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New source of resistance to *Aphis gossypii* in Tunisian melon accessions using phenotypic and molecular marker approaches

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Abstract *Aphis gossypii* (Glover) is one of the major pests of melon crops as well as an efficient vector of non-persistent virus such as *Cucumber Mosaic Virus* and *Zucchini Yellow Mosaic Virus* among others. Host-plant resistance is one of the best strategies that can be used to control this pest. In this study 14 Tunisian melon accessions were screened to identify new sources of resistance/tolerance to *Aphis gossypii* using phenotypic and molecular approaches. Antixenosis, antibiosis and tolerance tests were carried out to phenotype those accessions which were also analyzed by molecular markers linked to the *Vat* gene which confers resistance to both *A. gossypii* colonization and virus transmission. Results evidenced that only the accession TUN-7 showed antixenosis, antibiosis and tolerance (no leaf curling), at a similar level to that of the resistant control PI414723. Although plants of the accession TUN-13 did not show leaf curling either, the presence of the *Vat* gene was only detected in TUN-7; its fruit characteristics, of Ananas type, makes this accession as a valuable source of resistance to this aphid that can be used in breeding programs to develop new aphid resistant melon cultivars.

Keywords *Cucumis melo* · Aphid · Antixenosis · Antibiosis · Tolerance · *Vat* gene · Breeding program

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Introduction

Melon (*Cucumis melo* L., 2n = 24) is an outcrossing horticultural crop of economic importance that belongs to the family Cucurbitaceae. One of the main pests affecting this species around the world is *Aphis gossypii* Glover, the only aphid species able to colonize melon plants. Feeding on melon causes direct damage to the plant by removing photoassimilates producing stunting and severe leaf-curling, and heavy colonization can result in plant death. Aphids also excrete honeydew onto the leaves and fruits which acts as an ideal growth medium for sooty mold, which greatly decreases fruit quality (Dogimont et al. 2014). However the most dangerous damage produced by *A. gossypii* is indirect since this species is also an efficient vector of many non-persistent viruses such as *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Papaya ring spot virus* (PRSV) and *Watermelon mosaic virus* (WMV), causing important losses in melon crops (Sarria-Villada et al. 2009). Besides, this aphid species is also a vector of CABYV (*Cucurbit aphid-borne yellows virus*), a persistent virus affecting melon crops in

many production areas (Lecoq and Desbiez 2012; Kassem et al. 2015).

To decrease the harmful effects of this pest, the most efficient and environmentally acceptable option is the use of aphid resistant melon varieties. A dominant gene, *Ag* (*Aphis gossypii* tolerance), was reported to control antixenosis and antibiosis under controlled no-choice tests and free-curling tolerance in LJ 90634, later called PI 414723 (Kishaba et al. 1971, 1976). Antixenosis or non-acceptance consists of disturbance in aphid behavior leading to the rejection of the plant for feeding. Antibiosis corresponds to a modification of the physiology of the insect, affecting demographic parameters (Panda and Khush 1995), resulting in a decrease in the intrinsic growth of the *A. gossypii* population (Boissot et al. 2010).

Later on, Pitrat and Lecoq (1980, 1986) reported resistance in PI 161375 and PI 414723 to several viruses when they are transmitted by *A. gossypii* and the gene controlling this character was named *Vat* (Virus aphid transmission resistance). The gene *Vat* controls the resistance to the aphid itself by non-acceptance and antibiosis mechanisms (Pitrat and Lecoq 1980; Chen et al. 1997) and, as a consequence, controls resistance to virus transmission by this aphid (Sarria-Villada et al. 2009).

The gene was shown to encode a cytoplasmic protein that has a nucleotide binding site and leucine-rich repeat domains CC-NBS-LRR (Pauquet et al. 2004; Dogimont et al. 2008, 2014), belonging to the R gene family, which is widely associated with active defence mechanisms against pathogens and insects (Shinoda 1993). Consequently, an active defence mechanism against *A. gossypii* would be present in melon genotypes that carry the *Vat* gene. Shinoda (1993) described early deposits of callose in leaf tissues of resistant melon plants in response to *A. gossypii* infestation. Also, Sarria-Villada et al. (2009) showed that the resistance conferred by this gene is associated with a microscopic hypersensitive response specific against *A. gossypii*. Soon after aphid infestation, phenol synthesis, deposits of callose and lignin in the cell walls, disturbing aphid behavior, were detected in genotypes carrying the *Vat* gene (Sarria-Villada et al. 2009). Four additive and two couples of epistatic QTLs affecting behaviour and biotic potential of *A. gossypii* were mapped in GL5 using a population of recombinant inbred lines derived from the cross Védrantais x PI 161375; among them, a major QTL, which affects both behavior and biotic potential of *A. gossypii*, corresponds to the *Vat* gene (Boissot et al. 2010). Several genomics studies focused on the region containing *Vat* showed that the density of genes conferring resistance to various pathogens in melon is highest in this region (Brotman et al. 2002; García-Mas et al. 2012).

The *Vat* gene has been sequenced and tightly linked markers have been reported (Dogimont et al. 2004, 2007) which allows their use in the selection of *A. gossypii* resistance. Dogimont et al. (2009) showed that these markers can be used in particular in the context of conventional techniques for varietal selection, in order to facilitate the identification of resistant varieties, and/or the monitoring of the introgression of the resistance characteristic in varieties of agronomic interest. These markers allow detecting the presence or the absence of an allele of the *Vat* gene that promotes resistance to colonization by the aphid *Aphis gossypii* and/or resistance to viral transmission by said aphid (Dogimont et al. 2009).

To date, several sources of resistance carrying the *Vat* gene have been used in melon breeding programs to prevent *A. gossypii* colonization and aphid virus transmission (Dogimont and Boissot 2014; Boissot et al. 2016); however, most of these sources are quite different from commercial types which, makes it difficult to eliminate undesirable melon characteristics introgressed into the commercial melon lines together with the *Vat* gene (Palomares-Rius et al. 2018). In spite of the difficulties, several melons varieties, mostly of Charentais type, carrying the *Vat* gene, have been released (Boissot et al. 2016). The identification of new sources of aphid resistance with agronomic traits and fruit characteristics similar to commercial types of melons is still of interest in breeding programs.

In this study, we screened Tunisian melon germplasm to identify new sources of resistance to aphid infestation using phenotypic screening (antixenosis, antibiosis and tolerance tests) and further molecular marker validation.

Material and methods

Insect and plant material

Aphis gossypii individuals collected from infested melon crops in a greenhouse (IHSM-La Mayora, Málaga, Spain) were kindly provided by Dr. de la Peña. One nymph from a female aphid was chosen to create the

clone used in the bioassays. Aphid colonies were reared on plants of the aphid susceptible Spanish melon cultivar 'ANC-57' and maintained in a glasshouse at 25 °C (light) and 20 °C (dark) with a photoperiod of 16:8 h (light:dark).

The plant materials used in the experiments were the Tunisian accessions listed in Table 1 and the Indian melon line PI414723 in which resistance to *A. gossypii* is controlled by the *Vat* gene (Sarria et al. 2008); the Spanish cultivar 'Bola de oro' was used as susceptible control. The Korean melon line PI161375, carrying the *Vat* gene, was also used in the molecular marker validation.

Melon plants were grown in plastic pots (12 cm diameter) filled with soil-substrate composed of peat (60%), litonite (10%), and compost (30%) and placed in a glasshouse under insect-proof muslin net, in the same environmental conditions as aphid colonies.

Phenotypic evaluation

To assess the plant response to *A. gossypii*, five plants per accession at the 4th–5th true leaf stage were infested on the second expanded leaf from the apex with 10 apterous aphids (5 to 7 days old), which were previously starved for 2 h. Plants in pots were placed on dishes with water and carefully separated from each other to avoid contact between leaves and pots. Plants were then randomly distributed on the glasshouse bench.

Table 1 Tunisian melon cultivars used in the experiments

Register number	Accession name	Botanical group*
TUN-1	Stambouli	*inodorus*
TUN-2	Maazoun Menzel Chaker	*inodorus* (*cazaba*)
TUN-3	Trabelsi	*reticulatus*
TUN-4	Maazoun Mehdia	*inodorus*
TUN-5	Galaoui	*reticulatus*
TUN-6	Sarachika	*inodorus*
TUN-7	Chamem (Ananas type)	*reticulatus*
TUN-8	Rupa	*cantalupensis*
TUN-9	Asli	*inodorus*
TUN-10	Dziri	*inodorus*
TUN-12	Fakous	*flexuosus*
TUN-13	Arbi13	*inodorus*
TUN-14	Maazoul	*inodorus*
TUN-15	Maazoun Chott Meriem	*inodorus*

*According to Pitrat et al. (2000)

Forty-eight and 72 h after infestation the numbers of aphids remaining on the plant were recorded to evaluate antixenosis. Since in previous experiments aphid disturbance behavior has been observed in resistant lines, which resulted in the aphids tending to leave the site of infestation (Sarria et al. 2008), the numbers of aphids that remained on the infested leaves 48 and 72 h after the infestation were also recorded. The average number of aphids on plants and on the infested leaves was considered as the phenotypic value of each accession.

Garzo et al. (2002) reported that the number of nymphs produced by aphids on resistant plants is lower than on susceptible plants and it could be considered as an antibiosis parameter; therefore the number of nymphs produced on the infested leaf 48 h after the release of the aphids was also scored.

Tolerance to *A. gossypii* was evaluated in young plants at the first true leaf stage under conditions of massive aphid infestation (10 adults/plantlet). Aphids used in the experiment were young wingless adults 5–7 days old. Tolerance was recorded seven days after infestation in accordance with Ivanoff (1945); plants with curled leaves were considered susceptible to aphids whereas plants with uncurled leaves were considered tolerant.

Molecular marker analysis

The presence of the *Vat* gene is associated with antixenosis, antibiosis and tolerance mechanisms. Therefore, if any resistant accession was found through the bioassays, molecular analysis based on molecular markers linked to *Vat* gene were also carried out. For PCR assays, two molecular markers D and E previously reported to be linked to the *Vat* gene were used (Dogimont et al. 2004). The sequences of the used primers and size fragment are shown in Table 2. The susceptible cultivar 'Bola de oro' and the resistant lines PI414723 and PI 161375 were used as controls.

DNA extraction and PCR amplification

Tunisian melon accessions were used to provide template DNA for PCR amplification. Fresh young leaf tissues, of 14-day old plantlets, was collected from each accession and stored at −20 °C till DNA extraction. The genomic DNA was extracted using the CTAB extraction protocol (Doyle and Doyle 1987) with some modifications (Oumouloud et al. 2012). The quality and quantity

of extracted DNA was determined using Nanodrop Spectrophotometer ND-100 (Nanodrop Technologies, Delaware, USA). For PCR technique, the DNA concentration was adjusted to 10 ng/μl.

Polymerase chain reaction (PCR) for Marker-D and Marker-E of the *Vat* locus was carried out following the indications of Dogimont et al. (2007). PCR was performed in 50 μl solution containing 3.52 μl of distilled H_2O, 5 μl 5xReaction buffer, 16.5 μl 15 mM $MgCl_2$, 16.5 μl 5 mM dNTP, 3.3 μl of 0.5 μM primer, 10 ng of DNA and 1.37 μl of Taq Polymerase. PCR reactions were performed in a thermocycler (model 9700; Perkin-Elmer Corp., Norwalk, CT, USA). The optimization of PCR is done by a multiple choice of temperature for the annealing phase to set the required temperature.

The PCR products were separated in 2.2% agarose gel pre-stained with ethidium bromide and visualized using an UV transilluminator. The image was photographed and gel images were used for scoring.

Data analysis

To evaluate antixenosis the average number of aphids on both, plants and infested leaves 48 and 72 h after infestation was recorded; to estimate antibiosis, the average number of nymphs on the infested leaves 48 h after infestation was considered as the phenotypic value of each accession. In the tolerance test the occurrence or absence of leaf curling was used to evaluate the character. Data were subjected to analysis of variance (ANOVA) using SPSS for Windows (Version 20). The effect of different melon accession on different parameters was analyzed by one-way-ANOVA. For each parameter, differences among accessions were determined by Student- Newman- Keuls (SNK) test.

Table 2 Molecular markers used to study *Aphis gossypii* resistance in Tunisian melon accessions (Dogimont et al. 2009)

Primer name	Primer sequence	Size fragment
Marker-D	F: AACAACTTAGAACCATCTCC CAGC R: GTTGTTGAGAGCAATAGTGT ACCC	1549 bp
Marker-E	F: CCTTAGAAGAAGATGAAGTC TCCC R: CTCCACTCAGAATTGGTAGG TGCC	1722 bp

Results

Phenotypic evaluation

Most of the aphids remained on plants of all the accessions 48 and 72 h after aphid infestation except on plants of PI414723, where only 5–6 aphids were found (Table 3). The melon cultivar 'Bola de oro' was susceptible to aphids, as expected.

However, significant differences among accessions were observed for the number of remaining aphids on the infested leaf. PI414723 was clearly different from the rest since only 2–3 aphids were found on that leaf regardless of the time of evaluation. The number of aphids remaining on the infested leaf of the accession TUN-7 ranged from 5 to 6 at 72 and 48 h after infestation respectively; therefore, TUN-7 showed an antixenotic effect on *A. gossypii.* The remaining accessions were considered as susceptible as the control 'Bola de oro' (Table 3).

The number of nymphs on the infested leaf 48 h after infestation showed significant differences among accessions. Aphid reproduction was very high in most of the accessions and the highest number of nymphs was recorded in the accessions TUN-6 and TUN-12. PI414723 followed by TUN-7 showed the lowest values for this parameter (Table 3); therefore, these genotypes seem to have an antibiotic effect on the aphid.

Seven days after massive aphid infestation the accessions PI414723, TUN-7 and TUN-13 did not show any leaf curling symptoms; therefore, these Tunisian accessions could be considered as tolerant to the aphid. The susceptible cultivar 'Bola de oro' and the remaining Tunisian accessions showed leaf curling symptoms, which demonstrated their susceptibility to *A. gossypii* (Table 3).

Molecular marker analysis

Tolerant melon accessions were further screened for presence of *Vat* gene conferring resistance to *A. gossypii*. The two markers (Marker-D and Marker-E) gave reproducible bands with known resistant and susceptible accessions (Fig. 1). The marker-D amplified, in the resistant controls (PI414723 and PI161375) and in TUN-7 a 1549 bp fragment corresponding to the *Vat* gene. In the susceptible 'Bola de oro', the marker amplified a fragment of approximately 750 bp. In TUN-13 only the susceptible allele was also amplified (Fig. 1).

Table 3 Antixenosis (aphids number remained on the infested leaf and on the whole plant 48 h and 72 h after infestation), antibiosis (number of nymphs on the infested leaf 48 h after infestation) and tolerance (leaf curling) bioassays carried out with Tunisian melon accessions

	Antixenosis				Antibiosis	Tolerance
	Aphid number on plant (48 h)	Aphid number on infested leaf (48 h)	Aphid number on plant (72 h)	Aphid number on infested leaf (72 h)	Nymph number (48 h)	Leaf curling (after 7 days)
TUN-1	9,4 ± 0,24a	8,8 ± 0,20a	9,4 ± 0,40a	7,8 ± 0,33a	96,8 ± 4,41ab	+
TUN-2	9,2 ± 0,37a	7,9 ± 0,40a	8,8 ± 0,37a	7,6 ± 0,40a	104,8 ± 4,22a	+
TUN-3	9,8 ± 0,20a	9,2 ± 0,37a	9,2 ± 0,58a	8,7 ± 0,57a	115,0 ± 7,01a	+
TUN-4	9,4 ± 0,37a	8,4 ± 0,81a	8,8 ± 0,80a	8,4 ± 0,97a	97,3 ± 5,95ab	+
TUN-5	9,0 ± 0,31a	8,0 ± 0,66a	9,0 ± 0,63a	7,8 ± 0,42a	112,6 ± 6,35a	+
TUN-6	10 ± 0,00a	8,8 ± 0,58a	9,6 ± 0,24a	8,8 ± 0,37a	124,0 ± 5,34a	+
TUN-7	8,2 ± 0,58a	6,0 ± 0,80b	8,0 ± 0,54a	5,0 ± 0,87b	70,00 ± 3,64c	–
TUN-8	9,0 ± 0,31a	8,2 ± 0,58a	9,0 ± 0,32a	7,3 ± 0,30a	92,4 ± 7,83ab	+
TUN-9	9,4 ± 0,24a	7,6 ± 0,24a	8,8 ± 0,20a	7,4 ± 0,28a	93,5 ± 7,75ab	+
TUN-10	9,2 ± 0,48a	8,4 ± 0,73a	9,0 ± 0,44a	8,5 ± 0,74a	110,7 ± 5,02a	+
TUN-12	8,7 ± 0,51a	8,0 ± 0,34a	8,0 ± 0,66a	7,8 ± 0,28a	122,0 ± 17,78a	+
TUN-13	8,8 ± 0,37a	7,8 ± 0,77a	8,8 ± 0,49a	7,4 ± 0,47a	90,0 ± 9,45b	–
TUN-14	9,6 ± 0,24a	8,6 ± 0,50a	8,2 ± 0,20a	7,9 ± 0,24a	106,7 ± 5,17a	+
TUN-15	9,0 ± 0,54a	8,5 ± 1,04a	8,2 ± 0,86a	8,1 ± 1,10a	110,0 ± 14,18a	+
PI414723	6,0 ± 0,88b	3,0 ± 1,52c	5,0 ± 0,57b	2,2 ± 1,80c	43,0 ± 10,21d	–
Bola de Oro	9,5 ± 0,28a	8,5 ± 0,86a	9,2 ± 0,47a	8,8 ± 0,70a	96,7 ± 12,35ab	+

Values in each column are the mean of each parameter ± Standard Error (SE)

Different letters within the same column indicated significant differences ($P < 0.05$) among melon accessions

(+) and (−) indicating the occurrence or absence, respectively, of leaf curling

The marker-E amplified a 1722 bp fragment corresponding to the *Vat* gene in PI161375, PI414723 and TUN-7. The amplification product obtained from the genomic DNA of the susceptible 'Bola de oro' had a length of approximately 1.3 kb, and the same amplification product was obtained for TUN-13 (Fig. 1).

The *Vat* gene was then identified in the resistant control melon lines (PI414723 and PI1611375) as well as in the Tunisian melon accession TUN-7.

Discussion

A. gossypii is an efficient vector for many non-persistent viruses, and also transmits CABYV in a persistent, non propagative manner, contributing to the spread of virus diseases. One of the most economically and environmentally options to control this pest is the use of resistant melon varieties. So, there is a need to screen germplasm accessions to identify strong sources of resistance to aphids that will serve as resistant donors in breeding programs. Selection of aphid resistance can be greatly improved by integrating molecular markers with the phenotypic screening.

In the antixenosis test, a high number of aphids remained on both resistant and susceptible plants at 48 h and 72 h after aphid infestation, except on plants of PI414723. These results showed that the methods described by Pitrat and Lecoq (1980) and Martín and Fereres (2003) did not enable us to discriminate between aphid resistance and susceptibility. Following their methodology, all the plants tested in our experiment should be considered susceptible to aphids. However, by scoring the number of aphids only on the infested leaves, significant differences between accessions were detected. Besides, scoring the number of aphids remaining on the infested leaf 72 h after infestation was a more accurate method for discrimination between resistant and susceptible plants than scoring after 48 h. This behavior was also observed by Sarria et al. (2008,

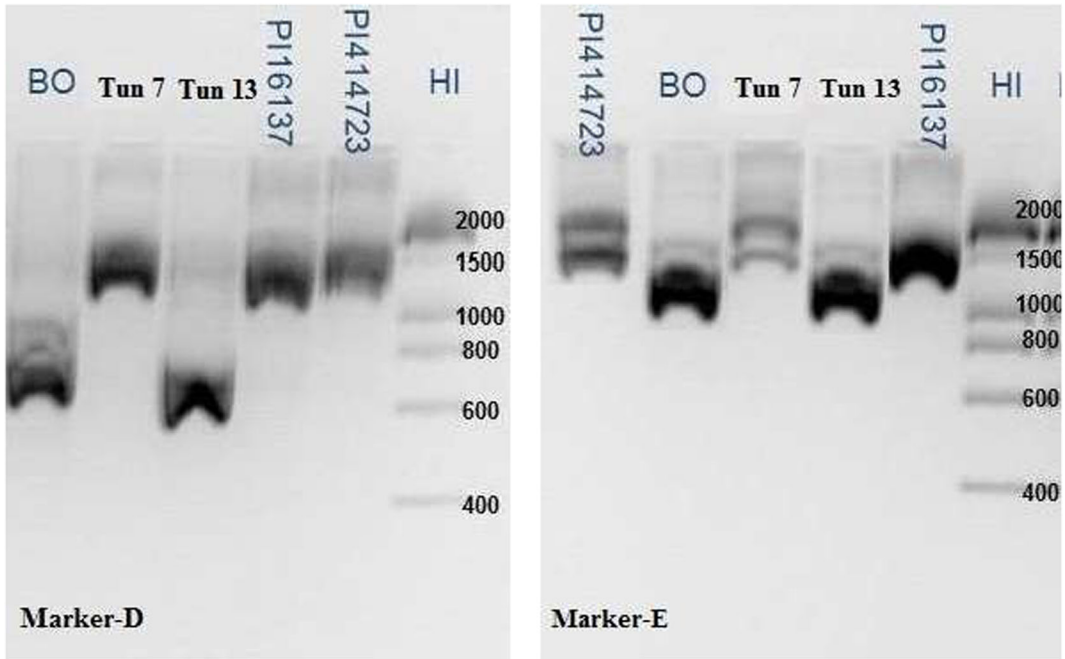

Fig. 1 Genotyping results of markers D and E linked to the *Vat* gene which confers resistance to both *A. gossypii* colonization and the viruses it transmits. The marker-D showed the resistance alleles of the *Vat* gene in PI161375, PI414723 and in TUN-7, whereas 'Bola de oro' (BO) and TUN-13 showed the susceptible alleles. The marker-E showed the resistance alleles of the *Vat* gene in PI161375, PI414723 and in TUN-7

2010). The observed differences between these results and the results obtained by Pitrat and Lecoq (1980) and Martín and Fereres (2003) could be related to differences in the experimental conditions. For example, the age of the plants could influence the expression of resistance to aphids and other insect species, as reported for other plant species (Van Dam et al. 1999; Gurr and McGrath 2001; Leite et al. 2001; Nair et al. 2003; Goggin et al. 2004). In our experiments, the plants used were at the 4th–5th true leaf stage, whereas Pitrat and Lecoq (1980) and Martín and Fereres (2003) used plants at the first true leaf stage. In addition, the aphid clones used by Pitrat and Lecoq (1980) and Martín and Fereres (2003) and the clones employed in our experiments could exhibit different patterns of behavior upon exposure to resistant plants, which might influence the antixenosis and antibiosis results. As reported by Lombaert et al. (2009) and Boissot et al. (2016), different strains/clones of *A. gossypii* showed phenotypic variability for behavioral traits upon exposure to melon plants that carry the *Vat* gene.

Many accessions have been tested for their effect on the aphid life-parameters (Pitrat et al. 1996; Fergany et al. 2011). Those large screenings suggested that about 5% of accessions display resistance to colonization by *A. gossypii*. Among them, only a small number have been also tested for resistance to virus transmission. Up to now, the double phenotype (aphid tolerance and resistance to virus transmission) has been identified in several melon lines (Pitrat and Lecoq 1980; Soria et al. 2000; Thomas et al. 2012; Boissot et al. 2016) coming from Asia, Africa, America, and Europe. However, most of those accessions are quite different from commercial types, which make it difficult to eliminate the undesirable melon characteristics introgressed together with the *Vat* gene (Palomares-Rius et al. 2018).

Bioassays carried out in this work revealed that TUN-7 carried antixenotic and antibiotic resistance to *A. gossypii*. Seedlings of this accession grew normally without any curling symptoms after massive aphid infestation, which indicated its aphid tolerance. The accession TUN-13 showed no curling symptoms and also

seemed to be tolerant to this aphid. The remaining Tunisian accessions were susceptible. On susceptible plants, aphids established a feeding site on the infested leaf; however, on resistant plants, as observed in previous experiments, most of the aphids left the infested leaf and moved to the apex. Similar findings had been reported by Martín and Fereres (2003).

Breeding programs, for pest resistance, are slow and laborious when based on conventional plant selection methods, since rearing healthy insect colonies and carrying out infestation tests are required. Therefore, the identification of molecular markers tightly linked to a resistance gene allows their use in marker assisted selection, making any breeding program much easier. Resistance to *A. gossypii* has been genetically characterized: the *Vat* gene controls antixenosis, antibiosis and *A. gossypii* tolerance and, resistance to virus transmission restricted to this aphid species (Dogimont et al. 2009; Sarria-Villada et al. 2009). The *Vat* gene has been sequenced and tightly linked markers have been reported (Dogimont et al. 2004, 2007). These markers allows detecting the presence or the absence of an allele of the *Vat* gene that promotes resistance to colonization by the aphid *Aphis gossypii* and resistance to viral transmission by said aphid (Dogimont et al. 2009). The usefulness of those markers in the selection of *A. gossypii* resistance has been demonstrated in the assays carried out herein. The results obtained support also their effective use in any breeding program leading to the introduction of the *Vat* gene in melons of commercial value.

Among all the Tunisian accessions the *Vat* gene was only identified in the TUN-7 accession. This accession belongs to the Ananas type, with ovate-elliptical fruits of yellow and netted skin, weighing around 1500 g, of yellow-orange flesh color. With these fruit characters, TUN-7 is a very interesting and suitable accession to be used as a source of aphid resistance to develop commercial melons of the Ananas type, which are highly appreciated not only in Tunisia but in other Mediterranean countries. The accession TUN-13 was considered as tolerant since non-curling of the infested leaf was observed 7 days after infestation; however, molecular analysis showed that this accession does not carry the *Vat* gene. To confirm the existence of any other genetic control different to the *Vat* gene in that accession a more careful study of its behavior against *A. gossypii* should be carried out. The confirmation of its tolerance by bioassays would lead to the DNA sequencing of TUN-13 looking for molecular polimorphisms that could be associated with aphid tolerance. The localization of resistance genes different from the *Vat* gene and their use in breeding could reinforce aphid resistance in melon crops.

Conclusion

In this work a new source of resistance to *A. gossypii* has been found. The TUN-7 accession carries antixenosis, antibiosis and tolerance to *A. gossypii* controlled by the *Vat* gene which could avoid the transmission of the virus transmitted by this aphid. Its fruit characteristics, of the Ananas type, make this accession an interesting donor of resistance to commercial melons. On the other hand, the TUN-13 accession should also be explored to clarify whether its *A. gossypii* tolerance is controlled by other gene different from *Vat*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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