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1 **Ergothioneine production with *Aspergillus oryzae***

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Ergothioneine production with *Aspergillus oryzae*

To establish a reliable and practical ergothioneine (ERG) supply, we employed fermentative ERG production using *Aspergillus oryzae*, a fungus used for food production. We heterologously overexpressed the *egt-1* and *-2* genes of *Neurospora crassa* in *A. oryzae* and succeeded in producing ERG (231.0 mg/Kg of media, which was 20 times higher than the wild type).

Keywords: ergothioneine; molecular breeding; heterologous expression; *Aspergillus oryzae*; *Neurospora crassa*

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Ergothioneine (ERG), a histidine (His) betaine derivative with a thiol group at the C2 position of the imidazole ring (Figure 1), is known as a very stable antioxidant. [1)] ERG is a natural compound and is found in the human body such as in red blood cells, the liver, the kidneys, and semen at high concentrations. However, humans cannot biosynthesize ERG and ingest it from diet via an organic cation transporter specific for ERG (OCTN1). [2)]

ERG was first isolated from an ergot fungus, *Claviceps purpurea*, more than a century ago, and mushrooms, fungi, fission yeast, actinobacteria, cyanobacteria, and a methylobacterium have recently been shown to synthesize ERG. [3,4,5,6,7)] Mushrooms are major dietary sources but their slow growth, low contents, and time-consuming purification procedures lead to a high manufacturing cost. Therefore, alternative and sustainable sources of ERG are required.

A reliable and practical method for ERG production is a fermentative process using ERG-producing microorganisms, but the productivities are reported to be very low. We recently succeeded in heterologous production of ERG in *Escherichia coli* utilizing ERG biosynthetic genes identified in *Mycobacteria smegmatis*. [8)] In this study,

40 we tried to produce ERG in a fungus with fungal biosynthetic genes. The biosynthetic
41 pathway in *Neurospora crassa* was recently reported (Figure 1). [9,10] Egt-1 is a bi-
42 functional enzyme catalyzing successive reactions; the formation of hercynine (HER)
43 with L-His and *S*-adenosylmethionine (SAM), followed by synthesis of
44 hercynylcysteine-sulfoxide (Cys-HER) with HER, L-cysteine (L-Cys), and O_2. Egt-2, a
45 pyridoxal phosphate-dependent C–S lyase, catalyzes ERG formation from Cys-HER
46 with concomitant release of pyruvate and ammonia as side-products.

47 *Aspergillus oryzae* is a filamentous fungus used for traditional Japanese fermented
48 beverages and seasonings, such as sake, soy sauce, and soybean paste, and is considered
49 to be "generally recognized as safe" by the U.S. Food and Drug Administration. [11] In
50 addition, genetic engineering tools for *A. oryzae* have been established and utilized for
51 heterologous expression of various foreign genes to produce many useful compounds.
52 [12] Therefore, we tried to heterologously produce Egt-1 and Egt-2 in *A. oryzae.*

53 We first examined whether *A. oryzae* produces ERG because it has *egt-1* and *-2*
54 orthologs, *AO090012000265* and *AO090026000291*, which have 47% and 45% amino
55 acid sequence identities, respectively (https://www.genome.jp/kegg-
56 bin/show_organism?org=aor). Mycelia of *A. oryzae* NSAR1 [13] (Table 1) were
57 inoculated into a solid medium containing polished rice (20 g) and adenine (10 mg) in a
58 50 mL glass petri dish and then cultured at 30°C for 5 days. After the whole culture was
59 extracted with 100 mL methanol, an aliquot (1.2 mL) of the extract was centrifuged at
60 20,000×*g* for 10 min to remove insoluble components. The supernatant (1 mL) was
61 dried in vacuo and dissolved in 300 µL of water. Part of the solution (20 µL) was mixed
62 with 0.05% (v/v) heptafluorobutyric acid (HFBA) solution (180 µL) and used for liquid
63 chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis.
64 Analytical conditions were as follows: UPLC, Waters ACQUITY system equipped with

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65 a photodiode array and a SQ Detector2 (Tokyo, Japan); column, XBridge BEH C18 XP
66 (150 mm L × 2.0 mm; ID, 2.5 μm; Waters); flow rate, 0.15 mL/min; temperature, 35°C;
67 mobile phase, water containing 0.05% HFBA and 7% methanol; injection volume, 2
68 μL; detection, 258 nm for ERG. [8] As shown in Figure 2, only a small amount of ERG
69 production (11.5 ± 1.0 mg/Kg of media) was detected. Therefore, we attempted to
70 enhance the ERG productivity using a genetic engineering approach.

71 We first attempted to overexpress the *N. crassa egt*-1 gene in *A. oryzae*. DNA
72 fragments encoding *egt*-1 were amplified by PCR with *N. crassa* JCM 19069 genomic
73 DNA as the template, the primers 5′-
74 AACAAACTAGTATGCCGAGTGCCGAATCCATGACCCCAAG-3′/5′-
75 TCACACTAGTCAACGACTCACAAATCCCTAACAACTCTCGC-3′ (*Spe*I
76 restriction sites are underlined), and Tks Gflex DNA polymerase (Takara Bio Inc.,
77 Shiga, Japan). The PCR products (3,491 bp) were cloned into the same restriction site in
78 the expression vector pUARA2 [14] to express the *egt*-1 gene under the control of the
79 *amyB* promoter. The plasmid thus constructed, pUARA2-Nc1 (11,546 bp, Table 1), was
80 introduced into *A. oryzae* according to a method described previously. [15] After the
81 transformants were subcultured twice on a selection medium, integration of the *Ncegt-1*
82 gene was confirmed by PCR with genomic DNA of the NSA-Nc1 strain and the same
83 primers used for gene cloning (Figure 3A). Moreover, Southern blot analysis using a
84 DNA fragment carrying *Ncegt-1* as the probe showed only one strong band at
85 approximately 11 Kbp after digestion of the genomic DNA with *Xho*I, which has a
86 unique restriction site in the *Ncegt-1* gene. In addition, only one band was detected by
87 digestion with *Kas*I or *Bpl*I, which have unique restriction sites in the vector. Taking
88 these results together, the *Ncegt-1* gene was suggested to be multiply integrated into the
89 genomic DNA in a similar manner as previously reported. [16] ERG productivity was

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90 then examined by the same methods as described above. As shown in Figure 2, the ERG
91 productivity of the transformant (strain NSA-Nc1) drastically increased to 124.5 ± 5.0
92 mg/Kg of media. This result suggested that the *egt-1* gene was efficiently expressed in
93 *A. oryzae.*

94 Therefore, we next introduced the *N. crassa egt-2* gene into the strain NSA-Nc1.
95 DNA fragments were amplified by PCR with *N. crassa* genomic DNA and the primers
96 5′-AACAAACTAGTATGGTCGCCACCACCGTCGAGCTGCCTCTG-3′/5′-
97 CAAATACTAGTTCAGGCGCTCTCCTTGTACTCCCCCTTAGCCAC-3′ (*Spe*I
98 restriction sites are underlined). The amplicon was digested with *Spe*I and inserted into
99 the *Spe*I site of pAdeA2 [17] to construct pAdeA2-Nc2 (8,086 bp, Table 1). After the
100 plasmid was introduced into the strain NSA-Nc1, integration of the *Ncegt-2* gene into
101 the genomic DNA was confirmed by PCR and Southern blot analysis (Figure 4). In this
102 case, Southern blot analysis showed at least three major bands after digestion of the
103 genomic DNA with *Nsi*I, which has a unique restriction site in the *Ncegt-2* gene,
104 suggesting that at least two copies of the plasmid were integrated into the genome.
105 Accordingly, two major bands were detected by digestion with *Hin*dIII or *Sal*I, which
106 could cut the vector at unique sites. The ERG productivity of the transformant (strain
107 NS-Nc12) was then investigated by the same methods as described above. As shown in
108 Figure 2, ERG productivity was further increased up to 231.0 ± 1.1 mg/Kg of media,
109 suggesting that the *egt-2* gene was also efficiently expressed in *A. oryzae*.

110 In conclusion, fermentative ERG production by a fungus was carried out to
111 establish a reliable and practical method for ERG production. By heterologous
112 overexpression of the *egt-1* and *-2* genes of *N. crassa* in *A. oryzae*, we achieved 20-fold
113 higher ERG production (231.0 ± 1.1 mg/Kg of media) than the wild type. To date,
114 screening of ERG producers from fungi [18] and production of ERG by engineered fungi

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115 [19] have been carried out. Besides these approaches, our system will also be an
116 alternative method for ERG supply.

117

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121 pAdeA2), respectively. We thank Robbie Lewis, MSc, from Edanz Group
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Author contributions

T. Dairi conceived and designed the experiments. S. Takusagawa, Y. Satoh, and I. Ohtsu performed the experiments and analyzed the data. Y. Satoh and T. Dairi wrote the paper.

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Abbreviations used

ERG, ergothioneine; HER, hercynine; Cys-HER, hercynylcysteine-sulfoxide; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; L-His, L-histidine; L-Cys, L-cysteine; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry.

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Table 1. Strains and plasmids used in this study.

Strains or plasmids	Description	Source
Strains		
A. oryzae		
NSAR1	sC^-, $niaD^-$, $\Delta argB$, $adeA^-$	13
NSA-Nc1	NSAR1 transformed with pUARA2-Nc1; $Ncegt\text{-}1^+$	This study
NS-Nc12	NSA-Nc1 transformed with pAdeA2-Nc2; $Ncegt\text{-}1^+$, $Ncegt\text{-}2^+$	This study
N. crassa		
JCM 19069	ERG producer	JCM
E. coli		
XL-1 Blue	*hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *supE*44, *relA*1, *lac*[F', *proAB*, $lacI^q$ZΔM15, Tn10(Tc^R)]	Nippon Gene
Plasmids		
pUARA2	Vector; PamyB, TamyA, Ap^R, *argB*	14
pUARA2-Nc1	pUARA2 derivative with *Ncegt1*	This study
pAdeA2	Vector; PamyB, TamyA, Ap^R, *adeA*	17
pAdeA2-Nc2	pAdeA2 derivative with *Ncegt2*	This study

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225 **Figure captions**

226 Figure 1. The ERG biosynthetic pathway in fungi.

227 SAH, *S*-adenosylhomocysteine

228

229 Figure 2. ERG production by *A. oryzae* NSAR1 and its recombinants.

230

231 Figure 3. Analysis of *egt-1* gene integration into the *A. oryzae* NSA-Nc1 strain.

232 (A) Integration of the *Ncegt-1* gene was checked by PCR with genomic DNA of the
233 NSA-Nc1 strain and the same primers used for gene cloning. The amplicon size was
234 estimated at 3.5 Kbp. (B) Southern blot analysis was carried out using the DIG system
235 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's
236 protocol. A DIG labeled-probe (3.5 Kbp) was prepared by PCR with the pUARA2-Nc1
237 plasmid as a template and the same primers used for gene cloning. Genomic DNA
238 digested with *Kas*I, *Bpl*I, or *Xho*I, which have unique restriction sites in pUARA2-Nc1,
239 was electrophoresed on a 0.5% agarose gel, blotted and then detected with the probe.

240

241 Figure 4. Analysis of *egt-2* gene integration into the *A. oryzae* NS-Nc12 strain.

242 (A) Integration of the *Ncegt-2* gene was checked by PCR with genomic DNA of the NS-
243 Nc12 strain and the same primers used for gene cloning. The amplicon size was
244 estimated at 1.5 Kbp. (B) Southern blot analysis was carried out using the DIG system.
245 A DIG labeled-probe (1.5 Kbp) was prepared by PCR with the pAdeA2-Nc2 plasmid as
246 a template and the same primers used for gene cloning. Genomic DNA digested with
247 *Hin*dIII, *Sal*I, or *Nsi*I, which have unique restriction sites in pAdeA2-Nc2, was
248 electrophoresed on a 0.5% agarose gel, blotted and then detected with the probe.

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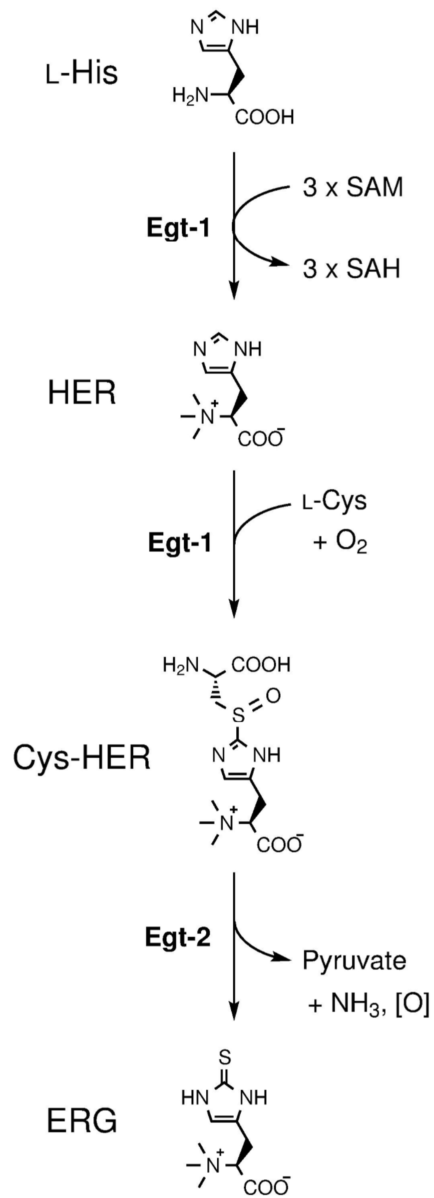

Figure 1. The ERG biosynthetic pathway in fungi.
SAH, S-adenosylhomocysteine

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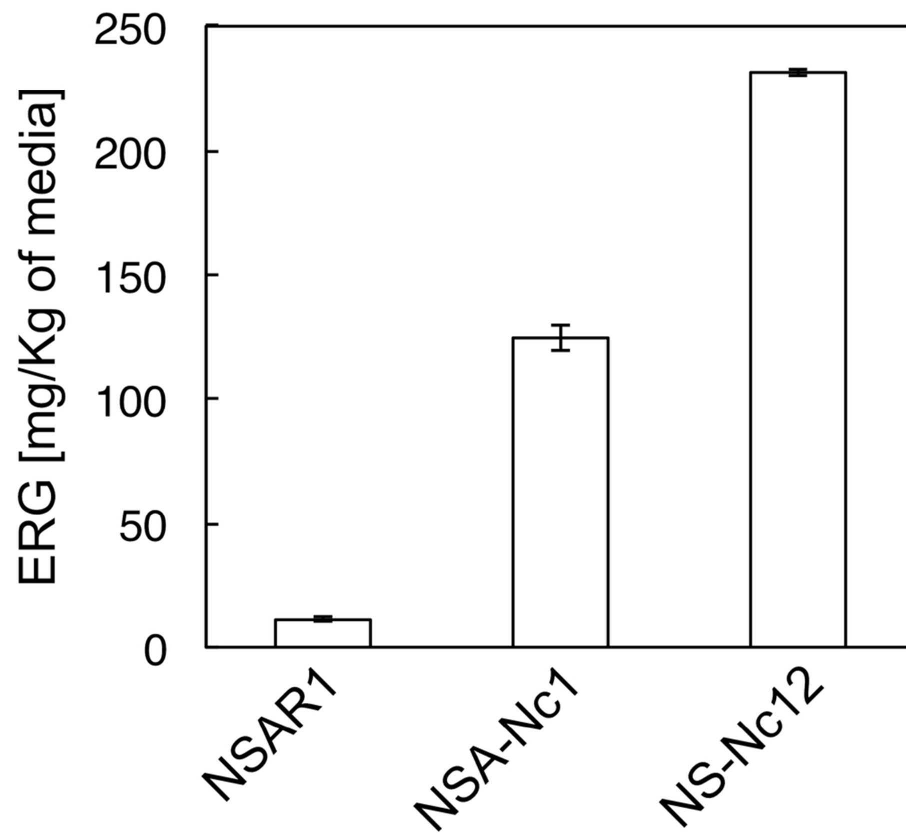

Figure 2. ERG production by A. oryzae NSAR1 and its recombinants.

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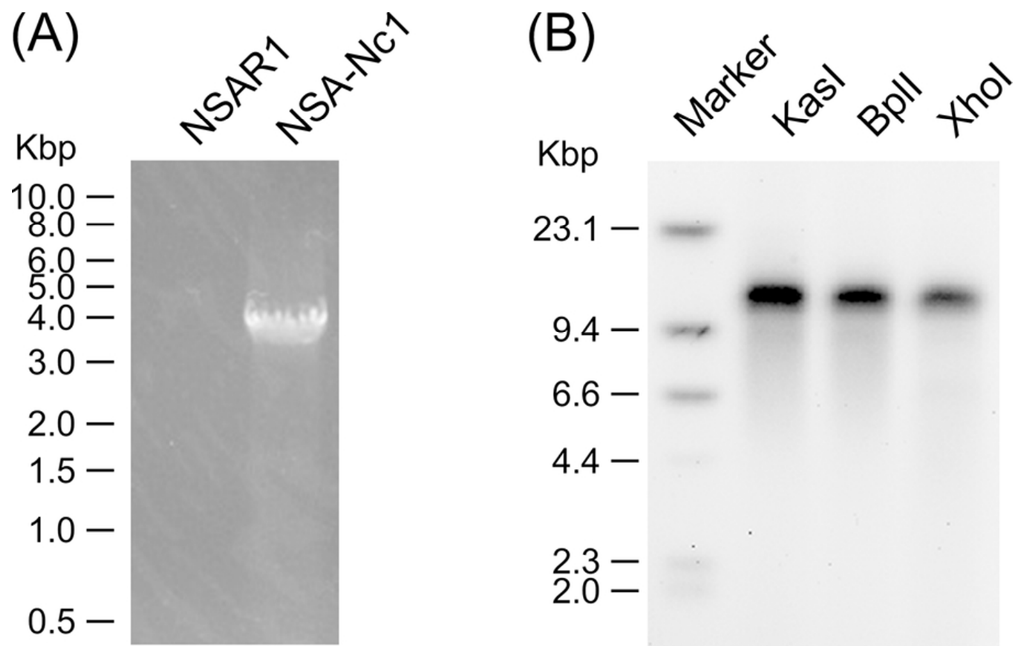

Figure 3. Analysis of egt-1 gene integration into the A. oryzae NSA-Nc1 strain.
(A) Integration of the Ncegt-1 gene was checked by PCR with genomic DNA of the NSA-Nc1 strain and the same primers used for gene cloning. The amplicon size was estimated at 3.5 Kbp. (B) Southern blot analysis was carried out using the DIG system (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. A DIG labeled-probe (3.5 Kbp) was prepared by PCR with the pUARA2-Nc1 plasmid as a template and the same primers used for gene cloning. Genomic DNA digested with KasI, BplI, or XhoI, which have unique restriction sites in pUARA2-Nc1, was electrophoresed on a 0.5% agarose gel, blotted and then detected with the probe.

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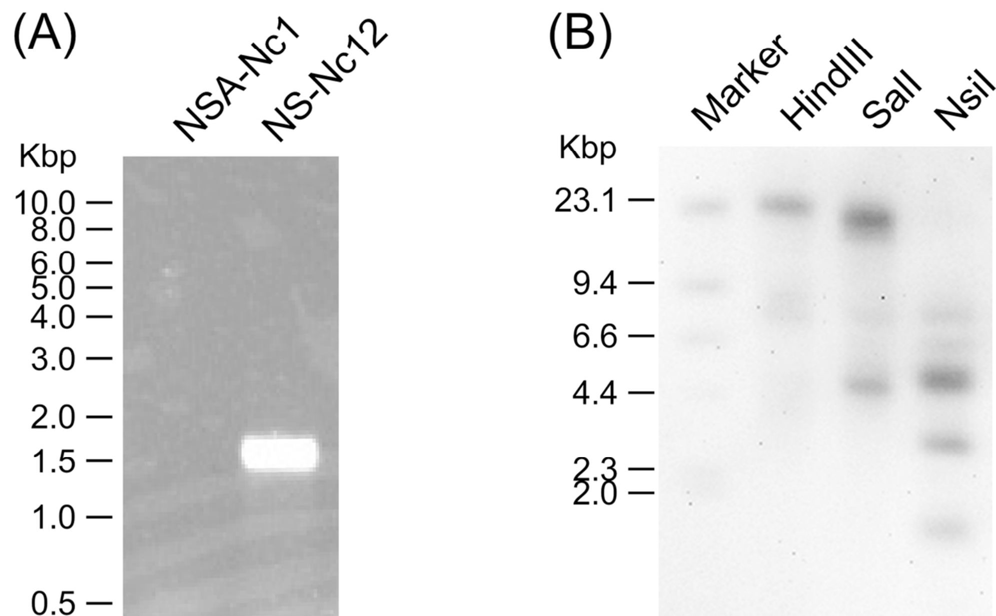

Figure 4. Analysis of egt-2 gene integration into the A. oryzae NS-Nc12 strain.
(A) Integration of the Ncegt-2 gene was checked by PCR with genomic DNA of the NS-Nc12 strain and the same primers used for gene cloning. The amplicon size was estimated at 1.5 Kbp. (B) Southern blot analysis was carried out using the DIG system. A DIG labeled-probe (1.5 Kbp) was prepared by PCR with the pAdeA2-Nc2 plasmid as a template and the same primers used for gene cloning. Genomic DNA digested with HindIII, SalI, or NsiI, which have unique restriction sites in pAdeA2-Nc2, was electrophoresed on a 0.5% agarose gel, blotted and then detected with the probe.

116x106mm (300 x 300 DPI)