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# Biochemical and molecular characterization of *Alternaria alternata* isolates highly resistant to procymidone from broccoli and cabbage

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## Abstract

*Alternaria alternata*, a causal agent of leaf blights and spots on a wide range of hosts, has a high risk of developing resistance to fungicides. Procymidone, a dicarboximide fungicide (DCF), has been widely used in controlling *A. alternata* leaf blights in China for decades. However, the resistance of *A. alternata* against DCFs has rarely been reported from crucifer plants. A total of 198 *A. alternata* isolates were collected from commercial fields of broccoli and cabbage during 2018–2019, and their sensitivities to procymidone were determined. Biochemical and molecular characteristics were subsequently compared between the high-level procymidone-resistant (Pro<sup>HR</sup>) and procymidone-sensitive (Pro<sup>S</sup>) isolates, and also between Pro<sup>HR</sup> isolates from broccoli and cabbage. Compared with Pro<sup>S</sup> isolates, the mycelial growth rate, sporulation capacity and virulence of most Pro<sup>HR</sup> isolates were reduced; Pro<sup>HR</sup> isolates displayed an increased sensitivity to osmotic stresses and a reduced sensitivity to sodium dodecyl sulfate (SDS); all Pro<sup>HR</sup> isolates showed a reduced sensitivity to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) except for the isolate B102. Correlation analysis revealed a positive cross-resistance between procymidone and iprodione, or fludioxonil. When treated with 10 µg/mL of procymidone, both mycelial intracellular glycerol accumulations (MIGAs) and relative expression of *AaHK1* in Pro<sup>S</sup> isolates were higher than those in Pro<sup>HR</sup> isolates. Sequence alignment of *AaHK1* from ten Pro<sup>HR</sup> isolates demonstrated that five of them possessed a single-point mutation (P94A, V612L, E708K or Q924STOP), and four isolates had an insertion or a deletion in their coding regions. No significant difference in biochemical characteristics was observed among Pro<sup>HR</sup> isolates from two different hosts, though mutations in *AaHK1* of the cabbage-originated Pro<sup>HR</sup> isolates were distinct from those of the broccoli-originated Pro<sup>HR</sup> isolates.

**Keywords:** *Alternaria alternata*, Broccoli, Cabbage, Resistance, Dicarboximide fungicides, Fitness, *AaHK1*

## Background

A leaf spot and blight disease, caused by the filamentous fungus *Alternaria alternata* (abbreviated as ALSB), is among the most common diseases of numerous economic crops, including crucifers (such as cabbage,

cauliflower and broccoli), bean, cotton, citrus and tomato (Nowicki et al. 2012; Mukesh et al. 2016). The pathogen can infect a wide range of hosts and produce mycotoxins, posing a severe threat to safe food and vegetables worldwide, for instance, the reduced quality of cauliflower heads and brassicaceae oil (Guillemette et al. 2004; Surviliene & Dambrauskiene 2006; Meena & Samal 2019). Owing to few commercially-satisfactory ALSB-resistant varieties of brassicaceae vegetables available, chemical control is relied to control ALSB in the

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field. Currently, dicarboximide fungicides (DCFs) (Dry et al. 2004), quinol-oxidizing inhibitors (QoIs) (Ma et al. 2003), succinate dehydrogenase inhibitors (SDHIs) (Avenot & Michailides 2007) and sterol demethylation inhibitors (DMIs) (Avenot et al. 2016) are intensively used to control ALSB.

The DCFs, including procymidone and iprodione, have been registered to control ALSB of cabbage and broccoli for decades in China (Ma & Michailides 2004). The biochemical mechanism of DCFs against phytopathogenic fungi is documented to regulate high osmolarity glycerol (HOG) MAP kinases, interfering with the phosphorylation of transduction-associated proteins and glycerol biosynthesis (Cui et al. 2002; Lin & Chung 2010). DCFs and phenylpyrroles are reported to target the same enzyme, a fungal two-component histidine kinase (HK), which belongs to group III hybrid histidine kinases involved in an osmotic-regulatory signal transduction cascade of mitogen-activated protein kinase (MAPK) signaling pathway, i.e., HOG-MAPK (Avenot et al. 2005; Lin & Chung 2010; Ren et al. 2016). This cascade not only perceives extracellular stimuli, but also maintains intracellular osmotic homeostasis. Positive cross-resistance between DCFs and phenylpyrroles is frequently observed in phytopathogenic fungi highly resistant to fludioxonil, including *Botrytis cinerea* (Sang et al. 2018), *Bipolaris maydis* (Han et al. 2017), *Stemphylium solani* (Wu et al. 2015) and *Sclerotinia sclerotiorum* (Duan et al. 2013). The typical structure of fungal group III HKs from major phytopathogenic fungi is composed of three domains: Histidine kinases-Adenylate cyclases-Methyl accepting proteins and Phosphatases (HAMPs, functionally named as “sensor domain”) at a highly variable N-terminus, a central transmitter domain composed of histidine kinase A (HisKA) and cognate histidine kinase-like ATPase catalytic subdomains (HATPase\_c), and a C-terminus receiver domain harboring a three amino-acid signature (DDK) (Herivaux et al. 2016).

Due to the extensive application of DCFs, the resistance against this type of fungicides has been reported in several phytopathogenic fungi, including *Alternaria* spp. (Dry et al. 2004; Avenot et al. 2005; Luo et al. 2008, 2012), *Sclerotinia* spp. (Ma et al. 2009; Kuang et al. 2011; Duan et al. 2013), *B. cinerea* (Fraile et al. 1986; Oshima et al. 2002; Cui et al. 2004; Grabke et al. 2014), *Magnaporthe grisea* (Motoyama et al. 2005), *Stemphylium vesicarium* (Alberoni et al. 2010), *Neurospora crassa* (Fujimura et al. 2000; Miller et al. 2002; Ochiai et al. 2010) and *Cochliobolus heterostrophus* (Yoshimi et al. 2003, 2004). The molecular mechanisms of DCF-resistance include amino acid substitution in group III HK proteins, deletion or insertion mutations in their coding regions (Cui et al. 2004; Dry et al. 2004; Luo et al. 2008). Mutations in *AaHK1* (insertion or deletion)

of *A. alternata* lead to premature termination of the AaHK protein (Dry et al. 2004). Similarly, diverse mutations are found in AbNIK1 of *Alternaria brassicicola*, including nonsense mutations, a frameshift and a single amino acid substitution (E753K) (Avenot et al. 2005), while deletions and single amino acid substitutions are detected in AIHK1 of *Alternaria longipes* (Luo et al. 2008, 2012). In brief, it is intriguing to observe a high degree of diversity among mutations in HKs of the DCF-resistant isolates from *Alternaria* spp..

The mutations of HK1s are involved in the development of resistance to DCFs in fungal pathogens under certain selective pressure (Dry et al. 2004). Recently, growers has raised concerns about the decreased efficacy of DCFs against ALSB on cabbage and broccoli in Jiangsu Province of China. The objectives of this study were to (i) investigate whether procymidone-resistant (Pro<sup>R</sup>) populations of *A. alternata* has developed in commercial fields of cabbage and broccoli that were repetitively exposed to procymidone; (ii) compare biochemical characteristics between Pro<sup>S</sup> and Pro<sup>HR</sup> isolates, and also between Pro<sup>HR</sup> isolates from broccoli and cabbage; (iii) reveal possible molecular resistant mechanisms of *A. alternata* against procymidone.

## Results

### Frequencies of resistance against procymidone in natural populations of *A. alternata* from broccoli and cabbage

The 198 isolates of *A. alternata* were obtained during 2018–2019, including 114 isolates from broccoli and 84 isolates from cabbage in Yancheng City, Jiangsu Province, China. Of the 114 isolates from broccoli, 7 high-level procymidone-resistant (Pro<sup>HR</sup>) isolates and 60 low-level procymidone-resistant (Pro<sup>LR</sup>) isolates were detected with resistance frequencies of 6.14 and 52.63%, respectively (Table 1). Of the 84 isolates from cabbage, in contrast, 3 Pro<sup>HR</sup> isolates and 45 Pro<sup>LR</sup> isolates were found with resistance frequencies of 3.57 and 53.57%, respectively (Table 1). The data indicate that DCF-resistant populations of *A. alternata* has developed in commercial fields of broccoli and cabbage.

The EC<sub>50</sub> values of all Pro<sup>HR</sup> isolates from broccoli and cabbage were more than 100 µg/mL, while the values for Pro<sup>S</sup> isolates were less than 1 µg/mL (Table 2).

Fourteen representative single-spore isolates, including ten Pro<sup>HR</sup> isolates (C5, C13, C40, B30, B35, B46, B53, B88, B102 and B108) and four Pro<sup>S</sup> isolates (C10 and C28 from cabbage; B16 and B21 from broccoli), were selected for phylogenetic analyses base on ITS and KOG1058 sequences (Fig. 1) and for further experiments.

**Table 1** Sensitivities of *Alternaria alternata* isolates to procymidone from Yancheng City, Jiangsu Province, China during 2018–2019

Location <sup>a</sup>	Host	Total <sup>b</sup>	Number of isolates			Frequency of resistance (%)	
			S	LR	HR	LR	HR
Xiangshui, JS	Broccoli	114	47	60	7	52.63	6.14
Xiangshui, JS	Cabbage	84	36	45	3	53.57	3.57

<sup>a</sup> The samples were collected from Yancheng City, Jiangsu Province (JS), China. <sup>b</sup> The number of total isolates. S, procymidone-sensitive isolates, LR, low-level procymidone-resistant isolates, HR, high-level procymidone-resistant isolates

### Cross-resistance

All the Pro<sup>HR</sup> isolates from broccoli and cabbage were also highly resistant to fludioxonil and iprodione based on their EC<sub>50</sub> values (Table 2). The Spearman's rank correlation coefficient between the sensitivity against procymidone and fludioxonil, or iprodione was 0.9870 or 0.9824 ( $P < 0.001$ ), respectively, suggesting a positive correlation (a positive cross-resistance) between procymidone and fludioxonil or iprodione was present in these tested *A. alternata* isolates.

### Mycelial growth, sporulation and virulence in Pro<sup>HR</sup> and Pro<sup>S</sup> isolates of *A. alternata*

The mycelial growth rates of the Pro<sup>HR</sup> isolates on fungicide-free potato dextrose agar medium (PDA) were significantly reduced compared with those of the Pro<sup>S</sup> isolates ( $P = 0.05$ ) (Table 3). No significant difference in mycelial growth was observed among the Pro<sup>HR</sup> isolates from cabbage and broccoli (Table 3). Compared with Pro<sup>S</sup> isolates, the sporulation capacity of the Pro<sup>HR</sup> isolates (except for isolates B46 and B88) was significantly reduced ( $P = 0.05$ ) (Table 3). Furthermore, the virulence of the Pro<sup>HR</sup> isolates (except for isolates B35 and B53) on broccoli detached leaves was also significantly

weakened compared with that of the Pro<sup>S</sup> isolates ( $P = 0.05$ ) (Fig. 2 and Table 3). Intriguingly, all Pro<sup>HR</sup> isolates from cabbage and broccoli fluctuated markedly in sporulation capacity and virulence.

### Sensitivities of Pro<sup>HR</sup> and Pro<sup>S</sup> isolates of *A. alternata* to stress agents

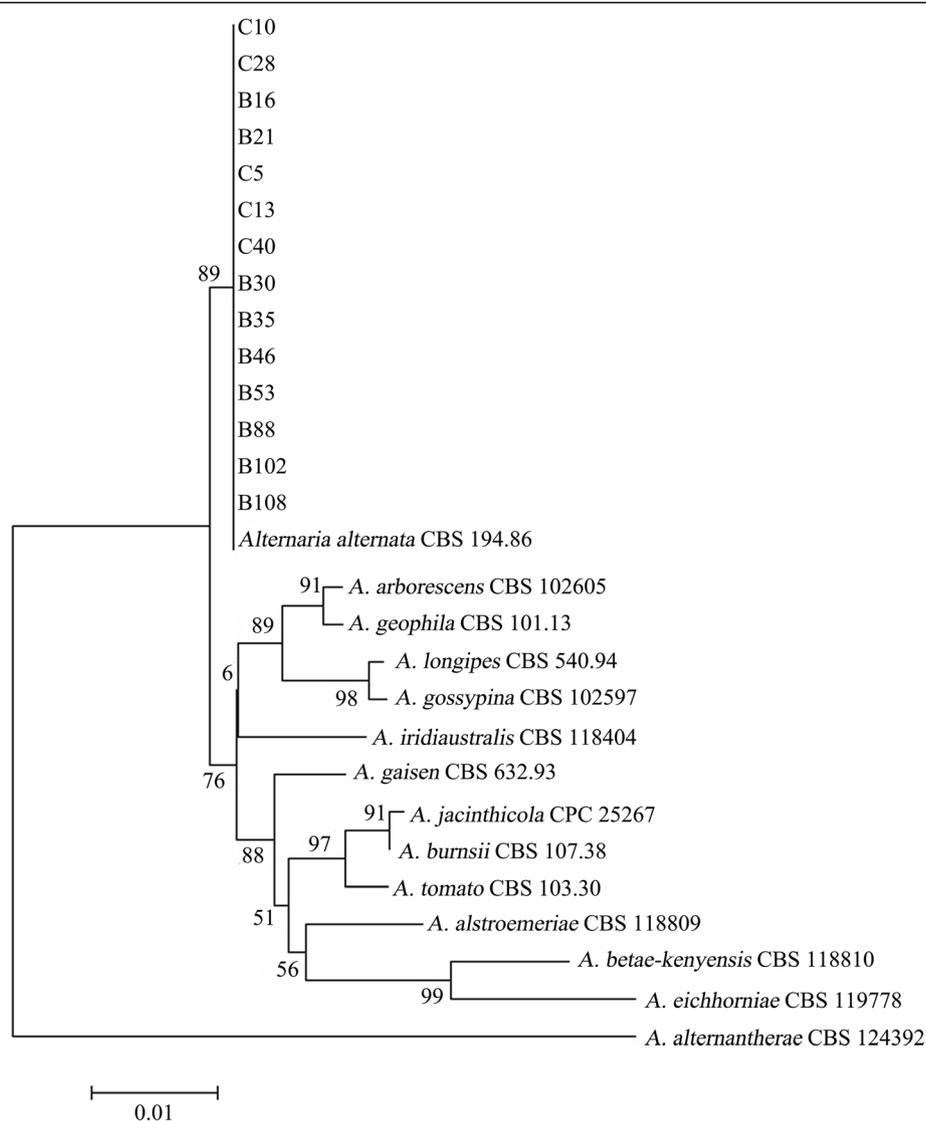
The group III HK is involved in osmotic-regulatory signal transduction cascade of MAPK signaling pathway (Lin & Chung 2010). To determine whether mutations in *AaHK1* affect the sensitivity of *A. alternata* to osmotic and oxidative stresses, cell membrane permeability or cell wall integrity, the mycelial growth inhibition rates of the Pro<sup>HR</sup> and Pro<sup>S</sup> isolates on PDA supplemented with an osmotic stress agent (1.2 M KCl or 1.2 M NaCl), an oxidative stress agent (10 mM hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>), a cell membrane stress agent (0.05% Sodium dodecyl sulfate, SDS) or a cell wall damage agent (0.05% Congo red, CR) were determined.

The data showed that all Pro<sup>HR</sup> isolates were strongly inhibited by 1.2 M KCl or 1.2 M NaCl, with mycelial growth inhibition rates ranging from 84 to 95% or from 85 to 95%, indicating that they were more sensitive to osmotic stresses than Pro<sup>S</sup> isolates (51–78% and 64–78%

**Table 2** EC<sub>50</sub> values of Pro<sup>S</sup> and Pro<sup>HR</sup> isolates of *Alternaria alternata* from broccoli and cabbage

Isolate	Sensitivity	Origin	EC <sub>50</sub> (µg/mL)		
			Procymidone	Iprodione	Fludioxonil
C10	Pro <sup>S</sup>	Cabbage	0.73 ± 0.13d	0.14 ± 0.07e	0.068 ± 0.005 cd
C28	Pro <sup>S</sup>	Cabbage	0.92 ± 0.07c	0.53 ± 0.03c	0.075 ± 0.006c
B16	Pro <sup>S</sup>	Broccoli	0.95 ± 0.11c	0.58 ± 0.12c	0.073 ± 0.006c
B21	Pro <sup>S</sup>	Broccoli	0.98 ± 0.08c	0.43 ± 0.04d	0.085 ± 0.004c
C5	Pro <sup>HR</sup>	Cabbage	>100b	>100b	>100b
C13	Pro <sup>HR</sup>	Cabbage	>100b	>100b	>100b
C40	Pro <sup>HR</sup>	Cabbage	>100b	>100b	>100b
B30	Pro <sup>HR</sup>	Broccoli	>100b	>100b	>100b
B35	Pro <sup>HR</sup>	Broccoli	>100b	>100b	>100b
B46	Pro <sup>HR</sup>	Broccoli	>100b	>100b	>100b
B53	Pro <sup>HR</sup>	Broccoli	>100b	>100b	>100b
B88	Pro <sup>HR</sup>	Broccoli	>100b	>100b	>100b
B102	Pro <sup>HR</sup>	Broccoli	>200a	>200a	>200a
B108	Pro <sup>HR</sup>	Broccoli	>200a	>200a	>200a

Means in a column followed by the same letter are not different according to Fisher's least significant difference ( $P = 0.05$ ). Pro<sup>S</sup>, procymidone-sensitive isolates; Pro<sup>HR</sup>, high-level procymidone-resistant isolates; EC<sub>50</sub>, effective concentration for 50% inhibition of mycelial growth



**Fig. 1** Phylogram generated from maximum parsimony analysis based on sequences of ITS and KOG1058, showing the phylogenetic relationship of the tested isolates with *A. alternata* and other *Alternaria* species

mycelial growth inhibition by 1.2 M KCl and 1.2 M NaCl, respectively) ( $P = 0.05$ ) (Fig. 3a, b). However, the mycelial growth rates of the Pro<sup>HR</sup> isolates were higher than those of the Pro<sup>S</sup> isolates when treated with 0.05% SDS ( $P = 0.05$ ) (Fig. 3c). Under 0.05% CR treatment condition, the radial growth rates of the Pro<sup>HR</sup> isolates displayed different degrees of inhibition relative to those of the Pro<sup>S</sup> isolates (Fig. 3d). Additionally, except for the Pro<sup>HR</sup> isolate B102 (having no mutation in *AaHKL1*), the mycelial growth rates of the Pro<sup>HR</sup> isolates were higher than those of the Pro<sup>S</sup> isolates when treated with 10 mM H<sub>2</sub>O<sub>2</sub> (Fig. 3e). Furthermore, the sensitivity to stress agents varied greatly among the Pro<sup>HR</sup> isolates from broccoli or cabbage.

#### Mycelial intracellular glycerol accumulations (MIGAs) in Pro<sup>HR</sup> and Pro<sup>S</sup> isolates of *A. alternata*

DCFs are reported to affect glycerol biosynthesis and phosphorylation in HOG-MAPK pathway (Cui et al. 2002). Under procymidone-free condition, no significant difference was observed in MIGAs between the Pro<sup>S</sup> and Pro<sup>HR</sup> isolates ( $P > 0.05$ ). After treated with 10 µg/mL procymidone for 5 h, MIGAs in Pro<sup>S</sup> isolates increased significantly and were 2.70–3.04 folds of those under procymidone-free condition, higher than that of 1.02–1.27 folds by the ten Pro<sup>HR</sup> isolates under the same conditions ( $P = 0.05$ ) (Fig. 4). Furthermore, no significant difference was observed in MIGAs among the Pro<sup>HR</sup> isolates from broccoli and cabbage ( $P > 0.05$ ) (Fig. 4).

**Table 3** Mycelial growth rate, sporulation and virulence in Pro<sup>S</sup> and Pro<sup>HR</sup> isolates of *Alternaria alternata* from cabbage and broccoli

Isolate	Sensitivity	Origin	Mycelial growth (cm)	Sporulation ( $\times 10^5$ spores/mL)	Virulence (lesion area, cm <sup>2</sup> )
C10	Pro <sup>S</sup>	Cabbage	7.49 $\pm$ 1.15a	11.58 $\pm$ 1.97bc	1.56 $\pm$ 0.08bc
C28	Pro <sup>S</sup>	Cabbage	7.53 $\pm$ 0.85a	10.15 $\pm$ 1.03c	1.68 $\pm$ 0.23b
B16	Pro <sup>S</sup>	Broccoli	7.63 $\pm$ 0.13a	9.95 $\pm$ 0.95 cd	1.53 $\pm$ 0.26bc
B21	Pro <sup>S</sup>	Broccoli	7.35 $\pm$ 1.54a	12.63 $\pm$ 1.32b	1.51 $\pm$ 0.43bc
C5	Pro <sup>HR</sup>	Cabbage	7.02 $\pm$ 1.46b	0.28 $\pm$ 0.09gh	0.58 $\pm$ 0.27de
C13	Pro <sup>HR</sup>	Cabbage	5.82 $\pm$ 0.78d	2.59 $\pm$ 0.15e	1.05 $\pm$ 0.64c
C40	Pro <sup>HR</sup>	Cabbage	6.68 $\pm$ 1.06c	0.61 $\pm$ 0.12 g	0.62 $\pm$ 0.07d
B30	Pro <sup>HR</sup>	Broccoli	5.37 $\pm$ 0.53ef	0.87 $\pm$ 0.13 g	0.55 $\pm$ 0.14de
B35	Pro <sup>HR</sup>	Broccoli	5.53 $\pm$ 0.84e	1.25 $\pm$ 0.81f	2.38 $\pm$ 0.84a
B46	Pro <sup>HR</sup>	Broccoli	6.84 $\pm$ 1.12bc	23.25 $\pm$ 1.85a	0.46 $\pm$ 0.12e
B53	Pro <sup>HR</sup>	Broccoli	6.51 $\pm$ 0.25c	4.97 $\pm$ 1.02d	2.05 $\pm$ 0.23ab
B88	Pro <sup>HR</sup>	Broccoli	6.91 $\pm$ 0.66b	12.57 $\pm$ 0.76b	0.63 $\pm$ 0.04d
B102	Pro <sup>HR</sup>	Broccoli	6.24 $\pm$ 1.18 cd	4.52 $\pm$ 1.18de	0.48 $\pm$ 0.12e
B108	Pro <sup>HR</sup>	Broccoli	5.65 $\pm$ 0.57de	0.97 $\pm$ 0.08 g	0.45 $\pm$ 0.05e

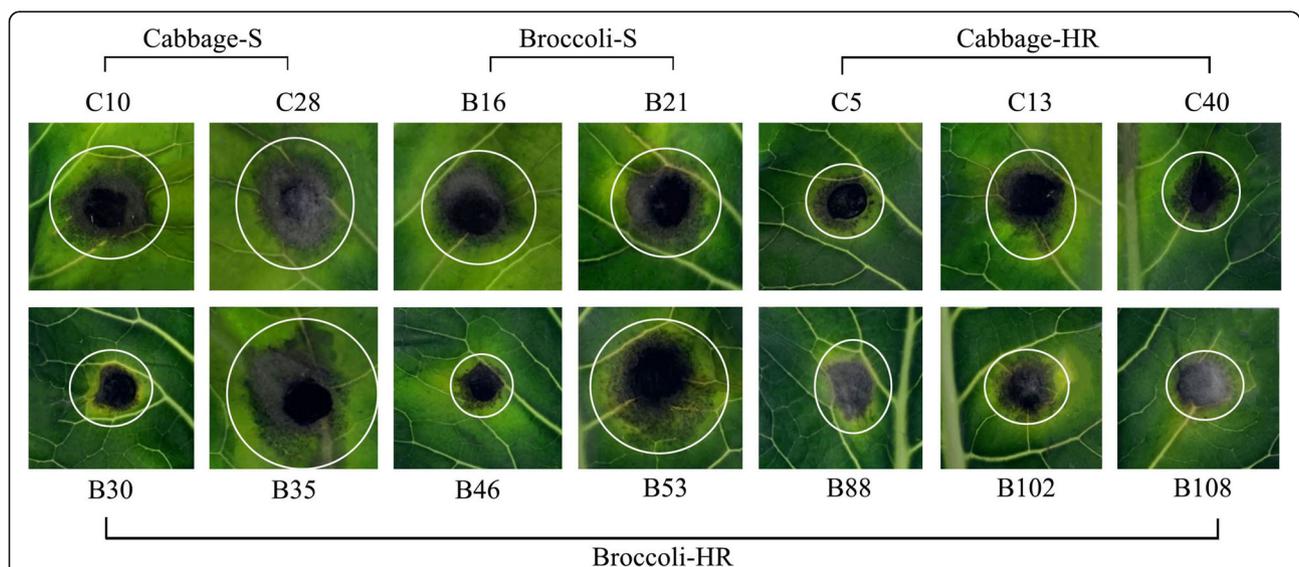
Radial mycelial growth of *A. alternata* isolate was measured after 7 days of incubation on potato dextrose agar (PDA) plates at 25 °C. Spores were harvested from the surface of 7-day-old PDA colony with sterile water (0.1% Tween 20). Lesion areas were determined on broccoli leaves at 7 days post-inoculation (dpi). The mean value  $\pm$  SD of each isolate was calculated from three independent experiments. Means in a column followed by the same letter are not different according to Fisher's least significant difference ( $P = 0.05$ ). Pro<sup>S</sup>, procymidone-sensitive isolates; Pro<sup>HR</sup>, high-level procymidone-resistant isolates

### Sequence alignments of *AaHK1* from Pro<sup>S</sup> and Pro<sup>HR</sup> isolates of *A. alternata*

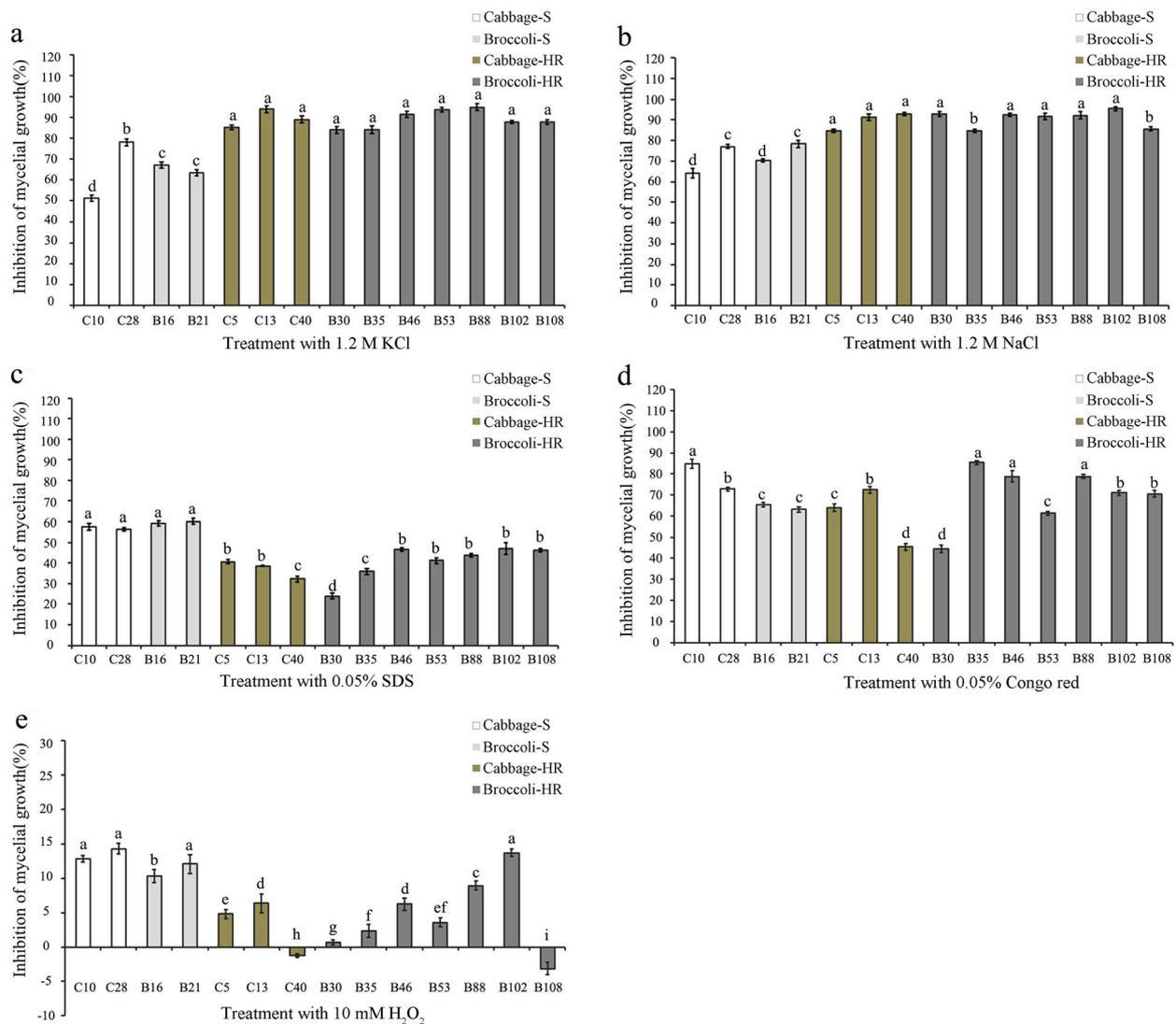
To characterize mutations in *AaHK1* proteins, the complete nucleotide sequences of *AaHK1* in all Pro<sup>HR</sup> and 20 arbitrarily selected Pro<sup>S</sup> isolates from broccoli and cabbage were sequenced and aligned. The results showed that the deduced *AaHK1* protein is 1330 amino acids (aa) in length, possessing six HAMP repeat domains at N-terminus, followed by a His Kinase A (phospho-acceptor)

domain (HisKA), an HK-like ATPase domain (HATPase<sub>c</sub>), and a response regulator domain at C-terminus (Fig. 5). The twenty Pro<sup>S</sup> isolates possessed identical *AaHK1* sequences with no genetic diversity, as illustrated by the four representative isolates (C10, C28, B16 and B21). No mutation was observed in *AaHK1* of all Pro<sup>LR</sup> isolates from broccoli and cabbage (data not show).

All mutations in the coding regions of *AaHK1* from Pro<sup>HR</sup> isolates are presented in Table 4. Except for the



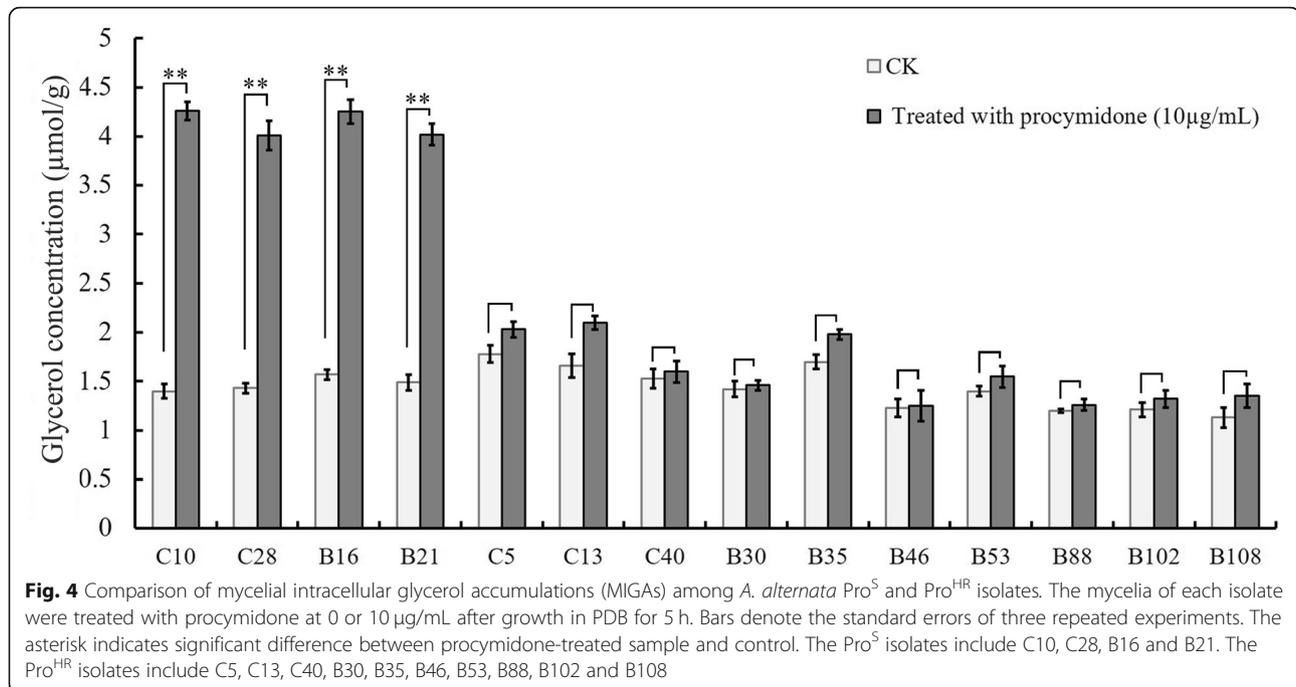
**Fig. 2** Virulence analysis of *A. alternata* Pro<sup>S</sup> and Pro<sup>HR</sup> isolates. The tested isolates were inoculated onto broccoli leaves. Disease lesions were determined at 7 days post-inoculation (dpi). The Pro<sup>S</sup> isolates include C10, C28, B16 and B21. The Pro<sup>HR</sup> isolates include C5, C13, C40, B30, B35, B46, B53, B88, B102 and B108



**Fig. 3** Sensitivity comparison among *A. alternata* Pro<sup>S</sup> and Pro<sup>HR</sup> isolates to osmotic stress agents generated by 1.2 M KCl (**a**); 1.2 M NaCl (**b**); 0.05% SDS (**c**); 0.05% Congo red (**d**) and 10 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (**e**). Bars denote the standard errors of three repeated experiments. Values on bars followed by the same letter are not significantly different from each other at  $P = 0.05$ . The Pro<sup>S</sup> isolates include C10, C28, B16 and B21. The Pro<sup>HR</sup> isolates include C5, C13, C40, B30, B35, B46, B53, B88, B102 and B108

isolate B102, all broccoli-originated Pro<sup>HR</sup> isolates contained abundant amino acid mutations in AaHK1, which could be divided into two groups. Group I possessed an amino acid substitution: E708K (a substitution of glutamic acid by lysine at codon<sup>708</sup> within the HAMP6 domain) in isolates B88 and B108; V612L (a substitution of valine by leucine at codon<sup>612</sup> within the HAMP6 domain) in isolate B30; Q924STOP (a precocious stop of translation at codon<sup>924</sup> within the HATPase\_c domain) in isolate B35. Group II had a 2-bp deletion between domains of HATPase\_c and Rec in isolate B53 (which caused a frame-shift, leading to premature termination at codon<sup>1104</sup> of AaHK1), or a 381-bp deletion (from

nt<sup>1707</sup> to nt<sup>2089</sup>, causing a 127-AA deletion from HAMP4 to HAMP6) in isolate B46. In contrast, mutations in AaHK1 from three cabbage-originated Pro<sup>HR</sup> isolates could be categorized into three groups: group I presented an amino acid substitution (P94A, proline was changed to alanine at codon<sup>94</sup> localized between N-terminus and HAMP1) in isolate C5; group II had an 8-bp insertion at the Rec domain in isolate C40, resulting in a premature termination at codon<sup>1204</sup>; group III had a 552-bp deletion, causing a 184-AA deletion from HAMP1 to HAMP3 in isolate C13. In brief, diverse mutations were found in Pro<sup>HR</sup> isolates, and the resistant genotypes of Pro<sup>HR</sup> isolates from cabbage were distinct



from those from broccoli. Nucleotide sequence accession numbers corresponding to *AaHK1* from the tested Pro<sup>HR</sup> and Pro<sup>S</sup> isolates are presented in Table 5.

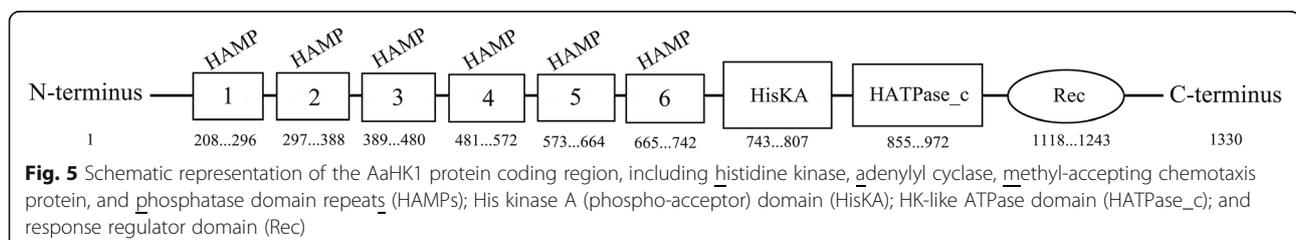
#### The relative expression level of *AaHK1* in Pro<sup>S</sup> and Pro<sup>HR</sup> isolates of *A. alternata*

The relative expression level of group III HK gene in *B. cinerea* is correlated with its resistance against histidine kinase inhibitors (Ren et al. 2016). Here, we investigated changes of *AaHK1* expression in Pro<sup>S</sup> and Pro<sup>HR</sup> isolates against procymidone. In the absence of procymidone, the expression of *AaHK1* was relatively low in the Pro<sup>S</sup> and Pro<sup>HR</sup> isolates (Fig. 6). When treated with procymidone at 10 μg/mL for 12 h, the relative expression level of *AaHK1* in the Pro<sup>S</sup> isolates was 3.36–3.65 folds of that of untreated Pro<sup>S</sup> isolates, much higher than that of 0.89–1.22 folds in the ten Pro<sup>HR</sup> isolates under the same condition (Fig. 6). Furthermore, no significant difference in the relative expression of *AaHK1* was observed among Pro<sup>HR</sup> isolates from two different hosts (broccoli and cabbage).

#### Discussion

Procymidone, a DCF fungicide for ALSB control, has been extensively used for decades in China, however, there is a growing concern about its decreased efficacy nowadays. Our data in this study indicate that DCF-resistance has developed in natural populations of *A. alternata* from commercial fields of broccoli and cabbage in Yancheng, Jiangsu Province, China. Pro<sup>LR</sup> populations developed faster than Pro<sup>HR</sup> populations in these fields during 2018–2019, and Pro<sup>HR</sup> populations from broccoli were larger than those from cabbage, which may be attributed to different selective pressures exerted by the use of a fungicide in different ways in the field. Long-term monitoring of population change should be performed to detect the dynamic of Pro<sup>HR</sup> population and formulate corresponding management strategy in time. To our knowledge, there has been no report so far on DCF-resistance in *A. alternata* from commercial fields of broccoli and cabbage.

DCF-resistance has been reported in several *Alternaria* species, including *A. alternata* (Dry et al. 2004), *A. longipes* (Luo et al. 2008), *A. brassicicola* (Avenot et al. 2005)



**Table 4** Mutations in the coding regions of *AaHK1* from *Alternaria alternata* Pro<sup>HR</sup> and Pro<sup>S</sup> isolates

Isolate	Origin	Nucleotide variation in coding region	Mutation in amino acid sequence	Structural domain
C10, C28	Cabbage /Pro <sup>S</sup>	WT (N)	N	N
B16, B21	Broccoli /Pro <sup>S</sup>	WT (N)	N	N
C5	Cabbage /Pro <sup>HR</sup>	CCC to GCC at nt <sup>280</sup>	P94A	Between N-terminus and HAMP1
C13	Cabbage /Pro <sup>HR</sup>	552-bp deletion between nt <sup>791</sup> – nt <sup>1344</sup>	A deletion of 184-AA residues	Between HAMP 1 and HAMP 3
C40	Cabbage /Pro <sup>HR</sup>	8-bp insertion between nt <sup>3294</sup> – nt <sup>3295</sup>	Frameshift mutation leading to premature termination at the codon <sup>1204</sup>	Rec
B30	Broccoli /Pro <sup>HR</sup>	GTG to CTG at nt <sup>1834</sup>	V612L	HAMP5
B88, B108	Broccoli /Pro <sup>HR</sup>	GAG to AAG at nt <sup>2122</sup>	E708K	HAMP6
B35	Broccoli /Pro <sup>HR</sup>	CAG to TAG at nt <sup>2770</sup>	Q924STOP	HATPase_c
B46	Broccoli /Pro <sup>HR</sup>	381-bp deletion between nt <sup>1707</sup> – nt <sup>2089</sup>	A deletion of 127-AA residues	Between HAMP4 and HAMP6
B53	Broccoli /Pro <sup>HR</sup>	2-bp deletion between nt <sup>3249</sup> – nt <sup>3252</sup>	Frameshift mutation leading to premature termination at the codon <sup>1104</sup>	Between HATPase_c and Rec
B102	Broccoli /Pro <sup>HR</sup>	N	N	N

Pro<sup>S</sup>, procymidone-sensitive isolates; Pro<sup>HR</sup>, high-level procymidone-resistant isolates; WT, wild type; nt, nucleotide; N, no mutation

and *A. arborescens* (Ma & Michailides 2004). However, the resistance mechanism of these fungal pathogens to DCFs and phenylpyrroles has not yet been determined. To elucidate the possible molecular mechanism of DCF-

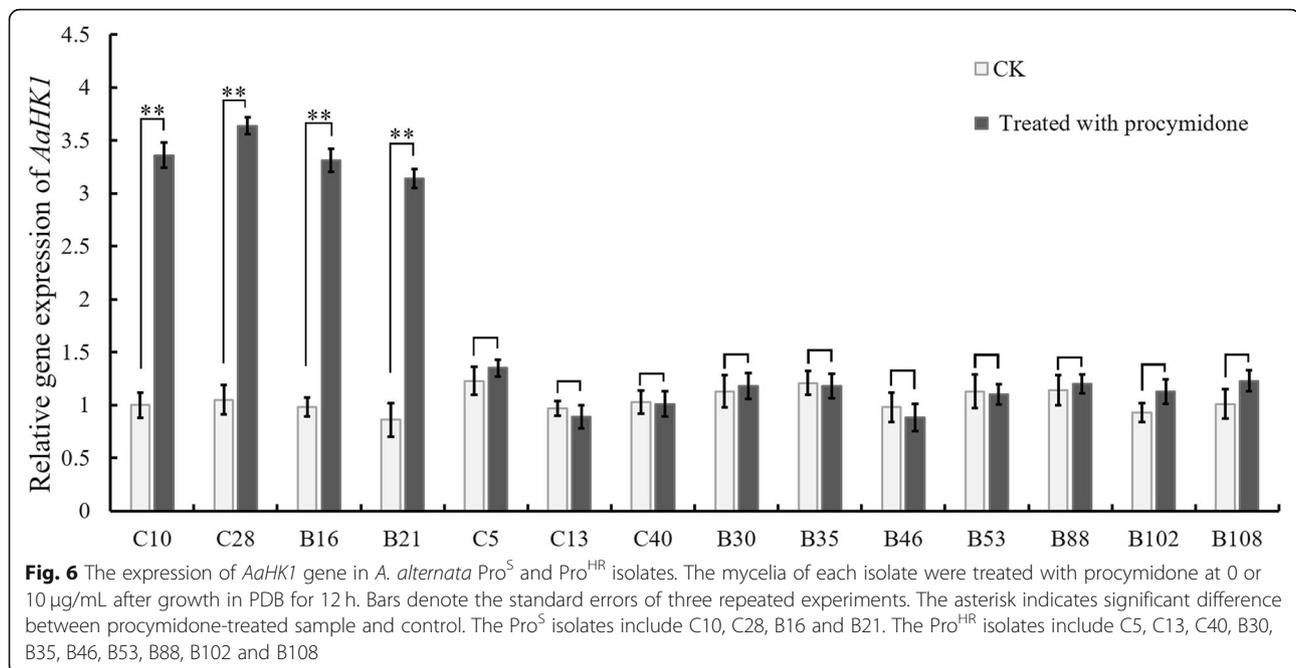
resistance, the nucleotide sequences of *AaHK1* of *A. alternata* Pro<sup>HR</sup> isolates from broccoli and cabbage were sequenced and aligned in this study. The results demonstrate that a high level of nucleotide sequence homology exists between *AaHK1* and *os-1* from *N. crassa* (Ochiai et al. 2010), *B. cinerea* (Ren et al. 2016), *A. brassicicola* (Avenot et al. 2005) and *A. longipes* (Luo et al. 2008).

**Table 5** Nucleotide sequence accession numbers corresponding to *AaHK1* from Pro<sup>HR</sup> and Pro<sup>S</sup> isolates of *Alternaria alternata* in this study

Isolate	Origin	Accession number	Reference
C10	Cabbage/Pro <sup>S</sup>	MZ268605	This article
C28	Cabbage/Pro <sup>S</sup>	MZ268606	This article
B16	Broccoli/Pro <sup>S</sup>	MZ268607	This article
B21	Broccoli/Pro <sup>S</sup>	MZ268608	This article
C5	Cabbage/Pro <sup>HR</sup>	MZ268609	This article
C13	Cabbage/Pro <sup>HR</sup>	MZ268610	This article
C40	Cabbage/Pro <sup>HR</sup>	MZ268611	This article
B30	Broccoli/Pro <sup>HR</sup>	MZ268612	This article
B88	Broccoli/Pro <sup>HR</sup>	MZ268613	This article
B108	Broccoli/Pro <sup>HR</sup>	MZ268614	This article
B35	Broccoli/Pro <sup>HR</sup>	MZ268615	This article
B46	Broccoli/Pro <sup>HR</sup>	MZ268616	This article
B53	Broccoli/Pro <sup>HR</sup>	MZ268617	This article
B102	Broccoli/Pro <sup>HR</sup>	MZ268618	This article

Pro<sup>S</sup>, procymidone-sensitive isolates; Pro<sup>HR</sup>, high-level procymidone-resistant isolates

Abundant mutation types were detected in *AaHK1* of the Pro<sup>HR</sup> isolates from cabbage and broccoli, including deletions at HAMPs or between HATPase\_c and Rec domains, an insertion at the Rec domain, amino acid substitutions at HAMPs or HATPase\_c domain. The observed premature termination mutations in *AaHK1* were generated by a 2-bp deletion, an 8-bp insertion or C to T transition. Intriguingly, no mutation was detected in *AaHK1* of B102 (a Pro<sup>HR</sup> isolate from broccoli), with the same situation occurring in DCF-resistant isolates of *A. alternata* (from pistachio), *A. arborescens* (Ma & Michailides 2004) and *A. brassicicola* (Avenot et al. 2005). Additionally, in DCF-resistant *A. alternata* isolates from passionfruit, frameshift mutations caused by a 4-bp and an 11-bp insertion at HAMP5 and HAMP1 of *AaHK1*, respectively, confer resistance to DCFs (Dry et al. 2004). The diverse mutations are also found in homologous HK1s of other *Alternaria* species. The iprodione-resistant *A. brassicicola* isolates possess an amino acid substitution (E to K) at the HisKA domain, a frameshift caused by a 2-bp deletion at HAMP4, or nonsense



mutations at HAMPs or HATPase\_c domains in *AbNIK1* (Avenot et al. 2005), while the dimethachlon-resistant *A. longipes* isolates possess amino acid substitutions at HAMPs, nonsense mutation generated by amino acid substitution at HAMP5, or a 321-bp deletion between HAMP5 and HAMP6 of *AIHK1* (Luo et al. 2008). All the mutations detected at different domains of HKs in DCF-resistant isolates might affect their corresponding biochemical characteristics, including the resistance against DCFs. In this study, although not all *A. alternata* Pro<sup>HR</sup> isolates possessed mutation in *AaHK1*, this might be the dominant mechanism involved in their DCF-resistance.

In yeast and *B. cinerea*, cellular responses to osmotic stress are controlled by the HOG-MAPK signaling pathway through which a range of osmotic responses including glycerol synthesis are regulated (Brewster et al. 1993; Cui et al. 2002). In this study, when treated with 10 µg/mL procymidone, MIGAs and the expression level of *AaHK1* were significantly increased in the Pro<sup>S</sup> isolates compared with those under procymidone-free conditions, while this increase in the ten Pro<sup>HR</sup> isolates was at a relatively low level, the same as that observed in fludioxonil-resistant *B. cinerea* isolates (Ren et al. 2016). The results indicate that *AaHK1* plays a role in the HOG-MAPK pathway, and a negative correlation exists between the resistance level of *A. alternata* to procymidone and the increase of MIGAs under procymidone-stressed condition. It was reported that the high level iprodione- or fludioxonil-resistant isolates of *A. alternata* from passionfruit exhibited moderate sensitivity to osmotic stress, identical to that of *A. brassicicola* (Dry

et al. 2004; Avenot et al. 2005). Nevertheless, in this study, all the Pro<sup>HR</sup> isolates from broccoli and cabbage were highly sensitive to osmotic stresses. The difference in sensitivity to osmotic stress agents in *Alternaria* spp. suggest that mutations in *HK1s* might affect signal transduction of the HOG-MAPK signaling pathway, consequently resulting in distinct responses to stress agents.

For other regulatory functions of HK1s in the development of DCF-resistance, Steel (Steel 1996) reported that the resistance to DCFs is positively correlated with the activity of an anti-oxidant enzyme, catalase. Compared with Pro<sup>S</sup> isolates, the sensitivity of Pro<sup>HR</sup> isolates (with mutations in *AaHK1*) to oxidative stress (H<sub>2</sub>O<sub>2</sub>) was reduced significantly, whereas, no significant difference in sensitivity to H<sub>2</sub>O<sub>2</sub> was observed in B102, a Pro<sup>HR</sup> isolate having no mutations in *AaHK1*. This suggests that mutations leading to inactivation of HK1 may result in constitutive induction of catalase, as observed in DCF-resistant *B. cinerea* and *A. alternata* isolates from passionfruit (Steel & Nair 1995; Steel 1996). In contrast, the sensitivity to oxidative stress in HK mutants of *A. longipes* from tobacco was not affected (Luo et al. 2012). These results indicate that HKs from *A. alternata* and *A. longipes* have a different regulatory function in signal transduction during oxidative stress. In addition, all Pro<sup>HR</sup> isolates were more resistant to cell membrane agent (0.05% SDS) than those of Pro<sup>S</sup> isolates, indicating that the group III HK of *A. alternata* is probably associated with membrane permeability. The enhanced tolerance to H<sub>2</sub>O<sub>2</sub> and SDS in Pro<sup>HR</sup> isolates is suggested to protect their cells from the deleterious effects of DCFs.

Fitness is an extremely essential parameter for evaluation of the potential risk of fungicide-resistant populations. Many DCF-resistant isolates display a fitness penalty relative to DCF-sensitive isolates, as those found in *S. sclerotiorum* (Kuang et al. 2011), *B. cinerea* (Ren et al. 2016) and *Penicillium expansum* (Li & Xiao 2008). Although most Pro<sup>HR</sup> isolates of *A. alternata* in current study was also reduced in mycelial growth rate, sporulation capacity and virulence, the Pro<sup>HR</sup> isolates B46, B35 and B53 were increased significantly in sporulation capacity or pathogenicity compared with Pro<sup>S</sup> isolates. This suggests that Pro<sup>HR</sup> populations of *A. alternata* from commercial fields of broccoli and cabbage as a whole possessed no superiority over Pro<sup>S</sup> populations (wild type), and was in a stage of slow development in these fields during 2018–2019. However, the Pro<sup>HR</sup> isolates with high fitness may have the potential to develop into a large group if no reasonable control methods are adopted. Therefore, the growers should be encouraged to use mixed fungicides with different modes of action (e.g., QoIs, DMIs or SDHIs) to delay the development of DCF-resistant *A. alternata* populations in these fields.

## Conclusions

The resistance to procymidone has developed in natural populations of *A. alternata* infecting broccoli and cabbage crops in Jiangsu Province of China. Most Pro<sup>HR</sup> isolates displayed a fitness penalty in mycelial growth rate, sporulation and virulence compared with Pro<sup>S</sup> isolates. The Pro<sup>HR</sup> isolates were highly sensitive to osmotic stress. In addition, a positive cross-resistance was observed between procymidone and fludioxonil or iprodione. Mutations in *AaHK1* were involved in the resistance of *A. alternata* isolates to DCFs. No significant differences in biochemical characteristics was observed between Pro<sup>HR</sup> isolates from broccoli and cabbage.

## Methods

### Sample collection, causal agent isolation and identification

All the 198 isolates of *A. alternata* were isolated from symptomatic leaves during 2018–2019. Diseased leaves were collected from different commercial fields of broccoli and cabbage at Yancheng City, Jiangsu Province, China, where procymidone (a member of DCFs), alone or mixed with other fungicides with different mode of action, had been extensively used to control ALSB twice or three times in a growing stage since 1980s. Any two neighboring samples were separated from each other at least 50 m, with each sample placed in an individual envelope. To obtain *A. alternata* isolates, small tissue pieces cut from lesion edges were sterilized in 1% NaClO for 3 min, washed three times with sterile distilled water,

and transferred onto Petri dishes containing PDA medium supplemented with 100 µg/mL streptomycin sulfate (Solarbio Science & Technology Co., Ltd.) (Ren et al. 2016). After incubation at 25 °C for 6 days, a mycelial plug was cut from the margin of an actively growing colony, and transferred to the center of a fresh PDA plate. Conidia were harvested using sterile distilled water after 6 days of incubation at 25 °C, and the single-spore isolate was obtained by picking a single conidium under a microscope (Zhang et al. 2017). In total, 198 single-spore isolates were obtained. The representative isolates were further identified by polymerase chain reactions (PCR) with two primer pairs ITS1/ITS4 and KOG1058F/KOG1058R (Table 6) (Woudenberg et al. 2015). The ITS and KOG1058 sequences of each isolate were blasted and congeneric sequences were obtained from the National Center for Biotechnology Information (NCBI) GenBank database. Phylogram analysis was processed by MEGA7 software based on ITS and KOG1058 sequences to further identify the isolates.

## Fungicides

All fungicides used are technical grade. Fludioxonil and iprodione (98% active ingredient; generously provided by Yangzhou Younuo Chemicals Co., Ltd., Yangzhou, China) were dissolved in methanol to obtain a stock solution of  $2 \times 10^4$  mg/L. Procymidone (99.2% active ingredient; provided by Jiangsu Xinyi Chemical Co., Ltd., Xuzhou, China) was dissolved in acetone to obtain a stock solution of  $2 \times 10^4$  mg/L. All stock solutions were kept at 4 °C before use.

## Determination of resistance frequencies of *A. alternata* isolates to procymidone

Sensitivities of 198 single-conidium isolates of *A. alternata* to procymidone were determined by discriminatory doses of mycelial growth inhibition method. In brief, procymidone at 0, 10, 50 and 100 µg/mL were used to determine sensitivities of all isolates for low-resistance (LR), moderate-resistance (MR) and high-resistance (HR). The isolate was considered procymidone-sensitive (Pro<sup>S</sup>) when the MIC (minimum inhibitory concentration) value was less than 10 µg/mL. Similarly,  $10 < \text{MIC} < 50$  µg/mL for low-level procymidone-resistant (Pro<sup>LR</sup>) isolates,  $50 < \text{MIC} < 100$  µg/mL for moderate-level procymidone-resistant (Pro<sup>MR</sup>) isolates, and  $\text{MIC} > 100$  µg/mL for high-level procymidone-resistant (Pro<sup>HR</sup>) isolates. Mycelial plugs (5 mm in diameter) were cut from the margin of a 6-day-old colony, and transferred to the center of Petri dishes containing PDA medium (one plug per dish) amended with procymidone at the above-mentioned concentrations. At 6 days of incubation, the sensitivity of each isolate to procymidone was determined based on the mycelial colony growth

**Table 6** Primers used in this study

Primer set	Forward primer (5'-3')	Reverse primer (5'-3')	Description <sup>a</sup>
1	TGTGGCTTGGCGTTATTTC	CGACGGCAGTGACGATG	Amplification of <i>AaHK1</i> from -128 to + 820
2	AGCCAGGAAGTGAATAAGCA	TTCGTGAGTCTCATAAC	Amplification of <i>AaHK1</i> from + 726 to + 2097
3	CAACAATGTCAACACTATGG'	CTTCTCCGAGCTCAGTAAAC	Amplification of <i>AaHK1</i> from + 1914 to + 2705
4	GAATGACCCAACCTTACGCT	CATAAGTACGCAATCGTAAAC	Amplification of <i>AaHK1</i> from + 2598 to + 3672
5	ATGACGACGCCCTGTCTACC	ATCCAATACACAAAAATG	Amplification of <i>AaHK1</i> from + 3551 to + 4505
6	TCCGTAGGTGAACCTGCGG	TCCTCCGCTTATTGATATGC	Amplification of ITS to identify <i>A. alternata</i>
7	GAGTCACGTTAYCGCASC	TGGCTKACGGARACG	Amplification of KOG1058 to identify <i>A. alternata</i>
8	ACGCTTCTCATCTCCAAGATCCGT	AGAGAGCCTCGTTGTCAATGCAGA	Amplification of $\beta$ -tubulin for qPCR
9	CTTACAAGGTGCCCGACTAC	GATCTGGGTGCGATTCTTGT	qPCR primers for analysis of <i>AaHK1</i> expression

<sup>a</sup> For primer set 1–5, the first nucleotide of the start codon in the *AaHK1* gene was considered as position + 1

inhibited by procymidone. The resistance frequency was determined using the following formula: resistance frequency (%) = (the number of resistant isolates/the number of the total isolates tested)  $\times$  100%. The experiment was performed three times with three replications for each isolate.

#### Determination of sensitivities of *A. alternata* isolates to procymidone

To determine the sensitivity of *A. alternata* isolates to procymidone, the fungicide was prepared in a serial concentrations of 0, 25, 50, 100, 200, 400 and 800  $\mu$ g/mL for Pro<sup>HR</sup> isolates, and another serial concentrations of 0, 0.03, 0.1, 0.3, 1, 3 and 9  $\mu$ g/mL for Pro<sup>S</sup> isolates. The 6-day-old mycelial plugs (5 mm in diameter) were separately transferred to the center of PDA plates with above-mentioned concentrations of procymidone. Percentage of inhibition was evaluated and EC<sub>50</sub> (effective concentration for 50% mycelial inhibitive growth) was calculated for each tested isolate using Data Processing System (DPS 7.05). The experiment was performed three times with three replications for each isolate.

#### Cross-resistance test among different fungicides

The sensitivity of *A. alternata* isolates to fludioxonil and iprodione were determined by measuring mycelial growth rate. One serial concentrations (0, 25, 50, 100, 200, 400 and 800  $\mu$ g/mL) were set up to test the sensitivity of resistant isolates to fludioxonil or iprodione, and another serial concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6  $\mu$ g/mL) for sensitive isolates. EC<sub>50</sub> values were determined with the same method as above-mentioned. Spearman's rank correlation coefficient for

the log<sub>10</sub>-transformed EC<sub>50</sub> values was used to analyze cross-resistance pattern between procymidone and fludioxonil or iprodione. The experiment was performed three times with four replicates per concentration.

#### Determination of radial growth rates and sporulation capacities of *A. alternata* isolates

Six-day-old mycelial plugs (5 mm in diameter) of *A. alternata* isolates were transferred to the center of Petri dishes containing PDA medium (one plug per dish), then placed in an incubator at 25 °C in darkness. At 6 days of incubation, the average colony diameter for each isolate was measured in two perpendicular directions. The experiment was performed three times with three replications for each isolate.

For sporulation test, conidia were harvested from the surface of six-day-old PDA colonies washed with 10 mL sterile water (containing 0.1% Tween 20), filtered through four layers of sterile lens papers, and centrifuged at 5000 rpm for 1 min. The pellets were re-suspended in 1 mL sterile distilled water. The concentrations of conidial suspensions were measured with a hemacytometer under microscope. Six replicates were performed for each isolate, and the experiment was repeated three times.

#### Virulence assay

Broccoli (var. Hanxiu) leaves were used to determine the pathogenicity of *A. alternata* isolates. Leaves were surface-disinfected with 75% alcohol for 10 s, and inoculated with six-day-old hyphal plugs (5 mm in diameter) of each isolate. Before inoculation, a sterilized needle was used to produce wound. All the inoculated leaves were transferred onto three layers of moistened filter

paper, and incubated at 25 °C with 85% relative humidity (RH) and a 12-h light/12-h dark cycle. The lesion areas were calculated at 7 dpi (days post-inoculation). The experiment was performed three times.

#### Determination of responses of *A. alternata* isolates to various environmental stresses

To evaluate responses of *A. alternata* isolates to different stress agents, all isolates were incubated at 25 °C for 6 days. Then, mycelial plugs (5 mm in diameter) were cut from the edge of actively growing colonies, and transferred onto PDA plates amended with 0.05% sodium dodecyl sulfate (SDS) (w/v), 0.05% Congo red (CR) (w/v), 1.2 M KCl, 1.2 M NaCl or 10 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (v/v). The isolates incubated in PDA plates without stress agents were served as controls. All treatments were incubated for 6 days at 25 °C, with three replicate plates for each agent. The inhibition percentages of mycelial growth by these stress agents were determined for each tested isolate. This experiment was performed three times.

#### Determination of intracellular glycerol accumulations in *A. alternata* isolates

A commercial assay kit (Applygen Technologies Inc., Beijing) was used to determine intracellular glycerol accumulations in mycelia of *A. alternata* isolates in accordance with the manufacturer's instruction. In brief, all isolates were incubated in potato dextrose broth (PDB) in a shaker (175 rpm) at 25 °C for 2 days. At 5 h after treatment with 10 µg/mL procymidone, mycelia of each isolate were collected with sterile distilled water, lyophilized and grounded in mortars amended with liquid nitrogen (Ren et al. 2016). Mycelial powder (0.1 g) was then transferred into a 2 mL centrifuge tube, and mixed in 1 mL glycerol extraction buffer (Applygen Technologies Inc., Beijing, China). The mixture was vortexed for 5 min and centrifuged at 5000 rpm for 20 min, and the supernatants were used to analyze mycelial intracellular glycerol concentrations (Duan et al. 2013). This experiment was repeated three times with four replicates for each isolate.

#### DNA extraction and sequence analysis of *AaHK1*

Genomic DNAs were extracted from mycelia of *A. alternata* isolates with a commonly used cetyltrimethylammonium bromide (CTAB) method. Based on the sequence of *AaHK1* (GenBank accession No. GQ414508.1) deposited in NCBI GenBank database, five primer pairs (Table 6) were designed to amplify the complete nucleotide sequence of *AaHK1*. Polymerase chain reactions (PCR) were conducted in a 25 µL volume, containing 0.25 µL of high-fidelity LA polymerase (5 U/mL) (TaKaRa, Japan), 1 µL of each primer (10 mM),

2.5 µL of 10× LA polymerase buffer (Mg<sup>2+</sup> free), 50 ng of genomic DNA, 2.5 µL of MgCl<sub>2</sub> (25 mM), 4 µL of dNTP mixture (2.5 mM of each dNTP) and 12.75 µL of ddH<sub>2</sub>O. The PCR program consisted of an initial denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 50s; and a terminal extension at 72 °C for 10 min. Each PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, MD, USA), and sequenced by the Sangon Biotech Co., Ltd. (Shanghai, China). The amino acid sequences were analyzed by DNAMAN software (version 6.0; Lynnon Biosoft Bioinformatic Solutions). PCR amplifications were performed three times for each isolate to avoid sequence mismatches during PCR amplification and sequencing.

#### Determination of the gene expression level of *AaHK1*

To analyze the relative gene expression level of *AaHK1* in *A. alternata* isolates, mycelial plugs were incubated in flasks containing PDB, with six replicate flasks for each isolate. After 2 days of incubation at 25 °C in a shaker, three flasks were treated with 10 µg/mL of procymidone, and the other three were added with the same volume of sterile water and used as controls. After 12 h, the mycelia of each isolate were harvested for RNA extraction using Total RNA Isolation kit (Promega, USA). Further, cDNA was synthesized using PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Japan). Primers used for quantitative PCR (qPCR) are shown in Table 6. qPCR was performed in an ABI 7500 Real-Time Detection System (Applied Biosystems) using SYBR Green I fluorescent dye detection. Amplification was performed in a 20-µL volume containing 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), 1 µL of each of the forward and reverse primers (10 µM), 0.4 µL of 50 × ROX Reference Dye 2, 1 µL of reverse transcription product, and 6.6 µL of ddH<sub>2</sub>O. The expression of *AaHK1* was normalized to that of β-tubulin gene. The transcription level of *AaHK1* in each isolate relative to that of β-tubulin gene was calculated using the 2<sup>-ΔΔCt</sup> method (Wei et al. 2019). The experiment was repeated three times.

#### Statistical analysis

The SIGMA-STAT Statistical Software Package (SPSS Science, version 11) was used to analyze the data. Fisher's protected least significant difference ( $P = 0.05$ ) test was calculated to evaluate statistical significance.

#### Abbreviations

ALSB: Alternaria leaf spots and blights; DMIs: Sterol demethylation inhibitors; DCFs: Dicarboximide fungicides; DDK: C-terminus receiver domain harboring a three amino-acid signature; EC<sub>50</sub>: Effective concentration for 50% mycelial inhibitive growth; HAMPs: Histidine kinases-Adenylate cyclases-Methyl accepting proteins and Phosphatases; HATPase\_c: Cognate histidine kinase-like ATPase catalytic subdomains; HiskA: Histidine kinase A; HK: Histidine

kinase; MAPK: Mitogen-activated protein kinase; MIGAs: Mycelial intracellular glycerol accumulations; Pro<sup>HR</sup>: High-level procymidone-resistant; Pro<sup>LR</sup>: Low-level procymidone-resistant; Pro<sup>MR</sup>: Moderate-level procymidone-resistant; Pro<sup>R</sup>: Procymidone-resistant; Pro<sup>S</sup>: Procymidone-sensitive; Qols: Quinol-oxidizing inhibitors; RH: Relative humidity; SDHIs: Succinate dehydrogenase inhibitors; MIC: Minimum inhibitory concentration

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#### Authors' contributions

BRW, TCL, LLW and WCC conducted the experiments. All authors analyzed the data. BRW, TCL and CCJ wrote and revised the manuscript. All authors read and approved the final manuscript.

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#### Availability of data and materials

Not applicable.

#### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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