Analysis of protein constitution of *Neurospora* cultured in various calcium concentrations: comparison of calcium homeostasis of wild-type and defective mutant strain

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Abstract

Because mycelial growth of filamentous fungus, *Neurospora crassa*, is dependent on the extracellular calcium concentration, calcium condition during the culture stage may affect the structural onstitution of *Neurospora* cell. In this study, we examined the effect of extracellular calcium concentration on the protein constitution of *Neurospora* cells, and found four proteins in wild-type strain which varied in quantity depending on extracellular calcium concentration. Two of four candidate proteins were highly expressed even at low extracellular calcium concentration in the case of defective mutant strain in calcium homeostasis, *ca*-1(19), which could not grow in high extracellular calcium concentration. These results suggest that two proteins of which molecular mass are 91 and 32 kDa are candidates for marker proteins of calcium stress in *Neurospora* cell.

Key words : calcium stress, vacuole, circadian rhythm
2010.11.17 accept

Introduction

Calcium is an essential element for growing and plays a crucial role in a variety of cellular processes such as secretion, intracellular transport and movement and the communication of signals. In filamentous fungus, *Neurospora crassa*, mycelial growth is dependent on the calcium concentration of the culture medium. The optimum calcium concentration of culture medium for growing of wild-type strain is about 1 mM. Nakashima and Ohnishi had isolated various *Neurospora* mutants that had defect in calcium homeostasis[1,2]. These mutants could grow well only in a medium in which concentration of calcium is lower than that used for culture of wild-type strain. *Neurospora* mutant strain *ca*-1(19) lost the function of vacuolar calcium transport system, and could not grow in the medium containing a normal concentration of calcium ions[1] (Fig. 1). The loss of storing calcium in vacuoles causes cell damage when the cells are cultured in the medium with a high calcium concentration because the intracellular calcium concentration of *Neurospora* cell should be kept in low level around 10^{-8} to 10^{-6} mol/l[3].

Protein constitution of *Neurospora* cell should be changed in response to an alteration of environmental condition such as extracellular calcium concentration. In this study, we examined the effect of extracellular calcium concentration on the protein constitution of *Neurospora* cell. We determined which proteins respond to the alteration of extracellular calcium concentration and revealed that amount of four proteins was changed by extracellular calcium stress. We further examined the protein constitution in the defective mutant strain in calcium homeostasis, *ca*-1(19).

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Materials and Methods

1. *Strains and culture medium*

The *band* (*bd*) strain, which was used as wild-type and *ca-1* (19) strain of *Neurospora crassa* was obtained from Fungal Genetic Stock Center. Conidia of *Neurospora* were collected from 7- to 14-day-old slants, suspended in distilled water and filtered through glass wool. Vogel's salts[4] were modified to divalent cation-free modified Vogel's salts (DCF-Vogel's salts), which contained 8.5 mM tri-sodium citrate, 36.7 mM KH_2PO_4, 25 mM NH_4NO_3, trace elements and biotin. A concentration of calcium ions was adjusted by an addition of them to a medium before autoclaving.

2. *Growth condition and harvesting*

Mycelial discs were prepared by the method reported by Johnson and Nakashima[5]. Conidia (1.3×10^6) were inoculated into petri dishes (90 mm in diameter) that contained 25 ml of liquid medium prepared with DCF-Vogel's salts, 0.3% (w/v) glucose and 0.5% (w/v) arginine. After 36 h in constant light, the uniform mycelial mat that had formed on the surface of the medium was cut into discs with a cork-borer (16 mm in diameter). In the case of an examination using *ca*-1(19), the concentration of calcium ions was adjusted at 0.01 mM in the standard medium described above. Specimens were transferred into new liquid medium that contained DCF-Vogel's salts, 0.03% (w/v) glucose and 0.05% (w/v) arginine and cultured at 26 ℃ with reciprocal shaking (100 rpm). The mycelia discs were harvested at 12 — 52 h after the start of liquid cultivation for determining the protein concentration and at 24 and 48 h for analyzing the protein composition of *Neurospora* cells.

3. *Protein content analysis, SDS-PAGE and silver staining*

Protein concentration of the cultured mycelial disc was determined by the Bradford protein assay system provided by Bio-Rad Laboratories, Inc. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 13 % polyacrylamide gel according to the standard method. The separated proteins were visualized by the method of rapid silver staining[6].

Results and Discussion

We examined the effect of extracellular calcium concentration on the protein constitution of *Neurospora* cell using liquid culture method[5], which is suitable for investigating effect extracellular calcium concentration because of excluding the effect of growth differences in various culture conditions. The apparent mycelial growth of *Neurospora* was stopped when it was transferred into the liquid medium that contained less concentration of glucose and arginine as a carbon source. We firstly determined the protein content of mycelial discs grown in the various concentration of extracellular calcium using wild-type and *ca*-1(19) strains (Fig. 2). In both strains, the protein content increased to some extent during the culture up to 52 h, but the increase rate was very small as compared to that in normal culture condition. These results showed that alteration of the protein content of mycelial discs was very slight. Variation of the protein content in *ca*-1(19) mycelia was somewhat bigger than that in wild-type strain when the cells were cultured in various concentration of the extracellular calcium.

We determined which proteins respond to change of extracellular calcium concentration by SDS-PAGE analysis (Fig. 3). Visualization method of protein is important for determining the candidate proteins, thus we tried various methods of protein staining such as Coomassie Brilliant Blue staining and silver staining. Finally, we found that silver staining using sodium thiosulfate in pretreatment and developing solution[6] was suitable for recognizing the difference of protein amount in this study. Comparing lanes 1 and 2 in Fig. 3, the amount of four proteins marked by a - d altered in wild-type *Neurospora* cell in response to change of extracellular calcium concentration. The apparent molecular mass and the behavior of these proteins were summarized in Table 1. The amount of three proteins of which molecular weight were 91, 35, and 32 kDa increased in higher calcium concentration, and 36 kDa protein decreased.

We further examined the alteration of protein constitution using the defective mutant strain in calcium homeostasis, *ca*-1(19). All candidate proteins amount of

which were altered by extracellular calcium concentration change were recognized in *ca*-1(19) strain. Comparing lanes 3 and 4 in Fig. 3, the alterations of two proteins molecular weight of which were 36 and 35 kDa were same as that in wild-type strain, whereas two proteins of which molecular mass were 91 and 32 kDa showed constantly high intensity regardless of extracellular calcium concentration. These behaviors were also observed in the samples obtained at 48 h after the transfer (comparing lanes 5 and 6 in Fig. 3).

Neurospora mutant strain *ca*-1(19) lost the function of vacuolar calcium transport system, and hardly grows in the medium containing a 1 mM $CaCl_2$. This result suggests that the stress of 0.01 mM $CaCl_2$ against *ca*-1(19) was similar to that of 1 mM $CaCl_2$ against wild-type strain because of losing ability that kept cytosolic calcium concentration low in *ca*-1(19) strain. Since amounts of the two proteins marked 'a' (91 kDa) and 'd' (32 kDa) were highly expressed in *ca*-1(19) even at 0.01 mM $CaCl_2$, these two proteins were candidate for marker proteins that display calcium stress of *Neurospora* cells. Further experiments are necessary for identifying the candidate proteins.

Nakashima have been reported that calcium ionophore A23187 shifted the phase of the circadian conidiation rhythm of *Neurospora*[7]. Ohnishi et al. have isolated other defective mutants in calcium homeostasis and found that certain mutant lost an ability of light-induced phase shifting of circadian conidiation rhythm[2]. These results suggest that calcium ions may involve in the mechanisms of the circadian clock of *Neurospora*. It is interesting to examine the interaction between molecular mechanism of circadian rhythm and the stress marker proteins revealed in this study.

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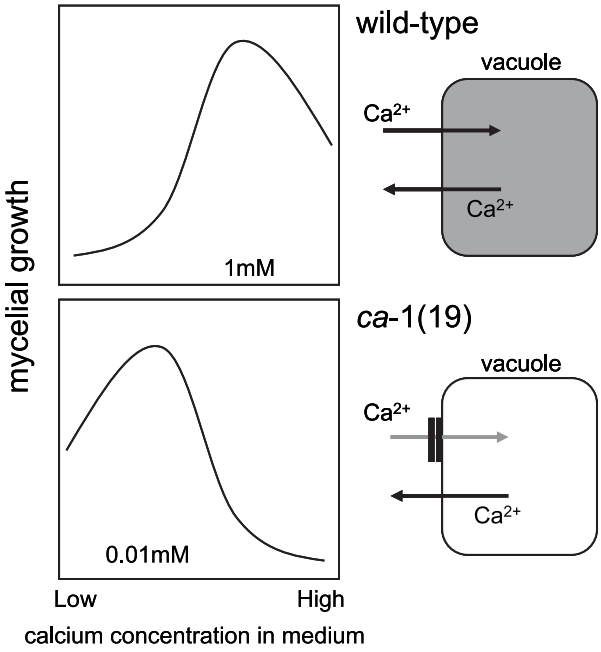

Fig. 1. **Properties of *Neurospora* mutant strain *ca*-1(19).** The mutant strain loses the function of calcium transporting activity into vacuoles. The reducing ability of storing calcium in vacuoles generates the toxic condition of cytosolic calcium when the cells are transferred in a medium containing high concentration of calcium. Thus, the optimum concentration of calcium is 0.01 mM for mycelial growth of *ca*-1(19).

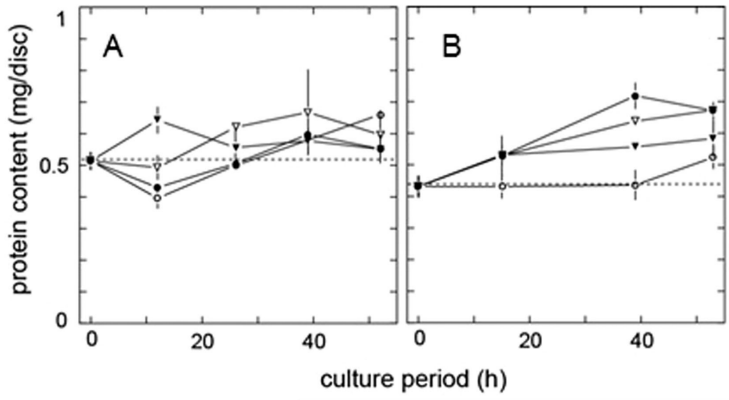

Fig. 2. **Protein contents of the cultured mycelial discs of wild-type (A) and *ca*-1(19) (B) grown in the culture medium containing various concentrations of calcium.** The mycelial discs were cultured in the medium containing 0 (open circles), 0.01 (closed circles), 0.1(open triangles), or 1 mM $CaCl_2$ (closed triangles) for 52 h. Cultured mycelial discs were harvested at 12, 26, 40, 52 h after the transferring to new medium with low carbon source, and protein content of mycelial discs were determined by Bradford protein assay system.

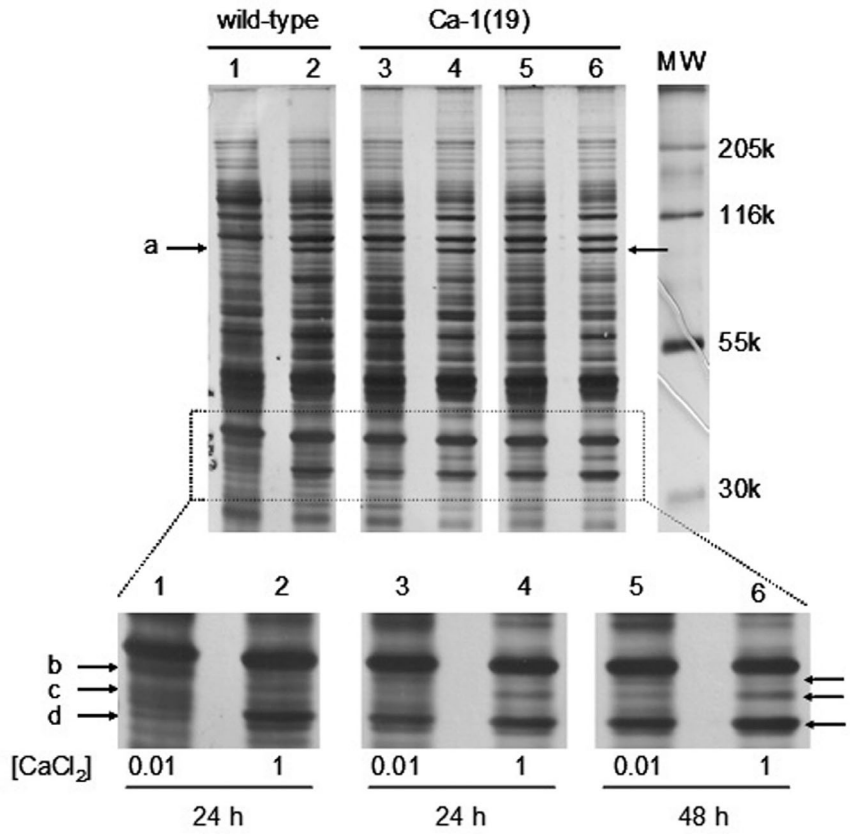

Fig. 3. Comparison of protein constitution of *Neurospora* cells grown in the medium containing 0.01 mM (lanes 1, 3, 5) and 1 mM $CaCl_2$ (lanes 2, 4, 6). The protein extract obtained from wild-type mycelial discs (lanes 1 and 2) and from *ca*-1(19) discs (lanes 3 - 6) cultured for 24 h (lanes 1 - 4) or 48 h (lanes 5 and 6) were analyzed by SDS-PAGE and visualized by silver staining. The arrows (a - d) indicate the proteins intensity of which were altered depending on extracellular calcium concentration. The altered proteins were summarized in Table 1. The lane indicated by MW shows molecular mass markers. The photograph below was an enlargement of the region around 30 - 40 kDa.

Table 1. **Summary of alterations of protein band intensity.**

protein (size)	wild-type	*ca*-1(19)
a (91kDa)	increase	constantly high
b (36kDa)	decrease	decrease
c (35kDa)	increase	increase
d (32kDa)	increase	constantly high

The amount of protein of mycelial discs cultured in the medium containing 1 mM $CaCl_2$ were compared with that cultured in the medium containing 0.01 mM $CaCl_2$.

アカパンカビ・カルシウムホメオスタシス異常突然変異株を用いた細胞外カルシウム濃度変化による細胞タンパク質パターン変化の解析

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要旨

アカパンカビ（*Neurospora crassa*）は培養液中のカルシウム濃度の変化により大きくその成長率が変わる．そのため培養中のカルシウム濃度が異なると，アカパンカビの細胞内の組成も変化すると考えられる．今回，培養中のカルシウム濃度がアカパンカビの細胞タンパク質の組成にどのような変化を与えるか調べた．その結果，野生株で細胞外カルシウム濃度に依存して量が変化する４種類のタンパク質の存在が明らかになった．カルシウムホメオスタシスに欠陥があり，高濃度カルシウム存在下では成長が抑制される突然変異株*ca*-1(19)を用い同様に調べた結果，低濃度カルシウムで培養したときにも２種類のタンパク質の量が増加していることが明らかになった．これら分子量91 と 32 kDa の２つのタンパク質は，アカパンカビのカルシウムストレスを示すマーカータンパク質になる可能性がある．

キーワード：カルシウムストレス，液胞，概日リズム
2010.11.17　受理