advanced Image Data Analyzer, ver. *3.23.001* (*Raytest*, Germany).

Results

On the 19 October the sky was clear and when the early vapour disappeared, the irradiance sharply increased, and at 11:00 of summer time it reached its daily maximum (747 μmol m^{-2} s^{-1}). At 13:00, the irradiance was 600 μmol m^{-2} s^{-1}, and then the sky clouded over and irradiance decreased sharply, so at 15:00 it was only 94 μmol m^{-2} s^{-1} (Fig. 1*A*). The initial activity was extremely low at 06:00 prior to sunrise, hence the activation state (measured by comparison of initial and total activity) was only 62 %. With increasing irradiance both initial and total activities increased up to 09:00 and then decreased up to 13:00 (midday depression). We observed the second maximum of activities at 17:00 (Fig. 1*B*). At 07:00, at very low irradiance, the activation state of RuBPCO was 88 %. From 07:00 till 17:00 the activation state remained high (80–90 %), but at 11:00 and 13:00 we observed its midday depression (75 %, Fig. 1*C*). At 19:00 the activation state was 65 %, *i.e.* was approximately the same as at 06:00. RuBPCO content decreased during the day from 3.1 g m^{-2} (07:00) to 1.4 g m^{-2} (15:00).

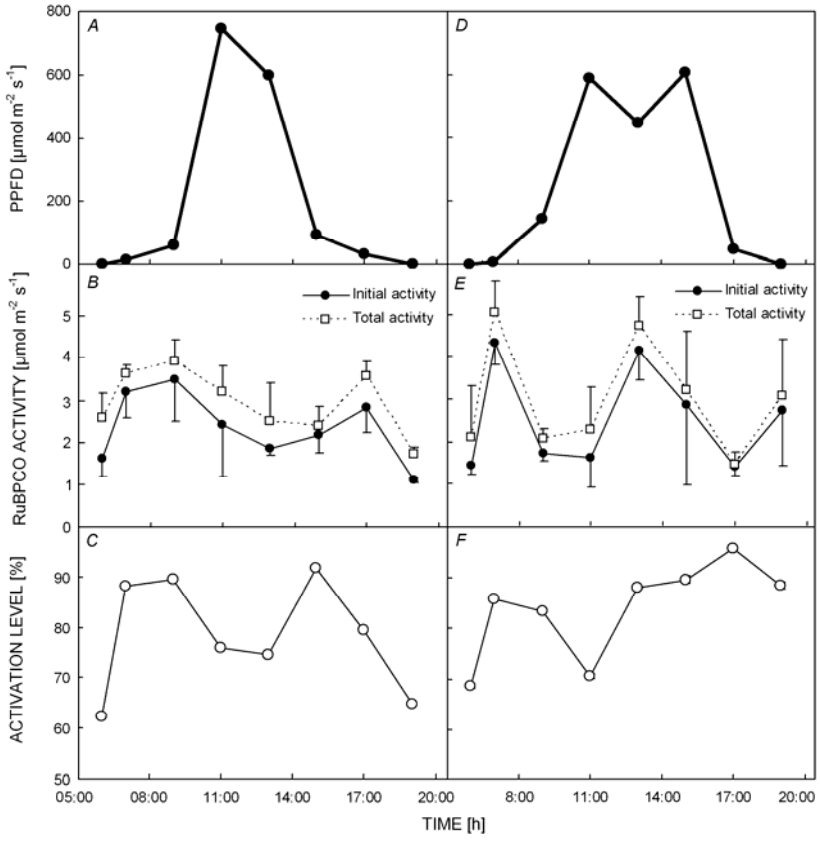

Fig. 1. Daily courses of photosynthetic photon flux density, PPFD (*A, D*), RuBPCO activity (*B, E*), and activation level determined as the ratio between initial and total RuBPCO activity (*C, F*) on the 19 (*A–C*) and 20 (*D–F*) October 2005. Means and standard deviations (*bars*), $n = 5$.

On 20 October at 09:00 the irradiance was higher than on the 19 October. At 11:00 and 13:00 there was a weak mist, therefore the irradiance was lower in comparison with 19 October. Only at 15:00 when the mist disappeared the irradiance reached its daily maximum of 604 μmol m^{-2} s^{-1} (Fig. 1*D*). On 20 October at 06:00 both initial and total activities corresponded with activities on 19 October, but at 07:00 they were substantially higher. At 09:00 and 11:00 we found very low activities (midday depression) and at 13:00 a second daily maximum of activities. Whereas on 19 October there was a second daily maximum of activities at 17:00, on 20 October the daily minimum was at 17:00 (Fig. 1*E*) and, surprisingly, the activities increased from 17:00 till 19:00. The time course

of the activation state was very similar to that of 19 October. Only at 06:00 the activation state was higher (69 %), the midday depression took place sooner (at 11:00), and even at 19:00 the activation state was very high (88 %, Fig. 1*F*). RuBPCO content was similar both in the morning and in the afternoon (2.7 g m^{-2}).

Discussion

RuBPCO activity may be modulated by the content of this enzyme and by the carbamylation of its active site. RuBPCO active site, more exactly its side chain Lys201, exists at least in the following forms: E (E–NH_2), ER (E–NH_2–RuBP), EC (E–NH–CO_2), ECM (E–NH–CO_2Mg^{2+}), ECMR (E–NH–CO_2Mg^{2+}–RuBP), and ECMI (E–NH–CO_2Mg^{2+}–inhibitor). Catalytic competent are only ECM and ECMR forms. The binding of inhibitors to carbamylated RuBPCO (EC) prevents catalysis, whilst binding of the substrate RuBP to the non-carbamylated enzyme prevents carbamylation of the lysine residue that is essential for activity (Parry *et al.* 2002). The extent of this carbamylation depends on: (*1*) the concentrations of CO_2 and Mg^{2+}, (*2*) the concentrations of RuBP, 2-carboxy-D-arabinitol-1-phosphate (CA1P), or some other phosphorylated sugars which can block the active site, and (*3*) the activity of RuBPCO activase. The activity of this latter enzyme is controlled by the ratio ATP/ADP, mainly modified by irradiance. The relative contribution of each mechanism of regulation varies with time and species (Parry *et al.* 2003).

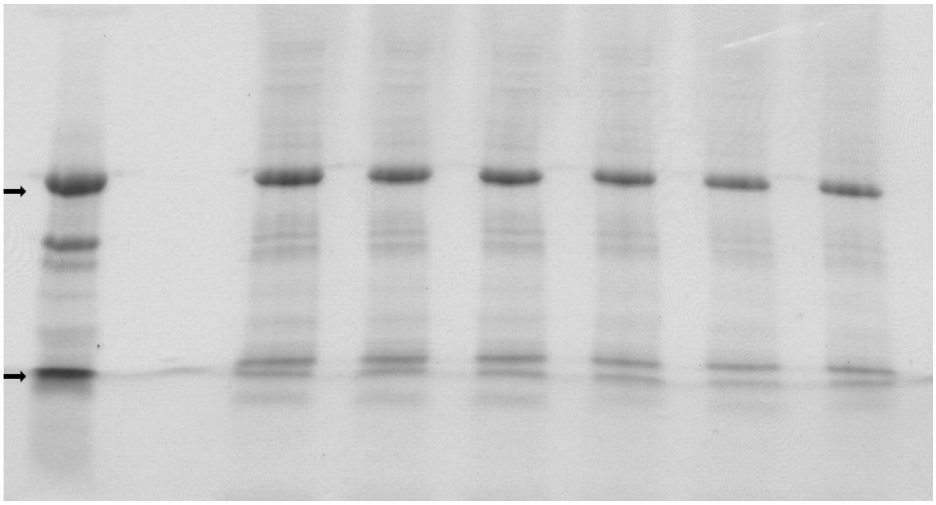

Fig. 2. A representative SDS-PAGE gel with separated proteins from Norway spruce needles (six *right columns*) and RuBPCO standard 1 mg cm^{-3} (*the first column from the left*). *The upper arrow* indicates the large subunit of RuBPCO protein and *the lower arrow* indicates the small one.

Relatively low morning and evening values of total activities indicate that the nocturnal inhibitor CA1P is important in Norway spruce. Because total activity was not constant during the day, but approximately copied the daily course of initial activity, we conclude that there is a various quantity of tight binding inhibitors in the active site of RuBPCO during the day. These are: (*1*) D-xylulose-1,5-bisphosphate and 3-keto-D-arabinitol-1,5-bisphosphate which are misformed during the catalytic reaction (Portis 1995), and for that reason this inhibition is called catalytic inactivation, (*2*) some other, little characterised daily inhibitor (Keys *et al.* 1995).

In our measurements neither initial nor total activities of RuBPCO copied irradiances. Whereas irradiance increased at least till 11:00, both initial and total activities increased only till 08:00 or 09:00, respectively, and than decreased. At noon we measured a midday depression of enzyme activities and in the afternoon we observed an increase of activities again. So, both the initial and total RuBPCO activities had the typical pattern with two peaks in the morning and afternoon, respectively, and a midday depression (Sage *et al.* 1990, Pathre *et al.* 1995, Singh *et al.* 1996, Sinha *et al.* 1997, Jiang 2001, Wang *et al.* 2003). Diurnal measurements indicate that trees often exhibit a midday depression in net photosynthetic rate and stomatal conductance, g_s (Sage *et al.* 1990, Pathre *et al.* 1995, Sinha *et al.* 1997, Jiang 2001, Wang *et al.* 2003). This phenomenon is usually associated with high vapour pressure deficit (Prado *et al.* 1995), high temperature (Singh *et al.* 1996, Sinha *et al.* 1997, Wang *et al.* 2003), and strong irradiance (Palanisamy 1996, Pons and Welschen 2003). In several cases, during midday depression the apparent carboxylation efficiency decreased while internal CO_2 concentration (C_i) remained constant (Gunasekera and Berkowitz 1992). On the other hand, the patchy stomatal closure during midday can decrease local C_i and subsequently inhibit photosynthesis (Beyschlag *et al.* 1992).

However, we observed the midday depression of RuBPCO initial and total activities *in vitro* even at low temperatures and at very low irradiances. Because we assayed RuBPCO total activity in extract after incubation

with saturating concentrations of CO_2 and Mg^{2+}, this phenomenon cannot be caused either by g_s or by high VPD or by decrease of local C_i but it must be caused only by the presence of the tight binding inhibitors in the active sites of RuBPCO. While on the leaf level the midday depression offers a well controlled mechanism that trees seemingly have evolved to overcome radiation and temperature stresses, the cause of midday depression on the molecular level is still not satisfactorily elucidated. High concentrations of daily inhibitors of RuBPCO may belong to the reasons of midday g_s decline.

The activation state of RuBPCO was calculated as the ratio of initial activity to total activity and reflects the extent of carbamylation of its active sites. RuBPCO is never decarbamylated in the dark and the activation state is between 30 and 40 % depending upon species (Caemmerer and Quick 2000). The carbamylation level of RuBPCO rarely limits steady state rates of CO_2 assimilation when RuBP regeneration capacity is low and carbamylation decreases (Sassenrath-Cole *et al.* 1994). In our experiment we found surprisingly high activation state in the morning before sunrise (62 and 69 %) and in the evening after the sunset (65 and 88 %).

The RuBPCO content of a leaf is dynamic and subject to rapid changes in response to changing environment. In an elegant set of experiments Prioul and Reyss (1988) demonstrated that within a single leaf of tobacco, localised changes in irradiance both up and down brought about parallel changes in the content of RuBPCO protein within several hours and days. The mechanisms by which these signals affect RuBPCO abundance involve altered rates of gene transcription (Gallagher and Ellis 1982), post-transcriptional mRNA regulation (Peters and Silverthorne 1995), mRNA translation (Berry *et al.* 1990), and protein turnover (Cheng *et al.* 1998).

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