Etude du rôle des cytokinines végétales et fongiques dans l'interaction riz-Magnaporthe oryzae

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C'EST DANS L'INDISCIPLINE
DE NOS COEURS
QUE SE TROUVE
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SOMMAIRE

Abréviations

ABA : Abscisic acid, acide abscissique

ACC : L'acide 1-aminocyclopropane-1-carboxylique

ACS : ACC Synthase

ADP : Adénosine Diphosphate

ADN : Acide Deoxyribonucléïque

AHK : Arabidopsis Histidine Kinase

AIA : Acide Indole-Acétique

AK : Adenosine Kinase

AM : Arbuscular Mycorrhiza

AMP : Adénosine MonoPhosphate

AMPc : Adénosine MonoPhosphate cyclique

AOS : *Allene Oxide Synthase 2*

ATP : Adénosine Tri-Phosphate

AUX : Auxine

Avr : protéine d'Avirulence

BA : Benzyl Adénine/adénosine

BAP : Benzyl Adénine/adenosine Phosphate

BAS : Biotrophic Associated Secreted protein

BED : BEAF et DREF

BIC : Biotrophic Interfacial Complex

BR : Brassinostéroïdes

CaM : Calmodulines

CBL : Calcineurin B-Like protein

CCR : Carbon Catabolite Repression

CDPK : Calcium-Dependent Protein Kinase

CEBiP : Chitin-Elicitor Binding Protein

CERK1 : Chitin Elicitor Receptor Kinase

CNL : Coil-Coil NLR

CIPK : CBL-Interacting Protein Kinase

cisZ : cisZéatine

cisZR : cisZéatine Riboside

cisZOG : cisZéatine-O-glycosylée

cisZROG : cisZéatine Riboside- O-glycosylée

CKs : Cytokinines

CK-N-GT : Cytokinine-N-glucosylée

CKS1 : Cytokinin Synthesis 1

CKX : Cytokinin OXidase

CNGC : Calcium Nucleotide-Gated Channel

CRF : Cytokinin Response Factor, Facteur de réponse aux CKs

CWDE : Cell Wall Degrading Enzyme

CYCD3 : Cyclin D3

DAMPs : Damage-Associated Molecular Patterns

DMAPP : Dimethylallyl Pyrophosphate

DPI : Diphenylene Iodonium

DZOG : DihydroZéatine-O-glycosylée

EIHM : Extra-Invasive Hyphal Membrane

ET : Ethylène

ETI : Effector Triggered Immunity

ETS : Effector Triggered Susceptibility

EUI : Elongated Uppermost Internode 1

FLS2 : Flagellin Sensing 2

G6P : Glucose-6-Phosphate

G6PDH : G6P DésHydrogenase

GAs : Gibberellic Acids, Gibbérellines

HK : Histidine Kinase

HMA : Heavy Metal Associated domain

HPt : Histidine Phosphotransfer protéine

HR : Hypersensitive Response

HRGP : Hydroxyproline-Rich GlycoProtéine

ICL : Isocitrate Lyase

iH : invasive Hyphae

iP : isoPentenyl-adenine

iPNT : isoPentenyl-adenine Nucléotide

IPT : isoPentenyl Transferase

iPR : isoPentenyl-adenine Riboside

JA : Jasmonate

Kin : Kinétine

LOG : Lonely Guy

LRR : Leucine Rich-Repeat

MAMPs : Microbe-Associated Molecular Patterns

MAPK : Mitogen-Activated Protein Kinase (Kinase MAPKK ou MAP2K/ Kinase Kinase MAPKKK ou MAP3K)

MEP : Methylerythritol Phosphate

NADPH : Nicotianamide Adenine Dinucleotide Phosphate

NB-ARC : Nucleotide-Binding ; APAF-A, R-proteins, CED-4

NH1 : NPR1 Homolog 1

NLR : NOD-Like Receptor

PAL : Phénylalanine Ammonia-Lyase

PAMPs: Pathogen-Associated Molecular Patterns

PBZ1: Probenazole 1

PCD : Programmed Cell death, mort cellulaire programmée

Protéine R : Protéine de résistance impliquée dans la reconnaissance d'effecteurs

PR: Pathogenesis Related

PRR: Pattern Recognition Receptor

PTI: PAMPs-Triggered Immunity

RBOH: Respiratory Burst Oxidase Homolog

RLK: Receptor Like Kinase

RLP: Receptor Like Protein

ROS: Reactive Oxygen Species

RR: Response Regulator, Régulateur de Réponse

RuBisCO: Ribulose 1,5-Bisphosphate Carboxylase Oxigenase

SA: Salicylic Acid, Acide Salicylique

TNL: Tir-NLR

TPS1: Trehalose Phosphate Synthase 1

tRNA: transfer Ribonucleic Acid

tRNA-IPT: tRNA-Isopentenyl Transferase

tZ : transZéatine

tZ9G : traasZéatine-N9-glycosylée

UDP: Uridine Diphosphate

UGT: UDP Glucosyl Transferase

VPE: Vacuolar Processing Enzyme

WT: Wild Type

Xoo: Xanthomonas oryzae pv.*oryzae*

ZBED: Zinc finger BEAF DREF domain protein

ZOGT: Zeatine-O-Glycosyl Transferase

INTRODUCTION

Les végétaux sont constamment menacés par une pléthore d'organismes pathogènes : virus, champignons, oomycètes, bactéries, nématodes… Certains agents pathogènes se nourrissent de tissus vivants : les biotrophes ; d'autres de tissus morts : les nécrotrophes. Certains présentent des modes de vie intermédiaires et sont dits hémibiotrophes.

I. Les interactions plantes-pathogènes : stratégies de défense

Les plantes ont acquis des récepteurs capables de percevoir la présence d'organismes étrangers et potentiellement pathogènes. Cette reconnaissance active une cascade de signalisation qui aboutit à la mise en place de mécanismes de défense pouvant limiter la pénétration et/ou l'invasion des organismes pathogènes.

1. La perception de l'agent pathogène par l'hôte

La perception des agents phytopathogènes par les plantes peut se faire par la reconnaissance de motifs généraux conservés chez les microorganismes appelés PAMPs ou MAMPs (Pathogen- ou Microbe-Associated-Molecular-Patterns, (Zipfel, 2009; Liu *et al.*, 2013). Des récepteurs transmembranaires, les PRRs (Pattern Recognition Receptors), assurent la reconnaissance de ces motifs conservés ainsi que celle de motifs issus de produits de dégradation cellulaires de l'hôte normalement non accessibles, les DAMPs (Damage Associated Molecular Patterns). Les PRRs connus sont des RLK (Receptor Like Kinases) ou RLP (Receptor Like proteins). Leur domaine extracellulaire assure la perception des ligands. Parmi ces domaines, on retrouve principalement des domaines LRR (Leucine Rich-Repeat), LysM et Lectine. Les PRRs de type RLK présentent un domaine Ser/Thr kinase intracellulaire qui assure la propagation du signal de perception à d'autres partenaires membranaires /cytoplasmiques. Certains PRRs, qui ne possèdent pas de domaine Ser/Thr kinase (RLP, (Zipfel, 2009)), interagissent avec des corécepteurs munis de ce domaine qui initient alors la cascade de phosphorylation nécessaire à la signalisation. Cette signalisation aboutit à la mise en place du système de défense de l'hôte appelé PTI (PAMPs Triggered Immunity, Figure 1A (Jones and Dangl, 2006)).

Parmi les PRRs du riz, OsCEBiP est une protéine transmembranaire possédant deux domaines LysM extracellulaires affins pour la chitine, un polymère de *N*-acetylglucosamide constituant en partie la paroi cellulaire des champignons au sens strict (Hayafune *et al.*, 2014). Cette protéine participe à la reconnaissance des organismes fongiques en interaction avec un autre PRR, OsCERK1. OsCERK1 est, lui, constitué de motifs LysM extracellulaires ainsi que d'un domaine kinase cytoplasmique requis pour la transduction du signal de la présence d'un organisme fongique

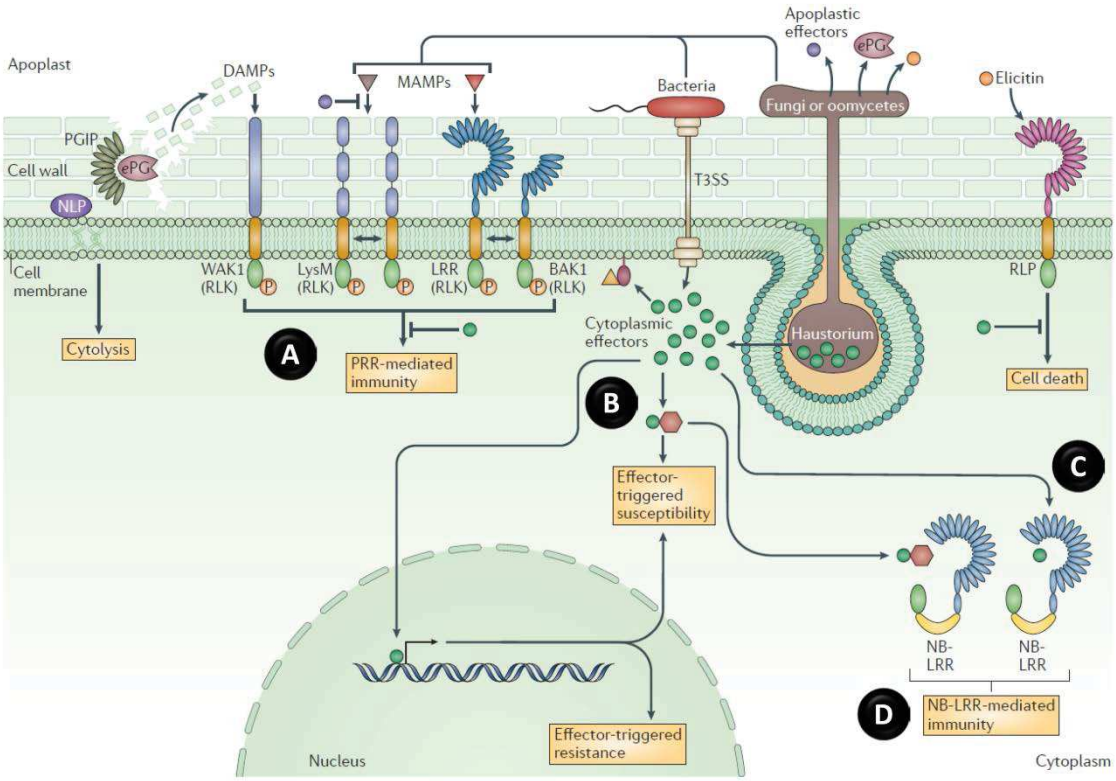

Figure 1. Schéma général de l'immunité végétale

Les plantes sont capables de reconnaitre des motifs généraux conservés au sein des microorganismes appelés PAMPs/MAMPs (Pathogen-/Microbe-Associated Patterns), grâce à des récepteurs membranaires, les PRRs et Co-PRRs (Pattern Recognition Receptors). Cette reconnaissance permet la mise en place des défenses basales. Si ces défenses conduisent à la résistance on parle de PTI (PAMPs-Triggered Immunity) (A). Les organismes pathogènes sécrètent de petites protéines appelées effecteurs (B) qui contribuent à leur virulence en inhibant la PTI. On parle d'ETS (Effector Triggered Susceptibility). Toutefois les plantes ont développé des "sentinelles", les récepteurs NLR, capables de reconnaitre ces effecteurs de manière directe ou indirecte (C). La perception de la présence de l'effecteur entraine la résistance de l'hôte : l'ETI (Effector Triggered Immunity) (D) via l'activation de mécanismes de défense supplémentaires, dont la réponse hypersensible. Modifiée à partir de Wirthmueller et *al*, 2013.

(Shimizu *et al.*, 2010; Hayafune *et al.*, 2014). En présence de chitine, OsCEBiP et OsCERK1 forment un hétérodimère alors qu'en absence de chitine, les formes homodimériques semblent être favorisées. D'autres RLK classiques comme OsFLS2 et Xa21 permettent la reconnaissance de PAMPs bactériens, respectivement la flagelline et Ax21 (une protéine présente chez la plupart des souches de *Xanthomonas oryzae* pv. o*ryzae* : *Xoo*).

Face à cette pression, les agents pathogènes ont développé des facteurs de virulence (voir II.) qui affectent la mise en place des défenses des plantes et permettent leur invasion, on parle alors d'ETS (Effector Triggered Susceptibility Figure 1B (Jones and Dangl, 2006)). Pour contrer les effets de ces effecteurs, les plantes ont développé des récepteurs cytoplasmiques et spécifiques, les NLR (NOD-like receptors ; Figure 1C). Cette reconnaissance engendre une signalisation plus rapide et plus forte des mécanismes de défense et aboutit souvent à une mort cellulaire programmée des cellules au site d'infection, également appelée réponse hypersensible (HR: Hypersensitive Response). Cette réaction est très efficace pour limiter la propagation d'agents pathogènes. Si la reconnaissance d'un effecteur aboutit à la résistance de l'hôte on parle d'ETI (Effector-Triggered Immunity, Figure 1D). L'effecteur reconnu par la protéine de résistance est alors appelé protéine d'avirulence ou Avr.

La plupart des gènes de résistance clonés à ce jour chez les plantes codent des protéines NLRs. Ces protéines sont caractérisées par la présence d'un domaine N-terminal variable entre Monocotylédones et Dicotylédones, suivi d'un domaine central NB-ARC (Nucleotide-Binding ; APAF-A, R-proteins, CED-4) puis un domaine LRR (Leucine-Rich Repeat) en position C-terminale. Ces trois domaines assurent la spécificité de reconnaissance, l'activation de la protéine NLR ainsi que la transduction du signal. Chez le riz 23 gènes codant une protéine NLRs ont été clonés (Liu *et al.*, 2014). Récemment, près d'une centaine de NLR de riz conférant une résistance ont été identifiées, expliquant une partie de la résistance à large spectre et potentiellement durable (Zhang *et al.*, 2015).

La reconnaissance des effecteurs par les protéines de résistance peut être directe ou indirecte (Figure 2 ; (van der Hoorn and Kamoun, 2008; Cesari *et al.*, 2014)). Dans le cas d'une reconnaissance directe, l'interaction physique entre la protéine de résistance et la protéine d'avirulence (Avr) induit la mise en place de l'ETI (Figure 2A). C'est le cas notamment de la reconnaissance d'Avr-Pita de *M. oryzae* par la protéine de résistance du riz correspondante, Pita (Jia *et al.*, 2000). Mais cette configuration n'est pas la plus courante. Dans le cas d'une reconnaissance indirecte, la protéine R reconnait les modifications apportées sur la (les) protéine(s) cible(s) de l'effecteur ou le complexe Avr/cible ; c'est le modèle de garde (Figure 2B). Dans ce contexte la modification de la protéine ciblée par l'effecteur est nécessaire à la virulence optimale de l'agent pathogène (van der Hoorn and Kamoun, 2008; Win *et al.*, 2012; Wu *et al.*, 2015; Cesari *et al.*, 2014). On observe dans ce cas que l'agent pathogène est moins virulent en l'absence simultanée de la protéine R et de la protéine gardée.

Figure 2.

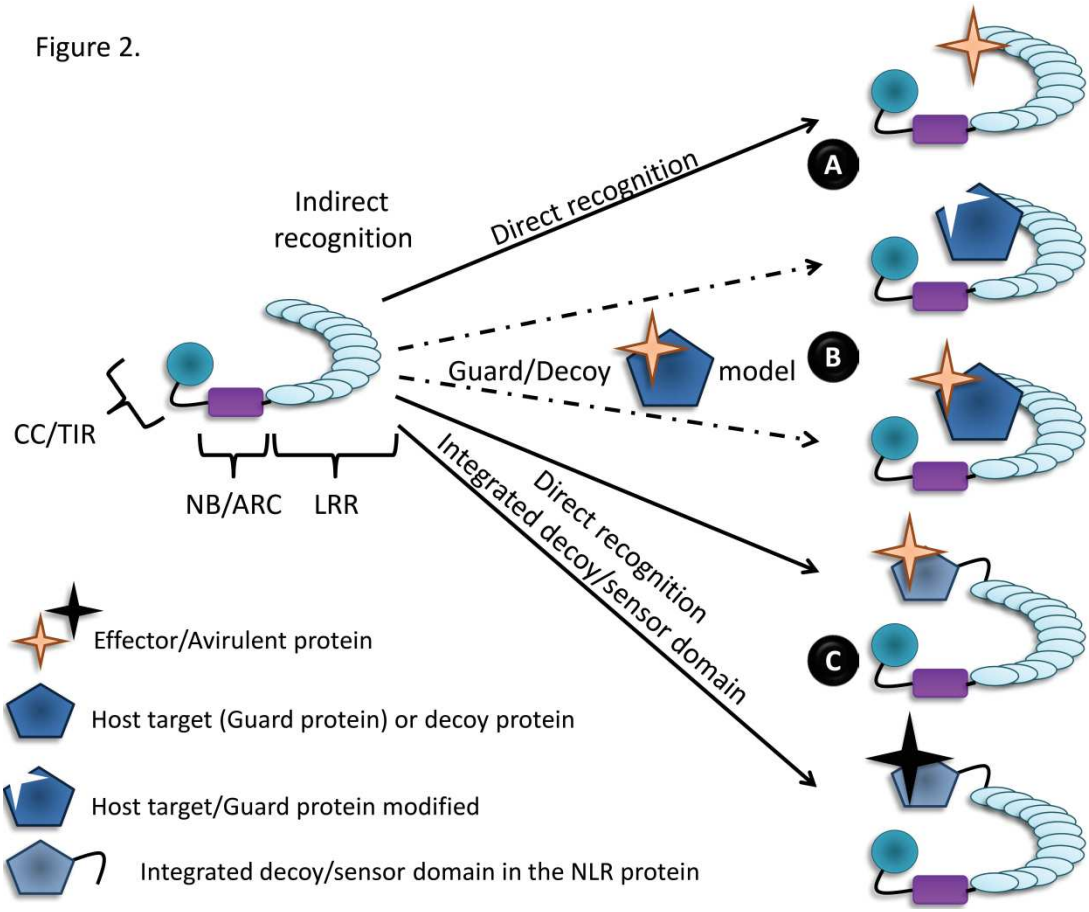

Figure 2. Reconnaissance des effecteurs par les protéines NLR

L'interaction entre la protéine effecteur et la protéine NLR (NOD-Like Receptors) de l'hôte peut être directe (A et C) ou indirecte (B). Dans le second cas, la protéine NLR reconnait la protéine de garde ou « decoy » (Leurre) modifiée ou en interaction avec l'effecteur. Certains NLR possèdent des domaines supplémentaires atypiques appelés « integrated decoy » ou « sensor domain » (C). Ce domaine peut permettre de reconnaitre un ou plusieurs effecteurs.

CC : Coil-Coil ; TIR : Toll/Interleukin Receptor; NB: Nucleotide Binding; ARC : APAF-A, R-proteins, CED-4; LRR : Leucin-Rich-Repeat

Les plantes ont évolué d'autres stratégies pour leurrer les agents pathogènes notamment des protéines pièges appelées « decoys » (leurres). Ces protéines présentent une certaine affinité pour un effecteur particulier qui va donc se lier à elles et se retrouver « séquestré», limitant ainsi l'interaction entre l'effecteur et sa protéine cible. Contrairement au modèle de garde, dans ce contexte, l'interaction entre la protéine « decoy » et l'effecteur ne contribue pas à la virulence de l'agent pathogène (van der Hoorn and Kamoun, 2008) et dans ce cas l'absence simultanée des protéines R et « decoy » ne provoque pas de perte de virulence d'un isolat possédant l'effecteur correspondant. Au sein de certaines protéines R, il est possible de retrouver des domaines protéiques atypiques qui semblent jouer un rôle de « decoy » particulier. Par exemple la protéine RRS1 *d'Arabidopsis* contient un domaine WRKY dont la fonction semble être de détecter un effecteur qui cible normalement plusieurs protéines WRKY impliquées dans la défense (Deslandes *et al.*, 2002). Chez le riz, il a été montré que les gènes de résistance *Pi-Co39* (Cesari *et al.*, 2013) et *Pi-k* (Maqbool *et al.*, 2015) possèdent un domaine supplémentaire HMA (Heavy Metal-Associated) qui détecte l'activité de plusieurs effecteurs dont le rôle pourrait être de perturber le fonctionnement de protéines possédant des domaines HMA. Sur la base de ces observations, un nouveau modèle dit de leurre intégré (« integrated decoy ») émerge (Figure 2C) (Cesari *et al.*, 2014). Toutefois la dénomination de ces domaines comme « integrated decoys » n'est pas clairement définie, le terme de « sensor domain » est également suggéré en fonction du rôle que jouerait le domaine en question (Wu *et al.*, 2015). Un inventaire de ces « decoys » intégrés reste à faire chez les plantes. De même, le rôle de la plupart des protéines contenant des domaines similaires aux « decoys » en tant qu'acteur des défenses et/ou cible d'effecteurs reste inconnu.

Cette question fait l'objet du travail présenté dans le Chapitre I.

Les mécanismes de défense sous-jacents à la perception de PAMPs et/ou d'effecteurs (PTI et ETI) sont souvent similaires (Tsuda and Katagiri, 2010). Toutefois le temps de réponse suite à la perception d'un organisme étranger est plus court et les mécanismes de défense sont également induits plus fortement lors de l'ETI que lors de la PTI. De plus, comme mentionné précédemment, l'ETI est également souvent caractérisée par la mise en place d'une mort cellulaire programmée visant à limiter la propagation de l'agent pathogène, la HR.

2. Mécanismes de signalisation rapide chez l'hôte

Parmi les mécanismes précocement affectés par la présence d'un agent pathogène, les flux calciques et la production d'espèces réactives de l'oxygène (ROS - Reactive Oxygen Species) jouent un rôle

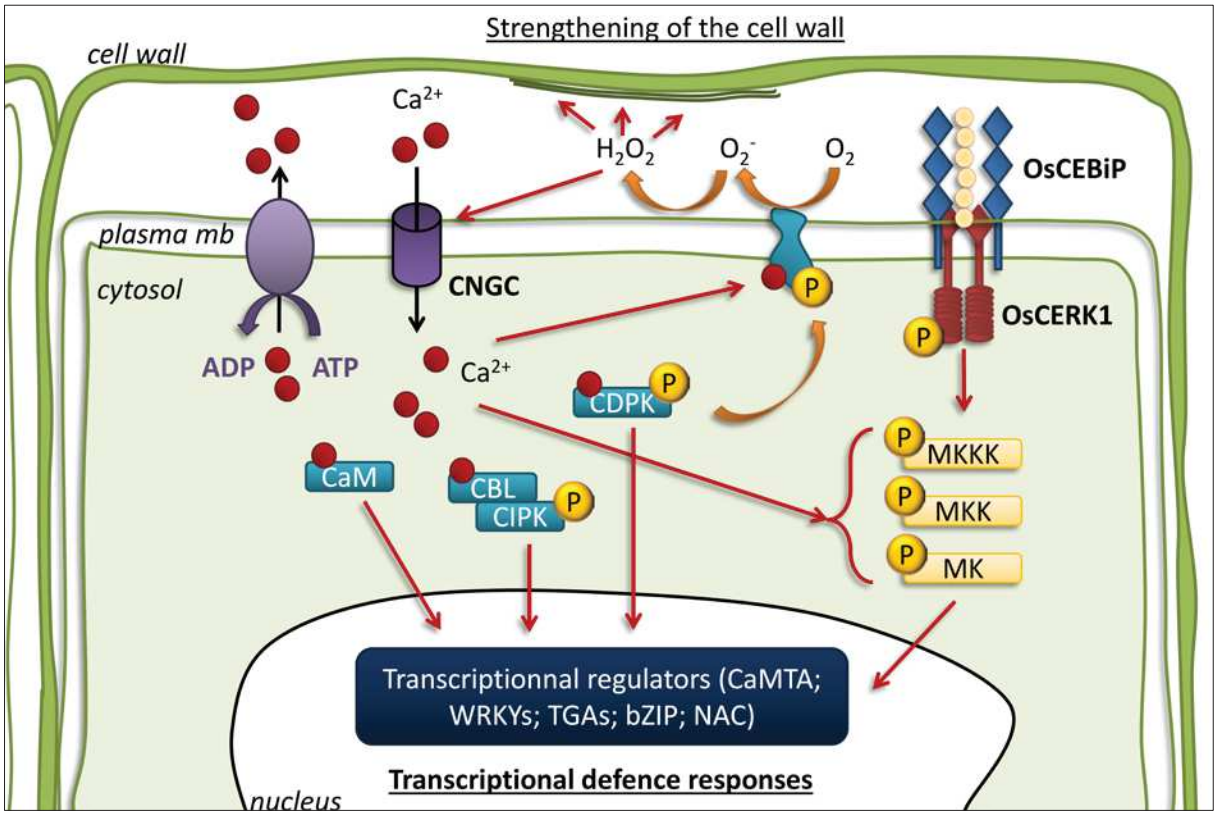

Figure 3. Signalisation générale de la mise en place des défenses

Suite à la perception des PAMPs, comme la chitine, par les PRRs (dont OsCEBiP et OsCERK1) une cascade de phosphorylation impliquant des MAPKs (Mitogen Activated Protein Kinases) est activée et aboutit à l'activation de facteurs de transcription qui participeront à l'induction de la réponse transcriptionnelle nécessaire aux défenses.

Les entrées calciques, via les CNGC (Cyclic Nucleotide-Gated Channel), conséquentes à l'attaque d'un agent pathogène contribuent également à l'activation de protéines impliquées dans la mise en place des défenses (CaM: Calmodulines, CBL: Calcineurin B-like protein, CIPK: CBL-interacting Protein Kinase, CDPK: Calcium Dependent Protein Kinase). Le calcium participe à l'induction de la production de ROS par les NADPH membranaires et est ensuite libéré dans l'apoplasme via des ATPases membranaires.

important en participant à l'induction de réponses locales et systémiques (Figure 3 ; (Gilroy *et al.*, 2014; Romeis and Herde, 2014)). La question des hormones impliquées dans la signalisation des défenses est traitée plus loin (I. 4. Les phytohormones dans la mise en place des défenses).

2.1. Les molécules messagers

2.1.1. Les flux calciques

Chez les plantes, le calcium est impliqué dans de nombreuses voies de signalisation et contrôle des processus physiologiques comme le cycle cellulaire, la division cellulaire et les réponses aux stress biotiques et abiotiques (Bose *et al.*, 2011). Le Ca^{2+} ne diffuse pas au travers les membranes, son transport est assuré par des transporteurs/canaux dont l'ouverture est régulée. La concentration en Ca^{2+} cytosolique est beaucoup plus faible que dans l'apoplasme ou que dans les organites intracellulaires (Pugin *et al.*, 2006). En fonction des stimuli, de la durée, de l'amplitude du flux et de la provenance du Ca^{2+}, les cellules végétales déclenchent les réponses spécifiques. Plusieurs études ont mis en évidence que la présence de PAMPs entraine une accumulation de Ca^{2+} dans le cytoplasme (Hashimoto and Kudla, 2011). Toutefois l'origine de ce Ca^{2+} est mal connue. Les influx calciques impliquent des protéines de type « cyclic nucleotide-gated channels » (CNGC) qui assurent le transport de cations de manière non-sélective et participent à la mise en place des défenses chez les plantes (Urquhart *et al.*, 2007; Moeder *et al.*, 2011; Nawaz *et al.*, 2014). Le calcium libre peut être perçu par des protéines contenant un motif « EF-hand » (Figure 3). Des études ont montré que des mutations dans la signalisation et/ou dans le transport du calcium affectaient la mise en place de la PTI et de l'ETI, suggérant que les signalisations calciques sont requises pour l'induction des mécanismes de défenses des plantes en général (Gao *et al.*, 2014; Boudsocq *et al.*, 2010).

2.1.2. Les espèces réactives de l'oxygène (ROS)

Tout comme le calcium, les ROS jouent le rôle de molécules signal chez les plantes et sont impliqués dans diverses réponses et processus développementaux (Foyer and Noctor, 2005). Les ROS sont des molécules pouvant être chargées ou non (H_2O_2, O_2^-, OH^-), très mobiles à travers les membranes et qui contribuent à la signalisation de cellule à cellule (Gilroy *et al.*, 2014). La production de ROS en réponse à une attaque pathogène est principalement assurée par des NADPH oxydases appelées Respiratory Burst Oxidase Homologs (RBOHs) (Egan *et al.*, 2007). Ces protéines membranaires présentent des motifs EF-hand affins pour le Ca^{2+} qui module ainsi leur activité. D'autres éléments de la voie de signalisation calcique affectent l'activité des NADPH-oxydases comme les CDPKs qui participent à leur phosphorylation (Figure 3) (Pugin *et al.*, 2006). Parmi les ROS, l'ion OH^- est le plus oxydant. Son accumulation est rapidement néfaste pour les cellules végétales car elle entraine la peroxydation des lipides, affecte dramatiquement l'activité de certaines enzymes ainsi que l'intégrité

des acides nucléiques. Le peroxyde d'hydrogène (H_2O_2) peut être métabolisé par des catalases ainsi que par des ascorbate ou glutathione peroxydases (Apel and Hirt, 2004). Il est également utilisé par des peroxydases pariétales pour former des liaisons covalentes entre les glycoprotéines de la paroi, les rendant ainsi insolubles. De même, il sert d'oxydant pour la synthèse de callose et de lignine. En plus de leur rôle dans la signalisation, les ROS contribuent donc également au renforcement de la paroi (Cf 3.2. Le renforcement de la paroi, (Torres *et al.*, 2006)).

L'accumulation de ROS au cours d'une infection fût décrite dans un premier temps chez le tabac et le soja, respectivement en interaction avec les bactéries phytopathogènes *Pseudomonas syringae pv. syringae* et *P.syringae* pv. g*lycinea.* Dans le cas d'une interaction compatible, une accumulation faible et rapide de ROS a été mise en évidence. En revanche, dans le cas d'une interaction incompatible, une deuxième vague de production de ROS plus importante a été mesurée. Cette deuxième vague de « burst » oxydatif joue un rôle crucial dans la mise en place de la HR (Cf 3.5. La réponse hypersensible, (Lamb and Dixon, 1997)).

Lors d'une attaque pathogène, le calcium déclenche la production de ROS. De même, l'accumulation de ROS entraine une accumulation de calcium. Ce système de boucle de régulation entre ROS et Ca^{2+} participe à la propagation du signal aux cellules adjacentes et *in fine* à toutes les cellules de l'organisme même à distance du site infectieux (Gilroy *et al.*, 2014).

2.2. Les autres facteurs moléculaires

2.2.1. Les Mitogen-Activated Protein Kinases (MAPKs)

Les modifications post-traductionnelles comme les phosphorylations permettent une régulation rapide des activités protéiques. Après la perception de l'agent pathogène par les PRRs et/ou par les protéines R, la signalisation est assurée par des MAPKs (Mitogen Activated Protein Kinases) (Pitzschke *et al.*, 2009; Tsuda *et al.*, 2013; Kishi-Kaboshi *et al.*, 2010). Les cascades de phosphorylation impliquant des MAPKs forment un réseau complexe qui contribue à une intégration de différents signaux – de stress abiotiques/biotiques et développementaux (Knight and Knight, 2001; Shi *et al.*, 2010; Zhang and Klessig, 2001; Wang *et al.*, 2007). Chez le riz certaines MAPKs – BWMK1, MAPK4, MAPK5, MAPK6 – ont été caractérisées pour affecter les défenses. BWMK1 active le facteur de transcription OsEREBP1, connu pour participer à la résistance à *M. oryzae* et *Xoo* (Cheong *et al.*, 2003). Le gène *OsMAPK5* est induit au cours de l'infection par *M. oryzae.* Des mutants de *OsMAPK5* silencés par RNAi montrent une résistance accrue à plusieurs pathogènes potentiellement due à une accumulation d'éthylène (ET) qui affecte l'expression de gènes de défense appelés gènes *PR* (Pathogenesis-Related genes). Cependant, les mutants *Osmapk5* présentent également une sensibilité accrue aux stress abiotiques (hydrique et osmotique) certainement due à une sensibilité plus faible à une autre hormone végétale, l'acide abscissique (ABA), requis pour la résistance à ce

type de stress. Ces phénotypes suggèrent qu'OsMAPK5 aurait un rôle de régulateur négatif dans la mise en place des défenses du riz potentiellement *via* la voie de l'ET, ainsi qu'un rôle de régulateur positif de la voie ABA impliquée dans la résistance aux stress abiotiques. OsMAPK5 serait donc au carrefour entre l'induction de réponses à des stress biotiques ou abiotiques (Xiong and Yang, 2003; Bailey *et al.*, 2009). La cascade de signalisation des MAPKs permet l'activation de facteurs de transcription qui contribuent à la reprogrammation transcriptionnelle nécessaire à la mise en place de l'immunité végétale (Liu *et al.*, 2014; Chen and Ronald, 2011).

2.2.2. Les régulateurs transcriptionnels

Au cours d'une infection, une reprogrammation transcriptionnelle massive a lieu. Pour cela des régulateurs transcriptionnels spécifiques sont induits ou réprimés en fonction du stimulus perçu. Parmi ces régulateurs on retrouve principalement des facteurs de transcription appartenant à la famille des WRKYs, des TGAs, des bZIP ou encore des NACs (Moore *et al.*, 2011; Pandey and Somssich, 2009; Nuruzzaman *et al.*, 2013). Chez le riz une centaine de facteurs de transcription de type WRKYs sont prédits, parmi eux beaucoup sont régulés transcriptionnellement au cours d'un stress biotique. Certains gènes *WRKYs* sont spécifiquement induits en réponse à *Xoo,* comme *WRKY28-62-71-76.* D'autres sont impliqués dans la résistance à divers pathogènes (WRKY30, WRKY45). OsWRKY45 est un élément clé de l'activation des défenses dépendante de la voie de l'acide salicylique (SA) (Shimono *et al.*, 2007). Cette protéine semble être à l'interface entre les réponses aux stress biotiques et abiotiques comme OsWRKY13 (Cheng *et al.*, 2015). Ce dernier est impliqué dans la régulation des gènes de défense au carrefour entre l'acide jasmonique (JA) et le SA (De Vleesschauwer *et al.*, 2013). Tout comme les MAPKs, les facteurs de transcription forment un véritable réseau qui permet l'intégration des différents signaux perçus par la plante et la mise en place des réponses optimales.

3. Les réponses de défense

Suite à la perception et à la transduction du signal de la présence d'un micro-organisme, la plante met en place différents mécanismes de défense visant à limiter la pénétration de l'agent pathogène et/ou son invasion (Figure 4).

3.1. La fermeture des stomates

Etant des ouvertures naturelles, les stomates sont couramment utilisés comme point d'entrée par les parasites foliaires L'ouverture stomatique est conséquente à une augmentation de la turgescence des cellules de garde causée par à un influx d'ions K^+ couplé à une sortie d'ions H^+ *via* des H^+-ATPases (Karlsson and Schwartz, 1988). Certaines phytohormones, comme l'ABA ou le JA, ou certaines

toxines, comme la fusicoccine, affectent l'ouverture des stomates et peuvent être manipulées par les agents pathogènes dans le but de faciliter leur pénétration (McAinsh *et al.*, 1990; Hossain *et al.*, 2011; Turner and Graniti, 1969). Par exemple, la bactérie phytopathogène *Pseudomonas syringae* pv. *syringae* produit un analogue du JA, la coronatine, qui entraine la ré-ouverture des stomates et optimise sa pénétration à l'intérieur des tissus foliaires. Cette molécule est requise pour la virulence optimale de ce parasite (Zeng *et al.*, 2010).

3.2. Le renforcement de la paroi

La paroi cellulaire végétale est principalement composée de glycoprotéines, de cellulose, d'hémicellulose et de pectines. Parmi les défenses pré-invasives, le renforcement et l'épaississement de la paroi végétale jouent un rôle essentiel car elle est la barrière physique ultime entre l'agent pathogène et le contenu intracellulaire de la cellule de l'hôte (Bradley *et al.*, 1992).

Suite à la perception d'un agent pathogène un dépôt d'un polymère de glucose, la callose, a pu être observé (Hématy *et al.*, 2009). Chez le riz, la synthèse de callose est assurée par des callose synthases membranaires (Chen and Kim, 2009). Ce dépôt est particulièrement visible au niveau du site d'infection et forme des structures appelées « papillae » (Hückelhoven, 2007; Hématy *et al.*, 2009). Les « papillae » sont également formées de lignine, de peroxydases et de glycoprotéines liées par des liaisons covalentes (Hückelhoven, 2007; Hématy *et al.*, 2009) .

La lignine est un composé phénolique dérivé de la phénylalanine. Très résistante à la pression mécanique et à la dégradation enzymatique, l'activation de la biosynthèse de lignine est directement corrélée à la quantité de lignine accumulée et au niveau de résistance des plantes (Vance *et al.*, 1980).

La paroi végétale comporte des protéines dont les hydroxyproline-rich glycoprotéines (HRGP). L'H_2O_2 généré pendant le « burst » oxydatif est utilisé par les peroxydases pariétales (de classe III) pour former des liaisons covalentes entre ces protéines, les rendant ainsi insolubles (Brisson *et al.*, 1994).

Les mécanismes décrits ci-dessus participent conjointement au renforcement de la paroi et limitent efficacement la pénétration de divers agents pathogènes (bactériens ou fongiques). De nombreux mutants affectés dans ces processus sont affectés dans leur résistance (Jacobs *et al.*, 2003; Kawasaki *et al.*, 2006; Tronchet *et al.*, 2010).

3.3. La production de phytoalexines

En réponse à une attaque pathogène, les plantes produisent des métabolites secondaires aux propriétés antimicrobiennes appelées phytoalexines. Chaque espèce végétale produit un panel plus ou moins spécifique de ces molécules (Ahuja *et al.*, 2012).

Figure 4.

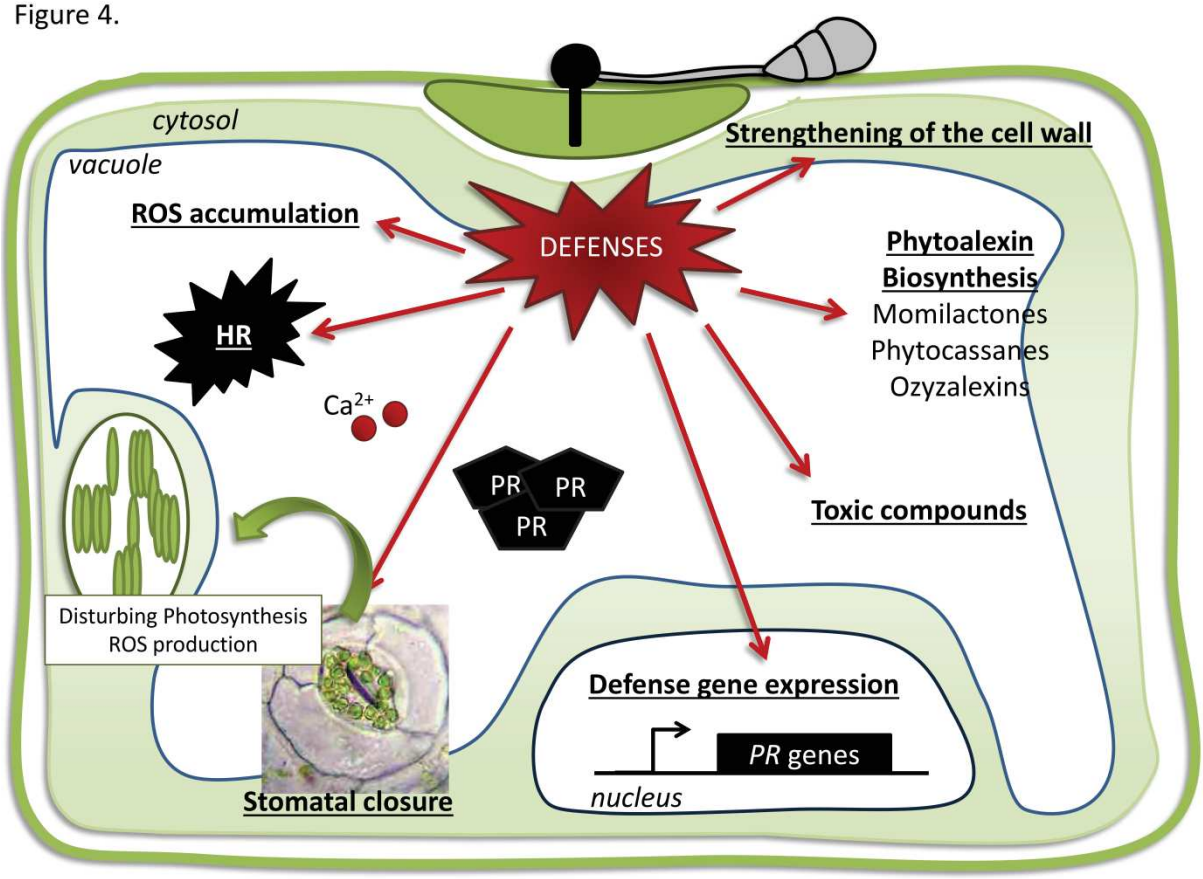

Figure 4. Défenses basales mises en plante par l'hôte

Suite à l'attaque d'un agent pathogène fongique, les plantes se défendent en mettant en place différents mécanismes. Une polarisation de la cellule végétale est observée avec un dépôt de callose au niveau du site d'infection afin de renforcer la paroi et d'empêcher la pénétration du champignon. Les ROS (Reactive Oxygen Species) produits au cours des défenses contribuent également au renforcement de cette barrière physique. La production de ROS est également affectée par la fermeture des stomates qui est l'une des réponses de défenses les plus précoces. Les reprogrammations transcriptionnelles qui ont lieu pendant les défenses participent à la production de protéines PR (Pathogenesis Related), de phytoalexines et autres composés phénoliques toxiques. Induits fortement, ces mécanismes peuvent entrainer une mort cellulaire programmée de la cellule infectée, appelée réponse hypersensible (HR : Hypersensitive Response).

On retrouve parmi elles la camalexine très étudiée chez *Arabidopsis* et autres Brassicassées, le resvératrol issu de la vigne, la scopolétine produite par certaines Solanacées… (Ahuja *et al.*, 2012). Parmi les phytoalexines produites chez le riz on distingue les momilactones, les phytocassanes et les oryzalexines dérivées de la voie des diterpénoïdes et la sakuranetine appartenant aux flavonoïdes (Shimura *et al.*, 2007).

Leur production est induite suite à la perception d'éliciteurs comme la chitine, les cérébrosides fongiques et l'acide cholique (Ahuja *et al.*, 2012; Koga *et al.*, 2006). L'activation de la voie du MEP (Methylerythritol phosphate), une voie métabolique chloroplastique, est requise pour former les précurseurs des phytoalexines comme l'isopentenyl diphosphate (Okada *et al.*, 2007).

Les variétés de riz résistantes montrent une accumulation de phytoalexines plus rapide que les variétés sensibles. Cette accumulation contribue à l'induction de la réponse hypersensible (HR) et limite efficacement la propagation de l'agent pathogène fongique *M. oryzae* (Hasegawa *et al.*, 2010).

Des études de mutants de riz affectés dans leur production en phytoalexines ont permis de mettre en évidence certains facteurs moléculaires impliqués dans leur biosynthèse ou dans leur signalisation. Parmi eux, les facteurs de transcription OsTGAP1 et OsWRKY45, des MAPKs comme OsMAPK4 (MAP2K) et OsMAPK6 ainsi que des protéines impliquées dans l'intégration des signaux calciques comme OsCIPK14 et OsCIPK15 (Okada *et al.*, 2009; Akagi *et al.*, 2014; Kishi-Kaboshi *et al.*, 2010). Ces éléments participent à l'induction de production de phytoalexines, à la mise en place de la HR qui en découle et à l'expression de gènes *PR* nécessaire aux défenses végétales (Kurusu *et al.*, 2010).

3.4. Les gènes *PR*

Parmi les gènes transcriptionnellement induits suite à une attaque pathogène, les gènes *PR* jouent un rôle essentiel. Ces gènes sont classés en 17 familles en fonction des propriétés biologiques et biochimiques des protéines qu'ils codent, des plantes chez lesquelles ils ont été identifiés, leurs conditions d'induction… (L C van Loon *et al.*, 2006). Certaines sont conservées entre différentes familles végétales d'autres peuvent être spécifiques. Les PR-protéines sont produites suite à la perception d'un agent pathogène, par des stimuli hormonaux comme le SA ou le JA mais peuvent être également présentes dans les organes sains (pièces florales, pollen) ou produites en réponse à des stress abiotiques comme le froid. Parmi les PR protéines certaines présentent des activités chitinases efficaces contre les champignons pathogènes et insectes herbivores (PR3, -4, -8, -11). D'autres sont des endoprotéinases (PR7) ou des inhibiteurs de protéases (PR6). Elles peuvent participer au renforcement de la paroi *via* leur activité peroxydase (PR9), à la formation de ROS *via* une activité superoxyde dismutase (spécifiques des graminées PR15, -16) ou contribuer à la mise en place de la HR comme les PR10 caractérisées par une activité ribonucléase (Kim *et al.*, 2008; Zubini *et*

al., 2009; Edreva, 2005). Chez le riz une protéine PR10, PBZ1, a été décrite pour présenter une activité RNase et participer à l'induction de la HR chez le riz et le tabac (Kim *et al.*, 2011). De même chez le piment, une interaction entre une protéine PR10 et un LRR est nécessaire pour l'induction de la mort cellulaire (D., S., Choi *et al.*, 2012). Chez plusieurs espèces végétales, il a été mis en évidence que ces protéines PR10 étaient capables de lier les cytokinines (CKs) (Fernandes *et al.*, 2008; Zubini *et al.*, 2009). Toutefois le rôle des CKs sur l'activité de ces enzymes reste à démontrer. Chez le riz, l'expression constitutive (hors infection) de gènes *PR* est corrélée au niveau de résistance des plantes à *M. oryzae*, on parle de défenses préformées (Vergne *et al.*, 2010).

3.5. La réponse hypersensible (Hypersensitive Response – HR)

La réponse ou réaction hypersensible (HR - hypersensitive response) est un processus de mort cellulaire programmée (Programmed Cell Death - PCD) enclenché suite à une attaque pathogène. Cette réponse est caractéristique de l'ETI et permet de limiter efficacement la propagation de l'agent pathogène. Chez les végétaux, la mort cellulaire programmée est également impliquée dans des processus développementaux comme la différenciation des vaisseaux conducteurs et la sénescence.

Ces processus sont contrôlés par la cellule (Ma and Berkowitz, 2007; Jones, 2014), leur induction requière la présence du noyau ainsi qu'une traduction effective. Les différentes PCD étudiées chez les végétaux présentent des caractéristiques communes avec des similarités avec la mise en place de l'apoptose des animaux. Toutes sont initiées par des flux calciques et la plupart impliquent la vacuole (Ma and Berkowitz, 2007). Chez le riz, un canal calcique putatif, OsTPC1, est impliqué dans la sensibilité de réponse aux éliciteurs (PAMPs, MAMPs). Les mutants surexpresseurs *d'OsTPC1* montre un « burst » oxydatif plus important, une activation des MAPKs plus forte et plus de HR (Kurusu *et al.*, 2005).

Au cours d'une infection, la vacuole, qui représente environ 90% du volume cellulaire, accumule des composés phénoliques et protéines PR et devient une bombe à retardement (Bol *et al.*, 1990). Des protéines vacuolaires ont été identifiées pour avoir des activités similaires aux caspases, décrites pour intervenir dans l'apoptose chez les animaux (Zhang *et al.*, 2010; Hara-Nishimura *et al.*, 2005). Suite à un signal calcique, le tonoplaste est altéré et la vacuole explose, libérant ainsi ces substances et enzymes antimicrobiennes. La HR est également caractérisée par un gonflement des mitochondries, une dégradation des chloroplastes, une condensation et une dégradation de la chromatine (Mur *et al.*, 2008). Les mitochondries et les chloroplastes participent à l'intégration du signal de la mort cellulaire (Lam *et al.*, 2001).

4. Les phytohormones dans la mise en place des défenses

Lors d'une attaque par un agent pathogène, le temps de réponse, entre la perception de l'agent pathogène et la mise en place des défenses, est crucial pour l'issue de l'interaction.

Les phytohormones constituent un véritable réseau et selon leur homéostasie générale et locale, contribuent à l'établissement des réponses optimales. Certaines sont historiquement décrites pour participer à des processus développementaux, d'autres d'avantage pour leur rôle dans les interactions biotiques. Au cours de ces dernières années, de plus en plus d'études mettent en évidence des « cross-talk » entre les différentes voies de signalisation et/ou métaboliques des phytohormones. Une meilleure connaissance de ces réseaux pourraient permettre la mise au point de variétés pouvant être tolérantes à plusieurs stress.

4.1. Les hormones de défense

La façon qu'ont les micro-organismes fongiques de perturber l'homéostasie hormonale est décrite dans la partie III de l'Introduction. Ci-dessous sont rassemblés des éléments côté plante concernant le fonctionnement des hormones en réponse à un stress biotique (Figure 5).

4.1.1. Le modèle impliquant l'acide salicylique, l'acide jasmonique et éthylène

Historiquement, les premières études menées sur la dicotylédone modèle *A.thaliana* ont montré une accumulation d'acide salicylique (SA), acide jasmonique (JA) et d'éthylène (ET) au cours d'une attaque pathogène (Robert-Seilaniantz *et al.*, 2011). Chez cet organisme, les voies SA et JA/ET sont antagonistes et participent respectivement à la résistance contre les agents pathogènes biotrophes (SA) et contre les nécrotrophes et les herbivores (JA/ET).

Le SA est un composé phénolique connu pour réguler un panel de réponses suite à la perception de PAMPs/MAMPs ou d'effecteurs, notamment l'induction transcriptionnelle des gènes *PR* (Lee *et al.*, 1995). L'accumulation de SA en réponse à une attaque est dans un premier temps locale, puis se propage à distance du site infectieux de manière systémique. Cette accumulation systémique contribue à la mise en place de défenses plus rapides et plus intenses si un autre stress biotique se manifeste. On parle alors de SAR (Systemic Acquired Resistance ; (Mou *et al.*, 2003)). Le SA affecte également la production de ROS et est impliqué dans la HR (Herrera-vásquez *et al.*, 2015). Les organismes pathogènes biotrophes sont donc privés des ressources vivantes nécessaires à leur développement. En réponse aux agents nécrotrophes, qui se nourrissent de tissus morts, les stratégies de défenses développées par l'hôte sont différentes et font intervenir le JA et l'ET. Des facteurs clés de l'antagonisme des voies SA et JA/ET ont été mis en évidence, notamment AtNPR1, un régulateur transcriptionnel essentiel à l'expression des gènes de défense régulés par le SA (Kinkema *et al.*, 2000; Johnson *et al.*, 2003).

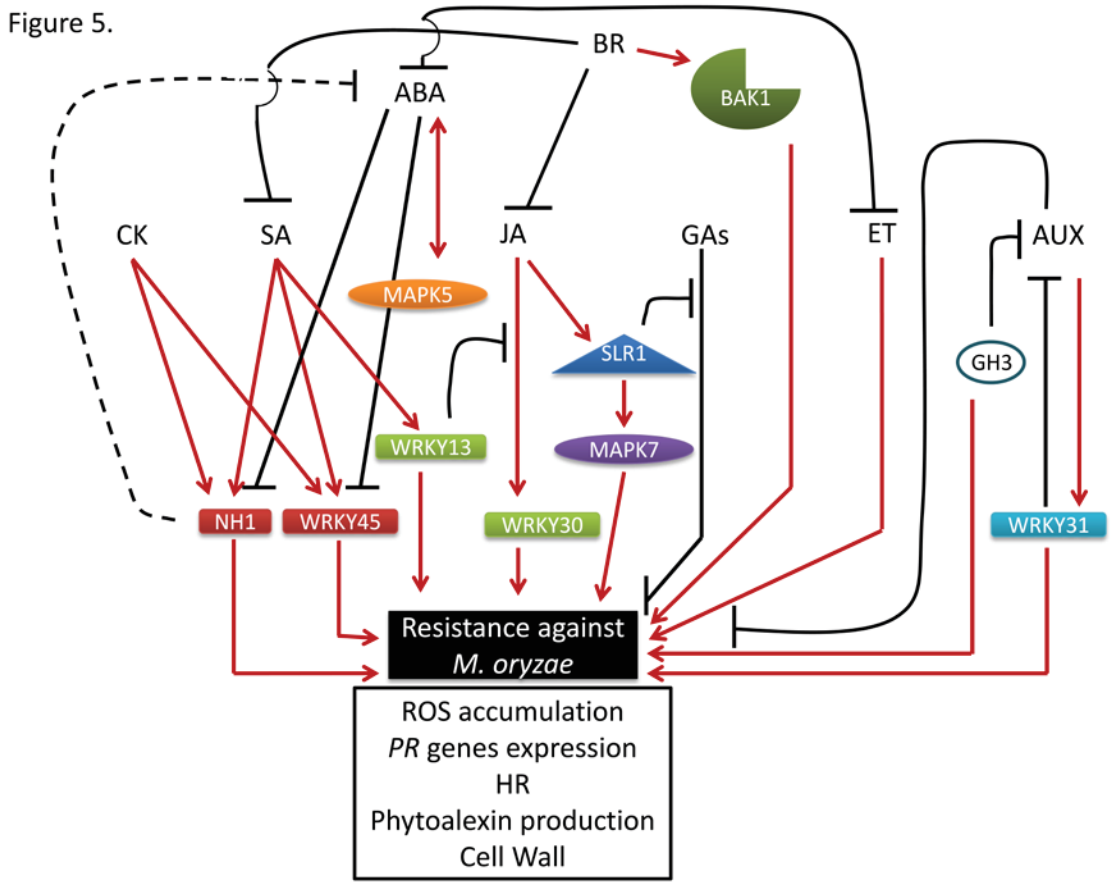

Figure 5. Effets des différentes hormones sur la résistance du riz à *Magnaporthe oryzae* et principaux facteurs impliqués

Les flèches rouges représentent un effet positif et les « T » noirs une inhibition.

CK: Cytokinines, SA: Acide Salicylique, ABA: Acide abscissique, JA: Jasmonates, BR: Brassinostéroïdes, GAs: Gibbérellines, ET: Ethylène, AUX: Auxines

BAK1 est un récepteur des BRs impliqué dans la mise en place des défenses et dans certains processus développementaux, SLR1 est une protéine DELLA impliquée dans l'inhibition de la signalisation des GAs requise pour la mise en place des défenses et stabilisée par le JA. GH3 est une enzyme qui intervient dans l'inactivation de l'auxine en assurant la conjugaison de cette hormone à des acides aminés. Certaines MAPKs, comme MAPK5 et MAPK7 sont également impliquées dans les réponses hormonales au cours des défenses, de même que certains facteurs de transcription (WRKY45-13-30-31) ou autres régulateurs transcriptionnels (NH1). La surexpression de NH1 entraine une répression des gènes normalement induit par l'ABA suggérant un effet inhibiteur de NH1 sur cette voie (indiqué en pointillé). Références clés récentes : De Vleesschauwer et *al*, 2014 ; 2013.

Cet antagonisme SA-JA/ET ainsi que le rôle clé de NPR1 ont été décrits chez plusieurs plantes représentant une large diversité du règne végétal. Cependant, chez *Oryza sativa* la régulation hormonale des défenses semblent être différente (Figure 5) (Chern *et al.*, 2001). Tout d'abord le riz présente un niveau basal de SA très élevé par rapport à des dicotylédones comme le tabac ou *Arabidopsis* (Silverman *et al.*, 1995). L'accumulation au cours d'une infection est faible et ceci indépendamment du mode de vie de l'agent pathogène (De Vleesschauwer *et al.*, 2013). Il semblerait que ce soit davantage la voie de signalisation du SA qui soit requise pour la mise en place des défenses que la production de SA elle-même. De plus, l'induction de gènes de défense par le BTH (un analogue du SA) est dépendante d'OsNPR1(OsNH1), l'orthologue d'AtNPR1, et également d'OsWRKY45. De même, l'analyse de données transcriptomiques a montré que la moitié des gènes affectés par le BTH (dont 2/3 des gènes réprimés) sont régulés par OsNPR1, notamment des gènes intervenant dans la photosynthèse et dans la synthèse de protéine (Nakayama *et al.*, 2013; Sugano *et al.*, 2010; De Vleesschauwer *et al.*, 2014). Ceci suggère des rôles distincts et plus ou moins complémentaires d'OsWRKY45 et d'OsNPR1, ce dernier étant potentiellement impliqué dans un « switch » énergétique visant à favoriser la mise en place des défenses.

Contrairement à la dichotomie observée chez *Arabidopsis*, chez le riz l'implication du JA ne semble pas être dépendante du mode de vie de l'agent pathogène. En effet, en plus de participer aux défenses en réponse aux agents pathogènes nécrotrophes et aux insectes herbivores, le JA permet également une résistance accrue aux organismes hémibiotrophes comme *Xoo* et *M. oryzae* (Mei *et al.*, 2006; Yamada *et al.*, 2012), ainsi qu'à l'agent pathogène racinaire biotrophe *Meloidogyne graminicola* (Nahar *et al.*, 2013). Pour autant, l'antagonisme entre les voies SA/JA semble être en partie conservé chez le riz mais reste ambigu (De Vleesschauwer *et al.*, 2014) : (i) une plus forte expression des gènes de réponse au JA a été détectée dans des plants de riz mutant NahG, déficient en SA (De Vleesschauwer *et al.*, 2014), (ii) une accumulation de JA plus importante de même qu'une plus forte induction des gènes de la voie de biosynthèse sont observées chez les mutants anti-sens *OsNPR1*. Enfin, (iii) une accumulation de JA suite à une blessure entraine une diminution de la quantité de SA.

Un autre facteur de transcription, OsWRKY13, participe à l'activation des défenses dépendante du SA et à l'inhibition de la voie JA en amont de WRKY45 et d'OsNPR1 (Qiu *et al.*, 2007). Toutefois, 50% des gènes induits par BTH ou par le SA sont également induits par le JA. Des études de mutants de riz ont montré qu'une accumulation et/ou une activation simultanée des voies SA et JA entraine une activation des gènes de défenses sous leur contrôle (De Vleesschauwer *et al.*, 2013).

Un nouveau modèle de régulation de ces voies a donc été proposé suggérant un effet synergique des deux hormones à de faibles concentrations mais antagonistes à concentration élevée (De Vleesschauwer *et al.*, 2014).

4.1.2. Les voies de défense JA-dependante SA-indépendante

Le JA et les formes conjuguées comme le MeJA, sont issus de la dégradation des lipides. Ils sont impliqués dans la résistance du riz à différents organismes pathogènes aux modes de vie différents. Des mutants sur-expresseurs *d'OsWRKY30* ou *d'OsAOS2 (Allene Oxide Synthase 2),* codant une enzyme clé de la biosynthèse du JA, montrent une accumulation de JA plus importante, ainsi qu'une expression plus forte des gènes *PR* JA-dépendante aboutissant à une résistance accrue à *Xoo* et à *M. oryzae* (Yamada *et al.*, 2012; Mei *et al.*, 2006). OsWRKY30 est également requis pour la résistance aux agents nécrotrophes, aux nématodes et aux insectes herbivores, de façon dépendante du JA (Ryu *et al.*, 2006). La synthèse de JA a été mesurée sur des cultures cellulaires de riz élicitées par de nombreux MAMPs et semble être une réponse commune (Desaki *et al.*, 2012).

4.1.3. L'éthylène (ET)

L'ET est une hormone gazeuse impliquée dans des processus développementaux mais également dans des réponses aux stress biotiques et abiotiques (Bleecker and Kende, 2000; Payton *et al.*, 1996; Grbic *et al.*, 1995). Dans les interactions plants-pathogènes, l'ET est décrit pour participer, avec le JA, à l'établissement de la résistance aux pathogènes nécrotrophes. En fonction des conditions environnementales, l'ET peut être un régulateur positif ou négatif des défenses (Broekaert *et al.*, 2006; Leendert C. van Loon *et al.*, 2006; Iwai *et al.*, 2006). Une accumulation plus précoce d'ET a été mesurée dans le cas d'une interaction incompatible entre le riz et *M. oryzae* que lors d'une interaction compatible. De même des mutants sur-exprimant le gène *ACS* impliqué dans la biosynthèse de l'ET montrent une production accrue d'ET et une résistance accrue à *M. oryzae.* Un métabolite dérivé de l'ET, le cyanide, est également requis pour la mise en place de l'ETI médiée par le gène de résistance *Pii* (Iwai *et al.*, 2006). D'une manière générale, cette hormone semble jouer un rôle important dans la mise en place des défenses basales du riz en réponse aux champignons mais compromettre la résistance à des agents pathogènes bactériens comme *Xoo* (Shen *et al.*, 2011). Des mutants perte de fonction *OsEDR1*, un régulateur de la voie éthylène, montrent une expression plus faible *d'ACS* ainsi qu'une plus forte accumulation de SA et de JA suggérant ainsi l'implication de ce régulateur dans un « cross talk » entre ces différentes voies hormonales (Shen *et al.*, 2011). La voie de l'ET semble également être connectée à celle de l'ABA : (i) des lignées RNAi *OsMAPK5* montrent une accumulation d'ET plus importante ainsi qu'une résistance accrue à de nombreux agents pathogènes hémibiotrophes dont *M. oryzae* (Bailey *et al.*, 2009) et (ii) chez des plantes RNAi pour le facteur de réponse à l'ET, OsEIN2, on observe une résistance accrue à *Cochliobolus miyabeanus* mais une sensibilité aux pathogènes hémibiotrophes, à l'ABA et à des stress abiotiques (De Vleesschauwer *et al.*, 2010).

4.2. L'acide abscissique, les auxines, les gibbérellines et brasinostéroïdes et leurs effets sur les défenses

Seuls quelques éléments généraux sont indiqués ici. L'implication des cytokinines dans les défenses est plus détaillée dans la sous-partie 4.3. Cytokinines et mécanismes de défense.

4.2.1. L'acide abscissique (ABA)

L'acide abscissique (ABA) est bien décrit pour son rôle dans l'induction de la fermeture des stomates et donc dans la résistance à des stress abiotiques comme la sécheresse (McAinsh *et al.*, 1990). Il a été montré que l'ABA augmentait la résistance du riz au champignon nécrotrophe, *C. miyabeanus* en limitant sa progression via une inhibition de la voie ET (De Vleesschauwer *et al.*, 2010). D'autres études montrent que l'ABA favorise l'infection par des agents pathogènes hémibiotrophes comme *Xoo* ou *M. oryzae* (Xu *et al.*, 2013; Koga *et al.*, 2004; Jiang *et al.*, 2010). Il agirait au niveau d'OsWRKY45 et d'OsNPR1 ou en amont, et inhiberait la mise en place des défenses dépendantes du SA. La sur-expression *d'OsWRKY45* ou *d'OsNPR1* abolit la sensibilité médiée par l'ABA (Jiang *et al.*, 2010). Des analyses d'expression ont montré que la surexpression d'*OsNPR1* entrainait la répression des gènes normalement induit par l'ABA (Sugano *et al.*, 2010). L'ABA participe à l'induction de gène codant des MAPKs comme *OsMAPK5* (Bailey *et al.*, 2009).

4.2.2. Les auxines

Les auxines sont des hormones dérivées du Tryptophane et sont impliquées dans la plupart des processus développementaux des végétaux (Zhao, 2010; Benjamins and Scheres, 2008). L'AIA (Acide indole 3-acétique) est l'auxine majeure détectée chez le riz et favorise, comme chez les dicotylédones (Navarro *et al.*, 2006), la sensibilité aux organismes (hemi)biotrophes comme *M. oryzae* ou *Xoo*. C'est l'AIA sous forme libre qui semble être responsable de cette sensibilité accrue car des mutants sur-exprimant *GRETCHEN HAGEN 3.8 (GH3.8),* qui code une enzyme nécessaire à la conjugaison de l'AIA avec des acides aminés, sont plus résistants à ces deux agents pathogènes (Fu *et al.*, 2011; Ding *et al.*, 2008; Domingo *et al.*, 2009). Cette résistance semble être indépendante des voies SA et JA et découler de l'effet de l'AIA sur la rigidité pariétale (Ding *et al.*, 2008). L'AIA est connue pour accroitre la plasticité de la paroi végétale, *via* les expansines. Ceci facilite ainsi la pénétration des organismes pathogènes et également leur accès aux nutriments. De même l'expression constitutive *d'OsGH3.1* conduit à une activation des gènes de défense (Domingo *et al.*, 2009). *OsWRKY31* est induit par un traitement à l'auxine et également pendant l'infection par *M. oryzae*. Ce facteur de transcription serait impliqué dans l'induction des gènes de réponse à l'auxine et des gènes de défense contribuant ainsi à une diminution de la sensibilité à l'AIA et à une résistance à

M. oryzae (J., Zhang *et al.*, 2008). D'une manière générale, l'AIA libre augmente la sensibilité du riz à diverses maladies.

4.2.3. Les brassinostéroïdes (BR)

Les BR composent l'unique famille d'hormones stéroïdes trouvée à ce jour chez les plantes. Des études ont montré qu'un traitement BR contribuait à la résistance du riz à divers organismes pathogènes (champignons, bactéries, virus) avec des modes de vie différents et ceci de manière SA-indépendante (Nakashita *et al.*, 2003). Toutefois, des études plus récentes menées sur la résistance du riz à des organismes pathogènes racinaires suggèrent un mode d'action des BR plus complexe. Il semblerait que l'activation de la voie BR rende le riz hypersensible aux attaques pathogènes de *Pythium graminicola.* Cet oomycète manipulerait la voie BR pour supprimer les défenses contrôlées par le SA et les GAs normalement requise pour la résistance à ce pathogène (De Vleesschauwer *et al.*, 2012). De même, les BRs compromettent la résistance médiée par le JA en réponse au nématode à galles *Meloidogyne graminicola* (Nahar *et al.*, 2013).

Comme mentionné précédemment, la perception extracellulaire des agents pathogènes nécessite parfois des co-récepteurs, parmi lesquels OsBAK1 (Macho and Zipfel, 2014). OsBAK1 est impliqué dans la perception des BRs, contribue à l'immunité végétale et est requis pour la régulation de processus développementaux (Park *et al.*, 2011). Des lignées RNAi montrent une sensibilité faible aux BRs et sont également affectés dans leur résistance à *M. oryzae* (Park *et al.*, 2011). Un traitement avec la brassinolide, une des BRs majeures, induit la résistance à *M. oryzae* ainsi qu'à *Xoo*. Ceci suggère un rôle intermédiaire d'OsBAK1 entre immunité et développement (Nakashita *et al.*, 2003).

4.2.4. Les gibbérellines (GA)

Les GAs ont été découvertes à l'origine chez le champignon pathogène du riz *Gibberella fujikuroi.* Cette maladie se manifeste par des plants de riz anormalement grands. Des mutants de riz affectés dans le gène *ELONGATED UPPERMOST INTERNODE* 1 (*Eui1*), codant une enzyme d'inactivation des GAs, accumulent une quantité plus importante de GAs et présentent de longs entre-nœuds. Les mutants KO-*eui1* présentent une sensibilité accrue à *Xoo* et à *M. oryzae*. Inversement, les lignées surexpresseurs EUI1 accumulent peu de GAs, sont de petite taille et sont plus résistants à ces deux agents pathogènes (Yang *et al.*, 2008; Y., Zhang *et al.*, 2008). D'autres études de mutants affectés dans la perception ou la biosynthèse des GAs suggèrent que ces hormones compromettent la mise en place de la résistance du riz aux agents pathogènes (hemi)biotrophes (Tanaka *et al.*, 2006; Qin *et al.*, 2012). Chez les mutants *gid1,* codant un récepteur aux GAs, PBZ1, une protéine PR10 aussi induite par le SA et le JA, est sur-accumulée (Tanaka *et al.*, 2006). Les mutants GAs sont affectés dans l'expression des gènes de défense, la production de phytoalexines et montrent des différences dans

la régulation/la production de SA et de JA (Tanaka *et al.*, 2006; Qin *et al.*, 2012). La biosynthèse des GAs implique des ent-kaurene oxydases. Certaines de ces enzymes, OsKOL4 et OsKOL5, semblent également être impliquées dans la biosynthèse des phytoalexines (Itoh *et al.*, 2004). De même des mutations dans la voie de signalisation des GAs, comme SLR1, l'unique protéine DELLA identifiée chez le riz, affecte la résistance à la pyriculariose. Chez les plantes, les protéines DELLA interviennent dans la répression de la signalisation des GAs. SLR1 est requise pour l'expression des gènes de défense JA dépendants permettant la résistance à *Xoo* et à *M. oryzae* comme *OsMAPK7* (Yang *et al.*, 2012). Des études montrent que SLR1 est stabilisé par le JA. Ceci suggère que cette protéine occupe une place charnière entre les voies JA et GAs (Yang *et al.*, 2012; Shimada *et al.*, 2006).

4.3. Les Cytokinines (CKs)

Les CKs sont impliquées chez les plantes dans divers processus physiologiques développementaux et adaptatifs en réponses à l'environnement biotique et abiotique.

4.3.1. Rôles chez la physiologie des plantes

Les CKs ont été initialement identifiées comme favorisant la division cellulaire (cytokinèse) (Skoog and Armstrong, 1970). Avec l'auxine, elles contribuent à la croissance et à la différenciation des tissus aériens et racinaires (Werner and Schmülling, 2009). Les CKs modulent la répartition des nutriments dans la plante et sont étroitement liées aux métabolismes carboné et azoté (Figure 6) (Peleg *et al.*, 2011; Sakakibara *et al.*, 2006). Comme l'ABA , les CKs sont impliquées dans la réponse au stress hydrique (Alvarez *et al.*, 2008). Elles semblent accroitre la tolérance des plantes à la sécheresse en participant à la redistribution des nutriments (Peleg *et al.*, 2011) et en affectant photosynthèse/photorespiration (Rivero *et al.*, 2009). Les CKs induisent la différenciation des chloroplastes, la synthèse de chlorophylle et de RuBisCo stimulant ainsi l'activité photosynthétique et la production de carbohydrates (Seyer *et al.*, 1975; Fletcher and McCullagh, 1971; Ohya and Suzuki, 1991). Il a été montré que cet effet stimulant sur la photosynthèse tend à retarder la mise en place de la senescence chez *Nicotiana* (Wingler *et al.*, 1998; Singh *et al.*, 1992; Jordi *et al.*, 2000). Le retardement de la senescence par les CKs semble également impliqué une régulation différente des flux/signaux calciques (Huang *et al.*, 1997; Elliott, 1983).

Tout comme l'ATP (Chivasa *et al.*, 2005), certaines CKs ont été caractérisées pour leur effet pro-apoptotique sur les cellules végétales (Carimi *et al.*, 2003; Carimi *et al.*, 2004; Vescovi *et al.*, 2012) et animales (Choi *et al.*, 2008; Mlejnek and Dolezel, 2005) suggérant que l'effet des CKs sur l'avenir de la cellule dépend de la molécule et de la concentration testée.

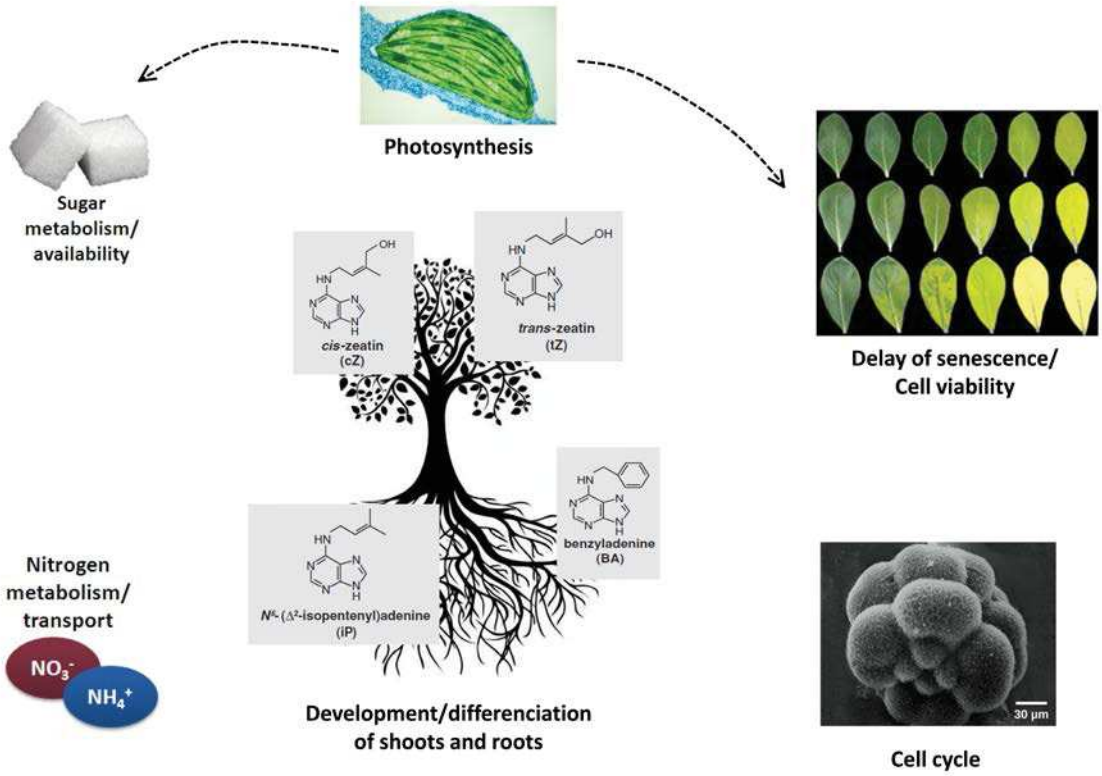

Figure 6. Principaux effets des CKs sur la physiologie des plantes

Les CKs sont des composés hormonaux dérivés d'adénine qui diffèrent par leurs chaines latérales pouvant être cyclique (BA) ou linéaire, hydroxylée (zéatines) ou non (isopentenyladenines). Elles participent à la croissance et à la différenciation de l'appareil aérien et racinaire. Les CKs affectent la photosynthèse en induisant la différenciation des chloroplastes et la synthèse de chlorophylle et de RuBisCo. Cet effet sur l'activité photosynthétique se répercute sur le métabolisme et la disponibilité des carbohydrates. Les CKs interviennent également dans le retardement de la sénescence (notamment en préservant la photosynthèse) ainsi que dans la viabilité des cellules et le cycle cellulaire. Ces hormones sont également connectées au métabolisme de l'azote en contribuant à l'expression de certaines enzymes ou transporteurs. Compte-tenu de leurs effets sur les métabolismes nutritionnels, directs ou indirects, les CKs modulent la répartition des nutriments chez les plantes.

4.3.2. Métabolismes des CKs

Les CKs sont des dérivés d'adénine, modifiés en position N^6 par la présence d'une chaine isopentenyl ou d'un cycle aromatique, respectivement appelés CKs isoprénoïdes ou aromatiques. Les CKs aromatiques ont été détectées dans certaines espèces végétales, mais les voies de biosynthèse et de dégradation restent à ce jour inconnues. La biosynthèse de CKs isoprenoïdes est assurée par des isopentenyl transférases, IPT ou tRNA-IPT en fonction de leur substrat (Figure 7) (Sakakibara, 2006). Ces enzymes assurent, respectivement, le transfert de la chaine isopentenyl sur l'ATP, ADP, AMP ou sur l'adénine 37 des ARNt dont l'anticodon commence par un U (Miyawaki *et al.*, 2006; Armstrong *et al.*, 1969). Les IPT contribuent à la biosynthèse de N^6-(Δ^2-isopentenyl)adenine (iP) et de *trans*-zéatine (*t*Z) ; les tRNA-IPT participent à la production de *cis*-zéatine (*c*Z) (Sakakibara, 2006). Chez *Arabidopsis*, iP et *t*Z semblent être majoritaires mais chez les monocotylédones, les formes *cis*-zéatines prédominent (Kudo *et al.*, 2012). Les CKs dites « libres » peuvent subir différentes modifications qui affectent leur affinité pour les récepteurs ainsi que pour les enzymes de dégradation (Sakakibara, 2006; Spíchal *et al.*, 2004; Lomin *et al.*, 2011; Strnad, 1997). Les CKs peuvent être glucosylées en *N3*, *N7*, *N9* du cycle purine et deviennent alors irréversiblement inactives. La chaine hydroxylée des zéatines peut également être modifiée par l'ajout d'un glucose ou d'un xylose par des zéatine-*O*-glycosyltransférase (Mok *et al.*, 2000; Veach *et al.*, 2003). Les *O*-glycosylzéatines et *O*-xylosylzeatines sont biologiquement inactives mais leur inactivation est réversible, elles serviraient de réservoir de CKs libres. Les formes libres (et ribosylées) peuvent être irréversiblement clivées par des cytokinines oxydases (CKX), libérant ainsi adénine/adénosine et la chaine latérale(Schmülling *et al.*, 2003; Ashikari *et al.*, 2005; Werner *et al.*, 2001). Les gènes du métabolisme des CKs semblent être conservés chez les plantes.

Chez *Arabidopsis* la voie impliquant des tRNA ne semble pas être la source majoritaire de CKs libres. Toutefois, les tRNA-IPT sont conservées chez d'autres organismes comme les bactéries (Motorin *et al.*, 1997), les champignons (Dihanich *et al.*, 1987), les oomycètes (Schwelm *et al.*, 2015; Argueso *et al.*, 2012), les nématodes (Siddique *et al.*, 2015) et les animaux (Golovko, 2000; Et and Acta, 1972). Les tRNA-IPT décrites, ne modifient que les tRNA dont l'anticodon commence par un U (Persson *et al.*, 1994; Phizicky and Hopper, 2010; Armstrong *et al.*, 1969). Chez *Saccharomyces cerevisiae,* les mutants *ΔMOD5* (KO pour un gène codant une tRNA-IPT) sont affectés dans l'efficacité de leur traduction, sont plus sensibles au stress osmotique et à certains fongicides (Suzuki *et al.*, 2012). Bien que ces modifications des tRNA aient été observées chez différents organismes, elles restent peu étudiées.

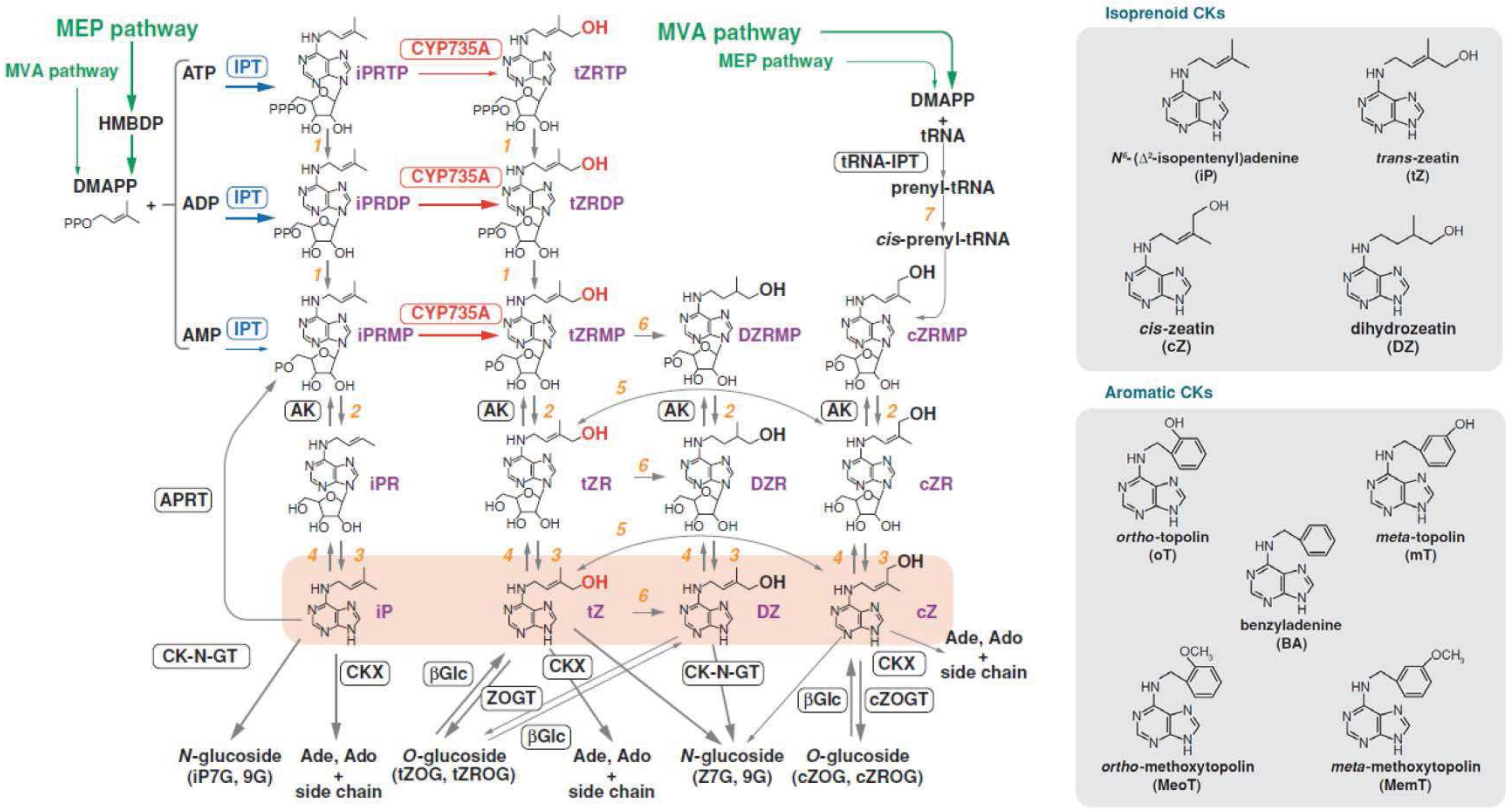

Figure 7. Schéma de la voie de biosynthèse des CKs isoprènes chez *A. thaliana*

Les CKs sont des composés hormonaux dérivés d'adénine qui diffèrent par leurs chaines latérales pouvant être aromatiques ou linéaires, hydroxylée ou non. Les chaînes latérales isoprènes de la *N6*-isopentenyl adenine (**iP**) et la trans-zeatine (tZ) issues du Dimethylallylpyrophosphate (DMAPP) de la voie du methylerythritol phosphate (MEP), contrairement à celle de la cis-zeatine (cZ) qui provient de la voie du mevalonate (MVA) (flèches vertes).

Les phospho-isopentenyl transférases (IPT) végétales préfèrent l'ATP ou l'ADP comme accepteurs des chaines isoprènes pour former respectivement iPRTP et iPRDP (flêches bleues).

La déphosphorylation des iPRTPs et iPRDPs par des phosphatases (1), la phosphorylation des iPRs par des adénosine kinases (AK) et la conjugaison de phosphoribosyles à l'iP, possible grâce à des adénine phosphoribosyltransferases (APRT), créent un pool métabolique d'iPRMP et d'iPRDP. L'APRT utilise également d'autres CKs nucléobasiques. Les CKs nucléotidiques sont converties en tZ-nucléotides par CYP735A (flèches rouges). Les iP, tZ et les nucléosides peuvent être catabolisés par des CKX (cytokinine oxydases) en adénine (Ade) ou adénosine (Ado). La conversion de la cZ en tZ est assurée par une zéatine cis-trans isomérase (5). La tZ peut être convertie de manière réversible en *O*-glycoside par les zéatine-*O*-glycosyltransferases (ZOGT) (reconvertie en zéatine par des β-glucosidases (βGlc)). Les CK nucleobasiques peuvent aussi être converties en *N*-glucosides par des CK *N*-glycosyltransférases (CK-*N*-GT). L'épaisseur des flèches vertes, bleues et rouges indique la force du flux métabolique. Les flèches noires représentent des mécanismes peu caractérisés à ce jour.

tZRDP, tZR 5-diphosphate; tZRTP, tZR 5-triphosphate; 2,5-ribonucleotide phosphohydrolase; 3, adenosine nucleosidase; 4, purine nucleoside phosphorylase; 6, zeatin reductase; 7, CK cis-hydroxylase. Modifiée à partir de Sakakibara, 2006.

4.3.3. Signalisation des CKs

La signalisation des CKs se fait selon un système de phospho-relai similaire au système de transduction du signal à deux composantes des bactéries (Figure 8) (West and Stock, 2001; Hwang and Sheen, 2001; Pareek *et al.*, 2006; Tsai *et al.*, 2012). La perception est assurée par des récepteurs histidine-kinase (HK) membranaires (Lomin *et al.*, 2011) dont les spécificités de substrats ont été étudiées (Lomin *et al.*, 2011; Spíchal *et al.*, 2004; J., Choi *et al.*, 2012). Cependant ces études sont souvent réalisées en systèmes hétérologues et peuvent présenter des biais, les microorganismes utilisés dans ces études étant également capables de produire des CKs (Mizuno and Yamashino, 2010). Certains récepteurs ont été caractérisés. Chez *Arabidopsis*, il a été mis en évidence qu'en culture cellulaire, le récepteur CRE1/AHK4 est nécessaire pour la mise en place de la PCD induite par les CKs (Vescovi *et al.*, 2012). AHK4 intervient également dans la reconnaissance des CKs produites par la bactérie phytopathogène *Rhodococcus fascians* qui ont la particularité d'être méthylées (Pertry *et al.*, 2009). Le récepteur orthologue identifié chez *Medicago truncatula* est impliqué dans la mise en place de la symbiose avec *Sinorhizobium meliloti* (Gonzalez-Rizzo *et al.*, 2006).

L'activation du récepteur HK entraine la phosphorylation de protéines navettes appelées, Histidine-Phospho transfer (HPt) protéines, qui assurent le transfert du phosphate du cytosol aux régulateurs de réponse nucléaires (RR). Parmi les RR on distingue, les RR-A (constitué uniquement d'un domaine RR) et les RR-B qui possèdent un domaine supplémentaire d'interaction à l'ADN. Les RR-B participent à l'induction des gènes de réponse aux CKs et les RR-A permettent un rétrocontrôle de la signalisation en inhibant les RR-B (Ito and Kurata, 2006; Hwang and Sheen, 2001). D'autres types de RR ont été mis en évidence (RR-C et pseudo RR) ainsi qu'un autre groupe de facteurs de transcription appelés CK-Response Factors (CRF) mais leurs rôle dans la signalisation des CKs reste peu connu (Pareek *et al.*, 2006; Zwack *et al.*, 2013; Rashotte and Goertzen, 2010). Les gènes *RR* sont rapidement induits suite à la perception de CKs (Brenner *et al.*, 2005; Jain *et al.*, 2006). Chez *Arabidopsis* certains RR ont été mis en évidence pour interagir avec des éléments de défense de la voie du SA (Choi *et al.*, 2010; Argueso *et al.*, 2012).

Les systèmes à deux composantes sont également impliqués dans la signalisation de l'éthylène et dans la réponse à certains stress abiotiques comme la sécheresse chez les plantes (Bertheau *et al.*, 2012; Bertheau *et al.*, 2013) ou au stress osmotique et résistance aux fongicides chez les champignons (Li *et al.*, 1998; Posas *et al.*, 1996; Motoyama *et al.*, 2005). Toutefois, l'affinité de ces récepteurs pour les CKs n'a jamais été testée.

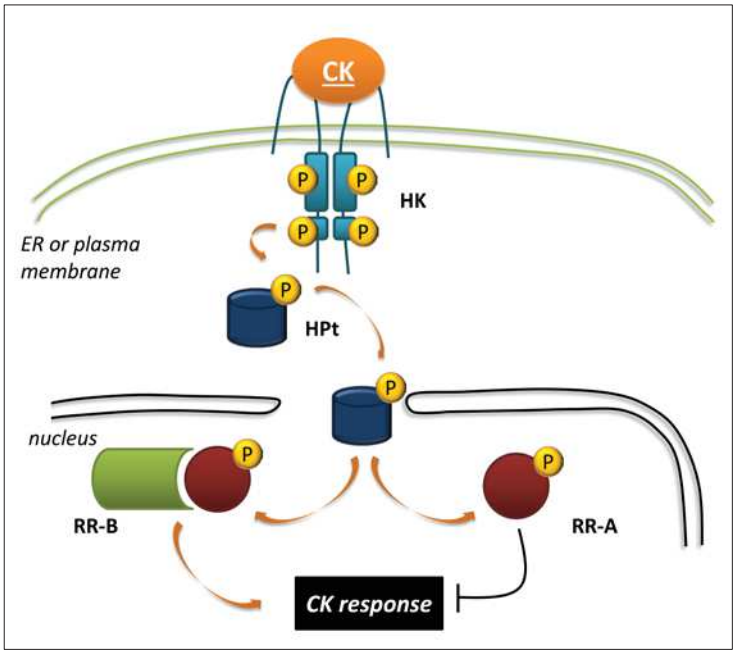

Figure 8. Voie de signalisation des CKs chez les plantes

Les CKs sont perçues par des récepteurs de type Histidine Kinases (HK) membranaires. La perception des composés hormonaux initie une cascade de phosphorylation du récepteur, aux régulateurs de réponses nucléaires (RR-A et B) *via* des protéines navettes appelées Histidine Phosphotransfert protein (HPt). Les RR-B possèdent un domaine de liaison à l'ADN et sont impliqués dans l'induction des gènes de réponse aux CKs. Les RR-A ne possèdent pas de domaine de liaison à l'ADN et participent au rétrocontrôle du signal.

4.3.4. Cytokinines et mécanismes de défense

Des études récentes montrent que les CKs participent à la mise en place des défenses chez les plantes (De Vleesschauwer *et al.*, 2013) en réponse à tous type d'agents pathogènes (Giron *et al.*, 2013). Chez *Arabidopsis,* les deux CKs *trans*-zéatine et BAP affectent respectivement la résistance à *Pseudomonas syringae* pv. *tomato* et à l'oomycète *Hyaloperonospora arabidopsidis* en intéragissant avec des facteurs impliqués dans la mise en place des défenses dépendantes du SA (Argueso *et al.*, 2012). Cette synergie entre les CKs et le SA a également été mise en évidence chez le riz (Figure 5) (Jiang *et al.*, 2013; Akagi *et al.*, 2014). Les CKs affectent l'induction de gènes *PR* et la biosynthèse de phytoalexines (Akagi *et al.*, 2014; Grosskinsky *et al.*, 2011; Jiang *et al.*, 2013; Ko *et al.*, 2010). Cependant comment les CKs affectent les mécanismes de défense des plantes reste peu documenté. Choi et *al*, 2010, ont mis en évidence que le régulateur de réponse aux CKs, ARR2, interagissait avec le facteur de transcription TGA3 pour induire l'expression du gène *PR1* (Choi *et al.*, 2010). Chez *Arabidopsis* la surexpression de la protéine NB-LRR UNI entraine une induction de l'expression de certains gènes *PR* qui nécessite une production de SA et altère le développement des plantes *via* une dérégulation de la voie des CKs (Igari *et al.*, 2008). Toutefois le lien avec la voie des CKs est mal défini. Chez les plantes modèles *Arabidopsis* et *Oryza sativa,* les CKs semblent être connectées à différentes voies hormonales impliquées dans les défenses (Naseem *et al.*, 2012; De Vleesschauwer *et al.*, 2013) et contribuer ainsi à l'équilibre entre les défenses et les autres processus physiologiques (croissance, nutrition, stress abiotiques…). Cependant ces connexions restent encore obscures.

L'étude des effets des CKs sur la résistance du riz fait l'objet du travail présenté dans le Chapitre II de la thèse.

II. Mécanismes de virulence : le cas de *Magnaporthe oryzae*

1. Généralités et cycle de développement

1.1. *Magnaporthe* et biotrophie

Magnaporthe oryzae est un champignon phytopathogène hémibiotrophe appartenant à la classe des Ascomycètes. Cet agent est responsable de la pyriculariose qui se manifeste par la formation de lésions sur différents organes : feuilles, gaines, tiges, panicules (Figure 9ABC). Les organismes pathogènes hémibiotrophes ont un mode de vie intermédiaire entre les biotrophes, qui cherchent à maintenir leur hôte en vie afin de se nourrir de tissus vivants, et les nécrotrophes qui sécrètent des toxines et des enzymes de dégradation visant à tuer activement les cellules infectées pour puiser les ressources qui leur sont nécessaires (Divon and Fluhr, 2007; Horbach *et al.*, 2011; Mendgen and Hahn, 2002; Walters and McRoberts, 2006). *M. oryzae* présente une hémibiotrophie particulière. Le mycélium envahit chaque nouvelle cellule de manière biotrophe puis évolue vers une activité nécrotrophe. Ainsi un individu se comporte simultanément (i) de manière biotrophe en bordure de lésion, correspondant à la zone récemment envahie et (ii) de manière nécrotrophe, au centre de la lésion, caractérisé par une nécrose (Ribot *et al.*, 2008; Talbot, 2003). C'est au sein de ces zones nécrosées que les spores seront générées. Ces spores issues de la reproduction asexuée, appelées conidies, contribuent à la propagation de cet agent pathogène.

1.2. La formation de l'appressorium nécessaire pour la pénétration de l'hôte

Une fois sur leur hôte, les conidies forment un tube germinatif (Figure 10). A l'extrémité du tube germinatif se différentie une structure indispensable pour la pénétration de *M. oryzae* : l'appressorium (Figure 9E). Au cours de la formation de cet organe vont être accumulés glycogène et corps lipidiques nécessaires pour l'augmentation de la turgescence. Lorsque l'appressorium est mature, il est caractérisé par une paroi mélanisée robuste qui permet de contenir cette très forte pression (8 MPa) et d'exercer une force sur la paroi cellulaire végétale jusqu'à sa rupture, contribuant ainsi à la pénétration du champignon. En condition *in vitro* la formation de l'appressorium a lieu entre 4 et 8h après la récolte des spores et entre 2 et 20h *in planta*. La perception de l'environnement hydrophobe nécessaire à l'initiation de la germination et de l'appressorium implique le récepteur membranaire PTH11, appartenant à la famille des récepteurs couplés aux protéines G (Talbot, 2003). Le développement de l'appressorium est crucial pour une virulence optimale. La plupart des mutants affectés dans leur virulence sont affectés dans la formation de l'appressorium. Par exemple les mutants *Δmac1,* mutés pour une adenylate cyclase, sont affectés dans la formation de l'appressorium suggérant que la production d'AMPc est requise pour le développement de cet organe (Talbot, 2003).

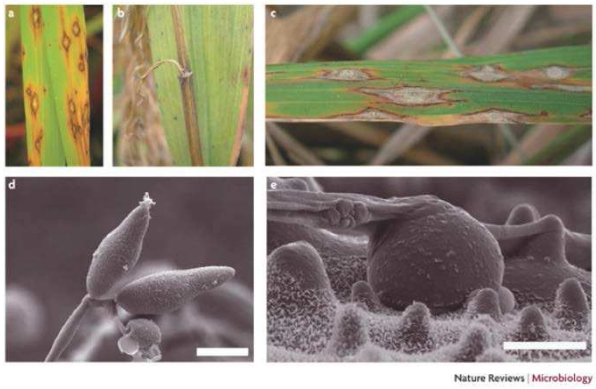

Figure 9. La pyriculariose : symptômes et cytologie

Symptômes causés par *Magnaporthe oryzae*, sur les feuilles, des lésions en losange pouvant être nécrosées au centre (a) et sur les panicules (aussi appelé « Neck blast ») (b). Au fur et à mesure que le champignon se propage, le centre des lésions se nécrose et devient sporulant (c). Les spores produites, les conidies (d), sont entourées d'un mucilage qui contribue à leur adhésion. Après germination, ces spores forment un appressorium, un organe qui permet d'accumuler une forte pression nécessaire pour rompre la paroi végétale (e). Issue de Talbot & Wilson, 2009.

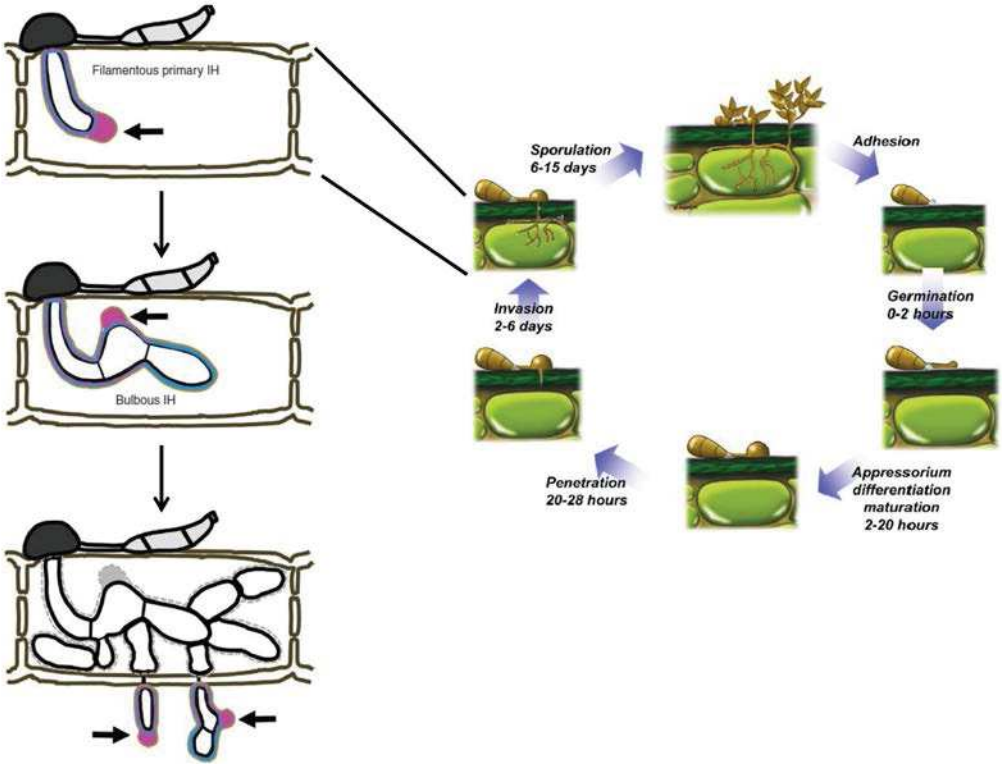

Figure 10. Cycle d'infection de *Magnaporthe oryzae* et croissance des hyphes *in planta*

Après son attachement à la surface de la feuille, la spore (conidie) germe rapidement. Le tube germinatif différencie ensuite à son extrémité l'appressorium, un organe nécessaire pour perforer la paroi végétale des cellules épidermiques. Une fois à l'intérieur de l'hôte, le champignon développe des hyphes bulbeux (IH, Invasive Hyphae) qui se divisent et finissent par envahir la cellule et les cellules voisines sans dommages visibles. Ces hyphes assurent la nutrition et la sécrétion de facteurs nécessaires à la virulence notamment *via* le « Biotrophic Interfacial Complex » (BIC) représenté par les excroissances roses et les flèches noires sur le schéma de gauche. Environ 4 à 5 jours après infection le champignon induit la nécrose des tissus au centre des lésions et sporule. Modifiée à partir de Giraldo et *al,* 2013 et Ribot et *al,* 2008.

Cette hypothèse est confirmée par la restauration de la formation de l'appressorium chez mutants *Δmac1* par un apport exogène d'AMPc. De même, de fortes concentrations d'AMPc engendrent la formation d'appressorium chez *Magnaporthe* dans des conditions d'hydrophobicité normalement non favorables (Choi and Dean, 1997; Lee and Dean, 1993).

En plus de l'AMPc, le Ca^{2+} et les ROS participent également à la signalisation de la mise en place de l'appressorium (Egan *et al.*, 2007; Li *et al.*, 2012). Des mutants affectés dans des canaux calciques sont affectés dans leur virulence (appressorium, sporulation, croissance des hyphes, (Nguyen *et al.*, 2008)). Le calcium induit également le facteur de transcription MoCRZ1 requis pour l'induction de gènes impliqués dans l'intégrité de la paroi (Rispail *et al.*, 2009). Bien que les ROS soient requis pour le développement de *Magnaporthe,* ils contribuent également à la mise en place des défenses de la plante et peuvent avoir un effet antimicrobien. Le gène *HYR1*, codant une protéine présentant un domaine glutathione peroxydase, est requis pour l'inhibition de l'accumulation de ROS au cours de l'infection, pour la tolérance au stress oxydatif *in vitro* et *in planta* et par conséquent pour la virulence optimale de ce champignon pathogène (Huang *et al.*, 2011).

1.3. Croissance mycélienne

A la base de l'appressorium se développe un hyphe de pénétration. Cet hyphe étroit s'enfonce dans les cellules épidermiques de l'hôte et devient ensuite bulbeux. Ces hyphes bulbeux, spécialisés dans la nutrition et dans les processus d'évasion de reconnaissance par l'hôte, se développent au contact de la membrane plasmique de la cellule végétale qui reste dans un premier temps intègre (phase biotrophe; Figure 10) (Yi and Valent, 2013; Oliveira-Garcia and Valent, 2015).

2. Nutrition et virulence

De nombreuses études menées *in vitro* ont mis en évidence que *M. oryzae* était capable de métaboliser une large gamme de substances carbonées et azotées (Fernandez *et al.*, 2014). Certains composés comme la biotine et la thiamine sont essentiels à son développement. D'une manière générale, le glucose est préférentiellement utilisé par les champignons comme source de carbone et l'ammonium comme source d'azote (Divon and Fluhr, 2007). Lorsqu'il atteint l'apoplasme, *M. oryzae* se retrouve dans un environnement riche en carbohydrates mais pauvre en azote. La perception du glucose, plus particulièrement du G6P, contrôle les voies métaboliques carbonées et azotées, de même que l'expression de certains facteurs de virulence. Cette régulation est dépendante de Tps1 (Tréhalose-6-Phosphate Synthase 1) (Figure 11) (Wilson *et al.*, 2007).

Tps1 est un facteur de régulation central entre le métabolisme carboné et azoté en affectant la voie de pentose phosphate et en permettant la régénération du NADPH essentiel à la régulation du redox (Wilson *et al.*, 2007; Wilson *et al.*, 2010). Les mutants *Δtps1* sont affectés dans le développement de

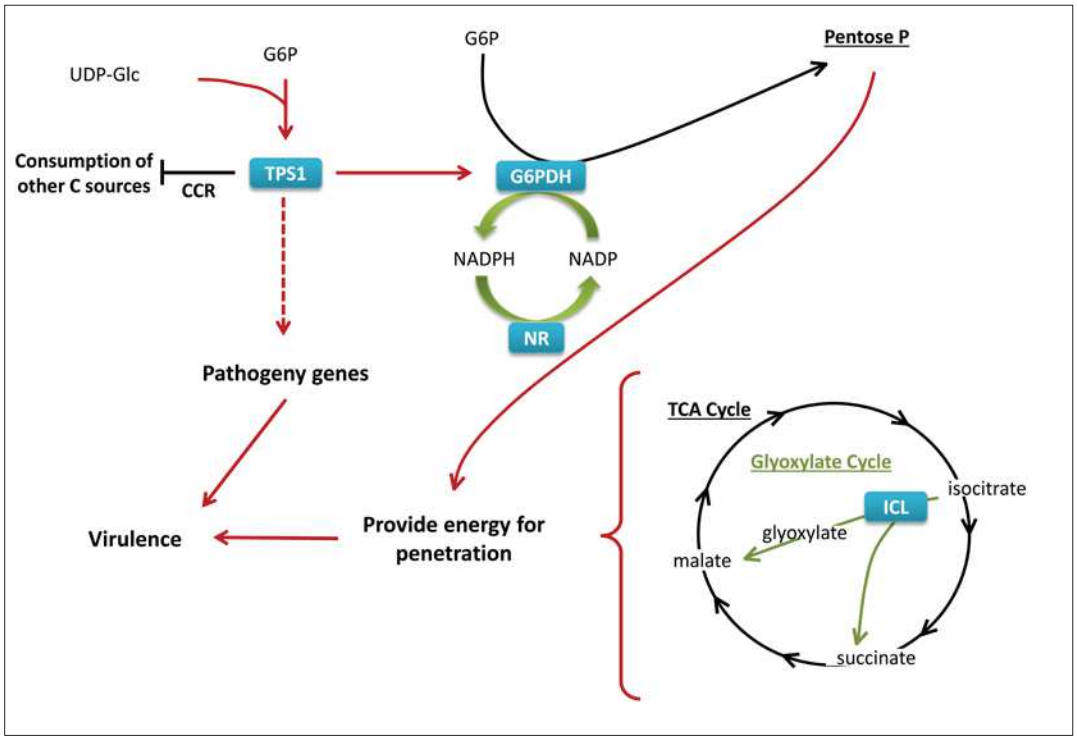

Figure 11. Voies métaboliques impliquées dans la virulence de *Magnaporthe oryzae*

Le G6P (glucose-6-P) est perçu par TPS1 (Trehalose-6-P Synthase 1). Cette enzyme est également impliquée dans l'activation d'une G6PDH (G6P deshydrogenase) contribuant ainsi à la génération de pouvoir réducteur (NADPH) nécessaire à la conversion des nitrates en nitrites par les nitrates réductases (NR). Le G6P est ainsi dans la voie des pentoses phosphates qui contribue à l'approvisionnement en pouvoir réducteur ainsi qu'à la synthèse d'acides aminés aromatiques et de nucléotides. En inhibant la consommation d'autres sources de carbone en présence de G6P, TPS1 participe à la CCR (Carbon Catabolite Repression). De même, TPS1 est requis pour l'expression de certains gènes impliqués dans le pouvoir pathogène. Le cycle du glyoxylate est également important pour la virulence de *M.oryzae*. Ce cycle fait intervenir une isocitrate lyase (ICL) qui convertit l'isocitrate en glyoxylate et qui est requise pour une virulence optimale.

leur appressorium et donc dans leur virulence (Wilson *et al.*, 2007). Il a été mis en évidence que Tps1 est requis pour l'expression de certains gènes impliqués dans le pouvoir pathogène (par ex. *MPG1, SPM1* et *PTH1*)*, via Nut1*, un régulateur du métabolisme azoté requis pour l'assimilation du nitrate (mais pas de l'ammonium). Tps1 assure donc l'expression des gènes de réponse à l'azote uniquement en présence de glucose et l'induction précoce des gènes de pathogénie au cours de l'infection (Fernandez and Wilson, 2012).

In vitro, les éléments du cycle de Krebs comme le succinate, le malate et le citrate sont d'excellents stimulants de croissance. Chez *M. oryzae,* une isocitrate lyase (ICL1), une des principales enzymes du cycle du glyoxylate (un court-circuit du cycle de Krebs) est requise pour sa virulence (Wang *et al.*, 2003). Cette enzyme est également requise pour la virulence de deux agents pathogènes de l'Homme, *Candida albicans* et *Mycobacterium tuberculosis* (Wang *et al.*, 2003; Muñoz-Elías and McKinney, 2005).

3. Effecteurs et pouvoir pathogène

Afin de permettre sa nutrition, d'éviter sa reconnaissance et de limiter la mise en place des défenses de l'hôte, *M. oryzae* sécrète des effecteurs (Kamoun, 2007). En général, les effecteurs sont de petites protéines sécrétées qui présentent une très faible homologie de séquence avec d'autres protéines. Elles peuvent être sécrétées dans le cytoplasme de la cellule végétale ou dans l'apoplasme. Parmi les effecteurs apoplastiques de *Magnaporthe,* on compte Slp1 (Figure 12) (Mentlak *et al.*, 2012). Slp1 est une petite protéine composée de domaines LysM. Cet effecteur interagit avec les composés de la paroi fongique limitant donc leur reconnaissance avec les récepteurs OsCEBiP et OsCERK1 de l'hôte et les réponses de défense qui en découlent. Le mutant *Δslp1* est moins virulent que la souche contrôle toutefois sa virulence est restaurée lorsqu'il se développe sur les mutants *cebip* de riz, affectés dans la perception de la chitine (Mentlak *et al.*, 2012). Chez *Magnaporthe* les effecteurs apoplastiques sont sécrétés par le système de sécrétion classique dépendant de l'appareil de golgi, alors que les effecteurs cytoplasmiques sont sécrétés par des structures particulières appelées BIC (Biotrophic Interfacial Complex (Giraldo *et al.*, 2013)). Les effecteurs sont souvent identifiés et caractérisés comme protéines d'avirulence, c'est-à-dire lorsqu'ils sont spécifiquement reconnus (directement ou indirectement) par une protéine R dans le cadre de l'ETI. Cependant leur rôle initial est bien de contribuer au pouvoir pathogène des microorganismes qui les produisent. Par exemple l'effecteur cytoplasmique de *Magnaporthe* AvrPiz-t interagit avec APIP6, une RING3 ubiquitine ligase impliquée dans la mise en place de la PTI.

Le complexe protéique AvrPiz-t/APIP6 est dégradé, les signaux de défenses normalement initiés par APIP6 sont alors altérés et l'agent pathogène est virulent (Park *et al.*, 2012). *Magnaporthe* progresse ensuite de cellule en cellule *via* les plasmodesmes (Kankanala *et al.*, 2007). Certains effecteurs

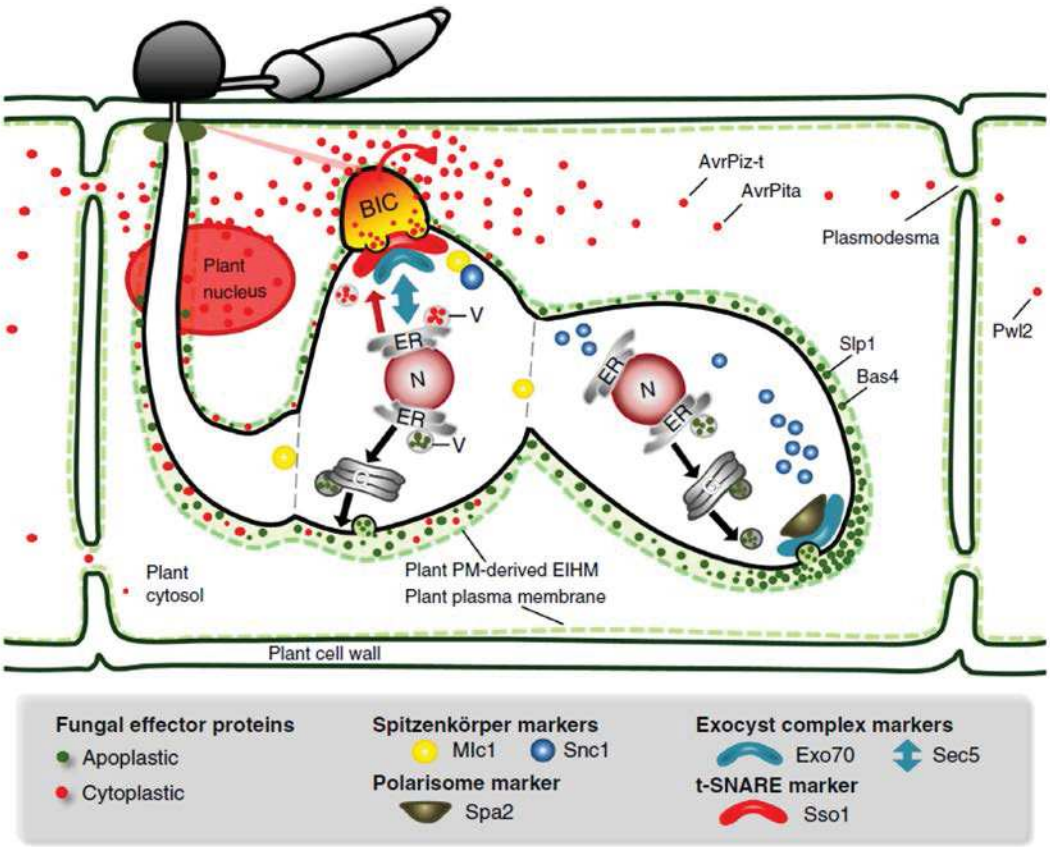

Figure 12. Schéma de la sécrétion d'effecteurs par *Magnaporthe oryzae*

La membrane plasmique de la cellule végétale envahie reste intègre, les hyphes du champignon sont entourés d'une membrane d'origine végétale appelée « Extra-invasive Hyphal Membrane » (EIHM). Certains effecteurs sont sécrétés dans l'espace apoplastique entre la paroi du champignon et la « EIHM » comme Bas4 et Slp1. D'autres sont cytoplasmiques et sont sécrétés par le « Biotrophic Interfacial Complex » (BIC) comme AvrPiz-t, AvrPita et AvrCo39. Pwl2 est un effecteur cytoplasmique détecté dans les cellules voisines d'une cellule infectée. Cette protéine est supposée contribuer à la virulence de *Magnaporthe* en préparant l'invasion des cellules non infectées aux alentours du site infectieux. D'après Oliveira-Garcia & Valent, 2015.

comme Pwl2 et BAS1 (Biotrophy Associated Secreted Protein 1) sont détectés dans les cellules adjacentes aux cellules infectées (Khang *et al.*, 2010). Ces protéines permettraient de préparer ces cellules saines à l'infection et de faciliter ainsi la progression *in planta* du champignon.

Récemment les effecteurs IUG6 et IUG9 (inhibiteur de cystéine-protéase) ont été caractérisés pour leur capacité à inhiber la mort cellulaire induite par la protéine pro-apoptotique BAX chez *Nicotiana benthamiana* (Dong *et al.*, 2015). La surexpression des gènes *Iug6* et *Iug9* dans le fond génétique Guy11 entraine une plus faible expression des gènes *PR* marqueurs des voies SA et ET, respectivement *PR1a* et *Cht1*, au cours de l'infection (Dong *et al.*, 2015). Les auteurs de cette étude suggèrent que ces effecteurs pourraient contribuer à la biotrophie de *Magnaporthe* en affectant la signalisation des hormones SA et ET de l'hôte et inhiber ainsi les défenses sous leur contrôle.

4. Cell-Wall Degrading Enzymes et toxines

Comme les autres pathogènes hémibiotrophes et nécrotrophes, *Magnaporthe* sécrète des enzymes de dégradation de la paroi (CWDE ou Cell-Wall Degrading Enzymes) comme des cutinases ou des xylanases caractéristiques de la phase nécrotrophe de son cycle (Sweigard *et al.*, 1992; Wu *et al.*, 1995). Au cours de cette phase, ce champignon produit également des toxines. Parmi elles, l'acide α-picolinic et la piricularine (Zhang *et al.*, 2004). Ces deux molécules ont un effet drastique sur la croissance du riz et une application exogène sur les feuilles entraine l'apparition de lésions nécrotiques très similaires à celles observées lors du développement de la pyriculariose.

En dehors des effecteurs protéiques et des toxines, *Magnaporthe* produit des composés hormonaux qui peuvent être nécessaires à son pouvoir pathogène notamment l'ABA (Jiang *et al.*, 2010), l'AIA (Jiang *et al.*, 2013), du 12OH-JA (Patkar *et al.*, 2015) et les CKs (Jiang *et al.*, 2013). Toutefois, l'implication de ses molécules dans la biologie ou le pouvoir pathogène de ce champignon n'a pas encore été démontrée.

La question du rôle des CKs chez *Magnaporthe* dans le pouvoir pathogène fait l'objet du travail présenté dans le Chapitre III.

III. Les phytohormones produites par les champignons

Les agents pathogènes ont développé de nombreux effecteurs protéiques pour perturber la signalisation hormonale des plantes (Figure 13). Chez de nombreuses bactéries, il a pu être mis en évidence une production d'hormones. Par exemple, *Rhodococcus fascians*, une bactérie phytopathogène qui induit la formation de tumeurs, secrète des CKs pour induire l'expression du gène *CYCD3* impliqué dans la division cellulaire *via* la voie de transduction du signal CKs de l'hôte

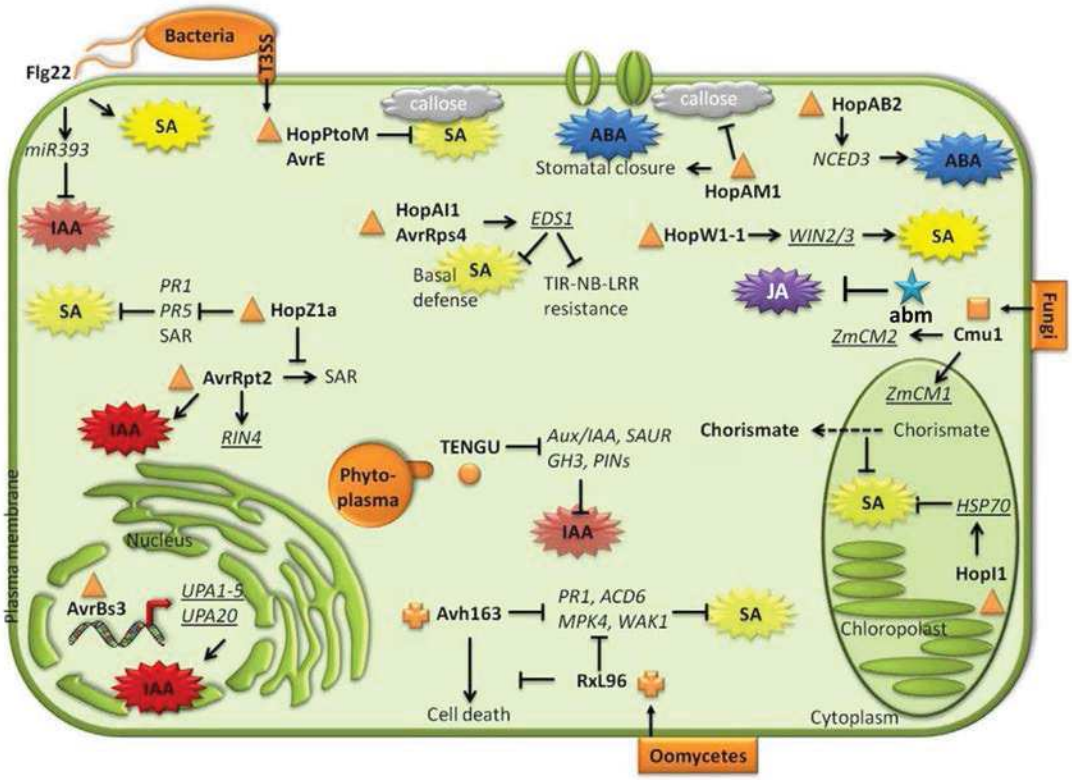

Figure 13. Stratégies élaborées par les agents pathogènes pour interférer avec les voies de biosynthèse des hormones végétales ou leur signalisation

Pendant infection, les bactéries, phytoplasmes, champignons et oomycètes sécrètent différents effecteurs à l'intérieur de la cellule hôte. Certains d'entre eux se lient spécifiquement à certaines protéines végétales (soulignés). De manière directe ou indirecte, les effecteurs peuvent induire (flèches) ou réprimer (« T »), l'expression de certains gènes cibles ou l'activité de certaines protéines. Ces modifications affectent les mécanismes de défense sous-jacents régulés par le SA, le JA, l'ABA et l'auxine. Modifiée à partir de Denancé et *al,* 2013.

(Choi *et al.*, 2011; Pertry *et al.*, 2009). La plupart des champignons eux aussi sont capables de produire des composés hormonaux similaires ou identiques aux phytohormones (Morrison *et al.*, 2015). Cependant, le rôle des hormones d'origine fongique dans les interactions pathogènes et symbiotiques reste peu décrit. Bien que les voies métaboliques soient relativement bien décrites chez les champignons, peu de mutants ont été générés puis testés en interaction avec leur hôte. Certains champignons n'interagissant pas avec les plantes, de même que d'autres organismes comme les oomycètes, les bactéries (saprophytes ou agents pathogènes d'animaux) produisent ces hormones dont certaines ont des effets sur la physiologie de ces organismes et sur leur hôte. La revue ci-après se concentre principalement sur la production des phytohormones par les champignons mais suggère que ces métabolites seraient conservés au sein des différents règnes du vivant et pourraient être potentiellement impliqués dans la communication entre les différents organismes.

Les questions du rôle des CKs fongiques dans la virulence et la physiologie de *Magnaporthe* sont développées dans le chapitre 3.

Plant hormones: a fungal point of view

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Abstract

Most classical plant hormones are also produced by pathogenic and symbiotic fungi. The way these molecules favor the invasion of plant tissues and the development of fungi inside plant tissues is still largely unknown. In this review, we examine the different roles of such hormone production by pathogenic fungi. Converging evidence suggest that these fungal-derived molecules have potentially two modes of action: (i) they may perturb plant processes, either positively or negatively, to favor invasion and nutrient uptake and (ii) they may also act as signals for the fungi themselves to engage appropriate developmental and physiological processes adapted to their environment. Indirect evidences suggest that abscisic acid, gibberellic acid and ethylene produced by fungi participate to pathogenicity. There is now evidence that auxin and cytokinins could be positive regulators required for virulence. Further research should establish whether or not fungal-derived hormones act like other fungal effectors.

Introduction

Many fungi interact with plants in a beneficial manner as in mycorrhizal symbiosis (Sanders, 2011) or in an harmful manner in the case of fungal diseases (Dean et al., 2012). In order to obtain nutrients, both symbiotic and most pathogenic fungi penetrate their host without breaking the plant cell plasma membrane. The fungal membrane is protected by a cell-wall composed of chitin that can be recognized by plants through membrane receptors which then activate basal immunity. Chitin perception modulates responses during both mutualistic and pathogenic fungi-plant interactions (Gust et al., 2012). Fungi have evolved a repertoire of tools such as protein effectors and metabolites to impede such plant immunity and/or to establish favorable conditions for their establishment in plant tissues (Kamoun, 2007). Besides the production of canonical effectors, fungi also produce compounds that are similar to plant hormones like auxins, cytokinins (CKs), gibberellic acids (GAs), ethylene (ET), abscisic acid (ABA), jasmonates (JA) and salicylates (SA). These hormones are well described to control plant development and to trigger important plant signaling events during biotic and abiotic stresses (reviewed in (Spence and Bais, 2015; Pozo et al., 2015; De Vleesschauwer et al., 2013; Robert-Seilaniantz et al., 2011; Peleg and Blumwald, 2011)).

There are now many examples showing that some pathogen protein effectors trigger hormone regulation to favor infection (Robert-Seilaniantz et al., 2007). By contrast, the involvement of hormonal compounds derived from microorganisms in plant-fungus interactions is poorly documented. Fungal-derived hormones were first suspected to be involved in the virulence of gall-forming pathogens (Robert-Seilaniantz et al., 2007; Denancé et al., 2013). For symbiotic fungi, such production of hormones is consistent with root modifications often required in these interactions (Hirsch et al., 1997). However, many pathogens that do not induce organ deformations can also produce and secrete plant hormones, suggesting a role of these molecules in other biological processes than organ deformation.

The role of plant-derived hormones in plant disease resistance has been extensively reviewed (Robert-Seilaniantz et al., 2011; De Vleesschauwer et al., 2014). In this review, we summarize the current knowledge on the role of fungal-derived plant hormones in plant-pathogen interactions with a focus on their putative role in virulence. When relevant, some information on plant-mycorrhiza interactions is also provided as it often sheds some light on the role of these molecules in plant-fungus interactions.

Auxins from fungi play a positive role in plant-fungus interactions

Auxins are indole-derived hormones involved in plant development processes such as cell division differentiation and organ formation (Oka et al., 1999; Vanneste, 2005; Benjamins and Scheres, 2008) and senescence (Kim et al., 2011). Auxins also control biotic and abiotic stress responses in plants (Peleg and Blumwald, 2011). In plants, auxins is synthetized from tryptophan which is converted into indole-3-acetamide by tryptophan-2-monooxygenase enzymes (Zhao, 2010). Indole-3-acetamide is hydrolyzed to form indole-3-acetic acid (IAA) which is the major auxin active form in plants. These genes were also identified in fungi, e.g. *Fusarium* sp., and were confirmed for being involved in fungal auxin production (Tsavkelova et al., 2012). However, several auxin synthesis pathways were described in fungi. In some of them, like *Fusarium sp.* and *Colletotrichum gloeosporioides* (Tsavkelova et al., 2012; Gruen, 1959; Robinson et al., 1998), auxins is synthesized from the same precursor than in plants (indole-3-acetamide) but as observed in other fungal genus, for instance *Ustilago* (Reineke et al., 2008) and *Rhizoctonia* (Furukawa et al., 1996), auxins can also be produced from indole-3-pyruvate. Auxins could also be produced in a tryptophan-independent manner but the corresponding pathways are still not well described.

A lot of fungal species, and not only plant-interacting fungi, produce and secrete auxins, suggesting that these hormones could have an endogenous role in these organisms (Gruen, 1959; Ulrich, 1960). A negative correlation between the speed of fungal growth and auxin production was shown in several species (Gruen, 1959). By contrast, auxin treatment promotes cellular elongation and sporulation in the yeasts *Saccharomyces cerevisiae* and *S. ellipsoideus* (Yanagishima, 1965; Kamisaka et al., 1967). Similarly, an aberrant production of auxins leads to morphological transition in *S. cerevisiae* as well as in the human pathogen *Candida albicans*, in which auxin triggers the transition into hyphal growth, a known virulence trait (Rao et al., 2010). The auxin IAA also promotes spore germination of the filamentous fungus *Neurospora crassa* (Nakamura et al., 1978, 1982). When tested on the tomato pathogen *F. oxysporum lycopersici*, auxin reduced spore germination (Sharaf and Farrag, 2004). The effect of auxin on growth could be concentration-dependent: in the case of *F. delphinoides*, a chick pea pathogen, low concentration of exogenous auxin increased fungal growth whereas high concentration decreased it (Kulkarni et al., 2013). Thus the effects of auxins on fungal physiology can strongly differ from one species to another and depends on the dose tested.

Auxins are involved in the symbiotic interactions between plant and bacteria or fungi. They are required for the initiation of nodule formation in the nitrogen-fixative bacterial symbiosis (Hirsch and Fang, 1994) and for the invasion of mycorrhizal fungi (Hanlon and Coenen, 2011; Etemadi et al., 2014). For instance, mutants of the ectomycorrhizal species *Hebeloma cylindrosporum* overproducing auxin showed an increased ability to invade root tissues of *Pinus pinaster* (Gay et al., 1994; Laurans et al., 2001). However, there was no difference between the growth of plants colonized with the mutant and wild-type strains, suggesting that fungal auxin is involved in host

invasion but not in the beneficial effects of symbiosis on host development. In most cases, plants interacting with mycorrhizal fungus contain a higher content in auxins than non-mycorrhized ones (Barker and Tagu, 2000; Meixner et al., 2005). However, the origin of these auxins, whether from the host or the fungal symbiont, is still unclear.

Auxin involvement in plant-pathogen interactions were early suspected and studied when symptoms, like organ deformation, were reminiscent to responses to high auxin level. For instance, *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* are well-known plant pathogenic bacteria that induce tumor formation in their hosts and auxins actively contribute to the virulence of these bacteria (Glass and Kosuge, 1988). Like *Agrobacterium*, some fungi are able to induce tumors, such as the corn smut causal agent, *U. maydis.* However, fungal mutants affected in auxin production were still able to induce tumors in a similar way than wild-type strain even if tumors contained a lower level of auxins (Reineke et al., 2008). This suggests that auxin production by *U. maydis* is not required for the virulence of this pathogen.

In the case of fungal pathogens not triggering organ deformations, functional evidences suggest a role for auxins. By measuring fungal biomass and auxins in plant tissues, it was suggested that *C. gloeosporioides* f. sp. *aeschynomene* produces auxins during early, biotrophic stages of plant colonization (Maor et al., 2004). In *F. oxysporum,* an enhanced expression of auxin biosynthetic genes (tryptophan-2-monooxyngenase and indole-3-acetamide hydrolase) triggers over-accumulation of IAA and an hypervirulent phenotype on Orobanche (Cohen et al., 2002). Consistent with these observations, the transient silencing in *Puccinia graminis* f. sp. *tritici* of a gene required for auxin biosynthesis was obtained in wheat infected leaves. The two-fold reduction of the transcript led to a decrease of pustule formation. Although auxins were not measured after silencing, these results suggest that auxins were required for full fungal pathogenicity (Yin et al., 2014). Thus auxins seem to play a role in pathogenicity and more functional studies with fungal mutants should help better understand how they participate to virulence.

Cytokinins from fungi: a now clear-cut positive function in virulence

Cytokinins (CKs) are diversified plant hormones derived from ATP/ADP/AMP or from the tRNA degradation pathway. CKs are well described for their role in plant development processes such as root and shoot formation through the regulation of cell cycle and cell differentiation (Barciszewski et al., 1999; Riou-Khamlichi et al., 1999; Carimi et al., 2003; Fosket and Torrey, 1969). CKs are also involved in delaying senescence and in source/sink nutrient distribution (Wingler et al., 1998; Peleg et al., 2011). The first step of CK biosynthesis in plants involves Isopentenyl Transferase enzymes (IPT

or tRNA-IPT) which perform the transfer of the isopentenyl chain from the methylerythritol phosphate (MEP) on the adenosine phosphate substrate leading to the formation of the ribosylated phosphorylated CK forms (Sakakibara, 2006). Then, these CKs are activated, in part by the LONELY GUY (LOG) enzymes, into CK free active forms like trans-zeatin and isopentenyladenine (Kurakawa et al., 2007; Frébort et al., 2011). The putative *IPT* and *LOG* genes are present in several fungal genomes and some of them have been recently characterized (see below (Hinsch et al., 2015; Morrison et al., 2015b; Chanclud et al., 2016).

A large diversity of fungal species, whether saprophytic, pathogenic or symbiotic, were shown to produce CKs (Murphy et al., 1997; Cooper and Ashby, 1998) and several studies suggest that they could play a role in several physiological processes in fungi themselves especially in hyphal development and nutrient uptake (LeJohn and Stevenson, 1973). For instance, CKs promote *in vitro* branching of ectomycorrhizal mycelia (Barker and Tagu, 2000) and affect in a dose-dependent manner hyphal membrane viscosity and therefore influence ion and water transport (Gogala, 1991; LeJohn and Stevenson, 1973). Pohleven et al, 1986, demonstrated that some CKs modify the content of K, Ca, P and Na in the mycelia of the basidiomycete *Suillus variegatus* (Gogala, 1991). The effect of CKs on hyphal growth seems to depend on the concentration and on the kind of CK molecule tested (Gryndler et al., 1998). CKs could also be involved in growth optimization under adverse conditions. For instance, the inhibition of the mycelial growth of *Amanita muscaria* caused by aluminium is significantly correlated with a decrease in CKs amount (Kovač and Žel, 1995). In a recent report, we have shown that endogenous and exogenous CKs are required for oxidative stress tolerance in the rice blast fungus *Magnaporthe oryzae* (Chanclud et al., 2016). In the 60's Lee et al reported that CKs also affect sexual reproduction in the ascomycete *N. crassa* suggesting a role in communication within fungi (Lee, 1961; Elliott, 1967).

During mycorrhizal symbiosis, CKs promote growth of the host and of the symbiont (Allen et al., 1980; Drüge and Schonbeck, 1993; Barker and Tagu, 2000). CK accumulation in the host, root and shoot, were shown in many fungal symbiotic interactions (Allen et al., 1980). A model has emerged since the early 90's about the role of CKs in plant symbiotic interactions, proposing that plants secrete CKs that (i) promote growth of symbiotic microbes that are thus able to detect them, then (ii) this contributes to a better absorption of nutrients through the symbiont and (iii) leads to increase the photosynthesis process in the host leaves (Wullschleger and Reid, 1990; Drüge and Schonbeck, 1993). It is possible that CKs produced by mycorrhizas may initiate this whole process but this awaits the study of CK-deficient symbiotic fungal mutants to confirm this hypothesis.

During interaction with fungal pathogens, CKs content is often affected (Jiang et al., 2013; Devos et al., 2006). Since most of the necrotrophic fungi analyzed do not seem to secrete CKs, in contrast to

(hemi)biotrophic ones, it was suggested that fungal CK production and secretion could depend on pathogen lifestyle. CKs are involved in many diseases caused by pathogens that induce tumor formation in their host: protists (e.g. *Plasmodiophora brassicae* (Siemens et al., 2006)), nematodes (e.g. *Heterodera schachtii* (Siddique et al., 2015)), bacteria (e.g. *P. savastanoi* (Barciszewski et al., 2000)*, Agrobacterium* sp.(Barciszewski et al., 2000), *Rhodococcus fascians* (Pertry et al., 2009)) and fungi (*U. maydis* (Mills and Van Staden, 1978), *Claviceps purpurea* (Hinsch et al., 2015)). In the tumor-inducing pathogen *C. purpurea,* deletion of two genes partially abolished CK *de novo* synthesis but did not affect virulence of the fungus. By contrast the mutants exhibited a hyper-sporulating phenotype, implying that CKs are environmental factors influencing fungal development (Hinsch et al., 2015). Recently it was shown that CK accumulation in *U. maydis* infected tissues is correlated to the virulence of this pathogen but there was no direct genetic evidence that fungal-derived CKs are required for full virulence of this pathogen (Morrison et al., 2015a).

Fungal pathogens that do not induce tumors also produce CK compounds and their role in virulence is still poorly understood (see for instance (Murphy et al., 1997; Jiang et al., 2013)). CKs are probably involved in “green island” formation, a photosynthetically active zone often found around lesions caused by biotrophic fungi (Angra and Mandahar, 1991; Choi et al., 2011). In plants CK production is thought to occur in the roots (Rani Debi et al., 2005) and experiments with detached leaves could indirectly address the question of the origin of the CKs in green islands. Using this assay in wheat and maize leaves respectively infected with *Pyrenophora teres* and *Dreschslera maydis*, the increase of CKs content was attributed to the pathogen. The increase in CKs levels in susceptible hosts was also correlated with increased metabolite contents around infection sites (Angra-Sharma and Sharma, 1999). CK secretion was shown by immuno-detection in plant tissues in the case of *P. recondita* f.sp. *tritici* during wheat infection (Hu and Rijkenberg, 1998) but as for most hormones found during infection, it is not possible to unambiguously assign this accumulation to the plant or to the pathogen without characterization of mutants impaired in CK production or perception. A recent study mentioned that CK production by fungi, especially the *cis*- zeatin forms (which seems to be the main one produced by filamentous fungi), could involve tRNA-IPT enzymes that perform modification on tRNA which will then release free CKs after degradation (Morrison et al., 2015b). Among non-tumor inducing fungal pathogens, *M. oryzae* produces and secretes CKs (Jiang et al., 2013). Knock-out mutants impaired in the only *tRNA-IPT* gene identified in *M. oryzae* were also impaired in CK production, thus confirming the hypothesis of Morrison et al., 2015b that tRNA-IPT are involved in fungal CK production (Chanclud et al., 2016). The interaction between rice and the CK-deficient strain of *M. oryzae* was characterized. This analysis demonstrated that *M. oryzae* derived CKs are required for full virulence by affecting rice defenses, nutrient distribution and fungal oxidative stress tolerance

(Chanclud et al., 2016). Since the tRNA-IPT gene identified in *M. oryzae* is well conserved, this mutation could be studied in other fungi as a nice tool for distinguishing fungal CKs to plant CKs in other plant-fungus interactions. Recently, the deletion of a *tRNA-IPT* gene has also been performed in the nematode *H. schachtii* confirming the conservation of the role of this enzyme in CK production among different organisms (Siddique et al., 2015).

Ancient but limited direct evidence for a role of Gibberellic acids from fungal origin

GAs are terpenoid hormonal compounds identified for the first time as being produced by *Gibberella fujikuroi.* This fungus is the causal agent of the "bakanae" or "foolish seedlings" disease of rice in which infected plants are abnormally tall. Following this discovery, the role of GAs on plant physiology started to be studied. GAs have been involved in the control of germination, flowering, cell division and internode elongation (Brian and Elson, 1954; Pimenta Lange and Lange, 2006; Swain and Singh, 2005). The first steps of GA biosynthesis pathways identified in fungi are almost identical with those known in plants. The complex GA biosynthesis pathways were well described by Tudzynski, 2005. GA production was found in several fungal species but their effects on fungal biology are not well described.

In liquid culture, GAs were shown to increase conidial germination and to improve growth of young hyphae of the ascomycete fungus *N. crassa* (Nakamura et al., 1978; Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984)(Tomita et al., 1984) . These effects are additive to the effects of auxins, suggesting that these two hormones could act independently to affect *Neurospora* germination and growth (Tomita et al., 1984).

Few studies have reported their role during interaction between fungi and plants (Tsavkelova et al., 2006). In mycorrhizal interaction, GAs content is increased in plants (Barker and Tagu, 2000). Blee and Anderson (1998) have suggested a model where production of fungal GAs is possibly required to initiate a signal leading to enhanced carbon-sink activity of the infected cell (probably combined with CKs and auxins).

In plant pathogen interactions, the role of GAs has been less studied compared to other plant hormonal pathways. Different strains of *G. fujikuroi* were analyzed for their production of GAs and a correlation was found between the quantity of GAs produced and the virulence of the strain (Desjardins et al., 2000). However, in another study, there was no clear link between the production of GAs and the pathogenicity of *Fusarium* (Mańka, 1980). *G. fujikuroi* mutated for two histone

deacetylases showed a reduction of GA production. Plants infected with the double deletion mutant resembled the uninfected control plants, suggesting that GA production is required for bakanae disease on rice (Studt et al., 2013). However, the possibility that the introduced mutations have affected other virulence factors cannot be ruled out.

Abscisic acid: converging evidence for ABA used as a virulence factor

In plants, ABA is well known to induce stomatal closure and thus to contribute to plant drought tolerance (Beardsell and Cohen, 1975). ABA is the key hormone for abiotic stress responses in plants (Peleg and Blumwald, 2011). ABA is also involved in seed dormancy by acting antagonistically with the GAs pathway (Debeaujon and Koornneef, 2000). In plants, ABA is synthesized from both the MEP and the mevalonate pathway (Nambara and Marion-Poll, 2005).

In fungi, it is thought that the mevalonate pathway is mostly involved and that different ABA precursors could be used (Morrison et al., 2015b; Oritani and Kiyota, 2003). Fungal production of ABA was first shown in *Cercospora risicola* (Norman et al., 1983). Since then a lot of fungi with different lifestyles (saprophytic, symbiotic and pathogenic) were described as producing ABA (Crocoli et al., 1991; Esch et al., 1994; Jiang et al., 2010; Morrison et al., 2015b). There are only two reports that ABA affects mycelium growth. In *Ceratocystis fimbriata*, exogenous application of ABA slightly promoted fungal growth. In *M. oryzae*, ABA increased germination and the formation of appressorium, a specialized infection structure differentiated for breaking down the plant cell wall and allowing invasion (Spence and Bais, 2015 and references inside).

The arbuscular-mycorrhizal fungus *Glomus sp.* produces ABA and ABA concentration in the xylem sap is different between mycorrhized plants compared to non-mycorrhized ones. However the origin of this increase of ABA is not established yet (Esch et al., 1994).

In several plant-pathogen interactions, ABA was described to affect plant disease resistance in a positive or a negative manner, depending on the host-pathogen interaction studied (Jiang et al., 2010; Xu et al., 2013; De Vleesschauwer et al., 2010). Kettner and Dorffling, 1995 have inoculated tomato plants with two strains of *Botrytis cinerea* presenting differences in ABA production and showed that ABA increase is higher in leaves inoculated with the ABA-producing strain than with the less-producing ones. This suggests that ABA accumulation in the host during infection could result from or be initiated by this pathogenic fungus. Similarly, ABA was accumulated during the early stages of infection by *U. maydis* and this accumulation could be correlated with the virulence of the fungus (Morrison et al., 2015a). Even though exogenous ABA triggered a faster development of the necrotic lesion, the role of fungal ABA in virulence was not described until recently. Knocking-out one

gene homologous to the *B. cinerea ABA4* gene responsible for ABA biosynthesis, reduced by two-fold ABA levels in *M. oryzae* (Spence et al., 2015; Siewers et al., 2006). Appressorium formation *in vitro* was severely reduced in the *M. oryzae Δaba4* mutant, a phenotype that could be reverted by exogenous application of ABA. The virulence of the *Δaba4* mutant was also strongly compromised suggesting that ABA contributes to the virulence of this fungus. One may then speculate that this production of ABA by *M. oryzae* inhibits the SA-dependent defense response (Jiang et al., 2010), as observed in many other biological situations (Ton et al., 2009). However, since the *Δaba4* mutant did not form appressoria and infect plants at all, it is difficult to conclude on a role of fungal-produced ABA on the plant itself.

Ethylene: a gaseous hormone involved in plant physiology and defenses which also affects fungal development

Ethylene (ET) is a gaseous compound first discovered for its role in fruit maturation (Bleecker and Kende, 2000; Payton et al., 1996). ET was later shown to be involved in senescence, germination, flowering as well as in the inhibition of root and shoot growth (Bleecker and Kende, 2000; Grbic and Bleecker, 1995). In *Arabidopsis*, ET has first been described to contribute, with JA, to the induction of defenses against necrotrophic pathogens. However, this dichotomy of responses, to biotrophic and necrotrophic pathogens, is not always clear in the other plants, like in rice, in which hormonal regulation of defenses is slightly different (De Vleesschauwer et al., 2013, 2014). In plants and fungi, ET biosynthesis occurs from methionine that is transformed in ACC (1-aminocyclopropane-1-carboxylic acid) via ACC-synthase enzymes (Esser et al., 2002). Moreover, fungi also produce ET from the 2-keto-4-methylthiobutyric acid, deriving from methionine, and/or from the 2-oxoglutarate then requiring Ethylene-Forming enzymes (Bockhaven et al., 2015; Hottiger and Boiler, 1991 and references inside). Altogether, studies about fungal ET production show that it is strongly dependent on growth media and confirmed that several pathways exist among fungi (Esser et al., 2002; Strzelczyk et al., 1994). Since the first report of ET production by *Penicillium digdatum* in 1940, ET production has been measured in a lot of fungal species, in both hyphae and spores (Dasilva et al., 1974). These fungi belong to different phylum, have different lifestyles and range from pathogenic like *B. cinerea* to symbiotic ones like *F. oxysporum* f. sp. *pini* (Graham and Linderman, 1980; Dasilva et al., 1974; Arshad and Frankenberger, 1991). Several *in vitro* experiments demonstrated that ET or some precursors (ethephon and ACC) affect spore germination and hyphal growth of the pathogenic filamentous fungi, *Alternaria alternata* and *B. cinerea*, and the symbiotic ones, *Gigaspora ramisporophora* and *G. mosseae* (Kępczyńska, 1994; Chagué et al., 2006). The effects of ET on fungal

development seem to be dose-dependent with a promoting effect observed at concentrations below 1mM and a negative effect at or higher than 1mM (Ishii et al., 1996).

In the case of mycorrhiza, the role of ET depends on the type of symbiotic interaction. A low content of ET was measured in mycorrhized roots (McArthur and Knowles, 1992) and an exogenous supply of ET suppressed AM development (Geil et al., 2001; Zsögön et al., 2008). Therefore, it was suggested that a repression of the ET pathway by AM fungi is required to allow the establishment of symbiosis. Indeed, the AM fungus *G. intraradices* secretes a protein (SP7: secreted protein 7) which interacts with an ethylene response factor to suppress ethylene signaling (Kloppholz et al., 2011). In contrast, ET seems to promote ectomycorrhizal symbiosis. Two species of truffles (*Tuber melanosporum* and *Tuber borchii*) were showed to produce ET (and auxin) for manipulating these hormonal pathways in the host and inducing root morphological modifications, a plant developmental process in which these hormones are involved (Splivallo et al., 2009). Given the roles of ET in plant defense, this fungal ET production by symbionts could also be required for counteracting the establishment of host immunity.

During plant fungal pathogen interactions, ET content often increases at the beginning of the interaction (Broekaert et al., 2006). However, its origin, from plants or fungi, is still unclear. In the case of the *Colletotrichum* sp. pathogens, ET is required for the formation of appressorium (Flaishman and Kolattukudy, 1994). Indeed appressoria formed on ripening tomato whereas none formed in plant mutants affected in ET production. An exogenous supply of ET restored appressorium formation on these plant mutants suggesting that ET produced by fruits during the ripening is perceived by the pathogen and is beneficial to initiate the development of specialized structures required for penetration and thus for full virulence. Furthermore ET production by fungi could be required to disturb host defense induction by affecting the plant hormonal homeostasis, essential for plant immunity establishment(Broekaert et al., 2006). This hypothesis was recently investigated in the interaction between rice and the necrotrophic pathogen, *Cochliobolus miyabeanus,* in which ET increases rice susceptibility (Bockhaven et al., 2015). In this study, the authors have used a specific inhibitor of fungal ET biosynthesis (2,2-bipyridyl), that abolishes fungal ET production and leads to a higher resistance of the host (or a lack of virulence of the fungus). Combined with other results showing that *C. miyabeanus* affects the 2-oxoglutarate (an ET precursor for microbes) pool in rice, the authors suggested that ET accumulation is mainly initiated and caused by the fungus and then contributes to the symptom development (Bockhaven et al., 2015). However, exogenous supplies of hormonal production inhibitors or signaling inhibitors could have side effects on the host and on the fungus, therefore the study of fungal mutants affected in ET perception or production is still missing for understanding the different roles of ET in plant-fungus interactions.

Pathogenic fungi also produce defense-related hormones SA and JA

In most plants, SA and JA trigger defenses against fungal biotrophic and necrotrophic pathogens respectively, in an antagonist manner (Bari and Jones, 2009; Robert-Seilaniantz et al., 2011). Some fungal pathogens may produce one hormone in order to inhibit the defense pathway which is the most detrimental to their growth. In plants, SA is synthesized from chorismate and the corresponding pathway is for instance targeted by the fungal pathogen *U. maydis* which secretes a chorismate mutase that channels chorismate into the phenyl propanoid pathway, preventing SA accumulation during infection and then contributing to its virulence (Djamei et al., 2011). However, the chorismate pathways identified in fungi do not lead to SA biosynthesis. Thus, even if SA (or SA-derivatives) production was measured in different species, to date this pathway is still unknown in fungi (Packter and Steward, 1967).

Some pathogens produce both SA *and* JA, like for instance *Moniliophthora perniciosa* which causes the witches' broom disease of cocoa. In this case, the production of these hormones could (i) contribute to manipulate the hormonal pathways involved in the host defense responses throughout its invasion i.e. causing abnormal shoot development and necrosis and (ii) could have a direct effect on this fungus since both SA *and* JA promote *in vitro* growth (Kilaru et al., 2007; Chaves and Gianfagna, 2006). A few other studies mentioned the effects of SA or JA on fungal physiological processes. SA had a moderate suppressive effect on spore germination and colonial growth rate of *Harpophora maydis* (Degani et al., 2015). In *Aspergillus flavus,* the results obtained from *in vitro* experiments showed that SA significantly reduced hyphal growth at all concentrations tested (Panahirad et al., 2014).

Several studies mentioned the production of JA by pathogenic fungi like *G. fujikuroi* and *Botryodiplodia theobromae* (Miersch et al., 1992, 1991). JAs are derived from lipid peroxidation, and thus belong to the oxylipins. Some fungal oxylipin biosynthesis pathways were identified and characterized. JA and the other oxylipins could affect both host and fungal physiological processes (Tsitsigiannis and Keller, 2007). The *in vitro* application on *F. oxysporum* f.sp. *lycopersici* of the methyl-JA reduced spore germination and mycelium growth (Król et al., 2015). Recently, *M. oryzae* was shown to secrete a monooxygenase that converts rice endogenous JA into hydroxylated JA (12OH-JA). This 12OH-JA may then inhibit JA signaling and thus impairs JA-dependent host defenses and resistance (Patkar et al., 2015).

Although the number of pathogenic fungi characterized for producing SA and/or JA increases, there is no direct evidence that fungal SA or JA are required for their virulence.

366 **Conclusions**

367 Thus far, most fungi have been shown to produce almost all plant-like hormones *in vitro*. It is
368 noteworthy that some growth medium used in many studies are made with potatoes dextrose agar
369 and/or yeast extract, two compounds that already contain some plant derived hormones including
370 auxins, CKs, ABA and the others, in unknown concentrations. Thus, some confusion exists between
371 the ability of *de novo* production of plant hormones by fungi and their ability to metabolize them
372 from the growth medium. This should be carefully addressed in future studies conducted *in vitro*. The
373 sequencing of many genomes may also help to shed light on the presence of the hormonal
374 biosynthesis pathways already described in some fungal species (Esser et al., 2002).

375 This overview shows that most known plant-hormonal compounds are produced and perceived by
376 fungi. To date the involvement of fungal hormonal compounds, and the way that they are secreted
377 and act in the plant cell are still poorly understood. Although most of the biosynthesis pathways of
378 hormones in fungi are well described (reviewed in Esser et al., 2002), studies on fungal mutants
379 affected in hormonal production are strikingly missing to confirm the involvement of fungal derived
380 plant hormones in such interactions. In particular, the origin of hormones in colonized tissues is
381 unclear and needs to be established to understand the complex relationships between fungal-
382 derived and plant-derived hormones.

383 Some plant hormones have been shown to affect fungal development, nutrition and reproduction
384 processes suggesting that these molecules trigger some signals in fungi (Nakamura et al., 1982;
385 Gryndler et al., 1998; Esch et al., 1994; Elliott, 1967; Nakamura et al., 1978; LeJohn and Stevenson,
386 1973; Kępczyńska, 1989). The Figure 1 non-exhaustively summarizes the main effects of hormones
387 known to date on fungal biology. However, the perception systems of hormones by fungi, as well as
388 the signaling pathways triggered and the physiological responses induced, still remain to be
389 discovered.

390 These plant hormone compounds are also produced, and very probably perceived, by other microbes
391 including bacteria and nematodes (Denancé et al., 2013; Siddique et al., 2015; Kisiala et al., 2013).
392 Moreover, some of these compounds have some effects on animal cells (Jiang et al., 2002;
393 Slaugenhaupt et al., 2004; Ishii et al., 2003), suggesting that “plant” hormones not only participate to
394 plant-microbe dialogue but might also contribute to communication in other host-microbe
395 interactions involving widely different organisms (animals, plants and all kind of pathogenic,
396 saprophytic and symbiotic microbes). However, to date there is no report on the involvement of such
397 compounds in these interactions.

398

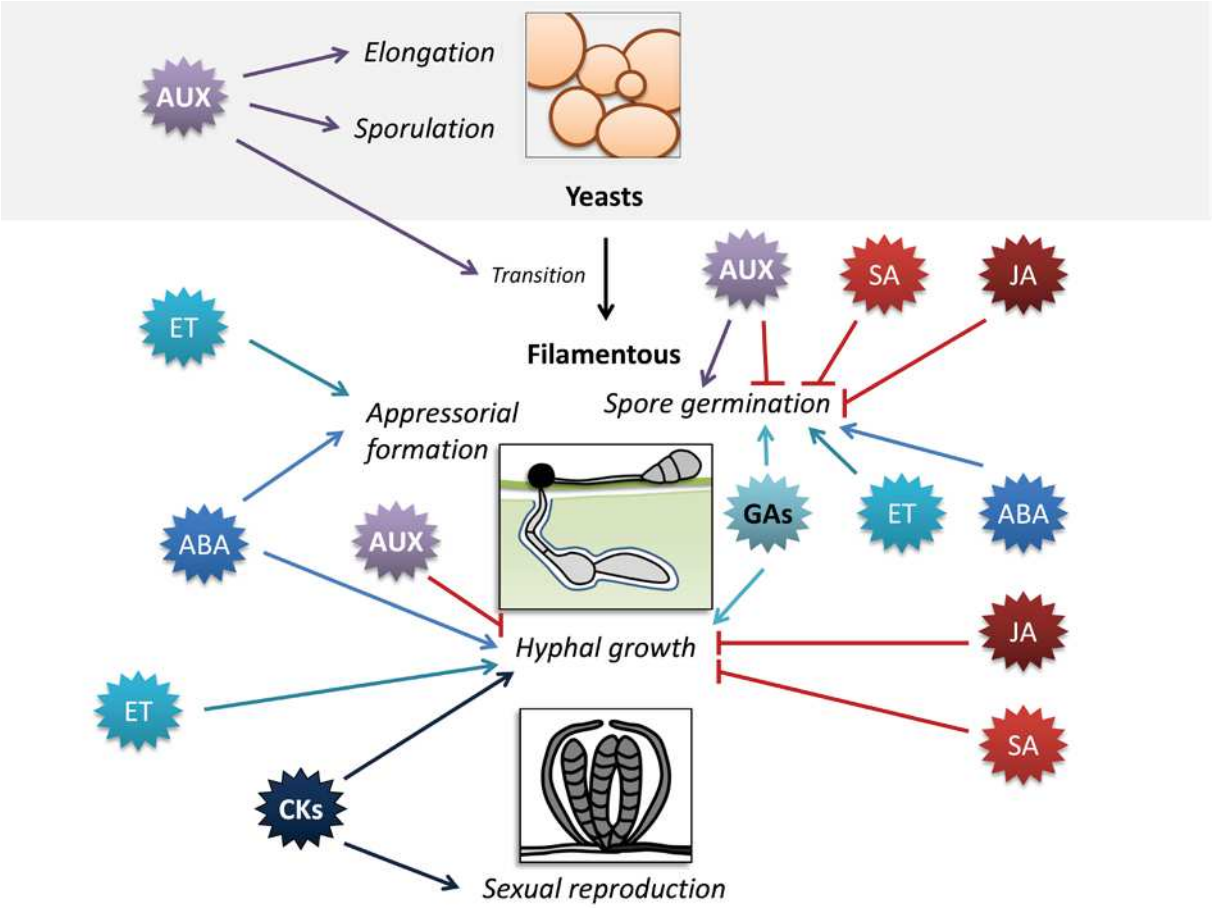

Figure 1. Schematic representation of the effects of hormones on fungal biology.
A schematic view of yeast-like (light grey background), infecting and sporulating filamentous fungus is given. This model summarizes the effects of hormones on fungal biological processes reported to date; the effects due to the dose, the molecule tested, other environmental factors as well as the specie studied are provided in the text and could differ among fungi. Arrows and « T » bars respectively represent positive and negative effects. AUX : Auxins, ABA : Abscisic Acid, ET : Ethylene, CKs : Cytokinins, GAs : Gibberellic Acids, JA : Jasmonic acid, SA : Salicylic Acid.

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PROJET DE THESE

Magnaporthe oryzae est l'agent phytopathogène qui cause le plus de dommages sur les cultures de riz [232]. L'identification de facteurs qui affectent la résistance de l'hôte ou la virulence de l'agent pathogène contribue à optimiser les techniques de résistance actuelles i.e. des traitements exogènes ou les améliorations variétales/modifications génétiques. Le pathosystème riz-*M. oryzae* est étudié depuis plusieurs décennies. De nombreuses données moléculaires et expérimentales sont disponibles et facilement accessibles (génomes, données d'expression, protéome…). Cette interaction est devenue l'un des pathosystèmes modèles pour étudier les maladies fongiques.

Avant mon arrivée, des approches de génétique inverse menées au laboratoire et décrites par Grand et *al,* 2012 [328] avaient identifié un gène, appelé *ZBED*, dont le rôle dans la mise en place des défenses du riz en réponse à *Magnaporthe* était suspecté. Parallèlement à ces analyses, les travaux de l'équipe sur les récepteurs immunitaires de type NLR ont abouti à suggérer que le domaine BED pourraient être un domaine « decoy » ou « sensor ». En effet, parmi les domaines atypiques (« decoy » intégrés ou « sensor domain ») identifiés dans les protéines NLRs, les domaines BED sont surreprésentés. Les domaines BED sont des domaines putatifs de liaison à l'ADN de type Zn-Finger C_2H_2. Les résultats portants sur les domaines « decoy » et la caractérisation des mutants ZBED sont présentés dans le chapitre I.

Durant mon stage de Master, l'analyse de mutants ZBED avait suggéré un rôle de la voie des cytokinines dans ces mutants. C'est la raison initiale qui nous a conduits à poser la question du rôle de ces hormones dans la résistance du riz. Les travaux publiés au début de ma thèse indiquaient que dans des conditions *in vitro* la kinétine (une CK) induit l'expression des gènes marqueurs des défenses de SA. Aucun phénotype de résistance n'avait été observé chez les plantes traitées 24h avant inoculation avec 50µM de kinétine [195]. Pourtant, dans nos conditions expérimentales, les plantes traitées avec 50µM de kinétine 48h avant inoculation étaient plus résistantes à *M. oryzae*. Ces données nous ont amené à poser la question suivante : Comment les CKs affectent-elles la résistance du riz ? Cette question est abordée dans le 2ème chapitre de la thèse.

Comme d'autres agents pathogènes, *M. oryzae* produit et sécrète des CKs [195]. Cependant la question de savoir si les CKs fongiques sont requises pendant le processus infectieux restait ouverte. Afin de tenter de répondre à cette question, le rôle des CKs d'origine fongique a été évalué. Ces travaux font l'objet du chapitre III.

Les CKs affectent la biologie de certains ascomycètes comme *Neurospora crassa* et *Saccharomyces.* Ces hormones pourraient avoir des effets similaires chez *M*. *oryzae*. Les résultats préliminaires obtenus pour tester cette hypothèse sont traités dans le chapitre III.

CHAPITRE I : Etude du gène *ZBED* dans la résistance du riz à *Magnaporthe*

Une analyse des séquences de 2344 protéines NLRs disponibles dans la base de données Greenphhyl présentant les trois domaines protéiques classiques (Coil-Coil ou Tir ; NB et LRR) a révélé que 4.3% de ces protéines contenaient des domaines atypiques supplémentaires. Ces domaines pourraient être des domaines « decoy » intégrés, comme déjà caractérisé pour les domaines WRKY [329,330] et HMA [9]. Parmi ces domaines atypiques certains sont sur-représentés, notamment les domaines kinase, WRKY et BED. Le gène *ZBED* a précédemment été identifié dans l'équipe au cours de la thèse de X. Grand pour être négativement corrélé au niveau de résistance partielle à *M. oryzae* dans un panel de variétés de riz.

Les domaines BED de la protéine ZBED partagent de 44 à 58% d'homologie avec le domaine BED de la protéine de résistance Xa1 impliquée dans la résistance à *Xanthomonas oryzae* pv. *oryzae*. Toutefois le rôle dans les défenses des protéines, autres que NLRs, contenant des domaines BED reste inconnu. Il s'avère que des mutants de riz sur-exprimant le gène *ZBED* sont plus résistants à *M. oryzae.*

Ces résultats renforcent l'hypothèse que les protéines contenants des domaines identifiés par ailleurs comme des « decoy » pourraient être des acteurs des mécanismes de défense dont les modes d'actions restent souvent à découvrir.

Le domaine BED est un domaine en doigt de zinc de type C_2H_2 particulier qui a été initialement mis en évidence chez les protéines régulatrices de la structure de la chromatine BEAF et DREF (BED) de la drosophile [331]. Ces domaines protéiques dériveraient des transposases d'éléments transposables et auraient été intégrés dans les protéines pour réguler la structure de la chromatine et/ou la transcription. A ce jour, peu de protéines contenant des domaines BED ont été caractérisées mais de récentes analyses faites chez l'animal indiquent qu'elles jouent des rôles variés dans le développement et la mort cellulaire (voir par exemple [332,333]. Chez le riz, une analyse *in silico* a permis de mettre en évidence que les gènes dont l'expression est corrélée à celle de *ZBED* sont prédits pour majoritairement intervenir dans le transport, la phosphorylation et la signalisation des CKs. C'est ce résultat initial nous a amenés à étudier le rôle des CKs dans la résistance du riz à *M. oryzae.*

Integration of decoy domains derived from protein targets of pathogen effectors into plant immune receptors is widespread

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Figures: 3 (should be published in colour)

Tables in supporting information: 5

Figures in supporting information: 4

SUMMARY

- Plant immune receptors of the class of NLR proteins can contain additional domains besides canonical NB-ARC and LRR domains. Recent research suggests that these additional domains act as integrated decoys recognizing effectors from pathogens. Proteins homologous to integrated decoys are suspected to be effector targets and involved in disease or resistance.
- Here, we scrutinized 31 entire plant genomes to identify putative integrated decoy domains in NLR proteins using Interpro search. The involvement of the ZBED protein containing a putative decoy domain, called BED, in rice resistance was tested by evaluating susceptibility to the blast fungus *Magnaporthe oryzae* in rice over-expression or knock-out mutants.
- This analysis shows that all plants tested have integrated various atypical protein domains into their NLR proteins (with an average of 3.5% of NLR). We also demonstrate that modifying the expression of the *ZBED* gene modifies disease susceptibility.
- This study suggests that integration of decoy domains in NLR immune receptors is widespread and frequent in plants. The integrated decoy model is therefore a powerful concept to identify new proteins involved in disease resistance. Further in-depth examination of additional domains in NLR proteins promises to unravel many new proteins of the plant immune system.

Key words: BED domain, decoy, genome, NLR, plant immunity

INTRODUCTION

In plants, disease resistance is frequently conferred by nucleotide-binding and leucine-rich repeat domain (NLR) proteins. Indeed, the majority of cloned dominant resistance (*R*) genes broadly used by breeders code for NLR proteins. NLRs constitute huge and highly diverse gene families in plant genomes that can be further subdivided into CNLs that contain an N-terminal coil-coil (CC) domain and TNLs that have a Toll/interleukin-1 (TIR) domain and are absent from Monocot genomes (Takken and Goverse, 2012). NLR proteins act as immune receptors that recognize pathogen effectors in the cytosol. Recognition can occur directly by physical binding of the effector or indirectly by the detection of modifications effectors cause on plant target proteins or mimics of these targets named decoys. The vast majority of the effector targets identified so far act in plant immunity, and all levels of the immune response such as signal perception, signal transduction, regulation of gene expression or defence execution are targeted by effectors. Accordingly, effector targets are involved in a broad range of cellular pathways and have many different molecular functions such as e.g. protein kinases, transcription factors or proteases (Presti et al., 2015; Deslandes and Rivas, 2012; Martin and Kamoun, 2011). A third intermediate mode of effector recognition relies on the integration of decoy domains mimicking effector target proteins into NLRs (Césari et al., 2014; Nishimura et al., 2015; Wu et al., 2015b). Integration of decoy domains was fist suspected in poplar where 32 NLR proteins were found to carry the same additional domain (Germain and Séguin 2011). This additional domain, called BED (for BEAF and DREF Drosophila proteins containing this domain; Aravind, 2000), was also found in 9 rice NLRs, including one which was found to code for the Xa1 functional resistance protein (Das et al., 2014). The authors of this study questionned why both rice and poplar have independently acquired this gene architecture and raised the hypothesis that the integrated domain may act as a sensor for pathogen effectors.

Experimental support for the integrated decoy model has been provided in the rice blast model system. The RATX1/HMA domain in the rice R proteins RGA5 and Pik-1 that ressembles to the product of the blast susceptibility gene *Pi21*interacts physically and confers specificity to the *Magnaporthe oryzae* effectors AVR-Pia and AVR-Pik (Cesari et al., 2013; Césari et al., 2014; Kanzaki et al., 2012). Recent studies on the *Arabidopsis thaliana* NLR RRS1 came also to the conclusion that the WRKY domain integrated into RRS1 acts as an integrated decoy that recognizes the effectors AvrRps4 and PopP2 by monitoring the perturbation they induce in WRKY transcription factors that are major regulators of plant defense (Le Roux et al., 2015; Sarris et al., 2015).

By specifically analyzing the C-terminal region of homologs of RGA5 and RRS1 from different plant species, a huge number of NLRs was identified that carry non-canonical domains frequently related

to signal transduction, regulation of transcription and defense responses (Césari et al., 2014). This observation suggested that integration of additional domains could be a common feature of plant NLRs and serve as a powerfull lead for the identification of novel actors in plant immunity that may represent targets for pathogen effectors. In this study, we addressed more systematically the question whether the integration of additional domains in plant NLRs is a general feature and occurs in all plant lineages, which fraction of plant NLRs carry integrated domains that may act as effector sensors and which type of domains are integrated and at which frequencies.

We show, by exploring large sets of plant NLRs from 31 genomes that unusual domains are integrated with a mean frequencty of 3.5% into NLRs. NLRs carrying unusual domains are present in all analyzed plant lineages, including mosses, and correspond to all major groups of NLRs. A huge number of functionally diverse domains was found in NLRs and they are integrated at different positions indicating that the integration of unusual domains has occurred frequently and repeatedly during plant evolution. All these features support the model that these unusual domains in NLRs represent integrated decoys that allow plants to detect pathogen effectors that target other proteins carrying such domains. By analyzing the rice ZBED protein containing the BED zinc finger domain frequently integrated into NLRs, we confirmed the hypothesis that integrated decoys are a lead for the identification of novel players in plant immunity and confirmed a role of ZBED in rice blast resistance.

MATERIAL AND METHODS

In silico analysis of NLR protein domains

To analyze a representative sample of NLR proteins from all available plant genomes, we used the NLR repertories defined by the Greenphyl database (http://www.greenphyl.org/; Conte et al., 2008; Rouard et al., 2011). Using TribeMCL software, Greenphyl has initially produced gene family clusters for rice and Arabidopsis that were manually curated (Conte et al., 2008). The protein sequences of other plant genomes were allocated to these protein clusters using BlastP (Rouard et al., 2011) and Interpro annotation was finally included for each protein. Thus Greenhpyl has the advantage to provide gene cluster families that were defined in a unified manner across the plant kingdom. We retrieved the Greenphyl protein clusters and the corresponding Interpro domains corresponding to approximately 155000 NLR proteins (GP000012, GP015056, GP015065 and GP015132) as well as >2500 Receptor-Like Kinases (GP039790). It is noteworthy that the Interpro database contains several, different, accessions for a given molecular function (see for instance kinase and LRR below).
The criterion used to select canonical NLRs (Table S1) was the presence of both the NB-ARC domain IPR002182 and an LRR domain (any of IPR001611, IPR011713, IPR003591, IPR025875, IPR006553, IPR026906, IPR008615, IPR021929, IPR013210, IPR013101). According to the same logic, a set of 1393 canonical RLKs (Table S2) was defined by the presence of an LRR domain (same Interpro domains as above) and the protein kinase domain IPR000719. Other domain were frequently found in the NB-ARC parts of the NLR proteins (AAA+ ATPase domain IPR003593) or the kinase domain of LRR-RLK proteins (IPR000719, IPR008271, IPR002290, IPR017441, IPR001245, IPR020635); these frequently found domains were therefore considered as usual components of these proteins and not as putative decoys (See Table S2 and S3).
For re-annotation of cloned R proteins, we used Interpro database (http://www.ebi.ac.uk/interpro/search/sequence-search) or the ncbi domain (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) search tools. In Table S3, the Gene Ontology terms (http://www.ebi.ac.uk/interpro/download.html) were assigned (or manually when no GO term was available) to each Interpro domain to establish global molecular function of decoy domains.

Over-expressor and knock-out mutant production

The ZBED cDNA from Nipponbare was PCR amplified (Fig. S1b) and cloned into the pBIOS2300OX transformation vector (provided by JC Breitler, CIRAD) under the control of the constitutive maize Ubiquitin promoter (Fig. S1d). Nipponbare and Kitaake cultivars were transorfmed as described (Grand et al, 2012). For Nipponbare, the number of T-DNA insertions was estimated in T1 and T2 families using PCR for the Geneticyn/Kanamycin selection marker (Fig. S1d) as a diagnostic for T-DNA

presence. Only lines carrying single copy insertions (showing 3:1 positive PCR, n>20 plantes analyzed) were conserved for further analysis. Siblings that were negative by PCR (thus not containing the transgene) were used as controls (NS: null-segregant controls). Individual T2 plants (homozygous OX and NS) from three independent monolocus lines were selfed and *ZBED* over-expression was confirmed in the T3 progeny. In the case of transgenic plants in the Kitaake background (Fig. S2), transformation was also done using the pBIOS2300OX empty vector in parallel to the *ZBED* over-expression vector. The expression of the ZBED gene was measured in the first generation (T0: Fig. S2a) and T1 seeds were amplified. For both vectors, T1 plants were selected on Genetycin and then inoculated (Fig. S2b and S2c).

For knock-out (KO) mutant, we retrieved the ASFH06 insertion line (Nipponbare background) from the OryzaTagline collection (Larmande et al, 2008) (Fig. S1a). Primers flanking the insertion site were used to identify homozygous plants (*ko-zbed*) as well as siblings showing no insertion (wt-zbed).

Gene expression analysis and disease resistance assays

Rice plant growth and inoculation were done as described (Vergne et al., 2010). Three week-old plants were inoculated with the moderately virulent isolate GUY11 of *Magnaporthe oryzae*. Five to seven days after inoculation, susceptible lesions (spots characterized by grayish center) were counted as a measure of susceptibility. Differences in total lesion numbers between Fig. 3b and 3e only reflect differences in inoculum quality.

RNA and gene expression by qRT-PCR (expression data were normalized with the *Actin* gene) were done as described in Grand et al (2012). The qRT-PCR primers used for ZBED (accession LOC_Os01g36670) were CCGTTGATTCAGAGGTGCTGAC and TCGTAACAACATGGTAGCCTTGG.

RESULTS

Hypothetical integrated decoydomains are found in 15% of the cloned NLR R proteins

First, we retrieved the sequences of cloned R proteins and expert-annotated R protein analogs of the NLR class from PRGdb (Plant Resistance Gene Data Base) (Sanseverino et al., 2013; Table S4) and searched this dataset of 98 NLRs for additional Interpro domains, not present in canonical NLRs (see Methods). In addition to the 9 previously reported R proteins with putative decoy domains (Cesari et al., 2014) (Supplemental Table 1), there are three known *R* proteins with putative decoy domains: the auto-immune NLR CHS3 that has a LIM-type Zinc-finger domain (Bi et al., 2011), the major splice variant of the rice blast R protein Pi-ta containing a thioredoxin domain (Costanzo and Jia, 2009), and the rice bacterial blight resistance protein Xa1 that contains a BED-type Zinc-finger domain that had been overlooked when Xa1 had been cloned (Yoshimura et al., 1998) and was also described in several recent studies (Germain et al., 2011; Das et al., 2014). Moreover, we identified three R proteins with additional domains that had not been detected yet (Fig. 1a): the potato late blight resistance protein R3a carries a FAM75 domain of unknown function in its C terminus; the R1 protein from a wild potato species contains a domain of unknown function; the putative soybean R protein *KR1* (He et al., 2003) contains a DNA-binding domain. Thus in total, 15 of the 98 functional *R* proteins of the NLR class contain a putative decoy domain indicating that up to 15% of the NLRs acting as R proteins contain integrated decoy domains.

Integrated domains are present in NLR proteins from 31 plant genomes

The analysis of cloned R proteins and previous reports suggest that NLRs with integrated decoy domains may be common in many plant genomes (Césari et al., 2014). To adress this hypothesis in a global manner, we analysed the NLR repertoires in 30 complete genomes from higher plants and one Moss species provided by the Greenphyl database that generated protein family clusters from plant whole genome sequences by using Tribe MCL and Blast similarity searches (for details see materials and methods (Rouard et al., 2011)). Greenphyl DB records slightly more than 15500 NLRs in these 31 plant genomes belonging either to the TNL orthology group GP000012 or one of the three major CNL orthology groups GP015065, GP015132 or GP015056. This set of NLRs presents the advantage that the same criteria (see Methods) were used to retrieve proteins from such large and complex gene families in whole genome data. Comparing the Greenphyl NLR sets provided to the manually curated NLR repertoires from rice (Luo et al., 2010) and *A. thaliana* (Tan et al., 2007) showed that the Greenphyl repertoire contained 236 of the 276 published and manually curated rice NLRs (85%) and 177of the 201 manually curated *A. thaliana* NLRs (88%). From this, we concluded

Figure 1 Kroj et al

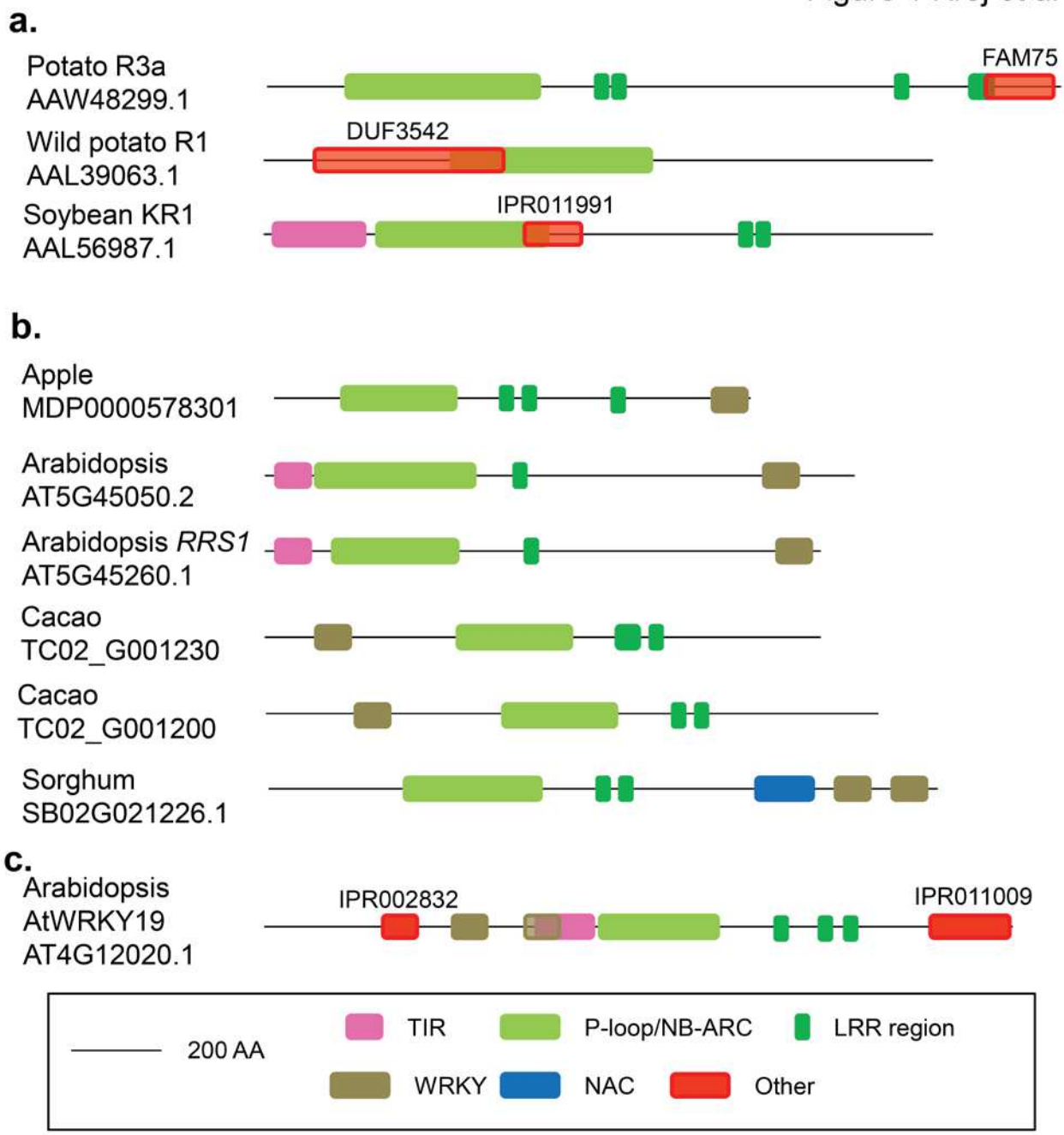

Figure 1. Examples of integration of unusual domains in NLR proteins in plants
Protein domains of the different NLRs were established by InterPro search (http://www.ebi.ac.uk/interpro/) or ncbi domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) along the full length protein (black line). Interpro search does not detect Coil-coil domains often found in NLR, thus explaining their absence here. Canonical NLR domains (TIR, P-loop/NB-ARC and LRR) are indicated in pink and green. Although these domains may be larger, the detection by Interpro search only indicates the borders defined by the Interpro domains. (**a**) The R3a, R1 and KR1 cloned resistance proteins contain unusual protein domains besides the canonical NLR structure. In KR1, IPR011991 represents a Winged helix-turn-helix DNA-binding domain whereas the FAM75 and DUF3542 are domains of unknown function. (**b**) The WRKY domain (brown) was integrated several times in plant NLR genes from different species and some examples are shown here. An additional NAC domain is also found in the Sorghum protein. (**c**) The *A. thaliana* NLR At4g12020 is highly modular and contains three types of additional domains besides the canonical TIR-NBS-LRR domains: a WRKY domain, a PAH domain of unknown function (IPR002832) and a kinase-like domain (IPR011009).

that the NLR repertoires provided by Greenphyl cover extensively the NLR diversity in plant genomes.

Inversely, we observed that the Greenphyl NLR dataset contained rice and *A. thaliana* proteins not present in the previously published curated NLR lists (Luo et al., 2011; Tan et al., 2007). This is partially explained by the fact that the Greenphyl NLR dataset contains proteins carrying either the LRR or the NB-ARC domain but not both of them. Therefore, we also selected among the more than15500 NLRs recorded by Greenphyl a subset of 2699 canonical NLRs based on the *combined* presence of the NB-ARC and the LRR Interpro domains (Table S1).

We retrieved the Interpro annotations for the approximately 15500 NLR proteins and searched for domains that do not correspond to canonical CNL or TNL domains. This identified 456 NLR proteins (3.6%) harboring at least one unusual domain (Fig. S3). The same analysis was performed with the more stringently defined dataset of 2699 canonical NLRs. This identified 94 NLRs (3.5%) with unusual domains (Fig. 2a), a value very similar to that obtained with the full Greenphyl set of more than15500 NLRs.

The two major classes of NLRs showed slight differences, with CNLs displaying less unusual domains (2.5%) than TNLs (4.7%). In addition, the frequency of NLRs with unusual domains varied between species (Fig. 2a), with a maximum of 6% in Poplar. Among the 10 NLRs of the moss *Phycomistrella patens,* two displayed an integrated domain, indicating that this phenomenon extends to lower plants.

Since many genomes are machine predictions, erroneous annotations leading to gene fusions could produce artefactual integrated domains. We thus tested whether another class of plant receptors, the Receptor-like kinases (RLKs), displayed similar occurence of unusual domain. Out of ~1400 RLK proteins tested, only 19 proteins (1.4%) displayed additional domains that do no correspond to canonical domains for RLK proteins (Fig. 2a; See Methods and Fig. S3). Although the frequency of additional domains in NLRs was two-fold higher than in RLK proteins, some of the integrated domains in NLRs could still result from wrong genome annotation like gene fusion. Taken together, the genomes of all 31 analyzed higher plants contained significant but varying numbers of NLR proteins with additional, yet unconventional, domains.

Kinase, WRKY and BED domains are most frequent among the myriad molecular functions hidden in NLR proteins

Automatic Gene Ontology assignment and manual annotation of the 94 Interpro domains identified in the 2699 canonical NLRs identified some molecular functions that are highly represented i.e. nucleic-acid binding activity (including transcription), signaling and oxidative metabolism (Table S3).

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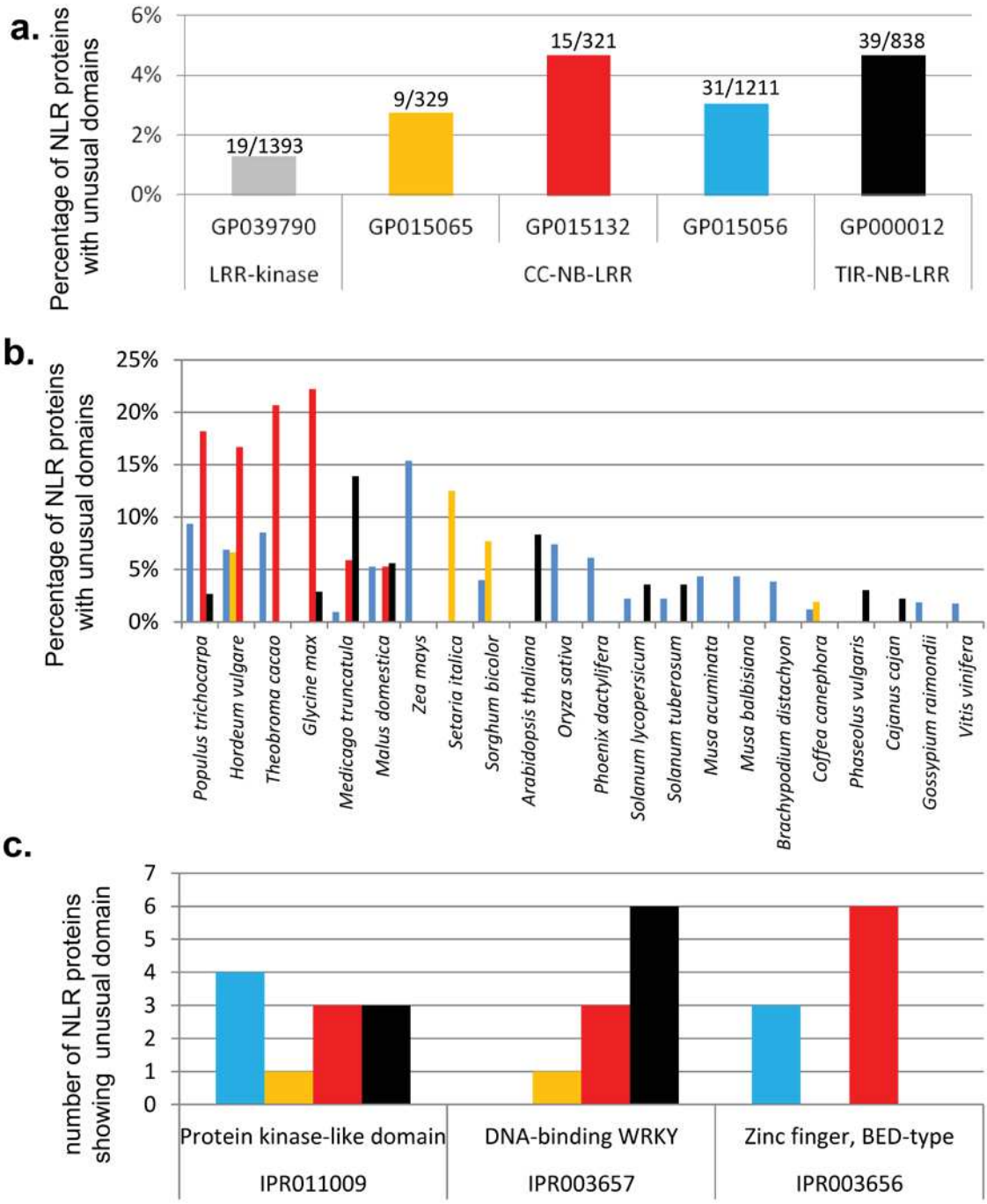

Figure 2. Most plant species show unusual domains in their NLR proteins
Additional domains, according to Interpro annotations, were searched in 2699 plant NLR proteins from differe of canonical NLRs. The NLR sub-groups defined by Greenphyl are shown with different colors: orange (GI red (GP015132), blue (GP015056) and black (TIR-NB-LRR: GP000012). (**a**) The number of unusual domair number of NLR analyzed is indicated above each bar (See Table S1 for more detail). The 1393 canonical F Greenphyl family GP039790 (grey bars) were used as a control to evaluate, on this different set of mu immune receptor proteins, the frequency of such unusual domains (See Table S2 for more detail). (**b**) Th genomes showing the highest frequencies and (**c**) the number of NLR proteins carrying the most frequen (IPR011009, IPR003657 and IPR003656) are shown.

However, most of the domains belonged to other very diverse classes that could not be regrouped, indicating that integrated domains encompass very diverse molecular functions.

Most domains occurred with low frequencies and were often detected only once. Only three domains were found in many NLRs and in different species (Fig. 2c): the protein kinase domain IPR011009 involved in protein phosphorylation, the WRKY domain IPR003657 involved in DNA-binding and transcription and the BED domain IPR003656 involved in DNA-binding. These domains were found respectively 11, 10 and 9 times and in 6, 4 and 4 different species. A particular feature of the BED domain is that it is particularly frequent in the N-terminus part of NLRs, , as described previously in Poplar (Germain and Séguin, 2011).

Uncommon domains have been integrated frequently and independently into NLRs during plant evolution

Acquisition of domains by NLRs seems to have occurred several times and independently during evolution since the same domain is present in NLRs from different phylogenetic clades. For instance, WRKY (IPR003657) domains were found in CNLs of the groups GP015132 and GP015056 and TNLs (group GP000012) (Fig. 2b; Table S1). Further support for repeated and independent acquisition of integrated domains comes from the observation that the same domain can be integrated at different locations. WRKY domains are for instance detected in the N- or the C-terminus of CNLs and the C-terminus of TNLs (Fig. 1b). This apparent flexibility in the integration of additional domains in NLRs had already been reported for the integration of the RAXT1/HMA domain in the rice blast resistance NLRs RGA5 and Pik-1 where it is located respectively at the C-terminus or between the CC and the NB (Kanzaki et al., 2012; Cesari et al., 2013).

In rare cases, several domains were integrated in one single NLR. For example, one Sorghum CNL contains a NAC and two WRKY domains (Fig. 1b) and an *A. thaliana* TNL (AT4G12020, also known as AtWRKY19; Fig. 1c), carries two WRKY domains, one kinase domain (IPR001109) and one PAH domain (IPR002832) involved in protein-protein interactions. Altogether, these examples suggest a high modularity of the NLR proteins.

A protein containing three BED domains affects disease resistance

The integrated decoy hypothesis predicts that some plant proteins similar to decoy domains are targeted by effector proteins because they act in disease resistance or susceptibility. The decoy domains in NLRs could therefore guide the identification of novel effector targets and new proteins involved in plant immunity. We challenged this hypothesis for the BED domain which is frequently integrated in NLRs but had, thus far, not been demonstrated to play a role *per se* in disease resistance. For this, we investigated the ZBED protein from rice which contains three BED domains

(Fig. S4a) that show 44% to 58% identity with the BED domains of Xa1 (Fig. S4b). *ZBED* had attracted our attention because its expression is negatively correlated to partial resistance to the blast fungus *Magnaporthe oryzae* across a panel of rice varieties (Fig. S2c). This type of expression pattern has been reported to be a hallmark of regulators of disease resistance (Grand et al., 2012). The *ZBED* gene is not strongly regulated during infection by the rice blast fungus *M. oryzae* (Fig. S4d). To analyze if ZBED has a role in immunity, rice plants over-expressing the *ZBED* gene were produced. These plants did not show any obvious visible growth phenotype (data not shown), however rice plants of the variety Nipponbare over-expressing *ZBED* (Fig. 3a) were less susceptible to *M. oryzae* and developed less disease lesions than control plants (Fig. 3b, c). This observation could be confirmed in a separate genetic background, Kitaake (Fig. S2), further supporting a role of ZBED in disease resistance. There was a weak correlation between transgene expression levels and disease symptoms (compare Fig. 3a with 3b and Fig. S2a with S2c). Since over-expression of many genes may artefactually lead to reduced susceptibility, we also analyzed *ZBED* T-DNA insertion mutants. We isolated an homozygous mutant for the *ZBED* gene (*ko-zbed*) as well as homozygous wild-type siblings (wt-bzed) (Fig. S1a) and inoculated them with *M.* oryzae. Loss of ZBED expression led to a significant increase in susceptibility (Fig. 3e and 3f) indicating that ZBED contributes to resistance to the rice blast fungus *M. oryzae* either by directly affecting fungal growth or by contributing to the induction of defense responses. These results on ZBED demonstrate that integrated decoy domains can serve as an extremely powerful lead for the identification of new actors in plant immunity.

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Figure 3 Kroj et al

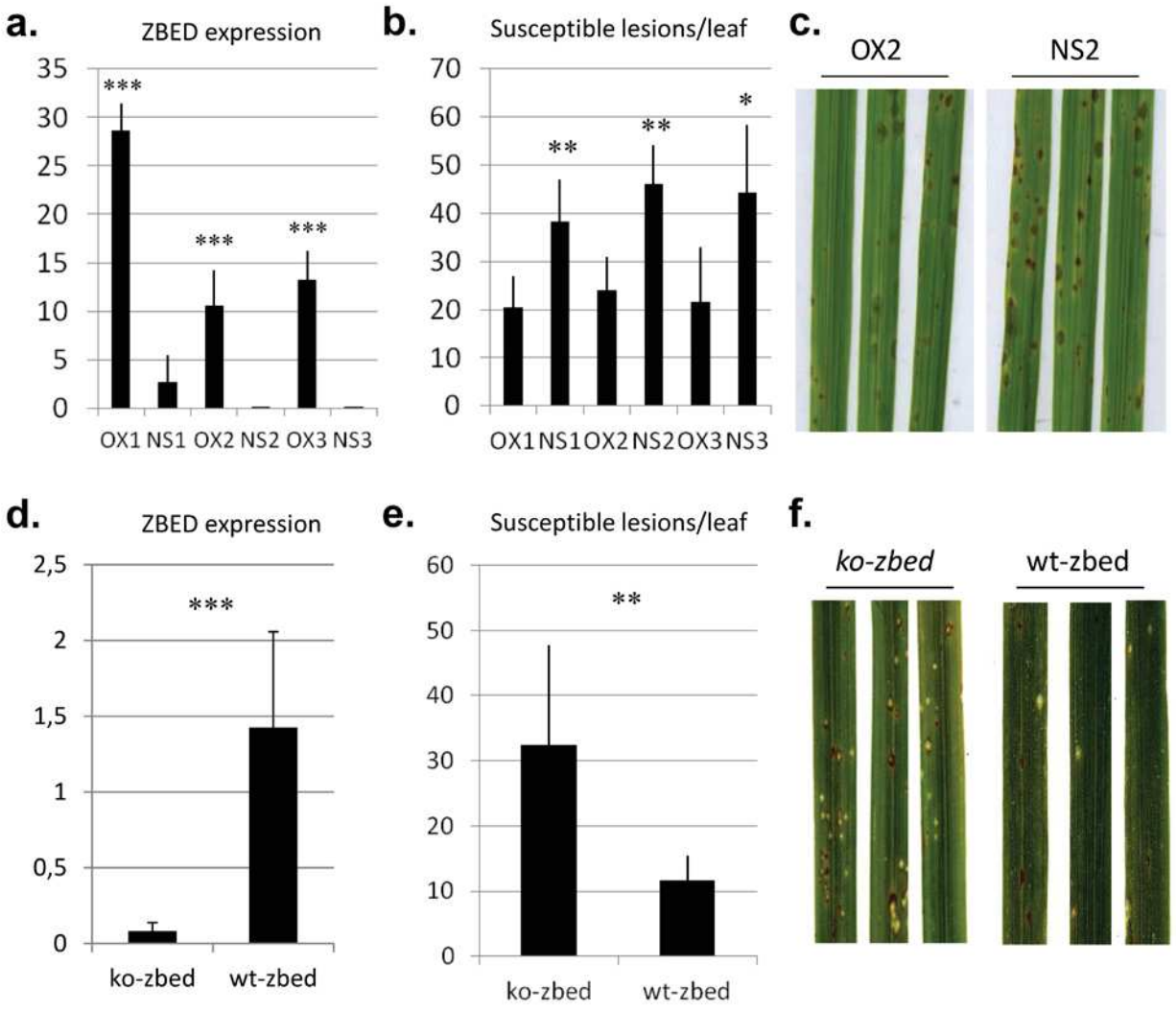

Figure 3. ZBED protein is involved in blast resistance

The role of the rice ZBED protein containing three BED domains in disease resistance against the blast fungus *M. oryzae* was evaluated by over-expressing (Fig. S1b,c,d) and knocking-out (Fig. S1a) the *ZBED* gene. The three homozygous, monolocus T3 transgenic rice lines over-expressing the *ZBED* gene (**a)** showed less disease lesions (**b** and **c**). By contrast the insertion mutant for the *ZBED* gene (**d**) showed more disease symptoms (**e** and **f**). ZBED gene expression was measured by qRT-PCR (normalized by *Actin*). Disease lesions are characterized by sporulation and a greyish center were quantified 7 days after inoculation with the *M. oryzae* strain GUY11 and representative symptoms are shown (**c, f**). Differences in symptom strength between panels **c** and **f** are frequent in this type of plant-pathogen interaction studies and is probably due to variability in plant growth-conditions. A Student t-test was used to compare the over-expresser lines (OX) and the ko mutant line with their respective null-segregant controls (NS- see Methods): *: P<0.05, **: P<0.01 and ***: P<0.001. The gene expression and disease values are the mean and SD from three independent

DISCUSSION

The discovery that unusual domains integrated into NLR immune receptors act as decoy domains is very recent. First experimental evidence has been provided by the investigation of the rice blast resistance NLR pairs RGA4/RGA5 and Pik-1/Pik-2 and further important validation has been gained by the detailed analysis of the *A. thaliana* NLR pair RPS4/RRS1 (Cesari et al., 2013; Sarris et al., 2015; Kanzaki et al., 2012; Le Roux et al., 2015). Occurrence of NLRs with decoy domains in phylogenetically unrelated immune receptors from mono and dicot plants suggested that it is a widespread phenomena and a first large scale analysis showed that the integration of unusual domains is frequent among cloned R proteins of the NLR class and RGA homologs similar to RGA5 and RRS1 (Césari et al., 2014). In the present study, we have analyzed more systematically the occurrence of integrated decoy domains in NLRs from 31 plant species from higher plants and one moss. For our systemic analysis, we used the publicly available data of the GreenPhyl database where a huge number of sequenced genomes have been analyzed with a common set of criteria for establishing protein family clusters (Rouard et al., 2011). Comparison of this NLR dataset with the published, expert-curated NLR repertoires of *A. thaliana* and rice showed for both species a good representativeness of the GreenPhyl database and suggested a good coverage for the other species. This dataset was also used to define a more stringently dataset of NLRs. Both NLR datasets were filtered according to the occurrence of Interpro domains leading to the definition of NLRs with additional, non-canonical domains. It is noteworthy that our approach using Interpro annotation has some limitations. For instance, the CNL domain found in almost half of the TIR-LRR proteins (Dodds et al., 2001) was not detected by our study since this domain is not defined in the Interpro database. Similarly, the coil-coil domain found in canonical NLR was not detected. Despite these limitations, our analysis clearly demonstrated that decoy integration is frequent and occurs in all analyzed plant lineages. Indeed NLRs from many different lineages of annual and perennial dicotyledonous plants, different lineages of monocotyledonous plants and one moss possess decoy domains at varying frequencies. Decoy domains are found in all classes of NLRs, i.e. in the different classes of CNLs and in TNLs and these NLRs seem tremendously flexible to the integration of decoys since integration can occur at the boundaries of all different canonical NLR domains and even multiple integrations at different positions in a single NLR are observed. The *A. thaliana* TNL At4g12020 is for instance highly modular and contains three types of additional domains, integrated C- or N-terminal to the canonical TIR, NBS and LRR domains (Figure 1).

Our analysis also revealed that integrated decoy domains are extremely diverse and cover many different molecular activities. Out of the 90 putative decoy domains identified here, 64 are novel (Table S3) compared to the initial study by Cesari et al (2014). Conversely, 34 of the 52 Interpro

domains found in RGA5 and RRS1 homologs were not detected in this analysis. Thus our analysis is probably not exhaustive and future analysis employing additional, eventually more sensitive approaches for domain detection or more complete datasets, including e.g. additional NLRs from re-annotated and expert-curated genomes, targeted NLR-sequencing by RenSeq (Jupe et al., 2013) or inclusion of additional species or more than one accession per species will further refine the picture.

For both analyzed datasets, the more loosely defined approximately 15500 and the more stringently defined 2699 NLRs, our analysis revealed a mean frequency of 3.5% of unusual domain integration (Fig. 1 and Fig. S3). This is much below the frequency of decoy domain integration in cloned R proteins of the NLR class that is 15% (Table S4). It could be that NLRs that are functional as immune receptors and confer resistance to pathogens contain more frequently integrated domains than NLRs that have no defined specificity and rather serve as a reservoir for the diversification of NLR receptors or act in other processes than pathogen recognition and disease resistance. The ongoing massive cloning of *R* genes and the increased reliability in the annotation of whole genome NLR complements by technical progress and i.e. the extremely powerful RenSeq approach will allow addressing this issue more thoroughly in the near future.

Integrated decoy domains have first been studied in paired NLRs and in the homologs of the paired NLRs RGA5 and RRS1 (Césari et al., 2014). This observation raised the question whether decoy domains are restricted to paired NLRs. Our study identified decoy domains in NLR type R proteins such as R3a and Xa1 that, according to current knowledge, are not paired and do not show the typical, tightly linked head-to head tandem arrangement with a second NLR in the genome (Huang et al., 2004; Yoshimura et al., 1998). In addition, our inspection of the genes coding NLRs with integrated decoys in rice revealed that many of them do not show the typical genomic head-to-head tandem organization and often even do not possess an adjacent neighbor NLR (Table S5). Therefore, integrated decoy domains seem not restricted to paired, mutually matching NLRs. Whether the non-paired NLRs that contain integrated decoy domains act in concert with downstream, helper NLRs (Wu et al., 2015a; Bonardi et al., 2011) will have to be elucidated in the future.

Three domains have been identified particularly frequently in NLRs, the WRKY and BED zinc finger domain and the protein kinase domain. For all these three domains, cloned R proteins are known, demonstrating that the corresponding chimerical NLR structures are functional as immune receptors in effector-mediated pathogen recognition. The WRKY domain is present in the RRS1 protein from *Arabidopsis thaliana* (Deslandes et al., 2002), where it has been convincingly shown to act as a decoy in the recognition of the effectors PopP2 and AvrRPS4 from the bacterial plant pathogens *Ralstonia solanacearum* and *Pseudomonas syringae* pv. *tomato* (Le Roux et al., 2015; Sarris et al., 2015). A protein kinase domain is present in the rust resistance protein RPG5 from *Hordeum vulgare* and in the susceptibility factor Tsn1 from wheat that confers, in an inverse gene-for-gene manner,

susceptibility towards isolates of the necrotrophic fungi *Pyrenophora tritici repentis* and *Stagonospora nodorum* carrying the ToxA effector protein (Wang et al., 2013b; Faris et al., 2010). The BED domain is present in the Xa1 resistance protein from *Oryza sativa* (Yoshimura et al., 1998) that recognizes an unknown factor from the bacterial blight pathogen *Xanthomonas oryzae* pv *oryzae*.

Besides the three most frequently found unusual domains, domains corresponding to a wide range of molecular functions have been integrated. This is in accordance with the hypothesis that these integrated additional domains serve to monitor modifications of the complex plant immune system. Integrated domains are unlikely to act in downstream signaling, triggered by NLR mediated effector recognition, which is supposed to be rather conserved between NLRs and between species. An activity of the integrated domains in other pathways and in the absence of the Avr effector cannot be completely excluded until experimentally demonstrated but seems, in particular in the cases where partial or non-functional domains are integrated, rather improbable (Wu et al., 2015b).

Some integrated domains are characteristic of well-known key players of plant immunity such as protein kinases and WRKY transcription factors. Others have been demonstrated more recently to be involved in resistance, targeted by effectors or guarded by NLRs. NAC transcription factors have for instance only very recently been shown to be targeted by an effector of the potato light bight pathogen *Pythophthora infestans* and to play an important role in resistance to this pathogen (McLellan et al., 2013). Corresponding NAC domains are integrated in different classes of NLRs from mono- and dicotyledonous plants (Table S1). Many other domains, not yet implicated in immunity and disease development are integrated in NLRs. This allows now to take a completely new and extremely exciting look on plant immunity and disease susceptibility by picking proteins containing these decoy domains and testing whether they are involved in resistance or disease-related processes.

The integrated decoy concept predicts that some proteins containing BED domains, the third most frequently integrated decoy domain, may be involved in disease resistance. Our analysis of the rice *ZBED* gene supports this hypothesis since a rice *ZBED* mutant is more susceptible to rice blast while ZBED over expresser lines show increased resistance. This suggests that BED domains in NLRs may indeed represent decoys mimicking BED proteins involved in some yet unknown immunity-related processes. However, the putative effector targeting the BED domains remains to be identified. In animals, proteins with BED domains were initially described in Drosophila where they have DNA-binding activities (Aravind, 2000). BED proteins in animals have diverse molecular functions; for instance the Zbed3 protein is a transcriptional regulator (Wang et al., 2013b) while Zbed6 is secreted to the membrane (Jia et al., 2014). To understand the role of BED domains in plant immunity, thorough investigation of ZBED and related BED proteins will be required.

394 The integrated decoy model adds one more level of complexity to our understanding of effector
395 recognition by plant NLR immune receptors and opens exciting new ways in the investigation of plant
396 immunity. Exploring the role of proteins containing the domains that correspond to the integrated
397 decoys promises to shed new light on the biological functions that phylogenetically unrelated
398 pathogens have chosen to target during evolution.

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K.T. and M.J-B. designed the research; C.E., M-R.C, X.G. and M.J-B. performed the research; C.E., M-R.C., K.T. and M.J-B. analyzed and interpreted the data; K.T., C.E. and M. J-B. wrote the manuscript

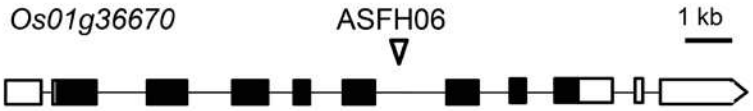
Integration of decoy domains derived from protein targets of pathogen effectors into plant immune receptors is widespread

Kroj Thomas, Chanclud Emilie· Michel-Romiti Corinne, Grand Xavier, and Morel Jean-Benoit

Accepted Dec 16th 2015

Suppl Figure 1 Kroj et al

a.



b. **ZBED protein corresponding to the cloned cDNA**

MDGVDKDKGNGKVHFRDSSPQEAFRTYKRRRQPRPEPQQQPQPQPQSEPEPQQQ PQ
PQPQPQPQPEAEAEAEAEAKAADVLARQVTETFWKSRDIGWKHGIMIDENRQHWKCMYCH
LTRYGGGVSRLKRHLAGDLDVKMCPKVPADVSEKIREHLRKKRERRKKRAAQNRDNCVTA
KSTSDDIKSGKDPLPVDSEVLTGVDTVLEEVTNQ TNHDNQDLTYPKATMLLRGIRDIGWE
HAVDLDGNKRRWKCKWCSLCRSGGVTTLKAHLTDSSCPNIPKEISKKVLNFIEEKRAARH
LFNSAAKSPFNVKFDEDAVNLSEIQVEGTPPLTDDRQPLGNSLHIQTSECTINEFEKVAA
GSNQQGAEHSNQLLNHGEQLMKSSDQPEEHCTLEHGRCQVLDNNKQQTMDNKTDNPEHKE
VLKHPKKTRFNIRKHIVIVDESARHWRCRYCGLDGYGKTSRLHFHLAAVFRHPKCPSVPK
EVFAKARHHIHLKRRLNVKKAGQQARSRPHILGQSSQQQQNNNPVLSNYPTRLRDNAWEH
SLIHDKEKGHWKCKWCSLEGYHGITRLKWHLVGWQNRPQCLNVPEDVAKTIRDKMISREK
QKEGRLNLDVIDSCNMPCSSESLQFDQENFAEVMQGKGSSEDFNQAERQSNTLNTVCNTT
HPPQNSNNYQGLQENGLYSSKNKSEKQTERYDCWSHWRYVLDGLMHLPGALEGPGIQSCI
RDVLLYGSAEFGTVGDKVEMDSNRKVSSDGNIAKCQSVLVDVLKSENFALLCNVLGRTVH
QDEQRTKYFDFTMIDSRMKNGDYGRAPLLFKHDLKMVTPLL*

c.

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                1        10        20        30        40        50        57
                |--------+---------+---------+---------+---------+------|
ZBED            MDGVDKDKGNGKVHFRDSSPQ---EAFRTYKRRRQPRPEPQQQPQPQPQSEPEPQQQ
KOME             MVLTRIKGMGRCISGIPLPRKLSEPTRGAGSHGRSRNSNRSRSRSQSRNHSNSHSQ
Consensus       .dgldrdKGnGrchfrdplPr...EafRgagrrrrpRnenrqrprpQprnep#pqqQ
```

d.

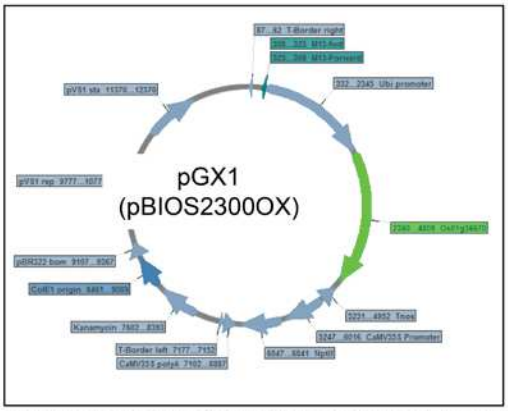

Fig. S1. *ZBED* over-expression and knock-out lines in the Nipponbare background

The genomic structure of the ZBED gene (intron/exon: white/black) is shown in (**a**). The triangle represents the position of the analyzed T-DNA insertion line (ASFH06). For over-expression, a full-length cDNA (**b**) was cloned from Nipponbare and used for transformation (see Methods). The underlined ZBED sequence cloned from rice cDNA is slightly different from the published sequence from the KOME database (**c**). The *ZBED* cDNA clone was inserted into the pBIOS2300OX vector under the control of the Ubiquitin constitutive promoter and the Kanamycin/Genetycin selection marker (**d**) as in Grand et al (2012).

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Suppl Figure 2 Kroj et al

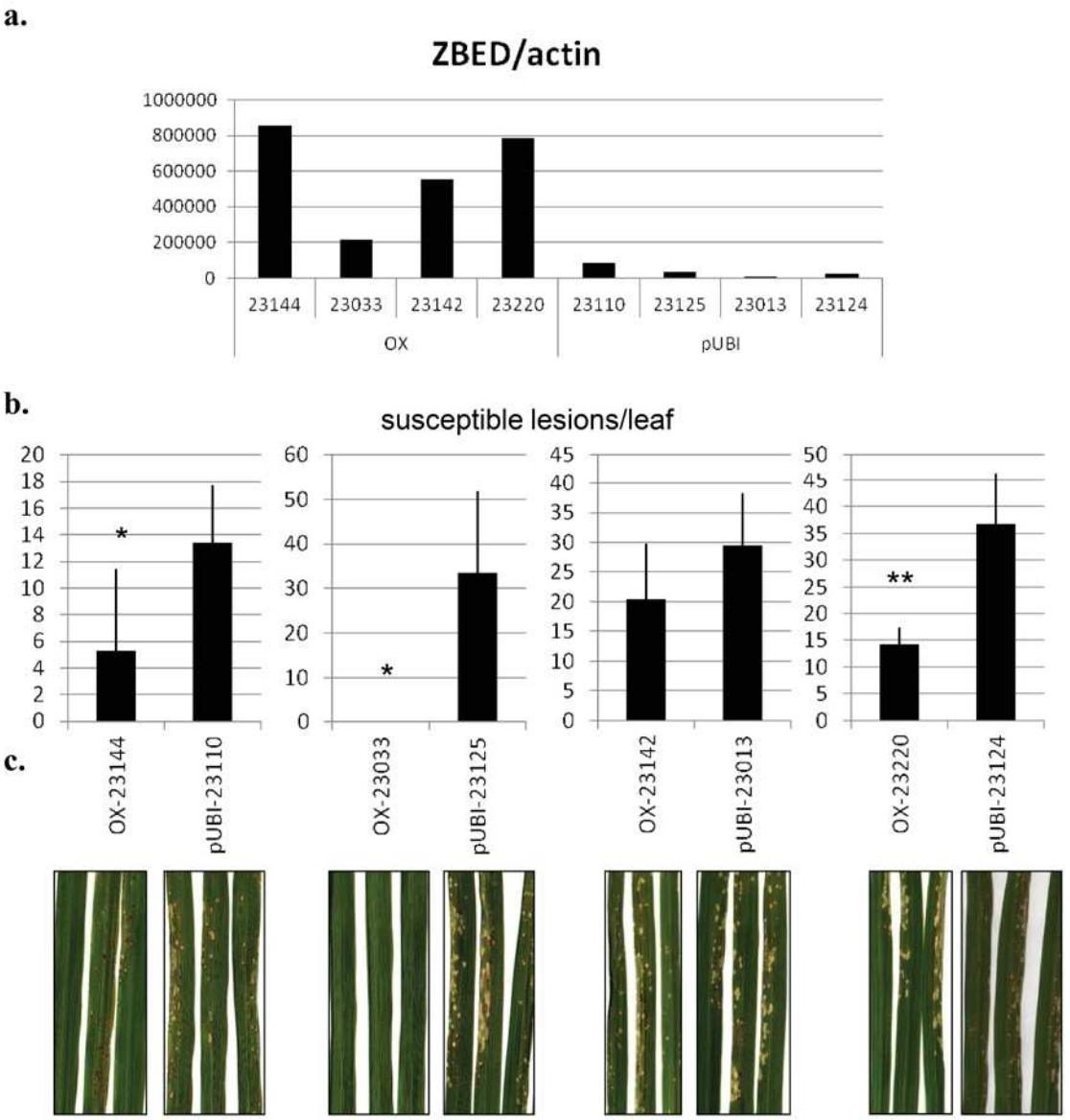

Fig. S2. ZBED-overexpressing transgenic lines (Kitaake background) are more resistant to the rice blast fungus

The vector shown Fig. S3d and the original empty vector (pUBI= pBIOS2300OX) were used to transform the rice Kitaake genotype. Individual T0 lines showing ZBED over-expression (**c**) were selected. T1 plants overexpressing *ZBED* or carrying the empty vector were sown on Kanamycin. Lines showing 3:1 Kanamycin resistance segregation (single locus insertion) were further analyzed. Pairs of Kanamycin resistant plants for *ZBED* over-expression vector and empty vector were transplanted for inoculation with the GY11 *M. oryzae* strain. The four pairs of *ZBED* over-expressor (OX) and empty vector (pUBI) lines were conducted independently. The ZBED gene expression (normalized with Actin) was measured on unique T0 plants (**a**). The symptoms (susceptible lesions) were counted 7 days after inoculation (**b**) and representative examples are provided (**c**). In panel (**b**), A T-test was used to compare OX and pUBI (*: P<0.05).

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Suppl Figure 3 Kroj et al

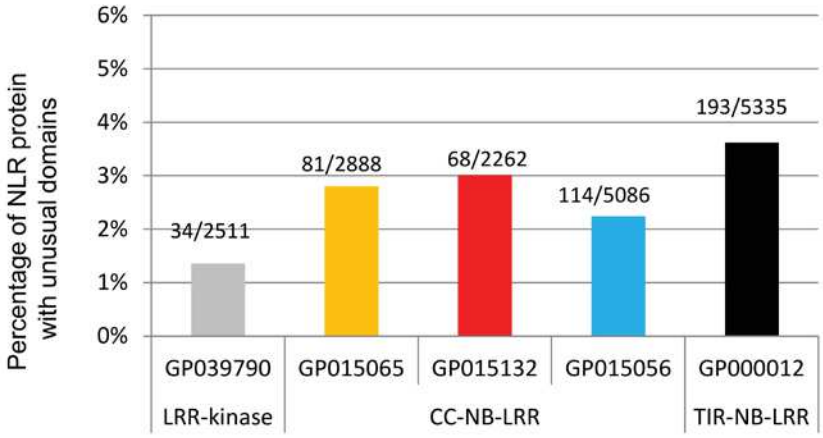

Fig. S3. Frequency of unusual domains in all Greenphyl NLR proteins
The approximately15500 NLR proteins from the Greenphyl database were analyzed (see Methods). Non-canonical, unusual domains were searched using InterPro annotation of the NLR proteins. The number of unusual domains and the number of analyzed NLRs per Greenphyl family is indicated above each bar. The 2511 RLKs from Greenphyl family GP039790 (grey bars) were used as a control and the frequency of unusual domains, other than LRR and kinase, was evaluated in this different set of multi-domain immune receptor proteins.

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Suppl Figure 4 Kroj et al

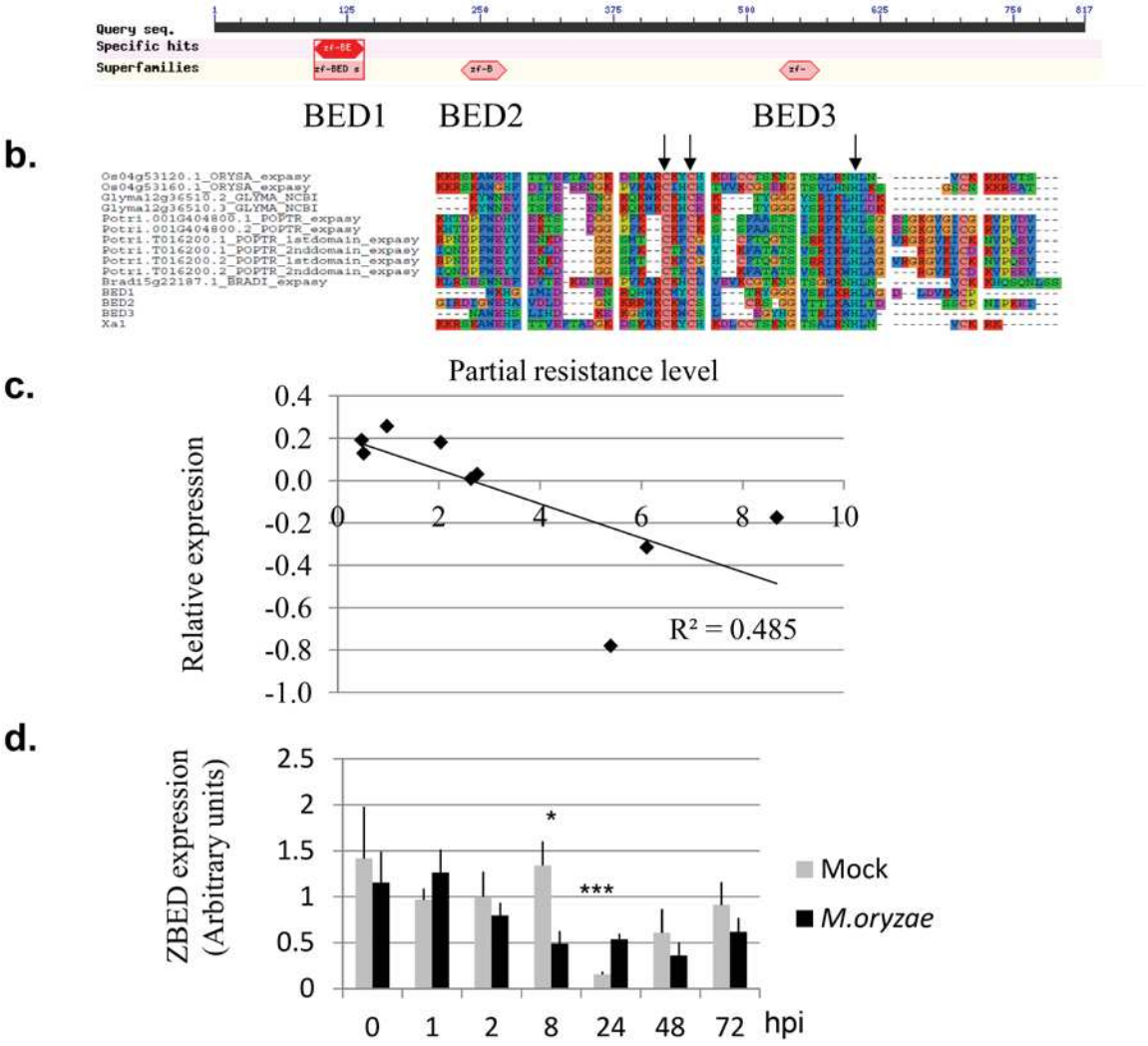

Fig. S4. Structure and expression of the ZBED protein and gene

(**a**) The ZBED protein contains three predicted BED domains (screenshot from ncbi domain search http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). (**b**) Alignment of the three BED domains from ZBED and the BED domain (IPR003656) identified in the Xa1 protein (Table S4 and Fig. 1a). Two Cysteine and one Histidine residues conserved across all proteins are indicated by arrows. (**c**) The constitutive expression of ZBED is negatively correlated to partial resistance level against *M. oryzae* as measured in Grand et al (2012). This measure relies on inoculations with several multivirulent isolates of M. oryzae that allow global evaluation of quantitative/partial blast resistance of a given rice variety. Each point in the graph represents this value for one rice variety for which ZBED expression was also measured. (**d**) The ZBED gene is slightly repressed by *M. oryzae* infection. The fact that the expression in mock treated plants also changes along time is classical in this type of experiments where the light regime and hygrometry are changing during the experiments (the first 8 h are in the dark for inoculation purposes). The data represent mean and SD from three independent biological experiments. A Student t-test was used to compare the infected plants with non-inoculated plants (Mock): *: P<0.05, ***: P<0.001.

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FIGURE LEGENDS

Figure 1. Examples of integration of unusual domains in NLR proteins in plants

Protein domains of the different NLRs were established by InterPro search (http://www.ebi.ac.uk/interpro/) or ncbi domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) along the full length protein (black line). Interpro search does not detect Coil-coil domains often found in NLR, thus explaining their absence here. Canonical NLR domains (TIR, P-loop/NB-ARC and LRR) are indicated in pink and green. Although these domains may be larger, the detection by Interpro search only indicates the borders defined by the Interpro domains. (**a**) The R3a, R1 and KR1 cloned resistance proteins contain unusual protein domains besides the canonical NLR structure. In KR1, IPR011991 represents a Winged helix-turn-helix DNA-binding domain whereas the FAM75 and DUF3542 are domains of unknown function. (**b**) The WRKY domain (brown) was integrated several times in plant NLR genes from different species and some examples are shown here. An additional NAC domain is also found in the Sorghum protein. (**c**) The *A. thaliana* NLR At4g12020 is highly modular and contains three types of additional domains besides the canonical TIR-NBS-LRR domains: a WRKY domain, a PAH domain of unknown function (IPR002832) and a kinase-like domain (IPR011009).

Figure 2. Most plant species show unusual domains in their NLR proteins

Additional domains, according to Interpro annotations, were searched in 2699 plant NLR proteins from different families of canonical NLRs. The NLR sub-groups defined by Greenphyl are shown with different colors: orange (GP015065), red (GP015132), blue (GP015056) and black (TIR-NB-LRR: GP000012). (**a**) The number of unusual domains and the number of NLR analyzed is indicated above each bar (See Table S1 for more detail). The 1393 canonical RLKs from Greenphyl family GP039790 (grey bars) were used as a control to evaluate, on this different set of multi-domain immune receptor proteins, the frequency of such unusual domains (See Table S2 for more detail). (**b**) The 22 plant genomes showing the highest frequencies and (**c**) the number of NLR proteins carrying the most frequent domains (IPR011009, IPR003657 and IPR003656) are shown.

Figure 3. ZBED protein is involved in blast resistance

The role of the rice ZBED protein containing three BED domains in disease resistance against the blast fungus *M. oryzae* was evaluated by over-expressing (Fig. S1b,c,d) and knocking-out (Fig. S1a) the *ZBED* gene. The three homozygous, monolocus T3 transgenic rice lines over-expressing the *ZBED* gene (**a)** showed less disease lesions (**b** and **c**). By contrast the insertion mutant for the *ZBED* gene (**d**) showed more disease symptoms (**e** and **f**). ZBED gene expression was measured by qRT-PCR

(normalized by *Actin*). Disease lesions are characterized by sporulation and a greyish center were quantified 7 days after inoculation with the *M. oryzae* strain GUY11 and representative symptoms are shown (**c, f**). Differences in symptom strength between panels **c** and **f** are frequent in this type of plant-pathogen interaction studies and is probably due to variability in plant growth-conditions. A Student t-test was used to compare the over-expresser lines (OX) and the ko mutant line with their respective null-segregant controls (NS- see Methods): *: P<0.05, **: P<0.01 and ***: P<0.001. The gene expression and disease values are the mean and SD from three independent experiments.

574 **Supporting Information**

575 **Fig. S1 *ZBED* over-expression and knock-out lines in the Nipponbare background**

576 **Fig. S2 ZBED transgenic lines (Kitaake background) are more resistant to the rice blast fungus**

577 **Fig. S3. Frequency of unusual domains in all Greenphyl >15500 NLR proteins**

578 **Fig. S4. Structure and expression of the ZBED protein and gene**

579

580 **Table S1. Unusual domains in canonical plant NLR proteins**

581 **Table S2. Unusual domains in canonical plant RLK proteins**

582 **Table S3. The 90 putative integrated decoys identified**

583 **Table S4. Curated cloned plant R genes**

584 **Table S5. Rice NLRs with integrated decoys and their neighborhood**

585

CHAPITRE II : Etude des cytokinines endogènes et exogènes sur la résistance du riz à *Magnaporthe oryzae*

Des résultats préliminaires sur le gène *ZBED* suggéraient que les cytokinines pouvaient être impliquées dans les mécanismes de défense du riz.

Une étude publiée en 2013 (Jiang et *al*, 2013) montrait l'effet synergique des CKs avec la voie de l'acide salicylique (SA) dans la mise en place des défenses chez le riz. Contrairement à nos résultats, aucun phénotype de résistance à *M.oryzae* n'a été observé dans leurs conditions expérimentales. Afin de mieux comprendre comment les CKs affectaient la résistance du riz, des CKs telles que la BAP et la kinétine (ainsi que des CKs naturelles, résultats non montrés) ont été pulvérisées sur les feuilles à différentes concentrations et à différents temps avant inoculation. L'effet de ces CKs exogènes sur la résistance a été mesuré.

Ces résultats montrent qu'un apport de kinétine 48h à 72h avant inoculation augmente la résistance du riz mais qu'aucune différence n'est observée chez les plantes traitées 24h avant inoculation (en adéquation avec les résultats obtenus par Jiang et *al,* 2013). L'effet de ces traitements sur l'expression des gènes de défense et la pénétration du champignon a été mesuré. Il s'avère que la kinétine induit une plus forte expression des gènes de défense au cours de l'infection confirmant *in planta* la synergie mesurée *in vitro* dans l'étude de 2013. La BAP, une autre CK, n'induit pas de résistance significative, suggérant que l'effet des CKs sur la résistance du riz est dépendant de la dose, du moment du traitement et de la molécule utilisée.

Afin d'évaluer le rôle des CKs endogènes, des mutants de riz présentant des profils altérés en CKs ont été produits et caractérisés pour leur résistance ainsi que leur réactions de défense. Ces mutants de riz montrent une résistance accrue à *M.oryzae.* Les mesures d'expression des gènes marqueurs des défenses montrent une expression plus forte chez les plantes mutantes non infectées et (pour certains) également pendant infection. Ces mutants montrent que les CKs endogènes pourraient participer à la mise en place des défenses de manière comparable aux réponses induites par la kinetine.

Modifying cytokinins levels in rice plants affects susceptibility to the rice blast fungus

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Key words: Cytokinin, *Magnaporthe oryzae*, UDP-glycosyl transferase, rice

Abstract

Cytokinins are plant hormones known to participate in cellular and biological processes like cell division and sink-source relationships. Their involvement in plant pathogen interactions is an emerging feature. We isolated rice lines mutated in a gene coding for a putative cytokinin UDP-glycosyl transferase (*CK-UGT*) that accumulated lower levels of the inactive CK *cis*Zeatin glucoside and higher levels of zeatin. These plants were more resistant to the rice blast fungus *Magnaporthe oryzae* and this could be associated to an enhanced induction of plant defenses. Furthermore, we show that exogenous application of the cytokinin kinetin at an appropriate time before infection could reduce susceptibility to *M. oryzae*, thus mimicking the effect of the *ck-ugt* mutation. These results support a model where cytokinins produced during (or accumulated before) rice blast infection act synergistically with classical defense pathways to amplify resistance to *M. oryzae*.

Introduction

Plant pathogen interactions rely on several regulation levels. Hormones play a key role in these regulations to orchestrate appropriate response to pathogens [1]. For instance, in rice and *Arabidopsis*, salicylic acid (SA) and jasmonic acid (JA) are crucial regulators to adjust plant inducible defense arsenal [2]. In contrast, ethylene (ET) has various effects on rice immunity compared to *Arabidopsis* where this hormone acts with JA for inducing defenses against necrotrophic pathogens [2]. Several crosstalks between different hormonal pathways are important for plants to engage the adapted response to microbes according to pathogen lifestyles and environmental conditions [3,4] . For instance, the abiotic responsive hormone abscissic acid (ABA) represses defenses mediated by SA, JA and ET leading to increase rice susceptibility against the hemibiotrophic rice fungal pathogen, *Magnaporthe oryzae* but increases resistance against the necrotrophic fungus *Cochliobolus miyabeanus* [5,6]. In rice, SA increases resistance against both (hemi)biotrophic and necrotrophic pathogens and this requires the transcriptional regulators NPR1 and WRKY45 [7,8].

Recently, cytokinins (CKs) were shown in rice to act with the SA pathway to induce rice defenses [9] like in *Arabidopsis*. The CKs are a group of N^6-substituted adenine derivatives that play important roles in cell division [10,11], cell viability [12,13] and sink-source relationships [14,15]. There is only very limited evidence of their implication in plant pathogen interactions, at least for those where tumors are not formed. In *Arabidopsis*, CKs are involved in the resistance against the bacteria *Pseudomonas syringae* or the oomycete *Hyaloperonospora arabidopsidis* and this requires some components of the SA pathway [16,17]. For instance, Choi et al (2010) showed that the CK *Arabidopsis* response-regulator ARR2 interacts with the defense-related transcription factor TGA3 to induce the defense gene *PR1*. In rice, simultaneous and exogenous application of a CK (kinetin) and SA (or BTH) induced a strong induction of defense-related genes [9]. The rice central regulator WRKY45 transcription factor is required for this synergistic effect [9,18]. Most of these conclusions were obtained by applying exogenous CK treatments. Jiang et al (2012) proposed a model where during early infection of rice by the fungus *Magnaporthe oryzae*, the CK produced by the fungus may drain nutrients towards the infection site. Later on, the normal activation of the SA-dependent defense pathway combined to CK accumulation (hypothetically derived from or initiated by the fungus) may act synergistically to activate defense response and block pathogen development. One prediction of this model is that exogenously supplied CKs could reduce susceptibility and this was not observed so far. Another prediction of this model is that increasing CK levels in plants could reduce susceptibility. Here we provide evidence supporting this model by demonstrating that either exogenously or endogenously elevated levels of CKs can reduce susceptibility to the rice blast fungus.

Results

Kinetin treatment 48h before inoculation affects susceptibility to the rice blast fungus

In their initial report, Jiang et al (2013) [9] observed that kinetin sprayed 24h pre-inoculation did not affect rice resistance against *M. oryzae*. By contrast, Argueso et al (2012)[17] observed that the susceptibility of *Arabidopsis* plants sprayed with the cytokinin BA (benzyl adenine) 48h before inoculation was affected. We thus further tested the possibility that the nature of cytokinins and/or the time for cytokinin pre-treatment may matter when applying exogenous cytokinins. The cytokinins kinetin and BAP (benzyl amino purine), two synthetic cytokinins commonly used to mimic respectively the two large classes of cytokinins, zeatin and isopentenyl adenine were exogenously applied before infection with the rice blast fungus *Magnaporthe oryzae* for measuring the induced level of resistance/susceptibility. Irrelevantly of the application time (from 24 to 72h before inoculation), treatment with BAP (50 µM) did not trigger any strong and reproducible change in rice susceptibility to the blast fungus (Suppl. Fig. 1AB). In addition, we could not detect any significant effect of kinetin treatment (50 µM) when applied 24h before infection. This last result is consistent with the findings of Jiang et al (2013) that kinetin does not affect susceptibility when applied in this way. By contrast, plants treated 48 and 72 h before inoculation with kinetin were more resistant to *M. oryzae* (Fig. 1A; Suppl. Fig. 1AB). Indeed the number of sporulating lesions caused by the moderately virulent isolate GY11 was reduced by 30% compared to untreated plants (Fig. 1B). This reduced susceptibility was also observed with the very aggressive isolate FR13 (Delteil et al. 2012) (Suppl. Fig. 2) and was associated with a reduced growth of the pathogen (Fig. 1C, Suppl. Fig.2C). By contrast, no effect was observed on an incompatible interaction (data not shown).

It was previously shown that different doses of cytokinins could trigger very contrasted effects [17]. Using the application time point that gave the most reproducible results (48h before inoculation), we tested the effect of different doses of kinetin (from 10 to 500 µM). All tested concentrations of kinetin above 10 µM tended to reduce rice susceptibility (Suppl. Fig. 3) and 50µM gave a very reproducible and significant reduction of susceptibility.

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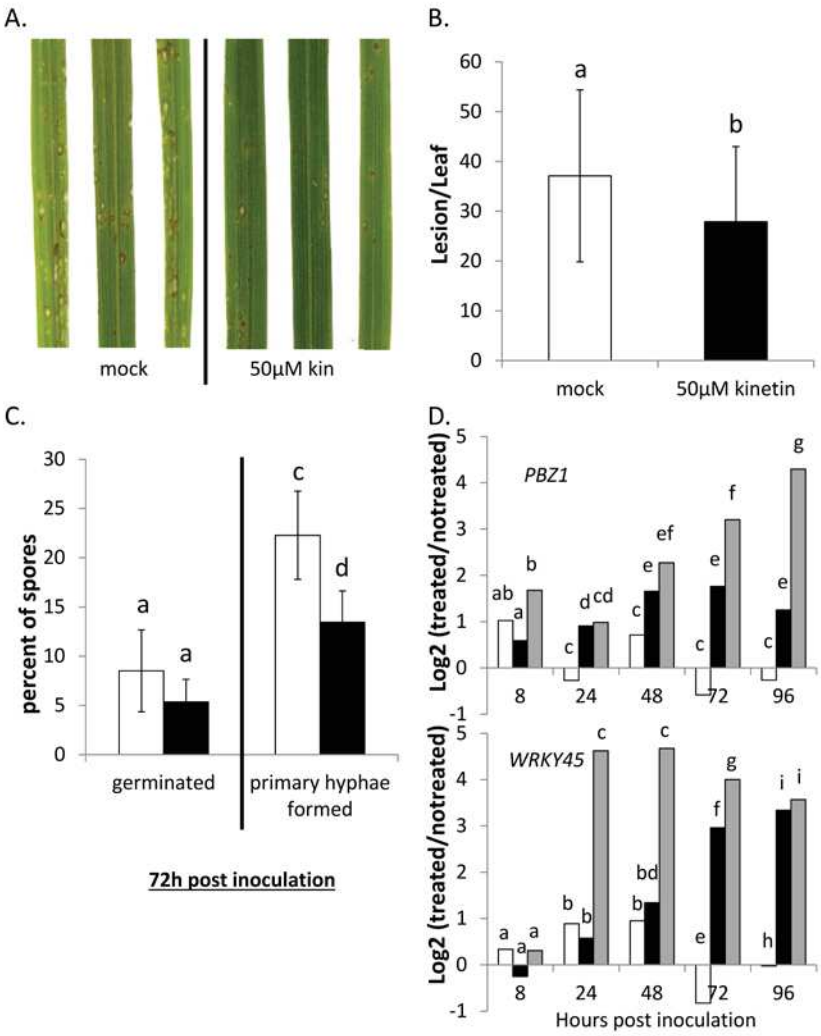
Figure 1.



(A) Symptoms caused on nipponbare plants by the strain GY11 treated with 50µM of kinetin 48h before infection or not (mock). (B) Symptoms quantifications. Statiscal analysis was performed with a t-test, p-value < 0,02. (C) Microscopic observations of spore germination and penetration 72h post inoculation on plants pretreated with 50µM of kinetin (black bars) or not (white bars), t-test, p-value = 0,02. (D) *PBZ1* and *WRKY45* marker genes expression after a kinetin treatement alone (white bars), during infection in untreated plants (black bars) and during infection in kinetin-treated plants (grey bars) (t-test, p-value < 0,04). Plants were inoculated with spore suspension (in gelatin 0.5%) of GY11 or sprayed with gelatin (0,5%). 50µM of kinetin were sprayed 48h before inoculation or control gelatin treatment. Like kinetin was previously diluted in EtOH 50%, EtOH 50% was added at the adjust concentration in the solution sprayed on control plants. The transcriptional regulation of defense marker genes *PBZ1* and *WRKY45* was evaluated by quantitative RT-PCR using the Actin gene for normalization. The values presented are the Log2 ratios compared to control plants which were treated with the mock solution 48h before to be sprayed with gelatin.

Kinetin pre-treatment synergistically induces rice defense

We first tested whether the cytokinin treatments performed induced the transcription of the cytokinin oxidase 2 (CKX2) gene as a marker which was previously reported to be induced by cytokinins [19]. Both kinetin and BAP induced *CKX2* (Suppl. Fig. 1C) but displayed differential efficiencies (see Discussion).

In their study, Jiang et al (2013)[9] reported that rice defense genes were more induced in detached leaves treated with SA *and* kinetin than with kinetin alone or SA alone. This suggested that there could be a synergistic effect of cytokinin and SA accumulation during infection [9]. We thus tested in our experimental system using intact plants whether such a synergistic effect was visible. As expected, the defense-marker genes tested (*PBZ1* [20]; *WRKY45* [8]) where induced upon rice blast infection (Fig. 1D). Similar results were obtained with most defense-markers tested (Suppl. Fig. 4). This induction was strongly enhanced when plants were pre-treated with kinetin, suggesting that in entire plants, CK and infection may act synergistically to induce plant defenses. In order to further strengthen this hypothesis, we tried to manipulate endogenous levels of cytokinins by using rice mutants.

Identification of rice mutants affected for their endogenous cytokinin levels

We had identified in previous transcriptomic analysis a putative cytokinin-UDP glycosyl transferase (*CK-UGT;* LOC_Os02g51930) that was transcriptionally regulated upon rice blast infection [21]. Two insertion lines (Fig. 2A) available in the Dongjin background where genotyped and homozygous mutants as well as null-segregant lines were produced. There was no visible phenotype associated with the mutations under normal growth conditions.

The levels of free and glycosylated CKs were measured on healthy plants at the stage where inoculations are usually performed. In the *ck-ugt1.1* mutant, we could measure a decrease of the cisZOG glycosylated form (Fig. 2B) whereas other glycosylated forms like tZ9G, DZOG and cisZROG did not seem affected. Consistent with a reduction of cisZOG, we measured that free zeatins were slightly increased in the mutant (Suppl. Fig 5). These elements suggested that the identified mutant is affected in zeatin-O-glucosylation.

The *ck-ugt* mutants display enhanced resistance to rice blast fungus

The exogenously applied cytokinin kinetin reduced susceptibility to the fungal pathogen *M. oryzae* (Fig.1). We used the *ck-ugt* mutants in order to test the effect of modified endogenous levels of cytokinins on blast susceptibility. When inoculated with the virulent GY11 isolate, the *ck-ugt1.1* mutants showed a significant reduction of disease symptoms (Fig. 3A) and a two-fold reduction of the number of susceptible lesions (Fig. 3B). This reduced susceptibility correlated with a reduction of pathogen growth inside the tissues (Fig. 3C). Similar results were found with the *ck-ugt1.2* mutant allele (Suppl. Fig. 6). We next tested if defense induction was modified in the *ck-ugt1.1* mutant. In the absence of the fungus, the transcription level of the tested genes was higher in *ck-ugt1.1* mutant compared to null-segregants (see not infected in Fig. 3D). This higher expression of defense markers was still visible two days after infection. This suggests that enhanced resistance in the *ck-ugt1.1* mutant is due to enhanced expression of defense before and after infection.

Discussion

Exogenous application of cytokinins has been used in several plant-pathogen interactions to evaluate the effect of this class of hormones in resistance [1,9,17,22–24]. In rice, first evidence by Jiang et al (2013) suggested that kinetin did not affect rice blast resistance. However these authors only tested the effect of a pre-treatment of kinetin one day before infection. Here we show that when treated with kinetin two or three days before inoculation, rice plants become less susceptible to the rice blast fungus. In addition, complete resistance was not affected (data not shown). Our finding is therefore similar to those obtained by Argueso et *al* (2012) [17] who showed that a pre-treatment of *Arabidopsis* plants with 100 µM BA two days before inoculation by *Hyaloperonospora arabidopsidis* reduced susceptibility. However, unlike in *Arabidopsis* where low doses of BA induced susceptibility and high doses induced resistance, we did not observe any induction of susceptibility by kinetin in the conditions tested. Even if it is not always statistically significant, kinetin also tended to affect resistance in a dose dependent manner (Suppl. Fig. 3).

Figure 2.

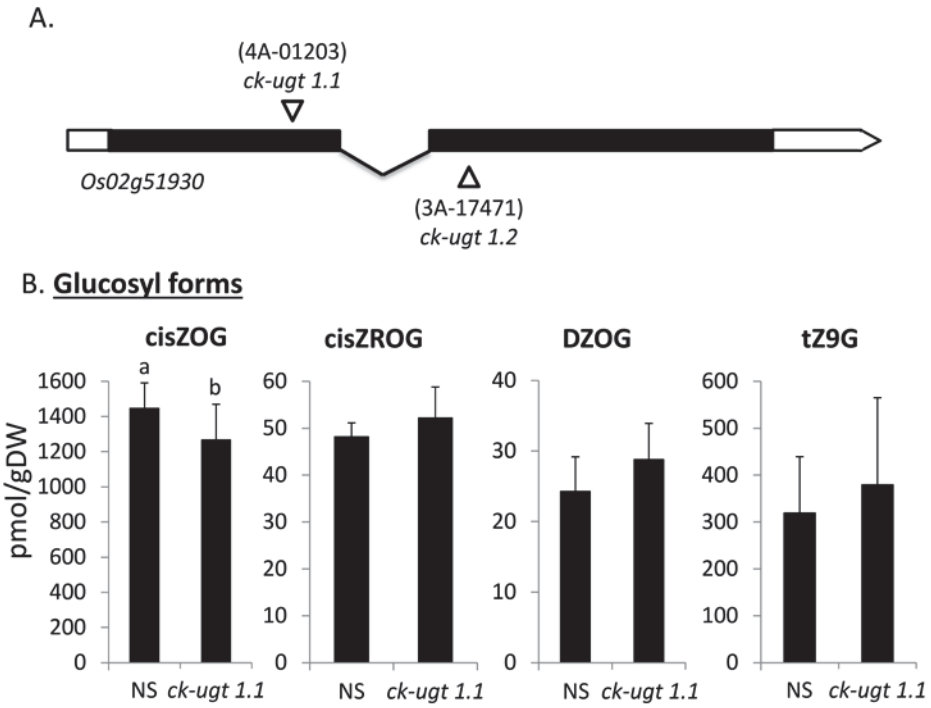


Figure 2. Knowk-out mutants *ck-ugt1.1* show reduced cisZOG content.

(A) Position of the T-DNA insertions leading to the disruption of the CK-UGT gene *LOC_Os02g51930*, (B) CK glucosyl forms contents in NS (Null Segregant) plants and *ck-ugt1.1* mutants (t-test; p-value<0,04).

cisZOG: *cis*-zeatin-O-glucosylated; tZ9G: *trans*-zeatin-N9-glucosylated; DZOG : dihydrozeatin-O-glucosylated; cisZROG : *cis*-zeatin riboside-O-glucosylated.

Plants were sampled at the same stage as they were normaly inoculated in other experiments.

Moreover, our results were obtained with kinetin whereas BAP, a molecule close to BA which was used in *Arabidopsis*, only slightly increased or did not affect susceptibility in rice. It is interesting to note that the *Cytokinin Oxidase 2* (*CKX2*) gene was strongly induced by BAP but less by kinetin (Suppl. Fig. 1C). It is therefore possible that the small effect of BAP in rice is due to the activation of CK catabolism through the CKX2 degradation enzyme. Thus the effects of exogenous treatment of CKs strongly vary with the timing, the type of molecule and the type of plant-pathogen interaction.

Previous studies suggested that CK and SA act synergistically during rice blast infection to induce defense [9,18]. However this hypothesis relies on exogenous treatment with CKs on detached leaves that could introduce many artefacts. Here the analysis of rice mutants potentially affected for CK inhibition allowed the evaluation of the role of endogenous CKs in resistance. Even if CK content is only slightly affected in these mutants, it seemed to be enough to affect resistance. That could also explain why these mutants did not show any abnormal developmental phenotypes in contrast with the over-expressor *CKX2* lines previously used for investigating the role of endogenous CKs in rice blast resistance [9]. Two independent insertion mutants in the *CK-UGT* gene were less susceptible to *M. oryzae*, consistent with the reduced susceptibility observed in this work with an exogenous application of kinetin. The reduced susceptibility of the *ck-ugt1.1* mutant could be correlated to enhanced induction of defense before and during infection. Thus, whereas exogenously applied or endogenously produced, CKs can activate rice defense.

Our results and previous reports [9,18] that CKs reduce susceptibility are leading to a paradox for the role of cytokinins in the interaction between rice and *M. oryzae*. Indeed, Jiang et al (2013) [9] reported that the blast fungus produces and secretes cytokinins (both zeatin and IP forms) and suggested that CKs may facilitate blast infection by increasing the sink strength in infected tissues. Recently we demonstrated that the fungal-derived CKs were required for full virulence of the blast fungus, further supporting the later hypothesis (Chanclud et al. 2015). Several non-exclusive explanations could explain this paradox. First our data suggest that, although endogenous zeatin forms increase resistance to *M. oryzae*, exogenously applied BAP (an isopentenyl form of cytokinin) could increase plant susceptibility in *Arabidopsis*.

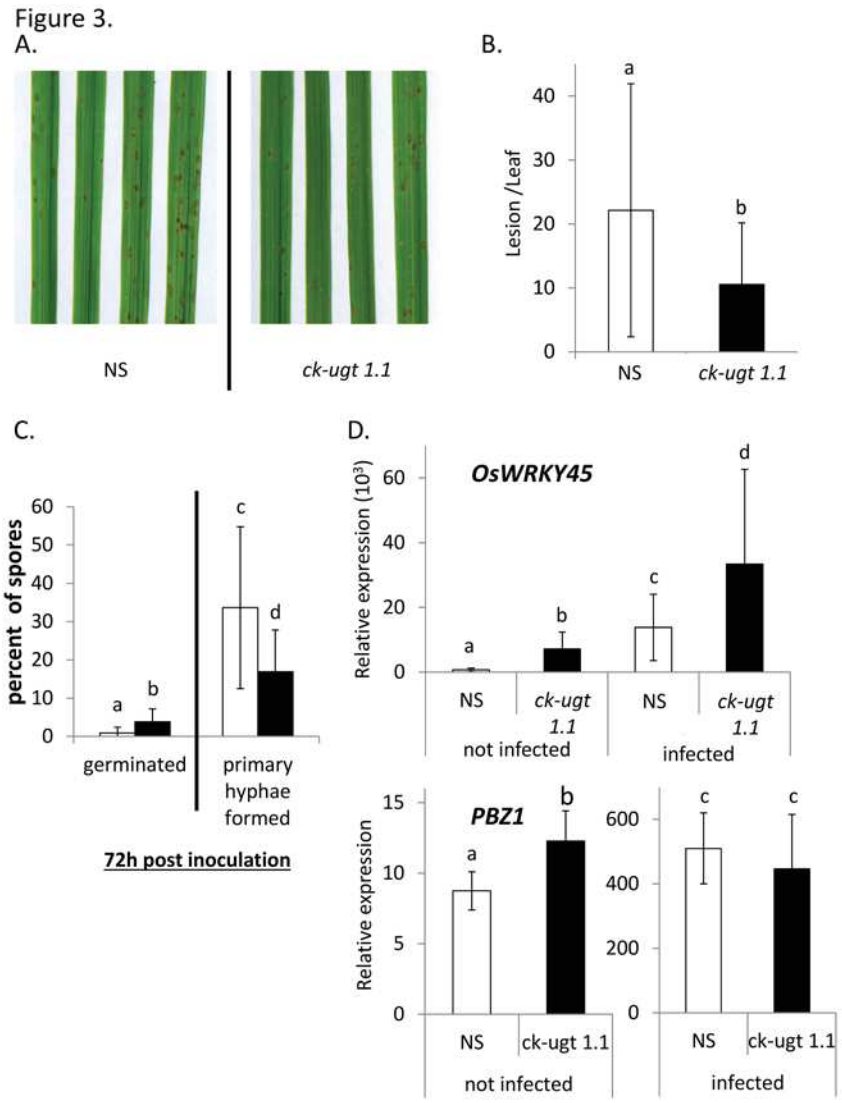


Figure 3. *ck-ugt1.1* plants are more resistant to *M. oryzae*.

(A) Symptoms caused by the virulent strain GY11; (B) Symptoms quantifications (t-test, p-value :); (C) Microscopic observations of different fungal stages of infection (t-test, p-value < 0,03) on NS plants (white bars) and *ck-ugt1.1* mutants (black bars); (D) Relative expression of *OsWRKY45* out infection (no inoc) and 48h post inoculation (inoc), in NS (Null Segregant) and *ck-ugt1.1* plants (t-test, p-value < 0,03) The transcriptional regulation of defense marker genes *WRKY45* and *PBZ1* was evaluated by quantitative RT-PCR using the Actin gene for normalization. The values presented are the Log2 ratios compared to control plants which were sprayed with a solution with 0,5% of gelatin.

Thus the outcome of the interaction may vary depending on the dominant form of CKs secreted *in planta* by the fungus. This hypothesis is however not consistent with the finding that zeatin forms are the major ones secreted by some *M. oryzae* strains (Jiang et al. 2012; Chanclud et al. 2015). Second, as indicated by our time course experiments with kinetin pre-treatments, the time when CKs are applied is critical for the outcome of the interaction. This suggests that the well-timed production of CKs by the fungus may have beneficial effects on susceptibility. For instance, the production of CKs by the fungus before the SA pathway is engaged by the plant may block further induction of defense and abolish the synergistic effects of SA and CKs on defense.

Our results further support the idea that in rice and *Arabidopsis*, CKs are connected to other defense pathways in response to a wide range of pathogens [25,26] and potentially contribute to the balance between defense and other physiological processes like abiotic stress [27]. Since affecting CKs homeostasis also leads in rice to increase grain production [28], these hormones present many features of key regulators useful for crop improvement.

Materials and Methods

Fungal, plant growth and disease assessment

Fungal isolates were grown on rice flour agar for spore production [29]. For the determination of interaction phenotypes and cytology analysis, a suspension of fungal conidiospores ($5x10^4$ sp/mL) was spray-inoculated on the leaves of 3-week-old plants. For gene expression analysis, an inoculum of $2x10^5$ sp/mL was used. Nipponbare and Dongjin japonica rice plants were grown during three weeks as described previously in [30]. In each experiment, disease was evaluated on at least 6 leaves from 3 to 4 different biological repetitions and all experiments were repeated at least 3 times.

RNA extraction and quantitative RT–PCR analysis

RNA extraction was performed as mentioned in Delteil et al., 2012 [63]. Quantitative PCR was performed using LC 480 SYBR Green I Master Mix (Roche, Basel, Switzerland) and a LightCycler 480 instrument (Roche). Amplification was performed as follows: 95°C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 20sec and 72 °C for 30 s; then 95°C for 5 min and 40°C for 30 s. Primers used are indicated table S1.

Cytokinin treatment and measurements

Glycosylated Cytokinins in Fig. 2 and in Suppl. Fig. 5 were measured as in Chanclud et al (2015) [31]. Kinetin and BAP were dissolved in ethanol 50% and appropriate concentrations of ethanol 50% were used in control (Mock) treatments.

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Supporting information

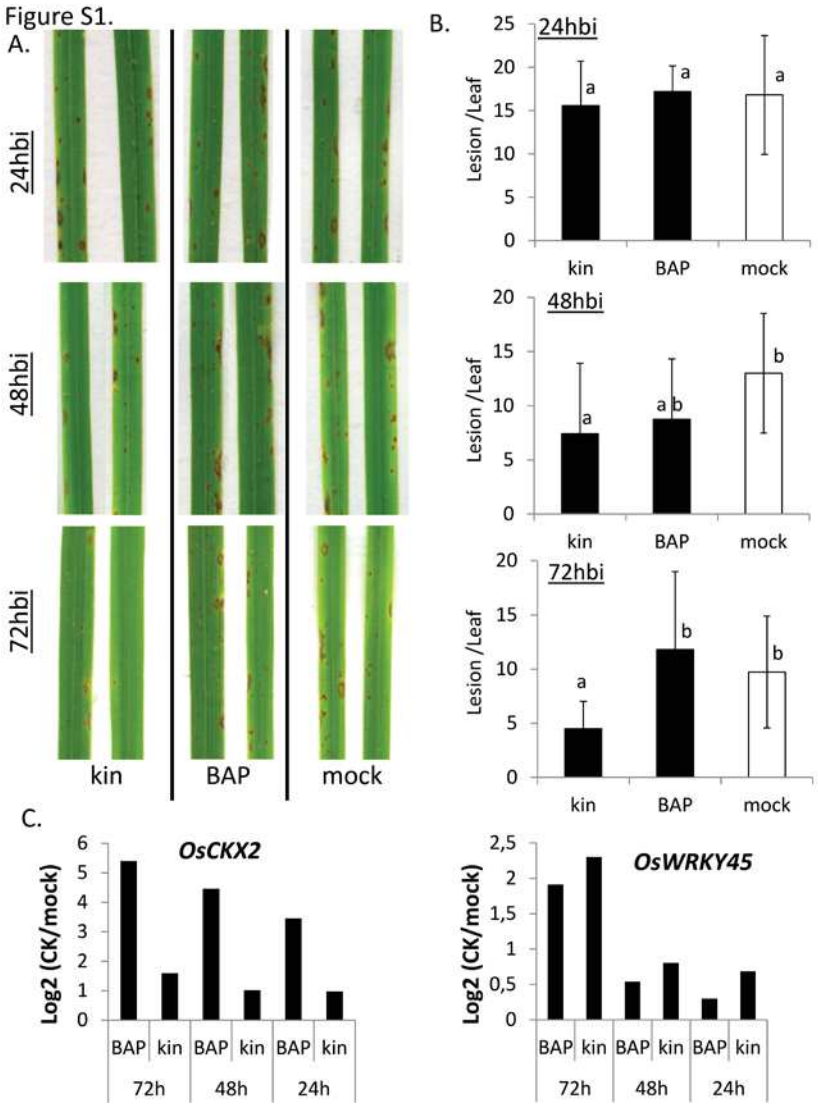

Figure S1. Kinetin and BAP differently affect rice resistance and gene expression.

(A) Symptoms caused on nipponbare plants by the strain GY11 treated with 50µM of kinetin or BAP 72h, 48h or 24h before infection or not (mock). (B) Symptoms quantifications. Statiscal analysis was performed with a t-test, p-value<0,02. (C) Gene expression 48h post inoculation in plants pre-treated with 50µM kinetin or BAP before infection (time indicated) (t-test, p-value < 0,04). The transcriptional regulation of the CK responsive gene *OsCKX2* and the defense regulator *WRKY45* was evaluated by quantitative RT-PCR using the Actin gene for normalization. The values presented are the Log2 ratios compared to control plants which were treated with the mock solution 72h, 48h or 24h before inoculation.

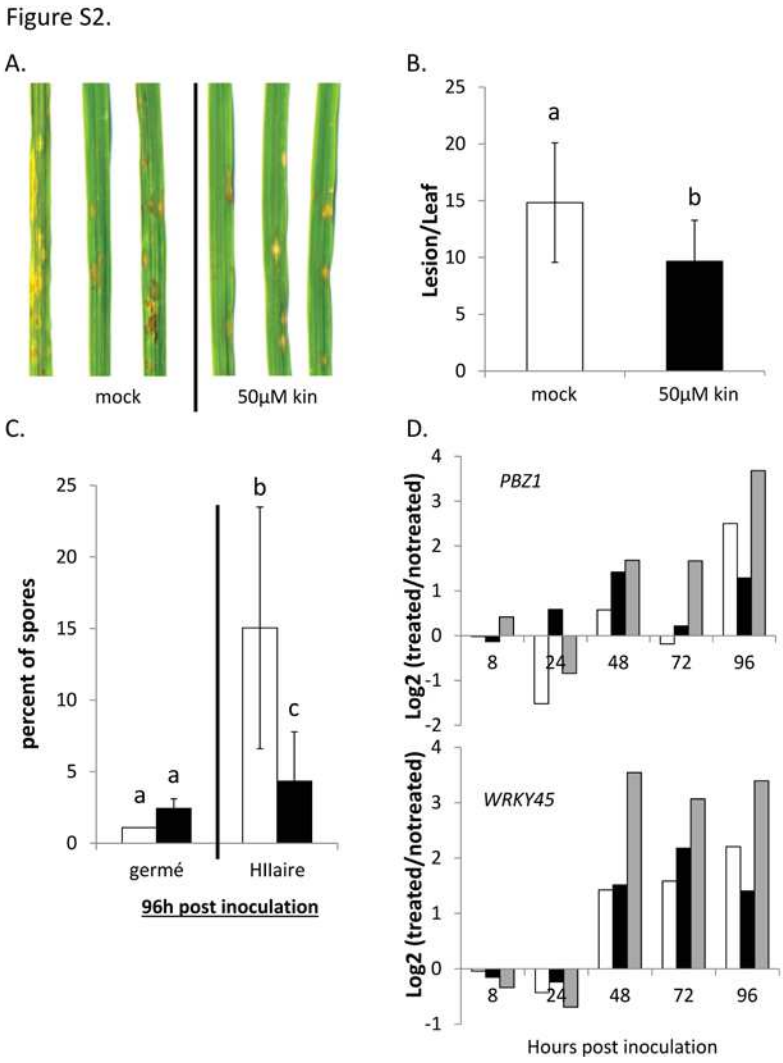

Figure S2. Kinetin reduced susceptibility to the virulent isolate FR13

(A) Symptoms caused on nipponbare plants by the virulent strain FR13 treated with 50µM kinetin 48h before infection or not (mock). (B) Symptoms quantification. Statistical analysis was performed with a t-test (p-value<0,03). (C) Microscopic observations of spore germination and penetration 96h post inoculation on plants pretreated with 50µM of kinetin (black bars) or not (white bars) (t-test, p-value<0,002). (D) Defense marker gene expression after kinetin treatment alone (white bars), during infection in untreated plants (black bars) and during infection in kinetin-treated plants (grey bars). All ratio were made compared to untreated (Mock) plants. The transcriptional regulation of defense marker genes *PBZ1* and *WRKY45* was evaluated by quantitative RT-PCR using the Actin gene for normalization. The values presented are the Log2 ratios compared to control plants which were treated with the mock solution 48h before to be spayed with gelatin.

Figure S3.

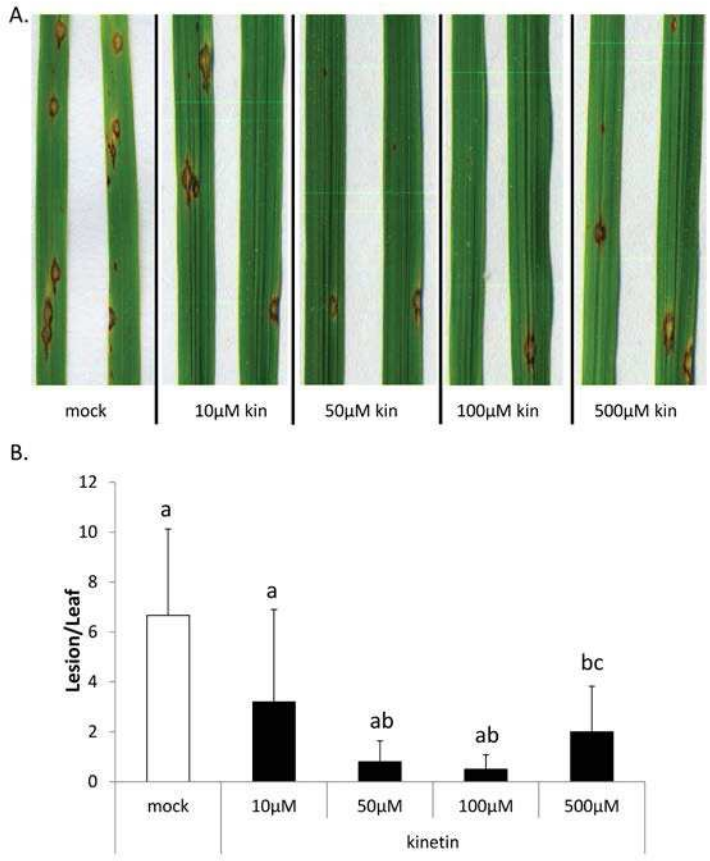

Figure S3. Kinetin reduces susceptibility at all concentrations tested

(A) Kinetin was sprayed at the indicated different concentraitons 48 h before inoculation with the virulent isolate GY11 of *M. oryzae*. Symptoms are shown (**A**) and the number of susceptible lesions per leaf was counted (**B**). (B) Symptoms quantifications. Statiscal analysis was performed with a t-test, p-value = 0.03. Kinetin was diluted in EtOH% so EtOH concentration was adjusted in each solution.

Figure S4.

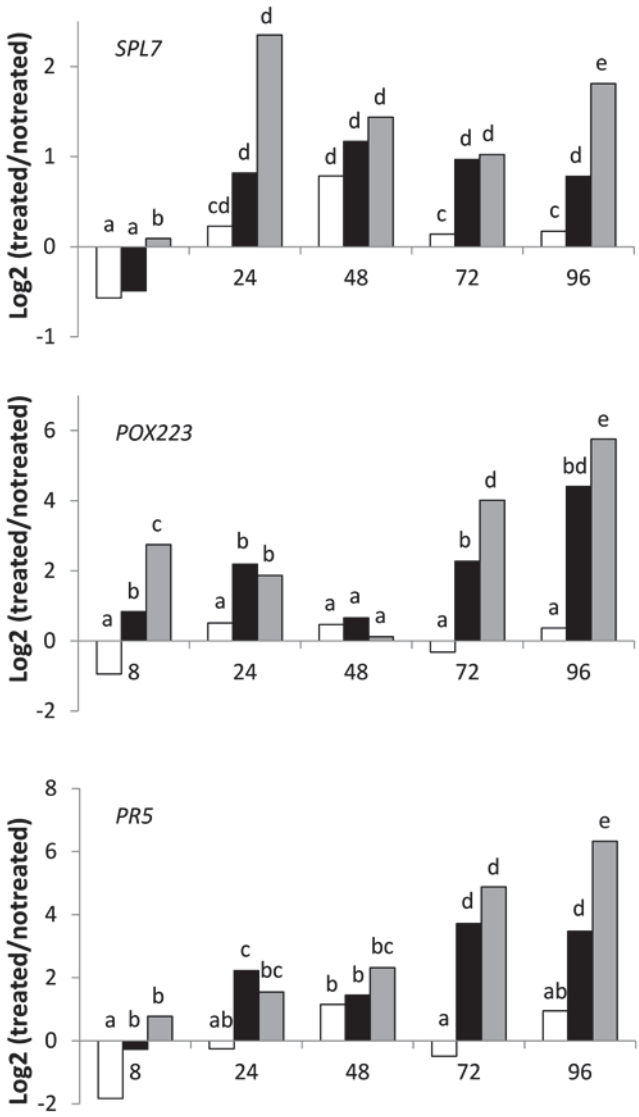

Figure S4. Defense marker genes expression after kinetin treatement

Defense marker genes expression after a kinetin treatement alone (white bars), during infection (hours post inoculation are indicated) in untreated plants (black bars) and during infection in kinetin-treated plants (grey bars) (t-test, p-value < 0,04). Plants were inoculated with spore suspension (in gelatin 0.5%) of GY11 or sprayed with gelatin (0,5%). 50µM of kinetin were sprayed 48h before inoculation or control gelatin treatment. Like kinetin was previously diluted in EtOH 50%, EtOH 50% was added at the adjust concentration in the solution sprayed on control plants. The transcriptional regulation of defense marker genes *Spl7*, *POX223* and *PR5* was evaluated by quantitative RT-PCR using the Actin gene for normalization. The values presented are the Log2 ratios (infected/not infected) of the means calculated from four independent replicates.

Figure S5.

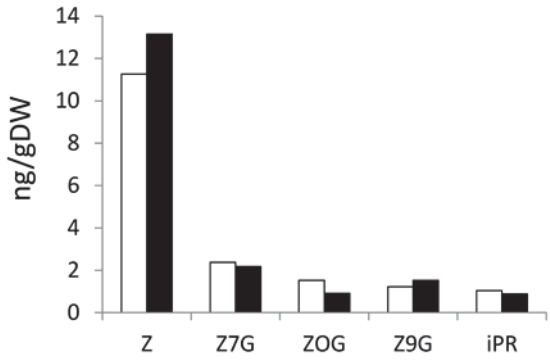

Figure S5. CK contents is differently altered in KO-*ugt* mutants.

CK contents in WT plants (white bars) and in KO-ugt mutants (black bars)

Z : zeatin; Z7G: zeatin-N7-glucosylated; ZOG: zeatin-O-glucosylated; Z9G: zeatin-N9-glucosylated; iPR : isopentenyladenosine

See Figure 2 and Materials and Methods

Figure S6.

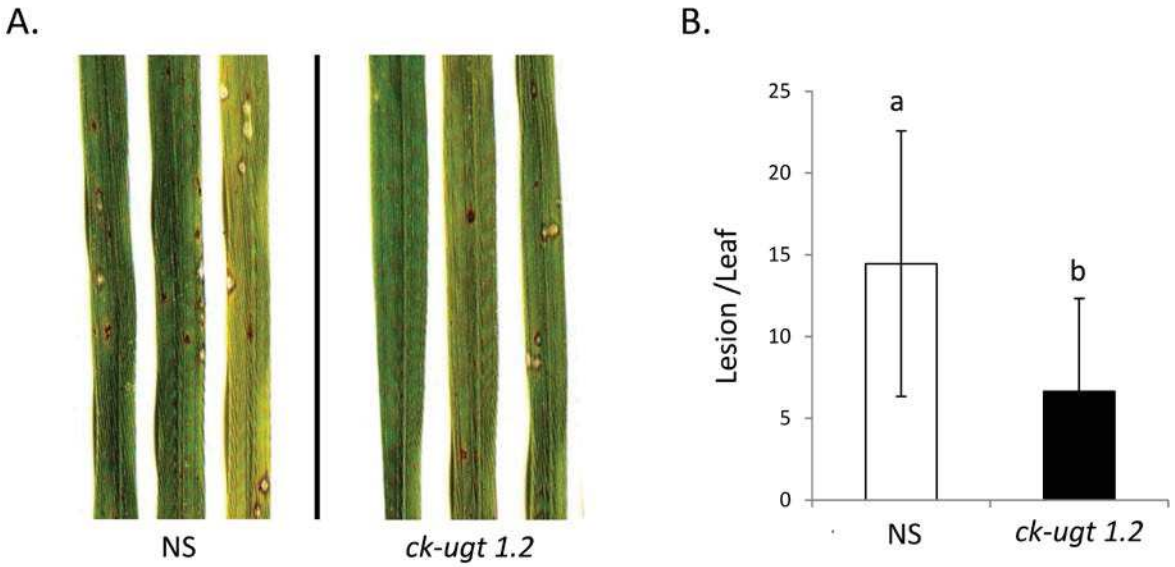

Figure S6. *ck-ugt1.2* plants are less susceptible to *M. oryzae*

(A) Symptom caused on *ck-ugt* mutants and control plants (NS) the virulent GY11 strain. (B) Symptom quantification *ck-ugt* (black bars) and Null Segregant plants (white bars) were inoculated with spore suspension (in gelatin 0.5%) of GY11.

Figure S7.

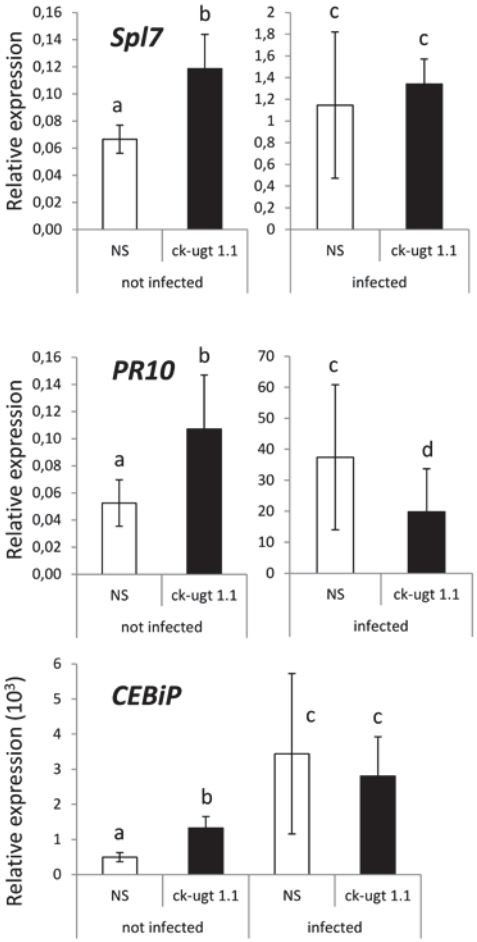

Figure S7. Relative expression of defense marker genes in *ck-ugt* mutants

The transcriptional regulation of defense marker genes *Spl7*, *PR10* and *CEBiP* was evaluated by quantitative RT-PCR using the Actin gene for normalization. *ck-ugt* (black bars) and Null Segregant plants (white bars) were inoculated with spore suspension (in gelatin 0.5%) of GY11 (infected) or not (not infected). The values presented are the Log2 ratios (infected/not infected) of the means calculated from four independent replicates. Uninfected plants were sprayed with gelatin 0.5% but without spore suspension.

Table S1.

Accession	Annotation	Sequence
Os12g36880	*PBZ1*	F-AGGCATCAGTGGTCAGTAGAG
		R-CGGGTCTTGTATGTGCTTCC
Os05g25770	*OsWRKY45-1*	F-ACGACGAGGTTGTCTTCGATCTG
		R-GCCCGTGTCCATCCATGATTCTTC
Os07g48020	*POX223*	F-CTCAGCTGCTCCAAGGTGAA
		R-TGTGGCCCGTTTATGTCGTC
Os12g36850	*PR10*	F-CAGATGATCGAGGCGTACCT
		R-CCACGCCACAGTAACATGAC
Os12g43430	*PR5*	F-AGCCAGGACTTCTACGACCT
		R-GCGTGTGTCTTGGTGTTGTC
Os05g45410	*SPL7*	F-CGGATTAGAGGCTTGCGTGTTAC
		R-GCACAGTAGTCAGCGGATAGAAC
Os12g36880	*PBZ1*	F-AGGCATCAGTGGTCAGTAGAG
		R-CGGGTCTTGTATGTGCTTCC
Os03g04110	*CEBiP*	F-CACTTGTACGGCTGCTTGAA
		R-GGAAGGTGGGAAGTCCATTC

Table S1. Primers of rice defense marker genes used for qRT-PCR

<u>CHAPITRE III</u> : Implication des cytokinines fongiques dans le pouvoir pathogène et la physiologie de *Magnaporthe oryzae.*

Afin de d'étudier l'implication des CKs fongiques dans le développement de la pyriculariose, des souches de *M. oryzae* mutantes affectées dans leur production en CKs ont été générées.

Comme mentionné en introduction (I.4.3.2. Métabolismes des CKs), chez les plantes la biosynthèse de CKs peut être assurée par des isopentenyl transferases (IPT) ou des tRNA-IPT. Une unique tRNA-IPT a été identifiée chez *M. oryzae* en se basant sur les homologies des séquences protéiques. Bien que chez *Arabidopsis* la voie de biosynthèse faisant intervenir une tRNA-IPT semble être secondaire, c'est cependant la seule que nous ayons identifiée chez *M. oryzae.* Des mutants d'insertion ont été générés par recombinaison homologue ainsi que des souches complémentées dans lesquelles la séquence génomique sous le contrôle du promoteur endogène a été réintroduite. La production de CKs en condition *in vitro* a été mesurée chez les différentes souches. Les mutants d'insertion ne produisent plus de CKs alors que la production des souches complémentées est similaire à celle de la souche sauvage. Compte-tenu de ce phénotype, le gène identifié chez *M. oryzae* a été nommé *CK-synthesis 1 (CKS1).*
La virulence des mutants *cks1* et des souches complémentées *cks1*^{CKS1} a été testée sur le cultivar Nipponbare. La souche *cks1* présente un retard de pénétration et une capacité d'invasion réduite par rapport aux souches témoins. Ce manque de virulence peut toutefois être restauré par un apport exogène de CKs comme la kinétine et la trans-zéatine.

Des expériences complémentaires ont permis de mettre en évidence que certaines réponses de défense étaient plus fortes chez les plantes inoculées avec la souche *cks1* comparé aux plantes infectées par les souches témoins. La virulence de *cks1* est également restaurée sur des plantes dont l'immunité est affectée ainsi que par l'ajout d'un inhibiteur de la production de ROS sur des plantes sauvages. De plus, le contenu en sucres et en certains acides aminés au site de pénétration ainsi que dans les zones non infectées alentour sont différentiellement affectés au cours de l'infection par la souche *cks1*. Des expériences *in vitro* ont également permis de mettre en évidence un rôle potentiellement endogène des CKs fongiques dans la résistance de *Magnaporthe* au stress oxydatif. Ces résultats suggèrent que les CKs fongiques seraient donc requises pour participer à l'inhibition des défenses, pour affecter la répartition des métabolites azotés et carbonés au site d'infection et dans les tissus non infectés proches et également participerait à la protection du champignon contre le stress oxydatif.

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Cytokinin production by the rice blast fungus is a pivotal requirement for full virulence

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Short title: Fungal cytokinins are required for *M. oryzae* virulence

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Key words: Cytokinin, *Magnaporthe oryzae*, rice, effector

Abstract

Plants produce cytokinin (CK) hormones for controlling key developmental processes like source/sink distribution, cell division or programmed cell-death. Some plant pathogens have been shown to produce CKs but the function of this mimicry production by non-tumor inducing pathogens, has yet to be established. Here we identify a gene required for CK biosynthesis, *CKS1*, in the rice blast fungus *Magnaporthe oryzae*. The fungal-secreted CKs are likely perceived by the plant during infection since the transcriptional regulation of rice CK-responsive genes is altered in plants infected by the mutants in which *CKS1* gene was deleted. Although *cks1* mutants showed normal *in vitro* growth and development, they were severely affected for *in planta* growth and virulence. Moreover, we showed that the *cks1* mutant triggered enhanced induction of plant defenses as manifested by an elevated oxidative burst and expression of defense-related markers. In addition, the contents of sugars and key amino acids for fungal growth were altered in and around the infection site by the *cks1* mutant in a different manner than by the control strain. These results suggest that fungal-derived CKs are key effectors required for dampening host defenses and affecting sugar and amino acid distribution in and around the infection site.

Author Summary

The role of plant-like hormonal compounds produced by fungal pathogens during infection has not been elucidated. Here we identified a conserved gene in most fungi, required for cytokinin production by the rice blast fungus and for its full virulence. Fungal-derived cytokinins are likely potent inhibitors of plant immunity. They are also needed to maintain elevated sugar contents at the site of infection and to drain or consume essential amino acids at, and around, the infection site. Thus, cytokinins represent the second example, after the bacterially-produced coronatine, of plant hormones hijacked by pathogens to successfully invade plant tissues. These findings also suggest that this invasion strategy could be widely conserved among fungi.

Introduction

Plant pathogens have evolved sophisticated strategies to manipulate host biological processes during infection [1,2]. (Hemi)biotrophic pathogens produce and secrete effector proteins and metabolites to hijack cellular metabolism of the infected tissues to their own benefit. For instance, the bacterial pathogen *Xanthomonas oryzae pv. oryzae* produces Transcription Activator Like effectors that specifically induce the expression of genes coding for sugar transporters and thus enhance bacterial nutrition [3]. The virulence factors can also participate to the inhibition of plant defenses that lead to cell death, thus contributing to maintaining infected cells alive [4]. Plant-pathogen interactions shaped the pathogen virulence arsenal and the host immune response system. A first layer of plant defenses is induced by the perception of pathogen- or microbe-associated molecular patterns, like flagellin from bacteria or chitin from fungi [5]. These basal defense responses consist of an early accumulation of reactive oxygen species (ROS), a thickening of the cell wall, and production of metabolites/enzymes with antimicrobial activities. To limit these defenses triggered by chitin perception by the plant's chitin receptor, fungal pathogens like *Magnaporthe oryzae* secrete chitin-binding effectors that enable escape from the host recognition system [6]. Pathogens also interfere with other steps of plant immunity like signaling cascades following recognition and transcriptional regulators of host defenses [2,7].

Plant pathogens manipulate components of hormonal pathways, whether the corresponding hormones are involved in disease resistance (i.e. salicylic acid, jasmonic acid and ethylene [8]) or in the control of plant developmental processes (i.e. auxin, cytokinins and gibberellic acid [8–11]). Pathogens can affect hormonal homeostasis by targeting/secreting enzymes involved in hormone metabolism or by producing hormonal/mimicking compounds and thereby inhibit defenses, modify nutrient flows and/or induce symptom development. For instance, the fungal pathogens *Ustilago maydis* and *Magnaporthe oryzae* secrete respectively a chorismate mutase and a monooxygenase affecting salicylic acid or jasmonic acid homeostasis during infection and then contributing to their virulence [12,13]. Plant pathogens can also directly produce hormones or compounds with similar

biological activities. The bacterial pathogen *Pseudomonas syringae* produces coronatine to mimic jasmonic acid, which actively opens stomata and counteracts salicylic acid accumulation [14,15]. As a consequence, these combined effects on several processes required for infection facilitate host invasion. In the case of pathogenic fungi, there is, however, no direct evidence that hormonal compounds produced are required for virulence.

Fungi and bacteria produce hormonal compounds that are chemically identical or very close to plant hormones involved in plant developmental processes such as cytokinins (CKs) [16–22]. CKs are adenine derivatives that differ in their side chains (aromatic or isoprenoid). In plants, isoprenoid CKs are mainly synthesized through a *de novo* biosynthesis pathway from adenosine phosphate. In this pathway, Isopentenyl transferase (IPT) enzymes perform the first step of biosynthesis. However, another minor CK biosynthesis pathway involving tRNA modification is also described in plants and yeast. This minor pathway requires tRNA-Isopentenyl Transferases (tRNA-IPT), enzymes that perform tRNA modification leading to CK production after tRNA degradation [23–25]. In plants, CKs were originally studied for their effects on cell division/differentiation [26]. CKs also modulate nutritional source/sink distribution and programmed cell-death processes like xylem differentiation, senescence and hypersensitive response [27–30]. Tumor-forming pathogens are striking examples of microbes which are able to produce CKs, like the fungi *U. maydis* [31] and *Claviceps purpurea* [32] or the protist *Plasmodiophora brassicae* [33]. Morrison et al., (2015a) [31] have recently shown that CK accumulation in *U. maydis* infected tissues is correlated to the virulence of this pathogen. However, in this latter case the evidence that these hormones are produced by the pathogen is still unclear. Likewise, the pathogenic bacterium *Rhodococcus fascians* secretes CKs to interfere with host CK signaling pathways by affecting the transcriptional regulation of key cell cycle genes leading to tumor development [34]. In addition to having potential roles in the virulence of tumor-inducing pathogens, CKs have long been suspected to participate to virulence of pathogens that do not trigger tumors or organ deformations. However, it has not been demonstrated that the CKs produced by this type of pathogens are key virulence factors.

Because CKs delay plant senescence by limiting oxidative burst and maintaining photosynthesis activity [35], they are at the cross-road of several pathways of interest for manipulation by pathogens that need to keep host cells alive in order to drain nutrients for their own growth. Consistent with this role, CKs are accumulated in “green islands”, which are tissues corresponding to photosynthetically active zones maintained around pathogenic lesions caused by (hemi)biotrophic pathogens [36–39]. CK compounds have been measured in several different fungal species (even in non-plant interacting ones [40]) and CK secretion was mostly observed in the case of (hemi)biotrophic microbes [41,42]. For instance, Hu & Rijkenberg (1998) immuno-detected CKs in and around fungal hyphae of *Puccinia recondita* f.sp. *tritici* during wheat infection [43].

The hemibiotrophic fungus *Magnaporthe oryzae* responsible for the rice blast disease, also produces CKs *in vitro* [44] but the CK biosynthesis pathway in this fungus has not been established. Moreover, the role of CKs in the virulence of filamentous fungi that, like *Magnaporthe,* do not form tumors has never been demonstrated. In this study we identified a gene coding for a putative tRNA-Isopentenyl Transferase (tRNA-IPT) in the genome of *M. oryzae* and in all Ascomycete genomes tested. Mutants deleted for this gene, named *Cytokinin Synthesis 1* (*CKS1*), were generated and were found to be impaired in CK production confirming that the tRNA degradation pathway is involved in fungal CK production in *Magnaporthe* as was suggested for other fungi by Morrison et al., (2015b) [40]. Deleted *cks1* mutants were not affected in their *in vitro* growth or asexual development in standard minimal conditions however they showed severely reduced virulence on rice. Remarkably, the CK-deficient mutant was not able to maintain the levels of several key nutrients at the infection site and induced early and strong plant defenses, suggesting that fungal CKs may contribute to metabolite mobilization and to rice defense inhibition. Our work confirms the view that, in *M. oryzae*, and possibly in many other fungi, this putative tRNA-IPT, contributes to CK production. Since *CKS1* is required for the full virulence of *Magnaporthe*, we propose that CKs from fungal pathogens could act as effectors combining functions in defense inhibition and nutrient mobilization.

Results

In silico analysis of *M. oryzae* genome revealed putative orthologous genes of yeast and plant cytokinin pathways

To investigate the role of CKs in the virulence of the blast fungus *M. oryzae*, homologs of plant and yeast genes involved in CK metabolism or signaling were searched in the *M. oryzae* genome using BLASTp. Potential orthologous genes of some important CK biosynthesis, degradation and signaling component coding genes were identified (S1 Table), further extending previous reports that the CK signaling and metabolic pathways are present in *M. oryzae*. As previously mentioned, independently of the *de novo* biosynthesis pathway, CKs can be also produced through tRNA degradation. In this second pathway the first step of CK production involves tRNA-Isopentenyl transferases (tRNA-IPT), as previously described in *A. thaliana* (AtIPT2 and AtIPT9) and *Saccharomyces cerevisiae* (MOD5) [23–25,45,46]. A putative tRNA-IPT protein, coded by MGG_04857, was identified in *M. oryzae* which shared 36% identity with AtIPT2 or MOD5 and the sequences of the different known interaction sites were highly conserved (S1A Fig.). No other gene containing the coding sequence of the IPT domain was identified, suggesting that there is only one orthologous gene in *M. oryzae.* We modeled the three-dimensional structure of MGG_04857 (S1B Fig.) by protein threading, a method based on fold recognition [47] and using the experimentally determined MOD5 structure as a template. The model produced suggested a conserved structure, but also confirmed a high conservation of primary sequences of interaction sites i.e. the ATP, DMAPP as well as tRNA binding sites (S1B Fig. [48,49]) and indicated that MGG_04857 shows all the features of a *bona fide* tRNA-IPT. Taken together, these results support the hypothesis that the blast fungus can produce CKs [44], potentially perceive them, and suggest that MGG_04857 could act as a key enzyme in fungal CK production.

Production of *MGG_04857 (CKS1- Cytokinin Synthesis 1)* mutants and their growth *in vitro*

To test the involvement of the putative tRNA-IPT coded by the *MGG_04857* gene in fungal CK production, knock-out mutants of this gene, later called *cks1* (see below), were generated by homologous recombination in the *M. oryzae* GY11 genetic background (S2 Fig.). In addition, a complemented strain (*cks1*^{CKS1}) was built by transformation of the *cks1* mutant strain with a construct carrying the genomic sequence of *MGG_04857* under control of its own promoter. Gene disruption and complementation were confirmed by PCR on genomic DNA and qRT-PCR and showed that the expression of *MGG_04857* was similar between wild type and *cks1*^{CKS1} isolates, but not detected in the *cks1* mutants (S2E Fig.).

Since yeast MOD5 mutants are known to display altered phenotypes [50], we measured the growth of *cks1* mutants under normal and environmental stress. The *in vitro* growth of the *cks1* mutant was not different from the wild type or *cks1*^{CKS1} strains under standard minimal growth conditions (S3A Fig.). Moreover, the development of infection structures like the appressorium was not impaired by the *cks1* mutation on glass slides (S3B Fig.) or on rice leaf surface (S3C Fig.). This suggests that the early steps of fungal growth are not significantly modified by the *cks1* mutation. By contrast, the *cks1* mutant showed slight but significant and reproducible reduced growth when grown under 1mM H_2O_2 (S4A Fig.). This phenotype could be complemented by the addition of the CK kinetin to the medium (S4B Fig.), suggesting that this defect could be related to a reduced CK production due to the *MGG_04857* deletion.

MGG_04857 (CKS1- Cytokinin Synthesis 1)* is required for CK biosynthesis in *M. oryzae

To investigate the role of MGG_04857 in CK production, CK levels were determined by liquid chromatography-positive electrospray ionization tandem mass spectrometry (LC(ESI+)-MS/MS) [19,51] both in the culture supernatant and mycelia of the different strains (Table 1).

	Mycelia (pmol/g FW)				Supernatant (pmol/30mL)			
	Nucleotide forms		Riboside forms		Nucleotide forms		Riboside forms	
	cisZNT	iPNT	cisZR	iPR	cisZNT	iPNT	cisZR	iPR
cks1	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0
cks1^{CKS1}	10.7 +/- 1.5	0.4 +/- 0.3	216 +/- 45.1	13.2 +/- 6.1	16.7 +/- 3.2	1.1+/- 0.1	1.7 +/- 0.5	0.4 +/- 0.1
GY11	7.8 +/- 5.3	0.7 +/- 0.2	164 +/- 126	13.0 +/- 9.9	14.8 +/- 11.9	1.2 +/- 0.5	2.9 +/- 1.7	1.1 +/- 0.3

Table 1: The *cks1* mutant does not produce cytokinins.
CKs quantification was performed by liquid chromatography-positive electrospray ionization tandem mass spectrometry. *cks1* corresponds to mutant deleted for MG_04857, *cks1*^{CKS1} to the complemented strain and GY11 to the original wild type strain.
The CKs detected were: cisZNT (cis-zeatin nucleotide), iPNT (isopentenyladenine), cisZR (cis-zeatin riboside) and iPR (isopentenyladenosine). For each strain, four replicates were analyzed; the mean and SD are indicated. This experiment was repeated twice with similar results.

Four major isoprenoid CKs, cisZR (*cis*-zeatin riboside), iPR (isopentenyl adenosine), cisZNT (*cis*-zeatin nucleotide) and iPNT (isopentenyladenine nucleotide) were detected in mycelia and supernatant of the wild type GY11. The riboside forms, cisZR and iPR, were the most abundant CKs in mycelia while the nucleotide precursor forms, cisZNT and iPNT, were the main secreted ones. The other CK forms, trans-zeatin and dihydrozeatin were not detected. Thus, in the minimal medium, cisZR and cisZNT are the major CKs produced and secreted by the wild-type strain. This is consistent with CK measurements made on several other fungi where cis-zeatin forms were the main CKs detected [40]. The *cks1* mutant was not able to produce and/or secrete any detectable CKs whereas the complemented strain, *cks1*^{CKS1}, produced CKs at similar levels as the wild type strain GY11 did. Thus, *MGG_04857* appears to be required for CK biosynthesis in the rice blast fungus (therefore called *CKS1* for *Cytokinin Synthesis 1*) and is likely coding for an active tRNA-IPT protein, although this activity needs to be established.

***M. oryzae cks1* mutants have reduced virulence on rice**

To test for involvement of fungal CKs in the interaction between rice and the blast fungus, we inoculated mutant and control strains on the susceptible rice cultivar Nipponbare. The *cks1* mutant strain was less virulent than *cks1*^{CKS1} or GY11 wild-type strains as shown by a reduction of disease symptoms (Fig. 1A).

The CK-deficient strain produced less grayish, sporulating lesions per leaf (Fig. 1B) and these lesions were smaller than those caused by control strains (Fig. 1C). These results suggest that leaf penetration (reflected by lesion number) and invasion (reflected by lesion size) of the CK-deficient strain are both impaired, although not completely abolished. Impaired penetration was confirmed by microscopic observation of individual interaction sites which showed that *cks1* failed to penetrate as efficiently as *cks1*^{CKS1} during the early times of infection (<24 h, Fig. 1D). This is likely due to defective steps after appressorium formation since this developmental stage was unaffected by the *cks1* mutation (S3C Fig.).

Figure 1

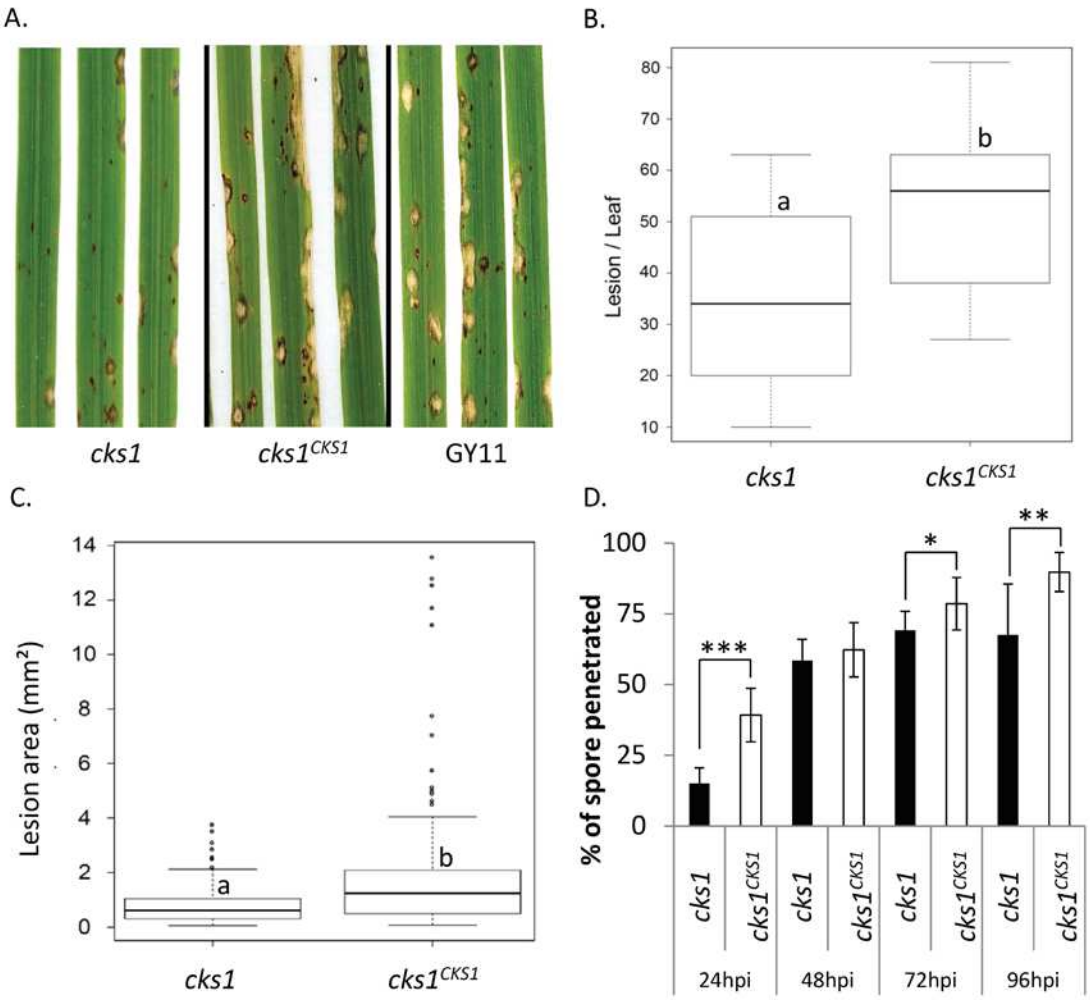


Fig. 1. The *cks1* mutant strain is less virulent than wild-type and complemented control strains. Nipponbare plants were inoculated with *Magnaporthe cks1* mutants and control strains to evaluate virulence. The results for control GY11 and the complemented strain were similar and are only shown for symptoms. (A) Disease symptoms were observed 6 days after inoculation. Grey spots represent susceptible lesions whereas brown spots represent failed penetration events. The number of susceptible lesions per leaf (generalized linear model, p-value = 0.02) and size of lesions (mixt model, p-value = 0.003) were measured as shown in B and C respectively (for more details see materials & methods). The values represent the mean and SD from four biological replicates each composed of 6 plants. The percentage of spores from the *cks1* and *cks1*^{CKS1} that penetrated the leaf was measured (D) under the microscope at different time points (hpi: hour post-inoculation). The data presented is the mean and SD of three biological replicates (>100 infection sites/replicate). A t-test was used to compare the penetration of the mutant and control strains, *, p-value < 0.01; **, p-value < 0.002; ***, p-value < 3.10^{-5}. All experiments were repeated three times with similar results and one representative experiment is shown here.

The reduced virulence of *cks1* mutant is linked to the absence of cytokinins

As a test for biological activity of fungal-produced CKs, we performed qRT-PCR and quantified the expression of rice Response Regulator (RR) genes *OsRR6* and *OsRR1* (Fig. 2) that were previously described to be transcriptionally regulated by CKs [52,53] and can thus be used as CK bio-sensors [44].

During early contact of fungal conidial spores with the plant surface (2h, 4h, 6h), and at a stage where all strains showed similar growth *in vitro* or on the plant surface (S3C Fig.), the two CK response markers tested had significantly lower expression in plants inoculated with *cks1* than with *cks1*^{CKS1} strains. Similar results were found for all other *RR* genes tested (S5A Fig.). This result supports the hypothesis that fungal CKs affect host CK signaling pathway and is consistent with the report made by Jiang et al., (2013) [44] that conidia, the first *M. oryzae* cells in contact with the host, can produce CKs.

To test if CKs could restore the virulence of the mutant strain, we exogenously applied the CK kinetin at 24h after inoculation (Fig. 3) since the delay of penetration of the mutant is noticed at this time (Fig. 1D). Kinetin was applied in the same conditions as previously described [44]. Kinetin treatment fully restored the virulence of *cks1* since the number and the size of lesions caused by *cks1* were similar to those caused by the *cks1*^{CKS1} complemented strain (Fig. 3). Similar results were obtained with an exogenous application of cis-zeatin (Fig. S6), which is the major cytokinin produced by *M. oryzae* (92% of CKs in supernatant; Table 1). These data strongly support the hypothesis that the reduced virulence of the *cks1* mutant is directly due to a defect in CK production.

Figure 2

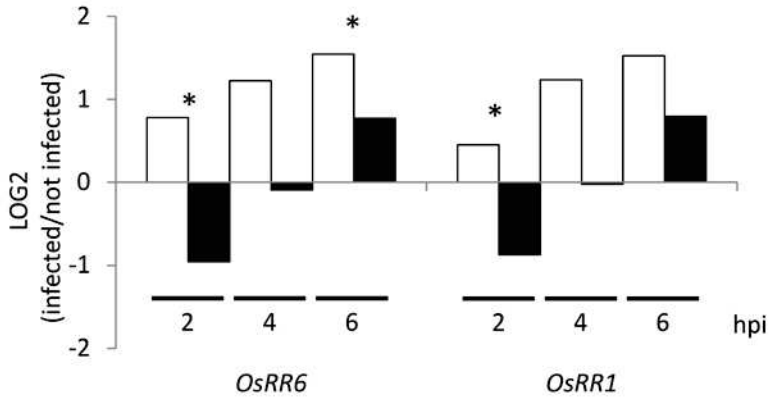


Fig. 2. Plant cytokinin signaling is differently affected during *cks1* infection. The transcriptional regulation of CK marker genes (*OsRR6* (Os04g57720) and *OsRR1* (Os11g04720) as named by Pareek et al., (2006)[54] was evaluated by quantitative RT-PCR using the Actin gene for normalization. Nipponbare plants were inoculated with spore suspension (in gelatin 0.5%) of either the *cks1* mutant (black bars) or *cks1*^{CKS1} control strain (white bars) and gene expression was measured at 2, 4 and 6 hours post inoculation (hpi), before penetration of the leaf tissues but at stages where there was no significant difference in growth of the *cks1* mutant compared to the complemented strain (S3C Fig.). The values presented are the Log2 ratios (infected/not infected) of the means calculated from four independent replicates. Uninfected plants were sprayed with gelatin 0.5% but without spore suspension. This experiment was repeated three times and showed similar results. A t-test was used to compare the means of expression quantified in *cks1* (black bars) and *cks1*^{CKS1} (white bars) inoculated plants. *: values significantly different at p-value < 0.05.

Figure 3

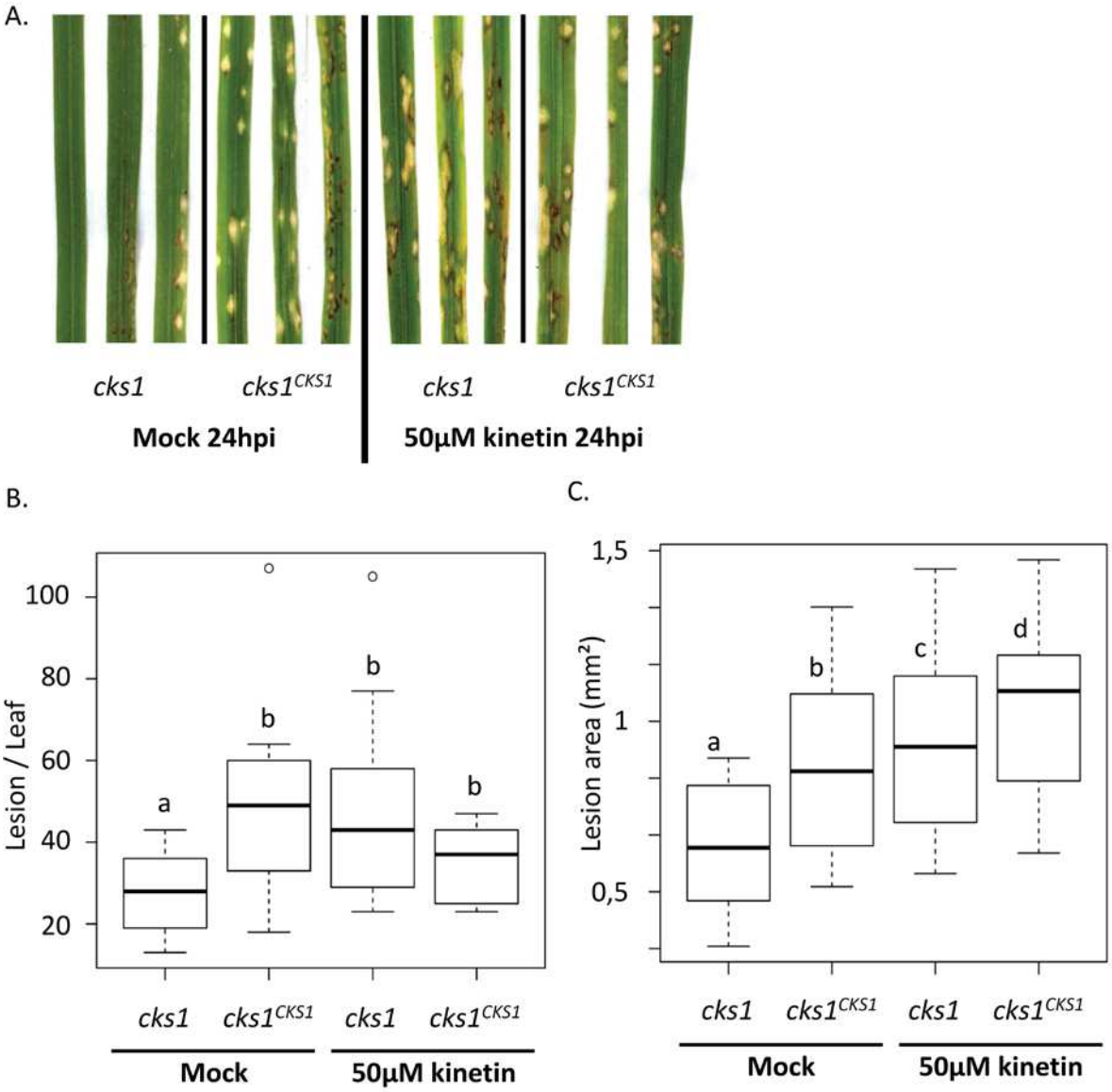


Fig. 3. The virulence of the *cks1* strain is fully restored by an exogenous application of cytokinin. One day after inoculation with the *cks1* mutant or the *cks1*^{CKS1} control strain, plants were treated with 50 µM of the CK compound, kinetin, or buffer alone. Kinetin alone without infection had no visible effect on leaf aspect. (A) The symptoms were observed 6 dpi. The number of lesion per leaf (B) and the size of lesions (C) were measured. The values represent the mean and SD of three biological replicates of 10 individuals. The entire experiment was repeated 3 times with similar results. The different letters indicate significant differences between values (p-value < 0.01) as estimated by a generalized linear model (B) or a mixed model (C) using ANOVA analysis (See Materials and Methods).

The *cks1* mutant induces a stronger oxidative burst than control strains

To evaluate the effect of fungal-derived CKs on early host basal defense responses, we measured ROS accumulation. Compared to *cks1*^{CKS1}, the accumulation of H_2O_2 (as revealed by DAB staining) was much more pronounced in response to the *cks1* mutant (Fig. 4A) and extended well beyond penetration sites (punctuated brown spots in complemented mutant controls) since the DAB staining was visible throughout all leaf tissues.

In order to test if the *cks1* mutant was able to infect the host more efficiently if ROS production was impaired, we treated inoculated plants with DPI (Diphenylene Iodonium), an inhibitor of flavor-enzymes like the NAD(P)H oxidase involved in H_2O_2 production [55]. The virulence of *cks1* mutant was partially restored when plants were treated with DPI (Fig. 4B and C). This suggests that the capacity of the *cks1* mutant to invade the host cell is restored, although only partially, when ROS production is reduced.

Figure 4

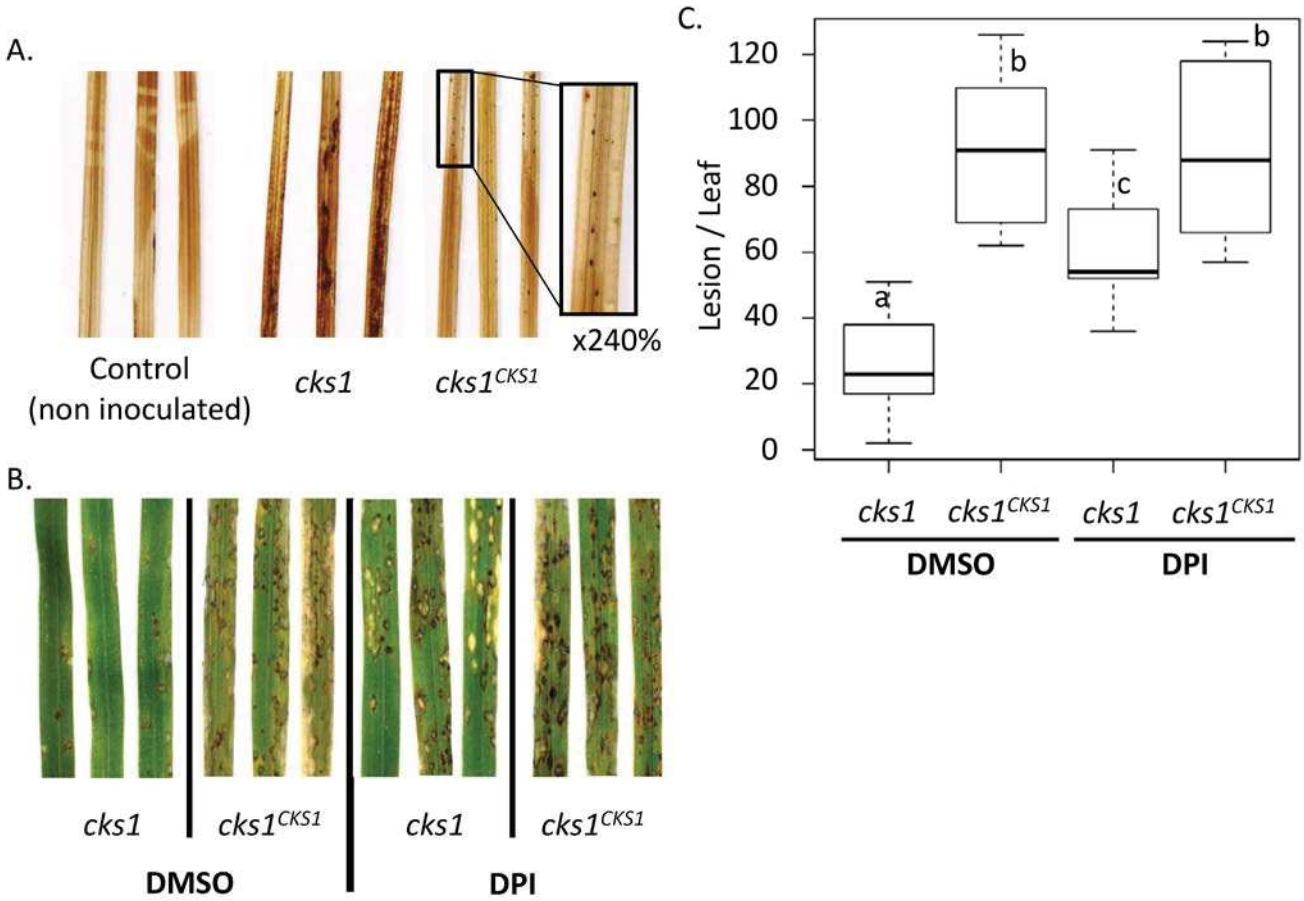


Fig. 4. The impaired virulence of *cks1* correlates with an enhanced induction of the oxidative burst and can be partially restored by inhibiting NAD(P)H oxidase activity. The relationship between virulence and reactive oxygen species accumulation was evaluated in the *cks1*-infected leaves. (A) The oxidative burst was detected 48 h after inoculation using DAB stain that turns brown upon reaction with H_2O_2. Brown spots correspond to sites where the wild-type blast fungus penetrated (see inlet), whereas a global browning was visible with infection with *cks1* mutant. This experiment was repeated two times and gave similar results. (B, C) DPI, an NAD(P)H oxidase inhibitor partially restores the virulence of the *cks1* mutant**.** One day after inoculation (once appressorium formation was initiated), plants were treated with DPI (0.5µM diluted in DMSO as previously described [56]. The symptoms were observed 6 dpi (B) and the number of lesions per leaf was measured (C). The letters indicate significantly different values according to a generalized linear model and ANOVA analysis (p-value < 0.04), see Materials and Methods.

The *cks1* mutant induces an early and strong transcriptional response of rice defense markers

We measured the expression of some well-established rice defense-marker genes during infection with the different strains. It first appeared that the defense-marker genes were differentially expressed earlier (2 to 6 hpi; Fig. 5A and S5B Fig.) in plants infected with *cks1* - before fungal penetration that mostly occurs after 24h (Fig. 1D). After penetration of the fungus (>24 hpi) and very strikingly, many defense-markers tested also showed a stronger induction in plants infected with *cks1* as compared to those infected with the control *cks1*^{CKS1} (e.g. *CHI*, *PBZ1*, *PR10* and *PR5* in Fig. 5B).

In the next step, we tested whether this over-induction of defense-markers by *cks1* was affected when the *cks1* virulence was complemented by exogenous application of the CK kinetin. This was the case for most genes that initially showed an over-induction (e.g. *CHI*, *PBZ1* and *PR5*; Fig. 5B). It indicates that complementation by exogenous cytokinin treatment reverts penetration and growth of the fungus as well as plant it brings back defense-marker expression to normal levels. Altogether these results support the hypothesis that the loss of virulence and the over-induction of defense associated with the *cks1* mutation are linked to an absence of CK production by the mutant strain.

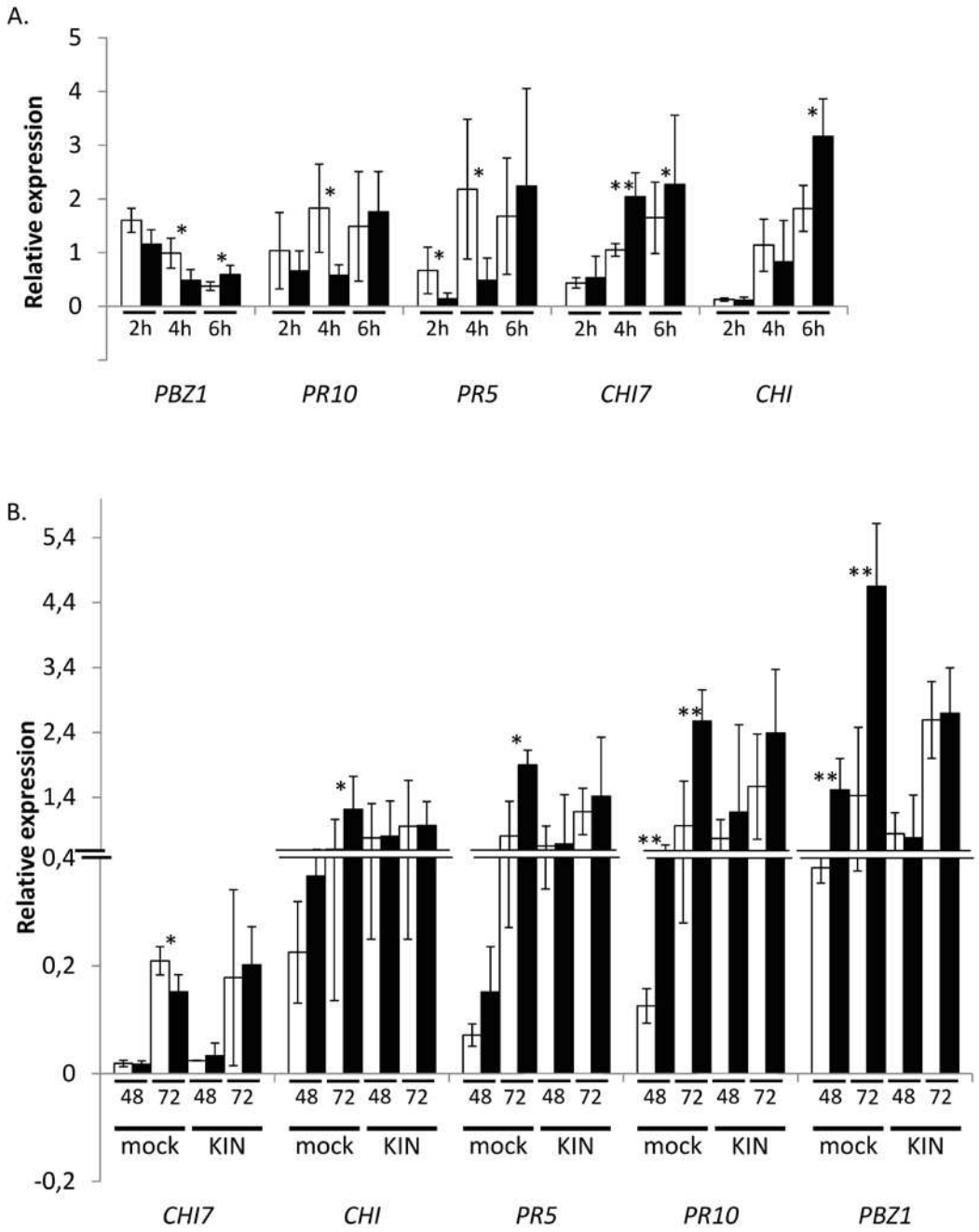


Fig. 5. The impaired virulence of *cks1* correlates with enhanced induction of defense genes and can be partially restored by exogenous cytokinin. The transcriptional regulation of defense-marker genes was evaluated upon inoculation with *cks1* mutant and complemented control strain. Nipponbare plants were inoculated with spore suspension (in gelatin 0.5%) of either the *cks1* mutant or *cks1*^{CKS1} control strain. Gene expression (normalized by plant Actin gene) was measured at different times after inoculation. *PBZ1* is a classical disease-related marker coding for a PR10 protein [57], *PR5* and *PR10* are classical disease-related markers [58], *CHI* and *CHI7* are chitinases [59]. (A) Gene expression was measured before the first indications of fungal penetration (< 6hpi). (B) Kinetin (50 µM) was applied (KIN) or not (mock) at 24 hpi and gene expression was also measured at 48 hpi. A t-test was used to compare the means between *cks1* and *cks1*^{CKS1}; for one given gene * indicate significant differences between *cks1* and complemented strain; for (A) *, p-value < 0.04 and **, p-value<0.002 and for (B) *, pvalue<0.03; **, p-value<0.008. The values presented are the means calculated from four independent replicates. The experiments were repeated twice with similar results.

The virulence of the *cks1* mutant can be restored in immuno-depressed plants

The enhanced oxidative burst and defense-genes expression is consistent with the reduced virulence phenotype observed with the *cks1* mutant. This suggests that fungal CKs normally produced by *M. oryzae* may inhibit key plant defense reactions. To further support this hypothesis, we tested the capacity of the *cks1* isolate to infect rice mutants which are defective for the chitin receptor, CEBiP, and the master transcriptional regulator NH1 that are both known to be immuno-depressed [58,60].

The fungal *cks1* mutant was more virulent on *cebip* and *nh1* rice mutants than on the wild-type plants (Fig. 6). The restoration of virulence of the *cks1* mutant on rice mutant plants impaired for defense responses strongly supports the hypothesis that the *cks1* mutants have the capacity to infect rice as long as defenses are inhibited.

Elevated fertilization can restore virulence of the *cks1* mutant

CKs affect different key metabolic processes in plants, like Calvin-Benson or tricarboxylic acid cycles and mediate source/sink modifications [29]. Therefore, we hypothesized that the lack of virulence of the CK-deficient strain could be also due to a reduced capacity to exploit or drain nutrient resources. Under this hypothesis, we reasoned that high levels of fertilization could enhance *cks1* virulence. Indeed high fertilization levels were previously shown to increase amino-acid and sugar contents in rice leaves [61] as well as rice blast susceptibility [62]. The protocol described in [62] was used to fertilize plants 24h before inoculation with *cks1* (Fig. 7). Under high fertilization regime, plants were more susceptible to the *cks1* mutant (Fig. 7A), supporting the hypothesis that this mutant is able to infect rice tissues but likely requires external complementation with essential nutrients.

Figure 6

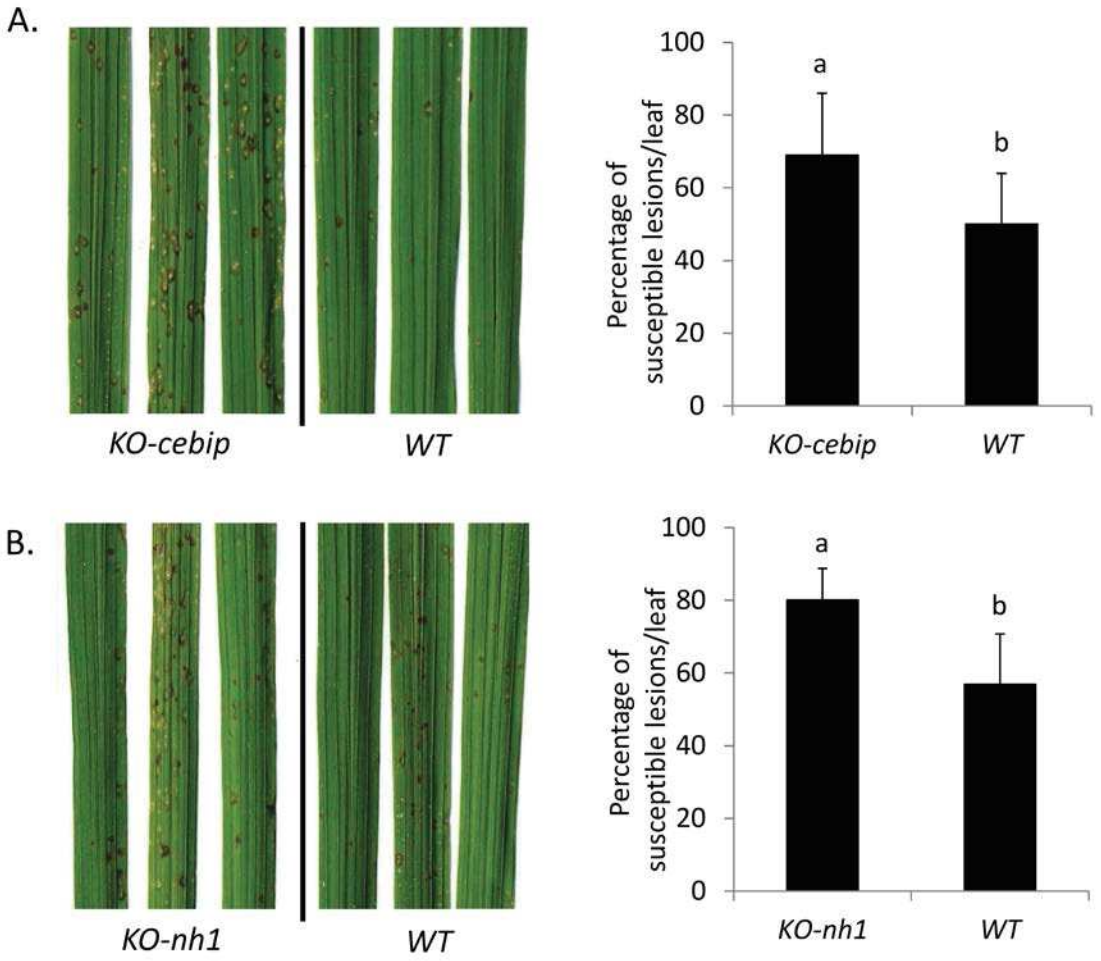
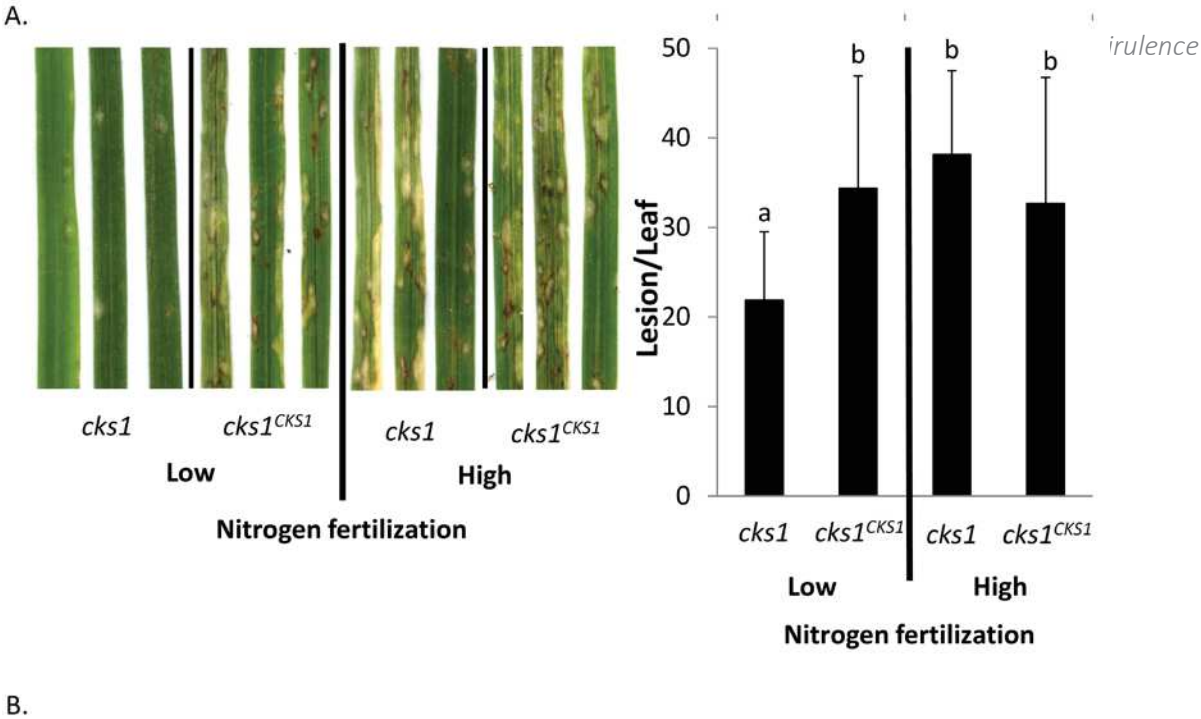


Fig. 6. The virulence of *cks1* can be restored in immuno-deficient rice mutants. The *cks1* strain is more virulent on rice mutants deficient for basal defenses. KO-*cebip* (A) and *KO-nh1* (B) rice mutants and control plants (WT) [58], all in Nipponbare genetic background, were spray-inoculated with *cks1*. Symptoms were measured 6 days after inoculation on three replicates containing 6 plants. The values are the mean and SD from three biological replicates. A t-test was used to compare the percentage of susceptible lesions of *cks1* on immune-deficient mutant and respective control plants, p-value < 0.02.

Figure 7



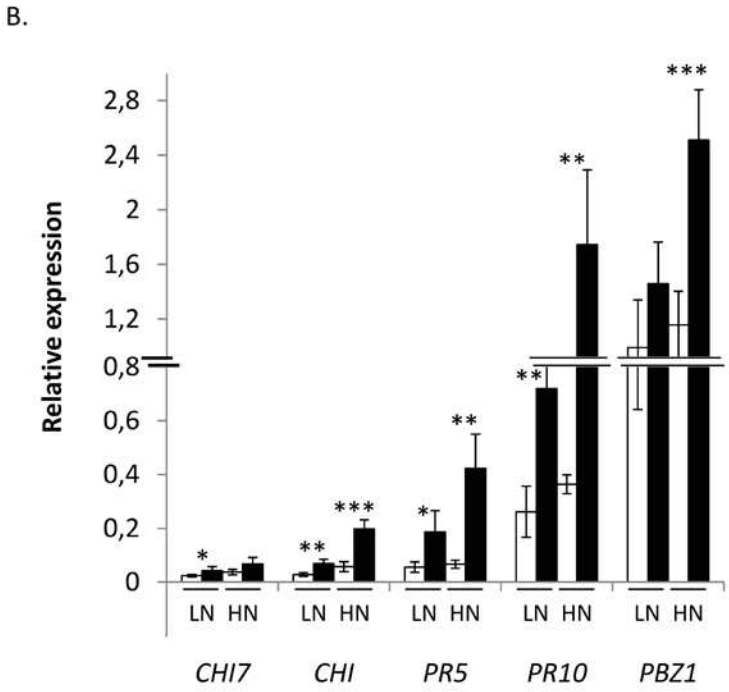


Fig. 7. High fertilization levels restored *cks1* virulence without inhibiting defense induction. Plants were fertilized (high fertilization) or not (low fertilization) 24h before inoculation. Fertilization was done as in Ballini et *al*, 2013 [62] to test the effect of plant nutritional status on *cks1* virulence. (A) Symptoms 6 days after inoculation and the number of lesion per leaf in plants inoculated with *cks1* mutant or *cks1*^{CKS1} under low or high nitrogen fertilization. Three biological replicates composed of 10 plants were analyzed per strain/condition. The different letters indicate significant differences between values (p-value < 0.03) as estimated by a t-test. (B) The expression of defense-marker genes was measured 48hpi and first normalized with *Actin* in Nipponbare plants inoculated with *cks1*^{CKS1} (white bars) or with *cks1* (black bars), fertilized (High Nitrogen, HN) or not (Low Nitrogen, LN) 24h before inoculation. For each gene, the mean and the SD of relative expression obtained from 4 biological replicates (each of 3 plants) are presented. A t-test was done on raw data to compare relative expression in *cks1* and *cks1*^{CKS1} inoculated plants. *, p-value<0.04; **, p-value<0.003; ***, p-value<0.0005.

We then evaluated if the over-induction of defense-markers, observed in plants infected with the *cks1* mutant (Fig. 5B), was still visible when the virulence was reversed by fertilization. Quite remarkably, the over-induction of defense-marker genes tested did not revert under high fertilization (Fig. 7B) in *cks1*-infected plants, despite the fact that the virulence of this mutant strain was restored (Fig. 7A). The data strongly support the idea that the over-induction of defense-markers by *cks1* is not due to an arrest of growth itself (which would subsequently trigger enhanced plant defense) but rather to another defect that leads to reduced virulence, likely in CK production. The virulence of the *cks1* mutant despite high expression of defense is likely compensated by high nutrient availability under high fertilization.

The *cks1* mutation affects sugar and amino acid contents in and around rice infected tissues

Previous metabolomic analysis of plants infected by the rice blast fungus showed that some amino acids (glutamate and aspartate) as well as sugars (fructose and glucose) are nutrients which are drained towards the infection site [63]. We therefore tested whether *CKS1* is required to efficiently maintain nutrient levels and/or modify nutrient fluxes at and around the penetration site. In order to test this hypothesis, after local deposition of a droplet of spore suspension of the different strains on the leaf surface, we measured the levels of different sugars and amino acids in the blast-infected and the neighboring non-infected zones (Fig. 8 and S7 Fig.).

During the infection by the cks^{CKS1} control strain, glucose (Fig. 8A) and fructose (S7 Fig.) contents progressively increased in and around the infected zone, which is consistent with previous observations that the accumulation of sugar is associated with successful pathogen invasion [64]. Initially, glucose and fructose contents were significantly higher 48 hpi in plants infected with the *cks1* mutant compared to plants infected with the control strain, an observation that can be related to enhanced induction of defense (see Discussion). By contrast, at later time points (after 48hpi), soluble sugars contents were significantly lower in plants infected with the *cks1* mutant. Given the

known effects of CKs on maintaining photosynthesis active [65], this observation supports the idea that CKs produced by *Magnaporthe* could contribute to maintain sugar production during infection.

For most amino acids, there were no strong differences between the tissues infected by *cks1* mutant and those infected by *cks1*^{CKS1} except for aspartate and glutamate (Fig. 8A). Away from the infected zone, the concentration of these two amino acids transiently decreased at 48hpi in plants infected with the control strain and increased at the site of infection at 72 hpi, suggesting that these amino acids can be drained towards the infection site or accumulated. By contrast, their level remained almost stable during infection with the *cks1* mutant. This suggests that the *cks1* mutant strain is not able to drain or consume aspartate and glutamate as efficiently as the control strain during infection.

Figure 8

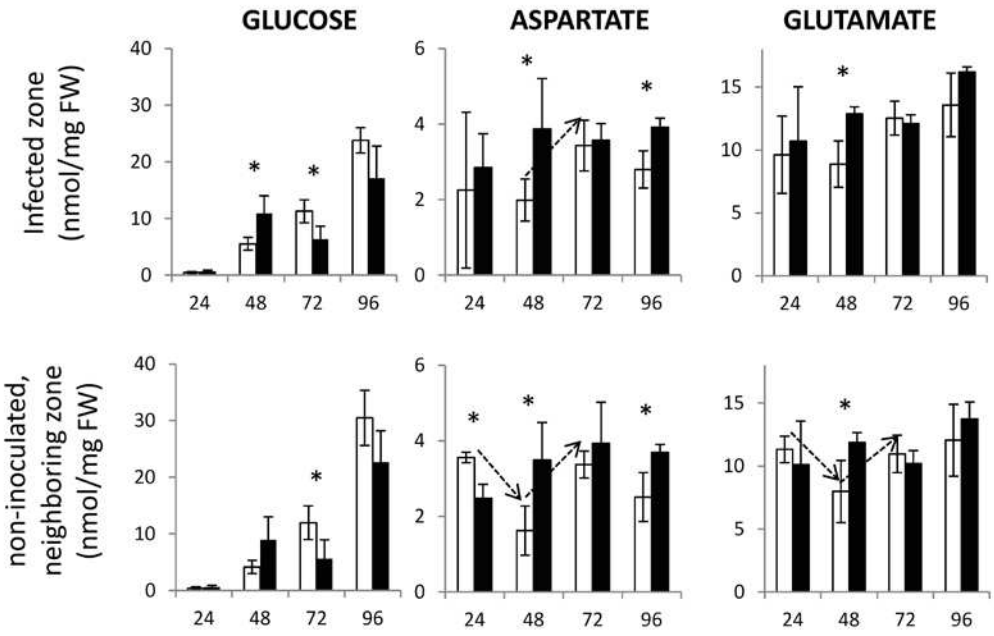


Fig. 8. The impaired virulence of *cks1* is associated with altered contents of nutritional elements essential for the fungus. Metabolomic analysis of plants infected with *cks1* and complemented mutant strains. Glucose, aspartate and glutamate contents were quantified (nmol/mg of fresh weight) as well as other sugars and amino acids, during infection (times are indicated), at the site of inoculation corresponding to the "infected zone" and one centimeter apart on both sides (upper and lower) with respect to the inoculated zone (S7 Fig.). Only the lower "non-inoculated, neighboring part of the leaf" is shown but all data relative to the "upper non-infected zone" can be found in S7 Figure. For more details see also Materials and Methods. A t-test (*, p-value < 0.05) was used to compare amino acid contents in leaf fragments from plants inoculated with the *cks1* (black bars) and *cks1*^{cks1} control complemented strain (white bars). The dashed arrows point to the significant changes (*t-test, p-value < 0.05) of amino-acid contents in *cks1*-infected plants between 24 and 48 hpi. For each time point four replicates composed of three leaf fragments were analyzed, mean and SD are indicated.

Discussion

Identification of *CKS1*, a conserved gene required for fungal cytokinin production

The pathogenic fungus *Magnaporthe oryzae* produces and secretes CKs [44] but its biosynthesis pathway had remained unknown. Moreover, the involvement of CKs in virulence of pathogenic fungi that do not induce tumors was still undetermined. Recently, a cluster including two genes (including one coding for a IPT-LOG) involved in the *de novo* CK biosynthesis pathway, was characterized in the ergot fungus *Claviceps purpurea* [41]. In mutants deleted for these two genes, CK production was partially affected but virulence was not. In the present study, we identified a gene in the rice blast fungus, *CKS1*, required for CK biosynthesis. The protein encoded by this gene presents all the features of a tRNA-IPT enzyme, the type of which is known in plants and yeast, and suspected in many fungi, to perform the first step of one of the CK biosynthesis pathways [25,40,46]. Phylogenetic analysis of tRNA-IPT protein sequences suggests that this gene is highly conserved among Ascomycete fungi (S8 Fig.) and beyond [48,66]. Our work sets the basis for functional analysis of this pathway in several other plant associated fungi known to produce CKs [42,67,68].

We generated a *cks1* mutant strain and demonstrated that this strain does not produce any of the CK types secreted by the wild type GY11 and *cks1*^{CKS1} complemented strain (Table 1). Moreover, the CKS1 protein is the only one found to contain a IPT domain in the rice blast fungal genome (S1 Table). These results suggest that the CK biosynthesis pathway controlled by CKS1 is probably the only one in the rice blast fungus. Nucleotide forms, which are known to be precursors to riboside, free base and glycosylated CKs [45], seem to be the major type of CKs secreted by control strains (*cks1*^{CKS1} and GY11). This contrasts with the yeast *Δmod5* mutant which was found to still produce CKs, and suggested that tRNA turnover is not mainly involved in yeast CK production [69]. However, the *Δmod5* mutant was grown on medium composed of yeast extract that already contains hormonal compounds. Thus, the free CK production by yeast observed in that study could have come from the recycling of CK compounds provided by the medium.

Like the yeast *Δmod5* mutant, the *M. oryzae cks1* mutant had no obvious pleiotropic effects under standard growth conditions in minimal medium (S3 Fig.). By contrast, the growth of *cks1* was affected under oxidative stress and this could be reverted by exogenous CKs (S4 Fig.). This result suggests that CKs play a role in fungal processes, like in yeast, for which MOD5 has primary roles in translation and is required for antifungal drug resistance [50]. These processes may participate to the loss of virulence of the *cks1* mutants (see below).

In plants, CK signaling is mediated by a multistep phosphorelay system involving Histidine Kinase Receptors, Histidine Phospho-transfer proteins and Response Regulators [54,70]. This kind of transduction system is widespread among organisms [71–74]. Several studies mentioned its involvement in osmoregulation in yeast and in hyphal growth of *Neurospora crassa* [75]. Based on protein sequence homology, putative orthologous genes to those of the plant CK signaling pathway were found in the *M. oryzae* genome (Table S1). Two of these genes, *MoSLN1* (coding for an histidine kinase receptor) and *MoSSK1* (coding for type-A response regulator), were previously shown to be required for full virulence of *M. oryzae* [76,77]. This suggests that CKs could also be perceived by the blast fungus in order to trigger a signal potentially required for its virulence. However, the involvement of these proteins in CKs perception and/or in CKs signaling transduction in response to plant or fungal-derived CKs remains to be established.

Cytokinins are required for fungal virulence

In addition to becoming deficient in CK production, the *Magnaporthe cks1* mutant was less virulent than wild-type GY11 or *cks1*^{CKS1} control strains since its capacity for penetration and invasion was strongly impaired (Fig. 1). Mutations affecting fungal invasion are still scarce in *M. oryzae* as most mutations affect appressorium formation and penetration only [78]. Only very recent studies reported the role of protein effectors in virulence of *Magnaporthe* during host invasion [79]. The characterization of the CK deficient *cks1* strain demonstrates that CKs play a key role in virulence of

the rice blast fungus. Testing whether CKs play similar roles in other pathogenic fungi is now possible since *CKS1* homologs exist in most of them (S8 Fig.).

An exogenous supply of kinetin (Fig. 3) or cis-zeatin (S6 Fig.) post inoculation restored the virulence of the CK-deficient strain, suggesting that the lack of virulence of these mutants was due to their inability to produce CKs. Moreover, exogenous application of kinetin reverted part of the over-induction of defense by the *cks1* mutant (Fig. 5B). This suggests that although other defects due to the deletion of the tRNA-IPT gene may exist, they are not responsible for this over-induction of defense. The demonstration that these fungal-derived CKs are secreted *in planta* is technically challenging because of the presence of plant CKs and the difficulty to localize such small metabolites. However the observation that the expression of plant CK responsive genes is differentially affected by the *cks1* strain before penetration in the plant tissue (Fig. 2 and Fig. 5A) suggests that the CKs produced by *Magnaporthe* are also secreted and detected by the plant. The plant receptors and pathways engaged in CK detection remain to be identified and CK mutants in rice will have to be produced to address this question.

Fungal cytokinins affect plant defense and nutrient fluxes and fungal physiology

Several observations support the hypothesis that the impaired virulence of *cks1* is the consequence of an inability of the fungus to manipulate the plant defense pathways and metabolic fluxes rather than a consequence of a self-triggered growth arrest (caused by the *cks1* loss-of-function) that would in turn trigger enhanced defense. First, enhanced defense is already visible *before* fungal penetration (Fig. 5A and S5B Fig.) at a time where the *cks1* and control strains were indistinguishable in terms of growth (S4C Fig.). Second, reduced fungal growth could be partially restored by manipulating the plant in three ways: (i) by reducing plant defense chemically (Fig. 4) (ii) by impairing immunity genetically in two independent mutants (Fig. 6), (iii) by modifying plant fertilization (Fig. 7A). Third, the restoration of virulence under high fertilization (Fig. 7A) did not affect the over-induction of defense responses (Fig. 7B). This indicates that the arrest of fungal growth can be compensated by

high N-fertilization, although the associated over-induction of defense response cannot, presumably because CKs are still not produced. For these reasons, we propose that the impaired virulence of the *cks1* mutant is the consequence of the absence of CK production that would normally dampen defenses and modify nutrient fluxes for the pathogen's benefit. Since the *cks1* mutant is also more susceptible to oxidative stress *in vitro* (S4 Fig.), this may further reduce its global capacity to grow into plant tissues, especially if the plant ROS production is enhanced (Fig. 4A). In that sense the *cks1* mutant is similar to the *des1* mutant which is required for dampening ROS-mediated plant defense [56].

Possible effects of fungal CK on the plant metabolism

The transient and elevated sugar content in *cks1* compared to the *cks1*^{CKS1} strain at 48 hpi (Fig. 8A) is consistent with previous studies in other pathosystems that showed that an early and strong accumulation of soluble sugars, providing energy for the establishment of host defenses [64,80,81]. By contrast, the lower contents of glucose, fructose and sucrose found after 72 hpi with the *cks1* strain suggests that photosynthesis is reduced in plants infected by the CK-deficient strain. We propose that *Magnaporthe*-derived CKs contribute to prevention of photosynthesis breakdown during infection process, for instance by limiting oxidative stress generated through photorespiration and, in consequence, allowing the establishment of the biotrophic phase. Among the different amino acids quantified, the contents of two key amino acids (glutamate and aspartate), which are known to be essential for *M. oryzae* [63], were differently affected between mutant and control strains following infection (Fig. 8A). In *Arabidopsis,* CKs were described to alter transcription of genes like glutamate dehydrogenase, asparagine synthetase and aspartate aminotransferase [82]. The fungal-derived CKs could also alter transcription of these genes to re-channel these amino acids towards fungal hyphae. Furthermore, CKs were previously shown to modify amino acid uptake through fungal cell membranes [83]; therefore, *cks1* mutants could also be affected in their capacity to import these molecules into fungal hyphae. Altogether, these possible effects of CKs could explain why aspartate

and glutamate contents were differently affected during infection by *cks1* strain and the complemented *cks1*^{CKS1} strain. These results suggest that fungal CKs could be involved in pathogen nutrition during infection as hypothesized for many plant/fungi interactions by Greene (1980) [16].

Our results also suggest that the lack of virulence of *cks1* mutants is partially due to an inability to limit plant defense responses like the oxidative burst (Fig. 4) and transcription of defense-related genes (Fig. 5A). The enhanced oxidative burst could lead to the stronger induction of defense markers observed during mutant infection and could participate to the strengthening of the cell wall to limit fungal penetration [84,85]. This is consistent with the ROS scavenging activity of CKs demonstrated in transgenic tobacco by Pogány et al., (2004)[86]. Quite paradoxically, exogenously applied CKs have been shown to enhance, in combination with salicylic acid, rice defense marker genes expression and phytoalexin biosynthesis [44,87]. This synergistic effect depends on key defense transcriptional regulators like OsWRKY45, a pivotal factor in biotic and abiotic stress responses [88]. Similarly, in *Arabidopsis*, specific recognition of bacterial CKs by plant CK receptors leads to a stronger induction of plant defenses and host resistance, involving plant CK Response Regulators and the transcription factor TGA3 [89]. In this context, how the CKs produced by *Magnaporthe oryzae* can act as negative regulators of defenses remains to be elucidated. A tight temporal and spatial production of CKs by the blast fungus could be central to avoiding enhanced activation of defenses.

Concluding remarks

CKs play a key role in plant-microorganism communication [90] and it seems to be particularly true in plant symbiotic relations with fungi and bacteria [91,92]. Our work, showing that *Magnaporthe* requires CK production to be fully virulent, extends the key role that these hormones play in the interactions between plants and microbes to pathogenic fungi that do not trigger organ deformation. Our work suggests that CKs produced by *M. oryzae* act like classical effectors during host invasion as they significantly reduce defenses (e.g. [79]). Moreover, our study shows that fungal CKs can divert

and attract plant nutrients essential for fungal growth, much like the TAL effectors from bacteria [3]. Therefore, CKs, like other hormones, could represent metabolic effectors with several biological functions. Given their central role in several metabolic processes, plant hormones represent ideal factors to be acquired as effectors by pathogens. Accordingly, evolution probably led the rice blast fungus to include CKs into its weapons for successful colonization of rice plants.

Materials and Methods

In silico analysis on *M. oryzae* and protein modelling

Based on published studies on plant CK metabolisms (cited in Table S1), sequences from plant proteins were used to perform BLASTp on the *Magnaporthe* proteome at http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/Blast.html?sp=Sblastp. We used an E-value of 1e-3, with the comparison Matrix BLOSUM62 and gapped alignment. Similarly, BLASTp were performed on the yeast proteome available at http://www.yeastgenome.org/cgi-bin/blast-sgd.pl with default parameters. Hit proteins from yeast and *M. oryzae* were used to BLASTp back on *Magnaporthe* proteome to ensure Best Blast Mutual Hits were identified.

Protein structure predictions were realized with the on-line platform I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The primary sequences of proteins of interest were submitted (At2g27760 and MGG_04857) and secondary structures were predicted. Based on secondary structure predictions and primary sequences, the most similar proteins whose 3D structure was determined by NMR or X-ray crystallography were used as template for the model prediction. The model presented in S1B Fig. was obtained with the MOD5 yeast protein (PDB accessions: 3epjA and 3ephA) as a template. Similar models were obtained with 3foz (*E. coli*) and 3a8t (*Humulus lupulus*) proteins as templates. Afterwards, alignments between the protein of interest and templates were generated by the use of different threading programs (MUSTER, FFAS-3D, SPARKS-X, HHSEARCH I, Neff-PPAS, HHSEARCH, pGenTHREADER, wdPPAS, PROSPECT2). Finally, the 10 best alignments were used to generate five structural models characterized [47]. The quality of structures used as templates were evaluated by Qmean server as well as the models obtained (http://swissmodel.expasy.org/qmean/cgi/index.cgi).

Statistical analysis and experimental design

We analyzed results obtained for symptom quantification (Fig. 1B, 3B and 4C) using a generalized linear model with a quasi-Poisson error structure. Significance was determined using a Chi^2 test. In

each experiment, three biological replicates composed of 10 plants were analyzed per strain/condition. The size of the lesion trait was analyzed using a mixed model, with the "leaf" factor as random error, to compare the invasion of the different strains in the different conditions (Fig. 1C and 3C). Boxplots represent data distribution using the median (indicated by the black line) and approximate quartiles. Each experiment was replicated at least three times. Gene expression data were analyzed using a Student t-test on four biological replicates, with each replicate composed of five to six plants (Fig. 2, 5, 7B and S5). A student t-test was also used to analyze data presented in fig 1D, 6, 7A, 8, S3, S4, S6 and S7.

Fungal transformation

Transformation was performed as described by Ribot et al., (2013)[93]. Protoplasts of GY11 were prepared as described previously [94]. For the knock-out *cks1* mutant, 1.2kb upstream and 1.2 kb downstream regions of the gene of interest were amplified by PCR using genomic GY11 DNA (100 ng) as template. The strategy used for constructing the gene replacement cassettes is derived from Kämper (2004) [95] and presented in the S2 Figure. Primers used are shown in Table S2. Growing colonies were transferred to Tanaka-Hygromycin or Tanaka-Basta plates for assessing resistance. Resistant colonies were further grown on rice agar media for 7–10 days at 26°C, purified by single-spore isolation, tested for resistance, and stored at -20°C. At least 2 independent transgenic fungal lines were isolated for each plasmid. Resistant colonies were characterized by PCR using a Phire Plant Direct PCR kit (Thermo Scientific, Waltham, MA, USA).

Measurement of cytokinin content

Fungal isolates were grown in 50mL of minimal Tanaka liquid medium without yeast extract (10g/L Glucose, 2 g/L $NaNO_3$, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4$-$7H_2O$, 0.1 g/L $CaCl_2$-$2H_2O$, 4 mg/L $FeSO_4$-$7H_2O$, 1mg/L Thiamine, 5µg Biotin and microelements as Tanaka-B medium [96]) on rotary shaker for 10 days at 26°C. Yeast extract was excluded because this compound already contains hormones,

including CKs, which leads to misinterpretation between CKs really produced compared to those that are just taken from the media and metabolized by the fungus. Fungal cultures were centrifuged for 10 min at 2000×g, the supernatant was collected and quantified. Mycelia were rinsed with sterile Tanaka liquid medium, centrifuged for 10 min at 2000×g and pressed with absorbent paper to accurately quantify the fungal biomass. Samples were then frozen and lyophilized. CK extraction and measurements were performed as previously described [19,51].

Fungal and plant growth

Fungal isolates were grown on rice flour agar for spore production [97]. For the determination of interaction phenotypes and cytology analysis, a suspension of fungal conidiospores (5×10^4 sp/mL) was spray-inoculated on the leaves of 3-week-old plants. For gene expression analysis, an inoculum of 2×10^5 sp/mL was used. Radial mycelial growth was measured on minimal Tanaka solid medium (20g/L agar added to the recipe mentioned above), during 13 days at 26°C. Nipponbare plants (*O. sativa* ssp. *japonica*) were grown during three weeks as described previously [98]. In standard conditions, nitrogen fertilization was performed for three weeks and inoculation was done 4 days after fertilization. For the high/low nitrogen experiments, plants were fertilized for two weeks as in standard experiment, and in the third week, plants were fertilized (or not) one day before infection, as described in [62].

Measurements of amino acids and sugar content

Amino acids and sugar contents were quantified as described in Gravot et al., (2010) [99] in leaf tissue of plants locally inoculated with a drop (15µL) of inoculum at 20 000 sp/mL. One centimeter corresponding to the inoculation site, and one centimeter above and below was sampled to quantify amino acids on 4 replicates composed of three leaf fragments (see S7 Fig. for a picture of the experimental setting).

RNA extraction and quantitative RT–PCR analysis

RNA extraction was performed as mentioned in Delteil et al, 2012 [58]. Quantitative PCR was performed using LC 480 SYBR Green I Master Mix (Roche, Basel, Switzerland) and a LightCycler 480 instrument (Roche). Amplification was performed as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 30 s; then 95°C for 5 min and 40°C for 30 s. In Fig. 5 and 7, in order to compare genes with different expression levels, for a given gene, all values were normalized using the average value of this gene across the different conditions.

Cytokinin and DPI treatments

Kinetin (Sigma) and cis-zeatin (OlChemIm) were diluted in 50% ethanol to prepare a stock solution of 50 mM. The solution sprayed contained 50 µM of CKs (replaced by ethanol 50% in mock solution), Tween at 0.02% final, diluted in water. Kinetin was applied to plants as described before [44] with slight modifications. Diphenylene iodonium (DPI; SIGMA D23926) treatment was performed at 0.5 µM final concentration, prepared from a 50× stock solution diluted in 50% DMSO. Mock treatment was performed with the equivalent volume of 50% DMSO/water. The volume sprayed was calculated to saturate the leaf surface (5mL for 10 three week-old plants).

Tissue staining for confocal observation and H_2O_2 staining

Inoculated leaves were harvested, fixed and stained as described by Ballini et al., (2013) [62]**.** In order to show H_2O_2 accumulation, we performed a DAB staining as mentioned in Faivre-rampant et al., (2008) [98].

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Competing interest

None

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Supporting information

Suppl Table 1

Function	Family	Number of genes in *A.thaliana*	Number of genes in *O.sativa*	Number of genes in *M.oryzae*	Number of gene in *S.cerevisiae*
Biosynthesis	IPT	7[1,2,3]	8[11,12]	not found	not found
	tRNA-IPT	2[4,5]	2[11]	1[b]	1[c]
	LOG	9[6]	11[11]	1[b]	1[c]
	CYP735A	2[7]	2[11]	difficult to identify	2[c]
	AK	2[8] (3 TAIR)	4[a]	1[b]	1[c]
Degradation	CKX	7[9]	11[11]	a lot of enzymes with a putative oxidase activity were found but their role in CKs metamolism is still unclear in fungi	
Transduction signaling	HKs and related proteins	16[10]	14[13]	10[14, 15, 16]	1[18,c]
	HPts	6[10]	5[13]	1[b]	1[18, 19c]
	RR-A	11[10]	22[13]; 10[11]	1[17, b]	1[18, 20,c]
	RR-B	12[10]	7[13]; 13[11]	1[17, b]	1[20,c]

S1 Table. Putative genes involved in CK metabolisms and signaling pathways, in *Oryza sativa*, *Arabidopsis thaliana, Magnaporthe oryzae* and *Saccharomyces cerevisiae*. CK biosynthesis in rice and *Arabidopsis* is well described. Based on homology between primary protein sequences some putative orthologous could be identified in *M. oryzae* and yeast by BLASTp. By the same way, primary sequences of plant enzymes already described to be involved in degradation of CKs were used to find orthologous in fungi. Finally, in plants, CK transduction signaling is performed by a multistep phosphorelay system, involving Histidine Kinase (HK) receptors, Histidine Phosphotransfer Proteins (HPt) and Response Regulators (only RR-A and RR-B are shown). Multistep phosphorelay is also already described in yeast and is conserved in *M. oryzae.*

IPT : Isopentenyl Transferase ; tRNA-IPT: tRNA-Isopentenyl Transferase ; LOG: Lonely Guy ; CYP735A: Cytochrome P450 monooxygenase ; AK: Adenosine Kinase ; CK-N-GT: Cytokinin N-Glucosyltransferase ; ZOGT: Zeatin-O-glucosyltransferase ; CKX: Cytokinin Oxydase.

[1]Kakimoto, 2001 [100] ; [2]Kakimoto, 2003 [101] ; [3]Takei et al., 2001 [102] ; [4]Golovko et ., 2002 [24]; [5]Miyawaki et al., 2006 [25]; [6]Kuroha et al., 2009 [103]; [7]Takei et al., 2004 [104]; [8]Moffatt et al., 2000 [105]; [9]Schmülling et al., 2003 [106]; [10]Hwang & Sheen, 2001 [107]; [11]Tsai et al., 2012 [70]; [12]Sakamoto et al., 2006 [108]; [13]Pareek et al., 2006 [54]; [14]Dean et al., 2005 [109]; [15]Motoyama et al., 2005 [110]; [16]Zhang et al., 2010 (Zhang et al., 2010); [17]Motoyama et al., 2008 [76]; [18]Posas et al., 1996 [111]; [19]Xu & West, 1999 [112]; [20]Li et al., 1998 [113]

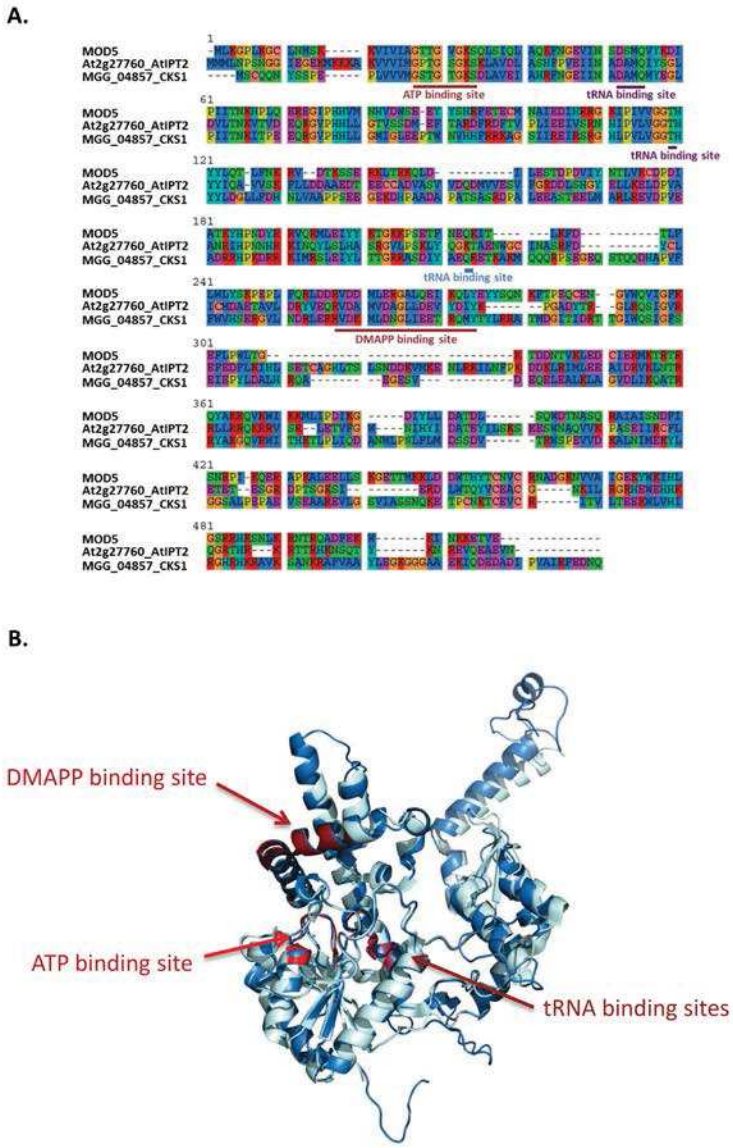
[a] BLASTp with AK from *A. thaliana* ; [b] identified by BLASTp from yeast and plant enzymes; [c] found annotated in yeast genome and publications associated (www.yeastgenome.org)

Suppl Table 2

Primer name	Accession	Annotation	Sequence
	MGG_04857	MoCKS1	F-GCAACAAGACGTGCGAAGTA
			R-TTATGGCGGTGACCTCTGAT
	Os11g04720	OsRR1	F-CTCAAACCAGTTCGGCTCTC
			R-CTGTTGTTGCTTTCGCTGTT
	Os12g04500	OsRR2	F-CTCAAACCAGTTCGGCTCTC
			R-TGTTGCTTTGCAGGTGTTGT
	Os01g72330	OsRR3	F-ATCTGCTCAAGAGGGTGAAGGG
			R-TGTCAGCGAGCTTGACAGGTTTC
	Os04g57720	OsRR6	F-TGTCAGGCATTTGCTTGTTC
			R-GGTGCCATTTCAACATTGTG
	Os02g35180	OsRR10	F-TCCAGAAGGCTGGATTGATT
			R-GCTGCACTCTTGCTTGATGA
	Os12g36880	PBZ1	F-AGGCATCAGTGGTCAGTAGAG
			R-CGGGTCTTGTATGTGCTTCC
	Os07g35560	CHI	F-TTAACGGCGCTGCTACCATT
			R-TCCCATCCTCTTACTGCCGA
	Os01g03390	BB trypsin inhibitor	F-ATCTGTGTCCGTCAATAAAACTCG
			R-TTGCTCTTGGTCACTGGCTAG
	Os07g48020	POX	F-GGATGCGTTCGTTGCTGGAAG
			R-GCTGCTCTGCTCCATACACTTG
	Os11g37970	HEL protein	F-AGTATGGATGGACCGCCTTCTG
			R-CGCAATTATTGTCGCACCTGTTC
	Os06g51050	CHI7	F-CAATGCACACGAGATTGTGA
			R-CCGCATTGTGTTAACGTCCA
	Os12g36850	PR10	F-CAGATGATCGAGGCGTACCT
			R-CCACGCCACAGTAACATGAC
	Os12g43430	PR5	F-AGCCAGGACTTCTACGACCT
			R-GCGTGTGTCTTGGTGTTGTC
1F	MGG_04857	promotor MGG_04857	GAGATGATGGGCGTGATACC
2R		hygromycin resistance gene	GGGATCAGCAATCGCGCATATGA
1R	MGG_04857	terminator MGG_04857	TTTGCTAGGCTTCGGTGAAT
3F	MGG_04857	Not I_ promotor MGG_04857	atatgcggccgcCCACATGATGGACTCGCAGAT
3R	MGG_04857	EcoRI_ 2nd exon MGG_04857	atatgaattcTTACTGGTTGTCCTCAAAACGGATAG

S2 Table. Primers used for RT-qPCR gene expression studies and for PCR genotyping. *MoCKS1: Magnaporthe oryzae Cytokinin Synthesis 1; OsRR1, 2, 3, 6, 10* corresponding to the annotation of response regulator coding genes published by Pareek et al., (2006) [54]. *PBZ1*: *Probenazole-inducible gene; CHI* and *CHI7* coding for chitinases; *PR: Pathogenesis Related genes*. The primers 1F, 2R, 1R, 3F and 3R are those used in S2 Fig.

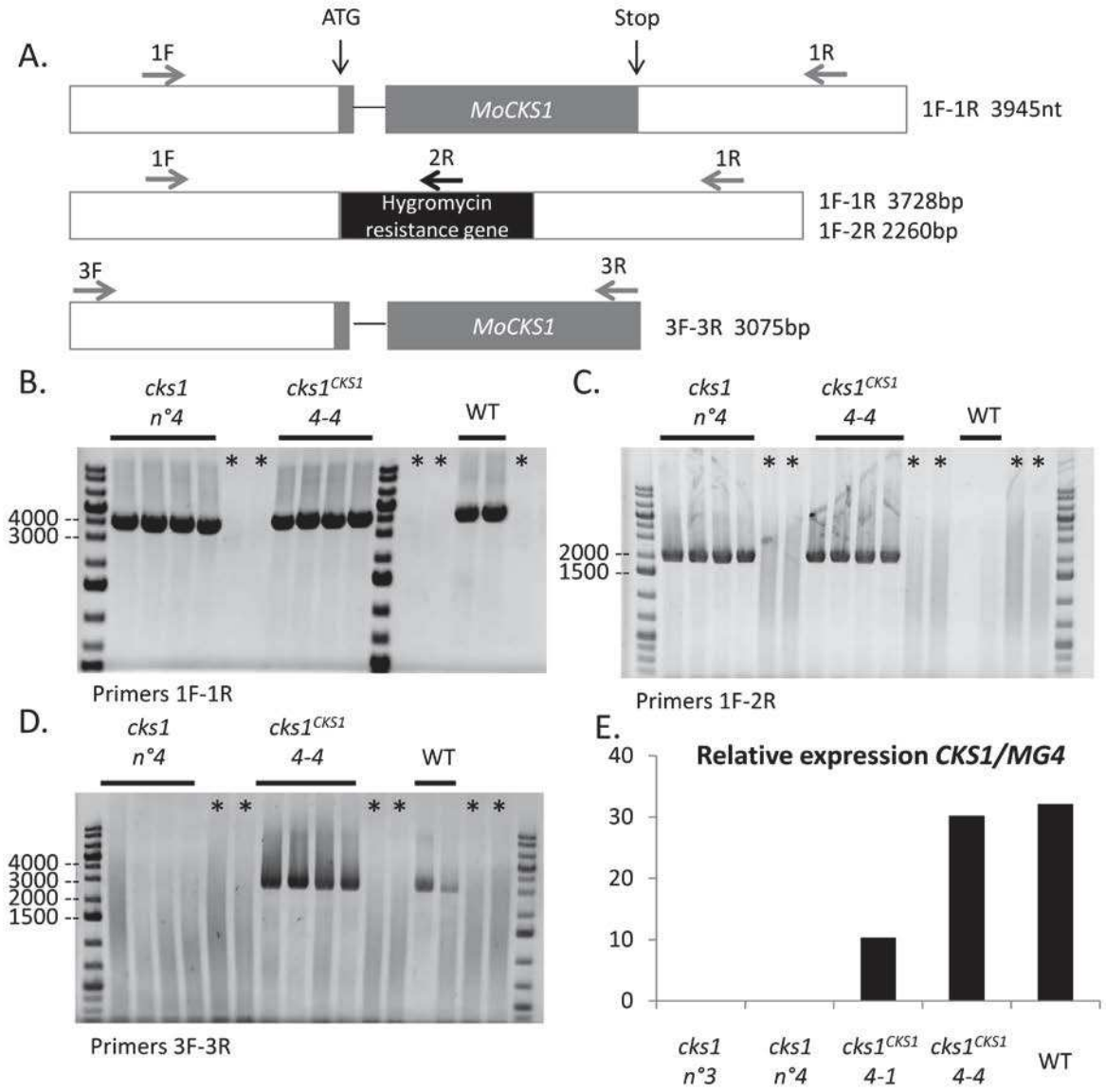
Suppl Figure 1



S1 Fig. The MGG_04857 protein is similar to a tRNA-Isopentenyl transferase.

(A) Primary sequence alignment of MOD5, AtIPT2 and the orthologous protein from *M. oryzae*. MOD5 and AtIPT2 are tRNA-isopentenyl transferases involved in CK biosynthesis in yeast [69] and *Arabidopsis* [24], respectively. Binding sites (underlined) are conserved in the putative tRNA-IPT from *M. oryzae* (MGG_04857, CKS1). The ATP binding site, from the amino acid 18 to 26 (GSTGTGKS), is conserved as well as tRNA binding sites (DAMQ 43-46 and T111) and DMAPP binding site (249-265). The percentage of identity and similarity between these proteins is higher than 30% and 50%, respectively (between MOD5 and AtIPT2: 32% identities, 55% positives (e-value 7.1^{e}-27); MOD5 and putative tRNA-IPT from *M. oryzae*: 36% identities, 60% positives (8.6^{e}-52); AtIPT2 and putative tRNA-IPT from *M. oryzae*: 36% identities, 54% positives (4^{e}-40). (B) The putative tRNA-IPT from *Magnaporthe* has a predicted structure similar to the yeast MOD5 protein. MOD5 structure (light blue) was determined by X-ray diffraction [114]. The MOD5 structure used for the alignment corresponds to the 3ephA and 3epjA accessions in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The predicted structural model of tRNA-IPT from *M. oryzae* (MGG_04857; dark blue) was obtained by threading on the on-line platform I-TASSER, C-score 0,02 (Cf Materials and Methods). The quality scores of the structural model obtained for MGG_04857 and MOD5 by the Qmean server were 0.610 and 0.656 respectively. Conserved substrate binding sites are indicated by red arrows.

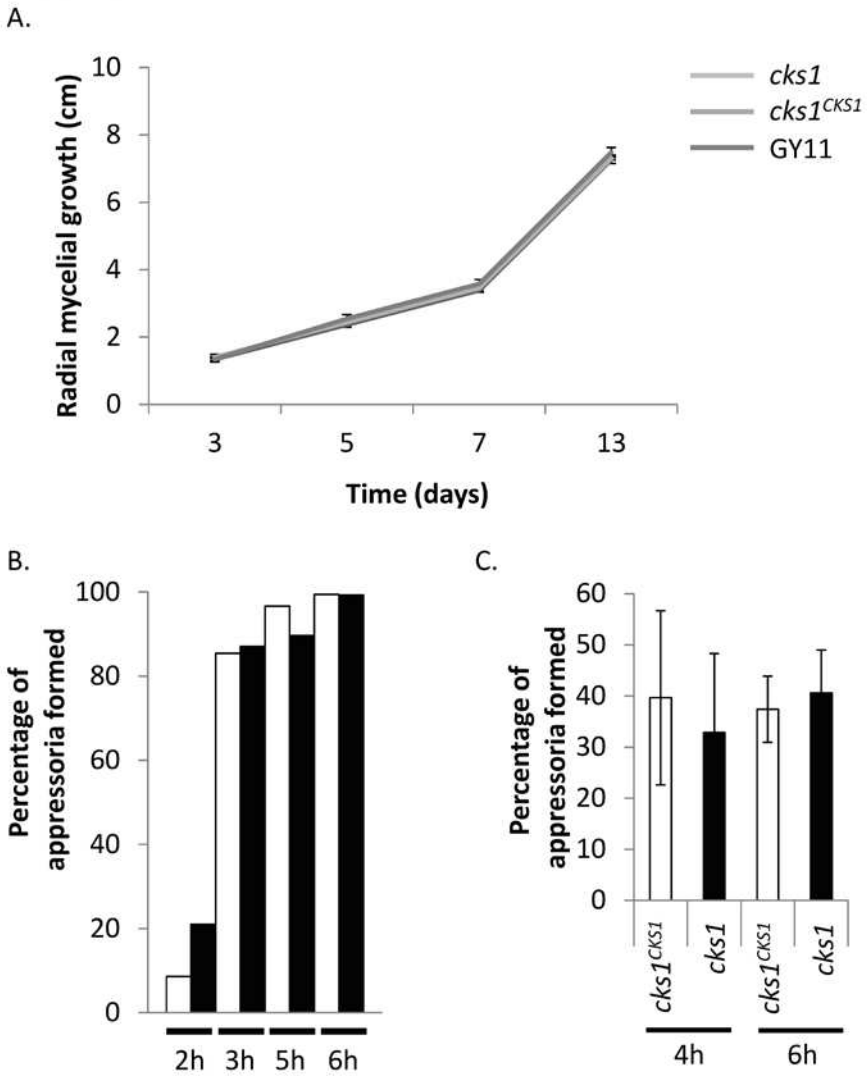
Suppl Figure 2



S2 Fig. Characterization of fungal *cks1* mutants and complemented strains.

(A) Genomic structure of *MoCKS1* (*MGG_04857*) gene. The *cks1* strain was generated by homologous recombination between the endogenous *CKS1* gene and a PCR fragment containing the hygromycin resistance gene. The knock-out mutation was complemented by a construct containing the genomic sequence of *MoCKS1* under its own promoter thus corresponding to the *cks1*^{CKS1} control isolate (genomic sequence 1100nt upstream and 1646nt downstream the ATG). The position of primers used for genotyping and the length of PCR products are indicated. Genotyping was established by PCR (1.2% agarose gel). The genotype of the strains is indicated and * corresponds to negative controls. (B) PCR products were obtained with primers 1F-1R showed in (A) on both sides of the insertion site. (C) PCR products obtained with primers 1F-2R, demonstrate the presence of the hygromycin resistance gene replacing *CKS1* endogenous gene. (D) PCR products obtained with primers 3F-3R showing the presence of *MoCKS1* genomic sequence under its own promoter. (E) *In vitro* relative expression of *MoCKS1*, *MGG_04857*, in *cks1* strains (*cks1* n°3, *cks1* n°4), potential complemented strains *cks1*^{CKS1} (*cks1*^{CKS1} 4-1 and *cks1*^{CKS1} 4-4; generated from *cks1* n°4 strain) and WT strain (GY11). The expression of *MoCKS1* was normalized with the expression of the *MG4* constitutive gene. The *cks1* n°3 and 4 strains do not express *MoCKS1*. We chose *cks1* n°4 as mutant strain and *cks1*^{CKS1} 4-4 as control complemented strain. In all experiments presented, these strains were named *cks1* and *cks1*^{CKS1}. GY11 corresponds to the wild type genetic background used to generate fungal mutants.

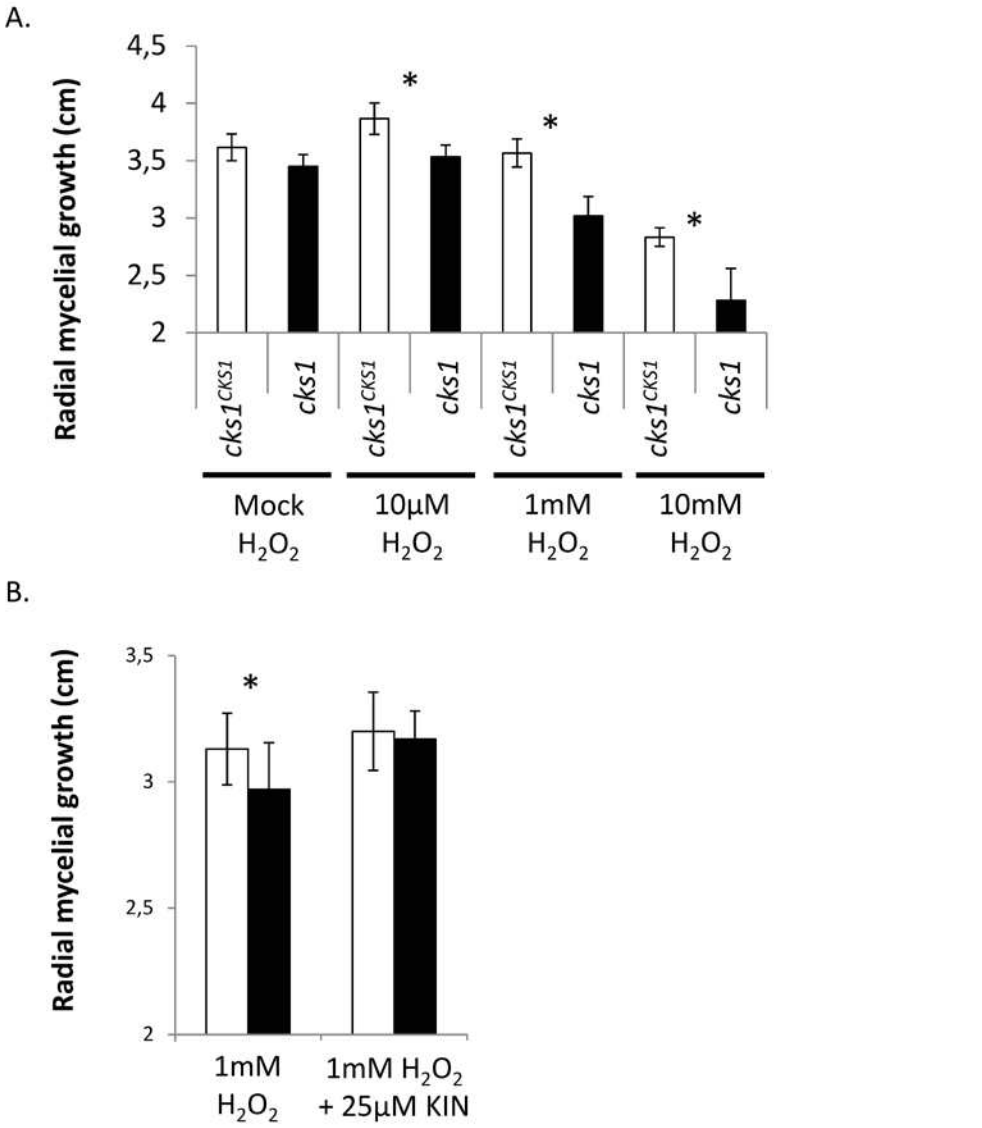
Suppl Figure 3



S3 Fig. Early developmental of *cks1* is not modified *in vitro* or on the plant surface.

(A) The mycelial growth of the different strains was initiated from a fungal disc of 1cm of diameter from a first plate where the fungi reached maximum growth. The diameter of mycelia was measured during 13 days on minimal medium. The values are the mean and SD of 5 replicates per strain. (B) The development of the appressorium was measured on glass slides at the indicated time points for each strain and (C) on the plant leaf surface. Plants were inoculated and the frequency of spores showing complete appressorial development was measured. There was no significant difference between *cks1* (black bars) and *cks1*^{CKS1} (white bars) strains as estimated with a t-test.

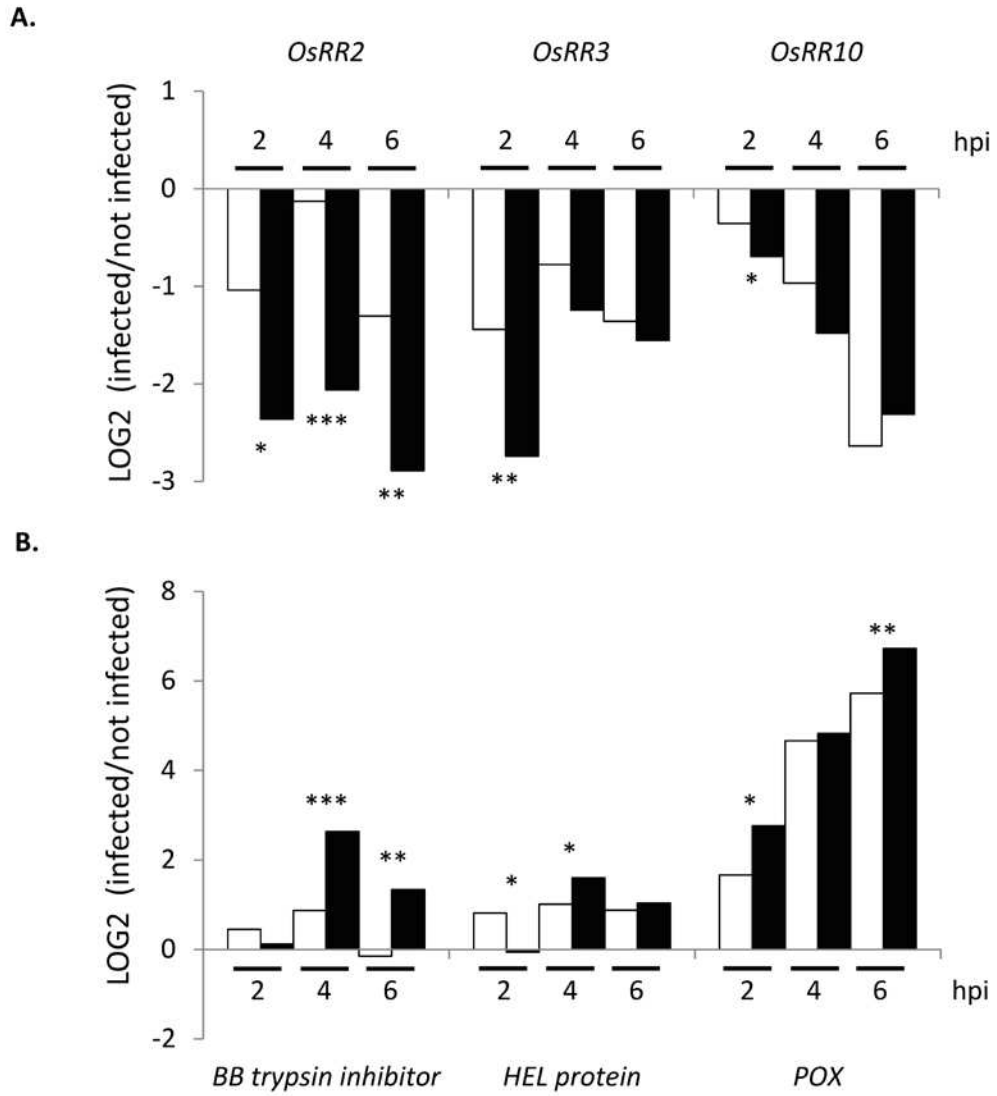
Suppl Figure 4



S4 Fig. The *cks1* mutant is less tolerant to oxidative stress

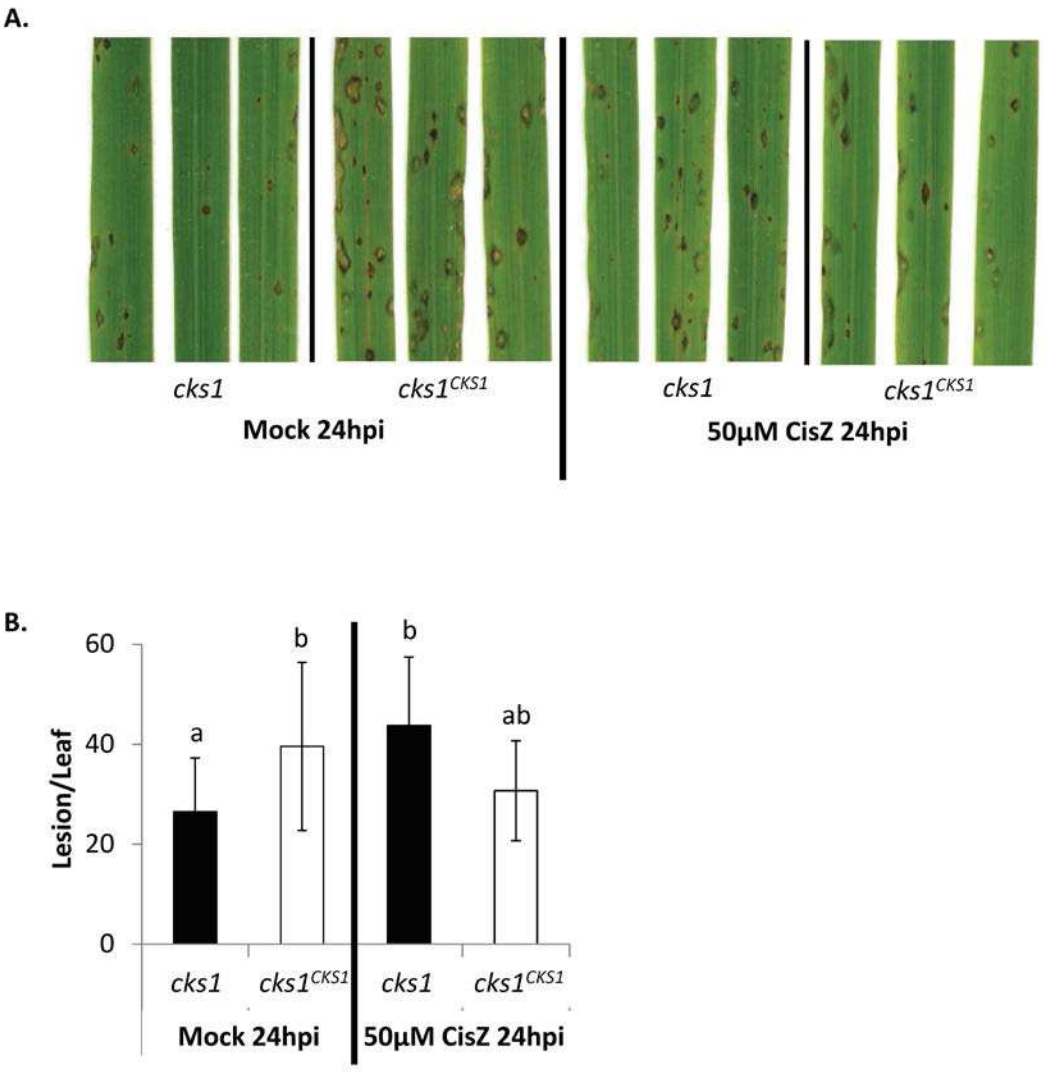
The *cks1* mutant and complemented strain were grown on minimal medium containing H_2O_2 and radial growth was measured as a read-out of fungal fitness. (A) The *cks1* mutant is hypersensitive to oxidative stress. (*: P<0.001; t-test comparing *cks1* and complemented mutant strains of 5 replicates). (B) The effect of 1 mM H_2O_2 was tested in the presence of 25µM of kinetin.

Suppl Figure 5

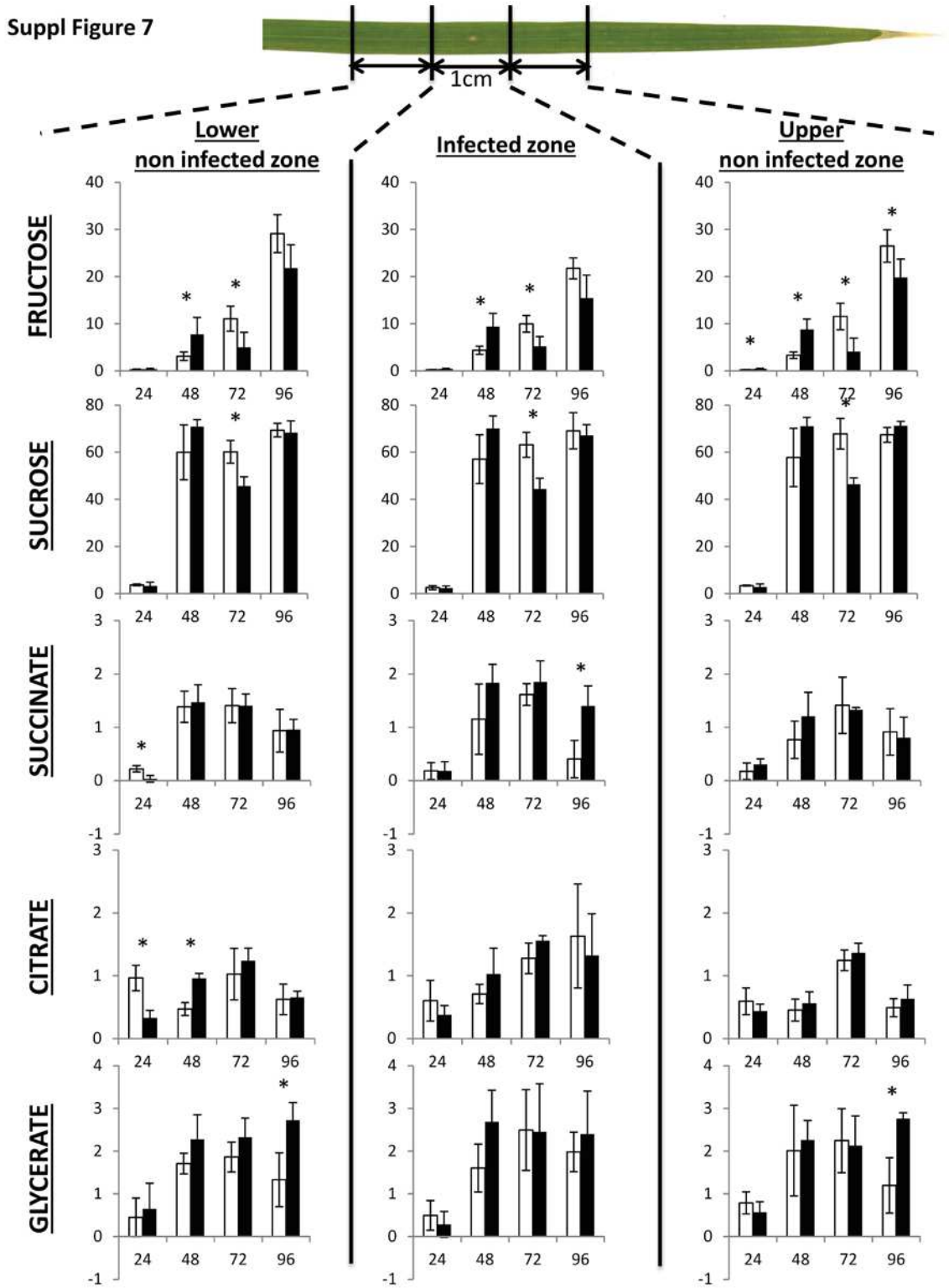


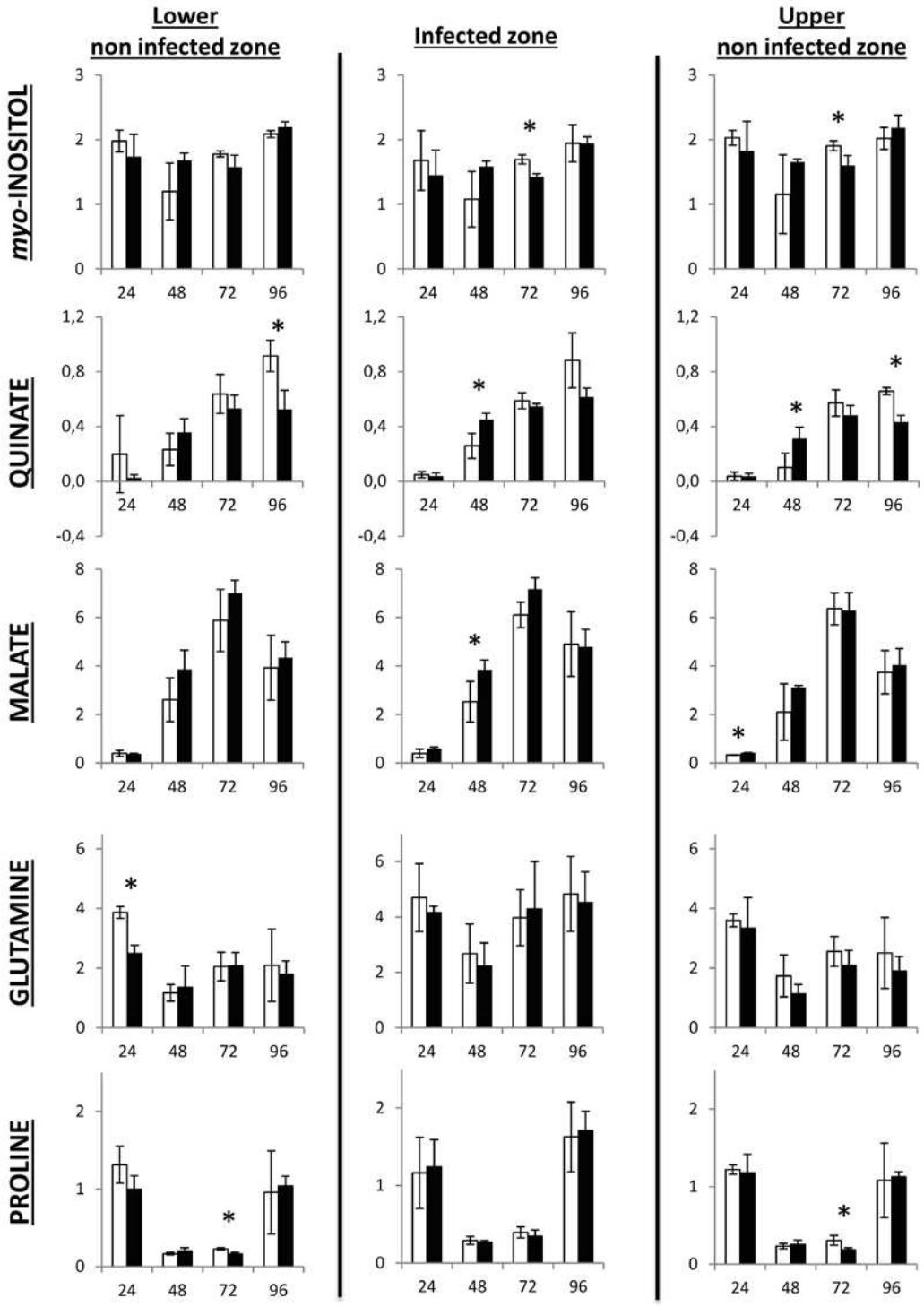
S5 Fig. The *cks1* mutant triggers different host CK signaling and defense transcriptional responses during early stages of infection. (A) The transcriptional regulation of CK marker genes (*OsRR2* (Os12g04500), *OsRR3* (Os01g72330) and *OsRR10* (Os02g35180) as named by Pareek et al., (2006) [54] [54]was evaluated by quantitative RT-PCR using the *Actin* gene for normalization. (B) The expression of defense marker genes was also measured: *Os01g03390 (BB trypsin inhibitor), Os11g37970 (HEL protein), Os07g48020 (Peroxidase).* Nipponbare plants were inoculated with spore suspension (in gelatin 0.5%) of either the *cks1* mutant (black bars) or *cks1*^{CKS1} control strain (white bars) and gene expression was measured at 2, 4 and 6 hours post inoculation (hpi), before penetration of the leaf tissues. The values presented are the Log2 ratios (infected/not infected) of the means calculated from four independent replicates. Uninfected plants were sprayed with gelatin 0.5% but without spore suspension. This experiment was repeated twice and showed similar results. A t-test was used to compare the means of expression quantified in *cks1* (black bars) and *cks1*^{CKS1} (white bars) inoculated plants. *: p-value < 0.05; **: p-value < 0.03; ***: p-value < 0.001.

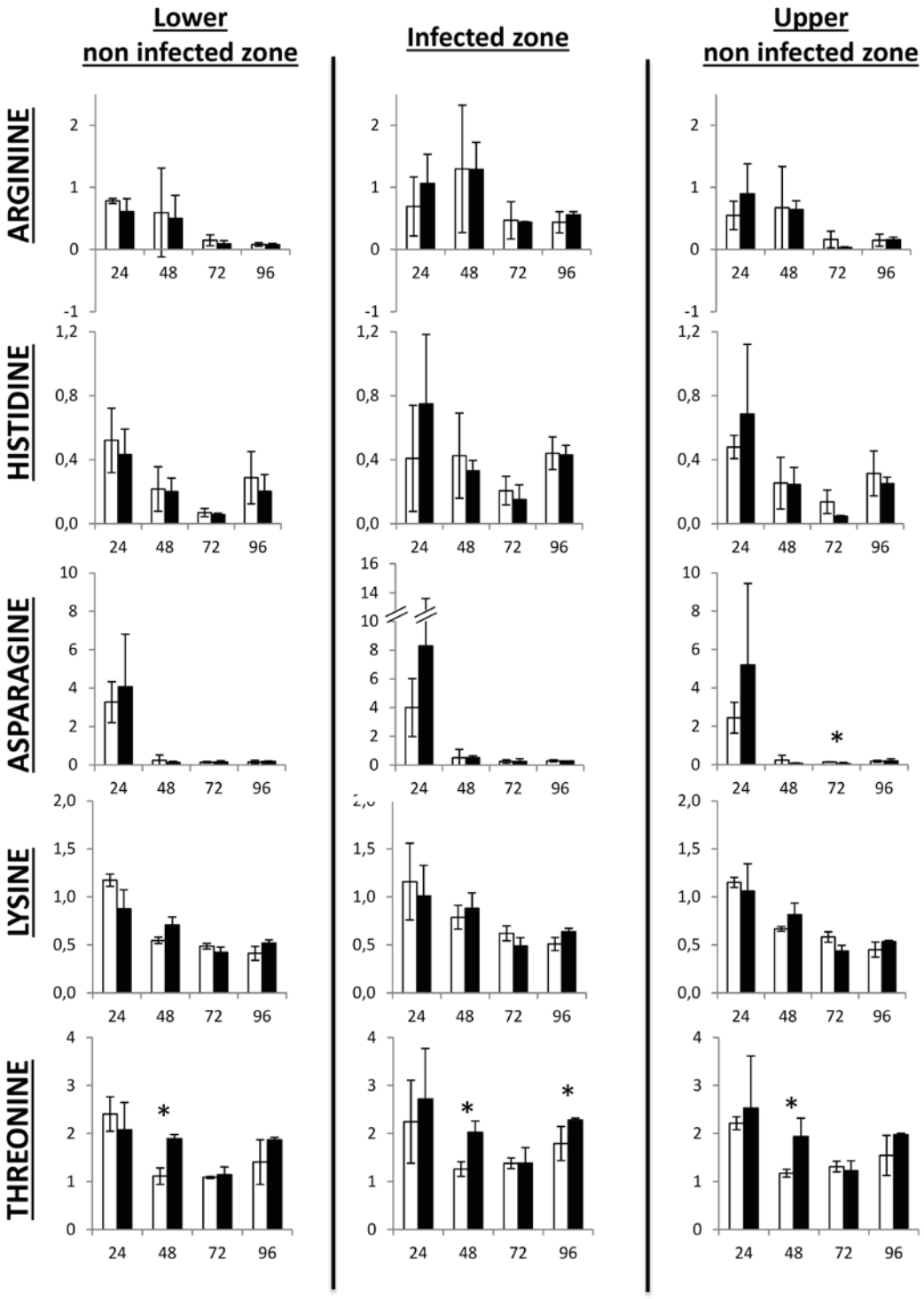
Suppl Figure 6

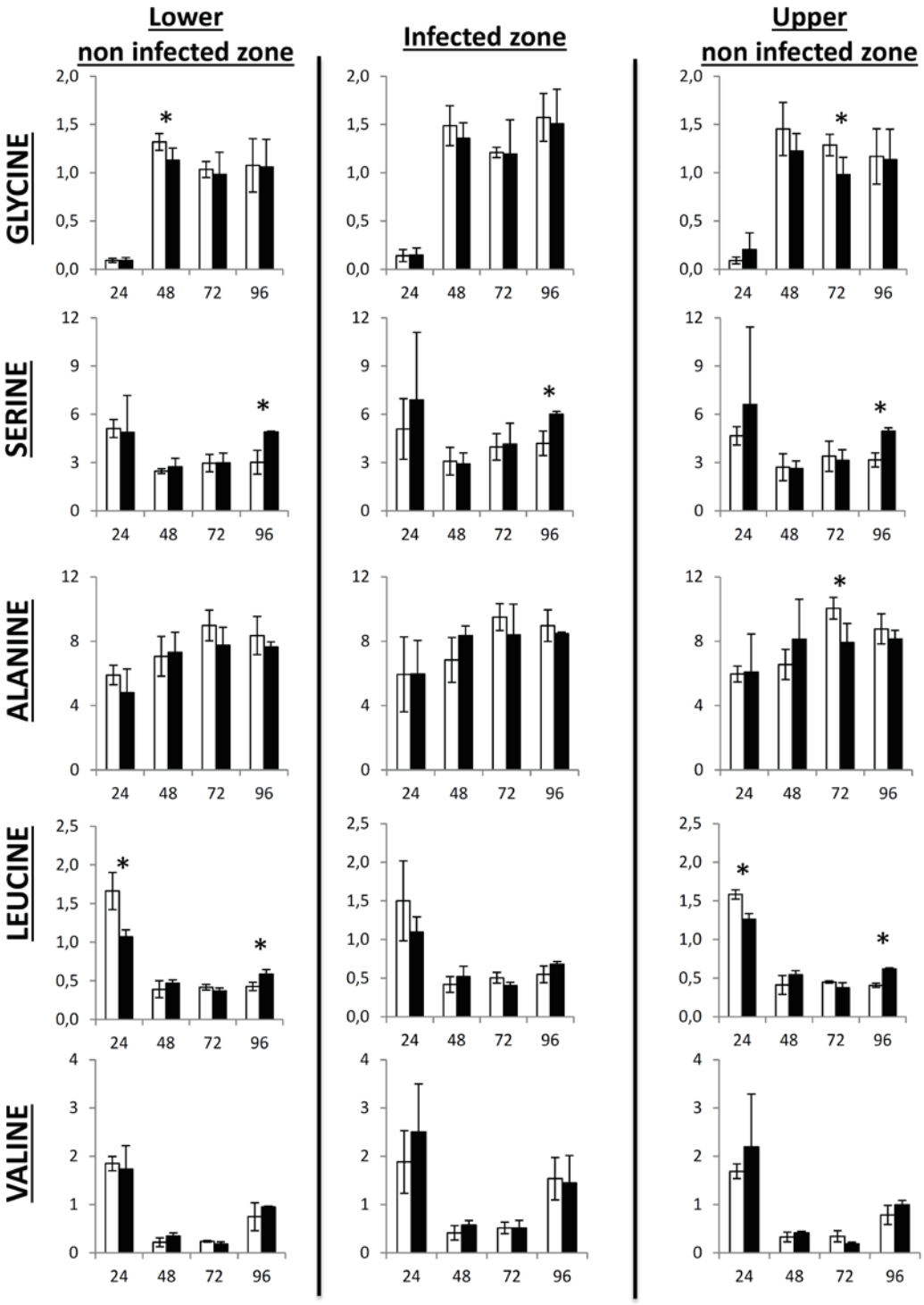


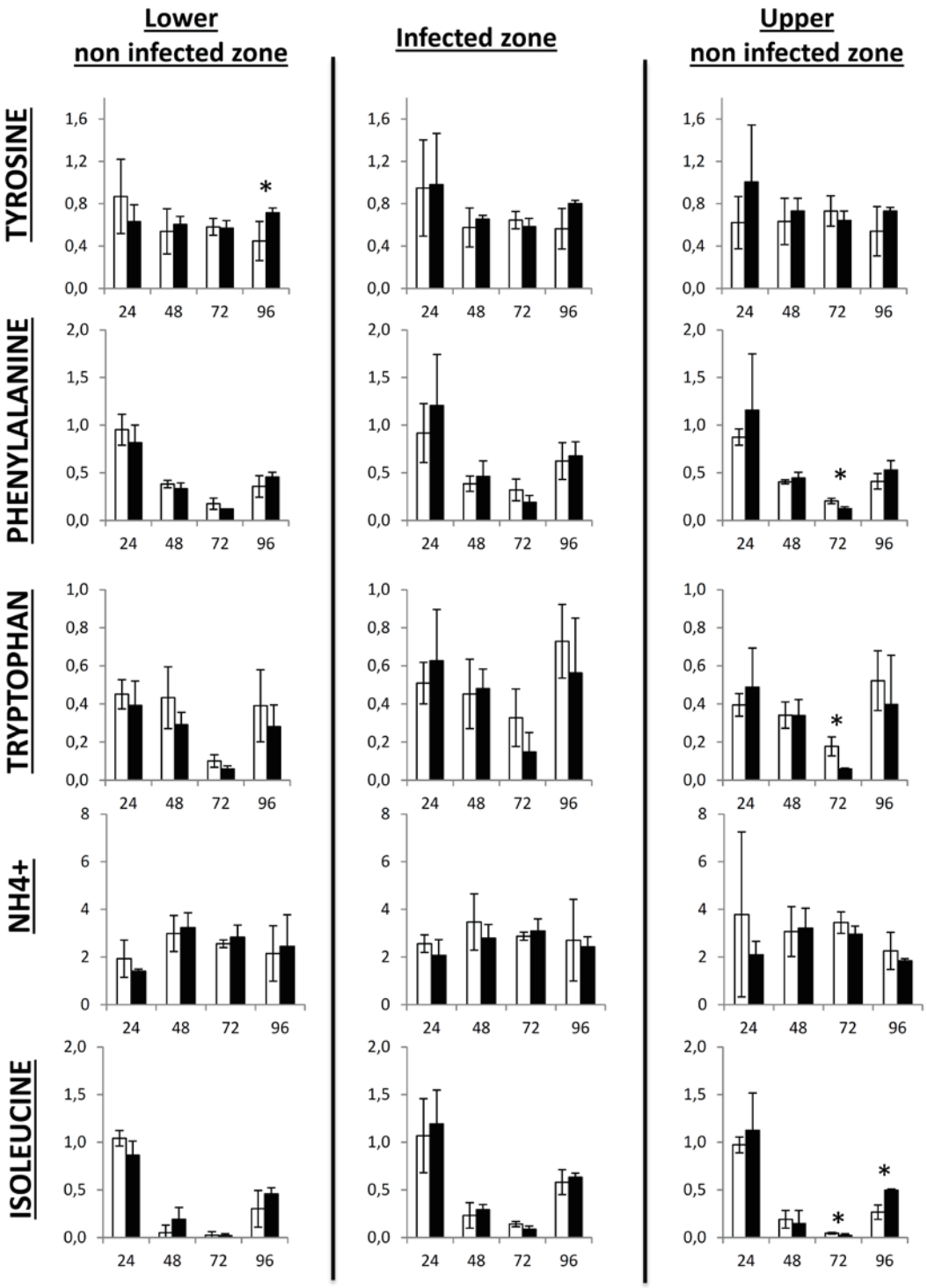
S6 Fig. The reduced virulence of *cks1* strain is restored by exogenous application of cis-zeatin.
Plants were treated with 50µM of cis-zeatin or buffer alone 24h after inoculation with *cks1* mutant and *cks1*^{CKS1} complemented strain. The symptoms were observed 6dpi (A) and the number of lesion per leaf is shown (B). The values represent the mean and SD of three biological replicates of 10 individuals. The different letters indicate significant differences between values obtained by t-test (p-value<0.04).


Suppl Figure 7
1cm
Lower
non infected zone
Infected zone
Upper
non infected zone
FRUCTOSE
SUCROSE
SUCCINATE
CITRATE
GLYCERATE
24
48
72
96


Lower
non infected zone
Infected zone
Upper
non infected zone
myo-INOSITOL
QUINATE
MALATE
GLUTAMINE
PROLINE
24
48
72
96

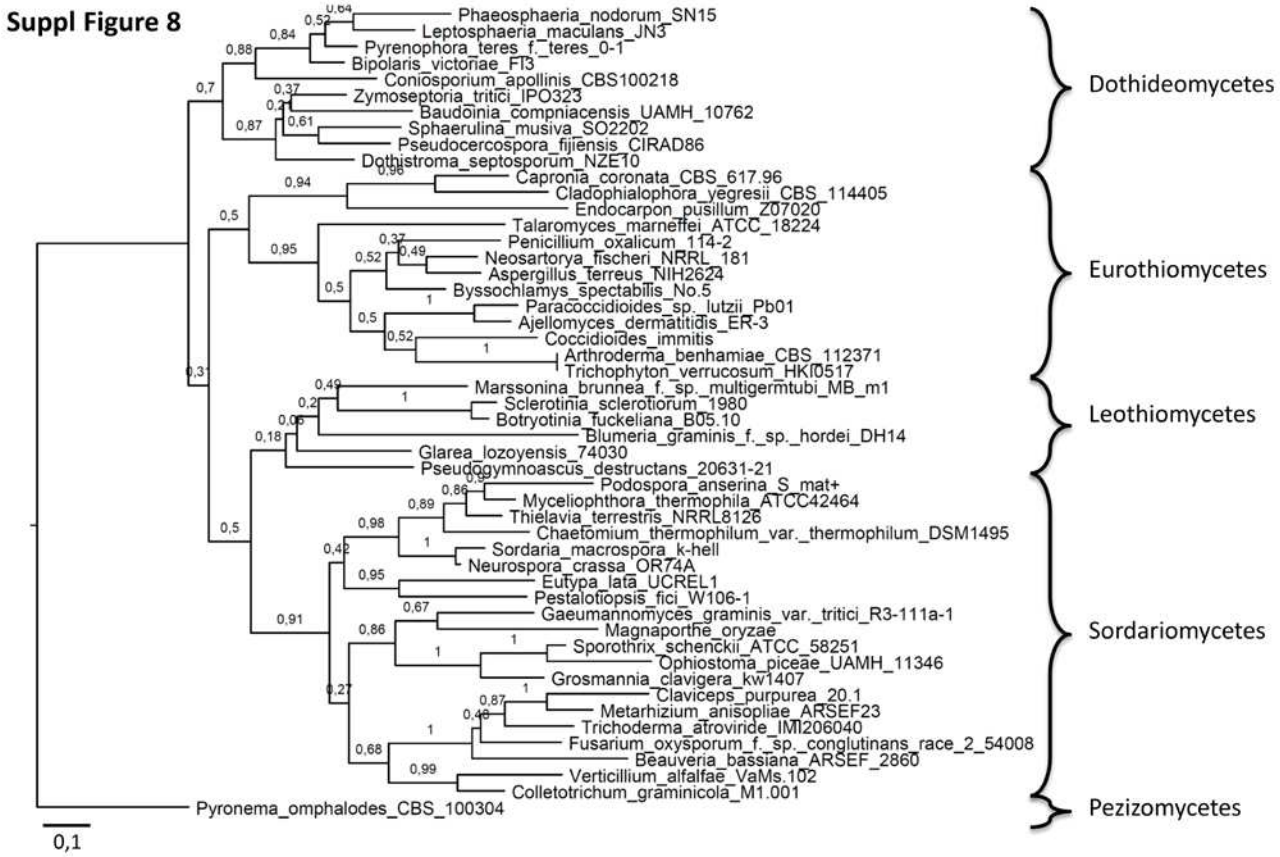

Lower
non infected zone
Infected zone
Upper
non infected zone
ARGININE
HISTIDINE
ASPARAGINE
LYSINE
THREONINE
24
48
72
96
*


Lower
non infected zone
Infected zone
Upper
non infected zone
GLYCINE
SERINE
ALANINE
LEUCINE
VALINE
24
48
72
96
*



S7 Fig. Accumulation of amino acids at the infected site and around.

Sugar and amino acid contents were quantified (presented in nmol/mg of fresh weight), during infection (times are indicated), at the site of inoculation corresponding to the ”infected zone” and one centimeter apart (respectively named “lower” and “upper non-infected zones”). For more details see Materials and Methods. A T-test (*, p-value < 0.05) was used to compare amino acid contents in leaf fragment from plants inoculated with the *cks1* (black bars) and *cks1*^{CKS1} (white bars).



S8 Fig. The tRNA-IPT protein is highly conserved across Ascomycetes.

Phylogenetic tree based on the primary sequence of putative orthologous tRNA-IPT proteins identified by BLASTp in Ascomycetes. Multiple protein sequences alignment was performed with MUSCLE and alignment curation with Gblocks. The phylogenetic tree was obtained using PhyML [115]. The phylogenetic tree was rooted with the farthest species, *Pyronema omphalodes*. The different classes of Ascomycetes and bootstrap values are indicated.

DISCUSSION GENERALE

I. Cytokinines végétales et défenses

Les cytokinines pourraient être connectées directement ou indirectement à plusieurs endroits avec les systèmes de défense du riz (Figure 1). Plusieurs connections éventuelles sont indiquées ci-dessous ainsi que les expériences qui pourraient permettre de tester ces hypothèses, afin de mieux comprendre comment ces hormones interfèrent avec les défenses.

Les voies des signalisations CKs de l'hôte directement impliquées dans la mise en place des défenses

Certains récepteurs de CKs et éléments de signalisation de l'hôte pourraient être requis pour l'induction des défenses via ces hormones. Par exemple chez *Arabidopsis* le récepteur AHK3 perçoit les CKs méthylées de la bactérie pour induire les défenses (Choi et al., 2011). Certains récepteurs chez le riz sont caractérisés (Choi et al., 2012b). Il serait possible de voir si, chez des mutants de riz affectés dans ces récepteurs de CKs, le phénotype de résistance à *M. oryzae* observé suite à une pulvérisation de kinétine est affecté, suggérant alors l'implication du ou des récepteurs en question dans la signalisation des CKs au cours des défenses. D'autres éléments de la voie de signalisation ont également été identifiés comme les protéines RR (Hirose et al., 2007; Jain et al., 2006; Ito and Kurata, 2006). Des expériences de double-hybride avec des éléments de la voie des CK du riz pourraient permettre d'identifier les partenaires protéiques impliqués dans la transduction du signal et d'identifier si des éléments connus des systèmes de résistance sont impliqués. En effet, il a été mis en évidence chez *Arabidopsis* que la protéine ARR2 interagit avec le facteur de transcription TGA3 et participe ainsi à l'induction du gène *PR1* (Choi et al., 2010; Argueso et al., 2012). Certains RR de riz pourraient également interagir avec des facteurs de transcription clés pour induire l'expression de gènes impliqués dans les défenses. L'identification et la caractérisation de ces partenaires permettrait de mieux comprendre comment les CKs sont liées aux défenses. Par ailleurs, des analyses de mutants de riz affectés dans des éléments de la voie de signalisation des CKs permettraient de tester si la régulation des phénotypes que nous avons observés passe par ces voies. Cependant de tels mutants ont souvent des phénotypes développementaux qui compliquent l'interprétation des résultats.

L'effet des CKs sur les défenses pourraient être indirect ou impliquer différentes signalisations

CKs et autres hormones

Il existe de multiples régulations entre les différentes voies hormonales végétales (Sharma et al., 2013).

Figure 1

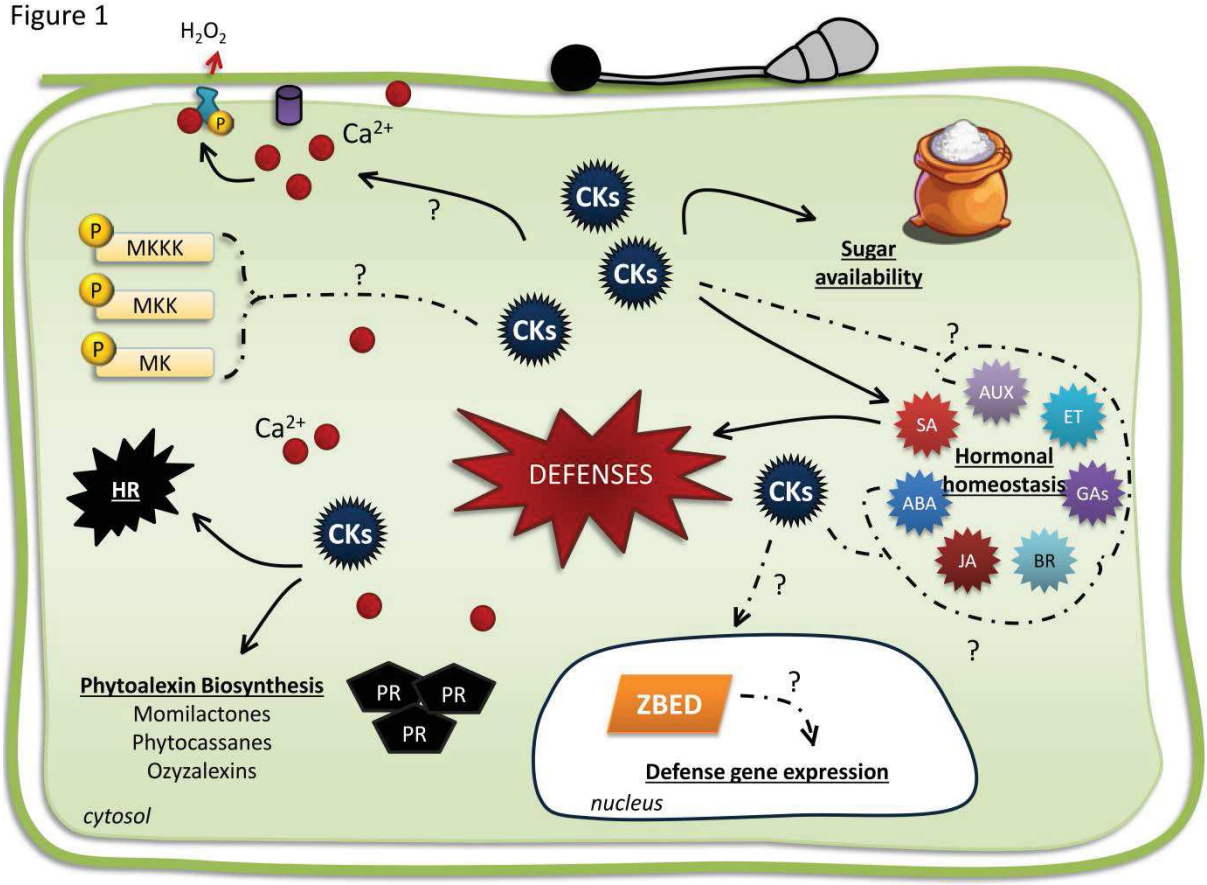


Figure 1. Schéma général des hypothèses sur le rôle des cytokinines et de ZBED dans la mise en place des défenses.

Chez le riz, des mutants exprimant un gène de biosynthèse de CKs (*IPT*) sous le contrôle d'un promoteur de réponse au stress hydrique (*pSARK*) montrent des régulations transcriptionnelles différentes des gènes impliqués dans la signalisation, les réponses ou le transport d'autres hormones comme les BR, le JA et l'auxine (Peleg et al., 2011). La résistance des mutants*ck-ugt* (chapitre II) pourrait être due à une répression ou une activation d'autres voies hormonales régulant les mécanismes de défense (autres que celle du SA). Des analyses transcriptomiques des mutants CKs hors et pendant infection, de même que des dosages hormonaux, renseigneraient sur les modifications générales qui ont lieu dans ces plantes.

Le calcium

Un traitement par des CKs semble augmenter la vitesse et l'amplitude des réponses moléculaires à *M. oryzae*. Ce type de phénotype a été observé chez des mutants de riz affectés dans le transport du calcium (Kurusu et al., 2005). Les CKs ont déjà été montrées pour (i) favoriser les entrée calciques dans les tissus jeunes et (ii) maintenir un niveau basal de calcium au cours de la sénescence chez *Petroselinum crispum* (Huang et al., 1997). Les entrées calciques jouent un rôle crucial dans l'induction des défenses, tant la fréquence que l'amplitude de ces flux, et pourraient être impliquées dans l'effet synergique observé (Urquhart et al., 2007; Barciszewski et al., 2000). Des mutants de riz affectés dans la signalisation calcique pourraient être testés pour leur réponse aux CKs. Par exemple, il serait intéressant de mesurer l'effet d'un traitement kinétine sur la résistance de lignées de riz, disponibles dans l'équipe, sur-exprimant une Calmodulin-binding protein (Grand et al., 2012).

Activation des MAPKs ?

Il est possible que l'induction plus rapide des défenses observée suite à un traitement kinétine 48h avant inoculation soit due à une « pré-activation » des voies de signalisation des MAPKs (Barciszewski et al., 2007, 1999). Les MAPKs sont activées par phosphorylation de certains acides aminés (Agrawal et al., 2003; Nühse et al., 2000; Huang et al., 2000). Des tests d'activité MAPK par immuno-marquage permettraient de révéler si certaines protéines sont accumulées sous forme active ou non dans les mutants CKs ou dans les plantes pré-traitées avec de la kinétine. L'effet éventuel des CKs sur l'activation des MAPKs pourraient également être une conséquence de la modification des flux calciques. L'analyse de mutants MAPK (comme par exemple des sur-expresseurs de *OsMAPK5* plus sensibles à *Magnaporthe* (Xiong and Yang, 2003)) qui sont disponibles permettrait de savoir si la résistance à *Magnaporthe* induite par les CKs nécessite des éléments de la voie de signalisation des MAPK.

Les effets des CKs sur les mécanismes de défenses

La HR

Les CKs pourraient agir sur la HR comme observé chez *A. thaliana* (Carimi et al., 2004) ou lors d'autres interactions plante-pathogène (Swartzberg et al., 2008). L'effet des CKs sur la HR pourraient impliquer des PR10. Ces PR protéines présentent une activité RNase et induisent la mort cellulaire (Choi et al., 2012a; Kim et al., 2011). L'une des plus étudiée chez le riz est PBZ1 dont l'effet sur l'induction de la mort cellulaire a été mis en évidence chez le tabac (Kim et al., 2011). Les gènes *PR10* peuvent être induits en réponse à l'acide salicylique, comme *PBZ1*, d'autres par l'acide jasmonique comme *RSOsPR10* (Nakashita et al., 2001; Hashimoto et al., 2004). Chez d'autres espèces, il a été mis en évidence que des protéines PR10 interagissent directement avec les CKs (Zubini et al., 2009; Fernandes et al., 2008). Toutefois, l'effet de l'interaction avec les CKs sur l'activité RNase ou la structure de ces protéines reste inconnu, de même que leur spécificité de reconnaissance (Fernandes et al., 2009). L'affinité des PR10 pour différentes CKs végétales ou fongiques pourrait être testée en condition *in vitro*. Des plantes de riz sur-exprimant le gène PBZ1 sont à présent disponibles au laboratoire et pourraient constituer un matériel utile pour répondre à la question des liens potentiels entre les protéines PR10, les CKs et la mort cellulaire.

CKs et homéostasie des sucres

L'effet des CKs sur les défenses pourraient faire intervenir le métabolisme des sucres. Les CKs assurent le maintien de la photosynthèse en induisant la synthèse de chlorophylle et de RuBisCo (Wingler et al., 1998). Une photosynthèse plus efficace tend à une production de carbohydrates plus importante. Au cours d'une infection par un pathogène fongique, une diminution de l'activité photosynthétique est souvent observée ; or les sucres agissent également comme des molécules signal pour la mise en place des défenses (Morkunas and Ratajczak, 2014; Herbers et al., 1996).

Les CKs affectent également l'homéostasie des sucres en activant des invertases pariétales qui convertissent le saccharose en glucose et fructose (Walters and McRoberts, 2006). Les invertases sont induites lors d'une attaque pathogène et certains agents pathogènes en sécrètent (Voegele et al., 2006; Walters and McRoberts, 2006). Suite à l'induction de l'activité invertase, la photosynthèse chute, probablement à cause de l'accumulation d'hexoses (Goldschmidt and Huber, 1992). Cependant les CKs contribuent à maintenir la photosynthèse au cours de certains stress ou lors de la senescence. Chez le tabac, l'induction d'un inhibiteur d'invertase sous le contrôle d'un promoteur sensible aux CKs entraine une perte du retardement de la sénescence par la kinétine, suggérant que l'activité invertase induite par les CKs est requise pour maintenir la photosynthèse [33, et refs citées]. Une meilleure gestion du métabolisme énergétique en présence de CKs pourrait permettre une mise en place plus efficace des défenses. Le phénotype de résistance observé chez les mutants *ck-ugt* pourrait être lié à une accumulation de sucres différente, pouvant être testée par dosages,

conséquente à une activité photosynthétique plus importante, elle aussi mesurable. Chez le riz, l'invertase GIF1 a été décrite pour jouer un rôle dans la mise en place des défenses basales et induites (Sun et al., 2014). L'activation de ces enzymes par les CKs pourrait être également requise pour l'induction des défenses (Roitsch et al., 2003).

Métabolisme azoté

La régulation du métabolisme primaire est cruciale pour la mise en place des défenses (Bolton, 2009). La plante a besoin d'énergie pour mettre en place des défenses efficaces avant la pénétration de l'agent pathogène car une fois à l'intérieur ce dernier cherchera à détourner le métabolisme de la cellule infectée à son profit. La vitesse de réponse énergétique est cruciale pour une mise en place de défenses efficaces.

Le lien entre les CKs et le métabolisme de l'azote a été mis en évidence chez plusieurs espèces. Les CKs modifient la répartition de l'azote chez le tabac (Jordi et al., 2000) et conduisent à des régulations transcriptionnelles des gènes du métabolisme et du transport de l'azote chez *Arabidopsis* (Sakakibara et al., 2006). Chez le riz, une fertilisation azotée active une biosynthèse *de novo* de CKs dans les feuilles (Kamada-Nobusada et al., 2013) et augmente la sensibilité à *M. oryzae* chez certains cultivars (Ballini et al., 2013). Des travaux précédents menés dans l'équipe montrent que suite à une fertilisation azotée les plantes sont plus sensibles à *M. oryzae,* un phénomène appelé « Nitrogen Induced Susceptibility », également observé au champ (Ballini et al., 2013). La synthèse *de novo* de CKs conséquente à l'apport d'azote (Kamada-Nobusada et al., 2013) est-elle impliquée dans la NIS ? Un dosage des CKs dans des plantes NIS pourraient apporter des réponses à cette question.

La protéine ZBED : un nouveau lien possible entre résistance et CKs

Enfin, l'analyse des plantes ZBED pourrait fournir des informations sur les liens entre les CKs et la résistance. En effet, plusieurs éléments suggèrent que ce gène est connecté à la fois aux CKs et aux défenses : (i) les plantes sur-exprimant *ZBED* sont plus résistantes à *Magnaporthe* (cf Chapitre I), (ii) des analyses *in silico* ont montré que l'expression du gène *ZBED* était corrélée à celle des gènes intervenant dans la signalisation des CKs et (iii) des expériences préliminaires montrent que les plantes OX-*ZBED* tendent à répondent plus fortement à une pulvérisation de kinétine avant inoculation. Enfin, les analyses menées au laboratoire (P. Bidzinski et A. Ducasse, communication personnelle) indiquent par ailleurs que la protéine ZBED interagit (données doubles hybrides et co-immuno précipitation) avec plusieurs protéines dont une potentiellement reliée aux défenses, le facteur de transcription OsWRKY4. L'analyse approfondie des plantes *OX-ZBED* pourrait donc nous renseigner sur les liens entre CK et résistance.

II. Cytokinines fongiques et virulence

Les fonctions putatives des CKs dans l'interaction entre le riz et *Magnaporthe* sont résumées dans la Figure 2.

A l'issue du chapitres III, nous ne pouvons pas totalement exclure la possibilité que le phénotype du mutant *cks1* ne résulte pas, au moins en partie, d'un effet pléiotrope de la mutation qui limiterait la croissance du champignon *in planta* (mais pas en condition *in vitro* standard). Ce retard éventuel de croissance aboutirait à une induction plus forte des défenses de la plante et à des modifications des métabolismes énergétiques différentes. Pour tester cette hypothèse, il serait par exemple intéressant de disposer de mutants de *Magnaporthe* sur-produisant des CKs (probablement toujours pathogènes) qui pourraient alors montrer une inhibition des défenses. A notre connaissance, la sur-expression d'une tRNA-IPT n'a jamais été effectuée chez un champignon et pourrait nous aider à tester cette hypothèse. Un test allant dans ce sens pourrait aussi consister à examiner le polymorphisme naturel de la production de CK par *Magnaporthe* (il semble en exister, (Jiang et al., 2013)). Il serait intéressant de mesurer la virulence et la production de CKs *in vitro* et *in planta* de plusieurs isolats.

D'autres traits comme la fitness des souches ainsi que leur spectre d'hôte permettraient de mieux comprendre l'implication des CKs fongiques dans le pouvoir pathogène. Toutefois dans cette approche, le polymorphisme lié au répertoire d'effecteurs protéiques classiques pourrait considérablement brouiller les analyses.

De même, l'existence des mutants *cks1* permet de reposer la question de la sécrétion des CKs dans les plantes. En effet, seuls quelques exemples de localisation de CKs dans les cellules de plantes infectées existent (Hu and Rijkenberg, 1998) mais n'apportent pas la preuve que les CKs observées proviennent de l'agent pathogène. Déterminer que les CKs fongiques sont bien sécrétées et identifier le (ou les) compartiments sub-cellulaires dans lesquelles on les trouve permettrait aussi d'apporter des éléments sur les éventuelles cibles de ces CKs. L'utilisation de milieux de croissance contenant les molécules précurseurs des CKs marquées permettrait de discriminer les CKs fongiques des CKs végétales pendant infection. Compte tenu du rôle paradoxal des CKs, à la fois participant à la résistance (Chapitre II) et à la fois à la virulence (Chapitre III), on peut émettre l'hypothèse que le timing de production des CKs par le champignon est déterminant pour l'issue de l'interaction. Des souches de *Magnaporthe* exprimant une construction RNAi ciblant *CKS1* à différents stades d'infections (grâce à des promoteurs spécifiques disponibles) pourraient être testées. De telles expériences, certes complexes, permettraient de mieux comprendre le rôle des CKs de *Magnaporthe* au cours des différentes phases d'infection (biotrophe et nécrotrophe).

Figure 2

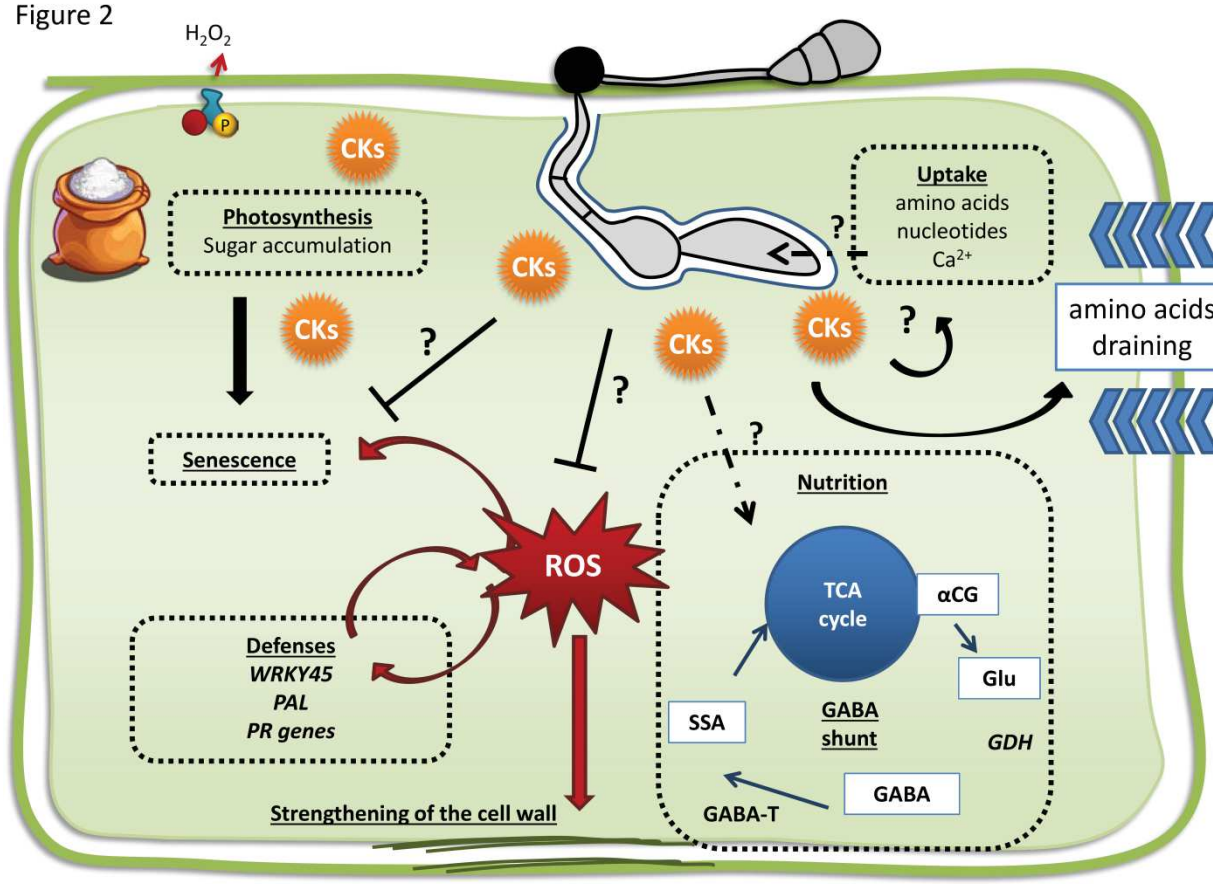


Figure 2. Hypothèses du rôle des cytokinines fongiques comme facteurs de virulence de *Magnaporthe oryzae*

Voie de signalisation des CKs chez *Magnaporthe*

Les CKs semblent affecter des mécanismes similaires entre plantes et champignons comme les flux calciques, la division et la différenciation cellulaire et certains mécanismes nutritionnels. Toutefois leur perception par les champignons ainsi que les voies de signalisation impliquées restent inconnues. Certaines protéines orthologues de la voie de signalisation des CKs ont été identifiées chez *M. oryzae* (Cf table S1 Chapitre III, p162). La signalisation des CKs implique un système de transduction à deux composantes (Sakakibara, 2006). Chez les plantes ces systèmes sont également impliqués dans la signalisation de l'éthylène et de certains stress abiotiques comme la sècheresse (Bertheau et al., 2012, 2013).

Chez la levure un seul récepteur transmembranaire de type histidine kinase est connu, SLN1. Cette protéine intervient dans la réponse à l'environnement osmotique avec la protéine navette YPD1 et le régulateur de réponse SSK1 (Figure 2) (Posas et al., 1996).

En condition d'osmolarité élevée, SSK1 engendre l'activation d'une cascade de MAPKs impliquant la MAPK HOG1. Toutes ces protéines sont conservées chez d'autres ascomycètes comme *Magnaporthe* (Zhao et al., 2007) et *Neurospora* où ils contribuent à la régulation de mécanismes similaires (Alex et al., 1996). Parmi eux, certains sont également requis pour la virulence de *Magnaporthe* et/ou la résistance à certains stress abiotiques. Par homologie, une dizaine de protéines présentant les caractéristiques de récepteurs HK ont été identifiées chez *Magnaporthe.* Certains présentent des caractéristiques communes avec les récepteurs de l'éthylène dont la signalisation semble également assurée par un système de transduction à deux composantes chez les champignons. L'orthologue du récepteur SLN1 chez *Magnaporthe* a été identifié et des mutants ont été générés. Chez ces mutants, l'expression des gènes associés à la biosynthèse de mélanine ou à l'intégrité de la paroi sont plus faiblement exprimés, la production de ROS est dérégulée et ils s'avèrent qu'ils sont plus sensibles à certains stress (osmotique, oxydatif) et avirulents. Le mutant *ΔMosln1* est affecté dans sa capacité de pénétration, un phénotype potentiellement associé à une défaillance dans l'accumulation de métabolites dans l'appressorium qui permet la montée de pression dans cet organe et donc la pénétration (Zhang et al., 2010b). Cependant, tout comme SLN1 de *S. cerevisiae*, aucun de ces récepteurs putatifs identifiés chez *Magnaporthe* ne semble posséder de domaine CHASE supposé requis pour la reconnaissance des CKs. D'autres domaines protéiques des HK putatifs identifiés pourraient intervenir dans la reconnaissance des CKs, mais restent toutefois à être identifiés et caractérisés pour confirmer l'implication de ce type de récepteurs dans la signalisation de ces hormones chez les champignons. Des régulateurs de réponse ont également été identifiés chez *Magnaporthe*, dont l'un possède un domaine putatif de liaison à l'ADN rappelant la structure d'un RR-B, SKN7, et l'autre non, comme les RR-A, SSK1.

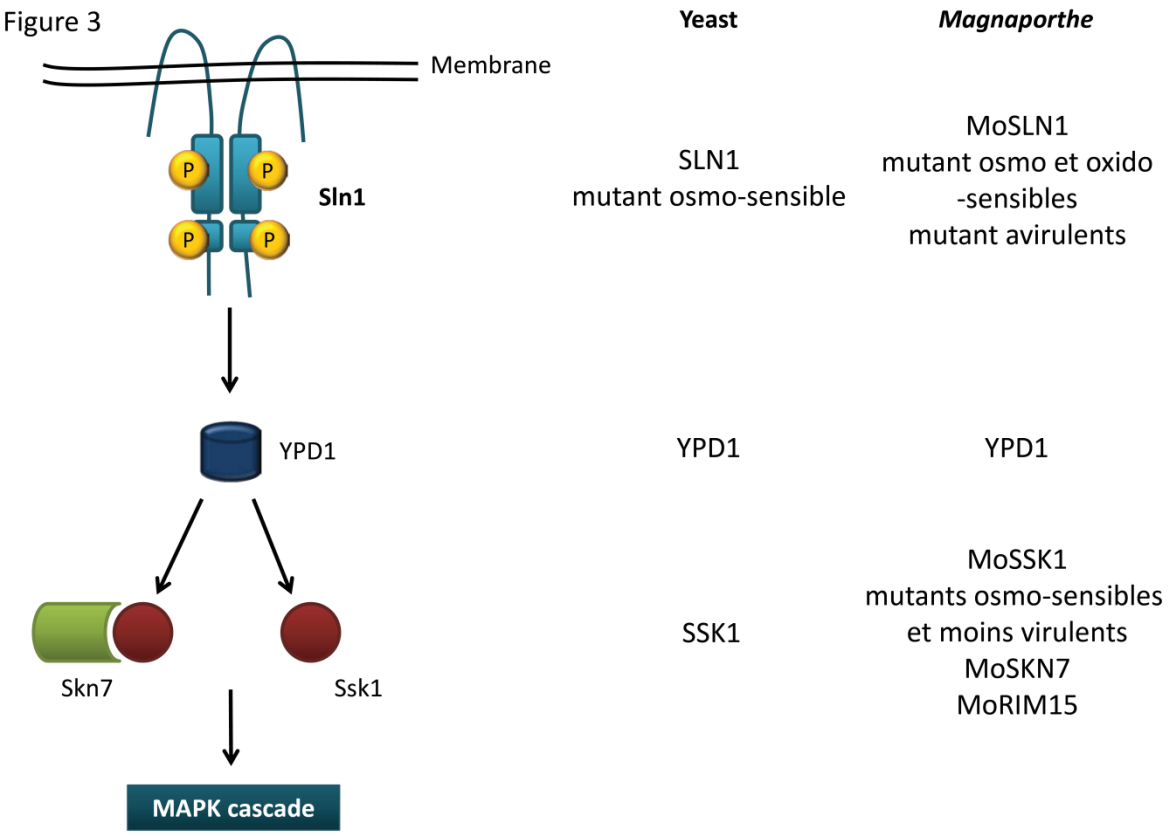


Figure 3. Voie de signalisation SLN1 chez *Saccharomyces cerevisiae* et *Magnaporthe oryzae*

Les mutants *ΔMoSSK1* sont plus sensibles à un stress osmotique et moins virulents, présentant donc des similitudes avec le phénotype des mutants *cks1*. MoSKN7 et MoSSK1 semblent tous deux être des régulateurs négatifs de la production de mélanine mais les mutants *ΔMoskn7* ne sont ni affectés dans leur virulence ni dans leur résistance au stress osmotique (Motoyama et al., 2008). Le domaine « response regulator » est également présent chez d'autres protéines en combinaison avec d'autres domaines protéique comme c'est le cas pour MoRIM15 où le domaine RR et couplé à un domaine ser/thr kinase (Motoyama et al., 2008). La plupart des mutants mentionnés montrent une sensibilité accrue aux stress oxydatif et osmotique. Analyser la réponse de ces mutants *Magnaporthe* en conditions de stress et en présence de CKs apporterait des indications sur leur implication dans la signalisation de cette voie hormonale.

CKs et reproduction sexuée chez *Magnaporthe*

La reproduction sexuée chez *Magnaporthe* nécessite la rencontre de deux hyphes de type sexuel différent (mat-1 ou mat-2) et au moins l'un des deux partenaires doit être femelle-fertile, c'est-à-dire capable de différencier les structures reproductrices appelées périthèces (Saleh et al., 2012). Les CKs ont été mise en évidence pour impacter la reproduction sexuée chez *Neurospora crassa* (LEE, 1961) et chez l'oomycète *Saprolegnia australis* (Elliott, 1967). Des expériences similaires en condition *in vitro* avec les différentes souches de *Magnaporthe* renseigneraient sur l'implication des CKs dans ce type de reconnaissance entre champignons.

LES CYTOKININES ET AUTRES INTERACTIONS BIOTIQUES

Cytokinines et interactions mutualistes

Les CKs sont impliquées dans l'induction des modifications racinaires (avec l'auxine) au cours de la mycorhization (Jane et al., 1998; Barker and Tagu, 2000). Tout comme l'ethylène, elles impactent la croissance de l'hôte et celle du symbiote (Gogala, 1991). Des plantes de luzerne, digitaire et de lin mycorhizés sont plus résistantes à la sécheresse que les plantes non-mycorhizées (Pedranzani et al., 2015; Goicoechea et al., 1995; Drüge and Schonbeck, 1993). L'accumulation de CKs observée chez les plantes en symbiose avec des champignons est corrélée à une meilleure tolérance à la sécheresse (Drüge and Schonbeck, 1993). De même les plantes mycorhizées sont d'une manière générale plus résistantes aux attaques pathogènes, on parle alors de Mycorrhizal Induced Resistance (MIR). Compte-tenu de leurs rôles dans les mécanismes de défenses chez les plantes, les CKs pourraient être requises pour la mise en place de la MIR. Morrison et *al*, 2015 ont mis en évidence que de nombreux champignons produisaient majoritairement de la *cis*Z, probablement issue de la voie de la dégradation des tRNA (Morrison et al., 2015). La mutation *cks1* pourrait être étudiée chez d'autres

organismes symbiotiques en vue de confirmer l'implication des CKs fongiques dans les interactions mutualistes et dans les effets positifs qui en découlent comme la tolérance à la sécheresse et aux attaques pathogènes. Ces effets sont également observés chez les plantes cultivées en présence de bactéries Plant Growth Promoting Rhizobacteria (PGPR) qui produisent et *a priori* perçoivent les CKs. Ces composés hormonaux pourraient donc intervenir dans la régulation des signaux issus et au sein de la rhizosphère et participer à l'intégration des signaux environnementaux par les plantes pour la mise en place d'une réponse physiologique optimale (Pozo et al., 2015). Les CKs contribuent à la mise en place des défenses des plantes en réponse à une large gamme de pathogène (Giron et al., 2013). Une étude récente montre qu'elles impactent également la physiologie d'insectes phytophages renforçant l'idée que les CKs pourraient être impliquées dans les interactions animales (Robischon, 2015)

Les tRNA-IPT sont conservées chez de nombreux organismes

Les tRNA-IPT, qui assurent l'ajout d'une chaine isopentenyl sur le tRNA, ont été décrites chez plusieurs organismes comme participant à la biosynthèse de CKs (Miyawaki et al., 2006; Siddique et al., 2015). Elles peuvent être libérées suite à la dégradation de ces tRNA modifiés. L'adénine modifiée (A37) est située juste à côté de l'anticodon qui commence par un U. Cette spécificité de reconnaissance a été observée chez procaryotes et eucaryotes. La modification de l'A37 contribue à la stabilisation du complexe ARN-ribosome-tRNA et faciliterait l'incorporation de certains acides aminés i.e. Ser, Tyr, Cys, Trp.

Chez *Agrobacterium* la délétion du gène *miaA*, codant pour une tRNA-IPT, entraine une réduction de l'expression des facteurs *vir* requis pour la virulence de cette bactérie (Gray et al., 1992). Cette diminution de l'expression des facteurs de virulence suite à la mutation *miaA* a également été observée chez des bactéries pathogènes de l'Homme (Durand et al., 1997). En accord avec ces données, l'analyse de plusieurs génomes bactériens d'agents pathogènes de l'Homme a permis de mettre en évidence un panel de gènes pouvant être associés à la virulence dont le gène *MiaA* (Garbom et al., 2004). Il est possible que chez les mutants *cks1* de *Magnaporthe* la traduction de certaines protéines requises pour le pouvoir pathogène soit également affectée. Le gène *MiaA* est impliqué dans la production de CKs chez les bactéries phytopathogènes. Pourtant, bien que les CKs affectent certains métabolismes cellulaires humains (Ishii et al., 2005; Slaugenhaupt et al., 2004; Rattan and Clark, 1994; Ishii et al., 2002; Griffaut et al., 2004), aucune étude n'a été menée sur la capacité de production de CKs chez les mutants *miaA* de bactéries pathogènes de l'Homme et le lien potentiel avec leur de virulence.

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Magnaporthe oryzae est un champignon filamenteux responsable de la principale maladie du riz, la pyriculariose. Ce pathosystème est très étudié, notamment dans le but de contribuer à l'identification de facteurs pouvant permettre le développement de résistances efficaces. Si certaines hormones végétales, comme l'acide salycilique, sont requises pour la mise en place des défenses de la plante, d'autres sont impliquées dans des processus développementaux. Parmi elles, les cytokinines (CKs) sont des dérivés d'adénine décrites pour participer à la croissance et la différenciation de l'appareil aérien et racinaire. Elles contribuent à la répartition des nutriments et impactent également la viabilité des cellules, en retardant la senescence ou en induisant la mort cellulaire. Des études précédentes ont montré que les CKs pouvaient perturber la résistance de la plante hôte dans différents pathosystèmes. Chez le riz, les CKs agissent en synergie avec l'acide salicylique pour induire l'expression des gènes marqueurs des défenses. Cependant aucun phénotype de résistance associé aux CKs n'a été observé *in planta*. Mes travaux montrent qu'un apport exogène de CKs (kinétine, BAP) affecte la résistance du riz à *Magnaporthe* avant infection, de manière dose dépendante. Le phénotype de résistance observé est corrélé avec une plus forte expression des défenses pendant infection, limitant la pénétration et l'invasion du champignon. Des plantes de riz mutées pour une probable cytokinine UDP-glucosyl transferase (CK-UGT) ont été obtenues. Ces mutants *ck-ugt* sont affectés dans le métabolisme des CKs et sont également plus résistants à *M. oryzae.* Hors infection, une plus forte expression des gènes de défense a été mesurée chez les plantes mutantes, confirmant que les CKs endogènes affectent directement ou indirectement les défenses de l'hôte. En parallèle de ces analyses sur la plante, mes travaux ont aussi porté sur le rôle des CKs produites par *M.oryzae.* En effet, leur rôle dans l'interaction ainsi que la voie de leur biosynthèse chez le champignon n'était pas caractérisé. Conservées au sein des différents organismes, les tRNA-IPT (isopentenyl transferase) sont décrites pour participer à la biosynthèse de CKs. Un seul gène homologue a été identifié chez *M. oryzae* et nommé *CKS1* car sa délétion abolit la production de CKs. Le mutant de *Magnaporthe cks1* est moins virulent (pénétration et invasion *in planta* réduites*)* que la souche témoin complémentée. Il induit une plus forte accumulation des espèces actives de l'oxygène et une plus forte expression des défenses chez la plante. Les dosages des acides aminés et des sucres pendant infection ont montré que les concentrations de ces nutriments étaient différemment perturbées par la souche déficiente en CKs. Ces résultats suggèrent que les CKs fongiques pourraient être requises pour affecter la répartition des acides aminés et contribuer une accumulation progressive de sucres au cours de l'infection. Ainsi, chez un champignon qui n'induit pas de tumeurs, les CKs pourraient agir comme des effecteurs qui auraient une double fonction d'inhibition des défenses et de drainage des nutriments. Chez les champignons, ces hormones induisent également des réponses physiologiques comme la résistance à certains stress, les processus de nutrition et la reproduction sexuée. Ces effets ont été étudiés chez *Magnaporthe* dans différentes conditions de croissance *in vitro* plus ou moins stressantes. Les résultats indiquent que les CKs augmentent la tolérance au stress osmotique et oxydatif et suggèrent qu'elles affecteraient aussi l'absorption des nutriments ainsi que la reproduction sexuée. Comme le gène *CKS1* est conservé, cette mutation peut être caractérisée chez d'autres organismes fongiques présentant des modes de vie différents de manière à mieux comprendre le rôle de ces hormones dans les interactions plante-microorganisme mais également au sein des interactions microbiennes.

The blast disease caused by *Magnaporthe oryzae* is one of the most devastating diseases on rice leading to important yield loss. Plant hormones, like salicylic acid, play a central role in plant resistance establishment. Among these hormones, cytokinins (CKs) are adenine derivatives well described to modulate root/shoot growth and differentiation, cell viability and nutrient distribution. Previous studies have shown that these hormonal compounds can also affect plant host resistance in different pathosystems involving monocot or dicot host plants and microbes (bacteria, oomycetes or fungi). In rice, CKs were described to act synergistically with the salicylic acid pathway to induce defense marker genes expression. However, no resistance phenotype associated with CKs was observed and the way that CKs could act *in planta* during infection is still unknown. In this work, a resistance phenotype induced by exogenous application of the CK kinetin was characterized and the role of endogenous CKs in rice resistance was investigated by phenotyping plant CK mutants. An exogenous supply of kinetin before infection led to a higher induction of defense marker genes that was associated with limited fungal penetration and invasion, suggesting. However the way CKs affected resistance or susceptibility (or virulence see below) depended on the timing at which they were applied (before or after inoculation). Rice lines mutated for a putative cytokinin- UDP-glycosyl transferase (CK-UGT) were produced. The *ck-ugt* mutants were more resistant, suggesting that endogenous CKs can also contribute to resistance. Defense marker genes were expressed higher in the absence of infection in the *ck-ugt* rice mutants, compared to the WT plants. In parallel of these analyses of CK on the plant side, we studied the possible role of CK produced by *Magnaporthe.* Indeed *M.oryzae* produces and secretes CKs. However, the way fungal CKs are involved in the rice blast disease development as well as the biosynthesis pathway in *M.oryzae* were not established. A putative tRNA-IPT (isopentenyl transferase) conserved across organisms was identified in *M.oryzae.* Mutant analysis of this gene confirmed that this enzyme, thus named *CKS1*, is required for CK production. Knock-out *cks1* fungal mutants were less virulent on rice, affected in penetration and invasion compared to the control complemented strain. They triggered a stronger accumulation of reactive oxygen species and a higher expression of defense marker genes. Aspartate and glutamate, two amino acids important for *M.oryzae* growth, were differently affected at and around the infected zone by *cks1* strain suggesting that fungal CKs could contribute to drain/consume nutrients during infection. Similarly, sugar accumulation was also differently disturbed, indicating that fungal-derived CKs may be required for maintaining a progressive sugar production during host invasion, probably by affecting photosynthesis process. Our results show that fungal CKs, in a non-gall forming fungal pathogen, could act as dual effectors by inhibiting defense and modifying nutrient fluxes. Furthermore, CKs are known to affect some physiological processes in fungi, like stress resistance, nutrition or sexual reproduction. In order to test whether CKs modulate *Magnaporthe* stress tolerance, the effect of CKs on the mycelial growth in different stressful conditions *in vitro* was tested. The results indicate that CKs increased osmotic and oxidative stress tolerance and suggest that they also affected nutrient acquisition as well as sexual reproduction. Since the *CKS1* gene is highly conserved, the effect of the *cks1* mutation could be studied in other fungi showing different lifestyles for improving our knowledge on the role these hormonal compounds play among microbes or in plant-microbe interactions.