Cytogenetic and Genome Research

Genomic and Cytogenetic Localization of the Carotenoid Genes in the Aphid Genome

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Key Words

Aphids · DNA secondary structure · Fragile site · Holocentric chromosomes · Karyotype rearrangements

Abstract

Data published in the scientific literature suggests a possible link between chromosomal rearrangements involving autosomes A1 and A3 and the presence of red morphs in the peach-potato aphid *Myzus persicae* (Sulzer). In order to begin a study of this relationship, we analysed the genomic and chromosomal location of genes involved in carotenoid biosynthesis in *M. persicae* and the pea aphid, *Acyrthosiphon pisum* (Harris), since carotenoids are the basis of the colour in many aphid species. Genomic analysis identified a DNA sequence containing carotenoid genes in synteny between the 2 species. According to the results obtained using in situ PCR, carotenoid genes were located in a subterminal portion of autosome 1 in both species. The same localization has also been observed in the onion aphid *Neotoxoptera formosana* Takahashi that, as *M. persicae* and *A. pisum,* belongs to the tribe Macrosiphini, thereby suggesting a synteny of this chromosomal region in aphids. In situ PCR experiments performed on 2 *M. persicae* asexual lineages bearing heterozygous translocations involving autosomes 1 and 3 revealed that carotenoid genes were located within chromosomal portions involved in recurrent rearrangements. We also verified by bioinformatics analyses the presence of fragile sites that could explain these recurrent rearrangements in *M. persicae*.

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The chromosome number and karyotype structure of aphids have been studied in a large number of species over the past few decades showing that chromosomal variations are relatively common within some species [Blackman, 1980; Manicardi et al., 2002, 2015a, b; Monti et al., 2011, 2012a, b; Rivi et al., 2012, 2013; Mandrioli et al., 2014a]. This is not surprising since aphids have holocentric chromosomes with kinetic activity spread along the whole chromosome axis [Blackman, 1980]. Hence, chromosomal fragments can contact the microtubules and move in the daughter cells during cell division. In contrast, fragments of monocentric chromosomes may be lost during mitosis and meiosis in the absence of centromeric activity in the chromosome fragment. Furthermore, aphids possess constitutive expression of telomerase, together with a reproduction mode based on apomictic parthenogenesis, so that chromosomal fragments may be stabilized and inherited in the offspring [Monti et al., 2011].

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The peach potato aphid *Myzus persicae* (Sulzer) is one of the most extensively studied aphid species in the world in terms of lifecycle, migration, population genetics, and cytogenetics, and several molecular and cytogenetic analyses have been performed in order to elucidate the structure of its genome and karyotype [Blackman, 1980; Manicardi et al., 2002, 2015a, b; Monti et al., 2011, 2012a, b; Rivi et al., 2012, 2013].

The standard karyotype of *M. persicae* comprises 12 chromosomes, including 5 pairs of homologous autosomes and 2 X chromosomes, but several variations in chromosome number and structure have been recently observed in Italy and Greece [Monti et al., 2011, 2012a, b; Rivi et al., 2012, 2013; Kati et al., 2014]. In particular, the most common *M. persicae* chromosomal variant consists of a reciprocal translocation between autosomes 1 (A1) and 3 (A3) resulting in females with 12 chromosomes with a marked structural difference in heterozygotes [Blackman, 1980; Manicardi et al., 2015b]. The worldwide presence of this chromosomal rearrangement is due to its linkage to the conferment of insecticide resistance, especially against organophosphates and carbamates [Blackman et al., 1978; Foster et al., 2007]. Indeed, biochemical and molecular approaches demonstrated that this resistance is based on an increased production of the carboxylesterase E4 involving both gene amplification and position-effect variegation [Blackman, 1971; Blackman and Devonshire, 1978; Takada, 1986; Devonshire, 1989; Blackman et al., 1996; Field et al., 1996]. As previously reported in the scientific literature [Blackman et al., 1999], the chromosomal location of the unamplified ('wild type') esterase genes is near a subtelomeric block of heterochromatic repetitive DNA on autosome 1, whereas after A1-A3 translocation, the esterase genes map within a euchromatic region [Blackman et al., 1978; Foster et al., 2007]. This hypothesis has been recently supported by data showing that the heterochromatin enriched in the subtelomeric repetitive DNA is silent since it is associated with the nuclear membrane through lamina-interacting proteins [Mandrioli et al., 2014b].

This chromosomal rearrangement has also been found in Italy, where it was not related to enhanced levels of insecticide resistance, since Italian *M. persicae* populations apparently do not have the E4 amplification [Rivi et al., 2013]. This observation indicated that this chromosomal rearrangement is not always associated with carboxylesterase amplification. Indeed, Rivi et al. [2013] suggested that rather it was due to a fragmentation occurring at a fragile site located on autosome 3 that was followed by the esterase gene amplification in some strains only. Interestingly, all individuals with the A1-A3 translocation were red morphs [Harlow et al., 1991; Yang and Zhang, 2000; Rivi et al., 2012] suggesting that this chromosomal rearrangement could influence the aphid body colour.

Aphids shows a range of colour polymorphisms, especially green and red (sometimes referred as pink), which influences their susceptibility to natural enemies [Caillaud and Losey, 2010]. According to literature data, aphid colour is based on the amount and type of the synthesized carotenoids which is due to genes that appear to have a fungal origin, but have been integrated and duplicated into the aphid genome [Jenkins et al., 1999; Moran and Jarvik, 2010]. In particular, the carotenoid torulene occurs only in red individuals in the pea aphids, whereas in *Sitobion avenae,* a brown and a green clone were shown to differ by the type of carotenoids present (4 carotenes for the brown clone against 1 form for the green clone) and the amount of carotenoid (the brown clone had 3 times more carotenoid material than the green clone) [Jenkins et al., 1999].

In view of this possible relationship between aphid colour and chromosome structure, we studied the chromosomal localization of genes involved in the carotenoid biosynthesis in the aphids *M. persicae*, *Acyrthosiphon pisum*, and *Neotoxoptera formosana*. The genes were mapped at a subterminal portion of autosome 1 in all 3 species tested here. In view of the current availability of genome sequences of both *M. persicae* and *A. pisum*, we also compared the gene map of the scaffold containing the carotenoid genes, thereby identifying a 246,000-bp-long fragment syntenic in the 2 species. Lastly, we investigated the presence of fragile sites in both *M. persicae* and *A. pisum* which, in the presence of chemicals able to stall the replication fork (e.g., nicotine), could be the underlying cause of the A1-A3 translocation repeatedly observed in *M. persicae*.

Materials and Methods

Specimens of *M. persicae* were obtained from 3 different aphid asexual lineages (= 'clones' sensu lato since lineages nevertheless rapidly accumulate mutational changes, as reported in Loxdale and Lushai [2003] and Loxdale et al. [2013]) maintained as a colony of parthenogenetic females on pea *(Pisum sativum)* plants. *M. persicae* clone 1 collected on the primary overwintering woody host peach, *Prunus persica,* and clone 64 sampled from a herbaceous secondary host tobacco, *Nicotiana tabacum,* were kindly supplied by Emanuele Mazzoni, Università Cattolica di Piacenza (Italy), whilst clone Re1 was collected on lavender plants, *Lavandula* sp., in Reggio Emilia (Italy).

Table 1. List of cyclase/synthase and desaturase coded enzymes according to data published in Moran and Jarvik [2010] for *A. pisum* and gene predictions according to the currently available version of the *A. pisum* genome

Enzyme type	Sequence in 2010	Sequence in 2015	Contig	Locus
Cyclase/synthase	XP_001943170	XP_001943170	NW_003383548.1	LOC100161104
Desaturase	XP_001943225	XP_001943225.2	NW_003383548.1	LOC100159050
Desaturase	XP_001950764	XP_001950764	NW_003383548.1	LOC100161380
Cyclase/synthase	np	XP_001950787	NW_003383548.1	LOC100159332
Cyclase/synthase pseudogene	np	XP_008178846	NW_003383548.1	LOC103308018
Cyclase/synthase	np	XP_003241668.1	NW_003383548.1	LOC100574964
Desaturase	XP_001946689	XP_001946689.2	NW_003383548.1	LOC100169110
Cyclase/synthase	XP_001350868	np	np	np
Desaturase	XP_001943938.1	NP_001171302	NW_003383967	tor

np = Gene not present in previous analysis or deleted in the update data set; tor = ▬▬▬. λ

The specimens of the pea aphid, *A. pisum*, used in the present research were obtained from the LSR1 laboratory lineage, kindly furnished by Manuel Plantagenest (INRA, France), and maintained asexually on broad bean *Vicia faba* plants.

Adults of the onion aphid, *N. formosana* were sampled on onion plants in Modena (Italy) and maintained asexually on onion plants.

Colonies of all 3 species were maintained asexually on the above plant hosts at 19°C, at a light-dark regime of 16 h light and 8 h darkness.

Chromosome preparations were obtained from parthenogenetic females by spreading embryo cells, as reported by Mandrioli et al. [1999]. CMA$_3$ staining was performed as described by Manicardi et al. [1994].

DNA extraction was done using the Wizard® SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions.

The desaturase genes were localized by in situ PCR experiments using the primers F (5′-ACTGGACACATTTTGACATCCT-3′) and R (5′-TCAATGTCGGGCGTAAATTACT-3′), designed according to the carotene desaturase gene sequence (Gene ID 100169245) available in GenBank.

In situ PCR experiments were performed according to Macas et al. [2006] with some modifications related to the use of a FITC-labelled dUTP solution in place of the Alexa Fluor-dUTP and counterstaining with propidium iodide in place of DAPI. PCR amplification was performed for 2 cycles at an annealing temperature of 60°C for 2 min with an extension time of 7 min at 68°C with the Long PCR Enzyme Mix (Thermo Scientific). In situ PCR slides were observed using a Zeiss Axioplan epifluorescence microscope. Photographs of the fluorescent images were taken using a CCD camera (Spot, Digital Instrument, Madison, Wis., USA) and the Spot software supplied with the camera and processed using Adobe Photoshop (Adobe Systems, Mountain View, Calif.).

RNA extraction and RT-PCR experiments were performed with the SV Total RNA Isolation System (Promega) and with the Access RT-PCR System (Promega), respectively, according to the supplier's recommendations.

Amplification of the desaturase gene was done using an Hybaid thermal cycler with the primers F-DES-RT (5′-GGCACTC-CAAACTGATTTCCA-3′) and R-DES-RT (5′-GATCGATGCG-GCTCGGTA-3′) at an annealing temperature of 53°C for 30 s and with an extension step at 68°C for 1 min. Cytoplasmic actin (primers F 5′-AGCAGGAGATGGCCACC-3′ and R 5′-TCCACATCT-GCTGGAAGG-3′) was amplified as a loading control in the RT-PCR experiments. For the cytoplasmic actin PCR reactions, 25 cycles were performed with annealing for 40 s at 58°C and elongation for 45 s at 68°C. Both primer sets have been designed according to the orthologous genes identified in the pea aphid *A. pisum* genome. After RT-PCR amplifications, done as triplicates, a semi-quantitative analysis of the desaturase gene expression was performed by densitometric gel image analysis using the Image J software (freely available at https://imagej.nih.gov/ij/). The expression levels were evaluated in arbitrary units.

Bioinformatic analyses were done by BLAST alignments in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) both at DNA and protein level. Later, a further search was performed by BLAST alignments of aphid genomes using AphidBase (http://www.aphidbase.com). The assembly 2.0 of the *M. persicae* clone G006 and the release 2.0 of the pea aphid genome were used for our analyses.

The secondary structure-forming potential was analysed using the Mfold software (freely available at http://unafold.rna.albany.edu/?q=mfold/dna-folding-form) that predicts the potential of single-stranded DNA to form a stable secondary structure with its free energy value [Zuker, 2003]. The secondary structure-forming potential of the aphid DNA sequences was analysed initially by inputting 6,000-bp-long segments with a 700-bp shift window into the Mfold program. Successively, a more precise analysis was performed on regions with putative folded regions only by inputting 600-bp segments with a 50-bp shift window. The free energy value of the most stable predicted secondary structure for each segment was used in the analysis. In particular, a more negative free energy value indicates a more stable DNA secondary structure. The predictions of the Mfold program have been validated in different published papers clearly assessing that energetically favourable free energy values predicted by Mfold correspond to sites with stable DNA secondary structure [Dillon et al., 2013].
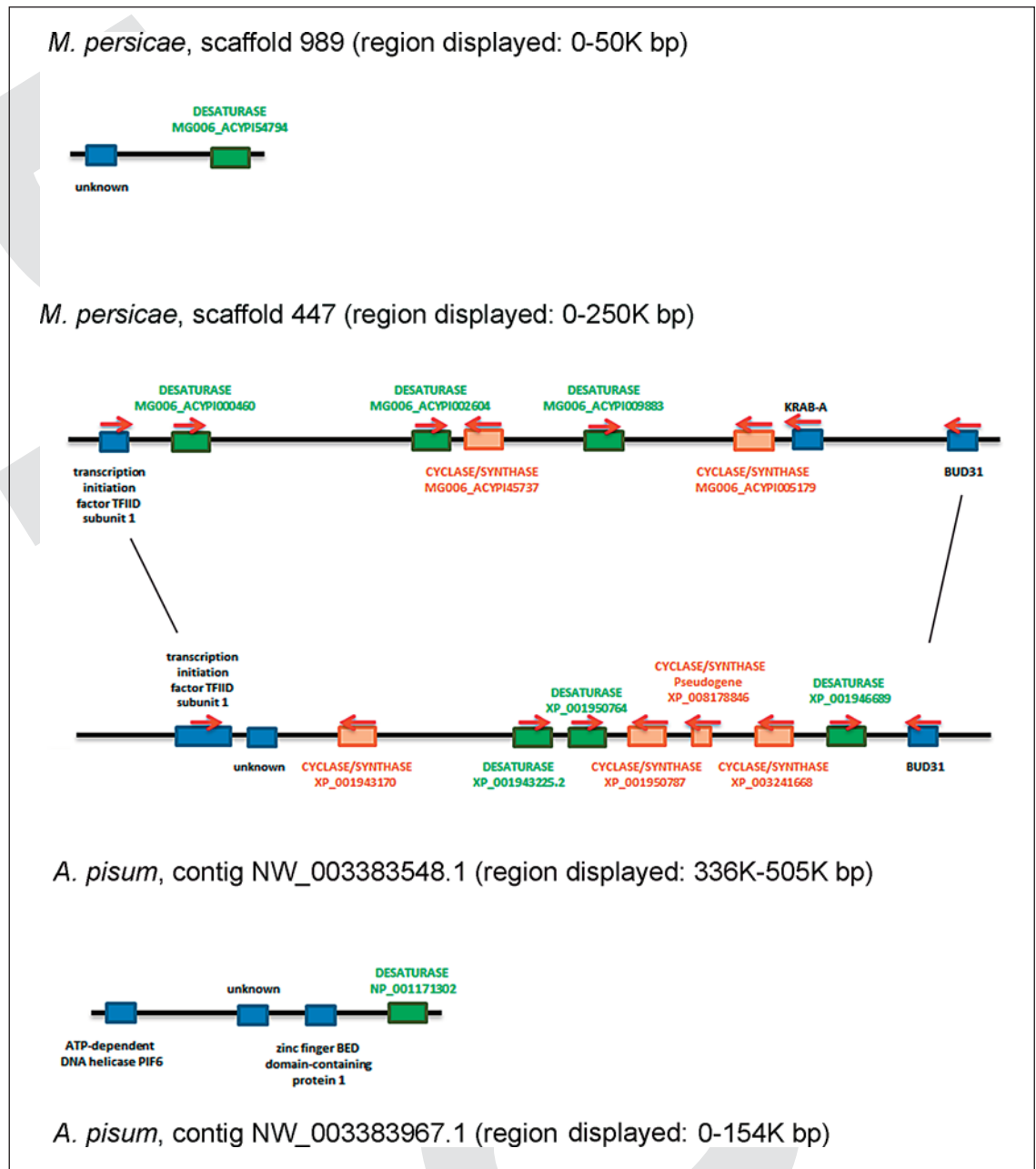
Fig. 1. Identification and localization (in scaffolds or contigs) of genes coding for cyclase/synthase and desaturase in the aphids *M. persicae* and *A. pisum* revealed in both species the presence of multiple copies for both genes that map to 2 different regions. The comparison of the gene map of *M. persicae* scaffold 447 and *A. pisum* contig NW_003383548.1 revealed the occurrence of synteny based not only on the common presence of carotenoid cyclase/synthase and desaturase genes but also on the presence of the genes *TFIID* and *BUD31* at the 2 termini of the DNA sequence.

Results

In order to map genes involved in the biosynthesis of carotenoid in the aphids *M. persicae* and *A. pisum*, we verified the currently available gene sequences in respect to those published in 2010 [Moran and Jarvik, 2010]. The search using GenBank and Aphidbase revealed that the release of the version 2.0 of the *A. pisum* genome brought a significant revision of the annotated cyclase/synthase and desaturase genes involved in the biosynthesis of ca-
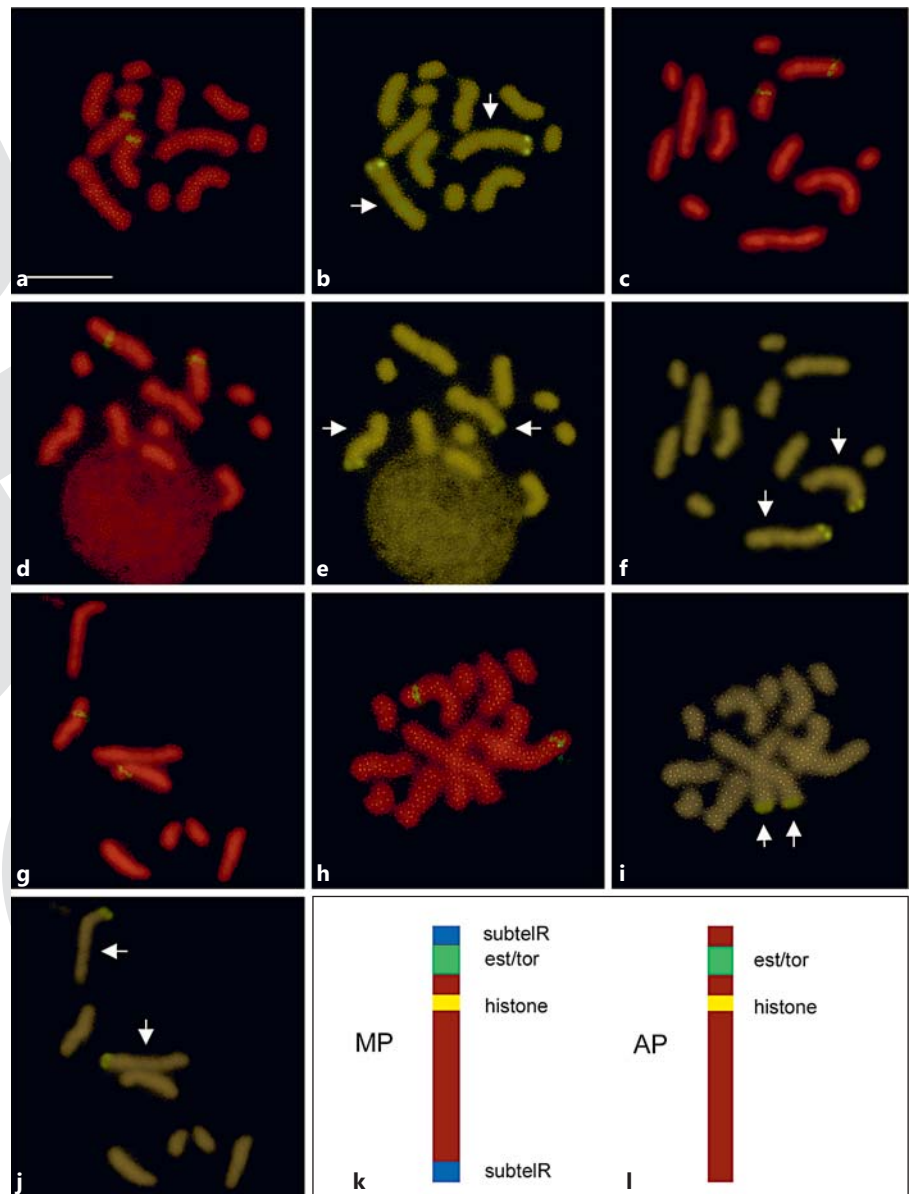
Fig. 2. Metaphase chromosomes after in situ PCR localization of the desaturase encoding genes in *M. persicae* clones 1 (**a**), 64 (**c**), and RE1 (**d**), in *A. pisum* (**g**), and in *N. formosana* (**h**). The same metaphase plates were stained with chromomycin A$_3$ (**b**, **e**, **f**, **i**, **j**). Arrows indicate the X chromosomes. Bar = 10 μm. **k**, **l** Schematic presentation of autosomes 1 in *M. persicae* (**k**) and *A. pisum* (**l**) including the currently available map information. subtelR = Subtelomeric DNA satellite; est = esterase coding genes; tor = carotenoid biosynthesis locus; histone = histone coding gene cluster.

rotenoid (table 1). In particular, 3 new and previously undetected cyclase/synthase genes are currently present (XP_0019507&, XP_008178846 and XP_003241668), whereas the cyclase/synthase XP_00 868 has been deleted in the present *A. pisum* genome release.

According to the available information about the loci, cyclase/synthase and desaturase genes map into 2 *A. pisum* contigs, but the contig NW_003383548.1 contains almost all the identified genes (fig. 1).

In view of the availability of data related to the ongoing *M. persicae* genome project, we identified 4 genes coding for a carotenoid desaturase and 2 coding for cyclase/synthase enzymes in the peach potato aphid. As previously reported for *A. pisum*, these genes were mapped in 2 scaffolds, but scaffold 447 contained almost all the identified genes (fig. 1).

The comparison of the map of *M. persicae* scaffold 447 and *A. pisum* contig NW_003383548.1 revealed synteny based not only on the common presence of carotenoid cyclase/synthase and desaturase genes (that were also oriented in the same direction), but also in the location of the genes *TFIID* and *BUD31* at the 2 termini of the scaf-
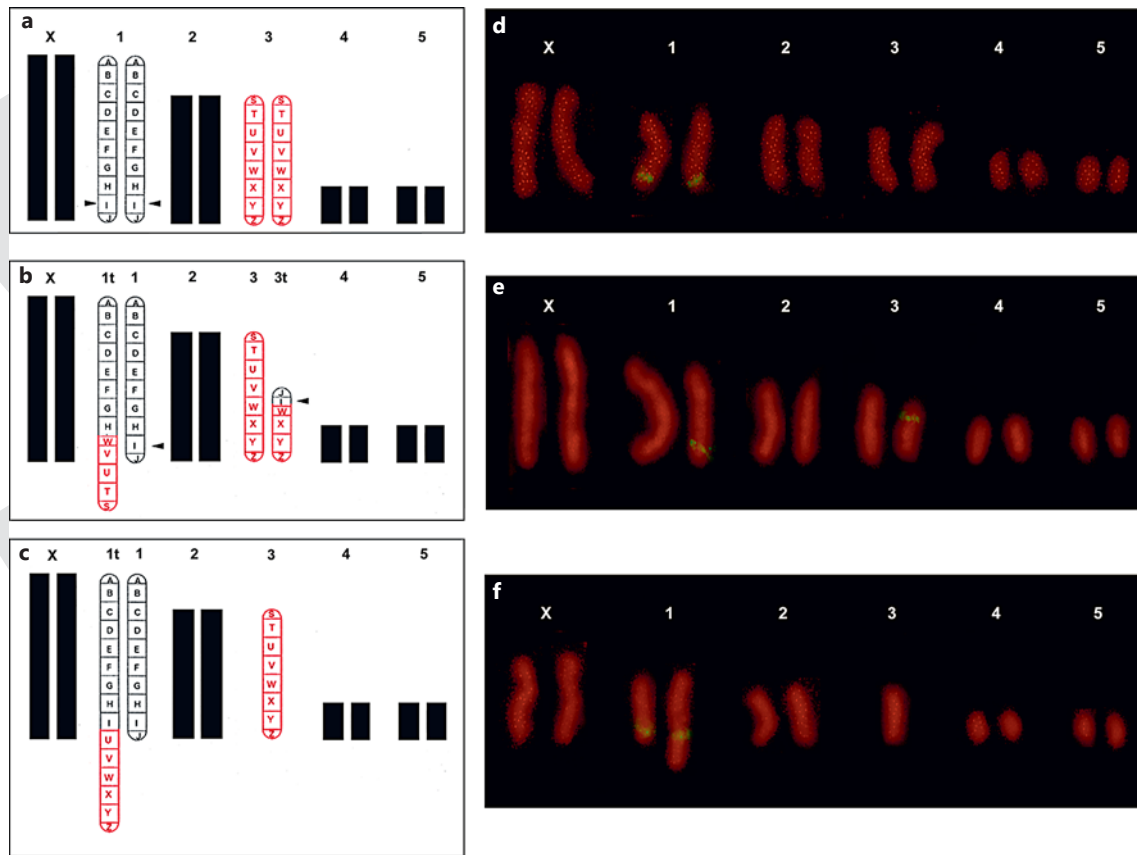
Localization of the Carotenoid Genes in the Aphid Genome

5

Fig. 3. Comparison of karyograms of *M. persicae* clones 1 (**a**), 64 (**b**), and RE1 (**c**) and the karyotypes of the same clones after in situ PCR localization of genes involved in the carotenoid biosynthesis (**d–f**). Arrowheads in **a** and **b** indicate the location of esterase genes according to Blackman et al. [1999].

fold/contig (fig. 1). These results clearly revealed the presence of a 246,000-bp-long syntenic genomic portion in the 2 species.

In order to further investigate this synteny, we mapped the desaturase genes on *M. persicae* and *A. pisum* chromosomes using in situ PCR (fig. 2). In particular, we compared the location of the desaturase genes in 3 different *M. persicae* clones (1, 64, and Re1) that have standard (as in clone 1) or rearranged karyotypes, with clone 64 possessing a reciprocal translocation involving autosomes 1 and 3, and clone Re1 bearing a complete translocation of an autosome 3 onto an autosome 1.

In *M. persicae* clone 1, in situ PCR mapped the desaturase genes to a subtelomeric portion of long chromosomes identified as autosomes 1 (figs. 2a, 3a) by CMA₃ staining which detected the GC-rich telomeres bearing the rDNA genes in the 2 X chromosomes (fig. 2b). In contrast to this situation, in *M. persicae* clone 64, which possesses a recip-

rocal A1-A3 translocation, desaturase genes were mapped to a subtelomeric portion of autosome 1 and a subtelomeric portion of autosome 3, as both are involved in the A1-A3 translocation (figs. 2c, f, 3b). In *M. persicae* clone Re1 (possessing a complete translocation of an autosome 3 onto an autosome 1), in situ PCR experiments mapped desaturase genes in an intercalary portion of the autosome 1 involved in the A1-A3 fusion and in a subtelomeric position of the other autosome 1 (figs. 2d, e, 3c).

In situ PCR showed that desaturase genes are located in a subtelomeric portion of autosomes 1 also in *A. pisum* (fig. 2g, j) and *N. formosana* (fig. 2h, i).

In order to evaluate if the different chromosomal positions of the desaturase genes influenced their expression, a semi-quantitative analysis was performed by RT-PCR showing that desaturase genes are more expressed in clones 64 and Re1 (bearing chromosomal rearrangements) than in clone 1 with standard karyotype (fig. 4).
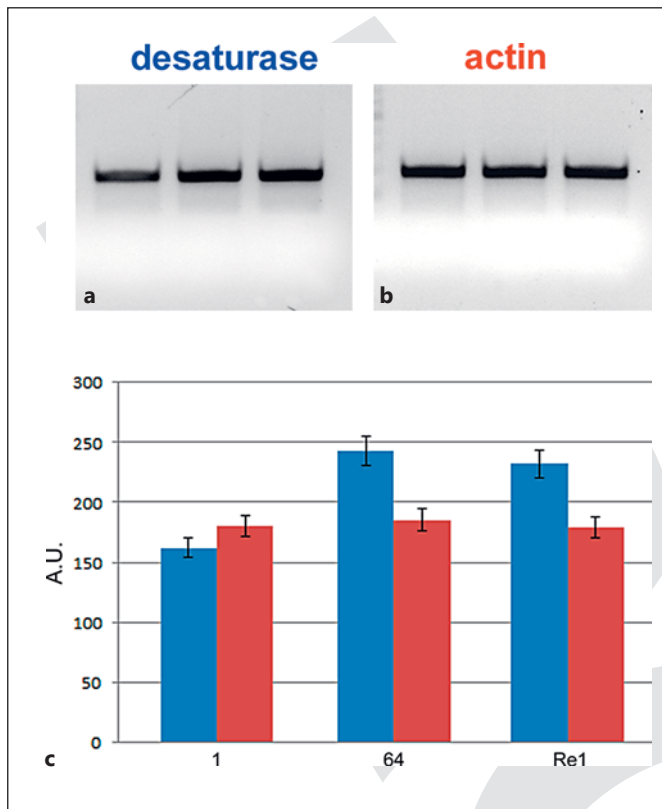
Fig. 4. Semi-quantitative analysis of desaturase gene expression (**a**) in comparison to actin (**b**) showed that desaturase genes (blue bars) are more expressed in clones 64 and Re1 with respect to clone 1 (**c**). The expression level was evaluated in arbitrary units (A.U.). Bars represent the standard deviation resulting from experiments done in triplicate.

Because of the localization of the carotenoid genes in a *M. persicae* chromosomal region frequently involved in recurrent chromosomal rearrangements, the DNA sequences of the *M. persicae* scaffold 447 and *A. pisum* contig NW_003383548.1 were searched in respect of DNA secondary structure-forming potential using the Mfold program. With this aim, the 246,000-bp-long syntenic region between the 2 species was divided into 41 arbitrary 6,000-bp-long sections, and for each section, the various potential DNA secondary structures with respect to the average free energy level were predicted. Sections that resulted energetically and hence more favourably folded into secondary structures were reanalysed in order to provide a more precise evaluation. Mfold analysis, as a whole, revealed in *M. persicae* the presence of 7 potential fragile sites with high ΔG free energy with respect to the average free energy value for the scaffold (–14.6 ± 2.16 kcal/mol). In *A. pisum*, 3 potential fragile sites with high

ΔG free energy were identified (*A. pisum* average free energy value: –24.2 ± 1.21 kcal/mol). All the identified predicted sites were located at the 3′ end of the *A. pisum* and *M. persicae* scaffold/contigs, where the presence of palindrome sequences allowed DNA to fold into highly stable hairpin structures (figs. 5, 6).

Discussion

Carotenoids form a large class of compounds present in many organisms and confer a variety of benefits, including protection from oxidative damage, light detection, photo-protection, and display coloration [Britton et al., 2004, 2006].

Aphids are quite unusual among metazoans since several species (principally *A. pisum* and *M. persicae*) possess functional carotenoid biosynthetic genes [Nováková and Moran, 2012]. This makes aphids the first identified animals with this unusual production and an exceptional case of an animal genome acquiring foreign genes with known function [Moran and Jarvik, 2010]. Interestingly, in both aphids and certain fungi, phytoene synthase and carotene cyclase are fused and are encoded in the same chromosomal region as carotene desaturase, in a distinctive bidirectionally transcribed arrangement not known from other carotenogenic organisms [Moran and Jarvik, 2010]. This unit has undergone several duplication events subsequent to its acquisition in aphids, resulting in multiple copies of the genes for both the carotene desaturase and the phytoene synthase/carotene cyclase [Moran and Jarvik, 2010]. The larger number of carotenogenic genes is typical for members of the Macrosiphini, which often show colour polymorphisms, strongly suggesting that aphid evolution has been accompanied by ongoing evolution of these genes, which have undergone duplication, recombination, and occasional positive selection to yield a wide variety of carotenoid profiles in different aphid species [Moran and Jarvik, 2010].

The present bioinformatic analyses identified a syntenic DNA sequence (consisting of more than 240 kb) in *A. pisum* and *M. persicae* containing the genes involved in the biosynthesis of carotenoids. Cytogenetic analyses, performed by in situ PCR (applied for the first time in aphids and more generally in organisms possessing holocentric chromosomes), revealed that carotenoid genes were also located in a subterminal position in autosome 1 in both aphid species.

The comparison of the karyotypes reconstructed after in situ PCR of *M. persicae* clones 1 and 64 is particularly
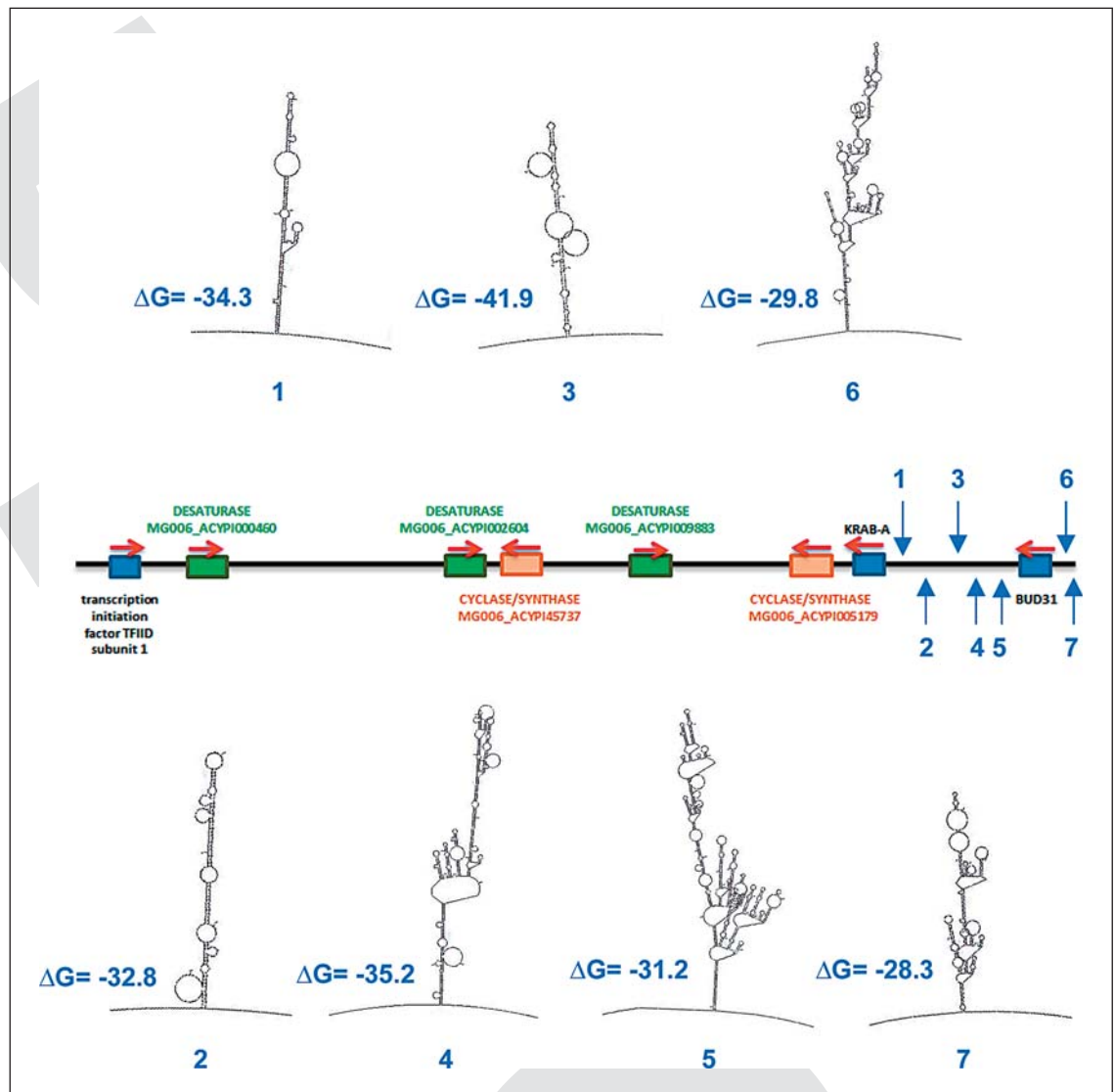
Fig. 5. Localization of the 7 most stable Mfold-predicted fragile sites (indicated by vertical blue arrows) in the *M. persicae* scaffold 447 with the predicted DNA secondary structures and calculated ∆G free energy at each site.

interesting since carotenoid genes map within a chromosomal region frequently involved in recurrent chromosomal rearrangements. Indeed, the carotenoid genes map to a locus adjacent to the esterase genes that is involved in a well-known reciprocal translocation among autosomes 1 and 3 [Blackman et al., 1999; Monti et al., 2012a; Mandrioli et al., 2014a, b].

According to data published in the scientific literature [Harlow et al., 1991; Yang and Zhang, 2000; Rivi et al., 2012], the A1-A3 translocation is generally found in red *M. persicae* morphs. The present chromosomal localization and the semi-quantitative RT-PCR analyses of carot-

enoid genes revealed complementary results suggesting that their chromosomal position influences their expression, so changing the amount and type of produced carotenoids.

This localization of the esterase genes prompted us to search for a molecular explanation of the karyotypic instability observed in *M. persicae* [Monti et al., 2012a; Mandrioli et al., 2014a, b]. In particular, we focused our attention on the presence of secondary DNA structures since they play a major role in different biological processes (such as transcription and telomere maintenance) and are also involved in genomic mutational events, in-
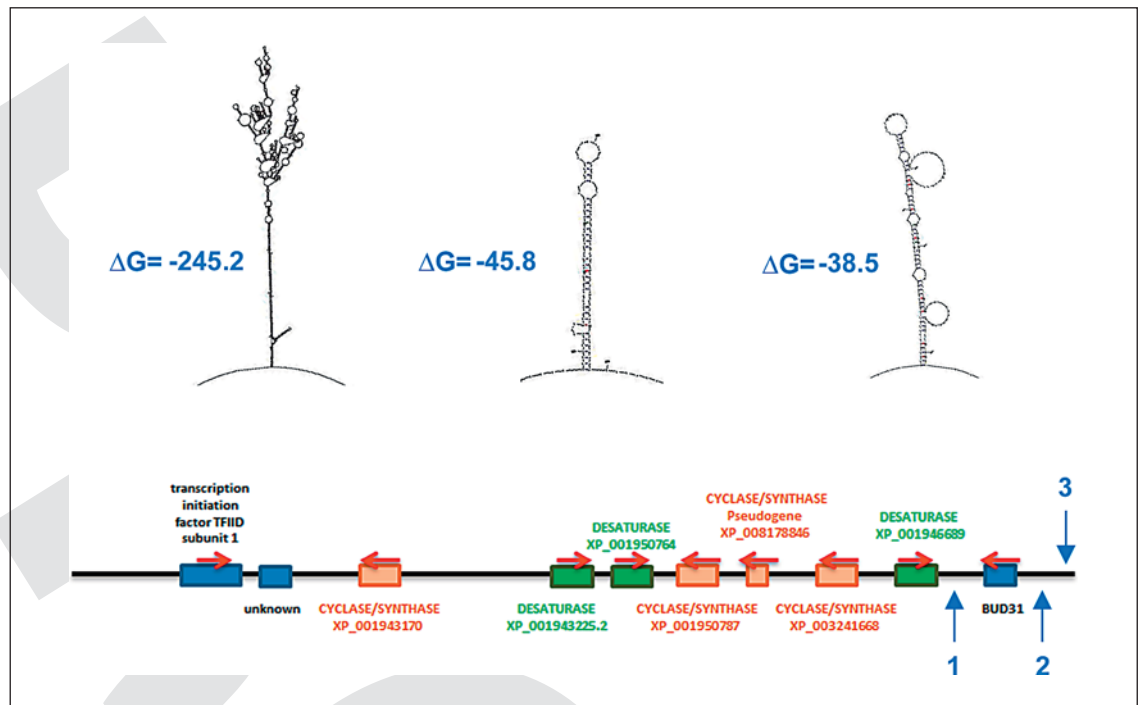
Fig. 6. Localization of the 3 most stable Mfold-predicted fragile sites (indicated by vertical blue arrows) in the *A. pisum* contig NW_003383548.1 with the predicted DNA secondary structure and calculated ΔG free energy at each site.

cluding deletions, amplifications, and chromosomal rearrangements acting as fragile sites [Bacolla and Wells, 2004; Dillon et al., 2010, 2013; Zhao et al., 2010; Thys et al., 2015].

Fragile sites are regions of the genome that are susceptible to breakage under conditions of replication stress (related for instance to the stalling of the DNA replication fork) and have been correlated with a specific architecture of the chromatin; but they can be also induced by numerous chemotherapeutic and environmental chemicals (such as nicotine and aphidicolin) able to further stall the fork progression [Zlotorynski et al., 2003; Dillon et al., 2010; Thys et al., 2015]. In particular, it has been postulated that during DNA replication, some chemicals cause the uncoupling of the DNA helicase-topoisomerase complex from the replicative polymerase; such uncoupling may lead to longer stretches of single-stranded DNA at the replication fork, where DNA forms stable secondary structures that in turn can cause a replication fork to stall and collapse, so bringing about DNA breakages [Thys et al., 2015].

Several studies have now shown a strong correlation between the location of fragile sites and the presence of breakage in cancer-specific chromosome aberrations (in-cluding specific chromosomal translocations, deletions, and amplifications), strongly arguing that fragile sites are involved in recurrent chromosomal rearrangements, and this relationship is undoubtedly also true in animals [Dillon et al., 2010].

The search for fragile sites, performed with the DNA secondary structure prediction program Mfold, revealed the presence of 7 potential fragile sites with high ΔG free energy, located at the 3′ end of the *M. persicae* scaffold. Similarly, Mfold showed 3 potential fragile sites at the 3′ terminus of the *A. pisum* scaffold. The predictions of the Mfold program have been validated by Dillon et al. [2013] who assessed that energetically favourable free energy values as predicted by Mfold correspond to DNA secondary structure formation and fragile sites in human chromosomes.

This is the first search for fragile sites in aphids (and the first analysis in insects as far as we are aware of) and the preliminary results reported here suggest that the observed rearranged *M. persicae* karyotype, in particular for translocations and deletions involving autosomes 1 and 3, could be related to the presence to fragile sites. Indeed, a similar colocalization of fragile sites and genes deleted,

amplified, and rearranged has been well documented in human cancers [Popescu, 2003]. At the same time, the presence of fragile sites in *A. pisum* (where karyotype rearrangements have never been observed) argues that chromosomal breakages could be favoured by the presence of chemicals such as nicotine. This view is supported by the finding that the A1-A3 translocation has been frequently reported in red morphs within *M. persicae* populations feeding on tobacco, a plant that typically produces nicotine, a molecule already known to favour DNA replication fork stalling and DNA damages.

Our findings therefore seem to support the hypothesis that the aphid chromosomal/genomic architecture could be most probably the basis of the observed karyotype rearrangements. But a larger set of chromosomal markers is necessary to identify other chromosomal loci recurrently rearranged in order to verify the presence of fragile sites within these regions.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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