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Nascent Polypeptide-Associated Complex Involved in the Development and Pathogenesis of *Fusarium graminearum* on Wheat

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ABSTRACT

Reliable knowledge on pathogenic agents contributes to effective plant protection. For most plant pathogens, maintaining protein homeostasis (proteostasis) is essential for unfolding the cellular functions to survive and thrive. However, the fungal proteins involved in proteostasis remain poorly characterized in the process of pathogenesis. In this study, we characterized the function of the nascent polypeptide-associated complex (NAC) in *Fusarium graminearum* (*F. graminearum*) (FgNAC), one of the top 10 fungal pathogens with predominant scientific/economic importance. We found that FgNACα, a subunit of FgNAC, manifests high structural and functional similarity to its homologous counterparts in yeast and other species. The mutants of *F. graminearum* lacking *NACα* are viable but suffer significant defects in vegetative growth, conidial production, and pathogenesis. In addition, we show here that FgNACα can interact with another subunit of NAC (FgNACβ) in a yeast-two-hybrid assay. The subcellular localization results show that FgNACα and FgNACβ are predominantly localized in the cytoplasm. Future studies should focus on deciphering the mechanism by which NAC orchestrates protein biogenesis and consequentially modulates development and pathogenesis.

1. Introduction

Plants in general, and crops in particular, are inevitably and continually exposed to attack from a diverse range of phytopathogenic organisms, each with a unique mode of pathogenesis. Plant diseases have a negative impact on human health directly through agricultural and economic loss, and indirectly through biodiversity collapse and environmental change [1]. In particular, it has been estimated that the losses due to persistent and epidemic outbreaks of fungal and oomycete infection in the five most important crops (i.e., corn, wheat, rice, potatoes, and cassava) would be sufficient to feed 8.5% of the world's population [2]. A reasonable strategy to cope with long-lasting and recurrent plant diseases should be effective protection and timely control. Characterization of the genes involved in pathogen development and pathogenesis contribute to novel strategies for plant disease control [3].

The nascent polypeptide-associated complex (NAC) is a functionally versatile protein complex that is involved in protein biogenesis, assembly, and transportation, and that plays a key role in maintaining protein homeostasis (proteostasis) [4]. In all eukaryotes, NAC is composed of a heterodimer consisting of an α- and a β-subunit [5–7]. The heterodimeric NAC associates with ribosomes in a 1:1 stoichiometry [8] and dynamically interacts with both ribosomes and nascent polypeptides in a reversible fashion [9], thereby performing a chaperone-like function [10–12]. In yeast *Saccharomyces cerevisiae*, NAC assists in the folding and maturation of newly synthesized proteins, prevents nascent polypeptides from inappropriate targeting to the endoplasmic reticulum [13,14], initiates protein targeting to mitochondria *in vivo* [15], and functionally connects to other chaperone networks such as the Hsp70 system [16]. In nematode *Caenorhabditis elegans*, NAC has a primary role—similar to its role in yeast—as a ribosome-associated chaperone to regulate translation and to assist in the folding of nascent polypeptides; it is also an essential and beneficial proteostasis sensor that not only detects proteotoxic stress, but also mediates translation upon proteotoxic stress, thus providing the cell with a regulatory feedback mechanism to maintain proteostasis

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[9]. These unique features of the NACs and their potential roles in cellular operation make them fascinating subjects of studies in a diverse range of organisms from various perspectives.

In the present study, we characterized the role of NAC in the ascomycete fungus *Fusarium graminearum* (*F. graminearum*; teleomorph: *Gibberella zeae*), with a focus on its pathogenesis on wheat. This fungus is a highly destructive plant pathogen that causes *Fusarium* head blight (FHB) in wheat and other small-grain cereals [17,18]. Infection by *F. graminearum* not only results in direct yield loss in the field, but also produces mycotoxins within grains during storage, which are detrimental to human and animal health; thus, this fungus poses a threat to food safety that is of global concern [19–22]. Investigation of the genes involved in pathogenesis may provide insights into the mechanism by which *F. graminearum* interacts with its host plants, thus promoting the development of new strategies for the effective control of FHB. Here, through targeted gene deletion, yeast two-hybrid assays, and subcellular localization combined with pathogenicity testing, we demonstrate that the NACα subunit plays an important role in regulating the development and pathogenesis of *F. graminearum* in wheat.

2. Materials and methods

2.1. Fungal strains and culture conditions

The wild-type strain PH-1 of *F. graminearum* was used for the construction of gene deletion mutants. Both the wild-type strain and its derived mutants were routinely cultured on potato dextrose agar (PDA) and V8 agar medium at 25 °C with a 12 h:12 h light:dark cycle for mycelial growth assays. The experiments were performed with three replicate plates. Asexual reproduction was induced in carboxyl methyl cellulose (CMC) [23]. All strains were preserved as conidial suspensions in 20% glycerol at −70 °C.

2.2. Bioinformatic analysis

The genome of *F. graminearum* PH-1 [24] was searched with protein basic local alignment search tool (BLASTp) using the sequence of the NACα subunit (NP_596361, EGD2) of fission yeast, *Schizosaccharomyces pombe* (*S. pombe*), as the query [25]. The whole-length sequencing of the *NACα* of the PH-1 was downloaded and then used as the query to search GenBank for homologous sequences from other species (Table S1 in Supplementary data). Homologous amino acid (aa) sequences were aligned using Clustal X version 2.0 [26]. A neighbor-joining phylogenetic tree was constructed using molecular evolutionary genetics analysis version 7.0 (MEGA7), with the node support being assessed through bootstrapping for 1000 replicates [27].

2.3. Generation of deletion mutants and complementary strains

The *NACα* gene deletion constructs were generated from the *F. graminearum* wild-type strain PH-1 using the split-marker approach [28,29]. The upstream 824 base pairs (bp) DNA fragment of *FgNACα* gene was cloned and inserted into the pUC19-G418^R vector to generate pUC19-A-G418, and the downstream 961 bp DNA fragment was cloned and sequentially inserted into pUC19-A-G418 to generate pUC19-A-G418-B (Fig. S1 in Supplementary data). The whole A-G418-B fragment was then amplified and transformed into *F. graminearum*, as described by Yuan et al. [30]. After checking for orientation, the correct gene replacement vector was linearized by digesting with *Hin*dIII and was transformed into PH-1 using the polyethylene glycol (PEG)–$CaCl_2$ method. The G418-resistant transformants were isolated and purified by means of the single-spore method, and their genotypes were examined by sequencing.

For complementation assays, a fragment containing the entire gene—including the open reading frame (ORF), the promoter region, and the terminator region—was amplified and cloned to the pMD18-T to generate the complementation construct pMD18-*FgNACα*. The insert sequence in the complementation construct was confirmed by DNA sequencing. The correct construct was linearized with *Eco*RI and introduced into the deletion mutants by cotransformation with the *Hin*dIII-linearized pUCATPH plasmid to generate the complementary strains. The G418-sensitive and hygromycin-resistant transformants were isolated and their genotypes were confirmed by polymerase chain reaction (PCR) using the primer pair ORFα-F/ORFα-R (Table S2 in Supplementary data).

2.4. Phenotypic assays

To measure the growth rate, 5 mm mycelium plugs cut from the edge of a 3 d-old colony were inoculated on PDA plates and cultivated at 25 °C. The diameter of the colony was measured daily for 4 d. To quantitatively analyze conidiation, four 5 mm mycelium plugs of the aerial hyphae of 2 d-old cultures were introduced into 100 mL of liquid CMC medium and cultured at 25 °C for 5 d. Conidia were harvested by centrifugation at 5000 $r \cdot min^{-1}$ for 5 min and washed twice with sterile distilled water. The concentration of conidia was determined with a hemocytometer in the microscope, as described by Hou et al. [31]. Morphological observation and measurement were conducted under an Olympus BH-2 microscope. The width of the middle intercalary cell and the length from the elongated apical cell to the pedicellate foot cell of 100 conidia were measured for each strain examined.

2.5. Plant infection assays

The wheat cv. Minxian169 was used for the infection assay on both spikelets and coleoptiles. Ten microliters of the conidia suspension (4×10^5 conidia$\cdot mL^{-1}$) collected from 5 d-old CMC medium was injected into the third full-sized spikelet from the base of the inflorescence of 35 to 42 d-old plants. The inoculated wheat heads were enclosed in small plastic bags for 48 h to maintain humidity. Symptomatic spikelets in each plant were counted 12 d after inoculation. The disease index scores were calculated as previous reported [32]. The number of inoculated wheat heads per treatment was 9 or 10 for each test, and all tests were repeated at least four times. Wheat coleoptiles were inoculated following the method described by Liu et al. [33]. Fifty coleoptiles were inoculated for each strain and then kept at room temperature in a growth chamber. The length of brown lesions was measured 7 d after inoculation.

2.6. Yeast two-hybrid assays and subcellular localization

The Matchmaker GAL4 two-hybrid system 3 (Clontech, USA) was used to test interaction between the two subunits of FgNAC. The *FgNACα* ORF was cloned into pGADT7 to create the vector AD-*FgNACα*. *FgNACβ* ORF was amplified from the complementary DNA (cDNA) of PH-1 and cloned into the pGBKT7 vector (BD-*FgNACβ*), which was used as the prey in the yeast two-hybrid assay. After confirmation by means of sequencing, the bait and prey vectors were co-transformed in pairs into the yeast strain AH109 with the Alkali-Cation yeast transformation kit (MP Biomedicals, USA). The Leu^+ and Trp^+ transformants were observed for growth on synthetic dextrose medium (SD–Leu-Trp-His, SD–LWH), and the expression of the *LacZ* reporter gene was measured as described by Zhang et al. [34].

To test the subcellular localization of FgNACα and FgNACβ in *F. graminearum*, the coding regions of *FgNACα* and *FgNACβ* were amplified separately. The vector pDL2 was used to generate the

C-terminal enhanced green fluorescent protein (eGFP) fusion constructs of *FgNACα* and *FgNACβ* that are under the control of the native promoter. The fusion constructs were directly used for *F. graminearum* protoplasts transformation with the general procedures described by Sweigard et al. [35]. All the samples were observed under an Olympus BX61 fluorescence microscope.

2.7. Statistical analysis

Statistical analyses were carried out using SPSS 19 software, and the significant difference between treatments was analyzed using one-way analysis of variance (ANOVA). Statistically significant differences p values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. FgNACα is orthologous to the yeast NACα subunit

Only a single NACα subunit gene (FGRAMPH1_01G10263) was found in the *F. graminearum* PH-1 genome in a BLASTp search with the fission yeast's NACα subunit EGD2 as the query. We designated this NACα subunit gene of *F. graminearum* as *FgNACα*. *FgNACα* contains 1166 nucleic acids with an ORF of 627 bp, which encodes 209 aas. Similar to EGD2, FgNACα consists of a conserved NAC domain (57 aas) at the N terminal and an ubiquitin-associated (UBA) domain (40 aas) at the C terminal (Figs. 1(a) and (b)). The neighbor-joining tree constructed from the *NACα* homologous sequences was completely consistent with the phylogeny of the analyzed species [36]. The aa sequence of FgNACα has greater than 70% similarity to that of *Magnaporthe grisea* (XP_366584.1), and together they formed a well-supported (bootstrap support 79%) subclade in the strongly supported (99%) clade including aa sequences of NACα or putative NACα, all from fungi (Fig. 1(c)). These results indicate that the gene *FgNACα* is the orthologous of the fungal *NACα*.

3.2. FgNACα gene disruption and complementation in F. graminearum

Transformants of the *FgNACα* deletion mutant (*fgnacα*) of *F. graminearum* that could grow in G418 medium were picked and identified by PCR, with the wild-type PH-1 used as the negative control (Fig. S1(b)). No *FgNACα* band (755 bp) was detected by primer pair ORFα-F/ORFα-R (Fig. S1(b), lane 3) compared with the bands amplified by primer pairs FgNACα-Ft1/Neo-F (2781 bp), indicating that *FgNACα* was successfully replaced by the *neo* gene (Fig. S1(b), lanes 1 and 2). To produce the complementary construct of the *fgnacα*, *fgnacα*::*FgNACα*, the 2993 bp of the full-length *FgNACα* gene, including the promoter and terminator, were cloned and transformed together with *Hin*dIII-linearized pUCATPH plasmid into deletion mutant strain *fgnacα* protoplast (Fig. S1(c)). Transformants that could grow in both G418 and hygromycin medium were selected and identified by PCR (Fig. S1(d)). The *FgNACα* band (755 bp) was detected by the primer pair ORFα-F/ORFα-R (Fig. S1(d), lane 4), but there was no corresponding band in the deletion mutant strain *fgnacα* (Fig. S1(d), lane 3), indicating that *FgNACα* was successfully restored in the deletion mutant strain *fgnacα*.

3.3. FgNACα regulates mycelia growth

In comparison with the wild-type strain PH-1, mycelia growth of the deletion mutant strain *fgnacα* was significantly reduced, as measured by colony diameter on both potato dextrose agar (PDA) and V8 medium (Fig. 2(a)). The deletion mutant strain *fgnacα* grows slowly during the cultural periods, and the colony diameter was only about 69% of that of the wild-type strain on the third day of inoculation on the PDA plates (Fig. 2(b)). In addition, the deletion mutant strain *fgnacα* produced fewer aerial hyphae with irregular colony margins (Fig. 2(a)). The phenotype was hardly observable between the wild-type strain PH-1 and the complementary strain *fgnacα*::*FgNACα* (Fig. 2). Similarly, in liquid CMC medium, the deletion strain produced much less mycelia biomass in comparison with the wild-type strain and the complementary strain throughout the whole of cultivation, and similar mycelia biomass was harvested from the wild-type and complementary strains (Fig. 2(c)). Taken together, disruption of the *FgNACα* gene dramatically restricted the vegetation growth of *F. graminearum*.

3.4. Disruption of FgNACα causes abnormal conidia production

To examine the role of the *FgNACα* gene in reproductive development, we compared conidia production and conidial germination of the mutant strains and wild-type strain. We did not find a

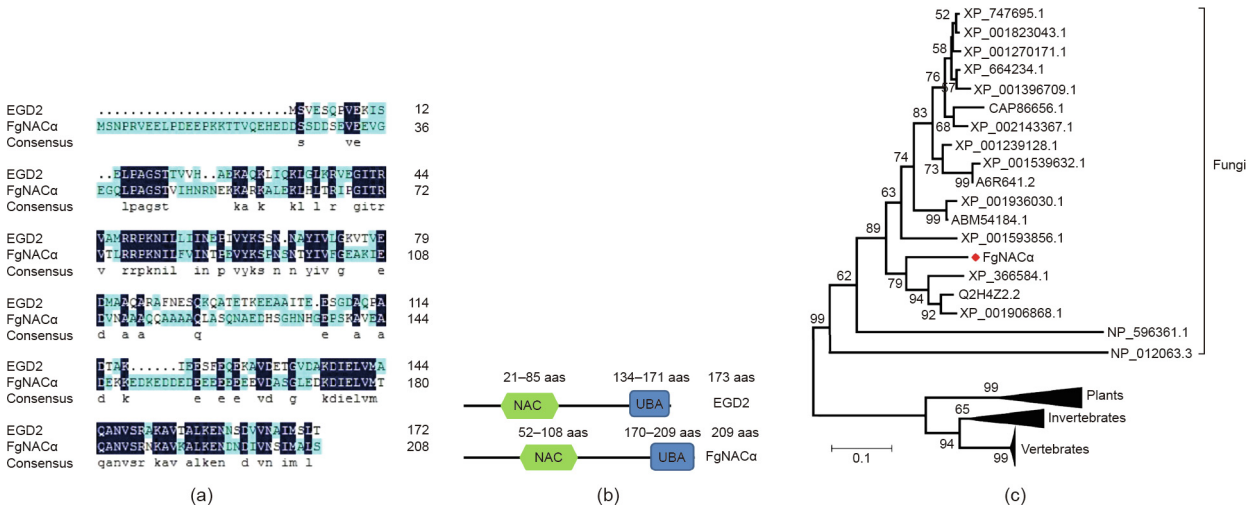

Fig. 1. Identification of the *FgNACα* orthologous gene. (a) Aa sequences comparison of NACα in *S. pombe* (NP_596361, EGD2) and *F. graminearum* (FGSG_08560.3, FgNACα); (b) functional domains of NACα in *S. pombe* and *F. graminearum*; (c) phylogenetic tree based on aa sequences of NACα. Numbers near the node represent the bootstrapping support value. The FgNACα from *F. graminearum* is indicated by the red diamond.

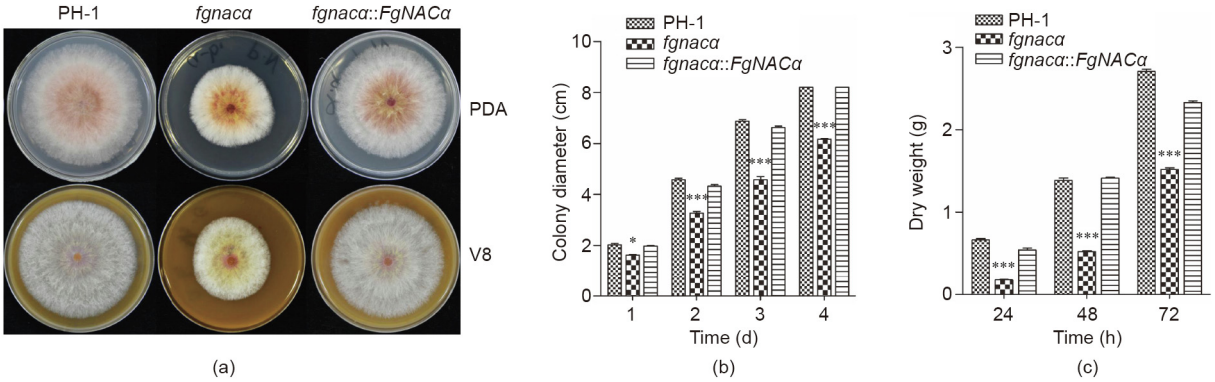

Fig. 2. Deletion of *FgNACα* causes deficit in mycelia growth. (a) Mycelium growth of the wild-type strain PH-1, the deletion mutant strain *fgnacα*, and the complementary strain *fgnacα*::*FgNACα* on PDA and V8 medium plates at 25 °C for 3 d; (b) the colony diameter of each strain was measured after incubation for 1, 2, 3, and 4 d in PDA medium at 25 °C; (c) the weight of the mycelia was calculated after culturing in liquid CMC medium at 25 °C for 24, 48, and 72 h. The symbol ∗ indicates statistically significant differences $p < 0.05$.

clear difference in conidial morphology between the deletion mutant strain *fgnacα* and the wild-type strain or between the deletion mutant strain and the complementary strain. While the number of spores produced by the deletion mutant strain *fgnacα* was significantly reduced ($p < 0.05$), only about 40% of those produced by the wild-type strain or about 42% of those produced by the complementary strain (Fig. 3(a)). In addition, the conidial germination of the deletion mutant strain *fgnacα* was significantly delayed, compared with the wild-type and complementary strains; the germination rate of the deletion mutant strain was only 32% of that of the wild-type strain PH-1 after incubating for 4 h in the CMC liquid medium (Fig. 3(b)). Interestingly, the germination rate of the deletion mutant strain caught up quickly, accounting for about 86% of that of the wild-type strain after 8 h of cultivation and more than 92% after 12 h of cultivation (Fig. 3(b)). The conidia development of the complementary stain was similar to that of the wild-type strain (Fig. 4). These results suggest that the *FgNACα* gene might be involved in the earlier stage of the conidiation process, and that its function might be compensated by those of other genes.

3.5. FgNACα is required for the infection of wheat

The results of the infection assays on wheat cv. Minxian169 are shown in Fig. 4. When the conidia were inoculated on the wheat heads for 12 d, the deletion mutant strain *fgnacα* only infected about 20% of the inoculated spikelet, whereas the wild-type strain PH-1 and the complementary strain caused over 47% infection rates (Fig. 4(b)). The disease symptom was much more serious when caused by the wild-type strain PH-1 and the complementary strain *fgnacα*::*FgNACα* (Fig. 4(a)). Similar patterns were also found when the conidia was inoculated on the coleoptiles (Figs. 4(c) and (d)). The brown lesions caused by the deletion mutant strains were much shorter (~0.54 cm) than those caused by the wild-type strain (1.92 cm) and the complementary strain (1.88 cm). These results suggest that *FgNACα* manipulates the virulence of *F. graminearum* on wheat.

3.6. Interaction between FgNACα and FgNACβ and their subcellular localization

It has been reported that the α and β subunits of NAC interact with each other and function as a heterodimer in order to facilitate the correct accumulation of the precursors of mitochondrial protein in the cytosol in yeast [37]. To test whether the two subunits functioned in the same way as they do in yeast, we investigated their interaction through yeast two-hybrid assays (Fig. 5). We found that FgNACα could interact with FgNACβ in yeast (Fig. 5(a)). This means that FgNACα is physically associated with and functionally related to FgNACβ. They may form a heterodimer and protect the nascent polypeptide from proteolysis.

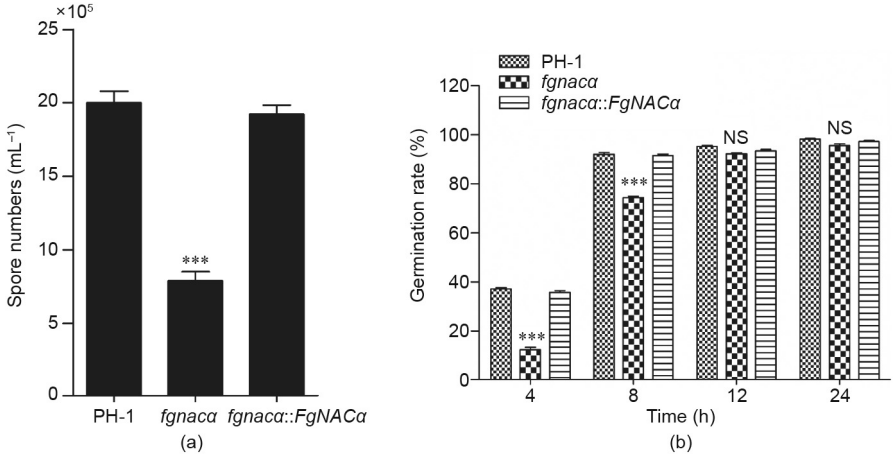

Fig. 3. Comparative difference of wild-type strain PH-1, deletion mutant strain *fgnacα*, and complementary strain *fgnacα*::*FgNACα* in spore number and germination rate. (a) The production of conidia in PH-1, *fgnacα*, and *fgnacα*::*FgNACα* strains. (b) Germination was measured by the percentage of germinated conidia in CMC liquid culture after incubating for 4, 8, 12, and 24 h. The symbol ∗ indicates statistically significant differences $p < 0.05$. NS: no significant difference.

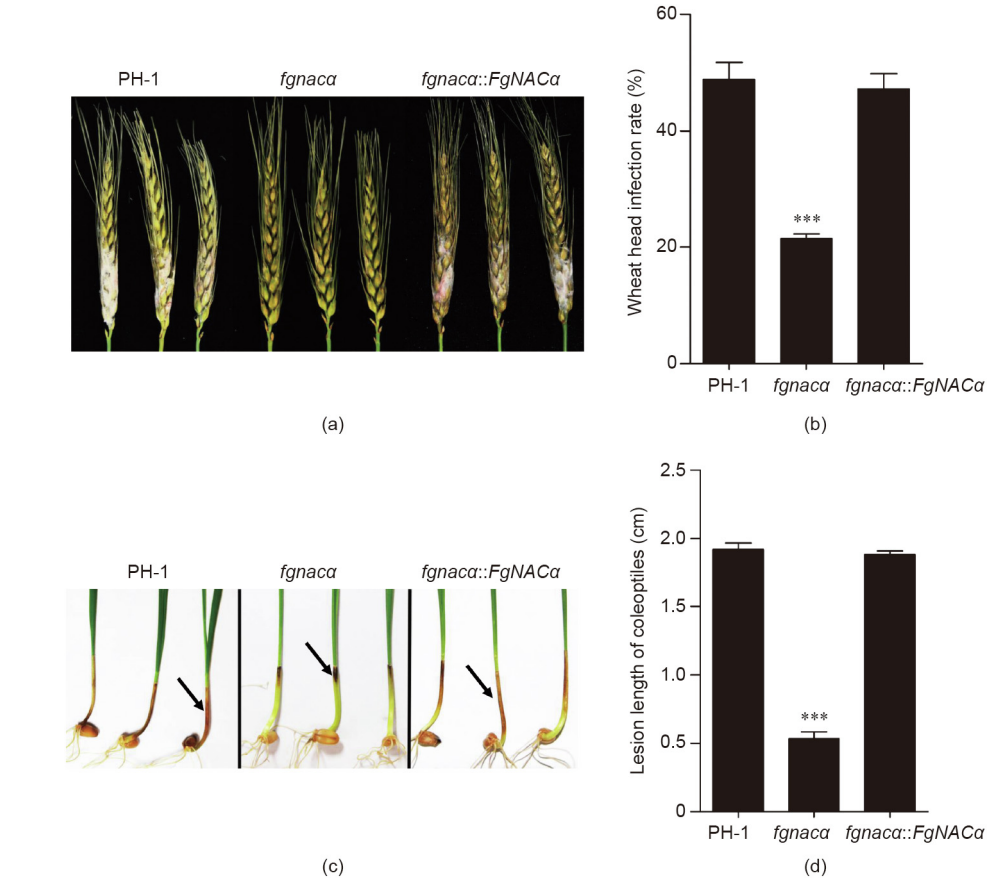

Fig. 4. *FgNACα* is required for *F. graminearum* pathogenicity. (a,b) Infection assays with flowering wheat heads. Wheat heads were inoculated with conidia of the wild-type strain PH-1, the deletion mutant strain *fgnacα*, and the complementary strain *fgnacα*::*FgNACα*. The pictures were taken 12 d post inoculation. (c,d) Deletion of *FgNACα* attenuates the virulence of *F. graminearum* on wheat coleoptiles. The representative brown lesions on the coleoptiles infected with PH-1, *fgnacα*, and *fgnacα*::*FgNACα* are indicated with arrows. The pictures were taken 7 d post inoculation. The symbol ∗ indicates statistically significant differences $p < 0.05$.

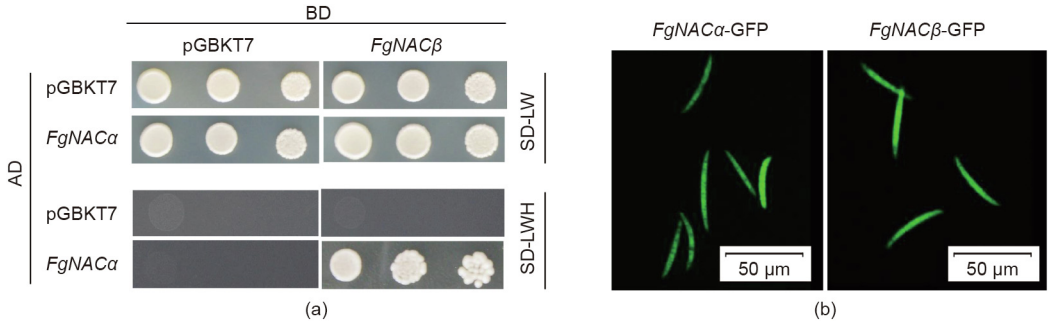

Fig. 5. FgNACα interacts with FgNACβ. (a) Yeast two-hybrid assays of FgNACα and FgNACβ. *FgNACα* was fused with the activation domain (AD) of *GAL4*; *FgNACβ* was fused with the binding domain (BD) of *GAL4*. The transformants of AH109 yeast were diluted 10, 100, and 1000 times, and grown on synthetic dextrose medium without Leu and Trp (SD–LW), and on the plate SD–LWH. (b) Subcellular localization of FgNACα and FgNACβ in *F. graminearum*. Conidia expressing the *FgNACα*-GFP or *FgNACβ*-GFP fusion construct were examined. All the samples were observed under fluorescence microscope.

In order to investigate the subcellular localization of FgNACα and FgNACβ, we generated the C-terminal GFP fusion constructs of the *FgNACα* and *FgNACβ* coding regions with the native promoters and transformed them to the wild-type strain PH-1 protoplasts. Under the fluorescence microscope, the green signals were detected in the cytoplasm of the conidia in transformation strains with *FgNACα*-GFP and *FgNACβ*-GFP (Fig. 5(b)). The results suggest that FgNACα and FgNACβ can have the same localization in the *F. graminearum* cells, which may provide chances for their physical interaction.

4. Discussion

NAC is the first discovered nonribosomal factor that cotranslationally interacts with newly synthesized polypeptides [14]. Together with signal recognition particles, NAC promotes the fidelity of protein targeting to the endoplasmic reticulum [38]. Since its discovery more than 20 years ago, various potential roles for NAC have been demonstrated in several evolutionarily dispersed model organisms, such as yeast [15,39,40], tobacco [41], and nematode [9]. From these studies, diverse roles in maintaining

proteostasis have been discovered [4,42]. In most cases, such functional multiplication was often companied by evolutionary diversification. However, we found that NACα is evolutionarily highly conserved in terms of both the primary sequence structure and the functional domains (Fig. 1). From animals to plants and to fungi, the aa sequences of NACα are very similar and harbor an NAC domain and a UBA domain (Figs. 1(a) and (b)). The phylogeny constructed from *NACα* sequences is highly congruent with the species evolutionary history (Fig. 1(c)). Given the fact that the α- and β-subunits of NAC form a heterodimer, which was found here (Fig. 5) and in other species [5,6], it is reasonable to predict that NACβ is also evolutionarily conserved. Nevertheless, this evolution-function paradox can be explained by the fact that NAC is a key factor in protein biogenesis and functionally connects to other metabolic networks, such as the Hsp70 system [16]. Such functional versatility and evolutionary conservatism imply that the results found in *F. graminearum* in the present study are likely to be transferable to other plant pathogens and vice versa, and that the disease management methods developed by targeting NAC will have trans-disease applicability.

Although the biological function of NACα has been extensively characterized in model organisms, its role in non-model organisms is still poorly understood. In the present study, we found that the NACα subunit gene of *F. graminearum* is not only involved in vegetative growth, but also regulates its pathogenicity positively. In other plant pathogens, it has been reported that *NACα* in *Sclerotinia sclerotiorum* regulates its pathogenicity negatively [43]. Such a difference may be explained by the different lifestyle of the two pathogens. *F. graminearum* is a hemi-biotrophic fungus, while *S. sclerotiorum* is a necrotrophic fungus. In animals, *NACα* is also involved in the innate immune response to pathogens; its expression was significantly up-regulated when a crab was exposed to *Vibrio anguillarum* challenges [44]. Similarly, in Japanese flounder, *NACα* gene expression was up-regulated in fish tissues in response to *Edwardsiella tarda* challenges, and overexpression of *NACα* enhanced resistant gene expression [45]. In plants, it has been demonstrated that silencing the *NACα* gene in tobacco (*Nicotiana benthamiana*) inhibits the cell-to-cell movement of the brome mosaic virus [41]. Moreover, NACα protein shows higher abundance in tomato resistant lines compared with susceptible lines after inoculation with *Ralstonia solanacearum* [46]. Clearly, the function of *NACα* in wheat or host plants in general and the molecular interaction between the pathogen–host systems need to be fully characterized before we can precisely determine the role of *NACα* in pathogenesis.

For fungal pathogens, the ability to invade the plant cell is a prerequisite for successful colonization and infection, while the ability to maintain proteostasis is essential for unfolding the cellular functions to survive and thrive. The fungal molecules involved in proteostasis remain poorly characterized in phytopathogens. In this study, we characterized *FgNACα*, an evolutionarily highly conserved regulator in maintaining proteostasis, which participates in fungal development and pathogenicity. In addition, our results suggest that the function of FgNACα is likely unfolded by forming a heterodimer with FgNACβ (Fig. 5). However, pathogenesis is unfolded through molecular interactions not only within pathogens and hosts, but also between factors from pathogens and from hosts. It will be very interesting to figure out the molecular mechanism of how NAC performs different functions in pathogen–host interaction systems.

5. Conclusion

Effective plant protection technologies often stem from a reliable understanding of pathogenic agents. Such basic knowledge will undoubtedly help to produce novel insight into pathogenesis, and will lead to innovative plant protection strategies. In the present study, we found that the NACα of the plant pathogenic fungus *F. graminearum* manifests high structural and functional similarity to its homologous counterparts from yeast and other fungal species. The mutants of *F. graminearum* that lack *NACα* are viable, but suffer significant defects in vegetative growth, conidial production, and pathogenesis on wheat. These functional consequences of *NACα* deletion might stem from the important role that NAC plays in proteostasis, as found in yeast and other model organisms. From the perspective of plant protection, NAC deserves recognition in the development of innovative control methods for the destructive plant disease, FHB.

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Compliance with ethics guidelines

Xuli Wang, Xin Xie, Jin Liu, Guo-Liang Wang, and Dewen Qiu declare that they have no conflict of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2019.07.025.

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