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RESEARCH ARTICLE

Establishment of GISH and multi-color GISH techniques to simultaneously discriminate different genomes in *Erianthus procerus* x *Saccharum officinarum* introgressed clones

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Abstract
An attempt was made to identify the chromosomes from different species genomes in BC_2 progenies of *Erianthus procerus x Saccharum officinarum* through multi-color GISH (MCGISH). The BC_2 population was derived from the cross between a BC1 hybrid (GU 12-14) and a commercial variety, Co 16018. The BC2 progenies were studied for their somatic chromosome number and two cytotypes were observed, i.e., 2n=92 (2) and 2n=96 (5). GISH revealed that the majority of progenies were having nine *Erianthus* chromosomes, except 2 clones (25-5 and 25-10) were having nine *Erianthus* chromosomes with one chromosome fragment. The methodology for multi-color GISH was standardized and it distinguished the chromosomes from different genomes. Interspecific recombination was also detected in somatic cells of one BC_2 clone, 25-10. The hybridity of the introgressed lines has been confirmed with amplification of *Erianthus* specific tandem repeat sequences.

Keywords: *Saccharum*; *Erianthus*; Introgression; Mitosis; GISH; Multi-color GISH

Introduction

In sugarcane breeding programme, significant attention has been given to wild hybridization to introgress genes for desirable characters like vigor, profuse tillering, ratoonability, adaptability as well as resistance to biotic and abiotic stresses from the related species and genera of the "*Saccharum complex.*" *Erianthus* is one of the members of "*Saccharum complex*" having all these valuable traits and is regularly being used in sugarcane hybridization programmes. Out of seven species in *Erianthus,* two species *viz., E. arundinaceus and E. procerus* were successfully utilized in the development of introgressed lines in sugarcane (D'Hont et al. 1995; Ram et al. 2001; Cai et al. 2005; Fukuhara, et. al. 2012; Nair et al. 2017; Mohanraj et al. 2019). Back cross derivatives (BC_1 and BC_2) were later developed from these F_1 hybrids by using the hybrid clones as the female parents and commercial varieties as the male parents. All commercial varieties are hybrids between *S. officinarum* and *S. spontaneum.* As we heve used varieties for back crossing, there is a chance for occurrence of *S. spontaneum* genome in all the back cross derivatives. In sugarcane during backcrosses the problem of flowering and synchrony makes the breeder use different sugarcane clones for back crossing rather than using one of their parents and it is recognized as a modified backcross and resultant progenies are referred to as BC progenies.

The major constraint in utilization of *Erianthus* germplasm in sugarcane breeding arises from the difficulties in distinguishing genuine hybrids and self's and sterility caused in the hybrids from F_1 generation onwards. The recent developments of efficient molecular tools in the identification of intergeneric hybrids like 5Sr DNA sequences (D'Hont et al. 1995). Simple Sequence Repeats (SSRs) (Cai et al. 2005) and AFLP (Aikten et al. 2007) have been utilized to develop introgressed lines from *Erianthus* germplasm.

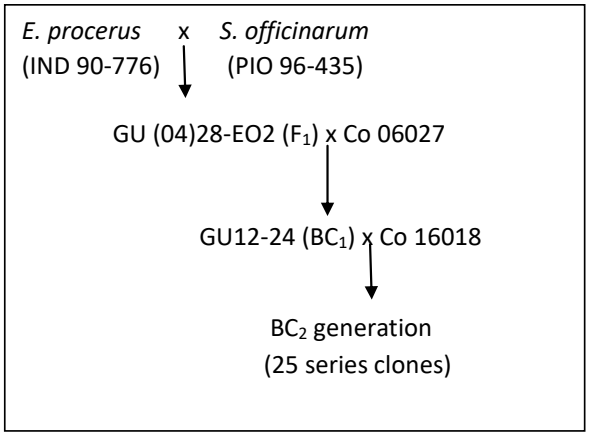
Though normal mitotic studies help to understand the chromosome behavior in intergeneric hybrids such as chromosome elimination and chromosome transmission pattern from the parental clones, it is necessary to understand the genome constitution of the hybrid for its utilization in further breeding programs. Genomic *in situ* hybridization (GISH) offers a relatively efficient way to visualize the genome constitution of a hybrid at the level of the whole chromosome or chromosome segment. GISH was shown to be an effective method for analyzing the genome constitution of the intergeneric hybrids involving *Saccharum* x *Erianthus* (D'Hont et al. 1995; Piperidis et al. 2000; Piperidis et al. 2010; Wu et al. 2014; Huang et al. 2015).

A special method called multi-color GISH (MCGISH) or simultaneous multiple target-sequence hybridization has been developed and has been used in many crops (Li et al. 2001 a, b). In this approach, alien chromosomes in the Oryza complexes were painted by the simultaneous hybridization of three probes with an unlabelled competitor. This technique enabled the simultaneous discrimination of parental genomes in allotetraploid *Triticum* spp. and *Agropyron* spp. and the painting of an extra chromosome from *T. tauschii* (Martin et al. 1999). This method has been successfully implemented in *Gossypium* (Guan et al. 2008) and in *Lilium* hybrids (Xie et al. 2010) to simultaneously identify three genomes in its hybrids. The multi-color genomic in situ hybridization was also performed for mitotic cells of the somatic hybrids of *Diospyros kaki* and *D. glandulose* to identify parental chromosomes (Choi et al. 2002). In sugarcane MCGISH has not yet been reported as it is a challenging technique to do with small and numerous chromosomes which shows a high degree of homology among different species.

In this article, we analysed the back cross progenies of intergeneric crosses involving *E. procerus* and *Saccharum.* We established the multi-color GISH technique in sugarcane introgressed lines to simultaneously distinguish different genomes. The hybridity of the clones was confirmed with the amplification of *Erianthus* specific sequences and the confirmed hybrids were subjected to mitotic analysis to determine their somatic chromosome number. Though GISH analysis determined the *E. procerus* chromosomes in the back cross population, MCGISH discriminate *E. procerus* and *S. spontaneum* genomes in the hybrids. A part of the total genome also contained the recombinants and other species genomes of *Saccharum.*

Materials and Methods

The plant materials used for the study included seven progenies of back cross derivatives (BC_2 generation) derived from intergeneric hybrid involving *E. procerus* (female parent) and *S. officinarum* (male parent). The details of the parentage of BC_2 progenies used in the study are described below.



In order to confirm the hybridity of the randomly selected BC_2 progenies they were screened using *Erianthus* specific primer, *Erianthus* specific Tandem repeat (ESTR) sequences (Yang et al. 2019). The amplified products were resolved in 2% agarose gel stained with ethidium bromide and the picture was documented using a gel documentation system.

The somatic chromosome number for *E. procerus* (female parent), *S. officinarum* (male parent), F_1, BC_1 and seven BC_2 clones was determined by the root tip squash technique described earlier (Sobhakumari and Asmita, 2014). For each clone, 10-15 well spread metaphase chromosome preparations were used to count the chromosomes using Carton Microsystem-CM 402T digital microscope. Photographs were taken using TS view software.

Mitotic chromosome preparations of BC_2 progenies for GISH analysis were performed as described by Sobhakumari et al. (2020). The frozen dehydrated slides were stored in moisture free slide box at room temperature. For GISH and MCGISH analysis the genomic DNA of *E. procerus* and *S. spontaneum* was isolated using CTAB extraction method (Paterson et al. 1993) and labeled with biotin-16 dUTP and digoxygenin-11 dUTP (Thermo Scientific, USA) respectively. The methodology followed for GISH analysis was as described previously by Sobhakumari et al. (2020). Genomic DNA of *E. procerus* labeled with biotin 11-duTP using random primed labeling method as described by the manufacturer (Thermo Scientific, USA) was used as probe. The hybridization mixture (30µl per slide) consisted of 50 ng of labeled probe *(Sorghum)*, 50% deionized formamide, 10% dextran sulphate, 0.5µg of sheared salmon sperm DNA in 20x SSC. This mixture was denatured by treating for 10 min in water bath at 100ºC and then cooled down quickly on ice. Hybridization was performed overnight in a moist chamber at 37ºC. Post hybridization washes were carried out in 2xSSC at 42ºC. Biotin labeled probe was then detected using avidin-FITC (Fluorescein isothiocyanate) by 1h incubation at 37ºC. Post detection washes were carried out in 2xSSC, 1% triton-x in 4xSSC followed by 2xSSC for 5 min each at 37ºC followed by 5 min incubation in 2xSSC at room temperature. Slides were mounted in Vectasheild (Vector labs,UK) mounting medium with DAPI (4,6-diamidino-2-phenylindole). The hybridization signals were observed on an AxioScope A1 Imager fluorescent microscope (Carl Zeiss, Gottingen, Germany). Images were captured digitally with an AxioCam 202 camera and processed with Zen 3.0 software (Carl Zeiss, Gottingen, Germany).

Multi-color GISH was performed in the GISH slides of one BC_2 clone (25-10) after the analysis of the first probing results. The hybridization mixture containing *S. spontaneum* probe was denatured for 10 min in a water bath at 100°C and then cooled quickly on ice to retain it as single stranded for hybridization. The immersion oil was blotted from the surface of the coverslip and the coverslip was carefully wiped with ethanol to remove any traces of oil from the slide. Wiped underneath of the slide also. The slides were incubated in 37°C for 10 min. to reduce the viscosity of the antifade mountant. With the edge of a razor blade the cover slip was removed by lifting slowly from the slide. Washed the slides in a coupling jar having 4XSSC with Tween 20 (0.1%) for 5 min followed by washes for two times for 1 hr each. Incubated the slides in 2XSSC for 2 times of 5 min each at room temperature. The slides were dehydrated in ice cold ethanol series and then air dried. These slides were used for reprobing with digoxigenin labelled *S. spontaneum* probe and detected with 20µg/ml rhodamine conjugated anti-digoxigenin. In situ hybridization steps were as in the first hybridization. After post detection washes, the slides were mounted in Vectasheild (Vector labs, UK) mounting medium with DAPI. Mitotic cells were analysed using AxioScope A1 Imager fluorescent microscope (Carl Zeiss, Gottingen, Germany) with appropriate filter sets. Images in different filter sets (blue, green & red) were captured with AxioCam 202 camera having Zen 3.0 software and merged using Adobe photoshop.

Results and Discussion

For the confirmation of the hybridity of BC_2 progenies PCR amplification was done using Erianthus specific 5S rDNA sequences as a preliminary evaluation. It was found that amplification was not obtained in some of the genuine hybrids of BC_2 progenies (data not shown). As 5S rDNA has one locus per set of basic chromosomes it is presents only on a few chromosomes in each genome. Due to unequal segregation of *Erianthus* chromosomes and its elimination at different stages, the advanced back cross progenies may not inherit the chromosomes that carry the 5S rDNA loci. Hence *Erianthus* specific 5S rDNA sequences may not be reliable for the identification of true hybrid progenies. The *Erianthus* specific tandem repeat sequences (ESTR) reported by Yang et al. (2019) was used as a marker to confirm the hybridity of the back cross progenies.

It was observed that the *Erianthus* specific bands (380bp and 760bp) were amplified in *E. procerus* (female parent) and all seven BC_2 clones (Fig. 1). In our study it was found that the ESTR sequences of *E. arundinaceus* is properly working in *E. procerus* and its BC_2 derivatives.

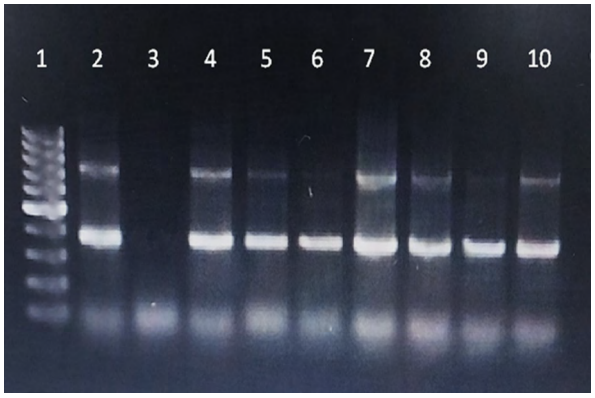


Figure 1. Electrophoretogram of BC2 progenies with its parents for amplification of ESTR primer in genomic DNA: 1) Marker 100bp ladder, 2) GU12-24, 3) Co 16018, 4) 25-2, 5) 24-4, 6) 25-5, 7) 25-8, 8) 25-10, 9) 25-12, 10) 25-13

The somatic chromosome number of female parent, *E. procerus* (IND 90-776), male parent *Saccharum officinarum* (PIO 94-435) and their F_1 (GU 04 (28) EO2) was 2n=40, 104 and 80 respectively (Sobhakumari et al. 2014). The BC_1 clone (GU 12-24) was derived from the cross F_1 (GU 04(28) EO2) x Co 06027 (2n=108) and its somatic chromosome number was determined as 2n=92. This BC_1 clone was crossed with a commercial variety Co 16018 and BC_2 progenies were derived. Randomly seven BC_2 progenies were selected for this study. The somatic chromosome number of these progenies was determined through root tip squash technique (Fig.2). Two different somatic chromosome numbers were observed among these progenies, five clones (25-2, 4, 10, 12 and 13) had 2n=92 and two clones (25-5 and 25-8) had 2n=96.

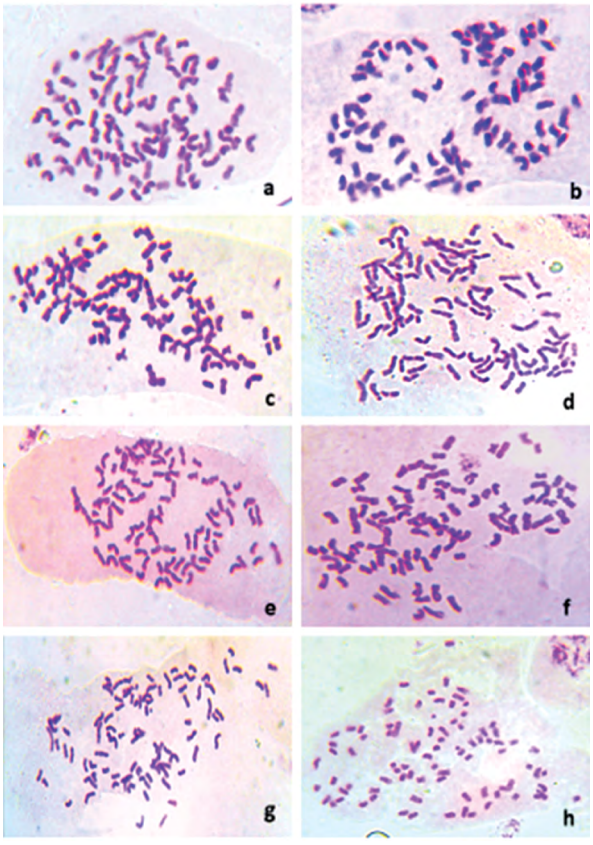


Figure 2. Somatic chromosome number of BC2 hybrids and BC1 parent: a) 25-2, b) 25-4, c) 25-5, d) 25-8, e) 25-10, f) 25-12, g) 25-13, h) GU 12-24

GISH analysis of F_1 hybrid from *E. procerus x S. officinarum* revealed 2n+n chromosome transmission with elimination of 12 chromosomes in our earlier report (Sobhakumari et al. 2020). In this study we analysed the introgression of *E. procerus* chromosomes in a BC_1 clone, GU 12-24, which was derived from the cross between F_1 hybrid and a commercial variety Co 06027. This clone was having 20 *E. procerus* chromosomes in its genome and this revealed n+n transmission in BC_1 generation. GU 12-24 was crossed with another commercial variety Co 16018 and BC_2 population was developed. Five BC_2 clones were analysed by GISH using *E. procerus* as the labelling probe. n+n chromosome transmission was observed in all the clones. GISH analysis revealed the presence of nine *E. procerus* chromosomes in five BC_2 clones. Out of these, two clones, 25-5 and 25-10 showed one *E. procerus* chromosome fragment in addition to nine *E. procerus* chromosomes (Fig.3). This was observed in all the cells of these clones which were analysed. While analysing the *E. procerus* introgression in three generations from *E. procerus* x *S. officinarum* cross we found that at each generation there is a reduction of *E. procerus* chromosomes by at least one half indicating that all *Erianthus* chromosomes will not be able to find a pairing companion at meiosis.

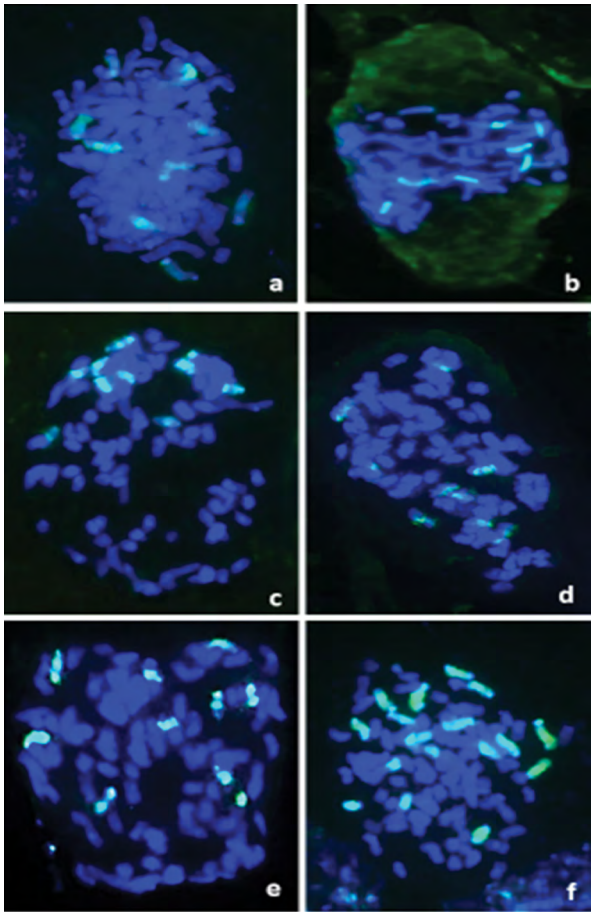


Figure 3. GISH analysis of BC_2 progenies of *E. procerus* x *S. officinarum* using *E. procerus* as the labeled probe. *E. procerus* chromosomes are shown in green color and *Saccharum* spp. chromosomes shown in blue. a) 25-2, b)25-4, c) 25-5, d) 25-8, e) 25-10, f) GU12-24

Among the species of *Erianthus* genus, *E. arundinaceus* has been utilized much more in the hybridization programmes of *Saccharum*. The GISH technique had been used to study the chromosomal structure, exchange, transmission pattern of chromosomes, alien chromosome introgression in different generations of hybrids between *Saccharum* x *E. arundinaceus.* n+n and 2n+n transmission at different generations have been reported (Wu et al. 2014; Huang et al. 2015; Piperidis et al. 2000 and 2010). This is for the first time the advanced back cross progenies of *E. procerus* x *S. officinarum* were subjected to GISH analysis. So far, the species like *E. arundinaceus* and *E. rockii* were used in breeding programmes of *Saccharum* and that too as the male parent. Intergeneric translocation was reported earlier in different cycles of *Saccharum* x *E. arundinaceus* (Wu et al. 2014; Huang et al.2015; Piperidis et al. 2010) whereas such translocations were not observed in F_1, BC_1 and BC_2 generations of *E. procerus* x *Saccharum.* Whole chromosome transmission through the conventional breeding method was observed in these hybrids. As the progressive elimination of *E. procerus* chromosomes was happening in

subsequent generations, it may need one or two more generations to get hybrids with minimum number of alien chromosomes in the *Saccharum* genome for further sorting and sequencing of *E. procerus* chromosomes.

In addition to GISH, an attempt has been made to differentiate *S. spontaneum* chromosomes from *E. procerus* chromosomes in a BC_2 clone 25-10. Reprobing of the GISH slide (which was already hybridized with biotin labeled *E. procerus* probe) with dig labeled *S. spontaneum* and its detection with anti-dig antibody showed the genome of *E. procerus, S. spontaneum* and other *Saccharum* species genome involved in this hybrid development (Fig.4). This clone was with 2n=92 and was having nine *E. procerus* chromosomes and a small fragment of *E. procerus* chromosomes. Multi color GISH determined the presence of nine *E. procerus* chromosomes with one chromosome fragment, 10 whole *S. spontaneum* chromosomes and 6 recombinant

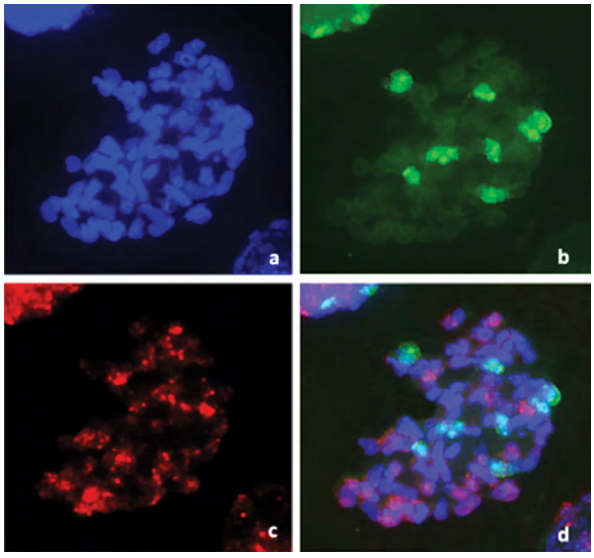


Figure 4. Multi-color Genomic in situ hybridization (MCGISH) simultaneously showed three genomes with a unique color in mitotic nuclei of *E. procerus* x *S. officinarum* BC_2 progeny, 25-10. a) Whole genome (DAPI) b) *E. procerus* chromosomes (FITC), c) *S. spontaneum* chromosomes (Rhodamine) d) Merged image simultaneously showing three genomes

chromosomes of *S. spontaneum*. The remaining part of *Saccharum* genome (67 chromosomes) counter stained with DAPI and it may include *S. officinarum* and other species genome involved in the development of commercial varieties.

Multi color GISH methodology for simultaneous analysis of the entire hybrid genome has improved the ability of researchers to recognise the chromosomal and genetic alterations on a genome wide scale. It is possible to rapidly identify the inter-chromosomal rearrangements and origin of unidentified extra chromosomal segments. With the inherent limitations of the multi color GISH /FISH technique this can be considered as a complementary system to the traditional cytogenetic tools. The contribution from cytogeneticists will continue to aid in the study of chromosomes, to answer difficult scientific questions about structural variations like substitution, translocation, localization, and distribution of alien chromosomes in the genomes of progenies from intergeneric hybrids and their role in genetic diversity, evolution, and origin, by using a combination of conventional cytogenetic techniques and advanced technologies including multi color GISH/FISH.

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