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Mahsa Farjad

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NNT : 2017SACLS215

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Sciences du Végétal : du Gène à l'Ecosystème  
Spécialité de doctorat ([Biologie](#))

Par

**Mme Mahsa FARJAD**

Impact de la nutrition azotée sur l'interaction entre *Arabidopsis thaliana* et la  
bactérie phytopathogène *Erwinia amylovora*

**Thèse présentée et soutenue à l'INRA de Versailles, le 30.08.2017 :**

**Composition du Jury :**

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## LISTE DES ABREVIATIONS

ABA	Acide abscissique
ADN	<i>Acide DésoxyriboNucléique</i>
AOC	<i>Allene Oxide Cyclase</i>
AOS	<i>Allene Oxide Synthase</i>
ARN	<i>Acide RiboNucléique</i>
AtNRT	<i>Nitrate Transporter</i>
ATP	Adénosine TriPhosphate
ATPase	Adénosine TriPhosPhatase
Avr	<i>Avirulence</i>
AWF	<i>Apoplast Washing Fluid</i>
CAMTA	<i>Calmodulin-binding transcription activator</i>
CC-NBS-LRR	<i>Coil-Coiled NucleotideBinding Leucine-Rich-Repeat</i>
CERK1	<i>Chitin Elicitor Receptor Kinase 1</i>
CFBP	Collection Française de Bactéries Phytopathogènes
CNGC	<i>Cyclic Nucleotide-Gated Channels</i>
COI1	<i>COronatine Insensitive 1</i>
CTR1	<i>Constitutive Triple Response 1</i>
CYP	<i>Cytochrome P</i>
DAB	<i>3,3'-DiAminoBenzidine</i>
DAMPs	<i>Damage Associated Molecular Patterns</i>
Ea	<i>E. amylovora</i>
EDS	<i>Enhanced Disease Susceptibility</i>
EFR	<i>EF-Tu Receptor</i>
EF-Tu	<i>Elongation Factor-Thermo unstable</i>
EIN	<i>Ethylene Insensitive</i>
EPS	<i>ExoPolySaccharide</i>
ERF	<i>Ethylene Response Factor</i>
ET	Ethylène
ETI	<i>Effector Triggered Immunity</i>
ETR	<i>Ethylene Response</i>
ETS	<i>Effector Triggered Susceptibility</i>
FLS2	<i>FLagelling Sensing 2</i>
GABA	<i>γ-Amino Butyrique Acid</i>
GS	<i>Glutamine Synthase</i>
H <sub>2</sub> O <sub>2</sub>	Peroxyde d'hydrogène
Hop	<i>Hrp Outer Protein</i>
HR	<i>Hypersensitive Response</i>
Hrc	<i>Hrp-conserved</i>
Hrp	<i>Hypersensitive Response and Pathogenicity</i>

ICS1	<i>Isochorismate Synthase 1</i>
IM	<i>Inducing Medium</i>
JA	<i>Jasmonic Acid</i>
JA-Ile	<i>Jasmonic Acid Isoleucine</i>
JAR1	<i>Jasmonate Resistant 1</i>
JAZ	<i>Jasmonate ZIM domain protein</i>
JMT	<i>Jasmonic Acid Carboxyl Methyltransferase</i>
JR1	<i>Jacalin lectin family protein</i>
LB	<i>Luria-Bertani</i>
LOX	<i>Lipoxygenase</i>
LRR	<i>Leucine Rich Repeat</i>
MAMPs	<i>Microbe Associated Molecular Patterns</i>
MAPK	<i>Mitogen Associated Protein Kinase</i>
MeJA	<i>Methyl Jasmonate</i>
NADPH	<i>Nicotinamide Adénine Dinucléotide Phosphate</i>
NBS-LRR	<i>Nucleotide-Binding Site/Leucine-Rich-Repeat</i>
NDR1	<i>Non-Race-Specific Disease Resistance1</i>
NIR	<i>Nitrite Reductase</i>
NO	<i>Nitric oxyde</i>
NPR	<i>Non expresser of PR genes</i>
NR	<i>Nitrate Reductase</i>
O <sub>2</sub> <sup>-</sup>	<i>Ion superoxyde</i>
OPDA	<i>12-Oxo-PhytoDienoic Acid</i>
ORA59	<i>Octadecanoid-Responsive Arabidopsis59</i>
PAD	<i>Phytoalexin Deficient</i>
PAL1	<i>Phenylalanine Ammonia Lyase 1</i>
PDF1.2	<i>Plant Defensin1.2</i>
PTI	<i>PAMP-triggered immunity</i>
PR	<i>Pathogenesis Related</i>
PRR	<i>Pattern recognition receptors</i>
R	<i>Résistance</i>
RLK	<i>Receptor-like kinase</i>
RLP	<i>Receptor-like proteins</i>
ROS	<i>Reactive Oxygen Species</i>
SA	<i>Acide Salicylique</i>
SAR	<i>Systemic Acquired Resistance</i>
SID2 (=ICS1)	<i>SA induction Deficient</i>
Sp.	<i>Species</i>
T3SS	<i>Type III secretion system</i>
TIR-NB-LRR	<i>Toll-Interleukin-1 Receptor Nucleotide-Binding Leucine-Rich-Repeat</i>
TRX	<i>Thioredoxin</i>
VSP	<i>Vegetative Storage Protein</i>
WR3	<i>Wound-Responsive gene 3</i>

## INTRODUCTION

### A. Généralités

Les plantes, dans leur milieu naturel, rencontrent une vaste gamme de micro-organismes pathogènes tels que les champignons, les oomycètes, les bactéries, les virus et les nématodes. Ces agents pathogènes délivrent des facteurs de pathogénie dans la plante, ce qui leur permet de promouvoir leur virulence et causer des maladies. Pour faire face à ces attaques, les plantes ont développé des mécanismes moléculaires leur permettant de percevoir les agents pathogènes et de mettre en place leurs défenses. Les grandes lignes des connaissances actuelles sur ce sujet sont présentées dans cette introduction.

### B. Reconnaissance de l'agent pathogène

La détection et la transduction de signaux permettant de faire face à des stress et des modifications environnementales sont des événements cruciaux pour l'adaptation des plantes et leur survie. Le système immunitaire de la plante se compose de deux voies de perception et de signalisation interconnectées, l'immunité déclenchée par les PAMPs<sup>1</sup>, la PTI<sup>2</sup>, et l'immunité déclenchée par les effecteurs, l'ETI<sup>3</sup>. La PTI est déclenchée par la reconnaissance de molécules microbiennes très conservées, les PAMPs, comme la chitine fongique, les lipopolysaccharides bactériens, les peptidoglycanes, les facteurs de *quorum sensing* et la flagelline. La perception de ces PAMPs se fait par des récepteurs de reconnaissance (PRRs) à la surface des cellules de la plante. De plus, certains PRRs reconnaissent les signaux de « danger » dérivés de l'hôte (*damage-associated molecular patterns* ou DAMPs) tels que des peptides de la plante ou des fragments de paroi cellulaire libérés pendant une infection ou une blessure. La reconnaissance des motifs PAMPs et/ou DAMPs initie l'activation de l'immunité de type PTI. Pour contourner la PTI, les microorganismes pathogènes adaptés à leur hôte ont acquis la capacité de réprimer la PTI. En outre, de nombreux agents pathogènes produisent des effecteurs dans les plantes hôtes, ce qui entraîne une virulence accrue grâce à la répression de la défense. En parallèle, certains effecteurs sont directement ou indirectement reconnus par des protéines de résistance (R) de la plante qui sont des récepteurs intracellulaires contenant des domaines de liaison

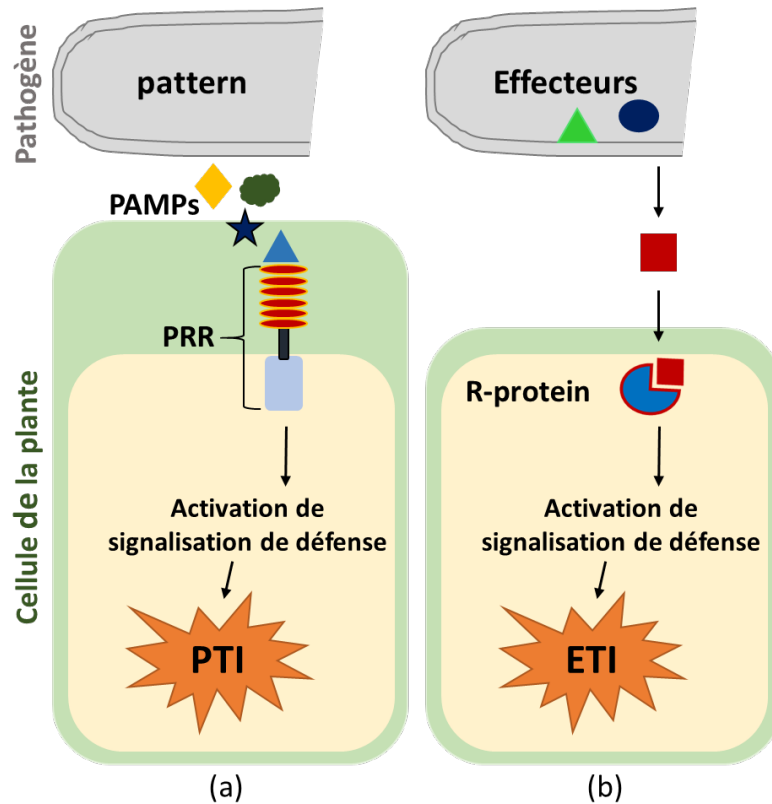
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<sup>1</sup> PAMP : *pathogen-associated molecular pattern*

<sup>2</sup> PTI: *PAMP-triggered immunity*

<sup>3</sup> ETI: *Effector-triggered immunity*

nucléotidique (NB) et de répétition riche en leucine (LRR). Cette reconnaissance active ensuite l'immunité de type ETI. L'ETI constitue un programme robuste de défense, aboutissant souvent à une mort cellulaire localisée (HR) (Monaghan and Zipfel, 2012) (Figure 1).



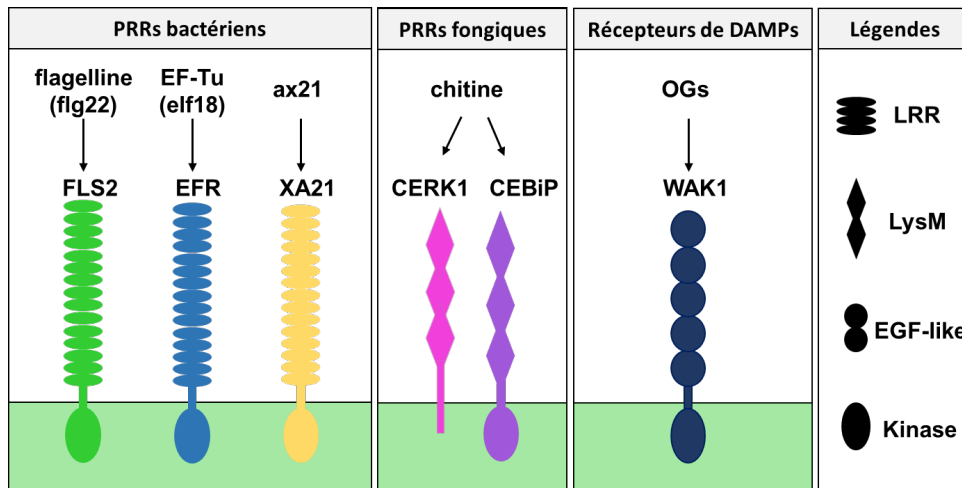
**Figure 1. Les réponses immunitaires des plantes aux agents pathogènes.** Le Schéma simplifié indique (a) la reconnaissance des PAMPs par les PRRs et l'activation des réponses immunitaires déclenchées par les PAMPs (PTI), (b) la reconnaissance des effecteurs par les protéines R et l'activation des réponses de défense déclenchées pas les effecteurs (ETI) (Mengiste, 2012).

### 1. PTI et la reconnaissance des PAMPs

Tous les PRRs connus de plantes sont des récepteurs kinases membranaires (*receptor-like kinases* ou RLKs) ou des protéines de type récepteur (*receptor-like proteins* ou RLPs) avec des domaines fonctionnels modulaires. Les PRRs les plus étudiés sont les RLKs FLS2, EFR et XA21 qui appartiennent à la sous-famille XII des RLK contenant de LRR. L'épitope flg22, présent à l'extrémité N-terminale conservée de la flagelline, est reconnu par la plupart des espèces végétales et le récepteur de la flagelline, FLS2 a été identifié chez *Arabidopsis thaliana*, la tomate, *Nicotiana benthamiana* et le riz. Comparativement, l'EFR est un PRR

spécifique des Brassicaceae qui reconnaît le facteur d'élongation bactérien Tu (EF-Tu). XA21 est codé par un locus conférant une résistance à *Xanthomonas oryzae* pv. *oryzae* chez le riz. Le ligand de XA21 a été identifié comme étant une protéine sécrétée, Ax21, conservée chez les espèces de *Xanthomonas* (Lee, et al., 2013).

Certains RLKs et RLPs contiennent un domaine LysM de liaison aux sucres (Figure 2). La première preuve d'un rôle des protéines contenant un domaine LysM dans la reconnaissance de PAMPs chez les plantes a été apportée par l'identification du LysM-RLP CEBiP comme une protéine de liaison et de reconnaissance de la chitine chez le riz (Kaku, et al., 2006). CEBiP forme un complexe hétéromérique avec la chitine et le LysM-RLK CERK1. Bien qu'il n'a pas été établi de liaison entre OsCERK1 et la chitine, OsCERK1 est nécessaire pour la réponse à la chitine chez le riz (Shimizu, et al., 2010). Chez *A. thaliana*, cependant, AtCERK1 est la principale protéine de liaison à la chitine nécessaire pour les réponses induites par la chitine (Monaghan and Zipfel, 2012). Certains PRRs ont été identifiés pour la reconnaissance de DAMPs. Par exemple WAK1 (Figure 2), un RLK contenant le domaine EGF-like permet la perception des oligogalacturonides libérés des parois de cellules de plante lors d'une infection fongique ou d'une blessure (Brutus, et al., 2010).

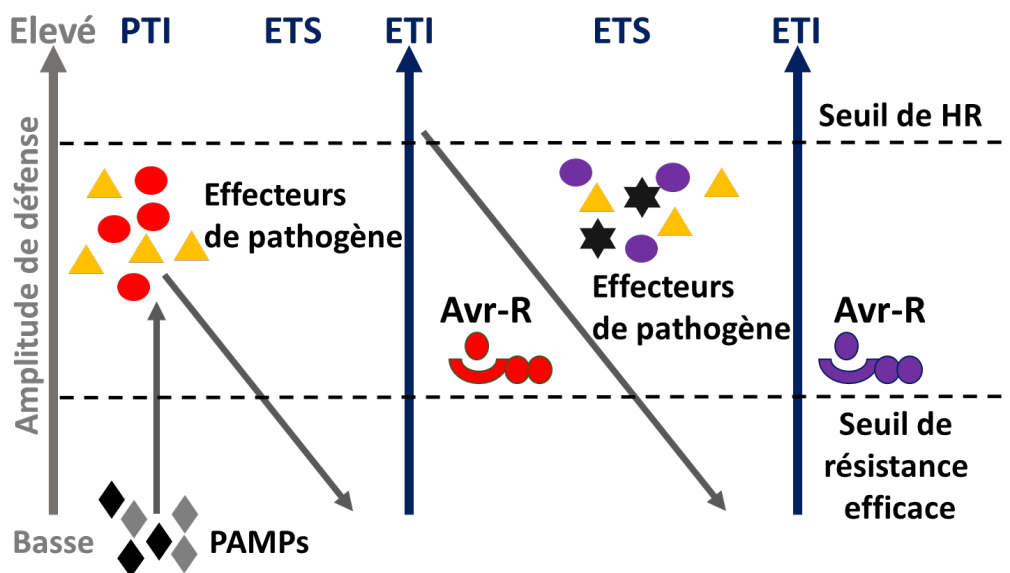


**Figure 2. Les récepteurs de type PRR avec des ligands connus.** Le LRR-RLK FLS2 reconnaît la flagelline bactérienne (ou l'épitope actif de flg22). EFR reconnaît le facteur d'élongation bactérien Tu (ou l'épitope actif d'elf18), et XA21 se lie à un peptide appelé Ax21. Le LysM-RLP CEBiP se lie à la chitine et interagit avec le LysM-RLK CERK1. Le RLK WAK1 est un récepteur pour les oligogalacturonides dérivés de la paroi cellulaire (Monaghan and Zipfel, 2012).



## 2. ETI et la reconnaissance des effecteurs de pathogènes dans les cellules

La signalisation par l'ETI correspond à un système de reconnaissance des agents pathogènes génétiquement déterminé par les gènes de résistance de la plante (gènes R). Dans ce système de résistance appelé « gène-pour-gène », un gène R donné confère la résistance aux souches pathogènes portant le gène d'avirulence (Avr) correspondant. L'événement de reconnaissance impliquant les produits des gènes R et Avr déclenche une réponse rapide et forte de défense de l'hôte, incluant généralement une réponse hypersensible (HR<sup>4</sup>), qui limite la propagation de l'agent pathogène. La plupart des protéines R connues contiennent un domaine de liaison nucléotidique (NBS<sup>5</sup>) et des répétitions riches en leucine (LRR<sup>5</sup>), ce dernier domaine étant impliqué dans la reconnaissance de l'agent pathogène. Les protéines NBS-LRR<sup>5</sup> se répartissent en deux grandes sous-classes en fonction de leurs domaines N-terminaux : celles qui ont un domaine *Toll-interleukine 1* (TIR) sont appelées TNL et celles qui ont un domaine *coiled-coil* (CC) sont appelées CNL (Cui, et al., 2015). En revanche, les protéines Avr sont plus diverses en termes de fonction, qu'elle soit connue ou supposée. La relation antagoniste entre les gènes R et Avr entraîne un conflit co-évolutionnaire car la sélection favorise l'évolution de la résistance des plantes et la virulence de leurs agents pathogènes (Dodds, et al., 2006).



**Figure 3 : Le modèle en zigzag du système immunitaire des plantes.** Dans la phase 1, les plantes détectent des molécules associées aux microbes / pathogènes (PAMP, diamants) via les PRRs pour déclencher l'immunité de type PTI. Dans la phase 2, les agents pathogènes

<sup>4</sup> *hypersensitive response*: HR

<sup>5</sup> *nucleotide-binding site/leucine-rich-repeat*: NBS-LRR

adaptés produisent des effecteurs qui interfèrent avec la PTI entraînant une sensibilité déclenchée par les effecteurs (ETS). Dans la phase 3, un effecteur (rond rouge) est reconnu par une protéine NB-LRR, activant l'immunité de type ETI, une version amplifiée de la PTI qui induit souvent la mort cellulaire hypersensible (HR). Dans la phase 4, les isolats pathogènes qui ont perdu l'effecteur rouge sont sélectionnés, et peut-être gagné de nouveaux effecteurs à travers le flux de gène horizontal (en violet), ce qui peut aider les agents pathogènes à supprimer l'ETI. La sélection privilégie les nouveaux allèles de NBS-LRR de plantes capables de reconnaître l'un des effecteurs nouvellement acquis, résultant de nouveau dans l'ETI, d'après (Jones and Dangl, 2006).

### 3. Modèle zigzag du système immunitaire des plantes

Le point de vue actuel sur le système immunitaire de la plante peut être représenté sous la forme d'un modèle en zigzag consistant en quatre phases (Figure 3). Dans la phase 1, les PAMP sont reconnus par les PRRs, ce qui entraîne le déclenchement de la défense de type PTI et peut empêcher une nouvelle colonisation de l'agent pathogène. Dans la phase 2, les agents pathogènes adaptés déploient des effecteurs qui contribuent à leur virulence. Ces effecteurs peuvent réprimer la PTI et permettent que la plante soit sensible à l'agent pathogène (ETS<sup>6</sup> ; Figure 3). Dans la phase 3, un effecteur donné est spécifiquement reconnu par l'une des protéines NBS-LRR, ce qui entraîne une immunité déclenchée par cet effecteur (ETI ; Figure 3). Dans la phase 4, la sélection naturelle entraîne l'apparition d'agents pathogènes ayant acquis des effecteurs supplémentaires qui suppriment l'ETI (Jones and Dangl, 2006).

## C. Les réponses de défense des plantes aux agents pathogène

Les plantes possèdent un système de défense très sophistiqué comprenant des barrières physiques et chimiques préformées qui entravent l'entrée de pathogène. Les plantes ont également développé une grande variété de mécanismes de défense induites déclenchées lors de la reconnaissance des pathogènes.

### 1. Défenses préformées

Les défenses préformées comprennent à la fois des restrictions physiques mais aussi des inhibitions chimiques des agents pathogènes.

La couche de cuticule, l'épiderme et la paroi cellulaire sont les obstructions naturelles à l'entrée des agents pathogènes présentes dans toutes les espèces végétales et dont l'efficacité est renforcée par la faible fréquence des ouvertures naturelles (stomates et hydatodes).

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<sup>6</sup> *effector-triggered susceptibility*: ETS

L'existence de cire cuticulaire sur la surface des feuilles agit à la fois comme barrière physique contre l'entrée des agents pathogènes et freine leur accès aux nutriments. Chez *A. thaliana* les mutants *lacs2*, déficients dans la biosynthèse de la cutine, présentent une forte sévérité de symptômes après l'inoculation de la souche avirulente de la bactérie *Pseudomonas syringae* pv. *tomato* DC3000 avrRpt2 en comparaison aux plantes sauvages (Tang, et al., 2007).

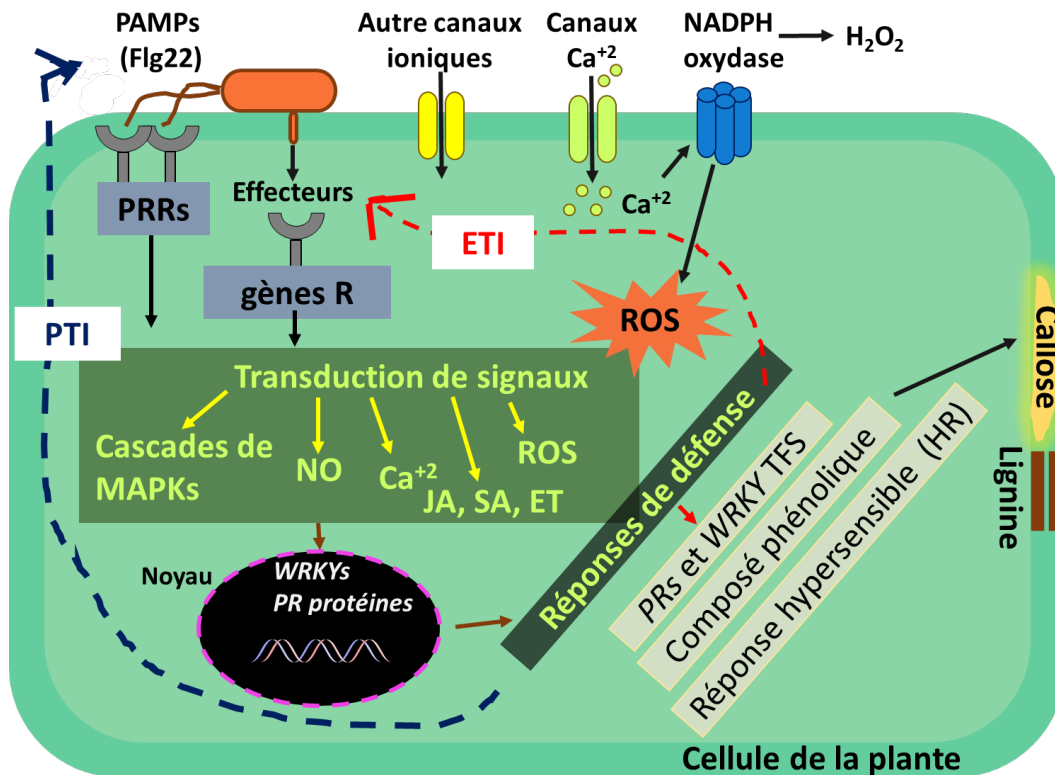
Par ailleurs les plantes produisent des composés chimiques antimicrobiens qui inhibent la multiplication des agents pathogènes. Ce sont généralement des métabolites secondaires présents constitutivement dans la plante. Par exemple chez les Brassicacées comme *A. thaliana* le glucosinolate, un métabolite secondaire composé d'azote et de soufre, est connu pour jouer un rôle dans la défense chimique préformée des plantes contre une large gamme d'agents pathogènes. Les mutants *gsm1-1* d'*A. thaliana*, déficients dans la biosynthèse des glucosinolates, présentent une multiplication plus élevée de *P. syringae* pv. *phaseolicola* comparativement aux plantes sauvages, montrant l'impact des glucosinolates sur le taux de survie des bactéries dans les feuilles (Mishina and Zeier, 2007).

## 2. Les défenses induites

La perception de molécules dérivées de l'agent pathogène conduit à l'induction des défenses de la plante. Ces réactions de défense arrêtent la multiplication de l'agent pathogène en produisant des obstacles structurels, mais aussi *via* la biosynthèse de produits chimiques antimicrobiens. Le renforcement de la paroi cellulaire par le dépôt de callose, de lignine et de subérine est par exemple induit après la reconnaissance de certains agents pathogènes. Le gène *Phenylalanine Ammonia Lyase 1 (PAL1)*, impliqué dans la biosynthèse de la lignine, a un rôle important dans la résistance aux agents pathogènes. Par exemple, chez le poivrier, *Capsicum annuum*, les mutants *pall* présentent une sensibilité accrue à l'infection par les souches virulentes et avirulentes de *Xanthomonas campestris* pv. *vesicatoria* par rapport aux plantes sauvages (Kim and Hwang, 2014). A part les barrières physiques induites, plusieurs composés chimiques sont synthétisés et agissent comme des agents antimicrobiens. Des métabolites secondaires, tels que les phytoalexines et les composés phénoliques mais aussi la formation de radicaux libres comme les espèces réactives de l'oxygène ou de l'azote, les *reactive oxygen species* (ROS) et les *reactive nitrogen species* (RNS), sont bien connus pour être synthétisés au cours des réponses de défense des plantes (voir plus loin pour une description détaillée).

#### D. La signalisation des défenses et les processus en aval

La reconnaissance de l'agent pathogène par la plante active des cascades de signalisation et plusieurs réponses précoces liées à la défense. Un des premiers événements de signalisation en réponse aux PAMPs et aux DAMPs correspond à une augmentation des flux d'ions  $\text{Ca}^{2+}$  (Cheval, et al., 2013). Ces flux ioniques agissent en amont d'autres événements de signalisation intracellulaire. Les autres événements de signalisation précoce communément détectés en réponse aux agents pathogènes correspondent à l'activation de *Mitogen-activated protein kinases* (MAPKs), à l'activation de NADPH oxydases et à la production des ROS appelée *oxidative burst* (Gomez-Gomez, et al., 1999). Après quelques minutes, une synthèse des phytohormones telles que l'acide salicylique (SA), l'éthylène (ET) et l'acide jasmonique (JA) va avoir lieu, contribuant ainsi aux mécanismes de défense (Malamy, et al., 1990, Pieterse, et al., 2014).



**Figure 4. La reconnaissance de l'agent pathogène et l'élicitation de la réponse de défense par la plante.** Lors de l'attaque par un agent pathogène, les PAMPs et/ou effecteurs sont reconnus par la plante grâce à des récepteurs spécifiques (PRRs et protéines R) conduisant à la réponse de la défense (PTI/ ETI) via l'activation de multiples voies de transduction du signal. Cette reconnaissance va conduire à un changement de potentiel de la membrane pour des échanges d'ions, avec notamment une rentrée de calcium  $\text{Ca}^{2+}$ , mais également à une activation de MAPKs menant à une activation du complexe de NADPH oxydase et la production de ROS intracellulaires et à l'activation des voies hormonales SA, JA et ET. Ces voies de signalisation vont ensuite activer de facteurs de transcriptions tels que les WRKYs qui se fixent sur les promoteurs des gènes de défenses permettant leur expression. L'expression de ces gènes de défense va mener à la synthèse de composés phénoliques, de protéines PR et à un renforcement de la paroi cellulaire (callose) ainsi qu'une mort cellulaire programmée (HR). Adapté de (Swarupa, et al., 2014).

## 1. Flux de Ca<sup>2+</sup>

L'un des événements les plus précoces de signalisation observés après la reconnaissance des agents pathogènes par les PAMPs est le flux d'ions à travers la membrane plasmique, y compris l'influx de Ca<sup>2+</sup> dans le cytosol. La signalisation par le Ca<sup>2+</sup> se manifeste par différentes amplitudes du flux de Ca<sup>2+</sup>, ce qui va ensuite stimuler les voies de signalisation en aval. Une augmentation de flux de Ca<sup>2+</sup> a été observée après l'exposition de plantules d'*A. thaliana* aux PAMPs bactériens flg22 et elf18, au PAMP fongique N-acetylchitooctaose (ch8), et à un DAMP dérivé de plante, Pep1 (Ranf, et al., 2011).

Chez *A. thaliana* les canaux ioniques de type *Cyclic Nucleotide-Gated Ion Channels* (CNGCs) forment une grande famille composée de 20 membres. Ils sont impliqués dans la signalisation Ca<sup>2+</sup> liée à divers processus physiologiques, tels que la défense aux agents pathogènes, le développement et la tolérance thermique. Les CNGCs sont des canaux cationiques non sélectifs pouvant conduire le flux de Ca<sup>2+</sup> à travers la membrane plasmique de la plante. Il a été suggéré que chez *A. thaliana*, AtCNGC2 aurait un rôle dans la réponse de défense. En effet, lors de l'infection par la souche avirulente de *P. syringae* pv *glycinea* exprimant le gène avrRpt2 les mutants *Atcngc2* montrent une absence de HR alors que celle-ci était visible à 24 heures post-infection (hpi) chez les plantes sauvages (Chin, et al., 2013).

## 2. Rôle de la signalisation par les MAPKs dans la défense

Les cascades MAPKs sont des modules très conservés chez tous les eucaryotes. Chez les plantes, les voies de MAPK sont impliquées dans la régulation, le développement, la croissance, la mort cellulaire programmée et dans les réponses aux différents stimuli environnementaux, y compris le froid, la chaleur, les ROS, les UV, la sécheresse et l'attaque de pathogène *via* un mécanisme de phosphorylation de ces cascades. Une cascade de MAPK est composée de trois protéines kinases liées fonctionnellement. Les MAPKs sont phosphorylées au niveau de résidus thréonine et tyrosine (TXY) et activées par une MAPK kinase (MAPKK), qui elle-même est phosphorylée au niveau de résidus sérine et thréonine et activée par une MAPKK kinase (MAPKKK).

Les cascades de MAPKs chez les plantes jouent un rôle central dans la voie de signalisation de la PTI par la transduction des signaux provenant des PRRs vers des composants en aval. Les PAMPs bactériens flg22 et elf18 déclenchent en effet une forte activation transitoire de MAPKs chez *A. thaliana*, y compris MPK3, MPK6, MPK4 et MPK11 (Meng and Zhang,

2013). En plus de la PTI, il a été démontré que les cascades de MAPKs sont également des composantes fondamentales de l'ETI. L'infection d'*A. thaliana* par la bactérie *P. syringae* portant l'effecteur AvrRpt2 déclenche une activation prolongée de MPK3 et MPK6 par rapport au traitement avec une souche contrôle portant un vecteur vide (Underwood, et al., 2007). Ces travaux démontrent l'implication de MPK3 et MPK6 d'*A. thaliana* dans l'ETI. Les cascades de MAPKs sont impliquées dans la régulation de divers événements de signalisation en aval. La phosphorylation par MPK3 et MPK6 des enzymes ACS2 et ACS6, impliquées dans la biosynthèse d'ET, stabilise ces protéines *in vivo*, entraînant une augmentation de l'activité ACS cellulaire et la production d'ET (Han, et al., 2010). Les MAPKs régulent également des facteurs de transcription. Par exemple chez *A. thaliana*, il a été démontré que la phosphorylation d'*Ethylene Response Factor 6 (ERF6)* par la cascade MPK3/MPK6 active les gènes liés à la défense comme *PDF1.1* et *PDF1.2* améliorant ainsi la résistance des plantes au champignon nécrotrophe *Botrytis cinerea* (Meng, et al., 2013). Chez *N. benthamiana* la liaison MAPK-WRKY est nécessaire pour l'activation du gène codant la NADPH oxydase (*NbRBOHB*) impliqué dans l'accumulation des ROS au cours de réponses de type ETI et PTI (Adachi, et al., 2015).

Les MAPKs ont également des rôles dans la biosynthèse et la signalisation des hormones SA et JA (voir plus loin), l'activation des gènes de défense, la biosynthèse de phytoalexine, l'accumulation des ROS et la HR.

### 3. Le rôle des ROS et des RNS dans la défense

Les ROS comprennent diverses molécules chimiquement réactives, tels que l'anion superoxyde ( $O_2^{\cdot-}$ ), le peroxyde d'hydrogène ( $H_2O_2$ ), le radical hydroxyle ( $OH^{\cdot}$ ), et le radical hydroperoxyde ( $HO_2^{\cdot}$ ). La production des ROS, souvent induite par des agents pathogènes avirulents, consiste en une accumulation de ROS biphasique avec une première phase courte et transitoire, le *burst*, suivie d'une phase continue d'une intensité beaucoup plus élevée qui est en corrélation avec la résistance aux maladies (Torres, et al., 2006). Plusieurs systèmes enzymatiques sont impliqués dans la génération des ROS suite à la reconnaissance des agents pathogènes tels que la NADPH oxydase, la Super Oxyde Dismutase (SOD), les oxalate oxydases, les peroxydases, les lipoxygénases et les amineoxydases. Les ROS jouent un rôle important dans le contrôle des interactions plantes-microbe et dans le développement de la plante en modulant différents processus cellulaires décrits ci-dessous.

L'accumulation des ROS peut entraîner le renforcement de la paroi cellulaire de la plante au niveau du site d'interaction, ce qui constitue une réaction de défense induite importante en réponse à un agent pathogène. Par exemple, chez *Arabidopsis*, la synthèse des composants de la paroi cellulaire comme la lignine et la subérine est régulée par les peroxydases de classe III (Almagro, et al., 2009). Les ROS régulent également différentes voies de transduction de signal chez la plante. La cooccurrence de l'augmentation de la production de ROS et de flux de  $\text{Ca}^{2+}$  transitoires est observée en réponse à des stress abiotiques et biotiques. En fait, les ROS et le flux de  $\text{Ca}^{2+}$  sont coproduits et corégulés, rendant l'analyse de la régulation de ces voies assez complexe (Lehmann, et al., 2015). Une des voies de signalisation de défense bien caractérisée pour être régulée par l'oxydation est l'induction des réponses SA-dépendantes *via* la régulation du récepteur *Non Expressor Of PRI (NPR1)* et du facteur de transcription *TGA* (voir paragraphe suivant). Enfin, la production de ROS module la mort cellulaire programmée au cours de la réponse hypersensible (HR), déclenchée par certaines interactions plante-pathogène. Yun et al. 2011 ont montré que chez *A. thaliana*, en réponse à la souche DC3000 de *P. syringae* pv. *tomato* et à l'oomycète *Hyaloperonospora arabidopsidis*, la S-nitrosation d'AtRBOHD diminue son activité, réduit l'accumulation de ROS, et perturbe la mise en place de la mort cellulaire au cours de la HR (Yun, et al., 2011).

Les plantes produisent plusieurs molécules de type RNS. Parmi elles, la plus étudiée est l'oxyde nitrique (NO), un gaz radical libre diatomique, molécule de signalisation impliquée dans un large éventail de processus physiologiques dans les plantes. En particulier, le NO se comporte comme un élément crucial de la réponse immunitaire des plantes et contribue avec l' $\text{H}_2\text{O}_2$  à l'activation de la réponse hypersensible (HR) lors d'interactions plantes-pathogènes incompatibles (Bellin, et al., 2013). Lors de la reconnaissance d'un agent pathogène par la plante, le NO peut réagir avec l' $\text{O}_2$  pour former un autre RNS, le peroxyde nitrique ( $\text{ONOO}^-$ ) (Scheler, et al., 2013). La biosynthèse du NO par la plante n'est pas complètement élucidée, même s'il a été montré que la nitrate réductase (NR) (Bellin et al 2013) participe à l'accumulation de NO en réponse à l'infection par des agents pathogènes fongiques nécrotrophes (Perchepped, et al., 2010) et des bactéries (Modolo, et al., 2006, Oliveira, et al., 2009).



#### 4. Les voies de signalisations hormonales

Les principales hormones produites par les plantes sont les auxines, les gibbérellines (GA), les cytokinines, les brassinostéroïdes, les strigolactones, l'acide abscissique (ABA), l'ET, le SA, et le JA. Parmi ces hormones, le SA, le JA et l'ET jouent un rôle majeur en réponse aux stress biotiques et leurs niveaux augmentent après l'infection par un agent pathogène. Le SA est généralement impliqué dans la réponse de défense contre les pathogènes biotrophes et héli-biotrophes, alors que le JA et l'ET sont souvent impliqués dans la défense contre les agents pathogènes nécrotrophes et les insectes herbivores (Verma, et al., 2016).

##### a) Voie du SA

Le SA est un composé phénolique qui peut être synthétisé à partir d'un métabolite primaire, le chorismate, par deux voies enzymatiques distinctes, (1) la voie de la PAL et (2) la voie de l'isochorismate synthase (*ICS1/SID2*). Une fois que la voie de signalisation par le SA est activée au niveau du site de l'infection, une réaction de défense est souvent déclenchée dans les parties distales des plantes afin de protéger les tissus encore sains. Cette défense induite, à longue durée et à large spectre, constitue la résistance systémique acquise (SAR).

La biosynthèse du SA est déclenchée au cours de la PTI et de l'ETI suite à des changements transitoires de niveaux  $Ca^{2+}$  induits par les microbes *via* les protéines de type lipase comme *Enhanced Disease Susceptibility1 (EDS1)* et *Phytoalexin Deficient4 (PAD4)*. Lorsque l'ETI est initiée par des protéines R de type TIR-NBS-LRR, la biosynthèse de SA est régulée par EDS1 et PAD4 au même titre que la PTI (Wiermer, et al., 2005). Par contre, lorsque des protéines de type CC-NBS-LRR déclenchent l'ETI, c'est le gène *Non-Race-Specific Disease Resistance1 (NDRI)* qui régule la production de SA (Pieterse, et al., 2012).

La signalisation en aval du SA est en grande partie contrôlée par la protéine *NPRI*, qui agit lors de la production de SA comme un coactivateur transcriptionnel des gènes liés à la défense. Les changements dans l'état redox cellulaire induits par le SA provoquent la monomérisation de *NPRI* par la réduction des ponts disulfures intermoléculaires. Des Thioredoxins, TRX-h5 et TRX-h3, catalysent la formation de monomères de *NPRI* qui se déplacent ensuite vers le noyau *via* les protéines des pores nucléaires, tels que *Modifier Of Snc1 (MOS)* 3, 6 et 7 (Pieterse, et al., 2012). Dans le noyau, *NPRI* interagit avec les facteurs de transcription *TGA*, de la classe des bZIP, qui à leur tour facilitent l'expression des gènes *PR*. Plusieurs facteurs de transcription *WRKY* jouent également des rôles importants, en aval de *NPRI*, dans la régulation des réactions de défense (Verma, et al., 2016). Par exemple le

gène *PtrWRKY73* de peuplier (*Populus trichocarpa*) est induit par le traitement exogène de SA et a un rôle dans la résistance à *P. syringae* (PstDC3000) chez *A.thaliana* (Duan, et al., 2015).

### b) Voie du JA

Le JA et les métabolites structurellement liés au JA sont des composés dérivés de lipides qui sont synthétisés par la voie de biosynthèse des oxylipines en réponse à l'attaque d'un agent pathogène ou d'un insecte (Figure 5). La phase initiale de la formation de JA a lieu dans les chloroplastes, où les acides gras de lipides membranaires comme l'acide linoléique, par exemple, sont métabolisés par des lipoxygénases pour générer les oxylipines y compris le *12-oxo-phytodienoic acid* (OPDA), précurseur du JA. Par la suite, l'OPDA est transporté vers les peroxyosomes où il subit trois étapes de  $\beta$ -oxydation pour produire le JA. Le JA peut être conjugué à des acides aminés, tels que la L-isoleucine, entraînant la production de JA-Ile, forme biologiquement active du JA. La protéine à F-box Coronatine Insensitive1 (*COI1*) est un régulateur clé de la voie de signalisation du JA, car il fait partie du complexe récepteur de JA (Vos, et al., 2013).

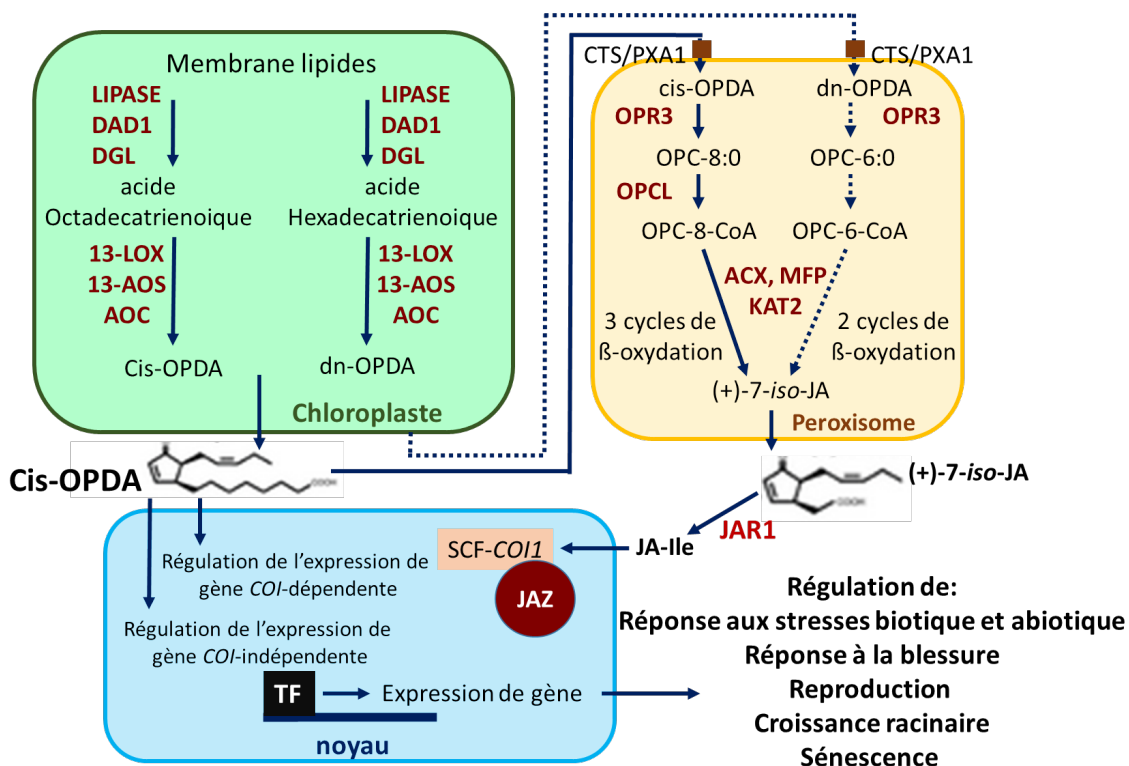
Chez *A. thaliana*, deux grandes branches de la voie de signalisation de JA sont connues : la branche MYC et la branche ERF. La branche MYC est activée lors d'une blessure ou d'une attaque par des insectes herbivores et contrôlée par des facteurs de transcription comme *Jasmonate Insensitive 1* (*JIN1/MYC2*). L'activation de la branche MYC conduit à l'induction de gène marqueur de *Vegetative Storage Protein2* (*VSP2*). La branche ERF est activée en cas d'attaque par des agents pathogènes nécrotrophes et régulée par la famille des facteurs de transcription *Apetala2/Ethylene-Responsive Factor* (*AP2/ERF*) tels qu'ERF1 et *Octadecanoid-Responsive Arabidopsis59* (*ORA59*). La branche ERF de la voie de réponse au JA nécessite également la production d'éthylène et cela active le gène marqueur *Plant Defensin1.2* (*PDF1.2*) (Pieterse, et al., 2012, Vos, et al., 2013).

Les protéines répresseur *Jasmonate-Zim-Domain* (JAZ), jouent également un rôle crucial dans la réponse au JA dans des conditions de stress. En absence de JA-Ile, la forme bioactive de JA, les protéines JAZ interagissent avec JIN1/MYC2 et inhibent la régulation transcriptionnelle des gènes de réponse au JA. Par contre dans les conditions stimulant la production de JA, JA-Ile se lie à son récepteur *COI1* ce qui conduit à la dégradation des protéines JAZ par le protéasome 26S, permettant ainsi à MYC2 d'induire des gènes cibles du JA. MYC2 est post-traductionnellement modifié par la phosphorylation au niveau du

résidu Thr328 pour stimuler son activité de transcription. Cependant, ce MYC2 modifié est instable et est dégradé par la *Protein U-Box* (PUB10) de la plante qui fonctionne comme une ligase E3. Cette facilité de changement de quantité de MYC2 accélère ainsi le dynamisme et l'affinement des réponses au JA par MYC2. (Verma, et al., 2016).

### c) Voie de l'ET

L'ET est une hormone gazeuse qui fonctionne comme un régulateur important de l'immunité des plantes. Les ERFs sont les principaux facteurs de régulation en aval de la voie de signalisation par l'ET lors des réponses aux stress. Le facteur de transcription *Ethylene Insensitive3* (*EIN3*) a été suggéré pour induire l'expression du gène *ERF1* en réponse à l'ET et l'activation des réponses de défense. Un autre régulateur positif de la signalisation par l'ET est *EIN2*. En absence d'ET, le facteur de transcription *Constitutive Triple Response* (*CTR1*) réprime *EIN2*. Par contre, lors de la perception d'ET par son récepteur *Ethylene Réponse 1* (*ETR1*), la répression d'*EIN2* est levée, activant ainsi la signalisation de l'ET (Verma, et al., 2016).



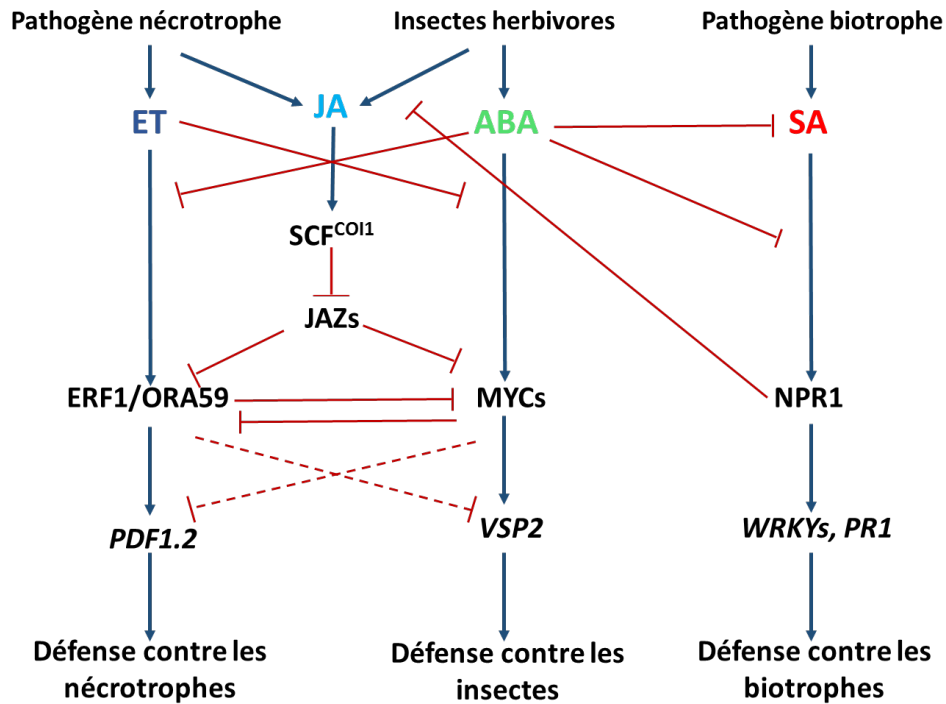
**Figure 5. La voie de biosynthèse des oxylipines et la transduction du signal chez *A. thaliana*.** La biosynthèse de JA commence dans le chloroplaste par la libération d'acide octadécatriénoïque ou d'acide hexadécatriénoïque libérés à partir de lipides membranaires suite à l'action de lipases telles que DAD1 et DGL. Le cis-OPDA et le dn-OPDA sont formés suivant des étapes séquentielles catalysées par 13-LOX, 13-AOS et AOC. Le cis-OPDA est transporté vers le peroxysome *via* le transporteur CTS, où après sa réduction par OPR3, il y a production d'OPC-8:0. OPC-8:0 est ensuite activé et transformé en son ester CoA par OPCL, qui subit ensuite trois cycles de  $\beta$ -oxydation catalysée par ACX, KAT et MFP pour former le (+)-7-iso-JA. JAR1 catalyse ensuite dans le cytosol la conjugaison du JA à un acide aminé pour former le JA-Ile, qui est la forme active de l'hormone impliquée dans la signalisation du JA. Les protéines JAZ répriment l'expression des gènes de réponse au JA. En réponse à JA-Ile, les protéines JAZ sont ciblées par SCF-CO11 pour la dégradation, conduisant ainsi à l'expression des gènes de réponse au JA et finalement la régulation de divers processus physiologiques. Le cis-OPDA peut également réguler l'expression de gènes, COI1-dépendante ou non.

Les enzymes sont en rouge. Les flèches en pointillé indiquent la voie de biosynthèse putative de JA via le dn-OPDA non prouvée expérimentalement. DAD1, DEFECTIVE IN ANTHWER DEHISCENCE 1 ; DGL, DONGLE; 13-LOX, 13-lipoxygénase; 13-AOS, 13-allène oxyde synthase; AOC, allène oxyde cyclase; OPR3, 12-oxophytodienoate reductase3; OPCL1, OPC-8:CoA ligase1; CTS, COMATOSE; ACX, acyl CoA oxydase; KAT, 3-l-ketoacyl-CoA-thiolase; MFP, multifunctional protein; JA, jasmonic acid; cis-OPDA, cis-(+)-12-oxo-phytyldienoic acid; dn-OPDA, dinor-oxo-phytyldienoic acid; JA-Ile, jasmonoyl-L-isoleucine; COI1, CORONATINE-INSENSITIVE1; JAZ, jasmonate ZIM domain. D'après (Dave and Graham, 2012).

#### d) Effets synergiques et antagonistes entre les voies

Il existe des liens importants entre les voies de signalisation des différentes phytohormones (Figure 6). Par exemple, l'ET peut avoir des effets synergiques ou antagonistes sur les voies du SA et du JA en fonction de l'interaction plante-pathogène. D'autre part, les différents types de phytohormones semblent contrôler l'établissement de défense contre des bioagresseurs ayant un mode de vie différent. En effet, la voie de réponse de SA est typiquement efficace contre les agents pathogènes biotrophes, celle de JA et de l'ET contre les agents pathogènes nécrotrophes et celle de JA contre des herbivores. Pourtant ces voies de signalisation ne sont pas indépendantes et des interactions antagonistes et synergiques entre les acteurs des différentes voies existent.

Par exemple, l'ET agit en synergie avec le SA sur le niveau de résistance de *Brassica napus* contre l'agent pathogène *Leptosphaeria maculans*. D'un autre côté, l'ET agit négativement sur la défense régulée par le SA chez *A. thaliana* infecté par *P. syringae* (Broekgaarden, et al., 2015). Concernant la voie du JA, l'ET a un effet synergique sur la branche ERF pour activer les gènes liés à la défense, comme *PDF1.2*, conduisant ainsi à une défense efficace contre les agents pathogènes nécrotrophes. D'autre part, la branche MYC est en synergie avec l'ABA pour activer les gènes liés à la défense, tels que *VSP2*, impliqués dans la défense contre les insectes. Cela suggère que l'équilibre entre les branches ERF et MYC de la voie de JA dépend des voies ET et ABA (Verhage, et al., 2011, Vos, et al., 2013).



**Figure 6. Modulation des voies de signalisation hormonales en réponse aux agents pathogènes et aux herbivores.** Voir le texte pour plus de détails. Les agents pathogènes nécrotrophes activent les voies de signalisation du JA et de l'ET ; Les insectes herbivores induisent les voies de signalisation du JA et de l'ABA ; Les agents pathogènes biotrophes induisent la voie de signalisation du SA. Les branches régulées par l'ET et l'ABA de la voie du JA sont antagonistes. L'acteur clé de la voie du SA est NPR1 qui régule négativement la voie du JA. L'ABA est impliqué indirectement dans la régulation de la défense en réprimant la voie du SA et de l'ET. Les lignes pleines indiquent les interactions établies, les lignes en pointillés représentent les hypothèses, les flèches indiquent les effets positifs et les lignes d'inhibition rouges représentent des effets négatifs. Abréviations: ABA, acide abscissique; ET, éthylène; JA, acide jasmonique et SA acide salicylique, selon (Pieterse, et al., 2012).

Les voie du SA et du JA sont souvent antagonistes et ceci de façon réciproque. Par exemple une activation élevée de la voie du SA contre un agent biotrophe est souvent accompagnée par une atténuation dans la résistance contre un agent nécrotrophe. De plus, des hormones qui ne sont pas directement impliquées dans la réponse de défense peuvent influencer les voies du SA, du JA et de l'ET : par exemple l'ABA réprime la voie du SA et de l'ET (Pieterse, et al., 2012).

## 5. La synthèse des protéines liées à la défense

Chez les plantes, les protéines dites *pathogenesis related* (PR) sont définies comme étant induites par divers types d'agents pathogènes tels que les virus, les bactéries et les champignons. Les protéines PRs ont été découvertes à partir de feuilles de tabac (*Nicotiana tabacum*) infectées par le virus de la mosaïque du tabac puis détectées plus tard dans de nombreuses plantes d'espèces différentes. 17 familles de protéines PR sont connues en fonction de leurs similarités de séquences en acides aminés, de leurs activités enzymatiques ou d'autres propriétés biologiques puis numérotées dans l'ordre où elles ont été découvertes (Tableau 1). Les protéines PR présentent des fonctions multiples. Certaines PRs présentent des propriétés antimicrobiennes connues, certaines ont des fonctions enzymatiques, telle que la chitinase et certaines ont un effet de perméabilisation de la membrane, comme les défensines et les protéines de transfert des lipides (Tableau 1). Parmi ces protéines PR, les chitinases et  $\beta$ -1,3-glucanases sont deux enzymes hydrolytiques importantes et abondantes chez de nombreuses espèces de plantes après infection avec différents types d'agents pathogènes. Les plantes de carotte (*Daucus carota*. L) co-transformées avec le gène de chitinase (*CHI-2*) de l'orge et un gène PR du blé, codant pour un protéine de transfert des lipides (LTP) ont été étudiés: après inoculation des deux agents pathogènes fongiques foliaires nécrotrophes, *Alternaria radicicola* et *B. cinerea*, les plantes transgéniques co-transformées ont résisté jusqu'à 90-95% aux attaques fongiques par rapport aux transformants individuels (40-50%) (Cletus, et al., 2013). Ces travaux montrent le rôle important de la combinaison des protéines PR pour la résistance des plantes face aux agents fongiques.

Plusieurs études montrent que ces protéines PR sont largement induites par des attaques d'agents pathogènes. En effet, le niveau transcriptionnel du gène *PR1* était 4,1 fois plus important en réponse à la bactérie *P. syringae* pv. *tomato* DC3000 chez *A. thaliana* par rapport aux contrôles non inoculés (Song, et al., 2015). Andargie et al. 2016 ont démontré que *Ustilaginoidea virens*, un champignon biotrophe important chez le riz, est capable de provoquer une interaction compatible chez *A. thaliana*. Suite à l'infection d'*A. thaliana* par les isolats de *U. virens*, la plante active différents mécanismes de défense incluant une augmentation de l'expression des gènes *PR1*, *PR2*, *PR5*, *PDF1.1* et *PDF1.2* (Andargie and Li, 2016).

Par ailleurs certains travaux ont montré que chez les plantes résistantes, ces protéines PR sont surexprimées suggérant leur rôle dans la résistance des plantes à divers agents pathogènes. Chez la lignée de blé Sumai-3 résistante à *Fusarium graminearum*, l'expression du gène *PR1* et la quantité de SA sont significativement plus importantes par rapport à la lignée sensible Bobwhite (Makandar, et al., 2012). La lignée sur-expresser de *WRKY46* d'*A. thaliana* est plus résistante à *P. syringae* par rapport aux plantes sauvages, et cette résistance est accompagnée d'une forte expression du gène *PR1* à 1 et 2 dpi comparativement aux plantes sauvages (Hu, et al., 2012). (Catinot, et al., 2015) ont montré que les plantes transgéniques d'*A.thaliana* surexprimant le gène *ERF96* ont une résistance accrue au champignon *B. cinerea* et à la bactérie *Pectobacterium carotovorum*. Ils ont ensuite démontré que *ERF96* améliore l'expression des gènes des voies JA/ET, *PDF1.2a*, *PR-3* et *PR-4* par une liaison directe aux éléments GCC présents dans leurs promoteurs.

Famille	Protéine	Activité antifongique ou fonction
<i>PR-1</i>	PR-1 a, PR-1 b et PR-1 c	Antifongique
<i>PR-2</i>	$\beta$ -1,3-glucanase	Cleaves $\beta$ -1,3-glucans
<i>PR-3</i>	Chitinase types I, II, IV, V, VI et VII	Endochitinase
<i>PR-4</i>	Chitinase types I et II	Antifongique et chitinase
<i>PR-5</i>	Thaumatococcus-like proteins	Antifongique
<i>PR-6</i>	Tomato proteinase inhibitor I	Proteinase inhibitor
<i>PR-7</i>	Tomato endoproteinase P	Endoproteinase
<i>PR-8</i>	Chitinase concombre	Chitinase III
<i>PR-9</i>	Tobacco lignin-forming peroxidase	Peroxydase
<i>PR-10</i>	Parsley "PR-1" Bet v 1, Mal d 1, Api g 1, and Dau c 1	Ribonuclease like
<i>PR-11</i>	Tobacco chitinase type V	peroxydase
<i>PR-12</i>	Radish Rs-AFP3	Perméabilisation de membrane
<i>PR-13</i>	Arabidopsis THI2.1	Perméabilisation de membrane
<i>PR-14</i>	Lipid transfer proteins	Lipid-transfer protein
<i>PR-15</i>	Barley OxOa	Oxalate oxydase
<i>PR-16</i>	Barley OxOLP	Oxalate-like oxydase
<i>PR-17</i>	Tobacco PRp27	Non connu

**Tableau 1. Liste des protéines liées à la pathogenèse dites protéines « PR » ; d'après (Sinha, et al., 2014)**



## 6. Métabolites secondaires

La production de métabolites secondaires antimicrobiens fait également partie des réponses induites de la plante en réponse à l'attaque d'un agent pathogène. Les métabolites secondaires induits par le stress, de faible masse moléculaire et ayant une activité antimicrobienne sont collectivement appelés les phytoalexines. Les phytoalexines constituent une partie importante du répertoire de défense des plantes. A chaque famille de plantes correspond une classe de phytoalexines. La camalexine (3-thiazol-2'-yl-indole) est la principale phytoalexine produite par *A. thaliana* et les espèces de la famille des brassicacées.

Chez *A. thaliana*, la biosynthèse de la camalexine nécessite deux enzymes, *Phytoalexin Deficient 2 (PAD2)* et *PAD3* (Ren, et al., 2008). Les mutants des gènes impliqués dans la régulation et la biosynthèse des phytoalexines présentent une forte sensibilité aux agents pathogènes, comme le mutant *phytoalexin deficient 3 (pad3)*, affecté dans la synthèse de camalexine, qui est plus sensible à *B. cinerea* (Ferrari, et al., 2003). D'autres travaux ont montré que le mutant *BcCHS3* de *B. cinerea*, dont la production en chitine est fortement diminuée est peu pathogène sur les plantes sauvages alors que ce mutant est capable de se développer sur les mutants *pad2* et *pad3* d'*A. thaliana*. Ces travaux indiquent que le gène *BcCHS3* de *B. cinerea* contribue à l'activation des défenses des plantes (Arbelet, et al., 2010). La biosynthèse de la camalexine semble être contrôlée par plusieurs voies de signalisation. Par exemple, l'absence de signalisation fonctionnelle de la voie du JA chez les mutants *coi1* d'*A. thaliana* aboutit à une forte diminution dans l'accumulation de camalexine en réponse à l'infection par *B. cinerea* (Rowe, et al., 2010). Plusieurs études ont également montré que la biosynthèse de camalexine est régulée par des cascades de MAPK. (Ren, et al., 2008) ont démontré que chez *A. thaliana*, l'induction de la camalexine par *B. cinerea* est précédée par l'activation MPK3/MPK6.

## 7. Facteurs de transcription de WRKY

La superfamille de facteurs de transcription de type WRKY est constituée de 74 et 109 membres respectivement chez *A. thaliana* et le riz. Les membres de cette famille contiennent au moins une région conservée de liaison à l'ADN comprenant la séquence peptidique hautement conservée WRKYGQK et un motif à doigt de zinc (CX<sub>4-7</sub>CX<sub>22-23</sub>HXH/C). Ce domaine se lie généralement à l'élément d'ADN appelé la boîte W

(C/TTGACT/C), bien que des sites de liaison alternatifs aient été identifiés (Pandey and Somssich, 2009).

La réponse des plantes à l'attaque par un agent pathogène nécessite une large reprogrammation transcriptionnelle, régulée par les facteurs de transcription comme les WRKYs. Les WRKYs ont été bien caractérisés et il a été démontré qu'ils peuvent avoir des rôles inducteurs ou réprimeurs au cours de la régulation des défenses des plantes. La régulation de l'activité des WRKYs par les protéines MAPKs est particulièrement bien connue. La phosphorylation de WRKY33 est régulée par MPK3/MPK6. Cette activation est nécessaire pour l'activation transcriptionnelle des gènes de biosynthèse de la camalexine pour contribuer à la défense d'*A. thaliana* vis à vis de *B. cinerea* (Mao, et al., 2011). Chez le riz, le facteur de transcription WRKY70 est régulé par MPK3/MPK6 et interagit physiquement avec les motifs *W-box* de l'ADN. OsWRKY70 priorise la défense, au détriment de la croissance, en régulant positivement la biosynthèse du JA et négativement celle des GA en réponse à l'attaque de l'insecte herbivore *Chilo suppressalis*. Cette biosynthèse de JA dépendant de WRKY70, est nécessaire pour l'activation d'inhibiteur de protéinase et la résistance contre *C. suppressalis* (Li, et al., 2015).

Pathogen	Host	Disease	References
<i>Corynebacterium sepedonicum</i>	Potato	Ring rot	Gallegly and Walker, 1949
<i>Erwinia amylovora</i>	Pear	Fire blight	Agrios, 1997
<i>Erwinia stewartii</i>	Corn	Stewart's wilt	McNew and Spencer, 1939
<i>Pseudomonas syringae</i> subsp. <i>savastanoi</i>	Olive	Olive knot	Balestra and Varvaro, 1997
<i>Streptomyces scabies</i>	Potato	Scab	Lapwood and Dyson, 1966
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	Tomato	Bacterial spot	McGuire et al., 1991
<i>Botrytis cinerea</i>	Grape	Botrytis bunch rot	Chérif and Boubaker, 1997
<i>Colletotrichum gloeosporioides</i>	Tomato	Fruit and root rot	Williams, 1965
<i>Erysiphe graminis</i>	Wheat	Powdery mildew	Last, 1953
<i>Magnaporthe grisea</i>	Rice	Rice blast	Teng, 1994
<i>Puccinia graminis</i>	Wheat	Stem rust	Daly, 1949
<i>Verticillium albo-atrum</i>	Potato, Tomato	Wilt	Wilhelm, 1950

**Tableau 2. Maladies favorisées par une augmentation de l'apport en azote à la plante hôte**

## E. Nutrition azotée et pathologie

D'une manière générale, l'azote est nécessaire pour la croissance et le développement de la plante. Cependant, l'azote affecte aussi la résistance des plantes aux maladies et peut avoir des effets positifs ou négatifs sur le développement des maladies.

### 1. Métabolisme de l'azote

L'azote est un élément clé de nombreux composants cellulaires des êtres vivants. En effet l'azote fait partie de la structure des alcaloïdes, des amides, des acides aminés et des protéines, des acides nucléiques (ADN, ARN), de certaines vitamines, de certaines hormones et d'autres composés cellulaires. Chez les plantes, l'azote est absorbé par les racines sous forme de nitrate ( $\text{NO}_3^-$ ) ou d'ammonium ( $\text{NH}_4^+$ ). Dans les sols aérobie, la principale forme d'azote inorganique est le nitrate ; dans les zones humides inondées ou dans les sols acides, la principale forme est l'ammonium. Pour les agents pathogènes, l'azote doit être obtenu au sein des tissus de la plante.

#### a) Chez les plantes

Une partie du nitrate absorbé par les racines est assimilé par les racines, et une partie importante est transportée vers les organes aériens. Une fois dans la feuille, le nitrate est transporté dans les cellules pour permettre la synthèse des acides aminés (Figure 7). Le nitrate est d'abord réduit en nitrite dans le cytosol par l'enzyme de nitrate réductase (NR) en utilisant le NADH comme pouvoir réducteur (Crawford, 1995). Dans les chloroplastes, le nitrite est ensuite réduit en ammonium par la nitrite réductase (NiR) qui utilise la ferredoxine réduite comme pouvoir réducteur (Wray, 1993). L'ammonium est ensuite assimilé grâce au cycle GS/GOGAT. La glutamine synthase (GS) existe sous une forme cytosolique, la GS1 majoritairement racinaire, et sous une forme plastidiale majoritaire dans les feuilles, la GS2 (Ishiyama, et al., 2004). La GS fixe l'ammonium à une molécule de glutamate à l'aide d'ATP pour donner de la glutamine. La glutamine et le glutamate sont les donneurs d'azote qui permettent ensuite la synthèse des autres acides aminés. La glutamate synthase (GOGAT), quant à elle, permet la synthèse de deux molécules de glutamate à partir d'une glutamine en utilisant de l' $\alpha$ -cetoglutarate, une molécule issue de la dégradation du citrate provenant du cycle de Krebs (Foyer, et al., 2011). La GOGAT est plastidiale et existe sous deux formes chez les plantes supérieures selon la spécificité du donneur d'électron : une GOGAT ferredoxine-dépendante (Fd-GOGAT) majoritaire dans

les feuilles et une GOGAT NADH-dépendante (NADH-GOGAT) majoritaire dans les racines (Lancien, et al., 2002).

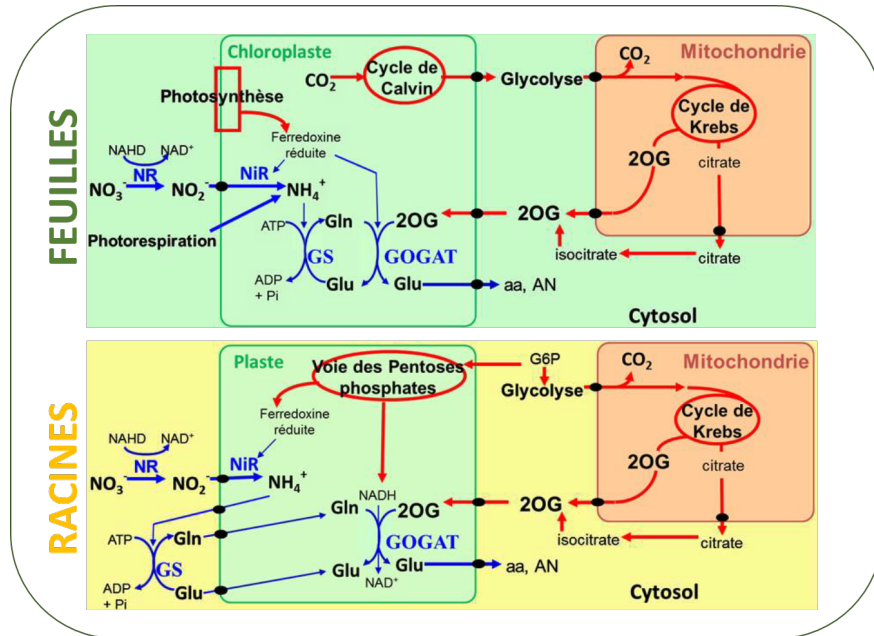


Figure 7 : Schéma de l'assimilation du nitrate dans les feuilles et les racines d'*A. thaliana*. L'assimilation de l'azote se fait grâce à une coordination du métabolisme carboné (en rouge) et du métabolisme azoté (en bleu). Les cercles noirs indiquent les transporteurs. Le nitrate ( $\text{NO}_3^-$ ) est réduit en nitrite ( $\text{NO}_2^-$ ) dans le cytosol *via* la nitrate réductase (NR) qui utilise le NADH comme pouvoir réducteur. Dans le plaste, le nitrite est ensuite réduit en ammonium ( $\text{NH}_4^+$ ) par la nitrite réductase (NiR) qui utilise la ferredoxine réduite issue de la photosynthèse dans les feuilles ou de la voie des pentoses phosphate dans les racines. Dans les feuilles, l'ammonium est issu de la photorespiration. La glutamine synthase (GS) forme de la glutamine (Gln) à partir d'ammonium, de glutamate (Glu) et d'ATP. La glutamine produite permet de synthétiser de nouveau du glutamate grâce à la glutamate synthase (GOGAT) qui utilise du 2-oxoglutarate (2OG) provenant du cycle de Krebs et du pouvoir réducteur de la ferredoxine réduite dans les feuilles ou du NADH dans les racines. Le glutamate participe ensuite à la synthèse d'acides aminés et d'acides nucléiques.

### b) Chez les agents pathogènes

Malgré l'importance de l'azote pour les plantes, les exigences de l'agent pathogène en azote sont tout aussi indispensables à étudier. En effet, l'acquisition de nutriments est un élément clé dans le cycle de vie de tous les agents pathogènes microbiens et cela est certainement crucial pour le maintien de la croissance au sein des tissus la plante hôte. De ce fait, l'apport d'engrais azoté à la plante peut augmenter la disponibilité en azote disponible pour le développement des agents pathogènes au sein des tissus végétaux (Tavernier, et al., 2007).

L'azote requis par les agents pathogènes pour leur croissance vient entièrement de la plante avec le  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  et les acides aminés, dont la nature exacte varie en fonction de la partie de la plante infectée (Mur, et al., 2017). Il a été montré qu'un nombre important d'acides aminés étaient présents dans l'apoplasme de feuilles de tomate, comme par exemple la glutamine, le glutamate, l'alanine et l'acide  $\gamma$ -aminobutyrique (GABA) à des concentrations millimolaires suffisantes pour permettre la croissance des agents pathogènes au cours des premiers stades de l'infection (Solomon and Oliver, 2001). Ces auteurs ont également observé une augmentation de la concentration en acides aminés entre 7 et 14 jours après l'infection corrélée à une augmentation de la biomasse fongique dans la feuille, ce qui suggère une dépendance sur le long terme du champignon envers la nutrition azotée de la plante hôte. Le cas du GABA est particulier puisqu'il se trouve habituellement à de faibles concentrations dans les plantes, mais ses niveaux augmentent en réponse à un certain nombre de stress, comme le froid, le choc thermique, la sécheresse et la réponse aux agents pathogènes. L'agent fongique *Cladosporium fulvum* est capable d'utiliser le GABA comme source d'azote, au même titre que l'aspartate ou le glutamate. Cela suggère que le GABA pourrait être une source efficace d'azote pour la croissance des agents pathogènes (Solomon and Oliver, 2001).

## 2. Impact de l'azote sur les maladies

### a) Effet des engrais azotés sur les maladies

Plusieurs études indiquent que l'application d'engrais azotés affecte les maladies des plantes. Des concentrations importantes d'azote augmentent souvent la sensibilité des plantes aux agents pathogènes (Tableau 2). Cependant, plusieurs études ont démontré que l'azote peut avoir un impact positif sur la résistance des plantes à certains agents pathogènes. Par exemple, il a été démontré que la disponibilité accrue en azote augmente la résistance de la tomate à *Fusarium oxysporum* f.sp. *lycopersici* et la résistance de plusieurs solanacées à *Alternaria solani*. Ainsi, plusieurs travaux montrent que des concentrations élevées d'azote augmentent la sensibilité des plantes aux maladies, alors que d'autres suggèrent que les plantes en limitation en azote deviennent plus faibles et plus sensibles aux agents pathogènes. Ces résultats contradictoires sont sans doute en partie dus aux modes de vie variées des agents pathogènes utilisés dans ces études. En effet, les apports azotés augmentent généralement la sensibilité des plantes aux biotrophes, alors qu'ils diminuent

généralement la sensibilité des plantes aux nécrotrophes (Ahmad, et al., 2011, Ballini, et al., 2013, Dordas, 2008, Snoeijers, et al., 2000). Cependant, il existe des contre-exemples comme le champignon nécrotrophe *B. cinerea* où l'on peut avoir les deux cas possibles suivant l'espèce hôte considérée (Daugaard, et al., 2003, Fagard, et al., 2014, Lecompte, et al., 2010).

Dans la littérature plusieurs exemples montrent également que la forme d'azote disponible pour la plante affecte aussi la sévérité des maladies. Par exemple, l'ammonium favorise les maladies provoquées par *Fusarium*, *Rhizoctonia* et *Sclerotium* sur les agrumes, le blé, le coton, la tomate et la betterave (Snoeijers, et al., 2000). Au contraire, la pourriture des racines de maïs, de pois et de coton ainsi que le flétrissement de tabac et de tomates causées par *Pythium*, *Phymatotrichum* et *Pseudomonas*, respectivement, sont favorisés par le nitrate par rapport à l'ammonium (Snoeijers, et al., 2000).

Au final, les connaissances actuelles indiquent que l'impact de l'azote sur la sensibilité des plantes aux maladies est complexe et nécessite plus d'études approfondies. L'azote peut en effet avoir des effets sur des facteurs macroscopiques, comme par exemple, dans le cas de la rouille jaune du blé. Dans ce cas, l'épidémie de rouille jaune semble être directement corrélée à la teneur en azote des feuilles qui affecte la structure de la canopée (Neumann, et al., 2004). Au niveau microscopique et/ou moléculaire, trois impacts majeurs de l'azote sur les interactions plante-pathogène sont possibles : l'azote peut affecter 1) la production des défenses de la plante, 2) la virulence de l'agent pathogène et 3) la disponibilité des nutriments pour l'agent pathogène au sein de la plante.

#### *b) Rôle de la nutrition azoté sur la défense*

Bien qu'il soit clair que la disponibilité en N a un impact sur la défense des plantes, il est encore difficile d'avoir une idée générale de cet effet au vu des exemples contradictoires dans la littérature (Fagard, et al., 2014). D'une part, certaines études indiquent qu'il existerait un compromis entre la croissance et la défense et qu'en cas de forte croissance peu de ressources seraient allouées à la mise en place des défenses (Walters and Heil, 2007) (Massad, et al., 2012). C'est le cas chez la légumineuse *Pentaclethra macroloba*, où les auteurs ont montré l'existence d'un compromis entre la production de biomasse et la production de flavane, un métabolite phénolique de défense (Massad, et al., 2012). Cependant, ces auteurs ont constaté que le niveau des saponines, métabolites de défense de

type terpénoïde, augmentait avec la biomasse, ce qui suggère que ce compromis entre la défense et la croissance peut ne pas être une caractéristique générale des interactions plante-pathogène.

D'autre part, certains auteurs suggèrent que la limitation en azote affaiblit les plantes et n'est donc pas favorable à la défense (Snoeiijers, et al., 2000). Une étude montre chez *A. thaliana* que des activités basales constitutives de trois enzymes associées à la défense, la chitinase, la chitosanase et la peroxydase, sont réduites dans les conditions de faible azote (Dietrich, et al., 2004). De plus, le taux d'induction de ces activités enzymatiques après un traitement par du BION®, un inducteur chimique de la défense des plantes, est réduit en faible azote. Néanmoins, ces études ne permettent pas de comprendre les mécanismes moléculaires contrôlant les défenses de la plante en fonction de la disponibilité en azote.

### c) Fertilisation azotée et virulence

Au cours des interactions plantes-pathogènes, l'azote peut également influencer la virulence de l'agent pathogène. En effet, une fois l'agent pathogène à l'intérieur de la plante, le transcriptome de l'agent pathogène répond à cet environnement différent et de nombreux gènes de virulence sont spécifiquement induits dans ces conditions. Plusieurs études ont démontré que les gènes codant les effecteurs bactériens et fongiques sont induits lors de la croissance *in planta* et dans des conditions limitantes en azote *in vitro* (Snoeiijers, et al., 2000). L'expression des protéines effectrices impliquées dans la virulence des champignons et des oomycètes peut être induite *in vitro* en condition d'azote limitant (Bolton and Thomma, 2008). C'est le cas du gène d'avirulence Avr9 de *Cladosporium fulvum*, fortement induit *in planta* et *in vitro* en azote limitant (Vandenackerveken, et al., 1994). Il a été également démontré que l'expression de certains gènes *hrp* bactériens, codant pour le système de sécrétion de type III (T3SS) et ses effecteurs, facteurs de virulence bactériens (voir §F.2.b), est régulée par l'environnement. Chez *Erwinia amylovora*, par exemple, l'expression de certains gènes codant pour des effecteurs, est régulée positivement *in vitro* dans un milieu minimal et réprimée en présence d'ammonium ou d'acides aminés tels que l'asparagine et l'histidine (Wei, et al., 1992). Enfin, l'azote affecte également les agents pathogènes de la pourriture molle qui sécrètent des enzymes de dégradation de la paroi végétale (CWDE<sup>7</sup>) qui conduisent à la désorganisation des tissus, à la mort cellulaire et à la libération des contenus cellulaires. Il a été proposé que la production de CWDE par la

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<sup>7</sup> CWDE: *cell wall degrading enzyme*



bactérie de la pourriture molle *P. carotovorum* serait induite *in vivo* dans les chicons en condition de fort azote (Schober and Vermeulen, 1999). Une autre étude révèle que la production de spores par le champignon de la rouille *Puccinia triticina* chez le blé était réduite jusqu'à 70% lorsque la plante est cultivée avec une faible teneur en azote (Robert, et al., 2002). Par contre, les auteurs ont observé que le pourcentage d'azote dans les spores était plus élevé que dans les feuilles, suggérant ainsi que l'agent pathogène est très efficace pour l'absorption de l'azote de l'hôte et que l'efficacité de ce mécanisme serait important pour la virulence de l'agent pathogène.

#### d) *Impact de la maladie sur le métabolisme azoté de la plante*

Plusieurs travaux montrent une modification dans le métabolisme azoté de la plante suite à l'infection par un agent pathogène. Par exemple pendant la pathogénèse des mécanismes similaires sont activés afin d'économiser l'azote (Pageau, et al., 2006), de la même manière qu'au cours de la sénescence. Les gènes *GSI*, codant la glutamine synthetase cytosolique et *GDH*, codant la glutamate déhydrogénase, impliquées dans l'assimilation primaire de l'azote pendant la sénescence sont induits également suite à une infection des feuilles de tabac avec *P. syringae* pv. *syringae*. En outre, l'analyse des niveaux d'acides aminés détectés dans les feuilles de tomate infectées par *P. syringae* pv. *tomato* indiquent que l'asparagine augmente significativement, or cet acide aminé est impliqué dans la remobilisation de l'azote lors de la sénescence des feuilles (Perez-Garcia, et al., 1998).

#### e) *Rôle des transporteurs de nitrate dans la défense*

La famille de gènes *NRT2* appartient à la grande superfamille des transporteurs de *major facilitator superfamily* (MFS). Chez *A. thaliana*, la famille *NRT2* comprend sept gènes, parmi lesquels quatre ont été caractérisés comme des transporteurs du nitrate à haute affinité (Orsel, et al., 2004). Récemment, deux gènes de cette famille, *NRT2.1* et *NRT2.6*, ont été identifiés comme ayant un rôle dans les réponses de défense de la plante. En effet, les mutants d'*A. thaliana* sont affectés dans leur réponse à *P. syringae* et à *E. amylovora*, respectivement (Camanes, et al., 2012, Dechorgnat, et al., 2012). Le mutant *nrt2.1* montre une sensibilité réduite à *P. syringae* accompagnée d'une réponse plus rapide de la voie de signalisation SA-dépendante et d'une sensibilité réduite à la toxine bactérienne coronatine (Camanes, et al., 2012). En outre, chez le mutant *nrt2.1*, l'état métabolique pourrait également contribuer à cette susceptibilité réduite puisque ce mutant accumule plus d'acides aminés aromatiques et de phénylpropanoïdes en comparaison de la plante sauvage. Le



mutant *nrt2.6* d'*A. thaliana* est plus sensible à *E. amylovora* que les plantes sauvages. Cette sensibilité accrue de mutant *nrt2.6* à la bactérie est corrélée avec une réduction dans l'accumulation de ROS, alors qu'aucune différence dans la réponse transcriptionnelle au pathogène ni dans d'autres réponses cellulaires telles que la synthèse de la callose et la production de NO n'a été mise en évidence (Dechorgnat, et al., 2012). Dans l'ensemble, ces données indiquent que la famille *NRT2* a un impact important sur les réactions de défense et joue un rôle important dans l'interaction entre la disponibilité en N et la réponse au stress biotique à travers un impact sur le voie du SA, la production de ROS ainsi que le statut de métabolites.

## F. L'interaction *E. amylovora*-*A. thaliana*

### 1. *E. amylovora* est responsable du feu bactérien

*E. amylovora* est une bactérie Gram négatif de la famille des enterobacteriaceae. *E. amylovora* provoque le feu bactérien ou brûlure bactérienne, maladie dévastatrice affectant une gamme restreinte d'espèces hôtes dans la famille des rosacées. Cette maladie est considérée comme une menace majeure pour la production commerciale des pommes et des poires. *E. amylovora* a été classée comme un organisme de quarantaine par l'Union européenne et, récemment, a été incluse parmi les 10 bactéries phytopathogènes les plus importantes à étudier selon des critères scientifiques et commerciaux (Mansfield, et al., 2012). Les bactéries pénètrent dans les plantes à travers les ouvertures naturelles situées au niveau des fleurs ou par les blessures et se propagent rapidement dans la plante au sein des vaisseaux du xylème provoquant ainsi des infections systémiques. Plusieurs déterminants de la virulence d'*E. amylovora* ont été caractérisés, dont le T3SS, l'exopolysaccharide amylovoran, la formation de biofilm et la motilité. Pour établir une infection avec succès, *E. amylovora* utilise un réseau complexe de régulateurs pour coordonner l'expression des facteurs de virulence en fonction des signaux environnementaux (Zeng and Sundin, 2014).

### 2. Les facteurs de virulence

Deux déterminants majeurs de la virulence sont nécessaires pour qu'*E. amylovora* infecte et provoque des maladies sur les plantes hôtes : la production de l'exopolysaccharide et le T3SS.

### a) *Exopolysaccharides : amylovorane et levane*

Il a été suggéré que les exopolysaccharides jouent un rôle clé pour contourner le système de défense des plantes, en perturbant et obstruant le système vasculaire de la plante et également en protégeant les bactéries contre la perte d'eau et de nutriments en conditions sèches. De plus ces exopolysaccharides sont des éléments importants dans la formation d'un biofilm par *E. amylovora*, permettant aux bactéries de se fixer sur les surfaces et entre elles (Koczan, et al., 2011, Koczan, et al., 2009). Le cluster de gènes *ams* comprend 12 gènes *ams* (*amsA* à *amsL*) impliqués dans la biosynthèse de l'amylovorane (Pique, et al., 2015). L'amylovorane est un polymère d'une unité répétitive de pentasaccharide qui se compose généralement de quatre résidus de galactose et d'un résidu d'acide glucuronique. L'amylovorane est nécessaire à la formation du biofilm et la quantité d'amylovorane produite par les souches individuelles d'*E. amylovora* est corrélée au degré de virulence (Koczan, et al., 2009, Vrancken, et al., 2013). En effet, les souches d'*E. amylovora* qui n'ont pas la capacité de produire de l'amylovorane ne sont pas pathogènes et sont incapables de se propager dans les vaisseaux de la plante (Bellemann and Geider, 1992, Vrancken, et al., 2013). Le levane est un autre exopolysaccharide produit par *E. amylovora*, considéré comme un facteur qui contribue à la virulence mais n'est pas indispensable. En effet, il a été montré que l'absence de synthèse du levane entraîne un développement plus lent des symptômes dans la plante hôte (Vrancken, et al., 2013). Cependant, le rôle spécifique du levane dans la virulence reste à établir.

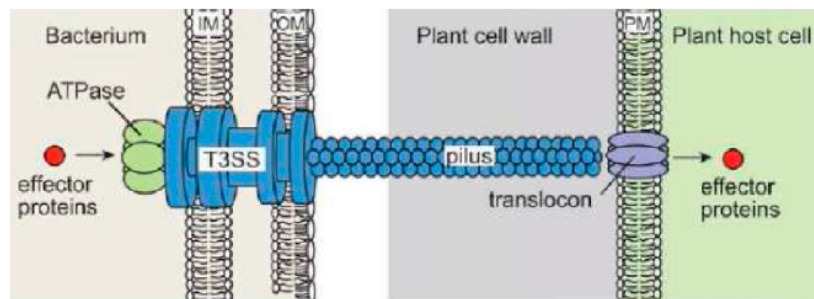
### b) *Le T3SS et les effecteurs*

Le T3SS est l'un des facteurs de virulence important utilisé par *E. amylovora* pour infecter ses hôtes. Comme d'autres bactéries Gram négatif phytopathogènes, *E. amylovora* utilise ce système de sécrétion hautement conservé pour exporter et délivrer des protéines effectrices dans le cytosol des cellules de la plante hôte grâce à une structure semblable à un pilus, qui forme l'élément central de T3SS (Pique, et al., 2015) (Figure 8). La pathogénicité d'*E. amylovora* repose sur le T3SS et sur un effecteur majeur DspA/E. Cet effecteur appartient à la famille d'effecteurs AvrE dont la fonction biologique n'est pas entièrement comprise (Jin, et al., 2001).

Le T3SS est composé d'un grand complexe macromoléculaire de forme cylindrique constitué de séries de structures annulaires avec des anneaux intérieurs, des anneaux extérieurs et un col. Il est intégré dans la membrane interne et externe de la bactérie, tout en

couvrant l'espace périplasmique et s'étendant dans l'environnement extracellulaire avec un filament de type pilus. Il a été démontré que le T3SS est produit uniquement au site de l'assemblage du pilus Hrp et que le pilus guide le transfert des protéines effectrices à l'extérieur de cellule bactérienne selon le modèle *conduit/guiding filament* (Figure 8) (Pique, et al., 2015).

Le T3SS des bactéries phytopathogènes est principalement constitué de plusieurs éléments : 1) les protéines Hrc, codées par des gènes *hrp*-conserved (*hrc*), 2) les protéines Hrp, codées par des gènes *hrp*<sup>8</sup>. Chez *E. amylovora*, les gènes *hrc* et *hrp* sont regroupés dans un îlot de pathogénicité, qui contient quatre régions : une région *hrc/hrp*, une région codant les effecteurs, une région codant des enzymes associées à Hrp et une région dite *island transfer region* (Vrancken, et al., 2013).



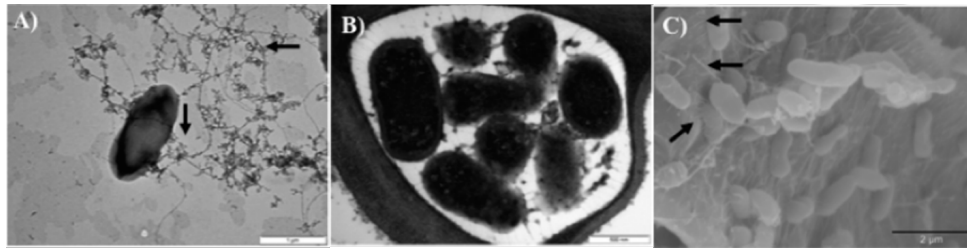
**Figure 8 : Représentation schématique du T3SS de bactéries phytopathogènes selon (Pique, et al., 2015).**

### c) La formation de biofilm

A part les exopolysaccharides amylovorane et levane qui jouent un rôle spécifique dans la formation du biofilm d'*E. amylovora*, une étude récente suggère que d'autres facteurs sont impliqués. En effet, plusieurs structures d'attachement comme les *fimbriae* de type I, les flagelles, le *pilus* de type IV et le *curli* d'*E. amylovora* peuvent contribuer à la formation de biofilm (Koczan, et al., 2011). L'utilisation de mutants bactériens affectés dans la production de ces structures dans des essais d'attachement *in vitro* et *in planta*, a permis de démontrer que ces multiples structures d'attachement jouent un rôle dans la formation de biofilm et qu'il s'agit d'un élément critique pour la pathogénicité et le mouvement systémique dans l'hôte (Koczan, et al., 2009, Pique, et al., 2015). En effet, les mutants déficients en biofilm comme  $\Delta$ hofC,  $\Delta$ fimD,  $\Delta$ fim, and  $\Delta$ flg-4 restent localisés dans une feuille inoculée et sont fortement altérés dans la capacité d'envahir le reste de la plante

<sup>8</sup> *hrp* : hypersensitive response and pathogenicity

(Koczan, et al., 2011). Bien que les détails mécanistiques de la formation de biofilm restent mal connus, les données actuelles suggèrent qu'ils sont formés en réponse à des déclencheurs environnementaux et des signaux de *quorum sensing* (Pique, et al., 2015) (Figure 9).



**Figure 9. Images de structures d'attachement putatives d'*E. amylovora*.** (A, B) images de microscope électronique à transmission d'*E. amylovora*. (A) Bactérie cultivée *in vitro* et colorée négativement. Les flagelles sont indiqués par des flèches (échelle : 1  $\mu$ m) ; (B) *E. amylovora in planta*. Des structures d'attachement putatives relient les cellules bactériennes aux cellules hôtes (échelle : 1  $\mu$ m) ; (C) Image de microscope électronique à balayage de cellules d'*E. amylovora* trouvées dans un biofilm, avec de multiples appendices qui se fixent à la surface de l'hôte, indiqués par les flèches (échelle: 2  $\mu$ m) selon (Koczan, et al., 2011).

### 3. L'interaction non-hôte *E. amylovora*-*A. thaliana*

*E. amylovora* est capable de se multiplier puis de déclencher des lésions nécrotiques et une mort cellulaire localisées dans les feuilles de la plante non-hôte *A. thaliana*. Ces processus sont dépendants du T3SS comme sur la plante hôte (Degrave, et al., 2008, Degrave, et al., 2013). Cependant, la multiplication bactérienne dans les feuilles d'*A. thaliana* est plus faible et transitoire par rapport à la plante hôte. Plusieurs réactions de défenses chez *A. thaliana* contre *E. amylovora* sont similaires à ce qui a été observé dans les plantes hôtes : il s'agit de l'activation de la voie de défense SA, de l'accumulation de ROS et d'une partie de la voie de défense JA. Par contre, l'accumulation de callose est une réaction de défense observée spécifiquement chez *A. thaliana* en réponse à *E. amylovora* qui n'existe pas chez les plantes hôtes. Aucun des mutants d'*A. thaliana* correspondant à ces différentes voies, y compris le mutant *pmr4* déficient pour la production de callose, ne s'est avéré plus sensible que les plantes sauvages suite à l'infection par *E. amylovora* (Moreau, et al., 2012). L'infection d'*A. thaliana* par *E. amylovora* provoque une large modification transcriptomique chez *A. thaliana* dans laquelle 20% de modifications correspondaient à l'induction de gènes liés à la défense et à la signalisation (Moreau et al, 2012). Ces auteurs ont également révélé que le gène *EDS1* est un régulateur positif de la résistance non-hôte d'*A. thaliana* contre *E. amylovora*. Ils supposent qu'elle pourrait contrôler la production de plusieurs défenses

efficaces contre *E. amylovora* via l'action de *WRKY46* et *WRKY54* tandis que *WRKY70* agit comme régulateur négatif. Les travaux menés sur cette interaction indiquent également qu'*E. amylovora* est capable de se multiplier fortement dans les tissus d'*A. thaliana* dans des conditions spécifiques, correspondant vraisemblablement à une répression des défenses de la plante (Moreau, et al., 2012). Plus récemment, des travaux ont montré que l'accumulation de ROS dans l'apoplasme semble jouer un rôle clé dans cette interaction (Launay, et al., 2016). Dans l'ensemble, l'état actuel des connaissances suggère que le manque d'adaptation d'*E. amylovora* à *A. thaliana* serait due à l'activation de plusieurs niveaux de défense par la plante auxquelles la bactérie ne serait pas capable d'échapper.

## G. Objectifs de la thèse

Des expériences préliminaires ont montré qu'un faible apport en nitrate permettait une meilleure multiplication d'*E. amylovora* chez *A. thaliana* (Fagard, et al., 2014). Mes travaux de thèse ont donc été effectués dans le but de mieux comprendre l'effet de la disponibilité en azote dans l'interaction *A. thaliana*-*E. amylovora*. Dans le cadre de cette thèse, deux objectifs principaux ont été définis.

Le **premier objectif** a été d'expliquer l'augmentation de la résistance non-hôte observée récemment chez *A. thaliana* vis à vis de la bactérie phytopathogène *E. amylovora* lorsque les plantes sont soumises à une forte concentration en nitrate (Fagard, et al., 2014). Pour cela, trois effets possibles de l'azote ont été envisagés : 1) premièrement, nous avons cherché à déterminer si l'azote pouvait affecter l'expression des défenses de la plante, ceci *via* la signalisation des voies hormonales efficaces contre cet agent pathogène, l'accumulation des ROS, le renforcement de la paroi de la cellule végétale avec le dépôt de callose, et l'expression de certains régulateurs de la défense comme les facteurs de transcription WRKY ; 2) deuxièmement, l'effet nutritionnel de l'azote sur le développement d'*E. amylovora* a été également étudié par le suivi de sa croissance *in vitro* en présence de différentes sources azotées (acides aminés, nitrate et ammonium) et à différentes concentrations ; 3) enfin, l'effet de la variation de la disponibilité en azote a été étudié sur l'expression des facteurs de virulence de la bactérie.

Le **deuxième objectif** de cette thèse a été d'analyser les réponses de la plante à des stress simples et multiples afin d'identifier les différents niveaux de régulation des gènes. Pour cet objectif nous avons réalisé une analyse de microarray par la technologie CATMA. Dans un

premier temps, l'effet de l'azote sur la réponse de la plante à la bactérie a été étudié. Dans un deuxième temps, les catégories de réponse des plantes aux stress individuels et combinés ont été définies. Enfin, la réponse des plantes aux stress simples et combinés a été réalisée en utilisant un autre agent pathogène bactérien, *P. syringae*, afin de savoir si l'azote agit de la même manière sur la réponse de la plante aux différents agents pathogènes bactériens.

## Matériel et méthodes

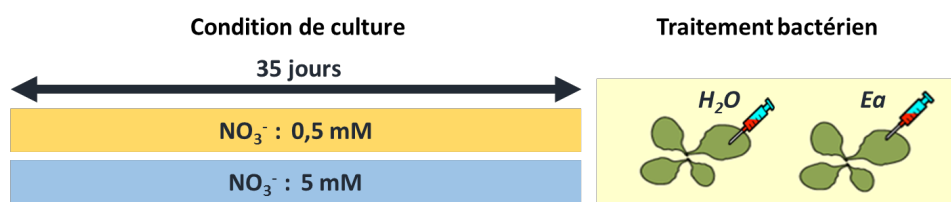
### 1. Culture des plantes et inoculations

Les plantes ont été cultivées dans une chambre de culture avec 8 h de lumière à 21° C /16 h de nuit à 18°C avec une radiation de 150 µE/m<sup>2</sup>/s irradiation et 65% d'humidité. Les plantes ont été arrosées trois fois par semaine avec une solution nutritive avec différentes concentrations de nitrate (NO<sub>3</sub><sup>-</sup>). Pour l'article présenté dans la partie A, les plantes ont été cultivées en mottes avec une solution nutritive à 0,5 ou 5 mM NO<sub>3</sub><sup>-</sup> (Figure 10). Pour l'article présenté dans la partie B, les plantes ont été cultivées en sable avec une solution nutritive à 2 ou 10 mM NO<sub>3</sub><sup>-</sup> (Figure 10).

A

Composition/Solution nutritive	NO <sub>3</sub> <sup>-</sup> 0,5 mM	NO <sub>3</sub> <sup>-</sup> 2 mM	NO <sub>3</sub> <sup>-</sup> 5 mM	NO <sub>3</sub> <sup>-</sup> 10 mM
KNO <sub>3</sub>	25,3 mg/L	176,8 mg/L	404 mg/L	505 mg/L
Ca(NO <sub>3</sub> ) <sub>2</sub> 4H <sub>2</sub> O	29,5 mg/L	29,5 mg/L	118 mg/L	590 mg/L
NaCl	-	-	11,7 mg/L	11,7 mg/L
CaCl <sub>2</sub>	18,5 mg/L	13,9 mg/L	-	-
MgSO <sub>4</sub> 7H <sub>2</sub> O	61,5 mg/L			
KH <sub>2</sub> PO <sub>4</sub>	34 mg/L			
Fer sequestrène	10 mg/L			
Oligoéléments	100 mg/L			

B



**Figure 10 : Dispositif expérimental.** **A** : composition des solutions nutritives utilisées. **B** : schéma des conditions expérimentales correspondant à la partie A des résultats de la thèse (analyse du transcriptome). Les plantes ont été cultivées 5 semaines en motte et arrosées avec une solution nutritive contenant 0,5 ou 5 mM de NO<sub>3</sub><sup>-</sup>. Au bout de 5 semaines, les plantes sont infiltrées par *E. amylovora* (*Ea*) ou avec de l'eau (feuilles mock). Pour la partie B des résultats de la thèse, les plantes ont été cultivées 5 semaines en sable et arrosées avec des solutions nutritives contenant 2 ou 10 mM de nitrate comme indiqué dans le tableau ci-dessus.

La souche sauvage d'*E. amylovora* CFBP<sup>9</sup>1430 est cultivée sur un milieu solide LB contenant du chloramphenicol (10 µg/ml) à 28°C. Au bout de 2 jours de croissance, les bactéries sont cultivées dans un milieu LB liquide sur la journée à 28°C sous agitation puis transférées sur milieu LB solide le soir afin d'effectuer les inoculations le lendemain matin. Les bactéries sont remises en suspension dans de l'eau stérile à une DO finale de 0,1 (10<sup>7</sup> CFU/ml). Les feuilles d'*A. thaliana* âgées de 5 semaines sont infiltrées avec l'eau, comme témoin négatif, ou avec *E. amylovora*. L'inoculation se fait à l'aide d'une seringue sans aiguille qui est appliquée sur la face abaxiale de la feuille. Les feuilles ont été collectées 6 h ou 24h après le traitement et immédiatement congelées dans de l'azote liquide. Deux à trois réplicas biologiques indépendants ont été prélevés pour chaque expérience et un minimum de deux expériences indépendantes ont été réalisées.

## 2. Approche transcriptomique

L'analyse du transcriptome a été réalisée selon la méthodologie *Complete Arabidopsis Transcriptome MicroArray* (CATMA) basée sur l'utilisation de puces à ADN (Crowe, et al., 2003, Hilson, et al., 2004). L'ARN total a été extrait à l'aide du TRIZOL® (Invitrogen Life Technologies, Saint-Aubin, France) et ensuite purifié en utilisant le kit Qiagen RNeasy selon les instructions du fournisseur. La vérification de l'intégrité des ARNs, la synthèse des ADNc et leur marquage au fluorochrome, l'hybridation sur les puces, la lecture des puces et l'analyse statistique des données ont été effectués par la plateforme POPS<sup>10</sup> située à Orsay. L'analyse statistique réalisée par la plateforme POPS est basée sur la réalisation de deux réplicas techniques avec un échange des fluorochromes cy3 et cy5 (Gagnot, et al., 2008). Pour identifier les gènes exprimés de manière différentielle en réponse à la bactérie par rapport aux conditions non infectées. Les points affichant une variance extrême, trop petite ou trop importante, sont exclus. Les *P-value* brutes ont été ajustées selon la méthode de Bonferroni. Les gènes ayant une *P-value* de Bonferroni  $\leq 0,05$  sont considérés comme différemment exprimés entre la condition « inoculé » et « contrôle non inoculé ». Cette analyse a été réalisée séparément pour les plantes cultivées à fort et faible azote puis les listes de gènes différentiels ont été comparés (voir article). L'enrichissement des catégories

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<sup>9</sup> CFBP: collection française de bactéries phytopathogènes

<sup>10</sup> <http://www.observatoire-vegetal.inra.fr/Les-plateformes-scientifiques/Plateforme-transcriptome>



fonctionnelles a été évalué en utilisant les catégories de la base de données FunCatDB<sup>11</sup>, sur la base d'une distribution hypergéométrique avec une *P-value* au seuil de  $5.10^{-3}$ .

### 3. Etude des modes de réponse transcriptionnels

Pour comparer la réponse des plantes aux stress simples et combinés, l'ensemble de transcrits significativement modulés en réponse à l'infection par *E. amylovora* ont été sélectionnés. Parmi ces gènes, nous avons sélectionné ceux ayant un log ratio supérieur à 1 ou inférieur à -1. Ensuite, nous avons considéré la réponse de manière binaire en ne considérant plus la valeur absolue du log ratio mais seulement si elle est inférieure à 1 (gène réprimé par l'infection), située entre -1 et 1 (gène non modulé par l'infection) ou supérieure à 1 (gène induit par l'infection). Cette opération a également été appliquée au log ratio de la comparaison des plantes contrôle en fort azote comparées aux plantes contrôle cultivées en faible azote. Puis cette opération a été appliquée à la combinaison des stress en comparant les plantes contrôle cultivées en fort azote aux plantes inoculées cultivées en fort azote. Tous les transcrits ayant le même profil d'expression en réponse au stress bactérie, au stress azote et à la combinaison des deux ont été regroupés en 20 profils d'expression prédéfinis (voir manuscrit). Chacune de ces 20 sous-catégories correspond à un motif d'expression potentiel qui peut se produire lors d'une application de stress simple et multiple. Les sous-catégories similaires ont ensuite été regroupées par type de réponse en fonction des 5 profils définis dans un travail antérieur, *combinatorial*, *cancelled*, *prioritized*, *independent* et *similar* (Rasmussen, et al., 2013). L'enrichissement des différentes catégories en catégories fonctionnelles spécifiques a été déterminée grâce à la base de données FunCatDB (Ruepp et al. 2004). Pour simplifier, nous nous sommes concentrés sur les catégories fonctionnelles déjà identifiées comme étant fortement modulées au cours de l'infection d'*A. thaliana* par *E. amylovora* (Moreau, et al., 2012).

### 4. Extraction d'ARN et RT

L'analyse de l'expression des gènes de plante a été effectuée à partir de quatre feuilles d'une plante âgée de 5 semaines infectée ou non par *E. amylovora*. L'ARN total a été isolé en utilisant le réactif TRIzol® (Life technologies) selon le protocole du fabricant. Les ADNc du premier brin ont été synthétisés à partir de 1 µg d'ARN en utilisant le kit RevertAid Reverse Transcriptase (Thermo Scientific®). Premièrement, la dénaturation de 5 µL

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<sup>11</sup> <http://mips.helmholtz-muenchen.de/funcatDB/>

d'ARNs et l'hybridation en présence de 1 µL d'oligo-dT à 0,5 µg/µL est effectuée à 65°C pendant 5 minutes dans un volume final de 12,5 µL. Deuxièmement, la reverse-transcription est effectuée en présence de 4 µL de tampon 5X, ®, de 2µL de dNTP à 10 mM, de 0,5 µL de Riboblock et de 1 µL de RevertAid pendant 1h à 42°C. La dernière partie correspond à l'inactivation de l'enzyme pendant 15 minutes à 70°C. Les ADNc sont ensuite dilués dans 180 µL d'eau osmosée.

Dans le cas d'expression de gène de virulence d'*E. amylovora in planta* toutes les étapes ont été effectués de manière similaire à l'analyse des gènes de plante à part pour la synthèse d'ADNc bactériens. Dans cette étape, au lieu des oligonucléotides oligo-dT on utilise des *random primer* c'est à dire des oligonucléotides de séquence aléatoire et d'une longueur de 6 nucléotides.

#### 5. Analyse de l'expression des gènes par qRT-PCR

Les analyses d'expression des gènes ont été déterminées par PCR quantitative (qPCR) en temps réel (Eppendorf Realplex MasterCycler) en utilisant le kit « LightCycler-FastStart DNA Master SYBR Green I » (Roche) kit. Cette réaction est préparée en *Master mix* puis réparti dans les puits. Chaque puits contient 2,2 µL d'eau osmosée, 0,3 µL des deux amorces spécifiques à 10 µM, 5 µL de mix pour qPCR (Applied biosystem®) contenant les dNTP, la Taq polymérase, le tampon 10X et le SYBR Green. La réaction est donc effectuée avec 2,5 µL d'ADN matrice dans un volume final de 10 µL. Les réactions de PCR quantitatives ont été réalisées sur le thermocycleur Biorad® CFX96. Le programme employé consiste en 5 étapes : 1) 5 minutes à 95°C ; 2) 15 secondes à 95°C ; 3) 1 minute à 60°C puis une lecture de la plaque est effectuée. Les étapes 2 à 3 sont répétées 39 fois à partir de la troisième étape. 4) 15 secondes à 95°C ; 5) réalisation de la courbe de dissociation des amorces. Elle commence à 60°C et par incréments de 0,5°C, la température monte à 95°C. Une lecture de plaque est effectuée à cette étape.

#### 6. Analyse du métabolome

Afin d'analyser le métabolome des feuilles de rosette de 5 semaines cultivées en fort ou faible nitrate, puis infiltrées à l'eau ou inoculées avec la souche sauvage d'*E. amylovora* ont été prélevées 24hpi. Pour chaque condition 5 à 6 feuilles ont été prélevées dans de l'azote liquide puis broyées. Entre 28 et 30 mg de poudre congelée ont été traitées par la plate-

forme de chimie du végétal de l'observatoire végétal par un chromatographe en phase gazeuse (GC) Agilent 7890A couplé à un spectromètre de masse (MS) Agilent 5975C selon le protocole décrit précédemment par (Amiour, et al., 2012). Pour la quantification, les standards ont été injectés au début et à la fin de l'analyse. Les données ont été analysées avec AMDIS<sup>12</sup> et le logiciel QuantLynx (Waters).

Pour l'analyse de clustering hiérarchique (HCA) nous avons utilisé la version 1.7.6 du logiciel Genesis<sup>13</sup> suite à la transformation des valeurs en Log2. Pour la visualisation, les données des métabolites ont été normalisées en divisant chaque valeur par la médiane de toutes les valeurs de l'expérience pour un métabolite donné. Les sous-groupes ont été identifiés en utilisant la méthode K-means disponible dans Genesis. L'analyse du métabolome a été faite à partir des proportions de chaque métabolite (pourcentage de chaque métabolite par rapport au total des métabolites [nmol / (mg FW)]).

#### 7. Mesure de la croissance *in vitro* d'*E. amylovora*

La souche sauvage d'*E. amylovora* CFBP<sup>14</sup>1430 est cultivée sur un milieu solide LB contenant du chloramphenicol (10 µg/ml) à 28°C. Au bout de 2 jours de croissance, les bactéries sont cultivées dans un milieu LB liquide sur la journée à 28°C sous agitation. La suspension bactérienne a été centrifugée à 3600 g pendant 10 minutes et lavée dans l'eau stérile deux fois afin d'éliminer complètement le LB. Après l'élimination du milieu LB, 500 µl de suspension bactérienne a été incubée dans 5 ml d'un milieu minimum M9 contenant 5 mM d'un acide aminé donné ou de nitrate ou d'ammonium comme source de N, du glucose (0,2 %) comme source de C et de l'acide nicotinique (2 mM). En parallèle, les bactéries ont également été incubées dans 5 ml de milieu minimum contenant du glucose et de l'acide nicotinique sans source de N (témoin négatif) et dans le milieu LB liquide (témoins positif). Des lectures de DO ont été effectuées à T0, 12, 24, 40 et 48 heures post incubation.

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<sup>12</sup> <http://chemdata.nist.gov/dokuwiki/doku.php?id=start>

<sup>13</sup> <http://genome.tugraz.at/>

<sup>14</sup> CFBP: collection française de bactéries phytopathogènes

#### 8. Pré-culture en milieu IM

Pour les expérimentations de pré-induction des gènes de pathogénie d'*Ea*, les bactéries ont été centrifugées deux fois comme décrit ci-dessus. Suite à l'élimination du milieu LB par deux centrifugations successives, les bactéries sont resuspendues dans de l'eau stérile puis sont étalées sur un milieu IM ou sur du LB pendant 6h avant de procéder à l'infection selon le protocole habituel. Le milieu IM correspond au milieu minimum M9 (Gannon, et al., 1988) complété par du galactose à 0,2 %. Ce milieu a été défini d'après des travaux antérieurs (Gaudriault, et al., 1997).

## Résultats

### A. Bases génétiques de l'impact de l'azote sur l'interaction *A. thaliana*-*E. amylovora* - Approche transcriptomique

#### 1. Introduction

##### a) Contexte scientifique

Les plantes sont menacées par un large éventail d'agents pathogènes microbiens et d'insectes herbivores (Oerke, 2006). Outre ces stress biotiques, les plantes sont également exposées à des conditions environnementales abiotiques telles que la sécheresse, la chaleur, le froid, la salinité élevée et la limitation nutritionnelle. Dans les milieux naturels et agricoles, les plantes doivent donc faire face simultanément à de multiples conditions de stress. Dans le contexte du changement climatique, il est très probable que la fréquence et la complexité de ces conditions dites de « multi-stress » menacent le taux de rendement des cultures (Coolen, et al., 2016). En effet, les stress abiotiques peuvent affecter de façon significative les réactions des plantes aux stress biotiques et vice versa, selon leur durée, leur nature et leur sévérité (Atkinson and Urwin, 2012). La façon dont les plantes régulent et hiérarchisent leur réponse adaptative lorsqu'elles sont exposées à de multiples stress est largement inconnue. Dans la littérature, les réponses des plantes à différents facteurs de stress ont été analysées simultanément ou séquentiellement (Atkinson and Urwin, 2012, Kissoudis, et al., 2014, Prash and Sonnewald, 2013, Rasmussen, et al., 2013, Rivero, et al., 2014, Santino, et al., 2013, Sewelam, et al., 2014). Ces études suggèrent que les différentes voies de signalisation liées au stress sont interconnectées dans un réseau qui est sous le contrôle de régulateurs clés tels que des MAPKs, des facteurs de transcription et les hormones de stress (Coolen, et al., 2016). Plusieurs études ont mis en évidence que les réponses aux stress doubles ne sont pas la simple addition de réponses à des stress simples (Atkinson and Urwin, 2012, Rasmussen, et al., 2013). Par conséquent, il est nécessaire de caractériser au mieux les réponses des plantes aux multi-stress afin d'identifier les mécanismes moléculaires sous-jacents. Ces données pertinentes fourniront des outils pour répondre à des questions fondamentales sur le *cross-talk* dans la signalisation en réponse aux multi-stress et pourront également, à moyen ou long terme, se traduire par des applications agronomiques (Mundy and Beresford, 2007).

## b) *Questions biologiques et stratégie expérimentale*

Afin de réaliser une analyse comparative des réponses des plantes aux stress individuels et combinés nous avons choisi de nous focaliser sur les modifications du transcriptome. Nous avons choisi d'étudier la réponse de la plante modèle *A. thaliana* à deux stress : 1) un stress biotique, représenté par l'infection avec la bactérie phytopathogène *E. amylovora*, et 2) un stress abiotique, avec une limitation de la nutrition azotée. L'azote est un élément essentiel pour la vie de la plante et peut devenir un facteur limitant de sa croissance. Le choix de ce stress abiotique est d'autant plus pertinent que de nombreuses données agronomiques indiquent l'importance de la disponibilité en azote sur l'incidence des maladies sur les grandes cultures (Dordas, 2008). Par contre, de nombreuses données sont contradictoires, suggérant que les mécanismes sous-jacents, encore mal connus, qui relie le statut azoté de la plante et sa réponse à l'infection sont complexes (Fagard et al, 2014).

La bactérie *E. amylovora* est l'agent causal du feu bactérien, maladie dévastatrice de certaines rosaceae, notamment chez les pommiers et les poiriers. L'équipe dans laquelle j'ai réalisé ma thèse étudie l'interaction *E. amylovora*-*A. thaliana* depuis plusieurs années, et a caractérisé cette interaction dans ses grandes lignes (Degrave, et al., 2008, Degrave, et al., 2013, Degrave, et al., 2013, Fagard, et al., 2014, Launay, et al., 2016, Moreau, et al., 2012, Zarattini, et al., 2016)). Bien qu'*A. thaliana* ne soit pas un hôte connu pour *E. amylovora*, les travaux antérieurs ont montré qu'*E. amylovora* est capable de se multiplier dans les tissus d'*A. thaliana* sans causer de symptômes systémiques du feu bactérien. De plus, l'inoculation de la bactérie en présence de cycloheximide, inhibiteur de la synthèse protéique chez l'hôte, a pour effet de favoriser la multiplication d'*E. amylovora* et de réprimer les défenses (Moreau et al, 2012). De plus, l'infection d'*A. thaliana* par *E. amylovora* entraîne des modifications profondes dans le transcriptome de la plante. En effet 20% des gènes induits suite à l'infection sont liés à la défense et à la signalisation (Moreau, et al., 2012). Dans le travail que je présente ici, les plantes ont été soumises à deux stress individuels, la limitation en azote d'une part et l'infection bactérienne d'autre part, puis à la combinaison de ces deux stress. Une analyse du transcriptome a été réalisée sur ces plantes et une analyse détaillée des profils de réponse des gènes est présentée dans le manuscrit joint, ce manuscrit ayant pour vocation d'être soumis à la revue *Frontiers in Plant Science*.

### 2. Article « Nitrogen limitation impacts the response of specific genes to biotic stress »

## **Nitrogen limitation impacts the response of specific genes to biotic stress**

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## ABSTRACT

Although it is now clear that the response of plants to a combination of stresses is not the simple addition of the response to each single stress, transcriptomic data in response to combined stresses are scarce. This is particularly true in the case of combined nutrient limitation such as nitrogen (N) and biotic stress. In order to determine whether there is an interaction between the responses to a biotic stress and to N limitation at the transcriptomic level, we performed a microarray analysis. Plants were grown in full (5 mM NO<sub>3</sub><sup>-</sup>) or limiting N (0.5 mM NO<sub>3</sub><sup>-</sup>) and subjected to infection by the bacterial phytopathogen *Erwinia amylovora* (*E. amylovora*), known to provoke a large reprogramming of the Arabidopsis transcriptome. N limitation had a visible effect on plant growth but impacted only a limited set of genes in response to infection, mainly linked to plant defense. We then considered the combination of the two individual stress treatments and found that when N limitation and biotic stress were combined, 32.5 % of transcripts responded in a manner that could not be deduced from their response to each individual stress, suggesting an interaction between these stress responses. These genes correspond to three categories: 1) genes that are not modulated in response to single stresses but that show repression or induction in response to the combination (combinatorial), 2) genes induced or repressed by one of the single stresses and not modulated in response to the combination (cancelled) and 3) genes that are repressed by N limitation, induced by biotic stress and remain induced in response to the combination of stresses (prioritized). Several defense-related genes were regulated in the prioritized manner, probably reflecting a dominance of the response to the biotic stress treatment over the abiotic stress response. Finally, we analyzed the impact of N supply on the response of selected defense marker genes to the bacterial phytopathogen *Pseudomonas syringae* and found similar results, suggesting that the interaction between nutritional supply and biotic stress are at least partly conserved between bacterial pathosystems.



## INTRODUCTION

Sustainable protection of crops is a worldwide necessity. Indeed, plants are often continuously exposed to a broad range of biotic and abiotic stresses in their natural habitats (Rasmussen *et al.*, 2013). Because biotic and abiotic stresses reduce significantly plant growth and productivity, considerable research has aimed to determine the responses of plants to single stresses (Chew and Halliday, 2011). However, the response of plants to a combination of stresses is not the simple addition of the response to each single stress (Suzuki *et al.*, 2014). In addition, it has also been reported that plant responses to different stresses are coordinated by complex and interconnected signaling pathways modulating numerous metabolic networks (Nakashima *et al.*, 2009). Apart from some recent reports the effects of combined biotic and abiotic stress have been little studied. Indeed, various abiotic stresses can affect negatively or positively plant-pathogen interactions. For example, the exposure of *Arabidopsis* plants to drought enhances plant susceptibility to an avirulent isolate of *P. syringae* while it decreases the susceptibility of tomato to the fungus *B. cinerea* (Atkinson *et al.*, 2013). One of the most important abiotic factors is the availability of nutrients that affects seriously plant disease severity (Dordas, 2008). Contradictory reports about the impact of nutrients on plant susceptibility to pathogens indicate that many factors affecting this process are not well understood.

Nitrogen (N) is an essential macronutrient and is a major limiting factor of plant growth and development (Masclaux-Daubresse *et al.*, 2010). Besides growth and developmental effects, many agronomical reports highlight the fact that N fertilizers can impact the plant's ability to cope with biotic stress (Ballini *et al.*, 2013; Hoffland *et al.*, 2000). However, contradictory data have been reported about the effect of N on disease development. On the one hand, the effect of N on this process seems partly dependent to the life style of the pathogen. Generally, high N availability increases the susceptibility of plants to biotrophs, while it generally decreases the susceptibility of plants to necrotrophs, although some exceptions have been reported (Fagard *et al.*, 2014). However, this process seems to be more complex and the impact of N can be dependent on the host plant for a given pathogen. For instance, in the case of *B. cinerea*, one of most important fungal plant pathogens, high N fertilization enhances the level infection in strawberry (Daugaard *et al.*, 2016) while it reduces susceptibility to this fungus in tomato (Vega *et al.*, 2015). The form of N available can also determine the effect of N on disease severity. For instance,  $\text{NH}_4^+$  fertilization enhances the

level of symptoms caused by *P. syringae*, while conversely  $\text{NO}_3^-$  reduces plant susceptibility to *P. syringae* (Mur *et al.*, 2016). These studies indicate the complexity of the relationship between N metabolism and plant resistance to pathogens.

Although the mechanisms are not well known, it is generally thought that N supply can affect plant–pathogen interactions through its impact on plant defense, pathogen virulence and the availability of nutrients for the pathogen (Fagard *et al.*, 2014). However, the effect of N limitation on the expression of defense remains unclear as the literature harbors contradictory reports concerning the effect of N limitation on the expression of defense. Indeed, on the one hand, some reports highlight that N limitation can influence constitutive or induced defense (Ward *et al.*, 2010). Indeed, Arabidopsis plants grown in low N show a reduced basal activity of three defense-associated enzymes (chitinase, chitosanase, and peroxidase). Furthermore, in plants grown in low N and treated with BION®, a chemical elicitor of plant defense, the level of these enzymes is also reduced compared to plants grown in high N (Dietrich *et al.*, 2004). A recent study on the other hand demonstrates that under low N, there is a decrease in the levels of polyamine, a compound known to increase plant resistance via the triggering of programmed cell death (Gupta *et al.*, 2013; Tiburcio *et al.*, 2014). Thus, many reports show that low N drives a decrease in defense (Snoeijsers *et al.*, 2000). However, other reports suggest that there could be a trade-off between plant growth and defense (Huot *et al.*, 2014; Walters and Heil, 2007). Thus, although it is obvious that N availability has an impact on plant defense, it is currently difficult to get a clear-cut idea of the effect of N on plant defense.

In this paper, we studied the impact of N limitation on the response of Arabidopsis to biotic stress. To do this, we analyzed the transcriptome of plants grown in limiting and non-limiting N and subjected to biotic stress. We found that most Arabidopsis genes were regulated similarly in the two contrasted N regimes in response to biotic stress. Further analysis of the expression data indicates that when two individual stresses were combined, 32.5 % of transcripts showed a profile that suggested an interaction between the two single stresses. A small fraction (2.6 %) of these transcripts were prioritized between antagonistic responses to individual stresses including known defence-related genes, reflecting a preservation of the plant defense program under N limitation treatment. Analysis of the microarray data indicated that Arabidopsis genes were affected in larger numbers by bacteria than by N limitation. Altogether, our data suggest a preponderance of the plants

response to biotic stress in terms of genes modulation over the response to N limitation. In order to determine the genericity of the effect of N supply on biotic stress, we analyzed the impact of N supply on the response of selected defense marker genes to a virulent and an avirulent strain of the bacterial phytopathogen *Pseudomonas syringae*. We found different results from the interaction between the response to *E. amylovora* and to N limitation, indicating that the interaction between stresses is not conserved between pathosystems.

## RESULTS

### **N limitation affects gene expression in response to biotic stress**

To determine to what extent the nutritional status of a plant affects its response to a bacterial pathogen, we grew *Arabidopsis* plants in limiting (0.5 mM NO<sub>3</sub><sup>-</sup>) or non-limiting (5 mM NO<sub>3</sub><sup>-</sup>) N in plugs of peat moss substrate for 5 weeks (Figure 1A), and infected them with the bacterial phytopathogen *E. amylovora*. In these conditions, rosette diameter was reduced by 38 % (Figure 1B) and nitrate content was reduced by 16 % (Supp. Figure 1). In order to determine if plant susceptibility to bacteria was affected by these growth conditions, plants were inoculated with *E. amylovora* wild-type strain and *in planta* titers of *E. amylovora* was analyzed 6 and 24 hours post inoculation (hpi). We observed that in leaves of *Arabidopsis* plants grown in limiting N (0.5 mM NO<sub>3</sub><sup>-</sup>), bacterial titers were slightly lower than in leaves of plants grown under non-limiting N (5 mM NO<sub>3</sub><sup>-</sup>) (Figure 1C). This indicated that in plants grown in 0.5 mM NO<sub>3</sub><sup>-</sup> bacterial survival *in planta* was compromised leading to reduce to bacterial titers *in planta*. These reduced bacterial titers in plants grown in limiting N (0.5 mM NO<sub>3</sub><sup>-</sup>) could be the consequence of a difference in the expression of the complex defense arsenal plants deploy in response to bacterial infection compared to plants grown in sufficient N (5 mM NO<sub>3</sub><sup>-</sup>).

In order to test the hypothesis that lower bacterial titers are due to a difference in the expression of defense in plants grown in 0.5 mM NO<sub>3</sub><sup>-</sup>, was analyzed the transcriptome of plants grown in 0.5 or 5 mM NO<sub>3</sub><sup>-</sup> and infected with *E. amylovora*. Leaves of five-week-old plants were *E. amylovora*- or mock-inoculated and sampled 6 hpi. Gene expression was analyzed using the CATMA microarray and log 2 of the ratio between expression in infected and mock plants was calculated. The Pearson correlation coefficient between the two sets of data was high ( $R^2 = 0,95$ ; Supp. Figure 2), indicating that response to infection of plants grown in contrasted N supply conditions was very close. Statistical analysis of the data indicated that the expression of 2982 and 3017 genes was significantly induced (Bonferroni,  $p_{val} < 0.05$ ) following infection with bacteria, in plants grown respectively at 0.5 mM and 5 mM NO<sub>3</sub><sup>-</sup>. Altogether, 2602 genes showed an increase in expression following infection in both N regimes (Figure 1D). We also found that 3248 and 3401 genes were significantly repressed (Bonferroni,  $p_{val} < 0.05$ ) following bacterial infection in plants grown respectively at 0.5 mM and 5 mM NO<sub>3</sub><sup>-</sup>. Altogether, 2799 genes showed a decrease in expression following infection in both N regimes (Figure 1D). Several hundreds of genes

were modulated in response to infection only in one N regime (Figure 1D). However, only a small number of these genes, described later, showed a strong difference in expression profile between the two N regimes (Supp. Table 1 and Supp. Figure 2).

We then analyzed the functional categories of the infection-modulated genes using the Munich Information Center for Protein Sequences database (Ruepp, 2004). We found, for infection-induced and infection-repressed genes, that the main functional categories represented were found both for plants grown at 0.5 mM and 5 mM  $\text{NO}_3^-$  (Figure 1E), which is not surprising given the similarity of the datasets obtained for plants grown at 0.5 mM and 5 mM  $\text{NO}_3^-$  (Supp. Figure 2). The largest categories represented unclassified and metabolism-related genes (Figure 1E). Several functional categories were significantly over-represented in our datasets compared to their representation in the whole genome (Categories with an asterisk in Figure 1E). In most cases, their over-representation was different between bacteria-induced and bacteria-repressed genes but was not affected by the N regime of the plants. Only the “cell fate” category, which mainly contains genes associated with cell growth, showed a difference according to the  $\text{NO}_3^-$  level as it was significantly over-represented only in plants grown in 5 mM  $\text{NO}_3^-$ . Among bacteria-induced genes, the “metabolism”, “protein fate”, cellular transport”, “cellular communication” and “cell rescue and defense” functional categories were over-represented. Among bacteria-repressed genes, the “metabolism” and “biogenesis of cellular components” categories were over-represented. These categories are consistent with the response of the plant to biotic stress, which is known to lead to strong metabolic readjustment, defense set-up and a shutting down of the photosynthesis apparatus.

Altogether, our data indicate that the very large transcriptional reprogramming previously observed in *Arabidopsis* leaves infected with *E. amylovora* (Moreau *et al.*, 2012) occurs whether plants are grown in limiting or non-limiting N. Despite the strong impact of the N limitation on the growth of the plants, these plants showed a response to biotic stress that was mostly similar to plants grown in non-limiting N with only strong differences in expression for a small subset of genes.

### **N modulates specific defense-related genes**

The response of plants to bacterial phytopathogens has been well studied (Pieterse *et al.*, 2012). Indeed, bacterial phytopathogens are known to activate the salicylic acid (SA)-, the

jasmonic acid (JA)- and the ethylene (ET)-dependent signaling pathways in Arabidopsis (Tao et al 2003; Moreau et al 2012). However, little is known on the impact of plant nutrition on the expression genes dependent on these signaling pathways following biotic stress. Thus, to analyze the impact of N on known defense-associated gene expression we compared the gene expression ratio (log<sub>2</sub>) between infected and mock plants grown at 0.5 mM or 5 mM NO<sub>3</sub><sup>-</sup> (Table 1). Most ET and SA-related genes analyzed were highly induced by bacterial infection in plants grown at both 0.5 mM and 5 mM NO<sub>3</sub><sup>-</sup>. In general, the level of modulation by infection of the SA and ET-associated genes analyzed here was not strongly affected by N availability. However, two SA-responsive genes, *PR2* and *PR5*, were induced only in plants grown upon N limitation (Table 1). Conversely, most JA-associated genes were either not modulated following bacterial infection or were repressed by bacteria. However, we found that two genes involved in JA biosynthesis, *AOS* (allene oxide synthase) and *AOC* (allene oxide cyclase) and the JA-responsive gene *JR1* (Jacalin lectin family protein) were repressed only in plants grown in non-limiting N. Only the JA-responsive *PR4* gene was induced by *E. amylovora*, and this was restricted to plants grown under limiting N conditions. Altogether, these data indicate that N mainly modulates the known plant defense response to pathogens through an impact on the JA signaling pathway.

We also analyzed the impact of N supply on the response to bacterial infection of known defense-related regulator genes. For example, WRKY transcription factors (TF) are well known regulators of the biotic stress response in plants. The majority of WRKY TFs were indeed modulated by *E. amylovora* infection but in most cases, there was no impact of N supply on this modulation (Supp. Table 2). Only five WRKY TFs showed a differential modulation in response to bacteria under different N regimes: four were induced only in non-limiting N (*WRKY42*, *WRKY47*, *WRKY64*, *WRKY67*) and two were repressed only in non-limiting N (*WRKY3* and *WRKY69*). However, the difference in modulation of these genes under different N regimes remains slight and the significance of these observations remains to be determined. Concerning MYB TFs, which have been in some cases associated with the defense response (Alves et al., 2014), most genes were not modulated by *E. amylovora* or repressed independent of the N regime. Only three genes showed a N-regime specific profile (AT1G25550, AT5G17300 and AT1G74840).

We hypothesized that the genes showing the strongest difference in the amplitude of the modulation by bacterial infection could be good candidates to explain the difference in

susceptibility to *E. amylovora* of *Arabidopsis* plants grown under different N regimes. We thus looked for genes with the highest differential in log<sub>2</sub> ratio in response to infection between plants grown in limiting N and non-limiting N. The forty genes with the highest differential in log<sub>2</sub> ratio showed mostly two types of profiles (Supp. Table 1) : genes repressed by bacterial infection in limiting N but not in non-limiting N, and genes induced by bacterial infection specifically or more highly in limiting N. Interestingly, genes which were repressed by bacteria only in limiting N were mainly associated to metabolism (Supp. Table 1) while genes were only induced in limiting N; interestingly most of them were linked to plant defense responses. For instance, two kelch repeat-containing F-box family proteins (AT1G80440, AT2G44130) involved in the regulation of the phenylpropanoid biosynthesis pathway (Zhang et al., 2014), an ankyrin repeat protein (AT5G54610) involved in regulation of immunity (Yang et al., 2012) and a leucine-rich repeat family protein (AT3G11010) involved in defense signaling (Galon et al., 2008).

Altogether, our data indicate that in plants grown in limiting N, bacterial infection provokes the induction of specific defense related genes, accompanied with a repression of metabolism-associated genes.

### **N limitation and biotic stresses interact**

In order to test the hypothesis of an interaction between the response of plants to N limitation and the response to biotic stress, we analyzed the response of plants to the combination of stresses in more detail. For this, we compared each single stress (N limitation or bacteria) to the combination of the two stresses (Figure 2A). We used the categories defined by Rasmussen et al (2013) to classify the genes according to their pattern of expression, using a log<sub>2</sub> (ratio) of 1 as a cutoff. Furthermore, the responses were considered in a simplified fashion as “induced/not modulated/repressed” without taking into account the level of expression. Comparison of the responses of single versus combined stresses showed that the vast majority of the genes modulated in our experiments (65.7 %) show an independent response (Figure 2B). Indeed, these genes show a response to the stress combination (N + B) that corresponds to the response of the gene to one of the single stresses. This was expected since the overlap between infection-modulated genes between plants grown in limiting and non-limiting N is very high (Figure 1D). However, 32.5 % of the genes showed

a response to the combination of the stresses that could not be deduced from their response to each individual stress, suggesting an interaction between the two single stress responses. These non-deducible gene patterns fall into three categories, as defined by Rasmussen and col., which are combinatorial, cancelled and prioritized (Figure 2C). Only 2.6 % of the transcripts responded in the prioritized manner (Figure 2C). The prioritized category corresponds exclusively to genes that are repressed by N limitation, induced by biotic stress in non-limiting N conditions and that remain induced by biotic stress in limiting N conditions. The majority of the genes showing a non-deducible pattern showed either a combinatorial pattern (16.4 % of total modulated genes) or a cancelled pattern (13.4 %, of total modulated genes). Furthermore, the expression profiles corresponded to a small number of specific expression patterns (Figure 2C). In the cancelled category, we found five sub-categories (C1- C5; Figure 2C) corresponding to genes induced or repressed by one of the single stresses and not modulated in response to the combination. Among these five categories, the most abundant correspond to genes induced by biotic stress and not modulated in response to the combination (C3) and genes repressed by N limitation and not modulated in response to the combination (C4). The most surprising category corresponds to the combinatorial pattern (Co1-Co2; Figure 2C). These genes correspond to genes that are not modulated in response to single stresses but that show repression (the most abundant sub-category; Co1) or induction (Co2) in response to the combination.

We then determined whether transcripts of each specific response mode could be linked to particular biological functions (Figure 2C). The prioritized mode primarily was associated to “cell rescue, defense” and “cellular communication”. These data suggested a repression of plant defense expression under abiotic stress treatment. In the canceled and combinatorial categories, the largest sub-categories (C3 and Co1) corresponded to a more diverse set of functional categories. In the Co1 pattern the “metabolism”, was very important while in the C3 category the “protein fate and cellular transport” functional categories were very important (Figure 2C). This may reflect a complex and specific adaptation of the plant to the combination of stresses.

Our data indicate that there is an interaction between the response of plants to N limitation and to biotic stress, which leads to a prioritization of the defense response and a more complex and specific response in terms of metabolism and cellular homeostasis in response to the combination of stresses.



### **Defense-associated genes are prioritized in response to the combination of N limitation with different pathogens**

Since our analysis of the transcriptome data indicated that defense-associated genes were over-represented in the prioritized mode, we analyzed the expression profile of specific defense-associated genes known to be expressed in response to biotic stress. We found a large number with a prioritized pattern. This is particularly true for the WRKY family of transcription factors (Birkenbihl et al., 2017) that have largely been described as being involved in the response of plants to stress (Table 2B). Another typical defense gene, *PR1*, was also found to be regulated in the prioritized mode. We compared these results to N metabolism-associated genes (Table 2A). Most N-related genes showed profiles that were independent in the stress combinations (Supp. Figure 3). Only five genes related to N metabolism showed a specific profile in response to the combination of the two stresses (Table 2A). Three of these genes showed a prioritized pattern while two showed a cancelled pattern. Interestingly, the three genes showing a prioritized pattern have been linked to defense responses. Indeed, *Lysine Histidine Transporter 1 (LHT1)* and *Ammonium Transporter 1 (AMT1)* have been shown to be involved in defense against pathogens (Liu et al., 2010; Pastor et al., 2014) and *Wound-responsive gene 3 (WR3/NAR1)* encoding a high-affinity nitrate transporter, involved also in jasmonic acid-independent wound signal transduction (Titarenko et al., 1997). Thus, our data show that genes known to play a role in Arabidopsis defense against pathogens were regulated in a prioritized manner, which is consistent with the fact that N limitation affected the response of the plant only for a limited subset of genes (Figure 2).

In order to determine how generic the interaction between stresses is, we analyzed the effect of N limitation on the response of Arabidopsis to another bacterial phytopathogen, *P. syringae* pv. *tomato*. We used the virulent strain DC3000 and the avirulent strain DC3000 *avrRpm1*. Arabidopsis plants were grown for five weeks in peat moss with 0.5 (limiting N) or 5 mM NO<sub>3</sub><sup>-</sup> (non-limiting N) as described above. After 5 weeks, rosette leaves were inoculated with the virulent or the avirulent strain of *P. syringae*. As a control, we also inoculated plants with *E. amylovora*. In order to study the kinetic of gene expression, plants were harvested at 6 and 24 hpi and gene expression was analyzed by qRT-PCR as above. We selected three defense-related genes *PR1*, *WRKY33* and *WRKY60*, which exhibited a

prioritized response and one, *PR5*, which exhibited a cancelled response. Interestingly, we found that *PR1* was not only more highly expressed in response to *E. amylovora* in plants grown under limiting N but also that *PR1* was induced by *E. amylovora* as early as 6 hpi only in plants grown in limiting N (Figure 3A). Furthermore, qRT-PCR analysis of these genes confirmed the prioritized response of these genes in combination of N limitation and *P. syringae*, both for the virulent and the avirulent strain tested (Figure 3B).

Our data show that defense-related genes respond in the same manner when N limitation is combined to different bacterial pathogens.

## DISCUSSION

Several studies have demonstrated that the availability of nutrients, in particular of nitrogen (N), influences the outcome of plant-pathogen interactions. Nevertheless, the mechanisms underlying this connection are poorly understood, in part because the effect of N on this biotic stress is dependent on the plant-pathogen interaction considered (Dordas, 2008; Fagard et al., 2014; Snoeijers et al., 2000; Walters and Bingham, 2007). Therefore, it is currently difficult to define general rules for the impact of N on the response of plants to biotic stress. Previous reports have suggested that defense activation in plants grown under N limitation is reduced (Dietrich et al., 2004). However, these data concerned a limited number of defenses and this did not allow to determine whether N limitation affected the response of a plant to biotic stress on a large scale. More recently, a transcriptomic analysis showed that the response of tomato to the fungus *Botrytis cinerea* is affected by N supply (Vega et al., 2015). However, data concerning combinations of biotic and abiotic stresses at the transcriptomic level remain scarce (Atkinson and Urwin, 2012; Ballini et al., 2013; Rasmussen et al., 2013; Vega et al., 2015) and no study has analyzed the impact of N supply on the response to biotic stress in *Arabidopsis*.

Bacterial infection leads to large modifications in transcriptomic profile of *Arabidopsis* plants at early time-points post inoculation (Eulgem, 2005). This was shown for virulent and avirulent *P. syringae* strains, the response to which mainly differs in timing and intensity (Tao, 2003), as well as for necrotrophic bacterial pathogens such as *E. amylovora* (Moreau et al., 2012). To test the hypothesis that the response of plants to *E. amylovora* infection is affected by N limitation, we analyzed the transcriptome of rosette leaves grown in limiting or non-limiting N and exposed to the phytopathogenic bacterium *E. amylovora*. Our data indicated, as expected, a large transcriptomic reprogramming following plant infection by *E. amylovora* with an important induction of defense-associated genes in both limiting and non-limiting N growth conditions. Close examination of the transcriptomic data indicated a large overlap in the plants' response to bacteria in plants grown in limiting or non-limiting N. Indeed, in our data-set, there was an 86 % overlap between genes modulated in limiting and non-limiting N, suggesting that, at least at the qualitative level the response of plants to biotic stress was close in limiting and non-limiting N. This was rather surprising given the important impact of N limitation on plant size in our experiments. Indeed, the commonly held idea of a tradeoff between plant growth and plant defense has

led to the widespread idea that plants have a lower capacity to defend themselves when growth is optimal, thus when nitrogen is abundant (Massad et al 2012).

We further analyzed our transcriptome data in response to single (N limitation or bacteria) and combined (N limitation and bacteria) stresses according to a previous analysis of transcriptomic data performed on several combinations of stresses (Rasmussen et al., 2013). As in this previous study, we found that three categories (independent, combinatorial and cancelled), among the five types of expression profiles identified, represent the most abundant transcript response modes (95.5% of the total transcripts; Figure 2B). Furthermore, as in this previous study, the deducible profiles (independent and similar) were much more frequent than the non-deducible profiles (combinatorial, canceled, and prioritized) which constituted 32.5% of the total transcripts (Figure 2B). In addition, we found that the response of plants to the combination of stresses was closer to its response to bacterial treatment than to N limitation alone (data not shown). This reflects a dominance of the response to biotic stress over the response to the abiotic stress in our experiments. On the other hand, a recent transcriptomic study on *Arabidopsis* response to sequential double stresses indicated that plants first subjected to drought or herbivory stress and then infected by *B. cinerea* responded similarly to *B. cinerea* treatment alone. The authors proposed that when two stresses are applied in sequence, plants display a transcriptome profile, which is very similar to the second stress, regardless of the nature of the first stress (Coolen et al., 2016). Since our experimental set-up involves applying N limitation before bacterial infection, one could imagine that the dominance of biotic stress is, at least partially, a result of the set-up.

The prioritized category corresponded exclusively to genes repressed by N limitation and induced by bacteria and combined stress (Figure 2C). The “cell rescue, defense” (Cr; Figure 2C) was over-represented among these genes. This indicates that signaling pathways regulating defense against bacteria are negatively regulated by abiotic stress. Previous studies showed that defense genes activated by *B. cinerea* were repressed under drought stress (Coolen et al 2016), suggesting that repression of defense expression occurs in response to different abiotic stresses and prioritized in response to different pathogens. We confirmed the prioritized mode for some selected defense genes in response to N limitation and another bacterial phytopathogen, *P. syringae*. N-related genes have been described to be affected by bacterial infection and recent reports show that some of these genes are

indeed involved in plant defense responses to pathogens (Camanes et al., 2012; Dechorgnat et al., 2012; Liu et al., 2010). Our transcriptomic data indicated that several genes related to N metabolism, such as *LHT1*, *NRT2.6* and *NRT2.5*, were strongly induced by bacteria (Table 2 and Supp. Table 3). Interestingly, we found that these genes showed a prioritized profile like many defense genes and not an independent mode like most N-associated genes. This suggests that although these genes are involved in N metabolism and/or transport, they are regulated by the plant as defense genes independently of N supply. On the other hand, *NRT2.1* was up-regulated by bacteria only in plants grown in non-limiting N. Interestingly, a recent transcriptomic study on tomato indicated that *NRT2.1* is also up-regulated by *B. cinerea* only in plants grown in non-limiting N (Vega et al 2015). This suggests that induction of *NRT2.1* is N-dependent in response to different pathogens, suggesting a conserved mechanism. *NRT2.1* has been shown to be a down-regulator of salicylic acid-dependent defenses in response to *P. syringae*. These results suggest that *NRT2.1* could play a role in controlling negatively defense activation in response to pathogens in high N in different pathosystems.

Infection with *E. amylovora* activates defense-related hormonal signaling pathways. The SA-signaling pathway is indeed strongly induced following *E. amylovora* infection in a T3SS-dependent manner both in host and non-host plants (Degrave et al 2008; Venisse 2002). Regulation of the JA signaling pathway in response to *E. amylovora* infection seems more complex. Indeed, several genes involved in JA biosynthesis are repressed following *E. amylovora* infection in Arabidopsis, but other JA-dependent genes are induced following infection by *E. amylovora* (Moreau 2012 and this study). In parallel, it was shown that T3SS-dependent down-regulation of the JA pathway is a critical element in the infection process of *Malus* spp. by *E. amylovora*, since the addition of methyl-jasmonate to susceptible plants increases their resistance to *E. amylovora*. In contrast, the SA pathway was similarly induced in both resistant and susceptible *Malus* spp by *E. amylovora* (Dugé De Bernonville et al 2012). Study of hormonal pathway related genes in our transcriptomic data indicated that SA and ET-associated genes were highly induced by *E. amylovora* while most of JA related genes were repressed by *E. amylovora*. Generally, in response to *E. amylovora*, ET and SA pathway were not significantly affected by N; however, some genes involved in JA biosynthesis and also JA-responsive genes were repressed only in plants grown in full N, suggesting that N modulates plant-pathogen interaction through the JA

pathway signaling. Thus, the higher bacterial titer in plants grown in non-limiting N could be linked to higher repression of JA-associated defense in these plants. Interestingly, these results are reminiscent of those observed with the *Botrytis*-tomato interaction for which expression of the JA pathway was clearly identified as being associated with the lower symptoms observed in plants grown in high N (Vega et al., 2015).

Our work thus suggests a main role for the JA signaling pathway in the impact of N supply on the adaptation of plants to biotic stress. It would be interesting to pursue the analysis of the role of the JA pathway in the interaction.

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## **MATERIALS AND METHODS**

### **Growth conditions of Arabidopsis plants**

Seeds of Arabidopsis Col-0 were obtained from the INRA Versailles collection. Plants were grown for 5 weeks in 4 cm plugs of peat moss substrate (70% blond peat, 20% perlite and 10% vermiculite) wrapped in a non-woven film (Tisé et al., 2013) and were subjected to an 8 h-light and 16 h-dark cycle at 21 °C (day)/18 °C (night) with 65 % relative humidity. Nitrogen limitation was performed as described in (Fagard *et al.*, 2014). Briefly: plants were grown for five weeks in soil and watered with a nutrient solution containing ample (5 mM NO<sub>3</sub><sup>-</sup>) or limiting nitrogen (0.5 mM NO<sub>3</sub><sup>-</sup>). In all cases, 5-week-old stressed or control plants were mock or pathogen-inoculated as described in the text.

### **Pathogen infections**

Rosette leaves of 5-week-old plants were infiltrated with *E. amylovora* CFBP1430 using a needleless syringe. Bacterial suspensions were prepared in sterile water (10<sup>7</sup> CFU ml<sup>-1</sup>). Six and twenty-four hours after infection (hpi) we performed bacterial counting by grinding infected leaves using glass beads in a TissueLyser (Qiagen/Retsch, Hilden, Germany). The bacterial suspensions were used to prepare serial dilutions, which were plated on LB medium and after 1 or 2 days the colonies formed were counted to evaluate the initial number of bacteria.

### **RNA isolation and qRT-PCR analysis**

For RNA extraction, twelve leaves of three plants (pathogen- or mock-treated) were collected at the indicated time-point after treatment, pooled and immediately frozen in liquid nitrogen. Total RNA was extracted from 100 mg of frozen ground leaves using Trizol® reagent (Invitrogen Life Technologies, Saint-Aubin, France) and then purified using the Qiagen RNeasy kit according to the supplier's instructions. RNA quality was evaluated by electrophoretic run on 1% agarose gel. For the qRT-PCR analysis, first-strand cDNA was synthesized using Superscript reverse transcriptase SSII (Invitrogen) from 1 µg of DNase-treated (Invitrogen) total RNA in a 20 µl reaction volume. qPCR reactions were performed using SYBR® Selected MasterMix 2x (Applied Biosystem), following the manufacturer's



protocol. The cycling conditions consisted of an initial 5 min at 95 °C, followed by 40 three-step cycles at 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Melting curve analysis was performed after cycle completion to validate amplicon identity. Relative expression levels were calculated following the standard curve based method (Larionov *et al.*, 2005). Expression of the *PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2A3)* reference gene (Ceccato *et al.*, 2013) was used for normalization of every target gene studied. For each treatment, three biological replicates, corresponding to a pool of 4 leaves from a single plant, were analyzed and each qRT-PCR reaction was carried out in duplicate. The gene-specific primers used in this analysis are indicated in Supplementary Table 4.

### **Transcriptome Studies**

Microarray analysis was carried out at the Institut of Plant Sciences Paris-Saclay (IPS2, Evry, France), using the CATMAv7 array (Lurin, 2004) based on AGILENT technology. The CATMAv7 design of *Arabidopsis thaliana* genome have been made with gene annotations included in FLAGdb++ (<http://tools.ips2.u-psud.fr/projects/FLAGdb++/HTML/index.shtml>), an integrative database around plant genome. The single high density CATMAv7 microarray slide contains four chambers, each containing 149 916 primers. Each 60 bp primer is triplicate in each chamber for robust analysis and in both strand. As part of all probes, 35 754 in triplicate correspond to gene TAIRv8 (among which 476 probes corresponding to mitochondrial and chloroplast genes) + 1289 probes corresponding to EUGENE software predictions + 658 probes for miRNA/MIR, and finally 240 controls. Two independent biological replicates were produced. For each biological replicate, RNA samples were obtained by pooling RNAs from more than three leaves. Leaves were collected on plants at 3.90 developmental growth stages (Boyes *et al.*, 2001) cultivated in short day conditions. Total RNA was extracted using Trizol® followed by a purification step on RNeasy column (Qiagen) according to the supplier's instructions. For each comparison, one technical replicate with fluorochrome reversal was performed for each biological replicate (i.e. four hybridizations per comparison). The labeling of cRNAs with Cy3-dUTP or Cy5-dUTP was performed as described in Two-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling manual (© Agilent Technologies, Inc.). The hybridization and washing were performed according to Agilent Microarray Hybridization Chamber User Guide instructions

((© Agilent Technologies, Inc.). Two-micron scanning was performed with InnoScan900 scanner (InnopsysR, Carbonne, FRANCE) and raw data were extracted using MapixR software (InnopsysR, Carbonne, FRANCE).

### **Statistical Analysis of Microarray Data**

Experiments were designed with the Genomic networks team of IPS2. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). For each array, a global intensity-dependent normalization using the loess procedure (Yang et al., 2002) was performed to correct the dye bias. The differential analysis is based on the log-ratios averaging over the duplicate probes and over the technical replicates. Hence the numbers of available data for each gene equals the number of biological replicates and are used to calculate the moderated t-test (Smyth, 2004). Under, the null hypothesis, no evidence that the specific variances vary between probes is highlighted by Limma and consequently the moderated t-statistic is assumed to follow a standard normal distribution.

To control the false discovery rate, adjusted *P*-values found using the optimized FDR approach of (Storey and Tibshirani, 2003) are calculated. We considered as being differentially expressed the probes with an adjusted *P*-value  $\leq 0.05$ .

Analysis was done with the R software (R Development Core Team, 2005). The function SqueezeVar of the Limma library has been used to smooth the specific variances by computing empirical Bayes posterior means. The library kerfdr has been used to calculate the adjusted *P*-values.

### **Data Deposition**

Microarray data from this article were deposited in the international repository GEO, Gene Expression Omnibus (Edgar R. 2002, <http://www.ncbi.nlm.nih.gov/geo/>), accession no. GSE97582) and All steps of the experiment, from growth conditions to bioinformatic and statistical analyses, were detailed in CATdb (Gagnot et al., 2007) (<http://tools.ips2.u-psud.fr/CATdb/>; Project: RA14-05\_Multipass) according to the “Minimum Information About a Microarray Experiment” standards.

### **Transcriptional response modes**

Transcript sets were created by grouping genes exhibiting similar expression patterns under single (N limitation or bacteria) and combined stress (N limitation and bacteria) treatments. The responses were considered in a simplified fashion as “induced/not modulated/repressed” without taking into account the level of expression: log-fold changes between -1 and 1 were considered as non-regulated genes, log-fold changes higher than 1 and lower than -1 were considered as induced and repressed genes respectively (Table 2). Plant response to N limitation corresponds to ratios (log<sub>2</sub>) between control plants grown in limiting N (0.5 mM NO<sub>3</sub><sup>-</sup>) and non-limiting N (5 mM NO<sub>3</sub><sup>-</sup>); plant response to bacteria corresponds to ratios (log<sub>2</sub>) between infected and control plants grown in non-limiting N (5 mM NO<sub>3</sub><sup>-</sup>); plant response to combined stresses corresponds to ratios (log<sub>2</sub>) between infected plant grown in limiting N (0.5 mM NO<sub>3</sub><sup>-</sup>) and control plants grown in non-limiting N (5 mM NO<sub>3</sub><sup>-</sup>).

Genes were grouped into 20 sub-categories, each representing a specific expression pattern. The 20 sub-categories were assembled into five larger categories (cancelled, combinatorial, prioritized, independent and similar) according to (Rasmussen et al, 2013). GO terms associated with each specific transcriptional response profile using the FunCatDB (Ruepp, 2004).

## FIGURE LEGENDS

**Table 1. Effect of N limitation on defense-related genes.** Values are log<sub>2</sub> signal ratios between infected and water-treated control plants. BS: genes involved in hormone biosynthesis, R: hormone-responsive genes, S: genes involved in hormone signaling. Two levels of significance threshold were considered according to the Bonferroni statistical test (a:  $P$ -value < 0.05; b:  $P$ -value <  $10^{-8}$ ).

**Table 2. Expression profiles of selected genes in response to single and combined stresses.** Non-deducible gene profiles of selected N metabolism and defense-associated genes are presented. Values represent log<sub>2</sub> signal ratios of the fold-changes upon single stresses (N: N limitation; B: *E. amylovora* infection) or combined stresses (N+B: N limitation and *E. amylovora* infection). The column at right indicates the transcriptional response mode according to the categories described by Rasmussen *et al.* 2013. N: N limitation, B: bacteria, N+B: combined stresses (N limitation and bacteria).

**Figure 1. Impact of N on physiological and transcriptional characteristics of Arabidopsis plants.** (A) Five-week-old Arabidopsis rosettes grown under limiting (0.5 mM NO<sub>3</sub><sup>-</sup>) and non-limiting (5 mM NO<sub>3</sub><sup>-</sup>) N. (B) Maximum rosette diameter quantified by ImageJ. (C) Bacterial titers of *E. amylovora* CFBP1430 in Arabidopsis rosette leaves at 6 hpi and 24 hpi. (B, C) Different letters indicate significant differences according to the Mann-Whitney test ( $P$ -value < 0.05); experiments were repeated twice with similar results. (D) Venn diagrams illustrating the overlap of up-regulated (red) and down-regulated (green) Arabidopsis genes in response to *E. amylovora* between limiting and non-limiting N. (E) Distribution of functional categories according to the FunCatDB (Ruepp, 2004). The pie charts represent: (a) 2982 genes induced and (b) 3248 genes repressed by *E. amylovora* in plants grown in limiting N; (c) 3017 genes induced and (d) 3401 genes repressed by *E. amylovora* in plants grown under non-limiting N. Asterisks (\*) indicate significant differences according hypergeometric distribution ( $P$ -value < 0.05).

**Figure 2. Gene expression patterns in response to single and combined stresses.** (A) Schematic representation of single stresses (N: N limitation; B: *E. amylovora* infection) and combined stresses (N+B: N limitation and *E. amylovora* infection). (B) Number of genes in

the different categories of responses to stress combination; for each category, the percentage among the total modulated genes is indicated. The dotted line indicates the three non-predictable categories, which represent 32.5 % of *E-amylovora*-responsive transcripts. (C) Detail of the expression patterns among the different non-predictable categories. The dotted line represents transcript level in control plants (mock/5mM NO<sub>3</sub><sup>-</sup>). For each sub-category, only the type of response is indicated with a full line (induced, repressed, no response). Enrichment in GO terms in each sub-category was determined according to the FunCatDB. Asterisks (\*) indicate a significant difference relative to the Arabidopsis genome (*P*-value <0.05). P: prioritized, C: cancelled and CO: combinatorial. Met: metabolism, Tr: transcription, Pf: protein fate, Ct: cellular transport, Cc: cellular communication, Cr: cell rescue, defense, Cf: cell fate, Bc: biogenesis of cellular components, Uc: unclassified.

**Figure 3. Impact of nitrate on defense-related genes expression in response to different bacterial pathogens.** (A) Time-course of *PR1* gene expression at 6 and 24 hpi following mock or *E. amylovora* treatment. (B) Response of selected defense-related genes in response to N, B and N+B at 6 hpi. (A, B) Col-0 plants were grown in high (5 mM NO<sub>3</sub><sup>-</sup>) or limiting (0.5 mM NO<sub>3</sub><sup>-</sup>) N. and infiltrated with bacteria (*E. amylovora* wild type, virulent and a virulent strain of *P. syringae*) or mock-inoculated. Expression is normalized to the *PP2a3* constitutive gene. Values are log<sub>2</sub> signal ratios between infected and mock plants. Similar results were obtained in a minimum of two independent experiments. The bars represent standard deviation. Different letters correspond to values that are significantly different according to the Mann-Whitney statistical test (*P*-value < 0.05).

**Supplemental Table 1. Genes with the most contrasted response to *E. amylovora* in low and high N.** Values are log<sub>2</sub> signal ratios between *E. amylovora*-infected and mock plants at low (0.5 mM NO<sub>3</sub><sup>-</sup>) and high N (5 mM NO<sub>3</sub><sup>-</sup>). Top: genes repressed by *E. amylovora* only in plants grown in low N; middle: genes induced by *E. amylovora* only in low N or more induced in low N; bottom: genes repressed by *E. amylovora* only in high N or more repressed in high N. Asterisk (\*) indicate a significant difference between mock and infected plants according to the Bonferroni test (*P*-value < 0.05).

**Supplemental Table 2. Modulation of *WRKY* TFs by *E. amylovora* in low and high N.** Values are log<sub>2</sub> signal ratios between infected and mock plants grown 0.5 and 5 mM NO<sub>3</sub><sup>-</sup>.

Two levels of significance threshold are presented according to the Bonferroni statistical test (a:  $P$ -value  $< 0.05$ ; b:  $P$ -value  $< 1 \times 10^{-8}$ ).

**Supplemental Table 3. Response to single and combined stresses of selected N-related genes showing an independent profile.** Values represent log 2 signal ratios of the fold-changes upon single stresses (N: N limitation; B: *E. amylovora* infection) or combined stresses (N+B: N limitation and *E. amylovora* infection). Most N-related genes that were modulated by at least one of the single stresses presented an independent profile in response to combined stresses (presented in this table). A few N-related genes presented non-predictable profiles in response to combined stresses and are presented in Table 2A. N: N limitation; B: *E. amylovora* infection; N+B: N limitation and *E. amylovora* infection.

**Supplemental Table 4.** Sequence of the gene-specific primers used in this analysis.

**Supplemental Figure 1. Nitrate content in Arabidopsis rosette leaves grown under limiting (0.5 mM) or non-limiting (5 mM) nitrate.** Nitrate content was measured using the Miranda method (Miranda et al., 2001) in 25 mg ground and frozen leaves. Each biological replicate corresponds to 4 leaves of a single plant. Values presented correspond to the mean and SE of three individual replicates. The asterisk (\*) indicates a significant difference between the two N nutritional conditions according to the Mann-Whitney test ( $P$ -value  $< 0.05$ ).

**Supplemental Figure 2. Correlation of transcriptome data.** Correlation between log2 values of the fold-changes upon *E. amylovora* infection in plants grown in limiting (0.5 mM  $\text{NO}_3^-$ ) and non-limiting N (5 mM  $\text{NO}_3^-$ ). Each black dot represents a single gene. Gray and orange dots correspond to genes with the most contrasted response to *E. amylovora* in low and high N; Grey: higher modulation by *E. amylovora* in 5 mM  $\text{NO}_3^-$ ; orange: higher modulation by *E. amylovora* in 0.5 mM  $\text{NO}_3^-$ .

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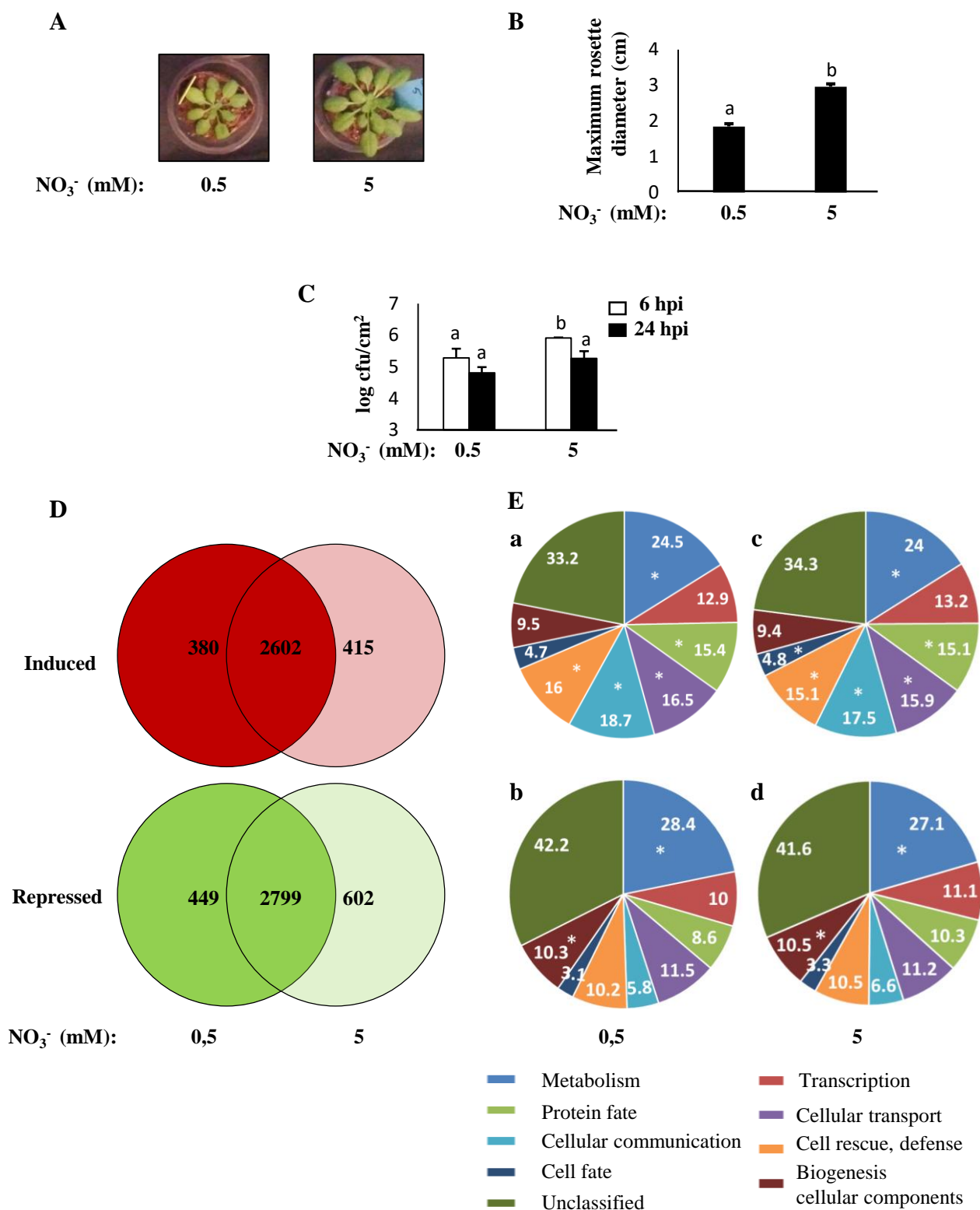


Figure 1. Impact of N on physiological and transcriptional characteristics of Arabidopsis plants.

<b>SA biosynthesis and responsive genes</b>			0.5 mM NO <sub>3</sub> <sup>-</sup> :5 mM NO <sub>3</sub> <sup>-</sup>	
AGI	Name	Function	Ea vs mock	Ea vs mock
AT1G74710	<i>ICS1</i>	BS	6.14 b	5.73 b
AT3G52430	<i>PAD4</i>	BS	5.15 b	4.51 b
AT3G48090	<i>EDS1</i>	S	3.85 b	3.82 b
AT4G39030	<i>EDS5</i>	S	6.33 b	5.67 b
AT1G64280	<i>NPR1</i>	S	2.14 a	1.66 a
AT2G14610	<i>PR1</i>	R	5.10 b	3.23 b
AT3G57260	<i>PR2</i>	R	1.50 a	0.03
AT1G75040	<i>PR5</i>	R	2.00 b	0.88
<b>ET biosynthesis and responsive genes</b>				
AT1G05010	<i>ACO</i>	BS	2.64 b	1.62 a
AT3G04580	<i>EIN4</i>	R	2.76 b	2.66
AT4G17500	<i>ERF-1</i>	R	1.93 a	1.92 a
AT2G40940	<i>ERS1</i>	R	1.42 a	1.05 b
AT1G66340	<i>ETR1</i>	R	1.19	1.14
<b>JA biosynthesis and responsive genes</b>				
AT3G25770	<i>AOC2</i>	BS	-1.17	-2.12 b
AT5G42650	<i>AOS</i>	BS	-1.13	-2.13 b
AT2G46370	<i>JAR1</i>	BS	-0.23	-0.51
AT3G45140	<i>LOX2</i>	BS	-0.35	-0.97
AT2G06050	<i>OPR3</i>	BS	0.16	0.26
AT1G19640	<i>JMT</i>	R	-0.60	-0.23
AT3G16470	<i>JR1</i>	R	0.08	-2.01 a
AT2G26020	<i>PDF1.2</i>	R	-1.77 a	-2.06 a
AT3G12500	<i>PR3</i>	R	0.06	-0.06
AT3G04720	<i>PR4</i>	R	2.43 b	0.35
AT5G24770	<i>VSP2</i>	R	0.14	-0.02
AT1G32640	<i>ATMYC2</i>	S	-1.70 a	-1.82 a
AT2G39940	<i>COI1</i>	S	0.25	0.10

Table 1. Effect of N limitation on defense-related genes.

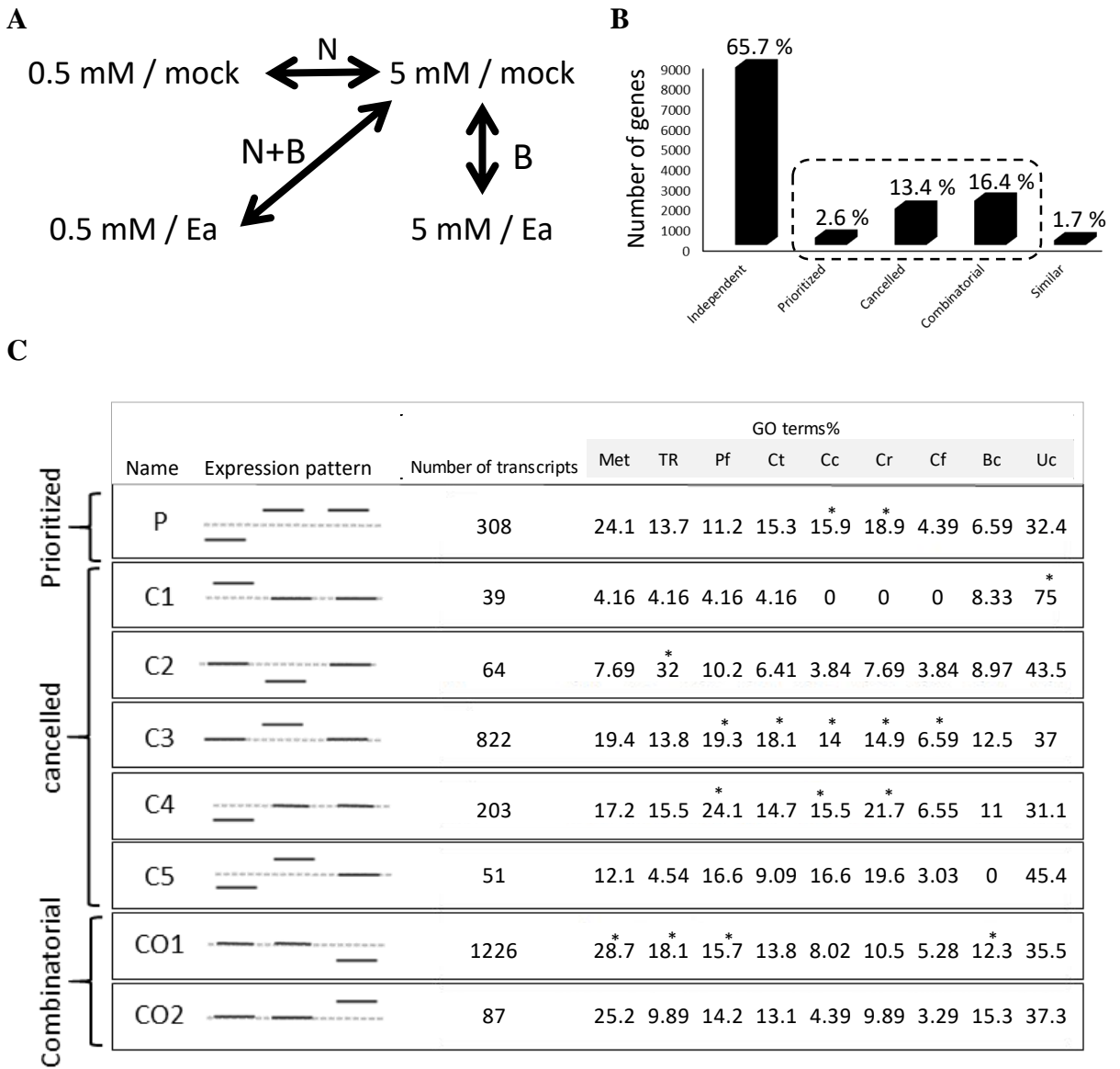


Figure 2. Gene expression patterns in response to single and combined stresses.

**A**

<b>N metabolism</b>	AGI	N	B	N+B	Response type
GLN1.3	AT3G17820	-0.38	-1.02	-0.90	cancelled
GDH3	AT3G03910	-0.06	1.21	0.24	cancelled
AMT1.1	AT4G13510	-1.13	2.40	1.98	prioritized
WR3	AT5G50200	-1.28	3.38	2.63	prioritized
LHT1	AT5G40780	-1.30	3.04	2.72	prioritized

**B****Response to salicylic acid**

Name	AGI	N	B	N+B	Response mode
<i>PR5</i>	AT1G75040	-2.07	0.88	-0.06	cancelled
<i>EDS5</i>	AT4G39030	-1.09	5.67	5.24	prioritized
<i>PR1</i>	AT2G14610	-2.71	3.23	2.40	prioritized

**ET/JA response and biosynthesis**

<i>JR1</i>	AT3G16470	-0.60	-2.01	-0.52	cancelled
<i>ETR1</i>	AT1G66340	-0.41	1.14	0.78	cancelled
<i>LOX3</i>	AT1G17420	-1.15	2.44	1.96	prioritized

**EDS1 pathway**

<i>FMO1</i>	AT1G19250	-1.28	5.85	5.02	prioritized
<i>AtNUDT7</i>	AT4G12720	-1.10	3.28	2.97	prioritized

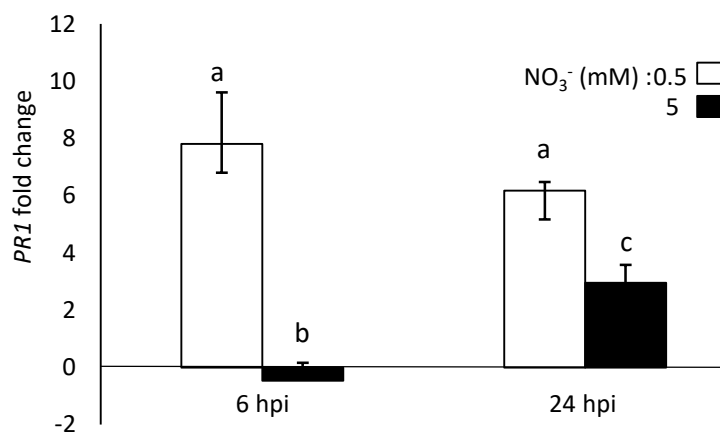
**WRKY transcription factors**

<i>WRKY75</i>	AT5G13080	-1.12	4.20	3.72	prioritized
<i>WRKY51</i>	AT5G64810	-1.50	5.93	5.47	prioritized
<i>WRKY38</i>	AT5G22570	-1.93	3.68	3.12	prioritized
<i>WRKY25</i>	AT2G30250	-1.23	2.53	1.96	prioritized
<i>WRKY54</i>	AT2G40750	-1.04	2.22	1.88	prioritized
<i>WRKY70</i>	AT3G56400	-1.30	1.69	1.46	prioritized
<i>WRKY53</i>	AT4G23810	-1.42	2.24	1.22	prioritized
<i>WRKY60</i>	AT2G25000	-1.15	1.77	1.34	prioritized
<i>WRKY50</i>	AT5G26170	-1.02	3.45	2.64	prioritized
<i>WRKY30</i>	AT5G24110	-1.15	6.11	4.90	prioritized
<i>WRKY33</i>	AT2G38470	-1.43	4.63	3.79	prioritized

**Resistance genes**

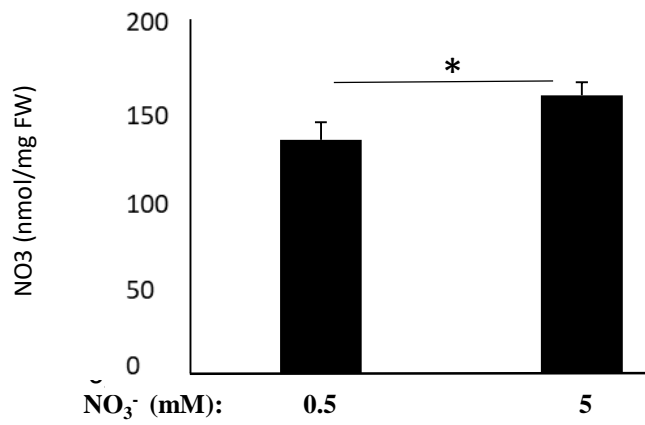
"LRR family protein"	AT5G45510	-1.24	1.87	1.43	prioritized
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Table 2. Expression profiles of selected genes in response to single and combined stresses.

**A****B**

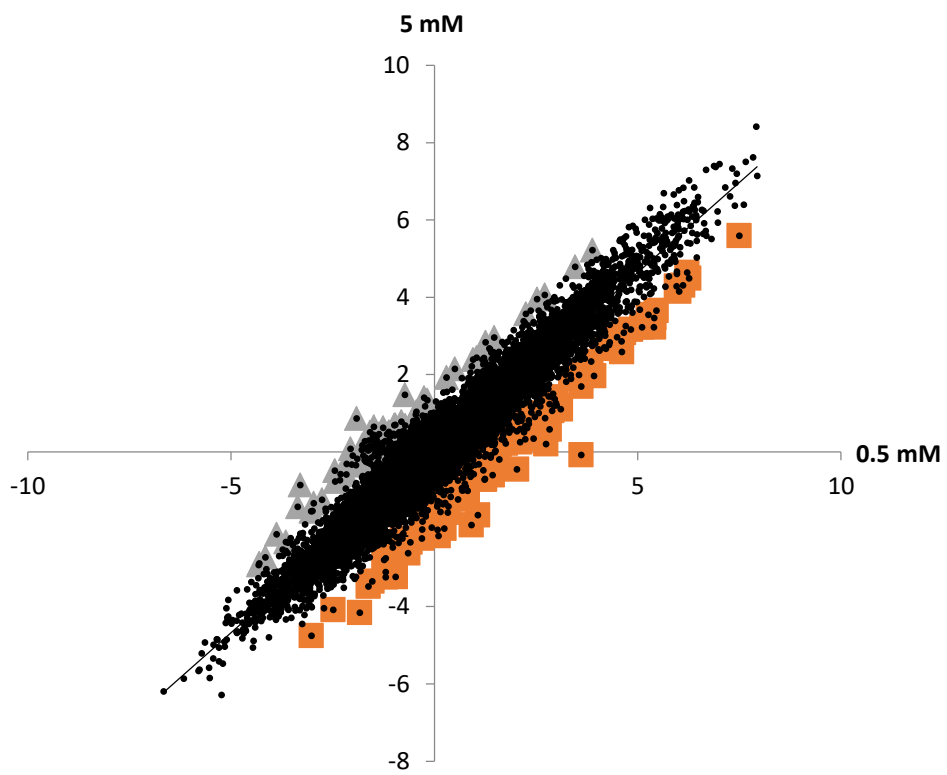
Gene	Treatment	N	B	N+B	Response type
<i>PR1</i>	Ea	-2.52	3.09	5.96	prioritized
	Ps vir	-2.43	0.81	1.89	prioritized
	Ps avir	-2.43	0.68	3.64	prioritized
<i>PR5</i>	Ea	-1.51	0.17	0.95	cancelled
	Ps vir	-1.02	-0.51	0.46	cancelled
	Ps avir	-1.02	-1.02	0.63	cancelled
<i>WRKY33</i>	Ea	-2.53	2.27	4.09	prioritized
	Ps vir	-0.57	0.61	1.67	prioritized
	Ps avir	-0.57	3.06	3.52	prioritized
<i>WRKY60</i>	Ea	-1.15	1.77	1.34	prioritized
	Ps vir	-0.51	1.48	2.33	prioritized
	Ps avir	-0.51	1.42	1.92	prioritized

Figure 3. Impact of nitrate on defense-related genes expression in response to different bacterial pathogens.



Supplemental Figure 1. Nitrate content in Arabidopsis rosette leaves grown under limiting (0.5 mM) or non-limiting (5 mM) nitrate.





Supplemental Figure 2. Correlation of transcriptome data.

AGI	Name	0.5 mM NO <sub>3</sub> <sup>-</sup>		5 mM NO <sub>3</sub> <sup>-</sup>	
		Ea vs mock	*	Ea vs mock	*
AT1G09350	GALACTINOL SYNTHASE 3	-3.37	*	-1.42	
AT3G19350	"polyadenylate-binding protein-related / PABP-related"	-3.31	*	-0.85	
AT1G26630	EUKARYOTIC ELONGATION FACTOR 5A-2	-1.79	*	0.06	
AT1G47450	"unknown protein"	-1.78	*	0.17	
AT2G07681	Cytochrome C assembly protein	-1.72	*	0.28	
AT1G24822	unknown protein	-1.69	*	0.10	
AT2G07681	Cytochrome C assembly protein	-1.62	*	0.29	
AT4G05631	"unknown protein"	-1.59	*	0.51	
AT2G07681	Cytochrome C assembly protein	-1.52	*	0.39	
AT2G07709	pseudogene	-1.27	*	0.63	
AT1G61120	"terpene synthase/cyclase family protein"	5.40	*	3.47	*
AT1G15670	"kelch repeat-containing F-box family protein"	5.39	*	3.23	*
AT3G16530	"legume lectin family protein"	4.61	*	2.59	*
AT5G35525	PLAC8 family protein	3.92	*	1.97	*
AT1G80440	"kelch repeat-containing F-box family protein"	3.60	*	-0.07	
AT3G46660	UDP-GLUCOSYL TRANSFERASE 76E12	3.10	*	1.11	
AT5G54610	"ANK (ANKYRIN); protein binding"	2.83	*	0.59	
AT1G26800	"zinc finger (C3HC4-type RING finger) family protein"	2.74	*	0.20	
AT3G04720	"PR4 (PATHOGENESIS-RELATED 4)"	2.43	*	0.35	
AT2G14560	"unknown protein"	2.02	*	-0.44	
AT3G06070	"unknown protein"	1.42	*	-0.59	
AT3G49940	LOB DOMAIN-CONTAINING PROTEIN 38	1.23	*	-0.71	
AT3G48360	BTB AND TAZ DOMAIN PROTEIN 2	-1.84	*	-4.16	*
AT5G19190	"unknown protein"	-1.20		-3.23	*
AT4G32480	"unknown protein"	-0.96		-3.23	*
AT5G66580	"unknown protein"	-0.65		-2.61	*
AT1G49130	"zinc finger (B-box type) family protein"	0.10		-2.17	*
AT3G16470	"JR1 (Jacalin lectin family protein)"	0.08		-2.01	*
AT1G23390	"kelch repeat-containing F-box family protein"	0.24		-1.97	*
AT5G03350	"legume lectin family protein"	0.91		-1.88	*
AT3G57240	"BG3 (BETA-1.3-GLUCANASE 3)"	1.06		-1.63	*

Supplemental Table 1. Genes with the most contrasted response to *E. amylovora* in low and high N.

AGI	Name	0.5 mM NO <sub>3</sub> <sup>-</sup>	5 mM NO <sub>3</sub> <sup>-</sup>
		Ea vs mock	Ea vs mock
AT3G58710	"WRKY69 (WRKY DNA-binding protein 69); transcription factor"	-1.21	-1.26 a
AT4G30935	"WRKY32 (WRKY DNA-binding protein 32); transcription factor"	-0.79	-0.30
AT2G03340	"WRKY3 (WRKY DNA-binding protein 3); transcription factor"	-0.58	-1.55 a
AT5G56270	"WRKY2 (WRKY DNA-binding protein 2); transcription factor"	-0.41	-0.01
AT4G26640	"WRKY20 (WRKY DNA-binding protein 20); transcription factor"	-0.28	-0.31
AT4G26640	"WRKY20 (WRKY DNA-binding protein 20); transcription factor"	-0.15	-0.04
AT2G47260	"WRKY23 (WRKY DNA-binding protein 23); transcription factor"	-0.15	-0.22
AT5G41570	"WRKY24 (WRKY DNA-binding protein 24); transcription factor"	-0.14	-0.04
AT1G64000	"WRKY56 (WRKY DNA-binding protein 56); transcription factor"	-0.13	0.11
AT5G28650	"WRKY74 (WRKY DNA-binding protein 74); transcription factor"	-0.09	-0.05
AT2G46130	"WRKY43 (WRKY DNA-binding protein 43); transcription factor"	-0.01	0.09
AT2G34830	"WRKY35 (WRKY DNA-binding protein 35. ); transcription factor"	0.00	-0.06
AT4G26440	"WRKY34 (MICROSPORE-SPECIFIC PROMOTER 3); transcription factor"	0.00	-0.07
AT4G29280	"WRKY65 (WRKY DNA-binding protein 65); transcription factor"	0.04	-0.51
AT1G30650	"WRKY14 (WRKY DNA-binding protein 14); transcription factor"	0.04	0.11
AT1G69310	"WRKY57 (WRKY DNA-binding protein 57); transcription factor"	0.05	-0.28
AT1G55600	"WRKY10 (MINISEED3); transcription factor"	0.05	0.17
AT3G62340	"WRKY68 (WRKY DNA-binding protein 68); transcription factor"	0.19	0.03
AT1G66600	"WRKY63 (WRKY DNA-binding protein 63); transcription factor"	0.24	0.14
AT3G01080	"WRKY58 (WRKY DNA-binding protein 58); transcription factor"	0.32	0.10
AT4G39410	"WRKY13 (WRKY DNA-binding protein 13); transcription factor"	0.46	0.25
AT4G12020	"WRKY19 (WRKY DNA-binding protein 19); transcription factor"	0.47	0.29
AT1G66550	"WRKY67 (WRKY DNA-binding protein 67); transcription factor"	0.63	1.33 a
AT1G66560	"WRKY64 (WRKY DNA-binding protein 64); transcription factor"	0.69	1.57 a
AT2G30590	"WRKY21 (WRKY DNA-binding protein 21); transcription factor"	0.92	0.51
AT1G13960	"WRKY4 (WRKY DNA-binding protein 4); transcription factor"	0.94	0.75
AT4G01720	"WRKY47 (WRKY DNA-binding protein 47); transcription factor"	1.09	1.49 a
AT4G04450	"WRKY42 (WRKY DNA-binding protein 42); transcription factor"	1.32	1.46 a
AT1G69810	"WRKY36 (WRKY DNA-binding protein 36); transcription factor"	1.65 a	1.73 a
AT5G52830	"WRKY27 (WRKY DNA-binding protein 27); transcription factor"	1.70 a	1.74 a
AT5G43290	"WRKY49 (WRKY DNA-binding protein 49); transcription factor"	1.80 a	2.49 b
AT4G31550	"WRKY11 (WRKY DNA-binding protein 11); transcription factor"	1.97 a	2.42 b
AT5G07100	"WRKY26 (WRKY DNA-binding protein 26); transcription factor"	1.99 a	2.51 b
AT1G80590	"WRKY66 (WRKY DNA-binding protein 66); transcription factor"	2.00 a	2.32 b
AT1G18860	"WRKY61 (WRKY DNA-binding protein 61); transcription factor"	2.31 b	2.81 b
AT5G01900	"WRKY62 (WRKY DNA-binding protein 62); transcription factor"	2.34 b	2.73 b
AT1G29860	"WRKY71 (WRKY DNA-binding protein 71); transcription factor"	2.37 b	2.34 b
AT2G21900	"WRKY59 (WRKY DNA-binding protein 59); transcription factor"	2.41 b	2.98 b
AT2G25000	"WRKY60 (WRKY DNA-binding protein 60); transcription factor"	2.49 b	1.77 a
AT2G24570	"WRKY17 (WRKY DNA-binding protein 17); transcription factor"	2.55 b	2.43 b
AT4G23810	"WRKY53 (WRKY DNA-binding protein 53); transcription factor"	2.63 b	2.24 b
AT4G24240	"WRKY7 (WRKY DNA-binding protein 7); transcription factor"	2.66 b	2.63 b
AT3G56400	"WRKY70 (WRKY DNA-binding protein 70); transcription factor"	2.76 b	1.69 a
AT3G04670	"WRKY39 (WRKY DNA-binding protein 39); transcription factor"	2.90 b	2.83 b
AT2G40750	"WRKY54 (WRKY DNA-binding protein 54); transcription factor"	2.92 b	2.22 b
AT2G23320	"WRKY15 (WRKY DNA-binding protein 15); transcription factor"	3.14 b	2.93 b
AT2G30250	"WRKY25 (WRKY DNA-binding protein 25); transcription factor"	3.18 b	2.53 b
AT5G46350	"WRKY8 (WRKY DNA-binding protein 8); transcription factor"	3.38 b	3.94 b
AT3G01970	"WRKY45 (WRKY DNA-binding protein 45); transcription factor"	3.51 b	3.45 b
AT4G11070	"WRKY41 (WRKY DNA-binding protein 41); transcription factor"	3.54 b	4.08 b
AT4G31800	"WRKY18 (WRKY DNA-binding protein 18); transcription factor"	3.65 b	4.09 b
AT5G26170	"WRKY50 (WRKY DNA-binding protein 50); transcription factor"	3.66 b	3.45 b
AT4G18170	"WRKY28 (WRKY DNA-binding protein 28); transcription factor"	3.71 b	3.00 b
AT5G49520	"WRKY48 (WRKY DNA-binding protein 48); transcription factor"	3.78 b	4.25 b
AT2G40740	"WRKY55 (WRKY DNA-binding protein 55)"	3.80 b	4.61 b
AT1G62300	"WRKY6 (WRKY DNA-binding protein 6); transcription factor"	3.85 b	3.03 b
AT1G68150	"WRKY9 (WRKY DNA-binding protein 9); transcription factor"	3.92 b	4.39 b
AT5G15130	"WRKY72 (WRKY DNA-binding protein 72); transcription factor"	3.94 b	4.11 b
AT4G22070	"WRKY31 (WRKY DNA-binding protein 31); transcription factor"	4.39 b	4.76 b
AT5G13080	"WRKY75 (WRKY DNA-binding protein 75); transcription factor"	4.83 b	4.20 b
AT5G22570	"WRKY38 (WRKY DNA-binding protein 38); transcription factor"	5.05 b	3.68 b
AT2G38470	"WRKY33 (WRKY DNA-binding protein 33); transcription factor"	5.22 b	4.63 b
AT4G01250	"WRKY22 (WRKY DNA-binding protein 22); transcription factor"	5.43 b	5.75 b
AT1G80840	"WRKY40 (WRKY DNA-binding protein 40); transcription factor"	5.59 b	5.29 b
AT4G23550	"WRKY29 (WRKY DNA-binding protein 29); transcription factor"	5.62 b	6.34 b
AT2G46400	"WRKY46 (WRKY DNA-binding protein 46); transcription factor"	5.65 b	5.38 b
AT5G24110	"WRKY30 (WRKY DNA-binding protein 30); transcription factor"	6.06 b	6.11 b
AT5G64810	"WRKY51 (WRKY DNA-binding protein 51); transcription factor"	6.97 b	5.93 b

Supplemental Table 2. Modulation of *WRKY* TFs by *E. amylovora* in low and high N.

Name	AGI	N	B	N+B	Response mode
AMT1.2	AT1G64780	0.17	-4.09	-4.18	independent
AAP3	AT1G77380	-0.46	3.33	3.47	independent
AMT2.1	AT2G38290	-0.73	3.55	2.91	independent
ASPG1	AT3G16150	-0.09	1.55	1.59	independent
ASPG2	AT3G16150	-0.09	1.55	1.59	independent
CARB	AT1G29900	-0.61	-1.15	-1.55	independent
GABA-T	AT3G22200	0.28	1.11	1.15	independent
GAD1	AT5G17330	-0.05	6.84	6.34	independent
GAD2	AT1G65960	-0.57	-1.46	-1.92	independent
GAT1	AT2G15570	-0.51	1.93	1.50	independent
GDH2	AT5G07440	-0.62	1.96	1.98	independent
GLN1.1	AT5G37600	-0.62	1.96	1.72	independent
GLN1.2	AT1G66200	-0.51	-2.42	-2.04	independent
GLN1.4	AT5G16570	0.29	3.43	3.57	independent
GLU1	AT5G04140	0.05	-2.23	-2.11	independent
GS2	AT5G35630	-0.48	-1.61	-2.51	independent
NIA1	AT1G77760	-2.37	-0.89	-1.60	independent
Nitr	AT1G68570	-0.03	-4.27	-4.08	independent
NRT1.1	AT1G12110	-0.69	-1.78	-1.92	independent
NRT1.5	AT1G32450	0.16	-1.58	-1.66	independent
NRT1.7	AT1G69870	-0.17	-1.67	-2.07	independent
NRT2.1	AT1G08090	0.40	2.34	1.64	independent
NRT2.5	AT1G12940	0.09	4.53	4.14	independent
NRT2.6	AT3G45060	-0.09	6.23	6.06	independent
NRT2.7	AT5G14570	-0.20	-2.62	-2.69	independent

Supplemental Table 3. Response to single and combined stresses of selected N-related genes showing an independent profile.

<i>A. thaliana</i> genes	LP	RP
<i>PR1</i>	TCTCCCTCGAAAGCTCAAGA	GTGCCTGGTTGTGAACCCTTA
<i>PR5</i>	GCCGTGGAGCTAACGATAAG	GCGTAGCTATAGGCGTCAGG
<i>WRKY33</i>	CATTCTGCCACCAAAGGAT	TTGATTGGTTTTATTCCGTGTTT
<i>WRKY60</i>	GGGTCACCCCATTTTATCT	GGCATCACATTCTTCTCTAATGG

Supplemental Table 4. Sequence of the gene-specific primers used in this analysis.

## B. Effet de l'azote sur l'interaction *A. thaliana*-*E. amylovora*

### 1. Introduction

L'azote est un macronutriment essentiel dont la disponibilité a un impact significatif sur la croissance et le développement des plantes mais qui peut avoir une incidence sur la capacité de la plante à faire face aux attaques d'agents pathogènes (Dordas, 2008, Fagard, et al., 2014, Snoeijers, et al., 2000, Walters and Bingham, 2007). L'effet de l'azote sur ces processus dépend fortement de la culture étudiée et du mode de vie de l'agent pathogène et par conséquent, il est difficile d'établir des règles générales pour le rôle de l'azote dans ce processus à l'heure actuelle.

Nous avons constaté que les apports élevés de nitrate augmentent la résistance de la plante modèle *A. thaliana* à la bactérie nécrotrophe *E. amylovora* (Fagard, et al., 2014). Nos résultats antérieurs au sein de l'équipe indiquent que la résistance d'*A. thaliana* contre *E. amylovora* est un processus actif (Moreau, et al., 2012) et cela suggère que le processus de défense pourrait être fortement affecté par l'approvisionnement en azote. Dans cette partie, nous nous sommes intéressés à des plantes cultivées à 2 et à 10 mM de nitrate puis infectées par *E. amylovora*. Dans cette partie, nous avons étudié l'impact de la nutrition azotée sur l'expression de la défense de la plante et sur la pathogénicité d'*E. amylovora*. Parallèlement, nous avons examiné l'effet des différentes conditions de nutrition azotée sur la physiologie de la plante et de la bactérie.

### 2. ARTICLE: Availability of nitrogen for plants affects the capacity of bacteria to repress defense through timely expression of bacterial virulence

**Availability of nitrogen for plants affects the capacity of bacterial pathogens to modulate JA and SA signaling through timely expression of their virulence**

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## ABSTRACT

Nitrogen (N) is a crucial element for plant growth, development, and defence against environmental abiotic and biotic stresses. The impact of N supply on disease development has been extensively described, nevertheless, to date the molecular mechanisms involved remain largely unknown. We have recently shown that the high availability of N enhances the resistance of *A. thaliana* plants to the necrotrophic bacterium *E. amylovora*. In this work, we show that plant resistance to bacteria in high N is correlated to higher apoplastic ROS accumulation and an increase in jasmonic acid pathway signaling. Interestingly, *in planta* titers of *t3ss* and *dspE/A-deficient* non-virulent *Ea* mutants was affected in comparison to *Ea* wild-type in low N but not in high N. Analysis of *Ea* virulence genes *in planta* indicated a lower expression of several *Ea T3SS*-encoding *hrp* genes. Pre-incubation of *Ea* in an *hrp*-inducing medium prior to infection enhanced significantly *Ea* titers *in planta* independently of plant N status. The induction of *Ea hrp* genes led to a significant repression of JA-associated genes while SA pathway was not affected. Our metabolomics data indicate a deep modification in sugar, amino acids and organic acids level following infection by *Ea*. We further found that linolenic acid the first plastid-located precursor of jasmonic acid, which disappeared completely upon *Ea hrp*-induced condition. This study suggests the N modulation of plant susceptibility to *Ea* through the regulation of *Ea* virulence factors which could target mainly JA pathway signaling in plants.



## INTRODUCTION

Plants are usually exposed simultaneously to multiple stresses and many reports have shown that one stress can affect the plant's response to another stress. In particular, abiotic stresses can interact with biotic stress, either negatively or positively. For example, high temperatures are known to inhibit R-gene mediated resistance to pathogens and thus increase infection to bacteria and viruses (Wang *et al.*, 2009) while drought has been described to decrease the severity of infection by the fungi *Botrytis cinerea* and *Oidium neolycopersici* (Atkinson *et al.*, 2013). Deficiencies in the soil in particular mineral elements can also trigger an abiotic stress, which in turn can affect plant susceptibility to pathogens (Dordas, 2008). In particular, a number of reports indicate that nitrogen (N) availability affects plant-pathogen interactions (Fagard *et al.*, 2014).

N is the most important nutrient for plant growth and thus large amounts of N are brought to crops through fertilization worldwide, which have both strong environmental and economic consequences. Several studies have reported an effect of N availability on disease development. However, both N supply and N deficiency have been found to increase disease, depending on the host-pathogen interaction, which suggests that complex mechanisms are at play. For example, addition of N fertilizer to rice cultures increases susceptibility to *Magnaporthe oryzae*, a phenomenon described by farmers as Nitrogen-Induced Susceptibility (Ballini *et al.*, 2013). On the contrary, tomato susceptibility to *B. cinerea* is known to increase in response to N deficiency (Lecompte *et al.*, 2010). Furthermore, N availability can have opposite effects for a given plant species depending on the pathogen. Indeed, low N concentration in tomato tissues is correlated with an increase in susceptibility to *B. cinerea*, however it is correlated with a decrease in the susceptibility to *Pseudomonas syringae* and *Oidium lycopersicum* (Hoffland *et al.*, 2000). To further complicate matters, the impact of N for a given pathogen depends on the plant species. For example, in tomato low N increases susceptibility to *B. cinerea* while in strawberry and *A. thaliana* it decreases susceptibility to *B. cinerea* (Fagard *et al.*, 2014; Daugaard *et al.*, 2016). Thus, the use by farmers of well adapted N supply to reduce pathogen attacks is hindered by the multiplicity of effects and by our lack of understanding of the mechanisms involved. Although the underlying mechanisms remain globally not well understood, it is known that

N availability can affect expression of plant defense, expression of plant virulence and availability of nutrients for the pathogen (Fagard *et al.*, 2014).

The literature bears contradictory reports about the impact of N availability on plant defense expression. In *A. thaliana*, the activities of three enzymes associated with defense, chitinase, chitosanase and peroxidase, were reduced in low N (Dietrich *et al.*, 2004). In tomato plants, the phenolic defense compound caffeoyl putrescine increased significantly lower in plants grown in low N following the infection by *P. syringae* than in plants grown in high N (Royer *et al.*, 2013). On the other hand, the form of N can also affect plant defense responses to pathogens. For example, it was reported that nitrate fertilization of tobacco enhanced nitric oxide (NO) production, expression of the defense associated gene *PR1* and polyamine-mediated HR in response to *Pseudomonas syringae* pv. *phaseolicola* while all three were low under ammonium nutrition (Gupta *et al.*, 2013).

N can affect pathogenicity by modulating the expression of virulence factors. In general, virulence factors of pathogens are more highly expressed in minimal growth medium than in rich growth medium. This is thought to reflect the fact that *in planta*, the pathogens are often confronted to a relatively limiting medium on several accounts. In the case of N, several studies have reported that N availability can affect both fungal and bacterial virulence genes expression *in planta* and *in vitro* (Snoeijers *et al.*, 2000). For example, N availability strongly affects the transcriptome of the *P. syringae* B728a strain. Indeed, *hrp* genes of *P. syringae* showed low expression in medium rich in N, while most virulence genes were highly induced under N limitation. Also, the expression of *Erwinia amylovora* (*Ea*) *hrp* genes is highly reduced in the presence of a high ammonium concentration as well as by some amino acids such as asparagine and histidine (Wei *et al.*, 1992).

*Ea* is a Gram-negative bacterium of the *Enterobacteriaceae* family, causal agent of fire blight, a very destructive disease for the *maloidae* sub-family of *Rosaceae*. The main virulence determinants of *Ea* are known and have been well studied (Holtappels *et al.*, 2016). They include an *hrp*-encoded type three secretion system (T3SS) and an exopolysaccharide (EPS) (Ancona *et al.*, 2014). On the plant side, several defense-pathways have been shown to be involved in defense against *Ea*, including the salicylic acid (SA)-

and jasmonic acid (JA)-dependent pathways and reactive oxygen species (ROS) accumulation. As no source of complete resistance has been found to date for *Ea* (Civetta *et al.*, 2009; PARRAVICINI *et al.*, 2011; Malnoy *et al.*, 2012), many studies have focused these past years on the development of elicitors including for the protection of apple seedlings and apple trees against fire blight (Dugé de Bernonville *et al.*, 2014). However, efficiencies of such protection methods in the field are irregular, in particular because they are influenced by environmental conditions. In the case of *Ea*, it is known that fast growing organs such as flowers and young leaves are more susceptible to *Ea* (Thomson and Gouk, 2003) but the physiological and molecular explanations for this are not known.

We have recently shown that low N availability increases the susceptibility of *A. thaliana* plants to *Ea* (Fagard *et al.*, 2014). In the model plant *A. thaliana*, *Ea* is able to transiently multiply in a T3SS-dependent manner and triggers similar defense pathways as in apple seedlings (Degraeve *et al.*, 2008). In order to understand the interactions between plant physiology and bacterial infection in a context of N limitation, we studied several aspects of the interaction in *A. thaliana* plants grown in low or high N and infected with *Ea*. We show that several physiological parameters of the plants were affected by the availability of N, and that defense expression (ROS and PR gene expression) is also affected. Most importantly, our data reveal that N availability to the plants strongly affects the *in planta* expression of bacterial virulence genes, revealing that this is the main limiting factor for infection of this non-host species by *Ea*. Finally, we show that low N or *in vitro* pre-induction of virulence genes correlate with low expression of the JA pathway and low apoplastic ROS, which both seem to be determinant for successful infection.

## RESULTS

### **Low N favors *in planta* bacterial multiplication independently of bacterial nutrition**

We have shown previously that wild-type *A. thaliana* plants grown in 2 mM NO<sub>3</sub><sup>-</sup> are more susceptible to *Ea*, based on *in planta* bacterial titers, in comparison to plants grown in 10 mM NO<sub>3</sub><sup>-</sup> (Fagard et al, 2014). We first tested the hypothesis that nitrate availability could affect nutrient availability for bacteria inside the leaves. Plants of the Col-0 accession were grown for five weeks in a growth chamber with an 8 h light/16 h dark cycle in sand culture irrigated with a nutrient solution containing 2 or 10 mM NO<sub>3</sub><sup>-</sup>. Although rosette fresh weight was slightly higher for plants grown in high N (10 mM NO<sub>3</sub><sup>-</sup>) this difference was not significant (Figure 1A). This is consistent with previous results obtained for the WS accession of *A. thaliana* for which no difference in rosette fresh weight was obtained 36 days after sowing (Lemaitre et al, 2010). Rosette diameter however was significantly higher in plants grown in high N (Figure 1B). Furthermore, as described previously for the WS accession (Lemaitre et al, 2010), nitrate content in leaves was significantly more important in plants grown in high N (10 mM NO<sub>3</sub><sup>-</sup>) than in low N (2 mM NO<sub>3</sub><sup>-</sup>) (Figure 1 C). Total amino acid content measurement showed that plants grown in low N had a slightly lower amino acid content than plants grown in high N, although these differences were not statistically significant (data not shown). A GC-MS analysis of the total metabolites indicated that there were also slight differences in the balance of amino acids between plants grown in high N (10 mM NO<sub>3</sub><sup>-</sup>) and low N (2 mM NO<sub>3</sub><sup>-</sup>) (Figure 1D). Furthermore, several organic acids accumulated more in leaves of plants grown in low N (2 mM NO<sub>3</sub><sup>-</sup>) namely citrate, fumarate, succinate and malate (Figure 1E), probably reflecting a modification of the metabolic fluxes in plants grown in low N. These results are consistent with previous results obtained for the WS accession (Fagard et al 2014).

Thus, the main impact of low N (2 mM NO<sub>3</sub><sup>-</sup>) on the physiology of Col-0 plants in our conditions is a reduced rosette diameter, a decrease in total leaf NO<sub>3</sub><sup>-</sup>, a slight decrease in total amino acid and a modification in the balance of amino acids and an increase in organic acids. Altogether, the low N condition impacted only moderately the physiology of five-week-old rosettes.

Previous reports indicate that *Ea* cannot reduce nitrate to nitrite and thus cannot use nitrate as a source of N, unlike most enterobacteriaceae (Paulin, 2000). Thus, the change in NO<sub>3</sub><sup>-</sup>

content in leaves (Figure 1) are unlikely to explain the difference in bacterial growth *in planta*. On the other hand, plants grown in low N (2mM NO<sub>3</sub><sup>-</sup>) showed a different balance in relative amino acid abundance (Figure 1D) and it was suggested previously that aspartate is an important source of N for *Ea* in host plants (Paulin, 2000). In order to determine whether *Ea* uses amino acids with different efficiencies and therefore whether the modification in the ratio of the different amino acids is responsible for the increase susceptibility of *A. thaliana* grown in low N, we studied *Ea* multiplication *in vitro* in a minimal medium (M9) containing various N sources. We tested nitrate, ammonium and several amino acids found in *A. thaliana* leaves, i.e. aspartate, glutamine, asparagine, and GABA. As expected *Ea* was not able to use nitrate as a source of N for growth *in vitro* but was able to use ammonium and all the amino acids tested (Figure 1F). Contrary to what was suggested previously (Paulin, 2000), we found that some N sources such as asparagine and glutamine were more favorable to *Ea* growth than aspartate or ammonium. In the case of GABA, although they can clearly be used by *Ea* as a source of N, it is difficult to conclude as to their quality as a nutritional source because of the latency period observed when bacteria are grown in the presence of the amino acids as sole source of N (Figure 1). Although we found slight differences in the use of amino acids by *Ea*, they are unlikely to be responsible for the increase in susceptibility of *A. thaliana* in low N.

Our data thus do not show any evidence that the modifications in N sources detected in leaves grown in low N explain *per se* higher plant susceptibility, as defined by the higher bacterial titers *in planta*.

### **Reactive oxygen species (ROS) accumulation is affected by N availability**

ROS accumulation is a common defense response to avirulent pathogens and to non-adapted pathogens such as *Ea* (Launay *et al.*, 2016). In order to determine whether N supply had an effect on ROS accumulation following infection, we used diaminobenzidine (DAB), which forms a brown precipitate in the presence of H<sub>2</sub>O<sub>2</sub>. Leaves of five-week-old *A. thaliana* plants, grown in low or high N (2 or 10 mM NO<sub>3</sub><sup>-</sup> respectively), were mock- or *Ea*-inoculated. As expected, the mock-inoculated control leaves did not display significant brown staining (Figure 2A), indicating that these leaves did not accumulate significant ROS. On the contrary, we observed strong brown staining in leaves inoculated with *Ea* (Figure 2A) in plants grown both in high and low N conditions. Recent data suggested that ROS

accumulation in the apoplast is negatively correlated with the level of susceptibility to *Ea* (Launay *et al.*, 2016). In order to determine if apoplastic ROS accumulation following inoculation by *Ea* is affected by N availability we observed DAB staining at the tissue level by light microscopy (Figure 2B). We detected a strong brown precipitate at the periphery of plant cells in infected leaves grown in 10 mM NO<sub>3</sub><sup>-</sup> (Figure 2B). In *Ea*-inoculated leaves grown in low N (2 mM NO<sub>3</sub><sup>-</sup>), this strong apoplastic ROS staining was observed only rarely. Altogether, our results indicate that higher susceptibility to *Ea* of plants grown in low N is associated with low apoplastic ROS accumulation.

### **N availability affects SA and JA-dependent defense**

To further study the impact of N supply on *A. thaliana* defense signaling pathways in response to bacterial infection, we monitored the expression of several genes dependent on the SA and JA signaling pathways shown previously to be induced in response to infection by *Ea*.

For the SA pathway, we studied the expression of *Isochorismate Synthase 1 (ICS1)*, involved in SA biosynthesis, *Phytoalexin Deficient 4 (PAD4)*, *Nonexpresser Of Pr Genes 1 (NPR1)* and *Enhanced Disease Susceptibility 1 (EDS1)*, regulators of SA-dependent signaling and finally *Pathogenesis-Related Protein 1 (PR1)*, a down-stream marker of SA signaling. We monitored the expression of these genes by qRT-PCR in five-week-old *A. thaliana* plants grown in low or high N (2 or 10 mM NO<sub>3</sub><sup>-</sup>) mock- or *Ea*-inoculated and sampled at 6 hpi. In general, we did not find strong differences in the level of expression of these genes in mock-inoculated leaves in the two nutritional regimes (Figure 3), however, there was one notable exception since *PR1* was significantly more expressed in control leaves of plants grown in low N than in those of plants grown in high N (Figure 3A). Genes involved in SA biosynthesis (*ICS1*) and regulation of the signaling pathway (*PAD4*, *EDS1*, *NPR1*) were induced following *Ea* infection in plants grown both in low and in high N (Figure 3A). However, the level of expression was slightly higher in plants grown in low N for *ICS1* and *PAD4*. Concerning the downstream *PR1* gene, previously shown to be induced by *Ea*, we found an induction following infection at this early time point only in leaves of plants grown in high N (Figure 3A). The expression level of this gene was indeed induced following *Ea* inoculation in plants grown in high N at the later 24 h time-point (not shown), however the expression level of *PR1* remained lower in *Ea*-inoculated plants grown in low

N (2 mM NO<sub>3</sub><sup>-</sup>) than in plants grown in high N as at the 6 hpi time-point (not shown and Figure 3).

For the JA signaling pathway we analyzed *Allene Oxide Synthase (AOS)*, *Allene Oxide Cyclase (AOC)* and *Jasmonate Resistant 1 (JAR1)*, the jasmonate-isoleucine synthase, *Coronatin insensitive 1 (COII)*, required for JA perception, and *Plant Defensin 1.2 (PDF1.2)*, a downstream marker of the JA pathway. In general, we did not find significant differences in the level of expression of these genes in mock-inoculated leaves of plants grown in low or high N (Figure 3B). Genes involved in JA biosynthesis and perception (*AOC*, *AOS*, *JAR1*, *COII*) were all repressed following *Ea* infection in plants grown in low N (Figure 3B) but in plants grown in high N only the *AOC* gene was significantly repressed following infection (Figure 3B). Following infection with *Ea*, the JA-downstream marker *PDF1.2* was less expressed in plants grown in low N than in high N (Figure 3B) although it was induced by *Ea* inoculation in both growth conditions.

In order to corroborate these results, we measured the level of hormone accumulation in leaves of five-week-old *A. thaliana* plants grown in 2 or 10 mM NO<sub>3</sub><sup>-</sup> following mock- or *Ea*-inoculation. SA and JA levels were measured in leaves by HPLC as described previously (Zarattini et al, 2014). In control plants grown in high N (10 mM NO<sub>3</sub><sup>-</sup>) both SA and JA accumulated slightly more than in control plants grown in low N (Figure 4A and B). Following inoculation with *Ea*, SA accumulated significantly and to comparable levels in plants grown in low and high N. Following inoculation with *Ea*, JA accumulation was slightly more important in plants grown in high N than in low N, however this was not significantly different. These data indicate that basal levels of SA and JA hormone levels are affected by N, but not hormone levels in response to bacteria.

### **N availability to the plant affects bacterial virulence gene expression *in planta***

As many phytopathogenic Gram- bacteria, *Ea* depends strongly on its T3SS for its virulence. Indeed, the T3SS allows bacteria to inject type 3 protein effectors inside plant cells to repress defense (He *et al.*, 2004; Galán and Wolf-Watz, 2006). In *A. thaliana*, the transient multiplication of *Ea* was found previously to be T3SS dependent (Degraeve *et al.*, 2008). In order to determine whether N availability affected the infection process, we analyzed bacterial titers of a T3SS-deficient *Ea* mutant *in planta* in comparison with the wild-type *Ea* strain. Leaves of *A. thaliana* grown in low or high N (2 or 10 mM NO<sub>3</sub><sup>-</sup>

respectively) were inoculated with a wild-type *Ea* strain or a T3SS-deficient strain and *in planta* bacterial titers were measured 24 hpi. For the wild-type *Ea* strain, *in planta* bacterial titers were lower in plants grown in high N, as described previously (Fagard *et al.*, 2014); Figure 5A). On the contrary, we found no difference in bacterial titers for the T3SS-deficient strain between plants grown in low and high N (Figure 5A). Indeed, the T3SS-deficient strain showed a lower titer than the wild-type *Ea* strain in low N but not in high N. This result suggests that in high N either the T3SS is not crucial for *in planta* bacterial survival and growth or that it is not functional or not expressed in these conditions.

Our first hypothesis to explain the lack of difference between the wild-type *Ea* strain and the T3SS-deficient strain in high N is that the T3SS is not expressed properly by the wild-type *Ea* strain in plants grown in high N. Indeed, it has been shown that environmental factors affect bacterial virulence gene expression (Snoeijers *et al.*, 2000) and that *Ea* virulence genes are highly expressed *in vitro* in minimal medium containing galactose (Wei *et al.*, 1992). Thus, in order to determine whether virulence gene expression was limiting in plants grown in high N, we pre-incubated the bacteria for 6 hours prior to infection in minimal medium containing galactose, hereafter referred to as “Inducing Medium” (IM). As a control, *A. thaliana* plants were inoculated with wild-type *Ea* pre-incubated in rich medium (LB). *In planta* bacterial numbers were quantified 24 hpi (Figure 5B). The pre-incubation of bacteria in IM led to a significant increase in the bacterial titers in comparison to bacteria pre-incubated in LB (Figure 5B). This increase of *in planta* bacterial titer following pre-incubation in IM was independent of N supply (Figure 5B).

The effect of N on the expression of *Ea* virulence genes was monitored in *A. thaliana* plants grown in low and high N, infected by *Ea* wild-type and harvested at 24 hpi. We chose the 24 hpi time-point because in flowers of *Malus*, the maximum gene expression of *Ea* virulence was reported at 24 hpi (Pester *et al.*, 2012). Our results indicated that *hrpN*, *hrpL*, *rpoN*, *dspA/E* and *amsG* were significantly more expressed in plants grown in low N (2 mM NO<sub>3</sub><sup>-</sup>) than in high N. However, we detected no significant effect of the N regime on the *in planta* expression of *hrpA* and *hrpS*. This result indicates that the low availability of N for plants could increase the expression of several genes involved in *Ea* pathogenicity during infection.

In order to verify that *Ea* virulence genes were induced by incubation in IM, we monitored several genes important for *Ea* virulence by qPCR. We analyzed the expression of three



sigma factors, *rpoN*, *hrpL*, and *hrpS*, that control *hrp* gene expression *in vitro* (Ancona et al 2014). We also analyzed *hrpA* which encodes a structural component of the T3SS, one gene encoding a type three secreted effectors (T3Es) *dspA/E*, *hrpN*, which encodes a harpin, and *amsG* involved in exopolysaccharide biosynthesis (Figure 5D). Most of these genes were significantly more expressed in bacteria incubated in IM than in bacteria incubated in LB with the exception of *hrpS* and *rpoN*, which were not affected by the incubation medium. In terms of fold induction, *hrpA* was the most highly induced by incubation in IM (Figure 5D).

Finally, we confirmed that the increase in *Ea in planta* titer is T3SS-dependent by analyzing the effect of IM-pre-incubation on *in planta* bacterial titers of a DspA/E-deficient *Ea* mutant and a T3SS-deficient *Ea* mutant. All three bacterial strains were incubated in IM and LB prior to plant infection and bacteria were quantified *in planta* 24hpi. Contrary to wild-type *Ea*, the T3SS- and DspA/E-deficient mutants were not affected by pre-incubation in IM (Figure 5E). These results confirmed that higher *Ea* titers following pre-incubation of *Ea* in IM is T3SS-dependent.

These results indicate that pre-incubation of *Ea* in IM *in vitro* and low N *in planta* leads to induction of *Ea* virulence genes that is limiting for *Ea* growth *in planta*, improving *Ea* pathogenicity through induction of the expression of T3SS and exopolysaccharides biosynthesis genes.

### **Pre-incubation of *Ea* in IM impacts defense and metabolite accumulation**

We hypothesized that IM pre-incubation, which led to a higher expression of the T3SS, led to the repression of defense. Five-week-old *A. thaliana* plants grown in low or high N were mock- or *Ea*-inoculated pre-incubated in LB and IM before infection. We monitored several SA- and JA-marker genes expression 6 hours following plant infection. The level of induction of SA pathway marker genes was only mildly affected by the pre-incubation of *Ea* in IM (Figure 6A). On the contrary the expression of JA pathway genes was significantly affected by the IM pre-incubation (Figure 6B). Indeed, *AOS* and *AOC* were more strongly repressed in response to bacteria pre-incubated in IM and *PDF1.2* was induced in response to bacteria pre-treated in LB but repressed in response to bacteria pre-treated in IM (Figure 6B). These results indicate that the main effect of pre-incubation of *Ea* in IM is on the JA

signaling pathway, suggesting that JA pathway signaling is one of the main target of *Ea* virulence genes.

Infection with virulent *Pseudomonas syringae* leads to large modifications in metabolic profile of *A. thaliana* including altered accumulation of defense-related compounds, disaccharides, amino acids and other nitrogenous compounds (Ward *et al.*, 2010). We hypothesized that when bacteria expressed adequately their T3SS, they could repress the accumulation of metabolites associated with defense to which the bacteria are susceptible. In order to determine whether IM-pretreatment led to a decrease in specific metabolites, a metabolomic analysis was carried out by GC/MS. For this purpose, five-week-old *A. thaliana* plants grown in low or high N were inoculated with *Ea*, pre-incubated in LB or IM. Leaves were harvested 24 hpi and metabolite levels was analyzed by GC-MS. Our results show an important modification in several plant metabolites including sugars, amino acids, organic acids and other compounds related to defense (Supplemental Figure 1). We performed hierarchical clustering analysis in order to group metabolites that accumulated under each treatment. Indeed, we found five major clusters (Supplementary Figure 2). To identify metabolites susceptible to have a role in plant resistance against *Ea* we studied cluster 3 which corresponds to 17 metabolites that accumulated less when plants were more susceptible (ie, following pre-incubation in LB and grown in 10 mM NO<sub>3</sub><sup>-</sup>). In this cluster, we found mainly organic acids (shikimate, sinapinate, dehydroascorbate, glycolate, 2-oxoglutarate, and threonate-lactone) and fatty acids (linoleic acid, linolenic acid, oleic acid, beta sitosterol and palmitelaidate). Some sugars (raffinose and glucose) and amino acids (aspartate and glutamate) were also found in this cluster (Figure 7A). In contrast, we found that cluster 5 contained metabolites that accumulated more when plants were more susceptible to *Ea*. In this cluster, we found amino acids (GABA, histidine, leucine, isoleucine, valine, lysine and proline) and sugars (maltose, ribose and xylulose) (Figure 7B).

Thus, pre-incubation of bacteria in *hrp*-inducing medium led to specific modifications in metabolite accumulation.

## DISCUSSION

It has been reported that generally, N fertilization increases the susceptibility of plants to biotrophs while it decreases the susceptibility of plants to necrotrophs (reviewed in Fagard et al, 2014). Indeed, we showed previously that the susceptibility of Arabidopsis to the necrotroph *E. amylovora* (*Ea*) is reduced by high availability of N (Fagard et al, 2014). Plants grown in 10 mM NO<sub>3</sub><sup>-</sup> supported lower titers of *Ea* than plants grown in 2 mM NO<sub>3</sub><sup>-</sup> (Figure 5B and Fagard *et al.*, 2014).

Our first hypothesis to explain the higher titers of *Ea* in Arabidopsis plants grown in low N was that in these conditions defense activation was weaker. In the present study, we show that the higher *Ea* titers found in low N were associated with lower JA signaling (Figure 3B). SA signaling was also slightly affected by N availability but the level of activation of this pathway could not explain the differences in bacterial titers (Figure 3A). It should be noted that, independently of their final expression levels, there was a strong difference between genes associated with these two pathways in their responsiveness to *Ea*. Indeed, genes associated with the SA pathway were induced by *Ea*. On the contrary, most genes associated with the JA pathway were repressed by *Ea*, with the exception of the downstream JA-responsive gene *PDF1.2* which was induced by *Ea*. However, this induction was very weak in low N, in conditions in which *Ea* titers were higher. Repression of the JA signaling pathway by *Ea* in plants grown in low N thus suggests that the JA pathway could be the main target of *Ea* to suppress plant defense. This hypothesis is supported by the observation that in susceptible *Malus* genotypes, a strong T3SS-dependent down-regulation of the JA pathway has been reported (De Bernonville *et al.*, 2012). It was proposed that repression of the JA pathway is a critical step in the infection process of *Malus spp* by *Ea* (DeBernonville:2012cw}. We also observed that higher *Ea* titers, observed in low N, were associated with low apoplastic ROS accumulation, suggesting a role of apoplastic ROS formation in non-host resistance of Arabidopsis grown in high N. This is consistent with our recent data showing that apoplastic ROS plays a critical role in Arabidopsis defense against *Ea* (Moreau et al 2012; Launay et al 2016). Thus, altogether, our data suggest that the higher bacterial titers observed in low N are linked to both a repression of the JA pathway and apoplastic ROS. However, whether or not there exists a link between apoplastic ROS accumulation and the JA pathway remains to be established.

The second hypothesis we tested was that N supply to plants affects pathogen nutrition *in planta*. Indeed, pathogens need to find a source of N once inside the plant tissue and modifications of N supply to the plant could affect N sources available for exploitation as a nutritional source by the pathogen. Our metabolomics analysis of Arabidopsis leaves showed that although we did find significant differences in the metabolite content of non-infected leaves, concerning amino acids, sugars and organic acids, these differences remained moderate (Figure 1D, E). It has been previously proposed that aspartate could be a potential source of N for *Ea in planta* since it was the major component of amino acids (58%) in apple shoots (Paulin *et al.*, 2000). In Arabidopsis leaves, aspartate was also one of the major amino acids in non-infected plants (Figure 1D). However, leaves grown in low or high NO<sub>3</sub><sup>-</sup>, showed very close aspartate contents and, *in vitro*, aspartate was not the best source of N for *Ea* growth. Thus, the differences in *Ea* titers in plants grown in low and high N are unlikely the result of a simple difference in N source availability for *Ea* growth *in planta* prior to infection.

Following plant infection with *Ea*, we found significant modifications in leaf amino acid content, indicating a specific metabolite reprogramming during plant infection (Figure 7 and Supp. Figure 2). The levels of the majority of amino acids increased (Supp. Figure 2), which is in accordance with a previous study that showed that several aromatic amino acids, tyrosine, tryptophan and phenylalanine, accumulated significantly following Arabidopsis infection by *P. syringae* pv. *tomato* DC3000 (Ward *et al.*, 2010). This was particularly true for the levels of GABA and lysine which increased strongly following *Ea* infection. Previous studies have reported that GABA accumulates in plants in response to a variety of abiotic and biotic stress as well as pathogen attack (Seifi *et al.*, 2013). For example, GABA is synthesized by Arabidopsis in response to infection by *P. syringae* pv. *tomato* DC3000 (McCraw *et al.*, 2016). In contrast, we observed that aspartate and glutamate levels decreased in response to *Ea* infection (Supp Figure 2). Similarly, Ward *et al* (2010) have shown that the levels of aspartate were reduced at 18 hpi in plants infected by *P. syringae* pv. *tomato* compared to mock plants. The disappearance of glutamate and aspartate could be linked to their role as precursors of GABA (Hildebrandt *et al.*, 2015) and lysine (Azevedo *et al.*, 2006), respectively. Conversely, the reduction in aspartate and glutamate levels could reflect their use by *Ea* as a nutritional source. It is also possible that the plant cells and bacteria compete for the use of aspartate and glutamate, for plant defense and for bacterial

nutrition and defense avoidance, respectively. Further investigation is required to distinguish between these hypotheses. However, although metabolic fluxes may play an important role during the interaction, the differences in amino acid levels we observed do not seem to explain the differences in *Ea* titers in low and high N.

The third hypothesis we tested was that *Ea* virulence gene expression *in planta* was affected by N supply to plants. This hypothesis was supported by previous data indicating that low N medium *in vitro* induces expression of the T3SS-encoding *hrp* gene cluster (Wei *et al.*, 1992; Gaudriault *et al.*, 1997) and by the observation that a T3SS-deficient *Ea* mutant showed similar titers in high N as the wild-type *Ea* strain instead of reduced titers as expected (Figure 5C). We observed that several *Ea* virulence genes were significantly more expressed *in planta* in low N than in high N (Figure 5C). This included the *hrpL* gene, known as the master regulator of the *hrp* gene cluster (Bush and Dixon, 2012). Interestingly, *rpoN*, which was previously found to play a role in N assimilation in addition to *hrp* gene regulation, through the regulation of *hrpL* (Bush and Dixon, 2012), was also more expressed in plants grown in low N. It was recently reported that *rpoN* is required for *Ea* virulence in pear (Ancona *et al.*, 2013). These results suggest that *rpoN* could play a key role *in planta* by regulating T3SS expression through the perception of reduced N availability. This hypothesis is consistent with a previous study indicating that several *Ea hrp* genes were repressed by elevated concentration of N *in vitro* (Wei *et al.*, 1992). However, the current state of the art has not allowed to determine precisely which factors allow *in planta* activation of *hrp* genes.

The last part of our study consisted in the pre-induction of *Ea* virulence genes prior to infection of Arabidopsis leaves. This pre-treatment led to higher *Ea* titers *in planta* independent of the N regime, indicating that *hrp* gene expression was likely to be limiting for *Ea* in Arabidopsis leaves. Furthermore, higher *Ea* titers *in planta* were associated with a strong repression of all the genes of the JA pathway that we tested, and a moderate increase in the expression of several genes of the SA pathway in low N (Figures 3A and 6A). This is consistent with a critical role for the JA pathway in the interaction of *Ea* with host plants and suggests that although the SA pathway is strongly induced by *Ea* infection it probably does not have a strong role in plant defense against *Ea*, as suggested by our previous results using the SA-deficient *sid2* Arabidopsis mutant (Degraeve *et al.*, 2008). Our results also strongly suggest that the T3SS of *Ea* mainly targets the JA signaling pathway, as previously

suggested by other studies (De Bernonville *et al.*, 2012). However, we cannot exclude the hypothesis that the repression of the JA pathway is the result of an increase in SA signaling in plants that are more susceptible to *E. amylovora*.

Our metabolomics analysis allowed us to identify a group of metabolites, cluster 3 (Figure 7A; Supp. Figure 1), that show an accumulation level that is negatively regulated with *Ea* titers *in planta*. In this cluster, we found mainly organic acids and fatty acids (Figure 7A), some of which are linked metabolic precursors of defense compounds. For instance, shikimate, sinapinate are known to be involved in biosynthetic pathways leading to phenylpropanoid products in plants (DIXON *et al.*, 2002). Both dehydroascorbate and glycolate are also involved in ROS accumulation processes in response to biotic stresses (O'Brien *et al.*, 2012). Linolenic acid is the first chloroplastic precursor of jasmonic acid biosynthesis (Schaller *et al.*) and oleic acid was also reported to be related to jasmonate pathway signaling (Gao *et al.*, 2011). We also identified a group of metabolites, cluster 5 (Figure 7B; Supp. Figure 1) that were positively associated with bacterial titers *in planta*. Thus, our data show that both defense-associated metabolites and primary metabolites show an accumulation level that is parallel to bacterial titers, however, whether these differential accumulations play a causal role or are the consequence of bacterial titers remains to be determined.

Altogether, our results show that varying N supply to plants affects *in planta Ea* virulence gene expression and that in turn, this allows sufficient repression of the JA signaling pathway to allow an increase in bacterial titers. Although previous reports have identified culture conditions that allow *in vitro* induction of T3SS virulence genes, the signals that allow timely activation of these genes *in planta* have not been identified (Haapalainen *et al.*, 2009). Interestingly, a previous report suggested that GABA uptake by *P. syringae* via *GabP* regulates T3SS expression *in planta* (McCraw *et al.*, 2016), which could also be the case for *Ea*. Furthermore, a recent study indicated that during incompatible interaction between an avirulent strain of *Pseudomonas syringae* Pv. *phaseolicola* (Pph 1302A) and *Phaseolus vulgaris* leaf, specific changes in apoplast composition have been observed, including increases in pH, conductivity, K<sup>+</sup>, citrate, and GABA (Leary, 2016). Since we have observed that apoplastic ROS was affected by *Ea* pathogenicity and they seem to be involved in non-host resistance of Arabidopsis to *Ea*, in this context it would be interesting

to study apoplast washing fluid (AWF) in Arabidopsis leaves and to measure enzymatic activities associated with ROS production in plants less susceptible to *Ea*.

*Ea* was initially identified in the USA and has now spread worldwide. It has been mentioned in the top 10 most important plant pathogenic bacteria to study. In the current worldwide context, in favor of using biological measures against plant microbial diseases to avoid the use of chemical compounds and pesticides in agriculture, it is essential to study the mechanisms that govern plant-pathogen interactions. In particular, understanding the precise molecular mechanisms underlying the impact of nutritional, as well as other abiotic stresses, on all aspects of pathogen life cycles *in planta* will hopefully provide a toolbox to set up new environment-friendly crop protection methods.

## **Experimental procedures**

### **Plant growth**

Seeds of *A. thaliana* Col-0 were obtained from the INRA Versailles collection. Plants were grown for 5 weeks in sand and were watered with a nutrient solution containing high (10 mM NO<sub>3</sub><sup>-</sup>) or low (2 mM NO<sub>3</sub><sup>-</sup>) nitrogen. During these 5 weeks, plants were subjected to an 8-h light and 16-h dark cycle at 21°C (day)/18°C (night) with 65% relative humidity. In all cases, 5-week-old plants were water- or *E. amylovora*-inoculated as described in the text.

### **Bacterial infection *in planta* and pathogenicity assay**

The bacterial strains used in this study are the wild-type strain *E. amylovora* CFBP1430 (Paulin and Samson 1973) and the following isogenic mutants: PMV6023 (*hrcV::MdiIPR13*) (Gaudriault et al. 1997) and *dspA/E* mutant (M81) (Degrave et al. 2013). For plant inoculations, the bacterial inoculums were prepared as follows. An aliquot of the glycerol stock culture was streaked on agar medium and grown for 48 h at 28°C. Bacteria grown on Petri dishes were used to inoculate a liquid culture under agitation at 28°C. After 8 h of growth, 100 µl of the culture were plated on agar medium without antibiotic and allowed to grow overnight at 28°C. A bacterial suspension of *E. amylovora* with an OD of 0.1 (10<sup>7</sup> CFU/mL) in water was syringe-inoculated into 5-weeks-old plants. Bacterial count procedure was performed as described in Degrave et al. (2008).

### **Pre-induction of *Ea* virulence genes**

Bacterial cells were grown overnight in LB medium. In order to remove LB, bacteria were centrifuged at 3600 g for 10 minutes and washed twice in sterile water. The bacteria were suspended in sterile water and plated on IM solid medium, corresponding to M9 medium supplemented with 0.2% galactose, for 8 hours prior to plant inoculation. For *in vitro* bacterial growth assays, bacterial cells were grown overnight in LB medium. Bacteria were separated from LB medium by two centrifugations and re-suspended in sterile water. 500 µl of bacterial suspension was added to liquid M9 medium supplemented with 0.2% glucose and 0.02% nicotinic and a nitrogen source at a concentration of 5 mM, as described in the text. OD was measured at T0 and at different time points post incubation.

### **RNA isolation and qRT-PCR analysis**



For plant RNA extraction, five leaves of each plant (Ea- or mock-treated) were collected at the indicated time point after treatment, pooled and immediately frozen in liquid nitrogen. Total RNA was extracted from 50 mg of frozen ground leaves using TrizolVR reagent (Invitrogen Life Technologies, Saint-Aubin, France). For the qRT-PCR analysis, first-strand cDNA was synthesized using Superscript reverse transcriptase SSII (Invitrogen) from 1 µg of DNase-treated (Invitrogen) total RNA in a 10 µL reaction volume. qPCRs were performed using SYBRVR Selected MasterMix 2x (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), following the manufacturer's protocol. Relative expression levels were calculated following the standard curve-based method (Larionov et al., 2005). Expression of the *PP2A* reference gene (Ceccato *et al.*, 2013) was used for normalization of each target gene studied. For each treatment, three biological replicates were analyzed. The gene-specific primers used in this analysis are indicated in Supplementary Figure 3. For the *Ea* virulence genes expression *in planta*, all the steps were carried out similarly as for plant gene expression, except that the reverse transcription was performed from 5µg of total RNA using random primers (SuperScript™) instead of oligo-dT. For qPCR, each *Ea* target gene was normalized to the *recA* reference gene (Pester et al., 2012).

### **Detection of ROS**

The intra and extracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) accumulation was detected by Diaminobenzidine (DAB) staining as described previously by (Launay *et al.*, 2016). Leaves were inoculated with *Ea* as described except that one half of each leaf was left uninoculated as a control. Leaves were sampled 2 hours post inoculation and infiltrated with a DAB solution (1 mg/ml). Leaves were placed in a Petri dish in a humid atmosphere in the dark overnight and discolored with alcohol the following morning. Stained leaves were observed with a Nikon MicrophotFXA photonic microscope at X400 magnification.

### **Metabolite analysis**

Lyophilized leaf material was used for metabolome analysis. Approximately 30 mg of the ground frozen leaf samples (mock or *Ea*-inoculated leaves, harvested at 24 hpi) was analyzed by an Agilent 7890A gas chromatograph (GC) coupled to an Agilent 5975C mass spectrometer (MS) according the protocol described previously (Amiour *et al.*, 2012). For quantification, standards were injected at the beginning and end of the analysis. Data were

analyzed with AMDIS (<http://chemdata.nist.gov/mass-spc/amdis/>) and QuantLynx software (Waters). Total free amino acids were measured from 30 mg of the ground frozen leaves by the Rosen colorimetric method with leucine as a standard (Rosen, 1957). Nitrate was determined from 20-25 mg of the ground frozen leaves by the method of (Cataldo *et al*, 1975).

For hierarchical cluster analysis (HCA) graphical representation of the metabolic changes, we used Genesis version 1.7.6 [<http://genome.tugraz.at/>] after Log2 transformation. For visualization, the metabolite data were normalized by dividing each value by the median of all measurements of the experiment for a given metabolite. The subgroups were identified using the K-means method available in Genesis.

### **SA and JA phytohormone measurement**

Rosette leaves of 5-week-old plants, *Ea*- or mock-treated were harvested 24 hpi and immediately frozen and ground in liquid nitrogen. For each treatment, 15 leaves from three plants were collected and 100 mg of fresh material was freeze-dried for hormone extraction. Hormone quantification was performed by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS), as described by (Le Roux *et al.*, 2014).

## Figure legends

**Figure 1. Comparison of physiological traits in plants grown under low (2 mM NO<sub>3</sub><sup>-</sup>) or abundant (10 mM NO<sub>3</sub><sup>-</sup>) nitrate conditions.** A. Rosette fresh weight (g); B. Maximum rosette diameter (cm); C. leaf nitrate content (nmol/ mg FW); D-E. Comparison of the raw values of amino acids and organic acids in the rosette leaves. Mean and SE of four individual plants are presented. Asterisk indicates a significant difference between two N conditions according to ANOVA statistical test, *P* value < 0.05. Ns indicates a non-significant difference between two different conditions. F. Dynamic of *E. amylovora* growth *in vitro*. Graph shows the optical density of *Ea* incubated in a minimal medium containing 5 mM of various N sources at different time-points. Each point corresponds to the mean and SE of two samples of bacterial suspension. Similar results were obtained for two independent experiments. LB: rich medium, Asn: asparagine, Gln: glutamine, NH<sub>4</sub><sup>+</sup>, aspartate, GABA, M9-(N) and N free-M9 medium.

**Figure 2. *E. amylovora* induces appoplastic accumulation of H<sub>2</sub>O<sub>2</sub> in plants grown in abundant N.** Leaves of 5-week-old plants grown upon low or abundant-N, were infiltrated on the upper half of the leaf blade with water (mock), *E. amylovora* (*Ea*) wild-type strain. A: Brown stains show accumulation of H<sub>2</sub>O<sub>2</sub> in leaves stained with DAB; B: Localization of H<sub>2</sub>O<sub>2</sub> deposits in DAB-stained leaves was observed by a photon microscope.

**Figure 3. Plant defense gene expression analysis.** A, B: SA- (A) and JA-dependent (B) gene expression in five-week-old wild-type plants grown under low (2 mM NO<sub>3</sub><sup>-</sup>) or abundant (10 mM NO<sub>3</sub><sup>-</sup>) nitrate. Plants were inoculated with water (mock) or *Ea*, and harvested 6 hpi for RNA extraction and qRT-PCR analysis. The numbers indicate Log<sub>2</sub> of fold changes between infected (black bar) and mock-treated plants (white bar) in high or low N. Arbitrary units were calculated relatively to the constitutive *PP2a3* gene expression level. Each bar corresponds the average of gene expression in three biological replicates. Different letters indicate a significant difference between two conditions according to Mann-Whitney tests, *P* value < 0.05. Experiments were repeated three times with similar results.

**Figure 4. Quantification of phytohormone contents in the leaves of Arabidopsis.** The amount of SA (A) and JA (B) was expressed in ng/gDW. Leaves of 5-weeks-old plants

grown upon low or abundant N, were infiltrated with water (white bar) or *Ea* wild-type strain (black bar) and harvested 24hpi. Columns with different letters are statistically different according to Mann-Whitney tests,  $P$  value < 0.05.

**Figure 5. High N availability attenuates T3SS expression.** A-B. Bacterial titers of wild-type *Ea* and *tts* mutant strains in *A. thaliana* leaves grown in low or abundant N at 24hpi. B. Bacteria were incubated for six hours in LB or inducing medium (IM) prior to infection. C. *Ea* virulence genes (*amsG*, *dspA/E*, *hrpA*, *hrpL*, *hrpN*, *hrpS*, and *RpoN*) expression *in planta* at 24hpi in *A. thaliana* leaves grown in low or abundant N and infected by *Ea* wild-type. D. *Ea* virulence genes expression *in vitro*, following *Ea* incubation in LB or IM. Normalized expression values of indicated genes represent mean values of two replicates of bacterial suspension. Expression is normalized to *recA*. Numbers below each gene represent the ratio between gene expression of bacteria incubated in IM relatively to LB. E. The titers of *Ea* wild-type (white bar), *tts* (gray bar) and *dspE* (black bar) mutant strains in *A. thaliana* leaves grown in low or abundant N at 24hpi. Bacteria were incubated in LB or IM prior to infection. Values correspond to the mean value of three biological replicates. A-E: Asterisks and different letters indicate a significant difference according to Mann-Whitney test,  $P$  value < 0.05. Similar results were obtained for two independent experiments.

**Figure 6. The fold induction plant hormone-associated genes in response to *Ea*.** A-B: Fold induction of SA- (A) and JA- (B) related genes. *A. thaliana* plants were grown in low or abundant N, infiltrated with water (mock), wild-type *Ea*. Bacteria were preincubated in LB or IM prior to infection. Leaves were sampled 6hpi for RNA extraction and qRT-PCR analysis. Asterisks indicate a significant difference according to Mann-Whitney tests,  $P$  value < 0.05.

**Figure 7. Heat map of relative metabolite levels in two specific clusters susceptible to have a role in plant resistance (A) or susceptibility (B) to *Ea*.** Plants were grown under low or full nitrate conditions for five weeks, inoculated by *Ea*. Bacteria were pre-incubated in LB or IM prior to infection and harvested 24 hpi. Metabolite analysis was performed by GC-MS. Heat map was based on the individual metabolites proportions for each biological replicate. The color of each cell corresponds to the log<sub>2</sub> ratio of each metabolite relative to

median center for a given metabolite. Ratio=1 (Yellow); ratio <1 (green); ratio> 1(red). Hierarchical clustering was performed using the GENESIS software.

**Supplementary Figure 1. Metabolomic analysis of *Arabidopsis* plants.** Heatmaps of amino acids, sugar and organic acids were performed by GENESIS software. Heatmap was based on the individual metabolites proportions for each biological replicate. The colour of each cell depicts corresponds to the log<sub>2</sub> ratio of each metabolite relative to median center metabolite. Ratio:1(Yello); ratio <1 (green); ratio> 1(red). Plants were grown under limiting and full N conditions for five weeks, inoculated by water (mock), *Ea* incubated for six hours in LB/IM prior to infection and harvested 24 hpi.

**Supplementary Figure 2. Cluster analysis of metabolomics data.** All the metabolites detected by GC-MS were divided into five major clusters by GENESIS software. Plants were grown under low or high N conditions for five weeks, inoculated by water (mock), *Ea* incubated for six hours in LB/IM prior to infection and harvested 24 hpi.

**Supplementary Figure 3: gene specific primers used in this study.**

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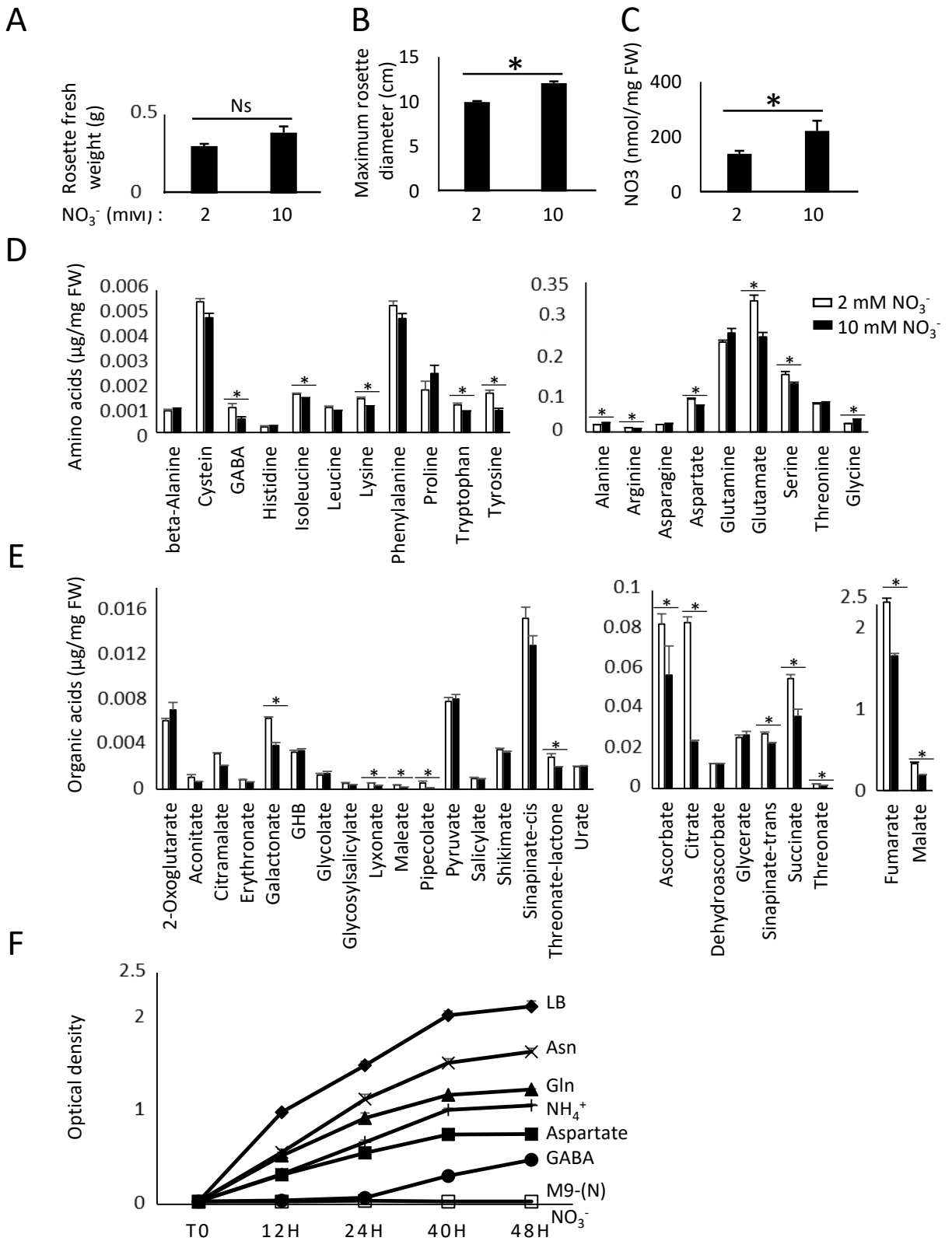


Figure 1. Comparison of physiological traits in plants grown under low (2 mM NO<sub>3</sub><sup>-</sup>) or abundant (10 mM NO<sub>3</sub><sup>-</sup>) nitrate conditions.

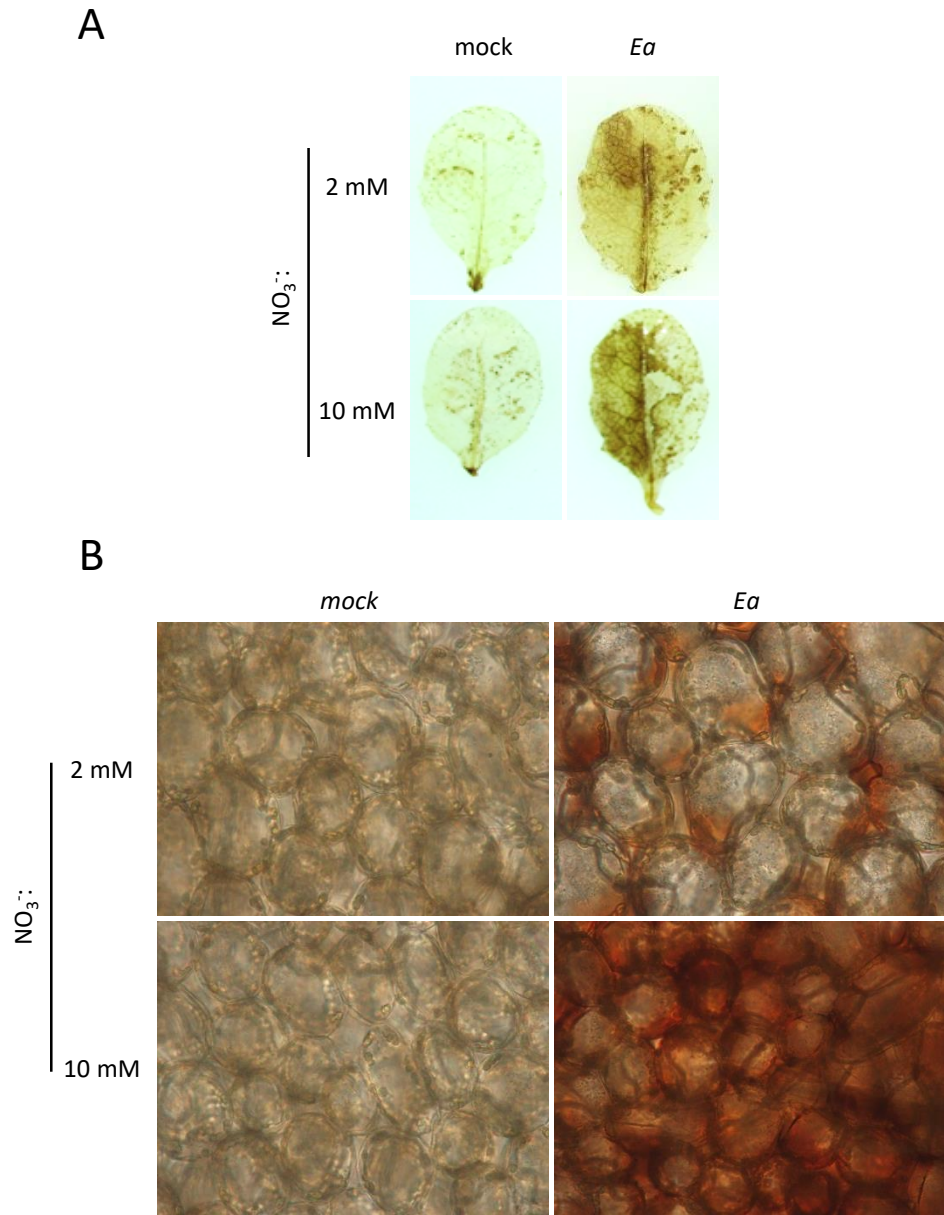


Figure 2. *E. amylovora* induces appoplastic accumulation of  $\text{H}_2\text{O}_2$  in plants grown in abundant N.

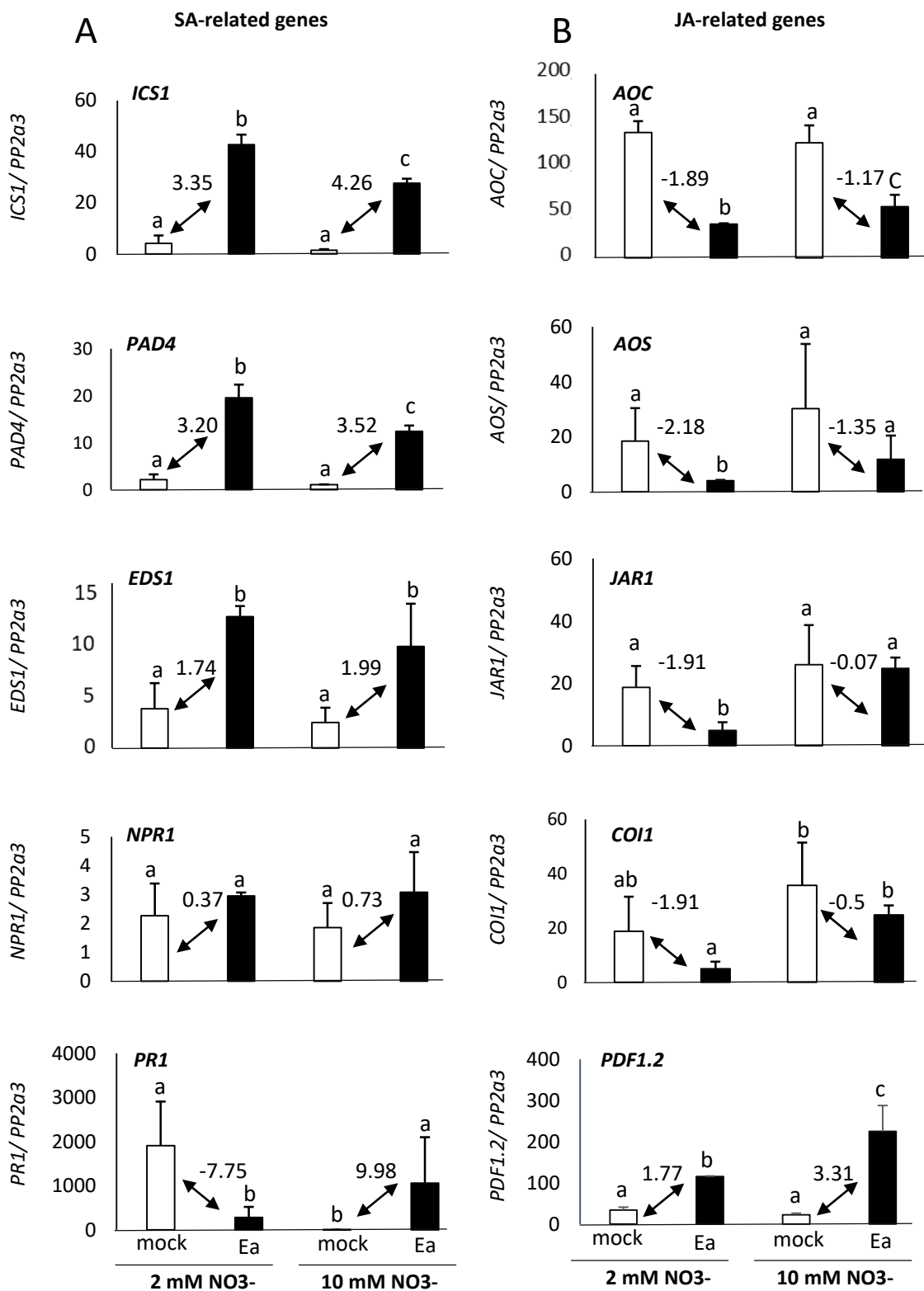


Figure 3. Plant defense gene expression analysis.

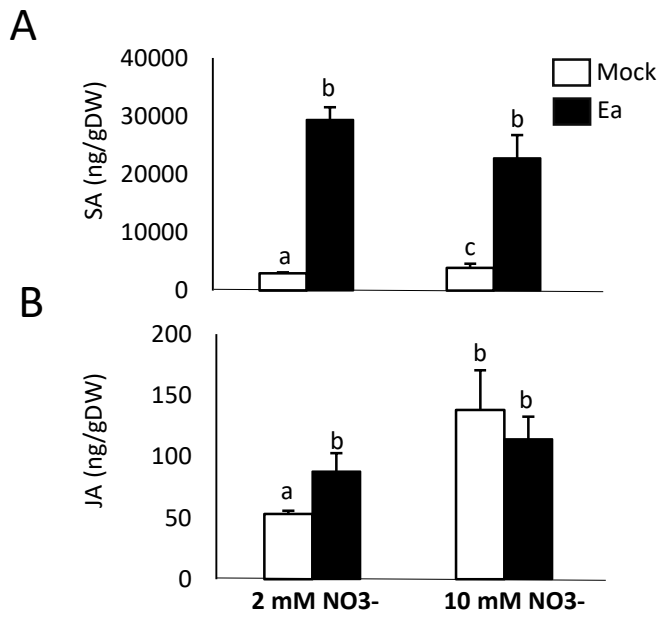


Figure 4. Quantification of phytohormone contents in the leaves of Arabidopsis.

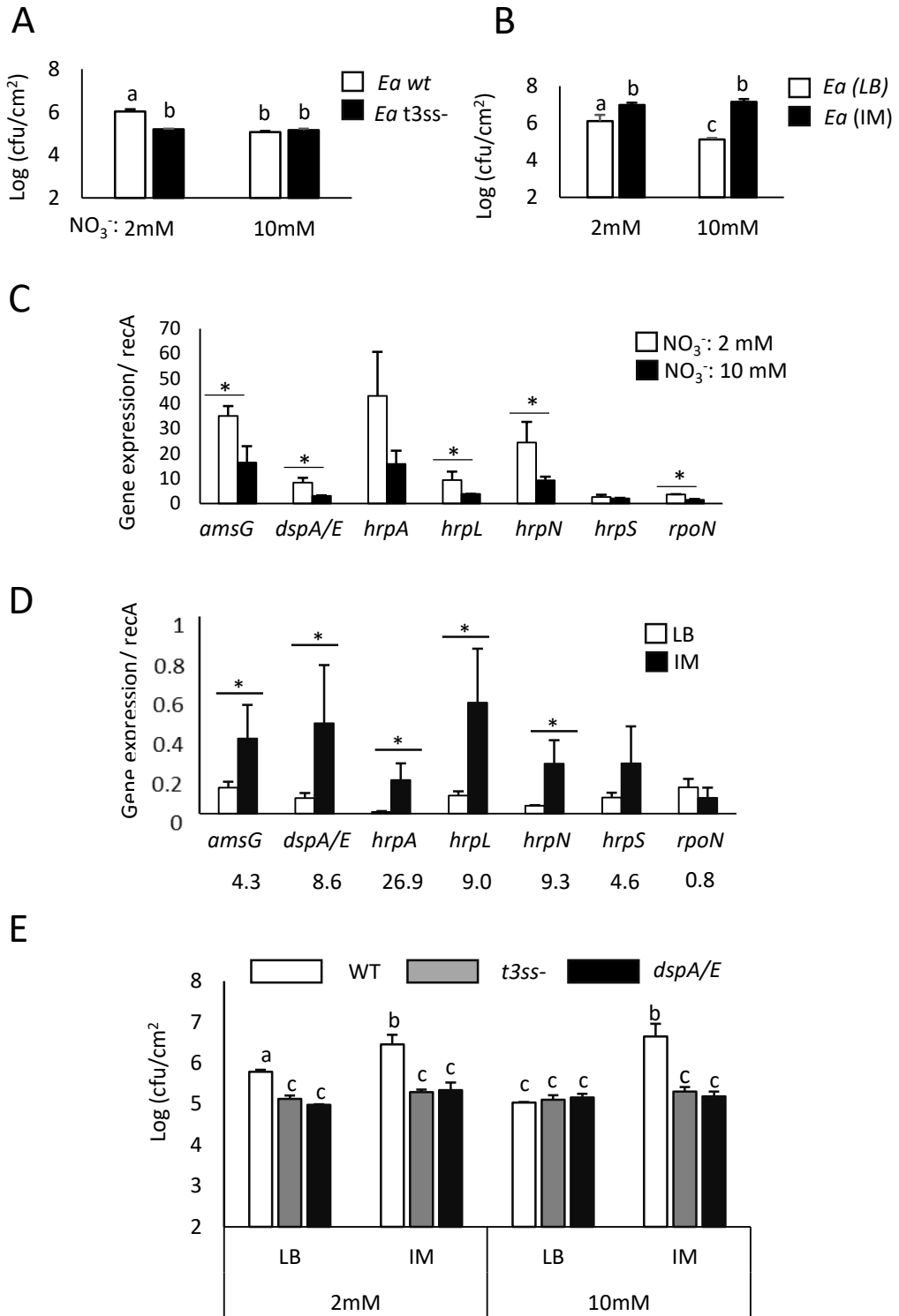


Figure 5. High N availability attenuates T3SS expression.

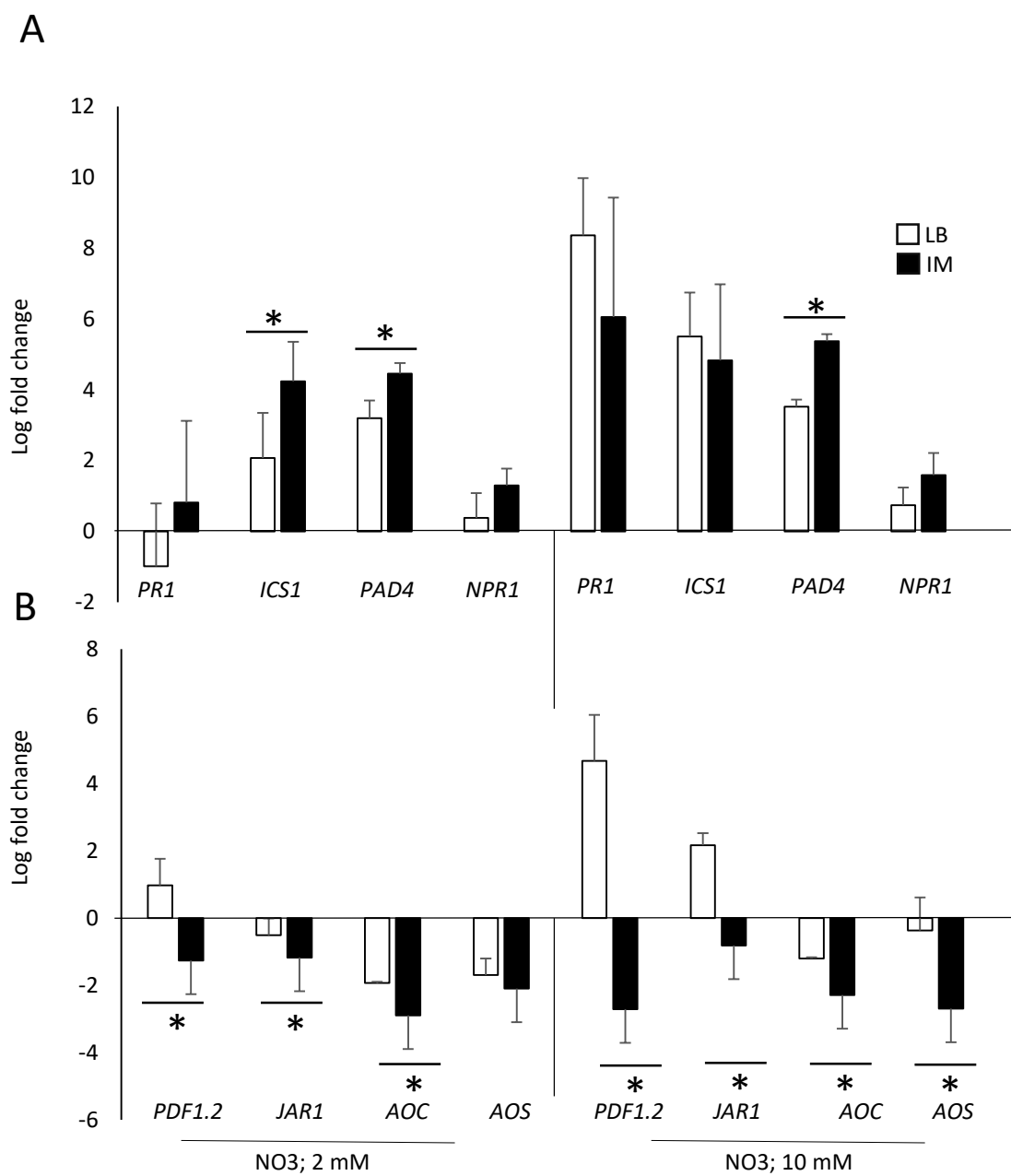


Figure 6. The fold induction plant hormone-associated genes in response to *Ea.*

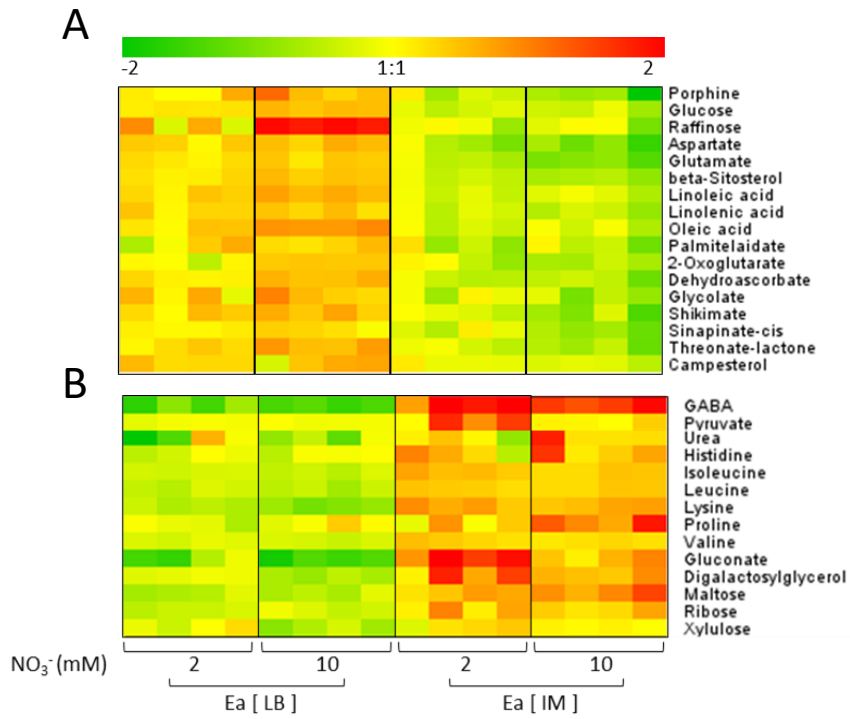
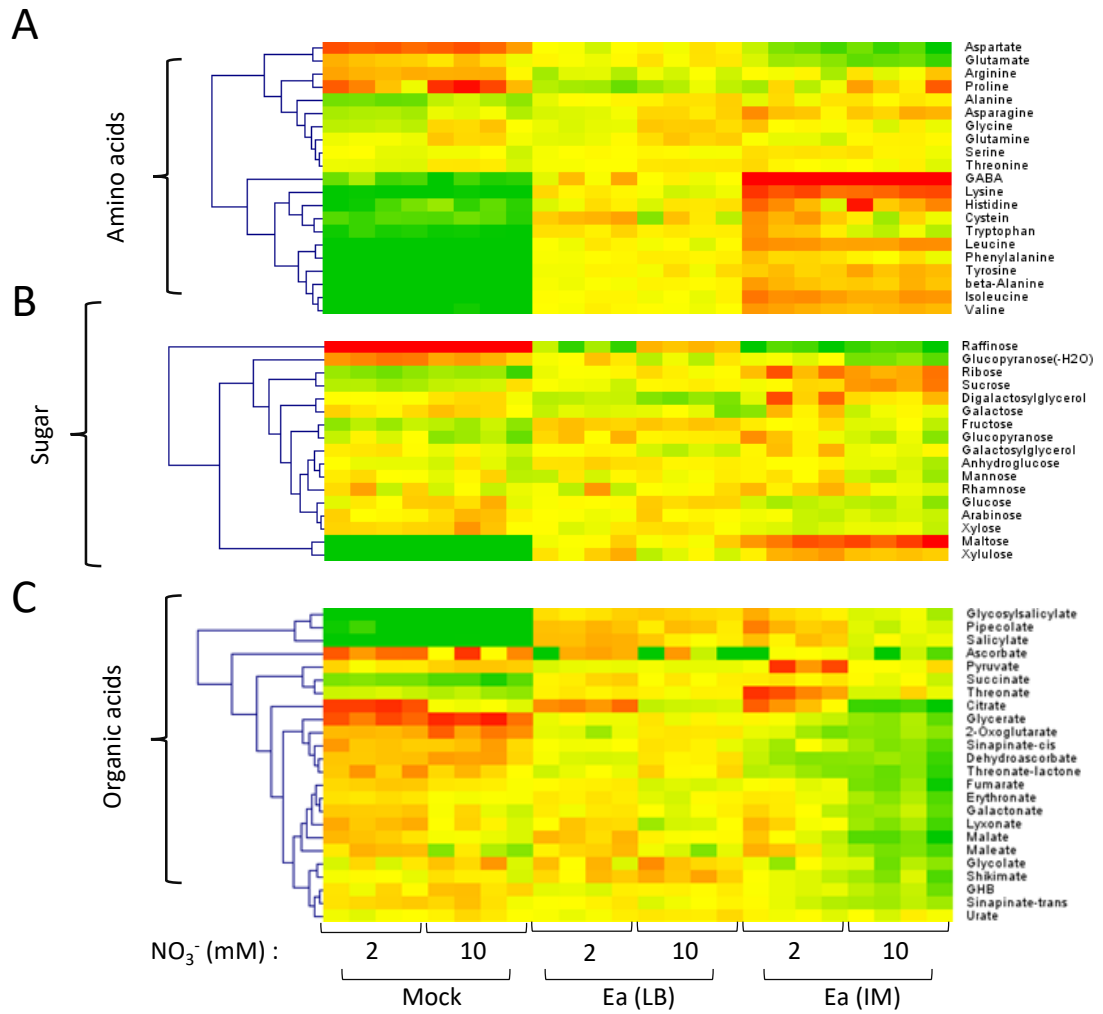
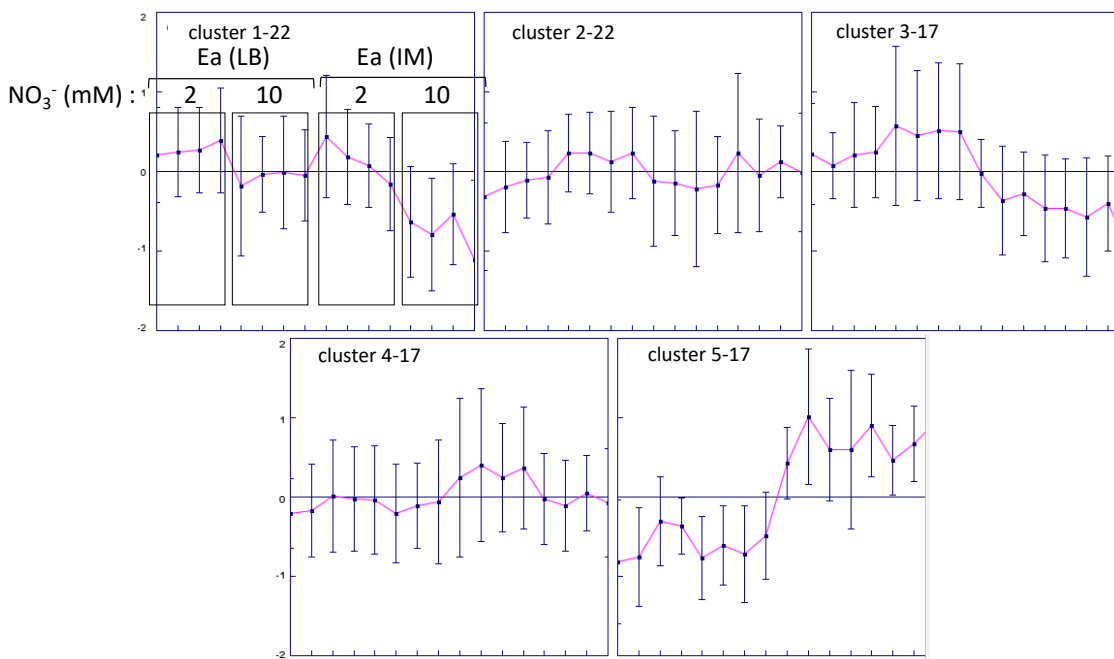


Figure 7. Heat map of relative metabolite levels in two specific clusters susceptible to have a role in plant resistance (A) or susceptibility (B) to *Ea*.





Supplementary Figure 1. Metabolomic analysis of *Arabidopsis* plants.



Supplementary Figure 2. Cluster analysis of metabolomics data

<i>A. thaliana</i> genes	LP	RP
<i>AOC</i>	GATTCGTTCTTGCCATCAC	AACCTGTCCGTAGGCACCTT
<i>AOS</i>	GACCAATCAAAGACCGTTGG	CGGATTCGTGATTTGAAGAAC
<i>COI1</i>	CTGACTTCCCTTAGGTA CTGTGG	CAACTCGTCTCGGAGGAATCAAC
<i>EDS1</i>	AGATCAATGGCGTTTGAAGCTCTTA	ACCCCATCATGAGACCATTTC AATC
<i>ICS1</i>	CAGTCCGAAAAGACGACCTCGAGTT	GCTGGAGTTGGATGCAGAGCAGCC
<i>JAR1</i>	CGTTCCTTCTCCACAATCC	GGGTCTTGGTGAATGTTGC
<i>NPR1</i>	AGGGGATATACGGTGCTTCA	ATGCACTTGACCTTTTTCC
<i>PAD4</i>	CATGGACGATTGTGCGATTCCG	ATTTGCGGTGTTGCATGAAC
<i>PDF1.2</i>	TGATCCATGTTTGCTCCTTCA	AAGCCAAGTGGGACATGGTCA
<i>PR1</i>	TCTTCCCTCGAAAGCTCAAGA	GTGCCTGGTGTGAACCCTTA

<i>E. amylovora</i> genes	LP	RP
<i>amsG</i>	GCTTTATGGCACGGATATGG	CCAACGAGATCGAAGGTACG
<i>dspA/E</i>	TCCAGCGAGGGCATAATACT	ACAACCGTACCCTGCAAAAC
<i>hrpA</i>	TACAAGCGCAAGCACTTCAG	GAGTCCATTTTGCCATCCAG
<i>hrpL</i>	TTAAGGCAATGCCAAACACC	GACGCGTGATCATTTTTATT
<i>hrpN</i>	GCTTTTGCCCATGATTTGTC	CAACCCGTTCTTTCGTC AAT
<i>hrpS</i>	AATGCTACGCGTGCTGGAAA	AACAATGGCGTTTGCGTTGC
<i>rpoN</i>	AAGCGGTA CTGAAACGGGTA	GCATCAGACTGCGAAAATCA

Supplementary Figure 3. gene specific primers used in this study.

## Conclusion et perspectives :

Généralement, il est admis que la disponibilité élevée en azote (N) diminue la sensibilité aux agents pathogènes nécrotrophes mais augmente la sensibilité des plantes aux biotrophes (Dordas, 2008, Gudesblat, et al., 2012). Malgré ces tendances générales, plusieurs travaux montrent des exemples contraires, suggérant une relation complexe entre le métabolisme azoté et les processus d'infection. Dans ce travail, nous avons étudié le rôle de la nutrition azotée de la plante sur l'interaction entre la bactérie nécrotrophe *E. amylovora* et la plante modèle *A. thaliana*.

### **La nutrition azotée module la réponse transcriptionnelle d'*A. thaliana* à *E. amylovora***

L'analyse de l'expression du génome complet d'*A. thaliana* nous a permis d'étudier les changements transcriptionnels en réponse à *E. amylovora*, à la limitation en azote puis à la combinaison de ces deux facteurs. Pour cette partie du travail (partie A), les plantes ont été cultivées en mottes à 5 ou 0,5 mM de nitrate, cette dernière concentration correspondant à une limitation en N.

Notre première observation est que globalement, la réponse de la plante à la bactérie est assez proche sous les deux conditions de nutrition azotée alors que la taille et la masse des rosettes étaient significativement diminués par la limitation en N. En effet, nous avons trouvé un coefficient de corrélation de Pearson très élevé entre les deux jeux de données. Malgré les fortes similarités entre les réponses à fort N (5 mM) et faible N (0,5 mM), certains gènes ne sont modulés en réponse à *E. amylovora* que dans une condition d'N. Cette modulation correspond notamment à l'activation des réponses de défense et à la répression de la photosynthèse en faible N (0,5 mM). Nous avons en effet observé que certains gènes de la voie du JA, comme *PR4* et *JRI*, n'étaient induits par *E. amylovora* qu'en faible N (0,5 mM NO<sub>3</sub><sup>-</sup>). L'impact de la disponibilité en N sur la voie du JA a été récemment observé dans une étude transcriptomique chez la tomate (*Solanum lycopersicum*) infectée par le champignon nécrotrophe *B. cinerea* (Vega, et al., 2015). Nos données vont dans le même sens que cette analyse transcriptomique et suggèrent que la voie du JA serait un élément clé dans la signalisation croisée entre la nutrition azotée de la plante et sa réponse à un agent pathogène nécrotrophe. En outre, le gène *PR4* pourrait être un bon marqueur pour caractériser l'effet de N sur la réponse d'*A. thaliana* à différents agents pathogènes

nécrotrophes (ce travail et Vega et al., 2015). En effet, chez le blé, le gène *PR4* est induit en réponse à l'infection par *Fusarium culmorum* et par d'autres agents pathogènes répandus au niveau du sol, par la SAR et au niveau des blessures (Bertini, et al., 2003).

Dans un deuxième temps, nous avons comparé les réponses transcriptomiques d'*A. thaliana* à chaque stress simple (bactérie ou limitation en N) et à la combinaison des deux stress. Notre analyse a révélé qu'environ 32% des transcrits ont répondu aux stress combinés de manière non-prévisible par rapport aux réponses aux stress individuels. Nous avons également montré que pour une catégorie de gènes présentant des réponses antagonistes aux stress individuels, la réponse à un des deux stress était favorisée pour une partie de ces gènes. Cette catégorie, dite « priorisée », représentait seulement 2,6% de transcrits et comprend une part importante de gènes de défense. En revanche une grande partie de gènes ont répondu de manière indépendante, c'est à dire ne répondant qu'à un des stress (bactérie ou limitation en N) sans être affecté par l'autre lors de la combinaison des stress. Ceci est probablement dû en partie à une régulation d'un faible nombre de transcrits par la limitation en N dans nos conditions expérimentales. En effet, dans cette expérience nous avons appliqué une limitation modérée en N, bien qu'ayant un impact net sur la croissance de la plante. L'effet de N pourrait probablement être plus important en ajoutant une concentration plus élevée de nitrate, par exemple  $\text{NO}_3^-$  à 10 ou 12 mM comme dans un travail précédent sur tomate (Vega, et al., 2015). De plus, nous avons récolté des feuilles de rosettes uniquement à 6 heures après le traitement, ce qui ne permet pas d'évaluer la dynamique temporelle des réponses aux stress simples ou des interactions entre les stress. Cela serait donc intéressant de réaliser une cinétique de réponse transcriptomique afin de suivre les événements en aval de l'interaction de stress biotique et abiotique.

Plusieurs gènes du métabolisme azoté, comme certains transporteurs du nitrate à haute affinité de la famille NRT2 et certains transporteurs d'acides aminés et de l'ammonium, sont modulés en réponse à *E. amylovora* alors que ces gènes ne sont pas significativement affectés par la limitation en N. Cela pourrait suggérer un rôle potentiel de ces gènes dans la régulation de la défense de la plante. De plus, différentes études transcriptomiques montrent qu'un nombre important de gènes du métabolisme azoté sont modulés en réponse aux agents pathogènes nécrotrophes (Fagard et al., 2014). Plusieurs hypothèses peuvent être avancées pour expliquer cette modulation au cours de l'infection. Ces changements d'expression de certains gènes du métabolisme azoté pourraient affecter le statut redox de la cellule, affecter

la mise en place de la mort cellulaire, affecter les voies de biosynthèse des défenses *via* leurs précurseurs, et affecter la disponibilité en nutriments pour l'agent pathogène. La modulation importante des gènes du métabolisme azoté de la plante suite à l'infection pourrait correspondre à une défense de la plante mais également à la manipulation du métabolisme de la plante hôte par l'agent pathogène.

### **Disponibilité en N et interaction *A. thaliana*-*E. amylovora***

Dans la deuxième partie du travail (partie B), nous avons utilisé des plantes cultivées à 2 et à 10 mM de  $\text{NO}_3^-$ , en sable. Ces conditions ont été sélectionnées initialement car elles ont un impact modéré sur la physiologie et le métabolisme des plantes tout en affectant la sensibilité d'*A. thaliana* à *E. amylovora*. En effet, nous avons démontré que les plantes cultivées à 2 mM  $\text{NO}_3^-$  supportent des titres bactériens plus importants que les plantes cultivées à 10 mM  $\text{NO}_3^-$ .

Notre première hypothèse était que les réponses de défense de plantes à *E. amylovora* étaient moins exprimées dans les plantes cultivées à faible N (2 mM  $\text{NO}_3^-$ ), plus sensibles à l'infection. Pour cela nous avons testé les défenses connues pour être déclenchées chez *A. thaliana* en réponse à *E. amylovora*. A faible N (2 mM  $\text{NO}_3^-$ ), dans les conditions où les plantes sont plus sensibles, l'accumulation de ROS apoplastique était nettement diminuée, la voie dépendante SA était fortement activée (comme à fort N), et la voie dépendante du JA était légèrement moins activée.

Il a déjà été suggéré que le SA ne jouerait pas un rôle crucial dans la défense contre *E. amylovora*, notamment du fait que chez le pommier la voie SA était similairement activée dans la feuille des génotypes résistants et sensibles en réponse à *Ea* (WT) (De Bernonville, et al., 2012). Ces auteurs ont proposé que dans l'interaction *E. amylovora*-pommier, la signalisation par le SA serait étroitement liée à la signalisation régulée par les ROS. En effet, dans le cas du pommier, ces auteurs ont proposé qu'*E. amylovora* utilise les ROS de la plante pour tuer les cellules de l'hôte. Toutes ces données indiquent qu'en réponse à *E. amylovora* chez la plante hôte (pommier) ainsi que non-hôte (*A. thaliana*), l'activation de la voie SA serait plutôt associée à la pathogénicité de la bactérie plutôt qu'à la défense contre *E. amylovora*. Nos résultats suggèrent qu'en condition d'azote limitant la répression de la voie du JA est accompagnée d'une forte induction des gènes de la biosynthèse du SA tels que *PAD4* et *ICSI*. Une hypothèse pourrait être que dans la condition de limitation en N,

les niveaux plus élevés d'expression des facteurs de pathogénie d'*E. amylovora* pourraient favoriser l'induction de la voie SA et par le biais de l'antagonisme entre les deux voies, favoriser la répression de la voie du JA (Figure 11). Une étude suggère que *EDS1* et *PAD4* et un autre partenaire de signalisation *in vivo* *SENESCENCE ASSOCIATED GENE 101* (*SAG101*) constituent un système de régulateur essentiel pour l'activation de la signalisation de SA qui régulerait positivement l'antagonisme entre les voies SA et JA/ET (Wiermer et al., 2005) ; ces gènes pourraient donc jouer un rôle clé dans l'effet de la disponibilité en N sur les interactions (Figure 11). Une hypothèse pourrait suggérer que dans la condition de la limitation en N, les niveaux les plus élevés d'expression des facteurs de pathogénies d'*Ea* pourraient renforcer l'effet antagoniste entre SA et JA en favorisant l'induction du SA et la mort cellulaire ainsi que la répression de la voie du JA. En effet ce renforcement de l'antagonisme SA/JA permettrait à *Ea* de se multiplier de façon la plus importante (Figure 11).

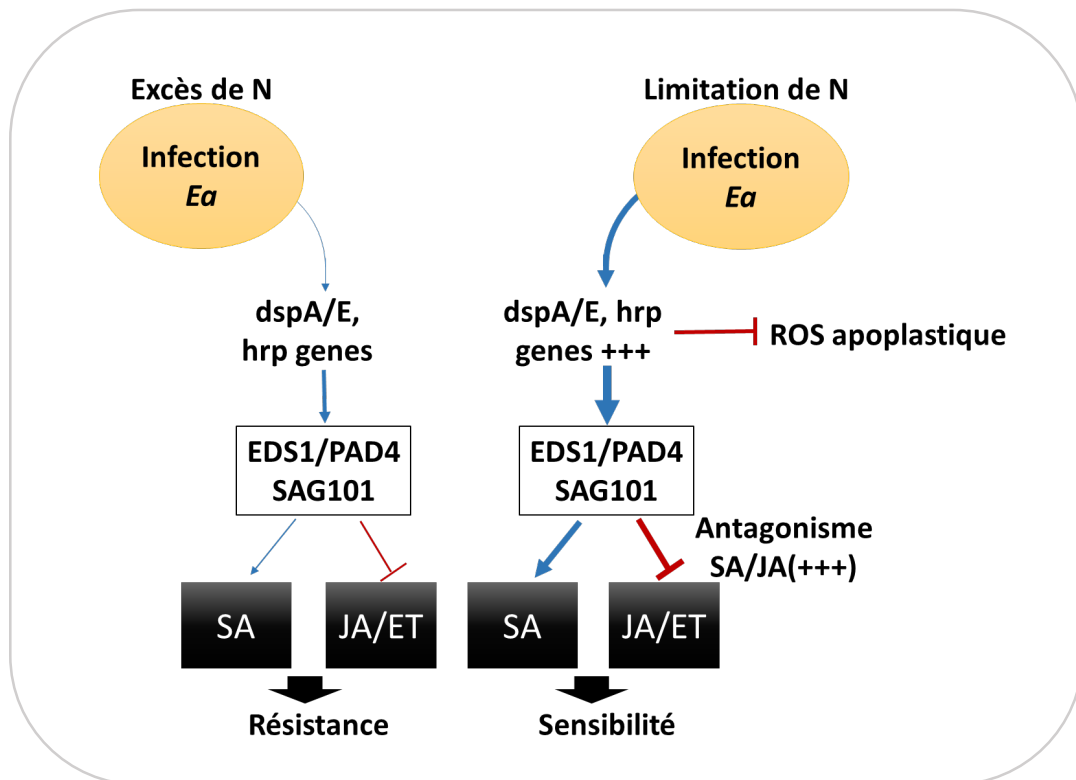


Figure 11. Schéma hypothétique représentant l'effet de N sur l'antagonisme SA/JA en réponse à *E. amylovora*. La limitation en N agirait sur le signal nutritif qui va activer les gènes de pathogénie d'*E. amylovora* (*dspA/E* et *hrp*) *in planta*. L'induction de *dspA/E* va ensuite réprimer l'accumulation des ROS apoplastiques. En effet le niveau élevé de virulence d'*E. amylovora* est accompagné d'une induction de trois gènes (*EDS1* et *PAD4* et *SAG101*) qui pourraient ensuite renforcer l'antagonisme SA/JA, conduisant à un envahissement et une multiplication bactérienne importante *in planta*.

En contraste de la voie du SA, nous avons détecté un niveau moins élevé d'expression de gènes impliqués dans la voie du JA dans les plantes cultivées à faible N (2 mM NO<sub>3</sub><sup>-</sup>), plus sensibles à *E. amylovora*. Dans l'interaction *E. amylovora*-pommier, la voie du JA est un élément de la défense contre *E. amylovora* (De Bernonville, et al., 2012). En effet, un traitement par le méthyl jasmonate dans les pommiers diminue la sensibilité à cette bactérie dans le génotype sensible (De Bernonville, et al., 2012). Chez *A. thaliana*, nous avons observé que les gènes *JARI*, *COII* et *PDF1.2* étaient moins exprimés en réponse à *E. amylovora* dans les plantes cultivées à faible N (2 mM NO<sub>3</sub><sup>-</sup>). Or *PDF1.2* est un gène marqueur des réponses au JA, connu pour avoir un rôle contre les agents pathogènes nécrotrophes (Pieterse, et al., 2012). D'ailleurs, nos données d'analyse métabolomique suggèrent également que les précurseurs clés du JA localisés dans le chloroplaste sont plus accumulés dans les plantes résistantes à *E. amylovora* à fort N (10 mM NO<sub>3</sub><sup>-</sup>). Nos différentes analyses suggèrent donc un rôle positif de JA dans la défense d'*A. thaliana* à *E.*



*amylovora*. Une hypothèse pour expliquer nos données serait une plus forte accumulation des protéines répresseurs de la voie du JA, les protéines JAZ, dans le noyau des plantes cultivées à faible N, plus sensibles à *E. amylovora*, ce qui entraînerait un contrôle négatif des défenses JA-dépendantes. Il serait intéressant d'étudier l'activité des protéines JAZ afin de confirmer l'effet de N sur le processus d'activation de la voie du JA en réponse à *E. amylovora*.

Malgré le rôle positif du JA dans la défense du pommier contre *E. amylovora*, nous n'avons pas pu démontrer un tel rôle chez *A. thaliana*. En effet, les mutants simples de la voie du JA tels que *coi1* et *jar1* n'ont perdu que légèrement leur résistance à fort N (10 mM NO<sub>3</sub><sup>-</sup>) par rapport aux plantes de génotype sauvage (résultats non montrés). Cela suggère que la résistance de la plante à *E. amylovora* à fort N n'est pas liée uniquement à l'activation de JA et que cette résistance pourrait être multifactorielle. Il faudrait tester des mutants doubles ou triples affectés dans différentes voies de défenses (ROS, callose, SA, JA) dans des conditions où les plantes sont moins sensibles à *E. amylovora*.

### **Le rôle de JA dans les autres interactions plante-pathogène :**

La manipulation de la voie du JA par les agents pathogènes et les parasites a déjà été démontrée. En effet le JA est impliqué à la fois dans la défense et dans la sensibilité aux agents pathogènes, selon les cas. Par exemple, *P. syringae* pv. *tomate* est capable d'activer la voie du JA *via* la production d'un analogue structural du JA appelé coronatine (Uppalapati, et al., 2007, Zhao, et al., 2003). Cela conduit à une réduction subséquente de la voie du SA et des défenses associées. Dans ce contexte, l'activation de la voie du JA est un facteur critique pour une infection réussie par l'agent pathogène. En effet, les mutants *coi1* d'*A. thaliana*, présentant une hyperactivation de la voie du SA, sont beaucoup plus résistants à *P. syringae* (Kloek, et al., 2001). Au contraire, une voie JA fonctionnelle est requise pour une résistance totale à *B. cinerea* et *Alternaria* (Glazebrook, 2005). Un autre rapport montre que l'acide jasmonique et son précurseur 12-Oxophytodienoic Acid (OPDA) contrôlent positivement les différents aspects des défenses constitutives et induites contre l'herbivore *Manduca sexta* chez *Solanum lycopersicum*. En effet les mutants *opr3* (OPR3-RNAi) n'accumulent pas de JA ou de JA-Ile, ce qui entraîne une réduction de la formation de trichomes et une diminution de la production de monoterpène et de sesquiterpène (Bosch et al., 2014).

## **Quel est l'impact de N sur l'expression de facteur de pathogénicité d'*E. amylovora* in planta ?**

Plusieurs études antérieures indiquent que les facteurs de pathogénie bactériens et fongiques sont activés par la limitation en N *in vitro* (Bolton and Thomma, 2008, Horst, et al., 2012, Wei, et al., 1992). Cependant, l'effet de la limitation en N sur l'expression de facteurs de pathogénie de l'agent pathogène *in planta* n'a pas été suffisamment caractérisé. Dans ce travail nous avons pu démontrer que les gènes de virulence d'*E. amylovora* sont plus exprimés en condition de N limitant, dans laquelle les plantes sont plus sensibles à *E. amylovora*. C'est le cas par exemple de son effecteur de type III principal, DspA/E, et de certains autres gènes *hrp* mais également de RpoN, codant un facteur sigma. RpoN est à la fois un régulateur positif de *hrpL* mais également de la réponse à la limitation en azote. Nous avons ensuite confirmé qu'une induction des gènes de pathogénie d'*E. amylovora* avant l'infection pourrait augmenter significativement la multiplication bactérienne *in planta*. Ces données indiquent que la disponibilité en N affecte principalement l'expression des gènes de pathogénie d'*E. amylovora*, et que cette expression est un facteur limitant pour la multiplication bactérienne *in planta*, surtout au sein de la plante non-hôte.

Chez plusieurs bactéries phytopathogènes, les protéines NtrB/NtrC régule l'activation transcriptionnelle de divers gènes impliqués dans l'assimilation de l'azote. Le domaine amino-terminal de NtrC agit comme un domaine régulateur. Dans des conditions de N limitant, NtrB phosphorylé interagit avec ce domaine pour activer NtrC par phosphorylation (Merrick and Edwards, 1995). Des gènes activés par la classe de protéines NtrC sont de type facteur sigma 54 (codé par le gène RpoN). En général, les facteurs sigma contrôlent un large éventail de gènes bactériens exprimés lors de la limitation en éléments nutritifs. Des motifs conservés du facteur sigma 54 ont été trouvés dans les promoteurs d'un certain nombre de gènes *hrp* et *Avr* de *P. syringae* (Snoeiijers, et al., 2000). Une étude récente montre que le facteur sigma alternatif RpoN est indispensable pour la virulence d'*E. amylovora* (Ancona, et al., 2014). Dans ce contexte, notre hypothèse est que la bactérie perçoit la disponibilité en N comme un signal et que les gènes bactériens seront ensuite régulés par ce signal nutritif. En effet en condition de la disponibilité élevée d'N, l'absence de la phosphorylation du NtrC par NtrB empêche la liaison de NtrC à des séquences d'amplificateur (*enhancer sequences*) pour augmenter la transcription des gènes de la bactérie en aval. Tandis que, sous la

limitation en N, NtrB est dans son état phosphorylé, il catalyse la phosphorylation et l'activation de NtrC. Le domaine central de NtrC contient un site de liaison nucléoside conservé qui va interagir avec le facteur Sigma RpoN pour activer la transcription de virulence de la bactérie.

Dans nos conditions expérimentales, nous avons pu observer une plus forte activation *in planta* de RpoN et de son gène cible *hrpL* en conditions de limitation en N. On peut donc faire l'hypothèse que c'est cette plus forte expression qui entrainerait ensuite une plus forte expression des gènes *hrp* et de *dspA/E* et finalement une plus forte sensibilité des plantes cultivées dans les conditions de N limitant. Pour confirmer cette hypothèse il faudrait étudier les évènements en amont de l'induction des gènes de virulence d'*E. amylovora*. Cela correspond à l'interaction NtrB/NtrC qui sont les premiers éléments influencés par l'abondance de N disponible *in planta*.

La Figure 12 représente le modèle de perception de la disponibilité en N ou « nitrogen-sensing » et l'induction des gènes de virulence azote-dépendent chez une bactérie phytopathogène.

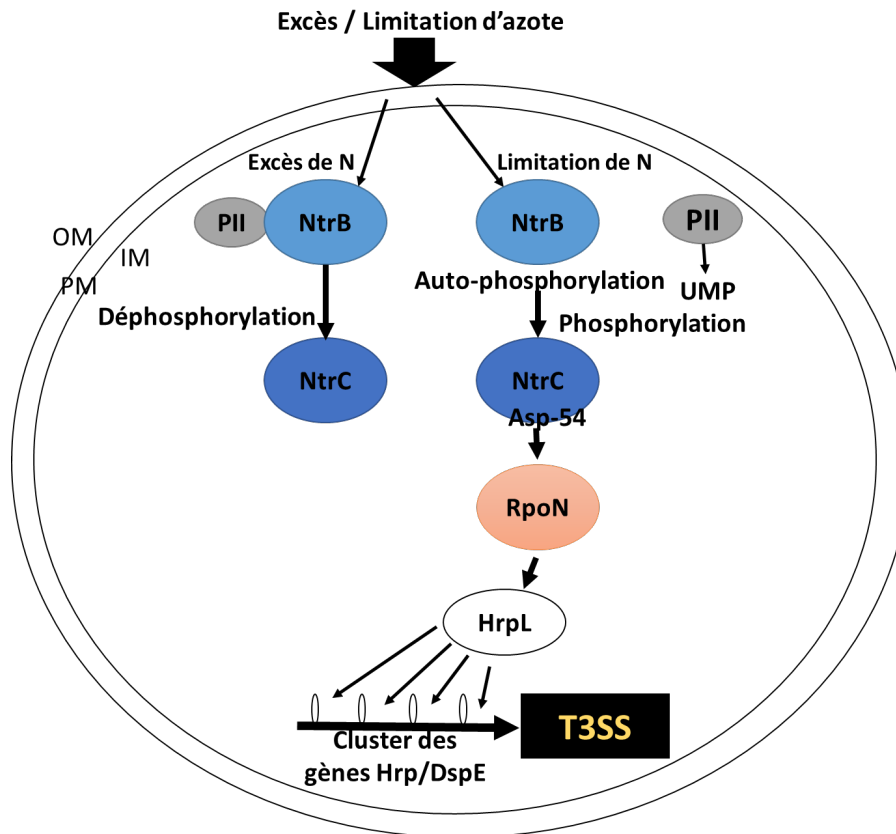


Figure 12 : Modèle pour la perception des niveaux d'azote ou « nitrogen-sensing » et l'induction des gènes Vir/Avr azote-dépendent de la bactérie phytopathogène. Dans des conditions d'excès de l'azote, la protéine PII se lie à NtrB pour activer son activité phosphatase. Lorsque cet évènement a lieu, NtrB déphosphoryle NtrC et donc ce dernier ne peut se lier à des séquences d'amplificateur (enhancer sequences) pour augmenter la transcription des gènes en aval. Cependant, PII-Ump, qui est présent pendant la limitation d'azote, ne peut pas se lier à NtrB. Dans cette situation, NtrB est autophosphorylé sur His-139. Lorsque NtrB est dans son état phosphorylé, il catalyse la phosphorylation et l'activation de NtrC. NtrC par son domaine aminoterminal agit comme récepteur régulateur. Le NtrB phosphorylé interagit avec ce domaine pour phosphoryler Asp-54 de la protéine NtrC. Le domaine central NtrC contient un site de liaison nucléoside conservé et est censé être le domaine responsable de l'interaction avec le facteur Sigma RpoN pour activer la transcription de virulence de la bactérie. OM, outer membrane; PM, plasma membrane; IM, inner membrane.

### Est-ce que la disponibilité en N joue un rôle nutritionnel pour le développement d'*Ea in planta* ?

Nous avons observé que les sources de N métabolisées par *E. amylovora in vitro* sont peu affectées par la disponibilité en N. En effet, dans les plantes non-infectées cultivées en faible

et fort N, les quantités relatives des acides aminés et des sucres métabolisables par *E. amylovora* étaient peu affectées. Cependant, nos données ne nous permettent pas d'exclure l'hypothèse d'un effet nutritionnel puisque nous avons réalisé nos analyses métaboliques sur les feuilles entières alors qu'*E. amylovora* est localisé dans l'apoplasme lors des premières étapes de l'infection. Une expérience qui pourrait mettre en évidence un éventuel effet nutritionnel de N au sein de la plante serait l'analyse d'extrait de fluide apoplastique. En effet l'extrait apoplastique pourrait révéler la présence de nutriments qui permettent de favoriser la croissance et le métabolisme des agents pathogènes pendant les premiers stades de l'infection. Pour évaluer les préférences nutritionnelles de *E. amylovora in planta*, une analyse métabolomique pourrait être réalisée avec des bactéries cultivées initialement dans un milieu LB ou IM et ensuite incubées dans l'extrait de fluide apoplastique (AWF) de feuilles saines d'*A. thaliana* (cultivées à fort et faible N). Une cinétique d'analyse métabolomique au cours de la culture d'*E. amylovora* dans l'AWF permettrait de déterminer la dynamique d'utilisation de nutriments par *E. amylovora* et d'une éventuelle source préférentielle pour *Ea in planta*. Cette expérience aiderait à mieux comprendre le métabolisme des bactéries *in planta*. Une étude récente a montré que le taux de la plupart des acides aminés diminue lors de l'incubation de *P. syringae* pv. *phaseolicola* dans l'AWF issu de feuilles haricot (*Phaseolus vulgaris*) par rapport aux AWFs témoin (O'Leary, et al., 2016). En effet le taux d'appauvrissement des acides aminés variait et les bactéries présentaient une préférence pour l'absorption et/ou le catabolisme de la glutamine, du glutamate, de l'aspartate, de la  $\beta$ -cyanoalanine ainsi que du glucose et du galactose comme source de carbone. Le profil métabolomique d'extrait d'apoplaste de feuilles infectées ou non-infectées, cultivées à fort ou faible N pourrait nous renseigner non seulement sur le métabolisme de la bactérie *in planta* mais également sur les composés impliqués dans la défense. La composition du fluide apoplastique des feuilles demanderait à être mieux connue car plusieurs travaux montrent qu'elle répond aux changements de conditions physiologiques, y compris la nutrition racinaire, le stress abiotique et biotique (Lopez-Millan, et al., 2000, Sattelmacher, 2001).

Pour conclure, nous avons réalisé deux études en parallèle (Partie A et Partie B) qui nous ont conduit à comparer deux à deux des concentrations de nitrate (0,5 et 5 mM  $\text{NO}_3^-$  dans la partie A ; 2 et 10 mM  $\text{NO}_3^-$  dans la partie B). Or l'effet de la diminution en nitrate dans

ces deux groupes de comparaison peut sembler opposé. En réalité, nous avons observé un maximum pour les titres bactériens *in planta* pour une disponibilité moyenne en nitrate (2 et 5 mM  $\text{NO}_3^-$ ), alors qu'un excès ou une forte limitation de  $\text{NO}_3^-$  diminuent significativement la sensibilité d'*A. thaliana* à *E. amylovora*. Dans tous les cas, l'augmentation de la sensibilité de plantes sous l'effet du  $\text{NO}_3^-$  est toujours accompagnée d'une réduction de la voie de signalisation de JA et d'une induction de certains gènes de la voie du SA. Nos résultats ne nous permettent pas actuellement de comprendre ces effets contrastés, cependant, ils démontrent clairement que la voie du JA joue un rôle clé dans l'effet de la disponibilité en  $\text{NO}_3^-$ .

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## Annexes

- A. Nitrogen metabolism meets phytopathology, Fagard *et al* (2014)

REVIEW PAPER

# Nitrogen metabolism meets phytopathology

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## Abstract

**Nitrogen (N) is essential for life and is a major limiting factor of plant growth. Because soils frequently lack sufficient N, large quantities of inorganic N fertilizers are added to soils for crop production. However, nitrate, urea, and ammonium are a major source of global pollution, because much of the N that is not taken up by plants enters streams, groundwater, and lakes, where it affects algal production and causes an imbalance in aquatic food webs. Many agronomical data indicate that the higher use of N fertilizers during the green revolution had an impact on the incidence of crop diseases. In contrast, examples in which a decrease in N fertilization increases disease severity are also reported, indicating that there is a complex relationship linking N uptake and metabolism and the disease infection processes. Thus, although it is clear that N availability affects disease, the underlying mechanisms remain unclear. The aim of this review is to describe current knowledge of the mechanisms that link plant N status to the plant's response to pathogen infection and to the virulence and nutritional status of phytopathogens.**

**Key words:** Disease, metabolome, nitrogen, pathogen, resistance, transcriptome.

## Introduction

Plant pathogens include oomycetes, fungi, bacteria, and viruses. Pathogens have developed different strategies to invade, feed on, and grow in the plant. Biotrophic pathogens need living plant tissue for growth and reproduction. In the case of hemibiotrophic pathogen attacks, the plant tissue will die in late stages of the infection process. Necrotrophic pathogens kill the plant tissue in early stages of the infection and are considered to feed on dead plant tissue. All viruses need living plant tissue to perform their life cycle, whereas bacteria, oomycetes, and fungi can be biotrophic or necrotrophic, depending on the species. The mechanisms of infection of viruses are very distinct from those of bacteria, oomycetes, and fungi, which share common features, and viruses trigger plants defences that are specific to these pathogens (Mandadi and Scholthof, 2013). The present review focuses on the interactions of plants with bacteria, oomycetes, and fungi and the importance of nitrogen (N) metabolism in the outcome of these interactions.

To resist pathogen attack, plants possess pre-formed defences such as cell walls, epidermal cuticles, and bark. Plants can also activate, both at the site of infection and systemically, an arsenal of inducible defences that includes massive transcriptional reprogramming, production of reactive oxygen species (ROS), reinforcements of the cell wall, such as accumulation of hydroxyproline-rich glycoproteins (HRGPs) and callose deposition, and synthesis of antimicrobial secondary metabolites and pathogenesis-related (PR) proteins involved in resistance (Glazebrook *et al.*, 2005; Bellincampi *et al.*, 2014). The molecular mechanisms underlying activation of plant defence responses are extremely complex and depend on the major defence hormones salicylic acid (SA) and jasmonic acid (JA); however, the phytohormones ethylene, gibberellins, auxins, abscisic acid (ABA), cytokinins, and brassinosteroids are also known to act as modulators of the immune response (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012).



## Recognition of pathogens by plants

Pathogen recognition by plants leading to defence activation occurs mainly at two levels (Jones and Dangl, 2006). The first line of inducible plant defence is formed by pattern recognition receptors (PRRs). These cell surface receptors recognize microbe- or pathogen-associated molecular patterns (MAMP/PAMPs) that are generally highly conserved molecules within a class of microbes and generally have an essential function in microbial fitness or survival, although counter-examples exist (Thomma *et al.*, 2011). Well-studied examples of PAMPs are bacterial flagellin and fungal chitin. Upon detection of PAMPs, PRRs activate an innate immune response called PAMP-triggered immunity (PTI). Successful pathogens are able to overcome PTI by secreting effectors that suppress PTI responses. Many phytopathogenic bacteria inject type three effectors (T3Es) directly into the host cytoplasm through their type three secretion system (T3SS), while many oomycetes and fungi secrete effectors in the inter-cellular space that can then be taken up by plant cells using specific recognition motifs (Kale and Tyler, 2011). During evolution, plants have responded to these effectors through the development of cytoplasmic resistance (R) proteins that recognize (the presence or activity of) single effectors and activate effector-triggered immunity (ETI). In general, ETI is considered to occur more rapidly and to be stronger than PTI; however, transcriptome analysis showed that the sets of defence genes induced during ETI and PTI are largely overlapping, the main difference between the two responses being in the timing and the intensity of the responses (Tao *et al.*, 2003). When the outcome of a plant–pathogen interaction is disease, the interaction is considered compatible, whereas when the outcome is resistance, it is considered incompatible.

The production of effectors by pathogens to suppress plant defence is a widespread virulence strategy. However, other virulence strategies exist. In particular, necrotrophic pathogens can produce pectin-degrading enzymes as well as cell death-inducing toxins, both of which can play major roles in virulence. For example, the virulence of the bacterial phytopathogens *Pectobacterium carotovorum* and *Dickeya dadantii* is strongly dependent on the production of pectin-degrading enzymes, whereas the virulence of the necrotrophic fungus *Botrytis cinerea* depends on both pectin-degrading enzymes and toxins (Choquer *et al.*, 2007; Davidsson *et al.*, 2013). The action of cell wall-degrading enzymes (CWDEs) leads to the production of pectin-derived oligogalacturonides (OGs). These host-derived molecules are called danger-associated molecular patterns (DAMPs) in reference to the MAMP/PAMPs cited above. Apart from pectin-derived OGs, AtPep1, a peptide derived from a cytoplasmic plant protein PROPEP1, has also been identified as a DAMP in plants. Plants have the capacity to detect the effect of infection via the detection of these host-derived molecules, and DAMP-induced defence shares many features with ETI and PTI (Mengiste, 2012).

The signalling pathways that result from pathogen attack and lead to defence activation have been widely studied, and many molecular players in the signalling pathways involved

have been identified. Interestingly, increasing evidence points to a strong convergence between signalling pathways involved in the response to biotic and abiotic stress (Fujita *et al.*, 2006). Indeed, many signalling components, including mitogen-activated protein (MAP) kinase and transcription factors, have now been shown to be involved in the response to biotic and abiotic stress. Moreover, recent data indicate that the response of plants to a combination of stresses is not a simple addition of the responses to the individual stresses (Prasch and Sonnewald, 2013). On the contrary, the plant's response to stress combinations at the transcriptional level is for the most part not predictable from the response to individual stresses (Rasmussen *et al.*, 2013).

## Plant metabolic status and pathogen infection are mutually inter-related

Superimposed on defence suppression, pathogens reconfigure host metabolism, and phytopathogen infection has a strong impact on both primary and secondary metabolism in plants (Ward *et al.*, 2010). These changes in primary plant metabolism notably can affect plant growth and development, but also lead to crop yield losses even when plant–pathogen interactions do not lead to disease and/or cell death (Berger *et al.*, 2007). There are three main aspects to the impact of pathogen infection on primary metabolism (Berger *et al.*, 2007): (i) defence is cost-intensive; (ii) the pathogen often tries to manipulate plant metabolism to its advantage creating a withdrawal of nutrients such as sugars and amino acids; and (iii) the development of chlorotic and necrotic areas following infection decreases photosynthetic activity and certainly also other chloroplast metabolism pathways locally.

Infection with both compatible and incompatible pathogens leads to a local decrease in chlorophyll fluorescence (Swarbrick *et al.*, 2006). As with defence activation (Tao *et al.*, 2003), the main difference between the reduction of chlorophyll fluorescence in response to these two types of pathogens lies in the kinetics, incompatible interactions leading to a more rapid decrease in chlorophyll fluorescence (Swarbrick *et al.*, 2006). In some cases, but not all, the observed decrease in chlorophyll fluorescence is correlated with a decrease in associated gene expression (Berger *et al.*, 2007). Finally, several data indicate that as a result of infection, reduction of the rate of photosynthesis is a fast and rapid process, while down-regulation of photosynthetic gene expression is a slower process. These changes in primary metabolism following pathogen infection lead to a reduction in photosynthetic assimilate availability which transform the originally source tissue into a sink tissue. One manifestation of this is an increase in cell wall invertases that cleave apoplastic sucrose into glucose and fructose, thereafter transported into the cell (Baker *et al.*, 2012). Repression of photosynthesis and induction of sink metabolism seem to be a general response to pathogen infection (Berger *et al.*, 2007). However, the effect of infection on the accumulation of macromolecules such as sugars depends on the pathogen (Berger *et al.*, 2007).

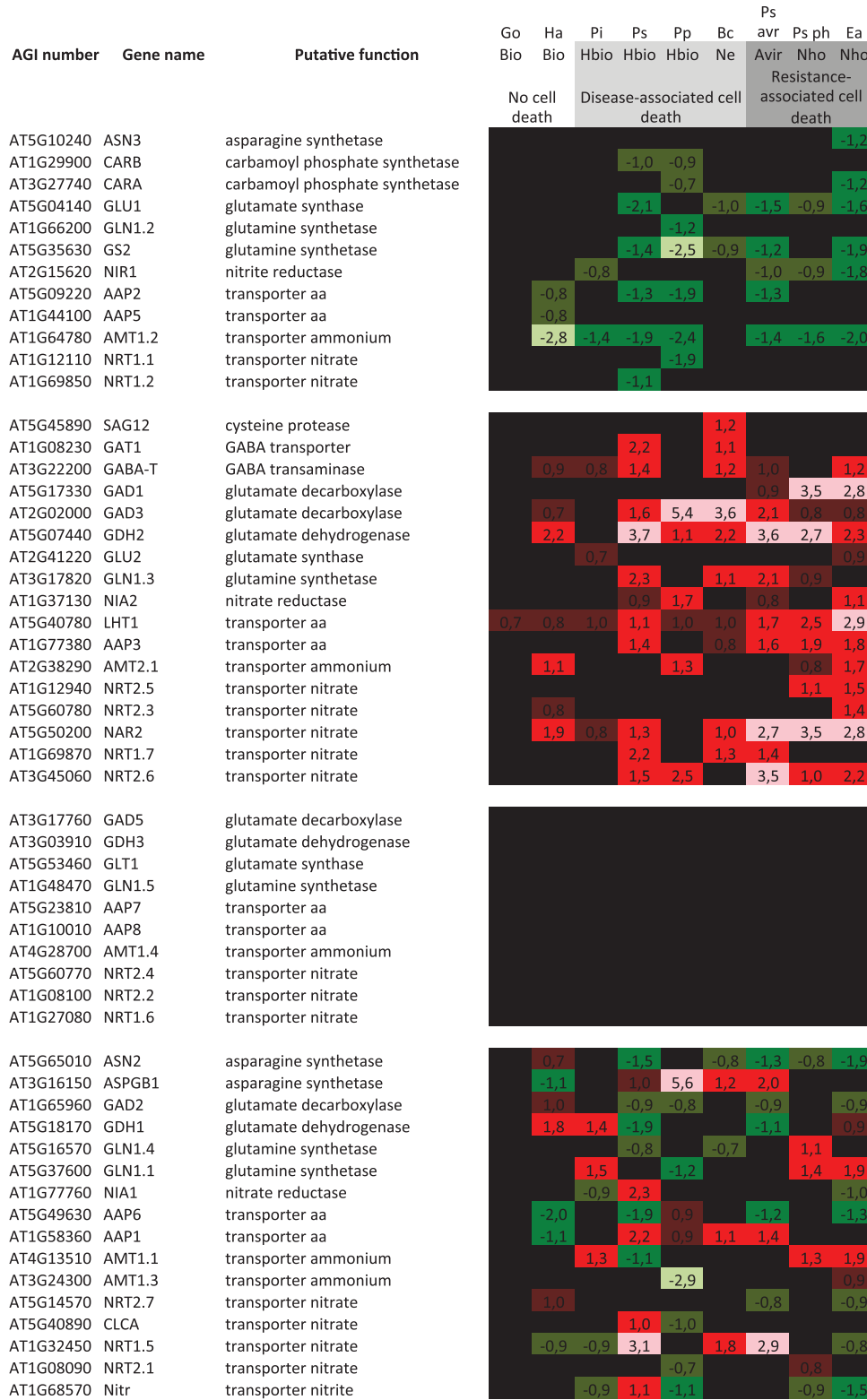
Several recent studies have focused on large-scale analysis of metabolic changes following plant infection by pathogens [reviewed in Balmer *et al.* (2013) for cereals]. One challenge in plant pathology is to be able to distinguish between defence-associated metabolites and disease-associated metabolites. Metabolic modifications associated with basal defence are rapidly activated, whereas disease-associated modifications, which require transcription and translation of virulence factors by the pathogen, occur at later time points. In response to the virulent bacterial phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000, *Arabidopsis* leaves display significant modifications starting from 8 h post-infection (Ward *et al.*, 2010). Many metabolite modifications detected by gas chromatography–mass spectrometry (GC-MS) were of low intensity, reflecting wide and subtle remodelling of the plant's metabolome following infection (Ward *et al.*, 2010). However, the accumulation of a number of metabolites displayed important changes following infection such as the phytoalexin camalexin, a non-pathogen-specific antimicrobial compound, and stigmaterol, which is known to reduce membrane permeability (Wang *et al.*, 2012). The amount of only one amino acid, aspartate, was found to be reduced in response to *P. syringae* DC3000 infection; the other amino acids were either not affected or accumulated to a higher extent than in non-infected *Arabidopsis* leaves, such as the three aromatic amino acids involved in the biosynthesis of the defence-associated secondary metabolites such as flavonoids (Ward *et al.*, 2010). In *Arabidopsis* leaves infected by the necrotrophic fungus *Alternaria brassicicola*, the amount of a large number of metabolites was also significantly altered (Botanga *et al.*, 2012). Several modifications were identical to those observed in response to *P. syringae*, such as stigmaterol and trehalose accumulation. Accumulation of the three aromatic amino acids also occurred in response to *A. brassicicola* in *Arabidopsis*, but the overall pattern of amino acid and sugar modifications retained specificities in response to each pathogen, indicating both common and specific metabolic patterns in response to different pathogens. This feature was confirmed by our GC-MS analysis of the non-host metabolic responses occurring in *Arabidopsis* Col-0 leaves, 24 h after inoculation with the phytopathogenic bacterium *Erwinia amylovora* (A. Launy and M. Fagard, unpublished). *E. amylovora* induces modifications shared with *P. syringae* (sucrose, valine, and isoleucine accumulation), *A. brassicicola* (homoserine and lysine accumulation), and both pathogens [stigmaterol, tyrosine, and  $\gamma$ -aminobutyric acid (GABA) accumulation, aspartate decrease] but also shows specific features such as a decrease in methionine content. Thus, metabolic reprogramming seems to be a general feature of plant–pathogen interactions and is a consequence of both defence (through the accumulation of secondary metabolites for example) and the requirement for pathogens to acquire nutrients, in particular carbon sources through sugar accumulation and N sources through amino acid accumulation (see below), which in turn can affect defence activation (Rojas *et al.*, 2014).

The metabolic state of the plant during the infection process, which is in part controlled through transcriptional regulation of N metabolism enzymes (Ward *et al.*, 2010), might

thus be important for the interaction. It was shown that N-related gene expression can be affected by pathogen infection (see ‘Impact of amino acid metabolism and recycling on plant–pathogen interactions’). For example, the cytosolic glutamine synthetase (GS1) isoform-encoding genes have been shown to be induced following infection by pathogens in tobacco (Pageau *et al.*, 2006), and we found in *Arabidopsis* that *GLN1.1* is strongly induced following *E. amylovora* infection (Fig. 1). In order to determine if the modification of N-related gene expression is specific for certain types of plant–pathogen interactions, we retrieved transcriptome data for *Arabidopsis* N metabolism genes (Masclaux-Daubresse *et al.*, 2010) from public databases (Fig. 1). Interestingly, most of the N metabolism genes modulated in response to infection by pathogens show a consistent pattern in response to different pathogens (either induced or repressed by several pathogens). A smaller proportion of genes displayed a variable pattern of modulation, both induced and repressed by pathogens, with no obvious pattern linked to either pathogen life style or compatibility of the interaction, perhaps as a result of specific manipulation by pathogens. No obvious pattern of modulation could be associated with the gene functions, apart from the fact that the chloroplastic GS (*GS2*) is repressed during most of the interactions associated with cell death (hemibiotrophs and incompatible interactions leading to resistance). *GS2* repression emphasizes the correlation between the chloroplastic decay leading to chlorosis and the cell death process occurring in these interactions. On the other hand, the cytoplasmic GS-encoding genes (*GLN1.1–GLN1.5*) show variable patterns of modulation in response to pathogens, which may also suggest specific manipulation by pathogens. Also, consistent with GABA accumulation in response to pathogens, GABA metabolism genes are mostly induced by infection (Fig. 1; see ‘Impact of amino acid metabolism and recycling on plant–pathogen interactions’). Several members of the NRT2 family of high-affinity nitrate transporters are also strongly induced in response to pathogens, consistent with previous reports (see ‘Nitrate uptake and plant–pathogen interactions’). Concerning pathogen life style (biotrophic, hemibiotrophic, or necrotrophic) and type of interactions (compatible or incompatible), it appears that there is a gradient showing an increase of the number of N metabolism genes up- or down-regulated during the infection. Indeed, in response to necrotrophic pathogens and pathogens triggering resistance, more N metabolism genes are up- or down-regulated than in response to biotrophic pathogens, suggesting that many of the observed gene modulations are associated with cell death and/or plant defence. Overall, N metabolism genes are strongly affected by pathogen infection, probably as a result of both defence activation and attempted pathogen manipulation of host metabolism for nutritional purposes.

## Nitrogen supply and disease

Many agronomical studies indicate that N fertilizer application affects plant disease (reviewed in Huber and Watson,



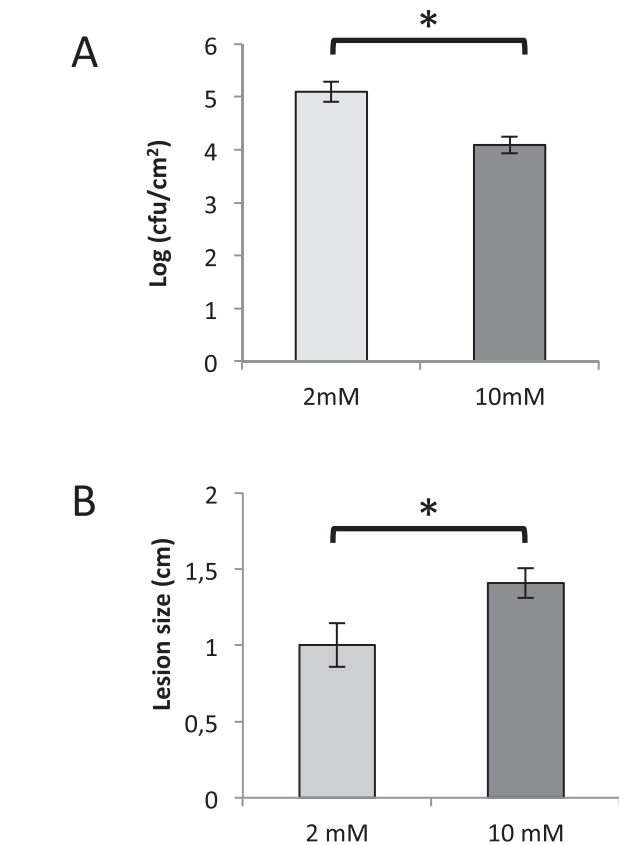
**Fig. 1.** Expression of selected N metabolism genes in response to biotic stress. Values represent the log<sub>2</sub> (ratio) of the fold modulation of *Arabidopsis* genes in response to infection compared with mock treatment. Data were collected from the Genevestigator database (Zimmermann et al., 2004) and correspond to Affymetrix analysis except for the Ea data that we generated by CATMA analysis (Moreau et al., 2012) and are present in the catDB database (Gagnot et al., 2008). Log ratios above 0.8 or below -0.8 are presented using a colour code according to the intensity of the modulation. The data set corresponds to a 24 h post-inoculation (hpi) time point except for Ha (12 hpi), Bc (18 hpi), and Pp (30 hpi). We selected a panel of compatible interactions leading to disease (Go, Ha, Pi, Ps, Bc, Pp) and incompatible interactions leading to resistance (Ps avr, Ps ph, Ea). We also selected representatives of the different pathogen life styles: biotrophic (Bio), hemibiotrophic (Hbio), necrotrophic (Ne), avirulent (Avir), and non-host (Nho) pathogens. Go, *Golovinomyces orontii*; Ha, *Hyaloperonospora arabidopsidis*; Pi, *Phytophthora infestans*; Ps, *Pseudomonas syringae* pv. *tomato* DC3000; Ps avr, *Pseudomonas syringae* pv. *tomato* avrRpm1; Ps, *Pseudomonas syringae* pv. *phaseolicola*; Ea, *Erwinia amylovora*; Pp, *Phytophthora parasitica*; Bc, *Botrytis cinerea*.



1974; Walters and Bingham, 2007; Dordas, 2008). However, these studies led to contradictory conclusions, probably in part because of different requirements for growth and sensitivity to defence metabolites of pathogens. It has also been suggested that obligate and biotrophic pathogens might have a different sensitivity to N fertilizers compared with necrotrophic pathogens. Indeed, N fertilizers generally increase the susceptibility of plants to biotrophs, whereas they generally decrease the susceptibility of plants to necrotrophs (Snoeijsers *et al.*, 2000; Dordas, 2008; Ballini *et al.*, 2013). However, this is clearly not the whole story. For example, the impact of N supply on the susceptibility of plants to the necrotrophic fungus *B. cinerea* depends on the virulence of the strain (Lecompte *et al.*, 2010), which could in part explain the contradictions observed in the literature as to the effect of N fertilization on disease. We analysed the impact of growth in N limitation conditions on the susceptibility of *Arabidopsis* Col-0 plants to the necrotrophic phytopathogen *E. amylovora* and to the necrotrophic fungus *B. cinerea*, two interactions during which cell death is thought to play an important role (Govrin and Levine, 2000). We found that N limitation reduced the resistance of *Arabidopsis* to *E. amylovora* (Fig. 2A; M. Farjad and M. Fagard, unpublished data), which is consistent with our previous results indicating that *Arabidopsis* resistance against *E. amylovora* is an active process (Moreau *et al.*, 2012) and suggesting that this defence process is strongly affected by N supply. On the other hand, N limitation reduced the susceptibility of *Arabidopsis* to *B. cinerea* (Fig. 2B; J. Courtial and M. C. Soulié, unpublished data). Altogether, current knowledge indicates that the impact of N supply on plant disease is complex and thus requires in-depth scientific investigation.

### Effect of N supply on defence

One way in which N supply may affect plant–pathogen interactions is through an impact on plant defence production. Contradictory generalities are found in the literature concerning the impact of nutritional status on plant defence production: on the one hand, it is generally thought that there is a trade-off between growth and defence (Walters and Heil, 2007), but on the other hand, some authors have suggested that low nutrition weakens plants and is thus not favourable for defence (Snoeijsers *et al.*, 2000). The growth–differentiation balance hypothesis (GDBH) and the carbon nutrient balance hypothesis (CNBH) both predict that under limiting growth conditions, available resources would be allocated to higher defence production (Massad *et al.*, 2012). This prediction was partly confirmed in the legume *Pentaclethra macroleoba* for which the authors found a trade-off between growth (biomass) and flavan production (phenolic defence metabolites) (Massad *et al.*, 2012). However, the authors found that saponins (terpenoid defence metabolites) increased with biomass, suggesting that the trade-off between defence and growth may not be a general feature of plant–pathogen interactions as is often believed. The monitoring of defence-associated enzymatic activities such as chitinase, chitosanase, and peroxidase in *Arabidopsis* plants showed that all three basal activities



**Fig. 2.** Effect of N limitation on the susceptibility of *Arabidopsis* to pathogens. *Arabidopsis* Col-0 plants were grown in sand on limiting nitrate (2mM NO<sub>3</sub><sup>-</sup>) or non-limiting nitrate (10mM NO<sub>3</sub><sup>-</sup>). As described previously, the 2mM condition was limiting for plant growth (Lemaître *et al.*, 2008) as the rosette biomass of 5-week-old plants grown at 2mM NO<sub>3</sub><sup>-</sup> was significantly lower than that of plants grown at 10mM NO<sub>3</sub><sup>-</sup> (not shown). Leaves of 5-week-old plants were infected with the bacterial phytopathogen *E. amylovora* (A; strain CFBP1430) or the necrotrophic fungus *B. cinerea* (B; strain B0510) as described previously (Degraeve *et al.*, 2013; Morcx *et al.*, 2013). *E. amylovora* bacterial growth is shown 24 h post-inoculation, and *B. cinerea*-induced lesion size is shown 3 d post-infection. Asterisks indicate a significant statistical difference between the two conditions according to Student's *t*-test (*P*-value < 0.05). The experiments were each repeated at least three times with similar results, and a representative experiment is shown.

were reduced in low N (Dietrich *et al.*, 2004). In addition, the induction level of these enzymes following treatment with BION<sup>®</sup>, a chemical elicitor of plant defence (Dietrich *et al.*, 2004), was reduced in low N. It has been shown recently that the form of N available to plants can also affect plant defence (see 'The role of NO').

Although it is clear that N limitation has an impact on plant defence, it is still difficult to obtain a general picture of this effect (Dietrich *et al.*, 2004). Furthermore, the molecular mechanisms underlying this control of defence by N limitation are globally unknown. One of the few interesting candidates is the RING-type ubiquitin E3 ligase BAH1/NLA, which is involved in regulating both SA accumulation and plant adaptation to N limitation (Yaeno and Iba, 2008). These authors suggest that BAH1/NLA could play a role in the regulation of SA levels under conditions of N starvation.

## Effect of N supply on pathogen virulence

N supply can impact plant–pathogen interactions through an effect on pathogen virulence. The perception of N nutritional status by pathogens can contribute to the signals controlling activation of virulence factors and metabolic adaptation (reviewed by [Snoeijs \*et al.\*, 2000](#)). Several infection processes are activated under N starvation *in vitro*. For instance, the development of the filamentous form of the basidiomycete *Ustilago maydis*, which is a prerequisite for plant infection, is stimulated by N starvation ([Horst \*et al.\*, 2012](#)). Expression of some bacterial *hrp* genes, encoding T3SS and T3Es, is up-regulated in minimal medium *in vitro* and repressed by ammonium or the amino acids asparagine and histidine ([Wei \*et al.\*, 1992](#)). Expression of effector proteins involved in virulence from fungi or oomycetes can be up-regulated *in vitro* under N starvation ([Bolton and Thomma, 2008](#)). The preferred N source ammonium was found to repress the capacity of the fungal species *Fusarium oxysporum*, *Fusarium graminearum*, and *Magnaporthe oryzae* to penetrate cellophane membrane, which is a well-established virulence factor ([Lopez-Berges \*et al.\*, 2010](#)). Soft rot pathogens secrete CWDEs, leading to tissue disorganization, cell death and cell content release. Interestingly, the production of CWDEs of the soft rot bacterium *P. carotovorum* is up-regulated *in vivo* in chicory heads under high N ([Schober and Vermeulen, 1999](#)). Likewise, *D. dadantii* production of pectinases is repressed under low ammonium ([Hugouvieux-Cotte-Pattat \*et al.\*, 1992](#)). The presence of ammonium probably represents favourable nutritional conditions that do not require expression of virulence factors.

How do pathogens sense the level of N and translate this into a fine-tuning of virulence factor expression? In several fungal species the transcription factor AreA/Nit2 belonging to the GATA family of transcription factors is a global N regulator that activates the expression of genes encoding proteins involved in the transport and catabolism of secondary N sources ([Bolton and Thomma, 2008](#)). The pathogenicity of *areA*-deficient mutants from several phytopathogenic fungal species is affected, indicating the requirement for a functional N perception for full pathogenesis ([Bolton and Thomma, 2008](#)). Interestingly, N starvation-induced filamentous growth of *U. maydis* is largely dependent on functional AreA/Nit2 ([Horst \*et al.\*, 2012](#)). In addition, the production of the mycotoxin fumonisin B1 by the maize pathogen *F. verticilloides* is also dependent of the AreA regulator ([Kim and Woloshuk, 2008](#)).

Recently, further investigation of the link between N signalling and virulence factors showed that the TOR kinase, which regulates eukaryotic cell growth in response to nutrient availability and in particular N ([De Virgilio and Loewith, 2006](#); [Rohde \*et al.\*, 2008](#)), and the bZIP MeaB protein, which acts as a negative regulator of the N catabolic response ([Wong \*et al.\*, 2008](#)), are both involved in the repression of virulence functions in *F. oxysporum*. Indeed, *F. oxysporum* repression of cellophane penetration by the favourable N source ammonium requires TOR and MaeB ([Lopez-Berges \*et al.\*, 2010](#)).

In Gram-negative bacteria, the global regulators of N sensing are the sigma 54 factor *rpoN* and the two-component system formed by NtrB–NtrC ([Weiss \*et al.\*, 2002](#)). The NtrB protein is anchored at the plasma membrane and senses environmental stimuli. In *Escherichia coli*, N metabolism regulation is in part controlled by the catabolic repressor protein CRP (cAMP receptor protein; [van Heeswijk \*et al.\*, 2013](#)). Knowing that genes encoding CWDEs in several *Pectobacterium* and *Dickeya* species are positively regulated by CRP ([Reverchon \*et al.\*, 1997](#); [Matsumoto \*et al.\*, 2003](#)), it would be worth investigating the cross-talk between CRP and N-sensing systems in regulation of CWDE-encoding genes. All these reports provide evidence that phytopathogens integrate signals concerning the source and availability of N to fine-tune the expression of their virulence functions. However, the exact underlying signalling mechanisms still remain to be elucidated.

## Nitrate uptake and plant–pathogen interactions

The NRT2 gene family belongs to the major facilitator superfamily (MFS) of transporters. In *Arabidopsis*, the NRT2 family comprises seven genes, among which four have been characterized as high-affinity nitrate transporters. NRT2.1 is the main high-affinity nitrate transporter in *Arabidopsis* roots under low nitrate availability ([Orsel \*et al.\*, 2004](#); [Li \*et al.\*, 2007](#)). Regarding the higher plant NRT2s characterized to date, no substrate other than nitrate has been identified so far. However, several NRT2 proteins from *Chlamydomonas reinhardtii* and *Hansenula polymorpha* are nitrate/nitrite bispecific transporters ([Machin \*et al.\*, 2004](#); [Fernandez and Galvan, 2007](#)). In addition to the nitrate transport function, nitrate transporters have recently been evidenced to be involved in nitrate sensing and act as so-called transceptors (transporter and receptor) ([Ho \*et al.\*, 2009](#); [Gojon \*et al.\*, 2011](#)). Of all the NRT2 family members, to date only AtNRT2.1 has been shown to modify lateral root development independent of nitrate transport and thus to act as a transceptor ([Little \*et al.\*, 2005](#)). Recently two NRT2 genes have been identified as players in plant defence responses: the *Arabidopsis* mutants *nrt2.1* and *nrt2.6* are modified in the responses to *P. syringae* and to *E. amylovora*, respectively ([Camanes \*et al.\*, 2012b](#); [Dechorgnat \*et al.\*, 2012](#)).

NRT2.1 represses responses to biotrophic pathogens, probably favouring abiotic stress resistance by safeguarding energy ([Camanes \*et al.\*, 2012a, b](#)). The reduced *P. syringae* susceptibility of the *nrt2.1* mutant was attributed to a faster response of the SA-dependent signalling pathway and a reduced sensitivity to the bacterial toxin coronatin. The ABA and JA signalling pathways were also modified, but probably as a result of SA priming. In an SA-deficient background the *nrt2.1* mutant was as susceptible as the wild type. In addition, the metabolic status of the *nrt2.1* mutant prior to infection might contribute to the reduced susceptibility. Indeed, the mutant accumulated higher levels of aromatic amino acids and phenylpropanoids ([Camanes \*et al.\*, 2012a](#)). Thus, priming, not

only by SA, but also by other secondary metabolites, seems to contribute to the reduced susceptibility of *nrt2.1* mutants to *P. syringae*. Transcriptomic analysis supported these results, as genes involved in SA and aromatic acid synthesis were differentially expressed between mutant and wild-type plants. In addition several ribosomal proteins are overexpressed in the mutant, which might be another factor contributing to resistance to bacterial infection (Camanes *et al.*, 2012a). No evidence for the mechanism of the altered coronatin sensitivity has been found yet, but a bacterial strain deficient for coronatin triggered clearly similar responses in wild-type and *nrt2.1* mutants (Camanes *et al.*, 2012a, b).

NRT2.6 has been shown to participate to the response to the necrotrophic bacterium *E. amylovora*. The *Arabidopsis nrt2.6* mutant was more sensitive to *E. amylovora* than wild-type plants. The hypersensitivity of the mutant was correlated with a reduced ROS accumulation, whereas no difference in the transcriptional response to the pathogen nor in other cellular responses such as callose synthesis and NO production has been evidenced (Dechorgnat *et al.*, 2012). The link between NRT2.6 and ROS production is not restricted to pathogen attack, but was also revealed after treatment with the redox-active methyl viologen herbicide. However, to date, no evidence for a function of NRT2.6 in nitrate transport has been obtained, and the molecular link between NRT2.6 and ROS production needs further investigation.

Altogether, these data indicate that nitrate sensing or nitrate transport has an important impact on defence reactions and suggest that the SA defence pathway, ROS production, and metabolite status play important roles in the cross-talk between N availability and biotic stress.

## The role of NO

NO is a highly reactive gas with high diffusion rates across membranes. Several molecules can derive from NO and are collectively called reactive nitrogen species (RNS) that comprise the radical NO $\cdot$ , its nitrosonium (NO $^+$ ), and ni-troxyl ions (NO $^-$ ). When NO is produced in conjunction with ROS, such as during plant–pathogen interactions, NO can react with the superoxide anion O $_2^-$  to generate peroxynitrite (ONOO $^-$ ). In animals, the generation of NO under infectious conditions is mainly due to an inducible nitric oxide synthase (iNOS), which catalyses the NADPH-dependent oxidation of L-arginine to L-citrulline and NO (Stuehr *et al.*, 2004). In plants several data suggest the existence of such an enzymatic activity, but we still do not know the enzyme involved in this process (Besson-Bard *et al.*, 2008; Bellin *et al.*, 2013). Production of NO in plants has been suggested to depend on several routes that can be divided into oxidative and reductive routes. Oxidative routes include the enzymatic activities polyamine and hydroxylamine oxidation (Tun *et al.*, 2006; Ruemer *et al.*, 2009). Accordingly, a copper amine oxidase was proposed to be involved in NO production *in planta* in response to ABA (Wimalasekera *et al.*, 2011). Reductive routes consist of nitrite reduction via mitochondrial electron transfer systems (Modolo *et al.*, 2005; Planchet *et al.*, 2005;

Gupta and Igamberdiev, 2011), a root-specific nitrite:NO-reductase (Ni-NOR), the peroxisomal xanthine oxidoreductase enzyme (Stohr *et al.*, 2001), and nitrate reductase (NR) (Rockel *et al.*, 2002; Moche *et al.*, 2010). NO is involved in several physiological processes in plants, including germination, development, stomatal closure, and immunity where it was shown to be involved in the hypersensitive response (HR) and during compatible interactions (Delledonne *et al.*, 1998, 2001; Durner *et al.*, 1998). The role of NO in plant–pathogen interactions has been reviewed recently (Bellin *et al.*, 2013). Here, we will only focus on aspects that link N nutrition and metabolism to NO production in plant–pathogen interactions.

In the context of plant–pathogen interactions, it seems that NR is an important source of NO. Modolo *et al.* (2005) were the first to show that NR activity is the major source of NO during the pathogenic interaction *Arabidopsis*–*P. syringae*. Other reports showed that NR participates in NO accumulation in plant–pathogen interactions (Asai and Yoshioka, 2009; Perchepped *et al.*, 2010) or in response to elicitors (Yamamoto-Katou *et al.*, 2006). However, decreased HR in *Arabidopsis* plants treated with *P. syringae* pv. *maculicola* in NR-deficient plants was correlated to a lack of L-arginine and NO $_2$ , two important endogenous substrates for NO synthesis (Modolo *et al.*, 2006). Conversely, it was later shown that the increased susceptibility to *P. syringae* of the NR-deficient plants was independent of amino acid accumulation and was more likely to be due to a reduced ability of these mutants to synthesize NO (Oliveira *et al.*, 2009). Interestingly, the activity of NIA2-encoded *Arabidopsis* NR enzyme was shown to be up-regulated through phosphorylation by the MAP kinase MPK6 (Wang *et al.*, 2010), involved in biotic stress responses (Pitzschke *et al.*, 2009); however, the role of this regulation during plant–pathogen interactions remains to be investigated.

Interestingly the nutrition of the plant can have an effect on NO production. Tobacco grown with nitrate was found to produce more NO than tobacco grown on ammonium when plants were inoculated with the pathogenic bacterium *P. syringae* pv. *tabaci* or the incompatible bacterium *P. syringae* pv. *phaseolicola* (Gupta *et al.*, 2013). The authors showed that NO accumulation was associated with increased resistance to the pathogens. Conversely, in soybean cotyledons, no difference was observed in NO production whether the plants were grown with nitrate or ammonium (Galatro *et al.*, 2013). Pathogens can contribute to the scavenging of NO. For instance, the flavohaemoglobin HmpX from the pathogenic bacterium *D. dadantii* was shown to contribute to the reduction of NO during HR (Boccaro *et al.*, 2005). Thus NO is a pivotal element in plant–pathogen interactions, and its production and turnover are strongly linked to N metabolism.

## Impact of amino acid metabolism and recycling on plant–pathogen interactions

Presumptions that amino acid metabolism can impact plant–pathogen interactions had been raised by the observations



that amino acid contents and relative concentrations are modified during plant disease or in response to pathogen attack. When peach tree leaves are infected by the Eastern X-disease pathogen, they accumulate proline (McKee, 1972). Kumar and Prasad (1992) observed that the whole amino acid pool increases in crucifers when infected by compatible or incompatible *Xanthomonas campestris* pv. *campestris*. Aubergines (eggplants) suffering little leaf disease, caused by *Candidatus* *Phytoplasma asteris*, accumulate specifically lysine and asparagine, whereas histidine and arginine are depleted (Das and Mitra, 1993). Reports of changes in amino acid composition during plant–pathogen interaction are numerous, and the more recent metabolomic technologies also confirm that amino acid metabolism is greatly modified during plant–pathogen interactions. However, none of these observations provided insight into the role of such amino acid modifications in the outcome of the interaction.

Relationships between amino acid accumulation and pathogen diet preferences suggested, however, that the co-evolutionary conflict between plant and pathogen has led, in compatible interactions, to pathogen adaptation to the host nutritional resources. As such it is interesting to note that GABA, which is the favourite N source of *Cladosporium fulvum*, accumulates in tomato leaves specifically during compatible interactions (Solomon and Oliver, 2001, 2002). Similarly, glutamine, which is preferentially used among all amino acids by *Colletotrichum lindemuthianum*, accumulates to high levels in French bean leaves during compatible interactions (leading to disease), but not in leaves that are infected by avirulent strains, which trigger resistance (Tavernier *et al.*, 2007). Nutritional specialization of the *P. syringae* pv. *tomato* for GABA, aspartate, glutamate, and glutamine also suggests in this case a trophic adaptation of this pathogen to its host N resources. Paradoxically, in the plant–pathogen interactions reported above, it can be observed that the amino acids preferentially used by pathogens are often those that accumulate specifically during disease. As it cannot be assumed that plants lay the table for the meal of their pathogens, it can be considered that pathogens have adapted to use amino acids that accumulate in their host under stress conditions. However, the possibility that pathogens manipulate plant metabolism to pump amino acids should also be considered.

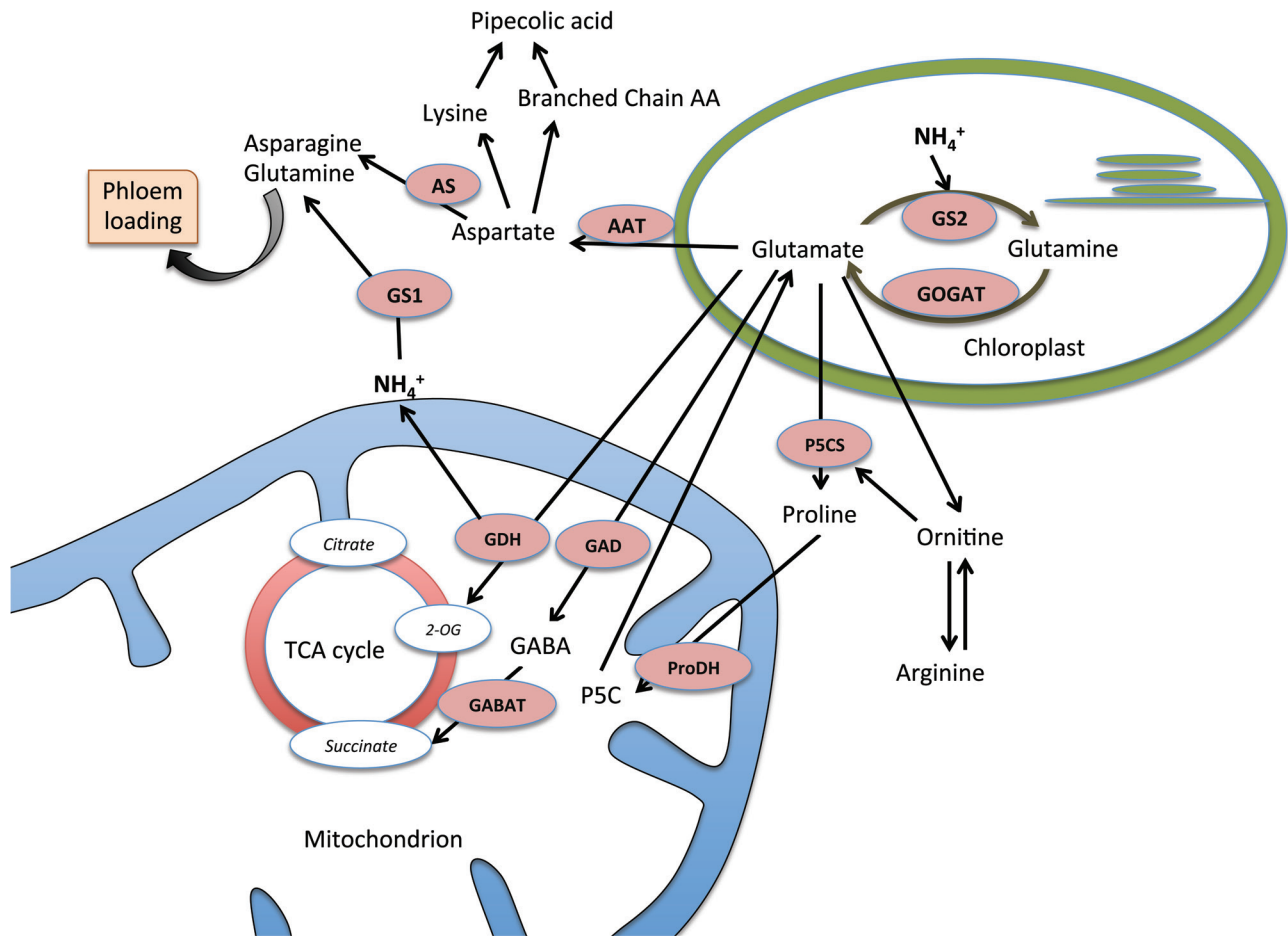
It has been known for a long time that amino acids such as GABA and proline have a role in plant tolerance to abiotic stresses (Snedden and Fromm, 1999; Sharma and Dietz, 2006). GABA and proline could protect cells against oxidative stress and osmotic stress, and regulate cytosolic pH (Bouche and Fromm, 2004; Szabados and Savoure, 2010). Together with arginine, they are also N storage amino acids known to accumulate during stress and to give back N and energy during recovery periods. GABA, proline, and arginine are direct products from glutamate metabolism (Fig. 3). The GABA shunt is a way to control the C:N status and to replenish the tricarboxylic acid (TCA) cycle with carbon to sustain mitochondrial respiration (Bolton, 2009; Michaeli *et al.*, 2011). Proline is known to accumulate to very high levels under drought conditions (Verslues and Juenger, 2011); however, its role in drought resistance is unclear, and, as for

GABA, it seems that the proline pool accumulated during the stress period is useful during the recovery time to provide new glutamate pools and energy (Szabados and Savoure, 2010).

Interestingly, GABA, proline, and arginine, as well as glutamine and asparagine are known in several plants to be involved in N recycling, remobilization, and translocation pathways. Their role during natural leaf senescence and sink/source N remobilization has been reported in several reports (for a review, see Masclaux-Daubresse *et al.*, 2010). We know that during leaf senescence chloroplast dismantling releases a large pool of amino acids from the degradation of stromal and photosynthetic apparatus proteins. Amino acids that are not directly loaded to the phloem sap for translocation are used to support mitochondrial respiration through the catabolism of GABA (GABA shunt) and glutamate (through glutamate dehydrogenase, GDH) in the mitochondria and to form asparagine and glutamine as the result of the condensation of ammonium on aspartate and glutamate molecules (Fig. 3). The synthesized glutamine and asparagine are then uploaded to the phloem sap. Induced expression of several of the senescence-associated N remobilization enzymes was observed during plant–pathogen interactions (Buchanan-Wollaston, 1997; Pérez-García *et al.*, 1998a, b; AbuQamar *et al.*, 2006; Pageau *et al.*, 2006; Tavernier *et al.*, 2007). In response to pathogen attack and to a large variety of stresses such as drought, salt stress, or heavy metal, and more globally in response to oxidative stress, plants induce N remobilization processes in order to translocate and safeguard nutrients in their non-infected tissues (Chaffei *et al.*, 2004; Olea *et al.*, 2004). It is thus possible that pathogens adapted to their host then take advantage of recycling metabolism for their own benefit.

Conversely, some evidence suggests that induction of N remobilization genes in response to plant pathogens is modulated in parallel to defence genes. Tavernier *et al.* (2007) showed that the expression of the GS1-encoding gene (*GS1-a*) paralleled the expression of the *PAL3* and *CHS* defence genes, suggesting a role for GS1 in plant defence. Similarly, the pepper asparagine synthetase 1 (*CaASI*) gene showed exactly the same expression pattern as the defence marker gene *CaBPRI* during compatible and incompatible interactions with *X. campestris* pv. *vesicatoria* strains (Hwang *et al.*, 2011). The pepper *CaAlaAT1* alanine aminotransferase gene was also induced in senescing leaves, in response to SA and ethylene but not JA, and was enhanced in the incompatible interaction with *Tobacco mosaic virus* (TMV-Po). In response to three different *P. syringae* strains, the tobacco *GS1* gene was also only found to be induced during incompatible interactions. Therefore it is likely that amino acid metabolism and plant–pathogen interactions have a more complex connection than simply a trophic relationship, and it is possible that N enzymes participate in plant defence signalling.

The role of amino acid metabolism in the control of plant–pathogen interactions is highlighted by recent studies that used mutants affected in amino acid metabolism enzymes or transporters. Hwang *et al.* (2011) showed that disease symptoms were higher in *CaASI*-silenced pepper leaves infected by *X. campestris* pv. *vesicatoria* than in controls

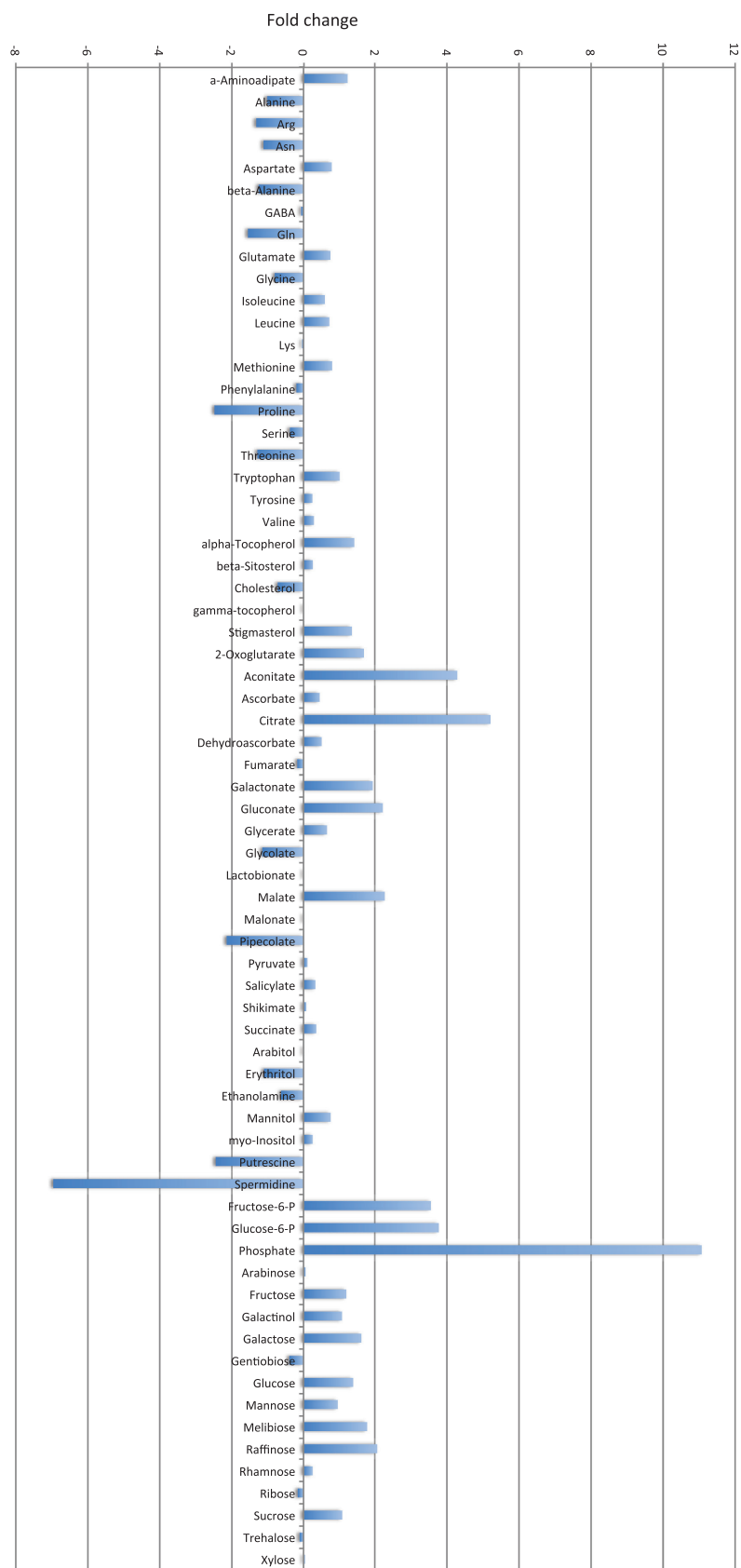


**Fig. 3.** Schematic representation of amino acid metabolism in plants. The major routes of amino acid metabolism and recycling are presented. Asparagine synthetase (AS), aspartate amino transferase (AAT), cytosolic glutamine synthetase (GS1), glutamate dehydrogenase (GDH), glutamate decarboxylase (GAD), GABA transaminase (GABAT), proline dehydrogenase (ProDH), 1-pyrroline-5-carboxylate synthetase (P5CS), chloroplastic glutamine synthetase (GS2), and glutamate synthase (GOGAT) are represented in pink ovals. In the tricarboxylic acid cycle (TCA),  $\alpha$ -ketoglutarate is represented as 2-OG. Adapted from Avila-Ospina L, Moison M, Yoshimoto K, Masclaux-Daubresse C. 2014. Autophagy, plant senescence, and nutrient recycling. *Journal of Experimental Botany* **65**, 3799–3812. With permission from the Society for Experimental Biology.

and that *Arabidopsis* plants overexpressing *CaAS1* exhibited enhanced resistance to *P. syringae* pv. *tomato* DC3000 and *Hyaloperonospora arabidopsidis*. Consistently, asparagine levels were associated with early defence responses such as electrolyte leakage and ROS accumulation. It remains unclear, however, how the conversion of aspartate to asparagine can modulate plant defence. Recently Seifi *et al.* (2014) showed that asparagine synthetase plays a role in the immune response of tomato to *B. cinerea* infection and that asparagine might also promote *B. cinerea* pathogenesis. Further evidence comes from the infection by *B. cinerea* of *Arabidopsis* plants overexpressing aspartate aminotransferase (AAT). AAT catalyses transamination between glutamate and oxaloacetate to produce aspartate and  $\alpha$ -ketoglutarate. The overexpression of the *Asp2* gene encoding AAT and known to be induced in *Arabidopsis* by several biotic stresses led to larger lesions spreading after *B. cinerea* infection and to changes in amino acid composition (Brauc *et al.*, 2011). More recently, the alterations in the GABA shunt, GS/GOGAT cycle, and phenylpropanoid pathways observed at transcriptional, enzymatic, and metabolic levels in the ABA-deficient *sitiens* mutant of tomato (*Solanum lycopersicum*) suggested

that glutamine and GABA could be important for the resistance of tomato to *B. cinerea* and for the rapid epidermal HR and phenylpropanoid pathway-derived cell wall fortification. Microarrays and further studies had revealed that in addition to genes involved in plant defence (*PRI*) and cell wall biogenesis, several genes, including AAT, glutamate decarboxylase, GABA transaminase (*GABAT*), and *GS1* were significantly up-regulated in the resistant tomato cultivars a few hours post-inoculation (Asselbergh *et al.*, 2007). As *GABAT* and *GS1* genes showed higher expression levels in the *sitiens* mutants, gene silencing was performed on these genes using virus-induced gene silencing (VIGS) technology on both wild-type and *sitiens* genotypes. While no effect of *GABAT* and *GS1* silencing can be observed at the symptom level on the wild type after *B. cinerea* infection, both the *GABAT*- and *GS1*-silenced *sitiens* mutants exhibited significantly higher susceptibility phenotypes compared with the *sitiens* controls transformed with empty vectors (Seifi *et al.*, 2013a). The authors thus proposed a model in which the overactivation of nutrient recycling through GS1 and the replenishment of the TCA cycle through the GABA shunt maintain cell viability in the areas surrounding invaded tissue. Cell survival





**Fig. 4.** Metabolomic analysis of *Arabidopsis* plants grown in limiting or non-limiting N conditions. Metabolite profiling of *Arabidopsis* plants grown in limiting or non-limiting nitrate (Lemaitre et al., 2008) was analysed by GC-MS. The results are shown as the ratio of accumulation of each metabolite between the two conditions ( $n=3$  plant repeats; only significant differences according to Student's  $t$ -test,  $P<0.05$  are shown). Positive values correspond to metabolites that accumulate more in high nitrate conditions (10 mM NO<sub>3</sub><sup>-</sup>), while negative values correspond to values that accumulate more in low nitrate conditions (2 mM NO<sub>3</sub><sup>-</sup>).

in these areas then allows efficient defence-associated HR, cell wall fortification, and the down-regulation of the pathogen-induced senescence and associated deleterious effects. Maintaining mitochondrial respiration and nutrient recycling that participate in cell viability would in that case result in the resistance to a necrotroph such as *B. cinerea*. The importance of GS activity in resistance is further supported by the fact that both bacterial and fungal toxins are known to repress GS activity directly (reviewed in Seifi *et al.*, 2013b).

In their report, Liu *et al.* (2010) show that knocking out a single amino acid transporter is sufficient to confer resistance of *Arabidopsis* against a large spectrum of pathogens. *LHT1* (Lysine Histidine Transporter 1) disruption in *Arabidopsis* enhanced disease resistance to *P. syringae* pv. *tomato* DC3000, *Colletotrichum higginsianum*, and *Erysiphe cichoracearum*. Disruption of *LHT1* decreased the concentrations of almost all amino acids, with a stronger effect on glutamine, alanine, and proline concentrations in leaves. This metabolic effect is consistent with the previous finding that *LHT1* is mainly involved in alanine and glutamine uptake (Svennerstam *et al.*, 2007). Using both exogenous glutamine administration onto leaves and glutamine synthetase inhibitor treatments, Liu *et al.* (2010) correlated glutamine deficiency or replenishment with the level of activation of defence responses. Interestingly, the *lht1* mutation-conferred phenotypes were SA dependent and associated with an altered redox status. The authors thus suggested that N metabolism, and more precisely glutamine metabolism, could modulate plant defence responses moderating cellular redox status as well as secondary metabolite pathways.

As shown in this review, the links between N assimilation, amino acid metabolism, and plant defence molecules are numerous. As glutamate is the first amino acid to be synthesized from inorganic N assimilation, all other amino acids are obtained from it, as well as the many molecules involved in plant defence that are dependent on amino acid metabolism. It is well known, for example, that phytoalexin, anthocyanin, and SA pathways are derived from the aromatic amino acid phenylalanine. Antioxidants such as glutathione derive from cysteine, vitamin E, also called  $\alpha$ -tocopherol, is derived from tyrosine, and ethylene is derived from methionine. Recently, Navarova *et al.* (2012) reported that the non-protein piperolic amino acid, which is a lysine catabolite, is an endogenous mediator of defence that plays a role in plant immunity. They showed that piperolate accumulates after plant infection in inoculated leaves, in the distal leaves, and in the petiole exudates. Defects in defence priming and systemic acquired resistance were observed in piperolate-deficient *Arabidopsis* mutants, as well as depletion of SA and camalexin molecules.

All these studies thus provide good evidence that amino acid anabolic and catabolic pathways are important for plant immunity.

## Conclusion

The question that remains to be fully explored is the generic character of the observations reported above. One way to

answer the question is certainly to combine transcriptomic and metabolic studies on a large set of pathosystems.

Another way is to determine how N availability can modulate plant response. We already know from previous studies that amino acid content is different in leaves of plants grown under N-rich and N-limiting conditions (Lemaître *et al.*, 2008). In a recent study, we have also found that other molecules vary between the two conditions (Fig. 4; C. Masclaux-Daubresse and G. Clément unpublished). Determining whether metabolite changes influence plant resistance or susceptibility and if pathogen aggressiveness is better if some molecules are present or absent in the plant organs it infects are other ways to investigate this difficult question. In addition, both systems biology and natural variation studies should provide new evidence to highlight the role of plant metabolites in the feature of plant–pathogen interactions as well as the impact of plant nutrition on plant defence and pathogen virulence.

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B. The bile acid deoxycholate elicits defences in Arabidopsis and reduces bacterial infection, Zarattini *et al* (2016)

# The bile acid deoxycholate elicits defences in Arabidopsis and reduces bacterial infection

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## SUMMARY

Disease has an effect on crop yields, causing significant losses. As the worldwide demand for agricultural products increases, there is a need to pursue the development of new methods to protect crops from disease. One mechanism of plant protection is through the activation of the plant immune system. By exogenous application, 'plant activator molecules' with elicitor properties can be used to activate the plant immune system. These defence-inducing molecules represent a powerful and often environmentally friendly tool to fight pathogens. We show that the secondary bile acid deoxycholic acid (DCA) induces defence in Arabidopsis and reduces the proliferation of two bacterial phytopathogens: *Erwinia amylovora* and *Pseudomonas syringae* pv. *tomato*. We describe the global defence response triggered by this new plant activator in Arabidopsis at the transcriptional level. Several induced genes were selected for further analysis by quantitative reverse transcription-polymerase chain reaction. We describe the kinetics of their induction and show that abiotic stress, such as moderate drought or nitrogen limitation, does not impede DCA induction of defence. Finally, we investigate the role in the activation of defence by this bile acid of the salicylic acid biosynthesis gene *SID2*, of the receptor-like kinase family genes *WAK1–3* and of the NADPH oxidase-encoding *RbohD* gene. Altogether, we show that DCA constitutes a promising molecule for plant protection which can induce complementary lines of defence, such as callose deposition, reactive oxygen species accumulation and the jasmonic acid and salicylic acid signalling pathways.

**Keywords:** bile acid, elicitor, *Erwinia amylovora*, plant defence, *Pseudomonas syringae*, *SID2*, *WAK*.

## INTRODUCTION

Plants have developed sophisticated protection mechanisms against the biotic and abiotic stresses to which they are exposed. The plant immune system consists of two closely interconnected defensive levels (Tsuda and Katagiri, 2010). The first line of defence is induced by the recognition of conserved molecular structures of pathogens, such as lipopolysaccharide (LPS), flagellin and chitin, which are collectively called microbe (or pathogen)-associated molecular patterns (MAMPs or PAMPs). This first line of defence is defined as PAMP-triggered immunity (PTI) and confers a wide-spectrum protection against phytopathogens. PTI is triggered on perception of MAMPs by plasma membrane pattern recognition receptors (PRRs) (Zipfel, 2008).

The second defensive level, defined as effector-triggered immunity (ETI), is specifically induced by pathogens that have evolved strategies to overcome PTI using protein effectors that function inside the plant cell. The recognition of such molecules, mediated by specific R proteins, is initiated by a more rapid and stronger type of response than PTI (Jones and Dangl, 2006). PTI and ETI are associated with common downstream responses (Tsuda *et al.*, 2009). In particular, transcriptional reprogramming, an early oxidative burst at the site of infection and changes in the concentration of hormones, such as salicylic acid (SA) and jasmonic acid (JA), have been observed. Other phytohormones, such as abscisic acid (ABA), cytokinins and ethylene (ET), are involved in the control of these signalling pathways (Pieterse *et al.*, 2012). Furthermore, another important defensive strategy related to ETI is the hypersensitive response (HR) (Jones and Dangl, 2006). The HR consists of the persistent production of several signal compounds, such as reactive oxygen species (ROS), nitric oxide (NO) and SA, coupled to Ca<sup>2+</sup> cytoplasmic influxes, leading to a programmed cell death (PCD) at the site of infection (Mur *et al.*, 2008). To date, ETI has been found to be effective almost exclusively against biotrophic and hemibiotrophic pathogens, but not against necrotrophic pathogens (Mengiste, 2012).

Pests and pathogens affect the potential yield of crops worldwide, in terms of both quantity and quality. It has been estimated

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that, in spite of current crop protection methods, pests and pathogens reduce crop production by 20%–40% (Oerke and Dehne, 2004; Savary *et al.*, 2012). Furthermore, the effect of disease on crop yield is heterogeneous in time and space as a result of environmental factors, such as edaphic and climatic factors. Because crop yields are significantly affected by disease and because the worldwide demand for agricultural products is increasing, there is a need to pursue the development of new methods to protect crops from disease, as well as to understand the underlying mechanisms. In parallel with classical and molecular breeding strategies to reduce crop losses caused by pathogen attacks, novel strategies are currently being developed to increase host recognition of the pathogen, interfering with the virulence arsenal and improving plant immunity (Gust *et al.*, 2010). One method of plant protection against disease is through the activation of its immune system. Indeed, the plant immune system can be activated by the exogenous application of molecules with elicitor properties (Noutoshi *et al.*, 2012c; Schreiber and Desveaux, 2008; Zarattini *et al.*, 2015). These defence-inducing molecules include pathogen-derived molecules, such as glucan, *N*-deacetylated chitin (chitosan) and pathogen effectors (Gust *et al.*, 2010), as well as other natural substances not derived from the pathogen, such as plant hormones. These molecules are collectively defined as 'plant activators' and represent a powerful and often environmentally friendly tool to fight pathogens (Noutoshi *et al.*, 2012a,b, c). Moreover, in contrast with antimicrobial compounds, such as fungicides or antibiotics, which limit microbe propagation, plant activators act on plant resistance, thus avoiding the occurrence of pathogen resistance (Noutoshi *et al.*, 2012c; Zarattini *et al.*, 2015). In this context, bile acids have recently attracted increasing attention in plant science for their capacity to elicit plant defences (Koga *et al.*, 2006). Bile acids are part of the steroid class of lipids that play key roles in animals. For example, it is well established that bile acids can dissolve cholesterol and other lipids (Rotunda *et al.*, 2004), enhance lipid absorption into the small intestine (Hofmann, 1999) and play a role in signalling to control certain aspects of metabolism (Li and Chiang, 2011).

It has been described by Koga *et al.* (2006) that bile acids, if applied directly on rice plants, can protect rice against the fungus *Magnaporthe grisea*. These authors showed that increased protection against the pathogen in rice plants was associated with the accumulation of an antimicrobial phytoalexin molecule, phytocassane, increased glucanase activity and electrolyte leakage. In soybean, bile acids trigger superoxide ( $O_2^{\cdot-}$ ) and hydroxyl radical ( $\cdot OH$ ) accumulation and increase the content of the antioxidant glutathione (GSH) and superoxide dismutase (SOD) enzymes (Keresan *et al.*, 2009; Malencic *et al.*, 2012), suggesting that bile acids trigger an HR in plant cells. These reports show that bile acids can elicit plant defences; however, the knowledge of their elicitor properties in plants is still limited.

Bile acids are divided into two categories based on their precursor. Primary bile acids are formed directly from cholesterol, whereas secondary bile acids are derived from primary bile acids (Okoli *et al.*, 2007). Deoxycholic acid (DCA) is a secondary bile acid that is preventatively removed during the industrial production of ursodeoxycholic acid (UDCA). Thus, DCA is a resulting industrial waste product (roughly 20% of bovine bile). The possibility to use an industrial waste product as a new tool to protect plants against pathogens, thanks to its elicitor properties, is appealing. Therefore, we decided to use *Arabidopsis* as a model plant to characterize the elicitor properties of DCA.

In this article, we show that DCA triggers defence activation in *Arabidopsis* and reduces the proliferation of two bacterial phytopathogens. We describe the global defence response triggered by DCA in *Arabidopsis* at the transcriptional level. Several induced genes were selected for further analysis by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and we describe the kinetics of their induction. Finally, we demonstrate that, in response to bile acid treatment, defence activation is affected in the SA-deficient *sid2-1* mutant and NahG transgenic line but not in the *wak1-1*, *wak2-1* and *wak3-1* mutants. We also show that ROS production in response to DCA is reduced in NADPH-defective *rbohD* mutant seedlings compared to wild-type seedlings.

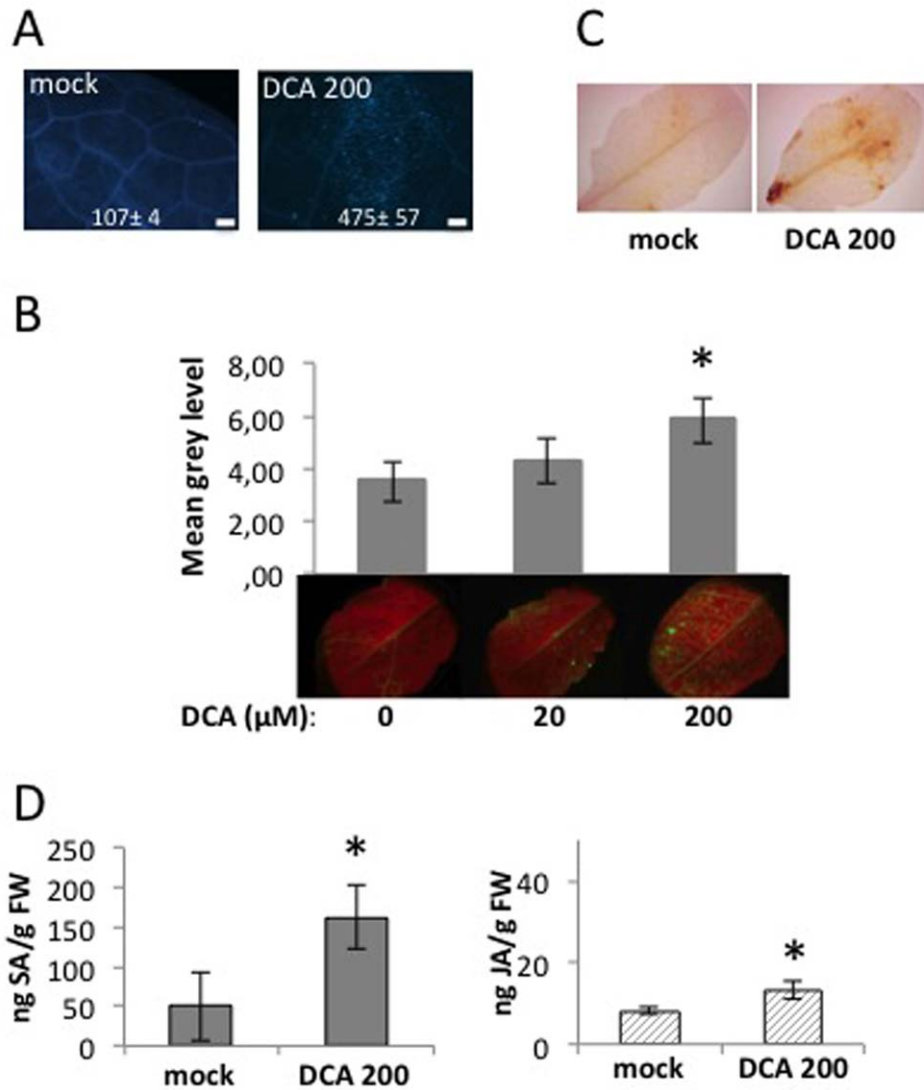
## RESULTS

### DCA elicits defences in *Arabidopsis*

To evaluate the potential of DCA to prevent disease in plants, we first analysed the defence reactions in plants sprayed with an aqueous solution of DCA. We chose two concentrations of DCA: 20  $\mu M$ , which is the concentration of cholic acid (CA) that is able to protect rice against *Magnaporthe grisea*, and 200  $\mu M$ , which is the lowest concentration of CA that induces the maximum defence response (Koga *et al.*, 2006).

The first mechanical barrier encountered by pathogens during plant invasion is the cell wall. To evaluate whether DCA treatment is able to reinforce the cell wall, we investigated the formation of callose deposits through aniline blue staining of DCA-treated leaves. Five-week-old *Arabidopsis* Col-0 plants were sprayed with 20  $\mu M$  DCA, 200  $\mu M$  DCA or mock solution, and then stained with aniline blue at 24 h post-treatment (hpt). In response to treatment with 200  $\mu M$  DCA, a visible amount of callose deposits could be observed in treated leaves compared with mock-treated leaves (Fig. 1A). The counting of callose spots using ImageJ software indicated that treatment with 200  $\mu M$  DCA induced significantly ( $P < 0.05$ ) more callose deposits than in mock-treated leaves (Fig. 1A). However, in response to treatment with 20  $\mu M$  DCA, no visible callose deposits were observed in leaves (Fig. 1A), except in the hydathodes (Fig. S1, see Supporting Information).





**Fig. 1** Deoxycholic acid (DCA) induces defence accumulation in Arabidopsis leaves. Arabidopsis leaves of 5-week-old plants were sprayed with DCA at the indicated concentration (20 or 200  $\mu\text{M}$ ) or mock-treated. (A) Leaves were stained with aniline blue at 24 h post-treatment (hpt) to detect callose deposition. The bar represents 200  $\mu\text{m}$ . The numbers in each photograph indicate the mean number of spots per photograph  $\pm$  standard error of the mean (SEM). (B) Leaves were sampled at 16 hpt and inoculated with 2',7'-dichlorofluorescein diacetate (DCFH-DA). The fluorescence level of DCFH-DA, which reveals the presence of  $\text{H}_2\text{O}_2$ , was measured using ImageJ ( $n = 9$ ). The bars represent the mean  $\pm$  SEM. (C) Leaves were collected at 6 hpt and infiltrated with 3,3'-diaminobenzidine (DAB). The brown colour reveals the presence of  $\text{H}_2\text{O}_2$ . (A–C) Nine leaves ( $n = 9$ ) from three plants were used for each condition. Three independent experiments were performed that gave similar results. Representative photographs are shown. (D) Quantification of salicylic acid (SA) and jasmonic acid (JA) contents using high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS). For each independent experiment, 20 leaves from four plants were collected ( $n = 4$ ) at 24 hpt and ground for JA and SA extraction. The bars represent the standard deviation (SD). For SA quantification, two independent experiments were performed that gave similar and statistically significant results. For JA quantification, the results of three independent experiments were pooled. FW, fresh weight. (B, D) The asterisk indicates a significant difference from mock-treated leaves according to the Mann–Whitney test ( $P < 0.05$ ).

One of the earliest cellular responses implemented by plants after pathogen recognition is the production of ROS, which is referred to as the oxidative burst. To estimate the effect of DCA treatment on ROS levels in Arabidopsis leaves, we first performed a specific histochemical assay using 2',7'-dichlorofluorescein diacetate (DCFH-DA) which fluoresces in the presence of intracellular

hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). DCFH-DA staining was carried out at 16 hpt. The leaves with the lowest concentration of DCA (20  $\mu\text{M}$ ) showed only a weak increase in DCFH-DA fluorescence compared with mock-treated leaves, indicating that 20  $\mu\text{M}$  DCA did not trigger a strong intracellular production of ROS. However, leaves treated with the highest DCA concentration tested (200  $\mu\text{M}$ )

showed stronger DCFH-DA staining than mock or 20  $\mu\text{M}$  DCA-treated leaves, indicating that 200  $\mu\text{M}$  DCA induced noticeable ROS production (Fig. 1B). Quantification of DCFH-DA fluorescence using ImageJ software confirmed that only 200  $\mu\text{M}$  DCA-treated leaves showed a significantly ( $P < 0.05$ ) stronger fluorescence than mock-treated leaves, indicating that 200  $\mu\text{M}$  DCA induced significant intracellular ROS production (Fig. 1B). These results indicate that, in Arabidopsis plants, DCA triggers significant ROS accumulation. As DCFH-DA has been shown to detect reactive nitrogen species, as well as ROS (Crow, 1997; Gomes *et al.*, 2005), we completed our analysis with a second histochemical assay using 3,3'-diaminobenzidine (DAB) which stains both intra- and extracellular  $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  production was examined at 6 hpt by DAB staining. Strong brown precipitates were observed in leaves treated with 200  $\mu\text{M}$  DCA, whereas, in mock- (Fig. 1C) and 20  $\mu\text{M}$  DCA-treated leaves (data not shown), only a light brown staining could be observed. These results confirm that DCA induces the production of  $\text{H}_2\text{O}_2$  in Arabidopsis leaves.

Many studies have shown the importance of the SA- and JA-mediated defence responses in plant-pathogen interactions (Okada *et al.*, 2015; Pieterse *et al.*, 2012). To determine whether leaf endogenous phytohormone content is altered by DCA treatment, JA and SA levels were quantified 24 h after 200  $\mu\text{M}$  DCA or mock application. As shown in Fig. 1D, a three-fold increase in SA concentration was observed in Arabidopsis plants treated with DCA compared with mock-treated plants. Moreover, a slight, but significant ( $P < 0.05$ ), increase in JA accumulation was observed following treatment with 200  $\mu\text{M}$  DCA (Fig. 1D).

As CA has been found to trigger necrosis in rice (Koga *et al.*, 2006), we determined whether DCA induced a similar reaction in Arabidopsis. To do so, we analysed necrosis induced by DCA treatment. Arabidopsis plants were treated with increasing DCA concentrations (0, 20, 100 and 200  $\mu\text{M}$ ). Necrosis was then estimated visually 24 h and 5 days after DCA treatment, assessing the symptom using the severity scale reported in Degraeve *et al.* (2008). After 24 h, no necrosis was observed in any of the treated leaves. After 5 days, no visible necrosis could be observed in water- or 20  $\mu\text{M}$  DCA-sprayed leaves; however, we observed slight necrosis symptoms in leaves sprayed with 100  $\mu\text{M}$  DCA and more extensive necrosis with 200  $\mu\text{M}$  DCA (Fig. S2, see Supporting Information).

Altogether, our results show that DCA is able to induce several lines of defence in Arabidopsis leaves.

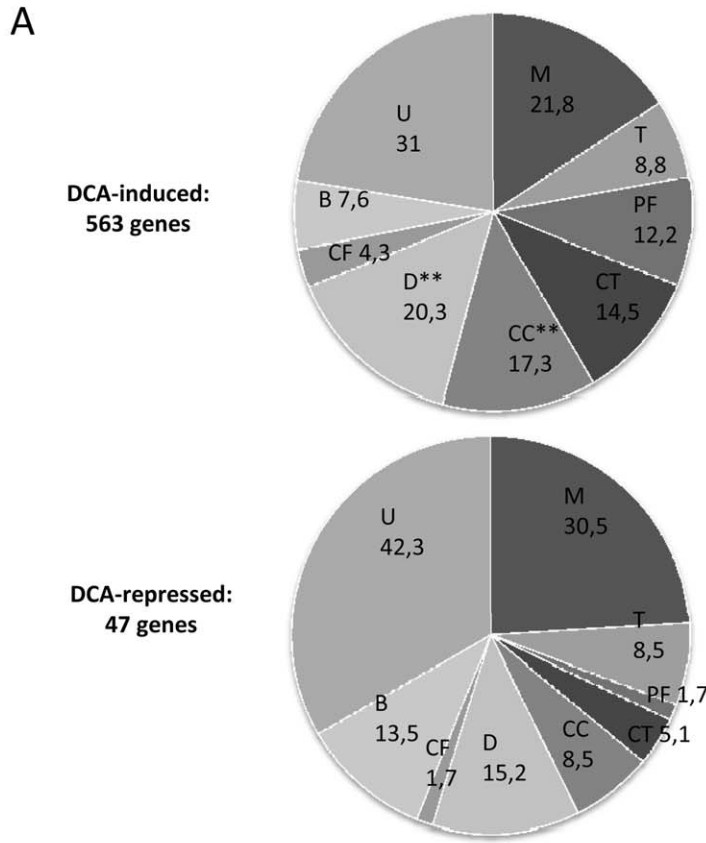
### DCA triggers changes in the transcriptome of Arabidopsis

In order to understand the molecular changes induced by DCA treatment, we analysed the transcriptome of Arabidopsis leaves following DCA treatment. Rosette leaves of 5-week-old Arabidopsis Col-0 plants were sprayed with 200  $\mu\text{M}$  DCA or mock-treated,

and collected at 24 hpt. RNA was extracted and the gene expression level was analysed using Complete Arabidopsis Transcriptome Microarray (CATMA) chips, as described previously (Moreau *et al.*, 2012). Two biological replicates and a dye swap were performed; a third biological replicate was performed to analyse the profile of selected genes by qRT-PCR as presented later (see Figs 4–6). Statistical analysis of the transcriptome data revealed that 563 and 47 genes were significantly up- and down-regulated, respectively, 24 h after DCA treatment (Table S2, see Supporting Information).

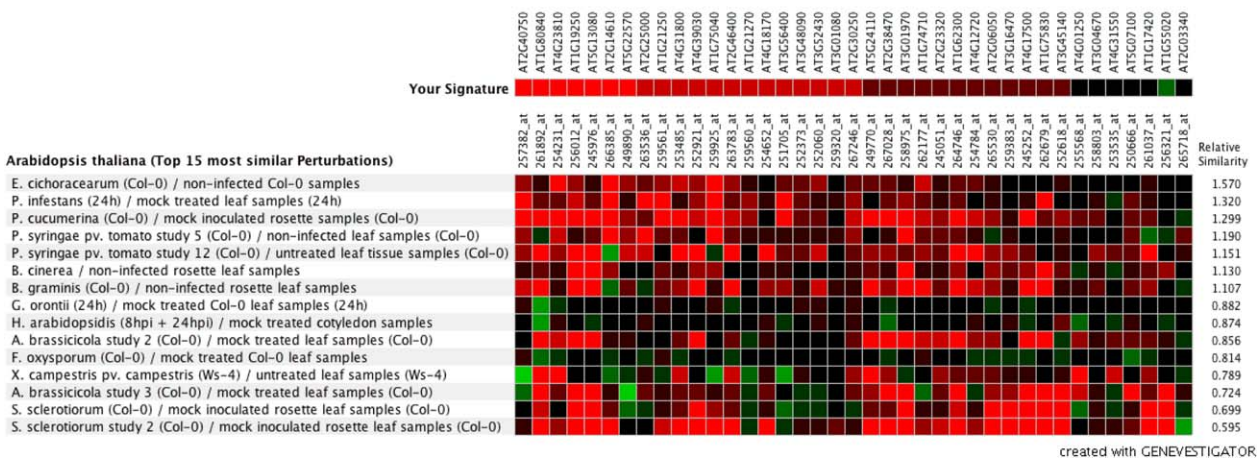
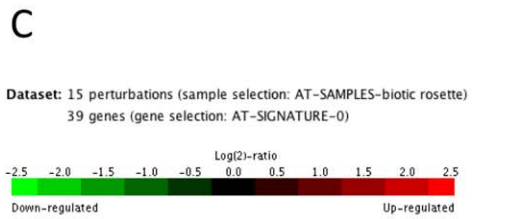
In order to gain an insight into the impact of DCA treatment on plants, we determined the putative functions of the genes modulated following DCA treatment using the MIPS Functional Catalogue Database (FunCatDB) (Ruepp *et al.*, 2004). This analysis revealed that a very large proportion of the 610 DCA-modulated genes (70%) were annotated as encoding proteins of known or predicted function (Fig. 2A), which is consistent with our previous analysis of pathogen response transcriptome datasets (Moreau *et al.*, 2012). Among the induced genes (Fig. 2A, top), the most represented functional categories were metabolism (21.8%), defence (20.3%), cellular communication (17.3%) and cellular transport (14.5%). The defence and cellular communication functional categories were both over-represented compared with the distribution of genes among the different functional categories in the Arabidopsis genome ( $P < 0.005$ ). Among the repressed genes (Fig. 2A, bottom), the most represented categories were metabolism (30.5%), defence (15.2%) and biogenesis of cellular components (13.5%); however, none of these categories was over-represented compared with the whole genome ( $P < 0.005$ ). Altogether, our results indicate that DCA triggers substantial changes in the gene expression of Arabidopsis and, in particular, induces changes in the expression of defence-associated genes.

As DCA induces the accumulation of defence-associated molecules, such as SA, JA, ROS and callose (Fig. 1), we analysed our transcriptome data to determine whether genes known to be associated with these defence responses were modulated by DCA treatment. *ICS1* encodes the SA biosynthesis enzyme isochorismate synthase and *EDS5* encodes the MATE-transporter required for the export of SA from the chloroplast (Serrano *et al.*, 2013). *LOX2* and *LOX3* encode lipoxygenases, and *OPR3* encodes a 12-oxo-phytodienoic acid (OPDA) reductase involved in JA biosynthesis (Dave and Graham, 2012; Wasternack and Hause, 2013). We also analysed genes that are known to be markers for the activation of these defence pathways, such as *PR1*, *PAD4*, *WAK1* and *WAK2* for the SA-dependent pathway and *PDF1*, *JR1* and *ATERF1* for the JA-dependent pathway. The results (Fig. 2B, DCA column) clearly indicate that the expression of both SA- and JA-associated genes is induced following DCA treatment, although the SA-associated genes appear to be more strongly induced. In particular, genes dependent on EDS1 are also induced by DCA. We also



**B**

SA response/biosynthesis		DCA	Ea
EDS5	AT4G39030	2,61	1,31
PAD4	AT3G52430	2,11	3,25
ICS1	AT1G74710	1,71	2,66
PR1	AT2G14610	3,12	#N/A
WAK1	AT1G21250	2,76	#N/A
WAK2	AT1G21270	2,45	1,52
WAK3	AT1G21240	1,73	3,00
PR2	AT3G57260	4,98	
PR5	AT1G75040	2,59	
ET/JA response and biosynthesis			
ATERF-1	AT4G17500	1,22	1,32
LOX3	AT1G17420	0,83	1,32
OPR3	AT2G06050	1,44	
JR1	AT3G16470	1,39	
LOX2	AT3G45140	1,00	
LCR67/PDF1.1	AT1G75830	1,16	
LOX1	AT1G55020	-1,66	2,31
EDS1 pathway			
FMO1	AT1G19250	3,43	2,70
EDS1	AT3G48090	2,26	1,20
AtNUDT7	AT4G12720	1,51	2,10
WRKY TFs			
WRKY22	AT4G01250	0,99	2,96
WRKY75	AT5G13080	3,40	2,94
WRKY51	AT5G64810	3,45	2,89
WRKY46	AT2G46400	2,52	2,68
WRKY18	AT4G31800	2,66	2,45
WRKY38	AT5G22570	3,01	2,43
WRKY40	AT1G80840	3,72	2,26
WRKY25	AT2G30250	2,03	2,01
WRKY54	AT2G40750	3,81	1,99
WRKY70	AT3G56400	2,34	1,98
WRKY45	AT3G01970	1,75	1,92
WRKY6	AT1G62300	1,52	1,61
WRKY15	AT2G23320	1,61	1,47
WRKY53	AT4G23810	3,52	1,41
WRKY39	AT3G04670	0,96	1,26
WRKY11	AT4G31550	0,94	1,25
WRKY60	AT2G25000	2,80	1,05
WRKY28	AT4G18170	2,45	0,80
WRKY50	AT5G26170	3,86	
WRKY30	AT5G24110	1,98	
WRKY26	AT5G07100	0,65	
WRKY58	AT3G01080	2,04	
WRKY33	AT2G38470	1,78	#N/A
WRKY3	AT2G03340	0,86	



analysed in more detail the transcriptome data of the WRKY family of transcription factors (TFs), one of the largest families of plant transcriptional regulators. Although WRKY TFs play a role in many

plant processes, the major role of this family is in the control of the response of plants to biotic stress (Rushton *et al.*, 2010). Our data indicate that DCA induced the expression of 23 WRKY-

**Fig. 2** Deoxycholic acid (DCA) induces major transcriptional reprogramming in Arabidopsis leaves. Five-week-old Arabidopsis leaves were sprayed with 200  $\mu\text{M}$  DCA or mock-treated. Leaves were collected at 24 h post-treatment (hpt) and RNA was extracted. Transcriptome analysis was performed using Complete Arabidopsis Transcriptome Microarray (CATMA) chips. (A) Functional categories of induced (563) and repressed (47) genes according to the MIPS database. B, biogenesis of cellular components; CC, cellular communication; CF, cell fate; CT, cellular transport; D, defence; M, metabolism; PF, protein fate; T, transcription; U, unclassified. Asterisks indicate an over-representation of the corresponding functional category compared with the Arabidopsis genome ( $P < 0.005$ ). The values correspond to the percentages of induced or repressed genes. (B) Microarray data for selected genes associated with defence. Values are  $\log_2$  signal ratios between DCA (DCA)- or *Erwinia amylovora* (*Ea*)-treated plants and mock-treated control plants. Positive (red and pink boxes) and negative (green boxes) values correspond to genes up- and down-regulated, respectively, in response to DCA or *E. amylovora* (Moreau *et al.*, 2012). Black boxes indicate that gene expression was not significantly changed according to the Bonferroni test ( $P < 0.05$ ). Grey boxes correspond to missing values. (C) Most similar transcriptome datasets, with respect to selected defence-related genes, according to the Genevestigator 'Signature' tool. The top line ('Your signature') corresponds to  $\log_2$  signal ratios between DCA (DCA)- and mock-treated plants (this study). All lines below correspond to  $\log_2$  signal ratios between pathogen-infected and mock-treated wild-type plants as indicated.

encoding genes, several of which have been shown previously to be involved in the plant's immune response, such as WRKY18, WRKY33, WRKY40, WRKY46 and WRKY70 (Pandey and Somssich, 2009; Rushton *et al.*, 2010).

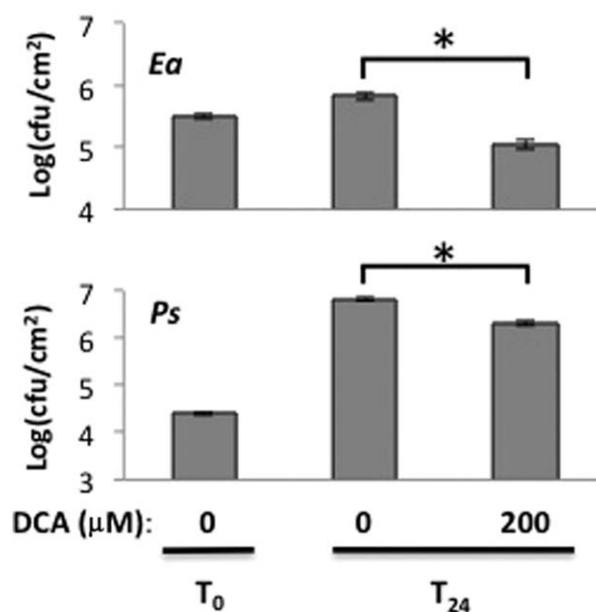
In order to determine whether the plant's transcriptional response to DCA resembled the plant's immune response, we compared the data obtained in response to DCA treatment with previously described data. We used data obtained in response to different pathogens available in the Genevestigator database (Fig. 2C), as well as our data obtained in response to *Erwinia amylovora* inoculation (Fig. 2B, *Ea* column; Moreau *et al.*, 2012). Values represent the  $\log_2$ (ratio) of the fold modulation of selected Arabidopsis genes in response to DCA treatment or pathogen inoculation (as described in the figure) compared with mock treatment (Fig. 2B,C). We compared the list of defence-associated genes identified as being induced by DCA (Fig. 2B, DCA column) with our previously obtained transcriptome data (Fig. 2B, *Ea* column) or with public datasets using the signature tool in the Genevestigator database (Fig. 2C). The results show an important similarity between the response of Arabidopsis to several pathogens, including the model pathogen *Pseudomonas syringae* pv. *tomato* (Fig. 2C, lines 4 and 5) and *E. amylovora* (Fig. 2B), and the response of Arabidopsis to DCA.

Altogether, our results indicate that treatment of Arabidopsis leaves with DCA triggers a transcriptional reprogramming that bears similarities to the plant's immune response.

### DCA protects against pathogen infection

Previous reports have indicated that CA is able to induce defence activation in rice and to protect rice plants against infection by the fungal pathogen *Magnaporthe grisea* (Koga *et al.*, 2006). To determine whether DCA treatment is also able to protect plants against pathogen infection, we analysed the capacity of DCA to reduce bacterial proliferation in Arabidopsis leaves. We chose *P. syringae* pv. *tomato* DC3000 and *E. amylovora* CFBP1430 as models of a compatible interaction and a non-host interaction, respectively, as our analysis indicates some similarity between the transcriptional response to DCA and the transcriptional response to both of these bacterial phytopathogens (Fig. 2B,C). Arabidopsis

leaves were treated with 200  $\mu\text{M}$  DCA or mock solution and, 24 h later, were inoculated with the virulent *P. syringae* DC3000 strain and the *E. amylovora* CFBP1430 strain. The bacterial count in the leaves was carried out at 0 and 24 h post-infection (hpi), as indicated in Fig. 3. Consistent with previous reports, we found that, in control plants, both *P. syringae* and *E. amylovora* were able to proliferate, although to a much lesser extent in the case of *E. amylovora* as it is not adapted to Arabidopsis (Moreau *et al.*, 2012). In plants pretreated with DCA, we observed a significantly ( $P < 0.05$ ) lower bacterial count *in planta* at 24 hpi for both



**Fig. 3** Deoxycholic acid (DCA) reduces the *in planta* growth of phytopathogens. Arabidopsis leaves were sprayed with 200  $\mu\text{M}$  DCA 24 h prior to infection with *Pseudomonas syringae* pv. *tomato* [*Ps*; optical density (OD) = 0.01,  $10^6$  colony-forming units (cfu)/mL] or *Erwinia amylovora* (*Ea*; OD = 0.1,  $10^7$  cfu/mL). For each experiment, 10 leaves ( $n = 10$ ) were collected immediately after infection ( $T_0$ ) or 24 h post-infection ( $T_{24}$ ), ground and cfu/cm<sup>2</sup> was determined. Bars show the mean number of cfu/cm<sup>2</sup> of leaf  $\pm$  standard error of the mean (SEM). Experiments were repeated three times with similar results. The asterisk indicates a significant difference from the mock-treated plants at the same time point according to the Mann–Whitney test ( $P < 0.05$ ).

bacteria tested. For both bacterial species, the bacterial count in plants pretreated with DCA was approximately 10 times lower than in plants that had not been pretreated with DCA. In the case of *E. amylovora*, which is not adapted to Arabidopsis and thus shows only weak bacterial multiplication in control plants, DCA pretreatment led to an absence of multiplication (Fig. 3). In the case of *P. syringae*, which is virulent on Arabidopsis, DCA pretreatment did not prevent bacterial multiplication, but reduced it significantly (Fig. 3).

Our results show that the treatment of plants with DCA before inoculation reduces the proliferation of two phytopathogenic bacteria.

### Dose response of gene modulation by DCA treatment and effect of abiotic stress

In order to better understand the response of Arabidopsis to DCA, we monitored, by qRT-PCR, the expression of selected defence marker genes in response to increasing concentrations of DCA. We chose *WRKY46* and *WRKY70*, two WRKY TFs, *PDF1.2a* and *CHI-B* as markers of the JA/ET-dependent pathway, and *PR1*, *WAK2* and *EDS1* as markers of the SA-dependent pathway. Five-week-old Arabidopsis leaves were sprayed with different concentrations of DCA or mock-treated. Leaves were collected at 24 hpt and gene expression was assayed using qRT-PCR. All genes analysed showed an induction of their mRNA accumulation in response to the highest concentration of DCA (200  $\mu\text{M}$ ), confirming our transcriptome data (Fig. 4). In response to 100  $\mu\text{M}$  only, the mRNA levels of *PR1* and *CHI-B* were induced significantly. However, exposure of Arabidopsis plants to 20  $\mu\text{M}$  DCA did not induce the expression of any of the tested genes (Fig. 4).

As plant growth conditions are not always optimal, especially water availability, we evaluated the impact of drought and nitrogen limitation on the induction of molecular defences by DCA. Five-week-old Arabidopsis plants subjected to water deficit were treated with 200  $\mu\text{M}$  DCA and gene expression at 24 hpt was evaluated by qRT-PCR. The expression of the drought marker genes *RAB18* and *RD29B* increased during water stress (Fig. S3, see Supporting Information), as expected (Harb *et al.*, 2010). In plants subjected to water deficit, most of the defence genes tested retained the ability to be induced by DCA, although the level of induction was affected slightly (Fig. 5). In contrast, the TFs *WRKY46* and *WRKY70* did not respond to DCA in plants subjected to drought. We also determined whether DCA was able to induce defence in Arabidopsis plants that were grown under nitrogen limitation. Five-week-old Arabidopsis plants subjected to nitrogen limitation (2 mM  $\text{NO}_3^-$ ) or not (10 mM  $\text{NO}_3^-$ ), as described in Fagard *et al.* (2014), were treated with 200  $\mu\text{M}$  DCA or mock-treated. Leaves were sampled at 24 hpt and defence gene expression was evaluated by qRT-PCR (Fig. 5B). We found that both the SA path-

way and JA pathway marker genes, *PR1* and *PDF1.2*, respectively, were induced by DCA in plants grown under nitrogen limitation.

In summary, we observed that DCA treatment induces defence marker genes in a dose-dependent manner, even in the presence of drought or nitrogen limitation.

### Kinetics of gene modulation by DCA treatment

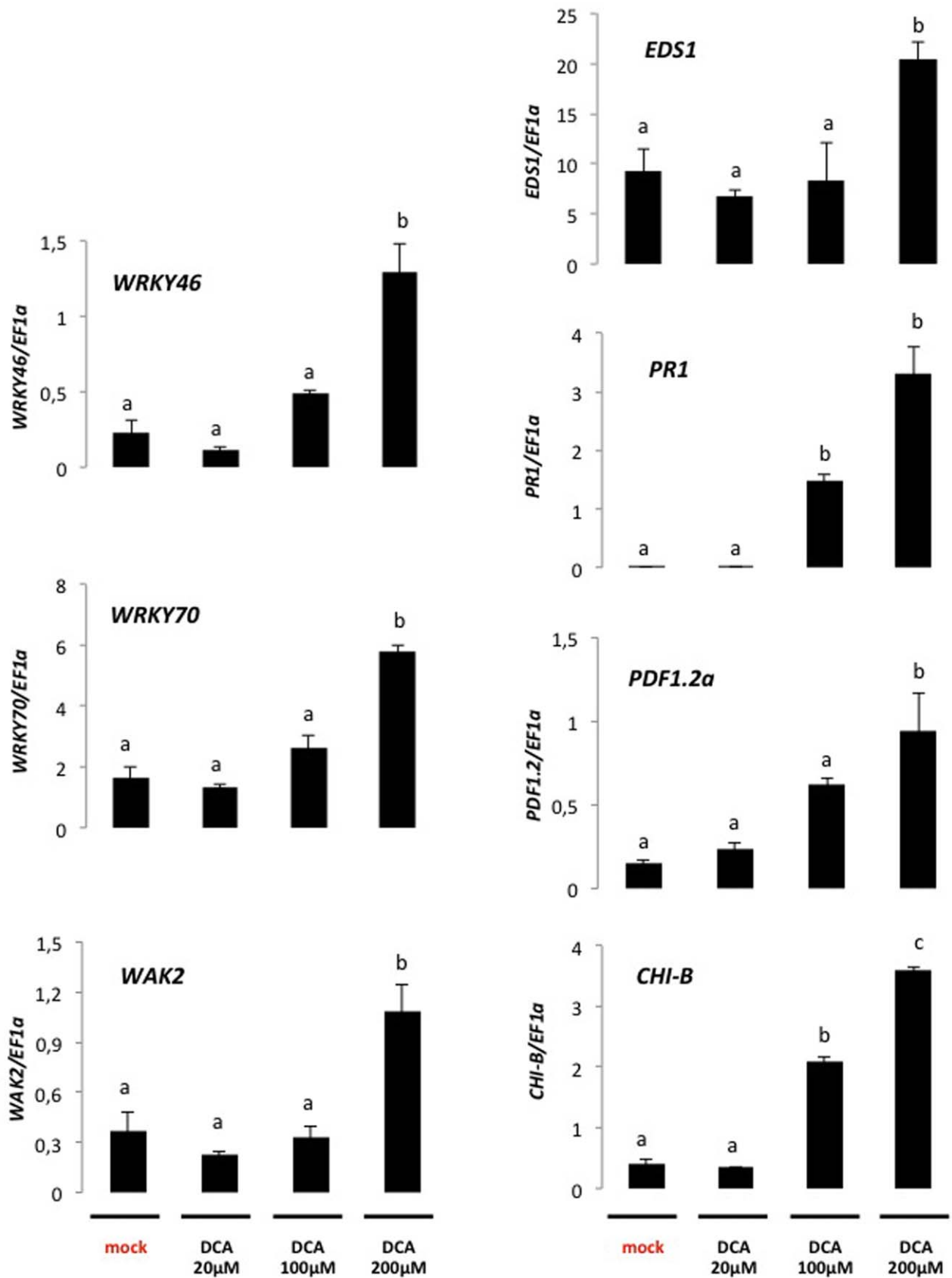
In order to assess the capacity of DCA to protect plants over time, we analysed gene expression over several time points from 6 hpi to 7 days post-infection. Five-week-old Arabidopsis leaves were sprayed with 200  $\mu\text{M}$  DCA or mock-treated. We observed a peak of mRNA accumulation between 24 and 48 hpt, depending on the gene, and thereafter the mRNA level decreased gradually within 72 h and then remained constant until 7 days after DCA treatment (Fig. 6). For the SA-dependent marker genes *EDS1* and *PR1*, the peak of mRNA accumulation was at 24 hpt, whereas it was at 48 hpt for the JA-dependent genes *CHI-B* and *PDF12a*. mRNA accumulation in response to DCA started at 6 hpt for *WRKY46*, *WRKY70*, *EDS1* and *WAK2*. The expression of *WAK2* showed the most rapid increase in mRNA accumulation in response to DCA.

Altogether, we show that gene induction following DCA treatment exhibits a peak between 6 and 48 h depending on the gene, whereas almost no gene induction remains 7 days after treatment.

### Defence induction by DCA requires *SID2* and *RbohD*

In order to further characterize the action of DCA, we analysed the induction of the SA marker gene *PR1* following DCA treatment in the SA biosynthesis-deficient *sid2-1* mutant (Nawrath and Métraux, 1999). As a control, we also used the NahG-B15 transgenic line that expresses a salicylate hydroxylase-encoding bacterial gene and displays very low levels of SA (Lawton *et al.*, 1995). Approximately 20 seedlings of each genotype were grown *in vitro* on one-fifth strength Murashige and Skoog medium and sprayed with 200  $\mu\text{M}$  DCA or mock-treated, and sampled at 24 hpt. The expression of the *PR1* gene was assessed by RT-PCR as shown in Fig. 7A. The expression of the constitutive gene *Elongation factor-1 $\alpha$*  (*EF1 $\alpha$* ) was used as a loading control. No expression of the *PR1* gene was detected in the mock-treated plants. Our results show that DCA treatment can induce the expression of the *PR1* gene in seedlings grown *in vitro* as it does in plants grown in soil. Furthermore, our results clearly indicate that the induction of the *PR1* gene by DCA is completely abolished in the *sid2-1* mutant, as well as in the NahG transgenic line, indicating that *PR1* induction by DCA requires *SID2*-dependent SA production.

*WAK* (wall-associated kinase) genes encode receptor-like kinase (RLK) genes that are thought to be involved in the response of plants to their extracellular environment. Among the five Arabidopsis *WAK* genes, DCA treatment induced the expression of *WAK1*, *WAK2* and *WAK3* (Fig. 2). Moreover, *WAK1* and

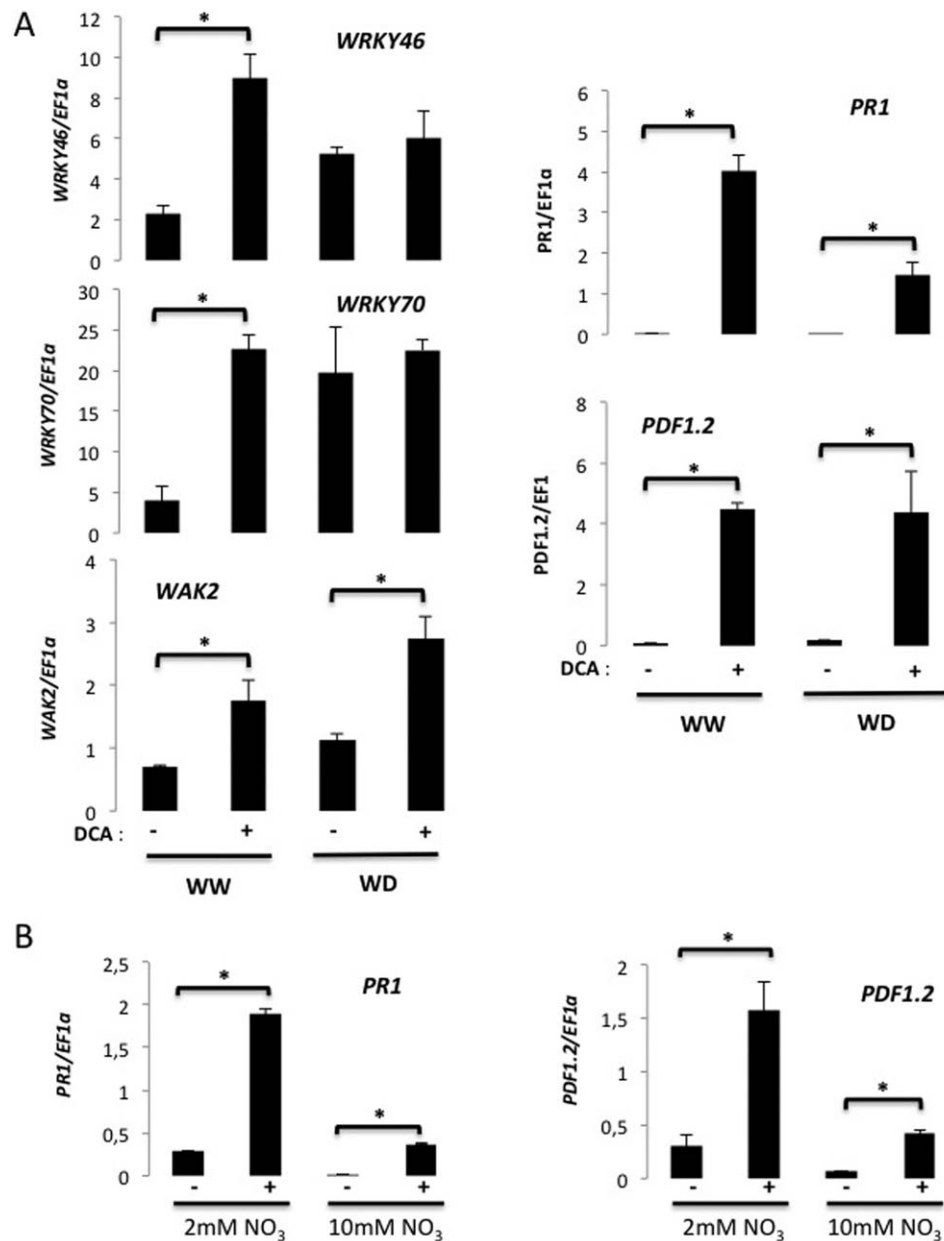


**Fig. 4** Deoxycholic acid (DCA) induces defence-related gene expression in a dose-dependent manner. The expression of selected defence genes was evaluated 24 h following mock treatment or treatment with increasing concentrations of DCA (as indicated). Nine leaves of three plants were collected for RNA extraction ( $n = 3$ ). Transcript accumulation was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Bars represent the mean expression  $\pm$  standard deviation (SD). Bars with the letter 'a' correspond to expression levels that are not significantly different from expression in mock-treated leaves according to the Mann-Whitney test ( $P < 0.05$ ). Bars with the letters 'b' and 'c' correspond to expression levels that are significantly different from expression in mock-treated leaves and from each other according to the Mann-Whitney test ( $P < 0.05$ ). The experiment was repeated twice with similar and statistically significant results.

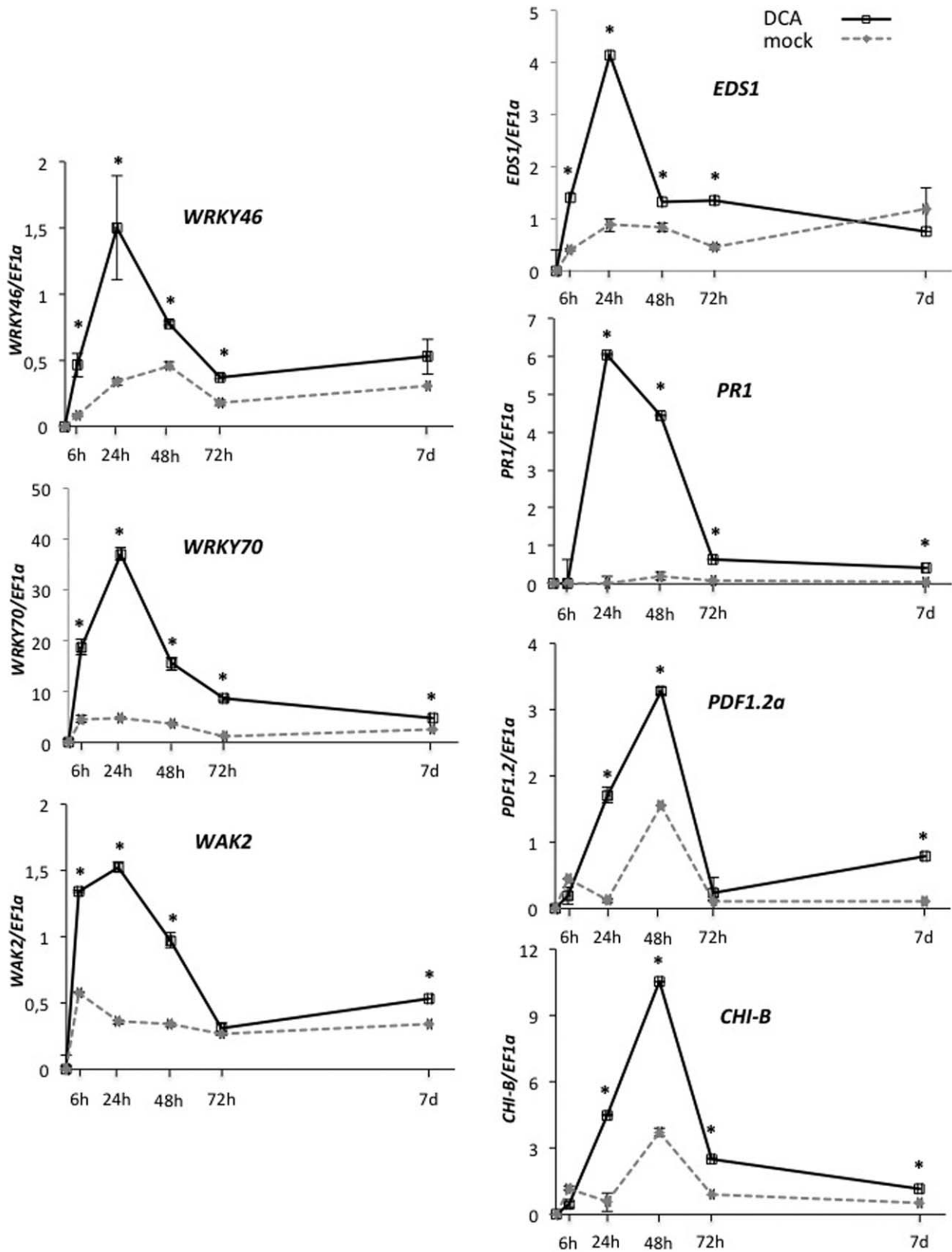
*WAK2* are involved in defence activation (He *et al.*, 1998; Kohorn *et al.*, 2009). In order to determine whether these receptors play a role in the induction of defence following DCA treatment, T-DNA insertion knock-out mutants were retrieved for *WAK1* and *WAK3*. The insertion of a T-DNA in the corresponding gene and the

absence of cDNA were confirmed for each line by PCR and RT-PCR, respectively (data not shown). For *WAK2*, we used the previously described *wak2-1* null allele (Kohorn *et al.*, 2006). Plants were treated with 200  $\mu\text{M}$  DCA or mock-treated, and sampled at 24 hpt. Gene expression was assessed using qRT-PCR. Our results

**Fig. 5** Effect of drought on the deoxycholic acid (DCA)-mediated induction of defence. (A) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of selected genes 24 h after mock (–) or 200  $\mu\text{M}$  DCA (+) treatment in Arabidopsis Col-0 plants under well-watered (WW) or water deficit (WD) conditions, as described in Bouchabke *et al.* (2008). (B) qRT-PCR analysis of selected genes 24 h after mock (–) or 200  $\mu\text{M}$  DCA (+) treatment in Arabidopsis Col-0 plants grown in limiting (2 mM  $\text{NO}_3^-$ ) or non-limiting (10 mM  $\text{NO}_3^-$ ) nitrate. (A, B) Nine leaves from three plants ( $n = 3$ ) were collected 24 h after DCA or mock treatment. Bars represent the mean expression  $\pm$  standard deviation (SD). The experiments were repeated twice with similar and statistically significant results. The asterisk indicates significant difference according to the Mann-Whitney test ( $P < 0.05$ ).





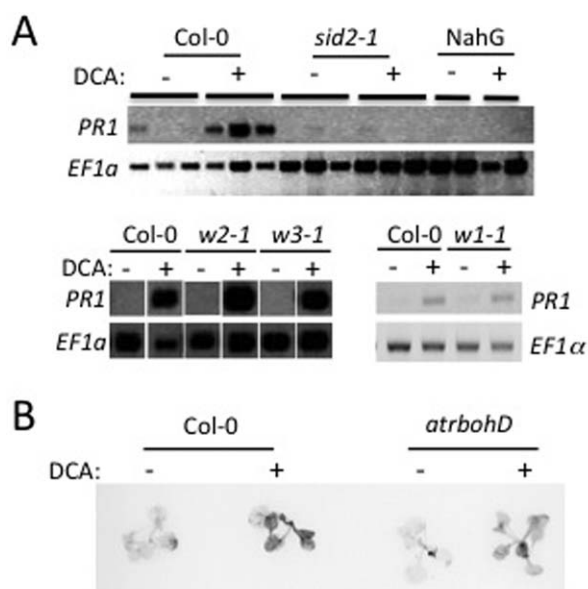




**Fig. 6** Kinetics of defence induction triggered by deoxycholic acid (DCA) treatment. The expression of selected defence genes was monitored at different time points between 6 h and 7 days following mock (broken line with diamonds) or 200  $\mu\text{M}$  DCA (full line with squares) treatment. Nine leaves from three plants ( $n = 3$ ) were collected at the indicated time point following mock treatment or treatment with 200  $\mu\text{M}$  DCA. Transcript accumulation was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Values represent the mean expression  $\pm$  standard deviation (SD). The experiments were repeated twice with similar and statistically significant results. The asterisk indicates a significant difference from mock-treated plants according to the Mann–Whitney test ( $P < 0.05$ ).

showed that the *wak1-1*, *wak2-1* and *wak3-1* mutant lines responded to DCA treatment in terms of defence gene induction, as did the Col-0 control plants (Fig. 7). These results indicate that none of the three *WAK* genes analysed is solely responsible for *PR1* induction following DCA treatment.

In Arabidopsis, the NADPH oxidase encoded by *RbohD* plays a predominant role in ROS production following pathogen infection (Torres *et al.*, 2002). We analysed  $\text{H}_2\text{O}_2$  production in seedlings grown *in vitro* following DCA or mock treatment using DAB staining. No DAB staining could be detected in mock-treated plants. In contrast, DCA induced ROS accumulation in wild-type plants grown *in vitro* (Fig. 7B), as it does in soil-grown plants (Fig. 1C). In the *rbohD* mutant, ROS induction following DCA treatment was much weaker than in wild-type plants, indicating that ROS induction by DCA is at least partly dependent on *RbohD*.



**Fig. 7** *SID2* and *RbohD* are required for defence activation by deoxycholic acid (DCA). (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the expression of *PR1* in 2-week-old *in vitro*-grown seedlings treated (+) or not (–) with 200  $\mu\text{M}$  DCA and sampled 24 h after treatment. The *EF1α* gene was used as an internal standard for the quantity of cDNA. Each lane corresponds to a pool of 10 seedlings and the experiment was conducted three times. *w1-1*, *wak1-1*; *w2-1*, *wak2-1*; *w3-1*, *wak3-1*. The figure was rearranged for clarity; the original image is available in Fig. S4 (see Supporting Information). (B) 3,3'-Diaminobenzidine (DAB) staining of 2-week-old seedlings treated with 200  $\mu\text{M}$  DCA (+) or mock-treated (–). The experiment was performed on 20 seedlings and the figure shows photographs of representative seedlings.

## DISCUSSION

Several studies have reported plant defence elicitor properties for bile acids when applied to rice or soybean plants (Kevresan *et al.*, 2009; Koga *et al.*, 2006; Malencic *et al.*, 2012). It has been shown that the primary bile acid CA elicits cell death, PR protein synthesis, phytoalexin accumulation, ROS accumulation and protects rice against the fungus *Magnaporthe grisea* (Kevresan *et al.*, 2009; Koga *et al.*, 2006; Malencic *et al.*, 2012). Although these studies focused on CA, it has been suggested that the secondary bile acid DCA, which is one of the three major constituents of bile, also has elicitor properties, as it induces phytoalexin accumulation in rice (Koga *et al.*, 2006). The present work focuses on the elicitor properties of DCA in plants. We investigated the effect of DCA treatment on defence elicitation in Arabidopsis. We first investigated the effect of DCA treatment on ROS accumulation and callose deposition, two representative cellular defence responses. Indeed, the first mechanical barrier encountered by pathogens during plant invasion is the cell wall and plants are able to reinforce their own cell wall by depositing callose, a  $\beta$ -(1-3)-glucan polymer, at the site of attack (Luna *et al.*, 2011). We found that DCA induces callose deposition and both intracellular and extracellular ROS accumulation in leaves (Fig. 1). Both callose and ROS accumulation were observed only in response to the highest concentration of DCA tested, consistent with previous reports that callose accumulation can be correlated with the oxidative burst (Luna *et al.*, 2011). DCA treatment was also able to induce the accumulation of SA and JA phytohormones (Figs 1 and S2). Consistent with the capacity of DCA to trigger defences in Arabidopsis leaves, we found that DCA reduces the capacity of two pathogenic bacteria to multiply in DCA-pretreated leaves (Fig. 3).

Previous studies have shown that CA can induce the gene expression required for phytoalexin biosynthesis in rice (Shimizu *et al.*, 2008), suggesting that defence activation by bile acids in plants could require transcriptional activation of the corresponding defence genes. However, no genome-wide analysis of the impact of bile acids on plants has been performed to date. In order to gain an insight into the elicitor properties of bile acids, we analysed the transcriptome of plants treated with DCA. Our results indicated that DCA strongly affected the plant's transcriptome, including many functional categories of genes (Fig. 2A). Interestingly, the vast majority of genes affected by DCA were induced (92%) and not repressed (8%), although it is not obvious whether this has any functional significance. Furthermore, an important number of genes from the WRKY family of TFs were induced following DCA treatment (Fig. 2B), which is consistent with a

transcriptional activation of defence by DCA following its perception by the plant by an as yet to be identified mechanism. Our data also showed that DCA induces the expression of a large number of defence-associated genes, including known defence genes that are induced in response to a wide set of pathogens (Fig. 2C). Thus, DCA has an important impact on the plant's transcriptome in a similar manner to two well-characterized PAMPs: the flagellin-derived flg22 peptide and oligogalacturonides (OGs). Indeed 359 of the 610 DCA-modulated genes were also modulated by flg22 or OGs (Denoux *et al.*, 2008). Furthermore, we showed that DCA affects gene expression as early as 6 hpi, as revealed by our qRT-PCR data (Fig. 6).

In accordance with our observation that SA and JA phytohormones accumulate following DCA treatment, transcriptome analysis of DCA-treated plants revealed an activation of genes dependent on the SA pathway and the JA pathway (Figs 3 and 4). In *Arabidopsis*, *PR2* encodes an SA-dependent pathogen-inducible  $\beta$ -1,3-glucanase (Gallego-Giraldo *et al.*, 2011). Koga *et al.* (2006) reported that CA treatment in rice increases  $\beta$ -1,3-glucanase activity. Consistent with the observations of Koga *et al.* (2006) on rice, *PR2* is induced in *Arabidopsis* by DCA (Fig. 2). Furthermore, although it is generally thought that SA strongly antagonizes the JA-mediated defensive pathway (Pieterse *et al.*, 2012), the interactions between SA and JA hormones are strongly dependent on the concentration of these molecules: applied exogenously at low concentrations, SA and JA act synergistically to activate defence gene expression, whereas antagonism is observed at higher concentrations (Mur *et al.*, 2006). Taken together, it appears that DCA treatment induces a balanced SA/JA accumulation that could be useful for plant protection against both necrotrophic and biotrophic pathogens (Okada *et al.*, 2015). Another interesting feature of DCA is that it can activate defence in plants grown under abiotic stress. Indeed, we showed that DCA induces defence in plants subjected to drought (Fig. 5A), as well as in plants grown under limiting nitrogen (Fig. 5B). Two genes, *WRKY46* and *WRKY70*, did not respond to DCA under moderate drought; however, this could be a result of the fact that both genes were more highly expressed in mock-treated plants subjected to drought stress than in untreated control plants.

*WAK* genes encode a family of RLKs that have attracted attention in recent years. *WAK2* has been shown to be necessary for normal cell enlargement, as a reduction in *WAK2* protein levels in transgenic plants results in dwarf plants (Wagner and Kohorn, 2001). The extracellular domain of *WAK1* and *WAK2* binds pectin *in vitro* (Kohorn *et al.*, 2009). Furthermore, the *wak2-1* mutant is affected in MAPK activation by pectin in protoplasts and the *wak2-1* mutant is defective in the activation of numerous genes in protoplasts treated with pectin (Kohorn *et al.*, 2009). Among the five *Arabidopsis WAK* genes, *WAK1–3* are induced by SA or its analogue 2,6-dichloroisonicotinic acid (INA) (He *et al.*, 1998,

1999). The expression of *WAK1* is also induced by *P. syringae* infection and its role in resistance to lethal SA levels has been demonstrated (He *et al.*, 1998). Our results showed that the single mutants *wak1-1*, *wak2-1* and *wak3-1* displayed an induction of defence in response to DCA similar to the wild-type plants. These results indicate that, although they are rapidly activated at the transcriptional level, *WAK1*, *WAK2* and *WAK3* are individually dispensable for defence activation by DCA. Interestingly, we found that the *wak1-1* knock-out mutant was not affected in root length, whereas the *wak3-1* knock-out mutant displayed a slight reduction in root length (data not shown), suggesting that *WAK3* could play a role in cell growth with *WAK2* (Kohorn *et al.*, 2006).

Altogether, we have shown that DCA constitutes a potent plant defence elicitor in *Arabidopsis*, and have demonstrated the role of *SID2* and *RbohD* in the activation of defence following treatment with this elicitor. The present work confirms the potential of bile acids as good candidate elicitors for a balanced defence response in both dicots and monocots, and in response to different classes of pathogen. Furthermore, our data indicate that defence activation by DCA occurs in plants grown in different conditions, including under abiotic stress, suggesting that bile acids could be robust elicitors that are not strongly affected by the physiology of the treated plant, and could be used in crop production. The development of sustainable agriculture will greatly benefit from the development of new plant defence elicitors, such as DCA, together with a better understanding of their mode of action for an optimal use of these molecules.

## EXPERIMENTAL PROCEDURES

### Plant growth and DCA treatment

Seeds of *A. thaliana* Col-0 were obtained from the INRA Versailles collection. The SA-deficient *sid2-1* null mutant (Nawrath and Métraux, 1999) and NahG-B15 transgenic line (Lawton *et al.*, 1995), the *wak2-1* null mutant (Kohorn *et al.*, 2006) and the *rbohD* mutant (Torres *et al.*, 2002) are all in the Col-0 background. The *wak1-1* and *wak3-1* alleles correspond to lines SALK\_107175 (Col-0) and SALK\_071999 (Col-0), respectively. These lines possess a T-DNA insertion in exon3 of AT1G21250 and exon2 of AT1G21240, respectively. We checked by qRT-PCR that both *wak1-1* and *wak3-1* are null alleles (data not shown).

Plants were grown for 5 weeks in soil and were subjected to an 8-h light and 16-h dark cycle at 21°C (day)/18°C (night) with 65% relative humidity (Figs 1–6), or were grown for 7 days on one-fifth strength Murashige and Skoog medium plus 1% sucrose in a 16-h light/8-h dark cycle at 21°C with 50% relative humidity (Fig. 7). Plants were sprayed with different concentrations of DCA (Industria Chimica Emiliana, Reggio Emilia, Italy) dissolved in 0.01% Tween-20 (as described in Koga *et al.*, 2006) or with a mock solution (0.01% Tween-20).

Drought treatment was performed as described in Bouchabke *et al.* (2008). Briefly, soil water content was fixed at 60% of maximal water content as a control in well-watered (WW) conditions. The water deficit (WD) treatment was fixed at 30% of the maximal water content. Pots

were weighed daily and adjusted to a fixed weight corresponding to the water contents described above. The two watering regimes were applied 2 weeks after sowing. Nitrogen limitation was performed as described in Fagard *et al.* (2014). Briefly, plants were grown for 5 weeks in sand and watered with a nutrient solution containing ample (10 mM NO<sub>3</sub><sup>-</sup>) or limiting (2 mM NO<sub>3</sub><sup>-</sup>) nitrogen. In all cases, 5-week-old stressed or control plants were mock- or DCA-treated as described in the text.

### Pathogen infections

Five-week-old Arabidopsis leaves were sprayed with 200 μM DCA or mock-treated and, 24 h later, inoculated with *E. amylovora* CFBP1430 or *P. syringae* pv. *tomato* DC3000. Bacterial suspensions were prepared in sterile water for *E. amylovora* (10<sup>7</sup> cfu/mL) or in 10 mM MgCl<sub>2</sub> for *P. syringae* (10<sup>6</sup> cfu/mL). At 24 hpi, we performed bacterial counting by grinding infected leaves using glass beads in a TissueLyser (Qiagen/Retsch, Hilden, Germany). The bacterial suspensions were used to prepare serial dilutions, which were plated on Lysogeny Broth (LB) medium and, after 1 or 2 days, the colonies formed were counted to evaluate the initial number of bacteria.

### Callose staining

Leaves of 5-week-old Arabidopsis plants were sprayed with the indicated concentration of DCA or mock-treated. Leaves were collected at 24 hpt and placed overnight in a lactophenol clearance solution. Callose deposits were stained using 0.01% aniline blue in 150 mM K<sub>2</sub>HPO<sub>4</sub> (pH 9.5) buffer for 30 min, as described previously (Degraeve *et al.*, 2008). Leaves were examined by stereofluorescence microscopy with an Azio Zoom V.16 (Carl Zeiss Inc., Oberkochen, Germany). Experiments were repeated three times with similar results and representative photographs are shown. The number of spots of callose deposit per photograph was determined using ImageJ software in 25–30 photographs corresponding to more than five independent leaves for each treatment.

### H<sub>2</sub>O<sub>2</sub> detection

Intracellular H<sub>2</sub>O<sub>2</sub> was detected using DCFH-DA. At 16 h after treatment with 20 or 200 μM DCA (0.01% Tween-20) or mock, leaves of 5-week-old Arabidopsis plants were cut off, immersed in 300 μM DCFH-DA solution and vacuum infiltrated. Whole-leaf images were taken using an Olympus SZX12 binocular (Olympus Corporation, Shinjuku, Japan). Green fluorescence was detected with an HQ510 1p emission filter. In parallel, we used DAB staining to detect both intra- and extracellular H<sub>2</sub>O<sub>2</sub>. Leaves of 5-week-old Arabidopsis plants were sprayed with 20 or 200 μM DCA or mock-treated. Leaves were then collected at 6 hpt and immediately vacuum infiltrated with DAB (1 mg/mL). We analysed 15–20 photographs per treatment and representative photographs were taken with a binocular (Leica MZFLIII, Wetzlar, Germany).

### SA and JA phytohormone quantification

Following treatment with DCA or mock treatment, rosette leaves of 5-week-old plants were immediately frozen after harvest and ground in liquid nitrogen. For each treatment, 20 leaves from four plants were collected and 100 mg of material was freeze-dried for hormone extraction.

Hormone detection and quantification were performed by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS), as described previously (Le Roux *et al.*, 2014).

### RNA extraction and qRT-PCR analysis

For RNA extraction, nine leaves of three plants (DCA- or mock-treated) were collected at the indicated time point after treatment, pooled and immediately frozen in liquid nitrogen. Total RNA was extracted from 100 mg of frozen ground leaves using Trizol® reagent (Invitrogen Life Technologies, Saint-Aubin, France). RNA quality was evaluated by an electrophoretic run on 1% agarose gel. For the qRT-PCR analysis, first-strand cDNA was synthesized using Superscript reverse transcriptase SII (Invitrogen) from 1 μg of DNase-treated (Invitrogen) total RNA in a 20-μL reaction volume. qPCRs were performed using SYBR® Selected MasterMix 2x (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), following the manufacturer's protocol. The cycling conditions consisted of an initial 5 min at 95°C, followed by 40 three-step cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. Melting curve analysis was performed after cycle completion to validate amplicon identity. Relative expression levels were calculated following the standard curve-based method (Larionov *et al.*, 2005). Expression of the *EF1α* reference gene (Czechowski *et al.*, 2005; Degraeve *et al.*, 2013; Moreau *et al.*, 2012) was used for normalization of each target gene studied. For each treatment, two biological replicates were analysed and each qRT-PCR was carried out in triplicate. The gene-specific primers used in this analysis are indicated in Table S1 (see Supporting Information).

### Transcriptome studies

Microarray analysis (Fig. 3A,B; Table S2) was performed with CATMA chips containing 24 756 gene-specific tags corresponding to 22 089 genes from Arabidopsis (Crowe *et al.*, 2003; Hilson *et al.*, 2004). Two independent biological replicates were produced. Leaves of 5-week-old plants were sprayed with 200 μM DCA or mock-treated, collected 24 h after treatment and immediately frozen in liquid nitrogen. Total RNA was extracted using the Qiagen RNeasy kit according to the manufacturer's instructions. RNA integrity, cDNA synthesis hybridization and array scanning were performed as described previously (Lurin *et al.*, 2004). Statistical analysis was based on two dye swaps (Gagnot *et al.*, 2008). To determine differentially expressed genes, a paired *t*-test was performed on the log ratios. Spots displaying extreme variance (too small or too large) were excluded. The raw *P* values were adjusted by the Bonferroni method. Genes with a Bonferroni *P* ≤ 0.05 were considered to be differentially expressed (pink, red and green boxes in Fig. 3B). Enrichment of FunCatDB categories (Fig. 3A) was assessed by employing hypergeometric distribution with a *P*-value cut-off of 5 × 10<sup>-3</sup> (<http://mips.helmholtz-muenchen.de/funecatDB/>). For selected defence-associated genes, the transcriptomic data obtained following DCA treatment (this study) were compared with the transcriptomic data obtained following *E. amylovora* inoculation (Fig. 3B; Moreau *et al.*, 2012) and with data available in the Genevestigator database (Hruz *et al.*, 2008) obtained following inoculation with other pathogens, including *P. syringae* pv. *tomato* (Fig. 3C). The 15 datasets most similar to the DCA dataset for the selected defence-associated genes were identified using the Genevestigator 'Signature' tool (<https://genevestigator.com/>).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Fig. S1** Deoxycholic acid (DCA) induces callose accumulation in the hydathodes.

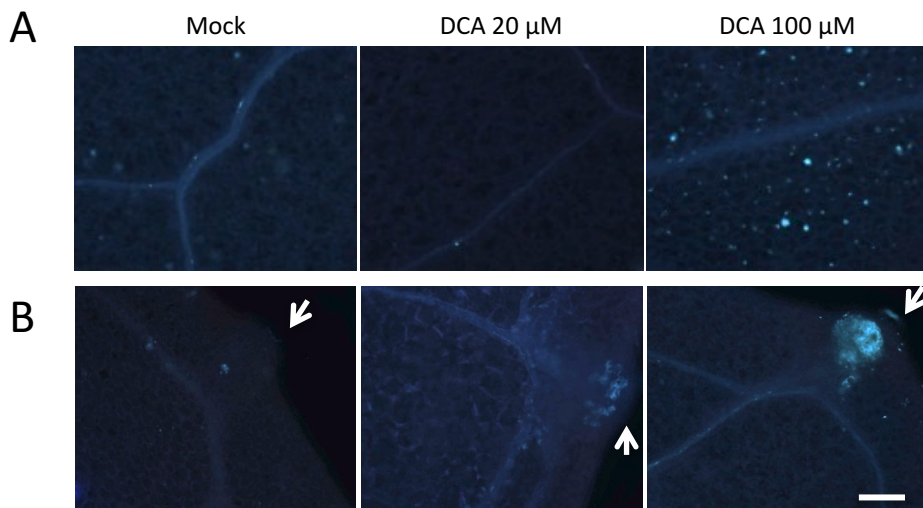
**Fig. S2** Symptoms induced by spraying increasing concentrations of deoxycholic acid (DCA) on Arabidopsis leaves.

**Fig. S3** Expression of drought marker genes in water-deprived plants.

**Fig. S4** Original images corresponding to Fig. 7.

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

**Table S2** Genes significantly up- and down-regulated 24 h after deoxycholic acid (DCA) treatment.



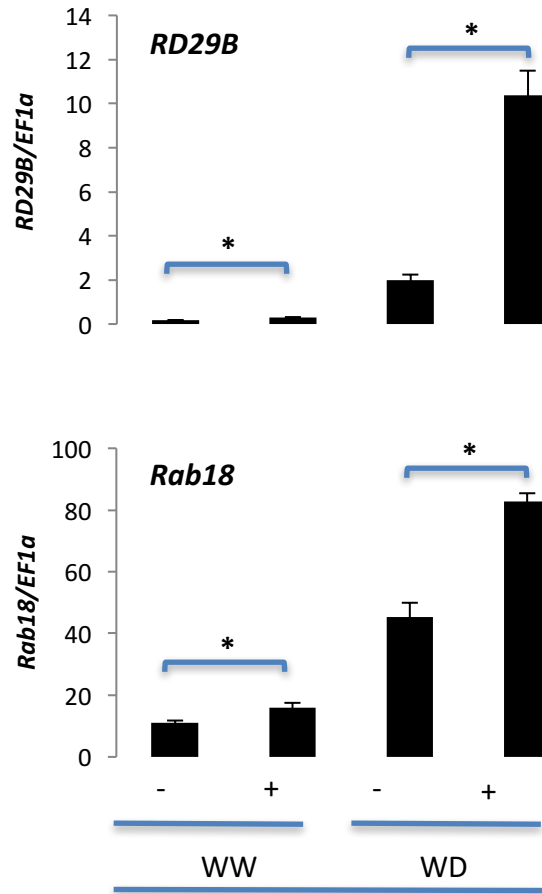
**Figure S1: DCA induces callose accumulation in the hydrotodes**

Arabidopsis leaves were sprayed with DCA at the indicated concentration or mock-treated. Leaves were stained with anilin blue 24 hpt to detect callose deposition. Experiments were conducted three times with similar results. Representative pictures of leaves (A) and leaf hydrotodes (B; pointed out by white arrows) are shown. The bar represents 200 μm.



**Figure S2: Symptoms induced by spraying increasing concentrations of DCA on Arabidopsis leaves.**

6 week-old Col-0 Arabidopsis plants were treated with mock-solution (A) or 20 (B), 100 (C), and 200  $\mu$ M DCA (D). For each conditions, six Arabidopsis plants were sprayed and five leaves per plant were collected for symptom analysis. All pictures were taken 5 days post-treatment. Representative pictures are reported. The experiment was repeated twice with similar results.

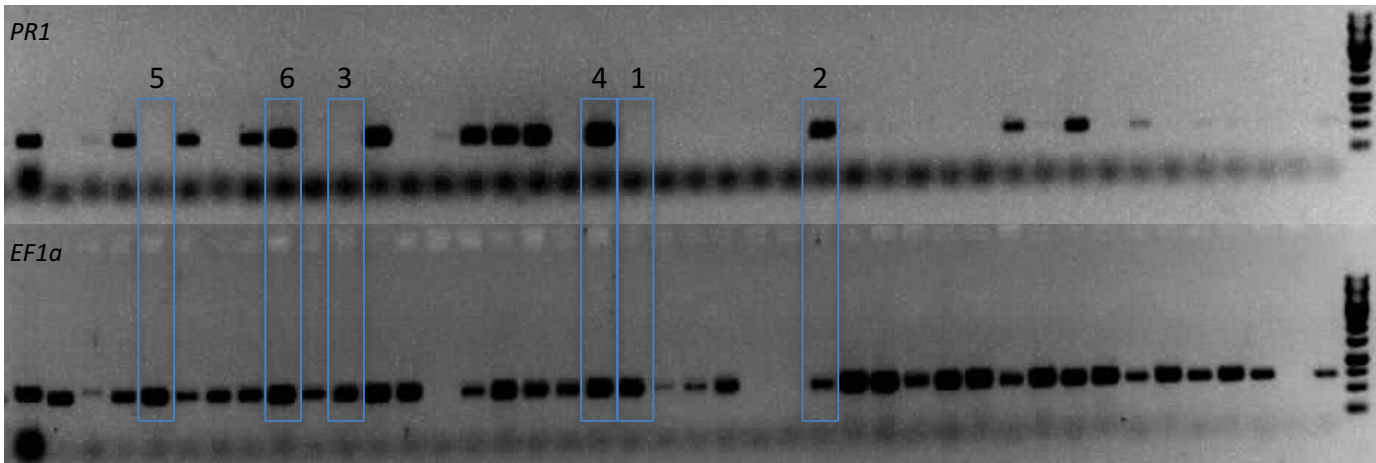


**Figure S3: Expression of drought marker genes in water-deprived plants**

**qRT-PCR** analysis of *RD29B* and *Rab18* genes 24 h after mock (-) or 200 μM DCA (+) treatment in *Arabidopsis Col-0* plants under well watered (WW) or water deficit (WD) as described in (Bouchabke *et al.*, 2008). The experiments were repeated twice with similar results. The star means significant differences according to Mann and Whitney's test ( $p < 0.05$ ). *Rab18* and *RD29B* are known markers of drought in *Arabidopsis* (Harb *et al.*, 2010)



	Col-0		w2-1		w3-1	
DCA:	-	+	-	+	-	+
<i>PR1</i>						
<i>EF1a</i>						
	1	2	3	4	5	6



**Figure S4: Original images corresponding to Fig. 7**

Upper panel: picture as presented in Fig. 7. Lower panel: original pictures of electrophoresis gels. 1-6: sample number in original pictures and modified version.

<b>gene</b>	<b>AGI</b>	<b>forward primer (5' --&gt; 3')</b>	<b>reverse primer (5' --&gt; 3')</b>
<i>CHI-B</i>	AT3G12500	ATCGTATAGGGTTTTATCAGAGGTATTGT	AGGAGGCCGTTAACGAAGGA
<i>EDSI</i>	AT3G48090	GAAGAAGCAGGAGCAGTCGT	CTCACAGCCATTTCCACAGA
<i>EF1a</i>	AT1G07940	CGTCACCCTAGCCGCTTTAC	CCCATGGTTAGAGACTGTCAAACA
<i>PDF1.2a</i>	AT5G44420	CACCGGCAATGGTGAA	CCCTGACCATGTCCCACTTG
<i>PRI</i>	AT2G14610	TCTTCCCTCGAAAGCTCAAGAT	TCTTCCCTCGAAAGCTCAAGAT
<i>Rab18</i>	AT5G66400	AGCTCGGAGGATGATGGACA	CACCGTAGCCACCAGCATCA
<i>RD29B</i>	AT5G52300	GCAAAGAACGTCGTTGCCTCA	GCCACTTTCTGCCCGTAAGC
<i>WAK2</i>	AT1G21270	TGAAGCTGGCCGTTGATATT	TTGATGAAGCAACAAGGAAATG
<i>WRKY46</i>	AT2G46400	TTGAGAACGGTGTGTGGAAA	CAACCAATCCTGTCCGAAAT
<i>WRKY70</i>	AT3G56400	GAGGACGCATTTTCTTGAG	TTGCTCTTGGGAGTTTCTGC

Supplementary Table 1: Detail of primers used in Zarattini *et al.* (2016) Mol. Plant Pathol.

Log2 (Ratio) color code:



AGI number	Gene description	log2 (ratio)	
		DCA vs	pval (bonferroni)
AT1G26390	"FAD-binding domain-containing protein"	5,41	0,00E+0
AT2G43570	"chitinase, putative"	5,29	0,00E+0
AT2G30770	"CYP71A13 (cytochrome P450, family 71, subfamily A, polypeptide 13); oxygen binding"	5,24	0,00E+0
AT1G13470	"unknown protein"	5,03	0,00E+0
AT3G57260	"BGL2 (PATHOGENESIS-RELATED PROTEIN 2); glucan 1,3-beta-glucosidase/ hydrolase, hydrolyzing O-glycosyl compounds"	4,98	0,00E+0
AT3G23120	"leucine-rich repeat family protein"	4,76	0,00E+0
AT1G73260	"trypsin and protease inhibitor family protein / Kunitz family protein"	4,76	0,00E+0
AT5G24200	"triacylglycerol lipase"	4,65	0,00E+0
AT2G14560	"unknown protein"	4,58	0,00E+0
AT3G13950	"unknown protein"	4,54	0,00E+0
AT5G26690	"heavy-metal-associated domain-containing protein"	4,48	0,00E+0
AT5G35525	"unknown protein"	4,41	0,00E+0
AT2G13810	"ALD1 (AGD2-LIKE DEFENSE RESPONSE PROTEIN1); transaminase"	4,39	0,00E+0
AT4G10500	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	4,38	0,00E+0
AT2G18660	"expansin family protein (EXPR3)"	4,33	0,00E+0
AT5E46700		4,22	0,00E+0
AT4G04490	"protein kinase family protein"	4,22	0,00E+0
AT2G47130	"short-chain dehydrogenase/reductase (SDR) family protein"	4,20	0,00E+0
AT5G55450	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	4,17	0,00E+0
AT3G11080	"disease resistance family protein"	4,12	0,00E+0
AT2G26400	"ARD/ATARD3 (ACIREDUCTONE DIOXYGENASE); acireductone dioxygenase (Fe2+-requiring)/ heteroglycan binding / metal ion	4,10	0,00E+0
AT1G21525	pseudogene	4,09	0,00E+0
AT3G11010	"disease resistance family protein / LRR family protein"	4,06	0,00E+0
AT3G57240	"BG3 (BETA-1,3-GLUCANASE 3); hydrolase, hydrolyzing O-glycosyl compounds"	4,06	0,00E+0
AT4G37370	"CYP81D8 (cytochrome P450, family 81, subfamily D, polypeptide 8); oxygen binding"	3,97	0,00E+0
AT2G24850	"TA3 (TYROSINE AMINOTRANSFERASE 3); transaminase"	3,96	0,00E+0
AT4G37990	"ELI3-2 (ELICITOR-ACTIVATED GENE 3)"	3,95	0,00E+0
AT1G10340	"ankyrin repeat family protein"	3,91	0,00E+0
AT4G35180	"LHT7 (LYS/HIS TRANSPORTER 7); amino acid permease"	3,91	0,00E+0
AT5G27060	"disease resistance family protein"	3,89	0,00E+0
AT1G02930	"ATGSTF6 (EARLY RESPONSIVE TO DEHYDRATION 11); glutathione transferase"	3,87	0,00E+0
AT5G26170	"WRKY50 (WRKY DNA-binding protein 50); transcription factor"	3,86	0,00E+0
AT5G38900	"DSBA oxidoreductase family protein"	3,86	0,00E+0
AT5G45380	"sodium:solute symporter family protein"	3,85	0,00E+0
AT2G39210	"nodulin family protein"	3,85	0,00E+0
AT5G54610	"ANK (ANKYRIN); protein binding"	3,84	0,00E+0
AT2G38240	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	3,83	0,00E+0
AT1G56120	"leucine-rich repeat family protein / protein kinase family protein"	3,82	0,00E+0
AT1G14870	"unknown protein"	3,81	0,00E+0
AT2G40750	"WRKY54 (WRKY DNA-binding protein 54); transcription factor"	3,81	0,00E+0
AT3G24982	"protein binding"	3,81	0,00E+0
AT5G39520	"unknown protein"	3,79	0,00E+0
AT3G01290	"band 7 family protein"	3,77	0,00E+0
AT3G46090	"ZAT7; nucleic acid binding / transcription factor/ zinc ion binding"	3,75	0,00E+0
AT1G28480	"glutaredoxin family protein"	3,75	0,00E+0
AT1G17745	"PGDH (3-PHOSPHOGLYCERATE DEHYDROGENASE); phosphoglycerate dehydrogenase"	3,75	0,00E+0
AT2G26020	"PDF1.2b (plant defensin 1.2b)"	3,73	0,00E+0
AT4G04500	"protein kinase family protein"	3,73	0,00E+0
AT3G48080	"lipase class 3 family protein / disease resistance protein-related"	3,72	0,00E+0
AT1G80840	"WRKY40 (WRKY DNA-binding protein 40); transcription factor"	3,72	0,00E+0
AT5G18470	"curculin-like (mannose-binding) lectin family protein"	3,67	0,00E+0
AT3G48640	"unknown protein"	3,67	0,00E+0
AT1G78410	"VQ motif-containing protein"	3,66	0,00E+0
AT5G10760	"aspartyl protease family protein"	3,61	0,00E+0
AT2G45550	"CYP76C4 (cytochrome P450, family 76, subfamily C, polypeptide 4); oxygen binding"	3,60	0,00E+0
AT1G57630	"disease resistance protein (TIR class), putative"	3,59	0,00E+0

AT1G15670	"kelch repeat-containing F-box family protein"	3.58	0.00E+0
AT4G23215	pseudogene	3.58	0.00E+0
AT2G26440	"pectinesterase family protein"	3.58	0.00E+0
AT1G03850	"glutaredoxin family protein"	3.57	0.00E+0
AT5G52760	"heavy-metal-associated domain-containing protein"	3.56	0.00E+0
AT4G39030	"EDS5 (ENHANCED DISEASE SUSCEPTIBILITY 5); antiporter/ transporter"	3.55	0.00E+0
AT2G15390	"FUT4 (fucosyltransferase 4); fucosyltransferase"	3.53	0.00E+0
AT4G23810	"WRKY53 (WRKY DNA-binding protein 53); transcription factor"	3.52	0.00E+0
AT2G04450	"ATNUDT6 (Arabidopsis thaliana Nudix hydrolase homolog 6); ADP-ribose diphosphatase/ NAD binding / hydrolase"	3.52	0.00E+0
AT2G44380	"DC1 domain-containing protein"	3.52	0.00E+0
AT3G50480	"HR4 (HOMOLOG OF RPW8 4)"	3.51	0.00E+0
AT3G47480	"calcium-binding EF hand family protein"	3.51	0.00E+0
AT3G26830	"PAD3 (PHYTOALEXIN DEFICIENT 3); oxygen binding"	3.51	0.00E+0
AT2G29350	"SAG13 (Senescence-associated gene 13); oxidoreductase"	3.50	0.00E+0
AT5G38900	"DSBA oxidoreductase family protein"	3.50	0.00E+0
AT5G60900	"RLK1 (RECEPTOR-LIKE PROTEIN KINASE 1); carbohydrate binding / kinase"	3.50	0.00E+0
AT1G48210	"serine/threonine protein kinase, putative"	3.49	0.00E+0
AT4G11890	"protein kinase family protein"	3.48	0.00E+0
AT5G55460	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	3.47	0.00E+0
AT3G26440	"unknown protein"	3.46	0.00E+0
AT4G03450	"ankyrin repeat family protein"	3.45	0.00E+0
AT2G39030	"GCN5-related N-acetyltransferase (GNAT) family protein"	3.45	0.00E+0
AT5G64810	"WRKY51 (WRKY DNA-binding protein 51); transcription factor"	3.45	0.00E+0
AT1G19020	"unknown protein"	3.44	0.00E+0
AT3G51860	"CA3 (cation exchanger 3); cation:cation antiporter"	3.44	0.00E+0
AT1G19250	"FMO1 (FLAVIN-DEPENDENT MONOOXYGENASE 1); monooxygenase"	3.43	0.00E+0
AT1G33960	"AIG1 (AVRRPT2-INDUCED GENE 1); GTP binding"	3.41	0.00E+0
AT1G56060	"unknown protein"	3.40	0.00E+0
AT3G22600	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	3.40	0.00E+0
AT5G13080	"WRKY75 (WRKY DNA-binding protein 75); transcription factor"	3.40	0.00E+0
AT3G26210	"CYP71B23 (cytochrome P450, family 71, subfamily B, polypeptide 23); oxygen binding"	3.40	0.00E+0
AT2G45760	"BAP2 (BON ASSOCIATION PROTEIN 2)"	3.38	0.00E+0
AT3G29252	pseudogene	3.38	0.00E+0
AT3G25020	"disease resistance family protein"	3.36	0.00E+0
AT3G51330	"aspartyl protease family protein"	3.35	0.00E+0
AT4G23140	"CRK6 (CYSTEINE-RICH RLK 6)"	3.34	0.00E+0
AT4G23150	"protein kinase family protein"	3.34	0.00E+0
AT1G08050	"zinc finger (C3HC4-type RING finger) family protein"	3.34	0.00E+0
AT2G35980	"YLS9 (YELLOW-LEAF-SPECIFIC GENE 9)"	3.34	0.00E+0
AT2G43510	"ATT1 (ARABIDOPSIS THALIANA TRYPSIN INHIBITOR PROTEIN 1)"	3.34	0.00E+0
AT1G48220	"serine/threonine protein kinase, putative"	3.32	0.00E+0
AT5G05600	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	3.32	0.00E+0
AT2G04495	"unknown protein"	3.32	0.00E+0
AT4G21850	"methionine sulfoxide reductase domain-containing protein / SelR domain-containing protein"	3.32	0.00E+0
AT3G13610	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	3.31	0.00E+0
AT5G25930	"leucine-rich repeat family protein / protein kinase family protein"	3.31	0.00E+0
AT2G47130	"short-chain dehydrogenase/reductase (SDR) family protein"	3.30	0.00E+0
AT3G60966	"protein binding / zinc ion binding"	3.29	0.00E+0
AT1G76960	"unknown protein"	3.28	0.00E+0
AT1G19320	"pathogenesis-related thaumatin family protein"	3.28	0.00E+0
AT3G21080	"ABC transporter-related"	3.27	0.00E+0
AT2G18690	"unknown protein"	3.27	0.00E+0
AT3G25010	"disease resistance family protein"	3.26	0.00E+0
AT1G24145	"unknown protein"	3.26	0.00E+0
AT2G45220	"pectinesterase family protein"	3.26	0.00E+0
AT5G45000	"transmembrane receptor"	3.26	0.00E+0
AT3G25882	"NIMIN-2 (NIM1-INTERACTING 2)"	3.24	0.00E+0
AT4G18250	"receptor serine/threonine kinase, putative"	3.24	0.00E+0
AT1G17745	"PGDH (3-PHOSPHOGLYCERATE DEHYDROGENASE); phosphoglycerate dehydrogenase"	3.22	0.00E+0
AT4G10860	"unknown protein"	3.22	0.00E+0
AT1G26400	"FAD-binding domain-containing protein"	3.21	0.00E+0
AT1G36622	"unknown protein"	3.21	0.00E+0
AT1G67980	"CCoAMT (caffeoyl-CoA 3-O-methyltransferase)"	3.20	0.00E+0
AT5G53110	"unknown protein"	3.19	0.00E+0
AT4G23210	"protein kinase family protein"	3.18	0.00E+0
AT2G29460	"ATGSTU4 (GLUTATHIONE S-TRANSFERASE 22); glutathione transferase"	3.17	0.00E+0
AT5G25260	"unknown protein"	3.16	0.00E+0
AT2G31865	"poly (ADP-ribose) glycohydrolase (PARG) family protein"	3.15	0.00E+0
AT1G65500	"unknown protein"	3.15	0.00E+0
AT4G23220	"protein kinase family protein"	3.15	0.00E+0
AT3G24900	"disease resistance family protein / LRR family protein"	3.14	0.00E+0
AT2G31880	"leucine-rich repeat transmembrane protein kinase, putative"	3.13	0.00E+0
AT1G07000	"ATEX070B2 (exocyst subunit EXO70 family protein B2); protein binding"	3.13	0.00E+0
AT2G22500	"mitochondrial substrate carrier family protein"	3.12	0.00E+0
AT3G55970	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	3.12	0.00E+0
AT5G42830	"transferase family protein"	3.12	0.00E+0
AT3G23550	"MATE efflux family protein"	3.12	0.00E+0
AT2G14610	"PR1 (PATHOGENESIS-RELATED GENE 1)"	3.12	0.00E+0
AT5G60950	"phytochelatin synthetase-related"	3.11	0.00E+0
AT5G44420	"PDF1.2 (Low-molecular-weight cysteine-rich 77)"	3.10	0.00E+0

AT1G65690	"harpin-induced protein-related / HIN1-related / harpin-responsive protein-related"	3.10	0.00E+0
AT1G35710	"leucine-rich repeat transmembrane protein kinase, putative"	3.09	0.00E+0
AT5G02780	"ln2-1 protein, putative"	3.09	0.00E+0
AT4G23160	"protein kinase family protein"	3.08	0.00E+0
AT5G48657	"defense protein-related"	3.07	0.00E+0
AT5G06860	"PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1); protein binding"	3.07	0.00E+0
AT5G25440	"protein kinase family protein"	3.06	0.00E+0
AT5G47220	"ATERF-2/ATERF2/ERF2 (ETHYLENE RESPONSE FACTOR 2); DNA binding / transcription factor/ transcriptional activator"	3.06	0.00E+0
AT5G24530	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	3.04	0.00E+0
AT3G50770	"calmodulin-related protein, putative"	3.03	0.00E+0
AT2G32680	"disease resistance family protein"	3.03	0.00E+0
AT3G57700	"protein kinase, putative"	3.03	0.00E+0
AT4G15610	"integral membrane family protein"	3.02	0.00E+0
AT3G50460	"HR2 (HOMOLOG OF RPW8 2)"	3.02	0.00E+0
AT5G22570	"WRKY38 (WRKY DNA-binding protein 38); transcription factor"	3.01	0.00E+0
AT1G26420	"FAD-binding domain-containing protein"	3.00	0.00E+0
AT5G64000	"SAL2; 3p(2p),5p-bisphosphate nucleotidase/ inositol or phosphatidylinositol phosphatase"	3.00	0.00E+0
AT2G20142	"unknown protein"	3.00	0.00E+0
AT5G44430	"PDF1.2c (plant defensin 1.2c)"	2.98	0.00E+0
AT1G02450	"NIMIN-1/NIMIN1; protein binding"	2.95	0.00E+0
AT5G35735	"auxin-responsive family protein"	2.95	0.00E+0
AT5G05460	"hydrolase, acting on glycosyl bonds"	2.95	0.00E+0
AT1G35230	"AGP5 (ARABINOGLACTAN-PROTEIN 5)"	2.93	0.00E+0
AT4G23130	"CRK5 (CYSTEINE-RICH RLK5)"	2.91	0.00E+0
AT4G11840	"PLDGAMMA3 (phospholipase D gamma 3); phospholipase D"	2.90	0.00E+0
AT5G50200	"WR3 (WOUND-RESPONSIVE 3)"	2.90	0.00E+0
AT1G23840	"unknown protein"	2.89	0.00E+0
AT2G18670	"zinc finger (C3HC4-type RING finger) family protein", "unknown protein"	2.89	0.00E+0
AT2G46150	"unknown protein"	2.88	0.00E+0
AT3G23110	"disease resistance family protein"	2.88	0.00E+0
AT3G09940	"MDHAR (MONODEHYDROASCORBATE REDUCTASE); monodehydroascorbate reductase (NADH)"	2.88	0.00E+0
AT3G29260	"short-chain dehydrogenase/reductase (SDR) family protein"	2.87	0.00E+0
AT5G26920	"calmodulin binding"	2.87	0.00E+0
AT3G25180	"CYP82G1 (cytochrome P450, family 82, subfamily G, polypeptide 1); oxygen binding"	2.87	0.00E+0
AT2G30750	"CYP71A12 (cytochrome P450, family 71, subfamily A, polypeptide 12); oxygen binding"	2.87	0.00E+0
AT1G11330	"S-locus lectin protein kinase family protein"	2.86	0.00E+0
AT1G01680	"U-box domain-containing protein"	2.85	0.00E+0
AT2G04515	"unknown protein"	2.85	0.00E+0
AT1G34420	"leucine-rich repeat family protein / protein kinase family protein"	2.85	0.00E+0
AT4G23610	"unknown protein"	2.85	0.00E+0
AT4G14365	"zinc finger (C3HC4-type RING finger) family protein / ankyrin repeat family protein"	2.84	0.00E+0
AT1G12290	"disease resistance protein (CC-NBS-LRR class), putative"	2.84	0.00E+0
AT4G25110	caspase	2.83	0.00E+0
AT1G30900	"vacuolar sorting receptor, putative"	2.83	0.00E+0
AT1G10070	"ATBCAT-2; branched-chain-amino-acid transaminase/ catalytic"	2.82	0.00E+0
AT5G57550	"XTR3 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 3); hydrolase, acting on glycosyl bonds"	2.81	0.00E+0
AT1G72900	"disease resistance protein (TIR-NBS class), putative"	2.80	0.00E+0
AT5G44568	"unknown protein"	2.80	0.00E+0
AT3G15518	"unknown protein"	2.80	0.00E+0
AT1G74440	"unknown protein"	2.80	0.00E+0
AT2G25000	"WRKY60 (WRKY DNA-binding protein 60); transcription factor"	2.80	0.00E+0
AT4G14630	"GLP9 (GERMIN-LIKE PROTEIN 9); manganese ion binding / metal ion binding / nutrient reservoir"	2.80	0.00E+0
AT5G01540	"lectin protein kinase, putative"	2.80	0.00E+0
AT4G21840	"methionine sulfoxide reductase domain-containing protein / SelR domain-containing protein"	2.79	0.00E+0
AT3G56710	"SIB1 (SIGMA FACTOR BINDING PROTEIN 1); binding"	2.79	0.00E+0
AT2G39210	"nodulin family protein"	2.79	0.00E+0
AT3G07195	"proline-rich family protein"	2.79	0.00E+0
AT2G26560	"PLP2 (PHOSPHOLIPASE A 2A); nutrient reservoir"	2.78	0.00E+0
AT1G74590	"ATGSTU10 (Arabidopsis thaliana Glutathione S-transferase (class tau) 10); glutathione transferase"	2.78	0.00E+0
AT4G12480	"pEARL1 1; lipid binding"	2.77	0.00E+0
AT3G26500	"leucine-rich repeat family protein"	2.77	0.00E+0
AT2G17040	"ANAC036 (Arabidopsis NAC domain containing protein 36); transcription factor"	2.77	0.00E+0
AT1G21250	"WAK1 (CELL WALL-ASSOCIATED KINASE); kinase"	2.76	0.00E+0
AT4G20110	"vacuolar sorting receptor, putative"	2.76	0.00E+0
AT5G05340	"peroxidase, putative"	2.75	0.00E+0
AT1G03660	"unknown protein"	2.75	0.00E+0
AT5G39670	"calcium-binding EF hand family protein"	2.75	0.00E+0
AT5G44585	"unknown protein"	2.75	0.00E+0
AT1G13340	"unknown protein"	2.75	0.00E+0
AT5G60280	"lectin protein kinase family protein"	2.75	0.00E+0
AT5G54860	"integral membrane transporter family protein"	2.74	0.00E+0
AT1G13990	"unknown protein"	2.74	0.00E+0
AT5G55170	"SUM3 (SMALL UBIQUITIN-LIKE MODIFIER 3)"	2.73	0.00E+0
AT3G11340	"UDP-glucuronosyl/UDP-glucosyl transferase family protein"	2.73	0.00E+0
AT2G32190	"unknown protein"	2.72	0.00E+0
AT1G61420	"S-locus lectin protein kinase family protein"	2.72	0.00E+0
AT1G43910	"AAA-type ATPase family protein"	2.72	0.00E+0
AT3G60540	"sec61beta family protein"	2.72	0.00E+0
AT3G09830	"protein kinase, putative"	2.71	0.00E+0
AT5G64510	"unknown protein"	2.71	0.00E+0

AT3G44300	"NIT2 (NITRILASE 2)"	2.69	0.00E+0
AT2G04430	"ATNUDT5 (Arabidopsis thaliana Nudix hydrolase homolog 5); hydrolase"	2.69	0.00E+0
AT1G72060	"serine-type endopeptidase inhibitor"	2.69	0.00E+0
AT3G48650	pseudogene	2.69	0.00E+0
AT5G20230	"ATBCB (ARABIDOPSIS BLUE-COPPER-BINDING PROTEIN); copper ion binding"	2.68	0.00E+0
AT3G50930	"AAA-type ATPase family protein"	2.68	0.00E+0
AT5G13320	"PBS3 (AVRPPHB SUSCEPTIBLE 3)"	2.68	0.00E+0
AT4G15233	"ATP binding / ATPase/ nucleoside-triphosphatase/ nucleotide binding"	2.68	0.00E+0
AT3G16565	"ATP binding / alanine-tRNA ligase"	2.68	0.00E+0
AT5G22380	"ANAC090 (Arabidopsis NAC domain containing protein 90); transcription factor"	2.67	0.00E+0
AT1G24140	"matrixin family protein"	2.67	0.00E+0
AT5G26340	"MSS1 (SUGAR TRANSPORT PROTEIN 13); carbohydrate transporter/ hexose:hydrogen symporter/ high-affinity hydrogen:glucose symporter"	2.67	0.00E+0
AT1G66690	"S-adenosyl-L-methionine:carboxyl methyltransferase family protein"	2.67	0.00E+0
AT3G50470	"HR3 (HOMOLOG OF RPW8 3)"	2.67	0.00E+0
AT2G43820	"GTUGT74F2 (UDP-GLUCOSYLTRANSFERASE 74F2); UDP-glucosyltransferase/ UDP-glycosyltransferase/ transferase, transferring"	2.67	0.00E+0
AT5G08760	"unknown protein"	2.66	0.00E+0
AT4G31800	"WRKY18 (WRKY DNA-binding protein 18); transcription factor"	2.66	0.00E+0
AT1G51860	"leucine-rich repeat protein kinase, putative"	2.66	0.00E+0
AT1G21520	"unknown protein"	2.65	0.00E+0
AT4G28490	"HAESA (RECEPTOR-LIKE PROTEIN KINASE 5); ATP binding / kinase/ protein serine/threonine kinase"	2.64	0.00E+0
AT5G52750	"heavy-metal-associated domain-containing protein"	2.63	0.00E+0
AT2G43000	"ANAC042 (Arabidopsis NAC domain containing protein 42); transcription factor"	2.63	0.00E+0
AT3G22235	"unknown protein", "unknown protein"	2.63	0.00E+0
AT4G38540	"monooxygenase, putative (MO2)"	2.63	0.00E+0
AT4G39030	"EDS5 (ENHANCED DISEASE SUSCEPTIBILITY 5); antiporter/ transporter"	2.61	0.00E+0
AT4G23230	"protein kinase family protein"	2.61	0.00E+0
AT4G34131	"UGT73B3 (UDP-glucosyl transferase 73B3); UDP-glycosyltransferase/ abscisic acid glucosyltransferase/ transferase, transferring"	2.60	0.00E+0
AT1G66700	"S-adenosyl-L-methionine:carboxyl methyltransferase family protein"	2.60	0.00E+0
AT3G11840	"U-box domain-containing protein"	2.60	0.00E+0
AT3G12220	"SCPL16 (serine carboxypeptidase-like 16); serine carboxypeptidase"	2.60	0.00E+0
AT1G06620	"2-oxoglutarate-dependent dioxygenase, putative"	2.60	0.00E+0
AT5G67450	"AZF1 (ARABIDOPSIS ZINC-FINGER PROTEIN 1); nucleic acid binding / transcription factor/ zinc ion binding"	2.59	0.00E+0
AT3G50260	"ATERF#011/CEJ1 (COOPERATIVELY REGULATED BY ETHYLENE AND JASMONATE 1); DNA binding / transcription factor"	2.59	0.00E+0
AT1G75040	"PR5 (PATHOGENESIS-RELATED GENE 5)"	2.59	0.00E+0
AT1G21120	"O-methyltransferase, putative"	2.59	0.00E+0
AT5G03350	"legume lectin family protein"	2.58	0.00E+0
AT5G44820	"unknown protein"	2.58	0.00E+0
AT5G40780	"LHT1 (LYSINE HISTIDINE TRANSPORTER 1); amino acid permease/ amino acid transporter"	2.57	0.00E+0
AT3G46080	"zinc finger (C2H2 type) family protein"	2.56	0.00E+0
AT1G45145	"ATTRX5 (thioredoxin H-type 5); thiol-disulfide exchange intermediate"	2.56	0.00E+0
AT1G02920	"ATGSTF7 (GLUTATHIONE S-TRANSFERASE 11); glutathione transferase"	2.55	0.00E+0
AT1G66090	"disease resistance protein (TIR-NBS class), putative"	2.55	0.00E+0
AT5G59670	"leucine-rich repeat protein kinase, putative"	2.55	0.00E+0
AT4G39670	"unknown protein"	2.55	0.00E+0
AT1G67810	"Fe-S metabolism associated domain-containing protein"	2.55	0.00E+0
AT1G72680	"cinnamyl-alcohol dehydrogenase, putative"	2.54	0.00E+0
AT4G11000	"ankyrin repeat family protein"	2.54	0.00E+0
AT5G35735	"auxin-responsive family protein"	2.54	0.00E+0
AT2G13800	"ATSERK5 (SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE 5); ATP binding / protein kinase/ transmembrane receptor"	2.53	0.00E+0
AT3G22160	"VQ motif-containing protein"	2.53	0.00E+0
AT5G25250	"unknown protein"	2.53	0.00E+0
AT3G09270	"ATGSTU8 (Arabidopsis thaliana Glutathione S-transferase (class tau) 8); glutathione transferase"	2.53	0.00E+0
AT3G48650	pseudogene	2.53	0.00E+0
AT2G46400	"WRKY46 (WRKY DNA-binding protein 46); transcription factor"	2.52	0.00E+0
AT5G06860	"PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1); protein binding"	2.52	0.00E+0
AT5G37600	"ATGSR1 (Arabidopsis thaliana glutamine synthase clone R1); glutamate-ammonia ligase"	2.51	0.00E+0
AT4G12490	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	2.51	0.00E+0
AT4G01750	"RGXT2 (RHAMNOGALACTURONAN XYLOSYLTRANSFERASE 2); UDP-xylosyltransferase"	2.51	0.00E+0
AT3G51450	"strictosidine synthase family protein"	2.51	0.00E+0
AT1G16420	"latex-abundant protein, putative (AMC8) / caspase family protein"	2.51	0.00E+0
AT1G15790	"unknown protein"	2.51	0.00E+0
AT1G04980	"ATPDIL2-2 (PDI-LIKE 2-2); thiol-disulfide exchange intermediate"	2.50	0.00E+0
AT4G15236	"ABC transporter family protein"	2.50	0.00E+0
AT4G11850	"PLDGAMMA1 (maternal effect embryo arrest 54); phospholipase D"	2.49	0.00E+0
AT2G33030	"leucine-rich repeat family protein"	2.49	0.00E+0
AT1G67920	"unknown protein"	2.47	0.00E+0
AT1G24147	"unknown protein"	2.47	0.00E+0
AT2G04070	"MATE efflux family protein"	2.46	0.00E+0
AT3G51440	"strictosidine synthase family protein"	2.45	0.00E+0
AT1G21270	"WAK2 (wall-associated kinase 2); protein serine/threonine kinase"	2.45	0.00E+0
AT4G18170	"WRKY28 (WRKY DNA-binding protein 28); transcription factor"	2.45	0.00E+0
AT1G32940	"subtilase family protein"	2.45	0.00E+0
AT3G22235	"unknown protein", "unknown protein"	2.45	0.00E+0
AT1G08450	"CRT3 (CALRETICULIN 3); calcium ion binding"	2.45	0.00E+0
AT2G23680	"stress-responsive protein, putative"	2.44	0.00E+0
AT3G15356	"legume lectin family protein"	2.42	0.00E+0
AT3G10930	"unknown protein"	2.41	0.00E+0
AT1G02360	"chitinase, putative"	2.41	0.00E+0
AT3G10114	pseudogene	2.40	0.00E+0
AT4G08470	"MAPKKK10 (Mitogen-activated protein kinase kinase kinase 10); kinase"	2.40	0.00E+0

AT2G26010	"PDF1.3 (plant defensin 1.3)"	2.39	0.00E+0
AT1G05340	"unknown protein"	2.39	0.00E+0
AT4G18250	"receptor serine/threonine kinase, putative"	2.39	0.00E+0
AT4G25070	"unknown protein"	2.38	0.00E+0
AT1G09932	"phosphoglycerate/bisphosphoglycerate mutase-related"	2.37	0.00E+0
AT5G15870	"glycosyl hydrolase family 81 protein"	2.37	0.00E+0
AT5G27420	"zinc finger (C3HC4-type RING finger) family protein"	2.36	0.00E+0
AT4G29520	"unknown protein"	2.36	0.00E+0
AT5G53550	"YSL3 (YELLOW STRIPE LIKE 3); oligopeptide transporter"	2.36	8.52E-12
AT4G38560	"unknown protein"	2.35	8.52E-12
AT1G07400	"17.8 kDa class I heat shock protein (HSP17.8-Cl)"	2.35	8.52E-12
AT5G50560	"unknown protein", "unknown protein"	2.34	8.52E-12
AT3G57700		2.34	8.52E-12
AT3G56400	"WRKY70 (WRKY DNA-binding protein 70); transcription factor"	2.34	8.52E-12
AT5G52740	"heavy-metal-associated domain-containing protein"	2.33	8.52E-12
AT2G29720	"CTF2B; monooxygenase"	2.32	1.70E-11
AT3G28540	"AAA-type ATPase family protein"	2.31	1.70E-11
AT2G24160	pseudogene	2.30	2.56E-11
AT3G51660	"macrophage migration inhibitory factor family protein / MIF family protein"	2.30	2.56E-11
AT5G04340	"C2H2; nucleic acid binding / transcription factor/ zinc ion binding"	2.29	3.41E-11
AT1G28370	"ATERF11/ERF11 (ERF domain protein 11); DNA binding / transcription factor/ transcriptional repressor"	2.29	3.41E-11
AT1G73805	"calmodulin-binding protein"	2.28	5.1E-11
AT5G39020	"protein kinase family protein"	2.28	5.1E-11
AT2G40095	"unknown protein"	2.27	5.1E-11
AT1G11330	"S-locus lectin protein kinase family protein"	2.27	6.82E-11
AT1G07900	"LOB domain protein 1 / lateral organ boundaries domain protein 1 (LBD1)"	2.26	8.52E-11
AT3G48090	"EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1); signal transducer/ triacylglycerol lipase"	2.26	8.52E-11
AT1G58225	"unknown protein"	2.24	1.53E-10
AT1G35513	pseudogene	2.24	1.53E-10
AT4G26070	"MEK1 (mitogen-activated protein kinase kinase 1); MAP kinase kinase/ kinase"	2.23	1.70E-10
AT1G61460	"S-locus protein kinase, putative"	2.23	1.96E-10
AT3G49120	"ATPCB/ATPERX34/PERX34/PRXCB (PEROXIDASE 34); peroxidase"	2.23	2.05E-10
AT4G22530	"embryo-abundant protein-related"	2.22	2.64E-10
AT4G17470	"palmitoyl protein thioesterase family protein"	2.22	2.90E-10
AT5G07770	"formin homology 2 domain-containing protein / FH2 domain-containing protein"	2.21	3.58E-10
AT1G27980	"pyridoxal-dependent decarboxylase family protein"	2.21	3.75E-10
AT4G01770	"RGXT1 (RHAMNOGALACTURONAN XYLOSYLTRANSFERASE 1); UDP-xylosyltransferase"	2.21	3.84E-10
AT4G23600	"CORI3 (CORONATINE INDUCED 1, JASMONIC ACID RESPONSIVE 2); transaminase"	2.20	4.77E-10
AT1G76970	"VHS domain-containing protein / GAT domain-containing protein"	2.20	4.77E-10
AT3G51430	"YLS2 (yellow-leaf-specific gene 2); strictosidine synthase"	2.19	5.29E-10
AT3G49620	"DIN11 (DARK INDUCIBLE 11); oxidoreductase"	2.19	5.46E-10
AT4G18880	"AT-HSFA4A (Arabidopsis thaliana heat shock transcription factor A4A); DNA binding / transcription factor"	2.19	5.89E-10
AT2G24600	"ankyrin repeat family protein"	2.19	6.05E-10
AT3G45860	"receptor-like protein kinase, putative"	2.18	6.99E-10
AT4G23170	"EP1; protein kinase"	2.18	7.93E-10
AT2G43590	"chitinase, putative"	2.18	8.35E-10
AT2G38940	"ATP2 (PHOSPHATE TRANSPORTER 2); carbohydrate transporter/ phosphate transporter/ sugar porter"	2.17	9.97E-10
AT1G76930	"ATEXT4 (EXTENSIN 4)"	2.17	9.97E-10
AT3G04720	"PR4 (PATHOGENESIS-RELATED 4)"	2.17	1.01E-9
AT1G20350	"ATTIM17-1 (Arabidopsis thaliana translocase inner membrane subunit 17-1); protein translocase"	2.17	1.05E-9
AT1G72910	"disease resistance protein (TIR-NBS class), putative"	2.17	1.1E-9
AT5G22520	"unknown protein"	2.17	1.12E-9
AT1G76600	"unknown protein"	2.16	1.25E-9
AT1G76980	"unknown protein"	2.15	1.57E-9
AT4G13510	"AMT1;1 (AMMONIUM TRANSPORT 1); ammonium transporter"	2.15	1.70E-9
AT2G46430	"ATCNGC3 (CYCLIC NUCLEOTIDE GATED CHANNEL 3); calmodulin binding / cyclic nucleotide binding / ion channel"	2.15	1.88E-9
AT1G14880	"unknown protein"	2.14	2.05E-9
AT5G33290	"XGD1 (XYLOGALACTURONAN DEFICIENT 1); catalytic"	2.14	2.07E-9
AT3G22231	"PCC1 (PATHOGEN AND CIRCADIAN CONTROLLED 1)"	2.14	2.21E-9
AT2G46440	"ATCNGC11 (cyclic nucleotide gated channel 11); calmodulin binding / cyclic nucleotide binding / ion channel"	2.13	2.76E-9
AT4G13510	"AMT1;1 (AMMONIUM TRANSPORT 1); ammonium transporter"	2.13	2.79E-9
AT2G02930	"ATGSTF3 (GLUTATHIONE S-TRANSFERASE 16); glutathione transferase"	2.13	2.97E-9
AT4G38550	"unknown protein"	2.13	3.24E-9
AT1G76040	"CPK29 (calcium-dependent protein kinase 29); calcium- and calmodulin-dependent protein kinase/ kinase"	2.12	3.78E-9
AT1G73500	"ATMKK9 (Arabidopsis thaliana MAP kinase kinase 9); kinase"	2.12	3.85E-9
AT5G02490	"heat shock cognate 70 kDa protein 2 (HSC70-2) (HSP70-2)"	2.12	3.91E-9
AT1G65845	"unknown protein"	2.12	4.07E-9
AT3G52430	"PAD4 (PHYTOALEXIN DEFICIENT 4); triacylglycerol lipase"	2.11	4.91E-9
AT2G38290	"ATAMT2 (AMMONIUM TRANSPORTER 2)"	2.11	4.93E-9
AT1G75030	"ATLP-3 (Arabidopsis thaumatin-like protein 3)"	2.10	5.72E-9
AT3G22235	"unknown protein", "unknown protein"	2.10	6.60E-9
AT3G22060	"receptor protein kinase-related"	2.09	7.73E-9
AT3G04717	pseudogene	2.09	8.47E-9
AT4G26270	"phosphofructokinase family protein"	2.09	8.58E-9
AT1G25400	"unknown protein"	2.09	8.88E-9
AT1G35210	"unknown protein"	2.09	9.08E-9
AT2G04260	pseudogene	2.09	9.21E-9
AT1G34750	"protein phosphatase 2C, putative / PP2C, putative"	2.09	9.33E-9
AT5G24780	"VSP1 (VEGETATIVE STORAGE PROTEIN 1); acid phosphatase"	2.08	9.68E-9
AT5G52810	"ornithine cyclodeaminase/mu-crystallin family protein"	2.08	9.71E-9



AT5G57220	"CYP81F2 (cytochrome P450, family 81, subfamily F, polypeptide 2); oxygen binding"	2.08	9.93E-9
AT5G19230	"unknown protein"	2.08	1.20E-8
AT3E15070		2.08	1.21E-8
AT2G31060	"GTP binding / translation elongation factor"	2.08	1.24E-8
AT3G60420	"unknown protein"	2.07	1.27E-8
AT2G34940	"vacuolar sorting receptor, putative"	2.07	1.34E-8
AT1G14370	"APK2A (PROTEIN KINASE 2A); kinase"	2.07	1.40E-8
AT3G08870	"lectin protein kinase, putative"	2.07	1.47E-8
AT1G65486	"unknown protein"	2.06	1.64E-8
AT3G23560	"ALF5 (ABERRANT LATERAL ROOT FORMATION 5); antiporter/ transporter"	2.06	1.67E-8
AT2G17120	"peptidoglycan-binding LysM domain-containing protein"	2.06	1.93E-8
AT3G14620	"CYP72A8 (cytochrome P450, family 72, subfamily A, polypeptide 8); oxygen binding"	2.06	1.95E-8
AT4G08850	"leucine-rich repeat family protein / protein kinase family protein"	2.05	2.21E-8
AT2G43590	"chitinase, putative"	2.05	2.24E-8
AT5G58940	"CRCK1 (CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASMIC KINASE 1); kinase"	2.05	2.42E-8
AT3G62780	"C2 domain-containing protein"	2.05	2.47E-8
AT3G01080	"WRKY58 (WRKY DNA-binding protein 58); transcription factor"	2.04	3.10E-8
AT2G41100	"TCH3 (TOUCH 3)"	2.04	3.21E-8
AT4G02220	"zinc finger (MYND type) family protein / programmed cell death 2 C-terminal domain-containing protein"	2.04	3.23E-8
AT5G42440	"protein kinase family protein"	2.04	3.31E-8
AT2G32140	"transmembrane receptor"	2.04	3.35E-8
AT2G30250	"WRKY25 (WRKY DNA-binding protein 25); transcription factor"	2.03	4.07E-8
AT3G09010	"protein kinase family protein"	2.02	4.86E-8
AT5G19240	"unknown protein"	2.02	5.16E-8
AT5G20400	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	2.02	5.19E-8
AT4G02380	"SAG21 (SENESCENCE-ASSOCIATED GENE 21)"	2.02	5.81E-8
AT4G29520	"unknown protein"	2.01	5.84E-8
AT5G48380	"leucine-rich repeat family protein / protein kinase family protein"	2.01	5.92E-8
AT3G29000	"calcium-binding EF hand family protein"	2.01	6.46E-8
AT1G53920	"GLIP5 (GDSL-motif lipase 5); carboxylic ester hydrolase"	2.01	6.81E-8
AT5G24210	"lipase class 3 family protein"	1.99	1.04E-7
AT5G47070	"protein kinase, putative"	1.98	1.28E-7
AT5G02290	"NAK; kinase"	1.98	1.53E-7
AT4G08770	"peroxidase, putative"	1.97	1.67E-7
AT3G05650	"disease resistance family protein"	1.97	1.90E-7
AT3G28210	"PMZ; zinc ion binding"	1.97	1.90E-7
AT4G04695	"CPK31 (calcium-dependent protein kinase 31); calcium- and calmodulin-dependent protein kinase/ kinase"	1.97	2.00E-7
AT1G53620	"unknown protein"	1.96	2.04E-7
AT4G11840	"PLDGAMMA3 (phospholipase D gamma 3); phospholipase D"	1.96	2.25E-7
AT5G08790	"ATAF2 (Arabidopsis NAC domain containing protein 81)"	1.96	2.27E-7
AT2G15080	"disease resistance family protein"	1.96	2.38E-7
AT2G44400	"DC1 domain-containing protein"	1.95	2.64E-7
AT1G69520	methyltransferase-related	1.95	2.65E-7
AT1G60730	"aldo/keto reductase family protein"	1.95	2.84E-7
AT1G27100	"unknown protein"	1.95	2.93E-7
AT3G26600	"armadillo/beta-catenin repeat family protein"	1.95	3.00E-7
AT3G45860	"receptor-like protein kinase, putative"	1.95	3.01E-7
AT1G33420	"PHD finger family protein"	1.95	3.26E-7
AT2G28940	"protein kinase family protein"	1.95	3.29E-7
AT1G72280	"AERO1 (ARABIDOPSIS ENDOPLASMIC RETICULUM OXIDOREDUCTINS 1)"	1.94	3.93E-7
AT3G47090	"leucine-rich repeat transmembrane protein kinase, putative"	1.94	4.19E-7
AT1G18570	"MYB51 (myb domain protein 51); DNA binding / transcription factor"	1.93	4.27E-7
AT4G23180	"CRK10 (CYSTEINE-RICH RLK10); kinase"	1.93	5.00E-7
AT4G13810	"disease resistance family protein / LRR family protein"	1.92	5.51E-7
AT5G61520	"hexose transporter, putative"	1.92	5.67E-7
AT1G01560	"ATMPK11 (Arabidopsis thaliana MAP kinase 11); MAP kinase/ kinase"	1.92	5.82E-7
AT5G45110	"NPR3 (NPR1-LIKE PROTEIN 3); protein binding"	1.92	6.10E-7
AT1G13750	"calcineurin-like phosphoesterase family protein"	1.92	6.36E-7
AT5G41750	"disease resistance protein (TIR-NBS-LRR class), putative"	1.92	6.68E-7
AT5G56870	"beta-galactosidase, putative / lactase, putative"	1.91	7.64E-7
AT2G15480	"UGT73B5 (UDP-glucosyl transferase 73B5); UDP-glycosyltransferase/ transferase, transferring glycosyl groups"	1.91	8.28E-7
AT3G03855	pseudogene	1.90	9.17E-7
AT3G16720	"ATL2 (Arabidopsis Thaliana 2); protein binding / zinc ion binding"	1.90	9.74E-7
AT5G12930	"unknown protein"	1.90	1.04E-6
AT4G14390	"ankyrin repeat family protein"	1.90	1.05E-6
AT1G02230	"ANAC004 (Arabidopsis NAC domain containing protein 4); transcription factor"	1.89	1.29E-6
AT1G51760	"IAR3 (IAA-ALANINE RESISTANT 3); metalloproteinase"	1.89	1.39E-6
AT4G33050	"EDA39 (embryo sac development arrest 39); calmodulin binding"	1.88	1.45E-6
AT3G08720	"ATPK19/ATPK2 (ARABIDOPSIS THALIANA SERINE/THREONINE PROTEIN KINASE 19, ARABIDOPSIS THALIANA SERINE/THREONINE PROTEIN KINASE 20); kinase"	1.88	1.66E-6
AT1G19960	"unknown protein"	1.87	1.85E-6



AT3G12230	"SCPL14 (serine carboxypeptidase-like 14); serine carboxypeptidase"	1.87	1.98E-6
AT1G67970	"AT-HSFA8 (Arabidopsis thaliana heat shock transcription factor A8); DNA binding / transcription factor"	1.87	2.7E-6
AT2G13790	"ATSERK4 (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 4); protein binding / protein kinase/ transmembrane recep	1.87	2.21E-6
AT2G37710	"RLK (RECEPTOR LECTIN KINASE); kinase"	1.86	2.55E-6
AT3G57460	"unknown protein"	1.86	2.55E-6
AT1G66880	"serine/threonine protein kinase family protein"	1.86	2.56E-6
AT1G13210	"haloacid dehalogenase-like hydrolase family protein"	1.85	2.89E-6
AT1G03210	"phenazine biosynthesis PhzC/PhzF family protein"	1.85	3.02E-6
AT3G49110	"ATPCA/ATPRX33/PRX33/PRXCA (PEROXIDASE 33); peroxidase"	1.85	3.15E-6
AT2G40140	CZF1/ZFAR1	1.85	3.33E-6
AT1G17147	"unknown protein"	1.85	3.48E-6
AT1G09210	"calreticulin 2 (CRT2)"	1.84	3.67E-6
AT3G04210	"disease resistance protein (TIR-NBS class), putative"	1.84	3.67E-6
AT5G17760	"AAA-type ATPase family protein"	1.84	4.26E-6
AT1G77510	"ATPDIL1-2 (PDI-LIKE 1-2); protein disulfide isomerase"	1.84	4.27E-6
AT3G20600	"NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE 1); signal transducer"	1.84	4.36E-6
AT3G25610	"haloacid dehalogenase-like hydrolase family protein"	1.83	4.59E-6
AT3G11820	"SYP121 (syntaxin 121); t-SNARE"	1.83	4.74E-6
AT1G56550	"unknown protein"	1.83	4.84E-6
AT5G43910	"pfkB-type carbohydrate kinase family protein"	1.80	103E-5
AT4G33300	"ADR1-L1 (ADR1-LIKE 1); ATP binding / protein binding"	1.80	104E-5
AT4G14400	"ACD6 (ACCELERATED CELL DEATH 6); protein binding"	1.79	120E-5
AT2G38470	"WRKY33 (WRKY DNA-binding protein 33); transcription factor"	1.78	155E-5
AT3G09020	"alpha 1,4-glycosyltransferase family protein / glycosyltransferase sugar-binding DXD motif-containing protein"	1.77	199E-5
AT2G18670	"zinc finger (C3HC4-type RING finger) family protein"	1.77	2.09E-5
AT1G59870	"PDR8/PEN3 (PLEIOTROPIC DRUG RESISTANCE8); ATPase, coupled to transmembrane movement of substances"	1.76	2.31E-5
AT4G36150	"disease resistance protein (TIR-NBS-LRR class), putative"	1.76	2.34E-5
AT5G42530	"unknown protein"	1.76	2.68E-5
AT3G60450	"unknown protein"	1.76	2.68E-5
AT4G16260	"glycosyl hydrolase family 17 protein"	1.76	2.69E-5
AT5G54490	"PBP1 (PINOID-BINDING PROTEIN 1); calcium ion binding"	1.75	3.27E-5
AT5G52540	"unknown protein"	1.75	3.33E-5
AT5G39450	"F-box family protein"	1.74	4.04E-5
AT5G19750	"peroxisomal membrane 22 kDa family protein"	1.73	4.50E-5
AT3G54960	"ATPDIL1-3 (PDI-LIKE 1-3); thiol-disulfide exchange intermediate"	1.72	6.16E-5
AT3G54150	"embryo-abundant protein-related"	1.72	6.68E-5
AT1G16670	"protein kinase family protein"	1.71	7.50E-5
AT1G74710	"ICS1 (ISOCHORISMATE SYNTHASE1); isochorismate synthase"	1.71	7.50E-5
AT1G66725	MIR163	1.71	7.99E-5
AT3G45620	"transducin family protein / WD-40 repeat family protein"	1.70	8.92E-5
AT5G52640	"HSP81-1 (HEAT SHOCK PROTEIN 81-1); ATP binding / unfolded protein binding"	1.70	9.98E-5
AT1G68585	"metal ion binding"	1.68	133E-4
AT1G52780	"unknown protein"	1.68	144E-4
AT5G47120	"ATBI-1 (ARABIDOPSIS BAX INHIBITOR 1)"	1.67	180E-4
AT1G03290	"unknown protein"	1.67	192E-4
AT1G55450	"embryo-abundant protein-related"	1.66	237E-4
AT1G16500	"unknown protein"	1.65	272E-4
AT5G04930	"ALA1 (AMINOPHOSPHOLIPID ATPASE1); ATPase, coupled to transmembrane movement of ions, phosphorylative mechanism	1.65	279E-4
AT4G11280	"ACS6 (1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6)"	1.64	330E-4
AT4G27740	"unknown protein"	1.64	342E-4
AT1G53625	"unknown protein"	1.63	415E-4
AT3G11402	"DC1 domain-containing protein"	1.63	420E-4
AT5G33439		1.62	438E-4
AT4G15280	"UDP-glucuronosyl/UDP-glucosyl transferase family protein"	1.62	439E-4
AT1G61420	"S-locus lectin protein kinase family protein"	1.62	50E-4
AT1G07135	"glycine-rich protein"	1.62	622E-4
AT4G11310	"cysteine-type peptidase"	1.62	625E-4
AT2G43150	"proline-rich extensin-like family protein"	1.62	647E-4
AT1G21130	"O-methyltransferase, putative"	1.61	672E-4
AT4G36090	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	1.61	732E-4
AT1G19180	"unknown protein"	1.61	6.12E-3
AT4G13900	pseudogene,pseudogene	1.60	6.78E-4
AT5G28540	"luminal binding protein 1 (BiP-1) (BP1)"	1.60	707E-4
AT2G31870	"TEJ (SANSKRIT FOR pBRIGHTp)"	1.60	744E-4
AT4G11830	"phospholipase D gamma 2 / PLD gamma 2 (PLDGAMMA2)"	1.59	862E-4
AT1G21750	"ATPDIL1-1 (PDI-LIKE 1-1); protein disulfide isomerase"	1.59	9.12E-4
AT4G04540	"protein kinase family protein"	1.59	9.66E-4
AT5G56960	"basic helix-loop-helix (bHLH) family protein"	1.59	9.90E-4
AT5G09290	"3p(2p),5p-bisphosphate nucleotidase, putative / inositol polyphosphate 1-phosphatase, putative"	1.58	1.0E-3
AT2G14580	"ATPRB1 (Arabidopsis thaliana basic pathogenesis-related protein 1)"	1.58	1.02E-3

AT5G45500	"unknown protein"	1.57	1.39E-3
AT3G19010	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	1.56	1.69E-3
AT5G46520	"ATP binding / nucleoside-triphosphatase/ nucleotide binding / protein binding / transmembrane receptor"	1.56	1.69E-3
AT5G61250	"glycosyl hydrolase family 79 N-terminal domain-containing protein"	1.55	1.89E-3
AT1G12210	"RFL1 (RPS5-LIKE 1); ATP binding / protein binding"	1.55	1.89E-3
AT1G16110	"WAKL6 (WALL ASSOCIATED KINASE-LIKE 6); kinase"	1.54	2.49E-3
AT3G45860	"receptor-like protein kinase, putative"	1.54	2.59E-3
AT3G50280	"transferase family protein"	1.54	2.69E-3
AT1G67800	copine-related	1.53	2.67E-3
AT3G55880	"unknown protein"	1.53	3.09E-3
AT2G39330	"jacalin lectin family protein"	1.53	3.29E-3
AT2G44180	"MAP2A (METHIONINE AMINOPEPTIDASE 2A); methionyl aminopeptidase"	1.52	3.59E-3
AT1G05630	"5PTASE13(AT5PTASE13; inositol or phosphatidylinositol phosphatase"	1.52	3.79E-3
AT5G61790	"calnexin 1 (CNX1)"	1.52	3.89E-3
AT1G21100	"O-methyltransferase, putative"	1.52	3.82E-3
AT1G11310	"MLO2 (MILDEW RESISTANCE LOCUS O 2); calmodulin binding"	1.52	3.99E-3
AT1G30010	"intron maturase, type II family protein"	1.51	4.49E-3
AT1G66380	"MYB114 (myb domain protein 114); DNA binding / transcription factor"	1.51	4.39E-3
AT2G32160	"unknown protein"	1.51	4.39E-3
AT4G12720	"AtNUDT7 (ARABIDOPSIS THALIANA NUDIX HYDROLASE HOMOLOG 7); hydrolase"	1.51	4.69E-3
AT4G25000	"AMY1 (ALPHA-AMYLASE-LIKE); alpha-amylase"	1.49	6.19E-3
AT4G29810	"ATMKK2 (MAP KINASE KINASE 2); MAP kinase kinase/ kinase"	1.49	6.60E-3
AT5G49570	"transglutaminase-like family protein"	1.49	6.84E-3
AT1G67850	"unknown protein"	1.48	7.59E-3
AT3G51890	"unknown protein"	1.47	9.89E-3
AT3G55450	"protein kinase, putative"	1.47	9.92E-3
AT4G02520	"ATGSTF2 (Arabidopsis thaliana Glutathione S-transferase (class phi) 2); glutathione transferase"	1.46	12.4E-3
AT1G49050	"aspartyl protease family protein"	1.45	12.9E-3
AT3G17910	"SURF1 (SURFEIT 1)"	1.45	14.0E-3
AT5G61900	"BON1 (BONZAI1); calcium-dependent phospholipid binding"	1.44	15.9E-3
AT5G61600	"ethylene-responsive element-binding family protein"	1.44	16.9E-3
AT2G27310	"F-box family protein"	1.44	16.9E-3
AT1G21110	"O-methyltransferase, putative"	1.44	17.9E-3
AT1G17990	"12-oxophytodienoate reductase", "12-oxophytodienoate reductase, putative"	1.44	17.9E-3
AT3G44870	"S-adenosyl-L-methionine:carboxyl methyltransferase family protein"	1.44	17.9E-3
AT5G04720	"ADR1-L2 (ADR1-LIKE 2); ATP binding / nucleoside-triphosphatase/ nucleotide binding / protein binding"	1.43	19.9E-3
AT1G14370	"APK2A (PROTEIN KINASE 2A); kinase"	1.43	2.09E-2
AT5G44070	"CAD1 (CADMIUM SENSITIVE 1)"	1.42	2.39E-2
AT1G01340	"ATCNGC10 (CYCLIC NUCLEOTIDE GATED CHANNEL 10); calmodulin binding / cyclic nucleotide binding / ion channel"	1.42	2.39E-2
AT1G57560	"AtMYB50 (myb domain protein 50); DNA binding / transcription factor"	1.42	2.42E-2
AT1G52040	"MBP1 (MYROSINASE-BINDING PROTEIN 1)"	1.42	2.49E-2
AT3G50800	"unknown protein"	1.41	2.39E-2
AT2G40600	"appr-1-p processing enzyme family protein"	1.40	3.42E-2
AT3G28930	"AIG2 (AVRRPT2-INDUCED GENE 2)"	1.40	3.62E-2
AT1G51660	"ATMKK4 (MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4); MAP kinase kinase/ kinase"	1.39	3.39E-2
AT2G15220	"secretory protein, putative"	1.39	4.29E-2
AT2G38860	"YLS5 (yellow-leaf-specific gene 5)"	1.39	4.39E-2
AT3G13000	"transcription factor"	-1.45	1.99E-2
AT1G20470	"auxin-responsive family protein"	-1.45	1.97E-2
AT2G34060	"peroxidase, putative"	-1.47	1.09E-2
AT1G20190	"ATEXPA11 (ARABIDOPSIS THALIANA EXPANSIN A11)"	-1.47	9.79E-3
AT1G61795	"unknown protein"	-1.47	9.49E-3
AT4G10150	"zinc finger (C3HC4-type RING finger) family protein"	-1.47	9.23E-3
AT5G11590	"TINY2 (TINY2); DNA binding / transcription factor"	-1.51	1.19E-2
AT3G16000	"MFP1 (MAR BINDING FILAMENT-LIKE PROTEIN 1)"	-1.52	3.40E-3
AT3G53530	"heavy-metal-associated domain-containing protein"	-1.54	2.59E-3
AT4G38660	"thaumatin, putative"	-1.55	1.99E-3
AT3G63200	"PLA IIIB/PLP9 (Patatin-like protein 9); nutrient reservoir"	-1.60	7.69E-4
AT5G57780	"unknown protein"	-1.61	5.63E-4
AT5G36910	"THI2.2 (THIONIN 2.2); toxin receptor binding"	-1.61	5.59E-4
AT2G37950	"zinc finger (C3HC4-type RING finger) family protein"	-1.63	4.42E-4
AT1G07450	"tropinone reductase, putative / tropine dehydrogenase, putative"	-1.63	4.19E-4
AT4G26790	"GDSL-motif lipase/hydrolase family protein"	-1.68	1.34E-4
AT4G39510	"CYP96A12 (cytochrome P450, family 96, subfamily A, polypeptide 12); oxygen binding"	-1.74	4.00E-5
AT1E70780		-1.77	1.97E-5
AT5G46690	"BHLH071 (BETA HLH PROTEIN 71); DNA binding / transcription factor"	-1.78	1.48E-5
AT3G18850	LPAT5	-1.79	1.30E-5
AT2G34170	"unknown protein"	-1.80	9.96E-6
AT1G02180	ferredoxin-related	-1.83	5.05E-6
AT3G22540	"unknown protein"	-1.83	4.93E-6
AT1G53520	"chalcone-flavanone isomerase-related"	-1.88	1.66E-6
AT2G44800	"oxidoreductase, 2OG-Fe(II) oxygenase family protein"	-1.89	1.40E-6
AT3G17640	"leucine-rich repeat family protein"	-1.89	1.29E-6
AT1G06980	"unknown protein"	-1.89	1.28E-6
AT1G52250	"dynein light chain type 1 family protein"	-1.92	5.75E-7
AT2G27385	"unknown protein"	-1.96	2.59E-7
AT2G21540	"SEC14 cytosolic factor, putative / phosphoglyceride transfer protein, putative"	-2.12	3.96E-9
AT5G24105	AGP41	-2.17	9.04E-10
AT5G26670	"pectinacetyltransferase, putative"	-2.25	1.02E-10
AT2G33330	"33 kDa secretory protein-related"	-2.31	2.56E-11
AT1G74890	"ARR15 (RESPONSE REGULATOR 15); transcription regulator"	-2.31	1.70E-11
AT5G13140	"unknown protein"	-2.31	1.70E-11
AT5G35740	"glycosyl hydrolase family protein 17"	-2.32	1.70E-11
AT1G06360	"fatty acid desaturase family protein"	-2.41	0.00E+0
AT3G14760	"unknown protein"	-2.48	0.00E+0
AT1G23205	"invertase/pectin methylesterase inhibitor family protein"	-2.55	0.00E+0
AT3G08770	"LTP6 (Lipid transfer protein 6); lipid binding"	-2.57	0.00E+0
AT1G62510	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	-2.60	0.00E+0
AT1G67750	"pectate lyase family protein"	-2.61	0.00E+0
AT4G28780	"GDSL-motif lipase/hydrolase family protein"	-2.75	0.00E+0
AT3G44990	"XTR8 (xyloglucan:xyloglucosyl transferase 8); hydrolase, acting on glycosyl bonds"	-2.97	0.00E+0
AT1G60590	"polygalacturonase, putative / pectinase, putative"	-3.05	0.00E+0
AT5G48490	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	-3.16	0.00E+0
AT5G20630	"GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir"	-3.53	0.00E+0



**Titre :** Impact de la nutrition azotée sur l'interaction entre *Arabidopsis thaliana* et la bactérie phytopathogène *Erwinia amylovora*

**Mots clés :** bactérie, plante, azote, stress, défense, virulence

**Résumé :** L'azote (N) est l'un des principaux éléments nécessaires pour la croissance des plantes et qui influence également la sensibilité des plantes aux agents pathogènes. En première partie de ce travail, nous avons réalisé une analyse transcriptomique de plantes d'*A. thaliana* infectées par *E. amylovora* (*Ea*), cultivées à fort et faible N. Malgré la différence de développement de ces plantes, leur réponse transcriptomique à la bactérie est globalement proche. Cependant, l'activation de certaines voies de signalisation, notamment la voie de l'acide jasmonique, suite à l'infection est affectée par le régime azoté. L'analyse de l'interaction entre les deux stress (N et bactérie) montre qu'en réponse à la combinaison des deux stress, 32.5% de gènes ont une réponse spécifique de la combinaison des stress, suggérant une interaction entre les réponses aux stress simples.

Plusieurs gènes liés à la défense, comme les facteurs de transcription de type *WRKY* et les gènes de résistance, ont répondu en priorité au stress biotique selon un mode défini comme « priorisé ».

Dans la deuxième partie de ce travail, nous avons démontré la bactérie *Ea* se multiplie mieux dans des plantes cultivées à faible azote (2 mM NO<sub>3</sub><sup>-</sup>) qu'à fort azote (10 mM NO<sub>3</sub><sup>-</sup>). Les facteurs de virulence d'*Ea*, sont significativement plus exprimés *in planta* à faible azote qu'à fort azote. De plus, le niveau d'expression des facteurs de virulence d'*Ea in planta* est inversement corrélé avec l'expression des gènes de la voie de l'acide jasmonique. Une analyse du métabolome par GC-MS montre que le niveau d'accumulation des précurseurs chloroplastiques de l'acide jasmonique est également inversement associé au niveau d'expression des facteurs de virulence d'*Ea*.

**Title :** Impact of nitrogen supply on the interaction between *Arabidopsis thaliana* and phytopathogenic bacterium *Erwinia amylovora*

**Keywords :** bacteria, plant, nitrogen, stress, defence, virulence

**Abstract:** Nitrogen (N) is one of the main nutrients required for plant growth, which also influences the susceptibility of plants to pathogens. In the first part of this work, we performed a transcriptomic analysis on *A. thaliana* plants grown under high or low N, infected with *E. amylovora* (*Ea*). Our results indicate that, despite the difference in development of these plants, their transcriptomic responses to the bacterium are close. Nevertheless, in response to infection, some signaling pathways have been affected, including the jasmonic acid pathway. Analysis of the interaction between the two stresses (N and bacteria) shows that in response to the combination of the two stresses, 32.5% of genes have a specific response to the stress combination, suggesting an interaction between simple stress responses. Several defense-related genes such as *WRKY* transcription factors and resistance proteins have prioritized between the

two responses by promoting response to biotic stress.

In the second part of this work, we demonstrated that the *Ea* multiplies better in plants grown in low nitrogen (2 mM NO<sub>3</sub><sup>-</sup>) than in high N (10 mM NO<sub>3</sub><sup>-</sup>). The experiments conducted indicate that *Ea* virulence factors are significantly more expressed *in planta* at low N than at high N. Moreover, the level of expression of virulence factor of *Ea in planta* is inversely correlated with the expression of the jasmonic acid-associated genes. A GC-MS analysis indicates that the level of accumulation of the chloroplastic precursors of jasmonic acid is also inversely correlated with the expression of virulence factor of *Ea in planta*.