

RESEARCH PAPER

Leaf cold acclimation and freezing injury in C₃ and C₄ grasses of the Mongolian Plateau

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Abstract

The scarcity of C₄ plants in cool climates is usually attributed to their lower photosynthetic efficiency than C₃ species at low temperatures. However, a lower freezing resistance may also decrease the competitive advantage of C₄ plants by reducing canopy duration, especially in continental steppe grasslands, where a short, hot growing season is bracketed by frost events. This paper reports an experimental test of the hypothesis that cold acclimation is negligible in C₄ grasses, leading to greater frost damage than in C₃ species. The experiments exposed six C₃ and three C₄ Mongolian steppe grasses to 20 d chilling or control pre-treatments, followed by a high-light freezing event. Leaf resistance to freezing injury was independent of photosynthetic type. Three C₃ species showed constitutive freezing resistance characterized by <20% leaf mortality, associated with high photosynthetic carbon fixation and electron transport rates and low leaf osmotic potential. One freezing-sensitive C₄ species showed the expected pattern of chilling-induced damage to photosynthesis and >95% leaf mortality after the freezing event. However, three C₃ and two C₄ species displayed a cold acclimation response, showing significant decreases in osmotic potential and photosynthesis after exposure to chilling, and a 30–72% reduction of leaf freezing injury. This result suggested that down-regulation of osmotic potential may be involved in the cold acclimation process, and

demonstrated that there is no inherent barrier to the development of cold acclimation in C₄ species from this ecosystem. Cold acclimation via osmoregulation represents a previously undescribed mechanism to explain the persistence of C₄ plants in cool climates.

Key words: C₃ photosynthesis, C₄ photosynthesis, chilling, cold acclimation, electron transport rate, freezing injury, leaf mortality, Mongolian Plateau, osmotic potential.

Introduction

C₄ grasses dominate the tropical and subtropical savannas, but the diversity and abundance of these species decline with temperature at higher latitudes and elevations (Sage *et al.*, 1999). This geographic distribution pattern is matched by temporal patterns within temperate grassland communities, where C₃ plants have superior growth during cool springs, whereas C₄ grasses dominate during mid-summer (Kemp and Williams, 1980; Monson *et al.*, 1983). Mechanistic explanations of these patterns focus on the efficiency and chilling sensitivity of photosynthesis. Photosynthetic efficiency is greater in the C₄ than in the C₃ type at temperatures >20–25 °C, where photorespiration accounts for a significant fraction of assimilated carbon (Ehleringer and Björkman, 1977). However, when photorespiration is limited by lower temperatures, the energetic requirements of the C₄ cycle make it less efficient than the C₃ type. Photosynthesis and growth of

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Abbreviations: A, net leaf CO₂ assimilation rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$); A_{max}, the CO₂-saturated value of A_{sat} ($\mu\text{mol m}^{-2} \text{s}^{-1}$); A_{sat}, the light-saturated value of A ($\mu\text{mol m}^{-2} \text{s}^{-1}$); C_i, intercellular pCO₂ (Pa); ETR, photosynthetic electron transport rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$); F_v/F_m, the maximum quantum efficiency of photosystem II after overnight dark adaptation (dimensionless); NPQ, fast-relaxing non-photochemical quenching (dimensionless); PFD, photon flux density ($\mu\text{mol m}^{-2} \text{s}^{-1}$); PSII, photosystem II; qP, photochemical quenching, the fraction of F_v/F_m realized under growth conditions (dimensionless); 1–qP, excitation pressure on photosystem II, the fraction of PSII reaction centres that are closed under ambient conditions; V_{c,max}, the apparent maximum carboxylation activity of Rubisco *in vivo* ($\mu\text{mol m}^{-2} \text{s}^{-1}$); Φ_{PSII}, quantum yield of PSII, the operating efficiency of photosystem II under growth conditions (dimensionless); Ψ_{leaf}, leaf water potential (MPa); Ψ_{osmotic}, leaf osmotic potential (MPa).

C₄ species may also be directly impaired by low temperatures in the chilling range (<15 °C), especially under high light fluxes (Long, 1983), when carbon assimilation is constrained by cold-labile enzymes such as pyruvate phosphate dikinase (PPDK; Sugiyama *et al.*, 1979; Potvin *et al.*, 1986), and the low capacity of Rubisco (Pittermann and Sage, 2000). This diminishes the photochemical sink for absorbed energy, increasing 'excitation pressure' on photosystem II (PSII; Huner *et al.*, 1998), and making C₄ leaves vulnerable to photoinhibition in cold, high-light conditions (Baker *et al.*, 1989).

Freezing resistance represents an additional potential difference between C₃ and C₄ species, but has been considered less often in studies over the past 30 years (reviewed by Larcher, 2001). Recent experiments with C₃ and C₄ subspecies of the African grass *Alloteropsis semialata* showed that the C₄ subspecies suffers from freezing-induced mortality of its leaves during winter, which the C₃ subspecies avoids via cold acclimation (Ibrahim *et al.*, 2008; Osborne *et al.*, 2008). Cold acclimation in the C₃ subspecies is associated with changes in the pattern of moisture release at low water potentials, compared with the C₄ type, indicating the possible involvement of water relations in the acclimation response. These data suggested that climatic extremes such as frost events, rather than photosynthetic responses to average climate conditions, may advantage the C₃ over the C₄ subspecies in cool climatic regions (Ibrahim *et al.*, 2008; Osborne *et al.*, 2008).

Injury to leaves caused by late spring and early autumn frosts significantly limits the growing season length, and exerts a strong influence over plant production and distribution (Woodward, 1987). Late spring frosts are particularly damaging, because they occur at a time when most plants have broken dormancy, and introduce significant costs for leaf replacement. Freezing injury is caused primarily by the physical disruption of cellular structures by ice crystals, and desiccation (Pearce, 2001), resulting from the higher water potential of cellular contents than extracellular ice (Larcher, 2001). Previous studies have suggested that only a few C₄ grass species have the capability for developing cold acclimation during exposure to chilling, thereby increasing leaf resistance to subsequent freezing events (e.g. Rowley, 1976; Stair *et al.*, 1998), but the underlying mechanisms of this response have not been reported. Despite its operation in some C₄ grass species, most do not develop cold acclimation and have minimal resistance to freezing. The reasons for this remain poorly understood, although they may be linked to the high energetic costs of freezing resistance, incurred via the synthesis of compatible osmolytes, membrane lipids, cell wall components, and cryoprotective proteins (Smallwood and Bowles, 2002).

Freezing events are critical to the ecology of the Mongolian steppe during springtime. The extreme conti-

mental climate of this region leads to an annual freeze-free period in Inner Mongolia of only 90–100 d, and significant diurnal temperature fluctuations during spring (Chen, 2000). During this season, daytime temperatures often exceed 20 °C after a night-time frost event, and leaf survival of freezing may therefore bring significant advantages for photosynthesis. However, C₃ grasses in Inner Mongolia usually begin to grow in early May, whilst their C₄ competitors do not initiate growth until early June (MZL, personal observations). Since the last freezing event generally occurs during May, these observations suggest that C₄ plants may be more sensitive than C₃ species to late spring frosts.

In the present study, controlled environment experiments with Mongolian steppe grasses were used to test the hypothesis that C₄ members of this plant community are inherently less resistant to freezing than co-occurring C₃ species. Four linked questions were addressed. (i) Does chilling cause a reduction in photosynthesis and greater photoinhibition in C₄ than C₃ species? (ii) Does osmotic adjustment accompany acclimation to chilling in C₃ but not C₄ grasses? (iii) Do freezing temperatures cause higher leaf mortality in C₄ than C₃ grasses? (iv) If the plants undergo a chilling treatment prior to freezing exposure, does this reduce leaf mortality?

Materials and methods

Plant materials

The experiments used steppe grasses of the Mongolian Plateau, an area occupied by Mongolia in the north and Inner Mongolia (an autonomous region of China) in the south (elevation 900–1500 m). Seeds of nine species (Table 1) were collected in autumn 2005 from the southern steppe of Inner Mongolia (42°39'–43°56'N, 116°08'–116°55'E). All frequently co-occur in this ecosystem, allowing direct, ecologically meaningful comparisons between C₃ and C₄ species. *Lolium perenne* and *Pennisetum clandestinum* were introduced as exotic pasture species in the 1980s, but the other species are indigenous.

Seeds were germinated in sterile nutrient agar for 1 week, and 12 plants of each species transferred to pots (18 cm×13 cm, height×diameter) containing four parts high nutrient compost (Levington M3, Scotts UK Professional, Suffolk, UK) to one part sand (Play Sand, William Sinclair Horticulture Ltd, Lincoln UK) and one part Perlite (Esoteric Hydroponics, Guildford, UK). Plants were watered daily with 40% Long Ashton Solution (Hewitt, 1966), to provide a nutrient-rich, moist soil environment. The plants were grown in a controlled environment chamber (Conviron BDR 16; Conviron Controlled Environments Ltd, Winnipeg, Manitoba, Canada) at the University of Sheffield and maintained under a 14 h photoperiod with a photon flux density (PFD) of 600 μmol m⁻² s⁻¹ measured at plant height, day/night temperature of 25/15 °C, and relative humidity of 60/80% for 12 weeks prior to the experiment.

At the initiation of the experiment, six plants of each species were maintained under these conditions for a further 20 d (control pre-treatment). The other half were transferred to an identical growth chamber with a day/night temperature of 15/5 °C for 20 d (chilling pre-treatment). The pre-treatments and plants were exchanged between cabinets mid-way through the experiment to

Table 1. Species involved in current experiments, and their photosynthetic subtypes and general habitats in the steppe ecosystem of Inner Mongolia

Species	Abbreviation	Subtype	Common habitats
<i>Lolium perenne</i>	Lp	C ₃	Naturalized exotic species, grown in pastures
<i>Festuca pratense</i>	Fp	C ₃	Meadow, forest edges
<i>Bromus inermis</i>	Bi	C ₃	Meadows, riverine, along roadsides
<i>Poa pratensis</i>	Pp	C ₃	Meadow, in thin forests
<i>Leymus chinensis</i>	Lc	C ₃	Steppe, in saline meadows, sand beds of river valleys.
<i>Achnatherum splendens</i>	As	C ₃	Saline meadows in arid/semi-arid regions
<i>Eragrostis minor</i>	Em	NAD-ME	Sand and pebble riverbeds, often as a weed in oases, along roadsides, wasteland
<i>Pennisetum clandestinum</i> cv 'Whittet' ^a	Pc	NADP-ME	Naturalized exotic species, grown in pastures
<i>Cleistogenes squarrosa</i>	Cs	NAD-ME	Desert steppes, especially in sandy and loamy soils; also found scattered in rocky habitats.

^a Commonly referred to as '*P. clandestini*' in China.

minimize the confounding effects of cabinet and pre-treatment. Temperature regimes were designed to match the average daily maximum/minimum temperatures of May (chilling pre-treatment) and July (control pre-treatment) in the field. Measurements of leaf gas exchange, chlorophyll fluorescence, and leaf osmotic potential were made at the end of the 20 d pre-treatments.

Gas exchange and chlorophyll fluorescence

Photosynthesis and chlorophyll fluorescence were measured on 2–4 tillers from a single plant, and four replicate plants in each pre-treatment. All measurements were made on the youngest fully expanded leaf on each tiller which, in the chilling plants, was completely developed under the control temperature regime.

Chlorophyll fluorescence was first measured using an integrated open gas-exchange and chlorophyll fluorescence system (LI-6400-40, LI-COR Biosciences, Inc., Lincoln, NE, USA). After dark adaptation overnight, the leaves were exposed to a weak modulated beam to determine the zero fluorescence level (F_0), and then a saturating pulse to obtain the maximum fluorescence level (F_m). Variable fluorescence (F_v) is the difference between F_m and F_0 , and was used to calculate F_v/F_m , the maximum quantum efficiency of PSII.

Steady-state measurements of CO₂ and H₂O exchange were made on the same part of the leaf, at the growth temperature of either 15 °C (chilling pre-treatment) or 25 °C (control pre-treatment), a leaf-to-air vapour pressure deficit of 1.0–1.2 kPa, $p\text{CO}_2$ of 38 Pa, and a PFD of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to simulate the growth environment. The steady-state value of fluorescence (F_i) was obtained next, followed by a saturating pulse to obtain the fluorescence maximum in the light (F_m'), and these were used to estimate the quantum yield of PSII (Φ_{PSII}) and photochemical quenching (qP). The minimum fluorescence emission of light-adapted leaves (F_0') was assessed by rapidly darkening the leaf in the presence of far-red light.

PFD was then increased to 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, which ensured light saturation in these plants and, after reaching a new steady-state value, the response of net CO₂ assimilation (A_{sat}) to intercellular $p\text{CO}_2$ (C_i) was used to estimate the Rubisco-limited photosynthetic capacity (Farquhar *et al.*, 1980; von Caemmerer, 2000). These measurements differed between C₃ and C₄ species. For the C₃ plants, four A – C_i points were obtained using a range of $p\text{CO}_2$ between 38 and 5 Pa. However, for the C₄ plants, only a single measurement of CO₂-saturated photosynthesis was made at 150 Pa. Gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981).

Following these measurements of A and chlorophyll fluorescence, leaf absorbance was measured using an imaging technique (Imaging-PAM Chlorophyll Fluorometer, Heinz Walz GmbH, Eichenring, Germany), based on the difference in leaf absorbance

of red and near-infrared photons. The apparent photosynthetic electron transport rate (ETR) was then estimated using Φ_{PSII} , PFD, and absorbance (Genty *et al.*, 1989), making the simplifying assumption of equal energy partitioning between PSII and PSI. Excitation pressure on PSII was defined as the fraction of PSII reaction centres that are closed under ambient conditions ($1-qP$; Huner *et al.*, 1998), and non-photochemical quenching (NPQ) was calculated using F_m' and F_m (Bilger and Björkman, 1990).

Maximum rate of carboxylation

In order to test the hypothesis that Rubisco capacity *in vivo* ($V_{c,\text{max}}$) exerts a higher degree of control over the C₄ than the C₃ photosynthetic rate at low temperatures (Pittermann and Sage, 2000), $V_{c,\text{max}}$ was approximated by fitting a biochemical model of C₃ photosynthesis to A – C_i curves at low values of C_i , and then used to calculate the potential Rubisco-limited CO₂ assimilation rate for each C₃ species, based on the C_i at light saturation (Farquhar *et al.*, 1980; Long and Bernacchi, 2003). For the C₄ species, it was assumed that Rubisco limitation of CO₂ assimilation would be indicated by a decrease in the CO₂-saturated value of photosynthesis (A_{max}), recognizing that A_{max} may also be limited by the capacity for ribulose biphosphate (RuBP) or phosphoenolpyruvate (PEP) regeneration (von Caemmerer, 2000). To investigate the limitation of A by Rubisco in the control and chilling pre-treatments, the modelled values of Rubisco-limited A for C₃ plants, and measured A_{max} for C₄ plants, were therefore plotted against the observed light-saturated value of A (A_{sat}) at the ambient CO₂ concentration.

Leaf water potential and osmotic potential

The leaf water potential (Ψ_{leaf}) and osmotic potential (Ψ_{osmotic}) were measured using psychrometry (C-30-SF chambers, Wescor Inc., Logan, UT, USA) following the principles outlined by Koide *et al.* (1989). All of the psychrometer chambers were calibrated with a series of known NaCl solutions prior to measurements. The plants were watered on the evening before sampling to allow leaf hydration overnight, and samples were taken at dawn (prior to the controlled-environment lights coming on). Ψ_{leaf} was first measured by equilibrating cut leaves in the psychrometer chambers for 60 min in an insulated box. The chambers were then flash-frozen in liquid nitrogen for 10 min to destroy cell integrity, and measurements repeated for an estimate of Ψ_{osmotic} . Four replicates were measured for each species and pre-treatment combination.

Freezing injury

After leaf physiology measurements, all plants were exposed to a high-light freezing treatment to test for constitutive freezing

resistance (control plants) and to investigate whether cold acclimation had developed (chilled plants). The freezing treatment was designed to simulate a frost in the natural environment, and was applied using a walk-in controlled-environment cabinet (BDW 40, Conviron Controlled Environments Ltd). The treatment was applied to a single block of plants (one of each species \times pre-treatment combination = 18 plants), and replicated on six consecutive days.

The protocol for each freezing event followed Osborne *et al.* (2008). Briefly, the root system of each plant was prevented from freezing by wrapping each pot in polythene, and immersing it in a water bath held at 15 °C. The plants were put into the cabinet at 14:00 h and exposed to 15 °C in the light until 19:00 h. The lights were then turned off, and the temperature was lowered gradually overnight to reach a minimum of -5 °C before dawn. The temperature was held constant at this level for 1 h, then the lights were turned on at 08:00 h to deliver a PFD of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and both temperature and PFD were then ramped gradually upwards to reach 15 °C and 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 13:30 h.

The light regime used for this freezing treatment represented a compromise between: (i) ensuring that the PFD was high enough to allow differential photodamage in C₃ and C₄ leaves; whilst (ii) not exposing the plants to a PFD too far from the value experienced during growth. The growth PFD was constrained by the type of controlled environment cabinets available, and PFD for the freezing treatment was therefore chosen as the minimum value required to saturate photosynthesis at 15 °C in these plants, based on prior gas exchange measurements. The remainder of this paper therefore refers to the 'high-light freezing treatment', recognizing that it had the potential to cause both freezing injury and light-mediated oxidative stress to the leaves.

The extent of leaf injury within the canopy was assessed for all of the plants exposed to this high-light freezing treatment. All dead leaves were cut off before the treatment, and the plants were returned to the control growth conditions after freezing. Over the subsequent 3–7 d, the total numbers of dead (>67% necrotic) and green leaves were counted, and used to calculate leaf mortality as the number of dead leaves divided by the total number of leaves.

Statistical analysis

Data for A , $V_{c,\text{max}}$, A_{max} , F_v/F_m , Φ_{PSII} , qP , NPQ , ETR , Ψ_{leaf} and Ψ_{osmotic} were taken from four replicates. For freezing injury, the data from six replicates were transformed by taking their natural logarithms to stabilize heterogeneous variances for statistic analysis. A linear mixed effects model (SPSS 14.0.1, Chicago, IL, USA) was used to examine the main effects and interactions of photosynthetic type and pre-treatment, with species included as a random effect. Ψ_{leaf} was added as a co-variate to the model for Ψ_{osmotic} . Variables were compared by least significant difference to determine whether they were significant at the 0.05 level.

Results

Photosynthesis

Values of A in the chilling pre-treatment were significantly lower than in controls (Fig. 1; Table 2). However, there were no differences between C₃ and C₄ species, and no differential effects of the chilling pre-treatment on plants with different photosynthetic types (Fig. 1; Table 2).

In C₃ leaves from the control pre-treatment, the modelled CO₂ assimilation rate was always equal to the observed value (Fig. 2a), suggesting that Rubisco exerted significant control over light-saturated photosynthesis. The

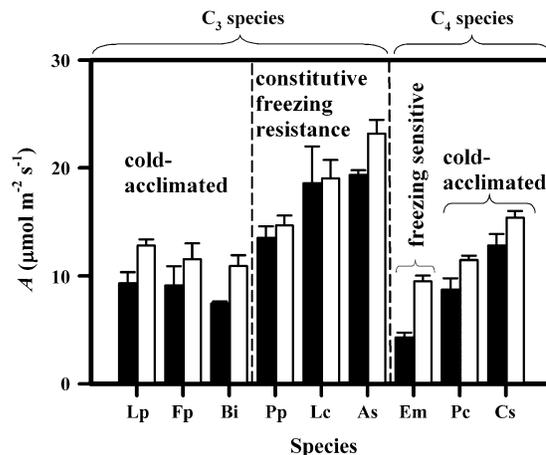


Fig. 1. Net leaf CO₂ assimilation rate (A) for C₃ and C₄ species on the 20th day of chilling (filled bars) or control (open bars) pre-treatments. Species are grouped according to hypothesized freezing resistance strategies (see Results), and abbreviations for species names are defined in Table 1. Each point represents the mean (\pm SE) of measurements on sets of leaves from four individual plants.

data indicated a similar relationship in the chilling pre-treatment (Fig. 2a), mediated by a significant decrease in $V_{c,\text{max}}$ (Table 2). These results suggest that the observed reductions in photosynthetic rates were due to temperature-related decreases in Rubisco activity or close co-ordination between the activity of this enzyme and another limiting process.

Values of A_{max} always exceeded A_{sat} in C₄ plants from the control pre-treatment (Fig. 2b), demonstrating that photosynthesis was not completely saturated with CO₂, and suggesting that Rubisco activity exceeded that of the C₄ cycle at 25 °C. However, the close correspondence of A_{max} and A_{sat} for *Eragrostis minor* and *P. clandestinum* in the chilling pre-treatment (Fig. 2b) indicated greater control of photosynthesis by the enzyme or the capacities for RuBP or PEP regeneration. Conversely, the greater value of A_{max} than A_{sat} for *Cleistogenes squarrosa* in the chilling pre-treatment suggests limitation of photosynthesis by the C₄ cycle (Fig. 2b). Overall, the chilling pre-treatment led to smaller decreases in A_{max} for the C₄ species than $V_{c,\text{max}}$ for the C₃ species, resulting in a significant interaction of photosynthetic type and pre-treatment (Table 2).

Electron transport

Photosynthetic type did not affect the excitation pressure, F_v/F_m , or ETR , but the effects of chilling pre-treatment were statistically significant for all (Fig. 3; Table 2). After 20 d growth at 15/5 °C, pre-dawn F_v/F_m of *E. minor* for the chilled plants decreased from 0.79 ± 0.006 in the control to 0.74 ± 0.002 , while there were no significant decreases in the other species (Fig. 3a). As with A , there was no significant interaction between photosynthetic type and pre-treatment (Table 2).

Table 2. Results of ANOVA (F-values and degree of freedom) testing the effects of photosynthetic type, chilling pre-treatment, 'freezing strategy', and their interactions on leaf mortality, CO_2 assimilation (A), the maximum carboxylation rate of Rubisco ($V_{c,max}$) ($\sim A_{max}$ in the C_4 species), electron transport rate (ETR), PSII excitation pressure ($1-qP$), non-photochemical quenching (NPQ), the maximum quantum yield of PSII (F_v/F_m), and leaf osmotic potential ($\Psi_{osmotic}$)

Analyses of leaf mortality were performed on \log_e -transformed values.

Variables	Photosynthetic type	Chilling pre-treatment	Chilling pre-treatment \times photosynthetic type	Freezing strategy	Chilling pre-treatment \times freezing strategy
A	1.10 (1, 64)	15.46 (1, 64)***	0.68 (1, 64)	28.88 (2, 64)***	0.57 (2, 64)
$V_{c,max}$	1.03 (1, 4)	23.68 (1, 40)***	12.35 (1, 40)***	3.80 (2, 4)	0.83 (2, 40)
F_v/F_m	2.42 (1, 5)	15.17 (1, 59)***	4.00 (1, 59)*	2.22 (2, 5)	2.88 (2, 59)
$1-qP$	0.75 (1, 5)	13.65 (1, 59)***	0.33 (1, 59)	2.57 (2, 5)	1.38 (2, 59)
NPQ	2.99 (1, 5)	9.95 (1, 59)**	1.06 (1, 59)	4.01 (2, 5)	5.24 (2, 59)**
ETR	0.59 (1, 64)	16.92(1, 64)***	0.14 (1, 64)	16.93 (2, 64)***	0.50 (2, 64)
$\Psi_{osmotic}$	1.50 (1, 5)	48.63 (1, 59)***	2.69 (1, 59)	0.67 (2, 5)	4.12 (2, 59)*
Leaf mortality	3.46 (1, 100)	3.0 (1, 100)	0.03 (1, 100)	26.21 (2, 100)***	8.51 (2,100)***

Significance level: $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4 shows the chilling-induced increase in excitation pressure and decrease in ETR relative to controls, plotted against the parallel decrease in A for each species. These plots represent the degree of coordination between photochemistry and CO_2 assimilation at low temperature. Chilling-induced decreases in CO_2 assimilation, increases in excitation pressure, and decreases in photosynthetic electron transport were typically coordinated, as indicated by the linear relationships between these variables across most of the species (Fig. 4). However, excitation pressure in *E. minor* and *Bromus inermis* showed a smaller than expected increase under the chilling pre-treatment relative to the other species (Fig. 4). A smaller than expected decrease in ETR was also observed for the cold-acclimating species *B. inermis* (Fig. 4). These results could be explained in terms of decreasing F_v/F_m for *E. minor*, but not *B. inermis*, where the data are consistent with a high alternative electron sink capacity.

Osmotic potential

Values of Ψ_{leaf} and $\Psi_{osmotic}$ for each species showed significant co-variation, and plants from the chilling pre-treatment had more negative values than controls (Fig. 5a). Relative to plants grown at 25/15 °C, the $\Psi_{osmotic}$ was significantly depressed by 4–43% in the chilling pre-treatment (Table 2; Fig. 5a), indicating osmoregulation in these leaves. There was no significant effect of photosynthetic type on $\Psi_{osmotic}$ (Table 2).

Leaf freezing injury

The high-light freezing treatment caused high leaf mortality in only one C_4 grass species, *E. minor*, killing >95% of the leaf canopy (Fig. 6). In contrast, leaves of the C_4 *P. clandestinum* and *C. squarrosa* exhibited significant resistance to this treatment, and cold acclimation

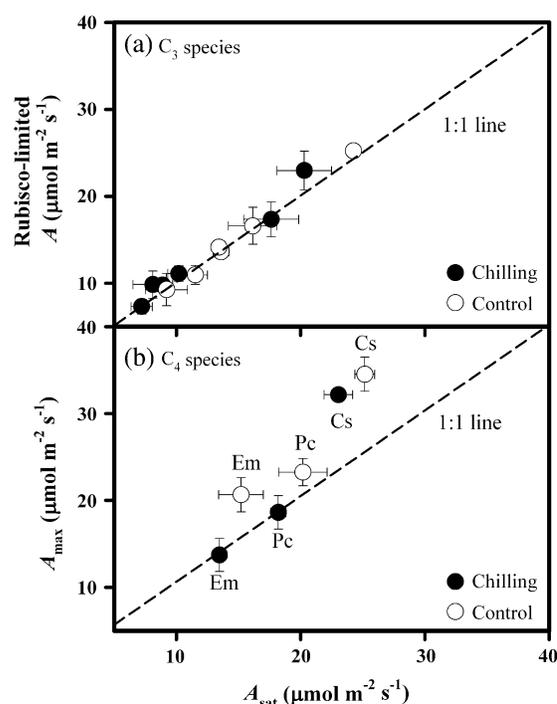


Fig. 2. Comparison of the light-saturated leaf CO_2 assimilation rate (A_{sat}) with: (a) the modelled Rubisco-limited CO_2 assimilation rate of C_3 species; and (b) the measured CO_2 -saturated rate of A (A_{max}) for C_4 species. Each point represents the mean (\pm SE) of measurements on sets of leaves from four individual plants.

responses more than halved leaf mortality in the chilling pre-treatment compared with controls (Fig. 6). Leaf mortality in these C_4 species was in the middle of the range observed for the C_3 grasses (Fig. 6), meaning that neither the effects of photosynthetic type nor its interaction with pre-treatment were significant (Table 2). If the values for *E. minor* were excluded, there was a negative correlation between leaf freezing injury and

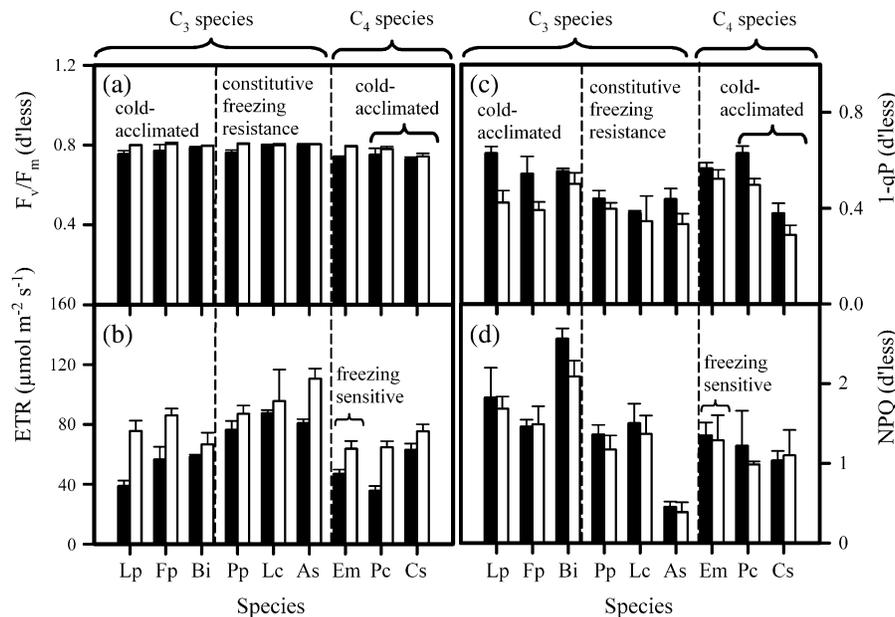


Fig. 3. Effects of the chilling pre-treatment on (a) maximum quantum yield of PSII (F_v/F_m); (b) PSII electron transport rate (ETR); (c) excitation pressure ($1-qP$); and (d) non-photochemical quenching (NPQ) for C_3 and C_4 species on the 20th day of chilling (filled bars) or control (open bars) pre-treatments. Species are grouped according to hypothesized freezing resistance strategies (see Results), and abbreviations for species names are defined in Table 1. Each point represents the mean (\pm SE) of measurements on sets of leaves from four individual plants.

Ψ_{osmotic} across both the control and chilling pre-treatments, with Ψ_{osmotic} explaining >50% of the variance in freezing mortality (Fig. 5b).

Based on a visual inspection of the data, it was hypothesized that ‘freezing strategies’ might provide a better explanation of the observed patterns than photosynthetic pathway, with species displaying either: sensitivity to the high-light freezing treatment (=‘freezing sensitivity’; Fig. 6, Em); cold acclimation (Fig. 6, Lp, Fp, Bi, Pc, and Cs); or constitutive resistance to the treatment (=‘freezing resistance’), with no cold acclimation (Fig. 6, Pp, Lc, and As). This *post hoc* hypothesis was supported by a statistical analysis incorporating ‘freezing strategy’ as a main effect, which demonstrated highly significant impacts of freezing strategy and its interaction with pre-treatment on leaf mortality, regardless of the photosynthetic type (Table 2). This result showed that the *post hoc* hypothesis was consistent with the data, and explained a significant amount of the variance.

Cold acclimation was observed in three C_3 and two C_4 species (Fig. 6). The largest effect was in the C_3 *B. inermis*, where plants in the chilling pre-treatment suffered leaf freezing mortality of only 1%, i.e. a 96% decrease compared with control plants. Mortality in the freezing treatment was 30% and 47% less in the chilling pre-treatment relative to the control of for the C_3 plants *L. perenne* and *Festuca pretense*, and 60% and 72% lower in the C_4 species *P. clandestinum* and *C. squarrosa* (Fig. 6). For the C_3 species where the data suggested constitutive resistance to the high-light freezing treatment, the chilling pre-treatment

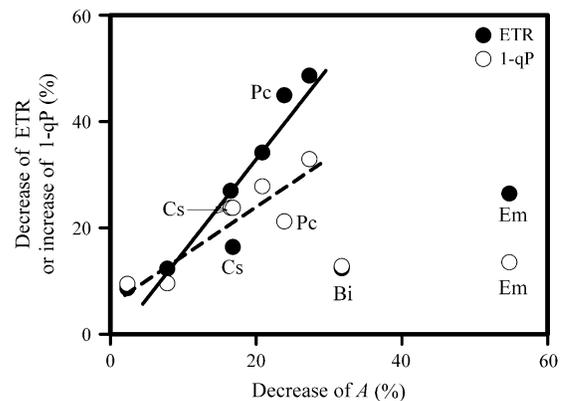


Fig. 4. Comparison of the increase in excitation pressure ($1-qP$) ($r^2=0.82$; $n=7$; $P<0.01$) and decrease of PSII electron transport rate (ETR) ($r^2=0.87$; $n=7$; $P<0.01$) with the decrease of CO_2 assimilation rate (A) for plants in the chilling compared with the control pre-treatment (mean \pm SE, $n=4$). The values for Em and Bi are excluded for both relationships.

had no effect on leaf mortality, and both the chilling and control plants displayed low leaf mortality after the freezing treatment, which varied from 5% to 22% (Fig. 6).

Freezing strategy

The factor ‘freezing strategy’ (based solely on the observed patterns of freezing injury) explained a highly significant amount of the variance in A (Fig. 1; Table 2). In other words, A measured prior to the freezing treatment emerged as a strong correlate of subsequent leaf injury patterns.

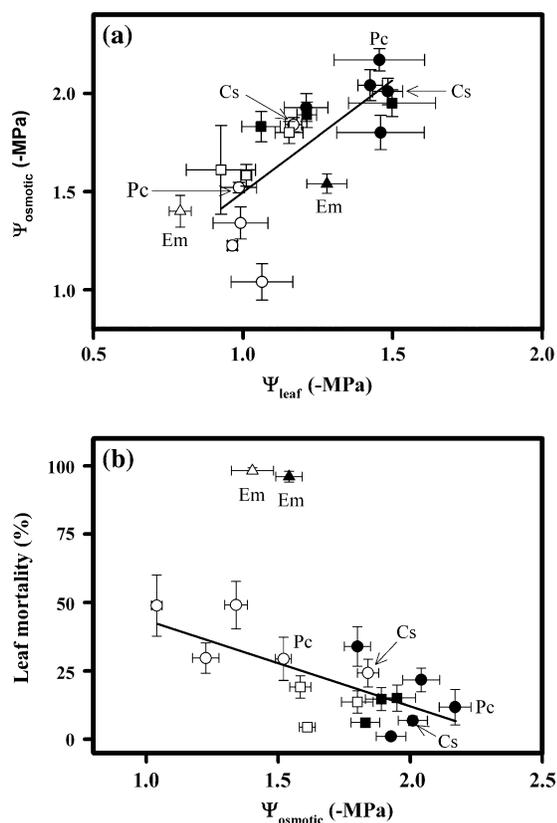


Fig. 5. Relationships of: (a) pre-dawn leaf osmotic potential (Ψ_{osmotic}) and water potential (Ψ_{leaf}) ($r^2=0.55$; $n=16$; $P < 0.01$); (b) leaf mortality and Ψ_{osmotic} for constitutive freezing resistance species (filled squares, chilling; open squares, control), cold-acclimated species (filled circles, chilling; open circles, control), and freezing-sensitive species (filled triangles, chilling; open triangles, control) ($r^2=0.53$; $n=16$; $P < 0.01$). The values for Em are excluded from both relationships. Each value represents the mean (\pm SE) of measurement from four individual plants.

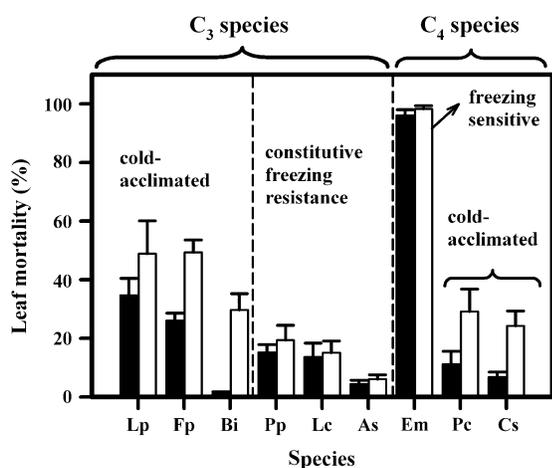


Fig. 6. Leaf mortality of C₃ and C₄ plants after 20 d of chilling (filled bars) or control (open bars) pre-treatments followed by a high-light -5°C freezing event. Species are grouped according to hypothesized freezing resistance strategies, and abbreviations for species names are defined in Table 1. Each value represents the mean (\pm SE) of measurements from six individual plants.

Values of A were the lowest in the ‘freezing-sensitive’ species, intermediate in the ‘cold-acclimated’ species, and highest in the species with ‘constitutive freezing resistance’ (Fig. 1). The freezing-sensitive species *E. minor* showed a significant, 50% depression of A in the chilling pre-treatment compared with controls ($P < 0.001$; Fig. 1). Values of A in the cold-acclimated group of plants were also depressed by the chilling pre-treatment compared with controls: by 27% in *L. perenne*, 32% in *B. inermis*, 21% in *F. pratense*, 17% in *C. squarrosa*, and 24% in *P. clandestinum* (Fig. 1). In contrast, A showed a tendency to be limited less by chilling in the species with constitutive resistance; *Achnatherum splendens* showed a slight (15%) decrease of A in the chilling pre-treatment, while no major change was observed for *L. chinensis* and *P. pratensis* (Fig. 1). However, there was no significant interaction between ‘freezing strategy’ and the chilling pre-treatment (Table 2).

‘Freezing strategy’ also explained a significant amount of the variance in ETR (Table 2). Species that displayed constitutive resistance to the high-light freezing treatment maintained the highest ETR under the chilling pre-treatment (Fig. 3b), and the lowest excitation pressure (Fig. 3c). As with A , there was no significant interaction between the chilling pre-treatment and freezing strategy (Table 2).

The magnitudes of Ψ_{osmotic} responses to the chilling pre-treatment were also consistent with the *post hoc* ‘freezing strategies’ hypothesis. The interaction of chilling pre-treatment and freezing strategy on Ψ_{osmotic} was highly significant, and independent of Ψ_{leaf} , which was included in the statistical analysis as a co-variate (Table 2). The effects of chilling were minimal for the freezing-sensitive species, decreasing the value of Ψ_{osmotic} by 9% relative to controls, and those with constitutive freezing resistance, where Ψ_{osmotic} was depressed by 4–16% (Fig. 5b). In contrast, Ψ_{osmotic} in the cold-acclimating species decreased by 8–43% in the chilling pre-treatment compared with controls (Fig. 5b).

Discussion

Freezing injury and osmoregulation

Two out of the three C₄ species in this experiment (*C. squarrosa* and *P. clandestinum*) developed significant cold acclimation under the chilling pre-treatment, manifested as a decrease in leaf osmotic potential, which reduced leaf mortality during a consequent high-light freezing event. These cold acclimation responses of C₄ grasses fell within the range observed for C₃ grass species from the same steppe ecosystem. This suggests that the photosynthetic pathway presents no inherent barrier to the survival of freezing followed by high light. This oxidative shock to leaves is typical of natural frost events, since low

night-time temperatures are commonly associated with clear sky conditions.

The development of cold acclimation in this experiment is atypical of C_4 species, which occur in tropical grass lineages (Edwards and Still, 2008), and have not usually evolved the complex set of genetic pathways required for frost protection (Beck, 2007). A mechanistic link between freezing sensitivity and the C_4 pathway is also suggested by the occurrence of cold acclimation in the C_3 but not in the C_4 subspecies of *A. semialata* (Ibrahim *et al.*, 2008; Osborne *et al.*, 2008). However, cold acclimation has been observed previously in a minority of C_4 species. A study in New Zealand showed that only two out of 11 C_4 species, *Paspalum dilatatum* and *Eragrostis curvula*, markedly increased leaf freezing resistance when the growth temperature was decreased gradually (Rowley, 1976). These two species, introduced from Africa, frequently occur in lawns or on roadsides and are drought-tolerant (Percival 1977; Robinson and Whalley 1991). Similar results were reported in winter-hardy genotypes of C_4 species in the southern USA; leaves of *Pennisetum ciliare*, *Pennisetum flaccidum*, and *Pennisetum mezianum*, which are very drought-tolerant perennials and can spread in dry years (Tellman, 2002), displayed increasing freezing resistance when they were grown in a low-temperature chamber of 10/5 °C for 4 weeks (Stair *et al.*, 1998). The results reported here demonstrate that some C_4 grass species from the Mongolian Plateau, which are drought tolerant and frequently occur in roadside or dry, rocky habitats (Table 1), also have the potential to acclimate to frost during the growing season.

Drawing upon these data, two complementary hypotheses may be put forward. First, that frost injury in the leaves of C_4 plants is the outcome of an adaptive trade-off between the energetic costs of cold acclimation and the photosynthetic benefits for a C_4 plant of leaf survival during freezing events (Sage and Percy, 2000). Since recent studies suggest that cold acclimation may be less effective or more costly in C_4 than in C_3 leaves (Sage and McKown, 2006; Osborne *et al.*, 2008), such a trade-off is most likely to favour cold acclimation in continental or high elevation environments experiencing large diurnal temperature ranges. Here, freezing temperatures at dawn are succeeded by daytime temperatures of >20–25 °C. The diurnal temperature range experienced by C_4 plants at high altitudes is further increased by the absorption of direct solar radiation, which may raise leaf temperature by 10–20 °C over that of the air (Sage and Sage, 2002). This adaptive interpretation is consistent with the evidence reported here and recent reports of freezing injury thresholds below –10 °C in C_4 grasses from high elevations in the Venezuelan Andes (Márquez *et al.*, 2006) and Californian White Mountains (Sage and Sage, 2002).

A second hypothesis, involving a linkage between frost and drought sensitivity, is also suggested by the results of

previous studies and the data reported here. Frost injury in freezing-sensitive species is induced mainly through extracellular ice formation, which dehydrates mesophyll tissues by extracting symplastic water, but may directly disrupt the bundle sheath cells of C_4 plants (Ashworth and Pearce, 2002). However, by altering their biochemical composition under chilling temperatures, cold-acclimated plants can accumulate compatible osmolytes in cells, which cause a decline in water potential and increase resistance to dehydration (Goldstein *et al.*, 1985; Aniško and Lindstrom, 1996), also improving their resistance to freezing (Smallwood and Bowles, 2002). The data reported here are consistent with this mechanism, showing that values of Ψ_{leaf} and Ψ_{osmotic} decreased in plants exposed to the chilling pre-treatment, indicating significant osmoregulation, especially for those in the cold-acclimating group (Fig. 5a, b). This was correlated with freezing resistance strategies that were defined via an independent method.

Leaf survival of high water deficits in drought environments is facilitated via similar desiccation protection mechanisms (Turner and Begg, 1981), which can directly improve the cold acclimation of species via biochemical changes (Mantyla *et al.*, 1995; Kacperska, 2004). Based on these results and the habitat requirements of individual species, it is hypothesized that cold acclimation in the C_4 steppe grass species from the Mongolian Plateau may be associated with drought adaptations. This inferred linkage is consistent with the observed cold acclimation responses in the C_4 grasses *C. squarrosa* and *P. clandestinum*, which occur in open grassland and desert steppe habitats (Table 1; Liang *et al.*, 2002), and the freezing sensitivity and failure to cold-acclimate in *E. minor*. The latter C_4 grass is confined to moist habitats of the Mongolian Plateau, including riverbeds and disturbed areas (Table 1; Ma, 1998), suggesting an obligate requirement for wet growth conditions. Furthermore, the three C_3 species showing the greatest constitutive freezing resistance are also those occupying the driest habitats in the steppe ecosystem (Table 1): *L. chinensis* is broadly distributed in the dry steppe regions of East Asia (Zhu, 2004), *P. pratensis* naturally occupies the open and high land, and *A. splendens* occurs naturally on saline soils where it survives low soil water potentials (Ma 1998). Clearly, the small sample of species used here precludes generalizations, but these observations suggest that the potential association of drought and frost resistance in steppe grasses could be a useful avenue for future research.

Photosynthesis under chilling conditions

At high temperatures, C_4 species have the potential to achieve a higher photosynthetic rate than their C_3 counterparts (Percy and Ehleringer, 1984). However, under the environmental conditions applied in this experiment,

constitutive freezing-tolerant C₃ species showed the highest rates of photosynthesis, followed by the cold-acclimating C₄ and C₃ species. This may be because of the PFD used, which limited rates of C₄ photosynthesis under the growth conditions. Despite the relatively low PFD (600 $\mu\text{mol m}^{-2} \text{s}^{-1}$), the chilling pre-treatment still caused a 50% decrease in the CO₂ assimilation rate in the freezing-sensitive C₄ species *E. minor* (Fig. 1), a decline in the pre-dawn F_v/F_m (Fig. 3a), and a small increase of excitation pressure (Fig. 3c), despite increased NPQ (Fig. 3d). Decreases of F_v/F_m and PSII photochemical efficiency following chilling have been noted previously for C₄ species (Baker *et al.*, 1983; Demmig *et al.*, 1987). The results for *E. minor* suggest the operation of slow-relaxing (>10 h) quenching mechanisms, indicative of photodamage, correlated with a greater excess energy flux through PSII (Fig. 3c) and high leaf mortality after the subsequent high-light freezing event (Fig. 6).

The inference of photodamage in *E. minor* is consistent with previous experiments applying similar PFD conditions to the tropical C₄ plants *Zea mays* and *Sorghum bicolor* (Taylor and Rowley, 1971). Interpretation of Fig. 2 using a biochemical model of C₄ photosynthesis (von Caemmerer, 2000) suggests a high degree of control by the capacities of Rubisco or components of the PEP regeneration pathway such as PPKK. Both have been implicated previously in the photoinhibition of C₄ photosynthesis (Long, 1983; Sage and Kubien, 2007), and represent key limitations that must be overcome in cold-tolerant C₄ species by dynamic quenching mechanisms (Ensminger *et al.*, 2006; Sage and Kubien, 2007) or repair processes mediated by protein expression (Wang *et al.*, 2007).

Chilling-induced photodamage did not occur in all of the C₄ species in the experiment. In the cold-acclimating species, the chilling pre-treatment lowered photosynthetic rates equally in C₄ and C₃ species (Fig. 1), and this was associated with lower, rather than higher, leaf mortality after the subsequent freezing event (Fig. 6). Photodamage was avoided in these species by coordinated decreases in the PSII electron flux and CO₂ assimilation, despite an increase in the excitation pressure on PSII (Fig. 3c). This acclimation response is common in C₃ species (Sage and Kubien, 2007), and suggests the operation of effective non-photochemical quenching mechanisms together with protective down-regulation of PSII efficiency in the cold-tolerant C₄ species studied here (Fig. 3d).

Conclusions

Resistance to a high-light freezing treatment was independent of photosynthetic pathway in the grasses of the Mongolian Plateau studied in this experiment, but differed among species according to ecological traits. The de-

pression of osmotic potential was associated with the acquisition of cold acclimation in some C₃ and C₄ species, and represents a previously undescribed mechanism to explain the persistence of C₄ plants in cool climate habitats. The one freezing-sensitive C₄ species in this experiment did not adjust osmotic potential, and was also susceptible to chilling-induced photodamage. Based on the habitat requirements of individual species in the natural ecosystem, it is hypothesized that freezing resistance in Mongolian steppe grasses may be more closely allied to the capacity for drought resistance than the photosynthetic pathway.

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