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Evaluation of the durability of the *Barley yellow dwarf virus*-resistant *Zhong ZH* and *TC14* wheat lines

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Abstract

Serial passage experiments (SPE) of a *Barley yellow dwarf virus*-PAV (BYDV-PAV) isolate were performed on *Zhong ZH* and *TC14* wheat lines to evaluate the durability of their resistance to BYDV. At different passage numbers (from the 2nd to the 114th), biological properties of the produced isolates were recorded either by monitoring infection percentages and virus titers of the first 3 weeks of viral infection or by measuring their impact on yield components. Statistical analyses using the area under pathogen progress curves and the area under concentration progress curves demonstrated that these two resistant lines induce, after only a few passages, a selection of variant(s) with significantly modified infection abilities. Isolates resulting from SPE performed on these lines induced important decreases of yield components. These results indicate that the use of *Zhong ZH* and *TC14* lines in BYDV-resistant breeding programmes should be approached with caution.

Introduction

Barley/Cereal yellow dwarf viruses (B/CYDVs, family *Luteoviridae*) correspond to a complex of eight virus species able to infect all members of the *Poaceae* family. These viruses induce barley yellow dwarf disease (BYDD), one of the most important viral diseases in small-grain cereals (D'Arcy and Burnett, 1995). The main symptoms of BYDD consist of dwarfing, yellowing and other physiological alterations. According to numerous biotic and abiotic factors (Collin et al., 1997; Gruntzig and Fuchs, 2000), BYDD induces yield losses ranging from 5 to 80% with an average of 30% in infected fields (Perry et al., 2000).

BYDV-PAV is the most widespread B/CYDV species in western Europe (Henry et al., 1993). The isometric BYDV-PAV particle of 25 nm in diameter contains a single-stranded positive-sense RNA of about 5.7 kb encoding six open reading frames (Miller et al., 1988). Like all *Luteoviridae* viruses, BYDV-PAV is transmitted by aphids in a persistent manner (Gildow and Rochow, 1980).

Control methods against BYDD are mainly based on insecticide treatments. However, the use of resistant or tolerant plants is both less costly and less environmentally damaging. High levels of B/CYDV resistance have not been found in wheat although thousands of accessions have been tested (Li et al., 1998). Only low levels of B/CYDV

resistance and some sources of tolerance (e.g. *Bdv1* gene in wheat) have been found in annual *Triticineae* (e.g. *Triticum*, *Aegilops*) (Singh et al., 1993). Some sources of B/CYDV resistance identified from perennial *Triticineae* (e.g. *Thinopyrum* spp., *Lophopyrum* spp.) have been transferred to bread wheat genotypes. *Zhong ZH*, a ditelosomic addition line derived from Zhong 5, a partial amphiploid, (Barloy et al., 2003) and *TC14*, a translocated line (Banks et al., 1995), are now available. Their biological and physiological properties are well documented in the literature (Nie et al., 1996; Tang et al., 2000; Ayala et al., 2001; Chain et al., 2005). However, prior to being extensively used in breeding programmes to produce BYDV-resistant bread wheat cultivars, resistance carried by *Zhong ZH* and *TC14* lines must be tested for durability. In this study, serial passage experiment (SPE) procedures were performed in controlled conditions, using *Zhong ZH* and *TC14* partial resistant hosts, to test the effect of successive passages of the pathogen on the resistant lines and to estimate the durability of their resistance. The biological properties of the host-dependent viral progenies produced at each passage were tested by infection kinetics and/or greenhouse trials.

Materials and methods

Virus isolates and host genotypes

BYDV-PAV 4, originally collected on oat cv. *Peniarth* in 1989 (Ille-et-Vilaine, France), has been maintained on barley cv. *Express* since its collection. BYDV-PAV 4 induces severe symptoms on the susceptible cv. *Express* and constitutes our BYDV-PAV reference isolate.

Four BYDV hosts, corresponding to the susceptible barley cv. *Express* (Sadeghi et al., 2000), the susceptible Australian wheat cv. *Sunstar* (Posadas and Henry, 2002) and the two BYDV-resistant wheat lines *Zhong ZH* (Barloy et al., 2003) and *TC14* (Banks et al., 1995) were used as hosts in BYDV inoculation experiments. Seeds were individually sown in plastic tubes containing vermiculite or in bulks of 30–40 seeds in plastic pots containing soil. Plants were grown in a temperature-controlled chamber at 20 °C with a light/dark period of 16/8 h.

Aphid clones, SPEs and infection kinetics

Rhopalosiphum padi RP1 clone (Simon et al., 1991), an efficient BYDV vector, was used in all transmission experiments. Viruliferous and virus-free aphids were obtained by parthenogenetic reproduction of RP1 aphid females on BYDV-infected and uninfected barley cv. *Express* plants, respectively. Viruliferous aphids were transferred on *Express*, *Sunstar*, *Zhong ZH* and *TC14* plants (bulks of 30–40 plants at 2nd leaf stage). Each aphid-infested genotype was separately maintained under confined conditions in mini insect-proof greenhouses. Two weeks after the aphid infestation, third or fourth instar larvae of viruliferous RP1 clones collected from infected *Express*, *Sunstar*, *Zhong ZH* and *TC14* were deposited respectively at the bases of 20 healthy *Express*, *Sunstar*, *Zhong ZH* and *TC14* plants (3 aphids/plant). Inoculated plants were then covered with micro-perforated cellophane bags. After a 5-day inoculation access period, aphids were killed by spraying plants with insecticide (lambda-cyhalothrine, 1 mg ml^{-1}, Karaté ®[Syngenta agro]). Infection parameters (percentage of infected plants and viral titers) of the inoculated plants were monitored using semi-quantitative ELISA performed 7, 11, 14, 17 and 21 days after inoculation (DAI) as described by Chain et al. (2005). Twenty-one DAI, virus-free aphids were deposited at the base of infected plants (4 plants per genotype) for a 3-day acquisition access period (AAP). The resulting viruliferous aphids were used to initiate a new inoculation/infection cycle as described above in order to produce data for passages 2 to 6.

To initiate passage 7, virus-free aphids were deposited at the base of infected plants (issued from passage 6) for a 3-day AAP and transferred according to the genotype used for the viral acquisition on either *Express*, *Sunstar*, *Zhong ZH* or *TC14* plants (bulks of 30–40 plants at 2-leaf stage) in a mini insect-proof greenhouse. One hundred and fourteen SPEs were performed for approximately 4 years by introducing (every 2 weeks) healthy plants of the corresponding genotype (bulks of 30–40 plants at 2-leaf stage) in the mini insect-proof greenhouses. This allowed viral transmission from infected to newly introduced plants. The number of aphids in each mini insect-proof greenhouse was comparable between SPEs. Starting with passage 2, BYDV-PAV 4 isolate

was renamed BYDV-PAV$_{Express}$, -PAV$_{Sunstar}$, -PAV$_{Zhong}$ or -PAV$_{TC14}$ according to the host on which the viral isolate was maintained. Data for passage 44–114 were gathered as follows: twenty plants from each of the four tested genotypes were separately inoculated with BYDV-PAV isolates obtained after passages 44, 62 and 110. In addition, 20 plants of each host genotype were inoculated with PAV$_{Express}$ from passages 66, 94, 106 and 114 (three aphids/plant) and infection kinetics was monitored as described by Chain et al. (2005).

Data analysis

Infected plant percentages and virus titers were compared at the different monitored DAI. Since each point of the infection kinetics was dependent on previous ones, the independence hypothesis required for classic ANOVA analyses was absent. Thus, an alternative approach was set up using the Area Under Pathogen Progress Curve (AUPPC). AUPPCs were calculated using a formula derived from the Area Under Disease Progress Curve (Jeger and Viljanen-Rollinson, 2001) where the variable Y_i is the percentage of infected plants and D_i is the number of DAI at the ith observation. In a similar manner, Area under concentration progress curve (AUCPC) was calculated using the same formula with Y_i being the virus load of the sampled leaves for each plant. ANOVA were performed using SAS software (v8.1, SAS institute Inc., Cary, NC, USA). For each dataset, residual normality was checked with the univariate procedure of SAS. Comparisons of variables were performed using Student-Newman-Keuls (SNK) options of the GLM procedure.

Greenhouse trial

BYDV-PAV isolates obtained at the 80th passage were separately inoculated on 42 plants of each of the four tested genotypes as described above. The experimental design of this trial included 6 randomised blocks each containing 7 replicates of all isolate/host combinations. Inoculated plants were grown under S2-type greenhouse confinement conditions from insecticide treatment to maturity. At 30 DAI, plants were sampled (5 cm of the youngest fully expanded leaf of each plant) and tested for BYDV-PAV using ELISA as described by Fabre et al. (2003). At maturity, height, dry weight and number of ears were measured for all inoculated plants.

Results

Effect of serial passages on BYDV-PAV infection efficiency

Raw data from ELISA allowed the calculation of the percentage of infected plants for each of the five sampling days of the monitored infection kinetics. Figure 1 illustrates the passage-dependent infection kinetics observed for *Sunstar* (Figure 1A), *Zhong ZH* (Figure 1B) and *TC14* (Figure 1C) inoculated with BYDV-PAV$_{Sunstar}$, -PAV$_{Zhong}$ and -PAV$_{TC14}$, respectively. At passage 2, infection patterns of the susceptible host *Sunstar* showed, as expected, an early detection of infected plants (Figure 1A, 2/20 at 7 DAI) and an almost complete infection of inoculated plants before the end of the monitored period (Figure 1A, 18/20 at 14 DAI). For *Zhong ZH* and *TC14*, the infection kinetics were delayed and the infectivity was reduced. Indeed, first infected plants were detected at 11 DAI and 14 DAI for *Zhong ZH* and *TC14*, respectively (Figure 1B, C, 3/20). At 21 DAI, almost complete infection (16/20) of inoculated plants was obtained for *Zhong ZH* while only 3 out of the 20 inoculated *TC14* were detected as infected plants. Moreover, the peak of infected plants occurred later in *Zhong ZH* (18 DAI) than in *Sunstar* (14 DAI). Patterns of infection on *Sunstar* were similar for all monitored passages. However, as the number of passages increased, *Zhong ZH* and *TC14* lines clearly showed a trend of increase in infection efficiency and a decrease in the time of detection of the first infected plant. Data corresponding to passage 110 for *Sunstar*, *Zhong ZH* and *TC14* showed detection of the first infected plant at 7 DAI, a rapid increase of number of infected plants from 7 DAI to 14 DAI and an infection of almost all inoculated plants at 21 DAI.

BYDV-PAV$_{Sunstar}$, -PAV$_{Zhong}$ and -PAV$_{TC14}$ isolates from passages 44, 62 and 110 were separately inoculated to *Sunstar*, *Zhong ZH* and *TC14*. BYDV-PAV$_{Express}$ from passages 44, 62, 66, 106 and 110 was inoculated on each of the four genotypes. The resulting data were used to calculate the mean inoculated host-dependent infection

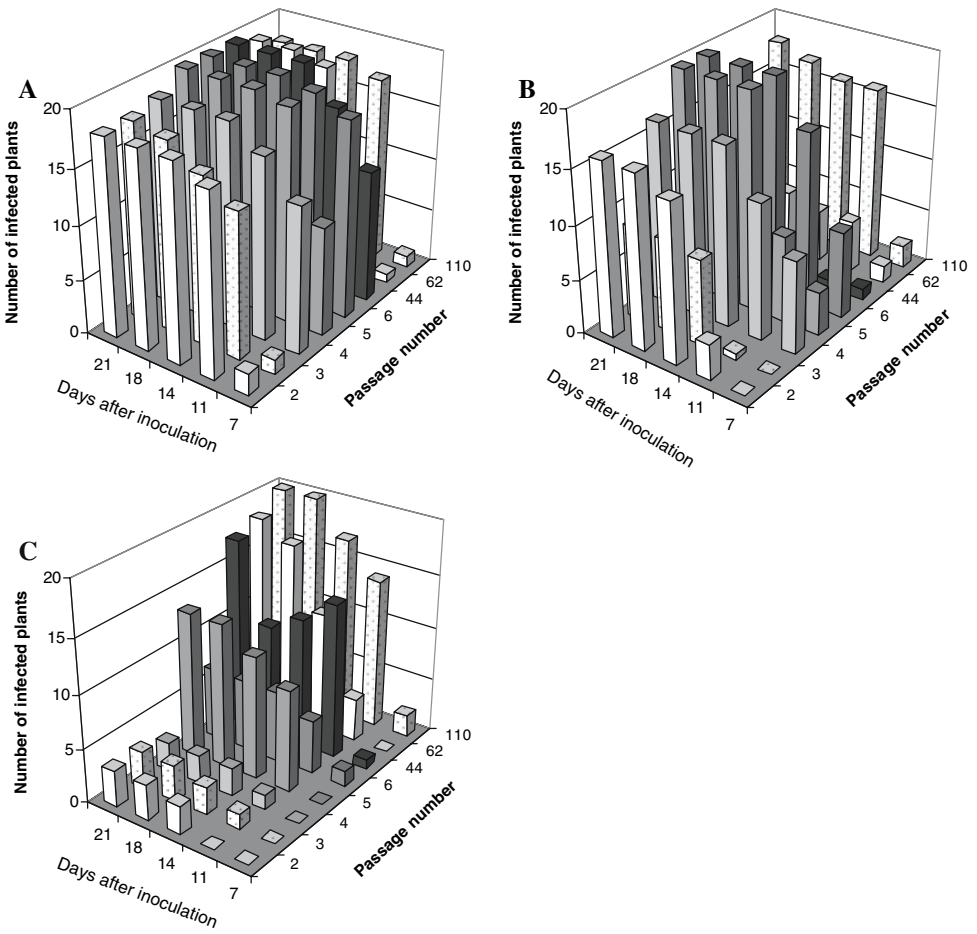

Figure 1. Increase in number of infected plants from 7 DAI to 21 DAI for passages 2–6, 44, 62 and 110. *Sunstar* (A), *Zhong ZH* (B) and *TC14* (C) plants were inoculated with BYDV-PAV$_{Sunstar}$, -PAV$_{Zhong}$ or -PAV$_{TC14}$ isolates, respectively.

percentage obtained for BYDV-PAV$_{Express}$, -PAV$_{Sunstar}$, -PAV$_{Zhong}$ and -PAV$_{TC14}$ (Figure 2A–D, respectively) at the five monitored days after inoculation. ANOVA performed on AUPPC calculated from percentages of infection kinetics indicate significant isolate ($F = 3.46$; $P = 0.0248$) and host genotype ($F = 19.30$; $P < 0.0001$) effects on the efficiency of infection. Susceptible hosts *Express* and *Sunstar* were efficiently infected regardless of the isolate while the two resistant genotypes seemed to be more efficiently infected by isolates resulting from SPEs performed with resistant genotypes (PAV$_{Zhong}$ and PAV$_{TC14}$) than by isolates maintained on susceptible genotypes (PAV$_{Express}$ and PAV$_{Sunstar}$) (Figure 2 and Table 1). Indeed, the SNK test ($\alpha = 0.10$) resulted in a ranking of mean AUPPC associated to isolates indicating that isolates maintained on resistant genotypes induced higher AUPPC (Table 1).

Effect of serial passages on BYDV-PAV titers in infected plants

The use of a semi-quantitative procedure for viral detection in collected samples enabled the calculation of viral titer of infected plants and the estimation of AUCPC values for each monitored kinetic. Applied to data corresponding to passages 2–6, the comparison of AUCPC reveals only a host genotype effect corresponding to the resistance behaviour of *Zhong ZH* and *TC14* lines (not illustrated). Moreover, the SPE procedure did not modify the

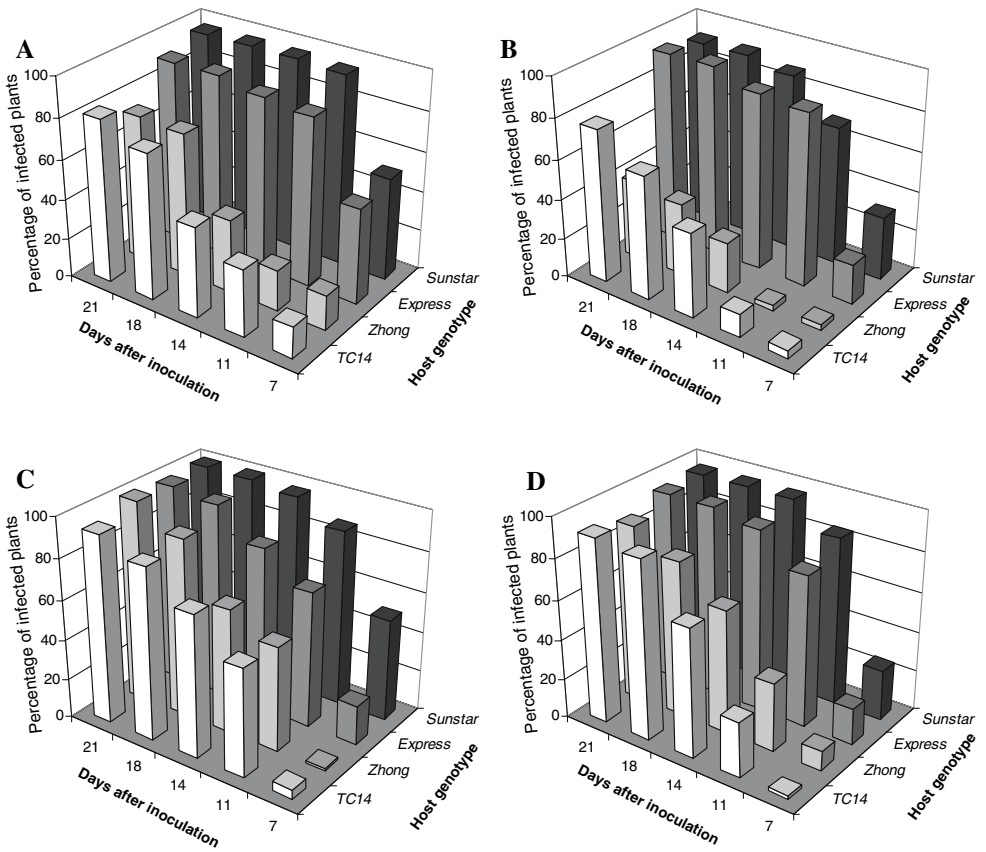

Figure 2. Increase in mean infection percentages from 7 DAI to 21 DAI for BYDV-$PAV_{Express}$ (A), -$PAV_{Sunstar}$ (B), -PAV_{Zhong} (C) and -PAV_{TC14} (D) inoculated on each host genotype. Data correspond to mean infection percentages from passages 44, 62 and 110. Results for $PAV_{Express}$ (A) include supplementary data corresponding to passages 66, 94, 106 and 114.

maximal level of viral accumulation in each infected genotype passage by passage. The inoculations performed using isolates from passages 44, 62 and 110 made it possible to test the host genotype and the isolate effects. Data showed strong plant genotype effects ($F = 123.34$; $P < 0.0001$). Moreover, a significant difference between tested isolates was noticed ($F = 11.66$; $P < 0.0001$). Interaction effects ($F = 3.41$; $P = 0.0004$) were also detected but the variances of the host and virus isolate effects were significantly larger than the variances of interactions. The SNK test ($\alpha = 0.05$) enabled the establishment of a ranking into three homogeneous groups corresponding to (a) BYDV-$PAV_{Sunstar}$,

Table 1. Area under pathogen progress curve (AUPPC) for inoculations at passages 44, 62 and 110

	BYDV-PAV isolate				Mean per host
	$PAV_{Express}$*	$PAV_{Sunstar}$	PAV_{Zhong}	PAV_{TC14}	
Inoculated host					
Express	1020	1127	1094	1046	1061 (a)
Sunstar	1147	1056	1164	1248	1152 (a)
Zhong ZH	571	291	763	845	610 (b)
TC14	564	531	776	885	664 (b)
Mean per isolate	828 (bc)	751 (c)	950 (ab)	1011 (a)	

*Results for $PAV_{Express}$ include data of the supplementary monitorings of infection kinetics performed at passages 66, 94, 106 and 114. Letters in brackets correspond to the groups defined by Student–Newman–Keuls test at 10% risk with the mean data.

Table 2. Area Under Concentration Progress Curve (AUCPC) for inoculations at passages 44, 62 and 110

	BYDV-PAV isolate				Mean per host
	$PAV_{Express}^{(*)}$	$PAV_{Sunstar}$	PAV_{Zhong}	PAV_{TC14}	
Inoculated host					
Express	2.20	2.64	1.92	2.05	2.18 (a)
Sunstar	2.30	2.44	1.89	2.21	2.21 (a)
Zhong ZH	1.23	1.69	1.26	1.31	1.31 (b)
TC14	1.30	1.18	1.21	1.42	1.29 (b)
Mean per isolate	1.82 (b)	2.06 (a)	1.61 (c)	1.75 (b)	

*Results for $PAV_{Express}$ include data of the supplementary monitorings of infection kinetics performed at passages 66, 94, 106 and 114. Letters in brackets correspond to the groups defined by Student–Newman–Keuls test at 5% risk with the mean data.

(b) -$PAV_{Express}$ and -PAV_{TC14}, and (c) -PAV_{Zhong} (Table 2).

Greenhouse trial

At 30 DAI, the greenhouse trial revealed low mean infection percentages for $PAV_{Sunstar}$ (38%), while higher percentages were obtained for PAV_{Zhong} (67%), $PAV_{Express}$ (76%) and PAV_{TC14} (92%) (Figure 3). As the greenhouse growth conditions used were not suitable for the production of high-quality grains, only height, ear number and plant dry weight were recorded. ANOVA analysis of these variables showed significant isolate and host genotype effects (Table 3). Although significant isolate × host genotype interaction and replicate effects were revealed, their variances were always significantly weaker than the variances of the isolate and host genotype effects. When the isolate effect on plant height, ear number and dry weight was analysed using the SNK test ($\alpha = 0.05$), a clear distinction between isolates was seen (Table 4). A comparison with data associated with non-inoculated control hosts indicated that BYDV-$PAV_{Express}$ and BYDV-$PAV_{Sunstar}$ isolates caused lower reductions of the monitored variables than isolates resulting from serial passages on BYDV-resistant hosts.

Discussion

SPEs associated with a sampling protocol developed to monitor the first 3 weeks of the viral infection process were used to test the impact of the BYDV-resistances present in *Zhong ZH* and *TC14* wheat lines on biological properties of the BYDV-PAV 4 isolate. As previously described

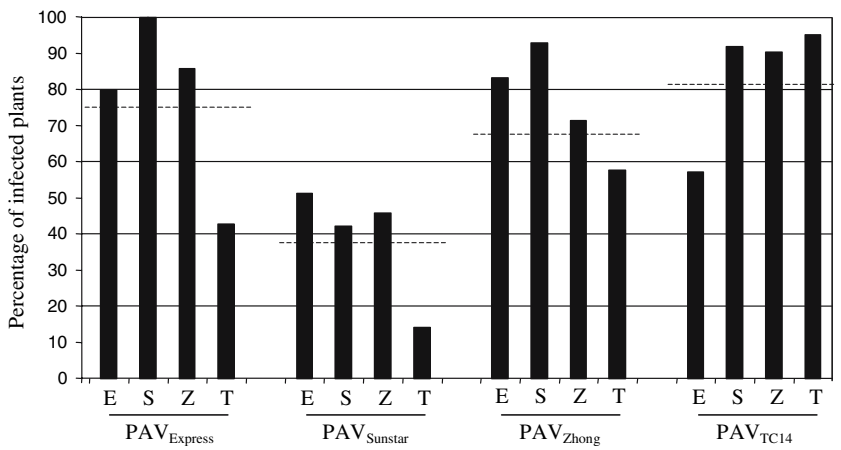

Figure 3. Percentage of infected plants in greenhouse trial at 30 DAI. Inoculated host genotypes are presented using a single letter code (E: *Express*, S: *Sunstar*, Z: *Zhong ZH*, T: *TC14*). Dotted lines symbolise the mean incidence for each isolate on the different hosts.

Table 3. Analysis of variance for isolate, host genotype and replicate effects on selected yield parameters in the greenhouse trial

Source of variation	DF	Height (cm)		Ear number		Dry weight (g)	
		F	*P*	*F*	*P*	*F*	*P*
Isolate	4	250.99	<0.0001	79.85	<0.0001	155.35	<0.0001
Host genotype	3	193.99	<0.0001	78.80	<0.0001	46.25	<0.0001
Replicate	5	31.89	<0.0001	6.63	<0.0001	19.72	<0.0001
Isolate × host genotype	12	60.78	<0.0001	7.41	<0.0001	17.99	<0.0001

(Barloy et al., 2003; Chain et al., 2005), the initial collected data (infection percentages and viral titers in infected plants) corresponding to passages 2 to 4 confirmed the partial resistance behaviour of these two wheat lines (reduced number of infected plants, lower virus titer in leaves and longer time between inoculation and initial detection of the virus). However, the use of these resistant lines as the single obligatory host in SPEs induced a rapid change of the biological properties of the used BYDV isolate. Changes were revealed by the progressive increase of infection efficiencies on resistant genotypes and the decreases in the recorded yield parameters (height, ear number and dry weight). Significant changes were observed, under our experimental conditions, after only a few passages. Such rapid changes in viral properties have already been described in SPE procedures applied to other plant viruses (Kurath and Palukaitis, 1990; Lawson et al., 1993; Chakraborty and Reid, 1999). Although virulence of the BYDV isolate maintained on resistant hosts increased with the number of passages performed, the accumulation of viral particles of BYDV in infected plants was shown to be stable from one passage to the next. This observation eliminates the possibility of bias in the biological data set due to a passage-dependent progressive change in the quantity of viral particles used as inoculum in plant-to-plant viral transmission.

Fitness of a viral isolate is considered to be a measure of its success in perpetuating its genes, i.e. to replicate extensively in a given environment and relative to a reference viral population (Domingo et al., 2005). Virus isolates that possess high fitness are likely to be maintained in natural conditions. Due to constraints imposed by the environment of resistant hosts, resistance-breaking isolates are often subjected to fitness loss in susceptible host infection when compared with reference isolates (Jenner et al., 2002). PAV_{Zhong} and PAV_{TC14} infection kinetics were associated with the lowest AUCPC values, meaning that over the period of titer measurement, PAV_{Zhong} and PAV_{TC14} viral components (coat protein and/or viral particles) accumulated to lower levels in aerial parts of infected plants than $PAV_{Express}$ and $PAV_{Sunstar}$. This fact would support considering PAV_{Zhong} and PAV_{TC14} isolates as possessing lower fitness than isolates maintained on susceptible hosts. However, PAV_{Zhong} and PAV_{TC14} induced more severe yield loss showing, as already described (Lapidot et al., 1997), that viral concentration in infected plants is not necessarily linked to the severity of symptoms. In natural conditions, BYDV-PAV mixed infections occur and bottlenecks caused by aphid transmissions could eliminate low-titer virus isolates (Elena et al., 2001). However, in addition to the dilution and bottleneck effects, the virulence of the isolate could also play an important role in

Table 4. Mean values of recorded yield parameters for each isolate used in the greenhouse trial

Recorded variables	Non-inoculated	Isolates			
		$PAV_{Express}$	$PAV_{Sunstar}$	PAV_{TC14}	PAV_{Zhong}
Height (cm)	73.8 (a)	70.78 (b)	71.33 (b)	59.91 (c)	43.94 (d)
Ear number	3.76 (a)	3.44 (b)	3.33 (b)	2.05 (c)	2.1 (d)
Dry weight (g)	6.31 (a)	4.84 (b)	5.25 (b)	2.2 (c)	1.39 (d)

For each recorded variable, different letters represent significant differences at 5% risk level according to Student–Newman–Keuls test.

virus propagation and should be considered when evaluating the possible spread of a low-accumulating isolate (Ebert and Bull, 2003).

Taken together, these data revealed that, in our experimental conditions, the two *Thinopyrum intermedium*-derived partial BYDV resistances of *Zhong ZH* and *TC14* genotypes induced, in only a few successive passages, the appearance of BYDV-PAV variant(s) that overcame the resistance phenotype and led to more important damage than isolates maintained on susceptible hosts. According to the dynamics of the BYDV field infection process (Irwin and Thresh, 1990), primary and secondary infections, including the spread of virus in the whole field, could correspond to at least three plant-to-plant transmissions, which would be equivalent to the role of passages in SPE assays. Hence, in less than two years of culture of these resistant lines, the virus would have the opportunity to perform the small number of infection cycles required to overcome the resistance phenotype. Thus, the *Zhong ZH* and *TC14* resistances do not seem to be durable when faced with the viral evolution process. Therefore, their use in BYDV-resistance breeding programmes should be considered with care. However, the numerous parameters used in SPEs, such as the length of time between passages, the transmission process, the development stage of the inoculated plants and the absence of both an alternative host for infection cycles and competition between isolates, are all different under natural conditions. Variation in these parameters might have an impact on the viral population in terms of production, selection and propagation of variants (Domingo, 1997; Tan et al., 2005). Under natural conditions, BYDV-PAV infects a wide range of *Poaceae* species including cultivated crops (e.g. wheat, barley, maize, oats, pasture grasses), volunteer and spontaneous species in non-cultivated areas. To perform successive infections of winter cereals, viral inoculum has to 'jump' several times within an agricultural landscape, from senescent infected hosts during spring to alternative hosts (Irwin and Thresh, 1990). In temperate climates, maize (Henry and Dedryver, 1989), cereal volunteers (Henry et al., 1993; Masterman et al., 1994; Plumb, 1995) and some pastures grasses (Henry and Dedryver, 1991) are important for BYDV to persist throughout the summer, with natural grasses being of lesser importance (Irwin and Thresh, 1990). Whatever the effective alternative host(s) used, the selection pressure(s) induced by the resistances of *Zhong ZH* and *TC14* lines is not operative during the time between two successive cultures of the resistant winter hosts. As was the case in the resistant hosts used in this study, the bridging of susceptible crops could allow the production of a new viral population in which less virulent isolate(s) could be positively selected. The effect of such alternate hosts on the selection and maintenance of virulent BYDV isolates must be investigated prior to excluding *Zhong ZH* and *TC14* lines from BYDV-resistant breeding programmes.

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