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MOLECULAR TAGGING OF SORGHUM DOWNY MILDEW *(Peronosclerospora sorghi)* DISEASE RESISTANCE LOCI IN MAIZE (*Zea mays* L.)

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Abstract- Sorghum downy mildew (SDM) caused by *Peronosclerospora sorghi* is a major constraint, which drastically affects maize production and productivity. Conventional breeding approaches to resolve the problem through evolving resistant varieties has resulted in limited success due to the longer breeding cycle. In order to exploit marker assisted selection (MAS) approach for developing resistant varieties, there is a paucity of information on markers linked with the trait. We report here the strategy for identification of markers linked to SDM resistance by selective genotyping, a first crucial step towards selection of resistant lines through MAS. The mapping population of maize Recombinant Inbred lines (158) of UMI 79 and UMI 936 (w) was developed and screened for their resistance levels, both in the field and glasshouse conditions, by spreader row technique and seedling spray inoculation technique, respectively. A total of 35 SSRs, 6 reported to be linked to SDM disease resistance QTL earlier with 29 other SSRs located on chromosome 3 was surveyed for parental polymorphism. Interestingly, of the six markers reported earlier, four were found polymorphic for the parents used in the present study also along with nine other markers. Through phenotypic screening, extreme phenotypes comprising of seven each of resistant and susceptible RILs, were selected and used for selective genotyping employing the polymorphic markers. Among the 13 markers surveyed, an SSR marker bnlg420 showed co-segregation with SDM resistant lines which proves to be a potential tag for introgression of the SDM resistant trait in UMI79 background.

Keywords- *Peronosclerospora sorghi,* Recombinant Inbred Line, Spreader row technique, co-segregation, Marker assisted selection.

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Introduction

Maize (*Zea mays* L.) is the third most important crop after rice and wheat. It is primarily utilized as food, feed, fodder and recently, as a fuel crop. Production of maize is increasing with the rate of 1.6% (non-compounding) per year globally, but this rate is not satisfactory to mitigate the worlds projected demand by 2050 [***1***]. Maize production is hampered by both biotic and abiotic stresses. Hundreds of pest and diseases affects it, contributing to major economic damages. Around 30% of economic losses in tropical lowland and subtropical highland maize is occurring because of a downy mildew [2]. Occasionally, heavy losses around 100% were also noticed in susceptible cultivars with one or other downy mildew [3,4]. It is considered as a top priority disease restraining the maize productivity.

In Asia, different species of genus *Peronosclerospora* and *Sclerophthora* are causing downy mildew [5]. Sorghum downy mildew (*P. sorghi* (Weston & Uppal) Shaw), Philippine Downy Mildew (*P. philippinensis*), Java Downy Mildew (*P. maydis*), Sugar cane Downy Mildew (*P. sacchari*) and Brown stripe DM (*S. rayssiaezeae*) and recently added Rajasthan downy mildew (RDM) caused by *P. heteropogoni* [6]. Among all, sorghum downy mildew (SDM) found to be prevalent in peninsular India in the state of Maharashtra, Karnataka, Tamil Nadu and Andhra Pradesh reportedly causing losses in susceptible cultivars [7].

Sorghum downy mildew occurs in the early stages of plant growth in warm and humid environment. Infected crop shows the "half leaf symptom"[8] where a chlorophyll bleaching starts at the base of the leaf and increase further, full leaf becomes yellowish at later stages thus distressing photosynthesis. If the plant is infected systemically, its reproductive organs get affected. The severely infected plant does not produce cob. If at all cobs are formed, it does not produce the seed. The tassel behaves crazily, forms bushy vegetative structure [9], and thus doesn't produce the pollens. Most of the plant shows the stunted appearance which does not yield well.

Metalaxyl a chemical pesticide found to be effective on diverse downy mildews. New pathotype of *P. sorghi* resistant to Metalaxyl is reported in Texas [10]. Thus, the mere use of chemical pesticide imposes a greater risk of evolving new pathotype [11]. A host plant resistance found to be an economical, environment friendly approach to control disease.

Resistance breeding requires persistent efforts to increase resistance genes/alleles pool to efficiently tackle the disease. In conventional breeding, disease screening is used to identify resistant genotype. Accurate and efficient SDM disease screening confined to conducive weather condition with heavy disease pressure, which imposes constraints for year round selection of the resistant genotype through disease screening. Marker assisted selection is an effective and environmental independent method to identify the resistant sources which can replace the laborious phenotyping that allow off-season selection and

speeding breeding cycle [12, 13].

QTL analysis of sorghum downy mildew resistance have been done previously with linkage mapping [14-18] and association mapping [19,20] that elucidated around 27 QTL regions located in all the chromosomes of the maize except chromosome number 8. QTLs located on chromosome 1,2,3,6 and 9 were reported in two or more than two studies. Hence, these QTLs can be used for the introgression by the marker-assisted selection with further validation [18].

Bulked segregant analysis [21] and selective genotyping [22,23] are two strategies through which a marker linked to large effect QTL or gene can be identified. Selective genotyping can be uni-directional (only one tail of phenotypic distribution is analyzed) or bi-directional. (Both the tail of phenotypic distribution is analyzed) [23]. Commonly, bi-directional genotyping is used as it is effective and problem of segregation-distorted region can be eliminated effectively. In this study, an attempt was made to identify marker linked to SDM disease resistance in maize with the selective genotyping from chromosome 3 where QTL for SDM disease resistance were consistently reported in bin region 3.04 – 3.05. [16-18].

Methodology

Plant Materials

Diverse inbred line, UMI 79 [highly susceptible] and UMI936 (w) [highly resistant], for SDM were crossed, the resultant F_1 hybrid was successively selfed for six generations to get F_7 population of Recombinant Inbred Lines (n=158). Developed F_7 RILs were screened for SDM resistant reaction in the field and glasshouse as given below. Based on phenotypic data, extreme RILs of the population (tails of phenotypic distribution) were carefully chosen further for selective genotyping

Screening of RILs for SDM

Phenotyping for SDM disease was done in the field by "spreader row technique" [*24,25*] CM500 a susceptible variety was used as spreader row and sown one month prior to sowing of the RILs on the border rows of experimental block. Naturally infected Maize leaves with visible conidial growth were collected from nearby fields and placed over moist gunny bags overnight in dark with the temperature maintained at 20^{0}C for sporulation. Conidial suspension was prepared by harvesting conidia of leaves in ice-chilled water and sprayed before sunrise on an eight day old seedlings (soon after emergence of flag leaf) of CM500. A month after the establishment of the disease in the spreader row, test entries, i.e. RILs along with parents were sown and similar process of spraying adopted again to achieve high pathogen pressure buildup.

Glasshouse screening was carried out by "Seedling spray inoculation technique" [*25,26*], where test entries were sown in portrays and conidial suspension was sprayed in two leaf stage of the test entries. Temperature (20^{0}C) and humidity (>90) was maintained to generate conducive weather. After thirty days of plant emergence, the number of infected plants and total number of plants in each entry were recorded from the field and glasshouse. Percent disease incidence was calculated from the field and glasshouse screening by standard procedure [27].

$$\text{Percent downy mildew incidence} = (\text{Number of plants infected}/\text{Total number of plants}) * 100$$

RILs were classified according to SDM reaction, 0 – 10 % as resistant (R); >10 – 30 % as moderately resistant (MR); >30 – 50 % moderately susceptible (MS); >50 % as susceptible (S) [28,29].

Genotyping and Identification of co-segregating marker

Genomic DNA was extracted from leaf sample of the parents and RILs by modified the CTAB method [30] with minor modification. Extracted DNA was quantified by using the Nanodrop (NanoDrop Spectrophotometer ND-1000) and diluted further to achieve concentration of 25 ng/ul. PCR reaction was set to 15 ul with 50 ng of genomic DNA, 10 mM of each forward and reverse primer, 200 uM each of dNTPs, 0.1 units of Taq DNA polymerase, 10 mMTris–HCl, 2 mM $MgCl_2$. The protocol was set to touch down in Bio-Rad PTC-200 DNA Engine® Thermal cycler manually and used. Polymorphism survey for parents UMI79 and UMI936 (w) were done with 35 markers covering all bins form chromosomes 3. Among 35 markers, six markers (phi053, umc1223, bnlg420, bnlg1035, phi073, umc2002) were reported earlier as flanking or within QTL regions of sorghum downy mildew [16-18].

Extreme resistant (<10% infection) and extreme susceptible RILs (>50% infection), showing the consistent SDM reaction in the field and glasshouse evaluation were chosen for a selective genotyping. SSR markers depicting polymorphism between parents UMI79 and UMI936 (w) were deployed to screen those extreme lines (selected based on phenotypic screening). Segregation pattern was observed by running amplified product in the 6% denaturing polyacrylamide gel electrophoresis. Alleles were scored as "A" for UMI79 type and "B" for UMI936 (w) allele type and "H" for the heterozygous allele and thus checked for co-segregation with SDM reaction.

Result and Discussion

Screening for SDM disease

F_7 RIL population (158) developed from a cross of UMI79 and UMI936 (W) was evaluated for SDM disease incidence in the field by spreader row technique. In this experiment, 100 % disease infection in susceptible parent i.e. UMI79 and 7.8 % infection in resistant parents i.e. UMI936 (w) were recorded. As per the standard scoring system for SDM, the percent disease incidence (PDI) was ranged from 6.7% to 95.8% in RILs. In glasshouse screening, UMI 79 showed 96.7%, whereas UMI 936 (w) showed 8.0% disease incidence. The PDI was ranged from 6.7 to 93.1 % in RILs

RILs were classified according to their level of resistance [Table-1] in both condition and it was evident from that, the RIL population did not follow the normal phenotypic distribution for SDM reaction. These results were at par with the observations made by earlier workers wherein they have reported significant deviation from normal distribution for downy mildew reaction for different mapping population [14-16].

Table-1 *Number of RILs showing different levels of resistance to sorghum downy mildew by spreader row techniqueand seedling spray inoculation technique in the field and glasshouse respectively.*

SDM Incidence (%)	Level of Resistance	No. of RILs	
		Field	Glasshouse
0 – 10	Resistant (R)	57	58
>10 – 30	Moderately Resistant (MR)	47	50
>30 – 50	Moderately Susceptible (MS)	28	24
>50	Susceptible (S)	26	26

Table-2 *List of SSR marker used for polymorphism survey between parent UMI79 and UMI936 (w)*

Yellow Highlighted SSR markers are polymorphic for the parent, UMI79 and UMI936(w)

S. No	Marker	Bin	Forward primer (5'-3')	Reverse primer (5'-3')	Earlier Report
1	umc2101	3	CCCGGCTAGAGCTATAAAGCAAGT	CTAGCTAGTTTGGTGCGTGGTGAT	-
2	umc2104	3	CTGCTGGCAGTGGCAGTATTC	TACTGCTACACCTTTGTCGTCACC	-
3	phi104127	3.01	CTTTGCTGCTGCTTCCTACG	AACCAGTGACGTACACAAAGCA	-
4	umc1746	3.01	ACCTTGCCTGTCCTTCTTTCTCTT	ACACGAGCATCCTACATCCTCCTA	-
5	phi193225	3.02	GCTCTTGGCGTGCTTCTT	GCGGGGAGGTGAAGAGCTA	-
6	phi374118	3.02	TACCCGGACATGGTTGAGC	TGAAGGGTGTCCTTCCGAT	-
7	umc2259	3.03	GGCTCGACTTCGAGGACACC	GAGGAGGAGAGGGACAGGGAAG	-
8	phi243966	3.04	CGACCGAAACGAATCAAAA	TACTAGGCTGACACGCACG	-
9	umc2263	3.04	CGTGCTTATATGGGTTCTTGGGT	GTTTGGTTGCTGCGACCTCTT	-
10	bnlg1904	3.04	AGGAGCATGCACTTGGTTCT	ACTCAACTGATGGCCGATCT	-
11	umc1030	3.04	TCCAGAGAATGAGATGACAAGACG	CAGAATAACAGGAGATGAGACGCA	-
12	umc1608	3.04	GTGTCGTGTTGGGAGAACATGAG	TAACTACTACACCACTCGCGCAAA	-
13	phi099	3.04	TACAAAAATCAGGACTGCGAAAAACCCAA	GTCGGTGTGTGATCCTTCCAC	-
14	phi029	3.04	TTGTCTTTCTTCCTCCACAAGCAGCGAA	ATTTCCAGTTGCCACCGACGAAGAACTT	-
15	umc2002	3.04	TGACCTCAACTCAGAATGCTGTTG	CACAAAATCCTCGAGTTCTTGATTG	Jamptaonget *al*., 2013
16	umc1772	3.04	ACATAATAACAAGCAGGCAGGAGG	AAATAACGACTACGGTCACACGGT	-
17	umc1223	3.04	TTCAACAGATTCAGAGAAAGCACA	TTGATAATTAATCCGCAGCTCTCTC	Nair *et al*.,2005
18	phi053	3.05	AACCCAACGTACTCCGGCAG	CTGCCTCTCAGATTCAGAGATTGAC	Nair et al.,2005
19	bnlg420	3.05	CTTGCGCTCTCCTCCCCTT	GGCCAGCTCACTGCTCACT	Nair et al.,2005
20	Bnlg1035	3.05	TGCTTGCACTGTCAGGAATC	CAGCTCTGACACACCACACA	Sabryet *al*., 2006
21	Phi73	3.05	GTGCGAGAGGCTTGACCAA	AAGGGTTGAGGGCGAGGAA	Sabry et al., 2006
22	bnlg1601	3.05	ATCGTGCGCTAGTCCAGAGT	CAGACCAGAGACCATCTGCA	-
23	umc1158	3.05	AATGCAACTGCTTCAGCTCCTACT	CGACGAATCGAGAAAAGATATTTGA	-
24	umc1973	3.05	CAGGCAGAAAAGGAACGGAAC	GTGCGAGAGAAGATGGATGATTG	-
25	bnlg197	3.06	GCGAGAAGAAAGCGAGCAGA	CGCCAAGAAGAAACACATCACA	-
26	umc1674	3.06	ACGAGGTCCACGACTATGGATCTT	AGTAGTACACGGCTGACGGCAC	-
27	umc1690	3.07	ACCTTAGTTACACAGGCACACGGT	GGTGATGGGATTTTCGCATTATTA	-
28	umc2050	3.07	CTCCTGCTGTGATTCTAGGACGA	CTGGATCTCGGCATGGTCTT	-
29	phi088	3.08	CTTCTGTTCCGCCATCCAGTATGT	GATTGCGATAAGCATTGCGGCAGTT	-
30	phi046	3.08	ATCTCGCGAACGTGTGCAGATTCT	TCGATCTTTCCCGGAACTCTGAC	-
31	umc2276	3.08	CTAGGTAGCCAGCTAGGTACGGGT	AGTGGAGCTTCTTCATCCTACCG	-
32	umc1273	3.08	GTTCGCTGCTGCTTCTTATATGCT	AATTGGCGCAGGCTATAGACATTT	-
33	umc1136	3.09	CTCTCGTCTCATCACCTTTCCCT	CTGCATACAGACATCCAACCAAAG	-
34	umc1594	3.09	CACTGCAGGCCACACATACATA	GCCAGGGGAGAAATAAAATAAAGC	-
35	umc1578	3.09	AAGCACTTCCAGTGGTACATGAGC	CGAGCAGCTAAGGTAGAGCAGCTA	-

Parental polymorphisms

For successful marker assisted selection, the marker needs to be tightly linked to the trait and should be polymorphic for the parents. Since, the present study aimed at tagging markers linked with the SDM resistant trait and no reports were available so far, it was therefore essential to identify the polymorphic markers for both QTL mapping and MAS. The parental polymorphism survey using 35 SSR markers located on chromosome three was done based on a report which stated the resistance trait loci is harbored in this chromosome [16-18]. Among the 35 SSR (6 reported earlier + 29 other) markers were screened, 13 markers exhibited polymorphism (37.14%) for the parents [Table-2]. Of the 6 previously reported SSR markers flanking SDM QTL that were used for polymorphic survey, four (bnlg420, phi053, bnlg1035, phi073) exhibited polymorphism in parents used in this study as well. These results evinced that these polymorphic markers could be efficiently used for selective genotyping.

Selective genotyping and identification of co-segregating marker

QTL detection by selective genotyping is easy, effective and rapid technique compared to interval mapping [31]. Extreme phenotypes, seven resistant (<10 percent infection) *viz*., RIL No. 6,18,113,114,16,95, and 102 and seven susceptible (>50 percent infection) *viz*., RIL No. 33, 12,31,25,21,262 and 255 which showed consistent expression for SDM reaction were selected for selective genotyping [Table-3]. Thirteen polymorphic SSR markers screened for co-segregation analysis on the selected extreme lines [14] revealed segregation distortion for three markers *viz*., umc1030, phi243966 and bnlg197 at the 5% level of significance. An SSR marker called bnlg420 segregated in Mendelian fashion [Fig-1], which found to be completely co-segregating with SDM reaction, whereas two markers *viz*., phi073 and bnlg1035 showed partial co-segregation pattern.

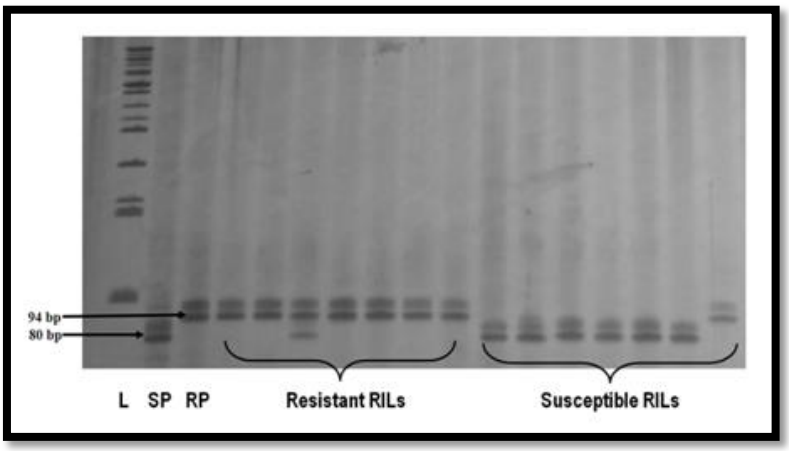

Fig-1 Selective genotyping with SSR marker bnlg420 depicting co-segregation of resistant and susceptible genotypes for Sorghum Downy Mildew (SDM) disease reaction on 6% Urea PAGE

L-100 base pair ladder, SP- SDM disease Susceptible Parent (UMI79), RP- Resistant Parent (UMI936 (w))

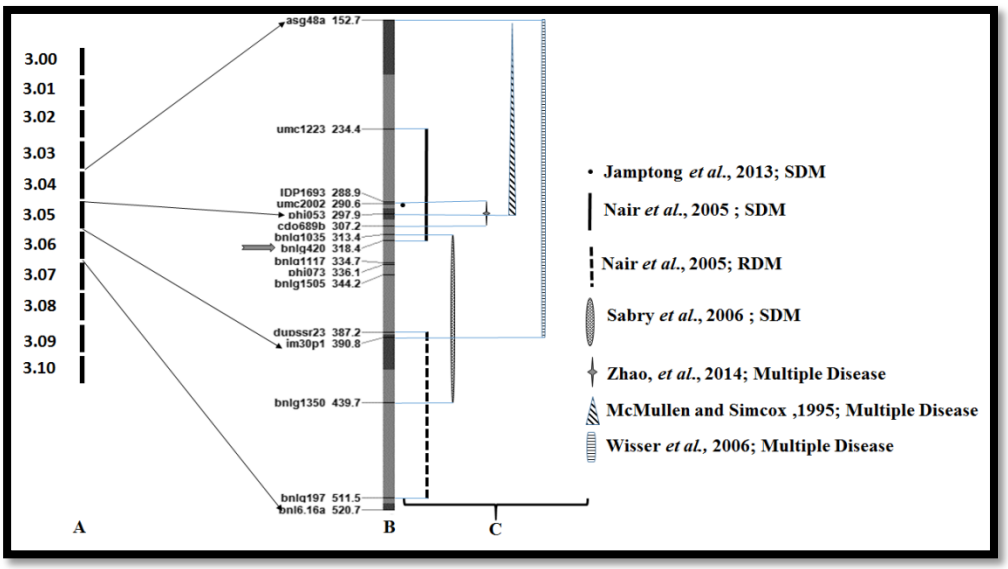

Fig-2 Comparative genetic map showing location of SSR marker, bnlg420, linked to SDM disease resistance locus identified in the present study through selective genotyping and previously reported QTL/Meta-QTL deciphering resistance to sorghum downy mildew (SDM), Rajasthan downy mildew (RDM) and several other diseases on chromosome 3.

A) Chromosome 3 genetic map is divided into 11 bins (approximately 20cM each) from 3.00 to 3.10)
B) Enlarged view of bin 3.04 - 3.06 that is delimited by core markers [40, 41] (shown as dark region on the chromosome). Markers and it cM distances adapted from IBM2 2008 Neighbors Frame 3) is depicted left side of the map. The position of SSR marker, Bnlg420 linked to SDM disease resistance locus, identified in present study is depicted by arrowhead.
C) QTL regions/Meta QTLs regions from various study is depicted

An integrated map of chromosome 3 was drawn by employing cM distances from (IBM2 2008 Neighbors Frame 3) reference map for a comparative analysis of results obtained in this study with the earlier reported QTLs for SDM resistance and other disease resistance [Fig-2].

According to IBM2 2008 Neighbors Frame 3, bnlg420, bnlg1035, phi073 are located at 318.4, 313.4 and 336.1cM respectively in the chromosome bin 3.04-3.05.

Interestingly, it was found that as identified in the present study bnlg420, the completely co-segregating SSR is located on bin 3.05 at 318.4 cM distance. This marker was earlier reported to be one of the markers flanking SDM resistant QTL region, in a backcross population of CM139 (susceptible) and NAI116 (highly resistant) [*16*]. This QTL (bin 3.04-3.05) explained 14.9% of phenotypic variation for SDM disease with a LOD score of 4.22.

Sabry and coworkers (2006) described a major QTL for SDM resistance on the chromosome 2, which explained 70% phenotypic variance, with two other minor QTLs one each positioned on chromosome 3 and 9. In particular, SDM resistant QTL located on chromosome 3 was found in the bin region of 3.04-3.05. Two SSR markers, phi073 and bnlg1035 were reported to be linked with the SDM QTL in one of the field and glasshouse study respectively. Both these markers, i.e. phi073 and bnlg1035 were also found to be polymorphic between parents used in the present study and showed partial co-segregation with SDM reaction in the selected genotypes suggesting the presence of a QTL harboring resistance to SDM. Earlier Jampatong et al. (2013) [18] used composite interval mapping in $F_{2:3}$ families of a cross Nei9008 (resistant) and CML289 (susceptible) and reported 9 QTL regions governing response to *P. sorghi*. An SSR marker umc2002 (bin 3.04) with a LOD value of 5.39 and R^2 of 5.6% was found to be linked with one of the QTLs in chromosome 3 [*18*]. SSR marker, umc2002 reported by Jampatong et al. (2013) [18] was included during polymorphism survey, but found to be monomorphic.

Table-3 *Percent of sorghum downy mildew (SDM) infection in selected RILs and parents at field and greenhouse condition*

Code	RILs	SDM disease percent under field condition	SDM disease percent under greenhouse condition
R1	6	7.1	7.4
R2	18	6.9	8.7
R3	113	6.9	7.4
R4	114	7.7	6.9
R5	16	6.9	7.4
R6	95	8.1	6.6
R7	102	7.5	7.4
S1	33	85.6	86.1
S2	12	43.0	42.2
S3	31	75.0	100.0
S4	25	65.3	85.1
S5	21	75.0	85.1
S6	262	50.0	37.0
S7	255	55.4	62.5
SP	UMI 79	100.0	96.6
RP	UMI936 (w)	7.7	8.01

The chromosomal location of the 34 QTLs and 19 major genes on disease and insect resistance have been classified by McMullen and Simcox (1995) [32], and positioned them according to chromosome bin to find eventual clustering of disease and insect resistance loci [*32*].The nonrandom distribution of resistant loci with tight clustering in bin regions 3.04 and 3.05 was recorded. Three dominant resistant genes, say, *rp3* [*33*], *wsm2* [*34*] , *mv1* [*35*]governing resistant against common rust, maize mosaic, and wheat streak mosaic, respectively were located within 5cM distance from marker umc102/phi053.The marker, umc102/phi053 depicted polymorphism for parents used in this study and depicted partial co-segregation with the SDM reaction.

QTL conferring resistance to European corn borer [*36*] and *Fusarium* stalk rot [*37*] are also been located in the bin region (3.04-3.05). Wisser and coworkers (2006) [*38*] compiled and documented 437 QTL, 17 major genes and 25 RGAs from 11 types of diseases or disease groups. They have reported that bin 3.04 and 3.05 is associated with 6 out of 11 diseases or disease groups compiled also agreed upon a previous report [*32*] that this region has a tight cluster of disease resistance gene.

Chromosome 3, bin 3.04-3.05 has been again demonstrated to be a hot spot for disease resistance region within interval IDP1693-cd0689b, when meta-analyzed with BioMercator 2.1 for 340 QTLs related to disease resistance in maize [*39*]. Thus, chromosome bin 3.04-3.05 is consensus locus, which shows resistance response against many diseases, and offers scope for their utilization in resistance breeding.

SSR marker bnlg420 identified through selective genotyping could be validated by introgression of SDM resistance QTL from UMI936 (w) to an elite inbred UMI 79 by marker-assisted backcross breeding strategy. Fine mapping and *in silico* mapping of the chromosome region, 3.04-3.05 may provide further information on potential candidate gene governing disease resistance. Such studies would enable us to hasten the maize improvement program for evolving varieties with enhanced disease resistant trait especially for sorghum downy mildew.

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