

Can miscanthus C₄ photosynthesis compete with festulolium C₃ photosynthesis in a temperate climate?

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Abstract

Miscanthus, a perennial grass with C₄ photosynthesis, is regarded as a promising energy crop due to its high biomass productivity. Compared with other C₄ species, most miscanthus genotypes have high cold tolerances at 14 °C. However, in temperate climates, temperatures below 14 °C are common and our aim was to elucidate cold tolerances of different miscanthus genotypes and compare with a C₃ perennial grass – festulolium. Eleven genotypes of *M. sacchariflorus*, *M. sinensis*, *M. tinctorius*, *M. × giganteus* as well as festulolium were grown under warm (24/20 °C, day/night) and three under cold (14/10 °C, 10/8 °C and 6/4 °C) conditions in a controlled environment. Measurements of photosynthetic light response curves, operating quantum yield of photosystem II (ΦPSII), net photosynthetic rate at a PAR of 1000 μmol m⁻² s⁻¹ (A₁₀₀₀) and dark-adapted chlorophyll fluorescence (Fv/Fm) were made at each temperature. In addition, temperature response curves were measured after the plants had been grown at 6/4 °C. The results showed that two tetraploid *M. sacchariflorus* and the standard triploid *M. × giganteus* cv. Hornum retained a significantly higher photosynthetic capacity than other miscanthus genotypes at each temperature level and still maintained photosynthesis after growing for a longer period at 6/4 °C. Only two of five measured miscanthus genotypes increased photosynthesis immediately after the temperature was raised again. The photosynthetic capacity of festulolium was significantly higher at 10/8 °C and 6/4 °C than of miscanthus genotypes. This indicates that festulolium may be more productive than the currently investigated miscanthus genotypes in cool, maritime climates. Within miscanthus, only one *M. sacchariflorus* genotype exhibited the same photosynthetic capacity as Hornum at both cold conditions and when the temperature was raised again. Therefore, this genotype could be useful for breeding new varieties with an improved cold tolerance vis-a-vis Hornum, and be valuable in broadening the genetic diversity of miscanthus for more widespread cultivation in temperate climates.

Keywords: C₃ photosynthesis, C₄ photosynthesis, chlorophyll fluorescence, cold tolerance, genotype difference, light response curves, quantum yield, temperature response curves

Received 31 October 2015; accepted 12 December 2015

Introduction

Bioenergy crops are a potential replacement source for fossil fuels. To make this option economically competitive and sustainable, major research issues include identifying crops that have a maximum biomass production and at the same time a minimum environmental impact when grown on marginal land under various abiotic stress conditions (Gabrielle *et al.*, 2014). Even though the yield of crops depends on light interception, conversion and partitioning efficiencies, an extension of the growing season has a significant positive impact on biomass production (Zhu *et al.*, 2010). To provide

sustainable biomasses/feedstocks in temperate climates, the ideal bioenergy crop should be perennial and have a high cold-tolerance to support a long growing season in which photosynthesis can take place, thereby promoting high annual yields and low losses to the environment (Karp & Shield, 2008).

An important basic physiological trait influencing cold sensitivity and productivity in crops is the difference between C₃ and C₄ photosynthesis. Photosynthetic activity in C₃ and C₄ species differs under cold conditions. For C₃ plants, the Rubisco capacity has been regarded as the predominant constraint to photosynthesis under a wide range of temperatures at CO₂ levels below the current ambient concentration (Farquhar *et al.*, 1980; Sage & Kubien, 2007). The C₄ photosynthesis is usually advantageous at low CO₂ concentrations and

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high temperatures, and thus its distribution is usually limited at daily mean minimum temperatures of 8 to 10 °C or below during the period of active growth (Teeri & Stowe, 1976; Long, 1983). However, it has been found that the C₄ *Miscanthus × giganteus* is capable of reaching high photosynthetic efficiency even in October with an average temperature of 10 °C in southern England (Beale *et al.*, 1996), and it is hypothesized that the C₄ sensitivity to low temperatures may not be a generic and unbreakable trait (Long & Spence, 2013).

The perennial grass crop, miscanthus, has therefore been regarded as one of the most ideal energy crops due to its high biomass potential and low fertilizer requirements (Lewandowski *et al.*, 2000) and because it can be harvested yearly with high average yields ranging from 13.1 to 14.4 Mg ha⁻¹ for a period of more than 15 years even in the cold temperate maritime climate of Denmark (Larsen *et al.*, 2014). Another productive perennial grass under temperate conditions, but with the C₃ photosynthetic pathway, is festulolium, which has been found useful for extending the autumn grazing season in continental conditions (Skladanka *et al.*, 2010). Festulolium is a cross between species from the genus *Festuca*, with a high level of general stress tolerance towards e.g. drought and heavy grazing, and species from the genus *Lolium*, which is the predominant forage grass in Europe (Wilkins & Humphreys, 2003). Several festulolium cultivars, in which the good forage quality of ryegrass species has been combined with the high persistency and stress tolerance of fescues, have been bred recently (Østrem & Larsen, 2008; Halling, 2012). The cultivar festulolium *cv.* Hykor has been shown to have a high yield potential of around 18 Mg ha⁻¹ and high persistency throughout the season (www.DLF.com). Thus, festulolium may be ideal as a sustainable feedstock for biorefineries (e.g. protein feed and bioenergy) in temperate climates (Parajuli *et al.*, 2015).

In previous studies, 14 °C has been chosen as an important temperature level for examining the cold tolerance of plants with C₄ photosynthesis (Haldimann, 1996; Farage *et al.*, 2006). We screened a range of miscanthus genotypes for their tolerance to a 14/10 °C growing environment to find improved genotypes compared with *M. × giganteus*, but found only a few with slightly improved characteristics (Jiao *et al.*, 2016). However, plants in northern Europe are frequently exposed to much lower temperatures, especially in the autumn and spring seasons. Therefore screening of the photosynthetic response to temperatures below 14 °C may be helpful to identify genotypes with a high tolerance to low temperatures, which are critical for growth in the spring and autumn in continental climates and throughout the growing season in cool temperate maritime climates.

In a side-by-side trial in Illinois, Dohleman & Long (2009) found that *M. × giganteus* was 59% more productive than maize (*Zea mays*) due to its larger leaf area and its longer growing season that was facilitated by superior cold tolerance. Photosynthesis in *M. × giganteus* has been found to still occur at 10 °C, although at a lower rate than in plants grown at 14 °C (Farage *et al.*, 2006). Photosynthetic CO₂ uptake of *M. × giganteus* and maize measured during short-term exposure to 5 °C after having been grown at 24 °C and 14 °C showed that *M. × giganteus* kept a photosynthetic level of around 4 to 5 μmol m⁻² s⁻¹, which was significantly higher than the 1 to 2 μmol m⁻² s⁻¹ of maize grown at 14 °C (Naidu *et al.*, 2003). However, it is unclear whether the photosynthetic apparatus in the miscanthus was damaged or not and whether the photosynthetic capacity would continue to function after long-term exposure to temperatures below 10 °C. The C₃ photosynthesis of festulolium is expected to be tolerant to low temperatures, but an exposure to 2 °C resulted in a dramatic decline in maximum quantum yield of PSII (Fv/Fm), in increased nonphotochemical quenching (NPQ) and in reduced efficiency of energy conversion in the photochemical processes (qP) (Pociecha *et al.*, 2010).

Previous studies on high-yielding and cold-tolerant miscanthus have focused mainly on *M. × giganteus cv.* Hornum (Larsen *et al.*, 2014), although this genotype is impossible to further improve by breeding since it is a sterile triploid hybrid of a tetraploid *M. sacchariflorus* and diploid *M. sinensis* (Greef & Deuter, 1993) with little genetic diversity (Hodkinson *et al.*, 2002; Clark *et al.*, 2014). However, *M. sinensis* and *M. sacchariflorus* could be used to breed new *M. × giganteus* cultivars for broadening the genetic base which would be useful in the breeding of high-yielding genotypes with improved cold-tolerance or for growing on a wider scale (Clifton-Brown & Lewandowski, 2002; Jorgensen, 2011). Other miscanthus species may also contribute cold tolerance to breeding, one possibility being *M. tinctorius*, which often grows at high altitudes or latitudes.

Exploring the background for high cold-tolerance in plants with C₄ photosynthesis may be helpful before introducing them in cool regions and broadening their planting area. An earlier study has found that low-temperature photoinhibition associated with the xanthophyll cycle (zeaxanthin accumulated from violaxanthin under high light conditions) is reversible and is a strategy for C₄ species to tolerate cool climates, even though it was more detrimental to carbon gain in the C₄ grass *Muhlenbergia glomerata* than in the C₃ species *Calamagrostis canadensis* grown at 14/10 °C (Kubien & Sage, 2004a). Photo damage is characterized by a reduced photosynthetic capacity, which cannot be redeemed by a rising temperature. For example, low temperatures

affect both the light-harvesting apparatus and the CO₂-fixing enzymes of maize (Baker *et al.*, 1983). A previous study has reported that the ability to sustain the photosynthetic rate at a relatively high level at 14 °C in some genotypes might be dependent on maintenance and increase of Rubisco (Spence, 2012) and PPDK concentrations in leaves (Wang *et al.*, 2008a,b).

C₃ plants usually have a lower optimal temperature and lower maximum photosynthetic rates, but have the advantage of higher photosynthesis at low temperatures than C₄ plants (Pearcy *et al.*, 1981; Yamori *et al.*, 2014). Still, it is unclear at which temperature level miscanthus can compete with C₃ species in cool climates, and to what degree there is genetic variation within the genus that may help improve the competitiveness of miscanthus. Increased knowledge of this will also be helpful for guiding site selection and adapting the cultivation practices for these two types of crop in cool climates.

The aim of this work was to examine the cold tolerance of different C₄ miscanthus genotypes through studies of net photosynthetic rate (A_n), PSII operating efficiency (ΦPSII), maximum quantum yield (F_v/F_m)

and light response under various growth temperatures down to 6 °C. Furthermore, the cold tolerance of C₄ miscanthus was compared with one well-known, highly-productive festulolium cultivar. Our hypothesis was that we would be able to identify genotypes with a higher photosynthetic performance under cold conditions than the *M. × giganteus* cv. Hornum and that these genotypes may even compete with a C₃ grass under the variable, temperate, maritime conditions of northwestern Europe.

Materials and methods

Plant material

One C₃ species, the grass festulolium (cultivar Hykor), which is a hybrid of *Lolium multiflorum* and *Festuca arundinacea* (Cernoch *et al.*, 2004; Zwierzykowski, 2004; Kopecky *et al.*, 2008), and 11 genotypes of the C₄ genus *Miscanthus* representing four different species (*M. sinensis*, *M. sacchariflorus*, *M. tinctorius* and *M. × giganteus*) were selected for climate chamber experiments (Table 1).

Table 1 Genotypes measured in the climate chamber experiments. The genotypes numbers 1 to 14 are from a previous climate chamber experiment (Jiao *et al.*, 2016); genotypes 15 to 19 are new material for this study

Identification	Identification in previous publication	Species	Origin	Altitude (m)	Latitude (°N)	Ploidy
Tin-1	133/1*	<i>M. tinctorius</i>	Ainukura, Honshu†	350	36	2x¶¶
Sin-3	56/1*	<i>M. sinensis</i>	Kamiyoshino (5 km SE of Kanazawa), Honshu†	280	36	2x¶¶
Sac-10		<i>M. sacchariflorus</i>	Hakusan National park, Honshu†	900	36	4x¶¶
Sac-11		<i>M. sacchariflorus</i>	South of Shirakawa, Honshu†	600	36	4x¶¶
Sac-12	EMI-5‡	<i>M. sacchariflorus</i>	Japan			4x¶¶
Gig-13	EMI-1‡	<i>M. × giganteus</i>	cv Hornum§, Larsen, Denmark			3x‡
Gig-14	M114¶	<i>M. × giganteus</i>	Tinplant, GmbH, Klein Wanzleben, Germany**			2x
Sin-15		<i>M. sinensis</i>	South of Shirakawa, Honshu†	600	36	
Sac-16		<i>M. sacchariflorus</i>	Japan†			4x
Sac-17		<i>M. sacchariflorus</i>	Near highway M58, outside of Birobidzhan turnoff, Russia	67	49	2x¶¶
Sac-18		<i>M. sacchariflorus</i>	Near M60 North of Lermontavka	86	47	2x¶¶
Fest-19	Hykor††	<i>L. multiflorum</i> × <i>F. arundinacea</i>	Bred in Czech Rep.‡‡			6x§§

*Identification used in Glowacka *et al.* (2015b).

†Seed plants collected in Japan 1995 and grown in Denmark since 1996 (Kjeldsen *et al.*, 1999).

‡Clones from 'European *Miscanthus* Improvement' project (Clifton-Brown *et al.*, 2001).

§EMI-1 was equivalent to Hornum, due to extreme high genetic similarity (Glowacka *et al.*, 2015a).

¶(Carrie *et al.*, 2012).

** (Kim *et al.*, 2012).

††Identification used in Cernoch *et al.* (2004); Østrem & Larsen (2008); Ambye-Jensen *et al.* (2013); Ostrem *et al.* (2013).

‡‡Acquired by DLF-Trifolium, Denmark (Zwierzykowski, 2004; Kopecky *et al.*, 2008).

§§(Cernoch *et al.*, 2004).

¶¶Ploidy levels were determined by flow cytometry described in Jiao *et al.* (2016).

The miscanthus genotypes Tin-1, Sin-3, Sac-10, Sac-11, Sac-12, Gig-13 and Gig-14 were selected for this study because they showed the most interesting results for growth under cold conditions in a previous climate chamber experiment (Jiao *et al.*, 2016). These were supplemented with two genotypes selected from the 166 genotypes that were previously evaluated in field experiments by Jiao *et al.* (2016): Sin-15 was selected because it had a late blossom and senescence in the autumn, and Sac-16 was chosen because it had a high photosynthetic rate in field experiments (data not shown). Two final *M. sacchariflorus* genotypes (Sac-17 and Sac-18) were selected from 222 accessions collected during a 2012 joint USDA-Vavilov Institute expedition in Eastern Russia led by Erik J. Sacks (L. V. Clark, D. Elena, D. Nikolay *et al.*, unpublished data). They grew at high latitude (Table 1) and had tall and relatively thick stems compared with the other collected genotypes (Erik Sacks, personal communication). In contrast with Japanese *M. sacchariflorus*, which is tetraploid, these Russian *M. sacchariflorus* are diploid. The Russian genotypes were planted in pots in the greenhouse in Denmark between 8 and 16 October 2012.

Plant cultivation

All miscanthus rhizomes, except Sac-17 and Sac-18, were dug up from the field on 15 November 2013 before onset of frost. They were stored at 2 °C until five days before planting and then at approx. 15 °C for acclimatization until planting out. The rhizomes were planted on 18 February 2014 in pots measuring 25 × 15 × 30 cm (length, width, height) and each filled with 2.2 kg peat (Pindstrup Substrate no. 4, 10–30 mm, pH 6.0, containing NPK fertilizer 1.0 g l⁻¹ and micro fertilizer 0.05 g l⁻¹). The rhizomes were planted in six replicates of each genotype. The Russian Sac-17 and Sac-18 were planted on 25 February using rhizomes from the plants grown in the greenhouse. Whole plants of Hykor were dug up from a field experiment established in 2012 (Manevski *et al.*, 2015) on 7 November 2013. They were irrigated immediately and kept outside the greenhouse during the winter next to the wall to protect them from severe frost. They were moved to the greenhouse and cut down to 6–8 cm height on 12 March 2014. All plants were cultivated in the greenhouse for 7 weeks. The temperature in the greenhouse was kept at approx. 15/10 °C day/night (d/n). Artificial light was turned on between 06:00 and 18:00 if the solar radiation during that time was below 670 μmol m⁻² s⁻¹. The intensity of the artificial light was about 350 μmol m⁻² s⁻¹ provided by 400 W metal-halide lamps (Osram Powerstar HQI-BT Daylight).

Irrigation and fertilization

The plants in the greenhouse were irrigated every two or three days to field capacity. After one month's growth in the greenhouse, 1% inorganic fertilizer (Prima Væksthusgødning, NPK 3-1-4) was added to the irrigation water every second or third day (in 0.3 l water to each pot). During the period of measurements in the climate chamber all the plants were irrigated every evening and fertilizer was added every second or third day.

Gas exchange measurements

On 15 April, all plants except Sac-18 were moved from the greenhouse into two climate chambers with same controlled environmental conditions. The genotype Sac-18 was moved from the greenhouse into the climate chamber on 5 May 2014 just after the temperature had been decreased to 14 °C.

Three replicates of each genotype were cultivated in each of the two chambers. The plants were randomly arranged on trolleys. They were grown in a constant temperature of 24/20 °C d/n for the warm conditions and 14/10 h d/n under a PAR of 670 μmol m⁻² s⁻¹. The relative humidity for warm conditions was set to 85/85% d/n and the CO₂ concentration was set to 400 ppm. After 12 days of growth in the warm conditions, light response curve measurements were performed (see details below) on four selected genotypes of miscanthus and festulolium, i.e. Tin-1, Sin-15, Sac-12, Gig-13 and Fest-19. The photosynthetic rate at a photosynthetic active radiation (PAR) of 1000 μmol m⁻² s⁻¹ (*A*₁₀₀₀) and chlorophyll fluorescence were measured on all the plants on 30 April as described below.

After 20 days in the warm conditions, the temperature in the chamber was reduced to 14/10 °C d/n with the same day/night cycle as for the warm conditions. The relative humidity was set to 75/85% d/n, while all other conditions were set as described above for 24/20 °C. Nine days later, the temperature in the chamber was reduced to 10/8 °C d/n and the relative humidity was set to 85%/90% d/n, while other conditions were left unchanged. After nine days at 10/8 °C, the temperature in the chamber was reduced to 6/4 °C d/n cycle and the relative humidity was set to 85%/95% d/n. Other conditions were left unchanged. After 5 days' growth at each temperature level, light response curve measurements were performed on the five miscanthus and festulolium genotypes. In addition, *A*₁₀₀₀ as well as chlorophyll fluorescence were measured on all the plants at the end of each cold temperature level.

Gas exchange measurements were performed on the youngest fully developed leaf (ligule present) of six replicates of each genotype from 8:30 to 15:00 hours using an open-flow gas exchange system, CIRAS-2 (PP Systems, Amesbury, MA, USA). The conditions inside the cuvette were set to reflect controlled environmental conditions (i.e. the CO₂ concentration was set to 400 μmol mol⁻¹ and the mean leaf temperature was maintained at the same level as the growth temperature). The vapor pressure deficit (VPD) in the leaf cuvette was 1.0 kPa (24 °C), 0.6 kPa (14 °C and 10 °C) and 0.5 kPa (6 °C) and the airflow through the chamber was 250 ml min⁻¹; PAR was set to 1000 μmol m⁻² s⁻¹.

For the light-response curve measurements, the leaves were acclimatized to a PAR of 1500 μmol m⁻² s⁻¹ (24 °C, 14 °C and 10 °C) and 1000 μmol m⁻² s⁻¹ (6 °C) until the photosynthetic rate was stabilized (Wang *et al.*, 2012). The PAR was then decreased from 1500 to 20 μmol m⁻² s⁻¹ in twelve steps (1500, 1200, 1000, 800, 500, 300, 200, 150, 100, 80, 50 and 20). For 6 °C, there were only 10 steps as the steps at 1500 and 1200 μmol m⁻² s⁻¹ were skipped. The measurements were logged after the photosynthetic rates had stabilized, which was usually after two minutes.

Temperature response curves

The temperature response of photosynthesis was measured after seven days of growth at 6 °C in the climate chamber. The temperature in the leaf cuvette was set at five steps: 5 °C, 10 °C, 15 °C, 20 °C and 25 °C. It was measured on three to four replicates on the youngest fully developed leaf with each leaf measured at all temperatures, and the CO₂ concentration was controlled at 400 ppm. For each temperature measurement, leaves were light and temperature-acclimated until steady state had been achieved in the gas exchange cuvette at a PAR of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Attempts were made to keep a similar leaf-to-air vapor pressure deficit at all temperatures by modifying the relative humidity in the leaf cuvette. Still, an increase in VPD could not be avoided with increasing temperatures.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was determined on Day 6 of each temperature settlement under the cold conditions using a Mini-PAM fluorometer (Walz, Germany). The leaves were dark-adapted with Dark Leaf Clips for 30 min before the measurements were performed (Bolhar-Nordenkamp *et al.*, 1989). The minimal fluorescence (F_0) was determined at very low PPFD, where the PSII reaction centers are in the 'open' state. The maximal fluorescence (F_m) was measured by applying a 0.8 s pulse at a high light level of approx. 4000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, which forces the closure of the reaction centers (Krause & Somersalo, 1989). The maximum quantum yield of PSII (F_v/F_m) was calculated from F_0 and F_m , as $F_v/F_m = (F_m - F_0)/F_m$. All measurements were repeated on the six replicates of each genotype.

When an actinic light is applied on the leaf, maximum fluorescence in the light (F_m') can be measured with a saturating flash, and steady-state fluorescence (F_s) can also be recorded just prior to the flash. The operating quantum yield of photosystem II (ΦPSII), calculated as $(F_m' - F_s)/F_m'$ (Genty *et al.*, 1989) was measured immediately after the A_{1000} had reached steady state using the chlorophyll fluorescence module (CFM) in CIRAS-2 on the ninth day after the growth temperature was decreased. It was always measured between 8:30 and 15:00.

The instantaneous quantum cost of assimilation can be approximated by the ratio of ΦPSII to quantum yield of CO₂ assimilation (ΦCO_2) (Oberhuber & Edwards, 1993). The ΦCO_2 parameter is the efficiency of CO₂ assimilation, which is calculated as $A / (\text{PAR} * \alpha)$. Leaf absorptance (α) was assumed to be 0.85 for all the genotypes in this study for the four temperature treatments, since generally 85% of incident light can be absorbed by leaves (Singsaas *et al.*, 2001) and also because apparently no dramatic changes in leaf absorptance (approximately 0.85) take place between 450 and 700 nm for *M. × giganteus* grown at 10, 14 and 25 °C (Farage *et al.*, 2006).

Photosynthetic response curves and calculation

Data from the light response experiment were fitted to a non-rectangular hyperbola model (Marshall & Biscoe, 1980) by means of the nonlinear least squares curve-fitting procedure of R for Windows (Team RC, 2015).

The AQY values were obtained from linear regression of the relationship between net CO₂ assimilation and PAR across five points at incident light intensities from 20 to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Long *et al.*, 1996). A_{1500} was the rate of photosynthesis at a PAR of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (A_{1500}).

Statistical analysis

Data for each temperature treatment were analysed separately using Analysis of Variance. If a significant effect was observed, multiple comparisons were performed using Tukey's HSD test. The difference in $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio between temperature treatments for each genotype was analysed using the same methods as mentioned above. All the analyses and tests were done in R version 3.1.2 (Team RC, 2015). The R package 'lsmeans' (Lenth & Hervé, 2005) was used for multiple comparisons.

Results

Photosynthetic performance and operating quantum yield of PSII

The A_{1000} of Sac-12 grown at 24/20 °C was 25.7 $\mu\text{mol m}^{-2} \text{s}^{-1}$, which was significantly higher than other genotypes except Sac-16, Sac-11 and Gig-13 (Fig. 1a). The lowest values of 18.0 and 9.8 $\mu\text{mol m}^{-2} \text{s}^{-1}$ were measured in Sin-15 and Tin-1, respectively, and this was significantly lower than for Sac-11, Sac-12, Gig-13, Sac-16 and Fest-19. Concerning ΦPSII , Fest-19 and Sac-12 had significantly higher values (0.379 and 0.366, respectively) than Sac-10, Sin-15, Gig-14 and Tin-1 (Fig. 1b).

For all genotypes, A_{1000} was reduced when the temperature was decreased, albeit to different extents (Fig. 1a). At the 14/10 °C temperature, Fest-19 showed the highest A_{1000} at 15.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$. This C₃ species as well as Sac-12 and Sac-11 had significantly higher A_{1000} values than *M. tinctorius*, the two *M. sinensis* genotypes, Sac-17 (the Russian genotype) and Sac-16 (Fig. 1a). Fest-19 also had the highest ΦPSII at 14/10 °C. Sin-3 and Gig-14 had significantly lower ΦPSII values than Sac-11, Sac-12, Gig-13 and Fest-19.

When the temperature was reduced to 10/8 °C, Fest-19 showed significantly higher A_{1000} (14.3 $\mu\text{mol m}^{-2} \text{s}^{-1}$) values than all the miscanthus genotypes of which Sac-11, Sac-12 and Gig-13 had significantly higher values (9.8, 8.0 and 8.9 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively) than all the other miscanthus genotypes (Fig. 1a). A similar trend was observed at the 6/4 °C conditions, where A_{1000} in Fest-19 was 7.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ but 3.8 and 3.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in Sac-11 and Gig-13, respectively, which is significantly higher than for the other miscanthus genotypes except for Sac-12 and Sin-15. The highest ΦPSII value was observed in Fest-19 at both 10/8 °C and 6/4 °C (Fig. 1b). Within the

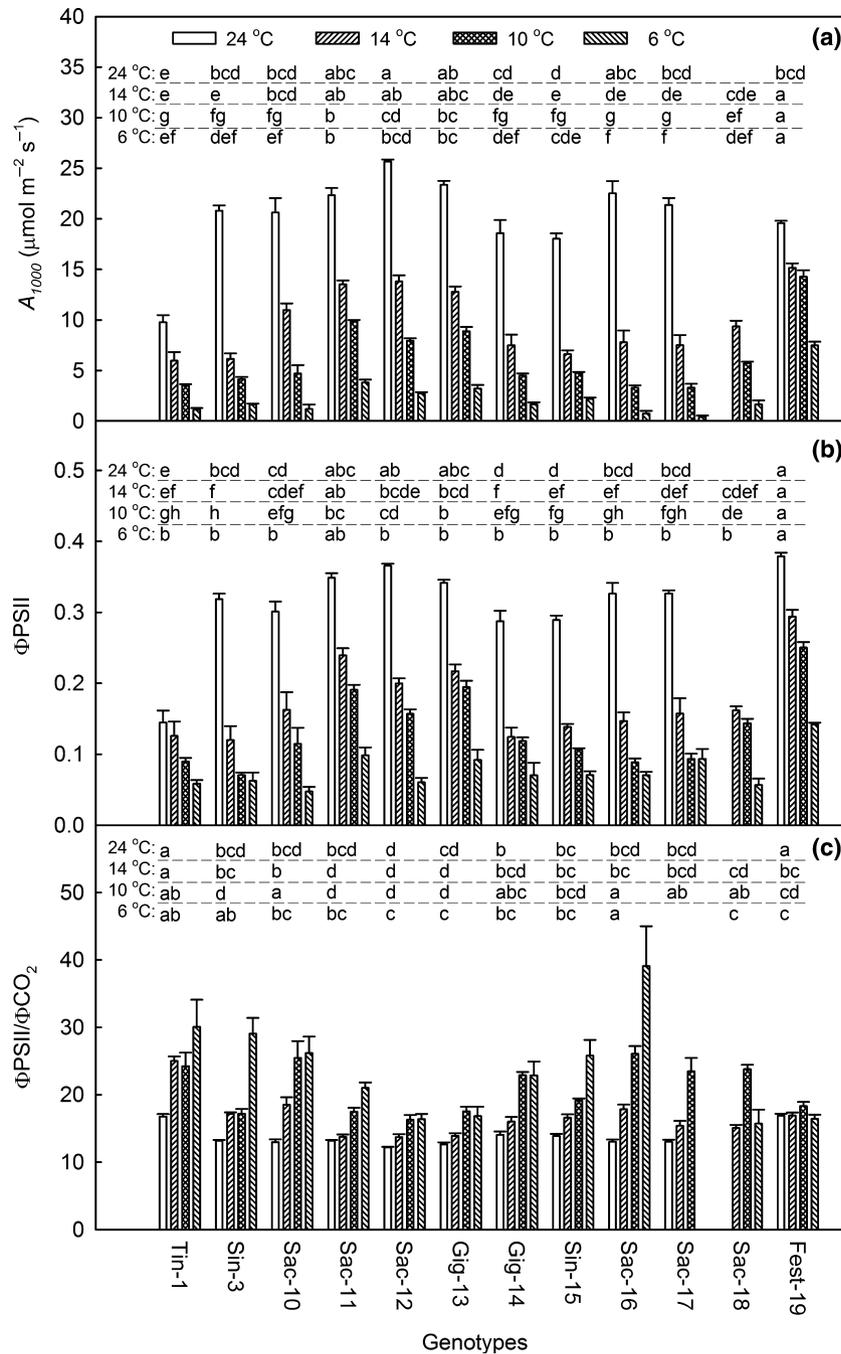


Fig. 1 (a) Net photosynthetic rate at 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (A_{1000}), (b) Quantum yield of photosystem II (Φ_{PSII}) at 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, (c) The ratio of operating efficiency of PSII to apparent quantum yield of CO₂ assimilation ($\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$) under warm day/night (24/20 °C) and three cold (14/10 °C, 10/8 °C and 6/4 °C) growing conditions. Error bars represent SE ($n = 6$). Values without letters in common are significantly different at the $P = 0.05$ level within each temperature treatment.

miscanthus genotypes, Sac-11, Sac-12 and Gig-13 showed significantly higher Φ_{PSII} values (0.191, 0.157 and 0.195, respectively) than other genotypes at 10/8 °C. There was no significant difference in Φ_{PSII} values between the miscanthus genotypes at 6/4 °C (Fig. 1b).

The $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ ratio

The ratio $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ increased after temperature reduction for the miscanthus genotypes. However, there was no significant change in Fest-19 when the temperature was decreased to 14/10 °C, 10/8 °C and even

6/4 °C. The Φ_{PSII}/Φ_{CO_2} ratio for Tin-1 and Fest-19 was significantly higher than for the other miscanthus genotypes at 24/20 °C (Fig. 1c). At 14/10 °C, Sac-12, Sac-11 and Gig-13 had the lowest values compared to the other genotypes, but they were not significantly different from other genotypes except Tin-1. When the temperature decreased from 14/10 °C to 10/8 °C a significant increase was found in Sac-11, Sac-12, Gig-13, Gig-14 and Sac-17. At 10/8 °C, Sin-3, Gig-13, Sac-11 and Sac-12 had significantly lower values than all other genotypes except Sin-15 and Sac-17 (Fig. 1c). The Φ_{PSII}/Φ_{CO_2} ratio in Sin-3, Sac-11 and Sin-15 increased significantly from 10/8 °C to 6/4 °C, whereas for Sac-18 it decreased (not significantly). At 6/4 °C, the ratio was significantly higher in Sin-3, Tin-1 and Sac-16 than in Sac-12, Gig-13, Sac-18 and Fest-19.

Chlorophyll fluorescence

The C₃ crop Fest-19 had an F_v/F_m value of 0.82 when it was grown at 24/20 °C, while in the miscanthus genotypes values were between 0.73 and 0.80 (Fig. 2). Three genotypes, Sac-10, Sac-11 and Sac-16, had significantly higher F_v/F_m values of 0.80, 0.79, and 0.80, respectively, than the 0.74 and 0.73, respectively, of Gig-14 and Tin-1 at 24/20 °C.

A reduction in F_v/F_m was observed in all miscanthus genotypes during the temperature decrease, but was less clear for Fest-19 (Fig. 2). Tin-1 and Gig-14 had significantly lower values than other genotypes at 14/10 °C. When the temperature was further decreased to 10/8 °C, there was a slight decrease in Fest-19, but it

was much greater in miscanthus. The F_v/F_m values of 0.68, 0.65 and 0.67, respectively, for Gig-13, Sac-11 and Sac-12 were significantly higher than the 0.43, 0.44, 0.51, 0.48 and 0.51 for Sin-3, Tin-1, Sac-16, Gig-14 and Sac-18.

At 6/4 °C, the F_v/F_m value in Fest-19 F_v/F_m was 0.71. This was significantly higher than all the miscanthus genotypes. At this temperature there was no significant difference between Gig-13, Sac-11, Sac-12 and Sin-15, but they had significantly higher values than Sin-3, Sac-10, Sac-16 and Gig-14 (Fig. 2). For both Fest-19 and the miscanthus genotypes, the largest reduction was observed when the temperature dropped from 10/8 °C to 6/4 °C.

Light response curves at different temperature levels

The responses of CO₂ assimilation to light showed significant effects of temperature on genotypes at each measured light level (Fig. 3). Sac-12 and Fest-19 showed significantly higher AQY (0.043 and 0.041) than other measured genotypes at 24/20 °C (Fig. 4). A significant reduction of AQY was observed in the measured genotypes when the temperature was decreased from 24/20 °C to 14/10 °C. However, there was no significant reduction of AQY for Sac-11, Sac-12, Gig-13 and Fest-19 when the temperature was further decreased from 14/10 °C to 10/8 °C, while Tin-1 and Sin-15 had a decrease of 38% and 52%, respectively. No significant difference in AQY was observed between Sac-11, Sac-12, Gig-13 and Fest-19 grown at 14/10 °C and 10/8 °C. At 6/4 °C, Fest-19 maintained the same AQY level as when it was grown under 14/10 °C, while the miscanthus genotypes

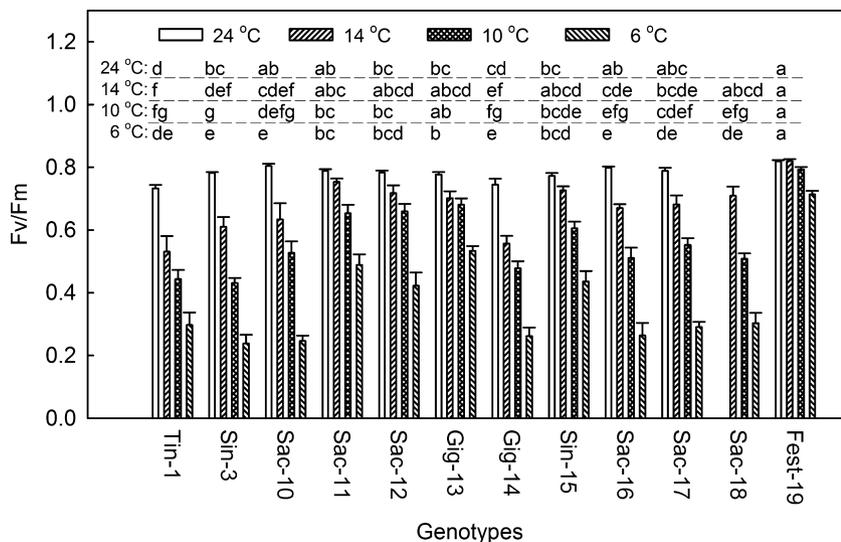


Fig. 2 Maximum quantum yield of PSII (F_v/F_m) measured under warm day/night (24/20 °C) and three cold (14/10 °C, 10/8 °C and 6/4 °C) growing conditions. Error bars represent SE ($n = 6$). Values without letters in common are significantly different at the $P = 0.05$ level within each temperature treatment.

experienced a significant reduction when the temperature fell from 10/8 °C to 6/4 °C.

Sac-12 had the highest A_{1500} value of 30.6 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 24/20 °C (Fig. 4b). This was significantly higher than for Fest-19 (25.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and the other genotypes, except Gig-13 (27.9 $\mu\text{mol m}^{-2} \text{s}^{-1}$). However, Fest-19 maintained the highest A_{1500} compared to the miscanthus genotypes at the three cold temperatures. Of the miscanthus genotypes at 14/10 °C, Sac-11 had a significantly higher A_{1500} (14.8 $\mu\text{mol m}^{-2} \text{s}^{-1}$) than Sin-15 and Tin-1 (11.1 and 6.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively). When the temperature was reduced to 10/8 °C, Sac-11 as well as Sac-12 and Gig-13 had significantly higher A_{1500} values than Sin-15 and Tin-1. Furthermore, Sac-11 was significantly higher than Sin-15 and Tin-1 at 6/4 °C.

Short-term temperature response curves at 6/4 °C growing temperature

Gig-13 and Sac-11 showed a significant increase in A_{1000} when the temperature was increased from 5 °C to 25 °C, while Sac-12 only increased when the temperature rose from 5 °C to 10 °C and remained steady during further temperature increases (Fig. 5). However, the A_{1000} of Fest-19, which showed the highest level at 5 °C, decreased significantly when the temperature was increased to 15 °C, as the leaf-to-air vapor pressure deficit increased during the temperature increase (from 0.4 to 2.5 kPa), inducing stomatal closure (Fig. S1, A).

Discussion

Photosynthetic performance at 14 °C

The constraint to C₄ photosynthesis in miscanthus in cold conditions might differ depending on the temperature. We observed a decrease in A_{1000} and ΦPSII in all the miscanthus genotypes and in festulolium for each temperature reduction. However, *M. × giganteus* has reportedly markedly higher photosynthetic rates at 14 °C compared to other C₄ species like maize (Naidu & Long, 2004; Wang *et al.*, 2008b) and *Cyperus longus* L. (Farage *et al.*, 2006). In the present study, Sac-11, Sac-12 and Gig-13 maintained similar photosynthetic rates to the C₃ grass Fest-19 at 14 °C (Fig. 1a). This indicates that some genotypes of miscanthus might be useful for cultivation in maritime temperate conditions. Our previous study suggests that a reduced photosynthetic activity in miscanthus due to a temperature decrease to 14 °C could be reversed in these cold-tolerant genotypes once the temperature is increased again (Jiao *et al.*, 2016). Another study shows that the effect of a low growth temperature (14 °C) on photosynthesis in the C₄ grass *M. glomerata* could be reversed by a 24-h exposure to higher temperatures (Kubien & Sage, 2004a). This suggests that such a reversible photoinhibition at 14 °C is lacking in maize (Naidu *et al.*, 2003), but is a strategy for some C₄ species to tolerate cool climates (Kubien & Sage, 2004a).

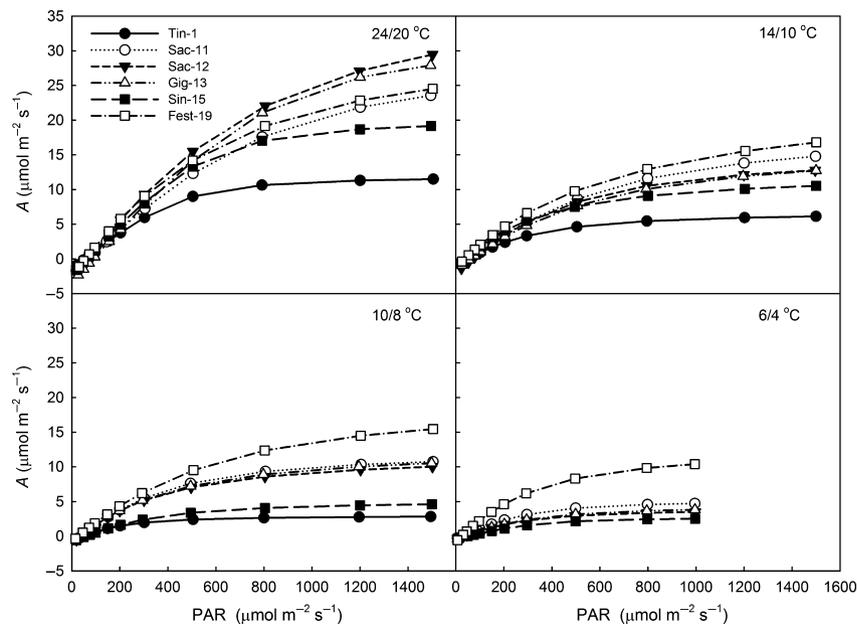


Fig. 3 Light response curves (net photosynthesis, A_n , vs. photosynthetic active radiation, PAR) of selected miscanthus genotypes and festulolium under warm day/night (24/20 °C) and three cold (14/10 °C, 10/8 °C and 6/4 °C) growing conditions. Each point is the fitted mean value from a nonrectangular hyperbolic model of six measurements. Tin-1 was not included in 6/4 °C.

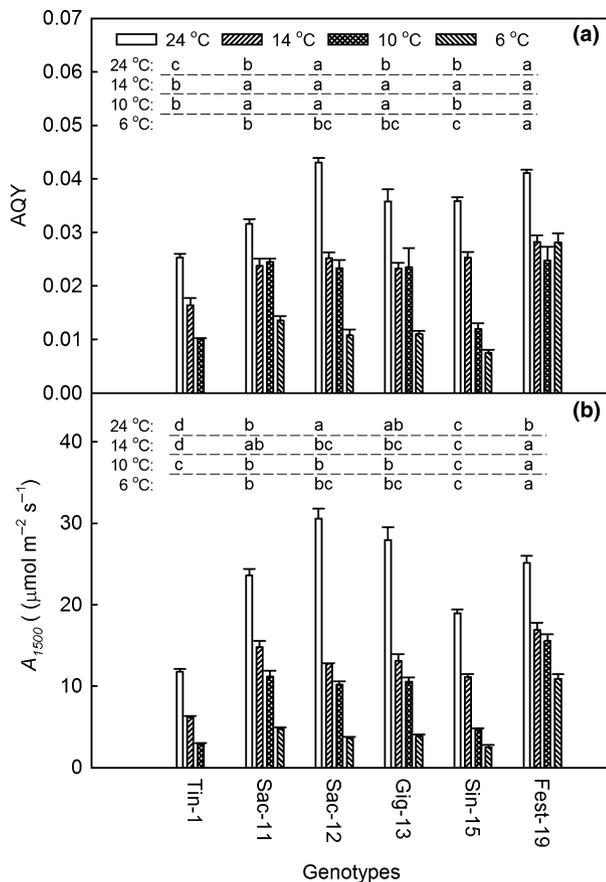


Fig. 4 (a) Apparent quantum yield of CO₂ uptake (AQY), and (b) Net photosynthetic rate at a PAR of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (A_{1500}). The parameters are measured on five miscanthus genotypes and festulium under warm day/night (24/20 °C) and three cold growing conditions (14/10 °C, 10/8 °C and 6/4 °C). Error bars represent SE ($n = 6$). Values without letters in common are significantly different at the $P = 0.05$ level within each temperature treatment.

Generally, C_4 plants show their photosynthetic advantage over C_3 plants at temperatures above 20 °C (Yamori *et al.*, 2014). However, here we find that some miscanthus genotypes can compete with C_3 festulium at a temperature of 14 °C.

For C_4 plants there is linear relationship between ΦPSII and ΦCO_2 under nonstressed conditions over a range of light intensities (Genty *et al.*, 1989; Krall & Edwards, 1990). No significant increase in $\Phi\text{PSII}/\Phi\text{CO}_2$ was found for any of the genotypes in this study when the temperature was lowered from 24/20 °C to 14/10 °C (Fig. 1c). This is consistent with previous findings that no significant changes happen to the $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio in *M. × giganteus* or *Z. mays* leaves when the temperature falls from 25/20 °C to 14/11 °C (Naidu & Long, 2004). This indicates that the electron transport through PSII is just sufficient for the CO₂ assimilation

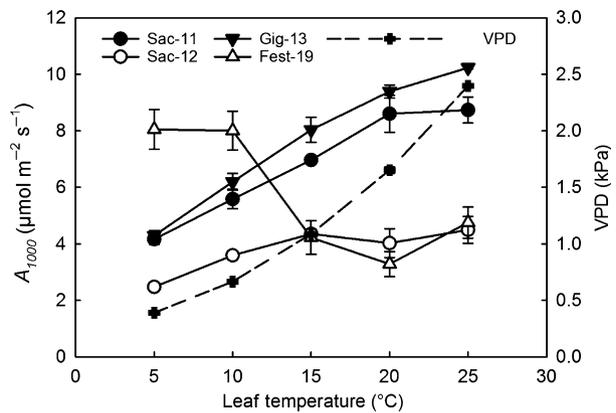


Fig. 5 Net photosynthetic CO₂ uptake at a PAR of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ vs. temperature for Sac-11, Sac-12, Gig-13 and Fest-19 grown at 6/4 °C (day/night) for 7 days, and the change of leaf-to-air vapor pressure deficit with temperature. Error bars represent SE ($n = 3$ or 4).

requirement, and that no alternative electron sinks exist at 14 °C for any of the miscanthus genotypes tested in this study.

Another interesting parameter is the ratio of F_v/F_m , which gives a relative measure of the maximum quantum efficiency of PSII photochemistry (Genty *et al.*, 1989). Decreases in F_v/F_m can be due to the development of nonphotochemical quenching processes or photo damage to PSII reaction centers (Baker & Rosenqvist, 2004). We observed no decrease in F_v/F_m for Fest-19 at 14 °C, but a slight decrease (within 10%) compared with the values measured at 24 °C was detected in Sac-11, Sac-12 and Gig-13 (Fig. 2). This suggests that a temperature of 14 °C had no damaging effect on the photosynthetic apparatus in the C_3 grass, but that a minor low-temperature-induced photo inhibition occurred in the three genotypes of miscanthus, even though the A_{1000} values for the other tested genotypes, except Sac-10, were significantly lower than the three apparently cold-tolerant genotypes (Fig. 1a). Interestingly, these other genotypes also had a significantly lower ΦPSII (Fig. 1b), which means that markedly less light absorbed by PSII antennae is used for photochemistry. Reduced A_{1000} could be the direct result of reduced PSII efficiency due to PSII photo inhibition.

The largest decrease in A_{1000} when the temperature was lowered from 24/20 °C to 14/10 °C occurred in Sac-16. The plants of this genotype turned reddish violet when they were transferred from 15 °C in the greenhouse to the 24/20 °C climate chamber conditions. This could be due to anthocyanin synthesis during temperature changes (Pietrini & Massacci, 1998). Like Sin-3 and Sin-15, the Sac-17 genotype suffered a large reduction in A_{1000} after the temperature decrease to 14/10 °C and

turned yellow after the repositioning from the greenhouse to the climate chamber, i.e. there was a visible chlorophyll reduction. These two genotypes thus seemed to be stressed both by the warm temperatures and by subsequent cold conditions.

Photosynthetic performance at 10 and 6 °C

When the temperature was lowered to 10/8 °C, further decreases in A_{1000} and Fv/Fm values were found in miscanthus but not in festulium (Fig. 2), which indicates that additional increases in nonphotochemical quenching and/or photo damage of the PS II reaction center occurred in miscanthus. One of the major mechanisms for miscanthus to get rid of excess energy absorbed by the leaves is heat dissipation. For *M. × giganteus* grown at 10 °C high levels of nonphotochemical quenching of excitation energy has been associated with a 20-fold increase in zeaxanthin content in dark-adapted leaves, as a photo-protective mechanism (Farage *et al.*, 2006). Sac-11, Sac-12 and Gig-13 showed the same AQY values at 10/8 °C as at 14/10 °C (Fig. 4a), which further indicates that no photo damage happened to these three genotypes when the temperature was decreased from 14/10 °C to 10/8 °C. The significantly higher AQY values and A_{1500} in the three genotypes obviously would make them more suitable for cold climates than Tin-1 and Sin-15 at 10/8 °C (Fig. 4), due to their significantly higher CO₂ uptake under both light-limited and light-saturated conditions. No significant decrease was observed in A_{1000} in Fest-19 during the temperature reduction from 14/10 °C to 10/8 °C; the relatively high ΦPSII and Fv/Fm in Fest-19 might have played a major role but it was not the only possible reason for maintaining the high photosynthetic rate. An alternative explanation might be the Rubisco content since C₃ species generally have three to six times as much Rubisco as C₄ species (Ku *et al.*, 1979).

Interestingly, all the miscanthus genotypes continued to display photosynthetic activity at 6/4 °C, though A_{1000} was quite low for several of them (Fig. 1a). The values of A_{1000} in Gig-13 grown for 6 days at, respectively, 10/8 °C and 6/4 °C were 8.9 and 3.2 μmol m⁻² s⁻¹, which is similar to previously reported values for *M. × giganteus* where the photosynthetic rate was measured during short-term exposure to low temperatures. Measured values in that study were approximately 9 μmol m⁻² s⁻¹ at 10 °C and 4 μmol m⁻² s⁻¹ at 5 °C for plants that had previously been grown at 14/11 °C and 25/14 °C, respectively (Naidu *et al.*, 2003). Thus, long-term exposure to cold temperatures down to 6 °C in *M. × giganteus* did not have a detrimental effect on the photosynthetic apparatus. This suggests that the decrease in photosynthetic capacity in Gig-13 and

Sac-11 at low temperatures (above 5 °C) could be reversed if the temperature was raised again, and our result of the temperature response curve also supported this (Fig. 5). This could be one of the reasons for the remarkable capacity of *M. × giganteus* to grow in cool climates and produce a much higher yield compared with another C₄ plant, *Spartina cynosuroides*, in the United Kingdom (Beale & Long, 1995). An earlier miscanthus growth model, MISCANMOD, uses a base temperature of 10 °C, which was considered as the starting temperature for leaf growth (Hastings *et al.*, 2009). Our results suggest that the base temperature for miscanthus could be lower than 6 °C.

The reduction in AQY in Sac-11, Sac-12 and Gig-13 from 10/8 °C to 6/4 °C might be because of a further accumulation of zeaxanthin even at the low light intensities, which reduce the light-harvesting efficiency under light-limiting conditions (Long *et al.*, 1994; Fryer *et al.*, 1995). However, this phenomenon was not observed in Fest-19, which showed no significant changes in AQY from 14/10 °C to 6/4 °C (Fig. 4a). This significantly higher tolerance to the cold in Fest-19 was also observed at 2 °C. A_{1000} for Fest-19 grown at 6/4 °C and measured at 2 °C was 5.72 μmol m⁻² s⁻¹ (data not shown), which was similar to data measured for *Lolium perenne* L. grown and measured at 2 °C (Hoglund *et al.*, 2011).

The significantly higher AQY and A_{1500} in Fest-19 than in the miscanthus genotypes even at 6/4 °C (Fig. 4a) and high A_{1000} at 2 °C can support the early emergence and longer growing season of Fest-19. The daily mean temperature in Denmark in 2013 and 2014 was around 8.7 °C, ranging from -8.6 to 23 °C, and there were about 200 days each year with a mean temperature below 10 °C (data collected from climate station in Foulumgaard, Denmark, 56°30'N, 9°35'E). Thus, the significantly higher photosynthesis in Fest-19 in the 2–10 °C temperature range can be the reason for its high biomass production when grown in temperate conditions. Yields in Denmark of more than 20 T DM ha⁻¹ for this cultivar have been recorded (Manevski *et al.*, 2015).

However, we observed a tendency of an increasing ΦPSII/ΦCO₂ ratio when lowering the temperature from 14/10 °C to 6/4 °C for all the miscanthus genotypes, but not for Fest-19. This indicates that the quantum efficiency of the electron flux through PSII relative to the quantum efficiency of CO₂ assimilation in miscanthus increased as the temperature decreased, due to additional electron sinks. It has been suggested that elevated ΦPSII/ΦCO₂ ratio in mature maize leaves under cold conditions is due to O₂ being used as an alternative electron acceptor (Fryer *et al.*, 1998), which is also a mechanism for preventing photo damage to the

photosynthetic apparatus (Farage *et al.*, 2006). A previous study also found an increased $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio for *M. × giganteus* grown at 10 °C compared with 24 °C and 14 °C (Farage *et al.*, 2006). Photoinhibitory damage has been found to be a major challenge for C_4 plants grown at cold temperatures, for example, maize grown at 17 °C or below (Long *et al.*, 1983). It seems that *M. × giganteus* has the ability to minimize this damage by a massive increase in zeaxanthin content to facilitate heat dissipation in conjunction with induction of electron transport to an acceptor other than CO_2 (Farage *et al.*, 2006).

However, CO_2 leakiness from the bundle sheath cells back to mesophyll is an alternative energy expenditure in the C_4 photosynthetic pathway under high light conditions. Especially at low temperatures, where the Rubisco capacity is an important limitation for sustaining C_4 photosynthesis (Sage, 2002; Kubien *et al.*, 2003), the $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio will increase (Von Caemmerer, 2000). Increased values of $\Phi\text{PSII}/\Phi\text{CO}_2$ at cool temperatures have also been observed in *Muhlenbergia glomerata* measured at 10 and 5 °C by Kubien & Sage (2004b), who concluded that leaking of CO_2 through the bundle sheaths was increased due to limited Rubisco capacity (Rubisco content) at low temperatures. The significantly higher $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio at 6/4 °C in our study might indicate that Rubisco posed a major limitation to photosynthesis in all the miscanthus genotypes but not in Fest-19. However, the underlying reasons for the increase of $\Phi\text{PSII}/\Phi\text{CO}_2$ were not determined in this study.

Gig-13 and Sac-11 grown at 6/4 °C in the present study showed much higher rates of photosynthesis at all measured temperatures from 5 to 25 °C than maize grown at 14/11 °C of 2–5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Naidu *et al.*, 2003) (Fig. 5). Even though the photosynthetic rate measured at temperatures from 10 to 25 °C in Gig-13 and Sac-11 grown at 6/4 °C was much lower than that for *M. × giganteus* grown at 14/11 °C (8–25 $\mu\text{mol m}^{-2} \text{s}^{-1}$, (Naidu *et al.*, 2003)), this could to some extent be caused by the increased VPD (Fig. 5), which significantly decreased stomatal conductance (Fig. S1, A) in combination with limited Rubisco content as mentioned above. The increase in VPD resulting in higher stomatal closure was probably the major reason for the low *A* in Fest-19 during the short-term temperature increase.

Typically, the ratio between intercellular CO_2 concentrations and ambient CO_2 concentrations (c_i/c_a) is about 0.7 in C_3 plants (Drake *et al.*, 1997) and 0.3–0.4 in C_4 plants (Jones, 1983). The c_i/c_a in Fest-19 decreased to 0.50 ± 0.01 at 25 °C, which is significantly lower than the typical value. With similar levels of c_i/c_a in Fest-19 and miscanthus genotypes at 15 °C, the C_4 pathway was more efficient in carbon fixation. The miscanthus

genotypes Gig-13 and Sac-11 had c_i/c_a values that were in the 0.3–0.4 range even at 25 °C (Fig. S1, B), while the c_i/c_a of Sac-12 decreased to almost 0.23 ± 0.08 at 25 °C, indicating that stomatal closure was limiting *A* in this genotype (Fig. S1, A).

Conclusions

We found that miscanthus maintains photosynthetic activity after growing for a longer period at 6/4 °C, and that several genotypes increased their photosynthesis immediately when the temperature was increased. As expected, the photosynthetic apparatus of C_3 Fest-19 was clearly more tolerant at 10/8 °C and 6/4 °C than the C_4 genotypes tested. However, also at 24/20 °C Fest-19 was as efficient as most of the miscanthus genotypes and only Sac-12 had a significantly higher A_{1000} (Fig. 1a). Fest-19 may thus overall be more productive than current miscanthus genotypes in maritime, cool, temperate climates even though not only leaf photosynthesis but a number of other physiological factors such as respiration and partitioning to roots are decisive for productivity as well. We are currently growing Fest-19 and Gig-13 in side-by-side replicated field trials at two Danish locations to obtain data on seasonal PAR interception in green leaves as well as yield data over several years. Also, we will use the leaf photosynthesis data as well as data on partitioning between plant organs to model seasonal productivity against the harvested yield data.

Of the miscanthus genotypes, only Sac-11 seemed to have photosynthetic capacity at a similar high level as Gig-13 (Hornum) in cold conditions and to recover quickly at enhanced temperatures. Thus, Sac-11 will be a good parent for breeding new varieties with an even better cold tolerance than Gig-13 (Hornum) and for broadening the genetic diversity of miscanthus if more widespread production in cool climates is to take place. Tin-1 did not have a high tolerance to cold conditions, as also previously found by Jiao *et al.* (2016).

Acknowledgements

We thank Finn Henning Christensen for help in operating the climate chambers and Jens Bonderup Kjeldsen for collecting genotypes in Japan and establishing the Danish miscanthus gene pool in 1996. The work was supported by the EU Seventh Framework Programme (FP7/2007–2013) as part of the GrassMargins Project (grant agreement No. 289461), and by the Danish Council for Strategic Research as part of the BIORESOURCE Project (Grant agreement No. 11-116725).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (a) stomatal conductance (G_s), and (b) the ratio between intercellular CO₂ concentrations and ambient CO₂ concentrations (c_i/c_a) vs. temperature for Sac-11, Sac-12, Gig-13 and Fest-19 grown at 6/4 °C (day/night), Error bars represent SE ($n = 3$ or 4).