**Saccharum × Miscanthus** intergeneric hybrids (miscanes) exhibit greater chilling tolerance of C₄ photosynthesis and postchilling recovery than sugarcane (*Saccharum* spp. hybrids)

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**Abstract**
Although commercial sugarcane (*Saccharum* spp. hybrid) produces large biomass yields, its lack of cold tolerance limits its cultivation to the tropics and subtropics. In contrast, sugarcane’s close relative, *Miscanthus*, tolerates low temperatures. We studied 18 miscane genotypes, derived from hybridizations between two genotypes of sugarcane and two genotypes of *Miscanthus* (one each of *M. sinensis* and *M. sacchariflorus*). In an initial greenhouse experiment on long-duration chilling stress (12–13°C day/7–9°C night), photosynthetic rates of the *Miscanthus* parents were significantly higher than the sugarcane parents after 7 days of chilling and were more than double by 14 days. The *Miscanthus* also retained more of their prechilling (22–25°C day/13–15°C night) photosynthetic rates (68%–72% 7 days, 64%–66% 14 days) than the sugarcanes (27% 7 days, 19%–20% 14 days). Seven of 18 miscanes exhibited higher photosynthetic rates than their sugarcane parents after 7 days of chilling, whereas after 14 days only four miscane genotypes had significantly higher photosynthetic rates than their sugarcane parents, but notably two of these did not differ from their highly tolerant *Miscanthus* parents. In a subsequent growth chamber experiment to evaluate short-duration chilling stress and postchilling recovery, three miscanes representing the range of responses observed in the greenhouse experiment were compared with their parents. After 4 days of chilling (12/7°C day/night), the miscanes retained between 45% and 60% of their prechilling photosynthetic rate, with the best entry not significantly different from its *Miscanthus* parent (66%), and all three miscanes performed significantly better than the sugarcane parents (32%–33% for sugarcanes). After 7 days of postchilling recovery (26/18°C day/night), the *Miscanthus* parents and two of the miscanes fully recovered their prechilling photosynthetic rates but the sugarcane parents only recovered 69%–73% of their prechilling rates. Thus, genes from *Miscanthus* can be used to improve chilling tolerance of sugarcane via introgression.

**Keywords**
carbon dioxide partial pressure, chlorophyll fluorescence, cold tolerance, gas exchange, heritability, selection
1 | INTRODUCTION

Chilling (0–12°C) is an important climatic factor that can adversely impact plant growth, particularly at higher latitudes (Bongi & Long, 1987; Powles, Berry, & Björkman, 1983). Untimely chilling, especially in the early stages of plant growth, may cause irreversible damage to the plant’s photosynthetic system, resulting in severe decreases in seedling establishment and survival. Although the effects of chilling depend on its intensity, duration, the growth stage of the plant, and the associated environment of the plant, chilling is one of the leading challenges for establishment of warm-season annual crops in temperate zones (Friesen & Sage, 2016; Glowacka et al., 2014; Long, 1999; Long & Spence, 2013; Sage, Melo Peixoto, Friesen, & Deen, 2015). Additionally, the success of perennial crops in temperate environments depends on their survival and vigor when exposed to low temperatures that are not optimally conducive to growth. In particular, having photosynthetically active leaves early and late in the season, when chilling temperatures are common at temperate latitudes, helps perennial crops take maximal advantage of the potential growing season and of available solar radiation, thereby facilitating high biomass yields (Dohleman & Long, 2009; Friesen, Peixoto, Busch, Johnson, & Sage, 2014; Glowacka et al., 2014, 2015).

Sugarcane is one of humanity’s most important and productive crops, but it is a tropical-adapted, warm-season, C₄, perennial grass that is especially vulnerable to chilling injury. Sugarcane currently produces more biomass worldwide than any other crop, with a total production of nearly 1.9 billion Mg/year, on 26.7 Mha (FAOSTAT, 2016), and a peak dry matter yield >100 dry Mg ha⁻¹ year⁻¹ (Waclawowsky, Sato, Lembke, Moore, & Souza, 2010). In addition to the production of purified sugar for human consumption, sugarcane can be used as a lignocellulosic biomass or sugar feedstock for bioethanol production (Ge, Burner, Xu, Phillips, & Sivakumar, 2011; Santiago, Rossetto, Mello Ivo, & Urquiaga, 2010). Currently, 102.4 billion liters of fuel ethanol is produced worldwide (RFA, 2017), with US leading the production with 58 billion liters of ethanol (AMIS, 2017) mainly produced from maize, whereas Brazil leads in sugarcane ethanol production with 28 billion liters (MAPA, 2018). If used as a dedicated energy crop, sugarcane is sometimes referred to as energycane (Matsuoka, Kennedy, Santos, Tomazela, & Rubio, 2014).

Modern sugarcane cultivars are interspecific hybrids consisting primarily of Saccharum officinarum L., with an additional minority percentage of genes introgressed from Saccharum spontaneum L. (typically for resistance to abiotic and biotic stresses) in a process historically referred to as nobilization (D’Hont et al., 1996; Fageria, Moreira, Moraes, Hale, & Viator, 2013; Li et al., 2017; Roach, 1989; Sreenivasan & Ahloowalia, 1987; Stevenson, 1965). Other Saccharum species, such as S. robustum Brandes & Jeswiet ex Grassl, S. barberi Jeswiet, and S. sinense Roxb. amend. Jeswiet, may also have contributed genes to modern sugarcane cultivars but the extent of these contributions is less than that of S. officinarum and S. spontaneum (Andru, Pan, Thongthawee, Burner, & Kimbeng, 2011; D’Hont et al., 1996; Hoarau et al., 2001; Piperidis, Piperidis, & D’Hont, 2010).

Although interspecific hybridization has emerged as an efficient tool for improving sugarcane (Fageria et al., 2013), insufficient adaptation under temperate conditions, especially temperatures <18°C has been a persistent problem (Du, Nose, & Wasano, 1999a, 1999b; Sage, Peixoto, & Sage, 2013), especially at the highest altitude and latitude extremes of its commercial production (e.g. Florida and Louisiana, USA). Glowacka et al. (2015) and Grantz (1989) described sugarcane as one of the most chilling-sensitive crops in the world. At temperatures below 20°C, sugarcane leaf production slows, and below 10–15°C growth ceases completely (Allison, Pammenter, & Haslam, 2007). Photosynthesis in sugarcane ceases between 8 and 12°C (Fageria et al., 2013; Nose, Uehara, Kawamitsu, Kobamoto, & Nakama, 1994) and severe frost (~5 to ~7°C) can completely kill the aboveground plant (Sloan & Farquhar, 1978). S. spontaneum has been used as a source of genes for improving tolerance to low temperatures in commercial sugarcane (Fageria et al., 2013; Jackson, 2013; Khan et al., 2013; Moore, 1987), but progress has been limited because the donor species is not typically adapted to cold temperate environments (Friesen et al., 2014; Hale et al., 2013; Knoll et al., 2013).

In contrast to S. spontaneum, the natural range of Miscanthus extends much further north, to ~50°N in eastern Russia and to environments as cold as USDA hardiness zone 3 (average annual minimum temperature of ~34.4 to ~40.0°C) (Clark et al., 2018; Clifton-Brown, Schwarz, & Hastings, 2015). Although the majority of C₄ species is of tropical origin and adapted to warm environments, the genus Miscanthus is among the few exceptions that are adapted to cold temperate environments (Heaton et al., 2010; Jiao et al., 2017; Jones, 2011; Long & Spence, 2013). In particular, Miscanthus, has a high degree of chilling tolerance, including exceptional photosynthetic capacity at low temperatures, compared to other warm-season C₄ perennial grasses, such as sugarcane (Beale, Bint, & Long, 1996; Fonteyne et al., 2016; Friesen et al., 2014; Glowacka et al., 2015; Long & Spence, 2013). In addition, Miscanthus rhizomes can tolerate freezing while dormant over the winter (Clifton-Brown & Lewandowski, 2000), and also show quick recovery of aboveground organs after chilling, which are both necessary for producing high biomass in cold temperate environments (Friesen et al., 2014; Glowacka et al., 2014). Thus, Miscanthus is considered one of the most suitable perennial grasses for biomass production in temperate environments (Lewandowski, 2013), which can be attributed largely to its chilling-tolerant C₄ photosynthesis.

The C₄ photosynthesis of Miscanthus is more efficient than C₃ species under warm temperatures (Beale & Long, 1995, 1997; Beale, Morison, & Long, 1999), yet more productive
than most C₄ species under chilling temperatures (Dohleman & Long, 2009). Because Miscanthus can maintain a high photosynthetic CO₂ assimilation rate under chilling temperatures, it can produce an active canopy early in spring and late in autumn, giving it the benefit of capturing solar radiation over a long growing season (Beale et al., 1996; Beale & Long, 1995; Dohleman, Heaton, Leakey, & Long, 2009; Dohleman & Long, 2009). For example, a Miscanthus hybrid evaluated in Germany was observed to produce shoots inside dark incubators at a temperature as low as 6°C (Farrell, Clifton-Brown, Lewandowski, & Jones, 2006) and survived after prolonged exposure to temperatures <−6.5°C (Clifton-Brown & Lewandowski, 2000; Farrell et al., 2006). Thus, we expect that Miscanthus has the potential to be a superior source of traits to S. spontaneum, for improving chilling tolerance of commercial sugarcane and energycane.

Molecular genetics studies indicate that Miscanthus and Saccharum are closely related (Amalraj & Balasundaram, 2006; Sacks, Juvik, Lin, Stewart, & Yamada, 2013; Sobral, Braga, LaHood, & Keim, 1994). The two genera were estimated to have separated from a common ancestor about 3.64 mya (Tsuruta, Ebina, Kobayashi, & Takahashi, 2017). Moreover, intergeneric hybrids of Saccharum and Miscanthus have been bred previously, and these intergeneric hybrids are often termed miscanes. Miscanes have been studied since the late 1940s for their biomass production and adaptive traits (Burner, 1997; Chen, Chen, & Lo, 2000; Chen & Lo, 1989; Fageria et al., 2013; Glowacka et al., 2015; Li, 1948, 1961; Loh & Wu, 1949; Price, 1965; Xiao & Tai, 1994). Miscanes show promise as a potential cellulosic biomass crop, given that they typically have strong, thick culms, long stem, and high biomass-yield potential (Burner, Hale, Carver, Pote, & Fritschi, 2015; S. Kar, T. Y. Weng, T. Nakashima, A. Villanueva-Morales, J. R. Stewart, E. J. Sacks, Y. Terajima, & T. Yamada, unpubl. data). Burner, Tew, Harvey, and Belesky (2009) reported that one miscane genotype studied in Arkansas, USA produced more biomass than Miscanthus × giganteus Greef & Deuter ex Hedkoston & Renvoieze, M. sinensis Andersson or the switchgrass (Panica virgatum L.) ‘Alamo’. Moreover, Burner et al. (2009) reported that miscanes overwintered in Boonville, Arkansas, USA, where they were subjected to a minimum winter air temperature of −14°C. Glowacka et al. (2016) reported that the photosynthetic rate and maximum operating efficiency of photosystem II of three miscanes were similar to that of Miscanthus × giganteus and greater than three sugarcane genotypes, when tested under chilling conditions in controlled environment chambers (10°C/5°C). Sacks et al. (2013) suggested that miscanes could also be a potential biomass crop especially under warm temperate or subtropical regions, through a combination of key traits from its parents, including high biomass and late flowering capacity from sugarcane and high culm density, low sugar, chilling tolerance, and dry down traits from Miscanthus. Thus, by incorporating high biomass traits from sugarcane and cold tolerance traits from Miscanthus, we expect that miscanes have the potential to become a valuable lignocellulosic biomass feedstock crop in warm temperate environments and a source of genes to confer chilling tolerance in sugarcane.

Little information is currently available on the photosynthetic response of miscanes to chilling temperatures. Although Glowacka et al. (2016) reported a promising chilling response for miscanes, they studied only three individuals. Moreover, the individuals studied previously were from crosses made in the 1980s, and thus, Glowacka et al. (2016) were able to compare the miscanes to only one of the three sugarcane parents and to none of the Miscanthus parents, which were of unknown provenance (even the species of the Miscanthus parent was unknown for two of the three progeny). Given that Miscanthus and sugarcane may perform very differently under chilling temperatures and that there may be variation for chilling tolerance within each genus, there is a need to evaluate a larger set of miscane progeny to determine what is typical and what range of variation might be expected. Moreover, there is a need to compare the photosynthetic response of miscanes with their respective parents to obtain an initial understanding of the trait’s inheritance. To address these gaps in knowledge, the present study evaluated the photosynthetic response to chilling of 18 miscane genotypes and their respective parental genotypes, including two species of Miscanthus, M. sacchariflorus, and M. sinensis.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Two sugarcane parents (‘KR 05-619’, and ‘KY 06-139’), 2 Miscanthus parents (M. sacchariflorus ‘Miyakonojo’, and M. sinensis ‘Shiozuka’), and 18 miscane F₁ progeny were studied (Table 1). The sugarcane parents were breeding lines developed in the Sugarcane Breeding Station, National Agriculture and Food Research Organization, Tanegashima, Japan (31°44′N, 131°4′E). The Miscanthus parents were selections from Hokkaido University. Miscanthus sinensis ‘Shiozuka’ was collected from Tokushima Prefecture, Japan (36°N, 138°E) and it is well adapted to Hokkaido (43°04′N, 141°20′E) conditions (data not shown). Miscanthus sacchariflorus ‘Miyakonojo’ was collected from Miyazaki Prefecture, Japan (31°43′N, 131°4′E) and it has survived over multiple winters in Hokkaido (data not shown). The miscanes were bred by Mr. Yoshihumi Terajima at the Tropical Agricultural Research Front of the Japan International Research Center for Agricultural Sciences in Ishigaki, Okinawa, Japan. Two of the miscanes were derived from M. sinensis, and the remaining 16 were derived from M. sacchariflorus.

Ramets of each genotype were obtained for replicated experiments by vegetatively propagating from belowground...
stems. For *Miscanthus*, rhizome pieces were cut to 5 cm length. For sugarcanes and miscanes, tillers with axillary buds were cut to 5 cm length. Stem divisions were established in plastic pots (dia. = 15 cm, h. = 15 cm, vol. = 2 L) containing soilless medium consisting of compost, vermiculite, calcined clay, and peat moss (Forex Mori Sangyo Co., Ltd., Hokkaido, Japan). At least three rhizome pieces or tillers of a single genotype were planted in each pot. Stem divisions were planted on October 28, 2016. At planting and again at the start of each experiment, 15 g of 12‐9‐12 slow‐release fertilizer (Kumiai Grassland No. 8; Hokkaido Fertilizer Co., Ltd., Japan) was added to each pot. Plants were established in a greenhouse at Hokkaido University in Sapporo, Japan (43.07°N, 141.33°E), with temperatures maintained at 22–25/13–15°C day/night, with natural photoperiod. Irrigation was provided each day as needed.

### 2.2 | Greenhouse experiment on long-duration chilling stress

The greenhouse experiment was a randomized complete block design, with pots randomly arranged within each of the three blocks. Each block included 1 pot of each of the 22 entries (Table 1). In order to limit edge effects, pots were rearranged randomly within each block, each day of the experiment.

From the start of the experiment, on December 9, 2016, 6 week old plants were given 21 additional days of warm conditions (22–25°C during the day, from 6:00 a.m. to 8:00 p.m., and 13–15°C during the night) with a 14 hr photoperiod (6:00 a.m. to 8:00 p.m. day). After the initial warm establishment, plants were subsequently challenged for 14 days with chilling temperatures (12–13°C during the day, from 8:00 a.m. to 6:00 p.m., and 7–9°C during the night). Considering the overcast nature of the sky along with short day length commonly observed during the months of December–January in Hokkaido, fluorescent lights (32-W, white; FHF 32EX-N-HX, NEC Lighting Ltd., Tokyo, Japan) supplemented sunlight to maintain the photoperiod. Fluorescent lights provided 100 μmol m⁻² s⁻¹ of photosynthetic photon flux density (PPFD) at canopy level. As the saturation light intensity for photosynthesis is determined by the light intensity at which the plants were grown (Friesen et al., 2014; Głowacka et al., 2014, 2016; Singh, Ogren, & Widholm, 1974; Usuda, Ku, & Edwards, 1985), during the warm establishment period,

| Genotype    | Type       | Female parent | Male parent | Experiment |
|-------------|------------|---------------|-------------|------------|
| ‘JM 14-06’  | Miscane    | ‘KY 06-139’   | ‘Shiozuka’  | 1          |
| ‘JM 14-09’  | Miscane    | ‘KR 05-619’   | ‘Shiozuka’  | 1, 2       |
| ‘JM 14-47’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-49’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-50’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-51’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-52’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-53’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-57’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-59’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-60’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-61’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-63’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-64’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-66’  | Miscane    | ‘KR 05-619’   | ‘Miyakonojo’| 1          |
| ‘JM 14-72’  | Miscane    | ‘KY 06-139’   | ‘Miyakonojo’| 1, 2       |
| ‘JM 14-76’  | Miscane    | ‘KY 06-139’   | ‘Miyakonojo’| 1          |
| ‘JM 14-88’  | Miscane    | ‘KY 06-139’   | ‘Miyakonojo’| 1, 2       |
| ‘KR 05-619’ | Sugarcane  | –             | –           | 1, 2       |
| ‘KY 06-139’ | Sugarcane  | –             | –           | 1, 2       |
| ‘Miyakonojo’| *Miscanthus*| –             | –           | 1, 2       |
| ‘Shiozuka’  | *Miscanthus*| –             | –           | 1, 2       |

**Note:** 1 – Greenhouse experiment. 2 – Growth chamber experiment.
initial prechilling measurements of net photosynthetic CO₂ assimilation rates at a PPFD of 1,000 μmol m⁻² s⁻¹ ($A_{1000}$) and a CO₂ concentration set to 400 μmol/mol were taken on all of the plants (pots) between 10:00 a.m. and 2:00 p.m. over a 2-day period on Days 20 and 21. Measurements of $A_{1000}$ were also taken on each plant on Days 7 and 14 of the chilling period. On each night following $A_{1000}$ measurements, maximum quantum yield of photosystem II ($F_v/F_m$) in dark-adapted leaves was measured on each plant between 0:00 and 2:00 a.m. Evaluations after 7 and 14 days of chilling treatment enabled us to mimic lengthy cold waves that occur during the winter in subtropical production environments.

All plant measurements were taken on the youngest fully expanded leaves of each pot. For $A_{1000}$ measurements, an individual leaf was enclosed in a controlled environment cuvette of a steady-state photosynthesis system (LI6400XT; LI-COR Bioscience, Lincoln, NE, USA). Based on the assumption that leaves are completely dark adapted (~6 hr) when measured during 0:00-2:00 a.m. in the morning, $F_v/F_m$ was measured with a chlorophyll fluorometer (Junior-PAM CFMG0700B; Heinz Walz GmbH, Effeltrich, Germany). Temperature at canopy height was recorded at 10 min intervals for the duration of the experiment with a data-logger (Thermo Recorder TR 72U; T&D Corporation, Matsumoto, Japan) and is shown in Figure S1. Irradiance inside the greenhouse was recorded with a quantum sensor at 10 min intervals (MIJ-14PARII; Environmental Measurement Japan Co. Ltd., Fukuoka, Japan) and is shown in Figure S2.

2.3 Growth chamber experiment on short-duration chilling stress and postchilling recovery

A total of seven genotypes were studied in this experiment, including the two sugarcane parents, the two Miscanthus parents, and three miscanes selected to represent the range of responses observed in the greenhouse experiment after 14 days of chilling (‘JM 14-09’, ‘JM 14-72’, and ‘JM 14-88’). The growth chamber experiment was a completely randomized design. Each genotype was represented by three pots containing healthy and vigorous 3-week old plants. Pots were randomly arranged on four 60 cm x 50 cm trays inside two 350-L growth chambers (BioTRON LH-350S, NK Systems, Nippon Medical & Chemical Instruments Co., Ltd. Osaka, Japan). Pots were rotated randomly inside and between the two chambers on a daily basis to minimize between-chamber and within-chamber environmental effects. The growth chambers provided $400 \pm 50$ μmol m⁻² s⁻¹ of photosynthetically active radiation with fluorescent lamps (Hitachi FLR40S-EX-N/ M36-A, Hitachi, Ltd., Tokyo, Japan), as measured with a quantum sensor (MIJ-14PARII, Environmental Measurement Japan Co. Ltd.) at the top of the plant canopy.

We measured net CO₂ assimilation rate at a PPFD of 1,500 μmol m⁻² s⁻¹ ($A_{1500}$), and intercellular to ambient CO₂ content ($C_i/C_a$) using a steady-state photosynthesis system (LI6400XT; LI-COR Bioscience); maximum quantum yield of photosystem II ($F_v/F_m$) using a chlorophyll fluorometer (Junior-PAM CFMG0700B; Heinz Walz GmbH) on the youngest fully expanded leaf; and SPAD with a chlorophyll meter (SPAD 502 Plus; Minolta Co., Ltd., Osaka, Japan) on the middle 5 cm of the top three fully expanded leaves of each plant. Measurements were taken at the end of a 14 day warm establishment period, after 4 days of chilling, and finally after 7 days of a postchilling warm recovery.

A warm establishment was provided with 26/18°C and 14 hr photoperiod for the first 14 days of the experiment. Initial prechilling measurements of $A_{1500}$, $F_v/F_m$, $C_i/C_a$, and SPAD were taken at ambient temperatures for each pot on Day 14. Initial prechilling measurements enabled estimation of percent retained values for the four traits after chilling and after postchilling recovery.

Subsequent to the warm establishment period, plants were challenged with 7 days of chilling at 12/7°C day/night. The chilling period began on Day 15 of the experiment. When the chilling was initiated, temperature was decreased at a rate of 1°C per 20 min until reaching the daytime set-point of 12°C. Photoperiod during the chilling period was 10 hr. After 4 days of chilling (Day 18 since the experiment began), we measured $A_{1500}$, $F_v/F_m$, $C_i/C_a$, and SPAD. Evaluation after 4 days of chilling treatment enabled us to mimic short-duration chilling stress, which is common during early spring and late autumn in subtropical and temperate production environments.

After 7 days of chilling (Day 21 since the experiment began), the environment was returned to warm pre-chilling conditions of 26/18°C and a 14 hr photoperiod. The temperature was transitioned at a rate of 1°C per 20 min until reaching the daytime set-point of 12°C. Photoperiod during the chilling period was 10 hr. After 7 days of postchilling recovery at warm temperatures (Day 28 since the experiment began), $A_{1500}$, $F_v/F_m$, $C_i/C_a$, and SPAD measurements were taken on all plants to evaluate plant recovery from potential chilling damage.

2.4 Data analysis

Statistical analyses for both experiments were performed with SAS procedure GLIMMIX (version 9.4; SAS Institute Inc., Cary, NC, USA). Data from each of the measured response variables were analyzed by fitting a repeated measures mixed model, with a covariance structure for the repeated measurements selected using the Akaike information criterion corrected (AICc) (Hurvich & Tsai, 1989), from several alternative candidate models. In case of the greenhouse experiment, the AICc selected a heterogeneous compound symmetry covariance structure for the photosynthesis response data and a first-order autoregressive covariance structure for the fluorescence ($F_v/F_m$) data. For the growth chamber experiment, a compound symmetry covariance structure provided an appropriate fit for the photosynthesis response data,
whereas an independent with unequal variances covariance structure was found to be appropriate for data from response variables $F/F_m, C_i/C_a,$ and SPAD. Means comparisons were implemented through the SAS LSMEANS statement with the ADJ = TUKEY option used to obtain a Tukey–Kramer multiple comparison adjustment of $p$-values for the differences in LS means. In all cases, significance for comparisons was tested at $p \leq 0.05$.

For the miscane $F_1$ full-sib family, sugarcane ‘KR 05-619’ × *Miscanthus sacchariflorus* ‘Miyakonojo’ ($n = 13$), and miscane $F_1$ half-sib family, sugarcane ‘KR 05-619’ or ‘KY 06-139 × M. sacchariflorus* ‘Miyakonojo’ ($n = 16$), completely random model analyses of variance were also conducted using restricted maximum likelihood via SAS procedure MIXED (version 9.4; SAS Institute Inc.) to test the effects of genotype and block in the greenhouse experiment:

$$Y = \text{Genotype} + \text{Block} + \text{Error}$$

where the response variable $Y$ was $A_{1000}$ or $F/F_m$. Variance components were estimated for each of the sources of variation in the model. Broad-sense heritability ($H^2$) on an individual plant basis was calculated using the variance components based on following the equation (Gusmini & Wehner, 2004; Tena, Mekbib, & Ayana, 2016):

$$H^2 = \frac{\sigma^2_G}{\sigma^2_Y} = \frac{\sigma^2_G}{\sigma^2_G + \sigma^2_E}$$

where $\sigma^2_G$ is the total genetic variance, $\sigma^2_Y$ is the total phenotypic variance, and $\sigma^2_E$ is the environmental variance. In this case, $\sigma^2_G$ represents the variation among miscane progeny genotypes, and $\sigma^2_E$ represents the interaction between genotype and block.

The Shapiro–Wilk’s test statistic for normality ($W$) was calculated using R v 3.5.1 (R Core Team, 2015), in addition, Pearson’s coefficient of skewness ($y_1$) and coefficient of kurtosis ($y_2$) for net CO$_2$ assimilation rate at 1,000 μmol m$^{-2}$ s$^{-1}$ photosynthetic photon flux density ($A_{1000}$) and maximum quantum yield of photosystem II ($F/F_m$) of dark-adapted leaves on the 14th day of chilling treatment for both full-sib and half-sib $F_1$s were calculated using ‘e1071’ package in R v 3.5.1 (R Core Team, 2015) to detect additive effects of alleles from parental genotypes and the presence of dominance genetic variation.

### RESULTS

#### 3.1 Performance of entries under long-term chilling (12–13°C/7–9°C day/night) in a greenhouse

In the greenhouse experiment, at the end of the warm (22–25°C/13–15°C day/night) establishment period, rates of net photosynthetic CO$_2$ assimilation were high for all entries, as expected; however, significant initial differences among entries were observed (Table 2). Similarly, initial values for $F/F_m$ were high (0.794–0.823) but there were no significant differences among the 22 entries. For the sugarcane parents, initial rates of $A_{1000}$ at warm temperatures (29.2–29.9 μmol m$^{-2}$ s$^{-1}$) were significantly greater than those for the *Miscanthus* parents (19.7–21.8 μmol m$^{-2}$ s$^{-1}$). For the miscanes, initial rates of $A_{1000}$ at warm temperatures ranged from 18.1 to 29.6 μmol m$^{-2}$ s$^{-1}$, and none were significantly higher than the sugarcanes or lower than the *Miscanthus* parents. Five of the miscanes (‘JM 14-47’, ‘JM 14-59’, ‘JM 14-60’, ‘JM 14-72’, and ‘JM 14-88’) had initial rates of $A_{1000}$ that were not significantly different from their sugarcane parent but significantly higher than their *Miscanthus* parent.

After 7 days of chilling treatment (12–13°C/7–9°C day/night) in the greenhouse, $A_{1000}$ of the *Miscanthus* parents (13.4–15.8 μmol m$^{-2}$ s$^{-1}$) were significantly higher than those of the sugarcane parents (7.9–8.1 μmol m$^{-2}$ s$^{-1}$), although all parents had significantly lower CO$_2$ assimilation rates than during the prechilling warm treatment (Table 2). However, the *Miscanthus* parents retained substantially more of their prechilling photosynthetic CO$_2$ assimilation rates after 7 days of chilling (68%–72%) than the sugarcanes (27%). Seven of the 18 miscane genotypes (‘JM 14-09’, ‘JM 14-49’, ‘JM 14-52’, ‘JM 14-55’, ‘JM 14-57’, ‘JM 14-59’, and ‘JM 14-60’) exhibited significantly higher CO$_2$ assimilation rates than their chilling-sensitive sugarcane parents, and were not significantly different from their chilling-tolerant *Miscanthus* parents, after 7 days of chilling. These seven best-performing miscanes retained 48%–77% of their prechilling photosynthetic CO$_2$ assimilation rates after 7 days of chilling.

$F/F_m$ of the *Miscanthus* parents after 7 days of chilling (0.803–0.808) was not significantly different from their values during the warm conditions (Table 2). In contrast, $F/F_m$ of the sugarcane parents after 7 days of chilling (0.748–0.750) was significantly lower than those of the *Miscanthus* parents and lower than the prechilling values of the sugarcane parents. Two miscane genotypes (‘JM 14-06’ and ‘JM 14-09’) had $F/F_m$ after 7 days of chilling that were not significantly different from their chilling-tolerant *M. sinensis* parent, and eight miscanes had values intermediate to and significantly different from both their sugarcane and *Miscanthus* parents (‘JM 14-50’, ‘JM 14-51’, ‘JM 14-52’, ‘JM 14-55’, ‘JM 14-57’, ‘JM 14-60’, ‘JM 14-61’, and ‘JM 14-72’).

After 2 weeks of chilling treatment (12–13°C/7–9°C day/night) inside the greenhouse, the differences between the *Miscanthus* and sugarcane parents were greater than after only 7 days of chilling (Table 2). $A_{1000}$ of the *Miscanthus* parents (12.7–14.4 μmol m$^{-2}$ s$^{-1}$) were more than double, and significantly higher, than the sugarcane parents (5.6–6.1 μmol m$^{-2}$ s$^{-1}$), after 14 days of chilling treatment, although the reductions in CO$_2$ assimilation from 7 to 14 days of chilling were not significant for each of the parents. The
TABLE 2 Results of greenhouse experiment showing least square means ± standard errors of net CO₂ assimilation rate at a photosynthetic photon flux density of 1,000 μmol m⁻² s⁻¹ ($A_{1000}$) and maximum quantum yield of photosystem II ($F_{v}/F_{m}$) in dark-adapted leaves of 18 miscanes, and their sugarcane and Miscanthus parents. Measurements were taken before chilling (22–25°C/13–15°C day/night) and after 7 and 14 days of chilling (12–13°C/7–9°C day/night).

| Genotype   | Type            | $A_{1000}$ (μmol m⁻² s⁻¹) | $F_{v}/F_{m}$ | Warm       | Chilling 7 days | Chilling 14 days |
|------------|-----------------|--------------------------|---------------|------------|----------------|-----------------|
|            |                 |                          |               | Warm       | Chilling 7 days | Chilling 14 days |
| 'JM 14-06' | Miscane         | 20.7 ± 0.5 CD a          | 11.4 ± 0.4 BC b | 10.3 ± 0.3 BC b | 0.823 ± 0.002 A a | 0.789 ± 0.002 B b | 0.773 ± 0.002 C c |
| 'JM 14-09' | Miscane         | 24.7 ± 0.5 BC a          | 14.4 ± 0.4 AB b | 12.1 ± 0.3 AB b | 0.820 ± 0.002 AB a | 0.790 ± 0.002 AB b | 0.780 ± 0.002 B b |
| 'JM 14-47' | Miscane         | 26.9 ± 0.5 AB a          | 10.7 ± 0.4 BC b | 8.4 ± 0.3 C b  | 0.808 ± 0.002 AB a | 0.749 ± 0.002 D b | 0.725 ± 0.002 DE c |
| 'JM 14-49' | Miscane         | 25.5 ± 0.5 BC a          | 12.3 ± 0.4 B b  | 11.2 ± 0.3 B b  | 0.817 ± 0.002 AB a | 0.745 ± 0.002 D b | 0.728 ± 0.002 DE c |
| 'JM 14-50' | Miscane         | 19.6 ± 0.5 CD a          | 15.0 ± 0.4 ABC b | 8.0 ± 0.3 CD c  | 0.794 ± 0.002 B a  | 0.773 ± 0.002 BC b | 0.746 ± 0.002 CD c |
| 'JM 14-51' | Miscane         | 18.1 ± 0.5 D a           | 11.0 ± 0.4 BC b | 7.9 ± 0.3 CD c  | 0.802 ± 0.002 B a  | 0.785 ± 0.002 BC b | 0.772 ± 0.002 B b  |
| 'JM 14-52' | Miscane         | 21.4 ± 0.5 CD a          | 12.6 ± 0.4 AB b | 10.8 ± 0.3 BC b  | 0.801 ± 0.002 B a  | 0.781 ± 0.002 BC b | 0.748 ± 0.002 CD c |
| 'JM 14-55' | Miscane         | 18.7 ± 0.5 CD a          | 14.4 ± 0.4 AB b | 13.4 ± 0.3 AB b  | 0.819 ± 0.002 AB a  | 0.780 ± 0.002 BC b | 0.768 ± 0.002 BC b |
| 'JM 14-57' | Miscane         | 25.4 ± 0.5 BC a          | 15.1 ± 0.4 AB b | 8.9 ± 0.3 BC c  | 0.819 ± 0.002 AB a  | 0.785 ± 0.002 BC b | 0.767 ± 0.002 BC c |
| 'JM 14-59' | Miscane         | 26.3 ± 0.5 AB a          | 12.8 ± 0.4 AB b | 10.9 ± 0.3 BC b  | 0.808 ± 0.002 AB a  | 0.738 ± 0.002 D b  | 0.712 ± 0.002 E c  |
| 'JM 14-60' | Miscane         | 28.4 ± 0.4 AB a          | 14.0 ± 0.5 AB b | 8.6 ± 0.4 BC c  | 0.808 ± 0.002 AB a  | 0.770 ± 0.002 C b  | 0.740 ± 0.002 CD c |
| 'JM 14-61' | Miscane         | 20.5 ± 0.5 CD a          | 10.8 ± 0.4 BC b | 8.0 ± 0.3 CD c  | 0.799 ± 0.002 B a  | 0.780 ± 0.002 BC b | 0.764 ± 0.002 BC c |
| 'JM 14-63' | Miscane         | 22.0 ± 0.5 BC a          | 9.4 ± 0.4 BC b  | 7.8 ± 0.3 CD b  | 0.803 ± 0.002 B a  | 0.747 ± 0.002 D b  | 0.750 ± 0.002 CD b |
| 'JM 14-64' | Miscane         | 18.3 ± 0.5 D a           | 10.7 ± 0.4 BC b | 9.1 ± 0.3 BC b  | 0.794 ± 0.002 B a  | 0.764 ± 0.002 CD b | 0.749 ± 0.002 CD c |
| 'JM 14-66' | Miscane         | 19.8 ± 0.5 CD a          | 7.4 ± 0.4 C b   | 6.4 ± 0.3 CD b  | 0.815 ± 0.002 AB a  | 0.750 ± 0.002 D b  | 0.733 ± 0.002 D c  |
| 'JM 14-72' | Miscane         | 29.6 ± 0.5 AB a          | 9.9 ± 0.4 BC b  | 9.0 ± 0.3 CD b  | 0.810 ± 0.002 AB a  | 0.782 ± 0.002 BC b | 0.774 ± 0.002 B b  |
| 'JM 14-76' | Miscane         | 22.2 ± 0.4 C a           | 8.6 ± 0.5 C b   | 6.6 ± 0.4 CD b  | 0.805 ± 0.002 AB a  | 0.759 ± 0.002 CD b | 0.754 ± 0.002 C b  |
| 'JM 14-88' | Miscane         | 25.9 ± 0.5 B a           | 8.6 ± 0.4 C b   | 7.2 ± 0.3 CD b  | 0.808 ± 0.002 AB a  | 0.756 ± 0.002 CD b | 0.745 ± 0.002 CD b |
| 'KR 05-619'| Sugarcane      | 29.9 ± 0.5 A a           | 8.1 ± 0.4 C b   | 6.1 ± 0.3 CD b  | 0.810 ± 0.002 AB a  | 0.748 ± 0.002 D b  | 0.726 ± 0.002 DE c |
| 'KY 06-139'| Sugarcane      | 29.2 ± 0.5 AB a          | 7.9 ± 0.4 C b   | 5.6 ± 0.3 D b   | 0.807 ± 0.002 AB a  | 0.750 ± 0.002 D b  | 0.725 ± 0.002 DE c |
| 'Miyakonojo’| M. sacchariflorus| 21.8 ± 0.5 CD a          | 15.8 ± 0.4 A b  | 14.4 ± 0.3 A b  | 0.812 ± 0.002 AB a  | 0.808 ± 0.002 A a  | 0.800 ± 0.002 A a  |
| 'Shiozuka’’| M. sinensis     | 19.7 ± 0.5 CD a          | 13.4 ± 0.4 AB b | 12.7 ± 0.3 AB b | 0.814 ± 0.002 AB a  | 0.803 ± 0.002 AB a  | 0.797 ± 0.002 AB b |

Note: Upper case letters indicate comparison among genotypes within a particular period of time (warm, 7th day of chilling treatment or 14th day of chilling treatment). Lower case letters indicate comparison across time (i.e., comparison between warm, 7th day of chilling treatment or 14th day of chilling treatment) within each genotype. A different letter indicates significant difference ($p < 0.0001$). Means separation was conducted by using the adjusted $p$-values from the Tukey–Kramer multiple comparison ($p \leq 0.05$).
Miscanthus parents continued to retain more of their pre-chilling photosynthetic CO$_2$ assimilation rates (64%–66%) than the sugarcane parents (19%–20%). Of the 18 miscane genotypes, only four (‘JM 14-09’, ‘JM 14-49’, ‘JM 14-55’, and ‘JM 14-72’) had significantly higher CO$_2$ assimilation rates than their chilling-sensitive sugarcane parents, but notably two of these (‘JM 14-09’ and ‘JM 14-55’) did not differ from their highly tolerant Miscanthus parents, after 14 days of chilling. After 14 days of chilling, ‘JM 14-09’ retained 49% of its pre-chilling photosynthetic CO$_2$ assimilation rate and ‘JM 14-55’ retained 72%. Also notable was that ‘JM 14-72’ was the only miscane genotype that had rates of CO$_2$ assimilation that were high and not significantly different from the sugarcanes under the initial warm conditions, yet remained among the top performers after 14 days of chilling. Miscane genotypes ‘JM 14-50’, ‘JM 14-51’, ‘JM 14-57’, ‘JM 14-60’, and ‘JM 14-61’ showed a significant drop, between 26% and 47%, in their photosynthetic rates compared to their CO$_2$ fixation levels after 7 days of cold treatment, but the remaining genotypes did not show any significant reductions in photosynthesis during the same period (Table 2).

$F_v/F_m$ of the $M. \text{sacchariflorus}$ parent after 14 days of chilling (0.800 ± 0.002) was not significantly different from its values after 7 days of chilling or during the warm conditions (Table 2). Although the $F_v/F_m$ after 14 days of chilling for the $M. \text{sinensis}$ parent (0.797 ± 0.002) was not significantly different than after 7 days of chilling, it was significantly lower than the prechilling values. In contrast, $F_v/F_m$ of the sugarcane parents after 14 days of chilling (0.725–0.726) was significantly lower than after 7 days of chilling and during the initial warm period, and significantly lower than those of the Miscanthus parents after 14 days of chilling. Of the 18 miscane genotypes, 7 (‘JM 14-06’, ‘JM 14-09’, ‘JM 14-51’, ‘JM 14-55’, ‘JM 14-57’, ‘JM 14-61’, and ‘JM 14-72’) had $F_v/F_m$ that did not differ significantly from the values of the $M. \text{sinensis}$ parent after 14 days of chilling, including the two $M. \text{sinensis}$ progeny, but none were as high as the $M. \text{sacchariflorus}$ parent. One miscane (‘JM 14-76’) differed significantly from and was intermediate to both parents for $F_v/F_m$ after 14 days of chilling. The remaining 10 miscane genotypes were not significantly different from the chilling-sensitive sugarcane parents for $F_v/F_m$ after 14 days of chilling.

### 3.2 Heritability estimates from a greenhouse experiment

Response of the miscane F$_1$ progenies to chilling temperatures varied among the 18 genotypes, with some performing as well as their chilling-tolerant Miscanthus parents, others performing as poorly as their chilling-sensitive sugarcane parents, and many performing intermediate to both parents (Table 2). Quantitative variation for a trait could be due to genotypic differences, interactions between genotypes and environment, or both. However, the estimates of broad-sense heritability for both $A_{1000}$ and $F_v/F_m$ on Day 14 of chilling were high (≥0.93), indicating that the observed variation in the miscane F$_1$ full-sib and half-sib families was primarily due to genetics and not environment (Table 3, Table S1). Additionally, broad-sense heritability estimates for the warm period and 7th day of chilling for $A_{1000}$ and $F_v/F_m$ were also high (≥0.79). These estimates of broad-sense heritability represent the upper potential limit of narrow-sense heritability, and the high values obtained suggest that phenotypic selection for chilling-tolerant photosynthesis in these miscane populations should be effective and efficient. These estimates are based on small population sizes ($n = 13$ or $n = 16$), conducted in control environments; however, we observed high heritability values for photosynthesis under field conditions in these genotypes (S. Kar, T. Y. Weng, T. Nakashima, A. Villanueva-Morales, J. R. Stewart, E. J. Sacks, Y. Terajima, & T. Yamada, unpubl. data). Thus, the heritability values obtained in our experiment are reliable. To the best of our

### TABLE 3

| Source | Full-sib family ($n = 13$) |  
| --- | --- | 
| | $A_{1000}$ |  
| | Warm period | 7th day of chilling treatment | 14th day of chilling treatment |  
| | Warm period | 7th day of chilling treatment | 14th day of chilling treatment |  
| Block | 0.00 | 0.00 | 0.003 | 0.000002 | 0.000000 | 0.000004 |  
| Genotype | 12.90 | 5.08 | 3.51 | 0.000074 | 0.000291 | 0.000335 |  
| Error | 0.69 | 0.74 | 0.21 | 0.000015 | 0.000014 | 0.000012 |  
| $H^2$ | 0.95 | 0.87 | 0.94 | 0.83 | 0.95 | 0.97 |
knowledge these are the first estimates of heritability for chilling-tolerant photosynthesis in miscane populations and thus are extremely valuable information for breeders.

### 3.3 | Performance of entries under short-term chilling (12/7°C day/night) followed by recovery after 7 days of rewarming (26/18°C day/night) in a growth chamber

At the start of the experiment in the growth chamber, under warm temperatures (26/18°C day/night), values of $A_{1500}$, $F_{i}/F_{m}$, $C_{i}/C_{a}$, and SPAD indicated that all plants were healthy and photosynthetically active, as expected; however, significant initial differences among entries were observed (Table 4). For the sugarcane parents, initial rates of $A_{1500}$ (28.3–29.3 μmol m$^{-2}$ s$^{-1}$) at warm temperatures were high and similar to the warm period rates observed in the greenhouse experiment. Similarly, initial rates of $A_{1500}$ for the *M. sacchariflorus* parent (29.9 ± 0.4 μmol m$^{-2}$ s$^{-1}$) and miscane ‘JM 14-09’ (28.8 ± 0.4 μmol m$^{-2}$ s$^{-1}$) were not significantly different from the sugarcane parents. However, initial $A_{1500}$ for the *M. sinensis* parent (24.2 ± 0.4 μmol m$^{-2}$ s$^{-1}$) and miscane ‘JM 14-88’ (23.4 ± 0.4 μmol m$^{-2}$ s$^{-1}$) were significantly lower than that for the sugarcane parents. Notably, miscane ‘JM 14-72’ had a significantly higher initial photosynthetic rate (33.2 ± 0.4 μmol m$^{-2}$ s$^{-1}$) than all the other entries, which was consistent with its high initial rate in the greenhouse experiment. Initial $F_{i}/F_{m}$ was high and similar among all entries (0.808–0.822), except for a significantly lower value for the *M. sinensis* parent (0.795 ± 0.001). No differences in initial $C_{i}/C_{a}$ among entries were observed. Initial SPAD among the sugarcane parents and *Miscanthus* parents were not significantly different.

A short 4 day exposure to chilling temperatures (12/7°C day/night) in the growth chambers negatively impacted photosynthesis of all entries tested, but some entries were more greatly affected than others. After 4 days of chilling, the *Miscanthus* parents had significantly higher $A_{1500}$ (16.0–19.7 μmol m$^{-2}$ s$^{-1}$) than the sugarcane parents (9.3–9.1 μmol m$^{-2}$ s$^{-1}$), and a similar significant difference was observed for $F_{i}/F_{m}$ (0.787–0.800 for the *Miscanthus*, in contrast to 0.728–0.731 for the sugarcanes). The *M. sacchariflorus* parent had a significantly greater $A_{1500}$ after chilling than the *M. sinensis* parent. Similarly, when exposed to 4 days of chilling temperatures, the *Miscanthus* parents retained a greater percentage of their prechilling $A_{1500}$ (66%) and $F_{i}/F_{m}$ (97%–99%) than the sugarcane parents (32%–33% $A_{1500}$, and 89%–90% of their prechilling $F_{i}/F_{m}$).

All three miscanes performed significantly better than the sugarcane parents for $A_{1500}$ and $F_{i}/F_{m}$ after 4 days of chilling. The miscanes were more similar to their *Miscanthus* parents than their sugarcane parents, retaining between 45% and 60% of their prechilling photosynthetic rate, and 97%–99% of their prechilling $F_{i}/F_{m}$. The best miscane entry, ‘JM 14-09’, did not significantly differ from its *M. sinensis* parent for $A_{1500}$ and $F_{i}/F_{m}$ after chilling. The performance of the three miscane genotypes in the growth chamber experiment in response to 4 days of chilling showed a similar pattern to their performance in the previous greenhouse experiment in response to 7 and 14 days of chilling. In each case, ‘JM 14-09’ had the highest CO$_2$ assimilation rate, followed by ‘JM 14-72’, then ‘JM 14-88’, although there was not a significant difference between the latter two entries at each time point.

Under chilling, $C_{i}/C_{a}$ of all entries in the growth chamber experiment increased relative to the prechilling values (Table 4), indicating that the intercellular CO$_2$ concentration increased, due to a decrease in CO$_2$ fixation. Means separation identified two groups of entries for $C_{i}/C_{a}$ under chilling temperatures but there was no clear distinction between the parental species, as there had been for $A_{1500}$ and $F_{i}/F_{m}$. Under chilling, miscanes ‘JM 14-09’, ‘JM 14-72’, sugarcane ‘KR 05-619’, and the *M. sinensis* parent had significantly higher $C_{i}/C_{a}$ (0.701 ± 0.011, 0.769 ± 0.011, 0.709 ± 0.011, and 0.754 ± 0.011, respectively) than the other genotypes.

Seven days after postchilling return to warm temperatures (26/18°C day/night) in the growth chambers, two types of responses were observed: (a) full recovery, and (b) partial recovery (Table 4). Both *Miscanthus* parents and miscanes ‘JM 14-09’ and ‘JM 14-88’ fully recovered or exceeded their prechilling values of $A_{1500}$ and $F_{i}/F_{m}$ after 7 days of chilling followed by 7 days return to warm temperatures. In contrast, the sugarcane parents had significantly lower $A_{1500}$ (20.3–20.5 μmol m$^{-2}$ s$^{-1}$) and $F_{i}/F_{m}$ (0.763–0.766) at the end of the experiment than during the warm period but the end values were significantly higher than after 4 days of chilling, indicating a partial recovery. Thus, the sugarcane parents recovered only 69%–73% of their prechilling photosynthetic CO$_2$ assimilation rates and 94% of their initial $F_{i}/F_{m}$ by the end of the experiment, whereas recovery for the *Miscanthus* parents was 97%–103% for CO$_2$ assimilation and 100%–103% for $F_{i}/F_{m}$. Similarly, recovery for the miscanes ‘JM 14-09’ and ‘JM 14-88’ was 103%–104% for CO$_2$ assimilation and 100%–101% for $F_{i}/F_{m}$. In contrast, miscane ‘JM 14-72’ had partial recovery for CO$_2$ assimilation (77%) but a full recovery for $F_{i}/F_{m}$ (100%). All entries fully recovered or exceeded their prechilling values of $C_{i}/C_{a}$.

### 4 | DISCUSSION

#### 4.1 | Physiology of photosynthetic chilling sensitivity in sugarcane and tolerance in *Miscanthus*

Although the experiment was a controlled environment experiment and light level was not sufficient enough to saturate
photosynthesis, it was enough to show the variation in CO₂ assimilation in genotypes as well as their response to the chilling treatment. As expected, the sugarcane parents were highly sensitive to chilling temperatures, with greatly reduced $A_{1000}$ and $F_i/F_m$ after only 7 days of exposure, whereas the Miscanthus parents were highly tolerant, even after 14 days of exposure (Table 2). Prior studies have also found that chilling temperatures severely reduced sugarcane photosynthesis (Friesen et al., 2014; Glowacka et al., 2014, 2016). In contrast to sugarcane, prior studies have documented exceptional chilling tolerance of photosynthesis in Miscanthus (Beale et al., 1996; Fonteyne et al., 2016; Friesen et al., 2014; Glowacka et al., 2015; Long & Spence, 2013). Thus, our results for the sugarcane and Miscanthus parents were consistent with these prior findings.

Photosystem II is the most sensitive component of the photosynthetic apparatus to light-induced chilling damage (Long, Humphries, & Falkowski, 1994). Decreases in $F_i/F_m$ in response to chilling temperatures can indicate damage to the PSII reaction centers, resulting in increased chilling sensitivity (Glowacka et al., 2014). Thus, the $F_i/F_m$ data from the greenhouse experiment indicated that after 7 days of chilling, the photosynthetic systems of the sugarcane parents were damaged, but the Miscanthus parents were undamaged, and after 14 days of chilling, the sugarcanes were further damaged, but M. sacchariflorus parent remained undamaged and the M. sinensis parent was little affected. Plants with undamaged PSII reaction centers are more likely to fully recover photosynthetic CO₂ assimilation rates upon postchilling return to warm temperatures than those with damaged PSII, a question we investigated in the subsequent growth chamber experiment. Photosynthetic carbon assimilation can be severely reduced under saturating light and chilling (<15°C) temperatures, and utilization of excitation energy leads to photo-inhibition and photo-oxidation (Baker et al., 1989; Long, 1983; Long et al., 1994). CO₂ assimilation can also be reduced if stomates close in response to cold. Additionally, decreases in chlorophyll fluorescence value ($F_i/F_m$) are typically associated with chilling-dependent photo-inhibition, which is one of the factors responsible for limiting CO₂ assimilation in mature leaves (Baker et al., 1989; Farage, Blowers, Long, & Baker, 2006; Farage & Long, 1987; Ortiz-Lopez, Nie, Ort, & Baker, 1990). Du, Nose, and Wasano (1999a) found that under chilling for 3 days, sensitive sugarcane genotypes retain about 22% of their prechilling photosynthetic rate, while tolerant genotypes retained between 69% and 74%. Our growth chamber experiment supports these findings. This chill-induced reduction in the capacity of the leaf to assimilate CO₂ is dependent on the duration of the chill and increases with increasing photon flux density (Long, East, & Baker, 1983) and its duration (Taylor & Rowley, 1971). However, in our experiment shortening the photoperiod during chilling may have induced a lesser extent of photo-inhibition and photo-oxidative damage to the tolerant genotypes.

Additionally, the substantial decrease in CO₂ assimilation we observed may also be explained by enzymatic regulation for photosynthesis under chilling. The differences in CO₂ assimilation between plants in the warm conditions and chilling treatment were not primarily due to the inhibition of the electron transport chain because the chilling stress did not cause a substantial reduction in $F_i/F_m$. In addition, observed differences also do not appear to be due to stomatal limitations because the increase observed in $C_i/C_a$ with chilling likely indicated that CO₂ entered the mesophyll, but was not consumed. As such, reductions in photosynthetic rate appear to have been due to PPDK or Rubisco limitations at low temperatures. The cold-labile C₄ photosynthetic enzyme pyruvate orthophosphate dikinase (PPDK) plays a limiting role under low temperature in cold-sensitive C₄ species (Burnell, 1990; Hatch, 1979; Potvin, Simon, & Strain, 1986; Sugiyama, Schmitt, Ku, & Edwards, 1979; Usami, Ohta, Komari, & Burnell, 1995; Wang, Naidu, Portis, Moose, & Long, 2008). In addition to PPDK, ribulose bisphosphate carboxylase/oxygenase (Rubisco), and phosphoenol pyruvate carboxylase (PEPc) also limit photosynthesis under chilling (Du et al., 1999a; Du, Nose, & Wasano, 1999b; Kingston-Smith, Harbinson, Williams, & Foyer, 1997; Wang, Naidu et al., 2008; Wang, Portis, Moose, & Long, 2008). Rubisco content and activity declines proportional to the rate of decline in photosynthetic rate (Kubien, Caemmerer, Furman, & Sage, 2003). Sage and McKown (2006) proposed that in C₄ plants, Kranz anatomy limits Rubisco to bundle sheath, hence C₄s are inherently more prone to Rubisco limitation under low temperature. Du et al. (1999a) found that a sensitive sugarcane genotype, determined by a greater reduction in photosynthetic rate after chilling, showed a significant reduction in activity of all three photosynthetic enzymes, while the tolerant genotypes showed a marked increase in activity of the enzymes, especially PPDK. An increased activity of photosynthetic enzymes in tolerant genotypes thus may have been essential in maintaining a higher rate of CO₂ assimilation during chilling in our experiments. Long et al. (1983) observed a steep increase in intercellular CO₂ content ($C_i$) along with photo-inhibition. Thus, the increased ratio of intercellular to ambient CO₂ concentration (i.e. $C_i/C_a$) along with the reduction in $F_i/F_m$, however small, observed in response to chilling in the growth chamber experiment (Table 4) indicated that some degree of light-induced chilling damage of PSII was likely a cause of the lower rates of photosynthesis observed. This conclusion is consistent with prior studies of Miscanthus and sugarcane (Glowacka et al., 2014, 2016; Jiao et al., 2017).

During recovery from chilling after 7 days of warming in the growth chamber experiment (Table 4), our results indicate that short-duration chilling resulted in lasting damage to
**TABLE 4** Results of growth chamber experiment showing least square means ± standard error for net CO₂ assimilation rate at a photosynthetic photon flux density of 1,500 μmol m⁻² s⁻¹ ($A_{1500}$), maximum quantum yield of photosystem II ($F_v/F_m$) in dark-adapted leaves, intercellular to ambient CO₂ content (C i/C a), and leaf chlorophyll content (SPAD value) for genotypes of miscane, sugarcane and Miscanthus. Prechilling measurements were taken after 14 days of a warm initial establishment period (26/18°C day/night). Subsequently, plants were challenged with a chilling treatment (12/7°C day/night) for 7 days, and measured on treatment Day 4. Recovery was measured after 7 days of postchilling return to warm temperatures (26/18°C day/night).

| Genotype       | Type       | A₁₅₀₀ (μmol m⁻² s⁻¹) | F_v/Fₐ | Warm | Treatment | Recovery | Warm | Treatment |
|---------------|------------|----------------------|--------|------|-----------|----------|------|-----------|
| ‘JM 14-09’    | Miscane    | 28.8 ± 0.4 A a        | 0.815 ± 0.001 A a | 0.795 ± 0.004 AB b |          |          |      |           |
| ‘JM 14-72’    | Miscane    | 33.2 ± 0.4 A a        | 0.817 ± 0.001 A a | 0.765 ± 0.004 B b  |          |          |      |           |
| ‘JM 14-88’    | Miscane    | 23.4 ± 0.4 A a        | 0.814 ± 0.001 A a | 0.747 ± 0.004 BC b |          |          |      |           |
| ‘KR 05-619’   | Sugarcane  | 28.3 ± 0.4 A a        | 0.808 ± 0.001 B a | 0.731 ± 0.004 C c  |          |          |      |           |
| ‘KY 06-139’   | Sugarcane  | 29.3 ± 0.4 A a        | 0.814 ± 0.001 A a | 0.728 ± 0.004 C c  |          |          |      |           |
| ‘Miyakonojo’  | *M. sacchariflorus* | 29.9 ± 0.4 A a | 0.822 ± 0.001 A a | 0.800 ± 0.004 A b  |          |          |      |           |
| ‘Shiozuka’    | *M. sinensis* | 24.2 ± 0.4 C a | 0.795 ± 0.001 C b | 0.787 ± 0.004 AB b |          |          |      |           |

Note: Upper case letters indicate comparison between genotypes within each treatment period (warm, chilling treatment and recovery). Lower case letters indicate comparison across time (i.e., comparison between warm, chilling treatment, and recovery period) within each genotype. A different letter indicates significant difference at $p < 0.0001$.

Means separation was conducted by using the adjusted $p$-values from the Tukey–Kramer multiple comparison ($p ≤ 0.05$).

PSII in the sugarcane genotypes but that the *Miscanthus* and miscanes were resilient. Glowacka et al. (2014, 2016, 2015) also found that chilling-susceptible sugarcane accessions had very low rates of recovery upon rewarming after chilling treatment, concluding that chilling resulted in a damaged photosynthetic system for sugarcanes but not for some *Miscanthus* genotypes. Wang, Naidu, et al. (2008) also observed that in *Miscanthus × giganteus*, a cold-tolerant biomass cultivar shows increased amount and activity of PPDK during chilling treatment which helps it maintain a stable photosynthetic rate, whereas in maize, a chilling-sensitive species, the PPDK activity reduces with duration of chilling. In our experiment we also observed a higher photosynthetic rate in miscane and *Miscanthus* genotypes compared to sugarcane. This increase in the activity of photosynthetic enzymes in tolerant genotypes during chilling may have caused the observed postchilling increment in photosynthetic rate in these genotypes over their respective prechilling values. In the field, resiliency to early-season and late-season temperature fluctuations is arguably more valuable than high rates of photosynthesis during chilling events per se, as the potential for CO₂ assimilation upon return to warm temperatures is substantially greater than during short-duration chilling events.

### 4.2 Inheritance of chilling-tolerant photosynthesis in miscanes

Both the *Miscanthus* and sugarcane parents were highly heterozygous (noninbred), thus segregation among the F₁ progeny could be expected. For both $A_{1000}$ and $F_v/F_m$ traits on Day 14 of chilling, the F₁ miscane full-sib and half-sib families show an approximately normal distribution (Table S2), which along with the high heritability estimates suggests that the *Miscanthus* and sugarcane parents each contributed alleles with predominantly additive effects from multiple loci that conferred chilling-tolerant photosynthesis to their progeny (Tables 2 and 3, Tables S1 and S2).

Segregation among F₁ progeny is typical in general for crosses of highly heterozygous parents. For example, in a study on the genetics of overwintering ability in *Miscanthus*, Dong et al. (2019) identified positive and negative QTL from both winter-hardy and non-hardy parents. In previous studies, quantitative variation among *Miscanthus* and among sugarcane genotypes had been observed for CO₂ assimilation and $F_v/F_m$ (Du et al., 1999a; Friesen et al., 2014; Glowacka et al., 2014, 2016, 2015; Tang, Li, & Yang, 2015). Nogueira, De Rosa, Ulian, and Arruda (2003) reported more than 50 genes in sugarcane that were responsive to low temperatures based on gene expression. Thus, chilling-tolerant photosynthesis is likely to be a complex trait in miscanes, and chilling stability of photosystem II is undoubtedly a necessary but perhaps insufficient component of overall photosynthetic tolerance to low temperatures in these populations.

In addition to being highly heterozygous, commercial sugarcane is highly polyploid (8× or more) and also typically aneuploid, so differences in chromosome number among miscane progenies, could also result in variable performance among the progenies. Dosage effects and interactions among alleles from both parents could affect progeny performance, especially given that the tolerant *Miscanthus* parent would be expected to contribute to the F₁ progeny fewer chromosomes than the intolerant sugarcane parent. In *Miscanthus*, ploidy differences have been
observed to affect photosynthetic chilling tolerance (Głowacka, Jezowski, & Kaczmarek, 2010; Glowacka et al., 2014; T. Yamada, personal communication) and other traits, even when advantageous gene combinations are present (Głowacka et al., 2010; Yu, Kim, Rayburn, Widholm, & Juvik, 2009).

Whatever the cause(s), chilling-tolerant photosynthesis in \( F_1 \) miscanes appears to be a quantitative trait, with nonsimple inheritance in a highly heterozygous, polyploid and likely aneuploid genetic background. Nevertheless, we identified highly promising selections from 18 \( F_1 \) miscanes, indicating that introgression of chilling tolerance from \textit{Miscanthus} into sugarcane should be feasible. Although \textit{M. sacchariflorus} is typically more cold tolerant than \textit{M. sinensis} (Clifton-Brown et al., 2008; Glowacka et al., 2014; Sacks et al., 2013), of the two best-performing miscanes we observed after 14 days of chilling temperatures, ‘JM 14-09’ and ‘JM 14-55’, the former was derived from \textit{M. sinensis} and the latter from \textit{M. sacchariflorus}, demonstrating that both species can be useful sources of genes for improving chilling tolerance in sugarcane.

In summary, to the best of our knowledge, this is the first study to compare miscane progeny with their \textit{Miscanthus} and sugarcane parents for photosynthetic chilling tolerance. Substantial variation was observed among the progeny for CO\(_2\) assimilation rate under chilling temperatures but there was no evidence of transgressive segregation. Duration of chilling had a large effect on the performance of the sugarcane parents and their miscane progeny but not for the \textit{Miscanthus} parents. From 4 to 14 days of chilling, the \textit{Miscanthus} parents were relatively stable, maintaining ~2/3rd of their prechilling CO\(_2\) assimilation rate. In contrast, the sugarcane parents kept only ~1/3rd of their prechilling CO\(_2\) assimilation rates after 4 days, and this dropped to ~1/5th after 14 days. Moreover, the sugarcane parents were unable to fully recover CO\(_2\) assimilation and \( F_m/F_0 \), upon return to warm temperatures for 7 days following a 7 day chilling stress, whereas the \textit{Miscanthus} parents and two of three miscanes fully recovered. Seven of 18 miscanes maintained prechilling CO\(_2\) assimilation rates that were greater than their chilling-sensitive sugarcane parent after 7 days of chilling, but by 14 days, only four miscanes were superior to their sugarcane parent. Notably, two miscanes, ‘JM 14-09’ and ‘JM 14-55’, had CO\(_2\) assimilation rates after 14 days of chilling that were not significantly different from their chilling-tolerant sugarcane parent after 7 days of chilling, but by 14 days, both species were superior to their sugarcane parent. Notably, two miscanes, ‘JM 14-09’ and ‘JM 14-55’, had CO\(_2\) assimilation rates after 14 days of chilling that were not significantly different from their chilling-tolerant \textit{Miscanthus} parent, demonstrating the value of early-generation selection and the potential to improve chilling tolerance of sugarcane via introgression. High estimates of broad-sense heritability indicated that differences among miscane genotypes for chilling-tolerant photosynthesis could be detected with little bias from genotype by environment interactions, and suggest that phenotypic selection should be effective. With genes from \textit{Miscanthus} conferring high levels of chilling tolerance in \( F_1 \) miscanes, it should be feasible to use these same genes to breed sugarcane and energycane cultivars that are well-adapted to colder climates than existing cultivars of these crops are, enabling an expansion onto lands that are currently economically off-limits to commercial sugarcane production. Such an advance would be expected to have a large impact on agriculture, energy, and the bioeconomy.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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