An Efficient Homologous Recombination-based in situ Protein-labeling Method in Verticillium dahliae

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Article

An Efficient Homologous Recombination-Based *In Situ* Protein-Labeling Method in *Verticillium dahliae*

Jie Yang [1,2,^], Mengran Liu [1,2,^], Yue Jiao [3], Hui-Shan Guo [1], Chun-Min Shan [1,*] and Haiting Wang [1*]

[1] State Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, China; yangjie@im.ac.cn (J.Y.), r15163001873@163.com (M.L.), guohs@im.ac.cn (H.-S.G.)

2 University of Chines and Academy of Sciences, Beijing 100049, China.

[3] Development Center of Science and Technology, Ministry of Agriculture and Rural Affairs, Beijing 100176, China. jiaoyue@agri.gov.cn (Y.J.)

* Correspondence: Correspondence: shancm@im.ac.cn (C.-M.S.); wanght@im.ac.cn (H.-T.W.)

^ These authors contribute equally.

Abstract: Accurate determination of protein localization, levels, or protein-protein interactions is pivotal for the study of their function, and *in situ* protein labeling *via* homologous recombination has emerged as a critical tool in many organisms. While this approach has been refined in various model fungi, the study of protein function in most plant pathogens has predominantly relied on ex-situ or overexpression manipulations. To dissect the molecular mechanisms of development and infection for *Verticillium dahliae*, a formidable plant pathogen responsible for vascular wilt diseases, we have established a robust, homologous recombination-based *in situ* protein labeling strategy in this organism. Utilizing *Agrobacterium tumefaciens*-mediated transformation (ATMT), this methodology facilitates the precise tagging of specific proteins at their C-termini with epitopes, such as GFP and Flag, within the native context of *V. dahliae*. We demonstrate the efficacy of our approach through the *in situ* labeling of *VdCf2* and *VdDMM2*, followed by subsequent confirmation *via* subcellular localization and protein-level analyses. Our findings confirm the applicability of homologous recombination for *in situ* protein labeling in *V. dahliae* and suggest its potential utility across a broad spectrum of filamentous fungi. This labeling method stands to significantly advance the field of functional genomics in plant pathogenic fungi, offering a versatile and powerful tool for the elucidation of protein function.

Keywords: protein-labeling; *in situ*; homologous recombination; *Agrobacterium tumefaciens*-mediated transformation (ATMT); *Verticillium dahliae;* filamentous fungi

1. INTRODUCTION

Verticillium dahliae, a soil-borne phytopathogen, is notorious for causing wilt disease across a wide range of plant species by colonizing their vascular systems, predominantly *via* root infection (Hu et al., 2017). This fungus is capable of producing microsclerotia, a type of dormant structure, enabling it to persist in soil for extended periods, even in the absence of a host plant. (Xie et al., 2017 ; Klosterman et al., 2009). Remarkably, *V. dahliae* can affect over 400 species of dicotyledonous plants, inclusive of vital crops such as cotton, tomato, potato, tobacco, and sunflower, which are all of high economic significance (Snelders et al., 2020; Chen et al., 2021; Wang et al., 2021). Among these, the impact of Verticillium wilt on cotton is particularly devastating, as it is the most destructive disease affecting this crop, causing substantial reductions in both yield and quality (Zhu et al., 2023). The control of *V. dahliae* presents increasing challenges due to its remarkable environmental adaptability, genetic variability, and intricate pathogenic mechanisms. Despite considerable efforts to elucidate the molecular mechanisms underlying *V. dahliae*'s development and infection processes (Wen et al., 2023; Wu et al., 2023; Zhang et al., 2022; Sun et al., 2019), many of these aspects remain largely elusive. One possible reason for this challenge may be the limited availability of genetic manipulation tools available for this particular fungus, compared with other filamentous fungi.

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Understanding the molecular basis of growth, development, and infection processes in plant pathogenic fungi, particularly through the functional analysis of key pathogenicity genes, is fundamental for enhancing crop resistance to fungal invasions (Song et al. 2020). Therefore, genome manipulation techniques that facilitate the direct modification or tagging of specific proteins, to study their functions within *V. dahliae* are recognized as potent tools for achieving this objective. However, current methods for protein labeling in *V. dahliae* often result in ex-situ manipulation or over-expression (Wen et al., 2023; Sun et al., 2019), which can lead to several adverse effects. Ectopic accumulation of tagged proteins beyond physiological levels may disrupt normal cellular function, induce negative feedback regulation, and may be toxic. Consequently, current protein tagging methods may not accurately represent the native structure or function of protein complexes, nor the true *in vivo* gene expression profiles in *V. dahliae*.

Homologous recombination offers a precise and efficient method for minimally invasive tagging at endogenous loci, as evidenced in various model organisms including *Saccharomyces cerevisiae* (Miao et al., 2023; Teubl et al., 2022; Kira et al., 2021; Khmelinskii et al., 2011; Sung et al., 2008), *Drosophila* (Kanca et al., 2022; Ghosh et al., 2018; Baena-Lopez et al., 2013), and others. This technique facilitates *in vivo* targeting and manipulation of genes on their normal chromosomal locations, and leads to the attachment of tags, such as fluorescent proteins or epitope tags, to these specific proteins. Tagged proteins enable the tracking of protein location within cells or tissues and the monitoring of protein expression and dynamics under native conditions in real-time, aiding in the visualization of their spatial distributions (Xiang et al., 2019; Khmelinskii et al., 2011). Furthermore, epitope tags (e.g., Flag or HA) can enhance the detection accuracy and purification efficiency of target proteins, and diminish the costs associated with synthesizing specific antibodies for different proteins (Shan et al., 2016; Shan et al., 2021). In *V. dahliae*, homologous recombination has been widely used for gene knockout, but the currently used protein labeling method often results from ex-situ or overexpression manipulation (Wang et al. 2016; Wen et al., 2023).

To address the challenges associated with elucidating the intrinsic characteristics of pathogenic proteins, and to facilitate more accurate biochemical and genetic studies in *V. dahliae*, we focus on the development of an efficient protein *in situ* tagging method in *V. dahliae*. We harnessed the endogenous homologous recombination machinery of *V. dahliae* to integrate specific tags at precise genomic loci, enabling the *in situ* C-terminal tagging of proteins of interest. This approach ensures the accurate placement of tags within the genome, thereby optimizing the fidelity of protein tagging.

To leverage the homologous recombination-based *in situ* tagging approach in *V. dahliae*, it is important to utilize a highly efficient transformation system, that can accurately introduce homologous DNA fragments into the host cells. A highly efficacious *Agrobacterium tumefaciens*-mediated transformation (ATMT) system for gene targeting in *V. dahliae* has been established (Wang et al., 2016), considering the critical role of ATMT in gene targeting across numerous filamentous fungi (Santhanam et al., 2012; Kramer et al., 2021). ATMT, an inherently biological process originally designed for transferring genes in plants, offers several distinct advantages over alternative methods, including (1) high transformation efficiency; (2) the capacity to introduce large DNA fragments with precision; and (3) the introduction of genes at a low copy number. Despite these benefits, ATMT is not without its limitations, which include: (1) occasional occurrences of multi-copy insertions; (2) a time-intensive transformation process; and (3) an inherently limited host range. To mitigate non-specific insertions during the transformation process, the implementation of the herpes simplex virus thymidine kinase (HSVtk) as a negative selection marker has been proposed, which has been shown to enhance the specificity of the transformation (Wang et al. 2016).

This study centered on two proteins: VdCf2 (*VDAG-08721*), which has been documented to localize to the nucleus (Liu et al., 2022), and another protein VdDMM2 (*VDAG-00626*), a candidate for nuclear localization. We developed an *in situ* tagging strategy to append GFP and Flag tags to the C-terminus of VdCf2 and VdDMM2, respectively, within the *V. dahliae* V592 strain. The resultant constructs, VdCf2-GFP, VdCf2-Flag, VdDMM2-GFP, and VdDMM2-Flag, were confirmed for

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genomic integration *via* PCR and sequencing. Confocal microscopy facilitated the visualization of the subcellular localization of the native GFP-tagged proteins, while the functionality of the Flag-tagged proteins was confirmed through Western blot analysis using anti-Flag antibodies, thus validating the efficacy of the *in situ* Flag-tagging approach.

2. MATERIALS and METHODS

2.1. Fungal and Bacterial Strains.

Verticillium dahliae strain V592， provided by Huishan Guo's Lab (isolated from cotton originating in Xinjiang, China, Gao et al., 2010)，was the fungal specimen utilized in this study. *Escherichia coli* strain DH5α was employed for plasmid propagation. For fungal transformation, the *Agrobacterium tumefaciens* strain EHA105 was used. Transformants were incubated and selected on Potato Dextrose Agar (PDA) medium, supplemented with 5-fluoro-2′deoxyuridine (5FU) at a concentration of 20 μg/L and hygromycin B at 50 μg/ml. Competent cells of *Escherichia coli* DH5α and *Agrobacterium tumefaciens* EHA105 were sourced from Beijing Qingke Biological Company.

2.2. Vectors.

The foundational vector, pGKO-HPT, provided by Huishan Guo's Lab containing the herpes simplex virus thymidine kinase (*HSVtk*) negative selection marker, to inhibit ectopic integration and the hygromycin B resistance gene. GFP/Flag cassettes were PCR amplified from pHM1-GFP and pFA6a-3flag vector, respectively, and then cloned into the StuI-digested pGKO-HPT vector *via* homologous recombination, to generate the pGKO-tag-HPT vector for *in situ* tagging.

2.3. Construction and Transformation.

In situ tagging vectors were constructed by PCR-amplifying 1 kb upstream (5') and downstream (3') flanking sequences adjacent to the target protein's stop codon， excluding the stop codon itself. These sequences were then inserted into the PacI restriction cleavage sites flanking the tagging box (Flag or GFP) and the hygromycin resistance cassette within the pGKO-tag-HPT vector *via* homologous recombination. The completed *in situ* tagging vectors were then introduced into *Agrobacterium* strain EHA105 which was subsequently co-cultivated with the *V. dahliae* V592 strain. Transformants were selected on potato dextrose agar (PDA) plates supplemented with 5-fluoro-2′deoxyuridine (5FU) and hygromycin. Ultimately, the primer pairs V1/Hpt-R and V2/Hpt-F were used to PCR confirm the successful integration of tags for target proteins in *V. dahliae* transformants.

2.4. Confocal Laser Scanning Microscopy (CLSM).

V. dahliae conidia grown on Potato Dextrose Agar (PDA) medium were harvested and rinsed once with double distilled water (ddH_2O), to remove any residual medium. A suitable volume of the conidial solution was placed on a silicified glass slide, air-dried, and then sealed with a DAPI dye solution. After a 10-minute incubation, samples were examined under a Leica TCS SP8 confocal microscope. GFP fluorescence was detected at an excitation wavelength of 488 nm, while DPAI-stained nuclei were visualized at 350 nm.

2.5. Protein Detection via Western Blotting.

Spores of *V. dahliae* were harvested and washed with PBS and then resuspended in 3ml of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton, 1×protease inhibitor cocktail). The cell suspension jelly was fast-frozen in liquid nitrogen and pulverized with a grinder (Xianfeng Scientific Instrument, 70 Hz, 1.5min, 3 times). The lysate was then diluted to 10 mL with lysis buffer and incubated with 150 μL Flag M2 agarose slurry (Sigma, A2220) at 4 ℃ overnight. The resin was washed three times with 1 mL TBS buffer each, and the tagged proteins were eluted with 100 μL 0.1M glycine-HCl (pH2.5) for 5-10min. The eluate was neutralized with 7.2 μL Tris-HCl (pH 8.0). Detection

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of VdCf2-Flag and VdDMM2-Flag was performed using Western blot analysis with anti-Flag antibodies (Sigma, F3165).

All primers used are listed in Supplementary Table S1.

3. RESULTS

3.1. The General Strategy for in situ C-terminusTagging in V. dahliae

To develop an *in situ* tagging system for *V. dahliae via* ATMT, our initial step was to engineer appropriate vectors. The foundational vector, pGKO-HPT, was equipped with a herpes simplex virus thymidine kinase (*HSVtk*) negative-selection marker to curtail ectopic integration and confer hygromycin B resistance for selection. We modified this vector by inserting the desired tag upstream of the HPT gene *via* homologous recombination, thus creating the pGKO-tag-HPT vector for protein tagging. Using this strategy, we synthesized the pGKO-Flag-HPT and pGKO-GFP-HPT vectors, containing the Flag and GFP tags, respectively (Figure 1A).

To generate the *in situ* tagging vector for a specific protein, we PCR-amplified the 1 kb upstream (5') and downstream (3') flanking sequences adjacent to the target protein's stop codon, excluding the stop codon itself, for insertion into the PacI restriction cutting sites flanking the tagging box (Flag or GFP) and the hygromycin resistance cassette within the pGKO-tag-HPT vector. To ensure proper orientation, the primers were designed to include the flanking 23bp homology sequence, with the PacI site sequence for both sides. The assembled *in situ* tagging vectors were then introduced into *Agrobacterium* strain EHA105. Following co-cultivation with the V592 strain cells of *V. dahliae*, the positive transformants were selected on potato dextrose agar (PDA) plates supplemented with 5-fluoro-2′deoxyuridine (5FU) and hygromycin B (Figure 1B). The ultimate result is the *in situ* integration of the tag and the hygromycin-resistant cassette at the C-terminus of the target protein, mediated by *V. dahliae*'s intrinsic homologous recombination system (Figure 1C).

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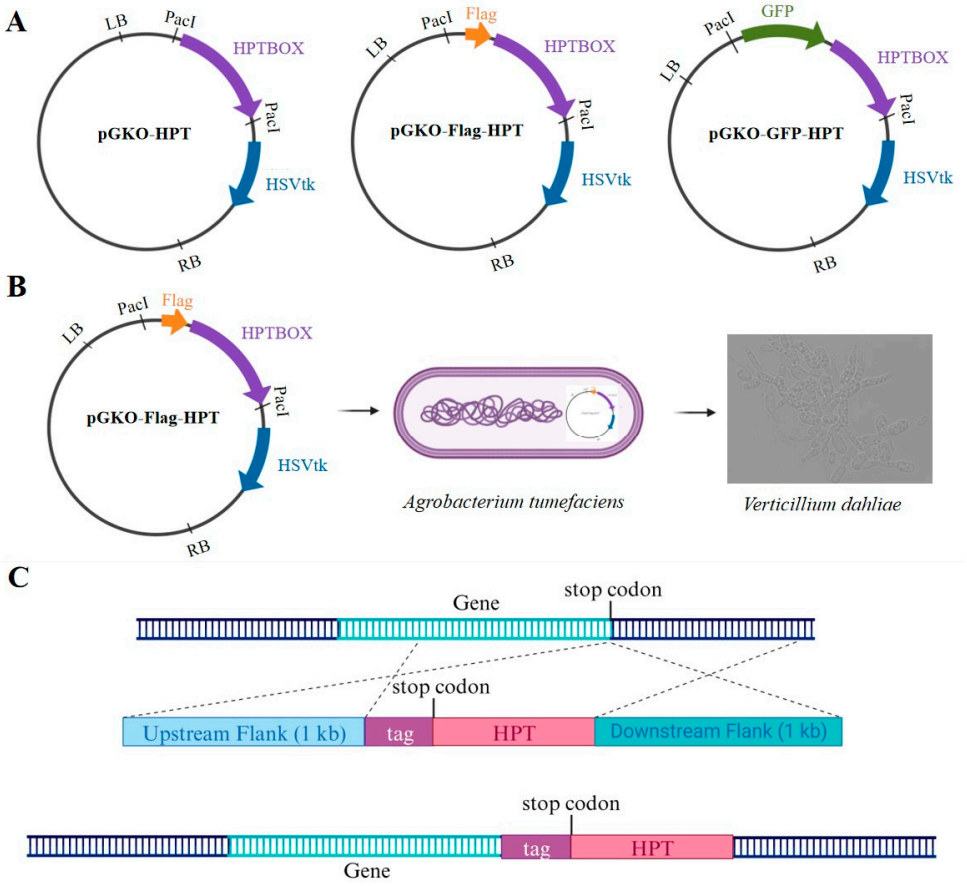

Figure 1. Overview of the *in situ* Protein Labeling Methodology. (A) Construction of the tagging vectors utilized in this study. (B) Stepwise protocol for *Agrobacterium tumefaciens*-mediated transformation (ATMT). (C) Schematic of the design strategy for *in situ* tagging and the corresponding genomic integration resulting from the process.

3.2. In situ GFP Tagging of VdCf2 and VdDMM2 using pGKO-GFP-HPT vector

To assess the capability of the pGKO-GFP-HPT vector in mediating *in situ* GFP tagging of specific proteins, we engineered the *in situ* tagging constructs for *VdCf2* and *VdDMM2*, incorporating the flanking sequences both upstream and downstream of the respective genes. Using *VdCf2* as a representative example, we commenced by synthesizing primer pairs VdCf2-G1 / VdCf2-G3 and VdCf2-G4 / VdCf2-G2 (Table S1) to PCR amplify the 5′ and 3′flanking regions respectively based on the reference genome sequence of *V. dahliae* strain *VdLs.17* (Klosterman et al., 2011) (Figure 2A). The pGKO-GFP-HPT vector was subjected to PacI digestion to liberate the vector backbone along with the GFP-HPTBOX. Subsequent to this step, the amplified flanking sequences (Figure 2 B) were seamlessly integrated into the PacI sites through homologous recombination, generating the pGKO-VdCf2-GFP-HPT construct (Figure 2C). Verification of the construct was meticulously performed using PCR with the primer pairs pGKO-seq-F / Hpt-R and Hpt-F / pGKO-seq-R (Figure 2C and Table S1). Upon confirmation, the resultant *in situ* tagging vectors were finally introduced into *V. dahliae via* ATMT, as described above. The methodology adopted for the construction of the *VdDMM2 in situ* tagging vector mirrored that of *VdCf2*, ensuring consistency in our approach.

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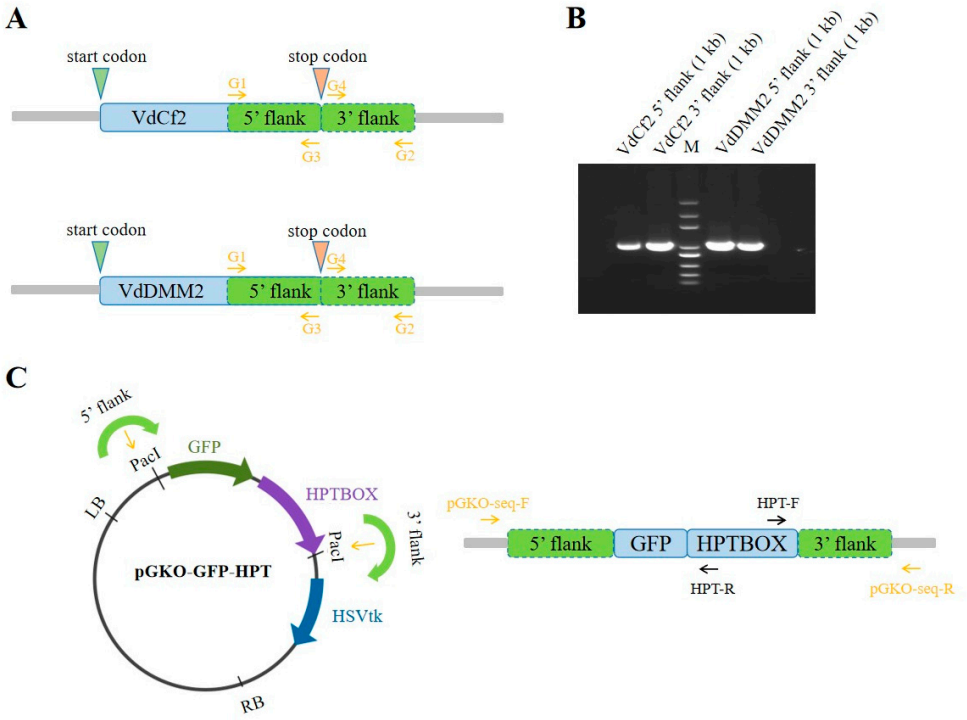

Figure 2. Construction of *in situ* GFP Tagging Vectors for *VdCf2* and *VdDMM2*. (A) Schematic of primer design specific to the *in situ* GFP tagging approach. (B) Agarose gel electrophoresis confirmation of the PCR-amplified 5′ and 3′ flanking regions of insertions at *VdCf2* and *VdDMM2* gene locus. (C) Schematic of the *in situ* GFP tagging vector construction, including the primer sets within the HPTBOX, employed for validating the integration of 5′ and 3′ flanking sequences into the pGKO-GFP-HPT vector.

3.3. Molecular Confirmation of C-terminal GFP Tagging for VdCf2 and VdDMM2 in V. dahliae

To confirm the successful *in situ* GFP tagging of *VdCf2* transformants, we isolated the genomic DNA from the candidate transformants (Figure 3A), and then designed the verification primer pair, to bind approximately 200 bp upstream of 5' flanking sequence (referred to as VdCf2-V1) and 200 bp downstream of 3' flanking sequence (referred to as VdCf2-V2) respectively. The primer pair VdCf2-V1 / Hpt-R was utilized to ascertain the insert of the correct upstream integration site, while the VdCf2-V2 / Hpt-F primer pair was applied for downstream validation (Figure 3B and Table S1). The methodology adopted for the verification of the *VdDMM2 in situ* GFP tagging was similar to *VdCf2*. Our PCR verification results demonstrated a remarkably high efficiency of *in situ* GFP tagging for *VdCf2* and *VdDMM2*, with success rates of 100% and 90% respectively (Figure 3C). To further validate the fidelity, sequencing of PCR products from the positive transformants was conducted, confirming the accurate integration of the *in situ* GFP tags within the *VdCf2* and *VdDMM2* genes.

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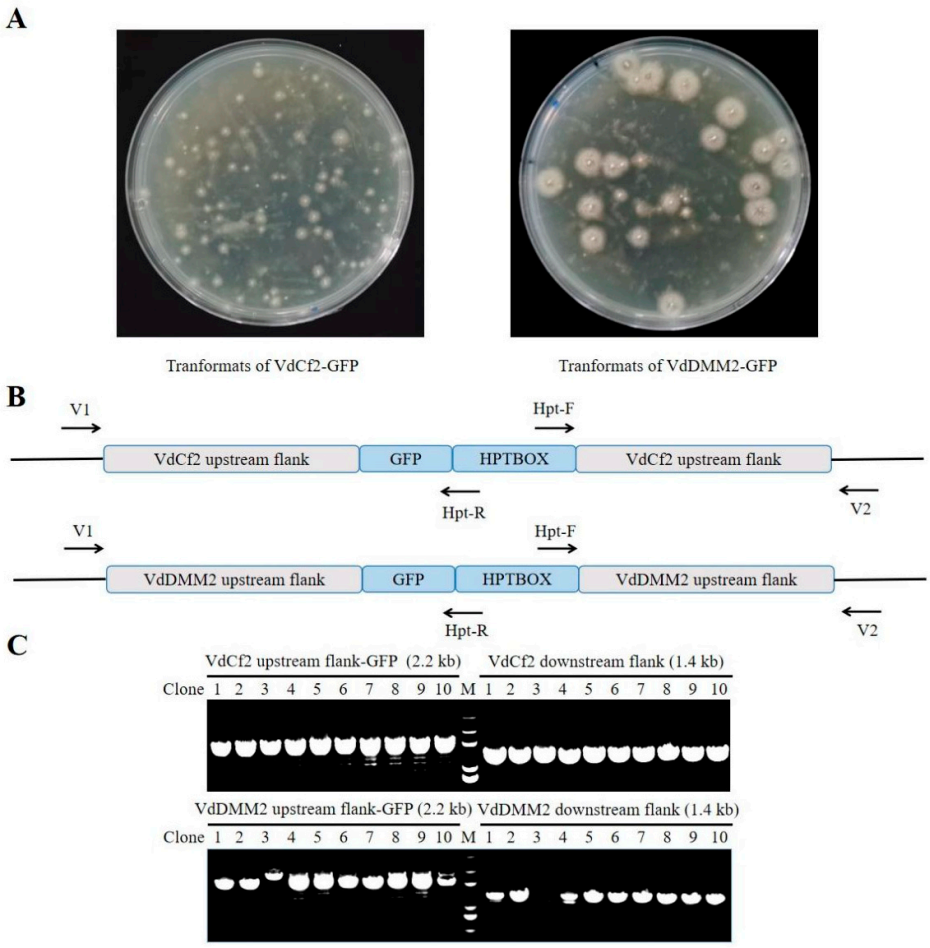

Figure 3. The Verification of *in situ* GFP Tagging for *VdCf2* and *VdDMM2* in *V. dahliae*. (A) Transformants of VdCf2-GFP and VdDMM2-GFP grown on potato dextrose agar (PDA) plates with 5-fluoro-2'deoxyuridine (5FU) and hygromycin B. (B) Schematic of verification primer pairs specifically designed for the assessment of successful tagging. (C) PCR validation results for demonstrating the successful *in situ* GFP tagging of *VdCf2* and *VdDMM2*, utilizing primer pairs V1 / Hpt-R, and V2 / Hpt-F.

3.4. Subcellular-localization for in situ Tagged Endogenous VdCf2-GFP and VdDMM2-GFP in V. dahliae

To explore the subcellular localization of VdCf2-GFP and VdDMM2-GFP in *V. dahliae*, we observed the *in situ* tagged VdCf2-GFP and VdDMM2-GFP proteins within *V. dahliae* cells using a confocal microscope (Leica TCS SP8). Previous studies have indicated nuclear localization for both proteins. Hence, we employed 4',6-diamidino-2-phenylindole (DAPI) staining to facilitate the identification of the nuclei in the cells. Confocal microscopy revealed distinct bright green foci within the positive transformants (Figure 4), indicative of the successful GFP-tagging of the VdCf2 and VdDMM2 proteins. Notably, colocalization of the GFP fluorescence with the DAPI-stained nuclei was observed (Figure 4), conclusively demonstrating that the endogenous VdCf2-GFP and VdDMM2-GFP fusion proteins indeed localized to the nucleus.

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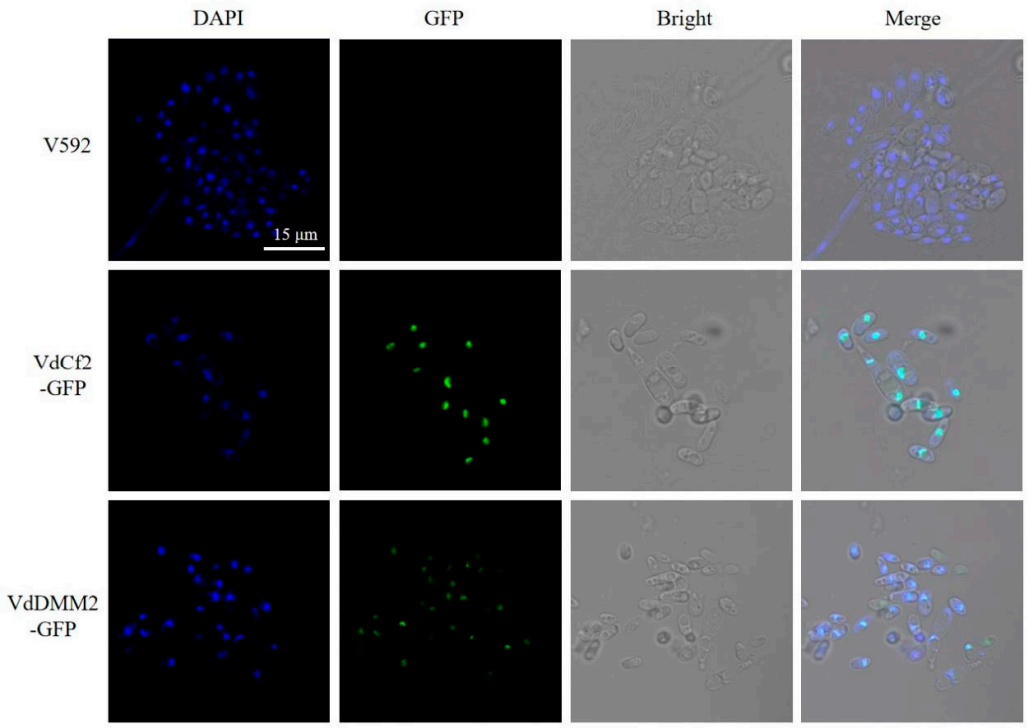

Figure 4. The Subcellular Localization for *in situ* GFP-tagged *VdCf2* and *VdDMM2* in *V. dahliae* cells. The fluorescence imaging of VdCf2-GFP and VdDMM2-GFP under confocal microscope (Leica TCS SP8). Scale bar = 15μm. .

3.5. Assessment of Versatility for in in situ Tagging System within V. dahliae

To evaluate the broad applicability of our *in situ* tagging strategy for the incorporation of various epitope tags into *V. dahliae* proteins, we extended our approach to include the Flag epitope. Utilizing the pGKO-Flag-HPT vector, we performed *in situ Flag* tagging for the *VdCf2* and *VdDMM2* genes, mirroring the methodology employed for GFP tagging. The procedure entailed the integration of upstream and downstream flanking sequences adjacent to the Flag tag and HPT selection marker, followed by transformation into *V. dahliae.* To molecularly verify the transformants (Figure 5A), we also designed the verification primers V1 and V2, which were paired with Hpt-R and Hpt-F respectively for PCR (Figure 5B and Table S1). And the products were then sequenced for further verification. The efficiency of generating VdCf2-Flag and VdDMM2-Flag tagged strains was still impressively high, with success rates of 100% and 80% , respectively (Figure 5C).

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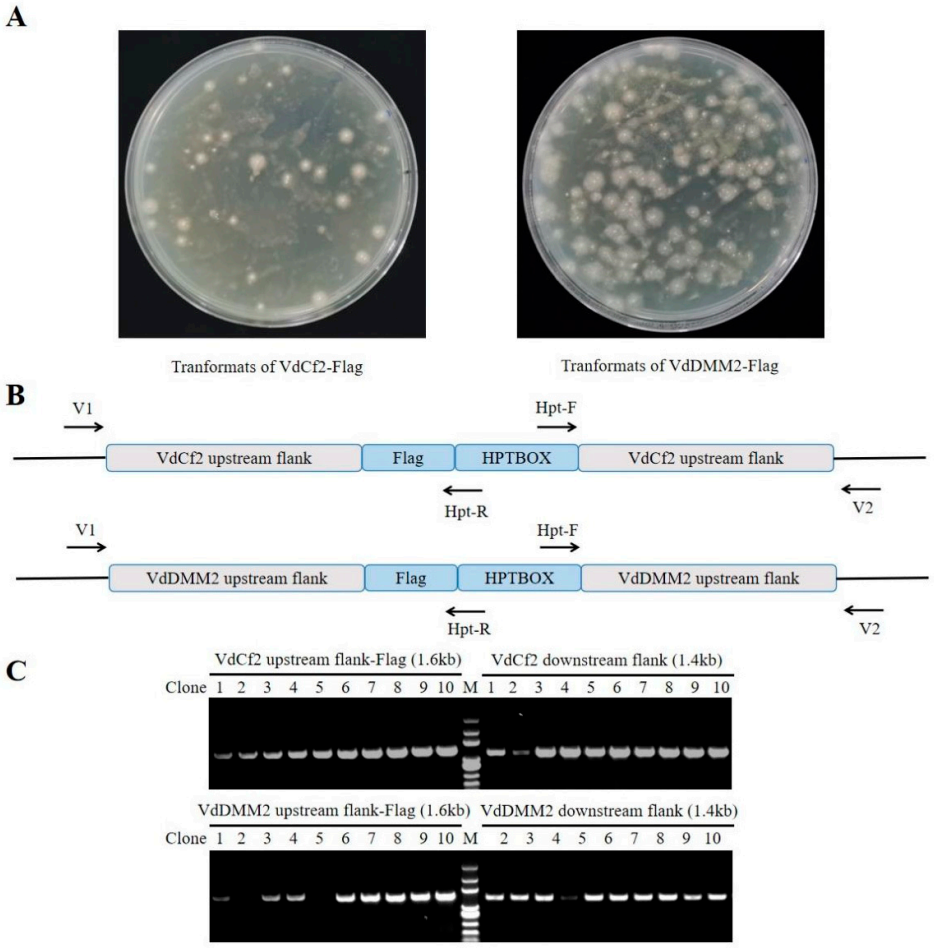

Figure 5. The Verification of *in situ* Flag Tagging for *VdCf2* and *VdDMM2* in *V. dahliae*. (A) Transformants of VdCf2-Flag and VdDMM2-Flag grown on PDA plates with 5FU and hygromycin B. (B) Schematic of verification primer pairs specifically designed for the assessment of successful tagging. (C) PCR validation results for demonstrating the successful *in situ* Flag tagging of *VdCf2* and *VdDMM2*, utilizing primer pairs V1 / Hpt-R, and V2 / Hpt-F.

3.6. Validation of Endogenous VdCf2-Flag and VdDMM2-Flag Fusion Proteins in V. dahliae by Western Blot Analysis

To confirm the *in vivo* expression of Flag-tagged VdCf2 and VdDMM2 fusion proteins in *V. dahliae*, we isolated proteins from positive transformants expressing VdCf2-Flag and VdDMM2-Flag, using anti-Flag agarose beads (Sigma, A2220). Subsequent Western blot analysis was conducted utilizing an anti-Flag antibody (Sigma, F3165), to detect the presence of the fusion proteins. The resulting immunoblots revealed distinct bands corresponding to VdCf2-Flag and VdDMM2-Flag, thereby verifying their successful expression in the transformants. In contrast, no such bands were detected in the wild-type strain, confirming the specificity of the Flag-tagging and the absence of endogenous Flag-tagged proteins.

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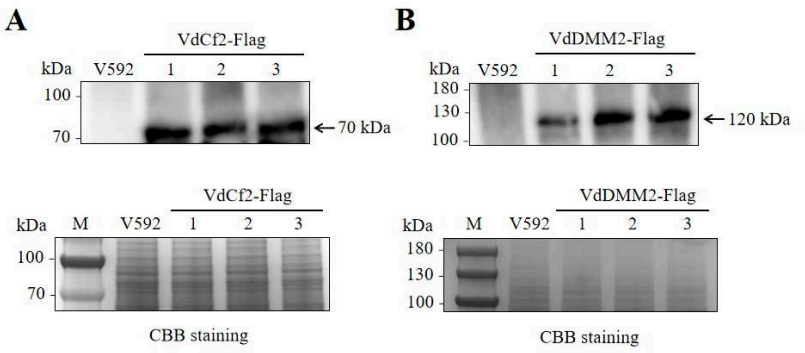

Figure 6. Western Blot Analysis of *VdCf2* and *VdDMM2 in situ* Flag tagging in *V. dahliae*. (A)Western blot confirmation of VdCf2-Flag protein expression, with total protein loading visualized by Coomassie brilliant blue (CBB) staining. (B) Western blot confirmation of VdDMM2-Flag protein expression, with total protein loading visualized by CBB staining.

4. DISCUSSION

The soilborne fungal pathogen *V. dahliae* presents a significant challenge to agriculture due to its high virulence, broad host range, and the difficulty associated with its management. In recent years, Host-induced gene silencing (HIGS), a technique derived from trans-kingdom RNA interference (RNAi), has emerged as an innovative strategy for the mitigation of *V. dahliae* infections (Hua et al., 2018). This approach hinges on the identification and utilization of fungal genes that are pivotal for its growth and pathogenicity, underscoring the importance of functional genomics in the control of this pathogen.

Protein tagging is a cornerstone in the elucidation in the study of gene function and localization. Currently, A suite of gene tagging methodologies has been developed and refined, including homologous recombination (Davidson et al., 2002), transposon tagging (Vandenbussche et al., 2013), CRISPR/Cas9-mediated endogenous gene tagging (Wang et al., 2019) and epitope tagging (Barnard et al., 2008), each contributing valuable insights into fungal studies.

In the study of gene function within fungal systems, ectopic expression has been proven invaluable. For example, in *Saccharomyces cerevisiae*, researchers have extensively utilized the Yeast two-hybrid system for ectopic protein tagging, to decipher protein interactions. Following advances include the epitope tagging of each protein's open reading frame (ORF), facilitating the construction of a comprehensive library of hybrid proteins, to dissect protein-protein interactions (Uetz et al., 2000). While gene localization studies have predominantly relied on overexpression systems to yield more robust signals (Wu et al., 2023; Wen et al., 2023), the use of ectopic or overexpression tags is not without its detriments. Such approaches can inadvertently alter the native expression levels and functionality of the protein, disrupt complex assembly, lead to gene silencing, or even result in lethality of the cells. Therefore, the pursuit of more refined gene tagging methods that preserve the endogenous context of target genes, remains a critical objective for the accurate interpretation of protein function and interaction networks within *V. dahliae*.

In contrast to ectopic or overexpression of proteins, *in situ* tagging offers a methodological advantage by maintaining the native levels of endogenous proteins. This method has been successfully implemented in various model fungal organisms, including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Miao et al., 2023; Khmelinskii et al., 2011; Shan et al., 2016; Shan et al., 2021), setting a precedent for its application in fungal study. Despite its proven efficacy in model systems, the adoption of *in situ* tagging system in plant pathogenic fungi, particularly in *V. dahliae*, remains scant. This situation significantly constrains our capacity to study the structural, functional, and physiological aspects of this pathogen. The integration of *in situ* tagging methods into the research toolkit for these filamentous pathogen fungi represents a formidable approach to elucidate the localization, interactions, and dynamic behavior of intracellular molecules. Its application is poised to yield new insights into the cellular biology of *V. dahliae*, thereby advancing our

understanding of its pathogenic mechanisms and informing the development of novel control strategies.

In this study, we validated the effectiveness of the homologous recombination-based *in situ* tagging method in the filamentous pathogen fungus *V. dahliae* V592, exemplified by the successful tagging of *VdCf2* and *VdDMM2* with Flag and GFP, respectively. After *in situ* tagging, the resultant fusion proteins were expressed at normal levels within *V. dahliae*, and their intracellular localization was consistent with previously reported observations. The application of Flag tagging, possibly followed by affinity purification and mass spectrometry analysis, has set the stage for probing the interacting partners of these proteins, thereby facilitating a deeper understanding of protein complexes, which is a pivotal aspect of functional proteomics. Moreover, this methodology is not confined to Flag or GFP tags. It also enables the integration of alternative tags or inducible promoters into *V. dahliae via* homologous recombination. This advancement is particularly useful as it provides a strategy to surmount the inherent autofluorescence often encountered in fungal cells, and it paves the way for the regulable expression of target proteins.

Looking ahead, future work should concentrate on enhancing the efficiency of transformation techniques, given the intricacy and laborious nature of ATMT for *V. dahliae*. Additionally, the expansion of the tagging types is imperative to fully harness the capabilities of this method and to propel the functional genomics of *V. dahliae*, or other plant pathogens into new frontiers.

Author Contributions: J.Y.: methodology; investigation; data curation; formal analysis; writing—original draft; writing—review and editing.; **M.L.**: methodology; investigation; data curation; formal analysis; writing—original draft; writing—review and editing.; **Y.J.**: resources; investigation; data curation. ; **H-S.G.:** supervision; funding acquisition; resources; writing—review and editing.; **C-M.S.**: Conceptualization; supervision; funding acquisition; methodology; resources; writing—original draft; writing—review and editing.; **H.W.**: Conceptualization; supervision; project administration; methodology; resources; investigation; data curation; writing—original draft; writing—review and editing.

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