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A single-stranded conformational polymorphism (SSCP)-derived quantitative variable to monitor the virulence of a *Barley yellow dwarf virus*-PAV (BYDV-PAV) isolate during adaptation to the *TC14* resistant wheat line

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SUMMARY

A standardized single-stranded conformational polymorphism (SSCP) procedure is proposed as an alternative to the time-consuming biological characterization of *Barley yellow dwarf virus*-PAV (BYDV-PAV) isolates. Using this procedure, six of 21 overlapping regions used to scan the viral genome gave patterns specific to '4E' (avirulent) or '4T' ('4E'-derived virulent) isolates. The calibration of samples and integration of SSCP patterns corresponding to the nucleotide region 1482–2023 allowed the estimation of P_T values that reflect the proportions of a '4T'-specific band. Analysis of the biological (area under the pathogen progress curve) and molecular (P_T) data suggested a positive linear relation between these variables. Moreover, sequence analysis of the nucleotide region 1482–2023 highlighted the presence of a nucleotide polymorphism (C/A_{1835}) which can be considered as a candidate for virus–host interactions linked to the monitored virulence. According to these parameters, P_T values associated with '4E'- and '4T'-derived populations show that: (i) long-term infection of a BYDV-PAV isolate on the '*TC14*' resistant host leads to the fixation of virulent individuals in viral populations; and (ii) the introduction of susceptible hosts in successive '*TC14*' infections results in the maintenance of low virulence of the populations. Thus, the presented study demonstrates that SSCP is a useful tool for monitoring viral populations during the host adaptation process. The described impact of host alternation provides new opportunities for the use of the '*TC14*' resistance source in BYDV-resistant breeding programmes. This study is part of the global effort made by the scientific community to propose sustainable alternatives to the chemical control of this viral disease.

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INTRODUCTION

Barley yellow dwarf virus-PAV (BYDV-PAV) is the type member of the genus *Luteovirus* (family *Luteoviridae*; D'Arcy *et al*., 2000) and infects a wide host range belonging to the Poaceae family (e.g. wheat, barley, maize, oat and pasture grasses). BYDV-PAV is transmitted by aphid vectors (mainly by *Rhopalosiphum padi* L. and *Sitobion avenae* Fabricius; Plumb and Johnstone, 1995) in a persistent, circulative and nonpropagative manner (Gray and Gildow, 2003; Miller and Rasochova, 1997). The isometric BYDV-PAV particle of 25 nm in diameter contains a single-stranded positive-sense RNA genome of about 5.7 kb (Miller *et al*., 1988) with six open reading frames (Fig. 1A; ORF 1–ORF 6). ORFs 1 and 2 encode the viral polymerase (Miller and Rasochova, 1997). ORFs 3 and 5 code for the coat protein and read-through protein, respectively. In addition to their structural functions in the formation of particles (Martin *et al*., 1990), these two proteins are involved in several other functions, such as aphid-mediated transmission (Chay *et al*., 1996; Wang *et al*., 1995) and long-distance movement in the plant (Ziegler-Graff *et al*., 1996). ORF 4, an embedded ORF 3 sequence in a different frame, codes for a cell-to-cell movement protein (Miller and Rasochova, 1997). Finally, ORF 6 encodes a small protein with an as yet unknown function.

BYDV-PAV induces symptoms such as dwarfing, yellowing and reddening, and is responsible for important yield losses (5%–80% with an average of 30% during epidemic outbreaks) (Perry *et al*., 2000). Control methods against BYDV-PAV are mainly based on insecticide treatments to prevent plant infections from viruliferous aphids visiting cultivated fields. Indeed, epidemiological surveys have correlated the occurrence of severe epidemic outbreaks and aphid infestation on plants in early growth stages (Irwin and Thresh, 1990; Plumb, 1995). Moreover, the detection of viruliferous aphids (Fabre *et al*., 2003) can be performed in autumn on alate aphids caught in fields or

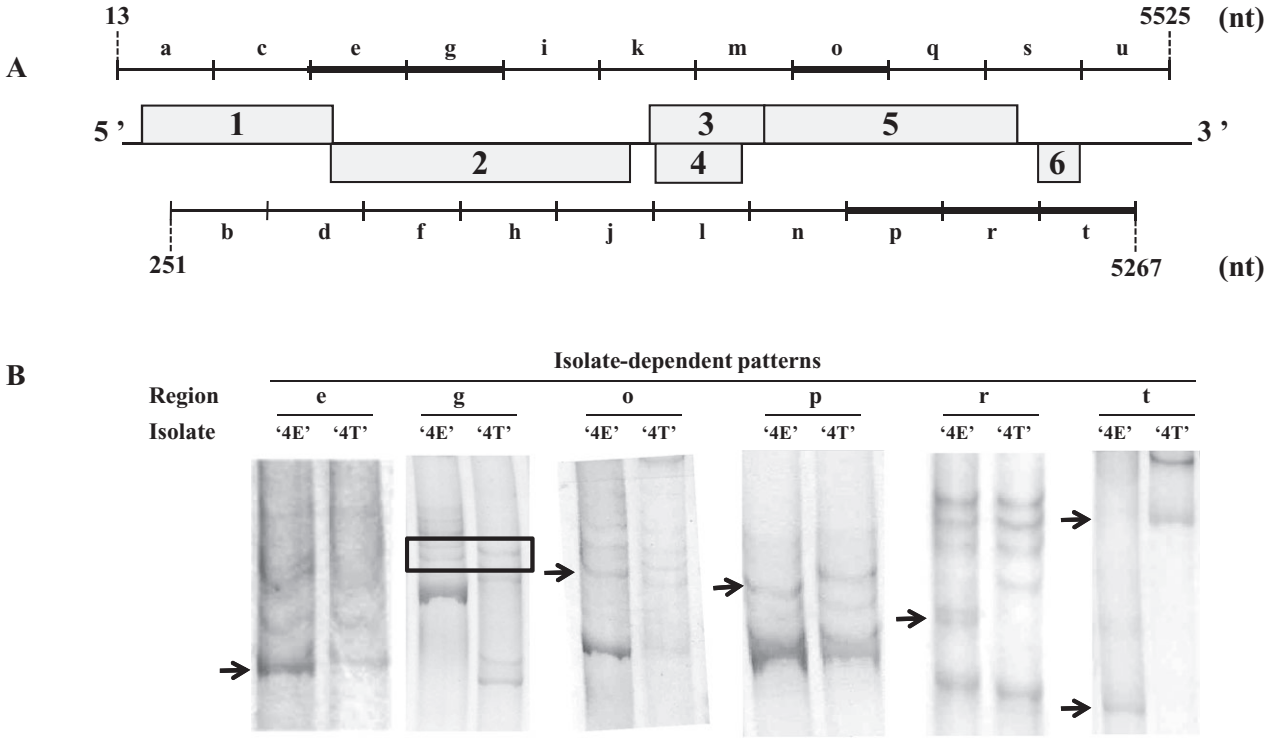

Fig. 1 Schematic representation of the *Barley yellow dwarf virus*-PAV (BYDV-PAV) genome and localization of regions (a–u) analysed using the single-stranded conformational polymorphism (SSCP) procedure (A), and illustration of isolate-dependent SSCP patterns (B). Open reading frames (ORFs) encoding BYDV-PAV proteins, presented in boxes, correspond to RNA polymerase (1 and 2), coat protein (3), movement protein (4), read-through protein (5) and P6 protein (6). The 5.7-kb RNA genome is illustrated by a thin line framed by the 5′ and 3′ ends. SSCP patterns associated with the six polymorphic regions (thick lines in A) are presented in (B) to illustrate band intensity variations (e.g. arrow in pattern e), addition or deletion of specific bands (e.g. arrows in patterns o, p and r) or band shifts (e.g. arrows in pattern t). The box in pattern g indicates the localization of the bands targeted for quantitative analyses.

through suction traps (Fabre *et al*., 2005). The resulting data can be efficiently used to: (i) build simulation models to predict the impact of treatment(s) on disease spread (Thackray *et al*., 2009); and (ii) inform cereal growers on the opportunity for chemical treatments. Together, these procedures help to reduce the amount of insecticides released to the environment. However, breeding for resistant or tolerant plants constitutes an alternative approach which is both less costly and less environmentally damaging than chemicals to manage plant diseases. Unfortunately, BYDV-PAV resistance has not been found in wheat, although thousands of accessions have been tested (Francki *et al*., 2001; Li *et al*., 1998; Qian *et al*., 1993). The only identified sources of BYDV-PAV resistance are from perennial Triticineae (e.g. *Thinopyrum* sp., *Lophopyrum* sp.) (Comeau and Plourde, 1987; Larkin *et al*., 1990; Xu *et al*., 1994). Transfers of these resistance sources have been initiated into bread wheat genotypes, and the '*TC14*' translocated line (Banks *et al*., 1995) carrying alien resistance genes is now available and well documented in the literature (Chain *et al*., 2005; Jahier *et al*., 2009). However, by monitoring the biological properties of a BYDV-PAV isolate during serial passages, Chain *et al*. (2007) observed that the '*TC14*' resistant line induces a selection of variant(s) with modified infection abilities, as demonstrated by the increase in the virulence of the adapted '4T' BYDV-PAV isolate obtained after as few as five passages on '*TC14*'. Moreover, this increased virulence of '*TC14*'-adapted BYDV-PAV was observed on both resistant and susceptible hosts. This conclusion results from statistical analyses of data (area under the pathogen progress curve, AUPPC) linked to biological properties of BYDV-PAV isolates. However, as the acquisition of such biologically linked data requires the monitoring of the kinetics of viral infections at each infection passage, the whole procedure is both time- and space-consuming. Consequently, several important questions (e.g. the fitness of virulent BYDV populations on susceptible hosts and the effect(s) of host alternations on the selection/maintenance of virulent BYDV populations) associated with the possible use of the '*TC14*' resistant line in BYDV-resistant breeding programmes need to be addressed before the deployment of improved material derived from the '*TC14*' resistant wheat line.

In this work, we argue that the modification of the biological properties of an isolate resulting from the host adaptation process could be considered as the phenotypic expression of

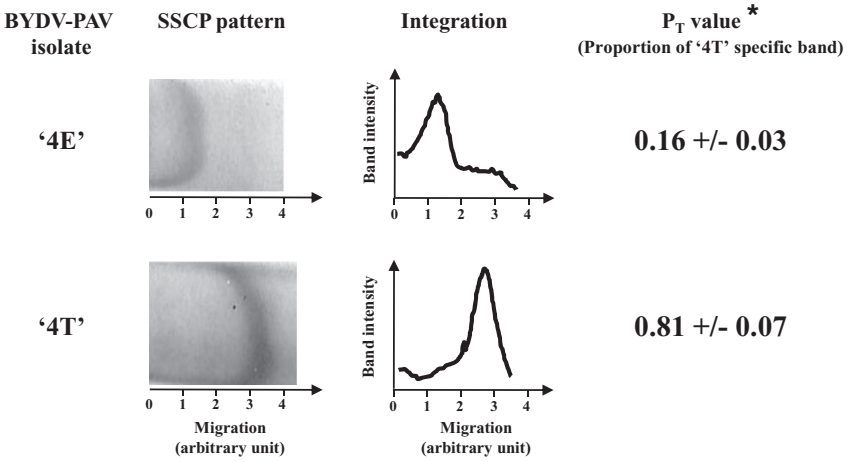

Fig. 2 Analysis of the '4E'- and '4T'-specific bands present in the single-stranded conformational polymorphism (SSCP) patterns obtained for the nucleotide region 1482–2023 of the *Barley yellow dwarf virus*-PAV (BYDV-PAV) genome. The presented SSCP patterns focus on a small area containing isolate-specific bands. Integration of the signals produced by the analysis of the SSCP area makes possible the calculation of the P_T value. *Presented P_T values result from replicates performed with independent samples.

modification(s) in the genetic background of the 'ancestral' isolate. Therefore, adaptation of viral populations could be efficiently described using molecular tools able to detect mutation(s). The monitoring of this phenomenon requires the use of detection/characterization tools that allow the identification of small variations within genomic sequences. During the last 20 years, numerous methods have been published for the detection of mutations (Fraile *et al*., 1996) and for the description of genomic variations (Lim *et al*., 2005; Rubio *et al*., 2001) and allelic differentiations (Kuhn *et al*., 2008; Monckton and Jeffreys, 1994; Sunnucks, 2000). These techniques are often based on time-consuming methods and are rarely adapted to the screening of numerous samples. Thus, the selection of the most appropriate method to study the genetic diversity of an organism is mainly determined by the characteristics of the molecular polymorphisms to be described. Indeed, selected technical approaches would not be necessarily the same for the localization of mutations in a whole genome (Padidam *et al*., 1995), the quantification of the number of polymorphic nucleotides (Kearney *et al*., 1999; Orita *et al*., 1989a) or the identification of specific mutations in targeted sequences (Balme-Sinibaldi *et al*., 2006; Rolland *et al*., 2008). Among these tools, single-stranded conformational polymorphism (SSCP) can be considered as an attractive method for this objective and was used to monitor both the kinetics of BYDV adaptation to the '*TC14*' line and the consequences of host alternations (resistant/susceptible) on the monitored adaptation process. The results of this study must be considered in the breeding programmes performed with BYDV resistance gene(s) present in the '*TC14*' wheat line.

RESULTS

SSCP analyses of the BYDV-PAV '4E' and '4T' genomes

The quantification of nucleic acids, standardization of electrophoresis and staining of single-stranded DNA molecules were used to describe additional or deleted bands, shifts in band positions and the modifications of band intensities in SSCP patterns. Applied to the BYDV-PAV genome, the SSCP procedure used in this study produced distinct patterns for each of the 21 overlapping tested regions (Fig. 1A). In addition to these region-specific patterns, isolate-specific polymorphisms were observed for six regions (Fig. 1A,B). Among the observed differences, analyses were focused on the SSCP patterns associated with DNA fragments corresponding to a region (nucleotides 1482–2023) that overlaps with ORF 2 (polymerase) of the viral genome (Fig. 1A, region g). Indeed, patterns associated with this region present, in a small area of the polyacrylamide gel, '4E'-specific and '4T'-specific bands which can be used to assign SSCP patterns to the corresponding BYDV-PAV isolate (Fig. 1B, pattern g). This small area of the SSCP patterns was integrated using Image J® software (National Institutes of Health, Bethesda, MD, USA) to generate a line graph of band intensity (Fig. 2). The intensities of the peaks located at the positions of the two isolate-specific bands were calculated. The data produced could be used to estimate the value P_T, which describes the viral population according to the proportion of '4T'-specific band in the sample. Therefore, the increase in P_T values during the serial passage experiments (SPEs) initiated with '4E' isolate indicates the increase in the proportion of the '4T'-specific band in the viral population. The P_T values associated with '4E' and '4T' isolates were 0.16 ($\pm$0.03) and 0.81 ($\pm$0.07), respectively. This observation could be considered as the molecular expression of the adaptation of the initial '4E' isolate to the BYDV-resistant '*TC14*' host.

Biological and molecular characterization of BYDV-PAV '4E'- and '4T'-derived populations

The 14 '4E'- and the 14 '4T'-derived BYDV-PAV populations produced in SPE procedures were characterized at both the biological (AUPPC) and molecular (P_T value) levels. The

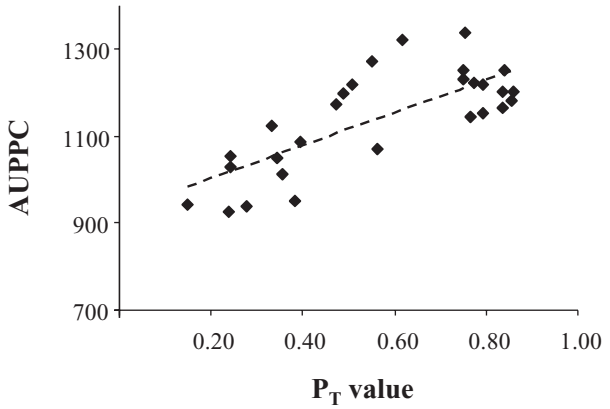

Fig. 3 Correlation between biological characterization (area under the pathogen progress curve, AUPPC) and molecular analysis (P_T value) performed with *Barley yellow dwarf virus*-PAV (BYDV-PAV) populations resulting from serial passage experiments (SPEs). Each point corresponds to data associated with a viral population. The broken line illustrates the linear regression between the variables ($P < 0.001$).

AUPPC data for the viral populations issued from host alternation-based SPE procedures ranged from 900 to 1320. P_T values estimated for these viral populations ranged from 0.15 to 0.86. These data, which partially described the characteristics of the pathogens present in infected samples, were analysed to explore the possible relationship between the two calculated variables (Fig. 3). Using a linear regression, a significant relationship was found ($R^2 = 0.5731$, $P < 0.01$), illustrating that the calculated P_T values seem to be linked to the virulence of the corresponding viral populations in the '*TC14*' resistant wheat line. Finally, the analysis of the AUPPC values associated with populations described by P_T values below or above 0.5 (mean AUPPC = 1040 $\pm$ 93 and 1214 $\pm$ 67, respectively) revealed a significant difference ($P < 0.001$) between these two groups of samples. This suggested that $P_T = 0.5$ could be considered, under our experimental conditions, as a threshold for the analysis of the virulence of the monitored BYDV-PAV populations.

Identification of molecular determinants linked to the monitored virulence

The correlation between biological characterization (AUPPC) and molecular analysis (P_T value) performed with BYDV-PAV populations suggested the presence of molecular determinant(s) linked to virulence in the 1482–2023 region of the viral genome. Alignment of the '4E' and '4T' nucleotide sequences revealed only two nucleotide polymorphisms (C/T_{1727} and C/A_{1835}) in this region (Table 1). These nucleotide variations in the viral genome did not induce amino acid changes (isoleucine$_{192}$ for C/T_{1727} and glycine$_{228}$ for C/A_{1835}) in the RNA polymerase protein encoded by the BYDV-PAV ORF 2. The sequence obtained for the '4E' isolate ($P_T = 0.16 \pm 0.03$) contained C_{1727} and C_{1835} nucleotides. Nucleotides T_{1727} and A_{1835} have been observed in the genomes of the '4T' isolate ($P_T = 0.81 \pm 0.07$), a '4T'-derived population ($P_T = 0.73$) maintained in the susceptible host (barley cv. Express) and in the genome of a '4E'-derived isolate obtained after four passages on the resistant wheat line '*TC14*' ($P_T = 0.73$). To complete this molecular analysis, the 1482–2023 genomic region from a viral population produced during SPE procedures based on host alternations has been sequenced. The selected '4E'-derived BYDV population was associated with $P_T = 0.53$. Despite the high quality of the sequencing procedure, the identity of nucleotides 1727 and 1835 in the genome of this BYDV-PAV population could not be assigned. Indeed, the corresponding positions in the electropherograms were associated with double peaks corresponding to C/T at position 1727 and C/A at position 1835. This result was consistent with the P_T value of this population, as $P_T = 0.53$ reflects the presence of the '4E'- and '4T'-specific bands in equivalent proportions.

Monitoring of viral adaptation using the P_T values

P_T values were calculated for the viral populations produced passage by passage with BYDV-PAV '4E' and '4T' isolates maintained on the resistant wheat line '*TC14*' and on susceptible barley cv. Express, respectively (Fig. 4A). The molecular characterization of the '4E' and '4T' isolates and of the viral populations produced after the first two passages was repeated 3–11 times using infected materials from independent SPE procedures for each characterization. The molecular descriptions of the viral populations produced at passages 3, 4, 7 and 8 were only performed once, except for the '4T'-derived population obtained after three passages on barley, which was subjected to a triplicate analysis. The mean P_T value of the '4E' isolate maintained on '*TC14*' was not modified after the first passage on the '*TC14*' resistant host ('4E': $P_T = 0.16$ (± 0.03); '4E'-derived population at passage 1: $P_T = 0.20$ (± 0.09); $P = 0.531$), whereas a second passage induced an increase in the P_T value to 0.64 (± 0.10; $P < 0.0001$), thus rising above the previously defined $P_T = 0.5$ threshold. The next passages of the '4E'-derived populations on the '*TC14*' line were associated with P_T values close to 0.8 (ranging from 0.74 at passage 4 to 0.88 at passage 8). Similar analysis performed with '4T' and '4T'-derived populations resulting from passages performed on the susceptible barley cv. Express showed P_T values close to 0.8 for all tested samples. No statistical differences ($P = 0.328$) were noted between P_T values associated with the '4T' isolate ('4T': $P_T = 0.81 \pm 0.07$) and '4T'-derived populations produced after the first ($P_T = 0.75 \pm 0.11$) and second ($P_T = 0.79 \pm 0.06$) passages on barley. During SPE procedures based on host alternations, infected leaves were collected for the molecular characterization of the '4E'-derived populations produced at

Table 1 Comparison of P_T values and polymorphisms of nucleotides 1727 and 1835 for *Barley yellow dwarf virus*-PAV (BYDV-PAV) isolates and populations resulting from serial passage experiments (SPEs).

BYDV-PAV isolates or populations*	P_T value	Nucleotide identity	
		Position 1727	Position 1835
'4E'†	0.16 ± 0.03	C	C
'4T'†	0.81 ± 0.07	T	A
'4T'-derived (from 'Express')	0.73	T	A
'4E'-derived (from '*TC14*')	0.79	T	A
'4E'-derived (from host alternation)	0.53	C/T	C/A

*'from "Express" ', 'from "TC14" ' and 'from host alternation' correspond to the maintenance of the viral population on 'Express', '*TC14*' line or 'Express'/'*TC14*' host alternation during SPE procedures, respectively. †Molecular characterization of original '4E' and '4T' isolates results from at least three independent experiments performed during a 2-year period.

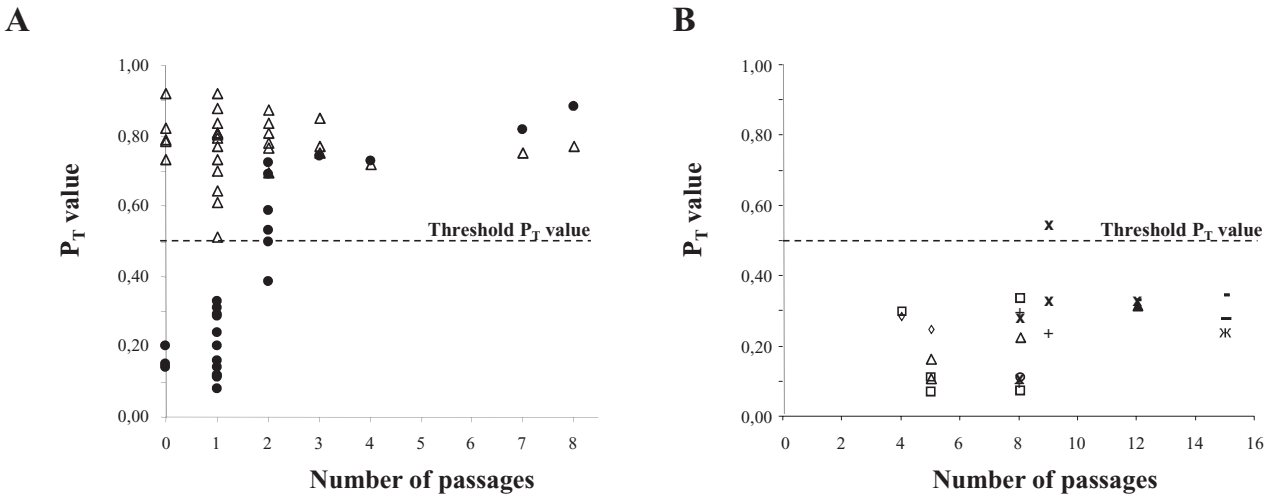

Fig. 4 P_T values calculated for *Barley yellow dwarf virus*-PAV (BYDV-PAV) populations obtained at different passages during serial passage experiments (SPEs) based on the use of one type of host (A) or on host alternations (B). The P_T values of the initial isolates '4E' and '4T' and of '4E'- and '4T'-derived viral populations produced after one to four, seven and eight passages on '*TC14*' and 'Express', respectively, are presented in (A). ●, data associated with '4E' and '4E'-derived populations; Δ, data associated with '4T' and '4T'-derived populations. The P_T values of the '4E'-derived populations produced during host alternation procedures are presented in (B). The different symbols indicate the total number of passages performed on '*TC14*' (◇, one passage; □, two passages; Δ, three passages; X, four passages; ○, five passages; +, six passages; –, seven passages, —, eight passages; ж, nine passages; ▲, 10 passages) according to the host alternation procedure used. The broken lines correspond to the previously defined threshold P_T value ($P_T = 0.5$).

different passages on the '*TC14*' line from passages 3–14. P_T values associated with these populations ranged from 0.07 to 0.55 (Fig. 4B). According to the SPE protocols, these '4E'-derived populations resulted from host alternation pathways with 1–10 passages on the '*TC14*' resistant host. However, statistical analysis showed that the P_T values associated with these populations were not linked to the number of passages on the '*TC14*' host. Moreover, the mean P_T value [0.257 (±0.116)] obtained for '4E'-derived populations resulting from host alternation SPE procedures with three or more passages on the '*TC14*' host was significantly different from the P_T value observed after three consecutive passages of the '4E' isolate on the '*TC14*' resistant line ($P < 0.001$).

DISCUSSION

A standardized SSCP procedure was developed as an alternative to the time-consuming biological characterization of viral isolates during the host adaptation process [e.g. estimation of AUPPC (Chain *et al*., 2005), expression and severity of symptoms (Ebert, 1998; Li and Roossinck, 2004; Mandal *et al*., 2006; Tan *et al*., 2005)]. As shown in our results, SSCP patterns can be made up of numerous bands. These bands can either result from equilibria between different stable conformational structures for a DNA fragment or reflect the presence in the analysed sample of heterogeneous molecules (Ayllón *et al*., 1999; Orita *et al*., 1989b; Rubio *et al*., 2001; Turturo *et al*., 2005). To improve the

reliability of SSCP analyses and to make possible the efficient qualitative and quantitative description of emerging sequences in nucleic acid populations, the presented standardized SSCP procedure includes the calibration of the amount of DNA molecules loaded on a polyacrylamide gel. Moreover, the integration of the resulting SSCP patterns using the software Image J® allows both a confident analysis of the distance migration and the estimation of the quantity of each band in the patterns produced. This quantitative approach of the SSCP protocol, not frequently applied in viral phytopathology, has long been used in medical biology. As an example, Liu *et al*. (2000) took advantage of both a scanning method with a gel densitometer to compare distance migrations in SSCP patterns and an estimation of the optical density of peak heights to control the quality of X-ray exposure. Similarly, Gelfi *et al*. (1997) compared, using a quantitative approach, the proportion of wild-type and mutated p53 gene in tumour cells. Thus, the quantification of the intensity of bands in SSCP patterns allows the monitoring of the progress of molecular changes in the tested samples which fits perfectly with our objectives. Applied to our genetically linked BYDV-PAV isolates, this technique made it possible to identify polymorphisms between initial (low virulent '4E') and adapted/evolved (virulent '4T') BYDV-PAV isolates in six of the 21 overlapping regions of 500 bases in length used to scan the 5700 bases of the viral genome. These polymorphic regions are localized in the polymerase gene (ORF 2), the read-through coding sequence (ORF 5) and the 3′-end part of the genome (including ORF 6). Moreover, except for two regions in ORF 5, the polymorphic regions do not overlap with each other, illustrating the importance of different screening scales to detect isolate-specific patterns.

Among the isolate-specific patterns obtained in this study, we decided to go further in the analysis of the nucleotide region 1482–2023 located in the polymerase ORF 2 coding sequence. The BYDV-PAV resistance source present in the '*TC14*' genotype is known to reduce: (i) the efficiency and development rate of infection; and (ii) the virus load in infected plants (Chain *et al*., 2005). Thus, the polymerase is a good candidate for genetic modifications, supporting the increased virulence of the '4T' isolate through an improved amplification of the viral genome during the infection process. However, the involvement of other described polymorphic regions in the monitored host adaptation process cannot be ruled out prior to being tested. As an example, this can be considered for polymorphisms observed for regions in the ORF coding for the read-through protein, which is well known in *Luteoviridae* to be involved in long-distance virus movement in plants (Ziegler-Graff *et al*., 1996). Indeed, previous studies have demonstrated that the resistant behaviour of '*TC14*' plants could also be partly linked to virus movement in plants, as suggested by the efficient detection of BYDV-PAV in roots of almost all inoculated '*TC14*' plants (Chain *et al*., 2005). Thus, variations in this ORF could produce variants with increased ability to migrate within the plant, allowing the spread of the infection from roots to aerial parts of the host.

According to our SSCP results, the nucleotide region 1482–2023 should contain genetic variation(s) that could support the modification of the biological properties observed between '4E' and '4T' isolates. As demonstrated by the sequence data produced, the polymorphism of this region is linked to synonymous point mutations (C/T_{1727} and C/A_{1835}) in the coding sequence of the 5′-terminal part of ORF 2. In the absence of amino acid changes, the impact of these nucleotide variations on the biological properties of the BYDV-PAV isolate cannot correspond to the modification of polymerase function. However, in addition to changes in coding sequences, silent mutations have already been reported to be involved in the biological properties of viral pathogens (Cuevas *et al*., 2002). Thus, the described mutations C/T_{1727} and C/A_{1835} could, as revealed by the SSCP data, have an impact on the folding of the viral RNA, which is known to be extremely important for the different transcription/translation steps during the viral cycle (Miller *et al*., 2002). To complete this analysis, it is important to note that, in 2003, the original '4E' and '4T' isolates were included in a BYDV-PAV full-length sequencing programme. The sequences produced were submitted in 2005 to the GENBANK database with the accession numbers DQ079609 and DQ079612, respectively. Alignment of these sequences in the nucleotide region 1482–2023 revealed, as observed with the '4E' and '4T' isolates used in this study, two nucleotide polymorphisms. However, the locations of the latter were conserved only for C/A_{1835}, as the second denoted nucleotide modifications in the analysed sequence changes from A/G_{1798} (in 2003's isolates; full-length sequencing project) to C/T_{1727} (in 2006's isolates; this study). The observed difference in nucleotide variation suggests that, of the two nucleotide variations identified in the nucleotide region 1482–2023, the C/A_{1835} polymorphism should be considered as a candidate for virus–host interactions linked to BYDV-PAV virulence monitored in wheat, and constitutes a favourable target for future studies on viral adaptation/durability of resistance involving '*TC14*' plants as hosts.

The analyses of the recorded biological (AUPPC) and molecular (SSCP-derived P_T values associated with '4E'- and '4T'-specific bands) data suggest a positive linear relation between virulence and polymorphism observed in the nucleotide region 1482–2023 of the corresponding viral populations. Moreover, the analysis of the dataset makes it possible to propose that a BYDV-PAV population with a P_T value above the 0.5 threshold can be considered as virulent (or adapted to '*TC14*' resistance). According to these parameters, the serial passage of the BYDV-PAV '4E' isolate on the '*TC14*' host, monitored with the P_T value, is associated with the emergence of a specific DNA molecule that becomes prevalent (above the virulence threshold $P_T = 0.5$) in the viral population after three passages (P_T close to 0.8 from passages 3–8).

This is consistent with a previously published study on the evaluation of the durability of the BYDV-resistant '*TC14*' line (Chain *et al*., 2007). Indeed, in this paper, the authors reported that the modification of the biological properties (increase in AUPPC) of the viral populations can be observed from the fifth serial passage of BYDV-PAV '4E' on '*TC14*' plants until the last (number 110) monitored passage. In addition to the '*TC14* adaptation process', our data from serial passages of the '4T' isolate on the susceptible barley cv. Express described the maintenance of the '4T'-specific band in the viral populations for eight passages. This suggests that the molecular changes in the viral population (from the emergence to the prevalence of the '4T'-specific band), induced by passages on resistant '*TC14*' hosts, are not reversible in the absence of the resistance gene of '*TC14*'. This contrasts with similar studies performed with other host–virus pathosystems, where the variants selected on resistant hosts are generally replaced by the avirulent isolate when the resistance pressure is removed from the passage experiments (Acosta-Leal *et al*., 2008; Desbiez *et al*., 2003; Giraud *et al*., 2001; Poulicard *et al*., 2010). However, one of the main differences between these types of data and our study is linked to the fact that the '4T' isolate used as initial source in our experiments results from long-term maintenance involving more than 100 serial passages of the initial '4E' isolate on the '*TC14*' host. Thus, the maintenance (more than 5 years) of the '4T' (i.e. '4E'-derived) viral population on the resistant '*TC14*' host could have selected for epistatic mutation(s) in the viral sequence that increases the fitness of the emerging virulent variant. As a consequence, the impact of host alternations (resistant/susceptible) on the modifications of BYDV-PAV properties was analysed using the '4E' isolate as initial viral source. Under these experimental conditions, the proportion of the '4T'-specific band in the '4E'-derived viral populations produced during SPE was maintained below the defined threshold P_T value ($P_T = 0.5$) whatever the number of passages (up to 10) on the resistant '*TC14*' host. This suggests that, rather than the total number of passages, the number of successive passages performed on the resistant '*TC14*' host seems to be the important parameter for the maintenance of virulent BYDV-PAV 'entities' in viral populations at an acceptable level when '*TC14*'-derived resistant hosts are introduced in the history of BYDV infections.

In conclusion, this study demonstrates that SSCP is a useful tool to monitor, using the polymorphism of the viral sequences, the evolution of populations during the host adaptation process. The very simple and rapid protocol proposed is particularly adapted to this type of investigation as it saves both time and money for the description of characteristics of numerous samples (e.g. series of related mutants resulting from different maintenance histories). Indeed, a simple SSCP scan of the targeted genomes makes possible the identification of semiquantitative molecular marker(s). After the validation of a correlation between the defined molecular marker(s) and virulence with a set of reference isolates, numerous isolates can be analysed easily in few SSCP runs. Moreover, as it is based on a molecular analysis of the pathogen, the procedure makes it possible to simultaneously test samples collected at different periods of time (e.g. from different passages), which facilitates the analyses of kinetics in the evolutionary processes. Thus, applied to our biological model, the proposed SSCP-based analysis of the evolution of BYDV-PAV biological properties has demonstrated that: (i) long-term maintenance of a BYDV-PAV isolate on the '*TC14*' resistant host leads to the fixation of virulent individuals in the population; and (ii) the introduction of susceptible hosts between successive '*TC14*' infections seems to maintain the virulence of the populations produced at a low level. The observed impact of alternate (BYDV-susceptible) hosts on BYDV virulence provides new opportunities for the use of the '*TC14*' resistance source in BYDV-resistant breeding programmes. Indeed, according to the fact that, in addition to '*TC14*', only three resistance sources against BYDV are available in wheat ['*P29*', a substitution line (Sharma *et al*., 1995); '*Zhong ZH*', a ditelosomic addition line (Barloy *et al*., 2003); '*OK721154*', a partial amphiploid line (Chen *et al*., 1998; Comeau *et al*., 1994)], it is very important to characterize in detail these resistance sources. Moreover, we need to evaluate the impact associated with their possible deployment in the field on the virulence/aggressiveness of BYDV isolates prior to including/excluding them from wheat improvement projects. The work presented in this paper is part of the global effort made by the scientific community working on BYDV to propose a sustainable alternative to the chemical control of this viral disease.

EXPERIMENTAL PROCEDURE

Viral isolates and SPEs

Two BYDV-PAV isolates were used as 'initial' material in the presented work. BYDV-PAV 4E, originally collected on oat cv. Peniarth in 1989 (Ille-et-Vilaine, France), has been maintained on barley cv. Express since its collection. BYDV-PAV 4T, a more aggressive (Chain *et al*., 2006) and virulent variant of BYDV-PAV, has been obtained from long-term maintenance of the BYDV-PAV 4E isolate on the resistant wheat line '*TC14*' (Chain *et al*., 2007). These BYDV-PAV isolates ('4E' and '4T') were individually used to initiate SPE procedures including host alternations. Each SPE procedure was based on 30 passages performed on either BYDV-susceptible barley cv. Express or resistant wheat line '*TC14*'. In host alternation experiments, the number of successive passages on barley or on wheat was different for each SPE procedure, and ranged from one to three. The different SPE procedures applied to '4E' and '4T' isolates made it possible to produce 14 '4E'- and 14 '4T'-derived BYDV-PAV populations,

each resulting from a particular biological (host alternation) pathway. In addition to the 'host alternation' procedures, SPE corresponding to 30 serial passages of '4E' isolate on '*TC14*' wheat line and to 30 serial passages of '4T' isolate on barley cv. Express were performed to reproduce previously published '*TC14*' adaptation (Chain *et al*., 2007), and to test the effect of serial passages of the adapted '4T' isolate on the susceptible barley host, respectively. Viral transmissions (host transfers) were performed every 3 weeks, as described by Chain *et al*. (2007), using the efficient BYDV vector *R. padi* RP1 clone. Experimental procedures were carried out under controlled conditions (18 °C, 16 h–8 h light–dark cycle) in a growth chamber.

Determination of AUPPC

The biological property (virulence) of the BYDV-PAV populations produced by SPE procedures was estimated on the resistant wheat line '*TC14*' using the AUPPC parameter. Briefly, third or fourth viruliferous instar larvae of *R. padi* RP1 clone, obtained by parthenogenetic reproduction of RP1 aphid females on BYDV-infected plants (from passages of the SPE procedures described above), were deposited at the base of 20 healthy '*TC14*' plants (three aphids per plant). The inoculated plants were then covered with microperforated cellophane bags. Five days later, aphids were killed by spraying plants with insecticide (λ-cyhalothrine, 1 mg/mL; Karaté®, Syngenta agro, Basel, Switzerland). The percentages of infected plants were monitored using semiquantitative enzyme-linked immunosorbent assay (ELISA) performed 7, 11, 14, 17 and 21 days after inoculation (DAI), as described by Chain *et al*. (2005). Using data from the infection kinetics, the AUPPC associated with the first 21 days of infection was calculated for each tested population. AUPPC values were calculated using a formula $\sum_{i=1}^{n}\left[\frac{Y_{i-1}+Y_i}{2}\right][D_i - D_{i-1}]$ derived from the area under the disease progress curve (Jeger and Viljanen-Rollinson, 2001; Nutter, 1997), where the variable Y_i is the percentage of infected plants and D_i is the number of DAI at the *i*th observation. This biological characterization was performed: (i) at least three times using materials from independent experiments for both '4E' and '4T' isolates, employed as reference isolates in this study; and (ii) only once for '4E'- and '4T'-derived BYDV-PAV populations produced during the 14 different SPE procedures with host alternations.

Nucleic acid extraction and BYDV genome amplification

In addition to the infection kinetics used to estimate the virulence of the BYDV-PAV populations (see above), leaves from infected plants were sampled at different passages of the SPE procedures for molecular analyses. Total RNAs were extracted from each sample of material using the SV-RNA extraction kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Five microlitres of the RNA fractions produced were used as template for reverse transcription of the viral genome. Final concentrations in the reverse transcription mix were 2 × buffer (provided with enzyme), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 2 U/μL RNasine (Promega), 1 U/μL AMV Reverse Transcriptase (Promega) and 1 mM 3ter-end primer ($_{5565}$-5′-CAGGATTGCTATGGTTTATGTCCATCTCCTG-3′-$_{5535}$). Equal volumes of sample and mix solution were pooled and incubated for 1 h at 42 °C. The cDNA produced was stored at −20 °C or immediately used for amplification reactions. Twenty-one overlapping regions (500 nucleotides in length; regions defined between nucleotides 13–558, 251–805, 497–1051, 751–1321, 987–1539, 1247–1789, 1482–2023, 1731–2306, 1953–2542, 2230–2808, 2471–3026, 2734–3270, 2969–3522, 3215–3798, 3467–4043, 3732–4284, 3983–4531, 4228–4784, 4457–5015, 4730–5267, 4951–5525) of the viral genome were amplified (Fig. 1A, regions a–u) using appropriate primer pairs (Table S1, see Supporting Information) according to the sequence published by (Miller *et al*. (1988), available in the GenBank database with the accession number X07653. As an example, the forward primer $_{1482}$-5′-GTAGGGGATAAATTCAAACTCGACATTG CAAGCA-3′-$_{1505}$ and the reverse primer $_{2023}$-5′-GATCCTTCCT TGTTTGAAGTTGTCATAGCCAG-3′-$_{2000}$ were used to amplify the region defined between nucleotides 1482 and 2023.

Polymerase chain reactions (PCRs) were performed with 2 μL of cDNA added to 23 μL of a mix solution containing 1 × buffer (provided with enzyme), 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 2 U/μL AmpliTaq® DNA Polymerase (Applied Biosystems, Carlsbad, CA, USA) and 0.8 mM of a primer pair. Amplification conditions corresponded to 5 min at 94 °C followed by 40 cycles of 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C. A final elongation step was added (10 min, 72 °C) after the last PCR cycle. Five microlitres of each PCR product and 5 μL of calibrated DNA smart ladder© (Eurogentec, Seraing, Belgium) were loaded onto 1% agarose gel in 1 ×TBE (Tris-Boric-EDTA buffer, pH 8.2, Invitrogen, NV Leek, The Netherlands) in the presence of 0.6 mg/mL SybrSafe™ (Invitrogen) to confirm, under ultraviolet transillumination, the presence of the expected 500-bp DNA products. Using Image J® software, integration of the fluorescent signals associated with each fragment present in the DNA ladder used made it possible to produce a 'fluorescent vs. concentration' calibration curve. This calibration curve allows the quantification of amplified DNA fragments present in PCR fractions.

Molecular characterization using SSCP analysis and estimation of the P_T value

SSCP analyses were performed using a calibrated protocol. Briefly, the polymorphism detection was realized on a 3.5% nondenaturing polyacrylamide gel (40% acrylamide–

bisacrylamide, 19 : 1; Eurobio, Gentilly, France). Running conditions were fixed at 10 V/cm at 4 °C in 1 × TBE. The acrylamide gel was run for 1 h before loading the samples. According to the gel quantification results, the concentrations of the 500-bp DNA fragments in PCR fractions were adjusted to 0.75 ng/μL in formamide (Merck, Darmstadt, Germany) containing 5 M EDTA, xylene cyanol (5 mg/L) and bromophenol blue (5 mg/L). The fractions produced were denatured for 5 min at 95 °C and then immediately placed in wet ice. Eight microlitres of each sample were loaded on the gel. After 18 h of migration, DNA molecules present in the gel were stained using silver nitrate. Then, the gels were dried and the SSCP patterns produced were digitized and analysed using Image J® software. Integration of the signals produced by the analysis of the SSCP area makes possible the calculation of the P_T value (P_T = intensity of peak$_{(\text{position for '4T'-specific band})}$/(intensity of peak$_{(\text{position for '4T'-specific band})}$ + intensity of peak$_{(\text{position for '4E'-specific band})}$)), which represents the proportion of the '4T'-specific band in the analysed sample.

Sequencing of BYDV isolates

PCR products corresponding to the amplification of the 1482–2023 region from '4E' and '4T' BYDV isolates were sent for sequencing to Eurofins-MWG Operon (Ebersberg, Germany). Sequencing reactions were performed using forward (nucleotide 1482–1505) and reverse (nucleotide 2023-2000) primers. Each sequenced region was obtained from at least three independent experiments (from '4E'- or '4T'-infected plants sampled to sequencing steps). A BYDV isolate resulting from a SPE procedure with host alternations was introduced into the sequencing procedure. Sequence analyses (nucleotides and amino acids) were performed with Bioedit® software (Hall, 1999). Nucleotide positions listed in the article refer to those described in Miller *et al*. (1988).

Statistical analyses

Statistical analyses were performed using XLstat-Pro (v 6.02; Addinsoft, Paris, France). The effects of the number of SPE passages were evaluated by comparison of the distribution of the respective P_T values with the Kolmogorov–Smirnov test.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Sequences of forward and reverse primers used to amplify the 21 overlapping regions (regions a–u, see Fig. 1) of the *Barley yellow dwarf virus*-PAV (BYDV-PAV) genome. For each region, the characteristics of the primers used during polymerase chain reaction (PCR) are indicated. Nucleotide positions are according to Miller *et al*. (1988).

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