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# Ploidy level and reproductive organ abnormality in interspecific hybrids between tetraploid *Miscanthus sacchariflorus* and diploid *M. sinensis* bred from a single cross

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ABSTRACT

*M.* × *giganteus,* a hybrid of tetraploid *M. sacchariflorus* and diploid *M. sinensis,* is a good bioenergy crop because of its high biomass yield and non-invasiveness in non-native countries. However, this species has a single cultivar that was derived from the same clone, and would, therefore, have higher susceptibility to biotic and abiotic stress. To breed *M.* × *giganteus* cultivars, we developed an interspecific crossing method and assessed the breeding efficiency of this method. From the hybrid seeds of 14 crossing combinations, we obtained 323 young plants, of which 290 plants became necrotized during the seedling stage. Of the surviving 33 plants, 12 were assumed to be triploid based on their stomatal length, relative fluorescence intensity values, rhizome elongation, and growth habit, which were similar to those of 'legacy *M.* × *giganteus*' but were different from *M. sacchariflorus* and *M. sinensis*. In the first planting year, four plants headed during the first 10 days of October. Pollen grain size in some hybrid plants was not different from that in the fertile, diploid *M. sinensis*. The percentage of abnormal pollen and the coefficient of variation of pollen size in all hybrid plants and legacy *M.* × *giganteus* were different from those in tetraploid *M. sacchariflorus* and diploid *M. sinensis*. At 10 days after flowering, ovules were viable in diploid *M. sinensis* and tetraploid *M. sacchariflorus* but aborted in the interspecific hybrid and legacy *M.* × *giganteus*. Owing to its sterility, the newly bred *Miscanthus* interspecific hybrid has low invasion potential, posing minimal threat to the native flora, and is, therefore, suitable for cultivation in non-native countries. These results can be applied to future *Miscanthus* breeding programs designed to secure the genetic diversity of *M.* × *giganteus* and to develop a range of *Miscanthus* cultivars that can produce high biomass yield under diverse biotic and/or abiotic conditions.

## 1. Introduction

Species of the genus *Miscanthus* (of which there are 14–17) are perennial $C_4$ plants, belonging to the subtribe Saccharinae Griseb., within the tribe Andropogoneae Dumort of Poaceae (Sun et al., 2010). Owing to their high biomass productivity, low fertilizer requirement, and relatively wide adaptability, including cold tolerance, *Miscanthus* species are considered promising bioenergy crops in temperate regions (Clifton-Brown et al., 2008; Tamura et al., 2016). Among these species, *M. sacchariflorus, M. sinensis*, and *M.* × *giganteus* are cultivated for biomass production. *M. sacchariflorus* and *M. sinensis* are native to East Asia, including Japan, Korea, and China (Moon et al., 2013), and *M.* × *giganteus* is an allotriploid ($2n = 3x = 57$) interspecific hybrid between the tetraploid *M. sacchariflorus* and the diploid *M. sinensis* (Moon et al., 2013). In 1935, the first clone of *M.* × *giganteus* was discovered in Japan by the Danish nurseryman Aksel Olsen and was subsequently introduced into Europe and northern America (Linde-Laursen, 1993). To distinguish clones of the same species with other genotypes, Głowacka et al. (2015) named the cultivar 'legacy *M.* × *giganteus*'; in this study, we used this nomenclature for convenience.

Typically, triploid plants have larger organs, greater biomass, and superior stress resistance when compared to diploid plants (Wang et al., 2016). Thus, legacy *M.* × *giganteus* is of particular interest as a bioenergy crop because it has high biomass yield despite low fertilizer requirement (Cadoux et al., 2014). However, legacy *M.* × *giganteus* is unable to produce high quantities of biomass under all climatic conditions because of its sensitivity to heavy frost and drought (Cosentino et al., 2007; Zub et al., 2012).

*Abbreviations:* WNWF, white non woven fabric; WRA, weed risk assessment; DAPI, 4′,6-diamidino-2-phenylindole

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Given that legacy *M.* × *giganteus* is a single cultivar that was derived from the same clone, it may have enhanced susceptibility to diseases and pests (Clifton-Brown et al., 2001; Głowacka et al., 2015; Prasifka et al., 2009). Although there are few reports regarding the damage from diseases and pests in the cultivation of legacy *M.* × *giganteus*, the susceptibility to pathogenic attack is expected to be increased in monocultures of *Miscanthus* that lack genetic diversity (Ahonsi et al., 2013). In this regard, Beccari et al. (2010) reported *Miscanthus* rhizome rot caused by *Fusarium avenaceum*, *Fusarium oxysporum*, and *Mucor hiemalis*, resulting in a failure to establish a *Miscanthus* experimental field in Italy.

Some genotypes of *M. sinensis* are known to be superior to legacy *M.* × *giganteus* in terms of tolerance to drought and cold temperature (Zub and Brancourt-Hulmel, 2010). Furthermore, *M. sinensis* can be established in the field with seeds using conventional sowing methods, whereas legacy *M.* × *giganteus*, which is a sterile allotriploid hybrid, can only be established via vegetative propagation using rhizome cuttings (Christian et al., 2005).

It is commonly acknowledged that plants native to one region can become invasive when established elsewhere. *Miscanthus* species are regarded invasive plants or weeds in non-native countries because their seeds can be transported to long distances by wind and can germinate and proliferate extensively given their excellent environmental adaptability (Raghu et al., 2006). Accordingly, several reports have expressed concern that potential bioenergy crops, such as *Miscanthus*, could escape production areas to become invasive species (Quinn et al., 2010). Warnings regarding the invasiveness of bioenergy crops are based on widely accepted weed risk assessment (WRA) protocols (Barney and DiTomaso, 2008). Such undesirable spread of non-native invasive crops into natural areas can, however, be reduced or eliminated by the use of triploids, because these plants tend to be sterile and seedless (Wang et al., 2016). Thus, legacy *M.* × *giganteus* was determined to be "acceptable" in a WRA performed in the United States because of its sterility under the test conditions. By contrast, *M. sinensis*, which is fertile and can produce large quantities of seed, "failed" and is listed as an invasive species in several US states (Barney and DiTomaso, 2008). Although *M. sacchariflorus* produces smaller quantities of seed compared to *M. sinensis*, Bonin et al. (2014) suggested that it should also be monitored comprehensively with regards to invasiveness because it has abundant and aggressively spreading rhizomes and can escape from cultivation. By virtue of its low invasive potential, legacy *M.* × *giganteus* is a major species grown for biomass production in many non-native countries that wish to protect their natural ecosystems from invasion by imported bioenergy crops (Moon et al., 2013).

The above-mentioned facts, thus, highlight the necessity to develop *Miscanthus* breeding programs to secure genetic diversity in cultivating *M.* × *giganteus* and to develop a range of *Miscanthus* cultivars that can produce high biomass yield under various biotic or abiotic conditions (Arnoult and Brancourt-Hulmel, 2015). Many studies in a number of countries have accordingly focused on generating new genotypes of *M.* × *giganteus*. In Korea and Japan, in which *Miscanthus* species are native, some workers have collected plants intermediate in morphology between *M. sacchariflorus* and *M. sinensis* and have identified these as putative triploids of *M.* × *giganteus* by flow cytometric analysis of the nuclear DNA content (Moon et al., 2013; Nishiwaki et al., 2011; Tamura et al., 2016). To date, however, there have been no reports of commercial cultivars derived from naturally growing *M.* × *giganteus*. To generate genetic diversity in *M.* × *giganteus*, Deuter poly-crossed one clone of tetraploid *M. sacchariflorus* with several clones of diploid *M. sinensis* in an isolated green house, and developed a new triploid *M.* × *giganteus* genotype called 'Nagara' that is genetically distinct from the 'legacy *M.* × *giganteus*' clones (Deuter, 2009; Dierking et al., 2016).

Of the various crossing methods in plant breeding programs, single crosses can create hybrids that have higher yield than three-way, double-crossed, or poly-crossed hybrids because they can combine mother plant and pollen donor with breeder's intention in the breeding program, although the financial costs entailed in performing the cross tend to be higher because of the preliminary steps involving creation of a crossing garden and investigation of flowering date and growth characteristics (Nielson et al., 1970). Accordingly, when seeking to produce a new genotype of triploid *M.* × *giganteus* by crossing of tetraploid *M. sacchariflorus* and diploid *M. sinensis*, we anticipate that single-crossed hybrids will be superior to poly-crossed hybrids in various growth characteristics including biomass yield.

Generally, counting chromosome number is the most exact method for examining the ploidy levels of plants. Measuring nuclear DNA content by flow cytometry analysis of propidium iodide-stained nuclei has been used to examine genome size and to estimate the ploidy level of plants (Rayburn et al., 2009); however, counting chromosome number is a time- and labor-consuming procedure for examining polyploidy in plants. Measuring nuclear DNA content also demands careful standardization of tissue samples and fixation, reaction, and flow-cytometry conditions (Price, 1988). Moon et al. (2013) described a method for estimating the ploidy level of *Miscanthus* species by measuring the relative fluorescence intensity of 4′,6′-diamidino-2-phenylindole (DAPI)-stained nuclei with flow cytometry and stomatal length in *Miscanthus* species.

With the aim of breeding new *M.* × *giganteus* cultivars, we generated interspecific hybrids between tetraploid *M. sacchariflorus* and *M. sinensis* using a new single crossing method and describe the ploidy level and reproductive organ abnormalities in interspecific *Miscanthus* hybrids developed using the new crossing method.

## 2. Material and methods

### 2.1. Plant materials for interspecific single crossing

We collected approximately 1000 accessions of *Miscanthus* germplasm from across the Korean Peninsula and planted them in a preservation garden located in Cheonggye, Muan, Jeonnam, South Korea (34.97°N, 126.45°E). To prevent the shuffling of germplasm by tangling of the rhizomes of different accessions, each accession was planted in a bottomless PVC container of dimensions 102 × 72 × 35 cm (length × width × height).

**Table 1**
Ploidy level and growth characteristics of the accessions of *Miscanthus* species used for interspecific crossing. Ploidy level and growth characteristics were evaluated in the previous year.

| Accession | Species | Utilization | Ploidy level | Stem length (cm) | Stem diameter (mm) | Heading date |
|---|---|---|---|---|---|---|
| BM 00240 | *M. sacchariflorus* | Mother plant | 4*X* | 359 | 8.5 | Sep. 23 |
| BM 00242 | " | " | " | 350 | 8.4 | Sep. 15 |
| BM 00677 | *M. sinensis* | Pollen donor | 2*X* | 220 | 7.8 | Sep. 23 |
| BM 00720 | " | " | " | 256 | 8.7 | Sep. 23 |
| BM 00726 | " | " | " | 223 | 8.9 | Sep. 23 |
| BM 00734 | " | " | " | 262 | 7.7 | Sep. 23 |
| BM 00741 | " | " | " | 241 | 7.7 | Sep. 23 |
| BM 00803 | " | " | " | 272 | 8.9 | Sep. 15 |
| BM 00810 | " | " | " | 206 | 8.3 | Sep. 23 |