Theor Appl Genet (2004) 108:759–764
DOI 10.1007/s00122-003-1487-6

ORIGINAL PAPER

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Targeted mapping of a sugarcane rust resistance gene (*Bru*1) using bulked segregant analysis and AFLP markers

Received: 28 May 2003 / Accepted: 28 August 2003 / Published online: 28 October 2003

Abstract The presence of a major resistance gene (*Bru*1) for brown rust in the sugarcane cultivar R570 (2n about 115) was confirmed by analyzing segregation of rust resistance in a large population of 658 individuals, derived from selfing of clone R570. A subset of this population was analyzed with AFLP and bulked segregant analysis (BSA) to develop a detailed genetic map around the resistance gene. Four hundred and forty three primer pairs were used resulting in the identification of eight AFLP markers surrounding the resistance gene in an interval of 10 cM, with the closest markers located at 1.9 and 2.2 cM on each side of the gene. Efficiency of the AFLP/BSA applied to the complex polyploid genome of sugarcane is discussed, as well as the potential of the newly identified AFLP markers for developing a map-based cloning approach exploiting, synteny conservation with sorghum.

Introduction

The common rust of sugarcane, recently renamed brown rust (http://www.isppweb.org/names_common.asp), is a fungal disease caused by *Puccinia melanocephala* and present in almost all sugarcane growing areas. The development of the disease affects yield, and losses as high as 50% have been registered in Mexico (1981–82) for the susceptible cultivar B4362 (Purdy et al. 1983, cited by Comstock et al. 1992a). The most efficient way to control the propagation of the disease is to use resistant cultivars. Sugarcane cultivars have a complex genome structure. They are highly polyploids, aneuploids with around 120 chromosomes, among which the majority derive from the sugar-producing species *S. officinarum* (2n=80) and 15 to 25% derive from the wild species *Saccharamyces spontaneum* (2n=40 to 128) (D'Hont et al. 1996). Despite this complex genome structure, rust resistance has a moderate (Hogarth et al. 1993; Ramdoyal et al. 2000) to high heritability (Comstock et al. 1992b), and breeding for resistance was successful and rapid. Genetic determinism of rust resistance was studied on a population of 141 progenies, derived from the selfing of the resistant cultivar R570, and revealed a 3:1 segregation ratio (resistant:susceptible) indicative of a monogenic and dominant control involving a single copy of the resistance allele in R570 (Daugrois et al. 1996). The major gene was located on the R570 RFLP genetic map developed by Grivet et al. (1996). It was linked to one marker revealed by the sugarcane probe CDSR29, itself left unassigned to any co-segregation group.

This source of resistance is of particular interest. It is the first well characterized Mendelian trait described in the complex genomic context of sugarcane. Furthermore, tests under controlled conditions demonstrated that this gene provides resistance against various rust isolates collected in different geographic areas (Asnaghi et al. 2001), and resistance breakdown has never been observed despite an intensive culture of R570 in Réunion Island and various other places for 20 years.

This gene is used as a target to experience map-based cloning in the particularly complex genome of sugarcane. This issue implicated the development of a BAC library of cultivar R570 (Tomkins et al. 1999) and a fine map focused on the region surrounding the gene. For this latter purpose, a large population composed of 695 individuals derived from R570 selfing was produced and evaluated in

Communicated by H. F. Linskens

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the field for rust resistance. One of the main challenges in developing a map-based cloning approach in such a high polyploid is that the target area for fine mapping, around 2 cM, represents around 1/10,000 of the estimated size of the 17,000-cM genetic map (Hoarau et al. 2001) as compared to around 1/1,000 for a typical diploid species, such as sorghum, with a genetic map of 1,500 cM.

Two strategies were used to saturate the target area with markers. The first one exploited the conservation of synteny and colinearity between sugarcane, sorghum, maize and rice (D'Hont et al. 1994; Grivet et al. 1994; Dufour et al. 1996, 1997; Glaszmann et al. 1997; Ming et al. 1998). This approach resulted in locating the gene at the edge of a co-segregation group of 19 restriction fragment length polymorphism (RFLP) markers belonging to homology group VII (Asnaghi et al. 2000). However, none of these mapped loci were located closer to the gene than CDSR29, which thus appears to reside at the frontier of a 'synteny cluster' used to describe the different grass genomes.

The second strategy, described in this paper, was to take advantage of the high throughput amplified fragment length polymorphism (AFLP), genotyping technology and bulked segregant analysis (BSA) (Michelmore et al. 1991). In addition, AFLP markers were provided indirectly in the target area by positioning the rust locus on a high density map constructed for the genetic analysis of sugar yield components (Hoarau et al. 2001).

Materials and methods

Plant material

R570 is the main cultivar grown in Réunion Island, and it is included in many breeding programs worldwide. This cultivar was developed by the Centre d'Essai de Recherche et de Formation (CERF) in Réunion Island from a cross between H32-8560 and R445, both resistant to rust. A first population (population A) derived from the selfing of R570 was used originally to demonstrate the existence of a major resistance gene to brown rust in R570 (Daugrois et al. 1996), and to construct an RFLP map (Grivet et al. 1996).

A new population (population B), of 695 individuals derived from the selfing of R570, was developed to confirm the presence of a major resistance gene and for the fine linkage analysis. This population was divided in two subsets (population B1:295 individuals and population B2:400 individuals), evaluated for rust resistance separately in two different trials. In addition, progenies from four bi-parental crosses involving R570 as the male parent and four susceptible cultivars as the female parents were studied in order to confirm the Mendelian inheritance of the R570 resistance gene. These progenies include 240 clones from the cross M555/60×R570, 642 clones from the cross Triton×R570, 851 clones from the cross BT 64/134×R570 and 369 clones from the cross R94/167×R570, a non-commercial clone from CERF.

Field evaluation of rust resistance

The field reaction to rust was determined on Réunion Island using natural infestation. Both populations were evaluated in a randomized complete block design with three replicates on the CERF experimental stations, station Le Gol for Population 1 and station La Mare for population B2. For Population B1, each basic plot was composed of one row 1.25 m long (four cuttings). For population B2, each basic plot was composed of a single row 2 m long. Scores were noted in the plant-cane and first ratoon in 1996 and 1997 for both populations.

Rust resistance was scored on each plot on a 1 (the most resistant) to 9 (the most susceptible) scale, according to Tai et al. (1981). This scale is based on visual assessment of the disease symptoms. A score of 1 indicates resistant plants with no sporulation of the fungus, but some necrotic or chlorotic spots may appear on the leaves. A score of 2 indicates very rare sporulating lesions on old leaves. From grade 2 to 9, the density of sporulating lesions increases, as well as their apparition on younger leaves. Segregation analysis was performed on the mean score computed over replications for each progeny clone.

The bi-parental progenies were studied in the field without any particular statistical design, with seedlings planted 50 cm apart. In these crosses, rust resistance was scored for each individual seedling on the presence/absence of sporulations. Seedlings bearing sporulating pustules were classified as susceptible, otherwise they were classified as resistant.

AFLP protocol

Total genomic DNA was extracted from fresh leaves according to the method described by Hoisington (1992). AFLP analysis (Vos et al. 1995) was performed using the GIBCO BRL kit genome I and additional designed primers. DNA bulks were composed of 10 ng of DNA extracted from five different individuals (2 ng per individual) and were treated as a unique sample in the different steps of the AFLP process. The procedure followed manufacturer's recommendations with a few modifications (Hoarau et al. 2001). Genomic DNA was digested using restriction enzymes *Eco*RI and *Mse*I or *Pst*I and *Mse*I. Pre-amplification products were diluted (in water, Merck) 1:25 for individuals or 1:20 for bulks. Gels were exposed to Kodak Biomax X-ray film. Marker names are composed of six letters corresponding to two sets of the three selective nucleotides used in the PCR reactions. Some marker names are also supplemented with a number, corresponding to the band of the AFLP profile.

Genetic mapping

AFLP markers were mapped according to the following procedure: (1) each band was encoded as a dominant marker (presence vs absence); (2) single-dose markers, used for genetic mapping in polyploid species (Wu et al. 1992), were selected based on their segregation ratio as described in Grivet et al. (1996). (3) linkage analysis of the single-dose markers were performed with the Mapmaker 3.0 program (Lander et al. 1987). Co-segregation groups were generated at a LOD score of 5 and ordered with multipoint analyses. Distances between markers were calculated with the Haldane mapping function. Sugarcane cultivars are highly heterozygous, so progenies derived from selfing can be treated as F2 progenies in mapping analysis.

Results

Field evaluation of rust resistance

Eleven clones from population B1, and 26 clones from population B2, were excluded from the analysis since they had too many missing data. The design was complete for 222 progeny clones out of 284 in population B1 and for 309 progeny clones out of 374 in population B2, with three replicates observed over 2 years. Mean scores are plotted on Figs 1A and 1B for this subset of progeny

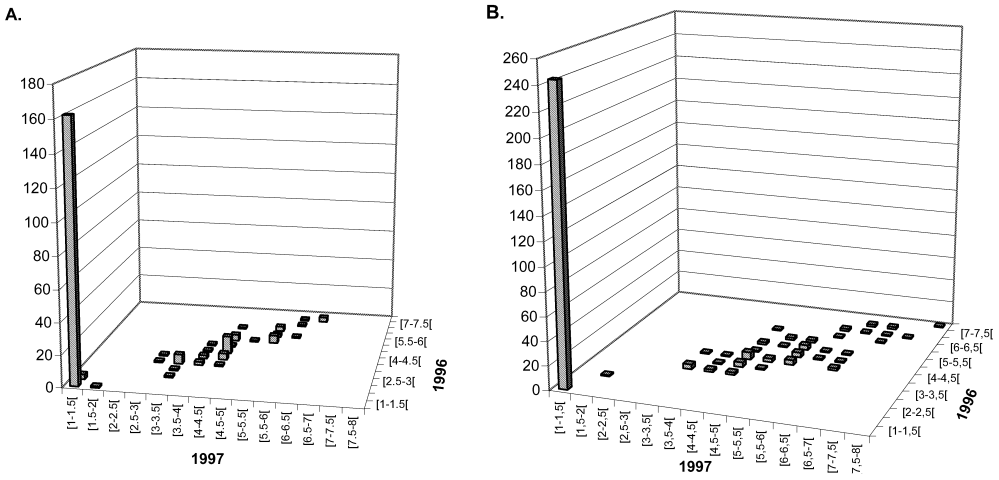

Fig. 1 Distribution of mean rust resistance scores in 1996 and 1997 for a subset of the populations with no missing data. **A** Population B1 (222 clones), **B** Population B2 (309 clones)

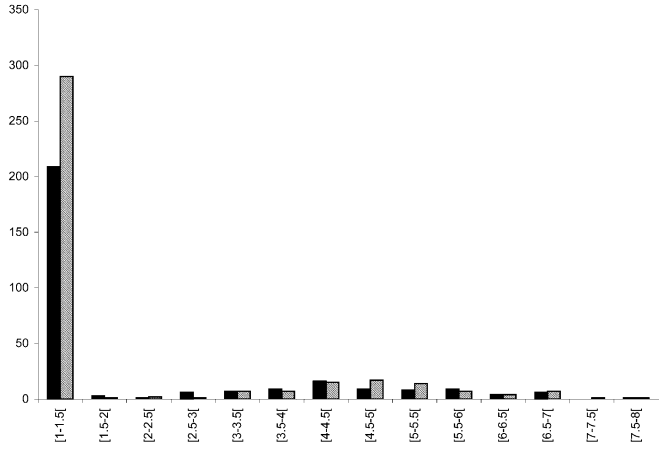

Fig. 2 Distribution of rust resistance mean scores for the complete population. In *black* B1 (284 clones) and in *gray* B2 (374 clones)

clones. Mean scores are scattered from 1.0 to 8.0. The distribution in both populations presents a clear discontinuity. Three-quarters of the progeny clones (167/222 for population B1; 244/309 for population B2) display mean scores equal or lower than 1.5, and one quarter (55/222 for population B1; 65/309 for population B2) display mean scores equal or higher than 2.5. The repeatability of the scoring procedure is demonstrated by observed values being scattered along the diagonal of the plot on Fig. 1. The distribution of the mean scores for the two complete populations, B1 (284 clones) and B2 (374 clones), is given in Fig. 2. A similar segregation is observed allowing the progeny to be classified in two groups, one homogeneous with highly resistant clones and the other with clones of a variable level of susceptibility. Figures 1 and 2 also show the important variability existing inside the 'susceptible' class, which is probably due to the segregation of several minor resistance factors. The consequence of this variability is that a minor proportion of clones that do not have the resistance allele at the major gene, may present a phenotype close to those that have it. When considering the whole population that has missing replicates for some clones, this was conducted to ambiguous scores (between 1.5 and 2.5) for seven individuals, representing around 1% of the total progeny size (4 in B1 and 3 in B2). We further discarded these seven clones in order to avoid any misclassification that could have dramatic consequences for the fine mapping study.

Considering as resistant, the clones with a mean score equal or lower than 1.5, and as susceptible, the clones with a mean score equal or higher than 2.5, the segregation ratio in populations B1 and B2 were 207 resistant : 73 susceptible and 290 resistant : 81 susceptible, respectively, which is not different from 3:1 at the threshold 5% and confirms the presence of a major resistance gene in R570.

The segregation ratio observed in three crosses between a susceptible clone and R570 was 1:1 (resistant:susceptible) as expected for a major dominant resistance gene (Table 1). In the case of the cross R94/167 x R570, the 1:1 ratio was observed in 1997 but not in 1998 (Table 1). This could be explained by the fact that R94/167 was the least susceptible tester we used and that environmental conditions in 1998 were less favorable to rust infection as suggested by the overall drift toward resistance.

According to these results, the presence of a major dominant resistance gene conferring resistance to brown rust was confirmed. It was named *Bru*1 for Brown Rust 1.

AFLP and bulked segregant analysis

Four bulks were designed: two resistant bulks each composed of DNA from five resistant individuals from population B, and two susceptible bulks each composed of DNA from five susceptible individuals from population B.

The four bulks were screened simultaneously with 34 AFLP primer pair combinations among the 64 possible pairs permitted by the GIBCO BRL commercial kit genome I. Two markers appeared as present in both

Table 1 Observed ratios of resistant and susceptible seedlings in four biparental families from crosses between a susceptible clone and the resistant clone R570, and the Chi-square test of the expected 1:1 segregation corresponding to the hypothesis of a major gene

Biparental progeny	1997			1998			1998
	Susceptible	Resistant	Chi-square[a]	Susceptible	Resistant	Chi-square[a]	Susceptible parent score[b]
M555/60×R570	122	118	0.067 ns	121	112	0.348 ns	7
R94/187×R570	176	193	0.783 ns	120	246	43.37*	3
Triton×R570	319	323	0.025 ns	245	273	1.51	4
BT64/134×R570	448	413	1.423 ns	396	396	0 ns	8

[a] Chisquare = 3.84 at the 5% level
[b] Scores on a 1 (the most resistant) to 9 (the most susceptible) scale (Tai et al. 1981)

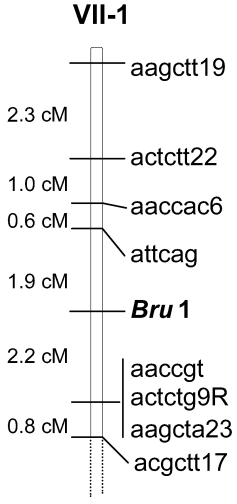

Fig. 3 AFLP genetic map of the region surrounding the *Bru*1 resistance gene in R570. Distances between markers using the Haldane mapping function are given in centiMorgans (cM)

'resistant' bulks and absent in both 'susceptible' bulks. Their segregation with the resistance gene was further checked on 10 resistant and 10 susceptible individuals from population B. This resulted in the identification of two AFLP markers (actctg9R and actctt22) linked to the resistance gene (Fig. 3).

In parallel to the BSA, an AFLP map was being constructed for the genetic analysis of the sugar-yield components with 295 individuals from population B. This map is described in detail by Hoarau et al. (2001). Thirty seven primer pairs from the GIBCO BRL commercial kit, including the 30 ones not used for the BSA, were employed. They revealed a total of 939 simplex markers, from which 887 were involved in a co-segregation group and 52 remained unlinked. The rust resistance scoring data were combined with the AFLP marker matrix to allow the mapping of *Bru*1 on the AFLP map. The resistance gene fell into co-segregation group 60, and was surrounded with six AFLP markers in a window of 10 cM. Two of the markers were already detected by the BSA (actctt22 and actctg9R) and four were new (aagctt19, aagcta23, aaccac6 and acgctt17) (Fig. 3).

New AFLP primers were then designed to be tested by BSA. For that purpose, several modifications were applied at different steps of the AFLP process: (1) modification of the last two nucleotides of amplification primers (16 new primers, *Eco*RI-A+2 and *Mse*I-C+2), (2) modification of the last nucleotide of pre-amplification primers (three new primers, *Eco*RI-G+2), (3) modification of the restriction enzyme (16 new primers, *Pst*I-A+2).

These new primers, ordered from different companies and purified with different methods (Polyacrylamide Gel Electrophoresis or High Pressure Liquid Chromatography), were tested on the same bulks used to test the GIBCO BRL primer pairs. An important proportion of these primer pairs (30%), whatever their origin of fabrication, were obviously not fully efficient since they produced low quality AFLP profiles characterized either by a very limited number of discrete bands, or by the absence of low intensity bands, which often characterize the single-dose markers.

Modification of selective nucleotides of amplification primers was permitted to add 192 potentially new primer combinations. Among those, 170 were tested, and 24 produced a low quality profile. The 146 remaining primer combinations allowed identifying 16 markers present in the resistant bulks and absent from the susceptible ones. Subsequently, they were further tested by individually genotyping 10 resistant and 10 susceptible individuals from population B, and only two markers appeared really linked to the resistance gene (markers attcag and aaccgt).

Among the new primer combinations generated by modification of the last nucleotide of pre-amplification primers, 17 were tested and 13 produced a standard quality profile. One marker was polymorphic between the 'resistant' and 'susceptible' bulks. However, individual genotyping of 10 resistant and 10 susceptible individuals did not confirm the linkage.

Sixteen *Pst*I-A primers were combined with 12 *Mse*I-C primers. The 192 possible combinations were tested with BSA, and examination of 110 standard quality profiles produced 16 markers potentially linked to the gene of interest. However, none was confirmed after individually genotyping 10 resistant and 10 susceptible individuals from population B.

Fine mapping

The eight AFLP markers linked to resistance, four derived from the BSA and four from the AFLP map, were then precisely mapped by individually genotyping 316 individuals from population B. Four markers were located on each side of the resistance gene, in a window of 10 cM, the closest markers being at 1.9 and 2.2 cM on each side of the gene (Fig. 3).

Discussion

Segregation of the resistance

In 1996, Daugrois et al. reported a 3:1 segregation ratio for resistance to brown rust in the self-progeny of cultivar R570, typical of the presence of a major resistance gene. This work was based on the analysis of 141 clones. In the present study, we have confirmed this result on the basis of 658 additional clones from the same progeny. The high, repeatable variation observed in the 'susceptible' class, also confirms that other genetic factors are involved in this trait segregation, as already suggested by Daugrois et al. (1996). The segregation of the resistance in four crosses between susceptible clones and R570 also confirmed the presence of a major dominant gene conferring resistance to brown rust in R570.

Targeted mapping

The combination of AFLP technology and BSA proved to be efficient for mapping the highly polyploid sugarcane genome. Eight markers now surround the gene in an interval of 10 cM. However, the efficiency of the method was not equivalent, depending on the origin of the primers, the GIBCO BRL kit versus other designed primers.

Among the 443 primer pairs tested, the 64 primer pairs from the GIBCO BRL kit generated six markers linked to the gene, and no false positive was detected with the 34 primer combinations tested by BSA. In contrast, the 379 other primer combinations that we designed and tested with BSA, generated only two markers linked to the gene and 31 false positives. Exploitation of this set of primers has led to a relatively high rate of low quality AFLP profiles. The origin of this low quality is not fully understood, but it may be in part due to the fact that GIBCO BRL primers had been selected for providing high quality profiles. These quality problems have affected the yield of the method and the number of false positives. Since sugarcane is highly polyploid, only single-dose markers can be used for genetic mapping, and they often appear as the weakest bands requiring higher quality profiles for their detection compared to what is necessary for diploid species analysis.

Among the 316 individuals genotyped, 26 (15 susceptible and 11 resistant) presented one recombination between the two extreme markers (aagctt19 and acgctt17) of the 10-cM chromosome segment surrounding the resistance gene. These 26 recombinant individuals represent an essential resource for further fine mapping in the target area.

The two closest AFLP markers from the resistance gene are located at 2.2 and 1.9 cM on each side of *Bru*1. Considering the estimated mean relationship between genetic and physical distances in sugarcane (500 kb/cM), these markers are too distant from the targeted gene to initiate a chromosome walk with the sugarcane BAC library. Furthermore, the recent availability of high-density genetic maps (Boivin et al. 1999; Bowers et al. 2000) and partially ordered bacterial artificial chromosome (BAC) libraries in sorghum (http://www.genome.arizona.edu/fpc/sorghum) may offer valuable resources to carry on the fine mapping, and assist the chromosome walking toward the resistance gene.

Previous global comparisons performed with unsaturated maps with limited precision did not reveal frequent ruptures of colinearity between sugarcane and sorghum (Grivet et al. 1994; Dufour et al. 1996, 1997; Guimares et al. 1997; Ming et al. 1998). At a finer scale, contrasted situations have been observed from micro-colinearity studies between different grasses, suggesting no general rule and situations depending on the species compared and the regions involved (Dunford et al. 1995; Kilian et al. 1995; Foote et al. 1997; Chen et al. 1997; Gallego et al. 1998; Bennetzen 2000; Bennetzen and Ramakrishna 2002). The comparative mapping approach with rice, maize and sorghum, to saturate the target area in markers described by Asnaghi et al. (2000), did not result in the identification of any marker in the distal part of the gene. This could be due to either a local rupture of synteny or to a poor RFLP marker saturation in the corresponding distal homoelogous regions. Cloning the four distal AFLP markers (aagctt19, actctt22, aaccac6 and attcag) and mapping them on sorghum will help refining the map comparison between sugarcane and sorghum in this area, and guide the further comparative approach.

Acknowledgments We thank the International Consortium for Sugarcane Biotechnology for the financial support of this work, and J.C. Glaszmann for critical reading of the manuscript.

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