MAPPING OF SUGARCANE VARIETY M 134/75 USING EST-SSR MARKERS

By

Y. PARMESSUR, S. ALJANABI AND A. DOOKUN-SAUMTALLY

Mauritius Sugar Industry Research Institute, Réduit, Mauritius
yparmessur@msiri.intnet.mu

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Abstract

THE CONSTRUCTION of sugarcane linkage maps has been largely limited to the use of non-genic marker systems like AFLP and genomic SSRs. Moreover, map comparison among the *Saccharum* genus has been hampered by the lack of common markers. In this study, an AFLP- and genomic SSR-based linkage map of sugarcane variety M 134/75 consisting of 95 linkage groups (LGs) was enhanced with genic marker systems in order to further dissect the sugarcane genome and to enable intra- and inter-species genome comparisons. The ultimate aim of this project is to identify markers linked to the yellow spot disease resistance QTL and their introgression into sugarcane marker assisted breeding. The mapping parents M 134/75 and R 570 were screened with 25 sugarcane and 425 sorghum EST-SSR primers. On average, 1.5 polymorphic bands were detected for M 134/75 with sugarcane EST-SSR primers. Two hundred and ninety-one sorghum EST-SSR primers were found polymorphic for the mapping parent M 134/75, with an average of 2.6 polymorphisms per primer. The progeny population of 226 individuals was screened with five sugarcane and 37 sorghum EST-SSR primers. One hundred and two single dose coding markers were combined with 985 available non-coding markers and analysed by GMendel to produce a partial map of M 134/75 containing 143 linkage groups (LGs). This enabled integration of 58 sorghum EST-SSR markers into 26 LGs. Based on common SSRs, LGs were grouped into ten homologous groups (HGs), among which four contained sorghum EST-SSRs. LG 68 containing the yellow spot resistance QTL was equated to HG VIII and was enhanced by three sorghum markers. Comparative mapping will be exploited to fine-map the yellow spot disease resistance QTL.

Introduction

The construction of sugarcane linkage maps has been largely limited to the use of non-genic marker systems like AFLP and genomic SSRs (gSSR). Intra-species and intra-genus comparisons have not been possible using these markers, as they were non-coding and locus unspecific.

Over the past decade, there has been an exponential increase in the availability of expressed sequence tags (EST) sequence data from a wide variety of taxa.

This abundance of EST sequences has become an attractive potential source of microsatellite markers after the findings that gene transcripts can also contain SSRs (Kantety *et al*., 2002). Deriving SSR markers from EST sequences (EST-SSRs) has an intrinsic advantage over gSSR because they can be easily obtained by data mining of EST databases.

This is a much cheaper alternative to the conventional isolation and characterisation of gSSRs via DNA libraries and is also less time consuming. The usefulness of these genic SSRs also lies in their cross-species transferability because the primers are from coding sequences that are more conserved across species. These EST-SSRs are also useful as anchor markers for comparative

mapping and have the added advantage of being directly associated with traits of interest. Hence, they provide a more targeted approach to identification of markers linked to genes or quantitative trait loci (QTL).

Over the past few years, several projects for the sequencing of sugarcane ESTs have been initiated in South Africa (Carson *et al*., 2000), Brazil (http: //sucest.lad.ic.unicamp.br/en/) and Australia (Casu *et al*., 2001), allowing the development of EST-SSRs for the *Saccharum* genus (Cordeiro *et al*., 2001; Pinto *et al.*, 2004).

The application of sugarcane EST-SSR for diversity studies has been previously reported (Cordeiro *et al*., 2001), but its use in genetic mapping of sugarcane has only been recently reported (Oliveira *et al*., 2007). In this work, 149 EST-SSRs were used in the construction of an integrated sugarcane map derived from a cross between Brazilian hybrid cultivars SP80-180 and SP80-4966. Combined with RFLP and AFLP marker data, a linkage map was constructed containing 149 functionally associated EST-SSR markers scattered among 79 LGs.

At the Mauritius Sugar Industry Research Institute (MSIRI), emphasis is being placed on the integration of marker assisted selection (MAS) into its sugarcane breeding program. The aim of this study is to identify markers linked to putative sugarcane yellow spot (YS) disease resistance gene(s).

This is a major disease of sugarcane and can account for 10–25% reduction in sucrose content among susceptible varieties grown in the superhumid zones of Mauritius. It is caused by the fungus *Mycovellosiella koepkei.* The selection for resistant varieties in the current breeding program is costly, time-consuming and not fully reliable as disease expression is dependent on abiotic factors such as weather conditions and high disease pressure.

In a previous study (Aljanabi *et al*., 2007), a population of 227 progenies derived from a bi-parental cross between a yellow spot disease resistant parent (M 134/75) and a susceptible parent (R 570) was screened with AFLP and gSSR. A genetic linkage map was constructed for variety M 134/75 containing 95 LGs onto which 567 single dose markers were mapped.

Field disease resistance data of the mapping population scored over two ratoon crops coupled with molecular marker data enabled the identification of a major quantitative trait locus (QTL) responsible for 23.8% field phenotypic variation in the trait. This QTL is flanked on one side by an AFLP marker actctc10 and an SSR marker mSSCIR12284 at 14 cM and 18 cM respectively. These markers are, however, not close enough to the yellow spot QTL to validate their use in sugarcane MAS. The ultimate aim of this project is to identify markers located at approximately 5 cM to this QTL that can be used for MAS.

Map enhancement of M 134/75 was thus considered from a set of sugarcane EST-SSR primers (sequences, kindly provided by E. Ulian). The use of orthologous EST-SSR was also investigated to facilitate the comparative mapping of sugarcane to closely related species. The most appropriate candidate is sorghum, which diverged from a common ancestor 8–9 million years ago (Jannoo *et al*., 2007). Sorghum RFLP probes have been previously used in sugarcane mapping and have shown perfect synteny with sugarcane (Guimares *et al*., 1997; Ming *et al*., 1998). The application of EST-SSR marker systems to the linkage mapping of sugarcane is thus being reported.

Materials and methods

DNA was extracted from mapping parents M 134/75 and R 570 and their 226 mapping progeny population as described by Aljanabi *et al*. (1999). Resuspended DNA was diluted to 20 ng /µL for further use.

Sugarcane EST-SSR PCR

The mapping parents M 134/75 and R 570 were screened with 25 sugarcane EST-SSR primers by PCR in 20 µL reaction volumes using the following conditions; 1X reaction buffer

(Roche), 0.4 µM dNTPs (Roche), 1 unit Taq polymerase (Roche), 0.4 µM forward primer, 0.4 µM ^{33}P labelled reverse primer and 50 ng DNA template. PCR conditions were as follows: an initial denaturation at 94°C for 3 min followed by 32 cycles at 94°C, annealing at 54°C and extension at 72°C each for 1 min and a final extension at 72°C for 5 min.

Selected polymorphic primers (5) were used to genotype the mapping population. PCR products were run for 2 h on 6% polyacrylamide gels, pre-warmed at 60°C. Gels were blotted on filter paper, vacuum dried and exposed to X-ray films (Kodak X-OMAT). Depending on the intensity of the ^{33}P signal, films were developed 2–5 days after exposure.

Sorghum EST-SSR primer testing

Two sets of sorghum EST-SSR were used, 30 primer pairs (SOR1-30) previously used in a cross-species transferability study (Wang *et al*., 2005) and 600 primer pairs (SAT0100-SAT1250) from ICRISAT (sequences kindly provided by Dr T. Hash) were initially tested for cross-species transferability at an annealing temperature of 50°C by PCR in 20 µL reaction volumes using the same reaction mix as above.

PCR conditions were as follows: an initial denaturation at 94°C for 3 min followed by 32 cycles at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C each for 30 s and a final extension at 72°C for 5 min. The PCR products were run on agarose gels (2%) and visualised over a UV transilluminator after staining with ethidium bromide.

The SOR primers were screened for polymorphism with the mapping parents and five selected primers were used for population genotyping. The SAT primers were also tested for cross-species transferability and 425 initially selected primers were used to assess the level of polymorphism between the mapping parents.

Thirty-two out of 134 primers showing distinct profiles with prominent bands were further used for population genotyping on polyacrylamide gels.

Marker scoring and nomenclature

A pseudo-testcross strategy was followed to score the polymorphisms (Grattapaglia and Sederrof, 1994). The bands were scored for the presence or absence when heterozygous in M 134/75, null in R 570 and segregating progeny population.

Each marker was given a unique identifier as follows: the first three letters referred to the origin of the primer; SOR or SAT). For SOR markers, the next digit or two digits referred to the primer number (1–30) and the last digit referred to the allele number (eg SOR 202 refers to allele number 2 from primer SOR20). For ICRISAT markers, the 4 digits next to SAT referred to the primer number and the last digit referred to the allele number.

Alleles from the same primer were numbered according to the position of the marker on the gels depending on their molecular weights. The highest molecular weight polymorphic marker was named as *Primername*1 and sequentially increased by one unit with decreasing sizes.

Likewise, sugarcane EST-SSR markers were named according to the name of the primer ESTSC or ESTD followed by the allele number. AFLP and gSSR markers nomenclature was as described as Aljanabi *et al*. (2007).

Linkage map construction and homology grouping

A chi-square test was performed to select for single dose markers i.e those segregating in a 1:1 ratio. GMendel (Holloway and Knapp, 1994) from iMAS platform (an integrated marker assisted selection software http://localhost:8080/iMAS) was used for linkage map construction.

All markers with more than 15% missing values and genotypes (5) missing at >15% were excluded from the analysis. Two-point analyses between single dose markers were performed at a LOD score threshold of five and recombination fraction threshold of 0.30. Markers within each

linkage group (LG) were then ordered by using the Kosambi mapping function. The LGs were graphically represented using Mapchart 2.1 (Voorrips, 2002). LGs were pooled into the same HGs when they had at least two common SSR markers.

A number of LGs with only one common SSR marker were grouped into unassigned HGs (UAHG). The nomenclature of the HGs was in accordance to Rossi *et al*. (2003) whenever possible.

Results

Among the twenty-five sugarcane EST-SSR primers, only 12 primers (48%) were found polymorphic. The average level of polymorphism for the resistant parent M 134/75 was only 1.5 as compared to 2.0 for gSSR (unpublished data). Twelve markers were scored after genotyping with five EST-SSR primers.

All SOR primers were transferable to sugarcane as observed on agarose following amplification, whereas 95% of SAT primers correspondingly amplified an orthologous target in sugarcane.

As compared to sorghum, sugarcane amplification with sorghum EST-SSR primers showed a more complex profile.

A higher number of amplicons and a wider distribution of amplicons along the gels were observed when sorghum primers were used on sugarcane. In most cases, the level of polymorphism is higher in sugarcane than in sorghum probably due to the higher ploidy level of the sugarcane genome.

There was also no apparent size correlation between sorghum and sugarcane amplicons (data kindly provided by R. Punna, ICRISAT).

Sixteen SOR primers produced markers polymorphic to M 134/75 revealing an average 2.3 polymorphisms. Among the 425 SAT primers screened, 275 primers were found polymorphic for the resistant parent M 134/75. These revealed 712 polymorphisms averaging 2.6 polymorphisms per primer.

The 32 SAT primers used to genotype the mapping population revealed 102 polymorphisms. These were combined with 13 SOR markers and 12 sugarcane EST-SSR markers. The 127 markers were trimmed to 102 markers segregating into a 1:1 ratio. These data were pooled with existing 985 non-coding AFLP and gSSR primers to produce a combined marker data set of 1087 markers.

The final data were analysed by GMendel from iMAS. A partial genetic map of sugarcane hybrid cv M 134/75 was produced containing 640 markers, 143 LGs and with a total map length of 9530 cM. Among these, 58 EST markers were mapped into 26 LGs, 8 of which (30%) contained solely EST-SSR markers.

Grouping of LGs into homologous groups was carried out according to Rossi *et al*. (2003) whenever applicable. Using this nomenclature system, LGs belonging to HG I, II, III, VI, VII and VIII were identified and assigned to their respective HG.

No grouping was possible into HG IV and HG V since no mSSCIR marker (Table 1) has been assigned to these HGs. Using the system of grouping of LGs containing at least two common SSR markers, four additional HGs were identified, HG IX –HG XII. LGs containing only one common SSR marker were classified into five unassigned HGs (UAHG 1-UAHG 5).

This method of nomenclature enabled classification of EST-SSR from 26 primers into four HGs, II, III, VIII and X.

Markers from only two primers were shared between HGs i.e markers SAT0918 and SAT0203 were present in both HG II and HG VIII.

Twenty-two LGs were classified into HG VIII, which represented the highest frequency found in any HG.

Table 1—Classification of LGs according Rossi *et al*. (2003) and identification of EST-SSR markers belonging to their respective HG. (Classification according to: a = Rossi *et al*., 2003, b = Aljanabi *et al*. 2007, na = not available).

	mSSCIR	Initial M 134/75 HG (b)	New M 134/75 HG	M 134/75 mSSCIR (if any)	M 134/75 EST-SSR
I	14,19,27,42, 52, 53	II	I	19	–
II	34, 35, 39, 41, 48	XII	II	35	SAT0635, SAT0606, SAT0345, SOR17, SAT0604, SAT02032, SAT0918, SAT0632, SAT0134, SAT0341
III	64	VII	III	64	ESTD16, SOR1, SAT0502, ESTD72
IV	n.a	IV	na		
V	n.a	V	na		–
VI	10,37,47, 54, 60	I	VI	60	–
VII	21,36,43	I	VII	21	–
VIII	12, 17, 18, 26, 28, 38, 46	II	VIII	12	SAT609, SOR20, SAT0918, SAT0623, SAT0632, SAT0118, SAT0604, SAT0617, SAT0619, SAT0203, SAT1225, SAT0612, ESTSC23,
–	–	XV	IX	M1011 M1288 M2019 M1084 M1825	
–	–	VI	X	11, 39	SAT0103
–		XX	XI	M1075HA, 1237FL, M1282FL	
		V	XII	M 765	
–	–	XIV	UAHG1	M1807	
–	–	XVI	UAHG2	M1069	
–	–	XI	UAHG3	16	
–	–	XIII	UAHG4	M1116	
–	–	XI	UAHG5	M119G	

In a previous work (Aljanabi *et al.* 2007), a putative QTL for resistance to yellow spot was identified on LG87 flanked by markers Actctc10 and CIR12284 (Figure 1). In this study, this LG has been enhanced with three sorghum EST-SSR markers SAT1225, SAT0203 and SAT0612 and sugarcane ESTSC2 of newly named LG68 classified into HG VIII (Figure 1).

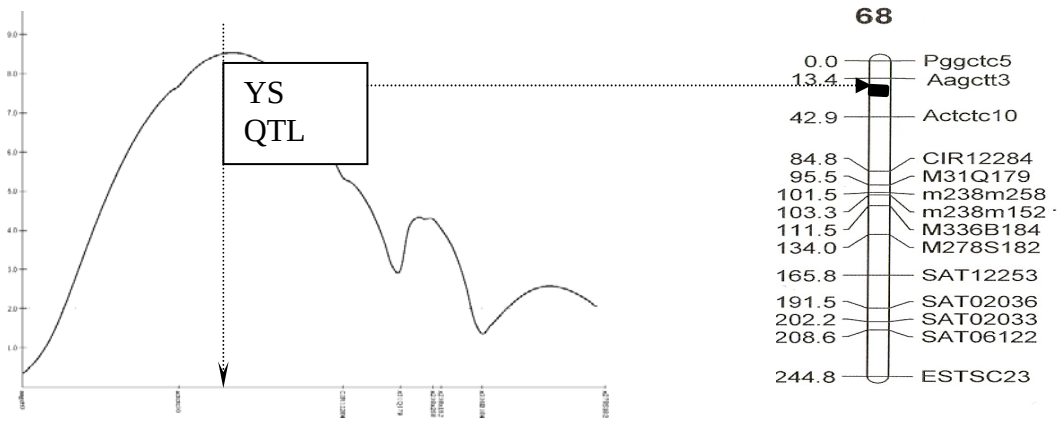

Fig. 1—Map enhancement of LG 68 harbouring the YS resistance QTL with sorghum EST-SSR primers SAT1225, SAT0203 and SAT0612 and sugarcane derived EST-SSR primer ESTSC2.

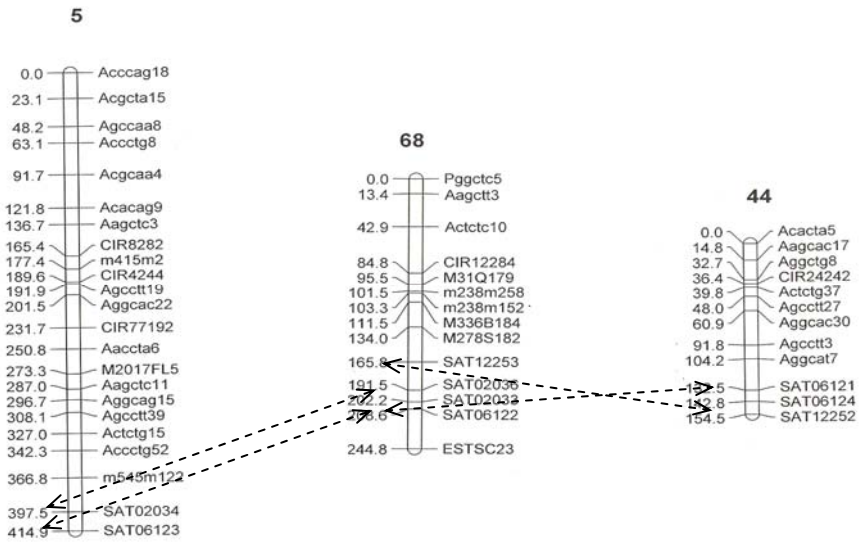

Fig. 2—Synteny across sugarcane LG based on sorghum EST-SSR markers. Arrows shows marker linkage conservation between markers SAT1225, SAT0203 and SAT0612 on LG 5, LG 68 and LG 44.

Synteny across the LGs is exemplified between markers of LG 5, LG 44 and LG 68 (Figure 2). SAT0203 and SAT0612 are linked on LG 5, SAT0612 and SAT1225 are linked on LG 44. All three markers are linked on LG 68.

Discussion

The use of sugarcane EST-SSR in the genetic mapping of sugarcane has so far been reported by Oliveira *et al*. (2007) whereby an integrated map of sugarcane was constructed from a cross between hybrid cultivars SP80-180 and SP80-4966.

The application of sugarcane EST-SSR mapping to variety M 134/75 was investigated from a set of 25 primers. However, a low level of polymorphisms was detected between the mapping parents. From 12 sugarcane EST-SSR SDMs scored, six (50%) were successfully assigned to LGs. On average, the same percentage of integration was observed by Oliveira *et al*. (2007).

The use of sorghum EST-SSR in the mapping of sugarcane has not been described before. Documentation is available mostly on cross-transferability studies and their potential use in diversity studies and linkage mapping. In a similar study (Wang *et al*., 2005), the transfer rate EST-SSR from hexaploid wheat to tetraploid wheat was found to be 80% despite them belonging to the same genus.

The transfer rate from sorghum to maize (both belonging to the same family –Panicoideae) was only 61%. The few sorghum EST-SSR primers (less than 5%) that could not be transferred to sugarcane could be attributed to sequence divergence between the two genera or to experimental errors. Average polymorphism level of 2.3 and 2.6 for SOR and SAT primers respectively indicated excellent potential of such orthologous marker systems applied to sugarcane mapping.

Sorghum EST-SSR primers produced a complex PCR profile with several bands when amplifying sugarcane as compared to sorghum where only 1–2 amplicons can be observed. This complexity can be attributed to the polyploid genome of sugarcane. With eight genomes contributed by *S. officinarum* and combined to the genome contributed by *S. spontaneum*, at least nine 'homoeologous' regions can be targeted by the primers.

Also, since target regions may vary in sequence between sorghum and sugarcane combined with a low annealing temperature (50°C), primers could be targeting non-orthologous regions adding to the complexity of the profile. Finally, variation in the number of repeats coupled with the

presence of indels/introns of different sizes across species could explain the lack of correlation in fragment size between sorghum and sugarcane.

Data from 102 single dose EST-SSR (sugarcane and sorghum) markers were combined with existing 985 non-coding AFLP and gSSR markers to produce a partial genetic map of sugarcane variety M 134/75 of 143 LGs. By grouping together LGs carrying common SSR markers as described by Rossi *et al*. (2003), it has been possible to equate the LGs into their respective HGs. Using this approach, LGs were assigned to HGs I, II, III, VI, VII and VIII. LGs belonging to HG IV and V were not identified due to lack of common gSSR markers. LG 68, harbouring the yellow spot disease resistance QTL was thus assigned into group VIII.

Of interest is that a sugarcane rust resistance gene was located on LG 3 of an R 570 map, which also belongs to HG VIII and where three resistance gene analogue clusters are also present (Raboin *et al*., 2006). Now that the location of the yellow spot disease resistance QTL has been confined to HG VIII, more effort can be channelled into the mapping of markers known to be located in this HG.

A framework of markers common to HG VIII is already available (Piperidis *et al*., 2008) after comparison of genetic maps of varieties Q 165, Q117 and MQ77-340 to R 570. This will be used to enhance LGs assigned to HG VIII of M 134/75 instead of targeting the sugarcane cv M 134/75 entire genome.

LG 68 harbouring the yellow spot disease resistance QTL has also been enhanced by three SAT markers i.e SAT1225, SAT0203 and SAT0612. The nomenclature of these SSR markers is based on their location on the rice chromosomes, i.e the first two digits of the primer name represents the EST location on the rice chromosome (R. Punna, personal communication).

In this respect, fragments of rice chromosomes 12, 2 and 6 could be collinear to the regions within LG 68. Channelling efforts into the mapping of a maximum of sorghum EST-SSR primers along these 3 series of primers (i.e SAT1201-1250, SAT0601-0650 and SAT0200-0250) could further enhance this LG and identify markers closer to the yellow spot gene.

However, synteny between rice and sugarcane is known to have been disrupted by a number of rearrangements and translocations (Asnaghi *et al*., 2000). A more accurate alternative is to identify the region into sorghum harbouring the SAT1225, SAT0203 and SAT0612 markers and eventually to describe the collinear region in the vicinity of the yellow spot gene.

A detailed study of LGs 5, 44 and 68 belonging to HG VIII provides an interesting example of the conservation of gene order among different chromosomes. LG 5 harbours markers SAT0203 and SAT0612, LG 44 links SAT1225 and SAT0612. LG 68 harbours all three SAT markers i.e SAT1225, SAT0612 and SAT0203 thus showing the conserved gene order between these three markers.

Therefore, through the use of sorghum EST-SSR markers, it has been possible to demonstrate the existence of collinearity among sugarcane homoeologous chromosomes and confirms the fact that comparative genetics remains a powerful tool to study genome organisation especially in complex polyploids like sugarcane.

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L'UTILISATION DES MARQUEURS EST-SSR POUR CARTOGRAPHIER LA VARIÉTÉ M 134/75 DE CANNE À SUCRE

Par

Y. PARMESSUR, S. ALJANABI et A. DOOKUN-SAUMTALLY
Mauritius Sugar Industry Research Institute, Réduit, Mauritius
yparmessur@msiri.intnet.mu

MOTS-CLÉS: EST-SSR, Carte Génétique, Canne à Sucre, Taches Jaunes.

Résumé

LA CONSTRUCTION de cartes génétiques de canne à sucre a été largement limitée à l'utilisation des marqueurs non-géniques tels les AFLP et les SSR génomiques. En outre, la comparaison entre les cartes génétiques du genre *Sacchurum* pose des difficultés en raison de l'absence des marqueurs en commun. Dans cette étude, une carte génétique de la variété M 134/75 de canne à sucre réalisée avec les marqueurs AFLP et SSR-génomiques et composée de 95 groupes de liaison a été couplée avec des marqueurs géniques dans le but de disséquer le génome de la canne à sucre et de permettre des comparaisons génomiques intra- et inter- spécifiques. Le but ultime de ce projet consiste à identifier des marqueurs liés au QTL de résistance de la maladie de taches jaunes (yellow spot) et à leur application dans la sélection assistée par marqueurs. Les parents M 134/75 et R 570 ont été criblés avec 25 amorces EST-SSR dérivées des séquences de canne à sucre et 425 du sorgho. En moyenne, 1.5 bandes polymorphes ont été observées pour la variété M 134/75 avec les amorces EST- canne à sucre. Au total 291 amorces EST-SSR-sorgho ont produit des bandes polymorphes dans la variété M 134/75, avec une moyenne de 2.6 polymorphismes par amorce. Une descendance de 226 progénitures a été évaluée avec cinq amorces EST-SSR dérivées de la canne à sucre et 37 du sorgho. Cent deux marqueurs EST-SSR qui ségréguaient selon un ratio de 1:1 (simplex) ont été couplés avec 985 marqueurs non codant et les résultats analysés à l'aide du logiciel Gmendel. Une carte partielle de la variété M 134/75 comprenant 143 groupes de liaison a été réalisée. Cela a permis l'intégration de 58 marqueurs EST-SSR du sorgho dans 26 groupes de liaison génétique. En comparant les marqueurs SSR communs, les groupes de liaison ont été rassemblés en dix groupes homologues, parmi lesquels quatre comprenaient des marqueurs EST-SSR du sorgho. Le groupe de liaison 68 sur lequel le QTL pour la résistance aux taches jaunes est présent, a été aligné avec le groupe homologue VIII (HG VIII). Trois marqueurs EST du sorgho ont aussi été placés sur le groupe de liaison 68. La cartographie comparative sera exploitée pour une cartographie fine du QTL responsable pour la résistance aux taches jaunes.

CARTOGRAFÍA DE LA VARIEDAD M 134/75 DE CAÑA DE AZÚCAR USANDO MARCADORES EST-SSR

Por

Y. PARMESSUR, S. ALJANABI y A. DOOKUN-SAUMTALLY

Instituto de Investigación de la Industria del Azúcar de Mauricio, Réduit, Mauricio
yparmessur@msiri.intnet.mu

PALABRAS CLAVE: EST-SSR, Mapa de Ligamiento, Caña de Azúcar y Enfermedad de la Mancha Amarilla.

Resumen

LA CONSTRUCCIÓN de mapas de ligamiento de caña de azúcar ha sido limitada en gran medida por el uso de sistemas no génicos, como los AFLP y los genómicos SSR. En adición, la comparación de mapas entre el género *Saccharum* ha sido obstaculizado por la falta de marcadores en común. En este estudio, el mapa de ligamiento de la variedad M 134/75 de la caña de azúcar (construído con marcadores AFLP y SSR) consistente de 95 grupos de ligamiento (GL) fue mejorado con sistemas de marcadores génicos a fin de disectar, subsecuentemente, al genomio de la caña de azúcar y hacer posible comparaciones genómicas dentro y entre especies. El objetivo esencial de este proyecto es identificar marcadores asociados con los QTL de la resistencia a la enfermedad de la mancha amarilla y su introgresión en el fitomejoramiento asistido con marcadores de la caña de azúcar. La criba de los progenitores usados en la cartografía, M 134/75 y R 570, fue efectuada con 25 iniciadores (*primers*) EST-SSR de la caña de azúcar y 425 del sorgo. En promedio, 1.5 bandas polimórficas fueron detectadas para el M 134/75 con iniciadores EST-SSR de la caña de azúcar. Doscientos noventa EST-SSR iniciadores de sorgo fueron identificados como polimórficos para el progenitor M 134/75, con un promedio de 2.6 polimorfismos por iniciador. La progenie que constituyó la población de 226 individuos fue evaluada con cinco iniciadores EST-SSR de la caña de azúcar y 37 de sorgo. Ciento dos marcadores que codifican para una sola secuencia fueron combinados con 985 marcadores de no codificación que se encontraban disponibles, los cuales fueron analizados con la aplicación GMendel para producir un mapa parcial de M 134/75 constituído por 143 GL. Esto hizo posible la integración de 58 marcadores EST-SSR de sorgo con 26 GL. Con base en los SSR en común, los GL fueron agrupados en diez grupos homólogos (GH), entre los que se encontraban cuatro EST-SSR de sorgo. Los 68 GL que contenían los QTL de resistencia a la mancha amarilla fueron equiparados al GH VIII, el cual fue adicionado con tres marcadores del sorgo. La cartografía comparada será aprovechada en la cartografía fina de los QTL de la resistencia a la enfermedad de la mancha amarilla.