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ORIGINAL ARTICLE

Detection of barley- and wheat-specific forms of *Wheat dwarf virus* in their vector *Psammotettix alienus* by duplex PCR assay

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Abstract

Wheat dwarf virus (WDV) has been one of the most common viruses on cereal crops in Poland in the last years. This single stranded DNA virus is transmitted by the leafhopper spec, *Psammotettix alienus* (Dahlb.) in a persistent manner. It induces yellowing and streaking of leaves, dwarfing or even death of infected plants. The presence of barley- and wheat-specific forms of WDV (WDV-B and WDV-W) and their vector were previously reported in the country, however the literature data did not include any information on the infectivity of the vector in Poland. A duplex polymerase chain reaction (PCR) procedure was developed and optimized for simultaneous detection and differentiation of both forms in the vector. Two sets of primers amplify 734 bp and 483 bp specific fragments for WDV-W and WDV-B, respectively. The results were verified by a sequencing method. The studies were carried out on insect samples collected in autumn from four different locations in Greater Poland. The results confirmed the presence of WDV-W in the tested samples. They also suggested the concomitant of both forms of the virus in the vector. Additional studies to determine virus-vector relationships should be undertaken.

Key words: duplex PCR, leafhopper, WDV-B, WDV-W, wheat dwarf virus

Introduction

Wheat dwarf virus (WDV) is a circular singlestranded (ss) DNA virus, which belongs to the genus *Mastrevirus* in the family Geminiviridae (Vacke *et al.* 2004). The virus was first reported in former Czechoslovakia (Vacke 1961) and subsequently in several countries in Europe, Asia, the Near East and Africa (Schubert *et al.* 2014). WDV infects economically important plant species of the family Poaceae such as: wheat (*Triticum aestivum* L.), barley (*Hordeum vulgaris* L.), rye (*Secale cereale* L.) and triticale (×*Triticosecale* Wittm. ex A. Camus). The main symptoms induced by WDV are dwarfing caused by a strong shortening of internodes and the inhibition of the development of the roots and yellowing (or streaking) of leaves of infected plants. WDV causes yield losses of as much as 80% (Lindbland *et al.* 1999). The virus is transmitted by the leafhopper species, *Psammotettix alienus* (Dahlb.) (Vacke 1962) and *P. provincialis* (Ekzayez *et al.* 2011) in a persistent manner. WDV is transferred by both the larval and the imago stages. Individuals of the next generation acquire the virus after feeding on infected plants. Minimal time of acquisition feeding is 5 min, and minimum time of inoculation feeding is 15 min. The incubation period in the vectors lasts from 1 to 4 days (Vacke 1962). The virus cannot be transmitted by mechanical inoculation, or contact between plants, soil, pollen, or seeds (Vacke *et al.* 2004).

WDV was first divided into wheat (WDV-W) and barley (WDV-B) adapted forms, (Lindsten and Vacke 1991). The phylogenetic analysis of both forms indicated their separation into the strains A to E (Muhire

et al. 2013) and recently into A to F (Wu *et al.* 2015). Nevertheless, results of the studies on the sequence similarity and the phylogenetic relationships confirmed the significant separation of WDV-wheat and WDV-barley specific groups. WDV A and F strains were mainly originated from barley while B, C, D and E were mainly originated from wheat (Wu *et al.* 2015).

In Poland WDV was found for the first time in 1999 (Jeżewska 2001). Later studies confirmed virus infections in different parts of Poland and the presence of both virus forms was found (Jeżewska *et al.* 2010). Based on published results the Polish isolates Pol-WDV-W (KM079154) and WDV-B (KM079155) are classified as WDV-E and WDV-F strains, respectively (Trzmiel 2017, unpublished data).

The first studies on leafhoppers in cereal crops and grasses in Poland (conducted from 1968 to 1975) showed that *P. alienus* was present among caught insects, however the dominant species was *Macrosteles laevis* (Nowacka 1982). Recent studies indicate an increase in the number of *P. alienus* (Klejdysz and Wałkowski 2008; Klejdysz 2013), especially during the growing season, in winter cereal crops and self-sown cereal. Extending the growth period by a long and warm autumn gives the leafhoppers the opportunity for longer feeding on the plants. Moreover, climate change may also cause changes in the biology of the leafhoppers. It was found that they appear in spring cereal crops much earlier, even up to a month, than 40 years ago (Klejdysz and Wałkowski 2008). Furthermore, leafhoppers, appearing earlier on plants, have more time to grow and according to the literature can probably produce more than two generations per year. The high number of *P. alienus* in the autumn can also be indirect evidence of a third generation in a year.

The existing literature data do not contain any information about the infectivity of *P. alienus* in Poland. Therefore, the aim of this study was to develop a method using duplex polymerase chain reaction (PCR) for the simultaneous detection and discrimination of WDV-W and WDV-B forms. The results were verified by sequencing method.

Materials and Methods

Leafhopper sources

Four insect samples were collected in autumn (September, October 2013) from winter cereal fields in four different locations in Greater Poland: Borowo (Bor), Choryń (Ch), Szelejewo (Sze) and Winna Góra (WG) (Fig. 1). The leafhoppers were captured and stored in plastic tubes with 96% ethanol solution at 4°C before DNA isolation.

Fig. 1. Locations of collected leafhopper samples tested by duplex PCR

DNA isolation

Total DNA from four samples (one from each location), created by five leafhoppers were extracted using the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The concentration of total DNA was measured with a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Delaware, USA), and stored at –20°C.

Primer design

Two groups of complete nucleotide sequences of WDV-W and WDV-B forms were aligned using ClustalW software (Thompson *et al.* 1994) to search their specific regions. Two primer pairs WDV-H-F (CAAGGGGCGAGATCACACA)/WDV-H-R (CCACAACTACTACAACAGCC) and WDV-T-F (CGAGTAGTTGATGAATGACTCG)/WDV-T-R (GGCTGTTTCAACTCCAGGTCG) for duplex PCR were designed by Primer3 software (Rosen and Skaletski 2000) based on the short intergenic region (SIR) of WDV-B and on the replicase gene (RepB) of WDV-W. The potential cross-reactivity of the oligonucleotides and their specificity were verified by the basic local alignment search tool (BLAST) available on the website of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

Duplex PCR

The reactions were performed in thermal profile as follows: initial denaturation at 94°C for 2 min, 35 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C and a final cycle of 7 min at 72°C. The reaction mix (10 µl)

contained: 0.5 µl of each forward and reverse primers (10 µM); 1 µl of buffer Allegro*Taq* pH 8.6; 0.4 µl of dNTP Mix (10 mm), 0.1 µl of Allegro*Taq* polymerase DNA (Novazym, Poznań, Poland). The duplex PCR assays were carried out with positive controls: Pol-WDV-W (KM079154) and WDV-B (KM079155). The PCR products were separated by 1% agarose gel electrophoresis and stained with Midori Green DNA Stain (NIPPON Genetics Europe GmbH, Düren, Germany) for UV light visualization.

Specificity and sensitivity of duplex PCR

In order to confirm the specificity of this differentiating test additional PCR reaction with WDVcp-F (GAG GACCGAGGAAATTGGTT)/WDVcp-R (CGGACG GCGTACAGTTTCTA), amplifying a specific product (988 bp), was performed. The obtained PCR products of expected sizes were excised from the agarose gel and purified using Wizard®SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, USA), according to the manufacturer's instructions. Random samples of each amplicon were subsequently sequenced by Genomed S.A. (Warsaw, Poland) with specific primers. The nucleotide sequences were analyzed using BlastN online tool, and edited in BioEdit software (Hall 1999). The sensitivity of duplex PCR was determined using a range of 10-fold dilutions (from 60 ng · µl^{-1}) of the total DNA extracted from a random selected insect sample.

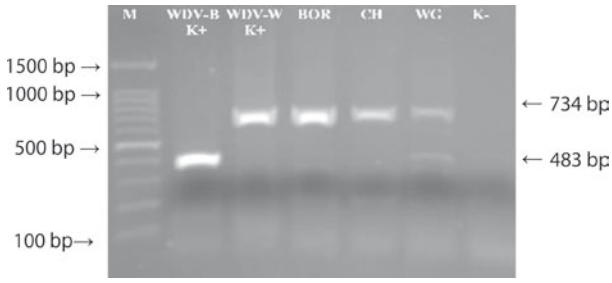

Fig. 2. Detection of WDV-W (734 bp fragment) and WDV-B (483 bp fragment) in insect samples collected from Greater Poland by duplex PCR. Amplified products were analyzed in 1% agarose gel. Lanes: M – 100-bp DNA ladder (Novazym); WDV-B K+ – plant sample infected with WDV-B; WDV-W K+ – plant sample infected with WDV-W; BOR, CH – insect samples infected with WDV-W; WG – insect sample co-infected with WDV-W/WDV-B; K- – no-template control

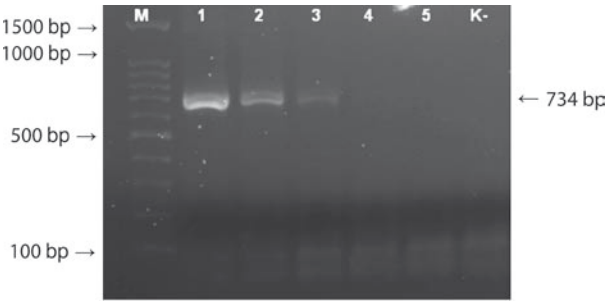

Fig. 3. Sensitivity of duplex PCR using a series of dilutions of total DNA (starting at 60 ng · µl^{-1}). Electrophoretic separation of PCR product in 1% agarose gel. Lanes: M – 100-bp DNA ladder (Novazym); 1–5 – 10-fold dilutions of total DNA; K- – no-template control

Results and Discussion

Information about viruliferous insects in local fields is very important for forecasting outbreaks of the wheat dwarf disease. In the literature both the classical transmission test (Mehner *et al.* 2003; Manurung *et al.* 2004) as well different molecular diagnostic techniques (Commandeur and Huth 1999; Schubert *et al.* 2007; Gadiou *et al.* 2012) have been described to determine the infectivity of the vector.

The duplex PCR assay presented here was developed for simultaneous detection and differentiation of WDV-B and WDV-W forms in insect samples, in one tube. The duplex PCR products of different length were visualized by electrophoresis. DNA amplicons of the expected size for WDV-W (734 bp) were detected in three out of four tested samples. Additionally, in the sample from Winna Góra both predicted DNA amplicons 734 bp and 483 bp were present (Fig. 2). WDV was not detected only in the insects from Szelejewo. The sequence data confirmed the specificity of the PCR products and the presence of WDV-W and WDV-B in tested insect samples. The obtained nucleotide sequences were consistent with nucleotide sequences of corresponding genome fragments of Polish isolates Pol-WDV-W (KM079154) and WDV-B (KM079155). They also showed high similarity (99%) with nucleotide sequences of other known WDV-W and WDV-B forms from GenBank database (JQ647455, FJ546188, KJ473704-05) and (HG422312-14, AM411651-52, FJ546181), respectively. Moreover, PCR with WDVcp-F/WDVcp-R primers generated specific (988 bp in size) amplicons for all leafhopper probes from four locations (data not shown). The sequencing results were consistent with those obtained for duplex PCR. The results indicated that the specific PCR product was detected in duplex PCR with the concentration of the template as low as 0.6 ng · µl^{-1}.

The developed assay is a sensitive and reliable method enabling the simultaneous detection and discrimination of the two main WDV forms, both in plants as well as directly in the vector. Furthermore, the duplex PCR is faster than an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (Rabenstein *et al.* 2005), rolling circle amplification combined with restriction fragment length

polymorphism (RCA-RFLP) (Schubert *et al*. 2007) or polymerase chain reaction combined with restriction fragment length polymorphism (PCR-RFLP) (Kundu *et al*. 2009) and more affordable than real-time PCR (Gadiou *et al*. 2012). In conclusion, the proposed assay is a useful diagnostic tool for the identification and differentiation of both WDV forms in studies to determine the infectivity of the virus vectors.

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