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Agroinfection-Mediated Virus Induced Gene Silencing of Fungal Pathogen *Fusarium verticillioides CYP51* Gene to Reduce its Pathogenicity during Maize (*Zea mays*) Germination

Yaprak NARMANLI[1], Buse Didem PERENDİ[2], Fatma AYDINOĞLU[3] ✍

[1]Department of Molecular Biology and Genetics, Faculty of Science, Gebze Technical University, Kocaeli, Türkiye, [2] Department of Molecular Biology and Genetics, Faculty of Science, Gebze Technical University, Kocaeli, Türkiye, [3] Department of Molecular Biology and Genetics, Faculty of Science, Gebze Technical University, Kocaeli, Türkiye

[1]https://orcid.org/0000-0002-1820-0460, [2]https://orcid.org/0009-0008-9789-9361, [3]https://orcid.org/0000-0002-9974-045X

✉: faydinoglu@gtu.edu.tr

ABSTRACT

Fusarium verticillioides is a fungal pathogen, resulting in devastating diseases in cereals, especially in maize (*Zea mays*) and causing massive economic losses in agriculture. *F. verticillioides* infections in the field are managed by chemical fungicides, mainly azoles which target cytochrome P450 lanosterol C-14α-demethylase (CYP51). Alternative to chemical fungicide, this study evaluated the potential of agroinfection-mediated Virus Induced Gene Silencing (VIGS) approach in controlling *F. verticillioides* pathogenicity, which is based on naturally occurring RNA interference (RNAi) mechanism. For this purpose, *F. verticillioides* was co-cultivated with *Agrobacterium tumefaciens* transformed with a Brome Mosaic Virus (BMV3) derived vector carrying a 313 nucleotide length fragment common to the three existing *F. verticillioides CYP51* genes and comparatively evaluated with the mock group treated similarly but with empty vector at morphologically, biochemically and transcriptionally. It was detected that agroinfection-mediated VIGS treatment of *F. verticillioides* reduced expression levels of *CYP51A* by 49%, *CYP51B* by 65% and *CYP51C* by 51% and the growth rate by 13% while the germination rate was 20% and ROS amount 56% higher compared to mock-treated *F. verticillioides*. These findings pointed out that silencing of *CYP51* caused ROS accumulation in cells leading to inhibition of the pathogenicity of *F. verticillioides*. This study represented the potential of targeting *CYP51* gene by agroinfection-mediated VIGS treatment as an agriculturally sustainable and environmentally friendly alternative method to control *F. verticillioides*-caused plant diseases.

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Mısır (*Zea mays*) Bitkisinin Çimlenmesi Sırasında Fungal Patojen *Fusarium verticillioides*'in Patojenitesini Azaltmak için Fungal *CYP51* Geninin Agroenfeksiyon Aracılığı ile Virüs Kaynaklı Gen Susturulması

ÖZET

Fusarium verticillioides, tahıllarda özellikle mısır (*Zea mays*) bitkisinde yıkıcı hastalıklara neden olan ve tarımda büyük ekonomik kayıplara yol açan bir fungal patojendir. Günümüzde, *F. verticillioides* enfeksiyonları ile mücadelede başlıca, sitokrom P450 lanosterol C-14α-demetilazı (CYP51) hedef alan azol içerikli kimyasal fungisitler kullanılmaktadır. Buna alternatif olarak, bu çalışmada, patojenin kontrolü için endojen RNA interferans (RNAi) mekanizmasına dayanan ve agroenfeksiyon aracılıklı Virüs Kaynaklı Gen Susturma (VIGS) yaklaşımının potansiyeli değerlendirilmiştir. Bu amaçla, *Agrobacterium tumefaciens, F. verticillioides*'in üç *CYP51* geninde ortak olan 313 nükleotidlik fragmenti taşıyan Brome Mozaik Virüs (BMV3) türevi bir vektörle transforme edildikten sonra fungal patojen ile birlikte kültüre alınmıştır. Sonuçlar, benzer şekilde fakat boş vektörle muamele edilmiş kontrol grubuyla morfolojik, biyokimyasal ve transkripsiyonel olarak karşılaştırmalı olarak analiz edilmiştir. Buna göre, *CYP51A*, *CYP51B* ve *CYP51C* genlerinin ifade

Mikrobiyoloji

Araştırma Makalesi

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Brome mozaik virusü
Agrobacterium tumefaciens
Sitokrom P450 lanosterol C-14α-demetilaz
VIGS

seviyelerinde, sırasıyla, %49, %65 ve %51 oranında azalış saptanmıştır. Fungal büyüme oranının %13 oranında azaldığı; çimlenme oranının %20 ve ROS miktarının %56 oranında kontrole göre daha yüksek olduğu tespit edilmiştir. Bu bulgular, CYP51'in susturulmasının hücrelerde ROS birikimine neden olarak *F. verticillioides*'in patojenitesinin inhibisyonuna yol açtığını göstermektedir. Bu çalışma, *F. verticillioides* kaynaklı bitki hastalıklarını kontrol etmek için tarımsal olarak sürdürülebilir ve çevre dostu bir alternatif yöntem olarak agroenfeksiyon aracılı VIGS uygulamasıyla CYP51 geninin hedeflenmesinin potansiyelini ortaya koymuştur.

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INTRODUCTION

Fusarium verticillioides, an endophytic fungal plant pathogen, belongs to the Ascomycota phylum of fungi kingdom (Omotayo *et al.*, 2023). *F. verticillioides* is phylogenetically diverged from the African clade of the monophyletic *Fusarium fujikuroi* species complex (FFSC) group (Blacutt *et al.*, 2018). Seedling blight, Fusarium ear rot (FER), crown root rot and stalk rot may occur due to *F. verticillioides* infection in maize (*Zea mays*), which is one of the most valuable crops worldwide (Lanubile *et al.*, 2017; Kılınç *et al.*, 2018; Zhang *et al.*, 2023). *F. verticillioides* infection significantly reduces maize yield in the field. Its pathogenicity arises from a mycotoxin, fumonisin B1 (FB1) production, which blocks the ceramide synthase and leads to changes in sphinganine and sphingosine levels (Chen *et al.*, 2023). FB1-contaminated maize consumption may have hepatotoxic, neurotoxic, and possibly carcinogenic effects in animals and humans (Voss *et al.*, 2001; Stockmann-Juvala & Savolainen, 2008).

Control of infections caused by plant fungal pathogens is strongly based on commercial chemical fungicides which are harmful to the environment and humans. Azoles comprise the largest fungicide class and they are sterol 14α-demethylase inhibitors (DMI) (Fan *et al.*, 2013; Jørgensen & Heick, 2021). DMIs catalytically restrict the activity of both 14α-demethylase and cytochrome P450 lanosterol C-14α-demethylase (*CYP51*) (Doughty *et al.*, 2021*). CYP51* belongs to the cytochrome P450 monooxygenase (CYP) superfamily, and is involved in the fungal kingdom-specific ergosterol biosynthesis. Catalytic restriction of CYP51 inhibits eburicol and lanosterol demethylation and ergosterol biosynthesis, deficiency of which leads to disruption of cell membrane fluidity and active transport system in fungi (Price *et al.*, 2015). In the fungi kingdom, five CYP51 gene families have been known up to now, which are *CYP51, CYP51A, CYP51B, CYP51C* and *CYP51D* (Pintye *et al.*, 2024). However, *Fusarium spp.* contain only *CYP51A, CYP51B* and *CYP51C* paralogs, sequences of which have nearly 60% identity (van Rhijn *et al.*, 2021).

Virus-induced gene silencing (VIGS) was revealed as an RNA-mediated defense mechanism against viruses (Voinnet, 2001). It has been developed as a method to downregulate gene expression by a modified viral vector for insertion of the gene of interest. Viral vector infection triggers RNA interference (RNAi) mechanism due to double-stranded RNA (dsRNA) that is released from the vector (Pooggin, 2017). RNA interference (RNAi), which is a naturally occurring post-transcriptional gene silencing mechanism, is highly conserved among eukaryotes (Koeppe *et al.*, 2023; Padilla-Roji *et al.*, 2023). RNAi not only regulates gene expression levels in a sequence-specific way with the help of small RNAs, which are microRNAs or small interfering RNAs (siRNAs), but also defend cells against transposons, retroelements and viruses (Buchon & Vaury, 2005).

Many plant viruses have been developed as vectors for transient gene expression or silencing in plants (Yang *et al.*, 2018). Brome mosaic virus (BMV) belonging to the Bromoviridae family has been modified for genetic modification of monocot species, which is difficult to stably transform (Ding *et al.*, 2017). RNAi also occurs via the bidirectional exchange of RNA between host and pathogen, which is called "Cross-kingdom RNAi" (Huang *et al.*, 2019). RNAi mechanism initiates with dsRNA cleavage by Dicer, thereby producing small-interfering RNAs (siRNAs) (Halder *et al.*, 2022). The guide strand of siRNA separates from the passenger strand and is integrated into an RNA-induced silencing complex (RISC) together with Argonate (AGO) proteins to bind complementary mRNA, which is degraded by endonuclease activity within the RISC causing gene silencing (Waheed *et al.*, 2021).

In this study, an agroinfection-mediated VIGS approach based on RNAi was used to silence the *Fusarium verticillioides CYP51 (Fv-CYP51)* gene to inhibit fungal growth and pathogenicity on maize seeds (Mascia *et al.*, 2014; Zulfiqar *et al.*, 2023). To this end, a fragment conserved in three existing *Fv-CYP51* gene paralogs was cloned into a derived vector and transformed into *Agrobacterium tumefaciens*. Transformed-*A. tumefaciens* and *F. verticillioides* co-cultivated and the transcript level of *CYP51* paralogs, endogenous ROS production and fungal growth, and pathogenicity inhibition were analyzed at morphological, biochemical, and transcriptional levels. This study aims to contribute to the development of a VIGS-based fungicide that can avoid the use of chemical azoles for the control of *F. verticillioides* infection.

MATERIAL and METHOD

Culture Conditions of Bacterial and Fungal Strains

Bacterial *E. coli* DH5α and *A. tumefaciens* EHA105 strains were used and cultured in Luria Bertani (LB) broth (Caisson Labs, England) at 37ºC and 28ºC, respectively. *Fusarium verticillioides* isolates were cultured on potato dextrose agar (PDA, Biolife, Italy) for 1-2 weeks or in potato dextrose broth (PDB) for 1-3 days at 28 ºC. PDB was prepared by boiling 200 g L^{-1} potato in distilled water, supplementing 200 g L^{-1} D-glucose and autoclaving at 121ºC for 20 min. Bacterial and fungal strains are stored at -80ºC in 15% glycerol.

Cloning *CYP51* into BMV3 Vector

The VIGS method was followed according to Ding *et al.* (2017) to clone *Fv-CYP51* (NCBI Sequence ID: XM_018901738.1) fragment into the BMV3 vector. For this end, total *F. verticillioides* RNA was isolated according to TRIzol (Invitrogen, Thermo Fischer Scientific, USA) method and cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) using 2 µg of the extracted total RNA as a template (Rymen *et al.* 2007). A 313 bp fragment of conserved in *Fv-CYP51* paralogs was amplified by forward (5'-CCTCCTAGGCCCTTGACGTGGGAGAATTTA-3') containing *AvrII* restriction site and reverse (5'-CTTCCATGGTGACGGACTTGCTCATCATAC-3') containing *NcoI* restriction endonuclease site by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Fisher). PCR condition was followed by an initial denaturation step at 97ºC and 30 s, then 30 cycles of denaturation at 98ºC for 10 s, annealing at 57ºC for 20 s, extension at 72ºC for 20 s, and final extension at 72ºC for 10 min.

The amplified insert fragment was ligated into pC13/F3CP5 (abbreviated as BMV3 in this article) vector linearized by *AvrII* and *NcoI* restriction enzymes (New England Biolabs). The vector and PCR product were subjected to overnight ligation with the T4 DNA ligase (Invitrogen) at +4°C. To confirm the positive clone, the BMV3: *CYP51* vector was subjected to restriction digestion with *AvrII* and *HindIII* (New England Biolabs) and 2% agarose gel electrophoresis. The unraveled insert fragment was extracted with the PCR Clean Up & Gel Extraction kit (Thermo Fisher) and sequenced. The obtained sequence aligned to the database with BLASTn algorithm (Altschul *et al.* 1990). The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method with Molecular Evolutionary Genetics Analysis Version 11 (MEGA 11) based on comparing nucleotide sequences (Tamura *et al.*, 2021).

BMV3 vector contains RNA3 of BMV encoding 3a protein and a capsid protein (CP) required for movement of virus, while pC13/F1+2 (abbreviated as BMV1 in this article) plasmid contains genomic RNA 1 and 2 of BMV, encoding proteins 1a and 2a, respectively (Ding *et al.*, 2017; Wang *et al.*, 2021). These proteins are required for virus replication processes. BMV1 and BMV3 vectors containing the kanamycin resistance gene were used to transform *A. tumefaciens*. Firstly, *E. coli* and *A. tumefaciens* EHA105 competent cells were prepared as described in (Sambrook and Russell, 2006). *Escherichia coli* (*E. coli)* and *A. tumefaciens* competent cells were transformed separately with BMV1, BMV3 and BMV3:CYP51 vectors by heat shock method.

Sensitivity of *F. verticillioides* to Kanamycin

To test the resistance of *F. verticillioides* to bactericide kanamycin; 10^3, 10^4, and 10^5 spores mL^{-1} *F. verticillioides* were cultivated in PDB for 1 d and on PDA for 3 d supplemented with 5, 10, 50, and 100 µg mL^{-1} kanamycin. The resistance of *F. verticillioides* to kanamycin was confirmed (Poudyal *et al.*, 2024); therefore, this antibiotic could be supplemented in a fungal selective PDA medium.

Agroinfection-Based VIGS Treatment of *Fusarium verticillioides* to Silence *Fv-CYP51*

Agroinfection applied based on previous studies of Bundock *et al.* (1995), Sayari *et al.* (2019), Dong *et al.* (2020) and Yoon *et al.* (2023). *A. tumefaciens* EHA105 strain was transformed with BMV1, BMV3 or BMV3:CYP51 and grown in 5 mL LB medium containing 10 µg mL^{-1} gentamicin and 100 µg mL^{-1} kanamycin antibiotics overnight at 28ºC with shaking at 150 rpm. Agrobacterium transformant cultures were diluted to 20 mL with LB medium and

grown to an optical density (OD) of 0.6 at 600 nm measured with a UV-1800 Spectrophotometer (Shimadzu, Japan). 500 µL aliquots of each transformant culture were centrifuged for 5 min at 3000 rpm and resuspended in 25 µL of induction buffer (containing 10 mM MES (2-(N-morpholino) ethanesulfonic acid); pH:5,6), 10 mM MgCl2, 0.1 mM Acetosyringone). 25 µL of BMV3:CYP51 agrotransformant culture was mixed with 25 µL of BMV1 agrotransformant culture (1:1, v:v). Subsequently, 50 µL of resuspended agrotransformant mixture and 25 µL of $10x10^4$ spore mL^{-1} *F. verticillioides* cultures were mixed and spread on sterile parchment paper placed on minimal medium (MM) (containing 2.05 K2HPO4 g L^{-1}, 1.45 KH2PO4 g L^{-1}, 0.15 NaCl g L^{-1}, 0.50 MgSO4.7H2O g L^{-1}, 0.1 CaCl2.6H2O g L^{-1} and 0.5 g L^{-1} (NH4)2SO4), 40mM MES (ph:5.3), 200 µM acetosyringone, 10 mM glucose, 0,5 % glycerol and 1.5% agar) (Bundock *et al.* 1995). For mock treatment, *F. verticillioides* was co-cultured similarly but with BMV1 and empty BMV3 transformed Agrobacterium.

After 2 d co-cultivation at 25ºC, the parchment papers were transferred to a fungal selective PDA medium containing 100 µg mL^{-1} kanamycin and 200 µg ml^{-1} timentin to kill bacterial cells and incubated for 7 d at 25ºC in the dark (Sayari *et al.* 2019). Lastly, the parchment papers were suspended in 20 mL PDB and grown overnight. These fungal cultures were used in ROS production assay, fungal growth zone measurement, transcriptional analysis of *CYP51* paralogous genes and seed germination assay.

Transcriptional Analysis of *Fv-CYP51* Paralogous Genes

Primers specific to *CYP51* paralogs which are *CYP51A* (XM_018899353.1), *CYP51B* (XM_018887915.1), and *CYP51C* (XM_018901738.1) and *ß-tubulin* (FVEG 05512) *(F. verticillioides* 7600; assembly ASM14955v1; https://www.ncbi.nlm.nih.gov/genome) were designed with IDT OligoAnalyzer 3.1 (https://eu.idtdna.com) by taking into account specific parameters (Melting temperature (Tm) between 58-61ºC; hairpin and self- or hetero- dimer formation at 50ºC with G> -3 kcal mol^{-1} and -6 kcal mol^{-1}, respectively). The specificity of primers was evaluated with the BLASTn tool (www.blast.ncbi.nlm.nih.gov).

Triplicate 1 mL aliquots were taken from the agroinfected *F. verticillioides* cultures and centrifuged at 13,000 rpm for 15 min. The supernatant was discarded. Total RNA and cDNA synthesis was conducted similarly to those represented above. RT-qPCR reactions were prepared in triplicate biological and duplicate technical replicates with SYBR Green qPCR Mix (AidLab, China); 10 µM forward and reverse primers (Table 1) and 3 µd cDNA as template. The conditions for RT-qPCR run on StepOne Real-Time PCR (ABI, Foster City, CA, USA) machine were as follows: 95ºC for 3 min (initial denaturation); 40 cycles of 10 s at 95ºC (denaturation), 20 s at 60ºC (hybridization) and 15 s at 72ºC (elongation) and for melting curve analysis 15 s at 95ºC, 1 min at 58ºC and 15 s at 95ºC. The results were normalized with endogenous control ß-tubulin. $2^{-\Delta\Delta CT}$ values were evaluated to assess relative gene expression levels (Pfaffl, 2001).

Table 1. The list of forward and reverse primers used in transcriptional analysis
Çizelge 1. Transkripsiyonel analizde kullanılan ileri ve geri primerlerin listesi

Gene (*Gen)*		Primer sequence (5'→3) (*Primer sekansı (5'→3))*
CYP51A (XM_018899353.1)	Forward primer *İleri primer*	TCTATTCTGCGAAAGGTCAAGAG
	Reverse primer *Geri primer*	CAGTTGGTGAAGCGAGGATAA
CYP51B (XM_018887915.1)	Forward primer *İleri primer*	GTGGCGATGACGAAGAGAAG
	Reverse primer *Geri primer*	AGCACCAAAGGGCAGATAAG
CYP51C (XM_018901738.1)	Forward primer *İleri primer*	CCCTTGACGTGGGAGAATTTG
	Reverse primer *Geri primer*	ACTTGACCTGTCGGAGAATAG
ß-tubulin (FVEG 05512)	Forward primer *İleri primer*	GCAGGGCTTCCAACATCTTA
	Reverse primer *Geri primer*	TATCGACCGTTGCGGAAATC

ROS Production Assay

Fluorometric 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining assay was conducted to measure the amount of intracellular reactive oxygen species (ROS) in agroinfected-*F. verticillioides* cultures produced in "Agroinfection-

Based VIGS Treatment of *Fusarium verticillioides* to Silence *Fv-CYP51*". 10^6 spores mL^{-1} of cultures were incubated in 10 µM DCFH-DA (Sigma Aldrich) at 30ºC for 30 min in the darkness with shaking at 150 rpm (Chen *et al.*, 2010). Fluorescence levels of cultures were measured every 10 min for 4 h with excitation wavelength at 488 nm and emission wavelength at 525 nm with Varioskan Flash Microplate Reader (Thermo Scientific) (Wang & Joseph, 1999). Intracellular ROS production level was calculated by subtracting the fluorescence levels of DCFH-DA non-treated experimental groups from fluorescence levels of DCFH-DA treated ones (Kobayashi *et al.*, 2002). This experiment was done in five replicates.

Fungal Growth Zone Measurement

Fungal growth zone assay was conducted according to Aydinoglu *et al.* (2024). PDA plates were divided into 6 parts and round wells of equal size were created in the middle of divisions. 10 µL from *F. verticillioides* cultures agroinfected with *BMV3:CYP51* or BMV3 agrotransformants was loaded into one well in six replicates. After 48 h incubation at 28ºC, the fungal growth zones were measured from the longest diameter with ImageJ&Fiji software (https://imagej.net/software/fiji/downloads). Experimental data was shown as average ± standard deviation (X±SS) and Student t-test analysis was applied in Microsoft Excel 365 for significance level between variances of groups (P-value<.05 was considered as a significant difference.). This experiment was done in six replicates for each experimental group.

Seed Germination Assay

Maize (*Zea mays*) hybrid line PEHLIVAN developed by Maize Research Institute (Sakarya, Türkiye) was used for seed germination assay. Maize seeds were sterilized in 5% sodium hypochlorite (NaOCl) solution for 5 min and then in 70% ethanol solution for 1 min and 3 times in an Erlenmeyer flask. They were washed five times with autoclaved distilled water. For inoculation, ten seeds per experimental group were shaken for 1 hour with *F. verticillioides* spore solutions (5×10^3 spores mL^{-1}) which were not agroinfected, agroinfected with BMV3 or *BMV3:CYP51 Agrobacterium* transformants. Non-inoculated seeds were used as the control group. For seed germination assay, seeds were placed between sterile filter papers moistened with autoclaved dH2O in glass plates for 7 d at 25ºC/ 20 °C (day/ night), 15.000 lux light intensity and 70% humidity (Khaeim *et al.*, 2022; Akgul & Aydinoglu, 2025). After 7 d, the germinated seeds were counted and compared. This experiment was done in ten replicates for each experimental group.

Statistical Analysis

Two-tailed *t*-test analysis was performed in Microsoft Excel 365 by assuming unequal or equal variances depending on the result of *F*-test analysis for variances of two samples. Data represent average ± standard deviation (X±SS). *P*-value<.05 was accepted as a statistically significant difference.

RESULTS and DISCUSSION

Cloning *Fv-CYP51* Gene Fragment into BMV3 Vector

BMV3:CYP51 clone was confirmed by restriction digestion followed by electrophoresis and sequencing analysis. Restriction digestion revealed the expected 313-bp-long inserted fragment and 3 vector fragments bigger than 1000 bp (Figure 1B). After being extracted from the gel and sequenced, the BLASTn results confirmed the sequence displayed 91% similarity to *F. verticillioides* sterol 14-demethylase (XM_018901738.1) with 439 coverage out of 486 (Figure 1A) (P<.01). This gene is highly conserved between Fusarium species. The sequence fragment showed 99% similarity to *Cytochrome P450 lanosterol C-14α-demethylase* (GU785046.1) gene of *F. proliferatum*, while it showed 95%, 91%, 91%, 88%, 88% similarity with *F. proliferatum* (GU785047.1), *F. mangiferae* (XM_041826468.1), *F. musae* (XM_044825370.1), *F. oxysporum* (XM_018393093.1), *F. subglutinans* (GU785054.1), respectively (Figure 1C).

The Transcript Level of *Fv-CYP51* Paralogs Decreased in Response to Agroinfection- Mediated VIGS Treatment of *Fusarium verticillioides*

The relative transcript levels of *Fv-CYP51* paralogous genes, *CYP51A, CYP51B,* and *CYP51C,* were determined to determine whether cross-kingdom gene silencing between *Agrobacterium* and *F. verticillioides* via RNAi induction was achieved or not in response to agroinfection mediated VIGS treatment. The results showed that, the transcript amounts of *CYP51A*, *CYP51B* and *CYP51C* were decreased by 49% ($t(4) = -2.87$, $p = .022$) , 65% ($t(4) = -2.24$, $p = .044$), 51% ($t(4) = -3.37$, $p = .014$) in *BMV3:CYP51* agroinfected-*F. verticillioides* compared to mock-treated *F. verticillioides*, respectively (Figure 2). This finding represented the successful targeting of *Fv-CYP51* paralogs by selecting the conserved region of three paralogs to clone into the VIGS vector. Mao *et al.* (2023) showed that

deletion of *CYP51A*, *CYP51B*, and *CYP51C* in *F. fujikuroi* had no obvious effect on morphology, conidia germination, or pathogenicity but displayed differential sensitivity to DMI fungicide. These findings revealed the silencing of *CYP51* genes as an efficient management strategy for pathogenic *Fusarium spp*. The transcriptional analysis of *Fv-CYP51* paralogs proved that the VIGS mechanism served the *CYP51* gene silencing, which is the purpose of the present study. Agroinfection-mediated VIGS treatment of *F. verticillioides* with *BMV3:CYP51* resulted in the silencing of *CYP51A, B* and *C* genes, as determined by a statistically significant reduction of the expression profiles of these genes by nearly 50% compared to mock.

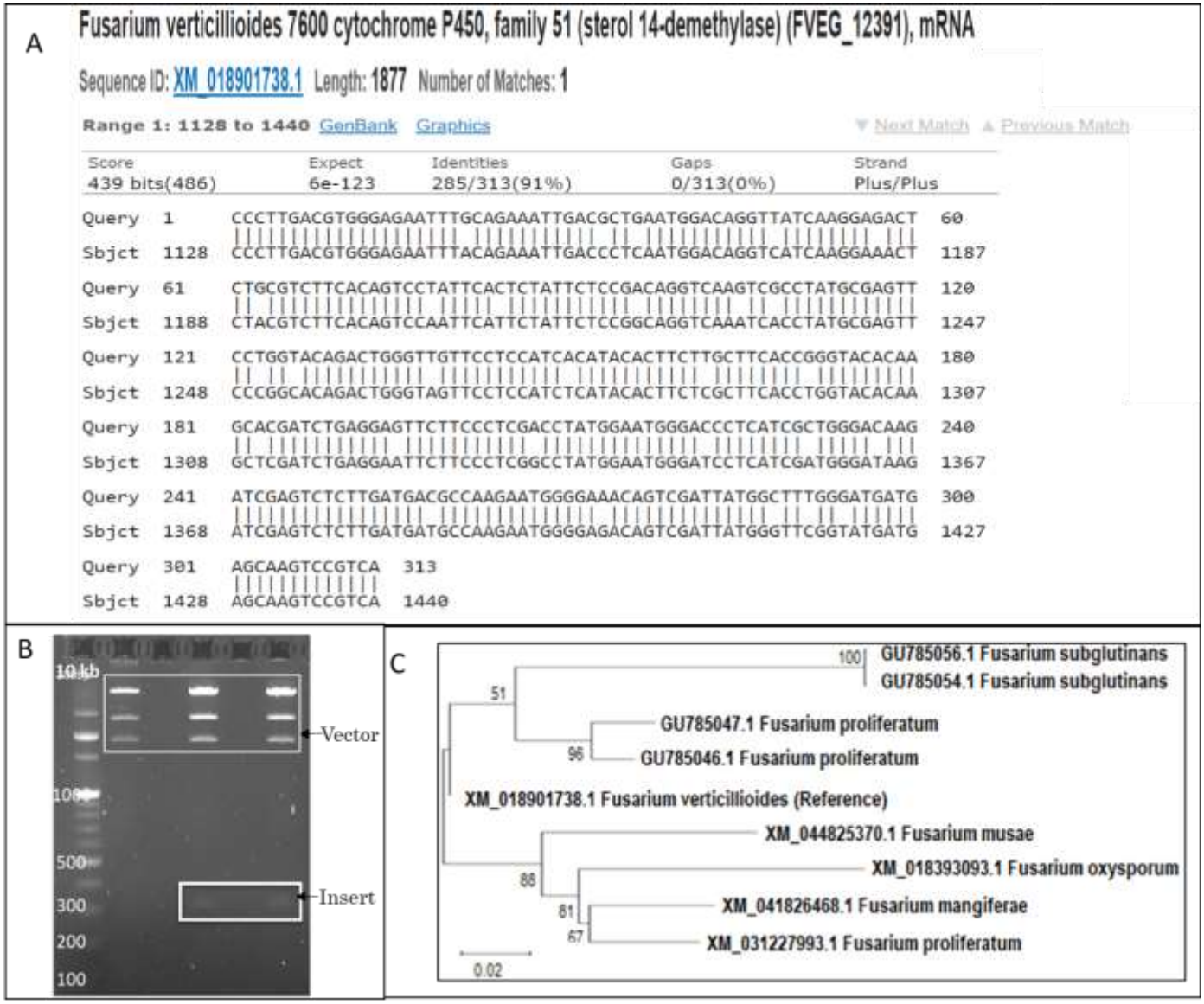

Figure 1. Confirmation of *BMV3:CYP51* clone. A) BLASTn alignment result of the sequenced fragment (Query) that was extracted from the gel. B) Restriction endonuclease (RE) digestion followed by gel electrophoresis of *BMV3:CYP51* clone treated to *Avr*II and *Hind*III. RE confirmed the insert fragment that was 313 bp long. Vector revealed 3 bands longer than 1000 bp. C) Phylogenetic tree constructed with the sequenced insert fragment using the Neighbor Joining method showing evolutionary relation within *Fusarium* species. The percentage of replicate trees where related taxa clustered together by bootstrapping (500 replicates). The branch lengths proportional to evolutionary distances were calculated using the MCL (Maximum Composite Likelihood) method and shown as a percentage.

Şekil 1. BMV3:CYP51 klonunun doğrulanması. A) Jelden izole edilen dizilenmiş parçanın BLASTn hizalama sonucu. B) AvrII ve HindIII'e tabi tutulan BMV3:CYP51 klonunun jel elektroforezini restriksiyon endonükleaz (RE) kesimi izledi. RE, ek parçanın 313 bp uzunluğunda olduğunu doğruladı. Vektör, 1000 bp'den daha uzun 3 bant ortaya çıkardı. C) Neighbor Joining yöntemi kullanılarak dizilenmiş ek parça ile oluşturulan filogenetik ağaç, Fusarium türleri içindeki evrimsel ilişkiyi gösteriyor. İlgili taksonların önyükleme yoluyla (500 tekrar) birlikte kümelendiği tekrar eden ağaçların yüzdesi. Evrimsel mesafelere orantılı dal uzunlukları, MCL (Maximum Composite Likelihood) yöntemi kullanılarak hesaplandı ve yüzde olarak gösterildi.

The result pointed out the possibility of dsRNA of *Fv-CYP51* transferred from *A. tumefaciens* to *F. verticillioides* in a cross-kingdom way and processed by host fungal RNAi machinery to release viral small-interfering RNAs (siRNAs) targeting endogenous fungal *CYP51* paralogs (Qin *et al.*, 2017). *Agrobacterium tumefaciens* is well known as a bacterial plant pathogen resulting in tumorigenic crown gall disease and has been long since utilized for Agrobacterium-mediated transformation of plants (Hooykaas, 2023). However, Bundock *et al.* (1995) reported that *Saccharomyces cerevisiae* transformed by T-DNA of *A. tumefaciens* in acetosyringone containing co-cultivation medium. de Groot *et al.* (1998) performed Agrobacterium-mediated transformation in a wide range of filamentous fungal species such as *Fusarium venenatum* and *Neurospora crassa*, thereby approving that this transformation technique application is not unique to the plant kingdom.

In this study, *F. verticillioides* and *A. tumefaciens* were co-cultivated for 48 hours and at 25℃ on a specific co-cultivation medium containing 200 µM acetosyringone. These co-cultivation conditions were determined based on common usage in previous studies (Sayari *et al.*, 2019; He *et al.*, 2021). In the study of Visentin *et al.* (2012), which focuses on the *Agrobacterium*-mediated transformation of *F. verticillioides*, the co-cultivation conditions were also 48 hours and at 25℃. Nevertheless, the acetosyringone concentration in the medium was 100 µM in this study. In the present study, 200 µM acetosyringone was preferred in the co-cultivation medium, as it has been the general usage of concentration since the study of Bundock *et al* (1995).

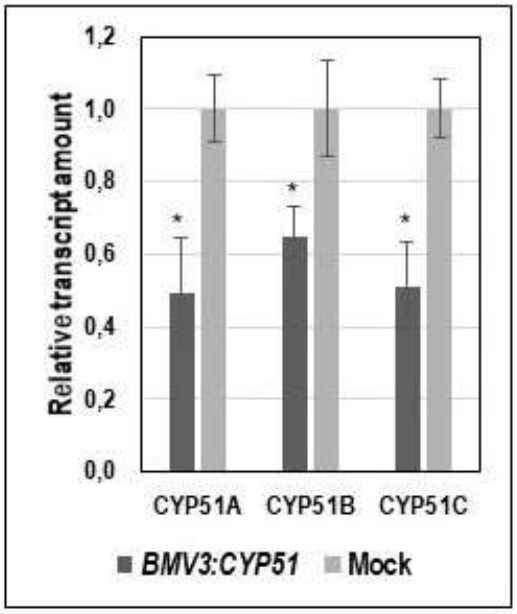

Figure 2. The transcript levels of *CYP51*A, *CYP51*B, and *CYP51C* in *BMV3:CYP51* and mock-treated *Fusarium verticillioides*. CYP51= cytochrome P450 lanosterol C-14α-demethylase, BMV: Brome mosaic virus, n=3, average ±standard deviation (X±SS), *=P<.05 according to Student T-test.

*Şekil 2. BMV3:CYP51 ve boş vektör ile agrotransformasyona uğratılmış A. tumefaciens ile birlikte inkübe edilmiş Fusarium verticillioides'teki CYP51A, CYP51B ve CYP51C transkript düzeyleri. CYP51= sitokrom P450 lanosterol C-14α-demetilaz, BMV: Brom mozaik virüsü, n=3, ortalama ± standart sapma (X±SS), *= Student T-testine göre P<.05.*

Endogenous ROS Level of *F. verticillioides* Cell Increased in Response to *Fv-CYP51* Silencing

To observe the oxidative status of the *Fv-CYP51* silenced *F. verticillioides* cells, total ROS production in agroinfected and VIGS-treated-*F. verticillioides* was determined by DCFH-DA staining assay. The results showed that the endogenous ROS level increased linearly over time of DCFH-DA incubation in both agroinfected *F. verticillioides* with BMV3 (mock) or *BMV3:CYP51* (Figure 3). Moreover, the latter has statistically significantly higher ROS level than the former in each measurement ($F(1, 8) = 9.73$, $p = .02$). At the end of 4 h incubation with DCFH-DA, it was measured as 70.9 OD in *BMV3:CYP51* agroinfected-*F. verticillioides* and 39.8 OD in mock-treated- *F. verticillioides*. This data represented that *CYP51* gene silencing increased total ROS production by 78% in cells ($t(8) = 18.78$, $p < .001$).

ROS (Reactive oxygen species) such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) are a common part of oxygen metabolism at low levels; however, at excessive levels, it disrupts cellular signaling

mechanisms and leads to oxidative stress (Collin, 2019). For total cellular ROS measurement, DCFH-DA staining has been a commonly used method due to its capability of detecting a wide range of ROS including nitrogen dioxide (•NO2) (Kim & Xue 2020). Non-fluorescent DCFH-DA can freely diffuse through cell membranes and intracellular esterases hydrolyze DCFH-DA to dichlorodihydrofluorescein (DCFH) which is also non-fluorescent (de Haan *et al.*, 2022).

ROS lead to the oxidation of DCFH to green fluorescent 2 ',7'-dichlorofluorescein (DCF), the fluorescence intensity of which provides quantification of cellular ROS production (Yu *et al.*, 2021). The increased level of ROS production in *F. verticillioides* infected with *BMV3:CYP51* agrotransformant compared to mock indicated the inhibition of *F. verticillioides* growth and the elevated intracellular oxidative stress conditions, which is an indicator of disrupted redox homeostasis and increased apoptosis rate (Kannan & Jain, 2000). It can be explained by the formation of *CYP51* dsRNA from viral vector (Padmanabhan & Dinesh-Kumar, 2009) in *A. tumefaciens* and its transfer to *F. verticillioides* due to agroinfection of *F. verticillioides* with the help of acetosyringone in the co-cultivation medium, inducing the expression of vir (virulence) genes in Ti-plasmid of *A. tumefaciens* (Sharma & Kuhad, 2010).

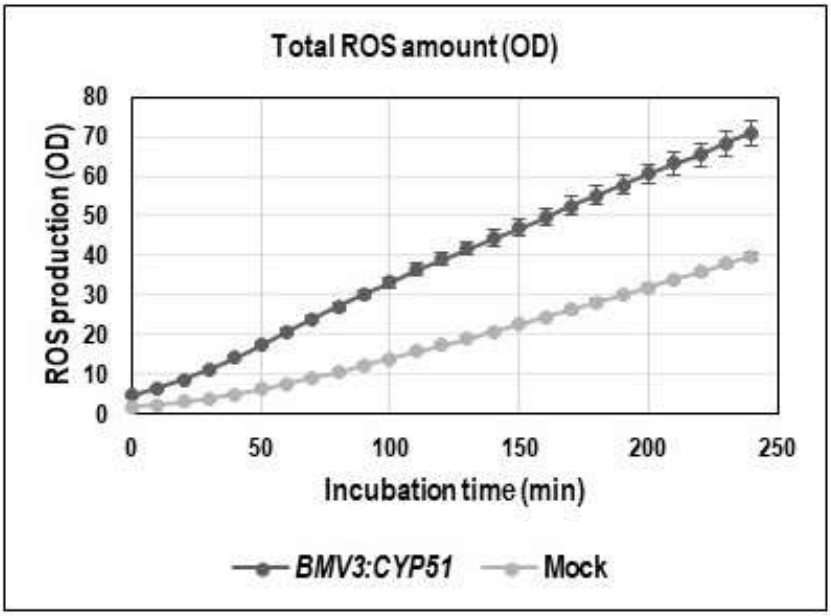

Figure 3. Total ROS production level of *F. verticillioides* infected with *BMV3:CYP51* and BMV3 Agrotransformants. The total ROS production amount of cultures was measured with fluorometric 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining assay every 10 minutes for 4 hours with 488 nm excitation wavelength and 525 nm emission wavelength with a microplate reader. Error bars represent standard deviation. ROS production is expressed as optic density (OD). n=5, average ± standard deviation (X±SS).

Şekil 3. BMV3:CYP51 ve BMV3 Agrotransformantları ile enfekte edilmiş F. verticillioides'in toplam ROS üretim seviyesi. Kültürlerin toplam ROS üretim miktarı, 488 nm uyarım dalga boyu ve 525 nm emisyon dalga boyu ile 4 saat boyunca her 10 dakikada bir florometrik 2',7'-diklorofluorescein diasetat (DCFH-DA) boyama testi ile mikro plaka okuyucu ile ölçüldü. Hata çubukları standart sapmayı temsil eder. ROS üretimi optik yoğunluk (OD) olarak ifade edilir. n=5, ortalama ± standart sapma (X±SS).

Agroinfection with *BMV3:CYP51* Reduces The Growth Rate of *Fusarium verticillioides*

To evaluate the fungal growth rate of agroinfected *F. verticillioides*, the fungal growth zone diameters on PDA were measured comparatively (Figure 3A, B). The growth zone of *BMV3:CYP51* agroinfected *F. verticillioides* was 1.4±0.07 cm, while it was 1.6±0.07 cm in mock. Therefore, *CYP51* silencing resulted in 13% decrease in the fungal growth rate which was a statistically significant result according to applied Student's t-test between VIGS untreated and treated *F. verticillioides* fungal growth zone measurements ($t(10) = -2.14$, $p=.029$) (Figure 3C).

F. verticillioides can generate a high number of spores that can easily spread through the air. *F. verticillioides* spores create a hazardous risk for not only cereals but also animals and humans. In the case of maize production, which is annually 1127 million tons on average in the world (Hoppe *et al.*, 2024), *F. verticillioides* spores infect pre-harvest nearly half of the crops in maize fields (Akanmu *et al.*, 2020). Because azole fungicides pose dangerous risks to humans such as disruption of steroid and androgen hormone balance and signaling (Draskau & Svingen,

2022), *F. verticillioides* infection in crops should be controlled more safely for the well-being of the whole environment. In this study, the procedure for growth inhibition of *F. verticillioides* pathogenesis was achieved by transient cross-kingdom gene silencing of *Fv-CYP51* with Agroinfection based on VIGS treatment. Apart from VIGS, other approaches used for gene silencing with the help of dsRNA are Host-induced gene silencing (HIGS) and Spray-induced gene silencing (SIGS). In several previous studies, *CYP51* gene silencing was achieved with both HIGS and SIGS in *Fusarium graminearum* (Koch *et al.,* 2018; Koch *et al.*, 2019; He *et al.*, 2019) and these studies have been successful in reducing virulence and infection in *Brachypodium distachyon* (He *et al.*, 2019), *Arabidopsis thaliana* and *Hordeum vulgare* plants (Koch *et al.*, 2019).

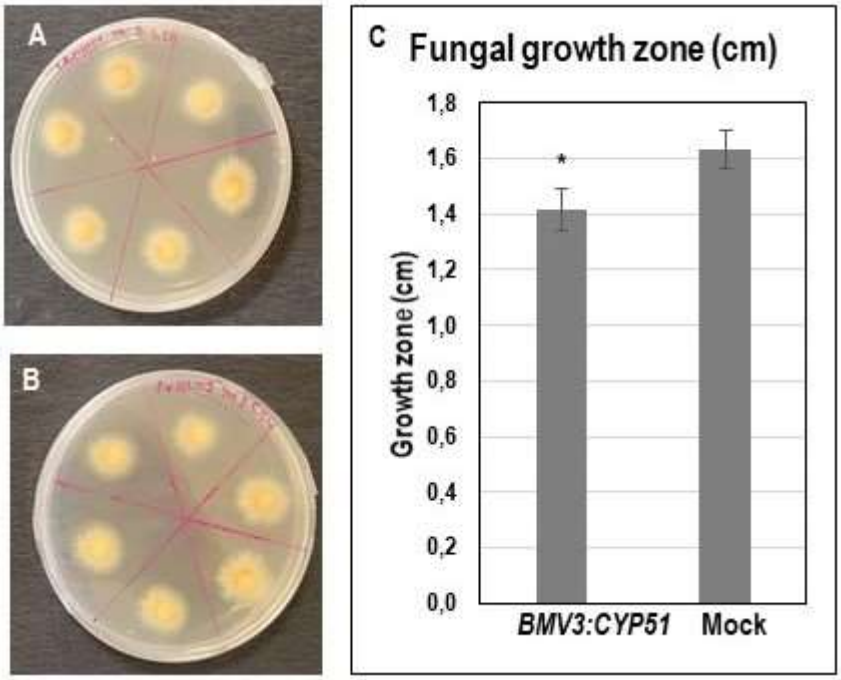

Figure 4. A) The fungal growth zone assay of *BMV3:CYP51* and BMV3 Agroinfected *Fusarium verticillioides* cultures. B) The graph representing the mean of growth zones (cm) measured with Image J. *BMV3:CYP51* is attributed to *F. verticillioides* Agroinfected with *BMV3:CYP51* transformant and BMV3 is attributed to *F. verticillioides* Agroinfected with BMV3 transformant. Error bars show standard deviation. n=6, average ± standard deviation (X±SS), *p=.029 according to Student t-test.

*Şekil 4. A) BMV3:CYP51 ve BMV3 Agroinfected Fusarium verticillioides kültürlerinin fungal büyüme çapı analizi. B) Image J ile ölçülen büyüme çaplarının (cm) ortalamasını gösteren grafik. BMV3:CYP51, BMV3:CYP51 transformant F. verticillioides Agroinfected'e atfedilir ve BMV3, BMV3 transformantlı F. verticillioides Agroinfected'e atfedilir. Hata çubukları standart sapmayı gösterir. n=6, ortalama ± standart sapma (X±SS), *Student t-testine göre p=.029.*

Silencing *CYP51* Gene in *Fusarium verticillioides* Increased Seed Germination Rate of *Zea mays*

The seed germination assay was conducted to observe whether the agroinfection of *Fusarium verticillioides* with *BMV3:CYP51* transformant decreases the pathogenicity of the fungus. To test this, seeds of PEHLIVAN maize hybrid were inoculated with $5x10^3$ spore mL^{-1} *F. verticillioides* alone and agroinfected with BMV3 or *BMV3:CYP51*-agrotransformants. In addition, non-inoculated maize seeds were included as the control group. Finally, it was detected that the non-inoculated seeds germinated 100%, while seeds inoculated with *F. verticillioides* alone germinated 40% (Figure 5). BMV3-agroinfected-*F. verticillioides* inoculation of maize seeds increased the germination rate by 10% compared with only *F. verticillioides* inoculation. Furthermore, it rose to 70% in seeds inoculated with *BMV3:CYP51* Agroinfected-*F. verticillioides*. These findings revealed that both the agroinfection approach and VIGS treatment against *Fv-CYP51* gene alleviated fungal pathogenicity and rotting in maize, thereby raising the seed germination rate compared to non-agroinfected or non-VIGS-treated *F. verticillioides*. *CYP51* gene silencing is more effective than Agroinfection in the decrement of fungal pathogenesis. In a previous study, *CYP51* gene silencing with the HIGS method in *Fusarium graminearum* resulted in diminished fungal pathogenesis on *Arabidopsis thaliana* and barley plant leaves (Höfle *et al.*, 2020). van der Linde *et al.* also applied BMV vector-based VIGS method by silencing *terpene synthase 6/11* (*tps6/11*) gene for the

identification of *Zea mays* genes in the interaction between maize and fungus *Ustilago maydis,* forming tumors on corn smut, leaves and stem (van der Linde *et al.*, 2010).

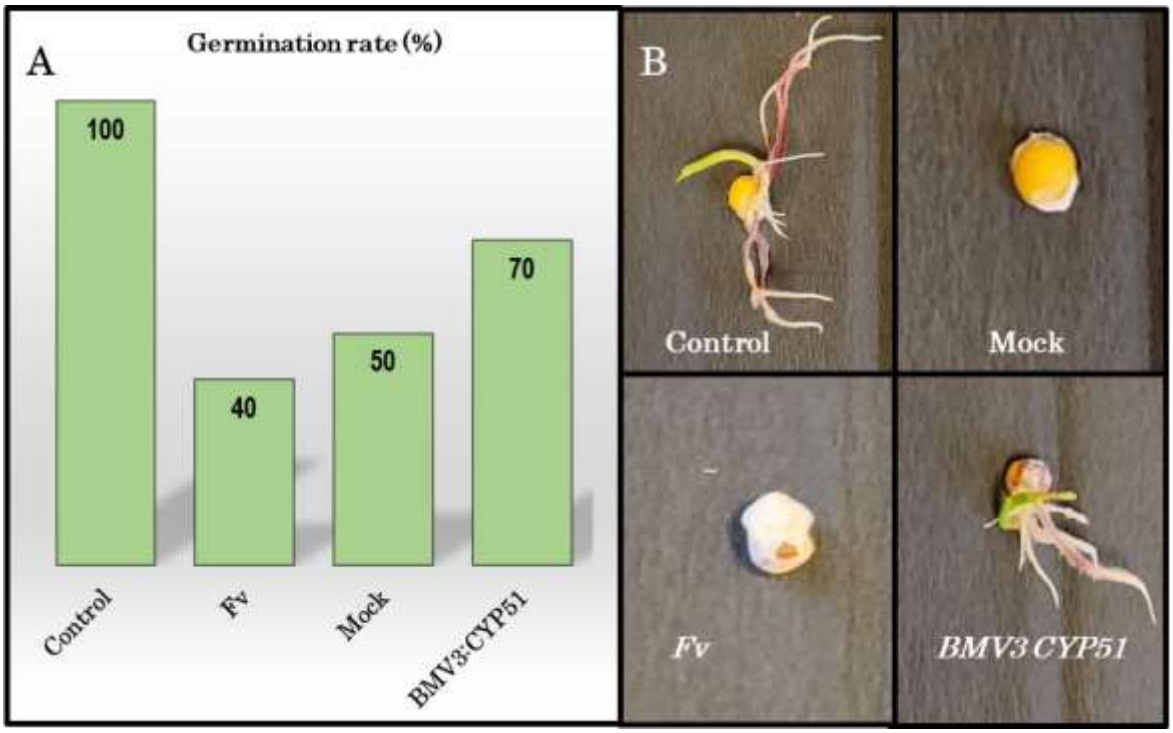

Figure 5. A) The germination rates of *Zea mays* hybrid line PEHLIVAN seeds that were infected with 5x10^3 spore mL^{-1} of non-treated *F. verticillioides* (Fv) and agroinfected with 5x10^3 spore mL^{-1} *F. verticillioides*-treated to *BMV3:CYP51* or BMV3 (Mock) agrotransformants. Control groups were not inoculated with *F. verticillioides*. Germination rate of the seeds in each experimental group as a percentage. B) Representative photographs of seeds after germination in the experimental groups. n=10.

Şekil 5. Mısır (Zea mays) hibridi PEHLIVAN tohumlarının, boş BMV3 (Mock) vektörüyle veya BMV3:CYP51 vektörü ile agrotransformasyona uğratılmış veya uğratılmamış 5 x 10^3 spor mL^{-1}'lik F. verticillioides ile enfeksiyonu sonucunda çimlenme oranları. Kontrol grubu F. verticillioides ile enfekte edilmemiştir. A) Her deney grubundaki tohumların çimlenme oranı yüzde olarak gösterilmiştir. B) Deney gruplarındaki tohumların çimlendikten sonraki temsili fotoğrafları. n=10.

CONCLUSION

This study has shown that cross-kingdom RNA exchange occurs between *Agrobacterium tumefaciens* and *Fusarium verticillioides* and that the transferred dsRNA produced in *A. tumefaciens* suppresses *CYP51* gene expression by stimulating RNAi mechanism in *F. verticillioides*. Our results showed that agroinfection-mediated VIGS based on the RNAi approach successfully decreased the transcript level of *CYP51A, CYP51B and CYP51C.* The silencing of *CYP51* genes significantly increased cellular ROS levels in *F. verticillioides*. In addition, the fungal growth zone assay represented a significant reduction in *F. verticillioides* growth by agroinfection-based VIGS approach. It was also observed that the silencing of *CYP51* genes decreased the pathogenicity of *F. verticillioides* on maize seeds. Our results suggested that *CYP51* gene silencing in *F. verticillioides* thanks to VIGS treatment altered the cell membrane lipid content and disrupted its integrity, resulting in suppression of fungal growth and pathogenicity in germination of maize seeds. In this context, agroinfection-mediated VIGS based on the RNAi approach is an applicable and profound procedure in silencing fungal genes. Hence, this research forms the basis of future studies to develop antifungal VIGS-based systems for *Fusarium verticillioides* in the field.

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Contribution Rate Statement Summary of Researchers

YN and BDP conducted the experimental works. YN wrote the manuscript. FA was responsible for designing and performing experiments and writing and reviewing the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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