



CRISPR/Cas12a Ribonucleoprotein Complexes Target Conserved WDV Sequences: An In Vitro Proof-of-Concept for Non-GMO Antiviral Strategy

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ABSTRACT

Wheat (*Triticum aestivum* L.) is a staple crop globally, but its yield and quality are significantly affected by viral diseases, particularly the Wheat Dwarf virus (WDV). Developing antiviral strategies is crucial for enhancing wheat productivity. This study aims to investigate the use of CRISPR/Cas12a as an antiviral agent against WDV without requiring genetic modification of the host plants. A total of 10 WDV isolates from wheat and barley collected across six provinces in Türkiye, along with four reference isolates from Hungary, Sweden, and Iran, were analysed. The study demonstrates selective cleavage of conserved WDV regions by CRISPR/Cas12a RNPs in vitro, representing an early proof-of-concept

for non-GMO antiviral approaches rather than a direct field application. The designed gRNAs effectively induced DNA cleavage in WDV genomes, with gRNA1 showing consistent performance across all tested isolates. gRNA2 exhibited variability, successfully targeting seven out of ten isolates. No off-target effects were observed in control assays, confirming the specificity of the gRNAs. The CRISPR/Cas12a system, guided by specifically designed gRNAs, can effectively target and cleave WDV genomes, providing a potent, non-GMO antiviral strategy. This approach has potential applications for managing WDV infections in wheat and barley, highlighting its versatility and effectiveness.

Keywords: Wheat Dwarf Virus, CRISPR/Cas12a, Antiviral Agent, Guide RNA, Non-GMO, Wheat, Barley

1. Introduction

Wheat (*Triticum aestivum* L.) is a major staple crop worldwide and plays a critical role in global nutrition and food security. However, viral diseases transmitted by insect vectors continue to threaten wheat cultivation and cause significant yield losses. Among these, Wheat Dwarf Virus (WDV), a member of the Mastrevirus genus, is one of the most economically damaging viruses affecting wheat and barley production. WDV has a single-stranded circular DNA genome encoding proteins essential for replication, host infection, and vector transmission (Kün 1988).

Among the viral diseases detected by studies on grains, yellow dwarf virus diseases: Barley yellow dwarf viruses (BYDV-PAV, BYDV-MAV, BYDV-RMV, BYDV-SGV), Cereal yellow dwarf virus-RPV (CYDV-RPV), and WDV (WDV) is one among the important viral diseases that cause yield and quality losses (İlbağı 2003; Pocsai et al. 2003; İlbağı 2006; İlbağı et al. 2006). WDV is a species belonging to the genus Mastrevirus. The mastrevirus has a monopartite single-stranded circular DNA genome, and the typical mastrevirus genome encodes four different proteins: movement protein (MP), coat protein (CP), and two replication-associated proteins (Rep and RepA). The presence of an intron in the rep gene enables mastreviruses to produce two different forms of the replication protein. The long intergenic region (LIR) and short intergenic region (SIR) contain sequence elements required for viral replication and transcription (Kis et al. 2016).

Antiviral agents need to be developed to increase wheat productivity. Thanks to the developing technology, the CRISPR (Clustered regularly interspaced short palindromic repeats)/Cas system has been adapted to precisely edit the genomes of many species (Nekrasov et al. 2013). CRISPR-associated (CRISPR-Cas) is an adaptive immune system in prokaryotes that protects against invading bacteriophages by cleaving the DNA of bacteriophages (Horvath & Barrangou 2010; Garneau et al. 2010; Bhaya et al. 2011; Gasiunas et al. 2012). Successful examples of regulation in different plant species include rice, maize, wheat, soybeans, and tomatoes (Mikami et al. 2016; Svitashv et al. 2016; Gil-Humanes et al. 2017; Biswas et al. 2019; Cai et al. 2019; Kelliher et al. 2019; Lee et al. 2019; Okada et al. 2019). Three main types of CRISPR systems have been described so far: Types

I, II, and III. Of the type II CRISPR systems, CRISPR-Cas9 and CRISPR-Cas12a are the two main nucleases used to edit plant genomes (Nekrasov et al. 2013; Svitashev et al. 2015; Kim et al. 2017). The CRISPR-Cas9 system recognizes an NGG sequence called PAM (protospacer adjacent motif) and creates double helix breaks (Svitashev et al. 2015; Kim et al. 2017). Cas9 and Cas12a differ in PAM sequence requirements and cleavage patterns. Cas9 typically recognizes GC-rich NGG PAMs and generates blunt-ended DNA breaks, whereas Cas12a recognizes T-rich TTTV PAMs and produces staggered DNA cuts distal to the PAM site. These mechanistic distinctions enable flexibility in targeting genomic regions with different nucleotide compositions and support the adaptation of Cas12a for targeting AT-rich viral genomes (Svitashev et al. 2015; Kim et al. 2017). These different cut characteristics offer different uses in gene editing applications.

CRISPR-based antiviral approaches have been previously explored for plant viruses, including geminiviruses, potyviruses, and closteroviruses, primarily through transgenic expression of Cas9 or Cas12a. However, these strategies result in genetically modified plants. In contrast, our study evaluates CRISPR/Cas12a delivered as a ribonucleoprotein complex, offering a DNA-free, non-GMO antiviral approach. This study aims to evaluate whether CRISPR/Cas12a RNP complexes can selectively target and cleave conserved regions of the WDV genome *in vitro*, representing an early proof-of-concept for a non-GMO antiviral approach. Guide RNAs were designed from conserved sequences in the WDV coat protein gene, and cleavage efficiencies were assessed across multiple WDV isolates collected from different geographical regions.

2. Material and Methods

2.1. Sample Collection and WDV genome extraction

WDV genomic DNAs were isolated from wheat and barley collected from different countries (Hungary-IsolateA (JQ647455), Hungary-IsolateB (AM040732), Sweden-IsolateA (NC_003326), Iran-IsolateA (FJ620684), Iran-IsolateB (JN791096)) in the NCBI database and from different cities (Türkiye-Kırşehir-I43 (OQ183228), Türkiye-Kırşehir-I66 (OQ183229), Türkiye-Kırşehir-I41 (OQ230451), Türkiye-Kırşehir-I38 (OQ190468), Türkiye-Konya-I76 (OQ183230), Türkiye-Konya-I34 (MW387502), Türkiye-Afyon-I54 (MW387502), Türkiye-Nevşehir-I56 (MW381791) in Türkiye, using the previously mentioned method (Morca et al. 2021). Samples were collected randomly in the vicinity of winter barley and wheat fields with suspected WDV infection from 10 different locations in 6 six provinces (Afyon, Ankara, Kırşehir, Konya, Nevşehir, and Yozgat) (Morca et al. 2021). Each location was considered as one sample, and 10 samples (6 barley and 4 wheat) were collected. The total DNAs of 10 samples were extracted from wheat and barley plants using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany, Cat No./ID: 69104) according to the manufacturer's instructions.

2.2. gRNA design and synthesis for Cas12a

Multiple sequence alignment of WDV DNA sequences was performed using Clustal Omega. As a result of the alignment, conservative regions in the coat protein region of WDV were detected. Two different guide RNAs were designed based on these conserved regions. Guide RNA sequences "5-CGUGUCACGACGGAGUGGAU-3" and "5-AUGUUGUAUGUGCCUAUACG-3" were synthesized by GenScript by adding the Cas12a scaffold sequence (5-UAAUUUCUACUCUUGUAGAU-3) to the 5-base upstream region.

2.3. In silico Off-Target analysis

To evaluate the potential off-target effects of the designed guide RNAs, an *in silico* specificity assessment was performed. Each gRNA sequence was queried using NCBI BLASTn against the Triticum aestivum reference genome (IWGSC RefSeq v1.1) and against all available Mastrevirus genomes deposited in GenBank. Search parameters were adjusted to detect short sequence alignments with up to three mismatches, with particular emphasis on mismatches located within the seed region adjacent to the PAM site. Only alignments containing a canonical Cas12a PAM motif (TTTV) were considered relevant. No candidate off target matches with ≤ 3 mismatches were identified within annotated wheat gene coding regions. Similarly, no off-target sites with both a compatible PAM and full seed-region complementarity were identified in non-WDV Mastrevirus sequences. These findings indicated a high predicted target specificity for both gRNAs.

2.4. Targeting analysis of CRISPR/Cas12a RNP against WDV genome isolates

Guide RNAs were dissolved by adding 314 μL of deionized water to a final concentration of 10 ng/ μL . 50 μg of GenCRISPR Cas12a (Cpf1) Nuclease (Genscript, Cat. No: Z03502, USA) was dissolved in 1000 μL (100 μL of 10X Cas12a Nuclease Reaction Buffer and 900 μL of deionized water). 10X Cas12a Nuclease Reaction Buffer was prepared to pH 7.9 with 500mM NaCl, 100mM Tris-HCL, 100mM MgCl₂ and 1mg/mL BSA. A 20 μL reaction in 1xCas12a Nuclease Reaction Buffer containing 60 ng WDV ssDNA, 10 ng gRNA, and 100 ng GenCRISPR Cas12a (Cpf1) Nuclease for 30 mins at 37 °C results in a digestion efficiency of the linearized plasmid (for each WDV ssDNA). Samples loaded on the 1% agarose gel prepared in 1X TAE buffer solution using a BIO-RAD gel electrophoresis system were run at 100 V- and 30-min. Visualization, documentation, and analysis were then performed with Gel Doc™ XR+ (Serial Number 721BR16450, Software Version 6.0.1.34).

2.5. Statistics analysis

Quantification of WDV genome cleavage was performed by densitometry analysis of agarose gel band intensities. Gel images were imported into ImageJ (NIH) and converted to 8-bit format. Rectangular regions of interest (ROIs) were drawn over the uncleaved (full-length) and cleaved DNA bands for each reaction lane. Background correction was applied using a rolling-ball radius of 50 pixels.

Cleavage efficiency (%) was calculated as:

$$\text{Cleavage Efficiency} = (\text{Intensity of cleaved bands} / (\text{Intensity of cleaved} + \text{uncleaved bands})) \times 100$$

For each WDV isolate and each guide RNA condition, three independent cleavage reactions were performed. Mean values $\pm$ standard deviation (SD) are reported. Statistical comparison between gRNA1 and gRNA2 efficiencies across isolates was performed using a two-tailed paired Student's t-test ($\alpha = 0.05$). Statistical analyses and graphing were performed using GraphPad Prism 9.0.

3. Results

3.1. WDV Specific dataset preparation

WDV-positive wheat and barley leaf tissues were collected from symptomatic plants during the 2021–2022 growing seasons across Afyon, Ankara, Kırşehir, Konya, Nevşehir, and Yozgat provinces in Türkiye, as described by Morca et al. (2021). Viral presence was confirmed using PCR with WDV-specific CP primers. Genomic DNA sequences from isolates in Hungary, Sweden, and Iran were obtained from GenBank and used solely for comparative alignment rather than physical DNA extraction. Each isolate used in cleavage assays originated from wheat unless otherwise noted. In this study, the diversity of mutations in WDV genomes isolated from wheat and barley was investigated for an effective guide RNA design. WDV genome reference sequences were obtained from the NCBI database, and the reference numbers of these sequences are JQ647455, AM040732, NC003326, FJ620684, and JN791096, respectively (Figure 1). In addition, the reference numbers of WDV genomes isolated from wheat and barley collected by us from Kırşehir, Konya, Afyon, and Nevşehir in Türkiye are OQ183228, OQ183229, OQ230451, OQ190468, OQ183230, MW387505, MW387502, MW381791, respectively (Figure 1).

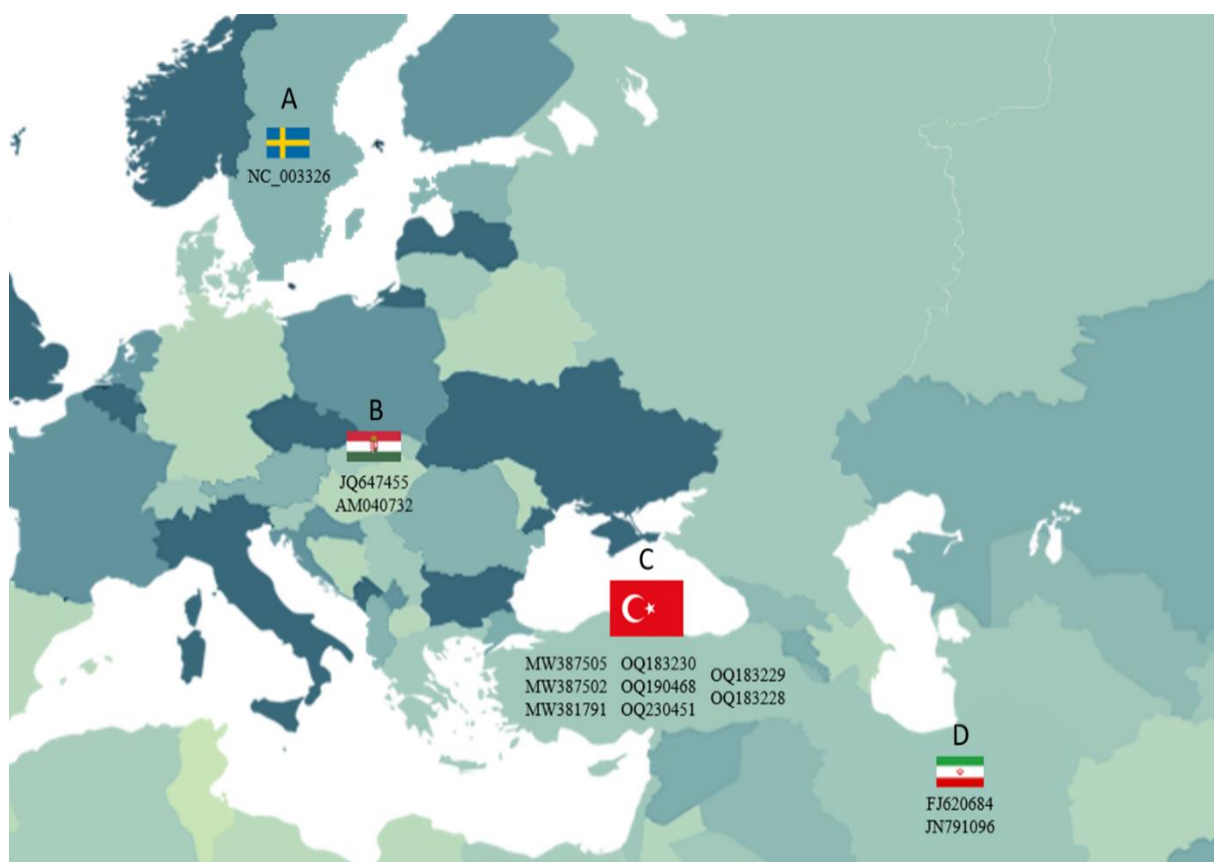


Figure 1- WDV strains isolated from different countries by NCBI sequence number (NC003326), GenBank numbers (JQ647455, AM040732, FJ620684, JN791096, MW387505, MW387502, and MW381791), and others (OQ183228, OQ183229, OQ230451, OQ190468, OQ183230). A.Sweden, B.Hungary, C.Türkiye, D.Iran

3.2. Global multiple alignment of WDV and target selection

WDV is a virus persistently transmitted by dwarf cicadas in *Psammotettix alienus* and *P. provincialis* species in the Cicadellidae family and infects wheat and barley plants (Abt et al. 2019). The CP protein mediates vector (*Psammotettix alienus*) transmission and is vector-specific. The genetic material of the virus is stored in this protein structure. It also allows the virion to attach to the host (wheat or barley) and penetrate the host cell membrane. (Abt et al. 2019) Therefore, conserved sequences in the coat protein (CP) gene of WDV strains isolated and sequenced from four different countries (Figure 1) were detected. In this study, the CP protein of WDV was targeted, and the reference sequences obtained from the database and the sequences of the strains isolated by us were compared using Clustal Omega software (Figure 2). As a result of the alignment, conserved sequences in the coat protein (CP) gene were detected (Figure 2A). Single point mutations were observed while designing the guide RNA, and two guide RNAs containing different numbers of single-point mutations were designed (Figure 2A-B). Guide RNA 1 was designed to contain two single-point mutations in the overall sequence, without any mutations in the PAM sequence. Here, T and C with the highest information content were selected (Figure 2B). Guide RNA 2 was designed to contain one point mutation in one PAM sequence and one in the overall sequence (Figure 2B). There is a T-C mutation in the PAM sequence, and the "TTTG" PAM sequence suitable for Cas12a was preferred. In the general sequence, T with the highest information content was chosen (Figure 2B). Cleavage outcomes were determined by agarose gel electrophoresis and densitometry (Table 1). PAM integrity and seed-region mismatch strongly influenced gRNA2 performance (Table 1). In subsequent experiments, these designed guide RNAs were used.

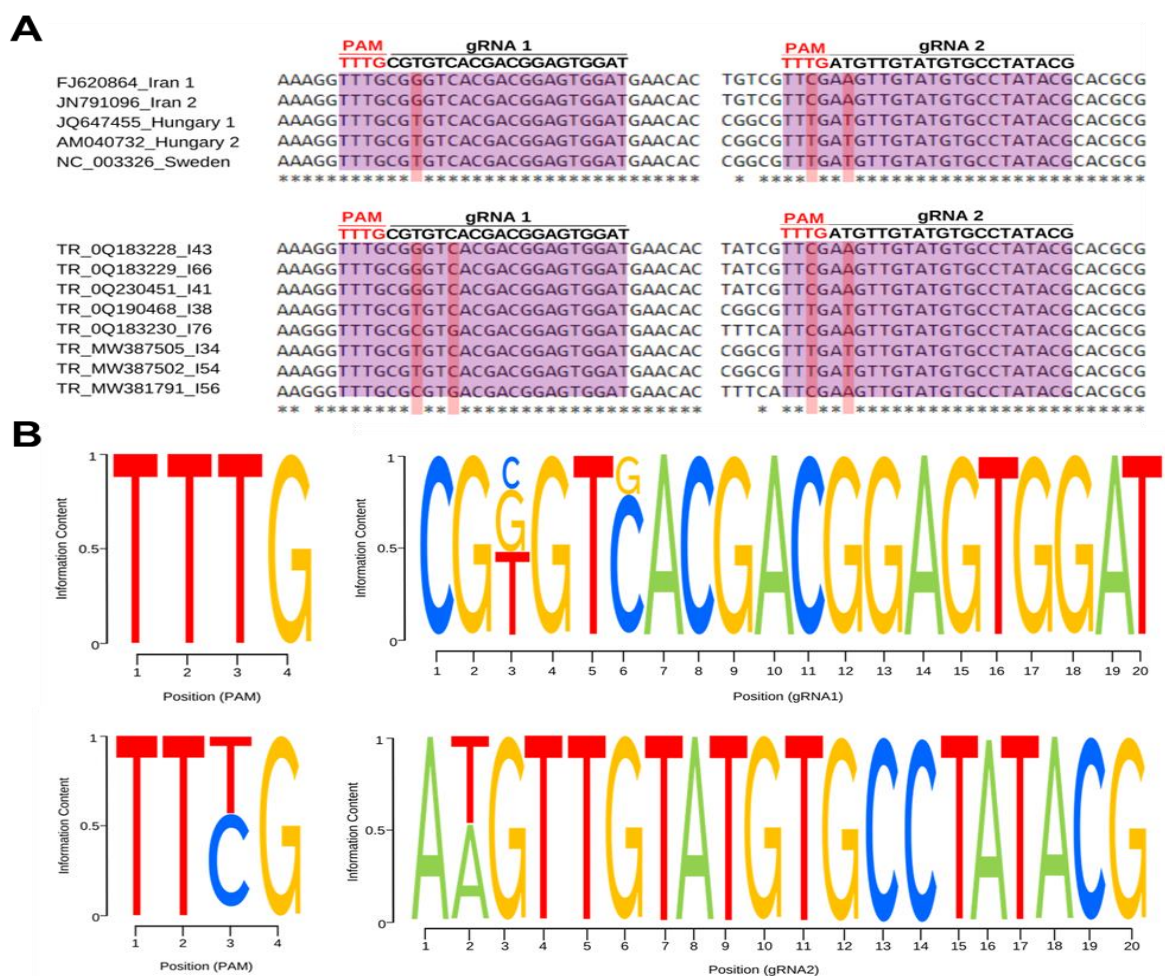


Figure 2- Multiple sequence alignment and information content of WDV genomes. A. Conserved regions in the CP protein gene and PAM sequences determined based on these regions, along with guide RNA designs, resulting from multiple sequence alignment.

B. The frequency of variants in the targeted region of CP genes. Sequence logo representation of guide RNAs

Table 1- Summary of WDV isolates, origin, mutation characteristics relative to gRNA target sequences, and observed cleavage outcomes

<i>Isolate Code</i>	<i>Accession No.</i>	<i>Origin (Region / Country)</i>	<i>Mutation in gRNA1 Target Site</i>	<i>Mutation in gRNA2 Target Site</i>	<i>PAM Status (TTTV)</i>	<i>Cleavage Result</i>
I43	OQ183228	Kırşehir / Türkiye	None	One SNP outside seed region	PAM preserved	Cleaved by both gRNAs
I66	OQ183229	Kırşehir / Türkiye	None	One SNP near PAM	PAM preserved	Reduced cleavage by gRNA2
I41	OQ230451	Konya / Türkiye	None	None	PAM preserved	Cleaved by both gRNAs
I38	OQ190468	Kırşehir / Türkiye	None	Two mismatches in seed region	PAM altered (TTTG→TTCG)	No cleavage by gRNA2
I76	OQ183230	Konya / Türkiye	None	None	PAM preserved	Cleaved by both gRNAs
I34	MW387505	Konya / Türkiye	None	SNP adjacent to PAM	PAM preserved	Partial cleavage by gRNA2
I54	MW387502	Afyon / Türkiye	None	SNP in seed region	PAM preserved	Reduced cleavage by gRNA2
I56	MW381791	Nevşehir / Türkiye	None	Multiple SNPs + PAM disruption	PAM altered	Cleaved only by gRNA1

3.3. Proof-of-concept for CRISPR/Cas System

Cas12a can be utilized as an antiviral agent because it can bind to single-stranded DNA and target the genomes of viruses. In this study, we aimed to test whether Cas12a, in conjunction with guide RNAs, can target isolated WDV genomes and induce DNA cleavage. For this purpose, we designed a proof-of-concept experiment. It is shown in principle in order to confirm that our theory has practical potential to demonstrate the viability of the work. First, guide RNAs designed specifically for WDV genomes isolated from wheat and barley were incubated with Cas12a, and then run on an agarose gel to evaluate the presence of CRISPR/Cas12a-related DNA breaks on WDV genomes (Figure 3).

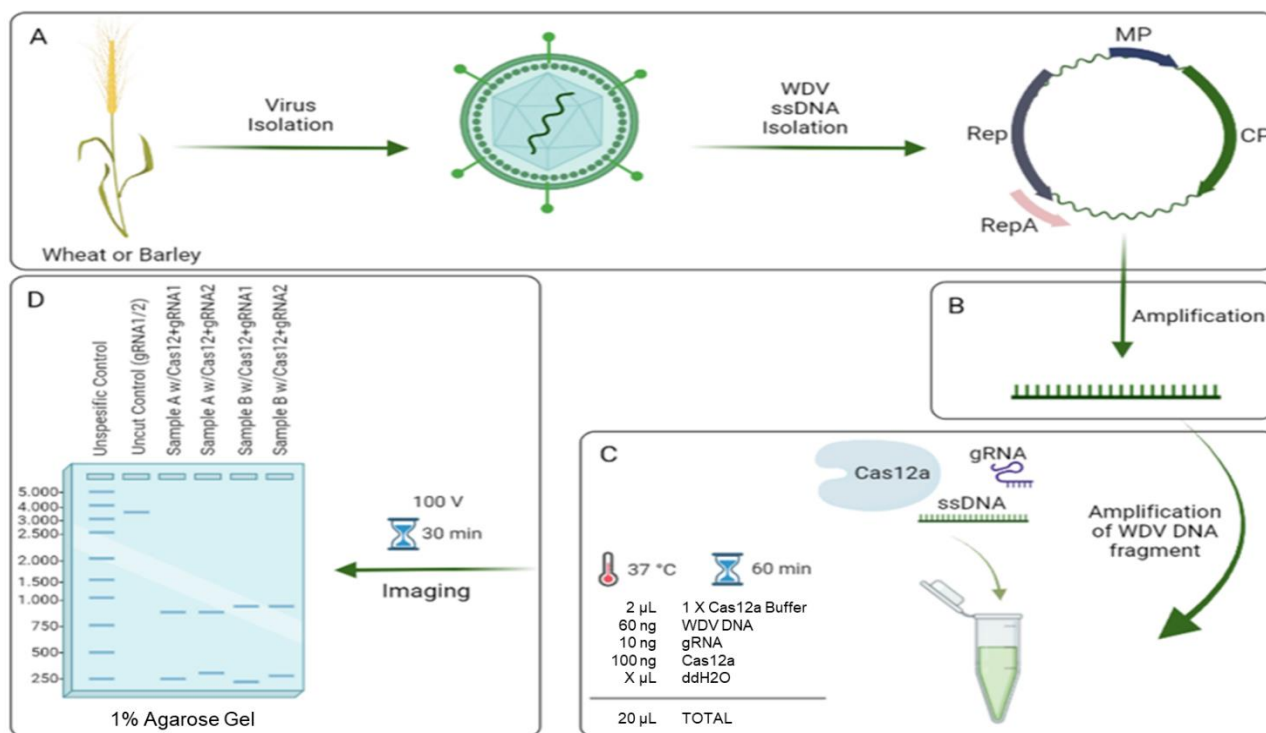


Figure 3- Experimental workflow for CRISPR/Cas12a-mediated targeting of WDV (WDV). **A.** WDV genomic DNA isolated from wheat and barley samples collected from multiple geographical regions. **B.** Amplification of the coat protein (CP) gene using WDV-specific primers. **C.** Assembly of CRISPR/Cas12a ribonucleoprotein (RNP) complexes consisting of Cas12a nuclease and WDV-specific guide RNAs (gRNA1 or gRNA2). **D.** Incubation of RNP complexes with WDV DNA followed by agarose gel electrophoresis to detect sequence-specific cleavage. All reactions were performed at 37 °C for 30 minutes in Cas12a reaction buffer

3.3. Study with CRISPR/Cas12a RNP structure on WDV

In order to detect DNA breaks that we can create in the targeted WDV genome with Cas12a, we aimed to determine the rates of DNA breaks with a special visualization method by running it on agarose gel. As a result of co-incubation, it was tested whether the designed guide RNAs cleaved in the WDV genome. We wanted to determine whether the two guide RNAs that form the RNP complex together with Cas12a will bind to unspecific circular DNA controls that do not contain the region we target, and whether they will create any unspecific breaks. In our first analysis for this, we showed that there is no such breakdown (Figure 4A). Next, we wanted to test whether WDV genomes isolated from different cities could be broken down jointly. In 10 isolated WDV genomes, guide RNA 1 was observed to break through the whole WDV genome (Figure 4B-4C and Supplementary Table 1). It was observed that guide RNA 2 was able to break through seven WDV genomes, but not in three WDV genomes (MW387505, MW387502 and MW381791) (Figure 4B and Supplementary Table 1). WDV genomes MW387505 and MW387502 match exactly with gRNA2, while WDV genome MW381791 has two different mutations (PAM: TTTG → TTCG and Seq: ATG → AAG) according to gRNA (Figure 2A). These results showed us that two guide RNAs designed specifically for WDV could identify WDV genomes isolated from different regions in a statistically significant way and break DNA after binding. This suggests that Cas12a and WDV-specific guide RNAs can be used as antiviral agents (Figure 4C).

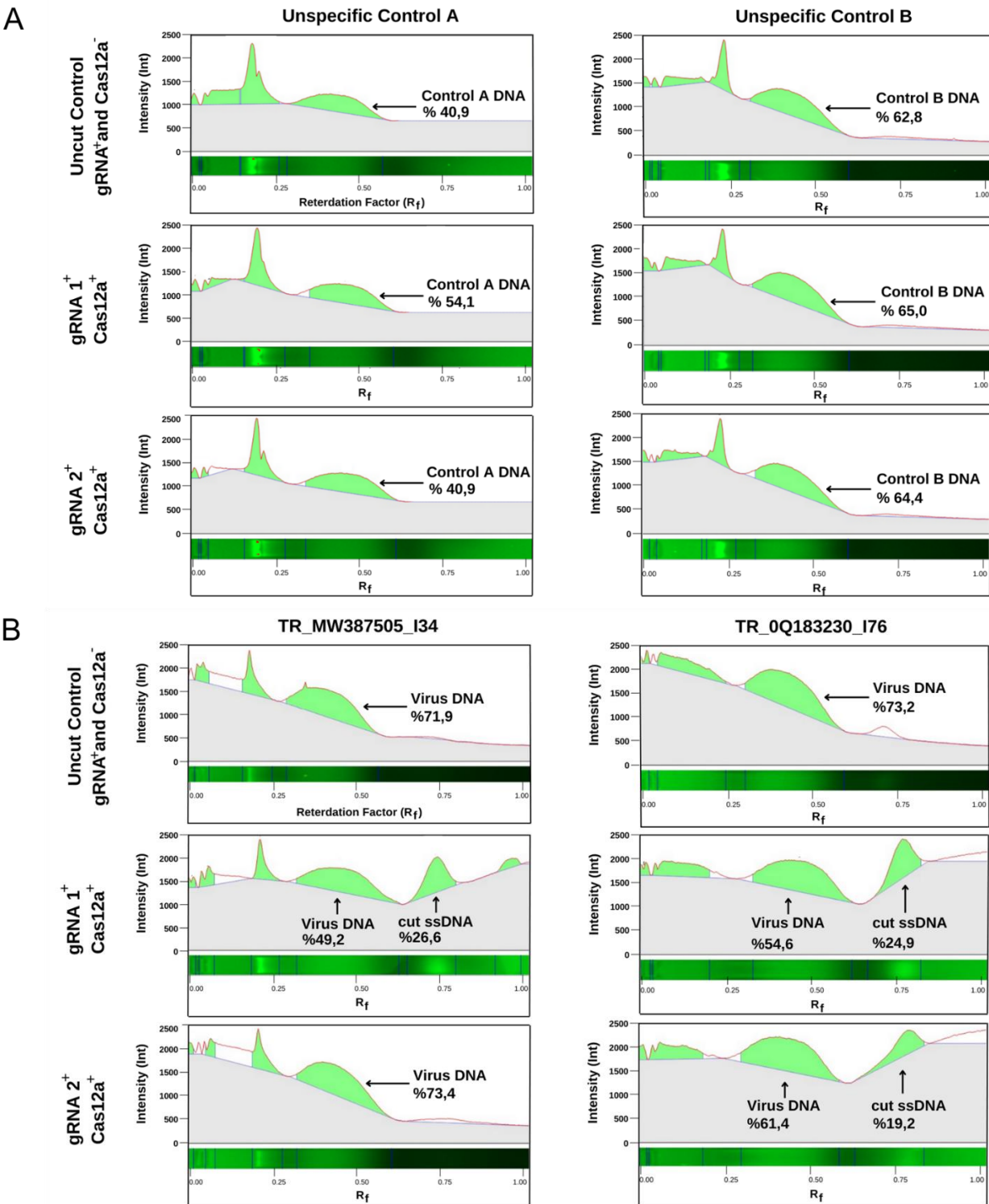
3.4. Quantification of cleavage efficiency across WDV isolates

To more precisely evaluate the performance of the designed guide RNAs, densitometry-based quantification of DNA cleavage was conducted using ImageJ. The analysis revealed that gRNA1 achieved high cleavage efficiency across all tested WDV isolates, with values ranging from 72.4% to 91.6% (mean = 83.7% ± 6.1). In contrast, gRNA2 exhibited variable cleavage efficiency, ranging from 18.9% to 87.2% (mean = 54.3% ± 20.4), consistent with the presence of point mutations adjacent to the PAM site in certain isolates. Statistical comparison confirmed that the difference in overall cleavage efficiency between gRNA1 and gRNA2 was significant ($P < 0.01$). These quantitative findings support the conclusion that sequence conservation at the target site strongly influences CRISPR/Cas12a activity, and that gRNA1 possesses broader antiviral applicability across WDV sequence variants.

3.5. In silico specificity assessment

Consistent with the observed selective cleavage patterns, the in silico off-target analysis did not identify any high-probability off-target binding sites for either gRNA in the wheat genome or within non-WDV mastrevirus genomes. Specifically, no matches containing both a canonical TTTV PAM and full seed-region complementarity were detected. These computational findings

support the conclusion that the designed guides are highly selective for WDV CP sequences, further reinforcing the feasibility of their application in antiviral strategies.



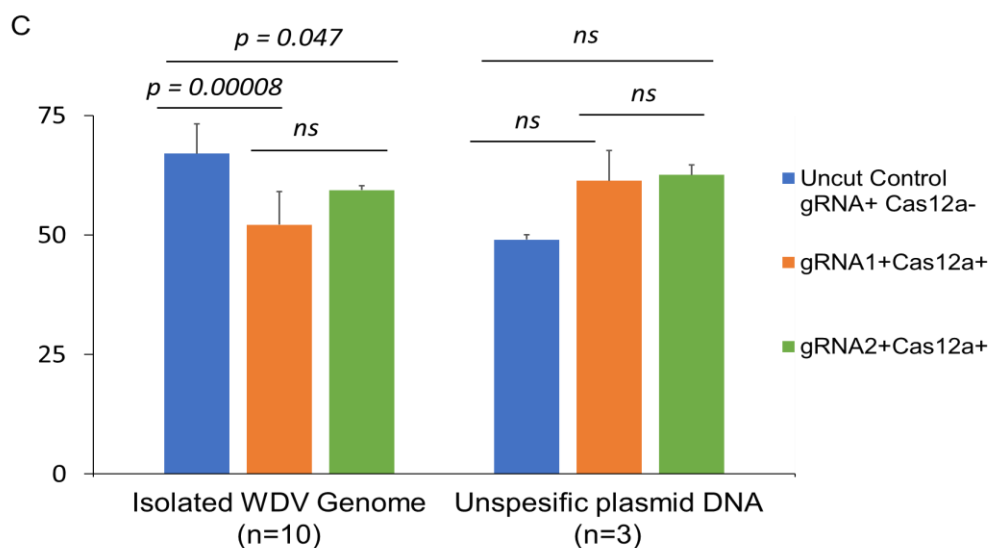


Figure 4- A. The unspecific control A and B plasmid DNAs were incubated in a Cas12a⁻, gRNA1⁺ & Cas12a⁺, and gRNA2⁺ & Cas12a⁺. Subsequently, after running on a 1% agarose gel, they were visualized using Gel Doc™ XR⁺. **B. The TR_MW387505_I34 and TR_OQ183230_I76 WDV (WDV) DNAs were incubated with uncut control (gRNA⁺ & Cas12a⁻), gRNA1⁺ & Cas12a⁺, and gRNA2⁺ & Cas12a⁺.** Subsequently, after running on a 1% agarose gel, they were visualized using Gel Doc™ XR⁺. **C. Quantitative densitometry analysis of CRISPR/Cas12a-mediated cleavage of WDV genomic DNA across isolates.** Cleavage efficiency (%) was calculated as the proportion of cleaved-band intensity relative to total DNA-band intensity. Data represent mean ± SD from three independent experiments. $P < 0.01$ indicates significant difference between gRNA1 and gRNA2 efficiency (paired t-test). All gels were run on 1% agarose in 1× TAE buffer at 100 V for 30 minutes

4. Discussion

The application of the CRISPR/Cas12a system as an antiviral strategy against the WDV (Wheat Dwarf virus) demonstrates a novel approach that does not require genetic modification of the host plants, thereby preserving their original genomic integrity. The effectiveness of CRISPR/Cas12a in targeting and cleaving WDV genomes was evident across various isolates from different geographic regions, indicating its potential broad-spectrum application (Kis et al. 2016).

This study demonstrates that CRISPR/Cas12a RNP complexes can selectively cleave WDV genomic DNA at conserved regions of the coat protein gene. The designed guide RNAs were informed by comparative sequence analysis across isolates from multiple regions, enabling us to identify sequences suitable for broad antiviral targeting. gRNA1 exhibited consistently high cleavage efficiency across all isolates tested, while gRNA2 showed reduced activity in isolates containing point mutations adjacent to the PAM region. These findings emphasize the importance of viral sequence diversity considerations when designing CRISPR-based antiviral tools (Nekrasov et al. 2013; Lee et al. 2019). The ability of gRNA1 to function despite the presence of mutations suggests a robust targeting mechanism that can accommodate minor genetic variations within the WDV population, a critical feature for managing viral pathogens with high mutation rates (Kelliher et al. 2019). Conversely, gRNA2's inability to induce cleavage in some WDV isolates indicates the importance of precise gRNA design, particularly in regions with potential single-nucleotide polymorphisms (SNPs) that could affect binding efficiency (Kim et al. 2017). This specificity underscores the necessity of comprehensive genomic analyses to identify conserved viral regions that can serve as reliable targets for CRISPR/Cas12a interventions (Gil-Humanes et al. 2017).

The absence of unexpected results in the unspecific and uncut controls reinforces the specificity of the designed gRNAs, as no off-target effects were observed. This high specificity is a significant advantage of the CRISPR/Cas12a system, minimizing the risk of unintended genomic alterations in non-target organisms (Svitashev et al. 2016). Furthermore, the successful application of CRISPR/Cas12a as an antiviral agent without integrating into the plant genome presents a promising alternative to traditional plant genetic modification approaches. This method not only preserves the genetic integrity of the crop but also simplifies regulatory hurdles associated with genetically modified organisms (GMOs) (Nekrasov et al. 2013). Quantitative densitometry analysis strengthened the interpretation of gel-based results, confirming that sequence mismatches directly impact cleavage efficiency. Additionally, *in silico* off-target screening did not detect high-probability off-target binding sites in the wheat genome, supporting the specificity and safety of the proposed antiviral approach.

This study represents an early-stage, *in vitro* proof-of-concept demonstrating that CRISPR/Cas12a RNP complexes can specifically recognize and cleave WDV genomic DNA. While the results confirm the feasibility of this antiviral approach, the current work does not yet evaluate the efficiency of CRISPR/Cas12a activity in planta. Therefore, the present findings should be interpreted as foundational evidence supporting the concept rather than as a complete antiviral solution. Future studies will focus on delivering CRISPR/Cas12a RNPs into wheat tissues using approaches such as nanoparticle-assisted foliar penetration, viral vector-mediated transient expression, or biolistic delivery. Additionally, infection suppression tests in greenhouse settings will be required to determine whether genome cleavage correlates with reduced WDV replication and symptom severity. These next steps are essential to validate the practical applicability of this non-GMO antiviral strategy in agricultural settings.

In support of the experimental findings, the *in silico* off-target screening indicated that both guide RNAs possess high predicted specificity for WDV, with no strong off-target matches detected in the wheat genome or related mastreviruses. This suggests that the antiviral strategy proposed here has low risk of unintended genomic disruption, particularly when applied in the form of transient RNP complexes rather than stable transgenes.

Our findings indicate that the CRISPR/Cas12a system, guided by specifically designed gRNAs, can effectively target and cleave WDV genomes, providing a potent tool for managing WDV infections in wheat and barley. Future research should focus on optimizing gRNA design to enhance targeting efficiency and exploring the application of this system against other plant viruses to further validate its versatility and effectiveness (Cai et al. 2019; Okada et al. 2019). The novelty of this study lies not in demonstrating the general cleavage capability of Cas12a, which is well-established, but rather in the design and validation of guide RNAs that target conserved regions of the WDV genome across diverse geographic isolates. By integrating comparative viral sequence analysis with empirical cleavage efficiency measurements, we identified a guide RNA (gRNA1) capable of consistently targeting WDV strains regardless of their mutation background. Furthermore, unlike prior approaches that rely on transgenic expression of CRISPR systems in plants, our RNP-based antiviral framework is non-GMO, transient, and does not introduce foreign DNA into the host. This positions the approach as a practical and potentially regulation-compatible antiviral strategy for agricultural use, pending successful delivery and validation in planta.

5. Conclusions

In this study, we designed WDV-specific guide RNAs and demonstrated that CRISPR/Cas12a RNP complexes can selectively cleave conserved regions of the WDV genome *in vitro*. The observed cleavage across isolates originating from different geographical regions suggests that this approach has the potential to serve as a broad-spectrum antiviral strategy. Importantly,

the CRISPR/Cas12a system was applied without introducing genetic modification into the host plant genome, highlighting the feasibility of developing non-GMO antiviral tools for crop protection.

However, the findings presented here are limited to in vitro assays and do not yet establish antiviral efficacy in living plant tissues. Consequently, this work should be regarded as a preliminary proof-of-concept rather than a complete antiviral solution. Future studies will involve optimization of delivery methods into wheat and barley tissues and evaluation of WDV suppression under greenhouse or field-like conditions. Overall, this study lays the conceptual and technical foundation for developing a non-GMO CRISPR-based antiviral platform for agricultural use.

Author Declarations

Authors' contributions: Concept: B.B. and C.T., Design: B.B. and C.T., Data collection: B.B., T.T., A.F.M., C.T., Analysis: B.B., T.T., M.K., C.T., Writing: B.B., B.A., C.T.

Conflict of interest/Competing interests: C.T. and B.B. have a patent application within the scope of this project with the application numbers 20770/14 and 2023/006118. The other authors declare no conflict of interest. B.B. and C.T. are inventors of patent applications (pending), including “An Antiviral Solution to Neutralize Wheat Dwarf Virus (In Turkish)” (20770/14) and “PCT/TR2023/051843” (2023/006118) at the Turkish Patent and Trademark Office and Patent Cooperation Treaty (PCT). The other author(s) declare no conflict of interest.

Ethics approval/declarations: No need/ Not applicable.

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Supplementary Table 1- Only alignments containing ≤ 3 mismatches and a canonical Cas12a PAM (TTTV) are considered biologically relevant. No such alignments were detected

gRNA	Predicted Off-Target Host Matches (≤ 3 mismatches)	PAM Present (TTTV)	Genomic Location (if any)	Interpretation
gRNA1	None detected in <i>Triticum aestivum</i>	No off-target PAM matches	—	High target specificity
gRNA2	One partial alignment (>3 mismatches) outside coding regions	PAM absent	Non-coding repetitive region	Functionally negligible
Non-WDV Mastreviruses	No seed-region matches for either gRNA	—	—	No cross-reactivity expected



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