A Possible Mechanism of Control of Rice Blast Disease by a Novel Alkoxyiminoacetamide Fungicide, SSF126

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We thank Y. Sowa for her help during the experiments and H. Takenaka, Shionogi Aburahi Laboratories, for supplying sakuranetin. We also thank H. Ichinari, Shionogi Aburahi Laboratories, for assistance in the preparation of this manuscript.
Accepted for publication 16 November 1995.

ABSTRACT

Mizutani, A., Miki, N., Yukioka, H., Tamura, H., and Masuko, M. 1996. A possible mechanism of control of rice blast disease by a novel alkoxyiminoacetamide fungicide, SSF126. Phytopathology 86:295-300.

When the mycelial cells of *Pyricularia grisea* were pulse-labeled with [^{35}S]-methionine in the presence of (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide (SSF126), the radioactivity was time-dependently incorporated into a 40-kDa mitochondrial protein. The appearance of the pulse-labeled 40-kDa protein paralleled the SSF126-dependent induction of cyanide-resistant respiratory activity in the mycelial cells. The SSF126-dependent induction of cyanide-resistant respiration was reversibly blocked by carbonylcyanide-*p*-trifluoromethoxyphenyl-hydrazone (FCCP). When the mycelial cells were pulse-labeled with radioactive methionine in the presence of SSF126 and FCCP, the radioactivity was incorporated into a 41.4-kDa mitochondrial polypeptide. Upon removal of FCCP, this polypeptide was converted into a 40-kDa protein and cyanide-resistant respiratory activity was induced in the mycelial cells. These findings suggested that the 41.4-kDa polypeptide is processed into the 40-kDa mature protein responsible for the cyanide-resistant respiration in *P. grisea*. Furthermore, superoxide anion (O_2^-) was suggested to be involved in the SSF126-dependent induction mechanism of the cyanide-resistant respiration in the mycelial cells. Flavonoids found in plants had the ability to scavenge O_2^- generated by blockage of electron flux through the cytochrome bc_1 segment in the mitochondrial respiratory chain and they inhibited SSF126-dependent induction of cyanide-resistant respiration. Consequently, it is feasible that SSF126 controls rice blast caused by *P. grisea* in conjunction with rice plant components.

Additional keywords: bc_1 complex, chemiluminescence, mitochondrial protein import, respiratory inhibitor, superoxide dismutase.

(*E*)-2-Methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide (SSF126) has been developed as a systemic fungicide for control of rice blast caused by *Pyricularia grisea* (Cooke) Sacc. (7). This compound inhibits fungal respiration by blockage of electron flux through the cytochrome bc_1 segment in the mitochondrial respiratory chain. However, fungal respiration recovers after inhibition by SSF126 because of oxygen consumption insensitive to potassium cyanide, indicating that the mycelial cells of *P. grisea* have the ability to induce a cyanide-resistant pathway in response to the blockage of cytochrome-mediated electron transport by SSF126 (20).

The cyanide-resistant pathway branches from the cytochrome-mediated pathway at the ubiquinone pool (1,27). Since the cyanide-resistant pathway per se does not contribute to the formation of a proton gradient at the cytochrome bc_1 complex or at the cytochrome a-a_3 complex, much of the free energy generated by the pathway is not conserved as chemical energy and is lost as heat (21). Although the role of cyanide-resistant respiration in thermogenesis is well known in the appendix tissue of Araceae, where heat is utilized to volatilize foul-smelling compounds to attract insect vectors for pollination (16), the primary role of the alternate pathway remains unknown in microorganisms.

In some microorganisms, cyanide-resistant respiration can be induced rapidly with respiratory inhibitors (13,18), in contrast to higher plants, in which its expression is under strict developmental control (4,5,14). The mycelial cells of *P. grisea* induced cyanide-resistant respiration to recover respiratory activity when the cytochrome-mediated pathway was blocked by SSF126 (20). However, SSF126 completely suppressed the development of rice blast in field trials, suggesting that some components of rice plants might interfere with the SSF126-dependent induction of the cyanide-resistant respiration in *P. grisea*.

Although the mechanism of induction of cyanide-resistant respiration has not yet been clarified in any organism, the following phenomena gave us a cue to seek for factors involved in the induction mechanism. The lack of oxygen prevents the normal development of cyanide-resistant respiration in *Cicer arietinum* seeds (3) and also prevents antimycin A-dependent induction in the yeast *Hansenula anomala* (18). Furthermore, antimycin A is capable of generating superoxide anion (O_2^-) at the site of the ubiquinone-cytochrome *b* segment (2,29). These suggest involvement of O_2^- in the mechanism of induction of cyanide-resistant respiration. It appeared feasible that SSF126-dependent induction of the respiration is also triggered by O_2^-. If true, the induction could be blocked by radical scavengers such as flavonoids.

Here we described a mitochondrial protein induced by SSF126 and the involvement of O_2^- in the induction mechanism in *P. grisea*. Furthermore, we proposed an explanation for the control of rice blast by SSF126.

MATERIALS AND METHODS

Fungus and culture conditions. *P. grisea* (race 003) was grown on a medium containing 5% (wt/wt) oatmeal and 2% (wt/wt) sucrose. Conidia were grown aerobically at 25°C for 24 h (20).

Chemicals. 5,4-Dihydroxy-7-methoxyflavanone (sakuranetin) was kindly supplied by H. Takenaka, Shionogi & Co., Aburahi

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Publication no. P-1996-0129-01R

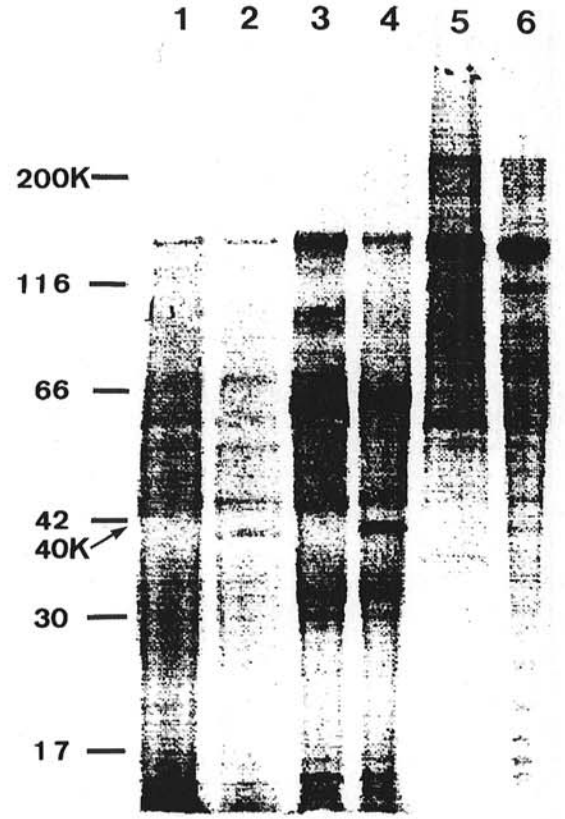

Fig. 1. Autoradiograph of sodium dodecyl sulfate (SDS) gel containing subcellular fractions from mycelial cells incubated in the presence or absence of (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide (SSF126). The cells labeled with [^{35}S]-methionine were disrupted and fractionated as described in the text, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 20% polyacrylamide gradient gel; 40 µg of protein was applied to each lane) and autoradiography for 5 days. Lanes 1, 3, and 5: untreated; lanes 2, 4, and 6: SSF126-treated (cyanide-resistant respiration = 19.2 nmol of O_2/min/20 mg). Lanes 1 and 2: 1,500 × *g* pellets; lanes 3 and 4: 12,000 × *g* pellets; and lanes 5 and 6: 12,000 × *g* supernatant.

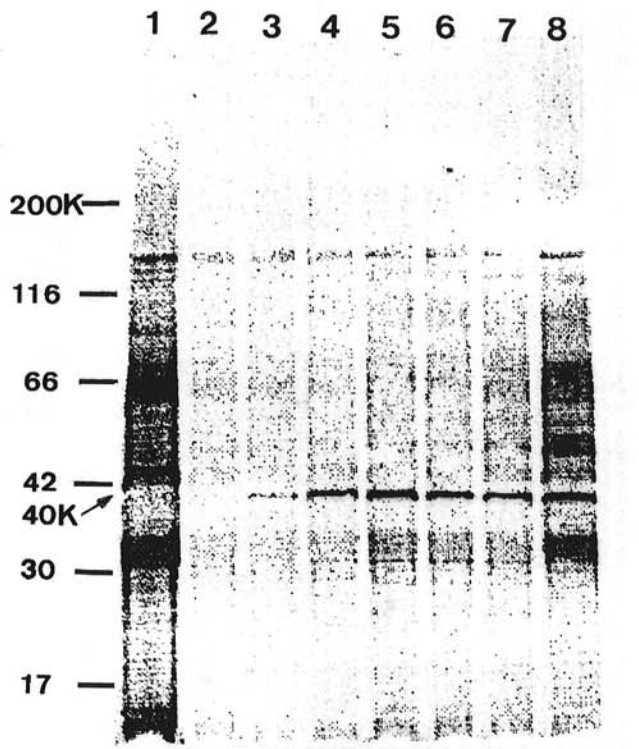

Fig. 2. Autoradiograph of sodium dodecyl sulfate (SDS) gel showing mitochondrial protein profile for mycelial cells pulse-labeled with [^{35}S]-methionine for the indicated periods after the addition of (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide (SSF126). Aliquots each of 30 µg of protein were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 20% polyacrylamide gradient gel). Lane 1: untreated; lane 2: 0 to 10 min; lane 3: 10 to 20 min; lane 4: 20 to 30 min; lane 5: 30 to 40 min; lane 6: 40 to 50 min; lane 7: 50 to 60 min; and lane 8: 0 to 60 min.

Laboratories (Shiga, Japan). The following chemicals were purchased from Sigma Chemical Co. (St. Louis): antimycin A, carbonylcyanide-*p*-trifluoromethoxyphenyl-hydrazone (FCCP), myxothiazol, apigenin, quercetin, naringenin, NADH (reduced form), and superoxide dismutase (SOD) (Mn type). 2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazole [1,2-a] pyrazin-3-one (MCLA) was purchased from Tokyo Kasei Co. (Tokyo), and L-[^{35}S]-methionine (37 TBq/mmol) was purchased from Amersham International (Amersham, United Kingdom). All other reagents were analytical grade and purchased from Wako Chemical Co. (Kyoto, Japan). SSF126 (99% purity) was synthesized by Aburahi Laboratories, Shionogi & Co.

Subcellular fractionation of labeled cells. Mycelial cells (1 g of fresh weight) from 1-day-old cultures of *P. grisea* were suspended in a culture medium containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–Tris buffer (pH 7.0) and 100 mM glucose, and preincubated aerobically for 10 min in an orbital incubator (R100; Yamato Co., Tokyo) at 25°C and 80 rpm. The intact cells were labeled with [^{35}S]-methionine (1.5 MBq) for 1 h in the presence of 50 µM SSF126, and subsequently chased with unlabeled methionine (2 mM) and cycloheximide (20 µM) to prevent further incorporation of radioactivity into proteins. The labeled cells were collected by centrifugation at 1,500 × *g* with a Hitachi 20PR 52D (Hitachi Koki Co., Ibraki, Japan) equipped with an RPR 20-2 rotor, and suspended in 30 ml of an enzyme solution containing 0.6 M KCl, 1% Driselase (Kyowa Co., Tokyo), 1% Cellulase Onozuka R-10 (Yakult Co., Tokyo), and 20 µM cycloheximide to prepare protoplasts according to the method of Teraoka et al. (28). The suspension was incubated in the orbital incubator for 1 h at 25°C and 80 rpm, disrupted by sonication, and then fractionated by centrifugation at 4°C (20). The pellet at 1,500 × *g* contained cell debris and unbroken cells. The pellet at 12,000 × *g* corresponded to the mitochondrial fraction, and the supernatant contained microsomes and soluble components. Fractions from centrifugation were treated with lysis buffer (15% [wt/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 2% [wt/vol] sodium dodecyl sulfate [SDS], and 62.5 mM Tris-HCl buffer [pH 6.8]) and subjected to heat treatment at 100°C for 2 min, followed by electrophoresis on SDS-polyacrylamide gels according to the method of Laemmli (12) at 40 mA for 1 h. After staining with Coomassie brilliant blue R-250, the gel was dried and autoradiographed on Hyperfilm β-max (Amersham International).

Pulse-labeling of cells. After aerobic preincubation for 10 min as described above, 50 µM SSF126 was added to the mycelial suspension, and then the cells were pulse-labeled with [^{35}S]-methionine (0.85 MBq) at the times indicated in the figure legend. The chase was initiated by adding an excess of unlabeled methionine after pulse-labeling for 10 min. One hour after the addition of SSF126, mitochondria were prepared from the labeled cells in the presence of 20 µM cycloheximide (20), and then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Effects of FCCP and flavone on expression of a protein induced by SSF126. After aerobic incubation for 30 min with 50 µM SSF126 and 0.5 µM FCCP, the mycelial cells (1 g of fresh weight) were pulse-labeled with radioactive methionine (0.85 MBq) for 10 min at 25°C, followed by chasing as described above. The pulse-labeled cells were harvested under vacuum 1 h after treatment with SSF126 and FCCP, washed with culture

TABLE 1. Increase in cyanide-resistant respiration of *Pyricularia grisea* mycelial cells following removal of FCCP[v]

Treatment	Cyanide-resistant respiratory activity				
	1 h[w]	5[x]	10[x]	20[x]	30[x]
SSF126[y] (50 μM) + FCCP (0.5 μM)	0.7 ± 0.4 b[z]	9.8 ± 2.0 (1.2 ± 0.1)	18.5 ± 1.5 (1.4 ± 0.4)	21.7 ± 1.8 (1.2 ± 0.4)	21.2 ± 2.2 b (1.6 ± 0.2)
SSF126 (50 μM)	19.2 ± 2.2 c	ND	ND	ND	20.0 ± 3.2 b
FCCP (0.5 μM)	0.6 ± 0.2 b	ND	ND	ND	0.4 ± 0.1 c
Untreated	0.5 ± 0.1 b	ND	ND	ND	0.4 ± 0.1 c

[v] FCCP = carbonylcyanide-*p*-trifluoromethoxyphenyl-hydrazone.
[w] Cyanide-resistant respiratory activity of mycelial cells incubated for 1 h under the conditions indicated.
[x] Time in minutes after removal of FCCP.
[y] SSF126 = (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide.
[z] Numerals show the cyanide-resistant respiratory activity of the cells expressed as nanomoles of O_2/min/20 mg of fresh weight mycelia. Numerals in parentheses show the respiratory activity of the cells incubated under the indicated conditions without removal of FCCP. Results are means and SE of two separate experiments. Values followed by different letters (within a column) differ significantly ($P < 0.01$) according to Duncan's new multiple range test. ND = not determined.

medium to remove FCCP, resuspended in 30 ml of the medium containing 20 μM cycloheximide, and then incubated at 25°C. At 0, 10, 20, and 30 min after removal of FCCP, the cells were harvested and mitochondria were prepared in the presence of 20 μM cycloheximide and 0.5 μM FCCP to prevent further translocation of precursor polypeptides into mitochondria, followed by SDS-PAGE and autoradiography.

To determine the effect of flavone on expression of a protein induced by SSF126, the mycelial cells were labeled with radioactive methionine for 1 h in the presence of 50 μM SSF126 and appropriate concentrations of flavone. Mitochondria were prepared, and analyzed by SDS-PAGE and autoradiography.

Estimation of O_2^- generation in submitochondrial particles. Generation of O_2^- was determined by monitoring MCLA-dependent chemiluminescence according to the method of Nakano (22). Submitochondrial particles were prepared from cyanide-sensitive mycelial cells (20). The particles (50 μg of protein) were suspended in 1 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 0.3 mM NADH, 250 mM sucrose, and 4 μM MCLA. The reaction was initiated by the addition of 10 μM SSF126, 3 μM myxothiazol, 3 μM antimycin A, or 100 μM flavone at 25°C. To determine whether light emission originated from O_2^--dependent chemiluminescence, 0.5 μM SOD was added to the reaction system at 40 s after the addition of the chemicals. Chemiluminescence was measured using a bioluminometer equipped with an injector (LB9505; Laboratorium Prof. Dr. Berthold GmbH Co., Wildbad, Germany).

Effects of flavonoids, FCCP, and nitrogen on induction of cyanide-resistant respiratory activity. Mycelial cells (0.6 g of fresh weight) of *P. grisea* from 1-day-old cultures were suspended in 90 ml of culture medium and incubated at 25°C with 50 μM SSF126 and appropriate concentrations of flavonoids dissolved in dimethyl sulfoxide. After incubation for 1 h, 3 ml of the mycelial suspension was extracted from the cultures and transferred into a reaction vessel. Immediately, 1 mM potassium cyanide was added, followed by incubation for 30 s, and then oxygen consumption by the cells was measured for 1 min. After recording, 1 mM salicylhydroxamic acid (SHAM), a potent inhibitor of cyanide-resistant respiration, was added to the suspension and the rate of oxygen consumption sensitive to SHAM was calculated from derivative recordings of oxygen electrode signals. The rate of oxygen consumption was measured polarographically at 25°C in a sealed reaction vessel in a circulating water bath, using a Clark-type oxygen electrode (YSI5300; Yellow Springs Instrument Co., Yellow Springs, OH). Cyanide-resistant respiration was determined as the oxygen consumption sensitive to SHAM in the presence of potassium cyanide.

To determine the effect of FCCP on the induction of cyanide-resistant respiration, the mycelial cells were suspended in culture medium as described above, and incubated with 50 μM SSF126 and 0.5 μM FCCP at 25°C. After incubation for 1 h, the cells were harvested under vacuum, washed with culture medium to remove FCCP, resuspended in medium containing 20 μM cycloheximide, and then incubated under aerobic conditions. At 5, 10, 20, and 30 min after removal of FCCP, 3-ml aliquots of the mycelial suspension were extracted from the cultures and cyanide-resistant respiratory activity of the cells was measured as described above.

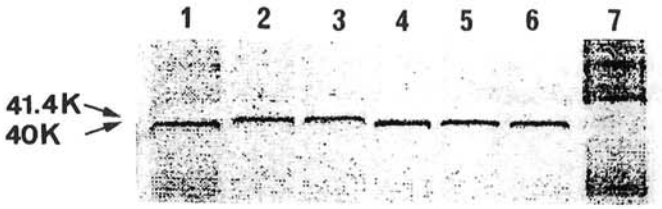

Fig. 3. Effect of carbonylcyanide-*p*-trifluoromethoxyphenyl-hydrazone (FCCP) removal on pulse-labeled mitochondrial polypeptide. At the indicated times after removal of FCCP, mycelial cells were harvested to prepare mitochondria as described in the text. Aliquots, each of 5 μg of protein, were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 to 20% polyacrylamide gradient gel). Lane 1 shows an autoradiograph of a mitochondrial fraction from mycelial cells incubated with (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide (SSF126) for 1 h and lane 2 shows an autoradiograph of a mitochondrial fraction from mycelial cells incubated with SSF126 + FCCP for 1 h. Lane 3: 0 min (immediately after removal of FCCP); lane 4: 10 min after FCCP removal; lane 5: 20 min after FCCP removal; lane 6: 30 min after FCCP removal; and lane 7: untreated.

To evaluate the contribution of oxygen to the induction of cyanide-resistant respiration, the mycelial cells suspended in culture medium as described above were incubated with 50 μM SSF126 under anaerobic conditions. Oxygen was removed by repeated degassing with a vacuum desiccator and resaturation with N_2, and the mycelial cells were kept under a stream of nitrogen. After various incubation periods, the rate of oxygen consumption sensitive to SHAM was measured as described above.

To examine the effect of removal of nitrogen on the induction of cyanide-resistant respiration, the mycelial cells were harvested after anaerobic incubation for 45 min, resuspended in air-saturated culture medium, and then incubated under aerobic conditions. After various incubation periods, the cyanide-resistant respiratory activity of the cells was measured as described above. The rate of oxygen consumption was expressed as nanomoles of O_2/min/20 mg.

RESULTS

Mycelial protoplasts labeled with [^{35}S]-methionine in the presence or absence of SSF126 were disrupted by sonication and analyzed by SDS-PAGE (Fig. 1). A distinct radioactive band corresponding to a 40-kDa protein was detected on the protein profile for the mitochondrial fraction (12,000 × *g* pellet) only from

the cells treated with SSF126 (Fig. 1, lane 4). No incorporation of radioactive methionine into the protein was observed in the untreated cells. The appearance of the weakly radioactive protein band corresponding to 40 kDa detected in the pellet at 1,500 × *g* from the cells treated with SSF126 (lane 2) was probably due to contamination by unbroken cells.

To examine SSF126-dependent induction of the 40-kDa protein, the mycelial cells were pulse-labeled with radioactive methionine (Fig. 2). No radioactive protein band corresponding to the 40-kDa species was detected in the mitochondrial protein profile for cells pulse-labeled for 10 min immediately after treatment with SSF126 (Fig. 2, lane 2). A faint protein band (40 kDa) appeared in the protein profile for the cells pulse-labeled between 10 to 20 min after treatment (lane 3). A distinct radioactive protein band of molecular mass corresponding to 40 kDa was detected on the protein profile for the cells pulse-labeled from 20 to 30 min after treatment (lane 4), and the appearance of the protein remained stable thereafter (lanes 5 to 7), indicating that the induction of the 40-kDa protein is SSF126-dependent.

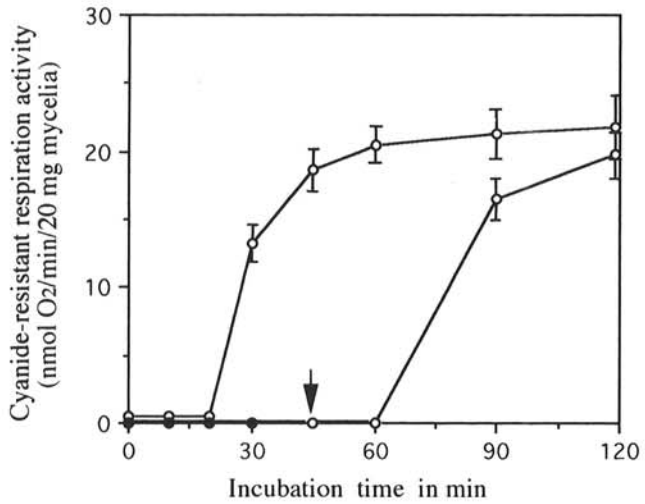

Fig. 4. Effect of nitrogen on induction of cyanide-resistant respiration. Mycelial cells were incubated with 50 μM (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide (SSF126), and the rate of oxygen consumption sensitive to salicylhydroxamic acid was measured at the indicated times. Open circles show the rate of oxygen consumption by the cells incubated under aerobic conditions; closed circles show the rate in the cells incubated under a N_2 atmosphere. The arrow indicates the time at which N_2 was replaced with air. Control activity was 20.6 nmol of O_2/min/20 mg of fresh weight mycelia. Results are means of two separate experiments with bars showing the standard errors.

FCCP, which disrupts transmembrane proton electrochemical gradients, inhibited SSF126-dependent induction of cyanide-resistant respiration in the mycelial cells (Table 1). However, after removal of FCCP, the mycelial cells that had been incubated with SSF126 and FCCP showed cyanide-resistant respiratory activity. At 5 min after removal of FCCP, the respiratory activity of the cells increased up to 50% of the activity of those cells incubated with SSF126 alone, increasing up to more than 90% at 10 min. Thus, the respiratory activity of the cells increased time-dependently after removal of FCCP.

The radioactive 40-kDa protein was detected on the mitochondrial protein profile for the cells pulse-labeled in the presence of SSF126 alone (Fig. 3, lane 1). In contrast, the radioactivity was incorporated into a mitochondrial polypeptide corresponding to 41.4 kDa in the protein profiles for the cells that were harvested after incubation with SSF126 and FCCP, and immediately after removal of FCCP (lanes 2 and 3). Neither radioactive 40-kDa nor 41.4-kDa protein was detected on the mitochondrial protein profile for control cells without any addition (lane 7). At 10 min after removal of FCCP, the mitochondrial polypeptide (41.4 kDa) accumulated in the presence of FCCP was converted to a 40-kDa protein (lane 4), and the appearance of the protein remained stable thereafter (lanes 5 and 6).

Cyanide-resistant respiration was induced time-dependently by SSF126 under aerobic conditions. In contrast, the induction was completely suppressed under anaerobic conditions (under nitrogen) (Fig. 4). The inhibitory effect of anaerobiosis was reversed upon subsequent aeration, indicating that oxygen is involved in the induction mechanism of the respiration in *P. grisea*.

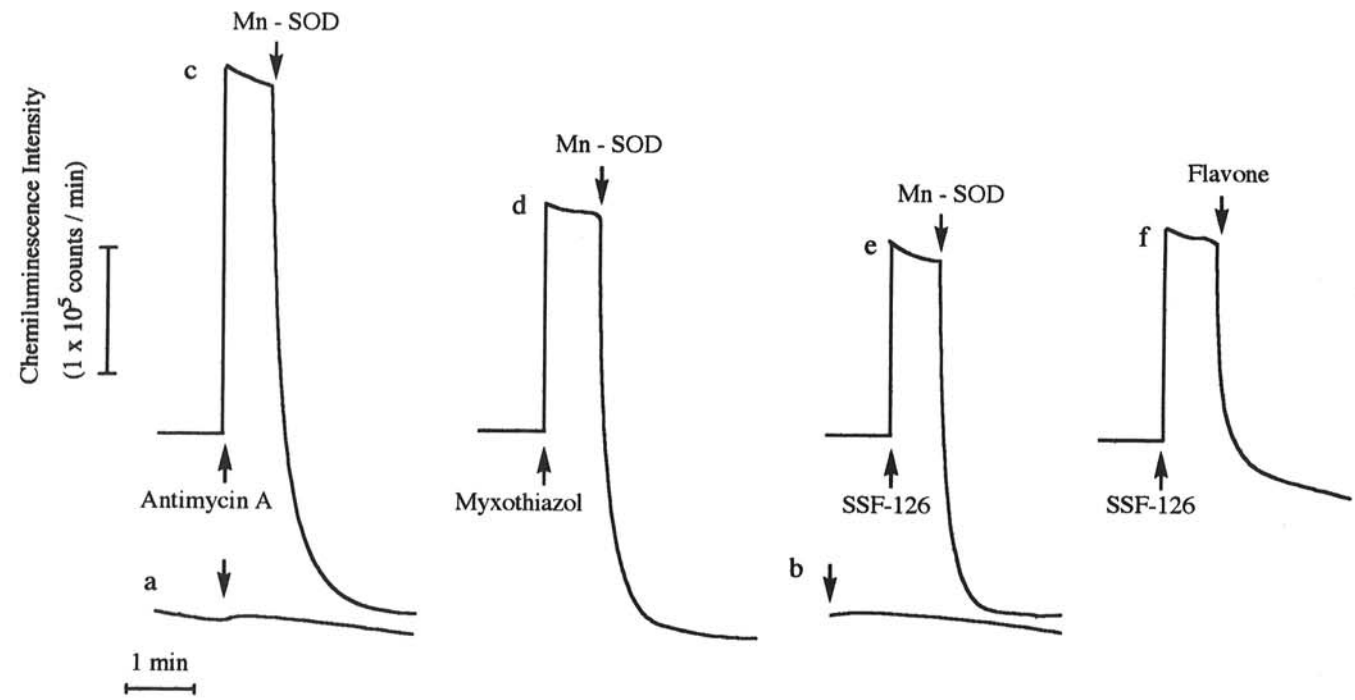

Fig. 5. Respiratory inhibitor-dependent superoxide anion generation in submitochondrial particles of *Pyricularia grisea*. Submitochondrial particles (50 μg of protein) prepared from cyanide-sensitive mitochondria were suspended in the reaction system with (a and b) or without (c, d, e, and f) superoxide dismutase. NADH, 0.3 mM; 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazole [1,2-a] pyrazin-3-one (MCLA), 4 μM; (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide (SSF126), 10 μM; antimycin A, 5 μM; myxothiazol, 5 μM; flavone, 100 μM; and superoxide dismutase (Mn type) (Mn-SOD), 0.5 μM.

Respiratory inhibitors for mitochondrial bc_1 complex (SSF126, myxothiazol, and antimycin A) increased MCLA-dependent chemiluminescence in a cyanide-sensitive submitochondrial particle system containing NADH as a respiratory substrate (Fig. 5c, d, e, and f). The enhanced light emission was significantly quenched by the addition of SOD (Fig. 5c, d, and e). The light emission was not activated when the respiratory inhibitors were added to the system containing SOD (Fig. 5a and b), indicating that the increment of the light emission originated from O_2^--dependent chemiluminescence. Flavone, known as a radical scavenger, also suppressed the light emission enhanced by the addition of SSF126 (Fig. 5f).

Six flavonoids were tested for their ability to inhibit SSF126-dependent induction of cyanide-resistant respiration in the mycelial cells. All the flavonoids (flavone, flavanone, naringenin, quercetin, sakuranetin, and apigenin) inhibited the induction of the respiration at a concentration of 1 mM. The flavonoids (1 mM) tested had no effect on oxygen consumption by the cyanide-sensitive mycelial cells (data not shown). Selected flavonoids (flavone, flavanone, and naringenin) dose-dependently inhibited the induction of the respiration in the cells (Fig. 6). Among these, flavone was most effective for the inhibition; the EC_{50} value of flavone was 37.1 μM, the EC_{50} value of flavanone was 110 μM, and the EC_{50} value of naringenin was 156.2 μM. These observations indicated that flavonoids existing naturally as plant components have the ability to inhibit SSF126-dependent induction of cyanide-resistant respiration.

To determine the effect of flavone on expression of the 40-kDa protein, the mycelial cells were pulse-labeled with radioactive methionine in the presence of SSF126 and appropriate concentrations of flavone (Fig. 7). The expression of the protein, absent in untreated cells (Fig. 7, lane 1), was obvious in the mitochondrial protein profile for the cells treated with SSF126 alone (lane 2). In mitochondria from the cells incubated with SSF126 and flavone, the expression of the protein was dose-dependently suppressed by flavone (lanes 3 to 5); 60 μM flavone completely inhibited its expression.

DISCUSSION

We demonstrated that a 40-kDa protein was specifically induced by SSF126, an inhibitor of the cytochrome bc_1 segment, and that this protein was detected exclusively in the mitochondrial fraction of the mycelial cells of *P. grisea*.

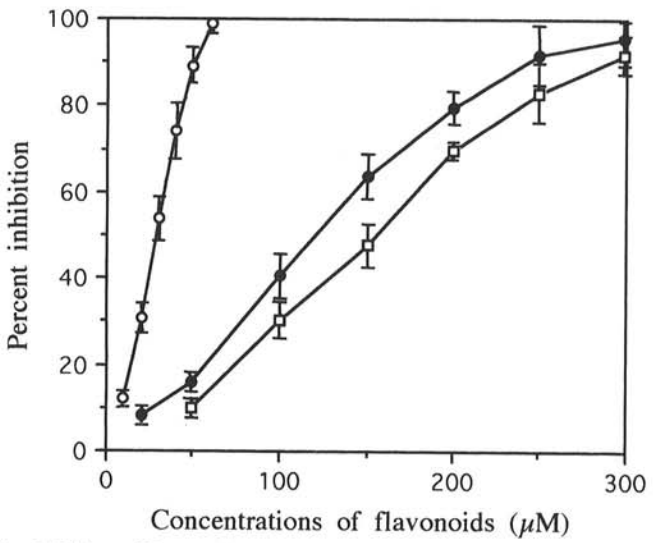

Fig. 6. Effects of flavone, flavanone, and naringenin on induction of cyanide-resistant respiration in *Pyricularia grisea*. Open circles show the percent inhibition for flavone, closed circles show the percent inhibition for flavanone, and squares show the percent inhibition for naringenin. Control activity was 19.8 nmol of O_2/min/ 20 mg of fresh weight mycelia. Results are means of three separate experiments with bars showing the standard errors.

In microorganisms and higher plants, the alternative oxidases responsible for cyanide-resistant respiration are encoded by nuclear genes (11,25,26,30). Judged from amino acid sequences deduced from nucleotide sequences, the gene products are expected to be precursors of alternative oxidases. The import of precursor polypeptides, which are synthesized on free cytoplasmic polysomes, into mitochondria is dependent on the electrochemical potential across the mitochondrial inner membrane (6).

In *P. grisea*, accumulation of a pulse-labeled 41.4-kDa polypeptide in place of the 40-kDa protein was observed when the mycelial cells were incubated with SSF126 and FCCP. The accumulation of this polypeptide could have been due to blockage of translocation into mitochondria for processing to the corresponding mature protein because FCCP disrupts the electrochemical potential across the membrane needed for translocation, suggesting that the accumulated polypeptide is the precursor of 40-kDa protein. The polypeptide was detected exclusively in the mitochondrial fraction, with none observed in the cytosolic fraction. As precursor polypeptides bind specifically to proteinaceous receptors on the surface of mitochondria (6,33), the accumulated 41.4-kDa polypeptide was expected to be bound to the outer membrane of the mitochondria.

In *P. grisea* mycelial cells, SSF126-dependent induction of cyanide-resistant respiration was completely inhibited by incubation with FCCP. At 10 min after removal of FCCP, the respiratory activity of the cells increased up to more than 90% of the activity in cells treated with SSF126 alone for 1 h. In contrast, cyanide-resistant respiration was not detected in the cells within 10 min after treatment with SSF126. The induction of cyanide-resistant respiration was also blocked by 1,10-phenanthroline, which inhibits the mitochondrial processing peptidase (data not shown) (15,19,24). These suggested that accumulation of the precursor polypeptide is accomplished without converting to the corresponding mature protein responsible for cyanide-resistant respiration in the presence of FCCP, and that the precursor can be converted to the mature protein within 10 min after removal of FCCP. Furthermore, the expression of the 40-kDa protein paralleled SSF126-dependent induction of cyanide-resistant respiratory activity in the cells (Figs. 2 and 4). From these, it appeared feasible that the pulse-labeled 40-kDa protein was the alternative oxidase responsible for cyanide-resistant respiration in *P. grisea*, and that the 41.4-kDa precursor polypeptide was synthesized de novo, imported into mitochondria, and subjected to processing for conversion to the corresponding mature protein (40 kDa).

Our findings suggested that SSF126 is capable of inducing O_2^- generation by blockage of electron flux through the bc_1 segment, and that O_2^- is involved in the mechanism of induction of cya-

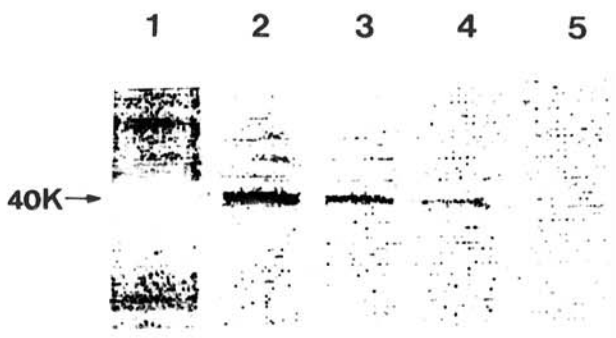

Fig. 7. Effect of flavone on a labeled mitochondrial protein. Mycelial cells were labeled with [^{35}S]-methionine in the presence of (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide (SSF126) and the indicated concentrations of flavone. Aliquots, each of 15 μg of protein, were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 to 20% polyacrylamide gradient gel). Lane 1: untreated; lane 2: SSF126; lane 3: SSF126 + 20 μM flavone; lane 4: SSF126 + 40 μM flavone; and lane 5: SSF126 + 60 μM flavone.

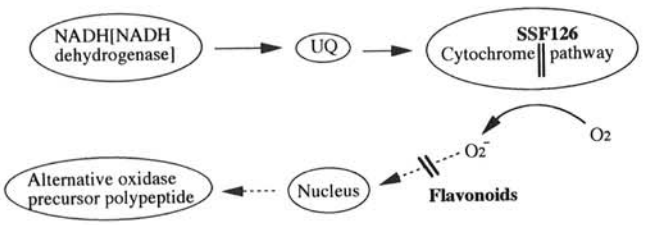

Fig. 8. Hypothetical diagram for a mechanism of control of rice blast by (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide (SSF126). UQ = ubiquinone.

nide-resistant respiration in *P. grisea*. Some microorganisms have the ability to induce cyanide-resistant respiration in response to respiratory inhibitors (13,18,20). Minagawa et al. (17) suggested that O_2^- is involved in antimycin A-dependent induction of the respiration in the yeast *Hansenula anomala*. Although O_2^- seems to be a common intermediate in the mechanism of induction of cyanide-resistant respiration in microorganisms, the mechanism by which it is capable of initiating the induction is still unknown.

It is well known that flavonoids are widespread in the plant kingdom and work as scavengers of oxygen radicals (23,32). In fact, flavone was capable of scavenging O_2^- generated by the submitochondrial particles of *P. grisea* in the presence of SSF126. All the flavonoids tested showed inhibitory effects on SSF126-dependent induction of cyanide-resistant respiration in the mycelial cells of *P. grisea*. Flavonoids work as respiratory inhibitors in bovine heart mitochondria (8,9) and also inhibit fungal growth (10,31). However, none of the flavonoids tested here had any effect on the cyanide-sensitive oxygen consumption by the mycelial cells as noted above. Accordingly, the inhibitory effect of the flavonoids on the induction was not due to the blockage of electron transport to ubiquinone. Furthermore, flavone dose-dependently blocked the expression of the 40-kDa protein induced by SSF126 in *P. grisea*. The inhibitory effect of flavone on the expression of this protein paralleled that on the SSF126-dependent induction of cyanide-resistant respiratory activity in the mycelial cells. These also suggested that the 40-kDa protein is an alternative oxidase and that flavonoids block SSF126-dependent induction of cyanide-resistant respiration through scavenging O_2^- generated by blocking electron flux through the bc_1 segment in the mitochondrial respiratory chain in *P. grisea*.

These findings offered an explanation for the control of rice blast by SSF126 (Fig. 8). *P. grisea* mycelial cells were capable of generating O_2^- at blockage of the cytochrome-mediated respiratory pathway by SSF126. In response to the O_2^- generated, the fungus could switch from respiration via the cytochrome-mediated pathway to that via the alternative pathway to survive. On the contrary, it is feasible that flavonoids in plants block the induction of the cyanide-resistant pathway by scavenging O_2^- and lead to complete suppression of fungal respiration. If this occurs, SSF126 would be able to control rice blast caused by *P. grisea* in conjunction with natural components of rice plants.

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