Characterization of mutations in the membrane-anchored subunits AaSDHC and AaSDHD of succinate dehydrogenase from Alternaria alternata isolates conferring field resistance to the fungicide boscalid

Avenot, H.; Sellam, A.; Michailides, T.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1111/j.1365-3059.2009.02154.x
Plant Pathology, 58, 6, pp. 1134-1143, 2009-09-30

Plant Pathology (2009) **58**, 1134–1143 Doi: 10.1111/j.1365-3059.2009.02154.x

Characterization of mutations in the membrane-anchored subunits AaSDHC and AaSDHD of succinate dehydrogenase from *Alternaria alternata* isolates conferring field resistance to the fungicide boscalid

H. Avenot[a]*, A. Sellam[b] and T. Michailides[a]

[a]*University of California Davis, Kearney Agricultural Center, Parlier, CA 93648, USA; and* [b]*Biotechnology Research Institute, National Research Council of Canada, Montréal, Québec, H4P 2R2, Canada and Department of Anatomy and Cell Biology, McGill University, Montréal, Québec, H3A 1B1, Canada*

The molecular basis of resistance to the fungicide boscalid in 25 *Alternaria alternata* field mutants exhibiting resistance to boscalid and previously tested negative for *AaSDHB* mutations conferring boscalid resistance was investigated by cloning and sequencing the *A. alternata SDHC* (*AaSDHC*) and *SDHD* (*AaSDHD*) genes from a boscalid-sensitive isolate. The *SDHB* and *SDHC/SDHD* genes encode the iron sulphur and two membrane-anchored subunits of succinate: ubiquinone oxidoreductase (SQR) that constitute the boscalid fungicide molecular targets. The deduced amino acid sequences exhibited low similarities with SDHC and SDHD peptides from other organisms, but residues essential to form the ubiquinone binding site or important in SQR assembly were particularly conserved. Sequence comparisons of the *AaSDHC* and *AaSDHD* genes between resistant mutants and wild-type isolates revealed that two highly conserved histidine residues implicated in the heme b ligation and located at codon 134 in AaSDHC (22 mutants) and codon 133 in AaSDHD (two mutants) were replaced by arginine residues (H134R and H133R). In another mutant, a substitution of an aspartate by a glutamic acid occurred at amino acid position 123 (D123E) in AaSDHD. Additional tests revealed that mycelial growth of boscalid-resistant isolates was reduced when isolates were subjected to oxidative stress. The identified mutations were confirmed using PCR-RFLP assays. This is apparently the first report of mutations located in the heme b ligands of the cytochrome II gene associated with carboxamide resistance.

Keywords: *Alternaria alternata,* carboxamides, oxidative stress, pistachio, ubiquinone-binding site

Introduction

Alternaria late blight, caused by *Alternaria* spp. in the *alternata*, *tenuissima* and *arborescens* species groups (Pryor & Michailides, 2002), is one of the most common fungal diseases of pistachio. The disease can cause severe premature defoliation, staining of nutshells and moulding of the kernels, reducing fruit quality. On foliage, the disease is characterized by the development of large necrotic lesions that eventually coalesce and consume the entire leaf (Pryor & Michailides, 2002). Alternaria late blight is mainly managed via multiple fungicide sprays. However, the chemical management of this disease is continuously challenged by the emergence of resistant isolates among *Alternaria* spp. populations in California pistachio, particularly with site-specific fungicides. For instance, as a strategy to control the widespread resistance to quinone outside inhibitors (QoIs) among populations of *Alternaria* spp. of pistachio (Yong *et al*., 2007), the new active ingredient boscalid [2-chloro-*N*-(4'-chlorobiphenyl-2-yl)-nicotinamide)] was introduced in California pistachio orchards in 2003, in mixture with the QoI pyraclostrobin in the product Pristine® (BASF Corporation). Because of the activity of boscalid, Pristine® has performed very well in controlling alternaria late blight. However, reductions in its efficacy have been observed in some fields only 2 years after its registration and commercial use (Avenot & Michailides, 2007). These losses were associated with the development of *A. alternata* populations resistant to both fungicides in the mixture (Avenot *et al*., 2008b). In contrast with the resistance to the QoI pyraclostrobin which is a pervasive occurrence in pistachio orchards, the quick emergence of boscalid-resistant isolates is a new phenomenon that could affect the longevity of this new compound as a tool to combat alternaria late blight.

Boscalid shows a broad-spectrum fungal activity on various crops (Stammler *et al*., 2007); it belongs to the carboxamide class of fungicides, which are inhibitors of succinate dehydrogenase (SDH) activity. SDH, also referred to as complex II or succinate:ubiquinone

*E-mail address: havenot@uckac.edu

Published online 30 September 2009

Plant Pathology

oxidoreductase (SQR), is a functional part of the Krebs cycle and the aerobic respiratory chain of mitochondria (Hägerhäll, 1997). This enzyme complex couples the oxidation of succinate to fumarate in the mitochondrial matrix (or cytoplasm in bacteria) with the reduction of ubiquinone (UQ) to ubiquinol (UQH2) in the membrane during aerobic respiration (Horsefield *et al*., 2006). The SDH complex is composed of a membrane-peripheral domain and a membrane-anchor domain. The peripheral domain, which consists of two hydrophilic subunits SDHA and SDHB, forms the soluble part of the complex and possesses the succinate dehydrogenase activity (oxidation of succinate to fumarate). SDHA is a flavoprotein (Fp) with a covalently bound flavin adenine dinucleotide (FAD), whereas SDHB is an iron-sulphur protein (Ip) containing three iron-sulphur clusters [2Fe-2S], [4Fe- 4S] and [3Fe-4S]. The membrane anchor domain is composed of two hydrophobic membrane-spanning subunits SDHC and SDHD that form the large and small subunits of cytochrome b. This latter integral membrane domain anchors the catalytic subunits (Fp and Ip) to the inner mitochondrial membrane and facilitates the transfer of electrons to ubiquinone (Ackrell *et al*., 1992; Hägerhäll & Hederstedt, 1996; Yankovskaya *et al*., 2003). The reduction of ubiquinone takes place in a specific UQ-binding site located within the membrane anchor subunits region and in close proximity to the [3Fe-4S] cluster and heme b. The UQ-binding site is highly conserved between bacteria and eukaryotes and is formed by residues from subunits B, C and D (Yankovskaya *et al*., 2003; Sun *et al*., 2005; Horsefield *et al*., 2006).

Carboxamide fungicides specifically interrupt the electron transport from the [3Fe-4S] cluster to ubiquinone by blocking the ubiquinone binding site (Horsefield *et al*., 2006; Stammler *et al*., 2007). In mutants of various fungi and bacteria, resistance to complex II inhibitors such as carboxin or flutolanil is reportedly conferred by a mutation in a highly conserved histidine residue located in the third cystein-rich cluster of the mitochondrial iron sulphur SDHB subunit (Broomfield & Hargreaves, 1992; Skinner *et al*., 1998; Honda *et al*., 2000; Matsson & Hederstedt, 2001). In the case of boscalid, field resistant mutants have been reported in *Botrytis cinerea* from grapes and strawberry (Stammler *et al*., 2007) and in *Corynespora cassiicola* isolates from cucumber (Miyamoto *et al*., 2009). In *Botrytis cinerea*, these isolates and mutants generated in the laboratory were found to bear mutations corresponding to the replacement of a Pro residue at position 225 by either Leu, Phe or Thr, and a His at position 272 by either Tyr or Arg in the SDHB subunit (Stammler *et al*., 2007). In a previous study to elucidate the mechanism of resistance to boscalid in *A. alternata* the iron sulphur gene *AaSDHB* from this fungus was targeted. Polymorphism analysis of the sequence of this gene between sensitive and resistant isolates showed that a conserved histidine residue at position 277 in the AaSDHB protein was mutated to either tyrosine (H277Y) or arginine (H277R) in some boscalid-resistant isolates (Avenot *et al*., 2008a). The histidine residue at amino acid position 277 in *A. alternata* corresponds to the histidine at position 272 in *B. cinerea* (Stammler *et al*., 2007), 267 in *Mycosphaerella graminicola* (Skinner *et al*., 1998), 257 in *Ustilago maydis* (Keon *et al*., 1991) and 229 in *Xanthomonas campestris* (Li *et al*., 2006). Interestingly, polymorphism analysis of the *AaSDHB* gene sequence from 23 *A. alternata* boscalid-resistant isolates did not reveal any difference relative to the wild type (Avenot *et al*., 2008a). This last observation suggests that other mutation(s) are likely to contribute to boscalid resistance outbreaks and is also in agreement with previous studies that demonstrated that carboxin resistance is reportedly conferred by mutations in the genes encoding the SDHC and SDHD subunits (Ito *et al*., 2004; Matsson *et al*., 1998).

In this study, the *A. alternata SDHC* (*AaSDHC*) and *SDHD* (*AaSDHD*) genes encoding the membrane-anchored subunits from a wild-type boscalid-sensitive isolate were cloned and their sequences compared with corresponding sequences from the undetermined boscalid-resistant mutants. In addition, since several studies also demonstrated that mutations in *SDHC*, *SDHD* and *SDHB* genes affecting the QP site in mutants of different organisms, correlated with hypersensitivity to hyperoxia or to oxidative stress (Ishii *et al*., 1990; Baysal *et al*., 2000; Szeto *et al*., 2007), the phenotypic characterization of the *A. alternata* boscalid-sensitive and -resistant mutants was supplemented by testing their sensitivity to oxidative stress. All the resistant isolates had a unique phenotype characterized by their sensitivity to paraquat. Finally, these results were considered in relation to the mode of action of boscalid and its interaction with the membrane-anchoring QP proteins in complex II.

Materials and methods

Fungal isolates and fungicide media

All the *A. alternata* isolates used in this study (listed in Table 1) were isolated from pistachio leaves showing putative *Alternaria* infection collected during the pistachio growing seasons from 2005 to 2006 from different orchards with a history of Pristine® (boscalid + pyraclostrobin) sprays. Three additional isolates (Aa1or, Aa2or and Aa3or) isolated in 2007 were also included in the study. Sensitivity to boscalid was estimated by measuring radial mycelial growth on potato dextrose agar (PDA) media amended with several concentrations of the fungicide as described by Avenot *et al*. (2008b). Single-spore isolates were obtained according to the procedure described by Pryor & Michailides (2002) and maintained on PDA at 4°C until use. Fresh subcultures were made by transferring hyphal plugs to dishes of PDA medium.

Sensitivity to oxidative stress

Paraquat is an herbicide believed to induce oxidative stress through the generation of superoxide ions, either in the cytoplasm (Yanase *et al*., 2002) or in the

Table 1 Nucleotide mutations and deduced alterations in the membrane-anchored subunits AaSDHC and AaSDHD in *Alternaria alternata* boscalid-resistant isolates as compared to four wild-type isolates

Isolate			EC_{50} boscalid	Mutation		
Code	Origin	Year	(μg mL^{-1})	Codon (position)	Type	Location
AaY16	Kern Co, CA	2005	0·28	CAC	-	AaSDHC
Aa4	Kern Co, CA	2005	0·26	CAC	-	
Aa6	Kern Co, CA	2006	0·28	CAC	-	AaSDHD
Aa61	Kern Co, CA	2006	0·45	CAC	-	
Aa65	Kern Co, CA	2005	>100	CAC to CGC (134)	H to R	AaSDHC
Aa122	Kern Co, CA	2005	>100	CAC to CGC (134)	H to R	AaSDHC
AaY1	Kern Co, CA	2005	>100	CAC to CGC (134)	H to R	AaSDHC
Aa2	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa26	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa51	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa44	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa41	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa13	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa48	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa3	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa68	Kern Co, CA	2005	>100	CAC to CGC (134)	H to R	AaSDHC
Aa40	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa10	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa37	Kern Co, CA	2006	>100	CAC to CGC (133)	H to R	AaSDHD
Aa32	Kern Co, CA	2006	>100	CAC to CGC (133)	H to R	AaSDHD
Aa47	Kern Co, CA	2006	>100	GAC to GAA (123)	D to E	AaSDHD
Aa54	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa19	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa94	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa102	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa103	Kern Co, CA	2006	>100	CAC to CGC (134)	H to R	AaSDHC
Aa1or	Tulare Co, CA	2007	>100	CAC to CGC (134)	H to R	AaSDHC
Aa2or	Tulare Co, CA	2007	>100	CAC to CGC (134)	H to R	AaSDHC
Aa3or	Tulare Co, CA	2007	>100	CAC to CGC (134)	H to R	AaSDHC

mitochondria (Betarbet *et al*., 2002). The sensitivity to paraquat dichloride (commercial formulation of paraquat, a.i. 43·8%) (Syngenta Crop Protection Inc.) of *A. alternata* boscalid-sensitive isolates (eight isolates) and boscalid-resistant mutants carrying mutations in the AaSDHC (eight mutants) and AaSDHD (three mutants) subunits was tested in PDA media amended with 0, 1, 10 or 100 μg mL^{-1} a.i. paraquat. Six *A. alternata* boscalid-resistant mutants carrying H277Y and H277R in the AaSDHB subunit (Avenot *et al*., 2008a) were also included in this study. The mycelial growth of each isolate was measured after 7 days' incubation of the cultures in the dark at 24°C; the effect of paraquat was then estimated by calculating the inhibition of radial mycelial growth relative to the untreated control. Two replicate plates were used per concentration for each isolate. Since the value from each replicate did not differ statistically ($P > 0·05$), the mean value was used for data analysis.

DNA procedures

Genomic DNA was extracted from mycelium of *A. alternata* using the FastDNA® kit (Qbiogene) according to the manufacturer's instructions. Primers used initially to amplify *AaSDHC* and *AaSDHD* (Table 2) were designed by aligning conserved sequences of the succinate dehydrogenase genes from *A. brassicicola* (http://genome.wustl.edu/pub/organism/Fungi/Alternaria_brassicicola/assembly/Alternaria_brassicicola-1·0/) and *Stagonospora nodorum* (syn. *Phaeosphaeria nodorum*) genome sequences. These primers were used to amplify the putative *AaSDHC* and *AaSDHD* gene fragments from *A. alternata* genomic DNA from the boscalid-sensitive isolate AaY16. PCR reactions were performed in 50-μL volumes containing 50 ng DNA, 50 mM KCl, 1·5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9·0), 0·1% Triton X-100, dNTPs at 0·2 mM each (Promega), primers at 0·2 μM each and 1 U Pfu DNA polymerase (Stratagene). PCR was carried out in a Mastercycler® (Eppendorf®) with an initial pre-heat for 3 min at 95°C, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 51°C for 50 s and extension at 72°C for 1 min, and terminated with a final extension at 72°C for 10 min. PCR products were separated by electrophoresis in 1·2% agarose gel in Tris-acetate (TAE) buffer. The PCR products from each isolate were purified from the gel using the Geneclean® Kit (MP Biomedicals, LLC), cloned into a pGEM-T Easy vector (Promega) and subsequently sequenced. The 5′ and 3′ regions of *AaSDHC* and *AaSDHD* were obtained by PCR amplification using two sets of primers (Table 2) designed from both the obtained fragment and conserved

Table 2 Primer sets used for amplification of the *AaSdhC and AaSdhD* genes from *Alternaria alternata* genomic DNA

Primer	Primer sequence	Amplified DNA fragment [a]	Purpose
SdhC F1	GAGATCCTCGCGAAACAGCGTATCAAC	+284 to +491	Cloning of *AaSdhC*
SdhC R1	GTGGAAGAAGAAGGGGAAAGCGTAGAAAG		
SdhC F2	ATGGCTTCTCAGCGGGTATTTCAG	+1 to +284	Cloning of *AaSdhC*
SdhC R2	GAAGGTGTAGTAAAGGCTGCAGG		
SdhC F3	CCAAATCACCTGGTACGCCTCG	+284 to +623	Cloning of *AaSdhC*
SdhC R3	TCATCCGAGGAAGGTGTAGTAAAGGCTG		
SdhD F1	TAGGGCACCACCAACGAC	+220 to +530	Cloning of *AaSdhD*
SdhD R1	CACATGGCGGCTGTTCGAGT		
SdhD F2	GTTCGTCGTTCCGATATTGCATC	−23 to +306	Cloning of *AaSdhD*
SdhD R2	AGAGATGATGCGCTCAAAACTCCA		
SdhD F3	CGCCGGTCCCGAACCCAGACTACT	+464 to +634	Cloning of *AaSdhD*
SdhD R3	CAGCCAAAGGACACAAACTCG		
SdhC SeqS1	ATGGCTTCTCAGCGGGTATTTC	+1 to +362	5′ ORF sequencing of *AaSdhC*
SdhC SeqR1	GATACCGAAGAGGTAGAGGGAAC		
SdhC SeqS2	CCATCTACAAGCCCCAAATC	+271 to +625	3′ ORF sequencing of *AaSdhC*
SdhC SeqR2	TCATCCGAGGAAGGTGTAGTAAAG		
SdhD SeqS1	CATCCAAGTCGTTCCGATATT	−20 to +459	5′ ORF sequencing of *AaSdhD*
SdhD SeqR1	CGTTAGCCACGGTAACCACT		
SdhD SeqS2	AGATCATCGGAACCACCAAC	+215 to +636	3′ ORF sequencing of *AaSdhD*
SdhD SeqR2	TATCTATGCGTGCCACAACC		
SdhC-(A-G) R1	TGGTTCTTGAAACCAATACCG	+264 to +547	SNP detection
SdhC-(A-G) F1	CACCTGGCCATCTACAAGC		
SdhD(A-G) R1	GAAGTAGTCGATGATGCAGGAC	+307 to +489	SNP detection
SdhD(A-G) S1	GCTGGTCTGATTCCCTTGAC		
SdhD(C-A) S1	CCACTGGAGCTTCGAGAGGA	+279 to +521	SNP detection
SdhD(C-A) R1	GCTGTTCGAGTCTTGGGAAC		

[a]Fragment numbering corresponds to nucleotide positions in the *AaSDHC* or *AaSDHD* gene, with the first nucleotide of the start codon considered as position +1.

sequence of corresponding regions in *A. brassicicola* homologous gene. PCR products were cloned and sequenced as described above.

PCR-RFLP assays

Portions of the *AaSDHC* and *AaSDHD* genes potentially carrying mutations were PCR-amplified using relevant primers (Table 2) and subsequently digested with different restriction enzymes (New England BioLabs Inc.) according to the manufacturer's instructions. Digestion products were then separated on 3% agarose gels in 1× TAE buffer.

Results

Characterization of field boscalid-resistant isolates of *A. alternata*

The sensitivity of the *A. alternata* isolates to boscalid was analysed by measuring radial growth on boscalid-amended media. Although a slight growth of wild-type isolates was observed at high doses of boscalid, the *A. alternaria* isolates were clearly distinguished as either sensitive or resistant to boscalid. Indeed, EC_{50} values exceeding 100 μg mL^{-1} were obtained for all the 25 tested isolates, which were classified as highly resistant to boscalid (Table 1). By contrast, the EC_{50} values of eight selected wild-type sensitive isolates were lower than 1 μg mL^{-1}. Values for four representative sensitive isolates are shown in Table 1.

Cloning and sequence analysis of the *AaSDHC* and *AaSDHD* genes of succinate dehydrogenase from *A. alternata*

The complete open reading frame (ORF) sequence of *AaSDHC* and *AaSDHD* were obtained following the strategy described by Avenot *et al.* (2008a). Primer sets (Table 2) designed on the basis of conserved regions of *SDHC* and *SDHD* from *A. brassicicola* and *S. nodorum* were successfully used to amplify fragments from the genomic DNA of *A. alternata* AaY16, a boscalid-sensitive isolate. These fragments were cloned and sequenced. The resulting sequence was used to search the GenBank non-redundant database using the BLASTX algorithm. Significant matches were obtained with SDHC and SDHD orthologues from several filamentous fungi. The complete coding sequence of *AaSDHC* (accession no. FJ437067) and *AaSDHD* (accession no. FJ437068) were obtained by PCR amplification using primers designed both on the basis of the obtained DNA fragment and *A. brassicicola SDHC* and *SDHD* DNA sequences. The assembled DNA sequences of *AaSDHC* and *AaSDHD* contained ORFs coding for 177 and 193 amino acids, respectively, each interrupted by one putative intron.

AaSDHC and AaSDHD were highly similar to the well-characterized succinate dehydrogenase cytochrome b560 subunits of phylogenetically close fungi such as *Pyrenophora tritici repentis* (85 and 87% identity, respectively), *P. nodorum* (67 and 79%) and *Aspergillus fumigatus* (59 and 64%). Conserved domain database (CDD) searching revealed the existence of the functional heme and quinone binding domains in the two proteins.

Polymorphism analysis of *AaSDHC* and *AaSDHD* sequences from boscalid-resistant and sensitive field isolates

To identify the mutation responsible for boscalid resistance, *AaSDHC* and *AaSDHD* DNA sequences from resistant isolates were compared with the corresponding gene of the sensitive isolates Aa4, Aa6 and AaY16. The complete nucleotide sequence of the *AaSDHC* and *AaSDHD* genes was determined for the 25 boscalid resistant isolates. Single nucleotide substitution was detected in *AaSDHC* sequence of 22 boscalid resistant isolates (Table 1). These isolates contained an A to G transition at position 490, resulting in the substitution of the conserved proximal heme-binding site His with Arg (H134R). Single nucleotide substitutions were also found in the *AaSDHD* sequences of three resistant isolates (Table 1). Isolates Aa32 and Aa37 contained an A to G transition at position 398 in *AaSDHD*, resulting, as in *AaSDHC*, in the substitution of the conserved proximal heme-binding site His with Arg (H133R). The *AaSDHD* sequence of isolate Aa47 isolate had a C to A mutation at position 369, resulting in the substitution of D with E (D123E). The exact alignment of AaSDHC and AaSDHD amino acid sequences, indicating the positions of mutations and the position of introns, is shown in Fig. 1. *AaSDHC* and *AaSDHD* sequences of boscalid-resistant isolates containing mutations in the iron-sulphur subunit AaSDHB (Avenot *et al*., 2008a) were also analysed and no polymorphism was detected in the 15 tested isolates. Six representative isolates are shown in Table 3.

Single nucleotide polymorphism typing of boscalid-resistant isolates

The A to G transition in the *AaSDHC* sequence in some resistant isolates resulted in the creation of a new *Aci*I restriction enzyme site. A specific primer (Table 2) was designed to amplify the mutation-containing fragment of *AaSDHC* that was subsequently digested with *Aci*I. The PCR products from resistant isolates carrying the mutated sequence were digested into 210- and 90-bp fragments, whereas products from sensitive isolates and nine other mutants remained undigested (Fig. 2). The C to A transition in the *AaSDHD* sequence from isolate Aa47 generated a new1 *Xmn*I site. A specific primer (Table 2) was used to amplify the mutation-containing fragments for all boscalid-resistant and -sensitive tested isolates. The PCR products were then treated using the mutation-specific enzyme. The PCR product from Aa47 carrying the mutated sequence was digested into 125- and 118-bp fragments, whereas products from sensitive isolates and other mutants remained undigested (Fig. 3). The A to G transition in the *AaSDHD* sequences in isolates Aa37 and Aa32 eliminated an *Xcm*I site. A specific primer (Table 2) was used to amplify the mutation-containing fragments for all boscalid-resistant and -sensitive tested isolates. The PCR products were then treated using the mutation-specific enzyme. The PCR products from isolates Aa37 and Aa32 carrying the mutated sequence remained undigested, whereas products from sensitive isolates and other mutants were digested into 97- and 85-bp fragments (Fig. 4).

Sensitivity to oxidative stress

Mycelial growth of both boscalid-sensitive and -resistant isolates was not affected by low concentrations (1 and 10 $\mu g\ mL^{-1}$) of paraquat (data not shown). However, on medium containing 100 $\mu g\ mL^{-1}$ paraquat, the boscalid-sensitive isolates (Aa4, Aa6, Aa61, AaY16, Aa35, Aa62, Aa61 and Aa127) and boscalid-resistant isolates (Aa65, Aa26, Aa13, Aa10, AaY1, Aa54, Aa30, Aa122, Aa47, Aa37, Aa29, Aa20, Aa111, AA33, Aa58 and Aa45) displayed different degrees of oxidative sensitivity (Table 3). Indeed, the sensitive isolates were only slightly affected by oxidative stress, with inhibition ranging from 5·48 to 11·62%, whereas the growth of the resistant isolates was moderately inhibited (15·61–47·97%). The mean values obtained for the groups of SDHC mutants (27·72 ± 8·58) and SDHB and SDHD mutants (26·43 ± 8·93) were significantly higher ($P < 0·05$, two-tailed *t*-test) than that obtained for the wild-type boscalid-sensitive isolates (8·94 ± 2·23).

Discussion

In the current study the AaSDHC and AaSDHD genes encoding the membrane anchored subunits of succinate dehydrogenase were cloned and sequenced. Low conservation of the deduced protein sequences of both *AaSDHC* and *AaSDHD* was observed when compared with SDHC and SDHD homologous proteins from model organisms, namely *Saccharomyces cerevisiae* and *Escherichia coli*. This finding was in agreement with other studies reporting a greater variation in the primary amino acid sequence of both cytochrome *b* subunits among species (Hägerhäll & Hederstedt, 1996; Baysal *et al*., 2001; Cecchini, 2003). In order to determine whether the presence of mutations in the *AaSDHC* and *AaSDHD* genes is associated with boscalid resistance in *A. alternata* boscalid-resistant mutants, the polymorphism of corresponding sequences from wild-type isolates and mutants characterized by the absence of mutations in the *AaSDHB* sequence (Avenot *et al*., 2008a) was then analysed. Two resistant isolate groups were identified based on the presence of point mutations affecting conserved amino acid residues in the AaSDHC and AaSDHD proteins, with

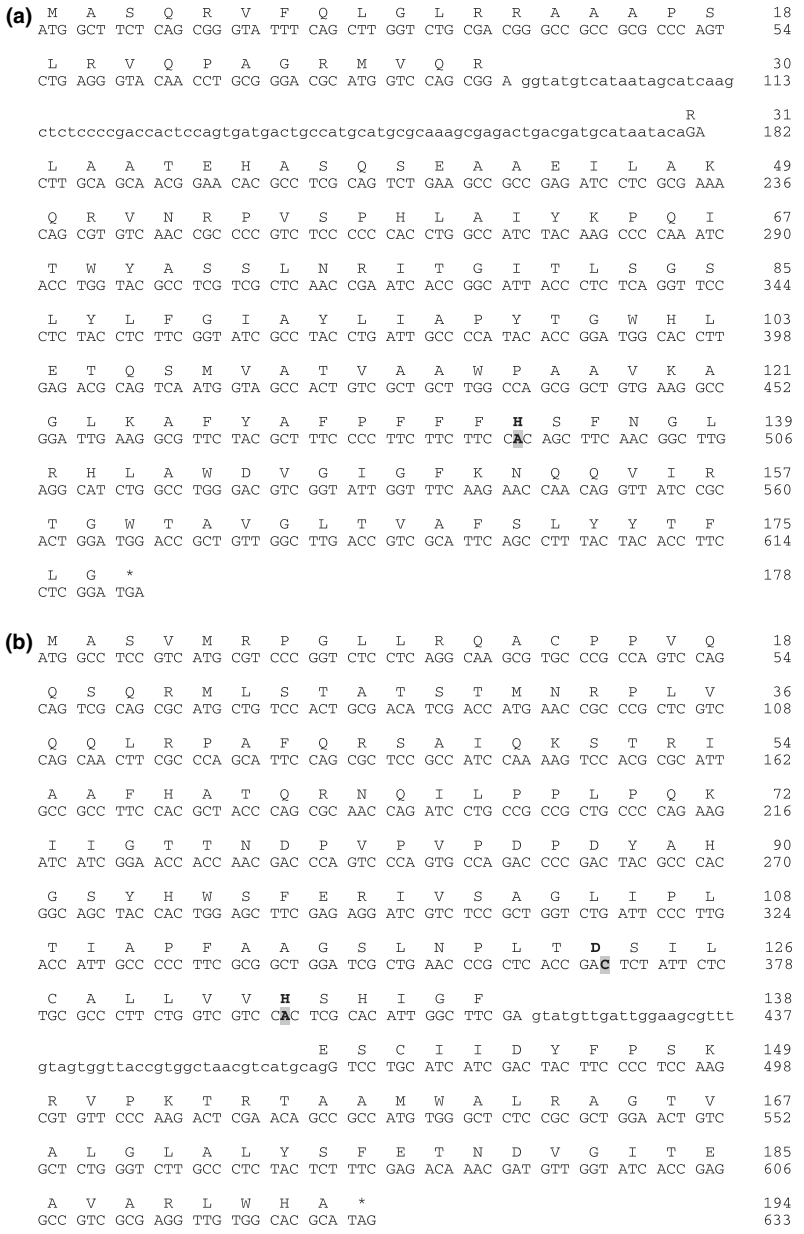

Figure 1 Genomic sequences of the *AaSDHC* (a) and *AaSDHD* (b) from boscalid-sensitive isolates of *Alternaria alternata* and the deduced amino acid sequences. Grey shading indicates locations of nucleotide substitutions and bold letters indicate the amino acid substitutions found in boscalid-resistant isolates.

these alterations potentially changing the boscalid binding site. Two distinct mutations in the *AaSDHD* gene corresponding to the replacement of a histidine by arginine (H133R) and an aspartate by a glutamic acid (D123E) were found, but in only three mutants. The mutated aspartate residue D123 in *A. alternata* is conserved in the human SDHD homologous protein and is equivalent to His-D92. In humans, mutation of this Asp92 residue to Tyr is related to hereditary paraganglioma (Baysal *et al.*, 2002). This residue was suggested to play a role in the protonation of ubiquinone upon reduction (Yanase *et al.*, 2002). In relation to carboxamide resistance, a mutation of an aspartate residue located at position 89 in the SDHD of *Paracoccus denitrificans* conferred resistance to carboxin (Matsson *et al.*, 1998). Analysis of the *AaSDHC* gene revealed a unique mutation leading to the substitution of a highly conserved histidine residue by arginine (H134R). Curiously, several resistant isolates displayed this mutation. This result is in accordance with a previous published study involving the basidiomycete *Coprinus cinereus*, in which the replacement of an asparagine at position 80 in the SDHC subunit by a lysine residue

Table 3 Effect of paraquat on the mycelial growth of *Alternaria alternata* boscalid-senstive (BS) and boscalid-resistant (BR) isolates with and without mutations in the SDH C, D and B subunits

Isolate	Phenotype	Genotype			Mycelial growth inhibition (%) in presence of paraquat (100 μg mL^{-1})
		AaSDH B locus	*AaSDH C* locus	*AaSDH D* locus	
Aa4	BS	Wild-type (wt)	Wild-type (wt)	Wild-type (wt)	10·58
Aa6	BS	wt	wt	wt	7·47
Aa61	BS	wt	wt	wt	9·66
AaY16	BS	wt	wt	wt	10·81
Aa35	BS	wt	wt	wt	6·41
Aa62	BS	wt	wt	wt	11·62
Aa61	BS	wt	wt	wt	5·48
Aa127	BS	wt	wt	wt	9·48
Aa65	BR	none	H134R	none	24·81
Aa26	BR	none	H134R	none	34·31
Aa13	BR	none	H134R	none	40·11
Aa10	BR	none	H134R	none	15·61
AaY1	BR	none	H134R	none	25·74
Aa54	BR	none	H134R	none	19·32
Aa30	BR	none	H134R	none	36·77
Aa122	BR	none	H134R	none	25·06
Aa47	BR	none	none	H133R	21·79
Aa37	BR	none	none	D123E	47·97
Aa29	BR	H277Y (Avenot *et al.*, 2008a)	none	none	22·03
Aa20					24·50
Aa111					21·67
Aa33	BR	H277R (Avenot *et al.*, 2008a)	none	none	27·55
Aa58					22·12
Aa45					23·81

resulted in resistance in mutants of this fungus to flutolanil and carboxin (Ito *et al.*, 2004).

It should be noted that the two histidine residues substituted in the boscalid-resistant mutants of *A. alternata*, namely His-C134 and His-D123, correspond to the residues His-C84 and His-D71 in the membrane anchor of the *E. coli* SQR. These residues are conserved throughout complex II and function as the axial ligands for heme b_{556}, which is known to be essential for the assembly and

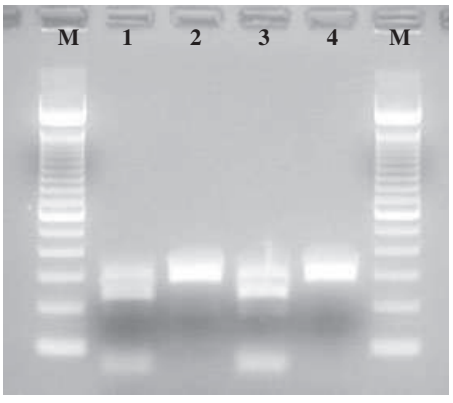

Figure 2 RFLP analysis of *Aci*I–digested 300-bp PCR products corresponding to the portion of the *AaSDHC* gene containing the A to G mutation. Amplification products were obtained using genomic DNA from AaSDHC mutants Aa65 (lane 1) and Aa122 (lane 3), sensitive isolate AaY16 (lane 2), AaSDHB-mutant Aa29 (lane 4) of *Alternaria alternata*. Lane M was loaded with a 100-bp DNA ladder (Invitrogen).

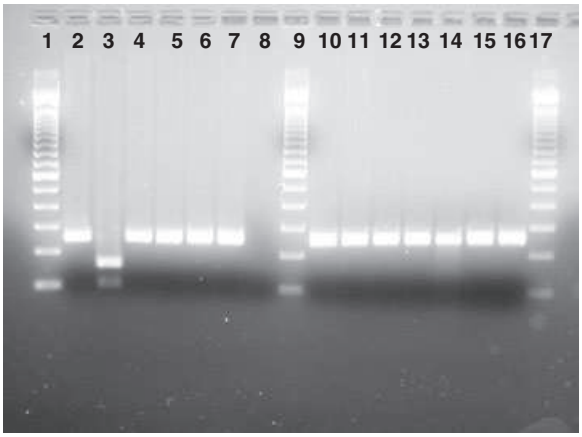

Figure 3 RFLP analysis of the *Xmn*I–digested 243-bp PCR products corresponding to the portion of the *AaSHD* gene containing the C to A mutation. Amplification products were obtained using genomic DNA from *Alternaria alternata*: AaSDHD-mutants Aa37 (lane 2), Aa47 (lane 3) and Aa32 (lane 4); AaSDHC-mutants Aa122 (lane 5), Aa10 (lane 6), AaY1 (lane 7) and Aa65 (lane 10); sensitive isolates AaY16 (lane 11), Aa127 (lane 12) and Aa4 (lane 13); AaSDHB-mutants Aa29 (lane 14), Aa20 (lane 15) and Aa111 (lane 16). Lanes 1, 9 and 17 were loaded with a 100-bp DNA ladder (Invitrogen).

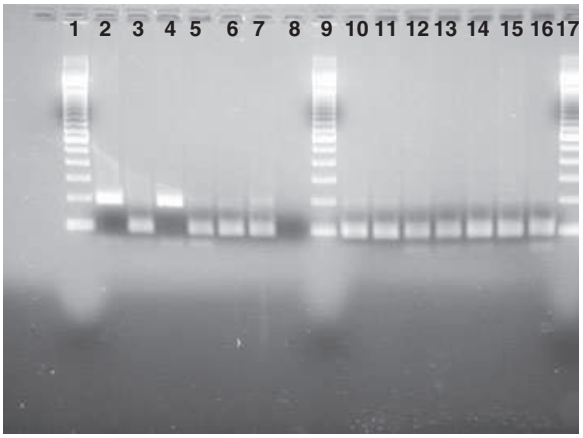

Figure 4 RFLP analysis of the *Xcm*I–digested 183-bp PCR products corresponding to the portion of the *AaSdhD* gene containing the A to G mutation. Amplification products were obtained using genomic DNA from the *Alternaria alternata*: AaSDHD-mutants Aa37 (lane 2), Aa47 (lane 3), Aa32 (lane 4); AaSDHC-mutants Aa122 (lane 5), Aa10 (lane 6), AaY1 (lane 7) and Aa65 (lane 10); sensitive isolates AaY16 (lane 11), Aa127 (lane 12) and Aa4 (lane 13); AaSDHB-mutants Aa29 (lane 14), Aa20 (lane 15) and Aa111 (lane 16). Lanes 1, 9 and 17 were loaded with a 100-bp DNA ladder (Invitrogen).

structural stability of the enzyme (Vibat *et al*., 1998; Maklashina *et al*., 2001). However, based on the *E. coli* SQR structure, heme b_{556} does not seem to be involved in the direct electron transfer pathway to ubiquinone (Nakamura *et al*., 1996; Vibat *et al*., 1998; Maklashina *et al*., 2001). In contrast with the mutations in SDHC and SDHD subunits reported to affect the sensitivity to other carboxamides fungicides in mutants of basidiomycetes species, the H134R and H133R mutations identified in AaSDHC and AaSDHD, respectively, appear unique, since they are located in the heme b ligands of the cytochrome II gene. This is apparently the first report of such mutations associated with carboxamide resistance.

In this study, in order to obtain additional insights regarding the mechanism of boscalid resistance, the phenotypic characterization of *A. alternata* boscalid resistant and -sensitive isolates was supplemented by studying their sensitivity to oxidative stress. The results obtained showed that the resistant isolates were more sensitive to oxidative stress than wild-type isolates. This effect was not entirely unexpected, as previous studies showed that mutants with SDH mutations in other organisms were hypersensitive to oxidative stress. For instance, in *Caenorhabditis elegans*, a mutation in the gene *mev-1*(*kn1*) homologue to *AaSDHC* conferred hypersensitivity to hyperoxia or to oxidative stress (Ishii *et al*., 1998). In *S. cerevisiae*, the R471K mutation in the *SDH3* subunit gene rendered an isolate hypersensitive to paraquat, whereas the mutations D88E and D88N in *SDH4* conferred mild sensitivity to this agent (Szeto *et al*., 2007). It is anticipated that the sensitivity of *A. alternata* boscalid-resistant mutants to oxidative stress might affect their ability to compete with wild-type isolates, at least in the absence of boscalid exposure.

The results from sequencing were consolidated into PCR-based assays. These results, combined with the fact that the *A. alternata* mutants were also sensitive to oxidative stress, as previously described in model organisms such as *C. elegans* or *S. cerevisiae,* are strong evidence of the correlation between phenotype (resistance to boscalid) and genotype (presence of mutations in SDH genes). However, the transformation of a wild-type isolate with the mutated *AaSDHC* and *AaSDHD* loci and restoration of the mutants will conclusively demonstrate the role of these mutations as the causes of boscalid resistance. Notably, the molecular diagnostic methods developed constitute sensitive tools for the rapid characterization of resistant isolates and will be useful in boscalid resistance monitoring studies in *A. alternata*, as well as for determining strategies for the use of new molecules, assuming that molecular tests can be made easily and simultaneously for all detected mutations.

It should be mentioned that the *A. alternata SDHB* mutants previously characterized (Avenot *et al*., 2008a) carried no mutations in the AaSDHC and AaSDHD subunits. It was previously demonstrated that the AaSDHC and AaSDHD mutants characterized in the present study had no mutations in AaSDHB (Avenot *et al*., 2008a). These findings indicate that each mutation found in one particular target-encoding gene can independently confer the resistance to boscalid. Stammler *et al*. (2007) also reported that field and laboratory mutants of *B. cinerea* isolated from grapes and strawberries with mutations within the homologous iron sulphur gene had no mutations in the SDHC and SDHD subunits. More recently, mutations in the three loci (SDH) B, C and D subunits in mutants of *Aspergillus oryzae* exhibiting resistance to carboxin were identified to be independently responsible for the resistance (Shima *et al*., 2009).

In summary, the data presented here imply that amino acid substitutions in the SDHC- and SDHD-type mutants might alter the conformation of residues that could directly interact with boscalid, and consequently decrease the affinity of SDH with boscalid or diminish the space needed for boscalid fungicide binding. In previous work it was shown that boscalid-resistant mutants displayed cross-resistance to carboxin, another fungicide in the carboxamide class. The different mutations in boscalid-SDH mutants and carboxin-SDH mutants suggest that the binding sites for carboxin and boscalid might slightly differ from each other.

Acknowledgements

The authors thank David P. Morgan for technical assistance and for the collection of isolates from pistachio orchards. We also thank Dr G. Stammler (BASF Germany) for exchanging information with us.

References

Ackrell BAC, Johnson MK, Gunsalus RP, Cecchini G, 1992. Structure and function of succinate dehydrogenase and

fumarate reductase. In: Müller F, eds. *Chemistry and Biochemistry of Flavoenzymes*, vol. 3. London, UK: CRC Press, 229–97.

Avenot HF, Michailides TJ, 2007. Resistance to boscalid fungicide in *Alternaria alternata* isolates from pistachio in California. *Plant Disease* **91**, 1345–50.

Avenot HF, Sellam A, Karaoglanidis G, Michailides TJ, 2008a. Characterization of mutations in the iron-sulphur subunit of succinate dehydrogenase correlating with boscalid resistance in *Alternaria alternata* from California pistachio. *Phytopathology* **98**, 736–42.

Avenot H, Morgan DP, Michailides TJ, 2008b. Resistance to pyraclostrobin, boscalid and multiple resistance to Pristine® (pyraclostrobin + boscalid) fungicide in *Alternaria alternata* causing alternaria late blight of pistachios in California. *Plant Pathology* **57**, 135–40.

Baysal BE, Ferrell RE, Willett-Brozick JE *et al.*, 2000. Mutations in *SDHD*, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* **287**, 848–51.

Baysal BE, Rubinstein WS, Taschner PE, 2001. Phenotypic dichotomy in mitochondrial complex II genetic disorders. *Journal of Molecular Medicine* **79**, 495–503.

Baysal BE, Willett-Brozick JE, Lawrence EC *et al.*, 2002. Prevalence of *SDHB, SDHC*, and *SDHD* germline mutations in clinic patients with head and neck paragangliomas. *Journal of Medical Genetics* **39**, 178–83.

Betarbet R, Sherer TB, Greenamyre JT, 2002. Animal models of Parkinson's disease. *Bioessays* **24**, 308–18.

Broomfield PLE, Hargreaves JA, 1992. A single amino-acid change in the iron sulphur protein subunit of succinate dehydrogenase confers resistance to carboxin in *Ustilago maydis*. *Current Genetics* **22**, 117–21.

Cecchini G, 2003. Function and structure of complex II of the respiration chain. *Annual Review of Biochemistry* **72**, 77–109.

Hägerhäll C, 1997. Succinate:quinone oxidoreductases. Variations on a conserved theme. *Biochemica et Biophysica Acta* **1320**, 107–41.

Hägerhäll C, Hederstedt L, 1996. A structural model for the membrane-integral domain of succinate:quinone oxidoreductases. *FEBS Letters* **389**, 25–31.

Honda Y, Matsuyama T, Irie T, Watanabe T, 2000. Carboxin resistance transformation of the homobasidiomycete fungus *Pleurotus ostreatus*. *Current Genetics* **37**, 209–12.

Horsefield R, Yankovskaya V, Sexton G *et al.*, 2006. Structural and computational analysis of the quinone-binding site of complex II (succinate-ubiquinone oxidoreductase): a mechanism of electron transfer and proton conduction during ubiquinone reduction. *Journal of Biological Chemistry* **281**, 7309–16.

Ishii N, Fujii M, Hartman PS, et al., 1998. A mutation in succinate dehydrogenase cytochrome b subunit causes oxidative stress and ageing in nematodes. *Nature* **394**, 694–7.

Ishii N, Takahashi K, Tomita S, et al., 1990. A methylviologen-sensitive mutant of the nematode *Caenorhabditis elegans*. *Mutation Research* **237**, 165–71.

Ito Y, Muraguchi H, Seshime Y, Oita S, Yanagi SO, 2004. Flutolanil and carboxin resistance in *Coprinus cinereus* conferred by a mutation in the cytochrome b560 subunit of succinate dehydrogenase complex (complex II). *Molecular Genetics and Genomics* **272**, 328–35.

Keon JPR, White GA, Hargreaves JA, 1991. Isolation, characterization and sequence of a gene conferring resistance to the systemic fungicide carboxin from the maize smut pathogen, *Ustilago maydis*. *Current Genetics* **19**, 475–81.

Li J, Zhou M, Li H, Chen C, Wang J, Zhang Y, 2006. A study on the molecular mechanism of resistance to amicarthiazol in *Xanthomonas campestris* pv. *citri*. *Pest Management Science* **62**, 440–5.

Maklashina E, Rothery RA, Weiner JH, Cecchini G, 2001. Retention of heme in axial ligand mutants of succinate:ubiquinone oxidoreductase (complex II) from *Escherichia coli*. *Journal of Biological Chemistry* **276**, 18968–76.

Matsson M, Hederstedt L, 2001. The carboxin-binding site on *Paracoccus denitrificans* succinate:quinone reductase identified by mutations. *Journal of Bioenergetics and Biomembranes* **33**, 99–105.

Matsson M, Ackrell BA, Cochran B, Hederstedt L, 1998. Carboxin resistance in *Paracoccus denitrificans* conferred by a mutation in the membrane-anchor domain of succinate: quinine reductase. *Archives of Microbiology* **170**, 27–37.

Miyamoto T, Ishii H, Seko T, Kobori S, Tomita Y, 2009. Occurrence of *Corynespora cassiicola* isolates resistant to boscalid on cucumber in Ibaraki Prefecture, Japan. *Plant Pathology* **58**, 1144–51.

Nakamura K, Yamaki M, Sarada M, *et al.*, 1996. Two hydrophobic subunits are essential for the heme b ligation and functional assembly of complex II (succinate-ubiquinone oxidoreductase) from *Escherichia coli*. *Journal of Biological Chemistry* **271**, 521–7.

Pryor B, Michailides TJ, 2002. Morphological, pathogenic, and molecular characterization of *Alternaria* isolates associated with Alternaria late blight of pistachio. *Phytopathology* **92**, 406–16.

Shima Y, Ito Y, Kaneko S, *et al.*, 2009. Identification of three mutant loci conferring carboxin-resistance and development of a novel transformation system in *Aspergillus oryzae*. *Fungal Genetics and Biology* **46**, 67–76.

Skinner W, Bailey A, Renwick A, Keon J, Gurr S, Hargreaves J, 1998. A single amino-acid substitution in the iron-sulphur protein subunit of succinate dehydrogenase determines resistance to carboxin in *Mycosphaerella graminicola*. *Current Genetics* **34**, 393–8.

Stammler G, Brix HD, Glättli A, Semar M, Schoefl U, 2007. Biological properties of the carboxamide boscalid including recent studies on its mode of action. In: *Proceedings of the 16th International Plant Protection Congress, Glasgow, UK, 2007*. Alton, UK: BCPC, 16–21.

Sun F, Huo X, Zhai Y *et al.*, 2005. Crystal structure of mitochondrial respiratory membrane protein complex II. *Cell* **121**, 1043–57.

Szeto SSW, Reinke SN, Sykes BD, Lemire BD, 2007. Ubiquinone-binding site mutations in the *Saccharomyces cerevisiae* succinate dehydrogenase generate superoxide and lead to the accumulation of succinate. *Journal of Biological Chemistry* **37**(Suppl. 282), 27518–26.

Vibat CR, Cecchini G, Nakamura K, Kita K, Gennis RB, 1998. Localization of histidine residues responsible for heme axial ligation in cytochrome *b556* of complex II (succinate:ubiquinone oxidoreductase) in *Escherichia coli*. *Biochemistry* **37**, 4148–59.

Yanase S, Yasuda K, Ishii N, 2002. Adaptive response to oxidative damage in three mutants of *Caenorhabditis elegans* (age-1, mev-

1 and daf-16) that affect life span. *Mechanisms of Ageing Development* **123**, 1579–87.

Yankovskaya V, Horsefield R, Tornroth S *et al.*, 2003. Architecture of succinate dehydrogenase and reactive oxygen species generation. *Science* **299**, 700–4.

Yong L, Ma Z, Reyes HC, Morgan DP, Michailides TJ, 2007. Using real-time PCR to survey frequency of azoxystrobin-resistant allele G143A in *Alternaria* populations from almond and pistachio orchards in California. *Pesticide Biochemistry and Physiology* **88**, 328–36.