Cytogenetic and Molecular Approaches to Detect Alien Chromosome Introgression and Its Impact in Three Successive Generations of *Erianthus procerus* × *Saccharum*

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Summary *Erianthus* is a wild related genus of sugarcane. Its introgression is expected to impart better biomass production, multi ratoonability, and disease and pest resistance to the sugarcane cultivars. In this study, the introgression pattern of the wild genome, *E. procerus*, has been analyzed in the three consecutive generations. The field performance concerning important agronomic traits and reaction to one of the major diseases, red rot, also has been studied. We report here for the first time the chromosome composition of fertile *E. procerus* × *S. officinarum* F₁, BC₁ (F₁ × sugarcane cultivar, Co 06027), and BC₂ (BC₁ × sugarcane cultivar, Co 775) hybrids via genomic *in situ* hybridization (GISH). The F₁ resulted from 2n+n chromosome transmission, while BC₁ and BC₂ from n+n transmission. The hybridity of these clones was confirmed with the presence of both *Erianthus* and *Saccharum* specific amplified bands of 5S rDNA. The field evaluation revealed that though BC₁ and BC₂ hybrids recorded significantly higher stalk number and comparable stalk yields, the juice quality traits need further improvement with few more backcrosses. These *Erianthus* introgressed hybrid clones proved to be a potential source for red rot resistance in future sugarcane cultivars. This study is aimed to identify the agronomic value of chromosomes from *E. procerus* and to conduct targeted breeding based on these information.

Keywords *Erianthus*, *Saccharum*, Introgression, Cytology, Genomic *in situ* hybridization, Intergeneric hybrid, Sugarcane.

Sugarcane which belongs to the genus *Saccharum* in the family Poaceae consists of six species including *S. officinarum* L., *S. spontaneum* L., *S. robustum* E. W. Brandes & Jeswiet ex Grassl, *S. barberi* Jeswiet, *S. sinense* Roxb. and *S. edule* Hassk. The *Saccharum* together with the four related genera namely *Erianthus*, *Miscanthus*, *Narenga* and *Sclerostachya*, comprises of the “*Saccharum* complex” (Amalraj and Balasundaram 2006). Modern sugarcane cultivars are complex inter-specific hybrids between the sugar-producing species *S. officinarum* (2n=80) and the wild species *S. spontaneum* (2n=40–128). Presently available sugarcane cultivars are derived from intercrossing between the first nobilized hybrids of a few potential clones of *S. officinarum* and *S. spontaneum*. While analyzing the pedigree of modern cultivars it has been revealed that only a limited number of cultivated and wild species have contributed towards the parentage of these cultivars (Arceneaux 1965). Hence breeders are concerned about the potentially narrow genetic base of the presently available commercial cultivars of sugarcane and have shown considerable in-

terest in exploiting other germplasm resources to enlarge the genetic base and introduce new genes for yield as well as biotic and abiotic stresses.

Erianthus is a wild relative genus of sugarcane and it has been considered as a valuable genetic resource of the sugarcane cultivars as it is showing genetic divergence with *Saccharum*. The species of *Erianthus* has been the focus of several breeding programs as a potentially valuable contributor with many desirable characters like high fiber content, excellent vigor, ratooning ability, and tolerance to abiotic and biotic stresses (Ram *et al.* 2001, Jackson and Hentry 2011, Fukuhara *et al.* 2013, Jayabose *et al.* 2017). Breeders are facing problems in using *Erianthus* species in sugarcane breeding programs due to the incompatibility that often occurs between *Erianthus* species and *Saccharum* and in identifying the genuine hybrids based on the morphological characters. The *Erianthus* specific molecular markers to overcome the difficulty in identification of intergeneric hybrids were developed (D'Hont *et al.* 1995, Besse *et al.* 1996, 1997, Piperidis *et al.* 2000, Cai *et al.* 2005). In addition to this GISH was shown to be an effective method for analyzing the genome constitution of the intergeneric hybrids involving *Saccharum* and *Erianthus* (D'Hont *et al.* 1995,

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Piperidis *et al.* 2000). At ICAR-Sugarcane Breeding Institute, Coimbatore, *Erianthus* introgression program has been going on for the last two decades and several hybrids have been developed and characterized (Ram *et al.* 2001, Nair *et al.* 2006, Lalitha and Premachandran 2007). In most cases, the *E. arundinaceus* has been the focus of several breeding programs as a source of potentially valuable traits.

In sugarcane during backcrosses the problem of flowering and synchrony makes the breeder use different sugarcane clones rather than using one of their parents and it is recognized as modified backcross and resultant progenies are referred as BC progenies. In this article, we analyzed the F_1 and back cross progenies of intergeneric crosses involving a different species of *Erianthus*, i.e., *E. procerus* and *Saccharum*. Here we report on the genomic characterization and chromosome transmission pattern of *Erianthus* in three generations of intergeneric hybrids (F_1 , BC_1 , and BC_2) derived from crosses between *E. procerus* and *Saccharum*. As a valuable source for abiotic and biotic stress tolerance, the molecular cytogenetic characterization and agronomic trait evaluation of hybrids involving *E. procerus* will be helpful for the planning of breeding strategies for further utilization of the traits from *E. procerus*.

Materials and methods

The plant materials used for the study are F_1 , BC_1 , and BC_2 of an intergeneric hybrid involving *E. procerus* (female parent) and *S. officinarum* (male parent). *E. procerus* clone, IND 90-776, was collected from Arunachal Pradesh in North East India. PIO 96-435 is an intraspecific hybrid of *S. officinarum* developed through poly-crossing between typical ($2n=80$) and atypical ($2n=\text{other than } 80$) clones of *S. officinarum*. The F_1 seedlings were screened morphologically and cytologically. GU 04(28) EO2 was one of the F_1 that was confirmed as a genuine hybrid and it was taken for GISH analysis. This hybrid was backcrossed to a commercial sugarcane cultivar Co 06027 and the selected seedlings (BC_1 progenies) were subjected to study. Confirmed BC_1

hybrid, GU 12-21 was backcrossed with a commercial sugarcane cultivar Co 775, and one BC_2 progeny, GU 15-3 was subjected to GISH analysis to understand the parental chromosome segregation pattern. The details of the materials used in the study are described in Fig. 1. In order to confirm the hybridity, F_1 , BC_1 , and BC_2 were screened with PCR that amplifies the 5S rDNA of *Erianthus* genome (D'Hont *et al.* 1995). Amplified products were resolved on 2% agarose gels, stained with ethidium bromide, and documented using a gel documentation system.

The somatic chromosome number of the *Erianthus*, *S. officinarum*, F_1 , BC_1 , and BC_2 clones was determined by root tip squash technique (Sobhakumari and Asmita 2014).

The parents, intergeneric hybrid, BC_1 , BC_2 hybrid along commercial cultivar Co 86032 were evaluated in a randomized block design with a plot size of two rows of 6 m for determining stalk yield and juice quality traits (in 10th month) during 2016–18. Data analysis has been done with the statistical software R 3.3.2 (<http://www.R-project.org>). The clones were screened for red rot resistance under artificially controlled condition testing against the red rot pathogen *cf.* 671 (Mohanraj *et al.* 1997).

Mitotic chromosome preparation for GISH was performed as previously described (D'Hont *et al.* 1996) with minor modifications. In brief, the hybrids were grown in pots for root tip harvesting. Excised root tips of about 1.0 cm were treated with 2 mM 8-hydroxyquinoline at room temperature for 2 h, rinsed in water, and fixed in ethanol–acetic acid (3:1) for about 16 h at 4°C. The root tips were hydrolyzed in 0.25 M HCl and digested in an enzyme solution (2% cellulase Onozuka and 20% pectinase in citrate buffer) at 37°C for 45 min. After washing in distilled water meristematic tissues were squashed in ethanol–acetic acid (3:1). Cells can be separated by gentle pressing over the coverslip with a filter paper. Slides were then frozen on dry ice or dipping it in liquid nitrogen. The coverslip was removed and the frozen slide was immediately dehydrated in absolute ethanol and stored in moisture free slide box.

Dried slides were treated with $1 \mu\text{g} \mu\text{L}^{-1}$ RNase at 37°C for 45 min and washed 3 times in $2\times\text{SSC}$ for 5 min each at room temperature. After drying, the slides were denatured in 70% formamide for 2 min at 72°C and dehydrated in ice-cold ethanol series. Genomic DNA of *E. procerus* was labeled with biotin-11-dUTP using a random primed labeling method described by the manufacturer (Thermo Scientific, USA) and used as a probe. The hybridization mixture (30 μL per slide) consisted of 50 ng of the labeled probe, 50% deionized formamide, 10% dextran sulfate, 0.5 μL of sheared salmon sperm DNA and $2\times\text{SSC}$. This mixture was denatured for 10 min in a water bath at 100°C and then cooled quickly on the ice. Hybridization was performed overnight in a

Fig. 1. Parentage of intergeneric and back cross hybrids used in the study.

moist chamber at 37°C. Post hybridization washes were carried out in 2×SSC at 42°C. Biotin-labeled probe was then detected using avidin-FITC by 1 h incubation at 37°C. Post detection washes were carried out in 2×SSC, 1% Triton X-100 in 4×SSC followed by 2×SSC for 5 min at 37°C followed by 5 min incubation in 2×SSC at room temperature. Slides were mounted in Vectasheild (Vector labs, UK) mounting medium with 4,6-diamidino-2-phenylindole. Mitotic cells were analyzed using a Carl Zeiss fluorescence microscope with appropriate filter sets. Images in different filters were captured with Progress Capture Pro software and merged using Adobe Photoshop.

Results

The somatic chromosome number of the parents and F₁, BC₁ and BC₂ progenies of the hybrid involving *Saccharum* and widely divergent genus *Erianthus* was determined in root tip mitosis (Fig. 2). The somatic chromosome number of the *E. procerus* (IND 90-776) and *Saccharum* (PIO 94-435) parents was 2n=40 and 104 respectively (Fig. 2a, b) and the chromosome number of the F₁ hybrid [GU04 (28) EO2] evolved from these parents was 2n=80. The hybrid flowered normally and was utilized for further backcrosses as the female parent and Co 06027 (2n=108) was used as a male parent. Four back cross progenies were subjected to cytological analysis to determine the somatic chromosome number. In these hybrids, the chromosome number ranged from 2n=88–94. One of the confirmed BC₁ hybrids,

GU 12-21, was again backcrossed with a commercial cultivar Co 775 (2n=110) and the obtained BC₂ progeny which showed 2n=103.

The 5S rDNA profiles of intergeneric hybrid, back cross hybrids, and their parents are shown in Fig. 3. The true hybridity has been confirmed by 5S rDNA markers and bands were in the size of approximately 400bp in *Erianthus* and 230bp in *Saccharum*. The F₁ [GU 04(28) EO-20], BC₁ (GU 12-21) and BC₂ (GU 15-3) had both the bands and confirmed as genuine hybrids of *Erianthus*.

The agronomic evaluation of F₁, BC₁, and BC₂ along with their parents and the commercial cultivar Co 86032 for cane yield and juice quality traits are given in Table 1. The F₁ hybrid GU 04(28) EO-2 recorded the highest stalk number 151,250 ha⁻¹) followed by GU 15-3 (127,140) and GU 12-21 (121,140). The intergeneric hy-

Fig. 3. Amplification of *Erianthus* specific 5S rDNA in F₁, BC₁, and BC₂ hybrids. Lane 1 IND90-776 (*E. procerus*), Lane 2 PIO96-435 (*S. officinarum*), Lane 3 GU04(28) EO2 (F₁ hybrid), Lane 4 GU 12-20 (BC₁), Lane 5 Co 06027, Lane 6 Co 775, Lane 7 GU15-3 (BC₂), Lane M 100bp ladder Marker.

Fig. 2. Somatic chromosome number of parents and hybrids. a) *E. procerus* (IND 90-776) 2n=40, b) *S. officinarum* (PIO 94-435) 2n=104, c) F₁ [GU 04(28) EO-2] 2n=80, d) Co 06027 2n=108, e) BC₁ (GU12-21) 2n=94, f) Co 775 (2n=110), g) BC₂ (GU 15-3) 2n=103. Scale bar=2 μm.

Table 1. Performance of intergeneric and backcross hybrids for stalk yield, juice quality traits, and red rot resistance.

Clone	NMS* (ha ⁻¹)	Stalk height (cm)	Stalk diameter (cm)	SSW** (kg)	Juice brix (%)	Sucrose (%)	CCS (%)	Purity (%)	Stalk yield (t ha ⁻¹)	Red rot rating
GU 04(28) EO2	151.25	235.33	1.26	0.65	12.51	7.88	4.40	62.99	98.31	R
PIO 96-435	70.45	190.10	2.45	0.98	19.10	16.28	11.06	85.24	69.04	S
GU 12-21	121.14	230.50	2.10	0.90	17.56	14.18	9.36	80.75	109.02	R
GU 15-3	127.60	245.60	2.34	1.01	18.25	14.90	9.90	81.64	128.87	MR
Co 06027	88.89	260.35	2.92	1.33	19.26	17.31	12.07	89.88	118.22	MR
Co 775	57.78	200.50	3.05	1.14	18.24	16.10	11.13	88.27	65.87	MS
Co 86032	102.22	240.42	2.78	1.08	20.67	18.69	13.07	90.42	110.39	MS
CD (0.05)	15.01	18.16	0.45	0.18	1.98	1.84	1.73	6.18	16.24	

*Number of millable stalks, **single stalk weight

Fig. 4. GISH in F₁, BC₁ and BC₂ progeny of *E. procerus* × *S. officinarum* a) Mitotic chromosomes of F₁ [GU 04(28) EO2] with *E. procerus* as the probe, b) Mitotic chromosomes of BC₁ (GU 12-20) followed by GISH with *E. procerus* as the probe, c) Mitotic chromosomes of BC₂ (GU 15-3) *E. procerus* as the probe. Scale bar=2 μm.

brid recorded the lowest stalk diameter (1.26 cm), single stalk weight (0.65 kg), and juice quality traits compared to BC hybrids and the commercial clones. The F₁ hybrid and BC₁ were red rot-resistant and BC₂ was moderately resistant under controlled condition testing against the highly virulent strain cf. 671.

The GISH analysis of the F₁ hybrid, GU 04(28) EO2 revealed that out of 80 chromosomes, 40 chromosomes were from *E. procerus* and 40 chromosomes from *S. officinarum* (Fig. 4a). Among the BC₁ progenies GU 12-21 (2n=94) was subjected to GISH analysis with the biotin labeled genome DNA of *E. procerus* revealed that out of 94 chromosomes, 20 chromosomes were derived from *E. procerus* (Fig. 4b). In the next generation, the BC₂ (GU 15-3) displayed 2n=103, of which 10 chromosomes were derived from *Erianthus* species (Fig. 4c).

Discussion

In recent years, sugarcane breeders are giving more attention towards the use of *Erianthus* spp. in several sugarcane breeding programs as a potential source for valuable traits like high biomass, excellent vigor, multi ratoonability, disease, and pest resistance and tolerance to environmental stresses (Piperidis *et al.* 2000, Jackson and Hentry 2011). Due to high genetic diversity between the two genera, limited success could only be achieved in the breeding programs because of low compatibility. Difficulties are also faced to identify the genuine progeny produced from the cross between these two genera

on account of a large proportion of selfed progeny and identification of hybrids based on morphological characters is very challenging. Molecular markers had been utilized for the identification of true hybrids involving *Saccharum* × *Erianthus* (Besse *et al.* 1996, 1997, Alix *et al.* 1999, Nair *et al.* 2006, Piperidis *et al.* 2000, Cai *et al.* 2005). Particularly 5S rDNA markers are being successfully used in the identification of *Erianthus* hybrids (D'Hont *et al.* 1995, Fukuhara *et al.* 2013, Pachakkil *et al.* 2019). In our study also the F₁, BC₁, and BC₂ hybrids of *Erianthus* were undoubtedly confirmed as genuine hybrids using 5S rDNA markers. Though the hybridity could be confirmed in intergeneric hybrids with markers, the presence of alien chromosomes, their number, and possible recombinant chromosomes could be confirmed only through GISH techniques (D'Hont *et al.* 1995, Piperidis *et al.* 2000, 2010a).

The classical cytological techniques revealed the somatic chromosome number of the F₁ hybrid, GU 04(28) EO-2 as 2n=80. The expected exact chromosome number from n+n transmission is 2n=72 (20+52). In our studies, the confusion regarding chromosome transmission has been cleared with GISH. We observed that the F₁ hybrid showed 2n+n transmission with the elimination of 12 chromosomes from *Saccharum* parent. The hybrid showed 40 chromosomes of *E. procerus* on using *E. procerus* genome DNA as the probe during GISH has been suggested that when crosses involved distantly related species there is a chance for 2n+n chromosome transmission as in the case of *S. officinarum* × *S. sponta-*

neum crosses (Bremer 1923, 1961, Parthasarathy 1948).

The male parent, PIO 96-435, is an intraspecific hybrid developed by intercrossing typical and atypical clones of *S. officinarum* in the germplasm. An atypical *S. officinarum* would have been originated by natural hybridization with related species at its place of origin. Hence there is a chance for *S. spontaneum* genome in it and its presence has been proved through molecular cytogenetic tools (Piperidis *et al.* 2010a, Yu *et al.* 2018). This genome might have induced the $2n$ chromosome transmission from the other parent (*E. procerus*). In crosses involving *Saccharum* and *E. arundinaceus* $n+n$ transmission had been generally reported in F_1 hybrids in which *Saccharum* spp. was used as the female parent and *Erianthus* as the male parent (Janaki Ammal 1941, Rao *et al.* 1963, Kandasami 1964, D'Hont *et al.* 1995, Piperidis *et al.* 2000, 2010b, Wu *et al.* 2014). This is the first report in which *Erianthus* was used as the female parent and its $2n$ gamete transmission in F_1 has been proved by GISH.

The high rate of aneuploidy in F_1 hybrid, due to the elimination of 12 chromosomes contributes to the production of unbalance gametes resulting in a high degree of pollen sterility in the F_1 hybrid. To obtain back cross progenies, the F_1 hybrid, GU 04(28) EO-2, was used as the female parent, and the cultivar Co 06027 ($2n=108$) used as the male parent. Mitotic studies revealed the somatic chromosome number of four BC_1 progenies ranging from $2n=88-94$ with $n+n$ chromosome segregation. Among these, the clone with the highest chromosome number, GU 12-21, was subjected to GISH analysis to understand the transmission pattern of alien chromosomes from *E. procerus*. Twenty chromosomes of *E. procerus* could be revealed in this BC_1 hybrid. This BC_1 progeny was later crossed with a cultivar Co 775 ($2n=110$) and root tip mitotic studies revealed that in BC_2 the somatic cell chromosome number as $2n=103$. GISH analysis revealed the $n+n$ segregation pattern with chromosomes of *E. procerus*.

In three consecutive generations, the GISH confirmed that there were no recombination events between *Saccharum* and *E. procerus* chromosomes. This is consistent with the earlier reports (Piperidis *et al.* 2000, 2010b, Lekshmi *et al.* 2017, Premachandran *et al.* 2017). As these genomes are distantly related it is suggested that *E. procerus* may be introgressed into cultivars as only in whole chromosomes by conventional breeding programs.

The F_1 hybrid, GU 04(28) EO-2, having *Erianthus* cytoplasm and whole-genome ($2n$) of *Erianthus* with *S. officinarum* genome is a potential source for diversifying the cytoplasmic and genomic base of the cultivars to be developed in the future. It is proven that in the F_1 hybrid, $2n+n$ transmission has occurred with chromosome elimination from *S. officinarum* to overcome a genetic imbalance that occurred due to the presence of two diverged genomes in the hybrid. In the earlier reports (Piperidis

2010b, Wu *et al.* 2014) where *Saccharum* was used as the female parent and *Erianthus* as the male parent, the F_1 showed $n+n$ transmission. In contradictory to these reports in our study with *Erianthus* as a female parent F_1 was showing $2n+n$ transmission and BC_1 was showing $n+n$ transmission. In both these cases at the stage of BC_1 , half the genome of the *Erianthus* is retaining. In our study either BC_1 or BC_2 progenies were not showing $2n+n$ chromosome transmission and this might be to avoid genomic imbalance by increasing the *Erianthus* components in the genome.

The F_1 , BC_1 , and BC_2 recorded significantly higher stalk number compared to commercial cultivar Co 86032. Though the backcross hybrid GU 15-3 recorded higher stalk yield but had significantly lower juice Brix%, Sucrose %, CCS%, and Purity% than the commercial cultivars which indicates further improvement is required for improving juice quality. There was an improvement in BC_2 for single stalk weight and juice quality over BC_1 and F_1 . For stalk yield also, BC_2 had shown an improvement of 31.02% over F_1 and 18.16% over BC_1 . There was a not significant difference between BC_1 and BC_2 for juice quality, however, BC_2 recorded a 60.27% improvement over F_1 hybrid and 5.02% over BC_1 . The F_1 and BC hybrids were resistant to red rot which is the most devastating disease in both tropics and subtropics of the Indian subcontinent. The *Erianthus* introgressed clones proved to be a potential source for diversifying red rot-resistance in sugarcane. The selection and utilization of promising back cross progenies in future hybridization programs will develop sugarcane cultivars with a broad genetic base and adaptability with better productivity.

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