INTRODUCTION

Fungal infections on susceptible plants are facilitated by hundreds of secreted proteins, collectively known as effectors, that modulate host cell structure, metabolism, and function (Giraldo & Valent, 2013; Sanchez-Vallet et al., 2018). The success and degree of infection is influenced by the precise control of effector gene expression. There is growing evidence that distinct sets of effector genes are expressed in a coordinated manner in successive waves during the course of infection while these genes are transcriptionally repressed during vegetative growth (Dong et al., 2015; Gervais et al., 2017; Hacquard et al., 2013; Kleemann et al., 2012; Lanver et al., 2018; O’Connell et al., 2012; Tan & Oliver, 2017). In hemibiotrophic pathogens, including the rice blast fungus *Magnaporthe oryzae*...
oryzae, some effector genes, called biotrophy-specific effector genes, are expressed during a biotrophic stage of infection, and other effector genes are expressed during a later necrotrophic stage of infection (Mosquera et al., 2009; O’Connell et al., 2012). What specific conditions trigger effector gene expression and how the expression is transcriptionally regulated in an infection stage-specific manner remain largely unknown. Answering these questions has the potential to reveal processes unique to fungal pathogens that can be exploited as novel targets for disease control.

M. oryzae causes devastating blast disease in rice and other economically important crops. Recent live-cell imaging studies, making use of a rice sheath infection assay and various fluorescent reporters, revealed the cellular dynamics associated with the early biotrophic stage of rice infection (Giraldo & Valent, 2013; Jones et al., 2017; Kankanala et al., 2007; Khang et al., 2010; Pfeifer & Kang, 2018; Sakulkoo et al., 2018; Shipman et al., 2017) (Figure 1a). M. oryzae biotrophic invasion begins when a single-celled appressorium produces a penetration peg that breaches the rice cell wall, allowing the fungus to enter a living rice cell. Once inside the first-invaded rice cell, the penetration peg expands to form a filamentous primary hypha. As the primary hypha switches from filamentous to depolarized growth, the nucleus in the appressorium begins mitosis, and one nucleus undergoes a long-distance migration to the swollen tip of the primary hypha, followed by a septation, to produce the first bulbous invasive hyphal cell (Shipman et al., 2017). The bulbous invasive hyphae (IH) continue colonizing the first-invaded host cell for 8–12 hr before moving into adjacent cells using IH pegs that co-opt plasmodesmata (Kankanala et al., 2007; Sakulkoo et al., 2018). The first-invaded rice cell is alive but dies when the fungus penetrates adjacent living cells, establishing a pattern of successive biotrophic invasion (Jones et al., 2017; Kankanala et al., 2007). M. oryzae secretes biotrophy-specific effector proteins, including PWL2, into the biotrophic interfacial complex (BIC) presumed to mediate effector translocation into host cells (Khang et al., 2010). The BIC first appears at the tip of the primary hypha (tip-BIC) but then is repositioned to the side of the first bulbous cell (side-BIC) in the first- and subsequently invaded cells (Khang et al., 2010; Shipman et al., 2017) (Figure 1a). PWL2 is one of the well-characterized blast effector genes. PWL2 was initially cloned as an avirulence gene, which prevented the fungus from infecting weeping lovegrass, and it belongs to a multigene family present in various host-adapted isolates of M. oryzae (Kang et al., 1995; Sweigard et al., 1995). Previous studies have demonstrated that PWL2 is highly expressed during infection, while its expression is rarely detectable in axenically grown cultures (Mosquera et al., 2009; Nishimura et al., 2016; Sweigard et al., 1995).

In this study, we analysed the PWL2 gene to understand how this and other effector genes are activated specifically during the early biotrophic invasion of rice cells. The PWL2 expression pattern and promoter activity were determined using confocal live-cell imaging of M. oryzae transformants with various PWL2 promoter fragments fused to sensitive green fluorescent protein (GFP) reporter genes (destabilized or nuclear-targeted), together with time-course quantitative reverse transcription PCR (RT-qPCR) analyses. We found that PWL2 expression was coupled with sequential biotrophic invasion and that the expression required fungal penetration into living plant cells of either host rice or nonhost onion. Deletion and mutagenesis experiments further revealed that the tandem repeats in the PWL2 promoter contain 12-base pair (bp) sequences required for expression. Taken together, these results show that biotrophy-specific PWL2 expression is activated by an unknown signal commonly present in living plant cells and requires 12-bp cis-regulatory sequences in the promoter.

## 2 | RESULTS

### 2.1 | Development of PWL2 promoter reporter strains of M. oryzae

To determine the PWL2 expression pattern, we generated a transcriptional reporter construct by fusing the PWL2 promoter (PWL2p, 872 bp) and 3′ terminator region (3′ter, 500 bp), respectively, at the 5′ and 3′ ends of a reporter gene that encodes the destabilized version of enhanced green fluorescent protein (EGFP). The rapid turnover of destabilized EGFP allows tracking of the transient increase and decrease of gene expression in living cells (Li et al., 1998). The resulting construct (PWL2p:EGFP) was introduced into an M. oryzae strain, constitutively expressing tdTomato under control of the M. oryzae ribosomal protein RP27 promoter (RP27p:tdTomato) (Figure 1b). Our initial confocal imaging of 10 randomly selected transformants consistently showed bright EGFP fluorescence mainly in IH growing inside rice cells, compared to tdTomato fluorescence in all developmental stages of M. oryzae (Figure S1). We identified one transformant (M. oryzae CKF3538) that showed brighter fluorescence than others. We used RT-qPCR to determine the expression patterns of the native PWL2 gene and the ectopically inserted EGFP under control of the PWL2 promoter in this strain (Figure 1b). We found that both the native PWL2 gene and the EGFP transgene (PWL2p:EGFP) displayed a similar pattern of expression. In mycelia from axenic culture, expression of both the native PWL2 gene and the EGFP transgene was not detectable (Figure 1c). During rice infection, expression of both genes peaked at 25 and 38 hours postinoculation (hpi) with lower, or basal, expression detected at 18 and 33 hpi (Figure 1c). We also noticed that the overall transcription level was higher for the EGFP transgene than the native PWL2 gene, probably due to a position effect of the transgene integration into the genome. The consistent expression pattern of the native PWL2 gene and the EGFP transgene confirmed that the PWL2 promoter reporter strain CKF3538 (PWL2p:EGFP and RP27p:tdTomato) can be used to monitor the transient transcriptional induction of PWL2 in real time during infection.

### 2.2 | Induced expression of PWL2 during appressorium-mediated penetration and hyphal cell-to-cell movement

To determine the expression pattern of PWL2 at the cellular level, we used confocal microscopy of M. oryzae transformant CKF3538...
**FIGURE 1** Induced PWL2 expression occurs during appressorium-mediated penetration and cell-to-cell movement of invasive hyphae (IH). (a) Schematic diagram of *Magnaporthe oryzae* invasion in rice cells. An appressorium on the rice cell surface produces a filamentous hypha that grows inside the living rice cell (c.25 hr postinoculation [hpi]). The hyphal tip is associated with a biotrophic interfacial complex (BIC or tip BIC; indicated in red). The hypha subsequently differentiates into branched bulbous IH, and the tip BIC becomes a side BIC positioned on the side of the first bulbous cell (c.33 hpi). After colonizing the first-invaded rice cell, IH invade adjacent living cells (c.38 hpi).

(b) Schematic diagram of the native PWL2 gene and two reporter transgenes, enhanced green fluorescent protein (EGFP) under control of the PWL2 promoter (PWL2p:EGFP) and tdTomato under control of the constitutively active RP27 promoter (RP27p:tdTomato), inserted ectopically in *M. oryzae* transformant CKF3538. (c) Quantitative reverse transcription PCR expression patterns of native PWL2 and transgene EGFP in CKF3538 with three biological replications. (d) Confocal images of CKF3538 invading rice cells at different stages of infection. (e, f) Time-lapse confocal images showing the activation of the PWL2 promoter immediately after appressorium-mediated penetration (e) and IH cell-to-cell movement (f). The inset in (e) shows a short filamentous hypha that grew inside the rice cell. Arrowheads indicate appressoria. Arrows indicate some IH in the first invaded cells, and double arrows indicate some IH that have moved to adjacent cells. Bars, 10 μm.
(PWL2p:EGFP and RP27p:tdTomato) invading living rice cells. EGFP fluorescence (PWL2p:EGFP) was barely detectable in mature appressoria that had not yet penetrated rice cells (18 hpi, n = 66). However, EGFP fluorescence was strongly detected in appressoria that had penetrated rice cells and in subsequently growing young IH (25 hpi, n = 178). As the fungus continued to grow in the first-invaded rice cell, EGFP fluorescence declined to a barely detectable level (33 hpi, n = 46) but strongly increased again in IH that had spread into adjacent cells (38 hpi, n = 36). We confirmed that lack of strong EGFP fluorescence at 18 and 33 hpi was not due to artifacts related to fungal cell death because there was consistent tdTomato fluorescence (RP27p:tdTomato) in all fungal cells (Figures 1d and S1). The expression pattern of EGFP fluorescence (PWL2p:EGFP) was consistent with the RT-qPCR results (Figure 1c). These results suggest that activation of the PWL2 promoter coincides with the timing of fungal penetrations into the first rice cells and adjacent rice cells. Consistent with this finding, we observed EGFP fluorescence in the appressorium that produced very short filamentous primary IH (c.11 μm) but not in the proximally located appressorium that had not yet penetrated the first rice cell (Figure S2).

Using time-lapse confocal imaging, we further demonstrated the transition from absence of EGFP fluorescence to strong EGFP fluorescence after the fungus penetrated rice cells and produced even c.10 μm of IH in the first invaded cell (Figure 1e) or 4–8 μm of IH in adjacent rice cells (Figure 1f). Taken together, our results revealed that induction of PWL2 expression repeatedly occurs immediately after appressorium-mediated penetration and hyphal cell-to-cell movement.

2.3 Induction of PWL2 expression requires penetration into living plant cells

Rice cells that are initially invaded by young biotrophic hyphae are viable, but the invaded rice cells subsequently lose viability when the hyphae are fully expanded (Jones et al., 2016, 2017). Given that PWL2 was expressed as the fungus penetrated into presumed living rice cells (25 and 38 hpi), but the expression was greatly reduced in the fully expanded hyphae in presumned dead rice cells (33 hpi) (Figure 1d), we hypothesized that living rice cells are required for PWL2 expression. To test this hypothesis, we examined EGFP expression using confocal microscopy of CKF3538 (PWL2p:EGFP and RP27p:tdTomato) inoculated on a heat-killed rice leaf sheath. After the fungus penetrated heat-killed rice cells, EGFP fluorescence was barely detectable, which was in stark contrast to strong EGFP fluorescence when the fungus penetrated living rice cells, while tdTomato fluorescence was comparable in both conditions (n = 42; Figures 2a and S3a).

To determine whether the induced PWL2 expression was specific to the host rice plant, we tested PWL2 expression using an onion peel penetration assay (Xu et al., 1997). Onion is not a natural host of M. oryzae, but the fungus penetrates onion epidermal cells using the appressorium. We observed similar results to those in heat-killed rice cells, that is, EGFP fluorescence (PWL2p:EGFP) was strongly detected when the fungus penetrated living onion cells, whereas the fluorescence was barely detectable when it penetrated heat-killed onion cells even observed at saturated fluorescence levels (Figures 2b and S3b). We concluded that highly induced PWL2 expression requires penetration into living plant cells, and the induction is not host specific.

2.4 Tandem repeats are required for PWL2 expression

The PWL2 promoter contains three imperfect repeat sequences whose role in transcriptional regulation was not yet studied (Sweigard et al., 1995). These repeats are located between −331 and −182 nucleotides relative to the translation start site, and occur three times in tandem, and thus are named R1 (48 bp), R2 (49 bp), and R3 (48 bp) in this study (Figure 3a). R1 shares 92% sequence identity with R2 or
**FIGURE 3** The tandem repeat sequences are required for PWL2 induction during plant infection. (a) The organization and DNA sequences of the three tandem repeats in the PWL2 promoter (PWL2p). The repeats are denoted as R1, R2, and R3 with red arrows. The numbers indicate the nucleotide positions relative to the translation start site. Black shading indicates sequences conserved in all three repeats, and grey shading indicates sequences conserved in two repeats. (b) Comparison of the repeat copy number in the PWL2 promoters of six different *Magnaporthe oryzae* strains, including four rice-pathogenic strains (red line) and two wheat-pathogenic strains (blue line). Red arrows indicate the repeats. DNA sequence alignment of the repeats are shown in Figure S4. (c) A schematic representation of the reporter constructs and expected localization of the fluorescent proteins (red tdTomato and green sfGFP). RP27p is a constitutive promoter. PWL2p is the PWL2 promoter, and PWL2pΔrepeats is the PWL2 promoter with deletion of all three tandem repeats. (d) Confocal images of *M. oryzae* transformants expressing sfGFP-NLS (green) under control of PWL2p (M. oryzae CKF3276) or PWL2pΔrepeats (M. oryzae CKF3700) at 25 hr postinoculation (hpi). Both transformants constitutively express tdTomato-NLS (red) as a control of visualizing nuclei of viable fungal cells. Arrowheads and arrows indicate, respectively, appressoria and filamentous invasive hyphae (IH) that just penetrated rice cells. Bars, 10 μm. (e) Comparison of normalized fluorescence intensities of tdTomato or sfGFP quantified from nuclei of CKF3276 and CKF3700 at 25 hpi. Data are presented as mean ± SD of more than 10 infection sites for each strain. Two-tailed Student’s *t* test was performed to determine statistical difference. ***p < .001, NS, no significant difference.
To determine the role of the tandem repeats in PWL2 expression, we compared the transcriptional activity of the PWL2 promoter (PWL2p) and the PWL2 promoter with deletion of all three tandem repeats (PWL2pΔrepeats). Each promoter was fused to a fast-folding superfolder GFP with a nuclear localization signal (sfGFP:NLS reporter). This construct permitted precise detection and quantification of the intensity of sfGFP fluorescence localized in nuclei when the PWL2 promoter was activated. Each reporter construct (PWL2p:sfGFP:NLS or PWL2pΔrepeats:sfGFP:NLS) was introduced into the M. oryzae strain constitutively expressing tdTomato fused to NLS as a control for visualizing nuclei of viable fungal cells (RP27p:tdTomato:NLS) (Figures 3c and S5). Using confocal microscopy, we first demonstrated that sfGFP fluorescence of the PWL2p:sfGFP:NLS reporter strain (CKF3276) strongly accumulated in the nucleus of the appressorium upon penetration (Figures 3d and S5). We also observed that the fluorescence intensity of this construct decreased in multibranched IH when invaded cells but then increased again when the hyphae moved into adjacent cells (Figure S5). This pattern of increase, decrease, and subsequent increase of PWL2 promoter activity was consistent with the data generated by RT-qPCR and another promoter reporter construct (PWL2p:EGFP) (Figure 1c,d,e,f). Given that PWL2 promoter activity changed during the course of infection, we compared fluorescence intensities of sfGFP driven by PWL2p or PWL2pΔrepeats at two comparable infection stages, specifically focusing on the appressorium that produced young IH (less than two hyphal cells) within the first-infected rice cell and in IH that spread into adjacent cells. Our confocal imaging clearly showed that there was no detectable sfGFP fluorescence in the PWL2pΔrepeats:sfGFP:NLS strain (CKF3700) at both infection stages while tdTomato fluorescence driven by RP27p in this strain was as strong as that in the PWL2p:sfGFP:NLS strain (CKF3276) (Figures 3d,e and S6). These results suggest that the tandem repeats contain a positive regulatory element required for activation of PWL2 expression during penetration into living rice cells.

2.5 | The tandem repeats in the PWL2 promoter contain cis-regulatory sequences

To determine if the PWL2 promoter activity is affected by a change of location or orientation of the tandem repeats in relation to the translation start site, we made a series of promoter constructs by inserting the repeat sequences back into the repeat-deleted promoter (Figure 4). These constructs were individually linked to the sfGFP:NLS reporter, and the promoter activity was measured by quantifying sfGFP fluorescence in the resulting transgenic M. oryzae strains during appressorium-mediated penetration, as described above for Figure 3c–e. We first confirmed that when the three copies of the repeats were inserted back into the original location, the promoter activity was fully restored (Original in Figure 4a). Next, we found that insertion of the repeats at a location 500 bp upstream from the original location (Non-original in Figure 4a) or in the reversed orientation at the original location (Reverse in Figure 4a) resulted in restoration of the promoter activity, although the expression was reduced when compared to the wild-type PWL2 promoter (reduction of c.50% or c.25%, respectively; Figure 4a). We further showed that the restoration of the promoter activity was specific to the repeats because when a random DNA of the same length as the repeats was inserted into the repeat-deleted promoter, there was no sfGFP fluorescence (Non-specific in Figure 4b). These results suggested that the repeats contain a cis-regulatory element controlling inducibility of the PWL2 promoter and also that the regulatory activity is not strictly dependent on the location or orientation of the repeats relative to the translation start site. Furthermore, we found that constructs with three repeats showed higher inducibility of the promoter compared to one or two repeats, suggesting that increasing the number of repeats increases promoter strength (Figure 4b). It is important to note that one repeat, designated as R, was generated by taking advantage of three HindIII (AAGCTT) sites, present in all three repeats at conserved locations. All three HindIII sites were digested and subsequently ligated to join the 5′ end region of the R1 repeat (14 bp) and the 3′ end of the R3 repeat (34 bp) (Figure 5a). The resulting single-copy repeat was sufficient for transcription, indicating the presence of a cis-regulatory element within each repeat.

2.6 | Identification of cis-regulatory sequences in the tandem repeat of the PWL2 promoter

Our initial BLAST search revealed that the repeat sequences in the PWL2 promoter shared some similarity with those in the upstream regions of other M. oryzae effector and candidate effector genes (Table S1). These shared sequences were short, ranging from 10 to 25 bp long, which we mapped on the single copy of the repeat and defined as Regions I to IV (Figures 5a and S8a). Regions I, II, and III are located within the first 24 bp (5′-end, Figure 5a), and Region IV is located at the 3′-end of the repeat (3′-end, Figure 5a). We used a sfGFP:NLS reporter to quantify promoter activity for a series of deletion or substitution mutations in the repeat. We first determined that deletion of the 5′-end (positions 1–24), but not the 3′-end (positions 25–48), completely abolished promoter activity (Figure 5a,b). This suggests the presence of a cis-regulatory element at the 5′ end. To further define the cis-regulatory element, we focused our fine-scale deletion and mutation analyses on Regions I, II, and III located at the 5′-end. We found that transversion substitution mutations in Regions I (11 bp), II (12 bp), or III (11 bp) reduced promoter activity, and particularly mutations in Region II resulted in the greatest reduction in promoter activity (Figures 5b and S8a). These results suggest that the 12-bp motif in Region II (5′-TTATGCAAGCTT-3′) is a cis-regulatory sequence. This finding was further supported because inserting the 12-bp motif back into the PWL2 promoter with the deletion of all three tandem repeats (PWL2pΔrepeats) restored promoter activity (Figure 5c,d).
The 12-bp-like motif is present in the upstream region of M. oryzae effector genes

We sought to determine if the 12-bp motif we found in Region II was present in the upstream regions of other M. oryzae effector genes. We first identified 540 predicted effector genes in the M. oryzae genome using EffectorP v. 1.0 (Sperschneider et al., 2016) and subsequently conducted motif scanning for the original 12-bp motif and for similar 12-bp motifs, that is, "12-bp-like motifs" within the 1-kb upstream regions of these genes using MEME suite (Grant et al., 2011). We found a total of 126 occurrences of the 12-bp or 12-bp-like motif \( (p < 0.0001) \) in the upstream sequences of 106 genes (19.6% of a total of 540 genes), and in some of these regions the motif occurs more than once (Table S2). The motif-containing genes include some known effector genes, such as AVR-Pik (MGG_15972), BAS4 (MGG_10914), MAX (MGG_08414), MAX (MGG_09425), MOCIDIP3 (MGG_07986), MoHEG9 (MGG_00043), and SPD10 (MGG_11991) (Chen et al., 2013; de Guillen et al., 2015; Mogga et al., 2016; Mosquera et al., 2009; Sharpee et al., 2017; Yoshida et al., 2009) (Table S2). A comparison of all 126 motif sequences suggests that the core sequence of the motif is 5’-TGCAAGCTT-3’ (Figure 5e).

To determine if the motif-containing effector genes are coexpressed with PWL2, we used a time-course RT-qPCR analysis for 10 selected genes that contain the 12-bp-like motif in the same orientation and at a similar location (−200 to −350 bp relative to the translation start site) as in the PWL2 promoter. We found that five genes showed similar expression patterns to PWL2 (Figures 1c and 6). In particular, the expression patterns of AVR-Pik and two predicted effector genes (MGG_01953 and MGG_08300) were strikingly similar to that of PWL2, exhibiting initial induction at 25 hpi during appressorium-mediated penetration into living cells and repression at 33 hpi when the fungus colonizes first-invaded dead cells, followed by subsequent reinduction at 38 hpi when the fungus penetrates into living adjacent cells during cell-to-cell movement.

![Diagram of promoter activity comparison](image-url)
FIGURE 5  The 12-bp motif in the tandem repeat sequence is essential for PWL2 promoter activity. (a) Schematic diagram of generating the PWL2 promoter with one repeat (R) by HindIII (AAGCTT) digestion and subsequent ligation to join the 5′ end region of the R1 (14 bp) and the 3′ end of the R3 (34 bp). The first 24 bp of the R is defined as the 5′-end, and the other 24 bp as the 3′-end. The 5′-end was further defined as Region I (11 bp), Region II (12 bp), and Region III (11 bp). (b) The PWL2 promoter activity with each of the regions defined in (a) being deleted was determined, along with the promoter with one repeat (1-repeat R generated from Figure 5a) and the promoter with no repeat [Δ repeats], as described for Figure 3c–e). More than two fungal transformants were randomly chosen for each construct, and at least 10 independent infection sites of each transformant were analysed. Data are presented as mean ± SD. Statistically significant differences were determined by the Tukey–Kramer HSD test. *p < .05, **p < .01, ***p < .001; NS, no significant difference at p < .05. (c) Schematic diagram of the PWL2 promoter with one repeat (1-repeat) or the 12-bp motif in place of the repeat (12-bp; red box corresponding to Region II in Figure 5a) or no repeat (Δ repeats) fused to the sfGFP:NLS reporter. (d) Confocal images of Magnaporthe oryzae transformants expressing sfGFP:NLS (green) under control of 1-repeat (M. oryzae CKF3736) or 12-bp (M. oryzae CKF3991) or Δ repeats (M. oryzae CKF3692) at 25 hr postinoculation. All transformants constitutively express tdTomato:NLS (red) as a control of visualizing nuclei of viable fungal cells. Arrowheads and arrows indicate, respectively, appressoria and filamentous invasive hyphae (IH) that just penetrated rice cells. Note that there are two nuclei (one in the appressorium and another one in the IH cell). More than five independent infection sites were observed for each of 11 random transformants for the 12-bp construct, and all showed the consistent result. Bars, 10 μm. (e) Consensus 12-bp motif sequence generated from 126 sequences similar to the 12-bp motif shown in Figure 5a using WebLogo (Crooks et al., 2004)
These results indicate that the 12-bp-like motif plays a role in regulating the biotrophy-specific expression of *M. oryzae* effector genes.

### DISCUSSION

#### 3.1 | PWL2 expression is coupled with sequential biotrophic invasion

In this study, we provide evidence that transcriptional regulation of PWL2 is coupled to the biotrophic phase of *M. oryzae* during colonization of the first two rice cells. In *M. oryzae*, biotrophy is characterized as sequential invasion into living rice cells, which begins with the appressorium initially penetrating a living rice cell (c.25 hpi), and then when highly branched IH penetrate adjacent living cells after colonizing the first-invaded cell until cell death (c.38 hpi) (Figure 1a) (Jones et al., 2017; Kankanala et al., 2007). Our time-course RT-qPCR analysis at the tissue level and EGFP reporter-based live-cell imaging at single-cell resolution consistently showed that PWL2 expression is induced immediately upon penetrating living rice cells from the appressorium or from IH moving to adjacent living rice cells (Figure 1c,d). This strong expression was in stark contrast to the barely detectable expression in the appressorium on the rice cell surface (prior to penetration into the first cell), and highly branched IH in the first-invaded dead rice cell (prior to penetration into the second cell). This two-peaked expression pattern of PWL2 coincides with localization of the PWL2 protein in BICs, which form when the fungus penetrates new living rice cells (Khang et al., 2010; Shipman et al., 2017). These results suggest that PWL2 transcription is tightly regulated in coordination with BIC development during biotrophic invasion. Shipman et al. (2017) made an intriguing observation that cytoplasmic effector proteins, including PWL2, are secreted into the tip BIC and the early side BIC, which can be located more than 32 μm away from the nearest nucleus in the appressorium where this study shows PWL2 promoter is activated. It remains to be determined how effector trafficking is regulated from the appressorium through the primary hypha to the distantly located BIC.

#### 3.2 | PWL2 expression is activated by an unknown signal in living plant cells

Fungal genes that are induced during infection are transcriptionally regulated presumably in response to a combination of nutrition conditions, plant-derived inducing compounds, or infection-related fungal development (Basse et al., 2000; Meyer et al., 2017; Van den Ackerveken et al., 1994; van der Does et al., 2008). Earlier studies by Sweigard et al. (1995) showed that PWL2 transcripts were not detectable when *M. oryzae* was grown in complete, minimal, or nitrogen-depleted medium, thus excluding these nutrition conditions as a cue to induce PWL2 expression. Our finding that PWL2 expression was
induced when the fungus penetrated living cells, but not dead cells, of both rice (host) and onion (nonhost) suggests that the presumed inducer is commonly present in living plant cells (Figure 2). Similar observations have been reported for biotrophy-specific effector genes in other fungi. For instance, expression of the effector gene Six1 of *Fusarium oxysporum* is induced upon penetration of the root cortex of living tomato (host) and also in response to cell cultures of tomato and tobacco (nonhost) (van der Does et al., 2008). The plant signals that induce Six1 expression remain unknown. The effector gene MISSL2 of *Laccaria bicolor* is expressed during interactions with poplar (host) and *Arabidopsis thaliana* (nonhost) roots, and the inducer has been identified as rutin and quercetin, commonly found flavonoids in the exudates of plant roots (Plett et al., 2011). Future studies will be needed to identify inducers and regulatory components for PWL2, which can be facilitated by using strategies such as mutagenesis and screening plant compounds using GFP-based reporters (Basse et al., 2000, 2002).

### 3.3 | Tandem DNA repeats within the PWL2 promoter contain cis-regulatory sequences

Tandem DNA repeat sequences are often associated with gene promoters and function as cis-regulatory sequences. For example, tandem repeats are found in 25% of all promoters in the *Saccharomyces cerevisiae* genome (Vinces et al., 2009). Some tandem repeats directly regulate expression of genes such as the maltose permease gene in *S. cerevisiae* (Bell et al., 1997) and the anthocyanin-regulating transcription factor MYB10 in apple (Espley et al., 2009). Approximately 52% of the *M. oryzae* genome consists of various repetitive sequences, and a genome-wide analysis is needed to determine how many *M. oryzae* gene promoters, particularly effector gene promoters, are associated with tandem repeats (Raffaele & Kamoun, 2012). The PWL2 promoter contains three 48-bp imperfect tandem repeats (Figure 3a) (Sweigard et al., 1995). Using dot plot analyses, we found that promoters of eight additional effector genes beside PWL2 contain tandem repeats in various numbers and lengths in their promoters (Figure 59). There is growing evidence that tandem repeat-containing promoters show higher transcriptional divergence (Vinces et al., 2009). In agreement with this, we found that the indubility of the PWL2 promoter varies depending on the number of the repeats within the promoter (Figure 4b). Intriguingly, this appears to be further implicated with the role PWL2 plays during plant infection. PWL2 confers avirulence against weeping lovegrass, containing a yet-to-be discovered resistance gene. PWL2 is also presumed to have a virulence role in other plants, lacking the resistance gene, based on the prevalent occurrence of PWL2 in diverse *M. oryzae* populations (Kang et al., 1995; Sweigard et al., 1995). While characterizing DNA sequences required for PWL2 avirulence activity, Sweigard et al. (1995) showed that avirulence was partially lost when the promoter was deleted to contain one and a half copies of the repeat or completely lost when deleted further to contain the half copy. In contrast, avirulence was fully retained with more than two and a half copies of the repeat. This impaired avirulence is probably due to the reduced transcription of PWL2 that we observed with the reduced copy number of the repeats (Figure 4b). It is possible that a certain level of PWL2 transcription, correlated with protein production, is required for the PWL2 protein being recognized as an avirulence factor. We suggest that fine-tuned expression of PWL2 through variations in the repeat copies could be a potential mechanism by which PWL2 avoids host recognition (losing avirulence activity) while retaining a presumed virulence function, thereby facilitating *M. oryzae* host adaptation. Consistent with this notion, we found that different host-adapted *M. oryzae* strains carry a varying copy number of the repeat in the PWL2 promoter (Figure 3b). It will be exciting to investigate how repeat copy number variations correlate with PWL2 expression in these strains and how these variations in copy number contribute to *M. oryzae* adaptability on different plant species.

We provide evidence that the tandem DNA repeats contain cis-regulatory sequences required for biotrophy-specific expression of PWL2. Deletion of these repeats resulted in a complete loss of PWL2 expression, which could be complemented when the repeats were inserted at the original location (Figure 4a). The complementation was specific to the sequences of the repeats because an unrelated DNA sequence in the same length as the repeats failed to complement (Figure 4a). We also determined that the single copy of the repeat contained all sequences sufficient for PWL2 expression (Figure 4b). These data suggest that each repeat contains cis-regulatory sequences, directly regulating gene expression. These cis-regulatory sequences are presumed to function as a transcription factor (TF) binding site rather than as a structural component of the promoter. The location and orientation of TF binding sites can have effect on promoter activity (Lis & Walther, 2016; Sharon et al., 2012). Consistent with these findings, we observed some level of promoter activity even when these repeats were inserted at a distal location or in reverse orientation (Figure 4a).

Further deletion and mutagenesis studies identified a 12-bp motif that is present within each repeat and is sufficient for PWL2 expression (Figure 5). Addition of the 12-bp motif to PWL2Δ repeats restored the GFP reporter expression upon appressorium-mediated penetration into living rice cells (Figure 5c,d). The 12-bp-like motif appears to be involved in regulating expression of other effector genes in *M. oryzae*. We found that at least 106 effector or effector candidate genes contain the 12-bp or 12-bp-like motif in their promoter regions, and five of these genes indeed showed similar expression patterns to PWL2 (Figure 6). These results suggest that they are transcriptionally co-regulated by common transcription factors (Lanver et al., 2018). Whether the motif directly regulates expression of these and other genes containing the motif remains to be determined.

Evidence is accumulating that distinct sets of effector genes are coordinately expressed in successive waves during the course of host infection, reflecting the complexity of effector gene regulation and the diversity of TFs and cis-regulatory sequences (Dong et al., 2015; Farfsing et al., 2005; Gervais et al., 2017; Hacquard et al., 2013; Kleemann et al., 2012; Lanver et al., 2018; O’Connell et al., 2012; Soyer et al., 2014; Wang et al., 2011). Fungal genomes collectively contain at least 36 different families of TFs, and 13 TFs from four families are known for their roles in effector regulation.
M. oryzae wild-type strain O-137 was used as a recipient strain to generate fungal transformants using Agrobacterium tumefaciens-mediated transformation (Khang et al., 2005). M. oryzae strains were cultured on oatmeal agar plates at 24 °C under continuous light and stored frozen at −20 °C to maintain full pathogenicity (Valent et al., 1991). See Tables S3 and S4 for the list of M. oryzae strains and plasmids used in this study. To monitor PWL2 expression at single-cell resolution, M. oryzae transformant CKF3538 was made by sequential transformation of pCK1292 and pCK1714 into wild-type strain O-137. pCK1292 was generated by cloning a 0.5-kb EcoRI-BamHI fragment of pBV167 (RP27 promoter) (Shipman et al., 2017) and a 1.7-kb BamHI-HindIII fragment of pAN582 (pBV359, tdTomato:Nos terminator) (Nelson et al., 2007) in EcoRI-HindIII sites of pBV141 (pBGt) (Kim et al., 2011). pCK1714 was generated by cloning a 1.7-kb EcoRI-BsrGI fragment of pCK1298 (PWLP2p:EGFP), a 0.12-kb BsrGI-NotI fragment of pBV118 (pd2EGFP-1) (Li et al., 1998), and a 0.5-kb NolI-XhoI fragment of pBV1102 (PWL2 3′ terminator region) in EcoRI-SalI sites of pBV1 (pBH2) (Mullins et al., 2001).

To identify the cis-element in the PWL2 promoter, fungal transformants with fluorescently labelled nuclei were generated. In particular, M. oryzae transformant CKF3276 was generated by sequential transformation of pCK1528 and pCK1586 into wild-type strain O-137. pCK1528 was generated by cloning a 1.0-kb EcoRI-BamHI fragment of pBV126 (RP27 promoter) (Khang et al., 2010), a 1.4-kb BamHI-BsrGI fragment of pAN582 (tdTomato:NLS), and a 0.4-kb BsrGI-HindIII fragment of pBV578 (Nos terminator) in EcoRI-HindIII sites of pBV141. pCK1586 was generated by cloning a 872-bp EcoRI-BamHI fragment of pCK1574 (PWLP2 promoter) and a 1.3-kb BamHI-XhoI fragment of pCK1576 (sfGFP without ATG-NLS:PWLP2 3′ terminator region) in EcoRI-SalI sites of pBV1. sfGFP was first cloned from sfGFP-Lifeact-7 (pCK1349), a gift from Michael Davidson (Addgene plasmid # 54739). A series of constructs with deletions, replacements, and mutations in the PWL2 promoter was generated by restriction enzyme digestion and ligation, or PCR using the primers in Table S5 (Figures S8 and S9). The intact or manipulated PWL2 promoter fragments and sfGFP-NLS:PWL2 3′ terminator region were first cloned into pJET1.2 (Thermo Fisher Scientific) to confirm the introduction of the desired alteration by DNA sequencing, and later the fragments were inserted into the binary vector pBV1.

After two rounds of selections on TB3 (0.3% yeast extract, 0.3% casamino acid, 20% sucrose) and V8 (8% V8 vegetable juice [Campbell’s], pH 7) media containing 200 µg/ml of hygromycin (Fisher BioReagents) or 800 µg/ml of G418 (Fisher BioReagents), and 200 µM of cefotaxime (Gold Biotechnology), at least 10 independent transformants for each construct were selected and purified by single spore isolation. All positive transformants showed similar fluorescence patterns and behaved indistinguishably when compared to the wild-type strain under microscope examination. Due to the position effect of transgenic genes (Chen & Zhang, 2016; Soanes et al., 2002), two or three independent transformants for each construct were randomly chosen for further analysis.

4 | EXPERIMENTAL PROCEDURES

4.1 | Strains, fungal transformation, and plasmid construction

4.2 | Infection assays

Rice sheath inoculations were performed using the susceptible rice YT16 as previously described (Jones & Khang, 2018). Briefly, excised leaf sheaths (5–8 cm long) from 19- to 21-day-old plants were inoculated with a spore suspension (10^5 spores/ml in distilled water). The inoculated sheaths were hand-trimmed and immediately used for confocal microscopy. Inoculations on onion epidermal peels were performed as previously described (Xu et al., 1997). In heat-killed inoculations, pretrimmed sheaths or onion epidermal peels were incubated in 70 °C water for 25 min, cooled down to room temperature, and then inoculated with a spore suspension.

4.3 | RNA isolation and RT-qPCR

For the RT-qPCR assay, 15 infected rice sheaths at each time point of 18, 25, 33, and 38 hpi were collected as described (Mosquera et al., 2009), frozen immediately in liquid nitrogen, and stored at −80 °C for RNA extraction. The TRizol method (Invitrogen) was used to extract total RNA. Genomic DNA was eliminated by Turbo DNase (Ambion) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using the ImProm II Reverse Transcriptase system (Promega) from 500 ng of total RNA extracted from infected tissue or mycelia grown for 5 days in complete medium (CM). RT-qPCR was performed with the MX3005P (Stratagene) systems using the VeriQuest SYBR Green qPCR Master Mix (2×; Thermo Fisher). Each reaction (final volume 14 µl) contained 7 µl of VeriQuest SYBR Green qPCR Master Mix, 1.5 µl of each the forward and reverse primers (3.3 nM concentrations for each, Table S5), 2 µl of cDNA, and 2 µl of distilled water. Thermocycler conditions were as follows: 2 min at 50 °C, 10 min at 95 °C; followed by 40 cycles of 95 °C for 30 s, 60 °C for...
30 s, and 72 °C for 30 s. The specificity of each primer pair was checked by incorporation of a final dissociation cycle. The relative expression level was calculated using the 2^−ΔΔCt method (Livak & Schmittgen, 2001) with the M. oryzae actin gene (MGG_03982) as the reference gene (Che Omar et al., 2016). Briefly, the average threshold cycle (Ct) was normalized to that of the actin gene for each sample as 2^−ΔCt, where ΔCt = (Ct_target gene − Ct_actin). Two technical replications for each of three biological replications were performed. Mean and standard deviation were calculated from three biological replicates.

4.4 | Confocal microscopy and quantification of fluorescence intensity

Confocal microscopy was performed on Zeiss LSM 510 Meta and Zeiss LSM 880 confocal microscopes. Excitation/emission wavelengths were 488 nm/496–544 nm for EGFP/sfGFP and 543 nm/565–617 nm for tdTomato. Images were processed using Zen Black software v. 10.0 (Zeiss). To quantify and compare fluorescence intensity for each experiment, pinhole size and detector gain were optimized to maximize detection range to avoid saturated pixels. Identical image acquisition and processing settings were used to analyse all images for each experiment. Quantification of fluorescence intensity was performed using ImageJ (https://imagej.nih.gov/ij/) as previously described (Hartig, 2013; Jensen, 2013). Briefly, an image was first maximum-intensity projected and split into different channels. The image threshold was adjusted until all fluorescent areas were selected. Intensity measurements were then performed for each nucleus. PWL2 promoter activity was defined as normalized sfGFP intensity from 0 to 1, with 0 being background intensity as determined in nuclei without fluorescence, and 1 being the highest intensity value in nuclei of the fungal transformant with the 872-bp PWL2 promoter as determined by subtraction of background intensity.

4.5 | Sequence analysis and effector prediction

DNA sequence analyses were performed using Geneious software v. 8.1.2 (https://www.geneious.com/) with sequences obtained from the NCBI database. The 1,000-bp upstream sequences of M. oryzae genes were obtained from the Broad Institute (https://www.broadinstitute.org/). The sequences were then imported into Geneious and used as the database for a BLASTn analysis. The 12-bp motif was used for a motif occurrence search by FIMO (MEME v. 4.12.0, http://meme-suite.org/tools/fimo) with default parameters (Grant et al., 2011) against the 1,000-bp upstream sequences of M. oryzae effector genes. The alignment of PWL2 sequences was conducted using ClustalW (Thompson et al., 2003) implemented in Geneious. The previously generated M. oryzae secretome data (Zhang et al., 2018) were used to predict effector genes using EffectorP v. 1.0 (Sperschneider et al., 2016).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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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