Mycobiology

Research Article

Generation of Reactive Oxygen Species via *NOXa* Is Important for Development and Pathogenicity of *Mycosphaerella graminicola*

Yoon-E Choi[1], Changsu Lee[2] and Stephen B. Goodwin[3,*]

[1]*Division of Environmental Science & Ecological Engineering, Korea University, Seoul 02841, Korea*
[2]*Department of Bioprocess Engineering, Chonbuk National University, Jeonju 54896, Korea*
[3]*USDA-Agricultural Research Service, 915 West State Street, Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907-2054, USA*

Abstract The ascomycete fungus *Mycosphaerella graminicola* (synonym *Zymoseptoria tritici*) is an important pathogen of wheat causing economically significant losses. The primary nutritional mode of this fungus is thought to be hemibiotrophic. This pathogenic lifestyle is associated with an early biotrophic stage of nutrient uptake followed by a necrotrophic stage aided possibly by production of a toxin or reactive oxygen species (ROS). In many other fungi, the genes *CREA* and *AREA* are important during the biotrophic stage of infection, while the *NOXa* gene product is important during necrotrophic growth. To test the hypothesis that these genes are important for pathogenicity of *M. graminicola*, we employed an over-expression strategy for the selected target genes *CREA*, *AREA*, and *NOXa*, which might function as regulators of nutrient acquisition or ROS generation. Increased expressions of *CREA*, *AREA*, and *NOXa* in *M. graminicola* were confirmed via quantitative real-time PCR and strains were subsequently assayed for pathogenicity. Among them, the *NOXa* over-expression strain, NO2, resulted in significantly increased virulence. Moreover, instead of the usual filamentous growth, we observed a predominance of yeast-like growth of NO2 which was correlated with ROS production. Our data indicate that ROS generation via *NOXa* is important to pathogenicity as well as development in *M. graminicola*.

Keywords *AREA*, *CREA*, Development, *Mycosphaerella graminicola*, NADPH oxidase, *NOXa*, Over-expression, Pathogenicity, ROS, *Septoria tritici* blotch, *Zymoseptoria tritici*

Mycosphaerella graminicola (anamorph: *Septoria tritici*; synonym: *Zymoseptoria tritici*) is one of the most important foliar fungal pathogens of wheat, causing a destructive disease known as septoria tritici blotch (STB). STB is prevalent worldwide wherever wheat is grown and is particularly severe in areas with wet and cold climates. Infection by STB can cause yield losses of up to 30~50% annually [1]. These losses are significantly increased in areas where environmental conditions are favorable to *M. graminicola* growth which includes many regions in the United States. Therefore, *M. graminicola* is considered to be one of the most important diseases affecting wheat production causing significant economic impacts [2, 3].

Efficient strategies to control STB have not been developed completely. So far, strategies to manage STB are based on the development of resistant cultivars and fungicide applications [4]. However, both of these strategies have limitations due to rapid changes in fungicide resistance [5] and pathogenicity [6] in rapidly recombining *M. graminicola* populations and by restrictions on fungicide use due to environmental concerns. A better understanding of *M. graminicola* biology will facilitate development of improved management strategies for STB.

Mycosphaerella graminicola is a plant-pathogenic ascomycete with a hemibiotrophic lifestyle consisting of an early, biotrophic phase followed by a late, necrotrophic stage. Early infection by *M. graminicola* is characterized by symptomless intercellular growth for 8~10 days. During this period, most of the total fungal biomass accumulates in the wheat mesophyll tissue [7], and the strategy of survival of *M. graminicola* largely employs a biotrophic mode of nutrient

Mycobiology 2016 March, **44**(1): 38-47
http://dx.doi.org/10.5941/MYCO.2016.44.1.38
pISSN 1229-8093 • eISSN 2092-9323

***Corresponding author**
E-mail: Steve.Goodwin@ARS.USDA.gov

Received November 12, 2015
Revised January 12, 2016
Accepted January 17, 2016

uptake. This biotrophic phase is followed by rapid collapse of wheat tissue during the late stage of infection, particularly for compatible interactions, resulting in clear necrosis of the wheat leaf [7]. For these reasons, many scientists have speculated that there might be involvement of fungal-originated toxic compounds or possible reactive oxygen species (ROS) in the late, necrotrophic stage of infection by *M. graminicola* [8].

Additional analyses of *M. graminicola* biology, specifically gaining a better understanding of the mechanisms of its biotrophic and necrotrophic growth, are of vital importance to developing enduring modes of host resistance to this important fungal pathogen. However, so far, little is known about the mechanisms of biotrophic and necrotrophic growth of *M. graminicola*. It is well accepted that carbon and nitrogen are major nutrient resources for most living species, including filamentous fungi. In many fungi, the *CREA* and *AREA* genes, encoding elements for catabolite repression and nitrogen metabolite repression, respectively, are major regulators of nutrient uptake signaling, which may have a pivotal role during the biotrophic stage of growth [9, 10]. In contrast, ROS are among the important determinants of the necrotrophic stage of fungal infection [11]. Fungal ROS production is catalyzed by NADPH oxidase (encoded by *NOXa*), which functions by transferring electrons from NADPH to oxygen molecules.

In an effort to better understand the biotrophic virulence strategies of *M. graminicola*, we over-expressed genes homologous to *CREA* and *AREA* in the fungus and assayed for altered pathogenicity. To understand the necrotrophic stage, we then over-expressed a homolog of *NOXa* in *M. graminicola* to elucidate the possible functional roles of *NOXa* during infection. Here, we describe our results of the over-expression analysis of *CREA*, *AREA*, and *NOXa* in *M. graminicola*.

MATERIALS AND METHODS

Fungal strains and culture media. Highly virulent *M. graminicola* strain IPO323 was used as wild type throughout this study and was the subject of gene over-expression experiments. All of the strains used or generated in this study were stored at −80°C after desiccation on strips of Whatman filter paper (Whatman Inc., Piscataway, NJ, USA) overnight in a lyophilizer. Cultures grown in YSB medium (10 g each of sucrose and yeast extract [Difco Laboratories, Detroit, MI, USA] per liter of distilled water) were used for genomic DNA extraction or *Agrobacterium tumefaciens*-mediated transformation (ATMT). For characterization of over-expression strains, we used various media: YSB; YSA (YSB with 15 g of agar per liter); PDA (4% potato dextrose agar; Difco Laboratories); and water agar (15 g of agar per liter) supplemented with or without 200 mM glucose or 200 mM ammonium sulfate.

Nucleic acid isolation and manipulation. All plasmid DNA was purified with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). All primers used for PCR are listed in Table 1. Ribosomal protein 27 (RP27) is a strong constitutive promoter that significantly increases expression in other fungal species [12, 13]. The RP27 promoter for filamentous fungi was obtained from the rice blast fungus, *Magnaporthe oryzae*, and previous analyses successfully demonstrated its effectiveness for gene expression [13]. For preparation of over-expression constructs, we first amplified the RP27 promoter using RP27-F1 & R1 primers and cloned

Table 1. Primers used to generate and analyze over-expression strains for the genes *AREA*, *CREA*, and *NOXa* in the wheat pathogen *Mycosphaerella gramincola*

No.	Name	Primer sequence (5′–3′)
1	RP27-F1	CAAGGCGATTAAGTT*aagctt*GGGTAACG
2	RP27-R1	CTTGCTCACCCTC*ctgcag*GAGTGAT
3	CREA-F1	CATGCTCTCTCAG*gaattc*CTGTTGAACC
4	CREA-R1	CGTCTCGTCCATCTA*ggtacc*TAGCGCTAG
5	AREA-F1	CAGAGTACATCCGTC*ggtacc*CGTGTGAG
6	AREA-R1	GTGTTCTCCAGAACG*gaattc*ACTACATTCG
7	NOXa-F1	CCATCCGATTCAGAT*ctgcag*TCTGCTCG
8	NOXa-R1	CAATTGAGAAGTGCG*tctaga*TTTGGTGTG
9	RP27-che-F1	GAATTGGGTACTCAAATTGGTTAT
10	RP27-che-R1	CATTTTGAAGATTGGGTTCCTAC
11	CREA-rt-F1	ACAACAGACGCCACCCACAACAC
12	CREA-rt-R1	GACGATACGATCCGTTCCCTTGC
13	AREA-rt-F1	GGCTGGAGAATCTGTCGTGGAGG
14	AREA-rt-R1	GCATTGCGATACCACTGGGAACA
15	NOXa-rt-F1	GACAGGCATCGGCGTAACACCAT
16	NOXa-rt-R1	ACTTTGCGCTTCCAGGCTTGAGA
17	Tub1-rt-F1	CAGACAACTTCGTGTTCGGTCAATC
18	Tub1-rt-R1	GGGAACTCCTCACGGATCTTGCT

Restriction enzyme sites introduced into the primers for cloning purposes are indicated by italics.

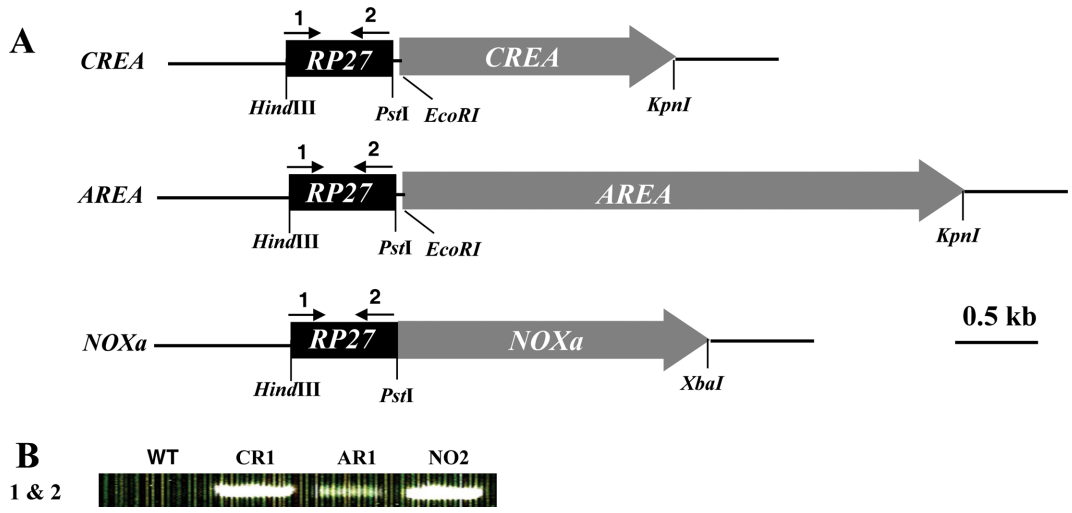

Fig. 1. Constructs for over-expression of the *CREA*, *AREA*, and *NOXa* genes in *Mycosphaerella graminicola* and PCR confirmation. A, Schematic drawing of the *CREA, AREA*, and *NOXa* over-expression constructs. The strong constitutive promoter RP27 was cloned into vector pBHT2 for *Agrobacterium tumefaciens*-mediated transformation [14] and subsequently used for making further individual gene over-expression constructs. Entire open reading frames of the *CREA*, *AREA*, and *NOXa* genes were amplified by PCR and cloned downstream of the RP27 promoter using the indicated restriction enzyme sites; B, PCR confirmation of fungal isolates integrating *CREA, AREA*, and *NOXa* over-expression constructs with the primers (1 and 2) described in Fig. 1A. WT, wild type; CR1, putative *CREA* over-expression strain; AR1, putative *AREA* over-expression strain; NO2, putative *NOXa* over-expression strain. The numbers on the left indicate the primer combinations used for PCR amplification. These amplification products are approximately 0.5 kb in size.

the product into the *Hind*III and *Pst*I sites of the ATMT vector, pBHT2 [14]. The resultant construct was designated as pOE01 (Fig. 1). Next, we produced individual PCR amplicons of the *CREA*, *AREA*, and *NOXa* genes by using primer pairs embedded with restriction enzyme sites for cloning purposes. All PCR procedures were performed with AccuSure DNA Polymerase (Bioline, Taunton, MA, USA), which has proof-reading activity. Primer pairs CREA-F1 & R1, AREA-F1 & R1, and NOXa-F1 & R1 were used to amplify approximately 1.5-, 3.0-, and 1.7-kb fragments of the *CREA*, *AREA*, and *NOXa* genes from *M. graminicola* genomic DNA, respectively. Recognition sites of restriction enzymes *Eco*RI and *Kpn*I were used for cloning the *CREA* and *AREA* amplicons into vector pOE01. The *NOXa* amplicon was cloned into the pOE01 vector with the *Pst*I and *Xba*I enzyme sites (Fig. 1A). The created vectors, designated as pOCR1, pOAR1, and pONO1, were individually transformed into competent cells of *Agrobacterium* by electroporation for further use in ATMT.

Agrobacterium tumefaciens-mediated transformation (ATMT). *Agrobacterium* strains containing the over-expression vectors pOCR1, pOAR1, and pONO1, were used for transformation. Recipient cells of *M. graminicola* for gene over-expression were grown in YSB at 18°C for 2 wk in an orbital incubator shaker. ATMT was carried out using fungal spores adjusted to 1×10^7/mL by following the protocol described previously [15]. PDA containing 100 μg/mL of hygromycin and 200 μg/mL cefatoxime was used for the selective medium. After 2~3 wk, hygromycin-resistant colonies appeared on the selective medium, and isolated colonies were sub-cultured on fresh plates of selective medium to verify incorporation of over-expression construct (OCR1, OAR1, or ONO1) into the *M. graminicola* genome.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) from 2-wk-old fungal cells grown in YSB according to the manufacturer's instructions and subsequently used for cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). All primers used for qRT-PCR analyses are listed in Table 1. All qRT-PCR reactions were performed in a 7900HT fast real time PCR System with RQ Manager ver. 1.2 software (Applied Biosystems, Carlsbad, CA, USA). Each cDNA sample was adjusted to 200 ng/mL before mixing with the SYBR Green PCR Master Mix (Applied Biosystems). qRT-PCR reactions consisted of 2 min at 5°C and 10 min at 95°C for activation, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Primer pairs CREA-rt-F1 & R1, AREA-rt-F1 & R1, and NOXa-rt-F1 & R1 were used to test levels of *CREA*, *AREA*, and *NOXa* gene expression, respectively. The *M. graminicola* β-tubulin-homologous gene, *TUB1* (located on *M. graminicola* chromosome 1, at the sequence from bases 5,604,084 to 5,605,941) was used as the internal control for normalization. To quantify *TUB1* expression levels, we used the Tub1-rt-F1 & R1 primer pair. Gene expression was calibrated by the $2^{-\Delta\Delta Ct}$ method [16]. The range of expression was calibrated

using $2^{-\Delta\Delta Ct-s} - 2^{-\Delta\Delta Ct+s}$ where s is the standard deviation of the DCt value (Ct = threshold cycle). At least four biological replicates were used to assay the expression of each gene.

Nitroblue tetrazolium (NBT) staining and microscopy. Microscopic observations were made with an Olympus BX41 microscope (Olympus America Inc., Melville, NY, USA) equipped with DP controller software and a DP70 camera. The NBT staining method was employed to detect and measure the amount of ROS produced according to protocols described previously [17, 18]. Cultures grown in YSB for 7 days were centrifuged and re-suspended in 0.2% NBT. After incubation for 12 hr, cells were centrifuged and re-suspended in 10 mL of 50% glacial acetic acid. The stained samples were examined under the microscope. For quantification, we used a sonicator to lyse the stained cells and measured absorbance at 560 nm.

Virulence assays. The susceptible wheat line, Taichung 29, was infected by stem inoculation. Briefly, we grew Taichung 29 in a greenhouse facility until the wheat seedlings reached the 3- to 5-leaf stage. Fungal inocula were prepared from 2-wk-old cultures of *M. graminicola* grown in YSB at room temperature in an orbital shaker adjusted to 120 rpm. Wheat stems were punctured with a sterile needle and 3×10^7 spores of each strain were inoculated into the wound. Subsequently, the inoculated wheat was incubated under a 12 hr light/12 hr dark cycle. After 21 days, when unmistakable STB disease symptoms of clear black pycnidia appeared, we analyzed the extent of the infections. All assays were repeated at least three times.

RESULTS

Identification of *CREA*, *AREA*, and *NOXa* homologs for over-expression analysis. We searched for homologs of the *CREA*, *AREA*, and *NOXa* genes in *M. graminicola* using the genomic database (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html). *Aspergillus nidulans CREA* (GenBank No. L03563), *Fusarium graminearum AREA* (GenBank No. FG08634.1), and *Magnaporthe oryzae NOX1* (GenBank No. MGG_00750) were used to search for homologous sequences in the *M. graminicola* genome. Open reading frames with the highest similarity were selected, and sequences were retrieved for further study. The putative *CREA* gene was located on *M. graminicola* chromosome 5, specifically at the sequence from bases 1,654,417 to 1,655,956. A putative *AREA* homolog was found also on chromosome 5, from bases 644,375 to 647,228, and the putative *NOXa* homolog was on chromosome 1, at the sequence from bases 1,949,198 to 1,950,919. The *NOXa* homolog in *M. graminicola* contains a well-conserved FAD-binding domain from 832 to 1,149 nt from its start codon (E value = 1.15e-17). We designated the identified genes in *M. graminicola* as *CREA*, *AREA*, and *NOXa*.

In silico analyses of the identified sequences revealed that the proteins encoded by *CREA*, *AREA*, and *NOXa* had significant similarities to various proteins involved respectively in catabolite repression, nitrogen metabolite repression, or functioning as NADPH oxidases across different fungal species. Specifically, *M. graminicola* CreA was highly similar to the *Cochliobolus carbonum* CreA protein, a probable carbon catabolite repressor (E value = 4e-62). BLAST searches also revealed that the *M. graminicola* AreA and NoxA proteins were highly similar to the GATA transcriptional activator AreA in *Aspergillus fumigatus* (E value = 1e-111) and NADPH oxidase isoform 1 in *Magnaporthe oryzae* (E value = 0.0), respectively. Based on these similarities, the identified *CREA*, *AREA*, and *NOXa* genes in *M. graminicola* were used for over-expression analyses throughout this study.

***M. graminicola* strains over-expressing *CREA*, *AREA*, and *NOXa* were confirmed.** Over-expression constructs (*OCR1*, *OAR1*, and *ONO1*) were individually integrated into the *M. graminicola* genome by ATMT. Initially, we identified *CREA*, *AREA*, and *NOXa* over-expression strains by isolating hygromycin-resistant transformants that were subsequently confirmed by PCR with primers RP27-che-F1 & R1 located within the RP27 promoter (Table 1, Fig. 1). In this study, the RP27 promoter was incorporated into all three over-expression constructs, driving the constitutive expression of *CREA*, *AREA*, and *NOXa* in transformed strains. Therefore, 0.5-kb bands originating from RP27 sequences could be generated from strains with integration of the over-expression constructs, whereas wild-type DNA did not result in any detectable PCR amplicons (Fig. 1B). We isolated more than 10 putative over-expression transformants for each targeted gene and these were subject to further verification of gene-expression levels. We employed qRT-PCR to test whether expression of the *CREA*, *AREA*, and *NOXa* genes increased in each of the putative over-expression transformants. Based on expression analysis, we selected over-expression transformants of *CREA*, *AREA*, and *NOXa*, designated as CR1 (*CREA* over-expression strain), AR1 (*AREA* over-expression strain), and NO2 (*NOXa* over-expression strain). Notably, the CR1, AR1, and NO2 strains showed greater than 3- to 5-fold transcriptional upregulation of each respective gene as compared to wild type (Fig. 2). We concluded that CR1, AR1, and NO2 are over-expression strains of the *CREA*, *AREA*, and *NOXa* genes, respectively, and they were phenotypically characterized further.

CR1 and AR1 are sensitive to nutrients and NO2 displays a yeast-like morphology. To test whether the over-expression events in CR1, AR1, and NO2 affect morphology as compared to the wild-type progenitor, we analyzed their growth patterns on various media (Fig. 3A). On both PDA and YSA media, CR1 showed increased invasive growth compared to wild type, whereas the growth of AR1 was significantly repressed on YSA (Fig. 3B). Exogenous carbon or nitrogen components in YSA or PDA may explain the observed radial growth rates of CR1

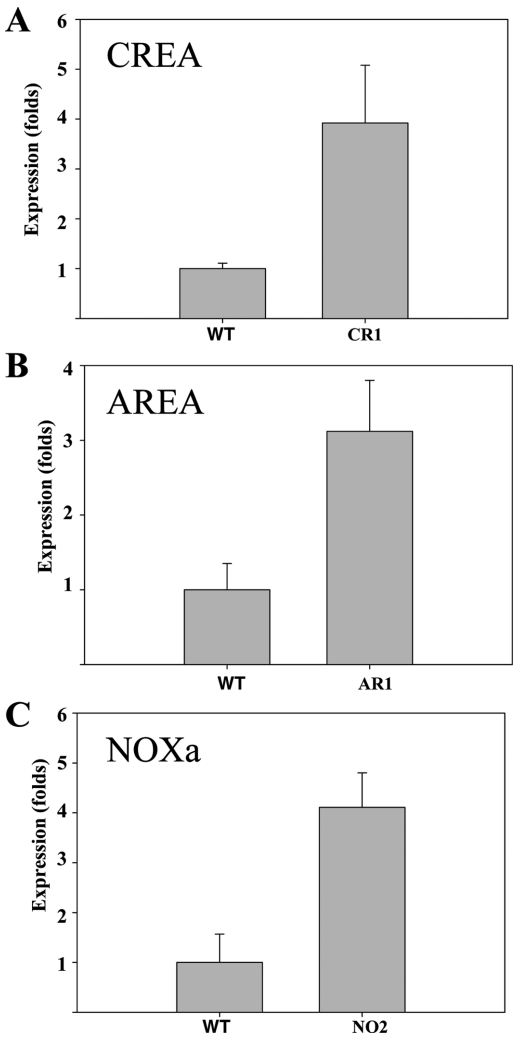

Fig. 2. Expression of the *CREA*, *AREA*, and *NOXa* genes in *Mycosphaerella graminicola* wild-type and putative over-expression strains. The transcription levels of the *CREA*, *AREA*, and *NOXa* genes were evaluated using the $2^{-\Delta\Delta CT}$ method with the *M. graminicola* β-tubulin gene as an endogenous control. Data represent the fold differences in gene expression. Three biological replications were performed to obtain standard deviations. A, B, and C indicate expression levels of the *CREA*, *AREA*, and *NOXa* genes between the wild-type and over-expression strains, CR1, AR1, and NO2, respectively.

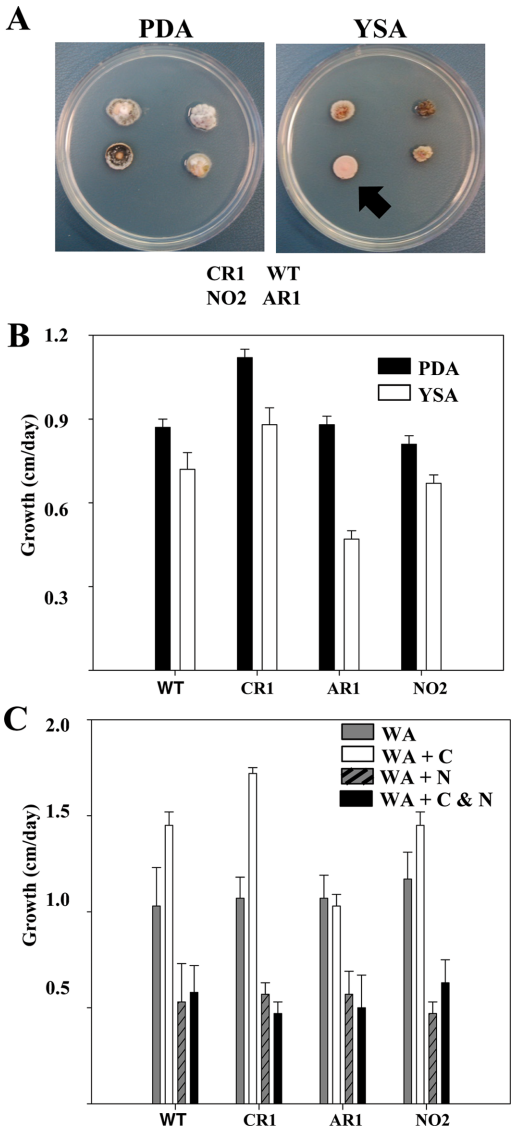

Fig. 3. Morphology and radial growth of *Mycosphaerella graminicola* strains on agar media. A, Growth on agar plates was tested with the wild type, *CREA*, *AREA*, and *NOXa* over-expression strains (WT, CR1, AR1, and NO2). All strains were grown on PDA or YSA for 10 days at 18°C. Yeast-like growth of strain NO2 on YSA is indicated by the black arrow. Locations of inoculated strains on agar plates are indicated below the photos; B, Radial growth on PDA or YSA was measured as the diameter of colonies between several time points. Growth per day was calculated and presented as a bar graph. Three biological replications were analyzed. Black bars, growth on PDA; white bars, on YSA; C, Radial growth on different carbon or nitrogen sourcesial growth was presented in the same way described in Fig. 3A. All media were based on WA medium with addition of the carbon or nitrogen sources indicated. PDA, potato dextrose agar; YSA, yeast extract agar; WA, water agar; gray bars, growth on WA; white bars, growth on WA with 200 mM glucose; hatched gray bars, growth on WA with 200 mM ammonium sulfate; black bars, growth on WA with 200 mM glucose and ammonium sulfate. The thin lines above the bars in B and C indicate the standard deviations.

and AR1. Therefore, we further tested the possibility of nutrient effects of carbon or nitrogen alone on the growth of CR1 and AR1 by incubating them on water agar supplemented with 200 mM glucose, 200 mM ammonium sulfate, or both. In response to carbon nutrients, AR1 displayed the opposite alterations in growth patterns as compared to wild type. CR1 showed the same pattern as wild type but the magnitude of the growth stimulation caused by glucose was much higher. Radial growth of *M. graminicola* was enhanced in the presence of exogenous carbon but was suppressed by nitrogen (Fig. 3C). However, the growth of CR1 was more stimulated, whereas that of AR1 was not

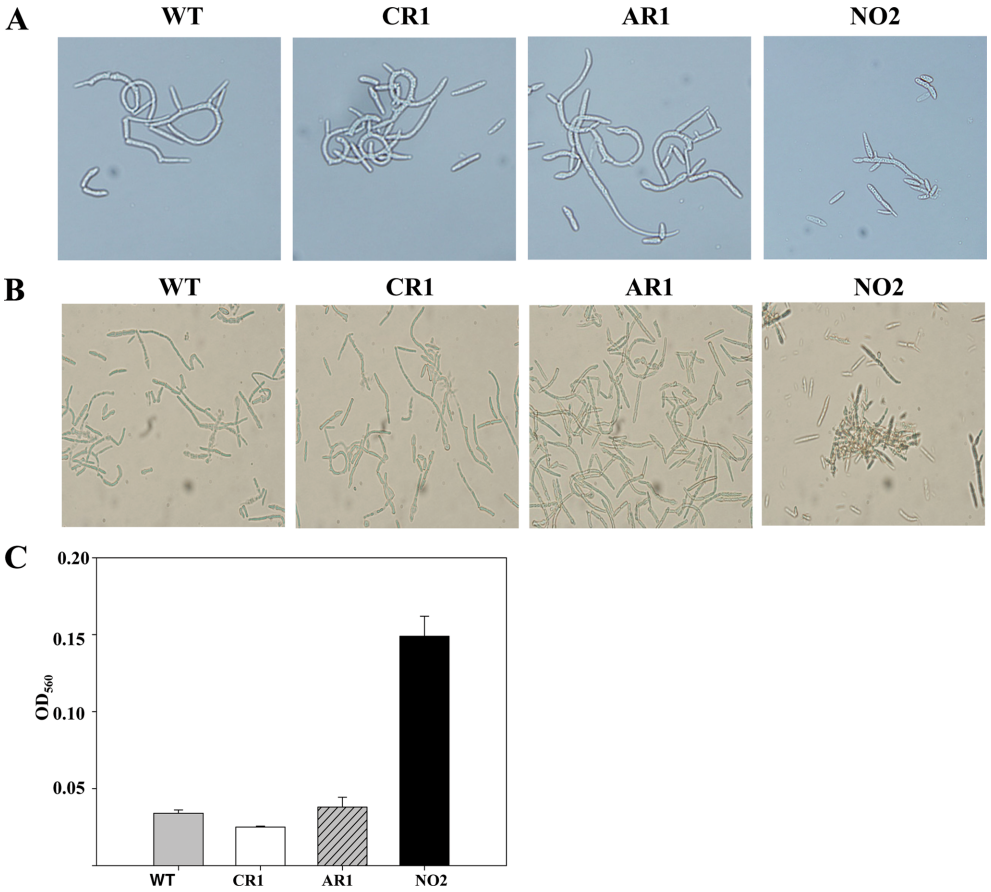

Fig. 4. Yeast-like morphology of *Mycosphaerella graminicola NOXa* over-expression strain NO2 and NBT staining analysis. A, Seven-day-old cultures in YSB were photographed under the compound microscope. Yeast-like growth was observed for strain NO2; B, Fungal cells in Fig. 4A were stained with NBT following published protocols and visualized with a compound microscope (40×). The NO2 strain showed dark blue coloration indicating increased ROS generation; C, NBT staining in Fig. 4B was quantified and represented as mean ± standard deviations in bar graph format. Three biological replications were included to obtain the standard deviations. WT, wild type; CR1, putative *CREA* over-expression strain; AR1, putative *AREA* over-expression strain; NO2, putative *NOXa* over-expression strain; NBT, nitroblue tetrazolium; YSB, yeast extract broth; ROS, reactive oxygen species.

increased with exogenous carbon relative to the other strains (Fig. 3C). Taken together, we concluded that *CREA* over-expression led to increased sensitivity to carbon, whereas the growth of AR1 was independent of exogenous carbon.

M. graminicola has unique morphology with both filamentous and yeast-like growth phases. In the yeast-like growth phase, *M. graminicola* has restricted colonial as well as invasive growth. We also observed a yeast-like growth predominance of the NO2 strain, although radial growth rate was not altered. Compared to the wild-type progenitor, the NO2 strain consistently showed more yeast-like growth on PDA as well as YSA (Fig. 3A). In addition, yeast-like growth became more evident as exogenous concentrations of supplemented H_2O_2 were increased (data not shown).

The NO2 strain developed yeast-like morphology in YSB, which correlates with ROS production. We attempted to determine whether the yeast-like morphology of the NO2 strain could be explained by increased *in vivo* generation of ROS in *M. graminicola*. To do so, we first grew the wild-type, CR1, AR1, and NO2 strains in liquid medium (YSB) and tested whether the yeast-like morphology of NO2 was consistent on agar as well as in liquid media. Again, we observed the increased yeast-like growth in NO2, compared to the other strains (Fig. 4A). Next, we stained with NBT to detect and quantify ROS generation from each of the strains *in vivo*. We followed the NBT staining protocol specifically designed to detect ROS [17, 18]. Once superoxide radicals react with NBT, they form a dark-blue, water-insoluble precipitate. No significant differences were observed in NBT-stained hyphae among the wild type, CR1, and AR1 strains, while strong blue precipitate formation was observed in the NO2 strain (Fig. 4B). Quantification demonstrated that NO2 had around a 4-fold increase in NBT staining as compared to the other strains (Fig. 4C). The level of NBT staining is a well known indicator of the extent of ROS generation in cells, thus indicating a significant correlation between the yeast-like

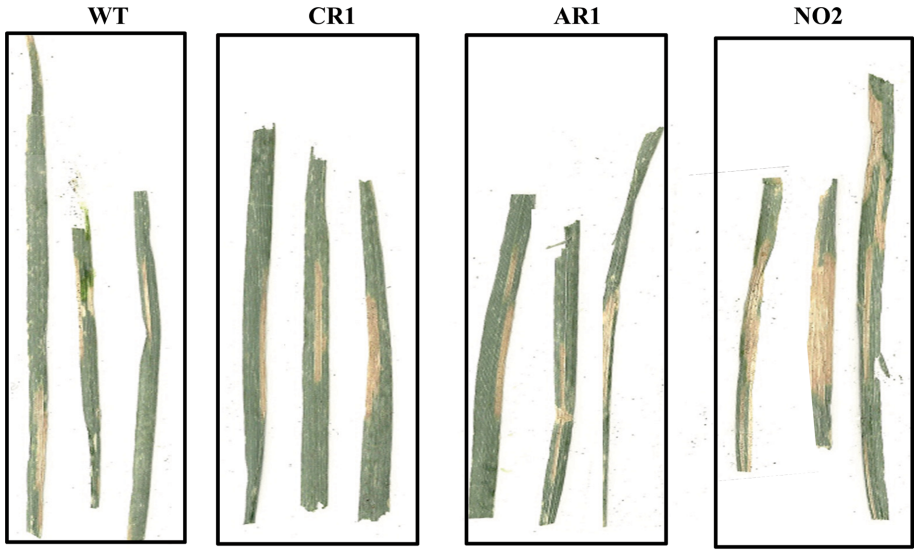

Fig. 5. The effect of over-expression of the *CREA*, *AREA*, and *NOXa* genes on pathogenicity of *Mycosphaerella graminicola*. Sheaths of the susceptible wheat cultivar, Taichung 29, were punctured with a sterile needle and infected with 3×10^7 spores of wild type (WT) or one of the over-expression strains, putative *CREA* over-expression strain (CR1), putative *AREA* over-expression strain (AR1), or putative *NOXa* over-expression strain (NO2). Inoculated wheat plants were incubated in a greenhouse for 21 days. The stem infection assays were performed with at least three biological replications for each fungal isolate.

morphology and ROS levels in NO2 (Fig. 4). Consequently, we conclude that increased ROS generation via *NOXa* may be responsible for yeast-like morphology in *M. graminicola.*

NOXa over-expression significantly increases pathogenicity. To determine the effect of *CREA*, *AREA*, and *NOXa* over-expression on virulence of *M. graminicola*, infection assays was performed on the highly susceptible wheat cultivar Taichung 29. Wheat seedlings at the 3~5 leaf stage were inoculated with 10^7 spores of the wild-type, CR1, AR1, and NO2 strains. After inoculation, plants were incubated until STB disease symptoms appeared, such as pycnidia formation indicated by black spots within tan lesions. We obtained consistent results that STB caused by NO2 was increased significantly compared to that caused by the wild-type, CR1, and AR1 strains (Fig. 5). We consistently observed that NO2 was capable of causing significantly greater symptoms than those caused by the other strains (data not shown). We initially hypothesized that the *CREA* and *AREA* over-expression strains might be more aggressive in the early stage of STB, whereas over-expression of *NOXa* may cause sudden disease development during the late stage of infection. However, we could not find any significant differences between wild type and CR1 or AR1, although NO2 showed consistently increased STB development. Therefore, we concluded that *NOXa*-mediated ROS generation, but not *CREA*- and *AREA*-mediated nutrient acquisition, is important for the development of STB.

DISCUSSION

M. graminicola is one of the most important fungal pathogens affecting wheat production worldwide. The pathogenic characteristics of *M. graminicola* suggest that its life cycle consists of an early biotrophic stage and a late necrotrophic stage [7, 8]. During the biotrophic stage of infection, *M. graminicola* colonizes living wheat host tissue by obtaining nutrients, such as carbon or nitrogen, available in the apoplastic and intercellular-associated spaces [7, 8]. This biotrophic stage is subsequently followed by a necrotrophic phase of infection associated with host cell death, suggesting the involvement of ROS or mycotoxins as a mechanism of virulence [7].

Fungal CreA is a well characterized protein involved in carbon catabolite repression. CreA has a Cys2His2 zinc-finger DNA-binding domain which has high similarity to yeast Mig1p and acts as a major regulator in carbon-response signaling [10, 19]. Fungal AreA is a GATA transcriptional activator regulating nitrogen metabolite repression. Many filamentous fungi are able to utilize other nitrogen sources via *AREA*-mediated gene regulation [9, 20, 21]. Therefore, *CREA* and *AREA* encode important fungal proteins that regulate many downstream nutrient-responsive genes. In this regard, we initially hypothesized that *CREA* and *AREA* in *M. graminicola* might have important functional roles during the early stage of STB by affecting nutrient acquisition. In addition, *M. graminicola* does not form nutrient-absorbing structures such as haustoria, suggesting the importance of putative unknown or CreA- and AreA-mediated signaling in biotrophic nutrient acquisition.

On the other hand, fungal *NOXa* encodes the membrane-bound enzyme NADPH oxidase, which plays a critical role in the production of ROS by transferring electrons from NADPH to oxygen molecules [22]. Filamentous fungi possess

three subfamilies of NADPH oxidases: NoxA, NoxB, and NoxC [23, 24]. Of these, NoxA and NoxB have very similar structures and play major roles in producing ROS, whereas NoxC is not commonly found across fungal taxa and does not have any clear functional roles [25]. Based on this, we selected a *NOXa* gene encoding the NoxA isoform as a target for over-expression in this study. Clearly, constitutive expression of *NOXa* led to increased ROS generation, as indicated by NBT staining (Fig. 4C), and we reasoned that *NOXa* over-expression is sufficient for testing the overall effects of ROS generation on *M. graminicola* pathogenicity. ROS are well known determinants of the outcomes during plant-fungal interactions. Several lines of evidence have shown that ROS-generated oxidative bursts are essential to plant defense responses as well as fungal pathogenicity [11, 26, 27]. We speculated that ROS generation via *NOXa* in *M. graminicola* may be associated with late stages of infection leading to the development of the characteristic necrosis associated with STB.

To understand the putative functional roles of *CREA*, *AREA*, and *NOXa* in the biotrophic or necrotrophic stages of the pathogenicity of *M. graminicola*, we employed over-expression strategies. Because multiple, redundant genes showed high similarity to each of the *CREA*, *AREA*, and *NOXa* genes in the *M. graminicola* genome, we adopted an over-expression strategy instead of time-consuming individual gene knock-outs. Due to the difficulties of loss-of-function genetic mutations or the inefficiency of single-gene deletions of redundant similar genes, we hypothesized that the over-expression strategy instead could provide information to understand the cellular function of the *CREA*, *AREA*, and *NOXa* genes.

Unfortunately, we found no differences in STB severity among the wild-type, *CREA* and *AREA* over-expression strains (CR1 and AR1). Previous analysis of *Alternaria citri* showed that over-expression of *CREA* resulted in severe symptoms of black rot of citrus fruit [28]. As we could not identify a significant difference in pathogenicity of CR1, we speculate that differences in carbon nutrient composition of different hosts, e.g., fruit juice sacs as compared to wheat leaves, could explain these observations, and that metabolism of carbohydrates is not important for early pathogenicity of *M. graminicola* as also hypothesized previously [29]. In *Colletotrichum lindemuthianum*, the AreA-like nitrogen regulator CLNR1 is indispensable for the infection cycle [30], although we could not demonstrate the same association for *M. graminicola AREA* with pathogenicity. It is possible that over-expression of *CREA* and *AREA* could lead to increased virulence of *M. graminicola*, even though it was not identified in this study. For example, perhaps the effects of *CREA*- and *AREA*-mediated nutrient signaling on pathogenicity in the biotrophic phase of *M. graminicola* occur too early to be detected clearly or led to differences that were too subtle to be observed. Further sophisticated investigations with the aid of more sensitive technologies may be necessary to understand the elusive molecular mechanisms of the biotrophic phase of pathogenicity by *M. graminicola*.

However, we could see an increased pathogenicity of the *NOXa* over-expression strain, NO2, compared to those of the other strains. NO2 was consistently more virulent as indicated by enhanced STB symptom development, suggesting that increased ROS generation via *NOXa* is an important factor for *M. graminicola* pathogenicity (Fig. 5). In the ergot fungus, *Claviceps purpurea*, NADPH oxidase Cpnox1 is required for full virulence [26]. In the rice blast pathogen, *Magnaporthe grisea*, NADPH oxidase Nox1 is responsible for the plant infection process [11]. Consistent with these reports in other filamentous fungi, we observed that *NOXa* was important for pathogenicity of *M. graminicola*. *Mycosphaerella graminicola* is a plant-pathogenic ascomycete with a hemibiotrophic lifestyle consisting of an early, biotrophic phase followed by a late, necrotrophic stage. During the necrotrophic growth, ROS might facilitate rapid collapse of wheat tissue, thereby increasing the severity of STB disease. Thus, we propose that ROS generation via *NOXa* is an important virulence determinant during the interaction between *M. graminicola* and its wheat host.

Previous microarray analyses also revealed that many genes related to oxidative stress mechanisms were upregulated during the late stages of STB development supporting ROS involvement in the wheat-*M. graminicola* interaction [7, 31]. In addition, H_2O_2 accumulation in wheat correlates with pycnidium formation and host cell death [32]. Taken together, we conclude that ROS accumulation, regardless of origin, either from wheat or from *M. graminicola*, is fundamental to STB disease development by aiding in necrosis and/or pycnidium formation (Fig. 5).

ROS production mediated by NoxA isoforms in filamentous fungi also has been implicated in important signaling roles during fungal development and differentiation [24, 25]. Consistent with previous reports, we observed a yeast-like growth preference of NO2, indicating a positive functional role(s) of *NOXa* in *M. graminicola* development (Figs. 3A and 4). We also provide evidence that yeast-like growth correlates with ROS production (Fig. 4), suggesting that *in vivo* generation of ROS via *NOXa* or *in vitro* ROS stress conditions can enhance yeast-stage growth of *M. graminicola*. Further serial gene knock-out studies of the *NOXa* and *NOXb* or genes of proteins physically interacting with NoxA will help elucidate ROS signaling associated with *M. graminicola* yeast-like growth.

We also observed that radial growth of *M. graminicola* responds differently to external carbon and nitrogen sources. The presence of readily usable nitrogen suppressed radial growth consistently across all strains tested in this study, indicating a suppressive role of nitrogen on *M. graminicola* radial growth (Fig. 3C). On the other hand, carbon in the absence of nitrogen significantly increased radial growth, and over-expression of *CREA* resulted in carbon sensitivity with increased growth compared to the other strains (Fig. 3C). In yeast, the CreA-homologous protein Mig1 recruits

the repressor proteins Ssn6 and Tup1 to inhibit expression of genes involved in carbon catabolite repression. However, Mig1 alone acts as a transcriptional activator of genes involved in this process [10, 32]. Hence, we assume that *M. graminicola CREA* over-expression led to increased amounts of the putative catabolite activator, CreA, which could explain our results of increased growth of CR1 with an available carbon source. Surprisingly, however, the radial growth of the *AREA* over-expression strain, AR1, was unaffected by exogenous carbon (Fig. 3C). We suspect that nitrogen nutritional cues act as radial growth inhibitors. Presumably, over-expression of *AREA* may lead to reduction in radial growth, due to increased inhibitory nitrogen signaling. In addition, as over-expression of *AREA* did not show any difference of radial growth in the presence of exogenous nitrogen, we speculate that detour pathway(s) independent of *AREA* may transduce nitrogen signaling. More studies will be necessary to understand *CREA*- or *AREA*-mediated signaling(s) in *M. graminicola*.

In conclusion, our results indicate that ROS production via *NOXa* in *M. graminicola* is an important signal for yeast-like development and pathogenicity. Additionally, *CREA*- or *AREA*-associated nutrient signaling pathway(s) are not crucial for STB pathogenicity, however they are important regulators of growth responses to extracellular nutrients.

ACKNOWLEDGEMENTS

We thank Drs. Larry Dunkle and Kristin Laluk for critical reading of the manuscript. We also thank Dr. Ian Thompson for assistance in the pathogenicity experiment. This work was supported by USDA-ARS CRIS projects 3602-22000-015-00D and 3602-22000-017-00D and by a USDA-ARS Administrator-Funded Postdoctoral position to Y.-E. Choi. This research was also financially supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014R1A1A2055300).

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