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Abstract

The resistance *Vat* gene of melon was isolated by map-based cloning. It encodes a protein with a coiled coil domain (CC), a nucleotide binding site (NBS) and leucine–rich repeats (LRR). Whereas most resistance genes confer resistance to a single pathogen, two alleles with different resistance specificities were identified at the locus. The *Vat* allele confers resistance to plant colonization by the aphid *Aphis gossypii* and to transmission of unrelated viruses by this vector specifically; the *Pm-W* allele confers resistance to the powdery mildew *Podosphaera xanthii*. Here we identified *Vat*-analogs in several melon genotypes using Long Range PCR, cloning and sequencing of full-length fragments. Several sources of variability were identified: (i) a different number of analogs were identified, suggesting a variable complexity of the gene cluster depending on the genotype, (ii) the number of repeats of a conserved motif of 65 amino acids within the LRR domain varied from two to five; this variation may be involved in the specific recognition of aphids or powdery mildew, (iii) finally, while the *Vat* allele seems to be shared by several aphid resistant accessions from various geographical origins, other alleles (genes) may be responsible for aphid resistance in some accessions; thus, a new putative allele was identified in the Indian accession 90625, which exhibits a resistance phenotype slightly distinct from that mediated by the *Vat* allele.

INTRODUCTION

The melon *Vat* gene is the second cloned resistance gene that mediates resistance to an aphid, the melon/cotton aphid *Aphis gossypii* (Kaloshian 2004). It encodes a protein which belongs to the coiled coil (CC) nucleotide binding site (NBS) - leucine-rich-repeat (LRR) family. The *Vat* gene was cloned by map-based cloning. Recombination events flanking the gene were screened using a 6000 plants back cross population and a physical map encompassing the gene was obtained by screening a melon BAC library. The *Vat* gene is 6 kb long, carries 5 exons and 4 introns and encodes a predicted 1473-amino acid protein, expected to be localized in the cytoplasm (Pauquet et al. 2004a, b). The *Vat* gene is a single functional locus, flanked at 17 kb by a paralogous gene, named *Vat-like*, not involved in the *Vat*-mediated resistance.

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Vat-mediated-aphid resistance is manifested by reduced feeding, fecundity, and survival. *A. gossypii* probe normally in the resistant melon leaves; they are able to insert their stylet intercellularly to access phloem tissue and initiate feeding. However, in a short time they stop feeding and withdraw their stylets. They finally die from starvation (Chen et al. 1997a, b). No hypersensitive reaction is detected in the resistant leaves after aphid feeding.

Besides the drastic effect towards aphid feeding, the *Vat* gene has the unique feature to trigger a resistance to transmission of unrelated viruses (*Cucumber mosaic virus* and Potyviruses such as *Zucchini yellow mosaic virus* and *Papaya ringspot virus*), when transmitted by the aphid species *A. gossypii,* specifically (Pitrat and Lecoq 1980; Lecoq et al. 1980). This double function mediated by the resistance *Vat* allele was demonstrated by map-based cloning - recombinant plants flanking the gene were either resistant or susceptible, both to aphid colonization and to virus transmission - and by complementation analysis using transgenic melon plants (Pauquet et al. 2004a and b; Pech et al. 2007).

A resistance gene usually confers resistance to a single pathogen or even to a single strain of this pathogen. Surprisingly we showed, using the same map-based cloning strategy, that the *Pm-W* gene, which confers resistance to the fungus *Podosphaera xanthii*, a major pathogen responsible for powdery mildew in melon, is a true allele of *Vat*. The *Pm-W* allele encodes a predicted 1538 amino-acid protein and shares a very similar sequence with the *Vat* allele (Dogimont et al. 2007).

The *Vat* allele was cloned from the Korean accession PI 161375. Several other sources of resistance to *A. gossypii* were reported in melon (Bohn et al. 1973 and 1996; Pitrat and Lecoq 1980; Pitrat et al. 1988; Soria et al. 2000 and 2003; Boissot et al. 2000 and 2008). Using small segregating populations, aphid resistance was shown to be located at the *Vat* locus in the Spanish accessions 'Anso' and 'Invernizo' and in the Indian accession PI 414723 (Pitrat et al. 1988; Klingler et al. 2001). Different phenotypes of resistance were observed on a set of melon accessions according to *A. gossypii* genotypes (Boissot et al. 2008).

Here we present data on the molecular diversity available at the *Vat/Pm-W* locus in a collection of melon accessions.

MATERIAL AND METHODS

Plant material

The diversity of *Vat* analogs was studied in four melon accessions: WMR 29, from which the *Pm-W* gene was isolated, which confers the resistance to races 1, 2 and 3 of *P. xanthii* is susceptible to aphids; the Indian accessions PI 414723 and 90625 are both resistant to *A. gossypii* and to the races 1, 2, 3, and 5 of *P. xanthii.* 'Védrantais' is a Charentais type variety (Vilmorin release), susceptible to both aphids and powdery mildew.

A collection of 31 melon accessions from various geographic origins and which exhibit resistance to *A. gossypii,* was studied for the presence of the *Vat* allele.

Phenotypes

Melon accessions were evaluated for their resistance to *A. gossypii* using four aphid clones belonging to the two main genotypic groups of clones (NM1 and C9) (Boissot et al. 2008).

PCR amplification of *Vat* analogs

Full-size *Vat* analog sequences were obtained from several melon accessions by amplification of a 6.5 kb genomic DNA fragment by Long Range-PCR (Sanchez and Bradeen 2006), using primers anchored in non-coding flanking regions of the *Vat* gene and designed to amplify the entire gene sequence. Primers amplified both *Vat* and *Vat-like* genes from PI 161375 DNA. DNA amplification was performed using a high fidelity DNA polymerase system, Takara LA Taq TM polymerase (Takara Bio Inc, Japan), which enables to amplify fragments of 6 kb. Amplified fragments were cloned. Clones were screened and selected on the basis of their restriction patterns. PCR primers pairs were designed along the gene to amplify partial fragments of the gene. The full-size sequence of each individual clone was obtained by aligning partial overlapping sequences. Sequence alignments and comparisons were performed using ClustalW software.

RESULTS

Comparison of the sequence of *Vat, Vat-like* and *Pm-W*

The full-length sequence of the genes *Vat, Vat-like* and *Pm-W* was obtained by map-based cloning, from a BAC clone from PI 161375 for *Vat* and *Vat-like* and from a BAC clone from WMR 29 for *Pm-W*.

The *Vat* gene encodes a 1473 amino acid predicted protein, comprising 6 domains (Tab. 1a). The CC domain comprises 175 amino acids and the NBS domain 342 amino acids. The C-terminal region is made up of 21 highly imperfect copies of a LRR motif containing 20-30 amino acids per repeat (LRR1 and LRR2 domains), interrupted by four near-perfect repeats of 65 amino acids (domain D). The four 65 amino acid repeats show from 83.1 to 89.2 % of identity between each other and each of them comprises two typical LRR motifs. The C-terminal domain comprises 52 amino acids.

The *Vat-like* gene shares a very similar structure with *Vat* but encodes a shorter protein of 1410 amino acids. The CC and the NBS domains are highly conserved (>99 % identity). *Vat* and *Vat-like* differ slightly in the LRR1 and LRR2 domains (89.8 and 85.7 % identity). The most striking difference between both of them is a reduced size of the *Vat-like* domain D, which lacks one repeat of 65 amino acids. Otherwise, the nucleic and protein sequence of three overlapping repeats are perfectly conserved between *Vat* and *Vat-like* (100 % identity).

The *Pm-W* gene encodes a 1538 amino acid protein, very similar to *Vat* in the CC and NBS domains, more divergent in the LRR1 and LRR2 domains (87.5 % protein identity). Strikingly, the domain D comprises five repeats of 65 amino acids.

Analogs of *Vat* in the three melon accessions, WMR 29, PI 414723 and 'Védrantais'

Using Long-range PCR, we amplified and obtained the full-length sequence of a *Vat* analog from the melon line WMR 29, from which the *Pm-W* gene was isolated. The analog gene, 5722 nucleotides long, is predicted to encode a 1410 amino acid protein, the same size as *Vat-like* and shares 98.9 % protein identity with *Vat-like*. The 195 amino acids of the domain D are 100 % identical to *Vat-like* (Tab. 1b).

Table 1. Molecular diversity of full-length sequence of analogs of the *Vat* gene from four melon genotypes.
a) Comparison of *Vat* analogs with the *Vat* gene and the predicted encoded-Vat protein, domain by domain.
b) Comparison of an analog sequenced from WMR 29 with the Vat-like gene and the predicted Vat-like protein, domain by domain.

(a)	**Nucleic size**	**Protein size**	**Nucleic identity**	**Protein identity**	**Protein identity by domain**						
					CC	**NBS**	**LRR1**	**D**		**LRR2**	**C-term**
Vat	**5896 bp**	**1473 aa**	**-**	**-**	**175 aa**	**342 aa**	**295 aa**	**260 aa**		**349 aa**	**52 aa**
Vat-like	5725 bp	1410 aa	93.8[x]	89.9 (82)[y]	99.4 (1)	99.7 (1)	89.8 (30)	**195 aa**	100 (0)[z]	85.7 (50)	98.1 (1)
Pm-W	6072 bp	1538 aa	93.1	87.5 (129)	99.4 (1)	90.4 (33)	88.8 (33)	**325 aa**	87.3 (33)	93.1 (24)	90.4 (5)
An_WMR 29	5722 bp	1410 aa	93.7	89.8 (86)	99.4 (1)	98.8 (4)	89.5 (31)	**195 aa**	100 (0)	85.7 (50)	100 (0)
An_PI 414723_1	5897 bp	1473 aa	99.8	99.6 (6)	100 (0)	99.7 (1)	99.0 (3)	**260 aa**	100 (0)	99.4 (2)	100 (0)
An_PI 414723_2	6093 bp	1538 aa	95.9	93.5 (34)	99.4 (1)	93.9 (21)	96.9 (9)	**325 aa**	99.6 (1)	99.4 (2)	100 (0)
An_PI 414723_3	6018 bp	1540 aa	92.1	91.0 (71)	99.4 (1)	93.9 (21)	96.9 (9)	**325 aa**	99.6 (1)	88.9 (39)+2aa	98.1 (1)
An_PI 414723_4	5487 bp	1345 aa	90.5	84.9 (99)	100 (0)	91.2 (30)	92.6 (22)+2aa	**130 aa**	83.8 (21)	93.4 (23)	94.2 (3)
An_Védrantais_1	4852 bp	1345 aa	78.9	83.6 (117)	98.3 (3)	90.6 (32)	90.6 (28)	**130 aa**	92.3 (10)	87.7 (43)	98.1 (1)
An_Védrantais_2	4852 bp	1345 aa	79.4	84.9 (96)	99.4 (1)	95.9 (14)	91.2 (26)	**130 aa**	91.5 (11)	87.4 (44)	100 (0)
An_90625	5802 bp	1478 aa	93.8	92.3 (107)	99.4 (1)	94.2 (13)	98.0 (6)+2aa	**260 aa**	89.2 (28)	84.9 (53)+2aa	88.5 (6)
(b)											
Vat-like	**5727 bp**	**1410 aa**	**-**	**-**	**175 aa**	**342 aa**	**295 aa**	**195 aa**		**349 aa**	**52 aa**
An_WMR 29	5722 bp	1410 aa	99.6	98.9 (15)	100 (0)	99.4 (3)	98.3 (5)	195 aa	100 (0)	98.3 (6)	98.1 (1)

[z] % of protein identity on the overlapping repeats
[y] % of protein identity in comparison with the Vat predicted protein (a), with the Vat-like predicted protein (b); in brackets, the number of divergent amino acids
[x] % of nucleic identity in comparison with *Vat* (a) with *Vat-like* (b)

Using Long range PCR on PI 414723 DNA, fragments of different sizes were amplified. Four analogs were cloned and fully sequenced. The sequence of the analog 1 is 5897 nucleotides long and shares 99.8 % identity with the *Vat* allele. It encodes a predicted protein of 1473 amino acids, which has the same size as the predicted Vat protein and differs from it by only 6 amino acids, none of them in the domain D. Analogs 2 and 3 encode a predicted protein of 1538 and 1540 amino acids, respectively; both have a domain D with five repeats of 65 amino acids. Analog 4 encodes a predicted protein of 1345 amino acids, which corresponds to the presence of two repeats of 65 amino acids in the domain D.

Two analogs were cloned and fully sequenced from DNA of the aphid and powdery susceptible line 'Védrantais'. They both encode a predicted protein of 1345 amino acids, exhibit two repeated motifs in the D domain and a large deletion within the second intron.

Presence of the *Vat* allele in a melon collection

Primers flanking the domain D from the *Vat* allele were designed and used to amplify DNA of 31 melon accessions, shown in our lab or reported in the literature to exhibit aphid resistance. PCR products of the same size as that expected for the *Vat* allele were obtained from 20 of the 31 aphid-resistant accessions tested (Tab. 2). Sequences of the amplified fragment were 100 % identical to *Vat* in all the accessions except in two of them; AR 5 had one nucleotide difference, which changes the encoded amino acid and SVI 0105 had one silent difference. Eleven aphid resistant accessions did not amplify any PCR product using the primers designed on the *Vat* allele. These accessions originated from various parts of the world, from Spain ('Anso 77', 'Escrito 8429', 'Invernizo 8427', 'Malaga AN-C-22', and 'Negro'), Yugoslavia ('Persiski BR5'), India (90625 and PI 164723) or Africa ('Fegouss 1', PI 224770 and PI 282448). We can conclude that they don't carry the *Vat* allele. Aphid resistance in these accessions is likely based on a different allele at the *Vat* locus or an independent aphid resistance gene.

A *Vat* putative allele in the aphid resistant accession 90625

Among the aphid resistant accessions, which did not amplify the *Vat* allele, the Indian accessions 90625 was chosen because it exhibits a phenotype different from that mediated by the *Vat* allele. 90625 is highly resistant to the colonization and virus transmission using NM1 *A. gossypii* clones but is susceptible to both colonization and virus transmission using C9 clones (Boissot et al. 2008). Using Long Range PCR, *Vat* analogs were cloned and sequenced from DNA of 90625. An analog was identified which has a similar size as *Vat* (5802 bp). It encodes a protein of 1478 amino acids, which shares 92.3 % identity with Vat. The domain D comprises four repeats of 65 amino acids, which share 89.2 % identity with Vat (Tab. 1a).

DISCUSSION

We previously identified two functional alleles at the melon *Vat/Pm-W* locus, one of them (*Vat*), which confers resistance to aphid colonization and to virus transmission by this specific aphid and the second one (*Pm-W*), which confers resistance to powdery mildew. Here, we identified *Vat* analogs in various melon genotypes.

Table 2. PCR amplification of the domain D of the *Vat* allele in 31 *A. gossypii* resistant melon accessions from various geographic origins. Sequence and % of identity with the Vat-encoded protein.

Accessions	Geographic origin	Size of the domain D	% protein identity
AR Hale's Best Jumbo	USA	260 aa	100 %
Chenggam	Korea	260 aa	100 %
Durgapura Madhu	India	260 aa	100%
Ginsen Makuwa	Japan	260 aa	100%
K5442	China	260 aa	100%
Kanro Makuwa	Japan	260 aa	100%
Margot	France	260 aa	100%
Meloncillo	Columbia	260 aa	100%
Miel Blanc	China	260 aa	100%
PI 164320	India	260 aa	100%
PI 164323	India	260 aa	100%
PI 255478	Korea	260 aa	100%
PI 266935	Japan	260 aa	100%
PI 414723	India	260 aa	100%
PI 482420 (TGR 1551)	Zimbabwe	260 aa	100%
Shiro Uri Okayama	Japan	260 aa	100%
Shirokawa Nashi Makuwa	Japan	260 aa	100%
SVI 0105	unknown	260 aa	100%
Virgos	France	260 aa	100 % (1 synonymous SNP)
AR 5	USA	260 aa	99.6 % (1aa change)
Anso 77	Spain	NA[z]	
Escrito 8429	Spain	NA	
Fegouss 1	Morocco	NA	
Invernizo 8427	Spain	NA	
Malaga AN-C-22	Spain	NA	
Negro	Spain	NA	
Persiski BR5	Yugoslavia	NA	
PI 164723	India	NA	
PI 224770	South Africa	NA	
PI 282448	South Africa	NA	
90625	India	NA	

[z] not amplified (NA)

Three major sources of variability were identified in *Vat* analogs, the complexity of the cluster of resistance genes, the number of repeats of a conserved motif within the LRR domain and putative allele variability.

The *Vat* gene was shown to be closely linked to the paralog *Vat-like* (17 kb), which may result from a duplication of the *Vat* gene. Here we showed that the accession WMR 29, from which the allele *Pm-W* was isolated, carries an analog 98.9 % identical to *Vat-like*. This suggests that the structure of the cluster is highly conserved between these two unrelated genotypes, PI 161375 (*Vat, Vat-like*) and WMR 29 (*Pm-W,* '*Vat-likeW'*). In contrast, four distinct analogs were identified in the Indian accession PI 414723 suggesting major rearrangements of the cluster in this accession. Variation in gene number and rearrangements events throughout a species has been shown in several R gene loci and gene duplication is assumed to be a major driving force for gene functional diversification (Baumgarten et al. 2003; Caicedo and Schaal 2004; Seah et al. 2004).

All the identified analogs exhibited a high percentage of identity with the Vat predicted protein (>80 %). The CC domain was highly conserved in all of them (>98 %). They mainly differed within the LRR region, which was shown to determine recognition specificity of several R proteins (Ellis et al. 2007). The main factor of divergence between *Vat* analogs was the size of the predicted proteins, which differ by the number of repeats of a conserved motif of 65 amino acids within the LRR domain: four repeats in *Vat* from PI 161375 and PI 414723 and in the putative *Vat* allele from 90625; five repeats in *Pm-W* and in two analogs from PI 414723, three repeats in *Vat-like*, the paralog of *Vat,* which does not confer aphid resistance neither any known resistance and in the *Vat-like* analog from WMR 29; finally two repeats in both analogs identified from the susceptible 'Védrantais' and an analog from PI 414723. In several NBS-LRR genes, variation in the number of LRR-coding units appears to be a major factor of variation between alleles or paralogs within a cluster (Caicedo and Schaal 2004). At the *Vat/Pm-W* locus, results obtained suggest that the number of repeats of a conserved motif within the LRR domain may play a major role in the specific recognition of aphids or powdery mildew. Functional analysis of analogs is needed to confirm this hypothesis.

We showed that the *Vat* allele is likely present in 20 of the 31 aphid resistant melon accessions tested. Among them, PI 414723, PI 482420 were previously described to exhibit a phenotype similar to that mediated by the *Vat* allele; slight phenotypic differences reported (Garzo et al. 2002; Soria et al. 2003) may result from other genetic factors (QTL). The *Vat* allele seems to be present in accessions from various geographic origins, from East Asia as well as from Africa and South America. In French ('Margot', 'Virgos') and American varieties ('AR Hales's Best Jumbo', 'AR 5'), the *Vat* allele was introgressed from PI 161375 and PI 414723, respectively. Among these 20 accessions, 14 were studied for resistance to NM1 and C9 genotypes of *A. gossypii*. All of them have the common feature to exhibit resistance to colonization by both NM1 and C9 aphids and to be resistant to virus transmission by both clones (Boissot et al. 2008). Our results also suggest that the *Vat* allele is absent from 11 of the aphid resistant accessions tested. Among them, seven accessions, which were phenotyped with both aphid genotypes, have the common feature to be susceptible to virus transmission by *A. gossypii* clones of the C9 genotype (Boissot et al. 2008). In the Indian accession 90625, a new aphid resistance putative allele was identified, which presents 92.3 % of identity with the *Vat* encoded protein. Preliminary results suggest that this allele is not present in the other nine accessions.

They may carry distinct alleles of resistance and new alleles may be identified in these resistance sources.

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