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Short-term growth of meadow fescue with atmospheric CO_2 enrichment decreases freezing tolerance, modifies photosynthetic apparatus performance and changes the expression of some genes during cold acclimation

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Abstract The objective of this study was to assess the effect of an elevated atmospheric CO_2 molar ratio on freezing tolerance, photosynthetic apparatus performance and expression of *CBF6*, *Cor14b* and *LOS2* in meadow fescue (*Festuca pratensis* Huds.). It was shown that cold acclimation under a CO_2 molar ratio of 800 µmol $mol(air)^{-1}$ decreased the freezing tolerance of meadow fescue when compared to the ambient CO_2 level. This effect was not related either to changes observed in PSII redox state or to photosynthetic acclimation to cold, which was in fact more effective at an elevated CO_2 level. The decrease in freezing tolerance was linked to changes in the expression of *CBF6* and *LOS2* genes, whereas the protective effect on photosynthetic apparatus was connected with the activation of a non-photochemical mechanism of photoprotection as well as upregulation of *FpCOR14b* expression.

Keywords Cold acclimation · Chlorophyll fluorescence · Elevated CO_2 · Freezing tolerance · Transcript level

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Abbreviations

CA	Cold acclimation
CBF/DREB	C-repeat binding factor/dehydration responsive element-binding protein
COR genes	Cold regulated genes
F_0 and F_0'	Fluorescence when all PSII reaction centres are open in dark- and light-acclimated leaves, respectively
F_d	Fluorescence decrease
F_m and F_m'	Fluorescence when all PSII reaction centres are closed in dark- and light-acclimated leaves, respectively
F_s,	Steady-state fluorescence in light-exposed leaves
F_v and F_v'	Variable fluorescence in dark- and light-acclimated leaves, respectively
F_v/F_m	Apparent quantum yield of PSII
PSII	Photosystem II
F_v'/F_m'	Photosystem II antenna trapping efficiency
HFT and LFT genotypes	Higher and lower freezing-tolerant genotypes at ambient CO_2 molar ratio, measured and described by Kosmala et al. (2009) as Fp37 and Fp13
LOS2	Low expression of osmotically responsive genes 2 (a bifunctional enolase with transcriptional repression activity)

t_{EL50}	Temperature at which 50 % of the plants were killed by frost
NPQ	Non-photochemical quenching coefficient of chlorophyll fluorescence
q_P	Photochemical quenching coefficient of chlorophyll fluorescence
R_{fd}	Chlorophyll fluorescence decrease ratio (vitality index)
Φ_{PSII}	The actual quantum yield of photochemical energy conversion in PSII

Introduction

The atmospheric concentration of carbon dioxide, the most important anthropogenic greenhouse gas, is rising (IPCC, fourth assessment report, 2007). It has been shown that CO_2 enrichment modifies plant freezing tolerance; however, contrasting effects have been observed (Obrist et al. 2001; Bigras and Bertrand 2006; Bertrand et al. 2007). Although the effects of CO_2 fumigation on plant growth and yield have been extensively studied, less is known about the effects of rising CO_2 concentration on the molecular basis of freezing tolerance.

Freezing tolerance seems to be closely related to tolerance to cold-induced photoinhibition of photosynthesis, which is a consequence of mechanisms of acclimation common to both stresses (Huner et al. 1996). As a result, plants with higher freezing tolerance are usually characterised by higher tolerance to cold-induced photoinhibition (Huner et al. 1993; Rapacz et al. 2011), but the correlation between these two traits is not strong (Rapacz et al. 2008, 2011). Increased reduction in PSII is observed along with a decrease in temperature under high irradiance. This may act as a signal triggering the increase in freezing tolerance and photoinhibition avoidance (Huner et al. 1998). In an elevated PSII reduction state, the upregulation of many genes can also be observed. These genes encode mainly proteins involved in photosynthetic acclimation to low temperatures (wheat Wsc19, showing homology with barley cor14b, and also genes encoding proteins involved in carbon fixation and biosynthesis of sugars and lipids) (Ndong et al. 2001).

CBF/DREB1 (C-repeat binding factor/dehydration responsive element-binding factor 1) genes encode a small family of transcriptional activators that play an important role in freezing tolerance and cold acclimation (CA) in Arabidopsis. Transient induction of CBFs occurs within 15 min of CA that results in the activation of multiple COR genes needed to protect plants against freezing stress (Gilmour et al. 1998; Vogel et al. 2005). It was shown by Steponkus et al. (1998) that the constitutive expression of the COR15a gene of Arabidopsis thaliana increases the freezing tolerance of isolated protoplasts. Moreover, the accumulation of COR14b in barley appears significant in relation to combined freezing tolerance and resistance to the photoinhibition of photosynthesis (Rapacz et al. 2008).

The proper induction of downstream genes and the development of freezing tolerance require the expression of CBFs to be transient and strictly controlled. CBF2/DREB1C acts as a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression, which in turn guarantees the proper induction of downstream genes and the proper development of freezing tolerance (Novillo et al. 2004). CBF-targeted genes are also regulated. CBFs induce the expression of ZAT10, a transcription factor which may downregulate the expression of COR genes. LOS2 (a bifunctional enolase) acts as a negative regulator of STS/ZAT10 and thus prevents direct downregulation of COR genes by CBFs (Lee et al. 2002; Chinnusamy et al. 2007).

Festuca pratensis (meadow fescue) is the most freezing-tolerant species within the Lolium-Festuca complex and may potentially be a source of freezing tolerance genes for closely related Lolium multiflorum and Lolium perenne (Kosmala et al. 2006).

The aim of the study was to determine the effect of an elevated CO_2 molar ratio on freezing tolerance, photosynthetic apparatus performance and expression of some genes involved in the CA process in F. pratensis. Three genes were selected for molecular studies: two of them acting as transcription factors that activate the expression of COR genes (CBF6 and LOS2) and one effector gene with a protective function (Cor14b) (Steponkus et al. 1998; Lee et al. 2002; Chinnusamy et al. 2007).

Materials and methods

Plant material and treatments

The experiments were performed on clones of two F. pratensis Huds. cv. Skra genotypes with contrasting freezing tolerance measured at ambient CO_2 molar ratio: the lower freezing tolerant genotype (LFT) and higher freezing tolerant genotype (HFT), described as Fp13 and Fp37, respectively, by Kosmala et al. (2009). These genotypes were selected from forty plants of F. pratensis, each one germinated from a single seed, as described in detail by Kosmala et al. (2009). Briefly, forty F. pratensis plants were tested for freezing tolerance, including an estimate of the plant's ability to regrow after freezing at −8, −11, and −14 °C using Larsen's (1978) visual score.

Next, the two genotypes with the highest and the two with the lowest levels of freezing tolerance were tested for freezing tolerance by estimating t_{EL50} (temperature causing a 50 % electrolyte leakage) (Flint et al. 1967). The level of freezing tolerance of four selected plants was determined before CA and after 2, 8, 26 h, and 3, 5, 7, 14 and 21 days of CA (4/2 °C, 10/14 h photoperiod, 200 µmol m^{-2} s^{-1} PPFD). Two genotypes with extreme values of t_{EL50} after 21 days of CA (Fp13 and Fp37 with $t_{EL50} = -15.9$ and -21.4 °C, respectively) were selected for the experiment. Prior to the beginning of the experiments, plants were grown in an open-air vegetation room. The experiments were performed using 3-year-old plants. In the autumn, the plants were transferred to an air-conditioned greenhouse and grown at 20 °C in daylight, which was (if necessary) increased to 12 h and supplemented automatically on cloudy days with PAR of 200 µmol m^{-2} s^{-1} using Agro HPS lamps (Philips). The experiment was repeated in two independent series. Ten clones of each genotype divided into two groups were transferred from the greenhouse to environment chambers controlled for CA (18 days at 6/4 °C, 10/14 h photoperiod, 190 µmol m^{-2} s^{-1} PAR provided by Agro HPS lamps, Philips). The chambers were maintained at CO_2 molar ratios of either 350 µmol mol^{-1} (ambient) or 800 µmol mol^{-1} (elevated). To maintain a constant elevated CO_2 molar ratio, a flow of synthetic air (800 µmol mol^{-1} CO_2) was supplied, exchanging the entire air volume every 2 h. The temperatures and light intensities listed above were measured on the upper surface of the leaves taken for sampling with a PAR/temperature micro-sensor, an integral part of the FMS2 chlorophyll fluorescence measuring system (Hansatech, King's Lynn, UK).

Assessment of freezing tolerance

Samples (*ca.* 3-cm fragments of the middle part of the youngest fully developed leaf) were collected from LFT and HFT plants after 6, 12 and 18 days of CA at the elevated CO_2 and after 6, 12 and 18 days of CA at the control molar ratio of CO_2. Leaf fragments were put on ice in plastic vials and frozen for 1.5 h at -6, -10, and -14 °C, chosen on the basis of a previous experiment (Kosmala et al. 2009), in the programmed freezer. The temperature was decreased at a rate of 2 °C h^{-1}. Freezing temperatures were maintained for 90 min, after which the temperature was increased up to 0 °C at a rate of 3 °C h^{-1}. After thawing, damage to the leaf tissue was estimated on the basis of electrolyte leakage (Radelkis OK-102/1 conductometer). To determine 100 % damage, control leaves were frozen in liquid nitrogen. Estimation of t_{EL50} was performed on the basis of the electrolyte leakage test (Flint et al. 1967). The results presented in this paper are the means from two experiments (14 replicates in total).

Analysis of photosynthetic acclimation to cold

Measurements of chlorophyll fluorescence parameters were performed before CA and after 6, 12 and 15 days of CA using the pulse amplitude modulation chlorophyll fluorescence imaging system FluorCam (PSI, Brno, Czech Republic). Six leaves from each genotype were detached and fixed with paper adhesive tape to black paper and placed inside a FluorCam measuring chamber. Chlorophyll fluorescence induction kinetics and quenching parameters were measured at 20 °C and a normal CO_2 molar ratio with an experimental protocol comprising 20 min of dark adaptation, measurements of F_0 (fluorescence of dark-adapted leaves, when all PSII reaction centres are open), F_m (fluorescence of dark-adapted leaves, when all PSII reaction centres are closed) after a light saturating pulse of about 2,000 µmol m^{-2} s^{-1}, F_s (steady-state fluorescence in light-exposed leaves) after 400 s of actinic light exposition (150 µmol m^{-2} s^{-1}) combined with saturating light pulses operated every 25 s, F_d (fluorescence decrease), F_m' (fluorescence of light-adapted leaves, when all PSII reaction centres are closed) during the last saturating pulse and F_0' (fluorescence of light-adapted leaves, when all PSII reaction centres are open) measured with an actinic light source switched off after the far-red light pulse. The photochemical quenching coefficient (q_P) was calculated in accordance with Schreiber et al. (1994): $q_P = (F_m' - F_s)/(F_m' - F_0')$. Non-photochemical quenching coefficient (NPQ) was calculated as: NPQ $= (F_m - F_m')/F_m'$ (Bilger and Bjorkman 1991). Apparent quantum yield of PSII (F_v/F_m) was defined as $(F_m - F_0)/F_m$. Photosystem II antenna trapping efficiency was calculated as F_v'/F_m'. The chlorophyll fluorescence decrease ratio (vitality index) was calculated in accordance with Lichtenthaler et al. (2004) as $R_{Fd} = F_d/F_s$. The actual or effective quantum yield of photochemical energy conversion in PSII (in the light-adapted state) was defined in accordance with Genty et al. (1989) as $\Phi_{PSII} = (F_m' - F_s)/F_m'$. The results presented in this paper are the means from two experiments (12 replicates in total).

Additionally, PSII excitation pressure $(1 - q_P)$ was measured after 15 days of CA in the growth chambers, where plants were cold-acclimated either at control or elevated CO_2 levels, on the middle part of the youngest fully developed leaf using the modulated fluorescence system FMS2 (Hansatech, King's Lynn, UK). The results are the means from two experiments (10 replicates in total).

Gas exchange

The net photosynthetic rate (P_N) and stomatal conductance (g_s) were measured using an infrared gas analyser (CIRAS-1, PP Systems, Hitchin, UK) integrated with a Parkinson leaf

chamber (PLC6, PP Systems, Hitchin, UK) automatically controlling measurement conditions. The irradiation system (PP Systems, Hitchin, UK) was equipped with three halogen lamps (3×20 W). The flow rate of air with a constant CO_2 molar ratio (400 μmol CO_2 mol^{-1} air) through the assimilation chamber was 350–400 cm^3 min^{-1}. Measurements were performed together with $1 - q_P$ measurements on the middle part of the youngest fully developed leaf. The results are the means from two experiments (8 replicates in total).

Analysis of the transcript levels of CBF6, Cor14b and LOS2 genes

Samples (*ca.* 0.04 g from the middle part of the youngest fully developed leaf) were collected from LFT and HFT plants after 1, 2 and 4 h of CA and on the 18th day of CA (1, 2 and 4 h after dawn) at ambient and elevated CO_2 molar ratios. Samples were immediately frozen in liquid nitrogen and stored at −80 °C. Next, RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The reverse transcription and the removal of genomic DNA contamination were performed with a QuantiTect Reverse Transcription Kit (Qiagen). The final concentration and quality of cDNA were determined spectrophotometrically (Ultrospec 2100 Pro, supplied with an ultramicrovolume cell, Amersham Biosciences, Buckinghamshire, UK).

Quantitative PCR analyses were performed using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) as described in detail by Jurczyk et al. (2012). We used the *Actin* gene as a reference gene to normalize the amount of cDNA added to each PCR (An et al. 1996). For each fragment of cDNA, we studied the expression of *CBF6*, *Cor14b*, *LOS2* and *Actin* genes using: in the case of *CBF6*, a specific forward primer (CTTCGCAGAACGAC AATTCG), a specific reverse primer (GGTCCCATCCC ATATCACTGA) and a specific probe (FAM-CGTTCG AGCTGGAAGT-MGB); in the case of *Cor14b*, a specific forward primer (AGACCCAGATCGATGGCTTCT), a specific reverse primer (GCACGGCCTGGGAAGAG) and a specific probe (FAM-TCGGAGGAGGCGCG-MGB); in the case of *LOS2*, a specific forward primer (AGATCG TAGGAGATGACCTTCTTGT), a specific reverse primer (TGCAGGTCTTCTCACTGATTGC) and a specific probe (FAM-CCCCACAGGGTTGCCA-MGB); and, finally, in the case of *Actin*, a specific forward primer (GTCGAG GGCAACATATGCAA), a specific reverse primer (CCAG TGCTGAGCGGGAAAT) and a specific probe (FAM-TT CTCCTTGATGTCACGGAC-MGB). Sequences of primers and probes were designed using Primer Express software version 3.0. on the basis of the appropriate *F. pratensis*: *Actin* EST (GenBank no. GO859520.1; the

sequence of the amplicon was checked and confirmed), the *LOS2* gene (not published, provided by Dr H. Rudi, Norwegian University of Life Sciences; the sequence of the amplicon was checked and confirmed), sequence of the *CBF6* gene (GenBank no. DQ996012.1), and the *Cor14b* gene (GenBank no. AJ512944.1). All primers and probes were supplied by Applied Biosystems. Data were analyzed using 7500 real-time PCR Sequence Detection Software version 1.3. (Applied Biosystems). We used the relative standard curve method (Applied Biosystems) to estimate relative gene expression. The results, based on six biological replicates (3 from each experiment), are presented as a fold change in the expression of a particular gene in treated samples relative to the endogenous control gene (*Actin*).

Statistical treatment

In addition to the calculation of standard error of relative gene expression, which was performed using Applied Biosystems 7500 System Sequence Detection Software version 1.3., variation analysis of the relative expression level was performed using the GLM module of Statistica 9.0. (StatSoft, Tulsa, OK, USA). The general effects of
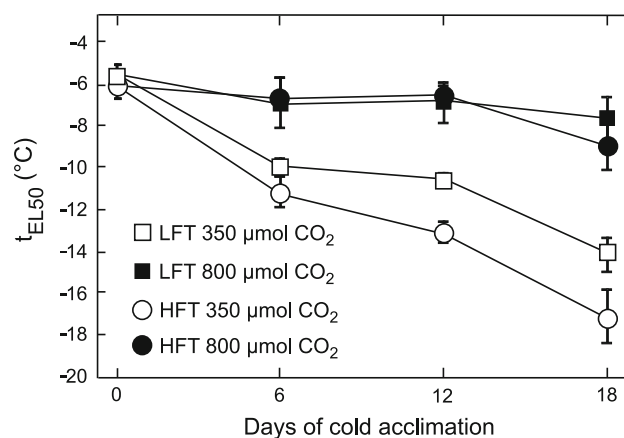

Fig. 1 The effect of CO_2 molar ratio on freezing tolerance in *F. pratensis*. Clones of each genotype divided into two groups were transferred from the greenhouse to controlled-environment chambers for cold acclimation (18 days at 6/4 °C, 10/14 h photoperiod, 190 μmol m^{-2} s^{-1} PAR provided by Agro HPS lamps, Philips). In vegetation chambers a CO_2 molar ratio of either 350 μmol mol^{-1} (ambient) or 800 μmol mol^{-1} (elevated) was maintained. To maintain a constant elevated CO_2 molar ratio, a flow of synthetic air (800 μmol mol^{-1} CO_2) was supplied. t_{EL50} is the temperature at which 50 % of the total electrolytes were released from leaf tissues. The values of t_{EL50} and the coefficient intervals for $P = 0.05$ were calculated on the basis of freezing tests made in two replicates at three temperatures (−6, −10, −14 °C). HFT and LFT genotype: higher and lower freezing-tolerant genotype at ambient CO_2 molar ratio (Kosmala et al. 2009)
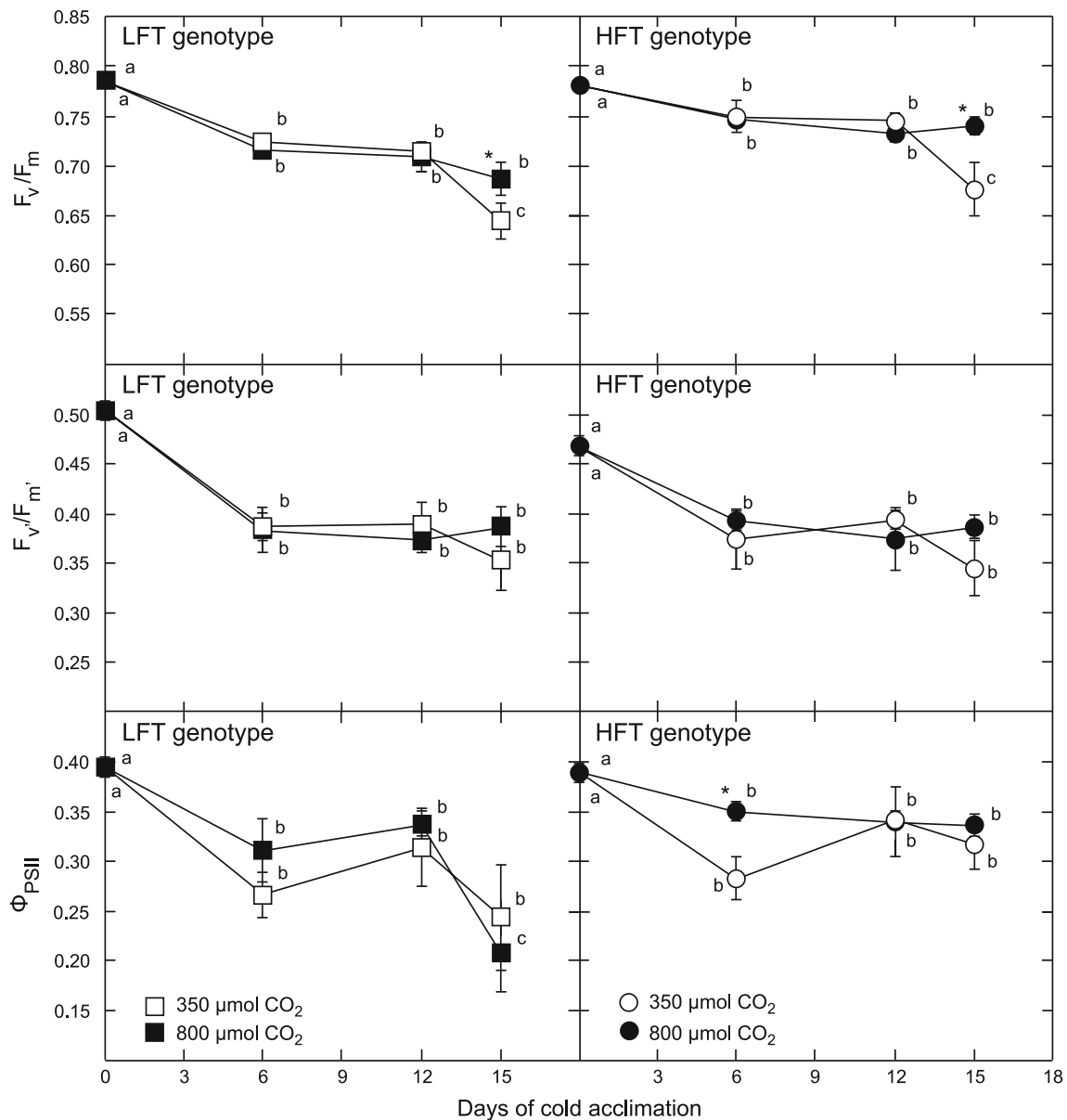
Fig. 2 Changes in chlorophyll fluorescence parameters: apparent quantum yield of PSII (F_v/F_m), photosystem II antenna trapping efficiency (F_v'/F_m'), actual quantum yield of photochemical energy conversion in PSII (Φ_{PSII}) during cold acclimation in *F. pratensis*. Clones of each genotype divided into two groups were transferred from the greenhouse to controlled-environment chambers for cold acclimation at 6/4 °C, 10/14 h photoperiod, 190 μmol m^{-2} s^{-1} PAR (Agro HPS lamps, Philips). In vegetation chambers a CO_2 molar ratio of either 350 μmol mol^{-1} (ambient) or 800 μmol mol^{-1} (elevated) was maintained. To maintain a constant elevated CO_2 molar ratio, a flow of synthetic air (800 μmol mol^{-1} CO_2) was supplied. HFT and LFT genotype: higher and lower freezing-tolerant genotype at ambient CO_2 molar ratio (Kosmala et al. 2009). The data represent the means of 12 replicates ± standard error. Values in the same treatment indicated with *different letters* differ significantly between days (Tukey's HSD test, $P = 0.05$). *Asterisked values* are different between CO_2 levels (*t* test, $P = 0.05$)

different factors on gene expression and photosynthetic acclimation to cold were tested using one-way (with treatment as a factor) or three-way ANOVA (genotype, treatment and time of sampling as factors) models at $P = 0.05$. The data of gas exchange and $1 - q_P$ were analyzed using a two-way ANOVA (genotype and treatment as factors). The t_{EL50} values were calculated using the simple regression module of Statistica 9.0. together with coefficient intervals for $P = 0.05$ as described elsewhere (Kosmala et al. 2009). The statistical significance of differences between means was tested with Tukey's HSD test ($P = 0.05$).
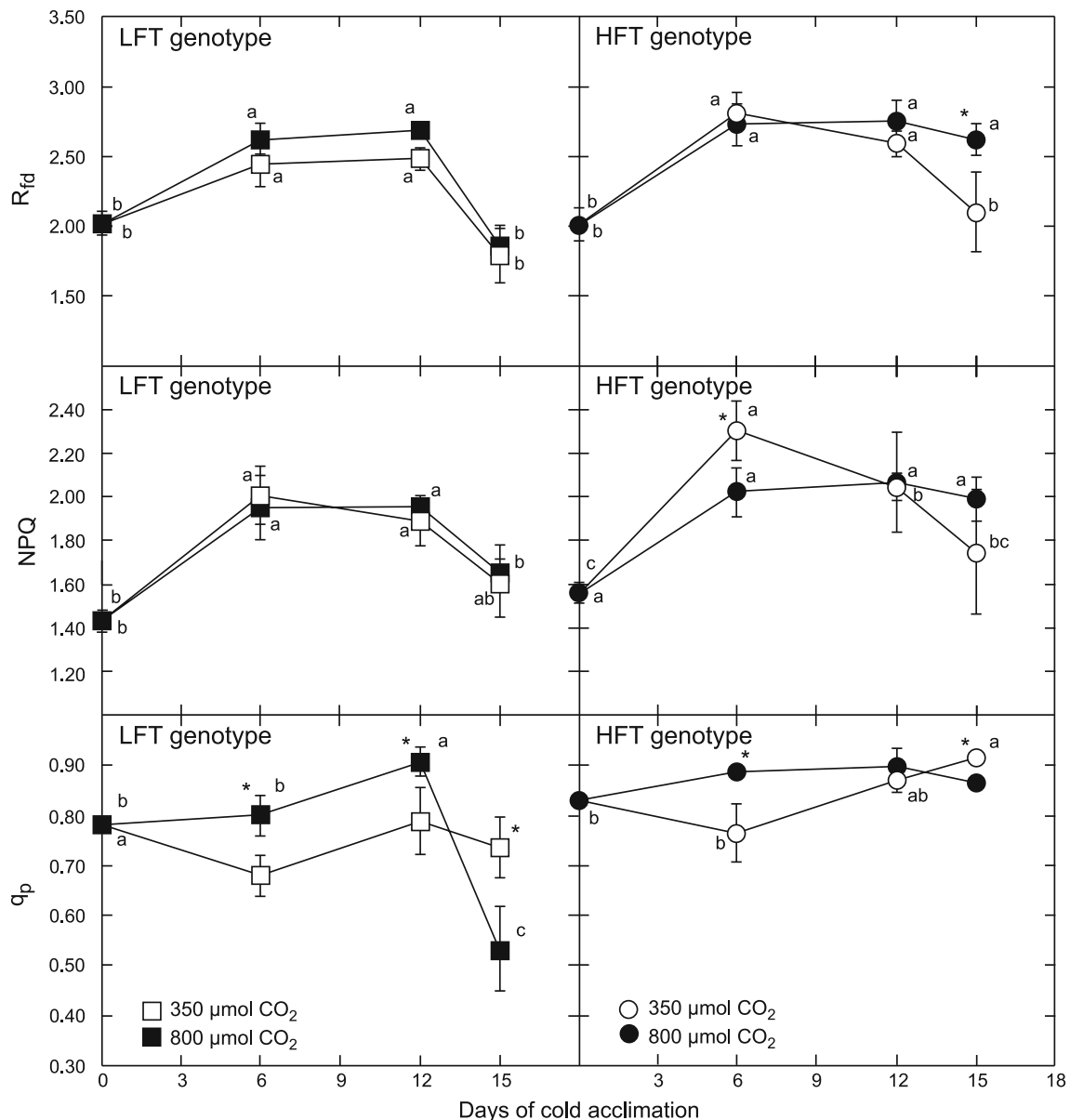
Fig. 3 Changes in fluorescence decrease ratio (R_{Fd}) and chlorophyll fluorescence quenching parameters: non-photochemical quenching coefficient (NPQ) and photochemical quenching coefficient (q_P) during cold acclimation in *F. pratensis*. Clones of each genotype divided into two groups were transferred from the greenhouse to controlled-environment chambers for cold acclimation at 6/4 °C, 10/14 h photoperiod, 190 μmol m^{-2} s^{-1} PAR (Agro HPS lamps, Philips). In vegetation chambers a CO_2 molar ratio of either 350 μmol mol^{-1} (ambient) or 800 μmol mol^{-1} (elevated) was maintained. To maintain a constant elevated CO_2 molar ratio, a flow of synthetic air (800 μmol mol^{-1} CO_2) was supplied. HFT and LFT genotype: higher and lower freezing-tolerant genotype at ambient CO_2 molar ratio (Kosmala et al. 2009). The data represent the means of 12 replicates ± standard error. Values in the same treatment indicated with *different letters* differ significantly between days (Tukey's HSD test, $P = 0.05$). *Asterisked values* are different between CO_2 levels (*t* test, $P = 0.05$)

Results

Freezing tolerance

Plants growing at the elevated CO_2 molar ratio showed a decreased CA rate as compared to plants growing at the control CO_2 molar ratio (Fig. 1). No decrease in t_{EL50} was observed in the LFT genotype (Fp13) during CA, whereas a slight decrease in t_{EL50} (−2.7 °C) was observed in the HFT genotype (Fp37). Also, there were no statistically significant differences in freezing tolerance between genotypes observed during the whole experiment at the elevated CO_2 molar ratio. On the other hand, in control plants, freezing tolerance increased gradually during 18 days of CA by −8.6 °C and almost −11 °C in the LFT and HFT genotypes, respectively. On the 12th and 18th day

Table 1 Changes in net photosynthesis rate (P_N), stomatal conductance (g_s) and excitation pressure of PSII ($1 - q_P$) at elevated CO_2 (800 µmol mol^{-1}) after 14 days of cold acclimation in HFT (Fp13) and LFT (Fp37) genotypes

Genotype	CO_2 molar ratio (µmol mol^{-1})	P_N (µmol m^{-2} s^{-1})	g_s (mmol m^{-2} s^{-1})	$1 - q_P$
LFT	350	3.600[a]	20.25[a]	0.6510[b]
LFT	800	3.100[a]	8.50[b]	0.7376[a]
HFT	350	2.650[a]	18.50[a]	0.6496[b]
HFT	800	3.325[a]	17.50[a]	0.5128[c]

Means of eight (P_N and g_s) or 10 ($1 - q_P$) replications. Values indicated with the same letter are not statistically significant ($P = 0.05$, Tukey's HSD test)
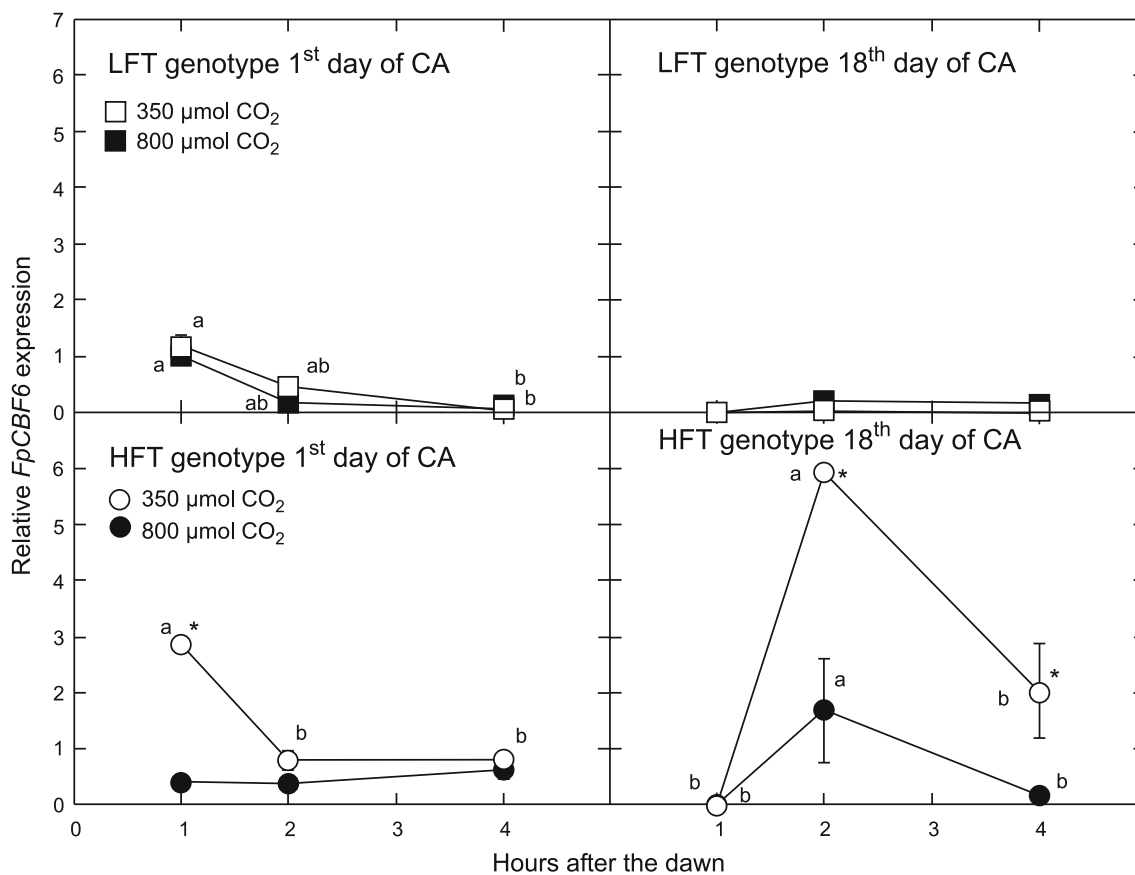

Fig. 4 Changes in the relative expression of the *CBF6* gene during cold acclimation (4 °C) in *F. pratensis*. The expression level was calculated relative to the *Actin* gene. Clones of each genotype divided into two groups were transferred from the greenhouse to controlled-environment chambers for cold acclimation at 6/4 °C, 10/14 h

photoperiod, 190 µmol m^{-2} s^{-1} PAR (Agro HPS lamps, Philips). Cold acclimation started with the dawn. In vegetation chambers a CO_2 molar ratio of either 350 µmol mol^{-1} (ambient) or 800 µmol mol^{-1} (elevated) was maintained. To maintain a constant elevated CO_2 molar ratio, a flow of synthetic air (800 µmol mol^{-1} CO_2) was supplied

of CA, the LFT and HFT genotypes showed differences in their freezing tolerances.

Acclimation of photosynthetic apparatus to low temperature

The after-effects of CA at different CO_2 molar ratios on photosynthetic apparatus were measured by means of chlorophyll fluorescence taken under unified conditions

(+20 °C, 350 µmol mol^{-1} CO_2). A very similar, slow reduction in the apparent quantum yield of PSII (F_v/F_m) was observed throughout CA regardless of the CO_2 molar ratio (Fig. 2). On the 15th day of CA only, a higher decrease rate was observed in plants cold-acclimated at normal CO_2 levels. The changes in antenna trapping efficiency (F_v'/F_m') observed during CA were very similar to those observed for F_v/F_m but without any effect from the CO_2 molar ratio (Fig. 2). The actual quantum yield of
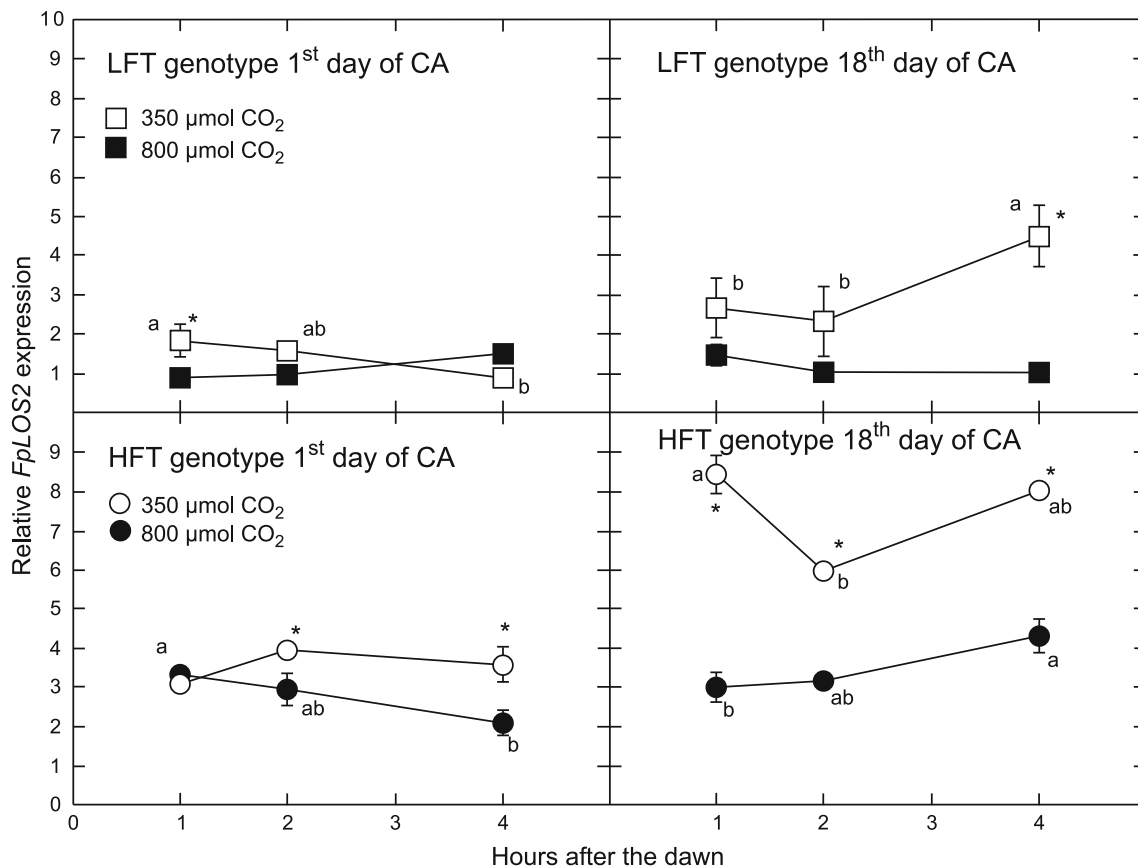
Fig. 5 Changes in the relative expression of the *LOS2* gene during cold acclimation (4 °C) in *F. pratensis*. The expression level was calculated relative to the *Actin* gene. Clones of each genotype divided into two groups were transferred from the greenhouse to controlled-environment chambers for cold acclimation at 6/4 °C, 10/14 h photoperiod, 190 μmol m^{-2} s^{-1} PAR (Agro HPS lamps, Philips). Cold acclimation started with the dawn. In vegetation chambers a CO_2 molar ratio of either 350 μmol mol^{-1} (ambient) or 800 μmol mol^{-1} (elevated) was maintained. To maintain a constant elevated CO_2 molar ratio, a flow of synthetic air (800 μmol mol^{-1} CO_2) was supplied. HFT and LFT genotype: higher and lower freezing-tolerant genotype at ambient CO_2 molar ratio (Kosmala et al. 2009). The data represent the means of six replicates ± standard error. Values in the same treatment indicated with *different letters* differ significantly between days (Tukey's HSD test, $P = 0.05$). *Asterisked values* are different between CO_2 levels (*t* test, $P = 0.05$)

photochemical energy conversion in PSII (Φ_{PSII}) decreased during CA; this decrease was unrelated to the CO_2 molar ratio (Fig. 2). After 6 days of CA, a greater decrease in Φ_{PSII} was observed in control plants of the HFT genotype (Fig. 2). In both genotypes, the vitality index (R_{fd}) increased at the beginning of CA and then decreased, with the exception of the HFT genotype growing under the elevated CO_2 molar ratio (Fig. 3), in which, on the 15th day of CA, the observed R_{fd} was higher than at the ambient CO_2 level. A very similar pattern of changes, but without statistically significant differences between treatments in the LFT genotype, was observed for the values of the non-photochemical quenching coefficient (NPQ) (Fig. 3). In addition, on the 6th day of CA in the HFT genotype, higher NPQ was measured in the control. During CA, only slight changes were observed in the values of the photochemical quenching coefficient (q_P), but the pattern of changes here was clearly different in the HFT and LFT genotypes (Fig. 3).

No change in q_P was observed in LFT plants cold-acclimated in lower CO_2, but a transient increase followed by a sudden decrease at the end of the experiment was observed under elevated CO_2. In contrast, in the case of the HFT genotype, an increase in q_P was observed in the end of CA at 350 μmol mol^{-1} CO_2.

The direct effects of the CO_2 molar ratio during CA on PSII excitation pressure ($1 - q_P$) and gas exchange were additionally measured under conditions of CA on the 15th day of the experiment, the day when the strongest effect of CO_2 on the photosynthetic apparatus was visible from measurements taken under unified conditions (Table 1). It was clearly visible that $1 - q_P$ increased during CA at elevated CO_2 levels in the LFT genotype and decreased in the HFT genotype. At the same time, a decrease in stomatal conductance (g_s) at elevated CO_2 was observed in the LFT genotype. No statistically significant differences were observed in the net photosynthesis rate (P_N).
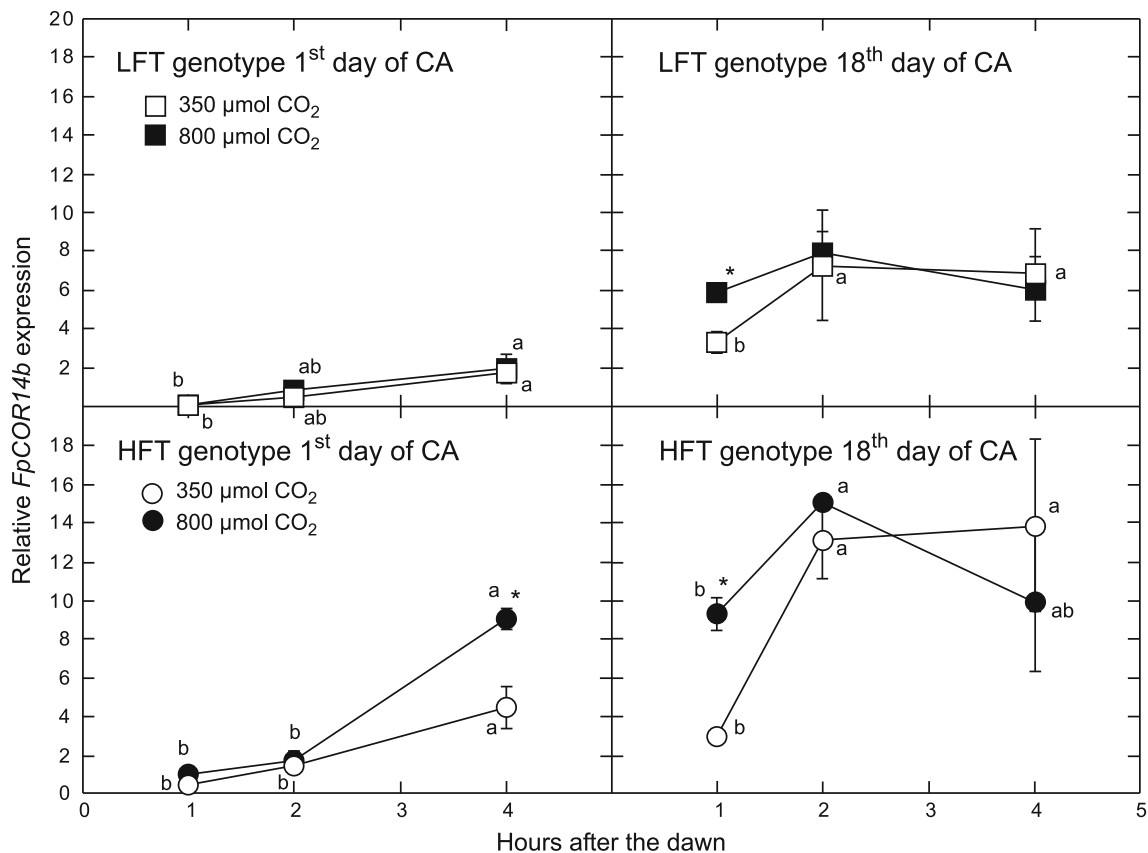
Fig. 6 Changes in the relative expression of the *Cor14b* gene during cold acclimation (4 °C) in *F. pratensis*. The expression level was calculated relative to the *Actin* gene. Clones of each genotype divided into two groups were transferred from the greenhouse to controlled-environment chambers for cold acclimation at 6/4 °C, 10/14 h photoperiod, 190 μmol m^{-2} s^{-1} PAR (Agro HPS lamps, Philips). Cold acclimation started at dawn. In vegetation chambers a CO_2 molar ratio of either 350 μmol mol^{-1} (ambient) or 800 μmol mol^{-1} (elevated) was maintained. To maintain a constant elevated CO_2 concentration, a flow of synthetic air (800 μmol mol^{-1} CO_2) was supplied. HFT and LFT genotype: higher and lower freezing tolerant genotype at ambient CO_2 molar ratio (Kosmala et al. 2009). The data represent the means of six replicates ± standard error. Values in the same treatment indicated with *different letters* differ significantly between days (Tukey's HSD test, $P = 0.05$). *Asterisked values* are different between CO_2 levels (*t* test, $P = 0.05$)

Expression profiles of *CBF6*, *LOS2* and *Cor14b* genes

The relative *CBF6* expression observed in the HFT genotype in the beginning of the day was higher than in the LFT genotype. This effect was observed on both the first (when light exposure was coupled with low-temperature shift) and the 18th day of CA (Fig. 4). In the HFT genotype, this effect was restrained by the elevated CO_2 molar ratio.

A slight decrease in relative *LOS2* expression was observed at the elevated CO_2 molar ratio after the first hour of CA in the LFT genotype and after the second and the fourth hour of CA in the HFT genotype. On the 18th day of CA, relative *LOS2* expression was reduced by the increased CO_2 molar ratio in the HFT genotype. A similar effect was observed in the LFT genotype after the fourth hour of the day (Fig. 5).

The increase in the CO_2 molar ratio did not significantly affect the relative *Cor14b* expression. Some transient increases were observed only in the HFT genotype after 4 h of CA and after the first hour of the 18th day of CA (Fig. 6).

Discussion

Contradictory conclusions were derived from the prior studies on the effects of elevated CO_2 concentration on freezing tolerance. Bertrand et al. (2007) observed that elevated CO_2 stimulated the plant growth and reduced the freezing tolerance in alfalfa. More severe frost damage was also observed in native temperate grassland by Obrist et al. (2001) and in *Ginkgo biloba* by Terry et al. (2000). On the other hand, higher CO_2 concentration increased the freezing tolerance of some species (Bigras and Bertrand 2006) and had no effects on the freezing tolerance of Norway spruce (Dalen et al. 2001). Thus, species-specific differences in the

CA process and freezing tolerance at elevated CO_2 concentrations probably exist. In the present paper, a CO_2 molar ratio which increased up to 800 μmol mol^{-1} reduced the freezing tolerance of *F. pratensis*. The freezing tolerance of both HFT and LFT genotypes was similar at the elevated CO_2 molar ratio, probably because CA had been almost completely prevented.

The results of the present paper suggest that an elevated CO_2 molar ratio affects photosynthetic apparatus performance at low temperatures. The measurements of gas exchange indicated that stomata were closed to a greater degree in plants cold acclimated under an elevated CO_2 level, which, according to Loveys et al. (2006), may cause a decrease in freezing tolerance through an increase in daytime leaf temperature. Furthermore, no increase in net photosynthesis rate was observed, which excludes the increased growth rate connected with higher photosynthetic productivity as a possible reason for the lower effectiveness of CA at elevated CO_2 as suggested by Dhont et al. (2006). During CA, the photosynthetic apparatus is not only the source of photoassimilates; PSII also acts as one of the putative temperature sensors involved in this process (Huner et al. 1998). The elevated CO_2 level could potentially disturb its function by changing the PSII redox state through an increase in the CO_2 assimilation rate. In our experiment, no such effect was observed. CA at an elevated CO_2 molar ratio only slightly changed the PSII excitation pressure; moreover, this change occurred in opposite directions in the studied genotypes, which taken together decreased their CA effectiveness under these conditions. Moreover, it seems that cold treatment at elevated CO_2 levels may increase the effectiveness of photosynthetic acclimation against cold-induced photoinhibition, which was clearly visible as halting the decline of F_v/F_m at the end of CA. In the HFT genotype, which was also more tolerant to cold-induced photoinhibition (the general effect of genotype on F_v/F_m is statistically significant during the whole experiment, data not shown), this protective effect was additionally confirmed by a higher R_{fd} ratio at the end of the experiment. In this case, the positive effect of elevated CO_2 at cold temperatures on photoinhibition avoidance is probably explained by the increase in non-photochemical quenching of excess light energy. NPQ values were slightly higher in the HFT genotype cold-acclimated at elevated CO_2 levels on the 15th day, when the effect of CO_2-induced photoprotection was observed, coupled with a clear decrease in photochemical quenching. A similar but partially (for q_P only) statistically insignificant effect was observed also for the LFT genotype, which may suggest the existence of a more general relationship. This supposed mechanism was perhaps to be expected, as the non-photochemical mechanism of photosynthetic acclimation to cold is a major means of protection induced during CA of meadow fescue, as was

reported earlier by Humphreys et al. (2007). The discussed protective effect of elevated CO_2 in cold against photoinhibition that accompanied by its detrimental effect on freezing tolerance may be rather surprising. This tolerance to cold-induced photoinhibition is closely related to freezing tolerance because of partially overlapping mechanisms of acclimation to both stresses (Sandve et al. 2011). Changes in the PSII redox state may affect the CA process by modifying the expression of many genes involved in CA (Ndong et al. 2001). On the other hand, many different mechanisms connected with, for instance, an altered carbohydrate metabolism may be involved in gene expression regulation at elevated CO_2 levels (Moore et al. 1999). In the present paper, considerable changes in the expression patterns of transcription factors of genes involved in CA were observed, which may be connected with a decrease in freezing tolerance at elevated CO_2 levels. We have shown that the induction of *CBF6* in the HFT genotype during the first hours of CA was downregulated at a higher level of CO_2. In *Arabidopsis*, more than 40 target genes of DREB1/CBF have been identified (Maruyama et al. 2004); therefore, CBFs act as master regulators that control gene clusters, which, when co-regulated, form a regulatory module (regulon). The expression of *CBF6* during the 18th day of CA at ambient CO_2 levels in the HFT genotype of *F. pratensis* was higher and demonstrated different induction kinetics than in the LFT genotype. Our results suggest that *CBF6* may act as a 'master switch' not only at the beginning of the CA process (Gilmour et al. 1998), but also after weeks of CA, which in turn may lead to changes in frost tolerance.

The proper induction of CBF-targeted genes and development of freezing tolerance requires regulation of the cold-responsive transcriptional network at the level of CBFs as well as at the level of CBF-targeted genes. LOS2 was shown to repress the *STS/ZAT10* transcription factor gene, which is upregulated by CBFs (Chinnusamy et al. 2007). We observed the same decreases in the accumulation of *LOS2* transcript at elevated CO_2 levels during the first and the 18th day of CA when compared to the control. Thus, the increase in CO_2, by decreasing the *LOS2* transcription level, may dampen the LOS2-dependent regulation of CBF-targeted genes and derange, at least partly, the functioning of the cold-responsive transcriptional network, thus contributing to lower freezing tolerance. We also showed increased *LOS2* accumulation in control plants after 18 days of CA relative to the first day of CA. This may suggest that the transcriptional repression activity of this bifunctional enolase expresses itself after a relatively long period of CA and plays an important role in sustaining the induction of CA after prolonged cold treatment.

The increase in CO_2 molar ratio also affected *FpCor14b* transcript accumulation, in which an increase

was observed both in the fourth hour of the first day of CA (in the HFT genotype only) and in the first hour of the 18th day of CA (in both genotypes). The observed changes may be connected with the protective effect of elevated CO_2 molar ratio against cold-induced photoinhibition observed in our study. This is quite likely, since the function of Cor14b is connected more closely with protection against cold-induced photoinhibition than the protection of thylakoids against freezing (Rapacz et al. 2008).

In conclusion, a rise in atmospheric CO_2 molar ratio during CA considerably decreases the freezing tolerance of *F. pratensis*. This effect is not related either to changes observed in PSII redox state or to photosynthetic acclimation to cold, which was in fact more effective in plants cold-acclimated at elevated CO_2 levels. The decrease in freezing tolerance may be connected with certain factors triggering changes in the expression of *CBF6* and *LOS2* genes, whereas the protective effect on photosynthetic apparatus is due to the activation of a non-photochemical mechanism of photoprotection as well as upregulation of *FpCOR14b* expression.

Author contribution B. Jurczyk ran the experiments, was responsible for data analysis and wrote the paper. M. Rapacz was responsible for the experimental design, was involved in interpretation of the results and in preparation of the manuscript. T. Krępski was involved in the molecular and physiological work and took part in preparation of the manuscript.

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