Pdel, Encoding a Low-Affinity cAMP Phosphodiesterase, Regulates Conidiation and Pathogenesis in *Alternaria alternata* Tangerine Pathotype

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Based on intracellular second messenger cAMP, the cyclic AMP-protein kinase A (cAMP-PKA) pathway transforms extracellular stimuli to activate effectors and downstream signaling components, mediating physiological processes in filamentous fungi. The concentration of intracellular cAMP was regulated by adenylate cyclase biosynthesis and cAMP phosphodiesterase (PDEs) hydrolysis, which mediate signal transduction and termination. In this study, we used a gene deletion and complementary strategy to characterize the functions of *AaPdel* and *AaPdeh* genes, which encoded low-affinity PDEs (Pdel) and high-affinity PDEs (Pdeh), respectively, in *Alternaria alternata. AaPdel*, but not *AaPdeh*, was found to be a key regulator in conidiation and pathogenesis in *A. alternata*. Δ*AaPdel* showed defects in conidiation, producing approximately 65% reduced conidiation and forming lowly pigmented aberrant structures. In response to osmotic stress, Δ*AaPdel* was more sensitive to non-ionic osmotic stress than ionic osmotic stress. Moreover, *AaPdel* deletion mutants had defects in vegetative growth and hyphal growth. Further analyses showed that the high chitin content of Δ*AaPdel* might account for the sensitivity to Congo red. Based on the attenuated pathogenicity and lowly pigmented aberrant structures, the laccase activity analysis found that both *AaPdel* and *AaPdeh* were involved in laccase activity regulation. Our data further support the PKA-mediated cAMP signaling pathway, as we have found that *AaPdel* was involved in intracellular cAMP levels in *A. alternata*.

Keywords: *Alternaria alternata* tangerine pathotype, cAMP phosphodiesterase, conidiation, cell wall integrity, osmotic stress, pathogenicity

INTRODUCTION

Seven pathotypes of *Alternaria alternata* have been identified in tangerine, Japanese pear, strawberry, apple, tomato, rough lemon, and tobacco (Hatta et al., 2002; Thomma, 2003). Alternaria brown spot disease (ABS) caused by the tangerine pathotype of *A. alternata* reduces quality and yield of tangerine production in China (Lee et al., 2015). Under the optimum condition of 28°C and high humidity, the *A. alternata* infected both leaves, twigs, and fruits, forming necrosis, and spread extensively (Solel and Kimchi, 1998). Infected leaves act as the primary infection source, and

conidia are derived from the lesions of infected leaves (Wang et al., 2018). As a necrotrophic fungus, the tangerine pathotype of *A. alternata* produced the host selective toxin *A. citri* toxin (ACT) to kill and colonize the host cells (Tsuge et al., 2013; Ma et al., 2019). In the infection process, the ACT toxin spreads along the veins of the leaves, forming irregular "v"-shaped or like-round lesions. By causing electrolyte leakage, tangerine and its hybrids are susceptible to the ACT toxin (Otani et al., 1995; Ito et al., 2004).

In filamentous fungi, cells respond to external stimuli through cell-surface receptors and complex signal transduction pathways. The cyclic AMP-protein kinase A (cAMP-PKA) pathway is one of the major studied pathways mediating physiological processes in filamentous fungi (Zhu et al., 2017). Based on the cAMP, intracellular second messenger, extracellular stimuli could be transformed to activate effectors and stimulate downstream signaling components (Calvo et al., 2002). The cAMP-PKA pathway consists of G protein-coupled receptors (GPCRs), heterotrimeric G proteins, adenylate cyclase (AC), cAMP, and protein kinase A (PKA) (Houslay et al., 2007). The concentration of intracellular cAMP was regulated by adenylate cyclase biosynthesis and cAMP phosphodiesterase (PDEs) hydrolysis, which mediated signal transduction and termination (Jin et al., 1992). Two identified GTP-binding proteins, Ras1 and Ras2, regulate cAMP production by activating adenylate cyclase (Ramanujam and Naqvi, 2010). And PDEs were directly acting on active site, mediating cAMP hydrolysis to $5'$-AMP (Conti and Beavo, 2007; Wilson et al., 2010). PDEs play an important role in the cAMP-PKA pathway, but their specific functions in *A. alternata* remain poorly understood.

In *Saccharomyces cerevisiae*, cAMP levels are modulated by two cAMP phosphodiesterase, low-affinity phosphodiesterase Pde1, and high-affinity phosphodiesterase Pde2. There is no obvious homology between Pde1 and Pde2. Simultaneously knockouting of *Pde1* and *Pde2* doubled the cAMP concentration, increased sensitivity to heat shock, and growth defects in non-fermentable carbon sources (Londesborough and Suoranta, 1983; Uno et al., 1983). *Pde2* regulates cAMP level induced by glucose stimulation, while there is no significant difference of cAMP level between the *Pde1* deletion mutant and the wild-type strain (Hu et al., 2010). *Pde1* does not regulate the cAMP level directly, but mediates a PKA negative feedback loop (Wera et al., 1997; Ma et al., 1999; Zhao et al., 2007; Caza and Kronstad, 2019).

In plant-microbe interactions, the fungus *Magnaporthe oryzae* *PdeH* (*Pde2*) has a dominant role in conidiation, pathogenicity, and intracellular cAMP level regulation. Compared with the wild-type strain, simultaneous knockout of *PdeH* and *PdeL* resulted in a 10-fold increase in cAMP level and completed disappearance of pathogenicity, whereas *PdeL* (*Pde1*) had no obvious function in *M. oryzae* (Zhang et al., 2011). The expression of GFP protein shows that PdeH localizes predominantly to the cytoplasm, while PdeL locates to the nucleus. *PdeH*-involved cAMP signaling is crucial for signal transduction and pathogenicity in *M. oryzae* (Ramanujam and Naqvi, 2010). In *Candida albicans*, the *PDE2* deletion mutant exhibits reduced invasion and virulence. In the simultaneous knockout of *CaPDE2* and *CaPDE1*, pathogenicity is abolished completely,

indicating that *Pde1* contributes to virulence as a secondary role (Wilson et al., 2010). In phytopathogenic fungus *Botrytis cinerea*, the Δ*bcpde2* exhibits a significant reduction in vegetative growth, spore formation, germination, and pathogenicity, but the Δ*bcpde1* displays a similar phenotype to the wild-type strain (Harren et al., 2013). In *Fusarium graminearum*, inactivation of *PDE2* but not *PDE1* results in activating PKA activities and increases DON production (Jiang et al., 2016).

In *A. alternata*, the cAMP-PKA pathway remains poorly understood, and no regulated genes have been identified. In this study, characterization of PDEs in *A. alternata* have been conducted. We have identified high and low-affinity cAMP phosphodiesterase in *A. alternata* and through targeted gene deletion, analyzed the gene function in vegetative growth, conidiation, stress response, cell wall integrity (CWI), intracellular cAMP level, laccase activity, and pathogenicity.

MATERIALS AND METHODS

Strains and Growth Condition

The *A. alternata* Z7 was used as a wild-type strain in this study (Wang et al., 2016). And the fungal strains defective of *AaPdel*, coding low-affinity cAMP phosphodiesterase, and *AaPdeh*, coding high-affinity cAMP phosphodiesterase, were used as deletion mutants (Δ*AaPdel* and Δ*AaPdeh*). For activating strains and determining characteristics of colonies, all strains were cultured on potato dextrose agar (PDA) plates for 4–8 days at 28°C. After targeted gene deletion, the resulting transformants were selected on TB3 medium (yeast extract 3 g, casamino acids 3 g, sucrose 200 g, and agar 7.5 g per liter of purified water) containing hygromycin (Kong et al., 2018). On the vegetative growth analyses, the wild type and deletion mutants were cultured on minimal medium (KCl 0.5 g, NaNO₃ 2 g, KH₂PO₄ 1 g, MgSO₄·7H₂O 0.5 g, FeSO₄ 0.01 g, sucrose 10 g, trace elements 200 µL and agar 20 g per liter of purified water) and complete medium (per liter of minimal medium supplied with yeast extract 1 g, casein hydrolysate 1 g, and peptone 2 g), respectively (Tang et al., 2020).

Targeted Gene Deletion and Complementation Analysis

Gene replacement through the hygromycin-resistance cassette released from pCX62 was used to generate the deletion mutants (Dong et al., 2015). The upper fragment of the target gene (S1) and hygromycin B gene (H1) were amplified and fused together by two-step overlapping PCR. And the lower fragment of the target gene (S2) and hygromycin B gene (H2) were fused to another fragment. The two resulting fragments with a hygromycin-resistance cassette were introduced into the protoplasts of the wild type by the polyethylene glycol (PEG)-mediated knockout technique (Rehman et al., 2016). To confirm the correct gene replacement, the resulting transformants resistant to hygromycin (TB3 medium with 250 mg/ml hygromycin), were identified by PCR assay and southern blot analysis.

Conidiation

The conidia of strains were harvested by blending with distilled water and scraping the colonies grown on V8 medium at 28°C for 8 days (Timmer et al., 1998). After filtering into two layers of sterile gauze, conidia were quantified by hemocytometer (QIUJING, China) and microscope (OLYMPUS, Japan). The conidia germinating rate and conidia diameter were assessed by microscopic examination of 200 conidia each for at least three repetitions. The statistical significance on conidiation of wild type, deletion mutants and complementary strains, was determined by one-way ANOVA and Duncan's new multiple range test ($P < 0.05$).

Nucleic Acid Manipulation

The homology of the targeted gene and protein sequences were searched in the Blast program and resources at NCBI[1] . Analyses of protein subcellular localization were predicted by WoLF PSORT[2]. Fungal genomic DNA and total RNA extraction were extracted from the mycelia grown in PDB medium for 36 h at 28°C. Fungal genomic DNA extraction was carried out using CTAB as previously described, and total RNA extraction was performed using Trizol (Invitrogen). Plasmid DNA was purified using the Plasmid DNA Miniprep Kit (Qiagen).

Vegetable Growth and Stress Treatment

Four kinds of growth medium [PDA, V8, minimal medium (MM), and complete medium (CM)] were configured to determine the involvement of vegetative growth between deletion mutants and wild-type strain. To analyse the involvement of *AaPdel* and *AaPdeh* in cell wall integrity and osmotic stress response, 3 days activated strains were inoculated on PDA amended with different chemicals. The same concentration of osmotic-stress inducer, 1M sorbitol, 1M NaCl, 1M KCl, or 1M sucrose was added to the PDA plate, respectively. And SDS or Congo red (CR) inducing medium was configured to analyse the cell wall integrity of strains. The diameter of colonies were measured in two perpendicular directions after 5 days of incubation at 28°C, and each treatment was replicated for at least three times. And the growth rates were presented as the mean ± SD of at least three repeats. Statistical significance of growth rates of mutants and wild type was determined by one-way ANOVA and Duncan's new multiple range test (MRT) ($P < 0.05$).

Quantification of Intracellular cAMP

Mycelia, grown in PDB medium for 36 h at 28°C and freeze-dried for 1.5 h, were quickly ground in liquid nitrogen and mixed with 200 µL of 6% trichloroacetic acid (TCA). Intracellular cAMP extraction was in accordance with the method described previously (Liu et al., 2007), and cAMP levels were measured using the AlphaScreen Assay Kit (PerkinElmer, Waltham, MA) according to the supplier's instruction.

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[1]https://www.ncbi.nlm.nih.gov

[2]https://www.genscript.com/psort/wolf_psort.html

Pathogenicity Assay

In the pathogenicity assay, the 10 days conidia harvested from V8 medium were diluted to a concentration of 1.0×10^5 conidia ml^{-1}. At least 20 leaves of tangerine (*Citrus reticulata* Blanco) with nearly identified size and maturity, were inoculated with 20 µL conidial suspension of wild type, deletion mutants, and complementary mutants (Solel and Kimchi, 1998). Distilled water was inoculated on the leaves as a positive control. The phenotype was recorded at 3–5 dpi (days post inoculation), with three repetitions in each treatment. According to Perina et al. (2019) the average scale levels based on the percentage of lesion area were quantified.

Laccase Activity Assay

Laccase activity was quantified as described previously (Mtibaa et al., 2018). Based on the oxidation of ABTS, the activated strains of wild type, Δ*AaPdel*, Δ*AaPdeh*, and complementary mutants were inoculated on the ABTS medium at 28°C. The morphology of these colonies was observed and photographed at 6 dpi. For the enzyme solution preparation, mycelial plugs of activated strains were cultured in 50 mL PDB liquid medium for 36 h. After filtration, mycelial pellets were harvested and mixed with 1 mL Tris-HCl. And the supernatant collected by centrifuge (10,000 rpm, 10 min) was the enzyme solution. Laccase activity was determined by 0.5 mM ABTS oxidation mixing in 0.1 M NaAc-HAc buffer, pH 4.0 at 28°C and measured the spectrophotometer absorbance at 420 nm. The resulted laccase activity was defined as the units U mL^{-1}, with 1 U being the amount of enzyme oxidizing 1 µmol of ABTS per min (Irfan et al., 2018).

Statistical Analysis

Data was presented as the mean ± SD of at least three independent experiments. Statistical significance was analyzed by one-way ANOVA and Duncan's new multiple range test (MRT). Different letters represent significant differences at $P < 0.05$.
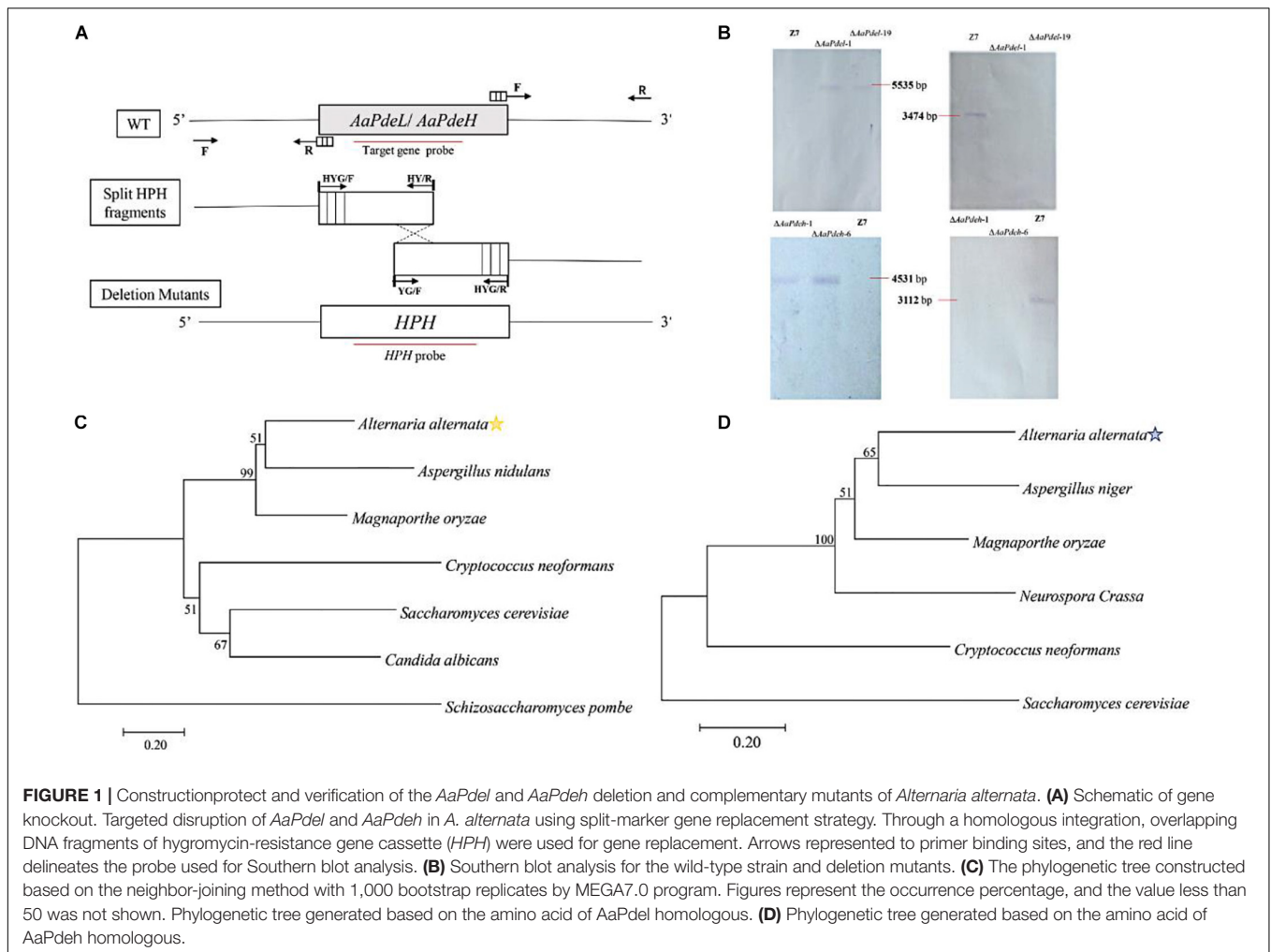
RESULTS

AaPdel, but Not *AaPdeh*, Is Involved in Mycelial Growth and Hyphal Growth

Through blasting *S. cerevisiae* Pde1 and Pde2 in the proteome of *A. alternata*, the corresponding OWY42401.1 and OWY42000.1 were identified by high protein homology, and named AaPdel and AaPdeh, respectively. The sequence alignment demonstrated that the AaPdel protein exhibited 18.50–52.59% similarity with *S. cerevisiae* ScPde1, *M. oryzae* MoPdel, *C. albicans* CaPdel, and the AaPdeh protein exhibited 30.26–69.59% similarity with *S. cerevisiae* ScPde2, *M. oryzae* MoPdeh, *C. albicans* CaPde2, and *Botrytis cinerea* Bcpde2. The phylogenetic tree of two identified proteins were shown in **Figures 1C,D**, respectively. The identified *AaPdel* gene encodes a 1,100-amino-acid polypeptide, and the identified *AaPdeh* gene encodes a 961-amino-acid polypeptide. As demonstrated in **Figure 1A**, the split-marker approach was used to generate deletion mutants. To confirm the correct

FIGURE 1 | Constructionprotect and verification of the *AaPdel* and *AaPdeh* deletion and complementary mutants of *Alternaria alternata*. **(A)** Schematic of gene knockout. Targeted disruption of *AaPdel* and *AaPdeh* in *A. alternata* using split-marker gene replacement strategy. Through a homologous integration, overlapping DNA fragments of hygromycin-resistance gene cassette (*HPH*) were used for gene replacement. Arrows represented to primer binding sites, and the red line delineates the probe used for Southern blot analysis. **(B)** Southern blot analysis for the wild-type strain and deletion mutants. **(C)** The phylogenetic tree constructed based on the neighbor-joining method with 1,000 bootstrap replicates by MEGA7.0 program. Figures represent the occurrence percentage, and the value less than 50 was not shown. Phylogenetic tree generated based on the amino acid of AaPdel homologous. **(D)** Phylogenetic tree generated based on the amino acid of AaPdeh homologous.

gene replacement, the resulting transformants were selected based on the resistance to hygromycin (TB3 medium containing 250 mg/ml hygromycin). And *AaPdel* transformants were verified by three primer pairs of PLP1F/H855R, H856F/PLP2R, and PLF/PLR. *AaPdeh* transformants were identified through PHP1F/H855R, H856F/PHP2R, and PHF/PHR (**Supplementary Figure 1**). Southern blot analysis was used for further analysis of the deletion mutants. Genomic DNA of *AaPdel* and *AaPdeh* transformants were respectively, digested with *Hin*dIII, *Bgl* II, *Xho*I, and *Sal*I. A 5,535-bp fragment in the deletion mutants of *AaPdel* and a 4,531-bp fragment in the deletion mutants of *AaPdeh* were probed with *HPH* gene. And when probed with homologous target genes, respectively, 3,474 and 3,112-bp fragments were only found in the wild type, indicating correct replacement of the target genes (**Figure 1B**).

The wild type, z7; deletion mutants, ∆*AaPdel*-1, ∆*AaPdel*-19, ∆*AaPdeh*-1, and ∆*AaPdeh*-6; and complement strains, *AaPdel-cp* and *AaPdeh-cp*, were inoculated on four vegetative mediums of PDA, V8, CM, and MM, respectively. The colony morphology and diameter were assessed after growing for 6 days and measured in two orthogonal directions. The ∆*AaPdel* displayed similar vegetative growth defects on the PDA, MM, CM, and

V8 media (**Figure 2A**). On the CM medium with sufficient nutrient elements, the growth rates of ∆*AaPdel* were decreased by 14%, significantly lower than the corresponding WT. On the MM covering the basic nutrient elements of fungal growth, the percentage growth rates of ∆*AaPdel* were decreased by 7.5%. The ∆*AaPdel* reduced growth on regular PDA medium by 6.6–7.8% compared with the wild type (**Figure 2B**). By light microscope observation, the wild type exhibited abundant and thick hyphal branch, while the hyphal growth of *AaPdel* deletion mutants was sparse (**Figure 2C**). However, the fungal strains defective of *AaPdeh*, coding high-affinity cAMP phosphodiesterase, exhibited no obvious growth defects in radical growth and colony morphology, which were in line with the wild type. The above results indicate that the *AaPdel* gene is involved in the mycelial growth of *A. alternata* while *AaPdeh* has a limited contribution.
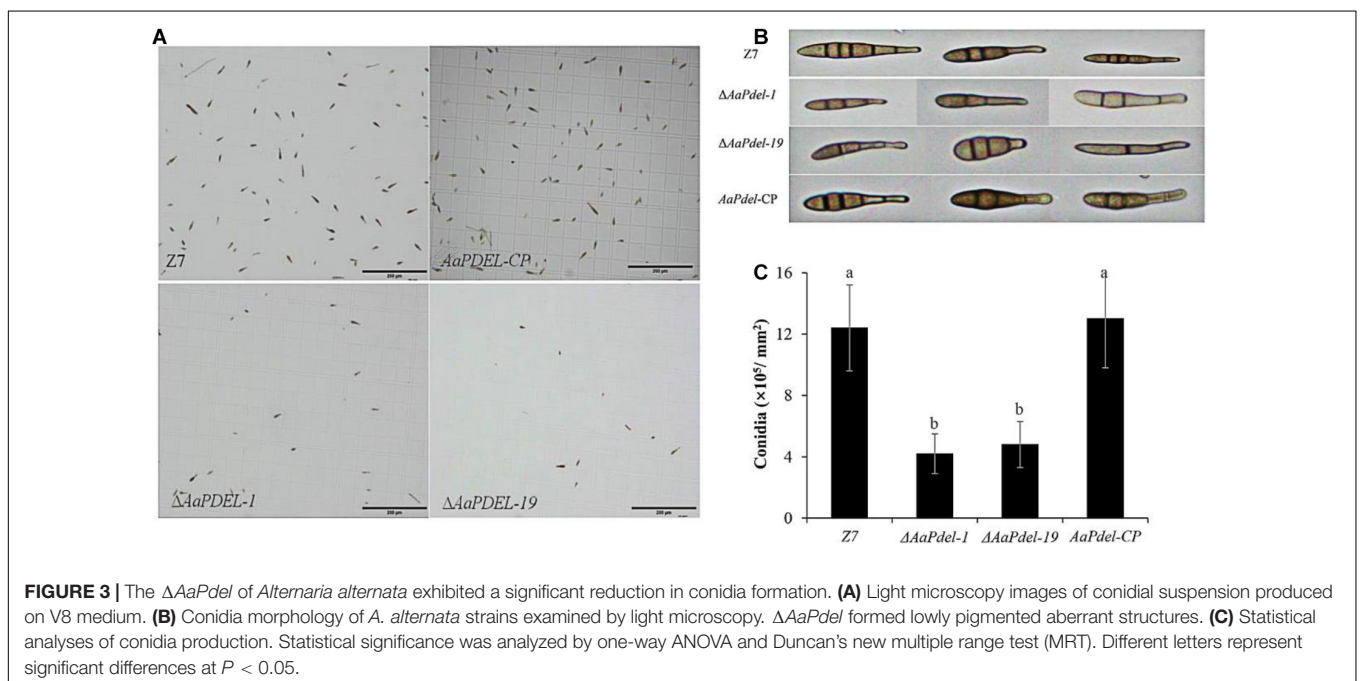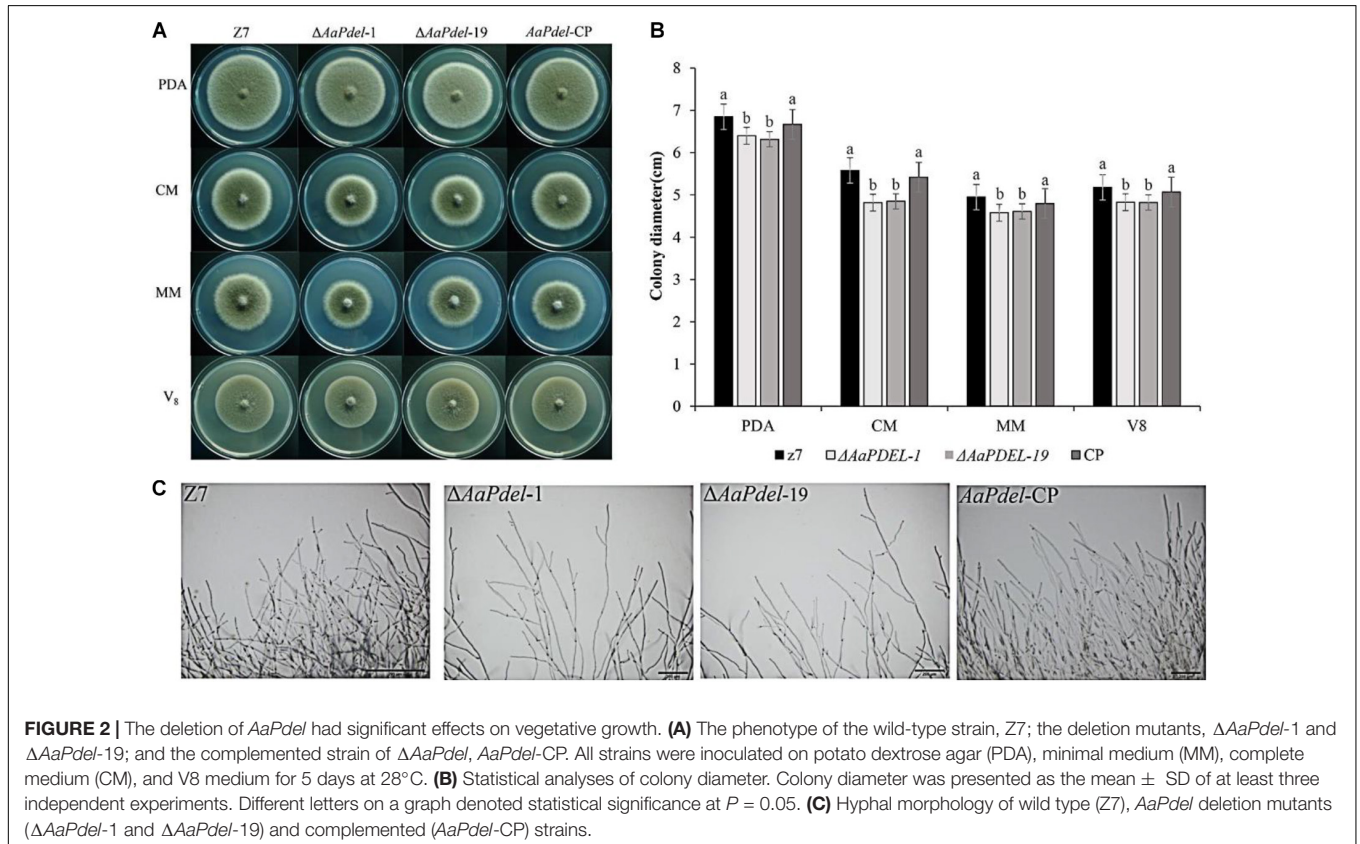
AaPdel Contributes to Conidia Formation and Morphology

To analyze potential gene functions in conidiation, the conidial suspension of wild type, deletion mutants, and supplementary strains were counted on a hemocytometer and photographed

under a microscope at 40 times magnification. On the V8 medium, the ΔAaPdel formed colony with 10% reduced-growth-rate and produced approximately 65% reduced conidiation compared to the wild type (**Figure 3A**). The sporulation of the

wild type Z7 was about 12.38×10^5, while the sporulation of the AaPdel mutant was about 4×10^5 (**Figure 3C**). Over 200 spore diameters of each strain were measured, and the spore morphology was observed under a microscope. The conidia of

FIGURE 2 | The deletion of AaPdel had significant effects on vegetative growth. **(A)** The phenotype of the wild-type strain, Z7; the deletion mutants, ΔAaPdel-1 and ΔAaPdel-19; and the complemented strain of ΔAaPdel, AaPdel-CP. All strains were inoculated on potato dextrose agar (PDA), minimal medium (MM), complete medium (CM), and V8 medium for 5 days at 28°C. **(B)** Statistical analyses of colony diameter. Colony diameter was presented as the mean ± SD of at least three independent experiments. Different letters on a graph denoted statistical significance at P = 0.05. **(C)** Hyphal morphology of wild type (Z7), AaPdel deletion mutants (ΔAaPdel-1 and ΔAaPdel-19) and complemented (AaPdel-CP) strains.

FIGURE 3 | The ΔAaPdel of Alternaria alternata exhibited a significant reduction in conidia formation. **(A)** Light microscopy images of conidial suspension produced on V8 medium. **(B)** Conidia morphology of A. alternata strains examined by light microscopy. ΔAaPdel formed lowly pigmented aberrant structures. **(C)** Statistical analyses of conidia production. Statistical significance was analyzed by one-way ANOVA and Duncan's new multiple range test (MRT). Different letters represent significant differences at P < 0.05.

$\Delta AaPdel$ had varied morphologies, forming lowly pigmented aberrant structures. The conidia of $\Delta AaPdel$ were light gray, while the wild type and the complementary strain produced typical dark brown conidia (**Figure 3B**). However, through statistical analysis, $AaPdel$ was not involved in conidia diameter and germination rate. There was no significant difference between $\Delta AaPdeh$ and wild type in terms of conidia production, germination rate, and morphology (**Supplementary Figure 2**). In summary, the $AaPdel$ gene is involved in regulating the conidia production and pigment synthesis of conidia in *A. alternata*.

AaPdel Plays a Role in Intracellular cAMP Level Regulation

Two cAMP phosphodiesterase-encoding genes were analyzed in the regulation of the intracellular cAMP level. Compared with the wild type, $\Delta AaPdel$ led to decreased accumulation of cAMP level, while the loss of $AaPdeh$ led to the similar accumulation of cAMP level with the wild type. The trend was further confirmed by the complementation assay (**Figure 4**). The intracellular cAMP level of $AaPdel$-CP and $AaPdeh$-CP was consistent with the wild-type strain. $AaPdel$ plays a role in intracellular cAMP level regulation.

AaPdel Is Involved in Response to Osmotic Stress and Oxidative Stress

To analyze the defects in response to the osmotic stress on the mutants, the activated strains were inoculated on PDA medium amended with 1 M sorbitol, 1 M NaCl, 1M KCl, or 1 M sucrose. Compared with the growth of the PDA medium with no stress factor present, the growth of all tested strains was restrained to the osmotic stress. The $\Delta AaPdel$-1 and $\Delta AaPdel$-19 mutants
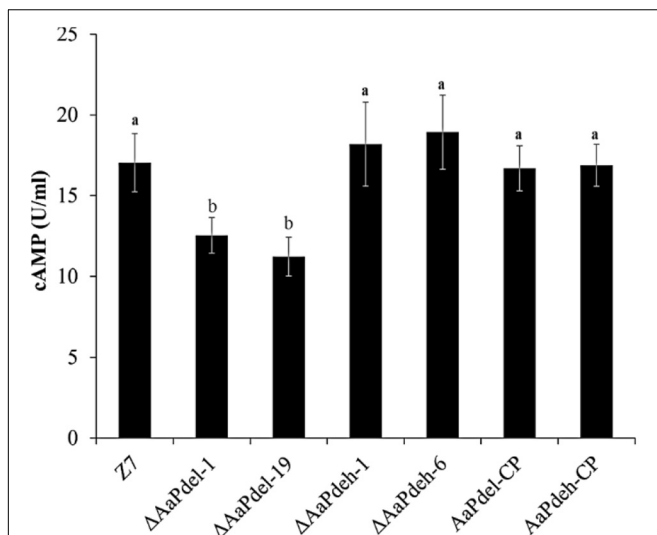
FIGURE 4 | Intracellular cAMP quantification of $\Delta AaPdel$ and $\Delta AaPdeh$ during the hyphal stage in *Alternaria alternata*. Bar graphs depicted the levels of intracellular cAMP in the mycelia of $\Delta AaPdeh$, $\Delta AaPdel$, wild type, and complemented strain, which were cultured in liquid PDB for 48 h. Bars represented standard error. Different letters mark significant differences at $P < 0.05$.

were hypersensitive to the osmotic stress inducers. The $\Delta AaPdel$ exhibited greater sensitivity to the non-ionic osmotic stress (sorbitol and sucrose) than the ionic osmotic stress (NaCl and KCl) (**Figure 5A**). Among these, the growth rate of $\Delta AaPdel$-1 in sorbitol or sucrose were decreased by 10.58 and 10.11%, respectively, compared with the wild-type strain. Whereas with the ionic osmotic stress, the inhibitory effect of $\Delta AaPdel$, $\Delta AaPdeh$ was similar compared with the wild type (**Table 1**). Online analysis of protein subcellular localization revealed that AaPdel protein was mainly located on the plasma membrane. According to the data, this different phenotype might be caused by the same concentrate Na^+ and K^+ treatment (**Figure 5B**).
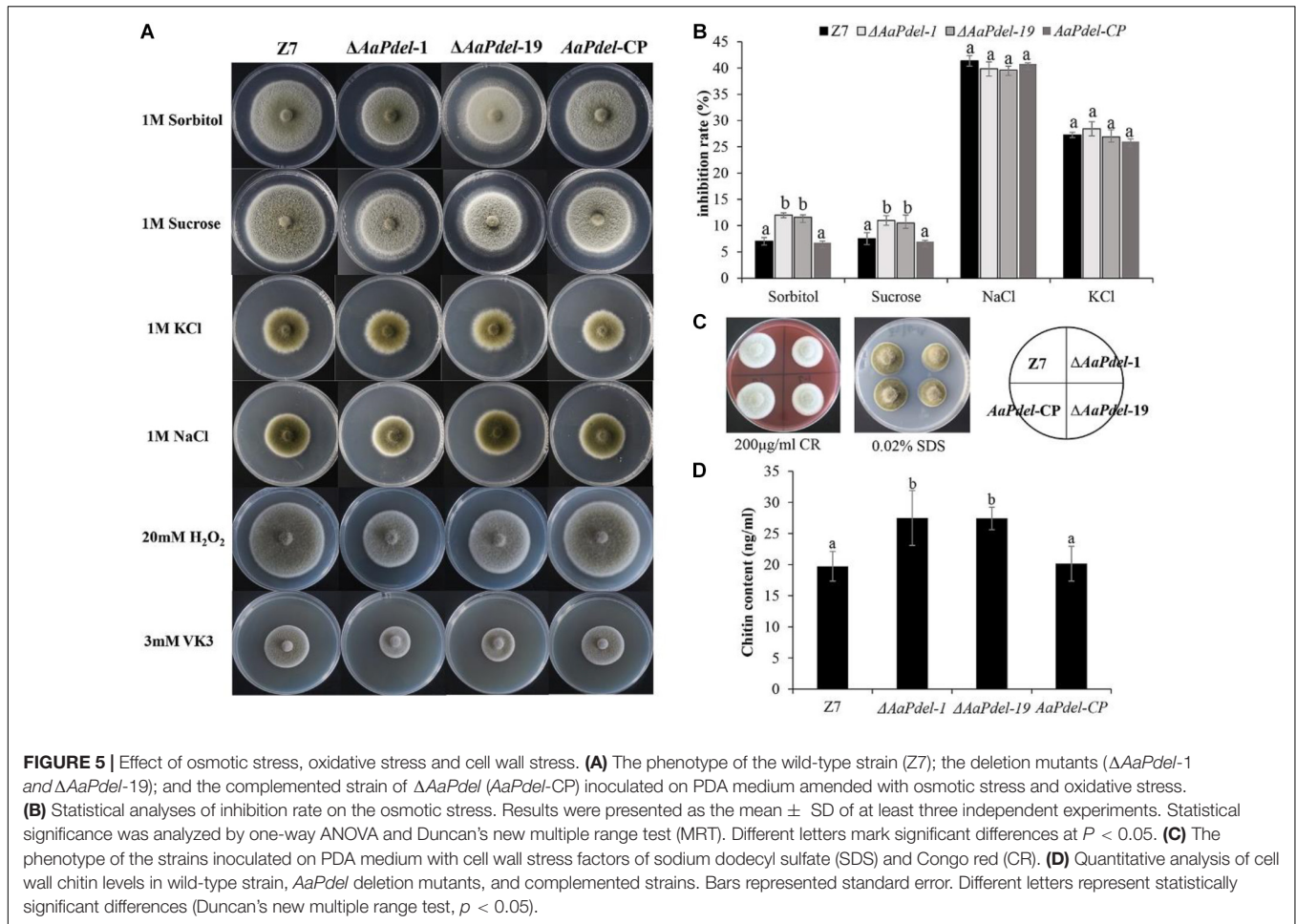
In order to analyse the defects in response to the oxidative stress, the activated strains were inoculated on PDA medium added with oxidative stress factors (20 mM H_2O_2 or 3 mM VK_3) after 5 days cultured at 28°C. The $\Delta AaPdel$ was hypersensitive to the oxidative stress inducers (**Figure 5A**). $\Delta AaPdel$-1 had 23.24 and 66.67% inhibition rate, respectively, on PDA supplemented with H_2O_2 or VK_3. The details of inhibition rate were presented in **Table 1**. However, under osmotic and oxidative stresses, the inhibition rates of $\Delta AaPdeh$-1 and $\Delta AaPdeh$-6 were similar to the wild type (**Supplementary Figure 3**).

AaPdel Played a Role in the Maintenance of Cell Wall Integrity

Cell wall stress tolerance assays were performed by inoculating WT and mutant strains onto PDA medium supplemented with either 0.02% SDS or 100 μg/ml of CR. By serving PDA as control, the growth of strains was restrained to both SDS and CR. And the $AaPdel$ mutants were hypersensitive to the cell wall stress reagents (**Figure 5C**). Compared with the wild type, the inhibition rate of the $AaPdel$ mutants was significantly increased in 0.02% SDS (**Table 1**). Chitin, the major component of the cell wall in *A. alternata*, was further quantified in deletion mutants and the wild-type strain. The chitin content of $\Delta AaPdel$ was higher than that of the wild type and complemented strain, indicating $AaPdel$ was involved in cell wall integrity and architecture (**Figure 5D**). However, the $\Delta AaPdeh$-1 and $\Delta AaPdeh$-6 were not more sensitive to the cell wall stress reagents. These stresses disturb cell wall biosynthesis in *A. alternata*, activating the CWI pathway. The CWI of the $AaPdel$ mutant was affected, indicating that $AaPdel$ is involved in the maintenance of CWI.

AaPdel Is Required for Pathogenicity

The pathogenicity was assessed by inoculating the leaves of tangerine with conidial suspension at a concentration of 1.0×10^5 conidia ml^{-1}. At 3 dpi, there were obvious lesions on the part of the leaves infected by wild type and complementary strains, but only mild lesions were observed on the leaves inoculated with $\Delta AaPdel$-1 and $\Delta AaPdel$-19. At 5 dpi, $\Delta AaPdel$-1 and $\Delta AaPdel$-19 conidial suspensions incited small necrotic lesions in the leave (**Figure 6**). According to Perina et al. (2019), the average scale levels quantified the disease severity of deletion mutants and Z7 were 4 and 6, respectively. It showed nearly

FIGURE 5 | Effect of osmotic stress, oxidative stress and cell wall stress. **(A)** The phenotype of the wild-type strain (Z7); the deletion mutants ($\Delta AaPdel$-1 and $\Delta AaPdel$-19); and the complemented strain of $\Delta AaPdel$ ($AaPdel$-CP) inoculated on PDA medium amended with osmotic stress and oxidative stress. **(B)** Statistical analyses of inhibition rate on the osmotic stress. Results were presented as the mean ± SD of at least three independent experiments. Statistical significance was analyzed by one-way ANOVA and Duncan's new multiple range test (MRT). Different letters mark significant differences at $P < 0.05$. **(C)** The phenotype of the strains inoculated on PDA medium with cell wall stress factors of sodium dodecyl sulfate (SDS) and Congo red (CR). **(D)** Quantitative analysis of cell wall chitin levels in wild-type strain, $AaPdel$ deletion mutants, and complemented strains. Bars represented standard error. Different letters represent statistically significant differences (Duncan's new multiple range test, $p < 0.05$).

TABLE 1 | Inhibition rate of osmotic, oxidative stress and cell wall integrity assay in the wild type Z7, $\Delta AaPdel$, $AaPdel$-CP in *Alternaria alternata*.

Strain	Inhibition rate (%)							
	Sorbitol 1M	Sucrose 1M	NaCl 1M	KCl 1M	Congo Red 200 μg/ml	SDS 0.02%	H$_2$O$_2$ 20 mM	VK$_3$ 3 mM
Z7	7.03 ± 0.71[A]	7.52 ± 0.46[A]	41.36 ± 0.32[A]	27.25 ± 0.45[A]	45.75 ± 3.37[A]	7.54 ± 0.61[A]	12.65 ± 3.17[A]	49.56 ± 5.22[A]
$\Delta AaPdel$-1	11.98 ± 1.15[B]	10.98 ± 0.92[B]	39.84 ± 0.25[A]	28.43 ± 1.51[A]	53.75 ± 2.32[B]	15.63 ± 1.25[B]	23.24 ± 5.86[B]	66.67 ± 7.29[B]
$\Delta AaPdel$-19	11.61 ± 0.98[B]	10.50 ± 1.35[B]	39.58 ± 0.25[A]	26.91 ± 0.80[A]	54.49 ± 4.51[B]	13.55 ± 0.95[B]	21.24 ± 3.57[B]	56.08 ± 2.80[B]
$AaPdel$-CP	6.75 ± 0.51[A]	6.95 ± 0.35[A]	40.75 ± 0.50[A]	26.00 ± 1.35[A]	44.98 ± 2.65[A]	7.50 ± 0.51[A]	11.55 ± 2.11[A]	52.26 ± 3.80[A]

*Different letters represent statistically significant differences (Duncan's new multiple range test, $p < 0.05$).*

75% reduced lesion area compared with WT. And *Pdel*-CP strain complemented the attenuated pathogenicity of $\Delta AaPdel$. In contrast, the necrotic lesions induced by $\Delta AaPdeh$-1 and $\Delta AaPdeh$-6 were as serious as the wild type in pathogenicity (**Supplementary Figure 4**). Taken together, *AaPdel*, coding low-affinity cAMP phosphodiesterase, was involved in regulating the pathogenicity of *A. alternata*.

AaPdel and *AaPdeh* Regulate Laccase Activity

Laccase is a copper-containing oxidase mainly involved in the catalysis of lignin degradation, pigment synthesis,

and fungal pathogenicity. To determine the involvement of PDEs in laccase activity, we used a specific substrate, 2,2′-azino-di-3-ethylbenzthiazoline-6-sulfonate (ABTS). The oxidized dark purple stain can be observed at the colonies of both the $\Delta AaPdel, \Delta AaPdeh$ mutants and the wild-type strain (**Figure 7A**). To further demonstrate the laccase activity difference between *AaPdel* and *AaPdeh* mutants, the crude enzyme solution was extracted and analyzed by spectrophotometer at 420 nm for 5 min. The statistical analyses showed that the laccase activity of both $\Delta AaPdel$ and $\Delta AaPdeh$ mutants were significantly lower than that of the wild type, and $\Delta AaPdel$ had a stronger inhibitory effect on laccase (**Figure 7B**). The above results indicate that both *AaPdel* and *AaPdeh* are
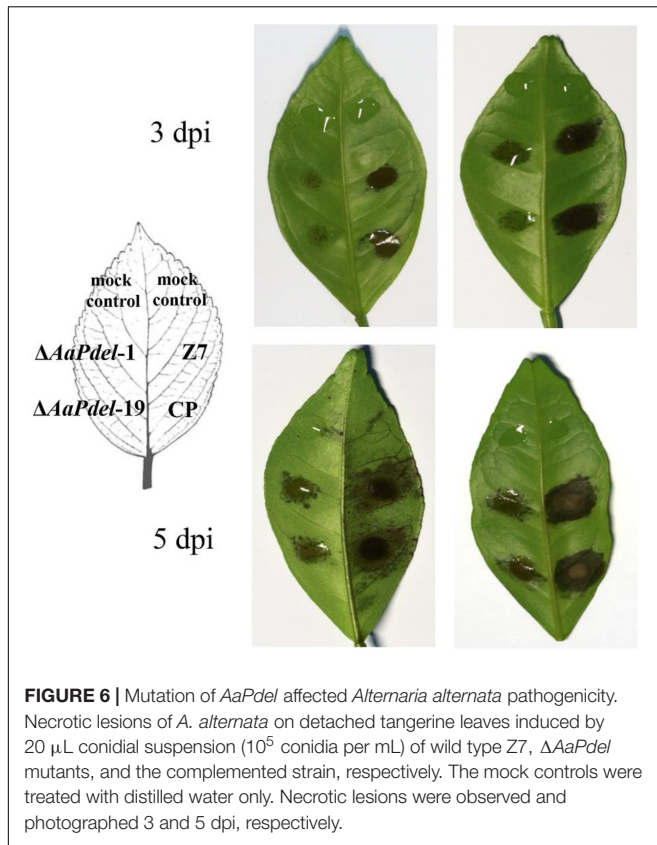
FIGURE 6 | Mutation of *AaPdel* affected *Alternaria alternata* pathogenicity. Necrotic lesions of *A. alternata* on detached tangerine leaves induced by 20 μL conidial suspension (10^5 conidia per mL) of wild type Z7, Δ*AaPdel* mutants, and the complemented strain, respectively. The mock controls were treated with distilled water only. Necrotic lesions were observed and photographed 3 and 5 dpi, respectively.

involved in regulating laccase activity, and *AaPdel* plays a primary role in this pathway.

DISCUSSION

In fungi and yeast, cAMP signaling cascade regulate a large amount of cellular and biological processes. Since the discovery of cAMP-PKA pathway, PDEs have been characterized as important phosphodiesterase for cAMP level regulation in plant pathogens, including the budding yeast (*S. cerevisiae*) and filamentous fungi (*F. graminearum*, *M. oryzae*, *C. albicans*, and so on). Two PDEs, low-affinity phosphodiesterase Pdel (Pde1) and high-affinity phosphodiesterase Pdeh (Pde2), are involved in cAMP level regulation (Bahn et al., 2003). *Pdeh* is widespread in fungi and mammals, while *Pdel* is only found in fungi. In this study, the biological functions of the *AaPdel* and *AaPdeh* in *A. alternata* were preliminarily explored through gene replacement strategy, and it was found that *AaPdel* but not *AaPdeh* played a dominant role in biological processes of *A. alternata*, which differed from previously reported findings. Our data further support the PKA-mediated cAMP signaling pathway by showing that the regulator is involved in intracellular cAMP level.

In filamentous fungi, identified *PDEL* gene functions are mainly concentrated on conidiation, as in *F. graminearum* and *Setosphaeria turcica*. Our result was similar to those described above. The conidia production of the *AaPdel* deletion mutant was 65% lower than that of the wild type and complemented

strain of Δ*AaPdel*. Also, the color of the Δ*AaPdel* conidia was relatively lighter. In *M. oryzae* and *Aspergillus flavus*, the *PdeH* deletion mutant also exhibited defects in vegetative growth and conidiation (Yang et al., 2017). In *S. turcica*, the deletion of the *StH-PDE* gene resulted in a loss of conidiation ability. Whereas the absence of *AaPdeh* did not result in the defects of conidia production, diameter, germination rate, and vegetative growth. The Δ*AaPdel* had growth defects on four vegetative growth media, and formed colonies with short, dense aerial hyphae. The growth defects of Δ*AaPdel* were extremely severe on the CM medium with sufficient nutrient elements. Comparing the utilization of basic nutrient elements, *AaPdel* might be involved in the utilization of certain nutrient elements. The *AaPdel* gene plays an important role in regulating conidiation and hypha growth.

The same concentration of osmotic stressors (sorbitol, sucrose, NaCl, and KCl) were used to analyze defects in stress response (Hanin et al., 2016). Δ*AaPdel* was more sensitive to non-ionic osmotic stress (sorbitol and sucrose) than ionic osmotic stress (NaCl and KCl). Among the analyzed osmotic stressors, sorbitol (a sugar alcohol), had the most significant inhibitory effect on the *AaPdel* deletion mutant. Whereas in the ionic osmotic stress, the inhibitory effect was the same when comparing the Δ*AaPdel*, Δ*AaPdeh*, and wild-type strain. According to the data, this phenotypic difference might be caused by the same concentration Na^+ and K^+ treatment. Analyses of protein subcellular localization revealed that the AaPdel protein mainly located on the plasma membrane. Hence, it can be hypothesized that *AaPdel* is involved in sodium-potassium pump regulation on the plasma membrane.

As the first barrier, the fungal cell wall and plasma membrane were of great significance to maintain the structural integrity and against external stresses. The fungal cell wall and plasma membrane are indispensable for maintaining the shape of the cell, and are also involved in signal transmission. In this study, Δ*AaPdel* was more sensitive to cell wall inhibitors CR and SDS, while Δ*AaPdeh* growth was similar to the wild type. SDS acts as a cell wall inhibitor by reducing the stability of the membrane to reveal the defect of the cell wall. CR inhibits binding to major components of the fungal cell wall, chitin and β-1,4-glucan (Gong et al., 2020). On the one hand, *AaPdel* appeared to be the sensor for signaling in response to the cell wall antagonist CR, which interferes with cell wall assembly by binding to chitin. Further analyses showed that the high chitin content of Δ*AaPdel* might account for the sensitivity to CR. However, the Δ*AaPdeh*-1 and Δ*AaPdeh*-6 were not more sensitive to cell wall stress reagents. Maintenance of cell wall integrity relies on the CWI pathway, a conserved cascade pathway, triggered by transmembrane sensors in response to extracellular stresses in fungus. These stresses disturb cell wall biosynthesis in *A. alternata*, activating the CWI pathway. The CWI of the *AaPdel* mutant was affected, indicating that *AaPdel* was involved in the maintenance of CWI.

Laccase is a copper-containing oxidase mainly involved in the catalysis of lignin degradation, pigment synthesis, and fungal pathogenicity (Mtibaa et al., 2018). The phenotype of lighter conidia color and attenuated pathogenicity led us to analyze the laccase activity. To determine the involvement of PDEs,
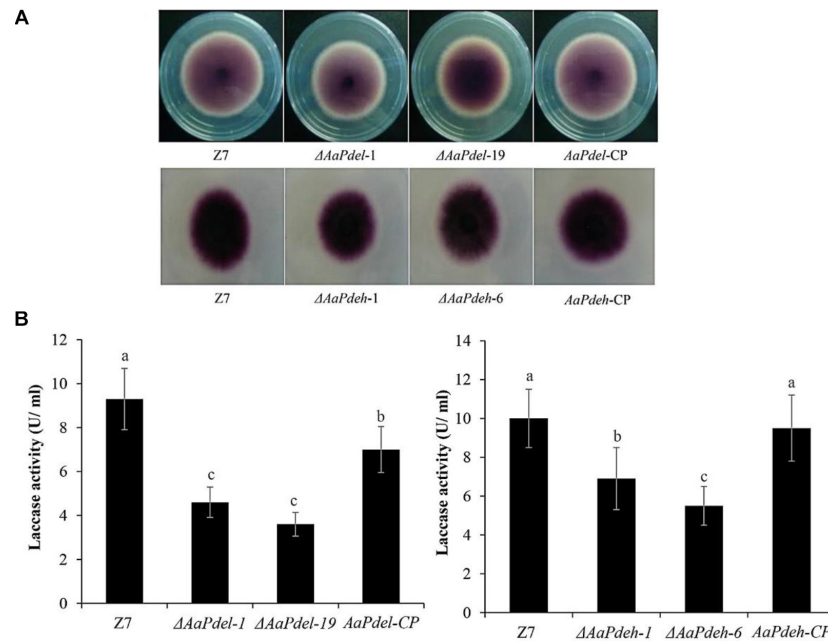
FIGURE 7 | The laccases activity assays of Δ*AaPdel* and Δ*AaPdeh* of *Alternaria alternata*. **(A)** Laccase activity was tested on PDA medium with 0.03% ABTS culture at 28°C. Discoloration was observed and photographed 6 dpi. **(B)** Statistical analyses of laccase activity, which was determined by the ABTS oxidizing test. Statistical significance was analyzed by one-way ANOVA and Duncan's new multiple range test (MRT). Different letters mark significant differences at $P < 0.05$.

the laccase activity of the Δ*AaPdel*, Δ*AaPdeh*, wild type, and complemented strain were tested and compared. We found that the wild type and complemented strain produced laccase, forming a dark purple hole on the ABTS medium, whereas the laccase activity of both Δ*AaPdel* and Δ*AaPdeh* was significantly lower than that of wild type and complemented strain. Consistent with our results, previous studies have demonstrated that the *MoPdeh* and *MoPdel* genes regulate intracellular laccase activity, and *MoPdel* plays a regulatory role in *M. oryzae*.

Research addressing gene functions in fungi has mainly focused on pathogens and those genes involved in pathogenicity or virulence. Among the studied PDEs, some genes encoding high-affinity phosphodiesterase affect pathogenicity. In *S. turcica*, the *StH-PDE* gene regulates secondary metabolism, melanin synthesis, and pathogenicity. The gene encoding high-affinity phosphodiesterase in *T. serrata* is involved in the regulation of mycelial growth and pathogenicity. *MoPdeH* (*Pde2*) has a dominant role in conidiation, pathogenicity, and intracellular cAMP level regulation, whereas *MoPdel* (*Pde1*) had no obvious function in *M. oryzae*. In this research, the finding was contrary to previous studies. In *A. alternata*, the Δ*AaPdel* mutant induced much smaller lesions than that of the wild type, whereas the Δ*AaPdeh* mutant induced lesions as drastic as those in the wild-type strain. Chitin is one of the fungal PAMPs, and the high content in Δ*AaPdel* may triggered rapid and strong plant parttern-triggered immunity. Above all, the attenuated pathogenicity of Δ*AaPdel* was probably caused by defects in the utilization of certain nutrient elements, osmotic stress response, plasma membrane, low laccase activity, high chitin content in the cell wall, and PKA-mediated cAMP signaling pathway.

PDEs target specific intracellular sites or signal transduction complexes and localize not only in the cytoplasm, but also in membrane, nucleus, and cytoskeleton locations (Houslay et al., 1998). Hu et al. (2010) reported that, like its low-affinity counterpart, the high-affinity phosphodiesterase may also play an important role in PKA negative feedback loop through the Pde2 localization assays. The localization of Pde2-GFP was affected by the carbon sources available and the cAMP-dependent PKA in the yeast *S. cerevisiae*. Pde2 is concentrated in the nucleus of cells grown on glucose, while it is distributed in the nucleus and cytoplasm of cells grown on some non-fermentable carbon sources. And PKA positively regulates the nuclear accumulation of Pde2. Elevated PKA activity increased the nuclear concentration of Pde2. Whereas in PKA attenuated strains, Pde2 is unable to concentrate in the nucleus of cells grown on glucose. Ramanujam and Naqvi (2010) reported the location of PdeH-GFP and PdeL-GFP during asexual and pathogenic development in *M. oryzae*. Although PdeH-GFP was cytoplasmic, it was dynamically associated with the plasma membrane and vesicle compartment, and the PdeL-GFP was predominantly localized in the nucleus. In *Aspergillus flavus*, PdeL-GFP shows a strong fluorescent signal in the nucleus of the hyphae, and PdeH-GFP is likely to be localized to punctate structures in the cytosol, rather than the nucleus. The localization of PDEs is complicated and dynamic. And limited to the resolution of the microscope, the reported location of the PDEs haven't been directly localized to the organelle. In this study, we use online software to analyze the gene sequence. Online analysis of protein subcellular localization revealed that the AaPdel protein was predominantly located on the plasma membrane

and the AaPdeh protein was located in the nucleus. From the perspective of protein subcellular localization, the AaPdeh protein was parallel to MoPdeL and AfPdeL, both in the nucleus. While AaPdel protein was similar to MoPdeH and AfPdeH. Further analysis of the signal peptides and transit peptides in AaPdel protein, revealed that the AaPdel protein was localized to the secretory pathway. We also investigate whether the two cAMP phosphodiesterase-encoding genes directly contribute to the intracellular cAMP level. Compared with the wild type, $\Delta AaPdel$ led to decreased accumulation of cAMP level, while the loss of AaPdeh led to the similar accumulation of cAMP level with the wild type. AaPdel regulated the intracellular cAMP level. This inverse trend of gene function in A. alternata was in accordance with the protein location difference between many fungi. We speculate that although PDEs homology has been identified in many filamentous fungi, their functions are not consistent, and there was no clear association between the conserved domain and physiological function. The inconsistent gene function may due to different protein location. It indicated that PKA-mediated cAMP signaling pathway was complicated in A. alternata, and needed further analysis.

In this study, the AaPdel and AaPdeh genes were identified to encode low-affinity PDEs (Pdel) and high-affinity PDEs (Pdeh) in A. alternata. We used a gene deletion and complementation strategy to characterize the gene function of AaPdel and AaPdeh. In A. alternata, AaPdel regulated intracellular cAMP levels during the hyphal stage. Deletion of AaPdel, but not AaPdeh, was found to play a role in conidiation and pathogenicity in A. alternata. $\Delta AaPdel$ showed a defect in conidiation, producing approximately 65% reduced conidiation and forming lowly pigmented aberrant structures. Upon exposure to osmotic stressors, $\Delta AaPdel$ had growth defects in response to osmotic stress, which was more sensitive to non-ionic osmotic stress than ionic osmotic stress. This different phenotype might be caused by the same concentrate Na^+ and K^+ treatment. Moreover, AaPdel deletion mutants had defects in vegetative growth, hyphal growth and pathogencity. Further analyses showed that the high chitin content of $\Delta AaPdel$ might account for the sensitivity to CR. Based on the attenuated pathogenicity and lowly pigmented aberrant structures, the laccase activity analyses found that both AaPdel and AaPdeh were involved in laccase activity regulation. Our data further support the PKA-mediated cAMP signaling pathway, as we have found the regulators of AaPdel is involved in intracellular cAMP levels in A. alternata. The inconsistent gene function may due to different protein location. In A. alternata, cAMP-PKA pathway played important roles in signal transduction, but the function of the genes involved in this pathway required further study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KT, XK, and WL conceived and designed the experiments. CZ contributed to reagents, materials, and analysis tools. WL and XK performed the experiments. WL, KT, and CZ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2020.597545/full#supplementary-material

Supplementary Figure 1 | PCR results of $\Delta AaPdel$ transformants were verified by three primer pairs of PLP1F/H855R, H856F/PLP2R, and PLF/PLR. AaPdeh transformants were identified through PHP1F/H855R, H856F/PHP2R, and PHF/PHR.

Supplementary Figure 2 | The conidia of AaPdeh deletion mutants exhibit similar phenotype with wild-type strain. **(A)** The conidia morphology examined by light microscopy. **(B)** Statistical analysis of conidia production. The quantity of conidia was quantified after cultured on the V8 medium in dark for 8 days. Bars indicate standard errors of at least three repetitions.

Supplementary Figure 3 | The sensitivity of the AaPdeh mutants to osmotic stress. Z7, $\Delta AaPdeh$-1, $\Delta AaPdeh$-6, and AaPdeh-CP strains were inoculated on PDA amended with osmotic stressors, NaCl, KCl, sorbitol or sucrose, respectively.

Supplementary Figure 4 | Similar necrotic lesions induced by $\Delta AaPdeh$ strain and wild type Z7 on detached tangerine leaves. The conidial suspension at 1.0×10^5 conidia/ml of Alternaria alternata wild type Z7, $\Delta AaPdeh$ and the complemented strains were inoculated on leaves, respectively. The purified water was used as mock control.

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