Mexican Journal of Phytopathology
e-ISSN: 2007-8080

Crossref Open access

Scientific Article

Identification of molecular markers associated with resistance genes to *Puccinia triticina*

Yérica Renata Valdez-Rodríguez[1], Julio Huerta-Espino[2], Luis Antonio Mariscal-Amaro[3]*, Héctor Eduardo Villaseñor-Mir[2], Elizabeth García-León[4], Eliel Martínez-Cruz[2]. [1]Colegio de Postgraduados, Km. 36.5, Montecillo, Estado de México, C.P. 56230, México. [2]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Campo Experimental Valle de México, Km. 13.5 carretera Los Reyes-Texcoco, Coatlinchán, Texcoco, Estado de México, C.P. 56250, México. [3]INIFAP, Campo Experimental Bajío, Km. 6.5 carretera Celaya-San Miguel de Allende, Celaya, Guanajuato, C.P. 38110, México. [4]INIFAP, Campo Experimental Valle del Fuerte, Km. 1609 Carretera Internacional México-Nogales, Juan José Ríos, Guasave, Sinaloa, C.P. 81110, México.

Corresponding Author:
Luis Antonio Mariscal-Amaro
mariscal.luis@inifap.gob.mx

Section:
Periodical Issue

Received:
May 01, 2024
Accepted:
August 21, 2025
Published:
August 27, 2025

Citation:
Valdez-Rodríguez YR, Huerta-Espino J, Mariscal-Amaro LA, Villaseñor-Mir HE, García-León E, *et al*. 2025. Identification of molecular markers associated with resistance genes to *Puccinia triticina.* Mexican Journal of Phytopathology 43(3): 84. https://doi.org/10.18781/R.MEX.FIT.2405-4

ABSTRACT

Background/Objective. Wheat is affected by *Puccinia triticina*, which causes leaf rust, with yield losses exceeding 80% in susceptible varieties. The most viable strategy for managing the disease is the use of varieties with partial resistance, which is expressed with low severity intensity. This requires parents with genes that confer this type of resistance, so wheat materials must be analyzed with molecular markers associated with these genes.

Materials and Methods. In this study, the presence of these markers was determined in advanced wheat lines and recently released varieties. Thirty-four genotypes were evaluated against leaf rust at seedling and adult plant stages, assessing disease severity. Different molecular markers were used to determine the presence of resistance alleles for five genes.

Results. Of the genes analyzed with the markers, *Lr34*, *Lr46*, *Lr68*, and *LrB1* were identified. These genes were found alone and in combinations, with lower levels of the disease observed in genotypes with a higher number of these genes.

Conclusion. The use of molecular markers associated with these genes will facilitate the selection of wheat genotypes that can be included in future crossing plans.

Keywords: *Triticum aestivum*, Rust, Parcial resistance, Slow-rusting

INTRODUCTION

Leaf rust caused by *Puccinia triticina* continues to be one of the most important wheat diseases in Mexico. One of the cheapest and environmentally appropriate control methods is the use of resistant varieties. Within the populations of this plant pathogen, the physiological races that preferentially attack durum wheat have evolved quicker than those that attack bread wheat. For example, in 2001, race BBG/BP (Singh *et al.,* 2004) was identified as virulent to the resistance gene *Lr72* (Herrera-Foessel *et al.,* 2014a), which sebsequently evolved and gave rise to the race BBG/BP with virulence to *Lr27+31* (Huerta-Espino *et al.,* 2009) and eventually, the pathogen evolved to overcome the resistance gene found in the variety Cirno C2008 (Huerta Espino *et al.,* 2017, Huerta-Espino *et al.,* 2024). However, among the races that preferentially attack bread wheats, evolution has been slow over the past two decades, partly due to the type of resistance used in the breeding process.

Resistance is generally classified in two non-exclusive types: race-specific and race-non-specific (Caldwell, 1968) and equivalent to the concept of vertical and horizontal resistance (Vanderplank, 1963). Race-non-specific resistance is also identified as partial resistance to late blight in potato (*Solanum tuberosum* L.) (Niederhauser *et al.,*1954), partial resistance to leaf rust in barley (*Hordeum vulgare* L.) (Parlevliet,1975), slow rusting to wheat (*Triticum aestivum* L.) leaf rust (Caldwell, 1968), or durable resistance to yellow wheat rust (Johnson, 1978). Race-specific resistance is generally qualitative and usually short-lived, due to the evolution of potentially virulent pathogens (Huerta-Espino *et al.,* 2011). On the other hand, adequate levels of race-non-specific resistance involves genes with minor to intermediate effects, so that plants carrying this type of resistance are susceptible at the seedling stage but display resistance in later growth stages (adult plant). This characteristic is named slow-rusting resistance and is associated with certain forms of adult plant resistance (Lagudah, 2011). It has been observed that near-immunity to fungi that cause rust can be achieved by combining minor genes with additive effects (Singh *et al.,* 2000). The most important genes belonging to the category of "slow-rusting" or "adult plant resistance" (RPA) are: *Lr34* (Singh *et al.,* 2012), *Lr46* (Singh *et al.,* 2013), *Lr67* (Herrera-Foessel *et al.,* 2014b) and *Lr68* (Herrera-Foessel *et al.,* 2012). These genes are related to leaf tip necrosis (*Ltn*), a morphological trait that appears after flowering (Huerta-Espino *et al.,* 2020). It has recently been discovered that the gene *Sr2*, which provides resistance to stem rust is related to a gene that provides resistance to leaf rust, temporarily named *LrB1* (Ye *et al.,* 2022). In Mexico there has been emphasis on the genes that provide partial resistance to the adult plant since the 1950s (Huerta-Espino *et al.,* 2020). Based on molecular markers, a recent study pointed out the presence of *Lr34*, *Lr46*, *Lr67*, *Lr68* and *Sr2/LrB1* genes, on their own or in different combinations in the improved wheat genotypes before the Green Revolution, such as Frontera, Supremo 211, Chapingo 48, Yaqui 50, Kentana 52, Bajío 52, Bajío 53, Yaqui 53, Chapingo 53, Mayo 54. These genes were also found in their offspring, the semi-dwarf wheats, developed after crosses and by the recombination of these parents, in recently released varieties (Huerta-Espino *et al.,* 2020).

Considering the importance of leaf rust and the availability of molecular markers associated with the genes for partial resistance, the aim of this study was to determine the presence of these markers in advanced wheat lines and recently released varieties.

MATERIALS AND METHODS

Genetic material. The wheat genotypes used in this study are shown in Table 1.

Table 1. Wheat genotypes used in the study.

1	Kronstad F2004	10	LinA[1] A	19	Texcoco F2016	28	Luminaria F2012
2	Urbina S2007	11	Conatrigo F2015	20	LinA D	29	Maya S2007
3	Roelfs F2007	12	LinA B	21	Canicula "s"	30	Urbina S2007
4	Norteña F2007	13	Fuertemayo F2016	22	LinA E	31	Elia M2016
5	Nana F2007	14	LinA C	23	LinA F	32	Faisán S2016
6	Onavas F2009	15	Noreste F2018	24	LinA G	33	Ibis M2016
7	Villa Juárez F2009	16	Altiplano F2007	25	Alondra F2014	34	Cisne F2016
8	Borlaug 100	17	Don Carlos M2015	26	Cortázar S94		
9	Bacorehuis F2015	18	Valles F2015	27	Bárcenas S2002		

[1]LinA= advanced lines: A = BECARD/KACHU; B = KACHU/DANPHE; C = ND643/2*WBLL1/4/WHEAR/KUKUNA/3/C80.1/3*BATAVIA//2*WBLL1; D = (Lucía "S") = ZCT/SLM//CHAZ/..../3/KITE/BOW" S" //MEX/ROM; E = PAMDOLY-PABG (C7).3; F = PAMDOLY-PABG (C7).1; G = PAMDOLY-PABG (C7).2.

Resistance tests. The resistance test on seedlings was carried out in the Laboratorio Nacional de Royas y otras Enfermedades de Cereales (LANAREC). The leaf rust race MBJ/SP was used, the avirulence/virulence formula of which is: *Lr2a*, *2b*, *2c*, *3ka*, *9*, *16*, *18*, *19*, *21*, *24*, *25*, *26*, *28*, *29*, *30*, *32*, *33*, *36/1*, *3*, *3bg*, *10*, *11*, *13*, *15*, *17*, *20*, *23*, *27+31* (Herrera-Foessel *et al.,* 2012). For this assay, 8 to 10 seeds of each genotype were placed in plastic trays, using sterile soil as a substrate; the differential leaf rust lines were also included (Huerta-Espino *et al.,* 2021). Thirteen days after planting, the plants were inoculated with a suspension of uredioniospores of the race in mineral oil (Soltrol 170®) in a concentration of 5 mg of urediniospores per mL of oil (Randhawa *et al.,* 2018) and they were watered using a sprinkler connected to an electric compressor (Valdez-Rodríguez *et al.,* 2020).

In an adult plant, the evaluation was performed in the field under rainfed conditions and in which the 34 bread wheat genotypes were established (Table 1) during the 2022 spring-summer cycle in the Valle de México Experimental Field of the (INIFAP-CEVAMEX). In the evaluation in the field, the 34 genotypes were planted in two furrows, each being one meter in length, with two repetitions. The genotypes were directly inoculated with the race MBJ/SP 45 days after planting, preparing an urediniospore suspension in mineral oil (5 mg of urediniospores mL^{-1} of oil) (Randhawa *et al.,* 2018) and sprayed using a manual sprayer. The evaluations of severity on the flag leaf were carried out following a scale that ranged from 0 to 100% (Huerta-Espino *et al.,* 2014).

Molecular analysis. The 34 genotypes listed in Table 1 were planted following the same procedure than the seedling tests, including the respective controls for each resistance gene.

The molecular analysis was carried out in the Biotechnology Laboratory of the Wheat Molecular Breeding Program in the International Maize and Wheat Improvement Center (CIMMYT). For the DNA extraction, leaf tissue samples were taken and placed into 1.1 mL tubes arranged in 96-well plates. The samples were then stored at -80 °C for 3 h, and then transferred to a lyophilizer and kept at -50 ºC under a vacuum level of 0.0 to 0.120

mbar for 48 h. The tissue was ground with 4 mm steel ball bearings for 2 or 3 minutes until it became a fine powder, using a Geno Grinder® (Metuchen, NJ, USA). The quantification and the valuation of the DNA quality were performed following the laboratory protocols for wheat followed by Dreisigacker *et al.* (2016) and Valdez-Rodríguez *et al.* (2020).

Molecular trial for the genotyping of slow-rusting genes. Molecular markers were used to determine the presence of resistance alleles of genes *Lr34*, *Lr46*, *Lr67*, *Lr68* y *Sr2/LrB1* (Table 2). For *Lr34*, an SNP marker was used, designed around the 3-bp indel in exon 11. Gene *Lr46* has not yet been cloned, so no functional markers were available. Therefore, two SNP markers located near *Lr46* were used. For *Lr67*, two diagnostic SNP markers were applied. The markers included the functional SNP found in exon 2 of the gene (*SNP_TM4*). An SNP marker derived from the linked CAPS marker cs7BLNLRR was used to determine the resistance allele for the *Lr68* gene. Similar to *Lr46* and *Lr68,* the *Sr2/LrB1* gene has not been cloned and functional markers are not available. To determine the resistance allele for *Sr2/LrB1* an SNP marker designed based on the linked CAPS marker csSr2 was applied.

The SNP polymorphisms were found using Kompetitive Allele Specific PCR (KASP) reagents (www.lgcgenomics.com) in reactions containing 2.5 µl of water, 2.5 µl reagent mixture 2×KASPar, 0.07 µl of reagent mixture and 50 ng of DNA. The marker for *Sr2/LrB1* was added 2.5 µl MgCl2. The program for the PCR was an inicial denaturation at 94 °C for 15 min, followed by 20 cycles at 94 °C for 10 s, 57 °C for 5 s and 72 °C for 10 s, followed by 18 cycles at 94 °C for 10 s, 57 °C for 20 s and 72 °C for 40 s. Fluorescence was read as an endpoint read at 25 °C. A BMG Pherastar Plus fluorescent plate reader (BMG LABTECH, Ortenberg, Germany) was used to read the PCR products, and the genotypic data were subsequently viewed graphically using KlusterCaller™ Software (LGC Biosearch Technologies).

RESULTS AND DISCUSSION

The results for seedlings in the greenhouse in response to race MBJ/SP helped identify susceptible genotypes and resistant genotypes. The resistant genotypes in seedlings maintained their resistance in adult plants in response to the same race, with the exception of Kronstand and Faisán S2016 (Table 3). The susceptible genotypes in seedlings had a differential behavior in the field from 1% of infection in Borlaug and Bacorehuis, up to high levels of 50% in Cortázar S94 and Bárcenas S2002, which are considered susceptible (Table 3).

Table 2. Molecular markers used to detect resistance alleles in genes *Lr34*, *Lr46*, *Lr67*, *Lr68* y *Sr2/LrB1*.

Gen	Marker	PEC[x]	SNP	A primer	B primer	common primer	FER[y]	Reference
Lr34	*Lr34_TCCIND*	7DS	Del/Ins	GGTATGCCATTTAAC ATAATCATGAA	GGTATGCCATTTAAC ATAATCATGAT	TACTATATGGGAGCATTATTTTTTTCC	Del	Lagudah *et al.* (2009)
Lr46	*Lr46_JF2-2*	1BL	G/C	ATTGTGTGAAGATAG AAGTTCTAATTGAAC	GTGTGAAGATAGAAG TTCTAATTGAAG	CTTGTTCTCTCTTGGAGCGTTGGTA	G	Huerta-Espino *et al.* (2020)
Lr46	*Lr46_SNP1G22*	1BL	A/G	ACCCATGGCTTTGGCT CCG	CTACCCATGGCTTTG GCTCCA	GAAATACGCTAAGACGCCTCCATCAT	A	Huerta-Espino *et al.* (2020)
Lr67	*csSNP856 (Lr67)*	4DL	A/G	GCTACTACTATTGGTA GCCTG	GCTACTACTATTGGT AGCCTA	CCAGTAGCTTATGGCACTCAAA	A	Forrest *et al.* (2014)
Lr67	*Lr67_TM4*	4DL	G/C	TCATCATCGGCAGGAT CCTGCTTC	TCATCATCGGCAGGA TCCTGCTTG	AACGTACGTAATCTTGCTTACTGA	C	Moore *et al.* (2015)
Lr68	*Lr68-2*	7BL	T/C	CGTGTCTTGGACCTGA GCAAT	CGTGTCTTGGACCTG AGCAAC	TGACCTGAGTCCCGTCAAGA	T	Herrera-Fossel *et al.* (2012)
Sr2/LrB1	*Sr2_ger93p*	3BS	G/A	GTGCGAGACATCCAAC ACTCAC	GTGCGAGACATCCAA CACTCAT	CTCAAATGGTCGAGCACAAGCTCTA	A	Mago *et al.* (2011); Ye *et al.* (2022)

[x]PEC= position in the chromosome. [y]FER= dwarf resistance phenotype.

Out of the five resistance genes analyzed with molecular markers, *Lr34*, *Lr46*, *Lr68* and *LrB1* were identified, but gene *Lr67* was not identified in any of the genotypes (Table 3). The marker related to *Lr34* was only identified in seven of the 34 genotypes, making it the least frequent. On the other hand, the marker related to *Lr46* was found in all of the evaluated genotypes. It was found only in the Kronstad variety and in advanced line B, as well as in combination with the *Lr34* marker in Texcoco F2016 and with the *Lr68* marker in advanced line A (Table 3). In combination with *LrB1*, the marker related to *Lr46* was identified in 26 of the genotypes, 13 of which carried the combination of *Lr46*, *Lr68* and *LrB1* (Table 3). Another three-gene combination (*Lr34*, *Lr46* and *Lr68*) was only identified in Valles F2015, Urbina S2007 and advanced line D. In the variety Norteña F2007, markers associated with *Lr34*, *Lr46*, *Lr68* and *LrB1* were identified, making it the only genotype in which the presence of four of the five markers used in this study was found.

The genotypes evaluated in the seedling stage in the greenhouse against the *P. triticina* race MBJ/SP that were resistant maintained their resistance in the field against the same race, with the exception of Kronstand, which has the gene *Lr16*. This gene only confers a moderate resistance to *P. triticina* race TCB/TD and at the adult plant stage behaves as a partial resistance gene, with a final leaf rust severity of 70 to 80% when the gene is on its own (Singh and Huerta-Espino, 1995). In other varieties, such as Francolin, the effect of *Lr16* is lower and resistance levels increase in the presence of non-race-specific genes (Lan *et al*., 2014), such as in the case of Kronstand in the presence of *Lr46*.

Table 3. Responses of the 34 genotypes evaluated to the race MBJ/SP *P. triticina* in the seedling and adult stages and the resistance genes.

No	Genotype	MBJ/SP	MBJ/SP	Genes present[x]	No	Genotype	MBJ/SP	MBJ/SP	Genes present
		Seedling	Adult plant				Seedling	Adult plant[y]	
12	Línea avanzada B	3+	5	*46*	25	Alondra F2014	3+	1	*34. 46, B1*
1	Kronstad F2004	1+3C	50	*46*	21	Canícula "s"	3+	10	*34. 46, B1*
28	Luminaria F2012	1	0	*46, B1*	13	Fuertemayo F2016	0	0	*46, 68, B1*
31	Elia M2016	11+	0	*46, B1*	24	Línea avanzada G	11+	0	*46, 68, B1*
34	Cisne F2016	11+	0	*46, B1*	6	Onavas F2009	1+3C	10	*46, 68, B1*
11	Conatrigo F2015	;	1	*46, B1*	23	Línea avanzada F	4	1	*46, 68, B1*
5	Nana F2007	11+	10	*46, B1*	14	Línea avanzada C	3+	5	*46, 68, B1*
17	Don Carlos M2015	1	10	*46, B1*	16	Altiplano F2007	3+	5	*46, 68, B1*
33	Ibis M2016	;	20	*46, B1*	22	Línea avanzada E	3+	5	*46, 68, B1*
15	Noreste F2018	0	10	*46, B1*	2	Urbina S2007	3+	10	*46, 68, B1*
32	Faisán S2016	1+	30	*46, B1*	3	Roelfs F2007	4	10	*46, 68, B1*
8	Borlaug 100	3+	1	*46, B1*	7	Villa Juárez F2009	4	10	*46, 68, B1*
9	Bacorehuis F2015	3+	1	*46, B1*	29	Maya S2007	3+	20	*46, 68, B1*
26	Cortázar S94	3+	50	*46, B1*	18	Valles F2015	3+	5	*34, 46, 68,*
27	Bárcenas S2002	3+	50	*46, B1*	20	Línea avanzada D	3+	5	*34, 46, 68,*
19	Texcoco F2016	3+	20	*34, 46*	30	Urbina S2007	4	10	*34, 46, 68,*
10	Línea avanzada A	3+	20	*46, 68*	4	Norteña F2007	4	5	*34, 46, 68, B1*

[x]Genes present by association with the molecular markers used in the study. [y]Percentage of infection in the flag leaf according Huerta-Espino *et al*. (2014).

For *Lr16*, certain molecular markers have been used, including *Lr16/Xgwm210* (Lan *et al.,* 2014). When this marker was used in this study (data not shown), it only coincided with the types of infection and the previous postulation of the gene *Lr16* (Huerta-Espino *et al*., 2021) in Nana F2007, Don Carlos M2015 and Luminaria F2012, although no association was found in the cases of Onavas F2009, Faisán S2016 and Cisne F2016, and the cases of Roelfs F2007, Borlaug 100 and Noreste F2018 were false positives. In the case of the genotypes susceptible to MBJ/SP in the seedling stage, its response in the adult plant can be explained by the association of the molecular markers with the resistance genes that include *Lr34*, *Lr46 Lr68* and *LrB1*, alone or combined. However, in the genotypes under evaluation, a higher number of adult plant genes would be expected to lead to lower

infection levels, but it was not the case of advanced line B (KACHU/DANPHE) with only *Lr46*, or in the case of Cortázar S94 and Bárcenas S2002, in which, alongside *Lr46*, the marker associated with *LrB1* was identified and the assumption was only fulfilled in the case of advanced lines C = (ND643/2*WBLL1/4/WHEAR/KUKUNA/3/C80.1/3*BATAVIA//2*WBLL1), D = [(Lucía "S") = ZCT/SLM//CHAZ/..../3/KITE/BOW"S"//MEX/ROM], E = [PAMDOLY-PABG (C7).3,] and F = [PAMDOLY-PABG (C7).1] and Norteña F2007. This would indicate that, along with the genes identified with the molecular markers used, these genotypes contain other resistance genes still uncatalogued and with possible additive effects due to the low infection levels displayed in the field in adult plants. Other studies with molecular markers associated to partial resistance or slow rusting have been reported, such as *Lr46* in Guarina and Huites (Huerta-Espino *et al*., 2020), and *Lr46*+*Sr2*/*LrB1* in Jaral 66, Curinda and Batan (Huerta-Espino *et al*., 2020). Although *Lr67*, alongside *Lr46* and *Lr68* was common in varieties of the 1950s, it was not identified in the semi-dwarf varieties and has not yet been found in combination with *Lr34* (Huerta-Espino *et al*., 2020), and not even identified in this study. The association of resistance gene *Sr2*, which gives stem rust partial resistance was also recently found to confer partial resistance to leaf rust (Diéguez *et al.*, 2014) (and designated as *LrB1*), yet to a lesser degree that the genes previously reported as *Lr34*, *LR46*, *Lr67* and *Lr68* (Ledesma-Ramírez *et al*., 2018 and Huerta-Espino *et al*., 2020). However, the QTL in chromosome 3BS, mapped to the genomic region *Sr2*/*LrB1* (Ye *et al*., 2022), has been reported as a unique locus that provides resistance to leaf rust in races found in the different locations and years in which mapping populations have been tested (Rauf *et al*., 2022). It has been reported that, with the combination of *LrB1* and *Lr46,* leaf rust infection levels decrease by up to 45% (Ye *et al*., 2022). Despite having proven that the strategy of clustering several partial resistance levels is the most effective to control wheat leaf rust (Singh *et al*., 2000), in practice, only in Norteña F2007 variety have genes *Lr34*, *Lr46*, *Lr68* and *LrB1* been able to be clustered, (present study), with *Lr67* still absent.

The results of the present study indicate a reduction in the frequency of *Lr34* and a considerable increase in *Lr46*. With the availability of the molecular markers associated to the resistance genes, an increase in the number of rust-resistant genes was to be expected, or at least, a certain number of combinations should be maintained. However, breeding programs need to select and prioritize the genes to be implemented or the combinations are most likely to provide durable resistance against the fungus that causes leaf rust and thus selection pressure on the pathogen, therefore limiting the emergence of new races. The results of this study support the notion that, ideally, multiple minor adult plant resistance genes and seedling resistance genes should be combined to optimize both the resistance level and its durability in wheat varieties to be released. Even so, a rigorous evaluation of the agronomic effect of the resistance gene combinations on phenotype and yield potential will be required.

CONCLUSIONS

With the analysis of the genotypes in seedlings and adult plants, susceptible and resistant materials were identified, which helped select them for future studies. The molecular markers used helped identify genes *Lr34*, *Lr46*, *Lr68* and *LrB1* in the wheat genotypes identified. In general terms, it was observed that, the greater the number of these genes in the genotypes, the lower the leaf rust levels became. The usefulness of molecular markers associated with the identified resistance genes will allow the selection of superior wheat genotypes that can be included in future crossing schemes of genetic breeding programs.

Acknowledgements

To the lab and field staff for their support during the development of this study. To the Secretariat of Science (SECIHTI) for the scholarship granted to the main author. To INIFAP and CIMMYT for the infrastructure and funding provided during different stages of the research.

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