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# Adaptation d'*Alternaria brassicicola* à son hôte: Etude de composantes moléculaires impliquées dans la protection du champignon pathogène vis-à-vis des phytoalexines indoliques des Brassicaceae

Aymeric Joubert

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**Adaptation d'*Alternaria brassicicola* à son hôte:  
Etude de composantes moléculaires impliquées  
dans la protection du champignon pathogène  
vis-à-vis des phytoalexines indoliques des  
*Brassicaceae***

**Thèse de doctorat**

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Ecole Doctorale VENAM

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**par Aymeric Joubert**

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*A mes parents.*

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## **Avant-propos**

Ce mémoire de thèse déposé à l'Ecole Doctorale Végétal Environnement Nutrition Agroalimentaire Mer (VENAM) de l'Université d'Angers résume le travail que j'ai réalisé depuis Octobre 2008 à l'UMR 77 PaVé dans l'équipe Complexes Fongiques Nérotrophes (CFN).

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Ce travail a donné lieu à la rédaction de quatre articles publiés, à trois communications orales lors de congrès et à l'encadrement de trois stagiaires (1 étudiant en Master 1 et 2 étudiants en 3<sup>ème</sup> année de Licence).

Cette thèse sur articles a pour but de présenter mes résultats des recherches autour des publications réalisées. Pour cela, l'introduction a été construite de manière à restituer les connaissances bibliographiques sur la thématique de recherche. Chaque article est présenté par une introduction générale qui rappelle le contexte d'étude suivi de résultats complémentaires non publiés et de perspectives. La discussion générale a été organisée afin de donner une vision globale des recherches présentées dans les différents chapitres. La fin du manuscrit est consacrée aux perspectives ouvertes par les résultats de mes travaux de thèse.

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## Liste des Abréviations

ADN	acide désoxyribonucléique
ADNc	ADN complémentaire d'un ARNm
ARN	acide ribonucléique
ARNm	ARN messenger
ARNr	ARN ribosomique
BET	bromure d'éthydiu
CR	CongoRed
CWF	CalcoFluor White
CWI	Cell Wall Integrity
DEPC	diéthyl pyrocarbonate
DHCA	DiHydroCAmalexique
DHN	1,8-DiHydroxyNaphthalene
DNase	désoxyribonucléase
dNTP	désoxynucléotide 5' triphosphate
DO	densité optique
DTT	dithiothréitol
EDTA	éthylènediamine tétraacétate
ERAD	Endoplasmic Reticulum Associated Degradation
FAD	Flavine Adenine Dinucléotide
g	accélération de la pesanteur
GS	glucosinolates
HOG	High Osmolarity Glycerol
HST	host-specific toxin
HPA	4-HydroxyPhenylacétique Acide hydroxylase
IAO	Indole-3-AcétaldOxime
IPTG	isopropyl- $\beta$ -D-thio galactopyranoside
ITC	isothiocyanate
kDa	kilo Dalton
LPI	Logarithmic Phenotypic Indice
MAP	Mitogen Activated Kinase
NHST	non host-specific toxin
ORF	cadre ouvert de lecture (« open reading frame »)
PCR	réaction de polymérisation en chaîne (« polymerase chain reaction »)
pH	potentiel Hydrogène
PKc	Protein Kinase c
qsp	quantité suffisante pour
RE	Reticulum Endoplasmique
RNase	ribonucléase
rpm	rotations par minute
ROS	Reactive Oxygen Species
RT-PCR	reverse transcriptase PCR
SDN	Stimulateur Défense Naturel

SDS	sodium dodécyl sulfate
SREBP	Sterol Regulatory Element-Binding Protein
SSH	Suppression Subtractive Hybridization
TAE	tris acétate EDTA
Tris	tris-hydroxyméthyl-aminoéthane
UPR	Unfolded Protein Response
UTR	Untranslated Region
UV	ultraviolets
v/v	volume par volume
X-Gal	5-bromo 4-chloro-3-indolyl- $\beta$ -D-galactopyranoside
YNBA	Yeast Nitrogen Base Agar

### **Unités internationales :**

°C	degré Celsius
Ci, $\mu$ Ci	Curie, microcurie
Da, kDa	Dalton, kilodalton
g, mg, $\mu$ g, ng	gramme, milligramme, microgramme, nanogramme
j, h, min, sec	jour, heure, minute, seconde
l, ml, $\mu$ l	litre, millilitre, microlitre
M, mM, $\mu$ M, nM	molaire, millimolaire, micromolaire, nanomolaire
mmol, pmol	millimole, picomole
V	volt

# **Introduction bibliographique**

# **1. Introduction générale**



L'équipe Complexes Fongiques Nérotrophes de l'Unité Mixte de Recherche de Pathologie Végétale A77 a pour objectifs d'apporter des connaissances sur le déterminisme des interactions plantes – pathogènes dans un contexte ciblé sur les maladies fongiques transmissibles par les semences. Ces recherches sont essentiellement conduites sur les espèces du genre *Alternaria* et, en particulier, celles responsables de maladies chez les *Brassicaceae*. Cette famille botanique comprend de nombreuses espèces cultivées d'importance économique comme par exemple, le chou (*Brassica oleracea*), le radis (*Raphanus sativus*) mais également une espèce sauvage modèle pour la communauté scientifique, *Arabidopsis thaliana*. Le genre *Alternaria* regroupe plus de 100 espèces ubiquitaires dont certaines sont des représentants quasi constants de la flore des semences en tant que parasites ou saprophytes. Sur les *Brassicaceae* agit un complexe de trois espèces à forte potentialité parasitaire, représenté par *Alternaria brassicae*, *A. brassicicola* et *A. japonica*, responsables d'une même maladie appelée « black spot » ou « taches noires » des *Brassicaceae*. Ces pathogènes sont transmissibles par les semences et provoquent d'importants dégâts dans ces cultures à travers le monde (Huang, 1995, Sivapalan, 1992), avec des incidences importantes sur les rendements et sur la qualité du produit qu'ils peuvent rendre invendable (Rude, 1999).

Afin de limiter les dégâts liés à ces champignons et d'éviter leur propagation, la qualité sanitaire des lots de semences est systématiquement analysée. Un autre moyen de lutte probablement efficace pour combattre ces pathogènes consisterait à utiliser des variétés résistantes. Néanmoins, les variétés commerciales actuellement disponibles sont toutes sensibles au « black spot ». En l'absence de variétés résistantes, les moyens actuels de protection des cultures contre les *Alternarioses*, passent essentiellement par la lutte chimique. Pour maintenir un niveau sanitaire satisfaisant des semences commercialisées et pour limiter de manière économiquement rentable les pertes dues aux attaques parasitaires, les traitements chimiques en végétation ainsi que le traitement des semences sont souvent utiles. Cependant, des travaux réalisés au sein de l'UMR PaVé ont montré l'existence de populations pathogènes résistantes à certains fongicides utilisés en traitement des cultures et semences (thèses B. Iacomi-Vasilescu, 2001 et H. Avenot, 2005 ; (Iacomi-Vasilescu, 2004)).

Il semble donc pertinent de mener une étude approfondie sur les mécanismes moléculaires régissant l'interaction entre l'hôte et son agresseur afin de proposer de nouvelles cibles pour la lutte phytosanitaire et l'aide au développement de nouvelles stratégies pour la sélection de variétés végétales résistantes. C'est dans ce contexte que s'inscrit le programme de recherche « Déterminisme du pouvoir pathogène des *Alternaria* » de l'équipe. Même si toutes les composantes du déterminisme du pouvoir pathogène ne sont pas, à ce jour, encore connues, il semblerait que deux composantes principales soient responsables de la pathogénie : l'une est nécessaire à l'installation du champignon dans les tissus hôtes et la mise en place du processus infectieux et l'autre, est nécessaire à la protection du champignon contre les défenses de la plante hôte. Les recherches antérieures menées au laboratoire illustrent ces deux composantes. En effet, la thèse de T. Guillemette (2003) s'est articulée autour de l'identification de gènes potentiellement impliqués dans la synthèse et la sécrétion de métabolites toxiques produits par le champignon et la thèse d'A. Sellam (2006), s'est articulée autour de la compréhension des mécanismes de protection du champignon face aux défenses chimiques de la plante (phytoalexines, phytoanticipines). Il a notamment pu mettre en évidence l'existence d'un mécanisme compensatoire qui permet au champignon de faire face à l'impact toxique des phytoalexines en renforçant son intégrité cellulaire.

La problématique de mon travail de thèse découle des travaux d'A. Sellam et s'articule autour de la mise en place des mécanismes de protection du champignon face aux défenses de la plante, et plus particulièrement face aux phytoalexines de types indoliques (camalexine et brassinine). Ce travail a pour objectifs d'explorer les événements cellulaires se situant entre le signal externe perçu par le pathogène (stress dû aux phytoalexines), et la réponse adaptative mise en place par le champignon (renforcement pariétal par exemple) et donc de caractériser les mécanismes de régulation (facteurs de transcription et voies de signalisation) de cette réponse.

L'étude approfondie du pouvoir pathogène d'*A. brassicicola* est devenue ces dernières années accessible notamment grâce aux avancées du séquençage du génome du champignon et à la possibilité de développer efficacement sur celui-ci les techniques de mutagenèse dirigée. Pour ce type d'étude, de nombreux mutants sont générés au laboratoire et leur phénotypage est nécessaire afin de comprendre les fonctions des gènes mutés.

Mon travail de thèse a débuté avec la mise au point d'une nouvelle méthode de phénotypage haut débit utilisable pour les champignons filamenteux basée sur l'analyse de leur croissance en microplaque. Aucune méthode fiable de ce type n'était disponible et par conséquent, il a été nécessaire de trouver une technologie adaptée permettant de générer des données quantitatives sur la croissance de ce type de champignon et ce, dans des temps courts, de faibles volumes et avec une bonne fiabilité.

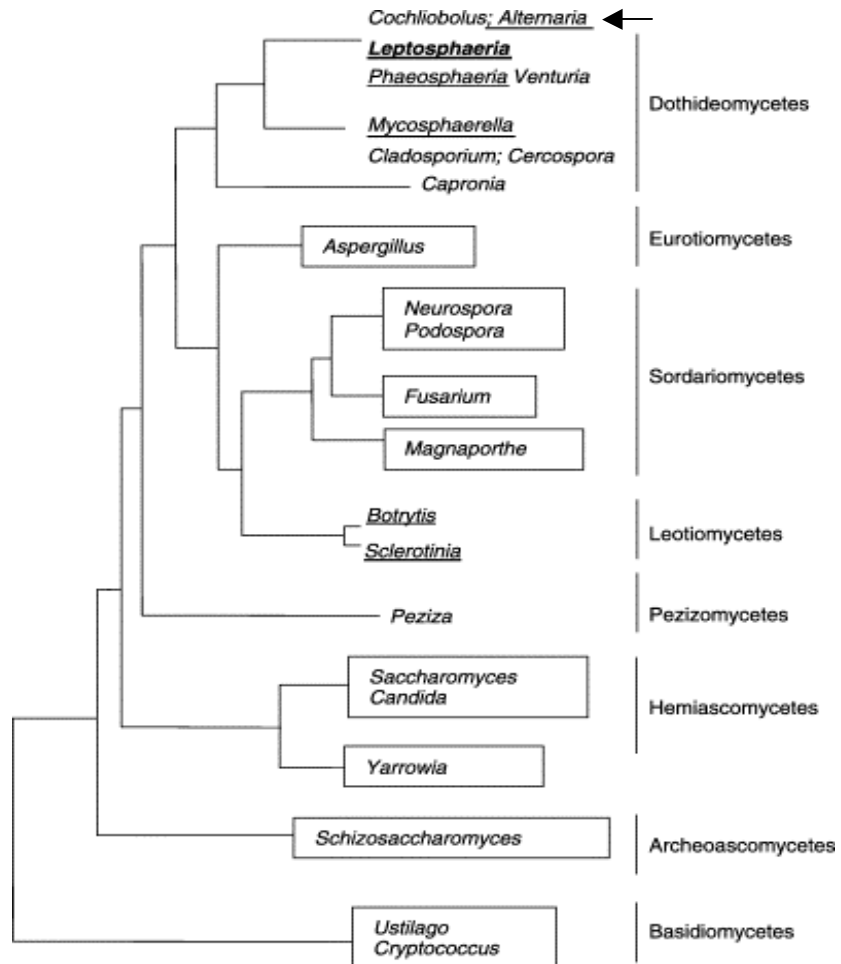
Le deuxième volet du travail de thèse porte sur la mise en évidence du rôle de certaines voies de signalisation cellulaires dans la réponse adaptative du champignon face aux phytoalexines. L'implication de voies de signalisation dans la réponse de champignons pathogènes à des molécules de défenses avait été plusieurs fois suggérée mais, jusqu'à présent, elle n'avait été démontrée que chez *Fusarium graminearum*, chez lequel la voie de l'intégrité cellulaire (ou CWI) intervient dans la réponse aux defensines (Ramamoorthy *et al.*, 2007). Notre étude a consisté à mettre en évidence l'implication de deux voies de signalisation, la voie de l'osmolarité HOG et la voie de l'intégrité cellulaire PKc (CWI), dans la réponse d'*A. brassicicola* à deux phytoalexines indoliques, la camalexine et la brassinine. Pour cela, des mutants nuls disruptés dans les MAP kinases de chacune de ces deux voies, les MAP kinases AbHog1p et AbSl2p, ont été obtenus et leurs phénotypes notamment au niveau du pouvoir pathogène mais aussi de leur sensibilité aux phytoalexines ont été analysés. Les résultats ont permis, pour la première fois, de mettre en évidence l'implication de ces deux voies de signalisation dans l'expression du pouvoir pathogène du champignon et de montrer que cette implication était, au moins en partie, liée à leur rôle dans la mise en place d'un mécanisme compensatoire visant à renforcer l'intégrité cellulaire lors de l'exposition *in planta* aux phytoalexines.

Le troisième volet de ce travail de thèse porte sur l'étude du facteur de transcription HacA et de son rôle dans le pouvoir pathogène d'*A. brassicicola*. Ce facteur de transcription intervient en cas de stress de sécrétion et est impliqué dans le repliement fonctionnel des protéines au niveau du réticulum endoplasmique. Son rôle dans le pouvoir pathogène a été récemment démontré chez *Aspergillus fumigatus* et a également été suggéré chez *Candida albicans* (Richie *et al.*, 2009, Wimalasena *et al.*, 2008). Chez *A. fumigatus*, le mutant nul disrupté dans le gène *HacA* montre un défaut dans la capacité de sécrétion et également une perte significative de la virulence. Nos travaux ont consisté à

étudier le rôle de ce facteur de transcription chez *A. brassicicola* par l'obtention et le phénotypage d'un mutant nul. Ce mutant est caractérisé par une absence de virulence *in planta*, mais aussi par une sensibilité très accrue aux phytoalexines camalexine et brassinine. Une nette diminution de la capacité de sécrétion et une altération de la composition pariétale sont également observées chez ce mutant. Le rôle de ce facteur de transcription dans le pouvoir pathogène d'un champignon phytopathogène n'avait jusqu'alors jamais été démontré.

Le quatrième volet présente divers résultats complémentaires obtenus au cours du travail de thèse. Ces résultats concernent deux approches visant respectivement à explorer d'autres voies possibles de protection du champignon contre ces molécules antimicrobiennes et à mieux comprendre le mécanisme d'action des phytoalexines. La première approche est basée sur l'étude d'une protéine potentiellement responsable de la détoxification intracellulaire de la camalexine. La protéine Mak1p d'*A. brassicicola* est l'homologue de la protéine Mak1p de *Nectria haematococca*, agent détoxifiant la maackiaine du pois (Covert SF, 1996, Miao VP, 1992). Sellam *et al.* (2007b) ont montré que le gène codant Mak1p est surexprimé chez *A. brassicicola* en présence de camalexine. Ces observations suggèrent que Mak1p d'*A. brassicicola* pourrait détoxifier la camalexine. Les résultats du phénotypage d'un mutant déficient pour cette protéine seront présentés. La seconde approche concerne l'étude des cibles cellulaires potentielles de la phytoalexine. Une proportion non négligeable de gènes liés à la synthèse des sphingolipides et stérols sont surexprimés en présence de camalexine (Sellam *et al.*, 2007b). Mon travail a consisté à obtenir des mutants nuls d'*A. brassicicola* disruptés dans différents gènes de la voie de biosynthèse des sphingolipides et stérols membranaires et à analyser leur comportement face aux phytoalexines.

## **2. Le pathosystème *Alternaria brassicicola*-*Brassicaceae***



**Figure 1** Phylogénie des champignons ascomycètes déduite de l'analyse des séquences protéiques de la sous-unité 2 de l'ARN polymérase II (d'après Eckert et al., 2005). Le genre *Alternaria* (indiqué par une flèche) appartient à la classe des Dothideomycètes.

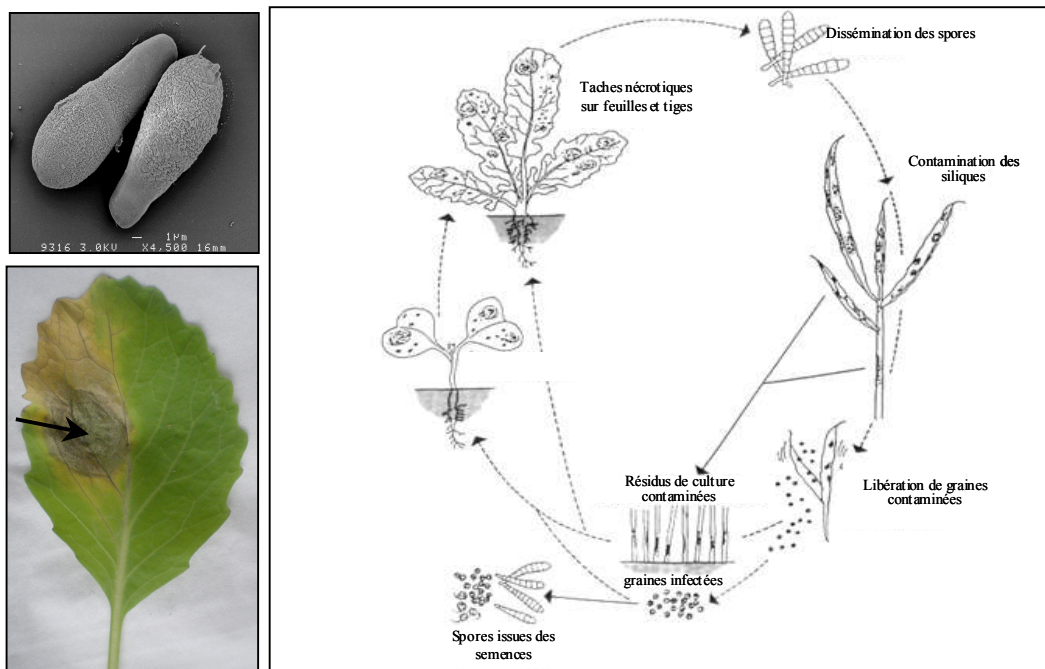
## 2.1 Présentation générale

### 2.1.1 Le champignon pathogène

L'ordre des *Pléosporales* est un large groupe monophylétique des *Dothideomycètes* et contient de nombreux genres économiquement importants pour l'agriculture. En effet, il comprend des genres de champignons pathogènes responsables de nombreux dégâts sur les cultures. Outre le genre *Alternaria*, on peut citer les genres *Cochliobolus*, *Phaeosphaeria* et *Pyrenophora*, pathogènes des céréales, ou encore *Lewia* et *Leptosphaeria* pathogènes des plantes herbacées. Les champignons du genre *Alternaria* peuvent également être responsables de pathologies chez l'Homme *via* des réactions allergiques ou des troubles pathologiques, notamment par l'action des mycotoxines (alternariol par exemple).

Le genre *Alternaria* est pleinement intégré aux *Pléosporales* même si sa classification a été remaniée de nombreuses fois (Christias, 2001). La taxonomie moderne basée sur la phylogénie moléculaire a fait disparaître la classe « fantôme » des *Deutéromycètes* qui regroupait, par pure convention, des champignons très différents les uns des autres, dont le genre *Alternaria* (Agrios, 1997, Ellis, 1971, Erickson, 1991, Simmons, 1986). A ce jour, l'ensemble des espèces du genre *Alternaria* est classé au sein des *Dothideomycètes* (ordre des *Pléosporales*) sur la base de critères moléculaires (séquences ITS, ADNr 18S, mtSSU) (Chou, 2002, Hong *et al.*, 2005, Jasalavitch, 1995, Kusaba, 1995, McKay, 1999, Pryor, 2000, Runa, 2009, Schoch *et al.*, 2009) (**Figure 1**).

Il existe une très grande diversité au sein du genre *Alternaria* : différents modes de vie, différentes répartitions ou encore différents cycles de vie en fonction des champignons (Agrios, 1997, Downs *et al.*, 2001). La centaine d'espèces composant ce genre renferme une grande majorité de souches saprophytes mais également quelques organismes pathogènes. Les attaques par des *Alternaria* peuvent se manifester de plusieurs façons : alternarioses cutanées et sinusites chez l'Homme, manque à la levée et fonte de semis chez les plantes (Ferrer *et al.*, 2002, Gene *et al.*, 1995, Schell, 2000).



**Figure 2** Caractéristiques du champignon *Alternaria* spp in vivo et in vitro (d'après Thomma, 2003).

(A) Conidies d'*A. brassicicola* au MEB.

(B) Symptôme typique (« Black spot ») d'*Alternaria* spp. avec les zones concentriques de sporulation sur feuille de chou.

(C) Cycle infectieux d'*A. brassicicola*.



### 2.1.2 *Les plantes hôtes*

La famille des *Brassicaceae* contient plus de 350 genres pour 2 500 espèces dont certaines sont des plantes de grandes cultures. Les *Brassicaceae* sont des dicotylédones, portant des fleurs à 4 sépales en croix. Ce sont des plantes vivaces, annuelles ou bisannuelles, au port herbacé. *A. brassicicola* s'attaque essentiellement aux *Brassicaceae* cultivées dont une grande partie est destinée à la consommation humaine, comme les choux *B. oleracea* et le radis *R. sativus*. Le spectre d'hôte englobe également d'autres *Brassicaceae* qui sont cultivées en tant que fourragères, comme le navet *Brassica campestris* subsp. *Rapifera*, et en tant que qu'oléagineuses, comme le colza (*Brassica napus*).

### 2.1.3 *La maladie*

*A. brassicicola* est l'agent responsable du « black spot » des *Brassicaceae*, maladie très répandue à travers le monde (Humpherson-Jones, 1989, Köhl J. *et al.*, 2010, Rotem, 1994). Comme toutes les alternarioses, le « black spot » apparaît sur les feuilles sous forme de lésions nécrotiques, souvent décrites comme des taches noires entourées chacune d'un halo chlorotique jaune (**Figure 2**) (Rotem, 1994). Néanmoins, *A. brassicicola* ne se limite pas à l'infection des feuilles mais à l'ensemble des parties aériennes. Le champignon peut en particulier contaminer les semences qui constituent un mode de conservation et de transmission de l'inoculum fongique. Des analyses de lots de semences ont montré que des certains lots contaminés par *A. brassicicola* présentent une diminution du taux de germination pouvant être supérieure à 20% (Köhl J. *et al.*, 2011). Aujourd'hui, en cas de contaminations importantes, les semences font l'objet d'un refus à la commercialisation et il s'avère souvent préférable de ne pas les récolter. La perte économique pour l'agriculteur est alors totale. Les pertes économiques occasionnées par *A. brassicicola* sont difficiles à chiffrer car la pathogénie du champignon s'exprime différemment selon les étapes de production des plantes et peut s'additionner à des attaques provoquées par d'autres agents pathogènes.

Des moyens de lutte sont mis en œuvre pour diminuer l'impact de la maladie comme l'application de fongicides, le contrôle sanitaire des semences ou encore des pratiques culturales adéquates. Toutefois, ces moyens de lutte sont souvent insuffisants ou

controversés dans le contexte écologique actuel, d'autant que des cas de résistances d'*A. brassicicola* vis-à-vis de certains fongicides sont apparus en France ces dernières années (Avenot *et al.*, 2005, Iacomì-Vasilescu, 2004). De plus, il n'existe à ce jour aucune variété de plantes cultivées résistantes à cet agent pathogène. C'est pourquoi il est nécessaire d'étudier les déterminismes du pouvoir pathogène d'*A. brassicicola* et de mieux connaître le pathogène afin de définir, sur la base de ces connaissances, de nouvelles stratégies de lutte.

## 2.2 Le pathosystème *A. brassicicola*/*A. thaliana*

Une meilleure compréhension des mécanismes moléculaires impliqués dans le pouvoir pathogène d'*A. brassicicola* passe notamment par des études sur le pathosystème modèle *Alternaria brassicicola*/*Arabidopsis thaliana*. En effet, *A. thaliana* est une *Brassicaceae* modèle dont l'utilisation et l'étude est simplifiée par la quantité de ressources génétiques et génomiques disponibles. Des données sont également disponibles sur les différences de sensibilités de divers génotypes d'*A. thaliana* vis-à-vis d'*A. brassicicola*. Il existe des variations naturelles de résistance au sein des différents écotypes et certains sont désormais caractérisés comme étant plus sensibles à l'alternariose (Mukherjee *et al.*, 2009, Thomma, 2003, Tierens *et al.*, 2002, Zheng *et al.*, 2006). Par exemple, l'écotype Col-0 d'*A. thaliana* présente une tolérance face aux pathogènes car l'agression entraînerait une réaction rapide de la plante, notamment par l'action de defensines (Thomma *et al.*, 1998). L'écotype DiG est par contre très sensible à *A. brassicicola* (Mukherjee *et al.*, 2009). La seconde raison qui pousse à l'utilisation des ressources génétiques de la plante modèle est la caractérisation de mutants déficients dans des voies de biosynthèse de métabolites de défense. Le mutant *Pad3*, déficient dans la biosynthèse de camalexine, est décrit comme étant plus sensible que l'écotype Col-0 parental à *A. brassicicola* démontrant qu'il existe un lien direct entre la production de camalexine et la tolérance de la plante hôte au pathogène. Afin de comprendre les mécanismes moléculaires mis en jeu dans la relation *A. brassicicola*/*A. thaliana*, des études transcriptomiques ont été réalisées. Van Wees *et al.*, (2003) ont identifiés 645 gènes induits par l'infection d'*A. brassicicola* chez *A. thaliana* écotype Col-0. Ces auteurs montrent que 265 des 645 gènes sont dépendants du régulateur de la voie de l'acide jasmonique *COLI-1* (Van Wees *et al.*, 2003). Cependant, il reste difficile d'établir un modèle de l'interaction même s'il apparaît clairement que l'acide

jasmonique et la camalexine jouent un rôle prépondérant dans la résistance de la plante à *A. brassicicola* (Cramer *et al.*, 2006).

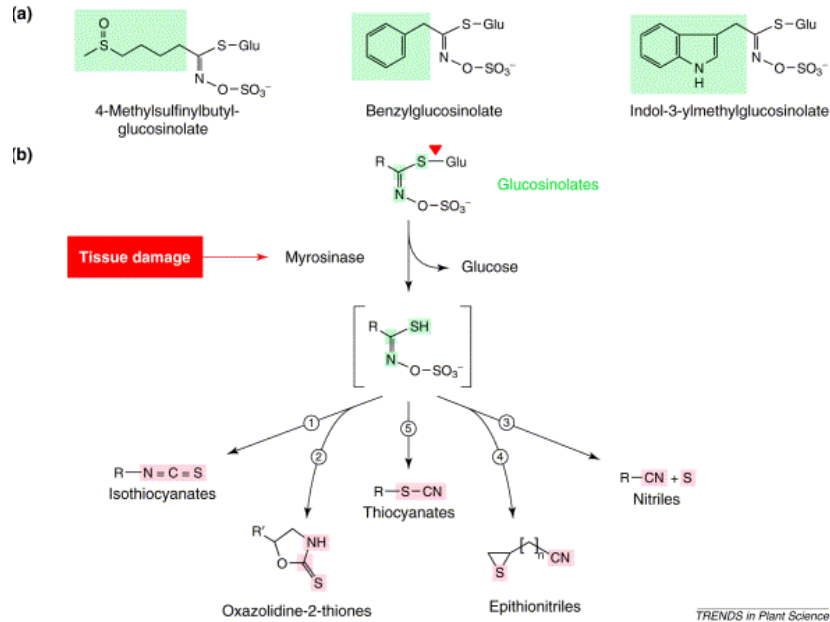
La disponibilité de la séquence génomique d'*A. thaliana* a permis depuis plusieurs années d'appréhender les interactions plante - hôte sous l'angle moléculaire. Dans la lignée des logiques contemporaines des séquençages en masse, le séquençage du génome d'*A. brassicicola* a été réalisé en 2005. Cette séquence est à présent disponible sous la forme de 838 supercontigs contenant 4,039 contigs pour 31 Mb. Les outils bioinformatiques ont prédit 10,688 gènes chez le champignon. D'autre part, il est désormais possible de transformer *A. brassicicola* et d'obtenir des mutants pour des gènes ciblés (Cho *et al.*, 2006). Le pathosystème *A. brassicicola/A. thaliana* peut ainsi être considéré comme un système modèle pour l'étude moléculaire des interactions de type nécrotrophe.

Au sein de notre laboratoire, l'obtention de mutant se fait par l'insertion d'un marqueur de sélection à l'intérieur du gène cible. Pour cela une cassette de disruption est obtenue par PCR et contient les régions flanquantes du gène cible et un gène de résistance à un antifongique (hygromycine B). Cette cassette, mise en présence de protoplastes d'*A. brassicicola*, s'insère dans le génome par recombinaison homologue et double « crossing over ». Cette « transformation » aboutira au final à la disruption du gène cible. L'utilisation de la « double-joint PCR » (Yu *et al.*, 2004) a augmenté considérablement l'efficacité de cette méthode et sa facilité de mise en œuvre par rapport à la technique initialement décrite par Cho *et al.* (2006) d'autant que le système de recombinaison homologue, très efficace chez *A. brassicicola*, permet d'obtenir des rendements de « disruption » proches de 100%.

## 2.3 Mécanismes de défense de l'hôte

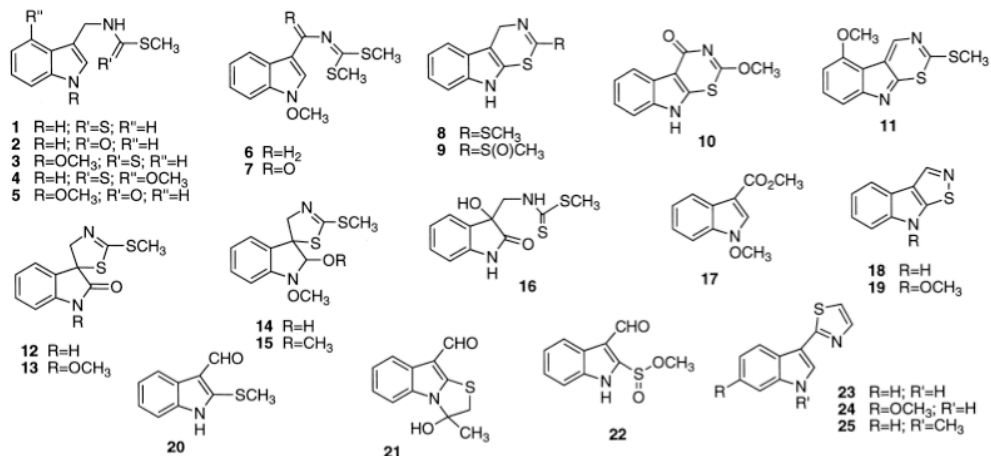
### 2.3.1 Présentation générale

Lorsqu'une plante est agressée par un agent pathogène, elle met en place des mécanismes de défense afin de faire face au parasite. Les métabolites antimicrobiens issus du métabolisme secondaire, comme les phytoalexines ou les phytoanticipines, constituent une barrière chimique contre une large gamme de pathogènes tels que les bactéries, les insectes, les nématodes ou encore les champignons (Kliebenstein, 2004). Par définition, les phytoanticipines sont synthétisées de manière constitutive tandis que les phytoalexines sont synthétisées *de novo* lors d'une attaque par un agent pathogène (Bailey *et al.*, 1982).



**Figure 3** Stratégie de défense Glucosinolates-Myrosinases (d'après Wittstock et al., 2002)

(a), Exemple montrant trois types de glucosinolates (b), L'endommagement des tissus de la plante hôte abouti à l'hydrolyse des GS par la myrosinase : la nature des produits résultants de l'hydrolyse des GS (①, ②, ③, ④ et ⑤) dépend des conditions du milieu et de la structure chimique des glucosinolates.



**Figure 4** Structure chimique de différentes phytoalexines des Brassicaceae (d'après Pedras et al., 2000).

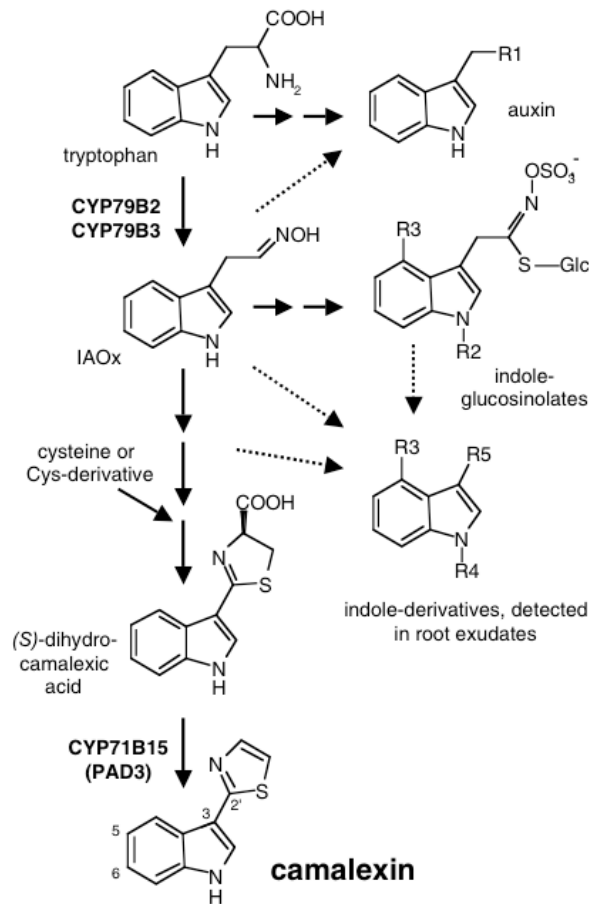
1 brassinine, 2 brassitine, 3 1-methoxybrassinine, 4 4-methoxybrassinine, 5 1-methoxybrassitine, 6 1-methoxybrassenine A, 7 1-methoxybrassenine B, 8 cyclobrassinine, 9 cyclobrassinine sulfoxide, 10 cyclobrassinone, 11 dehydro-4-methoxycyclobrassinine, 12 spirobrassinine, 13 1-methoxyspirobrassinine, 14 1-methoxyspirobrassinol, 15 1-methoxyspirobrassinol methyl ether, 16 dioxibrassinine, 17 methyl 1-methoxyindole-3-carboxylate, 18 brassilexine, 19 sinalexine, 20 brassicanal A, 21 brassicanal B, 22 brassicanal C, 23 camalexine, 24 6-methoxycamalexin, 25 1-methylcamalexine.

Les glucosinolates sont des phytoanticipines, issues du métabolisme secondaire, riches en soufre et dégradées par les enzymes myrosinases ( $\beta$ -thioglucosidases) après endommagement des tissus végétaux en plusieurs produits cyanogènes. Les produits de dégradation correspondent aux isothiocyanates, thiocyanates, nitriles, epithionitriles, et oxazolidine-2-thiones (**Figure 3**). Les composés issus de la dégradation des GS les plus dominants, après l'action de la myrosinase, sont les isothiocyanates (ITC) (Fenwick *et al.*, 1983). Plusieurs études montrent que les ITCs présentent une activité antimicrobienne *in vitro* vis-à-vis des bactéries, des insectes, des nématodes et également des champignons pathogènes de *Brassicaceae* (Brabban, 1995, Sellam *et al.*, 2007a, Tierens *et al.*, 2001, Wittstock *et al.*, 2002, Zasada *et al.*, 2003). Paradoxalement, Tierens *et al.* (2001) ont montré que le mutant *Δgsm1-1*, déficient dans la génération du 4-methylsulphinylbutyl ITC (ITC majeur d'*A. thaliana*), ne présente pas de différence de sensibilité face à *A. brassicicola*. Ce champignon nécrotrophe a donc certainement développé des mécanismes de contournement lui permettant de tolérer ces molécules.

Les phytoalexines ont été décrites pour la première fois en 1940 lors de l'interaction *Phytophthora infestans-Solanum tuberosum* (Müller *et al.*, 1940). Aujourd'hui, de nombreuses phytoalexines sont caractérisées et la diversité des molécules identifiées montre que leurs structures chimiques sont propres à chaque famille botanique.

Par définition, les phytoalexines s'accumulent au site d'infection en quantité nécessaire afin d'être utiles dans la résistance de la plante (Hammerschmidt *et al.*, 1999). L'agent pathogène peut alors mettre en place des mécanismes d'adaptation face à la charge toxique des phytoalexines ce qui va impacter sur son pouvoir pathogène. Des travaux montrent un lien direct entre la virulence et la capacité d'un pathogène à détoxifier les phytoalexines (Vanetten *et al.*, 2001).

Les premières phytoalexines des plantes de la famille des *Brassicaceae* ont été décrites en 1986 (Takasugi *et al.*, 1986). Depuis, plus de 40 phytoalexines ont été isolées et étudiées (**Figure 4**). D'un point de vue biochimique, la grande majorité des phytoalexines de la famille des *Brassicaceae* ont la particularité d'avoir une base soufrée avec, pour quelques unes, un groupement dithiocarbamate (base structurale de fongicides et herbicides synthétiques). Bien que les groupements puissent être différents, tous sont issus de la transformation du tryptophane. Cet acide aminé est le point de départ de la synthèse



**Figure 5** Biosynthèse de la camalexine et des composés dérivés chez *A. thaliana* (d'après Glawischnig, 2007).

*CYP79B2* et *CYP79B3* catalysent la conversion du tryptophane en indole-3-acetaldoxime (IAOx), un intermédiaire de l'indole glucosinolate et de la biosynthèse de la camalexine. La dernière étape de la biosynthèse de la camalexine est la décarboxylation de l'acide dihydrocamalexique par *CYP71B15* (*PAD3*). IAOx est un précurseur de l'indole-3-carbaldehyde ( $R5 = CHO$ ), l'acide indole-3-carboxylique ( $R5 = COOH$ ), et des dérivés de l'indole-3-aceto-nitrile ( $R5 = CH_2CN$ ), comme l'auxine (IAA, l'auxine prédominante,  $R1 = COOH$ ; l'acide indole propionique,  $R1 = CH_2COOH$ ; l'acide indole butyrique,  $R1 = (CH_2)_2COOH$ ).  $R2 = H$  or  $OCH_3$ ;  $R3 = H, OCH_3$ ;  $R4 = H, CH_3$ , or  $OCH_3$ .

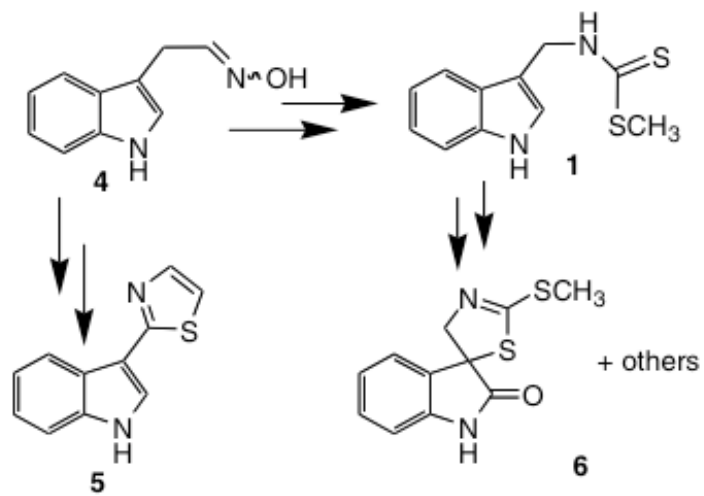
de plusieurs composés de défenses des *Brassicaceae* comme les phytoalexines et dérivés ou encore certaines phytoanticipines comme les glucosinolates indoliques. La répartition au sein des *Brassicaceae* et l'activation de ces molécules peuvent être variables. Certaines sont présentes chez plusieurs espèces de plantes et peuvent être élicitées par différents stress biotiques (agents pathogènes bactériens ou fongiques) ou même abiotiques. L'effet antimicrobien des phytoalexines camalexine et brassinine a déjà été démontré au laboratoire (Sellam *et al.*, 2007a, Sellam *et al.*, 2007b).

## 2.3.2 Les principales phytoalexines

### 2.3.2.1 La camalexine

La camalexine ou 3-thiazol-2'-yl-indole a été découverte chez *Camelina sativa* suite à son infection par le champignon *A. brassicae* (Browne *et al.*, 1991). *A. thaliana*, *Arabis lyrata* et *Capsella bursa-pastoris* sont également décrits comme producteurs de la phytoalexine (Jimenez, 1997, Tsuji *et al.*, 1992, Zook, 1998b). La synthèse de la molécule est possible dans les racines ou s'effectue dans les feuilles lorsque celles-ci sont infectées. La biosynthèse de la camalexine peut aussi être induite par des stress abiotiques. Ainsi, l'application d'acifluorfen (générateur de formes réactives d'oxygène) ou encore d'UV-B peut aboutir à la production de camalexine (Mert-Türk, 2003, Zhao *et al.*, 1998).

La voie de biosynthèse de la camalexine commence par la transformation du tryptophane en indole-3-acetaldoxime ou IAO (Tsuji *et al.*, 1992, Zook, 1998a). L'IAO est un précurseur de nombreuses molécules fongitoxiques comme la brassinine et ses dérivés ou encore les glucosinolates (Pedras *et al.*, 2001). L'IAO est alors condensé avec un groupement thiazole (issu de la cystéine) ce qui aboutit à la production de d'acide dihydrocamalexique ou DHCA. La dernière réaction de cette voie métabolique est catalysée par le cytochrome P450 CYP71B15 (*Pad3*). Le cytochrome va catalyser la formation de camalexine à partir de DHCA (**Figure 5**). Cette phytoalexine est un analogue structural du thiabendazole qui est lui-même un fongicide systémique utilisé dans le contrôle de plusieurs pathologies végétales. L'inactivation du gène *Pad3* d'*A. thaliana* altère significativement la résistance envers *A. brassicicola* suggérant le rôle primordial de ce métabolite dans le contrôle du pathogène par la plante lors de l'interaction (Thomma *et al.*, 1999).



**Figure 6** Biosynthèse de la brassinine (d'après Pedras et al., 2005).

*L'indolyl-3- acetaldoxime [4] est un précurseur commun de la camalexine [5] et de la brassinine [1], lui même précurseur de la spirobrassinine [6].*

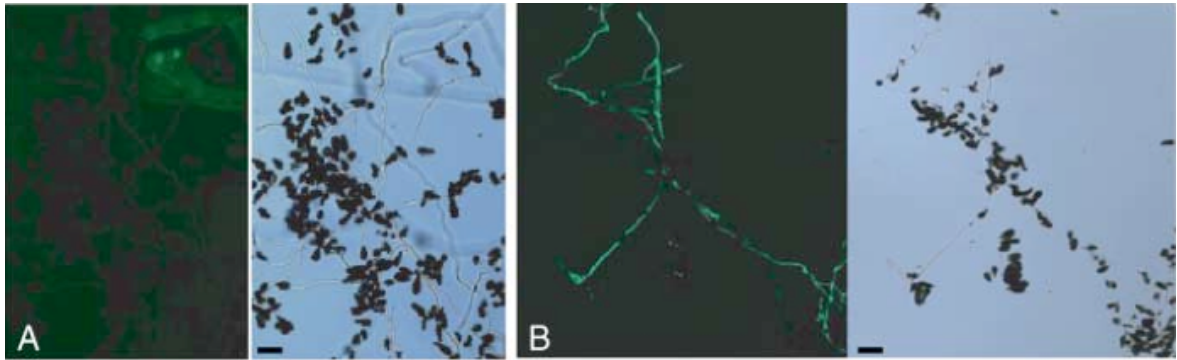


### 2.3.2.2 La brassinine

La brassinine est présente chez les *Brassicaceae* telles que *B. napus*, *B. oleracea* ou encore *R. sativus* (Pedras *et al.*, 2008b). Le rôle de cette phytoalexine est double dans la mesure où elle sert à la fois de composé antifongique mais aussi de précurseur à la biosynthèse d'autres phytoalexines. En effet, même si sa voie de biosynthèse (**Figure 6**) est encore peu étudiée, de récents travaux montrent que celle-ci est un dérivé de l'IAO et un précurseur de nombreuses autres phytoalexines (Pedras *et al.*, 2008a). C'est en subissant plusieurs transformations que l'IAO acquiert son groupement dithiocarbamate et aboutit à la brassinine. Ce groupement dithiocarbamate est certainement responsable de l'activité antifongique de la brassinine et les fongicides de synthèse, utilisés durant les années 1960, utilisaient cette base comme structure active (Leroux, 2003). Il est aussi décrit que la brassinine dispose d'un pouvoir antifongique moins prononcé que d'autres phytoalexines (Pedras *et al.*, 2008c, Sellam *et al.*, 2007a). Effectivement, la brassinine serait un précurseur d'autres phytoalexines à action fongitoxique plus élevée (Pedras *et al.*, 2007) comme la spirobrassinine, la cyclobrassinine ou encore la brassilexine qui présentent des activités antifongiques nettement plus prononcées notamment sur la croissance d'*A. brassicicola* ou *Botrytis cinerea* (Pedras *et al.*, 2009a, Pedras *et al.*, 2011). La brassinine reste cependant un composé très intéressant à étudier dans la mesure où elle se situe en amont de composés très fongitoxiques.

### 2.3.3 Les cibles cellulaires des phytoalexines

Le groupement dithiocarbamate, responsable de l'activité antifongique de la brassinine est utilisé comme fongicide de synthèse. Par exemple, le Manèbe<sup>®</sup> ou le Carbatène<sup>®</sup>, deux fongicides à base de dithiocarbamate, ont été montrés comme agissant à trois niveaux dans la cellule fongique : inhibition de l'oxydation du glucose, inhibition de la synthèse d'acide nucléique, inhibition de la dégradation des acides gras (Mohamed *et al.*, 2009). En ce qui concerne les fongicides comprenant un groupement thiabendazole (retrouvé dans la camalexine), le Mintezol<sup>®</sup> par exemple, des études ont montré qu'ils cibleraient les  $\beta$ -tubulines, principaux constituants des microtubules (Cabanas *et al.*, 2009). Contrairement aux descriptions faites sur les antifongiques de synthèse, les cibles cellulaires des phytoalexines sont encore peu étudiées. Il a été récemment montré que sous l'effet de la camalexine, *A. brassicicola* active l'expression de gènes impliqués dans le métabolisme de lipides membranaires (ergostérol et sphingolipides) (Sellam *et al.*, 2007b). Ces résultats



**Figure 7** Mise en évidence de la perte de l'intégrité cellulaire chez *A. brassicicola* en présence de la phytoalexine camalexine (d'après Sellam et al., 2007).

La perte de l'intégrité cellulaire est visualisée par l'incorporation du SYTOX par des conidies germées en conditions témoin (incubées en présence de DMSO 1 % v/v) (A) ou en conditions traitées par 125  $\mu$ M de camalexine (B) pendant 24 heures.

suggèrent que le système membranaire pourrait constituer une cible potentielle pour la camalexine chez les champignons. Ce mode d'action hypothétique est conforté par celui déjà mis en évidence chez la bactérie phytopathogène *Pseudomonas syringae* (Rogers *et al.*, 1996). Ces auteurs ont montré que l'activité antimicrobienne de la camalexine était la résultante de la perte de l'intégrité membranaire des bactéries. L'addition de camalexine aboutit très rapidement à la déstabilisation de la membrane cellulaire. Le criblage réalisé sur des mutants de *P. syringae* Psm ES4326 partiellement résistant à la camalexine a montré que ces souches sont également plus tolérantes à des antibiotiques (tétracycline et acide nalidixique). Ces molécules seraient incapables de pénétrer dans la cellule du fait du remodelage de la membrane bactérienne (Rogers *et al.*, 1996).

Chez *A. brassicicola*, l'hypothèse d'une altération des systèmes membranaires par la camalexine a pu être confortée par l'utilisation d'un marqueur fluorescent, le SYTOX Green (Sellam *et al.*, 2007b). Ce composé fluoresce une fois lié à l'ADN. La membrane plasmique étant imperméable à ce composé, l'observation d'une fluorescence dans les cellules fongiques traitées avec de la camalexine suggère une possible perturbation de la perméabilité cellulaire (**Figure 7**).

L'hypothèse que nous pouvons formuler est que la camalexine pourrait agir au niveau membranaire et plus précisément au niveau des sphingolipides ou des stérols. Les sphingolipides, tout comme les stérols et les glycérophospholipides, contribuent à la structure des membranes qu'elles soient plasmiques, vacuolaires, golgiennes ou endoplasmiques. Ces composés membranaires sont déjà répertoriés comme pouvant être la cible de composés antifongiques. Par exemple, la défensine RsAFP2 du radis interagit avec des glucosylcéramides (glycosphingolipides) et provoque la mort de la cellule fongique par génération d'un stress oxydatif (Aerts *et al.*, 2007). Autre exemple, la syringomycine E est un lipodepsinonapeptide cyclique (produit par *Pseudomonas syringae* pv. *syringae*) qui est capable d'inhiber la croissance de *Saccharomyces cerevisiae* par interaction avec la membrane plasmique (Stock *et al.*, 2000). Des analyses moléculaires indiquent que plusieurs gènes de la voie de biosynthèse des stérols et des sphingolipides sont impliqués dans la sensibilité de la levure à la syringomycine E (Stock *et al.*, 2000, Takemoto *et al.*, 1993).

### **3. Le déterminisme du pouvoir pathogène d'A.**

*brassicicola*

### 3.1 Facteurs de pathogénie liés à l'attaque de l'hôte

Les espèces pathogènes du genre *Alternaria* sont nécrotrophes et disposent de tout un arsenal de facteurs de pathogénie liés à leur capacité à envahir leurs hôtes. Les principaux mécanismes du pouvoir pathogène comme la production d'enzymes lytiques et de phytotoxines sont détaillés par la suite.

#### 3.1.1 Les enzymes lytiques

La pénétration du champignon dans les tissus de la plante hôte nécessite la destruction des parois cellulaires et autres barrières physiques. Parmi les mécanismes impliqués dans cette destruction, le rôle des enzymes fongiques de dégradation a été particulièrement étudié car elles sont nécessaires pour des champignons qui traversent la surface de l'hôte sans profiter des ouvertures naturelles (stomates ou blessures) et ne produisant pas d'*appressoria* permettant une pénétration mécanique (Idnurm *et al.*, 2001). Ces enzymes correspondent souvent à des pectinases, des cutinases ou encore des protéases.

Chez *A. brassicicola*, deux isoformes de cutinases codées par un seul gène (*Cutab1*) ont été identifiées (Yao, 1994). Il a été montré cependant que la disruption de ce gène n'a aucune influence sur le pouvoir pathogène, mais a des effets drastiques sur la capacité du champignon à utiliser la cutine comme source carbonée lors de sa croissance saprophytique (Fan *et al.*, 1998, Yao *et al.*, 1994).

Le rôle d'une lipase de type sérine estérase dans la pénétration et/ou l'adhésion d'*A. brassicicola* a été établi. Une lipase de 80 kDa a été identifiée chez *A. brassicicola* et son rôle dans le pouvoir pathogène a été démontré. L'addition d'anticorps anti-lipase, ou d'un inhibiteur chimique de l'activité sérine hydrolase, à une suspension de conidies, a pour effet de réduire considérablement (réduction de 90%) l'apparition des symptômes (Berto *et al.*, 1999).

Comme c'est le cas de plusieurs champignons phytopathogènes, *A. brassicicola* semble produire un "cocktail" de plusieurs enzymes lytiques (cutinases et lipases) afin de franchir les barrières physiques des tissus des plantes hôtes.

Plus récemment, des travaux montrent l'implication de voies de transduction du signal dans la synthèse et/ou la sécrétion d'enzymes hydrolytiques. Cho *et al.*, (2007a) ont mis en évidence le rôle de la MAP kinase Amk1p, homologue de Fus3p/Kss1p chez *S. cerevisiae*, dans le pouvoir pathogène d'*A. brassicicola* (Cho *et al.*, 2007). Chez certains champignons phytopathogènes, *Cochliobolus heterostrophus* ou *Magnaporthe grisea* par exemple, le gène *Amk1* est impliqué dans le pouvoir pathogène et notamment dans la formation des *appressoria* (Lev *et al.*, 2003, Xu *et al.*, 1996). Chez ces champignons, la mutation dans cette MAP kinase aboutit à une perte de virulence due à leur incapacité à pénétrer par voie mécanique au delà de l'épiderme végétal. Chez *A. brassicicola*, la disruption du gène *Amk1* aboutit à une perte de la virulence du champignon par absence de pénétration mais aussi à une diminution de l'expression de gènes codant des enzymes hydrolytiques (cutinases ou lipases) sur feuilles non blessées. Les auteurs ont également montré la restauration de cette virulence et la surexpression des gènes codant des enzymes lytiques sur une plante blessée (Jenczmionka *et al.*, 2005). Ces résultats suggèrent une implication de la MAP kinase Amk1p dans le pouvoir pathogène d'*A. brassicicola* par l'intermédiaire de la régulation de la production d'enzymes hydrolytiques en réponse à un signal « nutritionnel » produit par la plante.

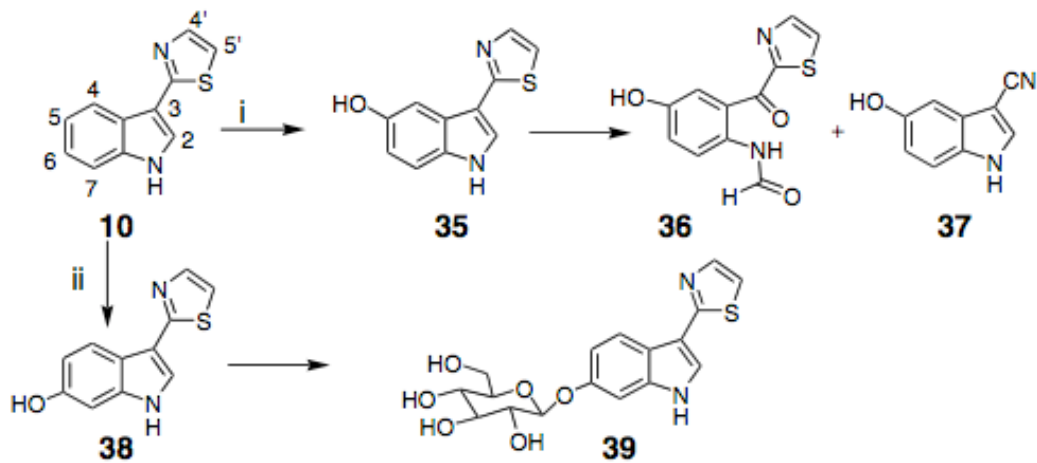
### **3.1.2 Les toxines**

La majorité des phytotoxines produites par les champignons sont des composés chimiques issus du métabolisme secondaire. Les *Alternaria* phytopathogènes sont répertoriés comme des champignons synthétisant deux catégories de toxines : les toxines spécifiques de l'hôte (HST) et des toxines non hôte spécifiques (NHST). Les toxines non spécifiques participent au développement des symptômes (facteurs d'agressivité) bien qu'elles aient un pouvoir toxique assez faible (Ballio, 1991). Chez *Alternaria*, de nombreuses toxines non spécifiques sont identifiées. Pour illustration, la brefeldine A, la (dehydro-) curvularin, l'acide tenuazonique et le zinniol (*A. solani*) sont des toxines non spécifiques qui diffèrent selon leur mode d'action (Thomma, 2003). Ainsi, la brefeldine A

agit comme un inhibiteur des mécanismes de sécrétion tandis que la curvularin agit comme un inhibiteur de la division cellulaire (Thomma, 2003). En raison de leurs larges spectres d'action, qui ciblent des fonctions « basiques » de la cellule, ces mycotoxines font souvent partie des molécules dangereuses pour l'alimentation humaine.

Parallèlement aux toxines non spécifiques, les HSTs sont indispensables pour le développement du symptôme (facteurs de virulence). Le genre *Alternaria* a également été décrit comme produisant des HSTs (Wolpert *et al.*, 2002). En effet, plus de 12 HSTs sont décrites chez divers pathotypes d'*A. alternata* (Kusaba *et al.*, 1995, Kusaba, 1997). Hormis la description biochimique des HSTs, les études montrent également une relation entre la présence, chez ces pathotypes, d'un petit chromosome surnuméraire et la capacité de produire les HSTs (Akamatsu *et al.*, 1997). Ce chromosome surnuméraire n'est pas présent chez toutes les souches et porte des gènes liés à la synthèse et à la sécrétion des toxines. Par exemple, les gènes de biosynthèse de l'AF toxine d'*A. alternata sp fragaria*, sont regroupés en clusters sur un chromosome surnuméraire de 1,05 Mb (Hatta *et al.*, 2002). Un autre exemple est celui de la toxine cyclique AM produite par *A. alternata sp mali* dont les gènes de biosynthèse se situent également sur un chromosome surnuméraire (Johnson *et al.*, 2000). Le regroupement en clusters des gènes de biosynthèse de toxines sur un chromosome surnuméraire ainsi que le fait que ces chromosomes sont retrouvés chez différents pathotypes suggèrent une possible acquisition de ces gènes par transfert horizontal (Cabanas *et al.*, 2009).

Contrairement aux différents pathotypes d'*A. alternata*, peu de données sont disponibles concernant les toxines d'*A. brassicicola*. L'AB toxine, seule HST caractérisée chez *A. brassicicola*, est produite uniquement au niveau du site d'infection et correspond à une protéine de 35 kDa (Otani *et al.*, 1998). Des études ultérieures ont démontré que la production de l'AB toxine est induite par des oligosaccharides provenant de la dégradation des tissus des plantes hôtes (Oka, 2005). Cependant, le ou les gène (s) impliqué(s) dans la biosynthèse de l'AB toxine ne sont toujours pas caractérisés.



**Figure 8** Détoxication de la phytoalexine camalexine (10) par deux champignons pathogènes.

(i) *Rhizoctonia solani* (d'après Pedras, 1997); (ii) *Sclerotinia sclerotiorum* (d'après Pedras et al., 2002).

[10] : camalexine ; [35] : 5-Hydroxycamalexine ; [38] : 6-hydroxycamalexine ; [39] : 1-β-D-Glucopyranosyl camalexine.



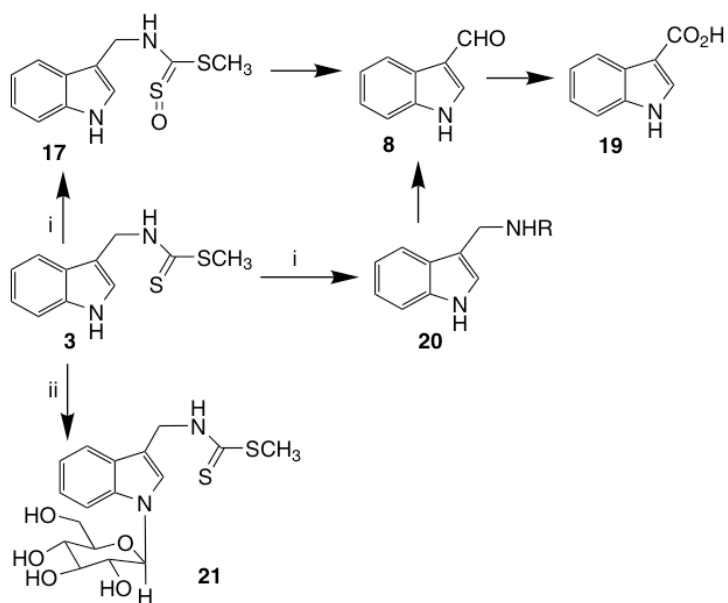
## 3.2 Facteurs de pathogénie liés à la protection du champignon face aux défenses de l'hôte

### 3.2.1 Les mécanismes de détoxification

Les mécanismes de détoxification des métabolites antimicrobiens des *Brassicaceae* par différents pathogènes ont fait l'objet de plusieurs investigations. La résistance de la plante modèle *A. thaliana* ainsi que d'autres *Brassicaceae* sauvages (*Camelina sativa* et *Arabis lyrata*) à des champignons phytopathogènes est généralement associée à la production de la camalexine (Conn, 1994, Thomma *et al.*, 1999, Zook, 1998b). *Rhizoctonia solani*, agent de la fonte des semis de certaines espèces cultivées de *Brassicaceae*, est capable de détoxifier la camalexine en la transformant en 5-hydroxycamalexine et en d'autres produits polaires dont la charge toxique est diminuée (Pedras *et al.*, 2000, Pedras *et al.*, 2007). La détoxification de la camalexine a été également mise en évidence chez *Sclerotinia sclerotiorum* (**Figure 8**) (Pedras *et al.*, 2002). Cette détoxification est réalisée en deux étapes. La camalexine est, tout d'abord, transformée en 6-hydroxycamalexine puis en 1- $\beta$ -D-glucopyranosyl camalexine, composé avec une charge toxique plus faible que celle de la camalexine (Pedras *et al.*, 2002). Cependant, le lien entre la capacité de détoxifier la camalexine et le pouvoir pathogène de ce champignon n'a pas été déterminé (Pedras *et al.*, 2007). Les champignons *A. brassicae* ainsi que *Leptosphaeria maculans* sont incapables de détoxifier la camalexine et sont *a priori* incapables d'infecter efficacement *A. thaliana* ou *C. sativa* (Pedras *et al.*, 1998)

Aucune enzyme de détoxification de la camalexine n'a été, à ce jour, identifiée chez *A. brassicicola*. Néanmoins, il apparaît que chez cette espèce, un gène homologue à *Mak1*, responsable de la détoxification de phytoalexines du pois (pisatine et maackiaine) chez *N. haematococca* (Funnell *et al.*, 2002, Mundodi *et al.*, 2001), est surexprimé en présence de camalexine (Sellam *et al.*, 2007b).

Concernant la brassinine, il existe trois différentes enzymes de détoxification mise en évidence à ce jour. Tout d'abord, une brassinine oxydase et une brassinine hydroxylase ont été identifiées chez *L. maculans* puis une brassinine glucosyltransferase (SsBGT1) a été



**Figure 9** Détoxification de la phytoalexine brassinine par des champignons filamenteux.

(i) *Leptosphaeria maculans/Phoma lingam* (d'après Pedras, 1993, Pedras, 1992, Pedras, 1991)); (ii) *Sclerotinia sclerotiorum* (d'après Pedras, 2004).

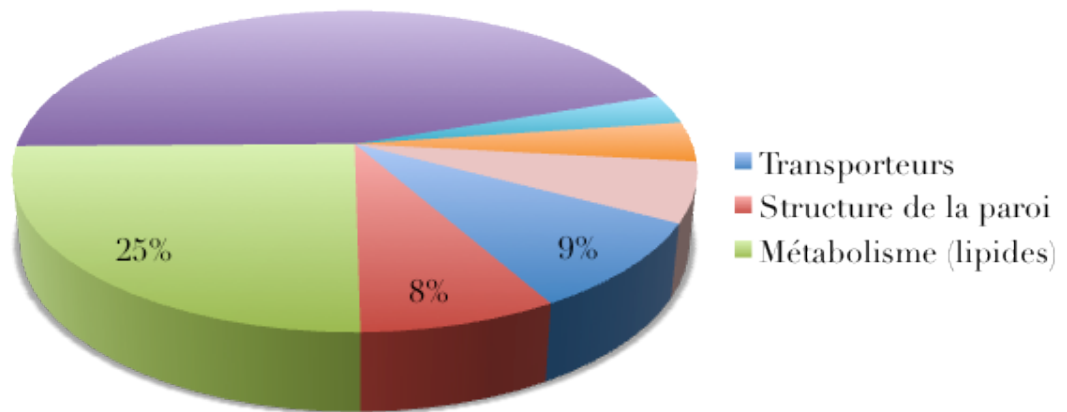
[3] : brassinine; [8] : brassicanate A; [17] : methyl 3- indolylmethyl dithiocarbamate-S-oxide; [18] : indole-3-carboxaldehyde [19] : indole-3-carboxylic acid ; [20] : indolyl-3-methanamine (R=H) ; [21] : 1-β-D-glucopyranosylbrassinine.

décrite chez *Sclerotinia sclerotiorum* (Pedras *et al.*, 2008b, Pedras *et al.*, 2009b, Sexton *et al.*, 2009) (**Figure 9**). Une brassinine hydroxylase (BH) vient d'être récemment découverte chez *A. brassicicola* (BHAb) et est capable de détoxiquer le groupement dithiocarbamate en indolyl-3-methanamine. De plus, l'activité BH est très fortement inhibée par l'action de la cyclobrassinine, dérivée de la brassinine. Ces données sont très importantes en matière de lutte contre le pathogène dans la mesure où elles permettent d'envisager un traitement des alternarioses à base de cyclobrassinine, composé naturel des plantes (Pedras *et al.*, 2009b).

Mise à part ces données récentes, peu d'études relatives aux mécanismes de détoxification des autres métabolites antimicrobiens des *Brassicaceae* sont disponibles. Des travaux ont identifié, chez *A. brassicicola*, un gène codant une cyanide hydratase différentiellement exprimé au cours de l'interaction avec *A. thaliana* (Cramer *et al.*, 2004). Les cyanides hydratases appartiennent à la famille des enzymes nitrilases impliquées dans la détoxification des produits cyanogènes. Ces auteurs ont suggéré que cette protéine pourrait être impliquée dans la détoxification des produits de dégradation des glucosinolates. Le mutant nul de ce gène montre une forte sensibilité vis-à-vis du produit cyanogène KCN, cependant, il garde entièrement son pouvoir pathogène, suggérant que ce gène n'est pas indispensable pour réussir la colonisation des plantes hôtes (Cramer, 2005).

### 3.2.2 L'exportation des molécules

Ce mode de détoxification consiste en la surexpression de systèmes d'efflux empêchant l'accumulation de métabolites potentiellement antifongiques à des taux toxiques au niveau cellulaire. Dans ce cas, le contournement des métabolites antimicrobiens est assuré par un système de transporteurs membranaires. Des transporteurs de type ATP-binding cassette (ABC) et les «Major Facilitator Superfamily» (MFS) sont impliqués dans ce type de détoxification du fait de leur capacité à exporter la molécule toxique à l'extérieur de la cellule. Le lien entre l'aptitude à contourner des métabolites antimicrobiens produits par les plantes hôtes *via* ce mécanisme d'efflux et le pouvoir pathogène a été clairement établi chez certains champignons comme *B. cinerea* (Schoonbeek *et al.*, 2001, Schoonbeek *et al.*, 2002, Stefanato *et al.*, 2009) et *M. grisea* (Urban *et al.*, 1999). Chez *B. cinerea*, le mutant nul du gène codant l'ABC transporteur *BcAtrB* est très sensible au resveratrol et au fongicide fenpiclonil suggérant un rôle important de ce transporteur dans l'efflux de ces xénobiotiques (Schoonbeek *et al.*, 2001). De plus, il a récemment été mis en évidence que



**Figure 10** Classification fonctionnelle des gènes surexprimés chez *A. brassicicola* en présence de camalexine (d'après Sellam et al., 2007).

ce transporteur était également responsable chez *B. cinerea* de l'export de la camalexine. Ainsi, le mutant nul *BcAtrB* est plus sensible à la camalexine et moins agressif sur *A. thaliana* écotype Col-0 (Stefanato *et al.*, 2009) que la souche sauvage de *B. cinerea* d'origine. De plus, ce mutant possède une agressivité comparable à la souche sauvage lors de l'infection du génotype *Pad3* (non producteur de camalexine) d'*A. thaliana*.

Chez *A. brassicicola*, plusieurs gènes impliqués dans la biosynthèse de transporteurs, MFS ou ABC, sont surexprimés en présence de camalexine (Sellam *et al.*, 2007b). Cette observation suggère que, comme pour *B. cinerea*, l'efflux de la camalexine pourrait constituer l'une des stratégies utilisée par ce champignon pour limiter l'effet toxique des phytoalexines. Néanmoins, aucune étude fonctionnelle de ces transporteurs n'a été réalisée à ce jour.

### 3.2.3 *La mise en place d'un mécanisme compensatoire*

La détoxification et l'exportation cellulaire des molécules de défense des plantes font partie des mécanismes mis en place par les champignons pour se protéger lors de l'infection de la plante hôte. Sellam *et al.*, (2007b) ont émis la possibilité d'une troisième stratégie de protection de la cellule fongique par l'intermédiaire d'un mécanisme compensatoire visant à renforcer l'intégrité cellulaire face à la phytoalexine camalexine (Sellam *et al.*, 2007b). Ces auteurs ont, tout d'abord, mis en évidence la perturbation cellulaire provoquée par cette phytoalexine. Ils ont également étudié la réponse du champignon (étude transcriptomique) en présence de la camalexine. Les résultats ont montré que des gènes impliqués dans le transport membranaire, la biogénèse pariétale, dans la biosynthèse des lipides et stérols membranaires mais aussi dans la biosynthèse de la mélanine sont surexprimés chez le pathogène en présence de camalexine suggérant ainsi l'intervention d'un mécanisme compensatoire (**Figure 10**).

Même si l'intervention d'un mécanisme compensatoire dans la protection des champignons phytopathogènes contre les molécules de défense des plantes est originale, le mécanisme compensatoire a déjà très bien décrit chez *S. cerevisiae* dans le cas de la réponse à des agents de stress pariétaux. Des études de type « microarrays » ont permis de mettre en évidence 132 et 101 gènes surexprimés chez *S. cerevisiae* soumis à des stress

avec respectivement le CongoRed et le Zymolyase (Garcia *et al.*, 2004). La plupart des gènes surexprimés montrent des motifs de régulation par les facteurs de transcription Rlm1p, Msn2p/Msn4p ou encore Ste12p. Ceci suggère une régulation complexe de ce mécanisme compensatoire avec l'intervention potentielle de plusieurs voies de signalisation.

Les voies de signalisation MAP kinases sont de véritables réseaux intracellulaires qui permettent aux champignons de répondre aux divers stress extérieurs. Elles sont liées les unes aux autres et agissent conjointement. La voie des phéromones, la voie de l'osmolarité, la voie de l'intégrité pariétale ou encore la voie de la filamentation participent toutes au pouvoir pathogène des champignons. Par exemple, des études ont montré que la voie de signalisation CWI serait nécessaire au pouvoir pathogène de plusieurs espèces phytopathogènes. Les mutants  $\Delta Mgs12$  et  $\Delta Bmp3$ , respectivement impliqués dans la voie CWI chez *M. grisea* et *B. cinerea*, montrent par exemple une entrée normale soit par un stomate ou soit par une blessure, mais le développement des lésions est stoppé ou très fortement ralenti. L'hypothèse émise mais non démontrée est une sensibilité accrue de ces mutants à des composés de défense comme des phytoalexines ou des PR-protéines (Pathogenesis Related Proteins) (Mehrabi *et al.*, 2006, Rui *et al.*, 2007). D'autre part, le rôle de la voie CWI dans la pathogénie est clairement démontré chez des champignons pathogènes des animaux, comme *C. albicans*, où le mutant déficient dans la MAP kinase Slt2p est incapable de croître à des températures élevées (proches de la température corporelle) et de se développer chez l'hôte (Navarro-Garcia *et al.*, 1995).

Par ailleurs, la voie de l'osmolarité HOG est également impliquée dans le pouvoir pathogène de certaines espèces fongiques. Des mutants nuls de cette voie montrent une sensibilité accrue aux stress osmotiques mais également une baisse significative de la virulence comme, par exemple, le mutant  $\Delta Osm1$  de *M. grisea* (Dixon *et al.*, 1999). Chez *B. cinerea*, le mutant  $\Delta Bcsak1$ , déficient dans la MAP kinase Hog1p est caractérisé par une déficience pour la pénétration dans la plante mais également par une diminution de sa capacité à former des sclérotés (Segmuller *et al.*, 2007).

Malgré des connaissances de plus en plus détaillées sur les voies de signalisation et leurs implications dans le pouvoir pathogène des champignons, à l'exception d'une étude réalisée chez *F. graminearum* (Ramamoorthy *et al.*, 2007), aucune publication ne démontre

l'implication de voie de signalisation dans l'adaptation des champignons à des molécules de défense de l'hôte. Chez *F. graminearum*, l'implication de la voie CWI a été mise en évidence dans la protection face à la défensine MsDef1. Le mutant nul de la MAP kinase Mgv1p, homologue de Slt2p de *S. cerevisiae*, est hypersensible à cette défensine et montrent une diminution de son agressivité *in planta*.

# Chapitre 1

**Mise au point d'une technique de phénotypage  
haut débit basée sur le suivi de la cinétique de  
champignons filamenteux**



## **1. Présentation de l'étude**

Ce chapitre est consacré à la mise au point d'une nouvelle technique de phénotypage haut débit basée sur le suivi de la croissance de champignon filamenteux. Cette croissance, en conditions optimales ou en conditions de stress, peut être classiquement étudiée sur des milieux nutritifs gélosés en boîte de Pétri. Le paramètre généralement mesuré est la vitesse de croissance radiale d'une souche. Cette méthode standard est simple à mettre en œuvre cependant elle présente certaines limites. Tout d'abord, elle ne permet pas un phénotypage à haut débit et ne donne pas directement accès à l'étude des phases précoces de la croissance (germination des conidies, élongation des tubes germinatifs). Les phases précoces de la croissance peuvent être étudiée en microscopie par l'intermédiaire de la mesure de la longueur des tubes germinatifs à différents temps. Néanmoins, cette méthode s'avère très couteuse en temps et relativement fastidieuse. La caractérisation de la croissance des champignons peut également être obtenue par absorptiométrie. La mesure est effectuée avec un spectrophotomètre qui permet de suivre une cinétique de croissance sur des temps très long (plusieurs jours) (Meletiadiis *et al.*, 2001a). Cependant, cette méthode, bien que donnant l'accès au haut débit, présente certains désavantages. Par exemple, l'absorptiométrie est régie par la loi de Beer-Lambert et présente une limite à la linéarité en cas de fortes concentrations. Le résultat de l'application d'une telle technique aux champignons filamenteux se traduit par des courbes avec une variation d'absorbance faible et un plateau de saturation rapidement obtenu (Meletiadiis *et al.*, 2003, Meletiadiis *et al.*, 2001b, Meletiadiis *et al.*, 2001a).

Pour faciliter le phénotypage des nombreux mutants d'*A brassicicola* générés au laboratoire, une méthode plus performante pour l'analyse de la croissance des champignons filamenteux a été développée. Cette nouvelle technique est basée sur l'utilisation d'un système de mesure par néphélométrie laser en microplaque. La néphélométrie est, au même titre que l'absorptiométrie, une technique de turbidimétrie qui consiste à mesurer l'intensité d'un trouble soit par mesure directe de la perte d'intensité entre le flux entrant dans une suspension et le flux sortant (cas de l'absorptiométrie), soit par mesure indirect des rayons déviés par les particules en suspension (cas de la néphélométrie). De manière générale, la néphélométrie présente l'avantage d'avoir une corrélation linéaire entre le nombre de particules en suspension et la valeur d'unité néphélométrique sans saturation. Ajouté à cela, la néphélométrie ne fait pas de ratio entre le flux entrant dans la suspension et le flux sortant. Ce phénomène évite, lorsque les

particules s'accumulent au fond d'une cupule, de saturer le système de mesure de l'appareil.

La néphélométrie se révèle bien adaptée à l'étude de la croissance des champignons filamenteux pour de nombreuses raisons. Tout d'abord, cette méthode permet l'étude des phases précoces de la croissance des champignons. En effet, les suspensions calibrées et mesurées sont composées de conidies et l'appareil mesure l'apparition et l'élongation des tubes germinatifs sur des temps très courts (moins de 30 heures). De plus, cette technique *in vitro* permet d'étudier le comportement de souches à haut débit dans des volumes très faibles. Les microplaques (96 puits) utilisées pour chaque tests contiennent seulement 300  $\mu\text{L}$  de suspension de conidies. L'avantage de disposer de faibles volumes est de pouvoir tester des composés, les métabolites secondaires par exemple, dont certains ne sont accessibles qu'en faibles quantités. Enfin, un dernier avantage de la méthode réside dans l'analyse des données. En effet, l'étude de la croissance d'un champignon filamenteux par néphélométrie aboutie à l'obtention d'une courbe de croissance. Celle-ci, bien qu'elle ne soit pas issue d'un phénomène de croissance exponentielle est très facilement assimilable à une sigmoïde caractéristique de la croissance d'une bactérie. Ainsi, il est possible de définir des paramètres quantitatifs sur les courbes de croissance tels que la vitesse de croissance ou encore le temps de latence. Ces données mathématiques permettent alors de comparer chaque souche et de mesurer l'impact d'un stress sur leur croissance.

L'article présenté dans ce chapitre explique la mise en place et le paramétrage de l'appareil néphélométrique mais également son utilisation pour mesurer la croissance de champignons filamenteux avec ou sans stress.

Dans un premier temps, l'étude a consisté à vérifier que la courbe de croissance obtenue avec l'appareil correspond bien à un état physiologique de la croissance du champignon. Des comptages et observations microscopiques, réalisés tout au long d'une cinétique de croissance d'*A. brassicicola*, assurent la corrélation entre la croissance du champignon et les valeurs néphélométriques. En effet, celles-ci augmentent avec le nombre de conidies germées ou l'allongement des tubes germinatifs. De plus, une corrélation linéaire entre la biomasse de champignon et les valeurs néphélométriques est observée. Un dernier point de paramétrage consiste à vérifier l'absence de limite à la linéarité lorsque les suspensions sont trop denses.

La seconde étape de l'étude a consisté à comparer les divers paramètres des courbes de croissance des champignons en présence ou non d'un stress. Pour cela, trois genres de champignons sont testés, deux filamenteux, *Aspergillus* (pathogène humain) et *Alternaria* (pathogène des plantes) et un levuroïde, *Candida* (pathogène humain). Chacun des tests fait sur ces champignons est réalisé avec la méthode néphélométrique et avec la méthode conventionnelle (croissance sur milieu nutritif en boîte de Pétri ou E-test). Le but est d'établir une correspondance entre la sensibilité ou la résistance de divers génotypes face à des antifongiques avec les deux méthodes.

La néphélométrie permet d'étudier les effets antifongiques d'une molécule sur la croissance des champignons. Par exemple, la sensibilité de la souche sauvage *Abra43* d'*A. brassicicola* vis-à-vis du fludioxonil (fongicide de la famille des phénylpyrroles) est mise en évidence par une modification de ses paramètres de croissance par rapport à la condition sans traitement (lag phase plus longue et croissance moins rapide). De plus, il est également possible de mesurer l'impact d'une mutation dans la réponse d'un champignon face à un composé antifongique. Par exemple, la souche mutante de *Candida glabrata* IHEM 21230 s'avère moins sensible à l'amphotéricine B que la souche sauvage. Il est important de noter que les tendances de résistance ou de sensibilité des souches observées avec les tests classiques sont également visualisées par néphélométrie.

La néphélométrie permet de différencier des comportements de souches face à des antifongiques de la même manière que les tests conventionnels avec les avantages de permettre une étude sur des temps de croissance courts et dans des volumes très faibles. Cette technique a pour but d'être utilisée en routine au laboratoire afin d'obtenir des phénotypes haut débit. L'article « Laser nephelometry applied in an automated microplate system to study filamentous fungus growth » paru dans la revue *Biotechniques* (vol. 48, N°5, 2010) présente les résultats détaillés de la mise en place et de l'utilisation de cette nouvelle méthode.

## 2. ARTICLE

### ***Laser nephelometry applied in an automated microplate system to study filamentous fungus growth***

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# Reports

## Laser nephelometry applied in an automated microplate system to study filamentous fungus growth

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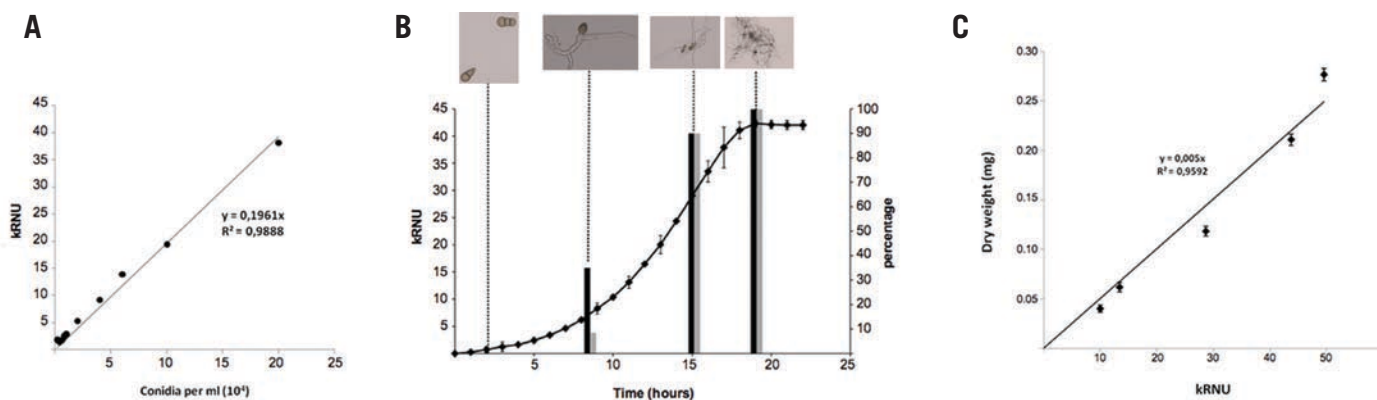
By contrast with photometry (i.e., the measurement of light transmitted through a particle suspension), nephelometry is a direct method of measuring light scattered by particles in suspension. Since the scattered light intensity is directly proportional to the suspended particle concentration, nephelometry is a promising method for recording microbial growth and especially for studying filamentous fungi, which cannot be efficiently investigated through spectrophotometric assays. We describe herein for the first time a filamentous fungi–tailored procedure based on microscale liquid cultivation and automated nephelometric recording of growth, followed by extraction of relevant variables (lag time and growth rate) from the obtained growth curves. This microplate reader technique is applicable for the evaluation of antifungal activity and for large-scale phenotypic profiling.

### Introduction

Since the yeast *Saccharomyces cerevisiae* was sequenced in 1996 (1), the number of available fungal genome sequences has increased by one order of magnitude. Over 100 complete fungal genomes have been publicly released or are currently being sequenced, thus representing the widest sampling of genomes from any eukaryotic kingdom (2). This fungal genomics boom has greatly expanded our view of the genetic and physiological diversity of these organisms. However, the function of many proteins encoded by the genome has not yet been experimentally determined. Targeted inactivation of the corresponding genes and subsequent phenotypic characterization of resulting mutants are frequently the first important step toward determining the cellular role of proteins. Deletion strain collections of all genes in the genome are now available for model organisms such as *S. cerevisiae* (EUROSCARF, Frankfurt, Germany) and the filamentous ascomycete *Neurospora crassa* (Fungal Genetics Stock Center, Kansas City, Missouri). These initiatives have paved the way to the development of phenomics: large-scale quanti-

tative phenotypic analysis of genotypes on a genome-wide scale (3). Procedures based on microscale liquid cultivation and optical recording of growth in automated microplate systems have been developed for high-throughput quantitative phenotypic profiling of yeasts (4,5). Concerning filamentous fungi, assays based on the analysis of colony expansion rates on solid media or on microscopic measurement of the length of hyphae can generate quantitative phenotypic data. However, such screenings are cumbersome and time-consuming, and therefore not suitable for large-scale phenotypic analysis. Moreover, the specific effects of a particular mutation and/or environment on different fungal growth features (such as the lag phase, rate of growth, and growth yield) cannot be deduced from data obtained on solid media. Despite the fact that they are theoretically only applicable for unicellular organisms (6), spectrophotometric assays have also been developed to monitor the growth of filamentous fungi in microbroth systems over time (7,8) [Q1]. However, the accuracy of spectrophotometric readings may be hampered by the presence of clumps of hyphae and the nonhomogeneous growth

of filamentous fungi in liquid media. Moreover, a major drawback of spectrophotometric methods, irrespective of the kind of cells under analysis, is that extinction in cell suspensions is proportional to the cell density only at rather low values. Usually, it is considered that this lack of proportionality is significant at OD values >0.5 and that only corrected photometric readings should be used for values beyond the range of proportionality (7,9,10). By contrast with spectrophotometry, which measures the transmission of light, nephelometry (another light-based technique for measuring medium opacity) uses light scattering. This parameter is directly proportional to the cell density and is suitable for a broad range of cell densities. Since its first description for counting yeast suspensions (11), nephelometry has been routinely used to study the growth of *S. cerevisiae*, and a microbroth kinetic system was recently developed to monitor the growth of some *Candida* yeast species (12,13). As it has also been successfully used for quantifying particles in nonhomogeneous suspensions, we assumed that nephelometry might be suitable for plotting accurate growth curves for filamentous



**Figure 1. Use of laser nephelometry for fungal propagule determination and growth curve monitoring.** (A) Correlation between the number of conidia of *A. brassicicola* and RNU values. RNU values were obtained from serial dilutions of conidia. Conidia were harvested in sterile water from PDA [Q12] plates and conidial densities were determined microscopically using a Thoma's counting chamber. Conidial suspensions were then diluted to obtain the desired concentrations (600–60000 conidia/well). RNU measurements were done immediately without incubation for each calibrated suspension. (B) Nephelometric growth curve of *A. brassicicola* in PDB. The initial suspension was adjusted to 10<sup>5</sup> conidia/mL and the nephelometric values were automatically recorded for each well every hour. The microscopic morphology was examined at different time points and the percent of conidia developing hyphae (black column) or branching (gray column) was estimated in duplicate at 2, 8, 15, and 19 h. (C) Regression curves of kRNU versus dry weight for *A. brassicicola*. The mycelial mass was collected at different time points of the growth curve and dry weight values were determined for the total biomass from 12 wells. [Q13]

fungi. In this study, we took advantage of the commercial availability of a laser-based microplate nephelometer to develop a method based on microscale liquid cultivation for automated recording of fungal growth. This procedure was evaluated with phytopathogenic (*Alternaria brassicicola*) or human pathogenic (*Aspergillus fumigatus* and *A. terreus*) filamentous fungi and the yeast *Candida glabrata*.

## Materials and methods

### Fungal strains and cultivation media

The *A. brassicicola* wild-type strain *Abra43* used in this study was isolated from *Raphanus sativus* seeds. Two disruptants—*Abhog1Δ1* and *Abnik1Δ3*—were obtained from the wild-type strain *Abra43* as previously described (14). Strains of *A. terreus* and *C. glabrata* were recovered from clinical samples (15) and deposited at the Institute of Hygiene and Epidemiology Mycology section (IHEM; Brussels, Belgium) culture collection. The reference strain 18963 of *A. fumigatus*, used for sequencing the genome of this species, was obtained from IHEM. *A. brassicicola* and *Aspergillus* strains were cultivated at 24°C on potato dextrose (PD) agar medium (Difco [Q2]). *C. glabrata* strains were grown at 30°C on YPD [Q3] (Sigma [Q4]) agar.

### Growth curve measurements

For inoculum preparation, conidia of filamentous fungi were collected from 8-day-old solid cultures by adding PD broth followed by gentle scraping of the agar plates. They were then counted in a Thoma's chamber and the conidial suspen-

sions were diluted to obtain the desired concentrations. Overnight precultures of *C. glabrata* isolates were harvested by centrifugation [Q5], washed in sterile water, and inoculated to OD 0.10–0.15 in fresh YPD medium. The microplate wells were filled with calibrated suspensions (300 μL/well).

Growth was automatically recorded for ≥30 h at 25°C for filamentous fungi and at 30°C for yeast using a nephelometric reader (NEPELOstar Galaxy, BMG Labtech, Offenburg, Germany), equipped with a 635-nm laser as radiation source. During incubation, the 96-well plates were subjected to shaking at 175 rpm for 5 min every 10 min. Measurements were done every hour with a gain value of 90 and a percentage of the maximum value of 20%. Each well was measured for 0.1 s with a laser beam focus of 2.5 mm.

### Analysis of growth data

Data were exported from Nephelostar Galaxy software in ASCII format and further processed in Microsoft Excel ([Q6] Microsoft, Redmond, Washington, USA) and R 2.6.1 (<http://cran.r-project.org> [Q7]). All data presented herein was obtained from two independent biological repetitions. Each repetition included three technical replicates. Both lag time and maximal growth rate variables were calculated from the growth curves using a calculation method derived from that reported for yeast cultures (5). First, an initial relative nephelometric unit (RNU) value was calculated as the mean of the initial three measurements and then subtracted from each curve value. For each point of

the curve, a slope was calculated using measurements made 2 h before and 2 h after that time point. The lag phase was defined as the time required to obtain a slope value of 1. The maximal growth rate was defined as the highest slope. Homoscedasticity and normality of residues were checked using Breusch-Pagan and Shapiro-Wilk tests, respectively.

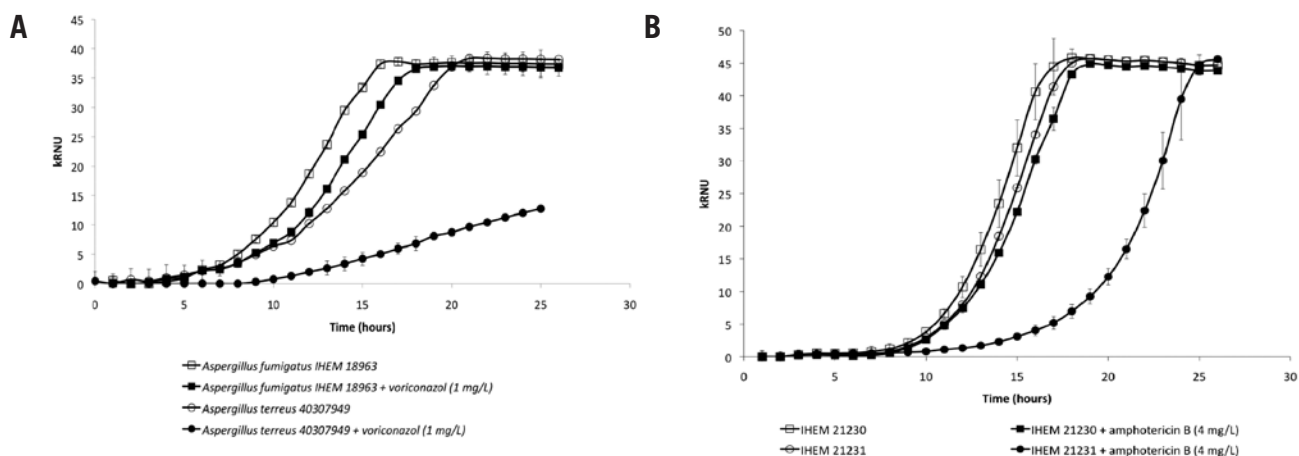
### Dry weight measurements

The initial conidial suspension was adjusted to 10<sup>5</sup> conidia/mL (i.e., 30000 conidia/well) in PDB [Q8]. The mycelial mass was collected at different time points of the growth curve of *A. brassicicola*. Dry weight values were determined for the total biomass from 12 wells after freeze-drying until constant weight.

## Results and discussion

### Proportionality between nephelometric values and cell densities

The nephelometric values were tested for linearity through serial dilution of conidia of *A. brassicicola*. At all tested densities (ranging 600–60,000 conidia/well), there was a linear relationship between the number of conidia and the RNU ( $R^2 = 0.988$ ) at least until saturation of the light detector [which was adjusted to 50,000 RNU [Q9] (Figure 1A)]. A direct consequence of the linear relationship between the number of fungal propagules and RNU is that the raw values do not need to be converted to corrected values before growth curve analysis. Moreover, the nephelometer can be used as an accurate tool for calibrating fungal inoculum or



**Figure 2. Nephelometric monitoring of growth for antifungal susceptibility testing.** (A) Growth curves describing the effect of voriconazole (1 mg/L) on isolates of *Aspergillus fumigatus* (IHEM 18963) and *A. terreus* (IHEM 23439). (B) Growth curves describing the effect of amphotericin B (4 mg/L) on two isolates of *C. glabrata* (wild-type IHEM 21231 and clinical isolate IHEM 21230). [Q14]

evaluating the capacity of mutants to sporulate.

### General growth characteristics of filamentous fungi in a laser-based microplate nephelometer

Figure 1B shows a representative growth curve for *A. brassicicola* plotted from the nephelometric assays. A similar growth pattern was obtained from *N. crassa* or *Aspergillus* inocula (data not shown) and was reproducible in both the biological and technical replicates. The initial suspension was adjusted to  $10^5$  conidia/mL (i.e., 30,000 conidia/well) in PDB and the nephelometric values were automatically recorded for each well every hour after shaking. In order to correlate the RNU changes with the morphology of the fungal colonies, the microplate well contents were harvested at given intervals and the hyphae and branching numbers were estimated by microscopic examination. Dry weight values were also determined for the mycelial mass at different time points of the growth curve.

Monitoring the filamentous fungi growth kinetics by nephelometry resulted in typical microbial growth curves containing a lag phase, a maximal growth rate phase [Q10], and a plateau. In our experimental procedure, this last step cannot be considered as a classical stationary phase; rather, it corresponds to the saturation value of the detector (adjusted to 50,000 RNU). This saturation step did not have an impact on shape of the growth curves or on extrapolated growth variables (lag time and maximal growth rate). A biological plateau could be reached by lowering the nutrient source (data not shown). However, the use of a poor medium might result in erroneous data (as reported

by Reference 8). Microscopic examinations revealed a correlation between the RNU variations and the extension and branching of hyphae from conidia. *A. brassicicola* conidia germination did not occur within 5 h of incubation, which was a period with low RNU variation (between 0 and 4000 RNU). After 18 h of incubation, all conidia had produced branched hyphae and a dense mycelium covered the bottom of the wells. Alongside the microscopy images, Figure 1C showed that dry weight values of mycelial biomass correlated with recorded nephelometry measures ( $R^2 = 0.959$ ), providing evidence that laser nephelometry is an accurate indicator of the fungal biomass and can be used as reliable tool for the monitoring of fungal growth.

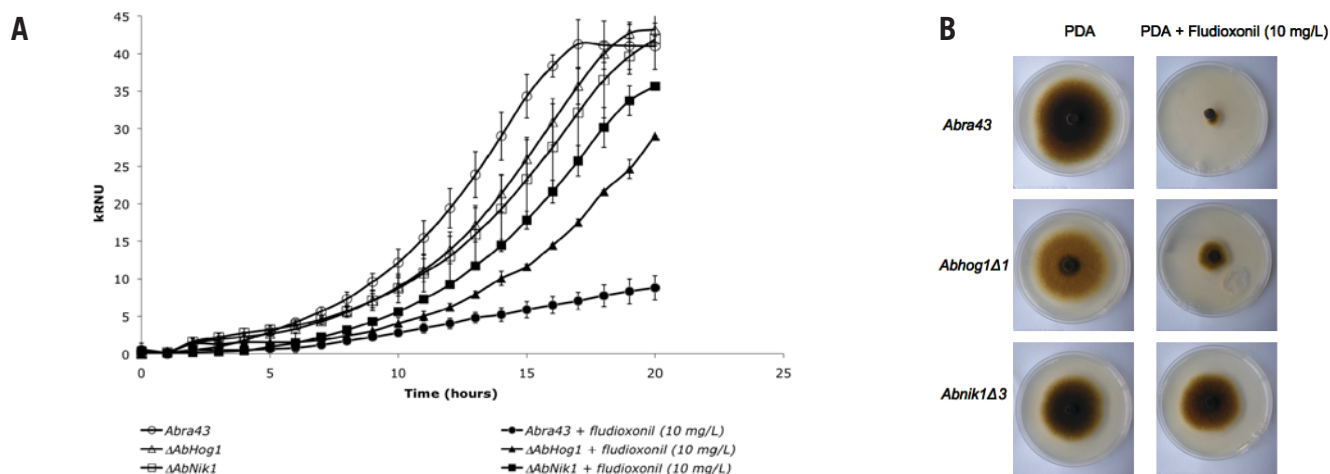
### Growth curves in the presence of antifungal compounds

As extrapolated from the yeast absorbance growth curves (5), two standard growth variables for filamentous fungi could be defined: the maximum growth rate and the length of the lag phase (or lag time). (For calculation details, see the “Materials and Methods” section.) These values provide precise parameters that can be used for analysis of growth inhibition curves and antifungal activity of natural or synthetic substances. Figures 2 shows two examples of antifungal susceptibility testing with important human pathogenic fungi. The sensitivity of a fungus to a drug could result in different types of growth aberration. A first effect is illustrated in Figure 2A. The sensitivity of a clinical *A. terreus* isolate (IHEM 23439) to voriconazole (a triazole molecule which is recommended for the treatment of invasive aspergillosis) resulted in a significant reduction of the maximum

growth rate (from  $3.66 \text{ kRNU/h} \pm 0.06$  for the control to  $1.91 \text{ kRNU/h} \pm 0.27$  after exposure to voriconazole at 1 mg/L). By comparison, the maximum slope ( $4.39 \text{ kRNU/h} \pm 0.40$ ) of the *A. fumigatus* reference strain (IHEM 18963) was only slightly affected when challenged with this drug at the tested concentration, which was in agreement with the minimum inhibitory concentrations (MICs) of voriconazole determined for these two *Aspergillus* strains using the E test method ( $0.064 \text{ mg/L}$  for *A. terreus*, IHEM 23439 and  $0.25 \text{ mg/L}$  for *A. fumigatus*, IHEM 18963). Figure 2B shows another type of inhibitory effect caused by the application of amphotericin B (4 mg/L) on *C. glabrata* suspensions. When using the E test method on Casitone agar, the clinical isolate IHEM 21230 was shown to be less susceptible to polyenes than the wild-type isolate IHEM 21231 with a MIC of amphotericin B more than twice that reported for the wild-type isolate (15). The resistance of isolate 21230 was perfectly illustrated by nephelometric recording, since the growth curves obtained with or without amphotericin B were almost identical [Q11]. By contrast, the sensitivity of the wild-type was clearly explained by a delayed entry into the log phase, while its maximum growth rate ( $6.48 \text{ kRNU/h} \pm 0.51$ ) remained unaffected. Lag times were estimated at  $6.50 \text{ h} \pm 0.71$  and  $14.50 \text{ h} \pm 0.71$  for the control and treated conditions, respectively.

In an attempt to create a methodology for large-scale phenotypic profiling, we applied a strategy similar to that used for antifungal susceptibility testing to compare the phenotypes of deletion and wild-type strains on the basis of growth behavior during micro-cultivation. In this study,





**Figure 3. Determination of fludioxonil susceptibility in null mutants of *A. brassicicola* deficient in genes from the HOG-related pathway.** (A) Nephelometric growth curves of wild-type and two deletion strains (*Abhog1Δ1* and *Abnik1Δ3*) following exposure to fludioxonil (10 mg/L) and under standard conditions. (B) Mycelium radial growth measurements on agar media. Agar disks were cut from the margin of a 7-day-old colony growing on PDA and were transferred onto the center of a PDA medium supplemented with fludioxonil at 10 mg/L. Growth was scored after 7 days of incubation at 24°C. [Q15]

we tested two *A. brassicicola* null mutants (*ΔAbHog1* and *nik1Δ3*) and the corresponding wild-type (*Abra43*) for susceptibility to the phenylpyrrole fungicide fludioxonil (Figure 3A). It was previously reported that this compound exerts its toxicity in filamentous fungi through targeting of the high-osmolarity glycerol (HOG) pathway, a mitogen-activated protein kinase (MAPK) cascade involved in the response to high osmotic stress (14,16). The genes *AbNIK1* and *AbHOG1* encode an osmosensor group III histidine kinase and the MAP kinase of the HOG pathway, respectively. The wild-type strain was highly susceptible to the fungicide, according to the nephelometric growth records. At 10 mg/L of fludioxonil, the lag time was increased by 4× and the maximal slope was reduced by 6×, compared with control conditions. By contrast, *Abnik1Δ3* was found to be highly resistant (roughly similar lag time and maximum growth rate under both control and treated conditions), while *Abhog1Δ1* exhibited moderate resistance to this fungicide (the lag time was increased by 1.5× and the maximum growth rate was decreased by 1.3× in the presence of 10 mg/L of fludioxonil). The growth patterns obtained by nephelometric recording were confirmed by mycelium radial growth measurements on agar media (Figure 3B). We also observed distinct nephelometric profiles revealing various levels of susceptibility toward fludioxonil in different *N. crassa* mutants deficient in genes from the Hog/Os-related pathway (data not shown). These fungicide susceptibility patterns are in accordance with results obtained with deletion mutants of orthologous genes in the southern corn leaf blight fungus *Cochliobolus heterostrophus*,

thereby confirming that some filamentous fungi have two pathways that additively control phenylpyrrole fungicide sensitivity (17). Both are regulated by a group III histidine kinase but only one controls HOG1-type MAPK phosphorylation.

In conclusion, we present evidence that a laser-based microplate nephelometer can be used as a reliable tool for monitoring filamentous fungus growth. In comparison with the current standard method based on radial growth measurements on solid media, the microplate reader of this system provides many advantages, such as fast and easy handling of large numbers of samples, a reduction in the amount of test substances, and a rapid and accurate measuring mode with computerized processing of the quantitative data. Moreover, the raw values do not need to be corrected for nonlinearity before quantitative analysis, and altered growth can be easily interpreted on the basis of a change in the length of the lag phase, the rate of growth, or both, which is not the case for colony-size measurements. We believe that this system could be successfully applied for a broad range of filamentous fungi, yeasts, and bacteria for large-scale screening based on quantitative changes in growth phenotypes under a wide variety of growth conditions. Thus, the method described herein paves the way for the development of a high-resolution quantitative phenomic approach for filamentous fungi, as is already available for yeast (18).

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## Competing interests

The authors declare no competing interests.

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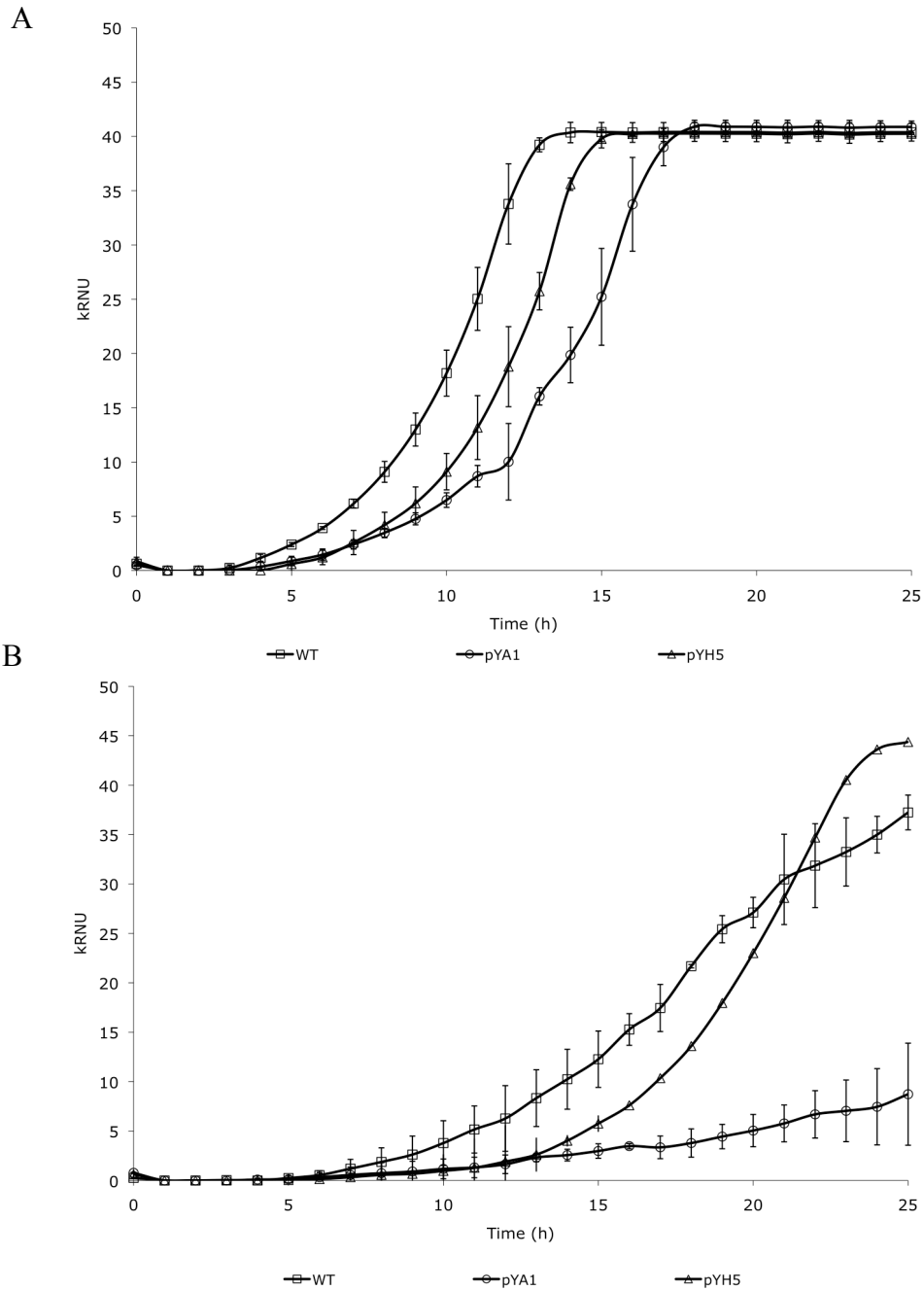
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### **3. Résultats complémentaires et perspectives**



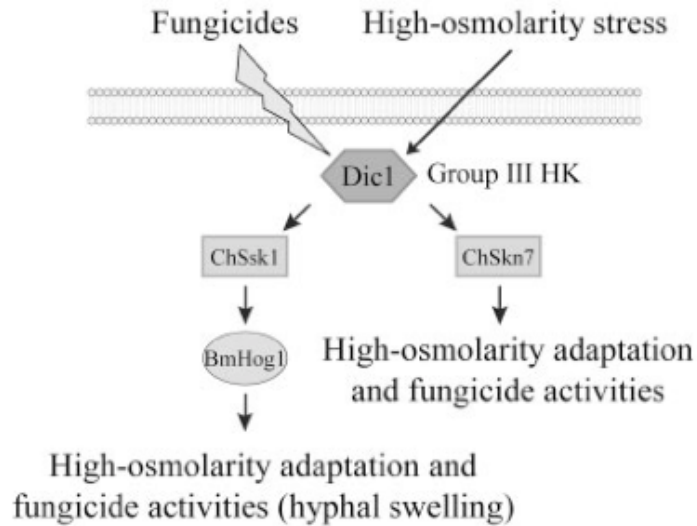
**Figure 11** Courbes de croissance de *S. cerevisiae* en présence de fludioxonil (10 mg/L) cultivées en milieu non inducteur (A) ou en milieu inducteur (B) et obtenues par néphélométrie.

La souche sauvage (WT) est représentée par des carrés ; la souche pYA1, contenant une forme tronquée d'AbNik1p, est représentée par des ronds et la souche pYH5, contenant une forme sauvage d'AbNik1p, est représentée par des triangles.

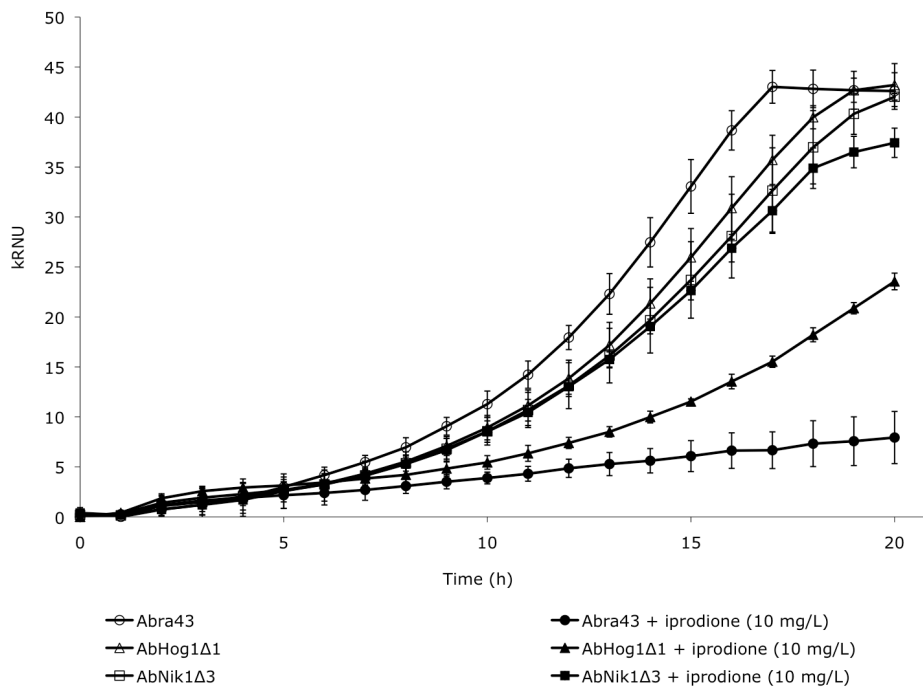
### 3.1 Intérêt de la néphélométrie pour le phénotypage de génotypes mutants

Les deux souches mutantes d'*A. brassicicola*, *AbNik1Δ3* et *AbHog1Δ1*, respectivement déficientes dans une histidine kinase de groupe III et une MAP kinase orthologue de Hog1p de *S. cerevisiae*, montrent des cinétiques de croissance différentes lors du traitement par un fongicide, le fludioxonil. Ce fongicide appartient à la famille des phénylpyrroles et a des effets similaires chez la plupart des champignons filamenteux : il provoque une diminution du taux de germination des conidies, une inhibition de l'élongation et des changements morphologiques des tubes germinatifs (Avenot *et al.*, 2005, Fujimura *et al.*, 2003). Plusieurs études montrent que l'activité antifongique du fludioxonil serait basée sur l'activation de la voie de l'osmorégulation (voie de signalisation HOG), en induisant une production massive de glycérol intracellulaire (Kojima *et al.*, 2004). Chez les champignons, l'adaptation aux fortes pressions osmotiques est régulée par une cascade de MAP kinase, voie HOG chez *S. cerevisiae* et voie OS chez *Neurospora crassa* et d'autres champignons filamenteux. Chez les champignons filamenteux, cette voie de signalisation est régulée en amont par une histidine kinase de groupe III (famille Os1) qui pourrait agir comme osmosenseur. Chez *A. brassicicola*, comme chez d'autres champignons filamenteux, cette histidine kinase est un régulateur positif de la voie HOG et serait la cible de certains fongicides dont les phénylpyrroles (Dongo *et al.*, 2009).

A la différence des champignons filamenteux, *S. cerevisiae* ne possède qu'un seul type d'histidine kinase classée dans le groupe VI (Sln1p) et est naturellement résistante au fludioxonil. De plus, l'expression dans la levure d'AbNik1p, une histidine kinase de groupe III d'*A. brassicicola*, lui confère une sensibilité au fongicide (**Figure 11**). En effet, comme montre cette figure et confirmant les résultats de Dongo *et al.* (2009), *S. cerevisiae*, exprimant la forme sauvage d'AbNik1p, devient sensible au fludioxonil tandis que la levure exprimant une forme tronquée de la protéine est, au même titre que la souche sauvage de référence, tolérante au fongicide.



**Figure 12** Illustration schématique d'une voie de signalisation régulée par une histidine kinase de groupe III et impliquant la MAP kinase *Hog1p* et le facteur de transcription *Skn7p* durant une adaptation à un stress hyper-osmotique ou à un stress dû à un fongicide de type phénylpyrrole chez *C. heterostrophus* (d'après Izumitsu, 2007).



**Figure 13** Sensibilité à l'iprodione de mutants d'*A. brassicicola* déficients dans des gènes de la voie HOG par néphélométrie.

La souche sauvage (*Abra43*) est représentée par des ronds ; le mutant *AbHog1Δ1*, disrupté dans la MAP kinase *Hog1p*, est représenté par des triangles et le mutant *AbNik1Δ3*, disrupté dans le gène *AbNik1*, est représenté par des carrés. La présence d'iprodione est indiquée par des symboles pleins.

Des observations similaires ont été rapportées chez un autre champignon filamenteux, *C. heterostrophus*, chez lequel l'histidine kinase Dic1p est responsable de l'adaptation osmotique et de la sensibilité à l'iprodione, un fongicide de la famille des dicarboximides dont le mode d'action suspecté est identique aux phénylpyrroles. En effet, le mutant délété dans le gène *Dic1* s'avère être très résistant à l'iprodione (Izumitsu, 2007). Contrairement à ce qui était attendu, ces auteurs ont montré qu'une souche délétée dans le gène *ChSsk1* codant un régulateur de réponse situé en aval de Dic1p et en amont de Hog1p (**Figure 12**) était seulement partiellement résistante au fongicide. En complément, Izumitsu *et al.* (2007) montrent qu'une souche déficiente pour le facteur de transcription ChSkn7, également situé en aval de Dic1, a un phénotype similaire aux souches  $\Delta Ssk1$  alors le double mutant délété dans les deux gènes est caractérisé par un niveau de résistance à l'iprodione similaire à celui du mutant  $\Delta Dic1$ . Ces résultats suggèrent que la sensibilité au fongicide n'est pas uniquement médiée par la voie HOG mais dépend également de l'activation de Skn7p. En effet, les réponses Ssk1-dépendante et Skn7-dépendante agiraient conjointement pour l'expression de la sensibilité de ce champignon à ces molécules antifongiques. Ces deux voies de réponses seraient régulées en commun par une histidine kinase de groupe III, Dic1p.

L'analyse néphélométrique de la sensibilité de différents génotypes d'*A. brassicicola* au fludioxonil mais également à l'iprodione (**Figure 13**) montre que le mutant *AbNik1Δ3* est hautement résistant aux deux fongicides alors que le mutant *AbHog1Δ1* a une sensibilité intermédiaire entre la souche sauvage (sensible) et le mutant *AbNik1Δ3*. Ces résultats sont à mettre en parallèle avec ceux décrits pour *C. heterostrophus*. Ils suggèrent que chez *A. brassicicola*, il existe également deux voies de réponses activées par ces fongicides : une voie de réponse régulée par AbHog1p et une autre régulée par AbSkn7p, les deux étant activées par l'histidine kinase de groupe III AbNik1p. Afin de vérifier cette hypothèse, l'obtention d'un mutant nul délété dans le gène *AbSkn7* est en cours. La suite des travaux consistera alors à tester la sensibilité de ce mutant vis-à-vis des deux familles de fongicides en comparaison avec celles obtenues pour les mutants *AbHog1Δ1* et *AbNik1Δ3*. En prolongement à ce travail il pourra également être envisagé d'obtenir d'un double mutant *AbHog1Δ1-AbSkn7Δ* afin de comparer son phénotype avec celui du mutant *AbNik1Δ3*. Une telle vérification expérimentale est indispensable car tous les champignons filamenteux ne semblent pas répondre à ce schéma. En effet, des différences notables entre *N. crassa* et *A. brassicicola* ont par exemple été observées. Chez *N. crassa*, les données

**Tableau 1** Sensibilité de différentes souches de *N. crassa* vis-à-vis de l'iprodione.

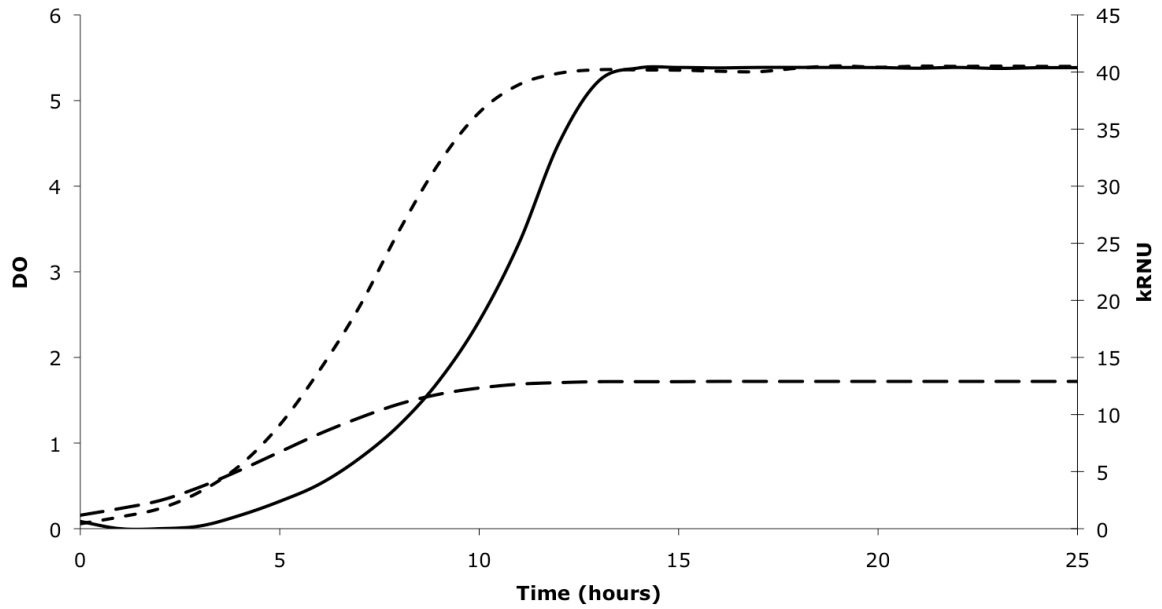
Les CI50 (en mg/L) sont calculées sur la base de la vitesse de croissances obtenues à partir de courbes néphélométriques.

<b>Souches</b>	<b>Génotype</b>	<b>CI 50 mg/L</b>
(988)	WT	2,6 ± 0,03
(824)	Os1	8,0 ± 0,99
(1509)	Os2	11,1 ± 2,61



néphélométriques montrent que le mutant déficient dans le gène *Hog* (*Os2*) est caractérisé par une forte tolérance à l'iprodione tout comme la souche déficiente dans l'histidine kinase de groupe III, *Os1p* (**tableau 1**). De plus, le mutant  $\Delta$ *rrg2* (orthologue de *Skn7*) de *N. crassa* est très sensible à ces fongicides contrairement au mutant  $\Delta$ *skn7* de *C. heterostrophus* (Dongo *et al.*, 2009).

Ces travaux ont une importance en termes de compréhension du mode d'action cellulaire et moléculaire de ces fongicides et des mécanismes de contournement de leurs effets observés au champ et doivent ainsi assurer une meilleure gestion de leur utilisation en protection des cultures.



**Figure 14** Courbe de croissance de la levure *S. cerevisiae* par néphélométrie (ligne pleine) ou par absorptiométrie avant (ligne avec grands tirets) ou après (ligne avec petits tirets) l'application d'un facteur de correction ( $Abs_{corrigée} = Abs + 0.8324(Abs)^3$ ).

### 3.2 Intérêt de la néphélométrie pour le suivi de croissance de champignons levuroïdes

Comme nous l'avons mentionné précédemment, les mesures néphélométriques sont très bien adaptées à l'étude de la croissance de champignons filamenteux. Cette technique est également intéressante pour l'étude des cinétiques de croissance des champignons levuroïdes. Notre étude présente le suivi de la croissance du champignon *C. glabrata*, pathogène humain, par néphélométrie. Les résultats donnent des courbes très similaires à celles obtenues avec un spectrophotomètre. En effet, les courbes de croissance en néphélométrie ou en absorptiométrie (après correction des valeurs) sont très comparables pour un champignon levuroïde (**Figure 14**). La néphélométrie évite l'application d'un facteur de correction qui est imposé par la limite de la linéarité de la loi de Beer-Lambert dans le cas de l'absorptiométrie.

De nombreux arguments montrent que la néphélométrie est une technique innovante qui permet à la fois l'analyse de champignons filamenteux et levuroïdes, mais également de visualiser les différences de sensibilité de diverses souches en calculant des indices quantitatifs. Cette méthode est très prometteuse pour une analyse à haut débit dans des faibles volumes de divers géotypes sous plusieurs types de stress sans être limité par les désavantages de l'absorptiométrie

# Chapitre 2

**Rôle des MAP Kinases dans la réponse  
d'*Alternaria brassicicola* aux phytoalexines  
indoliques**

## **1. Présentation de l'étude**

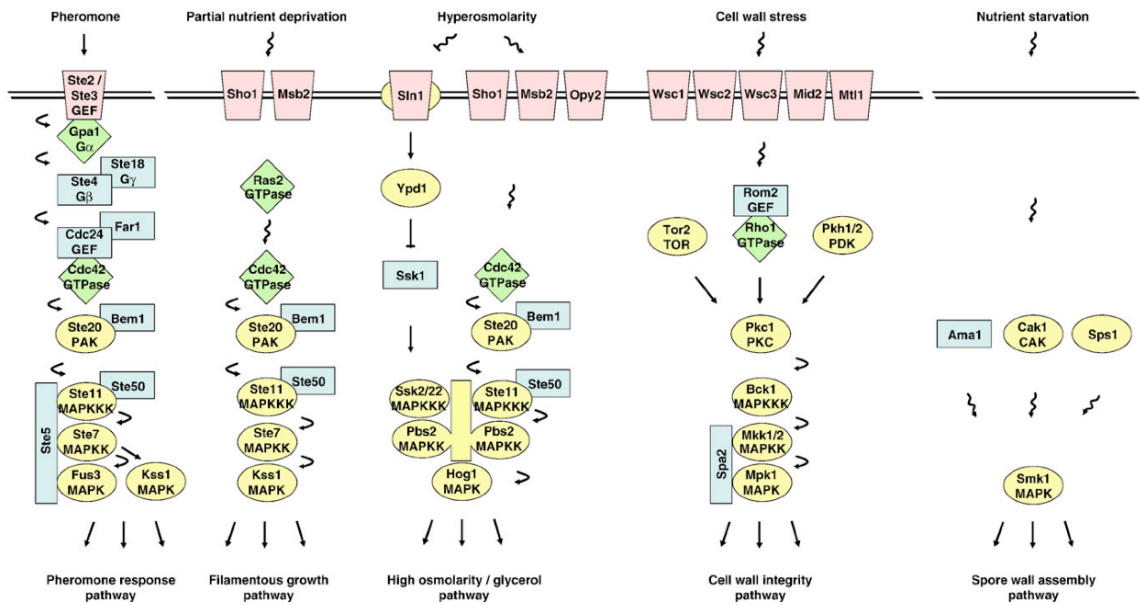
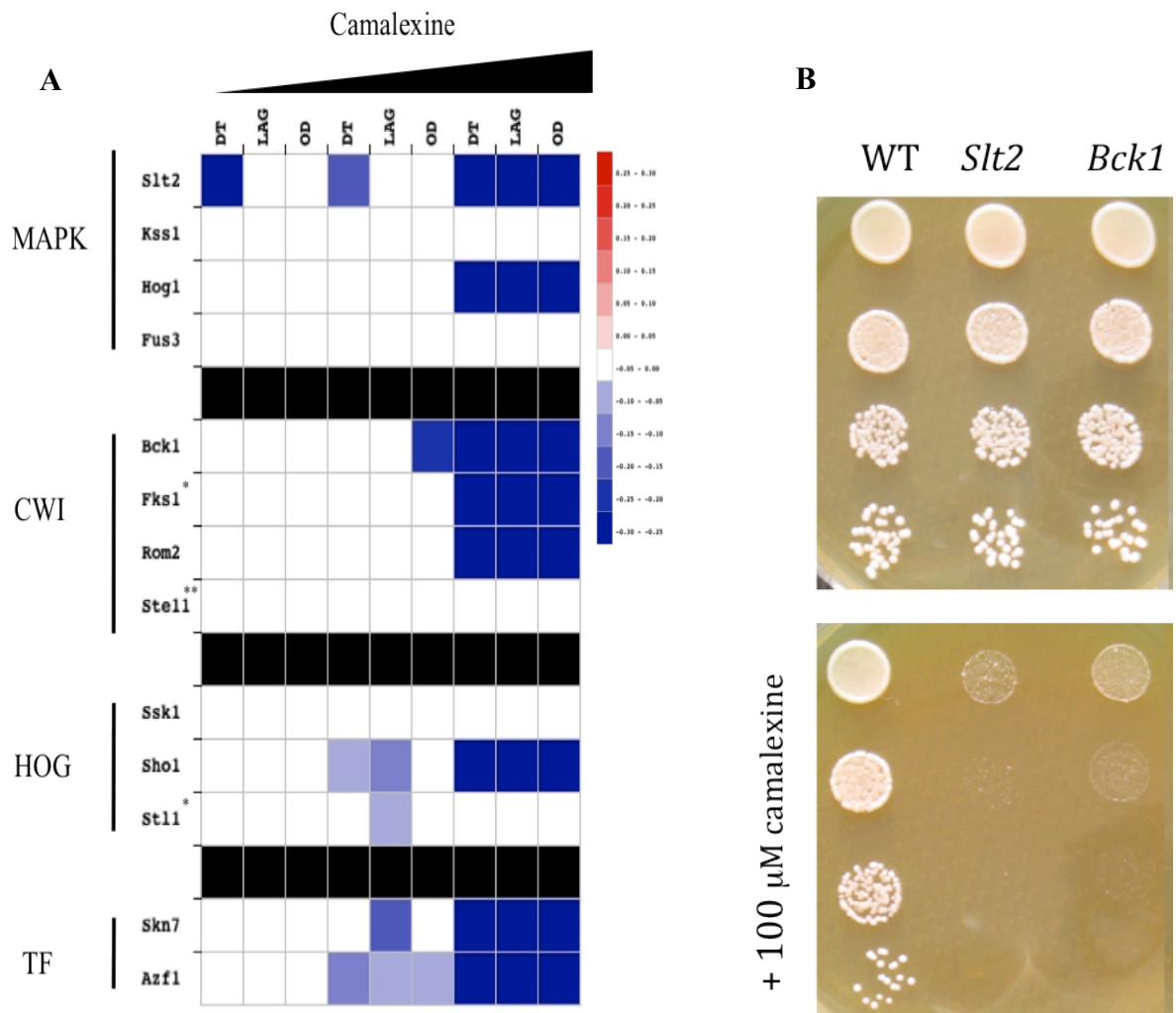


Figure 15 Les différentes voies MAP kinases chez *S. cerevisiae* (d'après Chen et al., 2007)

Les voies de signalisation médiées par des cascades de MAP Kinases (« Mitogen Activated Protein ») jouent un rôle prépondérant chez les champignons. Elles sont constituées par une succession de trois protéines kinases qui agissent en série : une MAP kinase kinase kinase (MAPKKK ou MEKK), une MAP kinase kinase (MAPKK ou MEK) et une MAP kinase (MAPK) (Cobb *et al.*, 1995, Cooper, 1994). Ces cascades sont très bien répertoriées chez *S. cerevisiae*, mais existent aussi chez les champignons filamenteux, les animaux et les plantes. Des analyses biochimiques et génétiques menées chez *S. cerevisiae* ont permis de mettre en évidence cinq cascades de MAP kinases (Hunter *et al.*, 1997, Xu, 2000, Zhao *et al.*, 2007) (**Figure 15**).

L'une des voies prépondérantes pour la survie d'un champignon est la voie de l'intégrité pariétale qui va lui permettre de s'adapter aux contraintes extérieures : hypo-osmolarité, changement de température ou encore protection contre les agents chimiques ciblant la paroi (Zarzov *et al.*, 1997). Cette voie fait intervenir la cascade Bck1-Mkk2/Mkk22-Slt2 mais aussi la protéine centrale de la voie de l'intégrité cellulaire, la protéine kinase C (Pk C) (Heinisch *et al.*, 1999). Il existe de forts liens entre cette voie de signalisation et la voie de régulation osmotique HOG (High Osmolarity Glycerol) qui permet à la cellule fongique de croître dans un environnement hypertonique en compensant la différence de pression avec une accumulation de glycérol (Attfield, 1997, Rodriguez-Pena *et al.*, 2010). La cascade de MAP kinase de la voie HOG est bien connue chez *S. cerevisiae* ; elle consiste en un enchaînement de cinq protéines kinases : trois MEKKs, Ssk2p, Ssk22p (Maeda *et al.*, 1995) de la voie dépendante de Sho1p et Ste11p de la voie dépendante de Sln1p ; une MEK, Pbs2p (Boguslawski *et al.*, 1987) et une MAP kinase Hog1p (Brewster *et al.*, 1993). La protéine Hog1p est à l'intersection de deux branches, l'une réagissant en cas de forte osmolarité externe (par l'intermédiaire des deux récepteurs Sho1p et Msb2p (O'Rourke *et al.*, 2002) et la seconde en cas d'une augmentation plus modérée de la pression osmotique (par l'intermédiaire du récepteur Sln1p). L'aboutissement de cascade de signalisation est, entre autre, la synthèse de glycérol avec l'induction des gènes *Gpd1* et *Gpd2* codant respectivement la glycerol-3-phosphate dehydrogenase et la glycerol-3-phosphatase (Rep *et al.*, 1999).

Chez différents champignons filamenteux, ces deux voies ont été mise en évidence comme étant impliquées dans le processus infectieux (**Cf. Introduction bibliographique 3.2.3**) (Dickson *et al.*, 2002, Mehrabi *et al.*, 2006, Ramamoorthy *et al.*, 2007, Rui *et al.*,



**Figure 16** Résultats du phénotypage de mutants de *S. cerevisiae*.

(A) Représentation des LPI (Indice Phénotypique Logarithmique) du phénotypage des mutants de *S. cerevisiae* en fonction de doses croissantes (75, 125 et 250  $\mu$ M) de camalexine. DT : temps de doublement, LAG : période de latence, OD : accroissement de la population, TF : Facteurs de transcription, \* : gènes induits par les voies de signalisation, \*\* : gène commun à *Fus3/Kss1* (B) Dépôt sur milieu YNBA additionné de camalexine de suspensions cellulaires de *S. cerevisiae* calibrées de différentes souches (WT,  $\Delta$ *Slt2* et  $\Delta$ *Bck1*). Les gènes *Slt2* et *Bck1* codent des kinases impliquées dans la voie *Pkc*.



2007, Segmuller *et al.*, 2007). C'est dans ce contexte que le **Chapitre 2** présente les résultats de l'étude des voies HOG et CWI et leurs implications dans la réponse du champignon *A. brassicicola* face à deux molécules de défenses des *Brassicaceae*, la camalexine et la brassinine.

La première étape du travail a été d'étudier la sensibilité à la camalexine de plusieurs mutants de *S. cerevisiae* déficients dans quatre des cinq voies de signalisation. Ce criblage a été effectué chez *S. cerevisiae* pour lequel une collection complète de mutants couvrant l'ensemble du transcriptome est disponible. Ce type d'approche est très utilisé pour phénotyper différents génotypes de souches (Cubillos *et al.*, 2011, Warringer *et al.*, 2003a, Warringer *et al.*, 2003b). L'analyse phénotypique à haut débit de collections de mutants de *S. cerevisiae* couvrant l'intégralité du transcriptome a ainsi reçu le nom de « Phenomic ».

Dans notre cas, l'étude a consisté à mesurer l'impact de la phytoalexine testée sur la croissance de divers mutants ciblés en fonction des données actuellement disponibles sur le mode d'action de la molécule (Sellam *et al.*, 2007b). La croissance des souches a été mesurée par turbidimétrie et, à partir de la courbe de croissance, trois paramètres sont évalués par des méthodes mathématiques : le temps de doublement de la souche, l'accroissement de la population et la période de latence. Ces paramètres permettent de déterminer la tolérance ou la sensibilité d'une souche mutante vis-à-vis d'une phytoalexine donnée (Warringer *et al.*, 2003a). Après l'analyse statistique des indices mathématiques issus des courbes de croissance des mutants, plusieurs types de comportements des mutants testés vis-à-vis de la camalexine sont observés (**Figure 16**).

Les résultats de ce phénotypage montrent que parmi les quatre souches déficientes pour des MAP kinases testées, les mutants  $\Delta Slt2$  et  $\Delta Hog1$ , sont caractérisés par une forte sensibilité aux fortes concentrations de camalexine et ce pour les trois paramètres étudiés (**Figure 16 A**). A l'inverse, les mutants  $\Delta Kss1$  et  $\Delta Fus3$  ne présentent pas de comportements différents de la souche de référence face à la camalexine. Ces résultats suggèrent un rôle des voies HOG et CWI dans la réponse de la levure au stress provoqué par la phytoalexine. En accord avec ces observations, la plupart des mutants testés déficients pour des composantes de la voie CWI, comme  $\Delta Rom2$  ou  $\Delta Bck1$ , ou des protéines codées par des gènes cibles de cette voie, comme  $\Delta Fks1$ , sont également hypersensibles à la camalexine. Ce même phénotype est également observé pour certains

mutants affectés dans la voie HOG comme le mutant  $\Delta Sho1$ , alors que le mutant  $\Delta Ssk1$  à l'inverse a un phénotype identique à la souche de référence. Ceci suggère que l'activation de la voie HOG par la camalexine pourrait être Sho1-dépendante Sln1-indépendante comme cela a également été rapporté dans le cas de la réponse de *S. cerevisiae* à la Zymolase qui est également un exemple de réponse coordonnée des voies HOG et CWI (Bermejo *et al.*, 2008, Rodriguez-Pena *et al.*, 2010). Il convient cependant de noter que ni le mutant  $\Delta Ste11$  ni le mutant  $\Delta Stil$ , codant un transporteur de glycérol régulé par la voie HOG, ne présentent une sensibilité accrue à la camalexine. L'hypersensibilité à la camalexine observée pour les mutants  $\Delta Skn7$  et  $\Delta Azf1$  codant des facteurs de transcription connus pour réguler l'expression de gènes de maintenance de la paroi renforce l'hypothèse d'un rôle majeur de l'activation de la voie CWI en réponse à la camalexine. Les résultats obtenus par suivi de la croissance en milieu liquide ont été confirmés pour les souches mutantes présentant des phénotypes d'hypersensibilité significativement différents de la souche sauvage par dépôt de dilutions calibrées d'une suspension de levures sur du milieu solide contenant de la camalexine (**Figure 16 B**).

A la suite de ce travail préliminaire, notre étude chez *A. brassicicola* s'est donc naturellement axée sur l'implication potentielle des voies CWI et HOG dans sa réponse adaptative aux phytoalexines indoliques. Les résultats obtenus sont décrits dans l'article présenté dans ce chapitre et brièvement résumés ci-dessous.

La première étape du travail a été de caractériser les orthologues de *S. cerevisiae* *Hog1* et *Slt2* chez le champignon *A. brassicicola*. La séquence protéique prédite de AbHog1p est de 355 acides aminés pour une protéine d'environ 40 kDa. Le motif TGY caractéristique des MAPK de type p32 est présent en position 171-173. En ce qui concerne le gène *Slt2*, son orthologue correspond à un gène de 1248 pb avec 6 introns. La séquence protéique prédite est de 416 acides aminés pour une masse d'environ 47 kDa. La protéine contient le motif de phosphorylation TEY caractéristique des MAPK de la famille Slt2p. Afin de confirmer la présence des orthologues, une complémentation hétérologue dans la levure a été réalisée.

La seconde étape a été d'obtenir des mutants nuls pour les deux gènes par la technique de « double-joint PCR » (Yu *et al.*, 2004). Les mutants nuls ont été vérifiés par PCR, Western blot (absence de la protéine) et par Southern blot (confirmation de l'intégration).

Le phénotypage a montré que les mutations ont un impact sur la croissance végétative avec, par exemple, le mutant *AbSlt2Δ1* qui présente une croissance sur milieu standard nettement ralentie par rapport au mutant *AbHog1Δ1* et la souche de référence Abra43. La germination des spores est retardée chez les deux mutants par comparaison avec la souche de référence. La souche *AbSlt2Δ1* montre également un éclatement des hyphes et une hyper-filamentation caractéristiques de ce type de mutation (Eliahu *et al.*, 2007, Mehrabi *et al.*, 2006, Mey *et al.*, 2002). Le retard de croissance du mutant *AbSlt2Δ1* est probablement dû à une difficulté pour faire face aux conditions hypo-osmotique dans la mesure où sa vitesse de croissance est restaurée lors de l'ajout de sorbitol. De plus, les anomalies cellulaires visibles chez ce mutant suggèrent que la formation de la paroi est altérée.

Leurs sensibilités vis-à-vis de certains stress ont été étudiées et toutes les souches mutantes présentent une hypersensibilité à la ménadione et une tolérance au CalcoFluorWhite. Comme décrit au chapitre précédent, le mutant *AbHog1Δ1* présente une tolérance au fludioxonil. A l'inverse, le mutant *AbSlt2Δ1* montre une plus forte sensibilité à ce fongicide que la souche sauvage de référence. Cette hypersensibilité du mutant *AbSlt2Δ1* au fludioxonil est à rapprocher de l'altération pariétale observée chez cette souche, phénomène déjà décrit chez *M. graminicola* et *B. cinerea* (Mehrabi *et al.*, 2006, Rui *et al.*, 2007).

Les deux souches mutantes sont hypersensibles à la brassinine et à la camalexine. Ceci est démontré à la fois par suivi des effets de ces composés sur la croissance en milieu liquide et en milieu solide, mais aussi par des tests d'incorporation du SYTOX. Par ailleurs notre étude montre que les deux MAP kinases sont activées par phosphorylation lors de l'exposition du champignon à la camalexine. Chez le mutant *AbHog1Δ1*, la MAP kinase AbSlt2p n'est cependant plus activée en présence de camalexine alors que chez le mutant *AbSlt2Δ1*, la MAP kinase AbHog1p est toujours activée. Ces résultats indiquent que la réponse adaptative à la camalexine passerait en premier lieu par l'activation de la MAP kinase AbHog1p puis par l'activation de la MAP kinase AbSlt2p. Des observations similaires ont été rapportées concernant l'adaptation de la levure à un stress provoqué par la Zymolyase (Garcia *et al.*, 2004). Néanmoins, les connaissances sur la connexion qui relie ces deux voies MAP kinases restent encore incomplètes.

Les travaux présentés dans cet article montrent également le rôle de ces deux MAP kinases dans le pouvoir pathogène du champignon. En effet, les deux types de souches mutantes montrent une baisse très nette de leur agressivité *in planta*. Les deux mutants présentent une diminution du nombre de pseudo-appressoria formés et, pour le mutant *AbSl2Δ1*, une hyper-filamentation sur l'épiderme de la plante. Des blessures créées artificiellement sur la feuille avant inoculation ne permettent pas de restaurer la capacité de ces deux types de mutants à produire des symptômes comparables à la souche sauvage de référence. La diminution de la capacité à pénétrer dans les tissus hôtes ne peut, à elle seule, expliquer la faible agressivité des mutants qui pourrait être en partie liée à une sensibilité accrue aux phytoalexines. Cette hypothèse est confirmée par des tests d'inoculation d' *A. thaliana* génotype *Pad3* (non producteur de camalexine) montrant que la souche *AbSl2Δ1* est capable de produire des symptômes comparables à la souche de référence sur cet hôte. A l'inverse, dans les mêmes conditions, le mutant *AbHog1Δ1* ne produit toujours pas de symptôme, suggérant un effet pléiotropique de cette mutation avec un impact sur d'autres composantes du pouvoir pathogène.

## 2. ARTICLE

### ***Cell wall integrity and high osmolarity glycerol pathways are required for adaptation of *Alternaria brassicicola* to cell wall stress caused by brassicaceous indolic phytoalexins***

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# Cell wall integrity and high osmolarity glycerol pathways are required for adaptation of *Alternaria brassicicola* to cell wall stress caused by brassicaceous indolic phytoalexins

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## Summary

**Camalexin, the characteristic phytoalexin of *Arabidopsis thaliana*, inhibits growth of the fungal necrotroph *Alternaria brassicicola*. This plant metabolite probably exerts its antifungal toxicity by causing cell membrane damage. Here we observed that activation of a cellular response to this damage requires cell wall integrity (CWI) and the high osmolarity glycerol (HOG) pathways. Camalexin was found to activate both AbHog1 and AbSit2 MAP kinases, and activation of the latter was abrogated in a AbHog1 deficient strain. Mutant strains lacking functional MAP kinases showed hypersensitivity to camalexin and brassinin, a structurally related phytoalexin produced by several cultivated *Brassica* species. Enhanced susceptibility to the membrane permeabilization activity of camalexin was observed for MAP kinase deficient mutants. These results suggest that the two signalling pathways have a pivotal role in regulating a cellular compensatory response to preserve cell integrity during exposure to camalexin. AbHog1 and AbSit2 deficient mutants had reduced virulence on host plants that may, at least for the latter mutants, partially result from their inability to**

**cope with defence metabolites such as indolic phytoalexins. This constitutes the first evidence that a phytoalexin activates fungal MAP kinases and that outputs of activated cascades contribute to protecting the fungus against antimicrobial plant metabolites.**

## Introduction

The survival of fungi in their environment highly depends upon their ability to cope with various stresses. They have thus evolved a complex network of mechanisms to deal with unforeseen changes occurring in their natural habitats. Signal transduction pathways involving mitogen-activated protein kinase (MAPK) cascades probably constitute the major mechanisms by which the fungi sense such changes and develop adaptive responses.

In the non-pathogenic model yeast species, *Saccharomyces cerevisiae*, five characterized MAPK signalling pathways were identified and have been implicated in different processes, such as mating, adjustment to nutrient limiting conditions, survival under hyperosmotic conditions, repair of cell wall injuries and ascospore cell wall assembly (Chen and Thorner, 2007). In the past decade, homologues of most yeast MAPKs have been characterized in various pathogenic fungi and many studies have revealed that they can be essential virulence determinants. In human pathogenic fungi, MAPK cascades have been shown to regulate different events that occur during infection such as fungal recognition by the immune system, proliferation within host tissues, susceptibility to phagocyte oxidative-mediated killing mechanisms and morphological switches from yeast forms to hyphal forms (Roman *et al.*, 2007). Although plant and human pathogenic fungi have adapted different strategies to colonize and produce disease in their hosts, common features (development of infection structures, protection against host defences, tissue colonization) are found for the two types of pathogens. Not surprisingly, the relevance of MAPK signalling pathways in the control of key processes contributing to virulence has also been documented for plant pathogenic fungi. Functional homologues of the

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yeast MAPKs Fus3/Kss1 have thus been shown to be required for complete development of penetration structures in appressorium-forming pathogens (for review see Zhao *et al.*, 2007). Besides their functions in infection-related morphogenesis, signalling pathways in relation to their roles in physical and chemical stress responses may also contribute substantially to fungal virulence.

As regards to physical stresses, Viaud *et al.* (2006) reported that *Botrytis cinerea bos1* mutants, defective for a group III histidine kinase (GIII-HK) involved in the osmosensing signalling cascade, have reduced *in planta* development. Such reduced virulence was interpreted as a consequence of slower development of *bos1* mutants in the host because of stresses imposed by the intrinsic osmotic state of the host. Similarly, Iacomi-Vasilescu *et al.* (2008) reported that osmosensitive *Alternaria brassicicola* null mutants for a GIII-HK have a reduced capacity to colonize radish seeds. This was explained by their inability to overcome severe osmotic conditions consecutive to a gradual decrease in the water potential in maturing seeds.

During plant tissue colonization, fungal pathogens are also subject to chemical stresses caused by changes in nutrient availability and by exposure to host defence molecules that exert fungal toxicity. The regulatory function of the Fus3/Kss1 MAPK cascade on the expression of cell wall degrading enzymes by *A. brassicicola* during plant infection and its requirement for pathogenicity clearly illustrates the essential role of signalling pathways in the adaptation to limiting nutrients in the host environment (Cho *et al.*, 2007). The role of cellular signalling in the response to antifungal molecules has been mainly investigated in human fungal pathogens to study the evolution of resistance to drugs causing cell wall and membrane stress (for review see Cowen and Steinbach, 2008). However, recently Ramamoorthy *et al.* (2007) reported that basal resistance to antifungal plant defensins, which probably impair the fungal cell wall integrity (CWI), was mediated by two MAPK signalling cascades in the plant fungal pathogen *Fusarium graminearum*. Other plant defence compounds such as phytoalexins also likely exert similar stresses on fungi. However, to our current knowledge, there are no reports on the involvement of signalling pathways in the response of fungi to these antimicrobial molecules.

*Alternaria brassicicola* is an economically important seed-borne fungal pathogen causing black spot disease in a wide range of Brassicaceae species. The *Arabidopsis thaliana* – *A. brassicicola* pathosystem has often been used as a model to study interactions of plants with necrotrophic fungi (Schenk *et al.*, 2003; van Wees *et al.*, 2003; Brader *et al.*, 2007; Mang *et al.*, 2009; Pogany *et al.*, 2009; Trusov *et al.*, 2009). The indolic secondary metabolite camalexin is the major phytoalexin synthesized by this plant species and it was demonstrated to

highly contribute to the resistance to *A. brassicicola* (Thomma *et al.*, 1999). We have previously shown that camalexin, when applied to *A. brassicicola* conidia, activated compensatory mechanisms to preserve cell wall and membrane integrity (Sellam *et al.*, 2007a). In *S. cerevisiae*, such homeostatic mechanisms are mediated via a signalling network that communicates information about cell surface stresses to the biosynthetic enzymes involved in cell wall synthesis and repair (Walker *et al.*, 2008). The CWI pathway plays an essential role in this cell wall remodelling process (see Levin, 2005, for review). Evidence of a role for the high osmolarity glycerol (HOG) pathway in yeast cell wall biogenesis and a cooperative role for both pathways controlling cell integrity has recently been published (Bermejo *et al.*, 2008).

The objective of the present study was to evaluate the contribution of CWI and HOG signalling pathways in the activation of cell wall compensatory mechanisms during exposure of *A. brassicicola* to camalexin. We present here a phenotypical analysis of *A. brassicicola* mutants defective in the SlT2- and Hog1-type MAPKs. These mutants showed hypersensitivity to camalexin and brassinin, two structurally related indolic phytoalexins, and had reduced virulence in host plants. We also observed that these two MAPK signalling cascades were activated upon exposure to camalexin. These results suggest that, besides the already reported role of detoxifying enzymes and efflux mechanisms in fungal tolerance to indolic phytoalexins (Pedras *et al.*, 2009; Stefanato *et al.*, 2009), both HOG and CWI signalling pathways provide some protection to *A. brassicicola* against the antifungal activity of these defence metabolites.

## Results

### *Characterization in A. brassicicola of homologues of S. cerevisiae MAPKs Hog1 and SlT2*

Successful *Hog1* homologue amplification in *A. brassicicola* was achieved with primers derived from conserved regions of *Hog1* from various available fungal species (Table S1), with flanking regions obtained by polymerase chain reaction (PCR) walking (Siebert *et al.*, 1995). Analysis of the whole-coding sequence (GenBank Accession No. AY987486) revealed eight introns, whose positions were confirmed by sequencing cDNA and genomic DNA clones. This open reading frame (ORF) predicted 355 amino acid residues with a protein mass of 40 785 Da. A TGY motif characteristic of hyperosmolarity activated MAP kinases was found at amino acids 171–173. A conserved common docking domain motif of the MAPK family containing two acidic amino acids (Asp-304 and Asp-307) was also present. A sequence encoding an *SlT2* homologue was identified within contig 7.83 via BLAST

analysis on the *A. brassicicola* genome using conserved sequences in *Slit2* orthologues of filamentous fungi. Sequence analysis and comparison with data obtained by sequencing amplified cDNA fragments revealed a 1248 bp ORF interrupted by six introns. This ORF, which was previously registered in GenBank (Accession No. AY705975) and named Amk2, predicted 416 amino acid residues with a protein mass of 47 499 Da, which is highly similar to kinases from filamentous fungi belonging to the YERK2 family. The protein contains MAPK dual phosphorylation sites TEY (residues 186–188) typical of kinases in this family. Phylogenetic analysis using representative amino acid sequences from the three major fungal MAPK subgroups (SAPK, YERK1 and YERK2) indicated that the sequences deduced from AY987486 and from AY705975 clustered with Hog1 and Slit2 homologues respectively (Fig. 1A). These genes were thus named *AbHog1* and *AbSlit2*. To confirm that they correspond to true functional homologues of the yeast *Hog1* and *Slit2* genes, complementation of *S. cerevisiae hog1Δ* and *slt2Δ* null mutants was carried out with relevant recombinant pYES plasmids. As shown in Fig. 1B, *AbHog1* and *AbSlit2* successfully complemented the *S. cerevisiae hog1* and *slt2* phenotypes respectively. The growth of *hog1Δ* cells expressing *AbHog1* was comparable with that of the WT strain on media containing 1 M NaCl. Similarly, significant growth of the *slt2Δ* strain transformed with pYES2–*AbSlit2* and the WT strain was recorded on medium supplemented with 10 mg l<sup>-1</sup> Calcofluor White (CFW) while growth of the *slt2Δ* mutant containing the empty vector pYES2 was highly impaired under these conditions.

#### Construction of *AbHog1* and *AbSlit2* knockout mutants in *A. brassicicola*

To study the role of *AbHog1* and *AbSlit2* during exposure of *A. brassicicola* to indolic phytoalexins, loss-of-function mutants were generated by homologous integration of replacement constructs generated by double-joint PCR and containing the HygB resistance gene marker flanked by sequences specific to the targeted genes (Fig. 2A). After transformation of protoplasts of the *Abra43* WT strain and regeneration, approximately 50 and 200 Hyg B-resistant colonies were obtained with the *Hog1*- and *Slit2*-replacement constructs respectively. From each transformation experiment, six Hyg B-resistant strains were randomly selected and further purified by three rounds of single-spore isolation. A PCR screen, carried out using primers homologous to the hygromycin resistance cassette and genomic sequence outside the flank regions, confirmed that integration of the replacement constructs occurred by homologous recombination at the targeted loci for all of these mutants (not shown). Complete deletion of the transformant coding regions was

further confirmed using two internal primers. No ectopic transformant was obtained, thus confirming the high efficiency of homologous recombination in *A. brassicicola* already pointed out by Cho *et al.* (2006). Genomic Southern hybridization was performed to further confirm the mutant identities. Two Hyg B-resistant strains (named *Hog1Δ1* and *Hog1Δ2*) from the *Hog1* transformation experiment and two Hyg B-resistant strains (named *Slit2Δ1* and *Slit2Δ2*) from the *Slit2* transformation experiment were analysed. As shown in Fig. 2B, all the putative knockout mutants had lost the wild-type (WT) *AbHog1* or *AbSlit2* coding sequences, which were replaced by the corresponding disruption cassettes.

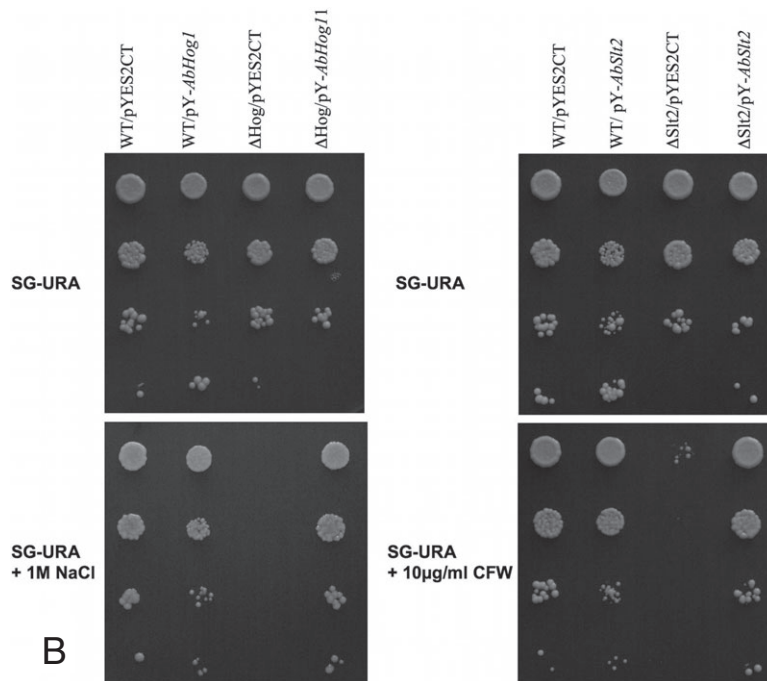
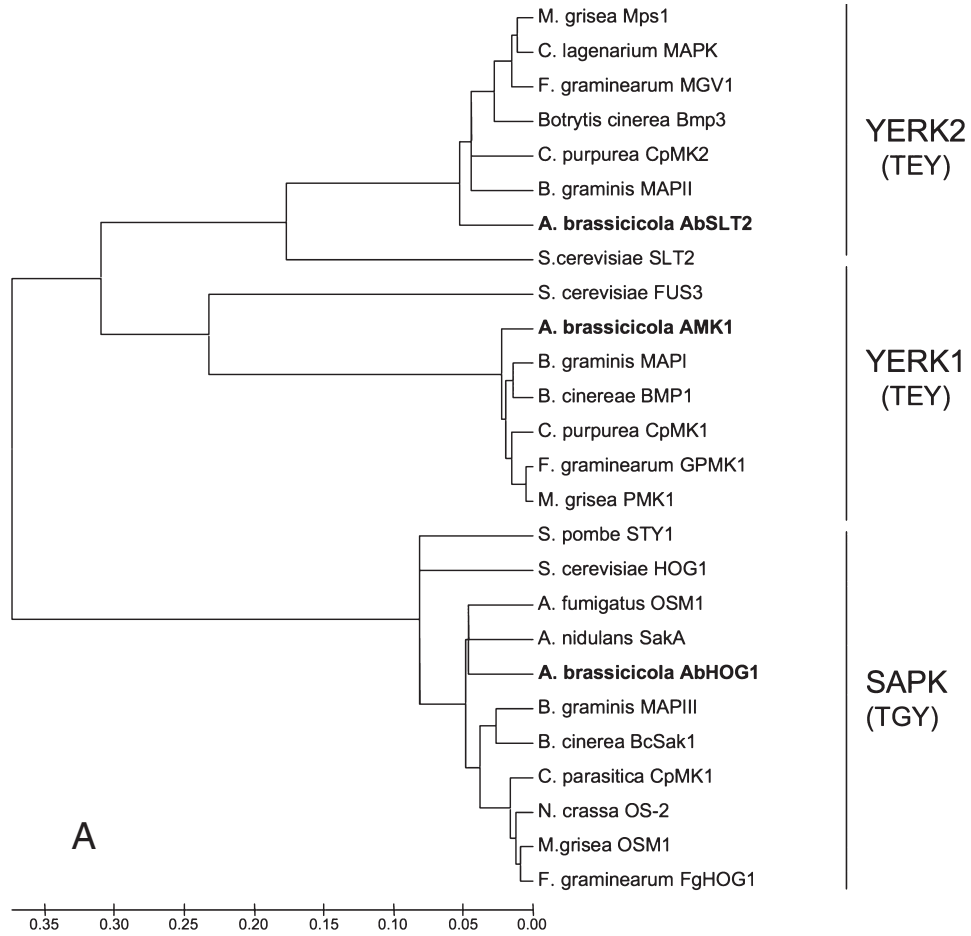
The *Hog1Δ* and *Slit2Δ* strains were confirmed to be null mutants by Western blot analysis with anti-Hog1 and anti-Mpk1 antibodies respectively (Fig. 2C). As expected the band at approximately 41 kDa corresponding to *AbHog1p* was absent in the *Hog1Δ* samples and the band at approximately 47 kDa corresponding to *AbSlit2p* was absent in the *Slit2Δ* samples. An additional signal at lower molecular weight was routinely observed with the anti-Mpk1 antibody in protein samples from both WT and *Slit2Δ* isolates. The cross-reacting signal intensity suggested that it might correspond to another YERK protein kinase.

Genetic complementation assays were performed to confirm that the observed mutant phenotypes were indeed the result of the deletions of *AbHog1* or *AbSlit2*. However, despite many attempts to modify the standard procedure used to generate competent protoplasts, no complemented transformants could be obtained, probably as a result of the low ability to maintain cellular integrity throughout the protoplasting process (Table S2).

#### Effects of *AbHog1* and *AbSlit2* on vegetative growth and stress tolerance

The *AbSlit2Δ* disruptants showed slightly reduced mycelial growth rates on potato dextrose agar (PDA) medium compared with *AbHog1Δ* disruptants and the WT *Abra43* strain, which showed almost identical growth (Fig. 3). The growth retardation observed with the *AbSlit2Δ* strains was alleviated in the presence of sorbitol as osmotic protectant. After 10 days post inoculation (dpi) on standard PDA medium, the different tested genotypes showed normal conidiation ( $2.2\text{--}4.1 \times 10^5$  conidia per mm<sup>2</sup>, data not shown). Conidia from the *AbHog1Δ* and *AbSlit2Δ* mutants were tested for their ability to germinate on potato dextrose broth (PDB). As shown on Fig. 4, germination of the two mutants was somewhat retarded compared with WT. After 5 h, their germination rates ( $7 \pm 2\%$  and  $16 \pm 2\%$ , for *AbHog1Δ* and *AbSlit2Δ* respectively) were significantly lower than that of WT ( $74 \pm 1.5\%$ ). However, at 15 h, differences in germination rates were no longer observed between the *AbSlit2Δ* and the WT strains. As





**Fig. 1.** Identification of structural and functional homologues of Hog1- and Slit2-related MAPKs in *A. brassicicola*.

A. Unrooted phylogenetic tree of fungal MAPKs constructed by the neighbour-joining method based on amino acid sequences using MEGA 4 software. Sequences from *A. brassicicola* are in bold letters. The conserved dual phosphorylation motif TXY of each subfamily (SAPK1, YERK1 and YERK2) is shown in parentheses.

B. Complementation of yeast *Slit2Δ* and *Hog1Δ* mutants with *AbSlit2* and *AbHog1* coding sequences. Identical volumes of 10-fold serial dilutions ( $10^{-1}$  to  $10^{-4}$ ) of exponentially growing *S. cerevisiae* (parental WT strain and deletion mutants) cells transformed with empty vector (pYESCT) or recombinant vectors (pYAbHog1 and pYAbSlit2) were spotted onto SG plates. Lower plates were supplemented with 1 M NaCl (lower left) or 10  $\mu\text{g ml}^{-1}$  CFW (lower right). Colony growth was observed after 48 h incubation at 30°C.

abnormal hyphal growth patterns were observed in *Slit2Δ* mutants from other fungal species, hyphal morphologies of *A. brassicicola* WT and mutants strains growing on glass slides covered with malt agar (MA) medium were compared (Fig. 5A). At 24 and 36 h of incubation, no obvious difference in the general hyphal growth patterns of the three tested genotypes was observed. In particular, the *AbSlit2Δ* strains had neither the 'coiled' nor the 'hyper-branched' phenotype reported for the CPMK2 (Slit2-related MAPK) deficient mutants of *Claviceps purpurea* (Mey *et al.*, 2002). However, as soon as 36 h post inoculation, microscopic observation of hyphae produced by *AbSlit2Δ* strains revealed abnormal polarized growth resulting in enlarged and swollen tip cells (Fig. 5B). Similar structures were rarely observed when growth was recorded on water agar medium (Fig. S1). The effects of toxic compounds (oxidizing agents, antifungal compounds, cell wall damaging drugs) on the colony expansion rates of the *A. brassicicola* mutants and the WT parental strain were also compared (Table 1). Increased sensitivities towards menadione (generation of  $\text{O}_2^-$ ) and tolerance towards CFW were recorded for all of the studied mutant strains compared with their parental strain. The *AbSlit2Δ* strains were found to be more sensitive than the other tested genotypes to  $\text{H}_2\text{O}_2$  and Congo red. As expected from previously published phenotypic characterization for similar mutants from other fungal species, the *AbHog1* deletion conferred resistance to the phenylpyrrole fungicide fludioxonil, albeit at lower level than the previously characterized *AbNik1* deletion (Dongo *et al.*, 2009), and the *AbSlit2Δ* strains were found to be hypersensitive to this compound. *Hog1Δ1* and *Hog1Δ2* on one hand, and *Slit2Δ1* and *Slit2Δ2* on the other, behaved

similarly in the phenotypic studies presented above. Unless otherwise stated, only the results obtained with *Hog1Δ1* and *Slit2Δ1* mutant strains are shown below.

#### *AbHog1* and *AbSlit2* MAPKs are activated by camalexin

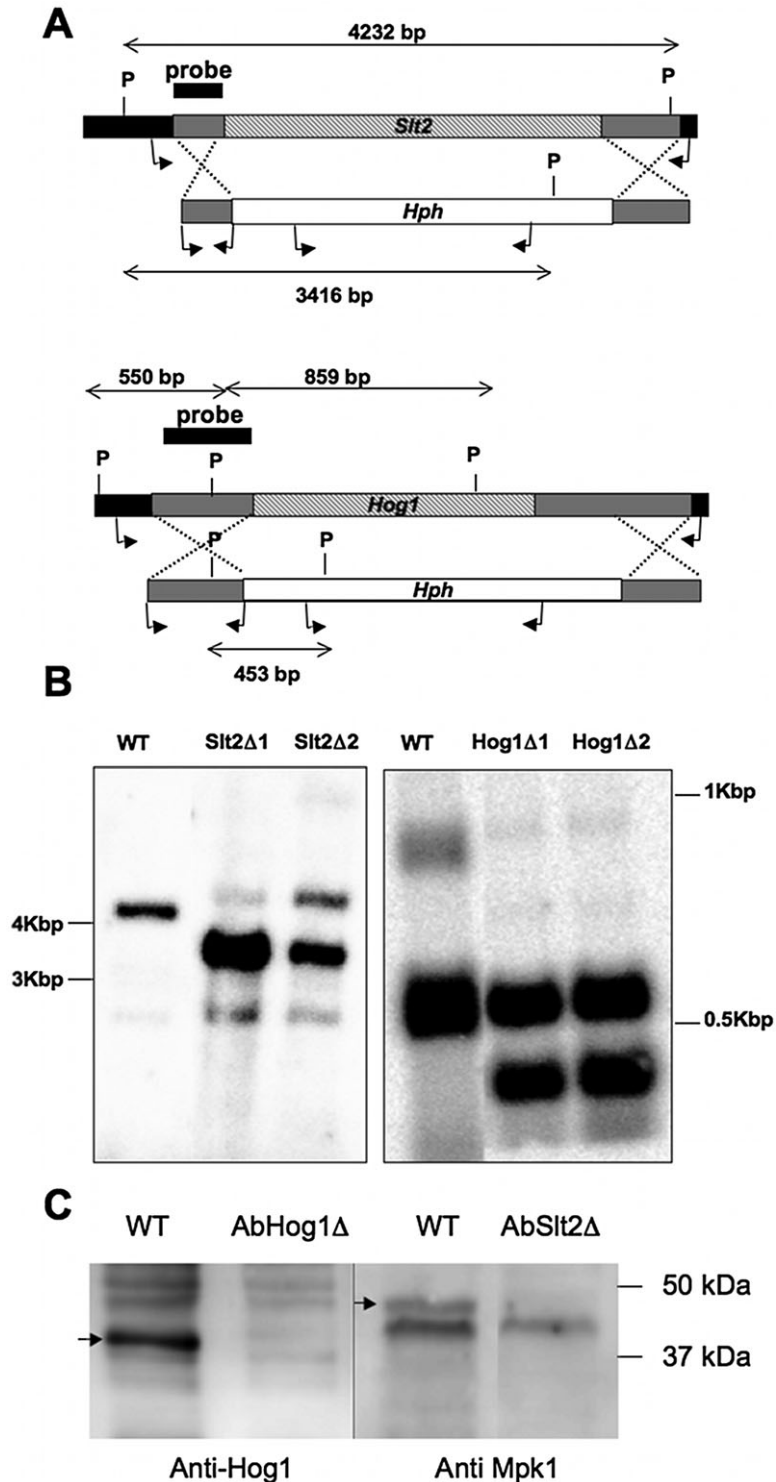
To investigate the phosphorylation status of Hog1- and Slit2-related MAPKs in *A. brassicicola* when exposed to indolic phytoalexins, a Western blot approach using antibodies that specifically recognize the phosphorylated form of p38- and p44/42-type kinases was used. *A. brassicicola* cells were grown in liquid medium, exposed to a sublethal concentrations of camalexin or brassinin for 10 min (Hog1 activation) or 20 min (Slit2 activation) and then harvested for total protein extraction. Exposures to fludioxonil and sodium orthovanadate, two potential activators of the Hog1 and Slit2 pathways respectively (Martin *et al.*, 2000; Dongo *et al.*, 2009), were also performed in parallel as positive controls. Immunoblot analysis using antiphospho-p44/42 and antiphospho-p38 antibodies revealed that, in the WT strain, both proteins were constitutively phosphorylated albeit at low levels (Figs 6 and S2). However, following camalexin and brassinin treatments, increased phosphorylation of both *AbHog1* and *AbSlit2* MAPKs was observed. Control blots challenged with antibodies recognizing Hog1- and Mpk1-type kinases confirmed equal loading of the protein samples. Similar analyses were conducted with proteins extracted from the *AbHog1*- and *AbSlit2*-replacement mutants. Increased phosphorylation of the *AbHog1* MAPK was still observed in the *AbSlit2Δ* mutant upon challenge with camalexin. By contrast, the *AbSlit2* MAPK phosphorylation level remained unchanged during the same treatment in the *AbHog1Δ* strain. The previously

**Table 1.** *In vitro* effects of oxidative stress, cell wall perturbing agents and the phenylpyrrole fungicide fludioxonil on radial growth rates of *A. brassicicola* WT and MAPK deficient mutants.

	Abra43	<i>AbHog1Δ1</i>	<i>AbHog1Δ2</i>	<i>AbSlit2Δ1</i>	<i>AbSlit2Δ2</i>
$\text{H}_2\text{O}_2$ (25 mM)	24 ± 2 <sup>a</sup>	25 ± 2	24 ± 3	32 ± 3 <sup>b</sup>	31 ± 4 <sup>b</sup>
Menadione (1 mM)	14 ± 2	37 ± 4 <sup>b</sup>	46 ± 2 <sup>b</sup>	32 ± 2 <sup>b</sup>	34 ± 2 <sup>b</sup>
CFW (100 mg l <sup>-1</sup> )	63 ± 2	41 ± 4 <sup>b</sup>	44 ± 6 <sup>b</sup>	54 ± 2 <sup>b</sup>	47 ± 2 <sup>b</sup>
CR (200 mg l <sup>-1</sup> )	34 ± 2	27 ± 1 <sup>b</sup>	27 ± 1 <sup>b</sup>	56 ± 2 <sup>b</sup>	46 ± 1 <sup>b</sup>
Fludioxonil (0.1 mg l <sup>-1</sup> )	44 ± 2	13 ± 2 <sup>b</sup>	8 ± 2 <sup>b</sup>	67 ± 5 <sup>b</sup>	61 ± 2 <sup>b</sup>
Fludioxonil (1 mg l <sup>-1</sup> )	75 ± 3	19 ± 1 <sup>b</sup>	19 ± 2 <sup>b</sup>	100 ± 0 <sup>b</sup>	100 ± 0 <sup>b</sup>

a. Values are means of three biological repetitions and represent the percentage growth inhibition under stress conditions compared with standard growth conditions (PDA medium without additive).

b. A significant difference between the mutant and the Abra43 WT parental isolate (Student's *t*-test,  $P < 0.01$ ).



**Fig. 2.** AbHog1 and AbSlt2 gene inactivation by homologous recombination.

**A.** Schematic representation of *AbHog1* and *AbSlt2* loci (hatched boxes), disruption constructs with the *HygB* resistance gene (*hph*) cassette (white boxes) and flanking sequences (gray boxes). P represents the position of the *Pst* 1 sites and arrows indicate the positions of primers used for PCR screening of deleted mutants and generation of hybridization probes.

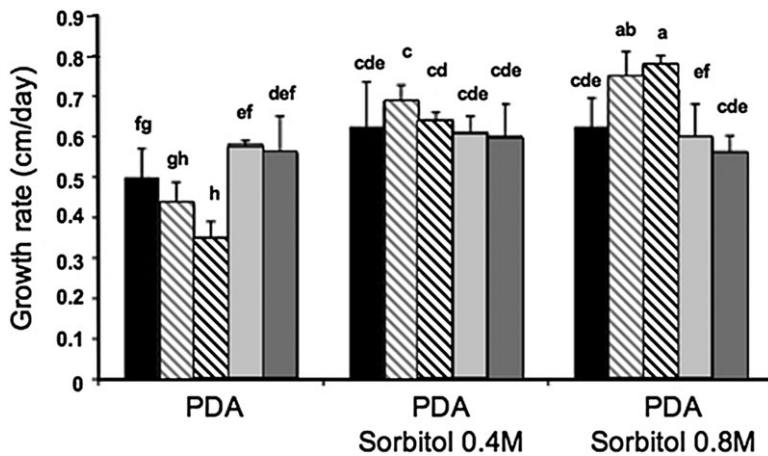
**B.** Southern hybridization of genomic DNA from WT isolates and transformants; each DNA was digested with *Pst* 1 and the blots were probed with  $^{32}\text{P}$ -labelled PCR fragments (shown by black boxes in panel A).

**C.** Immunodetection of AbHog1p and AbSlt2p in protein extracts from WT, *AbHog1Δ* and *AbSlt2Δ* isolates. Proteins were extracted from liquid cultures, analysed by SDS-PAGE and blotting with anti-Hog1 (left panel) and anti-Mpk1 (right panel) antibodies. Arrows indicate the position of immunoreactive polypeptides with sizes corresponding to AbHog1p and AbSlt2p. Molecular masses of proteins were determined using Precision Plus Proteins All Blue Standards (Bio-Rad Laboratories).

characterized GIII-HK AbNik1 was shown to act upstream of the HOG MAPK cascade in *A. brassicicola* (Dongo *et al.*, 2009). Parallel experiments with proteins extracted from the *AbNik1Δ* mutant revealed that the observed camalexin-induced phosphorylation of AbHog1p was independent of a functional GIII-HK.

*AbHog1* and *AbSlt2* MAPKs protect *A. brassicicola* cells from indolic phytoalexins

*AbHog1Δ* and *AbSlt2Δ* mutants were assessed for their susceptibility to camalexin exposure. This was performed both by analysis of colony expansion rates and monitoring

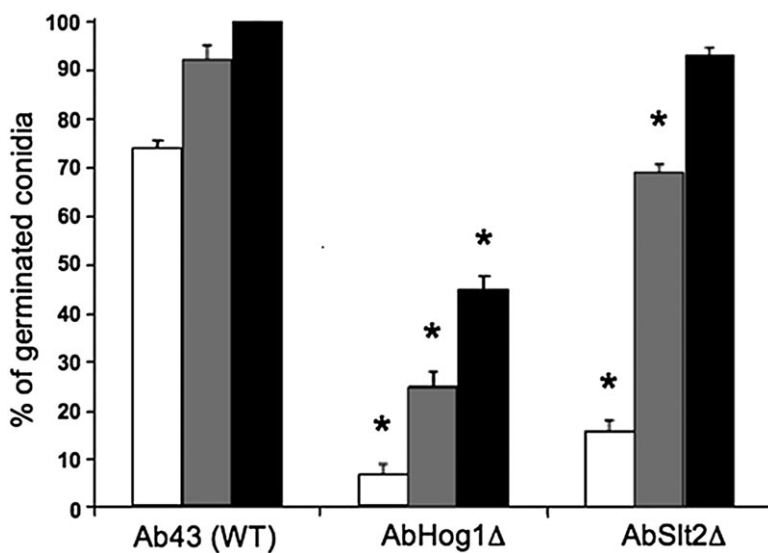


**Fig. 3.** Effects of increased medium osmolarity on the growth of WT, *AbHog1Δ* and *AbSlt2Δ* isolates. Growth rates of the WT strain Ab43 (black bars), two *AbSlt2Δ* (hatched bars) mutants and two *AbHog1Δ* (grey bars) mutants were determined at 24°C on standard solid medium (PDA) containing or not 0.4 and 0.8 M sorbitol. The data are means of three independent experiments. Different letters above the bars indicate significantly different values according to Waller–Duncan k-ratio test with k set at 100.

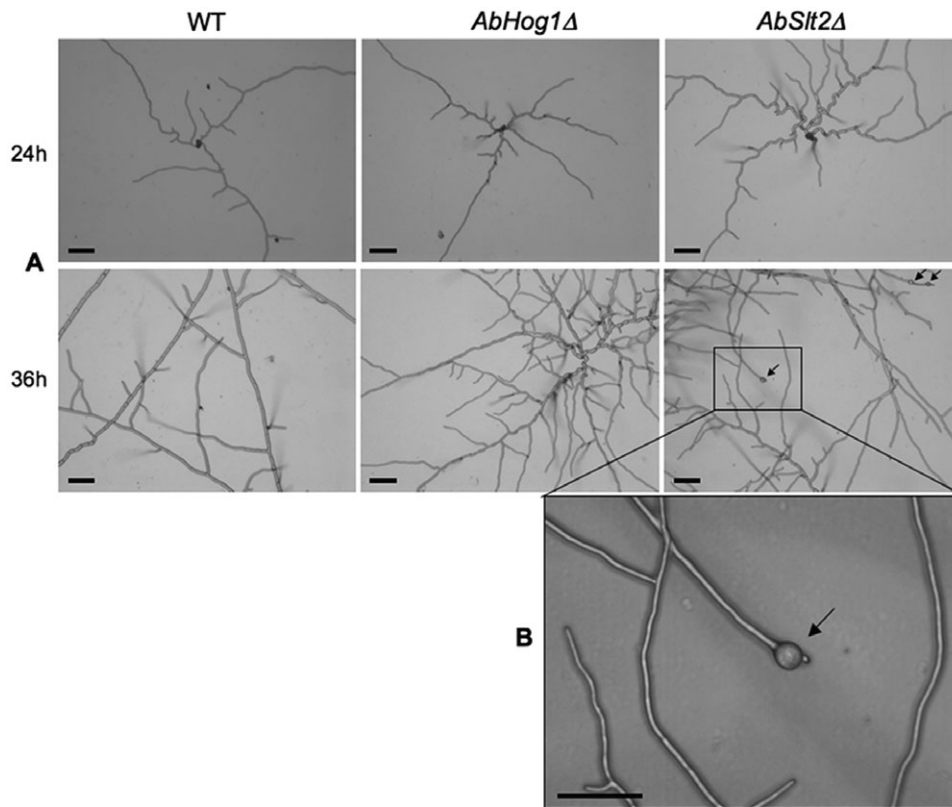
the initial growth stages in solid and liquid medium respectively. As illustrated in Fig. 7, comparison of the mycelial growth of the mutants with that of the WT parental strain on solid medium containing camalexin showed a more pronounced toxic effect of the phytoalexin on the *AbHog1*- and *AbSlt2*-replacement mutants than on the parental strain. In these growth conditions, the mean  $IC_{50}$  for camalexin ranged from 70 to 80  $\mu$ M for the *AbHog1* and *AbSlt2* mutants respectively, versus 148  $\mu$ M for the WT strain. In liquid medium, in the absence of phytoalexin, typical microbial growth curves containing a lag phase, a log phase and a plateau were recorded for the three genotypes tested (Fig. 8A). At 40  $\mu$ M camalexin, the maximum growth rate of the WT strain was only slightly affected (41% reduction). By contrast, a sharp reduction in this growth parameter was observed for the *AbSlt2Δ* and *AbHog1Δ* strains (81% and 77% reduction respectively) (Fig. 8B). Parallel experiments were conducted with the structurally related phytoalexin brassinin. As

shown in Fig. 8C, brassinin at 300  $\mu$ M had little effect on the maximum growth rate of the WT strain (21% reduction) but provoked delayed entry into the log phase (2.2-fold). A longer lag phase was also observed with the mutant strains (2.8-fold to fourfold for *AbSlt2Δ* and *AbHog1Δ* respectively), but in these cases, exposure to brassinin also resulted in an 82% (*AbHog1Δ* strain) and 46% (*AbSlt2Δ* strain) decrease in the maximum growth rate.

Sellam *et al.* (2007a) previously showed that camalexin induces fungal membrane permeabilization. An assay based on uptake of the fluorogenic dye SYTOX green was thus used to visualize the effect of this phytoalexin on membrane integrity of the different studied genotypes. As shown in Fig. 9, no green fluorescence was detected in WT hyphae upon exposure to 125  $\mu$ M of camalexin for at least 4 h. Under the same conditions, fluorogenic dye uptake was evident in samples from *AbSlt2Δ* and *AbHog1Δ* strains after 4 h camalexin exposure.



**Fig. 4.** Germination efficiency of conidia from WT, *AbHog1Δ* and *AbSlt2Δ* isolates. Germination rates were evaluated at 5 h (white bars), 10 h (grey bars) and 15 h (black bars) of incubation at 24°C. The percentage of spore germination (100 spores for each assay) was determined under a light microscope, using a micrometer. A spore was scored as germinated if the germ-tube length was equal in size to that of the conidium. Values are the mean of three independent experiments. Asterisks indicate percentages of conidia germination in mutant genotypes significantly different ( $P < 0.05$ , Student's *t*-test) from those measured at identical incubation time for the WT isolate.

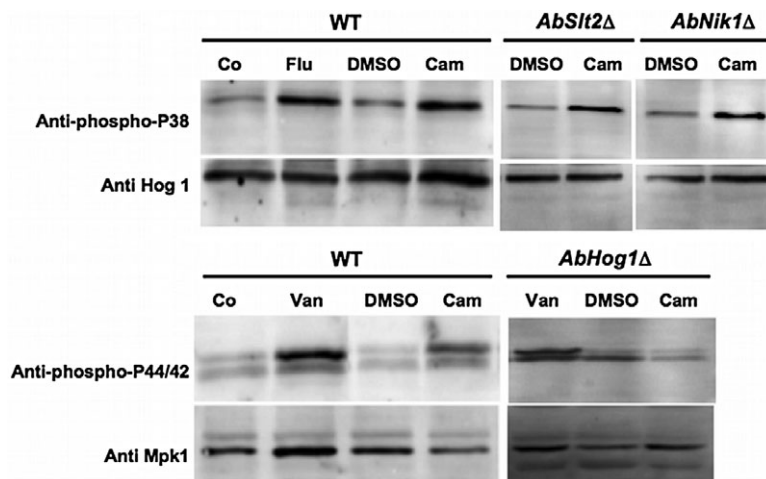


**Fig. 5.** Hyphal morphology of WT, *AbHog1Δ* and *AbSlt2Δ* isolates. (A) The different isolates were grown on glass slides covered with MA medium. The growing mycelium was directly observed every 12 h using light microscopy. Part of the photograph showing *AbSlt2Δ* hyphal morphology is enlarged in (B). Arrows indicate swollen tip cells. Scale bars = 30  $\mu$ m.

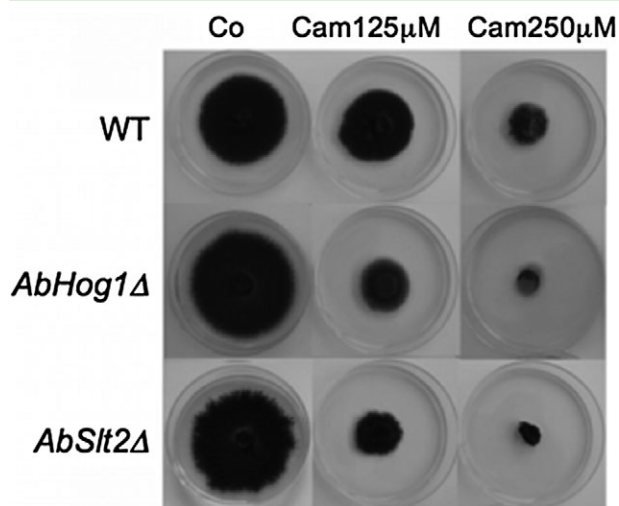
*Alternaria brassicicola* mutants disrupted in *AbHog1* and *AbSlt2* signalling cascades are highly compromised in their pathogenicity

The virulence of the WT, *AbHog1Δ* and *AbSlt2Δ* strains were compared on *Brassica oleracea* and *A. thaliana* host

plants by leaf inoculation experiments. On intact leaves of *B. oleracea* inoculated with the WT strain, small necrotic symptoms were already observed at 3 dpi, which continued to expand into large typical necrotic areas surrounded by chlorotic halos at 6 dpi. By contrast, only small necrotic lesions with very limited spread around the inoculation



**Fig. 6.** Phosphorylation of Hog1- and Slt2-like MAPKs in *A. brassicicola* after camalexin exposure. Germinated conidia from the WT strain, the *AbHog1Δ*, *AbSlt2Δ* and *AbNik1Δ* mutants were grown for 24 h (Hog1 blots) or 36 h (Mpk1/Slt2 blots) in PDB and exposed to 250  $\mu$ M camalexin (Cam) for 10 min (Hog1 blots) or 20 min (Mpk1/Slt2 blots). Negative controls (DMSO) were exposed to solvent only and positive controls for phosphorylation of *AbHog1* and *AbSlt2* were exposed to 100  $\mu$ g ml<sup>-1</sup> fludioxonil (Flu) and 5 mM Na vanadate (Van) respectively. Total protein extracts prepared from harvested mycelia were analysed by SDS-PAGE and blotting with either anti-dually phosphorylated p38 and anti-Hog1 C-terminus antibodies or anti-dually phosphorylated p44/42 and anti-Mpk1 antibodies.



**Fig. 7.** Effects of camalexin on the mycelium radial growth of *A. brassicicola* WT strain and MAPK deficient mutants. *In vitro* growth tests were carried out on PDA medium supplemented with camalexin (Cam) at 125 and 250  $\mu$ M. Growth was scored at 6 days (Abra43 WT and *Hog1* $\Delta$  strains) or 8 days (*Slt2* $\Delta$  strains) of incubation at 24°C.

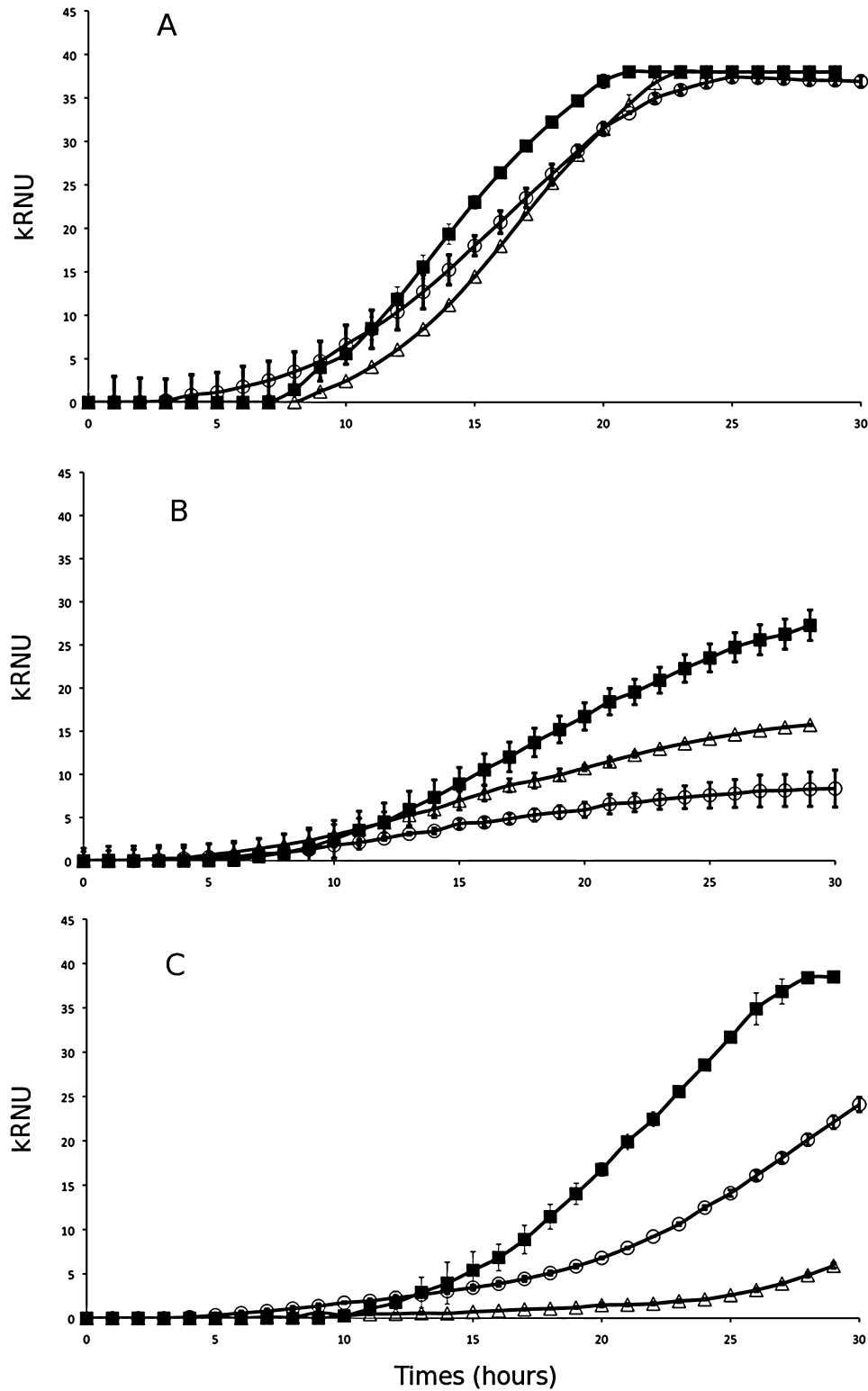
sites were observed at 6 dpi on tissues inoculated with the *AbHog1* $\Delta$  and *AbSlt2* $\Delta$  mutants (Fig. 10A). As it has been previously reported that inactivation of genes encoding Hog1- and Slt2-like MAPKs may infer penetration capacity (Xu *et al.*, 1998; Segmuller *et al.*, 2007; Liu *et al.*, 2008), we examined infection-related morphogenesis with a penetration assay of onion epidermal cells (Fig. 11A). At 24 h post inoculation, microscopic observations showed that 35% of the germinated conidia from the WT have already produced appressoria-like structures at the tips of short germ tubes to penetrate the epidermis. At the same time, the MAPK deficient mutants produced long non-penetrating hyphae at the surface of the onion epidermis with appressoria-like structures only differentiated by 8% and 2% of the germinated conidia from the *AbHog1* $\Delta$  and *AbSlt2* $\Delta$  mutants respectively. To test whether the reduced penetration efficiency was also observed on living host tissue, cabbage leaves were inoculated with conidia suspension as for infection tests. At 24 h post inoculation, plant samples were collected, stained with Solophenyl Flavine and observed using fluorescent microscopy. As shown in Fig. 11B, characteristic appressoria-like structures were observed in both the WT and mutant strains at the tips of germ tubes that were in contact with the leaf epidermis. However, quantitative analysis was difficult as only a few conidia were repeatedly (three independent experiments) observed on leaf tissues inoculated with the *AbHog1* $\Delta$  mutants suggesting that they poorly adhered to the leaf surface and were thus washed off during the staining procedure. Qualitative analyses performed by observing unstained samples

using environmental scanning electron microscopy revealed that the appressoria-like structures differentiated by the three tested genotypes had similar morphology (Fig. 11C).

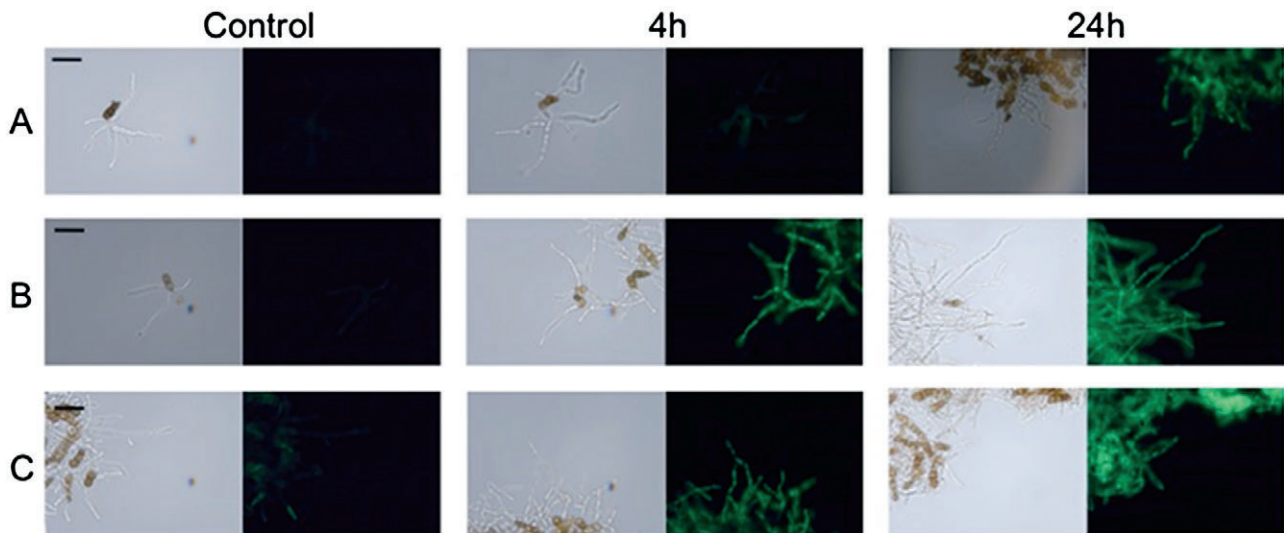
To check whether failure to penetrate plant cells constituted the primary pathogenicity defect of *AbHog1* $\Delta$  and *AbSlt2* $\Delta$  mutants, cabbage leaves were wounded prior to inoculation. Under these conditions, lesions induced by the MAPK mutants were still dramatically reduced in size compared with those produced by the WT strain (Fig. 10B). As, in *Arabidopsis*, resistance to *A. brassicicola* relies on the capability to produce camalexin (Thomma *et al.*, 1999), inoculation experiments were also performed on wounded leaves of either the WT Col-0 *A. thaliana* (camalexin producing and resistant genotype) or the *pad3* mutant (camalexin non-producing and susceptible genotype). Visual evaluation of the necrotic lesion area and quantification of the fungal biomass in inoculated leaves determined the state of infection at 6 dpi (Fig. 12). When using the Col-0 accession, the lesions obtained with the MAPK mutants did not progress beyond the boundaries of the inoculated regions and only faint slowly progressing lesions were formed by the WT strain. In the latter case, pathogen sporulation was repeatedly observed at the inoculation site. In agreement with previous studies, the *pad3* mutant supported significantly higher fungal growth when inoculated with the parental strain Abra 43 and more severe lesions were produced on this genotype in comparison with Col-0. Enhanced susceptibility of the *pad3* mutant was also observed with the *AbSlt2* $\Delta$  mutants that produced more severe symptoms that were the direct consequence of increased fungal colonization. There was indeed a more than 10-fold difference in fungal biomass between the *pad3* and WT Col-0 genotypes. In contrast, the *AbHog1* $\Delta$  mutants still failed to produce any symptoms when inoculated on wounded *pad3* leaves.

## Discussion

Camalexin plays a major role in the resistance of *A. thaliana* to necrotrophic pathogens (Thomma *et al.*, 1999). This phytoalexin was shown to strongly inhibit conidial germination, germ-tube elongation and to a lesser extent mycelial radial growth of *A. brassicicola* (Sellam *et al.*, 2007b). Although the exact mechanism by which camalexin exerts its toxicity is still unknown, we previously demonstrated that this plant defence metabolite probably causes membrane damage and activates a cellular response similar to the so-called 'compensatory mechanism' (Popolo *et al.*, 2001) to preserve cell integrity (Sellam *et al.*, 2007a). As the CWI pathway plays an essential role in the regulation of the cell wall remodelling process in yeast (Levin, 2005), here we sought to determine whether



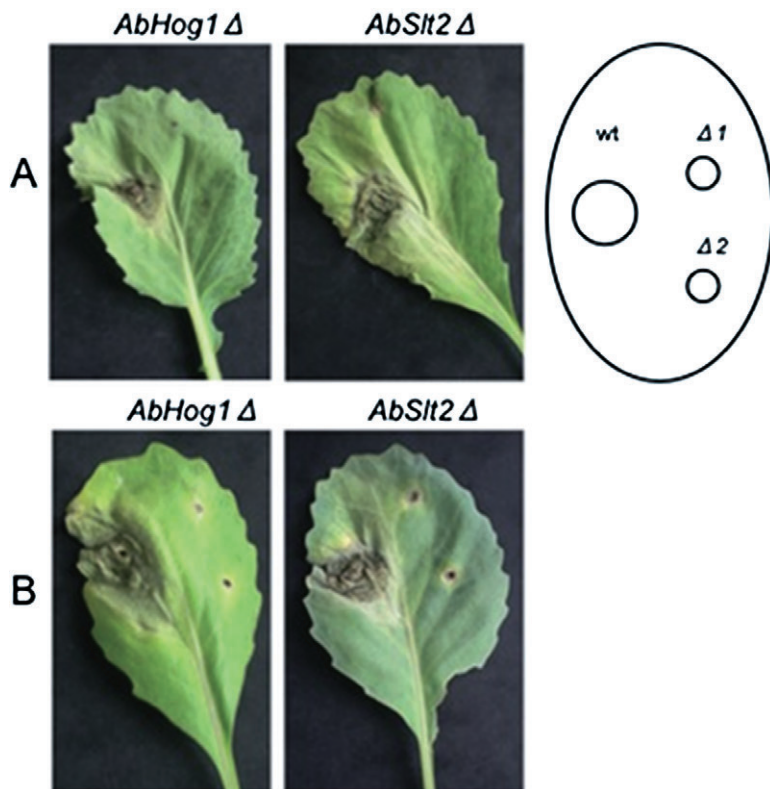
**Fig. 8.** Nephelometric monitoring of growth of WT strain and MAP kinase deficient mutants in the presence of indolic phytoalexins. Conidia from Abra 43 WT (black squares), *Hog1*Δ (open triangles) and *Sit2*Δ (open circles) strains were used to inoculate microplate wells containing standard PDB medium that was not supplemented (A), or supplemented with either 40 μM camalexin (B) or 300 μM brassinin (C). Growth was automatically recorded for 30 h at 24°C using a nephelometric reader. Each genotype was analysed in triplicate and the experiments were repeated three times per growth condition.



**Fig. 9.** Determination of membrane damage in *A. brassicicola* WT strain (A) *AbHog1Δ* (B) and *AbSlit2Δ* (C) mutants treated with camalexin. Membrane integrity was checked using SYTOX green uptake assays in 16 h germinated conidia treated for 4 and 24 h with 125  $\mu$ M camalexin. Controls were incubated for 24 h with DMSO 1% (v/v). For each panel, the right part corresponds to fluorescence microscopy and the left part to light-field microscopy. Scale bars = 20  $\mu$ m.

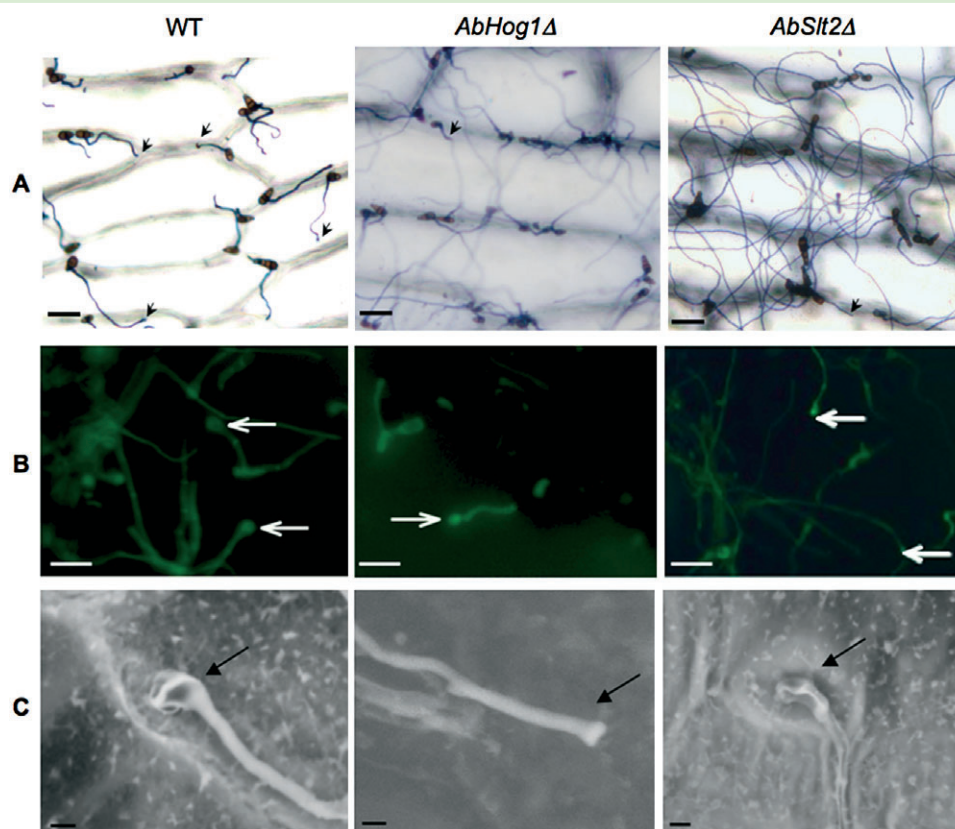
this signalling pathway is involved in regulation of the response of *A. brassicicola* to camalexin treatment. In *A. brassicicola*, we identified a homologue of the yeast Slit2 MAPK that functionally complements a *slt2Δ* *S. cerevisiae* strain. This MAPK, called *AbSlit2*, showed a rapid increase in phosphorylation in *A. brassicicola* cells challenged with

camalexin but also with the structurally related phytoalexin brassinin, which is produced by several crucifers including species within the genus *Brassica* (Pedras *et al.*, 2007). Gene replacement mutants were constructed to test the contribution of such activation in regulation of the fungus sensitivity towards indolic phytoalexins. The *AbSlit2Δ*



**Fig. 10.** Effects of the disruption of *AbHog1* and *AbSlit2* on the virulence of *A. brassicicola* on *B. oleracea*. Leaves of *B. oleracea* were inoculated with conidia suspensions of *Abra43* WT, *Hog1Δ* and *Slit2Δ* strains with (B) or without (A) artificial lesions. Two independent disruptants per mutant genotype were tested on *B. oleracea* leaves (right part of the central vein) and compared on the same leaf with the WT parental strain (left part of the central vein). Symptoms were observed at 6 dpi.

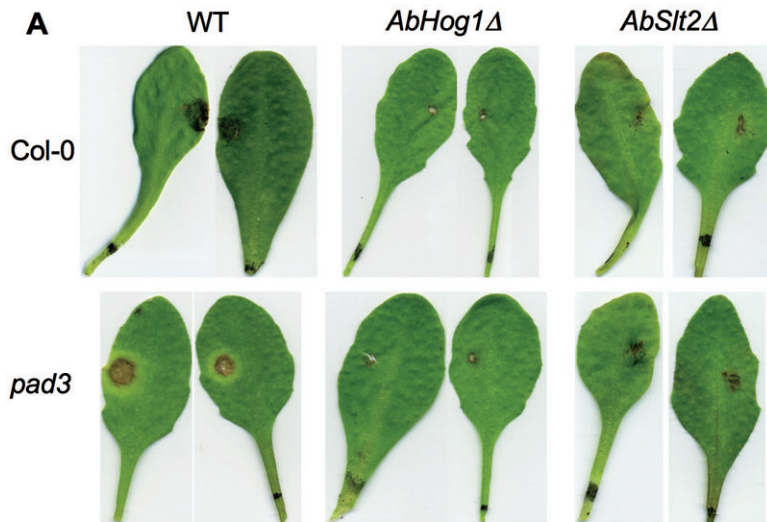




**Fig. 11.** Microscopic observations of the infection structure on onion epidermis (A) and on *B. oleracea* leaf surfaces (B, C) at an early infection with *A. brassicicola* WT strain, *AbHog1Δ* and *AbSlt2Δ* mutants. Inoculated plant tissue fragments were collected at 24 h dpi and stained with Trypan blue (A), Solophenyl Flavine (B) or directly processed for microscopic observations (C). Photographs correspond to either light-field (A), fluorescence (B) or electron scanning (C) microscopy. Appressoria-like structures, indicated by arrows, were differentiated by all the tested genotypes. On onion, germlings of the WT quickly penetrate the epidermis cells while germ tubes produced by the *AbSlt2Δ* mutant showed excessive elongation on onion epidermis and cabbage leaf surfaces. Scale bars = 20  $\mu\text{m}$  (A, B) or 2  $\mu\text{m}$  (C).

mutants showed slightly reduced vegetative growth on solid medium that might, at least partially, be due to problems in coping with hypo-osmotic conditions since this growth retardation was alleviated in the presence of sorbitol as osmotic protectant. Similar growth defects have already been reported for *F. graminearum* and *B. cinerea* *Slt2*-type MAPK mutants (Hou *et al.*, 2002; Rui and Hahn, 2007) and normal growth was also recovered in the latter fungal species in the presence of an osmotic stabilizer. Moreover, hyphae produced by *AbSlt2Δ* mutants on complex culture media have enlarged tip cells. The aberrant shape of tip cells eventually followed by a progressive autolytic process have also been observed in similar mutants of various filamentous fungi such as *Mycosphaerella graminicola*, *Magnaporthe grisea* and *Cochliobolus heterostrophus* (Xu *et al.*, 1998; Mehrabi *et al.*, 2006; Eliahu *et al.*, 2007). This phenomenon strongly suggests that cell wall formation is hampered in these mutants and may result in altered cell wall strength and thus in hypersensitivity towards some fungicides and xenobiotic compounds. Indeed the *MgSlt2Δ* mutant of *M. graminicola* and

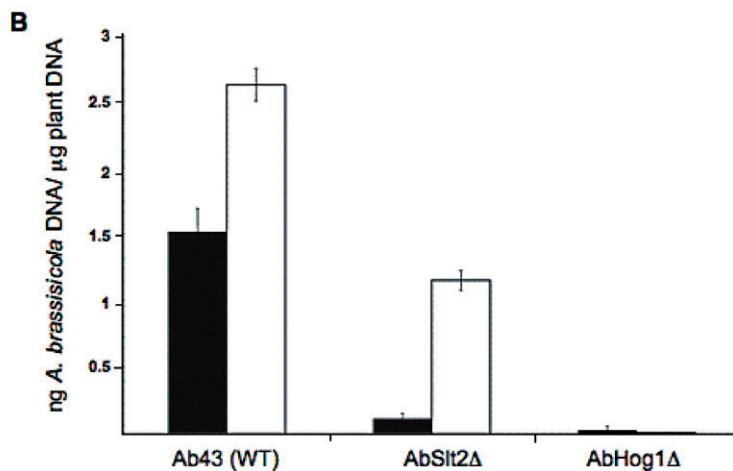
the *BcBmp3Δ* mutant of *B. cinerea*, which are deficient in *Slt2*-type MAPKs, have enhanced sensitivity to azole and phenylpyrrole fungicides respectively (Mehrabi *et al.*, 2006; Rui and Hahn, 2007). Similarly, *AbSlt2Δ* mutants were found to be more sensitive to fludioxonil than their WT parental strain. In line with a possible role of the CWI signalling pathway in regulating genes important for overcoming plant defence responses, *AbSlt2Δ* mutants were also found to be hypersensitive to camalexin and brassinin. Although the antifungal activity of brassinin has been partly attributed to its dithiocarbamate group that is absent in camalexin (Szolar, 2007), our results suggest that these two compounds may have toxic modes of action that at least partially overlap. Indeed, in both cases, activation of the *Slt2* signalling pathway probably results in cell wall straightening that may help the fungus to counteract their cell permeabilizing effects, as illustrated by the SYTOX assay. Interestingly, it was previously reported that the *Mgv1* MAPK (a yeast *Slt2* homologue) signalling cascade plays a major role in regulating the sensitivity of *F. graminearum* to plant defensins, which probably also act



**Fig. 12.** Effects of the disruption of *AbHog1* and *AbSlt2* on the virulence of *A. brassicicola* on *A. thaliana*. Wounded leaves of *A. thaliana* Col-0 and *pad3* were inoculated with conidia suspensions of *Abra43* WT, *Hog1Δ* and *Slt2Δ* strains.

A. For each plant-pathogen combination, two leaves are shown that correspond to representative symptoms obtained at 6 dpi with two independent *A. brassicicola* disruptants per mutant genotype (one per leaf).

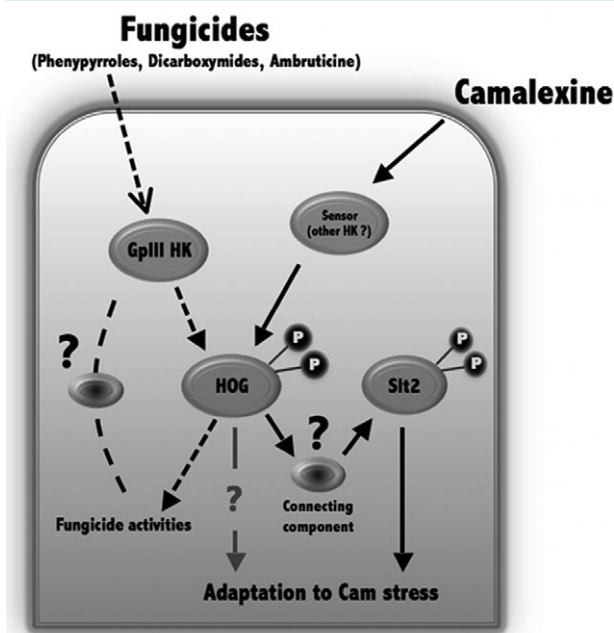
B. *A. brassicicola* biomass in WT Col-0 (black bars) and *pad3* mutant genotypes (white bars) measured by real-time PCR from leaf total DNA extracts at 6 dpi. Biomass of the fungal genotypes is shown as the ratio between fungal and plant DNA. Values are the means of two independent biological repeats (three plants per genotype per repeat) with each sample analysed in triplicate by real-time PCR.



by impairing fungal CWI (Ramamoorthy *et al.*, 2007). These authors reported that, in this fungal species, mutants deficient in a second MAPK called GpMPK1 (a yeast Kss1 homologue) have increased sensitivity to defensins, while the Hog 1 MAPK signalling cascade does not participate in the response to these antifungal proteins.

While the Slt2 MAPK pathway has been shown to be the main pathway responsible for the response to cell wall stress (Levin, 2005), additional signalling pathways, like the Kss1 MAPK pathway (Lee and Elion, 1999; Cullen *et al.*, 2000), have also been involved in CWI. Although we have not tested the sensitivity to camalexin of *A. brassicicola* mutants deficient for the previously described Fus3/Kss1 MAPK homologue (Cho *et al.*, 2007), our preliminary experiments revealed that neither the *Fus3Δ* mutant nor the *Kss1Δ* mutant of *S. cerevisiae* have increased sensitivity to this phytoalexin, which also has a membrane permeabilizing effect on yeast (Fig. S3). With regard to crosstalk between distinct MAPK pathways, it has been found that the Slt2 MAPK was activated by cell wall damage caused by Zymolase (Bermejo *et al.*, 2008)

but also by hyperosmotic shock (Garcia-Rodriguez *et al.*, 2005) in a Hog1-dependent manner. Moreover, *in silico* analysis of promoters of genes that are induced transcriptionally in response to cell wall damage suggests that the HOG pathway could also act as a regulator of the response to cell wall damage (Boorsma *et al.*, 2004; Garcia *et al.*, 2004). This prompted us to investigate the possible role of the HOG pathway in the response of *A. brassicicola* to indolic phytoalexins. As shown for *AbSlt2Δ* mutants, *AbHog1Δ* mutants were also found to be hypersensitive to camalexin and brassinin. Moreover, rapid activation of this pathway by the two phytoalexins was observed using anti-phospho-P38 antibodies in the WT *Abra43* strain. In some filamentous fungi, including *A. brassicicola*, a GIII-HK (Nik/Os1 family of two-component HK) was demonstrated to be a positive upstream regulator of Hog 1-type MAPK (Yoshimi *et al.*, 2005; Dongo *et al.*, 2009). We provide evidence here that camalexin-induced Hog1 MAPK activation was not dependent on this GIII-HK as increased phosphorylation of *AbHog1p* was still observed in a *AbNik1Δ* mutant challenged with



**Fig. 13.** Model illustration of fungal signalling pathways regulated by camalexin. The cell surface stress caused by camalexin triggered the Hog1 signalling cascade independently of GpIII-HK. Phosphorylated Hog1 MAPK was necessary for the activation of the cell integrity (Slt2) pathway that leads to cellular adaptation to membrane damage generated by the phytoalexin. Activation of Nik1-dependant signalling pathways by fungicides is also illustrated (dotted arrows).

camalexin. Similar evidence was obtained with a *AbSlt2Δ* mutant exposed to the phytoalexin. By contrast, activation of AbSlt2 MAPK was abrogated in a Hog1 deficient strain. These results suggest that yet to be identified upstream sensors are implicated in the detection and signalling of camalexin-induced cell damage leading to AbHog1 MAPK activation. Such activation would be necessary for proper AbSlt2 phosphorylation on the basis of a connection between HOG and CWI pathways (Fig. 13). Although they have not yet been identified, the existence of such elements connecting the HOG and Slt2 pathways was recently demonstrated in yeast (Bermejo *et al.*, 2008).

In line with several studies on fungal pathogens (for review see Zhao *et al.*, 2007), our study revealed that Hog1- and Slt2-like MAPKs were essential for full expression of *A. brassicicola* virulence in cabbage. With regards to other fungal pathogens with necrotrophic lifestyles, it has been shown that mutants defective in these two kinases failed to penetrate or colonize the host tissues. For instance, in *B. cinerea*, no penetration of unwounded host tissues occurred with BcSAK1 (Hog1-type) MAPK-null mutants and reduced penetration and retarded tissue colonization was observed with BcBMP3 (Slt2-type) MAPK-null mutants (Rui and Hahn, 2007; Segmuller *et al.*, 2007). We also observed excessive germ-tube elongation and weak adhesion of germinated conidia on

leaves surfaces with *AbSlt2Δ* and *AbHog1Δ* mutants respectively. Despite this, all the tested genotypes were able to develop typical penetration structures (i.e. appressoria-like) on host tissues, albeit with lower efficiencies for *AbSlt2Δ* and *AbHog1Δ* mutants when compared with WT, and the decreased ability to penetrate was probably not the single cause of the attenuated pathogenicity of *A. brassicicola* MAPK mutants. Indeed, only slight symptoms were observed in wounded tissues, suggesting that *AbHog1Δ* and *AbSlt2Δ* mutants were severely impaired in their tissue colonization capacity. In *M. graminicola*, similar observations were obtained with *MgSlt2Δ* mutants and it was suggested that increased sensitivity to plant defence metabolites may explain their failure to establish invasive growth *in planta* (Mehrabi *et al.*, 2006). In the same vein, it is likely that the reduced virulence of *A. brassicicola* MAPK mutants results at least partially from their hypersensitivity to indolic phytoalexins. In line with this hypothesis, *AbSlt2Δ* mutants were able to develop within tissues and produce necrotic symptoms when inoculated on wounded leaves of an *A. thaliana pad3* mutant deficient for camalexin synthesis. However, *AbHog1Δ* mutants did not produce any symptoms in such host plants, even on wounded tissues, suggesting a pleiotropic effect of this mutation that may affect other components of the infection process.

This study constitutes, to our knowledge, the first evidence that a plant phytoalexin activates fungal MAPKs and that outputs of at least one of the activated signalling cascades significantly contribute to the phytoalexin tolerance of the fungus. Future work will focus on the identification and characterization of upstream sensors involved in camalexin and brassinin detection by the fungus as well as additional elements involved in the link between HOG and SLT2 pathways.

## Experimental procedures

### *Fungal strains and growth conditions*

The *A. brassicicola* WT strain Abra43 used in this study has previously been described (Sellam *et al.*, 2007a). For routine culture, *A. brassicicola* was grown and maintained on PDA. Germination tests on conidia of WT and mutant strains were performed according to Sellam *et al.* (2007b). Light microscopy observations of hyphal morphology were performed on mycelium growing on glass slides covered with MA or water agar medium at 24°C under high humidity. Observations were done at 12, 24 and 36 h post inoculation. For radial growth assays, PDA plates amended with the test compounds were inoculated with mycelial plugs and incubated at 24°C. Colony diameters were measured daily and used for calculation of radial growth (mm day<sup>-1</sup>). To study hyphal growth in liquid media, conidial suspensions (10<sup>5</sup> spores ml<sup>-1</sup>, final concentration) were inoculated onto microplate wells containing appropriate test substances in PDB in a total volume of 300 μl. Microplates were placed in a laser-based

microplate nephelometer (NEPHELOstar, BMG Labtech) and growth was monitored automatically over a 30 h period. Data were expressed as relative nephelometric unit. Two variables, lag time and maximal growth rate, were calculated from the growth curves as described by Joubert *et al.* (2010).

The phytoalexin camalexin was synthesized according to Ayer *et al.* (1992) and brassinin according to Kutschy *et al.* (1998) and Takasugi *et al.* (1988). Stock solutions were prepared in dimethylsulfoxide (DMSO) and acetone respectively, and added to the medium at the desired concentrations.

#### DNA manipulation and analysis

Genomic DNA was extracted from mycelium according to Adachi *et al.* (1993). For Southern blot analysis, DNA fragments resulting from genomic DNA digestion with Pst1 were separated on 1% agarose gels and vacuum transferred to Hybond N membranes (Amersham Biosciences). Blots were then probed with PCR fragments obtained using the primer sets described below for the amplification of the 5' flanking regions of *AbHog1* and *AbSlt2* genes and <sup>32</sup>P labelled with a random primer labelling kit (Amersham Biosciences).

Phylogenetic tree generation, DNA and protein sequence alignment, editing and analysis were performed using MEGA 4 software (Kumar *et al.*, 2008), whereas homology searches were performed with the BLAST program (Altschul *et al.*, 1997) via the National Center for Biotechnology Information non-redundant database. Sequence comparisons with the *A. brassicicola* genome were conducted via (<http://genome.jgi-psf.org>).

#### Generation of targeted gene disruption constructs and fungal transformation

The gene replacement cassettes were generated using the double-joint PCR procedure described by Yu *et al.* (2004). The selectable marker inserted in the PCR construct corresponded to the HygB gene cassette (1436 bp) from pCB1636 (Sweigard *et al.*, 1997). For each targeted gene, two sets of primers (Table S1) were designed to amplify 805 bp (*AbSlt2* gene) and 838 bp (*AbHog1* gene) from the 5' flanking regions and 779 bp (*AbSlt2* gene) and 761 bp (*AbHog1* gene) from the 3' regions. All amplifications were performed with Phusion™ Hot Start High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). The final double-joint PCR products were purified and used to transform *A. brassicicola* protoplasts as described by Cho *et al.* (2006). Hygromycin-resistant transformants (six colonies) were pre-screened by PCR with relevant primer combinations to confirm integration of the replacement cassette at the targeted locus. The gene replacement mutants were further purified by three rounds of single-spore isolation and then confirmed by Southern blot analysis.

#### SYTOX assays

For membrane permeabilization assays, 16-h-old germinating conidia grown in PDB were exposed to 125 µM camalexin or 1% (v/v) DMSO as negative control for 2–24 h. After treatment, 20 µl of the incubation mixtures were mixed with a SYTOX green

solution (1 µM final concentration) and immediately observed under a Leica fluorescence microscope.

#### Yeast complementation assays

Fungal RNA was extracted, DNase treated and reverse transcribed as described by Guillemette *et al.* (2004). Sequences encoding the two MAPK *AbHog1* and *AbSlt2* of *A. brassicicola* were amplified by PCR with Phusion™ Hot Start High-Fidelity DNA polymerase from first-strand cDNAs with primer pairs *HindHog* (5'-**AAGCTTAATAATGGCGGAATTCGTACGC**-3') – *BamHog* (5'-**GGATCCGTTT**AGCTGCGGTTGTTCTTTG-3') and *EcoSlt2* (5'-**GAATCCATAATGGGCGACCTCGCCAAC**-3') – *XhoSlt2* (5'-**CTCGAGAATCATCGC**ATACGACCGTC-3') respectively. Unique *HindIII*–*BamH1* or *EcoRI*–*XhoI* restriction sites (indicated in boldface) were thus introduced at the upstream start and downstream stop codons (underlined) of the coding sequences respectively. The PCR products were cloned into pGEM-T (Promega) and then transferred into the yeast expression vector pYES2-CT (Invitrogen) after *HindIII*–*BamH1* or *EcoRI*–*XhoI* double digestion and ligation to yield the plasmids pYAbHog1 and pYAbSlt2. These plasmids can express full-length *AbHog1p* and *AbSlt2p* without a tag under control of the GAL1 promoter. *S. cerevisiae* cells (strains: parental BY4743 Acc#Y20000, ΔHog Acc#Y32724, ΔSLT2 Acc#Y30993, Euroscarf, Germany) were transformed using the S.c. EasyComp transformation kit (Invitrogen). Transformants were incubated under GAL1 promoter-repressive conditions [SD without uracil (SD–Ura)] or under inductive conditions [SG without uracil (SG–Ura)]. Sensitivity to reagents was analysed on plates. Transformants were precultured overnight in 5 ml of SD–Ura or SG–Ura. Serially diluted [1:10 (starting optical density at 600 nm of 0.1)] cell suspensions (5 µl) were spotted onto plates with each of the test reagents, and the plates were incubated for 60–240 h.

#### Infection assays

For plant infection assays on *B. oleracea* plants, 5 µl drops of *A. brassicicola* spore suspension ( $2 \times 10^5$  spores ml<sup>-1</sup> in water) were inoculated on intact and prewounded leaves from 5-week-old plants (three to four leaves per plant). Inocula were deposited on the left and right sides symmetrically from the central vein. *A. thaliana* Col-0 plants, WT and *pad3* genotypes, were grown to the 8- to 12-leaf stage in controlled environment rooms (21–19°C day and night temperature respectively) and a 8 h light photoperiod. For inoculations, 5 µl drops of *A. brassicicola* spore suspension ( $5 \times 10^5$  spores ml<sup>-1</sup> in water) were deposited onto six pre-wounded leaves per plant (one drop per leaf). Three plants were inoculated per fungal genotype and the experiment was repeated twice. The plants were then maintained under saturating humidity (100% relative humidity). Symptoms were observed at 1, 3 and 6 dpi. Quantitative PCR assays for the determination of fungal biomass in *A. thaliana* were performed on each *A. brassicicola* – infected leaf at 6 dpi as described by Sellam *et al.* (2006) using the *AbNik1* and 5.8S ribosomal RNA gene sequences as targets to assess fungal and plant DNA respectively.

#### Visualization of infection structures

Quantitative analysis of the development of infection-related structures was performed on onion epidermis. Conidial suspen-

sions ( $5 \mu\text{l}$  drops at  $5 \times 10^3$  spores  $\text{ml}^{-1}$ ) were placed on the hydrophobic face of the epidermis. After 24 h of incubation at high humidity in glass trays, tissues were stained by immersion into 0.25% Trypan Blue in lactophenol for 5 min (Doeblemann *et al.*, 2006), briefly rinsed with  $\text{H}_2\text{O}$  and observed under a light microscope to determine the number of germinated conidia that had differentiated appressoria-like structures. For each assay, at least 100 germinated conidia were evaluated. Infection structures differentiated on host tissues were also observed. For optical microscopic analyses, inoculated cabbage leaf fragments were discoloured, cleared and fungal structures were stained with Solophenyl Flavine 7GFE 500 (Ciba Specialty Chemicals, NC) as described by Hoch *et al.* (2005). Specimens were observed under a Leica fluorescent microscope. For electron microscopy analyses, the pictures of infection structures were acquired with an environmental scanning electron microscope Zeiss EVO 50 Variable Pressure SEM (Carl Zeiss SMT, New York), fitted with a high brightness LaB6 cathode. Pressure and humidity conditions were adjusted to 470 Pa and 30–40% respectively. The samples were directly imaged in their natural state without modification or preparation.

#### Western blot analysis

The phosphorylation status of Hog1- and Slr2-related MAPKs in *A. brassicicola* was studied by Western blot using antibodies directed against dually phosphorylated forms of p38 and p44/42 MAPK (Cell Signaling Technology, Beverly, MA) respectively. Total Hog1 and Slr2 proteins were detected using anti-Hog1 and anti-Mpk1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) respectively. Samples for study of Hog1-related protein phosphorylation status were prepared from mycelia obtained by growing conidial suspensions at  $25^\circ\text{C}$  for 24 h in PDB ( $200\,000$  conidia  $\text{ml}^{-1}$ ) and then exposed to  $250 \mu\text{M}$  camalexin,  $250\text{--}500 \mu\text{M}$  brassinin, 1% (v/v) DMSO or  $100 \mu\text{g ml}^{-1}$  fludioxonil for 10 min. Mycelia were collected by filtration on filter paper, ground with a mortar and pestle to a fine powder under liquid nitrogen and homogenized in ice-chilled buffer containing protease and phosphatase inhibitors (50 mM Na phosphate, pH 7.4, 1 mM EDTA, 5% (v/v) glycerol, 1 mM PMSF, 50 mM NaF, 5 mM Na pyrophosphate, 0.1 mM Na vanadate, 10 mM  $\beta$ -glycerophosphate). To study the phosphorylation status of Slr2-related protein, cultures obtained by deposition of  $100 \mu\text{l}$  of a conidia suspension ( $10^6$  conidia  $\text{ml}^{-1}$ ) on a cellophane membrane on PDA in a Petri dish and incubation for 36 h at  $25^\circ\text{C}$  were treated for 20 min by adding 20 ml PDB (control), 1% (v/v) DMSO,  $250 \mu\text{M}$  camalexin,  $250\text{--}500 \mu\text{M}$  brassinin or 5 mM Na vanadate in PDB. Membranes were broken under liquid nitrogen, then mycelia were recovered and homogenized by grinding with a mortar and pestle in the same ice chilled buffer as for the Hog1 study. Suspensions were centrifuged at  $10\,000 g$  for 10 min, and the resulting supernatants were stored at  $-80^\circ\text{C}$  until use. The protein concentration in the extracts was calculated using a BCA protein assay reagent (Pierce, Rockford, IL). Equal quantities ( $5 \mu\text{g}$ ) of protein samples were loaded on 10% polyacrylamide gels and blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). For each treatment, protein samples were prepared from at least three independent cultures and each sample was used to prepare at least three series of duplicated blots. Antibody binding was visualized using an ECL Plus Western

blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK) after binding of a horseradish peroxidase-conjugated secondary antibody.

#### Statistical analysis

Phenotypic characterizations of the *A. brassicicola* strains were subjected to at least two biological repetitions, each with three replicates. Statistical analyses were performed using R-2.9.1 software (Team, 2009). Growth rate and inhibition percentage did show homoscedasticity (Breusch–Pagan test,  $\alpha = 1\%$ ). A linear model from growth rate was made. Residuals were found to be normally distributed (normal Q-Q plot). Multiple comparisons were performed using a Waller–Duncan k-ratio test (Waller and Duncan, 1969; De Mendiburu, 2007) with k set at 100. For each treatment, inhibition percentage was compared for each mutant strain with inhibition percentage observed in wild strain using a Student's *t*-test. *P* was set at 0.0016 from  $\alpha = 0.05$  (Bonferroni correction, 30 comparisons).

#### Acknowledgements

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Hyphal morphology of WT, *AbHog1Δ* and *AbSlit2Δ* isolates on water agar. The different isolates were grown on glass slides covered with water agar. The growing mycelium was directly observed every 12 h using light microscopy. Scale bars = 30 μm.

**Fig. S2.** Phosphorylation of Hog1- and Slit2-like MAPKs in *A. brassicicola* after brassinin exposure. Germinated conidia from *A. brassicicola* (WT strain) were grown for 24 h (Hog1 blots) or 36 h (Mpk1/Slit2 blots) in PDB and exposed to 250 μM or 500 μM brassinin (Bra) for 10 min (Hog1 blots) or 20 min (Mpk1/Slit2 blots). Negative controls (DMSO) were exposed to solvent only and positive controls for phosphorylation of AbHog1 and AbSlit2

were exposed to 250  $\mu\text{M}$  camalexin (Cam). Total protein extracts prepared from harvested mycelia were analysed by SDS-PAGE and blotting with either anti-dually phosphorylated p38 and anti-Hog1 C-terminus antibodies or anti-dually phosphorylated p44/42 and anti-Mpk1 antibodies.

**Fig. S3.** Effects of camalexin on different yeast genotypes. A. Growth inhibition test of serially diluted ( $10^{-1}$  to  $10^{-3}$ ) *S. cerevisiae* cells (parental WT strain and  $\Delta\text{SLT2}$  Acc#Y30993,  $\Delta\text{KSS1}$  Acc# Y36981,  $\Delta\text{FUS3}$  Acc# Y03042, Euroscarf, Germany) spotted onto SG plates supplemented or not with 100  $\mu\text{M}$  camalexin. All strains had been transformed with empty vector (pYES2CT) and the *Slr2 $\Delta$*  mutant had also been transformed by pYAbSlr2. Colony growth was observed after 48 h of incubation at 30°C. At this concentration, camalexin did not significantly affect growth of the WT strain and the *Kss1 $\Delta$*  and *Fus3 $\Delta$*  mutants, but significantly altered growth of the *Slr2 $\Delta$*

mutant. Expression of AbSlr2 in this genotype restored normal growth on camalexin. B. Membrane permeabilization effect of camalexin on *S. cerevisiae* cells. Membrane integrity of  $\Delta\text{SLT2}$  strain was checked using SYTOX green uptake assays after 30 h exposure to 75  $\mu\text{M}$  camalexin. Controls (Co) were incubated with DMSO 1% (v/v). For each panel, the right part corresponds to fluorescence microscopy and the left part to light-field microscopy. Scale bar = 40  $\mu\text{m}$ .

**Table S1.** List of primers used in this study.

**Table S2.** Protoplasting efficiencies of the WT strain and MAPK deficient mutants.

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**Table S1: Protoplasting efficiencies of the WT strain and MAPK deficient mutants**

	Abra43 (WT)	AbHog1 $\Delta$	AbSlt2 $\Delta$
<i>Standard protoplasting conditions</i>			
initial step <sup>a</sup>	5. 10 <sup>7</sup> <sup>c</sup>	1.10 <sup>5</sup>	4.10 <sup>4</sup>
final step <sup>b</sup>	1.10 <sup>7</sup>	0	0
<i>Low salt protoplasting conditions</i>			
initial step <sup>a</sup>	1. 10 <sup>6</sup>	0	0
final step <sup>b</sup>	1. 10 <sup>5</sup>	0	0

<sup>a</sup> for standard conditions, germinated conidia were incubated at 32°C in lytic solution (0.7M NaCl, 20 mg/ml Driselase, 10 mg/ml Kitalase). The number of protoplasts was counted under a microscope after 4h incubation. For low salt conditions, the lytic solution contained 0.35 M NaCl.

<sup>b</sup> washed protoplasts were suspended in STC (1.2M sorbitol, 10 mM Tris pH 7.0, 50 mM CaCl<sub>2</sub>) and counted under a microscope

<sup>c</sup> numbers correspond to the number of protoplast. mL<sup>-1</sup>

**Table S2: List of primers used in this study**

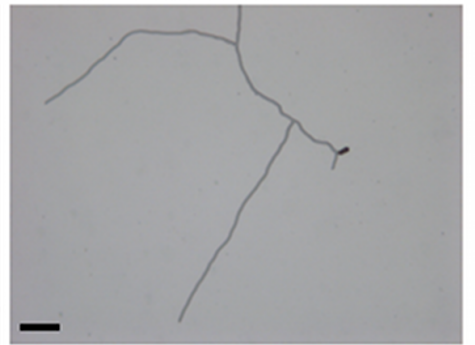
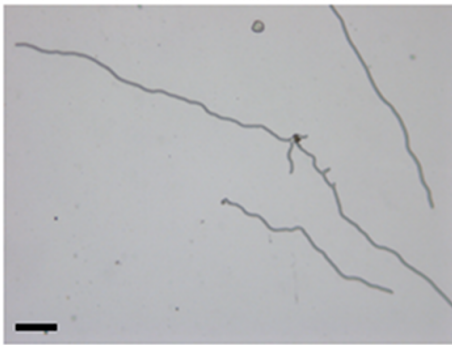
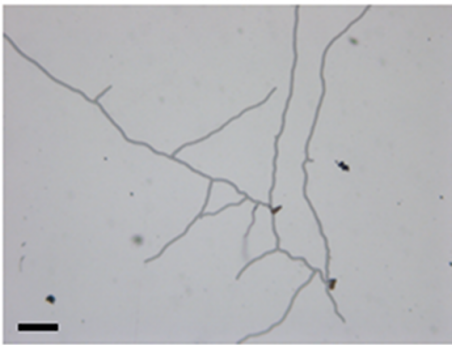
Name	Sequence	Description
FusHOG-F1	5' TGAGGTCATCCCGTCTTACC <sup>3'</sup>	Set of primers used to generate by double joint PCR the AbHog1 disruption cassette carrying the HygB resistance gene from pCB1636
FusHOG-R1	5' TCCTGTGTGAAATTGTTATCCGCTATGTCCCTCGAGGGGAGAGAT <sup>3'</sup>	
FusHOG-F2	5' GTCGTGACTGGGAAAACCTGGCGGAACGACAACCTTG TCCAAC <sup>3'</sup>	
FusHOG-R2	5' GCCGTTGACACAACAACAAC <sup>3'</sup>	
FusHOG-NF	5' AACCTGCCCTGATCTACCA <sup>3'</sup>	
FusHOG-NR	5' CAAACCCAAAGTCCCGTCTA <sup>3'</sup>	
FusSLT-F1	5' ATA ACTGGCGGGGTTCTCTT <sup>3'</sup>	Set of primers used to generate by double joint PCR the AbSl2 disruption cassette carrying the HygB resistance gene from pCB1636
FusSLT-R1	5' GTCGTGACTGGGAAAACCTGGCGGAACACCTGGGGTCCTGTAA <sup>3'</sup>	
FusSLT-F2	5' TCCTGTGTGAAATTGTTATCCGCTCACAAGAAGCGTTCAACCAG <sup>3'</sup>	
FusSKT-R2	5' CTCAGCGCTCTTTTGTCTCT <sup>3'</sup>	
FusSLT-NF	5' ACGGATATGGCGTTTGAGAG <sup>3'</sup>	
FusSLT-NR	5' AGCGGATAACAATTTACACAGGA <sup>3'</sup>	
NCL37	5' GGATGCCTCCGCTCGAAGTA <sup>3'</sup>	5' and 3' <i>HygB</i> specific primers
NCL38	5' CGTTGCAAGACCTGCCTGAA <sup>3'</sup>	
M13F	5' CGCCAGGGTTTTCCAGTCACGAC <sup>3'</sup>	Forward and reverse primers specific to the central part of <i>HygB</i>
M13R	5' AGCGGATAACAATTTACACAGGA <sup>3'</sup>	
Hog-sens1	5' ACA(AG)GA(CT)CC(AGCT)CA(AG)ATGAC <sup>3'</sup>	Degenerate primers for <i>Hog1</i> -related sequences
Hog-rev1	5' TC(AGCT)(GC)(AT)(AG)TACATCAT <sup>3'</sup>	

WT

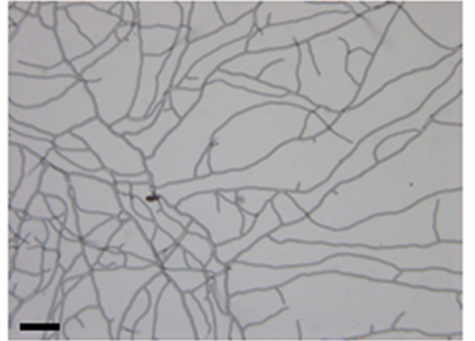
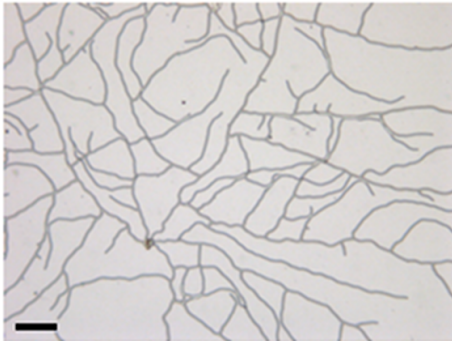
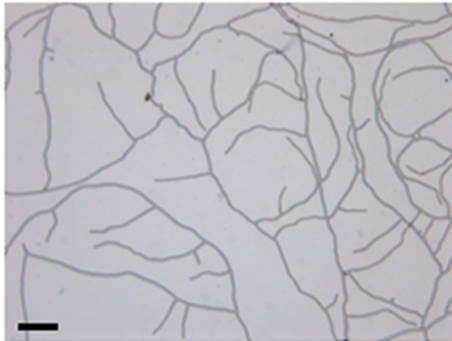
*AbHog1* $\Delta$

*AbSit2* $\Delta$

24h



36h



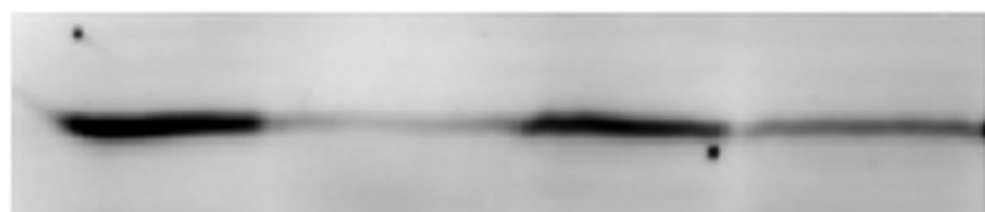
Cam

DMSO

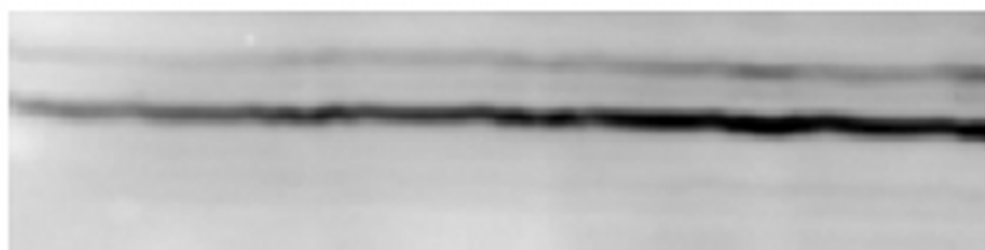
Bra 250  $\mu$ M

Bra 500  $\mu$ M

Anti-phospho-P38



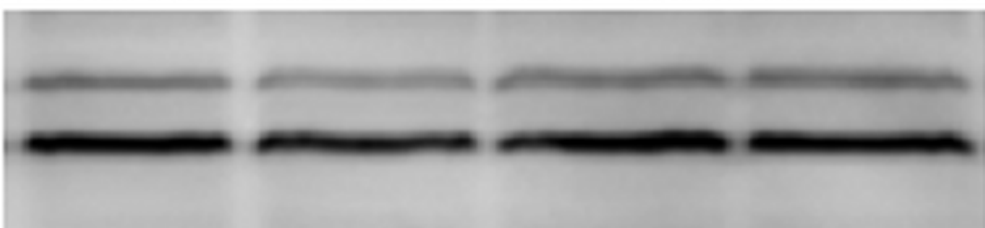
Anti Hog 1



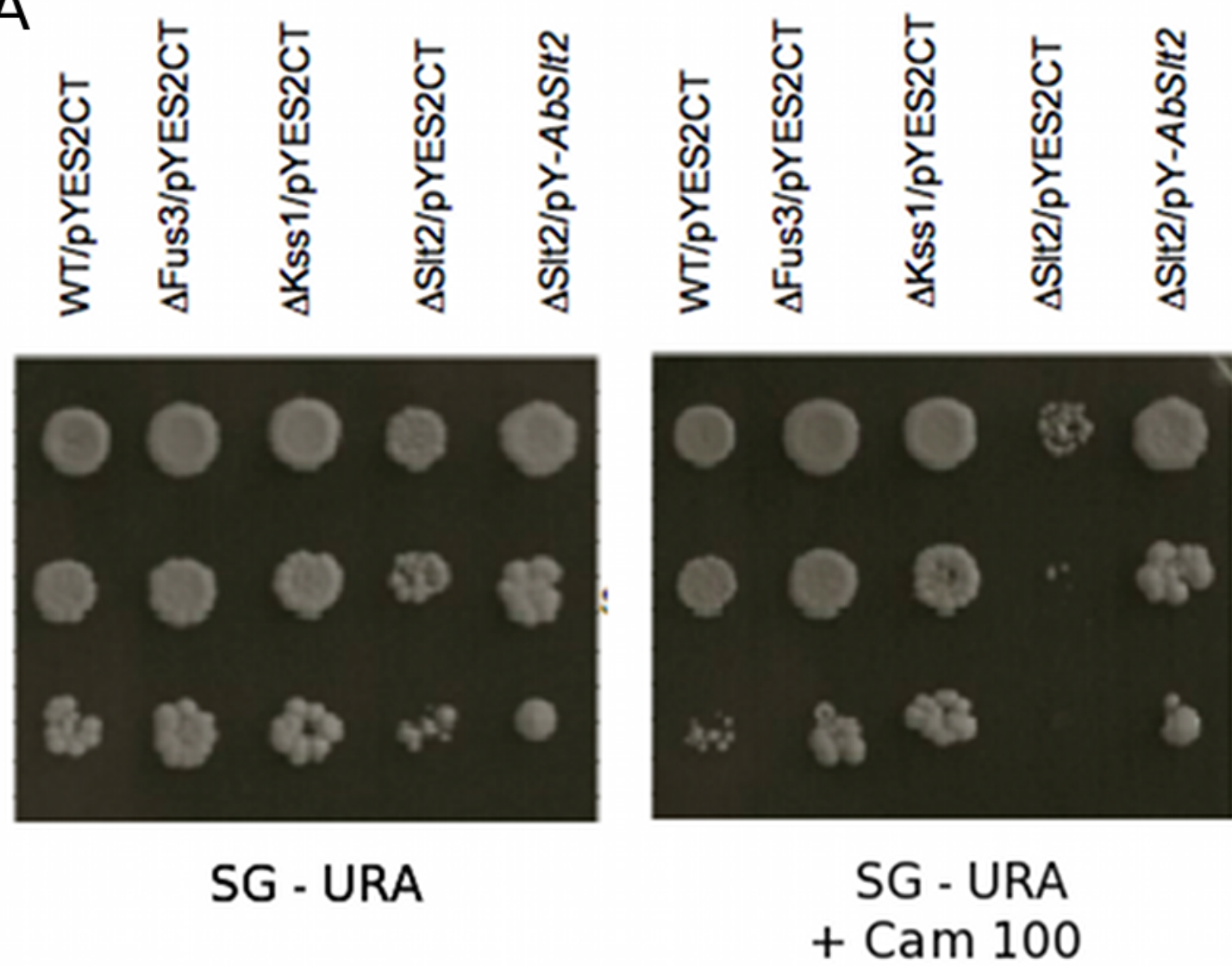
Anti-phospho-P44/42



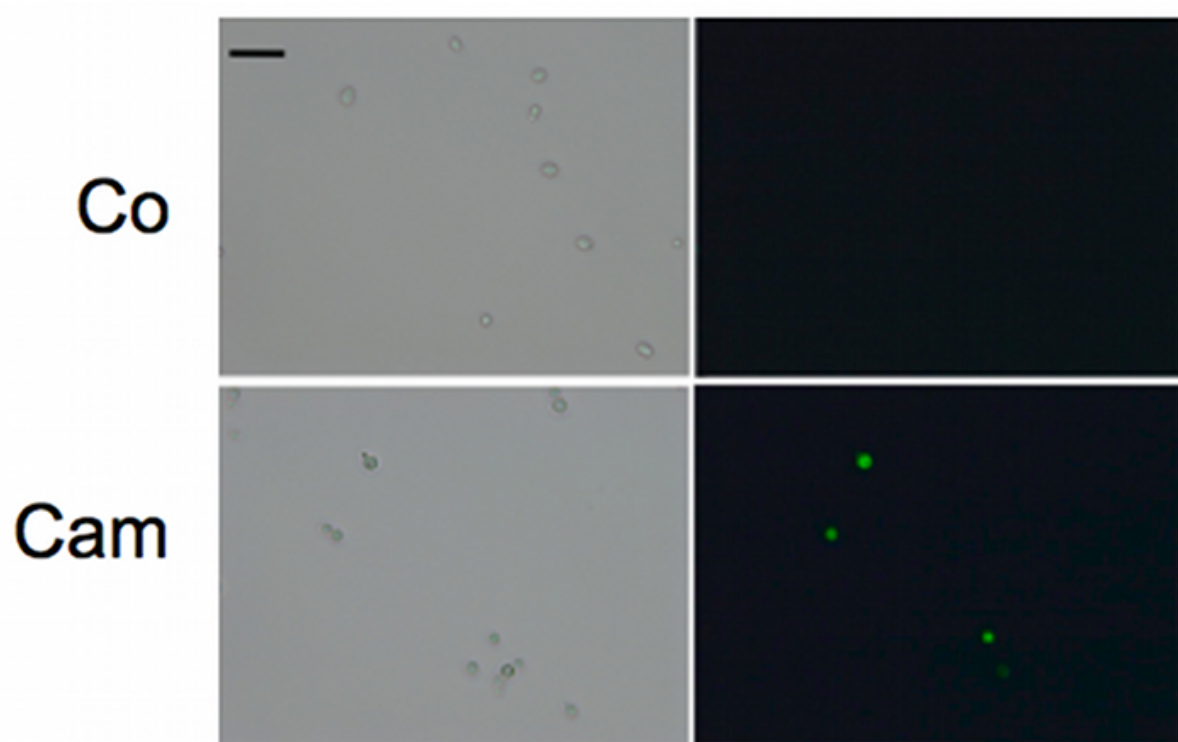
Anti Mpk1



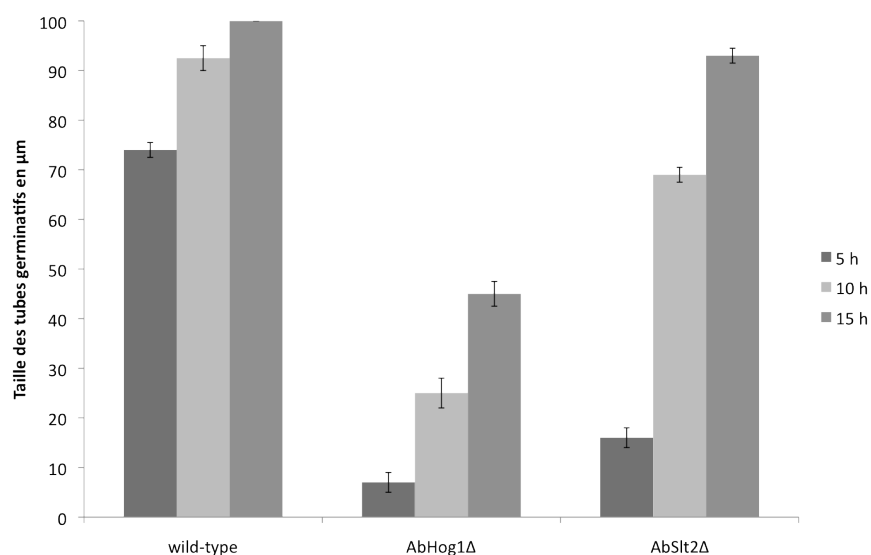
A



B



### **3. Résultats complémentaires et perspectives**



**Figure 17** Taille des tubes germinatifs en  $\mu\text{m}$  de la souche sauvage (*Abra43* wild-type) et des deux souches mutantes *AbSlt2Δ* et *AbHog1Δ* sur milieu nutritif Malt-agar à différents temps post-germination.

**Tableau 2** Effet du NaCl sur la croissance de la souche sauvage *Abra43* et sur les deux souches mutantes *AbSlt2Δ* et *AbHog1Δ*.

Les résultats sont exprimés en pourcentage d'inhibition par rapport à la condition non traitée. Les étoiles indiquent une différence significative par rapport à la souche *Abra43* (Test de student,  $P < 0,01$ ).

	<b>Abra43 (WT)</b>	<b>AbHog1Δ</b>	<b>AbSlt2Δ</b>
NaCl 0,3 M	34 ± 3	49 ± 2*	33 ± 3
NaCl 0,6 M	50 ± 3	65 ± 5*	36 ± 2*

Les travaux présentés ci-dessus montrent que les mutations dans les gènes *AbSlt2* et *AbHog1* ont un impact direct sur la morphologie et le développement des souches. En effet, celles-ci aboutissent à une diminution de la vitesse de croissance, une hyper-filamentation et un éclatement des hyphes à la base des apex pour le mutant *AbSlt2Δ1* ainsi qu'un retard dans la germination, également observé chez le mutant *AbHog1Δ1*. Ajouté à toutes ces différences morphologiques, les mutations impactent également sur la vitesse de développement des tubes germinatifs (**Figure 17**). Ce résultat est à mettre en parallèle avec le retard de germination des deux souches mutantes.

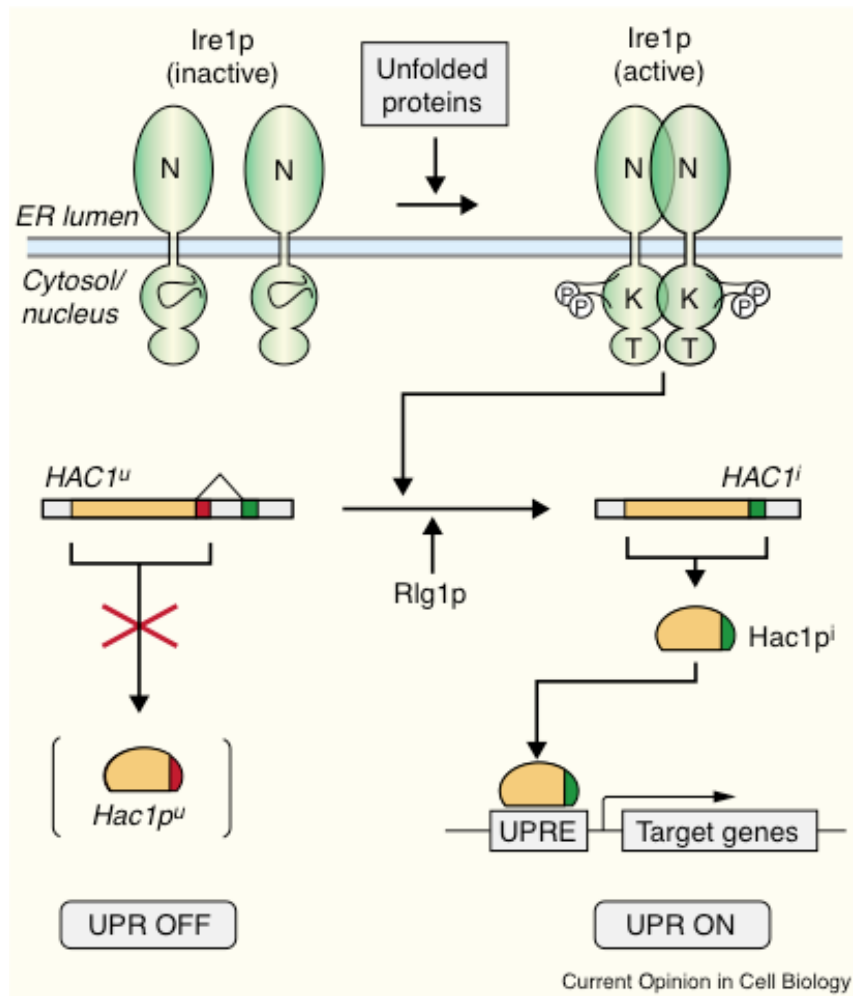
La mutation dans le gène *Hog1* entraîne des troubles du maintien de la pression osmotique chez *S. cerevisiae*. Chez *A. brassicicola*, nos travaux montrent que le mutant *AbHog1Δ1* est lui aussi plus sensible au stress provoqué par de fortes concentrations en NaCl (augmentation de la pression osmotique et de la force ionique du milieu de culture) que la souche sauvage d'origine (**Tableau 2**). Toutefois, ce mutant ne présente pas de différence de comportement par rapport à la souche sauvage lorsqu'il est cultivé en présence de fortes concentrations en sorbitol. Ce composé augmente la pression osmotique du milieu mais est neutre d'un point de vue ionique. A l'inverse le mutant *AbNik1Δ1* présente une sensibilité au sorbitol mais pas vis-à-vis du NaCl (Avenot *et al.*, 2005, Dongo *et al.*, 2009). Ces résultats nous indiquent que la réponse au stress osmotique (type sorbitol) serait dépendante d'une voie médiée par Nik1p et indépendante de Hog1p tandis que la réponse à un stress type ionique (NaCl) serait dépendante de Hog1p mais ne passerait pas par la kinase Nik1p. Afin de vérifier cette hypothèse, il serait intéressant de tester, chez *A. brassicicola*, la sensibilité aux stress ioniques de mutants nuls pour des orthologues des senseurs de la voie HOG, Sln1p et Sho1p.



# Chapitre 3

**Implication du facteur de transcription *HacA*  
dans la réponse du champignon *Alternaria  
brassicicola* aux phytoalexines indoliques**

## **1. Présentation de l'étude**



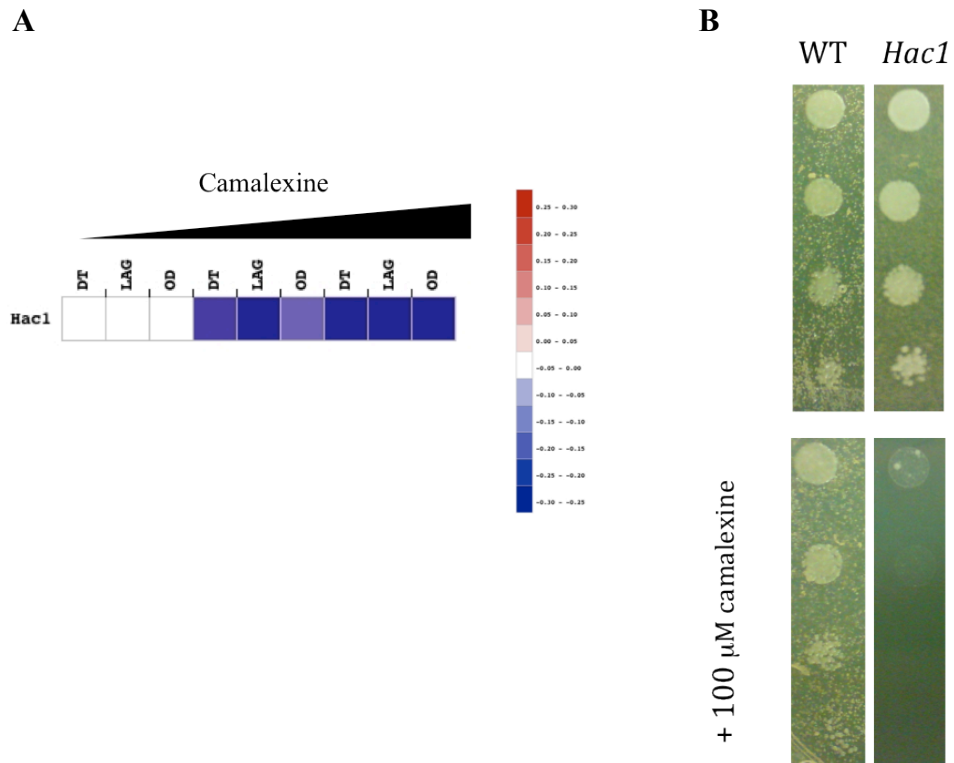
**Figure 18** Représentation schématique de l'UPR chez *S. cerevisiae* (d'après Patil et al., 2001)

Chez tous les eucaryotes, les protéines acquièrent leur conformation dans le réticulum endoplasmique (RE). Afin de s'assurer du bon repliement et de la bonne conformation des protéines, le RE possède des mécanismes de contrôle de la qualité des protéines, notamment l'Unfolded Protein Response (UPR), qui a pour rôle principal de prévenir la surcharge du RE en protéines non conformes (Schroder *et al.*, 2005).

Le mécanisme d'activation de l'UPR a été particulièrement bien étudié chez *S. cerevisiae* (**Figure 18**). Il dépend en particulier de l'activité ribonucléase de la protéine Ire1p. Il s'agit d'une protéine kinase transmembranaire située au niveau du RE et maintenue sous forme monomérique inactivée lorsque le RE est en condition non stressé. Avec l'accumulation de protéines non conforme, Ire1p est libéré et, par une homodimérisation, aboutit à l'activation de son activité RNase. Elle va permettre l'épissage alternatif d'un intron non conventionnel présent au niveau de l'ARNm codant Hac1p (Rueggsegger *et al.*, 2001). Cet épissage lève un bloc traductionnel et conduit à la production du facteur de transcription, Hac1p. Ce mécanisme d'activation de l'UPR par épissage alternatif est conservé parmi les organismes vivants et a été notamment décrit chez les champignons filamenteux (Mulder *et al.*, 2004, Saloheimo *et al.*, 2003).

Le facteur de transcription Hac1p est de type basic leucine zipper (bZIP) et se fixe au niveau de sites de fixation appelés UPR Element présents dans les promoteurs des gènes cibles de l'UPR. Plus de 381 gènes sont ainsi régulés durant l'UPR chez *S. cerevisiae* (Travers *et al.*, 2000), dont des gènes codant des chaperones, des foldases, des gènes de la voie de biosynthèse des phospholipides ou de l'ERAD (Endoplasmic Reticulum Associated Degradation) (Cox *et al.*, 1997). Hac1p va également réprimer des gènes du métabolisme afin de réduire l'accumulation de protéines dans le RE (Schroder *et al.*, 2004).

Les études sur les mécanismes de sécrétion chez certains champignons ont été essentiellement réalisées à des fins industriels et commerciaux pour la production d'enzymes par exemple (Machida *et al.*, 2005). De plus, ces connaissances cellulaires sur la sécrétion des champignons ont souvent été extrapolées à partir des travaux sur les cellules eucaryotes. Les phénomènes de stress de sécrétion chez les eucaryotes ont été très étudiés car ils interviennent dans le processus de certaines maladies tels que des maladies métaboliques, neurodégénératrices ou même dans les cancers (Patil *et al.*, 2001). Peu à peu



**Figure 19** Résultats du phénotypage de mutants de *S. cerevisiae*.

**(A)** Représentation des LPI (Indice Phénotypique Logarithmique) du phénotypage du mutant de *S. cerevisiae*  $\Delta$ Hac1 en fonction de doses croissantes (75, 125 et 250  $\mu$ M) de camalexine. DT : temps de doublement, LAG : période de latence, OD : accroissement de la population. **(B)** Croissance sur milieu YNBA additionné de camalexine de différentes dilutions de *S. cerevisiae* calibrées de la souche sauvage WT et de la souche mutante  $\Delta$ Hac1.

les travaux sur la compréhension de l'UPR chez *S. cerevisiae* se sont étendus chez les champignons filamenteux. Par exemple, Guillemette *et al.*, (2007) ont réalisé, pour la première fois, une analyse de l'expression du génome d'*Aspergillus niger* sous l'effet de stress de sécrétion. Ces auteurs ont notamment mis en évidence l'implication de l'UPR dans la régulation du métabolisme des lipides ou dans la biogénèse pariétale du champignon (Guillemette *et al.*, 2007). Très récemment, des travaux sur *A. fumigatus* ont mis en évidence le rôle de *HacA* dans le pouvoir pathogène du champignon. Aucun travail sur l'impact de ce mécanisme de régulation sur la virulence de champignons phytopathogènes n'existe à ce jour.

Plusieurs éléments permettent pourtant de supposer que l'UPR est un mécanisme clé dans le déterminisme du pouvoir pathogène. Le processus infectieux d'un champignon nécessite la synthèse de nombreuses protéines afin de pouvoir croître dans les tissus hôtes. L'UPR joue un rôle majeur dans la régulation de ces mécanismes de sécrétion. Le mutant nul  $\Delta HacA$  chez *A. fumigatus* présente ainsi une très forte diminution de sa capacité à sécréter des protéases (Richie *et al.*, 2009). L'UPR pourrait également jouer un rôle important dans les mécanismes d'adhésion aux tissus hôtes. Chez *C. albicans*, la mutation dans le gène *Hac1* a des répercussions sur la composition pariétale en adhésines et en agglutinines (Wimalasena *et al.*, 2008). Le champignon phytopathogène va également devoir mettre en place de nombreuses protections face aux défenses de la plante hôte. Comme nous l'avons vu dans **l'introduction bibliographique (3.2.3)**, les défenses de la plante hôte nécessitent la mise en place de protection notamment au niveau de l'intégrité cellulaire du pathogène (Rogers *et al.*, 1996, Sellam *et al.*, 2007b). Des travaux ont montré que l'UPR régulerait la biogénèse pariétale (Guillemette *et al.*, 2007, Richie *et al.*, 2009, Wimalasena *et al.*, 2008) et à ce titre pourrait impacter sur la mise en place du mécanisme compensatoire. Néanmoins, à ce jour, aucune donnée mettant en avant le rôle de l'UPR dans la protection d'un champignon phytopathogène face aux molécules de défense d'une plante n'a été clairement établi.

Afin de vérifier l'implication du facteur de transcription *HacA* dans la réponse des champignons aux phytoalexines, une stratégie identique à celle décrite dans le **Chapitre 2** a été entreprise. Le phénotypage du mutant nul  $\Delta Hac1$  chez *S. cerevisiae* en présence de camalexine a été réalisé par turbidimétrie et par dépôts sur boîte de Petri (**Figure 19**). Le phénotypage du mutant de *S. cerevisiae*  $\Delta Hac1$  en présence de camalexine montre une

forte sensibilité de ce mutant à la phytoalexine. Ainsi, l'étude de ce facteur de transcription s'avère très intéressante dans le but de comprendre l'implication de l'UPR dans la réponse d'*A. brassicicola* aux phytoalexines. Le gène *AbHacA*, orthologue du gène *Hac1* de *S. cerevisiae*, a été identifié dans le génome d'*A. brassicicola*

La première étape a été de visualiser l'activation de l'UPR par la camalexine. Comme il a été montré pour les voies de signalisation CWI et HOG où les phytoalexines camalexine et brassinine activent la phosphorylation des MAP kinases Slt2p et Hog1p correspondante, la camalexine active la transcription de *Hac1p*. Même si cette activation est plus tardive que la phosphorylation des MAP kinases (2 après addition de la camalexine), la camalexine va également activer l'épissage de l'ARNm de *AbHac1*. Ainsi, des gènes tel que *BipA* et *PdiA*, gènes codant respectivement des chaperones et foldases, nécessaire durant l'UPR vont être activés par la phytoalexine.

Afin de poursuivre cette étude, deux mutants nuls *AbHacA1* et *AbHacA2* ont été obtenus. Ces deux mutants sont incapables d'initier l'UPR. Ils présentent une hypersensibilité aux stress de sécrétion et des gènes cibles de l'UPR, tels que *PdiA* ou *BipA*, ne sont pas surexprimés chez les mutants en réponse à ces stress.

Les mutants *AbHacA1* sont incapables de provoquer des symptômes *in planta* avec ou sans blessures artificielles que ce soit sur chou ou sur les génotypes Landsberg (Ler), Col-0 et *Pad3* d'*A. thaliana*. Les génotypes Ler et *Pad3* sont pourtant connus pour être très sensible à l'alternariose. Au niveau morphologique, les mutants présentent des hyphes gonflées et des ramifications d'hyphes avortées ainsi qu'une réduction sévère de la vitesse de croissance et de la sporulation sur des milieux empiriques. Cette altération de la croissance *in vitro* ne peut expliquer l'absence de symptômes puisque des observations microscopiques montrent que les taux de germination des conidies *in planta* ainsi que le nombre de pseudo-*apressoria* observés chez les mutants sont quasiment identiques à ceux obtenus avec souche de référence. D'autre part, des tests de pouvoir pathogène sur choux ont été réalisés en présence de sorbitol qui stimule la croissance des mutants. Dans ces conditions, les mutants *AbHacA1* sont toujours incapables de produire des symptômes.

L'impact de la mutation sur la vitesse de croissance est annulé lorsque le champignon est cultivé sur milieu synthétique, ce qui suggère que le mutant présente un défaut dans sa capacité de sécrétion des enzymes lytiques aboutissant à un désavantage dans son mode de vie saprophytique. Cette diminution des capacités de sécrétion des mutants a pu être vérifiée par des dosages des activités protéase et lipase à partir de filtrats de culture. Il semble donc probable que cette altération de la sécrétion soit aussi un handicap pour la colonisation *in planta* du mutant.

De plus, il apparaît que le mutant *AbHacAA* présente une sensibilité accrue aux composés de défense hôtes. Les tests néphélométriques montrent que le mutant est effectivement plus sensible aux phytoalexines, camalexine, brassinine (ou encore resveratrol), mais également à un produit de dégradation des phytoanticipines, l'Allyl-ITC. La mutation dans le facteur de transcription va impacter sur la sensibilité de la souche aux composés à action antifongique. La sensibilité de la souche est alors un phénomène général montrant un impact large de la mutation. Des cultures fongiques réalisées en présence de SYTOX montrent une apparition plus précoce de la fluorescence chez la souche mutante que chez le sauvage en présence de camalexine. Nous émettons l'hypothèse cette hypersensibilité serait due à une altération de la structure pariétale des mutants, ce qui perturberait la mise en place du mécanisme compensatoire face à la camalexine. Cette modification pariétale est nettement visible en microscopie électronique et des dosages des composés pariétaux ont confirmé une nette diminution de la quantité des glucanes pariétaux chez les mutants. Ces résultats sont à mettre en parallèle avec les tests de sensibilité au CalcoFluor White (CFW) et CongoRed (CR). En effet, les mutant ne sont pas sensibles au CFW, composé ciblant la chitine tandis qu'ils sont sensibles au CR, composé ciblant les glucanes (résidu de glucoses) pariétaux. Chez *A. fumigatus*, le mutant *ΔhacA* présente également un défaut dans la fraction glucane qui est associé à une diminution de la résistance aux antifongiques (Richie *et al.*, 2009). Ces observations ont été également répertoriées sur le mutant *ΔDerA* d'*A. fumigatus*, impliqué dans l'ERAD (Richie *et al.*, 2011), mécanisme qui est fortement lié à l'UPR.

L'ensemble de ces données a permis pour la première fois de mettre en évidence l'importance de l'UPR dans le pouvoir pathogène d'un champignon phytopathogène. Il apparaît clairement que cette voie de sécrétion est une nouvelle cible à privilégier dans une stratégie de protection chimique contre les champignons phytopathogènes.



Les résultats de ces travaux sont présentés dans l'article : « Impact of the unfolded protein response on the pathogenicity of the necrotrophic fungus *Alternaria brassicicola* », paru dans *Molecular Microbiology* en 2011.

Un article basé sur les méthodes d'investigation et d'étude de l'UPR chez les champignons filamenteux est présenté dans l'**annexe 1**. Celui-ci, intitulé « Methods for investigating the UPR in filamentous fungi. » est paru en 2011 dans la revue *Methods in enzymology*.

## 2. ARTICLE

### ***Impact of the unfolded protein response on the pathogenicity of the necrotrophic fungus *Alternaria brassicicola****

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# Impact of the unfolded protein response on the pathogenicity of the necrotrophic fungus *Alternaria brassicicola*

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## Summary

The unfolded protein response (UPR) is an important stress signalling pathway involved in the cellular development and environmental adaptation of fungi. We investigated the importance of the UPR pathway in the pathogenicity of the plant necrotrophic fungus *Alternaria brassicicola*, which causes black spot disease on a wide range of *Brassicaceae*. We identified the *AbHacA* gene encoding the major UPR transcription regulator in *A. brassicicola*. Deletion of *AbHacA* prevented induction of the UPR in response to endoplasmic reticulum stress. Loss of UPR in mutants resulted in a complete loss of virulence and was also associated with a cell wall defect and a reduced capacity for secretion. In addition, our results showed that the UPR was triggered by treatment of mycelia with camalexin, i.e. the major *Arabidopsis thaliana* phytoalexin, and that strains lacking functional *AbHacA* exhibited increased *in vitro* susceptibility to antimicrobial plant metabolites. We

hypothesize that the UPR plays a major role in fungal virulence by altering cell protection against host metabolites and by reducing the ability of the fungus to assimilate nutrients required for growth in the host environment. This study suggests that targeting the UPR pathway would be an effective plant disease control strategy.

## Introduction

The endoplasmic reticulum (ER) orchestrates the processing of nearly all proteins that reside in, or pass through, the endomembrane system of eukaryotic cells. A variety of adverse physiological and environmental conditions can disturb protein folding and lead to the accumulation of misfolded proteins. The ER has evolved a highly specific signalling pathway, termed the unfolded protein response (UPR), to ensure that its protein-folding capacity is not overwhelmed (Schroder and Kaufman, 2005). Upon activation, the UPR restores ER homeostasis by increasing the protein-folding capacity, reducing the amount of new proteins translocated into the ER lumen and increasing retrotranslocation and degradation of ER-localized proteins.

During recent years, a considerable effort has been devoted to identifying components of the fungal UPR pathway, mainly with the aim of overcoming the bottlenecks to achieving industrial production of heterologous proteins (Conesa *et al.*, 2001; Archer and Turner, 2006; Lubertozzi and Keasling, 2009). In *Saccharomyces cerevisiae*, the basic leucine zipper (bZIP)-type transcription factor Hac1p is the major transcriptional regulator of the UPR. Hac1p synthesis is dependent on splicing of an unconventional intron in the *hac1* mRNA promoted by the ER-located transmembrane protein Ire1p (Ruegsegger *et al.*, 2001). This splicing event is activated in response to ER stress and overcomes a translational block, allowing for the translation of Hac1p that regulates the expression of UPR target genes. Most of the key elements constituting the signal transduction pathway of the yeast UPR have been identified in filamentous fungi and the central activation mechanism of the pathway, i.e. the stress-induced splicing of an unconventional intron, is conserved among filamentous fungi, yeast and even mammalian cells (Kohno, 2010). The predicted proteins

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encoded by most of the fungal UPR upregulated genes functioned as part of the secretory system including chaperones, foldases, glycosylation enzymes, vesicle transport proteins and ER-associated degradation proteins. Several upregulated genes were also involved in cell wall biogenesis (Travers *et al.*, 2000; Guillemette *et al.*, 2007; Wimalasena *et al.*, 2008). Among the repressed genes, several encode cell surface or extracellular proteins (Kimata *et al.*, 2006). In agreement with this result, a reduced capacity for protease secretion has been reported in an *Aspergillus fumigatus* strain lacking functional HacA (Richie *et al.*, 2009).

In fungal cells, loss of the UPR results in considerable morphological and biological changes. Hac1p impacts *Candida albicans* morphology, revealing a general reduction in polarized growth in *HAC1/HAC1* cells (Wimalasena *et al.*, 2008). In *A. fumigatus*, loss of *hacA* was found to be associated with impaired growth in complex substrate, attenuated virulence in mice and increased susceptibility to antifungal drugs used for the treatment of aspergillosis (Richie *et al.*, 2009). In both mutants, *hac* inactivation jeopardizes the integrity of the cell wall as revealed by an increased vulnerability to cell wall stress agents. In the blast fungus *Magnaporthe oryzae*, Yi *et al.* (2009) showed that the ER chaperone LHS1 and proper ER function play a role in asexual development and biotrophic invasion in susceptible rice. These results highlight the importance of ER homeostasis on different crucial aspects of the fungal life cycle and demonstrate that the UPR is a versatile and sensitive regulatory mechanism required for environmental adaptation of fungi.

*Alternaria brassicicola*, which causes black spot disease, is an economically important seed-borne fungal pathogen of *Brassicaceae* species. During host infection, *A. brassicicola* is exposed to high levels of defence compounds, such as phytoalexins and glucosinolate breakdown products, and the ability to overcome these antimicrobial metabolites is a key factor determining fungal virulence. Chemical defence systems have been well documented in the brassicaceous plant *Arabidopsis thaliana* and challenges with *A. brassicicola* constitute a model interaction for studying necrotrophic fungal pathogenesis and defence signalling pathways. The indolic secondary metabolite camalexin (3-thiazol-2'-yl-indole) is the major phytoalexin synthesized by this plant species and some other brassicaceous weeds (Glawischnig, 2007). Several *A. thaliana* mutants have been found in which camalexin production is attenuated and these alterations are correlated with the outcome of infection. For instance, mutation in the *PAD3* gene, encoding a plant cytochrome P450, abolishes camalexin production (Glazebrook and Ausubel, 1994) and results in enhanced susceptibility to necrotrophic fungi (Thomma *et al.*, 1999; Stefanato *et al.*, 2009). Moreover, resistance to *A. brassicicola* and *Botry-*

*tis cinerea* was shown to be correlated with high levels of camalexin in somatic hybrids of *Brassica oleracea* (Sigareva and Earl, 1999) or in plants overexpressing camalexin after UV treatment (Stefanato *et al.*, 2009).

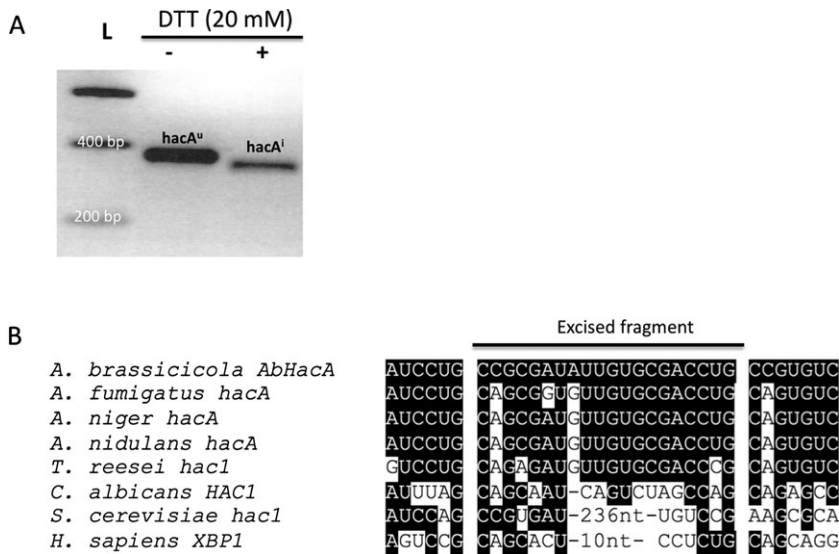
Sellam *et al.* (2007a) showed that camalexin probably causes damage to fungal membranes and activates a compensatory mechanism in fungal cells aimed at preserving membrane integrity. Transcriptional analysis also suggested that *A. brassicicola* is protected against camalexin via mechanisms, such as drug efflux and cell wall reinforcement, that limit intracellular accumulation of the metabolite. As in *A. brassicicola*, exposure to camalexin induces expression of genes encoding several transporters in *B. cinerea*. At least one of them, i.e. an ABC transporter called BcatrB, appears to be involved in camalexin export (Stefanato *et al.*, 2009). *A. brassicicola* also reacts to camalexin by upregulation of several genes involved in melanin biosynthesis and cell wall maintenance (Sellam *et al.*, 2007a). The authors suggested that the induction of these genes probably occurred in an attempt to decrease cell wall permeability and prevent intracellular accumulation of the antifungal metabolite.

In this study, we investigated the importance of the UPR pathway with respect to the pathogenicity of the plant necrotrophic fungus *A. brassicicola*. We identified the gene *AbHacA* that encodes the orthologue of the yeast Hac1p transcription factor in *A. brassicicola*, and generated deletion mutants unable to activate the UPR under ER stress. Deletion of *AbHacA* resulted in complete loss of virulence, which could be explained by an increased susceptibility to plant defence metabolites and a reduced ability to acquire nutrients from host tissues.

## Results

### *Isolation and sequence analysis of the AbHacA gene in A. brassicicola*

A presumptive *AbHacA* locus was identified in the unannotated *A. brassicicola* genome assembly (<http://genome.wustl.edu/>) via homology with *hacA* genes previously described in other filamentous fungi (Saloheimo *et al.*, 2003; Mulder *et al.*, 2004). In other fungal systems, an unconventional splicing event of the *hacA* mRNA occurs under ER stress, allowing for the translation of the transcription factor and the activation of the UPR. We examined splicing of the *AbHacA* transcript by performing RT-PCR from RNAs derived from cultures grown in the presence or absence of dithiothreitol (DTT)-induced ER stress. A comparison of the cDNA sequences with the *A. brassicicola* genome revealed an atypical 20 bp intron that was excised following ER stress. RT-PCR, with primers designed across the intron (Table S1), clearly showed an increase in the abundance of a shorter product



**Fig. 1.** Identification of the *AbHacA* unconventional intron.

A. RT-PCR analysis of *AbHacA* processing with primers (*AbHacAF1/AbHacAR1* in Table S1) designed across the intron in the presence (+) or absence (-) of 20 mM DTT for 1 h.

B. Alignment of the mRNA sequence surrounding the *hacA* atypical intron in various filamentous and unicellular fungi and in mammals. Black boxes denote identical nucleic acids.

following ER stress, indicating that DTT induced conversion of the unspliced *hacA* mRNA (*hacA<sup>u</sup>*) into the spliced form (*hacA<sup>s</sup>*) (Fig. 1A). This atypical intron was similar in size to other unconventional *hacA* introns from fungi and had highly conserved border sequences (Fig. 1B). A common feature also found in other fungi was the inability to clearly determine the exact splicing site by comparing cDNA and genomic sequences, because of the presence of a CTGCCG sequence on both sides of the intron. Moreover, the intron region and its flanking sequences were located in a region of strong predicted RNA secondary structure (organized in two loops of 7 nt each, data not shown), similar to those predicted in yeast *hac1* and *Aspergillus hacA* (Mulder *et al.*, 2004; Richie *et al.*, 2009).

The *hacA<sup>u</sup>* mRNA contained an ORF coding for a 423-amino-acid protein. The unconventional splicing event changed the reading frame, resulting in modification of the C-terminal amino acid sequence of the *AbHacA* protein. The first 236 amino acids encoded by both spliced and unspliced mRNAs were identical and shared a leucine zipper dimerization motif adjacent to a basic DNA binding domain (Fig. 2). Splicing replaced a portion coding for 187 amino acids with a different sequence coding for 113 amino acids. As already reported in *HacA* orthologues, the translated sequence displayed low sequence similarity to other fungal *HacA* protein sequences except in the DNA binding domain (Wimalasena *et al.*, 2008). The resulting *AbHacA<sup>s</sup>* protein had 32% and 26% identity to the corresponding proteins described in the *Aspergillus* genus and in *Trichoderma reesei*. A lower degree of identity was obtained by alignment with orthologues from yeasts (23% and 14% in *S. cerevisiae* and *C. albicans* respectively).

We determined that *AbHacA* was the orthologue of *S. cerevisiae* *hac1* by performing complementation tests using a *S. cerevisiae* *hac1* null mutant strain. Comple-

mentary DNAs corresponding to the unspliced and spliced forms of *A. brassicicola* *AbHacA* were cloned into the yeast expression vector pYESCT to generate pYAbHacA<sup>u</sup> and pYAbHacA<sup>s</sup> respectively. As already reported, the  $\Delta$ *hac1* strain was hypersensitive to DTT as compared with the isogenic wild-type parental strain. This phenotype was partially suppressed in a null strain expressing the spliced *AbHacA* sequence, but not in a null strain expressing the unspliced *AbHacA* sequence (Fig. 3).

#### Generation of *AbHacA* gene replacement mutants

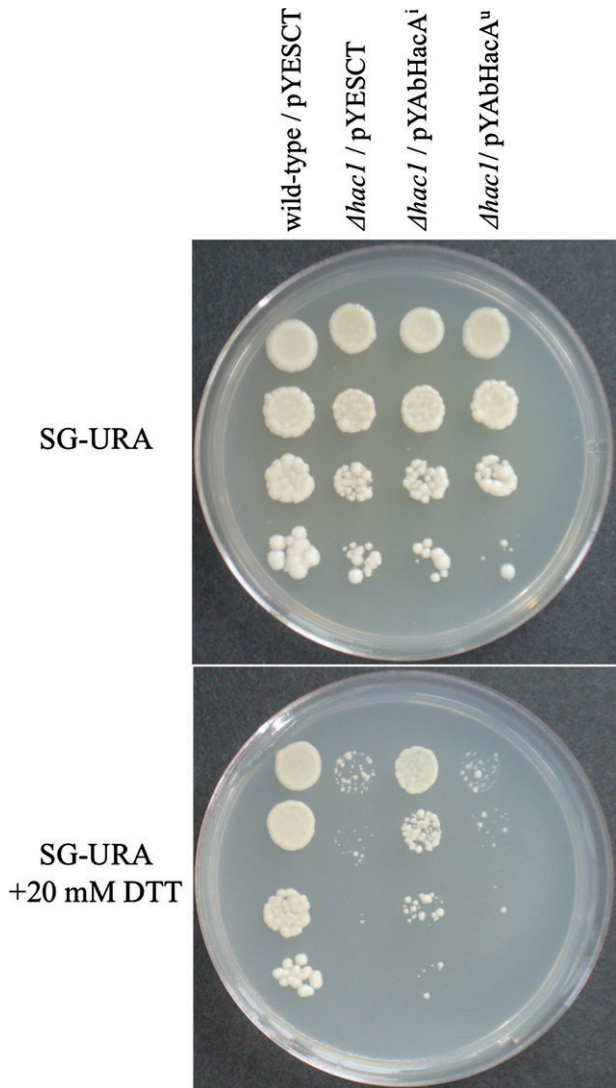
Deletion of *AbHacA* in *A. brassicicola* was accomplished by replacing the *AbHacA* ORF with a hygromycin B (Hyg B) resistance cassette (Fig. 4A). Two Hyg B-resistant transformants were generated ( $\Delta$ 1*abhaca* and  $\Delta$ 2*abhaca*) after transformation of protoplasts of the wild-type strain, regeneration and purification by single-spore isolation. A PCR screen, carried out using primers homologous to the hygromycin resistance cassette and genomic sequence outside the flank regions, confirmed that integration of the replacement constructs occurred by homologous recombination at the targeted loci for all of these mutants (Fig. 4B). Complete deletion of the coding region in the transformants was further confirmed using internal primers. No ectopic transformant was obtained, thus confirming the high efficiency of homologous recombination in *A. brassicicola* as already pointed out by Cho *et al.* (2009). Genomic Southern blot genotyping confirmed single-copy deletion of the *AbHacA* gene in both clones that were used for further phenotypic analysis (Fig. 4C). Complementation assays were performed to confirm that the observed phenotypes of the mutants were indeed the result of the deletion of *AbHacA*. However, despite many

<i>A. fumigatus</i>	-----MEDNFASVVESLSGTSAP-----ALPL	22
<i>T. reesei</i>	MAFQOSSPLVKFEASPAESFLSAPGDNFTSLFADSTPSTLNPRDMMTPDSVADIDSRLSV	60
<i>A. brassicicola</i>	-----MAQYTFDSSLFSQTMNNPAMTSTPLPPPSVEALPTTIKMEMIENYDH	47
<i>C. albicans</i>	-----MELTVDNNTTNSN-----IDDL	19
	. . .	
<i>A. fumigatus</i>	LTVSPADTSLKAPETKVQETKTEEEKPPKRRKSWGQELPIPKTNLPPRKRKRAKTEDEKEQR	82
<i>T. reesei</i>	IPESQDAEDDESHSTSATAPSTSEKKPVKRRKSWGQVLPEPKTNLPPRKRKRAKTEDEKEQR	120
<i>A. brassicicola</i>	SPSPAHSSTSSSEASHTPAPESNGSKPVKRRKSWGQVLPEPKTSLPPRKRKRAKTEDEKEQR	107
<i>C. albicans</i>	ATPTSILMTSTTTSPSMSTSTSSHSN-----TLDIDPATFKSTLPPRKRKRAKTEDEKEQR	72
	. . . : : . . . : . . . * : ***** : *****	
<i>A. fumigatus</i>	RIERVLRNRAAAQTSRERKRLEMEKLENEK-----IQMEQQNQFLQRLSQMEAENNR	135
<i>T. reesei</i>	RVERVLRNRAAQSSRERKRLEVEALEKRKKELETLLINVQKTNLILVEELNRFRRSSGV	180
<i>A. brassicicola</i>	RIERVKRNRLLAAHNSRERKRQEYEVLTQTEK-----ELEANMQAYKQKMAEMEAELRY	160
<i>C. albicans</i>	KIERILRNRAAAHASREKRRKHEVYLENYVVKLETNLMKLNNNYNQAFELLTKDNQELL	132
	: : * : * * : * * : * * . * * : . . . : : . . .	
<i>A. fumigatus</i>	LS-----QQLAQLTAEVRNSRNSTPKPGSPATASP	165
<i>T. reesei</i>	VTRSSSPDLSLQDSITLSQQLFGSRDQGTMSNPEQSLMDQIMRSAANPTVNPASLSPSLP	240
<i>A. brassicicola</i>	YR-----SKYPGEAPQRFVDFLATPSSDITLDTICP	189
<i>C. albicans</i>	SK-----LEVLDVSDLKEQIHSNMSGTRRSHNKKSNDE	166
	. . . : * . . .	
<i>A. fumigatus</i>	TLTPTLFKQ-----EGDELPLERIPFPTPSITDYSPTLKPSLAES--SDVTQHPAVSV	217
<i>T. reesei</i>	PISDKFQTKEEDEEQADEDEEMEQTWHETKEAAAAKEKNSKQSRVS--TDSTQRPVAVSI	298
<i>A. brassicicola</i>	AQIPTSFPS-----PPESMSMDSRPDSSCQPETPPSSFEASPEFDSTQYPVAVSV	239
<i>C. albicans</i>	DIEEEDDEDEHQEEGHVEKQEIKEEPVSKRKLNTKTKSKTKTKSSNKTMTSTPPSSVSS	226
	. . . . * . : **	
<i>A. fumigatus</i>	G-----GLEGPGSALPLFDLGSVGHDAANDIAAPLSDDDFHRLFNGD	260
<i>T. reesei</i>	GGDAAVPVFSDDAGANCLGLDPVHQDDGPFPSIGHSFGLSALDADRYLLESQLLASPNAS	358
<i>A. brassicicola</i>	DFG-----NFSTGDLGLPFSVQPFAPVDEKSVVMENFFDFEPFPK	279
<i>C. albicans</i>	LSP-----DVTNFGTNTTMSPIQIKKEFNIDNIFIKKEFSQSPLQP	269
	: . . : . . .	
<i>A. fumigatus</i>	SSTEPDSS-----VIEDGFSDILD--SGDLSAFPFDMSVNFSDSEPVALEGIEPAH	309
<i>T. reesei</i>	TVDDDYLAGDSAACFTNPLPSDYDFDINDFLTDDANHAAYDIVAASNYAAADRELDLEIH	418
<i>A. brassicicola</i>	CSPTTAP-----MEPAFMSVD-----SSSIDVFGPTPFHDSSFSNSY	317
<i>C. albicans</i>	THQPEQQE-----QQHEIYLKS----ESKDAFWNYPSPLSFHDSPLQIDIDTS	313
	: : . . . .	
<i>A. fumigatus</i>	GLPNETPYQTSGLQPSLGASTSRCDGQGIAGC-----	342
<i>T. reesei</i>	DPENQIPSRHSIQQPQSGASSHGCDGGIAGV-----	451
<i>A. brassicicola</i>	GFLDGFDAKFNDLQASAGATS--VSDEALAAGQL-----	349
<i>C. albicans</i>	SSSSTSPSSSSSLISSSGPTNHSIADLAEPFIESFLQDFDFFSILGDDVEVHKV	367
	. . . * : . . . ↑	

**Fig. 2.** Alignment of amino acid sequences of the induced forms of HacA from *A. fumigatus*, *T. reesei*, *A. brassicicola* and *C. albicans*. The underlined sequence represents the predicted DNA binding domain area. The symbols ↓ and ↑ indicate positions of the unconventional splice site in *A. brassicicola*, *A. fumigatus*, *T. reesei* and *C. albicans* respectively. The following symbols denote the degree of conservation: '\*' means that the residues or nucleotides in that column are identical in all sequences in the alignment, ':' means that conserved substitutions were observed, '.' means that semi-conserved substitutions were observed.

attempts and several modifications of the standard procedure used to generate competent protoplasts, no complemented transformants were obtained, probably because of the low ability to maintain cellular integrity throughout the protoplasting process (Table S2).

Microscopic observations revealed that  $\Delta abhacA$  mutants displayed abnormal hyphal morphology on standard potato dextrose agar (PDA) medium with enlarged and swollen cells (Fig. 5A). We also noticed the presence of structures that might be abortive branching. Deletion of



**Fig. 3.** Complementation of the yeast  $\Delta hac1$  mutant with *AbHacA* coding sequence. Identical volumes of 10-fold serial dilutions ( $10^{-1}$ – $10^{-4}$ ) of exponentially growing *S. cerevisiae* (parental wild-type strain and deletion mutants) cells transformed with the empty vector pYESCT or recombinant vector pYAbHacA [expressing the spliced (pYAbHacA<sup>i</sup>) or unspliced (pYAbHacA<sup>u</sup>) forms] were spotted onto SG plates complemented or not with 10 mM DTT. Colony growth was observed after 48 h incubation at 30°C.

*AbHacA* dramatically reduced the growth rates and conidiation on PDA plates at 24°C (Fig. 5B). On synthetic Vogel's medium N, the growth of the *AbHacA* null strains was similar to that of the wild-type, although the growth rates were markedly reduced as compared with those observed on PDA. This point will be further discussed in the section dealing with the reduced secretory capacity of the mutants. We also noticed that conidiation was partially restored on Vogel's medium N. The kinetics of germination of wild-type and mutant strains were compared on a glass surface in potato dextrose broth (PDB) (Fig. 5C).

Wild-type conidia were almost fully germinated after 15 h, while only 20% of  $\Delta abhaca$  conidia were germinated.  $\Delta abhaca$  conidia germination rates reached wild-type levels after 24 h.

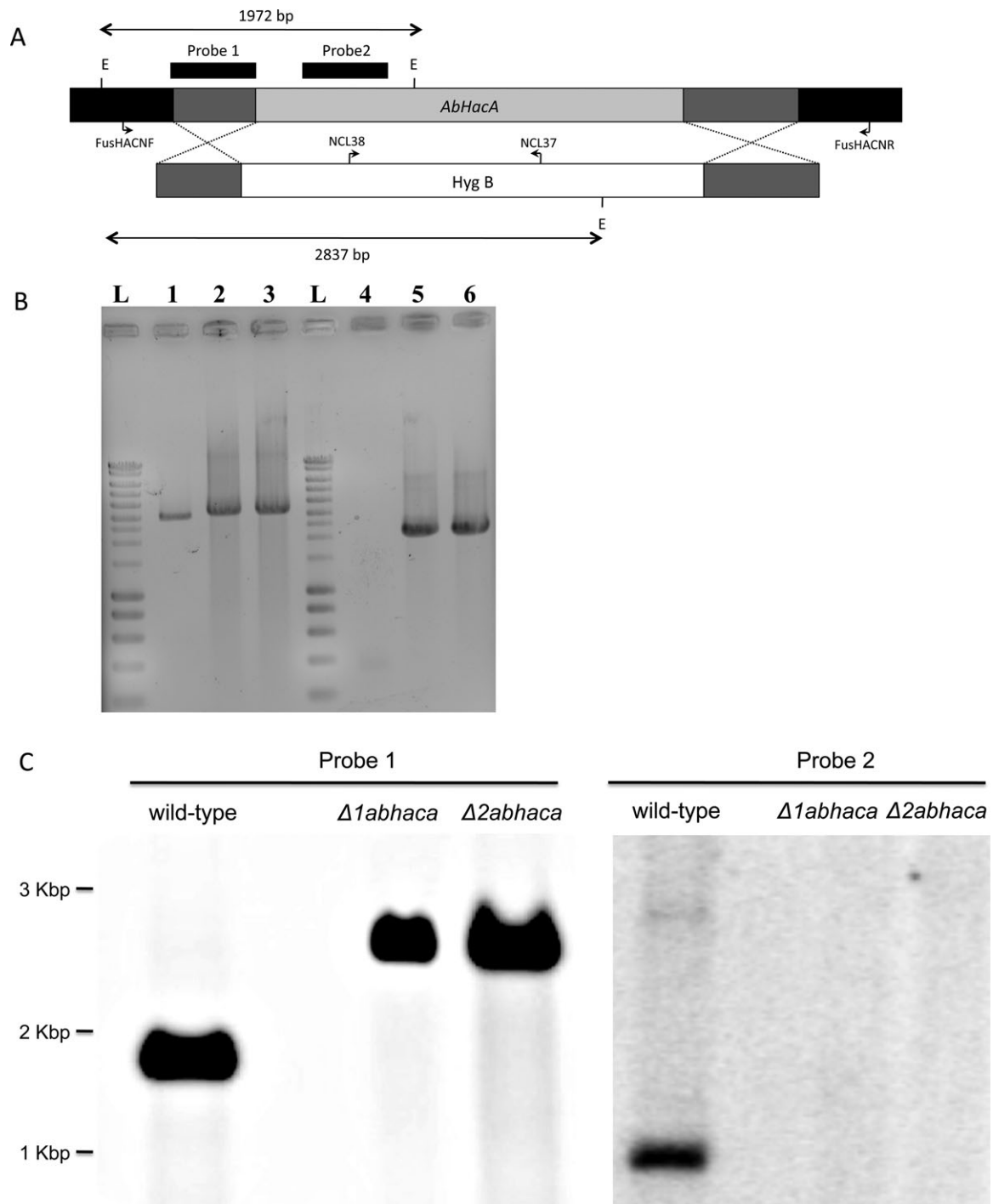
#### *AbHacA* mediates the UPR and secretion stress response in *A. brassicicola*

To determine whether the loss of *AbHacA* was efficient for disrupting UPR signalling in *A. brassicicola*, we treated mutant and wild-type strains with DTT and measured the fold change in the expression of two well-known UPR target genes compared with an untreated control. Each of these genes was identified by a homology search against the *A. brassicicola* genome database with the *A. niger bipA* (encoding an ER chaperone) and *pdiA* (encoding a protein disulfide isomerase). We previously showed that DTT treatment induced the conversion of the unspliced *AbHacA* mRNA into the spliced form, indicating activation of the UPR under this condition. As anticipated, UPR targets showed strong induction in wild-type *A. brassicicola* under DTT stress (Fig. 6A). In contrast to wild-type strain, the mutant strains were unable to increase the level of UPR target genes when treated with DTT, indicating a defect in UPR signalling in *AbHacA* null strains.

The effects of UPR disruption were analysed by monitoring the growth of transformants and wild-type strain following ER stress. Growth was recorded with a nephelometric reader for 30 h in conidial suspensions inoculated in microplate wells containing standard PDB medium (Joubert *et al.*, 2010a). Disruption of ER homeostasis was triggered by exposure to chemical agents, DTT and brefeldin A (BFA), which interfere with ER folding by different mechanisms and that are known to induce the UPR. Nephelometric monitoring confirmed that, even at early steps of the growth kinetics,  $\Delta abhaca$  mutants were growth-impaired in PDB (Fig. 6B). As expected, we also observed that *AbHacA* null strains were growth-impaired in the presence of concentrations of DTT and BFA that could be tolerated by the wild-type strain, indicating that *AbHacA* inactivation increased the sensitivity of *A. brassicicola* cells to these treatments.

#### The UPR is required for virulence in *A. brassicicola*

Virulence of the wild-type and  $\Delta abhaca$  mutants were compared on *B. oleracea* and *A. thaliana* (ecotype Col-0) host plants by inoculation experiments on leaves (Fig. 7A). On intact leaves of *B. oleracea* inoculated with the wild-type strain, small necrotic symptoms were already observed at 3 dpi that continued to expand into large typical necrotic areas surrounded by chlorotic halos at 6 dpi. During late stages of infection (9 dpi), necrotic



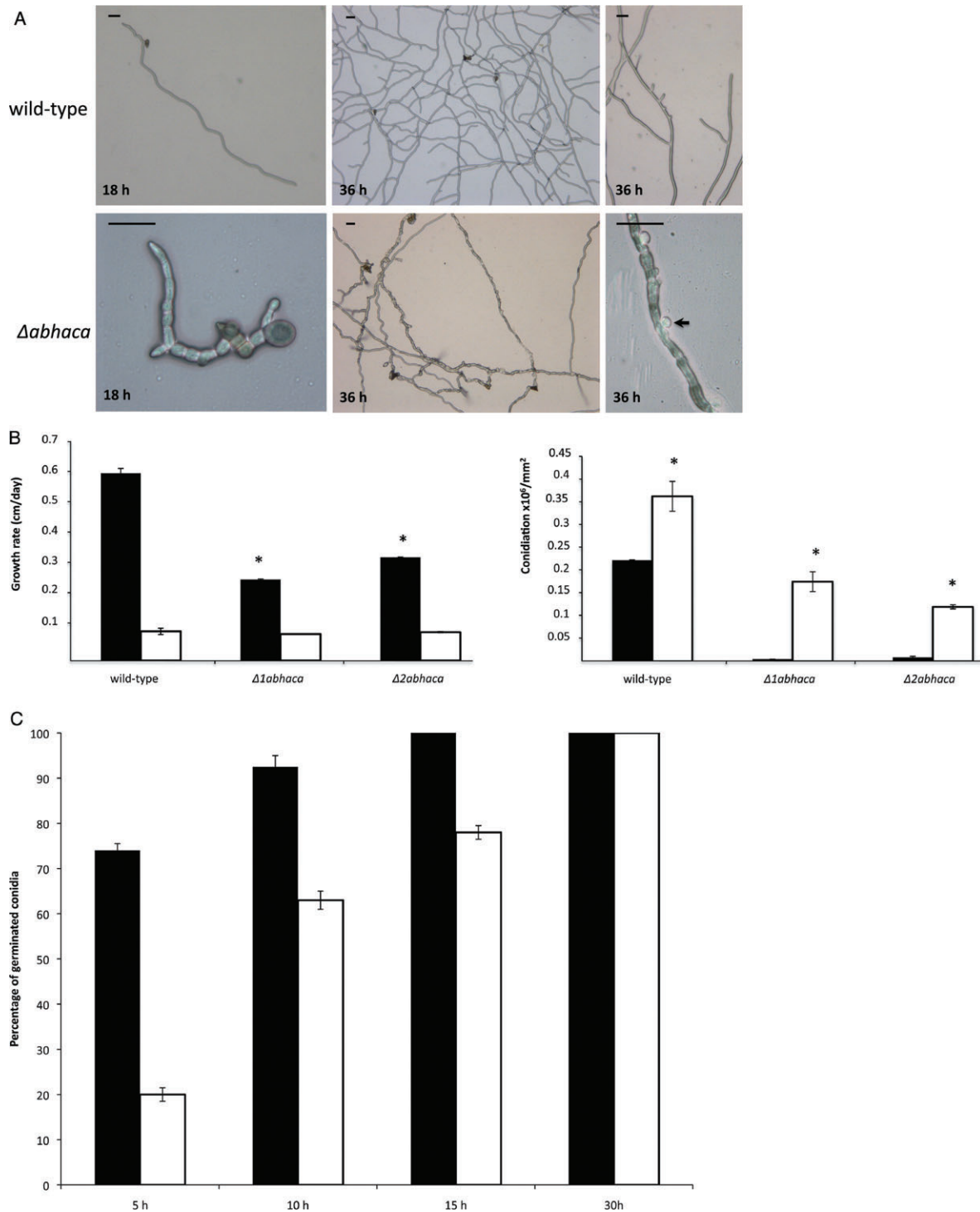
**Fig. 4.** Generation of  $\Delta abhaca$  by homologous recombination.

A. Schematic representation of the *AbHacA* locus (grey box), replacement constructs with the Hyg B resistance cassette (white box) and flanking sequences (dark grey box). E represents the position of EcoRI sites and arrows indicate the position of primers used for PCR screening of deleted mutants.

B. Gel electrophoresis of PCR products obtained from template DNA of the wild-type strain (lanes 1 and 4),  $\Delta 1abhaca$  (lanes 2 and 5) or  $\Delta 2abhaca$  (lanes 3 and 6). PCR products were amplified with primers designed at the 5' and 3' ends of the coding region (FusHACNF/FusHACNR, lanes 1–3) or inside the Hyg B sequence (FusHacANF/NCL37, lanes 4–6). Molecular sizes (kb) were estimated based on 1 kb ladder (lane L, Eurogentec, Seraing, Belgium).

C. Southern hybridization of genomic DNA from wild-type isolates and transformants. Each DNA was digested by EcoRI and the blots were probed with  $^{32}\text{P}$ -labelled PCR fragments (shown by boxes in A).



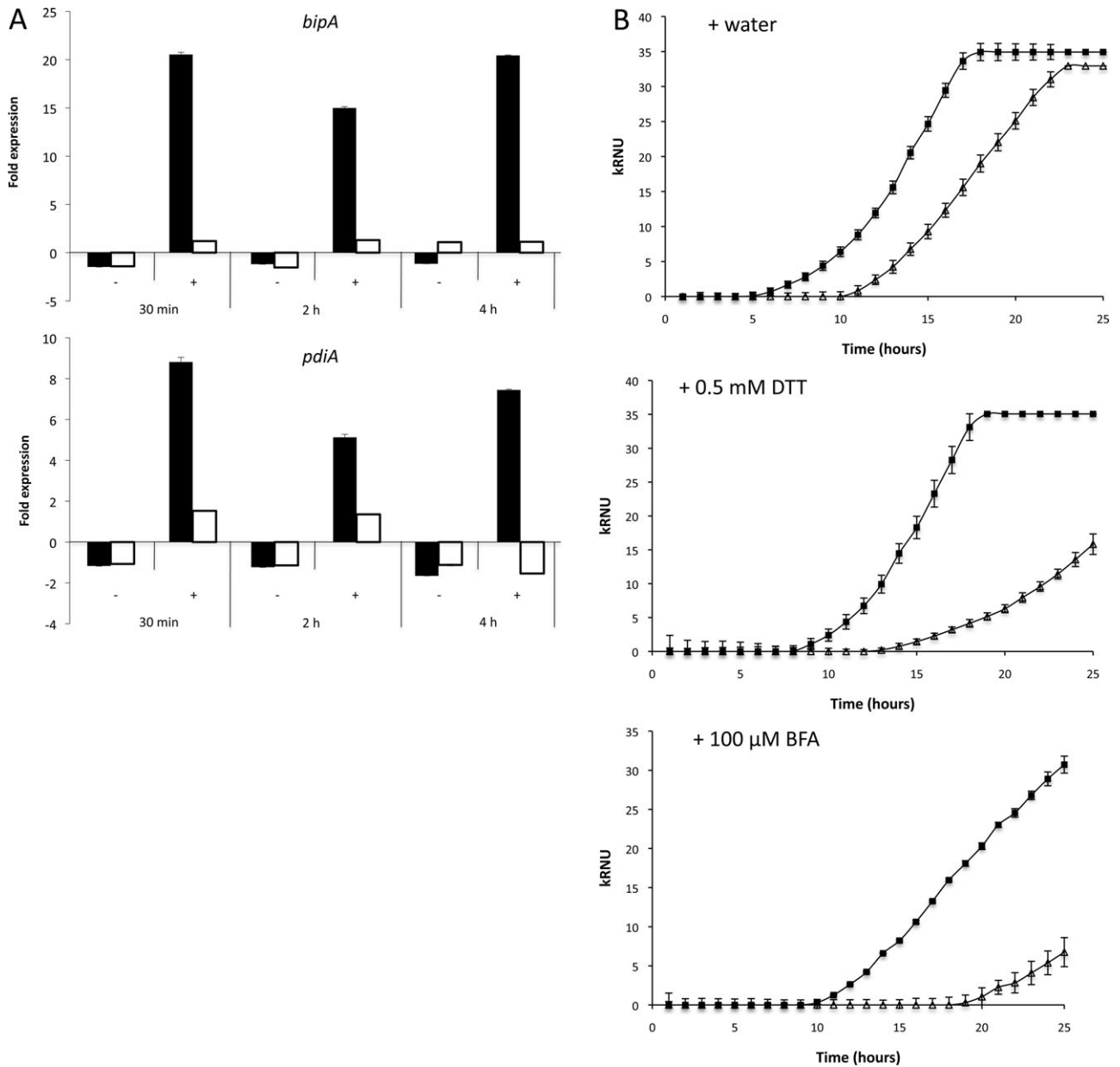


**Fig. 5.** Microscopic and growth phenotypes of  $\Delta abhaca$  strains.

A. Microscope images of wild-type and  $\Delta abhaca$  strains growing on PDA medium. Incubation times are indicated at the bottom left-hand corner of each image. Black arrows indicate potential abortive branches. The scale bars indicate 20  $\mu\text{m}$ .

B. Effect of the medium composition on the growth and conidiation of wild-type and *AbHacA* isolates. Growth rates and conidiation of the wild-type strain and *AbHacA* mutants were determined at 24°C on PDA solid medium (black bars) and Vogel's medium N supplemented with glucose (2%) (white bars). The data are the mean of three independent experiments. Asterisks indicate a significant difference between the mutant and the parental isolate (Student test,  $P < 0.01$ ).

C. Time-course of germination of wild-type (black bars) and  $\Delta 1abhaca$  strains (white bars). Conidial germinations were monitored on a glass surface cover with PDB. The data are the mean of three independent experiments. Asterisks indicate a significant difference between the mutant and the parental isolate (Student test,  $P < 0.01$ ).



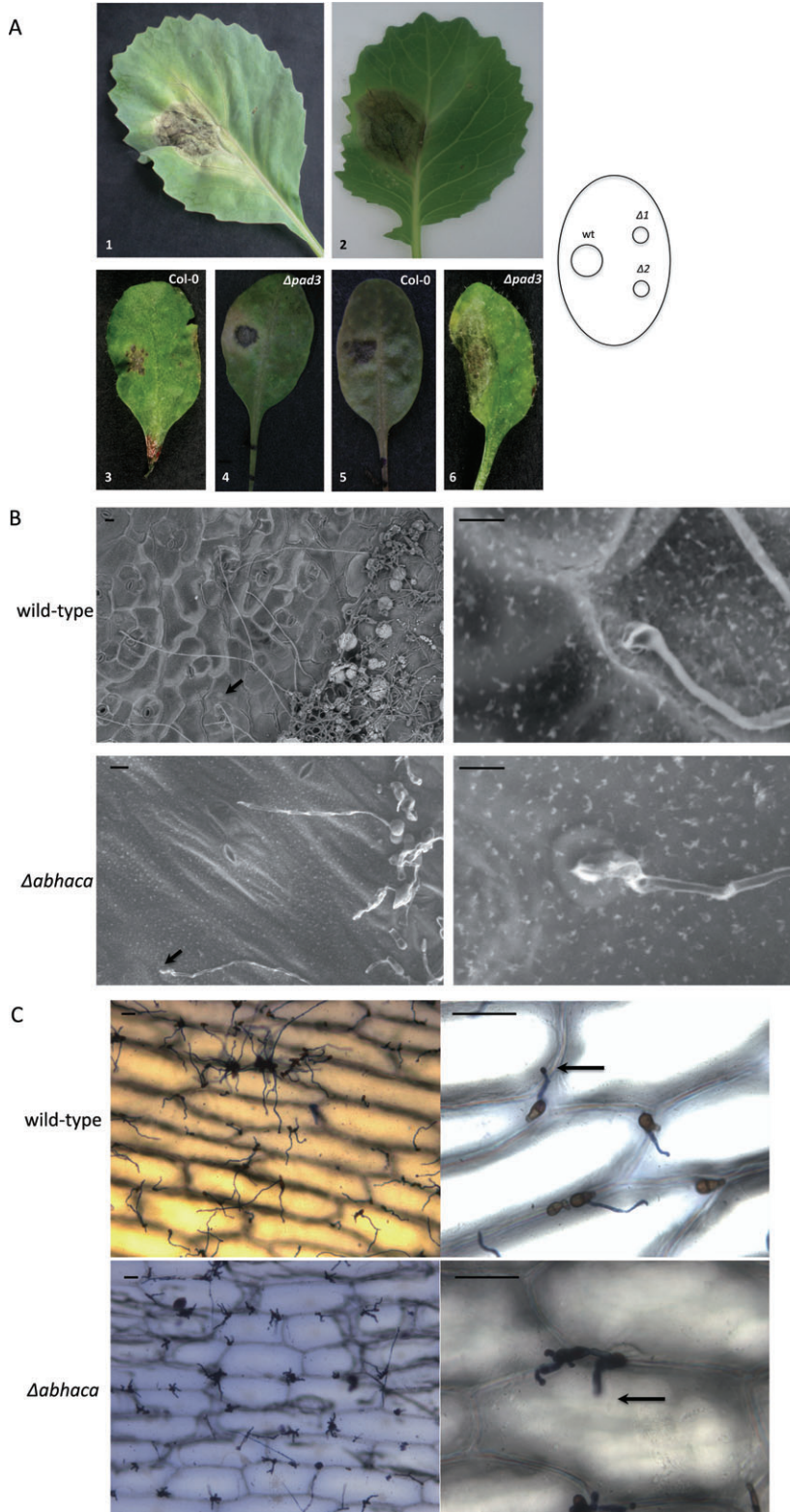
**Fig. 6.** Loss of AbHacA disrupts UPR signalling in *A. brassicicola*.

A. Quantitative RT-PCR results for the expression of two UPR target genes (*bipA* and *pdiA*) in wild-type (black bars) and  $\Delta 1abhaca$  (white bars) strains in the presence (+) or absence (-) of DTT (1 mM) for 0.5, 2 and 4 h. For each gene, induction of expression is represented as a ratio (fold expression) of its relative expression (studied gene transcript abundance/actin transcript abundance) in each inductive condition to its relative expression in the corresponding control. The data are the mean of three repetitions.

B. Nephelometric monitoring of growth of wild-type and  $\Delta 1abhaca$  strains in the presence of ER stress inducers. Conidia from *Abr43* wild-type (black squares) and  $\Delta 1abhaca$  (open triangles) were used to inoculate microplate wells containing standard PDB medium that was supplemented or not with 100  $\mu$ M BFA or 0.5 mM DTT. Growth was automatically recorded for 25 h at 24°C using a nephelometric reader (see *Experimental procedures*). Each genotype was analysed in triplicate and the experiments were repeated three times per growth condition. For clarity, we only present the results obtained with the  $\Delta 1abhaca$  strain, but similar results were obtained with  $\Delta 2abhaca$  strain.

spots exhibited a dense formation of conidia on the surface. When challenged with the same wild-type strain, Col-0 plants showed only small necrotic lesions at 6 dpi. By contrast, the  $\Delta abhaca$  mutants did not cause noticeable disease symptoms on healthy leaves of *A. thaliana* and *B. oleracea*. As few as 50 wild-type conidia in a water

drop were sufficient to cause blackspot symptoms on cabbage leaves (data not shown), while inoculations performed with up to 2500 times more  $\Delta abhaca$  conidia failed to induce typical symptoms. The lack of disease symptom development after inoculation of  $\Delta abhaca$  on intact leaves was also observed on mechanically wounded leaves



**Fig. 7.** Effects of targeted gene knockout in pathogenicity.

**A.** Effects of *AbHacA* replacement on the virulence of *A. brassicicola* on *B. oleracea* and *A. thaliana*. Leaves of *B. oleracea* (1, 2) and *A. thaliana* Col-0 (3, 5) and *pad3* mutants (4, 6) were inoculated with conidia suspensions of wild-type and *AbHacA* strains with (2, 5 and 6) or without (1, 3 and 4) artificial lesions. Both mutants were tested on (right part of the central vein) and compared on the same leaf with the parental strain (left part of the central vein). Symptoms were observed at 6 dpi.

**B.** Microscopic observations of the infection structures on *B. oleracea* leaf surfaces. Leaf fragments inoculated with *A. brassicicola* wild-type strain and  $\Delta abhaca$  mutants were collected at 24 h dpi and directly imaged with an environmental scanning electron microscope in their natural state without modification or preparation. Appressoria-like structures, indicated by arrows in the left panels, are enlarged in the right panels. Scale bars = 20  $\mu$ m.

**C.** Germination and early-stage infection on onion epidermal cell layers. Hyphae were stained with Trypan Blue after 24 h of incubation. Appressoria-like structures, indicated by arrows, were differentiated by the two genotypes. Scale bars = 10  $\mu$ m.

**Table 1.** *In vitro* effects of cell wall-perturbing agents on radial growth rates of *A. brassicicola* wild-type and both *AbHacA* mutants.

	Wild-type	$\Delta 1abhaca$	$\Delta 2abhaca$
CFW (200 mg l <sup>-1</sup> )	61 ± 3	60 ± 2	66 ± 2
CR (200 mg l <sup>-1</sup> )	17 ± 2	42 ± 1*	37 ± 3*
Caffeine (1 mM)	0 ± 1	27 ± 2*	33 ± 3*
Sorbitol (0.4 M)	5	0 (+23%)	0 (+26%)
Sorbitol (0.8 M)	28	0 (+28%)	0 (+31%)

Values are means of three biological repetitions and represent the percentage growth inhibition under stress conditions compared with standard growth conditions (PDA medium without additive). Asterisks indicate a significant difference between the mutant and the Abra43 wild-type parental isolate (Student test,  $P < 0.01$ ).

(Fig. 7A) or when the conidial drop was supplemented with 0.8 M sorbitol (data not shown), which stimulated mutants growth (Table 1). As, in *Arabidopsis*, resistance to *A. brassicicola* relies on the capability to produce camalexin (Thomma *et al.*, 1999), inoculation experiments were also performed on wounded leaves of the *pad3* mutant (camalexin non-producing genotype) (Fig. 7A). In agreement with previous studies, more severe lesions were produced on this genotype in comparison with Col-0 when inoculated with the parental strain. By contrast, the  $\Delta abhaca$  mutants still failed to produce any symptoms when inoculated on wounded *pad3* leaves.

At 24 h after inoculation of intact leaves, plant samples were collected, stained with Solophenyl Flavine and observed using fluorescent or light microscopy. As shown in Fig. S1, both the wild-type and mutant strains were able to produce characteristic appressoria-like structures at the tips of germ tubes that were in contact with the leaf epidermis. Observations of unstained plant samples using environmental scanning electron microscopy (SEM) revealed that the appressoria-like structures differentiated by both fungal genotypes had similar morphology (Fig. 7B). For a more in-depth analysis of germination and early-stage infection (amount of germlings and penetration structures), conidia were placed onto onion epidermal layers and tissues were stained with Trypan Blue after 24 h of incubation. Both wild-type and  $\Delta abhaca$  conidia germinated with similar efficiency on the epidermal surface (90%) and produced similar amounts of appressorial structures with swollen tips (35%) (Fig. 7C).

#### The UPR is required for proper cell wall construction

The growth rates of  $\Delta abhaca$  mutants were enhanced by osmotic stabilization of the medium with sorbitol, suggesting that their impaired growth on PDA plates was partly due to a cell wall defect (Table 1). This is consistent with growth phenotypes obtained in the presence of cell wall stress-inducing compounds such as Congo Red

(CR) and caffeine. Our results showed indeed that, although the wild-type strain was sensitive to these cell wall-perturbing agents at high concentrations, deletion strains exhibited a higher sensitivity, which is traditionally associated with defects in cell wall structure (Damveld *et al.*, 2005). Interestingly,  $\Delta abhaca$  strains were not hypersensitive to Calcofluor White (CFW). CFW and CR are known to interfere with normal cell assembly by interfering with chitin and glucan synthesis and cross-linking respectively. These results suggest that *AbHacA* could mainly impact on the cell wall glucan composition. To confirm this, we used the sulphuric acid procedure for determination of both mutant and wild-type cell wall polysaccharide composition. As shown in Table 2, the  $\Delta 1abhaca$  mutant revealed a significant decrease in glucose content of the cell wall, confirming a defect in glucan composition in the mutant cell wall.

We also investigated the morphology of the transformant conidia using scanning (SEM) and transmission (TEM) electron microscopy. SEM images of the conidial surface showed a typical ornamentation for the wild-type strain and smooth-walled conidia for *AbHacA* null strains (Fig. 8A). The aberrant morphology of the mutant conidia was confirmed on TEM images of the conidial wall ultrastructure (Fig. 8B). The conidial walls of wild-type strains were composed of several superimposed layers, with a thick electron transparent inner layer, a middle cell wall layer and a thin electron dense outermost layer. TEM showed that the outermost layer of the conidial cell wall of *AbHacA* mutants appeared fluffy and was in most cases separated from the middle electron dense layer. On the other hand, the most obvious cell wall feature in the  $\Delta abhaca$  strains was a lack of separation between the inner and middle layers.

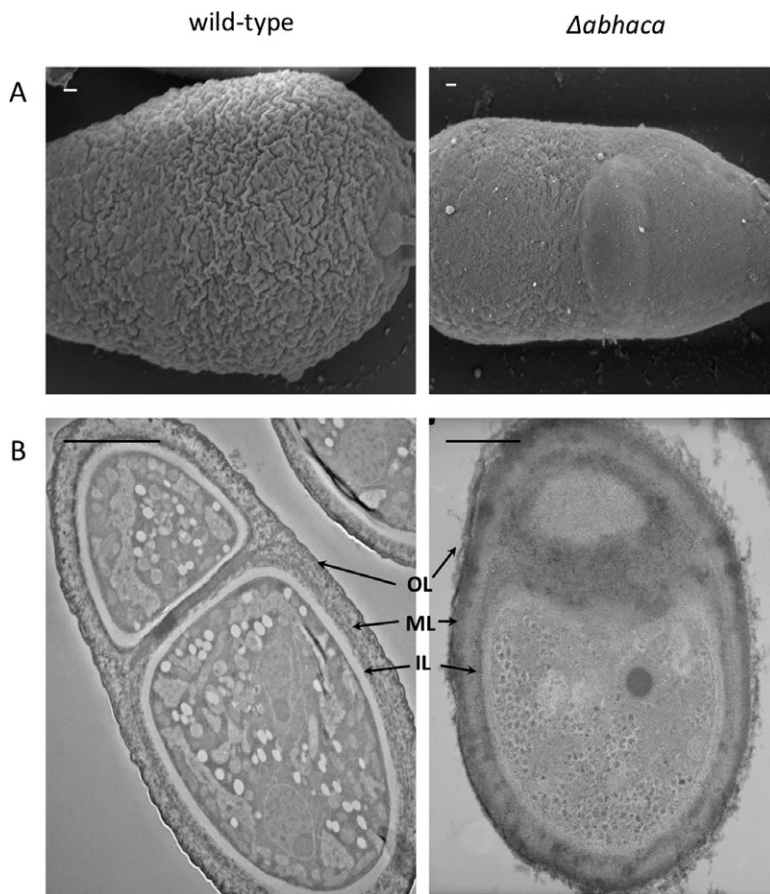
#### The $\Delta abhaca$ mutants exhibited increased susceptibility to host defence metabolites

Brassinin and camalexin are two indolic phytoalexins and allyl-isothiocyanates (AIITC) are glucosinolate breakdown

**Table 2.** Cell wall polysaccharide composition of wild-type and  $\Delta 1abhaca$  mutants after acid (H<sub>2</sub>SO<sub>4</sub>) treatment and quantification as monosaccharides (glucose, mannose, galactose and glucosamine) by high-performance ionic chromatography coupled to pulse amperometry detection (François, 2006).

	Wild-type	$\Delta 1abhaca$
Glucose* (µg ml <sup>-1</sup> )	303.1 ± 47.2	163.3 ± 59.5
Mannose (µg ml <sup>-1</sup> )	32.1 ± 5.4	21.0 ± 6.3
Glucosamine (µg ml <sup>-1</sup> )	20.4 ± 2.5	37.0 ± 11.8
Galactose (µg ml <sup>-1</sup> )	69.6 ± 7.6	50.1 ± 22.6

Asterisks indicate a significant difference between the mutant and the wild-type parental isolate (Student test,  $P < 0.01$ ).



**Fig. 8.**  $\Delta abhaca$  mutants show an aberrant conidial cell wall.

A. Scanning electron micrographs of 15-day-old wild-type and *AbHacA* mutant conidia (scale bars = 1  $\mu\text{m}$ ).

B. Transmission electron micrographs of 15-day-old wild-type and *AbHacA* mutant conidia (scale bars = 0.2  $\mu\text{m}$ ). OL, outermost cell wall layer; ML, middle cell wall layer; IL, inner cell wall layer.

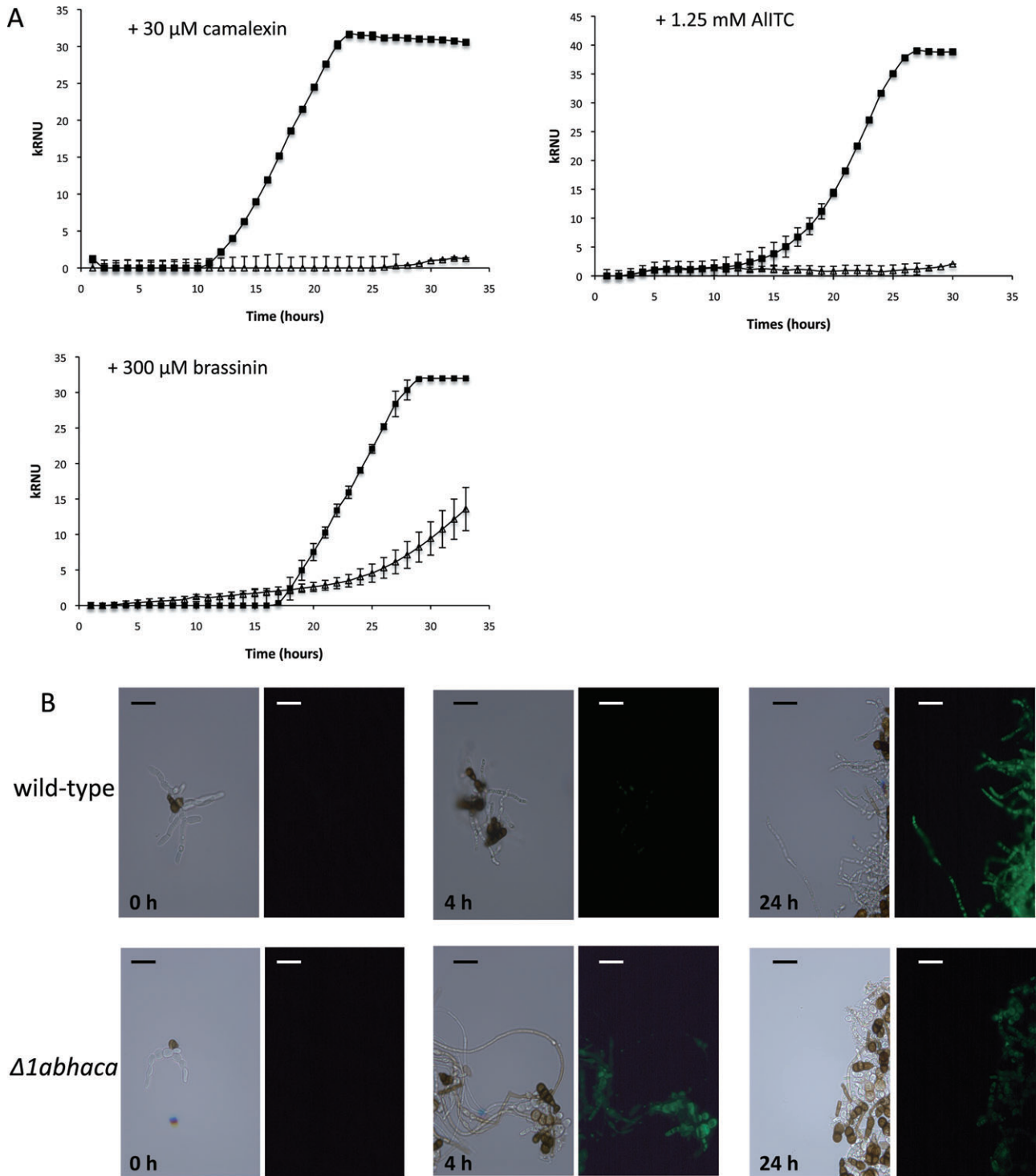
products. Brassinin and AIITC are produced in a variety of cruciferous vegetables such as cultivated *Brassica* species, while camalexin is synthesized by *A. thaliana* and some other brassicaceous weeds (Pedras *et al.*, 2000; Fahey *et al.*, 2001). Initial growth stages were monitored using laser nephelometry in liquid medium in the presence of different concentrations of each metabolite (Fig. 9A). At 30  $\mu\text{M}$  camalexin concentration, the maximum slope of the wild-type strain was not affected. By contrast, a severe reduction (84%) in this growth parameter was observed for  $\Delta abhaca$  strains and the lag time was also increased (2.5-fold). Similarly, the maximum slope of the wild-type strain was not affected when challenged with brassinin at 300  $\mu\text{M}$  but the lag time was increased (2.4-fold). A slight extension of lag time was also observed with the mutant strains but the major effect was a significant reduction (45%) in the maximum slope. At 1.25 mM AIITC concentration, growth of the wild-type strain was not significantly affected whereas a severe reduction (83%) in the maximum slope was observed for  $\Delta abhaca$  strains.

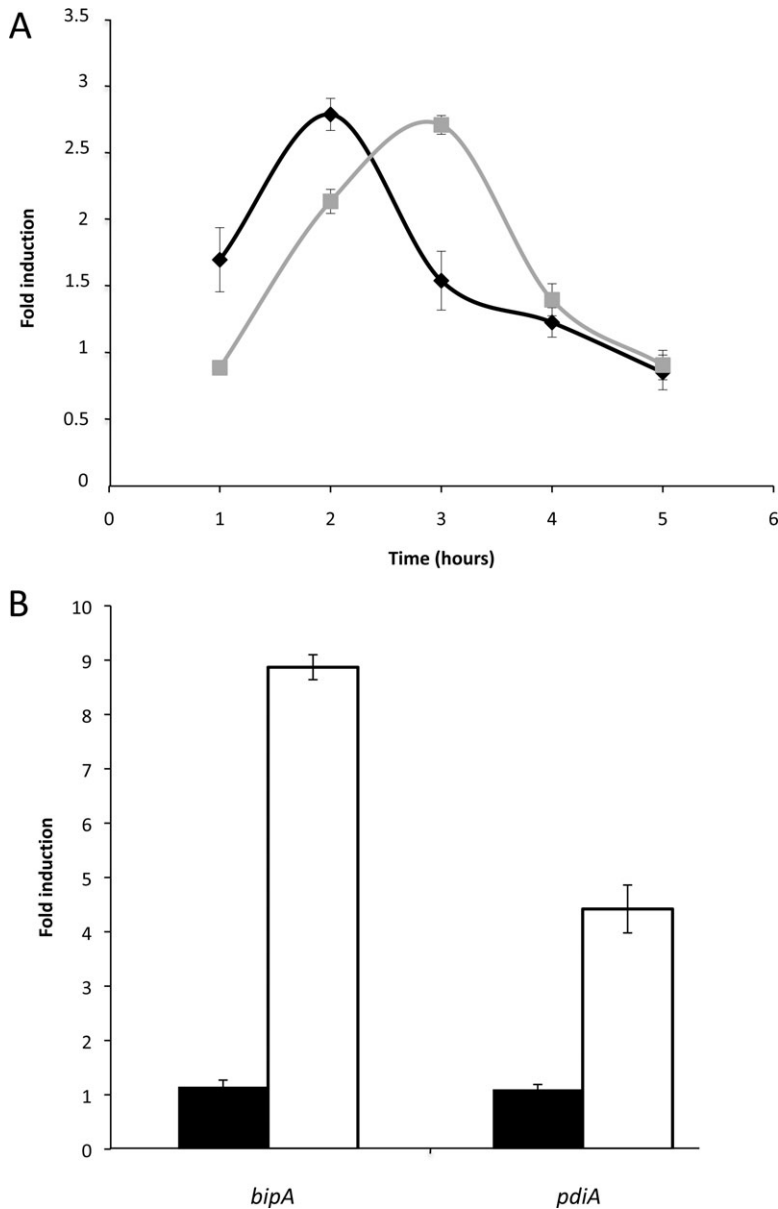
Sellam *et al.* (2007a) have previously shown that camalexin induces fungal membrane permeabilization. An

assay based on the uptake of the fluorogenic dye SYTOX green was thus used to visualize the effect of this phytoalexin on the membrane integrity of the different genotypes under study. As shown in Fig. 9B, no green fluorescence was detected in the wild-type hyphae upon exposure to 125  $\mu\text{M}$  camalexin for at least 4 h. Under the same conditions, uptake of the fluorogenic dye was evident in samples from the  $\Delta abhaca$  strains after only 4 h of exposure to camalexin. This result is consistent with the fact that null strains exhibited higher susceptibility to camalexin compared with the wild-type strain.

#### *The UPR is activated by camalexin*

In order to more thoroughly investigate the link between the UPR pathway and the response to camalexin, we quantified the spliced *AbHacA* mRNA using real-time RT-PCR. We adapted the method described by (Hirota *et al.*, 2006) that was initially developed to measure the splicing ratio of the mammalian XBP1 mRNA and was expected to be a good biomarker of ER stress. The cDNA derived from spliced *AbHacA* mRNA was thus specifically amplified using a specific primer designed to span the





**Fig. 10.** Camalexin activation of the UPR signalling pathway.

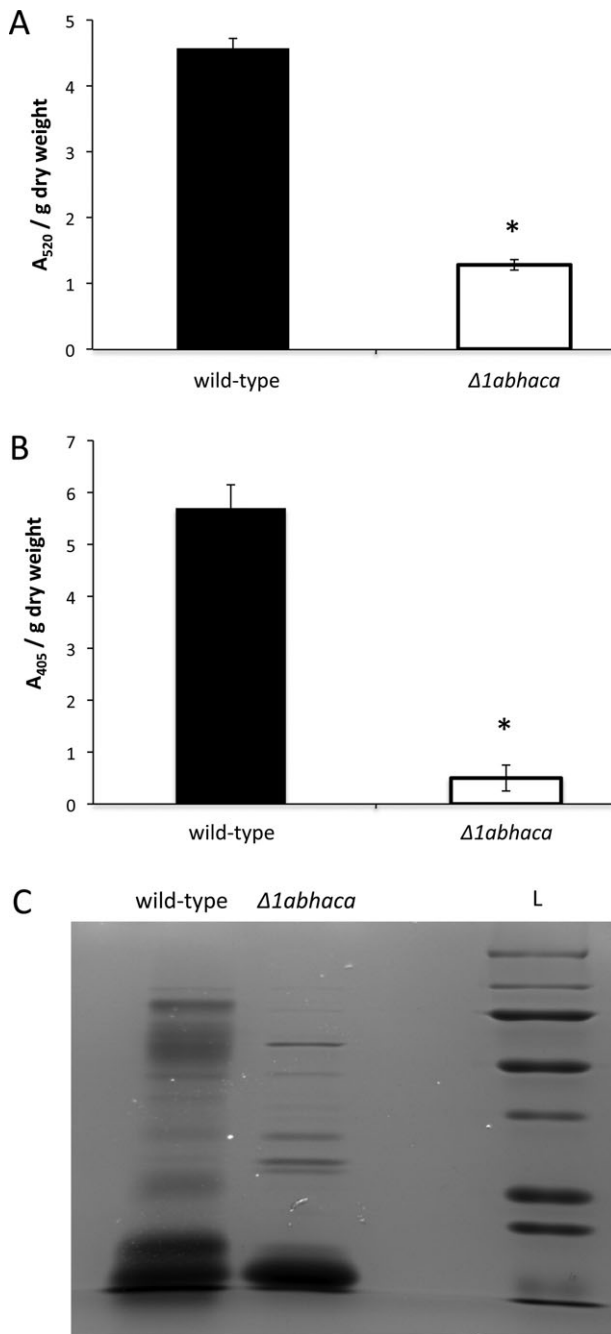
A. Analysis of *AbHacA* expression and processing in an *A. brassicicola* wild-type strain during camalexin exposure (125  $\mu$ M) for 1, 2, 3, 4 and 5 h. The black curve represents the fold induction observed with primers (called AbHacAF and AbHacAR in Table S1) designed for amplifying the cDNA derived from the spliced and unspliced *AbHacA* mRNA. The white curve represents the fold induction observed with primers specific of the spliced form (called AbHacAF<sup>i</sup> and AbHacAR in Table S1).

B. Quantitative RT-PCR results for the expression of two UPR target genes (*bipA* and *pdiA*) in the wild-type strain in the presence (white grey bars) or absence (dark grey bars) of camalexin (125  $\mu$ M) for 3 h. The data are the mean of three repetitions.

atypical 20 bp intron (Table S1). Real-time PCR analyses showed that exposure of the *A. brassicicola* mycelium to 125  $\mu$ M of camalexin induced splicing of the *AbHacA* mRNA intron and also led to simultaneous upregulation of the ER chaperone encoding gene *bipA* and the foldase encoding gene *pdiA* (Fig. 10). These results indicated that, like DTT treatment, camalexin treatment activated the UPR, although this effect appeared later than in DTT-treated mycelia. The *AbHacA<sup>i</sup>* cDNA yield reached peaked within 3 h after exposure to phytoalexin. This pattern was similar to that induced by tunicamycin treatment, which is a commonly used drug to induce an UPR response, or by production of a heterologous protein in other filamentous fungi (Mulder *et al.*, 2004).

#### *Loss of UPR signalling impairs the secretory capacity and saprophytic growth*

We hypothesized that the slow growth of *AbHacA* null strains on complex media (PDA or malt-agar) could be explained by a reduced secretory capacity and defects in assimilating nutrients from complex substrates. This hypothesis was consistent with the fact that growth of mutants on synthetic medium was restored to wild-type levels (Fig. 5B). To test this hypothesis, secreted proteolytic and esterase activities from wild-type and  $\Delta 1abhaca$  culture supernatants were quantified with the Azocoll and *p*-nitrophenyl butyrate (PNB) assays respectively. Azocoll is an insoluble collagen and its



hydrolysis by proteases from culture supernatants released soluble coloured peptides that can be quantified colorimetrically. PNB is a chromogenic substrate that is hydrolysed by esterases to produce *p*-nitrophenol that can be measured spectrophotometrically. As shown in Fig. 11A and B, culture supernatants derived from the  $\Delta 1abhaca$  mutant were significantly less efficient at hydrolysing Azocoll and PNB than wild-type cultures, indicating that protease and esterase secretions were impaired in the mutant. This result was consistent with the SDS-PAGE analysis, which revealed an alteration of

**Fig. 11.** Loss of UPR signalling impairs the secretory capacity. **A.** Azocoll hydrolysis by *A. brassicicola* proteases. An equal number of wild-type and mutant conidia were inoculated into Vogel's medium N supplemented with glucose 2%. After 15 days of culture, the supernatant was concentrated as described in the *Experimental procedures*. Culture supernatants from wild-type or  $\Delta 1abhaca$  were incubated in the presence of Azocoll for 3 h. Absorbances of the medium were determined at 520 nm. The experiment are the mean of three biological repetitions and values represent the mean  $A_{520}$  per gram dry weight  $\pm$  SD. Asterisks indicate a significant difference between the mutant and the parental isolate (Student test,  $P < 0.01$ ). **B.** PNB hydrolysis by *A. brassicicola* esterases. Culture supernatants from wild-type or  $\Delta 1abhaca$  were incubated in the presence of PNB for 1 h at 37°C and *p*-nitrophenol production was measured at 405 nm. The experiments are the mean of three biological repetitions and values represent the mean  $A_{520}$  per gram dry weight  $\pm$  SD. Asterisks indicate a significant difference between the mutant and the parental isolate (Student test,  $P < 0.01$ ). **C.** 1D gel analysis of wild-type and  $\Delta 1abhaca$  secreted proteins. A 5  $\mu$ l aliquot of each culture supernatant was fractionated by 1D SDS-PAGE and proteins were stained by methyl blue. L, ladder.

secreted protein patterns in  $\Delta 1abhaca$  mutant compared with wild-type culture supernatants (Fig. 11C).

## Discussion

The *A. brassicicola* *AbHacA* gene was isolated based on its similarity to other fungi *hac1* sequences. We showed that *AbHacA* was a functional homologue of *S. cerevisiae* *Hac1p* and mediated the UPR and secretion stress response in *A. brassicicola*. *AbHacA* mRNA carries an atypical intron whose length (20 bp) and sequence are very similar to that described in filamentous fungi and *C. albicans* (Mulder *et al.*, 2004; Wimalasena *et al.*, 2008). Its splicing occurs in response to ER stress, thus yielding a potent transcription factor and UPR activation, as visualized by upregulation of ER chaperones or foldases. Sequences at the boundaries of the intron and the predicted secondary RNA structure were well conserved, suggesting that the *AbHacA<sup>u</sup>* RNA is spliced by the action of an Ire1p1-like protein, as reported in yeast (Sidrauski and Walter, 1997; Gonzalez *et al.*, 1999). Mulder and Nikolaev (2009) showed that splicing of the 20 nt intron from *A. niger hacA* mRNA is sufficient to activate the UPR. They also showed that the 5'UTR of the *hacA* messenger loses 230 nt upon secretion stress, thus releasing an additional translational block via a switch to an alternative transcription start. We examined the truncation of the 5'-end of *AbHacA* mRNA under stress conditions by a RT-PCR approach. By using a forward primer defined in the 5'UTR, we clearly showed the disappearance of the PCR product following ER stress, indicating that the *AbHacA<sup>i</sup>* transcript also lack 5'-segment of the *AbHacA<sup>u</sup>* RNA (Fig. S2).

In this study, we generated UPR-deficient mutants by deleting the *AbHacA* ORF. Both mutants were unable to



increase the expression of well-known UPR target genes and were hypersensitive to agents that perturb ER homeostasis. Failure to induce the UPR following deletion of *AbHacA* resulted in a complete loss of *A. brassicicola* virulence. The loss of disease symptoms is not caused by the impaired growth of  $\Delta abhaca$  hyphae on the leaf surface.  $\Delta abhaca$  conidia were able to germinate and produce appressorial structures comparable to those of the wild-type strain. Moreover, the addition of 0.8 M sorbitol restored growth rates similar to those of the wild-type strain but did not restore the virulence.

Several hypotheses can be made to explain the impact of *AbHacA* upon pathogenicity. First, the UPR may contribute to virulence by supporting the high secretory load that occurs during host infection. Indeed, *A. brassicicola*, like many other filamentous fungi, secretes an arsenal of extracellular compounds required for pathogen penetration and nutrient consumption. In *AbHacA* null strains, failure to trigger the UPR under this situation, which requires an increased secretory capacity, would result in an overall reduction in secreted protein levels, including secreted pathogenicity factors, as suggested by the finding of SDS-PAGE analysis of culture supernatants and assays of esterase and protease activities. Multiple lines of evidence have indicated that esterases in *A. brassicicola* are crucially involved in cuticle penetration and in the saprophytic lifestyle (Fan and Köller, 1998). Alteration of the secretory profile and a decrease in proteolytic activity were already reported in an *A. fumigatus hacA* null strain that exhibited attenuated virulence in mouse models of invasive aspergillosis (Richie *et al.*, 2009). In *M. oryzae*, mutants produced by disruption of the ER chaperone LHS1 showed a defect in the translocation of proteins across the ER membrane and reduced activities of extracellular enzymes (Yi *et al.*, 2009). The  $\Delta lhs1$  mutants were thus severely impaired conidiation and in both penetration and biotrophic invasion in susceptible rice. This is consistent with a feedback mechanism called RESS (repression under secretion stress) that is activated in response to protein folding or transport impairment and leads to the selective transcriptional downregulation of genes encoding secreted proteins (Pakula *et al.*, 2003; Al-Sheikh *et al.*, 2004). Guillemette *et al.* (2007) also pointed out the possible existence of a feedback mechanism at the translational level. This reduced secretory capacity suggests that mutants have altered nutrient acquisition and is consistent with the fact that mutants became growth-impaired *in vitro* when forced to use a complex polymeric substrate. As shown in our study, Mulder and Nikolaev (2009) reported that growth was severely impaired and sporulation was almost absent in the *A. niger hacA* null strain.

In addition to the attenuated secretion, loss of *AbHacA* caused increased *in vitro* susceptibility to various plant defence metabolites. This result suggested that *AbHacA*

also functions as a virulence factor by enhancing the resistance of fungal cells against attack by host metabolites. Similarly, the  $\Delta abhaca$  mutant in *A. fumigatus* exhibited a dramatic increase in susceptibility to antifungal drugs that are used for the treatment of invasive aspergillosis (Richie *et al.*, 2009). The fact that *A. brassicicola*  $\Delta abhaca$  mutants did not produce any symptoms in *A. thaliana pad3* mutant, which is deficient for camalexin synthesis, might be partly explained by the antimicrobial activity of other compounds (such as rapalexin A and indole glucosinolates) at the inoculation site.

We hypothesized that the increased vulnerability of *AbHacA* mutants to plant defence compounds or antifungal drugs is partly or entirely caused by a cell wall defect. Indeed, alteration of the cell wall integrity of *AbHacA* mutants was observed, as indicated by their increased sensitivity to cell wall-perturbing agents, by electron microscopy observations of the conidial walls and by the fact that osmotic stabilization with sorbitol was able to restore growth to wild-type levels. Several transcript profiling studies have already revealed that *HacA* influences the transcription of genes involved in cell wall biogenesis (Kimata *et al.*, 2006; Wimalasena *et al.*, 2008). Moreover, strains lacking functional *HacA* in *A. fumigatus* (Richie *et al.*, 2009), *C. albicans* (Wimalasena *et al.*, 2008) or *S. cerevisiae* (Scrimale *et al.*, 2009) also displayed phenotypes associated with cell wall defects. In agreement with results obtained in *A. fumigatus hacA* null strain (Richie *et al.*, 2009), our biochemical cell wall analysis indicated a decreased glucose content in mutant cell walls, which is consistent with a defect of the glucan composition. By contrast, the *AbHacA* mutant did not exhibit significant difference in chitin levels as compared with the wild-type strain, suggesting that *HacA* mainly influences the glucan synthase activity. The plant and pathogen molecular interaction is far more elaborate than the production of attack molecules by the pathogen and the corresponding plant defence weaponry. In their long coevolution with hosts, fungal pathogens have evolved strategies to escape the defence responses and cell wall strengthening may be one common way to prevent intracellular accumulation of antifungal metabolites. Several studies, in particular in *Cryptococcus neoformans*, suggest that melanin may function *in vivo* to protect microbial pathogens against attack by the immune system (Casadevall *et al.*, 2000). Sellam *et al.* (2007a) showed that some genes involved in melanin biosynthesis and cell wall maintenance were upregulated in response to camalexin in *A. brassicicola*, suggesting the reinforcement of a physical barrier against the cellular penetration of toxic compounds.

Camalexin plays a major role in the resistance of *A. thaliana* to necrotrophic pathogens (Thomma *et al.*, 1999). This phytoalexin was shown to strongly inhibit conidial germination, germ-tube elongation and, to a lesser extent,

mycelial radial growth of *A. brassicicola* (Sellam *et al.*, 2007b). Although the exact mechanism by which camalexin exerts its toxicity is still unknown, we previously demonstrated that this plant defence metabolite probably causes membrane damage and activates a cellular response similar to the so-called compensatory mechanism (Popolo *et al.*, 2001) to preserve cell integrity (Sellam *et al.*, 2007a). Recently, we also reported that two mitogen-activated protein kinase (MAPK) signalling cascades, cell wall integrity (CWI) and high osmolarity glycerol pathways, were required for adaptation of *A. brassicicola* to cell wall stress caused by camalexin (Joubert *et al.*, 2010b). Camalexin was thus found to activate both AbHog1 and AbSit2 MAP kinases, and activation of the latter was abrogated in an AbHog1-deficient strain. Mutant strains lacking functional MAP kinases showed hypersensitivity to camalexin. Here we showed that the UPR pathway was also activated upon exposure to the phytoalexin, suggesting that stress responses mediated by UPR and MAPK signalling cascades are co-ordinated to buffer the cell against the toxicity of camalexin. Consistent with its hypothesis, Scrimale *et al.* (2009) showed that UPR and CWI pathway form an interdependent regulatory circuit that allows the response of yeast to both cell wall stress and ER stress. Description of the molecular mechanisms responsible for this complex interplay between UPR and CWI pathways will be investigated in future works.

In conclusion, these results highlight the importance of the UPR signalling pathway with respect to the ability of the fungus to grow *in planta*. We hypothesize that the UPR plays a major role in fungal virulence by impairing the secretory capacity and the protection mechanisms against host metabolites. In *A. fumigatus* the  $\Delta hacA$  mutant (Richie *et al.*, 2009) displayed increased susceptibility to various antifungal drugs, suggesting that pharmacologic inhibition of the UPR could synergize with current drugs in combination therapy. These results suggest that the UPR pathway is a crucial pathogenic factor and its inhibition, either alone or in combination with other antifungal drugs, could be envisaged as a promising strategy for disease control. Different approaches, involving pharmacological modulators of ER stress, were proposed to target the UPR as an anticancer strategy in mammalian cells (Healy *et al.*, 2009). Some of them, in particular the development of kinase inhibitors of Ire1p, could be applied to fungal cells. One of the major challenges is finding a therapeutic window where it is possible to specifically modulate fungal UPR without targeting plant cells.

## Experimental procedures

### Strains and growth conditions

The *A. brassicicola* wild-type strain Abra43 used in this study has previously been described (Sellam *et al.*, 2007b). For

routine cultures, fungi were maintained at 24°C by transferring hyphal plugs on 3.9% (w/v) PDA (Difco) or on agar-solidified Vogel's medium N supplemented with 1.2% (wt/vol) sucrose. For radial growth assays, agar disks were cut from the margin of a 7-day-old colony growing on PDA and were transferred onto the centre of PDA medium supplemented with the compounds under investigation (at concentrations specified in the *Results*) and incubated at 24°C. Colony diameters were measured daily and used for calculation of radial growth ( $\text{mm day}^{-1}$ ). To study hyphal growth in liquid media, conidial suspensions ( $10^5$  spores  $\text{ml}^{-1}$ , final concentration) were inoculated onto microplate wells containing the appropriate test substances in PDB in a total volume of 300  $\mu\text{l}$ . Microplates were placed in a laser-based microplate nephelometer (NEPHELOstar, BMG Labtech) and growth was monitored automatically over a 30 h period. Data were exported from Nephelostar Galaxy software in ASCII format and further processed in Microsoft Excel. Two variables, lag time and maximal slope, were calculated from the growth curves using the calculation method reported by Joubert *et al.* (2010a). At least three replicates per treatment were used.

To monitor growth under ER or cell wall stress, media was supplemented with DTT, BFA, CFW, CR and caffeine (Sigma). Stock solutions were prepared at the desired concentration using water as solvent. Phytoalexin camalexin was synthesized according to Ayer *et al.* (1992) and brassinin according to Kutschy *et al.* (1998) and Takasugi *et al.* (1988). Stock solutions were prepared in DMSO and acetone, respectively, and added to the medium at the desired concentrations. Solvent concentrations in controls and assays did not exceed 1% (v/v). The fungitoxic effects of camalexin were previously assessed (Sellam *et al.*, 2007b).

### DNA procedure and Southern hybridization

Genomic DNA was extracted from mycelium according to Moller *et al.* (1992). For Southern analysis, DNA fragments resulting from genomic DNA digestion with EcoRI were separated on 1% agarose gels and vacuum transferred to Hybond N membranes (Amersham Biosciences). Blots were then probed with a PCR product that was amplified from *A. brassicicola* genomic DNA and  $^{32}\text{P}$  labelled using the Random Prime Labelling System Rediprime II (Amersham Biosciences).

### RNA isolation and expression analysis by real-time quantitative PCR

Total RNA was prepared according to the TRIzol reagent protocol (Invitrogen). Additional cleanup and DNase treatment were performed using the Nucleospin RNA II kit (Macherey-Nagel) according to the manufacturer's protocol.

Complementary DNA was synthesized from 5  $\mu\text{g}$  of total RNA using the reverse-transcription system [50 mM Tris-HCl, 75 mM KCl, 10 mM DTT, 3 mM  $\text{MgCl}_2$ , 400 nM oligo(dT)<sub>15</sub>, 1  $\mu\text{M}$  random hexamers, 0.5 mM dNTP, 200 units M-MLV reverse transcriptase, Promega]. The total volume was adjusted to 30  $\mu\text{l}$  and the mixture was then incubated for 60 min at 42°C. Aliquots of the resulting first-strand cDNA were used for real-time PCR amplification experiments using

the ABI Prism 7000 sequence detection system (Applied Biosystems) and the SYBRgreen PCR master mix according to the manufacturer's instructions. After 10 min denaturation at 95°C, the reactions were cycled 40 times at 95°C for 15 s and 60°C for 1 min. The absence of contaminating genomic DNA in the RNA samples was checked by direct amplification of non-reverse transcribed samples. The synthesis of a single specific PCR product was verified by melting point analysis after the run. For each condition, all amplifications were performed in triplicate from two separate biological samples and the mean was determined for further calculations. The relative quantification analysis was performed using the comparative  $\Delta\Delta C_t$  method as described by Winer *et al.* (1999). To evaluate the gene expression level, the results were normalized using  $C_t$  values obtained from actin cDNA amplifications run on the same plate.

#### Expression of AbHacA in *S. cerevisiae*

Complementary DNAs containing spliced and unspliced versions of *AbHacA* were amplified by PCR with Phusion Hot Start High-Fidelity DNA polymerase (Finnzymes) using the primers presented in Table S1. Unique BamHI and NotI restriction sites (indicated in boldface) were thus introduced at the upstream start and downstream stop codons (underlined) of the *AbHacA* sequences respectively. The unspliced and spliced PCR products were cloned into pGEM-T (Promega) and then transferred into the yeast expression vector pYESCT (Invitrogen) after BamHI–NotI double digestion and ligation to yield the yeast expression vector pYAb-HacA<sup>u</sup> and pYAbHacA<sup>l</sup> respectively. These plasmids can express full-length *AbHacA* without a tag under control of the GAL1 promoter. *S. cerevisiae* cells (strains: parental BY4743 Acc#Y20000,  $\Delta Hac1$  Acc#Y35650, Euroscarf, Germany) were transformed using the *S. c.* EasyComp transformation kit (Invitrogen). Transformants were incubated under GAL1 promoter-repressive conditions [SD without uracil (SD/-Ura)] or under inductive conditions [SG without uracil (SG/-Ura)]. Sensitivity to DTT was analysed on plates. Transformants were precultured overnight in 5 ml of SD/-Ura or SG/-Ura. Serially diluted [1:10 (starting optical density of 0.1 at 600 nm)] cell suspensions (5  $\mu$ l) were spotted onto plates supplemented with DTT and the plates were incubated for 60–240 h.

#### Generation of targeted gene knockout mutant

The gene replacement cassettes were generated using the double-joint PCR procedure described by Yu *et al.* (2004). The selectable marker inserted in the PCR construct corresponded to the Hyg B resistance cassette (1436 bp) from pCB1636 (Sweigard *et al.*, 1995). Two sets of primers (Table S1) were used with Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) to amplify 872 bp and 818 bp from the 5' and 3' flanking regions of the *AbHacA* gene respectively. The double-joint final PCR was purified and used to transform *A. brassicicola* protoplasts as described in Cho *et al.* (2006). The hygromycin resistant mutants were selected and prescreened by PCR with relevant primer combinations to confirm integration of the

replacement cassette at the targeted locus. The gene replacement mutants were further purified by three rounds of single-spore isolation and then confirmed by Southern blot analysis.

#### Pathogenicity assays and light microscopic examination of infection structure

Plant infection assays were performed on leaves from whole plants of *A. thaliana* and *B. oleracea*. *A. thaliana* plants were grown to the 8- to 12-leaf stage in controlled environment rooms (21/19°C day/night temperature respectively) under an 8 h light photoperiod. *B. oleracea* plants were grown in a greenhouse for 5 weeks. For inoculations, 5  $\mu$ l drops of *A. brassicicola* spore suspension ( $5 \times 10^5$  spores ml<sup>-1</sup> in water) were deposited on intact and prewounded leaves on the left and right sides symmetrically from the central vein. Drops of sterile water were applied on control plants. The plants were then maintained under saturating humidity (100% relative humidity) in a plastic box. Symptoms were observed at 1, 3 and 6 days post inoculation (dpi).

For the microscopic analyses, *B. oleracea* leaf fragments were discoloured, cleared and fungal structures were stained with Solophenyl Flavine 7GFE 500 (Ciba Specialty Chemicals, North Carolina, USA) as described by Hoch *et al.* (2005). Specimens were observed under a Leica fluorescent microscope (using 480 nm excitation and 527 nm emission).

For the analysis of germination and penetration on epidermal cells, epidermal layers were peeled from onions and inoculated with 5  $\mu$ l droplets of conidial suspensions ( $5 \times 10^3$  spores ml<sup>-1</sup>) on the adaxial surface. After 24 h incubation at high humidity in glass trays, tissues were stained by immersion into 0.25% Trypan Blue in lactophenol for 5 min (Doehlemann *et al.*, 2006) and germination and penetration rates were evaluated. All assays were performed at least three times with at least 100 spores or germlings evaluated each time.

#### Electron microscopy

The conidial wall ultrastructure was investigated by TEM and SEM using conidial suspensions obtained from 7-day-old cultures on PDA. Concerning the TEM sample preparation, successive steps of fixation, post-fixation, dehydration and embedding in Epon were carried out as previously described (Tronchin *et al.*, 1993). Thin sections were contrasted with uranyl acetate and lead citrate and examined under a JEM-2010 transmission electron microscope (Jeol, Paris, France). SEM sample preparations were performed as described by Boedo *et al.* (2008). After drying by the critical-point method, specimens were then sputtercoated with a thin carbon layer and examined using a JEOL JSM 6301-F scanning electron microscope (Jeol, Paris, France).

The pictures of leaf infections were acquired with an environmental scanning electron microscope Zeiss EVO 50 Variable Pressure SEM (Carl Zeiss SMT, New York), fitted with a high brightness LaB6 cathode. Pressure and humidity conditions were adjusted to 470 Pa and 30–40% respectively. The samples were directly imaged in their natural state without modification or preparation.

### Cell wall analysis

The cell wall composition was determined according to the method described by François (2006). This protocol is based on the hydrolysis by sulphuric acid of  $\beta$ -glucan, mannan, galactomannan and chitin into their corresponding monomers glucose, mannose, galactose and glucosamine. The released monosaccharides are subsequently separated and quantified by high-performance ionic chromatography coupled to pulse amperometry detection.

### SYTOX green uptake assays

For membrane permeabilization assays, 16-h-old germinating conidia grown in PDB were exposed to 125  $\mu$ M camalexin or 1% (v/v) DMSO as negative control for 2–24 h. After treatment, 20  $\mu$ l of incubation mixtures were mixed with a SYTOX green solution (1  $\mu$ M final concentration) and immediately observed under a Leica fluorescence microscope (using 480 nm excitation and 527 nm emission).

### Analysis of protein secretion

Conidia were inoculated at  $1 \times 10^5$  ml<sup>-1</sup> in 50 ml of Vogel's liquid medium N plus 1% (wt/vol) sucrose. The cultures were incubated at 24°C for 15 days at 175 r.p.m. Under these conditions, the wild-type and  $\Delta$ abhaca generated a similar amount of dried biomass. The supernatants were removed from both cultures and concentrated to 250  $\mu$ l using the Amicon 8050 ultrafiltration system with a membrane cut-off of 10 kD. For gel analysis, each sample was mixed with buffer [200 mM Tris-HCl pH 6.8, 50% glycerol, 5% sodium dodecyl sulphate (SDS), 0.5% bromophenol blue and 5% (v/v)  $\beta$ -mercaptoethanol] and heated to 95°C for denaturation. Each sample (5  $\mu$ l) was loaded onto a 12% SDS-PAGE gel and run at 60 V for 2 h. After treatment in a fixing solution (50% methanol and 7% acetic acid) for 30 min and washing in water for 10 min, gels were stained for 2 h with Coomassie blue and destained in a solution containing 10% methanol and 7.5% acetic acid.

Protease and esterase activities were measured from wild-type and  $\Delta$ abhaca culture filtrates that were previously concentrated as described above. All assays were performed in triplicate and final values were normalized to the dry weight of the biomass obtained after 15 day incubation. Protease secretion was quantified using Azocoll (Sigma) hydrolysis (Gifford *et al.*, 2002). Azocoll substrate is composed of insoluble particles of collagen linked to a bright red azo dye. An Azocoll suspension (5 mg l<sup>-1</sup>) was prepared in a buffer containing 50 mM Tris-HCl pH 7.5, 1 mM CaCl<sub>2</sub> and 0.01% sodium azide. Aliquots of the culture filtrate (15  $\mu$ l) were added to 2.4 ml of the Azocoll suspension and the mixture was incubated for 3 h at 37°C on a rotator. The mixture was then centrifuged at 13 000 *g* for 5 min and azo dye release was determined by measuring the absorbance at 520 nm. Esterase activity was also determined spectrophotometrically using the artificial substrate PNB as described by Davies *et al.* (2000). Aliquots of culture filtrate (100  $\mu$ l) were added to 100  $\mu$ l of 0.4% Triton X-100 and 690  $\mu$ l of 50 mM sodium phosphate buffer pH 7.0, equilibrated to 37°C and 10  $\mu$ l of

1.76% (v/v) PNB in acetonitrile was added. *p*-Nitrophenol production was measured at 405 nm.

### Statistical analysis

Phenotypic characterizations of the *A. brassicicola* strains were subjected to at least two biological repetitions, each with three replicates. Data were analysed using the Student's test (*P*-value of < 0.01 was considered significant). Statistical analyses were done with the R software package (version 2.9.2 GUI 1.29).

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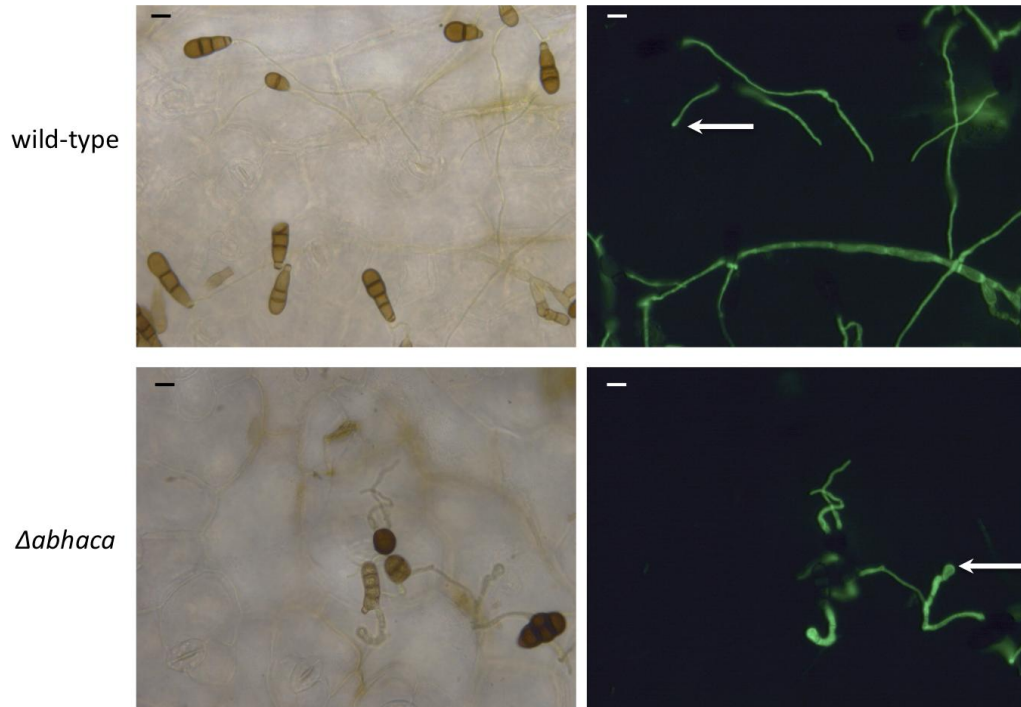
### Supporting information

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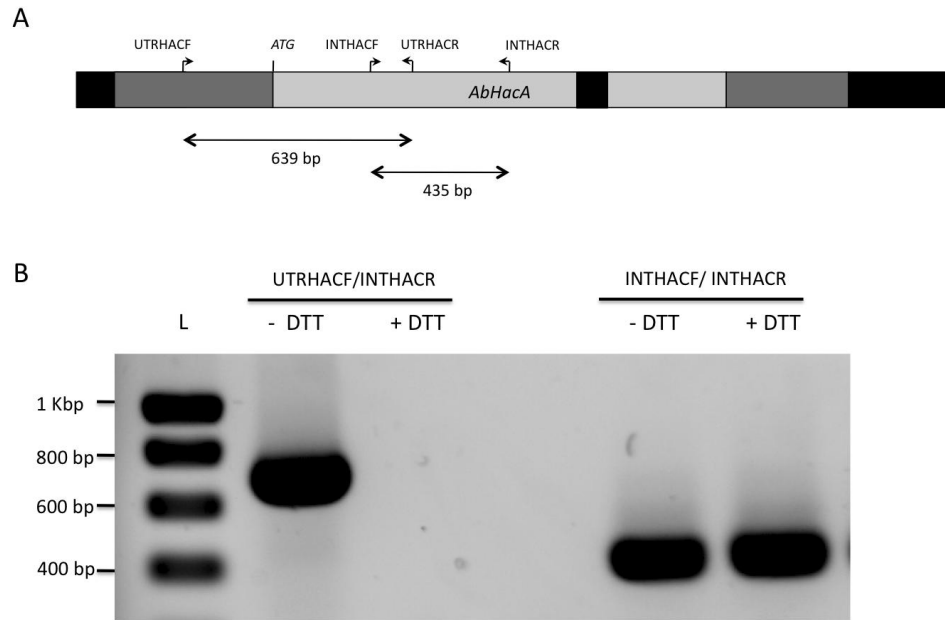
## Supplementary Information

Figure S1



**Fig. S1.** Microscopic observations of the infection structures on *B. oleracea* leaf surfaces.

Leaf fragments inoculated with *A. brassicicola* wild-type strain and  $\Delta abhaca$  mutants were collected at 24 h dpi and stained with Solophenyl Flavine. The right panels correspond to fluorescence microscopy and the left panel to bright-field microscopy. Appressoria-like structures, indicated by arrows, were differentiated by the two genotypes. Scale bars = 10  $\mu\text{m}$ .



**Fig. S2.** Examination of the 5'-truncation of *AbHacA* mRNA

A. Schematic representation of the *AbHacA* locus showing the positions of primers (Table S1) for RT-PCR analysis.

B. RT-PCR analysis of *AbHacA* processing in the presence or absence of 20 mM DTT for 1 h. Molecular sizes (kb) were estimated based on 1kb ladder (lane L, Eurogentec, Seraing, Belgium).



Table S1. List of primers used in this study.

Name	Sequence	Description
FusHAC5F	5'-CAAAAAGAGCCGGAGACAAA-3'	Set of primers used to generate the <i>AbHacA</i> replacement construct
FusHAC5R	5'-GTCGTGACTGGGAAAACCCTGGCGGTCGATAGTTGACGGGGCTA-3'	
FusHAC3F	5'-TCCTGTGTGAAATTGTTATCCGCTTAGGGCGGACAAGATACACAC-3'	
FusHAC3R	5'-ACTGACCCAGAACAGCCATC-3'	
FusHACNF	5'-ATGCACCTTCCCTGATGTTT-3'	
FusHACNR	5'-TCGAGGAAGGTAATCCGATG-3'	
M13F	5'-CGCCAGGGTTTTCCAGTCACGAC-3'	Primers for amplification of the Hyg B resistance cassette from pCB1636
M13R	5'-AGCGGATAACAATTCACACAGGA-3'	
NCL37	5'-GGATGCCTCCGCTCGAAGTA-3'	Hyg B specific primer
Hac1upbam	5'-GGATCCCAAAATGGCTCAATACACCTTCGACTCTAGTTTGTITTTCC-3'	Primers for the expression of <i>AbHacA</i> in <i>S. cerevisiae</i>
Hac1donot	5'-GCGGCCGCTCAGAACGCCGTCCCACGCCCGCAGTGGTGCCACA-3'	
AbHacAF1	5'-CGCAAACGACAAGAATACGA-3'	Primers used for the identification of the unconventional intron
AbHacAR1	5'-AAGTTCCCGAAATCGACTGA-3'	
UTRHACF	5'-GCTGGTGCCTATGTTTCGA-3'	Primers used to examine the truncation of the 5'-end of <i>AbHacA</i> mRNA
UTRHACR	5'-TATGTGATGCTCCGACGAG-3'	
INTHACF	5'-TTCCCAGACCATGAACAACC-3'	
INTHACR	5'-GCCATCTTTTGCTTGTAGGCC-3'	
AbHacAF	5'-ATGGACTCACCCCGTGACTC-3'	Primer pairs used for quantitative RT-PCR
AbHacAR	5'-CGCCCGTAGAGAAGTTCCC-3'	
AbHacAF <sup>i</sup>	5'-CGACACAATATCCTGCCGTG-3'	
bipAF	5'-GCTGTCCAGGGTGGTGTCT-3'	
bipAR	5'-AATGAGTTGGTCGGTAGC-3'	
pdiAF	5'-CCATCGAAGAGTTCAAGACGG-3'	
pdiAR	5'-CGAAGTAGGCGACGAGAACG-3'	
actinF	5'-GGCAACATTGTCATGTCTGG-3'	
actinR	5'-GAGCGAAGCAAGAATGGAAC-3'	

Table S2. Protoplasting efficiencies of *A. brassicicola* wild-type and both *AbHacA* mutants.

<sup>a</sup> For standard conditions, germinated conidia were incubated at 32°C in lytic solution (0.7 M

	wild-type	<i>Δ1-2abhaca</i>	NaCl
	<i>Standart protoplasting conditions</i>		, 20
Initial step <sup>a</sup>	5.10 <sup>7c</sup>	1.10 <sup>4</sup>	mg/
Final step <sup>b</sup>	1.10 <sup>7</sup>	0	ml
	<i>Low salt protoplasting conditions</i>		Drise
Initial step <sup>a</sup>	1.10 <sup>6</sup>	0	lase,
Final step <sup>b</sup>	1.10 <sup>5</sup>	0	10
			mg/

ml Kitalase). The number of protoplasts was counted under a microscope after 4h incubation. For low salt conditions, the lytic solution contained 0.35 M NaCl.

<sup>b</sup> Washed protoplasts were suspended in STC (1.2 M sorbitol, 10 mM Tris pH 7.0, 50 mM CaCl<sub>2</sub>) and counted under a microscope

<sup>c</sup> Numbers correspond to the number of protoplast. mL<sup>-1</sup>

### **3. Perspectives**

Ces travaux présentent l'importance du gène *HacA* dans la virulence d'*A. brassicicola* et dans sa réponse aux phytoalexines. En effet, le mutant *AbHacAΔ* est avirulent, sensible aux molécules de défenses et altéré dans sa composition pariétale. Ces travaux mettent en évidence pour la première fois, l'activation de l'UPR par une phytoalexine, la camalexine. Le **Chapitre 2** montre l'importance de deux MAP kinases Hog1p et Slk2p dans la réponse du champignon aux phytoalexines camalexine et brassinine. Ces deux protéines interviendraient dans le mécanisme compensatoire visant à renforcer l'intégrité cellulaire face à la molécule. Les travaux réalisés dans ce chapitre exposent l'importance de l'UPR dans la réponse à la camalexine et suggèrent un rôle commun des MAP kinases et de l'UPR dans la réponse adaptative. Des travaux ont montré que les voies HOG et CWI sont interconnectées pour répondre à des stress pariétaux mais également lors d'un stress du RE (Scrimale *et al.*, 2009). Néanmoins, les bases moléculaires de cette connexion ne sont pas encore connues. Une des perspectives de ce travail est de travailler sur la compréhension des connections entre les voies HOG, CWI et UPR, en s'aidant notamment des trois mutants nuls respectifs obtenus chez *A. brassicicola*. Ainsi, il serait intéressant de visualiser l'état de phosphorylation des deux MAP kinases dans le mutant nuls *AbHacAΔ* durant une exposition à la camalexine ou même à la brassinine et inversement, regarder l'activation de l'UPR dans les mutants des MAP kinases.

Finalement, ces résultats mettent clairement en évidence l'intérêt de considérer la voie de sécrétion et l'UPR comme de nouvelles cibles antifongiques dans une stratégie de lutte contre des champignons phytopathogènes. Les résultats montrent qu'une inhibition de l'UPR aboutit à une avirulence du champignon. De même, une inhibition de l'ERAD ou de l'UPR chez *A. fumigatus* conduit à une très forte diminution de l'agressivité, et une double mutation dans l'ERAD et dans l'UPR aboutit à une avirulence (Richie *et al.*, 2011). Néanmoins, l'une des principales difficultés consiste à identifier des inhibiteurs de ces voies, qui soient spécifiquement ciblés contre les cellules fongiques. Cette problématique est également retrouvée dans les stratégies de lutte contre les maladies humaines. Ainsi, la difficulté de trouver un inhibiteur spécifique des cellules tumorales, a été par exemple contournée par l'utilisation de la versipelostatine, molécule isolée de *Streptomyces verspellis*. Celle-ci inhibe l'UPR seulement chez les cellules tumorales pauvres en glucose (Scriven *et al.*, 2007). En effet, la versipelostatine va inhiber l'activation des facteurs de transcription XBP1 et ATF4 (régulateur de l'UPR) seulement dans les cellules dépourvu de glucose. A ce jour, aucun test de ce genre n'existe chez les champignons phytopathogènes.

# Chapitre 4

**Mode d'action des phytoalexines indoliques :  
résultats complémentaires**

Les travaux présentés dans les 2 chapitres précédents ont permis de mieux cerner l'implication des voies de signalisation HOG et CWI mais également de l'UPR dans le mécanisme compensatoire mis en place par *A. brassicicola* en réponse aux phytoalexines indoliques. Comme nous l'avons mentionné dans l'introduction, Sellam *et al.* (2007b) ont émis l'hypothèse que la protection du champignon contre la camalexine pourrait être également liée à une détoxification enzymatique. D'autre part, ces auteurs ont également suggéré dans leur modèle que les phytoalexines indoliques pourraient avoir pour cible cellulaire des sphingolipides et/ou des stéroïdes membranaires. Durant de cette thèse, nous avons initié des recherches visant à explorer ces deux hypothèses. Les résultats obtenus sont présentés dans ce chapitre.

# **1. AbMak1p: une protéine de détoxification de la camalexine ?**

A

```
MPHALTSRDSTMESKPLGSPNTGIDVLIVGTGLAGLVAALECTRKGHNVRLERNADINTAGDMYFMGLSATRFLKHWPPELLKEYKISLHNA
WIETFKHSGEVVIGPKKVADRLRAQGLDPPDPGGEFQMRPLVYKMFVSAVENLGVSVFENRRRVVDYFEDEQTKGGCTIDDGKRYEADVIAA
DGVGSKSQKLVGGQVRARPSGRAMWRAAHPREALDKDPEVKEFFKMMPGNEPIVRTWLGSTYALTLSDREDVMVWIMNHDTGTEKESWNNT
IEKEEVLGDGMNDMPGMETIKWAPIFKKLVEITPPNTIVNFELLWRNPOPSWTSPAARVIQIGDAAHSYLPASGNGATQAIEDAISIASCLQVGGKD
NIPOSVRVHIRFRFIRNSCAQKLGFSNAELLQDTDWKVKLDPRAAPKPKVWVSHDPEAYVYENYELSAQSLKEGIDMKDEDKFEPNYPKGY
KYEPWSIEKIMDDMRAGKPIDLGAGDWE
```

B

```
MPHALTSRDSTMESKPLGSPNTGIDVLIVTGLAFLVAALECTRKGHNVRLERNADINTAGDMYFMGLSATRFLKHWPPELLKEYKISLHNA
WIETFKHSGEVVIGPKKVADRLRAQGLDPPDPGGEFQMRPLVYKMFVSAVENLGVSVFENRRRVVDYFEDEQTKGGCTIDDGKRYEADVIAA
DGVGSKSQKLVGGQVRARPSGRAMWRAAHPREALDKDPEVKEFFKMMPGNEPIVRTWLGSTYALTLSDREDVMVWIMNHDTGTEKESWNNT
IEKEEVLGDGMNDMPGMETIKWAPIFKKLVEITPPNTIVNFELLWRNPOPSWTSPAARVIQIGDAAHSYLPASGNGATQAIEDAISIASCLQVGGKD
NIPOSVRVHIRFRFIRNSCAQKLGFSNAELLQDTDWKVKLDPRAAPKPKVWVSHDPEAYVYENYELSAQSLKEGIDMKDEDKFEPNYPKGY
KYEPWSIEKIMDDMRAGKPIDLGAGDWE
```

C

```
NhMAK1 MGDYVSSHTDPIS---MPRYPSSGLSVLVGGGIAGLSFAIEAYRKGHDVRIIERRPFDFH 57
AbMAK1 MPHALTSRDSTMESKPLGSPNTGIDVLIVGTGLAGLVAALECTRKGHNVRLERNADIN 60
* . . : : . : : . : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
NhMAK1 DYGDLIATQASALHSPSNWPGFVERCRDS-----PFSETHIHKYDGTLLGTIKFP- 108
AbMAK1 TAGDMYFMGLSATRFLKHWPPELLKEYKISLHNAWIETFKHSGEVVIGPKKVADRLRAQG 120
** : : ** : . : * : * : : . : . : . : . : * : * : * : * : * : * :
NhMAK1 LS-----MSRATFHGILLHEYAEKLCIDIQFSTVATYFFENE--DLAGVVLGDGTVLS 158
AbMAK1 LDPDTPPGEFQMRPLVYKMFVSAVENLGVSVFENRRRVVDYFEDEQTKGGCTTDDGKRYE 180
* . . : : . : : . : * : * : * : * : * : * : * : * : * : * : * : * :
NhMAK1 ADVVVAADGIGSKSWNLVSGYKEKPISSGFALFRSTYFVERAIQNPLVAKEF---EGCDQ 215
AbMAK1 ADVVIAADGVGSKSQKLVGG-QVRARPSGRAMWRAAHPREALDKDPEVKEFFKMMPGNEP 239
*** : *** : *** : * : * : * : * : * : * : * : * : * : * : * : * :
NhMAK1 KANLHLGPGAHIIIVAKSETIIVFMLTHRDNGNAEETWTKQTSVDNALP-----YVKG 267
AbMAK1 IVRTWLGSTYALTLSDREDVMVWIMNHDTGTEKESWNNTIEKEEVLGDGMNDMPGMETIK 299
.. *** : : : * : * : * : * : * : * : * : * : * : * : * : * :
NhMAK1 WAFYISEVIQAAFDRKAVDFKLMWRNFRDKWASFCARVIQIGDAAHTFLFTSASGATMAL 327
AbMAK1 WAFIFKKLVETTFPNTIVNFELLWRNPOPSWTSPAARVIQIGDAAHSYLPASGNGATQAI 359
*** : . : : : * . . * : * : * : * : * : * : * : * : * : * : * : * :
NhMAK1 EDAYSLATCLOLGGKSNAPLAVRVHNLRAERVAQAQKMGFKTRELHYHNTDWELHEKDPS 387
AbMAK1 EDAISIASCLQVGGKDNIPQSVRVHIRFRFIRNSCAQKLGFSNAELLQDTDWKVKLDP 419
*** : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
NhMAK1 RLLKMVGSVVINHDPEQYAYDNYGKCVQHILVGTFFKN-----TNAVPGYTYKPVTVQEL 442
AbMAK1 RAAPKPKVWVSHDPEAYVYENYELSAQSLKEGIDMKDEDKFEPNYPKGYKYEPWSIEKI 479
* : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
NhMAK1 LDASDRGVEVED-EGAWD 459
AbMAK1 MDDMRAGKPIDLGAGDWE 497
: * * * : : * * :
```

Figure 20 Séquences protéiques de la flavoprotéine AbMak1p :

- (A) Séquence protéique d'AbMak1p d'A.brassicicola. Les feuilletts bêta sont indiqués en vert et les hélices alpha en rouge.
- (B) Séquence protéique d'AbMak1p d'A.brassicicola. Le domaine Rossman est indiqué en bleu, le domaine FAD dépendant est indiqué en vert et le domaine monoxygénase est indiqué en rouge.
- (C) Alignement de séquences protéiques d'AbMak1p avec la protéine NhMak1 de N. haematococca. Les séquences homologues sont indiquées en bleu.

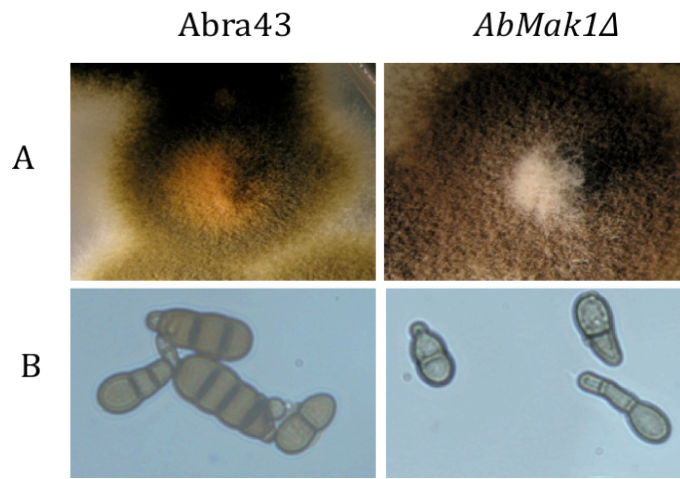


Le gène *AbMak1* a été caractérisé comme étant surexprimé chez *A. brassicicola* lors de l'exposition à la camalexine (Sellam *et al.*, 2007b). Il code une protéine qui présente de fortes homologues avec une enzyme produite par le champignon phytopathogène *N. haematococca*, permettant la détoxification de la maackiaine par déshydrogénation (Miao *et al.*, 1992). Il n'existe pas d'orthologue du gène *AbMak1* chez *S. cerevisiae* et le phénotypage d'un tel mutant n'a donc pu être réalisé chez la levure.

L'analyse bioinformatique de la séquence de la protéine AbMak1p montre qu'elle correspond à une flavoprotéine monooxygénase de classe A (van Berkel *et al.*, 2006), comme la salicylate hydroxylase de *Pseudomonas putida* (**Figure 20**) (White-Stevens *et al.*, 1972a, White-Stevens *et al.*, 1972b). Un mutant nul *AbMak1Δ1* déficient pour cette protéine a été obtenu chez *A. brassicicola*. Ce mutant différencie des conidies de coloration moins intense que celles de la souche sauvage suggérant une anomalie de la pigmentation et notamment de la mélanisation des conidies. En microscopie à balayage, les résultats montrent que le mutant présente des conidies très affectées au niveau de leur surface. En microscopie à transmission, les parois sont organisées comme la souche sauvage mais des granules noires de nature inconnue sont observées au niveau des parois primaires ou secondaires (**Figure 21 et 22**). L'hypothèse émise est que ces agrégats pourraient être de la mélanine.

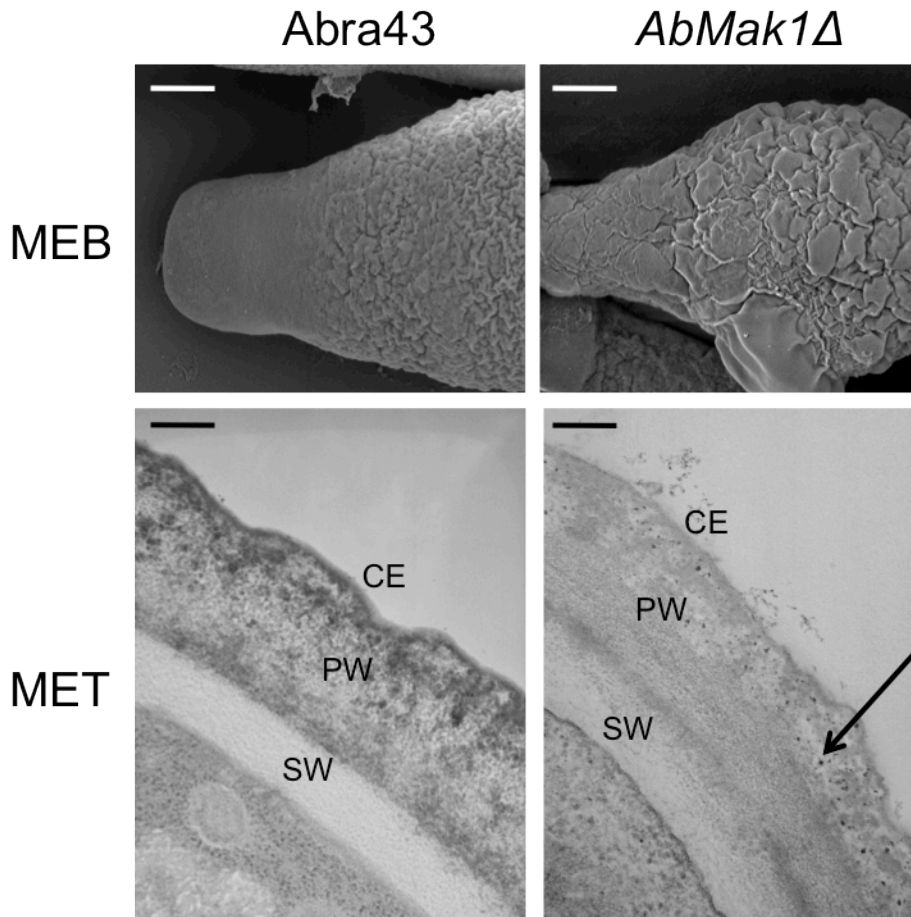
La sensibilité aux phytoalexines a été étudiée par des tests de croissance radiale. Les résultats montrent que le mutant *AbMak1Δ1* est plus tolérant à la camalexine que la souche sauvage. Le mutant ne présente toutefois pas de différence de virulence *in planta* sur chou ni sur *A. thaliana* (écotype Col-0) (**Figure 23**).

Ne connaissant pas la fonction du produit du gène *AbMak1*, il est actuellement difficile de relier ces observations aux hypothèses formulées précédemment. Toutefois le phénotype du mutant *AbMak1Δ1* montre que, contrairement à ce qui a été proposé par Sellam *et al.* (2007b) par analogie avec *N. haematococca* (Miao *et al.*, 1992), *AbMak1* n'est probablement pas impliqué dans une détoxification enzymatique de la camalexine. En effet le mutant nul est plus résistant à la camalexine que la souche sauvage. Le classement de la protéine AbMak1p parmi les flavoprotéines monooxygénases de classe A, dont fait également partie la squalene époxydase, permet d'envisager un rôle dans la biosynthèse des stérols membranaires. Cette hypothèse est supportée par le fait qu'un gène codant une



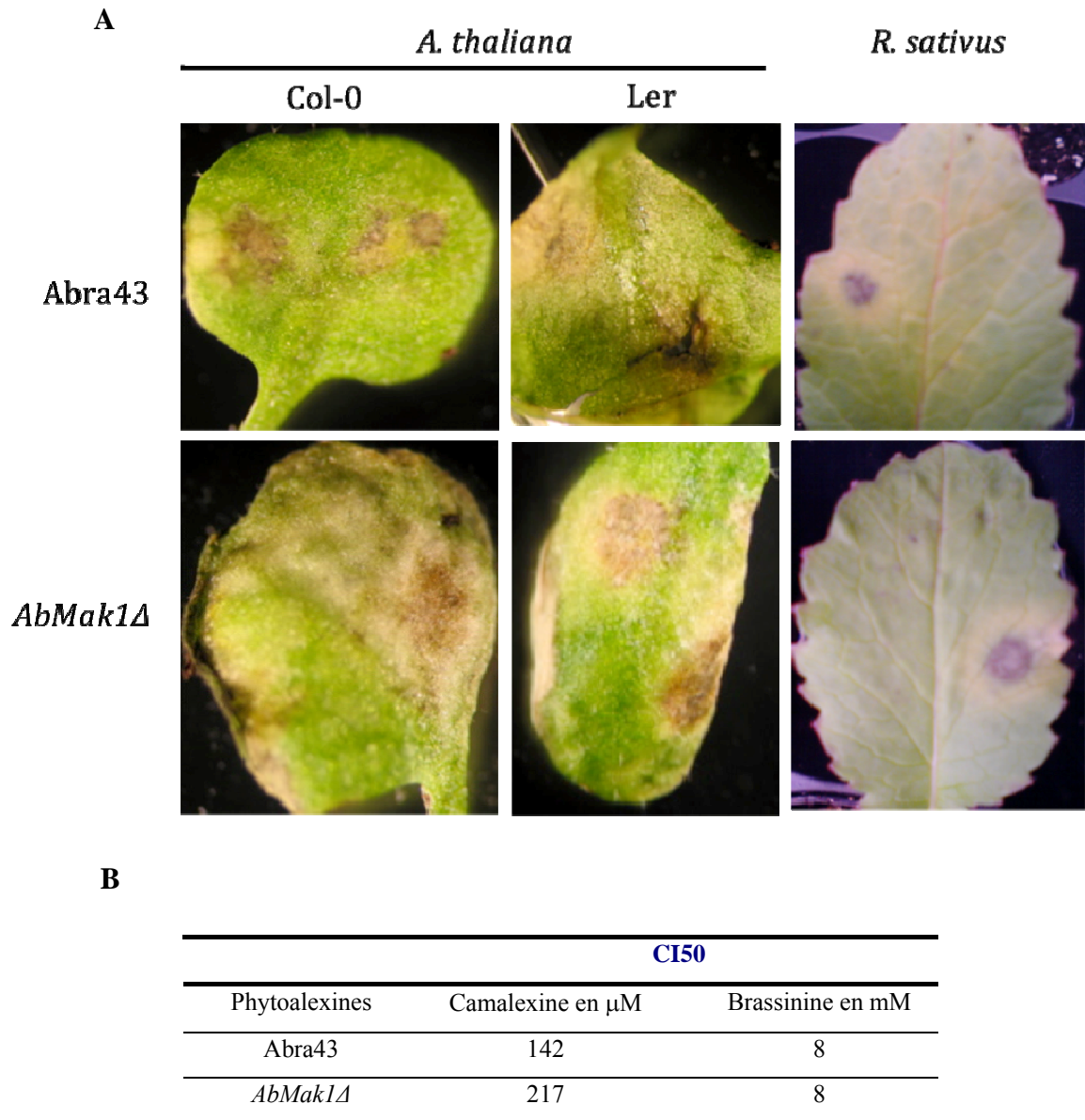
**Figure 21 :** Aspects macroscopiques et microscopiques de la souche sauvage (Abra43) et de la souche mutante AbMak1Δ.

(A) Colonies sur milieu PDA, (B) Conidies (x400),



**Figure 22 :** Images obtenues par microscopie électronique à balayage x10 000 (MEB) et à transmission (MET) de la souche sauvage Abra43 et de la souche mutante Abmak1Δ.

CE : Couche externe ; SW : paroi secondaire ; PW : paroi primaire. La flèche noire représente les incrustations potentielles de mélanine. trait blanc : 1 μm ; trait noir : 200 nm.



**Figure 23** : Analyse du pouvoir pathogène et de la sensibilité aux phytoalexines de la souche sauvage *Abra43* et du mutant *AbMak1Δ*.

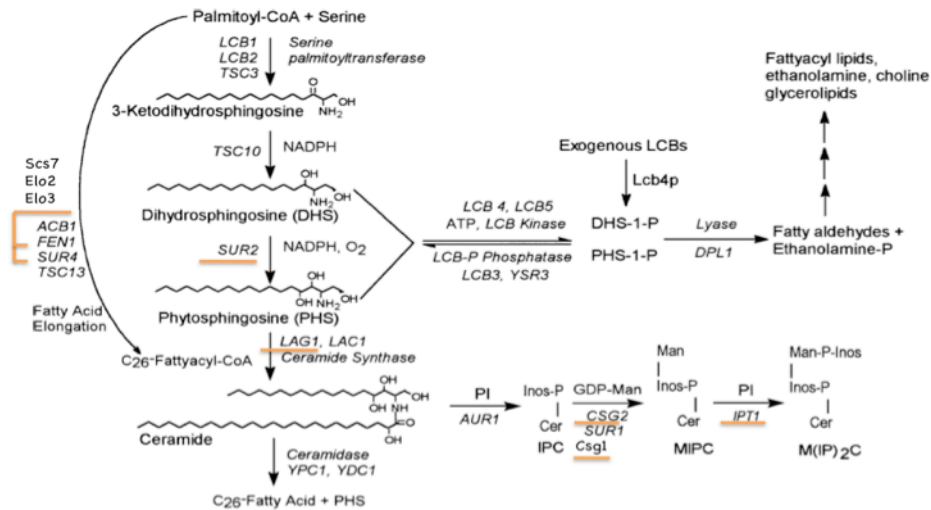
**A** : Analyse du pouvoir pathogène d'*Abra43* et de *AbMak1Δ* sur *A.thaliana* et sur *R.satvius* après 7 jours d'incubation. **B** : Analyse de la croissance radiale d'*Abra43* et *AbMak1Δ* en présence de concentrations croissantes en camalexine et brassinine.

enzyme potentiellement liée au métabolisme des stérols est situé à proximité du gène *AbMak1* sur le génome d'*A. brassicicola* mais également parce que certaines flavoprotéines et cytochromes P450 (CYP51 par exemple) sont nécessaires à la biosynthèse des stérols membranaires. Le mutant *AbMak1Δ1* pourrait donc avoir une composition anormale en stérols membranaires qui, de par leurs interactions avec les sphingolipides, seraient, selon Sellam *et al.* (2007b), impliqués indirectement dans la fixation de la camalexine sur les cellules fongiques.

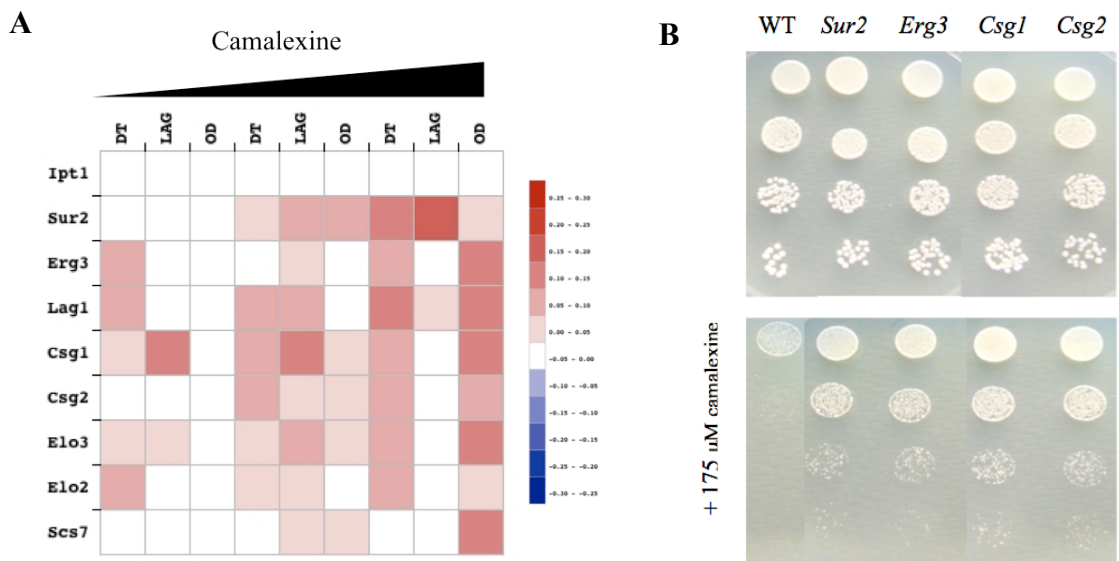
Sur la base de la faible pigmentation des conidies du mutant *AbMak1Δ1*, un rôle de ce gène dans la synthèse de mélanine est également envisageable. Des flavoprotéines sont impliquées dans cette voie métabolique comme la 4-hydroxyphénylacétique acide hydroxylase (HPA), protéine codée par le gène *HpaA* et responsable de la réduction de la mélanine chez la bactérie *Klebsiella pneumoniae* (Gibello *et al.*, 1995). Toutefois aucune des enzymes de synthèse de mélanine DHN (typique des champignons) décrites à ce jour n'est similaire à *AbMak1p*. D'autre part, d'après Sellam *et al.* (2007b), une surexpression de deux gènes clés de la synthèse de mélanine est observée lors de l'exposition d'*A. brassicicola* à la camalexine et ce pigment pourrait avoir un rôle protecteur par imperméabilisation. Un blocage dans cette voie de biosynthèse devrait dès lors se traduire par une hypersensibilité et non une résistance.

Le mutant *AbMak1Δ1* présente une résistance accrue à la camalexine ce qui suggère qu'il aurait un rôle dans l'action cytotoxique de cette molécule. Une des pistes proposées implique un lien avec les stérols membranaires. Nous envisageons donc de caractériser et doser les composés membranaires chez ce mutant. De plus, les tests du pouvoir pathogène sur chou et sur *A. thaliana* seront approfondis avec différentes conditions : différentes concentrations d'inoculum ou blessures des feuilles par exemple. Il serait également intéressant de visualiser le comportement du mutant sur le génotype d'*A. thaliana* ne produisant pas de camalexine, le mutant *Pad3*. De plus, un dosage de mélanine pourrait nous indiquer le lien entre cette protéine et la voie de biosynthèse de la mélanine. Ces données permettraient certainement de mieux comprendre le phénomène de résistance du mutant *AbMak1Δ1* face à la camalexine.

## **2. Cibles cellulaires de la camalexine**



**Figure 24 :** Voie de biosynthèse des sphingolipides chez *S. cerevisiae* (d'après Dickson et al., 2002). Les gènes surlignés en rouge représentent les gènes étudiés dans le phénotypage de mutants de *S. cerevisiae*.



**Figure 25** Résultats du phénotypage de mutants de *S. cerevisiae*

(A) Représentation des LPI (Indice Phénotypique Logarithmique) du phénotypage de mutants de *S. cerevisiae* impliqués dans la biogénèse des sphingolipides et stérols membranaires en fonction de doses croissantes (75, 125 et 250 μM) de camalexine. DT : temps de doublement, LAG : période de latence, OD : accroissement de la population. (B) Dépôt sur milieu YNBA additionné de camalexine de suspensions cellulaires de *S. cerevisiae* calibrées de la souche sauvage WT et de souches mutants.

Plusieurs gènes liés à la synthèse des sphingolipides ou des stérols membranaires sont surexprimés chez *A. brassicicola* après exposition à la camalexine (Sellam *et al.*, 2007b). Les sphingolipides pourraient dès lors être une cible directe ou indirecte de fixation de la phytoalexine. La voie de biosynthèse des sphingolipides chez *S. cerevisiae* est présentée **Figure 24**. Les sphingolipides, tout comme les stérols et les glycérophospholipides, contribuent à la structure des membranes qu'elles soient plasmiques, vacuolaires, golgiennes ou endoplasmiques. De plus, les sphingolipides sont déjà connus pour être la cible de diverses molécules antifongiques (Aerts *et al.*, 2007, Stock *et al.*, 2000, Takemoto *et al.*, 1993).

Dans un premier temps nous avons montré que plusieurs mutants de *S. cerevisiae* déficients pour des gènes de la voie de biosynthèse des sphingolipides présentent une tolérance accrue à la camalexine par rapport à la souche sauvage (**Figure 25**). Certains mutants, comme  $\Delta Sur2$ ,  $\Delta Lag1$ ,  $\Delta Elo3$  ou encore  $\Delta Csg1$  présente une tolérance accrue à la camalexine pour les paramètres étudiés à des doses élevées en camalexine (120 et 250  $\mu\text{M}$ ). Le mutant  $\Delta Ipt1$  ne présente pas de différence de sensibilité à la camalexine comparativement à la souche de référence. Le gène *Ipt1* intervient en fin de voie dans la synthèse du mannose-(inositol-P)<sub>2</sub>-ceramide ce qui pourrait expliquer le phénotype observé.

Dans un second temps, nous avons adopté une stratégie identique à celle décrite précédemment, c'est à dire l'obtention de mutants nuls chez *A. brassicicola* suivie d'un phénotypage. Deux gènes ont été sélectionnés : le gène *Sur2* codant la sphinganine C4-hydroxylase, et le gène *Scs7* codant une sphingolipide alpha-hydroxylase. La disruption de ces deux gènes a été tentée et malgré plusieurs répétitions, seul le mutant *AbScs7 $\Delta$*  a pu être obtenu. Néanmoins, celui-ci croît très lentement et présente une morphologie très altérée. L'étude phénotypique de ce mutant est dès lors très difficile. Le mutant  $\Delta Sur2$  chez *A. nidulans* présente des hyphes très gonflées mais aussi une croissance altérée (Li *et al.*, 2006). Le rôle primordial de ces gènes explique un effet très prononcé voir létal des mutations. Ainsi, nouveaux gènes candidats ont été choisis. Les gènes *Lag1*, *Gcs1*, *Erg5* et *SrbA* ont été sélectionnés pour la suite de l'étude. Le gène *Lag1* est un gène dont la mutation de l'homologue chez *S. cerevisiae* entraîne une résistance vis-à-vis de la camalexine. Il code une céramide synthase intervenant dans la voie de biosynthèse des sphingolipides. Le gène *Gcs1* code une glucosylcéramide synthase. Les cérébrosides

**Tableau 3** Représentation de la sensibilité de la souche sauvage *Abra43* et des souches mutantes dans les voies de biosynthèse des sphingolipides et stérols membranaires par le calcul de la CI50 obtenue avec les pentes des courbes néphélométriques.

Les étoiles indiquent une différence significative par rapport à la souche *Abra43* (Test de student,  $P < 0,01$ ).

<b>Souches</b>	<b>CI50 <math>\mu\text{M}</math></b>
WT	124,3
Lag1.1	132,7
Lag1.2	108,5
Erg5.1	104,3
Erg5.2	113,2
Gcs1.1	100,7
Gcs1.2	68,7*
SrbA.1	84,4*
SrbA.2	92,1*



(glucosylceramide, galactosylceramide) ne sont pas retrouvés chez la levure mais chez les champignons filamenteux, c'est pourquoi le gène *Gcs1* n'a pas été inclus au phénotypage de mutants de *S. cerevisiae*. Le gène *Erg5* code un cytochrome P450 impliqué dans la désaturation des stérols en C-22. Enfin, le gène *SrbA* code un facteur de transcription SREBP pour Sterol Regulatory Element-Binding Protein notamment impliqué dans la régulation de la biosynthèse des stérols et sphingolipides membranaires (Willger *et al.*, 2008). De plus, le gène *SrbA* chez *A. fumigatus* vient d'être très récemment répertorié comme étant impliqué dans la résistance aux composés azolés (Willger *et al.*, 2008).

Les gènes *Lag1*, *Gcs1*, *Erg5* et *SrbA* ont fait l'objet d'une disruption par mutagenèse dirigée. Tout d'abord, une étude macro et microscopique a été initiée et aucune différence notable n'est observée par rapport à la souche sauvage. Les tests de croissance radiale et néphélométrique en présence de camalexine ne montrent pas de différence significative entre la souche de référence et les différents mutants hormis pour les souches mutantes des gènes *Gcs1.2* et *SrbA* (**Tableau 3**).

Ces résultats sont encore préliminaires et devront être complétés notamment avec une analyse du pouvoir pathogène sur chou et sur *A. thaliana*.

Il sera également important de vérifier si ces mutations se traduisent effectivement par des modifications dans la composition en lipides et stérols de la membrane. Pour cela, des tests préliminaires de croissance en condition d'hypoxie pourront être réalisés. La synthèse de stérols membranaires est très coûteuse en oxygène et une mesure de la croissance en condition d'hypoxie pourrait être indicateur d'une perturbation dans la teneur en stérols du mutant. De plus, un dosage direct de la composition en stérols mais également sphingolipides membranaires nous permettrait de mieux préciser l'impact des mutations.

## **Discussion Générale et perspectives**

L'objectif de ce travail était d'étoffer les connaissances concernant les facteurs de pathogénie d'*A. brassicicola*. Parmi les différents déterminants de la pathogénie, nous nous sommes intéressés à ceux liés aux réponses du champignon à l'exposition aux phytoalexines de type indolique de l'hôte et aux mécanismes de protection contre ces défenses chimiques.

En préambule de cette étude, une nouvelle technique de phénotypage des champignons filamenteux a été mise au point. Les techniques utilisées préalablement en routine au laboratoire présentaient de nombreux inconvénients et il a été nécessaire de mettre au point une technique haut débit d'étude de la croissance des champignons. C'est pourquoi, la néphélométrie a été paramétrée de manière à pouvoir visualiser la croissance du champignon *A. brassicicola* mais également d'autres champignons, filamenteux ou levuroïdes, dans un système microplaque et sur des temps de culture très courts. Cette technique permet de comparer les courbes de croissance de différents génotypes dans différentes conditions de culture. Il a été ainsi possible de caractériser les effets de molécules antifongiques sur la croissance de divers mutants d'*A. brassicicola* obtenus au laboratoire. Les résultats issus de cette technique permettent une analyse statistique des courbes de croissance mais également de visualiser des différences fines de réactions de chaque génotype.

Mes recherches ont ensuite concernées l'étude de l'implication des voies de signalisation, dans la réponse du champignon *A. brassicicola* aux phytoalexines camalexine et brassinine. Sellam *et al.*, (2007a) ont montré l'action antimicrobienne de ces deux phytoalexines et ont émis l'hypothèse de la mise en place d'un mécanisme compensatoire visant à renforcer l'intégrité cellulaire du champignon face à la charge toxique de ces molécules. Les résultats obtenus durant cette thèse montrent l'implication des deux MAP kinases, Hog1p et Slt2p, dans la réponse à ces métabolites défense. Les deux phytoalexines activent par phosphorylation la MAP kinase Slt2p de façon Hog1-dépendante et les mutants nuls disruptés dans les gènes correspondants à ces MAP kinases sont hypersensibles à la brassinine et la camalexine. Ces observations sont à rapprocher de celles décrites pour la réponse de la levure à la Zymolase (Bermejo *et al.*, 2008) et suggèrent l'existence de connections étroites en ces deux voies. De plus, les deux types de mutants sont caractérisés par une baisse significative de leur agressivité *in planta* ce qui démontre le rôle prépondérant des deux MAP kinases dans le pouvoir pathogène d'*A.*

*brassicicola*. Pour conclure, nos travaux ont montré que les deux MAP kinases sont impliquées dans l'adaptation du champignon face aux phytoalexines de type indolique mettant en jeu l'activation d'un mécanisme compensatoire se traduisant par un renforcement des parois et de la membrane. Toutefois plusieurs questions restent posées : Y a-t-il un senseur spécifique du stress provoqué par les phytoalexines indoliques et si oui quel est-il ? Quels sont les éléments de connexion entre Hog1p et Slt2p ? quelles sont les cibles de cette réponse adaptative ?

Afin de répondre à certaines de ces questions, une stratégie couplant l'étude globale du phosphoprotéome et du transcriptome d'*A. brassicicola* est envisagée. Celle-ci sera conduite en comparant les niveaux de phosphorylation des protéines et d'expression des gènes en condition soit témoin soit de stress provoqué par la camalexine et ce dans différents fonds génétiques. Cette étude permettra de caractériser la réponse Hog1- et/ou Slt2- dépendante et ses régulations au niveau transcriptionnel et post-traductionnel. Une stratégie similaire est déjà en cours au laboratoire pour la caractérisation des phosphoprotéomes de réponse aux phénylpyrroles chez *A. brassicicola* et *B. cinerea* en collaboration avec l'UMR Bioger de Grignon et la plateforme PAPPSO du Moulon.

Une approche plus ciblée est également envisageable pour identifier d'éventuels senseurs des phytoalexines. Ainsi par exemple, par référence aux travaux réalisés sur le stress provoqué par la zymolase chez la levure (Garcia *et al.*, 2009), il est envisagé de caractériser chez *A. brassicicola* l'orthologue de Sho1p en lien avec son rôle possible dans l'activation des voies HOG et CWI par la camalexine. D'autre part, même si nous avons montré que l'activation de Hog1p par la camalexine était indépendante de l'histidine kinase de groupe III Nik1, il est possible d'émettre l'hypothèse d'un rôle d'une des 15 autres histidine kinases produites par *A. brassicicola* dans la perception de ce type de stress.

Les travaux réalisés dans le cadre de cette thèse ont également permis d'identifier un lien entre la réponse au stress de sécrétion chez *A. brassicicola* et l'adaptation face aux phytoalexines indoliques. Ainsi nous avons montré que AbHacAp, orthologue chez *A. brassicicola* du facteur de transcription Hac1p contrôlant l'UPR chez les champignons, était activé par épissage lors de l'exposition à la camalexine. Le mutant *AbHacAA* est avirulent, présente une très forte sensibilité aux phytoalexines, des hyphes déformées mais

## Camalexine

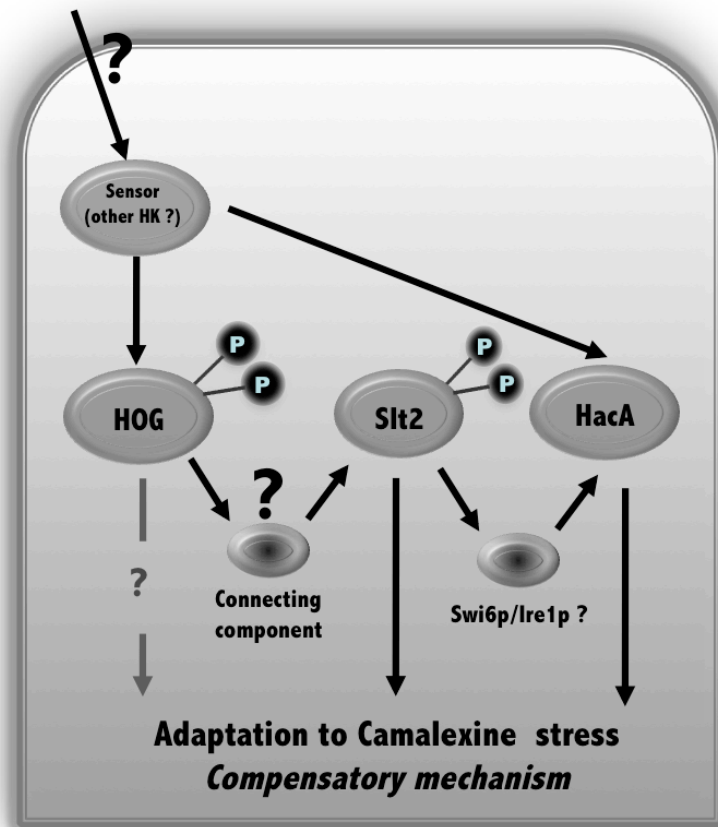


Figure 26 Schéma bilan du travail de thèse.

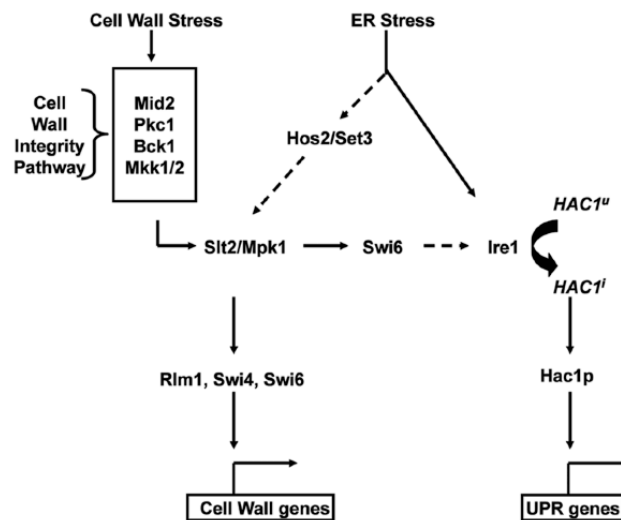


Figure 27 Schéma du lien qui existe entre la voie de l'UPR et la voie CWI (d'après Krysan, 2009).

également une paroi cellulaire déstructurée. De plus, il présente une très forte diminution de sa capacité de sécrétion suggérant une double implication de ce facteur de transcription dans le pouvoir pathogène du champignon : contrôle de la sécrétion d'enzymes lytiques nécessaires au développement du champignon dans les tissus hôtes et rôle dans le mécanisme compensatoire mis en place par le pathogène pour se protéger contre les défenses de l'hôte.

Là encore, il convient de se poser la question d'un éventuel lien entre la voie de signalisation UPR et les voies CWI et HOG (**figure 26**). L'activation de l'UPR en réponse à un stress pariétal a été caractérisée chez *S. cerevisiae* (Scrimale *et al.*, 2009) mais également chez *C. albicans* et *A. fumigatus* où l'inactivation de l'UPR entraîne un défaut pariétal et une forte sensibilité aux antifongiques (Richie *et al.*, 2009, Wimalasena *et al.*, 2008). Chez *S. cerevisiae*, le lien entre l'UPR et la voie CWI s'effectuerait essentiellement au niveau du facteur de transcription Swi6p et de la MAP kinase Ire1p (Krysan, 2009) (**Figure 27**). Dans un premier temps, il sera possible à l'aide des mutants dont nous disposons de vérifier si l'activation de *AbHacA* par la camalexine a toujours lieu chez un mutant *AbHog1Δ* ou un mutant *AbSl2Δ*. En complément il serait intéressant d'étudier l'impact de la mutation dans le gène *Swi6* chez *A. brassicicola* sur l'activation de *AbHacA* en présence de camalexine.

Nos recherches ont permis de mettre en évidence l'implication de la voie CWI dans le pouvoir pathogène d'*A. brassicicola*. L'agressivité des mutants *AbSl2Δ1* et *AbHog1Δ1* est significativement diminuée *in planta* en lien avec une hypersensibilité aux phytoalexines indoliques. Ces observations nous amène à envisager une approche appliquée sur l'utilisation d'inhibiteurs des voies de signalisation comme agents synergisant l'effet des phytoalexines et ainsi, mettre en place une méthode de lutte complémentaire des SDN (Stimulateurs de Défenses Naturels).

Des approches similaires ont déjà été réalisés chez *C. albicans* où l'effet synergique de la cercosporamide, inhibiteur de la voie CWI (Sussman *et al.*, 2004), et le fluconazole ou encore la micafongine aboutit à une très forte diminution de la croissance du champignon

(Sussman *et al.*, 2004, LaFayette *et al.*, 2010). Ces auteurs ont montré que l'addition de 100 µg/mL de cercosporamide et de micafongine (2 µg/mL) ou de fluconazole (8 µg/mL) aboutissait à une inhibition totale de la croissance du pathogène *C. albicans* confirmant leur effet synergique. Les tests ayant été réalisés à des doses où elles n'ont pas seules d'activité inhibitrice.

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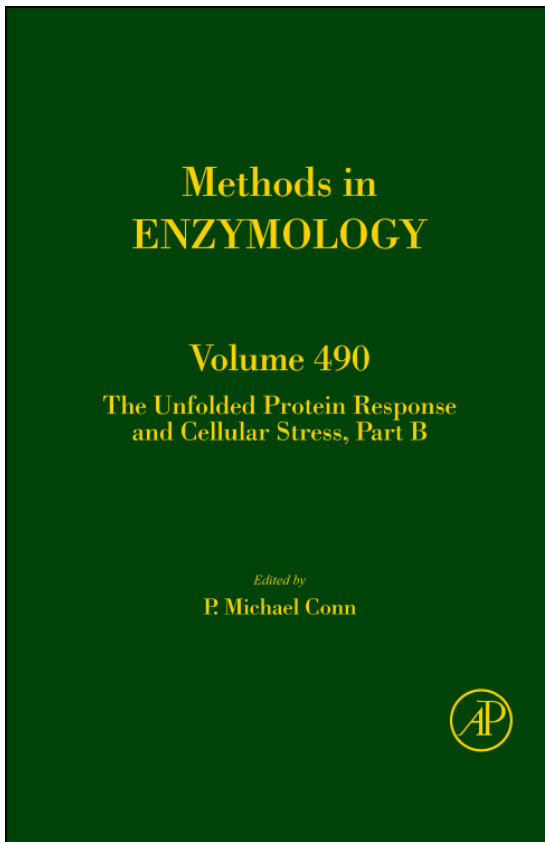
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# **Annexe**

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# METHODS FOR INVESTIGATING THE UPR IN FILAMENTOUS FUNGI

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## Abstract

Filamentous fungi have a high-capacity secretory system and are therefore widely exploited for the industrial production of native and heterologous proteins. However, in most cases, the yields of nonfungal proteins are significantly lower than those obtained for fungal proteins. One well-studied bottleneck appears to be the result of slow or aberrant folding of heterologous proteins in the ER during the early stages of secretion within the endoplasmic reticulum, leading to stress responses in the host, including the unfolded protein response (UPR). Most of the key elements constituting the signal transduction pathway of the UPR in *Saccharomyces cerevisiae* have been identified in filamentous fungi,

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including the central activation mechanism of the pathway, that is, the stress-induced splicing of an unconventional (nonspliceosomal) intron in orthologs of the *HAC1* mRNA. This splicing event relieves a translational block in the *HAC1* mRNA, allowing for the translation of the bZIP transcription factor Hac1p that regulates the expression of UPR target genes. The UPR is involved in regulating the folding, yield, and delivery of secretory proteins and that has consequences for fungal lifestyles, including virulence and biotechnology. The recent releases of genome sequences of several species of filamentous fungi and the availability of DNA arrays, GeneChips, and deep sequencing methodologies have provided an unprecedented resource for exploring expression profiles in response to secretion stresses. Furthermore, genome-wide investigation of translation profiles through polysome analyses is possible, and here, we outline methods for the use of such techniques with filamentous fungi and, principally, *Aspergillus niger*. We also describe methods for the batch and controlled cultivation of *A. niger* and for the replacement and study of its *hacA* gene, which provides either a UPR-deficient strain or a constitutively activated UPR strain for comparative analysis with its wild type. Although we focus on *A. niger*, the utility of the *hacA*-deletion strategy is also described for use in investigating the virulence of the plant pathogen *Alternaria brassicicola*.

## 1. INTRODUCTION

Filamentous fungi have conquered an astonishingly wide range of habitats, and individual species may be saprobic, pathogenic, or mutualistic partners (e.g., in lichenous or mycorrhizal associations). Their dispersal may be facilitated by the production and release of numerous spores (sexual or asexual), and colonization of food sources can also occur either more locally or over large areas by growth as a system of branched tubular bodies called hyphae. The expression and secretion of proteins underpin the saprobic lifestyle (e.g., by expressing hydrolase enzymes) and may also be important in virulence (e.g., by cell-surface presentation of adhesins and by secretion of lytic enzymes) and play an essential role in decomposition and recycling of organic matter in nature.

Although there are still deficits in our understanding of the fundamental mechanisms of the secretory pathway in filamentous fungi, the recent availability of the whole genome sequences of fungi (currently, several hundred yeasts and filamentous fungi, either sequenced or in progress), including *Aspergillus* species widely exploited commercially for their secreted enzymes (Machida *et al.*, 2005; Pel *et al.*, 2007), has provided a wealth of information. It is generally accepted that this pathway does not differ fundamentally from those in *S. cerevisiae* and higher eukaryotes, even though there are differences of detail (Geysens *et al.*, 2009). Key differences arise from the hyphal and branching phenotype of filamentous fungi and the



polarity of both growth and protein secretion that occurs primarily at the hyphal tips (Conesa *et al.*, 2001; Fischer *et al.*, 2008; Harris, 2008; Shoji *et al.*, 2008). As in other eukaryotes, the secretory route begins with the entry of secretory proteins into the endoplasmic reticulum (ER) either during or after translation. During transit through the ER, proteins undergo assisted folding and additional modifications such as signal peptide processing, glycosylation, disulfide bond formation, phosphorylation, and subunit assembly. Correctly folded proteins are sorted into coating-protein II vesicles (COPII) and then delivered to the Golgi (or fungal equivalent) for other modifications such as further glycosylation and peptide processing. Finally, the mature proteins are packed again into secretory vesicles and are delivered by exocytosis to the extracellular space at the hyphal tip.

This high-capacity secretory system has driven the commercial exploitation of filamentous fungi as cell factories for provision of native or heterologous enzymes that are used in a wide variety of applications. The progress in genetic manipulation and the availability of gene-transfer systems, combined with process improvements, have led to enhanced yields of native proteins and provided possibilities for improving the yields of heterologous proteins of both fungal and nonfungal origins. However, fungi often fail to secrete the heterologous proteins to the same high level as their own proteins, and this is especially so when the gene donor is not a fungus. Although factors negatively influencing the yield seem to be multiple (Gouka *et al.*, 1997), protein maturation in the ER is regarded as the major bottleneck to achieving high-secreted yields of at least some heterologous proteins from filamentous fungi. Many strategies have been applied to address those limitations and include manipulations of the secretory pathway and, in particular, the ER lumenal environment by overexpression of genes encoding foldases and chaperones or enzymes of the glycosylation machinery. Furthermore, increased gene dosage, optimized codons, use of protease-deficient host strains, expression as translational fusions with an efficiently secreted protein, and introduction of efficient secretion signals or prosequences—have all been used to some advantage (Archer and Turner, 2006; Conesa *et al.*, 2001; Lubertozzi and Keasling, 2009).

In yeast, the ER plays a pivotal role for quality control of proteins by ensuring that correctly folded proteins are delivered to subsequent cellular compartments. A variety of adverse physiological and environmental conditions can disturb ER homeostasis and lead to the accumulation of misfolded proteins. This is the case in expression systems, where there is a high flux of proteins being translocated into the ER. The folding, assembly, and secretion machinery may become saturated, leading to improperly folded structures or protein aggregates that are not secreted. To cope with ER stress, the intracellular signaling pathway, termed the unfolded protein response (UPR), is activated and triggers an extensive transcriptional response (Travers *et al.*, 2000) that increases the protein-folding capacity

within the ER. The UPR is also intimately linked to proteolytic systems that deliver misfolded proteins to vacuoles (autophagy) or to proteasomes (ER-associated degradation—ERAD) for degradation. In yeast, the basic leucine zipper (bZIP)-type transcription factor Hac1p is the transcriptional regulator of the UPR. Hac1p synthesis is dependent on the splicing of an unconventional (nonspliceosomal) intron in the *HAC1* mRNA initiated by the ER-located transmembrane kinase and endoribonuclease Ire1p (Rueggsegger *et al.*, 2001). This splicing event is activated in response to ER stress and relieves a translational block, allowing for the synthesis of Hac1p that regulates the expression of UPR target genes. Most of the key elements constituting the signal transduction pathway of the yeast UPR have been identified in filamentous fungi, and the central activation mechanism of the pathway, that is the stress-induced splicing of an unconventional intron, is conserved among filamentous fungi, yeast, and even mammalian cells (Kohno, 2010). An additional feedback mechanism that leads to selective transcriptional downregulation of some genes that encode secreted enzymes is termed repression under secretion stress (RESS) and has been reported in *Trichoderma reesei* and *Aspergillus niger* (Al-Sheikh *et al.*, 2004; Pakula *et al.*, 2003). Taken together, these ER-stress responses diminish the pool of newly synthesized proteins and provide homeostatic protection for the host cell. Thus, understanding more in detail about the process of protein maturation and secretion-related stress in filamentous fungi may hold the major key to improving their use as cell factories.

The genome sequences of filamentous fungi (including several *Aspergillus* spp.) and the availability of DNA GeneChips have provided an unprecedented resource for exploring expression profiles in response to particular environmental cues, including various secretion stresses (Breakspear and Momany, 2007). Transcriptomics to investigate the UPR in fungi was first used in *Aspergillus nidulans* and *T. reesei* (Arvas *et al.*, 2006; Sims *et al.*, 2005). In *A. nidulans*, the authors reported the effects of recombinant protein secretion by comparing a bovine chymosin-producing strain with its parental wild-type strain by using expressed sequence tag microarrays, which covered approximately one-third of the predicted open reading frames. In *T. reesei*, cultures expressing the heterologous protein tissue plasminogen activator (t-PA) and cultures treated with the reducing agent dithiothreitol (DTT) were analyzed with cDNA subtraction and cDNA-amplified fragment length polymorphism (AFLP). A genome-wide expression analysis of secretion stress responses in *A. niger* was reported by Guillemette *et al.* (2007). In that study, ER-associated stress was induced either by chemical treatment of the wild-type cells with DTT or tunicamycin, or by expressing t-PA. The predicted proteins encoded by most of the upregulated genes functioned as part of the secretory system including chaperones, foldases, glycosylation enzymes, vesicle transport proteins, and ERAD proteins. The authors also investigated translational regulation under ER stress by polysomal

fractionation. Combining proteomic and transcriptomic profiling of the events following protein secretion stress should lead to a better understanding of the molecular basis of protein secretion and provide targets for strain improvement. Indeed, further transcriptomic studies of *A. niger* in relation to hyphal development and branching, and to protein secretion, have been described and illustrate the value of transcriptomic data and, more broadly, integration of data from the transcriptome and proteome (Jacobs *et al.*, 2009; Jorgensen *et al.*, 2009; Meyer *et al.*, 2009).

We describe some of the methodologies that have been used in the genome-wide analysis of protein secretion stress in filamentous fungi, taking *A. niger* as our model organism. We give less emphasis to transcriptomic analyses because methods are well described in several papers already cited but are also evolving rapidly, for example, with the advent of deep sequencing. We therefore devote more coverage to the preparation of samples for analysis, and include cultivation and the construction and use of *hacA*-deletion strains and of constitutive *hacA* mutants. Deletion of *hacA* from *A. niger* has been reported (Carvalho *et al.*, 2010; Mulder and Nikolaev, 2009) and shown to induce a severe phenotype that emphasizes the important role for HacA in the biology of the fungus. Deletion of *HAC1* was also achieved in the dimorphic fungus *Candida albicans* (Wimalasena *et al.*, 2008), and the deletion strain was less able to produce filaments and was down-regulated for expression of some cell-surface adhesins, suggesting that Hac1p would have a role in the invasive virulence of *C. albicans*. The *hacA* gene was deleted from another fungal pathogen, *Aspergillus fumigatus*, and virulence tests showed that the knockout strain was indeed attenuated (Richie *et al.*, 2009). Because of the importance of Hac1/A in virulence, we also provide an example of deleting the gene from a plant pathogen, *Alternaria brassicicola*.



## 2. CONDITIONS TO STUDY THE UPR IN FILAMENTOUS FUNGI

In this section, we discuss the growth conditions and ER-stress-inducing methods that can be used with *A. niger* for producing fungal material destined for transcriptomic or polysome analyses.

### 2.1. Culture conditions

Filamentous fungi can grow either in pellets or as a dispersed mycelium. For our transcriptomic studies on UPR, a dispersed growth of the mycelium is preferred as this reduces heterogeneity of the mycelia. Growth in pellets is caused by an early aggregation of conidial spores and, after subsequent germination of the spores, micropellets are formed. Upon further growth,

these pellets increase in size which results quickly in low oxygen concentration in the center of the pellet, which increases heterogeneity. With the protocol indicated below, reproducible dispersed growth of mycelia can be obtained.

In our UPR studies, we have used two methods of cultivation to perform transcriptomic studies. The choice of cultivation, either a batch culture, or a chemostat culture, depends on the purpose. We have used controlled batch cultivations to examine the response of *A. niger* cells to drugs that interfere with protein folding. This protocol is of course not limited to study the effect of UPR-inducing compounds, but can also be used to study the effect of other compounds that affect fungal growth (Meyer *et al.*, 2007). For these studies, spores were germinated for 5 h which resulted in the formation of a small (20  $\mu\text{m}$  long) germtube, providing a more uniform population of cells than older, morphologically more heterogeneous, cells. At this stage, the antifungal compound is added.

Controlled batch cultivations can also be used to compare gene expression between different mutants. One important consideration here is that for a reliable comparison, the maximum growth rate during batch growth of both strains should be identical. Before performing transcriptomic studies on different mutants, it is important to establish that both mutants have an identical maximum specific growth rate. If not, the transcriptomic study will not only identify differences related to gene expression directly caused by the mutation, but will also identify differences related to a different growth rate. To prevent difference in growth rate to be reflected in the transcriptome, continuous cultivations should be performed in which the growth rate can be controlled (Jorgensen *et al.*, 2009). Here, we provide detailed conditions about controlled batch cultivation of *A. niger* in bioreactors.

### 2.1.1. Required materials

**2.1.1.1. Devices of the bioreactor apparatus** Bioreactor cultivations are performed in Bioflo3000 bioreactors (New Brunswick Scientific). Using the NBS Biocommand software, the pH, temperature-dissolved oxygen tension and agitation can be controlled and monitored. The pH is measured using an autoclavable glass electrode (Mettler Toledo) and the dissolved oxygen tension is measured with an InPro-6000 series  $\text{O}_2$  sensor (Mettler Toledo). For measuring the content of  $\text{CO}_2$  and  $\text{O}_2$  in the exhaust gas, a Xentra 4100C Gas Purity analyzer (Servomex BV, the Netherlands) is used. To minimize wall growth in the head space of the reactor, the glass surface of the head space is cooled by tubing through which cold water is flowing.

#### 2.1.1.2. Additional materials

- *Strains of interest:* The *A. niger* strains used in our UPR studies are all derived from the N402 strain. This strain is a UV-mutagenized derivative

of strain N400 that produces short conidiophores and is often used as a starting strain for genetic modifications (Guillemette *et al.*, 2007; van Hartingsveldt *et al.*, 1987). In our bioreactor controlled cultivations that are used for transcriptomic studies, only prototrophic strains are used to prevent effects on gene expression as a result from supplementation of any auxotrophy, for example, by uridine.

- **Growth medium:** A defined minimal medium (MM) is used to perform bioreactor cultivation. The growth-limiting component of the medium is the carbon source (0.75%, w/v). The MM contains the following: 7.5-g glucose, 4.5-g NH<sub>4</sub>Cl, 1.5-g KH<sub>2</sub>PO<sub>4</sub>, 0.5-g KCl, 0.5-g MgSO<sub>4</sub>·7H<sub>2</sub>O, and 1-ml trace metal solution per liter. The trace metal solution contains per liter, 10-g EDTA, 4.4-g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1.01-g MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.32-g CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.315-g CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.22-g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 1.47-g CaCl<sub>2</sub>·2H<sub>2</sub>O, and 1-g FeSO<sub>4</sub>·7H<sub>2</sub>O (modified from composition given by Vishniac and Santer (1957)). The pH is adjusted to 3. The carbon source is heat-sterilized separately from the MM. Germination of conidial spores is improved by adding 0.003% (w/w) yeast extract to the culture medium.

Complete medium plates contain per liter 10-g glucose, 6.0-g NaNO<sub>3</sub>, 1.5-g KH<sub>2</sub>PO<sub>4</sub>, 0.5-g KCl, 0.5-g MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0-g caseamino acids, 5.0-g yeast extract, 20-g agar, and 1-ml trace metal solution.

#### 2.1.1.3. Other reagents

- Detergent solution (Tween80 0.05%, w/v, NaCl 0.9%, w/v)

#### 2.1.1.4. Disposables

- Petri dishes

### 2.1.2. Inoculation

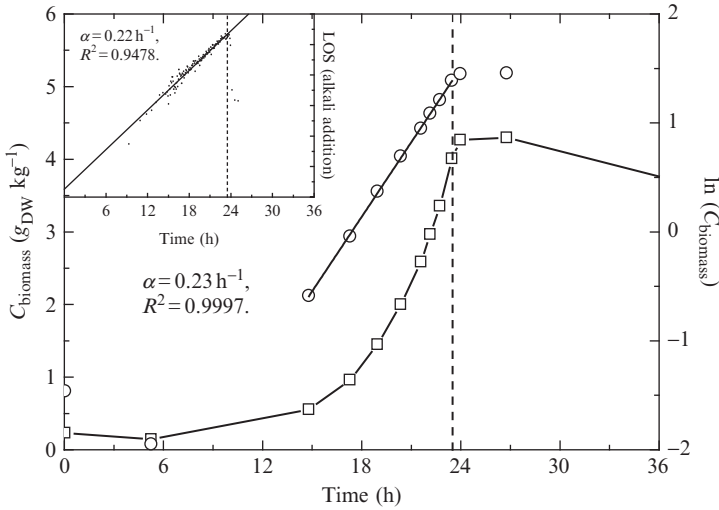
Conidia for inoculation of bioreactor cultures are harvested from solidified complete medium plates with sterile detergent solution and sterile cotton sticks to scrape off the spores. Bioreactors containing 5 l of MM are inoculated with  $1 \times 10^9$  conidia/l. During cultivation, the temperature is maintained at 30 or 37 °C and the pH is kept at 3, by computer-controlled addition of 2-M NaOH or 1-M HCl. During the first 6 h of cultivation, the culture is aerated through the head space of the reactor vessel and the stirrer speed is kept low at 250 rpm to prevent hydrophobic conidia escaping from the water. After 6 h, allowing for germination of the conidia (now hydrophilic), sterile air is sparged into the culture and mixing is intensified (750 rpm). During the growth, the dissolved oxygen tension is kept above 40% of air saturation at any time, ensuring sufficient oxygen for growth.

**2.1.2.1. Short batch cultivations** To examine transcriptomic responses to growth-disturbing compounds or antifungals, germlings are grown for 5 h at 37 °C before addition of the compound. Small volume sampling (10 ml) can be performed at various times after exposure, and we normally sample at 15-min intervals for microscopic analysis. Large sampling volumes (400 ml) of culture are taken after 1 or 2 h after addition of the antifungals, and mycelial samples are quickly harvested via filtration and frozen using liquid nitrogen. Sampling of a 400-ml culture yields enough biomass for RNA extraction to perform Affymetrix microarray analysis (Meyer *et al.*, 2007). DDT and tunicamycin are the most commonly used compounds to induce an UPR response. In common with other antifungals, the effect of the response is dependent on the concentration of the compound and the concentration of the cells. To study transcriptomics responses, we use sublethal concentrations of the antifungal compound and monitor the response of the cells shortly after adding the compound (within 1 h) to minimize secondary effects. Concentrations of the antifungal drug used should be empirically determined.

**2.1.2.2. Prolonged batch cultivation** After 6 h of cultivation and the start of sparger aeration and increasing the stirrer speed to 750 rpm, 0.01% polypropyleneglycol (PPG 2000, Fluka Chemika) is added to the medium as an antifoam agent. Acidification of the culture and the amount of NaOH used to maintain the pH at 3 is used as an indirect measure for growth. Dissolved oxygen tension is always above 40% of air saturation at any time ensuring oxygen-sufficient growth. During cultivation, samples are drawn regularly to monitor culture growth. Medium can be analyzed for residual carbon source and extracellular protein concentrations are determined to calculate protein production yields. Dry weight biomass concentration in the culture is determined by weighing lyophilized mycelia from a known weight of culture. Mycelium is separated from the culture broth by filtration through a GF/C glass microfiber filter (Whatman) and dried. The end of the batch phase (depletion of glucose) can be monitored by plotting biomass concentrations as well as monitoring the alkali addition. An example of a typical growth profile of a batch culture of *A. niger* is shown in Fig. 1.1.

## 2.2. Induction of ER-associated stress

The UPR is classically induced by treating mycelium cultures with two chemical agents which disrupt protein folding in the ER. DTT is a strong reducing agent that disturbs the oxidative environment of the ER and prevents disulfide bond formation. Tunicamycin is a drug produced by *Streptomyces lysosuperificus*, which inhibits N-linked glycosylation by preventing core oligosaccharide addition to nascent polypeptides and thereby blocks protein folding and transit through the ER. To assess the UPR under

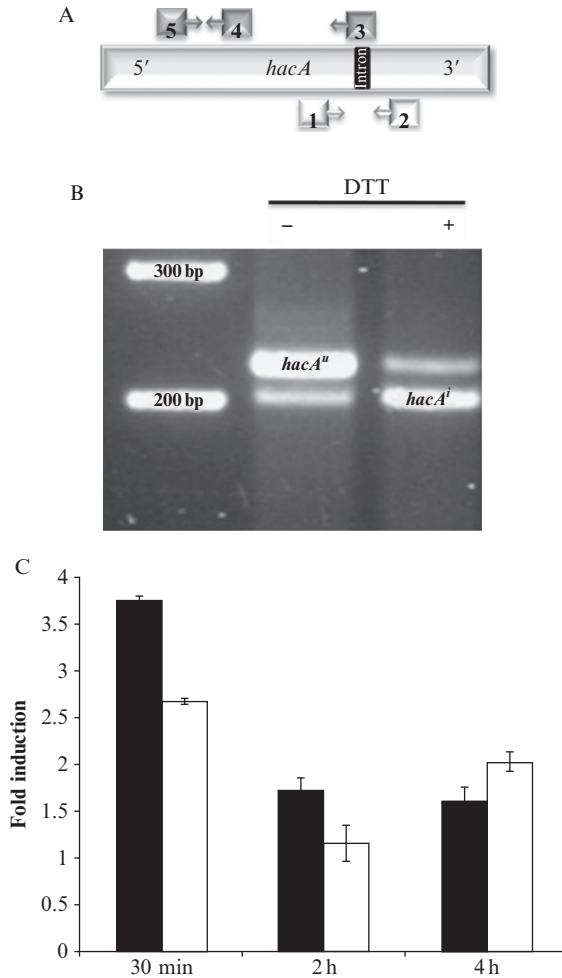


**Figure 1.1** Growth profile of *A. niger* wild-type batch culture. Dry weight biomass concentration ( $\text{g}_{\text{DW}} \text{kg}^{-1}$ ) as a function of time (h) illustrates the growth of the cultures. The maximum specific growth rate is determined from the slope ( $\alpha$ ) of the  $\ln$  transformation of biomass ( $C_{\text{biomass}}$ ) in the exponential growth phase as a function of time (h), as well from log transformation of alkali addition as a function of time (h) (see insert). Dash-line represents the end of the exponential growth phase (depletion of glucose).

conditions of heterologous protein production, expression profiles from a recombinant protein-producing strain and its parental wild-type strain can be compared. Thus, we have used a strain-producing recombinant t-PA, a serine protease. We recommend the use of at least two distinct UPR inducer treatments since discrepancy between responses is sometimes obtained. We know in particular that DTT has a variety of other effects on the cell and triggers a relatively large overall variation in gene expression levels compared to other treatments. The responses to each stress are then compared and the overlaps common to these conditions should lead to the identification of robust sets of UPR-regulated genes.

Before performing transcriptomic or polysome analysis, we confirm that each of the stress conditions leads to induction of the UPR by examining the transcriptional induction (by northern hybridization or real-time PCR) of genes known to be affected by ER stress, such as the ER chaperone-encoding gene *bipA* and the foldase-encoding gene *pdiA* (data not shown). The splicing of the *hacA* transcript also needs to be examined. This could be done from RNAs derived from treated or untreated cultures by using different methods: (i) by conventional RT-PCR with primers designed across the atypical intron (Guillemette *et al.*, 2007), (ii) by northern hybridization by using a cDNA fragment comprising the region from the start

codon up to the unconventional intron as a probe (Mulder *et al.*, 2004), (iii) by real-time RT-PCR by using a specific primer designed to span the atypical 20-bp intron (Fig. 1.2). A quantitative RT-PCR method was



**Figure 1.2** Analysis of the splicing of the *hacA* transcript in *A. niger* by PCR technology. (A) Schematic representation of the *hacA* ORF showing the positions of primers used for RT-PCR analysis. (B) Conventional RT-PCR analysis of *hacA* processing with primers 1 and 2 designed across the intron in the presence (+) or absence (-) of 20-mM DTT for 1 h. (B) Analysis of *hacA* expression by means of real-time RT-PCR in a wild-type strain during DTT exposure (20 mM) for 0.5, 2, and 4 h. The white box represents the fold induction observed with primers 4 and 5 designed for amplifying the cDNA derived from *hacA* mRNA (spliced and unspliced). The black boxes represent the fold induction observed with primers 1 and 3 designed to span the atypical 20-bp intron and are therefore specific for the spliced form, *hacA<sup>i</sup>*.



initially developed to measure the splicing ratio of the mammalian *XBP1* mRNA (Hirota *et al.*, 2006) and was also recently successfully applied to filamentous fungi (Joubert *et al.*, unpublished). The results in Fig. 1.2 clearly show an increase in the abundance of a shorter product following ER stress, indicating that treatment (DTT in this case) induces the conversion of the unspliced *hacA* mRNA (*hacA<sup>u</sup>*) into the spliced form (*hacA<sup>s</sup>*).

ER-stress reagents (DTT or tunicamycin) are added to fungal cultures. DTT stocks are prepared in water and the tunicamycin stock in DMSO. DTT or tunicamycin is added to the liquid medium at a final concentration of 20 mM or 10 µg/ml, respectively. Note that these concentrations are 10-fold higher than those routinely used with yeast. Control cultures have an equivalent volume of sterile water or DMSO added. In *A. niger*, expression analyses show that both upregulation of UPR target genes and appearance of the active *hacA* mRNA emerge within 30 min after exposure of the mycelium to DTT, and are still observed 2 h after adding the DTT (Guillemette *et al.*, 2007; Mulder *et al.*, 2004). Like DTT treatment, tunicamycin treatment and production of the recombinant t-PA lead to the activation of the UPR, although the effect appears later (1 h after induction of the ER stress) than in the DTT-treated mycelia.

### 3. ANALYSIS OF EXPRESSION DURING THE ER STRESS

#### 3.1. Transcriptomic analysis

The genome sequences of two different strains of *A. niger* have underpinned the construction of two different microarrays in the Affymetrix format (Andersen *et al.*, 2008; Pel *et al.*, 2007), and one of those arrays is a trispecies array affording comparisons between *A. niger*, *A. nidulans*, and *Aspergillus oryzae* (Andersen *et al.*, 2008). The Affymetrix protocols for transcriptomics and analysis are available on-line and have proved to be very effective for transcriptomic studies with *A. niger* (Guillemette *et al.*, 2007; Jorgensen *et al.*, 2009; Vongsangnak *et al.*, 2010). Despite the availability of GeneChips for *A. niger*, other array formats have been used for *A. niger* and many other species of filamentous fungi and so, it is not feasible to present a preferred method here. Instead, we advise that the cited papers are referred to for *A. niger*, and methods for other formats and fungal species are explored through the literature. The advent of nonarray methodologies for genome-wide transcriptomics brings new opportunities and these methods are beginning to be applied to filamentous fungi. There are various platforms available for the so-called deep sequencing (Bashir *et al.*, 2010) and, while such methods have been applied in the sequencing of fungal DNA, for example, in the comparison of mutant strains (e.g., Le Crom *et al.*, 2009), it is the use of RNA sequencing that concerns us here.

Transcript sequencing holds some advantages over array approaches by providing, for example, quantitative comparisons of the abundance of different transcripts and in the detection and quantification of transcript variations, for example, in splicing (Wang *et al.*, 2009). We anticipate reports of using such approaches to investigate further the UPR in filamentous fungi.

## 3.2. Polysome analysis

Expression profiling data are more meaningful when mRNA samples are enriched for transcripts that are being translated (Pradet-Balade *et al.*, 2001). This can be achieved by fractionation of cytoplasmic extracts in sucrose gradients, based on the methods described for polysome analysis by Arava (2003). This method involves size separation of large cellular components and monitoring the  $A_{254}$  across the gradient. It enables the separation of free mRNPs (ribonucleoprotein particles) from mRNAs fully loaded with ribosomes (i.e., polysomes). Polysomes represent actively translated transcripts and this fraction is directly correlated with the set of *de novo* synthesized proteins in a particular cellular state, enabling the determination of the translation efficiencies that are characteristic for each transcript in a cell (Smith *et al.*, 1999). In addition, changes in the distribution of a given mRNA indicate how this translational efficiency can vary under different conditions. Because it is generally accepted that translational control predominantly occurs at the initiation step (McCarthy, 1998), the number of mRNA molecules engaged in polysomes should be a robust indicator of the synthesis rate of the corresponding protein. To examine the effect of secretion stress on net translational activity in *A. niger*, we provide a detailed description of the method to analyze the global relative distribution of ribosomes between polysomes, 80S monosomes, and dissociated 40S and 60S subunits.

### 3.2.1. Required materials

**3.2.1.1. Devices and materials of the density gradient fractionator apparatus** RNA fractionation is performed with the Foxy Jr. Fraction Collector (TELEDYNE ISCO). RNA samples are loaded onto prepared gradients and spun in a ultracentrifuge. Once the spin is completed, the system allows you to fractionate and quantitate centrifuged zones with precision. The ISCO Density Gradient System produces a continuous absorbance profile as the gradient is collected in precisely measured fractions. Fractionation is performed by introducing a dense chase solution into the bottom of the centrifuged tube, raising the gradient intact by bulk flow. Chase solution is injected by piercing the bottom of the tube. System includes tube piercing stand, peristaltic pump, UA-6 Detector with 254

and 280 nm filters, density gradient flow cell, and Foxy Jr. Fraction Collector.

### 3.2.1.2. *Additional materials*

- Ultracentrifugation is performed in an Optima™ MAX-XP Benchtop Ultracentrifuge or an Optima™ MAX High-Capacity Personal Micro-Ultracentrifuge (Beckman Coulter) with an MLA-80 rotor. Alternatively, you can use a Beckman SW41 Ti rotor.
- Mortar and pestle, needle

### 3.2.1.3. *Other reagents*

- Polysome extraction buffer (20-mM Tris-HCl, pH 8.0, 140-mM KCl, 1.5-mM MgCl<sub>2</sub>, 1% Triton X-100, 0.1-mg/ml cycloheximide, 1.0-mg/ml heparin, 0.5-mM DTT)
- Chase solution (80% sucrose solutions prepared in 50-mM Tris-acetate, pH 7.0, 50-mM NH<sub>4</sub>Cl, 12-mM MgCl<sub>2</sub>, 1-mM DTT)
- TE (10-mM Tris-HCl, pH 8.0, 0,1-mM EDTA))

### 3.2.1.4. *Disposables*

- 8-ml centrifuge tubes, 2-ml (Eppendorf) tubes

## 3.2.2. *Sample preparation*

Ribosomal fractions are prepared according to the method described for polysome analysis (Arava, 2003) modified as follows: At the time of harvest, cycloheximide is added to a final concentration of 0.1 mg/ml to trap elongating ribosomes. The cultures are swirled rapidly and chilled on ice for 10 min. Fungal material is pelleted by centrifugation at 11,000×g for 10 min at 4 °C. The pellet is then resuspended in 5 ml of polysome extraction buffer and sedimented. This washing step is repeated, and cells are frozen in liquid nitrogen and stored at -80 °C. Approximately 0.25 g of cells are ground in liquid nitrogen with a mortar and pestle, and the powder is resuspended in 750 µl of ice-cold polysome extraction buffer. Excess cell debris are removed by sedimentation at 4000×g for 5 min at 4 °C. The supernatant is clarified by further centrifugation (12,000×g, 10 min, 4 °C).

## 3.2.3. *Sucrose gradient preparation*

We prepare 15–70% sucrose density gradients for ultracentrifugation without using a gradient maker. Sucrose solutions are prepared in 50-mM Tris-acetate (pH 7.0), 50-mM NH<sub>4</sub>Cl, 12-mM MgCl<sub>2</sub>, and 1-mM DTT. 1.3 ml of the lighter sucrose solution (15%) is laid on the bottom of the centrifuge tubes using a long needle. 2.6 ml each of 30% and 50% sucrose,

and finally, 1.3 ml of 70% sucrose are then carefully underlaid without disturbing the interfaces. The tubes are closed with parafilm and stored at 4 °C for at least 12 h before use to allow the gradients to thaw and diffuse to create a continuous gradient.

### 3.2.4. RNA fractionation

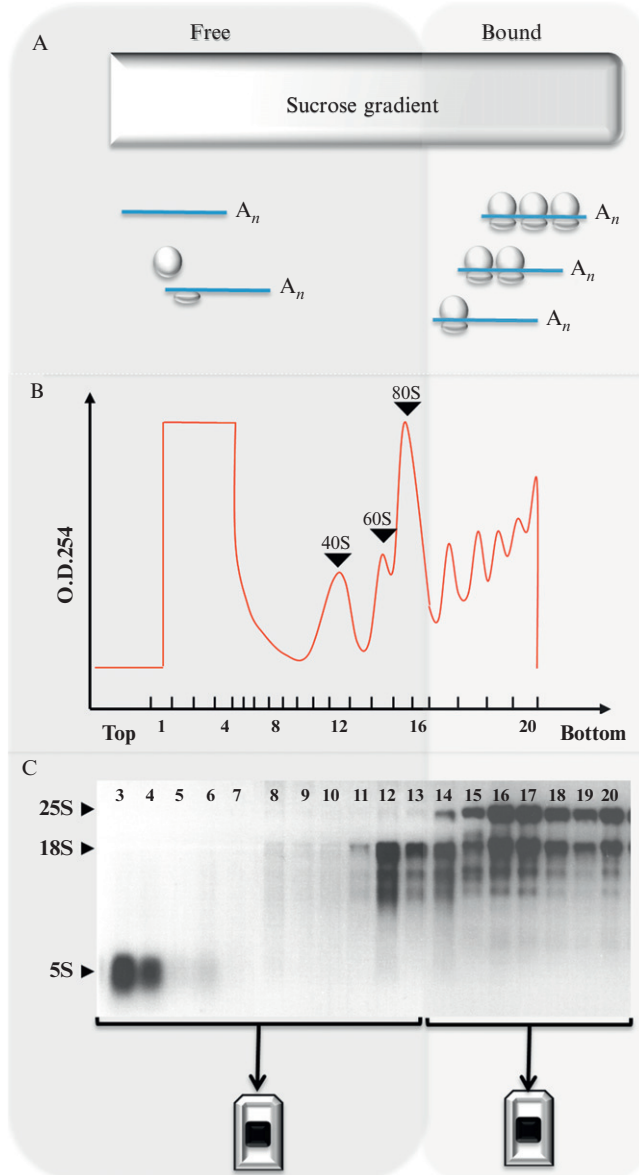
Each sample is loaded on an 8-ml 15–70% (w/v) sucrose gradient and sedimented at 150,000×*g* (55,000 rpm) and 4 °C in a Beckman MLA-80 rotor for 135 min. The gradient is fractionated with the density gradient fractionator while monitoring the absorbance at 254 nm. 0.5 ml fractions are collected from the top of the gradient directly into a 1-ml volume of 6-*M* guanidine hydrochloride. RNA is precipitated by adding an equal volume of 100% ethanol and resuspended in TE. The RNA is again precipitated by the addition of 50 µl of 3-*M* sodium acetate (pH 5.2) and 1 ml of 100% ethanol, and resuspended in TE. For RNAs destined for microarray analyses, the polysomal fractions and nonpolysomal fractions are pooled, respectively. Each fraction is treated with RQ1 RNase-Free DNase (Promega) and is again cleaned up by applying the samples to an RNeasy mini column (Qiagen). An aliquot of the RNA solution is extracted from each fraction and subjected to electrophoresis through a formaldehyde gel (Fig. 1.3).

## 4. MODIFYING THE UPR SIGNALING BY TARGETED GENE REPLACEMENT

### 4.1. Construction of UPR-deficient strains

As described in Section 1 in more detail, key proteins in UPR signaling are conserved in eukaryotic cells. They include the ER-localized transmembrane protein Ire1/IreA and transcription factor Hac1/HacA. Genes homologous to *IRE1* and *HAC1* are usually recognized by bidirectional Blast searches. Gene disruptions are often used to study the UPR, and genes encoding Hac1/HacA and Ire1/IreA are common targets for disruption. The most commonly used selection markers for making gene disruptions in *A. niger* include the counter selectable markers *pyrG* and *amdS*, and the hygromycin resistance marker (the *hph* gene from *Escherichia coli*). The use of hygromycin as a selection strategy is widely used for transformation of filamentous fungi as it does not require the isolation of an auxotrophic mutant (e.g., the isolation of a uridine/uracil requiring *pyrG* mutant).

Deletion of the *hacA* gene with the hygromycin resistance marker has been reported in both *A. niger* and *A. fumigatus* (Mulder and Nikolaev, 2009; Richie *et al.*, 2009) and in *A. niger* using the *pyrG* gene (Carvalho *et al.*, 2010). Several approaches are available for constructing gene deletion cassettes which include; (i) traditional PCR amplification and cloning of



**Figure 1.3** Schematic steps for polysome analysis. (A) Cytoplasmic extracts from *A. niger* cells are fractionated in 15–70% sucrose gradients. It enables the separation between free mRNAs and those loaded with ribosomes and engaged in translation. (B) Representative absorbance profile for RNA separated by velocity sedimentation through the sucrose gradient. For each fraction, absorbance at 254 nm is monitored using a UA-6 UV detector. The positions of the 40S, 60S, 80S, and polysomal peaks are indicated. The expected peaks for the tRNAs and other small RNAs in fractions 1–4 are

flanking regions on both sites of the selection markers, (ii) fusion PCR approaches to generate the entire deletion cassette by PCR, (iii) the use of Multisite Gateway recombination (Invitrogen) for the *in vitro* recombination of flanking regions and the selection markers or, (iv) the use of *S. cerevisiae* for *in vivo* recombination. It is beyond the scope of this review to discuss the different approaches to generate disruption cassettes, but in principle, all approaches are suitable. In this chapter, we include a Fusion PCR protocol which is routinely used by the authors to construct gene deletion cassettes and has been used to disrupt the *hacA* gene in *A. brassicicola* (Joubert *et al.* unpublished).

#### 4.1.1. Required materials

##### 4.1.1.1. Materials

- Thermocycler

##### 4.1.1.2. Other reagents

- High-Fidelity DNA Polymerase
- Plasmid pCB1636 (Sweigard *et al.*, 1995)

##### 4.1.1.3. Disposables

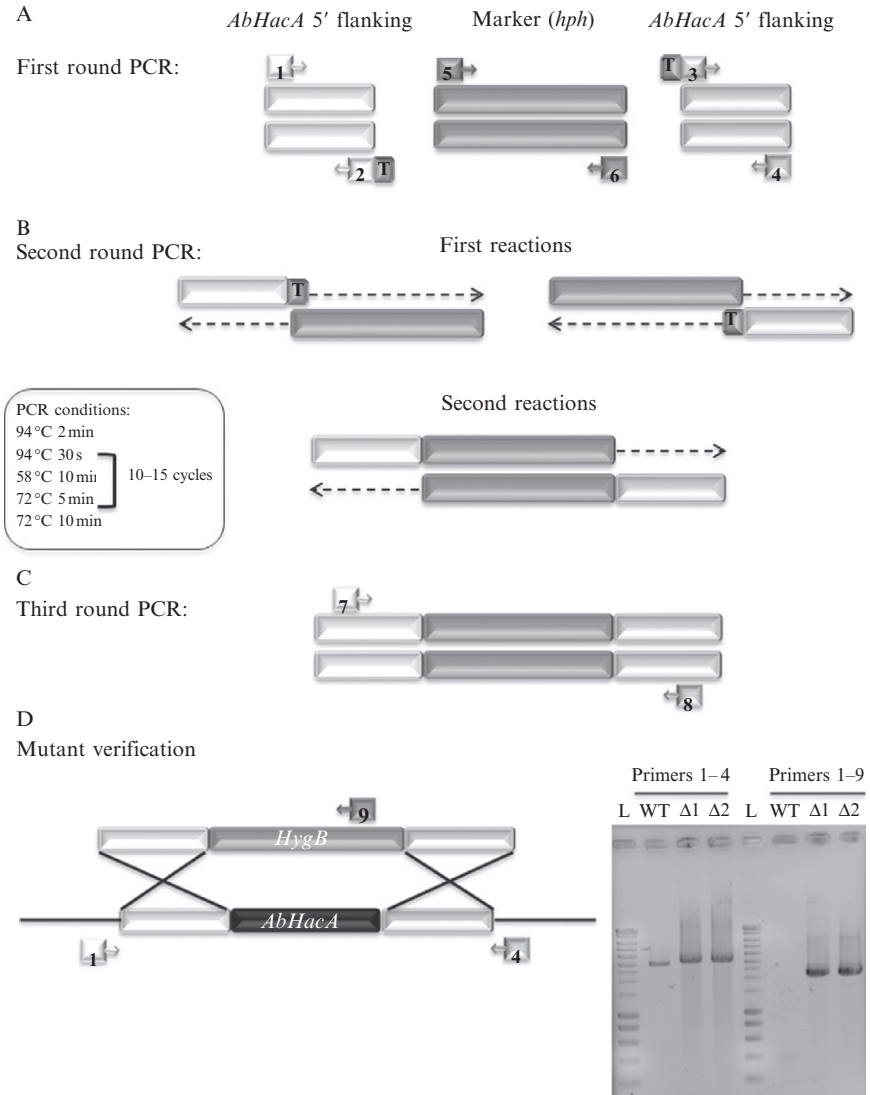
- 0.7-ml (Eppendorf) tubes

#### 4.1.2. Construction of a gene replacement cassette for use in *A. brassicicola*

Deletion of *hacA* in *A. brassicicola* was accomplished by replacing the *hacA* ORF with a hygromycin B resistance cassette. Gene replacement occurs via homologous double crossover between a linear construct and the target genomic locus. The linear construct flanks the resistance cassette (that contains the *hph* gene under control of the *trpC* fungal promoter) with two fragments representing 5' and 3' regions of the target gene, and is constructed by using a PCR-assisted DNA assembly called double-joint PCR (Fig. 1.4; Yu *et al.*, 2004). For all the PCR steps, we highly

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obscured by the high absorbance, presumably from proteins and detergents used in the preparation. (C) RNA is then extracted from each fraction and an aliquot is subjected to electrophoresis through a formaldehyde gel. As expected, 25-S and 18-S ribosomal RNAs were the prominent species. Before processing mRNA to cDNA, labeling, hybridization to *A. niger* Affymetrix GeneChips (Affymetrix, Inc., Santa Clara, CA), the polysomal fractions and nonpolysomal fractions can be pooled, respectively. Each pooled fraction is then treated with RQ1 RNase-Free DNase (Promega) and is again cleaned up by applying the samples to an RNeasy mini column (Qiagen).



**Figure 1.4** Schematic representation of the construction of a gene replacement cassette and verification of the mutants. The arrows numbered from 1 to 9 represent primers for the PCRs and primers 2 and 3 are 45–60 bases-long chimeric primers. (A) First-round PCR: amplification of the components using the specific and chimeric primers. A typical reaction will fuse DNA fragments of a 5' flanking sequence, a 3' flanking sequence, and a selectable marker (*hph* gene). Primers 2 and 3 carry 24 bases of homologous sequence overlapping with the ends of the selectable marker of choice. (B) Second-round PCR: the assembly reaction is carried out without using any specific primers, as the overhanging chimeric extensions act as primers. The first two cycles are shown in detail. (C) Third-round PCR: amplification of the final product using nested

recommend the use of a High-Fidelity DNA Polymerase. A typical reaction fuses DNA fragments of a 5' flanking sequence (amplified with the primers 1 and 2), a 3' flanking sequence (amplified with the primers 3 and 4), and the selectable marker. Here, the marker corresponds to the hygromycin resistance cassette (1436 bp) that is amplified with the primer M13F and M13R from the plasmid pCB1636 (Sweigard *et al.*, 1995). Primers 2 and 3 carry 24 bases complementary to M13F and M13R sequences, respectively, overlapping with the ends of the resistance cassette. During the first round of PCR, amplifications of 5' flanking region, marker, and 3' flanking region are separately carried out. The amplicons are then purified using a commercially available PCR cleanup kit. The three amplicons are mixed in 1:3:1 molar ratio, the total DNA amount of the three components being between 100 and 1000 ng. These three DNA fragments are specifically joined together during the second-round PCR. The assembly reaction is carried out without adding any specific primers, the overhanging chimeric extensions acting as primers. In the third round of PCR, the final PCR construct is amplified with a nested primer pair (primers 5 and 6). We can also use the first-round primer pair (primers 1 and 4) but the yield of amplicons is often lower and we also get PCR artifacts more easily. For round 3 fusion PCR, we generally use 1–2  $\mu$ l of the purified round 1 products as template. The final amplicon is purified and further concentrated in a volume of 10  $\mu$ l to a concentration of at least 1  $\mu$ g/ $\mu$ l.

## 4.2. Constitutive activation of the UPR

Activation of the HacA transcription factor includes the unconventional splicing of an intron from the *hacA*<sup>w</sup> mRNA, creating a transcriptionally active form of HacA. This mechanism allows a straightforward way to construct strains with a constitutively activated form of HacA and has been used in several *Aspergillus* species to generate strains with a constitutively activated UPR. Valkonen *et al.* (2003) reported the construction of an *Aspergillus awamori* strain which expressed the *hacA*<sup>i</sup> cDNA lacking the 20 nt unconventional intron and including a 150-bp truncation at the 5' end of the mRNA. In that study, the active form of *hacA* was expressed under

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primers 7 and 8. (D) Confirmation of gene replacement: transformants are randomly picked and examined for double crossover-mediated gene replacement pattern by PCR amplification of the *hacA* locus using the primer pairs 1/4 or 1/9. The primer 9 is designed inside the *hph* sequence. As shown, amplicons of wild-type and deletion alleles of *hacA* obtained with primer pairs 1/4 differ in size. No amplicon is obtained from wild-type matrix when using the primer 9. The results are shown for two mutants called  $\Delta 1$  and  $\Delta 2$ . Molecular sizes (kb) were estimated based on a 1-kb ladder (lane L, Eurogentec, Seraing, Belgium).



control of the highly expressed and inducible glucoamylase promoter. Mulder and Nikolaev (2009) constructed a constitutive HacA strain by expressing *hacA<sup>i</sup>*, lacking the 20 nucleotide intron, in a *hacA* deletion background. The site of integration of the introduced *hacA<sup>i</sup>* was controlled by using the *pyrG*\* approach (van Gorcom and van den Hondel, 1988). Finally, we recently used an approach to replace the wild-type *hacA* gene with the active *hacA<sup>i</sup>* form that lacks the 20 nucleotide intron at the *hacA* locus (Carvalho and Ram, unpublished). In all the three studies, the expression of the active form of HacA resulted in constitutive activation of the UPR pathway. Activation of the UPR pathway is often monitored by examining the expression level of UPR target genes such as *bipA* and *pdiA*. The latter approach in which the wild-type gene (*hacA<sup>u</sup>*) is replaced by a mutated and constitutively active form (*hacA<sup>i</sup>*) of the gene at the endogenous locus is an approach of general interest and is therefore described in this chapter.

#### 4.2.1. Required materials

##### 4.2.1.1. Materials

- Thermocycler

##### 4.2.1.2. Additional materials

- *Strain of interest*: Use of *Ku70* mutant dramatically increases the frequency of homologous recombination and reduces the number of transformants to be analyzed in order to find a transformant in which the cassette is integrated at the endogenous locus.

##### 4.2.1.3. Other reagents

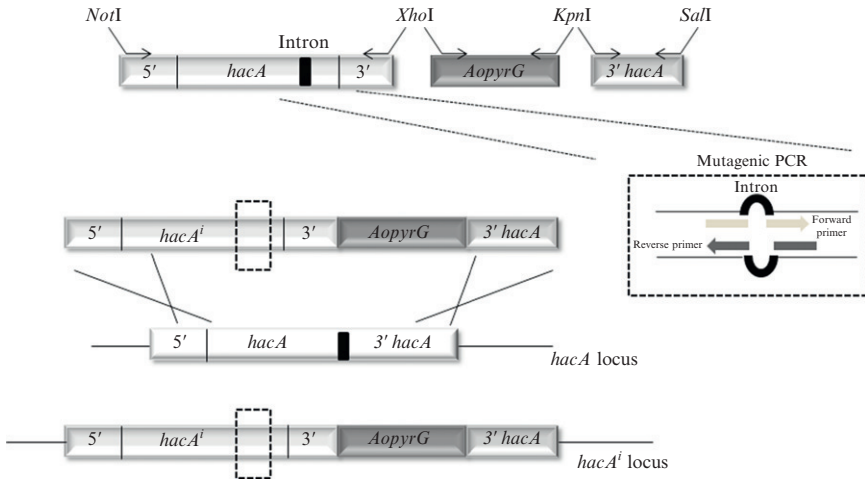
- High-fidelity DNA polymerase
- Plasmid PBluescript-SK
- Restriction enzymes

##### 4.2.1.4. Disposables

- 0.7-ml (Eppendorf) tubes

#### 4.2.2. Replacement of *hacA<sup>u</sup>* by a constitutive *hacA<sup>i</sup>* at the endogeneous locus in *A. niger*

To construct the replacement cassette, primers are designed to amplify various fragments, as depicted in Fig. 1.5. Three fragments, consisting of the *hacA<sup>u</sup>* gene, encoding promoter and terminator regions, the *Aspergillus oryzae pyrG* selection marker, and the *hacA* terminator region are amplified



**Figure 1.5** Schematic representation of the construction of a cassette for obtaining a constitutive UPR strain. Three fragments containing the *hacA* locus, the *Aspergillus oryzae pyrG* gene, and the *hacA* terminator region are amplified by PCR and ligated into a standard cloning vector (pBluescript-SK) using appropriate restriction enzymes. This plasmid is used as a template for site-directed mutagenesis using two complementary primers lacking the intron sequence. Transformants containing the construct lacking the intron (*hacA<sup>i</sup>*) at the *hacA* locus can be selected by Southern blot analysis and the lacking of the intron can then be confirmed by PCR analysis.

by PCR and cloned into pBluescript-SK using appropriate restriction enzymes. The *A. oryzae pyrG* gene and flanking regions (de Ruiter-Jacobs *et al.*, 1989) are used to prevent homologous recombination of the final construct at the *A. niger pyrG* locus. The plasmid containing the *hacA<sup>u</sup>* ORF is used as a template to introduce the *hacA<sup>i</sup>* gene by site-directed mutagenesis according to the Quick Change II site-directed mutagenesis protocol (Stratagene). Overlapping complementary primers lacking the intron sequence are used to obtain the *hacA<sup>i</sup>* gene in which the intron is deleted. To prevent single crossover of the construct, the construct should be linearized before transformation into an *A. niger ku70* mutant (Meyer *et al.*, 2007). Southern blot analysis should be performed to confirm integration of *hacA<sup>i</sup>* at the endogenous locus. As depicted in Fig. 1.5, the *A. oryzae pyrG* marker is flanked by repeats of the *hacA* terminator region. The direct repeat flanking the *AopyrG* gene allows efficient looping out of the *pyrG* marker after subjecting the strain to counter-selection using 5-fluoro-orotic acid (5-FOA). A detailed protocol for 5-FOA counter-selection has been published recently (Meyer *et al.*, 2010). The removal of the *pyrG* gene from the *hacA* locus completely restores the wild-type context of the *hacA* locus, and the *pyrG* auxotrophy can be used for transform additional constructs into this strain.

### 4.3. PEG-mediated transformation of protoplasts

The establishment of an effective transformation method for the filamentous fungi is of crucial importance to examine gene functions. At the moment, protoplast-mediated transformation and *Agrobacterium*-mediated transformation are the most commonly used techniques. As a detailed protocol for *Agrobacterium*-mediated transformation of *Aspergillus* sp. has been published recently (Michielse *et al.*, 2008), we describe here a protocol that we routinely use for the transformation of *A. brassicicola* protoplasts. This protocol can be used for many other filamentous fungi, providing that the enzyme solution is adapted to the targeted fungus. Indeed, the yield of protoplasts in fungi depends mainly of the composition of the lytic enzyme batch used. *Alternaria* spp. are members of the *Dothideomycetes* and they commonly present a highly melanized cell wall that requires specific enzyme mixture.

#### 4.3.1. Required materials

##### 4.3.1.1. Materials

- Thoma counting chamber
- 250-ml flasks

##### 4.3.1.2. Other reagents

- Enzyme-osmoticum is prepared in 0.7-*M* NaCl and contains kitalase (Wako Chemicals, Richmond, VA, USA) at 10 mg/ml and driselase (Interspex) at 20 mg/ml.
- STC buffer (1.2-*M* sorbitol, 10-*mM* Tris-HCl, pH 7.5, and 50-*mM* CaCl<sub>2</sub>)
- PEG solution (MW 3350–4000) 60%, 10-*mM* Tris-HCl, pH 7.5, and 50-*mM* CaCl<sub>2</sub>)
- *Regeneration medium*: you need to prepare the flasks A, B, C, and autoclave them separately. Combine them after autoclaving and hold at 55 °C.
  - *Flask A*: 1-g yeast extract, 1-g casein hydrolysate, water to 50 ml
  - *Flask B*: 342 g sucrose, water to 500 ml
  - *Flask C*: 16-g agar, water to 450 ml

##### 4.3.1.3. Disposables

- Petri dishes
- 50-ml tubes and 10-ml tubes

#### 4.3.2. Protoplast-mediated transformation of *A. brassicicola*

Transformation is carried out with linear PCR products based on the transformation protocol for *A. alternata* (Akamatsu *et al.*, 1997), with modifications. Approximately  $5 \times 10^6$  fungal conidia are harvested from a potato dextrose (PD) agar culture plate and introduced into 50 ml of PD broth media. They are cultured for 16 h at 25 °C with shaking at 100 rpm. The fungal material is harvested by centrifugation at 3500–4000×*g* for 10 min, washed twice with 0.7-*M* NaCl, followed by centrifugation again under the same conditions as before. During centrifugation, start making the enzyme-osmoticum. The enzyme-osmoticum is added to the mycelia in Falcon tubes and the cell walls of the germlings are digested for 3–4 h at 32 °C with gentle shaking every 30 min during incubation. The protoplasts are separated from undigested mycelia and cell-wall debris by filtering the suspension through 2–4 layers of cheesecloth. The protoplasts are then collected by centrifugation at 2500×*g* for 10 min. The enzyme mixture can be reused by collecting the supernatant in other tubes and storing at –80 °C. The pellet is washed twice with 10 ml of 0.7 *M* NaCl and then with 10 ml of STC buffer. The protoplasts are gently resuspended in 500-μl–1-ml STC to reach a concentration of  $10^6$ – $10^7$  protoplasts per ml. The counting is performed by using a Thoma chamber.

At least 10 μg of PCR products in 10 μl of ddH<sub>2</sub>O is added to the protoplast suspension in 12-ml Greiner tubes and gently mixed. The transformation mix is incubated on ice for 20 min. PEG solution is then added in three aliquots of 200, 200, and 800 μl each. The tubes are warmed by hand before each addition and drops must be added slowly (drop by drop). The tubes are also mixed after each addition by rolling. Incubations for 5 min on ice are required between each addition. Finally, the transformation mix is diluted with 1-ml STC. Then, 200 μl of the transformation mixture is added to 25 ml of molten regeneration medium in a 50-ml tube and subsequently poured into a 100 × 15-mm Petri dish. Allow the medium to solidify and then incubate the plates at 32 °C. After 24 h, the plates are overlaid with 10 ml of 1% agar containing hygromycin B (Sigma-Aldrich, St. Louis, USA). The final concentration of hygromycin B should be 15 μg/ml. Please note that sensitivity to hygromycin B varies according to strain, so strain sensitivity must be assessed in advance. Individual hygB-resistant transformants are transferred to a fresh hygB-containing plate between 5 and 15 days after each transformation. Each transformant is purified further by picking isolated conidia under the microscope and transferring them to a fresh hygB-containing plate. Three successive rounds of single-spore isolation are performed.

#### 4.4. Mutant verification

Two approaches, diagnostic PCR and Southern analysis of genomic DNA, are used to analyze mutants at the gene level and to verify that linear DNA products have inserted correctly. For both approaches, we routinely use high-throughput methods for isolation of genomic DNA from different fungal species. Detailed protocols have been reported by Meyer *et al.* (2010). We carry out a PCR screen by using primers homologous to the selection marker and genomic sequence outside the flanking regions, to confirm that integration of the replacement constructs occurred by homologous recombination at the targeted loci (Fig. 1.4D). The complete deletion of the coding region in mutants is further confirmed using two internal primers. Moreover, genomic Southern blot genotyping is performed to check single-copy deletion of the target gene in mutants that will be used for further phenotypic analysis (data not shown).

#### 4.5. Phenotypic analysis

Detailed analysis of the phenotypes of both the wild-type and mutants affected in the process under investigation is essential to determine gene function or investigate the importance of a pathway in a cell. We summarize in Table 1.1 the main phenotypic criteria that have been investigated in  $\Delta hacA$  UPR-deficient strains obtained in *A. niger* (Mulder and Nikolaev, 2009), *A. fumigatus* (Richie *et al.*, 2009), and in *A. brassicicola* (Joubert *et al.*, unpublished).

Among all the criteria that could be considered for establishing the phenotypic pattern of null mutants in filamentous fungi, growth characteristics and susceptibility to different drugs are key elements that we routinely monitor on agar plates as well as in liquid medium using laser nephelometry (NEPHELOstar Galaxy, BMG Labtech, Offenburg, Germany). By contrast with photometry (i.e., the measurement of light transmitted through a particle suspension), nephelometry is a direct method of measuring light scattered by particles in suspension. As the scattered light intensity is directly proportional to the suspended particle concentration, nephelometry is a powerful method for recording microbial growth and especially for studying filamentous fungi, which cannot be efficiently investigated through spectrophotometric assays. The advantages of nephelometry compared to analysis of colony expansion rates on solid media or spectrophotometric assays are discussed in a recent paper (Joubert *et al.*, 2010), in which we described a filamentous fungi-tailored procedure based on microscale liquid cultivation and automated nephelometric recording of growth.

**Table 1.1** Main phenotypic criteria investigated in fungal  $\Delta hacA$  strains

Phenotypic analysis	Fungal organism	Methods
Growth rate	<i>A. niger</i> <i>A. fumigatus</i> <i>A. brassicicola</i>	– Measure of colony diameter on different agar media (minimal or complete media, IMA, PDA, skim milk)
Sporulation	<i>A. niger</i> <i>A. brassicicola</i>	– Microscopic observation of conidia (using stereomicroscope or scanning electron microscopy and quantification of sporulation using a Thoma counting chamber)
Hypal morphology	<i>A. niger</i> <i>A. brassicicola</i>	– Microscopic observations of mycelia in liquid culture or grown between glass slides
Susceptibility to ER stress	<i>A. niger</i> <i>A. fumigatus</i> <i>A. brassicicola</i>	– Incubation in the presence of DTT, tunicamycin, or brefeldin A – Measure of colony diameter (from solid media) or growth monitoring using nephelometry (from liquid media)
Susceptibility to thermal stress	<i>A. fumigatus</i>	– Measure of colony diameter from solid media incubated at 37 and 45 °C – Measure of the percentage of surviving CFUs
Susceptibility to antifungal drugs	<i>A. fumigatus</i>	– Use of Etest strips impregnated with caspofungin, fluconazole, amphotericin B, and itraconazole
Expression of UPR target genes	<i>A. niger</i> <i>A. fumigatus</i> <i>A. brassicicola</i>	– Northern hybridization, real-time RT-PCR
Susceptibility to host defense metabolites	<i>A. brassicicola</i>	– Growth monitoring using nephelometry in the presence of cruciferous phytoalexins
Cell wall structure and composition	<i>A. fumigatus</i> <i>A. brassicicola</i>	– Treatment with calcofluor white and Congo red – Biochemical analysis of the cell wall – Observations with transmission electron microscopy
Protein secretion	<i>A. fumigatus</i> <i>A. brassicicola</i>	– SDS-PAGE analysis of culture supernatants – Quantification of secreted proteolytic activity with the Azocoll assay

**Table 1.1** (continued)

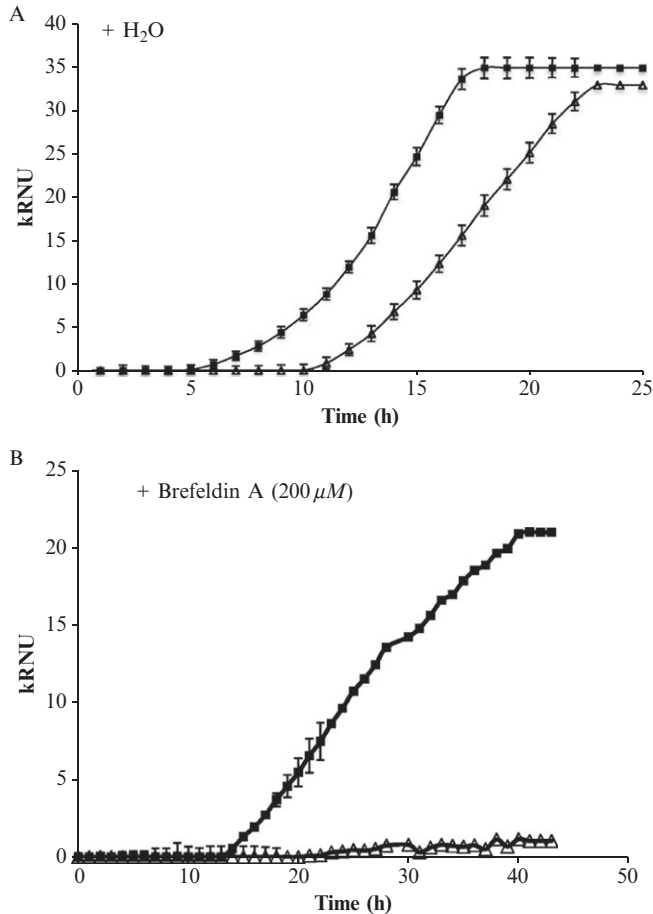
Phenotypic analysis	Fungal organism	Methods
Virulence	<i>A. fumigatus</i> <i>A. brassicicola</i>	<ul style="list-style-type: none"> <li>– Quantification of esterase activity with the artificial substrate <i>p</i>-nitrophenyl butyrate (PNB)</li> <li>– Use of an outbred and inbred mouse model of invasive aspergillosis</li> <li>– Inoculation of spore suspension on intact and prewounded leaves of <i>Arabidopsis thaliana</i></li> </ul>
Visualization of infection structures	<i>A. brassicicola</i>	<ul style="list-style-type: none"> <li>– Staining of fungal material with Solophenyl Flavine 7GFE 500 followed by microscopic observations</li> <li>– Observations with scanning electron microscopy</li> </ul>

We recommend this technique for the evaluation of antifungal activity and for large-scale phenotypic profiling.

Figure 1.6A shows a representative growth curve for *A. brassicicola* wild-type strain and  $\Delta hacA$  mutant plotted from the nephelometric assays. Nephelometric monitoring confirms that, at least for early steps of the growth kinetics, the  $\Delta hacA$  mutant is growth-impaired in PD broth. In another example (Fig. 1.6B), disruption of ER homeostasis is triggered by exposure to brefeldin A, which is an ER stress-inducing agent. As expected, we observe that the *hacA* null strain is growth-impaired in the presence of concentrations of brefeldin A that could be tolerated by the wild-type strain, indicating that *hacA* inactivation increased the sensitivity of *A. brassicicola* cells to these treatments.

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**Figure 1.6** Nephelometric monitoring of growth of wild-type and  $\Delta hacA$  strain. Conidial suspensions ( $10^5$  spores/ml, final concentration) of wild-type (black squares) and  $\Delta hacA$  (open triangles) are used to inoculate microplate wells containing a standard PDB medium that is supplemented (B) or not (A) with 200- $\mu$ M brefeldin A. Microplates are placed in a laser-based microplate nephelometer (NEPHELOstar Galaxy, BMG Labtech) and growth is monitored automatically over a 30-h period. Each genotype is analyzed in triplicate and the experiments are repeated three times per growth condition.

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## RESUME

*Alternaria brassicicola* est un champignon nécrotrophe responsable du « black spot » des *Brassicaceae*, causant d'importants dégâts culturels. Lors de l'infection, les *Brassicaceae* synthétisent divers composés de défense tels que les phytoalexines indoliques camalexine et brassinine. Une étude préalable avait suggéré une action de ces molécules sur l'intégrité de la membrane fongique et la mise en place chez le champignon d'un mécanisme adaptatif de type compensatoire visant à renforcer son intégrité cellulaire. L'objectif de la thèse est d'identifier les composantes moléculaires régulant l'induction de ce mécanisme. Les travaux réalisés montrent que les voies de signalisation HOG, CWI, et UPR impliquées respectivement dans la régulation de l'osmose cellulaire, la biosynthèse de la paroi et la réponse au stress de sécrétion sont activées chez *A. brassicicola* lors de l'exposition aux phytoalexines. Le phénotypage de mutants déficients pour des composantes clés de ces trois voies de signalisation montre qu'ils sont plus sensibles aux phytoalexines et présentent une baisse de leur agressivité *in planta* (*AbSlt2Δ* pour la voie CWI, *AbHog1Δ* pour la voie HOG) ou une perte de virulence (*AbHacAΔ* pour la voie UPR). Cette étude illustre le rôle crucial des voies de signalisation dans l'adaptation d'un champignon phytopathogène aux stress liés à la colonisation de son hôte et donc dans la réussite du cycle infectieux. Ce rôle clé en fait de bons candidats comme cibles pour la définition de stratégies de lutte contre l'alternariose.

**Mots clés :** *Alternaria brassicicola*, black spot, pouvoir pathogène, détournement des défenses, phytoalexines, camalexine, brassinine, UPR, CWI, HOG

## ABSTRACT

*Alternaria brassicicola* is a necrotrophic fungal pathogen responsible for the black spot disease of *Brassicaceae* that causes major damages in fields. During host infection, *A. brassicicola* is exposed to high levels of defence compounds, such as the indolic phytoalexins called camalexin and brassinin. Previous studies showed that these compounds probably cause damage to fungal membranes and activate a compensatory mechanism in fungal cells aimed at preserving membrane integrity. The aim of this thesis consists in investigating the molecular basis of this mechanism. The results show that the unfolded protein response (UPR) and two MAPK signalling pathways, the cell wall integrity (CWI) and the high osmolarity glycerol (HOG) were activated in *A. brassicicola* in response to phytoalexin exposure. Mutant strains lacking functional MAP kinases or AbHacA, the major UPR transcription regulator, showed *in vitro* hypersusceptibility to camalexin and brassinin. Replacement mutants exhibited a loss or an attenuation of the virulence on host plants that may partially result from their inability to cope with defence metabolites such as indolic phytoalexins. This study constitutes the first evidence that a phytoalexin activates fungal MAP kinases and UPR, and that outputs of activated pathways contribute to protecting the fungus against antimicrobial plant metabolites. A functional model of fungal signalling pathways regulated by camalexin is proposed and leads to consider new promising strategies for disease control.

**Key words:** *Alternaria brassicicola*, black spot, compensatory mechanism, phytoalexin, camalexin, brassinin, UPR, CWI, HOG