T.C.
USKUDAR UNIVERSITY
INSTITUTE OF SCIENCE

DEPARTMENT OF MOLECULAR BIOLOGY
MOLECULAR BIOLOGY MASTER'S PROGRAM

MASTER'S THESIS

INVESTIGATION OF CRISPR/CAS12A-BASED ANTIVIRALS TARGETING WHEAT DWARF VIRUS (WDV) FOR THE PREVENTION OF WHEAT INFECTIONS

BUSE BARAN

THESIS SUPERVISOR

ASSIS. PROF.DR. CIHAN TAŞTAN

ISTANBUL-2024

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ABSTRACT

Investigation of CRISPR/Cas12a-Based Antivirals Targeting Wheat Dwarf Virus (WDV) for the Prevention of Wheat Infections

Wheat (*Triticum aestivum L.*) is an essential crop worldwide, but its yield and quality are frequently reduced by viral infections, particularly Wheat Dwarf Virus (WDV). Effective antiviral strategies are critical for improving wheat productivity. This study investigates the use of the CRISPR/Cas12a system as an antiviral tool against WDV without the need to genetically modify the host plants. WDV genomic DNA was isolated from wheat and barley samples collected from different regions in Turkey and other countries. To target conserved regions of the coat protein (CP) gene, guide RNAs (gRNAs) were designed and synthesized. The CRISPR/Cas12a ribonucleoprotein (RNP) complex was tested in in vitro assays to assess its ability to cut the viral DNA. The outcomes were analyzed through agarose gel electrophoresis. The results showed that the gRNAs successfully induced DNA cleavage in the WDV genome. Specifically, gRNA1 exhibited consistent effectiveness across all isolates tested, while gRNA2 showed variability, successfully targeting seven out of ten isolates. Control assays confirmed the specificity of the designed gRNAs, as no off-target effects were detected. These findings suggest that the CRISPR/Cas12a system, guided by well-designed gRNAs, can accurately target and cut WDV genomes, providing an effective non-GMO antiviral strategy. This approach holds significant potential for managing WDV infections in wheat and barley, demonstrating both its adaptability and reliability in agricultural applications.

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

ÖZET

Buğday Enfeksiyonlarını Önlemeye Yönelik, Buğday Cüce Virüsüne (Wheat Dwarf Virus, WDV) Hedefli CRISPR/Cas12a Tabanlı Antivirallerin Araştırılması

Buğday *(Triticum aestivum L.),* dünya genelinde temel bir tarım ürünü olmasına rağmen, verim ve kalitesi viral hastalıklardan, özellikle Buğday Cücelik Virüsü'nden (WDV) önemli ölçüde etkilenmektedir. Bu bağlamda, antiviral stratejilerin geliştirilmesi, buğday verimliliğini artırmak açısından büyük önem taşımaktadır. Bu çalışma, WDV'ye karşı CRISPR/Cas12a sisteminin antiviral ajan olarak kullanımını, konukçu bitkilerin genetik modifikasyonuna ihtiyaç duymadan araştırmayı amaçlamaktadır. WDV genomik DNA'ları, Türkiye ve diğer ülkelerdeki çeşitli bölgelerden toplanan buğday ve arpa örneklerinden izole edilmiştir. WDV'nin kılıf proteini (CP) genindeki korunmuş bölgeleri hedefleyen kılavuz RNA'lar (gRNA'lar) tasarlanmış ve sentezlenmiştir. CRISPR/Cas12a ribonükleoprotein (RNP) kompleksi, WDV genomlarında DNA kesilmesini sağlamak amacıyla in vitro deneylerle test edilmiş ve sonuçlar agaroz jel elektroforezi ile analiz edilmiştir. Tasarlanan gRNA'lar, WDV genomlarında etkili bir şekilde DNA kesilmesine yol açmış; gRNA1, test edilen tüm izolatlarda tutarlı bir performans sergilemiştir. gRNA2 ise değişkenlik göstermiş olup, on izolatın yedisini başarılı bir şekilde hedeflemiştir. Kontrol deneylerinde hiçbir yan hedef etkisi gözlemlenmemiş ve böylece gRNA'ların özgüllüğü doğrulanmıştır. Özel olarak tasarlanan gRNA'lar tarafından yönlendirilen CRISPR/Cas12a sistemi, WDV genomlarını etkili bir şekilde hedefleyip kesebilmektedir ve bu durum, GDO içermeyen güçlü bir antiviral strateji sunmaktadır. Bu yaklaşımın, buğday ve arpa üzerindeki WDV enfeksiyonlarının yönetiminde uygulanabilirliği bulunmakta olup, yönteminin çok yönlülüğünü ve etkinliğini vurgulamaktadır.

Anahtar Kelimeler: Buğday Cüce Virüsü, CRISPR/Cas12a, Antiviral Ajan, Rehber RNA, GDO'suz, Buğday, Arpa

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DECLARATION

I hereby declare that all the information and materials presented in this study have been obtained in accordance with academic principles. I have presented all visual, auditory, and written data and results in adherence to the standards of scientific integrity, without any alteration or falsification. I have duly cited all sources in accordance with scientific norms, and except where sources are explicitly acknowledged, this thesis is original, produced by myself, and has been written in compliance with the Thesis Writing Guide of Uskudar University's Institute of Science.

20/09/2024

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ICONS AND INDEX OF ABBREVIATIONS

AT: Adenine-Thymine

BYDV: Barley Yellow Dwarf Virus

Cas: CRISPR Associated

CP: Coat Protein

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CYDV: Cereal Yellow Dwarf Virus

DNA: Deoxyribonucleic Acid

FAO: Food and Agriculture Organization

GC: Guanine-Cytosine

LIR: Long Intergenic Region

MP: Movement Protein

NGG PAM: Protospacer Adjacent Motif (NGG Sequence Motif)

PAM: Protospacer Adjacent Motif

RNP: Ribonucleoprotein

SIR: Short Intergenic Region

WDV: Wheat Dwarf Virus

WSMV: Wheat Streak Mosaic Virus

RdRp: RNA-Dependent RNA Polymerase

Cpf1 (Cas12a): CRISPR-associated protein of type V

sgRNA: Single-guide RNA

gRNA: Guide RNA

crRNA: CRISPR RNA

Rep and RepA: Replication-associated Proteins

T-DNA: Transfer DNA

Ti plasmid: Tumor-inducing plasmid

Ri plasmid: Root-inducing plasmid

ssDNA: Single-Stranded DNA

DSB: Double-Strand Break

cDNA: Complementary Deoxyribonucleic Acid

NHEJ: Non-Homologous End Joining

HR: Homologous Recombination

PCR: Polymerase Chain Reaction

RT-PCR: Reverse Transcription PCR

ELISA: Enzyme-Linked Immunosorbent Assay

SHERLOCK: Specific High-sensitivity Enzymatic Reporter Unlocking

PEG: Polyethylene Glycol

BSA: Bovine Serum Albumin

TAE: Tris-Acetate-EDTA

NCBI: National Center for Biotechnology Information

IPM: Integrated Pest Management

GMOs: Genetically Modified Organisms

MENA: Middle East and North Africa

SPSS: Statistical Package for the Social Sciences

SNP: Single Nucleotide Polymorphism

µl: Microliter

1. INTRODUCTION

Grains serve not only as essential components in human diets, such as bread, but also play a critical role in animal feed and industrial applications. Among these, wheat (Triticum aestivum L.) stands out as one of the most widely cultivated crops globally. Thanks to its broad ecological adaptability, wheat is recognized as a staple food in nearly 50 countries. Its popularity stems from its high nutritional value, long shelf life, and ease of processing (Kün, 1988). According to the FAO, 784 million tons of wheat were produced worldwide in 2023. However, production is continually threatened by biotic and abiotic stress factors, causing significant yield and quality losses. Climate change-induced temperature increases have exacerbated the spread of viral pathogens carried by aphids, making wheat production more vulnerable.

Research highlights that several viral diseases affect the yield and quality of cereals. Notable among these are yellow dwarf viruses, which include BYDV, CYDV, and WDV (Pocsai et al., 2003; İlbağı, 2003, 2006; İlbağı et al., 2006). WDV, belonging to the Mastrevirus genus, carries a monopartite, ssDNA genome. This genome encodes four proteins: MP, CP, Rep, and RepA. An intron in the rep gene allows for the production of two distinct isoforms. Additionally, the genome contains two critical regions, LIR and SIR, essential for replication and transcription (Kis et al., 2016).

The require for progressed biotechnological approaches to secure wheat from viral contaminations has ended up progressively apparent. CRISPR/Cas frameworks have risen as capable instruments for exact genome altering (Nekrasov et al., 2013). CRISPR capacities as an versatile safe component in microbes, focusing on and cleaving attacking DNA (Horvath and Barrangou, 2010; Gasiunas et al., 2012). This innovation has been effectively connected in crops such as tomato, rice, maize, wheat, and soybean (Kelliher et al., 2019; Biswas et al., 2019; Gil-Humanes et al., 2017; Svitashev et al., 2015, 2019; Cai et al., 2018).

CRISPR frameworks are categorized into Sort I, II, and III. Among these, Sort II frameworks, counting Cas9 and Cas12a, are broadly utilized in plant genome altering (Nekrasov et al., 2013; Kim et al., 2017; Svitashev et al., 2015). Cas9 recognizes the NGG PAM grouping and actuates double-stranded breaks close to the target (Svitashev et al., 2015; Kim et al., 2017). In differentiate, Cas12a recognizes the TTTV PAM and cleaves downstream. Cas9 works best in GC-rich locales, whereas Cas12a performs more effectively in AT-rich districts. In addition, the two nucleases contrast in their cleavage designs: Cas9 produces exact cuts close to the PAM, whereas Cas12a makes stunned breaks advance from the acknowledgment location (Kim et al., 2017; Svitashev et al., 2015). These contrasts permit for different applications in genome editing.

Whereas past investigations basically centered on genome altering or mutagenesis, our consider embraces a novel approach by maintaining a strategic distance from plant genome alterations. We outlined a CRISPR/Cas RNP complex focusing on the viral genome straightforwardly. As a confirmation of concept, we tried whether virus-specific RNP complexes may cleave WDV DNA without joining into the have genome. Our discoveries illustrate that the planned sgRNA groupings effectively cleaved WDV DNA separated from different districts in Turkey. This affirms that the Cas12a-sgRNA RNP complex proficiently targets the WDV genome and stifles viral replication.

This study offers crucial insights into the use of CRISPR/Cas RNP complexes as an antiviral strategy in agriculture. It demonstrates the potential of CRISPR to detect and control WDV infections without altering the genetic structure of the host plant. Our results pave the way for developing non-genetically modified antiviral solutions in crop protection, promoting more sustainable and resilient agricultural practices.

2. GENERAL INFORMATION

2.1. History of Wheat

Wheat (*Triticum vulgare*) has been a staple food crop in global agriculture and has shaped human civilization for thousands of years. Approximately 10,000 years ago, wheat was first domesticated in the Fertile Crescent region of the Near East, marking the beginnings of agricultural practices (Shewry & Hey, 2015). The process of domestication involved the selective gathering and cultivation of wild wheat populations, leading to the development of more productive and resilient varieties. This transition provided humans with a reliable food source and facilitated the shift from nomadic lifestyles to settled agricultural societies. The domestication of wheat played a crucial role in strengthening the economic foundations of various societies throughout history and has been instrumental in the development of civilizations (Heun et al., 1997).

Wheat's role in the formation of agrarian societies is well documented, with significant impacts on the economies of major civilizations such as Ancient Egypt, the Roman Empire, and the Ottoman Empire (Nesbitt, 2001). In Egypt, the fertile lands along the Nile River allowed for the extensive cultivation of wheat, which bolstered agricultural production and trade. The Roman Empire expanded wheat cultivation across the Mediterranean, establishing trade routes that sustained its armies. During the Ottoman Empire, wheat was a cornerstone of agricultural productivity and strategic agricultural policies. These examples illustrate that wheat has not only been a critical agricultural product but also a key factor influencing the economic and political structures of societies throughout history.

The influence of wheat extends beyond its agricultural importance, as it has also been a cultural and religious symbol in many societies. In Ancient Egypt, wheat was associated with Osiris, the god of agriculture and fertility, symbolizing rebirth and sustenance. Similarly, in the Roman Empire, wheat was linked to Ceres, the goddess of agriculture, and played a central role in religious ceremonies and rituals that celebrated the harvest. The significance of wheat is further evidenced by its central role in the development of trade routes, as well as in the establishment of early economic systems that facilitated the exchange of goods and the accumulation of wealth.

Over time, the cultivation and trade of wheat have not only shaped economies but also contributed to the spread of cultural practices and technological innovations across regions. This interconnectedness, fostered by the cultivation of wheat, highlights its enduring impact on both ancient and modern civilizations. The widespread cultivation and trade of wheat have been a driving force in the economic and cultural development of societies, making it one of the most influential crops in human history.

2.1.1. Economic Importance of Wheat

Wheat is right now the third most-produced cereal edit universally, after rice and maize (FAO, 2023). Global wheat production spans approximately 218 million hectares, with annual yields ranging between 770 and 780 million metric tons. This production provides a fundamental source of calories and protein for billions of people worldwide. The economic significance of wheat extends beyond its production volume; it also serves as a primary raw material for industries and labor markets. Wheat grains are essential in the production of flour, bread, pasta, biscuits, and cakes, among many other products. The global trade of wheat-based products generates a multi-billion-dollar market and provides employment for millions of individuals.

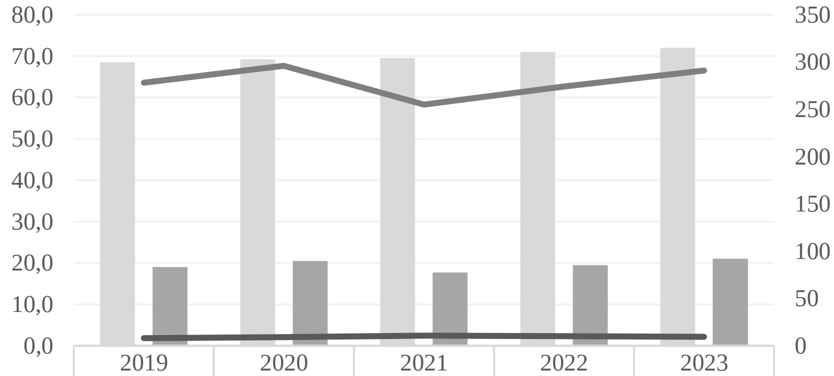
Table 1. Based on data from the FAO, wheat production across countries over the past five years (2019-2023).

Flag	Country	2023	2022	2021	2020	2019
	China	137.7M	137.7M	136.9M	134.3M	133.6M
	India	110.0M	107.7M	109.6M	107.9M	103.6M
	Russia	93.0M	104.2M	76.1M	85.9M	74.5M
	USA	46.7M	44.9M	44.8M	49.8M	52.3M
	Canada	33.0M	34.3M	22.4M	35.4M	32.3M
	France	34.5M	34.6M	36.6M	30.2M	40.6M
	Australia	37.0M	36.2M	31.9M	30.5M	23.5M
	Pakistan	27.5M	26.2M	27.5M	25.2M	24.4M
	Germany	22.8M	22.6M	21.5M	22.2M	23.1M
	Ukraine	18.0M	20.7M	32.2M	24.9M	28.4M
	Turkey	21.5M	19.8M	17.7M	20.5M	19.0M

Wheat occupies a central role in global trade and is essential to ensuring food security worldwide. Regions such as MENA are heavily reliant on wheat imports. Fluctuations in wheat prices and production challenges can have direct impacts on the economies and social stability of these countries. In developing nations, wheat is vital both as a staple food and a source of income. Variations in production can disrupt price stability in both national and international markets, placing significant pressure on agricultural economies. In this global context, Turkey emerges as a significant player in the wheat market, balancing its own domestic needs with the demands of international trade. As of 2023, Turkey produced approximately 21.5 million tons of wheat, which is vital for national food security and the agricultural economy. The major wheat-producing regions in Turkey include Central Anatolia, Thrace, and Southeastern Anatolia, which are key due to their extensive agricultural areas and favorable climate conditions.

Despite being a major wheat producer, Turkey imports wheat when domestic production falls short of meeting demand. This is particularly the case for producing higher-quality flour, which requires wheat with a higher protein content. The majority of Turkey's wheat imports come from countries like Russia, Ukraine, and Canada. In 2022, Turkey imported approximately 10 million tons of wheat, necessary to cover domestic production shortfalls and meet market demand.

Table 2. Wheat cultivation area, production, and yield data in Turkey (2019-2023) based on Turkish Statistical Institute (TUIK) reports.



	2019	2020	2021	2022	2023
Area (million decares)	68,5	69,2	69,5	71,0	72,0
Production (million tons)	19,0	20,5	17,7	19,5	21,0
Yield (kg/da)	278	296	255	274	291
Imports (million tons)	8,0	9,2	10,7	10,0	9,5

Moreover, the environmental impact of wheat production cannot be overlooked. Wheat cultivation covers vast areas and significantly affects soil health, water usage, and biodiversity. Sustainable agricultural practices are essential to mitigate these environmental impacts, preserve soil fertility, and efficiently utilize water resources. As the agricultural sector continues to evolve, another critical aspect that requires attention is the impact of climate change on wheat production. Rising temperatures, water scarcity, and shifting precipitation patterns pose a significant threat to global wheat yields, thereby challenging food security on a broader scale (Lobell et al., 2012). Addressing these challenges requires the development of wheat varieties adapted to changing climate conditions, which is essential for ensuring sustainable wheat production in the future.

In addition to these environmental and climatic challenges, wheat production is also threatened by a variety of viral diseases that can significantly reduce yields. These diseases are caused by pathogens such as *Barley Yellow Dwarf Virus* (*BYDV*), *Wheat Streak Mosaic Virus* (*WSMV*), and *Wheat Dwarf Virus* (*WDV*). These viruses lead to symptoms like stunting, leaf yellowing, and reduced yield in wheat plants (Murray et al., 2018). Addressing these biological threats, alongside environmental and economic factors, is essential for maintaining robust wheat production levels globally.

2.1.2. Important Viral Diseases in Wheat

One of the most significant challenges in wheat agriculture is the loss of yield and quality due to various viral diseases. These diseases, transmitted by insect vectors, can cause severe economic damage in wheat fields. Among the most prevalent and agriculturally significant viral pathogens affecting wheat are *Barley Yellow Dwarf Virus (BYDV)*, *Wheat Streak Mosaic Virus (WSMV)*, and *Wheat Dwarf Virus (WDV)*. Effectively managing these diseases is crucial for ensuring sustainable wheat production.

Barley Yellow Dwarf Virus (BYDV) is a positive-sense single-stranded RNA (ssRNA) virus belonging to the *Luteoviridae* family. This ssRNA genome, approximately 5.6-6 kb in length, encodes genes responsible for viral replication, capsid formation, and the synthesis of proteins associated with vector interaction (Miller & Rasochová, 1997). One of the key genes, the RdRp, directs RNA synthesis during viral replication. The gene encoding the capsid protein plays a crucial role in constructing the virus's structure, facilitating its transmission through vectors like aphids. The genome also contains MPs that assist in the cell-to-cell spread of the virus within the plant, promoting the progression of the infection throughout plant tissues.

There is significant genetic diversity among the different strains of *BYDV*, which plays a critical role in determining the virus's impact on wheat plants (D'Arcy & Domier, 2005). For example, some strains exhibit higher virulence, leading to more severe symptoms in wheat, while others may cause milder infections. These genetic differences manifest as single nucleotide polymorphisms (SNPs) or small indel mutations within the RNA genome. Such variations are crucial in understanding how the virus spreads within plant cells, targets specific tissues, and influences the plant's immune response. Thus, the genetic diversity within *BYDV* provides essential insights into the virus's pathogenicity and its transmission dynamics in wheat.

The virus is prevalent across Europe, North America, Asia, and Australia. In Europe, *BYDV* is a major concern in countries such as the United Kingdom, France, and Germany, where cool, moist climates favor the survival and activity of aphid vectors (Aradottir et al., 2021). In North America, the virus is prevalent in the United States and Canada, particularly in regions with significant wheat production like the Great Plains. Australia also faces significant challenges with *BYDV*, especially in New South Wales and Victoria, where the virus has led to considerable yield losses in wheat-growing areas. The widespread presence of *BYDV* in these regions underscores its global impact on wheat production.

Wheat Streak Mosaic Virus (WSMV) is a positive-sense ssRNA virus belonging to the *Potyviridae* family. This ssRNA genome, approximately 9.3 kb in length, encodes genes responsible for viral replication, the synthesis of the capsid protein, and the proteins that facilitate the virus's movement within the plant (Stenger & French, 2009). This genetic structure is crucial in determining the virus's spread and pathogenicity in wheat plants. The RdRp gene enables the replication of viral RNA, while the gene encoding the CP constructs the viral structure, allowing it to be transmitted through vectors. The genome also contains MPs that assist in the cell-to-cell spread of the virus within the plant, promoting the progression of the infection throughout plant tissues.

There is significant genetic diversity among *WSMV* strains, and these differences play a major role in determining the virus's impact on wheat plants. Mutations within the virus's RNA genome, particularly those affecting replication capacity and intracellular movement, can influence the severity of disease symptoms. For instance, certain strains may carry mutations that lead to more severe disease manifestations, while others result in milder symptoms. This genetic diversity influences the virus's regional spread and pathogenicity, necessitating tailored management strategies in different areas (Stenger et al., 1998; Tatineni et al., 2010).

WSMV is particularly prevalent in North America but also causes significant agricultural losses in other regions. The Great Plains region of the United States is one of the areas where the virus is frequently observed (Tatineni et al., 2010). The virus is also widespread in Canada's wheat-producing regions. In Australia, particularly in New South Wales and Victoria, *WSMV* has caused significant challenges for wheat production (Byamukama et al., 2014). In Europe, the virus is relatively less common, but localized outbreaks can occur in certain countries.

Wheat Dwarf Virus (WDV) is a virus belonging to the *Geminiviridae* family, genus *Mastrevirus*, that causes significant economic losses in small grain cereals. The *WDV* genome consists of a circular ssDNA approximately 2.75 kb in length. This genome encodes four main proteins: the MP, which facilitates cell-to-cell movement within the plant; the CP; and two replication-associated proteins (Pfrieme et al., 2023; Dallot et al., 2020).

WDV is genetically divided into two main strains: the wheat strain and the barley strain. These two strains typically infect their preferred hosts and are unable to infect the other's preferred host. However, in some cases, functional transcomplementation between these two strains can occur, allowing both strains to infect the same plant simultaneously (Dallot et al., 2020).

WDV is transmitted by the leafhopper *P. alienus,* which carries the infection in a determined, non-propagative way. The infection is shown in tainted plants through side effects such as overshadowing, leaf yellowing, streaking, and stifled heading. *WDV* contamination can lead to surrender misfortunes of up to 90% in wheat, coming about in extreme financial impacts depending on the virus's predominance in grain generation regions (Pfrieme et al., 2023).

The virus is widespread in Europe, the Middle East, Africa, Western Asia, and other parts of Asia, causing significant challenges for cereal production in these regions. Effective management of *WDV* requires integrated pest management strategies, the cultivation of resistant plant varieties, and vector control measures. However, knowledge about managing this virus is limited, and further research is needed (Dallot et al., 2020; Pfrieme et al., 2023).

The prevalence of these viruses varies by region, depending on factors such as climate conditions, agricultural practices, and the populations of vector insects. The Central Anatolia Region is one of Turkey's most critical wheat production areas, where *WDV* and *BYDV* are particularly widespread. The cold winter conditions of Central Anatolia, which are well-suited to vectors like aphids and leafhoppers, contribute to the spread of these viruses. *WDV* is frequently reported in major production centers such as Polatlı, Konya, and Eskişehir, while *BYDV* has a broader distribution and poses a significant threat to agricultural production in the region (Yıldırım et al., 2019; Demir & Güldalı, 2020).

The Southeastern Anatolia Region, on the other hand, is particularly affected by *WSMV*. The hot and dry conditions during the summer in this region increase the populations of aphids, which in turn facilitates the spread of the virus. *WSMV* has led to significant yield losses in provinces such as Diyarbakır, Şanlıurfa, and Mardin, adversely affecting the region's agricultural output. Additionally, irrigation practices and intensive farming contribute to the wider spread of the virus.

In the Thrace and Marmara Regions, located in northwestern Turkey, *BYDV* and *WSMV* are also prevalent. The Thrace Region is a significant wheat-producing area, and it's cool and humid climate supports aphid populations, promoting the spread of *BYDV*. *WSMV*, meanwhile, has caused sporadic outbreaks in provinces such as Edirne and Tekirdağ, impacting agricultural production in these areas (Kaya & Başer, 2018).

2.1.3. Wheat Dwarf Virus Infection Mechanism

The infection mechanism of WDV is a complex process involving the virus's entry into plant cells, replication, and systemic spread within the plant. The virus is persistently transmitted by dwarf cicadas such as *P. alienus* and *P. provincialis*, which play a crucial role in the virus's epidemic in the region. The prevalence of these vector insects directly influences the spread of WDV, with infection rates increasing proportionally to the abundance of these vectors (Dallot et al., 2020; Pfrieme et al., 2023).

The infection begins when the virus is introduced into a plant cell by its vector. Upon entering the plant cell, WDV targets the cell nucleus. In the nucleus, the virus's double-stranded DNA genome is replicated by the plant's DNA polymerase enzyme, initiating the replication process. During this process, the viral genome directs the synthesis of two key proteins, Rep (replication-associated protein) and RepA. These proteins enable the virus to replicate its genome and produce more viral particles by hijacking the plant cell's genetic machinery (Pfrieme et al., 2023).

The virus's spread within the plant is facilitated by the MP and CP, which allow the virus to move from cell to cell and maintain stability within the plant. MP facilitates the intercellular movement of the virus, while CP ensures the protection and stability of the viral particles within the plant. Through these processes, the virus exhibits systemic spread within the plant, reaching various tissues and advancing the infection. *WDV* infection results in symptoms such as stunting, leaf yellowing, and streaking, leading to significant yield losses (Dallot et al., 2020; Pfrieme et al., 2023).

Weeds also play a critical role in the spread of *WDV*, especially in transferring the virus to the next production season. Weeds, such as wild oats, which often go unnoticed in grain fields, are considered primary sources of the virus. Dwarf cicadas, the vectors of WDV, initially feed on these weeds before spreading the virus to the field (Pfrieme et al., 2023). In the later stages of infection, the virus suppresses the plant's heading and seed production processes, causing substantial agricultural losses. The virus's control over the plant cell disrupts the plant's growth and development processes, making WDV infection a major threat, particularly in agricultural areas, and necessitating the development of effective management strategies.

2.1.4. Diagnostic Tools for Wheat Viral Diseases

Accurate diagnosis of viral diseases in wheat plants is essential for developing effective management strategies. WDV, BYDV, and WSMV can cause similar symptoms, making it challenging to distinguish these viruses based on visual inspection alone. When visual symptoms are not conclusive, laboratory tests and molecular analyses are indispensable for accurate identification.

Figure 1. Symptoms and vector insects associated with viral diseases in wheat. **(1a)** Symptoms of BYDV infection, showing leaf yellowing and necrosis; **(1b)** Vector of BYDV, Bird Cherry-Oat Aphid and greenbug nymphs. **(2a)** Symptoms of WSMV infection, characterized by streaky mosaic patterns and leaf yellowing; **(2b)** Vector of WSMV, wheat curl mite. **(3a)** Symptoms of WDV infection, causing stunted growth and leaf yellowing; **(3b)** Vector of WDV, dwarf leafhopper (*Psammotettix alienus*).

The ELISA test is widely used in diagnosing viral diseases. This method detects the presence of a virus by using virus-specific antibodies. Different antibodies are used to detect the presence of *WDV*, *BYDV*, and *WSMV* (Clark & Adams, 1977).

PCR and RT-PCR methods amplify the viral genetic material to enable its detection. PCR is used for DNA viruses like *WDV*, while RT-PCR is preferred for RNA viruses such as *BYDV* and *WSMV*. These methods allow for the identification of the viruses based on their genetic differences (Mackay et al., 2002).

Sequencing is used to determine the genomic sequences of viruses, revealing how these viruses are genetically distinct from one another. This method is also used in phylogenetic analyses to ensure accurate diagnosis (Sanger et al., 1977).

2.1.5. Methods Used to Manage Viral Diseases in Wheat

Managing viral diseases in wheat requires a multifaceted approach that integrates various strategies to minimize the impact of these pathogens on crop yields. Effective management focuses on enhancing plant resistance, reducing the spread of viruses, and controlling the insect vectors that transmit these diseases.

Developing and utilizing disease-resistant wheat cultivars stands out as one of the most effective and sustainable strategies. Breeding programs have achieved substantial advancements by integrating resistance genes into wheat varieties, offering vital protection against viruses like BYDV, WSMV, and WDV. Advances in molecular biology, particularly marker-assisted selection and genetic engineering, have accelerated the development of resistant lines. However, the durability of resistance remains a challenge, as viruses can evolve to overcome these genetic defenses over time.

In addition to genetic resistance, cultural practices play a vital role in managing viral diseases by disrupting the life cycles of vectors and reducing the conditions that favor virus spread. For example, crop rotation can break the transmission cycle by interrupting the continuity of host plants. Delayed planting can minimize young wheat plants' exposure to vectors during critical periods. Moreover, removing volunteer wheat and infected plants from fields reduces the virus reservoir, lowering the overall infection pressure.

Chemical control, particularly the use of insecticides, is another crucial tool in managing viral diseases by targeting the insect vectors responsible for transmission. Vectors such as aphids and leafhoppers, which spread *BYDV* and *WDV*, can be controlled through systemic or contact insecticides. However, over-reliance on chemical controls can lead to insecticide resistance and has environmental and health implications. Consequently, chemical control is often integrated with other strategies to maintain long-term effectiveness (Dedryver et al., 2010).

Biological control methods also offer an environmentally friendly approach by utilizing the natural enemies of vector insects, such as predators, parasitoids, and pathogens, to reduce vector populations. For instance, lady beetles and parasitic wasps can significantly lower aphid populations, thereby limiting the spread of *BYDV*. While biological control alone may not fully prevent virus transmission, it is a valuable component of an integrated pest management (IPM) strategy (Van Emden & Harrington, 2007).

Integrated Pest Management (IPM) combines multiple strategies, including resistant cultivars, cultural practices, chemical control, and biological control, to manage viral diseases sustainably and effectively. The goal of IPM is to minimize the impact of viral diseases on wheat production while reducing reliance on chemical inputs and preserving the environment. IPM practices are tailored to specific regional conditions, considering the local ecology of vectors and the prevalence of various viral diseases (Kogan, 1998).

In conclusion, effective management of viral diseases in wheat requires a comprehensive approach that integrates various strategies. While developing resistant cultivars is fundamental to disease management, the combination of cultural practices, chemical and biological controls, and IPM offers a more sustainable and effective solution to combat these persistent threats.

2.2. History of CRISPR

The discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated Cas proteins marks a groundbreaking advancement in the field of genetic engineering. The history of the CRISPR/Cas system illustrates how scientific discoveries evolve over time and how an initially obscure finding can transform into a global research and application phenomenon.

CRISPR sequences were first discovered in 1987 by Japanese scientist Yoshizumi Ishino and his team in *E. coli* bacteria. These sequences were identified as short, repeating DNA sequences with an unknown function. At that time, the biological role of these sequences was unclear, and they were often regarded as anomalies (Ishino et al., 1987). However, this discovery opened the door to a new era in genetics and inspired numerous studies aimed at understanding how CRISPR functions in bacteria. In 1993, other researchers observed CRISPR sequences in different bacterial species and recognized that these sequences were widespread in bacterial genomes. However, there was no clear understanding of their function. Initially, it was thought that these sequences might be involved in the rearrangement of genetic material within the cell (Mojica et al., 2005).

In the early 2000s, Francisco Mojica and his team noticed a potential connection between CRISPR sequences and viruses. Mojica hypothesized that CRISPR sequences could be a defense mechanism against viral infections in bacteria. His hypothesis suggested that CRISPR sequences store DNA fragments from previous viral infections and use this information to defend against future infections (Mojica et al., 2005). In 2005, three independent research groups in Europe confirmed that CRISPR sequences are part of a bacterial defense mechanism against viruses. These researchers showed that CRISPR sequences contain short DNA fragments matching viral DNA and play a crucial role in the bacterial immune system (Bolotin et al., 2005).

Following these significant discoveries, research on the molecular mechanism of the CRISPR/Cas system intensified. In 2007, it was demonstrated that the CRISPR/Cas system could target and cleave foreign DNA in bacteria. This study suggested that the CRISPR/Cas system could be a potential tool in genetic engineering (Barrangou et al., 2007).

A pivotal study demonstrating the potential of the CRISPR/Cas9 system as a tool for modern genetic engineering was conducted by Jennifer Doudna and Emmanuelle Charpentier, published in 2012. This research showcased the CRISPR/Cas9 system's ability to function as a programmable gene-editing tool, enabling precise cuts at specific DNA sequences (Jinek et al., 2012). The versatility of CRISPR/Cas9 technology in genetic engineering facilitated its swift transition from research laboratories to industrial and agricultural applications. By 2013, the system had been successfully applied across various fields, including plant biotechnology and animal genetics. In plant biotechnology, CRISPR/Cas9 was particularly employed to enhance disease resistance and boost agricultural productivity (Mali et al., 2013; Cong et al., 2013).

The discovery of CRISPR/Cas9 also sparked ethical debates, especially concerning its potential use in human genome editing. The possibility of genetically modifying human embryos raised significant scientific and societal concerns. In 2015, experiments using CRISPR/Cas9 to edit human embryos in China further deepened these ethical discussions (Liang et al., 2015).

CRISPR/Cas technology offers tremendous potential in various fields, including genetic disease treatment, agricultural biotechnology, and biomedical research. However, ongoing research into the efficiency, specificity, and safety of the CRISPR/Cas system will further enhance its applications and broaden its use.

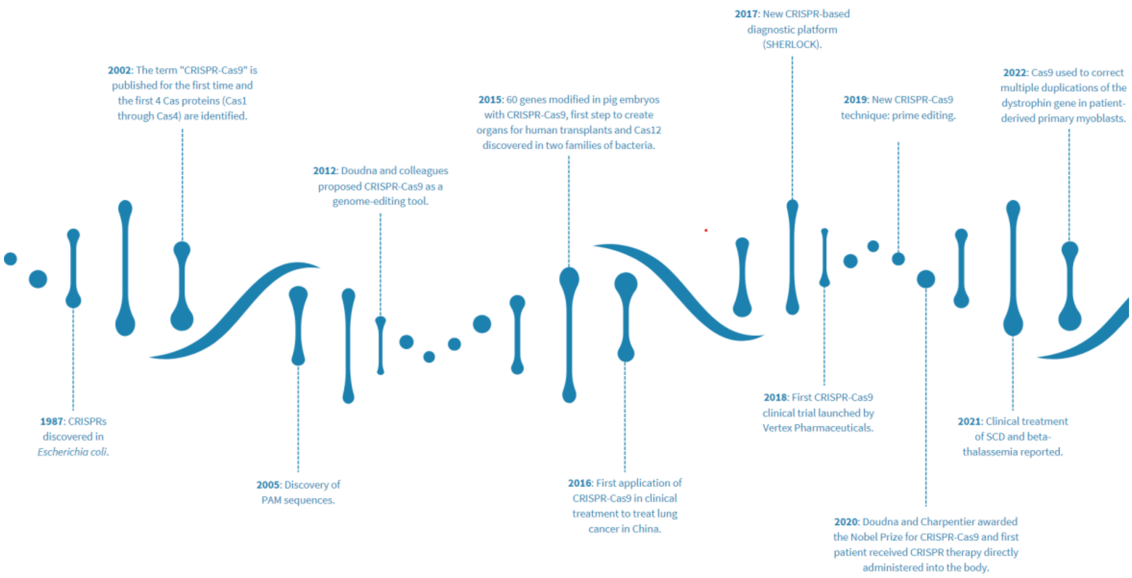


Figure 2. Timeline of key milestones in the development and application of CRISPR-Cas technologies, from their discovery in 1987 to clinical advancements in 2022.

2.2.1. Parts of the CRISPR/Cas System

The CRISPR/Cas system is regarded as a revolutionary tool in genetic engineering, with its fundamental components consisting of CRISPR sequences and Cas proteins. CRISPR sequences are short DNA sequences found in the genome of a microorganism, which recognize foreign DNA elements that the organism has previously encountered. These sequences serve as an immune memory, helping the microorganism defend against future infections.

Guide RNA (gRNA) is a critical component of the CRISPR/Cas system, playing a key role in directing the Cas protein to the specific target DNA sequence that needs to be modified. The gRNA is composed of two essential regions: the spacer region and the scaffold region. The spacer region is a short sequence of RNA, typically about 20 nucleotides long, that is complementary to the target DNA sequence. This complementary sequence allows the gRNA to precisely bind to the target DNA, ensuring that the Cas protein makes a cut at the correct location. The scaffold region, on the other hand, is responsible for binding to the Cas protein, typically Cas9. This interaction forms a ribonucleoprotein complex, which is directed to the target DNA by the spacer region of the gRNA. Once the gRNA pairs with the target DNA, the Cas protein, guided by the gRNA, induces a double-strand break at the specific site identified by the spacer region. For the gRNA-Cas9 complex to accurately recognize and bind to the target sequence, a short DNA motif called the Protospacer Adjacent Motif (PAM) is essential. The PAM sequence is positioned immediately downstream of the target DNA and enables the Cas protein to initiate the cleavage process. The precision and adaptability of the gRNA in guiding the Cas protein make the CRISPR/Cas system a highly effective tool for gene editing, facilitating the targeted modification of nearly any gene within a genome (Jinek et al., 2012; Doudna & Charpentier, 2014).

Cas proteins play a critical role in the functioning of CRISPR sequences. These proteins are enzymes that recognize and cleave foreign DNA targeted by the CRISPR sequences. The most widely recognized Cas protein is Cas9, which enables gene editing by making precise cuts in double-stranded DNA (Jinek et al., 2012). Cas proteins are divided into various types, each serving distinct functions. For instance, Cas1 and Cas2 play roles in incorporating foreign DNA fragments into CRISPR sequences, while proteins such as Cas3, Cas9, Cas12a (Cpf1), and Cas13 are involved in gene editing by cleaving DNA or RNA targets (Makarova et al., 2015).

2.2.1.1. Cas proteins (CRISPR-associated proteins)

The CRISPR/Cas system is a complex and diverse system that operates primarily through the action of various Cas proteins. Each Cas protein fulfills specific roles within different types of CRISPR/Cas systems, evolved to perform functions such as recognizing, binding, cleaving, and subsequently altering target DNA or RNA sequences.

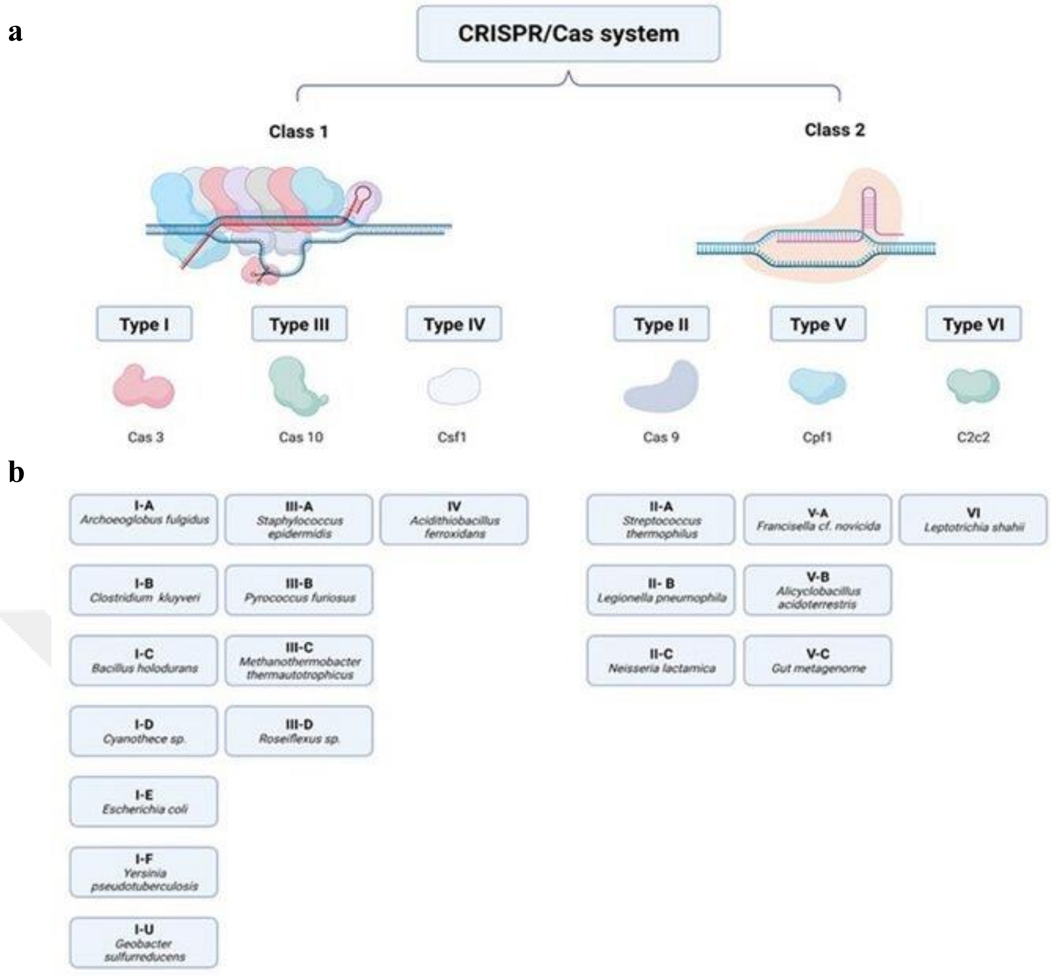


Figure 3. a-b. Schematic representation of classification of CRISPR-Cas systems. This figure was adapted from Aman Mohammadi, M., Maximiano, M.R., Hosseini, S.M. et al., (Bioprocess Biosyst Eng, 46, 483–497, 2023).

Cas1 and Cas2 proteins are involved in the CRISPR adaptation phase. During this phase, bacteria recognize foreign DNA and integrate it into CRISPR sequences. Cas1 is responsible for integrating foreign DNA into CRISPR sequences, while Cas2 assists this process as an endonuclease. Cas3 is a helicase-nuclease found in Type I CRISPR/Cas systems. This protein unwinds and degrades target DNA, making it an effective defense mechanism. Cas3 exhibits exonuclease activity against DNA targets, degrading foreign DNA from the protospacer region that matches CRISPR sequences (Sinkunas et al., 2011). Cas4 is another important protein involved during CRISPR adaptation. Cas4 guides the integration of foreign DNA into CRISPR sequences and recognizes PAM sequences. This function ensures the correct arrangement of CRISPR sequences, working in conjunction with Cas1 and Cas2 (Zhang et al., 2013).

Cas9 is among the most well-known and extensively studied components of the CRISPR/Cas system, particularly within Type II systems. It has attracted considerable attention for its effectiveness as a powerful tool in gene editing. Cas9 functions as a DNA endonuclease, inducing site-specific double-strand breaks (DSBs) in DNA, a key step in genome editing. These breaks are repaired by the cell's natural mechanisms, either through non-homologous end joining (NHEJ) or homologous recombination (HR), resulting in targeted mutations or the insertion of new genetic material (Jinek et al., 2012).

The function of Cas9 relies on its ability to recognize specific DNA sequences in conjunction with a gRNA. The gRNA binds to Cas9 and directs it to the complementary DNA sequence within the target genome. For Cas9 to successfully bind and cleave the target DNA, a short DNA sequence known as the Protospacer Adjacent Motif (PAM) is required. In the widely used *S. pyogenes* Cas9 (SpCas9), the PAM sequence is typically 5'-NGG-3', where 'N' represents any nucleotide, followed by two guanine bases (Gasiunas et al., 2012).

Cas9 functions through two nuclease domains, the HNH domain and the RuvC domain, which are responsible for cleaving the DNA strands. The HNH domain cuts the strand that is complementary to the gRNA, while the RuvC domain cleaves the non-complementary strand, creating a double-strand break. This precise cleavage mechanism makes Cas9 an essential tool for targeted gene editing, enabling specific modifications at nearly any site within the genome (Cong et al., 2013).

Beyond its use in basic research, Cas9 has found applications in various fields, including agriculture, where it is used to enhance crop traits, and medicine, where it holds promise for treating genetic disorders. However, its potential for editing the human genome has raised ethical concerns, particularly regarding the possibility of off-target effects, where Cas9 might inadvertently cut DNA sequences similar to the target site. This has led to ongoing research aimed at improving the specificity and efficiency of Cas9 to minimize such risks (Doudna & Charpentier, 2014).

Cas12a, also known as Cpf1, is another endonuclease in the CRISPR/Cas family that has gained prominence as an alternative to Cas9. Unlike Cas9, Cas12a is part of the Type V CRISPR/Cas system and has unique properties that make it particularly useful for certain gene-editing applications. Cas12a was discovered by the Zhang laboratory in 2015 and has since been widely adopted in genetic engineering for its ability to target and cleave single-stranded DNA in a staggered manner, producing sticky ends that are often easier to manipulate in genetic editing. One of the distinctive features of Cas12a is its recognition of a different PAM sequence compared to Cas9. Cas12a typically recognizes a 5'-TTTV-3' PAM sequence, where "V" represents A, C, or G nucleotides. This difference in PAM recognition expands the range of potential target sites for gene editing, especially in genomic regions where the Cas9 PAM sequence is not present (Zetsche et al., 2015).

Cas12a's mechanism of action differs from Cas9 in that it creates a single-stranded cut or nick in the DNA, rather than a blunt double-strand break. This leads to the creation of overhangs, which can be particularly useful for specific types of genetic modifications, such as those requiring the precise insertion of DNA sequences. Additionally, Cas12a has the ability to process its own guide RNA from a precursor transcript, allowing for the multiplexing of guide RNAs in a single expression vector, which is advantageous for editing multiple genes simultaneously (Tang et al., 2017).

The versatility of Cas12a has established it as a valuable tool across various fields, particularly in crop improvement, where it has been employed to boost disease resistance and enhance yield. In medicine, Cas12a's unique properties are being explored for developing new therapies, particularly in areas where precise and controlled gene editing is required. The ongoing development of Cas12a-based technologies continues to expand the possibilities of CRISPR/Cas systems in biotechnology (Li et al., 2018).

Cas13 is a unique member of the CRISPR/Cas family that targets RNA instead of DNA, setting it apart from Cas9 and Cas12. Cas13 belongs to the Type VI CRISPR/Cas system and functions as an RNA-guided RNA endonuclease. This capability makes Cas13 particularly useful for applications that involve RNA interference, RNA editing, and antiviral strategies targeting RNA viruses. Cas13 was first identified in 2016 and has since been explored for its potential in a variety of RNA-targeted applications (Abudayyeh et al., 2016).

Cas13's function is guided by a CRISPR RNA (crRNA) that directs the protein to specific RNA sequences, where it binds and cleaves the RNA. Unlike Cas9, which creates double-strand breaks in DNA, Cas13 cuts single-stranded RNA, which can disrupt the function of the RNA or degrade it entirely. This RNA-targeting capability has opened new avenues for developing antiviral therapies, particularly against RNA viruses like influenza and coronaviruses, where Cas13 could be used to specifically target and degrade viral RNA within infected cells (East-Seletsky et al., 2016).

One of the most promising applications of Cas13 lies in RNA editing, where it can correct point mutations directly at the RNA level. This approach presents a potential treatment strategy for genetic diseases caused by single nucleotide mutations, without modifying the underlying DNA sequence. Cas13's precision and ability to target RNA with minimal off-target effects make it a compelling tool for developing gene therapies that are both safer and more reversible than DNA-targeted methods (Gootenberg et al., 2017).

In addition to its role in gene editing, Cas13 has been employed in diagnostic applications, such as the SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) platform, which uses Cas13 to detect the presence of specific RNA sequences indicative of viral infections or genetic mutations. This technology has demonstrated the potential for rapid, point-of-care diagnostics that are both sensitive and specific, further expanding the impact of Cas13 beyond traditional gene editing (Konermann et al., 2018).

2.2.2. Transformation of CRISPR/Cas Components into Plants

The transformation of CRISPR-Cas systems into plants is a crucial process in genetic engineering, employing various techniques to introduce genetic material effectively. One such technique is PEG (*Polyethylene Glycol*)-mediated transformation, widely used for the genetic modification of protoplasts. PEG allows the direct introduction of genetic material into protoplast cells, which lack cell walls. This method has been successfully applied to various plants, including maize, soybean, rice, and wheat (Liang et al., 2014; Sun et al., 2016; Arndell et al., 2019). PEG-mediated transformation is particularly advantageous for generating vector-free or DNA-free edited mutants, making it more acceptable in terms of regulatory and ethical considerations. For example, in 2016, transgene-free polyphenol oxidase fungi were successfully edited using ribonucleoproteins and the PEG-mediated transformation method (Waltz, 2016). However, this technique presents challenges in terms of the suspension cell culture and protoplast isolation required, reflecting some of its limitations in efficiency.

The biolistic method, also known as the gene gun, involves the high-pressure shooting of metal particles, such as gold, tungsten, or silver, coated with genetic material into plant cells. This method is particularly effective for monocotyledonous plants like maize, where cell walls are intact. The biolistic method can be applied to a wide range of plant species and is effective for introducing both DNA and RNA sequences (Ismagul et al., 2014). However, the efficiency of this method may be limited by factors such as the tissue regeneration capacity and the effectiveness of gene transfer. For instance, the CRISPR/Cas9 vector targeting the *TaSal1* gene was successfully delivered into wheat via particle bombardment to enhance drought tolerance (Abdallah et al., 2022). While the biolistic method reduces genotype dependency, it also presents limitations such as multiple gene insertions and low transformation efficiency.

Agrobacterium tumefaciens-mediated transformation is one of the most commonly used methods for delivering genetic material into plants. Bacteria such as *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are frequently used due to their ability to integrate genetic material into plant cells. *Agrobacterium tumefaciens Ti* and *Agrobacterium rhizogenes Ri* plasmids are effective tools for genetic transformation, typically infecting wounded dicotyledonous plants and integrating bacterial T-DNA into the host genome (Li et al., 2012). This method generally results in higher single-copy transformation rates and is cost-effective. However, the efficiency of transformation can vary depending on the *Agrobacterium* strain used and the genetic makeup of the target plant.

2.2.3. CRISPR/Cas Implementations in Wheat

Genetic improvement has been a fundamental strategy for enhancing key traits in wheat, such as yield, quality, and disease resistance. In this context, the CRISPR-Cas9 technology has emerged in recent years as a powerful tool for precise genetic modifications in the wheat genome. This technology facilitates the rapid and accurate alteration of specific genes, representing a significant advancement in wheat breeding.

The initial application of CRISPR/Cas9 technology to the wheat genome demonstrated that, despite the complexity of wheat's hexaploid structure, this approach could be employed successfully. For example, a 2018 study targeted the α-gliadin genes in wheat to reduce gluten content, highlighting the effective use of CRISPR-Cas9 technology. Further research in 2019 showed that CRISPR-Cas9-mediated editing of genes such as TaGW2, TaGW8, and TaGLW7, which regulate grain size and yield, resulted in notable yield improvements (Zhang et al., 2019). By 2021, CRISPR-Cas9 applications to enhance tolerance to biotic and abiotic stresses in wheat had gained momentum. Specifically, modifications in the TaARE1 gene improved nitrogen use efficiency, promoting agricultural sustainability. Around the same time, mutations in the TaSBEIIa gene produced wheat lines with higher amylose content (Li et al., 2021). These studies have further emphasized the potential of CRISPR-Cas9 technology to improve agricultural productivity and sustainability.

The application of CRISPR-Cas9 technology to the wheat genome has marked a significant milestone in plant breeding. Since its initial implementation, substantial improvements have been achieved in critical traits such as nutritional quality, yield, and stress tolerance. CRISPR-based wheat breeding efforts hold considerable potential for enhancing sustainable agricultural practices and ensuring global food security.

Compared to traditional methods, the most significant advantage of the CRISPR-Cas system is its ability to achieve desired genetic modifications rapidly. However, the importance of CRISPR extends beyond mere genetic modifications; it also holds the potential to confer resistance to viral infections in plants without altering their genetic structure. For instance, the CRISPR-Cas system can target viral RNA necessary for viral replication within plants and cleave these RNA sequences, thereby inhibiting viral replication. This approach preserves the natural genetic integrity of the plants while providing protection against viral diseases. Additionally, the CRISPR-Cas system can also function by targeting genetic pathways that activate antiviral defense mechanisms within plants. This method strengthens the plant’s inherent defense systems, leading to more effective resistance against viruses. Thus, it is possible to develop plant species resistant to viral infections without altering their genome.

The potential of CRISPR in this regard is of great significance for sustainable agriculture and global food security. Developing virus resistance without genetic modification reduces environmental impacts and addresses concerns associated with genetic engineering. Furthermore, this approach has the potential to enhance crop productivity and quality while preserving natural ecosystems. In this context, the primary objective of my thesis is to utilize the CRISPR-Cas system as an antiviral agent to develop resistance against Wheat Dwarf Virus (WDV) without altering the genetic structure of wheat. This approach aims to protect the genetic integrity of wheat plants while preventing significant yield losses caused by WDV.

3. EQUIPMENT AND METHOD

The experiments were conducted at Uskudar University, Transgenic Cell Technologies and Epigenetics Application and Research Center (TRGENMER). In addition, this study was supported by TÜBİTAK 2209-A University Students Research Projects Support Program (2020/2nd).

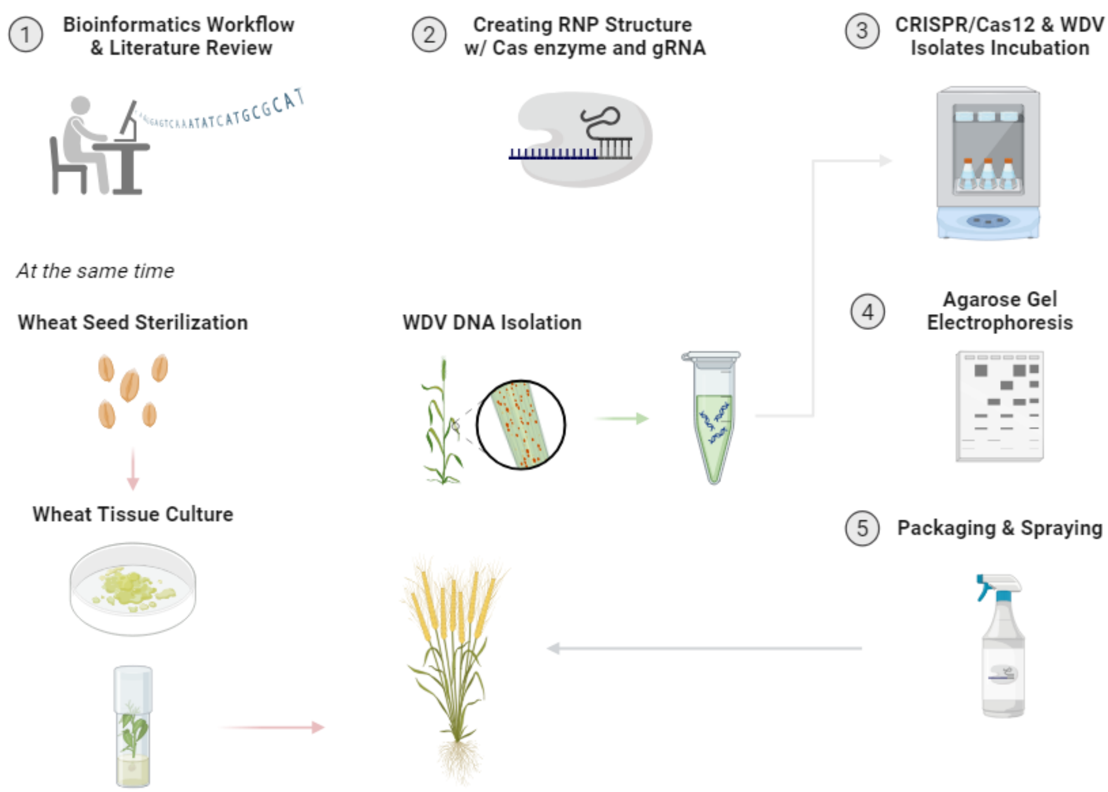


Figure 4. General steps of the project. The workflow starts with bioinformatics analysis and literature review **(1)** to identify optimal guide RNA (gRNA) sequences. The next step is the formation of the RNP complex **(2),** where the Cas12 enzyme is combined with the gRNA to specifically target WDV DNA. This complex, together with WDV isolates, is incubated **(3)** to allow effective binding and cleavage. Agarose gel electrophoresis **(4)** follows, confirming the successful cleavage of WDV DNA. Lastly, the resulting solution is packaged and prepared for application **(5)** to deliver the antiviral treatment to wheat plants.

3.1. WDV Specific Dataset Preparation

In this study, the mutation diversity within WDV genomes isolated from wheat and barley was analyzed to facilitate the design of effective guide RNAs. Reference sequences of WDV genomes were retrieved from the NCBI database, with accession numbers JQ647455, AM040732, NC003326, FJ620684, and JN791096, respectively (Figure 5). Furthermore, the accession numbers of WDV genomes isolated from wheat and barley in the Turkish regions of Kırşehir, Konya, Afyon, and Nevşehir are listed as OQ183228, OQ183229, OQ230451, OQ190468, OQ183230, MW387505, MW387502, and MW381791, respectively (Figure 5).

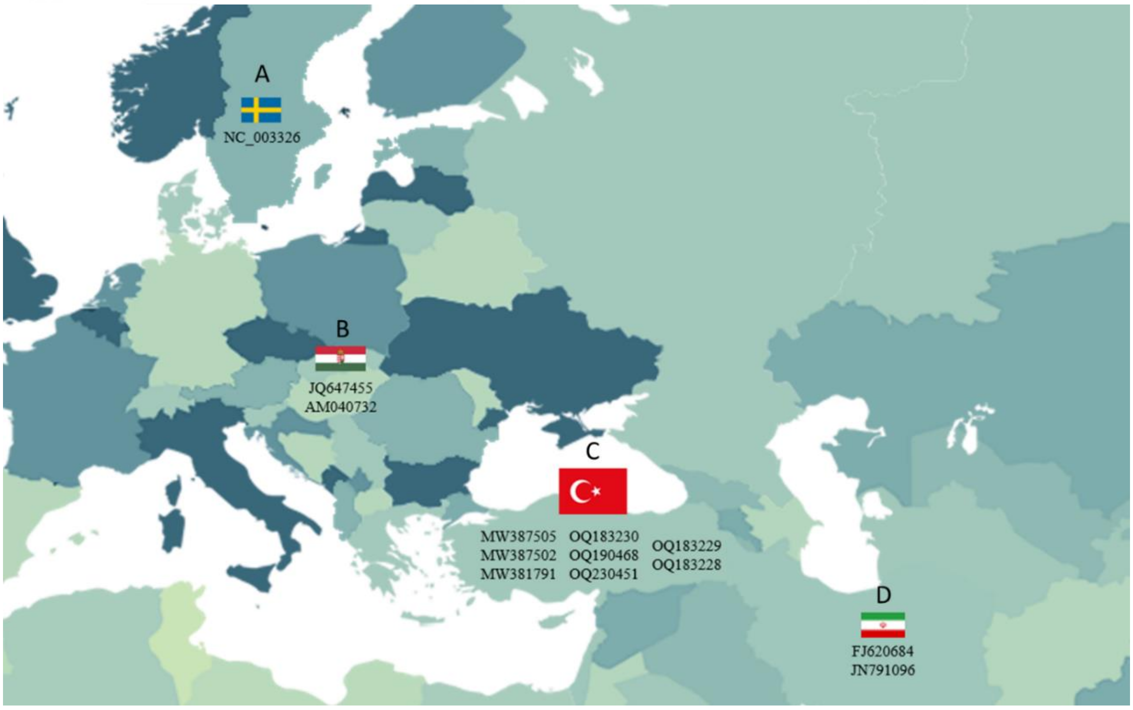


Figure 5. WDV strains isolated from various countries are identified by their NCBI sequence number: NC003326, GenBank accession numbers: JQ647455, AM040732, FJ620684, JN791096, MW387505, MW387502, and MW381791, and additional sequences: OQ183228, OQ183229, OQ230451, OQ190468, and OQ183230. These strains originate from the following countries: **A.** Sweden, **B.** Hungary, **C.** Turkey, and **D.** Iran.

Table 3. WDV genomes detail in the NCBI database.

Isolate	Accession number	RefSeq number	Host	Available sequence
Iran/2008/B	FJ620684		Hordeum vulgare	Complete genome
Iran/Bavanat/ 2010/D	JN791096		Hordeum vulgare	Complete genome
Hungary/Kompolt10/ 1/2010/C	JQ647455		Triticum vulgare	Complete genome
Hungary/B/ 2005/E	AM040732		Triticum aestivum	Complete genome
Sweden/WDV-[Enk1]		NC_003326	Triticum aestivum	Complete genome

Table 4. Detailed information of WDV genomes collected from Turkey.

Isolate	Host	Province	Generic primers	Barley-specifc primers	Wheat-specifc primers	Year
KNY-I34 (MW387505)	Wheat	Konya	+	−	+	2019
KNY-I38-I39 (2 Sample)	Wheat	Konya	+	+	−	2019
KRSH-I41-I43 (2 Sample)	Barley	Kırşehir	+	+	−	2019
AFY-I54 (MW387502)	Wheat	Afyon	+	−	+	2020
NVS-I56 (MW38791)	Barley	Nevşehir	+	+	−	2020
ANK- I66-I76-I77 (3 Sample)	Barley	Ankara	+	+	−	2020

3.2. gRNA design and synthesis for Cas12a

Multiple sequence alignment of WDV DNA sequences was performed using the 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.

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

Guide RNAs were reconstituted by the addition of 314 µL of deionized water, yielding a final concentration of 10 ng/µL. A 50 µg aliquot of GenCRISPR Cas12a (Cpf1) Nuclease (Genscript, Cat. No: Z03502, USA) was solubilized in 1000 µL of solution, consisting of 100 µL of 10X Cas12a Nuclease Reaction Buffer and 900 µL of deionized water. The 10X Cas12a Nuclease Reaction Buffer was formulated to pH 7.9, incorporating 500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, and 1 mg/mL BSA. A 20 µL reaction mixture, prepared in 1X Cas12a Nuclease Reaction Buffer, comprised 60 ng WDV ssDNA, 10 ng gRNA, and 100 ng GenCRISPR Cas12a (Cpf1) Nuclease. The reaction was incubated at 37°C for 30 minutes, achieving digestion efficiency on the linearized plasmid corresponding to each WDV ssDNA. The reaction products were subsequently resolved via electrophoresis on a 1% agarose gel, prepared using 1X TAE buffer. The gel was subjected to electrophoretic separation at 100 V for 30 minutes using a BIO-RAD gel electrophoresis system. Post-electrophoresis visualization, documentation, and analysis were conducted with the Gel Doc™ XR+ system (Serial Number 721BR16450, Software Version 6.0.1.34).3.3.1. Materials

- GenCRISPR Cas12a (Cpf1) Nuclease (Genscript, Cat. No: Z03502, USA)
- gRNA1
- gRNA2
- WDV's ssDNA
- Control plasmid (3)
- Agaroz LE (GENAXXON, Cat No: M3044.0100)

- TAE (BIOBASIC, Cat No: A800239-0500) (TAE contains Trisma base, Glacial Acetic Acid, 0.5 M EDTA (pH:8)
- EZView Stain (BIOMATIK, Cat No: A4205-1ML)
- NaCL
- Tris-HCL
- MgCl2
- Bovine Serum Albumin (BSA) Ultrapure

3.3.2. Cas12a Nuclease Reaction Buffer Preparation

To prepare a 50 mL solution of 10X reaction buffer, follow these steps:

Sodium Chloride (NaCl, 500 mM):

- Weigh out 1.46 grams of NaCl.
- Dissolve it in distilled water.
- This amount is calculated based on the formula:
  0.5M x 0.05L x 58.44g/mol = 1.46 grams NaCl.

Tris-HCl (100 mM, pH 7.9 at 25°C):

- Weigh out 0.61 grams of Tris-HCl.
- Dissolve it in distilled water.
- The calculation for this is:
  0.1M x 0.05L x 121.14g/mol = 0.61 grams Tris-HCL

Magnesium Chloride ($MgCl_2 \cdot 6H_2O$, 100 mM):

- Weigh out 1.02 grams of $MgCl_2 \cdot 6H_2O$.
- Dissolve in distilled water.
- This amount is determined by the following calculation:
  0.1M x 0.05L x 203.3g/mol = 1.02 gram $MgCl_2 * 6H_2O$

Bovine Serum Albumin (BSA, 1 mg/mL):

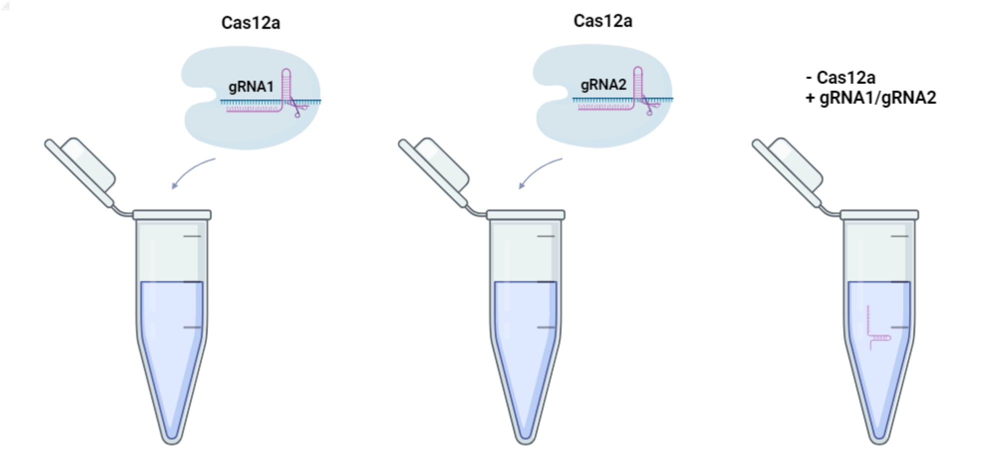
- Add 50 mg of ultrapure BSA to the solution.
- This is calculated as:

  1mg/ml x 50ml = 50mg BSA

Adjust the pH of the solution to 7.9 at 25°C using HCl or NaOH. After all the components are dissolved and the pH is adjusted, add distilled water to bring the final volume to 50 mL. Filter sterilizes the buffer using a 0.22 µm filter because it will be used for sensitive reactions.

3.3.3. Incubation

A total of 10 different WDV isolates were used, and for each isolate, three mixtures were prepared as shown in Figure 6: (guide RNA - Cas12a), (guide RNA1 + Cas12a), and (guide RNA2 + Cas12a). In addition, three different plasmid DNAs were used to test the specificity of the guide RNAs to WDV. Thus, a total of 39 distinct mixtures were prepared in the quantities specified below.



2 µl	1X Cas12a Nuclease Reaction Buffer
60 ng	WDV DNA (Plasmid DNA)
10 ng	Guide RNA (gRNA)
100 ng	Cas12a
X µl	Distilled water (ddH2O)
20 µl	TOTAL

2 µl	1X Cas12a Nuclease Reaction Buffer
60 ng	WDV DNA (Plasmid DNA)
10 ng	Guide RNA (gRNA)
X µl	Distilled water (ddH2O)
20 µl	TOTAL

Figure 6. Contents of the prepared mixtures.

Table 5. Detailed contents of the prepared mixtures.

No	Sample Name	Cas12a	gRNA 1	gRNA 2
1	Control A	-	+	-
2		+	+	-
3		+	-	+
4	Control B	-	+	-
5		+	+	-
6		+	-	+
7	Control C	-	+	-
8		+	+	-
9		+	-	+
10	I38	-	+	-
11		+	+	-
12		+	-	+
13	I66	-	+	-
14		+	+	-
15		+	-	+
16	I76	-	+	-
17		+	+	-
18		+	-	+
19	I43	-	+	-
20		+	+	-
21		+	-	+
22	I41	-	+	-
23		+	+	-
24		+	-	+
25	I77	-	-	+
26		+	+	-
27		+	-	+
28	I39	-	-	+
29		+	+	-
30		+	-	+

31	I56	-	-	+
32		+	+	-
33		+	-	+
34	I54	-	-	+
35		+	+	-
36		+	-	+
37	I34	-	-	+
38		+	+	-
39		+	-	+

3.3.4. Agarose Gel Electrophoresis

- 1% Agarose Gel will be prepared.
- Before preparing the gel, the system must be prepared.
- 50 ml for the small tank system, 100 ml for the large tank system should be prepared.
- Weigh 1 g of Agarose LE per 100 ml of Agarose gel.
- Add 100 ml of 1X TAE. (I have 10X TAE Buffers. Add 10 ml of 10X TAE to the graduated cylinder and add 90 ml of dH2O. Thus, the concentration is reduced to 1X.)
- It is also thawed in the microwave.
- If our hand does not burn when we touch it, 5 µl of EZView Stain is added.
- It is shaken and poured into the gel tray.
- It is waited for about 30 minutes for the gel to freeze.

3.4. Statistics Analysis

Two-tailed homoscedastic t-tests were conducted using SPSS software. No outliers were removed from the statistical analyses, and each data point corresponds to an independent measurement. Bar plots display the mean along with either the standard deviation or the standard error of the mean. A significance threshold of $p < 0.05$ was applied to all tests. "ns" indicates non-significant results.

3.5. Sample Collection and WDV genome extraction

Samples were randomly collected from areas surrounding winter barley and wheat fields showing potential WDV infection, covering 10 different locations across six provinces: Afyon, Ankara, Kırşehir, Konya, Nevşehir, and Yozgat (Morca et al., 2021). Each location was treated as a distinct sampling point, resulting in the collection of 10 samples—6 from barley and 4 from wheat. Genomic DNA was extracted from the plant material using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany, Cat No./ID: 69104), following the manufacturer's protocol. These isolates were kindly provided by Ali Ferhan Morca, a researcher at the Plant Protection Center Research Institute Directorate.

4. RESULTS

4.1. Global Multiple Alignment of WDV and Target Selection

WDV is a virus that is persistently transmitted by dwarf leafhoppers belonging to the species *P. alienus* and *P. provincialis* of the Cicadellidae family, infecting wheat and barley plants (Abt, I., 2019). The CP plays a crucial role in vector transmission, specifically mediating the interaction with *P. alienus*. Additionally, the CP protein encapsulates the viral genetic material, facilitating the attachment of the virion to the host plant (wheat or barley) and enabling its penetration through the host cell membrane (Abt, I., 2019).

In this study, conserved sequences within the CP gene of WDV strains isolated from four different countries were identified. The CP protein was targeted for guide RNA design, and multiple sequence alignments of reference sequences retrieved from the database were performed using Clustal Omega software (Figure 7). The alignment revealed conserved regions within the CP gene, with single-point mutations detected in these conserved sequences (Figure 7A). These mutations were carefully considered during the guide RNA design process, resulting in two distinct guide RNAs with varying numbers of point mutations (Figure 7A-B).

Guide RNA 1 was designed to include two single-point mutations across the general sequence while ensuring no mutations were present within the PAM sequence. For this guide RNA, nucleotides T and C, which exhibit the highest information content, were selected (Figure 7B). Guide RNA 2, on the other hand, incorporated a single-point mutation within one PAM sequence and another within the general sequence (Figure 7B). Specifically, a T-to-C mutation was present in the PAM region, and the "TTTG" PAM sequence, optimal for Cas12a, was chosen. In the overall sequence, the nucleotide T, which exhibited the highest information content, was utilized (Figure 7B). These designed guide RNAs were subsequently used in further experimental applications.

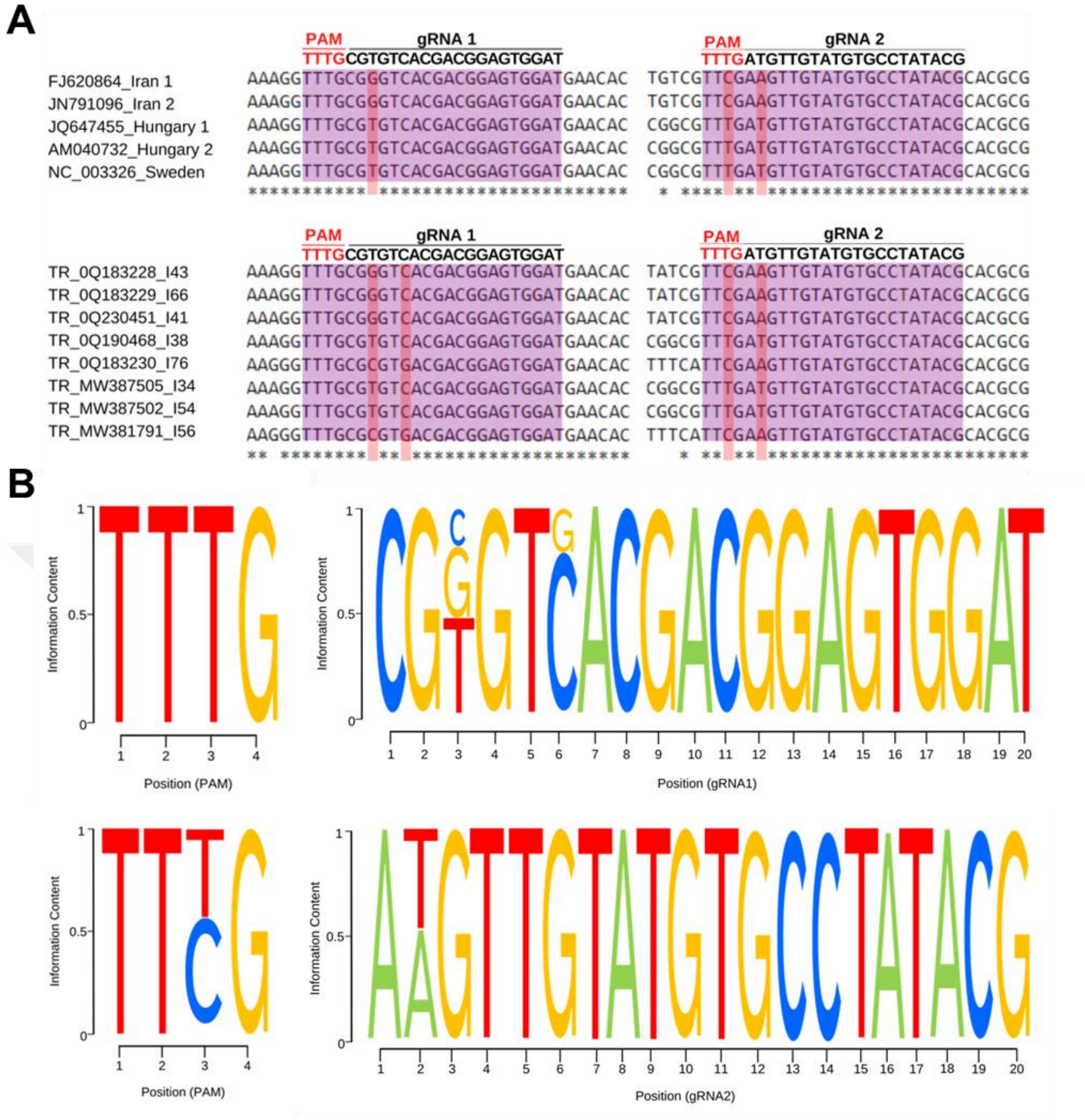


Figure 7. Multiple sequence alignment and information content analysis of WDV genomes. **A.** Conserved regions within the CP protein gene and the corresponding PAM sequences identified through multiple sequence alignment, along with the guide RNA designs based on these conserved regions. **B.** The frequency distribution of variants within the targeted region of CP genes, accompanied by a sequence logo representation illustrating the designed guide RNAs.

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

Cas12a can function as an antiviral agent due to its ability to bind single-stranded DNA (ssDNA) and target viral genomes. In this study, we aimed to evaluate whether Cas12a, in combination with guide RNAs, could effectively target isolated WDV genomes and induce DNA cleavage. To investigate this, a proof-of-concept experiment was designed. This experiment served to demonstrate the practical feasibility of our hypothesis and validate the potential application of our approach. Initially, guide RNAs specifically designed for WDV genomes isolated from wheat and barley were incubated with Cas12a. Following the incubation, the samples were subjected to agarose gel electrophoresis to assess the presence of CRISPR/Cas12a-induced DNA breaks in the WDV genomes (Figure 8).

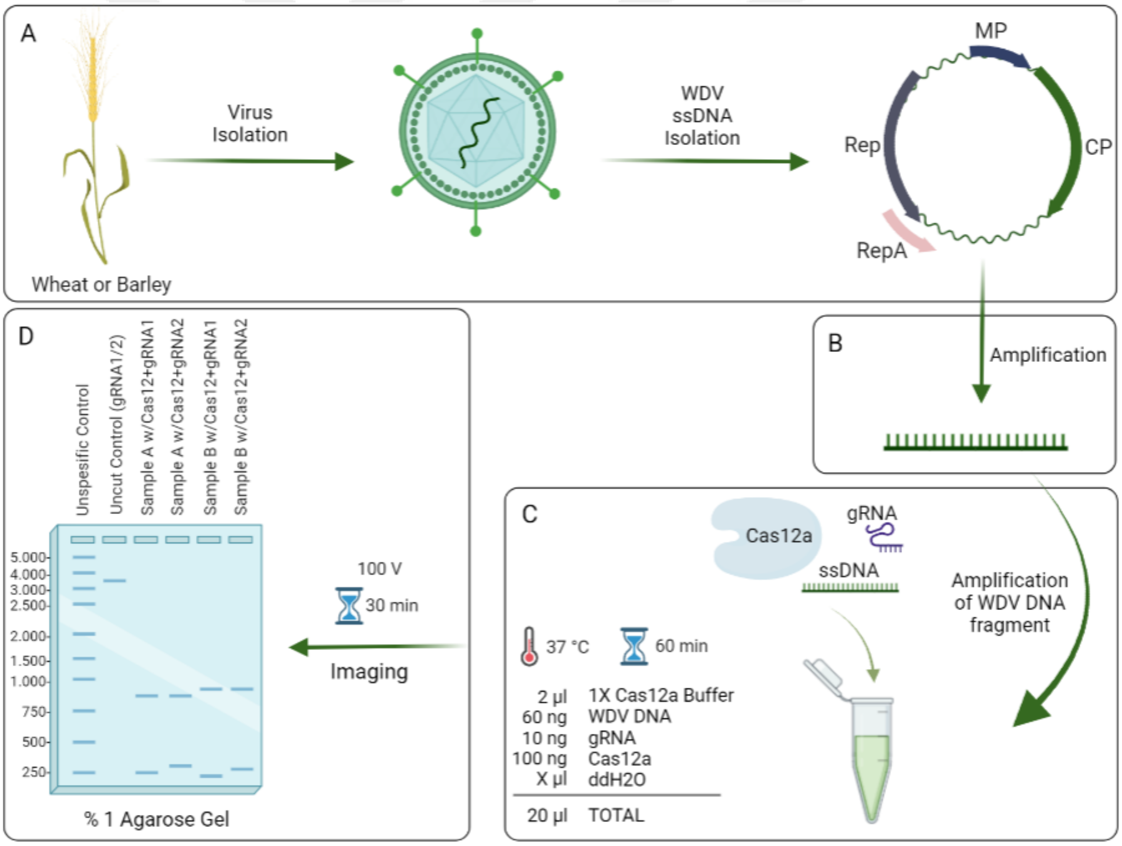
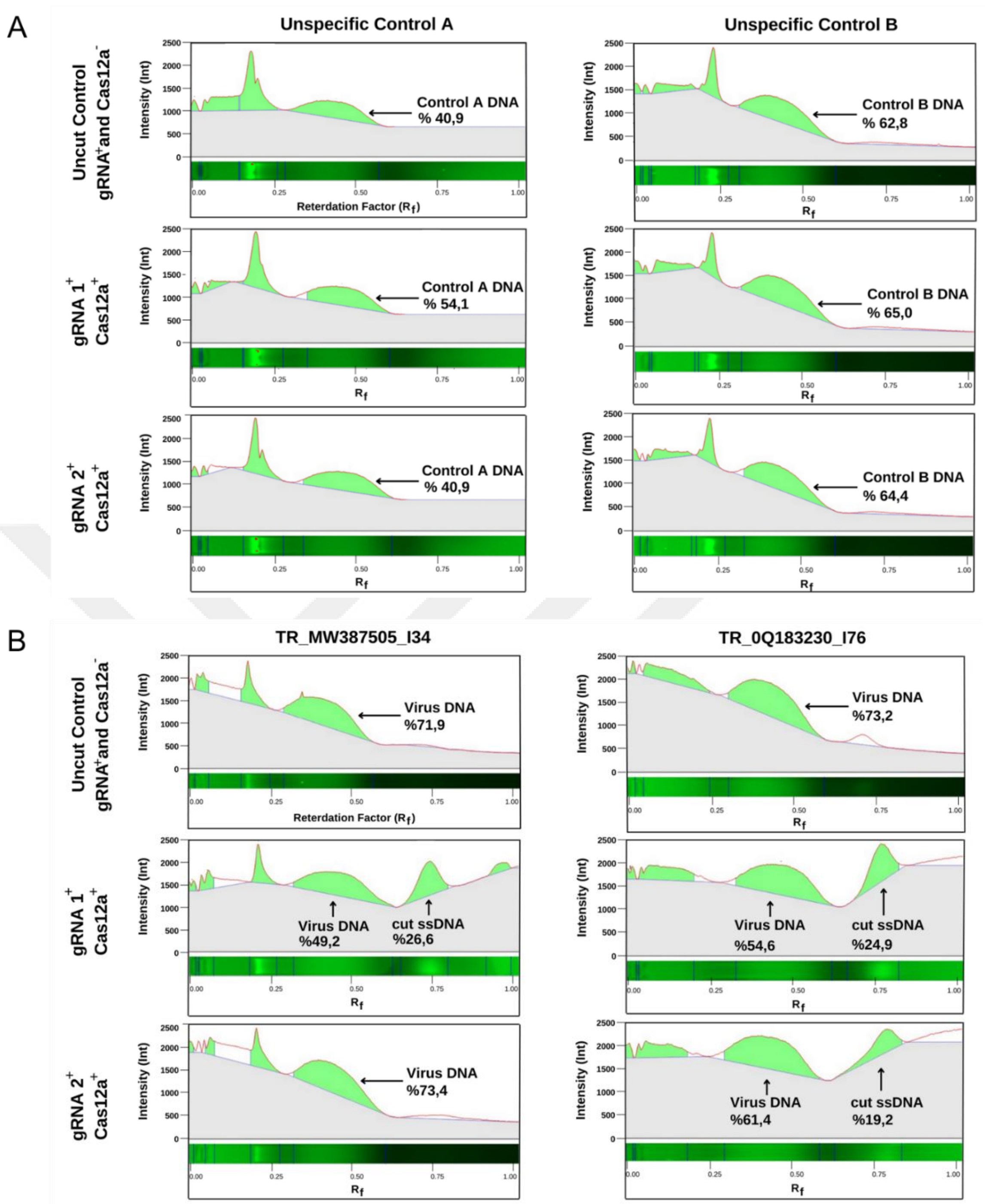


Figure 8. Experimental Set-Up of WDV specific Cas12a as an antiviral agent. **A.** WDV ssDNA genome isolated from wheat or barley. **B.** Amplification with CP primers using a thermal cycler. **C.** Co-incubation of WDV genomes with gRNA and Cas12a RNP complexes. **D.** Conducting gel electrophoresis after incubation.

4.3. 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 9A). 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 9B). 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 9B). 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. 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 9C).


A
Unspecific Control A
Unspecific Control B
Uncut Control gRNA+and Cas12a-
gRNA 1+ Cas12a+
gRNA 2+ Cas12a+
Control A DNA % 40,9
Control A DNA % 54,1
Control A DNA % 40,9
Control B DNA % 62,8
Control B DNA % 65,0
Control B DNA % 64,4
Intensity (Int)
Reterdation Factor (Rf)
Rf
B
TR_MW387505_I34
TR_0Q183230_I76
Virus DNA %71,9
Virus DNA %49,2
cut ssDNA %26,6
Virus DNA %73,4
Virus DNA %73,2
Virus DNA %54,6
cut ssDNA %24,9
Virus DNA %61,4
cut ssDNA %19,2

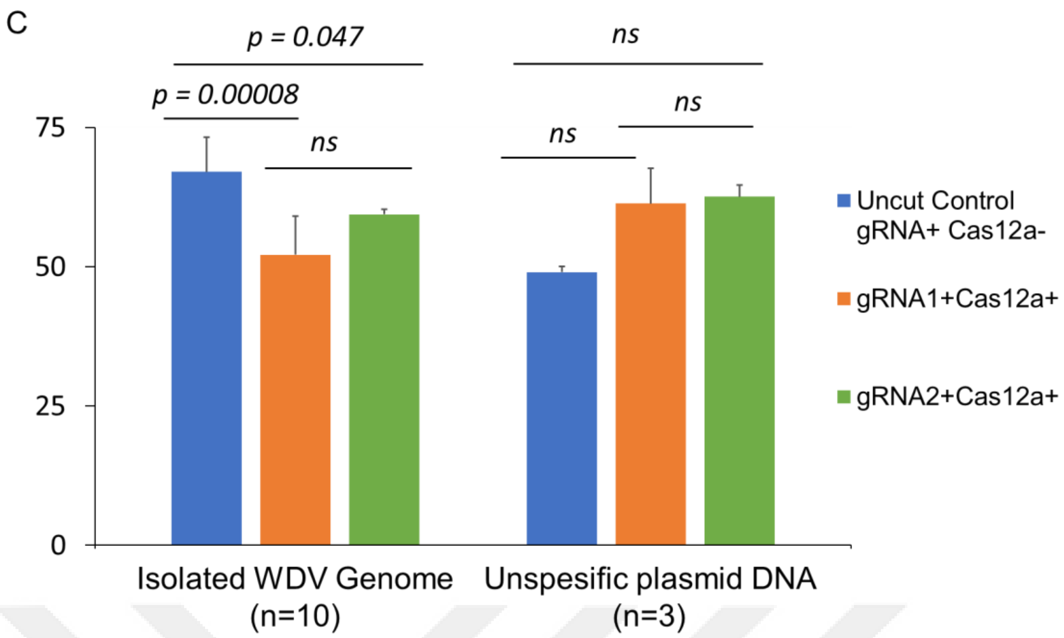


Figure 9A. The unspecific control A and B plasmid DNAs were incubated in a Cas12a$^-$, gRNA1$^+$ & Cas12a$^+$, and gRNA2$^+$ & Cas12a$^+$. Subsequently, after running on a 1% agarose gel, they were visualized using Gel Doc™ XR$^+$. **B.** The TR_MW387505_I34 and TR_OQ183230_I76 Wheat Dwarf Virus (WDV) 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.** Statistical analysis of the comparison of results obtained from the incubation of WDV DNAs and unspecific DNAs with gRNA1 and gRNA2, as well as their incubation with uncut control.

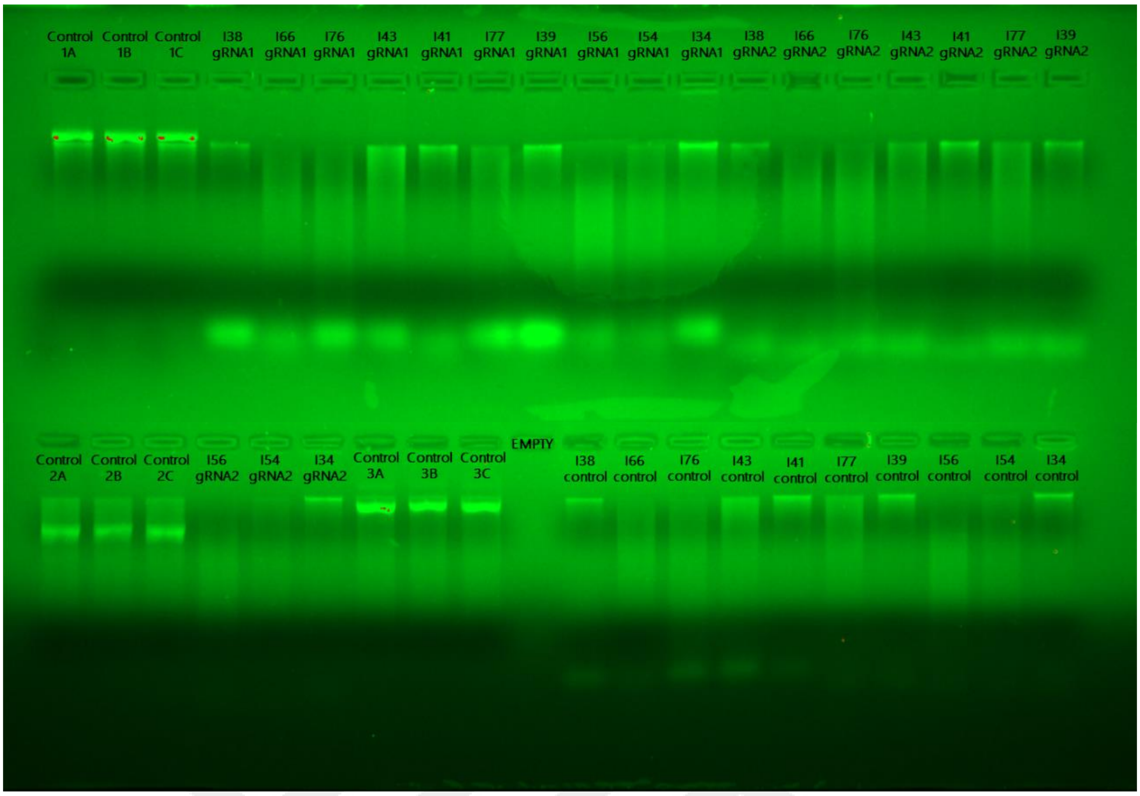
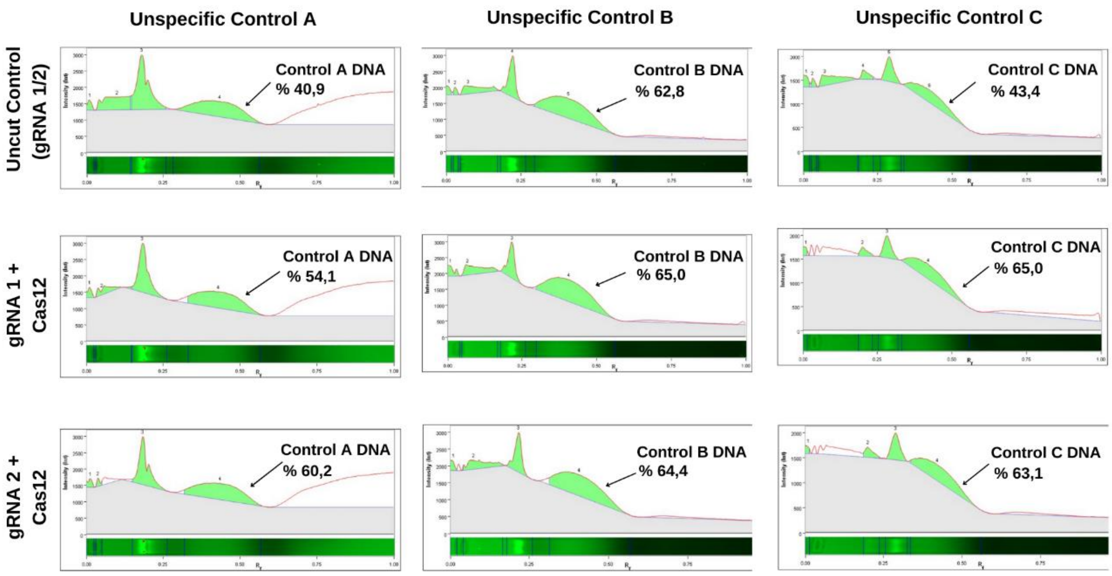
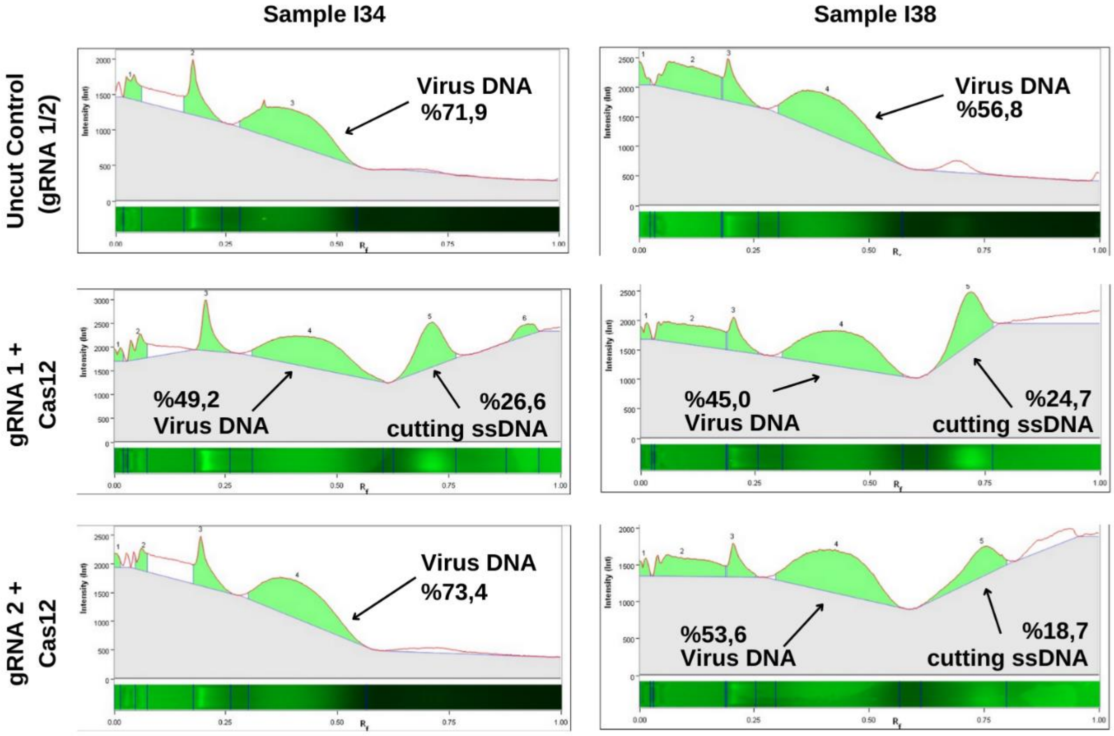
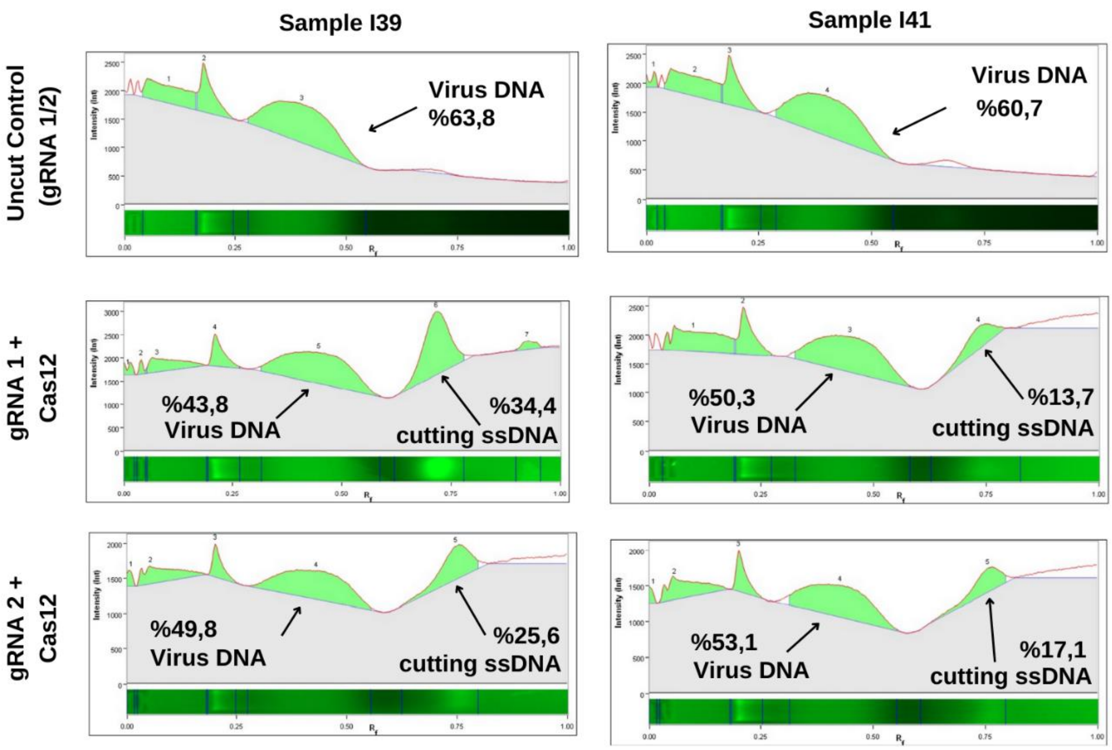


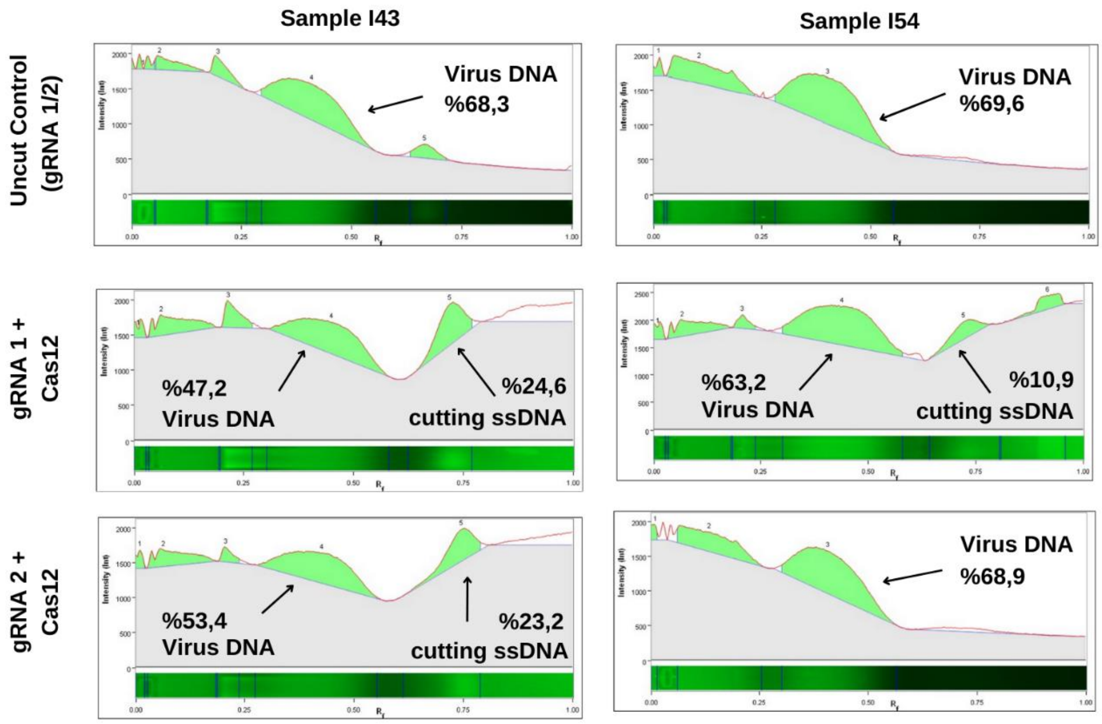
Figure 10. Pure gel image after agarose gel electrophoresis.

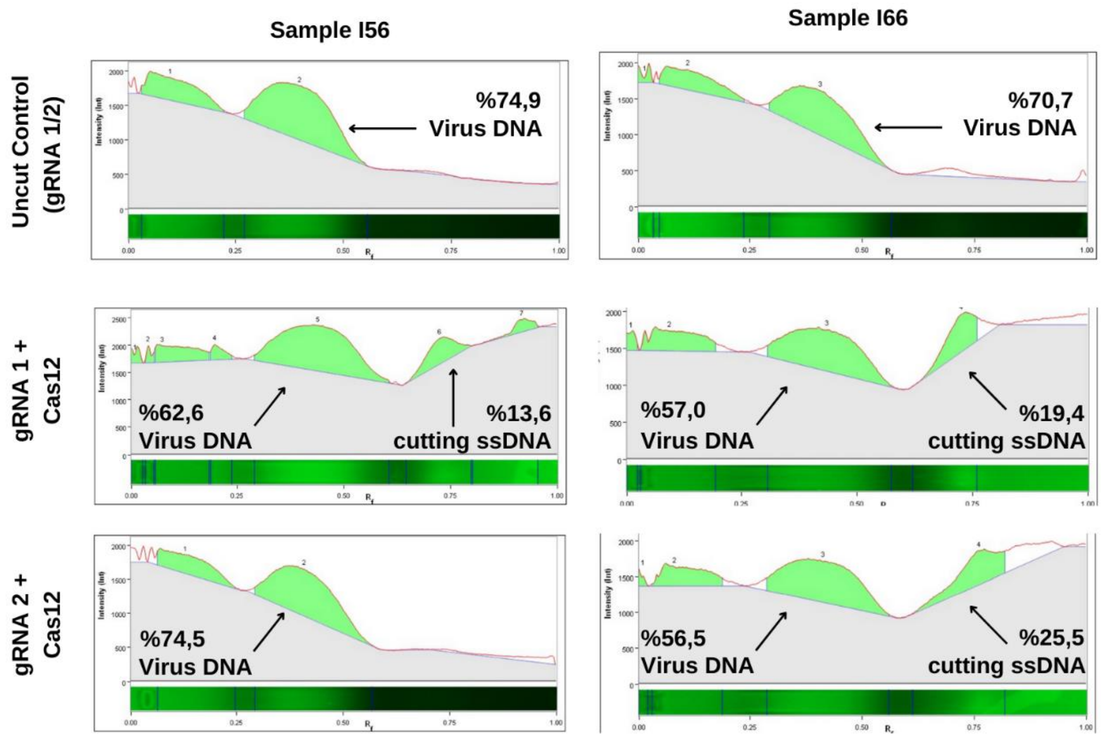
Figure 11 (below) shows the detailed analysis of all gel images.

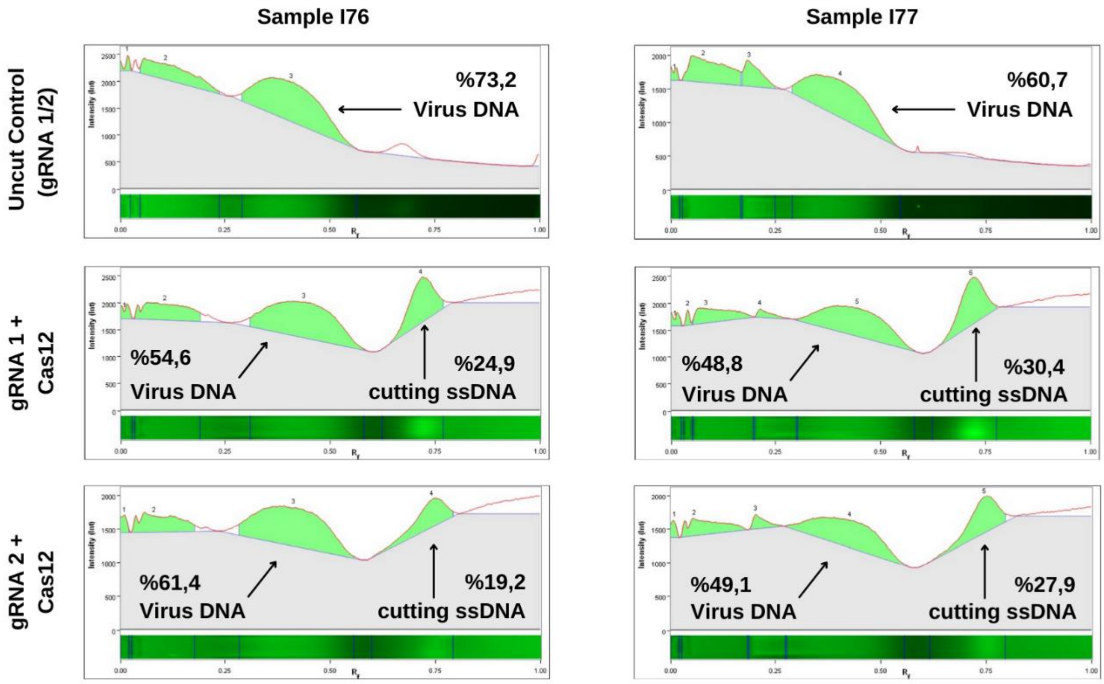



Sample I34
Sample I38
Uncut Control (gRNA 1/2)
gRNA 1 + Cas12
gRNA 2 + Cas12
Virus DNA %71,9
Virus DNA %56,8
%49,2 Virus DNA
%26,6 cutting ssDNA
%45,0 Virus DNA
%24,7 cutting ssDNA
Virus DNA %73,4
%53,6 Virus DNA
%18,7 cutting ssDNA


Sample I39
Sample I41
Uncut Control (gRNA 1/2)
gRNA 1 + Cas12
gRNA 2 + Cas12
Virus DNA %63,8
Virus DNA %60,7
%43,8 Virus DNA
%34,4 cutting ssDNA
%50,3 Virus DNA
%13,7 cutting ssDNA
%49,8 Virus DNA
%25,6 cutting ssDNA
%53,1 Virus DNA
%17,1 cutting ssDNA


Sample I43
Sample I54
Uncut Control (gRNA 1/2)
gRNA 1 + Cas12
gRNA 2 + Cas12
Virus DNA %68,3
Virus DNA %69,6
%47,2 Virus DNA
%24,6 cutting ssDNA
%63,2 Virus DNA
%10,9 cutting ssDNA
%53,4 Virus DNA
%23,2 cutting ssDNA
Virus DNA %68,9


Sample I56
Sample I66
Uncut Control (gRNA 1/2)
gRNA 1 + Cas12
gRNA 2 + Cas12
%74,9 Virus DNA
%70,7 Virus DNA
%62,6 Virus DNA
%13,6 cutting ssDNA
%57,0 Virus DNA
%19,4 cutting ssDNA
%74,5 Virus DNA
%56,5 Virus DNA
%25,5 cutting ssDNA



Sample I76
Sample I77
Uncut Control (gRNA 1/2)
gRNA 1 + Cas12
gRNA 2 + Cas12
%73,2
Virus DNA
%60,7
Virus DNA
%54,6
Virus DNA
%24,9
cutting ssDNA
%48,8
Virus DNA
%30,4
cutting ssDNA
%61,4
Virus DNA
%19,2
cutting ssDNA
%49,1
Virus DNA
%27,9
cutting ssDNA

5. DISCUSSION

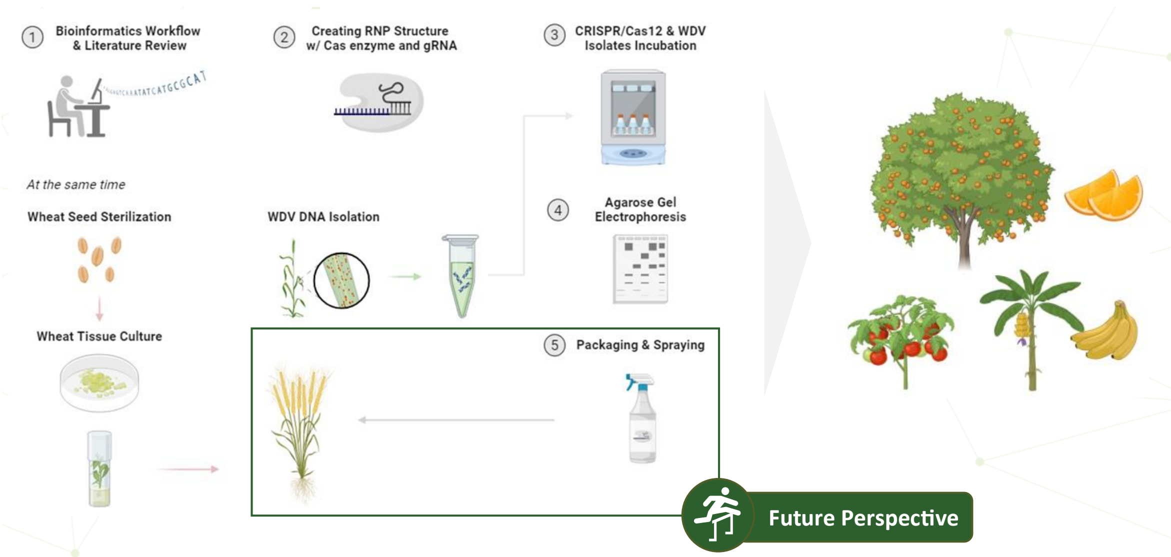
The application of the CRISPR/Cas12a framework as an antiviral procedure against Wheat Overshadow Infection (WDV) offers an imaginative approach that jam the genomic astuteness of have plants by circumventing the require for hereditary alteration. The system's viability in focusing on and cleaving WDV genomes was illustrated over numerous segregates from assorted geographic locales, recommending a potential for broad-spectrum application (Kis et al., 2016).

In this think about, we found that the outlined direct RNAs (gRNAs) displayed solid DNA cleavage movement inside WDV genomes, with gRNA1 demonstrating successful over all tried separates. This result proves past investigate emphasizing the proficiency of CRISPR/Cas frameworks in viral genome altering (Nekrasov et al., 2013). Strikingly, the capacity of gRNA1 to stay utilitarian in spite of minor transformations highlights a vigorous focusing on instrument competent of pleasing hereditary variability—a basic calculate in overseeing viral pathogens with tall change rates (Kelliher et al., 2019). In differentiate, the constrained adequacy of gRNA2 in certain confines underscores the significance of exact gRNA plan, especially in districts inclined to single nucleotide polymorphisms (SNPs) that may influence authoritative liking (Kim et al., 2017). This finding emphasizes the require for comprehensive genomic examination to distinguish preserved viral locales that serve as ideal targets for CRISPR/Cas12a intercessions (Gil-Humanes et al., 2017).

The nonappearance of unforeseen comes about in the unspecific and uncut controls advance approves the specificity of the planned gRNAs, as no off-target impacts were recognized. This tall specificity speaks to a key advantage of the CRISPR/Cas12a framework, relieving the hazard of unintended genomic changes in non-target life forms (Svitashev et al., 2015). Moreover, the utilize of CRISPR/Cas12a as an antiviral specialist, without integration into the plant genome, offers a promising elective to routine hereditary adjustment approaches. This methodology not as it were jam the hereditary personality of crops but moreover mitigates administrative challenges related with hereditarily altered living beings (GMOs) (Nekrasov et al., 2013).

In conclusion, our discoveries demonstrate that the CRISPR/Cas12a framework, guided by carefully outlined gRNAs, viably targets and cleaves WDV genomes, giving a effective apparatus for controlling WDV diseases in wheat and grain. Future inquiries about ought to centre on refining gRNA plan to progress focusing on effectiveness and investigating the application of this framework against other plant infections to advance approve its flexibility and adequacy (Cai et al., 2018).

6. FUTURE PERSPECTIVE



The potential applications of the CRISPR/Cas12a-based antiviral strategy developed in this research extend beyond the scope of wheat. This system, which offers a targeted and precise approach to combating Wheat Dwarf Virus (WDV), holds promise for application in a wide range of other crops, such as tomato (Solanum lycopersicum), maize (Zea mays), and other economically significant plants. The ability to directly target viral genomes without altering the host plant's genetic material represents a major advancement in plant biotechnology. In the future, this approach may help safeguard global food production systems by enabling effective, non-genetically modified (non-GMO) antiviral solutions across various crop species.

The primary goal of this research is to develop a CRISPR/Cas12a-based spray formulation for direct application on wheat to combat Wheat Dwarf Virus (WDV). By delivering CRISPR/Cas12a RNP complexes via foliar spray, the aim is to suppress viral infections efficiently without integrating the system into the plant genome, aligning with sustainable, non-GMO agricultural practices. Initial efforts will focus on optimizing the formulation, ensuring stability, and determining effective application protocols through greenhouse trials.

Once validated in wheat, this approach will be extended to other economically significant crops, such as tomato (Solanum lycopersicum) and maize (Zea mays), both of which are susceptible to various viral infections. Each crop's unique characteristics will require specific modifications, including targeted viral genome analysis and PAM site identification through bioinformatics tools. This gradual expansion will provide a broad-spectrum antiviral solution applicable across multiple agricultural systems.

The flexibility and scalability of this non-GMO CRISPR/Cas12a-based spray offer promising prospects for reducing reliance on chemical pesticides. As the technology advances, field trials and collaboration with agricultural stakeholders will facilitate its commercial adoption, ultimately contributing to more resilient, sustainable agricultural practices.

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