Microorganisms growing in natural habitats are constantly confronted with a wide variety of external stresses. Here we provide several lines of experimental evidence for the thesis that the filamentous fungus *Aspergillus nidulans* has a homolog of the AP-1-like bZip transcription factor, which is known to play general roles in oxidative responses in many types of yeast.

**Key words:** oxidative stress response; *Aspergillus nidulans*; bZip transcription factor; NapA

Microorganisms growing in natural habitats are constantly confronted with a wide variety of external stresses. To respond properly to such environmental stimuli, they have evolved a common and widespread signal transduction mechanism, which is generally referred to as the two-component system (or the histidine-to-aspartate phosphorelay). In this connection, we have been studying two-component systems in eukaryotic microorganisms, particularly with regard to fungi, including yeasts (e.g., *Schizosaccharomyces pombe*). Previously, we studied the model yeast *S. pombe*, which possesses two phospho-accepting response regulators, named Mcs4 and Prr1. These eukaryotic response regulators are common among fungi, and another model yeast, *Saccharomyces cerevisiae*, also has highly homologous counterparts, named Ssk1p and Skn7p respectively. Furthermore, we found recently that the filamentous fungus *Aspergillus nidulans* has also an orthologous bZip-type transcription factor, NapA. This bZip transcription factor, which plays important roles in oxidative stress responses in concert with the SskA and/or SrrA response regulators. The entire genome sequence of *A. nidulans* was inspected to identify a gene encoding a protein homologous to Yap1p/Pap1. A single gene (referred to as *AN7513* ) was found to encode a protein, the entire amino acid sequence of which is highly homologous to those of Yap1p and Pap1 as well as Cap1p, a bZip-type transcription factor of the model pathogenic yeast *Candida albicans*. (Fig. 1A). They have a common structural design, in which a bZip domain is followed by a conserved cystein-rich domain (CRD) at the C-terminal end. As far as the amino acid sequences are concerned, they appear to be orthologous to each other. In this study, this *A. nidulans* gene-product was designated NapA (*A. nidulans* AP-1 homolog). When compared the Yap1p and NapA amino acid sequences, for instance, they are 49% identical in the bZip domains, and 50% identical in the CRD domains.

In general, reactive oxygen species (ROS), including *H₂O₂*, damage cells seriously by reacting with cellular components such as protein, lipid, and DNA, causing oxidative stress and cell death. Accordingly, fungi have evolved efficient defense mechanisms to avoid such oxidative stress. As mentioned above, the common fungal response regulators (Mcs4/Ssk1p/SskA and Prr1/Skn7p/SrrA) appear to play prominent roles in oxidative stress responses. Other prominent regulators commonly implicated in the oxidative stress response in fungi are Yap1p for *S. cerevisiae* and Pap1 for *S. pombe*, both of which are yeast homologous proteins of the mammalian bZip (basic-region leucine zipper)-type transcription factor AP-1. An interesting fact is that, in the yeasts, Yap1p (or Pap1) cooperates intimately with the response regulator Skn7p (or Prr1) for the induction of many oxidative stress response genes. In this study, we raised the question whether the filamentous fungus *A. nidulans* has also an orthologous bZip-type transcription factor, which plays important roles in oxidative stress responses in concert with the SskA and/or SrrA response regulators.

To characterize the physiological function of NapA, an *A. nidulans* mutant strain was first constructed from the parental strain, ABPU1 (*argB*). The *napA* gene on
the A. nidulans chromosome was deleted by replacing it with the \textit{argB} \textsuperscript{+} maker gene, in accordance to the conventional gene-disruption methodology with homologous recombination (Fig. 1B). We confirmed the altered genomic structure of the resulting \textit{A. nidulans} mutant (designated \textit{\Delta napA}) by Southern blot hybridization analysis (Fig. 1C). More than two independent

\textbf{Fig. 1.} Structural Design of NapA and Deletion Construction of \textit{napA} Gene.  
A. Amino acid sequence alignment of the characteristic domain. The structural organization of \textit{AN7513.3} (gene ID in \textit{A. nidulans} genome database: http://www.broad.mit.edu/annotation/fungi/\textit{aspergillus}/), designated NapA, is shown. The bZip domain is shown as a dotted box, and cystein-rich domain (CRD) as a hatched box. The amino acid sequences of these domains of NapA are aligned with those of Yap1p, Pap1, and Cap1p. In these domains, the invariant amino acid residues were indicated as white letters on a black background, and the conserved region is boxed. B. Deletion construction of the \textit{napA} gene. The shaded arrow indicates the \textit{napA} gene on the \textit{A. nidulans} chromosome. To replace the \textit{napA} gene with the \textit{argB} gene, the plasmid containing the 5'-UTR (black heavy line) and 3'-UTR (heavy grey line) of the \textit{napA} gene inserted at both sides of the \textit{argB} gene (white arrow) was prepared as described previously.\textsuperscript{9} The fragments of 5'-UTR and 3'-UTR were obtained by genomic PCR using primers \textit{napA}-UU: CCGGGGCCGCGTC-GAATTCCGATGACC, \textit{napa}-UD: GTGATCCAGATGATGATCC, and \textit{napa}-DD: CCATGCGTGTCCCATCCTTTG. For the transformation, ABP1 (\textit{biA1}, \textit{pyrG89}, \textit{wA3}, \textit{argB2}, \textit{pyrA4}) was used as a parental strain. C. The genome structure of the transformants confirmed by Southern blot hybridization analysis. Through the transformation, the resulting strains showing \textit{argB} \textsuperscript{+} were obtained, and replacement of the \textit{napA} gene was confirmed by Southern blot hybridization analysis. Genomic DNA (1 \mu g) from the transformant \textit{\Delta napA} was digested with Bpu1,\textsuperscript{9} and a resulting transformant (\textit{\Delta napA} candidate) was digested with \textit{AccI}. These samples were electrophoresed and then transferred to Hybond-N+ nylon membranes. Labeling of the probes and detection of hybridization signals were carried out using an AlkPhos Direct (Amer sham, UK) and an LAS-3000MB (Fujifilm, Tokyo). The fragment of 5'-UTR was used as a probe.
type cells were resistant to these reactive oxygen species (ROS) under the conditions tested. Under the same growth conditions, the inoculated ΔnapA conidia did not grow on the stressfull media, particularly those containing H₂O₂, t-BOOH, or MD. However, the sensitivities to diamide and RB were not distinguishable as between the wild-type and the ΔnapA cells. These results suggest that the NapA transcription factor plays an important role in the protection of *A. nidulans* cells against certain harmful ROS, presumably by regulating (or activating) a set of genes involved in scavenging external and internal ROS. Nevertheless, perhaps NapA is not important for scavenging the singlet oxygen, which is often generated endogenously as a powerful oxidant in aerobically grown cells.

As mentioned above, *A. nidulans* has at least two important pathways for responding to oxidative stresses in which the response regulators play important roles. One is the SrrA-mediated pathway, and the other is the SskA-mediated pathway (see the introductory section). The latter pathway links immediately downstream with the HOGA-dependent MAPK (mitogen-activated protein kinase) cascade. In a previous study, we characterized a set of ΔsrrA and ΔsskA mutant strains with reference to oxidative stress responses (mainly to H₂O₂). Here we compared the phenotypes of ΔnapA, ΔsrrA, and ΔsskA cells on plates containing certain ROS (Fig. 2C). The results indicated that these regulatory genes are equally required for *A. nidulans* cells to survive against H₂O₂ and t-BOOH, but NapA was responsible for resistance to a considerably lower concentration of these ROS, as compared with SskA and SrrA. It was also found that NapA (but not SskA or SrrA) is uniquely implicated in the oxidative stress response against MD (i.e., superoxide radicals). Hence in *A. nidulans*, both specific and cooperative mechanisms appear to guarantee an optimal defense against a distinct ROS by adopting three different regulatory pathways (i.e., the SskA-, SrrA-, and NapA-dependent pathways). Among these, NapA serves as a distinct transcriptional regulator that is specifically responsive to certain ROS (e.g., MD, a superoxide radical generator).

To gain insight into the coordinate roles of NapA, SskA, and SrrA in the oxidative stress response at the molecular level, mRNA levels of the hallmark *catB* gene in response to H₂O₂ were examined for ΔnapA, ΔsskA, and ΔsrrA by semi-quantitative RT-PCR-aided Southern hybridization (Fig. 3A). The *catB* gene encoding a catalase is known to act as a major scavenger against H₂O₂ during the hyphal growth of *A. nidulans*. As expected, transcription of *catB*-mRNA was markedly enhanced in the wild-type background in response to H₂O₂, with which *A. nidulans* cells grown in liquid MM2G medium were treated. Such induction of *catB*-mRNA was severely attenuated not only in ΔsskA and ΔsrrA, but also in ΔnapA. This result suggests that in addition to SskA and SrrA the NapA transcription factor is also essential for the H₂O₂-induced expression of the *catB* gene, directly or indirectly. These observations at the molecular level (Fig. 3A) are consistent with the phenotypic observations (Fig. 2C). In this experiment, transcripts of *napA*, *sskA*, and *srrA* were also examined, and showed that expression of *napA* and *srrA* themselves is not induced in response to H₂O₂, while transcription of *sskA* is markedly enhanced by H₂O₂ (see the lower part of Fig. 3A). This event as to *sskA* appears to be so-called auto-regulation.

In addition to *catB*, we examined the expression of certain other oxidative stress-associated genes in ΔnapA, such as *trxB* (thioredoxin reductase, AN-3581.3), *thiO* (thioredoxin, AN0170.3), and *glaA* (gluthathione reductase, AN0932.3) (Fig. 3B). When we treated *A. nidulans* with H₂O₂ under the conditions described above, it was found that not only *catB*, but also other oxidative stress-associated genes (except for...
Essentially the same results were observed for cells treated with t-BOOH (Fig. 3B). When we treated *A. nidulans* with MD, the glrA gene was also markedly induced in a manner dependent on NapA. The basal level expressions of *catB* and *trxB* in the ROS-untreated cells were also significantly reduced in ΔnapA. This suggests that NapA is important for responses not only to externally applied ROS, but also to endogenously (or physiologically) generated ROS. Taken together, these results strongly support the view that NapA is important for the adaptive induction of a wide variety of ROS-associated genes.

As reported previously, the functions of the SskA and SrrA response regulators are most likely regulated by an as yet unidentified histidine kinase(s) through the phosphorelay signaling in response to ROS-signals.\(^5\) In this respect, it would be of interest to know the mechanism by which the activity of NapA is regulated in response to ROS-signals in *A. nidulans*. At this moment, we do not know anything about this particular issue as to NapA, but, we are aware of the current idea that the activity of Yap1p family proteins is regulated by the so-called nuclear localization mechanism.\(^4,6\)

According to the model proposed for Yap1p, oxidative stress-induced and intra-molecular disulfide bridge formation within Yap1p results in a vectorial trafficking of the modified protein from the cytoplasmic compartment to nuclear compartment. Consequently, the imported Yap1p is capable of acting as a transcriptional activator in response to ROS signals. As for this mechanism, it has been found that the C-terminal cysteine rich domain (CRD), containing three cysteine residues, is essential (see Fig. 1A). NapA of *A. nidulans* also has such a CRD, in which three cysteine residues are invariantly conserved. Hence a similar nuclear localization mechanism may be active in the NapA, but verification must await further examination in *A. nidulans*.

In summary, the results of this study indicate that the *napA* gene encoding a transcription factor homologous to mammalian AP-1 plays an important role in the oxidative stress response in *A. nidulans*. The conclusion of this study is consistent with the view that Yap1p family transcription factors are commonly essential for redox homeostasis in many fungi, including not only yeasts but also filamentous ascomycetes. Indeed, Yap1p family transcription factors have recently been identified and characterized in other ascomycetes.\(^5\) Considering that oxidative stresses are universal, the results of this study provide insight into this paradigm in the sense that *A. nidulans* is representative of interesting related species, such as *Aspergillus fumigatus* (a serious pathogen) and *Aspergillus oryzae* (a useful industrial fungus). In fact, both the *A. fumigatus* and *A. oryzae* genomes encode a gene that is highly homologous to the *napA* gene (gene IDs, Afu6g09930 for *A. fumigatus*, and A009-0001000627 for *A. oryzae*).

**Acknowledgment**

D.H. is a 21COE Program (14COEA2) Postdoctoral Fellow.

**References**