Analysis of the genitor origin of an intergeneric hybrid clone between 
Zea and Tripsacum for forage production by McGISH

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In this study, the chromosome number and composition of a novel perennial forage crop, ‘Yucao No. 6’ (Yu6), was revealed by chromosome spread and McGISH (multicolor genomic in situ hybridization) techniques to clarify its genitor origin. Cytogenetic analysis showed that Yu6, which has 56 chromosomes, is an aneuploid representing 12, 17 and 27 chromosomes from Zea mays ssp. mays L. (Zm, 2n = 2x = 20), Tripsacum dactyloides L. (Td, 2n = 4x = 72), and Z. perennis (Hitchc.) Reeves & Mangelsd. (Zp, 2n = 4x = 40), respectively. This finding indicates that Yu6 is the product of a reduced egg (n = 36 = 12Zm + 17Td + 7Zp) of MTP (a near-allohexaploid hybrid, 2n = 74 = 20Zm + 34Td + 20Zp) fertilized by a haploid sperm nucleus (n = 20Zp) of Z. perennis. Moreover, 3 translocated chromosomes consisting of the maize-genome chromosome with the segment of Z. perennis were observed. These results suggest that it is practical to develop perennial forage maize by remodeling the chromosomal architecture of MTP offspring with Z. perennis as a pollen parent. Finally, the overview of forage breeding in the Zea and Tripsacum genera was discussed.

Key Words: Zea, Tripsacum, perennial forage maize, McGISH, forage breeding.

Introduction
The novel forage maize cultivar, ‘Yucao No. 6’ (Yu6), is an intergeneric hybrid clone that has perenniality, multiple tillers, and high-biomass yield ability. The biomass productivity of Yu6 under three different densities (0.83, 1.0, 1.25 plants m–2) was evaluated in a field trial conducted at Chongzhou, Chengdu, China, during 2017 and 2018. The field data revealed that Yu6 can produce a dry matter yield of 14.44–22.40 Mg ha–1 (unpublished data), encouraging the exploitation of Yu6 as a very promising crop for fodder production. In 2018, Yu6 was approved as a forage crop in Sichuan Province, China. Yu6 can be vegetatively propagated via its aerial stems or mother stands. Its seed parent is a near-allohexaploid hybrid (called MTP, see Iqbal et al. 2019) involving maize (Zea mays ssp. mays L.), eastern gamagrass (Tripsacum dactyloides L.) and perennial tetraploid teosinte [Z. perennis (Hitchc.) Reeves & Mangelsd.]. MTP was created by successively undergoing autopolyploidization and allopolyploidization (Fig. 1A). However, the pollen parent of Yu6 is unclear. Owing to MTP’s infertile pollen, we suspect that its male parent may be a maize or perennial tetraploid teosinte plant planted adjacent to the mother MTP plant (Fig. 1B).

Multicolor genomic in situ hybridization (McGISH) is a powerful and effective tool for visualizing genomic constitution by hybridizing chromosomes on a microslide with a mixture of genomic DNA labeled by different fluorophores (Chester et al. 2010). McGISH can clearly discriminate the genomic constitution within a complex of Zea and Tripsacum genera. For example, McGISH was successfully used to confirm the hybrid status and chromosome translocation in the maize-Z. perennis hybrids (Cheng et al. 2016, Iqbal et al. 2018) and the maize-T. dactyloides-Z. perennis hybrids (Iqbal et al. 2019, Li et al. 2015). When MTP was backcrossed with maize, MTP showed its reproductive versatility by McGISH analysis, namely, 2n, 2n + n, n + n, and an irregular type (Iqbal et al. 2019). In the 2n type, one
The development of the polyploid marked in blue was mentioned by Kindiger et al. (1996) (A), and the allotetraploids marked in bold were generated in our laboratory (B). First, tetraploid maize was obtained by chromosome doubling. Second, an allotetraploid (maize-Tripsacum) was generated from crossing tetraploid maize with tetraploid Tripsacum dactyloides via an n + n mating and embryo rescuing. Next, MTP, which has 74 chromosomes with two missing Tripsacum chromosomes, was created from crossing a maize-Tripsacum plant with the perennial tetraploid teosinte (Z. perennis) by a 2n + n mating (Iqbal et al. 2019). Thereafter, among the progeny from MTP under open-pollination conditions (Z. mays or Z. perennis), one clone (Yu6) was outstanding and was approved as a forage crop. However, its genitor origin, reproductive mode and chromosome constitution are still uncertain.

Materials and Methods

Root tip collection

Seeding of Yu6 were planted in plastic pots with a diameter of 15 cm and a height of 20 cm in March 2017. Before adding soil to the plastic pots, plastic film (0.12 mm thick, 75 cm long and 15 cm wide) with several holes (1.5 cm diameter) in its central area was placed across the inner surface of each pot, with both ends of the plastic film left outside the pot. Four weeks after planting, the ends of the plastic film were pulled and lifted together with the plant out of the pot to expose the root tips of the soil surface on a sunny morning between 9:00–11:00 a.m. After obtaining the root tips that resembled the primary root from the germinating seeds, the plants were put back into the pot and were well watered. The steps by which the root tips were collected were repeated once every three days until the end of June.

Chromosome preparation

The fresh root tips obtained were pretreated in saturated α-bromonaphthalene for 3 h. The root tips were then fixed in freshly prepared Carnoy’s fixative (3:1 methanol:glacial acetic acid) at room temperature for 24 h and subsequently transferred to 70% ethanol for storage at 4°C. After washing the fixative solution with distilled water, the root meristems were transferred to a 1.5 ml Eppendorf tube that contained 20 μl of mixed enzyme solution [6% cellulase Onozuka R10 (Yakult):1% pectinase (Yakult), v/v = 1:1] per root tip in a water bath at 37°C for approximately 3 h.

The slide preparation for the McGISH procedure followed a flame drying protocol after the enzyme solution was washed with distilled water. The root tips were transferred to a microslide, and a drop of Carnoy’s fixative was added to the root tip. The slide was then dried over an alcohol flame. The slides featuring well-spread metaphase cells were selected under an Olympus BX41 microscope and stored at –20°C until the McGISH procedure was employed.

McGISH presentation

The total genomic DNA of Z. mays (inbred line Mo17) and Z. perennis (CIMMYT accession No. 9475) were isolated from young leaves using a modified 2× CTAB method (Fu et al. 2015), and then labeled with probes with a DIG-High Prime Kit and a Biotin Nick Translation Kit (Roche, Basel, Switzerland), respectively, according to the manufacturer’s instructions. The McGISH technique was performed according to the methods of Iqbal et al. (2019). The McGISH images were visualized via a phase video microscope (Olympus BX61, Tokyo, Japan) equipped with a charge-coupled-device camera (700 mm CCD) and via Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, USA).

Results

Of the 55 cells observed, 47 showed a mitotic chromosome number of 2n = 56 (Fig. 2B) by conventional staining in Yu6 (Fig. 2A), and the remaining cells contained 36–55 chromosomes. Its genomic origin was further revealed by fluorescence imaging to determine the genomic identity of its chromosomes as well as possible intergenomic structural changes. Slides featuring well-spread metaphase cells were selected for McGISH presentation, but only cells with 56 chromosomes were used for the recognition of the genomic source. Via the McGISH technique, three chromosome subsets were detected. The maize-, eastern gamagrass-, and tetraploid teosinte-genome chromosomes had green, blue, and red fluorescence (Fig. 2C), respectively. Apparently,
paternity testing of a Zea-Tripsacum perennial forage by McGISH

Fig. 2. Plant (A) of Yu6, chromosome spread (B) and McGISH pattern (C) of its root tip cells at mitotic metaphase. The green, blue and red colors represent Z. mays, T. dactyloides, and Z. perennis chromosomes, respectively. The arrows indicate translocated chromosomes between Z. mays and Z. perennis.

Yu6 contained 12, 17 and 27 chromosomes of the maize, eastern gamagrass and teosinte genomes, respectively. Notably, Yu6 contained three translocated chromosomes consisting of the maize-genome chromosome with the segment of Z. perennis. No translocated chromosomes between eastern gamagrass and Zea were observed by McGISH.

Discussion

Interspecific hybridization has been conducted in Zea L. and Tripsacum L. mainly for introgressing valuable genes from teosinte or T. dactyloides into maize. Teosinte from the genus Zea comprises eight species distributed in Mexico and Central America, i.e., two perennials (Z. diploperennis Ilitis, Doebley & Guzman and Z. perennis) and six annuals (Z. luxurians (Durieu & Asch.) Bird, Z. nicaaguensis Ilitis & Benz, Z. vespertilio Gómez-Laur, Z. mays ssp. mexicana (Schrad.) Ilitis, Z. mays ssp. parviglumis, and Z. mays ssp. huehuetenangensis (Ilitis & Doebley) Doebley) (Warburton et al. 2017). In addition to the only tetraploid species, Z. perennis (2n = 4x = 40), the others are diploid species (2n = 2x = 20). Recently, three new annual diploid species were discovered in Mexico (Sánchez et al. 2011). As a valued genetic resource for maize breeding, teosinte has been extensively studied and applied (Warburton et al. 2017). The genus Tripsacum, the closest relative to Zea, includes 16 species of warm-season, perennial, bunch-type grasses that are present throughout much of eastern Mesoamerica (Bidlack et al. 1999). One species of Tripsacum that has been proven valuable as a genetic resource for maize improvement is T. dactyloides (eastern gamagrass), originating from Mexico and the southern states of the USA (Coblentz et al. 1998). Hereinafter, we focused on the forage breeding of Yu6 and its relatives (i.e., teosinte and eastern gamagrass).

Previously, in the reported literature, teosinte species (especially Z. mexicana) have been largely mentioned as a forage crop in their native area (Collins 1921, Iltis and Benz 2000). Tolerance to various conditions ranging from moderate drought, heat stress, temporary flooding, and insect and pathogen attack have provided an increased interest in using teosinte for forage purposes (Niazi et al. 2015a). Moreover, teosinte has recently been used as a forage crop in nonnative countries, such as China (Wang et al. 2005 and India (Kumar et al. 2016). Screening superior cultivars from wild populations is the most common route due to the abundant genetic and phenotypic diversity among the wild resources. To our knowledge, interspecific hybridization between teosintes for improving teosinte has rarely been reported. Many reports have shown that the hybrids between maize and teosinte exhibiting biomass heterosis are accompanied by a reduction in fertility (Niazi et al. 2015b, Wang et al. 2012a) and that hybrid vigor severely declines in subsequently generations (Wang et al. 2012b). Moreover, the F1s have a higher fodder-crop quality than teosinte (Z. mexicana) and a longer stay-green period than maize (Niazi et al. 2015b). However, the cross incompatibility of maize and teosinte leads to low seed set (Ellstrand et al. 2007, Lu et al. 2014), limiting utilization of the F1 heterosis. Therefore, improving the seed setting rate is key to the application of maize × teosinte hybrids. In one case, a maize substitution line with three Z. perennis chromosomes was developed (Tang et al. 2005). Thereafter, interspecific hybridization between the substitution line and Z. perennis, which resulted in improved seed production, was implemented for the production of a new forage variety (‘Yucao No. 1’) that produces relatively high biomass and exhibits relatively high forage quality (Ren et al. 2007). The induction of Z. mays × Z. mexicana hybrid tetraploids represents another effective method for reducing heterosis breakdown and restoring reproductive fertility for the use of intersubgenomic heterosis for forage purposes (Niazi et al. 2015b).

However, annual forage production systems that aim for high yield and quality, as well as frequent soil disturbance, can result in considerable soil erosion on marginal and sloping croplands. Thus, owing to its high production potential, high feeding quality, and abundant leaf tissue, eastern gamagrass, a perennial species, has been recommended as an alternative to annual forage for fodder production and summer grazing on erosive croplands (Brejda et al. 1994, 1997, Coblentz et al. 1998). Its diverse ploidy level (2x, 3x, 4x, 5x and 6x) and reproductive modes (2n,
2n+n, and n+n) provide various avenues for creating genetic variation for improving eastern gamagrass (Kindiger and Dewald 1994, 1997). As such, there are four reported tactics to breed eastern gamagrass: selecting superior-fodder varieties from wild germplasm sources (Grabowski et al. 2005), selecting superior-fodder varieties from intraspecific crosses (Bidlack et al. 1999, Dewald and Kindiger 1996, Springer et al. 2006), selecting superior-fodder varieties from interspecific crosses (Eubanks et al. 2013), and the use of chromosome doubling (Salon and Pardee 1996). In particular, crossing eastern gamagrass with all other Tripsacum species is possible (Kindiger and Dewald 1997). With respect to the introgression breeding strategy, Eubanks et al. (2013) reported that two eastern gamagrass cultivars with high sugar contents were generated by introgressing Zea alleles from an eastern gamagrass-Z. diploperennis hybrid into eastern gamagrass.

In this study, conventional staining showed that a 2n = 56 chromosome number was the most frequent (85%), indicating that Yu6 is mitotically stable and characterized by a 2n chromosome number of 56. Because of cell disruption during chromosome preparation and overlapping chromosomes, not every cell displayed the somatic chromosome number. Owing to the reproductive versatility of the seed parent MTP (Iqbal et al. 2019), it is impossible to identify the pollen parent of Yu6 by the chromosome number. Slides featuring well-spread metaphase cells were subsequently selected for McGISH presentation, but only cells with 56 chromosomes were used for the recognition of the genomic source. The McGISH experiments distinguished each parental chromosome set in Yu6. Given the pollen abortion and 20Zp in the mther MTP, 27Zp in Yu6 indicates that the pollen parent of Yu6 is Z. perennis. Without apomictic reproduction, the hexaploid set of chromosomes in MTP would segregate into a variety of aneuploid eggs (Iqbal et al. 2019). This finding obviously suggests that Yu6 is the product of a reduced egg (n = 36 = 12Zm + 17Td + 7Zp) of MTP (2n = 74 = 20Zm + 34Td + 20Zp) fertilized by a haploid sperm nucleus (n = 20Zp) from Z. perennis. In addition to the genitor confirmation, McGISH again confirmed the occurrence of chromosome translocation between maize and Z. perennis. This is not a surprising result owing to allosyndesis between the chromosomes of maize and Z. perennis in MTP (Iqbal et al. 2019). Such chromosomal translocation between the maize chromosomes and those of Z. perennis has occurred in the previous maize × Z. perennis hybrids (Cheng et al. 2016, Iqbal et al. 2018).

Here, we provided an additional method to breed superior forage maize cultivars that had previously not been realized. MTP is a useful genetic bridge for augmenting maize diversity (Iqbal et al. 2019), and its use as a seed parent has also resulted in the release of a new clone cultivar [now called ‘Yucao No. 5’ (Yu5)] from crosses with Z. perennis (Li et al. 2015). Cytologically, the perennial forage maize cultivar Yu6 is also the result of an n+n mating generated from an MTP × Z. perennis cross. Yu6 outperforms its immediate (MTP) and ancestral parent species (Z. mays, T. dactyloides and Z. perennis) in terms of biomass production due to hybridization and allopolyploidization. The average fresh yield of Yu6 (12.86 kg plant⁻¹) was 35% higher than that of MTP (9.52 kg plant⁻¹) under 1.2 m × 1.5 m plant spacing at 130 days after planting at Wenjiang, Chengdu, China, in 2019 (unpublished data). Although trigenomic hybrids are ineffective at producing seed owing to pollen abortion, the asexuality of eastern gamagrass and tetraploid teosinte imparts the ability of MTP and its offspring (e.g., Yu5 and Yu6) to immediately fix biomass heterosis by asexual means. It is likely that clonal selection involving less sexual activity in the breeding process signifies a shortened breeding cycle and reduced financial costs. Perennial forage maize should be considered a highly potential crop for forage production in Sichuan and possibly elsewhere in the near future. The new maize form will augment biodiversity in forage fields by diversifying the forage types. An extra benefit of perennial forage maize is that it allows smallholder farmers to make perfect copies of these cultivars and sell seedlings on their own as well as the lack of need to repurchase propagules every year. Moreover, extension of the vegetative growth period would lead to relatively high biomass accumulation and would make forage quality relatively more stable (Niazi et al. 2015b). Collectively, the findings herein indicate that it is effective and workable to develop vegetatively propagated forage via an MTP × Z. perennis crossing scheme involving hybridization and allopolyploidization.

Author Contribution Statement

QT, XY and MC generated the material. YZL and ZW performed the plantlet cultivation and root tip collection. YZL, YL, ZW, XW, XL, YZ, and CY contributed to the McGISH experiments and chromosome analysis. RH and PZ created the figures. XY wrote the manuscript. EKS and TR revised the language. QT and JH organized and participated in the work.

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