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A Forward Genetics Approach Identifies Pathogenicity Genes in *Fusarium oxysporum* f. sp. *carthami,* A Fungus Causing Vascular Wilt Disease of Safflower

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ABSTRACT

Vascular wilt disease caused by *Fusarium oxysporum* f. sp. *carthami* (Foc) is one of the biggest constraints for safflower production in India. Understanding the basis of pathogenicity and molecular dissection of its complex processes is of immense economic importance for the effective management of the wilt disease in safflower. In this study, a forward genetic approach was employed as an unbiased tool to identify the candidate pathogenicity-related genes. *Agrobacterium* mediated random T-DNA mutagenesis in Foc resulted in the generation of 178 Foc transformants. A hydroponics-based pathogenicity screening of generated mutants led to the identification of 12 avirulent mutants. Genome walking with two of the single insertion mutants revealed T-DNA insertion in the intergenic region of one mutant, while in the other mutant T-DNA was inserted in the coding region of a transcription factor. The genes identified in the present study can be targeted by host-delivered RNAi to generate transgenic safflower lines resistant to Foc.

Key words: Vascular wilt disease, Safflower, T-DNA mutagenesis, Hydroponics, Avirulent, Effector protein

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) a member of the family Asteraceae, is cultivated as a rabi crop in India. Since ancient times safflower has been used as a medicinal plant for extracting coloring dyes and as an important source of high-quality oil. The oil extracted from the Safflower seeds is rich in oleic and linolenic acids (Gecgel et al. 2007, Matthaus et al. 2015), which reduces bad cholesterol and are considered beneficial for cardiac health (Nykiforuk et al. 2012). India was always at the forefront and a major contributor to the safflower production worldwide, however, in the last two decades, its annual production and area under cultivation have witnessed a continuous fall (FAOSTAT 2021). One of the reasons for reduction in safflower's cultivated area in India is shifting of farmers to more economically viable alternative crops. Amongst many other constraints, susceptibility of safflower to fungal diseases caused by *Alternaria carthami* and vascular wilt fungus *Fusarium oxysporum* f. sp. *carthami* (Foc) is a major factor affecting safflower productivity in India (Singh et al. 2019). Traditional methods to manage vascular wilt disease by chemical and/or physical means have had reasonable success but do not provide an effective long-term solution due to the persistence of recalcitrant and viable fungal spores in soil for years even in absence of the host (Juber et al. 2018, Pawar et al. 2013). Therefore, a long-term, environmentally friendly solution for management of wilt disease, would involve bolstering plant resistance to Foc by either conventional breeding approach or transgenesis (Kalpana Sastry and Chattopadhyay 2003).

F. oxysporum initiates its invasion life cycle as a hemibiotroph and shifts to necrotrophic mode after cellular death. Interaction of Foc with the host entails coordinated expression of several genes in both pathogen and host (Di Pietro et al. 2003). During the invasion, the pathogen secretes small effector proteins, majority of which are cysteine rich, which enable the pathogen to successfully invade the host plant (Tintor et al. 2020). The effector proteins can alter the structure and function of host cells to promote pathogenicity. Additionally, some effectors (also known as Avr proteins) are directly or indirectly

recognized by the host R-proteins resulting in mounting of Effector-triggered immunity (ETI) and an incompatible disease reaction (Takken and Rep 2010). Our understanding on the action of effectors largely stem from biotrophs and hemi biotrophs as the primary aim of necrotrophic fungi is to kill the host and derive nutrition from dead tissue (Faris and Friesen 2020). Similar to the action of effectors from biotrophs and hemi biotrophs, effectors from nectrophs hijack host immunity and induce cell death in the host plants (Shao et al. 2021).

Recent advances in genome and transcriptome sequencing technologies have allowed researchers to predict several effector proteins from necrotrophic plant fungi. Another approach to identify fungal effectors is by random mutagenesis followed by screening of mutants on susceptible host plants to identify avirulent mutants. With the availability of improved fungal transformation procedures (Idnurm et al. 2017, Mullins et al. 2001), a forward genetics approach involving random insertional mutagenesis by T-DNAs is now routinely used for the identification of pathogenicity genes in important fungal pathogens (Chen et al. 2011, Tayal et al. 2017). We therefore employed random T-DNA mutagenesis of Foc and identified 3 mutants that lost their ability to infect safflower seedlings. Genome-walking with the 2 single copy T-DNA insertion mutants revealed an intergenic insertion between two protein coding genes and insertion in a gene coding for Zn(II)2 Cys6 transcription factor. We believe that further functional analysis of these proteins will help us in devising suitable strategies to combat Foc-infection in safflower and increase its productivity.

MATERIAL AND METHODS

Fungal strain and culture condition

Fusarium oxysporum strain 'FOC-IARI-5175' was obtained from Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi. The fungal strain was maintained on potato dextrose agar plates and grown at 28±1°C in an incubator (New Brunswick Scientific, USA). *Agrobacterium tumefaciens* strain EHA105 was used for *Agrobacterium* mediated transformation of Foc and was grown in Luria Broth (LB) medium containing 30 mg/L rifampicin. Susceptible accession of safflower (PI-199897) was used as a host differential to screen the pathogenicity of mutated Foc strains.

ATMT

Agrobacterium mediated transformation of Foc was essentially performed as described by Pareek et al (2015) with minor modifications. An overnight culture of *A. tumefaciens* EHA105 strain harboring the binary vector pCAMBIA1300 (LB+50 mg/L Kanamycin +50 mg/L Rifampicin) was used to initiate a secondary culture. The secondary culture was devoid of antibiotics and was grown till the O.D. reached 0.4-0.5, following which cells were harvested by centrifugation for 10 min at 2000 x g. Harvested cells were resuspended in induction media [MM Salts, 40 mM MES buffer (pH-5.5), 5 mM Glucose, and 0.5 % Glycerol] containing 200 µM acetosyringone and adjusted to an O.D. of 0.15 with induction media, followed by incubation at 28°C, 200 rpm for 6 h. Foc was grown on potato dextrose agar (PDA) plates at 25°C for 7 days containing cefotaxime (100 mg/L) as a bacteriostatic agent. Fungal spores were collected by adding 2-3 ml autoclaved water and scrapping the colonies gently. Collected spores were filtered through 4 layers of sterile muslin cloth and the spore count was adjusted with sterile water to 10^6 spores/ml using a hemocytometer. For preparation of MM salts following components were added to final concentration: KH_2PO_4 (11.7 mM), K_2HPO_4 (10.6 mM), $(NH_4)_2SO_4$, (3.78 mM), NaCl (2.5 mM), glucose (1.1 mM) $CaCI_2 \cdot 6H_2O$ (456.4 ìM), $MgSO_4 \cdot 7H_2O$ (202.8 ìM), $FeSO_4 \cdot 7H_2O$ (9 ìM).

1 ml of *A. tumefaciens* culture was mixed with 1 ml of spore suspension and spread on sterile Whatman filter paper 1 placed on IM plates containing acetosyringone (200 µM). The plates were incubated at 28°C for 2 days for co-cultivation, following which the Whatman paper was transferred to PDA plates containing cefotaxime (300 mg/L) and hygromycin (100 mg/L) for selecting the transformants. Transformed fungal colonies were observed after 5-6 days. Putative Foc transformants were serially subcultured 2-3 times on PDA plates supplemented with hygromycin (100 mg/L) to ensure mitotic stability of the transgene.

Genomic DNA Extraction and Confirmation of transformants

Genomic DNA was extracted from mycelia of individual Foc samples using cetyltrimethyl-

ammonium bromide **(**CTAB) based procedure. 100 mg of mycelial tissue was ground in 500 µl of CTAB buffer [2% CTAB, 2.56 M NaCl, 0.1 M Tris–HCl (pH-8), 20 mM EDTA] and incubated at 65°C, 10 min. Post-incubation an equal volume of chloroform was added to the sample followed by mixing of contents and subjecting the tubes to centrifugation (RT, 12000 xg, 10 min). The upper aqueous layer was transferred to a fresh MCT and mixed with 2 volumes of chilled ethanol. Precipitation of DNA was accelerated by incubation at -20°C for 1 h followed by centrifugation (4°C, 12000 xg, 10 min). The supernatant was discarded after the centrifugation and pellet was washed twice with 500 µl of 70% ethanol (Incubation at RT for 5 min each), air dried and dissolved in 25 µl of sterile MQ water.

Foc transformants were confirmed by PCR using hygromycin specific primers. PCR was carried out in 20ìl and the mixture contained approximately 50 ng genomic DNA template, 1x reaction buffer, 2.5mM $MgCl_2$, 0.2mM dNTPs, 0.5 µM of each primer (Hygro FP/Hygro RP; Supplementary Table 1) and 1 u of Taq DNA polymerase (INtRON, South Korea). Thermal cycling conditions were initial denaturation at 98°C for 5 min followed by 30 cycles for amplification (30 sec at 98°C, 40 sec at 58°C and 40 sec at 72°C) and final extension at 72°C for 10 min.

Pathogenicity Assay

A hydroponics-based pathogenicity assay was employed for screening of generated Foc transformants (Kukreja et al. 2018). Safflower seeds of the Foc-susceptible accession (PI-199897) were surface sterilized and germinated on germination paper under the photoperiodic conditions of 16 h day and 8 h dark, 135µmol/m^2/s light intensity at 25±1°C. Individual Foc transformants were grown in 70 ml PDB media containing 100 mg/L cefotaxime (25±1°C, 3-4 days, 200 rpm). Cultures with optimum growth were filtered through 8 layers of sterile muslin cloth and spore count in the filtrate was adjusted to 10^6 spores/ml with sterile water. Fifteen days old seedlings were transferred to a sterile test tube filled with 40 ml of water and exposed to Foc infection for 40 h. Final spore count during the infection was maintained at $0.75x10^5$ spores /ml. Seedlings were subsequently placed in water for recovery and observed for development of phenotype. Discernible phenotype was observed between 15-17 days post-infection and at that time percentage disease incidence (PDI) was recorded. On the basis of PDI, Foc transformants were categorized either as virulent or avirulent. Phenotypes and PDI were recorded from 3 independent biological replicates, with each replicate consisting of at least 10 seedlings.

Growth rate analysis

For measurement of the growth rate, approximately a 5 mm stub of actively growing culture were excised from the PDA plates and inoculated in 20 ml of PDB medium supplemented with cefotaxime (50 mg/L). The inoculated medium was incubated in an incubator shaker (26±2°C, 200 rpm) for 3-4 days, thereafter, spore counting was done for each avirulent mutant. Spores ($1x10^4$ per ml) were inoculated at the center of fresh PDA plates (three replicates). Diameter of the growing colony was measured (five readings) on the 4th, 6th and 8th day post inoculation (dpi).

Southern Blot Analysis

Southern blot analysis was performed to confirm T-DNA integration and copy number. Extracted genomic DNA (from 10 g of mycelia) was treated with 50 ìl of RNase (10 mg/ml) to degrade contaminating RNA, followed by purification of DNA by two sequential phenol:chloroform: isoamyl alcohol (PCI, 25:24:1) extractions. DNA in the aqueous layer was precipitated by 0.1 volume of sodium acetate (3M, pH5.2) and 2 volumes of 100% ethanol followed by incubation at -20°C for 2 h. Sample was centrifuged (12000 x g, 4°C, 15 min) and the supernatant was discarded. Pellet was incubated with 2 ml of 70% ethanol for 5 min at RT and followed by centrifugation (12000 x g for 5 min) and removal of 70% ethanol. Pellet was air dried and dissolved in 100 µl of nuclease free water.

For Southern blotting, 10 µg of genomic DNA digested with HindIII at 37°C followed by resolving the DNA fragments on a 0.8% TAE-agarose gel (30 V, overnight). The resolved DNA fragments were transferred to a nylon membrane (Amersham Hybond N) by capillary transfer in 20x SSC (3 M Sodium chloride, 300 mM sodium citrate). Probe for hybridization was generated by amplifying hygromycin gene with primers (SB probe FP/SB probe RP primer set; Supplementary Table 2) using

PCR DIG probe synthesis kit (Roche). The blot was subjected to prehybridization, hybridization, washing and developing strictly as per manufacturer's instructions (DIG easy hyb and DIG Luminiscent Detection kit for nucleic acid, Roche).

Identification of T-DNA insertion site by genome walking

For isolation of genomic sequence flanking T-DNA in Foc mutants, genome walking was performed as described by Reddy et al (2002) with minor modifications. Genomic DNA (10 µg) was digested either with BamH1 or BglII (37°C for 16 h). Digested DNA fragments with 4 base pair overhangs were partially filled with dATP and dGTP using Klenow enzyme as follows: 0.25 mM of each dGTP and dATP was added with 2 units of Klenow enzyme and reaction volume was set up to 20 ul with sterile water and incubated at 37°C for 1 h, then at 75°C for 20 min. Adapters with 2 bp complementary overhangs were made by annealing WAP1 and WAP2 primers (Supplementary Table 1) by incubating 10 pM of each primer at 100°C for 2 min, at 25°C for 20 min. Digested genomic fragments were ligated to walking adapters by adding 50 ng of partially filled genomic DNA to 100 ng of walking adapter, 1x ligase buffer and 3 units of ligase enzyme (Promega). The reaction was incubated at 8°C for 36 h.

Approximately, 50 ng of adapter ligated DNA fragments were used as a template for initial PCR amplification with a biotinylated primer specific to the left border of T-DNA (LBB-bio-1) and adapter specific primer (wp1) primer set to enrich the genomic region adjacent to the known sequence of T-DNA. Thermal cycling conditions for initial amplification were 95°C, 1 min; 55°C, 1 min; and 72°C, 4 min for 30 cycles and final extension at 72°C, 7 min. To extract PCR products amplified by binding of wp1 on adapters at both ends, biotinylated amplicons were captured by streptavidin-linked paramagnetic beads. The captured amplicons were used for setting up two sequential nested PCR reactions with wp-2/LBB-2 and wp-2/LBB-3 primers (supplementary Table 1). The amplicons were gel purified with the help of a Quick DNA Fungal/ Bacterial mini prep kit (Zymo Research) and sequenced with LBB-2 primer. For *in silico* analysis, T-DNA sequence was removed and remaining sequence was aligned with the assembled Foc genome (unpublished data), to identify the site of integration.

The expression of identified candidate genes was analyzed using transcriptome data (unpublished) available in our lab for compatible and incompatible interaction of Foc with PI-199897 and Sharda respectively. Fold change in gene expression was determined corresponding to Log_2 transformed values for each gene.

RESULTS AND DISCUSSION

Agrobacterium mediated fungal transformation and pathogenetic screening

Agrobacterium mediated random T-DNA mutagenesis (ATMT) has routinely been used as an efficient and unbiased tool to tag the genes related to virulence in phytopathogenic fungi (Tayal et al. 2017). Several studies have also demonstrated the utility of ATMT for insertional mutagenesis in various economically important phytopathogenic fungi including selected formae speciales of *F. oxysporum* (Rho et al. 2001, Michielse et al. 2009, Lakshman et al. 2012, Tayal et al. 2017). With an aim to identify the genes whose mutation can affect the pathogenicity of Foc, we randomly mutagenized Foc with the help of ATMT and generated 178 Foc transformants. The efficiency of Foc transformation in our study was approximately 100-120 transformants with 10^6 spores/ml of fungal spores. The transformants were confirmed for the presence of transgene through PCR by amplifying 500 bp amplicon specific to the hygromycin gene (Figure 3a). No amplicon was observed in the untransformed Foc, whereas all the transformants showed an amplified band of the expected size. Earlier studies on ATMT in different formae speciales have reported a range of transformation efficiency such as 21- 24 transformants per 10^4 conidia in *F. oxysporum* f. sp. *cubanse* (MinHui et al. 2009), 1-5 transformants per 10^4 conidia in *F. oxysporum* f. sp. *gladioli* (Lakshman et al. 2012), 300-500 transformants per 10^6 conidia in *F. oxysporum* strain O-685 (Mullins et al. 2001). One of the primary barriers to the entry of naked DNA or the host carrying the DNA is the cell wall. The transformation efficiency in present study is lower than reported previously efficiencies and could

possibly be attributed to the differences in the composition of their cell walls. The efficiency of ATMT also depends on chromatin accessibility and therefore differences in the expression of host genes involved in modulating chromatin structure and cell wall rigidity also contribute to ATMT efficiency (Soltani et al. 2009, Zhu et al. 2003).

Next, we infected the safflower susceptible accession PI-199897 with the 178 confirmed Foc mutants. The accession PI-199897 starts displaying wilting symptoms after 12 days post infection (dpi) and is completely dead by 15 dpi (Singh et al. 2022). Subsequent pathogenicity screening of Foc transformants on the susceptible accession of safflower (PI-199897) led to the identification of 12 avirulent Foc mutants. These mutants were subsequently named as *Fusarium oxysporum* f.sp *carthami*-loss of virulence mutants (*Foc-lov1* to *Foc-lov12*). On the basis of consistent loss of virulence phenotype, we selected *Foc-lov5*, *-lov6* and *-lov8* for further analysis (Fig. 1). Complete loss of pathogenicity in these mutants suggested the integration of T-DNA in genes crucial for the pathogenicity of Foc against safflower.

To date, random mutagenesis of Fusarium and subsequent pathogenetic testing of mutants has unveiled multiple genes crucial for pathogenicity. Insertion mutations generated by restriction enzyme mediated integration (REMI) revealed that loss of function of Argininosuccinate lyase (Arg1) and Fow2 gene coding for Zn(II)2 Cys6 transcription factor severely compromised the virulence of *F. oxysporum* f. sp. *melonis* (Imazaki et al. 2007, Namiki et al. 2001). Similarly, T-DNA insertional mutants in *F. oxysporum* f. sp. *lycopersici* (Fol) discovered that Sge1 and a F-box protein (Frp1) are required for pathogenicity (Duyvesteijn et al. 2005, Michielse et al. 2009) ATMT has also been used as a reverse genetics tool to validate and characterize the candidate genes by generating targeted insertions. Loss of function mutants of G-protein subunit α and β (Fga1 and Fgb1) in *F. oxysporum* f. sp. *cucumerinum*, had reduced virulence (Guo et al. 2016) and knockout mutants of mitogen activated protein kinase in Fol displayed defects in root attachment and penetration (Di Pietro et al. 2001). Overall, T-DNA mutagenesis is a powerful approach for identifying and deciphering the function of genes contributing to the virulence of pathogenic fungi.

Morphological characterization of mutants

To check whether mutants had any pleiotropic effects, we compared the growth rate and pigmentation of the *Foc-lov5*, *-lov6* and *-lov8* mutants with the WT Foc strain. Accumulation of pigments in *Foc* and other formae speciales appear to be both genetic and environment dependent. (Palacio-Barrera et al. 2019, Santos et al. 2020). Pigments like carotenoids protect against photooxidation by light and melanin protect the fungus from environmental stress and have seldom been associated with pathogenicity. In our study, both *Foc-lov5* and *-lov6* displayed an orange pigmentation and were similar in appearance to that of wild type Foc (Fig. 2a). However, the *Foc-lov8* mutant was whitish and had reduced pigmentation. These results indicate that the mutated gene in *Foc-lov8* is either a gene involved in pigmentation or affects the pigmentation indirectly by regulating pigmentation related gene(s). To answer whether loss of virulence

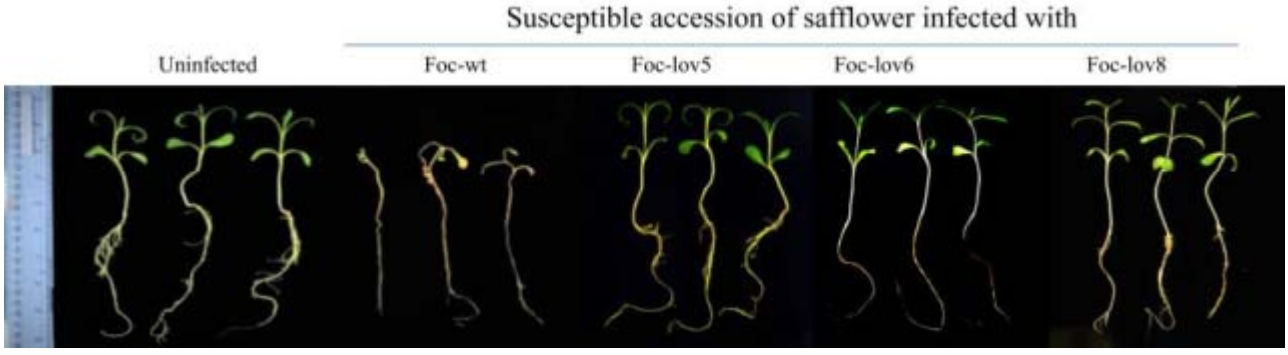

Figure 1. Phenotype of susceptible accession of safflower (PI-199897) uninfected and infected with Foc-wt, *Foc-lov5*, *Foc-lov6* and *Foc-lov8*

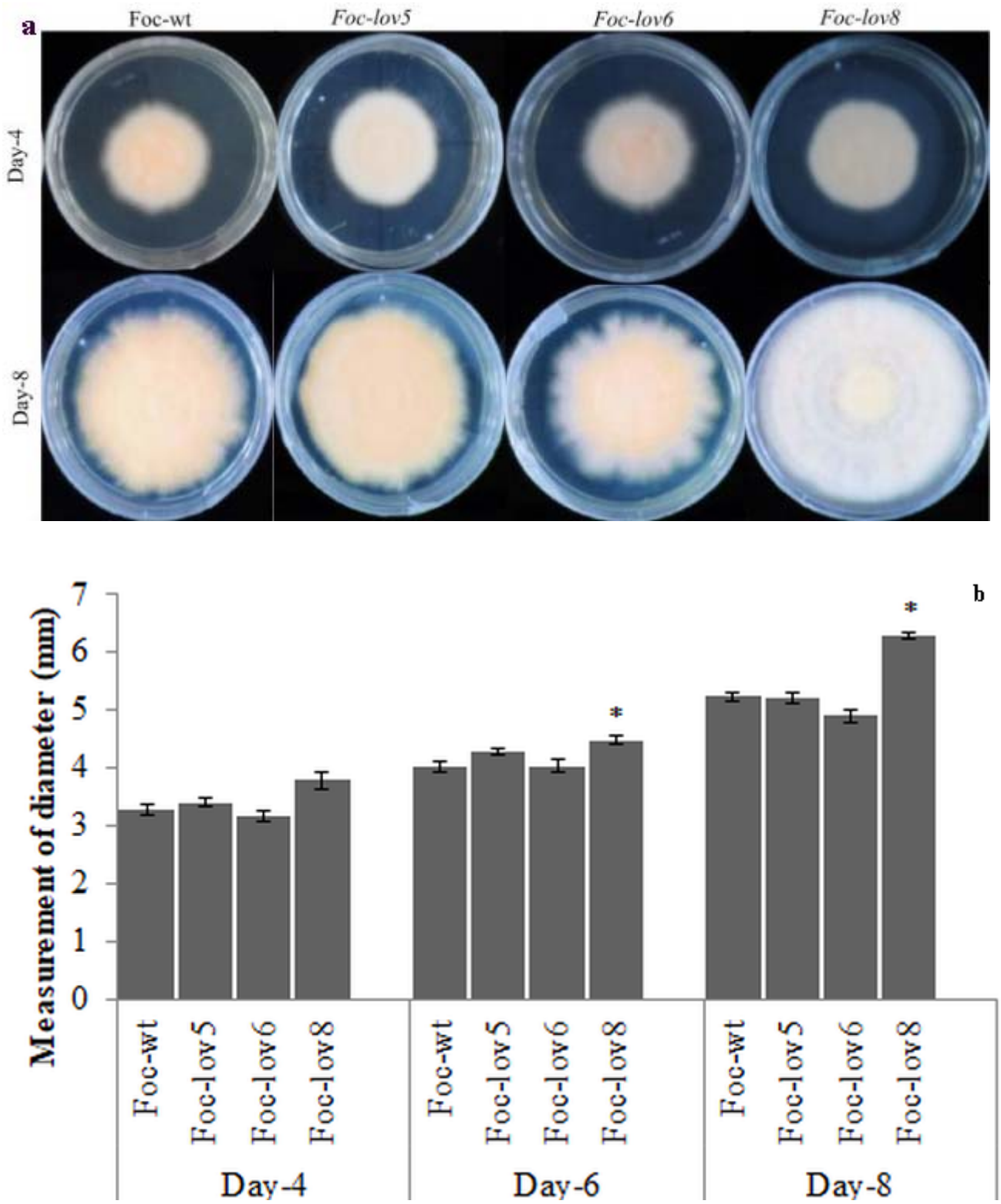

Figure 2**.** Morphological characterization of Foc-WT, Foc-lov5, foc-lov6 and Foc-lov8**.** (a) Colony morphology at 4 and 8 days after inoculation. (b) Growth as measured by colony diameter on 4, 6 and 8 days after inoculation. Each data represents the mean of 5 replicates ± standard error. Asterisk (*) on bars indicates the statistical significance by student's t-test ($P< 0.05$)

is due to lower amount of pigmentation in the *Foc-lov8* mutant, additional experimental evidence would need to be generated in future. It has previously been observed that RNAi-based suppression of peroxisome biogenesis factor6 (*Pex6*) gene in *Fol* resulted in reduced virulence and pigmentation (Tetorya et al. 2017). However, the loss in pathogenicity of Pex6 RNAi colonies could not be related to decreased pigmentation. In another contrasting report homology based targeted deletions of G-protein subunits (*FGA1* and *FGA2*) in *F.oxysporum* f.sp. *cucumerinum* led to reduced virulence but increased pigmentation (Guo et al. 2016). Further, Foc isolates of highly virulent Race 6 had natural variation in pigmentation ranging from dark orange to colorless (Singh et al. 2019). All these studies therefore indicate that the loss of pigmentation does not necessarily have a direct correlation with the loss of virulence in *F. oxysporum*. It is therefore likely that the mutated gene in *Foc-lov8* regulates pathogenicity and pigmentation by affecting independent pathways.

Additionally, the growth rate of mutants was determined to ascertain whether the loss of pathogenicity is due to a slow growth. Previously, different methods have been used by researchers to assess the growth related parameters for *F. oxysporum* such as measurement of fungal biomass, optical density of suspension and radial growth at different time points of fungal inoculation in culture (Davis et al. 1996; Srivastava et al. 2011). In the present study, we employed radial growth measurement for each pathogenicity mutant and wild type Foc. The growth of wild type *F. oxysporum* f. sp. *conglutinans* and *F. oxysporum* f. sp. ciceris ranges from 6 to 12.3 mm/day and we too observed that the wild type Foc had a growth rate (6.63 mm/day) within the reported growth rates (Liu et al. 2017, Singh et al. 2011). Till Day 4, *Foc-lov8* mutant had a similar growth as WT, however starting at Day 6, *Foc-lov8* showed a significantly higher growth as compared to the WT Foc (Fig. 2b). In contrast, the growth for *Foc-lov5* and *Foc-lov6* was not significantly different as compared to the WT.

Molecular characterization of T-DNA Foc mutants

Pathotypes of *Foc-lov5*, *Foc-lov6* and *Foc-lov8* mutants were confirmed by repeated rounds of hydroponics-based pathogenicity assay with the susceptible accession (PI-199897) of safflower. To assess the copy number of transgene we performed Southern analysis. While the mutants *Foc-lov6* and *Foc-lov8* had a single T-DNA insertion in their genome, *Foc-lov5* had 3 T-DNA insertions (Fig. 3c). Foc does not have a sexual cycle, segregation of transgene in *Foc-lov5* mutant is not possible, and therefore, *Foc-lov5* mutant was not used further for identification of flanking regions. However, T-DNA flanking genomic regions were identified for *Foc-lov6* and *Foc-lov8*. The sequence of the flanking regions was aligned with the Foc genome (unpublished data from the lab). The identity of the genes was ascertained on the basis of their corresponding annotations of the published Fol genome (Ma et al. 2010). For both the T-DNA mutants, sites of integration were on chromosome-2 (Table 1). The T-DNA insertion in the *Foc-lov6* mutant occurred between 2 convergent genes FOX_06124 and FOXG_06125. The left border of T-DNA was 805 bp from the stop codon of FOX_016124, while the right border was

Table 1. Details of T-DNA integration and site of insertion in genome of avirulent mutants identified by southern analysis and genome walking

S. No.	Mutant ID	Transgene copy number	Identified Locus	Functional annotation	Chromosome number
1	*Foc_lov5*	3	-	-	-
2	*Foc_lov6*	1	Between FOXG_06125 and FOXG_06124	-	Chr-2
3	*Foc_lov8*	1	FOXG_06378	Fow gene coding for Zn(II)2 Cys6 transcription factor	Chr-2

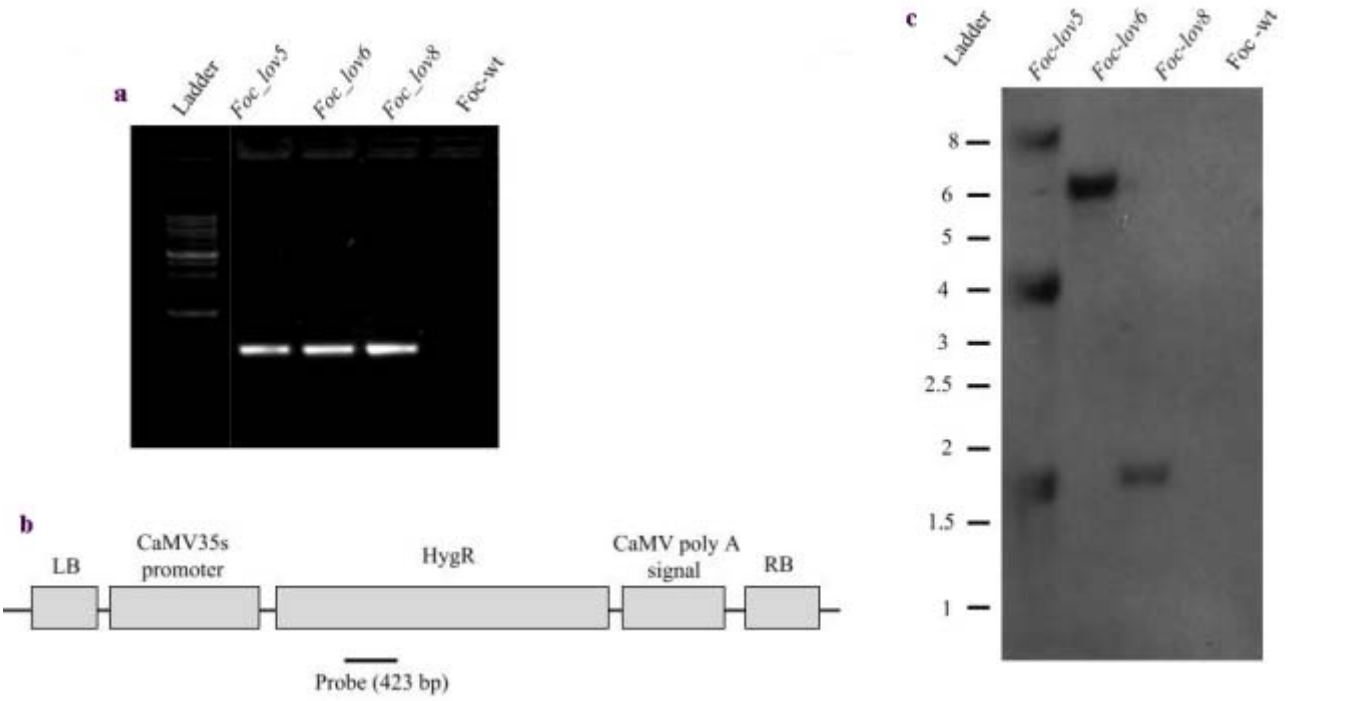

Figure 3. Molecular characterization of avirulent Foc mutant. (a) Confirmation through PCR with hygromycin specific primers. (b) Diagrammatic representation of T-DNA and probe used for southern analysis. (c) Southern blot showing the number of T-DNA integration in the Foc genome

approximately 6 kb from the stop codon of FOXG_06125 gene. The protein encoded by gene FOXG_06124 is similar to a protein with YjeF domain (Ma et al. 2010). YjeF domain resembles the Rossmann fold, which is generally associated with proteins that serve as enzymes involved in RNA metabolism. The YjeF domain has a putative catalytic site (Anantharaman and Aravind 2004). The FOXG_06125 codes for a hypothetical protein. Although the insertion was not disrupting the gene function directly, there are multiple possibilities of it changing the expression of one or both the genes. Firstly, the insertion is in the 3'UTR of FOX_06124, which can affect its transcript stability and expression. Secondly, hygromycin resistance in the T-DNA is driven by CaMV35S promoter. The strong constitutive promoter is known to regulate the expression of adjacent genes (Yoo et al. 2005). Another possibility for the intergenic insertion resulting in avirulence, could be the disruption of some epigenetic marks that possibly change the chromatin accessibility and hence expression of surrounding genes. Genome walking of *Foc-lov8* revealed insertion in 5' UTR (167 bp upstream to first exon) of a gene encoding a $Zn(II)_2$ Cys6 transcription factor. Earlier in a similar study, molecular analysis of REMI mutant strain of *F. oxysporum* f. sp. *melonis* (Fom) with loss of pathogenicity confirmed the insertion in the fifth exon of Fow2 gene. In Fom involvement of Fow2 transcription factor was further confirmed by generating knockout mutant using homologous recombination (Imazaki et al. 2007). Understanding the mechanism of Foc $Zn(II)_2$ Cys6 transcription factor in controlling virulence and pigmentation in Foc, will need additional studies. Nonetheless, $Zn(II)_2$ Cys6 transcription factor appears to be a major determinant of Foc virulence.

Expression of candidate genes during compatible and incompatible reaction

To discover the *Foc* genes that are differentially expressed in contrasting host accessions, we performed RNA-seq for Foc-infected roots of the susceptible (PI-199897) and resistant (Sharda) safflower accessions (unpublished data) and expression of the three candidate genes i.e, FOXG_06125, FOXG_06124 and FOXG_06378 was analyzed (Fig. 4). Although expression of two FOXG_06125 and FOXG_06124 was slightly

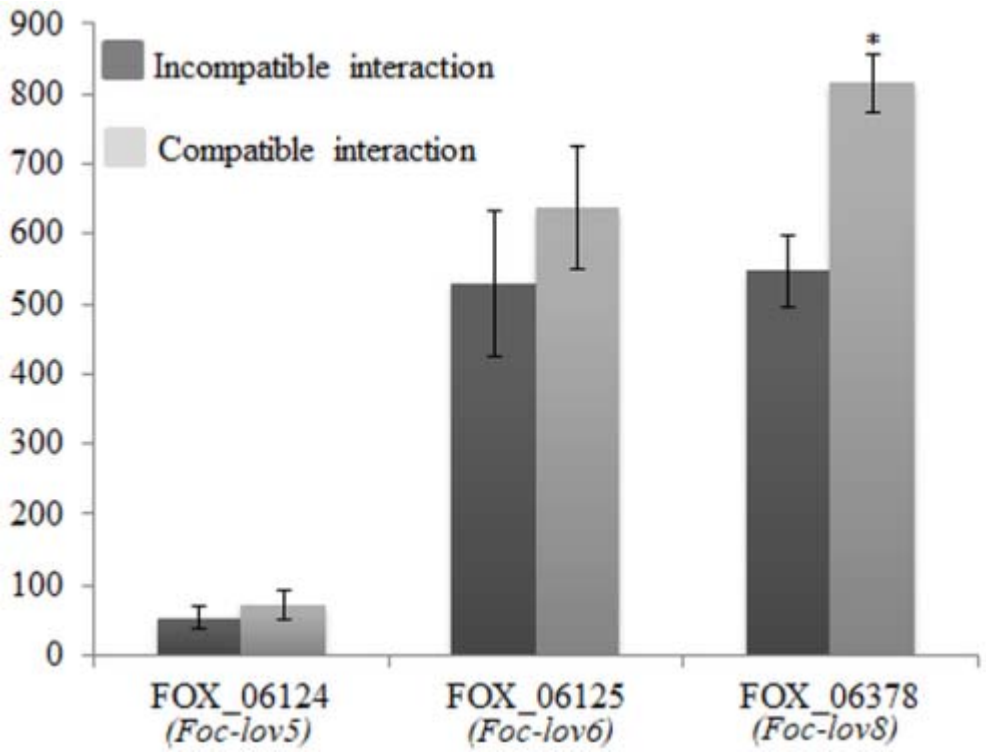

Figure 4. Expression of identified genes in Foc-lov6 (FOX_06124 and FOX_06125) and Foc-lov8 (FOX_06378) during compatible and incompatible interaction with PI-199897 and sharda respectively. Each data represents the mean of 2 replicates ± standard error. Asterisk (*) on bars indicates the statistical significance by student's t-test ($P< 0.05$)

upregulated in the susceptible host (1.2 and 1.3 folds respectively), the upregulation was not significant. The RNA-seq experiment was conducted at 7 dpi and the possibility of FOXG_06125/FOXG_06124 being transcriptionally regulated at an early stage of the infection cannot be ruled out. In contrast, the expression of gene FOXG_06378 encoding the $Zn(II)_2$ Cys6 transcription factor was significantly higher (1.49 folds) at the 7 dpi stage in the susceptible host as compared to the resistant host, further indicating that $Zn(II)_2$ Cys6 transcription factor is important for the virulence of Foc. Since TF regulates multiple downstream genes, chromatin immunoprecipitation based analysis together with expression analysis would be useful in identifying the targets regulated by the Foc $Zn(II)_2$ Cys6 TF.

CONCLUSIONS

We used *Agrobacterium* mediated random T-DNA mutagenesis in Foc and phenotypic evaluation of the mutants to identify novel pathogenicity genes. Hydroponics based screening of 178 Foc transformants led to the identification of 12 avirulent mutants. Gene tagging by genome walking of the 2 mutants having single copy T-DNA insertion revealed an intergenic insertion between FOXG_06124 and FOXG_06125 genes and an exonic insertion in FOXG_06378 gene encoding in the susceptible safflower accession as compared to the resistant accession. Overall, our results show that random T-DNA mutagenesis together with robust phenotypic evaluation is a powerful approach for the identification of virulence genes of *Fusarium oxysporum*. The proteins encoded by these genes and their host interactors will help in devising better and more effective strategies for the management of wilt disease in Safflower.

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REFERENCES

Anantharaman, V. and Aravind, L. 2004. Novel conserved domains in proteins with predicted roles in eukaryotic cell-cycle regulation, decapping and RNA stability. BMC Genomics, 5(1), 45. https://doi.org/10.1186/1471-2164-5-45

Chen, X.-L., Yang, J. and Peng, Y.-L. 2011. Large-Scale Insertional Mutagenesis in Magnaporthe oryzae by Agrobacterium tumefaciens-Mediated Transformation. pp. 213–224, In: Xu, J.-R. and Bluhm, B.H. (Eds.), Fungal Genomics: Methods and Protocols. Humana Press. https://doi.org/10.1007/978-1-61779-040-9_16

Davis, R., Moore, N. and Kochman, J. 1996. Characterisation of a population of *Fusarium oxysporum* f.sp. *Vasinfectum* causing wilt of cotton in Australia. Australian Journal of Agricultural Research, 47(7), 1143. https://doi.org/10.1071/AR9961143

Di Pietro, A., García-Maceira, F.I., Méglecz, E. and Roncero, M.I.G. 2001. A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. Molecular Microbiology, 39(5), 1140–1152. https://doi.org/10.1111/j.1365-2958.2001.02307.x

Di Pietro, A., Madrid, M.P., Caracuel, Z., Delgado-Jarana, J. and Roncero, M.I.G. 2003. *Fusarium oxysporum*: Exploring the molecular arsenal of a vascular wilt fungus. Molecular Plant Pathology, 4(5), 315–325.

Duyvesteijn, R.G.E., Van Wijk, R., Boer, Y., Rep, M., Cornelissen, B.J.C. and Haring, M.A. 2005. Frp1 is a *Fusarium oxysporum* F-box protein required for pathogenicity on tomato. Molecular Microbiology, 57(4), 1051–1063. https://doi.org/10.1111/j.1365-2958.2005.04751.x

Faris, J.D. and Friesen, T.L. 2020. Plant genes hijacked by necrotrophic fungal pathogens. Current Opinion in Plant Biology, 56, 74–80. https://doi.org/10.1016/j.pbi.2020.04.003

Gecgel, U., Demirci, M., Esendal, E. and Tasan, M. 2007. Fatty acid composition of the oil from developing seeds of different varieties of Safflower (*Carthamus tinctorius* L.). Journal of the American Oil Chemists' Society, 84(1), 47–54. https://doi.org/10.1007/s11746-006-1007-3

Idnurm, A., Bailey, A.M., Cairns, T.C., Elliott, C.E., Foster, G.D., Ianiri, G. and Jeon, J. 2017. A silver bullet in a golden age of functional genomics: The impact of Agrobacterium-mediated transformation of fungi. Fungal Biology and Biotechnology, 4(1), 6. https://doi.org/10.1186/s40694-017-0035-0

Imazaki, I., Kurahashi, M., Iida, Y. and Tsuge, T. 2007. Fow2, a Zn(II)2Cys6-type transcription regulator, controls plant infection of the vascular wilt fungus *Fusarium oxysporum*. Molecular Microbiology, 63(3), 737–753. https://doi.org/10.1111/j.1365-2958.2006.05554.x

Juber, K., Hassan, A. and Alhamiri, Y.N.H. 2018. Evaluation of biocontrol agents and chemical inducers for managing vascular wilt of tomato caused by *Fusarium oxysporum f.sp. Lycopersici*.

Kalpana Sastry, R. and Chattopadhyay, C. 2003. Development of Fusarium Wilt-resistant Genotypes in Safflower (*Carthamus tinctorius*). European Journal of Plant Pathology, 109(2), 147–151. https://doi.org/10.1023/A:1022502618887

Kukreja, B., Joshi, G., Sharma, E., Kapoor, R., Goel, S., Jagannath, A., Kumar, A. and Agarwal, M. 2018. Standardization of hydroponics based procedure for high-throughput screening and its application for identification of differential host response in Safflower against *Fusarium oxysporum carthamii*. Vegetos, 31(2), 5. https://doi.org/10.5958/2229-4473.2018.00049.6

Lakshman, D. K., Pandey, R., Kamo, K., Bauchan, G. and Mitra, A. 2012. Genetic transformation of *Fusarium oxysporum f.sp. Gladioli* with agrobacterium to study pathogenesis in Gladiolus. European Journal of Plant Pathology, 133(3), 729–738. https://doi.org/10.1007/s10658-012-9953-0

Liu, X., Ling, J., Xiao, Z., Xie, B., Fang, Z., Yang, L., Zhang, Y., Lv, H. and Yang, Y. 2017. Characterization of emerging populations of *Fusarium oxysporum* f. Sp. *Conglutinans* causing cabbage wilt in China. Journal of Phytopathology, 165(11–12), 813–821. https://doi.org/10.1111/jph.12621

Ma, L.-J., van der Does, H.C., Borkovich, K.A., Coleman, J.J., Daboussi, M.-J., Di Pietro, A., Dufresne, M., Freitag, M., Grabherr, M., Henrissat, B., Houterman, P. M., Kang, S., Shim, W.-B., Woloshuk, C., Xie, X., Xu, J.-R., Antoniw, J., Baker, S.E., Bluhm, B.H. and Rep, M. 2010. Comparative genomics reveals mobile pathogenicity chromosomes in Fusarium. Nature, 464(7287), 367–373. https://doi.org/10.1038/nature08850

Matthaus, B., Özcan, M.M. and Al Juhaimi, F.Y. 2015. Fatty acid composition and tocopherol profiles of safflower (*Carthamus tinctorius* L.) seed oils. Natural Product Research, 29(2), 193–196. https://doi.org/10.1080/14786419.2014.971316

Michielse, C.B., Wijk, R. van, Reijnen, L., Manders, E.M.M., Boas, S., Olivain, C., Alabouvette, C. and Rep, M. 2009. The nuclear protein Sge1 of *Fusarium oxysporum* is required for parasitic growth. PLOS Pathogens, 5(10), e1000637. https://doi.org/10.1371/journal.ppat.1000637

MinHui, L., Rong, Z., DaGang, H., PingGen, X., ChuXiong, Z. and ZiDe, J. 2009. *Agrobacterium tumefaciens*-mediated transformation of *Fusarium oxysporum* f. Sp. Cubense race 4. Acta Phytopathologica Sinica, 39(4), 405–412.

Mullins, E.D., Chen, X., Romaine, P., Raina, R., Geiser, D.M. and Kang, S. 2001. *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: An efficient tool for insertional mutagenesis and gene transfer.

Phytopathology, 91(2), 173–180. https://doi.org/10.1094/PHYTO.2001.91.2.173

Namiki, F., Matsunaga, M., Okuda, M., Inoue, I., Nishi, K., Fujita, Y. and Tsuge, T. 2001. Mutation of an arginine biosynthesis gene causes reduced pathogenicity in *Fusarium oxysporum* f. Sp. Melonis. Molecular Plant-Microbe Interactions, 14(4), 580–584. https://doi.org/10.1094/MPMI.2001.14.4.580

Nykiforuk, C.L., Shewmaker, C., Harry, I., Yurchenko, O.P., Zhang, M., Reed, C., Oinam, G.S., Zaplachinski, S., Fidantsef, A., Boothe, J.G. and Moloney, M.M. 2012. High level accumulation of gamma linolenic acid (C18:3Ä6.9,12 cis) in transgenic safflower (*Carthamus tinctorius*) seeds. Transgenic Research, 21(2), 367–381. https://doi.org/10.1007/s11248-011-9543-5

Palacio-Barrera, A.M., Areiza, D., Zapata, P., Atehortúa, L., Correa, C. and Peñuela-Vásquez, M. 2019. Induction of pigment production through media composition, abiotic and biotic factors in two filamentous fungi. Biotechnology Reports, 21, e00308. https://doi.org/10.1016/j.btre.2019.e00308

Pawar, S.V., Dey, U., Munde, V.G., Sutar, D.S. and Pal, D. 2013. Management of seed/soil borne diseases of safflower by chemical and biocontrol agents. African Journal of Microbiology Research, 7(18), 1834–1837. https://doi.org/10.5897/AJMR12.2010

Rho, H.-S., Kang, S. and Lee, Y. H. 2001. *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe grisea*. Molecules and Cells, 12, 407–411.

Santos, M.C.dos, Mendonça, M.de L. and Bicas, J.L. 2020. Modeling bikaverin production by *Fusarium oxysporum* CCT7620 in shake flask cultures. Bioresources and Bioprocessing, 7(1), 13. https://doi.org/10.1186/s40643-020-0301-5

Singh, K.N., Parveen, S., Kaushik, P., Goel, S., Jagannath, A., Kumar, K. and Agarwal, M. 2022. Identification and validation of in silico mined polymorphic EST-SSR for genetic diversity and cross-species transferability studies in Safflower. Journal of Plant Biochemistry and Biotechnology, 31(1), 168–177. https://doi.org/10.1007/s13562-021-00673-1

Singh, N., Anand, G. and Kapoor, R. 2019. Virulence and genetic diversity among Fusarium oxysporum f. Sp. Carthami isolates of India using multilocus RAPD and ISSR markers. Tropical Plant Pathology, 44(5), 409–422.

Singh, S.K., Singh, B. and Singh, V.B. 2011. Morphological, cultural and pathogenic variability among the isolates of *Fusarium oxysporum* f. Sp. Ciceri causing wilt of chick pea. Annals of Plant Protection Sciences, 19(1), 155–158.

Srivastava, S., Pathak, N. and Srivastava, P. 2011. Identification of limiting factors for the optimum growth of *Fusarium oxysporum* in liquid medium. Toxicology International, 18(2), 111–116. https://doi.org/10.4103/0971-6580.84262

Takken, F. and Rep, M. 2010. The arms race between tomato and *Fusarium oxysporum*. Molecular Plant Pathology, 11(2), 309–314.

Tayal, P., Raj, S., Sharma, E., Kumar, M., Dayaman, V., Verma, N., Jogawat, A., Dua, M., Kapoor, R. and Johri, A.K. 2017. A *Botrytis cinerea* KLP-7 kinesin acts as a virulence determinant during plant infection. Scientific Reports, 7(1), 1–16.

Tintor, N., Paauw, M., Rep, M. and Takken, F.L.W. 2020. The root-invading pathogen *Fusarium oxysporum* targets pattern-triggered immunity using both cytoplasmic and apoplastic effectors. New Phytologist, 227(5), 1479–1492. https://doi.org/10.1111/nph.16618.

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Supplementary material

List of all oligos used in the present analysis

Name of oligos	Sequence	Purpose
Hygro FP	TTTGAGATGGATGGTGTCAATGAG ACTTTTCAACAAAGGG	Confirmation of Foc transformants
Hygro RP	CATTAGATACCCGAGTCATCGATCT GGATTTTAGTACTGG	Confirmation of Foc transformants
WAP-1	CTAATACCACTCACATAGGGCGGC CGCCCGGGC	Walking adaptor primers
WAP-2	TCGCCCGGGCG	Walking adaptor primers
WAP-3	AGGCCCGGGCG	Walking adaptor primers
WP-1	CTAATACGACTCACTATAGGG	Walking primers
WP-2	GGGCGGCCGCCCGGGCGATC	Walking primers
WP-3	GGGCGGCCGCCCGGGCCTAG	Walking primers
LBB_Bio-1	AGGGTCGATGCGACGCAATCG	pCAMBIA1300 specific walking primers
LBB-2	GTGAGTAGTTCCCAGATAAGGG	pCAMBIA1300 specific walking primers
RBB_Bio-1	AGGCATGCAAGCTTGGCACTG	pCAMBIA1300 specific walking primers
RBB-2	AACAGTTGCGCAGCCTGAATG	pCAMBIA1300 specific walking primers
SB probe3 FP	CGCCGAATTAATTCGGGGGATCTG	Probe synthesis (Southern Blotting)
SB probe3 RP	TATGCTCCGCATTGGTCTTGACC	Probe synthesis (Southern Blotting)