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Research Article

Characterization of mutations in the two-component histidine kinase gene *AbNIK1* from *Alternaria brassicicola* that confer high dicarboximide and phenylpyrrole resistance

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Abstract Highly iprodione- and fludioxonil-resistant field and laboratory isolates of *A. brassicicola* were found to be either moderately sensitive or tolerant to osmotic stress. *AbNIK1*, a two-component histidine kinase gene, was isolated from a fungicide-sensitive strain. The predicted protein possessed the six tandem amino acid repeats at the N-terminal end, which is a landmark of osmosensor histidine kinases from filamentous fungi. A comparison of the nucleic acid sequences of the *AbNIK1* gene from fungicide-sensitive and fungicide-resistant isolates revealed the presence of mutations in six of the seven resistant strains analyzed. Null mutants were all found to be moderately sensitive to osmotic stress, indicating that they are similar to *Neurospora crassa* Type I *os-1* mutants. Only one mutation, corresponding to a single amino acid change within the H-box of the kinase domain, was found in an osmotolerant strain. These results suggest that AbNIK1p participates in osmoregulation and that expression of the fully functional enzyme is essential for dicarboximide and phenylpyrrole antifungal activities.

Keywords *Alternaria brassicicola* · Dicarboximides · Osmotic sensitivity · Phenylpyrroles · Two-component histidine kinase

U. Kück

Introduction

Alternaria brassicicola causes blackspot disease in cultivated *Brassica* and *Raphanus* spp throughout the world. This seed-borne fungal pathogen is responsible for considerable financial loss to growers. As commercial cultivars usually do not express resistance towards pathogenic *Alternaria* species, the disease is mainly managed via fungicide treatments. In particular, the dicarboximide fungicide iprodione is widely used as an active ingredient for seed dressing and spray programs to limit outbreaks of the disease on crucifers. However, field isolates of *A. brassicicola* highly resistant to dicarboximides were recently identified (Iacomi-Vasilescu et al. 2004). These isolates were also found to be highly resistant to phenylpyrroles. Laboratory mutants highly resistant to dicarboximides and phenylpyrroles have already been reported for some fungal species (Fujimura et al. 2000; Yoshimi et al. 2003). However, the phenotype of the *A. brassicicola* isolates was uncommon since field isolates resistant to dicarboximides were usually phenylpyrrole-sensitive (Oshima et al. 2002) . Moreover, in vitro tests revealed that, contrary to most laboratory mutants, *A. brassicicola* isolates were still pathogenic to host plants (Iacomi-Vasilescu et al. 2004).

The cellular targets of phenylpyrroles and dicarboximides have not yet been identified, but it was shown that they both stimulate glycerol synthesis in *Neurospora crassa* (Pillonel and Meyer 1997). This observation and the fact that osmosensitive (*os*) mutants of *N. crassa* were also found to be resistant to dicarboximides and fludioxonil (Fujimura et al. 2000) suggest that the response to osmotic stress and fungicide resistance might be linked. In this model filamentous fungus, the *nik1*/*os-1* gene encodes a two-component histidine kinase (Alex et al. 1996; Schumacher et al. 1997) that was predicted to be the sensor in an osmotic response signal transduction cascade constituted by MAP kinases encoded by other osmotic loci such as *os-2*,*os-4* and *os-5* (Fujimura et al. 2003; Zhang et al. 2002). Ochiai et al. (2001) showed that mutations in the 90 N-terminal amino acid repeats of the histidine kinase Nik1p/Os-1p confer fungicide resistance and osmotic sensitivity. Similarly, mutations within the amino acid repeat domains of BcOs1p and AaHK1p,

i.e. os-1p homologues from *Botrytis cinerea* and *A. alternata*, respectively, were found in iprodione-resistant field isolates (Oshima et al. 2002; Cui et al. 2004; Dry et al. 2004).

In this study, we supplemented the phenotypic characterization of highly fungicide-resistant isolates (field and laboratory mutant strains) of *A. brassicicola* by investigating their osmotic sensitivities. We also isolated *AbNIK1*, a *nik1*/*os1* orthologous gene, from a wild-type fungicide-sensitive *A. brassicicola* strain and compared its sequence with corresponding histidine kinase genes from fungicide-resistant strains. Two strain groups were identified on the basis of their responses to high osmotic pressure. All the fungicide-resistant strains displaying a moderately osmosensitive phenotype were found to have null mutations in the *AbNIK1* gene. Other resistant strains had a unique phenotype characterized by their tolerance to osmotic stress and either they were likely mutated at a locus not corresponding to *AbNIK1*, or they produced altered forms of the AbNIK1 protein.

Materials and methods

Strains and media

All strains used in this study are listed in Table 1. They were isolated in 1999 from radish or cabbage during quality controls of commercial seed lots (French isolates) or seeds from small local markets (Romanian isolates). All strains were purified by monospore isolation and maintained in tubes of malt agar medium (malt 20 g, agar 20 g in 1 l) at 4°C. The laboratory resistant Abra20M^{LR} and Abra43M^{LR} strains were obtained after transferring, onto selective medium, mycelial tips from fast-growing sectors observed with some sensitive isolates on iprodione-amended media. Fresh subcultures were made by transferring hyphal plugs to dishes of potato dextrose agar (PDA) medium amended or not with iprodione (dicarboximide) or fludioxonil (phenylyprrole) at a final concentration of 100 mg l^{1}. The effects of fungicides on spore germination and germ tube elongation were tested as described by Iacomi-Vasilescu et al. (2004). Mycelial plugs were transferred onto PDA containing 2–4% NaCl (w/v) in order to test sensitivity to osmotic stress.

[Table 1 will appear here. See end of document.]

DNA procedures

Genomic DNA was extracted from mycelium according to Adachi et al. (1993). For Southern analysis, DNA fragments resulting from genomic DNA digestion with *Eco*RI and *Hin*dIII were

separated on 1% agarose gels and vacuum-transferred to Hybond N membranes (Amersham Biosciences Europe, France). Blots were then probed with PCR fragments purified with Geneclean II (QBiogene Europe, France) and labeled with a random primer labeling kit (Amersham, Chalfont, UK). Fungal RNA was extracted, DNAse-treated and reverse-transcribed as described by Guillemette et al. (2004). PCR reactions using degenerated primers similar to those designed by Alex et al. (1996) and based on the conserved N and H boxes (N1A, 5′-GARAAY TTI AII GCR TT-3′; H1A, 5′-CAYGAI HTI MGI ACI CC-3′) were used to amplify a putative histidine kinase gene fragment from Abra43^{FS} genomic DNA. The amplification was performed in 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, containing 0.2 mmol of dATP, dCTP, dTTP and dGTP, 0.4 μmol of each primer, 50 ng of DNA and 1 unit of Taq polymerase (Promega, Madison, Wis.) in a final volume of 50 μl. PCR was carried out in a Thermojet (EquiBio, Seraing, Belgium) with 35 cycles at 95°C (1 min), 40°C (1 min) and 72°C (1 min), followed by a 30-min extension at 72°C. Amplification products were separated on 1.2% agarose gel, purified from gel by Gene-Clean II, cloned into a pGEM-T vector (Promega) and subsequently sequenced. Specific primers were designed from this sequence to perform PCR-walking according to the method described by Siebert et al. (1995). PCR amplifications (initial denaturation for 2 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 55°C, 1–4 min at 72°C, with a final extension for 10 min at 72°C) with specific primers were also performed on genomic DNA and first-strand cDNA in order to verify the presence of introns in coding sequences and to confirm the physical linkage between the 5 and 3′ amplified parts of the histidine kinase gene. A set of 11 primer pairs (Table 2) that span the whole coding sequence of the *AbNIK1* gene was designed for PCR amplification of corresponding portions of this gene using genomic DNA from three fungicide-sensitive strains (Abra43^{FS}, AbraCM^{FS}, Abra20^{FS}) and from the seven resistant mutants. Nucleotide sequences were obtained directly from these PCR products by Genome Express (France) and deposited in GenBank (accessions AY700092–AY00098). Sequences were compared using the Multalin program (Corpet 1988).

[Table 2 will appear here. See end of document.]

PCR-RFLP and heteroduplex mobility assays

Portions of the *AbNIK1* gene potentially carrying mutations were PCR-amplified using relevant primers and either directly digested with different restriction enzymes (Promega) according to the manufacturer's instructions or mixed for duplex formation as described by Sutra et al. (2001).

Digestion products or mixtures of hetero-duplexes and homo-duplexes were then separated on non-denaturing polyacrylamide gels.

Results

Osmosensitivity of fungicide-resistant isolates

Several *A. brassicicola* field isolates highly cross-resistant to dicarboximides and phenylpyrroles were described by Iacomi-Vasilescu et al. (2004). New laboratory and field resistant mutants were included in this study and all displayed a highly fungicide-resistant phenotype (Table 1). The sensitivity of these isolates to osmotic stress was evaluated by measuring their mycelial growth on PDA containing 2% and 4% NaCl (Fig. 1). The five fungicide-sensitive isolates (Abra43^{FS}, Abra20^{FS}, AbraRo10^{FS}, Abra1662^{FS}, AbraCM^{FS}) displayed different degrees of osmotic sensitivity on medium containing 4% NaCl, with only 62% inhibition for Abra43^{FS} and 68–71% inhibition for the other isolates. The fungicide-resistant isolates were separated into two groups based on their sensitivity to osmotic stress. The growth of isolates of the first group (Abra3^{FR}, Abra41^{FR}, Abra7407^{FR}, Abra20M^{LR}) was strongly inhibited (90–91%) on a medium containing 4% sodium chloride. In the second group, resistant isolates AbraCP^{FR}, Abra43M^{LR} and Abra40^{FR} were only moderately affected by osmotic stress (52.5–62.0% inhibition). The mean value obtained for the first group of resistant isolates (90.48±0.56% inhibition) differed significantly (P<0.001, two-tailed t-test) from that obtained for the second group (58.16±1.13% inhibition). The sensitivity of the latter isolates to osmotic stress was therefore not significantly different (P>0.05) from that of the fungicide-sensitive isolates (65.60±0.34% inhibition).

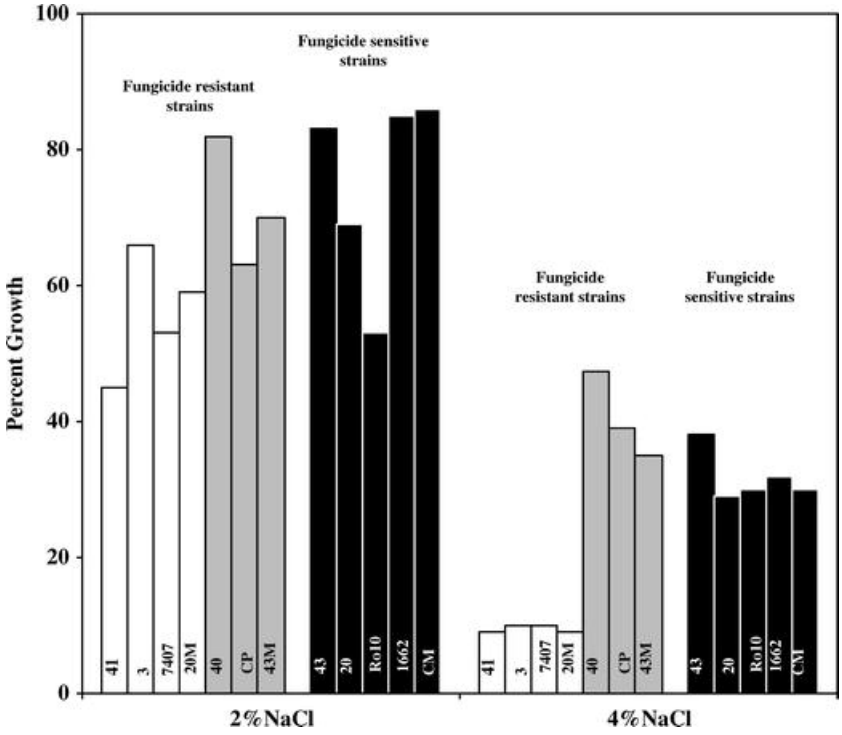

Fig. 1 Determination of linear growth rates of fungicide-resistant and fungicide-sensitive *A. brassicicola* strains on medium containing 2% and 4% NaCl. The linear growth rate of each strain was normalized to the growth rate on medium not containing NaCl. *White bars* Highly fungicide-resistant, moderately osmosensitive strains, *gray bars* highly fungicide-resistant, osmotolerant strains, *black bars* wild-type, fungicide-sensitive strains

Effect of fungicides on conidia and germ-tube morphology

We previously observed that fludioxonil strongly inhibited spore germination in wild-type *A. brassicicola*, even at low concentrations (Iacomi-Vasilescu et al. 2004). Examination of spores from a sensitive isolate after exposure to 10 μg ml^{1} fludioxonil revealed a majority of swollen conidia (Fig. 2a). At the same concentration, iprodione was less effective and 60% of the spores germinated. However, only short germ tubes with a tendency to swell, branch or even burst were observed (Fig. 2b). Fludioxonil or iprodione at the same concentrations did not inhibit spore germination or induce any morphological alterations in the germ tubes when applied to conidial suspensions from resistant strains (Fig. 2c,d).

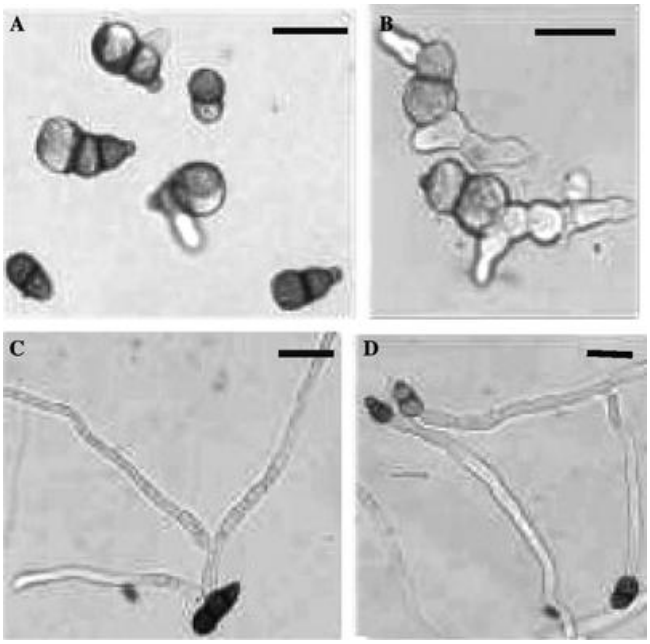

Fig. 2 Effects of fludioxonil and iprodione treatments on *A. brassicicola* conidial germination and germ-tube elongation. **a**, **b** Ungerminated and germinated conidia from the fungicide-sensitive strain Abra43^{FS} in the presence of 10 μg ml^{1} fludioxonil or iprodione, respectively. **c**, **d** Germinated conidia from the Abra3^{FR} fungicide-resistant strain in the presence of 10 μg ml^{1} fludioxonil or iprodione, respectively. *Bars* 20 μm

Cloning of a histidine kinase gene from *A. brassicicola*

Redundant oligonucleotide primers, based on those designed by Alex et al. (1996), were used to amplify putative histidine kinases from *A. brassicicola* strain Abra43^{FS} . A single 0.4-kb genomic DNA fragment was amplified under these conditions. This fragment was cloned and sequenced. The deduced amino acid sequence had high similarities with fungal histidine kinases. The PCR-walking procedure described by Siebert et al. (1995) was used to obtain the 3′ and 5′ sequences of the histidine kinase gene. The assembled 4,500-bp sequence contained the entire *AbNIK1* gene (Fig. 3a).

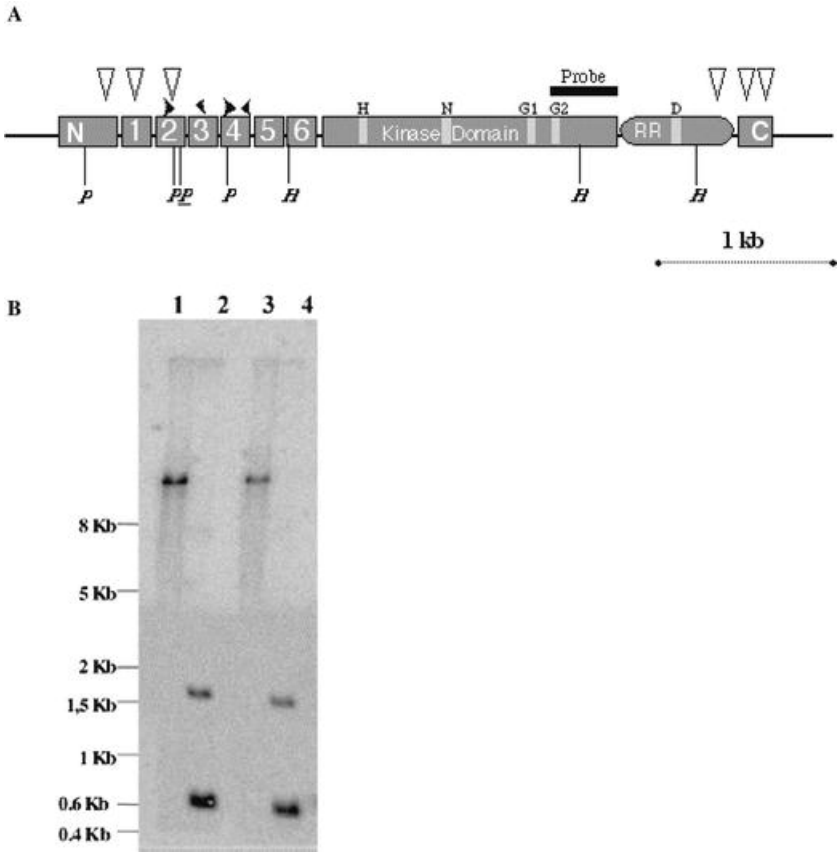

Fig. 3 Structure of the *AbNIK1* gene. **a** Schematic representation of the *AbNIK1* coding region in relation to the gene structure. Introns are indicated by *inverted triangles*. Protein-encoding domains are shown as *boxes*. The positions of the conserved H-, N-, G1- and G2-boxes in the kinase domain and the D-box in the response regulator (*RR*) domain are shown. The amino acid repeats are indicated by *numbers 1–6*. The restriction enzyme sites are denoted by the letters *H* for *Hin*dIII and *P* for *Pst*I. The *Pst*I site that is absent in strain Abra43M^{LR} due to a mutation is *underlined*. The *black arrows* indicate the position of the primer pairs used to amplify portions of the *AbNIK1* gene encoding amino acid repeats 2 and 4. The *bold line* indicates the fragment used for Southern analysis. **b** Southern analysis of chromosomal DNA isolated from the wild-type Abra43^{FS} strain (*lanes 1, 2*) and the mutant Abra40^{FR} strain (*lanes 3, 4*) digested with *Eco*RI (*lanes 1, 3*) and *Hin*dIII (*lanes 2, 4*). A 0.4-kb DNA fragment corresponding to the C terminal part of the kinase domain of the *AbNIK1* gene was used as probe

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Southern blot analysis of genomic DNA isolated from the fungicide-sensitive Abra43^{FS} strain and the fungicide-resistant Abra40^{FR} strain showed that *AbNIK1* is a single-copy gene (Fig. 3b). As expected from the *AbNIK1* gene sequence, two *Hin*dIII-DNA fragments (1.6 kbp, 0.6 kbp) and a single *Eco* RI-DNA fragment (13.0 kbp) hybridized with the probe.

The predicted 3,987-bp ORF was interrupted by six introns ranging from 50 bp to 58 bp in size (Fig. 3a). The *AbNIK1* genes from two other fungicide-sensitive isolates (Abra20^{FS}, AbraCM^{FS})

were also sequenced. Comparison of the three nucleotide sequences revealed only a few differences, but all were predicted to be silent.

The predicted 1,329-amino-acid protein sequence exhibited all the characteristic features of histidine kinases, including the conserved H-, N-, D-, G1- and G2-boxes (Fig. 4). The *A. brassicicola* protein, like other group III histidine kinases from filamentous fungi, possesses six 90-amino-acid repeat motifs near the N-terminus, putatively forming a coiled-coil domain (Catlett et al. 2003). The overall amino acid sequence identity of AbNIK1p with BmHK1p, BcOS1p and NcOS1p, three two-component osmosensing histidine kinases from *Cochliobolus heterostrophus*, *B. cinerea* and *N. crassa*, was 92, 64 and 60%, respectively. At the nucleotide level, more than 80% identity was noted when comparing the *AbNIK1* and *BmHK1* genes. Only five introns have been identified in the latter sequence (Yoshimi et al. 2004) that were mapped at exactly the same position as the first five intervening sequences found in the *A. brassicicola* gene. The presence of a sixth intron in the *AbNIK1* sequence only 24 bp upstream of the stop codon was demonstrated by cDNA sequencing.

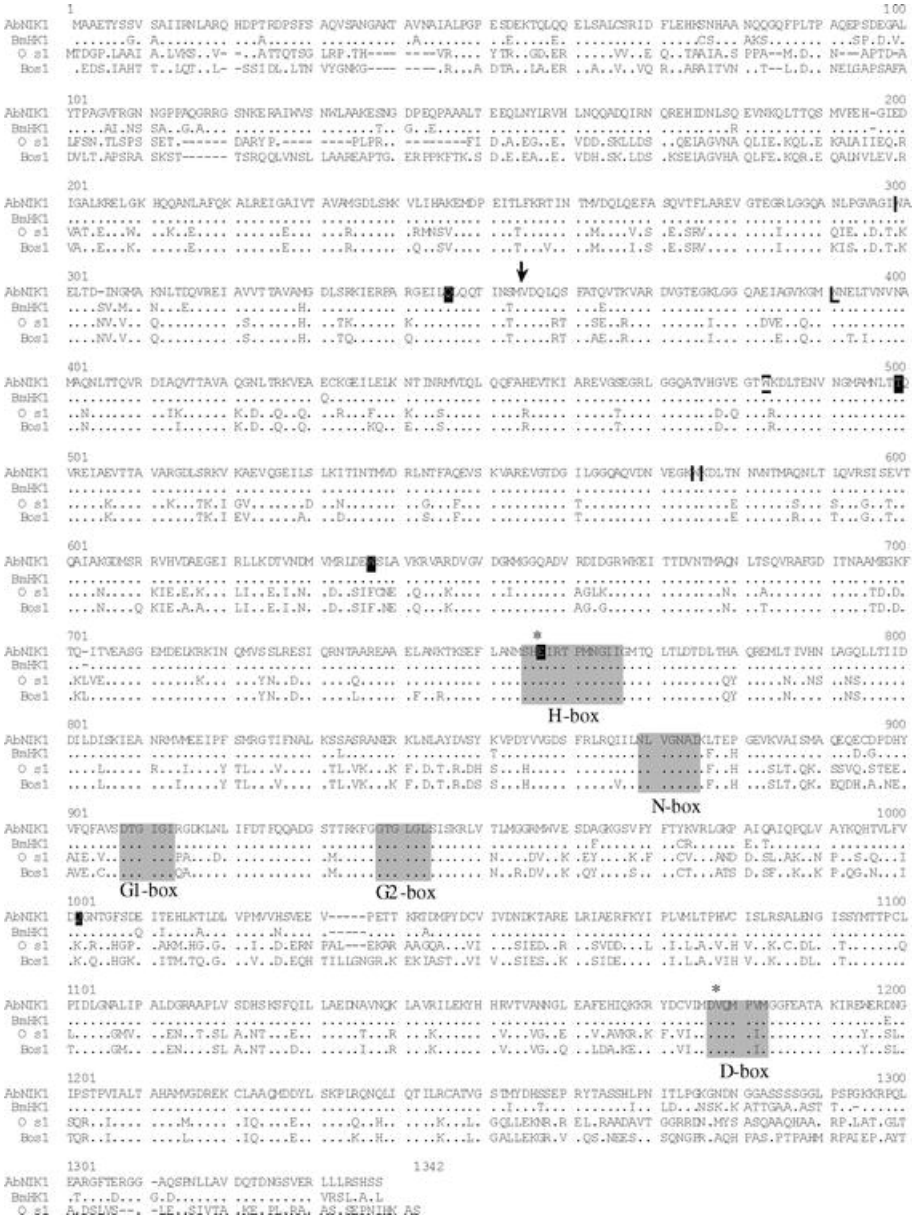

Fig. 4 Sequence alignment of AbNIK1p with BmHK1p (*C. heterostrophus*), Os1p (*N. crassa*) and Bos1p (*B. cinerea*). Amino acid identities between AbNIK1 and the other isolates are indicated by *dots*. The lack of a corresponding amino acid is indicated by a *hyphen*. *Asterisks* indicate putatively phosphorylated conserved histidine (*H*) and aspartic acid (*D*) residues. *Square letters* indicate the start of the amino acid repeats. The conserved H-, N-, G1-, G2- and D-boxes are marked and indicated by a *grey background*. *Residues* indicated in *white on a black background* correspond to positions of mutations in the *A. brassicicola* fungicide-resistant strains . The *arrow* indicates a possible start codon in repeat 2

Mutant strain characterization

Several pairs of specific primers (listed in Table 2) that span the whole *AbNIK1* gene sequence were designed and used to amplify parts of the corresponding gene from various fungicide-sensitive

and fungicide-resistant isolates. Mutations were localized in the *AbNIK1* gene for the four field isolates (Abra40^{FR}, 41^{FR}, 3^{FR}, 7407^{FR}) and the two laboratory mutants (Abra43M^{LR}, Abra20M^{LR}; Table 3). Among those analyzed, only one fungicide-resistant field isolate (AbraCP^{FR}) had no mutation in the *AbNIK1* gene. Half of the detected mutations were transitions generating stop codons in the second and fifth amino acid repeat (strains Abra43M^{LR}, Abra7407^{FR}, respectively), or in the kinase domain of the protein (strain Abra3^{FR}). An identical 2-bp deletion leading to a frameshift, and also located within one of the repeats, was found in two mutants: the Abra20M^{LR} laboratory strain and the Abra41^{FR} field strain. Finally, only one amino acid substitution, i.e. a glutamic acid at position 753 substituted for lysine, was found in strain Abra40^{FR}. This single amino acid change occurred within the H-box domain. These results contrast with those obtained for *B. cinerea* field and laboratory isolates (Cui et al. 2002, 2004; Oshima et al. 2002), where resistance to dicarboximide fungicides was exclusively associated with amino acid substitutions in the coiled-coil domain.

[Table 3 will appear here. See end of document.]

For several *A. brassicicola* mutants, sequence alterations led to restriction site modifications. This could ultimately be used to selectively detect the behavior of mutants in pathogenicity assays, in the presence or absence of a competitive wild-type population and with or without fungicide treatments. The C to T transition in strain Abra3^{FR} thus created a new *Dde*I site, while the G to A transition in strain Abra7407^{FR} generated a new *Sau3*AI site and the C to T transition in strain Abra43M^{LR} eliminated a *Pst*I site. Primer pairs were designed to amplify mutation-containing fragments that were subsequently digested with the appropriate enzyme. Restriction fragments were then separated on polyacrylamide gels. Concerning the 2-bp deletions (strains Abra41^{FR}, Abra20M^{LR}), heteroduplex mobility assays were used to highlight differences between mutant and wild-type sequences. Data given in Fig. 5 show the validity of the two types of assay for the detection of specific mutations in the *AbNIK1* gene.

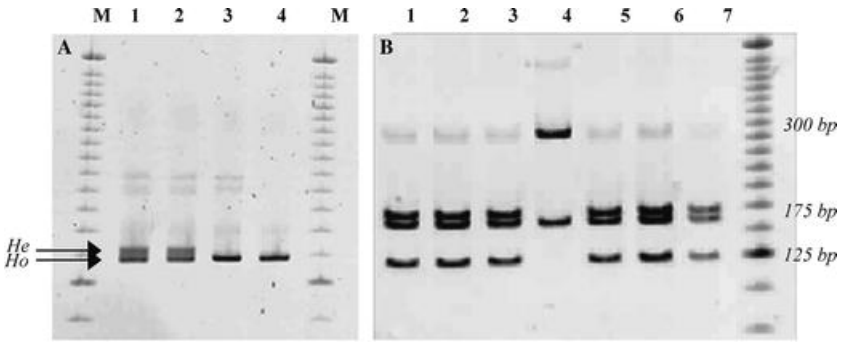

Fig. 5 Electrophoretic assays for the detection of specific mutations in the *AbNIK1* gene of *A. brassicicola*. **a** Heteroduplex mobility assays were conducted by mixing amplified DNA fragments corresponding to the portion of the *AbNIK1* gene encoding the amino acid repeat 4 from a wild-type AbraCM^{FS} strain and three mutant strains (Abra3^{FR}, Abra41^{FR}, Abra20M^{LR}). Samples contained amplified products from: Abra41^{FR} and AbraCM^{FS} (*lane 1*), Abra20M^{LR} and AbraCM^{FS} (*lane 2*), AbraCM^{FS} and Abra3^{FR} (*lane 3*). *Lane 4* was loaded with the amplified products from AbraCM^{FS} only. The positions of homoduplexes (*Ho*) and heteroduplexes (*He*) are shown. **b** RFLP analysis of *Pst*1-digested amplification products corresponding to the portion of the *AbNIK1* gene encoding the amino acid repeat 2. Amplification products were obtained using genomic DNA from strains Abra40^{FR} (*lane 1*), Abra41^{FR} (*lane 2*), Abra7407^{FR} (*lane 3*), Abra43M^{LR} (*lane 4*), Abra20M^{LR} (*lane 5*), Abra3^{FR} (*lane 6*) and AbraCM^{FS} (*lane 7*). In **a** and **b**, electrophoresis was carried out in 5% non-denaturing polyacrylamide Tris-borate-EDTA gels; and *M lanes* were loaded with a 25-bp ladder

Discussion

In *A. brassicicola*, all the resistant strains included in this study, either isolated from several fields in France or obtained spontaneously in our laboratory, displayed the same phenotype regarding their sensitivity to fungicides. All were found to be highly resistant to iprodione and fludioxonil. Laboratory dicarboximide-resistant mutants with positive cross-resistance to phenylpyrroles have already been obtained in other filamentous fungi, such as *N. crassa* (Fujimura et al. 2000; Miller et al. 2002), *B. cinerea* (Cui et al. 2002) and *C. heterostrophus* (Yoshimi et al. 2003). In contrast, field dicarboximide-resistant strains were found to be sensitive to phenylpyrroles in *B. cinerea* (Oshima et al. 2002). Recently, Dry et al. (2004) reported fungicide-resistant *A. alternata* field isolates that were considered to be highly resistant to both iprodione and fludioxonil. However, these field resistant strains should be registered as moderately resistant to phenylpyrroles on the basis of their growth characteristics. Indeed, the latter authors mentioned that their growth was reduced to 50% in the presence of 0.1 μg ml^{1} fludioxonil. As a comparison, strains that were scored as highly resistant in *C. heterostrophus* (Yoshimi et al. 2003), *N. crassa* (Fujimura et al. 2000; Miller et al. 2002) and *A. brassicicola* (this study) have an EC_{50} of above 25 μg ml^{1} or even 100 μg ml^{1}. When considering their sensitivity to fungicides, the *A. brassicicola* field strains depicted here resembled some previously described laboratory dicarboximide-resistant mutants. In general, these induced mutants were also described as being sensitive to high pressure, albeit with various levels of sensitivity (Table 4). In the *N. crassa* filamentous fungus model, osmosensitive *os-1* mutants were classified in two groups: type I *os-1* mutants were more resistant to fungicides and less sensitive to osmotic stress than type II mutants (Fujimura et al. 2000). In *B.*

cinerea, two groups of dicarboximide-resistant laboratory mutants that differed in their fungicide sensitivity were also reported, but all displayed high osmosensitivity (Cui et al. 2002). In *C. heterostrophus*, three groups of *dic-1* mutants could be distinguished: (1) highly resistant to fungicides and hyper-sensitive to osmotic stress, (2) moderately resistant to fungicides and hyper-sensitive to osmotic stress and (3) moderately resistant to fungicides with low osmosensitivity (Yoshimi et al. 2004). The *A. brassicicola* mutant isolates described here could also be divided into two groups, based on their response to osmotic stress. Some strains were phenotypically similar to *N. crassa* type I *os-1* mutants, i.e. highly resistant to fungicides and only moderately osmosensitive. The remaining strains had a more original phenotype, characterized by a high level of fungicide resistance and no significantly reduced tolerance to high osmoticum, as compared with wild-type strains.

[Table 4 will appear here. See end of document.]

The primary site of action of the dicarboximides and phenylpyrroles has not yet been determined, but it has been proposed that these fungicides could target a component of the osmosensing histidine kinase pathway (Ochiai et al. 2001). This hypothesis is supported by the observation that dicarboximides and phenylpyrroles stimulate glycerol production in wild-type *N. crassa* strains under low osmolarity growth conditions (Pillonel and Meyer 1997; Fujimura et al. 2000). It was hypothesized that this fungicide-induced intracellular glycerol accumulation results in abnormally high internal turgor pressure, leading to the rupture of fungal cells (Zhang et al. 2002). As these authors noted in *N. crassa*, treatment of *A. brassicicola* wild-type strains with fludioxonil and iprodione caused conidia and germ tube swelling. Moreover, it was recently reported that resistance to these fungicides is often the result of mutations in histidine kinases regulating osmotic signal transmission (Cui et al. 2002; Dry et al. 2004; Ochiai et al. 2001; Oshima et al. 2002; Yoshimi et al. 2004). In this study, we cloned a two-component histidine kinase *AbNIK1* gene from *A. brassicicola*. An analysis of the sequence of the corresponding predicted protein revealed that it is an apparent orthologue of group III histidine kinases (Catlett et al. 2003). Proteins belonging to this class, such as CaNIK1p (*C. albicans*), NcNIK-1/OS-1p (*N. crassa*), BcBOS1p (*B. cinerea*) and BmHK1p (*C. heterostrophus*), are all considered to be putative osmosensors and characterized by a unique N-terminal region consisting of HAMP domain repeats (PFAM000672; Aravind and Ponting 1999), as also noted in AbNIK1p. This region contains a total of 11 coiled-coils and is probably essential for the proper functioning of the histidine kinase (Miller et al. 2002). Consistent with their phylogenetic close-relatedness, high similarities at the amino acid and nucleotide levels were found when comparing histidine kinases from *A. brassicicola* and *C. heterostrophus*. In

particular, the first five introns that span these two coding regions were found at the same locations and only the length of the first intron differed between the two sequences. This difference was mainly due to the presence of a $CA(TA)_3CA(TA)_3CA(TA)_2CA(TA)_5$ microsatellite in the intervening *C. heterostrophus* sequence. Interestingly, two microsatellites, i.e. $(GTC)_8$ and $(GTT)_7$, which vary in the number of trinucleotide repeats depending on the *A. brassicicola* strain, were also found in the 3′UTR of *AbNIK1* (data not shown). The relevance of the presence of these nucleotide repeats in the non-coding regions of the two fungal histidine kinase genes is currently unknown, but modulation of gene expression related to microsatellite polymorphism in UTRs has already been demonstrated in eucaryotes (Ruggiero et al. 2003).

To elucidate the molecular basis of the osmosensitive and dicarboximide/phenylpyrrole-resistant phenotypes in *A. brassicicola*, we sequenced the *AbNIK1* mutant alleles and compared them with those of the wild-type strains. The four strains with a moderately osmosensitive and fungicide-resistant phenotype had null mutations resulting from premature termination of translation due to a frameshift within repeat 4 (strains Abra41^{FR}, Abra20M^{LR}) or the presence of stop codons within repeat 5 (strain Abra7407^{FR}) or near the G2-box (strain Abra3^{FR}). This observation is consistent with previously published results concerning *N. crassa* Type I *os-1* mutants (Table 4) that were also shown to be null mutants (Ochiai et al. 2001; Miller et al. 2002); and it also indicates that *os1/nik1* orthologues have an obvious role in osmotolerance and are essential for the antifungal activity of dicarboximides and phenylpyrroles. A definitive proof that the null mutations in *AbNIK1* are responsible for the observed fungicide resistance would require the introduction of a wild-type allele in mutants to reverse their phenotype. Unfortunately, despite many efforts, the efficient transformation of *Alternaria* species pathogenic to crucifers has not yet been obtained and it was therefore not possible to complement the *A. brassicicola* mutants.

One laboratory mutant, strain Abra43M^{LR}, also had a point-mutation that resulted in a stop codon in the second amino acid repeat and could therefore be regarded as a null mutant. However, in contrast to other *A. brassicicola* null mutants, this strain was not found to be osmosensitive. There is a Kozack sequence and a start codon 24 bases 3′ from the mutation and, as proposed by Miller et al. (2002) for the NM233t mutant strain of *N. crassa*, this may give rise to a protein lacking the first two amino acid repeats. If this assumption is correct, this truncated histidine kinase should still be active and able to regulate the downstream osmosensing pathway. Moreover, the fact that strain Abra43M^{LR} is highly resistant to phenylpyrroles and dicarboximides suggests that the N-terminal amino acid repeats have an important function in fungicide resistance in *A. brassicicola*, in agreement with results obtained with other filamentous fungi (Cui et al. 2002,

2004; Ochiai et al. 2001; Yoshimi et al. 2004). Since several mutants with amino acid substitutions in the N-terminal region of histidine kinases are highly resistant to dicarboximides (Table 4), it is even suggested that the coiled-coil domain might be the binding site for these fungicides (Cui et al. 2002). Surprisingly, none of the *A. brassicicola* mutants we analyzed had this type of mutation, whereas they all displayed a highly resistant phenotype. The single amino acid substitution we found was located within the H-box of the histidine kinase from the field mutant Abra40^{FR} strain. To our knowledge, the *C. heterostrophus dic1* mutant E9003 strain is the only other previously reported mutant with a single amino acid change within the kinase domain (Yoshimi et al. 2004). However, this mutant is only moderately resistant to iprodione and fludioxonil and is highly osmosensitive, while strain Abra40^{FR} is highly resistant to fungicides and tolerant to osmotic stress. These differences between the two mutants suggest that mutations within the kinase domain affect signal transmission in the osmosensing pathway resulting in different responses to stress compounds (Yoshimi et al. 2004). The amino acid substitution found in strain Abra40^{FR} occurred in the strictly conserved consensus H motif surrounding the histidine, which becomes phosphorylated when the kinase is activated (Loomis et al. 1998). Therefore, it is likely that this mutation greatly affects the enzyme activity, even though it does not lead to any apparent pertubation in the response of this strain to high osmoticum. The possibility that a mutation in another locus could confer the fungicide resistance phenotype in strain Abra40^{FR} cannot be ruled out, as is probably the case for the field resistant AbraCP^{FR} strain. Indeed, this strain had a phenotype similar to that of strain Abra40^{FR}, but the DNA sequence of its *AbNIK1* gene did not reveal any difference relative to the wild type. A similar observation was recently reported for some iprodione-resistant isolates of *Alternaria* spp in the *alternata* and *arborescens* species-group (Ma and Michailides 2004). Mutations in at least three other *os* loci were shown to result in cross-resistance to phenylpyrroles and dicarboximides in *N. crassa* (Grindle and Temple 1982). It was recently demonstrated that these loci correspond to genes encoding components of the MAP kinase cascade of the osmosensing pathway (Fujimura et al. 2003; Zhang et al. 2002). It would therefore be interesting to isolate orthologues of these genes in *A. brassicicola* in order to further analyze the resistant field mutant strains AbraCP^{FR} and Abra40^{FR} .

As discussed above, the *A. brassicicola* field mutants described in this study displayed high levels of dicarboximide and phenylpyrrole resistance, contrary to those previously reported for *B. cinerea* and *A. alternata* field resistant strains. We previously showed that, under controlled conditions, there were no differences in pathogenicity, hyphal growth rate or spore production between sensitive and resistant field isolates (Iacomi-Vasilescu et al. 2004). However, as some of

these isolates had a reduced tolerance to high osmotic pressure, probably due to the absence of functional two-component histidine kinase, they might also have a lower fitness, at least under field conditions. Competition experiments are underway between resistant and sensitive strains on host plants under controlled and field conditions to test this hypothesis. The PCR-based RFLP and heteroduplex mobility assays we have developed to type the different resistant mutants should greatly help us to analyze the collected infected plant materials and determine, for each tested strain combination, the relative fungal genotype ratios.

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Table 1 *A. brassicicola* used in this study. Strain names ending with *FR*,*FS* and *LR* indicate field resistant, field sensitive and laboratory resistant strains, respectively

Strain	Host (geographic) origin	Fungicide sensitivity (EC_{50}, μg ml^{-1})	
		Iprodione	Fludioxonil
Abra 43^{FS}	*R. sativus* (France)	1.56	3.36
Abra CM^{FS}	*B. oleracea* (France)	1.65	3.25
Abra 20^{FS}	*R. sativus* (France)	1.36	2.51
Abra $Ro10^{FS}$	*B. oleracea* (Romania)	1.24	3.07
$Abra1662^{FS}$	*R. sativus* (France)	2.12	0.50
Abra 40^{FR}	*R. sativus* (France)	>100	>100
Abra 3^{FR}	*R. sativus* (France)	>100	>100
Abra $43M^{LR}$	Parental strain: Abra43	>100	>100
Abra 41^{FR}	*R. sativus* (France)	>100	>100
$Abra7407^{FR}$	*B. oleracea* (France)	>100	>100
$Abra20M^{LR}$	Parental strain: Abra20	>100	>100
Abra CP^{FR}	*B. oleracea* (France)	>100	>100

Table 2 Primer sets used for amplification of the *AbNIK1* gene from *A. brassicicola* genomic DNA. Fragment-numbering corresponds to nucleotide positions in the *AbNIK1* gene, with the first nucleotide of the start codon considered as position +1

Primer set	5′ primer	3′ primer	Amplified DNA fragment
1	5′-TGCCAAAGGTTCTTCAGTCC-3′	5′-CAGCCCAAATACCAGCAACT-3′	−154 to +995
2	5′-TTGCCAGTCAAGTCACATTCC-3′	5′-CCATGGCGTTTACATTGACA-3′	+900 to +1,350
3	5′-AGAGATGTCGGAACTGAAGGCAAGC-3′	5′-ATTTCTCGCACCTGTGTTGTG-3′	+1,254 to +1,657
4	5′-CGAAGTCACAAAGATTGCGCGAG-3′	5′-TACTTCGTACTTGCAGCGTGAG-3′	+1,511 to +1,932
5	5′-TCGCACAAGAAGTCAGCAAGGTC-3′	5′-TGAATTTGCCCTCCATTGCAGC-3′	+1,780 to +2,247
6	5′-CGACATGGTGATGAGACTGGAT-3′	5′-GTTGCGCATGCGTCAGATCGGTG-3′	+2,030 to +2,487
7	5′-CGCAACGAGAGATGTTGAC-3′	5′-GATGAGGTTAAGCTTATCG-3′	+2,482 to +2,906
8	5′-CCAGATCATTATGTCTTCCA-3′	5′-GCACCTCCTCAACCGAGTGA-3′	+2,832 to +3,237
9	5′-CGGATGAGATTACTGAGCAC-3′	5′-ACTTCTCCAGAATCCTAC-3′	+3,166 to +3,573
10	5′-CGTTCCAGATTCTTCTGGC-3′	5′-GAGAGCCGAATTAGTAGGG-3′	+3,505 to +3,911
11	5′-CGTCATTGCACTAACGGCGCAC-3′	5′-TCAGCTACTGGACTCCCAGC-3′	+3,794 to +4,304

Table 3 Nucleotide mutations and deduced alterations in the two-component histidine kinase AbNIK1 from *A. brassicicola* fungicide-resistant strains

Strain	Origin	Phenotype[a]	Mutation (codon number)	Mutation type	Null mutation	Location
Abra 3^{FR}	Field	Ipr^{HR} Flu^{HR} Os^{M}	CAA to TAA (998)	Nonsense	Yes	Near G2-box
Abra 41^{FR}	Field		Δ CA (495)	Frameshift	Yes	Repeat 4
Abra7407^{FR}	Field		TGG to TGA (634)	Nonsense	Yes	Repeat 5
Abra20M^{LR}	Laboratory		Δ CA (495)	Frameshift	Yes	Repeat 4
Abra CP^{FR}	Field	Ipr^{HR} Flu^{HR} Os^{L}	none	?	–	–
Abra 43M^{LR}	Laboratory		CAG to TAG (343)	Nonsense	Yes (?)[b]	Repeat 2
Abra40^{FR}	Field		GAG to AAG (753)	E to K	No	H box

[a] Ipr and Flu stand for iprodione and fludioxonil, respectively; *HR* high level of resistance; Os^{L} and Os^{M} stand for low and moderate levels of osmosensitivity, respectively

[b] Potential Kozack sequences and start codons are located a few basepairs downstream from the mutations

Table 4 Comparison of phenotypes and histidine kinase gene mutations in dicarboximide-resistant strains of filamentous fungi

Phenotype [a]	Strain type[b]	Null mutation	Location	Reference
A. brassicicola				
Ipr^{HR} Flu^{HR} Os^{M}	Field, laboratory (S)	Yes	Near G2-box, repeats 4 and 5	This study
Ipr^{HR} Flu^{HR} Os^{L}	Field, laboratory (S)	No	Repeat 2[c], H box	This study
B. cinerea				
Ipr^{HR} $Flu^{?}$ Os^{H}	Laboratory (S)	No	Repeats 2–5	Cui et al. (2002)
Ipr^{LR} $Flu^{?}$ Os^{H}	Laboratory (S)	No	Repeats 2 and 4	Cui et al. (2002)
Ipr^{LR} Flu^{S} $Os^{L,M}$	Field	No	Repeat 2	Cui et al. (2002), Oshima et al. (2002)
A. alternata				
Ipr^{HR} Flu^{LR} Os^{M}	Field	Yes	Repeats 1 and 5	Dry et al. (2004)
C. heterostrophus				
Ipr^{HR} Flu^{HR} Os^{H}	Laboratory (EMS/NQO)	No	Repeats 1, 2–5	
		Yes	Repeat 1, 5 and near H-box	
Ipr^{MR} Flu^{MR} Os^{H}	Laboratory (EMS)	No	Near H box	Yoshimi et al. (2004)
Ipr^{MR} Flu^{MR} Os^{L}	Laboratory (NQO)	No	Near D box	
N. crassa				
Ipr^{HR} Flu^{HR} Os^{M} (*os-1* Type I)	Laboratory (UV)	Yes	Repeat 2 and near D-box	Ochiai et al. (2001), Miller et al. (2002)
Ipr^{HR} $Flu^{?}$ Os^{H} (*os-1* Type II)	Laboratory (S/UV)	No	Repeats 3–6	Miller et al. (2002), Ochiai et al. (2001)

[a] *Ipr* and *Flu* Iprodione and fludioxonil, respectively; *HR* high level of resistance, *MR* moderate level of resistance, *LR* low level of resistance; *S* sensitive. Os^{L}, Os^{M} and Os^{H} low, moderate and high levels of osmosensitivity, respectively

[b] *S* Spontaneous, *UV* ultraviolet, *EMS* ethyl methanesulfonate, *NQO N*-nitroquinoline oxide

[c] Potential Kozack sequences and start codons are located a few basepairs downstream from the mutations