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Wheat dwarf virus infectious clones allow to infect wheat and *Triticum monococcum* plants

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Abstract: We constructed *Wheat dwarf virus* (WDV) infectious clones in the bacterial plasmids pUC18 and pIPKb002 and tested their ability to inoculate plants using Bio-Rad Helios Gene Gun biolistic inoculation method and *Agrobacterium tumefaciens* agroinoculation method, and we then compared them with the natural inoculation method via viruliferous *P. alienus*. Infected plants were generated using both infectious clones, whereas the agroinoculation method was able to produce strong systemic infection in all three tested cultivars of wheat and *Triticum monococcum*, comparable to plants inoculated by viruliferous *P. alienus*. Infection was confirmed by DAS-ELISA, and WDV titres were quantified using qPCR. The levels of remaining bacterial plasmid DNA were also confirmed to be zero.

Keywords: WDV; *Triticum aestivum* L.; virus infectious clone; agroinoculation; biolistic inoculation; leafhopper; qPCR detection

Wheat dwarf virus (WDV), from the genus *Mastrevirus* (family *Geminiviridae*), is a pathogen affecting cereal crops that is transmitted by the leafhopper *Psammotettix alienus* (Dahlbom, 1850). It was described for the first time in former Czechoslovakia (Vacke 1961), and it has spread throughout Europe, Africa and Asia. The virus affects wheat, barley, oat, and some wild grasses (Vacke 1972; Lindsten & Vacke 1991). Infected plants are dwarfish, with many tillers, and shrunken or even lacking grains (Figure 1), which leads to a dramatically lower yield. It is one of the most dangerous cereal viral pathogens causing considerable commercial losses, especially in countries cultivating winter crops (Lindblad & Waern 2002; Širlová *et al.* 2005). WDV is transmitted by a leafhopper species, *P. alienus*, in a circulative, non-propagative manner (Lindsten *et al.* 1980; Lindsten & Vacke 1991). Two main strains are known – the wheat-adapted strain (WDV-W), affecting at least wheat, oat, rye, and some wild grasses, and the barley-adapted strain (WDV-B), affecting at least barley and oat (Lindsten & Vacke 1991). Oat and some wild grasses were identified as hosts of both strains (Lindsten & Vacke 1991; Vacke & Cibulka 1999; Ripl & Kundu 2015). There are also reports that WDV-W can infect barley and WDV-B can infect wheat in the field (Schubert *et al.* 2007; Kundu *et al.* 2009; Tobias *et al.* 2011) or using infectious clones (Ramsell *et al.* 2009).

Similar to other mastreviruses, WDV is a single-stranded DNA (ssDNA) virus with four coding proteins: replication protein (Rep), replication-associated protein (RepA), coat protein (CP), and movement protein (MP). The proteins are encoded both in a virion-sense orientation (CP, MP) in relation to ssDNA from the virion and in a complementary sense orien-

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Figure 1. Wheat (left) and barley (right) susceptible cultivars infected by *Wheat dwarf virus* in the field (Photo: J. Ripl)

tation (Rep, RepA) (Dekker *et al.* 1991) (Figure 2). The searching for cultivars with resistance to WDV is made difficult due to the complex ecology and life cycle of the virus, which is tightly bound to its natural insect vector and lacks mechanical transmissibility. No commercially available cultivars of wheat or barley resistant to this virus are known, and only a few experimental barley cultivars have been reported in the scientific literature (Habekuss *et al.* 2009; Kis *et al.* 2016). For partial resistance or tolerance to WDV, only a few cultivars are reported (Vacke & Cibulka 2000; Širlová *et al.* 2005; Benkovics *et al.* 2010). An important role may also be played by the proven natural resistance of some WDV proteins to gene-silencing mechanisms (Liu *et al.* 2014).

Many laboratory workflows for the preparation of infectious clones of WDV have been previously described (Hayes *et al.* 1988; Woolston *et al.* 1988; Bendahmane *et al.* 1995; Boulton 2008; Ramsell *et al.* 2009) together with different methodologies for age and part of the plant selected for inoculation (Hayes *et al.* 1988; Dale *et al.* 1989; Chen & Dale 1992; Ramsell *et al.* 2009), selected host cultivar (Woolston *et al.* 1988; Benkovics *et al.* 2010) or used agrobacterium strain (Marks *et al.* 1989). The different reached agroinoculation efficiencies (Dale

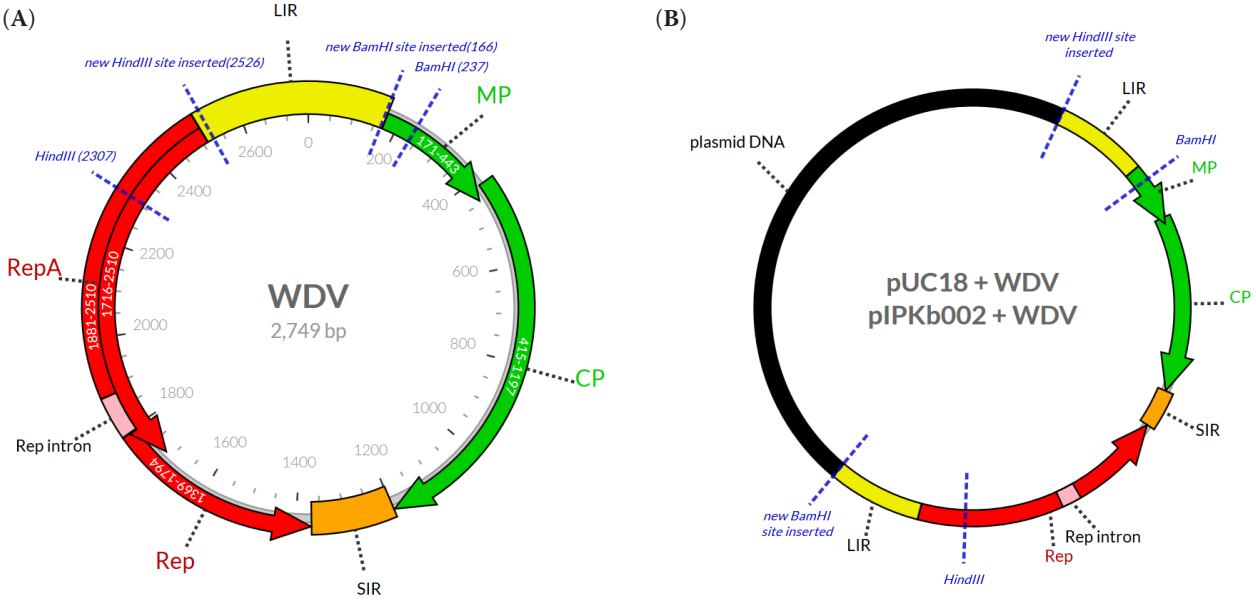

Figure 2. The structure of WDV genome and constructed WDV infectious clones: (**A**) structure and position of *Wheat dwarf virus* open reading frames and their orientation in the circular ssDNA WDV genome: LIR – long intergenic region with replication origin hairpin, MP – movement protein gene, CP – coat protein gene, RepA – replication-associated protein gene, Rep – replication protein gene, SIR – short intergenic region and (**B**) structure of pUC18 + WDV and pIPKb002 + WDV infectious clones

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et al. 1989; Bendahmane *et al.* 1995; Ramsell *et al.* 2009; Benkovics *et al.* 2010) are then also closely dependent on selected evaluation schemes for virus detection and quantification. The DNA of an infectious clone should contain a hairpin sequence of the circular DNA replication origin at the beginning and at the end, as this leads to the preferential release of viral DNA (Redinbaugh 2003; Ramsell *et al.* 2009). The infectious clone should also contain a replication origin for plasmid replication in bacteria (e.g., *E. coli*) and genes for plasmid selection. If used for agroinoculation, the plasmid must contain a replication origin targeted at replication in agrobacteria. However, a successful inoculation with an infectious clone in a prokaryotic bacterial plasmid was only achieved with the biolistic transformation of wheat (Jones & Shewry 2009) or even, with the closely related virus, *Maize streak virus* (MSV), via mechanical inoculation (Redinbaugh 2003). Following up on this finding in the literature, we constructed a WDV infectious clone in the bacterial plasmid pUC18, as well as in the plasmid pIPKb002, also containing the replication origin for replication in bacteria genera, and tested their ability to infect plants.

MATERIAL AND METHODS

Construction of infectious clones pUC18+WDV and pIPKb002+WDV. An infectious WDV clone was carefully constructed to contain a copy of a replication origin at the beginning and at the end but not overlapping the other regions outside. A 472 bp selected portion of the WDV genome of Czech wheat isolate (Kundu *et al.* 2009; WDV-W NCBI accession number FJ546188) was amplified by PCR from the beginning of circular replication origin hairpin sequence, adding a *HindIII* restriction site, following up to the existing *BamHI* restriction site (HindIIIREPcutF/WDVBamHIrv primer pair; Table 1). This product was then ligated into the target vector pUC18 (Takara Bio, Shiga, Japan) that had initially been extended with a *HindIII-KpnI* extender (a1fw/a1rw pair – Table 1), allowing for a blunt-ended digestion with the *EcoRV* restriction enzyme. The subsequent 2684 bp section of the WDV genome from the existing *BamHI* restriction site up to the end including the next copy of the circular replication origin hairpin sequence was amplified by PCR, appending a new *BamHI* restriction site to the end (WDVBamHIfw/BamHIMPcutR primer pair; Table 1). This product was then inserted into the pGEM-T Easy vector (Promega, Madison, USA), followed by redigestion with the *BamHI* restriction enzyme. This almost full copy of WDV was then inserted into the pUC18 target vector with the HindIIIREPcutF/WDVBamHIrv part from the previous reaction also being redigested with the *BamHI* restriction enzyme (Figure 2).

Table 1. Primers used for construction of WDV infectious clones derived from the WDV genome (NCBI accession number FJ546188, primers 1–4). The newly added restriction sites are underlined. Primers 5–6, a1fw, a1rv are synthesised extenders for the *HindIII-KpnI* region of pUC18 plasmid to allow blunt end cloning using *EcoRV* restriction digestion

Primer	Sequence (5'→3')
HindIIIREPcutF	AAGCTTTCCGGCAGGTCCTTAGCGAAA
WDVBamHIrv	GGATCCGGGATTGGAAGGGGTC
WDVBamHIfw	GGATCCTCCGACTACGCCTGGC
BamHIMPcutR	GGATCCTGGGCTACCACGCACTTCCT
a1fw	AGCTTCTGTTCGATATCTAGTACGGTAC
a1rv	CGTACTAGATATCGAACAGA

For the creation of an infectious clone in the pIPKb002 target vector (IPK, Gatersleben, Germany; Himmelbach *et al.* 2007), the WDV infectious clone was first constructed in the pENTR D-TOPO vector (Invitrogen, Waltham, USA) in the same way as for pUC18+WDV and then shuffled into the target vector using the LR-Clonase reaction (Invitrogen, USA). Both infectious clones were sequenced (GATC Biotech, Konstanz, Germany) to confirm their sequence and orientation.

Plant material. Experiments were performed on the *Triticum aestivum* L. winter wheat cultivars Alana and Svitava, as well as *Triticum monococcum* L. (accession No. 01C0106429), obtained from GenBank Prague-Ruzyně, Crop Research Institute, Czech Republic. The plants were grown in quantities of 4–5 plants per pot in autoclaved soil and were cultivated in a 16/8 h, 22/18°C day/night regime. On the inoculation day, the plants were kept in dark until inoculation. After inoculation, the plants were left to grow in the same conditions for the next 6 weeks. Leaf material from each tested plant was then disrupted in liquid nitrogen, and the samples were stored at –80°C until tested (up to 7 days).

WDV biolistic inoculation. The biolistic inoculation of the plants was performed using a Bio-Rad

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Helios Gene Gun (Bio-Rad Laboratories, Hercules, USA), and the optimal parameters for wheat were detected using control pIPKb002+GUS plasmid and subsequent GUS staining. Finally, 0.6 µm gold particles (Bio-Rad Laboratories, USA) with DNA of the selected infectious clone were prepared with 0.5 µg of DNA per shot and 0.5 µg of gold projectiles per shot.

Each tested plant from a given growth stage was shot twice from each bullet cartridge, two cartridges per plant. Two shots from one cartridge were targeted at the top of the leaf, and two shots from another cartridge were targeted at the base of the seedling. The shooting pressure was 150–180 psi (pounds per sq. inch, i.e. 1034–1241 kPa). All the shots were made with the diffraction screen included.

WDV agroinoculation. The selected pIPKb002+WDV clone was transferred together with the pSoup helper plasmid into *Agrobacterium tumefaciens* (AGL1 strain). The stock culture was cultivated in LB medium + streptomycin (50 µg/l) + rifampicin (50 µg/l) + tetracycline (5 µg/l), at 28°C with shaking at 150 rpm, for 40–48 h, and then centrifuged for 3 min (10°C, 1 200 *g*); the supernatant was removed from the pellet, and 1 ml of LB was added. The culture was centrifuged again for 2 min (10°C, 1 200 *g*), and then the pellet was diluted in 3 ml of sterile distilled water. Approximately 5-day-old plants were injected with a Hamilton 1801 RN 10 µl syringe (Hamilton Company, Reno, USA), 3 times at the base of the seedling (10 µl), twice vertically and one time horizontally, as described in Boulton (2008). The plants were growing in 25/25°C regime for the next two days and then switched to the regular 22/18°C regime.

WDV inoculation by Psammotettix alienus. The plants were inoculated at Zadoks stage DC12 or DC13 (Zadoks *et al.* 1974). Five days before inoculation, leafhoppers were left feeding on the wheat plants of cv. Ludwig infected with WDV-W (NCBI accession No. FJ546188) with significantly developed WDV infection. On the inoculation day, 10 leafhoppers were moved to each pot and left there for the next 5 days. Then, the leafhoppers were removed, and the plants were left to grow for the following 6 weeks.

Quantification of WDV titres. Plant DNA was isolated by adding 0.5 ml of extraction buffer (1 M guanidine thiocyanate, 20 mM Na_2H_2EDTA, 0.1 M MOPS, pH 4.6, 0.2% mercaptoethanol) to 50–100 mg of sampled tissue that had been disrupted and homogenised in liquid nitrogen. The solution was incubated for 30 min in a 60°C water bath with occasional vortexing followed by phenol-chloroform-isoamyl alcohol extraction (25 : 24 : 1; Affymetrix, Santa Clara, USA), chloroform extraction, isopropanol, and sodium acetate precipitation and two-step 70% ethanol purification. The qPCR for WDV detection was run on a 7300 Real-Time PCR System (Applied Biosystems by ThermoFisher Scientific, USA) using 6 µl of the Applied Biosystems™ PowerSYBR Green RNA-to-C_T™ 1-Step Kit without the reverse-transcriptase component, the U2WDV-fw/U2WDV-rv primer pair (in final concentration of 0.4 µM; Table 2), and 1 µl of tested sample and then filled with distilled deionised water up to a 12 µl reaction volume. The temperature parameters were set to 95°C for 10 min, 40 cycles of 95°C for15 s, 65°C for 1 min and, then, for the evaluation of a dissociation curve, 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 seconds. For all samples, the mean detected WDV concentration was calculated based on the testing of triplicate samples and subsequently normalised using the DNA sample concentration detected spectrophotometrically.

The amount of potentially remaining infectious clone plasmid vector DNA alone was quantified using forward and reverse primers (spec3RT-fw/spec3RT-rv; Table 2) targeted at a 178 bp amplicon in the spectinomycin/streptomycin resistance gene in pIPKb002 plasmid or using forward and reverse primers (betalac2-fw/betalac2-rv; Table 2) targeted at a 117 bp amplicon of the beta-lactamase gene in pUC18 or pGEM-T Easy plasmid. All other conditions were the same as for qPCR for WDV detection.

Table 2. Primers used for the detection of WDV and bacterial plasmids by qPCR

Primer	Sequence (5'→3')	Origin of the sequence
U2WDV-fw	CAGAGCCGAAACAGGCAAT	WDV coat protein gene
U2WDV-rv	GGTTCACGGTCCACTTCCTT	WDV coat protein gene
spec3RT-fw	GCAGTAACCGGCAAAATCGC	pIPKb001 spectinomycin/streptomycin resistance gene
spec3RT-rv	CGCCTTTCACGTAGTGGACA	pIPKb001 spectinomycin/streptomycin resistance gene
betalac2-fw	GCAACTTTATCCGCCTCCATC	pUC18 and pGEM-T Easy beta-lactamase gene
betalac2-rv	TGACACCACGATGCCTGTAG	pUC18 and pGEM-T Easy beta-lactamase gene

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As a qPCR standard, pGEM-T Easy plasmid was used with all detected sequences included (U2WDV-fw/U2WDV-rv primer pair amplicon inserted and ligated directly into the pGEM-T Easy plasmid and the spec3RT-fw/spec3RT-rv primer pair amplicon inserted into the plasmid *Sal I* restriction digestion site with primers containing the *Sal I* restriction digestion sequence GTCGAC; for the betalac2-fw/betalac2-rv differential qPCR, the amplicon is already included in the beta-lactamase gene sequence).

The efficiency factor of qPCR was 99.61% or better for U2WDV primer pair and spec3RT primer pair using the qPCR double standard, and the efficiency factor of qPCR for the betalac2 primer pair was 98.91%. qPCR was able to correctly detect the number of copies at least in the range of 6.68×10^1–6.68×10^8. All the samples with WDV infection detected by qPCR were also tested by DAS-ELISA (rabbit polyclonal antibodies to WDV, alkaline phosphatase-conjugated antibodies; SEDIAG, Bretenière, France) for the presence of viral proteins according to the manufacturer's instructions.

RESULTS

WDV infectious clone constructs. Two WDV-W infectious clones (pUC18+WDV and pIPKb002+WDV) for initiating WDV infection in plants were constructed. Both infectious clones contain the 1.1 length of WDV genomic sequence starting from the long intergenic region with the replication origin hairpin, going through all viral open reading frames up to the next added copy of the long intergenic region, with a terminating replication origin hairpin. The pUC18+WDV infectious clone also contains the *Escherichia coli* origin of replication pMB1 (ColE1 and pBR322) and the *E. coli* beta-lactamase/ampicillin resistance gene taken from the original pUC18 plasmid. The pIPKb002+WDV infectious clone also contained an *E. coli* ColE1 origin of replication, *Pseudomonas* pVS1 origin of replication for replication in other bacteria genera such as *Agrobacterium tumefaciens*, hygromycin phosphotransferase under the maize ubiquitin promoter for conferring hygromycin resistance in plants and streptomycin/spectinomycin adenyltransferase for streptomycin/spectinomycin resistance, where all added genes originated from pIPKb002 plasmid. For construction details see Materials and Methods and Figure 2.

Efficacy of WDV infectious clone using different inoculation methods. Four hundred sixty-one plants were agroinoculated in 11 experiments to allow for the optimisation of agroinoculation parameters and the testing of the agroinoculation method. No significant differences were detected when *Agrobacterium* for the inoculum was cultivated on the plate or in solution with all necessary antibiotics, only with the antibiotic for the agroinoculation plasmid (streptomycin) or completely without antibiotics. Using all of these different protocols, plants with WDV infection were detected by both qPCR and by ELISA in only 1–3 cases out of 25–35 tested samples, and another 1–4 WDV infected plants were detected only by qPCR. For wheat cv. Svitava and *Triticum monococcum,* 3 ELISA and qPCR positive cases out of 25 were reached, and for cv. Alana, only 1 ELISA and qPCR WDV infection out of 25 and 1 ELISA and qPCR WDV infection out of 35 inoculated plants were detected. This ratio was not improved even when agrobacterium was induced in induction buffer (acetosyringone 150 mM, 10 mM $MgCl_2$ in sterile distilled water) for 4 h in dark at room temperature just before the inoculation. If the plants were inoculated later than at Zadoks DC12 (approx. two weeks old), the inoculation did not lead to any positive results, and neither did the agroinoculation of young plants where the injection of the inoculation solution was replaced by the submersion of leaves into the inoculation solution with leaf tips injured with a razor blade (both 0 out of 16 tested plants). The visual control of agroinoculated plants does not allow clear discrimination of positively infected plants. Leaf yellowing, mottling or stunting of plants were often caused only by the agroinoculation treatment protocol. While dwarfing was detectable, the infected plants were higher than some wilting agrobacterium-treated controls.

Two hundred nine wheat plants were inoculated by biolistic inoculation in 15 different experiments with infectious clones placed in pUC18 or pIPKb002 plasmids. During the optimisation of the biolistic inoculation method using GUS staining, an optimal pressure for the shot was detected as 150–200 psi with the smallest golden particles (0.6 μm) and the youngest plants possible (Zadoks stage DC10–11). Shot particles with a larger diameter (1.0 μm) caused visible damage to target tissues. It was necessary to fix the Helios Gene Gun device in an additional rack to ensure that the target part of the plant was hit. We also tried to test older plants; however, with plants older than 7 days (Zadoks stage DC12–14), no positive results were obtained (0/16, pUC18 + WDV

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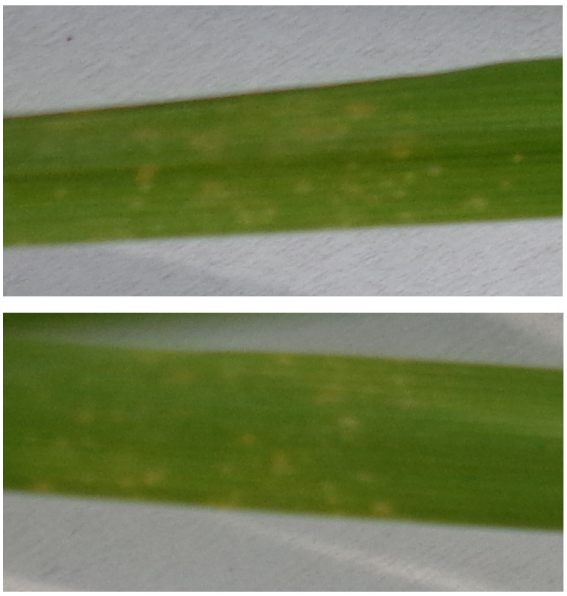

Figure 3. Yellowing spots appearing 2 days after successful inoculation of *Triticum aestivum* cv. Alana, 180 psi helium shot pressure, 0.6 µm gold particles (0.5 µg/shot), and 0.5 µg of WDV infectious clone DNA per shot

for 10, 14, and 21 days old plants). Using the biolistic inoculation method, we were able to get ELISA and qPCR-positive plants for both plasmids tested (for pUC18+WDV 1 positive case per 12 inoculated plants and 2 positive cases per 35 inoculated plants, for pIPKb002 1 positive case per 35 inoculated plants). In all infected plants, the amount of remaining DNA was quantified as at most 275 copies/ng of extracted DNA, i.e., several orders of magnitude less than the detected amounts of WDV DNA. Plants successfully inoculated with 0.6 µm gold particles showed yellowing spots in the shot area several days after inoculation in comparison with treated controls (Figure 3). However, for 1.0 µm or larger gold particles very tiny spots also appeared for treated controls.

Table 3. The best results obtained for different inoculation methods. The number of WDV infected plants out of all tested plants and the efficiency are depicted for the experiment with the highest reached efficiency

Method	Winter wheat cv.		*Triticum monococcum*
	Svitava	Alana	
Inoculation by *Psammotettix alienus*	10/10 (100%)	10/10 (100%)	10/10 (100%)
Agroinoculation	3/25 (12%)	1/25 (4%)	3/25 (12%)
Biolistic inoculation	1/12 (8.33%)	1/12 (8.33%)	0/35 (0%)

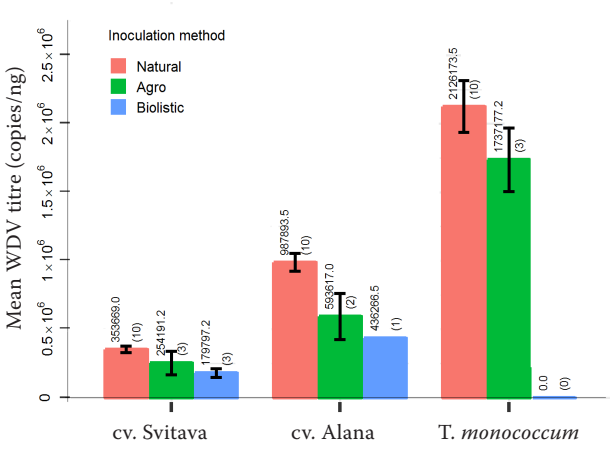

Figure 4. The mean WDV titres for each inoculation method and tested cultivar together with the interval of plus minus one standard error of the mean (SEM). The numbers in parentheses show the total number of infected plants positively detected by both qPCR and DAS-ELISA for the given method and cultivar

As a control experiment, 10 plants of each tested cultivar were inoculated by the leafhopper. Successfully inoculated plants were obtained with 100% efficiency of transmission (Table 3). All plants with qPCR detected WDV infection were also confirmed as infected by DAS-ELISA. The WDV titres detected for each inoculation method and tested cultivar are depicted in Figure 4. The high efficiency of the natural inoculation method allowed for us to estimate mean WDV titres for wheat cvs Svitava and Alana and for *Triticum monococcum* and to compare them with those obtained by agroinoculation and biolistic inoculation using the WDV infectious clone.

DISCUSSION

Wheat dwarf virus (WDV) is a leafhopper-transmitted virus, and the availability of the *P. alienus* virus vector is the only means of plant inoculation. The creation of an infectious clone of an insect-transmitted virus and its ability to infect plants independently of the insect vector may be a powerful tool for plant-virus interaction studies. Two infectious clones of WDV from a Czech wheat isolate have been described in this paper. These infectious clones, pUC18+WDV and pIPKb002+WDV, are able to infect wheat and *T. monococcum* plants. Two inoculation methods (biolistic and agroinoculation) were used, and the agroinoculation with an infectious WDV clone was

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demonstrated as an effective tool for virus inoculation in our hands. The success of agroinoculation with infectious *Maize streak virus* (MSV), a closely related mastrevirus, was described earlier in maize plants (Grimsley *et al.* 1987; Boulton *et al.* 1989). The agroinoculation of plants with pIPKb002+WDV using the *A. tumefaciens* AGL1 strain confirmed the suitability of the constructed infectious clone to initiate a WDV infection in plants. Promising results were obtained from the inoculation by the *A. tumefaciens* AGL1 strain regardless of the induction by acetosyringone prior to the agroinoculation or the agrobacterium cultivation method. The inoculation by *A. tumefaciens* AGL1 obtained from cultivation on plates gave similar results to those obtained with the culture of agrobacterium in a liquid medium. The reached agroinoculation efficiency for selected cultivars (4–12%) is similar to other reported results for wheat or barley (Bendahmane *et al.* 1995; Ramsell *et al.* 2009), however, the higher reached agroinoculation efficiencies were also reported using different agrobacterium strains (Marks *et al.* 1989; Benkovics *et al.* 2010). For MSV, comparison studies of its infectious clones show that the additional promoter sequence present (similarly to the used pIPKb002 plasmid) could increase the agroinoculation efficiency as well as the selected virus DNA orientation in the infectious clone plasmid (Martin & Rybicki, 2000). However, the similarity of WDV and MSV could be compromised, while the MSV infectious clone is known to be mechanically transmissible to its preferential host – maize (Redinbaugh 2003), and extremely high agroinoculation efficiencies were reported for maize (Grimsley *et al.* 1987, 1988).

Successful biolistic transformations of wheat or barley highlight the use of immature embryos or induced calluses cultivated on agar (Jones & Shewry 2009) or very young plant tissues for which the Bio-Rad Helios Gene Gun device is less convenient than, for example, the Bio-Rad Helios PDS1000/He with a vacuum chamber. Despite this, when the optimal parameters were chosen, WDV-infected plants were obtained using any of the two constructed infectious clones; however, the efficiency of transformation using the biolistic methodology was lower than the efficiency for agroinoculation. Our results demonstrate that a phloem-targeted virus such as WDV (Dinant *et al.* 2004; Tholt *et al.* 2018) can be successfully inoculated by a leafhopper free system using an infectious virus clone either by agroinoculation or in a biolistic manner. Many earlier reports have also shown that agroinoculation is a very effective system for delivery of infectious viral DNA into host cells (Grimsley *et al.* 1986) resulting in the induction of disease symptoms in plants (Czosnek *et al.* 1993; Kheyrpour *et al.* 1994). Similarly, biolistic inoculations with infectious viral DNA have a good efficiency to develop the disease in plants (Lapidot *et al.* 2007). In monocot plants such as cereals, tissue biolistic inoculation may be less effective (Helloco-Kervarrec *et al.* 2002), which correlates with our results. Quantitative analysis in our study has shown that in agroinoculated plants, the WDV titre is much higher than that of biolistic inoculation and is similar to plants naturally WDV inoculated by leafhopper.

CONCLUSION

We constructed two different infectious clones, in the bacterial plasmid pUC18 with a prokaryotic *E. coli* replication origin only and in the plasmid pIPKb002 having also the replication origin for *E. coli* and *A. tumefaciens*. We were able to obtain infected plants using both the constructed infectious clones either by biolistic inoculation or, for the pIPKb002+WDV infectious clone, by the agroinoculation method. Successfully infected plants then showed similar titres of WDV compared to plants inoculated by insect transmission.

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