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Chloroplast DNA polymorphism in *psbC-trnS* and *trnL* intron segments differentiate *Saccharum* and *Erianthus*

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Abstract

Broadening of the genetic base of sugarcane (*Saccharum* spp. hybrid) is done using wild related species *Saccharum spontaneum* and *Erianthus arundinaceus*. Intergeneric crosses of *Saccharum* with *E. arundinaceus* as female were made and putative hybrids were clonally maintained. The polymorphism in the chloroplast DNA segments *psbC-trnS* and *trnL* intron amplified by polymerase chain reaction and fragmented with restriction enzymes *HaeIII* and *TaqI*, respectively could differentiate the *Saccharum* and *Erianthus* cytoplasm. One *E. arundinaceus* (2n = 60) × *S. spontaneum* (2n = 64) hybrid with chromosome number 2n = 62 was found to have *Erianthus* cytoplasm, which is a first report.

Key words: Sugarcane, chloroplast DNA, *Erianthus* × *Saccharum*, *psbC-trnS*, *trnL* intron

Introduction

Sugarcane (*Saccharum* L. spp. hybrid, Family - Poaceae), is a major crop in the tropical and sub-tropical parts of the world cultivated mainly for sugar and ethanol. The commercial sugarcane varieties are derivatives of interspecific hybrids involving *Saccharum officinarum* L. and the wild related species *S. spontaneum* L. Only a limited number of *S. officinarum* clones were involved as cytoplasm donor in the pedigree of the sugarcane cultivars world-wide [1]. The genera *Saccharum* L., *Erianthus* Michx. section *Ripidium* Henrad, *Miscanthus* Anderss., *Sclerostachya* (Hack.) A. Camus and *Narenga* Bor., which are closely related, were considered to be part of '*Saccharum* complex' [2, 3], sharing a common gene pool. The involvement of *Erianthus arundinaceus* (Retz.) Jesweit in the origin of *S. officinarum* was suggested [4]. *E. arundinaceus* is a large grass with tall and thick stalks resembling sugarcane and having useful traits such as growth vigour, wide ecological adaptability, extensive root system, drought and water logging resistance, good ratoonability, high biomass and disease resistance [5, 6], The genetic base of sugarcane is considered to be very narrow and gene introgression studies are being done to transfer the genes from wild species such as *S. spontaneum* and *E. arundinaceus* [5].

The chloroplast genome in plants is highly conserved. The chloroplast DNA segments can be specifically amplified by polymerase chain reaction (PCR) using specific primer pairs, and by subsequent fragmentation with different restriction enzymes the sequence differences could be resolved by electrophoresis. The availability of universal primers which can amplify the chloroplast introns and intragenic spacers in a wide range of plant taxa made the chloroplast polymorphism an efficient tool for phylogenetic studies [7]. The chloroplast DNA diversity among the different species of *Saccharum* is limited and the commercial sugarcane varieties are having *S. officinarum* chloroplast DNA pattern [8]. The chloroplast DNA segments *psbC-trnS* and *trnL* introns, which are non-coding sequences, were found to have interspecific polymorphism in many plant taxa [9, 10, 11]. Maternal inheritance of chloroplast genome was generally observed in angiosperm species and biparental inheritance was also reported in few crop species [12]. Strict paternal inheritance of chloroplast DNA was found in *Actinidia* [13].

Intergeneric crosses involving *E. arundinaceus* as female were made with sugarcane commercial varieties and *S. spontaneum*. The present report is on the polymorphism in the chloroplast DNA between *Saccharum* and *Erianthus* by which the hybrids with *Erianthus* cytoplasm could be identified.

Materials and methods

E. arundinaceus clones IK 76-62 and IK 76-91 were crossed as female with *S. spontaneum* clone Iritty-2 and sugarcane clone Co 98007, respectively. From the progeny, putative hybrids were identified by the presence of the dewlap at the junction of leaf blade and leaf sheath, a *Saccharum* feature not present in *Erianthus*. One hybrid from IK 76-62 × Iritty-2 named as CYM 04-420 and two hybrids from IK 76-91 × Co 98007 named as GUK 00-1054 and GUK 00-1058 were clonally maintained. The parental clones and the three putative *Erianthus* × *Saccharum* hybrids were used for cytological and molecular analysis.

The somatic chromosome number of the parental clones and the hybrids were determined by root tip squash technique. Actively growing root tips from cane cuttings planted in pots with river sand were excised and treated in a saturated solution of α-bromo naphthalene for about 90 minutes. After thorough washing, the root tips were fixed in 3:1 ethanol-acetic acid at least for 16 hours. The root tips were hydrolyzed in 1 N hydrochloric acid at 60°C for 13 minutes and after washing in water were stained in leuco basic fuchsin under dark for 30 minutes. Well-stained root tips were excised and squashed in 1 per cent acetocarmine. Well-spread mitotic chromosomes were counted and photographed.

One gram of fresh leaf tissue was ground in liquid nitrogen and suspended into a buffer containing 100 mM Tris (pH 8.0), 100 mM NaCl, 20 mM EDTA, 0.1 % β-mercapto ethanol and 2 % Cetyl methyl ammonium bromide (CTAB) and incubated in a water bath at 60°C for 30 minutes. The genomic DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with 0.7 volumes of ice cold isopropanol. The pellet containing the DNA was dissolved in TE buffer and purified from RNase and proteins following standard procedures [14].

The chloroplast DNA region *psb*C-*trn*S was PCR amplified from the genomic DNA using a pair of universal primers : Primer 1: 5'-GGT CGT GAC CAA GAA ACC AC-3', Primer 2: 5'-GGT TCG AAT CCC TCT CTC TC-3' [9, 11]. The PCR profile consisted of 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 63°C and 2 min at 72°C and the final extension for 15 min at 72°C. The amplified PCR product was purified by using PCR purification kit (Qiagen). About 200 ng of PCR product in 10uL reaction mix was digested with enzyme *Hae*III (GENEI, Bangalore) as per manufacturer's instructions. The restricted samples were separated on 1.5 % agarose gel in TE buffer at 80 m. amps for 4 h. The chloroplast DNA region *trn*L intron was PCR amplified using a pair of universal primers : Primer 1: 5'-CGA AAT CGG TAG ACG CTA CG-3', Primer 2: 5'-GGG GAT AGA GGG ACT TGA AC-3' [10]. The PCR profile consisted of 5 min at 95°C, 30 cycles of 15 sec at 94°C, 1 min at 50°C and 1 min at 72°C and the final extension for 10 min at 72°C. The amplified PCR product was purified and digested with enzyme *Taq*I (GENEI, Bangalore) as per manufacturer's instructions and separated on 1.5 % agarose gel as in the case of *psb*C-*trn*S, given above.

Results and discussion

The polymerase chain reaction (PCR) carried out with the *psb*C-*trn*S specific primers gave a product of approximately 1.6 kb in all the clones used in the study (Fig. 1a), indicating that between *Saccharum* and *Erianthus* the *psb*C-*trn*S segments could not be easily distinguished by product size. The PCR product when digested with *Hae*III had restriction fragment length polymorphism (RFLP) among the clones. The restriction pattern in Iritty-2, Co 98007, GUK 00-1054 and GUK

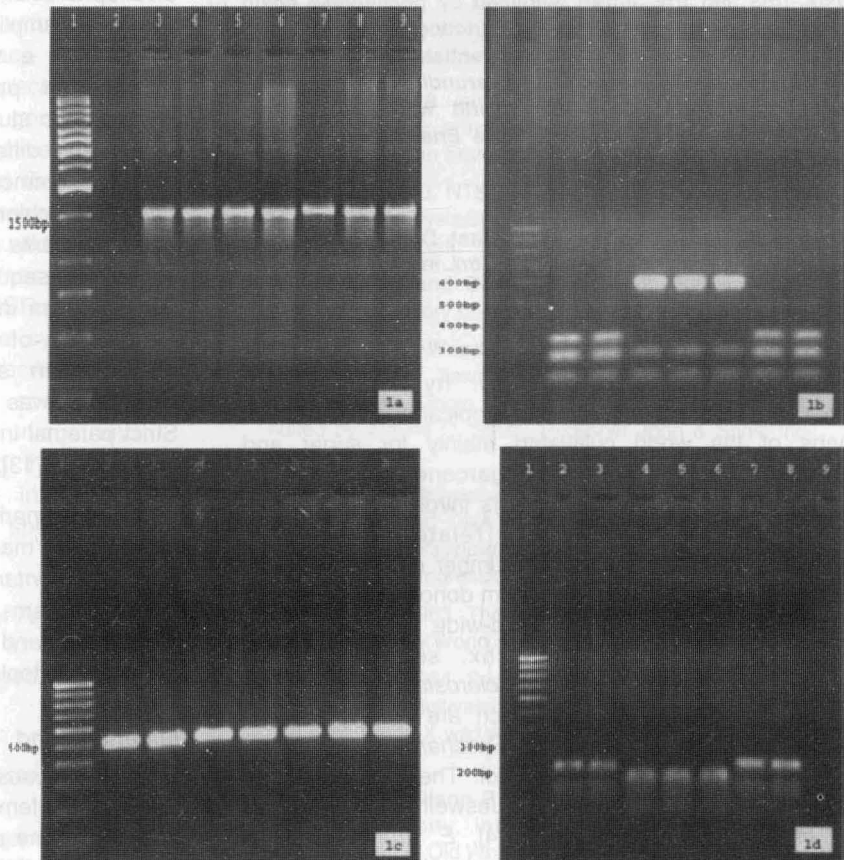

Fig. 1. PCR-RFLP of chloroplast DNA segments *psb*C-*trn*S and *trn*L intron: (a) PCR product of *psb*C-*trn*S specific primers. Lane 1 - marker, lane 2 - blank without plant DNA, lane 3 - Co 98007 (*Saccharum* spp. hybrid), Lane 4 - Iritty-2 (*S. spontaneum*), lane 5 - IK 76-62 (*Erianthus arundinaceus*), lane 6 - IK 76-91 (*E. arundinaceus*), lane 7 - CYM 04-420 (*E. arundinaceus* × *S. spontaneum* hybrid), lane 8 - GUK 00-1054 (*E. arundinaceus* × *Saccharum* hybrid), lane 9 - GUK 00-1058 (*E. arundinaceus* × *Saccharum* hybrid), (b) *psb*C-*trn*S segment restricted with *Hae* III. Lanes 1-8 as in Fig. 1a, excluding blank in lane 2. (c) PCR product of *trn*L intron specific primers. Lanes 1-8 as in Fig. 1b. (d) *trn*L intron restricted with *Taq* I. Lane 1-8 as in Fig. 1b.

00-1058 was different from that of IK 76-62, IK 76-91 and CYM 04-420 (Fig. 1b). The PCR product in the case of *trn*L intron specific primers was of approximately 520 bp in all the material studied (Fig. 1c). When digested with *Taq* I, the PCR product of Iritty-2, Co 98007, GUK 00-1054 and GUK 00-1058 had similar restriction pattern whereas in IK 76-62, IK 76-91 and CYM 04-420 the pattern was different (Fig. 1d). The restriction pattern in *Erianthus* clones IK 76-62 and IK 76-91 of both *psb*C-*trn*S with *Hae*III and *trn*L intron with *TaqI* are different from that of the *Saccharum* clones Iritty-2 and Co 98007, showing that these two chloroplast DNA segments have sequence differences to distinguish *Erianthus* and *Saccharum*. The chloroplasts are maternally transmitted in most of the angiosperms and in sugarcane also this is expected. The hybrid clone CYM 04-420 had the restriction patterns of *Erianthus*, the maternal parent. The clones GUK 00-1054 and GUK 00-1058 had the restriction patterns of *Saccharum* indicating that these clones do not have the *Erianthus* cytoplasm.

The chromosome number of the putative hybrid clones CYM 04-420, GUK 00-1054 and GUK 00-1058 were $2n = 62$, $2n = 98$ and $2n = 112$, respectively (Fig. 2). The clone CYM 04-420 is a product of $n + n$ transmission from the parental clones IK 76-62 (2n = 60) and Iritty-2 (2n = 64). Both GUK 00-1054 and GUK 00-1058 had chromosome number higher than that expected from $n + n$ transmission from IK 76-91 (2n = 60) and Co 98007 (2n = 110). The chromosome number in the interspecific and intergeneric hybrids involving *Saccharum* species can not be taken as proof for hybridity of the clones as in different crosses $n + n$, $2n + n$, or $n + 2n$ transmission may occur and chromosome elimination from both the parental clones also may happen [15, 16]. The chloroplast DNA studies and the chromosome number of the clone CYM 04-420 show that it is a true hybrid between *E. arundinaceus* ($2n = 60$) and *S. spontaneum* ($2n = 64$). The other two suspected hybrid clones may be contaminants as the cytoplasm is not of the maternal parent, *E. arundinaceus*. But, in the light of the observation by Kiang *et al.* [17] that a break down in the control mechanisms of maternal inheritance might happen when wide crosses between crop plants and distantly related wild species were made, it needs further probe.

Intergeneric hybrids between *S. officinarum* and *E. arundinaceus* with *S. officinarum* as female parent [6, 16, 17, 18] had $n + n$ transmission, often with chromosome elimination. In crosses involving *E. arundinaceus* with sugarcane hybrid clones as female parent, chromosome numbers higher than that expected from $n + n$ transmission was generally observed (data not presented here). When diploid male gamete from

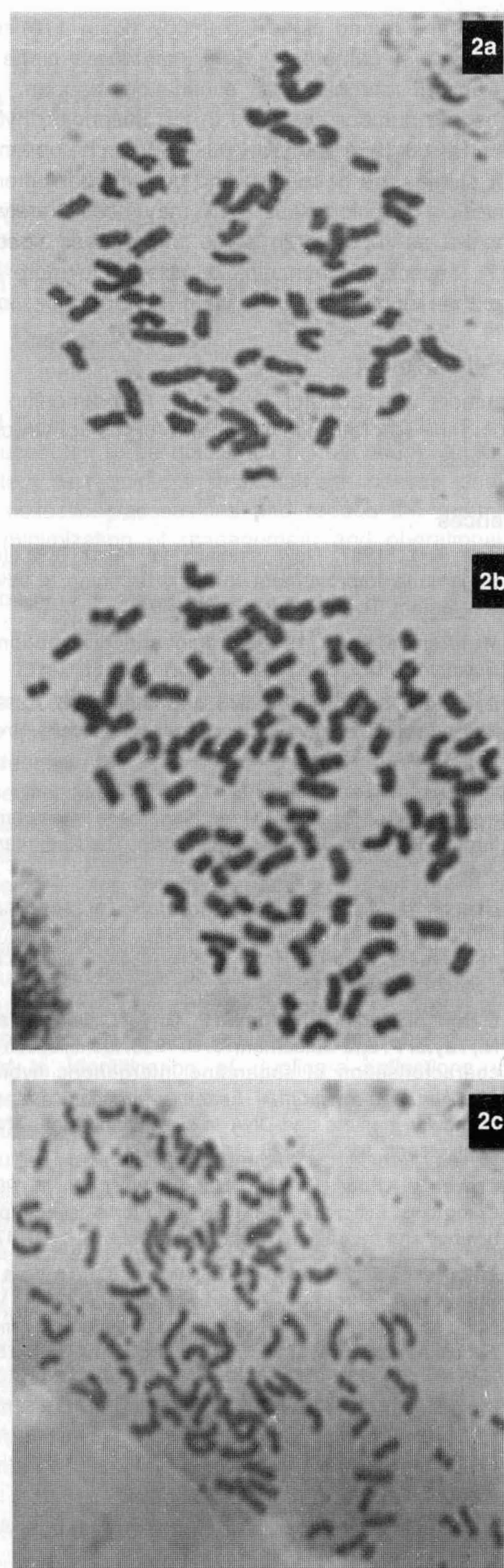

Fig. 2. Somatic chromosomes of *Erianthus* × *Saccharum* hybrids: (2a) CYM 04-420 (*E. arundinaceus* × *S. spontaneum* hybrid) 2n = 62, (2b) GUK 00-1054 (*E. arundinaceus* × *Saccharum* hybrid) 2n = 98, (2c) GUK 00-1058 (*E. arundinaceus* × *Saccharum* hybrid) 2n = 112

the sugarcane hybrid clone Co 98007 ($2n$ = 110) may function with n gamete of *E. arundinaceus* clone IK 76-91 and if chromosome elimination happen, the chromosome number observed in the suspected hybrid clones GUK 00-1054 and GUK 00-1058 can be obtained. Further studies are needed to find out whether paternal transmission of chloroplast DNA may occur rarely in *Saccharum* as reported in some other grass species [12, 17], especially when diploid pollen with more cell volume than normal pollen is involved in fertilization.

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