Assessment of drought tolerance in *Miscanthus* spp. and *Saccharum* x *Miscanthus* intergeneric hybrids (miscanes)

（ススキ属およびサトウキビ x ススキ属間雑種（ミスケーン）の耐乾性評価）

北海道大学　大学院国際食資源学院

国際食資源学専攻　博士後期課程

Hokkaido University　Graduate School of Global Food Resources

Division of Global Food Resources　　Doctor Course

WENG, Tzuya

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Chapter 1. General Introduction

Background of the present study

Global warming, which means temperature of the earth's surface increasing caused by atmosphere CO_2 or methane absorbing sunlight and solar radiation heat, makes big problems to agriculture in the world. High temperature caused by global warming reduces the yield of crops and water loss from soil and plants by evapotranspiration accelerates, which makes plants under drought. Water resources also becomes unbalance and unstable due to climate change. In general, developing countries gets more damage on agriculture than developed countries because most of them located near to the equator where temperatures are already close to thresholds for crops growth. Cline (2008) reported that, by the 2080s, the impact of global warming will a big reduction on agriculture production with the losses of 10-25% in developing country regions and 9-21% in developed countries, while the most affected countries will be in Africa, South America, and South Asia. Besides high temperature, rising sea levels caused by global warming also affected the reducing of agriculture cultivated area in low altitude countries such as Bangladesh and Egypt (Cline, 2008). The problems caused by global warming on agriculture is needed to be solved as soon as possible.

High level of atmosphere CO_2 and carbon emissions, which is the major reason of global warming, is a pressing problem in the world. Cline (2008) reported that annual carbon emissions amount will increase to around 16 billion by 2050 and 29 billion by 2100, while it is

about 7 billion tons of carbon in 2006. As a result, the average surface temperature will rise 5℃ by land area. According to Intergovernmental Panel on Climate Change (IPCC) Sixth Assessment Report (2021), average temperature has been risen 1.1℃ since 1990s and it will rapidly rise more than 1.5℃ even to 2℃ if greenhouse gas emissions are not reduced on a large scale immediately. Therefore, reducing or no increasing atmospheric concentrations of CO_2 is important for sustainable development for the earth. Most carbon emissions are caused by fossil fuel which is used to produce significant energy for human society. Developing renewable, sustainable energy such as bioenergy instead of fossil fuel seems to be a pressing mission.

Drought-induced response of plants

Drought stress, one kind of abiotic stress, limits plant growth and yield and acts as a barrier to the successful cultivation of bioenergy crops, such as sugarcane and maize, particularly in world arid and semi-arid regions (Morrow et al., 2014). Drought impairs plant metabolism, such that plants cannot provide sufficient photosynthetic energy for cell growth and maintenance, which sometimes results in death (Farooq et al., 2009). Under drought, leaf relative water content (RWC) decreases when water absorption became less than transpiration, causing stomatal closure with increasing abscisic acid (ABA) content (Ikegami et al., 2009). As a result, photosynthesis rate and chlorophyll content reducing, caused by low stomatal conductance and CO_2 assimilation, makes plants have energy shortage problem with low photosynthesis

performance. On the other hand, cell turgor decreases due to low RWC. In order to absorb water from low moisture soil, plants increase cellular contents concentration with soluble sugar/ amino acid content to make higher osmotic potential. Moreover, plants have developed mechanisms of reactive oxygen species (ROS) generation as an abiotic stress signal of cell-to-cell communication, which can improve plant tolerance to abiotic stress such as drought (Li et al., 2014).

To adapt and survive under drought stress, mechanisms involving drought resistance and drought recovery are key aspects of adaptations (Chen et al., 2016). Plants with drought tolerance generally express certain traits under stress, such as leaf area reduction to minimize transpirational water loss and maintenance of high chlorophyll content to enable high photosynthetic levels in order to produce enough energy for survival. Therefore, photosynthetic parameters, especially photosynthetic rate (Pn), are considered as an effective measure of drought tolerance in plants, such as in *Leucaena leucocephala* (Lam.) de Wit (Chen, et al., 2012). Liu et al. (2015) reported that the photosynthetic rate of drought-tolerant switchgrass (*Panicum virgatum* L.) genotypes was positively correlated with other physiological parameters, such as relative water content, transpiration rate (Tr), stomatal conductance (gs), and water-use efficiency (WUE) when subjected to low-water conditions. It means that the performance of Pn can be regarded as the physiological response of plants under drought.

Extreme drought will likely increase in the future due to global warming (Trenberth et al.,

2014). Consequently, there is a strong need for identifying crop accessions with high recovery capacity to drought stress. Such a trait enables crops to access water from the soil from short-term rain events and to maintain physiological function to survive drought. Lauenroth et al. (1987) observed that the warm-season perennial grass species, *Bouteloua gracilis* H.B.K. Lag. ex Steud., in response to low soil moisture, generated new root growth after the root zone was replenished with water, which led to increased soil water uptake. Also, lipid peroxidation and H_2O_2 content, which were generated in tea plants (*Camellia sinensis* (L) O. Kuntze) in response to drought, decreased after post-drought soil-water recharge (Upadhyaya, Panda, &Dutta, 2011). In addition, the catalase activity of pea (*Pisum sativum* L., cv. Progress 9), which is involved in removing H_2O_2 molecules, increased during drought (Mittler & Zilinskas, 1994). However, H_2O_2 molecules decreased to normal levels after re-watering. Moreover, Chen et al. (2016) reported drought adaptability of maize (*Zea mays* L.) seedlings was more associated with drought recovery (r = 0.714) than drought resistance (r = 0.332) in correlation analysis, suggesting recovery capacity is a key component of plant survival to drought stress. They also used it as a screening criterion to identify drought-tolerant genotypes.

Drought stress tolerance improvement has been succeed in other crops such as maize, rice and wheat (Campos et al., 2004; Manickavelu et. Al., 2006; Mwadzingeniet al., 2016). For lignocellulose crops, drought tolerance of 49 genotypes of switchgrass (*Panicum virgatum* L.) were evaluated in previous research and several drought-tolerant and drought-sensitive

switchgrass genotypes were successfully selected (Liu et al., 2015). It is necessary to evaluate drought tolerance genotypes of other bioenergy crops such as *Miscanthus*.

Given that there are considerable genetic differences among *Miscanthus* genotypes, even under well-watered conditions, assessment of drought tolerance, based only on photosynthesis data collected during periods of low SMC, can be fraught with limitations. To avoid this problem, the drought stress index (DSI) methodology of Liu et al. (2015) was employed. It shows promise in quantifying drought-induced effects in *Miscanthus* plants. The DSI can remove genetic differences among different genotypes and can be used as an indicator of drought tolerance throughout the *Miscanthus* genus.

Bioenergy development

As the increasing demands of energy due to population growth in the word, bioenergy has been considered as one solution response to energy crisis. The renewable, environment friendly energy from biological sources is also recognized as a potential energy to replace fossil fuel in the future (Yuan et al., 2008). Three major bioenergy product are ethanol, biodiesel and bio-gas, while ethanol can be used in transportation fuels and in chemical industry. For modern bioenergy industry, global biofuel production was around 16 billion gallons, while 43% of production was made in the United States and 32% was made in Brazil (Coyle, 2007). Most ethanol was made by Maize and sugarcane from their starch and sugar contents, which is called

as first-generation biofuel. Although the techniques of producing ethanol from starch or sugar has been established and convenient, it is easy to cause conflict of crops between food and energy. For example, world food prices rose 10 percent in 2006 mainly due to increasing demand of maize, wheat, soybean for bioenergy production (Coyle, 2007). Therefore, developing second generation bioenergy, which produce ethanol from non-food lignocellulose crops, is necessary for bioenergy industry. Besides avoiding crop competition between food and energy, lignocellulose bioenergy crops can be grown in marginal land where is not suitable for agriculture production. Moreover, lignocellulose crops such as switchgrass and *Miscanthus* have high CO_2 fixed capacity, which can help to mitigate the high concentrations of atmospheric CO_2 and global warming. However, the major problem of lignocellulosic ethanol production is the complicated structure of the cell wall, which is difficult to breakdown. The techniques of increasing efficiency and reducing the cost of lignocellulosic ethanol production is a pressing mission for second generation bioenergy. In addition, increasing tolerance to abiotic stress is important to enable lignocellulose crops to be cultivated in marginal land (Yuan, Tiller, Al-Ahmad, Stewart, &Stewart, 2008). Abiotic stress such as drought, salt, cold and heat stress largely reduce the biomass production of crops. Improvement tolerance to abiotic stress in lignocellulose crops can help plants maintain stable biomass production response to extreme climate change due to global warming.

***Miscanthus* spp.**

Miscanthus, a C_4 perennial rhizomatous grass originated in East Asia, has high biomass productivity in marginal lands and expresses high CO_2 fixation in low-temperature conditions, underscoring its potential as a non-food lignocellulose bioenergy crop (Heaton et al., 2008; Toma et al., 2010). *Miscanthus ×giganteus*, a single sterile triploid hybrid between tetraploid *M. sacchariflorus* (Maxim.) Hack. and diploid *M. sinensis* Andersson, has been adapted for commercial biomass production in Europe and North America with average 28.7ton biomass production per ha (Angelini, Ceccarini, Nassi o Di Nasso, &Bonari, 2009). Moreover, *Miscanthus* can mitigate carbon dioxide emissions produced by fossil fuels and sequestrate carbon in the soil, which is effective in mitigating greenhouse effect and global warming (Clifton-Brown et al., 2007).

Miscanthus species are considered to have stronger drought tolerance than another potential energy crop, switchgrass (Heaton et al., 2004; Mann et al., 2013). Under drought conditions, relative to maize and switchgrass, *Miscanthus* exhibited higher light-use efficiency, photosynthetic rate, and above-ground biomass (Joo et al., 2016). However, as a potential energy crop, selection needs to be made of drought-tolerant *Miscanthus* accessions (van der Weijde et al., 2017). Many cultivated *Miscanthus* genotypes, including the widely cultivated, high-yielding *Miscanthus × giganteus*, lack strong drought tolerance (Vanloocke et al., 2010). Moreover, *M. ×giganteus* uses more water than maize due to its longer growing season and

higher productivity (Vanloocke et al., 2010). Selecting for drought tolerance of *Miscanthus* is essential for wherever it may be cultivated as a bioenergy crop because the ubiquity of drought also happens even in high-rainfall areas (van der Weijde et al., 2017). Selecting for and developing drought-tolerant *Miscanthus* genotypes as breeding material increases the versatility of *Miscanthus* as a sustainable bioenergy crop.

Little research appears to have been done to characterize drought tolerance of *Miscanthus*. Previous research on the impact of drought on *Miscanthus* mainly focused on *M.* × *giganteus* (Ings et al., 2013). Most parameters, such as dry weight accumulation, leaf expansion chlorophyl content, decreased when *M.* × *giganteus* meet drought (Emerson et al., 2014). Moreover, there are many genetic resources of *Miscanthus* spp., which could be used as breeding stock to improve drought-adaptation capacity in high-yielding accessions. Consequently, there is a need to identify and evaluate drought-tolerant *Miscanthus* genotypes as breeding material from the core population.

***Saccharum* × *Miscanthus* intergeneric hybrids (miscane)**

Intergeneric hybrids of *Saccharum* and *Miscanthus*, which often named as miscanes, have been reported that they can be used for introgression of traits from *Miscanthus* into sugarcane by backcrossing (Chen & Lo, 1989; Tai et al., 1991). Hybridization of *Miscanthus* with sugarcane can occur in nature or artificial crossing (Price & Daniels, 1968). Although miscanes were

originally studied as a tool to introgress disease-resistance genes from *Miscanthus* into sugarcane, the high biomass potential of miscane made it become a high potential cellulosic bioenergy crop (Sacks et al., 2013). With the traits of high biomass productivity and chilling and drought tolerance, Sacks et al. (2013) suggested miscanes can be expected as a bioenergy crop under warm temperate or subtropical regions. In addition, miscane has been reported that it can produce more biomass than *M.* ×*giganteus*, *M. sinensis* Andersson, and the switchgrass (*Panicum virgatum* L.) in in Arkansas, USA (Burner et al., 2009). About drought tolerance in miscane, it has been reported that miscane showed much sensitive to drought and less biomass production under drought than *M.* × *giganteus* and giant reed (*Arundo donax*) (Burner et al. , 2015). However, little information is currently available on the photosynthetic response under drought and drought-induced gene expression of miscanes comparing to sugarcane. Therefore, clarify drought tolerance of miscanes compared with their sugarcane and *Miscanthus* parents genotypes is important for improving miscane as a promising lignocellulosic bioenergy crop and also help to transfer drought tolerant genes from *Miscanthus* to sugarcane through breeding.

Objectives and composition of this thesis

This study was conducted to assess drought tolerance of *Miscanthus* spp. and *Saccharum* × *Miscanthus* intergeneric hybrids (miscane) with a wide range of genetic clusters. Previous studies of drought tolerance in *Miscanthus* spp. were mainly focused on the commercial variety

M. × *giganteus*. In order to identify germplasm to use as future breeding-stock material in *Miscanthus* spp., evaluation drought tolerance with wide range genotypes is necessary. Moreover, two types of evaluation techniques of drought tolerance in plants, the dry-down and fixed soil-moisture-contents (SMC) methods, can manifest different types of drought stress in plants. In order to improve accuracy and utility of the data, it is necessary to compare changes in soil water content and the responses of plants to low-water availability via these two techniques. In chapter 2, total 29 *Miscanthus* genotypes of East-Asian origin were screened for drought tolerance with two methods, a dry-down treatment in two locations and a specific-soil-moisture-content using an automatic irrigation system in one location. Observation of photosynthesis parameters are important to evaluate drought tolerance capacity in plants and were used as selection parameters in this study. Chapter 3 evaluates the variation in drought tolerance of three *Saccharum* × *Miscanthus* intergeneric hybrids (miscane) and their parent genotypes. Photosynthetic rate and DSI were used as assessment parameter. To reveal the possible drought-induced response, gene expression level of three drought-associated genes were analyzed by real-time PCR. In chapter 4, the results of this study were comprehensively discussed and compared with previous researches. Limitations of this study and prospects of research were also discussed in this chapter.

Chapter 2. Assessment of drought tolerance of *Miscanthus* genotypes through dry-down treatment and fixed-soil-moisture-content techniques

Introduction

For evaluation of drought tolerance in plants under greenhouse studies, two types of techniques, the dry-down treatment (Blackman et al., 2019) and fixed-soil moisture content (SMC) methods (Bergsten & Stewart, 2013; Nemali & van Iersel, 2006), have been used to apply low-water conditions in potted plants. In the dry-down method, water is withheld from plants, often for several days, after initially being well watered. As evapotranspiration occurs, SMC will continue to decline, which often leads to gradually increasing levels of plant drought stress. The SMC of plants in dry-down treatments usually decreases quickly over a short period of time. Ad-vantages of the dry-down technique include cost-efficiency and ease of operation. However, the method affords little time for researchers to observe how plants respond to drought.

On the other hand, the fixed SMC technique is used to keep the SMC of target plants at fixed soil-moisture levels by regularly adding water through a computerized irrigation system to the rhizosphere of the potted plants based on the amount of water evapotranspired from the plant and medium (Bergsten & Stewart, 2013). In this technique, the rhizosphere of target plants can be maintained at a relatively constant SMC, thus allowing for the plants to experience continuous drought conditions. The disadvantages of the fixed-SMC method include the large amount of time and effort required for calculating evapotranspiration and for maintaining irrigation levels. However, comparison be-tween both techniques is warranted given that each method offers distinct advantages in terms of characterizing plant responses to drought stress.

Miscanthus spp. is a tropical C_4 perennial rhizomatous grass, which originated in East

Asia and has a wide geographical distribution in different latitudes areas. Two major important *Miscanthus* species are *Miscanthus sinensis* Andersson and *Miscanthus sacchariflorus* (Maxim.) Ben-tham. A single sterile triploid clone of *Miscanthus ×giganteus* Greef & Deuter ex Hodk. & Renvoize, a hybrid between *M. sacchariflorus* and *M. sinensis*, has been adapted for commercial biomass production in Europe and North America. Based on data generated from restriction site-associated DNA sequencing and Golden Gate technologies, *M. sinensis* is mainly comprised of 6 genetic clusters, which include South-eastern China plus tropical group, Yangtze-Qinling group, Sichuan Basin group, Korea, North China group, Southern Japan group, and Northern Japan group (Clark et al., 2014). On the other hand, *M. sacchariflorus* consists of Yangtze group diploids , Northern China group diploids, Korea/Northeast China/Russia group diploids, Northern China/Korea/Russia group tetraploids, Southern Japan group tetraploids, and Northern Japan group tetraploids (Clark et al., 2019). Relative to *M. sinensis* species, the diploid and tetraploid clusters of *M. sacchariflorus*, possibly play a role in stress-tolerance expression in the species complex, when used to breed *M. × giganteus* new genotype by crossing *M. sacchariflorus* with *M. sinensis* species. In the present study, a core population of several *Miscanthus* species, which were characterized by Clark et al. (2014, 2019), were included for evaluation of their response to drought.

The present study focused on two objectives to characterize the drought-tolerance capacity of *Miscanthus*. The first objective was to compare different techniques used to impose

drought stress in plants in terms of their suitable applications. The second was to screen *Miscanthus* genotypes for drought tolerance with the express purpose of identifying germplasm to use as future breeding-stock material.

Materials and Methods

1. Screening experiment for dry-down-imposed drought stress

1.1. Experiment in Hokkaido University, Japan

A population of 23 *Miscanthus* genotypes, which included 10 *M. sinensis*, one *M. sinensis var. condensatus*, 11 *M. sacchariflorus*, and one *M. floridulus* genotypes, which were collected from across East Asia, served as the source of the selection materials for this study (Table 2-1; Table 2-S1). The genotypes were divided into thirteen genetic clusters, based on analyses by Clark et al. (2014, 2019). Considerable genetic variation existed among the genotypes (Clark et al., 2014; Clark et al., 2019). As such, we considered that even with only 23 genotypes, which had limited representation (i.e., between 1–6 genotypes) of each genetic cluster, there was sufficient genetic variation to draw broad-based inferences for the genus at large. The experiment was conducted in a semi-open rain-shelter green-house at Hokkaido University (HU) in Sapporo, Japan (43°4'43"N, 141°20'19"E). The dry-down experiment ran from July to August 2018. All 23 *Miscanthus* genotypes were propagated from rhizomes. Rhizome pieces of each genotype were cut into 10 cm lengths and grown in plastic pots (diameter = 19 cm, height = 27 cm). All plants

were irrigated every day for 4 weeks before starting the experiment.

The screening experiment was arranged as a randomized complete block design. There are three blocks and each block consisted of two pots of each of the 23 genotypes. One pot represented the well-watered treatment and one pot was assigned to the drought-stress treatment for each of the 23 genotypes in one block. The well-watered treatment involved daily irrigation to saturate the rhizosphere of each of the treated plants, while the dry-down treatment was applied by withholding water for 7 days. After 7 days, plants were irrigated to container capacity. The dry-down period was re-peated four times.

The Soil Plant Analysis Development (SPAD) Chlorophyll meter (SPAD-502Plus, Konica Minolta, Osaka, Japan), used to measure chlorophyll content, is one of the simpler and quicker means to characterize drought stress due to its non-destructive nature and its close correlation with leaf-level photosynthesis (Kato et al., 2004; Takai et al., 2010). Measurements of SPAD value were taken on all plants between 10:30 am to 2:00 pm on days 0, 7, 14, 21, and 28. Rhizosphere conditions after 28 days of the dry-down experiment could be equated with what occurs in the field in the spring and/or summer in temperate regions, such as the east-central U.S. (Namias, 1966).

To evaluate drought tolerance in 23 *Miscanthus* genotypes during the dry-down experiment, DSI of SPAD value was plotted against coefficient of variance (CV) of SPAD value (Figure 2-1). The DSI of the HU screening experiment was calculated as fol-lows:

$$\text{DSI of SPAD value (HU screening experiment)} = (\text{value of traits on day 28 of drought})/(\text{value of traits on day 0 as well-watered treatment}) \times 100 \quad (1)$$

1.2. Experiment at Brigham Young University, USA

A population of 14 *Miscanthus* genotypes (Table 2-1), where each plant constituted the experimental units, were included in a drought-tolerance-evaluation experiment at Brigham Young University (BYU), Provo, Utah, USA (40°14' 59" N, 111°38' 57" W). The experiment was arranged in a completely randomized design. Due to independent *Miscanthus* genotype management at HU and BYU, six *Miscanthus* genotypes (JPN-2011-010, PMS-7, PMS-164, PMS-285, PMS-347, PI417947) were both evaluated in the HU screening experiment and BYU experiment. However, the remaining eight genotypes were only evaluated in the BYU experiment. The experiment was conducted under greenhouse conditions from 4 to 25 October 2019. Each genotype was replicated two times. All plants grown in plastic pots (diameter = 19 cm, height = 27 cm) were irrigated daily for one week prior to treatment initiation to keep them well watered be-fore the dry-down experiment started. After measurements were collected on day 0 of the experiment, the dry-down treatment was applied by withholding water for 7 days. Plants were then irrigated to container capacity. The dry-down period was repeated three times.

The SPAD value was measured in all plants between 1:00 am to 3:30 pm on days 0, 7, 14, and 21 with a SPAD chlorophyll meter (MC-100 Chlorophyll Concentration Meter, Apogee

Instruments, Inc., Logan, UT, USA). Photosynthesis parameters such as Pn, gs, Tr, intercellular CO_2 concentration (Ci), and leaf-level fluorescence (φPSII) were also measured for all genotypes with a portable photosynthesis system (LI-6400XT, LI-COR, Lincoln, NE, USA) with a 6400-40 leaf chamber fluorometer for use with the LI-6400 Portable Photosynthesis System (LI-COR, Lincoln, NE, USA). In addition, soil water potential was measured from collected soil samples on days 0, 7, 14, and 21 with a WP4C Dew Point Potentiometer (METER Group, Pullman, WA. USA).

Drought tolerance of *Miscanthus* genotypes was evaluated with the DSI data from the 14-day-dry-down data set from the BYU screening experiment, where the soil water potential (−2.6 MPa) led to slight levels of drought stress after the dry-down period.

$$\text{DSI (14-day dry-down data)} = (\text{value of traits from 14-day dry-down})/(\text{value of traits of 0-day dry-down}) \times 100 \quad (2)$$

In order to comprehensively assess drought tolerance of the different genotypes, principal component analysis (PCA) ranking values, which were based on DSI values, were used to assess drought-tolerance capacity in each *Miscanthus* genotype based on the methodology of Liu et al. (2015). Liu et al. (2015) reported that the PCA based on the DSI of physiological parameters is considered to be a reliable method for evaluating drought tolerance among plants genotypes.

The 14 *Miscanthus* genotypes were ranked based on the PCA ranking values, which are

based on DSI (14-day dry-down data) values. A significance test analysis done through SAS of the DSI data from the BYU screening experiment was used to complement the PCA results.

To understand the effect of different environments on Miscanthus genotype performance, SPAD value-based DSI values of the six *Miscanthus* genotypes were subject-ed to analysis of variance (ANOVA) in the HU screening and BYU experiments. As mentioned previously, six *Miscanthus* genotypes (JPN-2011-010, PMS-7, PMS-164, PMS-285, PMS-347, PI417947) were used in both the HU-screening and BYU screening experiments. Both experiments used the dry-down treatment to impose drought stress.

2. Precise-comparison experiment with automated irrigation system at HU

A total of ten *Miscanthus* genotypes, consisting of eight putatively drought-tolerant and two drought-sensitive *Miscanthus* genotypes, were selected based on preliminary results from the HU screening experiment. A scatterplot of SPAD value-based CV values and SPAD value-based DSI values in the HU screening experiment is shown in Figure 2-1. Relatively lower CV values and higher DSI values of some genotypes indicated that they had fewer variation between different drought levels and less differences between well-watered and drought conditions. Based on these results, eight putatively drought-tolerant genotypes (PMS-164, PMS-285, PMS-347, PMS-7, UI10-00008, UI10-00015, UI10-00020, UI10-00024) and two drought-sensitive geno-types (JPN-2011-004, UI11-00033) were selected to be included in the HU precise-

comparison experiment for further analysis of their photosynthetic performance under specific drought levels through an automated irrigation system. Among the eight drought-tolerant genotypes, there was only one representative from the *M. sacchariflorus* species group, UI10-00008, while the other seven were *M. sinensis* genotypes. On the other hand, the most drought-sensitive genotypes, JPN-2011-004 and UI11-00033, were *M. sacchariflorus*. The genotypes were evaluated for drought tolerance in a precise-comparison experiment using an automated irrigation system following the methodology of Nemali and van Iersel (2006). A simplified diagram of the irrigation system can be seen in Figure S1. The experiment was conducted in a semi-open greenhouse at Hokkaido University from 10 September to 10 October 2018.

The precise-comparison experiment was arranged in a completely randomized design. Each genotype had three replicates. Soil moisture sensors (GS3, Meter Group, Pullman, WA) were inserted, along with drip emitters, into each of the potted plants (diameter = 19 cm, height = 27 cm). The sensors and emitters were connected to an automatic irrigation system, which regulated the amount of water applied to each plant. Soil-moisture treatments (20, 25, and 30% SMC) were arranged by setting the set-point of the system at pre-determined soil-moisture levels. The lowest SMC treatment (20%) was defined as the severe drought treatment and the highest SMC treatment (30%) was considered the well-watered treatment. After all potted plants achieved their SMC set points for 5 days, Pn, gs, Ci, and Tr were collected on the youngest, fully expanded leaf of each plant with a portable photosynthesis system (LI-6400XT).

Leaf-level fluorescence (φPSII) and SPAD value, which were measured at the same time as photo-synthesis, were measured with a fluorometer (Junior-PAM, Heinz Walz GmbH, Ef-feltrich, Germany) and a SPAD chlorophyll meter (SPAD-502Plus), respectively.

Soil moisture of all pots was controlled by the automated irrigation system. Aver-age changes in SMC levels can be seen in Figure S2. The time taken for the potted media to reach the severe-drought-level set point required more time than media in the slight-drought-level treatment. For example, it only took 5 days for soil moisture to de-crease from 30% to 25%, while it took 8 days for soil moisture to reduce from 25% to 20% (Figure 2-S2).

Drought tolerance of these 10 Miscanthus genotypes was evaluated with the DSI data from the 25% SMC treatment in the HU precise-comparison experiment.

$$\text{DSI (25\% SMC treatment)} = (\text{value of traits of 25\% SMC})/(\text{value of traits of 30\% SMC}) \times 100 \quad (3)$$

A PCA-ranking value based on DSI from the 25% SMC treatment was calculated for each genotype following the method of Liu et al. (2015). The 10 *Miscanthus* genotypes were ranked as relatively drought tolerant based on PCA ranking values. A significance test analysis done through SAS of the DSI data from the HU precise-comparison experiment was used to complement the PCA results.

To understand how different drought-treatment methods affected evaluation results of drought tolerance in *Miscanthus* spp., DSI of four photosynthetic parameters (Pn, gs, Tr, φPSII) of four

Miscanthus genotypes (PMS-7, PMS-164, PMS-285, PMS-347), which were subjected to slight stress-level conditions (25% SMC in the HU precise-comparison experiment and −2.6 MPa of soil water potential on day 14 of the BYU experiment), were subjected to ANOVA. The fixed-SMC method was used as a drought-treatment method in the HU precise-comparison experiment, while in the BYU experiment, the dry-down method was used to subject plants to drought stress.

3. Post-drought recovery in the BYU experiment

After the 21-day BYU dry-down screening experiment finished, a 7-day post-drought recovery experiment was conducted with the same plants in order to evaluate the drought-recovery capacity of the *Miscanthus* genotypes. A population of 14 *Miscanthus* genotypes (Table 2-1) was used in the 7-day post-drought-recovery experiment, which was the same material used in the BYU screening experiment. The recovery experiment was arranged in a completely randomized design and conducted under greenhouse conditions from 25 October to 1 November 2019, with two replicates of each genotype. Plants were watered daily over the 7-day experiment. Instrumentation and measurement parameters were the same as those used in the screening experiment. Measurements were made on the seventh day of the recovery experiment.

To understand the degree of recovery capacity from drought stress in *Miscanthus*

genotypes, recovery DSI values were used to calculate PCA ranking values as assessment criteria.

$$\text{Recovery DSI} = (\text{value of traits of day 7 in BYU recovery experiment})/(\text{value of traits of day 21 in BYU screening experiment}) \times 100 \quad (4)$$

Moreover, to comprehensively assess drought-recovery capacity of the different genotypes, the PCA ranking value based on recovery DSI values was calculated. The 14 *Miscanthus* genotypes were ranked according to their relative drought recovery capacity levels, which were based on the PCA-ranking-value results.

4. Comparing drought tolerance of selected *Miscanthus* genotypes with commercial bioenergy genotype *Miscanthus* ×*giganteus*.

Five *Miscanthus* genotypes selected and the commercial bioenergy genotype *Miscanthus* ×*giganteus* were studied in this study. The selected 5 *Miscanthus* genotypes were selected as three levels to drought: tolerant (*M. sinensis* PMS-285, *M. sinensis* PMS-007), medium (*M. sinensis* PMS-347), and sensitive (*M. sinensis* PMS-014, *M. sinensis* PMS-586). All *Miscanthus* genotype were propagated from rhizomes with 10 cm lengths and grown in plastic pots (diameter = 19 cm, height = 27 cm) containing soilless medium consisting of compost, vermiculite, calcined clay, and peat moss (Forex Mori Sangyo Co., Ltd., Hokkaido, Japan). At planting, 5g of 13-18-4 slow-release fertilizer (Ekopu nigatsuchi S380, SunAgro 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 daily irrigation for 4 weeks before starting the experiment.

The experiment was conducted under greenhouse conditions in Hokkaido University from 1 Jun. to 26 July. 2021. The experiment was arranged in a completely randomized design. Each genotype had four replicates. The irrigation was controlled by an automatic irrigation system. Drought stress treatment was given as different level by setting the set point of the system, while 40% soil moisture was well-water condition as control treatment, 25% soil moisture was drought stress treatment and 40% soil moisture was recovery treatment. After plants well irrigated by the automatic irrigation system for 7 days, Pn, gs, Ci, Tr were taken on the youngest, fully expanded leaf of each plant with a portable photosynthesis system (LI-6400XT). Drought treatment was started after the measurement of control treatment finished, and the Pn measurement was taken on after 1, 9, 15 days to drought. After the Pn measurement of 15 days to drought finished, recovery treatment was conducted by the automatic irrigation system and the Pn measurement was made on after 11 days to re-watered.

5. Drought tolerance evaluation and statistical analysis

The DSI values and PCA ranking values, which were based on DSI values, were used to assess the drought-tolerance capacity in *Miscanthus* genotypes which was based on the methodology of Liu et al. (2015). In order to quantify drought-induced effects in *Miscanthus* plants, the DSI value of each photosynthesis parameter was calculated the formula below:

$$\text{DSI} = (\text{value of traits under stress condition})/(\text{value of traits under well-watered condition}) \times 100 \quad (5)$$

Moreover, to comprehensively assess drought tolerance of the different genotypes, the PCA ranking value based on DSI values was calculated using the formula below:

$$\text{PCA ranking value} = (\text{contribution of the first principal components (PC1) (\%)} \times \text{PC1}) + (\text{contribution of the second principal components (PC2) (\%)} \times \text{PC2}) + (\text{contribution of the third principal components (PC3) (\%)} \times \text{PC3}) \quad (6)$$

In the BYU post-drought recovery experiment, recovery DSI values were used as an evaluation parameter of the recovery capacity of different genotypes. The formula used Equation (4). In the HU screening experiment, DSI of SPAD value and CV of SPAD value were used as drought-tolerance-evaluation parameters.

Microsoft Excel (Microsoft Office 2016, Microsoft Corporation, Redmond, WA, USA) was used to perform ANOVA. R statistical software (R3.5.1 by R Development Core Team, 2018) and ggplot2 package of R software were used to perform PCA of drought tolerance of Miscanthus genotypes. Statistical Analysis System (SAS Institute Inc., Cary, NC, USA) was used to respectively perform a significance test analysis of the DSI data from the HU precise-comparison and BYU screening experiments.

Results

1. Comparison of *Miscanthus* genotype performance between HU and BYU experiments

Drought stress index values of 21-day dry-down of SPAD value of six *Miscanthus* genotypes (JPN-2011-010, PMS-007, PMS-164, PMS-285, PMS-347, PI417947) in the HU screening experiment and BYU experiment were subjected to ANOVA (Table 2-2). In the ANOVA results, there was no significant difference ($p > 0.05$) in DSI values of *Miscanthus* genotypes in the HU and BYU experiments.

Drought stress index values of four photosynthetic parameters (Pn, gs, Tr, φPSII) of four *Miscanthus* genotypes (PMS-7, PMS-164, PMS-285, PMS-347) under slight stress-level conditions (25% SMC in the HU precise-comparison experiment and soil water potential as −2.6 MPa on day 14 of the BYU experiment) were subjected to ANOVA (Table 2-3). The ANOVA results was significant ($p \leq 0.05$) in DSI values of *Miscanthus* genotypes in the HU precise-comparison and BYU experiments.

The DSI φPSII mean values of *M. sinensis* genotype PMS-285 in the slight stress-level treatment in both the HU precise-comparison (94.1) and BYU (75.9) experiments significantly differed from that of the *M. sacchariflorus* genotype UI11-00033 (39.2) and *M. sinensis* genotype UI10-00015 (37.8) in the HU precise-comparison experiment (Table 2-S2). In the BYU experiment, DSI φPSII of *M. sinensis* genotype PMS-007 (102.9) was higher than other genotypes and significantly differed from that of four *M. sinensis* genotypes (PMS-014, PMS-164, PMS-347, PMS-586) (Table 2-S2-1). However, compared to its performance in the BYU

experiment, the DSI φPSII of PMS-007 (71.5) was moderately high in the HU precise-comparison experiment (Table 2-S2-2). The DSI Pn of *M. sinensis* genotype PMS-007 was relatively higher in the HU precise-comparison (77.3) and BYU (94.4) experiments than other genotypes subjected to slight stress levels, with the exception of *M. sinensis* genotype UI10-00088 in the BYU experiment (Table 2-S3).

In addition, plants of *M. sinensis* genotype PMS-164 had higher DSI φPSII levels in the severe-stress-level treatment in the HU precise comparison (99.0) and BYU (67.8) experiments relative to those in slight stress-level treatment (HU: 42.9, BYU: 63.6) (Table 2-S2). The DSI gs of *M. sinensis* genotype PMS-164 in the slight-stress-level treatment of the HU precise-comparison (824) and BYU (195) experiments statistically differed from 9 genotypes in the HU precise-comparison experiment (Table 2-S4).

2. Changes in soil water potential across treatments in the BYU experiment

Average changes in soil water potential of each genotype across treatments in the BYU experiment are shown in Table 4. Across treatments, soil water potential in the BYU experiment decreased, on average, from day 7 to 21 with the gradual exposure of plants to different levels of SMC. At first, soil water potential did not differ between days 0 and 7, but then considerably decreased on days 14 and 21 (Table 2-4). Soil water potential was around −0.1 MPa on days 0 and 7 and then decreased to −2.6 MPa on day 14 and −10.2 MPa on day 21 (Table 2-4). The

soil water potential values on days 14 and 21 were more severe than those found at field capacity (−0.33 MPa) and permanent wilting (−1.5 MPa).

3. Performance of genotypes under dry-down experiment in BYU screening experiment

After water-deficit treatments were initiated, photosynthetic levels of all *Miscanthus* genotypes decreased after day 7 as soil water potential decreased (Table 2-4). Most genotypes showed higher Pn on day 7 than on day 0, which corresponded to no changes in soil water potential (Table 2-4). After soil water potential values exhibited a large drop from day 7 to day 14 (−0.14 to −2.6 MPa), the Pn performance of all genotypes also showed a sharp decline, particularly going from a 15% decrease to a 77% decrease in Pn (Table 2-4). Moreover, five genotypes (JPN-2011-010, JM11-006, JPN-2010-005, UI10-00048, UI10-00092) died after day 14 due to serious drought. In addition, the Pn performance of the *M. sinensis* genotype, PMS-285, when experiencing low-water availability, showed almost no differences with conspecific genotypes in the well-watered treatment (Table 2-4). Although Pn of *M. sinensis* PMS-285 was at relatively moderate levels on days 0 and 7, it dropped when low soil-water conditions became more severe on days 14 and 21 (Table 2-4). However, the Pn of other genotypes experienced sharp decreases due to low soil-water availability during this time period, such as *M. sinensis* genotype UI10-00048 (Table 2-4).

In order to understand how photosynthetic traits contributed to drought tolerance of

Miscanthus genotypes in the BYU experiment, we performed PCA using the DSI values (day 14) of six measured parameters (Pn, gs, Ci, Tr, φPSII, SPAD value) (Figure 2-2). The first (PC1) and second (PC2) principal components explained 76.6% of the variance among 14 *Miscanthus* genotypes. In addition, Pn and Tr had the largest contribution in PC1, suggesting Pn and Tr were the two most important photosynthesis param-eters to the PCA results (Figure 2-2).

According to the PCA ranking value based on the DSI (day 14 of the BYU screening experiment) (Table 2-5), *M. sinensis* genotype PMS-285 and *M. floridulus* genotype PI417947 had relatively high ranking values compared to other genotypes, suggesting that they were more tolerant to drought stress among the 14 *Miscanthus* genotypes. In contrast, three of the *M. sacchariflorus* genotypes (JPN-2011-010, JPN-2010-005, JM11-006), which originated from Japan, showed relatively poor performance under low-water conditions while *M. sinensis* genotype UI10-00048 had the lowest PCA ranking relative to the other 13 *Miscanthus* genotypes in the BYU experiment (Table 2-5).

4. Performance of genotypes under fixed drought level with automated irrigation system in the HU precise-comparison experiment

The PCA using the DSI (25% SMC) values of six parameters suggested that the PC1 and PC2 explained 76.9% of the variance among all 10 genotypes (Figure 2-3). Photosynthetic rate and Tr showed similar and strong influences on the PC1 axis. Stomatal conductance (gs), and Tr

were the most important photosynthesis parameters to the PCA result of the HU precise-comparison experiment because they provided the largest contribution to PC1 (Figure 2-3).

According to the PCA ranking value based on the DSI (25% SMC) data (Table 2-6), *M. sinensis* genotypes, PMS-007 and PMS-285, had relatively high ranking values than the other genotypes, suggesting that they were more tolerant to drought stress while *M. sacchariflorus* genotypes, JPN-2011-004 and UI11-00033, had relatively lower ranking values than the other genotypes and were found to be more sensitive to drought stress. It is noteworthy that *M. sinensis* genotype PMS-285 also had a higher PCA ranking than other genotypes in the BYU screening experiment, while *M. sinensis* genotype PMS-007 did not have a high PCA ranking in the BYU screening experiment (Table 2-5). In contrast, *M. sacchariflorus* genotypes, with the exception of genotype UI10-00008, appeared to be more sensitive to drought than *M. sinensis* genotypes in the HU precise-comparison experiment (Table 2-6), which was also observed in the BYU screening experiment (Table 2-5).

5. Drought recovery capacity of *Miscanthus* genotypes of post-drought recovery experiment in BYU

Upon rewatering plants daily for 7 days after a 21-day dry-down treatment, the average soil water potential of all *Miscanthus* genotypes on day 7 of the BYU recovery experiment increased to 0.04 MPa, which was similar to that on day 0 of the BYU screening experiment (Table 2-4).

This result suggests that the soil moisture level was high enough for plants to recover from drought (Table 2-4). Three *M. sacchariflorus* genotypes, JPN-2011-010, JM11-006, and JPN-2010-005, and two *M. sinensis* genotypes, UI10-00048 and UI10-00092, were nearly dead due to drought stress after a 21-day dry-down period in the BYU screening experiment (Table 2-4). Consequently, we were not able to characterize the recovery capacity of these genotypes.

On the other hand, the photosynthetic levels of *M. sinensis* genotypes PMS-014 and PMS-586 exhibited relatively quick recovery of Pn levels on day 7 in the BYU recovery experiment (Table 2-4). The Pn level of genotype PMS-014 on day 7 in the BYU recovery experiment was six times greater than its Pn performance on day 21 in the BYU screening experiment (Table 2-4). A similar pattern could be seen with genotype PMS-586, whose Pn level was four times greater than its Pn performance on day 21 in the BYU screening experiment (Table 2-4). In addition, these two genotypes had high recovery-PCA-ranking values, suggesting that they had the potential to recover from drought damage (Table 2-7). On the other hand, *M. sinensis* genotype PMS-285 had a relatively low recovery ranking value and was less capable of recovering from drought (Table 2-7), but it displayed higher Pn levels than other genotypes under low-water conditions in the BYU screening experiment (Table 2-4).

6. Comparison experiment of selected *Miscanthus sinensis* genotypes with *Miscanthus* ×*giganteus*

Average changes of Pn of selected five *Miscanthus sinensis* genotypes and *Miscanthus ×giganteus* in greenhouse experiment was shown as Figure 2-4. *Miscanthus ×giganteus* and *M. sinensis* PMS-007 had relatively high Pn values than other genotypes under control condition, while *M. sinensis* PMS-285 showed low Pn values. As drought treatment starting, the Pn performance decreased after 1 day to drought and continuously declined to 15 days of drought (Figure 2-4). Although *M. sinensis* PMS-014 and *M. sinensis* PMS-347 exhibited only little decreasing of Pn values on 1 day to drought, the Pn of two genotypes experienced sharp decreases due to long-term drought (15 days to drought). On the other hand, the Pn performance of the *M. sinensis* PMS-285 showed only little decline under long-term drought.

Drought stress index (DSI) of Pn of all *Miscanthus* genotypes decreased largely after 15 days to drought (Figure 2-5). Among 6 genotypes, *M. sinensis* PMS-285 showed the highest DSI Pn values on 15 days to drought, which is consistent with the selected result of HU experiment and BYU experiment. The DSI Pn performance of *Miscanthus ×giganteus* was similar with *M. sinensis* PMS-285 (Figure 2-5).

The photosynthetic levels of *M. sinensis* genotypes PMS-014 and PMS-586 exhibited relatively quick recovery of Pn levels after 11 days of re-watered and *M. sinensis* PMS-586 even had better Pn performance after rewatered than under control condition (Figure 2-4). A similar pattern could be seen of Recovery DSI Pn values (Figure 2-6). In addition, *M.×giganteus* exhibited relatively low Recovery DSI Pn values than other genotypes.

Table 2-1. List of *Miscanthus* genotypes included in screening experiments at Hokkaido University (HU), Sapporo, Japan and Brigham Young University (BYU), Provo, Utah, USA.

HU Screening Experiment (2017, 2018)			**BYU Screening Experiment (2019)**		
Species	Accession	Type	Species	Accession	Type
M. sacchariflorus	JM11-006	Wild	*M. sacchariflorus*	JM11-006	Wild
M. sacchariflorus	JPN-2011-004	Wild	*M. sacchariflorus*	JPN-2010-005	Wild
M. sacchariflorus	JPN-2011-006	Wild	*M. sacchariflorus*	JPN-2011-010	Wild
M. sacchariflorus	JPN-2011-010	Wild	*M. sacchariflorus*	UI11-00031	Wild
M. sacchariflorus	PMS-076	Wild	*M. sinensis*	PMS-007	Wild
M. sacchariflorus	RU2012-056.1WD (4x)	Wild	*M. sinensis*	PMS-014	Wild
M. sacchariflorus	RU2012-141	Wild	*M. sinensis*	PMS-164	Wild
M. sacchariflorus	RU2012-169	Wild	*M. sinensis*	PMS-285	Wild
M. sacchariflorus	RU2012-183	Wild	*M. sinensis*	PMS-347	Wild
M. sacchariflorus	UI10-00008	Cultivar	*M. sinensis*	PMS-586	Wild
M. sacchariflorus	UI11-00033	Wild	*M. sinensis*	UI10-00048	Cultivar
M. sinensis	PMS-164	Wild	*M. sinensis*	UI10-00088	Cultivar
M. sinensis	PMS-285	Wild	*M. sinensis*	UI10-00092	Wild
M. sinensis	PMS-347	Wild	*M. floridulus*	PI417947	Wild
M. sinensis	PMS-7	Wild			
M. sinensis var. condensatus	UI10-00015	Wild			
M. sinensis	UI10-00020	Wild			
M. sinensis	UI10-00024	Cultivar			
M. sinensis	UI10-00053	Cultivar			
M. sinensis	UI10-00080	Cultivar			
M. sinensis	UI10-00097	Cultivar			
M. sinensis	UI10-00100	Cultivar			
M. floridulus	PI417947	Wild			

Table 2-2. Analysis of Variance (ANOVA) result of six *Miscanthus* genotypes (JPN-2011-010, PMS-7, PMS-164, PMS-285, PMS-347, PI417947) between Hokkaido University screening experiment and Brigham Young University screening experiment using drought stress index of 21 days of SPAD value.

ANOVA					
Source of variation	SS	df	MS	F	*p*-value
Between Groups	0.0069	1	0.0069	0.1991	0.6650
Within Groups	0.3453	10	0.0345		
Total	0.3522	11			

Table 2-3. Analysis of Variance (ANOVA) result of four *Miscanthus* genotypes (PMS-7, PMS-164, PMS-285, PMS-347) based on their drought stress index of four photosynthetic parameters (photosynthetic rate, stomatal conductance, transpiration rate, and chlorophyll fluorescence) under slight drought stress† of Hokkaido University (HU) precise-comparison and Brigham Young University (BYU) screening experiments.

ANOVA					
Source of variation	SS	df	MS	F	*p*-value
Between Groups	3.2866	1	3.2866	18.3031	0.0002
Within Groups	5.3870	30	0.1796		
Total	8.6736	31			

† Slight drought stress was set as 25% volumetric water content in soil of HU experiment and soil water potential as −2.6 MPa in BYU experiment.

Table 2-4. Photosynthetic rate (Pn) of each *Miscanthus* genotype under each soil water potential in a screening experiment and a post-drought recovery experiment at Brigham Young University, Provo, Utah, USA.

Soil water potential (mPa)		**Day 0 −0.096**	**Day 7 −0.14**	**Day 14 −2.6025**	**Day 21 −10.25**	**Day 7 after re-watered 0.04**
Species	**Accession**	**Pn (µmol CO_2•m^{-2}•s^{-1})**				
M. sacchariflorus	JM11-006	11.281	10.355	2.310	NA	NA
M. sacchariflorus	JPN-2010-005	7.673	9.899	2.744	NA	NA
M. sacchariflorus	JPN-2011-010	8.087	8.930	3.927	NA	NA
M. sacchariflorus	UI11-00031	12.044	12.292	7.707	5.372	8.145
M. floridulus	PI417947	3.961	5.595	3.192	3.006	2.774
M. sinensis	PMS-007	6.268	8.006	5.824	3.488	3.367
M. sinensis	PMS-014	10.724	12.436	6.394	1.655	11.899
M. sinensis	PMS-164	6.294	11.962	3.107	7.393	7.330
M. sinensis	PMS-285	7.613	8.364	6.810	6.484	5.121
M. sinensis	PMS-347	8.624	10.411	2.919	1.886	5.144
M. sinensis	PMS-586	5.148	9.832	2.051	1.438	6.569
M. sinensis	UI10-00048	5.034	15.136	0.777	NA	NA
M. sinensis	UI10-00088	4.418	5.081	4.312	1.160	3.275
M. sinensis	UI10-00092	5.334	13.335	5.049	NA	NA

Table 2-5. Principal components analysis (PCA) ranking values† based on the drought stress index (Day 14) and the rank of drought-tolerance capacity of fourteen *Miscanthus* genotypes under slight drought stress‡ in a screening experiment at Brigham Young University (BYU), Provo, Utah, USA.

Species	Accession	Origin	Genetic Clusters§	PC1	PC2	PC3	Ranking Value	Rank
M. sinensis	PMS-285	China	Yangtze-Qinling Msi	2.2033	−0.3101	0.4616	1.2647	1
M. floridulus	PI417947	Cultivar	SE China Msi	1.5610	0.6848	0.1983	1.0543	2
M. sinensis	UI10-00088	Cultivar	C Japan Msi	2.2217	−1.0798	0.0110	1.0521	3
M. sinensis	UI10-00092	Cultivar	C Japan Msi	1.8232	−0.4588	0.4144	1.0117	4
M. sinensis	PMS-007	China	Yangtze-Qinling Msi	1.7714	−0.8379	0.8903	0.9825	5
M. sinensis	PMS-347	China	SE China Msi	−0.3521	2.5066	1.3918	0.5140	6
M. sinensis	PMS-164	China	Yangtze-Qinling Msi	0.6587	−0.1539	−1.8284	0.0560	7
M. sinensis	PMS-586	China	Sichuan Msi	−0.6486	1.7557	0.0789	−0.0098	8
M. sacchariflorus	UI11-00031	China	Yangtze diploids (ssp. lutarioripariu s) Msa	−0.2352	−0.1630	−0.0439	−0.1729	9
M. sinensis	PMS-014	China	Sichuan Msi	−0.3133	−0.6493	−0.3376	−0.3596	10
M. sacchariflorus	JPN-2011-010	Japan	N Japan 4x Msa	−0.9108	0.0087	−0.5782	−0.6075	11
M. sacchariflorus	JPN-2010-005	Japan	N Japan 4x Msa	−1.6686	0.1401	−1.2992	−1.1262	12
M. sacchariflorus	JM11-006	Japan	S Japan 4x Msa	−1.9928	0.2420	−0.9925	−1.2422	13
M. sinensis	UI10-00048	Cultivar	S Japan Msi	−4.1179	−1.6850	1.6335	−2.4174	14

†PCA ranking value was derived via calculation of first, second, and third principal components (PC1, PC2, and PC3).

‡Slight drought stress was set as soil water potential as −2.6 MPa in the BYU experiment.

§According to Clark et al. (2014) and Clark et al. (2019).

Table 2-6. Principal components analysis (PCA) ranking values† based on the drought stress index (25% soil moisture content) and the rank of drought-tolerance capacity of ten *Miscanthus* genotypes under slight drought stress‡ in a precise comparison experiment of a precise comparison experiment at Hokkaido University (HU), Sapporo, Japan.

Species	Accession	Origin	Genetic Clusters§	Leaf width (cm)	Leaf length (cm)	PC1	PC2	PC3	Ranking Value	Rank
M. sinensis	PMS-007	China	Yangtze-Qinling Msi	2.0	60	0.71	3.03	−0.57	1.232	1
M. sinensis	PMS-285	China	Yangtze-Qinling Msi	1.1	56	0.93	1.02	0.04	0.749	2
M. sacchariflorus	UI10-00008	Cultivar	NEChina/Korea/Russia diploids Msa	0.8	44	2.65	−1.90	0.55	0.618	3
M. sinensis	UI10-00020	Cultivar	S Japan Msi	0.4	18	0.89	−0.09	0.68	0.450	4
M. sinensis	PMS-164	China	Yangtze-Qinling Msi	1.1	25	1.29	−0.58	−0.36	0.333	5
M. sinensis	UI10-00024	Cultivar	S Japan Msi	0.6	27	−0.40	0.90	0.47	0.178	6
M. sinensis	PMS-347	China	SE China Msi	1.8	48	−0.04	−0.76	−0.52	−0.333	7
M. sinensis var. condensatus	UI10-00015	Cultivar	C Japan Msi	1.6	40	−2.27	0.34	1.39	−0.712	8
M. sacchariflorus	JPN-2011-004	Japan	S Japan 4x Msa	1.8	55	−1.35	−0.79	−1.80	−1.085	9
M. sacchariflorus	UI11-00033	Japan	S Japan 4x Msa	2.0	61	−2.41	−1.16	0.14	−1.425	10

†PCA ranking value was derived via calculation of first, second, and third principal components (PC1, PC2, and PC3).

‡Slight drought stress was set as 25% volumetric water content in the media of the HU experiment.

§According to Clark et al. (2014) and Clark et al. (2019).

Table 2-7. Recovery principal components analysis (PCA) ranking values† based on the recovery drought stress index and the rank of recovery capacity from drought stress of fourteen *Miscanthus* genotypes in post-drought recovery experiment at Brigham Young University, Provo, Utah, USA.

Species	Accession	Origin	Genetic clusters‡	PC1	PC2	PC3	Ranking value	Rank
M. sinensis	PMS-014	China	Sichuan Msi	4.3565	−1.4954	0.2379	2.8970	1
M. sinensis	PMS-586	China	Sichuan Msi	3.3939	0.4381	0.5461	2.5689	2
M. sinensis	PMS-347	China	SE China Msi	2.7456	1.2399	−1.0582	2.1484	3
M. sinensis	UI10-00088	Cultivar	C Japan Msi	1.2360	−0.6833	0.0990	0.7769	4
M. sacchariflorus	UI11-00031	China	Yangtze diploids (ssp. lutarioriparius) Msa	−0.2981	0.8091	0.6621	−0.0294	5
M. floridulus	PI417947	Cultivar	SE China Msi	−0.9572	2.3607	0.7977	−0.2173	6
M. sinensis	PMS-164	China	Yangtze-Qinling Msi	−0.5758	0.7901	−0.0801	−0.2786	7
M. sinensis	PMS-007	China	Yangtze-Qinling Msi	−0.5925	−0.3455	−0.4199	−0.5167	8
M. sinensis	PMS-285	China	Yangtze-Qinling Msi	−0.9592	−0.8056	0.0559	−0.8369	9

†PCA ranking value was derived via calculation of first, second, and third principal components (PC1, PC2, and PC3).

‡According to Clark et al. (2014) and Clark et al. (2019).

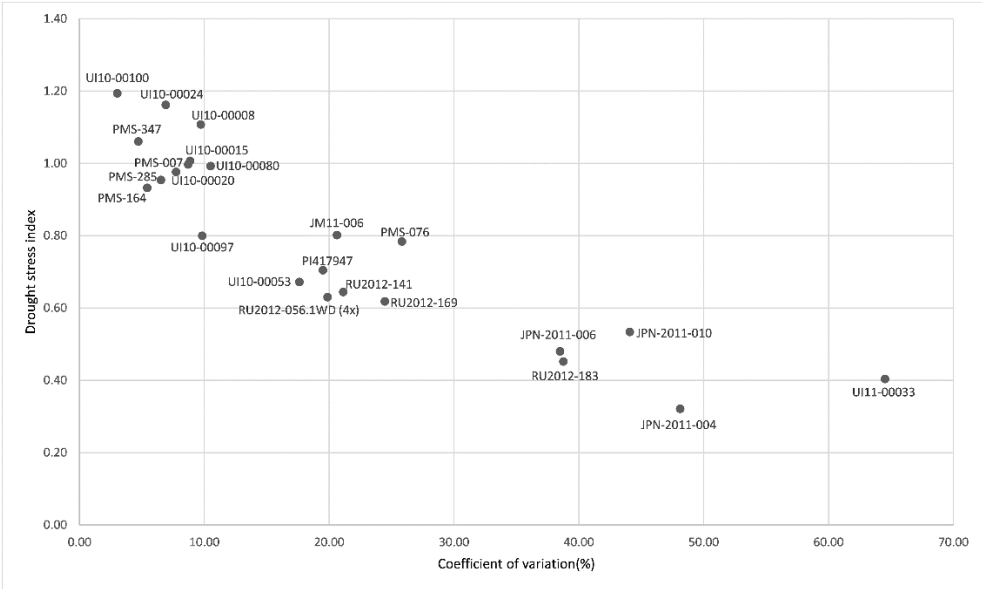


Figure 2-1. Scatter plot of coefficient of variation and drought stress index of the Soil Plant Analysis Development (SPAD) Chlorophyll meter value in screening experiment at Hokkaido University, Sapporo, Japan of drought-stress tolerance.

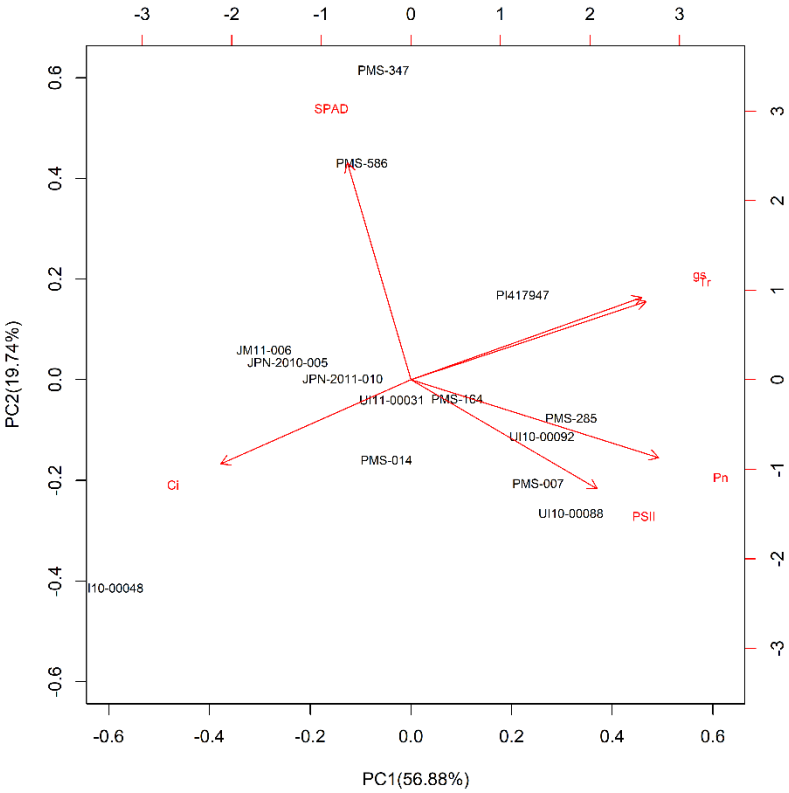


Figure 2-2. Principal component analysis (PCA) bi-plot of drought stress index (DSI) of six physiological traits (photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (Tr), intercellular CO_2 concentration (Ci), the Soil Plant Analysis Development (SPAD) Chlorophyll meter value, and chlorophyll fluorescence (PSII)) under drought over a 14-day period in screening experiment at Brigham Young University, Provo, UT, USA. Arrows represent physiological traits with various lengths, which were based on the impact of each trait on the separation of genotypes.

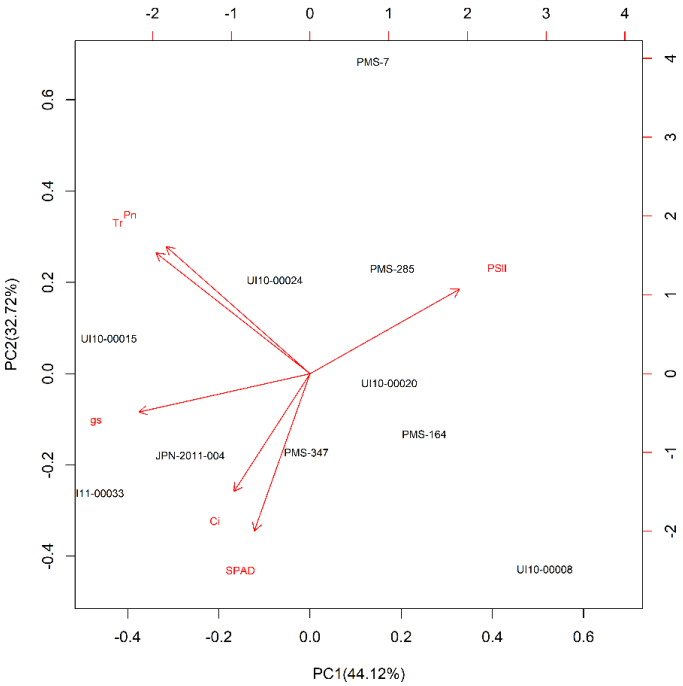


Figure 2-3. Principal component analysis (PCA) bi-plot of the drought stress index (DSI) of six physiological traits: photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (Tr), intercellular CO_2 concentration (Ci), the Soil Plant Analysis Development (SPAD) Chlorophyll meter value, chlorophyll fluorescence (PSII) under 25% soil moisture in a precise-comparison experiment at Hokkaido University, Sapporo, Japan. Arrows represent physiological traits with various lengths, which were based on the impact of each trait on the separation of genotypes.

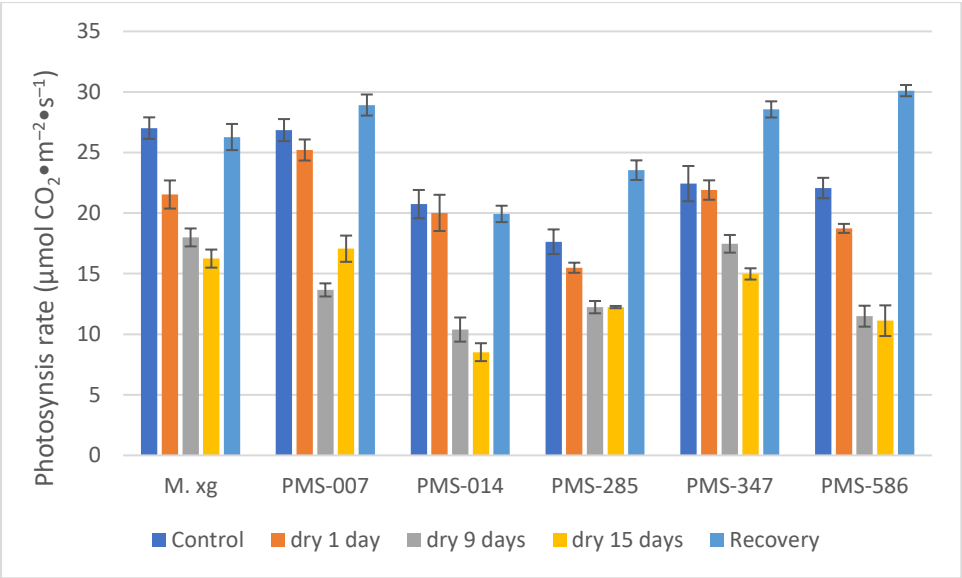


Figure 2-4. Photosynthetic rate (Pn) of selected five *Miscanthus sinesis* genotypes and *Miscanthus ×giganteus* on control condition, after 1, 9, 15 days of drought and re-watered 11 days in greenhouse experiment.

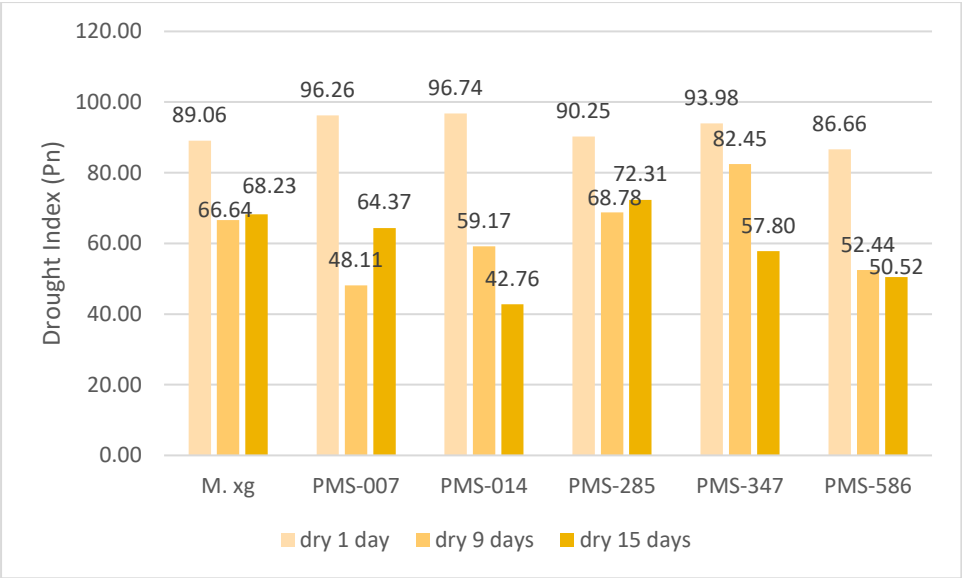


Figure 2-5. Drought stress index (DSI) of selected five *Miscanthus sinesis* genotypes and *Miscanthus ×giganteus* after 1, 9, 15 days of drought in greenhouse experiment.

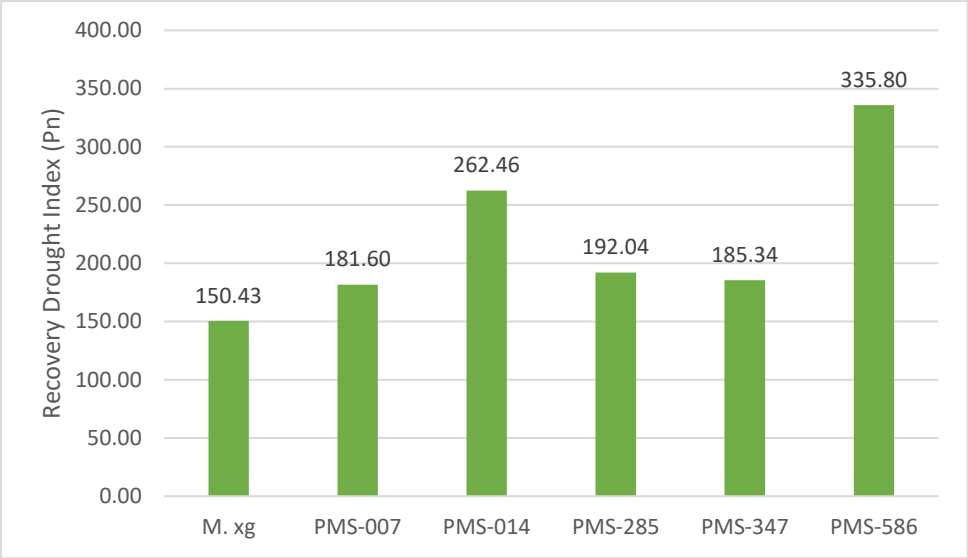


Figure 2-6. Recovery drought stress index (DSI) of selected five *Miscanthus sinesis* genotypes and *Miscanthus* ×*giganteus* after re-watered 11 days in greenhouse experiment.

Discussion

1. Comparison of different drought treatment methods for evaluation

Some plants species show different physiological responses under rapidly-imposed and slowly-imposed drought-stress conditions (Cornic et al., 1987). As we mentioned earlier, two drought-treatment methods, the dry-down technique, and the fixed-SMC technique, imposed different patterns of drought stress on plants in the experiments. The dry-down technique made a quick and sizable decrease in SMC over a short period of time, while the fixed-SMC technique-controlled SMC at a relatively constant level at a slower rate and for a longer period of time. Both drought-imposition techniques were used in previous research for studying drought tolerance in plants (Chen et al., 2012; Ganjeali et al., 2011; Li et al., 2014; Liu et al., 2015; Nazari & Pakniyat, 2010; Perciva et al., 2006).

Drought-tolerant genotypes, which were selected through the PCA ranking analysis, also showed different degrees of drought tolerance in the HU precise-comparison and BYU experiments. For example, *M. sinensis* PMS-007 showed high drought tolerance performance in the HU precise-comparison, but only medium-level performance in the BYU screening experiment. Environmental factors and methods of drought im-position could have been factors that influenced the results of the HU precise-comparison and BYU screening experiments. However, it appears that environ-mental factors did not influence the results of the two experiments. According to our results, there was no significant difference ($p > 0.5$) in DSI

values of *Miscanthus* genotypes in the HU and BYU experiments (Table 2-2), suggesting that there was no effect of environment between the HU and BYU experiments when both experiments used the dry-down technique to impose drought on *Miscanthus* plants. Therefore, the different evaluation results between the HU precise-comparison and BYU experiments were likely due to differences in how drought was imposed.

Decreases in SMC showed different patterns in the two drought-treatment methods used in this study. As reflected in changes in soil water potential values, drought stress conditions caused by the dry-down technique became more severe (i.e., soil water potential went below the permanent wilting point (−1.5 MPa) over a 14-day period (day 7 to day 21 in the BYU screening experiment) with a quick and sizable decrease in SMC (Table 2-4). In this case, plants had little time to adjust low-water conditions. On the other hand, with the fixed-SMC method, SMC changed slowly and could be con-trolled at a relatively constant level for plants to respond low-water availability. In the HU precise-comparison experiment, soil moisture controlled by an automated irrigation system took around 30 days to change from slight stress to severe stress, and at each stress level plants had 3–5 days to adjust the stress before measurement (Figure 2-S2). With the fixed-SMC method, plants had enough time to exhibit their responses to drought, presuming that there was some physiological regulation in their cells. Based on the different patterns we observed in decreases in SMC (Table 2-4; Figure 2-S2), the dry-down method is suitable for selecting drought-tolerant genotypes for cultivar or breeding development.

However, the fixed-SMC method can aid researchers in clarifying drought-induced response of plants, such as changes in cell-level osmotic potential changing or toxic ROS scavenging regulation (Rohollahi et al. 2018).

Under field conditions, drought can be defined as a condition where plants cannot get take up enough water from dry soil for normal physiological function over an extended period of time (Dracup et al., 1980). Large decreases in soil moisture over a short period of time during a dry-down are more similar to drought in the field, which leads plants to perform all steps of drought-caused physiological regulation in a short time (Cornic et al., 1987). This aspect of the dry-down method leads plants to respond to low-water availability as if they were subjected to field conditions. However, rapidly decreasing soil moisture makes it difficult to capture and characterize ephemeral physiological changes in plants (Cornic et al., 1987). On the other hand, the fixed-SMC method is controlled by a computer, which can regulate irrigation and thereby control SMC to maintain continuous drought conditions (Nemali & van Iersel, 2006). Therefore, plants generally have enough time in this method to physiologically respond to drought due to being subjected to constant, low-SMC conditions. In addition, physiological responses of plants to different soil-moisture conditions (e.g., well-watered, moderate, severe) with this approach seem more straightforward than in the dry-down method (Kim & Iersel, 2011). However, such constant soil-moisture conditions, even when water levels are fairly low, differ from drought in the field such that genotypes identified as drought tolerant through the fixed-SMC method may

not perform well when grown in the field.

2. Characteristics of drought stress in *Miscanthus* spp.

In general, *M. sinensis* appears to have stronger drought tolerance than *M. sacchariflorus* (Table 2-5 and 2-6). Based on the PCA ranking results of the BYU screening experiment, four *M. sacchariflorus* genotypes (UI11-00031, JPN-2011-010, JPN-2010-005, and JM11-006) ranked relatively low in terms of drought-stress tolerance (Table 2-5). Similarly, based on the PCA ranking results of the HU precise-comparison experiment, two *M. sacchariflorus* genotypes, JPN-2011-004 and UI11-00033 ranked 9 and 10, suggesting they were sensitive to drought stress (Table 2-6). These results correspond to their native habitats. *Miscanthus sinensis* usually grows in dry, upland areas, while *M. sacchariflorus* occurs in mesic, lowland areas (Tamura et al. 2016).

Miscanthus × *giganteus*, which is a triploid hybrid of tetraploid *M. sacchariflorus* and diploid *M. sinensis*, is considered as a potential high-yielding energy crop (29–38 Mg ha^{-1}) (Heaton et al. 2008). However, *M.* × *giganteus* expresses sensitivity to drought and needs more irrigation than maize under commercial cultivation conditions (Vanloocke et al., 2010). Genes inherited from *M. sacchariflorus* possibly influence the drought sensitivity of *M.* × *giganteus*.

Based on the PCA ranking results of the HU precise-comparison and BYU screening experiments, *M. sinensis* genotype PMS-285 had higher photosynthetic performance under

drought in both experiments, suggesting that it can be used as germplasm in breeding programs (Tables 2-5 and 2-6). *Miscanthus sinensis* genotype PMS-007 showed relatively higher photosynthesis performance than other genotypes in the HU precise-comparison experiment (Table 2-6), but it exhibited only relatively moderate photosynthesis performance in the BYU screening experiment (Table 2-5). Considering the two drought-imposition methods used in our study, *M. sinensis* genotype PMS-007 appeared to maintain high photosynthesis performance for stable and consistent responses to low-water availability in the fixed-SMC method, but the photosynthesis performance was relatively lower at rapidly decreasing SMC conditions caused by the dry-down method (Tables 2-4 and 2-S5). Considering the different photosynthetic performance of *M. sinensis* genotypes PMS-285 and PMS-007 under dry-down and the fixed-SMC treatments, there should be some differences between the drought-response mechanisms of *M. sinensis* genotypes PMS-285 and PMS-007, which allowed for genotype PMS-285 to be tolerant of both rapidly and slowly decreasing soil-moisture availability, which needs to be clarified in the future. In addition, the genotypes, *M. sacchariflorus* UI10-00008 and *M. sinensis* UI10-00020, which had relatively narrow leaves and smaller leaf area than other genotypes, were more tolerant to drought than other genotypes in the HU precise-comparison experiment, with the exception of *M. sinensis* genotypes PMS-285 and PMS-007 (Table 2-6). A relatively small leaf area can lead to low transpiration levels, which could allow for plants to maintain photosynthetic rates at levels to sustain moderate growth despite having low soil-water

availability (Ganjeali et al. 2011; Smith 1978).

The DSI φPSII of *Miscanthus sinensis* genotype PMS-285 in the slight-stress-level treatment in the HU precise-comparison (94.1) and BYU (75.9) experiments exceeded that of other genotypes in the study, except for *M. sinensis* genotype PMS007 and UI10-00088 in the BYU experiment (Table 2-S2). On the other hand, the DSI Pn of *M. sinensis* genotype PMS-007 is relatively higher than other genotypes under slight stress levels in both the HU precise-comparison and BYU experiments (Table 2-S3). The relatively high values of DSI φPSII of *M. sinensis* genotype PMS-285 and DSI Pn of *M. sinensis* genotype PMS-007 could help explain why these two genotypes showed stronger drought tolerance than other genotypes in this study. In addition, the DSI gs of *M. sinensis* genotype PMS-164 exceeded that of other genotypes in both experiments (Table 2-S4).

Interestingly, the *Miscanthus* genotypes with strong drought-recovery capacity (PMS-014, PMS-586) did not exhibit high drought tolerance (Tables 2-5 and 2-7). On the other hand, genotypes with high drought tolerance may not have sufficient drought-recovery capacity. Based on the recovery PCA ranking results (Table 2-7), *M. sinensis* genotypes PMS-014 and PMS-586 ranked relatively higher than other genotypes, but they only displayed moderate levels of tolerance under 14 days of being subjected to the drought treatment in the BYU screening experiment (Table 2-5).

Miscanthus sinensis genotype PMS-285 had a higher photosynthetic performance of Pn

and DSI φPSII than other genotypes under drought in both the HU precise comparison and BYU screening experiments (Table 2-4 and Table 2-S2). In addition, this genotype had a higher Pn value on day 21 of the BYU screening experiment than its Pn value on day 7 of the BYU recovery experiment (Table 2-4). In addition, *M. sinensis* genotype PMS-285 did not have a high recovery PCA ranking value (Table 2-7), which suggests it did not recover from drought stress after being rewatered. This is surprising given that it had a high PCA ranking value under slight drought stress in both the HU precise comparison and BYU screening experiments (Tables 2-5 and 2-6).

Miscanthus ×giganteus seems to have similar drought tolerance with *M. sinensis* genotype PMS-285 with relative lower recovery capacity than other selected *Miscanthus* genotypes. In comparison experiment, *Miscanthus ×giganteus* had similar the DSI Pn performance with *M. sinensis* PMS-285 on 1, 9, 15 days to drought, presuming *M.×giganteus* was relatively drought tolerant comparing to other 5 selected *Miscanthus* genotypes (Figure 2-5). On the other hand, *M.×giganteus* exhibited relatively low Recovery DSI Pn values than other genotypes (Figure 2-6), suggesting *M.×giganteus* has great potentials for improving post-drought recovery capacity. Moreover, *M. sinensis* PMS-586, which showed high recovery performance in both BYU recovery experiment and comparison experiment (Figure 2-4), can be considered for using to improve *Miscanthus* spp. recovery capacity and breed new strong recovery capacity *M.×giganteus* genotypes in the future.

Recovery capacity from drought is an important trait to help plants tide over from the effects of low SMC conditions (Chen et al., 2016). Several plant species, whose photosynthetic

machinery can often recover rapidly from drought stress, can absorb water when short-term rain events occur in the midst of a prolonged drought (Lauenroth et al., 1987; Chen et al., 2016). Lauenroth et al. (1987) reported that new root growth of *Bouteloua gracilis*, a warm-season perennial grass species, occurred nearly 40 h after being rewatered. Such root growth has the capability of increasing water availability for plants. Another study, which focused on water relations of sugarcane (*Saccharum officinarum* L.), found that high WUE and deep root systems enable sugarcane to recover from drought damage (Jangpromma et al., 2012). Such traits may be a possible reason for the strong recovery performance of *M. sinensis* genotypes PMS-014 and PMS-586. These traits could be effective screening criteria for drought-tolerant genotypes of *Miscanthus*. The WUE and rooting depth were not measured in our experiments but should be focused on in future research.

Genetic clusters and ploidy levels may be factors that have considerable influence on drought tolerance in *Miscanthus* spp. (Clark et al., 2019). Regarding the influence of genetic clusters on drought tolerance, genotypes in the *M. sinensis* Yangtze-Qinling genetic clusters group appear to have relatively stronger drought tolerance than other genetic clusters (Tables 2-5 and 2-6). In addition, genotypes in the *M. sinensis* Sichuan genetic cluster group can quickly recover from drought damage after being re-watered (Table 2-7). Both *M. sinensis* genotypes, PMS-007 and PMS-285, which align with the *M. sinensis* Yangtze-Qinling genetic cluster group (Tables 2-5 and 2-6), expressed relatively higher photosynthesis performance than other

genotypes under stress in the HU precise comparison experiment (Table 2-6). In contrast, in the BYU screening experiment, *M. sinensis* genotypes PMS-014 and PMS-586, which are associated with the *M. sinensis* Sichuan genetic cluster group (Table 2-5), displayed low photosynthetic levels when subjected to drought (Table 2-5). However, both exhibited relatively high recovery of photosynthetic levels after re-irrigation in the BYU recovery experiment (Table 2-7).

For different ploidy-type accessions, *M. sacchariflorus* diploid genotype UI10-00008 (Table 2-6) showed much higher photosynthesis performance than two other *M. sacchariflorus* tetraploid genotypes, JPN-2011-004 and UI11-00033, in the HU precise-comparison experiment (Table 2-6). *M. sacchariflorus* UI10-00008 has, on average, a small leaf area with only 0.8 cm leaf width, while genotypes JPN-2011-004 and UI11-00033 have an average leaf width of 2 cm (Table 2-6). In the BYU screening experiment, three *M. sacchariflorus* tetraploid genotypes were dead after a dry-down period of 21 days, but *M. sacchariflorus* diploid genotype UI11-00031 survived despite prolonged exposure to severe drought stress (Table 2-4). In addition, *M. sacchariflorus* diploid genotype UI11-00031 showed considerable recovery of Pn on day 7 in the BYU recovery experiment (Table 2-4). Therefore, ploidy level may reflect how leaf area and transpiration rate of *Miscanthus* genotypes contribute to drought tolerance. Moreover, drought-tolerant diploid *M. sacchariflorus* genotypes could be used as breeding material to produce high-yielding *M.* ×*giganteus* genotypes with strong drought tolerance.

Using genetic clusters and ploidy levels for genotype evaluation will help to improve the efficiency of the selection and breeding of stress-tolerant crops.

Ornamental *Miscanthus* cultivars exhibited relatively higher drought tolerance than most wild-type accessions in both the HU precise-comparison and BYU screening experiments (Tables 2-5 and 2-6). Although wild-type *Miscanthus* accessions usually express stronger stress tolerance to drought, disease, and insect pests than ornamental cultivars (Dougherty et al., 2015), we found that some cultivars (*M. floridulus* PI417947, *M. sinensis* UI10-00088, *M. sinensis* UI10-00092, and *M. sacchariflorus* UI10-00008) showed relatively higher drought tolerance than wild-type accessions (Tables 2-5 and 2-6). *Miscanthus sinensis* cultivars can be found in gardens and yards throughout the U.S., Canada, and Europe (Linde-Laursen, 1993). For ornamental plants, drought tolerance ranks high as an important selection criteria because drought stress is commonly encountered in managed landscapes.

Further studies are needed to characterize drought-stress-response mechanisms in *Miscanthus*. Few information exists regarding the drought-stress physiology of *Miscanthus* (Alvarez et al. 2007; Chen et al. 2013; Stavridou et al. 2019). Improvement of drought tolerance in *Miscanthus* spp. can enable them to survive when subjected to drought conditions caused by climate change. Such crops offer the opportunity to also generate biomass under low-soil-water conditions, which is important for developing *Miscanthus* as a sustainable energy crop.

Chapter 3. Evaluation of variation in drought tolerance of sugarcane (*Saccharum* spp. hybrids), *Miscanthus* spp. and their intergeneric hybrids (miscanes)

Introduction

Sugarcane, a warm-season C_4 perennial grass, is one of the most important crops for agriculture production in the world. The sugarcane's cultivation range between 31º N and S of the equator because it is adapted to warm temperature region with abundant sunlight and rainfall (Moore et al., 2013). Sugarcane is considered as the most biomass production crop in the world with nearly 1.9 billion tons production in the world and cultivated in approximately 100 countries (FAOSTAT, 2016). Although the main application of sugarcane is to produce purified sugar for human consumption, sugarcane also can be used as lignocellulosic biomass and bioethanol production for bioenergy (Ge et al., 2011), for example around 28 billion liters of sugarcane ethanol produced in Brazil per year (MAPA, 2018). In addition, production of bioethanol from sugarcane in Brazil successfully reduced its gasoline usage. However, sugarcane has been reported that yield of sugarcane decreased a lot under drought and sugarcane is more sensitive to drought than other bioenergy crop such as switchgrass and *Miscanthus*. It is necessary to improve tolerance of drought in sugarcane for both purified sugar and bioenergy production.

Intergeneric hybrids of *Saccharum* and *Miscanthus*, which often named as miscanes, are also considered as a high potential cellulosic bioenergy crop due to their high biomass potential with thick stems. Miscane has been reported that it can produce more biomass than *M.*

×*giganteus* Greef & Deuter ex Hodkinson & Renvoize, *M. sinensis* Andersson, and the switchgrass (*Panicum virgatum* L.) in in Arkansas, USA (Burner et al., 2009). Based on combining key traits from its parents such as high biomass productivity from sugarcane and high culm density, chilling and drought tolerance from *Miscanthus*, miscanes are considered as a bioenergy crop especially under warm temperate or subtropical regions (Sacks et al., 2013). Kar et al. (2019) reported that, in the chilling tolerance study, seven of 18 miscanes showed higher photosynthetic rates than their chilling-sensitive sugarcane parent after 7 days of chilling and two miscanes genotypes were not significantly different from their chilling-tolerant *Miscanthus* parent after 14 days of chilling. Similar performance of drought tolerance could be excepted in miscanes. Therefore, miscanes could be expected not only as a precious lignocellulosic biomass crop but also a source of genes to transform drought tolerance from *Miscanthus* to sugarcane. In addition, Ji et al. (2014) reported that 13 stress-responsive NAC ((NAM, ATAF1/2 and CUC2) genes were identified from *Miscanthus lutarioriparius*, and three genes (*MlNAC1*, *MlNAC11*, *MlNAC12*) exhibited more than 100-fold increases in salt and drought treatments. Moreover, six stress-responsive genes had significantly up-regulated expression in *MlNAC12* overexpression lines in in transgenic Arabidopsis (*Arabidopsis thaliana*) (Yang et al., 2018). In previous researches, a stress-responsive Mitogen-activated protein kinase gene from rice (*OsMAPK5*) increased tolerance to drought, salt, and cold stresses in overexpression lines of *OsMAPK5* of transgenic rice plants (Xiong &Yang, 2003).

Little information is currently available on the photosynthetic response under drought and drought-induced gene expression of miscanes comparing to sugarcane. The present study evaluated photosynthetic response to drought and three drought-associated gene expression of three miscanes genotypes and their respective sugarcane and *Miscanthus* parental genotypes.

Materials and Methods

Plant materials

One sugarcane parent (‘KR 05-619’), one *Miscanthus* parent (*M. sacchariflorus* ‘Miyakonojo’), and 3 miscane F1 progeny (‘JM14-52’, ‘JM14-57’, ‘JM14-60’) were studied. The sugarcane parent was breeding lines developed in the Sugarcane Breeding Station, National Agriculture and Food Research Organization, Tanegashima, Japan (31° 44′ N, 131° 4′ E). The *Miscanthus* parent was selection from Hokkaido University. *M. 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 Dr. Yoshifumi Terajima at the Tropical Agricultural Research Front of the Japan International Research Center for Agricultural Sciences in Ishigaki, Okinawa, Japan.

All sugarcane and miscane genotypes were propagated from tillers and *Miscanthus* genotype were propagated from rhizomes. Tillers and rhizome pieces of each genotype were cut into 10 cm lengths and grown in plastic pots (diameter = 19 cm, height = 27 cm) containing soilless medium consisting of compost, vermiculite, calcined clay, and peat moss (Forex Mori Sangyo Co., Ltd., Hokkaido, Japan). At planting, 5g of 13-18-4 slow-release fertilizer (Ekopu nigatsuchi S380, SunAgro 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 daily

irrigation for 4 weeks before starting the experiment.

Greenhouse experiment

The experiment was conducted under greenhouse conditions in Hokkaido University from 1 Jul. to 28 July. 2020 and from 9 Sep. to 24 Sep. 2020 as two continuous replicates experiments with around one month break between the first experiment and the second experiment. The experiment was arranged in a completely randomized design. Each genotype had three replicates. The irrigation was controlled by an automatic irrigation system. Drought stress treatment was given as different level by setting the set point of the system, while 40% soil moisture was well-water condition as control treatment and 25% soil moisture was drought stress treatment. After plants well irrigated by the automatic irrigation system for 7 days, Pn, gs, Ci, Tr were taken on the youngest, fully expanded leaf of each plant with a portable photosynthesis system (LI-6400XT). Drought treatment was started after the measurement of control treatment finished, and the Pn measurement was taken on days 8 and 19 of the first experiment and days 7 of the second experiment. In the second experiment, after the measurement of control and drought treatment finished, leaves of each plant, which were used to measure Pn, were sampled for RNA isolation and real-time reverse transcription-PCR (real-time RT-PCR) analysis. All leaves samples were immediately frozen in liquid nitrogen and stored in -80°C freezer until analysis.

RNA isolation and real-time reverse transcription-PCR analysis

Total RNA was isolated from frozen leaves based on the lithium chloride precipitation procedure (Napoli et al., 1990) and treated with DNase I (TaKaRa Bio, Shiga, Japan) in order to remove genomic DNA from the RNA fraction. According to Dwiyanti et al. (2011), cDNA was synthesized from purified RNA with oligo (dT) 20 primers and random hexamer primers and Invitrogen™ M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Two target drought-associated gene were one stress-responsive NAC genes of *Miscanthus lutarioriparius*

(*MINAC12*), and one mitogen-activated protein kinase gene of rice (*OsMAPK5*). The expression of three target gene were determined by real-time RT-PCR, following the method of Guo et al. (2021). The 4.6 μL of the cDNA synthesis reaction mixture, which was diluted 15 times with ultrapure water, 5 μL of 1.2 μM primer premix, 0.4 μL ROX Reference Dye (50 ×) and 10 μL of TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa Bio, Shiga, Japan) were contained in 20 μL PCR reactions. A StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to determent the expression level of target gene. The cycling conditions were 95°C for 5 minutes and followed with 40 cycles which were 95°C for 10 sec, 62 °C for 20 sec and 72 °C for 30 sec. The internal control was Ubiquitin. Relative changes in gene expression were estimated following the $2^{-\Delta\Delta Ct}$ method (Bookout and Mangelsdorf, 2003). Averages and standard errors of relative expression levels were calculated for three replicates of each genotype. The primer were designed based on the database of the National Center for Biotechnology Information (NCBI) and the Plant Comparative Genomics portal of the Department of Energy's Joint Genome Institute (Phytozome). The forward primer used for Ubiquitin gene expression was 5'-GACACCATCGACAACGTGAA-3' and the reverse primer was 5'-GCTGCTTGCCGGCGAAGAT-3'. The forward primer used for *MINAC12* gene expression was 5'-CAAGGAGGAGGCGATGGACA-3' and the reverse primer was 5'- CCTTCTTGGGCACCATCATCAT-3'. The forward primer used for *OsMAPK5* gene expression was 5'- CATAGGCATCAGGGACGTGA-3' and the reverse primer was 5'-GGTACAGGAAGTACTGGCAGT-3'.

Data analysis

Drought stress index (DSI) values of Pn were used to assess the drought tolerance capacity in total sugarcane, *Miscanthus* and miscane genotypes. In order to quantify drought-induced effects in different plants, the DSI value of Pn was calculated using equation 1. Microsoft Excel (Microsoft office 2016, Microsoft Corporation, USA) was used to analyze and make graph of

Pn, the DSI values of Pn, and relative changes in mean ± standard error of the mean (SE) gene expression of real-time RT-PCR result.

Results

Photosynthetic rate performance of genotypes in greenhouse experiment

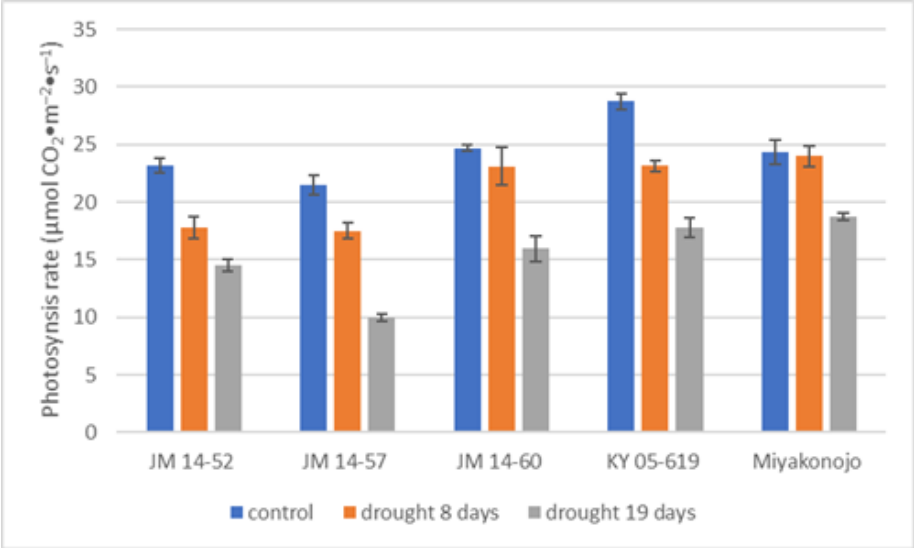
Besides Pn values, there was no significant different perfoemance of gs, Ci, Tr among all genotypes. Average changes of Pn of sugarcane, *Miscanthus*, and miscane of the first experiment and the second experiment were showed as Figure 3-1. Under control condition, sugarcane KY05-619 had highest Pn than other 4 genotypes in both two experiment, while *M. sacchariflorus* Miyakonojo had medium Pn values comparing to sugarcan and miscanes genotypes. Three miscane genotypes had similar Pn values under control condition. After drought treatments were initiated, photosynthetic levels of all genotypes decreased, except the Pn value of 'JM14-60' after 7 days of drought in the second experiment. The Pn performance of all genotypes showed a sharp decline from 8 to 19 days of drought in the first experiment, particularly going from a 18% decrease to a 43% decrease in Pn (Figure 3-1). *Miscanthus sacchariflorus* Miyakonojo performed only little decreasing of Pn on 8 days of drought in the first experiment and on 7 days of drought in the second experiment, while sugarcane KY05-619 showed a largely decline. Interestingly, the Pn performance of miscane 'JM14-60' increased on 7 days of drought in the second experiment, while other 4 genotypes decreasing (Figure 3-1).

Drought stress index (DSI) of all 3 miscane genotypes were higher than the sugarcane parent after 7 or 8 days of drought, although miscane 'JM14-57' showed lower DSI value than sugarcane KY05-619 after 19 days of drought in the first experiment (Figure 3-2). *Miscanthus sacchariflorus* Miyakonojo showed relatively higher DSI values than other 4 genotypes in both

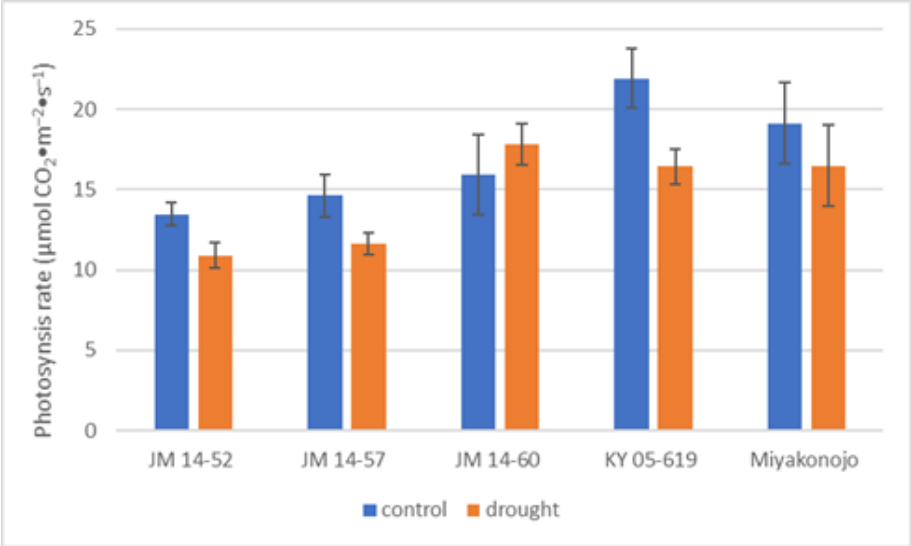
two experiment (Figure 3-2). One genotype, miscane “JM 14-60”, showed higher DSI values than other two miscane genotypes and the sugarcane parent under drought treatment, which was similar to its *Miscanthus* parent genotype.

Drought-associated gene expression of real-time reverse transcription-PCR analysis

For three genotypes, miscane ‘JM14-52’, miscane ‘JM14-57’ and sugarcane KY05-619, both the expression of *MINAC12* (assessed as the ratio of *MINAC12* / Ubiquitin mRNA transcript abundance), the expression of *OsMAPK5* (assessed as the ratio of *OsMAPK5*/ Ubiquitin mRNA transcript abundance) decreased under drought treatment (Figure 3-3). However, *M. sacchariflorus* ‘Miyakonojo’ had a 2.6-fold of the expression of *MINAC12* and a 1.8-fold of the expression of *OsMAPK5* in drought relative to control condition (Figure 3-3). Moreover, miscane ‘JM14-60’ also had a 1.7-times increasing expression of *MINAC12* and no different expression of *OsMAPK5* after 7 days of drought, which is similar to its *Miscanthus* parent genotype (Figure 3-3).

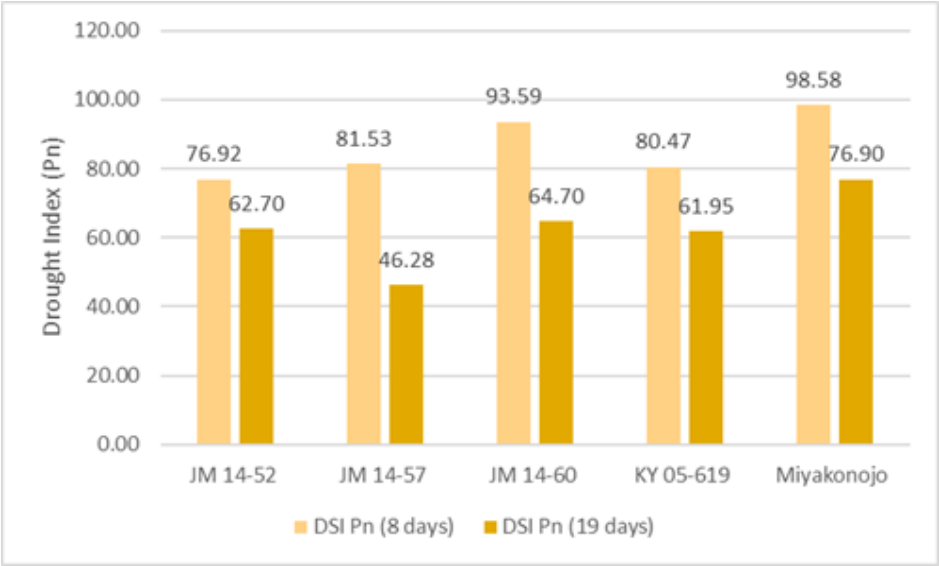


(a)

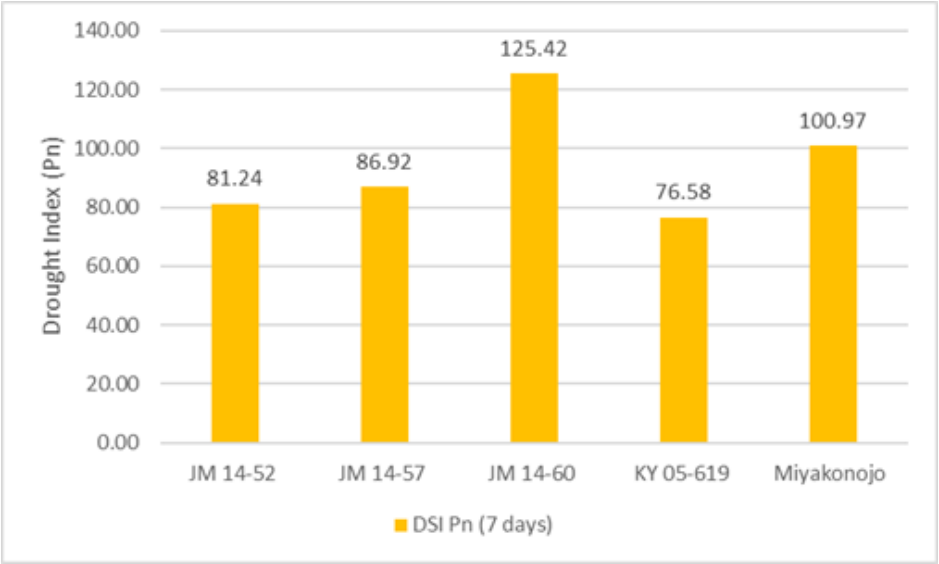


(b)

Figure 3-1. Photosynthetic rate (Pn) of three miscane genotypes and their sugarcane and *Miscanthus* parents in greenhouse experiment. (a) control condition and after 8 and 19 days of drought in the first experiment. (b) control condition and after 7 days of drought in the second experiment.

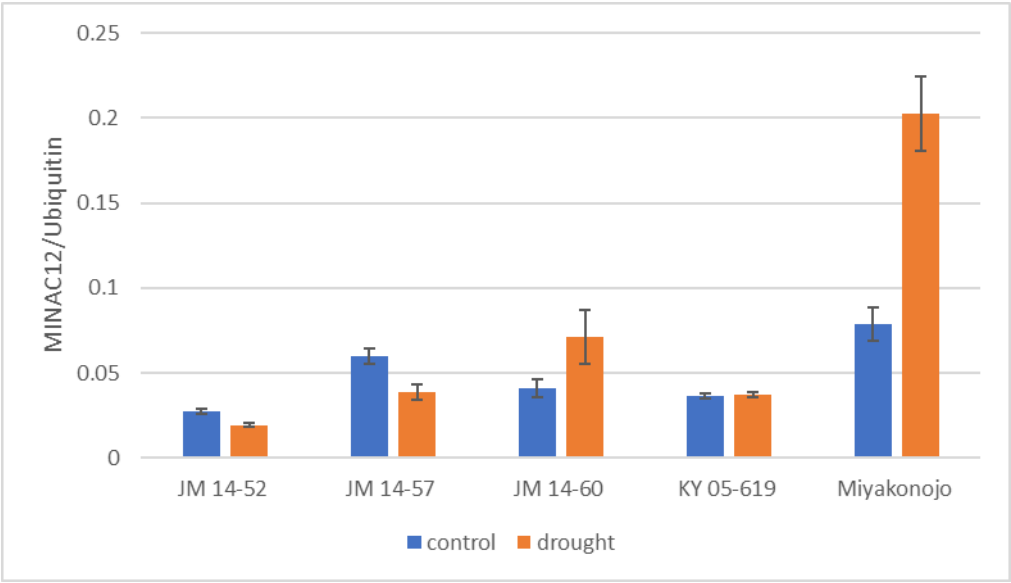


(a)

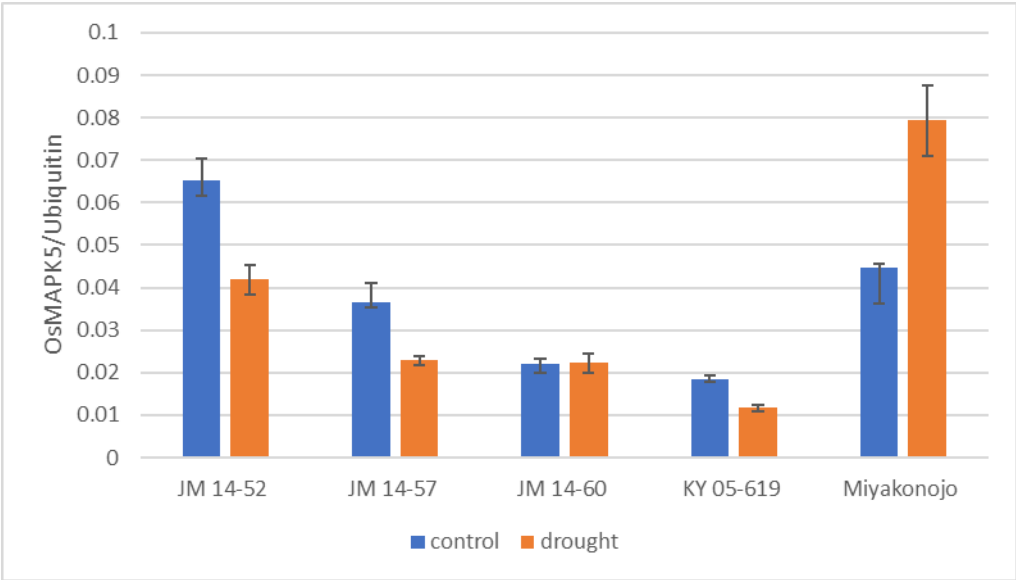


(b)

Figure 3-2. Drought stress index (DSI) of photosynthetic rate (Pn) three miscane genotypes and their sugarcane and *Miscanthus* parents in greenhouse experiment. (a) after 8 and 19 days of drought in the first experiment. (b) after 7 days of drought in the second experiment.



(a)



(b)

Figure 3-3. Expression of three drought-associated gene expression for two miscane genotypes and their sugarcane and *Miscanthus* parents in control and drought treatment in the second experiment. (a) Expression of *MINAC12*. (b) Expression of *OsMAPK5*. Relative mRNA levels are expressed as the ratios to Ubiquitin transcript levels. Mean ± 1SE for three replications are given for each data point.

Discussion

As expected, the sugarcane parent was highly sensitive to drought, with a sharp decline of Pn values under drought treatment and relatively low DSI values (Figure 3-1, 3-2), *Miscanthus* parent showed high tolerant to drought with relatively high DSI values especially after 7 or 8 days to drought (Figure 3-2). Our results for sugarcane and *Miscanthus* genotypes showed that *Miscanthus* is more tolerant to drought than sugarcane. Three miscane genotypes had similar Pn values under control condition.

All of three miscane genotypes showed higher DSI values than the sugarcane parent in drought (Figure 3-2), suggesting that miscane genotypes were more tolerant to drought than sugarcane. Although three miscane genotypes had similar Pn values under control condition, their Pn performance were very different under drought treatment (Figure 3-1). One genotype, miscane 'JM 14-60', exhibited high photosynthesis rate and high DSI under drought environment in both experiments, especially after 7 or 8 days to drought, suggesting that it is a drought tolerant genotype which can be used as germplasm in breeding programs and of genes to transform drought tolerance from *Miscanthus* to sugarcane. (Figure 3-1, 3-2). Comparing with *Miscanthus* parent, miscane 'JM 14-60' showed similar Pn and DSI performance with *Miscanthus* parent *M. sacchariflorus* 'Miyakonojo', presuming miscane 'JM 14-60' inherited drought tolerance traits from *Miscanthus* parent.

The two drought tolerant genotypes, *M. sacchariflorus* 'Miyakonojo' and miscane 'JM

14-60', exhibited increasing or no different expression of *MINAC12* and *OsMAPK5* in drought (Figure 3-3), suggesting high level of *MINAC12* and *OsMAPK5* expression seem to contribute to the drought tolerance in *Miscanthus* and miscane genotypes. The previous study reported that over 100-fold variations in transcript levels of *MINAC12* was observed in *Miscanthus lutarioriparius* under drought and overexpression of *MlNAC12* in Arabidopsis showed significantly enhanced drought stress tolerance than wild type with reducing stomata aperture and decreasing the accumulation of the reactive oxygen species (ROS) (Ji et al., 2014; Yang et al., 2018). Both *MINAC12* and *OsMAPK5* are ABA-induced in drought, suggesting that these two gene might participate in an ABA-dependent signaling pathway in response to drought stress (Xiong &Yang, 2003; Yang et al., 2018). Therefore, further research is necessary to evaluate ABA or ROS such as hydrogen peroxide (H_2O_2) content response to drought in *Miscanthus* and miscane, which will help to clarify drought-induced mechanism in these two plants as improving and developing drought tolerant bioenergy crops.

According to Clark et al. (2019), *M. sacchariflorus* spp. Yangtze group (ssp. *lutarioriparius*), which includes *Miscanthus lutarioriparius*, was derived from the N China group, with a substantial bottleneck. Clark et al. (2019) also reported that *M. sacchariflorus* ssp. *lutarioriparius* (Yangtze diploids) has the traits of tall and high yielding traits and ancestry from *M. sacchariflorus* ssp. *lutarioriparius* (Yangtze diploids) seems to have contribution to the high yield potential of the biomass cultivar *M.* × *giganteus* '1993-1780'. Therefore, research

of drought-induced performance or drought-associated gene expression in *Miscanthus lutarioriparius* will be useful and important for improving tolerance in commercial variety *M.* × *giganteus*. In this study, *MINAC12* exhibited high expression level under drought in drought tolerance genotypes *M. sacchariflorus* 'Miyakonojo' and miscane 'JM 14-60', which was also observed in *M. lutarioriparius* (Ji et al., 2014), suggesting the result of *M. lutarioriparius* research will be a useful information for clarifying genetic mechanism of drought tolerance in *Miscanthus* and miscane. In addition, Fan et al. (2015) reported that 39 genes, which were assigned to photosynthesis, protein metabolism, and abiotic stress responses, were expressed relatively at higher levels in tolerant *M. lutarioriparius* genotypes than in sensitive *M. lutarioriparius* genotypes under drought. In order to clarify genetic mechanism of drought tolerance in *Miscanthus* spp., it is necessary to study how these 39 genes performs under drought in *M. sacchariflorus*, *M.sinesis*, and *M.* × *giganteus* in further researches.

Substantial variation was observed among different miscane genotype based on their Pn performance and gene expression level of target gene under drought. Kar et al. (2019) also observed this kind of variation among miscanes genotypes in chilling tolerance research. It suggested that selecting strong tolerance to drought miscane genotypes is necessary for developing miscane as one potential bioenergy crop and can be useful sources of genes for improving drought tolerance in sugarcane.

Chapter 4. General discussion

Agriculture has been suffered serious damage by global warming since the 20th century and global climate change seems to be a major issue for crop production in the future. High temperature and unbalance water resource reduce yield of crop and limit plant growth, sometimes led to a dead end. On the other hand, to reducing carbon emissions caused by fossil fuel, developing renewable bioenergy is important for energy industry in the world. For example, in Denmark, they collect various organic wastes, including agriculture waste, livestock manure, and waste vegetables and fruits, to produce electricity, biogas and biogas fertilizer (Wandervogel Study in Global Food Resources I, Graduate School of Global Food resources, Hokkaido University). Moreover, global biofuel production was around 16 billion gallons in modern bioenergy industry in the world. In order to avoid crop competition between food and energy, developing non-food lignocellulose bioenergy crops which can be grown in marginal land is a potential solution response to energy crisis and global warming. Marginal land means the area is water or nutrition insufficient for cultivation of agriculture crops. Improvement of drought tolerance in bioenergy crops such as switchgrass and *Miscanthus* can make them be able to grow on water deficit area, putting aside of good farming land for cultivating food crops. Cline (2008) reported that global food demand will increase by three times because of higher world population. Thus, agriculture farm land with good environmental and soil condition should be used for food production. Therefore, improving tolerance of abiotic

stresses such as drought is important key for bioenergy crop to cultivate in marginal agriculture land in term of issue for conflict between food and bioenergy production.

Two types of drought treatment techniques, dry-down method and fixed SMC method, were usually used to evaluate of drought tolerance in plants in greenhouse experiment. In the dry-down method, SMC usually continuously decline by evapotranspiration and the decreases quickly over a short period of time. By contrast, the fixed SMC technique is used to keep the SMC of target plants at fixed soil-moisture levels by regularly adding water based on evapotranspiration amount. Although dry-down method is cost-efficiency and ease of operation, the fixed-SMC method needs more time and effort to conduct. In general, scientist only choose one technique to conduct in the experiment. However, different technique may affect evaluation results due to the different patterns of decreasing SMC. According to the result, dry-down method is suitable to selected drought tolerant genotypes grown in the field and the fixed-SMC method is good for clarifying drought-induced mechanism in plants.

Conducting experiments in two locations where has different level of humility is important to assessment of drought tolerance in *Miscanthus*. In this study, total 29 *Miscanthus* genotypes were evaluated in two locations experiments, HU experiment and BYU experiment. Japan, where is temperate monsoon climate, has high temperature and high precipitation in summer and it belongs to high annual precipitation regions in the world. With the high snowfall in winter, air humidity of Sapporo is around 70% over the year. On the other hand, Utah is

temperate continental climate and has 300ml average year precipitation, which is only one third of average year precipitation in Sapporo. As a result, air humidity of Utah is around 50% from April to October. To assess drought tolerance in *Miscanthus*, it is good to evaluate them in not only high humidity area such as Sapporo but also low humidity area such as Utah.

One genotype *M.sinensis* PMS-285 showed drought tolerance in both two location experiments, suggesting that it can used as a drought tolerant breeding material for the improvement of *Miscanthus* spp. Drought tolerance of *M.sinensis* PMS-285 is probably credited to ABA-dependent signaling pathway in response to drought stress. In this study, *M.sinensis* PMS-285 showed strong drought tolerance in both two location experiments, HU and BYU experiments. In order to avoid destructive measurement, only photosynthesis paraments of *Miscanthus* measured in this study. It is difficult to explain the reason of drought tolerance in *M.sinensis* PMS-285 relative to other genotypes. However, in Chapter 2 experiment, *M.sinensis* PMS-285 showed relative lower gs than *M.×giganteus* and other genotypes after 9 and15 days to drought (data not shown). Stomatal closure is stimulated by ABA-dependent signaling pathway, in order to reduce water loss by evapotranspiration. In addition, Fan et al. (2015) reported that several gene assigned to stomatal regulation regulated by ABA signal transduction of *Miscanthus lutarioriparius* located in the semiarid region expressed higher level than that located in the wet location. Moreover, based on the gene expression result in Chapter 3, *M. sacchariflorus* 'Miyakonojo' exhibited increasing expression of *MINAC12* and

OsMAPK5 in drought, which was contributed to drought tolerance in *M. sacchariflorus* 'Miyakonojo'. Both *MINAC12* and *OsMAPK5* are ABA-induced in drought, suggesting that these two gene might participate in an ABA-dependent signaling pathway in response to drought stress (Xiong &Yang, 2003; Yang et al., 2018). Therefore, it can be expected that drought tolerance of *M.sinensis* PMS-285 is possible caused by ABA-dependent signaling pathway response to drought stress.

Agave (*Agave* L.), a Crassulacean Acid Metabolism (CAM) plant which can grow in marginal and semiarid area, has been recognized as a potential bioenergy crop (Postgraduados, Biology, &Angeles, 2011). Agave species are mainly grown in arid and semiarid regions from the southwestern United States, through Central America, to north South America, especially in Mexico. The production of agave can be used for food, drinks, fiber, and ornamental plants, while the most famous products is Tequila (Gentry, 1982; Nobel, 2010). Moreover, the carbohydrates in the stems of agave can be used to produce ethanol and the lignocelluloses from leaves are a resource of biomass, which made it become a potential bioenergy crop (Borland et al., 2009). Comparing to *Miscanthus*, agave can be grown in more drought area with poor soil such as desert and chaparral. For example, *Miscanthus* is suitable for planting in the Midwestern United States, where is the most important agriculture area of USA. On the other hand, agave can tolerant high temperature of 60℃ and water deficit area so it is usually grown in the Southwestern United States such as Utah. The reason why agave can survival in drought regions

is its CAM photosynthetic mode, which absorb and fix CO_2 at night and close the stomata to provide water lost through transpiration with high temperature in daytime (Pimienta et al., 2006; Nobel, 2010). This character makes CAM plants have much higher water use efficiency (WUE) than C_3 and C_4 plants. For example, the WUE of CAM plants is 10-40g CO_2 $kg^{-1}H_2O$, while the WUE of C_3 is 1-3 g CO_2 $kg^{-1}H_2O$ and the WUE of C_4 is 2-5 g CO_2 $kg^{-1}H_2O$ (Nobel, 2009). In addition, agave has high capacity to assimilate and transform atmospheric CO_2, which can help to mitigate the high concentrations of atmospheric CO_2 and global warming (Nobel, 2010). However, the production system of agave has not been established well, which limited the development of agaves production. As water deficit and high temperature problems increasing by global warming, it is important to clarify mechanism of drought tolerance of agave and introduce the traits into *Miscanthus* or other biomass crops by genetic techniques such as gene editing.

Evaluation and selection drought tolerance genotypes from genetic resource of *Miscanthus* spp. is an important progress for both bioenergy and agriculture production. With genetic engineering technology, it is possible to transform drought tolerance gene from tolerance genotype such as *M.sinensis* PMS-285 to commercial variety *M.×giganteus* or other bioenergy crops. *Miscanthus sinensis* PMS-285 can also be used to breed *M.* × *giganteus* new genotype by crossing with *M. sacchariflorus* genotypes. Moreover, drought tolerance genetic clusters *M. sinensis* Yangtze-Qinling group and high post-drought recovery genetic clusters *M.*

sinensis Sichuan Basin group showed the potential genetic resources of *Miscanthus* spp. and supply a probability for help to improve the efficiency of the selection and breeding of stress-tolerant crops. It is also showed that miscanes had better photosynthetic performance than sugarcane under drought, suggesting that miscanes could be expected not only as a precious lignocellulosic biomass crop but also a source of genes to transform drought tolerance from *Miscanthus* to sugarcane.

The flow chart of improving drought tolerance in *Miscanthus* spp is shown as Figure 4-1. First, it is necessary to select superior genotypes of drought tolerance from genetic resource of *Miscanthus* spp., for example, *M.sinensis* PMS-285 was selected as a drought tolerant genotype and *M.sinensis* PMS-586 was selected as a rapid post-drought recovery genotype in this study. In this progress, it is better to use dry-down method to screen superior genotypes because dry-down method has the similar SMC pattern to the drought happened in the field. The selected superior genotypes can be used for clarify drought-induced mechanism in *Miscanthus* spp. With the fixed SMC method, plants generally have enough time to physiologically respond to drought due to being subjected to continuous drought conditions and it is convenient for scientist to observe and clarify drought-induced mechanism. Superior genotypes can also be used for breeding new tolerant genotypes. For example, *M. sinensis* PMS-285 can be used to breed new drought tolerant *Miscanthus* ×*giganteus* genotype by crossing with *M. sacchariflorus* for biomass production. New drought tolerant miscane genotypes also can be

breed by *M.sinensis* PMS-285.

The weak point of this study is that, even covering all genetic clusters of *Miscanthus* accessions, the genotypes used in this study was still small and limited. It is necessary to evaluate other *Miscanthus* genotypes or accessions such as genotypes of genetic clusters *M. sinensis* Yangtze-Qinling group in the further study. Next, further research is necessary to elucidate physiological & genetic mechanism of drought tolerance of superior genotypes. For example, it is necessary to work on physiological characteristic such as ABA or hydrogen peroxide (H_2O_2), which usually increase response to drought in plants. Moreover, *Miscanthus* is perennial grass so the drought tolerance in second or third year is also needed to be evaluated. In addition, relation between growth stage and drought tolerance in *Miscanthus* is also needed to be clarify. Tolerance of seeding stage or mature stage has a profound influence on biomass production. The result of this study can be data base for breeding program of improvement drought tolerance in *Miscanthus* spp. for developing non-food lignocellulose bioenergy crops as one potential solution of food resources problems in the world.

Finally, highlights of the research findings from the present study are as follows.

- Two drought treatment methods had different SMC pattern and were suitable for different applications.
- Superior genotypes of tolerance (*M. sinensis* PMS-285) and high recovery (*M. sinensis* PMS-586) for drought stress were successfully selected in this study.

- Miscane had stronger drought tolerance than sugarcane parent and miscane can be useful sources of genes for improving drought tolerance in sugarcane.
- Miscane can inherit drought tolerance and drought-associated gene from *Miscanthus*.

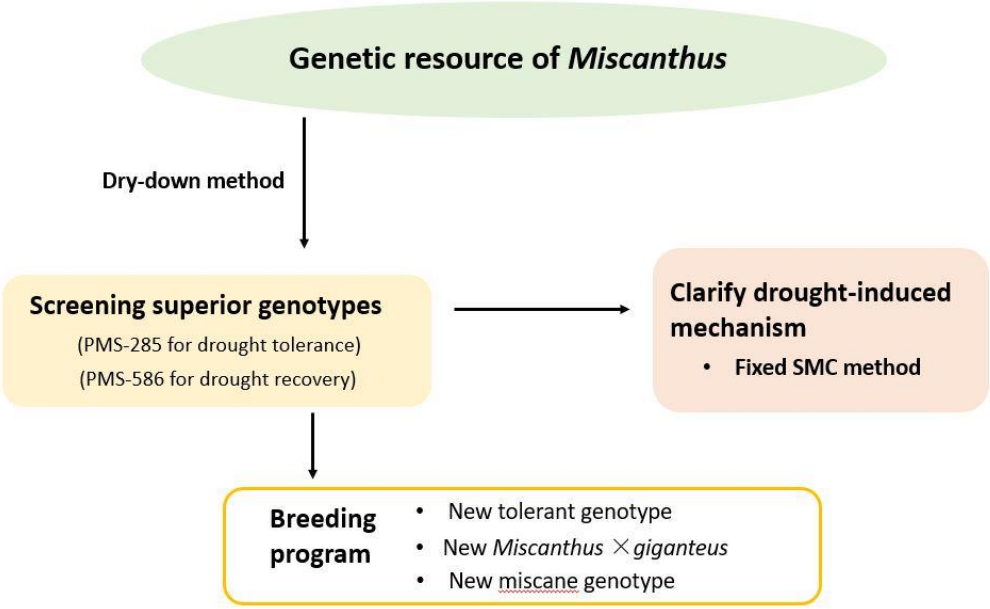


Figure 4-1. Flow chart of improving drought tolerance in *Miscanthus* spp. Dry-down method is suitable for screening superior genotypes and fixed soil moisture content (SMC) method is suitable for clarifying drought-induced mechanism in *Miscanthus* spp.

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Appendix I: Supplementary information of chapter 2

Supplemental Table 2-S1. Detailed information of 29 *Miscanthus* genotypes used for the evaluation of low-water-adaptability capacity in *Miscanthus* spp., including entry number, species, origin location, and genetic groups background. Some information is not available for some genotypes

Species	Accession	Genetic clusters†	Type	Country of origin	Cultivar/ Strain name	Place	Latitude	Longitude
M. sacchariflorus	UI11-00033	S Japan 4x Msa	Wild	Japan		Gifu		
M. sacchariflorus	JPN-2011-010	N Japan 4x Msa	Wild	Japan		Hokkaido	42.61618	141.8116
M. sacchariflorus	JPN-2011-004	S Japan 4x Msa	Wild	Japan		Gifu	36.16943	137.3095
M. sacchariflorus	JPN-2011-006	S Japan 4x Msa	Wild	Japan		Gifu	35.3246	136.693
M. sacchariflorus	JPN-2010-005	N Japan 4x Msa	Wild	Japan		Hokkaido	42.61475	141.8167
M. sacchariflorus	UI11-00031	Yangtze diploids (ssp.	Wild	China				

		lutarioriparius) Msa						
M. sacchariflorus	RU2012-169	NEChina/Korea/Russia	Wild	Russia		Primorsky Krai	45.34596	133.5559
		diploids Msa						
M. sacchariflorus	RU2012-183	NEChina/Korea/Russia	Wild	Russia			43.75422	132.0818
		diploids Msa						
M. sacchariflorus	RU2012-056.1WD (4x)	NChina/Korea/Russia	Wild	Russia			48.82444	135.948
		tetraploids Msa						
M. sacchariflorus	JM11-006	S Japan 4x Msa	Wild	Japan		Yamaguchi	34.1986667	131.2806
M. sacchariflorus	UI10-00008	NEChina/Korea/Russia	Cultivar	Unknown	Hortico			
		diploids Msa						
M. sacchariflorus	RU2012-141	NEChina/Korea/Russia	Wild	Russia			47.50762	134.7411

		diploids Msa						
M. sinensis	PMS-164	Yangtze-Qinling Msi	Wild	China		Hebei	37.3400167	114.281
M. sinensis	PMS-007	Yangtze-Qinling Msi	Wild	China		Hubei	30.79765	110.2624
M. sinensis	UI10-00092	C Japan Msi	Cultivar		Strictus			
M. sinensis	PMS-586	Sichuan Msi	Wild	China		Guizhou	27.0010333	108.699
M. sinensis	UI10-00024	S Japan Msi	Cultivar		Arabesque			
M. sinensis var. *condensatus*	UI10-00015	C Japan Msi	Cultivar		Cosmopolitan			
M. sinensis	PMS-014	Sichuan Msi	Wild	China		Hubei	29.6570167	109.1195
M. sinensis	UI10-00097	S Japan Msi	Cultivar		Variegatus			
M. sinensis	UI10-00088	C Japan Msi	Cultivar		Silberturm			
					(Silver Tower)			

M. sinensis	PMS-347	SE China Msi	Wild	China		Guangdong	24.1679833	115.8839
M. sinensis	UI10-00020	S Japan Msi	Cultivar		Adagio			
M. sinensis	PMS-285	Yangtze-Qinling Msi	Wild	China		Anhui	29.64345	118.1584
M. sinensis	UI10-00100	S Japan Msi	Cultivar		Yaku Jima			
M. sinensis	UI10-00053	S Japan Msi	Cultivar		Grosse Fontaine			
M. sinensis	UI10-00080	C Japan Msi	Cultivar		Roland			
M. sinensis	UI10-00048	S Japan Msi	Cultivar		Gracillimus			
M. floridulus	PI417947	SE China Msi	Wild	Papua New Guinea	NG77-022			

†According to Clark *et al*. (2014) and Clark *et al*. (2019)

Supplemental Table 2-S2-1. Least squares means of drought stress index (DSI) values of chlorophyll fluorescence (φPSII) of *Miscanthus* genotypes in a screening experiment at Brigham Young University, Provo, Utah, USA

Species	Accession	Stress level†	DSI of φPSII
M. sinensis	PMS-007	1	102.89 A‡
M. sinensis	UI10-00088	1	84.10 AB
M. sinensis	PMS-285	1	75.91 AB
M. sinensis	PMS-164	2	67.81 AB
M. sacchariflorus	UI11-00031	1	61.13 AB
M. floridulus	PI417947	1	59.20 AB
M. sinensis	PMS-347	1	52.71 AB
M. sinensis	PMS-014	1	52.58 AB
M. floridulus	PI417947	2	51.15 AB
M. sinensis	PMS-007	2	47.43 AB
M. sinensis	PMS-285	2	44.68 AB
M. sinensis	PMS-164	1	42.87 AB
M. sacchariflorus	UI11-00031	2	33.91 AB

M. sinensis	PMS-586	1	32.91 AB
M. sinensis	UI10-00088	2	16.86 AB
M. sinensis	PMS-014	2	0.019 AB
M. sinensis	PMS-586	2	-7.23 AB
M. sinensis	PMS-347	2	-17.19 B

†Stress level 1 represents the slight drought stress treatment and stress level 2 represents the severe drought stress treatment.

‡Least squares means with the same letter are not significantly different ($p < 0.05$).

Supplemental Table 2-S2-2. Least squares means of drought stress index (DSI) values of chlorophyll fluorescence (φPSII) of *Miscanthus* genotypes in a precise-comparison experiment at Hokkaido University, Sapporo, Japan

Species	Accession	Stress level†	DSI of φPSII
M. sinensis	PMS-285	2	163.24 A‡
M. sinensis	UI10-00024	2	114.06 AB
M. sinensis	PMS-164	2	98.95 ABC
M. sinensis	PMS-285	1	94.04 ABC
M. sacchariflorus	JPN-2011-004	2	80.80 ABC
M. sinensis	PMS-347	2	80.06 ABC
M. sinensis	UI10-00024	1	74.97 BC
M. sinensis	PMS-007	1	71.52 BC
M. sinensis	PMS-007	2	68.85 BC
M. sacchariflorus	UI10-00008	1	67.99 BC
M. sinensis	UI10-00020	2	65.80 BC
M. sinensis	PMS-164	1	63.64 BC
M. sinensis	UI10-00020	1	62.51 BC
M. sacchariflorus	UI11-00033	2	61.95 BC

M. sacchariflorus	UI10-00008	2	53.70 BC
M. sacchariflorus	JPN-2011-004	1	53.15 BC
M. sinensis var. condensatus	UI10-00015	2	51.87 BC
M. sinensis	PMS-347	1	47.97 BC
M. sacchariflorus	UI11-00033	1	39.22 C
M. sinensis var. condensatus	UI10-00015	1	37.81 C

†Stress level 1 represents the slight drought stress treatment and stress level 2 represents the severe drought stress treatment.

‡Least squares means with the same letter are not significantly different ($p < 0.05$).

Supplemental Table 2-S3-1. Least squares means of drought stress index (DSI) values of photosynthetic rate (Pn) of *Miscanthus* genotypes in a screening experiment at Brigham Young University, Provo, Utah, USA

Species	Accession	Stress level†	DSI of Pn
M. sinensis	PMS-164	2	124.48 A‡
M. sinensis	UI10-00088	1	99.62 A
M. sinensis	PMS-007	1	94.39 A
M. floridulus	PI417947	1	89.04 A
M. sinensis	PMS-285	1	86.75 A
M. floridulus	PI417947	2	82.02 A
M. sinensis	PMS-285	2	71.93 A
M. sinensis	PMS-007	2	66.02 A
M. sacchariflorus	UI11-00031	1	63.54 A
M. sinensis	PMS-014	1	56.38 A
M. sinensis	PMS-164	1	42.95 A
M. sinensis	PMS-586	1	39.28 A
M. sinensis	PMS-347	1	36.13 A
M. sacchariflorus	UI11-00031	2	35.86 A

M. sinensis	UI10-00088	2	21.59 A
M. sinensis	PMS-586	2	3.16 A
M. sinensis	PMS-014	2	-0.86 A
M. sinensis	PMS-347	2	-5.47 A

†Stress level 1 represents the slight drought stress treatment and stress level 2 represents the severe drought stress treatment.

‡Least squares means with the same letter are not significantly different ($p < 0.05$).

Supplemental Table 2-S3-2. Least squares means of drought stress index (DSI) values of photosynthetic rate (Pn) of *Miscanthus* genotypes in a precise-comparison experiment at Hokkaido University, Sapporo, Japan

Species	Accession	Stress level†	DSI of Pn
M. sinensis	PMS-007	1	77.31 A‡
M. sacchariflorus	UI11-00033	2	76.05 A
M. sinensis var. condensatus	UI10-00015	1	75.19 A
M. sinensis var. *condensatus*	UI10-00015	2	66.66 AB
M. sacchariflorus	JPN-2011-004	2	63.66 ABC
M. sinensis	UI10-00024	1	62.08 ABC
M. sacchariflorus	JPN-2011-004	1	61.20 ABC
M. sinensis	PMS-285	1	58.94 ABC
M. sinensis	PMS-007	2	56.27 ABC
M. sacchariflorus	UI11-00033	1	55.11 ABC
M. sinensis	PMS-285	2	54.60 ABC
M. sinensis	PMS-347	1	52.45 ABCD
M. sinensis	UI10-00020	1	51.97 ABCD
M. sinensis	PMS-164	1	37.64ABCD

M. sinensis	PMS-347	2	35.96 BCD
M. sinensis	PMS-164	2	34.95 CD
M. sacchariflorus	UI10-00008	2	15.46 D
M. sacchariflorus	UI10-00008	1	8.71 D

† Stress level 1 represents the slight drought stress treatment and stress level 2 represents the severe drought stress treatment.

‡Least squares means with the same letter are not significantly different ($p < 0.05$).

Supplemental Table 2-S4-1. Least squares means of drought stress index (DSI) values of stomatal conductance (gs) of *Miscanthus* genotypes in a screening experiment at Brigham Young University, Provo, Utah, USA

Species	Accession	Stress level†	DSI of gs
M. sinensis	PMS-164	2	223.67 A‡
M. floridulus	PI417947	1	205.71 A
M. sinensis	PMS-164	1	195.37 A
M. sinensis	UI10-00088	1	188.70 A
M. sinensis	PMS-007	1	181.12 A
M. sinensis	PMS-285	1	177.53 A
M. sinensis	PMS-007	2	117.85 A
M. floridulus	PI417947	2	109.87 A
M. sinensis	PMS-586	1	104.44 A
M. sinensis	PMS-285	2	96.88 A
M. sinensis	PMS-347	1	88.73 A
M. sacchariflorus	UI11-00031	1	79.86 A
M. sinensis	UI10-00088	2	53.99 A
M. sinensis	PMS-014	1	53.59 A

M. sacchariflorus	UI11-00031	2	31.96 A
M. sinensis	PMS-586	2	8.55 A
M. sinensis	PMS-014	2	-4.02 A
M. sinensis	PMS-347	2	-28.86 A

† Stress level 1 represents the slight drought stress treatment and stress level 2 represents the severe drought stress treatment.

‡Least squares means with the same letter are not significantly different ($p < 0.05$).

Supplemental Table 2-S4-2. Least squares means of drought stress index (DSI) values of stomatal conductance (gs) of *Miscanthus* genotypes in a precise-comparison experiment at Hokkaido University, Sapporo, Japan

Species	Accession	Stress level†	DSI of gs
M. sinensis	PMS-164	1	824.29 A‡
M. sinensis	PMS-007	2	148.33 B
M. sinensis var. condensatus	UI10-00015	2	105.52 B
M. sinensis	UI10-00024	2	97.24 B
M. sacchariflorus	UI11-00033	1	70.15 BC
M. sinensis	PMS-285	2	43.53 BC
M. sinensis var. condensatus	UI10-00015	1	27.49 BC
M. sinensis	PMS-007	1	27.32 BC
M. sinensis	PMS-164	2	26.25 BC
M. sinensis	PMS-285	1	24.51 BC
M. sinensis	UI10-00024	1	24.14 BC
M. sinensis	PMS-347	1	15.97 BC
M. sacchariflorus	JPN-2011-004	1	9.21 BC
M. sinensis	UI10-00020	1	7.47 BC

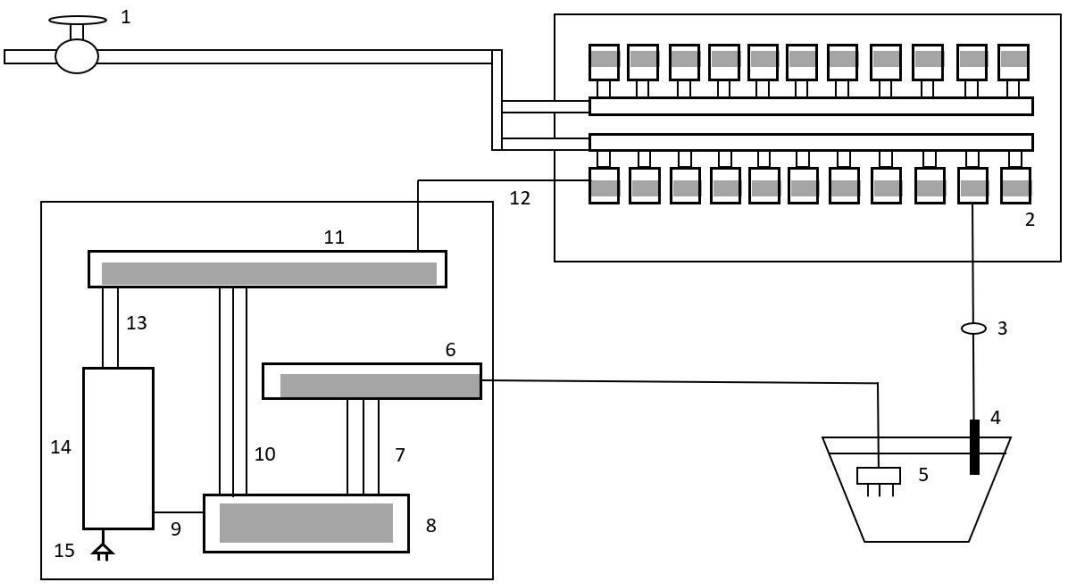
M. sacchariflorus	UI10-00008	1	5.49 BC
M. sacchariflorus	JPN-2011-004	2	-16.64 BC
M. sinensis	PMS-347	2	-43.31 BC
M. sacchariflorus	UI10-00008	2	-49.48 BC
M. sinensis	UI10-00020	2	-60.07 BC
M. sacchariflorus	UI11-00033	2	-118.04 C

†Stress level 1 represents the slight drought stress treatment and stress level 2 represents the severe drought stress treatment.

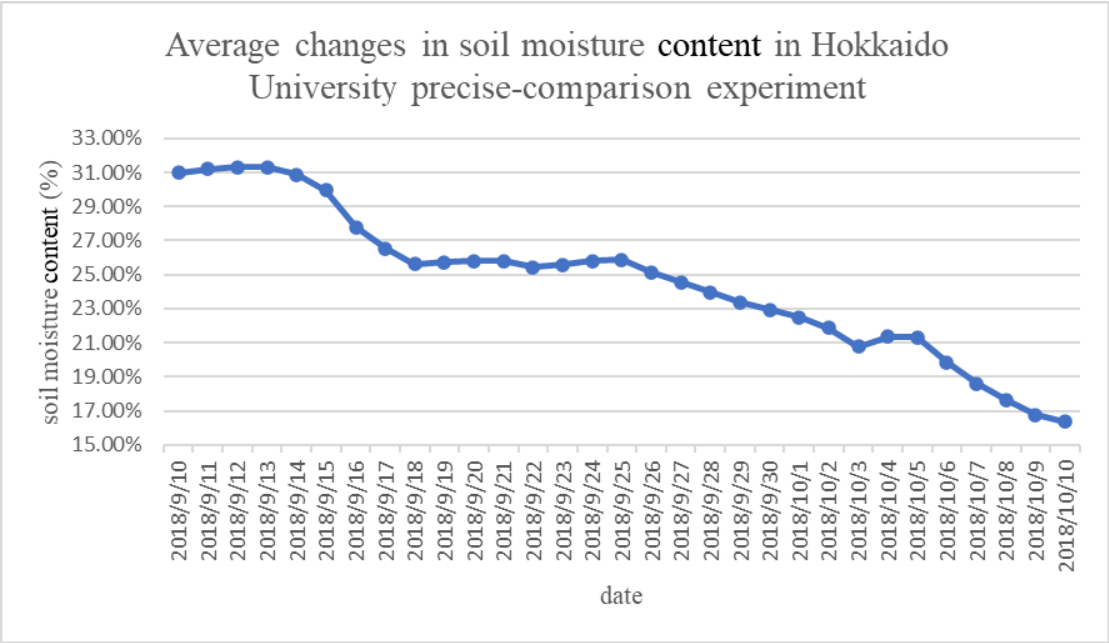
‡Least squares means with the same letter are not significantly different ($p < 0.05$).

Supplemental Table 2-S5. Photosynthetic rate (Pn) of each *Miscanthus* genotype under each soil water content level in a precise-comparison experiment at Hokkaido University, Sapporo, Japan

		Pn (μmol $CO_2 \bullet m^{-2} \bullet s^{-1}$) in each soil water content level			
Species	Accession	30%	25%	20%	15%
M. sinensis	PMS-007	13.608	9.684	7.475	0.258
M. sinensis	PMS-285	15.008	7.761	7.457	-0.158
M. sacchariflorus	UI10-00008	13.844	1.160	2.281	0.308
M. sinensis	UI10-00020	24.656	12.798	11.650	2.947
M. sinensis	PMS-164	13.528	4.497	4.513	-1.812
M. sinensis	UI10-00024	17.117	10.304	9.341	1.526
M. sinensis	PMS-347	11.377	5.666	4.212	0.352
M. sinensis var. condensatus	UI10-00015	9.672	7.493	5.768	-0.054
M. sacchariflorus	JPN-2011-004	12.280	7.737	8.077	0.684
M. sacchariflorus	UI11-00033	13.692	7.433	10.404	0.681



Supplemental Figure 2-S1 Simplified diagram showing various parts of the irrigation system, including the (1) water resource; (2) solenoid valve; (3) woodpecker emitter; (4) drip emitter; (5) soil moisture sensor; (6) thermocouple multiplexer; (7) connecting wires between CR6 datalogger and multiplexer; (8) CR6 datalogger; (9) power supply to CR6 datalogger; (10) connecting wires between CR6 datalogger and SDM 16 AC/DC relay controller; (11) SDM-CD16AC 16-Channel AC/DC relay controller; (12) power supply to solenoid valve; (13) power supply to SDM 16 AC/DC relay controller; (14) PS150 battery; and (15) main power supply. Only one pot is shown in detail (in the system, 30 pots can be independently irrigated).



Supplemental Figure 2-S2 Average changes in soil moisture content controlled by the automated irrigation system in a precise-comparison experiment at Hokkaido University, Sapporo, Japan.

Summary

Global warming is disrupting weather patterns, leading to extreme weather events, unpredictable water availability, exacerbating water scarcity. As a result, water scarcity has become a serious problem for agriculture production with reducing crop yield due to increasing drought in the field. Managing limited water resource becomes a pressing issue to human. On the other hand, reduction of greenhouse gas emission is necessary to stop global warming. Renewable energy to replace fossil fuels is urgent issue. Therefore, developing renewable bioenergy is necessary. To avoid conflict of crops between food and energy, the second-generation bioenergy, producing energy by non-food lignocellulose crops, seems to be a better option for bioenergy resources.

Miscanthus, a C_4 perennial rhizomatous grass, has high biomass productivity in marginal lands and expresses high CO_2 fixation in low-temperature conditions, underscoring its potential as a bioenergy crop in cool temperate environments. A single sterile triploid clone of *Miscanthus* ×*giganteus* has been adapted for commercial biomass production in Europe and North America, with average 28.7ton biomass production per ha. However, drought may restrain productivity of *Miscanthus*. Although *Miscanthus* exhibited higher light-use efficiency and above-ground biomass relative to maize and switchgrass under drought environment, as a potential energy crop, selection needs to be made of drought-tolerant *Miscanthus* cultivars, which have particularly robust production in marginal agriculture land. Therefore, selecting for

accessions and developing drought-tolerant *Miscanthus* cultivars are essential to increase the versatility of *Miscanthus* as a sustainable bioenergy crop.

On the other hand, intergeneric hybrids of *Saccharum* and *Miscanthus*, which often named as miscanes, are also considered as a high potential cellulosic bioenergy crop due to their high biomass potential with thick stems. Based on combining key traits from its parents such as high biomass productivity from sugarcane and chilling and drought tolerance from *Miscanthus*, miscanes are considered as a bioenergy crop especially under warm temperate or subtropical regions. Therefore, miscanes could be expected as a valuable lignocellulosic biomass crop and a source of genes to confer drought tolerance from *Miscanthus* to sugarcane.

The aim of the present study is 1) to screen *Miscanthus* accessions for drought tolerance with the express purpose of identifying superior genotypes to use as future breeding-stock material, and 2) to evaluate drought tolerance and drought-associated gene expression of miscane genotypes and their sugarcane and *Miscanthus* parents genotypes.

In chapter 2, a total of 29 *Miscanthus* accessions of East-Asian origin were screened for drought tolerance with two methods, a dry-down treatment at two locations (Hokkaido University, Sapporo and Brigham Young University, Utah, USA) and a system where soil moisture content (SMC) was maintained at fixed levels using an automatic irrigation system in one location (Sapporo). One genotype, *Miscanthus sinensis* “PMS-285”, showed relatively high drought-tolerance capacity under moderate drought stress. *M. sinensis* “PMS-285”, aligned

with the *M. sinensis* 'Yangtze-Qinling' genetic cluster, had relatively high principal component analysis ranking values at both two locations. Genotypes derived from the 'Yangtze-Qinling' genetic cluster showed relatively greater photosynthetic performance than other genetic clusters, suggesting germplasm from this group could be a potential source of drought-tolerant plant material. Diploid genotypes also showed stronger drought tolerance than tetraploid genotypes, suggesting ploidy could be an influential factor for this trait.

In chapter 3, three miscane genotypes, derived from hybridizations between one sugarcane genotype and one *Miscanthus* genotype were evaluated for their drought tolerance and gene expression of 3 drought-associated genes, which were *Hydroxyacid oxidase 1* (*HAO1*), one stress-responsive NAC genes of *Miscanthus lutarioriparius* (*MINAC12*), one mitogen-activated protein kinase gene of rice (*OsMAPK5*). In greenhouse experiments, drought stress index (DSI) of all 3 genotypes of miscane were higher than the sugarcane parent after 7 or 8 days of drought. One genotype, miscane "JM 14-60", showed high photosynthesis rate and high DSI under drought environment, which is similar to its *Miscanthus* parent genotype. Drought-associated gene expression experiment revealed that *Miscanthus sacchariflorus* "Miyakonojo" and miscane "JM 14-60" genotype, *MINAC12* and *OsMAPK5* expressed higher level in drought relative to control condition. As a result, miscane "JM 14-60" showed relatively high drought-tolerance capacity and it is considered as a superior genotype for introgression breeding programs.

The present study assessed drought tolerance in *Miscanthus* spp. and *Saccharum* x *Miscanthus* intergeneric hybrids (miscanes). The drought tolerant genotypes *M. sinensis* "PMS-285" and miscane "JM 14-60" could be valuable for elucidation of drought stress mechanism and improving drought tolerance in *Miscanthus* spp and a source of genes to confer drought tolerance from *Miscanthus* to sugarcane in the further studies.

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