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Control of reactive oxygen species (ROS) production through histidine kinases in *Aspergillus nidulans* under different growth conditions☆

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ABSTRACT

Sensor histidine kinases (HKs) are important factors that control cellular growth in response to environmental conditions. The expression of 15 HKs from *Aspergillus nidulans* was analyzed by quantitative real-time PCR under vegetative, asexual, and sexual growth conditions. Most HKs were highly expressed during asexual growth. All HK gene-disrupted strains produced reactive oxygen species (ROS). Three HKs are involved in the control of ROS: HysA was the most abundant under the restricted oxygen condition, NikA is involved in fungicide sensing, and FphA inhibits sexual development in response to red light. Phosphotransfer signal transduction via HysA is essential for ROS production control.

1. Introduction

His-Asp phosphorelay signal transduction helps cells adapt to environmental changes and is common among bacterial and some eukaryotic cells. Sensor histidine kinases (HKs) recognize external signals, autophosphorylate on their own histidine residues, transfer phosphoryl groups to their own aspartic acid residues, and subsequently transfer the phosphate signals to histidine-containing phosphor transmitter (HPt). Finally, response regulators (RRs) receive the phosphate signals on their aspartic acid residues and regulate gene expressions directly or by controlling the downstream signal transduction pathways. *Aspergillus nidulans* is a model filamentous fungus that contains 15 HKs, 1 HPt, and 4 RRs [1]. Several HKs have been studied in attempts to characterize the roles of His-Asp phosphorelay systems in *A. nidulans*. TcsA is important for the formation of conidia during asexual development [2]. Meanwhile, TcsB is an ortholog of Sln1, which is an osmosensor in *Saccharomyces cerevisiae* [3]. NikA is involved in sensitivity to fungicides [4,5]. FphA is a fungal phytochrome that negatively regulates sexual development in response to red light [6,7]. However, the roles of the other HKs remain unknown.

Reactive oxygen species (ROS) are produced during cell growth in living organisms. Uncontrolled ROS production causes severe cellular damage including DNA strand breakage, enzyme inactivation, and increased membrane permeability. Most ROS production occurs in the mitochondria, where cells generate ATP for survival via oxidative phosphorylation reactions [8]. On the other hand, ROS signals are also reported to be essential for the normal growth and development of *A. nidulans* [9].

In this study, 15 HKs in *A. nidulans* were characterized by analyzing their expression profiles by quantitative real-time PCR. ROS products were observed by microscopy in HK gene-disrupted strains.

2. Materials and methods

Additional information regarding experimental methods can be found in Supplementary methods.

2.1. Strains, media, and transformation

A. nidulans ABPU1, and *Escherichia coli* XL1-blue and BL21 (DE3) were cultivated as described in the Supplementary methods and used for transformation, RNA preparation, DNA manipulation, and protein purification. A *nikA* deletion strain was constructed by using ABPU1 as a host strain [4]. *ΔphkA* and *ΔphkB* were constructed from another

Abbreviations: DTT, dithiothreitol; FGSC, Fungal Genetics Stock Center; GSH, glutathione; HK, histidine kinase; HPt, histidine-containing phosphotransfer; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; RR, response regulator; t-BOOH, tert-butyl hydroperoxide.

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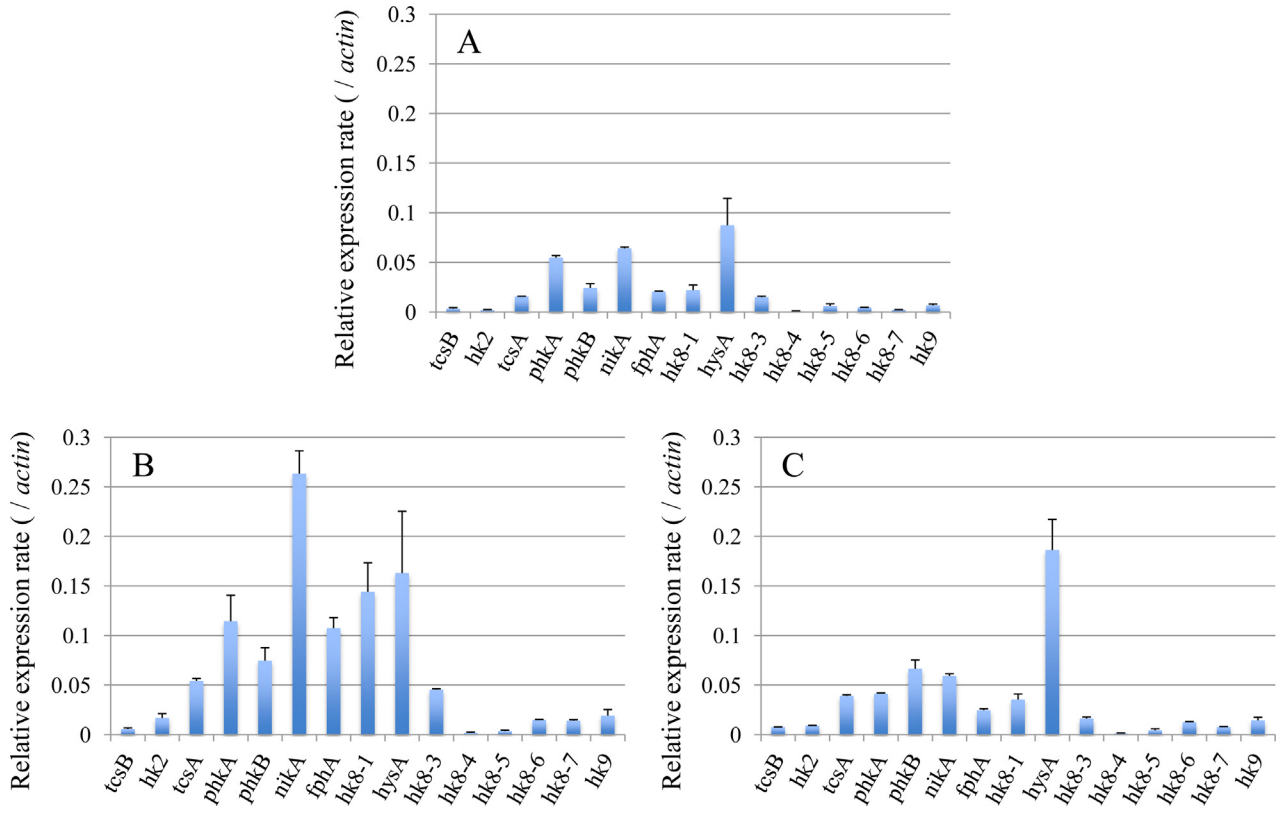

Fig. 1. Expression levels of *A. nidulans* signaling proteins determined by quantitative real-time PCR. The wild-type strain (ABPU1) was cultivated in liquid culture for 18 h (A). The cells were subsequently replaced on plates and incubated for 18 h (B). The replaced cells were also incubated under restricted oxygen conditions by sealing the plates for 18 h (C). RNA samples were isolated from the cells and used as templates for quantitative real-time PCR. Table S1 lists the primers used for PCR. Error bars represent the standard deviations of at least 3 independent experiments.

ABPU1 strain including *ligD* gene deletion to induce efficient homologous recombination [10]. *ΔnapA* was a kind gift from Dr. T. Mizuno [11]. A set of 14 HK deletion strains was purchased from Fungal Genetics Stock Center [12].

2.2. Construction of a hysA deletion and alcA promoter control strain

A *hysA*-deletion plasmid, pKK001, was constructed and introduced into ABPU1 by protoplast transformation. The wild-type *hysA* gene was connected to an *alcA* promoter to create the plasmid, pTM003, which was introduced into the *hysA*-deletion strain. The details of plasmid construction are described in the Supplementary methods.

2.3. Total RNA preparation

Total RNA was prepared, and relative transcription levels were determined by quantitative real-time PCR as described in the Supplementary methods.

2.4. Nitro-blue tetrazolium staining

A. nidulans cultivation and nitro-blue tetrazolium (NBT) staining were performed as described previously [9] with some modifications. To observe germling growth, conidia were inoculated in liquid culture on cover glasses and cultivated for 12 h at 37 °C. To observe sporulating hyphae, each strain was grown beneath cover glass crossed with square holes on agar plates for 2 days at 37 °C. *A. nidulans* strains growing along the cover glass were drifted and sunk into NBT solution (0.1% Nitro Blue Tetrazolium [Wako], 100 mM Sodium phosphate buffer [pH 7.0]). After 4 h in the dark, blue-colored precipitate, which is the reduction product of NBT by superoxide anion, was observed under a microscope (OLYMPUS BX51).

2.5. HysA protein purification

The construction of expression plasmids, cultivation for protein induction, and purification of recombinant HysA HR and HysA H are described in the Supplementary methods.

2.6. In vitro autophosphorylation experiment

The *in vitro* autophosphorylation reaction of purified HysA proteins was carried out according to the method of Azuma et al. [13]. Aliquots of HysA proteins (2 μg) were incubated with 0.05 mM [γ-^{32}P] ATP (37 kBq) in TEG buffer (50 mM Tris–HCl [pH 8.0], 0.5 mM EDTA, and 10% glycerol) containing 5 mM $MgCl_2$ and 200 mM KCl at 25 °C. The reaction was terminated by the addition of SDS–PAGE sample buffer (final, 20 mM Tris–HCl [pH 8.0], 1% β-mercaptoethanol, 1% SDS, 6% glycerol, and 0.02% bromophenol blue). Samples were subjected to SDS–PAGE. The gel was dried and analyzed with an imaging scanner (BAS-2500, Fuji Film, Tokyo, Japan).

3. Results and discussion

3.1. Expression levels of HK proteins in A. nidulans

To determine the roles of unknown signaling proteins in the cell development of *A. nidulans*, we analyzed the expression levels of all HK proteins at different stages of cell growth (Fig. 1) [14]. Only a few HKs were expressed during vegetative growth (Fig. 1A), whereas

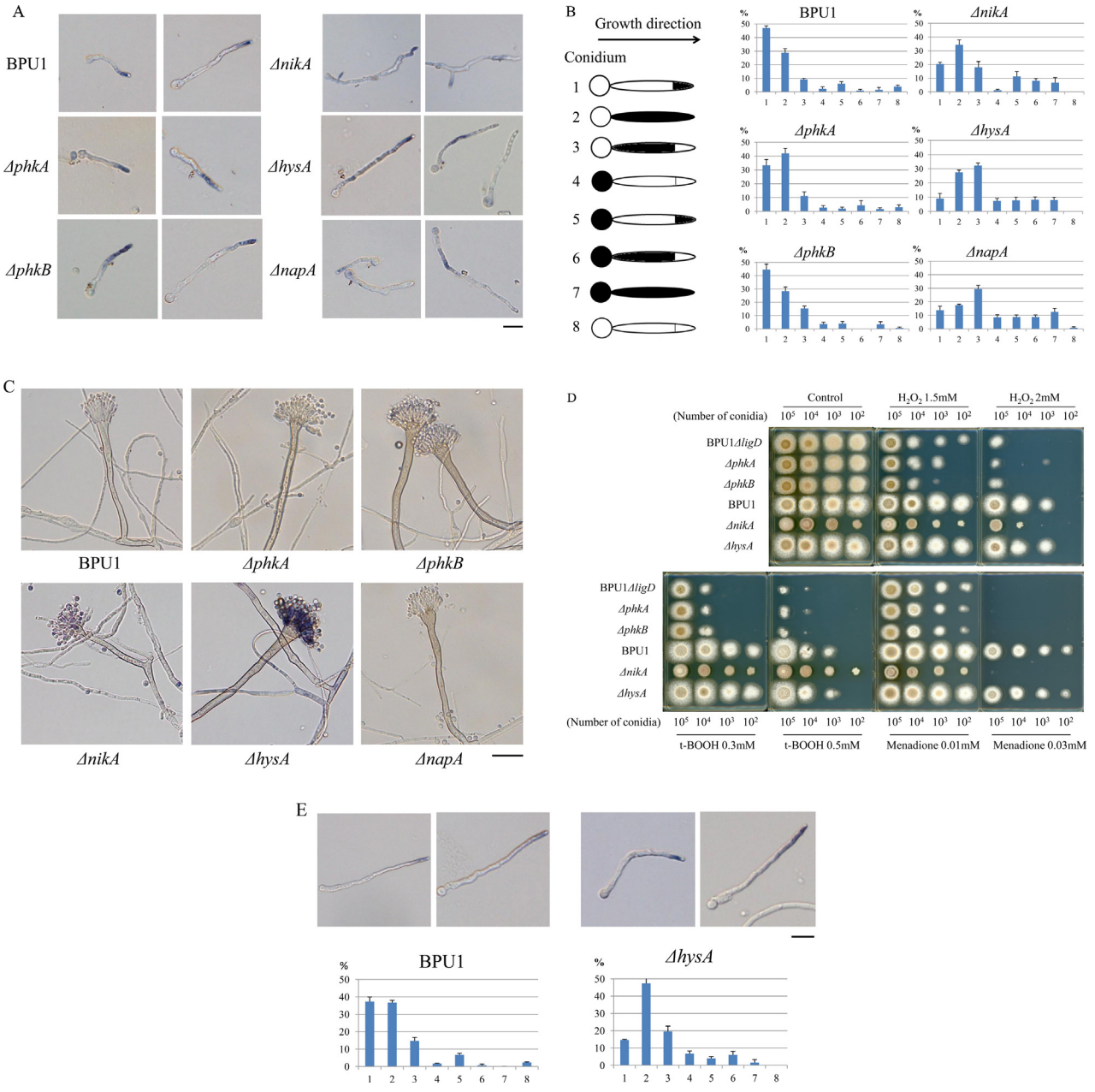

Fig. 2. Detection of ROS by NBT staining. (A) Strains were cultivated on cover glasses in liquid culture for 12 h at 37 °C. The cover glasses were subsequently stained in NBT solution for 4 h and observed under a microscope. (B) Left drawing: after the germination of conidia, hyphae grew in the direction of the arrow in liquid culture. Eight different patterns of NBT staining patterns were visible (black spots). Right graphs: approximately 50 growing cells for each strain were categorized according to the 8 patterns and counted. Experiments were repeated at least 3 times. (C) Each strain was grown along cover glass crossed with square holes on minimum medium plates. After 2 days at 37 °C, the cover glass was stained by NBT solution. (D) Growth on oxidative stress plates. The indicated numbers of conidia were spotted on minimal medium agar plates including H_2O_2, t-BOOH, and Menadione and incubated at 37 °C for 3 days. The control plate lacked oxidizing reagents. *ΔhysA* and *ΔnikA* were constructed by using a host strain (ABPU1) different from those of *ΔphkA* and *ΔphkB* (ABPU1 *ΔligD*). Two control strains (BPU1 and BPU1 *ΔligD*), which were constructed by introducing arginine gene into each host strain, showed the sensitivity to different concentrations of oxidizing regents. (E) Strains were cultivated as in (A) and (B), except the restricted oxygen condition, in which oxygen restricted by taping around the plates.

Table 1
Summary of NBT staining.

Strain	Germlings	Asexual development	Oxygen restriction	Gene's function [Refs.]
BPU1	Tip	–	Tip	–
ΔphkA	Tip	–	Tip	Oxidative stress response [1]
ΔphkB	Tip	–	Tip	Oxidative stress response [1]
ΔnikA	Hypha	Hypha	Hypha	Fungicide sensitivity [4,5]
ΔhysA	Hypha	Hypha Conidia	Tip	Hypoxia response [1]
ΔnapA	Hypha	–	Hypha	Transcription factor of oxidative stress response [11]

–, no NBT staining.

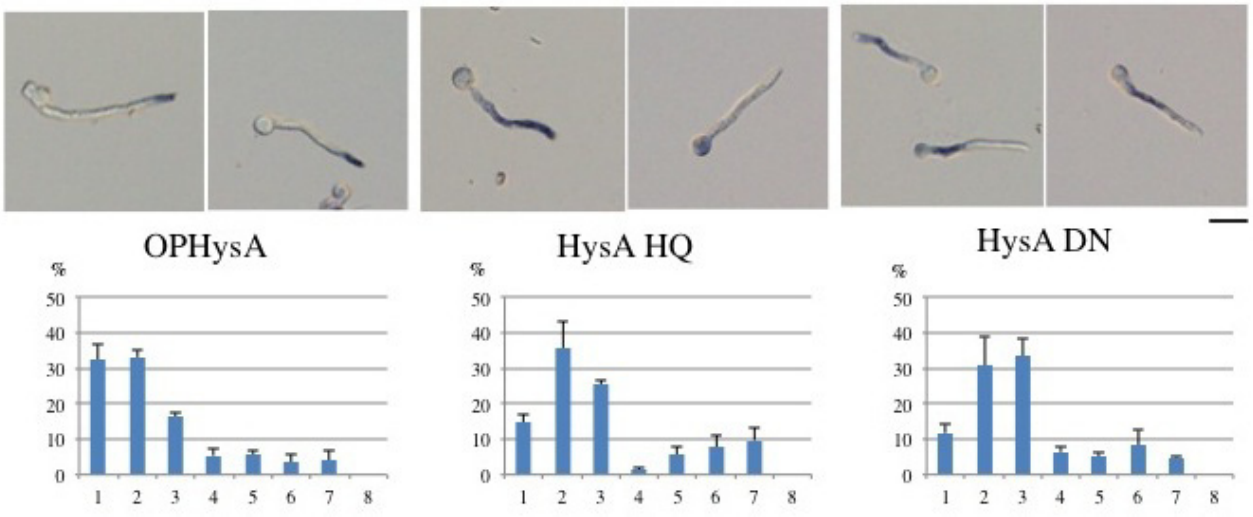

Fig. 3. The His-Asp phosphorelay function of HysA is essential for the control of ROS production. The OPHysA strain includes the *hysA* gene under the *alcA* promoter. HysA HQ and HysA DN are phosphotransfer-defective mutant strains. Cultivation, NBT staining, and categorization were the same as described in Fig. 2A and B.

most HKs were expressed with the progression of asexual development, even though their overall expression levels were low (Fig. 1B). These results indicate His-Asp phosphorelay signal transduction in *A. nidulans* mainly occurs during asexual development. However, under the restricted oxygen conditions by sealing the plates with tape (the induction of sexual development) (Fig. 1C), only 1 HK, originally called HK8-2, was markedly expressed; this implies a functionally important role of HK8–2 in response to low oxygen. Herein, we refer to HK8-2 as HysA (hypoxia expressed sensor protein A) and continued to analyze its functions along with other characteristic HKs.

3.2. Construction of the hysA-deletion strain (ΔhysA)

To determine the function of *hysA* in *A. nidulans*, the entire *hysA* region in the *A. nidulans* genome was replaced with auxotrophic marker, *argB* (see Supplementary methods). The deletion was performed in wild-type ABPU1, and confirmed by PCR and Southern blotting (data not shown). Functional growth defects of *A. nidulans* due to deletion were investigated by comparison with BPU1, which was constructed by introducing wild-type *argB* into the ABPU1 strain. However, there were no differences between the BPU1 and *ΔhysA* strains with respect to growth even under the restricted oxygen condition (data not shown).

3.3. ROS production in HK gene-deletion strains

Since ROS are important signals for cell growth in *A. nidulans* like all organisms [9], we determined whether differences in NBT staining were detected using microscopy in *ΔhysA*, including differences in other HK gene disruptants. After 12 h of incubation at 37 °C in minimal medium liquid culture, cells were stained with NBT, which reacts with superoxide anion, one of ROS. Wild-type BPU1 produced ROS at the tip of growing hyphae (Fig. 2A) as reported previously [9]. PhkA and PhkB are thought to play roles in oxidative stress responses in *A. nidulans* because they have functional domains similar to those of Phk1–3, which are histidine kinases that function in response to oxidative stresses in *Schizosaccharomyces pombe* [1]. However, both *ΔphkA* and *ΔphkB* exhibited the same sensitivity to oxidative stress as the wild-type (Fig. 2D) and almost the same level of NBT staining as the wild-type (Fig. 2A). On the other hand, *ΔnikA* [4,5] and *ΔhysA* exhibited abnormal NBT staining at various parts of growing hyphae, indicating both NikA and HysA are involved in the control of ROS production (Fig. 2A).

Approximately 50 growing cells were observed for each strain and categorized into 8 different NBT staining patterns (Fig. 2B, left 1–8, black representing NBT-stained areas). The resultant distribution of ROS production is summarized in Fig. 2B (right graphs). It indicates that *ΔnikA* and *ΔhysA* produce ROS at any parts of cells during the germling growth.

A. nidulans also developed conidiophores after hyphal growth on minimal medium agar plate. Wild-type BPU1 minimally produced ROS in some conidia and aerial hyphae (Fig. 2C). The *ΔnikA* strain exhibited poor formation of conidia as described previously [4,5] but still produced ROS at several parts of the hyphae. *ΔhysA* strongly produced ROS at both developed conidiophores and aerial hyphae (Fig. 2C).

The oxidative stress sensitivity of cells is thought to be linked to cellular ROS production. However, in the present study, the *ΔhysA* strain exhibited the same sensitivity as the wild-type but growth patterns different from those of *ΔnikA* on the oxidative stress plates (Fig. 2D; H_2O_2, t-BOOH, and Menadione). Thus, the results indicate there is no association between direct ROS production and sensitivity to oxidative stress.

To investigate the effects of other HKs on ROS production, we obtained 14 HK gene-deletion strains listed in the Fungal Genetics Stock Center (FGSC) except *ΔphkA*, which was not included in the list [12]. In addition, we observed specific ROS production in *ΔfphA* strain (Fig. S1).

These are the first findings indicating HKs are involved in ROS production as directly observed by NBT staining.

3.4. ROS production in napA gene-disrupted strain

As NapA is a transcription factor that controls several genes for oxidative stress response, the deletion strain (*ΔnapA*) exhibited sensitivity to oxidizing regents [11]. Curiously, the *ΔnapA* strain exhibited ROS production similar to that of *ΔhysA* during the germling (Fig. 2A and B) but not at the conidiophore development (Fig. 2C). These results indicate HysA and NapA play different roles in ROS production during the development of *A. nidulans*.

To determine whether the involvement of HysA in ROS production is dependent on the presence of oxygen in growing cells, we cultivated *ΔhysA* under the restricted oxygen condition and observed the resultant ROS production. ROS levels were reduced mostly in the hyphae (Fig. 2E) but not at the tips in *ΔhysA*. It must be mentioned that other

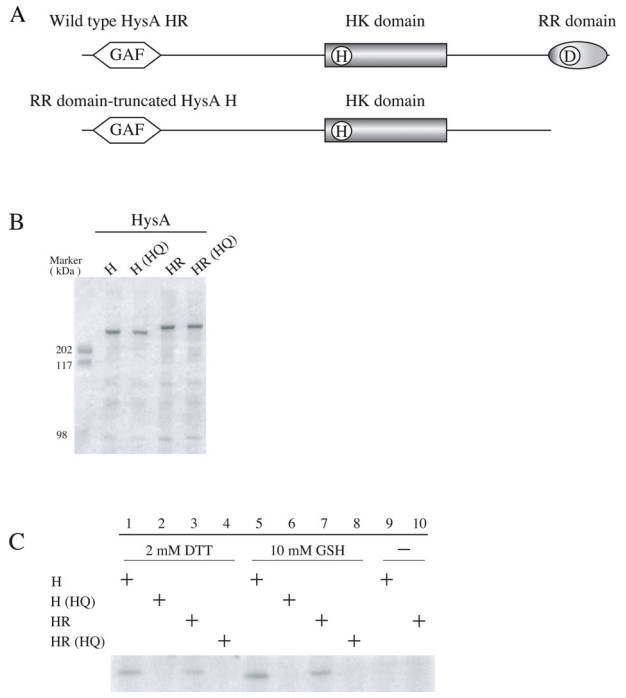

Fig. 4. *In vitro* autophosphorylation activity of the recombinant HysA protein. (A) The domain compositions of the wild-type HysA HR and RR domain-truncated HysA H are shown. GAF could be an important domain for receiving outside signals. H represents a histidine residue—a predicted autophosphorylation site in the HK domain. D represents an aspartic acid residue—a predicted phosphate-accepting site in the RR domain. (B) Aliquots (2 μg) of the purified HysA HR and H proteins were subjected to SDS–PAGE followed by detection with Coomassie Brilliant Blue. HysA HR (HQ) and H (HQ) are mutant proteins with an amino acid changes at their autophosphorylation sites (His566) in HysA HR and H, respectively. (C) *In vitro* autophosphorylation experiment: 2 μg of each purified protein was mixed with 0.05 mM [γ-^{32}P] ATP (37 kBq) in TEG buffer and incubated for 30 min. The reaction mixture included 2 mM DTT (lanes 1–4) or 10 mM GSH (lanes 5–8). After the reactions, samples were subjected to SDS–PAGE and subsequently analyzed with an imaging scanner (BAS-2500).

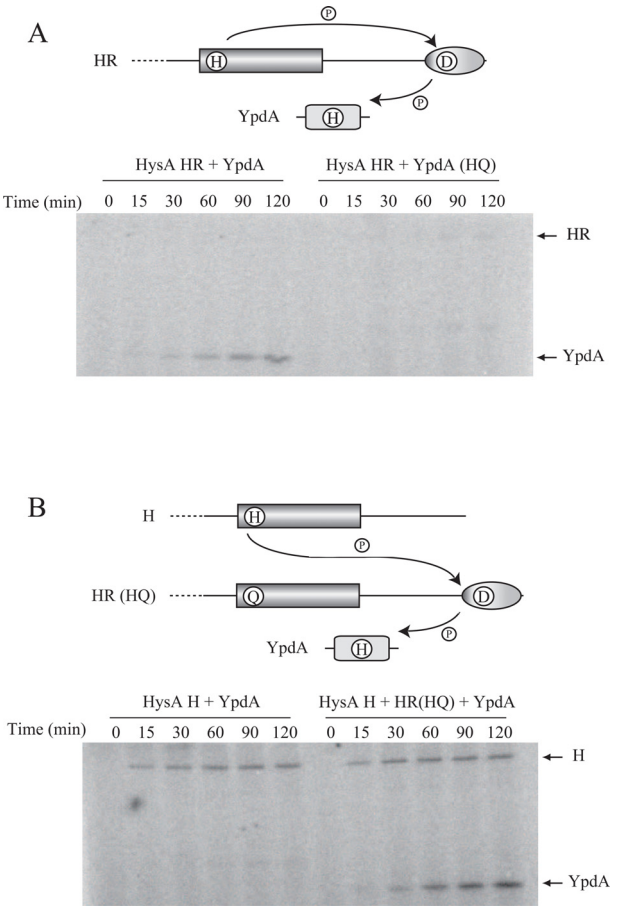

Fig. 5. *In vitro* multiple-phosphotransfer reactions among histidine and aspartic acid residues in the recombinant HysA and YpdA proteins. (A) Phosphotransfer from wild-type HysA HR to YpdA and (B) HysA H to YpdA via HysA HR (HQ). *In vitro* multiple-phosphotransfer reaction: 2 μg of each purified protein was mixed with 0.05 mM [γ-^{32}P] ATP (37 kBq) in TEG buffer and incubated for indicated times. All reaction mixtures included 2 mM DTT. After the reactions, samples were subjected to SDS–PAGE and subsequently analyzed with an imaging scanner (BAS-2500).

HK gene-disrupted strains and the *ΔnapA* strain including the wild-type did not exhibit different ROS production when oxygen was restricted, indicating they maintain their characteristic ROS production. Since ROS are mainly generated through the respiratory reactions at mitochondria under the presence of oxygen, HysA might be involved in the regulation of mitochondria. These results of NBT staining are summarized in Table 1.

3.5. Functional importance of the His-Asp phosphorelay system in the control of ROS production

To test whether His-Asp phosphorelay signal transduction via HysA is essential for controlling ROS production in *A. nidulans*, we constructed a strain in which *hysA* expression was controlled under the *alcA* promoter [15] by introducing the *alcA(p)::hysA* fusion at the *pyroA* locus in the *ΔhysA* strain (OPHysA). Histidine residue at position 566 (His566) of HysA is supposed to be an autophosphorylation site based on the homology among HKs, and aspartic acid residue at position 1134 (Asp1134) is a phosphate-receiving site. HysA HQ and HysA DN mutant strains were also constructed by means of 2 *alcA(p)::hysA* fusions: *alcA(p)::hysAHQ*, which included 1 point mutation (His566 → Glu), and *alcA(p)::hysADN*, which included another mutation (Asp1134 → Asn). These 3 strains were cultivated in minimal medium liquid culture and stained by NBT, and ROS production was observed by microscopy (Fig. 3). OPHysA produced ROS only on the tips of hyphae like the wild-type. However, HysA HQ and HysA DN exhibited the same abnormal ROS production as *ΔhysA*. In this experiment, we cultivated all strains in the non-inducing condition of *alcA* promoter including glucose, because the original expression of *hysA* promoter was similar to that of *alcA* promoter in this condition (data not shown). The induction condition of *alcA* promoter also resulted in similar NBT staining.

3.6. Autophosphorylation of purified HysA protein in vitro

Since it was unclear how HKs transmit phosphate signals in *A. nidulans*, we first examined the autophosphorylation activity of purified HysA protein *in vitro*. Plasmids were constructed in order to induce HysA HR and HysA H, i.e., the entire wild-type HysA protein and the HysA protein lacking the RR domain (Fig. 4A). Other plasmids were also prepared in order to introduce an amino acid change at the autophosphorylation site (i.e., His566) in HysA HR and H. The mutant proteins were purified in the same manner as the wild-type proteins (Fig. 4B). The phosphorylation reaction mixture included 2 mM DTT (Fig. 4C, lanes 1–4) or 10 mM GSH (Fig. 4C, lanes 5–8). ^{32}P-labeled protein bands were detected for HysA HR and HysA H (Fig. 4C, lanes 1, 3, 5, and 7) but not for the HysA HR (HQ) or H (HQ) mutant proteins (Fig. 4C, lanes 2, 4, 6, and 8). These *in vitro* results indicate HysA autophosphorylates its histidine residue at position 566. The autophosphorylation activities of HysA HR and HysA H were not detected without any reducing reagent (Fig. 4C, lanes 9 and 10). Because HysA controls ROS production, it is possible some redox conditions affect HysA activity.

3.7. Evidence of phosphotransfer from HysA to YpdA in vitro

YpdA is a histidine-containing phosphate transmitter in *A. nidulans*. YpdA (HQ) includes an amino acid change at His85 and does not receive phosphate signals from any histidine kinases or upstream sensor proteins [13]. We examined the *in vitro* phosphate signal transmission from HysA to YpdA in detail (Fig. 5). When purified HysA HR was mixed with YpdA (Fig. 5A, HysA HR + YpdA), a radiolabeled protein band of YpdA was observed, and it was confirmed by the lack of a protein band for YpdA (HQ) at the same position as YpdA (Fig. 5A, HysA HR + YpdA(HQ)). HysA H does not include the C-terminal RR domain of HysA HR. Therefore, HysA H exhibits autophosphorylation activity (Fig. 4C, lanes 1 and 5) but does not transfer its phosphate signal to YpdA (Fig. 5B, HysA H + YpdA). On the other hand, HysA HR (HQ) includes an amino acid change at a histidine residue for autophosphorylation but still has the ability to receive the phosphate signal at the aspartic acid residue in the C-terminal RR domain. As expected, HysA HR (HQ) did not phosphorylate YpdA (data not shown). However, a radiolabeled protein band of YpdA was detected when it was mixed with both HysA H and HysA HR (HQ) (Fig. 5B, HysA H + HR (HQ) + YpdA), indicating HysA H can transfer a phosphate signal to YpdA via the RR domain of HysA HR (HQ). These *in vitro* results collectively indicate phosphotransfer including wild-type HysA occurs from His (i.e., the HK domain of HysA) to Asp (i.e., the RR domain of HysA) and then to His (i.e., YpdA).

4. Conclusions

ROS are generated during cell growth in the presence of oxygen in all organisms. It makes sense that His-Asp signal transduction is involved in ROS production, because it should be an important system that controls cell growth and development. The lack of a direct link between ROS production and oxidative sensitivity was evidenced by the HK deletion mutant strains. Therefore, HysA might be a key HK controlling ROS production in response to redox conditions. Further studies are required to elucidate signal transduction after HysA-YpdA as well as the genes under the control of HysA.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2014.01.003.

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