Theor Appl Genet (1994) 89:959–963

S. M. Al-Janabi · R. J. Honeycutt · B. W. S. Sobral

Chromosome assortment in *Saccharum*

Received: 25 October 1993 / Accepted: 17 May 1994

Abstract Recent work has revealed random chromosome pairing and assortment in *Saccharum spontaneum* L., the most widely distributed, and morphologically and cytologically variable of the species of *Saccharum*. This conclusion was based on the analysis of a segregating population from a cross between *S. spontaneum* 'SES 208' and a spontaneously-doubled haploid of itself, derived from anther culture. To determine whether polysomic inheritance is common in *Saccharum* and whether it is observed in a typical biparental cross, we studied chromosome pairing and assortment in 44 progeny of a cross between euploid, meiotically regular, $2n$=80 forms of *Saccharum officinarum* 'LA Purple' and *Saccharum robustum* 'Mol 5829'. Papuan $2n$=80 forms of *S. robustum* have been suggested as the immediate progenitor species for cultivated sugarcane (*S. officinarum*). A total of 738 loci in LA Purple and 720 loci in Mol 5829 were amplified and typed in the progeny by arbitrarily primed PCR using 45 primers. Fifty and 33 single-dose polymorphisms were identified in the *S. officinarum* and *S. robustum* genomes, respectively (χ^2 at 98%). Linkage analysis of single-dose polymorphisms in both genomes revealed linkages in repulsion and coupling phases. In the *S. officinarum* genome, a map hypothesis gave 7 linkage groups with 17 linked and 33 unlinked markers. Four of 13 pairwise linkages were in repulsion phase and 9 were in coupling phase. In the *S. robustum* genome, a map hypothesis gave 5 linkage groups, defined by 12 markers, with 21 markers unlinked, and 2 of 9 pairwise linkages were in repulsion phase. Therefore, complete polysomic inheritance was not observed in either species, suggesting that chromosomal behavior is different from that observed by linkage analysis of over 500 markers in the *S. spontaneum* map. Implications of this finding for evolution and breeding are discussed.

Communicated by A. C. Kahler

S. M. Al-Janabi · R. J. Honeycutt · B. W. S. Sobral (✉)
California Institute of Biological Research,
11099 N. Torrey Pines Rd., Suite 300, La Jolla,
CA 92037, USA

Key words Sugarcane · Polyploidy · Genetics
Evolution · Breeding · DNA markers
Arbitrarily primed PCR · RAPD markers

Introduction

Saccharum L. is a polyploid grass in the Saccharinae subtribe of the Andropogoneae. Members of *Saccharum* and allied genera are thought to have arisen through polyploidization and hybridization events; the extended gene pool of the genus is known as the '*Saccharum* complex' (Mukherjee 1957; Daniels and Roach 1987). Chromosome numbers in the complex vary from $2n$=20, in some *Erianthus* Michx. sect. *Ripidium* Henrard and $2n$=38 in *Miscanthus* Anderss. sect. *Diandra*, to $2n$ = approximately 200 in some presumably hybrid *Saccharum* genotypes. Morphology is also variable, though many consider fewer genera within the complex than do sugarcane biologists (compare Clayton and Renvoize 1986, with Daniels and Roach 1987). Clayton and Renvoize (1986), for example, regard *Erianthus*, *Saccharum*, *Narenga* Bor, and even *Miscanthus* and *Sclerostachya* (Hack.) A. Camus ($2n$=30) as synonymous. Sugarcane (*Saccharum officinarum* L.) is a domesticated crop that has been cultivated for millennia because of its high sucrose content. *S. robustum* was identified by Brandes in a 1928 expedition to Papua New Guinea, and was proposed to be the "wild" progenitor of cultivated sugarcane (Brandes 1929). *S. officinarum* and most *S. robustum* contain $2n$=80 chromosomes by definition (Parthasarthy 1948; Vijayalakshmi 1967). Accessions of either species found to have a different chromosome complement are usually presumed to be of hybrid origin.

Saccharum and allied genera have presented difficulties for genetic, evolutionary, and taxonomic studies because of high levels of polyploidy. However, genetics, evolution and domestication processes need to be understood for breeding and germplasm conservation and use to be effective. Al-Janabi et al. (1993) and Da Silva et al. (1993, 1994) used genetic maps to show polysomic inheritance in

Saccharum spontaneum 'SES 208' (2*n*=64), a behavior typical of autopolyploids. Their data suggest that SES 208 is an auto-octoploid with a base chromosome number of *x*=8. This conclusion was based on lack of repulsion-phase linkages in over 500 markers mapped in the SES 208 genome (Da Silva et al. 1994) and on the proportion of single-dose to multiple-dose markers detected (Al-Janabi et al. 1993; Da Silva et al. 1993 1994). The cross analyzed was between SES 208 and ADP 85-0068, a doubled haploid derived from SES 208 by anther culture (Fitch and Moore 1983). To study the occurrence of polysomic inheritance in *Saccharum* and in sugarcane domestication, we used molecular markers to analyze meiotically regular, euploid progeny (2*n*=80) from a *S. officinarum* (2*n*=80)× *S. robustum* (2*n*=80) cross.

Materials and methods

Plant materials

S. officinarum 'LA Purple' (2*n*=80) was used as the female parent in a cross with *S. robustum* 'Mol 5829' (2*n*=80) and over 90 progeny were obtained at the Hawaiian Sugarcane Planters' Association (Aiea, Hawaii). The cytology of anther tissue from progeny and parents was determined (David Burner, USDA-ARS Houma, L., personal communication). Strict bivalent pairing and *n*=40 chromosomes were observed during meiosis, which was regular in both parents and in random samples of progeny. The mapping population consisted of 44 progeny from the LA Purple×Mol 5829 cross. Progeny are currently being scored for quantitative traits.

DNA extraction and manipulation

Genomic DNAs were extracted from mapping parents and progeny according to the method of Honeycutt et al. (1992). DNA concentration and quality were determined spectrophotometrically and standardized at 50 ng μl^{-1} in double-distilled, sterile H_2O. DNA quality and quantity were further checked on an agarose gel and final adjustments were made as required. A final dilution of 3 ng μl^{-1} was prepared in a 96-well format using an array of 1 ml tubes (Costar). This array was stored at 4°C and could be used indefinitely.

Arbitrarily primed PCR

The arbitrarily-primed PCR (Welsh and McClelland 1990; Williams et al. 1990) amplification protocol was used, as modified by Sobral and Honeycutt (1993) to include Stoffel fragment (Perkin-Elmer), a genetically-engineered variant of AmpliTaq (Perkin-Elmer). The PCR reaction mix was composed of 2 U of Stoffel fragment, 30 ng of template DNA, 0.22 μM of 10-mer primer, 0.1 mM of each dNTP, 4 mM $MgCl_2$ 10 mM KCl, and 10 mM Tris-HCl, pH 8.3, in a 30-μl reaction volume. Thermal cycling was done in a System 9600 Cycler (Perkin-Elmer) using the following thermal profile: 94°C/3 min, then 40 cycles of 94°C/1 min (denaturation), 35°C/1 min (annealing), increase to 72°C with a ramp of 0.41°C s^{-1}, and 72°C/2 min (extension). The temperature profile was terminated by a 72°C extension for 7 min and the amplified products were maintained at 12°C until ready to load onto an agarose gel. The 48-well array used with each primer contained two repetitions of genomic DNA from LA Purple and Mol 5829, and one repetition of each of the 44 progeny. Two repetitions of the parental templates were used to confirm the reproducibility of amplified fragments, and thereby avoid scoring of sporadic or faintly amplified fragments.

Table 1 Marker output for *S. officinarum* 'LA-Purple' × *S. robustum* 'Mol 5829'

Item	Parents	Progeny
Primers screened	160	45
Loci detected (45 primers) in LA Purple	738	–
Loci/primer	16.4	–
Polymorphisms scored	98	98
Single-dose markers (χ^2 @ 98%)	–	50
Loci detected (45 primers) in Mol 5829	720	–
Loci/primer	16.0	–
Polymorphisms scored	72	72
Single-dose marker (χ^2 @ 98%)	–	33

Primer screening and selection

One-hundred-sixty oligonucleotide primers of arbitrary sequence (10-mers, Operon Technologies, Inc.) were screened against the mapping parents. Forty five were selected and used on the progeny. The selection criteria were the number of polymorphisms per primer obtained from the primary screening of parents and the clarity (i.e., separation and intensity) of the amplified fragments. Marker output is summarized in Table 1.

Gel electrophoresis and scoring of amplified products

Amplified products were loaded in half-length (12.5 cm per tier) 1.7% LE agarose plus 0.3% NuSieve agarose gels (FMC). Gels were dissolved in 0.5×TBE (Maniatis et al. 1982) and run in a model HRH gel box (IBI) at 8 V cm^{-1} for 3 – 4 h. After electrophoresis, gels were stained with ethidium bromide (5 ng ml^{-1}) for 30 min, destained for 30 min (in 0.5×TBE), and photographed under UV light using an MP4 camera (Polaroid) and Polaroid type 55 film.

Polymorphic products were scored twice independently, directly from photographs. Polymorphisms were labeled according to the primer used for amplification, followed by a decimal point, then the number of base pairs of the amplified sequence (as estimated from a single gel measurement).

Segregation analysis and construction of linkage maps for *S. officinarum* and *S. robustum*

Single-dose markers were selected using a χ^2 test as described (Wu et al. 1992; Al-Janabi et al. 1993). The linkage relationships of single-dose markers were determined using Map Maker v 2.0 for the Mac (Lander et al. 1987). To search for repulsion-phase linkages, scores were inverted for single-dose markers; then, a joint database was established and used as input for Map Maker. Linkages between the first and second (inverted) groups of markers are in repulsion; their scores can be kept inverted, their original scores dropped, and subsequent Map Maker analyses will show linkages in repulsion (Al-Janabi et al. 1993; Da Silva et al. 1993, 1994). The process of inverting or flipping marker phase was also done using Map Manager v 2.5 (Manly 1993), which has a specific routine for phase inversion.

Results and Discussion

Linkage analysis of single-dose polymorphisms detected in progeny of a *S. officinarum*×*S. robustum* intercross showed that repulsion-phase linkages could be detected in both genomes using as few as 44 progeny, in contrast to results obtained for *S. spontaneum* 'SES 208' (Al-Janabi

et al. 1993; Da Silva et al. 1993, 1994). This would be a very unlikely result if these plants displayed polysomic inheritance because Wu et al. (1992) calculated that at least 750 progeny would be required to detect repulsion-phase linkages (at a 10 cM maximum detectable recombination fraction) in an auto-octoploid with strict bivalent pairing. So, our results indicate that *S. officinarum* and *S. robustum* do not behave like *S. spontaneum*. Table 2 shows mapping statistics for the *S. officinarum* and *S. robustum* genomes. Map distances using such a small number of progeny are tentative because they are associated with a high error (Table 2). Four of 13 pairwise linkages were in repulsion in *S. officinarum* as were 2 of 9 in *S. robustum*. Elimination of markers determined to be single dose using a significance level of 98%, but not considered single dose at a significance level of 90%, did not alter repulsion-phase linkages (data not shown), so it is unlikely that incorrectly classified markers were the cause of detection of repulsion-phase linkages.

The parents and progeny of this cross are euploid, $2n$=80 forms of these species, and meiosis is regular, with strict bivalent pairing and no aneuploidy (David Burner, USDA-ARS, Houma La, personal communication). We conclude that chromosome pairing is at least partially preferential, at least for some linkage groups. These genomes may display a mixture of disomic and polysomic inheritance, which suggests that both *S. officinarum* and *S. robustum* may be segmental allopolyploids. Detection of repulsion-phase linkages is determined by the type of meiotic chromosome pairing and assortment in each of the parents, as we scored the gametes of each parent directly. Disomic segregation with strict bivalent pairing is functionally a diploid situation. Our limited data does not suggest that disomic segregation is occurring in either of these species; however, complete polysomic inheritance can be ruled out by our results. It is possible that the genomes of these species are mosaic with respect to preferential pairing of chromosomes. If more progeny were studied, analysis of all linkage groups with a large number of markers could be used to determine the level of preferential pairing for each chromosome. The use of evenly spaced probes from the *S. spontaneum* map (Da Silva et al. 1994) would allow investigation of marker synteny across species as well.

Identification of repulsion-phase linkages with this few progeny and with so few markers is in stark contrast to the previous results of Al-Janabi et al. (1993) and Da Silva et al. (1993, 1994), who studied chromosome pairing and segregation in single-dose DNA-marker maps of *S. spontaneum* 'SES 208' ($2n$=64, from India). They failed to find a single linkage in repulsion phase using over 200 single-dose DNA markers (Al-Janabi et al. 1993), more than 200 single-dose RFLPs (Da Silva et al. 1993), or over 500 single-, double-, and triple- dose markers (Da Silva et al. 1994). Their mapping population was composed of 90 progeny (71 in common for the unified map) of a cross between ADP 85-0068×SES 208. ADP 85-0068 is a doubled haploid of SES 208, derived by anther culture (Fitch and Moore 1983). This means that repulsion-phase linkages, for perfectly-linked markers, would be undetectable because ADP 85-0068 is fixed for one or the other phase during chromosome doubling. However, because ADP 85-0068 is also a recombinational product of SES 208 (it is a doubled gamete), then all detectable repulsion phase linkages should be observed as a function of distance between markers (Da Silva et al. 1994). So, with over 500 markers and 71 common progeny, approximately 40 linkages in repulsion should have been observed if there were preferential chromosome pairing and assortment (Da Silva et al. 1994). The conclusion of polysomic inheritance in SES 208, with a possible autopolyploid origin, was reinforced by the observation of 16 bivalents and regular meiosis in haploids derived from SES 208 (Fitch and Moore 1983). The results presented herein suggest that *S. spontaneum* has a different genetic behavior from *S. robustum* and *S. officinarum*, because repulsion-phase linkages could be detected with so few progeny and such a small number of markers.

Chromosome pairing and assortment is of evolutionary and breeding interest. On evolutionary time scales, the maternal lineage of the '*Saccharum* complex' (Mukherjee 1957) shows little divergence; a common ancestor may have existed less than 5 million years ago (Sobral et al. 1994; Al-Janabi et al. 1994). However, many botanical characteristics of *Saccharum* are considered primitive (Stebbins 1956), which has resulted in the suggestion that it is an old genus (Celarier 1956; Burner 1991). In addition, the relative roles of autopolyploidy and allopolyploidy in the evolution of the complex and in the domestication of sugarcane have not been determined. Polysomic inheritance in *S. spontaneum*, if found to be generally in contrast to *S. robustum* and *S. officinarum*, would suggest evolutionary divergence between the lineages. It seems that the basic chromosome complement (x) for SES 208 is 8 (Al-Janabi et al. 1993; Da Silva et al. 1993, 1994); if additional data from *S. robustum* and *S. officinarum* genomes suggest x is different for these species that would also indicate evolutionary divergence.

From the breeder's perspective, modern sugarcane varieties are usually progenies of *S. officinarum* ($2n$=80)× *S. spontaneum* ($2n$=40-128) crosses, in which $2n+n$ transmission is regularly observed (known as "nobilization" by the sugarcane breeder because of rapid restoration of the "noble" or high-sucrose phenotype in backcrosses to *S. officinarum*). *S. spontaneum* chromosomes are preferentially lost in successive backcrosses (Parthasarathy 1948). Most current crosses are between modern commercial varieties that are at least three generations removed from the original interspecific hybridizations. Most modern commercial cultivars are interspecific hybrids with $2n$=100-160 and are frequently aneuploid (Burner and Legendre 1993). In such interspecific hybrids, both types of chromosome pairing and assortment may occur. Chromosome pairing and segregation have importance for QTL detection methods. Euploid *S. officinarum*×*S. robustum* progeny may frequently display regular pairing and disomic segregation for some linkage groups; in addition, such progeny may segregate for some of the most important agronomic traits of cultivated sugarcane, such as sucrose and fiber content, as well

Table 2 Statistical data for linkage of markers in LA Purple and Mol 5829 genomes[a]

Locus	Pre	Abs	X	N	Map	SE	95%	99%	LOD
S. officinarum 'LA Purple' Genome									
Linkage Group One									
U17.571	29	14	9	40	22.50	6.60	10.8–38.5	8.4–43.4	2.8
V17.818	26	15	9	40	22.50	6.60	10.8–38.5	8.4–3.4	2.8
V14.782	22	21							
Linkage Group Two									
P8.425	25	19	9	43	20.93	6.20	10.0–36.0	7.7–40.8	3.4
R14.1292	19	24	10	42	23.81	6.57	12.1–39.5	9.5–44.3	2.6
U18.682	28	15							
Linkage Group Three									
R10.302	20	24	2	44	4.54	3.14	0.6–15.5	0.2–19.4	9.7
T1.544	22	22	10	44	22.73	6.32	11.5–37.8	9.0–42.5	3.0
T9.487	24	20							
Linkage Group Four									
R19.603	24	20	3	42	7.14	3.97	1.5–19.5	0.8–23.8	7.9
V17.989	21	21							
Linkage Group Five									
U3.577	22	20	0	42	0.00	0.00	0.0–8.4	0.0–11.9	12.6
V8.910	22	22							
Linkage Group Six									
U12.945	19	24	1	41	2.44	2.41	0.1–12.9	0.0–16.8	10.3
V12.927	17	25							
Linkage Group Seven									
P5.620	20	23	9	43	20.93	6.20	10.0–36.0	7.7–40.8	3.4
I16.897	19	25							
S. robustum 'Mo5829' Genome									
Linkage Group one									
P20.565	22	22	12	42	28.57	6.97	15.7–44.6	12.7–49.4	1.7
U13.725	21	21	0	41	0.00	0.00	0.0–8.6	0.0–12.1	12.3
V13.749	21	22							
Linkage Group Two									
R15.331	19	24	8	43	18.60	5.93	8.4–33.4	6.3–38.1	4.0
V4.1384	24	20							
Linkage Group Three									
R15.214	26	16	9	42	21.43	6.33	10.3–36.8	7.9–41.6	3.2
U3.462	21	23							
Linkage Group Four									
R15.194	19	23	9	41	21.95	6.46	10.6–37.6	8.1–42.5	3.0
U10.556	17	26							
Linkage Group Five									
U18.1300	16	26	11	41	26.83	6.92	14.2–42.9	11.4–47.8	2.0
UA12.790	18	25	2	40	5.00	3.45	0.6–16.9	0.3–21.2	8.6
V12.750	20	21							

[a] Linkages were determined in Map Maker, as described (see Materials and methods), then exported into Map Manager v2.5 (Manly 1993) and statistics were calculated. Pre=# progeny showing present allele; Abs=# progeny showing absent allele; X=# of crossovers observed between adjacent loci; N=# of progeny for which data were analyzed (any remaining, that is, less than the 44 total typed, were missing data); Map=map distance, in % recombination; SE=standard error for map distance; 95%=range of map distance at the 95% confidence level; 99%=range of map distance at the 99% confidence level; LOD=L.O.D. score. Markers *V14.782* and *T9.487* were found to be linked to their respective groups only when their scores were inverted; therefore, they were in repulsion phase (Al-Janabi et al. 1993; Da Silva et al. 1993, 1993). Similarly, in *S. robustum*, marker *U18.1300* was linked in repulsion phase

as for other traits that differentiate *S. officinarum* from *S. robustum*. This would be particularly true if *S. robustum* were in fact the wild progenitor of sugarcane, as suggested by many (Brandes 1958; Grassl 1974 1977; Daniels 1973). Tagging of traits that differentiate *S. officinarum* from *S. robustum*, to understand their genetic basis, may be more easily accomplished in euploid, meiotically regular, *n+n* crosses, whereas it may be difficult to establish statistical correlations in existing commercial crosses with varying chromosome behavior. In this respect, metric data for sucrose and fiber phenotypes are currently being obtained for nearly 100 progeny of this cross. We will use these data to determine the genetic basis of sucrose accumulation and fiber phenotypes.

It is interesting to speculate about the possible involvement of genetic control of strict bivalent pairing in polyploid *Saccharum*. The promiscuity of *Saccharum* (de Wet et al. 1976; Gupta et al. 1978), along with the cytological observation that pairing is almost always in bivalents (Sreenivasan et al. 1987; Burner 1991), may suggest that genetic control was needed to stabilize auto- and allo-polyploidization events in such promiscuous species. Such a hypothetical gene would play a fundamental role in speciation within the *Saccharum* complex. However, the importance of vegetative reproduction in these species is not documented. If recruitment through seed is relatively unimportant under field conditions then the importance of sexual reproduction may be limited to allowing new combinations to arise so that gene flow between sympatric populations may be a small contributor to the genetic diversity of sugarcane germplasm. In fact, euploid ($2n$=80) genotypes of *S. officinarum* have never been observed in the "wild" and all germplasm of this species has been obtained from native Papuan gardens.

Acknowledgements We thank Paul Moore and K. K. Wu (Hawaiian Sugarcane Planters' Association, Aiea, Hawaii) for supplying cane tops from cross and shipping them to us for DNA extraction; Jorge da Silva (Copersucar, Brazil) for many fruitful discussions; David Burner (USDA-ARS Houma, LA.) for sharing unpublished data. This work was supported by a grant from the International Consortium for Sugarcane Biotechnology to B. W. S. S.

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