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Use of Molecular Markers to Identify Certain Stem Rust Resistance Genes in Thirteen Bread Wheat Cultivars Registered in Egypt

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Wheat stem rust caused by *Puccinia graminis* f.sp. *tritici* (*Pgt*) is one of the most destructive diseases of wheat all over the world. In Egypt, in-spite of the absence of the new stem rust pathotype TTKS (syn. Ug99) and its variants TTKSK and TTKST, the disease takes place as a serious situation in the last few years with high disease severities on most of the common wheat cultivars during 2012-2014 growing seasons. A modified North American *Pgt* differential set representing the resistance genes *Sr*5, 21, 9e, 7b, 11, 6, 8a, 9g, 36, 9b, 30, 17, 9a, 9d, 10, Tmp, 24, 31, 38 and McN, respectively, as well as 10 supplemental *Sr* genes were evaluated in natural field infection. Adult resistance plants were detected on *Sr* genes *Sr* 5, *Sr* 9g**,** *Sr* 11, *Sr* 13, *Sr* 14, *Sr* 21, *Sr* 24, *Sr* 28 and *Sr* 38. Four specific markers were selected to detect the presence of the resistant stem rust monogenic lines including *Sr*13, *Sr*25, *Sr*26 and *Sr*31 in thirteen Egyptian bread wheat cultivars. These markers were XBE403950EST, PSY-DI, *Sr*26#43 STS and IAG95-STS, respectively. Stem rust resistance gene *Sr*26 was absent in all the tested cultivars, whereas *Sr*13, *Sr*25 and *Sr*31 were presented at various frequencies. The highest frequency was observed for *Sr*13 (100%), followed by *Sr*25 (84.61%) and *Sr*31 (46.15%). These results revealed that DNA marker for *Sr*26 is needed in the Egyptian cultivars and more effective genes must be identified and incorporate in adopted germplasms to face such disease.

Keywords: DNA markers, molecular marker, *Puccinia graminis* f.sp. *tritici*, *Sr* genes, Ug99 and wheat.

Wheat (*Triticum aestivum* L.), is the largest cultivated crop in both cultivated areas and total production in the world. Stem rust caused by *Puccinia graminis* Pers. f.sp. *tritici* Eriks. and E. Henn., is one of the major diseases of wheat throughout the world. New *Pgt* strain Ug99 was detected by Pretorius *et al*. (2000) and was designated by Wanyera *et al*. (2006) as TTKS pathotype. It showed a virulence to *Sr*31 and its variants, TTKST and TTTSK which were detected in Kenya in 2006 and 2007 with virulence to genes *Sr24* and *Sr*36, respectively, (Jin *et al*., 2008).They pose a serious threat to bread wheat (*Triticum aestivum* L.). Controlling the disease through usage of chemicals besides being costly to the farmer is also harmful to the environment. Utilization of resistant cultivars is the most economical and environmental-friendly approach to control the diseases, enabling reductions in fungicide use. A diversified and effective resistant gene resource must be the basis

of breeding wheat cultivars with stem rust resistance. To date, 55 stem rust resistance genes have been identified and formally catalogued on different wheat chromosomes (McIntosh *et al*., 2011 and Ghazvini *et al*., 2013). Incorporation of the resistance genes is eco-friendly system which does not place any cost burden on the growers (Ejaz *et al*., 2012). The durability of effective resistance genes can be enhanced by deploying them as multiple genes in cultivars. *Sr*25 and *Sr*26 genes are among a few major genes effective against TTKS lineage that includes TTKST and TTTSK races (Singh *et al*., 2006 and Jin *et al*., 2007). Nowadays, various molecular markers have been widely used in plant genetic mapping and marker-assisted selection (MAS), such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), sequence tagged site (STS), simple sequence repeat(SSR), inter-simple sequence repeat (ISSR). Currently, microsatellites (simple sequence repeats, SSR) are the preferred type of molecular marker for marker-assisted selection (MAS) in wheat breeding.

In this study our objective was to check whether the stem rust resistance genes, *Sr*13, *Sr*25, *Sr*26 and *Sr*31 are presented in thirteen registered Egyptian bread wheat cultivars recently grown and to determine their frequencies using SSR technique.

Materials and Methods

1. Adult plant resistance to wheat stem rust:

Twenty modified North American *Pgt* differential set representing the resistance genes *Sr5*, 21, 9e, 7b, 11, 6, 8a, 9g, 36, 9b, 30, 17, 9a, 9d, 10, Tmp, 24, 31, 38 and McN, respectively, (Pretorius *et al*., 2000) as well as 10 supplemental stem rust monogenic lines *i.e*. *Sr* 7a, *Sr* 8b, *Sr* 13, *Sr* 14, *Sr* 15, *Sr* 22, *Sr* 25, *Sr* 26. *Sr* 27 and *Sr* 28 were used in this study (Table 3). These lines were obtained from Wheat Dis. Res. Dept., Plant Dis. Res. Inst., ARC, Giza. These lines were evaluated to natural infection of stem rust disease in the farm of Gemmeiza Res. St. in 2013/2014 growing season. *Sr* lines were sown in rows 5 m long, 50 cm apart between the rows and 5 cm within plants**.** All agricultural practices were performed as recommended. Disease severity was recorded at adult stage on the basis to the modified Cobb scale 0 to 100% (Peterson *et al*., 1948).

2. DNA extraction:

Thirteen Egyptian bread wheat cultivars adapted in Egypt, obtained from the National Wheat Program, Crops Res. Inst., ARC, Giza, as well as four stem rust monogenic lines, *i.e.* Sr13, Sr25, Sr26 and Sr31, were listed in Table (1) according to their responses at seedling and adult stages to stem rust seedlings of the previously mentioned bread wheat cultivars and monogenic lines were used at 15 days old for genomic DNA extraction (PCR analysis). Total DNA of each wheat cultivar and isogenic line was extracted from 200 mg leaf tissue which digested in liquid nitrogen with a mortar and pestle using Invisorb® Spin Plant Mini Kit (STRATEC Molecular, Germany) according to manufacturer's instructions.

Table 1. Bread wheat cultivars and stem rust monogenic lines (*Sr*'s) used in this study

Cultivar/*Sr* gene	Pedigree
Gemmeiza-7	CMH74A.360 / 5x // Seri82 /3/ Agent CGM4611-2GM-3GM-1GM-0GM.
Gemmeiza-10	Maya 74"S"/ On // 1160-174/3/ Bb /4/ Chal "S"/5/Ctow.
Gemmeiza-11	BUC"S"/ Kvz"S"// 7c/ Seri 82 /3/Giza 168/ Sakha 61 GM7892-2GM-1GM- 0 GM.
Gemmeiza-12	OTS /3/ SARA/ THB// Vee-CMss97Y0027S-5Y-010M-010Y-010M-2Y-1M-0Y-06M-06M-0GM.
Sids-12	BUC // 7c / Ald /5/ Maya 74 / On / 1160.147/3 / BB/ G11 /4/ Chat "S" /6/ Maya / vu1 // Cmh 74A.630/4* sx, SD7096- 4SD-1SD-0SD.
Sids-13	ALmaz 19= Kauz "S" // Tsi /snb "S" ICW 94-0375- 4AP-2AP-030AP-) APS- 2AP- 0APS- 050AP- 0AP- 0SD.
Sids-14	SW8488*2 / KUKUNA- CGSS01Y00081T- 099M-099Y-099M-099B-9Y-0B-0SD.
Giza-168	MIL /BUC // Seri CM93046-8M-0Y-0M-2Y-0M.
Giza-171	Gemmeiza-9 / Sakha-93.
Misr-1	Oasis /skauz // 4* BCN/3/2* Pastor CMss 00Y01881T-050M-030Y-030M-030WGY-33M- 0Y-0S.
Misr-2	SKAUZ / BAV 92. CMss 96 M03611S- 1M- 0105Y-010M-010SY- 8M- 0Y-0S.
Sahka-93	Sakha 92TR81032 S8871-1S-2S-1S-0S.
Gemmeiza-9	Ald"S"/ Huac"S" // CMH 74A.630/ 5x CGM 4583-5GM-1GM-0GM.
Sr13	Prelude*4 /2/ marquis*6/Khapstein.
Sr25	Agatha(CI 14048)/9*LMPG-6 DK16
Sr26	Eagle *Sr*26 .
Sr31	*Sr*31(Benno) / 6*LMPG-6 DK42.

3. Condition and amplification of PCR:

Amplification of Sr regions were conducted in an automated thermal cycler (C1000^{TM} Thermal Cycler, Bio-RAD) using the primers and conditions listed in Table (2) with one pre-denaturation cycle at 94ºC for 3 min. Each PCR mixture was 25µl as follow, 1µl of 25 ng nucleic acid, 1µl of each primer (10 pmol), forward and reverse, 12.5 µl of GoTag® Colorless Master Mix (Promega Corporation, USA) and 9.5µl of Nuclease free water (Promega). 15µl of all PCR products were analyzed by electrophoresis through a 1.5% agarose gel, stained with ethidium bromide, and DNA bands were visualized using a UV trans- illuminator and photographed.

Table 2. Primers and PCR conditions for markers associated with *Sr*13, *Sr*25, *Sr*26 and *Sr*31 monogenic lines

Primer name	Monogenic line	Primer sequence	PCR conditions
XBE403950 EST (Simons *et al*., 2011)	*Sr13*	F- 5`-GGAACATGTTGACGCTGTTG-3` R- 5`-AACACTGTTCCCGAAGTTGG-3`	Amplification step: (35 cycles): 94ºC, 20 sec., 53ºC, 20 sec, 72ºC, 30 sec & final extension at 72ºC, 7 min.
PSY-DI (Zhang & Dubcovsky, 2008)	*Sr25*	F- 5'-TTGCAGTGCAATGGTTTTCCA-3' R- 5'GACTCCTTTGACGATGTCTTC-3'	Amplification step:(35 cycles): 94ºC,20 ec.,53ºC,20 sec, 72ºC,30 sec & final extension at 72ºC, 7 min
Sr26#43 STS (Mago *et al*., 2005)	*Sr26*	F- 5'AATCGTCCACATTGGCTTCT-3' R- 5'CGCAACAAAATCATGCACTA-3'	Amplification step:(35cycles): 94ºC,60sec.,52ºC,60sec,72ºC,120 sec, and final extension at 72ºC,10 min
IAG95-STS (Mago *et al*., 2002)	*Sr31*	F-5'-CTCTGTGGATAGTTACTTGATCGA-3' R-5'-CTAGAACATGCATGGCTGTTACA-'3	Amplification step: (35cycles): 4ºC,20sec.,55ºC,20sec,72ºC,30 sec &final extension at 72ºC, 7 min

Results and Discussion

Data in Table (3) revealed the different levels responses of 30 *Sr* genes to stem rust natural infection at Gemmeiza Res. Station field in 2013/ 2014 growing season. High infection disease severity was recorded on *Sr* genes *Sr* 6, *Sr* 7a, *Sr* 9b,*Sr* 9d, *Sr* 17 and *Sr* McN (40-50-S), moderate disease severity *Sr*8a, *Sr* 10, *Sr* 15, *Sr* 25, *Sr* 30 and *Sr* Tmp (10 -30S). Low disease severity was observed with *Sr* 7b, *Sr* 8b, *Sr* 9a, *Sr* 9e, *Sr* 27 and *Sr* 31. However, complete resistance responses were recorded on *Sr* 5, *Sr* 9g, *Sr* 11, *Sr* 13, *Sr* 14, *Sr* 21, *Sr* 22, *Sr* 24, *Sr* 26, *Sr* 28, *Sr* 36 and *Sr* 38.

Similar results were obtained by Wanyera *et al*.(2006) and Jin *et al*. (2007) Who summarized the results of adult plant infection responses and seedling infection types for monogenic lines of designated *Sr* genes challenged with race TTKS. High infection types at the seedling stage and susceptible infection responses in adult plants were observed on monogenic lines carrying *Sr* 5, 6, 7a, 7b, 8a, 8b, 9a, 9b, 9d, 9g, 10, 11, 12, 15, 16, 17, 18, 19, 20, 23, 30, 31, 34, 38 and Wld-1. Monogenic lines of resistance genes *Sr' s* 13, 22, 24, 25, 26, 27, 28, 32, 33, 35, 36, 37, 39, 40, 44, Tmp and Tt-3 were effective against TTKS at seedling and adult plant stages.

Microsatellite PCR:

Four selected microsatellite markers were used to detect whether they reveal polymorphic bands between the stem rust genes, *Sr*13, *Sr*25, *Sr*26 and *Sr*31, and thirteen bread wheat cultivars commonly grown in Egypt using simple sequence repeat (SSR) technique.

Table 3. Response of thirty stem rust monogenic lines to natural infection of stem rust in 2013/2014 growing season

Sr genes	Disease severity	*Sr* genes	Disease severity
Sr 5	0	*Sr* 15	10-S
Sr 6	40-S	*Sr* 17	40-S
Sr 7a	50-S	*Sr* 21	0
Sr 7b	5-S	*Sr* 22	0
Sr 8a	20-S	*Sr* 24	0
Sr 8b	5-S	*Sr* 25	20-S
Sr 9a	5-S	*Sr* 26	0
Sr 9b	50-S	*Sr* 27	Tr-S
Sr 9d	50-S	*Sr* 28	0
Sr 9e	Tr-S	*Sr* 30	20-S
Sr 9g	0	*Sr* 31	5-S
Sr 10	10-S	Sr 36	0
Sr 11	0	*Sr* 38	0
Sr 13	0	*Sr* Tmp	30-S
Sr 14	0	*Sr* McN	40-S

Sr13:

Microsatalite dominant marker, XBE403950 EST amplified a DNA fragment of 700 bp known to be associated with *Sr*13 indicating the presence of *Sr*13 in all the tested bread wheat cultivars (Fig.1). Simons *et al*. (2011) reported that the XBE403950 EST amplified a DNA fragment of 691-723 bp for *Sr*13. The stem rust resistance gene *Sr13* presents in several *Triticum turgidum* sp. durum cultivars. Its main sources are the Ethiopian land race ST464 and the *T. turgidum* sp. *dicoccon* L. (emmer wheat) germplasm Khapli (Knott, 1962). Klindworth *et al*. (2007) stated that currently, *Sr*13 gene is the only one effective against the TTKS complex within the US durum wheat adapted cultivars. It is present in some common durum cultivars like Kofa, Kronos, Langdon, Medora and Sceptre. This gene confers a moderate type of resistance, so it is recommended to deploy this gene pyramided with other resistance genes, which will also extend the durability of the individual genes.

Sr25:

The dominant marker *PSY-D1* was used to detect stem rust resistance gene *Sr*25. This marker amplified a product of 175 bp DNA fragment (Fig. 2). Data revealed the presence of *Sr*25 in all the wheat cultivar except Misr-1 and Misr-2 bread wheat cultivars (Fig.2). *Sr*25 and the linked leaf rust resistance gene *Lr*19 were translocated onto the long arm of wheat chromosomes 7D (Friebe *et al*., 1994). Genes *Sr*25 and *Sr*26 transferred into wheat from *Thinopyrum ponticum* are effective against the *Sr*31-virulent race Ug99 (TTKSK) and its *Sr24*-virulent derivative (TTKST). So, it was then backcrossed into Australian wheat cultivars and has been used in the CIMMYT wheat breeding program (Bariana *et al*., 2007).

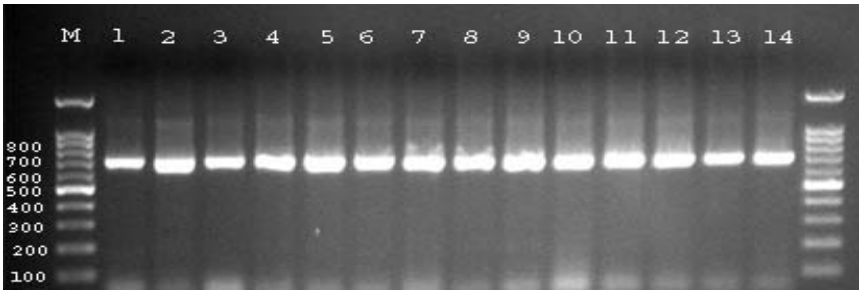

Fig.1. The fragment profile of the XBE403950 with one band labeled as 700 bp indicates the presence of *Sr*13 in all of the thirteen bread wheat cultivars. M: 1000bp DNA ladder RTU (Gene Direx), 1: Sr13 gene; 2: Gemmeiza-7, 3: Gemmeiza-10, 4: Gemmeiza-11, 5: Gemmeiza-12, 6: sids-12, 7: Sids-13, 8: Sids-14, 9: Giza-168, 10: Giza-171, 11: Misr-1, 12: Misr-2, 13: Sahka93, 14: Gemmeiza-9.

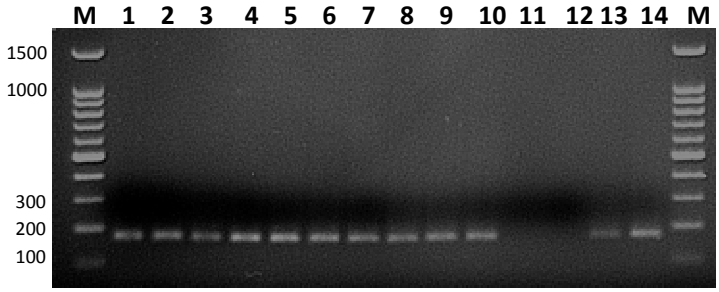

Fig.2. The fragment profile of the PSY-D1 with one band labeled as 175bp indicates the presence of *Sr*25 in 11 out of the thirteen bread wheat cultivars. M: 1000bp DNA ladder RTU (Gene Direx), 1: *Sr*25; 2: Gemmeiza-7, 3: Gemmeiza-10, 4: Gemmeiza-11, 5: Gemmeiza-12, 6: sids-12, 7: Sids-13, 8: Sids-14, 9: Giza-168, 10: Giza-171, 11: Misr-1, 12: Misr-2, 13: Sahka93, 14: Gemmeiza-9.

Sr26:

Sr26#43 marker (Mago *et al.*, 2005) was used to determine the Sr26 gene. This marker amplified DNA fragment of about 207bp .This fragment was absent in all the thirteen wheat cultivars (Fig.3). Dundas *et al.* (2007) stated that the effectiveness of this gene against the TTKS family of races, showed low frequency among modern cultivars, and its availability as donor lines with reduced alien segments makes Sr26 ideal for use by breeding programs. Bariana *et al*. (2007) reported that until now, Sr26 has been utilized as a source of resistance only in Australia. Liu *et al*. (2010) used PCR with marker Sr26#43 and 6A-specific marker BE518379 as a co-dominant marker for Sr26. The previously published dominant markers Gb for Sr25 and Sr26#43 for Sr26 were validated with eight wheat lines with or without Sr25 or Sr26. Sixin *et al*. (2010) stated that the co-dominant markers for Sr25 and Sr26 were validated with 37 lines with known stem rust resistance genes. A diverse set of

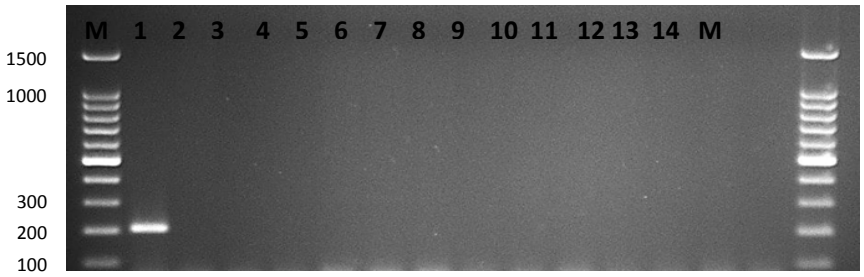

Fig. 3. The fragment profile of the *Sr*26#43 with one band labeled as 207bp indicates that *Sr*26 was absent in all the thirteen bread wheat cultivars. M: 1000bp DNA ladder RTU (Gene Direx).1: *Sr*26 gene; 2: Gemmeiza-7, 3: Gemmeiza-10, 4: Gemmeiza-11, 5: Gemmeiza-12, 6: sids-12, 7: Sids-13, 8: Sids-14, 9: Giza-168, 10: Giza-171, 11: Misr-1, 12: Misr-2, 13: Sahka93, 14: Gemmeiza-9.

germplasm consisting 170 lines from CIMMYT, China, USA and other countries were screened with the co-dominant markers for Sr25 and Sr26. Five lines with the diagnostic fragment for Sr25 were identified. None of the 170 lines tested had Sr26, as expected.

Sr31:

IAG95-STS marker specific to Sr31 (Mago *et al.*, 2002) amplified a DNA fragment of about 1100bp. This fragment was present in six wheat cultivars only Gemmeiza-7, Gemmeiza-10, Gemmeiza-11, Gemmeiza-12, Sids-13 and Gemmeiza-9 (Fig.4). Since 1980s, wheat cultivars with Sr31 were widely grown in nearly everywhere major wheat-producing region throughout the world. Wanyera *et al.* (2006) reported that during 2003 and 2004, a majority of current Kenyan cultivars and a large portion of CIMMYT wheat germplasm with gene Sr31 planted in Kenya were susceptible to stem rust. Recently, the resistance of Sr31 was finally overcome, which isolates of *Puccinia graminis* f.sp. *tritici* with high virulence to Sr31 were detected including Ug99 race in Uganda in 1999 and pose a worldwide threat to wheat production in areas where Sr31 resistance is important (Pretorius *et al.*, 2000).

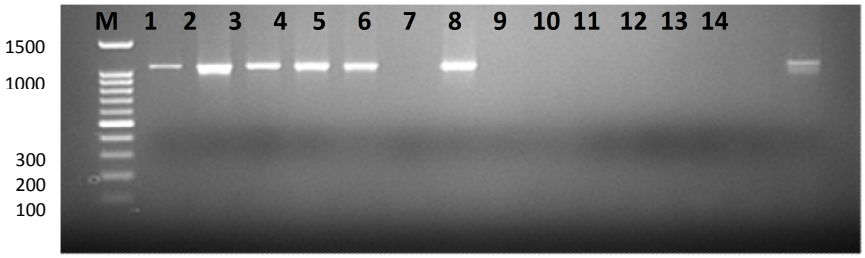

Fig.4. The fragment profile of the IAG95-STS with one band labeled as 1000 bp indicates the presence of *Sr* 31 in all the thirteen bread wheat cultivars. M: 100bp DNA ladder RTU (Gene Direx); 1: *Sr*31 gene; 2: Gemmeiza-7, 3: Gemmeiza-10, 4: Gemmeiza-11, 5: Gemmeiza-12, 6: sids-12, 7: Sids-13, 8: Sids-14, 9: Giza-168, 10: Giza-171, 11: Misr-1, 12: Misr-2, 13: Sahka93, 14: Gemmeiza-9.

Similar results were obtained by EJaz *et al*. (2012) who screened 117 Pakistani wheat varieties with 18 DNA markers to detect the presence of stem rust resistance genes Sr2, Sr6, Sr22, Sr24, Sr25, Sr26, Sr31, and Sr38. Stem rust resistance genes Sr22, Sr24, Sr25, and Sr26 were absent from all varieties, whereas Sr2, Sr6, Sr31, and Sr38 were present at various frequencies.

The detected *Sr* genes and their frequencies in the 13 bread wheat cultivars could be summarized in Table (4). *Sr* 13 gene was the most frequency one (100%) followed by *Sr*25 (84.61%), then *Sr*31 (46.15%).However, *the Sr*26 gene was absent in all of them (0 %). Similar results were proposed by Purnhauser and Bóna (2009) who stated that among156 Hungarian wheat's, a significant part (32.7%) had the 1RS.1BL wheat-rye chromosome translocation, the source of *Sr*31 gene. Also, EJaz *et al*. (2012) stated that in 117 Pakistani wheat varieties, the highest frequency was observed for *Sr*2 (9-79% by different markers), followed by *Sr*31 (35%), *Sr*6 (11%), and *Sr*38 (9%). They reported that these results indicated that Pakistani varieties are being protected by very few resistance genes and lack resistance genes potentially effective against new stem rust races.

Table 4. Percentage frequency of the four monogenic stem rust genes, *Sr* 13, *Sr* 25, *Sr* 26 and *Sr* 31 in thirteen bread wheat cultivars

Sr gene Cultivar	*Sr*13	*Sr*25	*Sr*26	*Sr*31
Gemmeiza-7	+	+	-	+
Gemmeiza-10	+	+	-	+
Gemmeiza-11	+	+	-	+
Gemmeiza-12	+	+	-	+
Sids-12	+	+	-	-
Sids-13	+	+	-	+
Sids-14	+	+	-	-
Giza-168	+	+	-	-
Giza-171	+	+	-	-
Misr-1	+	-	-	-
Misr-2	+	-	-	-
Sahk93	+	+	-	-
Gemmeiza-9	+	+	-	+
Frequency (%)	100%	84.61	0.00	46.15

Owing to the susceptible responses of the Egyptian bread wheat cultivars to Ug99 race and its variants under hot-spots of stem rust (Kenya, Ethiopia and Uganda) and to the local races. The understanding of the effectiveness of individual *Sr* genes against stem rust pathotypes will facilitate the utilization of these genes in breeding for stem rust resistance in wheat. More studies are needed to identify more resistance *Sr* genes and pyramiding these genes into our adapted germplasms.

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استخدام المعلمات الوراثية فى تعريف بعض الجينات المقاومة لمرض صداء الساق فى ثلاثة عشر صنفا من أصناف قمح الخبز المصرية

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يعتبر مرض صدأ الساق فى القمح والذى يسببة الفطر بكسينيا جرامينيز تريتيسای من أخطر أمراض محصول القمح فى العالم . على الرغم من عدم وجود سلالة صدأ الساق ت ت ك س (أوغندا ٩٩) فى مصر الا ان هذا المرض احتل موقعا خطيرا فى السنوات القليلة الماضية (٢٠١٢ – ٢٠١٤) حيث ظهر بدرجات عالية من الأصابة على معظم أصناف قمح الخبز المصرية المنزرعة حاليا .

تم تقييم ثلاثون سلالة من سلالات القمح أحادية الجين المقاومة لمرض صدأ الساق فى القمح تتضمن ٢٠ سلالة قمح من السلالات المفرقة بالأضافة الى ١٠ سلالات اضافية تحت ظروف العدوى الطبيعية بمزرعة محطة البحوث الزراعية بالجميزة موسم ٢٠١٣ - ٢٠١٤ أظهرت الجينات:

Sr 5 , *Sr* 9g, *Sr* 11, *Sr* 13, *Sr* 14, *Sr* 21,Sr22, *Sr* 24, *Sr* 28, Sr36,and *Sr* 38 مقاومة عالية للمرض. تم أستخدام أربعة من المعلمات الوراثية (ماركر) المتخصصة فى تعريف الجينات فى ثلاثة عشر صفا من أصناف قمح الخبز المصرية والتى تزرع حاليا فى مصر, *Sr*13, *Sr*25, *Sr*26, *Sr*31. هذة المعلمات الوراثية هى:

XBE403950EST- PSY-DI - *Sr*26#43 - IAG95-STS

ولقد أظهرت النتائج أن الجين أس أر ١٣ موجود فى جميع الأصناف المصرية – بينما الجين أس أر ٢٥ يوجد بنسبة ٨٤,٦١ % - الجين أس أر ٢٦ غير موجود فى جميع الأصناف –والجين أس أر ٣١ موجود بنسبة ٤٦,١٥ %. أوضحت هذة النتائج أهمية استخدام المعلمات الوراثية فى تحديد جينات المقاومة فى أصناف وسلالات القمح وأهمية ذلك فى عمل خطة لادخالها فى أصناف القمح لزيادة المقاومة لهذا المرض.