Siberian *Miscanthus sacchariflorus* accessions surpass the exceptional chilling tolerance of the most widely cultivated clone of *Miscanthus x giganteus*

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Abstract

Chilling temperatures (0–15°C) inhibit photosynthesis in most C₄ grasses, yet photosynthesis is chilling tolerant in the ‘Illinois’ clone of the C₄ grass *Miscanthus x giganteus*, a candidate cellulosic bioenergy crop. *M. x giganteus* is a hybrid between *Miscanthus sacchariflorus* and *Miscanthus sinensis*; therefore chilling-tolerant parent lines might produce hybrids superior even to the current clone. Recently a collection of *M. sacchariflorus* from Siberia, the apparent low temperature limit of natural distribution, became available, which may be a source for chilling tolerance. The collection was screened for chilling tolerance of photosynthesis by measuring dark-adapted maximum quantum yield of PSII photochemistry (*Fᵥ/ Fₘ*) on plants in the field in cool weather. Superior accessions were selected for further phenotyping: plants were grown at 25°C, transferred to 10°C (chilling) for 15 days, and returned to 25°C for 7 days (recovery). Two experiments assessed: (a) light-saturated net photosynthetic rate (*Aₛₜₐ*) and operating quantum yield of PSII photochemistry (*Φₚₛₛ*I), (b) response of net leaf CO₂ uptake (*A*) to intercellular [CO₂] (*ci*). Three accessions showed superior chilling tolerance: RU2012-069 and RU2012-114 achieved *Aₛₜₐ* up to double that of *M. x giganteus* prior to and during chilling, due to increased *ci* - saturated photosynthesis (*Vₘₐₓ*). RU2012-069 and RU2012-114 also maintained greater levels of *Φₚₛₛ*I during chilling, indicating reduced photodamage. Additionally, accession RU2012-112 maintained a stable *Aₛₜₐ* throughout the 15-day chilling period, while *Aₛₜₐ* continuously declined in other accessions; this suggests RU2012-112 could outperform others in lengthy chilling periods. Plants were returned to 25°C after the chilling period; *M. x giganteus* showed the weakest recovery after 1 day, but a strong recovery after 1 week. This study has therefore identified important genetic resources for the synthesis of improved lines of *M. x giganteus*, which could facilitate the displacement of fossil fuels by cellulosic bioenergy.

Keywords

bioenergy, biomass, chilling tolerance, cold tolerance, *Miscanthus sacchariflorus*, *Miscanthus x giganteus*, photosynthesis, photosystem II, Siberia
**INTRODUCTION**

The C₄ grass *Miscanthus x giganteus* Greef and Deuter ex Hodkinson & Renvoize is exceptionally productive in the US Midwest, yielding 59% greater peak biomass than *Zea mays* L. (Dohleman & Long, 2009). This is explained by its ability to develop and maintain leaves that are photosynthetically competent under the chilling conditions of early Spring and Fall, enabling a longer growing season than *Z. mays* (Dohleman & Long, 2009). In southern England where low temperatures prevent production of a *Z. mays* grain crop in most years, *M. x giganteus* produced a peak dry biomass yield of 30 t/ha, a record productivity for the UK (Beale & Long, 1995). This correlated with a lack of the chilling damage to photosynthesis observed in *Z. mays* (Beale, Bint, & Long, 1996).

Superior chilling tolerance and resulting higher yields compared to other candidate bioenergy grasses has garnered strong interest in developing *M. x giganteus* as a feedstock for cellulosic bioenergy production (Clifton-Brown, Chiang, & Hodkinson, 2008; Clifton-Brown et al., 2018; Heaton, Dohleman, & Miguez, 2010). A recent meta-analysis of over 1,000 published yields, showed that across temperature, fertility and water availability gradients, average yields of *M. x giganteus* were more than double those of *Panicum virgatum* under the same conditions, despite major breeding efforts to improve the latter (LeBauer et al., 2018). As a result *Miscanthus* could be closer than *P. virgatum* to commercial-scale viability (Clifton-Brown et al., 2018). Unlike *P. virgatum*, which includes adapted and improved ecotypes, yields of *M. x giganteus* in the US are largely for the single unimproved ‘Illinois’ clone (Głowacka, Clark, et al., 2015; Long & Spence, 2013). There is probably no or very little genetic diversity between the ‘Illinois’ clone and other *M. x giganteus* legacy clones in the US and Europe (Głowacka, Clark, et al., 2015). Genetic mapping of the parent species, however, finds very significant potential for breeding of high-yielding *Miscanthus* cultivars (Clark et al., 2014, 2018; Dong et al., 2018). This raises the likelihood that even more productive forms of *M. x giganteus* could be developed.

*M. x giganteus* ‘Illinois’ is a sterile, triploid hybrid between a tetraploid *Miscanthus sacchariflorus* (Maxim.) Hack. and the diploid *Miscanthus sinensis* Andersson (Hodkinson & Renvoize, 2001). *M. sacchariflorus* and *M. sinensis* are genetically diverse, distributed over a wide latitudinal and thermal range in eastern Asia from the sub-tropics to Siberia (Clark et al., 2014, 2016, 2018).

It was predicted that productivity of *M. x giganteus* could be improved by up to 25% with the greater chilling tolerance seen in a *M. sinensis* hybrid (Farrell, Clifton-Brown, Lewandowski, & Jones, 2006). A study of various *Miscanthus* accessions found that when abruptly exposed to an 11-day 10°C chilling treatment, some Japanese *M. sacchariflorus* accessions could match the photosynthetic rate of *M. x giganteus* ‘Illinois’ (Glowacka et al., 2014). A similar study of *Miscanthus* found two *M. sacchariflorus* accessions from Japan capable of surviving night-time frost and achieving light-limited and light-saturated photosynthetic rates >40% greater than *M. x giganteus* grown at 15°C (Głowacka, Jorgensen, et al., 2015). However, most of these plants originated from temperate regions. Even the highly studied ‘Illinois’ clone of *M. x giganteus* appears to originate from a cross of parental accessions from temperate southern Japan (Clark et al., 2014; Glowacka, Clark, et al., 2015). This raises the question of whether higher latitude germplasm, from the coldest regions that support *Miscanthus* growth, could show still greater chilling tolerance.

One hundred and sixty *M. sacchariflorus* accessions were recently collected across the eastern region of Russian Siberia (Clark et al., 2016); these represent the northern limit of wild *Miscanthus* and likely candidates for greater chilling tolerance. The hypothesis that this material would have greater chilling tolerance was tested in three successive physiological experiments, culminating in the discovery of three *M. sacchariflorus* accessions capable of achieving double the photosynthetic rate of *M. x giganteus* during chilling.

**MATERIALS AND METHODS**

Three experiments were conducted as successive cold-tolerance screens: Experiment 1: measurements of dark-adapted maximum quantum yield of PSII photochemistry ($F_{v}/F_{m}$) were used to screen for chilling tolerance in a field-grown population of 92 *M. sacchariflorus* accessions that originated from 43°N to 49°N in eastern Russia, representing USDA hardiness zones 5 to 3 (warmer to colder) (Clark et al., 2016). These accessions are the northernmost members of the extensive *M. sacchariflorus* Korea/NE China/Russia diploids genetic group, one of six *M. sacchariflorus* genetic groups (Clark et al., 2018). Seven accessions were chosen for further evaluation based on their field performance and availability of sufficient clonal material for further investigation: RU2012-114 (48.60930°N, 134.21509°E, 42.0 m, hardiness zone 3), RU2012-112 (48.58787°N, 133.93944°E, 45.0 m, hardiness zone 3), RU2012-069 (48.54032°N, 135.13335°E, 134.71992°E, 34.0 m, hardiness zone 3), RU2012-121 (48.54032°N, 134.71992°E, 34.0 m, hardiness zone 3), RU2012-073 (48.62415°N, 133.17572°E, hardiness zone 3), RU2012-073 (48.62415°N, 133.17572°E, 35.0 m, hardiness zone 3). Experiment 2: With these seven accessions, a controlled-environment experiment assessed operating light-saturated net rates of leaf CO₂ uptake ($A_{sat}$) and operating quantum yield of PSII photochemistry ($\Phi_{PSSII}$) during a 15-day chilling period. Three accessions, RU2012-114, RU2012-112, and RU2012-069, showed high chilling
tolerance. Experiment 3: With these three accessions, a controlled-environment experiment analyzed CO₂-saturated rate of photosynthesis (V\textsubscript{max}) and the maximum apparent rate of PEPc carboxylation (V\textsubscript{Pmin}) during a 15-day chilling period, followed by a 7-day recovery to warm temperature. In all experiments M. x giganteus ‘Illinois’ was included as a control.

2.1 | Experiment 1: Field screen of F\textsubscript{v}/F\textsubscript{m}

2.1.1 | Plant material and growing conditions

A collection of 160 accessions of M. sacchariflorus was made by the University of Illinois with the Vavilov Institute of Plant Genetic Resources in eastern Siberia in 2012 (Clark et al., 2016). Detailed description of the origin of each accession was given previously (Clark et al., 2016). These were then initially planted out on the Aarhus University Farm at Foulum, Denmark, in 2013 (56°30′N, 9°35′E). For this study the accessions were narrowed down to the most chilling-tolerant subset in terms of photosystem II maximum efficiency in the experiments described below. Soil at this site is a sandy loam (typic Fragiudalf; USDA soil taxonomy). Two clones of each accession were planted 150 cm apart in the summer of 2013 together with M. x giganteus ‘Illinois’ which was propagated from rhizome cuttings. Plants were rainfed and not fertilized. Air temperature at 20 and 150 cm was recorded every 10 min by a meteorological station located within the trial site.

2.1.2 | Dark-adapted chlorophyll fluorescence measurements

Between June 19th and July 2nd, 2014, a 13-day chilling period occurred at the Foulum field site where average daily air temperatures did not exceed 16°C. During this period about 40% of the collection became chlorotic; these accessions were not included in the screen. Ninety-two M. sacchariflorus accessions showing minimal leaf chlorosis were selected: measurements were taken from 11:30 p.m. to 4:00 a.m., on at least two shoots for each of the two plantings of selected accessions, along with M. x giganteus. This timing was to ensure that leaves were dark adapted and that functioning photosystem II (PSII) centers would be fully open (Baker, 2008). The youngest fully expanded leaf, as indicated by ligule emergence, was placed in the cuvette of a portable gas-exchange system incorporating infra-red CO₂ and water vapor analyzers (LI 6400; LI-COR, Inc., Lincoln, NE) and using a combined gas-exchange and pulse-amplitude fluorescence attachment (LI 6400–40; LI-COR). Once chlorophyll fluorescence stabilized (<30 s), dark-adapted maximum quantum yield of PSII photochemistry (F\textsubscript{v}/F\textsubscript{m}) was recorded using a rectangular saturating flash protocol (Baker, 2008).

This method follows that of the rapid screen for chilling tolerance in field grown plants used previously (Glowacka, Jorgensen, et al., 2015).

The mean F\textsubscript{v}/F\textsubscript{m} per accession in Experiment 1 was used to select seven accessions that were representative of the full range of variation in F\textsubscript{v}/F\textsubscript{m}, and were used for further screening in Experiment 2. These seven were among several accessions that were cloned and shipped for propagation in controlled environments at the University of Illinois.

2.2 | Experiment 2: Controlled-environment screen of A\textsubscript{sat} and ΦPSII

2.2.1 | Plant material and growing conditions

Seven M. sacchariflorus accessions, and the ‘Illinois’ clone of M. x giganteus, were grown for a controlled-environment experiment. Clonal divisions (n = 4) of each accession were grown from rhizomes in 1.6 L pots containing a peat/bark/perlite-based growing medium (Metro-Mix 900; Sun Gro Horticulture, Agawam, MA). After cloning by rhizome propagation, a slow release fertilizer was added according to the manufacturer’s instructions (Osmocote Pro, 8–9 mo 19–5–8 Minors; Everris NA, Inc., Dublin, OH). Plants were watered daily and grown for 53 days in a controlled-environment greenhouse at ~25°C, with high pressure sodium lamps ensuring a minimum photosynthetically active photon flux (Q) of 300 μmol m\textsuperscript{-2} s\textsuperscript{-1} and a 14-hr day length. Plants were transferred from the greenhouse to two controlled-environment growth cabinets for 23 days before measurements began (Model PCG20, Conviron, Winnipeg, MB R3H 0R9, Canada). The cabinets maintained a 14 hr /10 hr day/night cycle under 800 photons m\textsuperscript{-2} s\textsuperscript{-1}, 25°C daytime/20°C nighttime temperature, with a relative humidity of 75% throughout.

2.2.2 | Gas exchange and chlorophyll fluorescence measurements

A first day of measurement was taken at 25°C (day 0), then cabinet temperature was reduced to 10°C/5°C day/night for 15 days (days 1–15). This experimental timeline has been used previously to mimic the type of chilling (0–12°C) that might affect leaf emergence during spring or expanded leaves in the autumn (Allen & Ort, 2001; Baker, Bradbury, Farage, Ireland, & Long, 1989; Glowacka et al., 2014). Measurements were taken on days 0, 1, 2, 5, 6, 8, 10, 12, and 15. Pictures of plants were taken with a camera (Canon PowerShot SX50 HS, Canon, Tokyo, Japan) before and after the experiment to show differences in any visible damage sustained by plants during chilling.
The youngest fully expanded leaf from each plant's primary tiller was selected for measurement. Leaves were measured with a portable gas-exchange system (LI 6400; LI-COR, Inc.). The manufacturer's combined gas-exchange and fluorescence measurement attachment was used (LI 6400-40; LI-COR). Incident photon flux was set to 2,000 μmol m\(^{-2}\) s\(^{-1}\), block temperature to either 25 or 10°C on warm and chilling days, respectively, flow rate to 400 μmol/s, reference [CO\(_2\)] to 400 μmol/mol and leaf-to-air water vapor pressure deficit maintained at <2 kPa. Light was provided by the integrated red (635 nm wavelength) and blue (465 nm wavelength) light-emitting diodes (LED). Leaves acclimated in the measurement cuvette until the net rate of leaf CO\(_2\) uptake (A) reached a steady state, then measurements were initiated.

Gas-exchange data were recorded and ΦPSII, a and g\(_c\) calculated (von Caemmerer & Farquhar, 1981); followed by modulated fluorescence measurements to derive operating quantum yield of PSII photochemistry (ΦPSII) using a multiphase flash protocol (Loriaux et al., 2013). Because all measurements were taken under saturating photon flux (Q = 2,000 μmol m\(^{-2}\) s\(^{-1}\)) reached a steady state, then gas-exchange data were recorded. Because all measurements were taken under saturating photon flux (Q = 2,000 μmol m\(^{-2}\) s\(^{-1}\)) A\(_{sat}\), then gas-exchange data were recorded. Because all measurements were taken under saturating photon flux (Q = 2,000 μmol m\(^{-2}\) s\(^{-1}\)) A\(_{sat}\) was determined as A measured at [CO\(_2\)] = 400 μmol/mol.

A- c\(_i\) curves were fit to a nonrectangular hyperbolic function as in (Leakey et al., 2006), and the CO\(_2\)-saturated rate of photosynthesis (V\(_{max}\)) was estimated as the predicted value of each function for c\(_j\) = 2,000 μmol/mol. The response of A to c\(_j\) at c\(_j\) <100 μmol/mol was used to solve for maximum apparent rate of PEPc carboxylation (V\(_{pmax}\)) (von Caemmerer, 2000), with temperature-dependent estimates of the Michaelis–Menten constant of PEPc for [CO\(_2\)] (K\(_p\)) from the C\(_4\) grass Setaria viridis (Boyd, Gandin, & Cousins, 2015). All curve fitting was performed using non-linear regression (PROC NLIN, SAS v8.02; SAS Institute, Cary, NC).

### 2.3.1 | Plant material and growing conditions

Three M. sacchariflorus accessions, and an ‘Illinois’ clone of M. x giganteus, were grown in another controlled environment experiment. Growing conditions were similar to Experiment 2, except that: n = 6 clonal divisions of each accession were planted in 5.7 L pots containing a soil-free medium (LC1, Sungro Horticulture). Pots were fertilized twice; after cloning by rhizome propagation, and 2 days before the start of measurements with a slow release 17-5-11 fertilizer added according to the manufacturer's instructions (Osmocote Pro, Everris NA, Inc.) and supplemented with iron (Ferrous sulphate heptahydrate, QC Corporation, Girardeau, MO). Plants were grown for 1 month in a controlled-environment cabinet before measurements began (Model PCG20, Conviron) with growing conditions as in Experiment 2.

### 2.3.2 | Gas exchange measurements

Growth cabinet conditions during the experiment were similar to Experiment 2, except that on day 16 cabinets were returned to 25°C daytime/20°C nighttime for a week. Measurements were taken on days 0, 1, 3, 5, 7, 9, 11, 13, 15, 16, and 23.

A portable gas-exchange system (LI 6400; LI-COR, Inc.) was used for measurement as in Experiment 2, but a larger chamber without fluorescence capability was used to improve measurement accuracy of small gas fluxes (LI 6400-02B; LI-COR). Leaves acclimated in the measurement cuvette until the net rate of leaf CO\(_2\) uptake (A) reached a steady state, then A-c\(_i\) curves were measured by progressively decreasing [CO\(_2\)] in the reference cell (400, 350, 300, 250, 200, 150, 100, 60, and 0 μmol/mol). Leaves were allowed to acclimate to each step reduction in [CO\(_2\)] for 2–3 min, as assessed by a resumption of a steady-state A, then gas-exchange data were recorded. Because all measurements were taken under saturating photon flux (Q = 2,000 μmol m\(^{-2}\) s\(^{-1}\)) A\(_{sat}\) was calculated as A measured at [CO\(_2\)] = 400 μmol/mol.

A- c\(_i\) curves were fit to a nonrectangular hyperbolic function as in (Leakey et al., 2006), and the CO\(_2\)-saturated rate of photosynthesis (V\(_{max}\)) was estimated as the predicted value of each function for c\(_j\) = 2,000 μmol/mol. The response of A to c\(_j\) at c\(_j\) <100 μmol/mol was used to solve for maximum apparent rate of PEPc carboxylation (V\(_{pmax}\)) (von Caemmerer, 2000), with temperature-dependent estimates of the Michaelis–Menten constant of PEPc for [CO\(_2\)] (K\(_p\)) from the C\(_4\) grass Setaria viridis (Boyd, Gandin, & Cousins, 2015). All curve fitting was performed using non-linear regression (PROC NLIN, SAS v8.02; SAS Institute, Cary, NC).

### 2.4 | Statistical analysis

There were three main statistical tests used to compare the three top-performing M. sacchariflorus accessions to M. x giganteus: ANOVA on individual days, repeated measures ANOVA throughout chilling days, and non-linear regression throughout chilling days.

ANOVA (PROC GLM, SAS Institute Inc.) with a one-tailed Dunnett’s test (significant: p < 0.05 threshold, marginally significant: p < 0.1 threshold) was used to determine whether any of the M. sacchariflorus accessions surpassed M. x giganteus in terms of A\(_{sat}\), ΦPSII, V\(_{max}\), V\(_{pmax}\) and g\(_c\) on key measurement days: the prechilling day (day 0), the first (day 1) and last (day 15) day of chilling, and the first day of recovery to 25°C (day 16). For the last day of recovery to 25°C (day 23), a similar ANOVA was performed, but with a two-tailed Dunnett’s test, to account for the possibility of M. x giganteus surpassing the M. sacchariflorus accessions.

In order to assess photosynthetic traits throughout the chilling period (days 1 through 15), a repeated measures one-tailed ANOVA (significant: p < 0.05 threshold, marginally significant: p < 0.1 threshold) (PROC GLM, SAS Institute Inc.) was used to test whether each M. sacchariflorus accession surpassed M. x giganteus for A\(_{sat}\), ΦPSII, V\(_{max}\), V\(_{pmax}\), and g\(_c\).
FIGURE 1  Experimental time course of $A_{sat}$ in Experiment 2, for Miscanthus x giganteus and accessions of Miscanthus sacchariflorus. Plants were kept at 25°C daytime/20°C nighttime on day 0 and at 10°C daytime/5°C nighttime on days 1 through 15. Each point is the mean (±1 SE) of 4 plants. Lines from days 0–15 are best-fit curves, as explained in Figure S1. Arrows identify high-performing accessions, along with the control $M. x$ giganteus, which were selected to test in Experiment 3

In different accessions, $A_{sat}$, $\Phi_{PSII}$, $V_{max}$, $V_{pmax}$, and $g_s$ would either decrease throughout days 1–15, or decline to a minimum and then increase over several days. To quantify these different patterns, data from days 0–15 were fit by non-linear regression (PROC NLIN, SAS Institute Inc.) to the equation:

$$Y = b + (y_0 - b) \times e^{(-\lambda \times \text{day})} + m \times \text{day}$$

This describes an exponential decline of $Y$ during the transition from 25°C to 10°C, followed by a linear increase, decrease, or plateau over the course of days 1–15. $Y$ is either $A_{sat}$, $\Phi_{PSII}$, $V_{max}$, $V_{pmax}$, or $g_s$. $m$ is the key parameter describing the rate of linear increase ($m > 0$) or decrease ($m < 0$) of $Y$ in days 1–15. $y_0$ is the $Y$-intercept, $b$ is the lowest value of $Y$ reached during the chilling period, $\lambda$ describes the rate of change of $Y$ from $y_0$ to $b$. Figure S1 is a schematic example describing this fit equation, where $Y$ decreases to a minimum at day 4, then increases until day 15, leading to $m > 0$. ANOVA (PROC GLM, SAS Institute Inc.) with a one-tailed Dunnett’s test (significant: $p < 0.05$, marginally significant: $p < 0.1$) was used to determine whether any of the Miscanthus sacchariflorus accessions surpassed $M. x$ giganteus for $m$ fit to $A_{sat}$, $\Phi_{PSII}$, $V_{max}$, $V_{pmax}$, or $g_s$.

Measurements of $A_{sat}$ and $g_s$ were obtained for $M. x$ giganteus and the three top-performing Miscanthus sacchariflorus accessions in Experiments 2 & 3 under comparable experimental conditions, therefore data from both experiments were pooled and a fixed two-factor block for ‘Experiment’ added when analyzing these variables. In all ANOVAs, homogeneity of variance was verified graphically, and normality of studentized residuals tested by Shapiro-Wilk (PROC UNIVARIATE, SAS Institute) at $p = 0.01$ threshold.

3 | RESULTS

3.1 | Selection of three top-performing accessions

Air temperature varied between 5 and 25°C throughout June of 2014, and dipped at the end of the month as a cool spell set in with three successive days in which average air temperature was 12°C, with dawn air temperatures averaging 8°C (Figure S2). Field measurements of $F_v/F_m$ following these 3 days ranged from 0.62 to 0.74, and all but one of the Miscanthus sacchariflorus accessions achieved higher $F_v/F_m$ than $M. x$ giganteus. Based on these results and availability of plant material, seven accessions were selected for transfer to Illinois for further analysis in Experiment 2. These were accessions RU2012–114, RU2012–069, RU2012–073, RU2012–083, and RU2012–091, which achieved the highest $F_v/F_m$. RU2012–121 as an example of an accession with mid-range $F_v/F_m$ and RU2012–112 with the third-lowest $F_v/F_m$ (Figure S3).

In Experiment 2, average $A_{sat}$ over all chilling days (days 1–15) was lowest in $M. x$ giganteus at 6 µmol m$^{-2}$ s$^{-1}$, and greatest in accessions RU2012–069, RU2012–114 and RU2012–112, ranging from 8.5 to 12 µmol m$^{-2}$ s$^{-1}$ (Figure 1). Therefore, these three accessions were selected for further analysis in Experiment 3, and for statistical comparison to $M. x$ giganteus. All plants were somewhat chlorotic at the end of the chilling period, though damage appeared least pronounced in accession RU2012–069 (Figure S4).

3.2 | Improved chilling $A_{sat}$ in three top-performing $M. sacchariflorus$ accessions

Decline in $A_{sat}$ upon chilling was seen in Experiments 2 and 3, and was matched by decline in $\Phi_{PSII}$, $V_{max}$, $V_{pmax}$ and $g_s$ (Figures 2–4). $A_{sat}$ was significantly ($p < 0.05$), and up to 2-fold greater than $M. x$ giganteus in accessions RU2012–069 and RU2012–114 prior to chilling (day 0), on the first (day 1) and on the last (day 15) day of chilling (Table 1, Figure 2a), and throughout the entire chilling period (days 1–15) (Table 1, Figure 4a).

In accessions RU2012–069 and RU2012–114, $A_{sat}$ declined continuously over the course of days 1–15 ($m < 0$ for $A_{sat}$, Figures 2a, 4b) at a rate of 0.16–0.27 µmol m$^{-2}$ s$^{-1}$ per day. This caused $A_{sat}$ to drop by more than half in accession RU2012–069 over this time period (Table 1). In contrast, in RU2012–112, $A_{sat}$ declined from days 1–4, then increased from days 4–15 ($m > 0$ for $A_{sat}$, Figures 2a, 4b). $m$ for $A_{sat}$ was significantly greater than in $M. x$ giganteus ($p < 0.05$),
indicating RU2012-112 was more successful than *M. x giganteus* at avoiding continued loss of *A*$_{sat}$ throughout days 1–15 (Figure 4b).

### 3.3 Physiological basis for improved *A*$_{sat}$

*A*$_{sat}$ may be affected by ΦPSII, $V_{max}$, $V_{pmax}$, and $g_s$, and all of these variables showed similar declines throughout chilling (Figure 2). Throughout the chilling period (days 1–15), ΦPSII was marginally ($p < 0.1$) or significantly ($p < 0.05$) lowest in *M. x giganteus* relative to RU2012-069, RU2012-114 and RU2012-112 (Figure 4c).

Graphical analysis of *A-* $c_i$ curves clearly shows that $A_{sat}$ was not restricted by either $g_s$ or $V_{pmax}$ on chilling days (Figure 3). The operating point is the $c_i$ found when the external [CO$_2$] is equal to the current atmospheric level: 400 µmol/mol, i.e. the intersection of the response of $A$ to $c_i$ and the stomatal supply function described by $1/ g_s$ as shown in Figure 3. In all accessions, the operating point increased during chilling such that control markedly shifted to $V_{max}$, which is the plateau of the *A-* $c_i$ response (Figure 3; Days 1 & 15).

$V_{max}$ was marginally ($p < 0.1$) or significantly ($p < 0.05$) greater in RU2012-069 than in *M. x giganteus* prior to chilling (day 0), on the first (day 1) and on the last (day 15) day of chilling (Table 1, Figure 2d), and throughout the entire chilling period (days 1–15) (Figure 4a). Further, the decline in $V_{max}$ over the course of days 1–15 was most severe ($m < 0$) for $V_{max}$ in *M. x giganteus*; in contrast $m$ for $V_{max}$ was significantly greater ($p < 0.05$) in RU2012-114 and RU2012-112 than in *M. x giganteus*, indicating improved ability to maintain $V_{max}$ throughout chilling (Figure 4b).

Despite the apparently low sensitivity of $A_{sat}$ to $g_s$ (Figure 3) during chilling, $g_s$ was significantly ($p < 0.05$) greater in accessions RU2012-069 and RU2012-114 than *M. x giganteus* throughout days 1–15 (Figure 4c).

### 3.4 Recovery on return to 25°C

RU2012-114 showed the greatest initial recovery (Day 16), achieving $A_{sat}$ significantly greater ($p < 0.05$) and more than double that of *M. x giganteus* (Figure 2a, Table 1). However, recovery of *M. x giganteus* 1 week later (day 23) was markedly superior. Not only was $A_{sat}$ significantly ($p < 0.05$)
and up to 70% greater in *M. x giganteus* than any of the *M. sacchariflorus* accessions, it had also exceeded its own pre-chilling levels of $A_{\text{sat}}$ by 35%. *M. x giganteus* also showed the significantly ($p < 0.05$) greatest values for $V'_{\text{max}}$ and $g_s$ on day 23 (Figure 2b,e and Table 1). Graphical analysis of $A-c_i$ curves on day 23 shows the operating point in all accessions had returned to co-limitation by $V'_{\text{max}}$ and $V_{\text{max}}$ on day 23, as in day 0 (Figure 3); this suggests the improved $V'_{\text{max}}$ and $g_s$ of *M. x giganteus* on day 23 both contributed to its greater $A_{\text{sat}}$.

4 | DISCUSSION

This study has identified three *M. sacchariflorus* accessions, RU2012-114, RU2012-112, and in particular RU2012-069, with superior chilling tolerance of photosynthesis relative to *M. x giganteus* ‘Illinois’. RU2012-069 and RU2012-114 both maintained $A_{\text{sat}}$ up to two times greater than *M. x giganteus* throughout chilling. Although RU2012-112 showed a low initial $A_{\text{sat}}$ on chilling, consistent with its low $F'/F_m$ (Figure S3) in the field relative to other accessions, $A_{\text{sat}}$ improved with time in chilling, as did $g_s$ and $V'_{\text{max}}$ (Figure 2a,b,d). This accession therefore showed a superior ability to acclimate to the chilling conditions. Recovery of $A_{\text{sat}}$ to 25°C was lower in *M. x giganteus* than in these three *M. sacchariflorus* accessions, but it showed superior recovery 1 week later. However, this required 7 days of 25°C, and such continuous periods of high temperatures would be unlikely at the high latitude limits of *Miscanthus* spp. cultivation or during the spring and autumn of lower latitude temperate climates. Under these conditions the far more rapid recovery of photosynthesis in the three Siberian accessions, in particular RU2012-114, in the first 24 hr would be far more important (Figure 2a), i.e. allowing these accessions to take advantage of brief periods of warmer weather. This is the first published physiological analysis of Siberian *Miscanthus* germplasm representing the high latitude extreme of the native range of *M. sacchariflorus*, which demonstrates an ability to maintain and even increase photosynthetic capacity during chilling.
4.1 Exceptional chilling tolerance found in three Siberian *M. sacchariflorus* accessions

C₄ plants are highly vulnerable to chilling temperatures, i.e. above freezing but below 15°C (Friesen, Peixoto, Busch, Johnson, & Sage, 2014; Long, 1983; Long & Spence, 2013). Field experiments in southern England, near the northern limit of *Z. mays* production in Europe have shown that the combination of chilling temperatures and high light are particularly damaging to photosynthesis and early growth (Baker et al., 1989). The inter-specific hybrid C₄ grass *M. x giganteus* was long considered an exception to this rule, being able to avoid such damage and maintain photosynthetic efficiency during and following chilling events. This has been associated with the ability to up-regulate genes coding for key photosynthetic enzymes and chloroplast membrane components (Dohleman & Long, 2009; Friesen & Sage, 2016; Naidu, Moose, Al-Shoaibi, Raines, & Long, 2003; Spence et al., 2014; Wang, Naidu, Portis, Moose, & Long, 2008; Wang, Portis, Moose, & Long, 2008) and increasing capacity for non-photochemical quenching of PSII excitation energy upon chilling (Farage, Blowers, Long, & Baker, 2006; Spence et al., 2014).

We show the exceptional photosynthetic performance of *M. x giganteus* at low temperature can be surpassed. Field screening of Fv/Fm indicated a high potential for chilling tolerance within the Siberian *Miscanthus* germplasm (Figure S3).
Nearly all *M. sacchariflorus* accessions surpassed the $F_v/F_m$ of *M. x giganteus*, and achieved values as high as 0.74, remarkably close to the values of 0.77 achieved by healthy upper canopy leaves of *M. x giganteus* ‘Illinois’ grown in the warm summer of the US Midwest (Pignon, 2015). A previous study has identified *M. sacchariflorus* accessions which exceeded those of *M. sacchariflorus* nearly all *F_v/F_m* achieves a higher *A_sat* \( \text{sat} \) to 25°C (day 16) in *M. x giganteus* (Dunnett’s one-tailed test). For day 23, # and * identify marginally significant \( p < 0.05 \) different value between a *M. sacchariflorus* accession and *M. x giganteus* (Dunnett’s two-tailed test). Values in bold black are marginally significant or significantly different from *M. x giganteus* accession and *M. sacchariflorus* accessions during transient warm periods after 7 days at 25°C (day 23), such temperatures for such a prolonged period would be a rare event in the spring and autumn of the current regions of cultivation of this crop (Figure 2a, Table 1) (U.S.D.A, 2017). The discovery of accessions with far more rapid recovery on return to warm weather (day 16) than in the widely used ‘Illinois’ clone of *M. x giganteus*, suggests important genetic resources for producing new lines of *M. x giganteus* that can recover rapidly during warm spells interspersed with chilling events. Poor short-term recovery of *A_sat* to 25°C (day 16) in *M. x giganteus* suggests it would underperform relative to these *M. sacchariflorus* accessions during transient warm periods early in the growing season, particularly relative to the rapid recovery of RU2012-114 (Figure 2a, Table 1) (Glowacka et al., 2015).
are particularly damaging to photosynthesis in C₄ crops species under chilling conditions (Long & Spence, 2013). This might explain the higher photosynthetic rates it achieved a week after the end of the chilling treatment (day 23) (Figure 2a, Table 1). While the operating point of photosynthesis was Vₘₐₓ -limited at low temperature, control of Aₛₐₜ was shared with gₛ and Vₚₘₐₓ in M. x giganteus on day 23, contributing to the higher Aₛₐₜ (Figure 3) (Glowacka, Jorgensen, et al., 2015).

4.2 Low-temperature Aₛₐₜ primarily driven by Vₘₐₓ

For all accessions and prior to chilling (day 0), the operating point of the Aₐₛₜ response was at the point of inflexion between limitation by the rate of PEP carboxylation (Vₚₘₐₓ) and regeneration (Vₘₐₓ), when measured at 25°C prior to the chilling treatment (Figure 3). However, during chilling the operating point was on the plateau of the Aₛₐₜ response for all accessions, showing biochemical limitation by Vₘₐₓ and negligible stomatal limitation (Figure 3). Metabolic control analysis indicates that Vₘₐₓ is limited by Rubisco, PPDK or the two in combination (Furbank et al., 1997). The higher Aₛₐₜ of RU2012-069 achieved during chilling compared to the other accessions suggests a likely increase in these proteins and/or their activation (Table 1, Figure 2a, d, Figure 3).

Given the low stomatal limitation to A in all accessions, reducing gₛ should in theory reduce evapotranspiratory cooling and slightly warm the leaf, with minimal cost to A (i.e. shifting the operating point to the left of the A‐cₗₐₜ curve) (Figure 3). The fact this does not occur in any of the accessions studied here may be an indication of impaired stomatal control at low temperature, a commonly noted feature of C₄ species under chilling conditions (Long & Spence, 2013).

The combination of chilling temperatures and high light are particularly damaging to photosynthesis in C₄ crops resulting in a decline in the maximum efficiency of photosystem II measurable as a decrease in Fₚₗ/Fₘ and in the operating efficiency of PSII (Φₚₛₛᵢₐ) (Baker et al., 1989). At lower photon fluxes, the greater Φₚₛₛᵢₐ seen in M. sacchariflorus accessions would contribute to improved photosynthesis (Figure 4c) (Glowacka et al., 2014; Glowacka, Jorgensen, et al., 2015). While greater Φₚₛₛᵢₐ may be indicative of reduced photoinhibition, and therefore improved chilling tolerance, this by definition will not restrict photosynthesis under the light-saturating conditions used here during the controlled-environment experiments (Glowacka, Jorgensen, et al., 2015; Pignon, Jaiswal, Mcgrath, & Long, 2017).

M. sacchariflorus typically shows greater chilling tolerance (Glowacka et al., 2014; Glowacka, Jorgensen, et al., 2015) and genetic diversity (Clark et al., 2018) than M. sinensis. This is in accordance with the natural distribution of M. sacchariflorus, which extends further north into Asia than M. sinensis (Clark et al., 2014; Clifton-Brown et al., 2008). To maximize the effectiveness of breeding programs involving the M. sacchariflorus accessions described here, it will be necessary to pair them with high performance M. sinensis accessions: crosses with warmer temperate-adapted accessions could produce progeny able to grow under a wide range of climates and surpass both parent’s yield potential (Dong et al., 2018; Farrell et al., 2006). This result suggests a means to add to the recent discovery of genetic markers associated with biomass yield in Miscanthus (Dong et al., 2018).

In conclusion, this study, the first to investigate chilling tolerance of photosynthesis in Siberian Miscanthus sacchariflorus has identified accessions with greater tolerance than the widely grown M. x giganteus ‘Illinois’ clone. These accessions show both higher photosynthetic capacity during a 2-week period of chilling and faster recovery over the first 24 hr of return to non-chilling temperatures. The study has therefore identified important genetic resources for this key trait of low temperature performance for use in the breeding of improved lines of M. x giganteus for cool climates.

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