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Development of SNP markers closely linked to Sorghum Turcicum Leaf Blight Resistant Genes

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ABSTRACT
Exserohilum turcicum is a major fungal pathogen that causes turcicum leaf blight, foliar disease, for both sorghum and maize. The knowledge gained from the analysis of sorghum genes is beneficial to all aspects of plant research, including crop improvement. The knowledge gained from genomic studies on maize to identify quantitative trait loci associated with resistance to E. turcicum is beneficial to sorghum research and the crop improvement. Comparative analysis was undertaken of the maize remorin gene ZmREM6.3, which has been identified to have a significant contribution towards turcicum leaf blight resistance. The gene ZMREM6.3 was retrieved from the maize database. This was used to search the sorghum database in Phytozome. The application of high-resolution melting (HRM) of DNA, a method that allows detecting polymorphism in dsDNA by comparing profiles of melting curves, was used in this study. These SNP markers were developed using the orthologous sorghum remorin gene on linkage group two and validated across diverse Sudanese sorghum cultivars. This is the first evidence of a role for remorins in sorghum-fungal interactions. Quantitative disease resistance is frequently employed by breeders to protect crops from pathogen attack. And this remorin (ZmREM6.3) should be employed to underlying genes and mechanisms which remain largely a matter of conjecture in quantitative resistance against sorghum TLB.

Key words: Comparative genomics, *Exserohilum turcicum*, high-resolution melting (HRM), quantitative disease resistance, remorin, sorghum, ZmREM6.3

RÉSUMÉ
Exserohilum turcicum est un pathogène fongique majeur qui cause la brûlure des feuilles de turcicum, une maladie foliaire, à la fois pour le sorgho et le maïs. Les connaissances acquises grâce à l'analyse des gènes du sorgho sont bénéfiques pour tous les aspects de la recherche sur les plantes, y compris l'amélioration des cultures. Les connaissances acquises à partir d'études génomiques sur le maïs pour identifier les locus de caractères

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quantitatifs associés à la résistance à E. turcicum sont bénéfiques pour la recherche sur le sorgho et l'amélioration des cultures. Une analyse comparative a été entreprise sur le gène de la rémorine de maïs ZmREM6.3, qui a été identifié comme ayant une contribution significative à la résistance à la brûlure des feuilles de turcicum. Le gène ZMREM6.3 a été extrait de la base de données du maïs. Cela a été utilisé pour rechercher la base de données sur le sorgho dans Phytozome. L'application de la fusion à haute résolution (HRM) de l'ADN, une méthode qui permet de détecter le polymorphisme dans l'ADNdb en comparant les profils des courbes de fusion, a été utilisée dans cette étude. Ces marqueurs SNP ont été développés à l'aide du gène orthologue de la rémorine de sorgho sur le groupe de liaison deux et validés sur divers cultivars de sorgho soudanais. Il s'agit de la première preuve d'un rôle des rémorines dans les interactions sorgho-champignon. La résistance quantitative aux maladies est fréquemment utilisée par les sélectionneurs pour protéger les cultures contre les attaques de pathogènes. Et cette rémorine (ZmREM6.3) devrait être utilisée pour les gènes et mécanismes sous-jacents qui restent largement une question de conjecture dans la résistance quantitative contre le TLB du sorgho.

Mots clés: Génomique comparative, Exserohilum turcicum, fusion à haute résolution (HRM), résistance quantitative aux maladies, rémorine, sorgho, ZmREM6.3

BACKGROUND

Exserohilum turcicum (Pass) K.J. Leonard and E.G. Suggs (teleomorph: *Setosphaeria turcica* (Luttrell) Leonard and Suggs), formerly known as *Helminthosporium turcicum*, causes turcicum leaf blight (TLB) for both sorghum (*Sorghum bicolor* L.) and maize (*Zea mays* L.) that limits their productivity in Sub Saharan Africa (Reddy and Prasad, 2013). The *E. turcicum* pathogenic lifestyles colonizes sorghum living tissue, spreading through vascular tissue causing wilted lesions by plugging xylem vessels, and ultimately causing necrotic lesions (Beshir *et al*., 2016). Sorghum and maize and their fungal pathogens have continuously confronted each other through coevolution for growth and survival (Inghelandt *et al*., 2010). In this process, sorghum and maize have evolved array of structural and gene-based defense mechanisms designed to combat different pathogens (Taylor *et al*., 2006), and so have pathogens, by developing new pathotypes of races (Tesso *et al*., 2012).

LITERATURE SUMMARY

The large role of additive gene action in host resistance of both sorghum and maize to TLB suggests an evolutionary adaptation to a wide array of pathotypes. However sorghum and maize are close relatives, they react similarly to TLB (Ramathani *et al*., 2011). TLB was reported previously to be due to race O (Adipala *et al*., 1993), and more recently suggestions of new identified races have been reported (Ramathani *et al*., 2011). Resistant lines carrying Ht genes (for *Helminthosporium turcicum*) have been widely used in maize breeding programs (Welz and Geiger, 2000). Compared with Ht genes, quantitative trait loci (QTL) are now emphasized in maize breeding because they show durable resistance and are less likely to be overcome by evolution of novel resistant pathogen races (Parlevliet, 2002).

Recently, six orthologous resistant genes in maize, present in a cluster of three pairs, on chromosome five of sorghum have been reported (Martin *et al*., 2011). These genes are highly conserved among the Poaceae and may reflect wide adaptation to diverse fungi and their races as is expected under additive gene action. Resolution of QTL has subsequently been improved in maize using a large, multi-

parental population, confirming the highly complex genetic architecture for QTL (Jamann *et al*., 2016). In maize, 29 independent loci were implicated in quantitative resistance (Poland *et al*., 2011) and may be complex and conditioned by multiple genes (Jamann *et al*., 2014).

Phylogenetic analysis of all genes containing an annotated remorin domain in maize genome was previously classified remorin genes. The remorin of interest was grouped with other known group six remorin genes and was thus named ZmREM6.3 (Jamann *et al*., 2016). This group had a conserved C-terminal region and were classified as long remorins that have potential role in plant - microbe interactions (Raffaele *et al*., 2007). In the present study, we conducted fine mapping for the remorin gene ZmREM6.3 in sorghum using SNPs. The objectives were to detect the presence of this gene in sorghum and to identify molecular markers that co-segregate with diseases resistant loci. Results from this research could advance the use of marker-assisted selection in sorghum breeding programs.

STUDY DESCRIPTION

Experimental sites. Phenotypic characterization was carried out in in Shambat Research Station, Sudan. Genotypic characterization and HRM assay were carried out using aligned melting curve of the HRM analysis in The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Kenya.

Genetic materials and experimental design. The developed SNPs were validated across two diverse sorghum cultivar MUK007/009, a resistant to turcicum leaf blight, and cultivar Tabat, a susceptible to TLB. After validation, the polymorphic SNPs were selected to screen fifteen cultivars namely; six cultivars from Sudan, Yarwasha, Arfa gadamak, Butana, Gadam alhaman, HD1 and Wad ahmed, and nine cultivars from East Africa namely; Epuripuri, Jesu, KARI, Seredo, Sekedo, Siaya, IESV and GA06/106 and GA06/18 from East Africa. Sorghum cultivars were planted under field conditions using randomised complete block design (RCBD) with three replications during summer season of 2018 and 2019.

Technique for inoculation. *E. turcicum inoculum* was prepared as described by Ramathani *et al*. (2011). The plants were inoculated with E. turcicum at stage three (Vanderlip, 1993). Innoculation was done in the evening hours when dew and ambient temperature were optimal for successful infection (Ramathani *et al*., 2011) and was repeated three times at six day intervals to ensure successful infection (Carson, 1995).

Confirmation of occurrence of *E. turcuicum*. The *Exserohilum Turcicum* isolates were screened by polymerase chain reaction (PCR) using the sequence information from the internal transcribed spacer ribosomal DNA (ITS rDNA) of the 5.8S ribosomal RNA gene (GenBank accession number AF163067) (forward: 5′ - GCAACAGTGCTCTGCTGAAA-3′, reverse: 5′- ATAAGACGGCCAACACCAAG-3′). The isolates showed positive reaction and generated a 344 bp fragment indicating the presence of *E. turcuicum* (Beshir *et al*., 2017).

Gene selection strategy and SNP primers designing. The remorin gene ZMREM6.3 was retrieved from the maize database and used to search against sorghum database in Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Sbicolor). Prime3 (http://bioinfo.ut.ee/primer3/) software was used to design SNP primers.

Data collection and analysis

Phenotypic data. Cultivars were assessed for disease severity based on percentage scale (Ramathani *et al*., 2011) from flowering stage till physiological maturity at a weekly interval (Dube *et al*., 2010). Area under disease progress curves (AUDPC) were computed using the

weekly severity ratings (Madden *et al*., 2007). Percentages and residuals were normally distributed therefore parametric analysis was used. The analysis of variances was calculated using GenStat 12th Edition (VSN International Ltd., UK). Means were compared using the Fisher's Protected Least significance difference test (LSD) at P<0.05 (Steel and Torrie, 1997).

Genotypic data. DNA was extracted from three week old leaf samples using Zymo research extraction kit and was immediately stored at -20 ºC. HRM analysis starts with PCR amplification of the SNP primers in the presence of the dsDNA dye using total volume of 10µL and protocol presented in Table 1. This binding dye has a high fluorescence when bound to dsDNA and low inflorescence in the unbound state. Amplification is followed by high resolution melting step using instrumentation capable of capturing a large number of fluorescent data points per change in temperature, with high precision. When the dsDNA melts into single strands, the dye is released causing a change in fluorescence. This result in melt curve profile characteristic of the amplicon.

RESULTS AND DISCUSSION

Reaction of sorghum cultivars to E. turcicum . There was significant variation (P=0.00003) in disease severity while there was no significance in incidence indicating that reaction to TLB varied among what farmers prefer to plant every season. The reaction of cultivars to TLB is presented in Table 2. Unsurprisingly, Tabat showed low TLB severity (9.4%) and AUDPC (632.7) while MUK007/009 showed low TLB severity (54.8%) and AUDPC (632.7). Jesu 91-104DL, GAO6/18, Gadam elhamam and Arfa gadamak showed low TLB severities (9.94-12.4%) and AUDPC (206-258.7) therefore they were considered resistant. GAO6/106, KARI matama, Seredo, sekedo, Butana, HD1, Yarwasha and Wad ahmed showed high TLB severities (13.5-18.6%) between and AUDPC (269.8-447.2) and therefore were considered moderately resistant. Epuripuri, Siaya and IESV showed highest TLB severities (52-65) and AUDPC and therefore were considered highly susceptible.

Table 1. Protocol for high resolution melting analysis

Step	Type	Information
1	Temperature	Temperature: 95 °C; Duration: 7 minutes.
2	Cycle	Number of cycles to loop: 30; Number of steps inside loop: 2.
3	Temperature	Temperature: 95°C; Duration: 05 seconds.
4	Temperature	Temperature: 60°C; Duration: 30 seconds.
5	Temperature	Temperature: 60°C; Duration: 30 seconds.
6	Melting Curve	Start temperature: 60°C; End temperature: 95°C; Hold time: 1 seconds. Temperature increment after hold: 0.2°C.
7	Temperature	Temperature: 20°C; Duration: 10 seconds.

Table 2. The reaction of cultivars to Turcicum Leaf Blight screened under field conditions

Cultivar	Final severity	AUDPC	Resistant/ Susceptible#
Epuripuri	54.0	659.8	HS
Jesu 91-104DL	9.94	258.7	R
GA06/18	12.4	253.3	R
GA06/106	14.5	362.6	MR
KARI mtama	13.6	302.1	MR
Seredo	15.5	496.8	MR
Sekedo	13.5	296.8	MR
Siaya	65.0	759.9	HS
IESV	52.0	639.1	HS
Butana	15.9	398.4	MR
HD1	14.4	373.5	MR
Yarwasha	15.0	429.5	MR
Wad ahmed	18.6	447.2	MR
Gadam ehaman	11.0	206	R
Arfa gadamak	9.69	218.2	R
Checks:			
Tabat	54.8	632.7	HS
MUK007/009	9.40	168.8	R
Minimum	8.96	206,0	
Mean	14.3	281.3	
Maximum	24.8	368.8	
LSD 0.05	11.3	156.5	
SED	5.65	78,00	
±SE	4.00	55.20	

#=refers to the reviewed publications from Ngugi *et al.* (2002); Ramathani (2009) and Beshir *et al.* (2017). HS= highly susceptible; S= susceptible cultivar; MR= moderate resistance; R= Resistant cultivar;

Single nucleotide polymorphism. This research is based on comparative analysis selected maize resistant gene remorin which has been identified to have a significant contribution towards turcicum leaf blight resistance. Plate 1 shows sample of extracted DNA from young sorghum plants under study.

Many high throughput SNP genotyping technologies are currently available worldwide. SNP analysis was carried using aligned melting curve HRM analysis of five polymorphic SNP markers. However, only two SNPs showed clear and distinguished variation among sorghum cultivars as well as between the two checks (Figure 1).

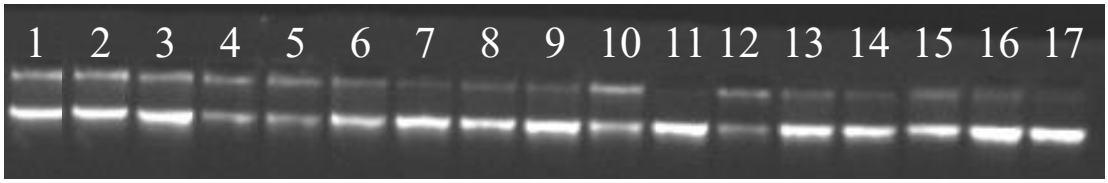

Plate 1. DNA band extracted from three week old sorghum leaves
1= MUK007/009 (resistant), 2= Tabat (susceptible), and 3 to 17 = sorghum cultivars

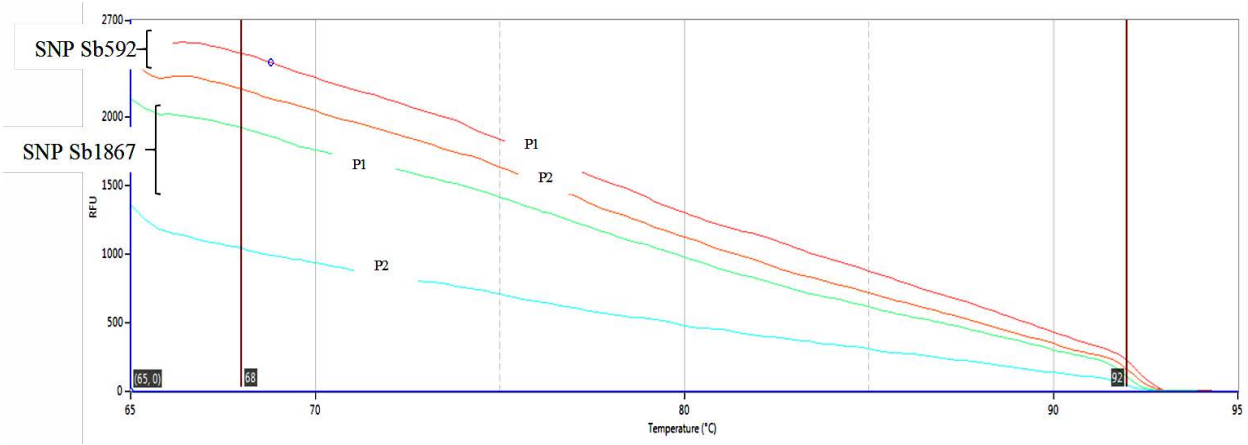

Figure 1. Aligned melting curve HRM analysis of SNP markers Sb592 and Sb1867 polymorphism
P1= MUK007/009 (resistant) and P2= Tabat (susceptible)

Table 3. HRM assay at melting temperature 60 ^{O}C for polymorphic SNP markers Sb592 and Sb1867

Name	Phenotypic response to foliar diseases from HRM*	Genotypic response to foliar disease from review#	Melting Temperature	Peak	Area of height the peak
Epuripuri	HS	-	91.15	458	148
Jesu 91-104DL	R	-	91.15	830	267
GA06/18	R	+	90.98	386	291
GA06/106	MR	-	91.17	942	275
KARI mtama	MR	-	91.16	1796	561
Seredo	MR	-	91.16	1332	414
Sekedo	MR	-	91.06	487	298
Siaya	HS	-	91.15	653	215
IESV	HS	-	91.14	432	154
Butana	MR	-	91.15	1932	621
HD1	MR	-	91.17	1105	328
Yarwasha	MR	+	90.49	383	722
Wad ahmed	MR	-	91.16	1620	498
Gadam elhamam	R	-	91.15	1328	425
Arfa gadamak	R	+	90.94	365	311
Checks:					
Tabat	S	-	91.17	1392	413
MUK007/009	R	+	90.98	367	270

#=refers to the reviewed publications from Ngugi *et al*. (2002); Ramathani (2009) and Beshir *et al*. (2017). HS= highly susceptible; S= susceptible cultivar; MR= moderate resistance; R= Resistant cultivar.
-= refers to the presence of the susceptible allele similar to the susceptible check Tabat.
+= refers to the presence of the resistant allele similar to the resistant check MUK007/009.

Table 3 shows the HRM analysis of two polymorphic SNP markers with fifteen cultivars and two checks. The results characterised the response of the selected sorghum cultivars into two distinct classes based on the melting temperature and peak height in comparison to the checks. The resistant cultivars Yarwasha, Arfa gadamak from Sudan and GA06/18 from East Africa showed similar melting temperature and peak height to the resistant cultivar MUK007/009. Butana, Gadam alhaman, HD1 and Wad ahmed from Sudan and Epuripuri, Jesu91-104DL, KARI, Seredo, Sekedo, Siaya, IESV and GA06/106 from East Africa did show similar melting temperature and peak height to the susceptible cultivar Tabat.

Despite its utility, the genetic and mechanistic basis of quantitative disease resistance is not well understood. In this study, we examined existence of region of the maize genome in sorghum that has been associated with resistance to pathogens using comparative studies. There is urgent need to utilize whole-genome sequencing for large-scale SNP identification and development of molecular markers for identifying novel sites that co-segregate with foliar disease resistant phenotypic traits. Therefore, there is need for performing whole-genome sequencing of the MUC007/009 and Tabat and genome-wide SNP identification using the recently published sorghum genome sequences as reference. However, reliable phenotypic evaluation is a prerequisite for genome-wide association mapping. Results from this study can advance the use of marker assisted selection in sorghum breeding national programs.

Successful utilization of whole-genome sequencing for large-scale SNP identification and development of molecular markers for identifying novel QTLs that co-segregate with TLB resistant phenotypic traits has previously been applied (Rostoks *et al*., 2005; Ganal *et al*., 2009; Deschamps and Campbell, 2010). To detect QTL for the disease resistance, there is need for performing whole-genome sequencing of the parent MUC007/009 and Epuripuri and genome-wide SNP identification using the recently published sorghum genome sequences as reference. However, reliable phenotypic evaluation is a prerequisite for QTL and genome-wide association mapping. In previous reports, disease severity has typically been used to evaluate phenotypic variation in resistance to TLB in maize and sorghum. Despite its utility, the genetic and mechanistic basis of quantitative disease resistance is not well understood. In this study, we examined existence of region of the maize genome in sorghum that has been associated with resistance to *E. turcicum* pathogen using comparative studies.

CONCLUSION

The detection and study of disease resistance is essential for the understanding of changes in sorghum phenotypic variation and evolution of species. This study provides opportunity for more comparative studies between maize and sorghum in tackling the devastating effect of TLB in the two globally important cereals (maize and sorghum). This study proved the presence of the maize remorin gene ZmREM6.3 in sorghum genome. This research provides two highly SNP markers showed polymorphism using HRM. This approach does not require previous knowledge of the possible haplotypes, does not need fluorescent labels, and multiple alleles can be detected in a single amplicon. This is likely to make a significant contribution towards developing cultivars with TLB resistance.

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STATEMENT OF NO CONFLICT OF INTEREST

The authors declare that there is no conflict of

interest in this publication.

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