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Molecular discovery of new allele associated with loose smut resistance gene *Ut-X* in spring wheat

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Abstract. Genes of resistance to loose smut incited by the fungus *Ustilago tritici* (Pers.) Rostr. are still unknown in the Egyptian spring wheat. Loose smut incidence (LSI) was assessed in ten wheat cultivars through a two-year field trial during 2018–2020. All of the tested cultivars exhibited various percentages of susceptibility (> 10–70% LSI) to the disease except cultivar Misr-3 which exhibited resistance. The most susceptible cultivars were Sakha-93 (60%), Giza-168 (42.1%), and Misr-2 (34.28%). However, the resistant cultivar Misr-3 recorded the least LSI amounting to 5%. The wheat cultivars were screened by the SCAR marker (Xcrc4.2) to identify the presence/absence of loose smut resistance gene *Ut-X*. Molecular data revealed that the SCAR marker (Xcrc4.2) generated two alleles in cultivars with PCR fragments size of 800-bp and ≃ 200-bp. The favorable allele 800-bp was generated only in the resistant Egyptian cultivar 'Misr-3' and the resistant check cultivar 'Biggar', indicating the presence of the gene. Meanwhile, another allele ≃ 200-bp was generated in seven Egyptian cultivars, Giza-168, Giza-171, Misr2, Sakha-93, Gemmeiza-12, N-95, and Shandweel-1, indicating the absence of the resistant gene. This is the first study to report resistance genes to loose smut in Egyptian spring wheat, by detecting *Ut-X* in cultivar Misr-3. In addition, the study documented the first report of another allele ≃ 200-bp associated with SCAR marker (Xcrc4.2). Findings also revealed that the race-specific resistance gene *Ut-X* confers effective resistance to local *U. tritici* races, including race T10 which could be widely incorporated in breeding programs to control the disease.

Key words: SCAR marker, resistance genes, *Triticum aestivum*, *Ustilago tritici*, *Ut-X* alleles.

INTRODUCTION

Wheat is the staple food for approximately one-third of the world population. More than 215 million hectares with an annual production of 700 million tons of wheat were estimated worldwide (FAOSTAT, 2018). In Egypt, wheat is one of the most important winter cereal crops in terms of the planted area and crop production. It provides more than 30% calorie intake of the population. The wheat area grown in Egypt is approximately 1.26 million hectares with a yield of approximately 8.1 million tons, but

there is still a big gap, about 50%, between production and consumption (Kishk et al., 2019). Wheat is liable to attack by many important diseases, causing great losses in grain yield and quality. Rusts, mildews, black point, and loose smut are among the most common and widespread diseases of wheat in Egypt (El-Gremi et al., 2017; Gad et al., 2019; Elkot et al., 2020; Draz Abd El-Kreem, 2021; Esmail et al., 2021). Loose smut incited by the basidiomycete fungus *Ustilago tritici* (Pers.) Rostr. commonly occurs in the majority of the wheat-growing countries (Nielsen & Thomas, 1996; Thambugala et al., 2020). The spike produced from infected germinated plants are converted into black powdery spore clusters in which grains are usually not formed, only the rachis remains intact. It is also common for some particles to form on a locally infected head. Contaminated seeds are the only source of perpetuation and loose smut causes yield losses up to 5–7% where farmers plant their harvested infected seeds again (Ramdani et al., 2004). The presence of loose smut infection cannot be predicted until the plant, which is impregnated with the inoculum, produces a spike characteristic symptom i.e., early emergence and blackening of the emerging spike. This seed-borne disease is commonly present in Egypt at different levels of incidence and unfortunately, none of the Egyptian wheat cultivars is known to be resistant against this disease.

The management of loose smut was achieved in wheat with a combination of resistant cultivars, certified seeds, and systemic fungicides applied as seed treatments, yet the absence of an effective control practice resulted in significant yield and economic losses (Nielsen, 1983). Although the use of pesticides to protect the production of crops may have an adverse impact on the environment and the consumers, most farmers still prefer to use chemical control for effective immediate results in disease control. The development of resistant cultivars is an effective eco-friendly approach to eradicate this problematic fungus particularly in organic wheat production and in countries where seed treatment is not readily available (Menzies, 2008; Menzies et al., 2009). Commercial wheat cultivars with effective resistance to loose smut have been developed as a result of the incorporation of loose smut resistance genes of the bread (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* var. *durum*) collected around the world (Nielsen, 1987; Menzies et al., 2003). *U. tritici* races of differing virulence have been reported from both bread and durum wheat worldwide, in which approximately fifty races of *U. tritici* have been identified from various regions of the world growing hexaploid wheat (Menzies, 2016). The virulence *U. tritici* population varies substantially globally (Kaur et al., 2014; Kassa et al., 2015). For instance, the occurrence of races such as T1, T17, T34, and T38 has been reported in Egypt which was virulent on many hexaploid wheat cultivars/lines (Knox & Menzies, 2012). Nowadays distinguishing wheat resistance genes is crucial, since new *U. tritici* races continue to be found in commercial wheat fields in Egypt (Gad et al., 2019).

Previous studies on the genetics or mechanisms of loose smut resistance in wheat have shown that resistance may be inherited as a qualitative or quantitative trait (Knox et al., 2014). Resistance to wheat loose smut is known to be under monogenic control and several resistance genes have been identified and localized in hexaploid wheat (Procunier et al., 1997). To date, at least eleven genes resistant to loose smut were recorded in the catalog of wheat gene symbols, *Ut1–Ut4*, *Ut-X*, *Ut6–Ut11* (Nielsen, 1977, 1982; McIntosh et al., 2013; Kassa et al., 2014; Thambugala et al., 2020). Although the gene originally named *Ut-X* located on chromosome 2BL has been recently identified as *Ut5* (Procunier et al., 1997; Knox et al., 2014). However, the presence of

the *Ut5* resistance gene, complementary to the *utv5* virulence gene is debatable (Syukov & Porotkin, 2015). We, therefore, decided to refer the respective resistance gene in the current study to the original named *Ut-X* here on. This gene mapped to the distal end of chromosome arm 2BL and conditioned resistance to *U. tritici* race T10 (Procunier et al., 1997). In wheat, several classes of molecular markers have been successfully used for linkages to resistance genes such as restriction fragment length polymorphisms (RFLP) (Autrique et al., 1995; Schachermayr et al., 1995), random amplified polymorphic DNA (RAPD) (Procunier et al., 1995; Schachermayr et al., 1995), simple sequence repeat (SSR) markers (Abou-Elseoud et al., 2014; Draz, 2017; Shahin et al 2020) and sequence characterized amplified region (SCAR) (Paran & Michelmore, 1993; Procunier et al., 1997; Cao et al., 2001; Gupta et al., 2006; Rai et al., 2017). SCAR markers are derived from a robust PCR and show a visually less complex banding pattern that have an advantage of high reproducibility and locus-specific (Procunier et al., 1997).

Wheat production in Egypt has been improved due to the development of breeding and cultivation techniques to avoid the huge negative impact of loose smut on wheat production. For successful breeding of cultivars resistant to this disease, the breeder and the pathologist, first of all, should have the information on the effective resistance genes for the local area of wheat cultivation. To date, resistance genes to loose smut in Egyptian spring wheat have not been identified yet. Hence, the present study aimed to identify loose smut resistance gene *Ut-X* based on SCAR flanking marker and to report alleles associated with the disease incidence in Egyptian spring wheat.

MATERIALS AND METHODS

Plant material

Seeds of ten Egyptian wheat cultivars were provided by the Wheat Disease Research Department, Plant Pathology Research Institute, ARC, Egypt. Seeds of the wheat material served as susceptible check cultivar 'Diamant' for disease evaluation (Nielsen & Tikhomirov, 1993) and as positive check cultivar 'Biggar' for a molecular assay (Procunier et al., 1997**)** were provided by the International Maize and Wheat Improvement Center (CIMMYT), Mexico. The tested wheat cultivars and their pedigree are provided in Table 1.

Table 1. List of the tested spring wheat cultivars and their pedigree

No.	Cultivar	Pedigree
1	Giza-168	MRL/BUC//Seri-82
2	Giza-171	Sakha-93/Gemmeiza-9
3	Sids-14	SW8488*2/KUKUNA
4	Misr-2	Skauz/Bav-92
5	Sakha-93	Sakha-92/TR810328
6	Beniswef-5	DIPPER-2/BUCHEN-3
7	Misr-3	Rolf-07*2/Kiritati
8	Gemmeiza-12	OTUS/3/SARA/THB//VEE
9	N-95	-
10	Shandweel-1	SITE//MO/4/NAC/TH.AC//3*PVN/3/MIRLO/BUC
11	Biggar	TOBARI-66/ROMANY-66
12	Diamant	YUBILEI/SADOVO-1

Evaluation of loose smut incidence

In a two-year field trial, loose smut incidence (LSI) was assessed in the tested wheat cultivars (Table 1) during 2018/19–2019/20 growing seasons at Sakha Agricultural Research Station, Agricultural Research Center (ARC), Egypt. During the 2018/19 season, cultivars were grown in three-row plots, each 1.5 m long with 30 cm distance between rows. The plots were arranged in a randomized complete block design (RCBD) with three replicates. All recommended cultural practices for wheat crops in the commercial fields were applied. The wheat cultivars were inoculated with a mixture of local *U. tritici* races including predominant race T10, according to the method described by Nielsen (1987). In which, florets of the plants were inoculated with a teliospore suspension of *U. tritici* races at mid-anthesis (GS 60-65, Zadoks et al., 1974). Independently of each cultivar, ten spikes were inoculated and each spike was tagged. Inoculated spikes were harvested at maturity and the grains from the inoculated spikes were collected in envelopes labeled with wheat cultivar identity. During the 2019/20 season, a minimum of 100 inoculated grains for each cultivar was planted in 1.5 m long with 30 cm between rows. Loose smut incidence (LSI) was assessed in each cultivar according to the method described by Menzies et al. (2009), and was calculated as follows:

$$\text{LSI (\%)} = \frac{\text{Number of smutted plants}}{\text{Total number of plants}} \times 100$$

Molecular assay of loose smut resistance gene *Ut-X*

The molecular assay was carried out in the Biological Laboratory of the Plant Pathology Department, Faculty of Agriculture, Alexandria University, Egypt. Genomic DNA was extracted using a commercial kit: Thermo Scientific™ GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, Cat. No. K0701) and quantified using a spectrophotometer (MaestroNano, Drop MN-913). The DNA samples were diluted for a final concentration of 100 ng/µL. The SCAR marker (Xcrc4.2) developed by Procunier et al. (1997) was used to detect the loose smut resistance gene *Ut-X* in the ten Egyptian wheat cultivars. Amplification of genomic DNA with SCAR primer pair 5'-TGGGCTCGCTTCATAAATTGGTTC-3' and 5'-TGGGCTCGCTGCTACCGGGGTGGA-3' was done in a thermocycler (Techne-Progene, UK). The 25-µL PCR reaction volume was prepared and the PCR program was optimized in the initial study at an annealing temperature of 68 °C according to Procunier et al. (1997). Amplification products were electrophoresed in 1.4% agarose gel with RedSafe™ Nucleic Acid Staining Solution. The tests were repeated twice. The DNA banding patterns were visualized using a UV-transilluminator (Herolab UVT 2020, Kurzwellig) and photographed. The obtained PCR fragments were scored to indicate alleles associated with the gene *Ut-X* and its presence/absence in cultivars.

RESULTS AND DISCUSSION

Loose smut incidence

The twelve wheat cultivars presented in Table 1, consisted of ten Egyptian wheat-tested cultivars and two check cultivars, Biggar as the resistant cultivar, and Diamant as the susceptible cultivar. All cultivars were evaluated for their reactions against wheat loose smut in the field trials during the growing seasons of 2018/19–2019/20. Data

illustrated in Fig. 1 revealed the variations in loose smut incidence (LSI) among cultivars ranged from 5 to 70%. The most affected cultivar among the tested Egyptian wheat cultivar was Sakha-93 which recorded 60% LSI, followed by Giza-168 (42.1%), and Misr-2 (34.28%). While Egyptian cultivar Misr-3 was the least affected with a disease incidence of 5%, followed by Gemmeiza-5 and N-95 (12.50% each). The susceptible check cultivar (Diamant) recorded the highest value of LSI with 70%, while the resistant check cultivar (Biggar) recorded only 5% LSI. Diamant is a loose smut differential line (D-6) from the former Soviet Union and susceptible to most races of the loose smut pathogen (Nielsen & Tikhomirov, 1993). It has been used as a susceptible check cultivar for loose smut pathology studies for over 30 years (Thambugala et al., 2020). The Biggar cultivar is a Canada Prairie Spring Red wheat that carries the resistance gene *Ut-X* to the loose smut race T10 (Procunier et al., 1997). Based on the loose smut incidence (%) results, the tested wheat cultivars were classified into resistant and susceptible classes according to (Nielsen, 1987; Kassa et al., 2014), which considered wheat cultivars with 0–10% LSI as resistant and wheat cultivars with > 10% LSI as susceptible. Data showed that all tested Egyptian wheat cultivars were susceptible to the disease with LSI values > 10%, except cultivar Misr-3 which exhibited resistance to the disease with only 5% LSI. Little data are available on the genetic resistance to loose smut of wheat that has not yet been studied in Egypt.

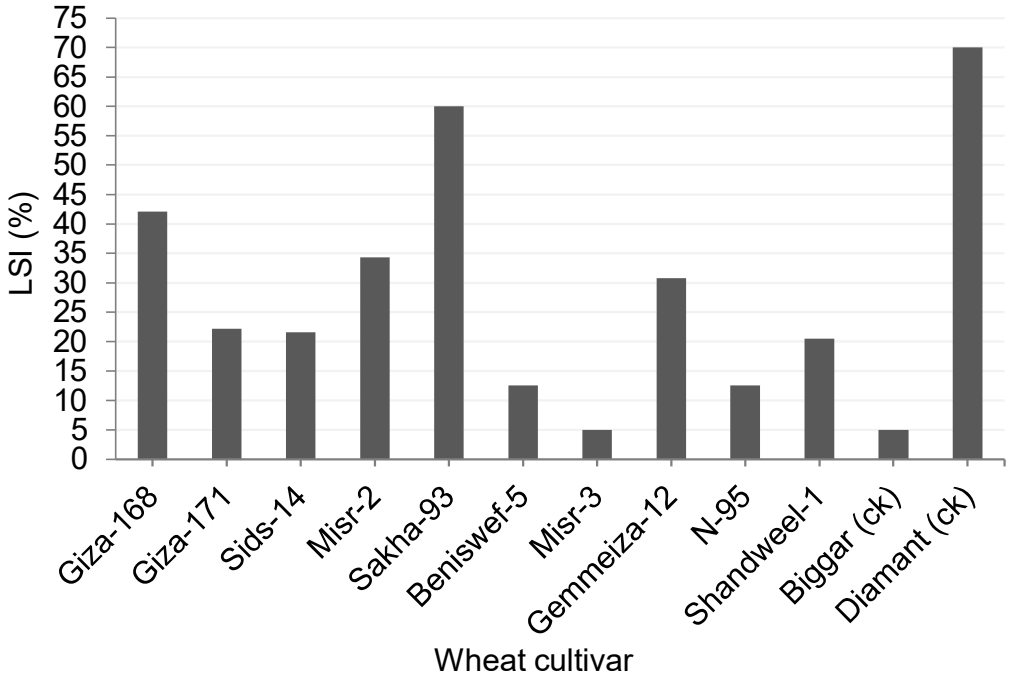

Figure 1. Loose smut incidence (%) in Egyptian spring wheat cultivars and check cultivars, Biggar (resistant) and Diamant (susceptible) in 2019/20 field growing season affected with artificial inoculation with a mixture of *Ustilago tritici* races, including T10.

Molecular detection of *Ut-X* and alleles associated

The tested wheat cultivars were screened by the SCAR marker (Xcrc.4.2) closely linked to the loose smut resistance gene *Ut-X*. The particular SCAR primer at an annealing temperature of 68 °C amplified intense DNA products with two alleles at PCR fragments sizes of ≃ 200 and 800-bp in different cultivars (Fig. 2). A favorable allele

with 800-bp was generated in only one Egyptian cultivar 'Misr-3' and the resistant check cultivar 'Biggar'. While, the other allele ≃ 200-bp was generated in seven Egyptian cultivars, Giza-168, Giza-171, Misr-2, Sakha-93, Gemmeiza12, N-95, and Shandweel-1. No amplification products were observed in two cultivars, Sids-14 and Beniswef-5. These findings indicated that the loose smut resistance gene *Ut-X* was present only in resistant Egyptian cultivar Misr-3 (allele 800-bp), while it was absent in nine susceptible Egyptian cultivars (≃ 200-bp or NIL). A loose smut resistance gene *Ut-X* to *U. tritici* race T10 was found to be located on chromosome 2B in "Chinese Spring" wheat using varietal substitution lines (Bernier et al., 1995). The use of longer and specific SCAR primers allows for a more robust PCR reaction and eliminates the multiple banding pattern which increases the advantages of its use over RAPD markers (Cao et al., 2001**)**. The SCAR marker (Xcrc4.2 locus) has been previously reported as a single genetic locus linked (14 cm) to the *Ut-X* locus at 800-bp (Procunier et al., 1997). In the current study, an allele of (≃ 200-bp) was amplified by the SCAR marker (Xcrc4.2) in susceptible cultivars. Also, obtained results revealed that *Ut-X* is an effective resistance gene against local *U. tritici* races, including T10 which should be considered in the breeding program to control the disease. Given the pedigree information, the origin of the loose smut resistance gene *Ut-X* in the cultivar Biggar derived from TOBARI-66/ROMANY-66, is unknown (Procunier et al., 1997**)**. However, the Egyptian cultivar Misr-3 derived from Rolf-07*2/Kiritati which has TOBARI-66 in previous crosses of Kiritati. Therefore, TOBARI-66 may be the origin of the loose smut resistance gene *Ut-X* in both cultivars, Biggar and Misr-3.

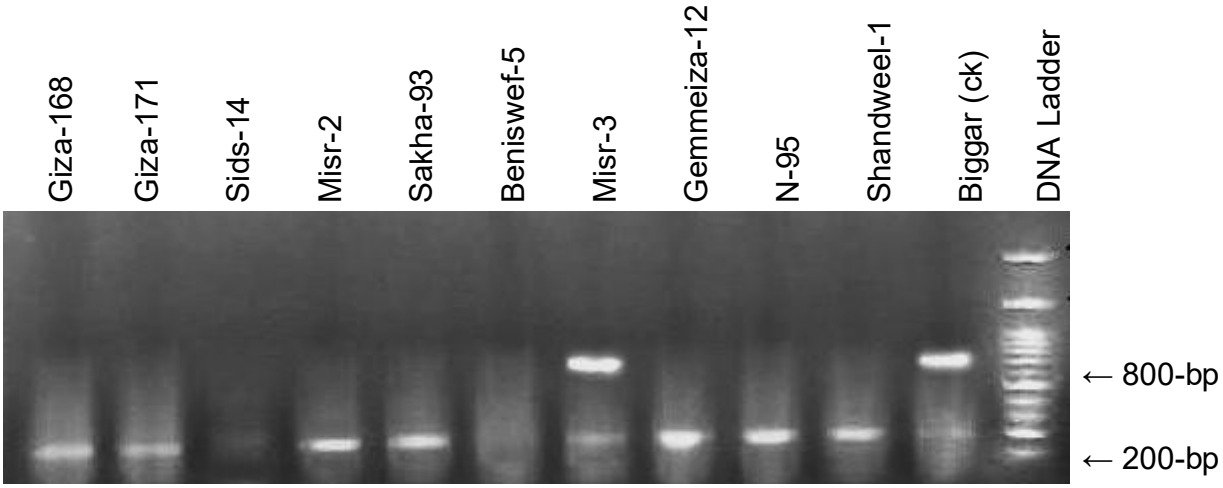

Figure 2. PCR amplification products generated by the SCAR marker (Xcrc4.2) linked to loose smut resistance gene *Ut-X* in Egyptian spring wheat cultivars and resistant check cultivar Biggar carrying *Ut-X*.

The majority of resistance studies carried out so far have also indicated a simple genetic basis for loose smut resistance, with resistance being governed by major genes (Knose et al., 2001; Thambugala et al., 2020). Biggar carries the race-specific resistance gene *Ut-X*. This gene mapped to the distal end of chromosome arm 2BL and conditioned resistance to *U. tritici* race T10 (Procunier et al., 1997). The broad loose smut resistance in the differential wheat line TD-14 (Sonop) is caused by multiple resistance loci (Thambugala et al., 2020**)**. The SCAR flanking marker (Xcrc4.2) linked to a loose smut

resistance gene *Ut-X* with resistant allele (800-bp) and susceptible allele (≃ 200-bp) would facilitate the pyramiding of other resistance genes and eliminate the time-consuming progeny testing of individual plants in a breeding program. Also, these markers can be used on seedlings, thus avoiding the lengthy two-generation disease testing time.

CONCLUSIONS

This is the first attempt to determine the genes of resistance to loose smut in Egyptian spring wheat. We identified a major loose smut resistance gene *Ut-X* in Egyptian cultivar Misr-3. *Ut-X* confers resistance to local *U. tritici* races, including T10. In addition, the study documented the first report to characterize the SCAR marker (Xcrc4.2) linked to *Ut-X* with two alleles that have the potential for use in marker-assisted selection in spring wheat breeding programs. Further studies based on quantitative trait locus (QTL) mapping to identify a major QTL controlling LSI in the *Ut-X* gene contributing multiple alleles are in demand.

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