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Can chilling tolerance of C_4 photosynthesis in *Miscanthus* be transferred to sugarcane?

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Abstract

The goal of this study was to investigate whether chilling tolerance of C_4 photosynthesis in *Miscanthus* can be transferred to sugarcane by hybridization. Net leaf CO_2 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 day in controlled environment chambers. Two of three hybrids (miscanes), 'US 84-1058' and 'US 87-1019', did not differ significantly from the chilling tolerant *M.* ×*giganteus* 'Illinois' (Mxg), for A_{sat}, and Φ_{PSII} measured during chilling. For Mxg grown at 10 °C/5 °C for 11 days, A_{sat} was 4.4 μmol m^{-2} s^{-1}, while for miscane 'US 84-1058' and 'US 87-1019', A_{sat} was 5.7 and 3.5 μmol m^{-2} s^{-1}, respectively. Miscanes 'US 84-1058' and 'US 87-1019' and Mxg had significantly higher rates of A_{sat} during chilling than three tested sugarcanes. A third miscane showed lower rates than Mxg during chilling, but recovered to higher rates than sugarcane upon return to warm conditions. Chilling tolerance of 'US 84-1058' was further confirmed under autumn field conditions in southern Illinois. The selected chilling tolerant miscanes have particular value for biomass feedstock and biofuel production and at the same time they can be a starting point for extending sugarcane's range to colder climates.

Keywords: bioenergy crop, chilling, cold tolerance, miscane, *Miscanthus* ×*giganteus*, photosynthesis, plant breeding, *Saccharum officinarum*, sugarcane

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Introduction

Sugarcane (*Saccharum* spp. hybrid) is one of the world's most important crops. In 2013, sugarcane produced 1.9 billion tonnes of biomass, more than any other single crop, for sugar and bioenergy via ethanol and electricity (Botha & Moore, 2014; FAOSTAT, 2014). Sugarcane is grown commercially in over 100 countries on a total of 26.5 million hectares (data for 2013; FAOSTAT, 2014). However, commercial sugarcane production is limited to tropical and subtropical environments, due to the crop's limited tolerance to cold; southern Louisiana, USA, is perhaps where commercial production is the most challenged by cold. Agronomic success of modern sugarcane varieties can be explained by effective introgression of genes from wild germplasm, particularly from *S. spontaneum* into *S. officinarum*, starting in the early 1900s (Daniels & Roach, 1987; D'Hont *et al.*, 1996; Hoarau *et al.*, 2001; Piperidis *et al.*, 2010; Andru *et al.*, 2011). Additional genetic contributions from *S. robustum*, *S. sinense* and *S. barberi* are most likely present in modern sugarcane varieties (Daniels & Roach, 1987; Lima *et al.*, 2002; Brown *et al.*, 2007). The introgressed genes provide sources of disease resistance, vigor, ability to ratoon, and better yields under abiotic stresses (Mangelsdorf, 1960; Chen & Lo, 1988; Chen, 1993).

Miscanthus is a potentially valuable genetic resource for improving sugarcane. Particularly, *Miscanthus* is a source of resistance to downy mildew (*Peronosclerospora sacchari*), culmicolus smut (*Sporisorium scitamineum*; Chen & Lo, 1988), lesion nematodes (*Pratylenchus* spp.; E. Sacks, personal communication), as well as tolerance to drought and cold (Lo *et al.*, 1978). Previously, the Taiwan Sugarcane Institute's collection of over 120 *Miscanthus* clones was evaluated to select parents for resistance to culmicolus smut and downy mildew and then to introduce the resistance into sugarcane by intergeneric hybridization with sugarcane and subsequent backcrossing to sugarcane (Chen & Lo, 1988). In the second backcross of the intergeneric hybrids to sugarcane (BC2), the downy mildew resistance inherited from *Miscanthus* was maintained and at the same time sugar content similar to the sugarcane parent's was restored.

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The hybrids of *Saccharum* × *Miscanthus* are sometimes named 'miscanes' and in addition to their use for sugarcane improvement, they also show promise as a highly productive cellulosic biomass crop (Park *et al*., 2011). Although there are many reports about hybrids between *Saccharum* and *Miscanthus* (Li *et al*., 1948, 1953, 1961; Loh & Wu, 1949; Price, 1965; Chen & Lo, 1988; Xiao & Tai, 1994; Burner, 1997; Chen *et al*., 2000), there has been little reported on tolerance of miscanes to abiotic stresses, particularly chill tolerance of photosynthesis (≤14 °C) (Burner *et al*., 2009).

C_4 is potentially more efficient than C_3 photosynthesis in its use of light, nitrogen, and water (Long, 1983; Long & Spence, 2013). However, in cooler environments, peak yields of most C_4 plants are markedly reduced, and only a few C_4 species (e.g., *Miscanthus ×giganteus, Spartina anglica,* and *Spartina pectinata*) can match the production of C_3 crops under cooler temperatures (Heaton *et al*., 2008; Long & Spence, 2013; Sage *et al*., 2014). In stark contrast to *Miscanthus*, sugarcane is noted to be one of the most chilling sensitive crop species. When grown at 10 °C/5 °C (day/night) for 11 days, sugarcane exhibited a >98% reduction in CO_2 assimilation relative to control plants grown at 25 °C/20 °C (day/night) (Głowacka *et al*., 2014). At 8–12 °C, sugarcane CO_2 assimilation extrapolates to zero (Nose *et al*., 1994) while its close relative *M. ×giganteus* retains most of its photosynthetic capacity (Farage *et al*., 2006). Below 20 °C, leaves of sugarcane grow slowly (Allison *et al*., 2007) and when grown below 10–15 °C leaf elongation is negligible (1.4 mm day^{-1}) (Głowacka *et al*., 2014) or absent (Allison *et al*., 2007). Studies of the effect of cool temperatures on field-grown sugarcane in Hawaii revealed seasonal differences in chilling injury. In winter, minimum leaf temperatures of ca. 14 °C were associated with inhibition, and in summer, minimum temperatures as high as ca. 20 °C were sufficiently cool to reduce the maximal photosynthesis capacity (Grantz, 1989). For these reasons, sugarcane is usually grown between latitude 30N and 35S, but its northern range limit has not been firmly established. In contrast to sugarcane, the exceptional chilling tolerance of *Miscanthus* allows it to be grown with success in the cooler climates of NW Europe (Beale & Long, 1995; Lewandowski *et al*., 2000; Clifton-Brown *et al*., 2001; Jeżowski *et al*., 2011) and the Midwest USA (Heaton *et al*., 2008). Thus, hybridization of sugarcane with chilling tolerant *Miscanthus* germplasm could theoretically provide a means to develop more chilling tolerant sugarcane.

For perennial plants adapted to temperate environments, overwintering requires survival at temperatures that are not conducive to growth, and especially tolerance to freezing. For *Miscanthus,* overwintering in temperate environments is facilitated by dormancy. After a perennial crop survives the winter, its next challenge is to establish photosynthetically competent leaves as early in the growing season as possible and maintain photosynthesis as late into the growing season as temperatures will allow, thereby maximizing carbon assimilation over the season (Long & Spence, 2013). As demonstrated by Farrell *et al*.'s (2006), productivity model, extending the growing season for *Miscanthus* by 30 days, would result in up to 25% higher yield. However, earlier canopy development will only result in higher yield if early growth and low temperature tolerance are combined. Leaf necrosis resulting from late frosts during the beginning of spring can greatly retard canopy establishment because few nutrients will remain in the rhizomes after initial growth for a second cohort of shoots (Kaiser, 2014; K. Głowacka data not published). For these reasons, early emergence of leaves that are photosynthetically competent at chilling temperatures is the crucial feature of highly productive perennial grasses in temperate climate.

For the present study, we chose three *Saccharum* sp. × *Miscanthus* sp. hybrids that had been previously observed to overwinter as far north as Booneville, Arkansas (35°05′N, 93°59′W), with a minimum winter air temperature of −14 °C and an average monthly temperature of −0.3 °C in the coldest month of 2000 (Burner *et al*., 2009). Although rhizomes of these three selected miscanes showed cold tolerance for overwintering, their ability to maintain photosynthetic capacity in aboveground tissues under chilling conditions (≤14 °C) has not been determined.

This study examines whether (i) the chilling tolerance of C_4 photosynthesis in *Miscanthus* is apparent in the hybrids under controlled and field conditions; (ii) the hybrids show improved recovery of photosynthesis upon return to warm conditions relative to sugarcane; and (iii) the hybrids retain the high photosynthetic capacity of sugarcane under warm conditions, that is, is chilling tolerance achieved at the expense of capacity under warm conditions?

Materials and methods

Plant material

Eight genotypes were studied (Table 1) as follows: three miscanes ('US84-1028', 'US84-1058', and 'US87-1019'), three sugarcanes (*Saccharum* sp. 'L79-1002', *S. officinarum* 'Louisiana Purple', and *Saccharum* hybr. 'NCo310'), the chill tolerant control *M. ×giganteus* (3x) 'Illinois' (Mxg), and a chilling sensitive control, *Z. mays* 'FR1064'. Miscane, 'US84-1028', was obtained from a cross between the elite sugarcane cultivar *Saccharum* sp. 'CP78-2042' (GRIN, 2008) and *M. sinensis* clone 'US58-2-1' (Burner *et al*., 2009). Miscane 'US84-1058' was a hybrid between the wild sugarcane *S. spontaneum* 'Saudi Arabia' and an unspeci-

Table 1 Accessions of miscanes and controls studied for photosynthetic response to low temperature, including three *Saccharum* × *Miscanthus* hybrids (miscanes), three controls from *Saccharum*, one from *Zea* and one from *Miscanthus*

Name	Accession identifier	Pedigree	Source
Putative miscanes (*Saccharum* × *Miscanthus* hybrid)*			
Miscane 'US84-1028'	US84-1028	*Saccharum* sp 'CP78-2042' × *M. sinensis* clone 'US58-2-1'	USDA-ARS Sugarcane Field Station, Canal Point, FL
Miscane 'US84-1058'	US84-1058	*S. spontaneum* 'Saudi Arabia' × unspecified *Miscanthus* sp.	USDA-ARS Sugarcane Field Station, Canal Point, FL
Miscane 'US87-1019'	US87-1019	*Saccharum* hybr. 'NCo310' × *Miscanthus* sp. clone '3905'	USDA-ARS Sugarcane Field Station, Canal Point, FL
Controls from *Saccharum*			
Saccharum sp. 'L79-1002'	PI651501		USDA-NPGS
S. officinarum 'Louisiana Purple'	PI495639		USDA-NPGS
Saccharum hybr. 'NCo310'	PI504672		USDA-NPGS
Negative controls from Zea			
Zea mays inbred line 'FR1064'	FR1064		S. Moose, UI, USA ← Illinois Foundation Seeds, IL, USA
Positive controls from *Miscanthus*			
M. ×*giganteus* 'Illinois'	UI10-00107		T. Voigt, UI, USA ← Chicago Botanic Garden, USA

UI, University of Illinois; USDA-ARS, United States Department of Agriculture – Agricultural Research Service; USDA-NPGS, United States Department of Agriculture – National Plant Germplasm System.
*Tai & Miller, 1988; Tai *et al*., 1991; Burner *et al*., 2009.

fied *Miscanthus* clone (Burner *et al*., 2009). The third miscane study, 'US87-1019', was from a cross between the commercial cultivar *Saccharum* hybr. 'NCo310', developed in KwaZulu-Natal, South Africa (GRIN, 2014), and *Miscanthus* sp. clone '3905' (Tai & Miller, 1988; Tai *et al*., 1991; Burner *et al*., 2009). The studied miscanes were first generation progeny (F1) of crosses made in the 1980s by USDA-ARS in Florida, USA. The *Miscanthus* parental lines of the three miscanes were no longer available from USDA, and similarly, the *Saccharum* sp. parent, 'CP78-2042', was also unavailable (J. C. Comstock, personal communication). *Saccharum* sp. 'L79-1002' is an energycane bred for Louisiana; it is an F1 hybrid of commercial sugarcane 'CP 52-68' and *S. spontaneum* (Bischoff *et al*., 2008). Previously, it was shown that *Saccharum* sp. 'L79-1002' grown in a location farther north (32.1°N latitude) than traditional sugarcane production can produce higher total yield (entire above ground biomass) than the commercial sugarcane standard, 'CP 65-357' (Bischoff *et al*., 2008).

Propagation of plant material

The sugarcanes and miscanes were propagated from 10 to 15 cm stem sections with mature buds at the nodes. With the sheathing leaves removed, bare stem pieces were planted vertically in cell trays (38-cell star trays; T.O. Plastics) with one stem piece per cell containing a peat-, bark-, and perlite-based growing medium (Metro-Mix 900; Sun Gro Horticulture, Agawam, MA, USA). Cells were kept initially in greenhouse under mist (VibroNet Mister Nozzle, 20.1 l h^{-1}; Netafime; Tel Aviv, Israel) for 10s every 10 min during daylight hours. The day/night cycle followed natural light with the temperatures 25 °C/21 °C. After the new shoots appeared, clonal divisions were transferred to 1 l pots of the same soil mix (mini-treepot # MT38; Stuewe & Sons, Tangent, OR, USA) for subsequent use in controlled environment chambers. Mxg was propagated from 3-cm-long rhizome pieces with visible roots and nodes, then grown in 1 l pots as described above. *Z. mays* seeds were sown directly into 1 l pots. When the plants were transferred to minitreepots, an all-purpose slow release fertilizer was added following the manufacturer's instructions (Osmocote Classic, 8–9 mo 13-13-13; Everris NA, Inc., Dublin, OH, USA), and one teaspoon of additional ferrous sulfate heptahydrate per pot was added (QC Corporation, Girardeau, MO, USA). Prior to transfer to controlled environment cabinets, plants were grown in a greenhouse at ~25 °C. Throughout, soil moisture content was maintained by watering to field capacity daily.

Gas exchange and chlorophyll fluorescence in controlled environment chambers

To mimic the type of chilling that might develop during spring after leaf emergence or to expanded leaves in the autumn, the plants were grown at: 25 °C day/20 °C night (warm) for 10 days, followed by 11 days at 10 °C/5 °C (chilling), and then returned to 25 °C/20 °C for one day. From three to six replicate, plants for each of the eight accessions were grown in two controlled environment chambers (Conviron PGC20; Controlled Environments, Winnipeg, Manitoba, Canada) equipped with an opened counter-balanced light canopy with ten high output dimmable metal halide bulbs (Mastercolor CDM_TP MV; PHILLIPS).

Leaf photosynthetic gas exchange and modulated chlorophyll fluorescence were measured on the most recently emerged leaf on the main stem, as judged by ligule appearance. The positions of plants within the chambers were changed every 2 days to avoid confounding any undetected variation in environment within the chamber with accessions. 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, with an open gas exchange system incorporating differential infrared CO_2 and water vapor analyzers (LI-6400, LI-COR, Lincoln, NE, USA). With 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 in ambient air (210 mmol mol^{-1} O_2 and 390 μmol mol^{-1} CO_2), 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 (Genty *et al*., 1989). These measurements were taken 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 days at 10 °C (except days 6, 8, and 10), and finally one day after transferring the plants back to 25 °C (12th day of the experiment – recovery). All measurements were taken during the daylight hours on light-adapted leaves when a steady state CO_2 and water vapor flux was obtained in the cuvette (20–50 min). For each accession, from three to six replicate plants were measured for each treatment. From these procedures, measurements of light-saturated leaf net CO_2 uptake per unit leaf area (A_{sat}), quantum yield of photosystem II (Φ_{PSII}), stomatal conductance to water vapor (g_s), and intercellular CO_2 concentration (c_i) were obtained as described previously (Bernacchi *et al*., 2003).

Field experiment

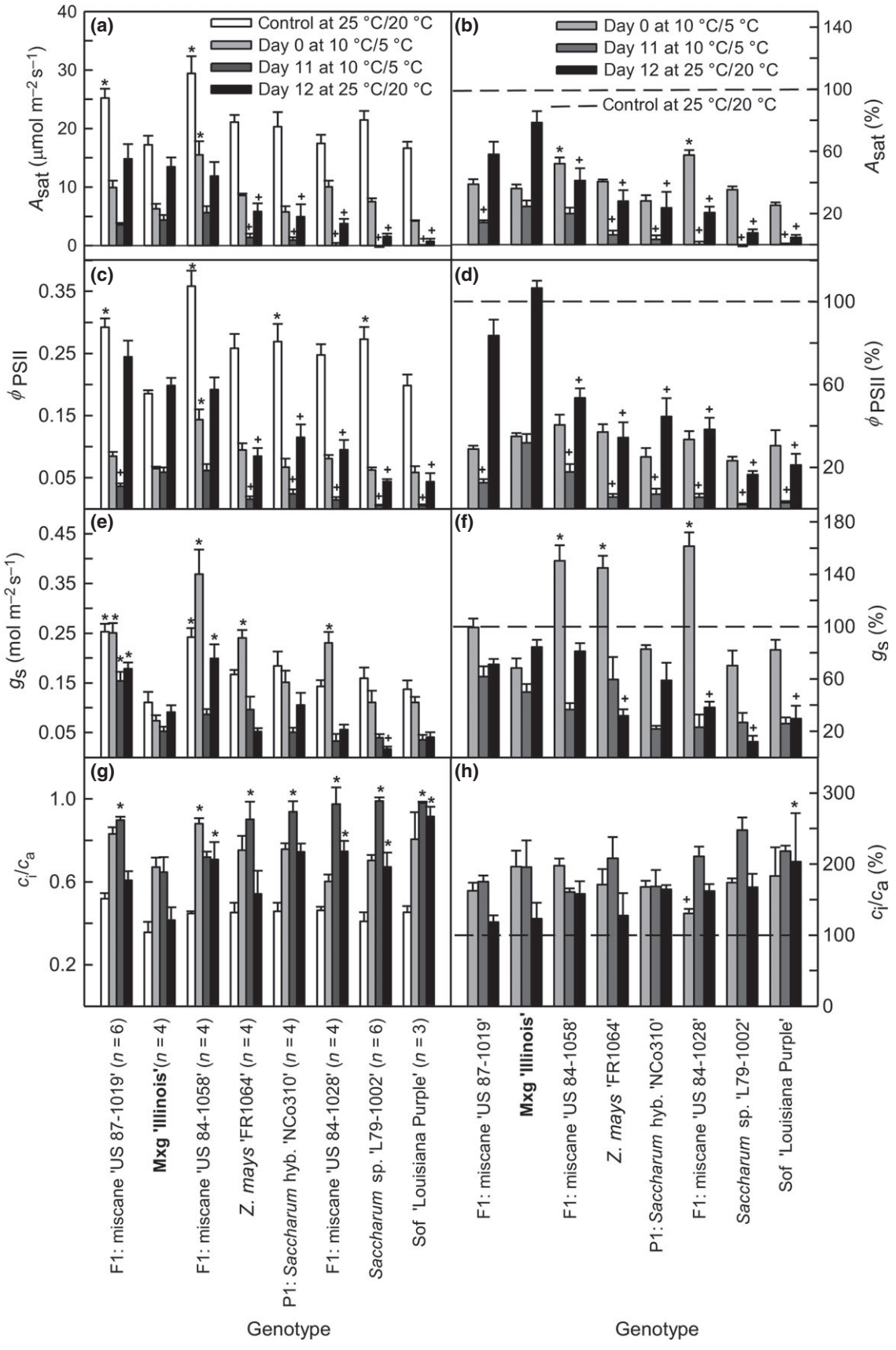
A field experiment was established on May 22, 2013 at the Dixon Springs Agricultural Center (37° 26′18″N, 88°39′56″ W; USDA hardiness zone 6/7 border) from plugs propagated in cells, as detailed above. Plots were single rows of eight plants (ramets), spaced on 0.9 m centers. The trial was a randomized complete block design with four replicates. The soil was a silt loam (fine-silty, mixed, active, mesic, Oxyaquic, Fragiudalfs, 1–3% organic matter). Air temperature at a height of 2 m above ground was recorded every 10 s by the meteorological station which was located 400 m away from the planting (WARM, 2014).

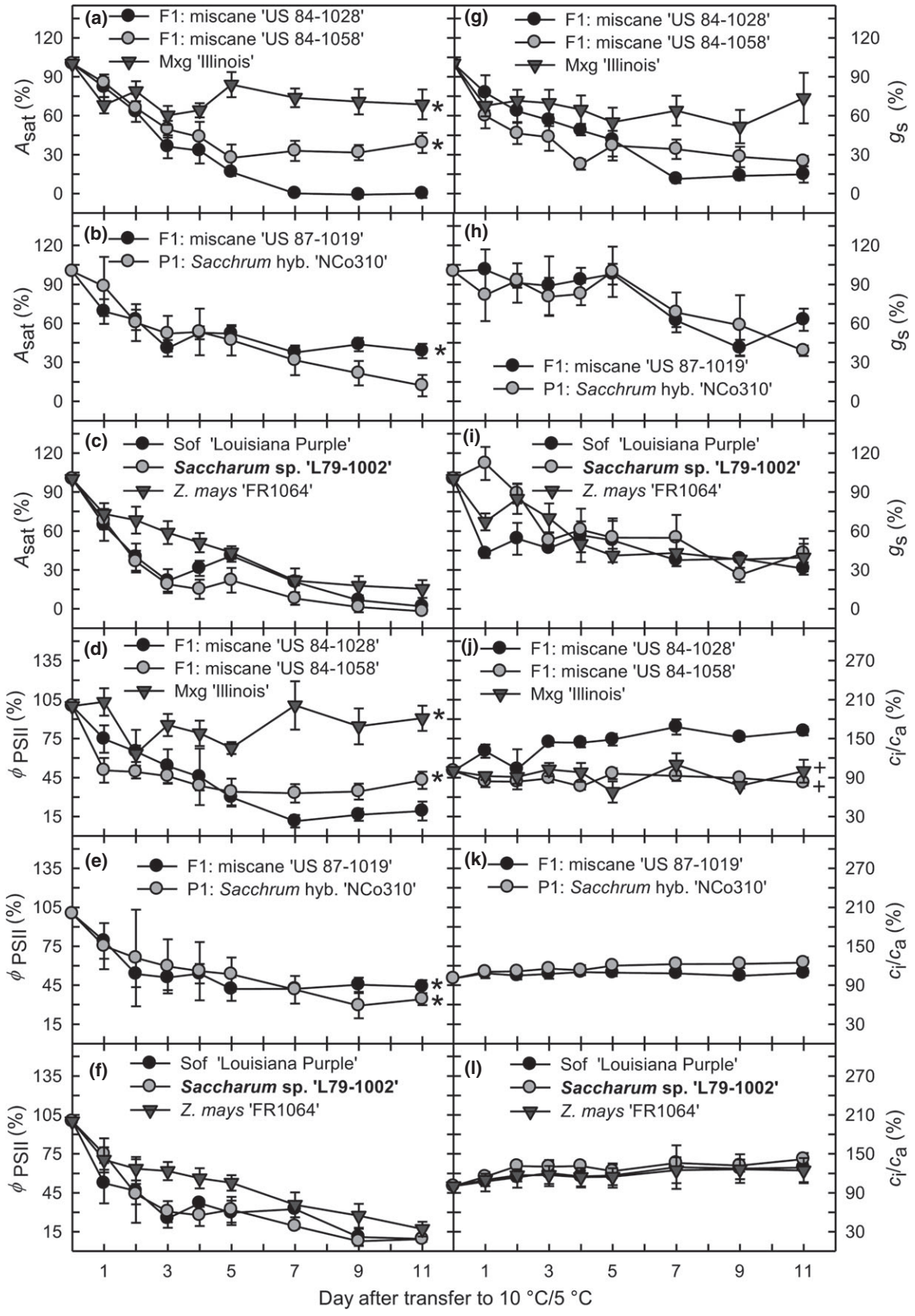
At the end of first growing season on October 23, 2013 and October 24, 2013, photosynthetic leaf CO_2 uptake was measured, as described above, in ambient air (210 mmol mol^{-1} O_2 and 400 μmol mol^{-1} CO_2), at 1500 μmol m^{-2} s^{-1} of photon flux and 65% relative humidity. Leaf temperature was maintained in the cuvette at the average ambient temperature of 13 °C.

Data analysis

Statistical analyses were performed with SAS PROCEDURE GLM (SAS v. 9.3, SAS Institute, Cary, NC, USA). For data from the growth chamber experiment, two-way analyses of variance were conducted to determine whether genotype and/or treatment had significant effects on A_{sat}, Φ_{PSII}, g_s, and c_i/c_a. Analyses were conducted for each day of the experiment. Dunnett's multiple comparison tests were used to compare each genotype with the chilling tolerant control, Mxg (Fig. 1), or with the chilling sensitive control, *Saccharum* sp. 'L79-1002' (Fig. 2). For the field experiment, in which data were collected during two consecutive days, two-way analyses of variance were performed to assess whether there were significant differences between days and tested genotypes. As the date of measurement in the field experiment did not significantly affect the measured parameters A_{sat}, g_s, and c_i/c_a ($P = 0.08$; $P = 0.29$; $P = 0.65$, respectively), the date factor was omitted in the final analyses. For comparisons of means between the Mxg control and other genotypes, Dunnett's was used (Fig. 3c–e).

Fig. 1 (a–b) Light-saturated leaf net CO_2 uptake rate (A_{sat}), (c–d) quantum yield of photosystem II (Φ_{PSII}), (e–f) stomatal conductance to water vapor (g_s), and (g–h) 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 11th day of chilling treatment and one day after transfer of plants back to the warm conditions (12th day of experiment – recovery). Left panels (a, c, e, and g) are absolute values; right panels (b, d, f, and h) indicate responses to treatments expressed as a percentage of rates observed in warm conditions before the chilling treatment (i.e., percentage of control, white bars in the adjacent left panels). Plants were grown at 25 °C/20 °C (warm) day/night, with 14-h-day/10-h-night cycle under 1000 μmol photons m^{-2} s^{-1}, excepting the 11 days at 10 °C/5 °C (chilling). In all panels, accessions are ordered according to A_{sat} on day 12 of the experiment (panel a; from highest to lowest; fourth bar (black fill) for each genotype). For each treatment stage, an asterisk indicates a significantly higher value and a cross indicates a significantly lower value in comparison with Mxg 'Illinois' based on Dunnett's test ($P \leq 0.05$). Time-point values for Mxg 'Illinois' were as follows: (a) 17.2, 6.3, 4.4, and 13.5 (μmol m^{-2} s^{-1}); (b) 36, 25, and 79 (%); (c) 0.19; 0.06; 0.06, and 0.20 (dimensionless); (d) 35, 32, and 107 (%) (e) 0.11, 0.07, 0.05, and 0.09 (mol m^{-2} s^{-1}); (f) 68, 50, and 84 (%); (g) 0.36, 0.67, 0.65, and 0.41 (dimensionless); (h) 197, 196, and 123 (%). Data are mean + SE (n = from 3 to 6, as indicated below panel g). F1 = the first generation of *Saccharum* × *Miscanthus* hybrids (miscane); Mxg = *M.* ×*giganteus*; P1 = parent 1 of miscane 'US 87-1019'; Sof = *Saccharum officinarum*.




(a) F1: miscane 'US 84-1028'
F1: miscane 'US 84-1058'
Mxg 'Illinois'
(b) F1: miscane 'US 87-1019'
P1: Sacchrum hyb. 'NCo310'
(c) Sof 'Louisiana Purple'
Saccharum sp. 'L79-1002'
Z. mays 'FR1064'
(d) F1: miscane 'US 84-1028'
F1: miscane 'US 84-1058'
Mxg 'Illinois'
(e) F1: miscane 'US 87-1019'
P1: Sacchrum hyb. 'NCo310'
(f) Sof 'Louisiana Purple'
Saccharum sp. 'L79-1002'
Z. mays 'FR1064'
(g) F1: miscane 'US 84-1028'
F1: miscane 'US 84-1058'
Mxg 'Illinois'
(h) F1: miscane 'US 87-1019'
P1: Sacchrum hyb. 'NCo310'
(i) Sof 'Louisiana Purple'
Saccharum sp. 'L79-1002'
Z. mays 'FR1064'
(j) F1: miscane 'US 84-1028'
F1: miscane 'US 84-1058'
Mxg 'Illinois'
(k) F1: miscane 'US 87-1019'
P1: Sacchrum hyb. 'NCo310'
(l) Sof 'Louisiana Purple'
Saccharum sp. 'L79-1002'
Z. mays 'FR1064'
Asat (%)
gs (%)
ϕ PSII (%)
ci/ca (%)
Day after transfer to 10 °C/5 °C

Results

Chilling experiment in controlled environment chambers

Two of three miscanes, 'US 84-1058' and 'US 87-1019', did not differ significantly for A_{sat} from the chilling tolerant control, Mxg, after being subjected to chilling conditions (10 °C/5 °C) for 11 days, and also after subsequently being returned to warm temperatures for one day (25 °C/20 °C) (Fig. 1a). For Mxg grown at 10 °C/5 °C for 11 days, A_{sat} was 4.4 μmol m^{-2} s^{-1}, while for miscane 'US 84-1058' and 'US 87-1019', A_{sat} was 5.7 and 3.5 μmol m^{-2} s^{-1}, respectively, which were not significantly different from that of Mxg. Moreover, 'US 87-1019' and 'US 84-1058' showed the highest rates of leaf CO_2 uptake prior to chilling treatment (Fig. 1a), indicating that their improved chilling tolerance was not at the expense of photosynthetic capacity under warm conditions. Additionally, at the beginning of the chilling treatment (day 0 of experiment when plants were transferred from 25 °C to 10 °C), miscane 'US 84-1058' had A_{sat} and Φ_{PSII} values twice (15.5 μmol m^{-2} s^{-1} and 0.14, respectively) and miscane 'US 87-1019' over 1.3 times (9.9 μmol m^{-2} s^{-1} and 0.08, respectively) as large as those of Mxg (6.3 μmol m^{-2} s^{-1} and 0.06, respectively) (Fig. 1a,c). In contrast, the remaining accessions after 11 day at 10 °C/5 °C exhibited up to 103% reduction in A_{sat} (*Saccharum* sp. 'L79-1002') and up to 90% reduction in Φ_{PSII} (*Saccharum* sp. 'L79-1002' and *S. officinarum* 'Louisiana Purple') relative to Mxg. Recovery values, one day after transfer back to 25 °C, were similar for the two miscanes 'US 87-1019', 'US 84-1058' and Mxg, with A_{sat} between 11.9 and 14.8 μmol m^{-2} s^{-1} and Φ_{PSII} between 0.20 and 0.24. Relative to the prechilling conditions, Mxg, miscane 'US 87-1019' and 'US 84-1058' showed 79%, 58%, and 41% recovery of A_{sat}, respectively (Fig. 1b), and 107%, 84%, and 54% recovery of Φ_{PSII}, respectively (Fig 1d). The lowest recovery of photosynthesis on return to warm conditions was observed for the sugarcane *S. officinarum* 'Louisiana Purple' (4% of the prechilling A_{sat} and 21% of prechilling Φ_{PSII}), which was significantly lower than the Mxg control ($P < 0.001$).

For the chilling tolerant control, Mxg, A_{sat}, and Φ_{PSII} declined for the first 2–3 day at 10 °C/5 °C, followed by a rebound over the following days to stabilize at 69% and 91% of the rates on initial transfer to 10 °C/5 °C, respectively (Fig. 2a,d). Two miscanes 'US 87-1019' and 'US-1058' also showed a rebound in photosynthesis starting from day 5 or 6, with the A_{sat} and Φ_{PSII} on the final day of 10 °C/5 °C treatment ending at ~39–44% of the rates recorded on day 0 of chill treatment (Fig. 2a, d). Only miscanes 'US 87-1019', 'US 84-1058', and the chilling tolerant control, Mxg, had significantly higher level of stabilization of A_{sat} than the cane *Saccharum* sp. 'L79-1002', which failed to stabilize readings of A_{sat} during 11 days of chilling treatment and ended at −2% of the day 0 rates, respectively (Fig. 2a–c). For $\Phi_{PSII,}$ four accessions, miscane 'US 87-1019', miscane 'US-1058', *Saccharum* hybr. 'NCo310', and Mxg, stabilized at significantly higher levels than *Saccharum* sp. 'L79-1002' (Fig. 2d–f). However, the commercial cultivar *Saccharum* hybr. 'NCo310' was not significantly different from energycane 'L79-1002' for A_{sat}, and c_i/c_a at the end of chilling period (after 11 day in 10 °C/5 °C). There were no significant differences between energycane 'L79-1002', *S. officinarum* 'Louisiana Purple' and *Z. mays* 'FR1064' for A_{sat}, Φ_{PSII}, g_s, and c_i/c_a after 11 days in 10 °C/5 °C (Fig. 2c,f,i,l).

All lines increased in c_i/c_a on transfer to 10 °C/5 °C (Fig. 1g–h). Over the 11 days of chilling, all genotypes except miscane 'US-1058' had significantly higher c_i/c_a than the Mxg control. In contrast to the observations for c_i/c_a, g_s of all genotypes decreased over the 11 days of chilling (Fig. 1e–f). For three of eight genotypes, g_s increased with the onset of chilling on day 0 and then decreased during the subsequent days of chilling treatment; the other five genotypes decreased in g_s after only 20 min in chilling. One day after, the plants were transferred from chilling to warm conditions, miscanes 'US 87-1019' and 'US-1058' had a similar recovery of g_s as Mxg, but the other genotypes did not (Fig. 1f).

Fig. 2 Changes in relative values for gas exchange and fluorescence parameters over 11 days of chilling treatment (10 °C/5 °C day/night). Values are expressed as a percentage of rates on day 0 measured immediately after transfer of plants from warm (25 °C/20 °C day/night) to chilling conditions. Light-saturated leaf net CO_2 uptake rate (A_{sat}; a–c), quantum yield of photosystem II (Φ_{PSII}; d–f), stomatal conductance to water vapor (g_s; g–i), and ratio of intercellular to atmospheric CO_2 concentration (c_i/c_a; j–l). Panels a, d, g, and j are miscanes 'US 84-1028', 'US 84-1058' and positive control Mxg 'Illinois', b, e, h, and k are miscane 'US 87-1019' and its cane parent *Saccharum* hyb. 'NCo310', c, f, i, and l are negative controls. Data are means ± SE (n = from 3 to 6, as indicted below Fig. 1g). An asterisk indicates a significantly higher value, and a cross indicates a significantly lower value in comparison with *Saccharum* sp. 'L79-1002' (bold) on the 11th day after transfer to 10 °C/5 °C based on Dunnett's test ($P \leq 0.05$). Values for *Saccharum* sp. 'L79-1002' on the 11th day of chilling treatment were as follows: (c) −2%, (f) 9%, (i) 43% (l) 142%. F1 = the first generation of *Saccharum* × *Miscanthus* hybrids (miscane); Mxg = *M.* ×*giganteus*; P1 = parent 1 of miscane 'US 87-1019'; Sof = *Saccharum officinarum*.

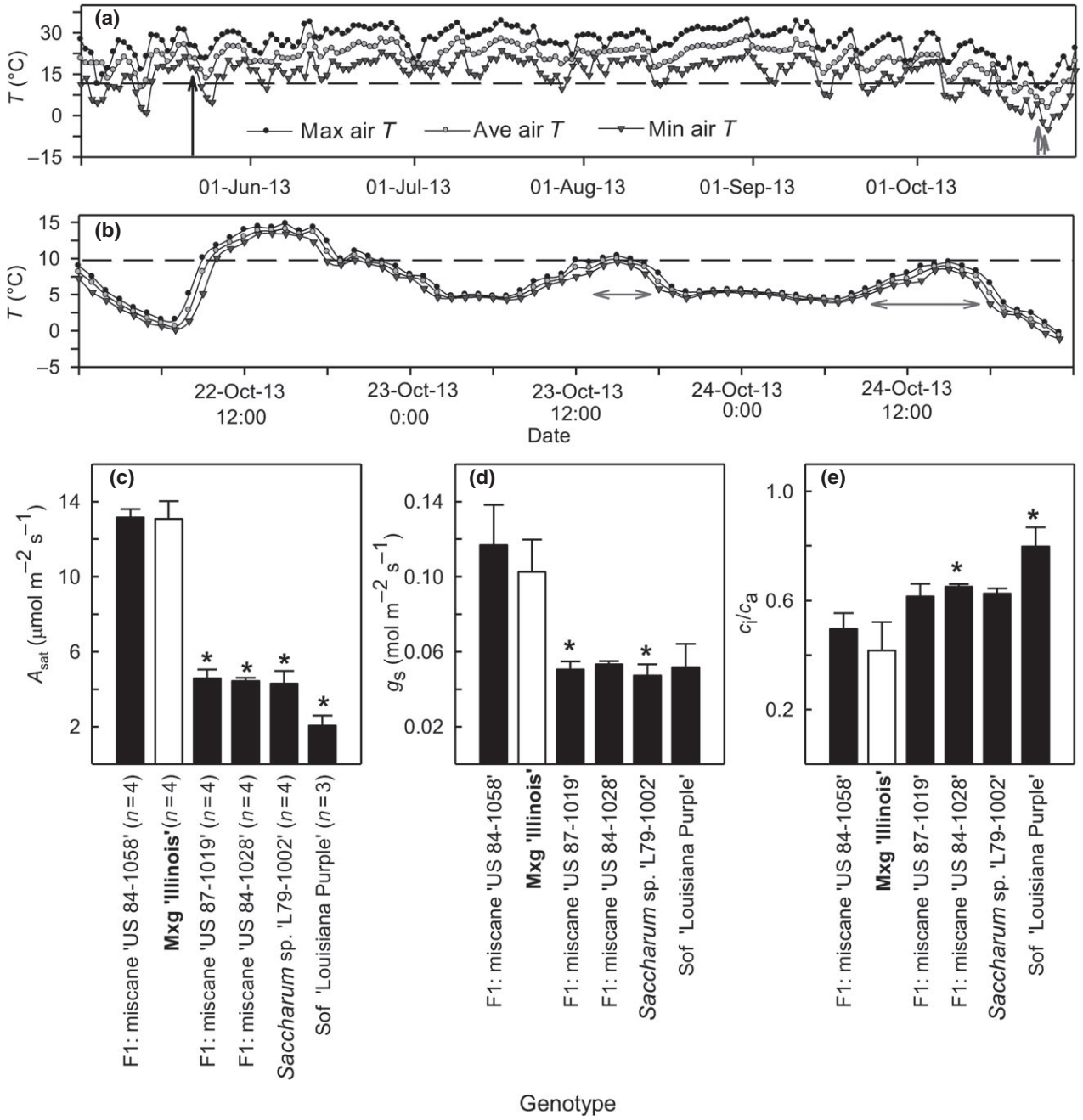


Fig. 3 Air temperatures during the 2013 growing season for a field trial at the Dixon Springs Agricultural Center in southern Illinois where on 23–24 Oct 2013 leaf gas exchange rates were measured. (a) Average daily temperatures for growing season; (b) average hourly temperatures in the period between day before and day after measurements; (c) light-saturated leaf net CO_2 uptake rate (A_{sat}); (d) stomatal conductance to water vapor (g_s); and (e) ratio of intercellular to atmospheric CO_2 concentration (c_i/c_a). The black arrow (panel a) indicates day of planting, and the gray arrows (panels a and b) indicate time when measurements of leaf gas exchange rates were taken. Dashed lines across panels a and b indicate the chilling threshold of 10 °C. (b) On 23 and 24 October during the part of day when measurements were taken, the average, low, and high temperatures were as follows: 9.2 °C (7.9–10.0 °C); 7.4 °C (4.9–9.1 °C), respectively. Measurements were taken at a leaf temperature of 13.4 °C (±0.4), photon flux of 1500 μmol m^{-2} s^{-1}, and 400 μmol mol^{-1} of CO_2 in air. An asterisk indicates significantly different values in comparison with Mxg 'Illinois' (bold) based on Dunnett's test ($P \leq 0.05$). Data are means + SE (n = from 3 to 4; as indicated below panel c). F1 = the first generation of *Saccharum* × *Miscanthus* hybrids (miscane); Mxg = *M.* ×*giganteus*; Sof = *Saccharum officinarum*.

When miscane 'US 87-1019' and its sugarcane parent 'NCo310' were grown at 25 °C (warm), no significant differences in A_{sat} and Φ_{PSII} between parent and progeny were observed. Specifically, 'US 87-1019' grown at 25 °C/20 °C for 10 days had A_{sat} and Φ_{PSII} values of 25 μmol m^{-2} s^{-1} and 0.28, respectively, and similarly for *Saccharum* 'NCo310' the values were only slightly lower at 22 μmol m^{-2} s^{-1} and 0.27, respectively (Fig. 1a, c). However, on the 11th day of chilling treatment, miscane 'US 87-1019' had a leaf CO_2 uptake rate that was three times that of its sugarcane parent. Additionally, miscane 'US 87-1019' had 2.8 times higher CO_2 assimilation than its sugarcane parent, 'NCo310', after transfer back to warm conditions (recovery).

Field experiment

Plants grown in the field at Dixon Springs, Illinois, in the autumn of 2013 experienced chilling temperatures below 10 °C during the 17 days (4 days in September and 13 days in October) prior to measurements of gas exchange (Fig. 3a). The average air temperature in first 22 days of October was 15.5 °C with the minimum at 0.1 °C and maximum at 29.7 °C. On 23 and 24 October during the part of day when measurements were taken, the average, low and high temperatures were as follows: 9.2 °C (7.9–10.0 °C), 7.4 °C (4.9–9.1 °C), respectively (Fig. 3b). Of three miscanes examined in late October in the field, one accession, 'US 84-1058', had comparable A_{sat}, g_s, and c_i/c_a to the chilling tolerant control, Mxg (Fig. 3c–e). A_{sat} for Mxg and miscane 'US 84-1058' was 13.1 μmol m^{-2} s^{-1} (Fig. 3c). The A_{sat} of Mxg was three time higher than A_{sat} of to two other miscanes, 'US 87-1019' and 'US 84-1928' and six times higher than A_{sat} of *S. officinarum* 'Louisiana Purple'.

Discussion

Can chilling tolerance of C_4 photosynthesis in Miscanthus be transferred to sugarcane?

To answer the question whether chilling tolerance of C_4 photosynthesis in *Miscanthus* can be transferred to sugarcane, we compared the gas exchange readings for three miscanes with results obtained for Mxg when grown in the same chilling conditions. As the *Miscanthus* parents of the miscane hybrids were not available, we chose for a chilling tolerant control the previously studied *Miscanthus* genotype, Mxg, which has been shown to have exceptionally efficient photosynthesis at low temperature for a high yielding C_4 plant (Long & Spence, 2013). Unexpectedly, we identified two *Saccharum* × *Miscanthus* hybrids (miscanes 'US 84-1058' and 'US 87-1019') that were not significantly different from the chilling tolerant Mxg when gas exchanges values were compared for plants grown at 10 °C/5 °C for 11 days in controlled environment chambers (Fig. 1). Furthermore, we were able to confirm photosynthetic chilling tolerance of miscane 'US 84-1058' under field conditions during the autumn in southern Illinois (Fig. 3), indicating that the chilling tolerance of C_4 photosynthesis in *Miscanthus* could be transferred to sugarcane. However, not all of the miscane genotypes that we tested had chilling tolerance. For example, miscane 'US 84-1028' lacked chilling tolerant photosynthesis, with A_{sat} after 11 days of growth at 10 °C/5 °C that was a fraction of that of the best miscane in our study, 'US 84-1058' (Fig. 1). Differences among miscane genotypes for chilling tolerance could be due to different levels of chilling tolerance contributed by the *Miscanthus* parents and/or interactions between genes from the *Miscanthus* and *Saccharum* parents. Moreover, the initial A_{sat} before cold treatment was higher for the miscanes 'US 84-1058' and 'US 87-1019' than for Mxg (Fig. 1a). Thus, even though the relative responses (compared to their initial A_{sat} values, Fig. 1b) of 'US 84-1058' and 'US 87-1019' to chilling were intermediate to Mxg (high) and the sugarcane (low) controls, because they started with higher per se values, their final A_{sat} values were similar to Mxg even after 11 days of chilling stress. Because the *Miscanthus* parents were not available for our current study, the comparison of F1 progeny with their non-*Saccharum* parents was unfortunately not possible. However, in a previous screen of *Miscanthus* germplasm, we found variation among species, and among genotypes within species for leaf extension rates and photosynthesis at low temperature (Głowacka *et al*., 2014). Thus, selection of parents and early generation miscane hybrids for chilling tolerant photosynthesis is advisable to successfully breed sugarcane for this trait.

The miscanes evaluated for photosynthesis at low temperature in the current study, 'US84-1028', 'US84-1058', and 'US87-1019', had previously been shown to resprout after being cut in the autumn and then allowed to over-winter in a field (overwintering ability when cut to 15 cm in the autumn) at Booneville, Arkansas (Burner *et al*., 2009). Interestingly, in the previous study 'US84-1028' and 'US84-1058' had acceptable overwintering ability and vigor in Arkansas, but 'US87-1019' lacked vigor after overwintering (Burner *et al*., 2009), whereas in the current study, photosynthesis of 'US84-1028' had poor tolerance to chilling, but 'US84-1058' and 'US87-1019' had good tolerance to chilling. Thus, chilling tolerant photosynthesis did not necessarily ensure good overwintering ability and vice versa. Cold tolerance is a complex set of component traits, and it will likely be advantageous to select for the different components when breeding sugarcane for adaptation to

more temperate environments than where this crop is currently grown commercially.

The primary source of germplasm to improve sugarcane for tolerance to biotic and abiotic stresses has been *S. spontaneum*. However, the potential of *S. spontaneum* for breeding cold tolerant sugarcane may be more limited than *Miscanthus* because the latter has a more northern and temperate natural distribution (to hardiness zone 3 in eastern Russia). Additionally, as *S. spontaneum* is listed on the federal noxious weed list, from the United States Department of Agriculture Plants Database it cannot be evaluated under natural field conditions; only tests under controlled conditions are allowed. Although the natural distribution of *S. spontaneum* does not extend as far north in Asia as *Miscanthus*, *S. spontaneum* populations occur in environments as diverse as tropical lowlands in South-East Asia to the temperate midlatitudes of Honshu, Japan. Thus, the cold tolerance of sugarcane progeny derived from *S. spontaneum* is expected to be strongly associated with a given *S. spontaneum* accession's adaptation to the environment in which it originated. Brandes (1940) reported selecting cold tolerant *S. spontaneum* clones able to survive 18 days of below freezing temperatures. In contrast, Breaux & Irvine (1976) observed that when actively growing young seedlings of *S. spontaneum* were frozen for 8 h, only 10% survived the test; moreover, under natural freezing conditions, these selected survivors were no more resistant to low temperatures than unselected populations. Recently, Hale *et al.* (2014) evaluated 41 *S. spontaneum* accessions for survivability of below ground (stubble) buds following exposure to freezing temperatures of −7 °C and identified four accessions that had more ratoon cold tolerance than the most tolerant commercial sugarcane variety tested (HoCP 96-540). However, when progeny of ten sugarcane × *S. spontaneum* hybrids was examined for stubble cold tolerance in Arkansas, none of the progeny survived (Burner *et al*., 2009). When the energycane variety 'Ho 02-113', a hybrid of *S. spontaneum* 'SES 234' from the Himalayan foothills of northern India and a leading commercial sugarcane variety LCP 85–384 (*S. officinarum* × *S. spontaneum* × *S. barberi* × *S. sinense*) (Milligan *et al*., 1994; Hale *et al*., 2013), was grown for six days at 12 °C/5 °C and then moved back to 25 °C/20 °C for one day, its ability to recover net CO_2 assimilation rate was 63% of the initial values at 25 °C on day 0 (Friesen *et al*., 2014); however, this was 21% less than the recovery of Mxg when grown in the same conditions. In our experiment, the difference between the recovery level of Mxg and the best miscane tested, 'US87-1019', was 20.5%. Thus, both *S. spontaneum* and *Miscanthus* accessions have potential to improve chilling tolerance in sugarcane. Choice of *S. spontaneum* and *Miscanthus* parents with exceptional levels of cold tolerance will be key to further improving sugarcane for this trait. To date, crosses between *Miscanthus* and sugarcane have used genotypes of *Miscanthus* that grew well in subtropical or tropical environments, where sugarcane crossing was routinely conducted. Thus, there is an opportunity to make additional genetic gains in cold tolerance of sugarcane by selecting donor *Miscanthus* parents with greater cold adaptation, such as those that originate from northern China, northern Japan, and eastern Russia.

Physiological mechanisms of chilling tolerance

All eight accessions studied showed decreases in A_{sat}, and Φ_{PSII} and increases in c_i/c_a, after transfer from warm to chilling (Fig. 1). In chilling, stomata reacted by reducing their aperture when the concentration of CO_2 increased in the intercellular compartment as a consequence of decreased of CO_2 fixation. The time needed for the stomata to compensate for new internal environmental conditions associated with chilling differed among genotypes but all eventually did compensate. For three of eight genotypes, g_s increased with the onset of chilling on day 0 and then decreased during the following chilling treatment; the other five genotypes decreased in g_s after only 20 min in chilling. Thus, differences among accessions for relative decreases in A_{sat} could not be explained by deficiency in CO_2 or by loss of stomatal function. On the other hand, comparisons of A_{sat} readings with values of Φ_{PSII} indicated that light-induced chilling damage of PSII was the primary reason for low CO_2 assimilation in chilling susceptible accessions. Similar patterns of change for physiological parameters in response to low temperature were previously observed in *Miscanthus* and sugarcane (Farage *et al*., 2006; Głowacka *et al*., 2014).

Typically, in warm-temperate environments at the beginning and end of the growing season significant fluctuations of temperatures are recorded (e.g., USDA hardiness zone 8 in the southern USA). At these times of the year, after chilling or frost events at night, days with bright sun and relatively high temperatures can occur, that causes a potential challenge for photosynthetic apparatus in C_4 plants mainly because of the risk of photodamage to PSII (Long, 1983; Long *et al*., 1994; Allen & Ort, 2001).

In our study, in comparison with the chilling sensitive sugarcane lines, two miscanes ('US 84-1058' and 'US 87-1019'), were better in recovery of A_{sat}, Φ_{PSII}, and g_s when returned to warm conditions for <24 h (Fig. 1). Additionally, 'US 84-1058' exhibited relatively high A_{sat} when grown in natural cycles of chilling and warm temperatures during autumn in a field trial in southern

Illinois. Thus, some miscanes appear to have an ability to protect the photosynthetic apparatus, permitting relatively high rates of photosynthesis at low temperature per se, and perhaps more importantly, enabling these undamaged plants to assimilate even more carbon on subsequent warm days.

Will chilling tolerant miscanes be less photosynthetically efficient in warm conditions than sugarcane?

A key challenge for plant breeders is to introgress desirable traits from wild or distantly related species into domesticated crops but at the same retain the favorable traits of the crop. When crossing a temperate-adapted species such as *M. sinensis* to a tropical crop such as sugarcane, there is the potential for tradeoffs associated with adaptation to different temperatures. Fortunately, however, we did not observe such a tradeoff for photosynthesis in the miscanes. For example, miscane 'US 87-1019' did not differ significantly from its sugarcane parent, 'NCo310', for A_{sat} and Φ_{PSII} when grown at 25 °C/20 °C but had significantly higher leaf photosynthetic gas exchange on the 11th day of chilling treatment (Fig. 1). Previously, studies on *Saccharum* × *Miscanthus* hybrids showed that values of agronomic traits for miscane were intermediate between the two parents (Chen, 1953; Chen *et al*., 1983). Whether backcrossing can be employed without losing chilling tolerant photosynthesis will depend on the number of genes that confer the trait, their interaction with sugarcane genes and the identification of marker–trait associations for marker-assisted selection.

We have shown that the chilling tolerance of C_4 photosynthesis in *Miscanthus* can be successfully transferred to sugarcane. The selected chilling tolerant miscanes, 'US 84-1058' and 'US 87-1019', have particular value for biomass feedstock and biofuel production, and at the same time they can be a starting point for extending sugarcane's range to higher latitudes and altitudes than current production regions. Although previous efforts to improve sugarcane with genes from *Miscanthus* have been few, these initial efforts point the way toward a bright future. In the last 30 years, our understanding of chilling tolerance in *Miscanthus* (Long & Spence, 2013; Głowacka *et al*., 2014), as well as the genetic diversity of this genus, has increased greatly (Hodkinson *et al*., 2002; Clark *et al*., 2014; Głowacka *et al*., 2015). This knowledge will facilitate improvement of sugarcane with genes from *Miscanthus*.

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