Oxalic Acid Metabolism Contributes to Full Virulence and Pycnidial Development in the Poplar Canker Fungus *Cytospora chrysosperma*

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ABSTRACT

Poplar *Cytospora* canker, which is mainly caused by *Cytospora chrysosperma*, is one of the most destructive and widespread tree diseases worldwide. Although oxalic acid (OA) is demonstrated as an important virulence determinant in several necrotrophic fungi, specific functions of OA during pathogenesis remain controversial. Here, we identified three genes (*CcOah*, *CcOdc1*, and *CcOdc2*) directly involved in OA biosynthesis and catabolism in *C. chrysosperma*. We demonstrated that *CcOah* is required for OA biogenesis. All three genes were found to be highly upregulated during early infection stages of the poplar stem. The deletion of any of the three genes led to an obvious reduction of pycnidial production but no abnormality of hyphal growth and morphology. Furthermore, the individual deletion strain exhibited significantly limited lesion sizes on poplar twigs and

leaves. Exogenous application of OA or citric acid can complement the virulence defects of $\Delta CcOah$ and $\Delta CcOdc1$ strains. We further found that the $\Delta CcOah$ strain strongly promoted reactive oxygen species burst of poplar leaves during infection. Finally, induced secretion of OA was observed by monitoring color change of the plates after poplar stem extracts were added in the cultures; however, we failed to quantify OA concentration by high-performance liquid chromatography. Taken together, the present results provide insights into the function of OA acting as an important virulence factor of *C. chrysosperma*.

Keywords: *Cytospora chrysosperma*, mycology, oxalic acid, poplar canker, ROS, virulence

Species of the genus *Cytospora* are ubiquitous pathogens that cause destructive canker or dieback of woody hosts around the world. Traditionally, sexual morphs of *Cytospora* have been classified into several genera, mainly including *Valsa* (Rossman et al. 2015). *Cytospora* or *Valsa* canker diseases can be devastating to perennial plants such as *Populus* (Adams et al. 2006), *Malus* (Wang et al. 2014), and *Prunus* (Farr and Rossman 2015). In China, poplar canker and apple canker occur annually and have resulted in huge economic and ecological losses in landscape greening and apple production; however, disease prevention and control measures are still limited (Fan et al. 2015). The fungus *Cytospora chrysosperma* is the main causal agent of poplar canker, which can cause gradual twig and branch mortality, or even entire tree death. As a necrotrophic fungus, the colonization of *C. chrysosperma* is first restricted to injured or dying bark in healthy poplar; then, the hyphae rapidly and expansively spread in poplar stems weakened by drought or freeze damage (Biggs et al. 1983). Previously, investigation of the fungal taxonomy, histopathology, and epidemiology has been extensive (Biggs et al. 1983; Fan et al. 2015; Tao et al. 1984; Zhang et al. 2017) but the molecular basis underlying the pathogenicity of the poplar canker fungus remains largely unknown.

Necrotrophic plant pathogens employ various phytotoxins and hydrolytic enzymes to induce cell necrosis and obtain nourishment (Mengiste 2012). Oxalic acid (OA), a nonspecific phytotoxin, is determined to be a primary arsenal in necrotrophic fungi such as *Sclerotinia sclerotiorum* (Dutton and Evans 1996; Liang and

Rollins 2018; Xu et al. 2018). OA is remarkably multifunctional and participates in numerous pathogenic processes. For example, OA is secreted in the early infection stage of *S. sclerotiorum* and acidifies the host environment (Kim et al. 2007), inducing plant cell death as an elicitor via regulating the generation of reactive oxygen species (ROS) in the host (Kim et al. 2008).

Biosynthesis and degradation of OA have been genetically defined in fungi (Liang and Rollins 2018). Biochemically, OA biogenesis is predominately generated from oxaloacetate via hydrolysis of oxaloacetate acetylhydrolase (*Oah*) in fungi (Chen et al. 2010; Gombert et al. 2011; Pedersen et al. 2000a). In addition, OA biosynthesis is regulated by the PacC-mediated pH signaling pathway which senses changes of ambient pH (Peñalva et al. 2008). With the increase of OA, ambient pH is drastically reduced and, in turn, OA secretion is induced. On the other hand, OA can be catabolized to produce formate and CO₂ by oxalate decarboxylase (*Odc*) after being secreted from fungal cells (Liang et al. 2015b). The involvement of *Oah* or *Odc* in virulence has been substantiated in several phytopathogenic fungi. For example, the deletion of the *Oah* homolog abolished OA accumulation and established very small restricted lesions in *S. sclerotiorum* (Liang et al. 2015a), *Botrytis cinerea* (Han et al. 2007), and *Cryphonectria parasitica* (Chen et al. 2010). Moreover, other supportive evidence of the importance of *Oah* in OA accumulation has been substantiated in other fungi such as *Aspergillus niger* (Pedersen et al. 2000b) and *Penicillium chrysogenum* (Gombert et al. 2011). Interestingly, the *S. sclerotiorum* genome contains two *Odc*-encoding genes; however, only disruption of *Ssodc2* reduces oxalate accumulation and fails to develop normal compound appressorium (Liang et al. 2015a).

In addition to a two-phase infection model and unknown pH regulators, recent updates of virulence theories of *S. sclerotiorum* put forward a complicated new insight into understanding the pathogenesis of necrotrophic fungi (Liang and Rollins 2018; Xu et al. 2018). Coupled with these advances, molecular elucidation of pathogenicity of canker fungi has been promoted. Recently, a large number of virulence-contributing factors (such as regulatory factors, cell wall degrading enzymes, effectors, and so on) were

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identified in the apple canker fungus *Valsa mali* (Nie et al. 2019; Wu et al. 2018; Zhang et al. 2018, 2019), and especially demonstrated that VmPacC is involved in the acidification of its environment and is required for full virulence (Wu et al. 2018). In *Cytospora chrysosperma*, CcPmk1 was characterized, which was required for fungal growth, cell wall integrity, and pathogenicity (Yu et al. 2019). However, it is not known whether OA metabolism affects virulence in these canker fungi. Therefore, understanding the function of OA during *C. chrysosperma* infection might broaden and provide a basis for illuminating the potential development of novel sustainable canker management strategies.

In this study, we showed the strongly induced expression of *Oah* and *Odc* during *C. chrysosperma* infection and determined the key roles of *Oah* in OA accumulation. Furthermore, we revealed that *Oah* and *Odc* are crucial for full virulence and affect pycnidial development. The observation that exogenous addition of OA or citric acid (CA) restored the virulence defects of the $\Delta CcOah$ and $\Delta CcOdc1$ strains suggested that acidification of host environment is required for pathogenesis of *C. chrysosperma*. Finally, the $\Delta CcOah$ strain elicited poplar ROS burst. These results demonstrated key roles of OA metabolism in full virulence and pycnidial development in *C. chrysosperma*.

MATERIALS AND METHODS

Fungal strains and culture conditions. The *C. chrysosperma* wild-type strain CN-1 was isolated from *Populus alba* var. *pyramidalis* in Gansu Province of China and was used as a recipient strain to generate the targeted gene deletion strains in this study. All strains were cultured on potato dextrose agar (1 liter PDA = 200 g of potato, 20 g of glucose, and 15 g of agar) at 25°C. Hygromycin- or geneticin-resistant strains were grown on PDA amended with hygromycin at 35 µg/ml (Sigma-Aldrich) or geneticin at 75 µg/ml (Sigma-Aldrich), respectively.

Identification of *Oah*, *Odc1*, and *Odc2*. The complete sequences of *Oah*, *Odc1*, and *Odc2* were retrieved from a local genome database of the *C. chrysosperma* CN-1 by using the BlastP program with their corresponding homologs of *S. sclerotiorum*. The domains of proteins were analyzed via InterPro (www.ebi.ac.uk/interpro).

The targeted disruption and complementation of *Oah*, *Odc1*, and *Odc2*. Targeted deletion strains of *CcOah*, *CcOdc1*, and *CcOdc2* were generated using the split-marker method (Goswami 2012). The entire open reading frame of each gene was replaced by a hygromycin resistance gene cassette. The upstream and downstream flanking sequences were amplified with primers (Supplementary Table S1); then, both flanking fragments were fused to the hygromycin resistance cassette by fusion PCR. All fragments were confirmed by sequencing analysis. The recombinant PCR fragments were used for polyethylene-glycol-mediated protoplast transformation, as described (Liu et al. 2017). The transformants were cultured on TB₃ medium (3 g of yeast extract, 3 g of casamino acids, 20% sucrose, and 0.7% agar) supplemented with hygromycin at 35 µg/ml; then, the transformants were screened using PCR amplification by external and internal primers to confirm gene replacement (Supplementary Table S1). Three individual deletion strains were obtained for each gene. For the complementation of the targeted deletion strains, a fragment containing the entire coding region of each gene along with its native promoter and terminator regions was amplified using PCR with the specific primers (Supplementary Table S1). The complementation strains were selected with geneticin at 75 µg/ml by reintroducing the wild-type copy of every gene.

Virulence analysis. Twigs and leaves of 1-year-old *Populus × beijingensis* from the Beijing Forestry University nursery garden were used for virulence assay. Twigs were cut into 20-cm-long segments, then washed with distilled water and sealed with wax. Every segment was subjected to scald wound and inoculated with

5-mm agar plugs of 2-day-old cultures of the wild-type CN-1, deletion, and complemented strains. Leaves were scalded and inoculated with mycelial plugs. The controls were mock inoculated with agar plugs. All inoculated twigs and leaves were placed in trays to maintain humidity at 25°C under day-and-night cycles. Lesions were outlined using a preservative cling film purchased at our local grocery store. The cling film specifically outlined the lesioned area, which was then placed against graph paper to quantify the area of the lesion. The graph paper measured the area of lesions by each square of 1 mm². This method was loosely based upon Yu et al. (2019). All assays were repeated three times with 25 twigs or leaves for each replicate.

Exogenous application of OA or CA was preformed to the scald wound of twigs and leaves using 20 µl of the buffer with different concentrations (100, 200, and 300 µg/ml). Then, pretreated twig and leaves were inoculated with mycelial plugs as mentioned above.

Extracellular OA detection assay. For detection of OA accumulation, the color change was observed on SM medium (10 g of glucose, 2 g of asparagine, 0.2 g of MgSO₄, 0.4 g of KH₂PO₃, 0.2 g of KCl, 0.01 g of FeSO₄, 0.01 g of ZnSO₄, and 0.01 g of MnSO₄ per liter) for 4 days, then amended with methyl red at 2 mg/ml (Beijing Chemical Factory), bromocresol purple at 1 mg/ml (Beijing Chemical Factory), and bromophenol blue at 1 mg/ml (Beijing Chemical Factory). For quantification of extracellular OA accumulation, the wild-type CN-1 and deletion strains were cultured in liquid SM medium without or with 20% poplar bark for 4 day; then, 1 ml of supernatant was filtrated with a 0.45-µm cellulose acetate membrane before was measured by high-performance liquid chromatography (HPLC) analysis at the School of Chemistry and Molecular Engineering, Peking University, Beijing, China. Standard OA (TMstandard) samples were prepared in pure water. HPLC was further performed using an Agilent 2100 series system with a C18 column using 0.1% HCl as a mobile phase, and ultraviolet detection at 210 nm.

ROS burst assay. For detection of H₂O₂ during infection, at 12 and 24 h postinoculation (hpi), leaves were stained in 3,3'-diaminobenzidine (DAB) buffer at 1 mg/ml (Sigma-Aldrich) by HCl shake for 12 h, then discolored with 95% alcohol and observed under a microscope (Leica DM 2500).

Quantitative real-time PCR analysis. RNA was extracted from poplar twigs inoculated by the wild-type CN-1 at 4, 12, and 24 hpi using TRIzol reagent (Invitrogen) and purified with the RNA Mini Kit (Ambion) in accordance with the manufacturers' instructions. RNA was reverse transcribed using the QuantScript RT Kit (TIANGEN Biotech, Beijing, China) for fluorescence quantitative PCR experiments. Quantitative real-time PCR (qRT-PCR) was performed with SuperReal Premix Plus (TIANGEN Biotech) using an ABI 7500 real-time PCR system (Applied Biosystems). The genes were tested independently and in triplicate. *CcActin* was used as an internal reference. Relative gene expression levels were calculated by the 2^{-ΔΔCt} method (Livak and Schmittgen 2001). All primers in this assay are listed in Supplementary Table S1.

Statistical analysis. Data were expressed as mean values ± standard error of the mean and analyzed with a one-way analysis of variance independent-samples Tukey's range test using SPSS for Windows, version 16.0. One asterisk indicated *P* value < 0.05 while two asterisks indicated significance at *P* value < 0.01.

RESULTS

Induced expression of *CcOah*, *CcOdc1*, and *CcOdc2* during early infection of *C. chrysosperma*. We identified *CcOah* (CCG_02905), *CcOdc1* (CCG_01318), and *CcOdc2* (CCG_01001) in the *C. chrysosperma* genome via a homolog search using BlastP with the corresponding genes from *S. sclerotiorum* as queries. The bioinformatics analyses showed that *CcOah* contains an isocitrate lyase/PEP mutase domain and *CcOdc1/2* contains two bipinin

domains (Supplementary Fig. S1). Phylogenetics revealed the three genes were well conserved in filamentous fungi.

The expression of the three genes during *C. chrysosperma* infection was determined. qRT-PCR data showed that their expression was significantly upregulated during early infection. Compared with 0 hpi, expression of *CcOah* and *CcOdc2* reached a peak (6.21 and 5.48 times, respectively) at 4 hpi, whereas *CcOdc1* reached a peak (35 times) at 12 hpi (Fig. 1). Interestingly, transcript levels of any of them at 36 and 48 hpi were comparable with that of 0 hpi (data not shown). These results suggest that OA metabolism might be involved in virulence of *C. chrysosperma*.

***CcOah*, *CcOdc1*, and *CcOdc2* are required for pycnidial development.** To investigate the roles of *CcOah*, *CcOdc1*, and *CcOdc2* in *C. chrysosperma*, we performed targeted gene replacement of the entire coding region of each gene with the hygromycin resistance cassette. Three individual deletion strains for *CcOah* ($\Delta CcOah$), *CcOdc1* ($\Delta CcOdc1$), and *CcOdc2* ($\Delta CcOdc2$) were acquired; moreover, the complemented strains were yielded by ectopically reintroducing the entire coding region of *CcOah*, *CcOdc1*, and *CcOdc2* into the corresponding deletion strains (Supplementary Fig. S2). The deletion strains displayed no obvious growth defects and colony morphology on PDA (Fig. 2A and

Fig. 1. Induced expression of *CcOah*, *CcOdc1*, and *CcOdc2* during plant infection of *Cytospora chrysosperma*. The relative expression of *CcOah*, *CcOdc1*, and *CcOdc2*, which are associated with oxalic acid metabolism, was analyzed during *C. chrysosperma* infection. RNA was extracted from mycelium-inoculated stems of poplar at the indicated time points. Their expression was normalized against the *CcAct1* gene and the values are the average of two biological replicates consisting of four technical replicates each. Error bars indicate standard deviation. Data were analyzed using the Duncan test ($n = 2$; ** indicates $P < 0.01$).

Fig. 2. Involvement of *CcOah*, *CcOdc1*, and *CcOdc2* in the vegetative growth and asexual reproduction. **A**, Colony morphology of the wild-type CN-1, $\Delta CcOah$, $\Delta CcOdc1$, $\Delta CcOdc2$, $\Delta CcOah$ -C, $\Delta CcOdc1$ -C, and $\Delta CcOdc2$ -C strains on potato dextrose agar (PDA) plates at 3 days postinoculation (dpi) at 25°C. **B**, Observations of pycnidia produced by each strain on PDA at 30 dpi. **C**, Relative mycelial growth of the indicated strain on PDA plates. **D**, Quantitative comparisons in pycnidia production of the indicated strains on PDA at 20 dpi. Error bars indicate standard deviation; * indicates $P < 0.05$.

C); however, pycnidial production was drastically reduced at 20 days postinoculation (dpi) compared with the wild-type CN-1 and complemented strains (Fig. 2B and D). Observations of the morphology of pycnidia produced by deletion strains and wild-type CN-1 showed no difference. The findings indicated that OA metabolism plays an important role in pycnidial production of *C. chrysosperma*.

***CcOah* is required for OA biogenesis in *C. chrysosperma*.**

To assess the importance of the three genes in OA biogenesis in *C. chrysosperma*, we first checked the color change of pH-indicating PDA plates caused by the acidification following fungal incubation. Unexpectedly, the plates supplemented with methyl red (yellow $\geq$ pH 6.2; red $\leq$ 4.4 pH), bromocresol purple (purple $>$ pH 6.8; yellow $<$ pH 5.2), and bromophenol blue (violet $>$ pH 4.6; yellow $<$ pH 3.0) failed to change color following fungal growth of the wild-type CN-1 and deletion strains (Fig. 3A). However, after the fungal cultures were coincubated by extracts of poplar barks on

plates, the plates supplemented with bromocresol purple rapidly changed color from violet to yellow following growth of the wild-type CN-1, whereas plates inoculated with the $\Delta CcOah$ strain remained violet (Fig. 3B). We also quantified OA accumulation of the wild-type CN-1 and deletion strains using HPLC profiles; however, no clear OA accumulation was quantitatively detected even after coincubation with extracts of poplar barks (Supplementary Fig. S3). These findings suggest that *CcOah* is required for OA biogenesis and its expression is induced by the host.

OA disruptants display significantly attenuated virulence.

We sought to determine whether the three genes contribute to the virulence of *C. chrysosperma*. Compared with the wild-type and complemented strains, loss of any of the three genes severely weakened fungal virulence on poplar twigs (Fig. 4A). Poplar twigs inoculated with $\Delta CcOah$, $\Delta CcOdc1$, and $\Delta CcOdc2$ exhibited largely limited lesions on poplar twigs whereas the twig inoculated with the wild-type CN-1 strains showed typical canker symptom

Fig. 3. Induced production of oxalic acid by poplar extracts. Detection of oxalic acid accumulation was performed after cultures of the wild-type CN-1, $\Delta CcOah$, $\Delta CcOdc1$, and $\Delta CcOdc2$ strain grown in liquid SM **A**, without or **B**, with poplar bark extracts. The representative color change was displayed on different plates supplied with methyl red, bromocresol purple, and bromophenol blue. Liquid SM was used as a mock treatment.

and expanded larger lesions, although the $\Delta CcOdc2$ -24-C did not significantly restore the pathogenicity. Furthermore, the size of lesions caused by any OA disruptant was much smaller than the wild-type and complemented strains (Fig. 4B). Similarly, the symptoms and sizes of lesions in leaves inoculated with the deletion strains were significantly different from the wild-type and complemented strains (Supplementary Fig. S3). These observations showed that *CcOah*, *CcOdc1*, and *CcOdc2* play essential roles in full virulence of *C. chrysosperma*.

Acidification of host cells is important for virulence of *C. chrysosperma*. To further evaluate the role of OA in the virulence, we assessed the effects of exogenous OA on the virulence defect of OA disruptants. Poplar twigs which were simultaneously treated with OA at 200 $\mu\text{g/ml}$ and inoculated with the $\Delta CcOah$ and $\Delta CcOdc1$ strains showed obvious canker symptoms similar to those of the wild-type CN-1 (Fig. 5A); however, exogenous application of OA did not affect the virulence defect of the $\Delta CcOdc2$ strain. Interestingly, the exogenous addition of OA slightly enhanced virulence of the $\Delta CcOah$ strain and the lesion length was about 1.3-fold as high as the wild-type strain (Fig. 5B). To investigate whether the exogenous addition of other acids could restore the virulence defect of OA disruptants, the addition of CA was performed. As expected, exogenous application of CA at 200 $\mu\text{g/ml}$ can also restore the virulence defects of the $\Delta CcOah$ and $\Delta CcOdc1$ strains similarly to OA, and CA did not affect the virulence defect of the $\Delta CcOdc2$ strain (Fig. 5C and D). These results demonstrated that acidification of the host environment is critical for the infection of *C. chrysosperma*.

The $\Delta CcOah$ strain elicits elevated poplar ROS burst. *S. sclerotiorum* secretes OA and establishes disease by suppressing host ROS (Williams et al. 2011); therefore, we examined ROS burst by staining with ROS indicator DAB during *C. chrysosperma* infection. A slight difference in ROS burst surrounding the infection sites of poplar leaves inoculated with the $\Delta CcOah$ strain was observed at 12 dpi compared with other strains (Fig. 6). Importantly, the $\Delta CcOah$ strain promoted much stronger ROS burst at 24 dpi compared with other strains (Fig. 6). We also found that the $\Delta CcOdc1$ and $\Delta CcOdc2$ strains showed weaker DAB staining relative to the wild type. Thus, we conclude that *CcOah* is involved in regulating host ROS burst during *C. chrysosperma* infection.

DISCUSSION

In order to establish successful infection, necrotrophic fungi secrete OA and acidify the host environment (Nagarajkumar et al. 2005; Xu et al. 2018). OA is a dicarboxylic acid produced by fungi, in which OA biosynthesis is primarily catalyzed by *Oah* and degradation is mediated by *Odc* (Xu et al. 2018). Here, we demonstrated the importance of *CcOah* and *CcOdc* in full virulence of *C. chrysosperma*. Our results and reports from other Ascomycota fungi suggested that *Oah* plays a critical role in OA biosynthesis and deletion of *Oah* homologs resulted in a significant virulence defect (Chen et al. 2010; Gombert et al. 2011; Liang et al. 2015a; Pedersen et al. 2000b). From our transcript profile, *CcOah*, *CcOdc1*, and *CcOdc2* appeared to be only induced during early infection of *C. chrysosperma*, because we hardly detected their expression at late infection stages and in hyphae and pycnidia. In agreement with previous studies, the strains lacking *CcOah* could not change the color of pH-indicating plates either (Liang et al. 2015b; Peñalva et al. 2008). Importantly, the $\Delta CcOah$ strain displayed significantly attenuated virulence, and hyphal growth was obviously restricted surrounding the infection sites, demonstrating that the important role of *CcOah* in full virulence is conserved.

OA disruptants showed virulence defects, then exogenous addition of OA or CA could restore the defects of the $\Delta CcOah$ strain, suggesting that acidification of the host environment during pathogenesis facilitates successful infection. In *S. sclerotiorum*, acidic pH, not OA per se, promotes the expression of virulence

factors (Xu et al. 2015). Furthermore, PacC is an alkaline pH-dependent transcription factor (Peñalva et al. 2008). PacC is involved in virulence in several fungi, including the apple canker fungus *V. mali*; for instance, VmPacC is required for acidification of its environment and for full virulence in *V. mali* (Wu et al. 2018), emphasizing the importance of acidic pH in fungal virulence expression. According to the hypothesis that PacC can directly bind to the promoter of *Oah*, the presence of four PacC putative binding sites was found in the promoter region of *CcOah1*, which is similar to the report from *S. sclerotiorum* (Liang et al. 2015a). However, OA disruptants did not show pH responsiveness in *C. chrysosperma* (data not shown). These data indicate that both a pH-dependent and -independent virulence regulatory mechanism might exist in *C. chrysosperma*. Thus, more studies are needed to clarify the mechanisms.

OA accumulation can also be modulated by OA-degrading enzymes, especially *Odc*. In this study, the expression of *CcOdc1* and *CcOdc2* was induced during *C. chrysosperma* infection, and their deletion strains were indistinguishable from the wild-type CN-1 in vegetative growth and colony morphology, except for pycnidia production and reduced virulence. Interestingly, the exogenous addition of OA or CA exclusively restored the virulence defect of the $\Delta CcOdc1$ strain but not the $\Delta CcOdc2$ strain. In addition, both $\Delta CcOdc1$ and $\Delta CcOdc2$ strains did not hyperaccumulate OA, which is contradictory to hyperaccumulation of OA in $\Delta ss-odc2$ in *S. sclerotiorum* (Liang et al. 2015b), indicating a dramatic difference of *Odc* among fungi.

ROS burst is one of the earliest responses of host tissue challenged by an invading microbe. Previous studies showed that secreted OA can modulate host redox to inhibit and promote ROS (Williams et al. 2011). Early stages of necrotrophic infection are

Fig. 4. Oxalic acid disruptants displayed significantly reduced virulence on poplar twigs. **A**, Pathogenicity of wild-type CN-1, $\Delta CcOah$, $\Delta CcOdc1$, $\Delta CcOdc2$, $\Delta CcOah$ -C, $\Delta CcOdc1$ -C, and $\Delta CcOdc2$ -C on poplar twigs. Agar plugs without fungal mycelia were used as a mock treatment. Disease symptoms were photographed 4 days postinoculation (dpi). **B**, Average lesion size of scabs measured on twigs at 4 dpi. Bars indicate the standard deviations of the mean of 25 individual poplar twigs; ** indicates $P < 0.01$.

involved in host cell death, and OA secreted from *Sclerotinia* spp. elicited plant programmed cell death (Kim et al. 2008). We noted that the $\Delta CcOah$ strain resulted in restricted growth and displayed strong DAB staining surrounding the infection point, supporting the idea that *C. chrysosperma* modulates host cell death through secretion of OA.

Given the importance of OA biosynthesis and catabolism in pathogenesis, fine regulation of OA accumulation is a key question in future research. In the closely related necrotrophic fungus *V. mali*, the pH-responsive transcription factor VmPacC has been

demonstrated to contribute to virulence through the generation of CA for acidifying the host (Zhang et al. 2018). In addition, we also demonstrated that exogenous addition of CA can restore virulence defects of the $\Delta CcOah$ and $\Delta CcOdc1$ strains. What the underlying bases and regulation of these phenomena are remains to be elucidated.

In conclusion, we determined the function of OA biosynthesis and catabolism in *C. chrysosperma* and the involvement of acidification during pathogenesis. Future research efforts to

Fig. 5. Exogenous application of oxalic acid (OA) or citric acid (CA) can restore the virulence defect of the $\Delta CcOah$ and $\Delta CcOdc1$ strains. Disease symptoms of poplar twigs inoculated with the wild-type CN-1, $\Delta CcOah$, $\Delta CcOdc1$, and $\Delta CcOdc2$ strains. Twigs were pretreated with A, OA at 200 μ g/ml or C, CA at 100 μ g/ml before inoculation. Pictures were taken at 4 days postinoculation. **B** and **D**, Average lesion size of scabs measured on twigs as mentioned above. Bars indicate the mean of 25 individual poplar twigs; ** and * indicate $P < 0.01$ and 0.05, respectively.

Fig. 6. CcOah is essential for suppressing the host reactive oxygen species (ROS) burst. Poplar leaves were inoculated with the wild-type CN-1, $\Delta CcOah$, $\Delta CcOdc1$, and $\Delta CcOdc2$. ROS production of detached leaves was stained with 0.01% 3,3'-diaminobenzidine (DAB) and decolorized by 95% alcohol at 12 and 24 h postinoculation. The red arrow indicates the ROS produced by heat wound and the blue arrow indicates the ROS activated by the strains. Bars = 20 μ m.

characterize more genetic or biochemical virulence determinants would provide a better understanding of *C. chrysosperma* pathogenesis and, thus, provide new targets of canker disease control.

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