

Scientific note

Biotype characterization and genetic diversity of the greenbug, *Schizaphis graminum* (Hemiptera: Aphididae), in north Tunisia

Caracterización de biotipo y diversidad genética del pulgón verde, *Schizaphis graminum* (Hemiptera: Aphididae), en el norte de Túnez

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Abstract: The greenbug *Schizaphis graminum*, is a major pest of wheat worldwide. Biotype screening of this pest is essential to develop pest management programs. In this research, eight greenbug clones, collected on wheat in the cereal-growing region of Béja (north Tunisia), were used to determine their damage on six reference wheat cultivars. All tested clones shared a unique biotypic profile, similar to biotype C. Moreover, DNA from the tested clones and that from seven reference clones of biotypes C, E, F, G, H, I and K, was analyzed, using 5 RAPD-PCR primers. The UPGMA method clustered samples into two distinct clades: a first one (I) included clones from north Tunisia, which were clearly associated to agricultural biotypes C, E, I and K, while a second clade (II) included non agricultural biotypes F, G and H. Results reported in this paper suggest that resistance genes *Gb2*, *Gb3*, *Gb4*, *Gb5* and *Gb6* in wheat would be the most efficient if used in wheat improvement programs for resistance against greenbug in Tunisia.

Key words: Aphid. Cereals. Resistance genes. Insect genotypes. Molecular markers.

Resumen: El pulgón verde *Schizaphis graminum*, es una plaga importante del trigo en todo el mundo. La detección de biotipos de esta plaga es esencial para el desarrollo de programas de manejo de plagas. En esta investigación se colectaron, ocho clones del pulgón verde, en el trigo en la región de cultivo de cereales de Béja (norte de Túnez), y se utilizaron para determinar el daño en seis cultivos de trigo de referencia. Todos los clones ensayados comparten un perfil único de biotipo, similar al biotipo C. Además, el ADN de los clones ensayados y de los siete clones de referencia de biotipos C, E, F, G, H, I y K, se analizaron, utilizando 5 RAPD PCR primers. El método UPGMA agrupó las muestras en dos cladros: el primero (I) incluye clones del norte de Túnez, que están claramente asociados a los biotipos agrícolas C, E, I y K, mientras que un segundo clado (II) incluyó los biotipos no agrícolas F, G y H. Los resultados sugieren que los genes de resistencia, la *Gb2*, *Gb3*, *Gb4*, *Gb5* y *Gb6* en el trigo serían los más eficaces si se utilizan en programas de mejoramiento de trigo para la resistencia contra el pulgón verde en Túnez.

Palabras clave: Pulgón. Cereales. Genes de resistencia. Genotipos de insectos. Marcadores moleculares.

Introduction

The greenbug, *Schizaphis graminum* Rondani (Homoptera: Aphididae) is an aphid pest of several graminaceous crops worldwide. It causes severe injuries to host plants in all growth stages and often kills the entire cereal plant. It is prejudicial to the host, either due to the large quantity of sap it extracts, causing water and nutrients depletion (Cruz *et al.* 1998) or due to vectoring Barley Yellow Dwarf Virus (BYDV, Gray *et al.* 2007), Mosaic Maize Dwarf Virus (MMDV, Nault and Bradley 1969) or Sugarcane Mosaic Virus (SCMV, Ingram and Summers 1938). Over twenty greenbug biotypes have been recognized (Porter *et al.* 1997; Shufran *et al.* 2000; Burd and Porter 2006); nearly all of them (except D, which has been identified on the basis of insecticide resistance) have been characterized based on the preference for a host plant species and/or ability to damage specific cultivars of a defined species.

The term "biotype" usually designates an infraspecific group of organisms that are not morphologically distinguishable, but differing by a biological function (Eastop 1973).

Within this definition, *S. graminum* biotypes could be described as a case of host race. In fact, host plant response remains the main criterion for recognition of greenbug biotypes. However, determination of host plant response is often laborious and time-consuming. Therefore, other methods based on morphological characters (Starks and Burton 1977), isozymes (Abid *et al.* 1989) and mitochondrial DNA (Shufran *et al.* 2000), have been used to assess genetic relationships among biotypes or to develop alternative identification procedures. These methods, however, have not fully distinguished all biotypes. Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) has been successfully applied to reveal distinctive patterns among some greenbug biotypes (Black *et al.* 1992; Aikhionbare *et al.* 1998; Lopes-Da-Silva *et al.* 2004). Because biotype identification of *S. graminum* should be the first step for any cereal-breeding program aiming to obtain resistant cultivars to this pest, the main objective of this work was to characterize the biotype(s) of this insect in a wheat-growing area in the north of Tunisia.

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Materials and Methods

Insect and plant material. Eight greenbug clones (Sg11-Sg18), collected over a three year-period, in March 2009, 2010 and 2011 (Table 1), from several wheat fields located in Béja, in the north of Tunisia (36°43'N 9°11'E), were used for infestation assays and subsequent molecular analysis. Seven clones of biotypes C, E, F, G, H, I and K, received from the USDA-ARS, Stillwater, Oklahoma (USA), were used in molecular analysis. All clones were reared for 3 generations on the susceptible barley cultivar 'Custer', in a growth chamber, under standard conditions (22-5°C, 50% relative humidity and 16L: 8D photoperiod, Shufran *et al.* 1992). In addition, 6 differential wheat cultivars ('DS28A', 'Amigo', 'Largo', 'CI 17959', 'CI 17882', and 'GRS1201', Burd and Porter 2006), containing each a resistance gene to *S. graminum* were used in monitoring greenbug biotype. A barley cultivar ('Custer'), containing no resistance gene was used as susceptible check. All these cultivars (Table1) were kindly provided by USDA-ARS, Stillwater, Oklahoma, USA.

Determination of biotype. The biotype for greenbug clones from Tunisia was determined by screening the feeding damage on the wheat differentials mentioned in table 1. As described in Starks and Burton (1977), 10 seeds of each wheat differential and 10 seeds of the susceptible check 'Custer' were planted in 15cm rows (replicated four times) in flats on greenhouse benches. Seedlings were grown at 25°C with a 16:8 (L:D) photoperiod. Flats were caged to avoid secondary infestation. Immediately after emergence, plants were infested by greenbug clones. Once the susceptible control plants (Custer) were killed (usually within 7 - 14 d), the test was terminated and the plants were scored as alive (resistant) or dead (susceptible).

RAPD-PCR analysis. Following the determination of biotype, Tunisian greenbug clones were freshly collected from plants and kept in Eppendorf® tubes; while other clones, belonging to selected biotypes, were stored in ethanol 95%. DNA was isolated, from all clones, according to the protocol described in Doyle and Doyle (1987). RAPD-PCR reactions were performed in a Thermal cycler 2720 (Applied Biosystem, USA), as described in Lopes-Da-Silva *et al.* (2004). Five single decamers (Operon Technologies, USA) were used as primers; OPC-09 (5'-CTCACCGTCC-3'), OPG-03 (5'-GAGCCCTCCA-3'), OPG-08 (5'-TCACGTCCAC-3'),

OPG-18 (5'-GGCTCATGTG-3') and OPH-05 (5'-AGTCGTCCCC-3'). Amplification products were analyzed on 1.5% agarose gel in Tris-borate-EDTA buffer, stained in ethidium bromide and visualized under UV light. A 100 bp ladder (Invitrogen) was used as molecular size standard. The RAPD-PCR patterns, for each clone, were identified visually by scoring the presence or absence of all reproducible bands and a single binomial genotype, for each of the 15 samples, was defined. A RAPD fragment was considered as polymorphic once it was present in at least one *S. graminum* clone and absent in the remaining clones. The percentage of polymorphic fragments (%P) was calculated as (number of polymorphic fragments / total number of fragments generated) x 100. Pairwise genetic distances (Nei and Li 1979) between clone pairs were calculated using the GENDIST program of PHYLIP software version 3.68 (Felsenstein 2008). Finally, a UPGMA dendrogram was constructed on the basis of pairwise Nei and Li's (1979) genetic distances, using the NEIGHBOR program of PHYLIP version 3.68 (Felsenstein 2008) and visualized by the software Treeview version 1.6.6 (Page 1996).

Results and Discussion

Biotype screening on a differential set of indicator cultivars showed that the studied greenbug clones from northern Tunisia exhibited profile similar to biotype C (Table 1). All DNA amplification products obtained were reproducible and 60 different markers were scored with the 5 primers used, ranging from 150 to 1100 bp in size. The percentage of polymorphic RAPD fragments generated by each primer alone varied from 60% with primer OP-C09 to 88.88% with primer OP-G18, with an overall percentage of 74.05%. The range of genetic distance was high, varying from 0.040 between clones Sg11 and Sg15, to 1.021 between biotype E and clone Sg11. The dendrogram yielded by the UPGMA method (Fig. 1) showed that the studied clones clustered into 2 distinct clades: a first clade (I), included *S. graminum* clones from north Tunisia, which were clearly associated to agricultural biotypes C, E, I and K (Shufran *et al.* 2000); while a second clade (II) included non agricultural biotypes F, G and H. These results do not contrast with the infestation assay; thereby tending to confirm the assignment of Tunisian clones to biotype C. Although the limited set of primers used in this study did not result in clear discrimination between the reference biotypes, the RAPD analysis was useful to distinguish between agricultural biotypes (C, E, I and K) and non-agricultural ones (F,

Table 1. Reactions of cereal differential cultivars to infestation by *S. graminum* clones from north Tunisia.

Greenbug clone		Sg11	Sg12	Sg13	Sg14	Sg15	Sg16	Sg17	Sg18
Date of collection		March 2009	March 2009	March 2010	March 2010	March 2010	March 2011	March 2011	March 2011
Reactions of wheat differentials (R gene)	Custer (none)	S*	S	S	S	S	S	S	S
	DS28A (Gb1)	S	S	S	S	S	S	S	S
	Amigo (Gb2)	R*	R	R	R	R	R	R	R
	Largo (Gb3)	R	R	R	R	R	R	R	R
	CI 17959 (Gb4)	R	R	R	R	R	R	R	R
	CI 17882 (Gb5)	R	R	R	R	R	R	R	R
	GRS1201 (Gb6)	R	R	R	R	R	R	R	R

*S: Susceptible, R: Resistant.

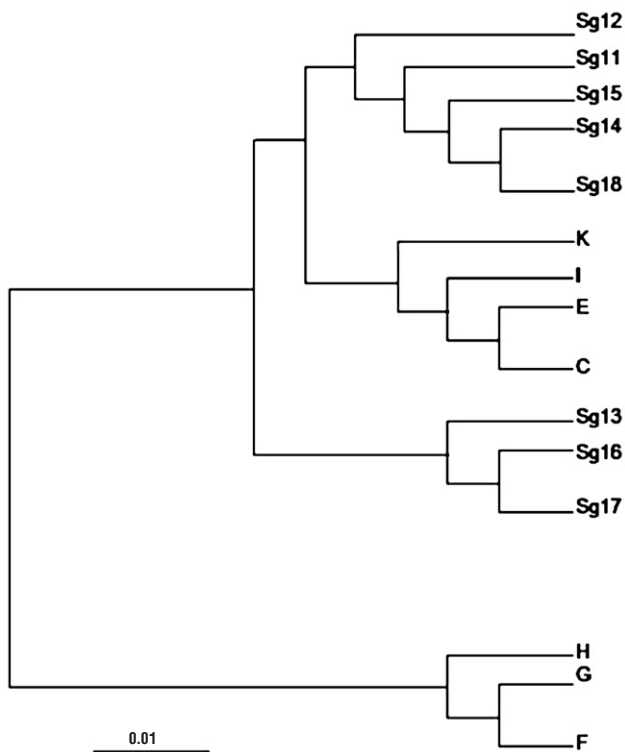


Figure 1. UPGMA dendrogram showing genetic relationships between 8 *S. graminum* clones from north Tunisia (Sg11-Sg18) and biotypes C, E, F, G, H, I and K, based on 60 RAPD markers generated by 5 primers.

G and H). In similar studies, Aikhonbare *et al.* (1998) with USA populations, and Lopes-Da-Silva *et al.* (2004) with Brazilian populations, could not distinguish biotypes C and E, by RAPD markers.

Although greenbug clones from Tunisia, used in this study, showed a homogeneous behaviour at the physiological level, when used for infesting wheat cultivars; they were heterogeneous in molecular analysis, as they were relatively discarded in the dendrogram generated based on RAPD-PCR. This observation is in agreement with several reports where genetic differences were detectable between and within greenbug biotypes (Black *et al.* 1992; Shufran *et al.* 1992; Black 1993; Anstead *et al.* 2002). While some authors (Black *et al.* 1993) have treated biotypes as evolutionary lineages/units, Anstead *et al.* (2002) suggested that a conceptual distinction should be made between three terms, which are “genotype”, “biotype” and “host race”. In other words, a biotype may be characterized by its pattern of virulence to resistance genes or associated with a particular host plant species; yet, it should not be treated as an evolutionary unit or be given taxonomic status and, therefore, it could potentially display genotypic diversity. For example, Shufran *et al.* (1992) found that biotype E in the field was genetically heterogeneous and concluded that it was comprised of many clonal lineages. In order to distinguish normal variation “between clones within a biotype” from differences “between biotypes”, Anstead *et al.* (2002) suggest that phylogeographical surveys should include large samples and integrate data from different genomic and mitochondrial markers. Anyway, biotype designation remains very useful to researchers, and especially to breeders, as it enables the identification of efficient resistant

genes. Biotype C, characterized in this study, implies that resistance genes *Gb2*, *Gb3*, *Gb4*, *Gb5* and *Gb6* in wheat would be efficient if integrated in wheat improvement programs in Tunisia. The transfer of these genes into commercial wheat cultivars in Tunisia could be facilitated by a marker-assisted selection (MAS), as several molecular markers associated with most genes have been characterized (Weng *et al.* 2005; Lu *et al.* 2010).

For a better efficiency of cereal breeding programs, the findings reported here should be continued by extensive surveys, to identify greenbug biotypes in Tunisia, at a greater scale encompassing the South of the country where barley and sorghum are usually grown. Besides, regular biotype surveys, through field and molecular assays, should be conducted, as biotype shifts may occur, rendering previously efficient genes, susceptible to the new biotypes.

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