A Point Mutation in the Two-Component Histidine Kinase *BcOS-1* Gene Confers Dicarboximide Resistance in Field Isolates of *Botrytis cinerea*

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Accepted for publication 24 September 2001.

ABSTRACT

Oshima, M., Fujimura, M., Banno, S., Hashimoto, C., Motoyama, T., Ichiishi, A., and Yamaguchi, I. 2002. A point mutation in the two-component histidine kinase *BcOS-1* gene confers dicarboximide resistance in field isolates of *Botrytis cinerea*. *Phytopathology* 92:75-80.

Partial DNA fragments of *Botrytis cinerea* field isolates encoding the putative osmosensor histidine kinase gene (*BcOS1*) were cloned by polymerase chain reaction amplification and the predicted amino acid sequences were compared between dicarboximide-sensitive and resistant field isolates. The predicted *BcOS1p* is highly homologous to osmosensor histidine kinase *OS1p* from *Neurospora crassa* including the N-terminal six tandem repeats of approximately 90 amino acids. Four dicarboximide-resistant isolates of *B. cinerea* (Bc-19, Bc-45, Bc-682, and Bc-RKR) contained a single base pair mutation in their *BcOS1* gene that

resulted in an amino acid substitution in the predicted protein. In these resistant isolates, codon 86 of the second repeat, which encodes an isoleucine residue in sensitive strains, was converted to a codon for serine. The mutation of *Botrytis* field resistant isolates was located on the second unit of tandem amino acid repeats of *BcOS1p*, whereas the point mutations of the fifth repeat of *OS1p* confer resistance to both dicarboximides and phenylpyrroles and also osmotic sensitivity in *Neurospora crassa*. These results suggest that an amino acid substitution within the second repeat of *BcOS1p* is responsible for phenotypes of field resistant isolates (resistant to dicarboximides but sensitive to phenylpyrroles, and normal osmotic sensitivity) in *B. cinerea*.

Additional keywords: fungicide, glycerol, two-component signal transduction.

Gray mold, caused by *Botrytis cinerea*, is an economically important disease in many kinds of vegetables, grapes, and flower crops. The dicarboximide fungicides, iprodione and procymidone, are used to control *B. cinerea* (25). However, the spread of *B. cinerea* strains resistant to dicarboximides has greatly reduced the usefulness of the chemicals in many countries (4,15,16). Despite many years of investigation, the mechanism of dicarboximide resistance in field isolates of *B. cinerea* is not known yet (5). The phenylpyrrole fungicides, such as fludioxonil and fenpiclonil, are a new class of fungicides with chemical structures quite different from those of the dicarboximides (9,22). However, the dicarboximides and phenylpyrroles induce similar morphological changes of the germination tubes, and their fungicidal toxicity is reverted by piperonyl butoxide and α -tocopherol in *B. cinerea* and other fungi (8,18). These fungicides stimulate glycerol synthesis in the mycelium of *Neurospora crassa* (8,14,24). Under laboratory conditions, dicarboximides easily select resistant strains in many kinds of fungi including *B. cinerea*. In general, most dicarboximide-resistant laboratory mutants acquire high resistance not only to dicarboximides but also to aromatic hydrocarbons and phenylpyrroles, and they are hypersensitive to osmotic stress (3,11,17). However, high-level resistant strains of *B. cinerea* have seldom been obtained in the field. Only moderately resistant strains without osmotic-sensitive phenotypes are recovered from the field, causing difficulties with disease control (4,17). In addition, dicarboximide-resistant field isolates of *B. cinerea* show cross-resistance to aromatic hydrocarbons but not to phenylpyrroles (9,18). Thus, fludioxonil controls gray mold effectively even in dicar-

boximide-resistant fields (9). These observations suggest that the resistance mechanism of field isolates differs from that of laboratory isolates.

Faretra and Pollastro have isolated and characterized phenylpyrrole-resistant mutants from *B. cinerea* (6). Genetic analysis indicates that most mutants resistant to phenylpyrroles are caused by the mutations at the *Daf1* locus responsible for dicarboximide field resistance. Their results suggest that *Daf1* confers phenotypes of both field resistance and laboratory resistance in *B. cinerea*. The *Daf1* gene is polymorphic; at least five classes of alleles have been identified for their effects on phenotype response to dicarboximides and phenylpyrroles in *B. cinerea*. Similar results were published by Hilber et al. (13).

We have a working hypothesis that *Daf1*, which confers the dicarboximide resistance in field isolates of *B. cinerea*, might be a homologue of the *os-1* gene of *Neurospora crassa*. In *Neurospora crassa*, osmotic-sensitive mutants, *os-1*, *os-2*, *os-4*, and *os-5*, are resistant to dicarboximides, aromatic hydrocarbons, and phenylpyrroles (8,11). Among them, only the *os-1* gene of *Neurospora crassa* is as highly polymorphic as the *Daf1* gene of *B. cinerea* (7). All *os* mutants other than the *os-1* strains are highly resistant to fungicides including phenylpyrroles, and their phenotypes are similar to each other. In contrast, some *os-1* mutant alleles of *Neurospora crassa* remain sensitive to fludioxonil. In addition, osmotic sensitivity in *os-1* mutants is not directly linked with fungicide resistance. All *os-1* mutants are sensitive to osmotic stress, because they have been isolated as osmotic-sensitive mutants but not as dicarboximide-resistant mutants. Thus, it might be possible that specific mutations within the *os-1* gene confer the field resistant phenotype. Furthermore, most mutations in laboratory resistant isolates of *B. cinerea* are mapped on the *Daf1* locus (6). Similarly, among the *os* mutants, *os-1* mutants are most easily obtained as dicarboximide-resistant mutants (10).

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Publication no. P-2001-1102-01R

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The *os-1* gene encodes a protein with homology to two-component histidine kinase (1,29). This protein is involved in the osmotic signal transduction pathway and needed for adaptation to high osmolality, although the kinase activity of the *os-1* gene product (OS1p) has not been established yet. Two-component histidine kinase SLN1p acts as osmosensor in *Saccharomyces cerevisiae* (12,20,26). However, N-terminal region of OS1p is quite different from that of SLN1p. OS1p has six repeats of about 90 amino acids at the N-terminal domain, the function of which was unknown. In our previous work, we identified the *os-1* mutations as conferring fungicide resistance and osmotic sensitivity (23). The null mutation of the *os-1* gene conferred high resistance to fungicides. In contrast, single amino acid substitutions on the fifth unit of six tandem repeats of OS1p conferred high sensitivity to osmotic stress but low resistance to fungicides.

In this work, we cloned the *os-1* homologue gene (*BcOS1*) from several field isolates of *B. cinerea* and compared their sequences to clarify the possibility that *BcOS1* gene mutations are responsible for field resistance to dicarboximides in *B. cinerea*.

MATERIALS AND METHODS

Strains, growth conditions, and antifungal assay. *B. cinerea* strains, dicarboximide-sensitive isolates (Bc-16, Bc-56, Bc-RKS, and Bc-BYS1), and dicarboximide-resistant isolates (Bc-19, Bc-45, Bc-682, and Bc-RKR) were obtained from several fields in Japan (Table 1). Strains Bc-SMX and Bc-ROV were selected on medium containing dicarboximide fungicides after UV light irradiation from strains Bc-16 and Bc-RKS, respectively. *B. cinerea* was maintained on potato dextrose agar (PDA) medium at 18°C under black light blue (BLB) light (FL20S, 20 W; Toshiba Co., Tokyo, Japan). To determine sensitivity to fungicides and osmotic stress, mycelial disks were inoculated on PDA containing fungicides or NaCl in petri dishes and cultured at 20°C for 3 days. The mean colony diameter minus the diameter of the inoculation disk was measured and expressed as the percentage of the mean colony diameter of untreated control. Fifty percent effective dose values were determined by regressing the relative growth against the log of the fungicide concentration. All experiments were repeated twice. To isolate the conidia, the mycelial disks were cultured on VNC medium (Vogel's N medium [31] containing 1.5% [wt/vol] sucrose, 0.25% [wt/vol] yeast extract, 0.1% [wt/vol] casamino acids, 0.5% [vol/wt] malt extract, and 1.5% [wt/vol] agar) at 20°C for 7 days under BLB light. For suspension culture, conidia (1×10^6 conidia per ml) of *B. cinerea* were inoculated into liquid VNC medium and cultured at 22°C for 24 h with a reciprocal shaker (125 rpm).

For determination of the glycerol content in cells of strains Bc-56, Bc-45, and Bc-SMX, the mycelial suspension was incubated

in liquid VNC medium with fungicides or 4% (wt/vol) NaCl for 4 h at 22°C. The incubated mycelium was then collected by filtration and extensively sonicated in distilled water, and the glycerol in the mycelium was extracted at 80°C for 5 min. After centrifugation, the glycerol concentration in the supernatant was measured by the UV-glycerol assay procedure (Roche Diagnostics KK, Tokyo, Japan) (8). After incubation, the cultures were vacuum-filtered and the same mycelia were dried for 24 h at 105°C for dry weight determination.

Cloning of *BcOS1* gene of *B. cinerea*. Mycelia of eight field isolates were harvested by filtration and frozen in liquid nitrogen. The genomic DNA of the *B. cinerea* strains was isolated with a fungal DNA miniprep kit (EZNA; Omega Biotek, Doraville, GA) according to the manufacturer's directions. The resultant nucleic acid was used as template for the polymerase chain reaction (PCR) and gene cloning. Two primers, H1A (CA[T/C]GAI[A/T/C]TI-[C/A]GIACICC) and N1A (GT[A/G]AA[T/C]TTIAIGC[A/G]TT) were synthesized as described by Alex et al. (1). These two primers correspond to the H box consensus H(E/D)(M/I/L/F)RTP and N box NLV(S/G)NA(I/V)KFT of histidine kinase, respectively. Another mix primer, BcOS7 (TGGAARCAAYCARAA-RGCNAAYGAR) was newly designed for the amino acid sequence WKHQKANE, which corresponds to the initial sequence of the first unit in the tandem 90-amino acid repeats of *Neurospora crassa os-1* (1,29) and *Nectria FIK* (U61838). Three primers, BcOS1R (TTGAAGACGGTCCTATAA), BcOS4R (CA-TACCAATGATACCGTTTCAT), and BcOS5 (GAGGCTTTCC-AAAAAGCTAT), were also synthesized based on the DNA sequencing of PCR fragments. These primers were used to amplify the genomic DNA from *B. cinerea*. All primers were purified by a Sep-Pak C18 column. The PCR mixtures contained 0.5 µM of each primer, 1 µg of genomic DNA, and PremixTaq (ExTaq Version, Takara Shuzo Co., Kyoto, Japan) in a total volume of 50 µl. After being preheated at 95°C for 10 min, the reaction mixtures were cycled 20 times at 94°C (1 min), 48°C (30 s), followed by a 5-min extension at 72°C. DNA fragments amplified by PCR reaction were cloned into TA cloning vector (pT7Blue-2; Novagen, Madison, WI).

DNA sequence. An analysis of the DNA sequence was conducted by an auto sequence system (Model 373A; Perkin-Elmer Applied Biosystems, Foster City, CA), the PCR cycle sequencing protocol, and fluorescent dye terminator dideoxynucleotides (Perkin-Elmer Applied Biosystems). The homology and alignment of the nucleotide sequences with those in the gene and protein databases were performed with GENETYX software (Software Development Co., Tokyo, Japan).

Southern blot analysis. Genomic DNA of Bc-56 strain of *B. cinerea* was digested by several restriction enzymes (*EcoRI*, *HindIII*, *XbaI*, *XhoI*, or *EcoRV*) and loaded for Southern blot

TABLE 1. Characterization of strains of *Botrytis cinerea*

Strains	Sensitivity to fungicide (µg/ml) ^a		Sensitivity to osmotic stress ^b		Region of Japan
	Iprodione	Fludioxonil	4% NaCl	2% NaCl	
Dicarboximide sensitive					
Bc-16	0.3	0.01	4	0	Hyogo
Bc-56	0.3	0.006	13	8	Gunma
Bc-RKS	0.4	0.006	4	0	Saitama
Bc-BYS1	0.3	0.006	32	8	Kochi
Dicarboximide resistant from field					
Bc-19	3.9	0.006	35	23	Kagawa
Bc-45	2.4	0.018	33	12	Gunma
Bc-682	5.1	0.05	40	7	Hyogo
Bc-RKR	1.4	0.006	35	23	Saitama
Dicarboximide resistant in laboratory					
Bc-ROV	>25	>25	100	100	...
Bc-SMX	>25	>25	96	89	...

^a Concentration to inhibit radial growth at the 50% level.

^b Percentage of growth inhibition on medium containing NaCl.

analysis. These enzymes do not digest DNA fragments encoding the amino acid repeats. The DNA was transferred to nylon membranes (Hybond-N+; Amersham Pharmacia Biotech, Tokyo, Japan). PCR fragments encoding the amino acid repeats of the *BcOS1* gene were digoxigenin labeled by random priming and hybridized and detected according to the method described in the manufacturer's recommendations (DIG DNA Labeling and Detection Kit; Roche Diagnostics).

Chemicals. Iprodione was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Fludioxonil was contributed by C. Pillonel (Syngenta Crop Protection AG, Basel, Switzerland). The chemicals were stored in the dark at 100 mg/ml in dimethylsulfoxide at 4°C.

RESULTS

Characterization of dicarboximide-resistant field isolates of *B. cinerea*. Four field isolates, Bc-16, Bc-56, Bc-RKS, and Bc-BYS1, were sensitive to both iprodione and fludioxonil; their growth was inhibited by iprodione and fludioxonil at a concentration of 0.3 to 0.4 µg/ml and 0.01 to 0.006 µg/ml, respectively

(Table 1). In comparison, four strains, Bc-19, Bc-45, Bc-682, and Bc-RKR, grew on medium containing 0.4 µg/ml of iprodione, but their growth was completely inhibited at 25 µg/ml, indicating moderate resistance to iprodione. All the dicarboximide field resistant isolates tested were sensitive to fludioxonil. In contrast, two mutants, Bc-ROV and Bc-SMX, that were isolated in our laboratory were highly resistant to both iprodione and fludioxonil. In addition, the growth of these two laboratory mutants was inhibited even on medium containing 2% (wt/vol) NaCl, whereas the field isolates did not exhibit such high osmotic sensitivity. On medium containing 4% (wt/vol) NaCl, field resistant isolates were slightly more sensitive to osmotic stress than the sensitive ones, except for BcBYS1 isolate. Although these field strains were collected from different areas in Japan, the phenotypes of the resistant strains were very similar.

Glycerol accumulation induced by iprodione and fludioxonil. Induction of glycerol synthesis was observed in Bc-56, the sensitive strain of *B. cinerea*, through the treatment of iprodione, even at 1 µg/ml. The resistant field isolate Bc-45 did not accumulate glycerol in the medium containing 1 µg of iprodione per ml, but stimulation of glycerol synthesis did occur at a higher concen-

Fig. 1. Gene structure of a partial *BcOS1* gene and alignment of six units of 90 amino acid repeats. **A**, Schematic representation of the *BcOS1* coding region in relation to gene structure. Arrows indicate the primer position used for polymerase chain reaction cloning of the *BcOS1* gene. Bold arrow indicates the fragment used for Southern analysis. Inverted triangles indicate the locations of predicted introns. Restriction enzyme sites are denoted by the letters P, *PvuII*; B, *BglIII*; K, *KpnI*; and F, *FbaI*. **B**, Alignment of six units of 90 amino acid repeats in the wild-type *BcOS1* gene. The lack of a corresponding amino acid position is indicated by hyphens. Sites of amino acid substitutions of BcOS1p are written in bold.

tration of iprodione (25 µg/ml). Fludioxonil induced the glycerol accumulation in both field isolates at concentration of 0.1 µg/ml. Conversely, both iprodione and fludioxonil did not stimulate glycerol synthesis in resistant laboratory isolate Bc-SMX at 25 µg/ml. In response to 4% (wt/vol) NaCl, both Bc-56 and Bc-45 strains accumulated glycerol at similar levels, whereas the laboratory resistant Bc-SMX strain accumulated less glycerol.

PCR cloning of two-component histidine kinase *BcOS1* gene. Genomic DNA of the Bc-56 strain was isolated and used for PCR cloning of the *os-1* homologue gene (*BcOS1*) with primers H1A and N1A. The amplification products, approximately 0.4 kb of DNA fragments, were cloned and sequenced. The deduced amino acid sequence of the partial putative histidine kinase showed high homology to that of *Neurospora crassa* OS1p. Based on the sequencing result of kinase domain, we designed two primers, BcOS1R and BcOS4R, to serve as the reverse primers to clone the N-terminal region containing the amino acid repeat domain. A second PCR was conducted with BcOS7 as the forward primer and BcOS1R as the reverse primer. The resulting PCR products were purified and used for nest PCR with primers BcOS7 and BcOS4R (Fig. 1A). The nested PCR products were cloned and sequenced. The sequencing results revealed that the cloned DNA was highly homologous to the *Neurospora crassa os-1* gene. In the same way, we designated a new forward primer, BcOS5, based on the sequencing data, which was completely homologous to the N-terminal of the amino acid repeats of the *BcOS1* gene. Finally, we used these two primers, BcOS5 and BcOS1R, which are completely homologous to the *BcOS1* gene, and cloned a partial *BcOS1* gene from the Bc-56 strain into pT7Blue-2 as pBcOS56 (Fig. 1A).

Approximately 2.2 kb of fragment of pBcOS56 was sequenced on both strands. Nucleotide sequence analysis indicated a predict-

ed open reading frame of approximately 1.9 kb that was interrupted by three introns. Introns were deduced by similarity to fungal intron consensus sequences. The predicted introns ranged in size from 61 to 203. The predicted 619 amino acids encoded by the partial *BcOS1* gene are very similar to that of OS1p of *Neurospora crassa* and consists of a domain of six 90-amino-acid repeats and a histidine kinase domain (Fig. 1A). Figure 2 shows an amino acid sequence alignment of BcOS1p with the OS1p of *Neurospora crassa* (1,29), FIKp of *Nectria haematococca* (U61838), and COS1p of *Candida albicans* (2,21). The overall amino acid sequence identity of the cloned region of BcOS1p with those of OS1p, FIKp, and COS1p is approximately 92, 87, and 59%, respectively. In addition, Sln1p from *S. cerevisiae* shared about 30% identity (data not shown). BcOS1p contains presumed phosphoryl group acceptor H in the histidine kinase domain, identical to the motif defined for bacterial and yeast histidine kinase module (Fig. 2). BcOS1p contains a 90-amino-acid motif that is repeated five times, followed by a truncated 60-amino-acid repeat (Fig. 1B). When the individual repeat units were compared with each other, a high degree of homology was revealed, ranging from 41 to 68% (Fig. 1B). By Southern blot analysis of *Eco*RI, *Hind*III, *Xba*I, *Xho*I, or *Eco*RV-digested genomic DNA of strain Bc-56 probed with the 1.4-kb partial gene fragment (Fig. 1A), we could detect only single signals (data not shown), suggesting that this gene is a single copy gene.

Identification of the point mutation in *BcOS1* gene of the dicarboximide-resistant isolates. The nucleotide sequences of the *BcOS1* gene fragments isolated from four dicarboximide-resistant field strains were determined and compared with those of four dicarboximide-sensitive strains. At least three independent PCR cloning processes were done for each genomic DNA to distinguish the original mutations from possible PCR mutations.

<i>BcOS1</i>	1:WKHKQKANEAFQKALREIGEIVTAVARGDLSKKVQIHSVEMDPEITTFKRVINTMMDQLQIFSSSEVSRVAREVGTGEGILGGQAKISGVDTGT
<i>os-1</i>	:.....RMN.....T.....V.....Q.E.....
<i>FIK</i>	:.....TM..RMNT.....T..A.....T.A.....L.....R.G.....
<i>COS1</i>	:E.SKN..K...Q..S...TV..IS..M.....E..T..N....LKV.IT.....T.AN..TK..T..ANGE-.....ND.SV.I
<i>BcOS1</i>	91:WKELTDNVNVMAQNLTQVREIASVTTAVAHGDLTQKIERPAQGEILQLQQTINTMVDQLRTFAAEVTRVARVDVGTEGILGGQAEIEGVQGM
<i>os-1</i>	:.....K.....K.....S.....DV.....
<i>FIK</i>	:.....K.....R.....S.....M.....DVG.....
<i>COS1</i>	:RS.....I..L...N....D..R..K...SR..NVH.....R.....F..SK.....VL.....L..N.E.I
<i>BcOS1</i>	183:WNTLIVNVNAMANNLTQVRDIAIVTTAVAKGDLTQKVQAECKGEIKQLKETINSMVDQLQQFAREVTKIAREVGTGEGRLGGQATVHDVEGT
<i>os-1</i>	:..E.T.....IK.....R...FE..K.....Q..
<i>FIK</i>	:..D.T.....IK.....D.R...FD..S.....
<i>COS1</i>	:EE.TD.....L.....N..N.....SK..T.D....LD..L...Q...R..N..LA..TLS....L.IW...N.Q...A
<i>BcOS1</i>	275:WRDLTENVNGMAMNLTQVREIAKVTTAVARGDLTKKIEVEVQGEIASLKDTINTMVDRLSTFAFEVSKVAREVGTGTLGGQAQVDNVEGK
<i>os-1</i>	:.....K.....G.....LD..N.....G.....
<i>FIK</i>	:.....K.....G...K...LE..N...Q...G...V.....A.....
<i>COS1</i>	:.KQV....L..T...N...S..T....H...SQ..D.HA...LQ..N...K...S..QL..S.....QD..IN.K..I...SD.D.L
<i>BcOS1</i>	367:WKDLTENVNTMARNLTQVRGISTVTQAIANGDMSQKIEVAAAGEILILKETINNMDRLSIFSNVQRVAKDVGVGDKMGQADVAGIGGR
<i>os-1</i>	:.....S...S.....R...EAKG.....C.....I.....LK..
<i>FIK</i>	:.....S...V...S.....K.G.N...QV.....S.CY.....A.....LD..
<i>COS1</i>	:.....
<i>BcOS1</i>	459:WKEITTDVNTMANNLTQVRAFGDITNAATDGDFTKLITVEASGEMDELKRKINQMVYNLRDSIQRNTLAREAAEFANRTKSEFLANMSHEI
<i>os-1</i>	:.....A.....VE.....K.....Q.....L..K.....
<i>FIK</i>	:.....S.....S...L.....VD.....IS.....Q.....L..K.....
<i>COS1</i>	:.....SN.....S...S...AQ..A.....RF.....A..T.....F...E..L...A.....L..SA.....
<i>BcOS1</i>	551:RTPMNGIIGMTQLTLDLTQYQREMLNIVHNLANSLLTIIDDILDLSKIEANRMIMEEIPYTLIGTVF
<i>os-1</i>	:.....NS.....R..VI.....R...
<i>FIK</i>	:.....N.....R..VI.....R...
<i>COS1</i>	:...L.....S...E.....S.....I.....TV.Q.DFS.R...

Fig. 2. Amino acid sequence alignment of partial BcOS1p with the corresponding region of OS1p (*Neurospora crassa*), FIKp (*Nectria haematococca*), and COS1p (*Candida albicans*). Amino acid identities between BcOS1p and others are indicated by a dot. The lack of a corresponding amino acid position is indicated by a hyphen. The asterisk indicates a putative phosphoryl group acceptor. The triangle (▲) indicates the site of the amino acid substitution of *Botrytis* BcOS1p. Sites of amino acid substitutions of *Neurospora crassa* OS1p are written in bold (23).

In strains Bc-19, Bc-45, Bc-682, and Bc-RKR, a T-to-G transition occurred at codon 86 of the second amino acid repeat (Table 2), resulting in a serine (AGC) being substituted for isoleucine (ATC). The amino acid isoleucine at codon 86 was conserved in all strains sensitive to dicarboximides. This amino acid change was located on the second unit of six tandem repeats of 90 amino acids at the N-terminal of BcOS1p. Alignment of the second unit of BcOS1p with OS1p, FIKp, and COS1 indicates that the amino acid at codon 86 was restricted in isoleucine or valine (Fig. 2). A comparison of the amino acid sequence in the second unit with those in other units of BcOS1p revealed that the amino acid residue at codon 86 in each unit was restricted in isoleucine or valine (Fig. 1B). These data strongly suggest that the change from isoleucine to serine confers field resistance to dicarboximides in *B. cinerea*.

DISCUSSION

In *B. cinerea*, the resistant strains isolated from several fields in Japan were moderately resistant to iprodione but sensitive to fludioxonil, and their phenotypes were very similar to each other (Table 1). The strains highly resistant to dicarboximides and phenylpyrroles that were isolated on the medium containing dicarboximides after UV irradiation displayed a clear osmotic-sensitive phenotype on the medium containing 2% (vol/wt) NaCl. These observations indicate differences between field resistance and laboratory resistance and were consistent with the results previously reported by Leroux et al. (17). Iprodione as well as fludioxonil stimulated glycerol synthesis in the wild-type strains of *B. cinerea*. In response to osmotic stress, the wild-type strain accumulated glycerol probably to counterbalance high external osmolality. Glycerol accumulation induced by iprodione was not observed in the highly resistant strain (laboratory resistant strain), whereas the moderately resistant strain (field resistant strain) accumulated glycerol only at a high dose of iprodione. That the levels of resistance in *Botrytis* strains correlate with the stimulation of glycerol synthesis by fungicide treatments suggests that abnormal glycerol accumulation led to cell death. Similar results have been obtained in *Neurospora crassa*. However, increased glycerol content was not directly linked with the fungal toxicity of dicarboximides and phenylpyrroles, because both fungicides did not induce glycerol biosynthesis in the fungicide-sensitive *cut* mutant strain of *Neurospora crassa* (8). These data suggest that dicarboximides and fludioxonil interfere with the osmotic signal transduction pathway, resulting in stimulation of glycerol biosynthesis, but that abnormal glycerol accumulation is not essential for their fungal toxicity.

In this study, we cloned a two-component histidine kinase *BcOS1* gene fragment from *B. cinerea* as a homologue of the *Neurospora crassa* osmosensor *os-1* gene. Although the *BcOS1* sequence is homologous with the kinase domain of the two-

component signal protein, the N-terminal end of BcOS1p is quite different from that of Sln1p of *S. cerevisiae*. BcOS1p possesses the tandem repeats of 90 amino acids and the putative autophosphorylated histidine residue in the H box (Fig. 2), as in the filamentous fungal histidine kinase *Neurospora* OS1p, *Nectria* FIKp, and *Candida* COS1p. There was no predicted membrane-spanning domain in BcOS1p, OS1p, FIKp, and COS1p in the N terminus, whereas Sln1p contains two. A typical histidine kinase contains an N-terminal input domain and a C-terminal transmitter domain. The input domain of the sensory histidine kinase detects environmental variables and selectively promotes autophosphorylation of a histidine residue within its transmitter domain. These results suggest that BcOS1p and OS1p regulate osmotic signal transmission in a manner different from that of Sln1p of *S. cerevisiae*. The repeat unit motif of BcOS1p and related kinases showed a structural similarity to that of the bacterial histidine kinase Htr1p, with a high probability of forming a coiled-coil structure, a feature that might be important in the transduction of extracellular signals (19).

In comparing the *BcOS1* gene of the dicarboximide-resistant isolates with those of the sensitive ones, we found a single amino acid substitution in the second unit of tandem amino acid repeats of BcOS1p. In four resistant field isolates, codon 86 of the second repeat was substituted with serine for isoleucine. Amino acid change from isoleucine (hydrophobic amino acid) to serine (hydrophilic amino acid) in field resistant strains is predicted to affect the function of the amino acid repeat domain. The 86th codon of each amino acid repeat of BcOS1p was restricted in isoleucine or valine (Fig. 1B). A comparison of the BcOS1p with other *os-1* homologue proteins revealed a conserved isoleucine or valine in position 86 (Fig. 2), suggesting that there is considerable constraint on this residue. These results strongly suggest that this amino acid substitution of serine for isoleucine is responsible for dicarboximide resistance in the field. A practical diagnostic method of detecting the population of dicarboximide-resistant strains in the field with a DNA probe is now under construction. Preliminary data show that all strains containing a mutation from isoleucine to serine are resistant to dicarboximide without exception. However, some isolates with isoleucine at codon 86 in the second unit are resistant to dicarboximides, suggesting the possibility of other types of resistant strains in the field.

The mutations conferring dicarboximide resistance were limited to codon 86 in the second amino acid repeat of BcOS1p in the field strains of *B. cinerea*. In contrast, we previously identified the point mutations of *Neurospora crassa os-1* mutant alleles; their mutations were located on the fifth amino acid repeat of OS1p, proline from leucine at codon 89, valine from alanine at codon 42, and arginine from glycine at codon 44 (23) (Fig. 2). A possible explanation for the reduced variation in mutations of the field strains is that the mutations in other codons might interfere with the fitness of mutants and impose a selective disadvantage under field conditions. The mutations found in *os-1* genes of *Neurospora* confer not only dicarboximide resistance but also increased sensitivity to osmotic stress. Osmotic sensitivity in laboratory resistant strains possibly affects the stability and virulence in field conditions. Even in the field resistant strains, negative correlations between survival of sclerotia and resistance to dicarboximides are found in *B. cinerea* (27,28). It is known that the population of the resistant isolates decreases rapidly after discontinuous application of dicarboximides (30). These observations suggest that most of the mutations within the *BcOS1* gene affect virulence or fitness in *B. cinerea* under field conditions.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (1066052) from the Ministry of Education, Science, Sports, and Culture of Japan. We thank C. Pillonel for his gift of fludioxonil.

TABLE 2. Amino acid substitution of BcOS1p in field strains of *Botrytis cinerea*

Strain	Codon 86 in second repeat ^a	Amino acid at codon 86
Dicarboximide sensitive		
Bc-16	ATC	Isoleucine
Bc-56	ATC	Isoleucine
Bc-RKS	ATC	Isoleucine
Bc-BYS1	ATC	Isoleucine
Dicarboximide resistant		
Bc-19	AGC	Serine
Bc-45	AGC	Serine
Bc-682	AGC	Serine
Bc-RKR	AGC	Serine

^a 86th Codon in second amino acid repeat of predicted *BcOS1* gene product.

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