

## Note

# 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 The field data revealed that Yu6 can produce a dry matter yield of 14.44–22.40 Mg  $ha^{-1}$  (unpublished data), encouraging the exploitation of Yu6 as a very promising crop for fodder production.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

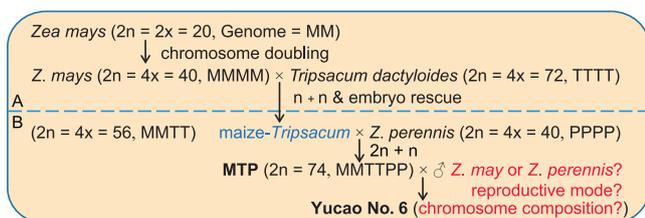
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**Fig. 1.** Genealogy of Yu6. The development of the polyploid marked in blue was mentioned by Kindiger *et al.* (1996) (A), and the allopolyploids marked in bold were generated in our laboratory (B). First, tetraploid maize was obtained by chromosome 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.

unreduced  $2n$  female gamete (having the somatic chromosome number) forms seed asexually. In the  $2n + n$  type, the  $2n$  female gamete will fuse with a reduced  $n$  male gamete (producing through a normal meiosis) to form a zygote. The irregular type was generated through the fusion of an  $n$  female gamete with an irregular male gamete. For these reasons, the aim of this study was to first identify the chromosome number and composition of Yu6 by chromosome counting and McGISH techniques, to further predict both the reproduction mode of MTP with respect to the generation of Yu6 and Yu6's genitor, and to finally review forage breeding within the genera *Zea* and *Tripsacum*.

## Materials and Methods

### Root tip collection

Seedling 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

$\alpha$ -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  $\mu$ l 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. After removing the enzyme solution with a Pasteur pipette and washing with distilled water twice (10 min each), the root tips were stained with a drop of magenta and squashed onto a slide with a coverslip. The chromosome number was observed by light microscopy (Olympus BX41, Tokyo, Japan), and at least 50 cells were identified. The chromosome number in more than 80% of the cells was the same value, and this value was the chromosome number of Yu6.

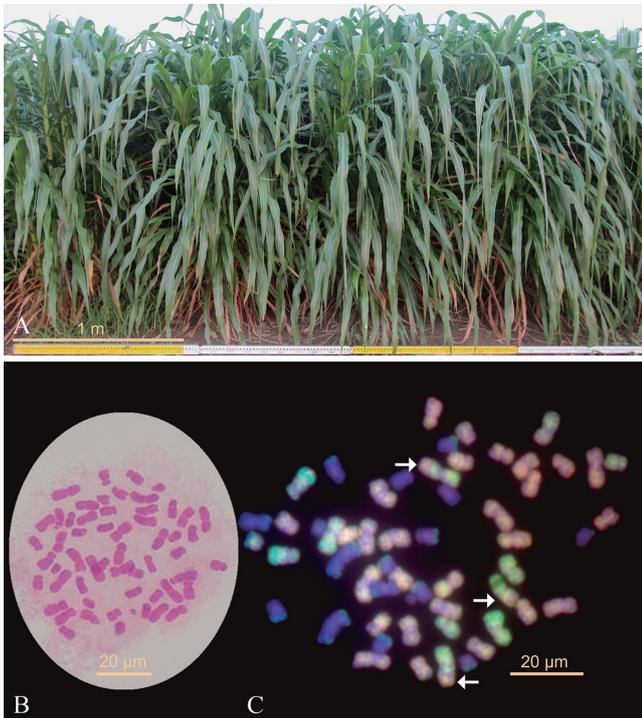
The slide preparation for the McGISH procedure followed a flame drying protocol after the enzyme solution was washed with distilled water. The root tips were transferred to a microslide, and a drop of Carnoy's fixative was added to the root tip. The slide was then dried over an alcohol flame. The slides 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  $2\times$  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,



**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* Iltis, Doebley & Guzmán and *Z. perennis*) and six annuals [*Z. luxurians* (Durieu & Asch.) Bird, *Z. nicaraguensis* Iltis & Benz, *Z. vespertilio* Gómez-Laur., *Z. mays* ssp. *mexicana* (Schrad.) Iltis, *Z. mays* ssp. *parviglumis* Iltis & Doebley, and *Z. mays* ssp. *huehuetenangensis* (Iltis & 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 taxa were discovered in Mexico, including an annual diploid, a perennial diploid, and a perennial tetraploid (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  $F_1$ s 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  $F_1$  heterosis. Therefore, improving the seed setting rate is key to the application of maize  $\times$  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 ('Yucaco No. 1') that produces relatively high biomass and exhibits relatively high forage quality (Ren *et al.* 2007). The induction of *Z. mays*  $\times$  *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, Coblenz *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 intra- and interspecific crosses (Bidlack *et al.* 1999, Dewald and Kindiger 1996, Springer *et al.* 2006), selecting superior-fodder varieties from intergeneric 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 intergeneric 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 straining 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 mother 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<sup>-1</sup>) was 35% higher than that of MTP (9.52 kg plant<sup>-1</sup>) under 1.2 m × 1.5 m plant spacing at 130 days after planting at Wenjiang, Chengdu, China, in 2019 (unpublished data). Although trigonomic 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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