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ORIGINAL PAPER

Haplotype structure around *Bru*1 reveals a narrow genetic basis for brown rust resistance in modern sugarcane cultivars

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Abstract Modern sugarcane cultivars (*Saccharum* spp., $2n = 100–130$) are high polyploid, aneuploid and of interspecific origin. A major gene (*Bru*1) conferring resistance to brown rust, caused by the fungus *Puccinia melanocephala,* has been identified in cultivar R570. We analyzed 380 modern cultivars and breeding materials covering the worldwide diversity with 22 molecular markers genetically linked to *Bru*1 in R570 within a 8.2 cM segment. Our results revealed a strong LD in the *Bru*1 region and strong associations between most of the markers and rust resistance. Two PCR markers, that flank the *Bru*1-bearing segment, were found completely associated with one another and only in resistant clones representing efficient molecular diagnostic for *Bru*1. On this basis, *Bru*1 was inferred in 86 % of the 194 resistant sugarcane accessions, revealing that it constitutes the main source of brown rust resistance in modern cultivars. *Bru*1 PCR diagnostic markers should be particularly useful to identify cultivars with potentially alternative sources of resistance to diversify the basis of brown rust resistance in breeding programs.

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Introduction

Sugarcane (*Saccharum* spp.) brown rust is caused by the fungus *Puccinia melanocephala* H. & P. Sydow that provokes reddish flecks on leaf surfaces. Since its first report in India in the 1950s (Patel et al. 1950), brown rust has spread over almost all sugarcane-growing areas of the world. It has been responsible for important yield losses reaching up to 50 % in Mexico in 1981–1982 (Comstock 1992; Purdy et al. 1983).

Progress in breeding for rust resistance has been rapid, since this trait is easy to evaluate, and shows high broad and narrow-sense heritability (Berding et al. 1984; Comstock et al. 1992; Hogarth et al. 1993; Ramdoyal et al. 2000). Although the development of resistant cultivars has resulted in the efficient control of brown rust epidemics in most sugarcane-growing areas, breeding for this trait remains an important objective (Butterfield 2007). Moreover, sudden outbreaks of potential economic importance have been reported recently in Louisiana (Hoy 2005; Hoy and Hollier 2009) on the leading cultivar previously considered as resistant (Hoy and Grisham 2000) and in South Africa on a major cultivar which showed only mild symptoms before (Cadet et al. 2003).

Genetic analyses are complicated in sugarcane due to the high polyploid ($2n = 100–130$), aneuploid and interspecific origin of modern cultivars (D'Hont et al. 1996; Grivet and Arruda 2002; Hoarau et al. 2007). Genetic mapping studies in self or biparental progenies have identified numerous small QTLs for most agronomic traits

surveyed (Hoarau et al. 2002; Ming et al. 2001; Aitken et al. 2008). Likewise, rust resistance has been considered for a long time to have a polygenic determinism (Tai et al. 1981; Hogarth et al. 1993; McIntyre et al. 2005). However, we recently identified two major brown rust resistance genes, *Bru*1 (Daugrois et al. 1996; Asnaghi 2000) and *Bru*2 (Raboin et al. 2006), which control leaf sporulation of the fungus. These genes were the first major genes identified in sugarcane. Important levels of linkage disequilibrium (LD) that decreases significantly beyond 5 cM was demonstrated in modern sugarcane cultivars (Jannoo et al. 1999; Raboin et al. 2008) leading to the development of genome-wide association mapping strategies (Wei et al. 2006, 2010; Pauquet et al. 2007). This LD results from the history of modern sugarcane breeding, that is characterized by the bottleneck effect of the crossings, a century ago, of a few clones of the sugar-producing species *S. officinarum* with very few clones of the wild species *S. spontaneum* and by a relatively small number of meioses (<10) since then, due to the vegetative propagation of this crop.

The major resistance gene *Bru*1 discovered in cultivar R570 (Daugrois et al. 1996) has been shown to control all the rust isolates collected from several geographic origins including the French West Indies and the Mascareign tested by Asnaghi et al. (2001). It is the focus of a map-based cloning approach that is complicated by the polyploidy of sugarcane but already resulted in the development of a high-resolution genetic map and a partial physical map with many molecular markers genetically linked to *Bru*1 in cultivar R570 (Asnaghi et al. 2000, 2004; Le Cunff et al. 2008).

In the present work, we used these markers to genotype a worldwide panel of modern sugarcane cultivars in order to (1) analyze the pattern of linkage disequilibrium (LD) in the *Bru*1 region, (2) establish diagnostic PCR molecular markers for *Bru*1 and (3) analyze the frequency distribution of *Bru*1 in modern sugarcane cultivars.

Materials and methods

Sugarcane accession panels

Three panels altogether gathering 380 sugarcane cultivars and breeding materials from over 30 different breeding centers around the world were analyzed in this study (Tables 1, 2). The REUa panel consisted of 84 accessions and the REUb panel consisted of 185 accessions from which 46 were common with REUa. Both REU panels were grown in Reunion in Ligne Paradis and in Bassin Martin CIRAD stations, respectively. The GUA panel consisted of 189 accessions, with 32 accessions in common with the REU panels (16 with REUa and 30 with REUb). This panel was grown in the Roujol station of CIRAD in Guadeloupe. Finally, 14 accessions were common to the three panels.

Field evaluation of rust resistance

The field reaction to rust was determined using natural infection in winter season, when inoculum pressure is high. For REUa, rust resistance was scored in year 2005 for each individual based on the presence/absence of sporulations in an unreplicated design consisting of individual row plots of 5 m length representing 20 clumps. Individuals showing sporulating pustules were classified as susceptible, otherwise they were classified as resistant. The field trials for REUb and GUA panels were designed for independent analysis of yield traits in addition to the present study on rust resistance. Both panels were planted in a randomized complete block design with three replicates. Each individual plot consisted of a 3 m length row of 12 clumps. In Reunion, each individual plot was planted between spreader rows of the cultivar B 34/104 which is highly susceptible to rust in order to maximize infection. Scoring of the rust reaction was performed in year 2007 in Reunion and in Guadeloupe, using a scale modified from Tai et al. (1981) (Online resource 1). Variance components were estimated using SAS Mixed procedure (SAS Institute 2008) as follows:

$$X_{ij} = \mu + R_i + G_j + \varepsilon_{ij} \quad (1)$$

where X_{ij} was the rust score measured on the jth accession in the ith replication, μ the general mean, R_i the effect of the ith replication, G_j the effect of the jth accession and ε_{ij} the residual error. The accession effect was considered as random. Broad-sense heritability of rust was calculated in REUb and GUA panels at experimental ($r = 3$) and individual plot ($r = 1$) levels, from the ratio between genetic variance (σ_g^2) and phenotypic variance (σ_p^2), with $\sigma_p^2 = \sigma_g^2 + \sigma_e^2/r$, where σ_e^2 is the error variance and r the number of considered replications.

For association mapping, rust resistance was considered as a qualitative trait, with the accessions scored 1 being resistant and the others, scored 2 and above, being susceptible.

Genotyping

Twenty-two markers linked to *Bru*1 in R570 were used in this study, i.e. 12 RFLPs, 6 AFLPs, 1 SSR, 2 PCRs and 1 CAPS markers. The RFLP and AFLP markers were previously developed and located on the genetic map of R570 (Asnaghi et al. 2004; Hoarau et al. 2001; Le Cunff et al. 2008). The SSR, two PCR markers and the CAPS markers were design from BAC sequences and plasmid sequences produced in the frame of the map-based cloning (Le Cunff

Table 1 List of the 380 sugarcane accessions studied and results of brown rust susceptibility evaluation in Guadeloupe and Reunion islands

Accession	Rust resistance			Accession	Rust resistance			Accession	Rust resistance			Accession	Rust resistance		
	R/S[a]	REUb[b]	GUA[b]		R/S[a]	REUb[b]	GUA[b]		R/S[a]	REUb[b]	GUA[b]		R/S[a]	REUb[b]	GUA[b]
B 37 172	R	1.0		LF 61 0005	R[c]		1.0	R 83 777	R a	1.0		BJ 82 118	S		4.3
B 41 227	R	1.0	1.0	LF 63 0033	R[c]		1.0	R 84 0813	R a			BJ 85 010	S		4.7
B 54 001	R		1.0	LF 65 3611	R[c]		1.0	R 84 1269	R		1.0	BJ 88 20	S a		
B 60 182	R		1.0	LF 65 3661	R[c]		1.0	R 84 413	R	1.0		BT 90 2495	S		4.3
B 62 163	R[c]		1.0	LF 74 4484	R[c] a	1.0		R 84 693	R	1.0	1.0	CB 36 14	S a	3.7	
B 64 277	R		1.0	M 1176 77	R[c]	1.0		R 84 75	R a	1.0		CB 45 6	S	3.0	
B 69 566	R	1.0		M 134 32	R	1.0		R 85 0449	R a			CL 41 223	S		3.0
B 70 466	R		1.0	M 134 75	R	1.0		R 85 1124	R a	1.0		CO 1148	S		3.7
B 70 531	R	1.0		M 165 38	R	1.0		R 85 1334	R a	1.0		CO 281	S	2.0	2.0
B 71 1001	R		1.0	M 2077 78	R	1.0		R 85 895	R	1.0		CO 290	S	3.0	
B 76 078	R		1.0	M 2173 63	R	1.0		R 86 595	R	1.0		CO 312	S	4.3	
B 78 124	R		1.0	M 3035 66	R	1.0		R 87 2159	R	1.0		CO 462	S	3.3	
B 79 045	R		1.0	M 31 45	R		1.0	R 90 2490	Ra	1.0		CO 527	S	3.3	
B 85 0747	R		1.0	M 40 85	R a	1.0		R 92 4378	R	1.0		CO 68 006	S		3.0
B 86 0049	R[c]		1.0	M 409 51	R		1.0	R 92 6545	R	1.0		CO 775	S		4.0
B 87 0001	R		1.0	M 447 67	R a	1.0		R 92 8027	R	1.0		CO 842	S		5.3
B 87 1296	R		1.0	M 695 69	R		1.0	R 92 8065	R	1.0		CP 29 116	S	3.3	
B 90 0246	R[c]		1.0	M 791 75	R[c]	1.0		R 93 2022	R	1.0		CP 34 120	S a		
BBZ 80 0219	R		1.0	MEX 71 1235	R		1.0	R 93 2034	R	1.0		CP 48 103	S a	3.3	
BBZ 81 0008	R[c]		1.0	MEX 73 523	R[c] a	1.0	1.0	REU-UN1	R	1.0		CP 57 0614	S		4.7
BJ 69 002	R		1.0	MQ 72 1175	R[c]	1.0		REU-UN2	R	1.0		CP 59 0073	S		2.3
BJ 79 038	R[c]		1.0	MQ 76 53	R[c] a	1.0	1.0	REU-UN4	R	1.0		CP 61 0084	S		4.7
BJ 82 031	R		1.0	N 13[c]	R[c]	1.0		ROC 1	R		1.0	CP 61 37	S	2.0	
BR 81 0077	R		1.0	N 14[c]	R[c]		1.0	ROC 2	R a	1.0		CP 63 306	S	3.3	
BV 93 30	R	1.0		N 17	R		1.0	ROC 3	R		1.0	CP 67 0413	S		2.7
CB 45 3	R	1.0		N 18	R	1.0		ROC 6	R		1.0	CP 70 1133	S	2.0	
CB 47 089	R		1.0	N 19	R		1.0	ROC 7	R		1.0	CP 72 355	S		3.0
CB 56 171	R	1.0		N 53 216	R	1.0	1.0	ROC 9	R		1.0	CP 72 356	S	1.7	
CO 1001	R[c]	1.0		NA 56 62	R	1.0		SM 81 16	R a	1.0		CP 74 383	S	4.0	
CO 1007	R	1.0		NCO 293	R	1.0		SP 70 1143	R	1.0		CP 75 361	S	4.3	4.0
CO 1186	R		1.0	NCO 310	R	1.0		SP 78 3137	R		1.0	CP 76 331	S	3.7	3.3
CO 1223	R		1.0	NCO 334	R	1.0	1.0	SP 79 1011	R	1.0		CP 85 1432	S		2.0
CO 213	R	1.0		NCO 376	Ra	1.0		TUC 68 18	R[c]	1.0		CP 86 1633	S		3.0
CO 214	R	1.0	1.0	NIF 4	R[c] a			TUC 74 0006	R[c]		1.0	D 84 84	S a		

Table 1 continued

Accession	Rust resistance			Accession	Rust resistance			Accession	Rust resistance			Accession	Rust resistance		
	R/S[a]	REUb[b]	GUA[b]		R/S[a]	REUb[b]	GUA[b]		R/S[a]	REUb[b]	GUA[b]		R/S[a]	REUb[b]	GUA[b]
CO 62 175	R		1.0	NIF 5	R[c] a	1.0		UBA	R	1.0		D 97	S	3.7	
CO 64 015	R		1.0	PHIL 66 007	R		1.0	US 1694	R[c]	1.0		DB 60 0377	S		6.0
CO 740	R		1.0	POJ 213	R[c]		1.0	VESTA	R a	1.0		DB 70 0047	S		5.0
CO 980	R	1.0		POJ 2878	R	1.0		WI 89 0034	R		1.0	DB 74 0208	S		2.3
CP 52 0043	R		1.0	PR 1028	R[c] a	1.0		69 F 775	S a			DB 82 0086	S		4.0
CP 69 1062	R		1.0	PR 1116	R[c] a	1.0		B 34 104	S	4.0		DB 82 0113	S		5.3
CP 72 2086	R	1.0		PS 56	R		1.0	B 34 39	S a			F 148	S a	3.0	2.0
CP 76 328	R	1.0		PS 60	R		1.0	B 43 62	S a			FR 80 237	S	3.7	
CP 84 1198	R[c]		1.0	PS 61	R		1.0	B 46 364	S	3.5		FR 80 806	S	3.0	
D 172	R	1.0		PT 43 52	R	1.0		B 47 44	S	3.0		FR 81 651	S	2.0	
D 88 004	R		1.0	Q 112	R		1.0	B 53 267	S		4.0	FR 89 0003	S		2.3
D 88 082	R		1.0	Q 119	R		1.0	B 54 215	S		3.3	FR 90 0253	S		5.3
DB 55 0005	R[c]		1.0	Q 122	R		1.0	B 60 267	S		3.0	FR 91 0699	S		2.3
DB 63 0237	R		1.0	Q 127	R	1.0	1.0	B 63 119	S a			FR 94 0119	S		3.7
DB 86 0124	R		1.0	Q 136	R a	1.0	1.0	B 63 758	S a			FR 94 1006	S		3.3
F 146	R a	1.0		Q 140	R		1.0	B 64 136	S		2.3	GUA-UN2	S		3.7
F 156	R		1.0	Q 175	R		1.0	B 65 220	S	4.3		GUA-UN3	S		2.7
F 160	R		1.0	Q 59	R	1.0		B 66 023	S		4.3	H 32 8560	S		1.7
F 175	R		1.0	Q 80	R	1.0		B 69 379	S	3.0	4.0	H 49 5	S	4.7	
FR 81 752	R	1.0		R 00 4178	R a			B 70 532	S	3.7		H 50 7209	S	2.3	
FR 84 178	R	1.0		R 336	R	1.0		B 70 533	S		4.0	H 59 3775	S	3.0	
FR 90 0027	R		1.0	R 365	R	1.0		B 70 571	S		5.3	H 68 1158	S		2.0
FR 90 0219	R[c]		1.0	R 366	R		1.0	B 74 010	S		4.7	KnH 80 412	S a		
FR 90 0771	R		1.0	R 397	R	1.0		B 74 060	S		3.7	L 66 097	S		4.0
FR 91 0253	R		1.0	R 445	R a	1.0		B 76 359	S		4.0	LF 52 3082	S		4.0
FR 94 0022	R		1.0	R 484	R[c] a	1.0		B 77 195	S		4.3	LF 54 6032	S	5.0	
FR 95 0025	R		1.0	R 526	R		1.0	B 78 527	S		4.0	LF 56 0051	S		2.0
GUA-UN1	R		1.0	R 570	R[c] a	1.0	1.0	B 79 136	S		3.7	LF 56 26	S	4.7	5.0
H 56 278	R	1.0		R 572	R	1.0	1.0	B 82 0126	S		3.3	LF 61 0000	S		3.3
H 56 4848	R	1.0		R 573	R a	1.0	1.0	B 85 0356	S		4.0	LF 62 1450	S	3.0	
H 57 5174	R	1.0		R 574	R	1.0		B 85 0764	S		3.3	LF 65 3712	S	2.0	
H 60 5657	R	1.0		R 575	R a	1.0	1.0	B 85 0902	S		3.0	LF 66 9657	S a	1.3	
H 63 1418	R	1.0		R 577	R a	1.0	1.0	B 86 0185	S		1.7	M 1022 86	S a		
H 69 9092	R		1.0	R 578	R	1.0		B 86 0517	S		5.0	M 1135 64	S a		

Table 1 continued

Accession	Rust resistance			Accession	Rust resistance			Accession	Rust resistance			Accession	Rust resistance		
	R/S[a]	REUb[b]	GUA[b]		R/S[a]	REUb[b]	GUA[b]		R/S[a]	REUb[b]	GUA[b]		R/S[a]	REUb[b]	GUA[b]
H 70 6957	R	1.0		R 579	Ra	1.0	1.0	B 86 0699	S		5.0	M 1156 66	S	2.7	
H 74 4527	R	1.0		R 78 698	Ra	1.0		B 89 0030	S		4.3	M 1227 62	S		3.7
H 72 8597	R a	1.0	1.0	R 79 106	R	1.0		B 93 0440	S		4.7	M 13 56	S		2.7
IAC 64 257	R		1.0	R 81 404	R	1.0		B 93 0873	S		4.3	M 1371 78	S a		
JA 64 0020	R		1.0	R 81 852	R	1.0		BBZ 85 0102	S		3.7	M 147 44	S	3.0	
Kassoer	R	1.0		R 81 972	R a	1.0		BH 10 12	S	3.0	3.7	M 1864 78	S a	2.7	
KN 91 28	Ra	1.0		R 83 1592	R a	1.0	1.0	BJ 70 003	S		5.0	M 2229 80	S	2.0	
LF 53 4789	R[c]		1.0	R 83 288	R a	1.0		BJ 70 013	S		4.0	M 292 70	S	3.3	3.3
LF 53 4795	R	1.0		R 83 444	R a	1.0		BJ 82 026	S		3.3	M 377 56	S		4.7
LF 53 4825	R[c]	1.0		R 83 547	R a	1.0		BJ 82 048	S		2.3	M 574 62	S a		
M 624 78	S	3.0		POJ 2725	S	3.0		Q 90	S	4.3		R 84 0408	S a		
M 658 51	S a			PR 1059	S		4.7	R 00 4222	S a			R 84 379	S	4.0	
M 675 74	S	3.3		PR 980	S	3.3		R 363	S	2.7		R 85 0358	S a		
M 836 75	S	3.0		PS 58	S		5.0	R 446	S	3.3		R 86 591	S	4.0	
M 907 61	S a		4.3	Q 100	S		2.3	R 576	S a	4.7	4.7	R 87 2133	S a		
M 99 48	S	4.0		Q 102	S		5.0	R 68 0080	S a			R 92 6246	S	3.0	
MEX 68 200	S		1.3	Q 108	S a		4.7	R 73 0094	S a			R 92 6261	S a	4.3	
MEX 68 P23	S	3.7		Q 110	S	1.7		R 75 0428	S a			R 96 0534	S a		
MEX 70 485	S	2.3		Q 115	S a			R 75 0434	S a			REU-UN3	S	3.7	
MY 51 005	S		3.0	Q 126	S	2.7		R 79 0419	S a			S 17	S a	1.3	1.3
MY 54 129	S		3.7	Q 130	S	3.3		R 80 0202	S a			SP 71 8210	S		4.7
MY 55 14	S	2.7		Q 134	S		3.7	R 80 175	S a			SP 72 4928	S	3.3	
N 11	S		2.0	Q 138	S		3.3	R 81 420	S a	3.0		SP 73 3108	S	1.7	3.3
N 15	S		3.0	Q 49	S	3.0		R 83 0525	S a			SP 79 1169	S	3.3	
N 22	S		3.7	Q 69	S a			R 83 0916	S a			SP 80 1842	S	3.0	1.3
N 55 805	S	4.3		Q 76	S a	4.0		R 84 0068	S a			TRITON	S a	3.7	
Pmango 1471	S	1.3		Q 84	S a	3.0	3.0	R 84 0099	S a			TUC 74 26	S	3.0	

[a] Resistance status to rust infection based on the presence (S) or absence (R) of sporulation, resulting from evaluations in REUb (Column 3) and/or GUA (Column 4) and/or REUa (indicated by a)

[b] Quantitative evaluation of rust infection based on the modified scale of Tai et al. (1981)

[c] Indicates sugarcane accessions that are resistant to brown rust and do not have R12H16 and 9O20-F4-PCR *Rsa*I diagnostic markers of *Bru*1 identified in this study

Table 2 Brown rust resistance in the three sugarcane accessions panels

	REUa	REUb	GUA	All panels
Number of accessions	84	185	189	380
% of rust resistant accessions	45.2	59.5	51.3	51.1
% of resistant accessions having the *Bru*1-bearing haplotype	92.1	90.9	81.4	85.6

et al. 2008; Garsmeur et al. 2011) and locate on the R570 genetic map using R570 mapping population described in Le Cunff et al. (2008). RFLP, AFLP and SSR genotyping were performed as described in Le Cunff et al. (2008), Hoarau et al. (2001) and Rossi et al. (2003) except that for REU panels, fluorescent labeling was used for AFLP and SSR, and electrophoresis was performed in a 3130*xl* Genetic Analyzer (Applied Biosystems). The primer for SSR m164H22 was Fw: CACACTCAGTTCACCCTGGA/ Rv: CATGGGTAAAGTGGGAAAGC. PCR markers R12H16, 9O20-F4 and cBR37 were amplified with 50 ng of DNA mixed with 1×PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM forward primer, 0.2 µM reverse primer, 0.5 U DNA polymerase in a final volume of 25 µl, for 9O20-F4 the final volume was 50 µl. The primer pairs for PCR markers R12H16, 9O20-F4 and cBR37 were Fw: CTACGATGAAACTACACCCTTGTC/Rv: CTTATGTT AGCGTGACCTATGGTC, Fw: TACATAATTTTAGTG GCACTCAGC/Rv: ACCATAATTCAATTCTGCAGGT AC, Fw: 3GTCCAACTATGGATTAATTAGACTC/Rv: GCCAATCCAAAGTCGGCGAGCTTC, respectively. The PCR profile used was: one step of 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. Then, followed a final elongation step at 72 °C for 5 min. Fifteen microliters of the 9O20-F4 PCR products was digested with 1× NEBuffer1 and 5 U *Rsa*I (New England Biolabs). Water (Merck) was added to a final volume of 25 µl. This digestion mix was incubated at 37 °C for 2 h. The PCR products of R12H16, cBR37 and 9O20-F4 were run on a 2 % agarose gel and of 9O20-F4-*Rsa*I on a 3 % agarose gel.

Pair-wise marker associations and marker-rust associations

The intensity of associations among markers and rust resistance phenotype (resistant vs. susceptible) was assessed as previously described in Jannoo et al. (1999) and Raboin et al. (2008) using Fisher's exact tests computed with SAS Freq procedure (SAS Institute 2008) and quantified with the $-\log_{10}(P)$ value (P = Fisher probability). Association with rust resistance was monitored using the evolution of LD intensity along the genetic map, in search for LD peaks rising above the global background. While population structure in sugarcane is considered limited by a common practice of exchange of germplasm among breeding programs (Tew 1987; Jannoo et al. 1999; Raboin et al. 2008), we made use of DArT genome-wide genotyping data available for the two populations REUb and GUA (unpublished data) in order to test the potential impact of limited structure with a mixed logistic model including genetic structure and polygenic background effects (Yu et al. 2006).

Results

Frequency distribution of rust resistance in the cultivar panels

Resistance to brown rust was evaluated in the fields under natural infestation. For REUa, this resulted in the identification of 45.2 % resistant among the 84 materials tested (Tables 1, 2). For REUb and GUA, the quantitative mode of scoring revealed a bimodal distribution (Online resource 2), as already observed in progenies of cultivar R570 (Daugrois et al. 1996; Asnaghi et al. 2004). Broad-sense heritabilities of the rust susceptibility score calculated for the REUb and the GUA panels were as high as 0.94 and 0.97 at the whole experiment level and 0.85 and 0.92 at an individual plot level, respectively, attesting for the robustness of the phenotypic evaluations. Converting the bimodal distribution to qualitative scores resulted in the identification of 59.5 and 51.3 % resistant accessions among the 185 and 189 accessions from the REUb and GUA panels, respectively (Tables 1, 2). Altogether, the 46 accessions that were evaluated in Reunion in 2005 and 2007 exhibited the same behavior in terms of resistance versus susceptibility (Table 1), confirming the previously observed repeatability of resistant versus susceptible scoring between years in Reunion (Daugrois et al. 1996; Asnaghi et al. 2004). The 32 accessions that were evaluated in Reunion and Guadeloupe also displayed the same behavior in terms of resistance versus susceptibility (Table 1), in line with the previous observation that the isolates from both islands, Reunion in the Mascareign and Guadeloupe in the Caribbean, react similar to the presence of the *Bru*1 resistance gene (Asnaghi et al. 2001). Across the three panels, 51.1 % of the clones were found resistant.

Marker frequency and associations

The REUa, REUb, and GUA panels were genotyped, respectively, with overlapping sets of 22, 10 and 6 markers linked to *Bru*1 within a 8.2 cM segment on R570 reference

genetic map (Fig. 1). The frequency distribution of these markers along the *Bru*1 region exhibited bimodal patterns in the three accession panels (Fig. 2). The first group of markers had medium range frequencies (MF 0.41–0.61) in the whole panel, in the same range as the frequency of rust resistant accessions. The second group corresponded to markers in a higher frequency range (HF 0.74–0.97) in the whole panel.

All markers of the MF group showed strong mutual associations, with association intensity increasing with proximity on the R570 reference genetic map (Fig. 1). The strongest associations involved four markers, namely cBR37-PCR, 9O20-F4-PCR-*Rsa*I, R12H16-PCR, m164H22, localized within 0.28–0.14 cM around *Bru*1 in R570, that displayed $-\log_{10}(P)$ values ranging from 17.0 to 54.2. The global scheme suggests that the R570 *Bru*1-bearing segment is the main haplotype for all MF markers, while HF markers are also present in several other haplotypes.

Associations between markers and brown rust resistance

Associations between markers of the *Bru*1-bearing haplotype and brown rust resistance ranged [$-\log_{10}(P)$ values] from 4 to 41 among the MF markers and 0–4 among the HF markers. The associations for MF markers at distances of 3 cM or above on either side of the *Bru*1 location are often high [$-\log_{10}(P)$ between 4 and 14] but this sole data set cannot exclude that these associations be due to the structure of the sample. Within 0.3 cM, however, the intensity of the associations increased considerably and formed sharp peaks in all three panels, thus discarding a potential artefactual impact of structure (Figs. 1, 3). The distribution of the four central markers that were genotyped in the three panels is shown in Table 3. The availability of genome-wide genotyping data for the REUb and GUA panels made it possible to apply a mixed logistic model including genetic structure and polygenic background effects (Yu et al. 2006), this confirmed the global patterns and pinpoint those markers in close vicinity with *Bru*1 as extremely associated with rust resistance (Online resource 3).

This pattern of associations suggests that *Bru*1 contributes importantly to rust resistance in the three panels. Interestingly, R12H16-PCR and 9O20-F4-PCR-*Rsa*I, that are completely linked with *Bru*1 in the R570 map, were completely associated with the global sample and were present in most resistant materials and absent from all the 185 susceptible accessions. This distribution suggests that *Bru*1 is consistently harbored by the R12H16/9O20-F4 chromosome segment while other sources of resistance are occasionally found in resistant materials that do not have this chromosome segment.

Prevalence of *Bru*1 in the panels

Among the resistant accessions, 86 % (166/194) displayed the R12H16-PCR and 9O20-F4-PCR-*Rsa*I markers and thus bear *Bru*1, whereas 14 % of the resistant accessions (28/194) did not have these two markers and thus potentially bear other sources of resistance to brown rust (Table 3). The proportion of resistant accessions that do not bear the *Bru*1-bearing haplotype was 7.9, 9.1 and 18.6 % in REUa, REUb and GUA panels, respectively (Table 2).

*Bru*1 appeared the main source of resistance in all breeding programs that were represented by at least three accessions in this study, with the sole exception of the peculiar LF origin. Among the 28 resistant accessions of the whole panel that do not have the *Bru*1-bearing haplotype and thus may have other sources of resistance, more than a third (including seven LF accessions from Fidji and two MQ accession from Australia) are derived from recent base broadening programs involving crosses between *S. officinarum* and the wild species *S. spontaneum* and *S. robustum.* Among them, accession MQ76/53 was already shown to bear another major gene, named *Bru*2 (Raboin et al. 2006). Except for these particular clones, alternative sources of resistance than the *Bru*1-bearing haplotype were not found (according to our limited samples by region) much more represented in particular breeding programs.

Discussion

Pattern of LD in the *Bru*1 region

Several markers linked to *Bru*1 in an interval of 8.2 cM in cultivar R570 (Le Cunff et al. 2008) were analyzed on a panel of 380 modern cultivars and breeding materials covering the world diversity. The results revealed that most of these markers are in tight LD in the accession panels and derived from a common *Bru*1-bearing haplotype contributed by one of the founder of modern cultivars. The main exceptions were in eight markers that are in high frequency in the accession panels; they probably correspond to markers that are part of the *Bru*1 haplotype, but that are also present in other haplotypes (not containing *Bru*1) in many accessions of the panels. These results are congruent with previous studies of Jannoo et al. (1999) and Raboin et al. (2008) that showed that LD among modern sugarcane cultivars is generally strong within the first 5 cM. In addition, the detailed mapping of the *Bru*1 locus in cultivar R570 (Le Cunff et al. 2008) revealed that the haplotype bearing *Bru*1 contains an insertion (including *Bru*1) that is specific to this haplotype and is thus absent in the other hom(e)ologous haplotypes. This insertion, yet of unknown

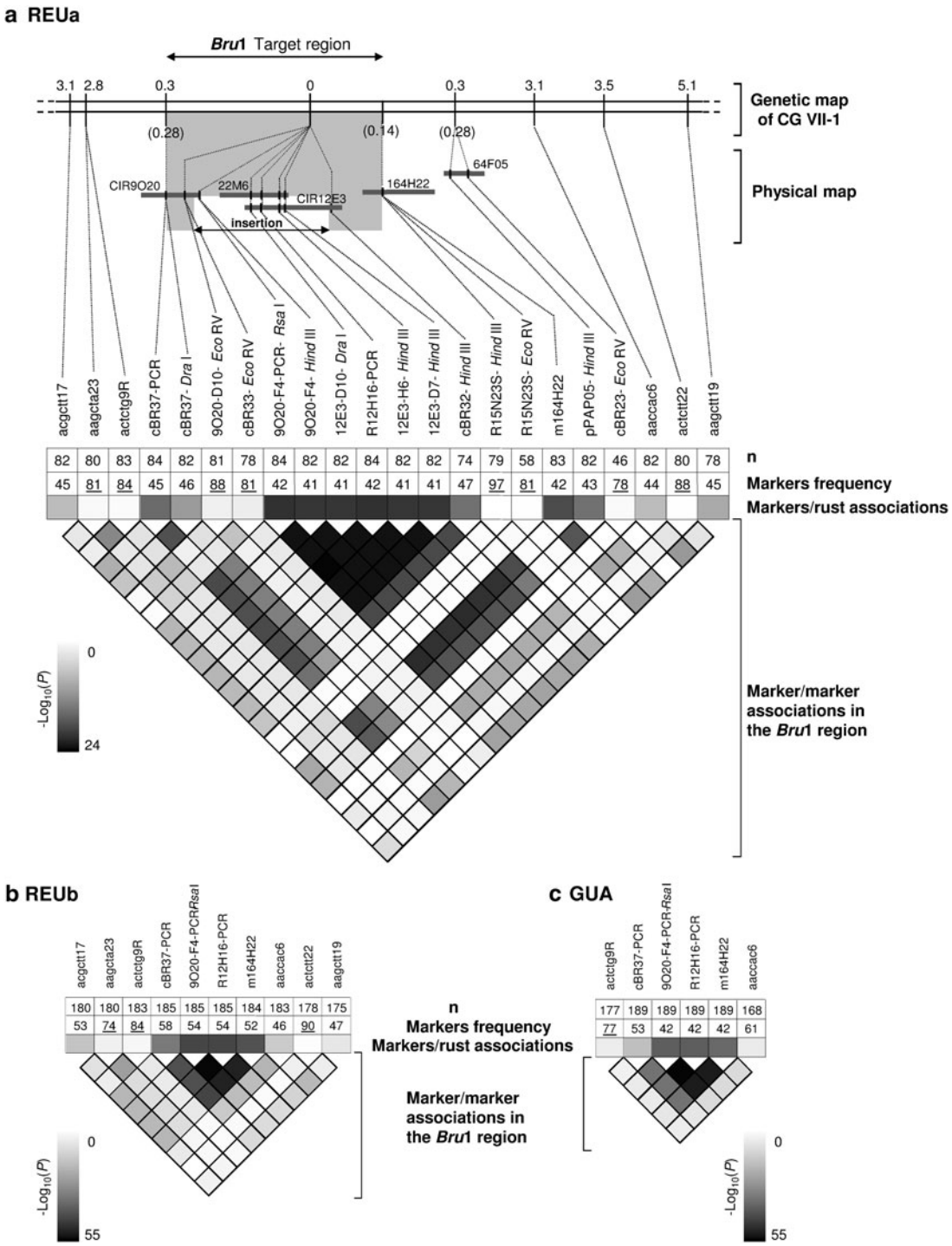

Fig. 1 Pattern of linkage disequilibrium in the *Bru*1 region and marker/rust associations for REUa (**a**), REUb (**b**), and GUA (**c**) *panels*. Schematic representation of genetic and partial physical map of the region bearing *Bru*1 in R570 derived from Le Cunff et al. (2008). Genetic distances (in cM between markers and *Bru*1 locus) above the genetic map resulted from the analysis of 312 self-progenies (Le Cunff et al. 2008), except for markers aagctt19 [112 progenies, Rossi et al. (2003); Hoarau et al. (2001)]. Distances under the genetic map (in *brackets*) were based on 712 progenies (Le Cunff et al. 2008). The physical map consists of BAC clones represented by horizontal lines. *n* number of accessions tested for the marker. Marker frequency represents the % of accessions bearing the marker; when *underlined*, it indicates marker in high frequency in the accession panel. Marker/marker and marker/rust association represents the association between markers pairs and between markers and qualitative rust phenotype (resistant vs. susceptible), respectively. The degree of association is expressed as $-\log_{10}(P)$ (Fisher's exact test) and colored related to value **a** from *black* for 24 to *white* for 0 and for **b** and **c** from *black* for 55 to *white* for 0

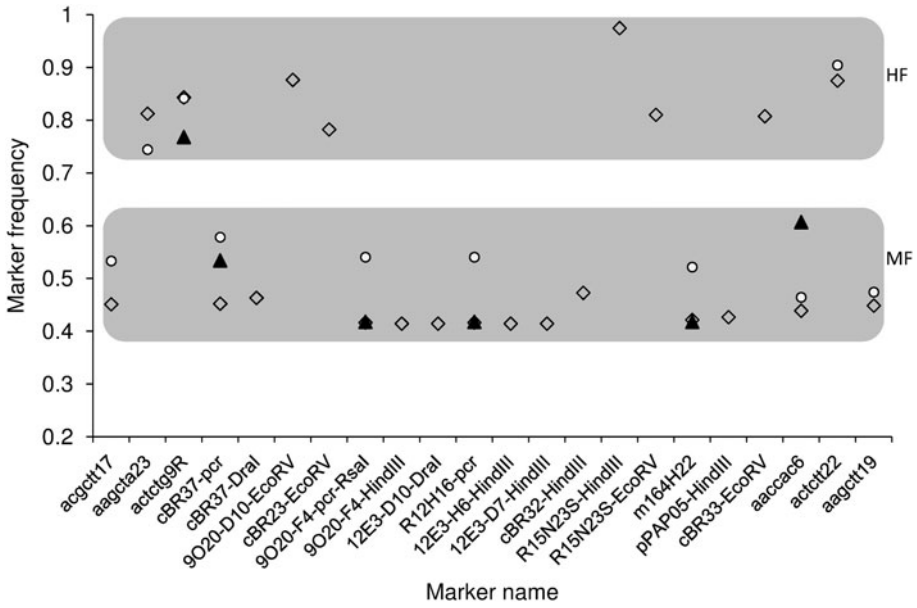

Fig. 2 Marker frequencies in REUa (*solid grey diamonds*), REUb (*open circle*), and GUA *panels* (*solid black triangles*). *MF* markers in medium frequencies, *HF* markers in high frequencies

size, induced a marked reduction of recombination in the *Bru*1 region (Le Cunff et al. 2008) that is responsible for the fact that many of the markers in the region co-segregated completely with *Bru*1 in the R570 mapping population. This reduction of recombination is accompanied by strong LD in the *Bru*1 region and by complete LD between the R12H16 and 9O20-F4 markers.

Resistance to brown rust in modern sugarcane cultivars relies largely on *Bru*1

A strong association between most markers associated with *Bru*1 in R570 and brown rust resistance was demonstrated. This strong association is due to the single origin of the target region and also to the prevalent role of *Bru*1 as a source of resistance to brown rust in the accession panels. The detailed analysis of the four markers common to the three panels and having the highest associations with *Bru*1 revealed that *Bru*1 is present in 86 % of the resistant accessions in the whole panel. This panel covers the modern sugarcane cultivar worldwide diversity. Our results thus clearly indicate that resistance to brown rust in modern sugarcane cultivars relies essentially on *Bru*1 and is thus dangerously narrow.

PCR diagnostic markers for *Bru*1 to search for new sources of resistance

The two markers, R12H16-PCR and 9O20-F4-*Rsa*I, that co-segregated completely with *Bru*1 in R570 progeny were found completely associated together in the panel of 380 sugarcane accessions, strongly associated with brown rust resistance and totally absent in susceptible accessions. They thus represent molecular diagnostic markers for the presence of *Bru*1 (Fig. 4). Their presence in any modern cultivar indicates the presence of *Bru*1 and predicts a resistant phenotype of the cultivar. Exceptions to this pattern, with dissociation between the two markers could indicate the presence of a very rare event of recombination that could be useful for our current map-based cloning project of *Bru*1. Exceptions with the presence of both markers in a susceptible accession could indicate the existence of different races of *Puccinia melanocephala* that would not be controlled by *Bru*1. Little is known about the existence of *P. melanocephala* races throughout the world, although several cases of change in cultivar susceptibility to brown rust have been reported in India (Srinivasan and Muthaiyan 1965), in Hawaii and Florida (Comstock et al. 1994; Dean and Purdy 1984; Liu 1980a, b; Raid 1989; Shine et al. 2005), in Australia (Taylor 1992) and in South Africa (Pillay et al. 2005), none of them has yet been validated through standardized experimentations and it is unclear whether the expression of the disease can be influenced by environmental conditions such as excessive fertilizing practices (Anderson and Dean 1986; Taylor 1992; Johnson et al. 2007). Conversely, the absence of the two diagnostic markers in a given resistant modern cultivar indicates the absence of the *Bru*1-bearing haplotype and makes it a good candidate for an alternative source of resistance.

Field evaluation of resistance to brown rust is relatively easy to perform in areas presenting high level of brown rust inoculums. However, Marker-Assisted Selection (MAS) with *Bru*1 PCR diagnostic markers could be very useful, in particular, in areas where the disease has not yet occurred such as Somalia or Sudan (Kelly et al. 2009) or does not

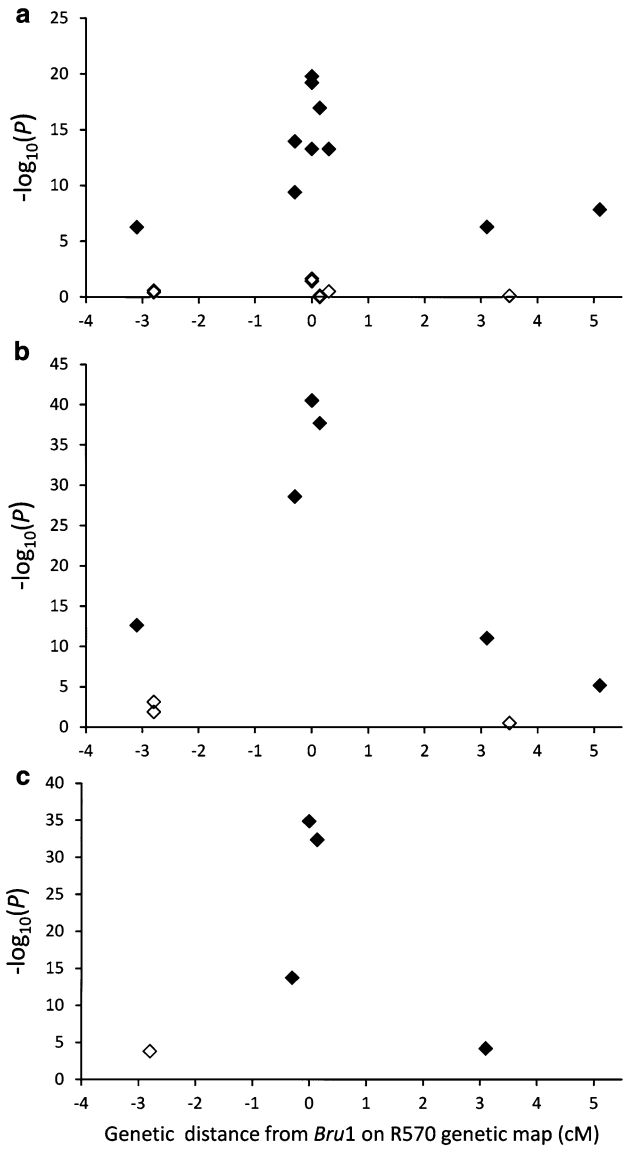

Fig. 3 Association between markers and brown rust resistance in REUa (**a**), REUb (**b**) and GUA (**c**) panels. Level of statistical association between markers and qualitative rust phenotype (resistant vs. susceptible) using a Fisher's exact test and expressed as $-\log_{10}(P)$. Markers in medium or high frequency in the accession panels are represented by *solid* and *open marks*, respectively

regularly develop and thus for which field evaluation is difficult to perform. More importantly, the *Bru*1 PCR diagnostic markers should be particularly useful to identify resistant cultivars, within germplasm collection, that do not bear *Bru*1 and thus may present alternative sources of resistance to brown rust. Tests under controlled conditions demonstrated that *Bru*1 confers resistance to diverse rust isolates collected in Brazil, Colombia, Florida, Guadeloupe, Reunion and Zimbabwe (Asnaghi et al. 2001). In addition, *Bru*1 resistance appears durable, since R570 has been intensively cultivated for 20 years in various regions of the world, including Reunion, Mauritius, and several West African (Burkina Faso, Gabon, Congo) or East African (Tanzania, Malawi, Kenya, Swaziland, Mozambique) countries, and resistance breakdown has never been observed. Nevertheless, alternative sources of resistance will be interesting to use in breeding programs to diversify the genetic basis of brown rust resistance in modern cultivars and develop more durable approaches to brown rust control.

Table 3 Distribution of the four markers that showed the strongest association with *Bru*1 in the whole sugarcane accession panel

Distance in R570 (cM)	Markers	Rust resistant haplotypes					Rust susceptible haplotypes		
0.14	m164H22	1	0	0	0	0	0	0	1
0	R12H16-PCR	1	1	1	0	0	0	0	0
0	9O20-F4-PCR-*Rsa*I	1	1	1	0	0	0	0	0
0.28	cBR37-PCR	1	1	0	1	0	0	1	0
	Nb of accessions[a]	162	1	3	1	27	149	34	2

[a] One accession out of the 380 analyzed had incomplete information and thus is not included

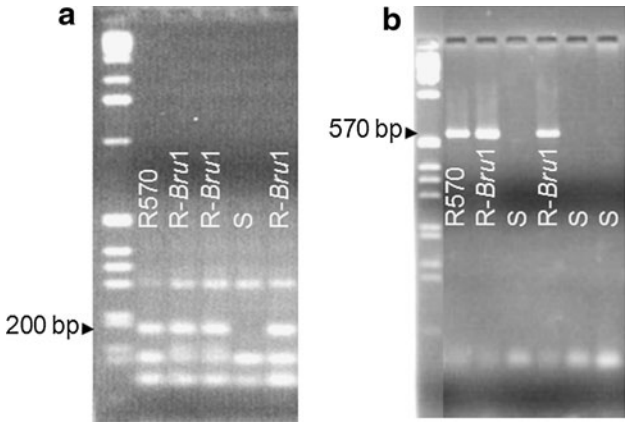

Fig. 4 DNA profiles of the two molecular diagnostic markers for *Bru*1 in a subset of sugarcane modern cultivars. **a** 9O20-F4-PCR-*Rsa*1, **b** R12H16-PCR. R-*Bru*1: rust resistant sugarcane cultivar bearing *Bru*1. *S* rust susceptible sugarcane cultivar

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that the experiments presented in this publication comply with the current laws of France.

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