

RESEARCH PAPERS

Barley yellow dwarf virus in barley crops in Tunisia: prevalence and molecular characterization

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Summary. A field survey was conducted in Tunisia in the North-Eastern regions (Bizerte, CapBon and Zaghouan), the North-Western region (Kef) and the Central-Eastern region (Kairouan) during the 2011/2012 growing season, in order to determine the incidence and the geographic distribution of *Barley yellow dwarf virus* (BYDVs) in barley fields. Tissue blot immunoassays (TBIA) showed that BYDV was most common in Zaghouan (incidence 14%), Cap Bon (14%) and Bizerte (35%), in randomly collected samples from these three locations. Among the different BYDVs identified, BYDV-PAV (64%) was the most common followed by BYDV-MAV (16%) and CYDV-RPV (3%). The coat protein gene sequences of six isolates collected from different regions shared >98% pairwise similarity. In comparisons with other BYDV sequences from around the world, the Tunisian sequences shared greatest homology with isolates 109 and ASL1 from the United States of America and Germany ($\approx 97\%$), and <90% with all other isolate sequences available in public databases.

Key words: Barley, BYDV, sequencing, serological tests, virus incidence.

Introduction

Barley is an important and widely cultivated field crop in Tunisia, covering an average area of 500,000 to 600,000 ha, mainly distributed in semi-arid and arid parts of the country. Barley grain is one of the main sources for human or animal food. Agronomic potential of the cultivated barley genotypes fluctuates among crop seasons due to abiotic or biotic constraints, and in particular virus diseases are known to reduce the yield potential of most barley cultivars. Barley yellow dwarf (BYD) is one of the most economically important diseases of cereals worldwide (D'Arcy, 1995; Svanella-Dumas *et al.*, 2013). It is caused by several viruses in the family Luteoviridae. The most important of these are *Barley yellow dwarf*

virus-PAV (BYDV-PAV), *Barley yellow dwarf virus*-MAV (BYDV-MAV, genus *Luteovirus*), and *Cereal yellow dwarf virus*-RPV (CYDV-RPV, genus of *Polerovirus*) (Van Regenmortel *et al.*, 2000). These single-stranded positive sense RNA viruses are phloem-limited and cause yellowing, reddening, and/or stunting of infected plants. They are specifically transmitted by aphids in persistent and circulative manners (Mayo and Ziegler-Graff, 1996; D'Arcy and Domier, 2005). Many isolates of BYDV have been sequenced, particularly their respective coat protein (CP) genes. BYD was first reported in Tunisia in 1990 and subsequently in 2000 (Makkouk *et al.*, 1990; Najjar *et al.*, 2000), with BYDV-PAV being found to be the predominant species (Makkouk *et al.*, 2001). Other BYDV species detected, in decreasing order, were BYDV-SGV, BYDV-RMV, BYDV-MAV and CYDV-RPV. As part of our continued effort to assess BYDV incidence, a field survey was conducted during April 2012 in the major barley growing areas of Tunisia.

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Materials and methods

Field surveys and sample collection

Surveys in Tunisia were conducted in the north-eastern (Bizerte; CapBon and Zaghouan), north-western (Kef) and central-eastern (Kairouan) regions. Thirty-six barley fields were randomly selected, and from each field two types of samples were collected; approx. 20 samples from symptomatic plants and 100-200 random samples. A total of 5,403 random samples and 611 samples from plants with symptoms suggestive of virus infection were brought to the Virology Laboratory at INRAT for tissue blot immunoassays (TBIA) (Table 1).

Serological assays

All samples were tested for BYDV presence, using the tissue-blot immunoassay (TBIA) (Makkouk and Kumari, 1996) with polyclonal antibodies provided by ICARDA's virology laboratory. Samples that were found to be positive by TBIA were further tested using DAS-ELISA (Clark and Adams, 1977) with three polyclonal antibodies raised against BYDV-PAV, BYDV-MAV and CYDV-RPV. These were obtained from BIOREBA.

RT-PCR assays

Six barley samples which reacted positively with BYDV-PAV polyclonal antibody and showed severe BYD symptoms in the field were further investigated to determine their CP gene sequences. These six samples included two isolates (D12 and D13) from Bizerte, two (D23, D24) from Zaghouan and two (D34, D35) from Cap Bon. Total RNAs were extracted using the Trizol (Invitrogen) and chloroform method (Kelly *et al.*, 1994). Complementary DNA to the CP

gene was synthesized using reverse transcription PCR amplification (RT-PCR) in a single tube using the specific BYDV-PAV primer pairs which amplifies the CP gene (744 nts): (BYDV-F: 5'-GTTCTGCTCAACATCGGAT-3' and BYDV-R: 5'-AATAGGTAGACTCCTCAACA-3') (Ratsgou *et al.*, 2005). For cDNA synthesis, 2 µL of total RNA extract and 0.2 µM primers were denatured for 5 min at 95°C and chilled on ice. RT-PCR was then performed in a single tube using a 20 µL reaction mixture that contained 3 mM MgCl₂, 0.4 mM each of dNTPs, 20 U of MMLV (reverse transcriptase), 4 U of RNase out recombinant ribonuclease inhibitor, 1 U of Taq DNA polymerase and 2.5 µL of reaction buffer (200mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen). PCR was performed in an automated thermal cycler (Applied Biosystems Gene) programmed for the following thermo-cycling conditions: one cycle of 50 min at 37°C; one cycle of 3 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, and one cycle of 10 min at 72°C. Amplification products were detected by electrophoresis in a 2% agarose gel followed by ethidium bromide staining (10 µg mL⁻¹).

Sequence analysis

Amplified products were purified using the PCR DNA purification Kit (Invitrogen) according to manufacturer's instructions. The PCR products were cloned into a pGEM-T vector using insert T/A clone PCR product Kit (Promega). Recombinant plasmids were used to transform DH5α *Escherichia coli* competent cells and the recombinant plasmids were purified using the High Pure Plasmid kit (Invitrogen). Positive clones of each isolate were Sanger sequenced using an ABI PRISM DNA sequencer 377 (Perkin Elmer). These six sequences were aligned together with all BYDV sequences that spanned this re-

Table 1. Location and number of barley samples collected from 36 fields in different regions in Tunisia in April 2012.

Sampling	Kef	Kairouan	Zaghouan	Cap Bon	Bizerte	Total
No. of fields surveyed	6	9	7	8	6	36
Random	930	1417	1055	762	1239	5403
With symptoms	90	140	131	120	130	611
Total	1020	1557	1186	882	1369	6014

gion (n = 84) available in GenBank, and two sequences BYDVNY-SGV and BYDV TX-SGV (U06865 and U06865) were used as an outgroup. The sequences were aligned using MUSCLE (Edgar, 2004), and this alignment was used to infer a maximum likelihood phylogenetic tree (1,000 bootstraps) using PHYML (Guindon *et al.*, 2010) with GTR + G determined to be the best model for substitution using the jModelTest (Darriba *et al.*, 2012). Branches with less than 60% support were collapsed using TreeGraph 2 (Stöver *et al.*, 2010). Sequence pairwise identified were determined using SDT v1.2 (Muhire *et al.*, 2014). Aligned sequences were analysed with DnaSP software version 4.0 (Rozas *et al.*, 2003) to estimate indices of haplotype diversity (Hd) (Nei and Tajima, 1983) and pairwise estimates of nucleotide divergence (Pi) (Jukes and Cantor, 1969) to assess the genetic diversity among Tunisian isolates.

Results

Field observations

The most commonly observed symptoms in barley crops were stunting of the affected plants and yellowing of the leaves. Virus disease incidence based on field inspections was estimated to range from 1 to 20%.

Incidence of BYDV from laboratory testing

TBIA results showed that BYDV incidence varied among locations, ranging from 2 to 35% for random samples, and from 19 to 83% for symptomatic samples. BYDV was most common in Zaghouan (14% incidence), Cap Bon (14%) and Bizerte (34%) regions (Figure 1). Among the different BYDV serotypes detected, BYDV-PAV (64%) was the most common followed by BYDV-MAV (16%) and CYDV-RPV (3%) (Table 2).

RT-PCR assays and sequence analysis

All six Tunisian samples (D12 and D13 detected from Bizerte, D23 and D24 from Zaghouan, and D34 and D35 from Cap Bon) generated amplicons of the expected size (744 nts) using specific BYDV-PAV primers (Figure 2). The six Tunisian isolates shared >98% pairwise similarity with each other and ≈97% with two BYDV sequences of isolate ASL1 (AJ810418) from Germany and isolate 109

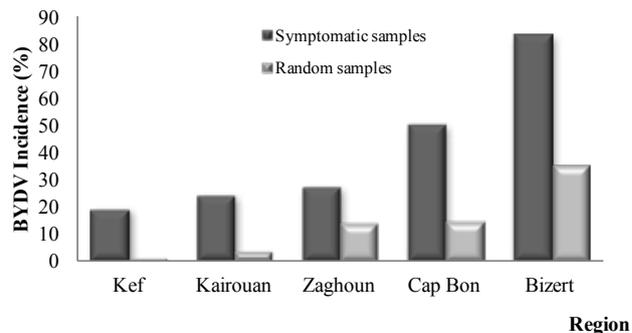


Figure 1. BYDV incidence based on TBIA tests of symptomatic and randomly selected samples collected from different barley regions of Tunisia during April 2012.

Table 2. BYDVs incidence (%) in barley fields of different regions in Tunisia based on virus testing by DAS –ELISA.

Region	No. of samples tested	BYDV-PAV	BYDV-MAV	CYDV-RPV
Kef	36	28	7	2
Kairouan	23	15	2	0
Zaghouan	30	15	6	0
Cap Bon	67	42	11	0
Bizerte	87	55	12	6
Total	243	155	38	8
BYDVs incidence (%)		63.78	15.63	3.29

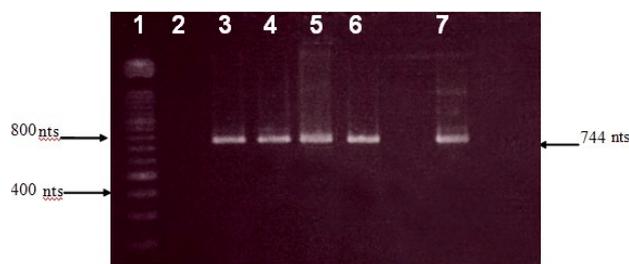


Figure 2. Detection of BYDV-PAV by RT-PCR. Lane 3 = barley from Cap Bon, lane 4 = barley from Bizerte, lane 5 = barley from Kef, lane 6 = barley from Kairouan, lane 7 = positive sample, lane 2 = negative control, and lane 1 = 100 nts ladder.

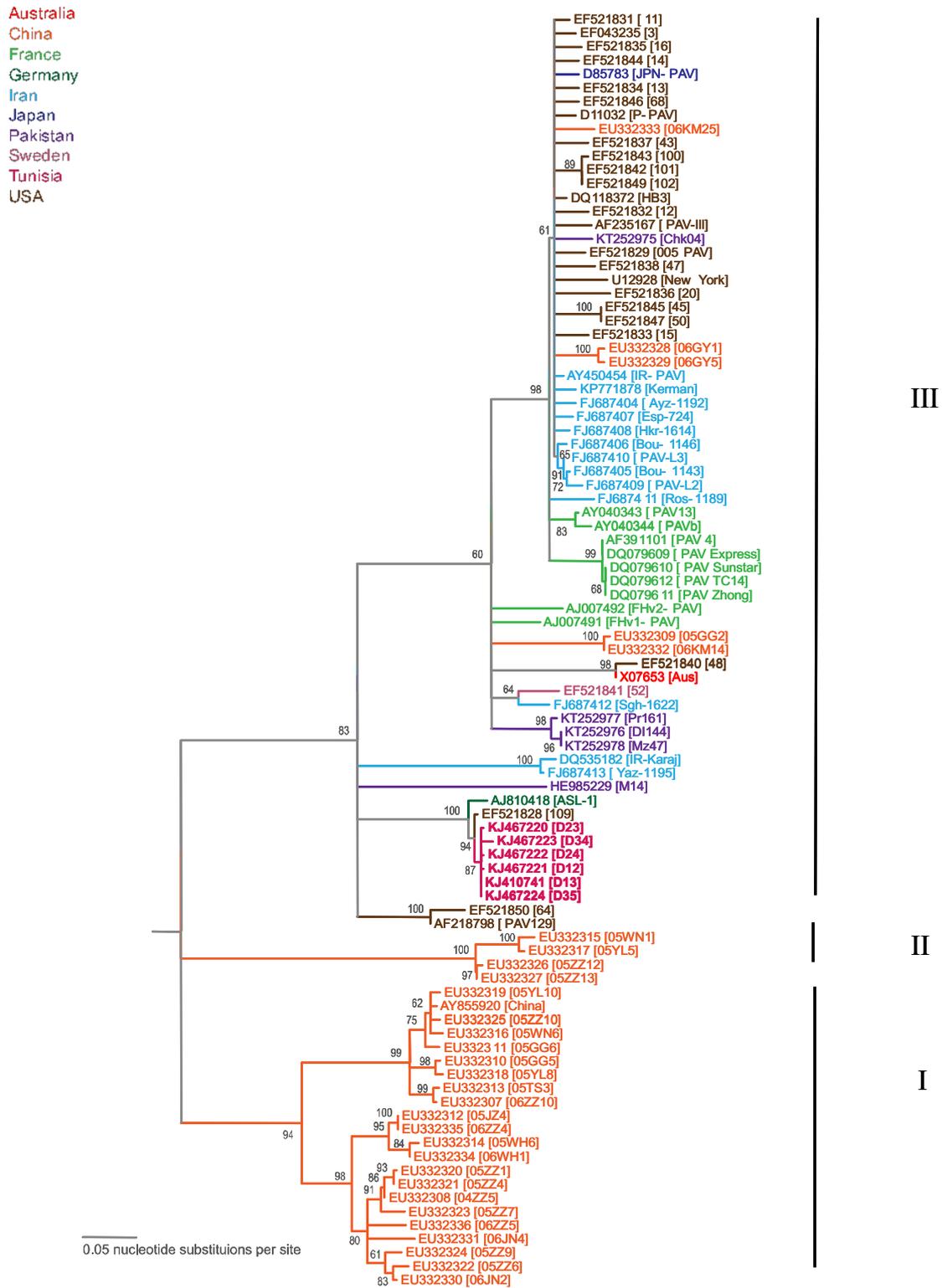


Figure 3. Maximum likelihood phylogenetic analysis of BYDV-PAV isolates. Taxa and branches are coloured based on sampling location.

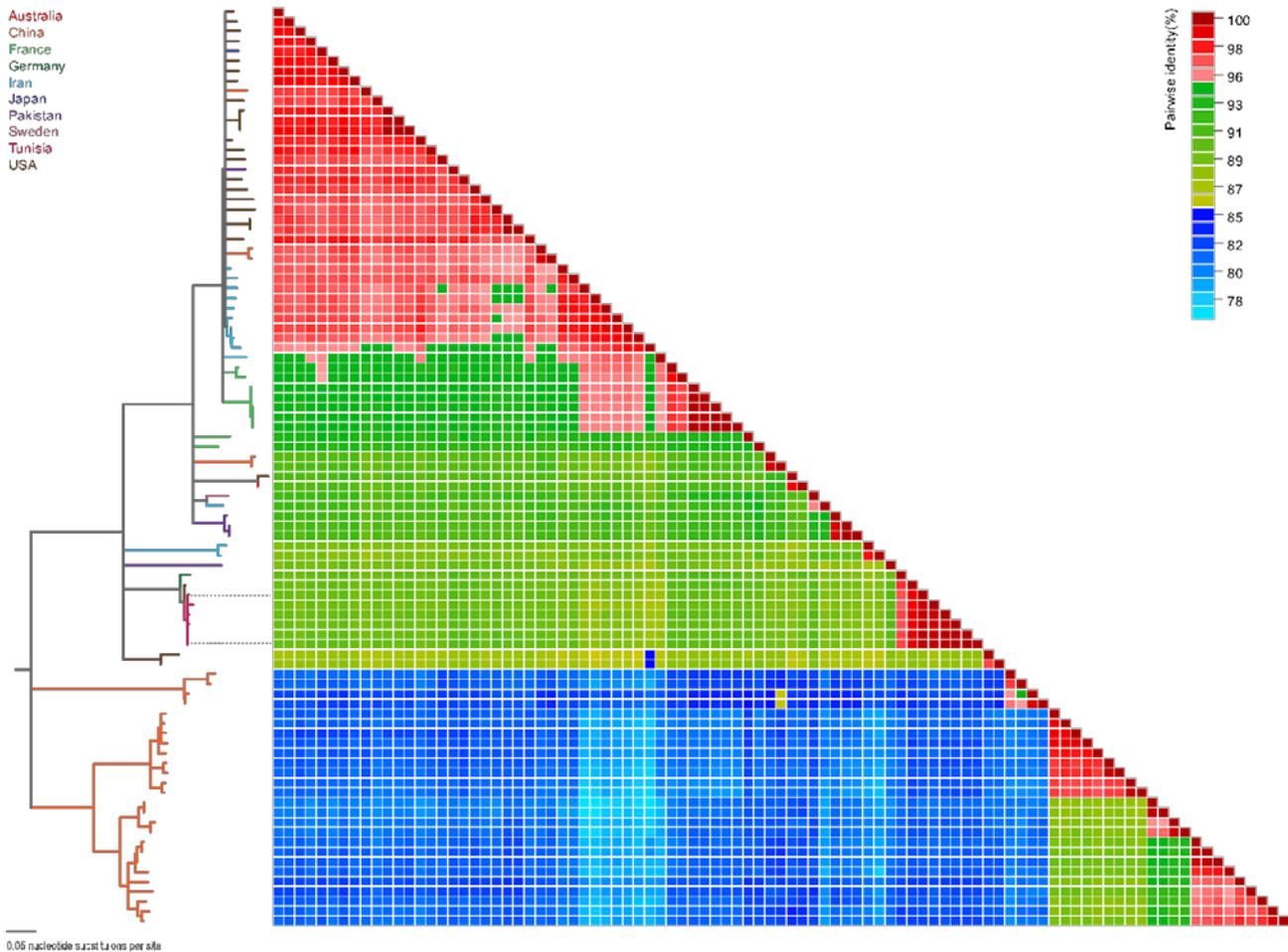


Figure 4. Maximum likelihood phylogenetic tree of BYDV-PAV sequences together with a pairwise identity matrix.

(EF521828) from the United States of America. Following the multiple alignments of Tunisian sequences, 740 conserved sites, nine variable sites divided into four informative sites and five unique sites have been identified. Figure 3 shows that the Maximum likelihood dendrogram placed the BYDV-PAV isolates into three groups. The first, labelled (I), is composed of exclusively Chinese isolates. The second, labeled (II) contains three Chinese isolates. All other isolates (from Australia, Japan, China, Sweden, Germany, Iran, the USA, Pakistan and Tunisia) form the group labelled (III). There was very close similarity between the six Tunisian isolate sequences (KJ410741, KJ467220 and KJ467224) together with isolates ASL1 and 109, forming a well-supported cluster of BYDV sequences (Figure 3). This cluster

shared <90.5% pairwise similarity with all other BYDV sequences available in GenBank (Figure 4, Supplementary data 1).

Maximum likelihood phylogenetic analysis of 93 BYDV-PAV sequences revealed two distant lineages of BYDV-PAVs in China (Figure 3; 26 sequences) that share <81% pairwise similarity with all other isolate sequences. The bulk of the sequences ($n = 67$) includes five Chinese isolates (06KM25, 06GY1, 06GY5, 05GG2 and 06KM14) and the six isolate sequences from Tunisia (Figure 3), all sharing >86% pairwise similarity (Figure 4).

These groupings reflect the presence of a continuous variability that characterizes the isolates studied. Variants were not grouped in accordance with their geographical origins.

Discussion

BYDV was reported in Tunisia during the 1985/1986 growing season, with incidence of 25% (Makkouk *et al.*, 1990), and was found to be of extremely low incidence (less than 1%) during the dry growing season of 1999/2000 (Najar *et al.*, 2000). During the 2011/2012 growing season, infection rates in barley fields reached 35% in the Bizerte region (a sub-humid zone), which probably provided a suitable environment for the activity of aphid vectors. These observations are similar to those of A'Brook (1981), Robert and Lemaire (1999), Thackray *et al.*, (2001) and Hall (2007), who reported the impact of climatic conditions, host species and sowing dates on the proliferation of insect vectors and, consequently, the dissemination of the virus. Results obtained have shown that visual field inspections underestimate the incidence of the virus disease. This underestimation could have resulted from the presence of the virus at very low concentrations and below detection levels during early infection and before the appearance of disease symptoms. Therefore, for accurate diagnoses, it is advisable to base field virus incidence on sensitive and robust laboratory assays.

Serological identification of the three main BYDVs (BYDV-PAV, BYDV-MAV and CYDV-RPV) showed that BYDV-PAV was predominant species with infection rate estimated at 66%, followed by BYDV-MAV (16%) and CYDV-RPV (3%). These proportions are comparable with those recorded in previous reports from Tunisia (Makkouk *et al.*, 2001; Bouallegue *et al.*, 2014), the west of France (Henry *et al.*, 1993; Leclercq-Le Quilicet *et al.*, 1995; Leclercq-Le Quilicet *et al.*, 2000), Great Britain (Barker, 1990; Plumb, 1990), Spain (Comas *et al.*, 1996) and Hungary (Pocsai *et al.*, 1995). The dominance of BYDV-PAV may be attributed to rapid population growth rate of its vector, *Rhopalosiphum padi*. Very limited infection with CYDV-RPV was also reported in this study. This could be due to two main factors: Firstly, this virus has a single aphid vector, *R. padi*, which restricts its competitiveness against BYDV-PAV, and secondly, the presence of small numbers of hosts for this virus. CYDV-RPV is mainly identified in ryegrass, as reported by Henry and Dedryver (1991), Henry *et al.* (1993), Kendall *et al.* (1996) and Mastari (1998). However, in Tunisia, ryegrass fields are rare.

The derived maximum likelihood phylogenetic tree illustrated in Figure 3 contains two large signifi-

cant clades (previously described as I and III), and two isolates (EU332330 and EF521850) were placed at the basis of these main clades. Isolate EU332330 from China was previously assigned to PAV-III subspecies, whereas isolate EF521850 was assigned to PAV-I (Wu *et al.*, 2011). The first large clade was heterogeneous in terms of origin of isolate sequences with isolates from Germany, China, Australia, Iran, Japan, Pakistan, Sweden and Tunisia. The second large clade is primarily populated by isolates of Chinese origin, all of which belong to subspecies PAV-III (formerly PAV-CN).

The Tunisian isolates formed a sub-cluster that is basal to the main Clade (identified by Wu *et al.*, 2011 as Clade I), together with ASL1 (AJ810418) from Germany and 109 (EF521828) from the USA. Wu *et al.* (2011) showed that isolates ASL1 (AJ810418) and 109 (EF521828) have highly mosaic genomes resulting from two recombination events (EF521828) or even three (AJ810418). Additionally, ASL1 (AJ810418) represents a particular case, since its 2862–3491 region came from viruses isolated from wheat and oat (Wu *et al.*, 2011). Thus, it is likely that Tunisian isolates are also recombinants.

As reported previously, recombination is a pervasive phenomenon among BYDV-PAV isolates (Bouli-la, 2011; Pagàn and Holmes, 2010; Wu *et al.*, 2011). The evolutionary process in BYDV-PAV is shaped by a combination of very frequent recombination and low rates of nucleotide substitution, itself a function of strong purifying selection operating on the three ORFs (Wu *et al.*, 2011).

Aligned sequences permitted identification of six haplotypes among six examined for the Tunisian BYDV-PAV CP gene. The haplotype diversity was estimated to be 1.000. The number of mutations = 9 with nucleotide diversity (Π_{JC}) reaching 0.00483. Although some Tunisian variants come from one locality, a high rate of polymorphism was observed. For example, in the case of D35 and D34 both coming from the “Cap bon” region, they had the greatest number of mutations (positions 305–307–406 and 422). This can be explained by host pressure. BYDV-PAV showed some evidence of population genetic structure, both at the geographic level and possibly at the host level. The current genetic structure can be explained by local geographic adaptation, and by host-driven adaptation (Wu *et al.*, 2011).

The similarity found between the six isolates can also be explained from the choice of the infected

samples for CP partial nucleotide sequencing. This was based on a common feature which is high field virulence levels. The severity of symptoms is characterized by severe dwarfism of host plants, yellowing of leaves, reduced tillering and abortion of ears (Qualset 1984). The viral transmission assays using these five PAV isolates on the sensitive Tunisian barley variety "Manel" have also led to the occurrence of the same types of symptoms under controlled conditions. These virulent strains will assure reliable selection of resistant lines in the national programme for barley improvement to select BYDV-PAV resistant genotypes.

To obtain a more complete picture of the diversity among isolates of BYDV-PAV isolates in Tunisia, more isolates should be sequenced and studied from different geographic locations and hosts, including cultivated and non-cultivated plants.

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