SEROLOGICAL AND MOLECULAR CHARACTERIZATION OF ISOLATES OF *PLUM POX VIRUS* STRAIN EL AMAR TO BETTER UNDERSTAND ITS DIVERSITY, EVOLUTION, AND UNIQUE GEOGRAPHICAL DISTRIBUTION

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SUMMARY

Sixteen isolates of *Plum pox virus* (PPV) were collected during a survey in the Egyptian areas of Sinro and Apoxa (El Fayoum) and El Amar (Nile Delta). All isolates reacted with the universal PPV monoclonal antibody MAb 5B and were identified as PPV strain EA by phylogenetic analysis of the full-length sequence of the coat protein (CP) gene. This classification was confirmed by detection with the strain-specific MAb EA24, except for an isolate denoted APR 50. Detailed analysis of the CP amino acid sequence of the EA isolates and epitope mapping revealed that histidine at amino acid position 65 of the CP sequence is an essential component of the epitope required for MAb EA24 recognition. APR 50 has an arginine substitution at this position. Five EA serogroups were identified, serogroup I being the prevailing one with 10 of the 14 isolates characterized. Moderate serological and relatively high genetic diversity was observed among isolates of PPV-EA. The most variable isolate, APR 48, contained a deletion of 33 nucleotides at the 5' terminus of the CP gene. The relatively high genetic diversity of PPV-EA suggests that it is not a recent introduction.

Key words: Plum pox virus, sharka, El Amar, monoclonal antibodies, RT-PCR, sequence analysis, epitope mapping.

INTRODUCTION

Sharka or plum pox, the most important disease affecting global production of *Prunus* spp, induces crop lossess as high as 95-100% (Németh, 1986; Garcia and Cambra, 2007). It was first detected in Bulgaria in 1918 (Atanasoff, 1932), and is now found in at least 41 countries, in five (Africa, Asia, Europe, North and South America) of the seven continents (Garcia and Cambra, 2007). Sharka is caused by *Plum pox virus* (PPV), a member of the genus *Potyvirus,* the largest and most economically important group of plant viruses (Shukla *et al*., 1991). The virus displays genetic, serological, and biological diversity (Marénaud and Massonie, 1977; Candresse *et al*., 1998; Bousalem *et al*., 2000; Myrta *et al*., 2001). The genome of PPV is a positive sense single-stranded RNA *ca*. 10 kb in size, encoding a polyprotein of 355.5 kDa that is cleaved post-translationally into 10 functional proteins (Riechmann *et al*., 1989; Shukla *et al*., 1991; Garcia *et al*., 1994), one of which is the genome-linked protein found at the 5' terminus. The genome contains a 5' and a 3' non-coding region, and a poly(A) tail at the 3' end (Garcia *et al*., 1994; Riechmann *et al*., 1989).

There are seven recognized strains of PPV denoted C, D, EA, M, Rec, T, and W (James and Glasa, 2006; Myrta *et al*., 2006; Ulubas Serçe *et al*., 2009). The strains are serologically distinct, and isolates of each strain can be identified by serology-based and/or by nucleic acid-based techniques. PPV strains differ in pathogenicity, host range, aphid transmissibility, and geographical distribution. Isolates of the strain PPV-EA are found only in Egypt (Wetzel *et al*., 1991a; Myrta *et al*., 2006).

The Mediterranean basin countries account for more than 50% of the world's apricot (*Prunus armeniaca*) production (Moustafa *et al*., 2001). The cultivated area of apricot in Egypt is about 15,585 ha with an approximate yearly production of 106,165 tons (Anonymous, 2008). In the past, apricot cultivation was based in the Nile Delta (in which El Amar village is located) and El Fayoum regions, but is now concentrated in new land reclaimed from the desert. There are many local apricot varieties grown in Egypt such as El Amar, Balady, Amal, and Hamawy, and also imported varieties such as Canino.

Our study focused on the characterization of the serological and molecular diversity of Egyptian PPV isolates since limited information is available on the variability of these isolates and only a few PPV-EA sequences are available in GenBank.

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MATERIALS AND METHODS

Plant material. PPV isolates used in this study were recovered from native and imported stone fruits in two different Egyptian areas that were found infected by PPV during an extensive survey of stone fruit orchards carried out by El Maghraby *et al.* (2007) (Table 1). All but one isolate came from apricots, the prevalent fruit species in the orchards visited. One isolate was from Japanese plum (*Prunus salicina*). PPV-infected samples were grafted onto GF305 seedlings and maintained in a climatized greenhouse for symptom observation, and serological and molecular analyses.

ELISA detection and serotyping. TAS-ELISA was used to detect virus presence using the PPV-specific universal monoclonal antibody (MAb) 5B (Cambra *et al.*, 1994). Strain typing was carried out using strain-specific MAbs: 4DG5 for PPV-D (Cambra *et al.,* 1994), AL for PPV-M (Boscia *et al.*, 1997), EA24 for PPV-EA (Myrta *et al.*, 1998) and AC for PPV-C (Myrta *et al.*, 2000). Additional 23 MAbs were used to serotype the various PPV isolates and determine the intra-strain serological variability of PPV-EA. These included EA2, EA4, EA5, EA7, EA8, EA11, EA12, EA13, EA14, EA16, and EA18 (Myrta *et al.*, 2001); 4DG11, 1EB6, 4F4, 4DB12, 3C6, and 4BD7 (Cambra *et al.*, 1994); 05, 06, and 07 (Navrátil *et al.*, 1992); B7C4 (Pasquini and Barba, 1994); EMA13/C (East Malling Research, UK) and V/8 (RIPF, Skierniewice, Poland). Absorbance values three times or more than those given by healthy controls were considered positive.

RT-PCR. Total nucleic acid (TNA) extraction was carried out as described (Foissac *et al.,* 2001). Seven µl of TNA preparations were mixed with 100 ng of random primers (Roche, Switzerland) and 26 µl of RNase-free water. The entire mixture was incubated at 95°C for 5 min, then chilled quickly on ice. cDNA was synthesized with 5X first-strand buffer (Invitrogen, USA), 0.5 mM dNTPs (Roche, Switzerland), 5 mM DTT, 200 U of MMLV reverse transcriptase (Invitrogen, USA) in a final volume of 50 µl. The synthesis proceeded at 37°C for 1 h. Two sets of primers were used in PCR for amplification of the CP region: (i) PPV-UN4F (5'-TCT TGA RCA RGC RCC RTA YAA TGC-3') and P1 (Wetzel *et al.*, 1991b) corresponding to nucleotides (nt) 8,396-9,585; or (ii) PPV-4CPF (5'-ATA CTT GAG TGG GAC AGA TCA AAT G-3') and PPV-4CPR (5'-GAG AAA AGG ATG CTA ACA GGA ATC-3') corresponding to nts 8,274-9,659 (nt positions were determined from the full-length sequence of PPV-EA isolate AM157175). Ten µl of cDNA were used for the PCR reaction which was performed with a mixture containing 10X Taq buffer (Eppendorf, Germany), 0.4 µM of each primer, 0.2 mM of dNTPs (Roche, Switzerland), 2.5 U Taq DNA polymerase (Eppendorf, Germany) and the final volume adjusted to 50 µl with sterile distilled water. The PCR program was initiated with a denaturation step of 95°C for 5 min, followed by 35 cycles at 94°C for 45 sec, 60°C for 45 sec and 72°C for 1 min, and a final extension step of 5 min at 72°C.

Table 1. List of Egyptian *Plum pox virus* isolates and their origins.

No.	Isolate code	Host plant	Cultivar	Location	Geographical area	Orchard (No.)	Accession No.
1	APR 1	Apricot	Balady	Sinro	El Fayoum	1	FN424165
2	APR 10	Apricot	Amar	Sinro	El Fayoum	1	FN424166
3	PL 18	Japanese Plum	Balady	Sinro	El Fayoum	2	FN424167
4	APR 22	Apricot	Amar	Sinro	El Fayoum	3	FN424168
5	APR 26	Apricot	Amar	Apoxa	El Fayoum	4	FN424169
6	APR 46	Apricot	Amar	El Amar	Nile Delta	5	FN424170
7	APR 48	Apricot	Amar	El Amar	Nile Delta	5	FN424171
8	APR 50	Apricot	Amar	El Amar	Nile Delta	5	FN424172
9	APR 53	Apricot	Balady	El Amar	Nile Delta	6	FN424173
10	APR 56	Apricot	Amar	El Amar	Nile Delta	7	FN424174
11	APR 60	Apricot	Amar	El Amar	Nile Delta	8	FN424175
12	APR 63	Apricot	Amar	El Amar	Nile Delta	9	FN424176
13	APR 65	Apricot	Amar	El Amar	Nile Delta	9	FN424177
14	APR II/2	Apricot	Amar	El Amar	Nile Delta	10	FN424178
15	APR II/3	Apricot	Amar	El Amar	Nile Delta	11	FN424179
16	APR II/4	Apricot	Amar	El Amar	Nile Delta	11	FN424180

Cloning and sequence analysis. Amplified cDNA fragments were gel-purified and extracted using a MinElute Gel Extraction kit (Qiagen, Germany). Purified cDNA fragments were ligated into the pCR-TOPO vector and cloned using the TOPO TA Cloning Kit as directed by the supplier (Invitrogen, USA). Sequencing and sequence analysis were carried out as described by James and Varga (2005) with bi-directional sequencing using the M13F and M13R primers. The CP coding sequence of each PPV-EA isolate was derived from 3 to 5 PCR clones and the complete sequences of 16 isolates were deposited in GenBank under accession Nos FN424165 to FN424180. Phylogenetic analyses were carried out using the Neighbour-Joining method described by (Saitou and Nei, 1987), within Clustal-X (version 1.81), and with a bootstrap of 1000 replicates. The Gonnet weight matrices in ClustalX (version 1.83), were used with the default parameters. Trees were visualized using NJPLOT (Perriere and Gouy, 1996).

Epitope mapping and analysis. Four peptides were selected for epitope analysis based on the serotyping data and alignments of the deduced amino acid (aa) sequences of all 16 PPV isolates from Egypt. Two methods were used for epitope identification. In method 1 analysis of the recognition site was achieved by inserting alanine substitutions at selected locations for three of the peptides (Fig. 2), to identify aa residues critical for epitope recognition. The peptides were synthesized on gears attached to polyethylene pins (Mimotopes International, Australia). The gears were processed as described by Croft *et al.* (2008). In method 2 the peptide PIFTPATTEPTTRTVP**H**TTTT (65**H**) common to most PPV-EA isolates and extending from aa 49 to aa 69 (Fig. 1A) was synthesized. A variant of this peptide was also synthesized and identified as 65**R**, with an aa substitution of arginine (R) for histidine (H) (PIFTPATTEPTTRTVP**R**TTTT) at aa position 65 (Fig. 1A). This substitution at position 65 is unique to PPV-EA isolate APR50. The peptides were custom synthesized (Bio Basic, Ontario), and used in a dot blot assay as described by Croft *et al.* (2008).

The aa sequence of PPV-EA (accession No. AM157175) was analysed using the antibody epitope prediction program (http://tools.immuneepitope.org), with the appropriate prediction model. The BepiPred linear algorithm was selected. This approach uses aa combination of predictions from a hidden Markov model and Parker propensity scale to evaluate and score the likelihood of B-cell recognition (Larsen *et al.*, 2006).

RESULTS AND DISCUSSION

Sixteen PPV isolates (15 from apricot and one from Japanese plum) were recovered from eleven orchards in two Egyptian regions including Sinro and Apoxa (El Fayoum) and El Amar (Nile Delta) (Table 1). Symptoms observed on the GF305 indicator grafted with these isolates consisted of vein yellowing, chlorotic mottle, and mild leaf distortion.

All 16 isolates reacted with the universal PPV MAb 5B and all but two were available for detailed serotyping. These isolates reacted with the strain-specific MAb EA24, except for isolate APR50 (Table 2) which did not react either with the M, D, and C strain-specific MAbs. Serological profiling of the 14 PPV-EA isolates resulted in the identification of five serogroups (Table 2). The main group, identified as serogroup I consisted of 10 isolates, whereas the other four serogroups contained one isolate each. Isolates of serogroup I originated from two locations (Sinro and El Amar), different *Prunus* species (apricot and Japanese plum), and different cultivars (Balady and Amar). Members of serogroup I were consistent in their reaction with all MAbs used for screening, and perhaps represent the native and dominant group. Interestingly, a minor serogroup (II) was composed of isolate APR22 from El Fayoum and the reference isolate of PPV-EA strain (AM157175) from El Amar in the Nile Delta. Serogroups III, IV and V comprised isolates from the Nile Delta. The finding of five serogroups among the 14 isolates serotyped indicates a moderate serological variability in the Egyptian PPV-EA isolates; and since the isolates from the Nile Delta belonged to four serogroups, it could be that a wider diversity of PPV-EA is present in that region, where the EA strain was reported initially (Wetzel *et al.*, 1991a).

All sequenced isolates had a CP coding region 996 nts in size, except for isolate APR48, which consisted of 963 nts with a 33 nt deletion. The deduced CP of all isolates consisted of 332 aa residues, except for APR48 for which the CP consisted of 321 residues with an 11 aa deletion (Fig. 1A). APR48 had the lowest identity (95%) at the nt and aa levels and is the first PPV-EA isolate described with a deletion event.

Analysis of the full-length CP gene sequences deposited in GenBank showed percentage identity ranges of 98-99% (nt) and 96-99% (aa) for PPV-D strains (50 isolates); 98-99% and 99-100%, respectively for PPV-M strains (28 isolates); 95-99% and 95-100% for PPV-Rec strains (19 isolates). A similar analysis of the 16 PPV-EA isolates characterized in this study and the four sequences deposited in GenBank showed 95-99% identity at the nt and aa levels.

Sequence alignment and phylogenetic analysis confirmed that all 16 isolates in this study are members of the PPV-EA strain, including isolate APR50 which did not react with the PPV-EA specific MAb EA24 (Table 2). Analyses including other PPV strains as outgoups did not alter the relationships observed (not shown). Figure 1B shows the phylogenetic analysis based on the deduced aa sequences. APR50 is most closely related to

A

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APR 50    1 adekeddeeevdagrplvtttqqpivttttqqtpmtgttlqatqamfnpiftpattepat
APR 60    1 adekeddeeevdagrplvtttqqptvttttqqtpitsatlqatqamfnpiftpattepat
APR 65    1 adekeddeeevdagrplvtttqqpivttttqqtpitsttlqatqamfnpiftpatteptt
APR 48    1 adekeddeeevdagrslvtttqqpivatttqqtpitsttlqatqamfnpiftpatteptt
APR 53    1 adekeddedevdagrplvtttqqpivttttqqtpitsttlqatqamfnpiftpatteptt

APR 50   61 rtvprttttppsfgvignentaptasnalvqtgrdrdvdagsigtftvprlkamtskls
APR 60   61 rtvphttttppsfgvignedtapnasnaliqtgrdrdvdagsigtftvprlkamtskls
APR 65   61 rtvphttttppsfgvignedtapnasnavvrtgrdrdvdagsigtftvprlkamtskls
APR 48   61 rtvphtttt----------dtapnasnalvqtgrdrdvdagsigtftvprlkamtskls
APR 53   61 rtvphttttppsfgvignedtapnasnalvrtgrdrdvdagsigtftvprlkamtskls
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B

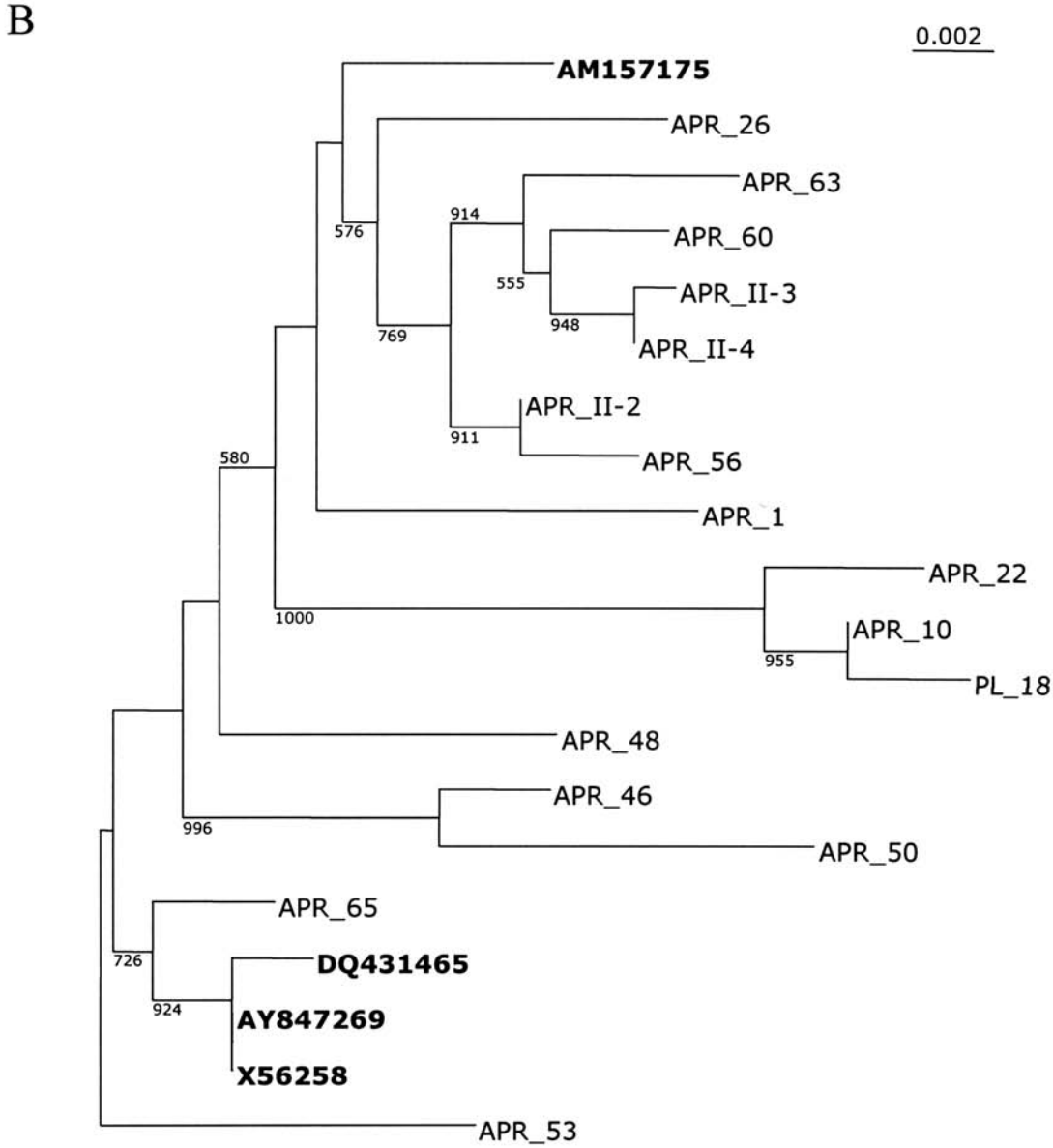

Fig. 1. A. Multiple sequence alignment of the N-terminus region of the coat protein of selected Egyptian *Plum pox virus* (PPV-EA strain) isolates. B. Phylogenetic analysis of the complete sequence (deduced amino acids) of the coat protein of 16 Egyptian *Plum pox virus* (PPV-EA strain) isolates and PPV-EA sequences previously deposited in GenBank (in bold). The tree was constructed using the N-J tree option of Clustal-X. Bootstrap values out of 1000 replicates are shown, and the scale bar indicates the number of substitutions per residue.

isolate APR46 originating from the same orchard. Three of the five isolates from the El Fayoum (PL 18, APR10, and APR22) formed a single clade, while the other two isolates (APR1 and APR26) grouped closer to the major clade consisting of isolates from the Nile Delta. This is unexpected since APR1 and APR10 originated from the same orchard, while PL 18 and APR22 came from orchards 2 and 3, respectively. Interestingly five of 11 isolates from the Nile Delta did not group with the major clade but formed several distinct branches. In only two of four cases did isolates from the same orchard group together, i.e. APR46 and APR50 from orchard 5, and APR II/3 and APR II/4 from orchard 11.

There was some correlation between the groupings derived from serological (Table 2) and phylogenetic analyses (Fig. 1B), but neither appeared completely predictive of the other. Nile Delta isolates showed higher serological and phylogenetic diversity than those from El Fayoum.

Alignments comparing the CP sequence of APR50 with two isolates (APR60 and APR65) that reacted with MAb EA24 (Table 2) revealed regions with aa variability unique to APR50 that may be involved in recognition (Fig. 1A, aa residues unique to APR50 are bolded and underlined). For isolate APR50, an isoleucine is substituted by a methionine at CP aa position 35, a serine by a glycine at position 37, an aspartic acid by an asparagine at position 81, and an asparagine by a threonine at position 85. Using Mimitope pin-bound synthetic peptides designed to flank both regions, peptides TQQTPITSATLQA and GNEDTAPNASNA deduced from the CP sequence of APR60 were not recognized by MAb EA24 (Fig. 2C). In dot blot analysis of the peptides 65H and 65R encompassing the unique aa substitution associated with APR50 at position 65, MAb EA24 recognized peptide 65H but not peptide 65R (not shown). This indicates that histidine at aa position 65 is essential for epitope recognition by MAb EA24. The 11 aa deletion of APR48 (Fig. 1A) did not affect recognition by the PPV-EA specific MAb EA24. This deletion is not associated with any of the epitopes evaluated in this study based on the recognition patterns of other isolates (Table 2).

Linear epitope prediction analysis revealed the region encompassing the peptide ADEKEDDEDEVDA as being the most antigenic region of the CP of PPV-EA (not shown). B-cell accessibility studies indicated that this region is the second most accessible region, hence most PPV-EA antibodies targeted this CP region (not shown). This peptide is identified as containing the epitope recognized by Mab EA2 and Mab EA11 (Fig. 2A and 2B).

Table 2. Serogroups of 14 Egyptian isolates and the reference *Plum pox virus* isolates determined by screening with a panel of monoclonal antibodies.

Serogroup	Isolate code	PPV strain	Reaction of different MAbs[a]								Frequency (No.)
			5B[b]	EA2[c]	EA(EA24)	EA11	4F4	D (4DG5)[d]	M (AL)	C(AC)	
I	APR 1*	EA	+	+	+	+	+	-	-	-	10
II	APR 22**	EA	+	+	+	+	-	-	-	-	2
III	APR 48	EA	+	+	+	-	+	-	-	-	1
IV	APR 50	EA	+	+	-	+	+	-	-	-	1
V	APR 53	EA	+	-	+	+	-	-	-	-	1
VI	X16415**	D	+	-	-	-	-	+	-	-	1
VII	AJ749995**	M	+	-	-	-	+	-	+	-	1
VIII	Y09851**	C	+	-	-	-	+	-	-	+	1
IX	AJ749998**	Rec	+	+	-	+	-	-	+	-	1

[a]MAbs EMA13/C, V/8, 1EB6, 4BD7, B7C4, 06 and 07 did not react with 14 Egyptian PPV-EA isolates;

[b]Mab 5B gave the same pattern as EA4, EA5, EA8, 4DB12, 3C6 and 05; [c]EA2 as EA7, EA12, EA13, EA14, EA16 and EA18; [d]4DG5 as 4DG11;

*APR10, APR56, APR60, APR63, APR65, APRII/2, APRII/3, APRII/4, and PL18 are included in the same serogroup (I);

**Reference isolate of PPV-EA strain (AM157175) is included in serogroup II, whereas other reference isolates of strains D (X16415), M (AJ749995), C (Y09851), and Rec (AJ749998) form separate serogroups (VI, VII, VIII, and IX, respectively).

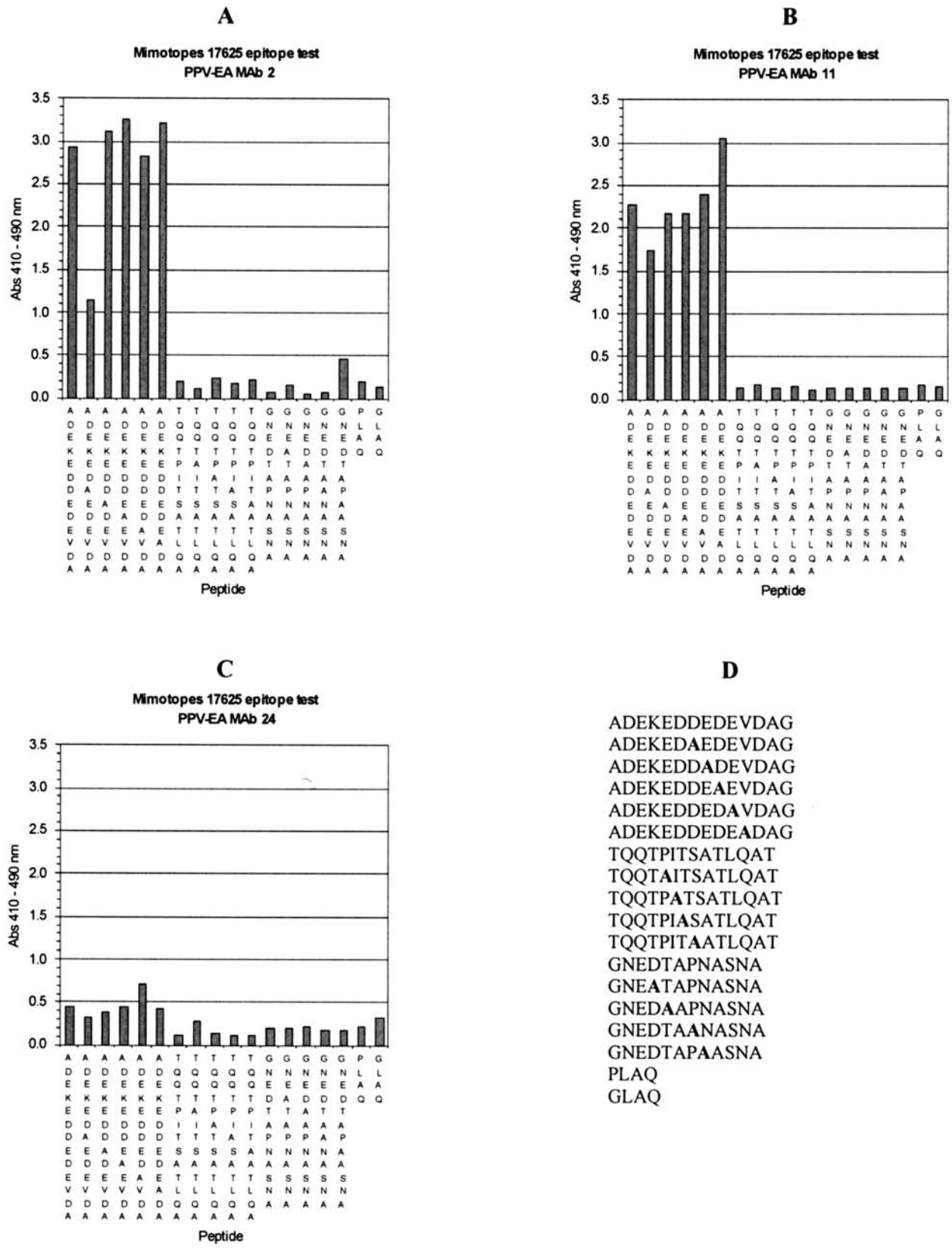

Fig. 2. Epitope mapping using synthetic pin-bound peptides (Mimotopes) derived from selected regions of *Plum pox virus* (PPV-EA strain) coat protein with apparent involvement in antibody recognition: ADEKEDDEDEVDA, TQQTPITSATLQA, and GNEDTAPNASNA. Tests were done using MAbs: EA2 (frame A), EA11 (frame B), and EA24 (frame C). Alanine (A) substitutions were used in attempts to identify critical amino acid residues. Frame D shows the position of the A substitutions. The unrelated peptides PLAQ and GLAQ were used to provide a baseline.

PPV-EA was detected in Egypt and described initially in 1991 (Wetzel *et al*., 1991a). One possibility is that PPV was introduced into Egypt a very long time ago from areas with different climatic conditions, and over time an evolutionary divergence occurred due to isolation and new conditions. Similar studies of PPV isolates from eastern Europe, especially the Balkan area, show high genetic diversity and serological variability (Myrta *et al*., 2001; Matic *et al*., 2006). After its introduction to Egypt, PPV did not spread further due to limited or no movement of germplasm out of the country.

In summary, the results of this study indicate that only isolates of PPV-EA were recovered from stone fruits in Egypt. The relatively high genetic diversity suggests that PPV-EA was not a recent introduction.

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