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Sequences of the 3′ halves of the genomes of barley yellow dwarf virus-PAV cpA isolates that vary in symptom severity

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Abstract

Barley yellow dwarf virus (BYDV)-PAV isolates from USA have been separated into two distinct clusters (Chay et al. (1996) Virology 219: 57–65; Chay et al. (1996) Phytopathology 86: 370–377). Following this finding we have shown that BYDV-PAV is divided into two groups cpA and cpB based on their coat protein gene sequence, and distinct host preferences (Mastari et al. (1998) Phytopathology 88: 818–821). We have sequenced the complete 3′ half of the genomes of two lethal and two mild cpA isolates and compared them with those of several known PAV cpA isolates to assess variability and locate potential determinants of severity. Open reading frames (ORFs) 3, 4, 5, 6 and the 3′ untranslated regions had different percent homologies between isolates: ORF5 (92–97%), ORF3 (88–98%) 3′-translational enhancer (87–100%) ORF4 (85–99%), 3′ untranslated region (72–97%) and ORF6 (61–99%). In contrast to the mild isolates, the field-lethal isolates (FHv1 and FHv2) fell into the same cluster, regardless of the genomic region analysed. The isolates FHv1 and FHv2 differed from mild isolates by eight amino acid substitutions in ORFs 3 and 4, and insertions in ORF5. Four amino acid substitutions in the 17-kDa protein encoded by ORF4 caused a change in local net charge in the field-lethal isolates. Two insertions of four amino acids were identified in the C-terminal half of ORF5 of the field-lethal isolates, but were not present systematically in all lethal isolates analysed. The potential relationships of these differences in predicted amino acid sequences to disease severity are discussed.

Introduction

The barley yellow dwarf (BYD) disease was first identified by Oswald and Houston (1951). It is associated with at least five viruses (PAV, MAV, RPV, RMV and SGV) of the luteovirus group which has been elevated to the *Luteoviridae* family including two genera (D'Arcy and Mayo, 1997). PAV and MAV belong to the *Luteovirus* genus and are referred as BYDVs. The RPV is called cereal yellow dwarf virus and belongs to the *Polerovirus* genus. The SGV and RMV are still not assigned. These single-stranded positive RNA viruses are limited to the phloem and cause yellowing, reddening, and/or stunting of infected plants. They are specifically transmitted by aphid in a circulative, non-propagative manner (Mayo and Ziegler-Graff, 1996). BYD disease causes large yield losses in cereals, and PAV is the most prevalent and globally distributed BYDV (Lister and Ranieri, 1995). It is transmitted mainly by three aphids species: *Rhopalosiphum padi* L., *Sitobion avenae* (Fabr.) and *Metopolophium dirhodum* (Walker). The BYDV-PAV genome is organised into six open reading frames (ORFs). ORF2 encode an RNA-dependent RNA polymerase protein (Mayo and Ziegler-Graff, 1996). ORFs 3, 4, 5, and 6 encode the 22-kDa coat protein, the 17-kDa protein implicated in systemic infection and intracellular movement (Chay et al., 1996b; Nass et al., 1998), the 50-kDa readthrough protein required for transmission (Chay et al., 1996b) and the 6.7-kDa putative protein (Miller et al., 1995), respectively.

Biological variants of BYDV-PAV have different capacities for transmission by *M. dirhodum* (Gildow and Rochow, 1983), different degrees of severity in barley (Chalhoub, 1994) and oats (Chay et al., 1996a), and/or distinct genetic and serological properties (Chalhoub et al., 1994; Chay et al., 1996a). Following the finding of Chay et al. (1996a), we have shown that BYDV-PAV is divided into two groups cpA and cpB based on their coat protein gene sequence, and distinct host preferences (Mastari et al., 1998). The present study was carried out to evaluate the differences in the 3′ half of the genome (ORFs 3, 4, 5, 6 and 3′-terminal regions) of BYDV-PAV group cpA isolates collected in different geographical areas. These isolates included lethal and mild isolates, enabling us to assess molecular variability of PAV group cpA and to identify potential determinants of severity.

Materials and methods

Disease severity

Non-viruliferous *Rhopalosiphum padi* were allowed to feed for 48 h on detached leaves of a barley genotype BYDV-susceptible (cultivar Plaisant) infected by different PAV isolates. These isolates were collected from a field of cultivar Plaisant at INRA Versailles (France) and were associated with different disease severities. Aphids were transferred to healthy seedlings (one leaf stage) of the cultivar Plaisant. Five days later, aphids were removed and the plants were maintained in a growth chamber for 30 days at 17 °C with 16 h of fluorescent light per day.

PAV isolates analysed

The references of isolates are listed in Table 1. The isolates FHv1, FHv2, FHm1, FHm2, and FH4 were collected from a barley (cultivar Plaisant) trial field. Isolates FL2 through FL9, previously reported by Mastari et al. (1998), were collected from ryegrass (Table 1).

Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) and direct sequencing

The optimal sequences for primer generation (Mj1–Mj8) were determined by the Prime program of the GCG software package (Table 2). Virions were recovered from homogenised leaf tissue by immunocapture in anti-PAV antibody-coated microtitre plates and subjected to two freeze–thaw cycles to expose the viral RNA (Wyatt et al., 1993). RNA was RT-PCR amplified as follows. The primer combinations, (P3, Lu4) and (M2, Z2) were used as described by Chay et al. (1996a) and Chalhoub (1994), respectively. Primers Mj1–Mj8 were used as follows: first strands were reverse transcribed from RNA using 4 pmol reverse primers (Table 2), buffer (250 mM Tris-HCl, pH 7.5; 0.2 M NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, and 50% glycerol,) (Promega), dNTPs (5 mM), water and 10 units of M-MLV reverse transcriptase (Promega). The mix was incubated at 42 °C for 1 h. Partial cDNAs obtained were amplified by PCR using 6 pmol reverse and sense primer, buffer (100 mM Tris-HCl pH 8; 500 mM KCl; 1% Triton X100) (Eurobio), dNTPs (5 mM), $MgCl_2$ (50 mM), water and 10 units of Taq DNA polymerase (Eurobio), and incubated in a Perkin-Elmer cycler programmed: initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 50–60 °C (depends on primer combination used), 1 min 30 s at 72 °C. The final elongation step was at 72 °C for 10 min.

Sequencing procedure

PCR products were purified using the JetSorb kit (GenoMed, Beverly Hills, CA). The sequencing reactions and analyses were carried out using Dye Terminator Cycle sequencing Kit (Perkin-Elmer/Applied Biosystems Inc.) in an automated Applied Biosystems 377 DNA sequencer.

Phylogenetic and sequence analyses

Sequences were analysed using ClustalW (1.7) software (Higgins and Sharp, 1989). The bootstrap option of ClustalW was used to assess the relationship between isolates. Physico-chemical parameters of protein sequences were determined using Expasy tools: Protparam (Kyte and Doolittle, 1982) and SAPS (Brendel et al., 1992).

Results

Disease severity

Thirty days after inoculation of the susceptible barley cultivar Plaisant, PAV cpA isolates studied

Table 1. References and symptom type of PAV group cpA isolates

Isolate name	Country of origin	Genomic region compared	Symptom type[2]		Database accession number of authors
			Field	Laboratory	
FHm1	France	ORF3/4/5/6, the 3′untranslated region	Mild	Mild	AJ223587
FHm2	France	ORF3/4/5/6, the 3′untranslated region	Mild	Mild	AJ223588
FHv1	France	ORF3/4/5/6, the 3′untranslated region	Lethal	Lethal	AJ007491
FHv2	France	ORF3/4/5/6, the 3′untranslated region	Lethal	Lethal	AJ007492
FH4	France	ORF3/4	n.d.	Lethal	Mastari et al. (1998)
FL4	France	ORF3/4, ORF6	n.d.	Lethal	Mastari et al. (1998)
FL5	France	ORF3/4	n.d.	Lethal	Mastari et al. (1998)
FL7	France	ORF3/4	n.d.	Lethal	Mastari et al. (1998)
FL8	France	ORF3/4	n.d.	Lethal	Mastari et al. (1998)
FL9	France	ORF3/4	n.d.	Lethal	Mastari et al. (1998)
FL6	France	ORF3/4	n.d.	Mild	Mastari et al. (1998)
FL2	France	ORF3/4	n.d.	Mild	Mastari et al. (1998)
FL3	France	ORF3/4	n.d.	Mild	Mastari et al. (1998)
FH3	France	ORF3/4	n.d.	Mild	AJ223589
Jpn	Japan	ORF3/4/5/6, the 3′untranslated region	n.d.	n.d.	D85783
NY	USA	ORF3/4	n.d.	n.d.	X56050
P	USA	ORF3/4/5/6, the 3′untranslated region	n.d.	n.d.	D11032
Vic	Australia	ORF3/4/5/6, the 3′untranslated region	Mild	Mild	X07653, Chalhoub (1994)
2t[2]	France	ORF6, the 3′untranslated region	Lethal	Lethal	Chalhoub (1994)
3b[2]	France	ORF6, the 3′untranslated region	Mild	Mild	Chalhoub (1994)
cloutier[2]	Canada	ORF6, the 3′untranslated region	Lethal	Lethal	Chalhoub (1994)
13t[2]	France	ORF6, the 3′untranslated region	Mild	Mild	X80050
RG[2]	France	ORF6, the 3′untranslated region	Lethal	Lethal	Chalhoub (1994)

[1]These isolates have been allocated to PAV group cpA following the RFLP and SSCP analyses reported in Mastari et al. (1998).
[2]Laboratory assessment of symptoms induced by FL2-FL9 and FH3-FH4 was carried out as for FHv1, FHv2, FHm1 and FHm2 isolates.

Table 2. Primers used for RT-PCR and direct sequencing

Primer designation[1]	Primer sequence	Position[2]
P3	GGTGAAATGAATTCAGTAGG	2858–2871
Lu4	GTCTACCTATTTGG	3455–3468
Mj1	CAGAGGCAATTAATGGG	3321–3337
Mj2	TCGTTTATCCAGTGCC	3753–3768
Mj3	CTGGCACTGGATAAACG	3770–3754
Mj4	TTCGTTCTGCCTCAAC	4187–4171
Mj5	ACGAATAACAAGGCACG	4198–4214
Mj6	ACCCAAGGAACCTGAAG	4681–4665
Mj7	TTGCCACTCTTCTTTGG	4681–4665
Mj8	AAACGGCGATAACGTG	4932–4917
M2	CGGTTCATAAGCTCGGGTAGGC	4862–4883
Z2	CCATGAGAATTGCGACTGTGAGC	5635–5657

[1]P3 and Lu4, were designed by Chay et al. (1996) and Robertson et al. (1991), respectively. M2 and Z2 were used by Chalhoub (1994). The primer couples used are as follows (P3, Lu4), (Mj1, Mj2), (Mj3, Mj4), (Mj5, Mj6), (Mj7, Mj8), (Mj1, Mj4), (Mj3, Mj6), (Mj5, Mj8), (M2, Z2) and (Mj5, Z1).
[2]PAV-Vic (Miller et al., 1988) complete sequence is used as reference.

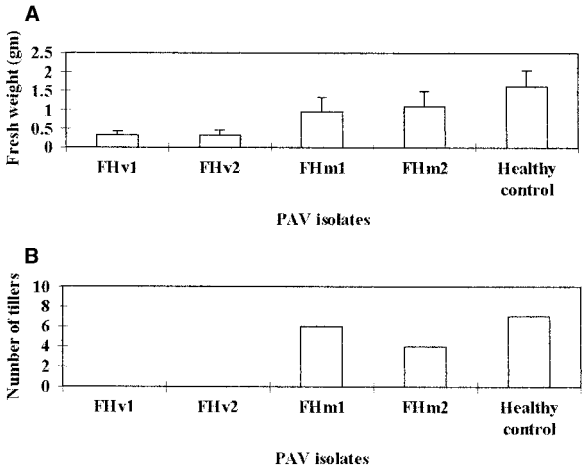

Figure 1. Effects of PAV cpA isolates on a BYDV-susceptible barley cultivar. Fresh shoot weight (A) and number of tillers (B) were determined at 30 days after inoculation in the growth chamber (means of 8 plants).

(FHv1, FHv2, FHm1 and FHm2) expressed distinctly different symptoms that were consistent with those observed in field conditions. The FHv1 and FHv2 isolates caused a great reduction in fresh weight. In contrast, the fresh weight of plants infected with FHm1 and FHm2 was slightly reduced (Figure 1A). Contrary to FHm1 and FHm2 isolates, FHv1 and FHv2 isolates inhibited the development of tillers (Figure 1B). Fifty to 70 days after inoculation, FHv1 and FHv2 isolates killed the plants.

Amino acid sequence analyses of ORF3, ORF4 and ORF5

Eighteen ORF3 amino acid sequences of PAV group cpA isolates with various severities were compared (Table 1). Of these, 16 had 90–98% similar amino acids (the lowest similarity (90%) occurred between FHm1 and FHm2 isolates) and fell in the cluster named $ORF3_1$ (Figure 2A). FHv1 and FHv2 were close (97% amino acid similarity) and fell in the cluster named $ORF3_2$ (Figure 2A). The amino acid similarity between clusters $ORF2_1$ and $ORF3_2$ ranged from 88% to 94%. As the whole cpA group, $ORF3_1$ and $ORF3_2$ clusters are distinguishable from cpB group by specific amino acid sequence at positions 51–58, amino acid identity from 82% to 88% and asymmetrical distribution between host plant species (Mastari et al., 1998).

Alignment of coat protein sequences showed that most amino acid changes were located in the

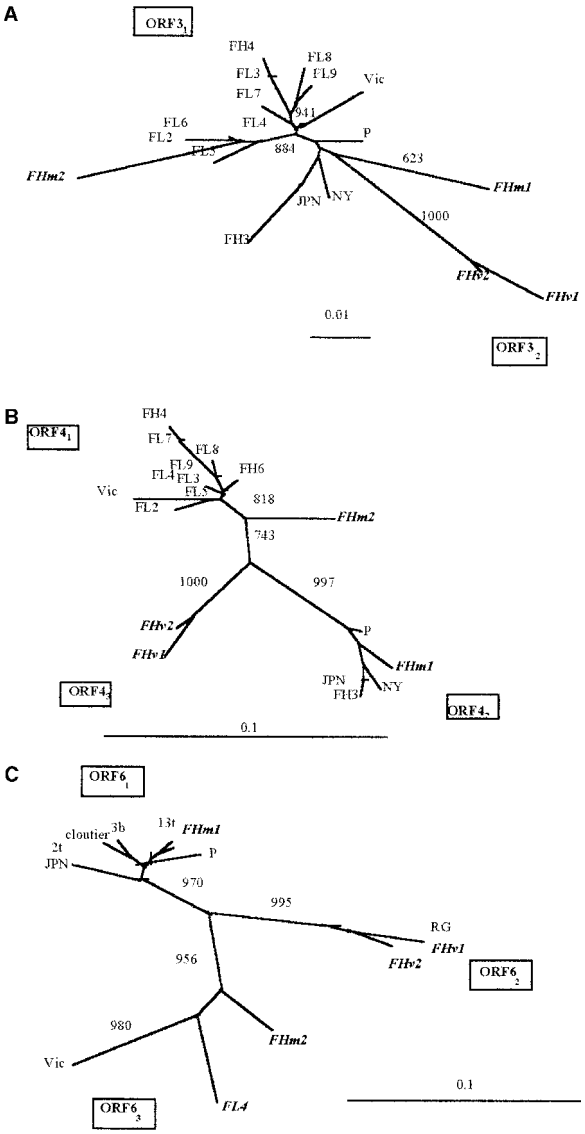

Figure 2. Unrooted phylogenetic trees describing relationships of amino acid sequences from ORF3 (A), ORF4 (B) and ORF6 (C). Cluster names are boxed. The newly sequenced isolates are in bolded italic. The scale representing nucleotide substitutions per position is shown. Only bootstrap values over 500 are indicated on different branches.

C-terminal part (Figure 3). There were eight amino acid substitutions specific to the two field-lethal isolates (FHv1 and FHv2) (Figure 3). Two of these substitutions alter the local net charge in the C-terminal part of the coat protein. Tyrosine (Y) at residue 156 in the lethal isolates was replaced by glutamine (E) in the mild ones, and tryptophan (W) at residue 165 was

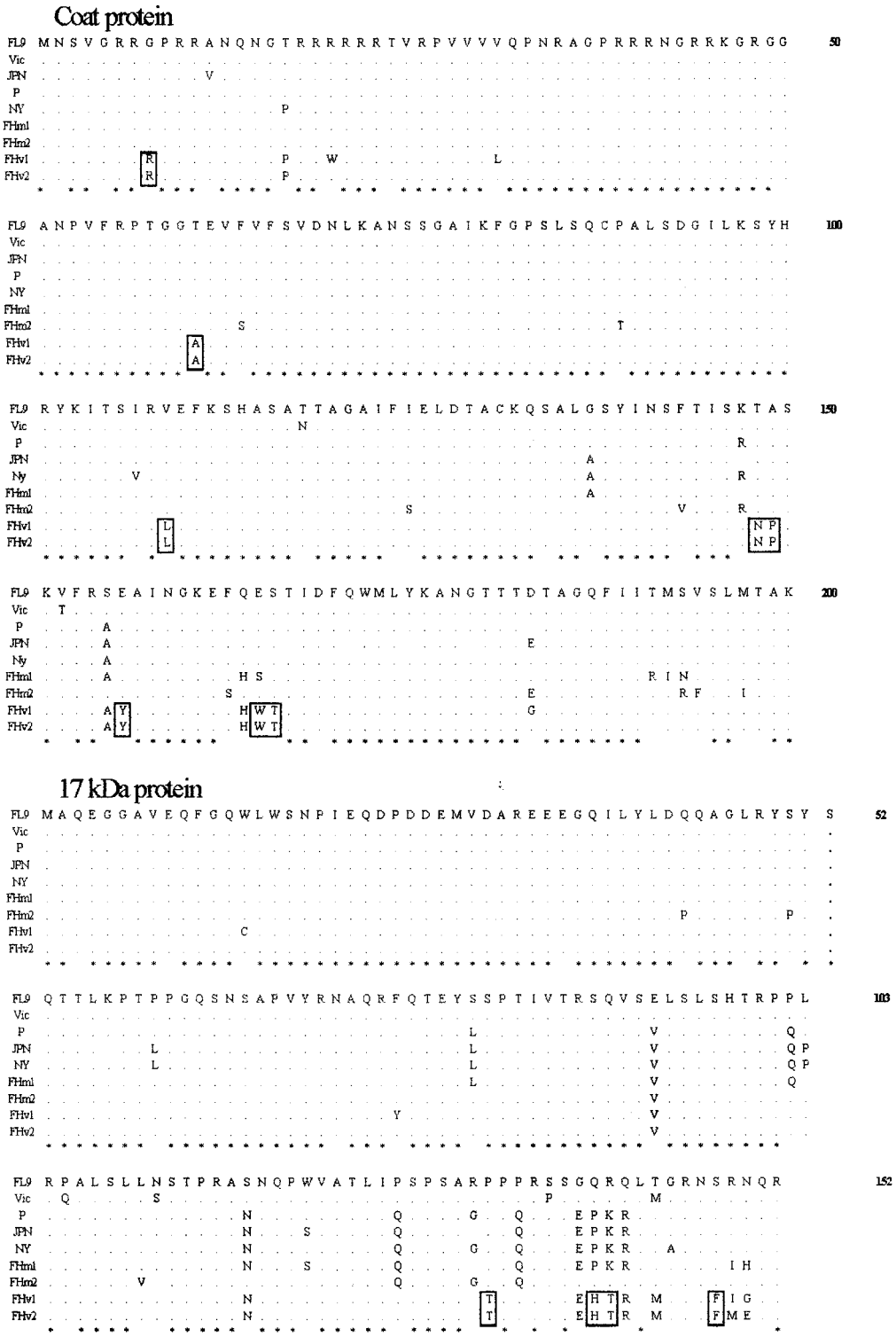

Figure 3. Multiple alignment of amino acid sequence of coat protein and 17-kDa protein of the PAV cpA group. The amino acids conserved only in FHv1 and FHv2 isolates are boxed. The '∗' designated conserved residue in the 18 isolates compared.

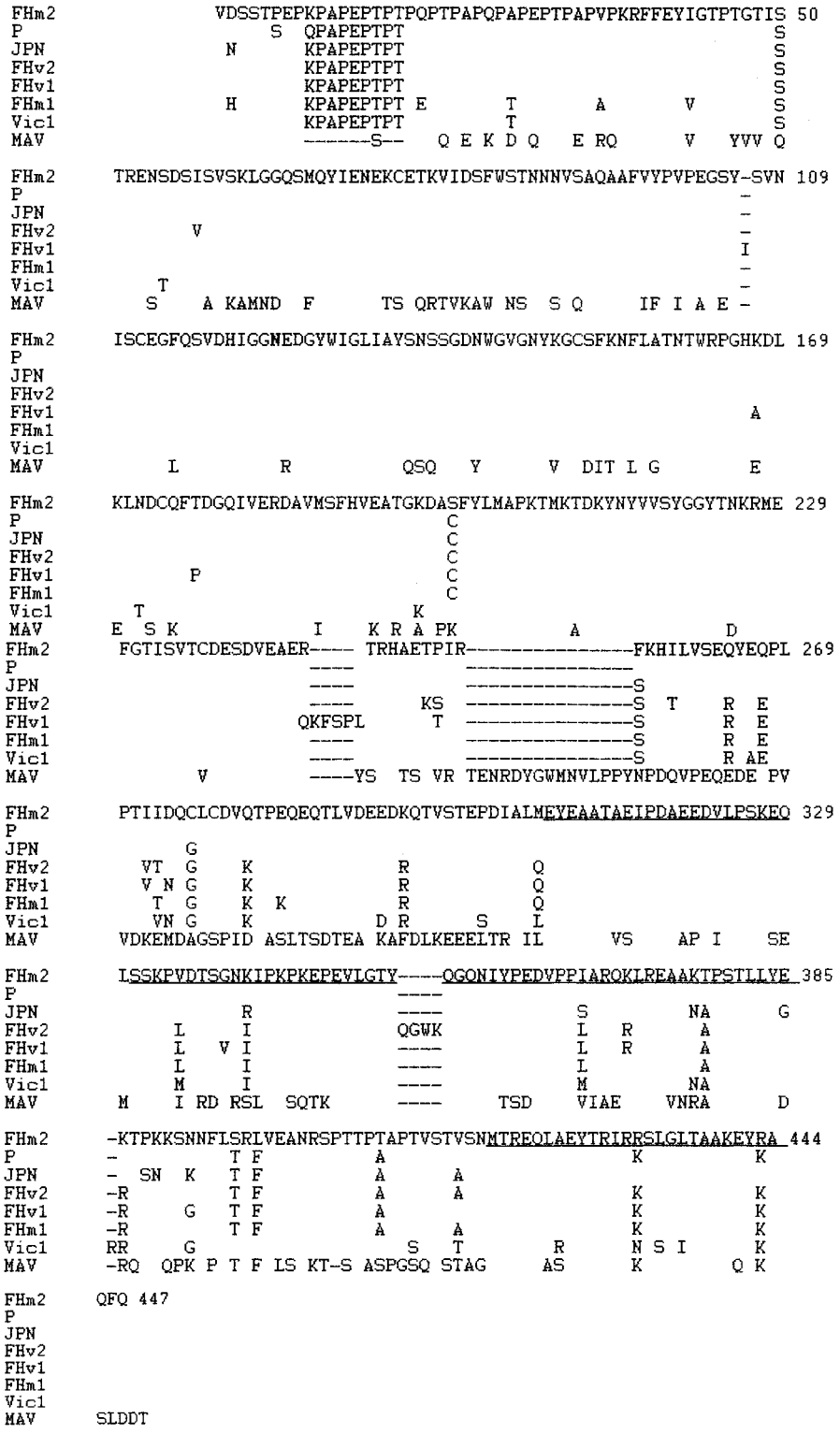

Figure 4. Multiple alignment of the amino sequences of the readthrough protein. Sequences conserved between PAV and MAV in the C-terminal part are underlined. Amino acids different from the top line are depicted.

replaced by glutamine (E) in the mild ones. The local net charge was calculated to be +1 and −1 or −2 for lethal and mild isolates, respectively when −1 is given to glutamine residue.

Phylogenetic analysis of the ORF4 amino acid sequences, revealed three major clusters (Figure 2B), where amino acid similarities were 91–99%, 96–98% and 96%. The amino acid similarities between clusters were 85–90%. Alignment of the deduced amino acid sequences of the ORF4-protein, showed that the C-terminal part varied more than the N-terminal part (Figure 3). Four amino acid substitutions specific to field-lethal isolates, were found between residues 134 and 149. There were no apparent changes in local net charge in this region, but the hydropathicity index was about 0.77 and 0.90 for field-lethal and mild isolates, respectively.

Phylogenetic analysis of the ORF5 amino acid sequences revealed three clusters but these were not supported by bootstrap data (data not shown). Amino acid sequence alignment of the encoded protein showed that 15 amino acid changes occurred in the 5′ half against 30 in the 3′ half (Figure 4). Two inserts were identified in isolate FHv1, one amino acid was inserted after residue 107, and four amino acids were inserted after residue 247. Isolate FHv2 had an insert of four amino acids after residue 357. Specific primers corresponding to the FHv1 and FHv2 insertions were used to determine frequency of isolates containing these insertions. Two-fifths (4/10) of the lethal isolates tested had one or both insertions, but none of the mild isolates had inserts (Table 3).

Table 3. Comparison of insertion type in PAV cpA and cpB isolates and severity of disease

Number of PAV isolates tested	Disease severity	PAV group	Insertion type
2	Lethal	cpA	FHv1
1	Lethal	cpA	FHv2
1	Lethal	cpA	FHv1 + FHv2
6	Lethal	cpA	0
10	Mild	7 cpA and 3 cpB	0

Nucleotide sequences analyses of the 3′ terminal region

The untranslated region between ORF5 and ORF6 (called 3′ TE for translation enhancer), was shown to confer efficient translation of PAV uncapped mRNA (Wang et al., 1997). The nucleotide identity in 3′ TE was 87–100%, 87% correspond to the similarity level between FHv1, FHv2 and the rest of the isolates. Nucleotide sequence alignment showed great sequence conservation between the most PAV cpA isolates (Figure 5).

The newly sequenced isolates FHm1, FHm2, FHv1, FHv2 and FL4 had short ORF6s (Table 4). This ORF was highly variable, with nucleotide sequence identities ranging from 61% to 99%. Phylogenetic analysis of ORF6 of 13 isolates identified three major clusters (Figure 2C).

Alignment of the nucleotide sequences of the 3′-untranslated region (3′UTR) downstream of ORF6 showed that this region was relatively well conserved within the first 226 nucleotides and that FHv1 and FHv2 isolates are very close (Figure 6). These isolates had only 72–81% nucleotide identity with all the rest of the isolates analysed where this value was 90–97%.

Discussion

We have compared the 3′ halves of 23 PAV group cpA isolates obtained from different geographical areas and inducing different disease severity. The amino acid differences that distinguished coat protein of cpA group and cpB group (Mastari et al., 1998) were perfectly conserved when the amino acid alignment included the newly sequenced isolates. Phylogenetic analyses based on amino acid sequences and nucleotides sequences were consistent for ORF3, ORF4 and ORF5, indicating a close correlation of nucleotide and amino acid substitution. As was found for beet mild yellowing virus and beet western yellows virus (two related virus of the *Luteoviridae* family) (de Miranda et al., 1994), but at different levels, the ORF4-protein of PAV cpA was more variable than the ORF3-protein, suggesting differential constraints on these overlapping genes.

Depending on the genomic domain analysed the isolates Vic and FL1-FL9 were grouped in the same or in different clusters. The grouping in different clusters probably reflects recombination or reassortment events.

The amino acid identity ranged from 82% to 88% between the two PAV groups cpA and cpB (Mastari et al., 1998). The coat proteins of all PAV cpA isolates examined had amino acid compositions that were 88–98% similar. Such low intra-group similarity level in the coat protein was observed recently for some

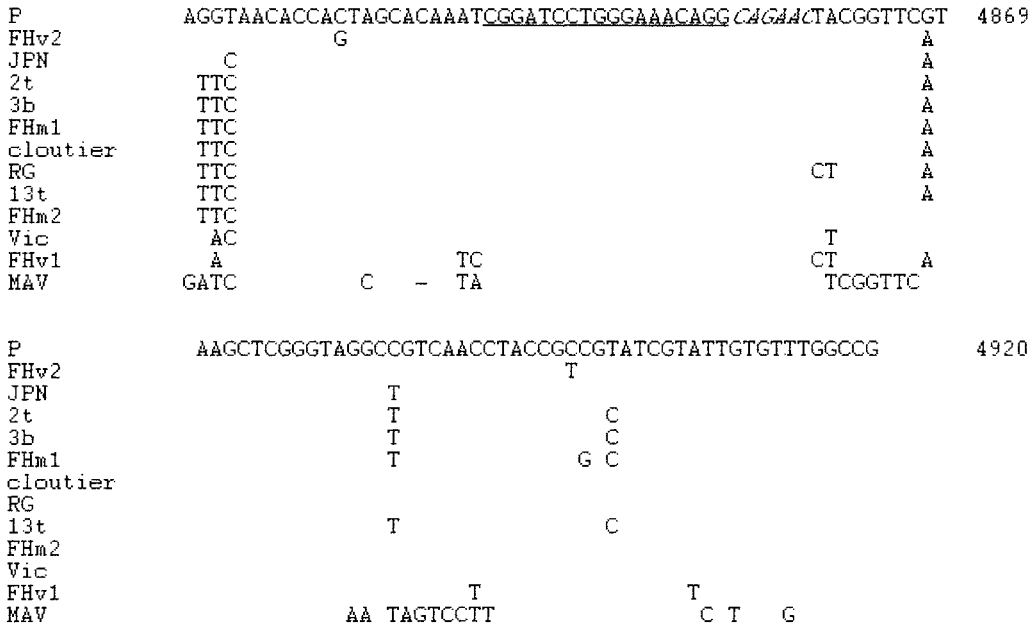

Figure 5. Nucleotide sequence alignment of 3′ TE. Nucleotides different from the top line are depicted. The first nucleotide correspond to the nucleotide 4814 of PAV-Vic is used here as reference. The nucleotides common to dianthovirus, luteovirus and necrovirus (Wang et al., 1997) are underlined. The sequence common only to PAV and MAV is in italics.

Table 4. Amino acid number and physico-chemical properties[1] of ORF6 putative protein

Isolates	Amino acid number	PI	Index of hydropathicity
JPN	41	5.19	1.134
FHm1	41	5.19	1.395
FHm2	41	5.30	1.356
Cloutier	41	5.19	1.334
2b	41	5.19	1.393
13t	41	4.20	1.617
FHv1	42	5.30	1.110
FHv2	42	5.19	1.143
RG	42	5.30	1.074
FL4	42	6.29	1.024
P	62	7.58	0.729
Vic	63	6.51	0.424
3b	67	7.68	0.479

[1]Physico-chemical parameters were assessed using ExPaSy Tools (Kyte and Doolittle, 1982; Brendel et al., 1992).

potyviruses: yam mosaic virus (Aleman-Verdaguer et al., 1997) and potato virus Y group C (Blanco-Urgoiti et al., 1998).

In the coat protein, amino acid substitutions specific to field-lethal isolates resulted in alteration of the local net charge. This alteration may influence the disease severity of PAV isolates, as demonstrated for tobacco mosaic virus where changes in local net charge of replicase and movement proteins were linked with overcoming Tm-1 and Tm-2 gene resistance, respectively (Meshi et al., 1988, 1989; Weber et al., 1993). Amino acid substitutions identified in the ORF4 protein of field-lethal isolates (FHv1 and FHv2) did not result in significantly different local net charges, but alter the hydrophobicity of this protein which was shown to be implicated in systemic infection (Chay et al., 1996b) and intracellular movement (Nass et al., 1998).

The repeat sequence (CCN NNN) located 3′ to this stop codon, and linked to readthrough processes (Brown et al., 1996; Miller et al., 1995) is highly conserved within all PAV cpA analysed. But the FHv1-insertion is located within a region containing the domain required for distal control of the readthrough processes (nucleotides residues 4154–4219 of PAV-Vic) (Brown et al., 1996). Chay et al. (1996b) suggested that the readthrough region is involved in virus transport via the aphid salivary glands and affects virus accumulation in infected plants cells. In long acquisition and transmission periods, *R. padi* transmitted FHv1, FHv2, FHm1 and FHm2 with similar efficiencies (data not shown). More investigations

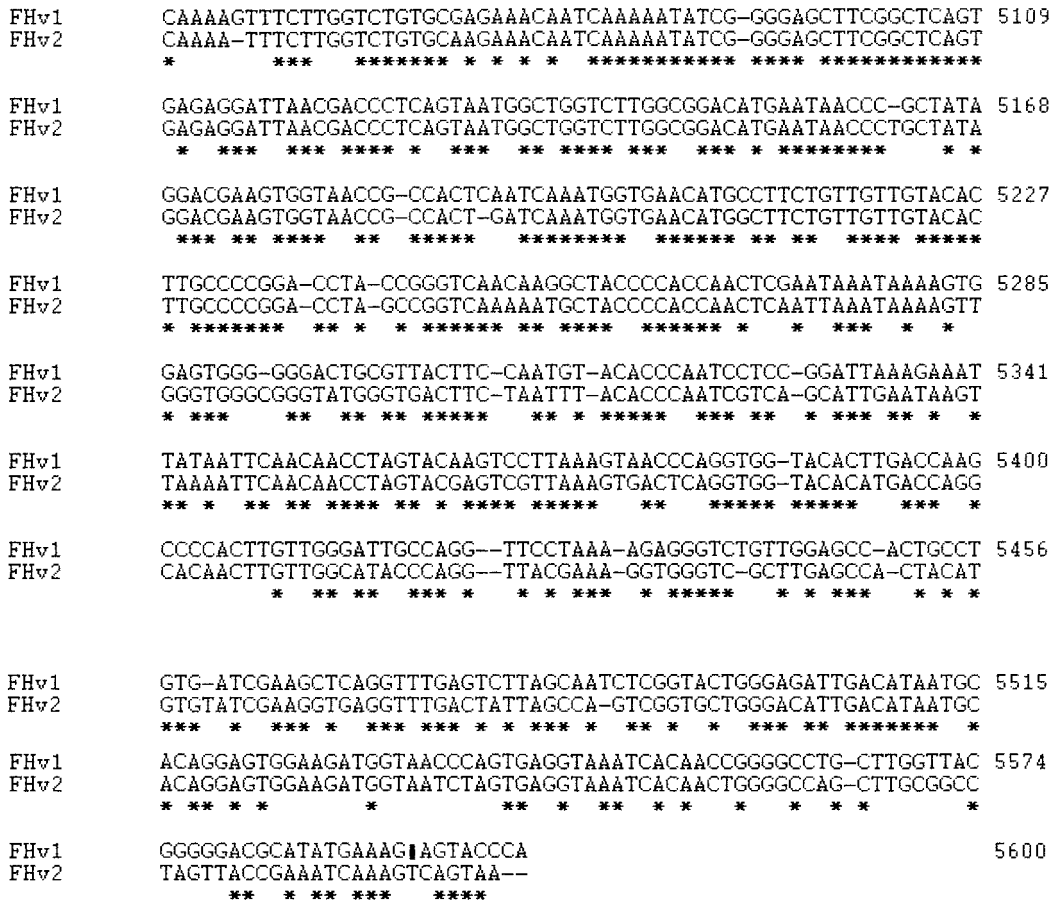

Figure 6. Nucleotide sequence alignment of 3′ UTR of FHv1 and FHv2. The first nucleotide of FHv1 corresponds to the nucleotide 5050 of PAV-Vic, used here as a reference (Miller et al., 1988). The '∗' designated conserved nucleotide in the 11 isolates compared (Cloutier, 3b, 2b, 13t, RG (Chalhoub, 1994), JPN (D85783), FHv1, FHv2, FHm1, FHm2 (this study) and Vic (X07653)).

are needed to evaluate the effect of both insertions on the translation and the activity of readthrough protein in the plant and aphid.

The four amino acid insertions identified both in FHv1 and FHv2, appeared to be rare and not necessarily associated with the severity of disease induced. Curiously, another insertion was identified in the readthrough protein of a virulent isolate of another yellow dwarf associated virus (RPV) (Beckett et al., 1997). The single amino acid insertions identified in PAV-Vic and FHv1, were absent in all PAV cpA isolates. The biological significance of such insertions is still unknown. The readthrough inserts, found only in PAV collected from cereals, may contribute to enhance our limited knowledge of PAV flux.

Hydrophobic domains of the readthrough protein of *Luteoviridae* viruses are assumed to interact with GroEL (a homologue to the protein produced by bacterial endosymbiotic in aphids vectoring these viruses). Except two, all amino acids implicated in the hydrophobic domains (including 52 amino acids in PAV) described by van den Heuvel et al. (1997), were conserved within mild and lethal isolates (data not shown), suggesting very similar constraints on these regions in this virus.

The significance of the very conserved sequences between PAV and MAV isolates in the highly variable C-terminal region of their readthrough protein, (Figure 4), has not been determined. Bruyère et al. (1997) showed that deletions in this domain in beet western yellows virus, another *Luteoviridae*, had no effect on aphid transmission, excluding a possible direct link between PAV/MAV conserved sequences and common transmission by *Sitobion avenae*.

An insert of 22 nucleotides was identified in the 3′TE region of a very severe PAV group cpB isolate (Beckett et al., 1997). A similar insertion was not found in any of the PAV group cpA isolates analysed. The sequence at position 4835–4853 within 3′TE region of PAV implicated in cap-independent translation and common to dianthovirus, *luteovirus* (former subgroup I) and necrovirus (Wang et al., 1997), is perfectly conserved. This sequence is followed by six nucleotides conserved in both PAV and MAV isolates (Figure 5). The implication of this extended domain in the translation mechanism in these two viruses has to be established.

The precise function of the ORF6 putative protein is still unknown (Miller et al., 1995; Mohan et al., 1995). The analysis of the predicted amino acid sequences of several isolates showed that the putative ORF6 products have diverse physico-chemical properties (Table 4), which could indicate that ORF6 is not translated. Nevertheless, the amino acid sequence of the N-part of this putative protein is well conserved. Thus we suggest that the C-part of ORF6 is truncated *in vivo* as in ORF5 encoded protein, and that only the N-part is functional after removal *in vivo* of the C-part.

Chalhoub et al. (1994) found a highly variable region located downstream of the ORF6, between nucleotides 5525–5552. Our results show that this variable region extends from 5512 nucleotide to 5583 when the analysis include the FHv1 and FHv2 sequences (Figure 6).

As suggested by genetic analyses of several viruses (Mangada and Igarashi, 1998; Blanco-Urgoiti et al., 1998), the potential determinants of disease expression in BYDV-PAV seem to be diverse. The comparison of sequences of the PAV cpA isolates with various disease severities has not clearly defined severity determinants, but gave good candidates for site-directed mutagenesis.

In summary, in all genomic regions of cpA isolates analysed, variations are unequally distributed. Most of the changes occurring in ORF3/4/5 and ORF6 are located in the C-terminal part. In contrast, the changes were evenly distributed in the ORF3/5 of two serotypes of SGV (Lei et al., 1995), suggesting distinct constraints on these closely related luteoviruses.

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