Variation in chilling tolerance for photosynthesis and leaf extension growth among genotypes related to the C₄ grass *Miscanthus ×giganteus*

Katarzyna Glowacka¹,²,*, Shivani Adhikari¹, Junhua Peng³, Justin Gifford¹, John A. Juvik¹, Stephen P. Long¹ and Erik J. Sacks¹

¹ Institute for Genomic Biology, University of Illinois, 1206 W. Gregory Dr., Urbana, IL 61801, USA
² Institute of Plant Genetics, Polish Academy of Sciences, ul. Strzeszyńska 34, 60-479 Poznań, Poland
³ Department of Soil and Crop Science, Colorado State University Fort Collins, CO 80523-1170, USA

* To whom correspondence should be addressed. E-mail: uszepti@tlen.pl

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**Abstract**

The goal of this study was to identify cold-tolerant genotypes within two species of *Miscanthus* related to the exceptionally chilling-tolerant C₄ biomass crop accession: *M. ×giganteus* ‘Illinois’ (Mxg) as well as in other Mxg genotypes. The ratio of leaf elongation at 10 °C/5 °C to that at 25 °C/25 °C was used to identify initially the 13 most promising *Miscanthus* genotypes out of 51 studied. Net leaf CO₂ uptake (A_sat) and the maximum operating efficiency of photosystem II (Φ_PSII) were measured in warm conditions (25 °C/20 °C), and then during and following a chilling treatment of 10 °C/5 °C for 11 d. Accessions of *M. sacchariflorus* (Msa) showed the smallest decline in leaf elongation on transfer to chilling conditions and did not differ significantly from Mxg, indicating greater chilling tolerance than diploid *M. sinensis* (Msi). Msa also showed the smallest reductions in A_sat and Φ_PSII, and greater chilling-tolerant photosynthesis than Msi, and three other forms of Mxg, including new triploid accessions and a hexaploid Mxg ‘Illinois’. Tetraploid Msa ‘PF30153’ collected in Gifu Prefecture in Honshu, Japan did not differ significantly from Mxg ‘Illinois’ in leaf elongation and photosynthesis at low temperature, but was significantly superior to all other forms of Mxg tested. The results suggested that the exceptional chilling tolerance of Mxg ‘Illinois’ cannot be explained simply by the hybrid vigour of this intraspecific allotriploid. Selection of chilling-tolerant accessions from both of Mxg’s parental species, Msi and Msa, would be advisable for breeding new highly chilling-tolerant Mxg genotypes.

**Key words:** Chilling, cold tolerance, electron transport, *Miscanthus ×giganteus, Miscanthus sacchariflorus, Miscanthus sinensis*, photosynthesis, plant breeding, *Saccharum officinarum*.

**Introduction**

During the last two decades, *Miscanthus ×giganteus* (Mxg) has become an important biomass crop for European and US bioenergy initiatives (*Scurlock, 1999; Clifton-Brown et al., 2004; Heaton et al., 2008; Somerville et al., 2010*). By combining perenniality, rhizome function, and cold-tolerant C₄ photosynthesis, Mxg is highly light, nitrogen, and water use efficient, which contributes to its exceptionally high biomass productivity even in cool temperate climates (*Lewandowski et al., 2000; Heaton et al., 2008; Long and Spence, 2013*). While many C₄ plants are now known to survive cold conditions, Mxg appears exceptional in its ability to achieve the high productivity of C₄ photosynthesis in cold climates. For example, in southeastern England, Mxg shoot productivity exceeded the highest values achieved by intensively managed C₃ crops (*Beale and Long, 1995*). A comparative field study with a high-yielding maize cultivar (Illinois, USA) revealed
that perenniality together with cold tolerance gave Mxg 59% more productivity than maize (Dohleman and Long, 2009). This was largely explained by the ability of Mxg to form photosynthetically competent leaves earlier in the year and maintain them later in the year. This allowed Mxg to capture 60% more solar energy than maize, and yet convert this into biomass with a similar efficiency, despite colder weather at the beginning and end of the growing season (Dohleman and Long, 2009).

A single genotype of Mxg collected from southern Honshu, Japan was initially introduced to Denmark in the 1930s and was then subsequently distributed throughout Europe and North America (Greef and Deuter, 1993; Hodkinson et al., 2002; Glowacka et al., 2014). This cultivar is a triploid sterile hybrid between diploid \( M. \text{sinensis} \) (Msi) and tetraploid \( M. \text{sacchariflorus} \) (Msa) (Greef and Deuter, 1993; Linde-Laursen, 1993; Hodkinson et al., 2002). Hodkinson and Renvoize (2001) defined the nothospecies, \( M. \text{xgigantues} \) Greef & Deu. ex Hodkinson & Renvoize, as a hybrid between Msi and Msa, a designation that could include an infinite number of genotypes. However, almost all trials and production fields of Mxg appear to be from the single clone first introduced to Denmark, though limited information about new natural Mxg accessions and those produced by controlled crosses is available (Rauh et al., 2014). This clone has been shown to avoid this particular chilling tolerance from selected Mxg accessions and those produced by controlled crosses is expected to come from polyploidy, since this could amplify genetic variation in relatives of Mxg that are investigated here. (i) What variation in chilling tolerance can be found in different genotypes of Mxg and in Mxg’s parental species? (ii) Can chilling tolerance equal to or better than that of the parental Mxg species be found? (iii) From which of the parental Mxg species is chilling tolerance most probably inherited? (iv) Is there an association between increasing ploidy level and chilling tolerance? To address these questions, low temperature leaf elongation of Mxg ‘Illinois’ together with 50 additional \( M. \text{xgigantues} \) genotypes belonging to seven species-ploidy groups were determined, and a subset were further evaluated for photosynthetic capacity during and after chilling conditions.

Materials and methods

Plant material

A total of 51 \( M. \text{oligostachyus} \) accessions were studied (Table 1): three diploid Msa, 10 tetraploid Msa, 27 diploid Msi, five triploid Mxg, three colchicine-induced polyploids (5x–6x) of Mxg ‘Illinois’ (Chae et al., 2013), one diploid Mxg, one \( M. \text{oligostachyus} \) ‘Purpurascens’, and a \( M. \text{xgigantues} \) accession of unknown provenance named \( M. \text{oligostachyus} \) sp. ‘PF1-7’. Most of the Msi genotypes were cultivars commercially available in the USA, and chosen based on spring and/or autumn hardiness, and other features related to cold tolerance, namely early emergence, late flowering, and remaining green after autumn frost (C. Kaiser and E. Sacks, unpublished data). Most of
Table 1. Miscanthus, sugarcane and maize accessions included in the leaf elongation study

| Name | Accession identifier | Source |
|------|----------------------|--------|
| **M. sacchariflorus (2x)** | | |
| ‘PMS-075’ | PMS-075 | J. Peng, Wuhan Botanical Garden, China |
| ‘Robustus-Bluemel’ | UI10-00009 | Kurt Bluemel, INC nursery, MD, USA |
| var. lutarioriparius ‘PF30022’ | UI11-00031 | New Energy Farms, Canada |
| **M. sacchariflorus (4x)** | | |
| ‘Bluemel Giganteus’ | UI10-00117 | Kurt Bluemel, INC nursery, MD, USA |
| ‘EMI-5’ | MATEREC11 | U. Jørgensen, Aarhus Univ., Denmark→ M. Deuter, Tinplant, Germany |
| ‘Gotemba Gold’ | UI11-00005 | Glasshouse Works nursery, OH, USA |
| ‘PF30150’ | UI11-00032 | Honshu, Japan by New Energy Farms, Canada |
| ‘PF30151’ | UI11-00033 | Honshu, Japan by New Energy Farms, Canada |
| ‘PF30153’ | UI11-00035 | Honshu, Japan by New Energy Farms, Canada |
| ‘PF30154’ | UI11-00036 | Honshu, Japan by New Energy Farms, Canada |
| ‘PF30155’ | UI11-00037 | Honshu, Japan by New Energy Farms, Canada |
| ‘PF30156’ | UI11-00038 | Honshu, Japan by New Energy Farms, Canada |
| ‘PF30157’ | UI11-00039 | Honshu, Japan by New Energy Farms, Canada |
| **M. sinensis (2x)** | | |
| ‘Autumn Light’ | UI10-00025 | Emerald Coast Growers nursery, FL, USA |
| ‘Blondo’ | UI11-00017 | Kurt Bluemel, INC nursery, MD, USA |
| ‘Burgander’ | UI10-00035 | Walla Walla Nursery Co., WA, USA |
| ‘Dixieland’ | UI10-00036 | Emerald Coast Growers nursery, FL, USA |
| ‘Emerald Shadow’ | UI10-00038 | Kurt Bluemel, INC nursery, MD, USA |
| ‘Emmanuel LePage’ | UI10-00039 | Kurt Bluemel, INC nursery, MD, USA |
| ‘Ferner Osten’ | UI10-00040 | Kurt Bluemel, INC nursery, MD, USA |
| ‘Gracillimus’ | UI10-00048 | Emerald Coast Growers nursery, FL, USA |
| ‘Huron Sentinel’ | UI10-00057 | Paradise Garden nursery, TN, USA |
| ‘Huron Sunrise’ | UI10-00058 | Paradise Garden nursery, TN, USA |
| ‘Kleine Silberspinne’ | UI12-00008 | Earthly Pursuits nursery, MD, USA |
| ‘Morning Light’ | UI10-00071 | Walla Walla Nursery Co, WA, USA |
| ‘Nippon’ | UI10-00074 | Emerald Coast Growers nursery, FL, USA |
| ‘November Sunset’ | UI10-00075 | Kurt Bluemel, INC nursery, MD, USA |
| ‘Roland’ | UI10-00080 | Earthly Pursuits nursery, MD, USA |
| ‘Sarabande’ | UI11-00010 | Emerald Coast Growers nursery, FL, USA |
| ‘Silberleder’ | UI12-00006 | Earthly Pursuits nursery, MD, USA |
| ‘Teshio’ | UI11-00003 | T. Yamada, Hokkaido Univ., Japan |
| ‘Tripple Brook Farm’ | UI10-00011 | Tripple Brook Farm nursery, MA, USA |
| ‘Undine’ | UI12-00009 | Earthly Pursuits nursery, MD, USA |
| ‘Uryu’ | UI11-00004 | T. Yamada, Hokkaido Univ., Japan |
| var. condensatus ‘Cabaret’ | UI10-00012 | Emerald Coast Growers nursery, FL, USA |
| var. condensatus ‘Cosmopolitan’ | UI10-00015 | Emerald Coast Growers nursery, FL, USA |
| var. purpurascens | UI10-00019 | Kurt Bluemel, INC nursery, MD, USA |
| var. transmorrisonensis | UI10-00106 | Walla Walla Nursery Co, WA, USA |
| ‘Variegatus’ | UI10-00097 | Emerald Coast Growers nursery, FL, USA |
| ‘Zebrinus’ | UI11-00016 | Kurt Bluemel, INC nursery, MD, USA |
| **M. × giganteus (3x)** | | |
| ‘Illinois’ | UI10-00107 | T. Voigt, UI, USA→ Chicago Botanic Garden, USA |
| ‘10UI-032.001’ | 10UI-032.001 | J. Gifford, J Juvik & E. Sacks, UI, USA |
| ‘10UI-032.002’ | 10UI-032.002 | J. Gifford, J Juvik & E. Sacks, UI, USA |
| ‘10UI-032.003’ | 10UI-032.003 | J. Gifford, J Juvik & E. Sacks, UI, USA |
| ‘10UI-032.004’ | 10UI-032.004 | J. Gifford, J Juvik & E. Sacks, UI, USA |
| **M. × giganteus colchicine-induced polyploids** | | |
| ‘Illinois-5x:02 (Mxg2-2)’ | UI10-00110 | W. Chae & J. Juvik, UI, USA |
| ‘Illinois-6x:01 (Mxg2-1)’ | UI10-00109 | W. Chae & J. Juvik, UI, USA |
| ‘Illinois-6x:07 (Mxg2-7)’ | UI10-00113 | W. Chae & J. Juvik, UI, USA |
| **M. × giganteus (2x)** | | |
| var. purpurascens ‘Herkules’ | UI10-00018 | Kurt Bluemel, INC nursery, MD, USA |
| **Other Miscanthus accessions** | | |
| Miscanthus sp. ‘PF1-7’ | UI11-00046 | New Energy Farms, Canada |
the tetraploid Msa studied originated from Gifu Prefecture, Honshu, Japan. Among the triploid Mxg accessions were the ‘Illinois’ clone, and four new tetraploid Mxg genotypes obtained from a cross [Msa (4x) ‘Bluemel Giganteus’×Msi (2x) var. *condensatus* ‘Cabaret’] made at the University of Illinois (Chae et al., 2014). Two sugarcane (*Saccharum officinarum*) cultivars and two *Zea mays* lines were included as controls.

Clonal divisions of *Miscanthus* and sugarcane accessions as well as *Z. mays* seedlings were grown in square pots of 1 litre volume (mini-treepot # MT38; Stuewe & Sons, Tangent, OR, USA) containing a peat-, bark-, and perlite-based growing medium (Metro-Mix 900; Sun Gro Horticulture, Agawam, MA, USA). After planting or sowing, an all-purpose slow release fertilizer was added following the manufacturer’s instructions (Osmocote Classic, 8–9 mo 13-13-13; Sun Gro Horticulture, Agawam, MA, USA). Prior to transfer to controlled-environment cabinets, plants were grown in a controlled environment greenhouse at ~25 °C. Plants were transferred to controlled-environment cabinets, three out of six plants from each accession were included as controls.

| Name                        | Accession identifier | Source                                |
|-----------------------------|----------------------|---------------------------------------|
| *M. oligostachyus* ‘Purpurascens’ | U110-00005           | Walla Walla Nursery Co, WA, USA       |
| Control sugarcane and maize lines |                       |                                       |
| *S. officinarum* ‘Louisiana Purple’ | P1495639             | USDA-NPGS, USA                        |
| *Saccharum* sp. ‘L79-1002’       | P651501              | USDA-NPGS, USA                        |
| *Z. mays* hybrid ‘DK065-44VT3’   | DK065                | Dekalb Brand Corn, USA                |
| *Z. mays* inbred line ‘FR1064’   | FR1064               | S. Moose, UI, USA— Illinois Foundation Seeds, IL, USA |

Leaf elongation

All 55 accessions (51 *Miscanthus*, two sugarcane, and two maize) were evaluated for leaf elongation under chilling temperatures, enabling those with the greatest and least chilling tolerance to be identified efficiently for further study. Plants were transferred to controlled-environment chambers (BioChambers GRC-40; Conviron, Winnipeg, Manitoba, Canada) with a 12 h day/12 h night cycle under 1000 μmol photons m⁻² s⁻¹, a constant temperature of 25 °C, and relative humidity of 50%. After 10 d acclimation to the controlled-environment chamber, three out of six plants from each accession were selected at random, and transferred to a second identical controlled-environment chamber, but with a 10 °C/5 °C day/night temperature. All other environmental conditions were unchanged. To avoid confounding genotype with any undetected variation, the position of each plant within the chamber was changed following a randomized design every second day. Data were collected on the uppermost leaf on the stem in which the ligule had not emerged from the sheath of the preceding leaf (i.e. growing leaves). The distance from the top edge of the pot to the tip of the chosen leaf was measured using a ruler. For each plant, leaf growth was calculated by subtracting the measurement on day 0 of the treatment from the measurements collected on subsequent days. In the 10 °C/5 °C treatment, developing leaves were measured every second day, and every day in the control.

Gas exchange and chlorophyll fluorescence

The high-throughput leaf elongation experiment served as a preliminary screen to select the most cold-tolerant genotypes for a more detailed but lower throughput assessment of photosynthetic response to chilling. A subset of 13 accessions, chosen from those which showed the greatest percentage of leaf elongation retained in chilling temperature relative to warm temperature and three negative controls (16 accessions in total; Fig. 1, grey- and yellow-marked accessions), was analysed for photosynthetic capacity under chilling conditions. Four plants of each accession were grown at 25 °C/20 °C (warm) in each of two controlled-environment chambers (Conviron PGR15; Controlled Environments) for 10 d, after which the temperature in both chambers was lowered to 10 °C/5 °C (chilling) day/night for 11 d, and then returned to 25 °C/20 °C. This period was chosen to mimic the type of chilling that might develop during spring after leaf emergence or expanded leaves in the autumn. Loss of efficiency during these periods greatly limits realization of the superior efficiency of C₃ relative to C₄ photosynthesis in temperate climates (Long and Spence, 2013). The position of plants within the chambers was changed, as described for the leaf elongation study. In both chambers a 14 h day/10 h night cycle with 1000 μmol photons m⁻² s⁻¹ and relative humidity of 65% was maintained. Leaf photosynthetic gas exchange and modulated chlorophyll fluorescence were measured *in situ* on the most recent fully expanded attached leaves, as judged by ligule emergence, with an open gas exchange system incorporating differential infrared CO₂ and water vapour analysers (LI-6400; LI-COR, Lincoln, NE, USA). In this system, the leaf was enclosed in a controlled-environment cuvette which tracked the light, temperature, and humidity in the controlled-environment chamber. Chlorophyll pulse amplitude modulated fluorescence was measured simultaneously with a fluorometer incorporated into the cuvette lid (LI-6400–40; LI-COR, Inc.). Measurements were conducted under ambient air (21% O₂) at 390 μmol mol⁻¹ CO₂ concentration, 1000 μmol m⁻² s⁻¹ photon flux, and 65% relative humidity. Leaf temperature was maintained at the growth temperature for each accession and treatment. Actinic light was supplied by light-emitting diodes (90% red light, 630 nm; 10% blue light, 470 nm). To maximize the fluorescence emissions, the fluorometer parameters (e.g. flash intensity and duration) were adjusted and the multiphase protocol was used. These measurements were made in warm conditions (25 °C) just prior to the chilling treatment, immediately after the temperature was reduced to 10 °C (day 0), during each of the 11 d at 10 °C (except days 6, 8, and 10 when the measurements were not taken), and finally 1 d after transferring the plants back to 25 °C (12th day of the experiment—recovery). All measurements were taken during the day on light-adapted leaves until steady state (20–50 min). On day 0, in order to obtain measurements for each plant immediately after reducing the temperature to 10 °C, each pot was individually transferred from the 25 °C growing chamber to a growing chamber at 10 °C; after the leaves reached steady-state readings, gas exchange parameter were recorded. For each accession, four replicate plants were measured for each treatment. From these measurements, leaf net CO₂ uptake per unit leaf area (A), stomatal conductance to water vapour (gₛ), intercellular CO₂ concentration (cᵢ), the quantum yield of photosystem II (Φₚₛᵢ), and rate of whole chain electron transport (J) were calculated, as described previously (Bernacchi et al., 2003).

Data analysis

All statistical analyses were performed with SAS v. 9.3 (SAS Institute, Cary, NC, USA). Leaf elongation was regressed against time to determine the best-fitting equation to describe the rate of
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leaf growth by using linear regression analysis in PROC REG. The significance of genotype and treatment on all measures (leaf elongation rate, $A_g$, $c/c_a$, and $\Phi_{PSII}$) was determined by two-way analysis of variance (ANOVA) in PROC GLM; genotype and treatment were fixed effects. Where a significant effect of genotype or species-ploidy group was detected, Dunnett’s test was used to determine which genotypes differed significantly from the Mxg ‘Illinois’. If significant interactions between genotype and treatment were observed, then treatment effects within each genotype were separately evaluated for significance by Tukey’s test.

**Results**

**Genotypic variation in leaf elongation**

Mxg (3x) ‘Illinois’, the reference chilling-tolerant accession, showed a 9-fold decrease in leaf elongation rate from 22.9 mm d$^{-1}$ at 25 °C to 2.5 mm d$^{-1}$ at 10 °C/5 °C (Fig. 1A, B). Of the 50 Miscanthus genotypes analysed, two groups were identified, one with 17 accessions that were not significantly different from Mxg (3x) ‘Illinois’ and the other with 32 accessions that showed a significantly greater reduction in leaf extension growth ($P<0.1$; Fig. 1B). Notably, one accession, Msa var. lutarioriparius ‘PF30154’, significantly exceeded the leaf elongation of Mxg (3x) ‘Illinois’ by 1.5-fold when grown at chilling temperature (Fig. 1B). There was considerable variation between Miscanthus accessions, with Msa var. lutarioriparius showing 43-fold greater leaf extension growth at 10 °C/5 °C than the worst performing accession, diploid Msi ‘Uryu’ (Fig. 1B). At 10 °C/5 °C, all 10 tested tetraploid Msa and two hexaploid Mxg were not significantly different from Mxg (3x) ‘Illinois’ (Fig. 1C). Two of the three diploid Msa were also not significantly different from Mxg (3x) ‘Illinois’. In contrast to the Msa accessions, approximately two-thirds

![Fig. 1. (A) Rate of leaf elongation at warm temperature and (B) chilling temperature, and (C) percentage of leaf elongation retained in chilling temperature relative to warm temperature for 51 Miscanthus accessions and four control sugarcane and maize lines. In chilling, developing leaves were measured during 14 d every other day, while for warm conditions data were collected during 7 d every day. The growing conditions were 10 °C/5 °C (chilling) or 25 °C/25 °C (warm) day/night and a 12 h day/12 h night cycle under 1000 μmol photons m$^{-2}$ s$^{-1}$. In all panels, accessions are ordered according to percentage of leaf elongation retained in chilling temperature relative to warm temperature (from the highest to the lowest, C). For each treatment stage, open symbols indicate no significant differences and filled symbols indicate significantly faster (black) or slower (red) elongation in comparison with M. ×giganteus (Mxg) (3x) ‘Illinois’ (yellow frame) based on Dunnett’s test ($P<0.1$). Rates of leaf elongation for Mxg (3x) ‘Illinois’ were: (A) 22.87 (mm d$^{-1}$); (B) 2.53 (mm d$^{-1}$); (C) 11.08 (%). Data are mean ±SE ($n$=3). Grey- and yellow-highlighted genotypes were selected for a subsequent experiment to study chilling-tolerant photosynthesis. F1, the first generation of Msa×Msi hybrids; Mol, M. oligostachyus; Msa, M. sacchariflorus; Msi, M. sinensis; Mxg, M. ×giganteus; cond., condensatus; P1 (high), parent 1 of interspecific Msa×Msi hybrids (Msa with high chilling tolerance); P2 (low), parent 2 of interspecific Msa×Msi hybrids (Msi with low chilling tolerance).
of the Msi accessions had significantly lower rates of leaf elongation at chilling temperatures than Mxg (3x) ‘Illinois’ (Figs 1, 2; Supplementary Fig. S1 available at JXB online).

Among four new triploid Mxg obtained from the cross, Msa (4x) ‘Bluemel Giganteus’×Msi (2x) var. condensatus ‘Cabaret’, three of the progeny and their Msa parent were not significantly different from Mxg (3x) ‘Illinois’ for percentage of leaf elongation retained in chilling temperature relative to warm temperature (Fig 1C). However, the Msi parent (P2 low; Fig 1), was among the least cold-tolerant accessions studied (similar to sugarcane and maize) as assessed by leaf elongation.

Genotypic variation in photosynthetic gas exchange and chlorophyll fluorescence

Tetraploid Msa ‘PF30153’ did not differ significantly from the cold-tolerant control, Mxg (3x) ‘Illinois’, for \( A_{\text{sat}} \) and \( \Phi_{\text{PSII}} \) in both warm and cool conditions (Fig. 3). For Mxg (3x) ‘Illinois’ grown at 25°C/20°C, \( A_{\text{sat}} \) was 18.3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and \( \Phi_{\text{PSII}} \) 0.212, while for Msa ‘PF30153’ the respective values were slightly higher at 20.52 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 0.231. After 11 d at 10°C/5°C, \( A_{\text{sat}} \) was 7.19 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and \( \Phi_{\text{PSII}} \) 0.072 for Mxg (3x) ‘Illinois’, whereas for Msa ‘PF30153’ the respective values were 4.72 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 0.066, which were not significantly different from those of Mxg (3x) ‘Illinois’. In contrast, the accessions of sugarcane and Z. mays showed a 94–99.5% reduction in \( A_{\text{sat}} \) and 88–91% reduction in \( \Phi_{\text{PSII}} \) relative to Mxg (3x) ‘Illinois’ after 11 d at 10°C/5°C.

Three main patterns of loss of photosynthetic capacity could be observed across the germplasm studied, over the 11 d following transfer to 10°C/5°C. These may be characterized as nearly full acclimatization, partial acclimatization, and failure to acclimatize (Fig. 4; Supplementary Fig. S3 at JXB online). For Mxg (3x) ‘Illinois’, \( A_{\text{sat}} \) and \( \Phi_{\text{PSII}} \) declined for the first 2–3 d at 10°C/5°C, followed by a recovery over the following days to stabilize at a level of 91–92% of the rates on initial transfer to 10°C/5°C (Fig 4A, C). Msa (4x) ‘PF30153’ was the only clone studied which showed a similar pattern of response and was not significantly different from Mxg (3x) ‘Illinois’ with respect to \( A_{\text{sat}} \) at the end of the chilling treatment. Both Mxg (3x) ‘Illinois’ and Msa ‘PF30153’ recovered fully 1 d after transfer back to 25°C, showing ~87% and 84%, respectively, of the pre-chilling \( A_{\text{sat}} \) (Supplementary Fig. S3A). Additionally, two other tetraploid Msa, ‘PF30151’ and ‘PF30154’, did not differ significantly from Mxg (3x) ‘Illinois’ with respect to relative \( A_{\text{sat}} \) at the end of the chilling treatment (\( A_{\text{sat}} \) calculated as a percentage of initial values at day 0; Fig 4). Compared with Mxg (3x) ‘Illinois’, all other Miscanthus clones showed a significantly lower \( A_{\text{sat}} \) over the 11 d of chilling (Figs 3, 4). Although seven other genotypes, six Msa and Msi ‘Nipon’, showed a significantly lower \( A_{\text{sat}} \) over the 11 d at 10°C/5°C compared with Mxg, on return to warm conditions they recovered an \( A_{\text{sat}} \) after 1 d that was not significantly lower than the value for Mxg (3x) ‘Illinois’ (Fig. 3A). All other lines showed a significantly greater loss of photosynthetic capacity during the 11 d at 10°C/5°C and on return to warm conditions, although none showed as large a loss as the Z. mays and two Saccharum spp. lines (Figs 3A, 4; Supplementary Fig. S2A). Several lines showed an increase in stomatal conductance on initial transfer to 10°C/5°C (Fig. 3; Supplementary Fig. S2). All lines showed an increase in \( c_{i}/c_{a} \) on transfer to 10°C/5°C, but only Z. mays and the two Saccharum spp. lines showed a significant increase in \( c_{i}/c_{a} \) over the 11 d of chilling compared with Mxg (3x) ‘Illinois’. Both Saccharum spp. lines also showed an increase in \( c_{i}/c_{a} \) on return to 25°C/20°C relative to pre-chilling levels. In general, the other Mxg genotypes tested, including the hexaploid derived from the Mxg (3x) ‘Illinois’, were among the least chilling-tolerant lines, with respect to gas exchange measures (Figs 3, 4; Supplementary Fig. S2). There was a close correlation between \( A_{\text{sat}} \) and whole chain electron transport (\( J \)) calculated from \( \Phi_{\text{PSII}} \) both before and after the chilling treatment (Fig. 5). However, the \( A_{\text{sat}}/J \) declined ~34% across the genotypes as a result of the chilling treatment (Fig. 5).
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Fig. 3. (A) Leaf CO\(_2\) uptake rate (\(A_{sat}\)), (B) quantum yield of photosystem II (\(\Phi_{PSII}\)), (C) stomatal conductance to water vapour (\(g_s\)), and (D) ratio of intercellular to atmospheric CO\(_2\) concentration (\(c_i/c_a\)) for warm conditions prior to chilling treatment, after transfer of plants to chilling (day 0), on the 11th day of chilling treatment and 1 d after transfer of plants back to warm conditions (12th day of the experiment—recovery). Plants were grown at 10 °C/5 °C (chilling) or 25 °C/20 °C (warm) day/night, with a 14 h day/10 h night cycle under 1000 μmol photons m\(^{-2}\) s\(^{-1}\). Measurements were taken during the daytime. In all panels, accessions are ordered according to \(A_{sat}\) on day 12 of the experiment [from the highest to lowest; A, fourth bar (black fill) for each genotype]. For each treatment stage, asterisks indicate significant differences in comparison with *M. ×giganteus* (3x) ‘Illinois’ based on Dunnett’s test (*\(P\) ≤0.05; **\(P\) ≤0.01).

Subsequent time point values for *Mxg* (3x) ‘Illinois’ were: (A) 18.29, 7.99, 7.19, and 15.63 (μmol m\(^{-2}\) s\(^{-1}\)); (B) 0.21, 0.08, 0.07, and 0.22 (dimensionless); (C) 0.13, 0.12, 0.08, and 0.13 (mol m\(^{-2}\) s\(^{-1}\)); (D) 0.40, 0.74, 0.60, and 0.44 (dimensionless). Data are the mean ±SE (\(n\) = 4). F1, the first generation of Msa×Msi hybrids; Msa, *M. sacchariflorus*; Msi, *M. sinensis*; Mxg, *M. ×giganteus*; P1 (high), parent 1 of interspecific Msa×Msi hybrids (Msa with high chilling tolerance).
Fig. 4. Changes in (A, C, G, I) leaf CO\(_2\) uptake rate (\(A_{\text{sat}}\)) and (B, D, F, H, J) stomatal conductance to water vapour (\(g_s\)) following transfer of plants from warm to chilling conditions. Values are expressed as a percentage of initial rates at time 0. (A and B) Accessions at different ploidy levels; (C and D) tetraploid M. sacchariflorus (Msa); (E and F) diploid Msa; (G and H) interspecific hybrids (F1) and their Msa parent (P1; high); (I and J) negative controls. Plants were grown at 25 °C/20 °C (warm) or 10 °C/5 °C (chilling) day/night, and a 14 h day/10 h night cycle under 1000 μmol photons m\(^{-2}\) s\(^{-1}\). Measurements were taken during the daytime. Data are the mean ±SE (\(n=4\)). Lower case letters indicate: 'a', non-significant differences; 'b', significant differences in comparison with M. ×giganteus (3x) 'Illinois' (bold) on the 11th day after transfer to 10 °C/5 °C based on Dunnett's test (\(P\leq0.05\)). Values for Mxg (3x) 'Illinois' on the 11th day of chilling treatment were: (A) 92.02%; (B) 68.18%. F1, the first generation of Msa×Msi hybrids; Msa, M. sacchariflorus; Msi, M. sinensis; Mxg, M. ×giganteus; P1 (high), parent 1 of interspecific Msa×Msi hybrids (Msa with high chilling tolerance).
Fig. 5. Electron transport rates (J) in relation to CO2 uptake rates (A\text{sat}) for: (A) plants grown in control warm conditions prior to chilling treatment and (B) for the recovery day when plants after 11 d chilling treatment were transferred back to warm conditions. Plants were grown at 10 °C/5 °C (chilling) or 25 °C/20 °C (warm) day/night, and a 14h day/10h night cycle under 1000 μmol photons m\(^{-2}\) s\(^{-1}\). Measurements were taken during the day. Data are mean ±SE (n=4). Msa, M. sacchariflorus; Msi, M. sinensis; Mxg, M. × giganteus.

Discussion

What variation in chilling tolerance can be found within Mxg?

Mxg (3x) ‘Illinois’, which is the same clone that has been widely trialled in Europe (Hodkinson et al., 2002; Glowacka et al., 2014), has exceptional chilling tolerance of photosynthesis and leaf elongation, underlying its high productivity in cool climates (Beale and Long, 1995; Beale et al., 1996; Wang et al., 2008b; Dohleman and Long, 2009; Purdy et al., 2013; Arundale et al., 2014). However, the basis of this chilling tolerance remains unclear. One possibility is that the performance of this clone at chilling temperatures results from hybrid vigour rather than simple inheritance from its Msa and Msi parents. To test this hypothesis, new triploid hybrids were generated by crossing Msa (4x) ‘Bluemel Giganteus’ and Msi (2x) var. condensatus ‘Cabaret’. It was known from previous experience that the ‘Cabaret’ parent was insufficiently hardy to over-winter undamaged in cold-temperate environments, such as in central Illinois. Four highly vigorous full-sibling triploid progeny were obtained, all of which had significantly lower leaf extension rates at low temperature relative to Mxg (3x) ‘Illinois’, and the two best siblings for leaf elongation also had significantly lower leaf CO2 uptake rates and whole-chain electron transport when grown at 10 °C/5 °C compared with Mxg (3x) ‘Illinois’. Thus, hybrid vigour appears to have been insufficient to account for chilling tolerance in Mxg.

Doubling the chromosome number in triploid Mxg ‘Illinois’ resulted in hexaploid lines with significantly lower chilling tolerance with respect to A\text{sat} and \text{Φ}_{\text{PSII}} (Figs 3–5; Supplementary Fig. S3 at JXB online). These results indicated that suboptimal ploidy can reduce chilling tolerance in Mxg even when advantageous genes and gene combinations are present. In previous studies, hexaploids of Mxg had slightly greater stem diameter, slower growth rates (Yu et al., 2009), and fewer shoots (Głowacka et al., 2010), compared with triploid Mxg. Thus, ploidy can affect a range of traits, including chilling tolerance.

What variation in chilling tolerance can be found in Mxg’s parental species?

Based on the present sampling, Msa was typically a better source of chilling tolerance than Msi (Figs 1, 2; Supplementary Fig. S1 at JXB online), though Msi from particularly cold environments in the northernmost part of the species natural range, such as northern China, and southeast Russia were not available for this study. The results were consistent with the fact that the natural range of Msa extends further north in Asia than Msi, indicating that Msa is generally better adapted to colder temperate environments than Msi (Clifton-Brown et al., 2008; Sacks et al., 2013).

Though only a few other studies of low temperature leaf elongation or photosynthesis have been conducted on multiple genotypes of Miscanthus, they all support the conclusion that there is genetic variation within Miscanthus for chilling tolerance (Earnshaw et al., 1990; Clifton-Brown and Jones, 1997; Farrell et al., 2006; Purdy et al., 2013). Farrell et al. (2006) compared four genotypes for leaf elongation of dormant rhizomes in the dark and found that Msi (2x) JESEL78 (a selection from the Jelitto population New Hybrid) was superior at all temperatures tested (7, 9, 11, 13, and 15 °C) to Mxg (3x), Msi (3x) ‘Goliath’ (GOFAL7), and Msa (4x) Sac-5 (=’EMI-5’=MATEREC11). Purdy et al. (2013) studied leaf elongation at 12 °C for 4 d after transfer from 28 °C for four Miscanthus genotypes: Mxg (3x), Msi (3x) ‘Goliath’, Msa (4x) Sac-5, and Msi (2x) EMI-11. Purdy et al. (2013) observed no significant decrease in leaf elongation for Mxg, which may reflect the shorter duration of the chilling treatment than in the present study. All other genotypes in the Purdy et al. (2013) study had a significant decrease in leaf elongation relative to Mxg, which was similar to 32 (65%) genotypes tested...
in the present study. It is noteworthy that Msa (4x) EMI-5 was neither the best nor worst performing Msa under chilling conditions in the present study, but it was the only Msa included in the studies of Farrell et al. (2006) and Purdy et al. (2013). Clifton-Brown and Jones (1997) compared leaf elongation of Mxg with 21 accessions of Msi and four of Msa, and they observed that the best and worst genotypes under the lowest temperatures studied (5 °C for 11.5 h) were Msi. It is unclear why the study of Clifton-Brown and Jones (1997) did not identify Mxg as a superior genotype under the lowest temperature treatment, in contrast to the study of Purdy et al. (2013) and the present study. Earnshaw et al. (1990) observed that M. floridulus originating from high altitude (3280 m) in Papua New Guinea had greater chilling tolerance than a low altitude (2600 m) accession, as evidenced by greater chlorophyll fluorescence after chilling of leaf samples at 0 °C, which indicated that natural selection for adaptation has resulted in genetic diversity of chilling tolerance in Miscanthus. An important contribution of the present study is that it was possible to evaluate more than twice as many Msa genotypes as all of the previous studies combined, which enabled the observation that both diploid and tetraploid genotypes of Msa typically have high, though variable tolerance to chilling.

In the present study, some Msa had chilling tolerance equivalent to Mxg (3x) ‘Illinois’. Tetraploid Msa ‘PF30153’, which was collected in Gifu Prefecture, did not differ significantly from Mxg (3x) ‘Illinois’ for chilling tolerance associated with leaf elongation and photosynthesis (Figs 1, 3, 4). Moreover, all Msa accessions studied, except the ornamental, chlorophyll mutant ‘Gotemba Gold’, did not show a significantly greater decline in the operating efficiency of photosystem II (ΦPSII) relative to Mxg (3x) ‘Illinois’ at the end of the 11 d chilling treatment and upon recovery to warm temperature (day 12) (Fig. 3; Supplementary Figs S2, S3). Photosystem II is considered the most sensitive part of the photosynthetic apparatus to light-induced chilling damage and often the first manifestation of chilling damage (Long et al., 1994; Maxwell and Johnson, 2000). Thus, stability of the photosynthetic apparatus under cold stress is necessary but insufficient for high levels of photosynthesis per se at low temperature, yet the former trait may be of considerable value on its own. The ability to recover higher rates of photosynthesis quickly upon return of warm temperatures may itself contribute to adaptation to fluctuating temperatures that are typical in temperate environments of the beginning and end of the growing season.

Given that Mxg (3x) ‘Illinois’ and most of the Msi included in the present study originated in southern Japan, yet chilling tolerance was highest for Mxg (3x) ‘Illinois’ and Msa, it is likely that Mxg’s chilling tolerance was obtained from its Msa parent. Furthermore, as the tetraploid parent to this triploid, Msa has double the influence of Msi on the genetic makeup of the Mxg hybrid. Given that chilling tolerance varies among and within Miscanthus species, careful selection of parents for developing new Mxg bioenergy cultivars will be needed to ensure optimal adaptation to temperate environments.

High productivity in a cool temperate climate requires a genotype to be able to develop photosynthetically competent leaves under the chilling conditions of early spring. Without this, a crop canopy will be unable to use the high insolation of April, May, and June fully in the northern hemisphere. This capacity is fundamental to explaining the higher productivity of Mxg (3x) ‘Illinois’ relative to Z. mays (Dohleman and Long, 2009; Long and Spence, 2013). Based on a productivity model, Farrell et al. (2006) predicted that a Miscanthus accession with greater cold and chilling tolerance than Mxg could extend the growing season an average of 30 d, which would result in up to 25% higher yield. Thus, understanding how to select for improved rates of photosynthesis under low temperatures is key to improving productivity and sustainability of crops grown in temperate environments, such as Miscanthus. For breeding Mxg, choice of both the Msa and Msi parents will be important for achieving gains in chilling tolerance and concomitant yield potential in temperate environments. Indeed, the four new triploid Mxg progeny [Msa (4x) ‘Bluemel Giganteus’×Msi (2x) var. condensatus ‘Cabaret’] that were tested in the leaf elongation experiment had inferior chilling tolerance relative to their Msa parent, because their Msi parent was poorly adapted to cold temperatures.

What are the physiological mechanisms of chilling susceptibility and chilling tolerance of Miscanthus accessions?

All 16 accessions studied (13 Miscanthus, two sugarcane, and one maize) showed decreases in Asat and ΦPSII, and increases in ε/εm, after transfer from 25 °C/20 °C to 10 °C/5 °C (Fig. 3; Supplementary Fig. S2 at JXB online). Stomatal conductance for most of the genotypes (81% of accessions) increased on day 0 and then decreased during the following 11 d of chilling treatment. Since in the intercellular compartment the concentration of CO2 increased as a consequence of a low rate of CO2 fixation, the stomata reacted by reducing their aperture. Thus, differences among accessions in relative decreases in Asat cannot be explained by deficiency in CO2 or by loss of stomatal function. Comparing the A sat reading with values of ΦPSII showed that light-induced chilling damage of photosystem II is the primary reason for low CO2 assimilation in chilling-susceptible accessions. The most chilling-tolerant accessions retained relatively high ΦPSII and A sat during 11 d of chilling treatment and, upon return to warm temperatures (on day 12 of the experiment), they also recovered their gas exchange and chlorophyll fluorescence to pre-chilling treatment levels. These results are consistent with those of Farage et al. (2006), which indicated that the main mechanism behind avoiding the photodamage under chilling temperatures is the capacity for non-photochemical quenching of absorbed light energy. In contrast to the chilling-tolerant accessions, the chilling-susceptible accessions in the present study showed very low levels of recovery of measured parameters upon return to warm temperatures at the end of the experiment. This indicates a damaged photosynthetic system in susceptible accessions, and suggests the absence of alternative pathways for dissipating absorbed excitation energy under chilling conditions, leading to irreversible photo-oxidation of photosystem II (Allen and Ort, 2001).
Conclusion

Based on the sampling carried out here, Msa is a better source of chilling tolerance than Msi; thus an even more extensive survey of tetraploid Msa collected from diverse environments would be advantageous for identifying superior Msa parents and breeding new chilling-tolerant Mxg cultivars. There is also a need to identify Msi parents with superior chilling tolerance, though this may be more challenging than for Msa. To the extent that lessons from breeding Miscanthus for increased photosynthetic capacity at low temperature can be applied to other crops, especially other Saccharinae, great gains in agricultural productivity and sustainability may be obtained in temperate environments.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Relationship between leaf elongation at warm and chilling temperatures for 51 Miscanthus accessions, two control sugarcane, and two maize lines.

Figure S2. Leaf CO₂ uptake rate, quantum yield of photosystem II, stomatal conductance to water vapour, and ratio of intercellular to atmospheric CO₂ concentration for warm conditions prior to chilling treatment, after transfer of plants from warm to chilling (day 0), on the 11th day of chilling treatment, and 1 d after transfer of plants back to warm conditions. Numbers are expressed as a percentage of rates observed in warm conditions before the chilling treatment.

Figure S3. Changes in quantum yield of photosystem II and intercellular to atmospheric CO₂ concentration following transfer of plants from warm to chilling conditions.

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