J. Microbiol. Biotechnol. (2008), **18**(1), 15–22

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of Mutations in *AlHK1* Gene from *Alternaria longipes*: Implication of Limited Function of Two-Component Histidine Kinase on Conferring Dicarboximide Resistance

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Received: May 16, 2007 / Accepted: July 14, 2007

Four series (S, M, R, and W) of *Alternaria longipes* isolates were obtained based on consecutive selection with Dimethachlon (Dim) and ultraviolet irradiation. These isolates were then characterized according to their tolerance to Dim, sensitivity to osmotic stress, and phenotypic properties. All the selected Dim-resistant isolates showed a higher osmosensitivity than the parental strains, and the last generation was more resistant than the first generation in the M, R, and W series. In addition, the changes in the Dim resistance and osmotic sensitivity were not found to be directly correlated, and no distinct morphologic characteristics were found among the resistant and sensitive isolates, with the exception of the resistant isolate K-11. Thus, to investigate the molecular basis of the fungicide resistance, a group III two-component histidine kinase (HK) gene, *AlHK1*, was cloned from nineteen *A. longipes* isolates. AlHK1p was found to be comprised of a six 92-amino-acid repeat domain (AARD), HK domain, and response regulator domain, similar to the Os-1p from *Neurospora crassa*. A comparison of the nucleotide sequences of the *AlHK1* gene from the Dim-sensitive and -resistant isolates revealed that all the resistant isolates contained a single-point mutation in the AARD of AlHK1p, with the exception of isolate K-11, where the AlHK1p contained a deletion of 107 amino acids. Moreover, the AlHK1p mutations in the isolates of each respective series involved the same amino acid substitution at the same site, although the resistance levels differed significantly in each series. Therefore, these findings suggested that a mutation in the AARD of AlHK1p was not the sole factor responsible for *A. longipes* resistance to dicarboximide fungicides.

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Keywords: *Alternaria longipes*, dicarboximide fungicides, two-component histidine kinase, mutation

Alternaria longipes is a worldwide pathogen of tobacco plants, causing brown spot disease on leaves that leads to considerable financial loss for growers. The major strategy used to prevent and control this disease is to spray fungicides, such as dicarboximide fungicides (DCFs), in tobacco fields. However, frequent use of DCFs has resulted in field resistance in many fungi, including *A. longipes* [16], *Botrytis cinerea* [5, 23], *Monilinia fructicola* [25], *Penicillium expansum* [27], *Sclerotinia homoeocarpa* [8], and *Sclerotium cepivorum* [17].

At present, the cellular targets of DCFs have not been identified, and the molecular basis of DCF resistance in fungi is not completely understood. However, results from studies with osmotic-sensitive (os) mutants of many filamentous fungi have shown that resistance to DCFs is related to the regulation of osmotic pressure and mutations in the group III two-component histidine kinase (HK) [7, 9–11, 20, 32]. Recently, several filamentous fungi group III HK genes, including *N. crassa Os-1/Nik-1*, *B. cinerea Bos1*, *Cochliobolus heterostrophus BmHK1*, and *A. alternata AaHK1*, were cloned and characterized using a unique five or six 92-amino-acid repeat domain (AARD), which putatively formed a coiled-coil domain at the N-termini, histidine kinase domain, and response regulator domain [1, 7, 9, 28, 32]. A nucleic acid sequence analysis of these group III HKs demonstrated that various mutations existed in the DCF-resistant isolates, and mainly occurred in the AARD, rather than the HK domain or response regulator domain [3, 7, 9, 21, 22].

Accordingly, to further characterize the molecular basis of DCF resistance, in this study, two field isolates (C-00

and K-10) of *A. longipes* were successively selected using the DCF Dimethachlon (Dim) and ultraviolet irradiation. The group III two-component HK gene (*AlHK1*) was then cloned from 19 Dim-sensitive and -resistant isolates of *A. longipes*, and the DNA sequences of these *AlHK1* genes were compared and the mutational sites analyzed, and finally the relationship between the two-component HKs and the morphological features were determined.

Materials and Methods

Isolates and Culture Conditions

The isolates of *A. longipes* used in this study (Table 1) were identified based on the morphological features of their conidia and conidiophores [30]. Single-spore isolates (C-00, C-12, C-25, K-10, and K-23) of the pathogen were obtained from tobacco fields according to the method of Ma *et al.* [18]. The isolates were maintained at 28°C on a potato dextrose agar (PDA) containing 50 μg/ml of ampicillin and 10 μg/ml of streptomycin. During the incubation of isolate C-00 on the PDA, a fan-shaped mutant was found along the edge of the colony. A mycelial plug of this sector was transferred to a new PDA plate and a single-spore isolate, designated K-11, was obtained after 7 days of incubation. The plugs of mycelia transferred in this study were all 6 mm in diameter and came from the edge of the colonies.

Selection of Resistant Isolates in Laboratory

Selection of Resistant Isolates using Various Concentrations of Dim. Two mycelial plugs of C-00 and one mycelial plug of K-10 were transferred to three new PDA plates and considered as the 0 generation for Dim selection. The isolates were then continuously transferred onto new PDA plates containing Dim, and the isolates from the 10th, 20th, and 30th transfers were named the 1st, 2nd, and 3rd generations, respectively. After 30 transfers, three series (S, M, and R) of selected isolates were obtained (Table 1), where the S series (including S-10, S-20, and S-30) and M series (including M-10, M-20, and M-30) originated from C-00 and were selected with 5 and 40 μg/ml Dim, respectively, whereas the R series (including R-10, R-20, and R-30) were obtained by selection with 400 μg/ml Dim from K-10.

Selection of Resistant Isolates using Ultraviolet (UV) Irradiation. Isolate C-00 was grown on a corn meal agar (CMA) medium for 15 days and its spores were collected and adjusted to a concentration of 1×10^6 spores/ml. Five ml of the spore suspension was poured into a 60-mm Petri dish, and then after 10 min of UV irradiation (wavelength: 254 nm; Power: 15W; vertical distance between samples and lamp-house: 45 cm), the spore suspension was spread onto the surface of PDA plates containing 300 μg/ml Dim and maintained at 28°C for 36 h. Three single colonies were obtained and transferred to new PDA plates. These three isolates, W-01, Z-16, and Z-26, were designated the first-generation products from C-00 by UV irradiation. W-01 was then considered the parent for the next selection experiment, with W-02 the product of further selection from W-01. Thus, W-01 and W-02 were named the W series.

Table 1. List of *A. longipes* isolates used in this study.

Isolate	Source	Phenotype[c]
C-00	Wenshan, China	FS
C-12	Qujing, China	FS
C-25	Yuxi, China	FS
K-10	Yuxi, China	FR
K-23	Wenshan, China	FR
K-11	Sector mutation of C-00	LR
S-10	First generation of C-00 selected by Dim[a]	LR
S-20	Second generation of C-00 selected by Dim	LR
S-30	Third generation of C-00 selected by Dim	LR
M-10	First generation of C-00 selected by Dim	LR
M-20	Second generation of C-00 selected by Dim	LR
M-30	Third generation of C-00 selected by Dim	LR
R-10	First generation of K-10 selected by Dim	LR
R-20	Second generation of K-10 selected by Dim	LR
R-30	Third generation of K-10 selected by Dim	LR
W-01	First generation of C-00 selected by UV[b]	LR
W-02	First generation of W-01 selected by UV	LR
Z-16	First generation of C-00 selected by UV	LR
Z-26	First generation of C-00 selected by UV	LR

[a]Dim, Dimethachlon.
[b]UV, ultraviolet light.
[c]FS, FR, and LR indicate field-sensitive, field-resistant, and laboratory-resistant isolates, respectively.

Calculation of Spore Numbers from Dim-Sensitive and -Resistant Isolates

The Dim-sensitive and -resistant isolates of *A. longipes* were incubated on a PDA medium for 12 days, and their spores collected by rinsing with 6 ml of sterile water. The number of spores was then counted using a direct microscopic count with hemocytometers.

Fungicide Resistance and Salt Tolerance Testing

The fungicide resistance and salt tolerance testing were determined using mycelial growth assays. The isolates were tested for resistance to Dim and phenylpyrroles fludioxonil by transferring mycelial plugs onto PDA containing a series of concentrations of Dim (from 1 to 600 μg/ml) or 1, 5, or 10 μg/ml fludioxonil. The 50% effective concentration (EC_{50}) for each isolate, which is the fungicide concentration that results in 50% mycelial growth inhibition, was calculated using the method of Ma *et al.* [19]. To test the sensitivity to osmotic stress, plugs of mycelia were transferred onto PDA containing 20 and 40 g/l NaCl, respectively. The percentage of mycelial radial growth (MRG) was then calculated using the formula MRG%=(T-6)/(C-6)×100, where T is the colony diameter of the NaCl treatment, and C is that of the non-NaCl control. Each experiment was performed four times and the results were averaged, and the SPSS software package (SPSS 11.01, U.S.A.) was used to determine any significant difference in the EC_{50} and MRG among all the isolates.

Extraction of Genomic DNA and Total RNA

The 19 isolates were inoculated onto sterile cellophane placed on the surface of PDA and incubated until mycelia covered the cellophane. The mycelia were then scraped off the cellophane and frozen in liquid nitrogen. Genomic DNA was extracted from

approximately 40 mg of fresh mycelia using the CTAB method [33]. The total RNA was extracted according to the manufacturer's instructions for the TRIzol Reagent (Invitrogen, U.S.A.), and stored at −70°C.

Cloning of *AlHK1* Gene and its cDNA Sequence

Amplification of Conserved Sequence of *AlHK1* Gene. A pair of degenerate primers, HKFwd and HKRev2 (Table 2), identical to the pair designed by Dry *et al.* [9] was synthesized to amplify the conserved regions of the *AlHK1* gene. The PCR amplifications were performed as described by Dry *et al.* [9], and then the PCR products were separated by electrophoresis in a 1% (w/v) agarose gel in a Tris-acetate (TAE) buffer, purified using a QIAquick Gel Extraction Kit (Takara, Japan), ligated into a pMD18-T vector (Takara, Japan), and finally sequenced using the M13+/− sequencing primers with an ABI PRISM 3730 automated sequencer (Perkin-Elmer, U.S.A.).

Amplification of Complete *AlHK1* Gene. The complete DNA sequence of the *AlHK1* gene from the Dim-sensitive isolate C-00 was obtained using a DNA Walking SpeedUp Premix Kit [14]. Three TSPs (target-specific primer; Table 2) were designed according to the known conserved sequence of the *AlHK1* gene, and the PCR amplifications performed according to the user's manual [14]. The PCR products were then cloned into a pMD18-T vector and sequenced. The complete *AlHK1* gene was obtained by assembling the sequences from the above PCR fragments using the DNAman software package (Version 5.2.2, Canada).

To amplify the complete *AlHK1* gene from the other Dim-sensitive and -resistant isolates, two pairs of specific primers were designed according to the complete *AlHK1* gene from isolate C-00. Thus, primers AlHKF1 and AlHKR2, and AlHKF3 and AlHKR4 (Table 2), were used to amplify the upstream and downstream sequences of the *AlHK1* gene, respectively. Performed in a 50 µl volume containing 50−100 ng genomic DNA, 5 µl of 10×super pfu PCR buffer, 10 mM dNTPs, 10 µM each primer, and 2.5 U super pfu high fidelity polymerase (BBI, Canada), the PCR used a touch-down program: 94°C for 5.0 min; 35 cycles at 98°C for 10 s, 66°C to 50°C for 35 s, and 72°C for 5 min, plus a final extension of 10 min at 72°C. The PCR products were then purified and directly sequenced.

cDNA Amplification. The first-strand cDNA was synthesized using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (Takara, Japan), and the complete cDNA amplified using the primers AlHKF1 and AlHKR4. The same PCR program as described above was also used to amplify the upstream and downstream sequences of the *AlHK1* gene, with a difference of 9 min instead of 5 min as the extension time. The PCR products were then purified and directly sequenced.

Sequence Comparisons and Analyses

The deduced amino acid sequences from the Dim-sensitive and -resistant isolates of *A. longipes* were aligned using the DNAman software package and BLAST of NCBI/GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). These programs were also used to align the deduced amino acid sequences for isolate C-00 and DCF-sensitive isolates from other phytopathogenic fungi.

The GenBank accession numbers for the sequences reported in this paper are DQ887538-DQ887546.

Table 2. Oligonucleotide primers used in this study.

Oligo pair	Oligo name	Oligo sequence (5'→3')	Description
1	HKFwd HKRev2	AAGGCCAACGAGGCCTTC/TCAA/GAAA/GGC CTCGCCGTGCTCGGTA/GAAC/TTT(A/G/T)AT	Degenerate primers for amplification of conserved sequence of *AlHK1*
2	ACP1/2/3/4 TSP1	ACP-AGGTC/ACP-TGGTC/ACP-GGGTC/ACP-CGGTC CTCAAGTCACCCATAGCG	Oligo pairs 2, 3, and 4 were used to amplify 5'-unknown sequence of *AlHK1* using DNA walking kit
3	ACPN TSP2	ACPN-GGTC TCACCCATAGCGACGGCAGT	
4	Uni-primer TSP3	TCACAGAAGTATGCCAAGCGA CATAGCGACGGCAGTGACGAT	
5	TSP1° ACP1/2/3/4	TAGCATCAAGAGCCAACGAG ACP-AGGTC/ACP-TGGTC/ACP-GGGTC/ACP-CGGTC	First round of Oligo pairs 5, 6, and 7 amplified 750 bp DNA fragment of 3'-unknown sequence of *AlHK1* using DNA walking kit
6	TSP2° ACPN	CCAACGAGCGGAAGTTGAA ACPN-GGTC	
7	TSP3° Uni-primer	CTTACAAGGTGCCCGACTACG TCACAGAAGTATGCCAAGCGA	
8	TSPa ACP1/2/3/4	GCACGGGAGCTTAGGATTG ACP-AGGTC/ACP-TGGTC/ACP-GGGTC/ACP-CGGTC	Second round of Oligo pairs 8, 9, and 10 amplified 900 bp DNA fragment upstream from 750 bp first-round PCR product using DNA walking kit
9	TSPb ACPN	TCAGGTCTGCGCTAGAAAACG ACPN-GGTC	
10	TSPc Uni-primer	TATGACGACGCCCTGTCTACC TCACAGAAGTATGCCAAGCGA	
11	AlHKF1 AlHKR2	CGAGCGTAAGTTGGGTCAT CTGTCCATCTTGCCCGTTCC	Specific primer for amplification of upstream sequence of *AlHK1*
12	AlHKF3 AlHKR4	CGGCTAAAACAGTTCTTTCGGTA GGCTTCTGGTGAGATGGATG	Specific primer for amplification of the downstream sequence of *AlHK1*

Table 3. Summary of resistance to Dimethachlon and sensitivity to osmotic stress of Dim-selected isolates and their parents.

Isolate	Dimethachlon			Radial growth (%)			
	EC_{50} (μg/ml)[a]	Significance level[b]		NaCl (g/l)[a]		Significance level[b]	
		0.05	0.01	0	20	0.05	0.01
C-00	2.5(±0.1)	a	A	100	91.0(±0.4)	a	A
S-10	10.1(±0.3)	b	B	100	56.4(±0.6)	b	B
S-20	12.1(±0.6)	b	B	100	53.6(±1.9)	b	B
S-30	15.0(±1.3)	b	B	100	53.0(±1.6)	b	B
M-10	54.2(±1.0)	c	C	100	42.8(±2.1)	c	C
M-20	56.5(±0.3)	c	C	100	41.8(±2.3)	c	CD
M-30	62.9(±2.2)	d	D	100	32.9(±1.0)	d	D
K-10	251.9(±7.1)	e	E	100	60.4(±1.2)	a	A
R-10	345.1(±4.9)	f	F	100	47.1(±1.4)	b	B
R-20	370.3(±6.8)	fg	F	100	45.7(±1.3)	b	BC
R-30	388.0(±12.9)	g	F	100	36.5(±1.5)	c	C

[a]Values represent the means (±SE) of four replicates for each isolate relative to the untreated control.
[b]For Tables 3, 4, and 5, lowercase and uppercase letters indicate significance level at 0.05 and 0.01, respectively. The Dim-resistant level and osmotic stress level for isolate C-00 are marked with the letters a and A, respectively, and then the levels of each two isolates are compared, where a significant difference is indicated by different letters, such as b, c, d, and B, C, D, respectively (Fisher's LSD test).

Results

Fungicide Resistance and Osmosensitivity

The Dim resistance and osmotic sensitivity of the *A. longipes* isolates (Table 1) are presented in Tables 3, 4, and 5, where the EC_{50} values for C-00, C-12, and C-25 were all low, at less than 3 μg/ml. However, the Dim resistance of the progeny of C-00 increased in varying degrees after being Dim selected, where the S and M series were above 4 and 20 times higher than the parent (isolate C-00), respectively. Significant EC_{50} increases were also obtained after UV irradiation of isolate C-00. As such, the EC_{50} values for W-01, W-02, Z-16, and Z-26 were 141.7, 173.6, 103.3, and 132.5 times higher than that for isolate C-00, respectively. In contrast, the resistance levels of the R series isolates did not exhibit obvious changes, as their EC_{50} values were only about 1.3–1.6 times higher than that of their parental isolate K-10. In addition, all the Dim-resistant isolates showed a cross-resistance to the fungicide fludioxonil, and exhibited an ability to grow normally on a medium under 5 μg/ml fludioxonil, with certain isolates such as the R and W series growing under 10 μg/ml, whereas the Dim-sensitive isolates could not grow even under 1 μg/ml (data not shown). As regards sensitivity to osmotic stress, all the *A. longipes* isolates showed a significantly reduced growth in 4% NaCl compared with the no NaCl control (data not shown). With 2% NaCl, all the selected Dim-resistant isolates grew slower than their parents. In particular, there was not much difference between each generation of the S series, whereas in the W series, W-01 was more sensitive than W-02. For the M and R series, the osmosensitivity was similar between the 1st and 2nd generations, yet both were less sensitive than the 3rd generation.

Phenotypic Properties of Dim-Sensitive and -Resistant Isolates of *A. longipes*

When comparing the phenotypic properties among the Dim-sensitive and -resistant isolates, with the exception of isolate K-11, there was no difference in the hyphal growth rate or spore production (Figs. 1 and 2). Isolate K-11 only had a few aerial hyphae and its colony color was much

Table 4. Summary of resistance to Dimethachlon and sensitivity to osmotic stress of UV-induced selected and their parents.

Isolate	Dimethachlon			Radial growth (%)			
	EC_{50} (μg/ml)	Significance level		NaCl (g/l)		Significance level	
		0.05	0.01	0	20	0.05	0.01
C-00	2.5(±0.1)	a	A	100	91.0(±0.4)	a	A
Z-16	258.3(±6.0)	b	B	100	81.4(±2.1)	b	A
Z-26	331.2(±1.1)	c	C	100	58.7(±2.5)	c	B
W-02	433.9(±9.9)	d	D	100	49.9(±1.3)	c	BC
W-01	354.2(±7.7)	ce	CE	100	39.0(±2.8)	d	C

Table 5. Summary of resistance to Dimethachlon and sensitivity to osmotic stress of field wild-type isolates and laboratory spontaneous mutation isolate.

Isolate	Dimethachlon			Radial growth (%)			
	EC_{50} (μg/ml)	Significance level		NaCl (g/l)		Significance level	
		0.05	0.01	0	20	0.05	0.01
C-25	1.9(±0.1)	a	A	100	91.9(±0.6)	a	A
C-00	2.5(±0.1)	a	A	100	91.0(±0.4)	a	A
C-12	1.6(±0.1)	a	A	100	76.1(±2.0)	b	B
K-10	251.9(±7.1)	b	B	100	60.4(±1.2)	c	C
K-23	398.7(±7.5)	c	C	100	56.4(±0.5)	c	CD
K-11	261.0(±10.4)	b	B	100	48.4(±2.2)	d	D

darker than those of the other isolates, plus the hyphae of K-11 did not form a branched multicellular network, but rather many short and crushed hyphae. In addition, K-11 had much greater ability in spore production than the other isolates, as after 12 days of incubation on the PDA medium, K-11 had a spore concentration of 5.5×10^5/ml, whereas the spore concentrations of the other isolates ranged from 3.0 to 4.0×10^4/ml (Fig. 1).

Cloning of *AlHK1* Gene from Dim-Sensitive Isolate C-00

A 2,000-bp DNA fragment was obtained from the genomic DNA of isolate C-00 using the degenerate primers HKFwd and HKRev2 (Table 2). The 3' and 5' flanking sequences of the *AlHK1* gene were cloned using a DNA Walking SpeedUp Premix Kit, and two fragments amplified in the 3' end (900 bp and 750 bp) of the *AlHK1* gene and one 1,100-bp fragment in the 5' end. The complete *AlHK1* gene was then obtained by assembling the sequences from these PCR fragments, yielding a 4,554 bp fragment. The predicted open reading frame (ORF) contained 4,302 bp, with six introns, and the intron positions were confirmed by aligning the DNA and cDNA sequences. The deduced protein contained 1,330 amino acids and shared all the properties of group III HK proteins, including an N-terminal 92-AARD, histidine kinase, and response regulator modules (Fig. 3). The AlHK1p exhibited a 98%, 64%, and 62% sequence identity to other group III HKs from *A. brassicicola* (AAU10313), *B. cinerea* (AAL30826), and *N. crassa* (AAB03698), respectively.

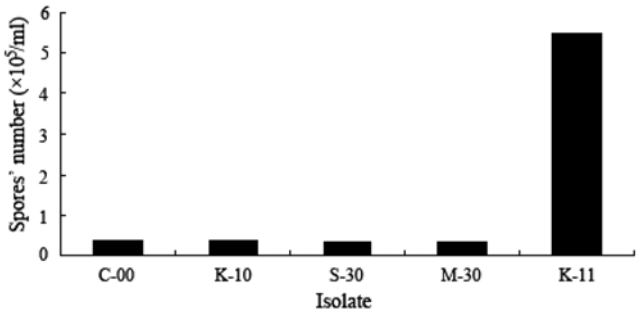

Fig. 1. Comparison of spore numbers from Dim-sensitive isolate C-00 and Dim-resistant isolates K-10, S-30, M-30, and K-11, when spores were collected after incubating isolates on PDA medium for 12 days.

Cloning and Mutational Site Analysis of *AlHK1* Gene from Dim-Sensitive and -Resistant Isolates

Based on the complete *AlHK1* gene from isolate C-00, two pairs of specific primers (Table 2) were used to amplify the *AlHK1* gene from the other isolates. All the PCRs were carried out with a high-fidelity DNA polymerase, and each PCR product was sequenced three times from individual clones to avoid any potential amplification errors, leading to a sequencing difference. A sequence comparison of the complete *AlHK1* genes from the different isolates (Table 1) revealed 10 and 4 bp differences between the Dim-sensitive isolates C-12, C-25, and C-00, respectively. However, all the base substitutions were silent, resulting in no amino acid changes among them. In addition, all but one of the resistant isolates showed just a single base substitution in the AARD region compared with the Dim-sensitive isolates, and the same mutations in the S, M, and W series occurred at the same site, whereas the R series had no amino acid change compared with their parent, although an aspartic acid (D) was substituted for a glycine (G) within the 3rd repeat when compared with the Dim-sensitive isolate C-00 (Table 6). The exception was isolate K-11, where the AlHK1p contained a 107-amino-acid deletion between the 5th and 6th repeats (Table 6).

Discussion

Two-component signal systems involving two conserved components, a histidine kinase and response regulator, have been found to exist in most eukaryotic and a few prokaryotic organisms. These systems use a His-Asp phosphorelay to sense and adapt to environmental signals and regulate diverse biologic processes, including

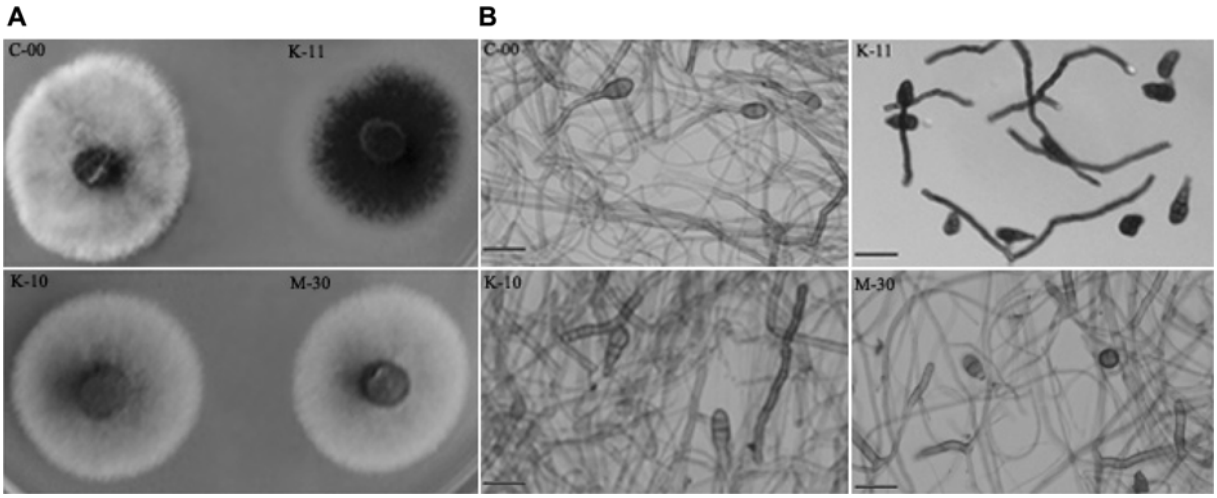

Fig. 2. Comparisons of (**A**) colony morphology among Dim-sensitive isolate C-00 and Dim-resistant isolates K-11, K-10, and M-30 incubated on PDA medium at 28°C for 3 days and (**B**) hyphal structure among Dim-sensitive isolate C-00 and Dim-resistant isolates K-11, K-10, and M-30, where hyphae were obtained from relative isolates that had been incubated for 12 days. Bars=40 μm.

differentiation, chemotaxis, photochromism, secondary metabolite production, and virulence [4, 12, 29, 31]. In *Saccharomyces cerevisiae*, the sole HK, Sln1p, has been predicted to be an osmosensor and a pivotal enzyme as regards the negative regulation of an osmosensing signaling pathway constituted by high osmolarity glycerol (HOG) MAP kinases [13, 15, 26]. In this study, a two-component HK gene (*AlHK1*) was cloned from the plant pathogenic fungus *A. longipes*. A structural analysis indicated that AlHK1p was an ortholog of group III two-component HKs, characterized by an N-terminal six 92-AARD followed by a conserved histidine kinase domain [1, 6, 9].

The mutant genotypes of HKs from DCF-resistant isolates can be divided into three groups based on the present results and previous reports [3, 7, 9]. The first group includes point mutation leading to amino acid substitution or a stop codon leading to premature termination of the translated protein. The second group includes a small deletion or duplication of a few base pairs, leading to a frameshift of the ORF. The third group includes a large deletion (Table 6). Although point mutation occurs constantly,

Fig. 3. Schematic diagram of the structure of fungal two-component HK genes showing the location of primers (Table 2) used in this study.
Triangles indicate intron positions, and H and D indicate putatively phosphorylated conserved histidine (H) and aspartic acid (D) residues.

a large deletion has rarely been found. At present, only two examples of a large deletion in HKs are known, one previously reported by Yoshimi *et al.* [32] and the other identified in the present study (isolate K-11).

Several recent studies have demonstrated a link between DCF-resistance and the regulation of osmotic stress in *os-1*, *Bos1*, and *HIK1* mutant isolates [7, 10, 20], plus the results from such studies have also suggested that mutations within the AARD of two-component HKs are responsible for the development of resistance to DCFs and phenylpyrrole chemical families [7, 21, 22]. In *N. crassa*, there are two types of *os-1* mutant; type I containing null mutations, which shows a high resistance to fungicides and moderate sensitivity to osmotic stress; and type II containing amino acid substitutions, which shows a moderate resistance to fungicides and high sensitivity to osmotic stress [11, 22]. However, the present results were inconsistent with the above phenotypic criteria. Whereas isolates K-10, K-23, Z-26, and the M, R, and W series were phenotypically similar to *N. crassa* type I mutants, their mutations were amino acid substitution instead of null mutation. Moreover, isolate Z-16 had a null mutation, yet a high level of fungicide resistance and low level of osmosensitivity. Furthermore, isolate K-11 was also phenotypically similar to type I mutants, yet its AlHK1p contained a 107-amino-acid deletion. Finally, the characteristics of the S series isolates were far from type I mutants, as they contained amino acid substitutions, yet showed a moderate level of both resistance to fungicides and sensitivity to osmotic stress. Hence, the mutational types and phenotypic characteristics of *A. longipes* were diverse, and the mutational type could not be determined according to the phenotypic characteristics and *vice versa*. Interestingly, the present study also indicated that most

Table 6. Comparison of phenotypes and *AlHK1* gene mutations among isolates of *A. longipes*.

Isolate	Phenotype[a]	Mutation	Position in coiled-coil domain
S series	Dim^{MR} Os^{M}	E 725 G	6th repeat
M series	Dim^{HR} Os^{M}	A 418 T	3rd repeat
R series	Dim^{HR} Os^{M}	G 420 D	3rd repeat
W series	Dim^{HR} Os^{M}	W 297 R	2nd repeat
K-10	Dim^{HR} Os^{M}	G 420 D	3rd repeat
K-23	Dim^{HR} Os^{M}	G 420 D	3rd repeat
K-11	Dim^{HR} Os^{M}	582-689 (107AA) deletion	Between 5th and 6th repeat
Z-16	Dim^{HR} Os^{L}	Q (599 nt) to stop codon	5th repeat
Z-26	Dim^{HR} Os^{M}	W 297 R	2nd repeat

[a]Dim, Dimethachlon; HR and MR, high and moderate levels of resistance, respectively; Os^{L} and Os^{M}, low and moderate levels of osmosensitivity, respectively.

isolates decreased their osmotic stress level with an increasing Dim resistance level. However, there were some inconsistent cases, as isolate W-02 was more resistant than W-01, yet its osmosentivity was lower than that of W-01 (Table 4). Additionally, both C-00 and C-12 were sensitive to Dim and their AlHK1p sequence had no amino acid difference, yet their osmosensitivity was significantly different (Table 5). Therefore, these results revealed that the change between the Dim resistance and osmotic stress of *A. longipes* was not directly correlated.

So far, the mode of action of DCFs on plant pathogenic fungi is still not fully understood. Recent studies have shown that DCFs may target the osmotic stress signal transduction pathway, and mutations in components of this pathway may result in the DCF resistance of filamentous fungi. In Tables 3, 4, and 6, the Dim-resistant levels of the S, M, W, and R series were significantly higher than those of their parental isolates C-00 and K-10, respectively. Furthermore, the HK mutations in all the M and W series progeny matched those in the parents, respectively, with the same amino acid substitutions occurring at the same position, although the last generations were more resistant to Dim than the first generation. In contrast, the R series had no mutation compared with the parental isolate K-10 when their resistant levels were significantly higher than that of K-10. Moreover, the R series showed the same point mutation as the Dim-sensitive isolate C-00. Therefore, these results suggested that (1) in a single selected series, there was no accumulation of amino acid substitutions in AlHK1p, and even without obvious mutations, the phenotypic effects differed among the examined isolates; and (2) a single amino acid mutation in the 92-AARD of AlHK1p could confer *A. longipes* resistance to DCFs, yet it was not the sole factor. Thus, alternative mechanisms other than mutations in the two-component HKs may also be responsible for DCF resistance in other fungal species, such as *N. crassa*, *U. maydis*, and *A. brassicicola*. For example, in *N. crassa*, *os-2*, *os-4*, and *os-5* have also been found to confer resistance to DCFs, aromatic hydrocarbons, and phenylpyrrole fungicides, in addition to *os-1* [10, 11]; in *U. maydis*, mutants with a disruption of the *ubc1* gene, which encodes the catalytic subunit of a cAMP-dependent protein kinase (PKA), have been shown to be resistant to DCFs [24]; and in *A. brassicicola*, a field-resistant $AbraCP^{FR}$ isolate produced a phenotype with a high resistance to iprodione and fludioxonil, yet the DNA sequence of its *AbNIK1* gene did not reveal any difference to that of the wild type [3].

Meanwhile, other functions of AlHKp have also been found, in addition to DCF resistance and the regulation of osmotic stress. Alex *et al*. [1, 2] previously reported that deletion of the *Nik-1* gene in *N. crassa* and the *Cos1* gene in *Candida albicans* resulted in an aberrant hyphal structure and defective hyphal formation. In this study, isolate K-11 also displayed an aberrant colony morphology and abnormal hyphal structure (Fig. 2). A molecular analysis of isolate K-11 showed that its *AlHK1* gene had a large deletion that could be considered another form of gene disruption. Therefore, AlHK1p may also be involved in hyphal development and spore production. In conclusion, the present results expanded current knowledge on the role of the *AlHK1* gene in conferring Dim resistance and sensitivity to osmotic stress, while providing a good basis for further studies on the DCF resistance mechanism of filamentous fungi.

Acknowledgments

The authors would like to thank Drs. Jianping Xu (McMaster University), Chenggang Zou, and Xiaowei Huang for their help and advice. We would also like to thank Zhengjiang Heyi Pesticide and Chemical Co. Ltd (China) for providing the fungicides. This work was jointly supported by the National Basic Research Program of China (2007CB411600) and the Scientific Foundation of the Yunnan Tobacco Corporation (04A19).

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