Bru*1 gene and potential alternative sources of resistance to sugarcane brown rust disease*

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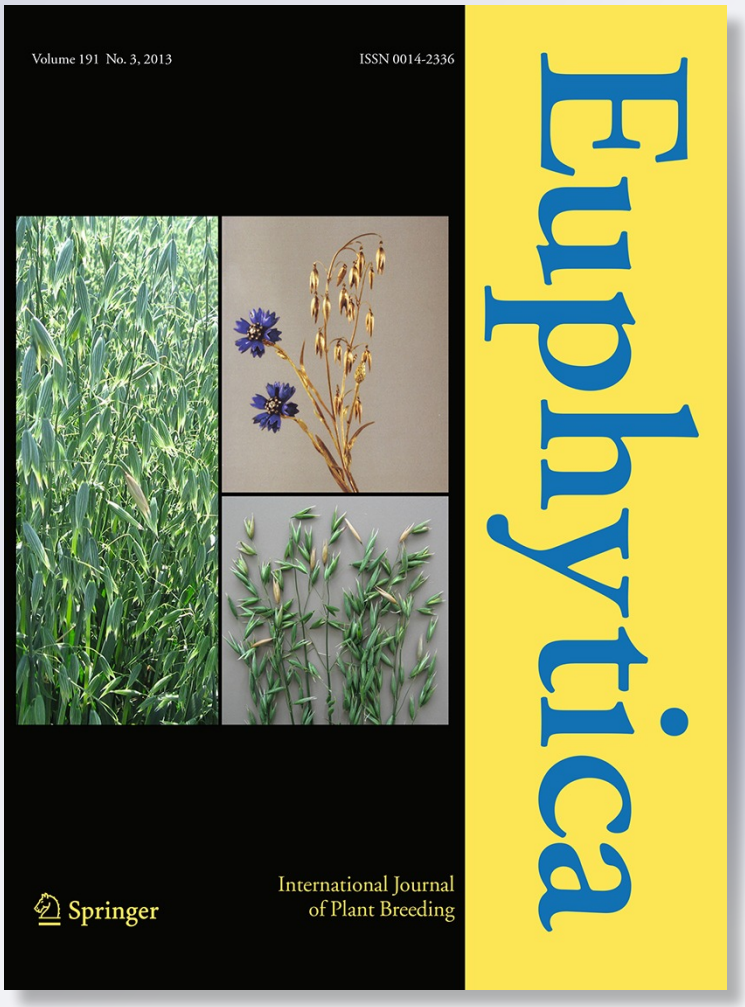

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*Bru*1 gene and potential alternative sources of resistance to sugarcane brown rust disease

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Abstract Brown rust, caused by the fungus *Puccinia melanocephala*, is responsible for important yield losses in sugarcane production globally and it is therefore an important objective to introduce resistance to this disease in breeding programs. A major gene, *Bru*1, has been shown to confer resistance to *P. melanocephala* strains from different parts of the world and two molecular markers, R12H16 and 9O20-F4, closely associated to this gene have been previously reported. The usefulness of these molecular diagnostic markers in order to predict a rust resistant phenotype under natural high pressure inoculums conditions was analyzed. A total of 129 sugarcane accessions were evaluated under field infection for resistance or susceptibility to brown rust and subsequently screened for presence or absence of the two *Bru*1 diagnostic markers. A total of 49 genotypes (38 %) were phenotyped as resistant to brown rust but only eight (16.3 %) of them were harboring the *Bru*1 gene. To determine overall frequency of the *Bru*1 in the local sugarcane germplasm collection, 190 additional genotypes were examined. Presence of *Bru*1, as determined by the diagnostic markers, was detected in only 7 % of the genotypes evaluated. In conclusion, *Bru*1 diagnostic markers enable positive selection for brown rust resistance in sugarcane and moreover allowed detecting at least one additional source(s) of resistance. Interestingly, whilst only little genetic variability of rust resistance independent of *Bru*1 has been reported previously, this alternative genetic resource(s) found in our local germplasm constitutes the predominant one and should be helpful in order to amplify the narrow genetic basis for brown rust resistance in sugarcane.

Keywords *Bru*1 · Disease resistance · Genetic resources · Molecular markers · *Puccinia melanocephala*

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Introduction

Puccinia melanocephala is the causal agent of brown rust, an important sugarcane disease in many production areas worldwide (Raid and Comstock 2000). Brown rust has been associated with significant reduction in yields

(over 50 % in some cases) and as a consequence important economic losses (Hoy and Hollier 2009).

The most effective method to control this disease is the use of resistant commercial varieties. However, the durability of the resistance is affected by a number of different factors, including, pathogen genetics, plant growth stage, weather conditions, plant nutrition, and soil characteristics (Anderson and Dean 1986; Anderson et al. 1991; Raid and Comstock 2000). Due to this, shifts in cultivar reactions from resistance to susceptibility have been observed in sugarcane (Hoy and Hollier 2009; Ramallo et al. 2005). Nevertheless, a brown rust resistant sugarcane cultivar, R570, one of the most widely cultivated varieties for 20 years on the Reunion island and other places (Asnaghi et al. 2004), has never lost its resistance to brown rust although it has been challenged with various isolates collected from several different parts of the world (Asnaghi et al. 2001). The basis of the brown rust resistance of this cultivar has been thoroughly studied and results showed a monogenic and dominant control involving a single copy resistance allele (Daugrois et al. 1996). This major resistance gene, *Bru*1, is the first well-characterized Mendelian trait described in the complex genomic context of sugarcane (Asnaghi et al. 2004). A recent study has reported that brown rust resistance in modern sugarcane cultivars relies essentially on the *Bru*1 gene (Costet et al. 2012). Genomic studies have shown that the *Bru*1 is included in an insertion of unknown size that is specific to *Bru*1-bearing haplotypes, being absent in other hom(e)ologous haplotypes (Le Cunff et al. 2008). This insertion induces a reduction of recombination, resulting in strong linkage disequilibrium (LD) in the *Bru*1 region and a complete LD between two flanking molecular markers, R12H16 and 9O20-F4. These represent valuable diagnostic markers for the presence of *Bru*1, since their presence predicts a resistant behavior of any modern sugarcane cultivar. In addition, the absence of these markers in a resistant cultivar would indicate the absence of *Bru*1, suggesting an alternative source of resistance (Costet et al. 2012).

The aims of the present work were (i) to study the usefulness of both markers associated with *Bru*1 to predict a rust resistant phenotype in the Sugarcane Breeding Program of Estación Experimental Agroindustrial Obispo Colombres (EEAOC) and their potential application in marker assisted selection (MAS); (ii) to determine the presence of alternative genetic sources for resistance to sugarcane brown rust; as well as (iii) to assess the frequency of the *Bru*1 gene in the local germplasm.

Materials and methods

Plant material

In order to determine the usefulness of *Bru*1, 129 sugarcane accessions, frequently used as parents in the EEAOC Sugarcane Breeding Program, were evaluated under field infection for resistance or susceptibility to local brown rust inoculum and subsequently screened for presence or absence of *Bru*1 by applying both markers R12H16 and 9O20-F4-*RsaI* associated to this gene (Costet et al. 2012). Out of the 129 sugarcane cultivars tested, 60 were HOCP varieties (46 %) obtained in crosses performed at Canal Point (Florida, USA) and later selected at Houma (Louisiana, USA) and 51 TUC varieties (40 %) obtained and selected in the EEAOC Sugarcane Breeding Program. The remaining 14 % of accessions is comprised by genotypes CP, HO, L (which were obtained and selected at Canal Point, Houma and Louisiana, respectively), LCP (obtained at Canal Point and selected at Louisiana), LHO (obtained at Houma and selected at Louisiana), RA (obtained and selected by the EEAOC-INTA former breeding program in the República Argentina) and TUCCP (obtained at Canal Point and selected at Tucumán, Argentina) (Table 1).

In order to determine the status, needs and future directions for breeding for resistance against brown rust, 190 additional sugarcane accessions of the EEAOC germplasm collection (Table 2) were studied to assess the overall frequency of appearance of the *Bru*1 gene.

Field evaluation of brown rust resistance

In order to evaluate susceptibility and resistance against brown rust, 129 sugarcane cultivars in plant-cane (Table 1) that were evaluated for the presence of the *Bru*1 were also examined in a field-test using natural infections in an unreplicated design consisting of individual 3-meters-long row plots. The field-trial was planted in Cerco Represa (Tucumán, Argentina) in July 2011 and evaluated for brown rust appearance in February 2012, i.e. under high pressure inoculums

Table 1 Brown rust severity and presence of molecular diagnostic markers for *Bru*1, in the 129 sugarcane accessions studied

Rust severity[a]	*Bru*1 diagnostic markers presence[b]	Accessions				
1	+	HO94-856	HOCP03-714	HOCP03-738	HOCP04-814	HOCP05-918
		HOCP05-920	L79-1002	TUC95-35		
	–	HO94-851	HOCP00-961	HOCP02-640	HOCP03-717	HOCP03-731
		HOCP03-744	HOCP05-931	HOCP85-845	HOCP92-631	HOCP95-951
		RA87-3	TUC00-56	TUC00-74	TUC92-10	TUC94-59
		TUC95-24	TUC96-46	TUC97-20	TUC98-44	TUC99-12
		TUC99-132				
2	–	HO02-653	HOCP01-517	HOCP02-636	HOCP02-652	HOCP03-739
		HOCP04-847	HOCP05-961	HOCP94-806	HOCP95-988	TUC00-019
		TUC00-165	TUC00-53	TUC01-14	TUC01-23	TUC89-28
		TUC95-07	TUC95-37	TUC97-08	TUC98-16	TUC98-24
3	–	CP91-523	HOCP01-523	HOCP03-711	HOCP03-713	HOCP03-730
		HOCP03-736	HOCP05-902	HOCP92-645	HOCP92-648	HOCP93-746
		HOCP93-750	L89-113	L91-281	L94-424	LCP86-454
		TUC00-008	TUC95-10	TUC95-23	TUC95-34	TUC95-46
		TUC96-01	TUC97-30	TUC98-018	TUC98-048	TUC99-05
4	–	CP89-2377	HOCP02-618	HOCP02-622	HOCP03-718	HOCP03-720
		HOCP05-903	HOCP91-555	HOCP92-624	L94-428	LHO83-153
		TUC94-47	TUC96-24	TUC96-60	TUC98-01	TUC98-20
		TUC99-10	TUCCP77-42			
5	–	HO95-985	HOCP00-950	HOCP01-551	HOCP02-610	HOCP02-625
		HOCP03-704	HOCP03-708	HOCP03-719	HOCP92-618	HOCP93-754
		HOCP96-540	TUC01-24	TUC95-18	TUC95-25	TUC95-36
		TUC96-52	TUC97-07	TUC98-02	TUC98-13	
6	–	HOCP01-561	HOCP01-564	HOCP02-632	HOCP03-725	HOCP05-923
		HOCP05-937	HOCP92-675	TUC97-101		
7	–	CP65-357	HOCP03-749	TUC01-11		
8	–	HOCP03-743	HOCP05-904	HOCP91-552	TUC01-29	TUC98-108
		TUC99-125				
9	–	LCP85-384	TUC95-22			

[a] Rust severity scored on a 1 (the most resistant) to 9 (the most susceptible) scale, according to Amorin et al. (1987)

[b] *Bru*1 presence detected by molecular diagnostic markers R12H16 and 9020-F4-*RsaI*. (+) indicates the presence of both markers. (–) indicates the absence of both markers

during the most favorable period for rust development. Brown rust reaction was scored in each plot on a 1 (no disease) to 9 (more than 50 % of the leaf blade tissue damaged) scale, according to Amorin et al. (1987), by two independent observers. This logarithmic scale is based on damaged leaf area (0; 0.5; 1; 5; 10; 25; 35; 50 and greater than 50 %). A score of 1–2 indicates resistant plants with no pustules of the fungus, though some necrotic or chlorotic spots may appear on the leaves. A score of 3–4 indicates moderate resistant plants with very few pustules lesions on old leaves but with no sporulating pustules. Grade 5–9 plants were considered as susceptible with increasing density of sporulating lesions on older leaves, with appearance on younger leaves and extensive leaf necrosis.

The LCP 85-384 variety, which is very susceptible to brown rust, was planted at the borders surrounding the trial, as well as repeatedly inside the plot in order to maximize infection.

It must be pointed out that the presence of *P. kuehnii*, the causal agent of orange rust, has not been detected in Tucumán (Bertani et al. 2012), so all

Table 2 Sugarcane accessions belong to the EEAOC germplasm bank studied for the presence of *Bru*1 gene

*Bru*1 presence[a]	Accessions					
+	CO419	CO421	CP52-68	CP53-17	POJ2878	R570
	TUC01-45	TUC02-38	TUC02-41	TUC03-32	TUC03-36	TUC04-4
	TUC94-58					
−	CO281	CP33-224	CP36-105	CP44-155	CP48-103	CP48-126
	CP52-1	CP53-16	CP57-614	CP61-37	CP67-411	CP72-370
	HO94-850	HOCP95-931	L65-69	LCP82-89	NA56-79	TUC00-15
	TUC00-16	TUC00-24	TUC00-26	TUC00-27	TUC00-33	TUC00-36
	TUC00-5	TUC00-55	TUC00-68	TUC00-72	TUC00-9	TUC01-17
	TUC01-22	TUC01-3	TUC01-38	TUC01-39	TUC01-40	TUC01-41
	TUC01-42	TUC01-43	TUC01-44	TUC01-46	TUC01-47	TUC01-48
	TUC01-49	TUC01-50	TUC01-51	TUC01-52	TUC01-53	TUC01-54
	TUC01-55	TUC01-56	TUC02-27	TUC02-28	TUC02-29	TUC02-30
	TUC02-31	TUC02-32	TUC02-33	TUC02-34	TUC02-35	TUC02-36
	TUC02-37	TUC02-39	TUC02-40	TUC02-42	TUC02-43	TUC02-44
	TUC02-45	TUC02-46	TUC02-47	TUC02-48	TUC02-49	TUC02-50
	TUC02-51	TUC02-52	TUC02-53	TUC02-54	TUC02-55	TUC02-56
	TUC02-57	TUC02-58	TUC02-59	TUC02-60	TUC02-61	TUC02-62
	TUC02-63	TUC02-64	TUC02-65	TUC02-66	TUC02-67	TUC02-68
	TUC02-69	TUC02-70	TUC02-71	TUC02-72	TUC03-17	TUC03-18
	TUC03-19	TUC03-20	TUC03-21	TUC03-22	TUC03-23	TUC03-24
	TUC03-25	TUC03-26	TUC03-27	TUC03-28	TUC03-29	TUC03-30
	TUC03-31	TUC03-33	TUC03-34	TUC03-35	TUC03-37	TUC03-38
	TUC03-39	TUC03-40	TUC03-41	TUC03-42	TUC03-43	TUC04-1
	TUC04-2	TUC04-3	TUC04-5	TUC04-6	TUC04-7	TUC69-2
	TUC71-7	TUC78-17	TUC79-9	TUC90-14	TUC92-3	TUC93-104
	TUC93-116	TUC93-58	TUC93-89	TUC94-55	TUC94-61	TUC95-02
	TUC95-1	TUC95-17	TUC95-26	TUC95-30	TUC95-33	TUC95-39
	TUC95-41	TUC95-59	TUC95-65	TUC96-17	TUC96-19	TUC96-21
	TUC96-23	TUC96-41	TUC96-43	TUC96-53	TUC96-55	TUC96-59
	TUC97-1	TUC97-19	TUC97-21	TUC97-22	TUC97-23	TUC97-24
	TUC97-25	TUC97-26	TUC97-27	TUC97-4	TUC97-9	TUC98-19
	TUC98-21	TUC98-36	TUC98-38	TUC98-49	TUC98-5	TUC98-54
	TUC99-11	TUC99-17	TUC99-3			

[a] *Bru*1 presence detected by molecular diagnostic markers R12H16 and 9020-F4-*RsaI*. (+) indicates the presence of both markers. (−) indicates the absence of both markers

rust symptoms observed were a direct consequence of *P. melanocephala* infection.

Molecular diagnostic test for *Bru*1 presence

To screen for presence of *Bru*1 gene, three young leaves from each genotype were collected and genomic DNA was extracted following the CTAB method as described by Aljanabi et al. (1999). The two molecular diagnostic markers, R12H16 and 9O20-F4, strongly associated to *Bru*1 and associated with brown rust resistance (Costet et al. 2012), were used in this study to test for presence of the *Bru*1 gene. All PCR reactions were carried out using the same set up: 20 µl final volume containing 50 ng template DNA, 0.4 µM of each primer; 0.4 mM of each dNTP, 2.5 mM $MgCl_2$, 0.5 units Taq Polymerase with 1× PCR buffer

provided with the enzyme. Thermocycling was performed in My Cycler thermocycler (Bio-Rad) as follows: 4 min denaturation at 94 °C followed by 35 cycles of 94 °C for 30 s, 55.5 °C for 45 s, 72 °C for 72 s and final elongation for 8 min at 72 °C. All DNA amplifications were repeated at least three times for each genotype. The R12H16 PCR marker, amplified with the primer pair: Fw: CTACGATGAAACTACACCCTTGTC/Rv: CTTATGTTAGCGTGACCTATGGTC, corresponds to a specific fragment whose presence is associated to the *Bru*1 gene. Determination of a positive result using the primer set 9O20-F4 (Fw: TACATAATTTTAGTGGCACTCAGC/Rv: ACCATAATTCAATTCTGCAGGTAC) requires digestion of the amplified product with the restriction enzyme *Rsa I*. Fifteen microliters of 9O20-F4 PCR products was digested to visualize the presence of a specific band associated to the *Bru*1 gene. R12H16 PCR products and 9O20-F4-*RsaI* restriction fragments were visualized following electrophoresis on 1.5 and 3 % agarose gels, respectively, by Gelred staining. Positive DNA control used in batch testing of the samples was DNA from the sugarcane cultivar R570; the genotype in which *Bru*1 was originally discovered.

Results

Association between the presence of *Bru*1 diagnostic markers and brown rust resistance

One hundred and twenty-nine parents frequently used in the EEAOC Sugarcane Breeding Program were evaluated for brown rust resistance and susceptibility in the field under natural infestation conditions. Genotypes were considered as resistant, collecting a score of 2 or lower and as susceptible when the score was equal to or higher than 3. Out of the 129 genotypes evaluated, 49 (38 %) were found to be resistant to brown rust (Table 1). Both molecular markers, R12H16 and 9O20-F4-*RsaI*, associated with the *Bru*1 gene were only detected in eight out of the 49 resistant genotypes tested (16.3 %) (Table 1). Both markers were present in all positive samples, confirming a high linkage between them, and were absent in all the 80 susceptible accessions. Interestingly, 83.7 % of all clones considered to be resistant to rust, did not contain *Bru*1 as inferred from analyses with the two diagnostic markers.

Screening for rust resistance gene *Bru*1 using molecular diagnostic markers

One hundred and ninety additional sugarcane accessions from the EEAOC sugarcane germplasm were analyzed using both molecular diagnostic markers in order to study the overall frequency of appearance of the *Bru*1 gene. Only 7 % (13/190) of these additional genotypes evaluated were found to contain the *Bru*1 gene (Table 2). The accessions studied include TUC-genotypes in the last selection stages within the EEAOC Sugarcane Breeding Program, as well as ancestors of CP-varieties (from Canal Point), among others.

Combining both studies, *Bru*1 was detected in only 21 of the 319 accessions studied, including ancestors, parents and clones from breeding populations, resulting in a low overall frequency of 0.07 (Fig. 1).

Discussion

Puccinia melanocephala was first described in Tucuman, Argentina, by Vazquez de Ramallo (1988) and became within a few years one of the most important sugarcane pathogens in the country. The most important sugarcane varieties grown in Argentina at that time were susceptible to the disease and had therefore to be replaced by more resistant cultivars. When the resistant cultivar LCP 85-384 was released in Argentina in 1999, it was rapidly expanded in acreage due to the improved yields. Thereby, this cv. has soon became the most wide-spread variety in Tucumán, occupying 76.6 % of the sugarcane acreage in 2011 (Ostengo et al. 2012), leading to a situation known as "boom" in a ''boom-and-bust'' cycle (Priestly 1978). Under such a situation with a rapid expanding acreage of a specific cultivar and of the resistance gene(s), the most virulent strains can take advantage within the pathogen population. As a consequence the resistance breaks down and since there are no pathogen strains with which to compete, the virulent one(s) rapidly proliferate over large acreages, and the ''bust'' occurs (Glynn et al. 2012). This disease outbreak occurred in Argentina in 2005 with the brown rust outbreak in the cv. LCP 85-384 (Ramallo et al. 2005), becoming one of the most serious sugarcane phytosanitary problems in the country (Ostengo et al. 2012). The same situation had already been observed in Louisiana

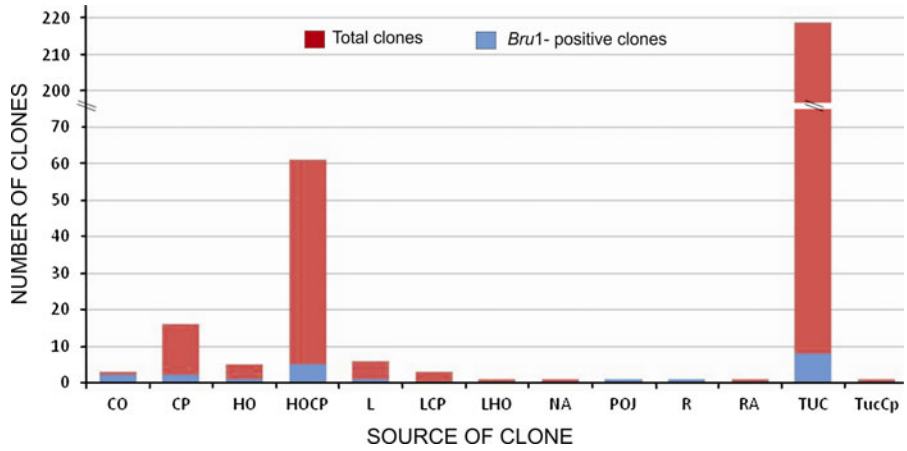

Fig. 1 Number and source of the 319 clones evaluated for the *Bru*1 presence

when cv. LCP 85-384, considered to be resistant to rust from its release in 1993, became increasingly popular with growers covering ≈71 % of the sugarcane production area in 2000, leading to a rust outbreak in this cultivar that year (Johnson et al. 2007). Although the rust epidemic was not as severe during the 2 following years at Louisiana, the incidence and severity of rust increased causing significant yield losses in LCP 85-384 during 2004 and 2005 particularly in the southern areas of the USA industry (Hoy 2005). The acreage of this cultivar was reduced rapidly (Hoy and Hollier 2009). However, in Argentina, growers refuse to replace LCP 85-384 because even tough this variety is showing the highest grade in rust incidence scale, its yield is not affected. This observation has been corroborated in recent studies concluding that there were no significant yield losses due to rust in LCP 85-384 during 2010 and 2011 in Argentina (Funes et al. 2012). The explanation for this is probably due to that the major incidence of the disease in this region takes place during January and February, and some years even in March, when the cane field is around 7 months of age and the cultural yield is already defined. The high productivity of LCP 85-384, which still is not affected by their high susceptibility to brown rust, make it the preferred cultivar and explain the rapid growth of its planted area in Tucumán, the main cane producing region in Argentina. However, taking into account the climate changes (Feijóo et al. 1997; Solomon et al. 2007), the incidence and severity of rust could increase and affect the plant in an earlier stage of growth which would cause significant yield losses. Moreover, an undiversified production scheme, as seen in Tucumán, is causal of a serious phytosanitary problem due to the increased presence of inoculum in the region. To shift this unsustainable situation the local breeding program of the EEAOC is working intensively in order to release new highly productive and brown rust resistant varieties.

In this work, the prevalence of *Bru*1 gene within the genetic base of the EEAOC Sugarcane Breeding Program was estimated by determining the frequency of *Bru*1 among 319 genotypes. Interestingly, the two molecular diagnostic markers associated with *Bru*1 were detected in only 21 genotypes (Fig. 1) although at least 49 accessions had been phenotyped as resistance to brown rust. This relatively low frequency of the *Bru*1 gene is probably due to the presence of an alternative source(s) of resistance and to the relatively recent selection pressure on sugarcane clones towards resistant phenotypes, as brown rust was not an important sugarcane disease in Argentina before 2005. The EEAOC Sugarcane Breeding Program constantly exchanges parents with the breeding program from Louisiana due to similar agro- ecological and growth conditions. As a consequence, these breeding programs have been shown to share a narrow gene pool (Perera et al. 2012). In accordance with this finding the low frequency of *Bru*1-positive genotypes in the EEAOC genotype collection is in agreement with the results obtained by Glynn et al. (2012), who found that the frequency of *Bru*1 in parental clones used in Louisiana sugarcane breeding programs was as low as 6 %.

Knowledge of the frequency of *Bru*1 and its performance against local inoculums of brown rust

within the sugarcane genetic base, will allow breeders to differentiate clones exhibiting resistance due to *Bru*1 from other genetic sources. The identification and integration of additional genetic sources of brown rust resistance in sugarcane breeding will broaden the brown rust resistance genetic base, avoiding the "bust" effect and helping to preserve the useful life of *Bru*1 for controlling brown rust. Actually, the brown rust resistance due to *Bru*1 in sugarcane is in a ''boom'' phase in Florida (USA) acreage, where the current three leading cultivars (CP 89-2143, CP 88-1762, and CP 80-1743), occupying 60–70 % of the acreage since 2004, all were tested positive for *Bru*1 (Glynn et al. 2012). Due to this, breeders should increase efforts to identify more sources of brown rust resistance to avoid the potential breakdown in resistance to *Bru*1 due to *P. melanocephala* race evolution. In contrast, the low frequency of *Bru*1 among the EEAOC sugarcane germplasm coupled with its performance against local rust inoculum make this gene a useful tool for breeders to control brown rust of sugarcane caused by *P. melanocephala* in Argentina.

In this study we have shown that *Bru*1 diagnostic molecular markers enable positive selection of brown rust resistant phenotypes under local conditions in the main sugarcane production area (Tucumán) of Argentina. Moreover, these diagnostic markers helped reveal the existence of additional source(s) of resistance in the EEAOC sugarcane germplasm. This alternative source(s) of resistance will help to diversify the dangerously narrow genetic base of brown rust resistance as been recently reported by Costet et al. (2012) and Glynn et al. (2012). Both studies concluded that *Bru*1 is the predominant source of brown rust resistance in the sugarcane germplasm that they analyzed. In contrast to these results, we conclude that the predominant source of resistance in the local breeding program of Tucuman would be a resistance source(s) independent of the *Bru*1 gene.

However, further analysis are necessary to characterize both the genetic diversity of the pathogen and the alternative sources of resistance, in order to develop additional molecular markers associated with resistance and to improve brown rust disease management.

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