The Zn(II)2Cys6-Type Transcription Factor ADA-6 Regulates Conidiation, Sexual Development, and Oxidative Stress Response in *Neurospora crassa*

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Conidiation and sexual development are critical for reproduction, dispersal and better-adapted survival in many filamentous fungi. The *Neurospora crassa* gene *ada-6* encodes a Zn(II)2Cys6-type transcription factor, whose deletion resulted in reduced conidial production and female sterility. In this study, we confirmed the positive contribution of *ada-6* to conidiation and sexual development by detailed phenotypic characterization of its deletion mutant and the complemented mutant. To understand the regulatory mechanisms of ADA-6 in conidiation and sexual development, transcriptomic profiles generated by RNA-seq from the *ada-6* mutant and wild type during conidiation and sexual development were compared. During conidial development, differential expressed genes (DEGs) between the *ada-6* mutant and wild type are mainly involved in oxidation-reduction process and single-organism metabolic process. Several conidiation related genes are positively regulated by ADA-6, including genes that positively regulate conidiation (*fluffy* and *acon-3*), and genes preferentially expressed during conidial development (*eas*, *con-6*, *con-8*, *con-10*, *con-13*, *pcp-1*, and NCU9357), as the expression of these genes were lower in the *ada-6* mutant compared to wild type during conidial development. Phenotypic observation of deletion mutants for other genes with unknown function down-regulated by *ada-6* deletion revealed that deletion mutants for four genes (NCU00929, NCU05260, NCU00116, and NCU04813) produced less conidia than wild type. Deletion of *ada-6* resulted in female sterility, which might be due to that ADA-6 affects oxidation-reduction process and transmembrane transport process, and positively regulates the transcription of *pre-2*, *poi-2*, and NCU05832, three key genes participating in sexual development. In both conidiation and the sexual development process, ADA-6 regulates the transcription of *cat-3* and other genes participating in reactive oxygen species production according to RNA-seq data, indicating a role of ADA-6 in oxidative stress response. This was
INTRODUCTION

Conidial production is critical for reproduction, dispersal and survival in many filamentous fungi. Sexual reproduction is a key feature that distinguishes eukaryotic organisms from prokaryotic organisms. It produces better-adapted progenies by driving genetic recombination and eliminating deleterious mutations (Ni et al., 2011; Heitman et al., 2013). *Neurospora crassa* is a multicellular ascomycete fungus in the family Sordariomycetes and has long been used as an excellent model organism for genetic and biochemical researches as well as the study of morphological development (Springer, 1993; Perkins and Davis, 2000; Davis and Perkins, 2002). From vegetative growth to conidiation or sexual reproduction, morphological changes were evident. Behind it, transcriptional levels of many genes are altered (Greenwald et al., 2010; Wang et al., 2012; Lehr et al., 2014).

For example, 25% predicted genes in the genome of *N. crassa* are differentially expressed during conidiation (Greenwald et al., 2010), as well as during sexual development (Lehr et al., 2014). Transcription factors play important roles in activating or repressing gene expression in response to developmental signals. Thus, identification of transcription factors, which are crucial to conidial and sexual development, and characterization of their mechanisms are critical steps toward deeper understanding of how fungal morphogenesis is regulated.

Several transcription factors required for basal hyphae growth, asexual sporulation, and sexual development have been reported in *N. crassa* (Colot et al., 2006; Carrillo et al., 2017). Among identified 273 transcription factor genes, 33 genes were found specifically affect asexual development (Carrillo et al., 2017). Some of these genes have been extensively studied. For example, *fl* is required for the formation of major constriction chains (Matsuyama et al., 1974; Bailey and Ebbole, 1998). Overexpression of *fl* under the control of a heterologous promotor is sufficient to induce conidiation in a liquid medium which is unfavourable for conidiation (Bailey-Shrode and Ebbole, 2004). Some transcription factor genes, such as *hsf-2/NCU08480* (Thompson et al., 2008) and *chc-1/NCU00749* (Sun et al., 2011), were found to regulate the extent of conidiation or conidiation in response to environmental conditions. Deletion of *hsf-2* does not affect hyphal growth and aerial hyphal development but dramatically reduces conidial yield (Thompson et al., 2008). *CHC-1* is involved in CO$_2$-mediated conidiation suppression, and *chc-1* deletion results in earlier conidial formation than wild type, especially at a higher CO$_2$ concentration (Sun et al., 2011).

*N. crassa* is heterothallic with two mating types, designated *mat a* and *mat A*. Both of the mating types can form protoperithecia when nitrogen source is depleted (Davis and de Serres, 1970). Among identified transcription factor genes, ten of them were found to specifically affect sexual development (Carrillo et al., 2017). For example, *ff-7/NCU04001* is required for initiation of sexual development, and deletion mutant of *ff-7* does not produce protoperithecia, perithecia as well as ascopores (Colot et al., 2006; Carrillo et al., 2017); Deletion of *bek-1/NCU00097* results in aberrant perithecia, which exhibit defective beaks and cannot produce ascopores (Colot et al., 2006; Carrillo et al., 2017).

In addition to these specific transcriptional regulators, some transcription factors were showed to participate in regulating both asexual sporulation and sexual development. Knockout mutants of 25 transcription factor genes display significant defects in both asexual sporulation and sexual development (Colot et al., 2006; Carrillo et al., 2017). Most of these genes were named as *all development altered* (ada) genes (Colot et al., 2006), including *ada-1/NCU00499*, *ada-2/NCU02017*, *ada-3/NCU02896*, *ada-4/NCU03320*, *ada-5/NCU03931*, *ada-6/NCU04866* and *ada-7/NCU09739*. These transcription factors play crucial roles in fungal growth and development by regulating gene expression on a global scale. However, the regulatory roles of most of these newly found transcription factors in growth and development needs further confirmation, and their molecular mechanisms are not addressed. Among them, *ada-6/NCU04866* is a representative gene, which codes a Zn(II)$_2$Cys$_6$ transcription factor (Borkovich et al., 2004). Deletion of *ada-6* results in slower growth, dramatic reduction in conidiation and female infertility during sexual development (Colot et al., 2006), suggesting *ADA-6* is an important transcription factor. However, the confirmation of its function is still required and their mechanism has not been investigated.

*ADA-6* orthologs are widely distributed in filamentous fungi by sequence alignment. The ortholog of *ADA-6* in *N. discreta* has a predicted function involved in embryonic development. In *Aspergillus oryzae*, the *ADA-6* ortholog has predicted role in hyphal growth, positive regulation of secondary metabolite and sporocarp development during sexual reproduction.

In this study, we confirmed the positive role of *ADA-6* in growth and development by detailed phenotypic characterization of its deletion mutant and the complemented strain. By comparing transcriptomic profiles generated by RNA-seq from the *Ada-6* mutant and wild type during conidiation and sexual development, as well as by analyzing the contribution of the genes or biological pathway influenced by *ada-6* deletion, we explored the mechanisms by which *ADA-6* promotes conidiation and sexual development. We also found that deletion of *ada-6* causes hypersensitive to oxidants H$_2$O$_2$ and menadione. Together, our

Keywords: conidiation, *ada-6*, sexual development, oxidative stress response, *Neurospora crassa*
results proved that ADA-6 is a global regulator of conidiation, sexual development and oxidative stress response in *N. crassa*.

**MATERIALS AND METHODS**

**Strains and Media**

Most strains of *N. crassa* used in this study, including FGSC#4200 (wild type), FGSC#11022 (Δada-6/NCU04866: a), and knockout mutants for genes responsive to ada-6 deletion, were purchased from the Fungal Genetics Stock Center. All the strains were cultured at 28°C if it's not mentioned.

Media used in this study include Vogel's slant medium (1 × Vogel's salts, 2% sucrose, and 1.5% Bacto Agar), Vogel's plate medium (1 × Vogel's salts, 2% glucose, and 0.75% Bacto Agar), liquid Vogel's medium (1 × Vogel's salts, 2% glucose), the agar medium for transformant regeneration (1 × Vogel's salts, 1 M sorbitol, 1 × FGS, and 1.5% Bacto Agar), and the agar medium for filling race tubes [1 × Vogel's salts, 2% carbon source (glucose, sucrose, xylose, xylan, or carboxymethyl cellulose sodium), and 1.5% Bacto Agar].

**Complementation of the ada-6 Deletion Mutant**

The plasmid pCB1532-ada6 used for complementation was created by inserting a 4547 bp DNA fragment, containing the ada-6 gene (2230 bp) flanked by a 1238 bp upstream regulatory region and a 1079 bp downstream region, into the plasmid pCB1532 which harbors a sulfonyleurea resistant allele of the *Magna\_porthe \_grisea ILV1* as a selective marker (Sweigard et al., 1997). Briefly, the DNA fragment was amplified from the wild-type strain FGSC#4200 using primers Ada6F-EcoRI: GGAGTTGAAAGTGAAGGTTGG and Ada6R-HindIII: CCAAGCTTATCATACCTGAGGCCTC (EcoRI and HindIII sites were underlined), digested by EcoRI and HindIII and ligated into plasmid pCB1532. The construct pCB1532-ada6 was transformed into the Δada-6 mutant FGSC#11022 according to the previously reported protoplast transformation method (Royer and Yamashiro, 1992). 15 μg/ml of chlorimuron ethyl (Sigma) was added to the top agar to inhibit the growth of non-transformed protoplasts. Obtained transformants were subjected to serial transfers on slants with 15 μg/ml chlorimuron ethyl to favor homokaryon formation (Ebbole and Sachs, 1990) and further verified by PCR.

**Analysis of Hyphal Growth, Conidiation, and Sexual Development**

Hyphal extension of wild type and the Δada-6 mutant were analyzed in race tube. Briefly, one piece of mycelium mat (2 mm × 10 mm) for each strain was separately inoculated on one end of the race tube containing solid Vogel’s medium with different carbon source [2% glucose, 2% sucrose, 2% xylose, 2% xylan, and 2% carboxymethyl cellulose sodium (CMC-Na), respectively]. Inoculated race tubes were incubated at 28°C and the leading edge of the colony were marked every 24 h. The hyphal extension was then documented and measured by a ruler.

For conidiation analysis, mycelium for each strain was inoculated on Vogel's slants and grown at 28°C with continuous light for 7 days. Conidia produced were washed by 5 ml distilled water and counted with a hemocytometer.

For protoperithecium and peritheciun formation analysis, the mycelial mat or conidia suspension of the strain used as female parent was first inoculated on solid synthetic crossing medium with 0.1% sucrose and grown for 5 days under constant darkness at 25°C. Then the opposite mating-type strain was inoculated as male parent and incubated at 25°C for another 7 days under constant darkness. The protoperithecium and peritheciun formation were checked and documented by an optical microscope equipped with a Zeiss CCD.

**Transcriptomic Profiling Analysis**

Genome-wide transcriptional profiles for wild type and the Δada-6 mutant during conidiation, and at the initiate stage of protoperithecium formation were obtained by RNA sequencing, while transcriptional profiles for vegetative growth were used as control. Briefly, *N. crassa* wild-type strain and the Δada-6 mutant were inoculated on Vogel's plates covered with cellophane and grown at 28°C in darkness for 24 h. The mycelia were then transferred into 150-ml flasks containing 75 ml of liquid Vogel’s medium. Cultures were incubated at 28°C with constant agitation at 180 rpm for 18 h, and the mycelia were harvested by vacuum filtration. For conidial development analysis, the mycelial mats were transferred onto the surface of agar plates (9 cm) to induce conidial development at 28°C under constant light. Cultures were sampled at 12 h intervals. For sexual development analysis, the mycelial mats were inoculated on solid synthetic crossing medium with 0.1% sucrose and grown for 4 days under constant darkness at 25°C. Then, the mycelia were harvested and total RNA was extracted according to the standard TRIzol protocol (Invitrogen Corporation, Carlsbad, CA, United States).

RNA samples were sent to Beijing Genomics Institute (BGI) for RNA-seq analysis using the Illumina Hiseq2000 with a 50 bp single-end module (Illumina, San Diego, CA, United States). The obtained raw data was treated, mapped to *N. crassa* genome and transformed into expression value following standard BGI workflow. The gene expression level was calculated by using RPKM (Reads per kb per million reads). The differences in gene expression between samples was compared by comparing RPKM values (Grabherr et al., 2011), and those with fold change more than 2 (FDR < 0.001) were thought to be differentially expressed genes (DEGs). In addition, genes with RPKM less than 12 at all time points were thought to be low abundant transcripts and removed from the DEGs lists. The expressions of some genes, crucial for development and oxidative stress responses, were verified by time course experiment using real time PCR.

**RT-qPCR Analysis**

Samples were prepared as described above. Then mycelia were harvested and immediately frozen and ground into fine powder in liquid nitrogen. Total RNA was extracted and treated with DNase I to remove genomic DNA according to the standard TRIzol protocol (Invitrogen Corporation, Carlsbad, CA,
Susceptibility Tests of the Strains to Oxidative Stress

N. crassa wild-type strain and knockout mutants (ada-6, nox-1, cat-2, and cat-3) were separately inoculated onto 90-mm plates (containing 15-ml liquid Vogel's medium) and allowed to grow at 28°C in darkness for 24 h. The mycelial mat were poured and the round mat (2-mm) were inoculated on the center of plates (90-mm) with or without oxidant (H₂O₂ or menadione), and incubated at 28°C for 22 h (control), 32 h (25 µM menadione) and 48 h (10 mM H₂O₂), respectively. Each test was repeated and the experiment was independently repeated at least three times. The relative growth inhibition rates (mutant growth under oxidant stress was compared to wild type grown under oxidant stress and normalized by growth under non- oxidant condition) of each strain were calculated based on colony diameters after 22 h of incubation.

RESULTS

Phenotypic Characterization of the Δada-6 Mutant

The phenotype of the Δada-6 mutant has been described by Colot et al. (2006). Deletion of ada-6 resulted in reduced hyphal growth and altered asexual and sexual development. However, the deletion of ada-6 only slightly affected colony growth. In race tubes containing solid Vogel's medium with different carbon sources, the colony growth of the Δada-6 mutant were slightly slower than that of wild type: with 2% glucose, 2% sucrose, 2% xylose, 2% xylan, and 2% carboxymethyl cellulose sodium (CMC-Na) as carbon sources, the growth rate of the Δada-6 mutant was 6.5, 6.6, 5.6, 7.2, and 4.9 cm/day, respectively, while the growth rate of wild type was 8.5, 8.3, 7.5, 8.3, and 5.6 cm/day, respectively (Figure 1A).

The most dramatic effects caused by ada-6 deletion were the defects in asexual sporulation and sexual development. On slants containing solid Vogel's medium with 2% sucrose, aerial hyphal growth of the Δada-6 mutant was only slightly shorter than that of wild type (Figure 1B), but the conidial production of the Δada-6 mutant was reduced by 93% as compared with that of wild type (Figure 1B). Unlike the deletion mutant of fl in which conidial development stops at the major constriction formation stage (Bailey and Ebbole, 1998), the Δada-6 mutant was capable to pass through all conidial development stages to produce mature conidia (Figure 1C).

To investigate the contribution of ADA-6 to sexual development, we analyzed the formation of protoperithecium and perithecium on solid synthetic crossing medium. When using the Δada-6 (a) as female parent and wild type FGSC#2225 (A) as male parent, only a very few and small protoperithecium formed but no perithecium was observed (Figure 1D). While using wild type 2225 (A) as female parent and the Δada-6 (a) as male parent, normal protoperithecium and perithecium were produced (Figure 1D). These results suggest that deletion of ada-6 resulted in female sterility.

To confirm the role of ADA-6 in growth and development, we generated a plasmid pCB1532-ada6 which carries the full length of ada-6 gene with its regulatory regions as described in Materials and Methods. By transforming this plasmid into the Δada-6 mutant FGSC#11022, complemented transformants (Δada-6; ada-6) were obtained and phenotypically compared the growth and development to wild type FGSC#4200 (WT). As expected, the complemented transformants (Δada-6; ada-6) displayed phenotypes resembling wild-type conidiation and sexual development (Figures 1B–D).

Genome-Wide Transcriptional Responses to ada-6 Deletion

To understand the regulatory roles of ADA-6 in growth, conidiation and sexual development, transcriptomic profiles generated by RNA-seq from the Δada-6 mutant and wild type during conidial development or sexual development were compared. Among 9403 detected genes, 330, 441, and 1547 genes were found to be transcriptionally changed for more than two folds upon ada-6 deletion after conidiation induction for 0, 12, and 24 h, respectively (Supplementary Data S1). For sexual development, 1024 genes were found to be transcriptionally changed for more than two folds upon ada-6 deletion after 4 days of induction of sexual development (Supplementary Data S1).

To functionally understand the DEGs, functional classification and gene set enrichment analysis were conducted. In the vegetative growth stage (or conidiation induction for 0 h), DEGs between the Δada-6 mutant and wild type were mainly enriched in carbohydrate metabolic process (14 up- and 2 down-regulated genes), xylan catabolic process (5 up- and 2 down-regulated genes), and glucose import (6 up- and 2 down-regulated genes) (Supplementary Table S2). After 12 h of conidiation induction, DEGs between the Δada-6 mutant and wild type were mostly enriched in oxidation-reduction process (30 up- and 6 down-regulated genes) and transmembrane transport process (21 up- and 3 down-regulated genes) (Supplementary Table S2). While after 24 h of conidiation induction, DEGs between the Δada-6 mutant and wild type mostly participated in oxidation-reduction process...
Deletion of ada-6 results in reduced hyphal growth, less conidial production, and female sterility in N. crassa. (A) Hyphal growth characterization of the ada-6 deletion mutant (\(\Delta ada-6\)) and wild type (WT) grown with different carbon source. Strains were grown in race tube containing solid Vogel’s medium with different carbon sources. Inoculated race tubes were incubated at 28\(^\circ\)C and the leading edge of the colony were marked every 24 h. The hyphal extension was then measured by a ruler. The means of hyphal extension rates from three race tubes are shown and standard deviations are indicated by error bars. (B) Conidiation characterization of wild type (WT), the ada-6 deletion mutant (\(\Delta ada-6\)) and it’s complemented transformant (\(\Delta ada-6\); ada-6). Strains were grown in Vogel’s slants at 28\(^\circ\)C with continuous light for 7 days and then imaged. Conidia produced on slants were counted with a hemocytometer. The means of conidial counts from three slants are shown and standard deviations are indicated by error bars. (C) Conidiophore structure of wild type (WT), the ada-6 deletion mutant (\(\Delta ada-6\)) and it’s complemented transformant (\(\Delta ada-6\); ada-6). Bar, 50 \(\mu\)m. (D) Protoperithecium and perithecium formation by crossing of the ada-6 deletion mutant (\(\Delta ada-6\), a) with wild type (#2225, A). The ada-6 deletion mutant (\(\Delta ada-6\), a) or wild type (#2225, A) were used as female parent and first grown on solid crossing medium for 5 days under constant darkness at 25\(^\circ\)C, then the opposite mating type strain was inoculated as male parent and incubated at 25\(^\circ\)C for another 7 days under constant darkness. Protoperithecium and perithecium formation was checked and imaged.

(41 up- and 66 down-regulated genes) and transmembrane transport process (42 up- and 34 down-regulated genes) (Supplementary Table S2).

Under all tested conditions, the expression of 14 genes (cat-3, NCU02910, NCU03323, NCU04917, NCU5126, NCU05230, sut-28, NCU06170, NCU07088, NCU07095, cdt-2, NCU08223, pho-3, adh-9) were commonly increased and the expression of 13 genes (NCU00496, NCU00719, NCU05629, NCU05762, NCU05859, NCU06140, NCU08455, cas, gao-1, NCU09210, NCU10610, NCU11340, NCU17271) were commonly reduced in response to ada-6 deletion (Tables 1–4). Among these genes, seven genes (sut-28, cdt-2, NCU17271, NCU10610, NCU05762, NCU05230, NCU05126) encode proteins as integral components of plasma membrane, five genes (cat-3, gao-1, NCU09210, NCU05762, adh-9) are involved in oxidation-reduction process, and nine genes (NCU11340, NCU08455, NCU08223, NCU07088, NCU06170, NCU05859, NCU05629, NCU03323, NCU02310) encode proteins with unknown function (Table 1).
TABLE 1 Genes transcriptionally response to ada-6 deletion under all tested conditions.

| Genes          | Function annotation                 | RPKM \(\Delta_{ada6-0\ h}\) | RPKM \(\Delta_{ada6-12\ h}\) | RPKM \(\Delta_{ada6-24\ h}\) | RPKM \(\Delta_{ada6-4\ d}\) | RPKM \(WT-0\ h\) | RPKM \(WT-12\ h\) | RPKM \(WT-24\ h\) | RPKM \(WT-4\ d\) |
|----------------|-------------------------------------|-------------------------------|-----------------------------|-------------------------------|--------------------------|-----------------|-----------------|-----------------|-----------------|
| NCU04866       | ada-6                               | 0.046                         | 0.0883                      | 0.0408                        | 7.1396                   | 40.4223         | 19.06           | 5.1192          |
| NCU00496       | hypothetical protein                | 1.2303                        | 5.8881                      | 3.8363                        | 1.6779                   | 2.8212          | 17.7147         | 17.029          | 5.7047          |
| NCU00719       | hypothetical protein, direct target of ADV-1 | 5.0315                        | 3.9651                      | 0.4172                        | 2.4077                   | 20.5478         | 9.5147          | 2.9538          | 4.8644          |
| NCU05629       | hypothetical protein                | 0.2144                        | 1.9056                      | 1.126                         | 0.3899                   | 4.8953          | 24.0909         | 7.9924          | 3.4413          |
| NCU05762       | hypothetical protein with oxidoreductase activity | 0.2                        | 2.7995                      | 0.768                         | 0.6382                   | 0.1248          | 12.6732         | 15.5484         | 48.4745         |
| NCU05859       | hypothetical protein                | 0.4292                        | 3.301                       | 1.0565                        | 14.3203                  | 0.9269          | 20.0974         | 10.7257         | 30.5766         |
| NCU06140       | Ribosome biogenesis protein – Nop58p/Nop5 | 0.4019                        | 4.3793                      | 0.3957                        | 1.7814                   | 2.0093          | 22.6131         | 1.4656          | 4.6589          |
| NCU08455       | hypothetical protein                | 2.7386                        | 48.0429                     | 4.5419                        | 14.6781                  | 8.6476          | 206.8643        | 76.1128         | 83.6734         |
| NCU08457       | as, rolet protein                   | 18.8768                       | 124.787                     | 270.4869                      | 25.5233                  | 82.9049         | 10027.32        | 17750.16        | 3584.603        |
| NCU09209       | gao-1, galactose oxidase-1          | 7.0906                        | 88.2666                     | 34.087                        | 6.212                    | 41.5932         | 229.2223        | 364.781         | 57.7112         |
| NCU09210       | dyp-type peroxidase                | 20.2343                       | 196.3718                    | 52.1181                       | 14.9053                  | 97.0414         | 549.4004        | 363.9373        | 146.3083        |
| NCU10610       | hypothetical protein, ADV-1 target gene | 2.8556                       | 17.463                      | 3.6971                        | 4.4715                   | 6.3875          | 38.4266         | 12.0849         | 10.6545         |
| NCU11340       | hypothetical protein                | 0                            | 0.0474                      | 0.1956                        | 3.2504                   | 0.1589          | 0.4009          | 0.7726          | 23.0234         |
| NCU117271      | hypothetical protein                | 0                            | 1.0222                      | 0.3569                        | 0.3707                   | 0.1087          | 7.2247          | 6.9613          | 15.6349         |

(1) WT, wild type; \(\Delta_{ ada6 }\), ada-6 deletion mutant; -0 h, samples of N. crassa in vegetative growth stage; -12, samples of N. crassa after 12 h of conidiation induction; -24, samples of N. crassa after 24 h of conidiation induction; -4 d, samples of N. crassa after 4 days of sexual development induction. (2) Locus numbers and function were annotated according to the N. crassa genome assembly (http://fungidb.org/fungidb/). (3) RPKM, Reads Per Kilobase of exon model per Million mapped reads.

Genes Regulated by ADA-6 Are Involved in Oxidation-Reduction Process

During both conidiation and sexual development, the most seriously affected biological process by ada-6 deletion is oxidation-reduction reaction. After conidiation induction for 12 and 24 h, 36 and 218 genes, respectively, which involved in oxidation-reduction process, were differentially expressed between the \(\Delta_{ ada6 }\) mutant and wild type (Supplementary Data S1). The expressions of some crucial genes were verified by time course experiment using real time PCR (Figure 2). During the conidiation induction, the transcriptional levels of 15 genes, involved in oxidation-reduction reaction, were increased in wild type. However, their transcriptional levels in the \(\Delta_{ ada6 }\) mutant were obviously lower than those in wild type (Table 2). Most of these 15 DGEs encode oxidase or dehydrogenase, including NCU08856 (myo-inositol oxygenase), NCU05858 (fatty acid oxidase), NCU09209 (galactose oxidase), NCU10015 (methanesulfonate monooxygenase), NCU04474 (sulfite oxidase), NCU01853 (choline dehydrogenase), NCU03893 (short-chain dehydrogenase/reductase SDR), NCU02287 (acyl-CoA dehydrogenase-1), and three peroxidase encoding genes (NCU09210, cat-1 and cat-4) (Table 2). There were 13 genes in wild type were down-regulated during conidiation, but their transcriptional levers in the \(\Delta_{ ada6 }\) mutant were higher than those in wild type. These 13 DGEs include three oxidase encoding genes (NCU06402, NCU04983 and NCU01546),...
two peroxidase encoding genes (cat-3 and NCU10051), two dehydrogenase encoding genes (NCU09798 and NCU01754), and two hydrolase encoding genes (NCU01720 and NCU05969), etc. (Table 2).

Oxidation-reduction reaction is involved in many processes. The foremost is oxidative stress response pathway, which affect fungal growth, development and stress responses. During the conidiation induction, 15 genes involved in oxidative stress response or with antioxidant activity were differentially expressed between the Δada-6 mutant and wild type (Table 2).

After 24 h of conidiation induction, the transcriptional levels of 11 genes (nox-1, cat-2, cat-3, NCU03646, NCU09534, NCU16942, ara-2, NCU16942, ccp-1, NCU11046, trx-4, trx-5 and trx-6) in the Δada-6 mutant were obviously higher than those in

### Table 2 | Transcriptional responses to ada-6 deletion by the genes involved in oxidation-reduction process.

| Genes          | Function annotation            | RPKM Δada6-0 h | RPKM Δada6-12 h | RPKM Δada6-24 h | RPKM WT-0 h | RPKM WT-12 h | RPKM WT-24 h |
|----------------|-------------------------------|----------------|----------------|----------------|-------------|-------------|-------------|
| NCU00355       | Cat-3                         | 506.5213       | 574.7889       | 75.34231       | 237.5218    | 167.5816    | 19.71859    |
| NCU00598       | trx-4, thioredoxin-4          | 35.076         | 62.3528        | 67.1768        | 37.2486     | 67.8260     | 25.4097     |
| NCU02110       | Nox-1                         | 16.33786       | 44.27099       | 22.52937       | 12.94278    | 22.54903    | 11.09398    |
| NCU03297       | ccp-1, thioredoxin c peroxidase| 179.2891       | 93.8437        | 369.5966       | 195.4672    | 82.2211     | 120.0121    |
| NCU03646       | L-ascorbate peroxidase         | 9.7929         | 48.4457        | 58.8551        | 5.0478      | 47.5253     | 27.9330     |
| NCU03714       | trx-5, thioredoxin-5          | 10.6542        | 11.0685        | 90.3788        | 11.0683     | 10.8925     | 16.1468     |
| NCU04268       | peroxidoxin 2 family          | 301.3897       | 58.4775        | 67.2506        | 274.8588    | 47.7068     | 203.4202    |
| NCU05169       | Cat-4                         | 6.19272        | 12.84018       | 10.87036       | 5.196066    | 12.67001    | 47.45783    |
| NCU05770       | Cat-2                         | 42.99698       | 259.8382       | 422.92         | 69.11465    | 264.3265    | 149.3483    |
| NCU06556       | trx-6, thioredoxin-6          | 424.7498       | 343.7312       | 1242.284       | 344.6572    | 251.2739    | 328.9048    |
| NCU06880       | pxr-1, peroxidoxin-1          | 145.9389       | 173.7802       | 36.7785        | 152.3986    | 176.3585    | 85.6079     |
| NCU07386       | mtp-1, Fe superoxide dismutase | 48.5932        | 37.0135        | 15.4648        | 49.4639     | 33.9387     | 33.3602     |
| NCU08791       | Cat-1                         | 159.6162       | 112.2809       | 200.6002       | 188.7525    | 172.519     | 456.6022    |
| NCU09210       | dyp-type peroxidase           | 20.2343        | 196.3718       | 51.1181        | 97.0414     | 549.4004    | 363.9373    |
| NCU09534       | ara-2, peroxidoxin HYR1        | 74.8098        | 299.9837       | 261.9622       | 77.8472     | 170.2767    | 87.7308     |
| NCU11046       | with predicted peroxidase activity | 13.1388       | 18.1692        | 128.2936       | 17.0875     | 27.9754     | 56.6314     |
| NCU16942       | Peroxidase/oxygenase          | 0.8903         | 7.5761         | 27.7938        | 0.7507      | 10.7828     | 12.9403     |

(1) WT, wild type; Δada6, ada-6 deletion mutant; -0 h, samples of N. crassa in vegetative growth stage; -12, samples of N. crassa after 12 h of conidiation induction; -24, samples of N. crassa after 24 h of conidiation induction. (2) Locus numbers and function were annotated according to the N. crassa genome assembly (http://fungidb.org/fungidb/). (3) RPKM, Reads Per Kilobase of exon model per Million mapped reads.
wild type. Among these 11 genes, the transcriptional levels of nox-1 and cat-3 in the Δada-6 mutant were higher than those in wild type by both 12 and 24 h after conidiation induction (Table 2 and Figure 2). NOX-1 participates in reactive oxygen species (ROS) production, and CAT-3 activity increases during exponential growth and is induced under various stress conditions (Chary and Natvig, 1989; Peraza and Hansberg, 2002). The gene cat-2 encodes catalase-2, which is mainly found in aerial hyphae and conidia (Peraza and Hansberg, 2002). The coding products of NCU03151, NCU08114, and NCU07966 (peroxidase), NCU11046 (peroxiredoxin-1), their transcriptional levels in the ada-6 mutant are obviously lower than those in wild type after 24 h conidiation induction (Table 2). The transcriptional levels of cat-1 and cat-4 were increased in wild type but not changed in the Δada-6 mutant during the late period of conidiation induction (Table 2). CAT-1 is highly abundant in conidia and function mainly in conidia germination (Peraza and Hansberg, 2002; Wang et al., 2007), CAT-4 is located in the cytosol (Schlies et al., 2006), and both CAT-4 and dyp-type peroxidase (NCU09210) were induced during the conidiation (Greenwald et al., 2010). As expression of these genes is correlated with conidiation, the lower transcription of the cat-1, cat-4, and NCU09210 in the Δada-6 mutant compared with those in wild type is consistent with the less sporulation phenotype of the Δada-6 mutant.

During sexual development, 107 genes involved in oxidation-reduction process were found differentially expressed between the Δada-6 mutant and wild type (Supplementary Table S2 and Table 4). After 4 days of sexual development induction, the transcriptional levels of 61 genes in wild type were increased or not changed, but were obviously lower in the Δada-6 mutant than in wild type. While the transcriptional levels of 26 genes were obviously higher in the Δada-6 mutant than in wild type. Among these 107 genes, 15 genes are involved in oxidative stress response or with antioxidant activity (Table 4). After 4 days of sexual development induction, the transcriptional levels of 7 genes (nox-1, nox-2, nox-R, cat-3, NCU03151, NCU08114, and NCU07966) in the Δada-6 mutant were obviously higher than those in wild type. NOX-1, NOX-2, and NOX-R participate in ROS production, and CAT-3 activity increases during exponential growth and is induced under various stress conditions (Chary and Natvig, 1989; Peraza and Hansberg, 2002). NCU03151 encodes a peroxisomal membrane protein, and NCU08114 encodes a hexose transporter. The higher expression of these seven genes in the Δada-6 mutant suggests that the Δada-6 mutant may be still metabolically active and produced more ROS than wild type after 4 days of sexual development induction. For the genes sod-1, cat-1, cat-4, NCU09210, NCU07966, NCU05858,

**Table 3** Transcriptional response to ada-6 deletion by the genes involved in conidiation and vegetative cell wall development.

| Genes     | Function annotation | RPKM Δada6-0 h | RPKM Δada6-12 h | RPKM Δada6-24 h | RPKM WT-0 h | RPKM WT-12 h | RPKM WT-24 h |
|-----------|---------------------|----------------|----------------|----------------|-------------|-------------|-------------|
| NCU04866  | Ada-6               | 0              | 0.045997       | 0.088329       | 7.139561    | 40.42227    | 19.05998    |
| NCU01116  | Aab-1, TF subunit   | 41.0876        | 104.1344       | 177.4739       | 48.6871     | 114.6335    | 560.9975    |
| NCU00269  | Set-2               | 1.868055       | 2.594838       | 22.58605       | 2.667073    | 3.150217    | 6.208719    |
| NCU00929  | hypothetical protein | 9.3544         | 4.7866         | 7.945482       | 7.22975     | 4.810735    | 25.61734    |
| NCU04813  | hypothetical protein | 13.3412        | 12.7783        | 9.8237         | 13.9730     | 15.9312     | 27.8968     |
| NCU05260  | Protein kinase      | 7.46533        | 3.77964        | 5.44357        | 6.425309    | 4.244103    | 19.1833     |
| NCU07617  | Acon-3              | 0.186927       | 1.389872       | 3.91145        | 0.635499    | 1.958488    | 1.672642    |
| NCU08726  | ft, fluffy          | 9.121856       | 17.91114       | 14.32059       | 17.76177    | 36.69432    | 45.58477    |

(1) WT, wild type; Δada6, ada-6 deletion mutant; -0 h, samples of N. crassa in vegetative growth stage; -12, samples of N. crassa after 12 h of conidiation induction; -24, samples of N. crassa after 24 h of conidiation induction. (2) Locus numbers and function were annotated according to the N. crassa genome assembly (http://fungidb.org/fungidb/). (3) RPKM, Reads Per Kilobase of exon model per Million mapped reads.
NCU11286, and NCU03651, their transcriptional levels in the Δada-6 mutant were obviously lower than those in wild type (Table 4). As expression of these genes is correlated with conidiation, the lower expression of these eight genes in the Δada-6 mutant than in wild type is consistent with their phenotypic characteristics, as asexual development is accompanied with sexual development after 4 days of sexual development induction.

**Genes Regulated by ADA-6 Are Involved in Conidiation and Vegetative Cell Wall Development**

Many genes are associated with conidiation in *N. crassa*. Among the genes positively regulating conidiation, *acon-3* and *fl* were differentially expressed between the Δada-6 mutant and wild type (Table 3). The result was verified by time course experiment, in which the expression profiles of *acon-3* and *fl* were analyzed by real time PCR (Figure 2). During the conidiation induction, the transcriptional level of *acon-3* and *fl* in wild type were significantly increased after conidiation induction. At 12 h, the *acon-3* and *fl* transcriptional level was 11.2 times and 6.1 times higher than that at the initial time point, respectively. However, the transcriptional increases of *acon-3* and *fl* were only 1.7-fold and 3-fold in the Δada-6 mutant after 12 h induction. Similar results were found at 24 h of conidiation induction. The transcriptional levels of *acon-3* and *fl* in the Δada-6 mutant were lower than those in wild type during the entire experiment (Figure 2).

For DEGs with unknown functions, we analyzed conidiation of the corresponding gene deletion mutants grown on Vogel’s slants and found that mutants for four genes, including NCU00929, NCU05260, NCU00116, and NCU04813, displayed reduced conidial production (Figure 3). NCU05260 encodes a protein kinase, NCU00116 encodes a CCAAT-binding transcription factor subunit AAB-1 (Chen et al., 1998), and both NCU00929 and NCU04813 encode hypothetical protein. The transcriptional levels of these four genes were increased dramatically at 24 h after conidiation induction, but their transcriptional levels were lower in the Δada-6 mutant than those in wild type (Table 3).

Some conidiation related genes, including *eas*, *con-6*, *con-8*, *con-10*, *con-13*, and NCU09357 (encoding stage V sporulation protein K), were highly expressed in wild type during the late period of conidiation (Table 3 and Figure 2). However, the transcriptional levels of these genes in the Δada-6 mutant were much lower than those in wild type in the mid-late period of conidiation (Table 3 and Figure 2). This result is consistent with the phenotype of reduced conidial production in the Δada-6 mutant. After the 24 h of conidiation induction, the wild type had produced many spores, while the Δada-6 mutant produced only a few spores (data not shown). In consistent with this phenotype, the genes encoding cell wall proteins in
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FIGURE 2 | The expressions of some DGEs, crucial for development and oxidative stress responses, were determined by time course experiment using RT-qPCR. Wild type (WT) and the ada-6 deletion mutant (Δada-6) were inoculated on Vogel’s plates and allowed to grow at 28°C in darkness for 24 h. The mycelia were then transferred into 150-ml flasks containing 75 ml of liquid Vogel’s medium (2% sucrose). Cultures were incubated at 28°C with constant agitation at 180 rpm for 18 h, and the mycelia were harvested by vacuum filtration and transferred onto the surface of agar plates (9 cm) to induce conidial development at 28°C under constant light. Cultures were sampled after induction for 6, 12, and 24 h. The total RNA was extracted and transcriptional levels of indicated genes were analyzed by RT-qPCR.

FIGURE 3 | Conidial production of the knockout mutants of genes regulated by ADA-6. (A) Wild type and knockout mutants were inoculated and grown on Vogel’s slants at 28°C with constant darkness for 1 day, and then transferred to constant light for another 6 days. Conidial production of each strain was documented as images. (B) Conidial production was measured as number of conidia per slant. Standard deviations from three replicates were marked by error bars.

vegetative hyphae, including acw-8 and mwg1 (Maddi et al., 2009), were higher expressed in the Δada-6 mutant than those in wild type in the mid-late period of conidiation (Table 3 and Figure 2).

Some genes negatively influence conidial development and their deletion resulted in earlier or enhanced conidial production (Michán et al., 2003; Sun et al., 2011, 2012). Among these genes, cat-3 was differentially expressed between the Δada-6 mutant and wild type (Table 3 and Figure 2). As shown in Figure 2, the expression of cat-3 was not increased during conidiation in wild type. However, the transcriptional level of cat-3 in the Δada-6 mutant was higher than those in wild type during the entire period of conidiation induction.

Regulation of the Genes Involved in Sexual Development by ADA-6

A large number of N. crassa genes have been identified to be required for sexual development. Among them, only
five genes (app, poi-2, pre-2, fbm-1, and NCU05832) were found differentially expressed between the Δada-6 mutant and wild type during sexual development according to RNA-seq data (Table 4). NCU05832 encodes a methyltransferase, whose homologue in A. fumigatus negatively regulates sexual sporation, and its deletion resulted in formation of a cellular spore. The transcription of NCU05832 was not induced in wild type, but induced in the Δada-6 mutant during sexual development. After 4 days of sexual development induction, the transcriptional level of NCU05832 in the Δada-6 mutant was 142% higher than that of wild type (Table 4). This result indicates that NCU05832 is negatively regulated by ADA-6.

The app (abundant perithecial protein) is an indicator of sexual development, and its transcripts occur only after the onset of sexual development (Nowrousian et al., 2007). pre-2 (NCU005758) is a pheromone receptor encoding gene, and plays a vital role during mating in N. crassa (Kim and Borkovich, 2006). poi-2 is essential for differentiation of female reproduction structures and perithecial development (Kim and Nelson, 2005). fbm-1 encodes fruiting body maturation-1, whose homologue in N. discreta is Kynurenine 3-monoxygenase and related to flavoprotein monooxygenases. The transcripts of app, poi-2, pre-2, and fbm-1 increased during sexual development, but were obviously lower in the Δada-6 mutant than those in wild type. After 4 days of sexual development induction, transcriptional level of app, poi-2, pre-2, and fbm-1 in the Δada-6 was only 0.6, 0.4, 38.5, and 2.8%, respectively, of that in wild type (Table 4). This result suggests that ADA-6 positively regulates the transcription of pre-2 and poi-2, which promote sexual development. The lower expression of app and fbm-1 in the Δada-6 mutant might be the consequence of female sterility of the Δada-6 mutant.

Deletion of ada-6 Causes Hypersensitivity to Oxidants

During both conidiation and sexual development, RNA-seq data suggest that ADA-6 regulate the transcription of cat-3 and genes participating in ROS production. If it is true, deletion of ada-6 might cause an alteration in the sensitivity to oxidants. To confirm this, we inoculated knockout mutants (ada-6, nox-1, cat-2, and cat-3) and wild type on plates with or without oxidants (10 mM H2O2 or 25 µg/ml menadione), and the relative growth inhibition rates of each strain were calculated after 22 h of incubation. The Δcat-3 strain displayed hypersensitivity phenotype to H2O2 (Figure 4A): the relative growth inhibition of the Δcat-3 strain (87%) is higher than that of wild type (70%) (Figure 4B), while, the sensitivity of the Δcat-3 mutant to menadione was similar to that of wild type (Figure 4). This result is consistent with previous report (Michán et al., 2003). The sensitivity of the Δcat-2 mutant to both H2O2 and menadione was similar to wild type, and the Δnox-1 mutant showed slight hypersensitivity to menadione and similar sensitive to H2O2 with wild type (Figure 4). The Δada-6 mutant was more sensitive than wild type to both H2O2 and menadione (Figure 4A). On the plates with 10 mM H2O2, the growth of the Δada-6 mutant and wild type was inhibited by 75 and 70%, respectively. On the plates with 25 µg/ml menadione, the growth of the Δada-6 mutant and wild type was inhibited by 63 and 58%, respectively. Above results, together with RNA-seq data suggest that ADA-6 might play a role in oxidative stress response.

DISCUSSION

Global transcription factors control fungal growth, development and stress responses by regulating gene transcription on a global scale. Several transcription factors encoding genes named as all development altered (ada) were previously identified, and

![Figure 4](image_url)
their deletion resulted in significant defects in basal hyphal growth, asexual sporulation, and sexual development (Colot et al., 2006; Carrillo et al., 2017). One of them is ada-6 (NCU04866), whose deletion resulted in slower growth, dramatic reduction in conidiation and female infertility (Colot et al., 2006). However, the regulatory role of ADA-6 in growth and development needs further confirmation by mutant complementation, and its mechanism has not been addressed. Here we first confirmed the positive role of ADA-6 in growth and development by detailed phenotypic characterization of its deletion mutant and the complemented mutant. Then, by comparing transcriptomic profiles and functional analysis of genes influenced by ada-6 deletion, we explored the mechanisms by which ADA-6 promotes conidiation and sexual development. Our results demonstrate that ADA-6 might play a role in conidiation by regulating oxidation-reduction process and single-organism metabolic process. Moreover, ADA-6 positively regulates the transcription of fluffy and acon-3, two key genes required for the initiation of asexual sporulation by controlling the formation of major constriction chains (Matsuyama et al., 1974; Springer and Yanofsky, 1989; Bailey and Ebbole, 1998; Bailey-Shrode and Ebbole, 2004; Chung et al., 2011). Deletion of ada-6 also resulted in female sterility. ADA-6 regulates some genes associated with sexual development, including pre-2, poi-2, and NCU05832, three key genes required for sexual development (Kim and Nelson, 2005; Kim and Borkovich, 2006).

Oxidation-reduction reaction is involved in many pathways. The foremost of these is oxidative stress response pathway, which affects fungal growth, development and stress responses. ADA-6 regulates the transcription of cat-3 and genes participating in ROS production according to RNA-seq data, indicating a role of ADA-6 in oxidative stress response. This was confirmed by the hypersensitivity phenotype of the Δada-6 mutant to oxidants H₂O₂ and menadione.

Numerous studies have found the role of ROS in the regulation of conidiation and sexual development (Michán et al., 2003; Aguirre et al., 2005; Cano-Domínguez et al., 2008). In N. crassa, formation of conidia from growing hyphae includes three morphogenetic developmental stages: growing hyphae to adherent mycelium, adherent mycelium to aerial hyphae, and aerial hyphae to conidia. A hypertoxicant state develops at the start of these morphogenetic transitions (Hansberg et al., 1993; Toledo et al., 1995). Oxidative stress due to lack of CAT-3 induces hyphal adhesion, and development of more aerial hyphae and conidia (Michán et al., 2003). NOX-1, NOX-2, and NOX-R participates in ROS production, and NOX-1 elimination results in complete female sterility, decreased asexual development, and reduction of hyphal growth in N. crassa (Cano-Domínguez et al., 2008). All these studies indicate that ROS, whose accumulation is induced by eliminating ROS-decomposion (CAT-3) or activating ROS-generation (NOX-1), is a critical cell differentiation signal promoting conidiation and sexual development. In our study, ADA-6 regulates the transcription of oxidative stress related genes, including nox-1, cat-3, etc., during both conidiation and sexual development. Based on above result, we speculate that more ROS may accumulate in cells of the Δada-6 mutant during development or stress response process. This was further confirmed by the results that deletion of ada-6 resulted in hypersensitive to oxidative stress inducers H₂O₂ and menadione. Based on previous studies, this ROS accumulation can promote conidiation and sexual development in N. crassa (Hansberg et al., 1993; Toledo et al., 1995; Cano-Domínguez et al., 2008). However, the Δada-6 mutant still exhibited reduced conidial production and female sterility. These results indicate that regulation of development and oxidative stress response by ADA-6 may be independent.

In summary, our study showed that ADA-6, as a global regulator, plays positive roles in conidiation and sexual development, and regulates oxidative stress response of N. crassa. Combining transcriptomic profiles and functional assay, we explored the mechanisms by which ADA-6 promotes conidiation and sexual development, and regulates oxidative stress response. This work has augmented our knowledge of the functions and mechanisms of global regulators that influence growth, development and stress responses in filamentous fungi.

AUTHOR CONTRIBUTIONS

XS designed the study and wrote the manuscript. XS, FW, NL, and CH performed the main experiments. SL, BL, WX, and ZZ contributed to the data analysis and the data interpretation.

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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.2019.00750/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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