Preliminary Communication

In vivo Visualization of the Distribution of a Secretory Protein in Aspergillus oryzae Hyphae Using the RntA-EGFP Fusion Protein

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A fusion gene encoding ribonuclease Ti-EGFP (rntA-egfp) was constructed and expressed to use it as a tool for studies on the secretory pathway in Aspergillus oryzae. The successful secretion of the intact RntA-EGFP fusion protein was detected by fluorescence measurement and Western analysis. With use of the RntA-EGFP system, we were able to see high fluorescence at hyphal tips and observe concentrated fluorescence at septa in basal cells during growth at optimal conditions. Cold or heat shock during growth caused the accumulation of EGFP fluorescence in vacuoles.

Key words: Aspergillus oryzae; secretory pathway; fusion protein; EGFP

Aspergillus oryzae, a fungal strain with a long history of usage in the Japanese food fermentation industry, has emerged recently as an attractive host for heterologous protein production due to its ability to secrete at the g l⁻¹ range of homologous proteins and the status as a Generally Recognized as Safe (GRAS) organism. However, heterologous protein production has not yielded at levels attained during homologous protein production. It has been shown that the bottleneck of low output of heterologous proteins does not lie on the low expression of the heterologous gene, but post-transcriptionally, in the path through the ER and the Golgi apparatus. Despite much research on improvements of heterologous protein production by modifying promoters and constructing fusion proteins with homologous proteins, studies on the secretory pathway of A. oryzae has not been given much attention. To undertake the problem of low heterologous protein production, it is imperative to understand the secretory pathway of this organism.

Much of the knowledge on the molecular processes and the genes involved in intracellular protein transport has come from studies on Saccharomyces cerevisiae, and it is believed that most of these processes are similar to those in other eukaryotes. However, A. oryzae, like other filamentous fungi, grows through polar extension of hyphae, and protein secretion in this organism is considered to be a process limited to growing hyphal tips (the bulk flow hypothesis). Due to morphological differences between filamentous fungi and S. cerevisiae, some aspects of the organization of secretory components are suspected to be different from each other. Therefore, the investigation into the organization of secretory components focusing only on filamentous fungi should prove invaluable for the food fermentation and protein production industries.

The use of the enhanced green fluorescent protein (EGFP) as a reporter in A. oryzae has made possible the in vivo analyses of organelle dynamics in hyphae, such as nuclei and vacuoles. Here, we report the first in vivo observation of the intracellular distribution of a secretory protein in A. oryzae hyphae using an EGFP-tagged fusion system. We also observed the effects of cold or heat shock on the distribution of the fusion protein inside hyphae.

In order to construct an EGFP-tagged fusion protein system, we selected the secretory protein ribonuclease Ti (RntA). The plasmid pUNARG was constructed; it carries the fusion gene rntA-egfp, which was expressed under the control of the strong inducing amyB promoter (Fig. 1A). Introduction of pUNARG into A. oryzae niaD300 (niaD⁻) strain was done by the protocol of Pun and van den Hondel with some modifications. Transformants were first selected on Czapek-Dox (CD) medium (0.3% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, 2% glucose, pH 5.5) and were further checked by fluorescence microscopy for EGFP bioluminescence. One of the transformants, NRG1, was used in all experiments done in this study. To use the RntA-EGFP fusion protein for studies on secretion, its secretion into the culture medium was investigated. The presence of

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RntA-EGFP in the culture medium was analyzed by fluorescence spectrophotometry and Western blotting. The fluorescence intensity of the NRGI culture medium was 42 times more than that of wild type RIB40 (Fig. 1B). Western analysis (Fig. 1C) also demonstrated the secretion of RntA-EGFP fusion protein in its intact and complete form; the size of the band at 38 kDa corresponded to the expected molecular weight of the fusion protein between the mature RntA (11.1 kDa) and EGFP (26.9 kDa). The faint signal detected at around 20 kDa was considered to be a product of proteolysis. The size of this band did not match that of EGFP alone, suggesting that the fusion protein was not cleaved at the fusion site. These results indicate that the fluorescence in the culture medium presumably came from the intact fusion protein and that RntA-EGFP was successfully processed, transported, and secreted into the culture medium. In Aspergillus niger's glucoamylase::sGFP (GLA::sGFP) system,10 the fusion protein was cleaved at the fusion site detaching the GFP from the glucoamylase protein in the culture medium. As a result, the cleavage at the fusion site would restrict the usage of GLA::sGFP, for example, when measurements of secretory protein in the extracellular medium is required. Hence, the RntA-EGFP system could be used to measure the amount of the fusion protein quantitatively and to analyze the process of protein secretion.
secretion in mutants of protein trafficking and/or secretion.

To determine the location of RntA-EGFP in hyphae of *A. oryzae* grown in optimal conditions, *in vivo* observations of the NRG1 strain was done under the fluorescence microscope. At the apex region, RntA-EGFP was located throughout hyphae and brighter fluorescence was detected at the tips (Fig. 1D). This observation suggests that the secretion process of RntA-EGFP is carried out at the tips, which is consistent with the bulk flow hypothesis. At basal cells, a distinctly high fluorescence was located at septa (Fig. 1E), and spots of fluorescence were also found throughout compartments. The accumulation of the fusion protein at septa was unexpected, and it would be of interest to determine if this localization is associated with the process of secretion. Similar fluorescence patterns were also observed by means of the GLA::sGFP fusion system in *A. niger*. These results indicate that the *in vivo* images of the flow of secretory proteins would be similar in other *Aspergillus* species; and thus, a visual standard of secretory proteins could be established in *A. oryzae*.

We further applied the RntA-EGFP system in *A. oryzae* to find the effects on the flow of secretory proteins in apical regions during conditions of stress. Sudden changes in growth temperature could affect cellular activities negatively and may also lead to serious consequences on the process of protein transport and secretion. To investigate the effect of changes in growth temperature on the distribution of secretory proteins *in vivo*, NRG1 cultures were transferred to either 4°C for cold shock or 42°C for heat shock for varying lengths of time after the initial 16-18 hour growth at 30°C, and mycelia were observed under the fluorescence microscope. After transferring the culture to 4°C, fluorescence at the apex region diminished gradually and accumulated in an uneven pattern, where there were parts of high fluorescence and low fluorescence. The change in the distribution of RntA-EGFP implies a disturbance in the flow of secretory proteins, possibly in the rate of protein transport. Subsequently, we observed tubular, network-like fluorescence in hyphae (data not shown). These observations indicate the accumulation of RntA-EGFP in specific areas of hyphae, which could be organelles and/or intermediate components in the secretory pathway. After 4 hours, fluorescence accumulated inside spherical structures (Fig. 2B). These structures were located at the positions of surface depressions observed in the DIC image of

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**Fig. 2.** Localization of RntA-EGFP during Cold Shock.

NRG1 mycelia grown in DPY medium on cover slips were transferred to 4°C after the initial incubation at 30°C for 16-18 hours. After 4 hours of cold treatment, hyphae were observed for EGFP fluorescence to detect the location of RntA-EGFP (B). Images of hyphae with depressions at the location of EGFP fluorescence were taken with Differential Interference Contrast (DIC) microscopy (A). Vacuoles were stained with Cell Tracker Blue CMAC (7-amino-4-chloromethylcoumarin, Molecular Probes, Eugene, OR, USA). CMAC was dissolved in DMSO to a stock concentration of 10 μM, and it was added directly to the 16-18 hour incubated culture to the final concentration of 10 μM. CMAC staining identified the locations of vacuoles, some of which are indicated by arrowheads (C). Bar, 10 μm.
hyphae and were believed to be vacuoles (Fig. 2A). To confirm this, CMAC, a fluorescent probe staining vacuoles, was added, and the location of vacuoles corresponded to that of EGFP fluorescence (Fig. 2C). Additionally, Western analysis on intracellular proteins of hyphae under cold stress showed that a 38-kDa band corresponding to the RntA-EGFP fusion protein was detected with GFP antibody (data not shown). As a result, it was suggested that RntA-EGFP was transported to vacuoles when hyphae encountered low growth temperature. Similarly, during heat shock at 42°C, fluorescence was also observed in vacuoles as early as 2 hours (data not shown). In conclusion, the pattern of RntA-EGFP fluorescence, which was observed in hyphal apices during growth at the optimal temperature, changes when the growth temperature is suddenly altered. Moreover, the fusion protein destined for secretion is presumably diverted toward vacuoles for degradation, recycling, and/or storage.

In this study, we constructed the RntA-EGFP system and observed the distribution of the secretory protein in A. oryzae hyphae. The application of the RntA-EGFP fusion system will allow the analyses of secretory mutants by measurement of protein secretion with EGFP fluorescence and by making the effects of these mutations visible in hyphae. We were also able to follow the changes in the distribution of RntA-EGFP fluorescence during cold or heat shock. Khalaj et al. (2001) has reported the accumulation of GLA::EGFP in tubular networks during cold shock in hyphae of A. niger, suggesting the retention of secretory proteins in the ER. We observed similar structures during cold shock in A. oryzae; however, this pattern was transitional, as we also detected EGFP fluorescence inside vacuoles at latter stages of the treatment. Our observation is significant because not only does this data provide new information about the effects of temperature stress on the distribution of secretory proteins, but it also completes the full picture of the pathway that secretory proteins take during cold or heat shock. The combination of both studies suggests that the ER may be acting as the control center, where it decides, in reaction to stress, whether proteins are transported for secretion or towards vacuoles. Therefore, it is important to pursue the detailed analyses of the intermediate structures before the accumulation of secretory proteins in vacuoles. In line with this, we are attempting the visualization of the ER and the Golgi apparatus in A. oryzae with fluorescent proteins (e.g., DsRed2). Furthermore, studies on secretory mutants and genes involved in protein secretion would be required to provide further knowledge of the molecular mechanism behind the control of the protein transport to vacuoles and the secretory pathway of A. oryzae in general. In the end, the overall information on the secretory pathway will enable industrial strain improvements at the molecular and genetic level, ultimately making the development of the ideal cell factory possible for homologous and heterologous protein production.

References

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