Convergent and distinctive functions of transcription factors VdYap1, VdAtf1, and VdSkn7 in the regulation of nitrosative stress resistance, microsclerotia formation, and virulence in *Verticillium dahliae*

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Abstract

Reactive oxygen/nitrogen species (ROS/RNS) play a fundamental role in plant–fungal interactions. How pathogenic fungi manipulate plant-derived ROS/RNS is of importance to the outcomes of these interactions. In this study, we explored the individual and combined contributions of three transcription factors, VdAtf1, VdYap1, and VdSkn7, in the response to ROS/RNS, microsclerotia formation, and virulence in the plant wilt pathogen *Verticillium dahliae*. We showed that VdYap1 is essential for ROS response. Additionally, mutants lacking any combination of the three genes shared significant hypersensitivity to nitro-oxidative stress like sodium nitroprusside dehydrate and double deletions lacking VdYap1 and VdAtf1 resulted in further increased sensitivity to ROS. Double deletion of VdAtf1 and VdSkn7 reduced melanin production and virulence while simultaneous lack of VdSkn7 and VdYap1 disrupted nitrogen metabolism and ROS resistance. Finally, comparison of transcriptional profiles of the respective single or double mutants in response to nitro-oxidative stress revealed that the three transcription factors are involved in denitrification of nitrated alkanes and lipids to protect against nitro-oxidative stress. Taken together, our results demonstrate convergent and distinctive functions of VdYap1, VdAtf1, and VdSkn7 in *V. dahliae*, and provide new data on their roles in response to ROS/RNS in fungi.

**KEYWORDS**
nitrosative stress, VdAtf1, VdSkn7, VdYap1, *Verticillium dahliae*, virulence

1 | **INTRODUCTION**

Vascular wilt disease caused by *Verticillium dahliae* impacts over 200 plant species worldwide and results in losses of agricultural production, as well as damage to ornamental plants and ecological habitats (Klosterman et al., 2009). In China, *V. dahliae* has not only posed a major threat to crop production, but has also taken a heavy toll on ornamental perennials like smoke trees (*Cotinus coggygria*), the leaves of which provide beautiful scenery at the Fragrant Hills Park in Beijing, China (Wang et al., 2013). *V. dahliae* penetrates plants through the roots via infectious hyphae that germinate from the long-lived soilborne microsclerotia (Fradin and Thomma, 2006; Klosterman et al., 2009). After penetration, the fungus continues to proliferate in the xylem, resulting in characteristic plant wilt symptoms (Beckman et al., 1976).
During plant-pathogen interactions, plants rapidly generate reactive oxygen species (ROS) like hydrogen peroxide (H$_2$O$_2$) and reactive nitrogen species (RNS) like nitric oxide (NO) that are important as signalling molecules and antimicrobial substances to prevent pathogen invasion and spread (Hong et al., 2007). For example, V. dahliae toxins can generate NO and H$_2$O$_2$ from cotton suspension cells to induce host responses (Jia et al., 2007). These compounds not only mediate plant signalling for several physiological processes, but also give rise to oxidative and nitrosative stress (Besson-Bard et al., 2008; Corporas and Barroso, 2013). H$_2$O$_2$ production has many adverse biological effects on cellular macromolecules such as lipids, DNA, and proteins, and NO also causes damage through lipid peroxidation and S-nitrosylation of proteins, which impact cellular activities, ultimately leading to cell death (Apel and Hirt, 2004; Martinez and Andriantsitohaina, 2009; Das and Roychoudhury, 2014). For survival and colonization, pathogens must overcome H$_2$O$_2$ and NO stresses generated by plants (Segal and Wilson, 2018). Magnaporthe oryzae responds to host nitro-oxidative stress to suppress elevated ROS and host innate immunity, and RNS mitigation is connected to nitrogen metabolism (Marroquin-Guzman et al., 2017). The necrotrophic fungus Botrytis cinerea exhibits complex cross-talk of NO signalling with its plant hosts, and NO has important physiological consequences in the establishment of disease (Turrión-Gomez and Benito, 2011). However, the roles of NO during the interaction between V. dahliae and its plant hosts have not been wellstudied.

In some pathogens, such as Penicillium expansum and Colletotrichum linden, NO can inhibit fungal growth by inducing the generation of ROS, which subsequently causes severe oxidative damage (Wang and Higgins, 2005; Lai et al., 2011). To overcome ROS stress, plant pathogens deploy both ROS scavenging enzymes and nonenzymatic antioxidants (Heller and Tudzynski, 2011; Segal and Wilson, 2018). Several fungal transcription factors are involved in the regulation of ROS-responsive genes to resist an oxidative burst (Segal and Wilson, 2018). Among these transcription factors, Yap1, Atf1, and Skn7 are well studied in fungi and play important roles in oxidative responses (Moye-Rowley, 2003; Fassler and West, 2011). Aside from their involvement of oxidative resistance, Yap1 and Atf1 are also responsive to RNS stresses. For instance, VdAtf1 is critical to an appropriate response to NO stress by regulating the transcription of genes involved in NO detoxification in V. dahliae, and an FgAtf1 mutant strain of Fusarium graminearum displayed increased sensitivity to NO (Tang et al., 2020). In Schizosaccharomyces pombe, Atf1 accumulates in the nucleus in response to nitrosative stress to promote stress tolerance (Kar et al., 2018), while in Saccharomyces cerevisiae, Yap1 also accumulates in the nucleus under nitrosative stress and mediates the changes in gene expression that correlate with RNS-induced responses (Horan et al., 2006; Lushchak et al., 2010). Nevertheless, little is known about how Skn7 homologs mediate responses to RNS.

There are examples of overlapping or synergetic functions of Atf1, Yap1, and Skn7-related homologs in fungi. FgAtf1 and FgSkn7 have overlapping functions in vegetative growth, sexual and asexual reproduction, stress responses, and pathogenesis in F. graminearum (Jiang et al., 2015). Both Atf1 and Pap1 (a fission yeast AP-1-like transcription factor) protect S. pombe against exogenous acute oxidative stress caused by glucose depletion (Madrid et al., 2004). In Cochliobolus heterostrophus, ChAP1 and Skn7 both function to regulate virulence and gene expression in response to oxidative stress (Shalaby et al., 2014). Nevertheless, it is not known whether these ROS-responsive transcription factors have synergistic or overlapping effects with NO stress responsiveness.

VdAtf1 is essential to virulence and nitrogen metabolism but not H$_2$O$_2$ resistance (Fang et al., 2017; Tang et al., 2020) and VdSkn7 regulates microsclerotial development, H$_2$O$_2$ responsiveness, and virulence in V. dahliae (Tang et al., 2017). The aim of the present study was to systematically integrate the role of the three transcription factors VdSkn7, VdAtf1, and VdYap1 in nitrosative stress resistance, melanized microsclerotia formation, and virulence of V. dahliae. To investigate overlapping functions, a set of strains lacking single genes individually or double mutants of these genes were generated. Our results demonstrate that VdYap1 regulates ROS tolerance and that VdYap1 has overlapping functions with VdSkn7 in regulating nitrogen metabolism. VdSkn7 and VdAtf1 coregulate melanin formation and nitrogen metabolism. RNA-Seq analyses of NO stress responses verified the overlapping and the distinct functions of the three transcription factors. Overall, the findings revealed convergent and distinctive functions of VdYap1, VdAtf1, and VdSkn7 in microsclerotial development, ROS/RNS responses, and virulence of V. dahliae, and shed light on the regulation of these functions in response to ROS/RNS in fungi.

2 | RESULTS

2.1 | The three V. dahliae transcription factors VdYap1, VdAtf1, and VdSkn7 differentially contribute to full virulence

The V. dahliae VdLs.17 genome (Klosterman et al., 2011) embraces VDAG_08676, VDAG_02250, and VDAG_01588, which are the orthologs of the transcription factors Atf1, Skn7, and Yap1 in yeast, respectively. Thus, these orthologs were named VdAtf1 (Fang et al., 2017), VdSkn7 (Tang et al., 2017), and VdYap1 in this study. To systematically reveal functions of these genes, single (ΔVdYap1, ΔVdAtf1, and ΔVdSkn7) or double deletion mutants (ΔVdYap1ΔVdAtf1, ΔVdYap1ΔVdSkn7, and ΔVdAtf1ΔVdSkn7) were generated herein or previously (Table 1). Successful gene deletion transfectants were confirmed by PCR and Southern blot (Figure S1). We chose one deletion strain from several putative mutants per deletion group for further experiments.

Pathogenicity assays on 1-year-old smoke tree seedlings were performed to evaluate their roles in virulence. Plants infected by the wild-type strain XS11 exhibited typical verticillium wilt disease symptoms, with a relative disease index of 61% (Figure 1a,b). Interestingly, plants infected with ΔVdYap1, ΔVdYap1ΔVdAtf1, and ΔVdYap1ΔVdSkn7 strains exhibited no distinguishable difference from those of the XS11 strain, whereas the double deletion
of VdSkn7 and VdAtf1 clearly reduced the relative disease index by more than half (25%). Moreover, the ΔVdAtf1ΔVdSkn7 strain was 11% lower than that of the ΔVdSkn7 strain but similar to that of the ΔVdAtf1 strain (Tang et al., 2017, 2020), indicating that the two transcription factors independently regulate pathogenicity. For further examination of the infection process, we used a cellophane membrane to observe infection structure formation. No differences were observed between any mutants and the XS11 strain in hyphopodia or penetration peg development (Figure 1c), and all strains penetrated the cellophane membrane at the same time (Figure 1d). These results suggest that VdYap1 is dispensable to virulence and VdSkn7 and VdAtf1 seem not to coregulate virulence in V. dahliae.

### 2.2 Deleting VdYap1 attenuates microsclerotia formation

At the later stage of the V. dahliae disease cycle, microsclerotia are typically produced in dying plant tissues (Fradin and Thomma, 2006; Klosterman et al., 2009). A previous study revealed that VdSkn7 is required for microsclerotia formation (Tang et al., 2017). In this study, all strains were cultured on potato dextrose agar (PDA) for 7 days, and the XS11 strain formed darkly melanized regions of microsclerotia production (Figure 2a). The ΔVdYap1 and the ΔVdYap1ΔVdAtf1 strain also exhibited the melanized regions, though these were smaller than those observed for the XS11 strain. In contrast, microsclerotia were completely absent in the ΔVdYap1ΔVdSkn7 and ΔVdAtf1ΔVdSkn7 strains. Examination of microsclerotia formation was undertaken in these strains using cellulose membranes overlaid on basal medium (BM) and a sample of the culture was removed for microscopic observations. Both the ΔVdYap1 and ΔVdAtf1 strains formed microsclerotia at 4 days postinoculation (dpi), similar to those formed in strain XS11, and formed mature black microsclerotia at 8 dpi, as did the ΔVdYap1ΔVdAtf1 strain (Figure 2b,c).

### TABLE 1 Fungal strains used in this study

| Strain name         | Description | Reference         |
|---------------------|-------------|-------------------|
| XS11                | Verticillium dahliae wild-type strain | Wang et al. (2013) |
| ΔVdAtf1             | Deleting VdAtf1 (VDAG_08676) in XS11 | Fang et al. (2017) |
| ΔVdSkn7             | Deleting VdSkn7 (VDAG_02250) in XS11 | Tang et al. (2017) |
| ΔVdYap1             | Deleting VdYap1 (VDAG_01588) in XS11 | This study         |
| ΔVdYap1ΔVdAtf1      | Deleting both VdYap1 and VdAtf1 in XS11 | This study         |
| ΔVdYap1ΔVdSkn7      | Deleting both VdYap1 and VdSkn7 in XS11 | This study         |
| ΔVdAtf1ΔVdSkn7      | Deleting both VdSkn7 and VdAtf1 in XS11 | This study         |

### FIGURE 1 Pathogenicity tests of the ΔVdYap1 strain of Verticillium dahliae.

(a) Smoke tree (Cotinus coggyria) seedlings were inoculated with 10⁶ spores/ml conidial suspension of the wild type XS11, ΔVdYap1, ΔVdYap1ΔVdAtf1, ΔVdYap1ΔVdSkn7, and ΔVdAtf1ΔVdSkn7 strains of V. dahliae. Controls (CK) were mock-inoculated with water. All seedlings were placed in the greenhouse, and mature plant symptoms were photographed at 35 days postinoculation (dpi). Thirty seedlings were inoculated per strain. Photographs of the analyses of discoloration of shoots inoculated with each strain were taken at 35 dpi. (b) The relative disease index of seedlings inoculated with all strains. Error bars represent the standard deviations based on 10 independent replicates. Asterisks indicate significant differences (**p < .01, *p < .05). (c) Observation of penetration peg development after 3 days. The penetration pegs (from the hyphopodia) are indicated by the black arrows. (d) Strains of V. dahliae were grown on minimal medium overlaid with a cellophane layer for 3 days and photographed (above), and the plates were further incubated for 3 days after removal of the cellophane layer and photographed (below).
reduced numbers of microsclerotia at 12 dpi (17% and 25% lower) and even at 30 dpi (18% and 33% lower) when compared with those of the ΔVdAtf1 and XS11 strains, respectively (Figure 2d,e). Among the remaining mutant strains, the ΔVdAtf1ΔVdSkn7 strain exhibited the slowest microsclerotial formation while the ΔVdYap1ΔVdSkn7 strain was similar in this regard to the ΔVdSkn7 strain. The number of microsclerotia in these strains was also reduced at 12 dpi, a decrease that remained significant at 30 dpi. After 30 days of culture, abundant mature melanized microsclerotia appeared in all strains except the ΔVdAtf1ΔVdSkn7 strain, the analysis of which revealed a large number of immature microsclerotia, which were light grey or hyaline in appearance (Figure 2f). Analyses of all strains in shake cultures for 12 days in liquid BM revealed that the XS11, ΔVdYap1, and ΔVdAtf1 strains formed melanized microsclerotia in similar colour and number while the ΔVdYap1ΔVdAtf1 strain showed the same black colour but reduced numbers of microsclerotia (Figure 2g). The ΔVdYap1ΔVdSkn7 strains formed microsclerotia at reduced numbers relative to the XS11 strain, and many of these were black like those of the wild type. The ΔVdAtf1ΔVdSkn7 strain formed the fewest microsclerotia and those observed were not melanized. These results indicate that VdYap1 contributes to regulation of mature microsclerotia formation and VdAtf1 and VdSkn7 would coregulate melanin formation.

2.3 | VdYap1 are involved in ROS resistance

To investigate the roles of VdYap1 with the VdAtf1 and VdSkn7 in ROS resistance, we examined the sensitivity of these strains to hydrogen peroxide (H$_2$O$_2$). We found that the ΔVdYap1 mutant was much more sensitive to H$_2$O$_2$ than that of XS11 treated with 15% and 30% H$_2$O$_2$ (Figure 3a,b). Furthermore, when double deletion mutants were treated with 15% and 30% H$_2$O$_2$, we found that the inhibition rate of the ΔVdYap1ΔVdAtf1 strain was 48% higher than that of the XS11 strain under 15% H$_2$O$_2$, and the ΔVdYap1ΔVdAtf1 strain did not grow under 30% H$_2$O$_2$. The ΔVdAtf1ΔVdSkn7 strain exhibited a 23% larger inhibition zone than that of the wild type XS11. However, the ΔVdYap1ΔVdSkn7 strain showed hypervulnerability when treated with 30% H$_2$O$_2$. Meanwhile, all strains were cultured on PDA on a glass slide, and hyphae were stained by 3,3'-diaminobenzidine (DAB) after culturing for 3 days for analysis of H$_2$O$_2$ degradation. All single and double mutants stained
red, while the XS11 strain did not (Figure 3c). This result indicated that double deletion of VdYap1 and VdSkn7 directly affected H$_2$O$_2$ tolerance in V. dahliae, and unlike the XS11 strain, these mutants failed to neutralize H$_2$O$_2$, suggesting that VdYap1, VdAtf1, and VdSkn7 share a role in the mediation of redox balance in V. dahliae.

### 2.4 All three transcription factors are required for RNS resistance

Our previous study demonstrated that VdAtf1 regulates nitrogen metabolism and NO resistance in V. dahliae (Tang et al., 2020). To examine the responsiveness of the other two transcription factors, we tested the sensitivity of all mutants to NO stress. In response to 5 and 10 mM sodium nitroprusside dehydrate (SNP) treatments, all of the mutant strains grew more slowly than the XS11 strain, and this was most evident in the ΔVdYap1 strain as its inhibition zones were 21% and 12% higher than that of the XS11 strain when treated with 5 and 10 mM SNP, respectively (Figure 4a,b). Interestingly, knockout of VdSkn7 also caused increased sensitivity to NO stress. The three double mutants showed significant hypersensitivity relative to the XS11 strain. The ΔVdAtf1ΔVdSkn7 strain exhibited lower sensitivity to NO stress than the ΔVdAtf1 and ΔVdSkn7 single mutants when treated with 5 mM SNP, but all double mutants exhibited no differences as compared to the single deletion mutant strains at the 10 mM SNP concentration (Tang et al., 2020). We also measured malondialdehyde (MDA) and nitrotyrosine (NT) levels in all strains because both are markers for the presence of high levels of RNS (Marroquin-Guzman et al., 2017). MDA and NT accumulated in the mycelia of all mutants compared to the XS11 strain (Figure 4c,d). This result illustrated that all three genes could regulate NO resistance and that they may have overlapping roles in NO resistance.
Comparative transcriptomic analysis of the XS11 strain exposed to NO stress

Strains ΔVdAtf1, ΔVdSkn7, and ΔVdYap1 exhibited sensitivity to NO stress, but how each of these genes influences this sensitivity is uncertain. Therefore, we performed whole transcriptome analyses of each V. dahliae mutant strain and the XS11 strain with or without 10 mM SNP treatment. Forty-two libraries, including all single and double mutants with or without NO stress treatment, as well as the strain XS11, were produced. After quality filtering, we obtained...
FIGURE 5  Analysis of differentially expressed genes (DEGs) in the wildtype XS11, ΔVdAtf1, ΔVdYap1, and ΔVdSkn7 strains of *Verticillium dahliae* in response to nitric oxide stress. (a) Bar chart illustration of gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichments \( (p < .01) \) of all DEGs expressed in the XS11 strain with sodium nitroprusside dehydrate (SNP) treatment compared to those from XS11 strain without SNP treatment. (b) The wildtype XS11, ΔVdSkn7, ΔVdYap1, ΔVdYap1ΔVdAtf1, ΔVdYap1ΔVdSkn7, and ΔVdAtf1ΔVdSkn7 strains of *V. dahliae* were grown for 10 days on complete medium and on defined minimal medium containing 1% (wt/vol) glucose (GMM) with 10 mM of the indicated sole nitrogen source \( \text{NO}_2^-, \text{NO}_3^- \) or \( \text{NH}_4^+ \) at 25°C. Photographs were taken after 10 days' incubation of the media. (c) Inhibition rates of the above colonies after 10 days' incubation. Error bars represent the standard deviation based on three independent replicates. Asterisks indicate significant differences (**\( p < .01 \), *\( p < .05 \))
a total of 1,887.96 million reads from the 42 libraries, with an average of 44.95 million reads per sample and ranging from 38.08 to 54.38 million reads in each sample (Table S2). In total, 77.72% of the reads were mapped to the *V. dahliae* VdLs.17 reference genome (Klosterman et al., 2011) and 9,901 of the predicted genes were expressed. A two-fold or greater change in expression and \( p < .05 \) was determined to be significant.

We identified the genes that were differentially expressed after NO stress in wild-type XS11 strain compared to the strain grown in the absence of stress (control), and analyses of these data revealed 656 genes up-regulated and 429 genes down-regulated. Gene ontology (GO) enrichment revealed five terms while the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment revealed two significant pathways mainly related to metabolism.
including pantothenate and CoA biosynthesis and nitrogen metabolism (Figure 5a).

The main inorganic nitrogen sources in nitrogen metabolism, such as nitrate (NO$_3^-$) and ammonium (NH$_4^+$), can also serve as by-products in NO metabolism, and NO is also a by-product produced in nitrate metabolism (Chen et al., 2013; Marroquin-Guzman et al., 2017). VdAtf1 is involved in nitrogen metabolism and NO response (Tang et al., 2020). Therefore, nitrogen utilization was tested in all strains. The growth on NO$_2^-$, NO$_3^-$, and NH$_4^+$ of the VdYap1 strain and the VdSkn7 strain was the same as that of the strain XS11 (Figure 5b,c) while the inhibition rate of the VdYap1 and VdSkn7 strain was higher than that of the XS11 strain (34%, 24%, and 25%, respectively) as well as the single mutant strains VdYap1 (35%, 32%, and 22%, respectively) and VdSkn7 (36%, 17%, and 15%, respectively). The VdYap1-VdAtf1 strain also exhibited reduced nitrogen utilization, like the VdAtf1 strain. Interestingly, the VdYap1-VdSkn7 strain restored the phenotype of the VdAtf1 strain, resulting in normal nitrogen metabolism. In summary, VdYap1 and VdSkn7 can coregulate nitrogen metabolism. However, deleting VdSkn7 in the VdAtf1 strain had no influence on nitrogen utilization, suggesting that VdSkn7 has a negative impact on nitrogen utilization and this ability requires the participation of VdAtf1.

2.6 | Differential gene expression individually regulated by VdAtf1, VdSkn7, and VdYap1 in response to NO stress

Because the three single mutants were sensitive to NO stress, we also wanted to identify genes dependent on their function under the nitrosative stress condition. Comparisons of gene expression in all strains with or without SNP treatment revealed 87 genes regulated by VdAtf1, 70 by VdSkn7, and 153 by VdYap1 under nitrosative stress (Figure 6a). The analysis showed that these genes were not expressed in the mutant strain with and without SNP treatment and in the XS11 strain grown in the absence of stress, but they were expressed in the XS11 strain with SNP treatment. Among these, 15 genes including VDAG_02945 (chromate transporter) and VDAG_05694 (copper chaperone for superoxide dismutase, CCS) were not expressed when any of the three transcription factors was knocked out, suggesting that the three transcription factors are equivalently important. In total, 19, 13, and 90 genes rely on the existence of VdAtf1, VdSkn7, and VdYap1, respectively, suggesting that VdYap1 has a major influence on NO stress response.

To further explore how the three transcription factors mediate NO stress responses in V. dahliae, we compared differentially expressed genes (DEGs) in the XS11 strain versus those of each single mutant strain, as shown in a Venn diagram (Figure 6b). GO enrichment analysis of down-regulated genes revealed membrane compartment enriched categories such as “integral component of membrane” (GO: 0016021) and “intrinsinc component of membrane” (GO: 0031224) in all single mutants, suggesting that the main changes regulated by all three transcription factors mostly happened on the membrane (Table S3). The terms related to catalytic activity and metabolic process were also shared in all single mutants despite different GO terms enriched in different mutants. Moreover, transcription processes such as “transcription, RNA-templated” (GO: 0006351), “RNA biosynthetic process” (GO: 0032774), and “nucleic acid-templated transcription” (GO: 0097659) were shared in the VdAtf1 and VdSkn7 strains, which seems to show both VdAtf1 and VdSkn7 repress transcription activities to respond to NO stress. Both VdSkn7 and VdYap1 repressed genes involved in “protein kinase activity” (GO: 004672). As for enrichments of significantly activated genes, “transmembrane transporter activity” (GO: 0022857) as well as “oxidoreductase activity” (GO: 0016491) were enriched in VdSkn7 strain, and 23 GO terms were significantly enriched in the VdYap1 strain, suggesting that VdYap1 up-regulates more genes that participate in NO stress responses and these genes mostly concentrate in nuclear part and ribonucleoprotein complex (Table S4).

KEGG pathway enrichment was also analysed. DEGs related to ATP-binding cassette (ABC) transporters and glycerol, serine, and threonine metabolism were enriched in all single mutant strains, revealing that their common points are repressing transporter activity and stimulating amino acid metabolism (Figure 6c,d). All of the transcription factors control genes involved in metabolism in response to NO stress, though diverse metabolic pathways were enriched in different mutants. DEGs were enriched in the meiosis pathway only after deletion of VdSkn7, indicating that VdSkn7 might respond to NO resistance via depressing meiosis. All data showed that VdYap1, VdAtf1, and VdSkn7 not only have distinct regulation patterns but also share the same pathways in response to NO stress.

2.7 | Transcriptomic profiles of the three double mutants VdAtf1 VdSkn7, and VdYap1 in response to NO treatment

To further probe the coregulation and overlapping functions of VdAtf1, VdSkn7, and VdYap1 in V. dahliae, gene expression in the double mutant strains VdAtf1 VdSkn7, and VdYap1 VdSkn7 was also examined. For comparison, we concentrated on genes expressed only in the XS11 strain treated with SNP rather than in the XS11 strain without treatment and other mutants, revealing 12 genes in the VdYap1 VdAtf1 strain, 23 in the VdYap1 VdSkn7 strain, and 14 in the VdAtf1 VdSkn7 strain that were only expressed when each set of the two transcription factors was present, illustrating the coregulatory activity of the transcription factors.

Comparisons of the DEGs in the XS11 strain versus every double mutant strain and the single mutants VdAtf1, VdSkn7, and VdYap1 is shown in the respective Venn diagrams (Figure 7a). Genes down-regulated in the VdAtf1 VdSkn7 strain were significantly enriched in seven GO terms belonging to biological process, including transcription and some biosynthetic processes (Table S5). Meanwhile, genes related to “localization” (GO: 0005179) that were down-regulated in the VdYap1 VdSkn7 strain were also covered by
the biological process. The ΔVdYap1ΔVdAtf1 strain down-regulated genes involved in molecular function such as "peptidase activity" (GO:0008233), "threonine-type peptidase activity" (GO:0070003), and "threonine-type endopeptidase activity" (GO:0004298). Among genes activated by knocking out double genes, GO enrichment revealed 11 GO terms in the ΔVdAtf1ΔVdSkn7 strain, including the organelle part and cellular component organization or biogenesis, and revealed four GO terms in the ΔVdYap1ΔVdSkn7 strain containing membrane part, which showed genes enriched in different locations in the two double mutants (Table S6). In the ΔVdYap1ΔVdAtf1 strain, only the GO term "oxidoreductase activity" (GO:0016491) was enriched. The data illustrate that VdAtf1 and VdSkn7 have a complex influence on the NO response, while VdAtf1 and VdYap1 affect NO resistance by employing genes related to ROS resistance.
Thirty up-regulated DEGs related to "oxidoreductase activity" were enriched in the ΔVdYap1ΔVdAtf1 strain while they were not significantly expressed in either the ΔVdYap1 strain or the ΔVdAtf1 strain, suggesting that VdAtf1 and VdYap1 coregulate these genes. After comparisons of up-regulated DEGs for oxidoreductase activity with the ΔVdSkn7 strain, the gene encoding a glycerol-3-phosphate dehydrogenase (VDAG_07740) was present in both lists, revealing that the three transcription factors have a complicated relationship in responding to NO stress. Ten DEGs related to "transcription, DNA-templated" and "nucleic acid-templated transcription" were enriched in the ΔVdAtf1 strain and the same 10 DEGs plus VDAG_00141 (RNA polymerase II transcription factor B subunit 5) were enriched in the ΔVdSkn7 strain (Figure 7b). Among them, seven of the same DEGs were enriched in the ΔVdAtf1ΔVdSkn7 strain. Six genes (VDAG_02961, VDAG_07886, VDAG_10045, VDAG_10428, VDAG_10430, and VDAG_10522) were highly expressed only when both VdAtf1 and VdSkn7 were present, and VDAG_00141 was expressed only in the presence of VdSkn7. Moreover, VDAG_03521 was remarkably expressed until both VdAtf1 and VdSkn7 were deleted. These data indicate that VdAtf1 and VdSkn7 play distinct and overlapping roles in NO response through genes related to nucleic acid biosynthesis.

Results of KEGG enrichment analyses were mixed for all three double mutant strains; none of the above pathways were enriched in the ΔVdYap1ΔVdSkn7 strain. Pathways enriched in the other two double mutants were mainly concentrated in KEGG pathways belonging to various metabolisms, and metabolism pathways in repression were more than those in stimulation (Figure 7c,d). In addition, enrichment of repressed genes in the ΔVdYap1ΔVdAtf1 strain included the terms of ABC transporters and protein export. All data demonstrated that VdYap1, VdAtf1, and VdSkn7 not only impact NO resistance, but also genetically interact with each other to regulate NO response.

In M. oryzae, Nmo2 (nitrate mono-oxygenase) is required for mitigating nitro-oxidative stress by repairing lipid damage either directly or indirectly from RNS (Marroquin-Guzman et al., 2017). We were then curious about whether the expression profile of VdNmo2 is linked to the responsiveness to NO stress in V. dahliae. The expression of VdNmo2 was up-regulated in all strains except the ΔVdYap1ΔVdSkn7 strain under NO stress (Figure 7e). The up-regulation of VdNmo2 in the ΔVdAtf1 and ΔVdSkn7 strains was lower than that of the XS11 and ΔVdYap1 strains, suggesting that deletion of VdAtf1 and VdSkn7 repressed the expression of Nmo2 while knock-out of VdYap1 induced the expression under NO stress compared with the XS11 strain.

3 | DISCUSSION

Fungi employ transcription factors to respond to many adverse environmental stresses, and the regulatory relationship among them is complicated. Herein, we found that VdYap1 is involved in ROS and RNS stress responses directly and contributes to microsclerotia development. Combined with our previous results (Tang et al., 2017, 2020), we demonstrated a general understanding of the overlapping and distinctive roles of three transcription factors, VdYap1, VdAtf1, and VdSkn7, in the regulation of nitrosative stress resistance, microsclerotia formation, and virulence in V. dahliae.

3.1 | Functional cooperation of VdYap1, VdAtf1, and VdSkn7 in V. dahliae

Atf1, Skn7, and Yap1 are typical ROS-responsive transcription factors whose mutual functional relationships in stress resistance have been extensively studied in fungi. In V. dahliae, we demonstrated that VdSkn7 is a key regulator of microsclerotia formation (Tang et al., 2017). In this study, these findings showed that VdYap1 and VdSkn7 both contribute to microsclerotia formation as well as H2O2 sensitivity, and VdAtf1 and VdSkn7 can both regulate virulence. These results suggest that the functions controlled by VdSkn7 are more diverse than the other two transcription factors, at least in relation to the few phenotypes examined. The phenotypes of double mutants were not more apparent than those of single mutants, revealing that these transcription factors have overlapping functions.

Apart from functional similarities, VdSkn7 interacts with VdYap1 to regulate nitrogen metabolism, and with VdAtf1 for melanin formation as well. VdAtf1 and VdYap1 also jointly affect the response to H2O2 stress in V. dahliae. In F. graminearum, glucose limitation in S. pombe induces oxidative stress, the detoxification of which depends on the cooperation of Pap1 and Atf1 (Madrid et al., 2004). Considering the oxidative stress response phenotypes of all strains in this study, it is difficult to explain the synergistic effect of VdYap1 and VdAtf1, though we may surmise two possible explanations. First,
some VdYap1-dependent genes essential for oxidative responses may also be activated by VdAtf1, in which case VdAtf1 is likely to partly offset oxidative sensitivity caused by deletion of VdYap1. In S. pombe, Atf1 can also regulate various Pap1-dependent genes containing those encoding catalase and sulfiredoxin responsive to oxidative stress (Chen et al., 2008; Veal et al., 2014). Another possibility is that VdAtf1 and VdYap1 combine to form a heterodimer for transcription under oxidative stress. They belong to the bZIP transcription factor family and the bZIP domains contain a leucine zipper dimerization motif regulating dimerization (Jakoby et al., 2002). In addition, c-Jun (Yap1 homolog) and ATF2 (Atf1 homolog) can form a heterodimer activating target gene expression to respond to many cellular and environmental signals in mammals (Liu et al., 2006).

### 3.2 Convergent and distinctive roles of VdYap1, VdAtf1, and VdSkn7 in RNS detoxification in V. dahliae

A high concentration of NO is toxic to cells by damaging proteins, lipids, and DNA directly or indirectly (Ischiropoulos and Gow, 2005). Fungi have developed detoxification strategies to counteract the damaging effects of NO and the ROS-responsive transcription factors also play protective important roles. For example, in S. pombe Atf1 is a key player for the initiation of the S-phase in the cell cycle, allowing a pause in replication to respond to nitrosative stress (Kar et al., 2018). In S. cerevisiae Yap1 is a key regulator in response to nitrosative stress by up-regulating expression of superoxide dismutase and catalase (Lushchak et al., 2010). The current work demonstrates that VdAtf1, VdYap1, and VdSkn7 all play roles in NO stress responses and RNA-Seq analyses indicated not only divergent but also shared regulation patterns.

The aberrant accumulation of MDA and NT in all mutants support the idea that the three transcription factors can denitrify nitrated alkanes and lipids to protect against nitro-oxidative stress. In M. oryzae, Nmo2 played a crucial role in coping with nitro-oxidative stress and the high concentration of MDA and NT may result from the misregulation of Nmo2 (Marroquin-Guzman et al., 2017). In this study, we found that deletion of VdAtf1 and VdSkn7 down-regulated expression of VdNmo2 under NO stress, and when combined with our previous results we can infer both transcription factors are likely to mediate lipid damage repair to resist NO stress (Figure 7e). VdYap1 failed to influence the expression of VdNmo2, suggestive of unimpaired lipid damage repair. Furthermore, DEGs in the ΔVdYap1 strain were enriched in metabolisms such as amino acid metabolism, lipid metabolism, and carbohydrate metabolism. We can infer that disturbances in metabolism may be responsible for NO sensitivity in the VdYap1 deletion mutant. In S. pombe, pathway categories of meiosis and spliceosome were most affected under nitrosative stress (Biswas and Ghosh, 2015). Herein, genes related to meiosis were only down-regulated in the ΔVdSkn7 strain and those involved in RNA transport and spliceosome were only down-regulated in the ΔVdAtf1 strain, suggesting a novel relationship between the Skn7 homolog and meiosis, and suggesting that VdAtf1 can participate in transcription by potentially regulating the spliceosome. All single mutants also regulated genes corresponding to the ABC transporter category and metabolism to respond NO stress. ABC transporters, being present in virtually every living cell from prokaryotes and eukaryotes, hydrolyse ATP to transport a wide variety of compounds like toxic metabolites across extra- and intracellular membranes, playing important roles in regulating various physiological processes (Del Sorbo et al., 2000). In humans, the three transporters ABCB1, ABCC1, and ABCC2 (pleiotropic drug resistance [PDR] subfamily) have significant roles in multiple drug resistance, and among fungal ABC transporters the PDR subfamily is the only one involved in clinical antifungal resistance as reported so far (El-Awady et al., 2017; Moreno et al., 2019). Thus, these types of genes encode products that can function in detoxification. When V. dahliae is exposed to NO stress, toxic metabolites would be induced because of disruptions to metabolism, requiring detoxification by transport out of the cell. Hence, VdAtf1, VdYap1, and VdSkn7 respond to NO stress by regulating ABC transporters.

Organisms have evolved subtle strategies to maintain redox balance within the cell for normal physiological activities (Segal and Wilson, 2018). Arbitrary changed expression of genes for oxidoreductase activity may cause cell damage owing to breaking the redox balance. Among genes related to oxidoreductase activity, VDAG_08462 was most strikingly up-regulated in the ΔVdSkn7 strain, encoding ornithine acetyltransferase (ArgJ) which is involved in the synthesis of l-arginine (Dou et al., 2011). Interestingly, stress-induced arginine production can be used for NO production (Nishimura et al., 2010). Therefore, the deviant activation of VdArgJ induced by NO stress not only leads to a disorder of arginine biosynthesis but also results in excess NO production, causing damage. Another gene (VDAG_07740) related to oxidoreductase activity encodes glycerol 3-phosphate dehydrogenase (GPD1), a key enzyme that plays an important regulatory role in the metabolism of glycerol (Albertyn et al., 1994). Single deletion of VdSkn7 or double deletion of VdAtf1 and VdYap1 leads to its up-regulation, causing abnormal glycerol accumulation. Over-accumulation of intracellular glycerol may cause cell swelling, thereby leading to various morphological defects and ultimately growth inhibition (Randhawa et al., 2019).

Taken together, our results demonstrate convergent and distinctive functions of VdYap1, VdAtf1, and VdSkn7 in V. dahliae. Because the primary structures of each of these proteins are well conserved in fungi, the results also provide new insight on the potentially conserved roles of each of these proteins in response to ROS/RNS in fungi.

### 4 EXPERIMENTAL PROCEDURES

#### 4.1 Fungal strain and culture conditions

The wild-type V. dahliae strain XS11 was isolated from a smoke tree in Fragrant Hills, Beijing (Wang et al., 2013) and was used as a recipient to generate the different mutant strains used in this study. Conidial suspensions of all strains were stored long term at ~80°C in 30% glycerol. Five-day-old vegetative hyphae collected from liquid...
compete medium (CM, 50 ml of 20 nitrate salts, 1 ml of 1,000 x trace element, 10 g glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acids, and 1 ml of vitamin solution per litre) were used for genomic DNA extraction. Selection of strains resistant to the antibiotics hygromycin (Hyg; Sigma) and geneticin (G418; Sigma) was carried out by plating the respective strains on PDA plates (200 g potato, 20 g glucose, 15 g agar per litre) amended with 25 μg/ml hygromycin or 50 μg/ml geneticin, respectively. All strains were grown from a single-spore culture on PDA at room temperature prior to experimentation, and mycelial masses used for inoculation were obtained using a punch (5 mm).

To test oxidative stresses, a 100 μl conidial suspension (10^7 spores/ml) of each strain was spread onto PDA plates, and filter paper discs containing 5 μl of 15% and 30% hydrogen peroxide (H_2O_2) were placed onto the centre of each plate. The zone of growth inhibition was measured after 2 days. To observe ROS production, a 100 μl conidial suspension of all strains were cultured on slides dipped on PDA at 25°C for 4 days. Observations were made under light microscopy (DM2500; Leica) after staining with 1 mg/ml 3,3′-diaminobenzidine (DAB; Sigma) solution (pH 3.8) in the dark for 8 hr at room temperature. For the nitrosative stress assay, all strains were grown for 10 days on CM amended with 5 or 10 mM NO donor sodium nitroprusside dehydrate (SNP; Sigma). As for growth tests on single nitrogen sources, all strains were cultured for 10 days on CM and defined minimal medium containing 1% (wt/vol) glucose (GMM, 1.52 g KH_2PO_4, 0.52 g KCl, 0.152 g MgSO_4.7H_2O, 3 μM thiamine.HCl, 1.98 g glucose, 15 g agar per litre) with 10 mM of these sole nitrogen sources including nitrate ([NO_3]_3), nitrite ([NO_2]_3), and ammonium ([NH_4]_3) at 25°C. The colony diameter was measured after 10 days. All the experiments were repeated three times.

To study microsclerotal production, conidia were harvested from cultures grown in liquid CM and 100 μl of a conidial suspension (10^6 spores/ml) was spread on a cellulose membrane (Whatman, 80 mm diameter; pore size 0.22 μm) that had been overlaid on solid basal medium (BM, 10 g glucose, 0.2 g sodium nitrate, 0.52 g KCl, 0.52 g MgSO_4.7H_2O, 1.52 g KH_2PO_4, 3 μM thiamine.HCl, 0.1 μM biotin, 15 g agar per litre), then these plates were placed in the dark at 25°C. The contents and concentrations of the components of the solid BM were provided by Dr Katherine Dobinson. University of Western Ontario, Ontario, Canada. Distinct developmental stages were observed under light microscopy (DM2500, Leica) after 4, 8, 12, and 30 days, similar to those described previously by Xiong et al. (2014). For melanin formation, a 100 μl conidial suspension (10^6 spores/ml) of each strain was added into liquid BM and shaken (150 rpm, 25°C) for 12 days. At 12 days, melanin formation was observed by an-
The restriction enzyme EcoRI was used to digest the genomic DNA extracted from the wild-type strain and double mutant strains.

### 4.3 Virulence assays

All strains were cultured in liquid CM medium for 5 days, then conidia were harvested by filtering through two layers of Miracloth (Millipore) and diluted to 10^6 conidia/ml by sterilized deionized water. Fifty 1-year-old smoke trees were root-dipped in conidial suspension and checked for morbidity with wilt symptoms. Shoots were cut after 35 days to observe vascular discoloration. Pathogenicity experiments were replicated three times.

### 4.4 Statistical analysis of melanized microsclerotia

The melanized area of the respective cultures was measured using ImageJ (Papadopulos et al., 2007) under the default settings (the threshold of all images was 178). Data were expressed as mean value ± standard error of the mean. Statistical analyses were performed using Student’s t test. The p value < 0.05 was considered statistically significant in this study.

### 4.5 Assays of malondialdehyde and nitrotyrosine levels

The mycelia of all strains were obtained after culturing for 3 days in liquid CM, and for 3 days in CM prior to switching to different treatments such as CM with 10 mM SNP for 24 hr, and ground to a fine powder. All biochemistry measurements were determined using the spectrophotometric procedure described by the manufacturer (Solarbio).

### 4.6 RNA-Seq and differential gene expression analysis

Fresh mycelia of all strains used for transcriptome profile analyses were grown in liquid CM at 25°C for 3 days before adding 10 mM SNP for 24 hr. The fungus was collected following one strain using a single-layer of Miracloth (Millipore). All of the fungal samples were ground to a fine powder using a mortar and pestle in liquid nitrogen. RNA extraction was performed using TRIzol reagent (Invitrogen) and purified with the RNA Mini Kit (Ambion) in accordance with the manufacturer’s instructions. After checking the quantity and quality of RNA using a NanoPhotometer spectrophotometer (Implen) and an RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies), three biological replicates were sequenced by a BGISEQ-500 platform (Beijing Genomics Institute). Raw reads were first processed using in-house Perl scripts and clean reads were obtained by removing reads containing adapter and poly-N sequences and low-quality reads from the raw data. Data filtering was performed using SOAPnuke software (Beijing Genomics Institute). The reads were aligned to the V. dahliae reference genome (Klosterman et al., 2011) using Burrows-Wheeler transform (Li and Dewey, 2011) to count the read numbers mapped to each gene and calculate gene expression. The assembled transcripts in the final transcriptome were annotated by mapping them to several public databases. The DESeq package (Wang et al., 2009) was used to provide statistical analyses for determining differential expression in digital gene expression data. Twelve libraries of deletion strains (ΔVdYap1, ΔVdAtf1, ΔVdSkn7, ΔVdYap1ΔVdAtf1, ΔVdYap1ΔVdSkn7, and ΔVdAtf1ΔVdSkn7) corresponding to the wild-type strain XS11 with and without SNP treatment were used to detect DEGs. Significantly expressed genes with a |log2(fold-change)| > 1 and p ≤ 0.05 were selected. The KEGG database (Hu et al., 2018) was used to identify enriched pathways by a two-tailed Fisher’s exact test to examine the enrichment of the differentially expressed individual gene products against all those identified. Significant enrichment was determined at p < 0.01. These pathways were classified into hierarchical categories according to the KEGG website (https://www.kegg.jp/kegg/kegg2.html).

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### AUTHOR CONTRIBUTIONS

Y.W. and C.T. conceived the experiments. C.T. and X.J. performed the experiments. C.T., Y.W., and S.J.K. analysed the data. C.T., Y.W., and S.J.K. wrote the manuscript. All authors read and approved the manuscript.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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