Characterization and role of nitric oxide production in Arabidopsis thaliana defense responses induced by oligogalacturonides

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par

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DEDICATED TO

MY SWEET MOTHER,

WHO IS HEAVEN FOR ME

WHOSE HANDS ALWAYS

RAISED FOR MY WELL-BEINGS

EVEN AT THIS MOMENT OF TIME

MY DEAREST FATHER

WHOSE LOVE IS MORE PRECIOUS

THAN THE PEARLS AND DIAMONDS

BY THE VIRTUE OF WHO PRAYS

I HAVE BEEN ABLE TO REACH

A THIS HIGH POSITION

MY BROTHERS AND SISTERS

WHO ARE THE WORLD FOR ME

WHOSE LOVE ENCOURAGE ME

AT EVERY STEP

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other activities I shall badly miss. I don't exactly know where I shall be in the next future but where ever it is if you're in the neighborhood don't hesitate to visit me.

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Sumaira RASUL

ABSTRACT

Characterization and role of nitric oxide production in *Arabidopsis thaliana* defense responses induced by oligogalacturonides

Nitric oxide (NO) regulates a wide range of plant processes from development to environmental adaptation. In this study, NO production and its effects were investigated in a plant-pathogen context. The production of NO following Arabidopsis treatment with oligogalacturonides (OGs), an endogenous elicitor of plant defense, was assessed using the NO sensitive probe 4, 5-diamino fluorescein diacetate. Pharmacological and genetic approaches were used to analyze NO enzymatic sources and its role in the *Arabidopsis thaliana* /*Botrytis cinerea* interaction. We showed that NO production involves both a L-arginine- and a nitrate reductase (NR)-pathways. OGs-induced NO production was Ca^{2+}-dependent and modulated RBOHD-mediated ROS production. NO production was also regulated by CDPKs activities, but worked independently of the MAPKs pathway. Using a transcriptomic approach, we further demonstrated that NO participates to the regulation of genes induced by OGs such as genes encoding disease-related proteins and transcription factors. The over-representation of certain regulatory elements (e.g. W-BOX) in promoter sequences of target genes also suggests the involvement of specific transcription factors in the NO response. Mutant plants impaired in several selected NO-responsive genes, as well as Col-0 plants treated with the NO scavenger cPTIO, were more susceptible to *B. cinerea.* Taken together, our investigation deciphers part of the mechanisms linking NO production, NO-induced effects and basal resistance to *Botrytis cinerea*. More generally, our data reinforce the concept that NO is a key mediator of plant defense responses.

Keywords: nitric oxide, oligogalacturonides, nitrate reductase, plant defense, *Arabidopsis thaliana*, *Botrytis cinerea*, calcium, reactive oxygen species, transcriptome.

RÉSUMÉ

Caractérisation et rôle de la production du monoxyde d'azote en réponse aux oligogalacturonidase chez *Arabidopsis thaliana*

Le monoxyde d'azote (NO) régule un grand nombre de processus physiologiques tel que le développement ou les réponses aux modifications des conditions environnementales. Dans ce travail, la production de NO et ses effets ont été étudiés dans le contexte des interactions plante – pathogène. La production de NO générée chez *Arabidopsis thaliana* par les oligogalacturonides (OGs), eliciteur endogène des réactions de défense, a été mesurée par la sonde fluorescente 4, 5-diamino fluoresceine diacetate. L'utilisation d'approches pharmacologiques et génétiques ont permis d'étudier les sources enzymatiques de la production de NO et son rôle dans l'interaction *A. thaliana*/*Botrytis cinerea.* Nous avons montré que le NO est produit par une voie dépendante de la L-arginine ainsi que d'une voie impliquant la Nitrate Réductase. La production de NO induite par les OGs est dépendante du Ca^{2+} et modulée par les formes activées de l'oxygène (produites par AtRBOHD). La production de NO est également régulée par les CDPKs mais est indépendante des activités MAPKs. A l'aide d'une approche transcriptomique nous avons ensuite démontré que le NO participe à la régulation de l'expression de gènes induits par les OGs tels que des gènes codant pour des protéines PR ou des facteurs de transcription. La sur-représentation de certains éléments régulateurs (par exemple de type W-box) dans les régions promotrices des gènes cibles du NO suggère également l'implication de facteurs de transcription spécifiques dans la réponse au NO. Enfin, des plantes mutantes, affectées dans l'expression de gènes cibles de NO, ainsi que des plantes de type sauvage (Col-0) traitées par le piégeur de NO, cPTIO, sont plus sensibles à *B. cinerea*. L'ensemble de ces résultats nous a permis de mieux comprendre les mécanismes liant la production de NO, ses effets et la résistance d'*A. thaliana* à *B. cinerea,* confirmant que le NO est un élément-clé des réactions de défense des plantes.

Mots clés : monoxyde d'azote, oligogalacturonides, nitrate réductase, réactions de défenses des plantes, *Arabidopsis thaliana*, *Botrytis cinerea*, calcium, formes activées de l'oxygène, transcriptome.

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[Ca $^{2+}$]cyt	Cytosolic free calcium concentration variation	**µE**	Micromole.m^{-2}.s^{-1}
^{32}P	Radioactive phosphorus	***A.thaliana***	*Arabidopsis thaliana*
ABA	Abscisic acid	**ABF**	ABRE (ABA-responsive element) binding factor
ADPRC	ADP-ribosyl cyclase	**AM**	Arbuscular mycorrhizae
ANOVA	ANOVA	**AOS**	Active oxygen species
APX	Ascorbate peroxidase	**Arg**	Arginine
AtMPK	*Arabidopsis thaliana* MAPK	**AtNOA1**	Arabidopsis Nitric Oxide Associated 1
AtNOS	Arabidopsis thaliana nitric oxide synthase	**Avr**	Avirulence genes
AvrB	Avirulence protein B from *P. syringae pv. Glycinea*	**AvrPphB**	Avirulence protein B from *P. syringae pv. Phaseolicola*
AvrRpt2	Avirulence protein 2 from *P. syringae pv. Tomato*	**bGlu**	β-1,3 glucanase
BH4	Tetrahydrobiopterin	**BSA**	Bovine serum albumin
BY-2	Nicotiana tabacum L. cv Bright Yellow 2	**Ca^{2+}**	Calcium
$CaCl_2$	Calcium chloride	**cADPR**	Cyclic ADP-ribose
cADP-Rib	Cyclic Adenosine diphosphate ribose	**CaM**	Calmodulin
CaMBD	CaM-binding domain	**CaMK/CBK**	CaM-binding protein kinase
CAT	Catalase	**CBL**	Calcineurin B-like protein
CBP	CBP: Ca^{2+}-binding protein	**CBRLK**	CBRLK: CaM-binding receptor-like kinase
CC	Coiled-coil	**CCaMK**	Ca $^{2+}$ /calmodulin-dependent protein kinase
CD	b-1,4 cellodextrins	**Cd**	Cadmium
cDNA	Complementary Deoxyribonucleic acid	**CDPK**	Ca^{2+}-dependent protein kinase
Cfu	Colony-forming unit	**cGMP**	Cyclic GMP

Chit	Chitinase	**CLSM**	Confocal laser scanning microscopy
CNGC	Cyclic nucleotide gated channel	**CNGC2**	Cyclic nucleotide-gated channel 2
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt	**CWD**	Cell wall-derived
DNA	Deoxyribonucleic acid	**DAB**	3,3'-diaminobenzidine tetrahydrochloride
DAF-2 DA	4,5-diaminoflorescein diacetate	**DEA/NO**	Diethylamine NONOate
DEPC	Diethyl pyrocarbonate	**DMSO**	Dimethylsulphoxide
dnd	defense no death	**dNTP**	Deoxynucleoside triphosphate
DP	Degree of polymerization	**dpi**	Days post-infection
DPI	Diphenylene iodonium	**DTT**	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid	**EF1-α**	Elongation factor 1-α
EGTA	Ethylene glycol-bis(b-aminoethyl ether)-N,N,N¢,N¢-tetraacetic acid	**ER**	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase	**ET**	Ethylene
FAD	Flavin adenine dinucleotide	**FMN**	Flavin mononucleotide
FW	Fresh weight	**GA**	Gibberellin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	**GC**	Guanylate cyclase
gDNA	genomic DNA	**GFP**	Green fluorescent protein
G-kinase	cGMP-dependent protein kinase	**GMP**	GMP: Guanosine monophosphate
GSH	Glutathione synthetase	**GSNO**	S-nitrosoglutathion
GSNOR	S-nitrosoglutathione reductase	**GSSG**	Glutathion disulfide
GSSH	Glutathione disulfide	**GST**	Glutathione transferase
H_2O_2	Hydrogen peroxide	**HAMPs**	Host-Associated Molecular Patterns
Hb	Hemoglobin	**HEPES**	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIIIS	Histone IIIS	**HPLC**	High-performance liquid chromatography or High pressure liquid

			chromatography
HR	Hypersensitive Response	**HSR**	Hypersensitive-related
IAA	Auxin	**ISR**	Induced systemic resistance
JA	Jasmonic acid	**LB**	Luria Broth
L-NAME	Nω-nitro-L-arginine methyl ester	**L-NMMA**	Nω-Methyl-l-arginine acetate salt
LOX	Lipoxygenase	**LP**	Left Primer
LPS	Lipopolysaccharide	**LRR-(RK)**	Leucine-rich repeat (receptor kinase)
LRR	Leucine-rich repeat	**LZ-CC**	Leucine-zipper/coil-coil domain
MAMPs	Microbe-Associated Molecular Patterns	**MAPK/MPK**	Mitogen-activated protein kinase
MBP	Myelin basic protein	**MES**	2-(N-morpholino) ethanesulfonic acid
Min	Minutes	**MKP**	MAPK phosphatase
MP	Methylphosphonate	**mRNA**	Messenger Ribonucleic acid
MS	Murashige et Skoog	**MTs**	Metallothioneins
$NADP^+$	Nicotinamide adenine dinucleotide phosphate	**NASC**	Nottingham Arabidopsis Stock Centre
NBS	Nucleotide-binding site	**Ni-NOR**	Nitrite-NO reductase
nm	Nanometer	**NMMA**	NG-monomethyl-l-arginine monoacetate
NO	Nitric oxide	**NO_2-Tyr**	3-nitrotyrosine
NO_3^-	Nitrate	**Nod**	Nucleotide-binding oligomerization domain
NOS	Nitric oxide synthase	**NOS-like**	Nitric oxide synthase-like
NOX	NADPH oxidase	**NR**	Nitrate reductase
NPR1	Non-expresser of PR-genes 1	**NR**	Nitrate Reductase
NR1	Nitrate Reductase isoform 1	**NR2**	Nitrate Reductase isoform 2
N-ter	N-terminal	**O_2^-**	Superoxide anion or Superoxide radical
OD	Optical density	**OGs**	Oligogalacturonides
OH	Hydroxyl radical	**$ONOO^-$**	Peroxynitrite anion
PAL	Phenylalanine ammonia lyase	**PAMPs**	Pathogen- -Associated Molecular Patterns
PAOX	Polyamine oxidase	**PAs**	Polyamines

PBITU	S,S′-(1,3-phenylenebis(1,3-ethanediyl))bis-isothiourea	**PBS**	Phosphate-buffered saline
PBS1	AvrPphB SUSCEPTIBLE1	**PCD**	Programmed cell death
PCR	Polymerase chain reaction ;	**PCs**	Phytochelatins
pep13	Peptide 13	**PEPR1**	Pep1 receptor 1
PGIP	Polygalacturonase inhibiting protein	**PKs**	Protein kinases
PLA2	Phospholipase A2	**PLAP**	Phospholipase A2-activating protein
PM	Plasma membrane	**pmol/mg**	Picomole per milligram
PMSF	Phenylmethylsulfonyl fluoride	**PPs**	Protein phosphatases
PR	PR:pathogenesis-related	***PR1***	Pathogenesis-related protein 1
PROPEP2	Pro-peptide 2	**PRR**	Pattern recognition receptor
PRs	Pathogenesis-related proteins	**PS3**	Sulfated laminarin;
Pst(avrRpt2)	Pseudomonas syringae pv. *tomato* carrying the *avrRpt2* gene	**Pst**	*Pseudomonas syringae pv. tomato*
PTIO	2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl	**PVPP**	Polyvinyl polypyrrol-idone
QTL	Quantitative trait loci	**R gene**	Resistance gene
RBOH	Respiratory burst oxidase homologue	**RbohD**	NADPH /Respiratory burst oxidase protein
Real Time qPCR	Realtime quantitative Polymerase Reaction	**RFU**	Relative Fluorescence unit
RIN4	RPM1-INTERACTING PROTEIN4	**RK**	Receptor kinases
RLP	Receptor-like protein	**RLU**	*Relative* luminescence unit
RNA	Ribonucleic acid	**RNAi**	RNA interference
RNS	reactive nitrogen species	**ROS**	Rective oxygen species
RP	Right primer	**rpm**	Rotation per minute
RPM1	RESISTANCE TO *P. syringae* Expressing AVRRPM1	**RPS2**	Resistance To *P. Syringae* Expressing Avrrpt2
RSG	Repression Of Shoot Growth	**RSNO:**	S-nitrosothiol
RT-PCR	Reverse Transcription – PCR	**Rubisco**	Ribulose 1,5 biphosphate carboxylase/oxygenase
Rx	Resistance To Potato Virus	**S.O.C**	Super Optimal broth with

	X		Catabolite repression
SA	Salicylic acid	**SAR**	Systemic acquired resistance
SDS	Sodium dodecyl sulfate	**SDS-PAGE**	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sGC	Guanylate cyclase soluble	**SIPK**	Salicylic acid-induced protein kinase
SNAP	S-nitroso-N-acetyl-dl-penicillamine	**SNC1**	SUPPRESSOR OF npr1-1 CONSTITUTIVE1
SNP	Sodium nitroprusside	**SOS**	Salt overly sensitive
TEV	Tobacco etch virus	**TFA**	Trifluoroacetic acid
TIR	Toll /interleukine-1 receptor	**TMV**	Tobacco mosaic virus
UV	Ultraviolet	**V**	Volts
VIGS	Virus-induced gene Silencing	**WIPK**	Wounding-induced protein kinese
Y2H	Yeast two hybrid		

A. brassicicola	*Alternaria brassicicola*	*P. infestans*	*Phytophthora infestans*
B. cinerea	*Botrytis cinerea*	*P. parasitica*	*Phytophthora parasitica*
E. coli	*Escherichia coli*	*P. sojae*	*Phytophthora sojae*
H. arabidopsidis	*Hyaloperonospora arabidopsidis*	*P. syringae*	*Pseudomonas syringae*
P. brassicae	*Phytophthora brassicae*	*P. viticola*	*Plasmopara viticola*
P. capsici	*Phytophthora capsici*	*S. littoralis*	*Spodoptera littoralis*
S. sclerotinium	*Selerotinia sclerotinium*		

CHAPTER 1

CHAPTER 1

"Introduction"

CHAPTER 1

INTRODUCTION

Plants are hosts to thousands of infectious diseases caused by a vast array of phytopathogenic fungi, bacteria, viruses, and nematodes. A relatively small proportion of these pathogens successfully invades the plant host and cause disease. Nevertheless, plant diseases have catastrophic effects on crops. Today, we can estimate that the loss of yields due to pests reach about 20-30% in most crops. These losses are observed despite the substantial increase in the use of pesticides (about 500 million kg of active ingredient worldwide). Although pesticides have successfully controlled disease, their increasing use will have harmful effects on our health and the environment. This indirect cost of pesticides use to the environment and public health has to be balanced against the benefits. Based on the available data, the environmental (impacts on wildlife, pollinators, natural enemies, fisheries, water and development of resistance) and social costs (human poisonings and illnesses) of pesticide use reach about $8 billion each year (Pimentel and Lehman, 1993).

In the past decade, growing concerns about the impact of fungicide applications has encouraged research scientists to develop strategies that could provide a safe and reliable method for improving crop protection. These alternative strategies of disease and pest management are based on the better understanding of the plant-pathogen interaction and plant's own defense mechanisms.

Over the past 60 years, mechanisms for the resistance of plants have received considerable attention. Plants recognize and resist many invading phytopathogens by inducing local and systemic defense response. These phenomena are known as hypersensitive response (HR) and systemic acquired resistance (SAR), respectively. They are induced after perception of the pathogenic microorganism (very specific, gene-for-gene recognition) or of microbial components (non-specific elicitors). Once triggered, SAR provides resistance to a wide range of pathogens for days. Mimicking pathogen attack with such non-specific elicitors could become an alternative strategy in crop plant protection. The term elicitor usually refers to molecules originating either from the host plant (endogenous elicitors) or from the plant pathogen (exogenous elicitors), which are capable of inducing responses associated with plant disease

resistance. Exploiting induced resistance meets with current needs for sustainable agriculture at low environmental cost.

A major objective of our laboratory, "Cellular and Molecular Signaling of Plant Defense Reactions" team of the Plante-Microbe-Environnement (UMR PME), is to understand how plants perceive a response to pathogen attack at the molecular and the cellular level. Major advances have been made in understanding the sequential events taking place during the induction and expression of plant defence responses. This process is mediated by the release of number of signaling components/messengers as described later (Chapter 1). Among these messengers, we attempt to elucidate the mechanisms of action nitric oxide (NO), a free radical reactive gas, which has been shown, in animals and in plants, to be involved in major physiological processes (Torreilles, 2001; Besson-Bard et al., 2008). Moreover, NO performs a vital role in the adaptive response to plant-pathogen interaction. In this context, during the past several years, the team demonstrated that NO is a signal molecule produced and mobilized quickly in cell signaling processes during defense responses (Besson-Bard et al., 2008). These data support a model in which NO produced in plant cells by pathogen-derived elicitors acts as an endogenous regulator of Ca^{2+} mobilization and protein kinase activation (Lamotte et al., 2004; Lamotte et al., 2005; Vandelle et al., 2006). These studies were carried out in tobacco and grapevine. More recently, we have identified in tobacco several proteins modified post-translationally by NO in response to cryptogein, an elicitor of the tobacco defense responses (Astier et al., in preparation). In the longer term, the expected outcome of our studies is to characterize the role of natural elicitors or to design chemical messengers capable of triggering an array of plant defense responses (Benhamou, 1996; Klarinsky and Fritig, 2001). Treatments of plants with such molecules could be an alternative strategy for crop protection with a more satisfactory preservation of the environment by reducing the use of chemical pesticides.

We estimate that an increased knowledge of the mechanisms underlying the plant response to pathogen attack is a first important step to achieve these objectives. My thesis work relates to this context.

Outline of the thesis (my project)

All the experiments were performed in *Arabidopsis thaliana*. This model plant was retained because of the knowledge of its genome, and the availability of public well-developed genomics tools like T-DNA insertion mutants library and microarray service facilities. In preliminary studies, our lab screened different elicitors for their ability to trigger NO synthesis in *A. thaliana* and selected Oligogalacturonides (OGs) as a powerful one.

The objectives of my research work were:

- To identify the mechanisms playing a role in NO synthesis and its regulation in response to oligogalacturonides (OGs) in *A. thaliana.* For this purpose, we analysed the ability of OGs to trigger NO synthesis using molecular genetics (mutants impaired in the expression of genes coding proteins putatively involved or associated to NO biosynthesis) and pharmacological approaches (mammalian NOS inhibitors as well as NR inhibitors).
- To analyse the NO-regulated transcriptomic response to OGs and characterize several NO target genes in a plant pathogen context. To achieve this objective, a microarray analysis was performed using cPTIO (which is a scavenger of NO) to search the genes modulated by NO in response to OGs. These data allowed us to select candidate genes to study functional analysis.

This research work was included in the ANR PIANO project coordinated by Pr David Wendehenne which aims to deciphering the molecular basis of NO signaling in plants challenged by both biotic and abiotic stresses.

This thesis document consists of five chapters.

- In chapter 1, the bibliographic context of my work has been described with brief surrounding of the current knowledge in the field of mechanisms governing plant-pathogen interaction. This review of literature is not exhaustive, and, faced with the huge amount of information published, I tried to focus on data introducing and illustrating the context of the following chapters.

- Chapter 2, NO signaling is exposed in a chapter entitled **"***Nitric oxide signaling in plants: cross-talk with Ca^{2+}, protein kinases and reactive oxygen species***".**
- Chapter 3, presents the material and methods that have been used in this study.
- Chapter 4 corresponds to the first objective of my thesis. This chapter includes:
  - ✓ an article submitted in plant cell and environments (PCE) entitled "*Nitric oxide production mediates oligogalacturonides-triggered immunity and resistance to Botrytis cinerea in Arabidopsis thaliana*"
  - ✓ Additional experiments related to the link between protein kinases (MAPK and CDPK) and NO production.
- Chapter 5 corresponds to the second objective and is entitled "*Nitric oxide-regulated transcriptomic response to oligogalacturonides in Arabidopsis thaliana – characterisation of NO-responsive genes.*"
- Finally, a conclusion and perspectives has been given to this work.

CHAPTER 1

“Bibliographic context”

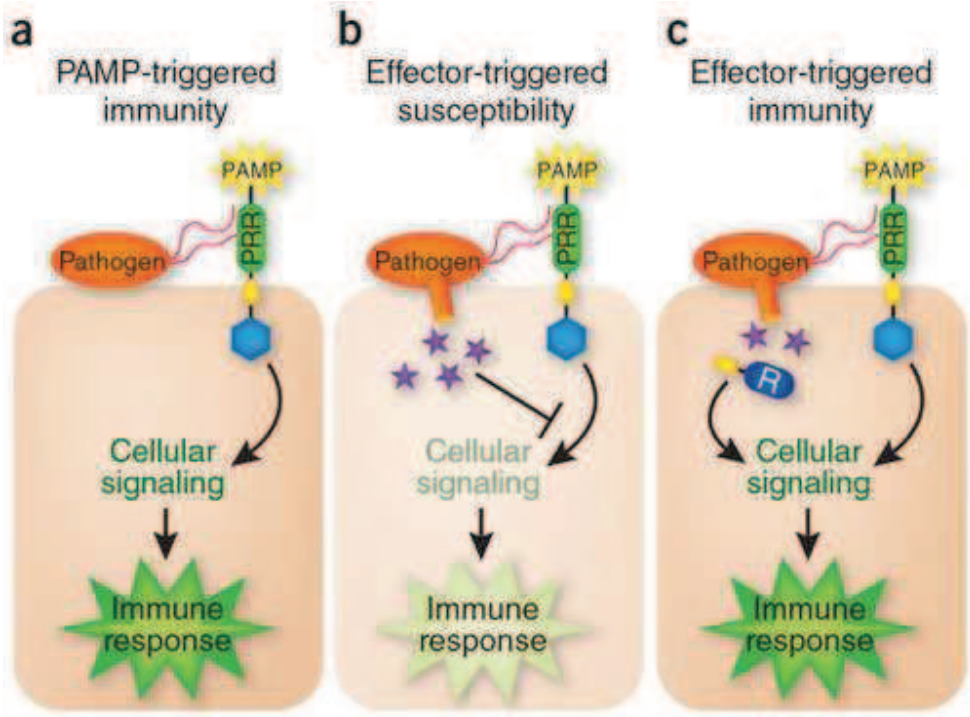

Figure 1.1: PAMP triggered Immune response A) Pathogen-associated molecular patterns (PAMPs) activate pattern-recognition receptors (PRRs) in the host after pathogen attack, resulting in the activation of downstream signaling cascade that leads to PAMP-triggered immunity (PTI). (**B**) Virulent pathogens have acquired effectors that suppress PTI, resulting in effector-triggered susceptibility (ETS). (**C**) In turn, plants have acquired resistance (R) proteins that recognize these pathogen-specific effectors, resulting in a secondary immune response called effector-triggered immunity (ETI; Pieterse et al., 2009).

CHAPTER 1

BIBLIOGRAPHIC CONTEXT

1. Plant responses against microbial pathogens

1.1. Plant immunity

Plants are constantly subjected to environmental variations that could harm their development and life cycle. Hence, they have developed particular physiological structures and effective strategies during evolution to protect themselves. First line of defense against microbial pathogens is made of physical and chemical barriers that hinder pathogen entry and colonization. In most cases, these constitutive defense structures are sufficient to protect plants from aggressors. However, microorganisms are sometimes able to overcome these first line barriers by infecting the plant through natural openings such as stomata or injury, or through the action of hydrolytic enzymes that degrade cuticle or cell wall. In addition, to prevent deleterious effects of colonization, some plants genotypes have evolved a wide variety of inducible defense mechanisms that are triggered upon pathogen recognition. Taking account of the similarities between plant and animal kingdoms, these defense mechanisms are called plant innate immunity (Jones and Takemoto, 2004; Chisholm et al., 2006). The current view of the plant immune system has been represented as a "zig zag" model (Jones and Dangl, 2006). This model identifies two components in the plant immune system. A first level of defense, defined as non-specific, is triggered by slowly evolving molecules which are molecular signatures of attackers, called PAMPs (Pathogen-Associated Molecular Patterns) and results in PAMP-triggered immunity (PTI; Figure 1.1A; Pieterse et al., 2009). Suppression of PTI by microbial effectors (effector-triggered susceptibility, ETS; Figure 1.1B; Pieterse et al., 2009) is a prerequisite for plant infection by adapted pathogens and is likely the cause for susceptibility of many crops to virulent microbial pathogens. A second level of immunity, named as Effector-triggered immunity (ETI), involves molecular recognition of pathogen effectors by protein encoded by resistance (R) genes present in plants (Figure 1.1C; Pieterse et al., 2009).

Table 1.1: Different classes of elicitors. List of elicitor commonly used to activate defense responses.

Nature	Elicitors	Origin	Host	References
Polysaccharides	**Oligogalacturans (especially 10 to 15-mers)**	Pectic fragments from plant cell wall	General	D'Ovidio et al., 2004
	Lipopolysaccharides	Gram-negative bacteria	General	Scheidle et al., 2005
Peptide	**Flg22**	22–amino acid N-terminal fragment of bacterial flagellin	General	Zipfel et al., 2004
	Pep-13	Oligopeptide of 13 amino acids within a 42-kDa transglutaminase secreted by *Phytophthora sojae*	Parsley	Brunner et al., 2002
	Chitosan (>hexamers)	Chitin fragments from fungus cell wall	General	Rabea et al., 2003
	Xylanase	*Trichoderma* spp.	General	Enkerli et al., 1999
	BcPG1	Endopolygalacturonase from *Botrytis cinerea*	Grapevine	Poinssot et al., 2003
	***beta*-glucans (especially heptaglucan), xylans**	Component of the mycelia cell walls of *Phytophthora megasperma* and other oomycetes	Soybean	Ebel 1998
	AvrPto	*Pseudomonas syringae* pv. *tomato*	Tomato race specific	Scofield et al., 1996
	Avr2, Avr4, Avr5, Avr9	Products of the corresponding *avr* genes of *Cladosporium fulvum*	Tomato race specific	Joosten and de Wit 1999

1.1.1. PAMP-triggered immunity (PTI)

In plants, PAMPs or general elicitors are most often surface-derived structural molecules and their compositions are diverse: (glyco) proteins, lipids and oligosaccharides (Table 1.1; Nürnberger et al., 2004; Boller and Felix, 2009). Among them, proteic elicitors, Flg22 (a 22 amino acids peptide corresponding to the N-terminus of bacterial flagellin) and elf18/elf26 (two peptides corresponding to the acetylated N-terminal portion of elongation factor EF-Tu from *Escherichia coli*) are the most commonly studied. These peptides lead to defense responses in *A. thaliana* (Felix et al., 1999; Kunze et al., 2004). Elicitins, proteins secreted by most *Phytophthora* species (oomycetes), also cause defense responses including localized cell death and systemic acquired resistance in tobacco and have been thought to function as oomycete PAMPs (Nurnberger et al., 2004). Another class of elicitors includes products resulting from the degradation of cellular structures of the plant or the pathogen by hydrolytic enzymes produced by the plant or the microorganism. These are called endogenous or elicitors DAMPs (Damage-Associated Molecular Pattern; Lotze et al., 2007). A classic example of such compound are oligogalacturonates (OGs), polymers of α-1,4-galacturonic acid, which are formed by mechanical tissue damage or released from cell wall pectin by the action of polygalacturonase (PG) enzymes into the wounding site (Miles, 1999; Boller, 2005).

The perception of these general elicitors (PAMPs/DAMPs) involves the patterns recognition receptors (PRRs) located on the surface of the plant cell (Nürnberger et al., 2004; Zipfel, 2009; Figure 1.2; Table 1.2). Two types of PRRs are found in plants: (1) receptor-like kinases (RLKs; proteins with an intracellular kinase domain), and (2) receptor-like proteins (RLPs; without cytoplasmic or intracellular domain; Pålsson-McDermott and O'Neill, 2007). These receptors are grouped in the LRR-RK (leucine-rich repeat receptor kinase) family and have structural similarities with the Toll-like receptors present in animal cells (Hayashi et al., 2001).

Several members of the PRRs have been identified in various plants such as Arabidopsis, rice and tomato (Boller and Felix, 2009; Nürnberger and Kemmerling, 2009). Two well studied examples of PRRs are FLS2 (flagellin sensing 2) and EFR (elongation factor Tu-receptor) that recognize flagelline/flg22 and EF-Tu/elf18 respectively in *A. thaliana* (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). Some other high-affinity sites are also involved in the recognition of elicitors, such as GBP (glucan binding protein) or CeBIP (chitin-binding protein) oligosaccharides in soybean or chitin in rice (Kaku et al., 2006), respectively. Finally, it

Table 1.2: Characteristic of selected Pathogen-associated molecular patterns (PAMPs) in plants. A) PAMPs from bacteria. **B)** PAMPs from oomycetes (*Phytophthora spp. and Pythium spp.*) and fungi (Nürnberger et al., 2004)

A

PAMP	Pathogen(s)	Minimal structural motif required for defense activation	Biological response	References
Lipopolysaccharides	Gram-negative bacteria (*Xanthomonas* and *Pseudomonas*)	Lipid A?	Oxidative burst, production of anti-microbial enzymes in pepper and tobacco, and potentiation of plant defenses in response to bacterial infection	(Dow et al., 2000; Meyer et al., 2001; Newman et al., 2002)
Flagellin	Gram-negative bacteria	Flg 22 (amino-terminal fragment of flagellin)	Induction of defense responses in tomato and Arabidopsis	(Felix et al., 1999; Asai et al., 2002)
Harpin	Gram-negative bacteria (*Pseudomonads and Erwinia*)	Undefined	Apoptosis-like cell death and induction of defense responses in various plants	(Wei et al., 1992; He et al., 1993; Lee et ., 2001)
Cold-shock protein	Gram-negative and gram-positive bacteria	RNP-1 motif (amino-terminal fragment of the cold-shock protein)	Oxidative burst and production of the plant stress hormone ethylene in tobacco, tomato, and potato	(Felix et al., 2003)
Necrosis-inducing proteins	Bacteria (*Bacillus spp.*) and fungi (*Fusarium spp.*),	Undefined	Apoptosis-like cell death and induction of defense responses in many dicot plants	(Bailey, 1995; Veit et al., 2001; Fellbrich et al., 2002; Qutob et al., 2002)

appears that the perception of elicitors could have the assistance of membrane molecules adapter to allow the initiation of signaling leading to defense responses, such as protein BAK1 (brassinosteroid receptor1-associated kinase 1) or CERK1 (chitin elicitor receptor kinase 1; Zipfel, 2009).

Perceptions of PAMPs by PRRs lead to induced resistance against various pathogens (Zipfel et al., 2004; Hann and Rathjen, 2007). In addition, Lacombe et al., (2010) have recently shown that the heterologous expression of EFR, a PRR islated form *A.thaliana* (AtEFR) in tomato and tobacco permits the establishment of a new response to EF-Tu (elf18) conferring increased resistance to a large spectrum of pathogens.

1.1.2. Effector-triggered immunity (ETI)

Effectors are secreted by different types of pathogens including bacteria and fungi (Chisholm et al., 2006; Schornack et al., 2008; Stergiopoulos and de Wit, 2009; Dodds and Rathjen, 2010). In plants, bacteria such as *P. syringae* are able to secrete approximately 20 to 30 effectors during infection (Chang et al., 2005). These effectors generally act by inactivating a target proteins of host involved in PTI or control signaling events crucial for the plant development (Jones and Dangl, 2006; Figure 1.1).

These effectors are proteins encoded by avirulence genes and could be recognized by the products of R-genes of the plant. Analysis of the products of R-genes has highlighted structural similarities and the presence of several protein motifs and conserved nature among different plant species (Table 1.3; Nürnberger et al., 2004). There are three major classes of R-genes in plants (Dangl and Jones, 2001). The largest class encodes nucleotide binding-site-leucine-rich repeat (NBS-LRR)–type R proteins with an NBS domain and LRRs. The NBS domain allows the binding and hydrolysis of ATP, via conformational changes of the protein and activation of signaling events necessary for the establishment of defense responses (Takken and Tameling, 2009). These proteins may have different N-terminal regions, defining two distinct classes: R protein CC-NBS-LRR and TIR-NBS-LRR (Meyers et al., 2003). The NBS-LRR class of R proteins is predicted to be intracellular. The two remaining classes of R-genes encode PRRs (see above), they do not encode R-protein *sensus stricto* as they do not recognize specific effectors but general elicitors.

B

PAMP	Pathogen(s)	Minimal structural motif required for defense activation	Biological response	References
Transglutaminase	Oomycetes (*Phytophthora spp.*)	Pep-13 motif (surface-exposed epitope of the transglutaminase)	Induction of defense responses in parsley and potato	(Nürnberger et al., 1994; Brunner et al., 2002)
Lipid-transfer proteins (elicitins)	Oomycetes (*Phytophthora spp. and Pythium spp.*)	Undefined	Apoptosis-like cell death, induction of defense responses in tobacco, and systemic acquired resistance to microbial infection	(Ricci et al., 1989; Osman et al., 2001)
Xylanase	Fungi (*Trichoderma spp.*)	TKLGE pentapeptide (surface-exposed epitope of the xylanase)	Apoptosis-like cell death and ethylene production in tobacco and tomato	(Hanania et al., 1997; Enkerli et al., 1999 ; Rotblat et al., 2002)
Invertase	Yeast	N-Mannosylated peptide (fragment of the invertase)	Activation of the phenylpropanoid pathway and ethylene production in tomato	(Basse et al., 1993)
β-Glucans	Fungi (*Pyricularia oryzae*), oomycetes (*Phytophthora spp.*), and brown algae	Tetraglucosyl glucitol-branched hepta-β-glucoside linear oligo-β-glucosides	Induction of defense responses in legumes, tobacco, and rice	(Klarzynski et al., 2000 ; Mithöfer et al., 2000; Yamaguchi et al., 2000)
Sulfated fucans	Brown algae	Fucan oligosaccharide	Induction of defense responses in tobacco and systemic resistance to viral infection	(Klarzynski et al., 2003)
Chitin	All fungi	Chitin oligosaccharides (degree of polymerization > 3)	Induction of defense responses in tomato, Arabidopsis, rice, wheat, and barley	(Baureithel et al., 1994 ; Barber et al., 1994; Ito et al., 1997 ; Peck et al., 2001)
Ergosterol	All fungi		Induction of ion fluxes in tomato	(Granado et al., 1995)
Cerebrosides A, C	Fungi (Magnaporthe spp.)	Sphingoid base	Phytoalexin production in rice	(Koga et al., 1998)

The perception of the pathogen effectors by the plant R-proteins involves different mechanisms (Chisholm et al., 2006; Dangl and Jones, 2006). First, the recognition may be direct by the physical interaction of R-protein with the pathogen effector (Figure 1.3). This kind of recognition has been demonstrated for different pathogens including bacteria, fungi and oomycetes, by two-hybrid experiments. First evidence comes from interaction between a NBS-LRR protein *Pi-ta* from rice and the effector protein AVR-Pita from the fungus *Magnaporthe grisea* (Jia et al., 2000). Other example of this type of interaction is the interaction between *P. syringae* effector AvrPto with the tomato R protein Pto (Tang et al., 2006). On the other hand, in many pathosystems, it was proposed that the recognition of the effectors is done indirectly (DeYoung and Innes, 2006; Figure 1.3). Plant cells express proteins may interact with complex formed between the effector and pathogenicity target (guard hypothesis; Dangl and Jones, 2001). For example, Mackey et al., (2002) identified RIN4, a protein that interacts with two *P. syringae* effectors AvrB and AvrRpm1 and the R protein RPM.

1.2. Signal transduction

The perception of the pathogen by elicitors and/or effectors, leads to the activation of intracellular signaling cascades (Figure 1.4; Felix and Boller, 2009), starting with the so called "early events". These early events could include changes in the plasma membrane permeability, ions fluxes, production of nitric oxide (NO) and ROS, and a cascade of phosphorylation of protein kinases including MAPKs (mitogen-activated protein kinases) and CDPKs (Ca^{2+}-dependent protein kinases). The mobilization of these actors leads to activation of transcription factors (TFs) that contribute to genome reprogramming and subsequent establishment of defense responses. These cellular mechanisms were described in different elicitors/plant models such as flagellin/*A. thaliana* (Gomez-Gomez and Boller, 2002), cryptogein/tobacco (Garcia-Brugger et al., 2006), *Pseudomonas* lipopolysaccharides and siderophores/tobacco (Van Loon et al., 2008), OGs, laminarin, or *Botrytis cinerea* endopolygalacturonase (BcPG1)/grapevine (Aziz et al., 2003; Aziz et al., 2004; Vandelle et al., 2006).

1.2.1. Ion fluxes

In plants, following the recognition of pathogen (elicitors/effectors), the ion fluxes through plasma membrane is one of the earliest events detected in the first five minutes after

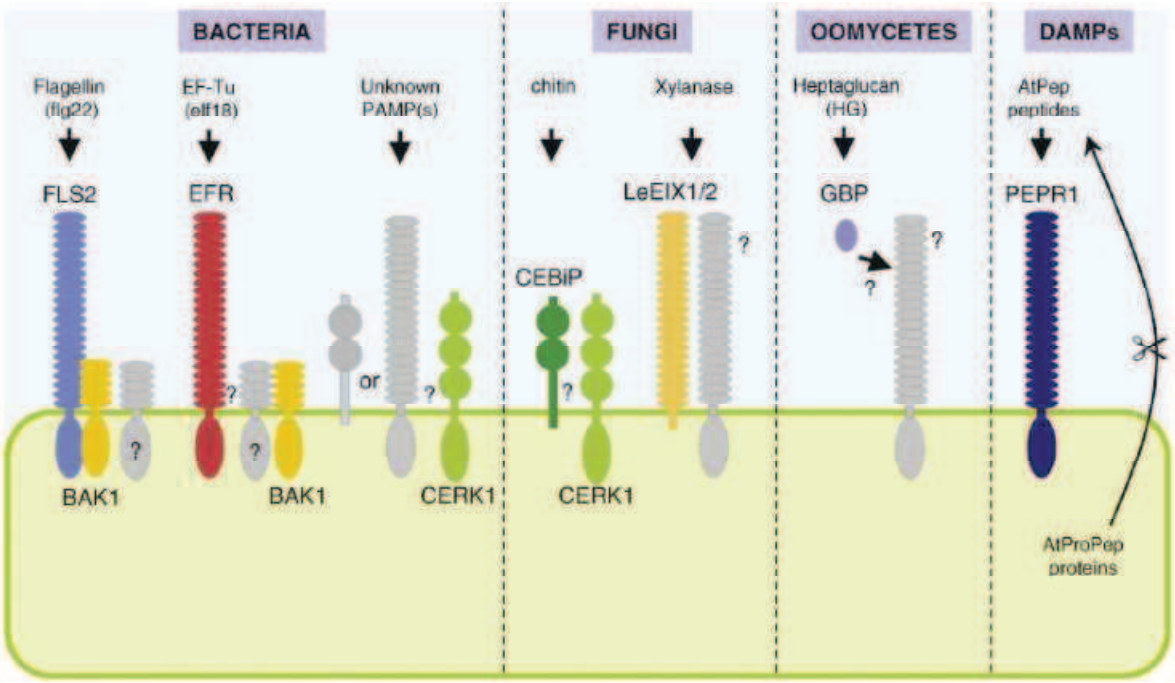

Figure 1.2: Plant pattern-recognition receptors (PRRs). Bacterial flagellin (flg22) and EF-Tu (elf18) are recognised by Arabidopsis LRR-RKs FLS2 and EFR, respectively. FLS2 Orthologues have been characterised in Arabidopsis, *N. benthamiana*, tomato and rice. The chitin high-affinity-binding site in rice corresponds to CEBiP, a transmembrane protein with two extracellular LysM domains. In tomato, xylanase is recognised by the RLPs LeEIX1/2. Although both LeEIX1 and LeEIX2 can bind to EIX, only LeEIX2 is able to trigger signalling. The Arabidopsis LRR-RK PEPR1 recognise the endogenous AtPep peptides that act as DAMPs (Zipfel et al., 2009).

Table 1.3: Classification of plant *R* genes Selected set of Avr/R gene pairs from various plant–microbe interactions including viruses, bacteria, fungi, and oomycetes. (Nürnberger et al., 2004).

Localisation of protein is indicated Intra or extracellular transmembran domain, ; protein kinases domain, ; Leucine-zipper/coil domain, ; transmembrane region, ; nucleotide- binding site, ; toll/ interleukine (TIR) leucine rich region.

Plant Species	Plant R gene	Structure	Locilization in plant	Pathogen	Mactching pathogen gene
Tomate	*Pto*			*Pseudomonas syringae pv. Tomato*	*AvrPto*
Arabidopsis thaliana	*RPW8*			*Erysiphe spp.*	*Avr RPW8*
Arabidopsis thaliana	*RPM1*			*Pseudomonas syringae pv. Maculicol*	*AvrRpm1, avrB*
Arabidopsis thaliana	*RPP8*			*Peronospora parasitica*	*AvrRpp8*
Arabidopsis thaliana	*RPS2*			*Pseudomonas syringae pv. Tomato*	*AvrRpt3*
Arabidopsis thaliana	*RPS5*			*Pseudomonas syringae pv. Tomato*	*AvrPphB*
Potato	*Rx*			Potato virus x	*Virual coat protein*
Barley	*Mla6*			*Blumeria graminis*	*Avr-M16*
Rice	*Pi-ta*			*Magnaporthe grisea*	*AvrPita*
Arabidopsis thaliana	*RPP5*			*Peronospora parasitica*	*AvrRPP5*
Arabidopsis thaliana	*RPS4*			*Pseudomonas syringae pv. Pisi*	*AvrRps4*
Flax	*L6*			*Melampsora lini*	*AvrL6*
Flax	*M*			*Melampsora lini*	*AvrM*
Tabac	*N*			*Tobacco mosaic virus*	*Replicase*
Tomato	*Cf-2*		Extracullular Transmembrane	*Cladosporium fulvum*	*Avr2*
Tomato	*Cf-4*		Extracullular Transmembrane	*Cladosporium fulvum*	*Avr4*
Tomato	*Cf-5*		Extracullular Transmembrane	*Cladosporium fulvum*	*Avr5*
Tomato	*Cf-9*		Extracullular Transmembrane	*Cladosporium fulvum*	*Avr9*
Rice	*Xa21*		Extracullular Transmembrane	*Xanthomonas oryzae pv. Oryzae*	*AvrXa21*

elicitation. Ion fluxes include influx of Ca^{2+} and H^+ and efflux of K^+ and anions (especially Cl^- or NO_3^-). The calcium and anionic fluxes trigger a depolarization of the plasma membrane; the kinetics and amplitude depend on the nature of the elicitor (Garcia-Brugger et al., 2006). Among these ions, Ca^{2+} is considered as one of the major second messengers, acting upstream numerous other signalling events in the plant response to pathogens (Dodd et al., 2010). Elicitors such as elicitins mobilize Ca^{2+} from both extracellular (apoplast) and intracellular pools (vacuole, ER). Several studies reported that the intracellular concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) increased from nM to mM range in just 2-5 minutes after elicitation (Zhao et al., 2005). It is proposed that each elicitor is able to induce specific changes in $[Ca^{2+}]_{cyt}$ in the cytosol defining the concept of "calcium signature" (Lecourieux et al., 2005). Rapid increase in cytoplasmic Ca^{2+} concentrations promotes the opening of other membrane channels (Blume et al., 2000; Brunner et al., 2002; Lecourieux et al., 2002; Ranf et al., 2008), or activates calcium-dependent protein kinases (Ludwig et al., 2005). Channels/transporters involved in the mobilization of Ca^{2+} into the cell include ATPases, interchanges Ca^{2+}/H^+ channels, TPC type (two pore channels), glutamate receptors and CNGC (cyclic nucleotide-gated channel; Dodd et al., 2010). These latter proteins were particularly studied in the plant defense context. In *Arabidopsis*, 20 CNGC members are subdivided into four groups (Figure 5; Maser et al., 2001). Mutants showed enhanced resistance to virulent and avirulent *P.syringae* strains but lacked the HR (Yu et al., 1998) named dnd "defense no death". Loss of CNGC4, with CNGC2 lead to enhanced resistance and lack of hypersensitive cell death (Balague et al., 2003; Jurkowski et al., 2004). In addition, Ali et al., (2007) identified CNGC2, as a key Ca^{2+} permeable channel that affect plant defense response through a Ca^{2+}-dependent NO production signalling cascade. *Arabidopsis dnd1* (cngc2) mutant displays no hypersensitive response to infection by some pathogens (Clough et al., 2000; Ali et al., 2007).

The involvement of anion fluxes in cell signaling in response to elicitors was supported mainly by pharmacological and biochemical approaches (Jabs et al., 1997; Zimmermann et al., 1998). The efflux of anions, especially Cl^- and NO_3^-, has been described as an early signaling event, as observed in the first five minutes of elicitor (cryptogein) treatment (Pugin et al., 1997; Wendehenne et al., 2002) and could trigger defense responses (Jabs et al., 1997; Nürnberger et al., 2004).

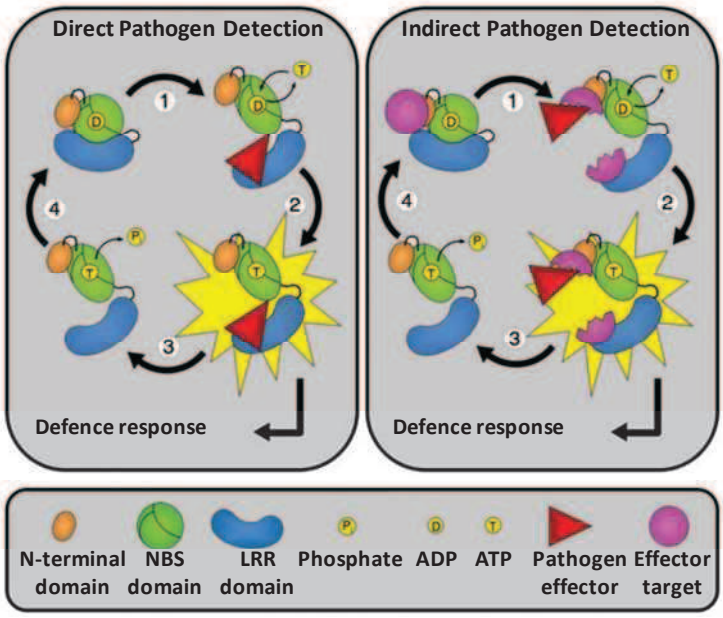

Figure 1.3: Models for plant NBS-LRR activation. Signaling is activated in a similar way for both modes of pathogen detection. Presence of the effector (1) change in the structure of NBS-LRR protein through direct binding (left) or alteration of additional plant proteins (right), allowing the formation of ATP from ADP. Binding of ATP to the NBS domain (2) activates signal transduction through the formation of binding sites for downstream signaling molecules and/or the formation of NBS-LRR protein multimers. Dissociation of the pathogen effector and modified effector targets (if present; 3) along with hydrolysis of ATP (4) returns the NBS-LRR protein to its inactive state (De young and Innes 2006).

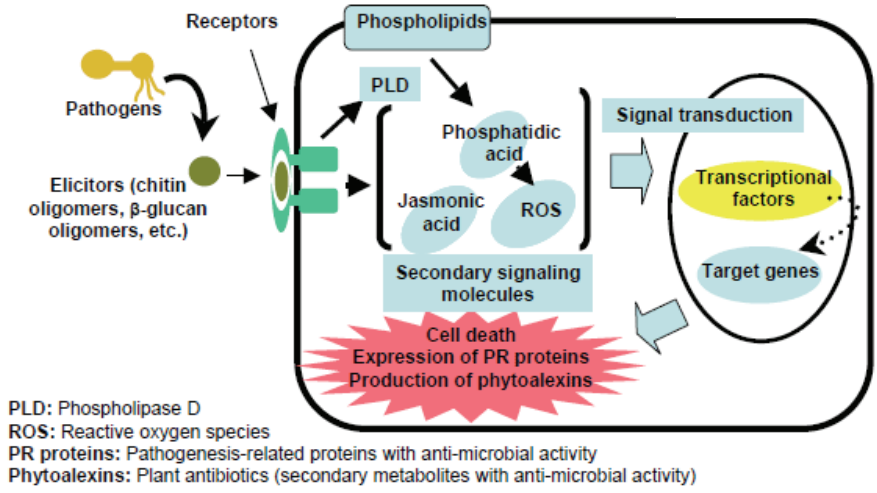

Figure 1.4: Signal transduction in plants. Pathogen-derived elicitors (e.g. chitin, polysaccharides and β-glucan oligomers) bind to specific receptors. This perception leads to defense responses, such as cell death, production of phytoalexins, and expression of pathogenesis-related (PR) proteins. These defense responses are caused via a series of signal transduction pathways including production of secondary signaling molecules (such as JA, ROS), expression and/or activation of transcription factors followed by activation of target genes {modified after Prof. H. Yamane plant research group (http://park.itc.u-tokyo.ac.jp/biotec-res-ctr/kampo/eng/research_plant.html)

1.2.2. Oxidative burst

Reactive oxygen species (ROS) play an important role in plant resistance. They contribute to limit the development of the pathogen, by acting directly on the pathogen, by helping to strengthen the cell wall and/or by participating in the cell signaling cascade leading to resistance (Lamb and Dixon, 1997).

ROS mainly superoxide anion ($O_2^{\cdot -}$) and H_2O_2 are produced after elicitation or pathogen attack (Torres et al., 2006; Lamb and Dixon, 1997). In cell suspensions, H_2O_2 production reached to its maximum after 10-30 min of treatment, depending the type of elicitor used (Simon-Plas et al., 1997; Aziz et al., 2003; Poinssot et al., 2003; Van Loon et al., 2008). This rapid and transient production of ROS is known as "oxidative burst".

Many studies were focused on identification of enzymatic sources of H_2O_2 production in plants defense mechanisms and reported the involvement of NADPH oxidase activity (Pugin et al., 1997; Simon-Plas et al., 2002; Torres et al., 2002; Torres and Dangl, 2005). Plant NADPH oxidases catalyze the reduction of dioxygen to $O_2^{\cdot -}$ from the oxidation of NADPH. $O_2^{\cdot -}$ undergoes a dismutation catalyzed by apoplastic superoxide dismutase (SOD), and produced H_2O_2. Identification of the gene encoding the NADPH oxidase in plants revealed that the protein corresponds to the catalytic subunit gp91-phox of mammalian (Torres et al., 1998). Plant NADPH oxidase contains six transmembrane domains, two binding domains for FAD and NADPH (C-terminal side), and two EF-hands motifs (N-terminal side; Keller et al., 1998; Torres and Dangl, 2005; Sagi and Fluhr, 2006). The activity of the protein is directly regulated by the binding of Ca^{2+} to EF hands but also by phosphorylation at the N-terminal domain by a CDPK-type kinase (Kobayashi et al., 2007). In *A. thaliana*, 10 Rboh forms (A to J) were identified (Torres et al., 1998; Sagi and Fluhr, 2006). In *A. thaliana*, RbohD and RbohF are essential for the accumulation of ROS and resistance during incompatible interaction with *Hyaloperonospora arabidopsidis* or the avirulent strain of *P. syringae* pv. tomato (Torres et al., 2002). Similarly, in other plant species such as tobacco, NtRbohD seems to be the major source of ROS. Cells elicited with cryptogein triggers a very fast ROS production and this production is concomitant with corresponding transcript accumulation (Simon-Plas et al., 2002).

ROS production is closely related to other cellular events. Pharmacological approaches show that it is dependent on Ca^{2+}, NO, phosphorylation of proteins in grapevine in response to

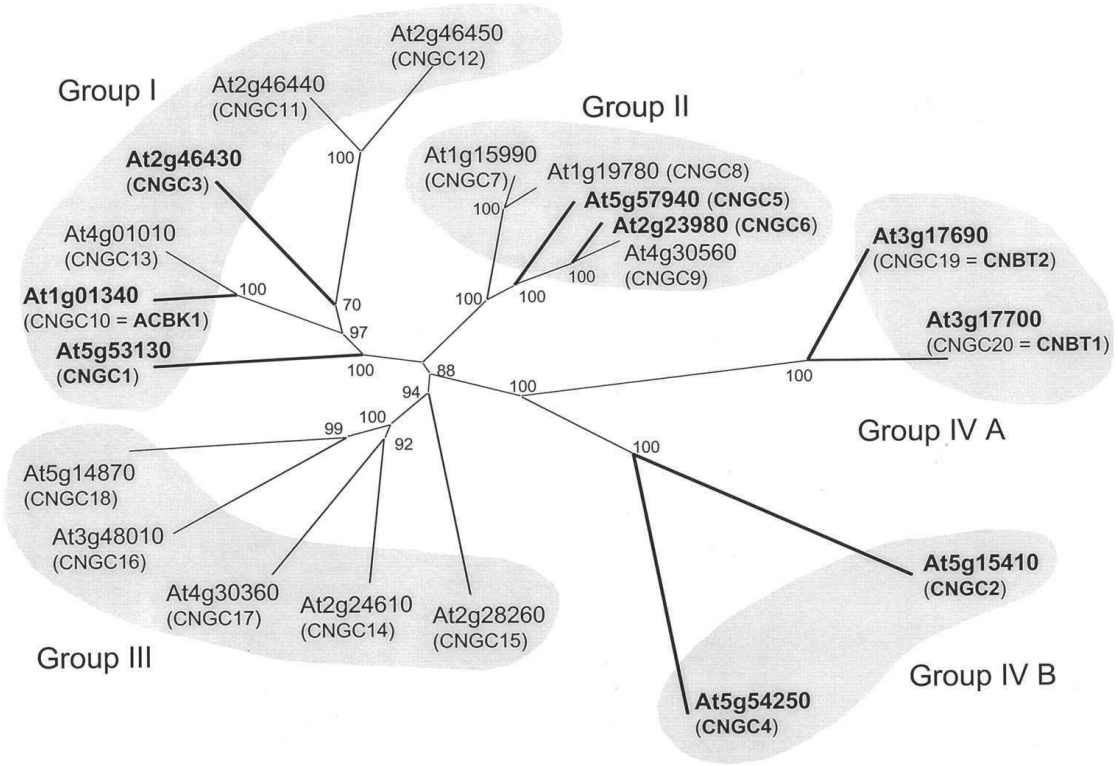

Figure 1.5: Phylogenetic tree of the *Arabidopsis* CNGC family. CNGCs are divided in 4 groups. The group four is subdivided in IV A and IV B. Group IV B contains two members CNGC2 and CNGC4 and the mutants for these are named dnd "defense, no death" (*dnd1* and *dnd2* respectively) because they displayed enhanced resistance and lack of hypersensitive cell death (Maser et al., 2001).

BcPG1 and it modulates $[Ca^{2+}]_{cyt}$ in tobacco in response to cryptogein (Lecourieux et al., 2002; Vandelle et al., 2006).

1.2.3. Activation of protein kinases (PKs)

1.2.3.1. Mitogen-activated protein kinases (MAPKs)

In response to elicitors in different species, analysis of the phosphoproteome revealed that phosphorylation of proteins are involved in the signaling cascade leading to defense response (Lecourieux-Ouaked et al., 2000; Nuhse et al., 2007). Among the protein kinases involved, MAPKs has been identified as proteins of major interest (Colcombet and Hirt, 2008; Pitzschke et al., 2009). They participate in a cascade of phosphorylation involving three types of protein kinases: the MAPK kinase kinases (MAPKKK), the MAPK kinases (MAPKK) and the MAPK (Figure 1.6). In response to a stimulus, a MAPKKK activates a MAPKK by phosphorylation, which in turn phosphorylates a MAPK that becomes active and transmits the signal (Table 1.4; Rodriguez et al., 2010). This cascade is involved in the activation of defense responses including the expression of genes encoding defense proteins such as PR (pathogenesis-related) proteins and secondary metabolism, and induction of HR (Pedley and Martin, 2005).

MAPK components are particularly abundant in plants: there are 80 putative MAPKK (MEKKs), 10 MAPKK (MKKs) and at least 20 MAPKs (MPKs) in *A. thaliana*, but functional informations are available for only few of them (Jonak et al., 2002; Nakagami et al., 2005). It was observed that MPK4 were specifically phosphorylated and activated both by biotic stresses, such as the bacterial elicitors flagellin or harpin, and by a variety of abiotic stresses (Ichimura et al., 2000; Desikan et al., 2001; Droillard et al., 2004; Teige et al., 2004). Two other MAPK (MPK3 and MPK6) are identified as being involved in the signaling of the peptide flg22 and in regulating phytoalexins synthesis in response to *B. cinerea* (Asai et al., 2002; Ren et al., 2008). More recently, it has been reported that OGs- and flg22-induced defense responses effective against *B. cinerea* are mainly MAPKs-dependent, and MAPK6 has greater contribution in these responses (Galletti et al., 2011).

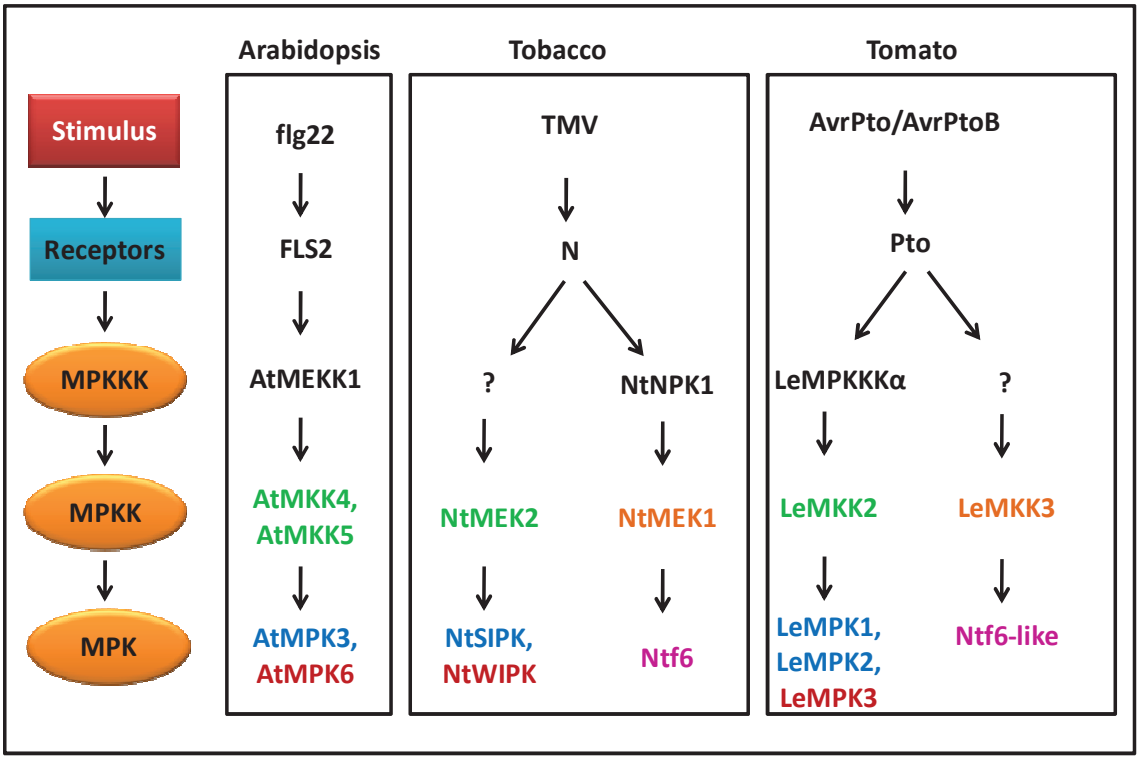

Figure 1.6: MAPK cascade in Arabidopsis, tobacco and tomato involved in defense-related signal transduction. Perception of flagellin by the FLS2 receptor activates AtMEKK1 which in turn activates AtMKK4 and AtMKK5 that activate two MPK, AtMPK3 and AtMPK6. In tobacco and tomato, two parallel paths have been defined that are activated downstream of the R-proteins N and Pto, respectively. In tobacco, the first path involves sequentially NtNPK1, NtMEK1 and Ntf6. The second cascade activation implies unknown MAPKKK then NtMEK2 and finally NtSIPK and NtWIPK. In tomato, a first cascade is represented by the MAPKKK, and MKK2 MPK1/MPK 2/MPK3. The other involves an unknown MAPKKK, MKK3 and Ntf6 (According to Pedley and Martin, 2005).

1.2.3.2. Calcium-dependent protein kinases (CDPKs)

Plant CDPKs may function as calcium sensors and play important roles in regulation of plant growth and development, responses to biotic and abiotic stresses (cold, salt, drought and wounding; Cheng et al., 2002; Ludwig et al., 2004; Klimecka and Muszyn´ska, 2007; DeFalco et al., 2010). *A. thaliana* genome encodes 34 CDPKs (or CPKs), which are subdivided in four groups (Boudscoq et al., 2010; Figure 1.7). CDPK families have also been identified in other plant species [In rice (31 members; Asano et al., 2005; Ray et al., 2007), poplar (30 members) and wheat (estimated 26 unique members in the diploid genome; Li et al., 2008)]. ABA-induced CDPKs activity has been reported in both tobacco and rice (Yoon et al., 1999; Li and Komastsu, 2000). The expression of many CDPKs is induced by stress-response and regulates plant abiotic stress responses (Rodriguez Milla et al., 2006). Moreover, under salinity stress AtCPK23 acts as a positive regulator of stomatal opening and regulation of K^+-acquisition (Ma and Wu, 2007).

In *Arabidopsis*, CDPKs are the convergence point of MAMPs triggered signaling. Boudscoq and colleagues (2010) identified a specific subgroup of CDPKs that regulate PAMP triggered immunity. They reported that in response to flg22, *cpk* mutant plants impaired in CDPKs activities, displayed gradual decline in oxidative burst and were more susceptible to pathogens.

1.2.4. Nitric oxide

NO regulates a wide range of plant processes from development to environmental adaptation and is an essential signalling molecule for plant stress responses particularly in the development of resistance against pathogens through the induction of defense responses (Wendehenne et al., 2004; Besson-Bard et al., 2008; see details in next section entitled “Nitric oxide”).

1.2.5. Role of Plant Hormones

Plant hormones [auxins, gibberellins (GA), abscisic acid (ABA), cytokinins (CK), salicylic acid (SA), ethylene (ET), jasmonates (JA), brassinosteroids (BR)] are molecules essential for the regulation of biological processes such as growth, development, reproduction, survival and plant defense mechanisms.

Table 1.4: MAPK signaling in plants. Putative MAPK signaling modules identified in plants based on genetic and biochemical studies (Rodriguez et al., 2010). Constitutive Triple Response 1 (CTR1), Enhanced Disease Resistance1 (EDR1), VirE1-Interacting Protein 1 (VIP1).

Pathways	MAPKKK	MAPKK	MAPK	Substrate	Reference
Defense responses and SA synthesis	MEKK1	MKK1/2	MPK4	MKS1/WRKY33	Petersen et al., 2000; Andreasson et al., 2005; Qiu et al., 2008
ROS homeostasis	MEKK1	MKK1/2	MPK4	?	Nakagami et al., 2006
Cold and salt stress	MEKK1	MKK2	MPK4/6	?	Teige et al., 2004
Ethylene synthesis	MEKK	MKK4/5	MPK3/6	ACS6	Asai et al., 2002; Liu and Zhang 2004
Pathogen signaling	YODA	MKK4/5	MPK3/6	VIP1	Djamei et al., 2007
Stomata development	YODA	MKK4/5	MPK3/6	SPH, SCRM, MUTE	Ohashi-Ito and Bergmann 2006; Kanaoka et al., 2008; Lampard et al., 2008
Pathogen and JA signaling	?	MKK3	MPK1/2/7/14	?	Doczi et al., 2007
Ethylene signaling and camalexin synthesis	CTR1/MAPKKK	MKK9	MPK3/6	EIN3	Yoo et al., 2008
Cytokinesis	NPK1	NtMEK2	Ntf6	NtMAP65-1a	Calderini et al., 2001; Sasabe et al., 2006

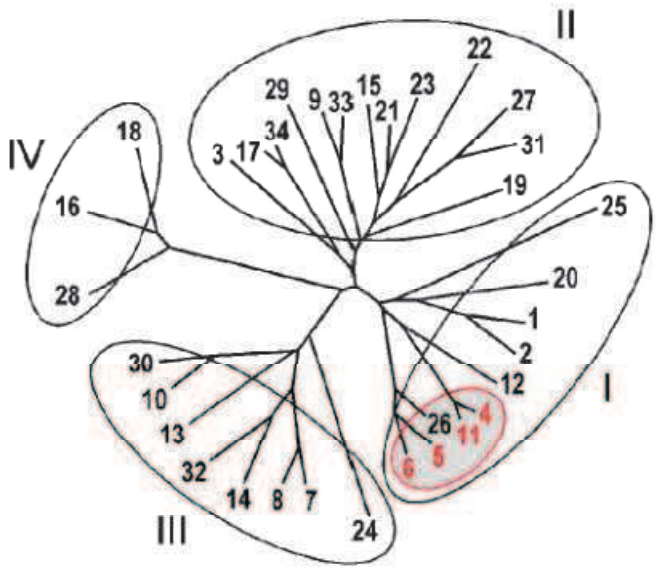

Figure 1.7: CDPK family in *A. thaliana*. *A. thaliana* CDPK family is subdivided in 4 groups. The subgroup I is involved in Flg22 signaling (Boudsocq et al., 2010).

Since last three decades, numerous studies highlighted that SA, ET and JA play a crucial role in defense responses to biotic stresses (Pieterse et al., 2009; Bari and Jones, 2009; Figure 1.8). SA is generally involved in the activation of defence against biotrophic and hemi-biotrophic pathogens as well as in the establishment of systemic acquired resistance (SAR; Grant and Lamb, 2006; Figure 1.10). In contrast, JA and ET are usually associated with defence against necrotrophic pathogens and herbivorous insects (Figure 1.8). Marker genes of these pathways have been identified. Thus, the marker gene of SA-dependent pathway is the PR-1 gene whose expression is activated by NPR1 (Lu, 2009). In contrast, VSP2 (vegetative storage protein 2) gene activation is observed only in the case of the JA pathway (Pieterse et al., 2009). Both JA and ET dependent pathways in the end lead to the expression of the PDF1.2 (plant defensin1.2) gene encoding a defensin (Guo and Ecker, 2004; Kazan and Manners, 2008). However, in some cases, such as induced systemic resistance (ISR), JA and ET pathways require the presence of NPR1 without inducing PR genes (Van Loon et al., 2006).

Interestingly, other phytohormones such as auxins, GA, CK, BR and ABA, mainly known as mediators of growth and development, and have been reported to participate in defense mechanisms however, even if their role are not clearly defined (Robert-Seilaniantz et al., 2007; Pieterse et al., 2009; Truman et al., 2010). Several studies showed that pathogen infection results in imbalances in auxin levels as well as changes in the expression of genes involved in auxin signaling. For example, infection with *Pst DC3000* resulted in increased auxins levels (IAA) in *Arabidopsis* (O'Donnell et al., 2003). However, the mode of action and how auxin levels affect the balances of other hormones for specific defence against pathogens remains to be discovered. Similarly, studies highlight that GA and CK and its signaling components play important roles in regulating defence response against various pathogens (Igari et al., 2008; Bari and Jones, 2009). BRs are a unique class of plant hormones that are structurally related to the animal steroid hormones. BRs are known to control various developmental processes, and can also improve tolerance against stresses such as cold, heat, drought, and nutrient deficiency throughout the plant kingdom (Clouse and Sasse, 1998; Bishop and Yokota, 2001). However, little is known about their role in plant responses to biotic stresses.

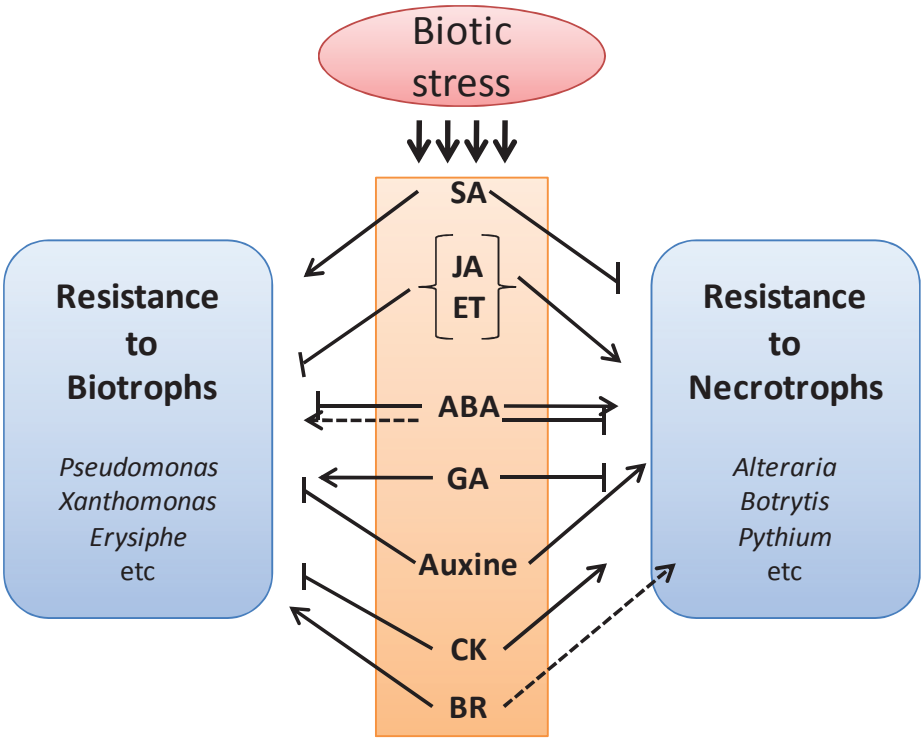

Figure 1.8: Involvement of hormones in the regulation of plant resistance to various pathogens. The arrows indicate activation or positive interaction and blocked lines indicate repression or negative interaction. Abbreviations used in figure SA: salicylic acid, JA: jasmonates, ET: ethylene, ABA: abscisic acid, GA: gibberellins, CK: cytokinins, BR: brassinosteroids (Bari and Jones, 2009).

1.3. Plant defense responses

The signaling pathways activated in plants following recognition of pathogens or elicitors lead to the activation of plant defenses (Figure 1.9). Defense induction involves genome reprogramming, defense protein accumulation (PR proteins, proteins associated with the strengthening of cell walls), biosynthesis of antimicrobial compounds and development of local and systemic resistance. These modulations are in part common to PTI and ETI (Tsuda and Katagiri, 2010). All these mechanisms could lead to the plant resistance.

1.3.1. Strengthening of the cell wall

The cell wall, composed of cellulose and pectin, is a first line of plant defense against attackers. However, most pathogens have a battery of enzymes capable of degrading the cell wall, such as pectinases, cellulases and polygalacturonases, to access nutrients from the plant. The strengthening of the cell wall results from a structural and chemical reorganization through the filling of newly synthesized molecules such as lignin and callose, accumulation of phenolic compounds and the recruitment of proteins at the cell wall. The act by catalyzing the lignification of plant cell walls (Huckelhoven, 2007). Cell wall lignification gives a better resistance to mechanical pressure and limits the spread of pathogen such as during the penetration of the appressorium of the fungus (Bechinger et al., 1999). The deposition of callose, a polymer of β-1,3-glucan, is often observed near the point of attack of the pathogen, including haustoria. Several studies have shown the involvement of callose in the defense of several plant species against different pathogens (Zimmerli et al., 2000; Hamiduzzaman et al., 2005; Ahn et al., 2007; Trouvelot et al., 2008).

1.3.2. Synthesis of antimicrobial compounds

Defense responses are characterized by the production of toxic compounds against the pathogens. These compounds are of various kinds and are synthesized after infection or elicitation. They are grouped under the term of phytoalexins (Hammerschmidt, 1999). More than 200 phytoalexins from different structural classes have been isolated and identified from more than 20 plant families (Coxon, 1982; Ingham, 1982; Kuc, 1982). The majority of these compounds are produced by members of *Leguminosae*, *Solanaceae*, and *Cruciferae* (Langcake and Pryce, 1977; Rogers et al., 1996). These phytoalexins include pterocarpans (e.g. glyceollin),

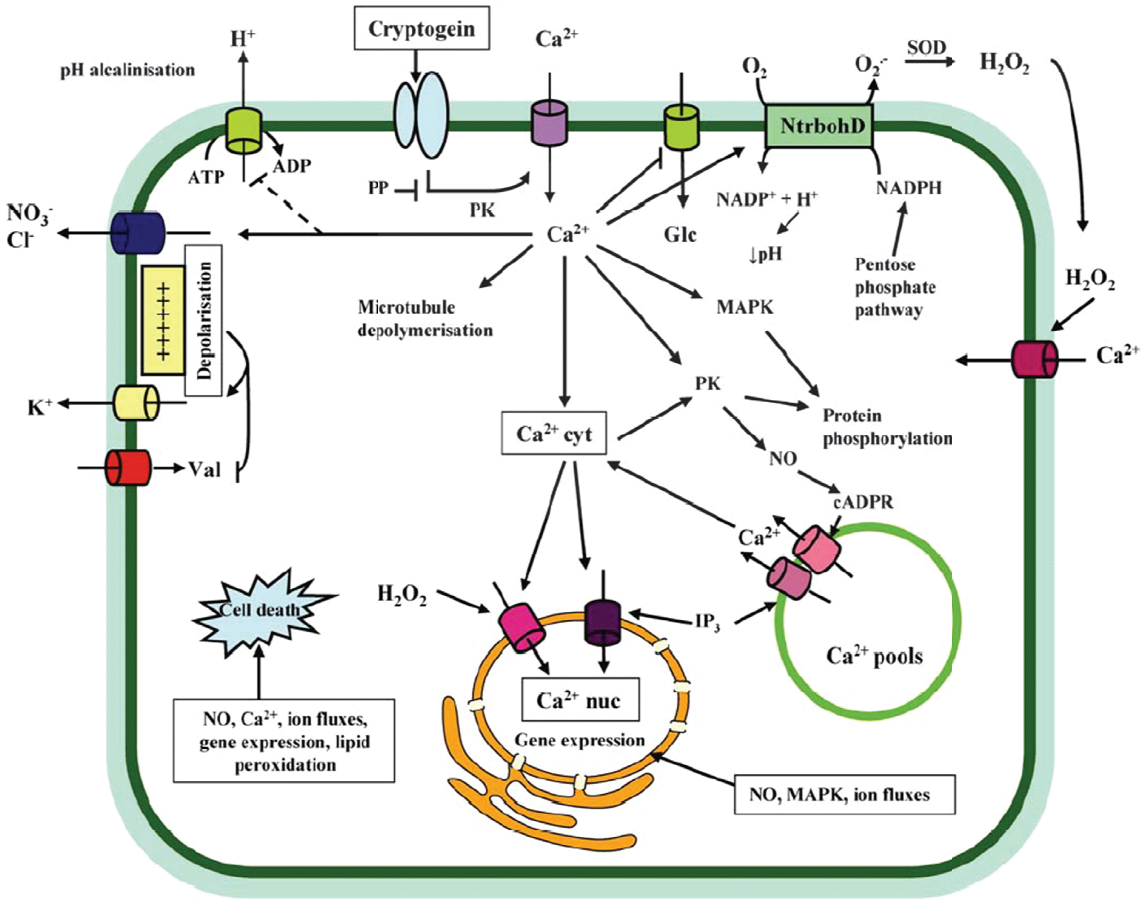

Figure 1.9: Cryptogein-induced signal transduction in plants. After the recognition of pathogens or elicitors, activation of signaling cascade leads to plant defense response (Garcia-Brugger et al., 2006). R: Receptor; NtrbohD: NADPH /Respiratory burst oxidase protein; SOD; Superoxide dismutase, PK; Protein kinases.

isoflavans, prenylated isoflavonoids (e.g. kievitone), stilbenes, psoralens, coumarins, 3-deoxyanthocyanidins, flavonols (e.g., quercetin, kaempferol), and aurones (Bailey and Mansfield, 1982; Dixon et al., 1995).

The main characterized phytoalexin of *A. thaliana* is camalexin. Camalexin belongs to the class of sulfur-rich compounds, tryptophan being the precursor for their biosynthesis (Rauhut and Glawischnig, 2009). The role of camalexin in resistance has been described through the use of camalexin deficient *A. thaliana* mutants (*pad* mutant, phytoalexin deficient; Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). *Pad3* mutant showed resistnce to virulent and avirulent strains of *P. syringae* (Glazebrook and Ausubel, 1994) but more susceptible to other pathogens such as *A. brassicicola* and *B. cinerea* (Thomma et al., 1999; Ferrari et al., 2003). In addition, the resistance induced by OGs/*B. cinerea* in *A. thaliana* requires PAD3 (Ferrari et al., 2007). PAD3 gene encodes the last enzyme involved in the biosynthesis of camalexin (Zhou et al., 1999; Nafisi et al., 2009). Several studies showed that *Arabidopsis thaliana* PAD2 mutant showed increased sensitivity to different types of necrotrophic pathogens such as *B. cinerea, A. brassicicola, or biotrophic P. brassicae, H. arabidopsidis* as well as to some insects such as *Spodoptera littoralis* (Schlaeppi et al., 2008).

1.3.3. Production of pathogenesis-related proteins (PR proteins)

PR proteins are synthesized in response to pathogens in many plant species. They were classified into 17 families on the basis of their biochemical and biological properties (Van Loon et al., 2006). Most of them have antimicrobial properties and act through hydrolytic activities, resulting in degradation of the wall of the pathogen.

For example, β-1,3-glucanase (PR-2) and chitinases (PR-3, PR-4, PR-8 and PR-11) break down the cell walls of fungi. The family of PR-7 includes endoproteases. The PR-12 (defensin), PR-13 (thionins) and some lipid transfer proteins (PR-14) have both antimicrobial and antifungal properties. Protein families PR-1 and PR-5 (thaumatin-like protein) seem to act against oomycetes and the family of PR-10 (ribonuclease-like protein) is involved in the defense against viruses. Class 6 protein contains protease inhibitors and their targets are nematodes and herbivorous insects. PR-15 (oxalate oxidase) and PR-16 (oxalate oxidase-like protein) have a superoxide dismutase activity that generates H_2O_2, which can be toxic or play a role in signaling. However, some seem rather involved in the development of resistance as PR-9 (anionic

peroxidase) which is likely peroxidases involved in strengthening the cell wall of the plant (Van Loon et al., 2006).

The expression of the PR genes is under the control of the phytohormones SA, JA and/or ET (Van Loon et al., 2006). Thus, these PR proteins can serve as a marker of the involvement of one of the three phytohormones. For example, PR-1 protein is used as a marker of SA-dependent defense pathway and for SAR.

1.3.4. Hypersensitive response (HR)

Many authors have described first hypersensitive response (HR) similar to apoptosis in animals (Greenberg and Yao, 2004). The HR is characterized by several cellular events including condensation of cytoplasm and chromatin, the release of cytochrome c from the mitochondria or the involvement of cysteine proteases (Wall et al., 2008).

In plant-pathogen interaction, the HR is defined as a localized cell death at the site of infection by the pathogens (fungi, bacteria and viruses), which causes the appearance of necrotic lesions. In responses to biotrophic or hemibiotrophic pathogens, this local resistance limits the development of the pathogen by reducing access to available nutrients (Dangl et al., 1996; Greenberg and Yao, 2004). In contrast, it was observed that the HR supports the development of necrotrophic pathogens that feed on dead tissue, such as the fungus *B. cinerea* (Govrin and Levine, 2000). The HR is considered by some authors as the final stage of development of resistance (Mur et al., 2008). It is often associated with resistance to race-specific type and can be triggered by general elicitors (Heath, 2000; Dangl and Jones, 2006). For example, some PAMPs, such as harpin, can induce HR responses (Jones and Dangl, 2006). In contrast polysaccharides (e.g. OGs, laminarin) do not induce HR and necrosis.

The precise molecular mechanisms contributing to the establishment of HR remain controversial. This probably reflects the fact that the events underlying its implementation vary depending on the pathosystem, and even considered the effector (Shapiro and Zhang, 2001). The signal transduction of programmed cell death (PCD) begins with an increase in free $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]$ of nuclear core, observed during cell death triggered by cryptogein in tobacco cell suspensions (Ma and Berkowitz, 2007; Lecourieux et al., 2006). The link between HR and transporting calcium into the cell was established using *A. thaliana* mutant *dnd1* to avirulent pathogens (Clough et al., 2000).

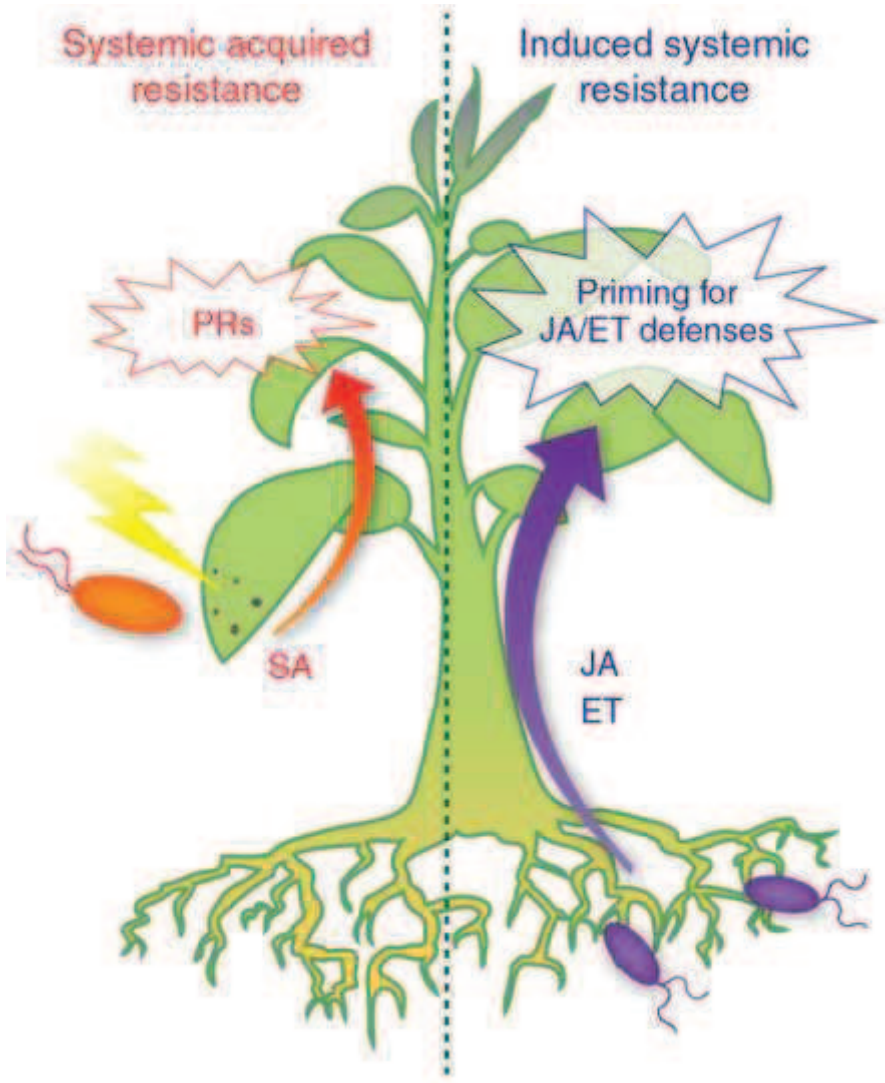

Figure 1.10: Systemic resistance in plants. After pathogen infection, a mobile signal travels through the vascular system to activate defense responses in distal tissues. SA (Salicylic acid) is an essential signal molecule for the onset of SAR, for the activation of a large set of genes that encode PR proteins with antimicrobial properties. In contract, ISR is commonly regulated by JA- (Jasmonic acid)- and ET(ethylene)-dependent signaling pathways upon colonization of plant roots by beneficial microorganisms. ISR is not associated with direct activation of *PR* genes but ISR-expressing plants are primed for accelerated JA- and ET-dependent gene expression (Pieterse et al., 2009).

1.3.5. Systemic resistance

Systemic resistance responses are generally grouped into two broad categories, systemic acquired resistance (SAR) and induced systemic resistance (ISR; Grant and Lamb, 2006).

1.3.5.1. Systemic acquired resistance (SAR)

SAR is a form of resistance set up after avirulent pathogens attack and spreads in the whole plant through the vascular system (Sticher et al., 1997). It should be noticed that elicitors such as polysaccharides or elicitins could also induce SAR. It allows protecting the plant against a subsequent attack by a broad spectrum of pathogens including viruses, bacteria, oomycetes and fungi and is effective at least for several weeks. Many studies have shown that the establishment of the SAR involved the SA-dependent pathway. It accumulates at the point of infection and in uninfected tissues (Figure 1.10). In addition, an increased expression of genes encoding some PR proteins is observed in the SAR, contributing to the maintenance of the state of plant resistance (Durrant and Dong, 2004). Although the mobile signal for SAR has been the subject of considerable research over years, its identity remained controversial (Liu et al., 2011).

1.3.5.2. Induced systemic resistance (ISR)

Among the bacteria in the rhizosphere, some rhizobacteria, called PGPR (plant growth-promoting rhizobacteria), are able to stimulate plant growth and improve its strength vis-à-vis many stress (Van Loon et al., 1998). The ISR is also observed in the case of plants colonized by mycorrhiza (Pozo and Azcona-Aguilar, 2007). ISR provides better resistance to the plant during subsequent attacks by pathogens (Pieterse et al., 1996). This resistance is used in different SAR signaling pathways because it is regulated by JA and ET, and is independent of SA (Van der Ent et al., 2009; Figure 1.10). ISR triggered by beneficial microorganisms is associated with priming rather than with direct activation of defence (Conrath et al., 2006; Pozo et al., 2008; Van Wees et al., 2008). ISR induced modulation of gene expression, mainly involved in the defense or the regulation of transcription in roots (Verhagen et al., 2004) against pathogen or insect that are sensitive/or respond to JA and ET (Ton et al., 2002; Van Oosten et al., 2008).

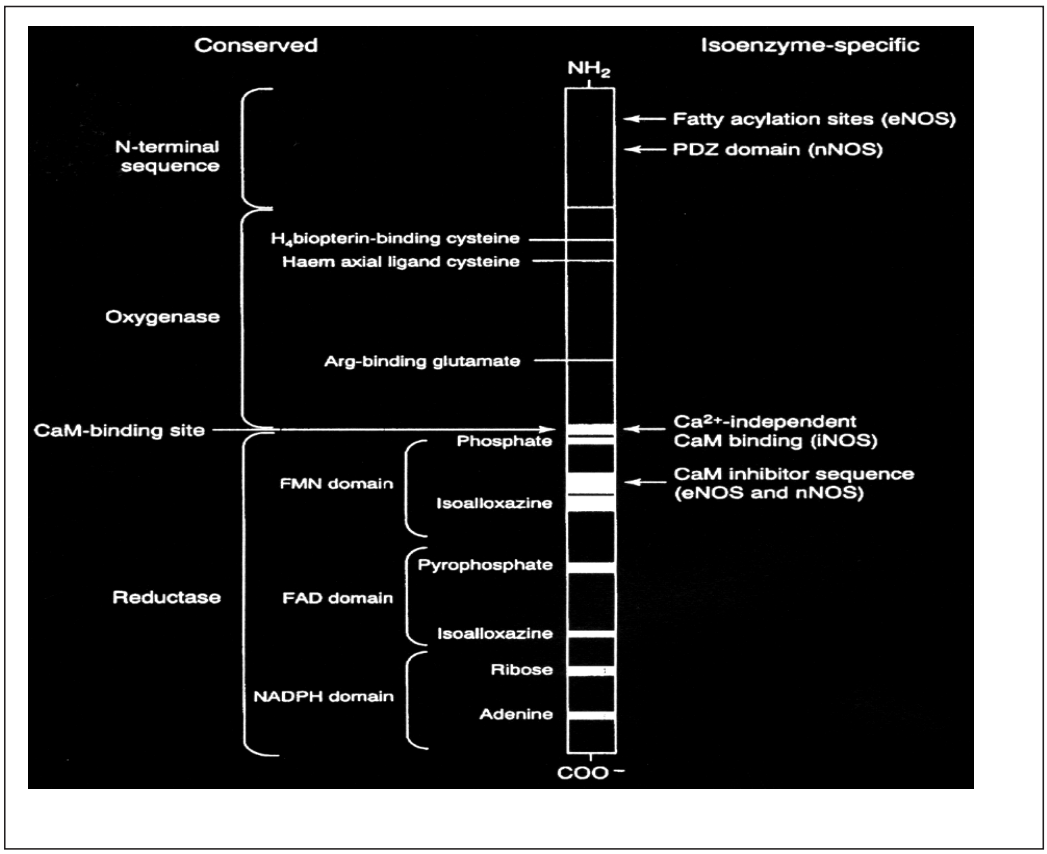

Figure 1.11: Structure of nitric oxide synthase (Wendehenne et al., 2003)

2. Nitric oxide (NO), a key player in plant defence signaling

Nitric oxide (NO) is a gaseous free radical involved in numerous reactions in all kingdoms of life (Torreilles, 2001; Besson-Bard et al., 2008). The chemical properties of NO have been studied for over 200 years (Gow et al., 2006). However, since the last 30 years, its biological implications have been considered. The knowledge of NO physiological functions in living organisms mainly comes from work done in mammals since 1980s. It is a major biological mediator involved in physiological processes. Indeed, when produced at high concentration by macrophages, NO exerts cytotoxic and genotoxic effects against invading pathogens. In addition, when produced at lowest concentrations by number of cell types including neurons and endothelial cells, it acts as a signalling molecule promoting blood vessels dilatation, smooth muscle relaxation and neurotransmission. In microorganisms, studies have shown that NO induces genes expression involved in responses to oxidative stress in signal transduction (Crane et al., 2010; Meilhoc et al., 2010). Even though NO research in plants is not as advanced as in animals, ability of plants to produce NO in the atmosphere has been corroborated since many years (Klepper, 1979). Many studies that indicate NO is as a major signaling event, involved in plant growth and development, germination, root growth, in opening and closing of stomata, gravitropism or cellular respiration as well as in responses to biotic stresses such as infections by bacteria or fungi, and abiotic stresses such as osmotic, salt or heavy metal exposure (Wendehenne et al., 2004; Besson-Bard et al., 2008; Wilson et al., 2008; Table 1.5).

At the molecular level, proteomic and transcriptomic analyses of plants tissues or cell suspensions exposed to artificially generated NO indicate that NO might convert its effects through gene expression and post-translational modifications. There is a statement that the molecular bases of NO signaling in physiological processes are still far to be understood. Another unresolved issue concerns the enzymatic sources of NO: although many efforts have been made, the mechanisms underlying NO synthesis in plants remain a black-box. This limitation severely hinders rapid progress in our understanding of NO physiological functions in plants.

In this chapter, I will firstly present basic concept of NO synthesis in animals and current advances in NO synthesis in plants. Secondly, I will give some details about NO signaling in the plants and its role in plant pathogen-interaction context.

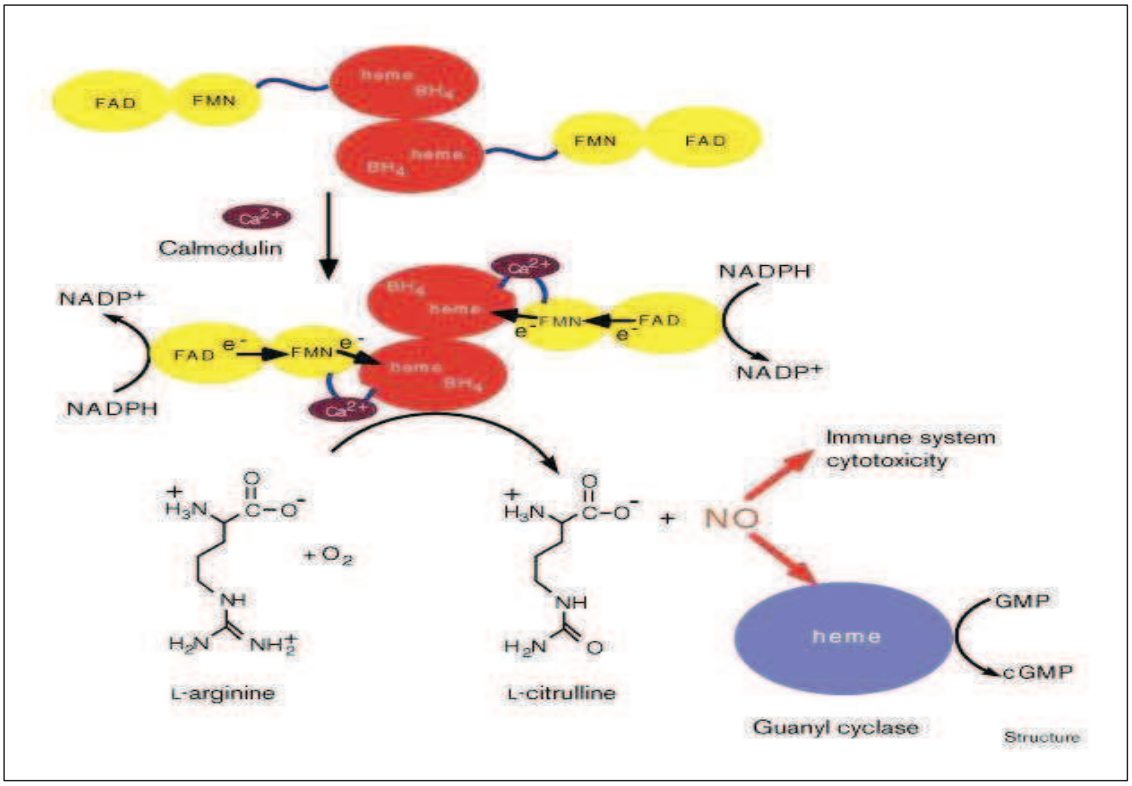

Figure 1.12: Biosynthesis of nitric oxide by nitric oxide synthase (Wendehenne et al., 2003).

2.1. NO synthesis

2.1.1. Basic concepts of NO synthesis in animals

In animals, NO is synthesized from L-arginine and oxygen by nitric oxide synthase (NOS). Three highly homologous mammalian isoforms of NOS have been identified: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS; Wendehenne et al., 2001). Each NOS is a modular enzyme that consists of a C-terminal reductase domain and an N-terminal oxygenase domain, both domains being separated by a short calmodulin (CaM) binding site (Poulos et al., 1998; Figure 1.11). In addition to the CaM binding site, NOS contains binding sites for NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4) and a heme group (Wendehenne et al., 2003; Figure 1.11). Functional NOSs are active as a homodimer and transfer electron from NADPH to their heme center *via* FMN and FAD, where L-arginine is oxidized to L-citrulline and NO (Poulos et al., 1998; Wendehenne et al., 2003; Figure 1.12).

The electron transfer between the reductase and oxygenase domains requires CaM binding. nNOS and eNOS are constitutively expressed and their activity are strickly Ca^{2+}/CaM-dependent and therefore transient (over a matter of minutes) (Mayer and Hemmens, 1997; Nathan and Xie, 1994). The context that is currently *de rigueur* states that NO produced by eNOS and iNOS acts as a signalling compound. iNOS is expressed in response to cytokines and microbial products. Remarkably, iNOS binds CaM in the absence of free Ca^{2+} (Griffith and Stuehr, 1995) and, consequently, produces large amount of NO for an extended period (hours to days), in accordance with its involvement as a toxic compound in the immune response. It should be specified that the classification constitutive versus inducible NOSs is not absolute as constitutive NOSs and inducible NOS were also shown to be regulated at the transcriptional and post-translational level, respectively.

NO is also produced in bacteria. Gram-positive bacteria encode smaller NOS proteins, containing only the oxygenase domain. Bacterial NOS uses non-specific cellular reductases to produce NO (Wang et al., 2007; Gusarov et al., 2008).

It should be noticed that in animals (Markert et al., 1994; Kouichi et al., 1997) and bacteria (Hooper and Terry, 1979), NO can also be produced by the oxidation of hydroxylamines.

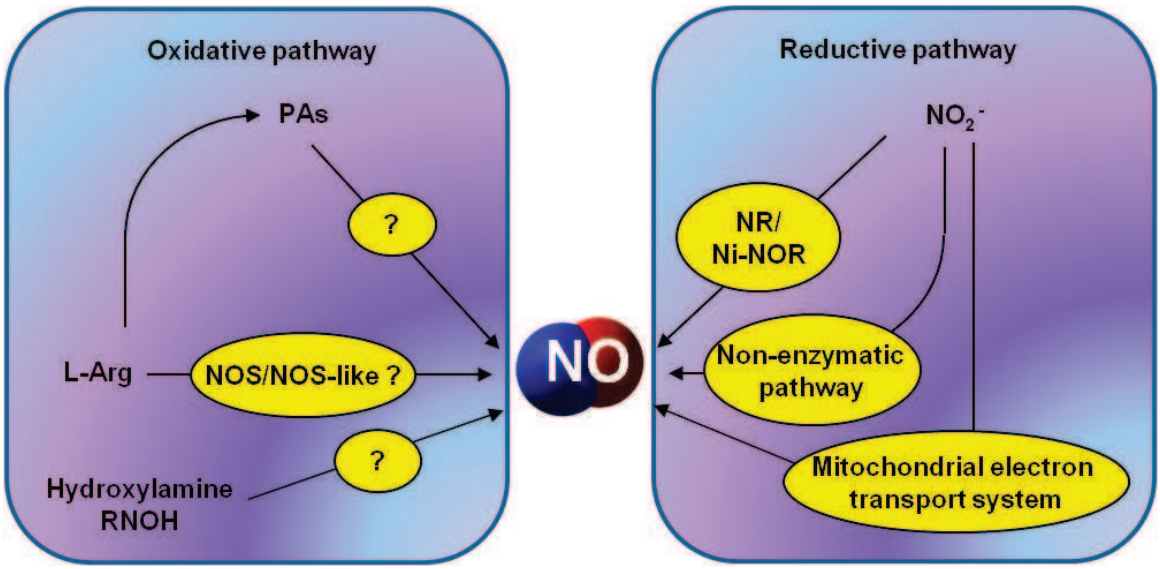

Figure 1.13: **Overview of nitric oxide synthesis in plants**. The figure shows schematic representation of NO production from two main pathways; the oxidative; and the reductive pathway. Detail descriptions of these pathways are presented in the text. NOS; Nitric oxide synthase , NOS-like; reactions sensitive to animal NOS inhibitors, L-Arg ; L-Arginine , PAs; Polyamines, NO_2^- ; Nitrite, NR; Nitrate reductase, NiNOR; nitrite-NO reductase (Adapted from Besson-bard et al., 2008; Moreau et al., 2010 and Gupta et al., 2011).

Finally, NO can be produced in a non-enzymatic chemical reduction of nitrite in an acidic environment (Weitzberg and Lundberg, 1998; Zweier et al., 1999). This is the case in certain disease states (Zweier et al., 1995) but also in acidic environments such as special light of the stomach (Benjamin et al., 1994).

2.1.2. Biosynthesis of NO in plants

Recently, several enzymatic sources of NO have been proposed for NO synthesis in plants. To date at least seven pathways of NO synthesis have been identified (Gupta et al., 2011). These pathways are classified into two groups: the oxidative pathway (L-arginine-dependent) and the reductive pathway (Nitrite-dependent).

2.1.2.1. Oxidative pathway

2.1.2.1.1. Arginine dependent NO production

There is no obvious homologues of mammalian NOS in the plant genomes sequenced so far. Various studies have failed to identify NOS genes in land plants encoding animal NOS homologue (Gupta et al., 2010; Moreau et al., 2010). Interestingly, the search for a NO synthase (NOS) sequence in the plant kingdom yielded two sequences from the recently published genomes of two green algae species of the *Ostreococcus* genus, belonging to Chlorophytae, one of the closest relative lineage of land plants (Foresi et al., 2011). However, constitutive as well as stimuli-inducible NOS activities have been measured in several land plant tissues as well as in cell suspensions (Cueto et al., 1996). These activities are sensitive to mammalian NOS inhibitors (Corpas et al., 2009). These inhibitors are able to block NO production measured in various physiological contexts such as development (Corpas et al., 2006; Wang et al., 2009), responses of plants to pathogens (Delledonne et al., 1998; Besson-Bard et al., 2008; Asai and Yoshioka, 2009) or abiotic stress such as exposure to cadmium (Besson-Bard et al., 2009; De Michele et al., 2009).

A putative *Arabidopsis* NOS gene, AtNOS1, was identified in roots through characterization of an *Arabidopsis* mutant that was defective in NO accumulation (Guo et al., 2003). Several groups have independently shown that AtNOS1 is not an NOS but might be associated with NO accumulation (Crawford et al., 2006; Moreau et al., 2008; Zemojtel et al.,

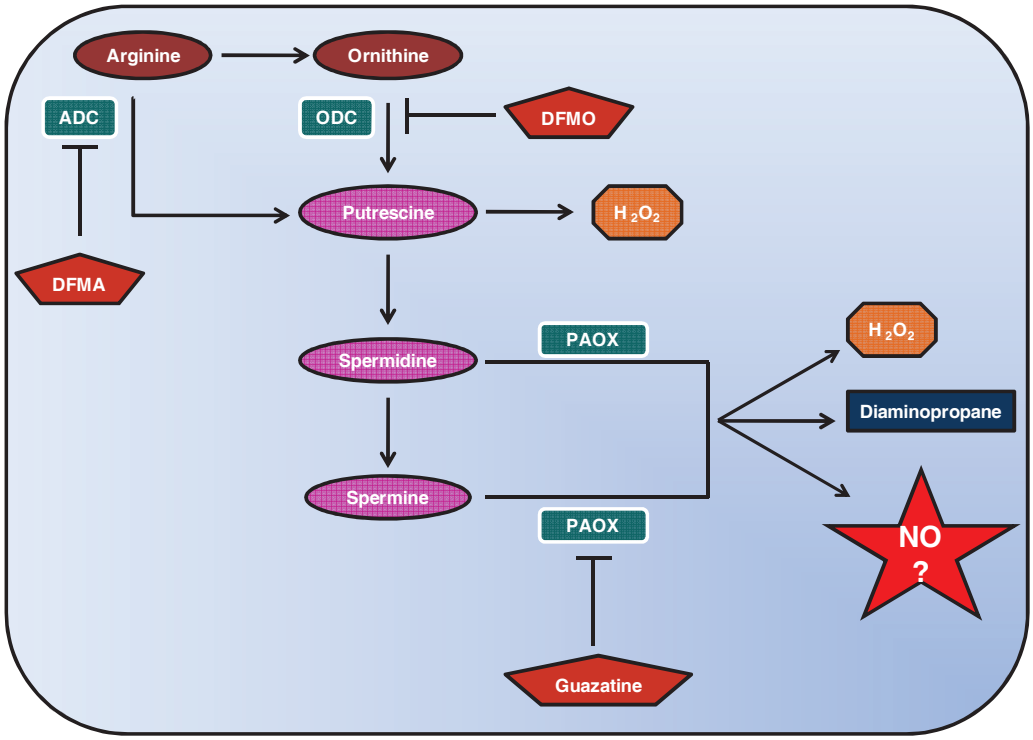

Figure 1.14: Polyamines metabolism in plants. Putrescine is synthesized biologically via two different pathways, both starting from arginine. In one pathway, arginine is converted into agmatine with a reaction catalyzed by the enzyme Arginine decarboxylase (ADC), after series of intermediate finally putrescine. In the second pathway, arginine is converted into ornithine and then ornithine is converted into putrescine by ornithine decarboxylase (ODC). Spermidine is synthesized from putrescine, using an aminopropylic group from decarboxylated S-adenosyl-L-methionine (SAM). The reaction is catalyzed by spermidine synthase. Spermine is synthesized from the reaction of spermidine with SAM in the presence of the enzyme spermine synthase. . ADC : Arginine decarboxylase, ODC : Ornithine decarboxylase, DFMA : Difluoromethylarginine, DFMO : Difluoromethylornithine, PAOX : Polyamine Oxidase.

2006). For this reason, AtNOS1 has been renamed NO-associated protein 1 (AtNOA1). *Atnoa1* mutant shows lower NO level under several stress conditions (He et al., 2004; Zeidler et al., 2004; Bright et al., 2006; Zottini et al., 2007) but accumulates as much NO as wild-type plants in other conditions (Arnaud et al., 2006; Bright et al., 2006; Kolbert et al., 2008; Shi and Li, 2008; Tun et al., 2008; Besson-Bard et al., 2009; Shi et al., 2009). In conclusion, the question of the NOS-like activity (L-arginine dependent) remains open.

2.1.2.1.2. PAOX pathway

The likely limited specificity of mammalian NOS inhibitors in plants is also a key parameter to take into account. Indeed, according to Besson-Bard et al., (2008) the possibility that these compounds affect the activities of other L-arginine metabolizing enzyme should not be excluded. This includes the first enzyme of the polyamine (PA) biosynthetic pathway (Figure 1.14), the arginine decarboxylase (ADC), as no gene coding for ornithine decarboxylase (ODC) is present in *A. thaliana* (Hanfrey et al., 2001). Supporting this assumption, Tun et al., (2006) reported that the PAs, spermine and spermidine, trigger a fast NO production in several tissues within *A. thaliana* seedlings. This raises the possibility that NO synthesis could be achieved through the polyamine synthesis (Figure 1.14) pathway and then the action of one or several polyamine oxidase (PAOX). At least 10 isoforms of PAOX have been identified in the *Arabidopsis thaliana* genome. In this scenario, the inhibitory effect of mammalian NOS inhibitors (Arginine analogs) on NO production might be related to their ability to suppress PAs synthesis and the subsequent PAs-dependent NO production (Figure 1.14). To our knowledge, enzymatic evidence that a PAOX indeed catalyses NO synthesis from L-Arg dependent PAs has not been clearly reported so far. Recently, NO production was reported by fluorescence microscopy and fluorometry by an *Arabidopsis thaliana* copper aminooxidase1 (CuAO1) from PA upon ABA treatment using knockout plant mutants. Plant mutant (*cuao1-1* and *cuao1-2*) impaired in copper aminooxidase1 (CuAO1) show low rate of NO production (Wimalasekera et al., 2011). The biological significance of this pathway remains unclear in the context of plant-pathogen interactions.

2.1.2.1.3. Hydroxylamine pathway

Finally, a recent study has shown that NO synthesis is possible from hydroxylamine (or R-NHOH) in tobacco cell suspensions (Rumer et al., 2009).

2.1.2.2. Reductive pathway

2.1.2.2.1. NR-dependent NO production

The nitrite-dependent NO synthesis involved mainly nitrate reductase (NR), a major cytosolic enzyme of nitrogen assimilation. NR catalyzes the reduction of nitrate (NO_3^-) into nitrite (NO_2^-) using NAD(P)H as electron donor (Crawford, 1995) which then are converted to ammonium by nitrite reductase (NiR) but could also reduce nitrite to NO both *in vitro* and *in vivo* (Yamasaki et al., 2000; Figure 1.13). The production occur in specific physiological context in which the cytosolic nitrite concentration reach high concentrations such as hypoxia (in the range of mM). NO produced by NR is involved in various physiological processes such as stomatal closure (Neill et al., 2008), the response to abiotic stress (Sang et al., 2008) or response to elicitors such as chitosan (Srivastava et al., 2009). In *A. thaliana*, two cytosolic isoforms have been identified (NR1 and NR2). For example, using the NR deficient *A. thaliana* mutants (*nia1 nia2 double mutant*), Bright et al., (2006) demonstrate that NR is the main enzymatic source in ABA-induced stomatal closure, highlighting a role for NR in NO-dependent signaling processes. As this NO production was shown to be H_2O_2 dependent, Neill et al., (2008) proposed a signaling cascade, ABA-H_2O_2-NO, leading to stomatal closure. Similarly, it was observed that the NR-deficient double mutant, which shows substantially reduced NO level after bacterial or fungal inoculation, showed no HR and was hyper-susceptible to *P. syringae* and to the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* (Modolo et al. 2006; Oliveira et al., 2009; Perchepied et al., 2010).

2.1.2.2.2. NI-NOR pathway

A plasma membrane-bound enzyme, nitrite:NO reductase (Ni-NOR), was discovered to be involved in NO formation from nitrite by plant roots (Stohr et al., 2001) using NADPH as electron donor (Figure 1.13). The activity of the NiNOR is induced by hypoxia catalyzing the

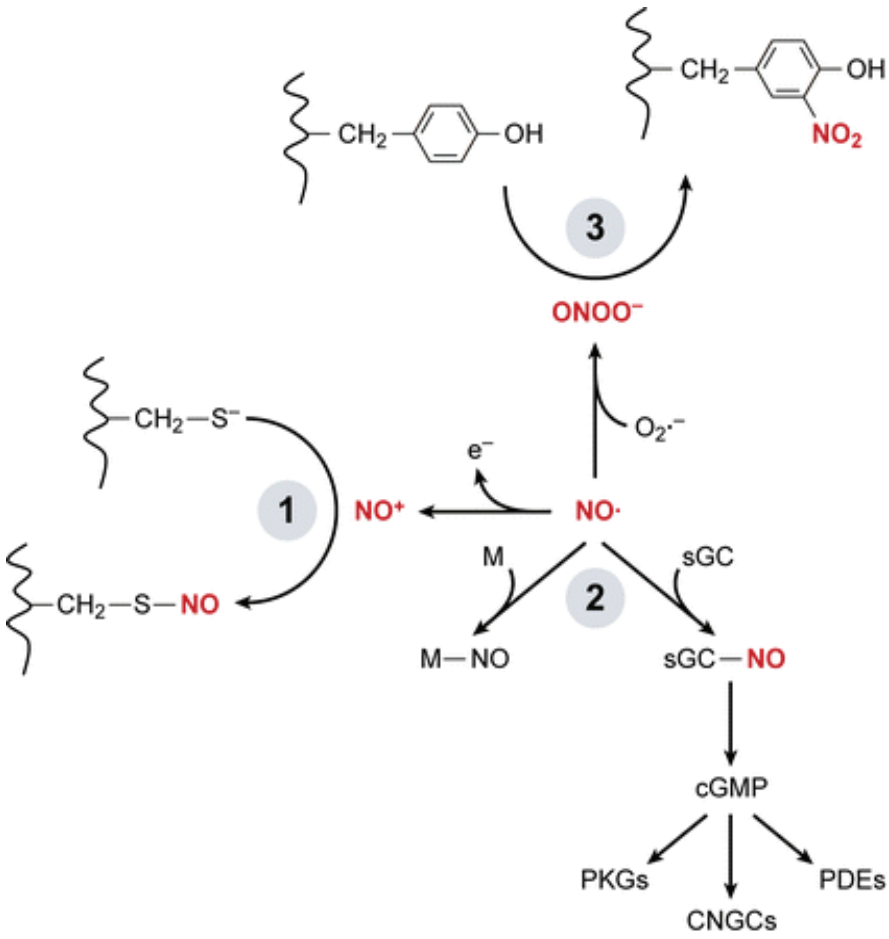

Figure 1.15: Post-translational modifications by NO. 1. S-nitrosylation is the process in which nitrosonium anion (NO^+) react with thiolate leading to the formation of an S-NO (S-nitrosothiol bond) in a protein. **2.** The NO radical can donate electrons and interact with iron sulfur clusters, heme, and zinc-finger proteins (M) to form a nitrosylated metalloproteins (M-NO). The reversible interaction of NO with soluble guanylate cyclase (sGC) increases the catalysis of cyclic GMP (cGMP) synthesis which in turn, acts on the downstream processes. **3.** Tyr nitration takes place when NO react with superoxide anions ($O_2^{\cdot -}$) and peroxynitrite (ONOO−) is formed (Besson-Bard et al., 2008).

reduction of nitrate to nitrite (Stohr and Ullrich, 2002). The identity of Ni-NOR is currently unknown.

2.1.2.2.3. Mitochorndrial pathway

In hypoxia and during the symbiotic bacteria, formation of NO by the reduction of nitrite can also be observed in the mitochondrial electrons from the mitochondrial respiratory chain (Planchet et al., 2005; Horchani et al., 2011). These two ways of production are limited to plant roots, where the oxygen partial pressure is low (Gupta et al., 2010).

2.1.2.2.4. Xanthine oxidoreductase (XOR) pathway

Nitrite can also be catalyzed by the peroxisomal enzyme xanthine oxidoreductase (XOR) to NO. XOR can reversibly convert xanthine dehydrogenase (XDH) into a xanthine oxidase (XOD; Corpas et al., 2008). Under anaerobic conditions, purified bovine milk XOD reduces nitrite to NO, using NADH or xanthine as reducing substrate (Godber et al., 2000).

2.1.2.3. Non enzymatic pathway

Finally, it has been mentioned that NO can be produced by an apoplastic nonenzymatic conversion of nitrite to NO at acidic pH, in the presence of reductants such as ascorbic acid (Bethke et al., 2004) (Figure 1.13).

2.1. Mechanisms of action of NO signaling

NO is a gaseous lipophilic free gaseous radical ($NO^{\cdot}$). It can pair to other radicals and it can be converted to oxidize and reduce form, nitrosonium cation (NO^{+}) or nitroxyl (NO^{-}) (Stamler et al., 1992). NO reacts directly with various chemical nature molecules including lipids, metals, proteins or nucleic acids and molecular oxygen and its derivatives. In animals, it has been shown that NO and its derivatives modulate the activity of proteins via post-translational modifications: S-nitrosylation, metal-nitrosylation and tyrosine (Tyr) nitration (Besson-Bard et al., 2008; Figure 1.15). In plants, microarray as well as cDNA-AFLP analyses of NO responsive transcripts, all indicate that NO governs the regulation of expression of numerous genes (Grun et al., 2006). The complete description of NO signaling will be described in the following chapter 2 entitled “Nitric oxide signaling in plants: cross-talk with Ca^{2+}, protein kinases and reactive

Table 1.5: Physiological functions of NO in plants.

Biological Process		Plant-microbe species	References
Auxin-regulated	Activation of the cell cycle	*Medicago sativa*	Otvos et al., 2005
	Development of root hairs	*Lactuca sativa; Arabidopsis thaliana*	Lombardo et al., 2006
	Formation of adventitious roots	*Cucumis sativus*	Pagnussat et al., 2003
		Cucumis sati-seen	Lanteri et al., 2006
	Involvement in the formation of lateral roots and growth inhibition of primary root	*Lycopersicon esculentum*	Aragunde-Correa et al., 2004
	oot growth	*Zea mays*	Gouvêa et al., 1997
	Response to gravitropism	*Glycine max*	Hu et al., 2005
ABA-regulated	Calcium mobilization	*Vicia faba*	Garcia-Mata et al., 2003
	Induction of antioxidant enzymes	*Stylosanthes guianensis*	Zhou et al., 2005
		Zea mays	Zhang et al., 2007
	Stomatal closure	*Arabidopsis thaliana*	Desikan et al., 2002
		Vicia faba	Garcia-Mata and LaMattina, 2002
		Arabidopsis thaliana	Guo et al., 2003
		Arabidopsis thaliana	Bright et al., 2006
Cytokinins -regulated	Accumulation of betalains	*Amaranthus caudatus*	Scherer and Holk, 2000
	Cell death	*Arabidopsis thaliana*	Carimi et al., 2005
Growth and development	Flowering	*Arabidopsis thaliana*	He et al., , 2004
	Seedlings induced by light	*Hordeum vulgare*	Zhang et al., 2006
	Leaf growth, lignification of the cell wall, pollen tube growth	*Lilium longiflorum*	Prado et al., 2004
	Fruit maturation		
	Senescence	*Fragaria-anannasa, Persea Americana*	Leshem and Pinchasov, 2000

		Arabidopsis thaliana	Guo and Crawford, 2005
	Germination	*Lactuca sativa*	Béligneux and Lamattina, 2000
		Arabidopsis thaliana	Guo et al., 2003
		Lupinus luteus-	Kopyra and Gwozdz, 2003
		Sorghum bicolor	Simontacchi et al., 2004
		Arabidopsis thaliana	Bethke et al., 2006
Symbiotic interactions	Nitrogen fixation	*Ycopersicon esculentum-L-Azospirillum brasilense*	Creus et al., 2005
		Lotus japonicus-Mesorhizobium loti-	Shimoda et al., 2005
	Regulation of symbiotic	*Medicago truncatula-Sinorhizobium meliloti*	Baudouin et al., 2006
		Medicago truncatula-Sinorhizobium meliloti	Ferrarini et al., 2008
		Medicago truncatula-Sinorhizobium meliloti	Pauly et al., 2011
		Medicago truncatula-Sinorhizobium meliloti	Horchani et al., 2010
Adaptation and responses to various abiotic stresses	Hypoxia	*Medicago sativa-*	Dorda et al., 2003
		Arabidopsis thaliana	Perazzolli et al., 2004
		Medicago sativa-	Igamberdiev et al., 2006
	Drought or osmotic stress	*Triticum aestivum, Vicia faba, and Tradescantia Salpichroa organifolia*	Garcia-Mata and LaMattina, 2001
		Nicotiana tabacum	Gould et al., 2003
		Triticum aestivum-	Xing et al., 2004
	Exposure to metals	*Lupinus luteus*	Kopyra and Gwozdz, 2003
		Arabidopsis thaliana	Arnaud et al., 2006
		Pisum sativum	Barroso et al., 2006
		Pisum sativum	Rodriguez-Serrano et al., 2006
		Arabidopsis thaliana	Besson-Bard et al., 2009
		Triticum aestivum	Gropp, et al., 2008
	Injury or wounding	*Ycopersicon esculentum-L*	Orozco-Cardenas and Ryan, 2002
		Arabidopsis thaliana	Huang et al., 2004

		Solanum tuberosum	Paris et al., 2007
	Mechanical stress	*Arabidopsis thaliana*	Garces et al., 2001
	Oxidative stress	*Phragmites communis-*	Zhao et al., 2008
	Response to ozone	*Phragmites australis*	Velikova et al., 2005
		Arabidopsis thaliana	Mahalingam, et al., 2006
	Salt stress	*Nicotiana tabacum*	Gould et al., 2003
		Lupinus luteus	Kopyra and Gwozdz, 2003
		Phragmites communis	Zhao et al., 2004
	Response to shear	*Taxus cuspidata*	Gong and Yuan, 2006
	Temperature changes	*Nicotiana tabacum*	Gould et al., 2003
		Arabidopsis thaliana	Cantrel et al., 2011
	Response to ultrasound	*Taxus-yunnannensis*	Wang et al., 2006a
	UV	*Zea mays*	An et al., 2005
		Vicia faba	He et al., , 2005
		Phaseolus vulgaris	Shi et al., 2005
Adaptation and responses to various biotic stresses	Cell death, HR	*A. thaliana-P. syringae*	Delledonne et al., 1998
		Avena sativa, Puccinia coronata	Tada et al., 2004
		A. thaliana-P. syringae pv. maculicola	Modolo et al., 2005
		A. thaliana-P. syringae; Nicotiana tabacum / Vicia faba-LPS	Ali et al., 2007
	Systemic resistance	*TMV-Nicotiana tabacum*	Song and Goodman, 2001
		Botrytis cinerea, Pelargonium peltatum	Floryszak-Wieczorek et al., 2007
		A. thaliana-Peronospora parasitica	Rusterucci et al., 2007
	NO production in plant tissues or cell cultures	*Glycine max-P. syringae pv. glycinea, A. thaliana-P. syringae pv. maculicola and avrRpml*	Delledonne et al., 1998
		TMV-Nicotiana tabacum	Durner et al., 1998
		A. thaliana-P. syringae pv. maculicola	Clarke et al., 2000
		Nicotiana tabacum-cryptogein	Foissner et al., 2000; Lamotte et

			al., 2004
		Nicotiana tabacum-P. syringae pv. tomato, Glycine max cell suspensions-P. syringae pv. glycinea	Conrath et al., 2004
		Nicotiana tabacum cell suspensions, cryptogein	Lamotte et al., 2004
		Nicotiana tabacum cell-suspensions-elicitin (INFI)	Yamamoto et al., 2004
		A. thaliana-and LPS	Zeidler, et al., 2004
		A. thaliana-P. syringae	Modolo et al., 2005
		Nicotiana tabacum-P. syringae pv. phaseolicola and tabaci	Mur et al., 2005
		Vitis vinifera cell suspensions endopolygalacturonase-1	Vandelle et al., 2006
		Lycopersicon-Solanum-cell suspensions xylanase	Laxalt et al., 2007
Metabolism	Photosynthesis		Hill and Bennett 1970
	Respiration		Zonttini et al., 2002
Others	Calcium mobilization	*Nicotiana plumbaginifolia*	Lamotte et al., 2006
	Iron homeostasis	*Zea mays*	Graziano et al., 2002
		Arabidopsis thaliana	Murgia et al., 2002
	Response to polyamines	*Arabidopsis thaliana*	Tun et al., 2006
	Response to SA	*Arabidopsis thaliana*	Zottini et al., 2007

oxygen species" published in 2011 in Annual Plant Reviews, Nitrogen Metabolism in Plants in the Post-genomic Era (Astier et al., 2011).

2.3. Role of NO in plant defense

Since the late two decades, scientists have focused on understanding the role of NO in Plant Physiology and particularly in physio-pathological context (Delledonne et al., 1998; Wilson et al., 2008; Leitner et al., 2009; Table 1.5).

2.3.1. NO production in different plant-pathogen context

NO was reported to be rapidly generated in several plant-pathogen/elicitors models (Table 1.6) by using different detection methods (Vandelle and Delledone, 2008). However, rates of NO production are often difficult to compare due to different methods used both for the application of stress and to measure NO production. Several examples were chosen from the literature to illustrate that NO production is a conserved event in plant pathogen interaction.

Firstly, elicitors from different origins and from different composition were able to initiate NO production. A rapid and intense intracellular NO production was detected in tobacco epidermal cells and cell suspensions treated with *Phythophthora cryptogea* proteic elicitin (cryptogein) using diaminofluorescein diacetate (DAF-2DA), a cell permeable NO specific fluorescent probe (Foissner et al., 2000) and by electrochemistry (Besson-Bard et al., 2008). Other elicitins from the related oomycete *Phytophthora infestans,* also induced an increase of NO level in tobacco Bright Yellow-2 cells or potato tubers (Yamamoto et al., 2003; Yamamoto et al., 2004). Bacterial PAMPs such as Lipopolysaccharides (LPS) isolated from different plant and animal bacterial pathogens exhibit a strong and quick burst of NO in cells suspension as well as in leaves in *A. thaliana* (Zeidler et al., 2004). A fungal elicitor prepared from the cell wall of *Penicillium citrinum* induced a rapid generation of NO (Xu and Dong, 2005). Moreover, polysaccharidic elicitors produced by cell wall during the plant pathogen interaction such as oligogalacturonic acid (OGs) stimulate NO in ginseng cells (Hu et al., 2003). Sulphated laminarin (PS3) was found to elicit a wider range of defense responses in tobacco and *Arabidopsis* (Ménard et al., 2004).

Secondly, NO production was also described in response to different pathogens in every type of interaction. Indeed, rapid accumulation of NO has been observed in response to avirulent

Table 1.6: NO production during plant-pathogen interaction. Evidence of NO production during plant-pathogen interaction in various systems. **Cry,** Cryptogein fungal elicitor from *Phytophthora cryptogea*; **Dpm,** fungal elicitor from *Diaporthe phaseolorum meridionalis*; **HWC,** hyphal wall component, fungal elicitor from *Phytophthora infestans*; **OGA,** oligogalacturonic acid, an elicitor from plant cell wall; **P. s. g.,** *Pseudomonas siringae glicinea*; ***P. s. g. avrA,*** *Pseudomonas siringae glicinea* carrying the *avrA* avirulence gene; ***P. s. m. avrRpm1,*** *Pseudomonas siringae maculicola* carrying the *avrRpm1* avirulence gene; ***P. s. m. m6***, *Pseudomonas siringae maculicola* race m6, avirulent strain for *Arabidopsis*; ***P. s. t. avrB or avrRpt2,*** *Pseudomonas siringae* tomato carrying either *avrB or avrRpt2* avirulence genes; **rust fungus**, avirulent crown rust fungus *Puccinia coronata avenae*; **TCV**, turnip crinkle virus; **TMV**, tobacco mosaic virus (Modified according to Romero-Puertas et al., 2004)

Plant	Pathogen/elicitor	Assay	References
Arabidopsis **leaves**	*P. s. m. avrRpm1*	NOS inhibitors, indirect method	Delledonne et al., 1998
Arabidopsis **cell suspension**	*P. s. m. m6*	Haemoglobin assay	Clarke et al., 2000
Arabidopsis **leaves**	TCV	NOS activity	Chandok et al., 2003
Arabidopsis **leaves**	*P. s. t. avrB or avrRpt2*	DAF detection	Zhang et al., 2003
Ginseng cells	OGA	NOS activity	Hu et al., 2003
Oat leaves	Rust fungus	DAF detection	Tada et al., 2004
Potato tuber disks	HWC	DAF detection in protein extract	Yamamoto et al., 2003
Soybean cell suspension	*P. s. g.*	Haemoglobin assay	Delledonne et al., 1998
Soybean cell suspension	*P. s. g. avrA*	Haemoglobin assay	Delledonne et al., 1998
Soybean cotyledons	*Dpm*	NOS activity	Modolo et al., 2002
Tobacco leaves	TMV	NOS activity	Durner et al., 1998
Tobacco epidermal section	Cry	DAF detection	Foissner et al., 2000
Tobacco leaves	TMV	NOS activity	Chandok et al., 2003

bacteria in *Arabidopsis* and soybean cell suspensions (Delledonne et al., 1998; Clarke et al., 2000; Zhang et al., 2003). Similarly, direct contact of avirulent crown rust fungus with oat plants induces NO production at an early stage in the defence response (Tada et al., 2004). In contrast, virulent bacteria cause only an extremely modest accumulation of NO in soybean or *Arabidopsis* cell suspensions (Delledonne et al., 1998; Clarke et al., 2000). Concerning necrotrophic fungal pathogens, inoculation with *Sclerotinia sclerotinium* triggered NO production and NO levels detected in the susceptible and resistant ecotypes were not significantly different (Perchepied et al., 2010). Notably, in response to *Botrytis cinerea*, *Pelargonium peltatum* leaves initiated a near-immediate NO burst, but the specificity of its generation was dependent on the genetic makeup of the host plant; a subsequent wave of NO generation enhance the resistance of *Pelargonium* to *Botrytis* (Floryszak-Wieczorek et al., 2007). Piterkova et al., (2009) also reported a specific two phase increase of NO production in moderately and highly resistant tomato genotypes to the biotrophic *Oidium neolycopersici* during pathogenesis. Interestingly, the authors also observed a systemic NO production in the extracts of adjacent and distant uninoculated leaves.

It should be noticed that NO production could be correlated with NOS-like activity. NO production could be also inhibited (totally or partially) by mammalian NOS inhibitors or in mutants impaired in NR activity (Modolo et al., 2002; Xu and Dong, 2005). No clear and established relationships could be found between sources of NO and the different biological models (different class of elicitors, avirulent/virulent or biotrophic/necrotrophic pathogens).

2.3.2. NO production mediates plant defense

It was shown that NO production could affect molecular responses and, therefore, could participate in plant disease resistance (Delledonne et al., 1998; Durner et al., 1998). Most studies on NO effects on biotic stress responses in plants have first involved the use of chemical NO donors. NO can regulate protein activities through post-translational modifications. For instance, treatment of Arabidopsis extract with NO donor (GSNO) led to the identification of more than one hundred S-nitrosylated proteins including stress-related (Lindermayr et al., 2005). It was reported that potato plants, treated with the NO-releasing compound 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC18) induced the accumulation of phytoalexin (rishitin) in response to *P. infestans* (Noritake et al., 1996). Similarly, treatment of wheat leaves with the NO donor SNP showed increase in the level of enzyme involved in the biosynthesis of defense molecules,

such as phenylalanine ammonia lyase (PAL; Guo et al., 2004; Manjunatha et al., 2008). Treatment of NO donor to tobacco plants also lead to a state of induced resistance and reduced lesion size caused by tobacco mosaic virus (TMV; Song et al., 2001). Finally, SNP and S-nitroso-N-acetylpenicillamine delayed GA-induced programmed cell death (Beligni et al., 2002), enhance papilla-based resistance and the HR in barley infected with powdery mildew fungus *Blumeria graminis f. sp. Hordei* (Prats et al., 2005).

Evidences for the involvement of NO in plant defense came also from the use of mutants impaired in enzymes involved or associated to NO production. For instance, it was observed that the NR-deficient double mutant (*nia1nia2*), which shows substantially reduced NO production after bacterial or fungal pathogens inoculation/infection, showed no HR and was hyper-susceptible to *P. syringae* (Modolo et al., 2006; Oliveira et al., 2009) and to the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* (Perchepied et al., 2010). Although, Modolo et al., (2006) have noticed that the levels of amino acids, and particularly L-arginine, are strongly reduced in *nia1nia2 A. thaliana* leaves. NO emission by *nia1nia2* leaves did not increase in the amino acids recovered mutants (Oliveira et al., 2009). These results suggest that the susceptibility to pathogen is a consequence of the reduced ability to synthesize NO. Similarly, plants affected in *AtNOA1* (NO-associated protein 1) expression exhibited a reduced endogenous NO level and were more susceptible to the virulent bacteria *P. syringae* pv tomato DC3000, to the fungi *Colletotrichum orbiculare, S. sclerotiorum* and *B. cinerea* (Zeidler et al., 2004; Asai et al., 2008; Asai and Yoshioka, 2009; Perchepied et al., 2010). Tobacco plants overexpressing AtHb1 (hemoglobins are considered as NO scavenger) had reduced necrotic lesions after inoculation (*P. syringae* or tobacco necrosis virus; Serege´lyes et al., 2003). Additionally, *A. thaliana* plants that express cotton GhHb1 gene showed enhanced resistance against *P. syringae* (Qu et al., 2006). In contrast, Perazzolli et al., 2004 did not observed using this mutant, any difference in HR after infection of *Arabidopsis thaliana* with *P. syringae* as compared to control.

NO, together with ROS, play an important role in HR (Levine et al., 1994; Zaninotto et al., 2006). Indeed, a balance NO /ROS is required to initiate cell death, knowing that NO alone is incapable to induced HR (Delledonne et al., 2001; De Pinto et al., 2002). It has been proposed that NO could also play a role in plant defense as an antioxidant. NO can strongly protect tomato plants from methylviologen damage by scavenging ROS (Beligni and Lamattina, 2001). In barley, similar effects have been observed where the ROS-dependent giberellin-induced

programmed cell death (PCD) has been delayed in the presence of NO donors (Beligni et al., 2002). Some of the antioxidant effects of NO may be due to its direct interaction with ROS such as superoxide to form peroxynitrite $ONOO^-$ that might then be scavenged by other cellular processes (Zanninotto et al., 2006). Peroxynitrite is able to induce apoptosis or necrosis in animals but in plants its role is controversial (Bonfoco et al., 1995; Alamillo and Garcia-Olmedo, 2001). Recently, it was shown that peroxynitrite accumulates in *A. thaliana* during the HR induced by avirulent strain of *P. syringae* but its role remains to be established (Gaupels et al., 2011). NO and ROS also exert indirectly reciprocal control on each other through the NO dependent inhibition of catalase and ascorbate peroxidase, two major H_2O_2-scavenging enzymes (Clarke et al., 2000; Arasimowicz et al., 2009).

Recently, Yun et al., (2011) revealed an intricate relationship between ROS and NO. They showed that S-nitrosylation facilitates the HR in the absence of the cell death agonist salicylic acid and the synthesis of reactive oxygen intermediates. Surprisingly, when concentrations of S-nitrosothiols were high, NO function also governed a negative feedback loop limiting the hypersensitive response, mediated by S-nitrosylation of the NADPH oxidase, AtRBOHD.

Finally, NO can control plant immunity by modifiying genes transcription (see Chapter 5).

3. The oligogalacturonides/*Arabidopsis thaliana* model

3.1. The oligogalacturonides (OGs)

OGs are structural components of the pectin homogalacturonan chains of plant cell wall. In *A. thaliana*, they are released during the interaction with pathogens, such as the necrotrophic fungus *B. cinerea*, which secretes polygalacturonases (PGs) as part of their cell wall degrading enzyme arsenal. OGs are non-specific elicitors; they cannot be considered true PAMPs, because these are not derived from the pathogen. However, they can be considered Damage-associated molecular patterns (DAMPs) or host-associated molecular patterns (HAMPs) that are produced by the host cell during the plant pathogen interaction (Galletti et al., 2009). Fully methylated OGs and oligomannuronides were unable to elicit a response, indicating that galacturonic acid residues are specifically required to activate a defense response (Navazio et al., 2002).

3.2. OGs and plant defense responses

The oligogalacturonides (OGs) are among the oligosaccharides that have been most intensively studied in term of biological activity. The structure-activity relationship of OGs depends on plant species and on assayed defense reaction. For instance, OGs with a degree of polymerization (DP) between 9 and 18 were most effective in increasing the intracellular Ca^{2+} concentration in soybean cells; however, a weak increase was also observed with those of a DP smaller or equal to 5. In particular, oligogalacturonides with a DP between 10 and 15 can accumulate when fungal polygalacturonases degrade the homogalacturonan component of plant pectin (Hahn et al., 1981). Production of this elicitor at the site of infection, where large amounts of PGs are secreted by the fungus, may contribute to activate defense responses.

Studies showed that OGs induce early events including protein phosphorylation and activation of MAPKs (Droillard et al., 2000), synthesis and accumulation of phytoalexins (Davis et al., 1986), glucanase, chitinase (Davis and Hahlbrock, 1987; Broekaert and Pneumas, 1988) activation of ion fluxes and membrane depolarization with H^+ influx and K^+ efflux, production of active oxygen species (H_2O_2, and O_2^-; Rouet-Mayer et al., 1997; Binet et al., 1998) and transcriptional activation of defense genes (De Lorenzo et al., 1997). OGs-induced oxidative burst is generated by the NADPH oxidase AtrbohD (Galetti et al., 2008). In *A. thaliana* or grapevine (*Vitis vinifera*), OGs treatment induces a variety of defense responses including

accumulation of phytoalexins, β-1,3-glucanase & chitinase and NO production (Hu et al., 2003). OGs also influence both Ca^{2+} influx and efflux and the activity of a plasma membrane Ca^{2+}-ATPase involved in the oxidative burst (Romani et al., 2004). OGs are not only involved in defence but also in plant growth and development. They induce the formation of flowers (Marf`a et al., 1991) and stimulate cell divisions leading to stoma formation (Altamura et al., 1998).

3.3. OGs and induced resistance

OGs increase resistance to the necrotrophic fungal pathogen *B. cinerea* independently of signaling pathways mediated by jasmonate, salicylic acid, and ethylene (Aziz et al., 2004; Ferrari et al., 2007). *B. cinerea* is the only species in the genus with a broad host range. It can infect more than 200 dicot plants species (Elad et al., 2004), whereas all other species are considered to be specific for single plant species. Similarly to OGs, *B. cinerea* can elicit defense responses such as NO accumulation, ROS production, or MAPKs activation (Pitzschke and Hirt, 2009). ROS burst is not required for the expression of OGs-responsive genes or for OGs-induced resistance to *B. cinerea*, whereas callose accumulation requires a functional AtrbohD (Galetti et al., 2008). Finally, *B. cinerea* virulence factor (BcPG1; Ten Have et al., 1998) induces MAPK activation, radical burst as well as cell death (Poinssot et al., 2003; Kars et al., 2005; Vandelle et al., 2006).

About half of the *A. thaliana* genes affected by OGs treatment displayed a similar pattern of expression after *B. cinerea* infection, suggesting that at least part of the responses activated by *B. cinerea* are mediated, directly or indirectly, by OGs (Ferrari et al., 2007). These results indicate that OGs and *B. cinerea* genes are activated through a common signaling pathway. The overall responses result in the relative contribution to overall defence. Therefore OGs represent a valuable tool to analyze the mechanisms involved in plant pathogen interaction.

CHAPTER 2

"Nitric oxide signaling in plants: cross-talk with Ca^{2+}, protein kinases and reactive oxygen species"

CHAPTER 2

Nitric oxide signaling in plants: cross-talk with Ca^{2+}, protein kinases and reactive oxygen species

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Nitric oxide (NO) is a gaseous free radical recognized as a ubiquitous signal transducer that contributes to various biological processes in animals. It exerts most of its effects by regulating the activities of various proteins including Ca^{2+} channels, protein kinases and transcription factors. In plants, studies conducted over the past ten years revealed that NO also functions as an endogenous mediator in diverse physiological processes ranging from root development to stomatal closure. Its biological role as an intracellular plant messenger molecule, however, remains poorly understood. Here, we review the molecular basis of NO signaling in animals and discuss current knowledge of NO signaling in plants, focusing on its interplay with Ca^{2+}, protein kinases and reactive oxygen species which are well established as widespread key regulators of signal transduction.

Nitric oxide (NO) is a noxious free radical gas which, in the late 1980s, was discovered to exist physiologically in mammalian systems. This discovery offered fresh perspectives on main processes including neurotransmission, immunity and relaxation of vascular smooth muscles (Schmidt and Walter, 1994). Notably, the idea that a simple gas could act as a messenger revolutionized researcher understanding of signal transduction. Recently, NO was also shown to mediate diverse plant physiological processes such as germination, root growth, flowering, stomatal closure and resistance to biotic as well as abiotic stresses (see reviews by Lamattina *et al*., 2003; Delledonne *et al*., 2005; Besson-Bard *et al*., 2008a; Wilson *et al*., 2008). Although evidences supporting NO as a plant physiological mediator are still growing, its functions at the molecular level remain poorly understood and, in some examples, are subjected to controversies. Research conducted over the past years has revealed that NO mediates part of its action in a concerted way with the second messenger Ca^{2+}, protein kinases and reactive oxygen species (ROS). The interplays between these molecules operate in cells challenged by biotic and abiotic stresses and modulate various cellular responses including gene expression and cell death. This review introduces the basic concepts of NO signaling in animals and discuss the mechanisms through which NO exerts its signaling activities in plants with a particular emphasis on Ca^{2+}, protein kinases and ROS signaling.

Basic concepts of NO signaling in animals

The field of research dedicated to NO signaling in animals has been extraordinary fruitful in the past two decades and has led scientists to introduce new concepts of signal transduction. NO is derived from the amino acid L-arginine by the enzymatic activity of nitric oxide synthase (NOS). Once produced, NO acts predominantly *via* the post-translational modifications of proteins. Three main processes have been described: *S*-nitrosylation, metal nitrosylation and tyrosine nitration. Well over a hundred proteins susceptible to these NO-dependent post-translational modifications and involved in all major cellular activities have been identified. In this section, we describe the principles of *S*-nitrosylation, metal nitrosylation and tyrosine nitration and discuss how these post-translational protein modifications influence Ca^{2+} and protein kinase signaling. Understanding these signaling concepts should facilitate a comprehensive analysis of the way NO acts as a signal in plants.

Metal Nitrosylation

As a radical, NO is capable of donating electrons and therefore reacts with transition metals. Covalent interaction of NO with the centers of iron-sulfur clusters, heme and zinc-finger proteins leads to an increase or a decline in protein activity. Amongst the proteins regulated through metal nitrosylation, a well-studied target for NO is soluble guanylate cyclase (sGC; Denninger and Marletta, 1999). sGC catalyses the conversion of GTP to pyrophosphate and 3',5'-cyclic GMP (cGMP), a well-defined second messenger. The interaction of NO with the sGC heme leads to the opening of the bond between ferrous iron and histidine 105 of the enzyme, thus triggering a conformational change that increases the catalysis of cGMP synthesis by several hundred-fold (Cary *et al.*, 2006; Roy and Garthwaite, 2006). Once produced, cGMP binds to target proteins: cGMP-dependent protein kinases (PKGs), cyclic-nucleotide-gated channels (cCNGCs) and cyclic-nucleotide phosphodiesterases, resulting in cell-specific downstream outputs (Beck *et al.*, 1999). Examples of physiological responses regulated through NO/cGMP signaling include neurotransmission, development, smooth muscle relaxation and blood pressure regulation (Denninger and Marletta, 1999; Krumenacker *et al.*, 2004).

S-nitrosylation

S-nitrosylation corresponds to the covalent modification of cysteine sulfurs of proteins by NO (or its derivatives) to form *S*-nitrosothiols (Stamler *et al.*, 2001; Hess *et al.*, 2005). It is not yet clear how NO *S*-nitrosylates target proteins. Candidate mechanisms include the electrophilic attack of the nitrosonium cation (NO^+, resulting from NO auto-oxidation) on thiolate, direct interaction of NO with thiolate in the presence of electron acceptors such as NAD^+ and complex chemical processes involving nitroxyl anions (NO^-, resulting from NO auto-reduction or dinitrogen trioxide decomposition) (Gow *et al.*, 1997; Hanafy *et al.*, 2001; Foster and Stamler, 2004). Interestingly, primary peptide sequences for motifs that might facilitate *S*-nitrosylation have been described, consisting of acidic/basic motifs, as well as hydrophobic motifs surrounding the cysteine residue (Hess *et al.*, 2005; Greco *et al.*, 2006). Similarly to metal nitrosylation, *S*-nitrosylation is a reversible form of post-translational modification. De-*S*-nitrosylation occurs chemically without the help of enzymes or enzymatically through thioredoxin and thoredoxin reductase (Benhar *et al.*, 2008; Jaffrey *et al.*, 2001).

Tyrosine nitration

Tyrosine nitration is mediated by two main NO-derived species including peroxynitrite ($ONOO^-$), resulting from the fast reaction between NO and ROS such as superoxyde ($O_2^{\cdot -}$), and nitroso-peroxocarboxylate ($ONOOCO_2^-$), an adduct formed following the reaction between $ONOO^-$ and CO_2 at a physiological concentration (Radi, 2004). Nitration occurs in one of the two equivalent carbon atoms in the ortho position (with respect to the hydroxyl group) of the phenolic ring of tyrosine residues and leads to protein 3-nitrotyrosine (3-NO_2-Tyr) (Radi, 2004). 3-NO_2-Tyr was first related to NO-dependent oxidative stress occurring during inflammatory diseases such as asthma (Schopfer *et al.*, 2003). Indeed, tyrosine nitration is usually associated with loss of protein functions and target proteins include Mn superoxide dismutase, cytochrome P450, tyrosine hydroxylase, glutamine synthase, glutathione reductase, actin and other cytoskeleton-related proteins (Greenacre and Ischiropoulos, 2001; Gow *et al.*, 2004). It is generally assumed that this process may be irreversible and increase the susceptibility of proteins to degradation by the 20S proteasome (Mannick and Schonhoff, 2002). However, the description of denitrase activities reversing protein nitration in several mammalian tissues suggests that tyrosine nitration might also be a reversible process (Gorg *et al.*, 2007). This latter finding opens the possibility that the formation of 3-NO_2-Tyr may play a role in signal transduction. Regarding this aspect, the relationship with protein tyrosine phosphorylation is particularly noteworthy. Indeed, according to several studies, the importance of tyrosine nitration on cell signaling would lie essentially in the inhibition of tyrosine residues to undergo phosphorylation and/or in the inhibition of phosphatases that allows protein kinases to become dominantly activated (Minetti *et al.*, 2002). A first mechanism has been proposed to explain the activation of tyrosine kinases c-Src by $ONOO^-$: *in vitro* nitration of a C-terminal tyrosine residue could prevent its phosphorylation which normally helps c-Src fold into an inactive conformation (Klotz *et al.*, 2002). At present, however, it is unclear if this process can occur *in vivo*.

Interplays between NO and Ca^{2+}

Currently, NO is recognized as one of the key messengers governing the overall control of Ca^{2+} homeostasis, and almost all types of Ca^{2+} channels and transporters are under its control. The effects of NO on Ca^{2+} channels and transporter activity can be divided into two mechanisms of action: a cGMP-dependent one and a cGMP-independent one. The molecular mechanisms

underlying the cGMP-dependent pathway are complex, and at least three processes have been reported. First, cGMP could directly activate CNGCs by virtue of their cyclic-nucleotide-binding sites, leading to an enrichment of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) (Hanafy et al., 2001; Ahern et al., 2002). Second, the effects of cGMP could be mediated via the activation of PKGs (Clementi and Meldolesi, 1997; Clementi, 1998; Ahern et al., 2002). PKGs have distinct effects on intracellular Ca^{2+}, increasing or decreasing $[Ca^{2+}]_{cyt}$, depending on the target channel, the stimuli and cell types. For example, in hepatocytes, phosphorylation of the 1,4,5-triphosphate (IP_3) receptor by PKGs potentiates IP_3-dependent Ca^{2+} release, whereas an opposite effect is observed in smooth muscle (Clementi, 1998; Murthy and Makhlouf, 1998). Besides the IP_3 receptor, Ca^{2+}-permeable channels and Ca^{2+} transporters whose activities appear to be modulated by PKGs include voltage-dependent Ca^{2+} channels (L-, N-, P/Q- and T-types), store-operated Ca^{2+} channels (SOCCs), mechano-sensitive Ca^{2+}-permeable non-selective cation channels (MS-NSCs), the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and the plasma membrane Ca^{2+} pump (PMCA) (Clementi, 1998; Wang et al., 2000; Chen et al., 2002; Yao and Huang, 2003; Grassi et al., 2004). The biochemical steps downstream of PKGs that are responsible for the modulation of these channels and transporters have not been completely clarified. Third, to add further complexity to these scenari, PKG activation has been found to be a crucial step in NO-induced cyclic ADP-ribose (cADPR) synthesis (Willmott et al., 1996; Reyes-Harde et al., 1999; Leckie et al., 2003). cADPR is synthesized from its precursor NAD^+ by ADP-ribosyl cyclase which might be activated through PKG-induced phosphorylation. cADPR is a Ca^{2+} mobilizing second messenger which promotes Ca^{2+} release from endoplasmic reticulum in a wide variety of animal cells *via* the activation of the ryanodine receptors (RYRs) (Fliegert et al., 2007). The cGMP/PKG/cADPR cascade is now recognized as a fundamental mechanism through which NO contributes to the generation and propagation of Ca^{2+} signals in various physiological processes including the induction of hippocampal long-term depression and fertilization in echinoderms (Willmott et al., 1996; Reyes-Harde et al., 1999; Leckie et al., 2003).

The cGMP-independent action of NO on Ca^{2+} homeostasis operates through the direct *S*-nitrosylation of Ca^{2+} channels and transporters. Voltage-dependent Ca^{2+} channels, RYRs, N-methyl-D-aspartate (NMDA) receptors, transient receptor potential channels (TRPC) and CNGCs were shown to be reversibly *S*-nitrosylated, with activation or inhibition as a consequence (Broillet, 2000; Stamler *et al.*, 2001, Yoshida *et al.*, 2006; Tjong *et al.*, 2007). For instance, the

skeletal muscle RyR1 (one of the three isoforms of RYRs) consists of four homologous 565 kDa subunits containing 100 cysteine residues. In the native protein, 50 of these residues appear to be in a reduced state (Aracena-Parks *et al.*, 2006). Remarkably, submicromolar NO concentrations were shown to activate RYR1 by *S*-nitrosylation of a single cysteine (Cys 3635), this reaction occurring only at low (e.g. physiological) pO_2 but not ambient pO_2 (Sun *et al.*, 2003). This specific *S*-nitrosylation reverses RYR1 inhibition by Ca^{2+}/Calmodulin (CaM) and may contribute to enhanced RYR1 activity. Interestingly, Cys 3635 can also be *S*-glutathionylated, suggesting that competition between *S*-nitrosylation and *S*-glutathionylation on Cys 3635 may occur in physiological processes (Aracena-Parks *et al.*, 2006). Another remarkable example of the influence of *S*-nitrosylation on Ca^{2+} channel activities concern TRPC5, one of the seven TRPC homologs in human. This plasma membrane Ca^{2+} channel was shown to induce Ca^{2+} entry into human embryonic kidney cells in response to NO released by several NO donors (Yoshida *et al.*, 2006). The molecular mechanism underlying NO-dependent TRPC5 activation may involve the nucleophilic attack of nitrosylated Cys 553 by the free sulfydryl group of Cys 558, thus leading to the formation of a disulfide bond between both cysteine residues. The disulfide bond might stabilize the open state of the channel.

The existence of both cGMP-mediated and direct *S*-nitrosylation pathways expands and enriches the possibilities for NO to modulate Ca^{2+}-dependent signaling processes including gene expression (Peunova and Enikolopov, 1993). Furthermore, because NO production by NOS requires an increase in $[Ca^{2+}]_{cyt}$, the ability for NO to attenuate Ca^{2+} influx by inhibiting some types of Ca^{2+} channels and/or to initiate cytosolic free Ca^{2+} removal by activation of SERCA and/or PMCA helps to protect cells from the deleterious effect of NO. The pathophysiological relevance of these processes is outlined in several examples such as the modulation of neuronal excitability or hypertension but has probably paved the way for new roles in normal and disturbed cell functions. From a mechanical point of view, the plasticity of the NO/Ca^{2+} pathways is particularly intriguing when both pathways act on the same channels. Several studies have provided support that the cGMP-dependent pathway generally occurs at low levels of NO whereas *S*-nitrosylation requires higher levels of NO and tends to proceed with slower kinetics than cGMP-induced actions (Denninger and Marletta, 1999; Hanafy *et al.*, 2001). However, this subject is still controversial and the issue is far from settled (Stamler *et al.*, 2001; Hess *et al.*, 2005).

NO signaling in plants

During the last decade, NO has been recognized as a versatile player in diverse plant physiological processes. Several routes for NO synthesis have been described: non-enzymatic as well as enzymatic pathways involving nitrate reductase and putative NOS-like enzymes (Besson-Bard *et al.*, 2008a, 2008b, 2008c; Kaiser and Huber, 2001; Crawford *et al.*, 2006; Corpas *et al.*, 2006; Wilson *et al.*, 2008). A major and still opened question concerns the molecular mechanisms of its signaling action. More than hundred proteins have been asserted to undergo regulation by *S*-nitrosylation and metal nitrosylation. Similarly, numerous genes up- and/or down-regulated in response to artificially produced NO have been identified based on microarray analyses. However, with few exceptions, the physiological significance of these post-transcriptional and post-translational modifications remains to be established. Parallel to these approaches, over the last years, evidence gathered from a number of studies has indicated that NO mediates part of its effects through the mobilization of free Ca^{2+}, *via* the modulation of protein kinases activities and by interacting with ROS. The aim of this section is to concentrate on the interplay between NO, Ca^{2+} and ROS and to describe what is known thus far concerning the physiological impact of the cross-talk between these signaling components.

Interplays between NO and Ca^{2+}

A large number of signals, including plant hormones, light, biotic as well as abiotic stresses cause transient and specific changes in intracellular [Ca^{2+}]. In the recent years, it has become increasingly appreciated that the signaling components that govern these changes include NO. Furthermore, because NO production is under the control of intracellular Ca^{2+} fluctuations, NO might also act as a Ca^{2+} sensor contributing to decoding the intracellular Ca^{2+} changes in plants.

NO acts as a Ca^{2+}-mobilizing messenger

The first conclusive evidence implicating NO as a Ca^{2+} mobilizing messenger in plant cells came from studies exploring the ability of NO donors to induce increases in intracellular [Ca^{2+}]. Using Ca^{2+}-sensitive dye fura 2 fluorescence ratio imaging, Garcia-Mata *et al.* (2003) showed that treating *Vicia faba* guard cells by the NO donor *S*-nitroso-*N*-acetyl-penacillamine (SNAP) induced stomatal closure by promoting an increase in intracellular [Ca^{2+}]. The ability of

exogenous NO to induce a rise of intracellular [Ca^{2+}] was further supported by the finding that the NO donor diethylamine NONOate (DEA/NO) triggers a transient rise of $[Ca^{2+}]_{cyt}$ in transgenic *Nicotiana plumbaginifolia* cell suspensions expressing the Ca^{2+}-reporter apo-aequorin (Lamotte *et al.*, 2004 and 2006). By contrast, the same NO donor did not induce any change in nuclear free Ca^{2+} concentration ($[Ca^{2+}]_{nuc}$) (Lecourieux *et al.*, 2005) suggesting that NO effects on Ca^{2+} homeostasis is restricted to specific cellular compartments.

The influence of NO on the cellular [Ca^{2+}] in physiological contexts was highlighted by the demonstration that NO scavengers and mammalian NOS inhibitors reduced stimulus-induced rises in $[Ca^{2+}]_{cyt}$. Notably, Lamotte *et al.* (2004) showed that the NO scavenger cPTIO and NOS inhibitors reduced the $[Ca^{2+}]_{cyt}$ increases induced in *N. plumbaginifolia* cells by the proteinaceous elicitor cryptogein secreted by the oomycete *Phytophthora cryptogea*. These pharmacological agents did not reduce the cryptogein-triggered $[Ca^{2+}]_{nuc}$ rises, confirming the observation made using NO donors (see above; Lecourieux *et al.*, 2005). Similar effects of NO were reported in grapevine cell suspensions exposed to the elicitor endopolygalacturonase 1 from *Botrytis cinerea* (Vandelle *et al.*, 2006). Another example emerges from studies investigating NO function in plant cells exposed to hyper-osmotic stress. Under this condition, addition of the NO scavenger cPTIO reduced the increase in $[Ca^{2+}]_{cyt}$ in *N. plumbaginifolia* cell suspensions expressing the calcium reporter apo-aequorin, highlighting again the role of NO in $[Ca^{2+}]_{cyt}$ elevations (Gould *et al.*, 2003; Lamotte *et al.*, 2006).

Underlying mechanisms

Research has been directed towards identifying the mechanisms through which NO triggers changes in intracellular [Ca^{2+}]. Pharmacological analyses of [Ca^{2+}] variations have indicated that NO might activate both plasma membrane and intracellular Ca^{2+}-permeable channels (Garcia-Mata *et al.*, 2003; Lamotte *et al.*, 2004; Lecourieux *et al.*, 2005; Lamotte *et al.*, 2006; Vandelle *et al.*, 2006). Whereas the putative identity of the NO-sensitive plasma membrane Ca^{2+}-permeable channels remains unknown, several lines of evidence have suggested that NO might target RYR-like channels. Indeed, the increase in $[Ca^{2+}]_{cyt}$ of *N. plumbaginifolia* cells treated by the NO donor DEA/NO was sensitive to ruthenium red (RR), an inhibitor of mammalian RYRs (Lamotte *et al.*, 2004). A similar inhibitory effect was obtained using the cADPR antagonist 8Br-cADPR, designing cADPR as possible intermediates of the NO signal

leading to changes in intracellular [Ca^{2+}]. According to Garcia Mata *et al.* (2003), cADPR might function together with cGMP as reported in animals (Willmott *et al.*, 1996; Reyes-Harde *et al.*, 1999). Indeed, they showed that ryanodine, an antagonist of RYR as well as ODQ (1-H-(1,2,4)-oxadiazole-(4,3-a)quinolxalin-1-one), an inhibitor of sGC, were able to suppress SNAP-mediated increase in intracellular [Ca^{2+}] in *Vicia faba* guard cells. The authors provided several arguments indicating that a similar NO/cGMP/cADPR/Ca^{2+} pathway might occur in response to abscisic acid (ABA).

Besides cADPR and cGMP, evidences that NO could also contribute to $[Ca^{2+}]_{cyt}$ increases *via* phosphorylation events were provided. Indeed, protein kinase inhibitors efficiently suppress NO donors-triggered elevation in $[Ca^{2+}]_{cyt}$ in *Vicia faba* guard cells and *N. plumbaginifolia* cells (Sokolovski *et al.*, 2005; Lamotte *et al.*, 2006), indicating that the signaling cascades relaying NO and Ca^{2+}-permeable channels could involve protein kinases, besides or together with cADPR. At present, only one protein kinase candidate, named NtOSAK (*Nicotiana tabacum* Osmotic Stress-activated protein Kinase) has been identified (Lamotte *et al*., 2006). This aspect is discussed farther. Finally, it should be specified that several arguments pointing out the involvement of NO in stimulus-induced plasma membrane depolarization have been reported (Lamotte *et al*., 2006; Vandelle *et al*., 2006). Such NO-dependent changes of the plasma membrane potential might modulate the activity of plasma membrane Ca^{2+}-permeable channels. The mechanisms underlying this effect are poorly understood.

Impacts of the NO/Ca^{2+} pathways

The first conclusive evidence of the biological significance of a NO/Ca^{2+} pathway came from studies in which the function of NO in plant defence responses was investigated (Durner *et al*., 1998; Klessig *et al*., 2000). Using *N. tabacum* plants and suspension cells treated with a recombinant mammalian NOS or NO donors, respectively, it was shown that NO was able to mediate the expression of the defence-related genes *PR* (*pathogenesis related-1*)-1 and *PAL* (*phenylalanine ammonia lyase*) through cGMP and/or cADPR. In addition to gene expression, compelling evidence suggests a role for the interplay between NO and Ca^{2+} on microorganism-triggered hypersensitive response (HR). In this context, NO appears to act as a Ca^{2+} sensor contributing to decode the intracellular Ca^{2+} changes in plants leading to cell death. Both pharmacological and genetic experimental data support this concept (Delledonne *et al.*, 1998;

Lamotte *et al.*, 2004; Ali *et al.*, 2007). For instance, in cryptogein-elicited tobacco cell suspensions, NO production is stimulated by an influx of extracellular Ca^{2+} (Lamotte *et al.*, 2004). In turn, NO partly contributes to the elicitor-triggered cell death. The plasma membrane cyclic nucleotide-gated Ca^{2+}-permeable channel CNGC2 was recently identified as one of the putative key component of this pathway in *Arabidopsis thaliana* (Ali *et al.*, 2007). Accordingly, the HR normally suppressed in the *A. thaliana dnd1* (*defence no death 1*) mutant impaired in CNGC2 expression, was shown to be partially restored by the NO donor sodium nitroprusside (SNP). Further research is needed to understand how NO, the production of which is stimulated by an influx of Ca^{2+}, can contribute to HR. Because, as discussed previously, NO also amplifies the mobilization of free Ca^{2+}, it is possible that the NO-dependent rise of intracellular [Ca^{2+}] facilitates cellular Ca^{2+} overload which, in turn, could cause cytotoxicity and could trigger cell death. Besides mediating defence responses, the NO/Ca^{2+} pathways might influence diverse cellular processes such as ABA-induced stomatal closing or auxin-mediated adventitious root formation (Garcia-Mata *et al*., 2003; Lamattina *et al*., 2003; Desikan *et al*., 2004).

Interplays between NO and protein kinases

Upon receiving a signal, cells often utilize multiple protein kinase cascades to transduce and amplify the information. Protein phosphorylation and dephosphorylation are very common intracellular signaling modes. Kinases and phosphatases regulate a wide range of cellular processes such as enzyme activation, assembly of macromolecules, protein localization and degradation. In animals, NO has been described to modify the activity of protein kinases involved in signal transduction, such as mitogen activated protein kinase (MAPK) cascades, Janus kinases or protein kinase C (Beck *et al*., 1999). Also, the activity of primary metabolism related kinases, for instance pyruvate kinase, were identified to be modified by *S*-nitrosylation (Gao *et al*., 2005).

It is presently known that serine/threonine protein kinases play a crucial role in the transduction of various extra- and intracellular signals in plants (Mishra *et al.*, 2006). However, although the identification of NO-modulated protein kinases is a major issue in the understanding of NO-dependent signal transduction, only few of them have been identified and studied. Moreover, these observations have been carried out using, for most part of them, artificially generated NO from NO donors, and not during a physiological plant process.

NO modulates MAPK activities

In all eukaryotes, MAPK pathways serve as highly conserved central regulators of growth, death, differentiation, proliferation and stress responses (Samaj *et al.*, 2004; Qi and Elion, 2005). MAPKs form the terminal components of the MAPK cascades (MAPKKK→MAPKK→MAPK). MAPKs are activated by MAPK kinases (MAPKKs/MEKs) *via* dual phosphorylation of conserved threonine and tyrosine residues in the motif TxY located in the activation loop. Some evidence shows that in plants NO also contributes to the activation of MAPK cascades.

Kumar and Klessig (2000) found a MAPK activated by NO in tobacco leaves and cell suspensions. Injection of tobacco leaves with recombinant rat neuronal NOS, together with its cofactors and substrate transiently activated a 48-kDa protein kinase phosphorylating MBP (myelin basic protein), an artificial MAPK substrate. Using specific anti-SIPK antibody in immuno-complex kinase activity assay, they identified this kinase as Salicylic Acid (SA)-Induced Protein Kinase (SIPK). The NO donors S-nitroso-L-glutathione (GSNO), DEA/NO and SNAP also transiently activated SIPK in tobacco cell suspension cultures (Kumar and Klessig, 2000; Besson-Bard *et al.*, 2008b). Depending on the NO-generating system, this activation was shown to be SA-dependent or SA-independent.

Although SIPK is the first and the only NO-dependent MAPK identified to date, there are more data indicating influence of NO on MAPK pathways. Working with *A. thaliana* shoots, Capone *et al*. (2004) demonstrated that brief oxidative or nitrosative stresses in the roots, using respectively H_2O_2 and the NO donor SNP, triggered the activation of a 38-kDa protein kinase able to phosphorylate MBP. It was confirmed that this kinase belongs to the MAPK family by using antibodies raised against the active (phosphorylated) form of a mammalian p38 MAPK, but no direct relation between this activation and NO production has been clearly demonstrated. Another example of NO ability to activate MAPK was provided by Clarke *et al*. (2000). The authors reported that a 47-kDa protein kinase, able to phosphorylate MBP, is activated within 5 minutes in response to the NO donor Roussin's black salt (RBS) in *A. thaliana* cell suspensions. A role for the 47-kDa protein kinase in mediating NO-induced cell death was tentatively assigned. However, pharmacological inhibition of this MAPK was inefficient in reducing cell death, leading opened the question of the cellular impact of the activation of this MAPK by NO.

More recently, a functional link between NO and MAPKs has been established in ABA signaling in mesophyll cells of maize leaves (Zhang *et al*., 2007). Using pharmacological approach, a linear interplay of these signaling components has been demonstrated: ABA treatment induces H_2O_2 production acting upstream NO synthesis. In turn, NO favours the activation of a 46-kDa MAPK. Induction of this MAPK results in an enhancement of the expression of genes encoding antioxidant proteins such as catalase, superoxide dismutase, gluthatione reductase or ascorbate peroxidase, thus improving the total antioxidative activity of the cells. This cascade of reaction could be triggered in response to stresses such as water-stress, thus highlighting a key role for NO in controlling MAPK involved in the plant adaptive response to abiotic stresses.

Finally, several lines of evidence suggest that NO and MAPKs act together in the auxin transduction pathway leading to adventitious root formation. More precisely, pharmacological-based experiments designed NO as a key regulator of an auxin-induced 48- kDa MAPK sensitive to the MAPK inhibitor PD098059 (Pagnussat *et al*., 2004). Cucumber explants co-treated with a NO donor and PD098059 showed a significant reduction in root length and root number, demonstrating firstly that NO is required for the activation of the 48- kDa MAPK, and secondly that this activation is essential for adventitious root formation. Interestingly, addition of a sGC inhibitor was not able to prevent the NO-dependent activation of the 48-kDa MAPK activation. This latter result suggested that this MAPK might be part of a NO-dependent/cGMP-independent signaling pathway which parallels a previously characterized NO/cGMP-dependent signaling cascade also acting in auxin-induced adventitious root formation (Pagnussat *et al*., 2003).

NO and Ca^{2+}-dependent protein kinases

Support for the hypothesis that NO promotes the activation of Ca^{2+}-dependent protein kinases (CDPKs) came through biochemical and pharmacological approaches that showed the ability of SNP and auxin to induce the activation of a 50-kDa protein kinase in a Ca^{2+}-dependent manner in cucumber hypocotyls (Lanteri *et al*., 2006). The auxin- or SNP-triggered activation of the 50-kDa protein kinase was also reduced by CaM antagonists including trifluoperazine dihydrochloride (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7). These results let to the assumption that the 50-kDa protein kinase may contain CaM-like Ca^{2+}-binding domain, a structural feature of CDPKs. However, it should be

noticed that both TFP and W-7 are not specific inhibitors of CDPKs. Indeed, these compounds also affect the binding of Ca^{2+} to proteins such as CaM and calcineurin B-like proteins (Anil and Rao, 2000) which normally regulate the activity, and therefore the function, of a variety of target proteins including protein kinases (e.g. CcaMKs, CaMKs, SnRKs3; Hrabak *et al*., 2003).

At a physiological level, the CaM antagonists TFP and W-7 were shown to negatively affect NO- or auxin-induced adventitious root formation in cucumber, suggesting the involvement of the 50-kDa protein kinase in this process (Lanteri *et al*., 2006). Interestingly, in contrast to the NO-dependent 48-kDa MAPK described above, the activity of the 50-kDa putative CDPK triggered by SNP or auxin was inhibited by sGC inhibitors, suggesting that this protein kinase is part of the NO/cGMP-dependent pathway leading to adventitious root formation (Pagnussat *et al*., 2003). Because the activity of the 50-kDa cucumbers CDPK was detected at the earlier stages of adventitious root formation, it was proposed that this NO-dependent protein kinase can be involved in cell dedifferentiation, division and/or differentiation (Lanteri *et al*., 2006).

NO and SnRKs

Plant SNF1 (sucrose nonfermenting 1)-related protein kinases (SnRKs) are classified into three subfamilies: SnRK1, SnRK2, and SnRK3. Available evidence indicate that SnRK1 might play an important role in the regulation of global metabolism, the disturbance of which might lead to developmental or adaptation defects (for reviews see Halford and Hardie, 1998; Halford *et al*., 2003; Hrabak *et al*., 2003). The SnRK2 and SnRK3 subfamilies are specific to plants and are involved in environmental stress signaling (for reviews see Hrabak *et al*., 2003; Boudsocq and Lauriere, 2005).

First evidence that NO modulates the activity of SnRKs was provided by Lamotte *et al*. (2006). These authors showed that application of the NO donor DEA/NO to tobacco cell suspensions resulted in a fast and transient activation of a 42-kDa protein kinase phosphorylating MBP and histone IIIS, another protein kinase substrate. Using specific antibodies in immuno-complex activity assay, this 42-kDa protein kinase was identified as NtOSAK, a member of the SnRK2 family (Kelner *et al*., 2004). Similarly to other SnRK2 members in *A. thaliana* (Droillard *et al*., 2002; Boudsocq *et al*., 2004) and rice (Kobayashi *et al*., 2004), NtOSAK is activated within minutes in response to hyperosmotic stress (Mikolajczyk *et al*., 2000), a process which

leads to a rapid increase in NO synthesis (Gould *et al*., 2003). Importantly, NtOSAK activation in response to osmotic stress was abolished by the NO scavenger cPTIO, highlighting the ability of NO in promoting SnRK2 activation during physiological processes.

These finding might be of general importance because it is consistent with the central roles of both NO and SnRK2s in the regulation of stomatal closure as well as defence responses. This assumption is exemplified by the involvement of NO and the *A. thaliana* SnRK2 protein kinase OST1 (open stomata 1) in common pathways. Indeed, OST1 was found to mediate the regulation of stomatal closure by ABA, a function also assigned to NO (Mustilli *et al*., 2002). Similarly, the pathogen-associated molecular pattern (PAMP) flagellin 22 and lipopolysaccharide (LPS) were shown to trigger stomatal closure through a NO- and OST1-dependent signaling cascade (Melotto *et al*., 2006). Although a mechanistic connection between NO and OST1 remains to be established, these data further support the hypothesis that NO and SnRK2 protein kinases act together in the plant adaptive responses to biotic as well as abiotic stresses.

Interplays between NO and ROS

Impacts of the NO/ROS balance in HR

The interplay between ROS and NO has long been recognised in the animal field (Curtin *et al*., 2002). In fact, many of the NO derived responses are believe to stem from the reaction between NO and ROS to form reactive nitrogen species (RNS) such as $ONOO^-$. In contrast to ROS, NO is exclusively produced by specific enzymes in animal cells (Turpaev and Litvinov, 2004). Conversely, plants can produce NO through a number of pathways: either enzymatic or not (Besson-Bard *et al.*, 2008a, 2008b, 2008c; Kaiser and Huber, 2001; Crawford *et al.*, 2006; Corpas *et al.*, 2006; Wilson *et al*., 2008). An interaction between both molecules during the HR was originally suggested by Delledonne *et al*. (1998 and 2001). The authors observed a strong NO burst accompanied by ROS generation following inoculation of soybean cell cultures with avirulent bacteria. However, the up-regulation of NO following this bacterial infection was not sufficient to activate the HR-cell death and, rather unexpectively, $ONOO^-$ was not responsible for cell death. Thus, although $O_2\cdot^-$ is not directly involved in this response, its conversion to H_2O_2 by superoxyde dismutase is critical for the H_2O_2/NO signaling pathway.

De Pinto *et al*. (2002 and 2006) also demonstrated that the NO/ROS couple is necessary for programmed cell death (PCD) in *N. tabacum* cv. BY-2 cells and a strong spatio-temporal correlation was reported between ROS and NO production during powdery mildew-dependent HR in barley (Mur *et al*., 2008). The HR elicited by *Pseudomonas syringae* pv. *phaseolicola* and pv. *tomato* harbouring the *avrRpm1* gene in tobacco and *A. thaliana* was preceded by an NO peak followed immediately by an H_2O_2 burst (Mur *et al*., 2005). Similarly, inoculation of *A. thaliana* cell cultures with *Pseudomonas syringae* pv. *maculicola* carrying the *avrRpm1* avirulence gene resulted in a rapid and sustained NO increase whereas the increase production of H_2O_2 was delayed (Clarke *et al*., 2000). Thus, although some discrepancy exist in the literature concerning the cooperation between NO and H_2O_2, the NO/H_2O_2 balance still seems crucial for many HR-dependent cell death events.

The question may thus arise as to how do cellular NO and H_2O_2 interact during the HR. A mechanistic answer was recently provided by Romero-Puertas *et al*. (2007). Employing a proteomic strategy based on the biotin-switch assay, the authors identified several proteins in which *S*-nitrosylation level is increased in *A. thaliana* leaves challenged by the incompatible pathogen *P. syringae*. The authors focused their attention on peroxiredoxin II E (PrxII E), a member of the peroxiredoxin family which catalyses the reduction of H_2O_2 but also $ONOO^-$, depending on the isoforms. Extensive biochemical and genetic approaches indicate that *S*-nitrosylation of PrxII E inhibits its capacity to detoxify $ONOO^-$. Based on these data, an interesting model was proposed in which *S*-nitrosylation of PrxII E impairs its peroxynitrite reductase activity, thus leading to an increased level of tyrosine nitration, a hallmark of NO/ROS-dependent oxidative stress.

Candidate sites of interaction between NO and ROS during the HR

In animals, mitochondria play a central role in PCD by releasing cytochrome *c* and activating caspases, and there is growing belief that the intracellular redox status is critical in mitochondria-dependent cell death in animals (Kowaltowski *et al.,* 2001). In particular, the interaction between mitochondrial cytochrome c and NO constitutes an important signaling pathway for the controlled production of H_2O_2 (Brookes *et al*., 2002). In plants, mitochondria have recently been identified as key players of cell redox homeostasis and signaling (Noctor *et al*., 2006), as well as important integrators of PCD (Jones, 2000; Swidzinski *et al.,* 2002; Lam,

2004; Swidzinski *et al.,* 2004). Transgenic tobacco cells lacking the alternative oxidase show enhanced susceptibility to various cell death inducers, including H_2O_2 (Robson and Vanlerberghe 2002; Vanlerberghe *et al.,* 2002) and H_2O_2-driven cell death occurs through a mitochondria-dependent pathway (Mur *et al.,* 2008). Interestingly, mitochondria are also considered as potential sites of NO action. Notably, although oxygen consumption *via* the cytochrome pathway is inhibited by NO in isolated soybean cotyledons (Millar and Day, 1996) and carrot cell suspensions (Zottini *et al*., 2002), in both cases the cyanide insensitive alternative oxidase is not significantly affected. Similarly, Yamasaki *et al*. (2001) found that the alternative pathway is resistant to NO in plant mitochondria isolated from mung bean. Thus, these data suggest that the NO effect on the respiratory pathway may play some role in maintaining mitochondrial homeostasis by limiting ROS release. Further support for this comes from the fact that mitochondria can support nitrite-dependent NO synthesis (Planchet *et al.*, 2005) and that AtNOA1 (Nitric Oxide Associated 1), an enzyme initially thought to display NOS activity, is targeted to the mitochondria (Guo and Crawford, 2005). Considering the hydrophobic and diffusible nature of NO, these data provide additional support for a potential interaction between NO and ROS in the mitochondria or its vicinity, thus potentially participating in mitochondria derived cell death signals.

The chloroplast has also recently been put forward as a critical player in the development of the HR under light (Zeier *et al*., 2004; Montillet *et al*., 2005; Mur *et al*., 2008). NO can inhibit chloroplast electron transport in a reversible manner (Takahashi and Yamasaki, 2002) and chloroplasts have also been identified as potential participant in NO synthesis and $ONOO^-$ production in plants (Gould *et al*., 2003; Jasid *et al*., 2006). In fact, a strong correlation between $ONOO^-$ and the presence of oxidatively modified proteins in both the stroma and the thylakoids was observed in soybean chloroplasts (Jasid *et al.*, 2006). Furthermore, it was suggested that $ONOO^-$ interacts with non-heme Fe^{2+} leading to PSII inhibition on the acceptor site (Gonzalez-Perez *et al*., 2008). Thus, *in situ* production of NO in the chloroplasts could play a protective role in preventing oxidation of chloroplastic lipids and proteins but alternatively, the reaction between $O_2{\cdot}^-$ and NO could lead to $ONOO^-$ production which could be responsible for the impairment of the photosynthetic machinery. Thus, the different HR cell death phenotypes observed in the light or in the dark during pathogen infection (Montillet *et al*., 2005) may

therefore depend on the effect of NO on chloroplastic homeostasis but also on the release of ROS by the chloroplast and their interaction with NO.

Finally, both ROS and NO can also be produced in the peroxisomes. In fact, peroxisomes are not only a major site of $O_2\cdot^-$ and H_2O_2 production (Del Rio *et al*., 2002) but they have also been proposed a major site of NO synthesis (Corpas *et al*., 2001). In a recent study, the possible interaction between NO and peroxisomal H_2O_2 production on gene regulation was analysed in transgenic catalase antisense tobacco plants (Zago *et al*., 2006). The different phenotypes obtained under various concentrations of H_2O_2 and NO clearly supported the idea that a tight balance between both molecules is necessary for HR-type cell death. Furthermore, this cDNA-AFLP analysis demonstrated that only 16 differentially expressed transcripts required both NO and H_2O_2. In contrast, 152 genes could be modulated by either NO or H_2O_2, thus demonstrating that the NO and H_2O_2 pathways may overlap to a greater extend than initially thought in HR induced cell death.

A protective molecule?

Finally, NO has been reported as both a cytotoxic and cytoprotecting molecule in plants (Beligni and Lamattina, 2001). This dual role may depend to a large extend on a tight spatio-temporal kinetic of cellular concentrations which will be governed by the production, displacement and removal of RNS (Noriega *et al*., 2007). NO was shown to function as an antioxidant and thus to protect plants from a variety of abiotic stresses such as drought, heat, salt or heavy metal stresses (Garcia-Mata and Lamattina, 2002; Uchida *et al*., 2002; Noriega *et al*., 2007; Tewari *et al*., 2008) and oxidative stress (Beligni and Lamattina, 2002; Dubovskaya *et al*., 2007). First, this protective effect may originate from direct detoxification of ROS by NO. Indeed, it is widely believed that NO can protect cells against oxidative stress by preventing the Fenton reaction by scavenging iron, thus avoiding the formation of hydroxyl radicals, one of the most phytotoxic oxygen radicals (Wink *et al*., 1995). Furthermore, the reaction between NO and $O_2\cdot^-$ which leads to $ONOO^-$ formation may help in reducing the adverse effects of oxygen radical accumulation. Second, the antioxidative properties of NO may rely on its ability to alter the plant antioxidant system. Indeed, it was recently demonstrated that NO could stimulate the expression of a heme oxygenase, which catalyses the conversion of heme to biliverdin IX with the concomitant release of CO and iron, and acts against oxidative stress in plants (Noriega *et al*.,

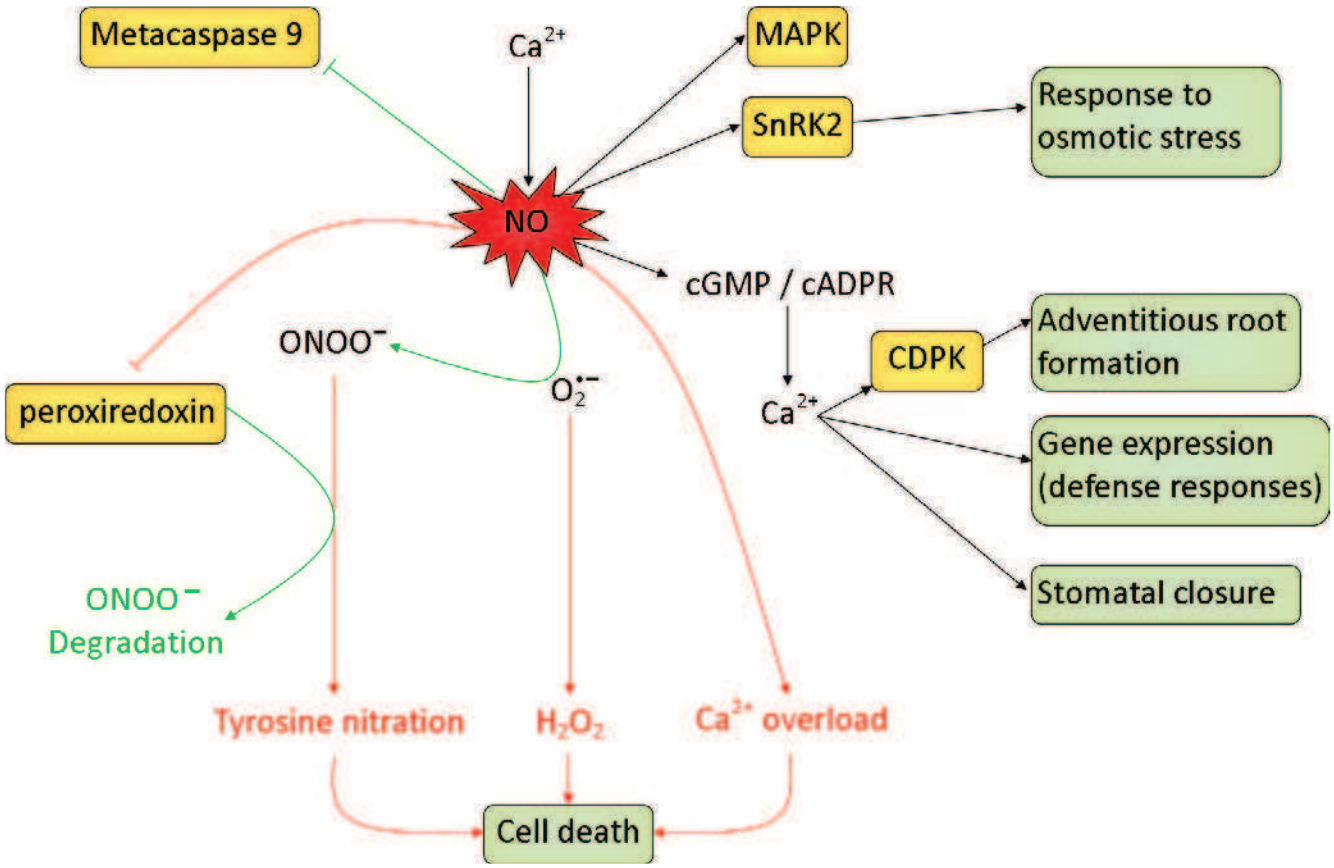

Figure 2.1: Schematic representation of the interplays between NO and Ca^{2+}, protein kinases and ROS in plant cells

NO production is up-regulated by Ca^{2+}-dependent processes. In turn, NO promotes increases in the cytosolic Ca^{2+} concentration through complex pathways involving cADPR and/or cGMP. The resulting rise in the cytosolic Ca^{2+} concentration contributes to CDPK activation and defense gene expression and represents a key signaling step in auxin-induced adventitious root formation and ABA-induced stomatal closure. NO also exerts part of its activities through MAPK and SnRK2. Whereas several arguments pointed out for a role of the NO/SnRK2 pathway in the plant adaptive response to osmotic stress, the cellular impacts of the NO-dependent activation of MAPK remain enigmatic.
NO signaling during cell death (red arrows) is in part understood by the requirement of H_2O_2 and tyrosine nitration and might involve a Ca^{2+} overload. Tyrosine nitration results as a consequence of peroxiredoxin inhibition through S-nitrosylation. Protective principle (green arrows) may partly arise from metacaspase 9 S-nitrosylation and peroxiredoxin activity.

2007). In addition, several studies have also reported an up-regulation of several antioxidant enzymes following treatment with low NO concentrations (Parani *et al*., 2004; Shi *et al*., 2005).

Finally, new insight into NO cytoprotective effects was recently provided by Belenghi *et al*. (2007). These authors reported that the *A. thaliana* metacaspase 9 (AtMC9) is constitutively *S*-nitrosylated *in vivo* at the catalytic Cys 147 residue. This posttranslational modification inhibits AtMC9 autoprocessing and proteolytic activity. This mechanism resembles those described for caspase 3 in mammalian cells. In resting cells, *S*-nitrosylation of the catalytic cysteine of caspase-3 maintains the enzyme in an inactive form (Mitchell *et al*., 2007). Upon apoptosis inducer action, thioredoxin mediates denitrosylation of mitochondria-associated caspase-3, a process required for caspase-3 activation that promotes apoptosis (Benhar *et al*., 2008). Whether a similar thioredoxin-dependent de-*S*-nitrosylation contributes to AtMC9 up-regulation remains to be established.

Conclusion

NO has undoubtedly been an area on intense research over the past years. While the number of physiological processes involving NO is likely to grow, understanding of how this gas exerts its effects at the molecular level is still in its infancy. Clearly, there is no simple and uniform picture of the signaling function of NO (Figure 2.1). Accumulating evidence now pointed out NO as one of the key messengers governing the control of Ca^{2+} homeostasis. The interaction between NO and Ca^{2+} operates in response to various stimuli in plants, suggesting that the cross-talk between both messengers is a basic transduction mechanism as reported in other organisms. Similarly, NO and ROS act in concert with protective or toxic effects as potential consequences, depending on the tight spatio-temporal kinetics of their respective production. It is however extremely difficult to predict the effects of the concerted action of NO and ROS, a main problem facing these studies being the current lack of drugs capable of selectively acting in one specie and our limited understanding of NO chemistry in plants. Finally, the ability of NO to modulate protein kinase activities represents another example of how NO mediates its action. The question of the physiological influence of NO/phosphorylation cascades remains, for the most, unanswered.

It is to be hoped that current and future studies will contribute towards the identification of *S*-nitrosylated, metal-nitrosylated and tyrosine nitrated proteins mediating NO signaling.

Deeper insight into these NO-dependent post-translational protein modifications will not only permit the detailed characterization of the biochemical steps involved in NO control of the Ca^{2+}, ROS and protein kinases systems, but also will allow us to understand the physiological significance of the heterogeneous behaviours of NO in plants.

Acknowledgements

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CHAPTER 3

"Materials and Methods"

Table 3.1: List of T-DNA mutant lines of *Arabidopsis thaliana* used in this study

Name	Gene ID	Description	References	Seed obtained from
nia1 nia2	At1g77760 At1g37130	Double mutant impaired in the expression of NR two isoforms (Bright et al., 2006).	(Bright et al., 2006)	Dr. Neill (University of the West of England, England).
nia1	At1g77760	Single mutant impaired in the expression of NR1 isoform.		Dr. Neill (University of the West of England, England).
nia2	At1g37130	Single mutant impaired in the expression of NR2 isoform.		Dr. Neill (University of the West of England, England).
dnd1	At5g15410	mutant impaired in gene encoding the Cyclic Nucleotide Gated Channel 2 (CNGC2)	(Ali et al., 2007)	Dr. Berkowitz (University of Connecticut, United States).
rbohD	At5g47910	Mutant impaired in the gene encoding NADPH/respiratory burst oxidase protein D (RbohD).		Dr. Torres (University of North Carolina, United States).
rbohD rbohF	At5g64060	Double mutant impaired in the gene encoding NADPH/respiratory burst oxidase protein homologue D and F	(Torres et al., 2002)	Dr. Torres (University of North Carolina, United States).
mpk3	At3g45640	Single mutant impaired in a mitogen-activated kinase whose mRNA levels increase in response to touch, cold, salinity stress and chitin oligomer.		Dr. Zhang (University of Missouri, United States)
mpk6	At2g43790	Single mutant impaired in a MAP kinase induced by pathogens, ethylene biosynthesis, oxidative stress and osmotic stress.		Dr Zhang (University of Missouri, United States)
cpk5.6.11	At4g35310 At2g17290 At1g35670	Cpk5.6.11: Triple mutant mutated for calmodulin-domain protein kinase CDPK isoform 5 (CPK5) for three isoforms 5, 6 and 11 CDPKs	(Boudsocq et al., 2010)	Dr. Boudsocq (CNRS, Gif sur Yvette, France).
Paox	At1g62830, At1g62830, At3g13682) and At3g13682	Single mutants impaired in the expression of four different Polyamines Oxidase isoforms		NASC (Nottingham Arabidopsis Stock Center)
per4	At1g14540	Mutants impaired in the gene encoding peroxidase superfamily protein.		NASC (Nottingham Arabidopsis Stock Center) (N655479/SALK_544730 and 661085/SALK_110617)

CHAPTER 3

Materials and Methods

1. Biological materials

1.1. Plant materials

Arabidopsis thaliana (ecotype Col-0) and T-DNA insertion mutant lines were used in this study (Table 3.1). Seeds were obtained from NASC (Nottingham *Arabidopsis* Stock Center; http://arabidopsis.info; http://www.arabidopsis.org/) or provided directly by laboratories (Table 3.1). All mutants lines used in this work were isolated from Col-0 background.

The plants were cultivated in commercial soil [Jiffy-7, Puteaux (SA, France)] in a climate-controlled growth chamber (KBW, Binder, Germany) with a 10 h light (175 µE.s $^{-1}$), 14 h dark cycle with the following settings: 20 °C light, 18 °C dark; 70 % relative humidity light / 95 % dark.

After 3 weeks, plants were watered with nutrient solution [0.25 mM ($Ca(NO_3)_2$; 1 mM KH_2PO_4; 0.5 mM KNO_3; 1 mM $MgSO_4$; 50 µM H_3BO_3; 19 µM $MnCl_2$; 10 µM $ZnCl_2$; 1 µM $CuSO_4$; 0.02 µM $NaMoO_4$; 100 µM Fe-Na-EDTA]. As *NR* double mutant could not assimilate nitrate, they were watered with ammonium citrate (20 mM) for better growth once a week.

1.2. Elicitor

The elicitor, oligogalacturonides (OGs; Figure 3.1), a polysaccharide derived from pectin was used in this study. The elicitor was obtained from GOEMAR (SA, France). Degree of polymerization (DP) for OGs is approximately 25 and dissolved in water at a working concentration of 2.5 mg.mL^{-1}.

1.3. Fungal pathogens

1.3.1. *Botrytis cinerea* culture and pathogen infection assay

Botrytis cinerea strain BMM (Zimmerli et al., 2000) was allowed to grow on Petri plates containing PDA (Potato dextrose agar, Becton Dickinson) for 10-12 days (light 10h, 20°C; dark

β,1-3 Glucanase	At3g55430	Mutant impaired in the gene encoding O-Glycosyl hydrolases family 17 protein.		NASC (Nottingham Arabidopsis Stock Center) (N642531/SALK_142531)
Chitinase-IV	At3g54420	Mutant impaired in the gene encoding an EP3 chitinase		NASC (Nottingham Arabidopsis Stock Center) (N872938/SAIL_102_E08)
tir	At1g52900	Mutant impaired in the gene encoding Toll-Interleukin-Resistance (TIR) domain family protein.		NASC (Nottingham Arabidopsis Stock Center) (N645664/SALK_145664; N813465/SAIL_290_F07; N816688/ SAIL_359_C09)
rlp7	At1g47890	Mutant impaired in the gene encoding receptor like protein 7 (RLP7).		NASC (Nottingham Arabidopsis Stock Center) N675914/SALK_080831; N861920/ SAIL_587_H10
gex3/srp	At5g16020	Mutant impaired in the gene encoding GEX3.		NASC (Nottingham Arabidopsis Stock Center) (N653488/SALK_131457; N502030 /SALK_002030)
propep2	At5g64890	Mutant impaired in the gene encoding elicitor peptide 2 precursor (PROPEP2);	(Yamaguchi, et al., 2010)	NASC (Nottingham Arabidopsis Stock Center) (N643805 /SALK_143805)
crf3	At5g53290	Single mutant impaired a member of the ERF (ethylene response factor) subfamily B-5 of ERF/AP2 transcription factor family.		NASC (Nottingham Arabidopsis Stock Center) (N862329/ SAIL_240_H09; N873186 /SAIL_ 325_H03)
bhlh	At1g10585	Mutant impaired in the gene encoding Basic helix-loop-helix (bHLH) DNA-binding superfamily protein.		NASC (Nottingham Arabidopsis Stock Center) (N810254/SAIL_221_H01)
wrky 41	At4g11070	Mutant impaired in the gene encoding member of Group III WRKY Transcription Factor.		NASC (Nottingham Arabidopsis Stock Center) (N573278/SALK_073278; N568648/SALK_068648; N612834 /SALK112834)
wrky 75	At5g13080	Mutant impaired in the gene encoding member of WRKY Transcription Factor.		NASC (Nottingham Arabidopsis Stock Center) (N674673/SALK_042481; N664405/SALK_004954)

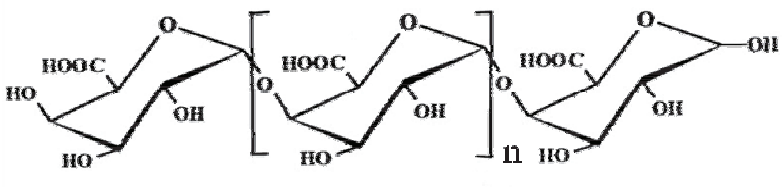


Figure 3.1: General structure of oligogalacturonides (OGs).

14h, 18°C). The spores were harvested in water and subsequent filtration through glass wool to remove hyphae, the spores were quantified under microscope and stored at 4°C.

Inoculation with *B. cinerea* was conducted on 4-week-old plants (wild type or mutants) by putting on leaves, droplets of 6 µL of the spore suspension diluted in ¼ PDB (potato dextrose broth) to obtain a final concentration of 5.10^4 spore.mL^{-1}. For assessment of symptoms in plants, lesion diameters were measured after 72 h (3 days) of inoculation. Results were analysed by one-way ANOVA on ranks, followed by Dunnett's test ($p<0.05$) using the SigmaPlot software.

To verify the effect of inhibitors and cPTIO on *B. cinerea* growth, spores were diluted to obtain the final concentration of 5.10^4 spore.mL^{-1} in PDB. 12 µL of spore suspension (5.10^4 spore.mL^{-1}) with inhibitors or cPTIO or water in PDB medium were placed on glass slides and allowed to grow under high humidity for different time. Fungal growth was observed under microscope after different time intervals.

2. Methods

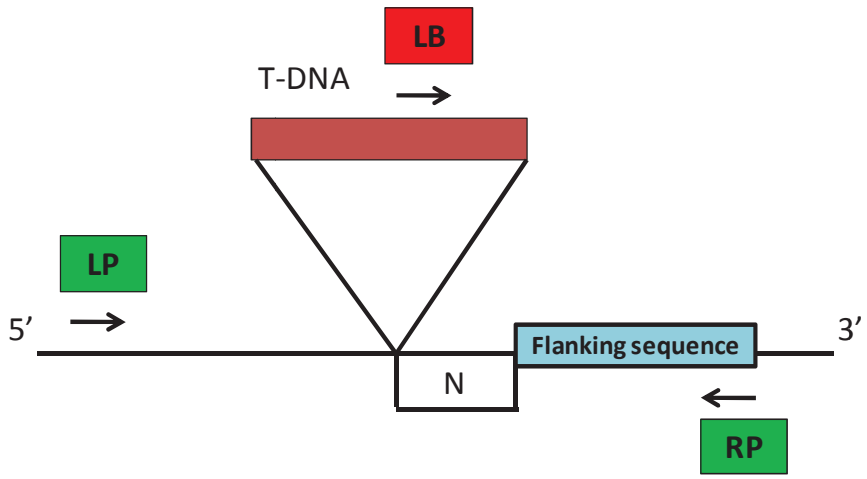
2.1. Genotyping

Genotyping is the process of determining the genotype of an individual by examining the individual's DNA sequence by using molecular tools. In our case genotyping is necessary because the T-DNA mutant lines could have different genotypes (segregating lines). Eight plants of each putative T-DNA mutant lines were screened to select homozygous genotypes for mutated allele.

Plant genomic DNA was extracted using the following protocol. Leaf tissue was ground by pestle in a 1.5 mL microtube in 350 µL of extraction buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 100 mM NaCl; 1% SDS; Edward et al., 1991). The sample was centrifuged (14,000 g, 10 min, RT). Supernatant (300 µL) was transferred to a new tube, and the DNA was precipitated with an equal volume of isopropanol. The DNA pellet was washed in 75 % ethanol and re-dissolved in 50 µL water. Genomic DNA from each genotype was quantified using UV spectrophotometry and run on 1.0 % agarose gel.

Two couples of primers were used for genotyping, LP/RP and Lb2/RP. Both LP and RP primers (left primer and right primer) were designed using the T-DNA primers software (http://signal.salk.edu/tdnaprimers.2.htmL). These two primers specifically amplified a fragment of the gene of interest and are located respectively downstream and upstream the T-DNA

A



B

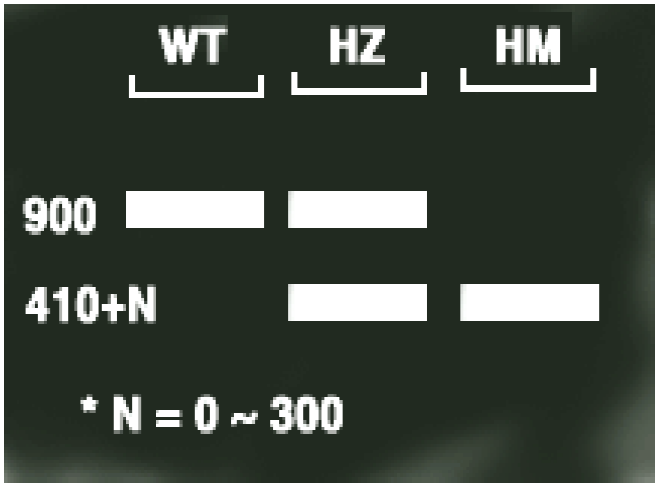


Figure 3.2: Screening of T-DNA mutants lines. A) Position of primers used for genotyping. LP and RP : Left and Right genomic primer. LB : T-DNA border primer; N - Difference of the actual insertion site and the flanking sequence position, usually 0 - 300 bases. **B) Possible PCR products visualized on agarose gel electrophoresis**. WT (Wild Type - no insertion) should get a product of about 900-1100 bps (LP-RP ; depending upon the size of of product); HM (Homozygous lines - insertions in both chromosomes) will get a band of 410+N bps (from RP to insertion site 300+N bases, plus 110 bases from LB to the left border of the vector), HZ (Heterozygous lines) will get both bands.

insertion (Figure 3.2). Amplification with LP/RP is possible for wild type allele (in the wild homozygous and the heterozygote genotypes). However, no fragment will be amplified with this primer pair for mutated allele because T-DNA fragment (typically 5 to 25 kb) prevents amplification in PCR conditions used (Figure 3.2).

A second PCR reaction is carried out with the RP/Lb primer combination. Lb is a universal primer complementary to the T-DNA inserted. No amplification will be observed for homozygous wild type. In homozygous plants for the mutated allele and heterozygotes, PCR amplification is possible (Figure 3.2).

PCR reactions were performed in a final volume of 25 µL including 2 µL gDNA, 200 nM of reverse and forward primers, 1 µM of dNTP, 2.5 µL 10 X *Taq* polymerase buffer and 0.75 U *Taq* Polymerase (GO *Taq,* Promega).

Amplifications were conducted in a thermocycler (MyCycler, Biorad). Forty cycles were performed, each consisting of a denaturation step of 30 s at 95 °C, an annealing step of 30 s at 50-58 °C (depending on primers ; list of primers is given in Table 3.2) and an extension step of 90 s at 72 °C. The last cycle was followed by 10 min at 72 °C to ensure that primer extension reactions proceeded to completion. The PCR products were run on 1.5% agarose gel electrophoresis (100 V, 30 min). The DNA fragments were stained with ethidium bromide and then revealed under UV using imaging system (Molecular imager GelDoc XR system, BioRad).

2.2. NO detection by spectrofluorometry

2.2.1. DAF-2DA detection method

NO production was mainly monitored in *A. thaliana* (ecotype Col-0) and mutant plant using 4,5-diaminofluorescein diacetate (DAF-2DA) (Sigma-Aldrich), a membrane-permeable derivative of the NO-sensitive fluorophore 4,5-diaminofluorescein. This method is indirect and relies on the measurement of reactive nitrogen species (notably N_2O_3 and NO^+) derived from NO autooxidation that nitrosate DAF-2 to yield the highly fluorescent DAF-2 triazole (DAF-2T; Figure 3.3; Jourd'heuil, 2002). This fluorophore has been successfully applied to detect NO production in plant tissues and cell suspensions.

Leaf discs excised from plants were infiltrated under vacuum for 3 min with an aqueous solution of 20 µM DAF-2DA and 2.5 $mg.mL^{-1}$ of OGs in 50 mM Tris-HCl (pH 7.5). For control

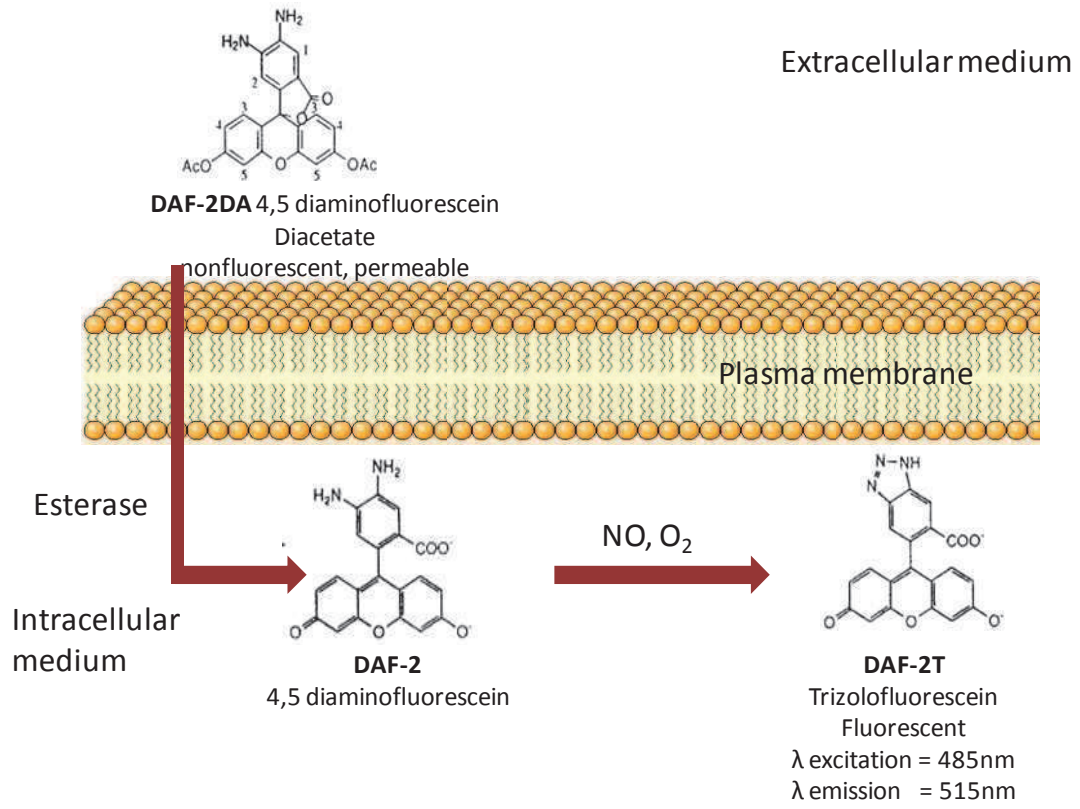


Figure 3.3: Principle of NO detection using Diaminofluorescein (DAF). DAF-2DA permeates through the cell membrane and is hydrolyzed by esterase to yield DAF-2 and this reacts with NO to form fluorescent complex, triazolofluorescein (DAF-2T). The fluorescent product can be quantified by fluorometry using an excitation filter of 492 nm and an emission filter of 515-535 nm.

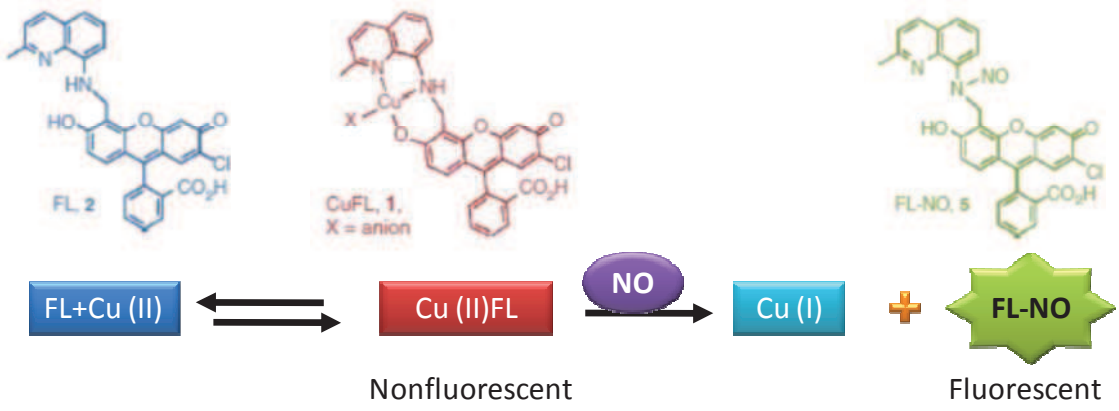


Figure 3.4: Principle of NO detection using CuFL. Cu(II) reacts with FL and gives a nonfluorescent product Cu(II)FL then Cu(II)FL reacts with NO to form fluorescent complex, (FL-NO). The fluorescent product can be quantified by fluorometry (Lim et al., 2006).

treatment, OGs were replaced by an equivalent volume of water. After infiltration the discs were incubated 1h in obscurity and washed with Tris-HCl (50 mM, pH 7.5), each disc was put in a separate well of 96 well plate (Microtest ^{Tm} flatbottom, Becton Dickinson, Europe) in 200 µL of Tris-HCl (50 mM, pH 7.5) with or without treatment solution in the dark. The increase of DAF-2T fluorescence triggered by OGs was measured using a spectrofluorometer (Mithras, Berthold Technologies). NO production was measured with 485 nm excitation and 535 nm emission filters for 12 h. Eight leaf discs were used for each treatment. Fluorescence was expressed as relative fluorescence units (arbitrary units: au).

L-NAME (inhibitor of NOS; ALEXIS Biochemicals), Tungstate (inhibitor of NR; Sigma-Aldrich), Guazatine (inhibitor of PAOX; Sigma-Aldrich) and DFMO (Difluoromethylornithine hydrochloride hydrate; inhibitor of Polyamines synthesis; Sigma-Aldrich), were infiltrated in 50 mM Tris-HCl (pH 7.5) and used at different concentrations.

2.2.2. CuFL detection method

Alternatively, NO production was measured in the leaf tissues through the use of CuFL (Strem Chemicals, Bischemi). It reacts with NO to form fluorescent complex, FL-NO (Figure 3.4). The fluorescent product can be quantified by fluorometry (Lim et al., 2006).

Plants leaf discs were infiltrated under vacuum for 3 min in buffer (Tris-HCl 10mM ; pH 7.5, KCl 10mM) with water (as a control) or OGs (2.5 mg.mL^{-1}). Filtrated discs were incubated 1 h in respective solution. After incubation, each disc was put in a separate well of 96 well plate (Microtest ^{Tm} flatbottom, Becton Dickinson) in 200 µL of detection buffer (Tris-HCl 10mM ; pH 7.5, KCl 10mM); with a probe final concentration of 5 µM, with or without treatment solution in the dark. Eight leaf discs were used for each treatment. The increase of fluorescence triggered by OGs was measured using spectrofluorometer (Mithras, Berthold Technologies). cPTIO was used to verify that this fluorescence was due to NO. NO production was measured with 485 nm excitation and 535 nm emission filters for 4 h. Fluorescence was expressed as relative fluorescence units (arbitrary units: au). For t0, leaf discs were infiltrated and fluorescence was measured directly in 200 µL of detection buffer with 5 µM final probe concentration.

Table 3.3: List of primer used for RT-qPCR

Primer name	Gene Name	Sequence (5'-3')	Annealing Temperature (℃)
At1g10585_F	bHLH-like	ATCATTAGTCGGATTGGC	55
At1g10585_R		AAAAACGAAACGACAACG	
At5g53290_CRF3_F	CRF3	TACAACATCTCTCATCTCCTAC	55
At5g53290_CRF3_R		ATAGTTGTCAAGAAACGGAG	
At2g14610_PR1_F	PR1	CACTACACTCAAGTTGTTTGG	55
At2g14610_PR1_R		TGATAAATATTGATACATCCTGC	
At3g23240_ERF1_F	ERF1	AATCCACTAACGATCCCTAAC	55
At3g23240_ERF1_R		ACTTTCTTGAGCTTACGG	
At1g52900_TIR_F	TIR	ATGAAGCCCGGAGATAAG	53
At1g52900_TIR_R		ATCAGAACATGTCACCCT	
At1g47890_RLP7_F	RLP7	CGACCTTGTTGGGATTGAGAA	55
At1g47890_RLP7_R		GGGGATGCGTGAGATATATAATGTG	
At5g16020_F	SRP/GEX3	CGATTGGTTCACTAGATGG	55
At5g16020_R		GAATAACTCTGAGACCAGTAGA	
At5g64890_PP2_F	PROPEP2	AGGAACAAGAGGAAGACTATGG	55
At5g64890_PP2_R		GACTGACTCATTGGCCTC	
At5g13080_W75_F	WRKY75	TGGAGGGATATGATAATGGGTC	55
At5g13080_W75_R		TGGCTCCTTGTTTGAAACGC	
At4g11070_W41_F	WRKY41	CACACAGTAGCAGTAAATTACCAGA	55
At4g11070_W41_R		GCCGTTGGATCAAATTGAAA	
At1g14540_PER4_F	PER4	CACTGGTTCAGATGGACAAA	55
At1g14540_PER4_R		AACAAACGAATTATCGCTGC	
AtUBQ10_4g05320_ F	UBQ10	GAGATAACAGGAACGGAAACATAG	55
AtUBQ10_4g05320_ R		GGCCTTGTATAATCCCTGATG	
At2g26560_ PLA2_F	PLA2	AAGAAAAGAAGATCCGAGAC	55
At2g26560_ PLA2_R		ATTCAAACGTACAAGTGACC	
At3g54420_ CHIV_F	CHIV	TGTTGACTCCCACCATTT	55
At3g54420_ CHIV_R		CGGTCGATCCAACTCTAC	
At3g55430_ glu_F	Glucanase	CTTCGCTGGAACTGGTATCT	55
At3g55430_ glu_R		GCAGAATCTCATTTCCGACT	
At1g77760_ Nia1_L	Nia1	ATCGTCAAAGAAACCGAAGTCA	55
At1g77760_ Nia1_R		ACGGAGCATGGATGAGTTAC	
At1g37130_Nia2 _L	Nia2	GTTACGCATATTCCGGAG	55
At1g37130_Nia2 _R		CATGCACGAACAGCAATA	
At3g26830_Pad3_F	PAD3	TGCTCCCAAGACAGACAATG	55
At3g26830_Pad3_R		GTTTTGGATCACGACCCATC	
At2g16500 _ADC1_F	ADC1	GTGGTGATAAGGGGAACGACA	53
At2g16500 _ADC1_R		CAACCGAAATAAGACCAATTCTCAT	
At 4g34710 _ADC2_F	ADC2	GCGATGGACCACACAGCTT	53
At 4g34710 _ADC2_R		AGGAACATCCGCTGAGGACTGA	

2.3. *In vivo* assay of Nitrate Reductase

In vivo NR activity was assayed by the protocol of Yu et al., (1998) with minor modifications. NR activity was measured in a spectrophotometric assay by determining the amount of NO_2^- released from the leaf tissue.

Nitrate + ß-NADH Nitrate Reductase $\longrightarrow$ Nitrite + ß-NAD + H_2O

Nitrite + Sulfanilamide + NED $\longrightarrow$ Nitrite Color Complex

ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form
NED = N-(1-Naphthyl) ethylenediamine Dihydrochloride

Leaf discs were weighed (≈ 50 mg), vacuum infiltrated with or without OGs in Tris-HCl pH 7.5 for 3 min and incubated for 4 h within the respected solutions. After incubation, leaf discs were transferred into 1 mL of reaction buffer (40 mM KNO_3, 0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, and 4 % [v/v] *n*-propanol, pH 7.5) and incubated in the dark for 2 h. The reaction was stopped by the addition of 200 µL of 1 % sulphanilamide (dissolved in 3 N HCl) and 200 µL of 0.05 % *N*-(1- napthyl) ethylenediamine hydrochloride. The concentration of NO_2^- was determined by spectrophotometer at OD=540nm.

2.4. Total RNA isolation

To extract RNA, plants were grown under same conditions as mentioned previously (Page 87). Leaves were infiltrated with OGs (2.5 $mg.mL^{-1}$) or water using a needleless syringe. After treatments leaf tissues were collected at different time intervals and were immediately frozen in liquid nitrogen. Leaf tissues (approximately 100 mg) were homogenized by grinding with liquid nitrogen using automatic tissue grinder system (Fisher Scientific). After addition of 1 mL of Trizole reagent (Molecular Research Centre Inc.) and 200 µL of chloroform, extracts were agitated vigorously for 15 s and centrifuged at 14,000 g for 15 min at 4 ^oC. The upper aqueous layer (approx. 500 µL) was collected in a new tube and precipitation of RNA was carried out 10 min following the addition of 500 µL of isopropanol. The samples were then centrifuged (14,000

g, 10 min, 4 ^{o}C). RNA pellets were washed with 75 % ethanol and centrifuged (14,000 g, 5 min, 4 ^{o}C). Finally, RNA pellets were dried, resuspended in 15 µL diethyl pyrocarbonate (DEPC-treated H_2O and then incubated 10 min at 55 ^{o}C to improve dissolution. RNA was quantified by UV spectrophotometer at 260 nm. Protein contamination was estimated by the ratio $OD_{260/280}$. The RNA concentration was determined by the following formula:

[RNA] (µg /µL) = Dilution Factor x OD260 x 40 (standard [RNA]).

RNA quality was analysed on 1.5 % agarose gel electrophoresis (50 V, 45 min). Gels were stained in ethidium bromide for 10 min. Gel pictures were obtained using imaging system (Molecular imager GelDoc XR system, BioRad).

2.5. Synthesis of cDNA

2.5.1. DNase treatment

Two µg of total RNA were treated with DNase1 (Sigma) in the following reaction mixture: 1 µL of 10 X reaction buffer, 1 µL of amplification grade DNase l (1 unit / µL) in a final volume of 10 µL. Reaction mixture was mix gently and was incubated at room temperature for 15 min. One µL of stop solution was added and the mixture was heated in water bath at 70 ^{o}C for 10 min to inactivate DNase l and inactivation of RNA.

2.5.2. First strand cDNA synthesis

First strand synthesis of cDNA was carried out using a cDNA synthesis kit (Superscript^{Tm} III Reverse Transcriptase, Invitrogen). One µg of DNase-treated RNA sample, oligo dT primer (13 µM), dNTP (760 mM) in a final volume of 13 µL were incubated at 65 ^{o}C for 5 min and then placed on ice at least 1 min. Four µL of 5X first strand buffer, 1 µL of 0.1 M DTT and 1 µL of reverse transcriptase (SuperScript III RT; 200 units / µL) were added. The reaction mixture was then incubated at 50 ^{o}C for 60 min. Reaction was stopped by heating at 70 ^{o}C for 15 min. cDNA were stored at -20 ^{o}C.

2.6. Analysis of transcript accumulation by Real Time qPCR

Gene-specific primers corresponding to OGs-responsive *A. thaliana* genes (Ferrari et al., 2007) and NO-responsive genes were designed using AmplifX 1.1 (http://ifrjr.nord.univ-

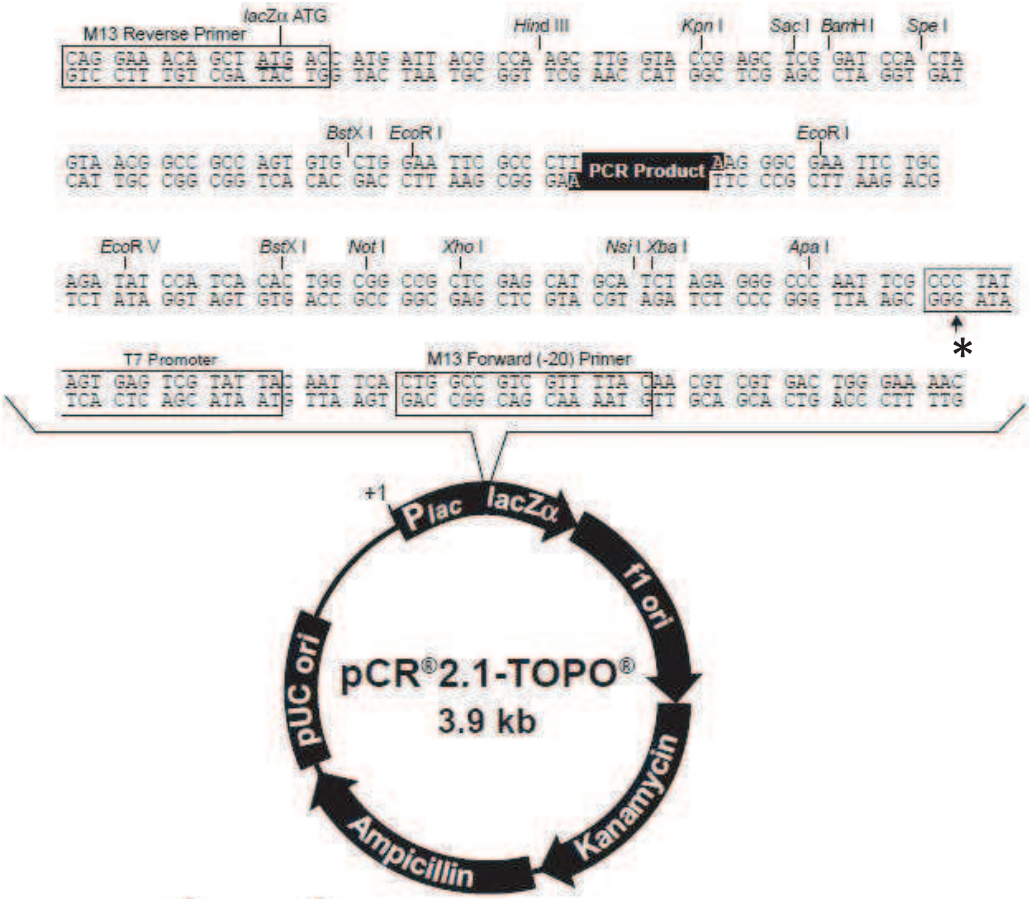


Figure 3.5: TOPO cloning vector. The figure shows the features of the TOPO cloning vector (pCR®2.1-TOPO®) and the sequence surrounding the TOPO® Cloning site. Vector has kanamycin and ampicillin resistance genes for selection. The black arrow indicates the start of transcription for T7 polymerase. Restriction sites are labeled to indicate the actual cleavage site. M13 forward and reverse priming site are presented in boxes.

mrs.fr/AmplifX) or selected from CATMA (http://www.catma.org/database/simple.htmL) (Table 3.3) for the gene expression analysis through qRT–PCR. The specificity of the primers was tested by sequencing the PCR products.

Gene expression was analysed by Real Time qPCR (RT qPCR). PCR amplification efficiency of target DNA was examined to measure linearity of dilution. For each gene a standard curve was generated with serial dilutions of plasmids containing the respective PCR product. These standard curves showed similar slopes demonstrating an efficiency of more than 90 %.

RT-qPCR was carried out with a Mastercycler (Sequence detection system, Applied Biosystem) in 96-well plate (Optical reaction plate with Bar code, Applied Biosystem). Reactions were performed in a final volume of 20 µL containing 8 µL cDNA sample (dilution 1/80), 200 nM forward and reverse primers and 10 µL realtime SYBR Green mix (Thermo Scientific) according to the following conditions:

- First denaturation step of 15 min at 95 ° C,
- 40 cycles consisting of three steps
    - 30 s at 95 °C,
    - 30 s at 50-58 °C (depending of primers; list of primer in Table 3.3),
    - 45 s at 72 °C,
- Final step consisting of 15 s at 95 ° C, 15 s at 72 ° C and 15 s at 95 ° C. Fluorescence was measured and results were analyzed using the SDS software (Applied Biosystems).

Alternatively, experiments were carried out in 384 wells plate in a Light- Cycler480 (Roche Applied Science). Reactions were performed in a final volume of 5 µL using Absolute ™ QPCR SYBR© Green ROX Mix (Thermo Fisher Scientific, Waltham, USA), with a 200 nM forward and reverse primer and 1 µL of 1/10 diluted cDNA using the same program mentioned above.

Expression levels were calculated relative to the appropriate housekeeping gene (HK; *UBQ10*: At4g05320) using the comparative threshold cycle method, where Ct represents the threshold cycle for target amplification: $\Delta Ct = \Delta Ct_{\text{gene of interest}} - \Delta Ct_{HK}$. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression from real-time quantitative PCR experiments (Livak and Schmittgen, 2001).

2.7. PCR fragment cloning

2.7.1. Ligation

PCR products were cloned using the TOPO cloning vector (Invitrogen; Figure 3.5) according to the manufacturer's recommendation. Briefly, 4 µL PCR product was used for ligation with 25 ng of vector (15 min at room temperature).

2.7.2. Preparation of competent cells

E. coli DH5α strain was grown overnight in 2 mL LB broth medium at 37 ^{o}C. From this culture, 5 µL bacteria culture was transferred into a pre-warmed culture flask containing 100 mL of LB broth medium. The culture was incubated (37 ^{o}C, 220 rpm) until it reached the OD 600 nm of 0.4 - 0.6. Culture was incubated in ice for 20 min and then centrifuged (3,000 g, 10 min, 4 ^{o}C). The pellet was resuspended in 10 mL 0.1 M $CaCl_2$ solution and centrifuged (3,000 g, 10 min, 4 ^{o}C). Finally, cells were re-suspended using the same conditions as described above in 0.1 M $CaCl_2$ containing 10 % glycerol and stored at 4 ^{o}C for 24 h - 48 h or at - 80 ^{o}C (Sambrook et al., 1989).

2.7.3. Transformation

Two µL of the ligation product were used to transform 200 µL of competent cells. Following the heat shock (42 ^{o}C for 45 s), SOC medium (500 µL) was added and cells were incubated at 37 ^{o}C for 1 h under agitation (220 rpm). Transformed cells (150 µL) were plated on LB agar containing ampicillin (50 $\mu g.mL^{-1}$) and X-Gal (40 $mg.mL^{-1}$). The plates were incubated overnight at 37 ^{o}C. Recombinant white colonies were selected and grown at 16 h (37 ^{o}C, 220 rpm) in 2.5 mL LB broth medium containing ampicillin (50 $\mu g.mL^{-1}$).

2.7.4. Plasmid isolation

Bacterial recombinant plasmids DNA were isolated using the UltraClean standard Mini prep kit (MolBio) and quantified using UV spectrophotometer. To check the presence of insert, plasmids were digested by *Eco*RI enzyme (37 ^{o}C for 1 h) (Biolabs inc.). Digested samples were separated on 2 % agarose gel electrophoresis. The presence of insert was also confirmed by PCR using M13 primers.

2.8. Immunodetection of phosphorylated mitogen activated protein kinases (MAPK)

Plant leaves (approximately 100 mg) were infiltrated with water or OGs and homogenized by automatic grinding system (Fisher Scientific). Two hundred fifty µL of extraction buffer (50 mM Hepes; 10 mM EGTA; 10 mM EDTA; 1 mM Na_3VO_4; 50 mM β- glycerol phosphate; 10 mM NaF; 5 mM DTT; leupeptine 5 $\mu g.mL^{-1}$; antipain 5 $\mu g.mL^{-1}$; 1 mM PMSF) was added in homogenized tissues and centrifuged (14,000 g, 15 min, 4 ^oC).

Protein quantity was determined using Bradford reagent (Bradford, 1974). For estimation BSA was used as standard protein (0-10 $\mu g.\mu L^{-1}$). Five µL of the supernatant was used for protein quantification by spectrophotometer at 595 nm.

For electrophoresis, total protein (supernatant) was diluted in 1X sample buffer (Laemmli, 1970) and then heated at 95 °C for 5 min. Gel plates were placed in a vertical gel electrophoresis system containing 10 % resolving and 4 % stacking gel with running buffer (25 mM Tris Base; 190 mM Glycine; 10 % SDS; 10 mL and distilled water quantity required for 1000 mL) in the reservoir. Fifteen µg protein samples were loaded on a 10 % polyacylamide gel electrophoresis. Gel was run at 80 V for 30 min and then at 150 V for 1 h. Gels were stained in Coomassie blue (0.25 % Coomassie R-250, 40 % methanol, 10 % acetic acid) for 2 h and destained with destaining solution (15 % methanol, 10 % acetic acid), followed by a washing with 5 % glycerol v/v solution and dried under vacuum drier.

For western blot analysis, 15 µg protein samples were loaded on a 10 % polyacrylamide gel electrophoresis as described previously, and transferred to nitrocellulose membrane in transfer buffer (48 mM Tris Base, 39 mM Glycine, 20 % methanol, 10 % SDS; pH 8.3) for 40 min at 15 V.

Nitrocellulose membrane was stained with ponceau red to verify the transfer and to check the equal amount of protein in each condition. Nitrocellulose membrane was put in TBST-1% BSA at 4 °C overnight. After three successive washes with TBST (each time 10 min), membrane was incubated with primary antibody [phosphor-p44/42 Map Kinase {Thr202/ Tyr204} antibody; dilution 1/1000; (Cell Signaling Technomogy, Inc.)] in 10 mL TBST-1 % BSA for 1 h 40 min at room temperature. Membrane was washed 3 times with TBST (each time 10 min). Nitrocellulose membrane was saturated with secondary antibody {dilution 1/60,000; Horseradish peroxidase antirabbit (Bio-Rad)}, in 20 mL TBST-BSA 1 % for 1 h at room temperature. Again nitrocellulose membrane was washed with TBST (3 times, 15 min each time).

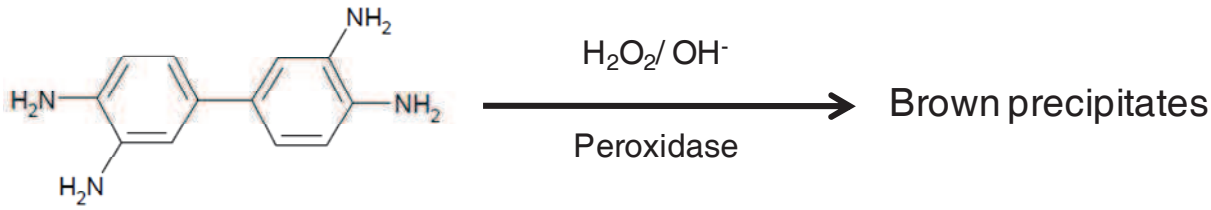
Substrate was prepared by diluting 20 X LumiGLO^{TM} reagent and peroxidase (Cell Signaling Technology) to 1 X in water. Nitrocellulose membrane was incubated with substrate for 1 min, wrapped in plastic sheet and expose to X-ray film for 5-30 min. After incubation, the film was revealed.

2.9. In gel kinase Assay

Infiltrated leaves (approximately 100 mg) were homogenized by grinding with liquid nitrogen. 250 µL of extraction buffer (50 mM Hepes; 5 mM EGTA; 5 mM EDTA; 1 mM Na_3VO_4; 50 mM β- glycerol phosphate; 10 mM NaF; 2 mM DTT; leupeptine 5 µg.mL^{-1}; antipain 5 µg.mL^{-1}; 1 mM PMSF) was added in grinded tissues and centrifuged at 14,000 g for 15 min at 4 ^{o}C.

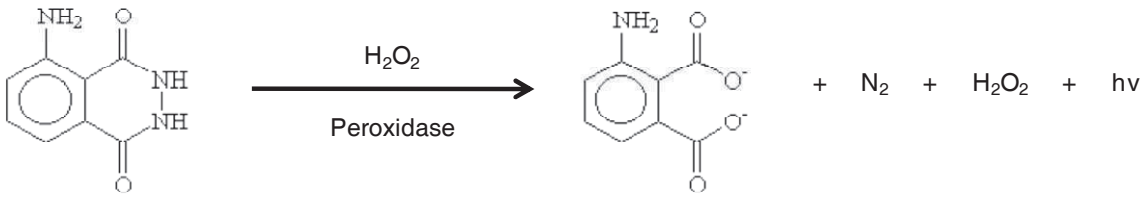
In-gel kinase assay was performed as previously described by Lebrun-Garcia et al., (1998) with minor modifications. Twenty µg of total proteins were electrophoresed on 10 % SDS-polyacrylamide gels embedded with 0.5 mg.mL^{-1} HIIIS in the resolving gel as a substrate for the kinases. After electrophoresis, SDS was removed by washing the gel for 1 h with washing buffer A (50 mM Tris–HCl; pH 8.0, 20 % 2-propanol), and then again for 1 h with washing buffer B (50 mM Tris–HCl; pH 8.0, containing 5 mM β-mercaptoethanol). The separated proteins were denaturated for 1 h in washing buffer C (6 M guanidine-HCl, 50 mM Tris–HCl; pH 8.0, 5 mM β-mercaptoethanol), and then allowed to renature by five successive washes at 4 °C over 16 h with buffer D (50 mM Tris–HCl pH 8.0, 5 mM β-mercaptoethanol and 0.04 % (v/v) Tween-40). The gels were then equilibrated for 30 min at room temperature in buffer E (40 mM HEPES; pH 7.5, 0.1 mM EGTA, 20 mM $MgCl_2$ and 2 mM DTT) and then for 1 h in the same buffer supplemented with 25 µM ATP and 0.37–0.925 MBq [ϒ-^{32}P]-ATP (Amersham). The reaction was stopped by extensive gel washing with washing buffer F [5 % (w/v) trichloroacetic acid, containing 1 % (w/v) potassium pyrophosphate].

The gels were dried on chromatography paper exposed to Kodak XAR-5 films. Kinase activity was revealed using a PhosphoImager (Molecular Dynamics Inc). Pre-stained size markers (Fermentas) were used to estimate the apparent molecular mass of the PK. Pre-stained molecular markers from Sigma or Bio-Rad were used to estimate the apparent molecular mass of the HIIIS kinases (protein kinase) after in-gel kinase assays.



3,3-Diaminobenzidine tetrahydrochloride (**DAB**)

Figure 3.6: Detection of H_2O_2 by reaction with 3,3 '-diaminobenzidine (DAB) in *Arabidopsis thaliana* leaves in the presence of peroxidase.



Luminol

Figure 3.7: Luminol based Chemiluminescence measurement for the production of H_2O_2 in *Arabidopsis thaliana* leaf discs in the presence of peroxidase.

2.10. Reactive oxygen species (ROS) measurement in *Arabidopsis* leaf discs

2.10.1. Diaminobenzidine (DAB) staining

3,3-Diaminobenzidine tetrahydrochloride (DAB) is a horseradish peroxidase substrate suitable for use in immunoblotting and immunohistological staining procedures. This substrate produces an insoluble, brown end product, which can be observed visually. The end product is not alcohol-soluble; therefore, a variety of counterstains and mounting media can be used (Figure 3.6).

To visualize H_2O_2 *in situ*, 3, 3-diaminobenzidine (DAB) staining was performed on *Arabidopsis* leaves (Col-0) with OGs or water vacuum-infiltrated in DAB solution. Leaves were placed in a plastic box under high humidity until brown precipitate was observed. Application of H_2O_2 directly to leaves was used as a negative control to verify that ATRBOH enzyme is impaired in the detection of ROS (H_2O_2) by this method. 8-10 leaves coming from different plants were used. After different time intervals of treatment (4 h, 6 h and 8 h) leaves were destained overnight in methanol to remove the chlorophyll.

The destained leaves were incubated overnight in chloral hydrate solution (0.25 $mg.mL^{-1}$) and finally washed with Na_2HPO_4 solution. Pictures were obtained by digital camera.

2.10.2. Detection by chemiluminescent assay

Luminol is a chemiluminescent horseradish peroxidase substrate. It is used for the detection of reactive oxygen species (Figure 3.7).

Two mm leaf discs of 4 weeks-old *Arabidopsis* were excised and 5 discs (≈ 10 mg) per condition were incubated in 200 µL H_2O overnight in assay tubes. For measuring the oxidative burst, active oxygen species released by leaf tissue were measured by a luminol-dependent assay (Keppler et al., 1989). Assay tubes were supplied with 60 µM luminol. Luminescence was measured in luminometer (Lumat LB 9507, Berthold) approximately for 5 min and then addition of the test solution (treatment) a total time of 50 min.

Measurements were integrated over 10 s periods. Inhibitor, DPI (10 µM, 20 µM and 50 µM), quinacrine (1 mM, 2 mM and 5 mM), L-NAME (5 mM), Tungstate (0.1 mM) and cPTIO (250 µM, 500 µM and 1 mM) were added 10 min prior to treatment.

2.11. Transcriptome analysis

Arabidopsis plants were grown under same conditions as described earlier (Page 51). Four weeks old plant leaves were infiltrated by syringe with water, OGs, OGs+cPTIO and cPTIO. OGs and cPTIO were used as a working concentration of 2.5mg.mL^{-1} and 500 µM respectively. Samples were collected at different time intervals (T0, 1h, 6h and 24h).

Three replicates containing three plants per replicate for each treatment were used for Nimblegen Array.

2.11.1. RNA extraction and cDNA synthesis and labeling

Total RNA was extracted from *Arabidopsis thaliana* frozen leaves with RNeasy Plant mini kit (Qiagen, Hilden, Germany) followed by manufacturer's instructions. RNA was quantified by nanodrop 100. Total RNA quality was checked by microchips on Agilent bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Total RNA (10 µg) was reverse transcribed in double stranded cDNA using SuperScript double-stranded cDNA synthesis kit (Invitrogen) followed by manufacturer's instructions. Then, double-stranded cDNA was labeled using NimbleGen One color DNA labeling kit (Roche NimbleGen,Inc) followed by manufacturer's instructions. Briefly, 1 µg of double stranded cDNA was incubated with Cy3-random nonamers primers at 98°C for 10 minutes (denaturation) followed by the incubation with dNTP and klenow enzyme for 2 hours at 37°C. Cy3 labeled cDNA was purified and quantified with nanodrop.

2.11.2. Array hybridization and scanning

Cy3-labeled cDNA was hybridized on *A. thaliana* Gene Expression 12x135K Array (Roche NimbleGen, Inc). The Array has 60 mers probes targeting 39,042 genes hybridization using Nimblegen hybridization kit (Roche NimbleGen, Inc). Each gene is targeted by 4 probes in one replicate. Briefly, 4 µg of Cy3-labeled cDNA was mixed with alignment oligonucleotides and samples tracking controls (STC), 2X hybridization buffer was provided in the Nimblegen Hybridization kit and incubated at 95°C for 5 minutes. Then the mixture was hybridized at 42°C for 20 hours using Nimblegen hybridization system and finally the slides were washed using Nimblegen wash buffer kit (Roche Nimblegen, Inc) followed by manufacturer's instructions.

The slides were scanned using a Genepix 4000B scanner (Axon, Union City, USA) and Genepix software fitted with the laser set at 532 nm. The laser power was set at 100%, and the photomultiplier tube voltage (PMT) was at 540. The scanned image files were analyzed using NimbleScan software version 2.6 which produced both a raw and normalized hybridization signal for each spot on the array.

2.13.3. Microarray data analysis

The relative intensity of the raw hybridization signal on arrays varies in different experiments. NimbleScan software was therefore used to normalize the raw hybridization signal on each array for better cross-array comparison using quartile normalization (Bolstad et al., 2003), and then gene calls are generated using Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003). A background correction was also performed. The complete set of gene called files was available at the GEO database under accession number **GSEXXXXX**.

Quality of processing was evaluated by two experimental metrics: the signal range and the coefficient of variation (Uniformity CV) of each array.

Statistical comparison and filtering were performed using Partek Genomics Suite.6.5 (Partek Inc., St. Louis, MO, US). Pair-wise comparisons were carried out by comparing each sample from one group with each sample from the other group and only genes showing a variation of 2-fold in all pair-wise comparisons were retained.

CHAPTER 4

"Results"

CHAPTER 4

"Part 1"

"Nitric oxide production mediates oligogalacturonides-triggered immunity and resistance to Botrytis cinerea in Arabidopsis thaliana"

CHAPTER 4

Nitric oxide production mediates oligogalacturonides-triggered immunity and resistance to *Botrytis cinerea* in *Arabidopsis thaliana*

Running title: NO production in response to OGs

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Abstract

Nitric oxide (NO) regulates a wide range of plant processes from development to environmental adaptation. In this study, NO production and its effects were investigated in a plant-pathogen context. The production of NO following Arabidopsis treatment with oligogalacturonides (OGs), an endogenous elicitor of plant defense, was assessed using the NO sensitive probes 4, 5-diamino fluorescein diacetate and CuFL. Pharmacological and genetic approaches were used to analyze NO enzymatic sources and its role in the *Arabidopsis*/*Botrytis cinerea* interaction. We showed that NO production involves both a L-arginine- and a nitrate reductase (NR)-pathways and correlates with an increased NR activity and NR transcript accumulation. OGs-induced NO production was Ca^{2+}-dependent and modulated RBOHD-mediated Reactive Oxygen Species (ROS) production. We further demonstrated that NO participates in the regulation of OGs-responsive genes such as anionic peroxidase (PER4) and a β−1,3−glucanase. Mutant plants impaired in PER4 and β−1,3−glucanase, as well as Col-0 plants treated with the NO scavenger cPTIO, were more susceptible to *B. cinerea.* Taken together, our investigation deciphers part of the mechanisms linking NO production, NO-induced effects and basal resistance to *Botrytis cinerea*. More generally, our data reinforce the concept that NO is a key mediator of plant defense responses.

Keywords: nitric oxide, oligogalacturonides, nitrate reductase, plant defense, *Arabidopsis thaliana*, *Botrytis cinerea*, calcium, reactive oxygen species.

INTRODUCTION

Defence responses of plants against microbial attack are initiated by signal molecules released during the infection process and mediated by a number of signalling components. The components include nitric oxide (NO), a widespread molecule in living organisms involved in major physiological processes (Torreilles, 2001; Besson-Bard et al. 2008a). In mammals, NO is mainly synthesized from L-arginine by NO synthase (NOS). In plants, NO is also derived from nitrogen-containing precursors but these precursors are more diverse than in animals. In broad outline, NO is generated in plants from two enzymatic pathways: a L-arginine-dependent pathway and a nitrite-dependent pathway (Besson-Bard et al. 2008a). Many studies highlighted the occurrence of an enzymatic process in which NO is produced from L-arg by an unidentified enzyme sensitive to mammalian NOS inhibitors (also named NOS-like enzyme) although there is no obvious homolog of mammalian NOS in the plant genomes sequenced so far (Cueto et al. 1996; Modolo et al. 2002; del Rio et al. 2004; Corpas et al. 2006). Tun et al. (2008) have reported the putative involvement of a polyamine-dependent production of NO related to L-arginine metabolism, this amino-acid being one key precursor of polyamines (PAs). *In vivo* and *in vitro* studies highlighted that NO is also a by-product of Nitrate Reductase (NR) activity when nitrite is used as a substrate (Yamasaki, 2000; Sakihama et al. 2002). NO production from NR occurs in specific context in which the cytosolic nitrite concentrations reach high concentrations, that is in the mM range. NR was shown to be involved in NO production in several physiological situations, such as abscisic acid (ABA)-induced stomatal closure, hypoxia, cold stress or in nitrogen-fixing nodules (Cantrel et al. 2010; Desikan et al. 2002; Dordas et al. 2004; Horchani et al. 2011; Zhao et al. 2009). In *Arabidopsis thaliana*, two cytosolic isoforms of NR (NR1 and NR2) have been identified. These isoforms have a high degree of coding sequence similarity and are 83.5% identical at the amino acid level. NR2 represents ~ 90% of the total NR activity regarding nitrate reduction (Wilkinson & Crawford 1991). Comparative analysis using the single mutants *nia1* and *nia2* suggested that the most committed NR isoform to NO production in response to ABA or cold stress was encoded by *NR1* (Bright et al. 2006; Zhao et al. 2009). Finally, it has been also reported that NO synthesis from nitrite occurs in mitochondria associated with mitochondrial electron transport (Horchani et al. 2011; Planchet et al. 2005).

In plants, NO was found, among different roles, to be involved in mediating defense reactions against microbial pathogens (Delledonne et al. 1998; Leitner et al. 2009). Firstly, NO was reported to be rapidly generated in several plant-pathogen models and using different detection methods (Vandelle & Delledone 2008). A rapid and intense intracellular NO

production was detected in tobacco epidermal cells and cell suspensions treated with the *Phythophthora cryptogea* elicitin cryptogein using diaminofluorescein diacetate (DAF-2DA), a cell permeable NO specific fluorescent probe (Foissner et al. 2000) and by electrochemistry (Besson-Bard et al. 2008b). In response to the necrotrophic fungus *Botrytis cinerea*, *Pelargonium peltatum* leaves also initiated a near-immediate NO burst but, interestingly, its generation was dependent on the genetic makeup of the host plant. Precisely, in resistant genotypes, a subsequent wave of NO generation was correlated with the resistance to *B. cinerea* whereas in susceptible cultivar, the second NO burst was absent (Floryszak-Wieczorek et al. 2007). Recently, Piterkova *et al.* (2009) also reported a systemic NO production in adjacent and distant uninoculated leaves of tomato plants challenged with the biotrophic fungus *Oidium neolycopersici*. Secondly, it was shown that pathogen-induced NO production or exogenous treatment with NO donors affect plant defense molecular responses, such as the expression of defense-related genes or phytoalexins biosynthesis and, therefore, could participate in plant disease resistance (Delledonne et al. 1998, Durner et al. 1998). More recently, it was observed that the NR-deficient double mutant (*nia1nia2*), which shows substantially reduced NO production after bacterial or fungal pathogens inoculation, showed no hypersensitive response and was hyper-susceptible to *Pseudomonas syringae* (Modolo et al. 2006; Oliveira et al. 2009) and to the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* (Perchepied et al. 2010). Although Modolo *et al.* (2006) have noticed that the levels of amino acids, and particularly L-arginine, are strongly reduced in *nia1nia2 A. thaliana* leaves, NO emission by *nia1nia2* leaves did not increase in the amino acids recovered mutants (Oliveira et al. 2009). These results suggest that the susceptibility to pathogen is a consequence of the reduced ability to synthesize NO. Similarly, plants affected in *AtNOA1* (NO-associated protein 1) expression exhibited a reduced endogenous NO level and were more susceptible to the virulent bacteria *Pseudomonas syringae* pv tomato DC3000, to the fungi *Colletotrichum orbiculare, Sclerotinia sclerotiorum* and *B. cinerea* (Zeidler et al. 2004; Asai et al. 2008; Perchepied et al. 2010; Asai & Yoshioka, 2009).

Research conducted over the past years has revealed that NO mediates part of its effect through modulation of protein kinase activities, post-translational modifications of target proteins and mobilization of free Ca^{2+} and other second messengers (Besson-Bard et al. 2008a). Particularly, it has been reported that the involvement of NO in plant immunity is related to its interplay with reactive oxygen species (ROS) and cell death occurring during HR might result from the simultaneous and balanced production of NO and ROS (Zanninoto et al. 2006). NO and ROS exert reciprocal control on each other, directly (reaction of NO and ROS

to form peroxynitrite anion $ONOO^-$; Cecconi et al. 2009) or indirectly (e.g. through the NO-dependent inhibition of catalase and ascorbate peroxidase, two major H_2O_2-scavenging enzymes by NO; Clarke et al. 2000; Arasimowicz et al. 2009).

In spite of these numerous evidences associating NO to plant defense reactions, little is known on the mechanisms that link NO production to its physiological activity. In order to further investigate the role of NO in a patho-physiological context, in the present study we analysed its functions in the plant defense responses triggered by oligogalacturonides (OGs) in *A. thaliana* plants. OGs are structural components of the pectin homogalacturonan chains of plant cell wall. In *A. thaliana*, they are released during the interaction with pathogens, such as the necrotrophic fungus *Botrytis cinerea*, which secretes polygalacturonase (PG) as part of their cell wall degrading enzyme arsenal. OGs, with a degree of polymerization between 10 to 25, are considered as endogenous elicitors as they are released from plant cell wall in response to the damage caused by invading pathogens. In *A. thaliana* or grapevine (*Vitis vinifera*), OGs treatment induces a variety of defense responses including accumulation of phytoalexins, β−1,3−glucanase and chitinase. Moreover, OGs have been shown to contribute to triggered immunity against fungal pathogens including *B. cinerea* (Aziz et al. 2004; Ferrari et al. 2007). About half of the *A. thaliana* genes affected by OGs treatment display a similar pattern of expression after *B. cinerea* infection, suggesting that at least part of the responses activated by *B. cinerea* are mediated, directly or indirectly, by OGs (Ferrari et al. 2007). Therefore OGs represent a valuable tool to analyze the mechanisms involved in plant pathogen interaction.

In this study, using genetic, biochemical and pharmacological approaches, we showed that OGs induced a NR- and L-arginine-dependent NO production together with an increased NR activity and NR transcripts accumulation. NO production was Ca^{2+}-dependent and we identified the plasma membrane cyclic nucleotide-gated channel CNGC2 as an upstream regulator of its synthesis. We further demonstrated that NO production modulated AtRBOHD-mediated ROS production and the transcriptional activation of defense-related genes encoding the anionic peroxidase PER4 and a β−1,3−glucanase. Finally, by pharmalogical and reverse genetic approaches we provided evidence that NO, as well as its target genes, contributes to the OGs-triggered immunity against *B. cinerea*. Taken together, our data reinforce the concept that NO is a key mediator of plant defense responses.

MATERIAL AND METHODS

Plant material and growth conditions

The *dnd1* (CNGC2) mutant line (Ali et al. 2007) was a gift from Dr Berkowitz (University of Connecticut, USA). The *rboh*D mutant was kindly provided by Dr Torres (Universidad Politecnica de Madrid, Spain). The double NR *nia1nia2* mutant (NR1-Ds and NR2 deletion mutant) was kindly provided by S. Neill (University of the West of England-UK). Mutants impaired in anionic peroxidase PER4 (*At1g14540*) were *Per4-1* (N655479/SALK_544730) and *Per4-2* (N661085/SALK_110617) and in β-1-3-glucanase (*At3g55430*) was *Glu* N642531/SALK_142531). Seeds of these mutant lines and of the wild type Col-0 were obtained from NASC (Nottingham Arabidopsis Stock Center). All mutants lines used in this work are in the Col-0 background.

The plants were cultivated in commercial soil [Jiffy-7, Puteaux (France)] in a climate-controlled growth chamber (KBW, Binder, Germany) with a 10 h light (175 $\mu E.s^{-1}$ light intensity), 14 h dark cycle with the following settings: 20 °C light, 18 °C dark; 70 % relative humidity light / 95 % dark.

Chemicals

All chemicals were purchased from Sigma-Aldrich except L-NAME (N^G-nitro-L-arginine-méthyl ester) which was from ALEXIS Biochemicals. Sodium Tungstate, Lanthanum chloride, L-NAME and cPTIO ((4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) were dissolved in water. DAF-2DA (4,5-diaminofluorescein diacetate) was received as a stock solution of 5 mM in dimethylsulphoxide (DMSO). DPI (diphenylene iodonium) was dissolved in DMSO.

OGs, with an average degree of polymerization (DP) of 25, were obtained from GOEMAR (Saint Malo, France). OGs were dissolved in water at a working concentration of 2.5 $mg.mL^{-1}$.

NO measurements

NO production was monitored using 4,5-diaminofluorescein diacetate (DAF-2DA; Sigma-Aldrich), a membrane-permeable derivative of the NO-sensitive fluorophore 4,5-diaminofluorescein. This method is indirect and relies on the measurement of reactive

nitrogen species (notably N_2O_3 and NO^+) derived from NO autooxidation that nitrosate DAF-2 to yield the highly fluorescent DAF-2 triazole (DAF-2T; Jourd'heuil, 2002).

Leaf discs excised from plants were infiltrated under vacuum for 3 min with an aqueous solution of 20 µM DAF-2DA and 2.5 $mg.mL^{-1}$ of OGs in 50 mM Tris-HCl (pH 7.5). For control treatment, OGs were replaced by an equivalent volume of water. After infiltration, the discs were washed with Tris-HCl (50 mM, pH 7.5). Each disc was put in separate well of 96 well plates (Microtest ^{Tm} flatbottom, Becton Dickinson, Europe) in 200 µL Tris-HCl (50 mM, pH 7.5) with OGs or water. The increase of DAF-2T fluorescence triggered by OGs was measured using a spectrofluorometer (Mithras, Berthold Technologies, Germany). Fluorescence was measured with a 485 nm excitation and a 535 nm emission filters for 12 h. Eight leaf discs were used for each treatment. Fluorescence was expressed as relative fluorescence units (arbitrary units: au).

Gene expression analysis by quantitative Real Time-qPCR

Treated leaves were immediately frozen in liquid nitrogen. Total RNA was isolated with Tri reagent (Molecular Research Centre Inc). Nucleic acid concentration was estimated at 260 nm. Purity of total RNA was assessed by determining the 260/280 ratio and the integrity was checked by agarose gel electrophoresis.

First-strand cDNA templates were produced by using the cDNA synthesis kit (Superscript^{Tm} III Reverse Transcriptase, Invitrogen) from 2 µg of total RNA treated with DNaseI (Sigma-aldrich, USA) as recommended by the manufacturer. Reactions were performed in a final volume of 20 µL containing 8 µL of cDNA (dilution 1/80) sample, 200 nM of forward and reverse primers and 10 µL of realtime SYBR Green mix (Abgene, Thermo Scientific, UK). qPCR was conducted with a Mastercycler (Sequence Detection System, Applied Biosystem) in 96-well plates (Optical reaction plate with Bar code, Applied Biosystem). Gene-specific primers were as follows: *PER4-At1g14540* (5'-cactggttcagatggacaaa-3' and 5'-aacaaacgaattatcgctgc-3'), *PLP2-At2g26560* (5'-aagaaaagaagatccgagac-3' and 5'-attcaaacgtacaagtgacc-3'), *CHI-IV-At3g54420* (5'-tgttgactcccaccattt-3' and 5'-cggtcgatccaactctac-3'), β–1,3-glucanase-*At3g55430* (5'-cttcgctggaactggtatct-3' and 5'-gcagaatctcatttccgact-3'). The specificity of the primers was tested by sequencing the PCR products. PCR amplification efficiency of target DNA was examined to measure linearity of dilution. For each gene a standard curve was generated with

serial dilutions of plasmids containing the respective PCR product. These standard curves showed similar slopes demonstrating an efficiency of more than 90%.

Expression levels were calculated relative to the housekeeping gene (HK) *UBQ10-At4g05320* (5'-ctatatgctcgctgctgagc-3' and 5'-aagccaggcagagacaactc-3') using the comparative threshold cycle method, where Ct represents the threshold cycle for target amplification: $\Delta\Delta Ct = \Delta Ct_{\text{gene of interest}} - \Delta Ct_{HK}$. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression from real-time quantitative PCR experiments (Livak & Schmittgen, 2001).

In vivo Nitrate Reductase (NR) activity

In vivo NR activity was assayed by the protocol of Yu *et al.* (1998). NR activity was measured in a spectrophotometric assay by determining the amount of NO_2^- released from the tissue. Leaf discs were weighed, vacuum infiltrated with or without OGs in Tris-HCl for 3 min and incubated for 4 h within the respected solutions. After incubation leaf discs were transferred into 1 mL of reaction buffer (40 mM KNO_3, 0.08 mM Na_2HPO_4, 0.02 mM NaH_2PO_4, and 4% [v/v] *n*-propanol, pH 7.5) and incubated in the dark for 2 h. The reaction was stopped by the addition of 200 µL of 1% sulphanilamide (dissolved in 3 N HCl) and 200 µL of 0.05% *N*-(1- napthyl)ethylenediamine hydrochloride. The concentration of NO_2^- was determined by measuring the OD of the solution at 540 nm.

H_2O_2 measurements

H_2O_2 was measured using a luminol-dependent assay (Keppler et al. 1989). Two mm leaf discs of 4 weeks-old Arabidopsis plants were excised and five discs per condition were incubated in 200 µL of H_2O overnight in assay tubes. Then, 4 µL of 3mM luminol solution (final concentration 60 µM) was added to the tubes 5 min before treatment. Luminescence was measured in a luminometer (Lumat LB 9507, Berthold) for a total time of 50 min. Measurements were integrated over 10 s periods.

Botrytis cinerea culture and infection method

Botrytis cinerea strain BMM (Zimmerli et al. 2000) was grown on Petri plates containing PDA (potato dextrose agar, DIFCO) for 10-12 days (light 10h, 20°C; dark 14h, 18°C). Spores were harvested in water and subsequent filtration through glass wool to remove hyphae. Spores were quantified under a microscope. About 1 h before infection, spores were diluted in ¼ PDB (potato dextrose Broth, DIFCO) to obtain a final concentration of $5x10^4$

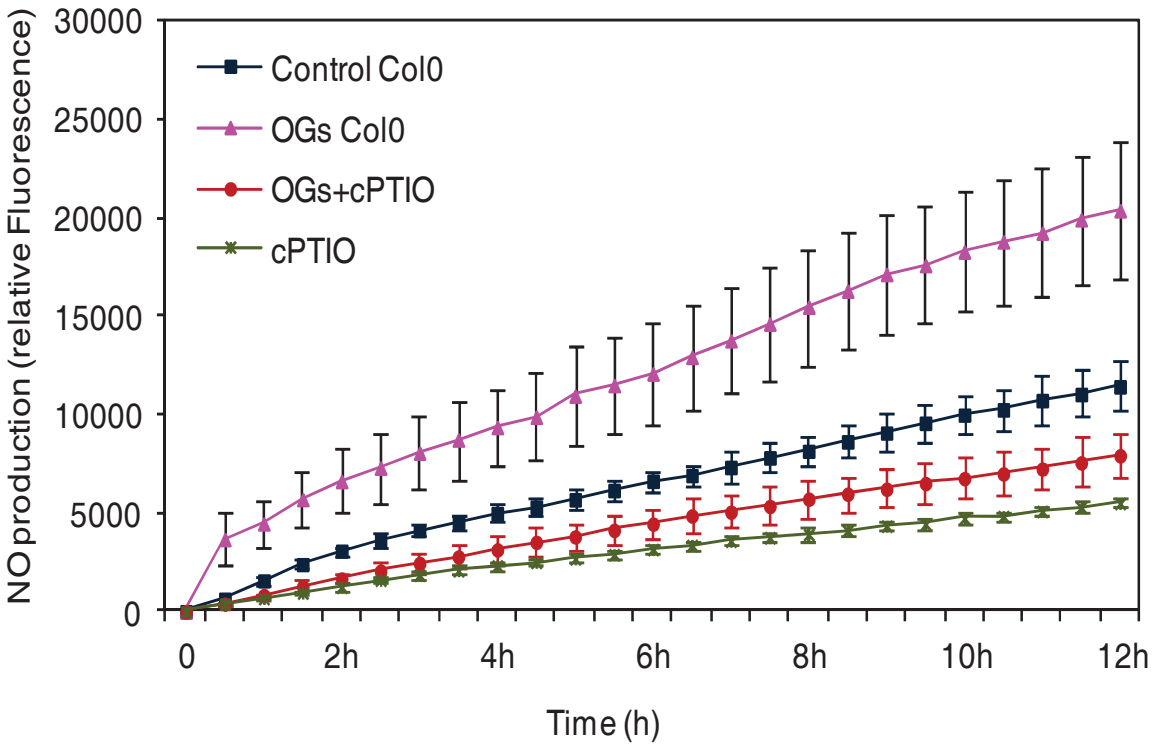

Figure 4.1: Time course of NO production in OGs-treated Col-0 leaf discs. NO production was monitored using DAF-2DA (20 µM) in the absence (control) or presence of OGs (2.5 $mg.mL^{-1}$), with or without NO scavenger (cPTIO 500 µM). Each value represents the mean of 8 measurements ± SD. This experiment is representative of 10 independent experiments using independent biological material.

spores mL^{-1}. For assessment of symptom in plants, droplets of 6 µL of spore suspension ($5x10^4$ spores mL^{-1}) were deposited on six leaves of 4-week-old plants (wild type and mutants). Plant infections were performed in covered plastic box to maintain high humidity and returned to the growth chamber. Lesions diameters were measured after 72 h of inoculation using callipers. Results were analysed by one-way ANOVA on ranks, followed by Dunnett's test ($p>0.05$), using the SigmaPlot software.

RESULTS

OGs induce NO production in *A. thaliana* (Col-0) leaf discs

The ability of OGs to induce NO production in Col-0 was investigated by monitoring the time course of DAF-2 triazole (DAF-2T) fluorescence accumulation in OGs-treated leaf discs pre-infiltrated with DAF-2DA (Fig. 4.1). DAF-2T is a fluorescent compound resulting from the nitrosation of DAF-2 by reactive nitrogen species derived from NO auto-oxidation (Besson-Bard et al. 2008b). The DAF-2-based assay for NO detection has been shown to be a suitable technique for the detection of NO (Vandelle & Delledonne, 2008). OGs triggered an increase in fluorescence which occurred after 1 h and remained constant thereafter. A slight increase in DAF fluorescence was also observed in control leaf discs. This background production might be related to constitutive NO production and/or to the wounding triggered during leaf discs preparation. Fluorescence increase in both OGs-treated and control leaf discs was markedly suppressed by the NO scavenger cPTIO (Fig. 4.1), arguing that the rise in the fluorescence was mainly related to NO production and not to NO-unrelated DAF-2 reacting compounds. NO production in response to OGs was confirmed through another approach based on the use of the CuFL fluorescent probe. The CuFL probe reacts rapidly with NO and is believed to be more sensitive than DAF-2A (Fig. S1; Lim et al., 2006). However, NO detection with CuFL is less convenient than DAF-2DA as freshly prepared probe is poorly stable and could not be used after 1h (manufacturer's instructions). Compared to control, OGs triggered a significant increase of CuFL fluorescence (Fig. S2). As expected, this fluorescence increase was completely suppressed by cPTIO, thus further confirming the ability of OG to trigger NO synthesis.

OGs-induced NO production is L-NAME sensitive

Several studies have suggested that production of NO from L-arginine occurred in plants by an unidentified NOS-like enzyme which activity is sensitive to mammalian NOS

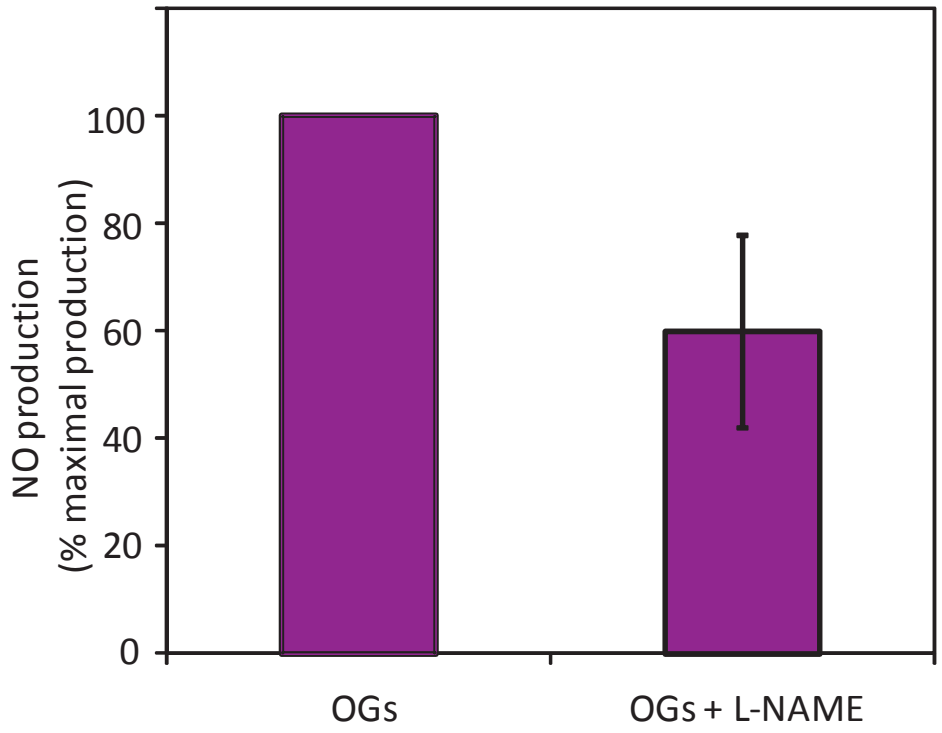

Figure 4.2: Effect of L-NAME on OGs-induced NO production in Col-0 leaf discs. Arabidopsis leaf discs from wild-type (Col-0) plants loaded with DAF-2DA (20 μM) were treated by OGs (2.5 mg.mL^{-1}) with or without a pretreatment with L-NAME (5 mM). NO accumulation was determined after 12 h of treatment and expressed as a percentage of the maximal response after subtracting background fluorescence of corresponding control. Each value is a mean ± SD of 3 independent experiments.

inhibitors (Cueto et al. 1996; Modolo et al. 2002; del Rıo et al. 2004; Corpas et al. 2006). To investigate the putative involvement of a NOS-like enzyme, we checked the sensitivity of OGs-induced NO production to L-NAME, a widely used mammalian NOS competitive inhibitor. L-NAME was reported to suppress NO synthesis, as well as NOS-like activities, in plant tissues and cell suspensions exposed to various stimuli. L-NAME reduced OGs-induced NO production in Col-0 leaf discs by 40 % after 12h of treatment (Fig. 4.2). This inhibition was observed at earlier time point (Fig. S3a).

NR is involved in OGs-induced NO production

We first investigated the putative contribution of NR as an enzymatic source for NO production during OGs treatment using the NR *nia1nia2* double mutant (Fig. 4.3a and Fig. S3b). OGs-induced increase of DAF-2T fluorescence was partially reduced by about 50% in *nia1nia2* as compared to wild type Col-0 leaf discs. The NO scavenger cPTIO completely suppressed the increase of fluorescence triggered by the elicitor in *nia1nia2*, indicating that the remaining fluorescence measured in the mutant was also due to OGs-induced NO production. Interestingly, L-NAME did not affect the remaining NO production observed in the NR mutant (Fig. 4.3a). To further confirm the involvement of NR as a NO source, we also examined the effect of the NR inhibitor tungstate on NO production. Pretreatment of Col-0 leaf discs with tungstate reduced OGs-triggered NO production by almost 30% (Fig. 4.3a and S3b). In contrast, pretreatment of *nia1nia2* with tungstate did not modify significantly the reduced NO production observed in the double mutant in response to OGs alone indicating that the remaining NO production in *nia1nia2* is not due to residual NR activity (Fig. 4.3a).

We next analysed whether OGs also triggered changes in NR activity and transcript accumulation. First, we quantified *in vivo* NR activity in *A. thaliana* Col-0 leaf discs. As shown in Fig. 4. 3b, OGs treatment induced a 2-fold increase of NR activity as compared to control after 4h of treatment. As expected, OGs-induced NR activity was completely suppressed by tunsgtate (Fig. 4.3b). In the *nia1nia2* mutant, a residual activity representing 2% to 3.3% of the activity measured in Col-0, was detected (data not shown). In order to better understand the link between Arg- and NR dependent pathways for NO production, we monitored NR activity in the presence of L-NAME (Fig. 4.3b). Surprisingly, L-NAME inhibited OGs-induced NR activity to a similar extend than tungstate. Second, we estimated *NR1* and *NR2* mRNA transcript accumulation by quantitative real time RT-PCR. A rise in the accumulation of both transcripts occurred in response to OGs (Fig. 4.3c). However, *NR2* *m*RNA showed noticeable higher and faster accumulation than *NR1* mRNA (Fig. 4.3c).

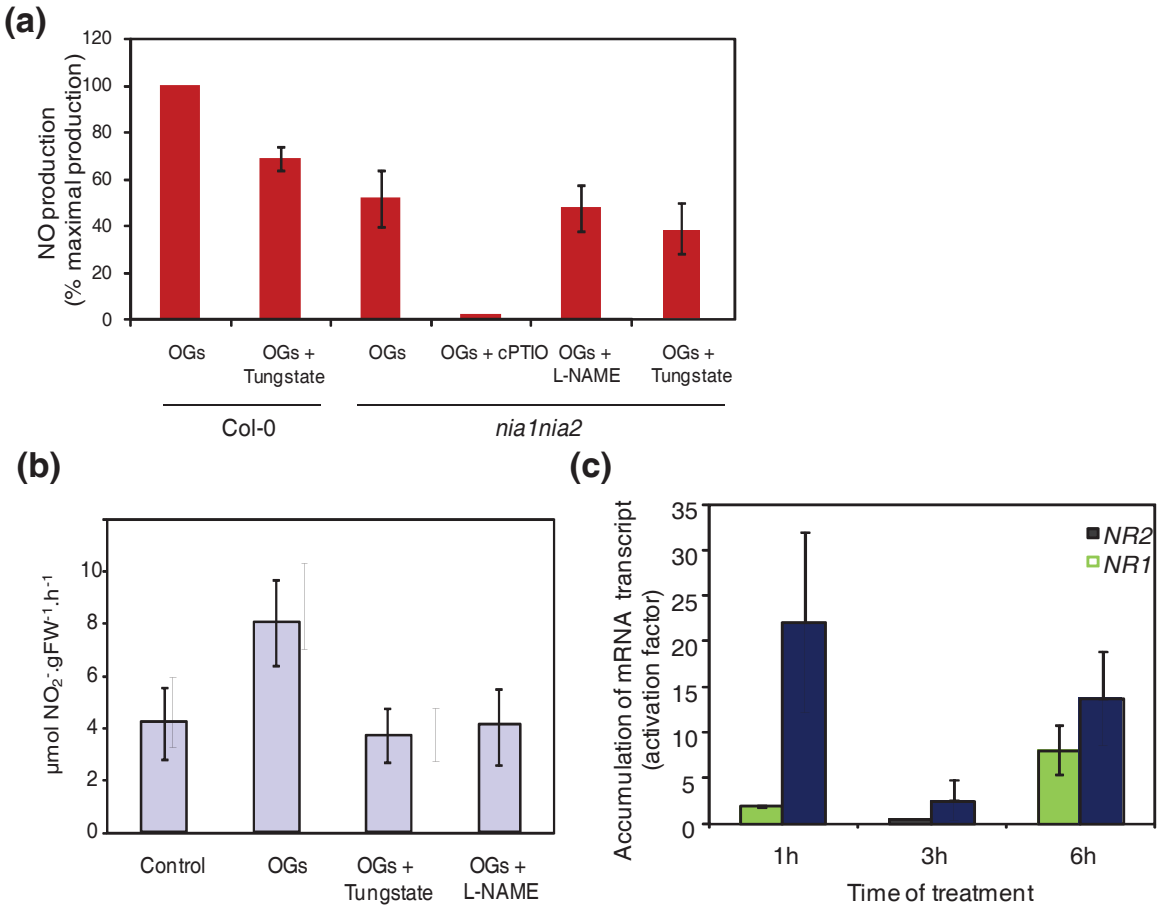

Figure 4.3: Involvement of nitrate reductase (NR) in OGs-induced NO production. **(a)** DAF-2DA-infiltrated (20 μM) Arabidopsis leaf discs from wild-type (Col-0), and *nia1nia2* plants were treated by OGs (2.5 mg.mL^{-1}) or water. The effect of Tungstate (0.1 mM) and/or L-NAME (5mM) on OGs-induced NO production was analysed on Col-0 and *nia1nia2* leaf discs. Bar graph is the mean of 5 independent experiments using independent biological material (8 replicates per experiment). NO accumulation determined after 12 h of treatment, is expressed as a percentage of the maximal response measured in wild-type Col-0 leaf discs treated with OGs, after subtracting background fluorescence of corresponding control. **(b)** *In vitro* NR activity in wild type (Col-0) leaf discs treated with water,OGs, OGs + Tungstate (0.1 mM) and OGs + L-NAME. Each value represents the mean ± SD of three independent experiments performed using independent biological materials. Tunsgtate and L-NAME alone did not modify significantly NR activity measured in control. **(c),** Time course of accumulation of *NR1* and *NR2* mRNA transcripts in Col-0 leaf discs treated with OGs or water. Transcribed mRNAs were analysed by real-time quantitative RT-PCR. Levels of transcripts were calculated using $2^{-\Delta\Delta CT}$ method. This experiment is representative of 3 independent experiments using independent biological material. Bars indicate average expression (± SD) of three replicates.

Furthermore, *NR2* transcripts showed a biphasic accumulation, the lowest accumulation being observed at 3h post-treatment. Taken together these results indicate that OGs elicit a NR-dependent NO production together with an increase of NR activity and transcript accumulation.

Ca^{2+} influx modulates NO production in response to OGs

The Ca^{2+}-dependency of NO production has been previously shown in several studies (Courtois et al. 2008). In particular, Ali *et al.* (2007) identified CNGC2, a plasma membrane cyclic nucleotide-gated channel (CNGC) member, as a key Ca^{2+}-permeable channel linking lipopolysaccharide (LPS)-triggered Ca^{2+} influx to downstream NO production in *A. thaliana*. Furthermore, OGs have been shown to trigger a fast and transient elevation in cytosolic free Ca^{2+} in *A. thaliana*, this process being suppressed by the calcium channel blocker lanthanum (La^{3+}; Moscatiello et al. 2006). Based on these data, we tested whether CNGC2 could act upstream of OGs-evoked NO generation using the *dnd1* (*defence no death1*) mutant impaired in functional CNGC2. In control leaf discs, Col-0 and *dnd1* mutant showed approximately similar level of basal DAF-2T fluorescence (data not shown). In response to OGs, *dnd1* leaf discs displayed a lower increased of DAF-2T fluorescence as compared to Col-0. Indeed, NO production in *dnd1* leaf discs reached 49 % of the value measured in Col-0 leaf discs (Fig. 4.4). Accordingly, a significant decrease in OGs-induced NO production occurred also in Col-0 leaf discs pretreated with La^{3+} (Fig. 4.4).Therefore, the reduced NO production in both *dnd1* and La^{3+}-treated Col-0 plants suggest a role for Ca^{2+} influx in mediating the OGs signal to NO production.

NO modulates OGs-triggered ROS production in *A. thaliana* leaf discs

Oxidative burst is a common early response of plant cells to pathogen attack and elicitor treatment. Galletti *et al.* (2008) demonstrated that OGs treatment induced an oxidative burst mediated by the NADPH oxidase AtRBOHD. In order to analyse a potential functional link between ROS and NO production in response to OGs, the role of NO in controlling the oxidative burst was first studied. ROS production was measured in leaf discs using a luminol-based assay. In response to OGs, ROS burst was detectable approximately 3 min after elicitation, reached a maximum after 10 min and declined thereafter (Fig. 4.5a). As expected, this production was sensitive to the NADPH oxidase inhibitors DPI (Fig. 4.5a) and also quinacrine, a general inhibitor of oxidases (not shown). Furthermore, it was completely abolished in the *RbohD* mutant as reported by Galetti *et al.* (2008; Fig. 4.5b). Pre-treatment of

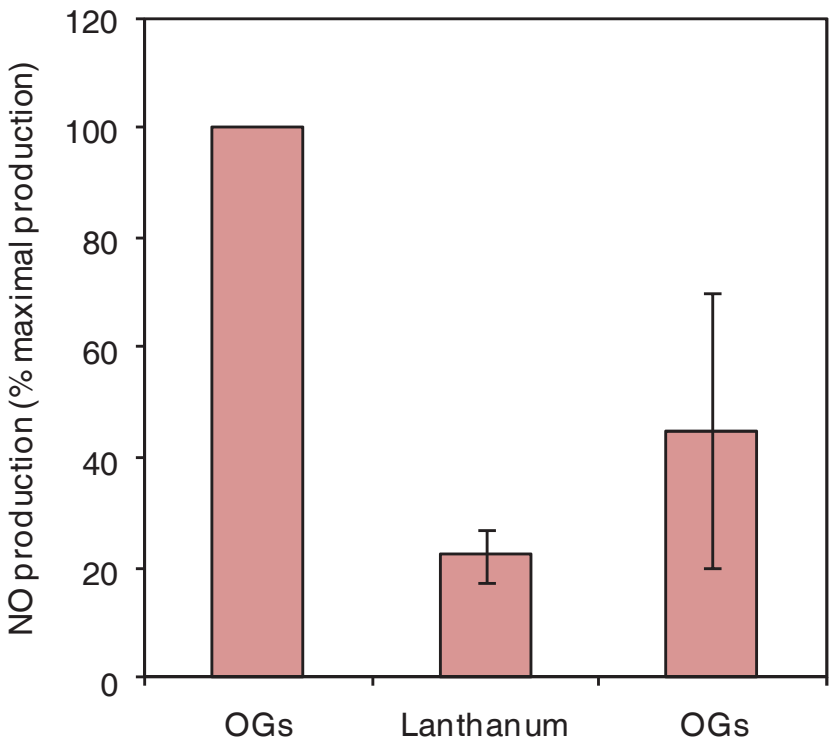

Figure 4.4: Involvement of Ca^{2+} influx in OGs-induced NO production. OGs-induced NO production was measured in Col-0 or *cngc2* leaf discs loaded with DAF-2DA (20 µM). Leaf discs were treated by OGs (2.5 $mg.mL^{-1}$) in the presence or absence of lanthanum (5 mM). Histogram represents the NO production measured after 12 h of treatment and expressed as a percentage of the maximal response after subtracting background fluorescence of corresponding control. Each value is a mean ± SD of 3 independent experiments.

leaf discs with cPTIO, L-NAME or La^{3+} 10 min prior OGs treatment led to an inhibition of OGs-stimulated ROS elevation (Fig. 4.5b). Surprisingly, the *nia1nia2* NR mutant, which displayed a reduced NO synthesis (Fig. 4.2), exhibited a similar increase of ROS production in response to OGs as compared to Col-0 (Fig. 4.5b).

To complete this analysis, we also verified NO level in response to OGs in *AtRbohD*. NO production in *rbohD* mutant was only 20% lower than Col-0 at 12h (Fig. 4.5c). Compared to Col-0, and taking into account variability, we considered this difference poorly significant, indicating that ROS produced by AtRBOHD do not, or slightly, control OG-induced NO production.

NO regulation of OGs-responsive genes

To demonstrate the involvement of NO in the plant defense responses induced by OGs, we monitored the expression of a set of four genes, selected according to previous studies, in leaf discs co-treated with cPTIO or L-NAME. The four genes correspond to *At1G14540*, encoding an anionic peroxidase (PER4), *At2G26560*, encoding a phospholipase A2 (PLP2), *At3G54420* encoding a chitinase IV (CHI-IV) and *At3g55430* encoding a β−1,3−glucanase. These four genes showed global transcriptional changes when *A. thaliana* plants were treated by OGs (Ferrari et al. 2007) and were found to be up regulated upon *B. cinerea* infection, a fungal pathogen known to release OGs from plant cell walls (La Camera et al. 2005; Doxey et al. 2007; Ferrari et al. 2007). Furthermore, *CHI-IV and β−1,3−glucanase* were identified as putative NO-responsive genes after analysis of publicly available expression data literature (Besson-Bard et al. 2009).

Compared to the control, a clear increase in *PER4* and *PLP2* transcript levels was observed at 1h and, to a lower extend, 3h after OGs treatment (Fig. 4.6a). The increase of *β−1,3−glucanase* transcript level occurred after 3h and decreased, while remaining significant, after 6h of treatment (Fig. 4.6a). In contrast, the expression of *CHI-IV* gene was not clearly up-regulated in our experiment (data not shown).

cPTIO reduced the OGs-induced up-regulation of these genes (Fig. 4.6a). However, only *PER4* and *β−1,3−glucanase* transcripts accumulation was affected by L-NAME treatment (Fig. 4.6b). Interestingly, the level of *PLP2* transcript was not significantly different in *nia1nia2* compared to Col-0 (Fig. S4). This result indicates that PLP2 expression, although cPTIO sensitive, is neither regulated by NR dependent pathway nor by Arg-dependent pathway.

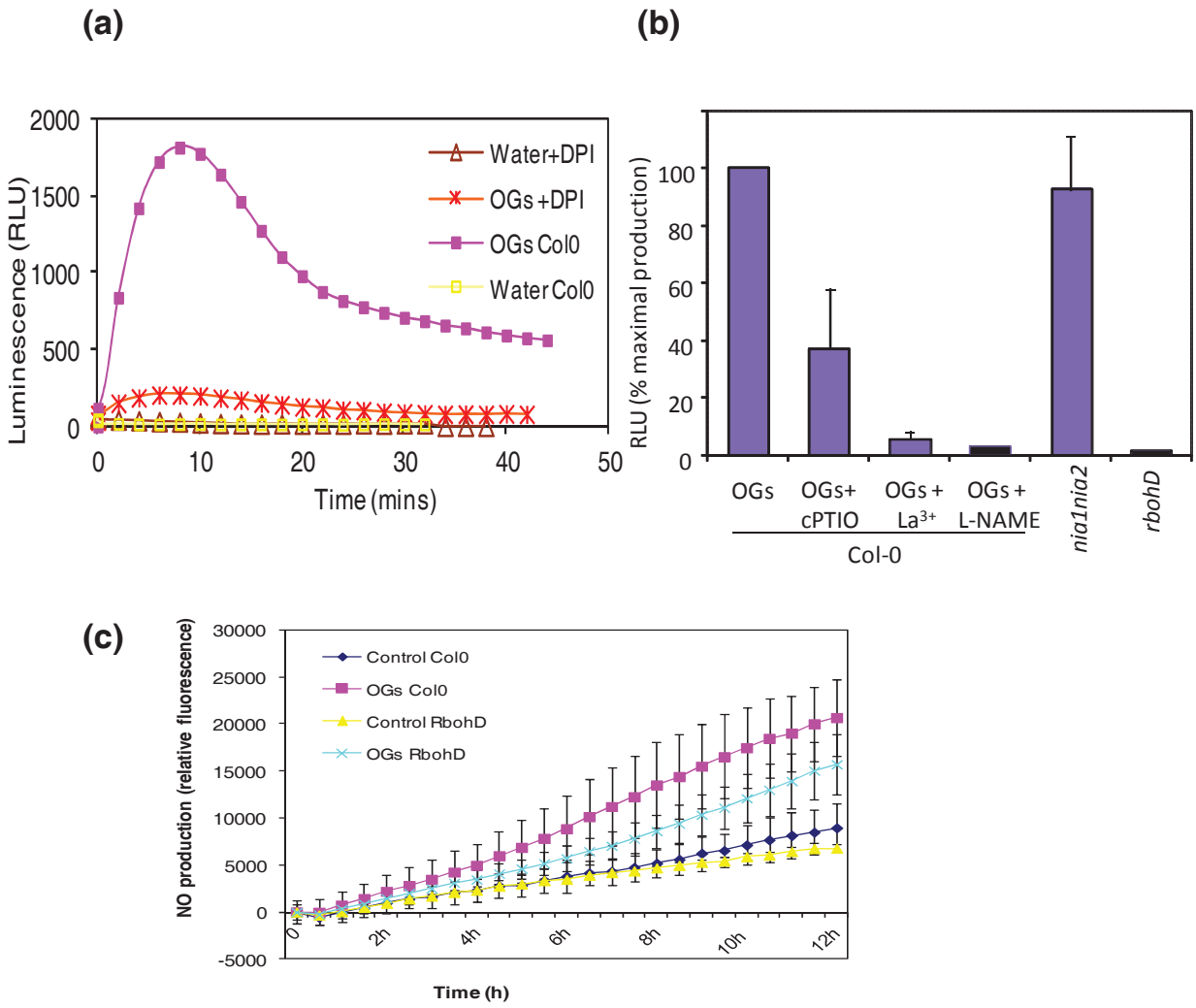

Figure 4.5: Relationships between ROS and NO production in plants elicited by OGs**. (a)** Accumulation of extracellular H_2O_2 in response to OGs. The production of ROS was measured with a luminol-based assay. Leaf discs from Col-0 plants were treated with water (control) or OGs (2.5 mg.mL^{-1}). DPI (10 µM) was added 10 min before treatment. Treatment with DPI alone did not induce luminescence. The data are representative of 3 experiments. **(b)** Effect of NO on H_2O_2 production in response to OGs. H_2O_2 production was measured as described in (a) and was represented as a percentage of the maximum ROS production observed in leaf discs of Col-0 and mutants elicited by OGs after subtracting background fluorescence of corresponding control. Luminescence values were recorded at 10 min of treatment (peak maximum). cPTIO (500 µM), L-NAME (5 mM) and lanthanum (5 mM) were added 10 min before treatment. **(c)** OGs-induced NO production in Col-0 and *rbohD* leaf discs. This experiment is the representative of three independent experiments using independent biological materials. Leaf discs were loaded with DAF-2DA (20 *µ*M) and treated by OGs (2.5 mg.mL^{-1}). Each value represents the mean of eight measurements ± SD.

NO participates to basal resistance to the fungal pathogen *Botrytis cinerea*

We used the *A. thaliana*/*B. cinerea* interaction model to determine the role of NO and NO/OGs responsive genes in plant defense. Indeed, it was previously demonstrated that OGs are released and accumulate during *B. cinerea* infection. OGs release results from polygalacturonase activity, this activity being required for pathogen virulence (Ten Have et al. 1998; Hahn et al. 2001). Arabidopsis leaves were infiltrated with water or different concentrations of cPTIO prior to inoculation with *B. cinerea*. The average diameter of necrotic lesions of plants pretreated with 500 µM cPTIO was significantly larger than in control plants (Fig. 4.7a). To rule out the possibility that the difference in disease lesions was due to an effect of cPTIO on fungal growth, we assessed the effect of cPTIO on the growth of *B. cinerea in vitro*. cPTIO has no effect on growth at the concentrations used in susceptibility analysis (Fig. S5).

Finally, we investigated *A. thaliana* basal resistance to *B. cinerea* in the different genetic background of interest by estimating macroscopic symptoms (*e.g.* diameter of lesions inflicted by the fungus). We used the double NR mutant *nia1nia2* affected in OGs-induced NO production and T-DNA insertion mutants impaired in the expression of the OGs/NO-inducible genes *PER4* and *β–1,3–glucanase* genes (*per4-1*, *per4-2* and *glu*, respectively). Mutants were challenged with *B. cinerea* and disease symptoms were assessed 3 days post-inoculation. Our results indicate that *per4-1*, *per4-2* and *glu* were more susceptible than Col-0, indicating that these two proteins are involved in basal plant resistance to *B. cinerea* (Fig. 4.7b). *Nia1nia2* double mutant was also more susceptible to *B. cinerea* (Fig. 4.7b).

DISCUSSION

Enzymatic sources of OGs-induced NO production

We demonstrated that OGs treatment triggered an increase of DAF-2T fluorescence related to NO synthesis. The ability of OGs to trigger NO synthesis was further confirmed using the fluorescence probe CuFL. NO production was partially suppressed by L-NAME, suggesting that NO synthesis involves a L-arginine-depend process. The ability of this compound to reduce NO synthesis as well as NOS-like activities in plant tissues and cell suspensions has been reported in many studies (Corpas et al. 2009). However, one must be cautious on interpretation based on L-NAME. Indeed, according to Besson-Bard *et al.* (2008a), the possibility that this compound could also affect the activities of other L-arginine

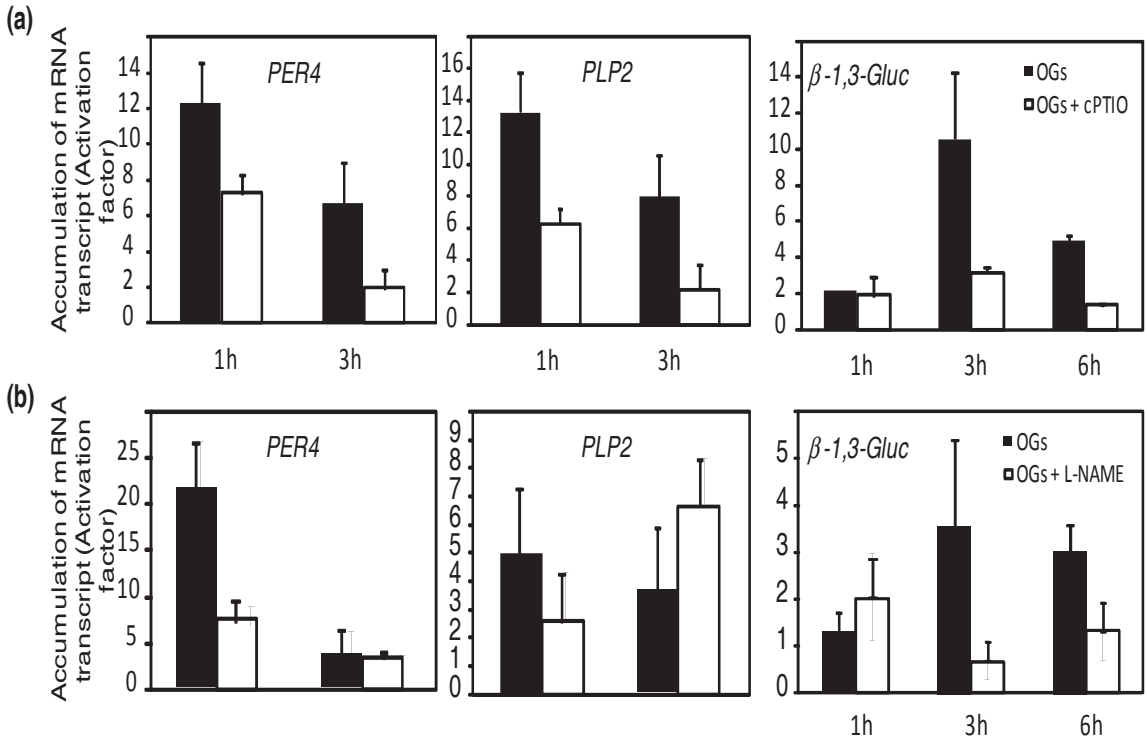

Figure 4.6: Role of NO on transcript accumulation of OGs-responsive genes. **(a)** Effect of cPTIO treatment on transcript accumulation of OGs-responsive genes. Col-0 leaf discs were infiltrated with water or OGs (2.5 mg.mL^{-1}) in the presence or absence of cPTIO (500 µM) and harvested for analysis after different time intervals. This experiment is representative of 3 independent biological experiments. Bars indicate average expression (± SD) of three replicates. **(b)** Effect of L-NAME on transcript accumulation of OGs-responsive genes. Col-0 leaf discs were infiltrated with water or with OGs (2.5 mg.mL^{-1}) in the presence or absence of L-NAME (5 mM) and were harvested for analysis after different time intervals. This experiment is representative of 3 independent biological experiments. Bars indicate average expression (± SD) of three replicates. Statistically significant comparisons are indicated by a star (Student's t-test, $p<0.05$).

metabolizing enzyme should not be excluded. Notably, the inhibitory effect of mammalian NOS inhibitors on NO production could be related to their ability to suppress enzymes activities such as enzymes related to PAs synthesis (such as arginase and arginine decarboxylase) and catabolism (PA oxidases), these latter being described as putative enzymatic source of NO (Tun et al. 2006).

Secondly, using both pharmalogical and genetic approaches, we provided evidence that NR is involved in OGs-induced NO production. The NR-dependent NO production was correlated with enhanced NR activity and up-regulation of *NIA1* and *NIA2* gene expression. Involvement of NR in NO production has been reported in interactions between plants and pathogen/elicitors such as necrotrophic fungal pathogen (Asai et al. 2008, Perchepied et al. 2010), bacteria (Modolo et al. 2006; Oliveira et al. 2009), chitosan (Li et al. 2009) and the elicitin INF1 (Yamamoto-Katou et al. 2006). However, Modolo *et al*. (2006) demonstrated that this double mutant has much lower L-arginine content in leaves (almost 10 times lower) as compared to wild-type plants, suggesting that L-arginine-dependent pathway for NO synthesis could be downregulated in the *nia1nia2* mutant. We observed that Arg supply did not restore NO production in the *nia1nia2* mutant (Fig. S6). This result confirms the previous observations of Oliveira et al. (2009) showing that no significant increase of NO emission was measured in response to inoculation with pathogen in *nia1nia2* leaves treated with 10 mM L-arginine. These results seem to exclude the possibility that the lower level of NO observed in *nia1nia2* in response to OGs is related to an Arg deficiency in the leave tissues. Induction of NR activity and/or up-regulation of NR genes have also been demonstrated in potato infected by *Phytophthora infestans* or in *A. thaliana* infected by *Sclerotinia sclerotorium* (Yamamoto et al. 2003; PerchePied et al. 2010). Interestingly, coincident NO production and NR activity induction have been also reported in response to other stimuli like cold stress (Zhao et al. 2009; Cantrel et al. 2010), H_2O_2 treatment (Wang et al. 2010) or the *Verticillium dalhiae* toxin (VD toxin; Shi & Li, 2008). Thus, our study strengthen the possibility that NR-mediated NO production participates in the mechanisms underlying the plant adaptive response to biotic and abiotic stresses through transcriptional regulation, *de novo* NR biosynthesis and enzyme activation.

Taken together our results suggest that a L-arginine-dependent activity as well as a NR activity could mediate NO production in response to OGs. If both processes are unique sources for NO and act independently, the application of L-NAME in the NR double mutant *nia1nia2* is expected to lead to an additive effect, that is a complete inhibition of OGs-induced NO production. In contrast, we observed that L-NAME did not further affect the remaining

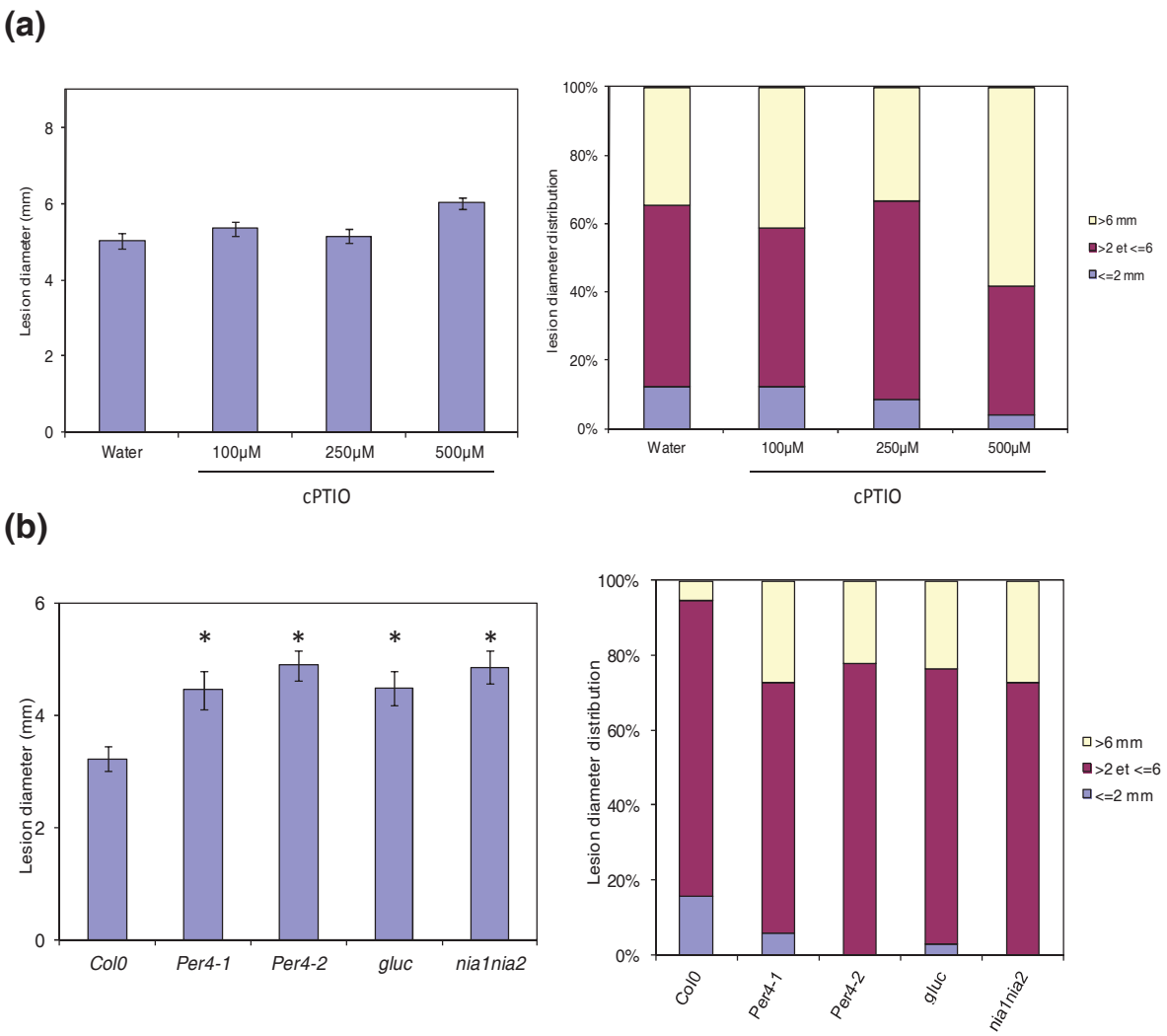

Figure 4.7: Role of NO in basal resistance to *Botrytis cinerea.* **(a)** Exogenous application of cPTIO decreases resistance against *B. cinerea* in *A. thaliana.* Col-0 leaves were infiltrated with different concentration of cPTIO or water and then inoculated with *B. cinerea* spores (6 µL droplets; 5.10^4 spores.mL^{-1}). Lesion diameters were measured 3 days after inoculation (left panel) and were grouped into three classes according to their sizes and the percentage of lesion distribution is shown (right panel). Data are the means (± SE) of three independent experiments performed on 10 plants for each genotype with 4 lesions per plant. Asterisks indicate statistically significant differences between WT and cPTIO treatment (see material and methods). **(b)** Basal resistance to *B. cinerea* in nitrate reductase (*nia1nia2*), peroxidase 4 (*per4-1* and *per4-2*) and β-1,3-glucanase (*glu*) mutant lines. Four week-old plants were inoculated on leaves with a 6 µL droplet (5.10^4 spores.mL^{-1}) and symptoms were scored 3 days later (left panel). Lesion diameters were grouped into three classes according to their sizes and the percentage of lesion distribution is shown (right panel). This experiment was performed on 10 plants for each genotype (four inoculations per plant) and was representative of 3 independent biological experiments. Data are the mean ± SE. Asterisks indicate statistically significant differences between WT and mutant lines (see material and methods).

OGs-induced NO production in the *nia1nia2* mutant. Moreover, we observed that L-NAME inhibits OGs-induced NR activity (Fig. 4.3b) but not clearly NR genes expression (Fig. S7). Firstly, these results suggest that the two enzymatic pathways do not work independently to produce NO in response to OGs: L-NAME-sensitive NO production could affect NR-dependent NO production. Supporting this assumption, two studies reported that NO donors and the NO scavenger cPTIO modulate NR activity *in Brassica chinensis* and *Solanum lycocarpum*, thus leading to the hypothesis that NR activity may be self-regulated rapidly by its product NO or by NO produced *via* other sources (Du et al. 2008; Jin et al. 2009). The effect of NO was shown to be dependent of N concentration (Jin et al. 2009). Based on these data, the authors hypothesized that NO can stimulate NR activity at the post-translational level though a direct interaction or, alternatively, by affecting the activity of proteins involved in NR regulation including protein kinases or phosphatases. However it cannot be excluded that NO produced by Arg-dependent pathway could be oxidized to nitrite, thus providing substrate for NR-triggered NO synthesis. Secondly, the remaining NO production might be mainly related to an alternative route to NR- and Arg-dependent pathways. This route could be involved in OGs-induced accumulation of PLP2 transcripts which appeared cPTIO sensitive but not affected by L-NAME or in the *nia1nia2* mutant. Numerous possible enzymatic sources for NO have been proposed (Neill et al. 2003, Planchet et al. 2005, Tun et al. 2008). Recently, PA-induced and ABA-induced NO production investigated by fluorometry and fluorescence microscopy showed that the plant mutant (*cuao1-1* and *cuao1-2*) impaired in copper aminooxidase1 (CuAO1) show low rate of NO production, suggesting a function of this protein in polyamine-mediated NO production (Wimalasekera et al. 2011). We tested the involvement of a polyamine oxidase (PAOX) pathway using AtPaox mutants and guazatine, an inhibitor of aminooxidase. Compared to Col-0, non-significant modification of OGs-induced NO production was observed (data not shown). Therefore at this step of knowledge, we are not able to assert the precise role of each enzymatic pathway. The identification of the enzymatic source producing NO from Arg (or L-NAME sensitive) and a detailed analysis of the molecular mechanism underlying L-NAME effect on nitrogen metabolism will be required to answer this question.

OGs-induced NO production depends on Ca^{2+} influx

Moscatiello *et al*. (2006) previously reported that OGs effects in *A. thaliana* cell suspensions are mediated through a La^{3+}-sensitive transient elevations of cytosolic Ca^{2+}. Interestingly, we measured a lower OGs-induced NO production in the *dnd1* mutant impaired

in the expression of the Ca^{2+}-permeable channel *CNGC2*. The reduced NO production observed in *dnd1* suggests a role for CNGC2 and Ca^{2+} in the transduction of OGs signal leading to NO production. The inhibition of OGs-induced NO production by La^{3+}, a calcium channel blocker, corroborates this conclusion. Interestingly, La^{3+} did not block *in vivo* OGs-induced NR activity, allowing us to suggest that calcium influx controls only Arg-dependent NO production (Fig. S6). In support of our findings, CNGC2 and associated Ca^{2+} influx were shown to act upstream of LPS-induced Arg-dependent NO production in *A. thaliana* guard cells (Ali et al. 2007). Similarly, pharmacological-based strategies pointed out the importance of Ca^{2+} influx in elicitor-triggered NO production (Courtois et al. 2008). However, the effect of Lanthanum on NR activity should be interpreted cautiously: whereas calcium influx is important for NO production, it is also known to contribute to NR inhibition trough CDPK phosphorylation and binding to 14-3-3 proteins (Buchanan et al. 2000 for review). Therefore, the observation that lanthanum did not affect NR activity might also reflect inhibitory role of Ca^{2+} on NR activity.

NO production modulates AtRBOHD-mediated oxidative burst

One of the earliest characterized events of defense mechanisms is the oxidative burst corresponding to the generation of ROS including H_2O_2 and $O_2^{\cdot -}$ (Lamb and Dixon, 1997; Wojtaszek *et al.,* 1997). There is considerable evidence that the elicitor- and pathogen-induced oxidative bursts in *Arabidopsis* are mediated by AtRBOH proteins, homologues of the gp91 sub-unit of mammalian NADPH oxidase (Torres *et al.,* 2002). Our results show that the OGs-induced oxidative burst in *A. thaliana* leaf discs was abolished using DPI and in the *Atrboh*D mutant (Figure 5a and c), confirming that plasma membrane NADPH oxidase was responsible for the OGs-induced oxidative burst as previously observed by Galetti *et al.* (2008).

We observed that DAF fluorescence was not significantly affected in *AtrbohD* mutant indicating that H_2O_2 was not sufficient for OGs-induced NO generation. This finding diverges from other studies assuming that ROS production acts upstream of NO production. Indeed, H_2O_2 was shown to be required for ABA- and UVB-induced NO production in guard cells of both *Vicia faba* and *A. thaliana* (Dong et al. 2005; He et al. 2005; Bright et al. 2006) and for chitosan-response in *Pisum sativum* (Srivastava et al. 2009). In contrast, we observed that the application of the cPTIO or L-NAME diminished the oxidative burst in response to OGs (Fig. 4.5b), suggesting a positive role for NO in regulating ROS production. These findings indicate that the induction of H_2O_2 accumulation may be a downstream component of OGs-

induced NO production. These results are in agreement with previous observations made in the context of the interaction between *B. cinerea* and *Vitis vinifera* which showed that NO, whether produced in response to BcPG1 or released by a NO donor, leads to ROS production in grapevine cells (Vandelle et al. 2006). In contrast, other studies reported that reducing endogenous NO level using cPTIO or plant mutants impaired in inducible NO production (*e.g*. NR double mutant) enhanced H_2O_2 accumulation (Tada et al. 2004; Asai et al. 2008), suggesting that part of the $O_2^{\cdot -}$ produced by NADPH oxidase is scavenged by NO. Finally, in contrast to cPTIO or L-NAME treatments, we observed that *nia1nia2* mutant behaves like wild type plants in terms of OGs-induced ROS generation, indicating that NR-mediated NO production is not essential for oxidative burst. These data reinforce the possibility that NO production might involve at least two enzymatic sources, NO resulting from the L-arginine-dependent pathway (50% of inhibition using L-NAME, Fig. 4.2) being involved in the control of the oxidative burst. This corroborates the assumption that the sources and the site of NO production could be of importance in its biological activity. Taken together, these findings illustrate the complex interplay between ROS and NO. We can speculate that NO (and relative RNS) induced by OGs modulates H_2O_2 level and cellular redox state through transcriptional regulation or post-translational modifications. For instance, it was observed that NO could S-nitrosylate redox-related proteins in plant and animal cells (Astier et al., 2011; Lindermayr et al. 2006; Sun et al. 2006; Tanou et al. 2009).

OGs-induced NO dependent genes are involved in *A. thaliana* basal resistance to *B. cinerea*

We analyzed, using Real Time qPCR, transcript accumulation of candidates genes in response to OGs in the presence of cPTIO and L-NAME. The reduced transcript level of *PLP2*, *PER*4 and *β–1,3–glucanase* observed in leaf discs co-treated with cPTIO or L-NAME and OGs indicates that their expression is, at least partly, dependent on NO. As *PER4* and *β–1,3–glucanase* genes were also induced by *B. cinerea*, we investigated the role of the corresponding proteins in the resistance to the pathogen using mutant lines. *PLP2* was previously identified as a modulator of *A. thaliana* resistance to *B. cinerea* (La Camera *et al.* 2005); we therefore focused our work on *PER4* and *β–1,3–glucanase.* The different mutant lines impaired in *PER4* and *β–1,3–glucanase* were more susceptible to *B. cinerea,* indicating that *PER4* and *β–1,3–glucanase* expression is required for *A. thaliana* resistance against *B. cinerea*. Because NO regulates the expression of these gene in response to OGs, these data

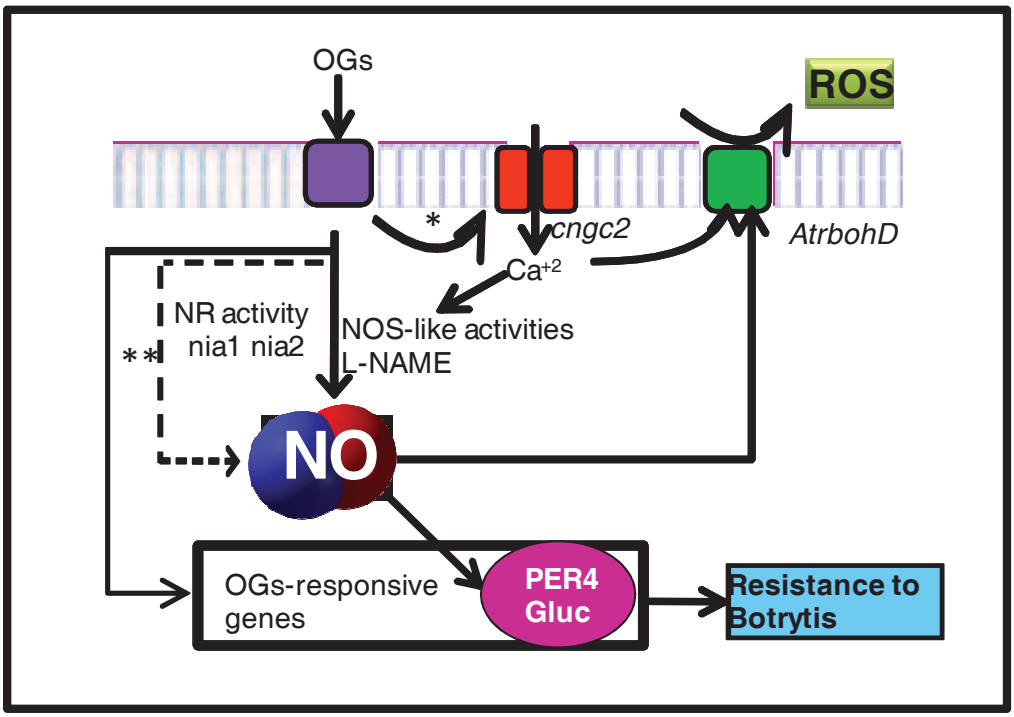

Figure 4.8: Schematic illustration of the signalling network involving NO that modulates OGs-triggered immunity against *Botrytis cinerea* in *Arabidopsis thaliana*. NO production involves both a L-arginine- and a nitrate reductase (NR)-pathways. OGs-induced Arg-dependent NO production was Ca^{2+}-dependent and modulated RBOHD-mediated ROS production. NO produced by Arg- and NR-pathways participates in the regulation of OGs-responsive genes such as anionic peroxidase (PER4) and a β–1,3–glucanase. Mutant plants impaired in PER4 and β–1,3–glucanase, as well as Col-0 plants treated with the NO scavenger cPTIO, were more susceptible to *B. cinerea*. (*): OGs elicit a rapid elevation of cytosolic free calcium in *A. thaliana* cells (Moscatiello et al. 2006). (**): not characterized alternative route for NO production.

also suggest that NO is involved in the processes controlling resistance through gene regulation. Accordingly, a higher susceptibility to *B. cinerea* was observed for Col-0 plants treated with cPTIO and in the *nia1nia2* mutant. Importantly, impairment of *PER4* and *β–1,3–glucanase* expression, as well as the suppression of NO synthesis by cPTIO, had a weak effect on plant resistance to *B. cinerea*. Indeed, disease susceptibility was only increased by 20% in those genotypes or in cPTIO-cotreated Col0 plants. However, we should mention that plant resistance to necrotrophic pathogens such as *B. cinerea* has a quantitative and complex genetic basis involving probably numerous genes. Indeed, 23 QTL with significant influence on *B. cinerea* lesion size or camalexin accumulation have been identified in *A. thaliana* (Rowe & Kliebenstein, 2008).

Our observations corroborate previous studies suggesting a role for NO in disease resistance to necrotrophic pathogen (Asai & Yoshioka, 2009; Perchepied et al. 2010). Notably, our investigation demonstrates that the effect of endogenously produced NO could be explained by the regulation of genes activated during fungal colonisation or elicitor treatment. These genes encode proteins related to plant defense. The anionic peroxidase PER4 is described as a lignin-forming peroxidase putatively located in the endomembrane system. This protein is related to the PR9 family (Van Loon et al. 2006) and closely similar to prxA3a involved in both lignin content and composition in hybrid aspen (Li et al. 2003). PR-9 is a specific type of peroxidase that could act in cell wall reinforcement and then enhance resistance to pathogens. β−1,3−glucanase is also referred as a PR protein of the PR2 family (Doxey *et al.* 2007). This protein, putatively localized in plant cell wall, could affect mycelium growth by hydrolysing the protective glucan sheath on the surface of the fungus such as *B. cinerea* (Gil-ad et al. 2001).

In conclusion, we proposed a role for NO in mediating OGs-induced effects in *A. thaliana*, including ROS production and the expression of genes encoding *PER4* and a β−1,3−glucanase (Fig. 4.8). The control of the expression of those genes by NO might constitute one of the mechanisms underlying *A. thaliana* basal resistance to *B. cinerea*.

Acknowledgements

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CHAPTER 4

"Supporting information"

"Nitric oxide production mediates oligogalacturonides-triggered immunity and resistance to Botrytis cinerea in Arabidopsis thaliana"

Supporting information

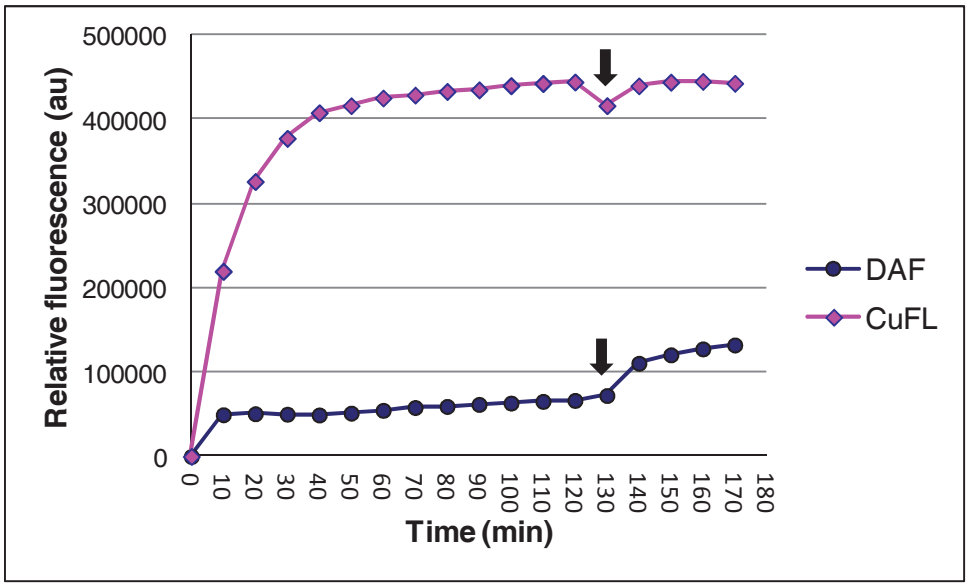


Fig. S1: Comparative analysis of NO detection using DAF-2 and CuFL fluorescent probes. The DAF-2 probe was added at a final concentration of 5 µM in 200 µL Tris-HCl (50 mM, pH 7.5). The CuFL probe (Strem Chemicals, Bischheim, France) was freshly prepared by adding 1:1 FL solution (1.0 mM) to the copper (II) solution (1.0 mM) and added at a final concentration of 5 µM in 200 µL Tris-HCl (10 mM, pH 7.5) KCl (10 mM). The increase of fluorescence triggered by GSNO (200 µL) was measured using a spectrofluorometer (Mithras, Berthold Technologies, Germany) with a 485 nm excitation and a 535 nm emission filters. A second addition of GSNO (200 µL) was performed after 120 min (arrows). Fluorescence was expressed as relative fluorescence units (arbitrary units: au) after subtracting background fluorescence of time zero (t0) and of the corresponding control (probe without GSNO). This experiment is representative of 3 independent experiments.

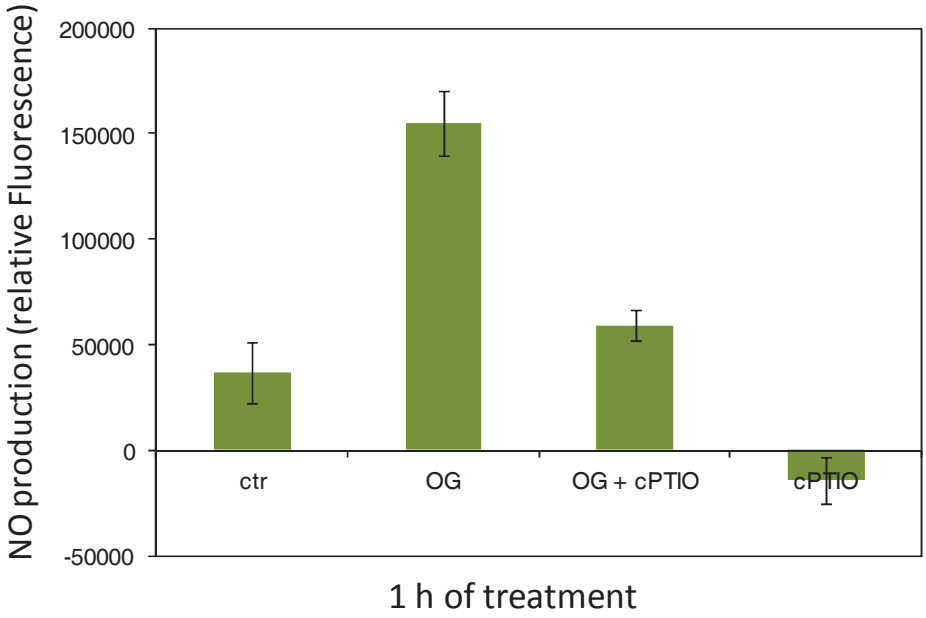


Fig. S2: NO detection in *A. thaliana* leaves discs by CuFL probe. NO production was monitored after 1 hour of treatment in the absence (control) or presence of OGs (2.5 mg.mL^{-1}), with or without NO scavenger (cPTIO 500 µM). Each value represents the mean of 8 measurements ± SD. Leaf discs excised from plants were infiltrated under vacuum for 3 min with an aqueous solution of OGs. For control treatment, OGs were replaced by an equivalent volume of water. After infiltration, discs were incubated for one hour with respective solutions and then each disc was put in separate well of 96 well plates (Microtest flatbottom, Becton Dickinson, Europe) in 200 µL Tris-HCl (10 mM, pH 7.5) KCl (10 mM). The CuFL probe (Strem Chemicals, Bischheim, France) was freshly prepared by adding 1:1 FL solution (1.0 mM) to the copper (II) solution (1.0 mM) and added in each well (final concentration of 5 µM). The increase of fluorescence triggered by OGs was measured using a spectrofluorometer (Mithras, Berthold Technologies, Germany) with a 485 nm excitation and a 535 nm emission filters. Fluorescence was expressed as relative fluorescence units (arbitrary units: au) after subtracting background fluorescence of time zero (t0). This experiment is representative of 3 independent experiments using independent biological material.

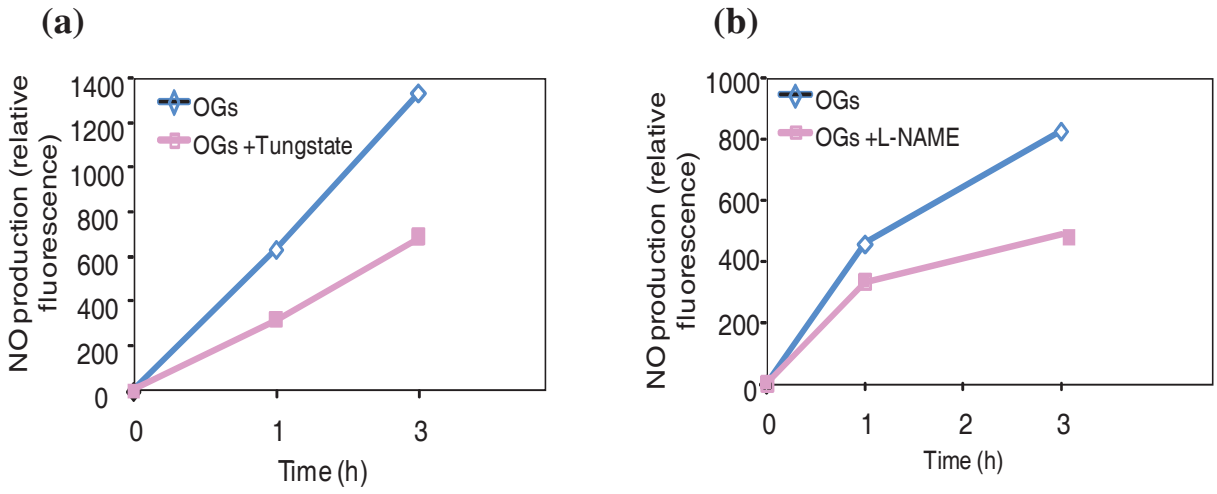


Fig. S3: Effect of Tungstate (a) or L-NAME (b) on OGs-induced NO production in Col-0 leaf discs. NO production was monitored as described in Figure 1 and visualized in the first 3 hours of treatment after subtracting fluorescence of corresponding control. Each value represents the mean of 8 measurements. This experiment is representative of at least 3 independent experiments using independent biological material.

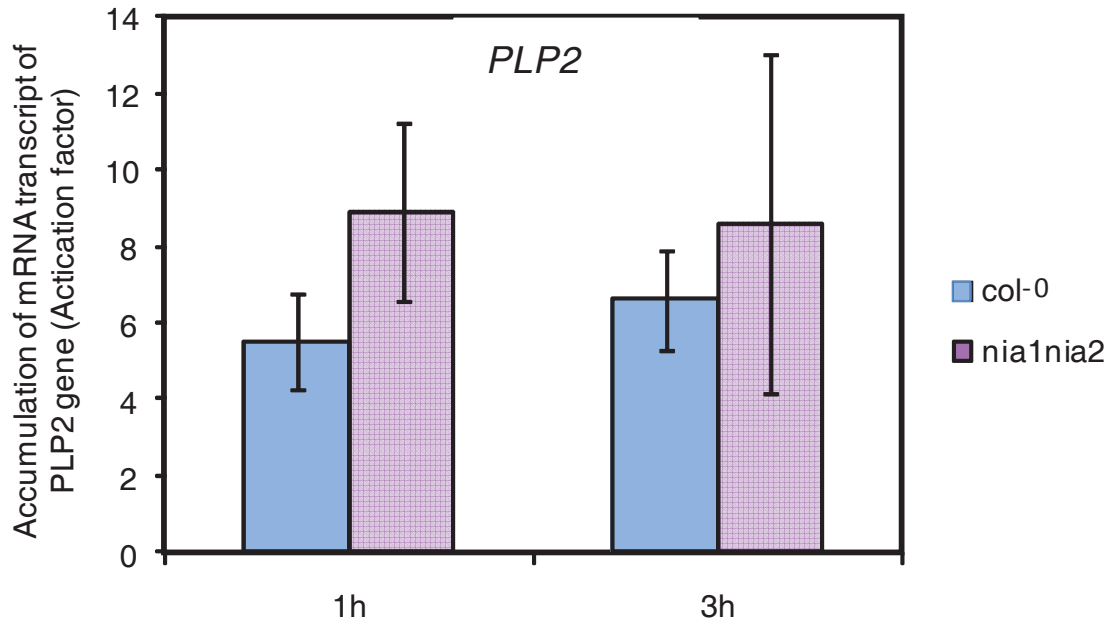


Fig. S4: *PLP2* transcript accumulation in response to OGs in the *nia1nia2* double mutant. Col-0 and *nia1nia2* leaf discs were infiltrated with water or OGs (2.5 mg.mL^{-1}) and harvested for analysis after different time intervals. Bars indicate average expression (± SD) of three biological replicates.

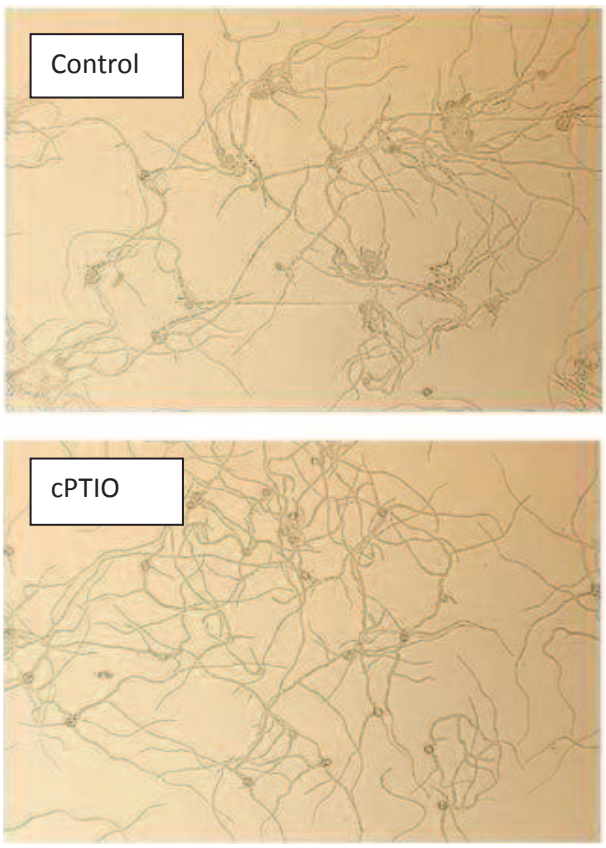


Fig. S5: Effect of cPTIO on *Botrytis cinerea* growth. *Botrytis cinerea* strain BMM (Zimmerli *et al.,* 2000) was grown on Petri plates containing PDA (potato dextrose agar, DIFCO) for 10-12 days. Spores were harvested in water and were quantified under a microscope. To verify the effect of cPTIO on *B. cinerea* growth, spores were diluted to obtain the final concentration of $5x10^4$ spores mL^{-1}. 12 µL of spore suspension ($5x10^4$ spores mL^{-1}) with cPTIO or water in PDB medium were placed on glass slides and allow to grown under high humidity for different time. Fungal growth was observed under microscope.

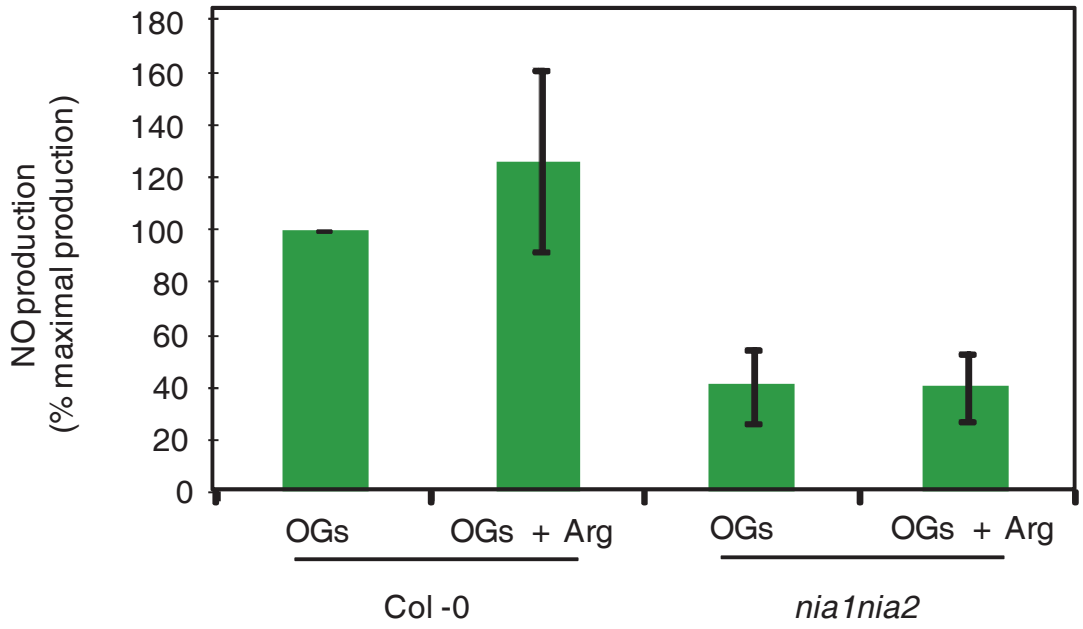


Fig. S6: Effect of Arginine on OGs-induced NO production in Col-0 and *nia1nia2* leaf discs. Arabidopsis leaf discs loaded with DAF-2DA (20 µM) were treated by OGs (2.5 mg.mL^{-1}) with or without Arginine (10 mM). NO accumulation was determined after 12 h of treatment and expressed as a percentage of the maximal response after subtracting background fluorescence of corresponding control. Each value is a mean ± SD of 3 independent experiments.

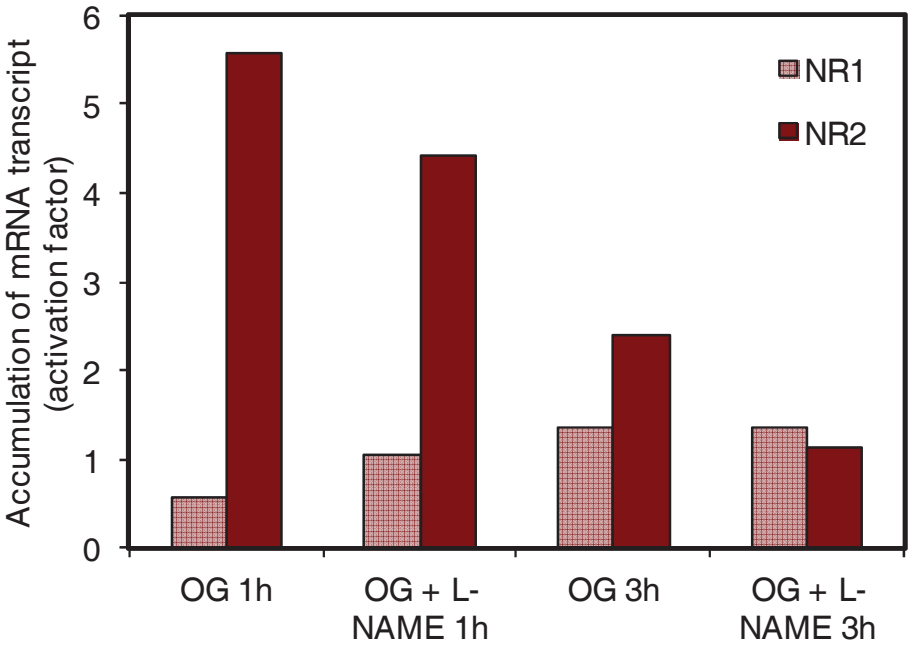


Fig. S7: Effect of cPTIO treatment on transcript accumulation of NR-genes. Col-0 leaf discs were infiltrated with water or OGs (2.5 mg.mL^{-1}) in the presence or absence of L-NAME (5 mM) and harvested for analysis after different time intervals. Transcribed mRNAs were analysed by real-time quantitative RT-PCR. Levels of transcripts were calculated using $2^{-\Delta\Delta CT}$ method. This experiment is representative of 2 independent experiments using independent biological material.

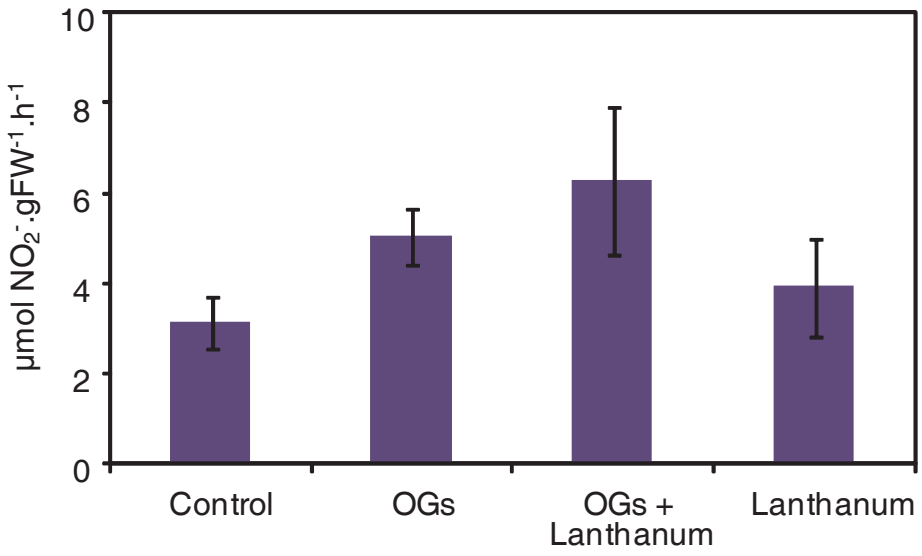


Fig. S8: *In vitro* NR activity in wild type (Col-0) leaf discs treated with water (control), OGs and OGs + Lanthanum (5 mM). Each value represents the mean ± SE of three independent experiments performed using independent biological materials.

CHAPTER 4

"Part 2"

"Relationship between MAPK and CDPK activities in response to oligogalacturonides"

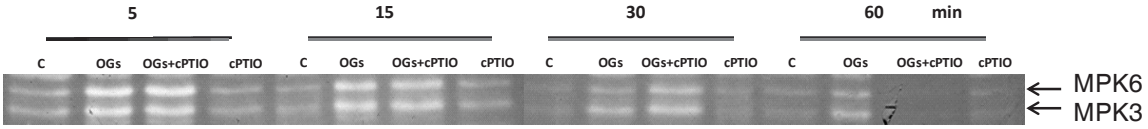


Figure 1: Effect of cPTIO on OGs-induced phosphorylation of MAPK in Col0. Leaf discs were taken after different time intervals. Phosphorylated MAPK were immunodetected from total protein extract using specific antibodies (Cell Signaling Technomogy, Inc.). Leaf discs were infiltrated in the absence (control) or presence of OGs (2.5 mg/mL) with or without cPTIO.

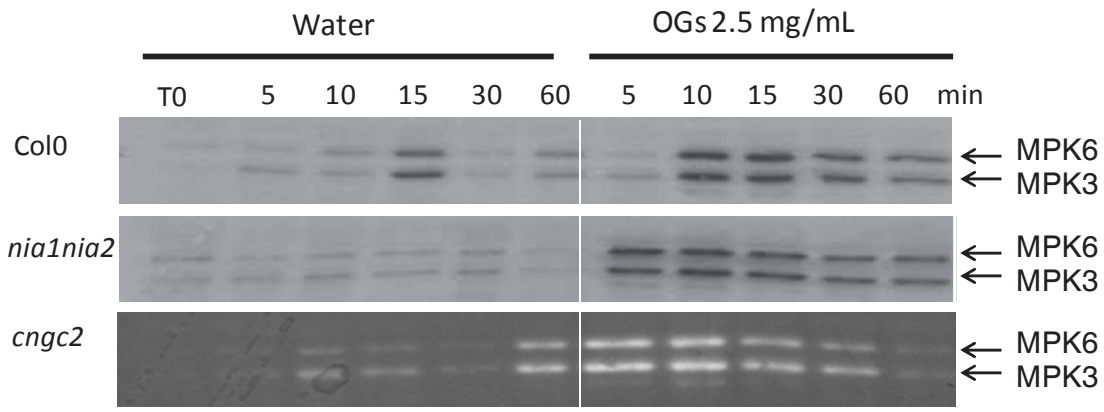


Figure 2: Phosphorylation of MAPK in Col-0 and mutants. Leaf discs were taken after different time intervals. Phosphorylated MAPK were immunodetected from total protein extract using specific antibodies (Cell Signaling Technomogy, Inc.). Leaf discs were infiltrated in the absence (control) or presence of OGs (2.5 mg/mL).

CHAPTER 4

Relationships between MAPKs/CDPKs activities and OGs-induced NO production

As already reported in the first part of this work (see Chapter 1), protein kinases play a central role in signaling during activation of plant defence mechanisms. Members of different kinase subfamilies, such as MAP kinases and calcium-dependent protein kinases are involved. Moreover, it was demonstrated that NO production is closely related to MAPKs and CDPKs by activating or mediating kinase activities (Astier et al., 2011, see Chapter 2) and that the NO-dependent gene PER4, identified in this work (see Chapter 4), was also modulated by CDPKs (Boudsocq et al., 2010).

In this context, the main objective of these experiments was to understand the link between MAPKs, CDPKs and OGs-induced NO production. For this purpose, in the first step, we demonstrated, using immunoblotting techniques, that OGs triggered MAPK phosphorylation. The use of the NO scavenger and mutants affected in NO production showed that MAPKs phosphorylation is independent of NO production. Reciprocally, NO production was not inhibited in T-DNA mutant lines impaired in MPK3 and MPK6, two MAPK isoforms involved in defense response. In the second step, we showed that, CDPKs partly control the NO production induced by OGs and that CDPKs seemed to be activated by OGs. Moreover, we demonstrated the implication of these CDPKs in the resistance against *B. cinerea* and in the regulation of several NO-responsive genes.

RESULTS

OGs triggered MAPKs phosphorylation, independently of NO production

MAPKs are crucial components of the signaling pathways underlying plant immune responses. In tobacco, the two MAPKs, SIPK and WIPK, are commonly mobilized in response to pathogens and elicitors (Zhang & Klessig, 1998). To analyse the potential relationships between NO and MAPKs, we tested the cross-reactivity of proteins extracted from OGs-treated *A.*

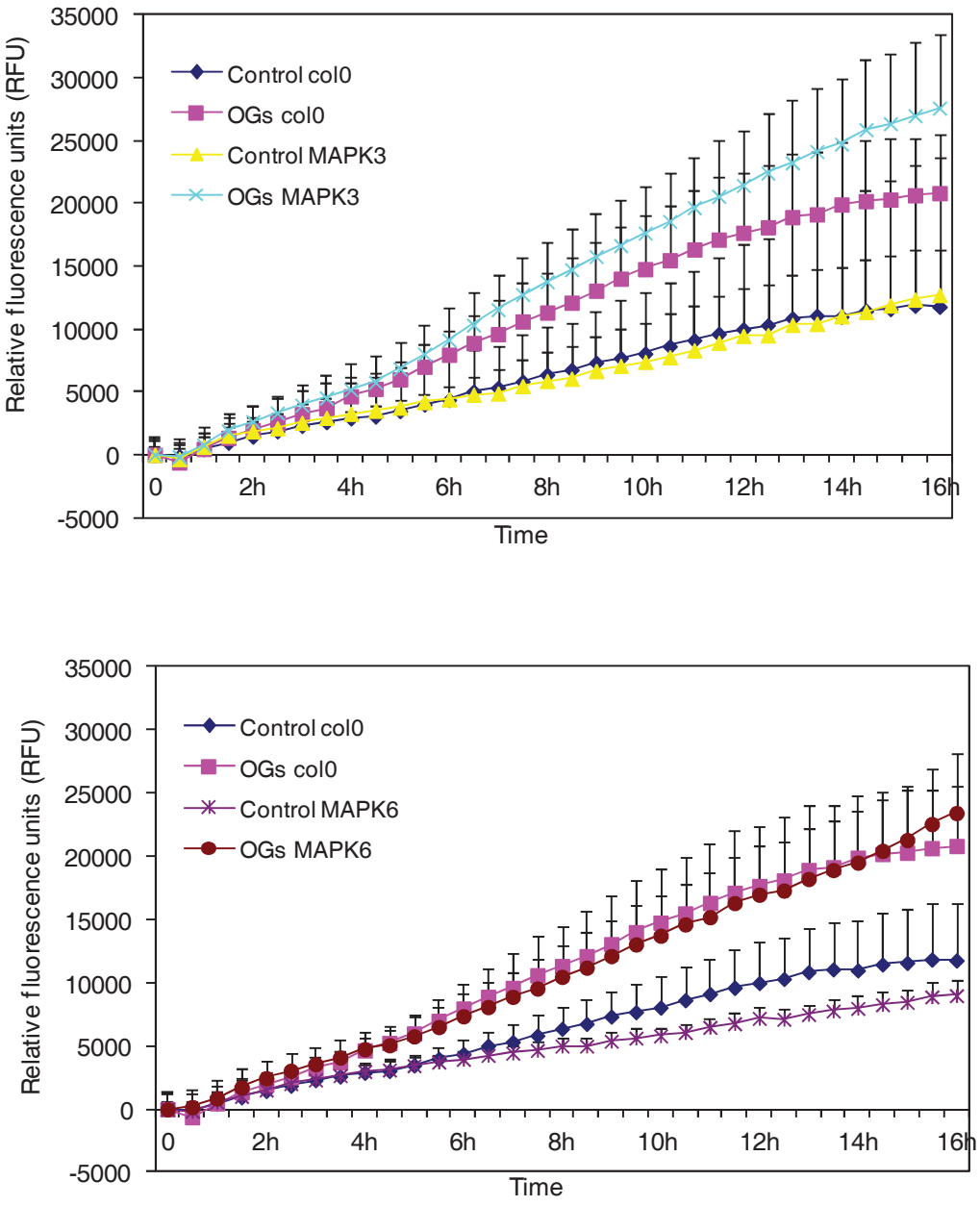


Figure 3: Time course of NO production in *mpk3 and mpk6* mutants. *Arabidopsis* leaf discs from wild-type (Col-0), *mpk3 and mpk6* plants were treated by OGs (2.5 mg/mL) or water with DAF-2DA (20 μM). This graph is the representative graph of 3 independent experiments using independent biological material (8 replicates per experiment). NO accumulation was measured up to 16h.

thaliana leaf discs with polyclonal antibodies raised against a phosphorylated form of human ERK1 (Extracellular signal-Regulated Kinase 1) and ERK2. These antibodies were previously shown to specifically react with the activated form of plant ERK-related MAPKs (Zhang and Liu, 2001). Westernblots revealed two bands rapidly and transiently detected in protein extracted from OGs-treated leaf discs. These bands had a relative molecular mass of 45 KDa and 47 KDa. Their apparent sizes suggest that they might correspond to MPK3 and MPK6, two MAPKs implicated in defense signaling in *A. thaliana* (Pitzschke and Hirt, 2009). In order to examine whether these MAPKs could be regulated by NO, similar experiments were performed in presence of cPTIO. We observed that MAPKs phosphorylation induced by OGs was not affected by cPTIO co-treatment (Figure 1). Activation of these MAPK3 and MAPK6 was analysed in mutants showing low level of NO in response to OGs (*nia1nia2*, *cngc2*, see Chapter 4) (Figure 2). MAPK phosphorylation appeared earlier but there is no significant difference in phosphorylation of these MAPKs. In contrast, in *cngc2* intensities of the bands were more pronounced as compared to control (Col-0).

To further investigate the putative interplay between NO and MAPKs, we monitored NO production in *mpk3* and *mpk6* mutants impaired in the expression of MPK3 and MPK6. In response to OGs, NO production measured in *mpk3* and *mpk6* genetic background was not significantly different from the one detected in Col-0 (Figure 3), suggesting that these two MAPKs are not involved in OGs-induced NO production. So, we can assume that MAPK pathway is either upstream or acts independently of the NO and both NO and MAPKs being partially under the control of Ca^{2+} influx.

CDPKs are regulated by OGs, independently of NO production

In *Arabidopsis thaliana*, CDPKs have been identified as regulatory proteins, involved in plant immunity. CDPKs are positive regulators in flg22 signaling (Boudsocq et al., 2010). The use of the triple mutant *cpk5.6.11* impaired in three CDPK isoforms (5, 6 and 11) revealed that CDPKs are necessary for ROS production and involved in *A. thaliana* resistance to *Pseudomonas*.

To reveal a possible activation of CDPKs by OGs, “In gel kinase” assay was performed. The results showed the activity of different kinases. One band was detected in OGs-treated leaf tissues and controls at a size of approximatively 55 kDa that is compatible with CDPKs (Figure

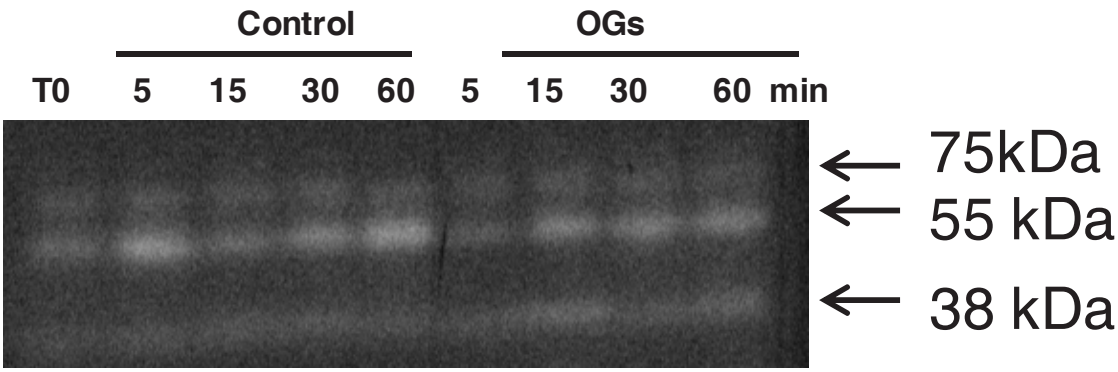


Figure 4: "In gel kinase" assay. Leaf tissues were infiltrated in the absence (control) or presence of OGs (2.5 mg/mL). Activation of CDPKs was detected by in-gel kinase assay with HISIII as a substrate in the presence of calcium.

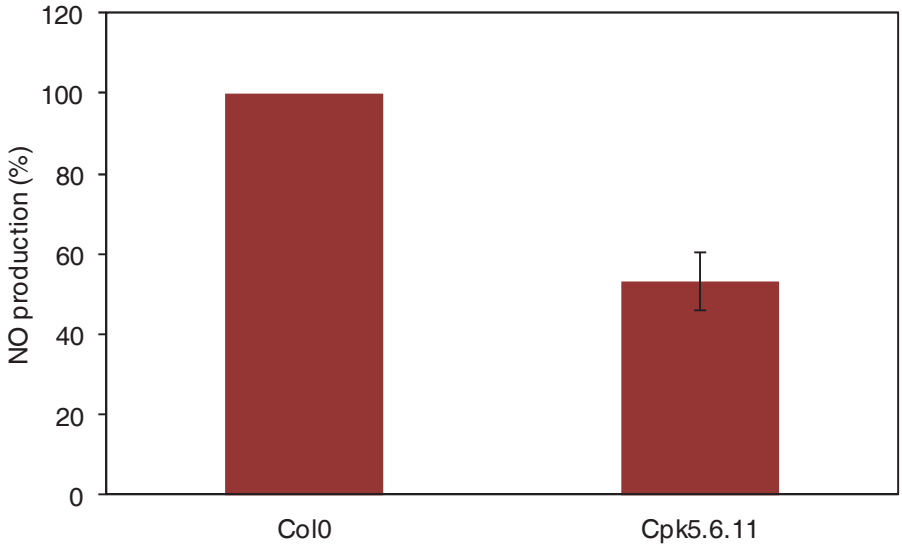


Figure 5: Effect of Ca^{2+} dependent protein kinases on OGs-induced NO production. OGs-induced NO production was measured in Col-0 and *cpk5.6.11* leaf discs with DAF-2DA (20 µM). Leaf discs were treated by OGs (2.5 $mg.mL^{-1}$) or water (control). Histogram represents the NO production measured after 12 h of treatment and expressed as a percentage of the maximal response after subtracting background fluorescence of corresponding control. Each value is a mean ± SD of 5 independent experiments.

4). This 55 kDa appeared to be more intense in response to OGs at 15min. The two other bands detected around 75kDa and 38 kDa could correspond to CCaMK and CK2 respectively.

Role of CDPKs in OGs-induced NO production

We investigated the putative contribution of CDPKs as regulators for NO production during OGs treatment using the triple mutant *cpk5.6.11*. OGs-induced increase of NO was partially reduced by about 50% in the mutant line as compared to Col-0 leaf discs (Figure 5). This suggests that NO production is partially controlled by CDPKs, in particular the isoforms 5, 6 and 11.

Role of CDPKs in *A. thaliana*/*B. cinerea* interaction

To determine the role of CDPKs in *B. cinerea/Arabidopsis* interaction, triple mutant *cpk5.6.11* was inoculated with *B. cinerea* (5.10^4spores.mL^{-1}). Symptoms were assayed 72h post infection. *cpk5.6.11* mutants had greater necrotic lesions and the average diameter of lesion is significantly larger than that observed in Col-0 (Figure 6). So, from these results we can conclude that triple mutant *cpk5.6.11* is more susceptible to *B. cinerea* and that CPK5,6 and 11 are involved the resistance to this pathogen.

Role of CDPKs in the target genes of NO

To explore the role of CDPKs in the regulation of NO target genes, the expression of NO-responsive genes (TIR, RLP7, PROPEP2, bHLH, CRF3, WRKY41, and WRKY75) was studied by RT-qPCR in the *cpk5.6.11* triple mutant in response to OGs. These genes were identified with the transcriptomic analysis described in Chapter 5.

Patterns of gene expression in Col-0 are relatively similar to those obtained by microarray. As compared to Col-0, the level of accumulation of all gene transcripts is lower except CRF3 and bHLH (Figure 7) indicating that NO responsive genes expression was modulated by CPK5,6 and 11 activities.

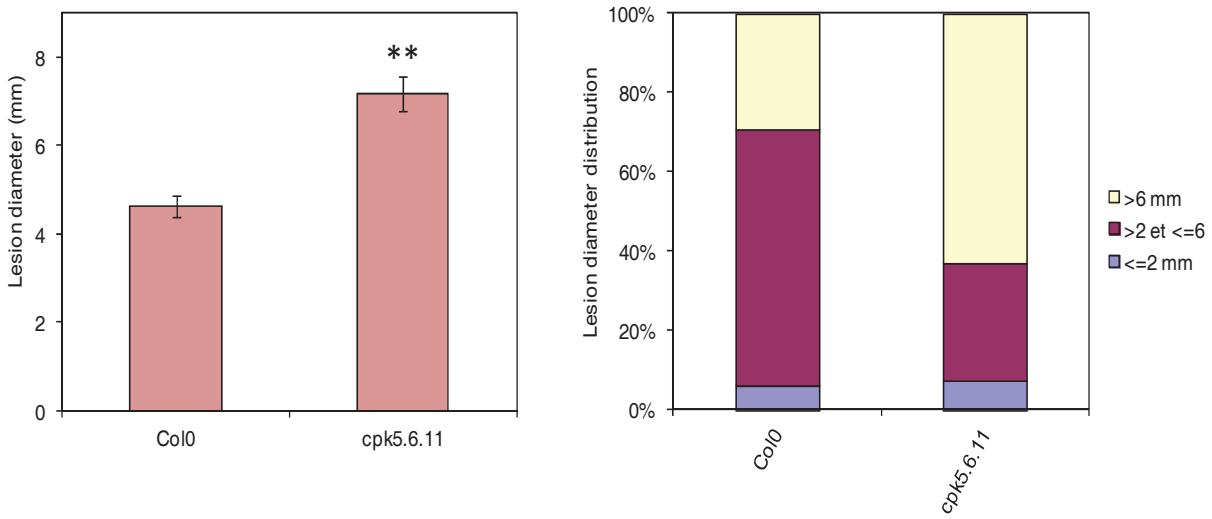


Figure 6: Basal resistance to *B. cinerea* in *cpk5.6.11* mutant lines. Four week-old plants were inoculated with a 6 µL droplet (5.10^4 spores.mL^{-1}) and symptoms were scored 3 days later Average lesion diameters were estimated (left panel). Lesion diameters were grouped into three classes according to their sizes and the percentage of lesion distribution was shown (right panel). The experiment was performed on 10 plants for each genotype (five inoculations per plant) and was representative of 3 independent biological experiments. Data are the mean ± SE. Asterisks indicate statistically significant differences between Col-0 and *cpk5.6.11* lines.

DISCUSSION

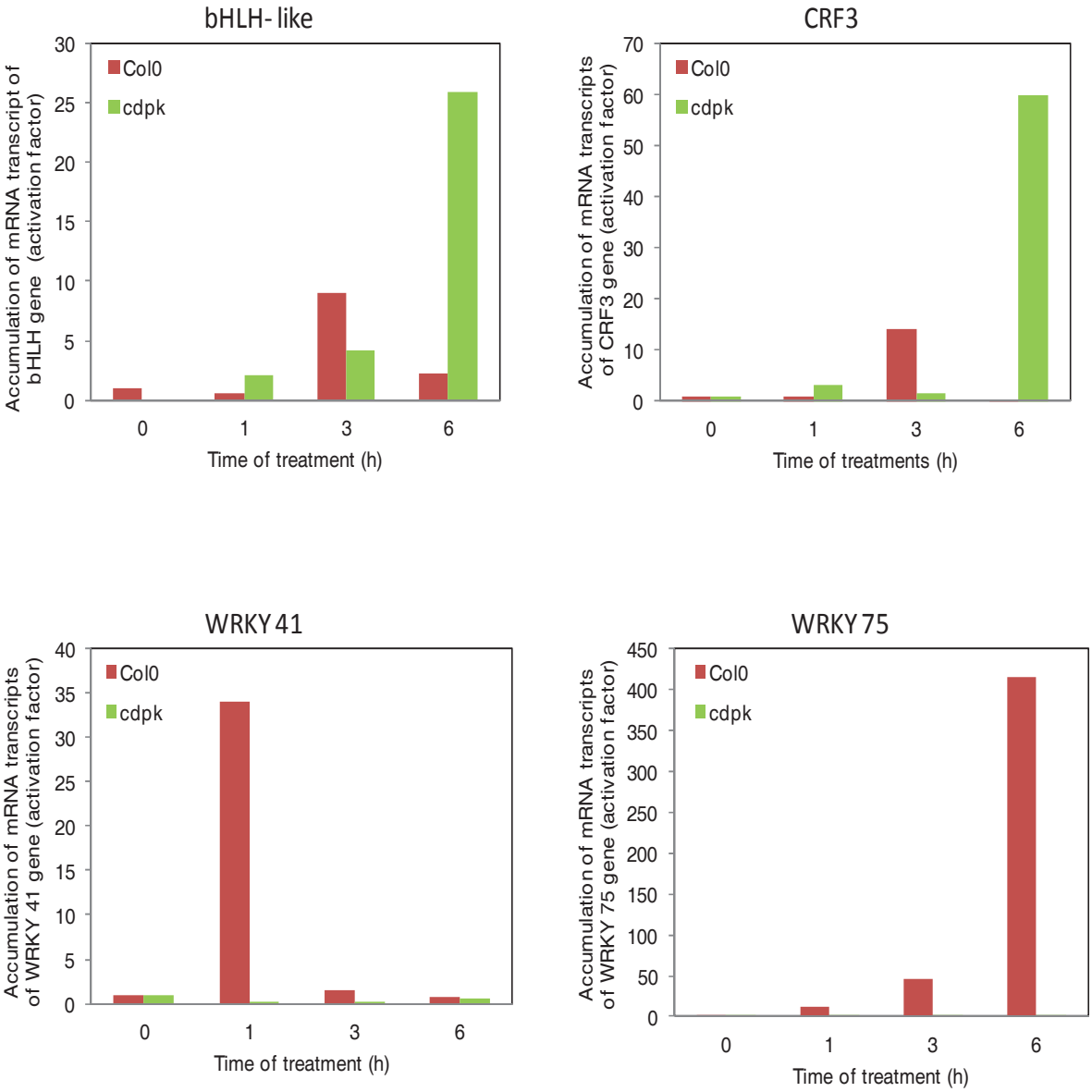
MAPKs activation is independent of NO production

OGs treatment (2.5 $mg.mL^{-1}$) allowed us to identified two MAPKs, MPK3 and MPK6. These two isoforms were also described recently by Galletti et al., (2011) in response to 100 µg mL^{-1} OGs and were commonly activated in defense against pathogens (Asai et al., 2002; Ichimura et al., 2006; Qiu et al., 2008). MPK3 and MPK6 play a positive role on the activation of Arabidopsis defense responses. Both regulate camalexin accumulation (Ren et al., 2008) and ET production during fungal infection, and to be required for chemically induced priming of stress responses (Beckers et al., 2009). Interestingly, Galletti et al., (2011) have investigated the role played by these MAPKs in elicitor-induced resistance against the fungal pathogen *Botrytis cinerea*. They showed that, using the single *mpk3* mutant, the lack of MPK3 increases basal susceptibility to the fungus, but did not significantly affect elicitor-induced resistance. In contract, lack of MPK6 had no effect on basal resistance but suppresses OGs- and flg22-induced resistance to *B. cinerea.*

Several studies point out that MAPKs signaling pathway and NO production are highly connected (Pitzschke and Hirt, 2009). It is reported that MAPKs activation can regulate INF1- or *B. cinerea*-induced NO production in *Nicotiana benthamiana* leaves (Asai et al., 2008; Asai and Yoshioka, 2009) but can be also induced by NO during adventitious root development (Pagnussat et al., 2004). Furthermore, NO released by NO donors was shown to induce the activity of MAPK in tobacco leaves and cell suspensions (Besson-Bard et al., 2008; Kumar and Klessig, 2000). In the same way, Zhang et al., (2007) found that ABA-induced H_2O_2 production mediates NO generation then activates a MAPK and results in the up-regulation of the expression and activities of antioxidant enzymes in maize leaves.

Our data showed that NO scavenging by cPTIO did not show any effect on MAPKs activation. A same result was obtained using *nia1nia2* and *dnd1* mutants in which OGs-induced NO production was lower (Figure 2). Moreover, we observed that NO production was not modified in the *mpk3* and *mpk6* mutants indicating that NO generation in response to OGs do not depend on MAPKs signaling. This data, which contrast with Wang et al., (2010) investigation showing that *mapk6* mutant is defective in H_2O_2-NO generation, imply that OGs-induced

Figure 7: Comparison of expression pattern of selected NO-responsive genes between Col-0 and *cpk5.6.11*. Induction profile of NO responsive genes (TIR, SRP, RLP7, WRKY41, WRKY75, CRF3, bHLH) after OG treatment in Arabidopsis leaf tissue. Leaves were treated with water or OGs with. The transcript accumulation was analyzed by real-time qPCR using *UBQ10*, as an internal control. Results are expressed as the fold changes in transcript level compared to the control (water-treated).



MAPKs activation and NO production result from independent pathways as previously observed in other plant/elicitor models (Vandelle et al., 2006).

CDPKs control NO production, NO-mediated response to OGs and resistance to *B. cinerea*

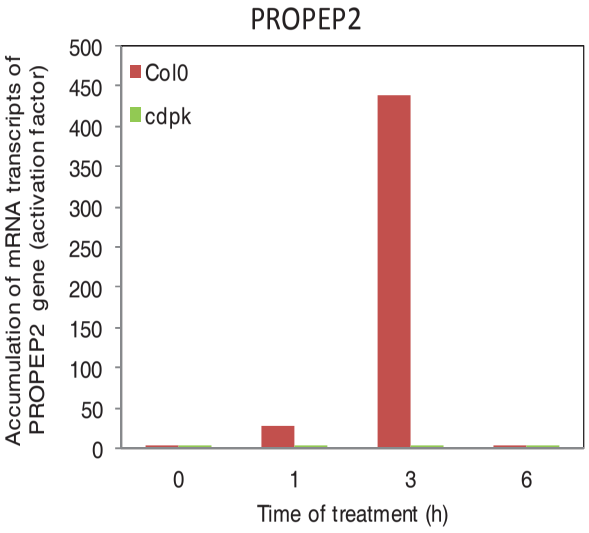
It was reported recently that the activity of CDPKs, induced by flg22, regulates the production of ROS and induce their effects through modulation of gene expression of genes (Boudsocq et al., 2010). Among the genes modulated by CDPKs, one of them, PER4 (for anionic peroxidase 4, At1g14540), attract our intention. Indeed, we observed that PER4 was induced by OGs, regulated by NO and involved in *A. thaliana* to *B. cinerea* interaction (see Chapter 4). In this context, we studied the role of CDPKs and their relation with NO.

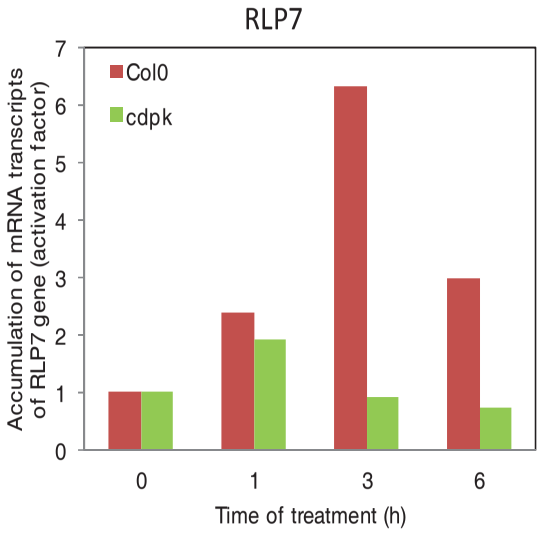
Firstly, we estimated the role of CDPKs in NO production using the triple mutant *cpk5.6.11*. Compared to Col-0, *cpk5.6.11* displayed 50 % reduction in NO production triggered by OGs. To our knowledge, this result was the first demonstration that CDPKs act on the upstream of elicitor-induced NO production. To verify the involvement of CDPKs in OGs-induced NO production, activation of CDPKs was tested by "in gel kinase" assay. The results indicate that calcium-dependent protein kinase having size of approximatively 55 kDa is activated in response to OGs, as previously observed in response to flg22 (Boudsocq et al., 2010). Activation of CDPKs protein could be a common response of plants to elicitors.

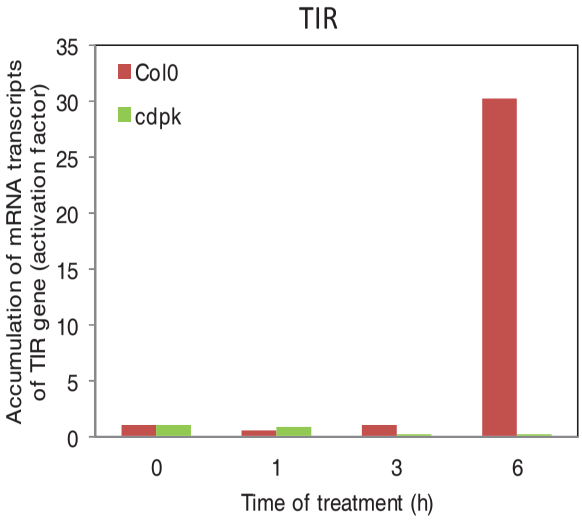
The triple *cpk5.6.11* mutant was significantly more sensitive to *B. cinerea* than Col-0. This indicates the involvement of CDPK 5, 6 and 11 in the establishment of plant defense reactions against pathogens as already reported by Boudsocq et al., (2010).

Finally, accumulation of transcripts of NO target genes (TIR, RLP7, SRP, PROPEP2, CRF3, bHLH, WRKY41 and WRKY75) in the mutant *cpk5, 6.11* was observed. All the NO-responsive genes were down-regulated in mutant except CRF3 and bHLH-like. Taken together, these results indicate that elicitors activated by CDPKs are important components of the resistance of Arabidopsis to *B. cinerea* and that this resistance may be dependent on NO through the regulation of NO target genes.

In conclusion, we observed that both MAPKs and CDPKs were activated in response to OGs. Previous studies and our work also demonstrated that both are important component of plant defense to pathogens. Boudsocq et al., (2010) showed different regulatory role for these two families of kinases. Some early flg22-responsive genes were MAPKs-specific, whereas others


PROPEP2
Accumulation of mRNA transcripts of PROPEP2 gene (activation factor)
Col0
cdpk
500
450
400
350
300
250
200
150
100
50
0
0
1
3
6
Time of treatment (h)


RLP7
Accumulation of mRNA transcripts of RLP7 gene (activation factor)
Col0
cdpk
7
6
5
4
3
2
1
0
0
1
3
6
Time of treatment (h)


TIR
Accumulation of mRNA transcripts of TIR gene (activation factor)
Col0
cdpk
35
30
25
20
15
10
5
0
0
1
3
6
Time of treatment (h)

seemed to be CDPK-specific. Other flg22 early genes were either activated equally by both CDPKs and MAPKs cascades, such as PER4. Few reports reported cross-talk between MAPKs- and CDPKs-dependent pathways. For example, ethylene-mediated crosstalk between CDPKs and MAPKs signaling, demonstrated that elevated CDPKs activities compromised stress-induced MAPKs activities (Ludwig et al., 2005). In contrast, Mehlmer et al., (2010) showed no evidence of cross talk between CDPK (CDPK3) kinase activity with MPK4 and MPK6 activities in response to salt stress and reported that CDPK3 and MAPKs act independently, and in parallel. According to our results, these two pathways can be differentiated according to their relationships with NO signaling. MAPKs are independent of NO, although CDPKs could act upstream of NO effects by controlling its production. Nevertheless, further investigations will be needed to clarify the role of CDPKs in NO signaling

CHAPTER 5

"Results"

"Nitric oxide-regulated transcriptomic response to oligogalacturonides in Arabidopsis thaliana – characterisation of NO-responsive genes"

CHAPTER 5

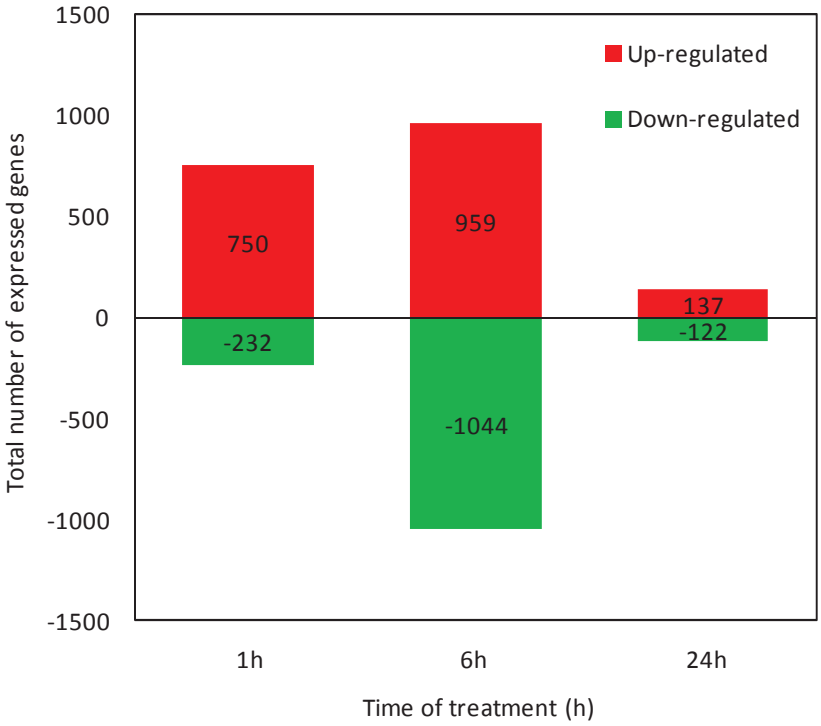
NO regulated transcriptomic response to OGs

INTRODUCTION

Nitric oxide (NO) is an important biological messenger in animals and in plants, involved in numerous physiological processes. One major challenges of NO biology is to determine how the correct specific response is evoked, despite shared use of the NO signal and, in some cases, its downstream second messengers. Many of NO biological functions arise as a direct consequence of chemical reactions between proteins and NO or NO derivatives such as metal or S nitrosylation, and tyrosine nitration (Besson Bard et al., 2008; Astier et al., 2011). However, besides the regulation of signaling pathways by post-translational modifications, NO can control physiological processes by modifying gene transcription (these two processes being not necessarily independent). For instance, during last years, the expression of several genes induced or repressed through NO-dependent processes has been identified in animals (Hemish et al., 2003; Turpaev et al., 2005). These genes are mainly involved in signaling, metabolism, cell cycle, transcription and protein degradation.

In *Arabidopsis thaliana*, large-scale transcriptional analyses in response to NO was reported in several studies (Parani et al., 2004; Badri et al., 2008; Palmieri et al., 2008; Ahlfors et al., 2009). These analyses of NO-responsive transcripts indicate that NO governs the regulation of expression of numerous genes (approx 100 to 600 depending on the study). These genes could be up- or down-regulated and putatively encode proteins related to phytohormones and lignin biosynthesis, to signaling such as protein kinases, to cellular transport, to transcriptional regulation, to (a)biotic stress responses, to protection against oxidative stresses and to photosynthesis (Grun et al., 2006; Besson-Bard et al., 2009). Comparative analysis of these transcriptomic data allowed the identification of a low number of genes commonly modulated by NO (Besson-Bard et al., 2009). These genes might represent candidate genes regulated by NO but these studies were mainly performed by the use of NO donor and NO-responsiveness evolved endogenously in the physiological context has been poorly defined. In conclusion, as written by

A



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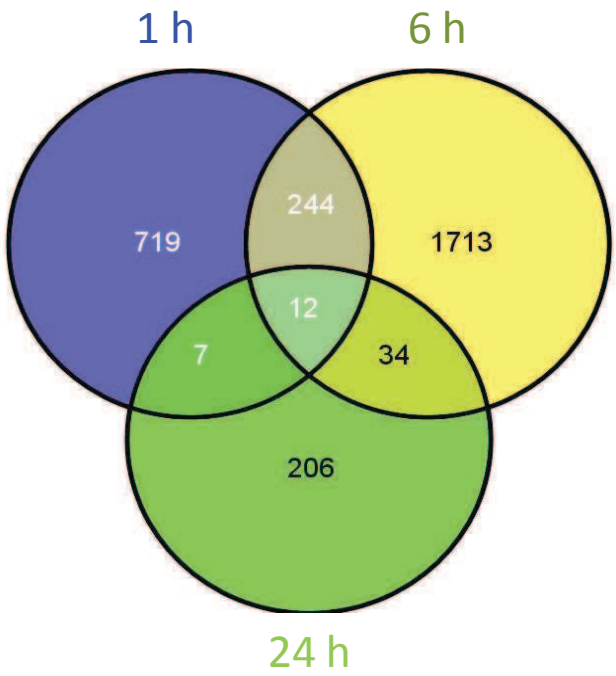


Figure 5.1: A: Differential expression of *Arabidopsis thaliana* genes in response to OGs after 1h, 6h and 24h of treatment. Up- and down-regulated genes are represented in red and green shades respectively. **B:** Venn diagram of overlapped and non-overlapped genes after OGs treatment at different time interval in Col-0 plants.

Grun et al. (2006) we still think that: "the 64000 dollar question is which genes are directly modulated by NO".

Modulation of the transcriptional network by NO raised the question of common NO targets in the regulatory regions of the genes. It is hypothesized that most of the effects of NO on gene expression are indirect. They are based on the modulation of several transcription factors (or upstream intermediates in the signaling pathways) as well as mRNA stability and processing of the primary gene product (Bogdan, 2001; Sha and Marshall, 2011). An *in silico* search for common transcription factor binding sites (TFBS) in the promoter region of genes induced by the NO donor NOR-3 was performed in *A. thaliana* by Palmieri et al. (2008). Eight families of TFBSs were found 15% more often in the promoter regions of the responsive genes as compared to promoter regions of 28447 *Arabidopsis* control genes. Most of the TFBS putatively correspond to the binding elements of stress-related transcriptional activators such as bZIP (basic region/leucine zipper motif), WRKY and MYCL (myelocytomatosis viral oncogene homolog L) transcription factors, strengthening a role for NO as a component of (a)biotic stress-related signaling pathways. TFBS elements found in the genes associated to jasmonate biosynthesis gave further evidences for the involvement of NO in jasmonate-associated processes.

OBJECTIVES

The aim of our present study was to identify network of genes regulated by NO endogeneously produced in response to oligogalacturonides in *Arabidopsis thaliana* leaf tissues using whole genome transcript analyses. Our analysis has pointed out the different cellular processes modulated by NO at the transcriptional level. Moreover, we investigated the role of some candidate genes in a plant-pathogen interaction context (*A. thaliana/B. cinerea* model) by using T-DNA mutant lines.

RESULTS

1. Transcriptomic response of *A. thaliana* leaves to OGs treatment

In this first part, the transcriptome response of *A. thaliana* to OGs will be described briefly. The characterization of the NO effects in this response will be given in the second part.

Table 5.1: OGs-responsive genes. List of genes commonly modulated after 1h, 6h and 24h treatment with OGs. Expression levels of up-regulated and down-regulated genes are presented in red and green shades, respectively.

Genes ID	Description	1h	6h	24h
AT1G30730.1	FAD-binding domain-containing protein	5.79173714	4.55102253	4.69019637
AT2G02310.1	AtPP2-B6 (Phloem protein 2-B6); carbohydrate binding	29.5171771	10.2913164	6.92006408
AT2G28210.1	ATACA2 (ALPHA CARBONIC ANHYDRASE 2); carbonate dehydratase/ zinc ion binding	6.94359583	58.9932707	19.1048304
AT3G28210.1	PMZ; zinc ion binding	7.93056961	14.4900308	8.64869972
AT3G49780.1	ATPSK4 (PHYTOSULFOKINE 4 PRECURSOR); growth factor	3.74283255	11.4029803	5.6035264
AT3G55150.1	ATEXO70H1 (exocyst subunit EXO70 family protein H1); protein binding	10.6147431	11.7729145	6.06337786
AT4G18170.1	WRKY28; transcription factor	3.76457983	11.0500259	5.26774698
AT4G35380.1	guanine nucleotide exchange family protein	11.6220518	5.88520084	11.0530966
AT4G36240.1	zinc finger (GATA type) family protein	-4.43486088	-3.65642673	-2.69089193
AT5G16020.1	stress protein-related	8.5598643	-6.09625282	9.33976337
AT5G25920.1	FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unkno	16.3169154	4.69361084	5.49293116
AT5G44990.1	FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unkno	48.1410164	19.3193188	4.97758508

1.1. *In silico* functional categorization of OGs-induced genes

Arabidopsis plants leaves were infiltrated with OGs for 1h, 6h and 24h. Transcriptomic analysis showed that a total of 1860 (2935 including splicing variants) different genes were significantly modulated by treatment with a predominant group at 6 h (2003 genes versus 982 genes at 1h and 259 at 24h of treatment). At 1 h, genes were mostly up-regulated in response to OGs (Figure 5.1A; list of common genes in Supplemental Table, in Annex). In contrast, at 6 h and 24 h, approximatively half of the genes were up-regulated (Figure 5.1A; list of common genes in Supplemental Table in Annex). Only 12 genes were commonly regulated on the three time intervals (Figure 5.1B and Table 1).

Identified genes were analysed for functional annotation using MapMan (Thimm et al., 2004; Usadel et al., 2005). Genes were classified into functional categories (BINs). MapMan software was used to test whether the expression values of genes belonging to each BIN differed significantly ($P<0.05$) from those in other BINs. Eleven BINs containing subgroups of genes (subBINs) that behave differently compared to other categories were identified (Table 5.2A, 5.2B and 5.2C). These categories were "PS" (photosystem) (BIN 1), "major CHO metabolism" (BIN 2), "cell wall" (BIN 10), "Lipid and hormone metabolism" (BIN 11 and 17), "stress" (BIN 20), "miscellaneous" (BIN 26), "RNA" (BIN 27), "DNA" (BIN 28), "protein" (BIN 29) and ""signaling" (BIN 30). BINs 1, 2 and 10 were specific to the 6h timepoint. BINs 11, 17, 26 and 28 were commonly identified at 1h and 6h and, in contrast, only BIN 29 was present at 6h and 24h, indicating a higher similarity of the transcriptome response between 1h and 6h rather than 6h and 24h. Eight of these categories were identified at two time intervals and three (BINs 20, 27 and 30) at all the three time intervals (Table 5.2D), highlighted the importance of these functional groups in the defense response of *Arabidopsis* in response to OGs. These three common BINs were explored in more details. In BIN20 "Stress", genes encoding proteins involved in plant immunity (classified in "biotic stress") were the most modulated by OGs at 6h and 24 h of treatment (Table 5.2B and 5.2C). In BIN27 "RNA", genes encoding transcription factors were clearly identified from different families. The "C2C2(Zn) TF" family (category 27-3-9 and 23-7-11) was significantly modulated at 1h and 24h of treatment whereas the "WRKY domain transcription factor family" (category 27.3.32) was most specific to 6h treatment (Table 2B). In the "Signalling" category (BIN 30), "light signaling" was the only commonly identified subBIN (1h and 6h of treatment).

Table 5.2: Categorization of statistically significant BINs into functional subgroups for OGs-responsive genes (**A:** 1h OGs treatment; **B:** 6h OGs treatment, **C:** 24h OGs treatment) using MAPMAN software. The expression ratio of genes within a BIN when compared to those in all the other BINs were statistically analyzed using the Wilcoxon rank sum test. BINs relating to main categories are shown in bold. **D :** Comparison of functional categories of OGs-responsive genes at 1h, 6h and 24h of treatment. NS = non significant.

A

BIN	Description	Elements	*p*-value
11	**Lipid metabolism**	14	NS
11.9	Lipid degradation	7	0.046
11.9.2	Lipases	3	0.025
11.9.2.1	Triacylglycerol lipase	2	0.039
17	**Hormone metabolism**	30	NS
17.2	Auxin	10	1.09 E-5
17.2.3	Induced-regulated-responsive-activated	10	1.09 E-5
20	**Stress**	47	0.036
20.1	Stress,biotic	40	0.027
26	**Misc**	54	8.71 E-5
26.1	Cytochrome P450	12	0.013
26.2	UDP glucosyl and glucoronyl transferases	7	0.020
26.9	Glutathione S transferases	4	0.027
27	**RNA**	94	NS
27.3.6	Regulation of transcription, Basic Helix-Loop-Helix family	4	0.045
27.3.7	Regulation of transcription,C2C2(Zn) CO-like TF family	3	0.016
27.3.9	Regulation of transcription,C2C2(Zn) TF family	4	0.003
27.3.27	Regulation of transcription, NAC domain TF family	3	0.015
28	**DNA**	17	NS
28.1	Synthesis/chromatin structure	16	0.036
30	**Signalling**	91	NS
30.1	Sugar and nutrient physiology	4	0.003
30.11	Signalling,light	3	0.019

B

BIN	Description	Elements	*p*-value
1	**PS**	34	3.4 E-7
1.1	Light reaction	21	1.1 E-5
1.1.1	Photosystem II	13	0.002
1.1.1.2	PSII polypeptide subunits	12	0.003
1.1.6	NADH DH	3	0.018
1.3	PS.calvin cyle	10	0.011
2	**Major CHO metabolism**	17	0.010
10	**Cell wall**	59	6.0 E-4
10.1.9	Precursor synthesis.MUR4	3	0.032
10.6	Degradation	16	2.6 E-4
10.6.3	Pectate lyases and polygalacturonases	11	1.5 E-4
10.7	Cell wall.modification	15	6.9 E-4
11	**Lipid metabolism**	29	0.017
11.1	FA synthesis and FA elongation	7	0.023
11.1.11	Fatty acid elongase	2	0.030
17	**Hormone metabolism**	63	NS
17.6.3	Gibberelin.induced-regulated-responsive-activated	3	0.020

20	**Stress**	78	0.002
20.1	Stress.biotic	51	4.3 E-7
20.1.7	PR-proteins	23	4.6 E-4
20.1.7.12	PR-proteins.plant defensins	3	0.006
20.2.99	Stress.abiotic.unspecified	9	0.033
26	**misc**	174	0.042
26.2	UDP glucosyl and glucoronyl transferases	20	0.046
26.9	Glutathione S transferases	9	6.5 E-4
26.12	Peroxidases	10	0.003
26.13	Acid and other phosphatases	3	0.016
27	**RNA**	160	NS
27.3.5	Regulation of transcription. ARR	4	0.040
27.3.27	Regulation of transcription. NAC domain TF family	3	0.010
27.3.32	Regulation of transcription. WRKY domain TF family	19	9.2 E-8
28	**DNA**	44	NS
28.1.1	DNA.synthesis/ retrotransposon/transposase	24	0.014
29	**Protein**	173	NS
29.5.9	Protein.degradation.AAA type	4	0.048
29.5.11.4.3.2	Ubiquitin.E3.SCF.FBOX	18	0.035
29.6	Protein.folding	4	0.014
30	**Signalling**	109	NS
30.1	In sugar and nutrient physiology	4	0.032
30.2.3	Receptor kinases.leucine rich repeat III	4	0.042
30.2.99	Receptor kinases.misc	12	2.9 E-4
30.3	Calcium	19	0.008
30.11	Light	10	0.003

C

BIN	Description	Elements	*p*-value
20	**Stress**	10	NS
20.1.7.12	Biotic.PR-proteins.plant defensins	2	0.043
27	**RNA**	28	NS
27.1	RNA.processing	2	0.034
27.3.11	Regulation of transcription.C2H2 zinc finger family	2	0.046
29	**Protein**	27	NS
29.5	Protein.degradation	17	0.017
29.5.11.4	Ubiquitin.E3	10	0.017
29.5.11.4.3	SCF	8	0.020
29.5.11.4.3.2	FBOX	8	0.020
30	**Signalling**	7	0.024
30.5	G-proteins	2	0.023

D

BINs	1h	6h	24h
1.PS		✓	
2.CHO metabolism		✓	
10. Cell Wall		✓	
11. Lipid metabolism	✓	✓	
17. Hormon metabolism	✓	✓	
20. Stress	✓	✓	✓
26. Miscellaneous	✓	✓	
27. RNA	✓	✓	✓
28. DNA	✓	✓	
29. protein		✓	✓
30. Signalling	✓	✓	✓

Table 5.3: Genes modulated in response after OGs treatment. Number of genes modulated in after OGs treatment in our study and previously published studies. Induction or repression represented number of genes significantly up-regulated or down-regulated with 2.0-fold change in treated plants as compared to control, respectively in different studies.

References	Biological model	Mode of treatment	Total number of genes modulated by OGs (FC>2)
OGs 2010 My Own data	*Arabidopsis* plants (4 weeks old)	OGs syringe infiltration in leaves	1860
OGs 2009 Dubreuil et al., in press	*Arabidopsis* leaf discs (8 weeks old)	OGs vacuum infiltration	145
OGs 2007 Ferrari et al., 2007	*Arabidopsis* seedlings (10 days old)	OGs in the medium	2699
OGs 2006 Moscatiello et al., 2006	*Arabidopsis* cell suspensions (10 days old)	Cell treated with OGs	271

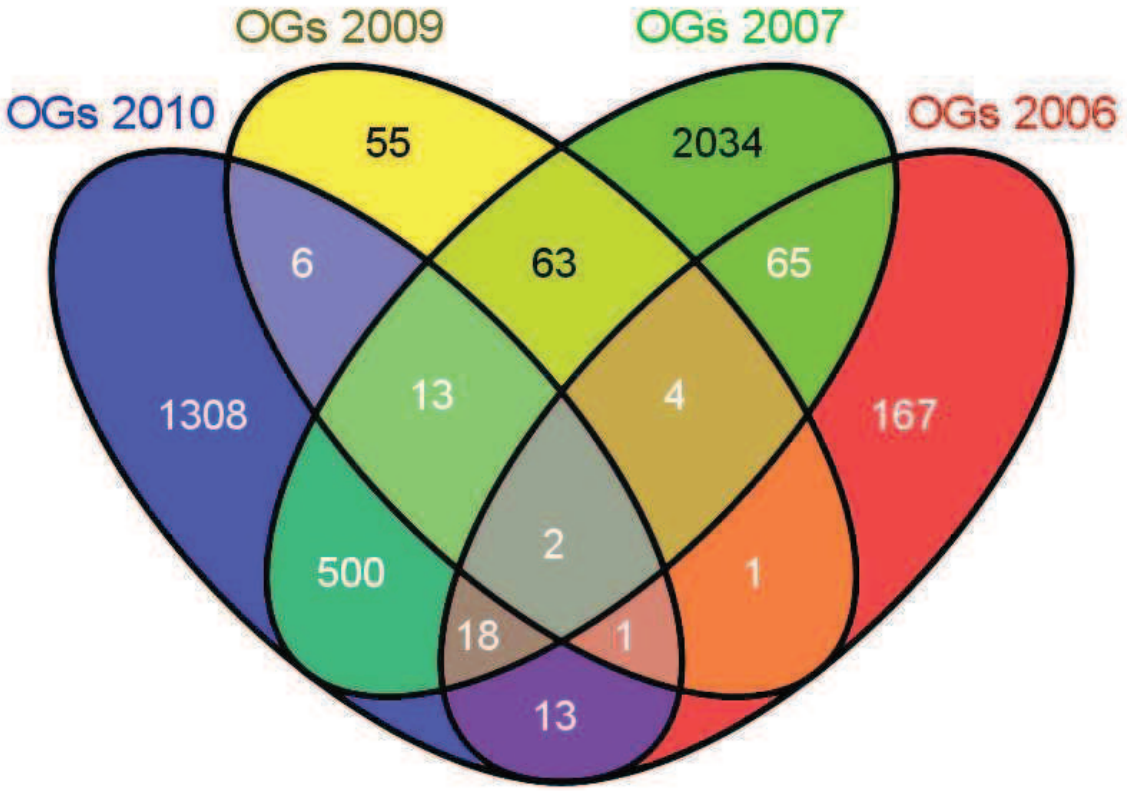


Figure 5.2: Venn diagram of overlapped and non-overlapped genes after OGs treatment in the different studies.

1.2. Comparative analysis of the *A. thaliana* transcriptomic response to OGs

Our results were compared with previously published transcriptomic data set in *A. thaliana* treated by OGs (Table 5.3). As already revealed by our study, OGs treatment modulates genes encoding stress- and disease-(defense) related proteins, signalling components, transcription factors and enzymes implicated in primary and secondary metabolism. Analyses were performed with pooled transcriptome response (different time intervals).

Overlap of significantly expressed genes has been presented in Figure 5.2.

- OGs 2010 vs OGs 2009: Twenty two (22) genes were common in the two experiments (leaf tissues infiltrated by syringe or using vacuum; Figure 5.2). Out of these 15 genes, 13 were up-regulated in both studies, but the two remaining genes behaved differently in the two studies (list of common genes in Supplemental Table in Annex). Out of these common genes, 23% genes were modulated in response to stress and 4% were from signal transduction (Figure 5.3).
- OGs 2010 vs OGs 2007: Five hundred thirty three (533) genes were commonly identified (Figure 5.2). Approximatly 10% genes were modulated in response to stress and only 3% genes were from signal transduction (Figure 5.3).
- OGs 2010 vs OGs 2006: Thirty four (34) genes were common (Figure 5.2). Approximatly 17 % genes were modulated in response to stress and only 2 % genes were from signal transduction (Figure 5.3). Among these genes, lipoxygenase (LOX) and allene oxide synthase (AOS) encoding genes were identified. These two enzymes required for jasmonate biosynthesis.

Finally, two genes were common in all these four studies (Figure 5.2). These genes encode a MAP kinase, ATMPK11 (AT1G01560) and the lipoxygenase 3 LOX3 (AT1G17420).

2. Nitric oxide-regulated transcriptomic response to OGs

2.1. Identification of NO-responsive genes

To identify genes modulated by NO in response to OGs, we performed microarray analyses using *A. thaliana* plants treated by OGs, in the presence of the NO scavenger cPTIO. Data from OGs+cPTIO treatment were analyzed and compared to data obtained from OGs treatment alone.

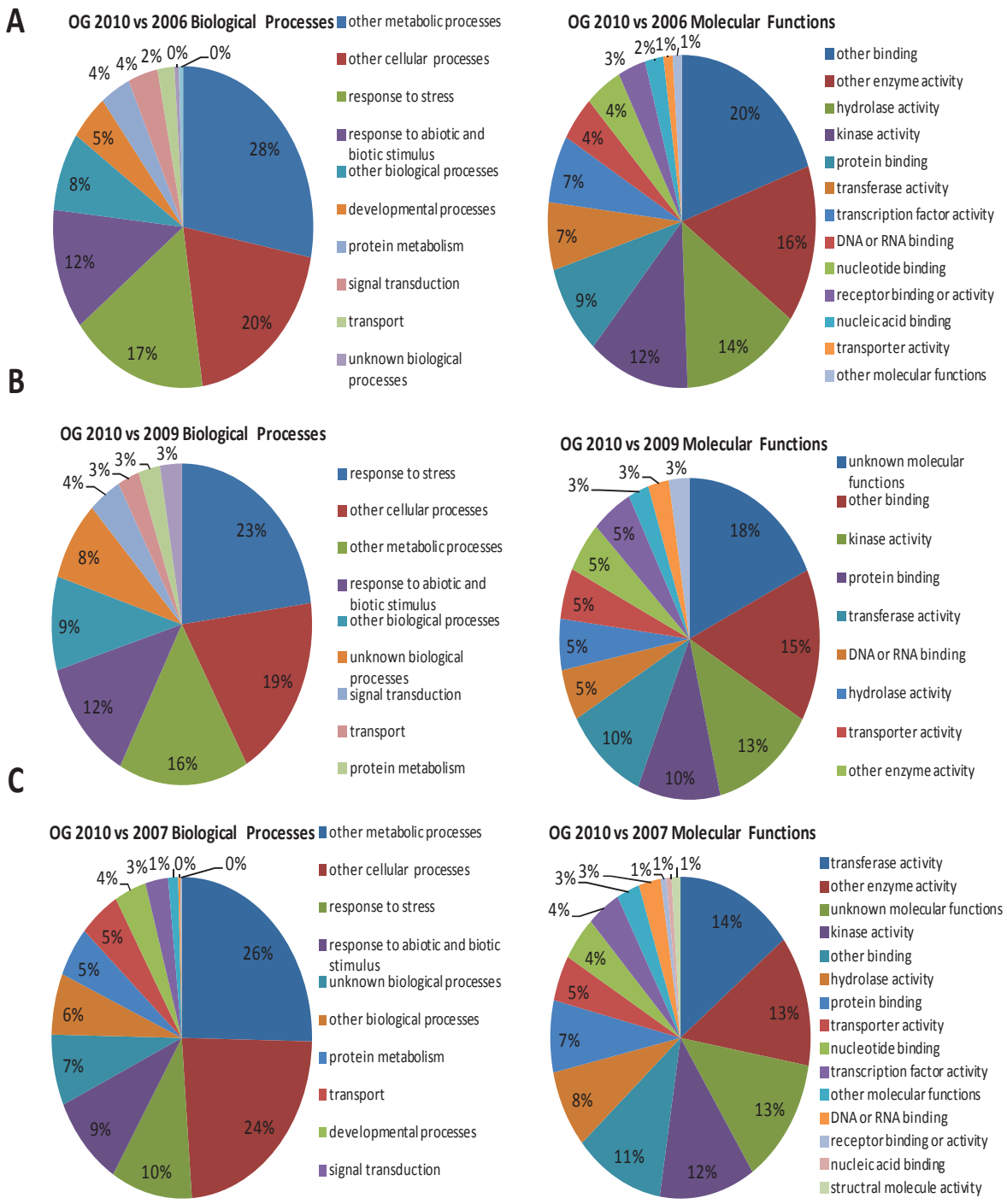


Figure 5.3: Distribution of common OGs-responsive genes with putative functions assigned through Gene Ontology (GO). A: Biological process and molecular function of common genes between OGs 2010 vs OGs 2006 , **B:** Biological process and molecular function of common genes between OGs 2010 vs OGs 2007, and **C:** Biological process and molecular function of common genes between OGs 2010 vs OGs 2007. The percentages shown indicate the abundance of each category within the whole dataset; Assignments are based on the data available at the TAIR and from the Gene Ontology Annotation Database.

OGs+cPTIO treatment modulated 765 genes at 1 h, 1550 genes at 6 h genes and 127 at 24 h treatment (fold-change ≥ 2). We compared this list of genes (genes modulated by OGs in the presence of cPTIO therefore independently of NO) with the list of OG-induced genes using the program FiRE (Garcion et al., 2006). NO-regulated transcriptomic response in the context of OGs elicitation corresponds to the list of genes regulated by OGs minus the list genes regulated by OGs in presence of cPTIO (modulated independently of NO). These genes were nammed as NO-responsive genes (list of genes in Supplemental Table in Annex).

At 1 h, 6 h and 24 h, 388, 1079 and 237 NO-responsive genes were found respectively (Figure 5.4A; list of genes Supplemental Table in Annex). NO responsive genes represent 40%, 50% and 90% of the genes induced by OGs alone at 1h, 6h and 24h respectively. As observed in response to OGs (Figure 5.4A), genes were mostly up-regulated at 1h and 24h of treatment, whereas they were mostly downregulated at 6h (Figure 5.4A). Comparison of the gene lists showed that the transcriptome response at the different time intervals was very dissimilar (Figure 5.4B; list of genes in Supplemental Table in Annex). Only one gene (AT5G16020) encoding GEX3, a plasma membrane localized protein expressed in the male gametophytes was common amongst the NO-responsive genes. This gene also plays a role in stress (previously identified as SRP for stress-related protein).

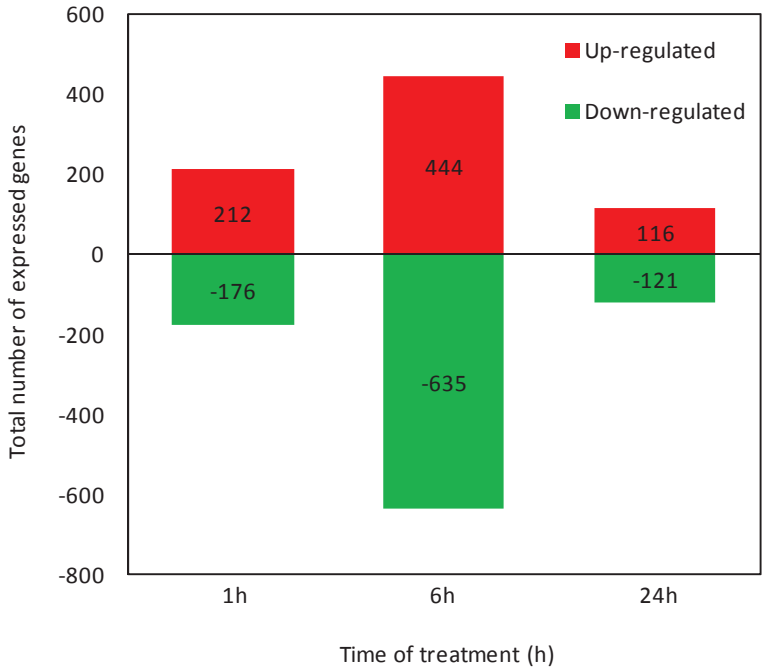
2.2. *In silico* functional annotation of NO-responsive genes

NO responsive genes were binned into categories based on functionnal annotation performed with GO annotation and MapMan.

Using MapMan, NO responsive genes were classified in seven different BINs: “PS” (photosystem) (BIN 1), “cell wall” (BIN 10), “Hormone metabolism” (BIN 17), “stress” (BIN 20), “misc” (BIN 26), “RNA” (BIN 27) and “protein” (BIN 29) (Table 5). Transcriptomic response at the different timepoints showed some similarities (e.g. genes from BIN 20 “Stress” (particularly “PR-proteins” subBIN) and BIN 27 “RNA” were identified at the three time points) but also some differences {(e.g. genes related to auxin metabolism were specifically modulated at 1h, related to PS (BIN 1) were specifically modulated at 6h or related to protein degradation (BIN 29) were specifically modulated at 24h)}.

MapMan could also display the transcriptome data onto pictorial diagrams that represent a biological function (pathway diagrams). Focusing on the stress pathway (“biotic stress” diagram),

A



B

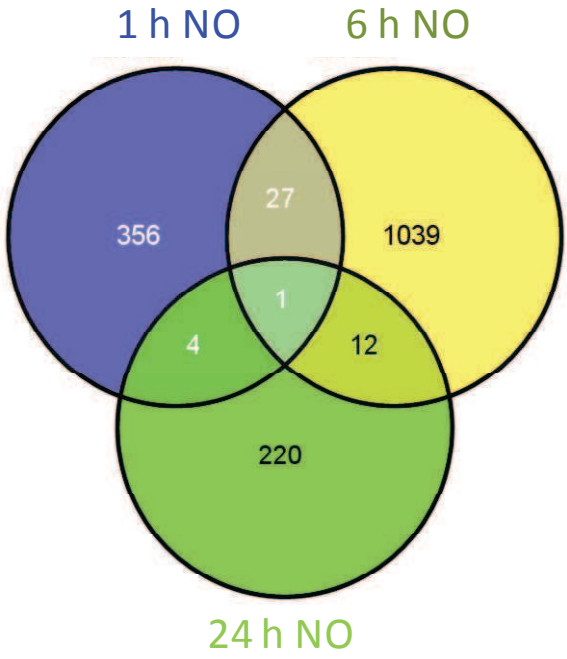


Figure 5.4: A: NO-regulated transcriptomic response. Differential expression of *Arabidopsis thaliana* NO-responsive genes after 1h, 6h and 24h of treatment. Up and down-regulated genes are represented in red and green shades respectively. **B:** Overlap of NO response. Venn diagram of overlapped and non-overlapped NO responsive genes at different time intervals in Col0 plants. Leaves were infiltrated with OGs (2.5 $mg.mL^{-1}$) or water (as a control). mRNA was subjected to transcriptomic analysis (Nimblegen array). Three independent biological replicates were carried out. Induction or repression represented number of genes significantly up-regulated or down-regulated with 2.0-fold change in treated plants as compared to control, respectively.

it was found that genes belonging to the transcription factors, the secondary metabolites, the hormone signaling, the signaling and the proteolysis groups were identified in a large extent (Figure 5.5). Many of the early up-regulated genes (1h of treatment) encode transcription factors which are members of large families, including WRKY, bZIPs, *ERF*s, basic helix-loop-helixes (bHLHs) and MYBs. More precisely, this diagram highlighted that within some categories, genes were coordinately modulated according to their function, differential activation, as well as repression was observed. For instance, genes involved in auxin signaling (BIN 17.2.3) were coordinately down-regulated at 1h of treatment (Figure 5.5A). In contrast, genes belonging to PR-proteins (BIN 20.1.7) and WRKY (BIN 27.3.32) categories were mostly up-regulated (Figure 5.5A and 5.5B).

NO-responsive genes were distributed in all the BINs identified with the OGs treatment (Table 5.5). However, it is interesting to note that among NO responsive genes, at 1h of treatment (Table 5.5A), the two categories "PR-Proteins" (20.1.17) and "WRKY " (27-3-32) were significantly affected whereas these categories were not in transcriptomic data from OGs treatment alone, at the same timeintervals (Table 5.5A).

2.3. Identification of transcription factor binding site (TFBS)

We explored the possibility of identifying common TFBSs in the promoter regions of the NO-responsive genes. Athena software package was used for putative transcription factor recognition site identification in the promoter region (-1000bp before TIS).

As a control, the occurrence of the TFBSs within the complete genome of *Arabidopsis* as a percentage was used. The analyses showed a large number of putative TFBSs in the promoter of the NO-regulated genes (Table 5.5). In total, 12 families of TFBSs are significantly over-represented in the promoter regions of the analysed groups of genes in comparison with the promoter regions of the control genes. We observed that W-box elements (WRKY binding sites) were enriched in the promoters of genes up-regulated at 1h and 6h of OGs treatment. In the down-regulated genes an increased occurrence of CARGCW8CAT motif, ARF, Myc, Myb4, DRE elements was observed in their promoters.

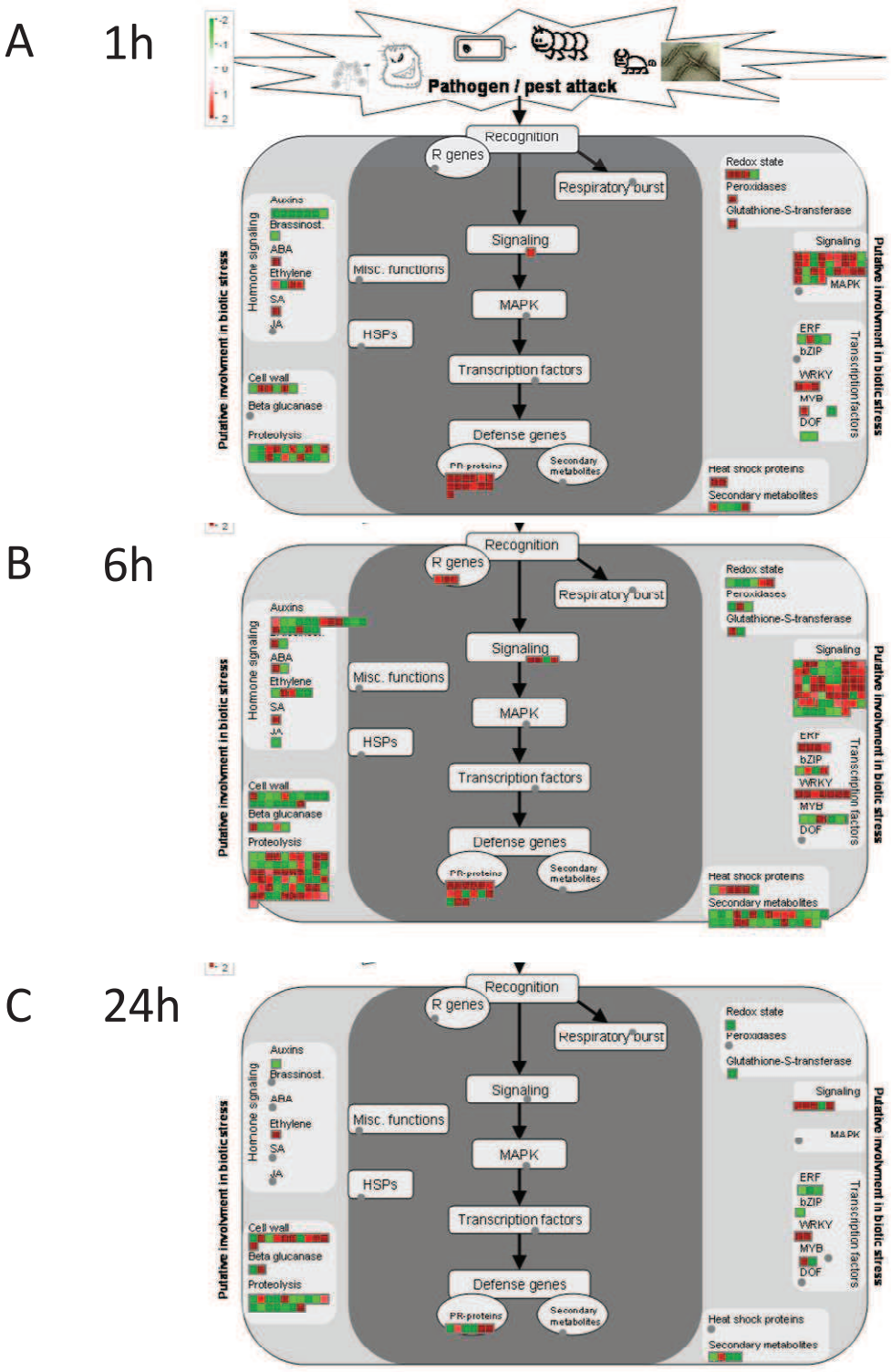


Figure 5.5: MapMan "Biotic stress" diagram for *A. thaliana* NO-responsive genes in response to OGs treatment (**A:** 1h, **B:** 6h and **C:** 24h). Single genes are represented by a square while the color indicates the direction of transcriptional change (up and down regulated genes are represented in red and green squares, respectively). Color intensity indicates the fold change ratio on log2 scale.

2.4. Functional characterization of NO-responsive genes

Seven candidate genes were selected for further investigations (Table 5.6). These genes were selected, firstly, according to their high fold-change value (Table 5.7), hypothesizing that up-regulation is indicative of a major role in the response and secondly, according to the *in silico* analysis (see above) and literature data. Four of these genes encoded transcription factors (CRF3, bHLH-like, WRKY 41 and WRKY 75), two were identified as putative resistance genes (TIR-At1g52900 and AtRPL7), and the last one (SRP2/GEX3) was the only gene modulated at all three three studied timeintervals.

2.4.1. Validation of microarray data by RT- qPCR

We analysed the transcript accumulation of selected genes by RT-qPCR to validate transcriptomic analysis. Transcripts accumulation were monitored at 1 h, 3 h and 6 h of treatment and then compared to the expression profiles determined with microarray. As expected, all the genes were up-regulated in response to OGs at 1h, 3h or/and 6h treatment, this induction was repressed with cPTIO treatment (Figure 5.6). Although fold induction and kinetics in gene expression have been shown to differ between the two methods, the relative expression patterns were similar (Table 5.8, Figure 5.4). As reported earlier, discrepancies between the results of these two different techniques were reported in literature with values ranging from 55 to 20 to 30% (Czechowski et al., 2004; Salzman et al., 2005; Svensson et al., 2006). In this context, we considered that our results showed a good correlation between microarray and real-time RT-PCR analyses and indicated that our microarray data were reliable.

2.4.2. Genotyping of T-DNA insertion mutant lines

T-DNA insertion mutant lines corresponding to the genes were genotyped to confirm their homozygocity. One example was shown in Figure 5.7. As expected, LP-RP primers allowed amplification of the wild-type allele (1400 bp) in the Col-0 genotype (Figure 5.7A). In contrast, primers Lb-RP gave one PCR product of 800 bp (mutant allele) in 8 plants out of the 8 plants tested. As expected, no amplification was observed in both wild Col-0 and negative control (Figure 5.7B). This result indicated that *wrky75* line was homozygous to the mutated allele. All the mutant lines were screened by this method before subsequent characterization.

Table 5.4: Categorization of statistically significant BINs into functional subgroups for NO responsive genes (**A:** 1h OGs treatment; **B:** 6h OGs treatment, **C:** 24h OGs treatment) using MAPMAN software. The expression ratio of genes within a BIN when compared to those in all the other BINs were statistically analyzed using the Wilcoxon rank sum test. Only BINs that were significantly different from other are shown. Numbers and groups relating to main categories are shown in bold. Categories identified with asterisk were not significantly stable in transcriptomic response to OGs alone (Table 5.2). NS = non significant.

A

BIN	Description	Elements	*p*-value
17	**Hormone metabolism**	14	0.036
17.2	Auxin	7	2.8 E-4
17.2.3	Induced-regulated-responsive-activated	7	2.8 E-4
20	**Stress**	24	5.3 E-4
20.1	Biotic	20	0.0016
20.1.7	PR-proteins	13	7.7 E-4*
26	**Misc**	16	0.018
26.8	Nitrilases, nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	3	0.046
27	**RNA**	40	NS
27.3.32	WRKY domain transcription factor family	3	0.038 *

B

BIN	Description	Elements	*p*-value
1	**PS**	19	4.7 E-4
1.1	Light reaction	13	9.6 E-4
10	**Cell wall**	17	0.0056
10.6	Degradation	5	0.0014
10.6.3	Pectate lyases and polygalacturonases	3	0.0040
20	**Stress**	49	0.0041
20.1	Stress.biotic	33	2.3 E-5
20.1.7	PR-proteins	15	0.0046
27	**RNA**	90	NS
27.3.32	WRKY domain transcription factor family	7	6.6 E-4

C

BIN	Description	Elements	*p*-value
20	**Stress**	9	NS
20.1.7.12	Biotic. PR-proteins. plant defensins	2	0.035
27	**RNA**	26	0.080
27.1	RNA.processing	2	0.029
27.3.11	Regulation of transcription.C2H2 zinc finger family	2	0.037
29	**Protein**	27	0.30
29.5	Protein.degradation	17	0.040
29.5.11.4	Ubiquitin.E3	10	0.030
29.5.11.4.3	SCF	8	0.032
29.5.11.4.3.2	FBOX	8	0.032

2.4.3. Role of candidate genes in Arabidopsis thaliana / Botrytis cinerea interaction

To understand the role of candidate genes selected from transcriptome analysis in a plant-pathogen interaction context, T-DNA mutant lines (*rlp7, tir, srp, crf3, bhlh, wrky41, wrky75*) and Col-0 as control were inoculated with the necrotrophic fungus *B. cinerea*. The symptoms were measured 72 h post infection. The experiments have been performed at least three times; but the results were considered preliminary as only one T-DNA line was studied for each gene.

In the *bhlh* and *crf3* mutants, lesion diameters were greater then that the ones of Col-0 plants. If we compared the lesion diameter distribution, there were more necrotic lesions that were larger than 6 mm (Figure 5.8A). These two mutant lines were considered as more susceptible to *B.cinerea*. In contrast, *wrky41* seems more resistant to *B. cinerea* infection: the average lesion diameter was less in *wrky41* as compared to Col-0 and also more lesions that were smaller then 6mm were observed (Figure 5.8B). Finally, we did not notice any significant difference between *wrky75* and Col-0 (Figure 5.8C).

The two mutants (*tir and rlp7*) impaired in the genes encoding receptors involved in innate immunity showed higher susceptibility (Figure 5.8D and 5.8E). Finally, stress-related protein (*srp*) mutant was more sensitive to *B. cinerea* infection and there were no necrotic lesions in class less than 2 and more no of lesions in the class larger than 6 mm (Figure 5.8F). The summary of basal resistance is shown in Table 5. 10. Mutants did not showed statistically significant differences compared to Col-0 for all experiments but showed the same trend.

DISCUSSION

Transcriptomic response to oligogalacturonides

Using our significant criteria around 3244 transcripts (around 8% of the *Arabidopsis* transcriptome), were found to be modulated by post OGs treatment. Most changes were observed after 1h and 6h of OGs treatment (2003 transcripts, approx. 7% of the whole *Arabidopsis* transcriptome, 90% of the total OGs response). Apart from a large number of hypothetical and unknown proteins, these transcripts correspond to genes predominantly encoding stress- and disease-related proteins (e.g. PR- proteins), signaling components, hormone metabolism and signalling, photosynthesis, protein degradation and transcription factors.

Table 5.5: Frequency of occurrence of different transcription factor- binding sites (TFBS) in the promoter region of NO-responsive genes. Promoter regions of NO-responsive genes were screened for common TFBS using Athena program tool. Only TFBS showing significantly different distribution compared to control (total transcriptome of *A. thaliana*) were presented in the table (highlighted in bold).

Motifs	**Control**	**Up-regulated genes**		**Down-regulated genes**	
		Genes 1h (n=194)	*p*-value	Genes 1h (n=156)	*p*-value
W-box	58%	**79%**	1 E-9	61%	0.21
T-box promoter	45%	**57%**	0.01	48%	0.27
CARGCW8GAT	53%	57%	0.238	**64%**	0.006
ARF bing site	30%	30%	0.567	**42%**	0.01
ATMycBS	29%	27%	0.77	**44%**	0.001
MycATERD	29%	27%	0.77	**44%**	0.001
DRE core motif	18%	16%	0.78	**29%**	0.01
		Genes 6h (n=404)	*p*-value	Genes 6h (n=561)	*p*-value
W-box	58%	**75%**	1 E-9	50%	0.99
MYB4 binding site	64%	**70%**	0.002	64%	0.98
Ibox	32%	31%	0.68	**44%**	1 E-7
ATMycBS	34%	28%	0.73	**29%**	0.008
MycATERD	34%	28%	0.73	**29%**	0.008
ABRE	18%	22%	0.02	**25%**	0.001
		Genes 24h (n=104)	*p*-value	Genes 24h (n=103)	*p*-value
CArG promoter motif	6%	**14%**	0.001	4%	0.763
MYB4 binding site	64%	67%	0.277	**76%**	0.003
Ibox	32%	28%	0.826	**45%**	0.004
DRE core motif	18%	12%	0.96	**28%**	0.01
RAV1-B binding site	9%	9%	0.509	**20%**	<0.001

We compared our data with previously published data. Strikingly, we identified a low percentage of common genes (1% to 20%). However, if we consider the comparison between the work of Ferrari et al., (2007) and our work (533 of common genes), MapMan classification performed on the two studies showed a high similarity between the two transcriptomic responses at the functional groups level. We can illustrate this observation with the "PR-proteins" group (BIN 20.1.7). Twenty four genes and 45 genes were classified in this BIN in our study and in the data from Ferrari et al., (2007) respectively. Only 15% of common genes were found when the two studies were compared, but the two global response of this BIN were very similar: the "PR-protein" genes correspond to 2.4% and 1.6% respectively of the transcriptome response, the genes were all up-regulated (except 3 genes in Ferrari et al., (2007) and the level of induction was very similar (2.8 ± 1.3 Vs 2.4 ± 0.95).

We have also observed that transcriptomic response did not follow the same kinetic. For instance, exposure to OGs for 1h has been demonstrated to activate jasmonate and ethylene signalling and biosynthesis pathways (Ferrari et al., 2007). The interaction between these hormones was proposed to determine the type of response to pathogen attack or wounding, including the expression of particular defence proteins such as PR1b, PR5 (osmotin) and PDF2.1. We also identified in our study, after 1h of treatment, transcripts involved in the ethylene biosynthesis and signaling. These included genes encoding different isoforms of aminocyclopropane1-carboxylic acid synthase (ACS) and different ethylene responsive elements; these transcripts being mainly up-regulated. However, transcripts for JA signaling were not identified at early time point but were induced later (6h of treatment).

Finally, we compared our data with data obtained with other polysaccharidic elicitors (data not shown). Again a low level of similarity when the genes were compared individually was observed but the global reponse are most conserved. The transcriptional modifications were predominantly elicitor-specific, but the processes affected surprisingly similar (e.g. hormone signaling, RNA regulation). This indicated that the different elicitors induced changes in similar plant processes through largely non-overlapping transcriptional alterations. In *Arabidopsis*, various transcriptome anyalyses with flg22, elf18, fungal MAMPs and responses to hairpin have display significance overlap between different elicitors (Ramonell et al., 2002; Zipfel et al., 2004; Moscatiello et al., 2006; Thilmony et al., 2006; Truman et al., 2006; Zipfel et al., 2006). This overlap suggests that all elicitors showed a conserved basal response resulting from the

Table 5.6: List of genes selected for functional analysis

Gene ID	Name of gene	DESCRIPTION	Reference
AT1G52900.1	TIR	Disease resistance protein (TIR class), putative ,Toll-Interleukin-Resistance (TIR) domain family protein; FUNCTIONS IN: transmembrane receptor activity; INVOLVED IN: signal transduction, defense response, innate immune response	(Blake et al. 2002; Cartieaux et al. 2008)
AT1G47890.1	RLP7	Receptor Like Protein 7; kinase/ protein binding ,INVOLVED IN: signal transduction, defense response	(Kobe and Kajava 2001; Wang et al. 2008))
AT5G16020.1	SRP (stress related protein)/GEX3	Encodes GEX3, a plasma membrane localized protein expressed in the male gametophyte. Required for micropylar pollen tube guidance. Also plays a role during early embryogenesis.	(Alandete-Saez et al. 2008)
AT5G13080.1	WRKY 75	WRKY75; transcription factor	(Rushton et al. 2010)
AT4G11070.1	WRKY 41	WRKY41; transcription factor	(Rushton et al. 2010)
AT1G10585.1	Transcription factor similar to bHLH	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein	(Bauer et al. 2007)
AT5G53290.1	CRF3 (Cytokinin response factor 3)	Encodes a member of the ERF (ethylene response factor) subfamily B-5 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 7 members in this subfamily.	(Rashotte et al. 2006)

convergence of a limited number of signalling pathways (Jones and Dangl, 2006). More recently, Denoux et al., (2008) showed that flg22 and OGs induced highly correlated early responses, but the response differs in late stages and kinetics.

Categorization of NO-responsive genes

Previous studies demonstrated that NO could modulate the expression of genes. In these studies, the role of NO on gene expression has been assessed by the use of NO donor. NO donor was delivered to the plants by mixing SNP with the irrigation water via the roots (Parani et al., 2004), by the spray of plants (Ahlfors et al., 2009), by treatment with NOR3 of a cell culture or by fumigation of the plant with gaseous NO (Huang et al., 2002; Palmieri et al., 2008). In contrast, in our study we tried to analyse the role of endogeneously produced NO in response to an elicitor of plant defense, the OGs.

About 1.0% of the genes in *Arabidopsis* responded to OGs treatment, and about 50%-80% of these genes behaved in a NO-dependent manner. The identified NO-responsive genes categorize most closely within the biotic stress category. Genes induced by OGs treatment were represented in all categories already identified in transcriptomic response to OGs indicating that NO did not clearly control a specific class of genes among OGs-responsive genes (it should be noticed that approximatively 50% of the transcriptome response correspond to gene encoding protein of unkwown function). However, MapMan analysis allowed to identify two BINs that were significantly affected in NO-regulated transcriptomic response at 1h of treatment (Table 5.5A), but not in transcriptomic data from OGs treatment alone, at the same timeintervals (Table 5.5A). These two BINS were 20.1.17 “PR-proteins” and “27-3-32 WRKY TF”. This result indicated that genes belonging to these categories were mainly regulated by NO (more often regulated by NO than others).

Seven genes were identified in this “PR-protein” group (BIN 20.1.17). It is important to notice that these genes were all up-regulated (Figure 5.5). These genes encode protein involved in different functions: PR-proteins such as defensin (PR-12), putative disease resistance protein (R-proteins or PAMPs receptors).

Identification of WRKY “27-3-32” among NO-responsive genes was especially interesting. Firstly, members of the WRKY gene family were known to regulate various physiological processes, including pathogen defence, plant responses to biotic stress, (Du and

Table 5.7 : Fold change of selected NO-responsive genes for functional study at 1h, 6h and 24h of treatment (X : non-induced or repressed gene)

Gene ID	Name of gene	1 h	6 h	24 h
AT1G52900.1	TIR	71.25	11.058	x
AT1G47890.1	AtRLP7	25.84	x	x
AT5G16020.1	SRP (stress reelated protein)/GEX3	8.56	-6.096	9.34
AT5G13080.1	WRKY 75	57.7	x	x
AT4G11070.1	WRKY 41	19.93	x	x
AT1G10585.1	Transcription factor similar to bHLH	x	17.13	x
AT5G53290.1	CRF3 (Cytokinin response factor 3)	x	10.444	x

Table 5.8: Comparison of expression pattern of selected NO-responsive genes using microarray and RT-qPCR analysis. Time points where the genes were identified as NO responsive (significantly repressed by cPTIO) are indicated for both analyses.

Gene ID	Name of gene	Microarray analysis	RT-qPCR
AT1G52900.1	TIR	**1h, 6h**	**x**
AT1G47890.1	AtRLP7	**1h**	**1h, 3h, 6h**
AT5G16020.1	SRP	**1h, 6h, 24h**	**1h, 6h**
AT5G13080.1	WRKY 75	**1h**	**3h, 6h**
AT4G11070.1	WRKY 41	**1h**	**1h, 3h, 6h**
AT1G10585.1	Transcription factor similar to bHLH	**6h**	**3h, 6h**
AT5G53290.1	CRF3 (Cytokinin response factor 3)	**6h**	**3h, 6h**

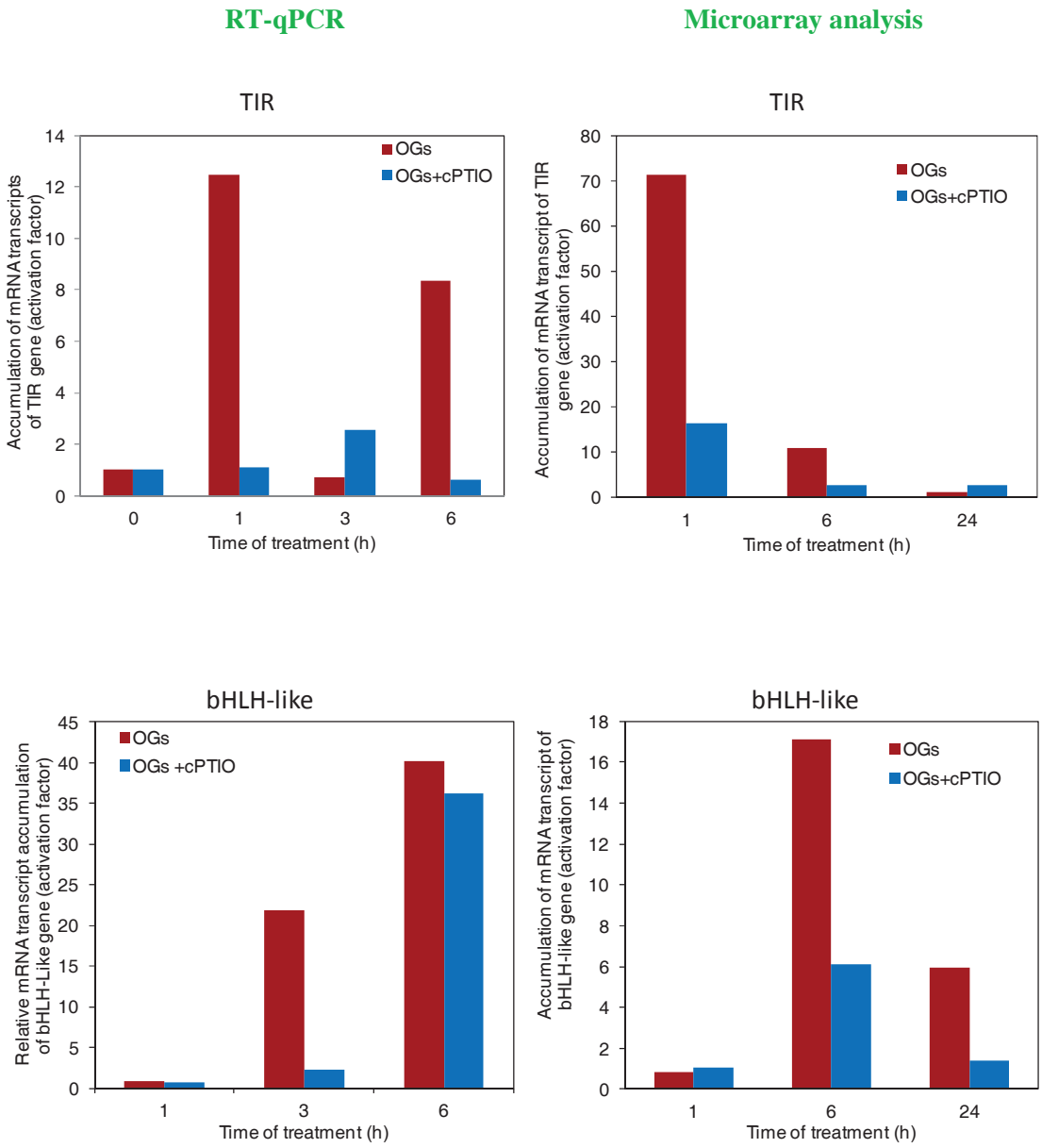
Chen, 2000; Robatzek and Somssich, 2001; Johnson et al., 2002; Dong et al., 2003; Eulgem and Somssich, 2007; Higashi et al., 2008; Pandey et al., 2010). Overall WRKY proteins structures have a 60 amino acids DNA binding domain (WRKY domain) and they are categorized into three groups which might reflect their different function (Eulgem et al., 2000). Secondly, transcription factors have the ability to regulate other NO target genes.

Many studies have reported the ability of WRKYs to bind the W-box element (TTGACC/T; Rushton et al., 2002; Yamasaki et al., 2005), which was found in the promoters of many plant defence genes (Maleck et al., 2000; Chen et al., 2002). Our *in silico* analysis revealed, among the target genes induced at 1 h and 6 h post OGs treatment, an enrichment of W-box binding motifs recognized by the WRKY family. In *Arabidopsis thaliana*, Palmieri et al., (2008) also reported enrichment in binding sites among the WRKY genes induced during treatment with an NO donor (NOR-3) and gaseous NO. These observations suggested that certain transcription factors, targets of NO and identified in this study, could control other NO target genes such as genes involved in the defenses. Studies demonstrated that expression of resistance gene RPP8 to mildew (*Hyaloperonospora arabidopsidis*) in *A. thaliana* was regulated by WRKY for the W-box motifs (Mohr et al., 2010).

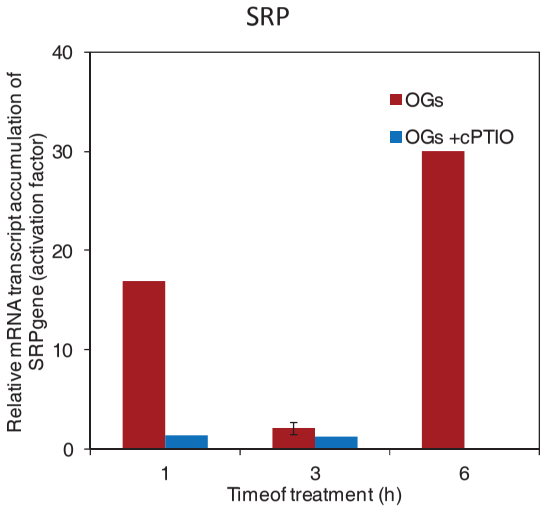
Another striking feature of the NO-regulated transcriptome response was the coordinated response of some groups of genes. For instance, we observed that, at 1h of treatment, all the genes belonging to Auxin and PR-protein categories were down- and up-regulated respectively. The co-expression of these genes can be explained by the cooperation of a set of TF that bind a common region in the promoter of the regulated genes. Corroborating this hypothesis, we observed that 100% of the "PR-proteins" contain W-box and 100% of Auxin down-regulated genes (1h) contain MycERD BS.

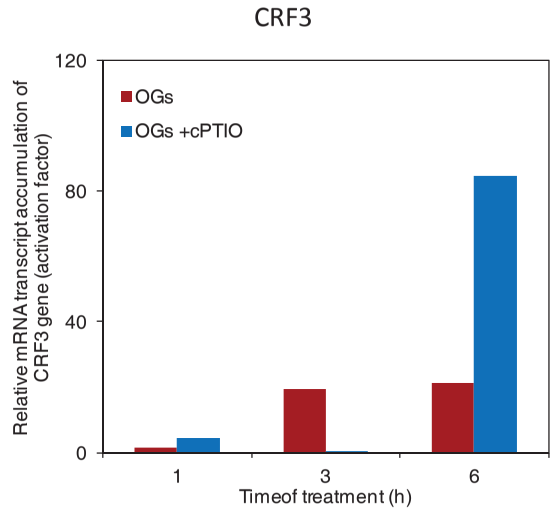
The overlap of NO-responsive genes was compared with the experiments from Parani et al., (2004), Badri et al., (2008), Palmieri et al., (2008) and Ahlfors et al., (2009) performed with NO donors. If we consider the data of Ahlfors et al., (2009), of the 614 genes regulated by SNP in *A. thaliana* plants, only 32 and 51 were identified in our study, at 1h and 6h of treatment respectively (approx. 6% of similarity). These genes were categorized in processes such as ethylene biosynthesis and signaling, disease or defense against pathogens, RNA regulation (TFs). Notably, genes invoved in auxin signalling were not identified with studies with NO donors and

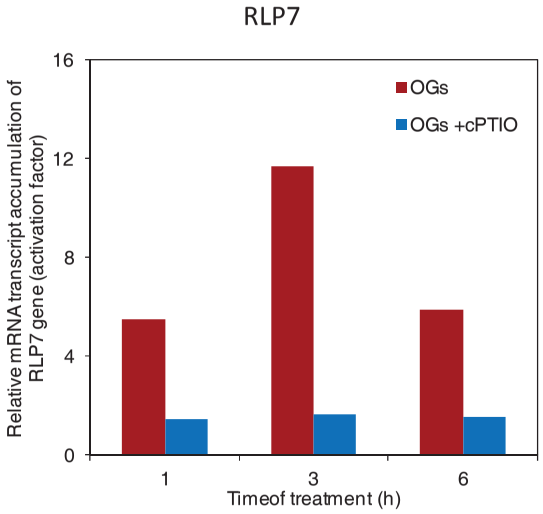
Figure 5.6: Comparison of expression pattern of selected NO-responsive genes (*TIR, SRP, RLP7, WRKY41, WRKY75, CRF3, bHLH*) using microarray and RT-qPCR analysis. Leaves were treated with water or OGs with in the presence or absence of cPTIO. The transcript accumulation was analyzed by real-time qPCR (left panel). After normalization with *UBQ10*, results are expressed as the fold changes in transcript level compared to the control (water-treated and cPTIO treated). The bar graph is the one representative of three biological repeats. On the right panel, the fold-change patterns from the microarray analysis (bar graphs are the mean of three biological replicates).



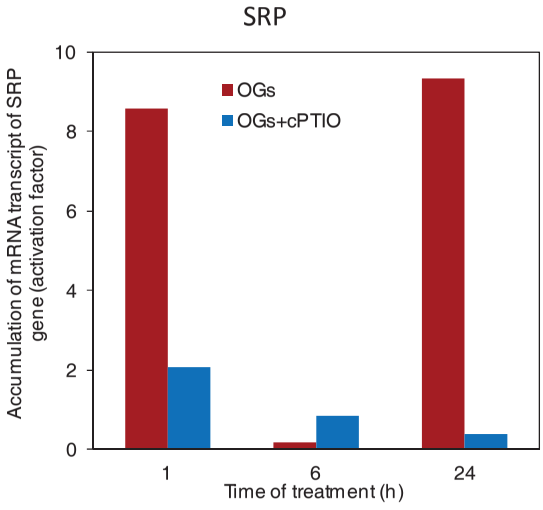
RT-qPCR

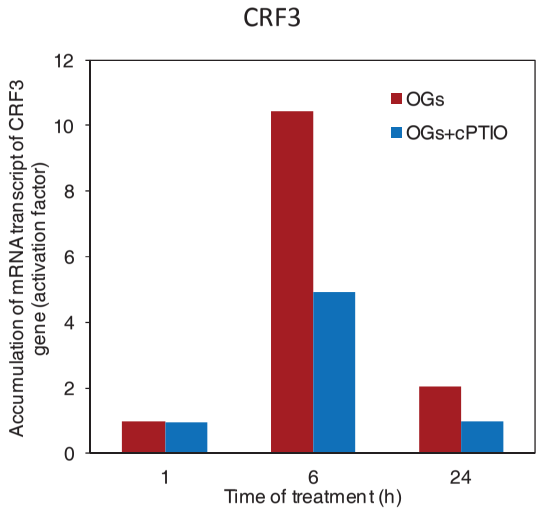


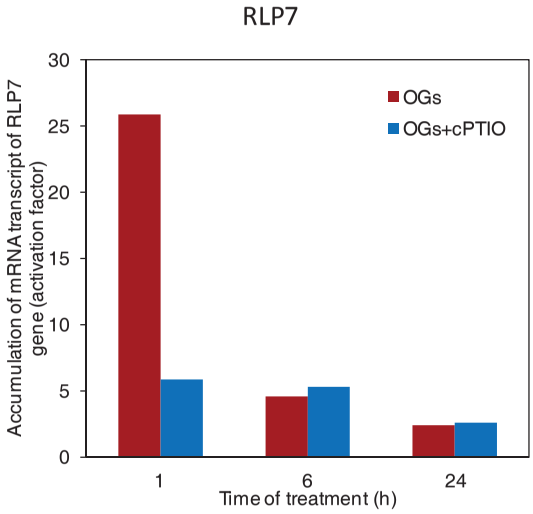




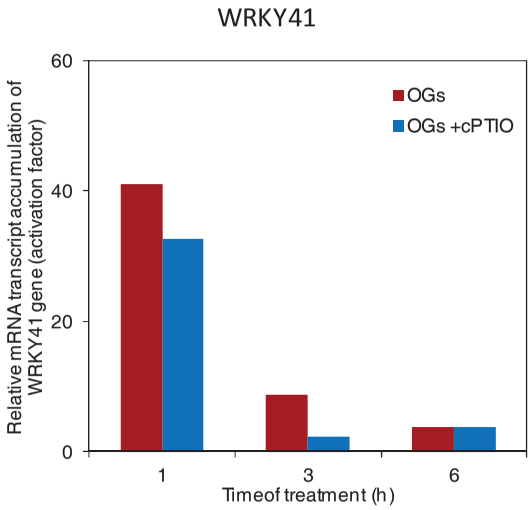
Microarray analysis

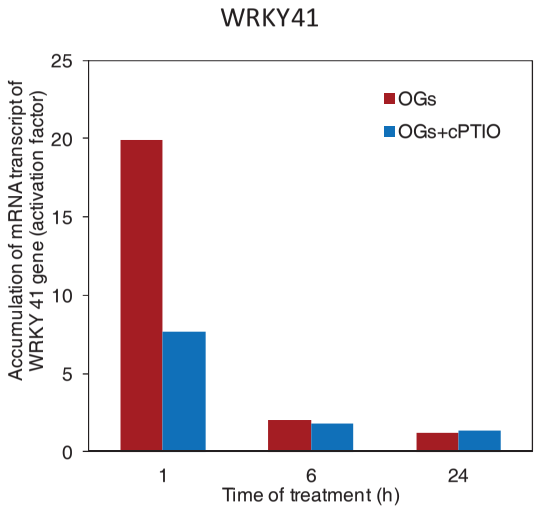


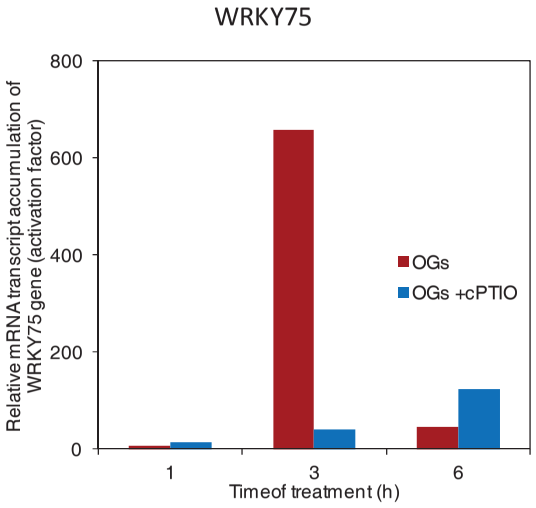


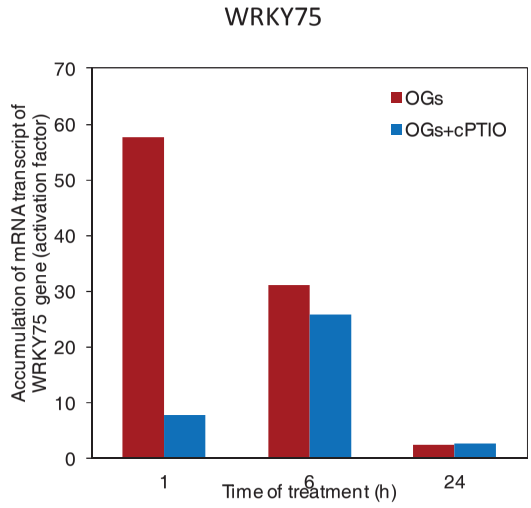


RT-qPCR **Microarray analysis**









could correspond to an example of NO-responsive genes in a biological context (elicitation with OGs).

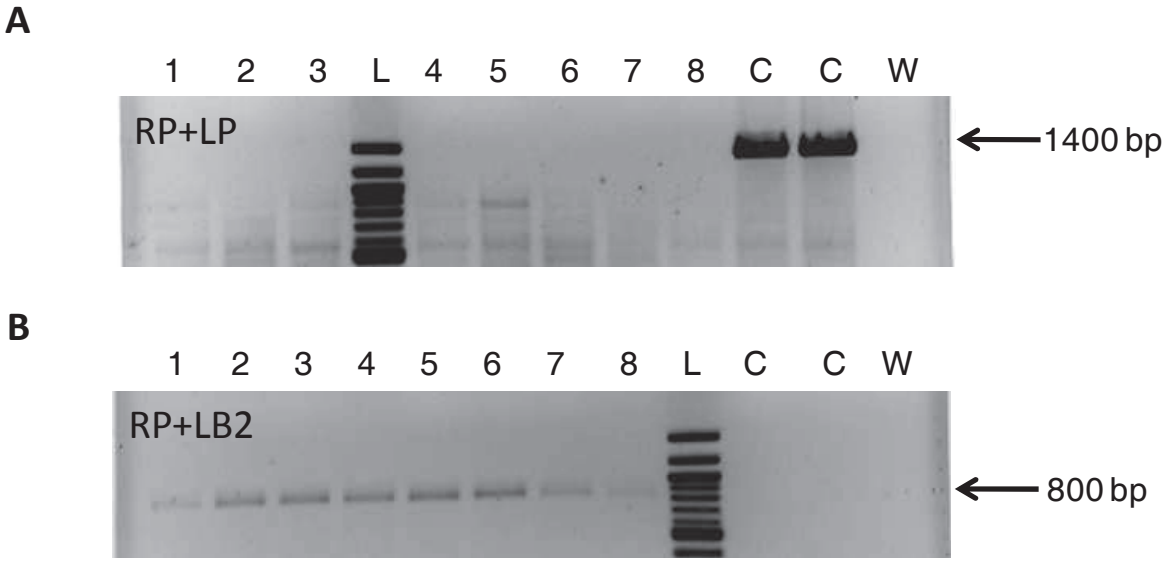
The effect of auxin in plant defense and particularly the antagonistic action between this hormone and elicitors have been firstly reported by Leguay and Jouanneau (1987) and Branca et al., (1988). These authors described that defense responses induced by a *Phytophthora* glucan preparation was inhibited by auxin in protoplasts and that auxin-induced growth was competitively inhibited by elicitor-active OGs in pea stem segments. OGs antagonize the action of auxins (inhibition of auxin-induced cell wall elongation and root formation; Bellincampi et al., 2000) and at the molecular level affect the expression of late but not early auxin-responsive genes (Bellincampi et al., 2000; Mauro et al., 2002). Recently it was reported that this inhibition did not require ethylene, JA or SA and was independent of AtRBOHD ROS production (Savatin et al., 2011). Interestingly, our transcriptomic analysis suggests that NO produce upon treatment with OGs was involved in the inhibition of auxin induced genes. The use of transgenic plants carrying an auxin responsive promoter-glucuronidase (GUS) gene fusion may help to verify the effect of NO as a mediator of the antagonistic effect of OGs on auxin response (Mauro et al., 2002).

Functional characterization of NO-responsive genes in the *B. cinerea/A. thaliana* interaction

Functional analysis of transcription factors

Four transcription factors (bHLH-like, CRF3, WRKY41 and WRKY75) were analysed during *A. thaliana* interaction with *B. cinerea*. Regarding the WRKY41 gene, we observed reduced necrosis in the mutant, indicating an increased resistance of the plant against *B. cinerea*. It could be hypothesized that the pathogen is able to modulate the expression of WRKY41, in order to promote its development. It was reported that WRKY41 was induced by the inoculation of *Arabidopsis* with the incompatible pathogen *Pseudomonas syringae* pv. Tomato strain pto (Higashi et al., 2008) and that plants over-expressing WRKY41 showed increased resistance to Pto strain but greater sensitivity in *Erwinia carotovora* EC1. Thus, these data indicated that the role of WRKY41 either used by the pathogen, or by the plant for defense reactions depends on the nature of the infecting pathogen.

Figure 5.7: Genotyping of mutant lines (WRK75). **A:** PCR amplification using RP-LB2 primer pair. **B:** PCR amplification using specific primer pair LP-RP. 1 to 8: Mutants plants tested, L: Ladder molecular weight marker 100 bp; C: Col-0 wild types negative control for A and control positive in B, W: water: negative control for both.



Mutants impaired in WRKY75 displayed no significant difference after *B. cinerea* infection. As reported earlier, *WRKY75* gene was strongly induced by environmental variations {phosphate deprivation (Devaiah et al., 2007), pathogen infection (Dong et al., 2003) or according to physiological stage (senescence, Guo et al., 2004)}. In constrast to our results, it was shown to act as a positive regulator of defense response in both compatible and incompatible interactions (Encinas-Villarejo et al., 2009).

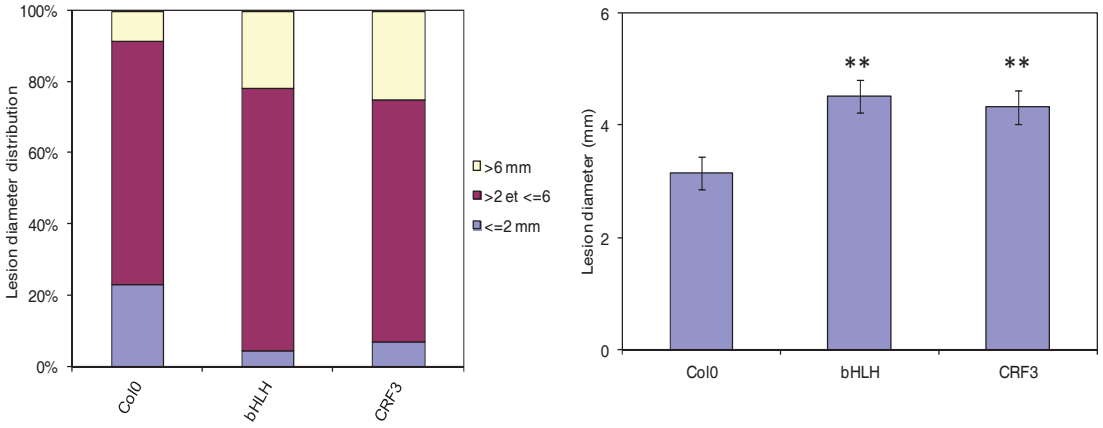
The functional link between WRKY protein and defence genes during defense response has been shown only for specific WRKY proteins. For example, AtWRKY70 was identified as a common regulatory component of SA- and JA-dependent defence signalling, mediating cross-talk between these antagonistic pathways. Over-expression and antisense lines indicated that WRKY70 plays a positive role in SA signalling and functions as a negative regulator of JA-inducible genes (Li et al., 2004). It was reported that AtWRKY70 regulated both senescence and plant defence by SA-mediated pathway. In contrast, WRKY18, may function as negative regulators in SA-dependent pathways but play a positive role in JA-mediated pathways (Xu et al., 2006). Additionally, two other AtWRKY53 and AtWRKY58 were identified as a positive and negative regulator of SAR (Wang et al., 2006). WRKY genes (AtWRKY18, -38, -53, -54, -58, -59, -66 and -70) were reported as a direct targets of NPR1 (Wang et al., 2006). WRKY are involved in response to a large spectrum of bioaggressors. For instance, AtWRKY6 were involved in resistance, over-expression of these increased resistance to both bacterial and fungal pathogens (Asai et al., 2002) and more recently some authors have demonstrated the role of WRKY6 as a positive regulator of the production of ROS in response to insects (Rushton et al., 2010).

As compared to Col-0, *bhlh* mutant appeared more susceptible to *B. cinerea*, highlighting that b*HLH*- like gene is important for setting the defense responses of the plant. We observed that inoculation of *A. thaliana* by *P. syringae, B. cinerea* and the application of MeJA, Flg22, and OGs induced *bHLH*-like transcripts accumulation; in contrast, other strain of *P. syringae* infection induced down-regulation of these transcripts {Genevestigator Expression Data (https://www.genevestigator.com/gv/)}. bHLHs activities are not restricted to biotic stress. These TFs are involved in transcriptional regulation of genes associated with phytochrome signaling, circadian clock, anthocyanin biosynthesis, globulin expression and fruit, carpel and epidermal development and tolerance to phosphate starvation (Heisler et al., 2001; Rajani and Sundaresan,

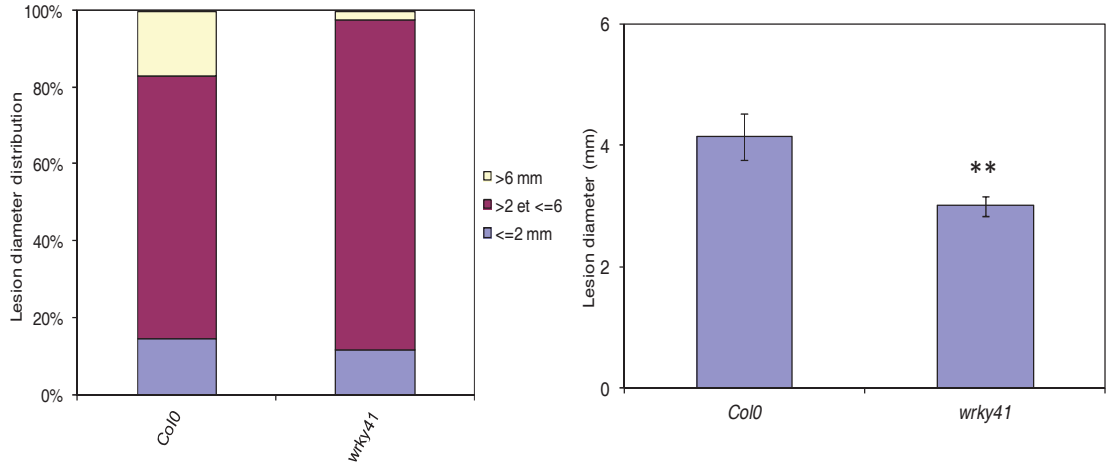
Figure 5.8: Basal resistance to *B. cinerea* in *Arabidopsis thaliana* mutant lines **A;** *crf3* and b*hlh*, **B;** *wrky41*, **C;** *wrky75* **D;** *tir*, **E;** *rlp7* and **F;** *srp*). Four weeks-old plants were inoculated with BMM (5.10^4 spores/mL) and symptoms were scored 3 days later. On the left panel, Bar graph with percentage lesion diameter distribution in three different categories and on the right panel, bar graph mean of lesion diameter. These graphs are the representative of 3 independent biological experiments. Data are the mean ± SE of 40 lesions per genotype.

Lesion diameters distribution **Average lesion diameters**

A) *bhlh* and *crf3*



B) *wrky41*



2001; Makino et al., 2002; Yi et al., 2005). Moreover, bHLH was also described as H_2O_2-responsive transcription factors (Inzé et al., 2011).

As observed with *bhlh*, the *crf*3 mutant showed greater necrotic lesions size suggesting that this mutant line was more sensitive to *B. cinerea*. Thus, this would highlight the role of CRF3 in setting defense responses of the plant. Inoculation of *A. thaliana* by *P. syringae* resulted in an increased level of CRF transcripts accumulation {Genevestigator Expression Data (https://www.genevestigator.com/gv/)}. In contrast, oomycete *Plasmopara parasitica* infection led to decrease CRF transcripts accumulation.

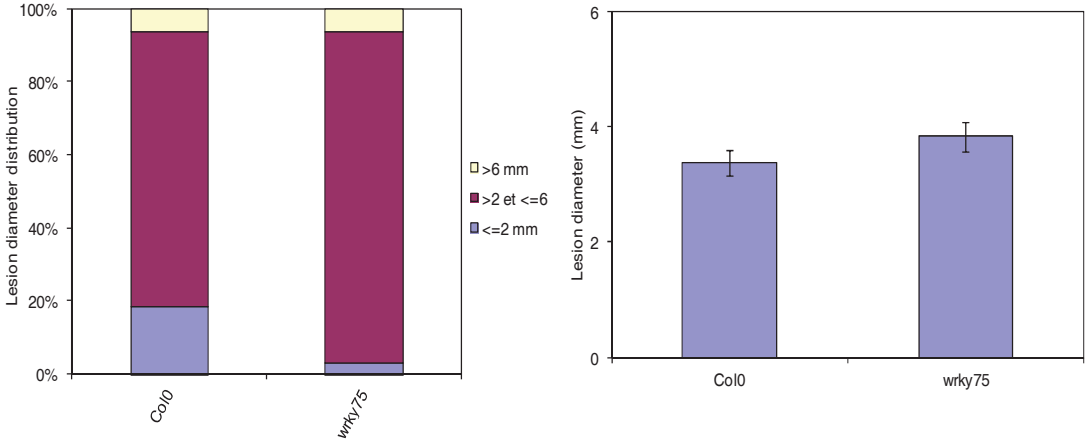
Functionnal analysis of disease related proteins

The role of SRP2 (stress related protein, At5g16020) and two putative disease resistance proteins involved in pathogen recognition (in broad sense), TIR (At1g52900) and RPL7 (At1g47890), were studied using corresponding T-DNA mutant lines, named respectively *srp2*, *tir* and *rpl7*. Contrary to *srp2* mutants, the *tir* and *rlp7* mutants showed more susceptibility to *B. cinerea*.

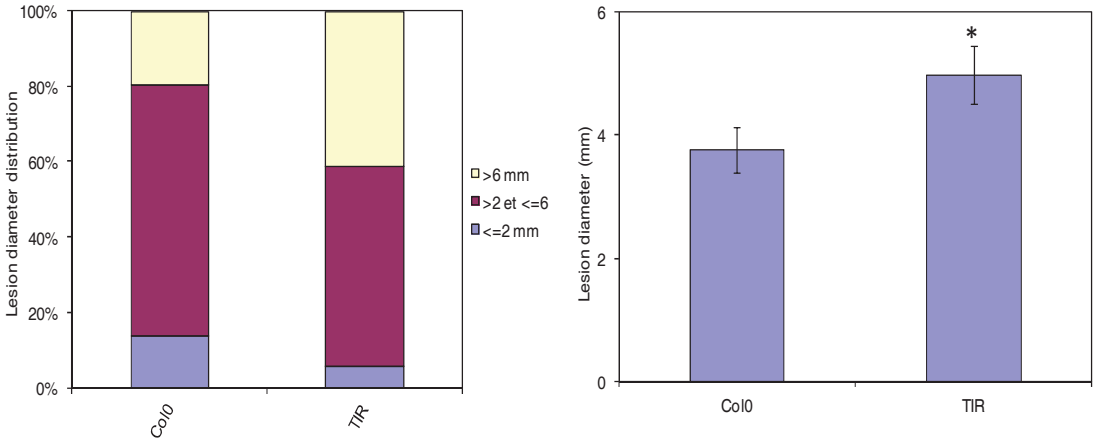
The NBS-LRR family belonging to the R-genes group has been subdivided further based on the presence or absence of an N-terminal Toll/Interleukin-1 Receptor (TIR) homology region : the TIR –NBS-LRR and the CC-NBS-LRR (Meyers et al., 2002). Besides the TIR-NB-LRRs, plants also have genes that code for 'incomplete' TIR-NB-LRRs: TX and TN genes. At1g52900, identified in our study, has been classified into the TX family (Meyers et al., 2002). The TX proteins possess an N-terminal TIR domain, but lack the NB-LRR domain. Whether TX proteins play a role in plant innate immunity is not clear. In addition to OGs, *TIR* gene was up-regulated in response to *B. cinerea, Pseudomonas syringae pv. Maculicola, Pseudomonas syringae pv. Tomato, Plasmopara parasitica* as well as ABA, and Flg22 {Genevestigator Expression Data (https://www.genevestigator.com/gv/)} confirming its role in biotic stress response. It was reported that the TX gene NRSA-1, was highly induced in the roots and leaves from the non-host plant marigold (*Tagetes erecta*) that is attacked by the parasitic weed *Strigia asiatica* (Gowda et al., 1999) NRSA-1: a resistance gene homolog expressed in roots of non-host plants following parasitism by *Striga asiatica* (witchweed; Gowda et al.,, 1999). Jasmonic acid (JA) treatment also resulted in the induction of NRSA-1 expression, but wounding or treatment with salicylic

Lesion diameters distribution **Average lesion diameters**

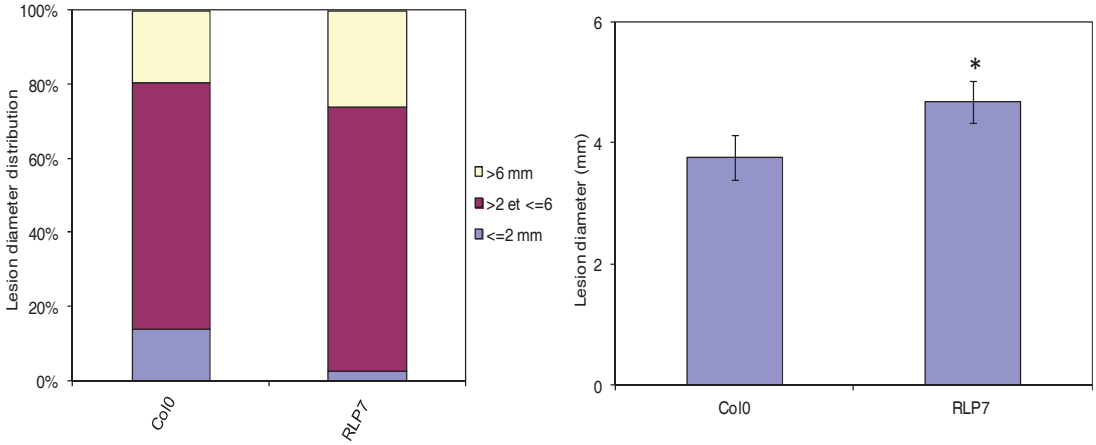
C) *wrky75*



D) *tir*



E) *rlp7*



acid (SA), paraquat or abscisic acid (ABA) did not. Further investigations will be needed to confirm the role of this protein in plant-pathogen interaction.

The T-DNA mutant line *rpl7* also showed more susceptibility to *B. cinerea*. Receptor-like proteins (RLPs) are cell surface receptors. They typically contain extracellular leucine-rich repeats (LRRs) and a short cytoplasmic tail linked by a single transmembrane motif. RLPs do not have an intracellular kinase domain (unlike RLKs). In *Arabidopsis thaliana*, there are 57 putative RLP genes (Wang et al., 2008) A genome-wide functional investigation into the roles s of receptor-like proteins in *Arabidopsis*). *Arabidopsis* RLP52 was implicated in resistance against the powdery mildew pathogen *Erysiphe cichoracearum* (Ramonell et al., 2005), whereas *Arabidopsis* RLP30 was suggested to influence nonhost resistance toward *Pseudomonas syringae* pv *phaseolicola* (Wang et al., 2008). *RLP* genes were induced by various stimuli. For instance, *in silico* analysis showed that, in addition to OGs, *RLP7* was induced under nitrate starvation, nitrogen depletion, Flg22 and Elf-26. In contrast, the gene was repressed upon LPS or hormones treatment and infection upon *Pseudomonas syringae pv. Tomato* (https://www.genevestigator.com/gv/)}. Recently, Zhang et al., (2010) demonstrated that the *snc2-1D* line, mutated for one RLP, in *A. thaliana* exhibit enhanced disease resistance against the virulent oomycete *Hyaloperonospora arabidopsidis* (Zhang et al., 2010). Moreover, mutations in WRKY70 suppressed the defense responses in *snc2-1D*, suggesting that WRKY70 functions downstream of *snc2-1D*.

Six out of the seven plant mutants impaired in NO-responsive genes seem to play a role in the *A. thaliana / B. cinerea* interaction. The four TFs (*wrky41*, *wrky75*, *bhlh* and *crf3*) showed different behavior and were classified, in our biological model, as positive or negative regulator of plant resistance. Previous studies reported that this behavior was also dependent of the pathogen nature. The confirmation of the involvement of these seven selected NO-responsive genes in plant-pathogen interaction would be assessed by analyzing the transcriptomic response (or the expression of marker defense genes) and plant defense response events (e.g. ROS production, MAPK activation…) of these mutants. Finally, Genevestigator data mining revealed that these TFs and these defense genes could be induced by different stimuli. It could be interesting to verify if these inductions were NO dependent.

Lesion diameters distribution **Average lesion diameters**

F) *srp*

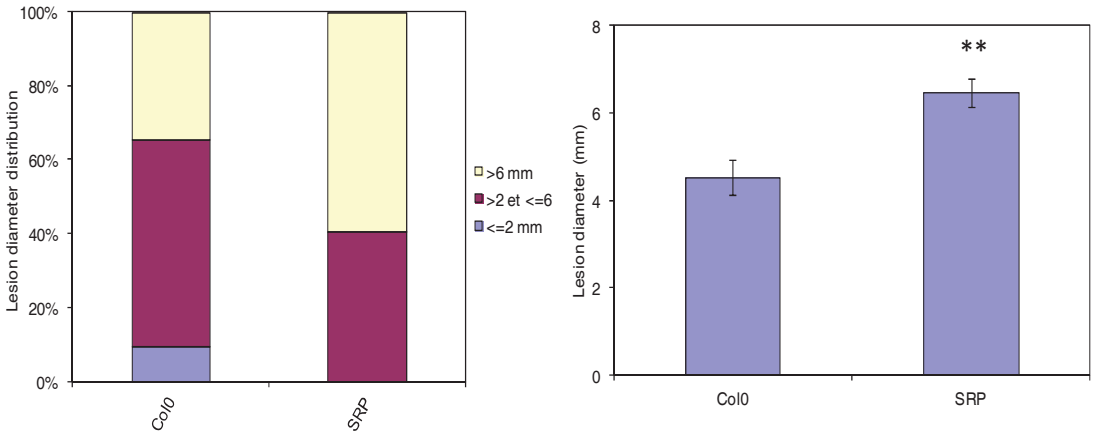


Table 5.9: Basal resistance to *B. cinerea* in *Arabidopsis thaliana* mutant lines impaired in NO-responsive genes. Data are the mean ± SE of 40 lesions per genotype for individual independent biological experiment. S= Susceptible, R= resistant, ND = not determined.

Gene ID	Gene name	Number of performed Experiments	Tendency	Statistically Significant experiments	Conclusions
At1g52900	TIR	3	S	1	S
At1g47890	RLP7	3	S	1	S
At5g16020	Stress related protein	3	S	2	S
At5g13080	WRKY 75	5	ND	0	ND
At4g11070	WRKY 41	5	R	3	R
At1g10585	Transcription factor similar to bHLH	3	S	1	S
At5g53290	CRF3 (Cytokinin response factor 3)	5	S	3	S

In conclusion,

- ✓ The NO-regulated transcriptome response mainly oriented towards response to biotic stress,
- ✓ the significant modulation of the WRKY family in the NO-regulated transcriptome response,
- ✓ the over-representation of TFBS or putative regulatory motifs in the promoters region of NO responsive genes,
- ✓ and the involvement of some identified NO-responsive genes in the resistance of *A. thaliana* to *B. cinerea* and particularly one WRKY (WRKY41),

led us to hypothesize that:

- ✓ NO could exert its effect by the transcriptional regulation of defense genes,
- ✓ WRKY family plays an important role in the NO effect,
- ✓ NO-responsive genes could be the target of TFs transcriptionaly regulated by NO (indirect effect of NO). Corroborating this hypothesis, we observed the presence of W-box (WRKY binding sites) in the regulatory region of the two "R-genes" analysed in our study. We can not exclude that NO responsive-genes could be the target of TFs activated by NO-induced post-translational modifications. WRKY transcription factors also regulate the expression of their own genes and/or other WRKY genes in addition to the defense-related genes, showing positive and negative feedback mechanisms (Eulgem and Somssich, 2007).

Finally, the low level of similarity between studies performed with NO donors or NO scavenger in a biological context reflect the difficulty to compare transcriptome studies in order to identify common responsive NO elements in plants. Comparison of NO-regulated transcriptome response in different biological contexts will be helpful to elucidate how NO could exert its activity in gene expression and in plant response to internal and external stimuli.

CONCLUSIONS AND PERSPECTIVES

CONCLUSIONS AND PERSPECTIVES

My thesis project aimed to understand the role of NO in cellular signaling processes activated in plants during biotic stresses and particularly in response to OGs, a plant defense elicitor.

Firstly, we investigated the putative enzymatic sources of NO production and its regulation. We demonstrated that, as already published in other physiological contexts, NR as well as L-arginine-dependent activities, were involved in NO production in response to OGs. Nevertheless, we hypothesized that at least one other unknown source of NO production could be involved. Interestingly, the enzymatic sources described do not work independently, as revealed by the effects of inhibitors of L-arginine dependent pathway on NR activity. We also demonstrated that this OGs-induced NO production is tightly related to other signaling events. NO production was partially regulated by Ca^{2+} influx and CDPKs activities, important for triggering ROS burst but worked independently of the MAPKs pathway.

Secondly, using a candidate gene approach, we identified two genes regulated by NO, that take place in the biological response of *A. thaliana* to *B. cinerera*. These genes encode proteins related to plant defense and include anionic peroxidase (PER4) and β-1,3-glucanase. These two proteins are classified in the PR family, PR9 and PR2 respectively and are suspected to have a direct effect on pathogens.

Thirdly, we have sought to investigate the "immunomodulatory" role of NO production at a more global gene expression level by microarray. This part of my thesis concerned the description of the NO-regulated transcriptome response and the identification of NO target genes (NO-responsive genes). Our results suggested that NO can induce its effects in plant-pathogen interactions context by altering the expression of certain genes such as genes encoding transcription factors and disease-related proteins. The over-representation of certain regulatory elements (e.g. W-BOX) in promoter sequences of target genes also suggests the involvement of specific transcription factors. NO can control the expression of genes by direct or/and indirect processes. We suspected that the expression of a major part of NO-responsive genes, involved in the establishment of plant defense reactions, was indirectly controlled by certain transcription factors (TFs), this latter being directly modulated by NO (via regulation of gene expression or post-translational modifications).

Taking together, our investigations decipher part of the mechanisms linking NO production, NO-induced effects and basal resistance to *Botrytis cinerea*. More generally, our data reinforce the concept that NO is a key mediator of plant defense response. Thus, the consideration of cellular NO production and its regulation could be an element of interest to control and manipulate the plant responses leading to resistance. It should be noticed that, independently of the enzymatic sources of NO, nitrogen, mainly supplied by NO_3^-, is the substrate for enzymatic NO production in plants. In this context, we can suspect that N plant nutrition (fertilization) could affect NO production and indirectly plant resistance.

In terms of perspectives, at a short term, some results need to be confirmed. The most important point concerns the role of some NO target genes in plant-pathogen interaction. The use of second mutant line for each selected genes will reinforce the role of the corresponding protein. Moreover, the use of a second plant pathogen model for instance *Arabidopsis / Hyaloperonospora arabidopsidis* will give a more general overview on the role of these proteins in biotic response to microbes.

At a longer term, we will investigate two directions.

The first one, we will try to better understand mechanisms involved in NO production, and particularly the link between nitrite dependent and L-arginine dependent pathways. We can hypothesize that the stimulating effect of NO on NR activity is regulated through the mechanism of a direct interaction between NO and NR protein. Both haem group and cysteine residues being incorporated in the NR, it will be interesting to test if the activating effects of NO on the NR could be due to NO binding to haem group and/or S-nitrosylation of cysteine.

The second direction is related to the analysis of the effect of NO in plant response to pathogens. It needs to be continued by analyzing the role of the identified NO-responsive TFs (such as WRKY41 and CRF3, if we confirm their involvement in resistance). Studies of physical interaction between transcription factors and promoter regions of resistance related proteins regulated by NO could be performed. Moreover, our team is generating, with different model, a catalog of proteins “S-nitrosylated” in response to pathogens or elicitors. Special attention will be given on the identification of TFs that could be involved in the regulation of disease-related genes. Finally, a comprehensive study of the expression of NO-responsive genes in the mutants

lines impaired in TFs in response to OGs will help us to demonstrate their role in modulating signaling controlled by NO.

CHAPTER 6

"References"

CHAPTER 6

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SUMMARY IN FRENCH

"RÉSUMÉ LONG"

"Caractérisation et rôle de la production de monoxyde d'azote induite par les oligogalacturonides chez Arabidopsis thaliana"

RÉSUMÉ LONG

Caractérisation et rôle de la production de monoxyde d'azote induite par les oligogalacturonides chez *Arabidopsis thaliana*

I. Introduction

Dans leur environnement, les plantes sont exposées à de nombreux microorganismes potentiellement pathogènes. Afin de se protéger, les plantes ont acquis la capacité de répondre aux agressions par l'activation d'une large gamme de mécanismes de protection. Ces réponses de défense induites sont initiées par la reconnaissance du pathogène et peuvent conduire à l'état de résistance de la plante. Les molécules libérées lors de l'interaction qui participent à la reconnaissance des pathogènes déclenchent une cascade de signalisation mettant en œuvre de nombreux messagers. Le monoxyde d'azote (NO) fait partie de ces messagers. Le NO est une molécule ubiquiste impliquée dans ne nombreux processus physiologiques dans le monde vivant (Toreilles, 2001, Besson Bard *et al.* 2008).

Chez les mammifères, le NO est synthétisé à partir de la L-arginine (L-arg) grâce à une enzyme, la NO synthase (NOS). Chez les plantes, le NO dérive également de précurseurs azotés, mais ces derniers sont plus divers que chez les animaux. Brièvement, le NO est généré selon deux voies enzymatiques différentes : la voie L-arginine dépendante et la voie nitrite-dépendante (Besson Bard *et al.* 2008). De nombreuses études ont démontré l'existence d'un processus grâce auquel le NO est produit à partir de la L-arg par une enzyme inconnue, sensible au inhibiteurs de NOS bien qu'aucun homologue de NOS de type animal n'ait été identifiée dans les génomes de plantes terrestres (del Rio *et al.* 2004). Tun *et al.* (2008) ont décrit la présence possible d'une voie impliquant les polyamine-oxydases (PAOx) et utilisant les polyamines (PA) comme substrat, la L-arg étant un précurseur des PA. Des études *in vivo* et *in vitro* ont démontré que le NO est également produit par la nitrate réductase (NR) quand les nitrites sont utilisés comme substrat (Yamasaki *et al.* 2000). Cette production de NO à partir de la NR est possible dans des conditions physiologiques particulières comme des concentrations en nitrite importantes. L'implication de la

NR comme source enzymatique du NO a été décrite dans des conditions physiologiques telles que la fermeture des stomates induite par l'ABA, le stress froid, l'hypoxie ou la fixation de l'azote atmosphérique dans les nodules (Cantrel *et al.* 2010; Desikan *et al.* 2002; Dordas *et al.* 2004; Horchani *et al.* 2011; Zhao *et al.* 2009). Enfin le NO peut être synthétisé à, partir des nitrites dans les mitochondries à partir de la chaine de transport des électrons (Horchani *et al.* 2011).

Le NO est capable de réguler, chez les plantes, de nombreux processus physiologiques tels que les réponses aux stress biotiques et abiotiques ou le développement de la plante (Wilson *et al.* 2008). Un des premiers rôles attribués au NO a été la mise en place de la résistance des plantes contre les pathogènes par l'induction de réponses de défense (Wendehenne *et al.* 2004). Dans plusieurs modèles, il a été montré que le NO était rapidement produit suite à l'interaction entre la plante et le pathogène (Vandelle & Delledonne, 2008). De plus, cette augmentation de la concentration intracellulaire de NO, induite par les pathogènes ou par un traitement exogène (donneurs de NO), est capable d'induire les réponses moléculaires de défense des plantes telles que l'expression de gènes codant pour les protéines PR (pathogenesis-related proteins) ou la synthèse de molécules à activités antimicrobiennes comme les phytoalexines et pourrait ainsi participer à la résistance des plantes (Delledonne *et al.* 1998 ; Leitner *et al.* 2009). Plus récemment, il a été démontré que des plantes d'Arabidopsis mutantes affectées dans la production de NO sont plus sensibles aux pathogènes (Perchepied *et al.* 2010 ; Rasul *et al.* soumis).

Les recherches conduites depuis plusieurs années indiquent que les effets biologiques du NO sont associés à la modulation d'activité protéine kinase, des modifications post-traductionnelles des protéines et/ou à la mobilisation du calcium ou d'autres messagers (Besson-Bard *et al.* 2008). Plus particulièrement, il a été démontré que les effets du NO dans les réponses immunes des plantes sont liés à son interaction avec les formes activées de l'oxygène (FAO) (Zaninotto *et al.* 2006).

Cependant, en dépit des nombreux résultats associant le NO aux réactions de défense des plantes, les mécanismes moléculaires de défense impliquant la production du NO et ses activités physiologiques sont très mal connus. Afin de comprendre le rôle du NO dans le contexte des interactions plante-pathogène, nous avons utilisé comme modèle d'étude la plante *Arabidopsis thaliana* traitée par les oligogalacturonides (OGs) ou inoculée par le champignon nécrotrophe

Botrytis cinerea. Les OGs sont des composants des parois cellulaires végétales. Ils sont produits durant l'infection des plantes par un pathogène suite à des activités polygalacturonases de l'agresseur (Ferrari *et al.* 2008). Les OGs sont considérés comme des éliciteurs endogènes, ou DAMPS pour Damage-Associated Molecular Patterns (Schwessinger & Zipfel 2008). Ils induisent chez Arabidopsis une variété de réponses de défense incluant la production de FAO et de NO (Galletti *et al.* 2008 ; Rasul *et al.* soumis). Des études transcriptomiques ont montré qu'approximativement 50% des gènes d'Arabidopsis régulés par les OGs le sont également lors d'une interaction avec le champignon nécrotrophe *B. cinerea*, suggérant qu'une partie des réponses activées par le champignon sont médiées, directement ou non, par l'accumulation des OGs (Ferrari *et al.* 2007). Etant donné qu'ils sont capables de mimer en partie les interactions plante-pathogène, les OGs représentent un outil intéressant pour analyser les mécanismes impliqués dans les interactions plante-pathogène.

II. Objectif de la thèse

Le cadre général de ce travail est l'étude du rôle du NO lors de la mise en place des mécanismes de défense des plantes contre les microorganismes pathogènes.

Les objectifs du travail étaient :

- l'identification des mécanismes jouant un rôle dans la biosynthèse du NO et dans sa régulation en réponse aux OGs chez *Arabidopsis thaliana*. Dans ce but, nous avons analysé la production de NO en réponse aux OGs grâce à des approches génétiques (lignées de plantes mutantes affectées pour l'expression de gènes codant des protéines impliquées dans la synthèse ou la régulation du NO) et pharmacologiques (inhibiteurs de NOS et NR).

- l'analyse de la régulation par NO de la réponse transcriptomique aux OGs et la caractérisation de gènes régulés par NO dans un contexte d'interaction plante - pathogène. Ainsi, nous avons réalisé une analyse par puces à ADN en utilisant le cPTIO (piégeur de NO) pour identifier les gènes dont le niveau d'expression est régulé

par le NO. Ces données nous ont permis de sélectionner quelques gènes candidats pour une analyse fonctionnelle.

Ce travail a fait partie du projet ANR « PIANO » coordonné par le Pr David Wendehenne et qui avait comme objectif de comprendre les bases moléculaires de la signalisation cellulaire impliquant le NO chez les plantes, soumises à des stress biotiques et abiotiques.

III. Matériel et méthodes

1. Matériels biologiques

1.1. Matériel végétal

Les expérimentations ont été menées sur *Arabidopsis thaliana* : l'écotype *Columbia 0* (Col0) et différentes lignées de plantes mutantes. Ces lignées ont été sélectionnées par interrogation de la base de données SIGnAL (Salk Institute Genomic Analysis Laboratory ; http://signal.salk.edu/cgi-bin/tdnaexpress) puis commandées au NASC (The European Arabidopsis Stock Centre ; http://arabidopsis.info/). Les plantes mutantes sont des mutants d'insertion obtenus par transformation *via Agrobacterium tumefaciens* qui a insérée son ADN de transfert (T-DNA) dans le génome de la plante.

1.2. Matériel fongique

Botrytis cinerea souche BMM a été utilisé pour réaliser les tests de pathogénicité. C'est un champignon phytopathogène nécrotrophe responsable de la pourriture grise. Pour l'évaluation des symptômes, les diamètres de lésions sont mesurés 72 h post inoculation sur feuilles de plantes *d'A. thaliana* agées de quatre semaines.

2. Méthodes

2.1. Génotypage des lignées d'*A. thaliana* mutantes par PCR

Le génotypage par PCR consiste à identifier, parmi les lignées mutantes, les individus homozygotes pour l'allèle muté. Cette étape est nécessaire car les lignées mutantes peuvent

présenter des génotypes différents même si nous avons privilégié l'étude de lignées préalablement sélectionnées par le SALK comme étant homozygotes. Un échantillon représentatif de huit plantes est utilisé pour le génotypage de chaque lignée.

2.2. Identification des gènes régulés par le NO

Une analyse transcriptomique a été réalisée par les puces à ADN (NimbleGen Gene Expression), pour identifier des gènes régulés par le NO en réponse aux OGs. L'expérience a été menée sur *A. thaliana* Col0 et a consisté en l'application, pendant différents temps (1 h, 6 h, 24 h), de quatre traitements : traitement OGs (2,5 $mg.mL^{-1}$) et son contrôle (traitement eau), traitement OGs en présence de cPTIO qui est un piégeur du NO (500 µM) et son contrôle (traitement cPTIO seul). Trois répétitions biologiques ont été réalisées. Les analyses statistiques ont permis de constituer, à chaque temps de cinétique, deux listes de gènes : une liste des gènes significativement induits par les OGs et une liste de gènes significativement induits par les OGs en présence du cPTIO. Ces deux listes ont été comparées afin de déterminer une liste de gène cibles du NO.

2.3. Traitement des disques foliaires par les oligogalacturonides (OGs)

Les plantes ont été traitées avec une solution d'OGs pendant 1 h, 3 h et 6 h afin d'analyser l'expression des gènes marqueurs de défense ou l'activité des protéines kinases en réponse à l'élicitation. Pour chaque échantillon, dix disques foliaires provenant de plusieurs plantes ont été utilisés. Ces disques sont infiltrés pendant 3 min avec une solution contenant soit des OGs (2,5 $mg.mL^{-1}$) dilués dans de l'eau, soit de l'eau pour le contrôle. Après 1 h, 3 h et 6 h, les tubes contenant les disques foliaires sont prélevés, immédiatement congelés dans de l'azote liquide dans des microtubes de 2 mL et enfin stockés à -80°C.

2.4. Extraction des ARNs totaux

Les échantillons de disques foliaires sont broyés dans de l'azote liquide à l'aide de deux billes de 3 mm de diamètre grâce à un broyeur de tissus automatisé (Fisher Bioblock Scientific-Retsch). Les ARN totaux sont ensuite extraits à l'aide de TriReagent (Molecular Research Centre Inc) selon les instructions du fournisseur.

2.5. Analyse de l'expression des gènes par PCR quantitative en temps réel (RT-qPCR)

La synthèse des ADNc est obtenue par reverse transcription (ImProm-II™ Reverse Transcription System, PROMEGA) à partir de 1,5 µg d'ARNs totaux préalablement traités à la DNaseI (Sigma-Aldrich) selon les instructions des fournisseurs. Les réactions de RT-qPCR sont réalisées dans un volume final de 15 µL contenant 2 µL d'ADNc dilué au $10^{ème}$, 0,2 µM de chaque amorce et le tampon GoTaq®qPCRMaster Mix contenant l'enzyme *Taq* polymérase (PROMEGA). Les réactions PCR ont été réalisées grâce à un Mastercycler (Sequence Detection System, Applied Biosystem) dans des plaques de 96 puits (Optical reaction plate with Bar code, Applied Biosystem).

2.6. Production de peroxyde d'hydrogène chez *A. thaliana*

La localisation de la production des FAO, en particulier du peroxyde d'hydrogène, est réalisée par la technique du DAB (3,3'-diaminobenzidine) ou du luminol.

2.7. Mesure de la production de NO par spectrofluorimétrie

La production de NO a été suivie à l'aide de la 4,5-diaminofluoresceine diacétate (DAF-2DA) ; (Sigma-Aldrich). Les disques foliaires sont infiltrés sous vide pendant 3 min avec une solution aqueuse de DAF-2DA (20 µM) et d'OGs (2,5 $mg.mL^{-1}$) La fluorescence émise du DAF-2T induite par les OGs est mesurée par un spectrofluorimètre (Mithras, Berthold Technologies) à une longueur d'onde d'excitation de 485 nm et une longueur d'onde d'émission de 535 nm.

2.8. Analyse de l'activité des protéines kinases

La visualisation des activités kinases en gel a été réalisée selon le protocole rédigé par Dahan (2008). La réaction de phosphorylation est réalisée en présence ou en absence de Ca^{2+} afin de mettre en évidence les protéines dont l'activité est dépendante ou indépendante du calcium.

IV. Résultats principaux

1. Le NO est produit par les voies enzymatiques dépendantes de la Nitrate Reductase et de la L-arginine.

La production du NO en réponse aux OGs à été mesurée sur des disques foliaires *d'Arabidopsis thaliana* grâce à la sonde fluorescente DAF-2DA. Les OGs déclenchent une augmentation de fluorescence à partir d'environ 1h de traitement, qui se poursuit pendant au moins 12h (Figure 1). Cette augmentation de fluorescence est inhibée par le cPTIO. Ce résultat permet d'affirmer que l'augmentation de la fluorescence de la sonde est liée à la production du NO.

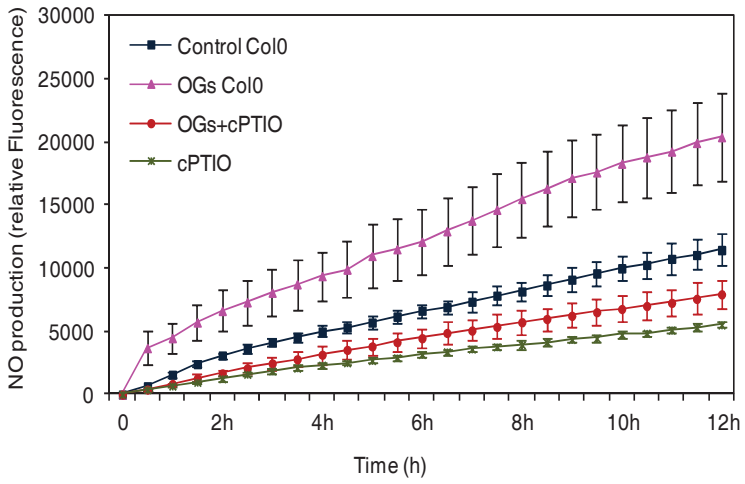


Figure 1 : Cinétique de production de NO en réponse aux OGs.

Afin d'étudier l'implication d'une voie enzymatique de biosynthèse de NO L-arg dépendante, nous avons étudié la production de NO induite par les OGs en présence du L-NAME, un inhibiteur de des activités NO synthase chez les mammifères. Un traitement par le L-NAME (5 mM) réduit d'environ 40% la production de NO après 12h de traitement.

L'implication de la Nitrate Réductase (NR) dans la biosynthèse de NO a été étudié à la fois par une approche génétique (utilisation du double mutant *nia1nia2*, montrant une activité NR réduite de 90%) et une approche pharmacologique (utilisation du tungstate, inhibiteur de la NR).

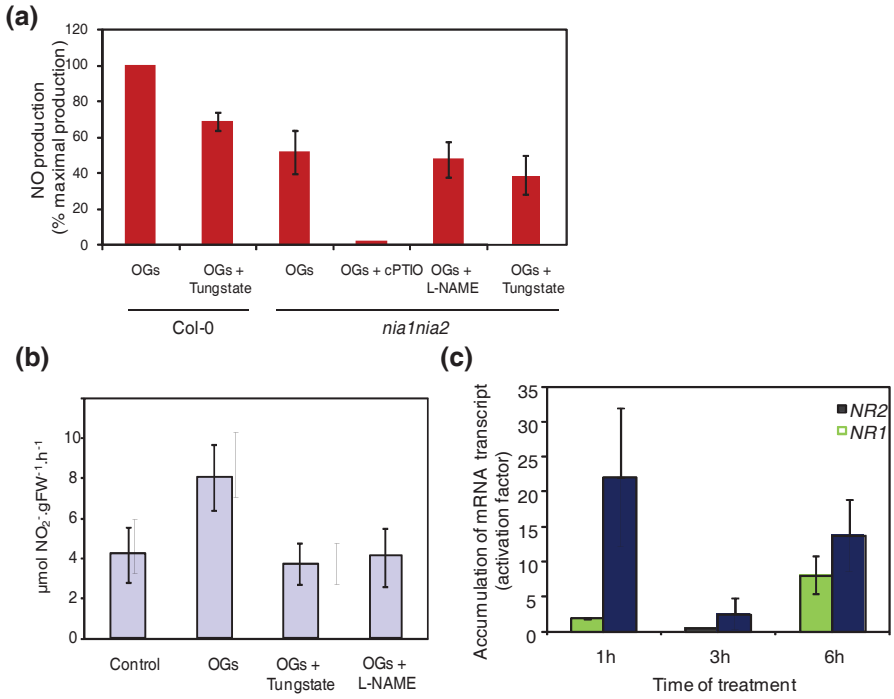


Figure 2 : Implication de la NR dans la production de NO induite par les OGs. (a) Production de NO détectée par le DAF 2DA. (b) Activité NR. (c) Accumulation des transcrits *NR1* et *NR2*.

Quelque soit l'approche utilisée, nous observons une diminution de la production du NO (de 30 à 50%) après 12h de traitement par les OGs. Nous avons pu démontrer également que les OGs stimulent de manière coïncidente l'activité NR et l'augmentation des transcrits des gènes *NR1* et *NR2* (Figure 2).

2. La production du NO en réponse aux OGS est régulée par l'influx de Ca^{2+}

L'utilisation du mutant *dnd1* affecté pour la protéine canal CNGC2 (impliquée dans le transport du Ca^{2+}) et du lanthane (bloquant les influx calciques) nous a permis d'observer une diminution de 50% environ de la production de NO en réponse aux OGs.

3. Le NO contrôle la production de ROS

La production des ROS (burst oxydatif) en réponse aux OGs est assurée chez *A. thaliana* par l'isoforme D de la NADPH oxydase (AtRBOHD). Nous n'avons pas observé de différence significative de la production de NO entre l'écotype sauvage Col0 et la lignée mutante *rbohD*

affectée dans la production de ROS, ceci suggérant que les ROS ne sont pas nécessaires à la production de NO.

Au contraire, il apparait qu'un traitement par le cPTIO ou le L-NAME réduit de manière significative la production de ROS induite par les OGs (Figure 3). La production de NO serait donc située en amont du burst oxydatif. Cette production de ROS est également dépendante de l'influx calcique (effet du lanthane) et de l'activité NR (utilisation du mutant *nia1nia2*) (Figure 3).

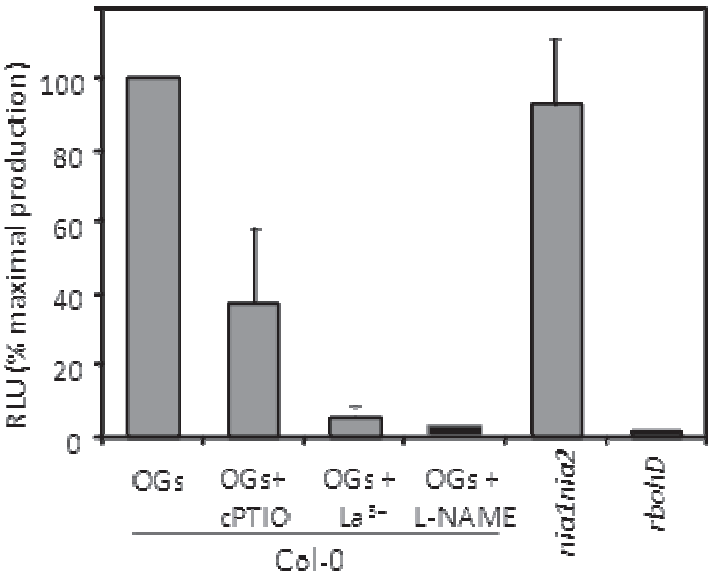


Figure 3 : Production de ROS par les OGS et sa régulation par le NO, le calcium et l'activité NR

4. Relations entre les protéines kinases (MAPK et CDPK) et la production de NO

4.1. Les CDPKs, et non les MAPKs, sont impliquées dans la production de NO

En considérant que la production observée chez Col0 est de 100%, on observe une diminution de la production de NO d'environ 50% chez le triple mutant *cpk5.6.11* (affecté pour les isoformes CDPK5, 6 et 11). Aucune différence n'est mesurée chez les mutants MAPK (isoformes 4 et 6).

4.2. Rôle des CDPKs dans l'interaction *A. thaliana* / *Botrytis cinerea*

Afin de déterminer le rôle des CDPKs dans la réponse d'Arabidopsis au champignon phytopathogène nécrotrophe *B. cinerea*, nous avons réalisé des infections du triple mutant

cpk5.6.11. Il apparait que les nécroses induites par le pathogène sont beaucoup plus importantes chez le triple mutant et que le diamètre moyen de lésion est significativement plus grand que celui observé chez Col0. Le triple mutant *cpk5.6.11* serait plus sensible à *B. cinerea*.

5. NO est impliqué dans la résistance d'*A. thaliana* à *B. cinerea*

Les feuilles d'Arabidopsis ont été infiltrées par différentes concentrations de cPTIO puis inoculées par *B. cinerea*. Le diamètre moyen des lésions des nécroses observées sur les plantes traitées par 500 µM de cPTIO est significativement plus important que chez les plantes « contrôle » (infiltration avec de l'eau). Ce résultat indique que les plantes traitées sont plus sensibles et donc que le NO est impliqué dans le processus de résistance à *B. cinerea* (Figure 4).

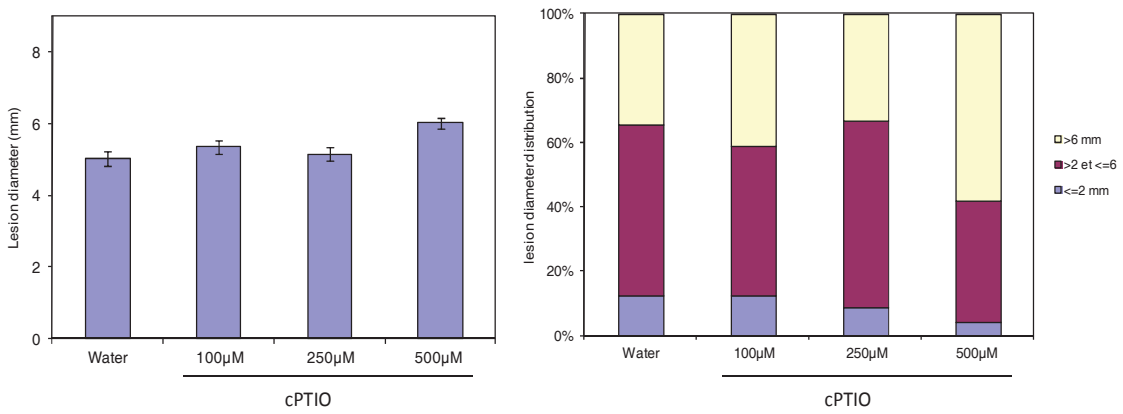


Figure 4 : Implication du piègeur de NO, cPTIO, dans la résistance basale d'*A. thaliana* à *B. cinerea.*

6. Analyse de la régulation par NO de la réponse transcriptomique aux OGs

Une première étude réalisée dans cette thèse sur quelques gènes candidats induits par le traitement OG (PER4, PLP2, ChiIV, β-1,3,-glucanase) a permis de démontrer que d'une part, le NO est un régulateur de l'expression de gènes induits par l'éliciteur (Figure 5), et que d'autre part ces gènes participent au fonctionnement de l'interaction entre *B. cinerea* (Figure 6).

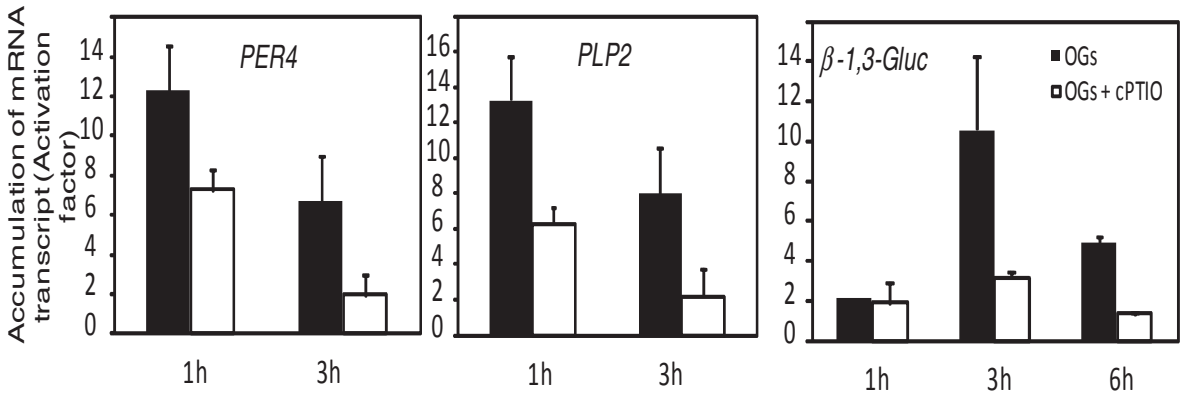


Figure 5 : Effet du cPTIO (piègeur de NO) diur le nivaeu d'accumulation des transcrits de trois gènes (*PER4, PLP2* et *β-1,3,-glucanase*) induits par les OGs.

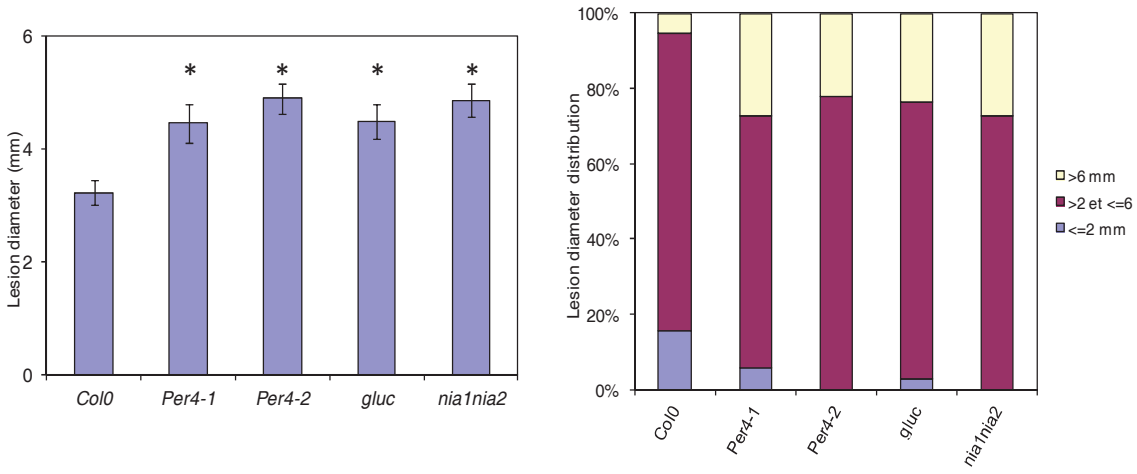


Figure 6 : Résistance basale à *A. thaliana* chez les lignées mutantes invalidées dans l'expression de PER4 (*per4.1, per4.2*) , α-1,3,-glucanase (*gluc*) et NR (*nia1nia2*).

Afin d'étudier de manière plus globale la régulation de gènes par le NO et le rôle de ces gènes dans les interactions plante-pathogène, nous avons réalisé une analyse du transcriptome d'*A. thaliana* par la technique des puces à ADN. Cette étude a permis d'étudier qualitativement et quantitativement l'expression des gènes en réponse aux OGs, à différents temps de traitement et en présence ou non de cPTIO. Seuls les gènes montrant des taux d'induction supérieurs à 2 ou inférieurs à -2 ont été conservés. Deux listes de gènes à chaque point de cinétique ont été obtenues : (i) l'ensemble des gènes positivement ou négativement régulés par les OGs ; (ii)

l'ensemble des gènes positivement ou négativement régulés par les OGs en présence de cPTIO, c'est à dire indépendamment du NO. A l'aide du programme FiRE développé sous Excel par Garcion *et al.* (2006), nous avons comparé les deux listes à chaque temps de cinétique. Les gènes régulés par le NO correspondent à l'ensemble des gènes régulés par les OGs auxquels les gènes régulés par les OGs indépendamment de NO ont été soustraits. Nous avons ainsi obtenu une liste de gènes appelée par la suite «gènes cibles du NO ». Cette analyse a été effectuée sur les trois points de cinétique. Le nombre de gènes cibles de NO est de 237 à 1 h, 1079 à 6 h et 389 à 24 h de traitement (Figure 7).

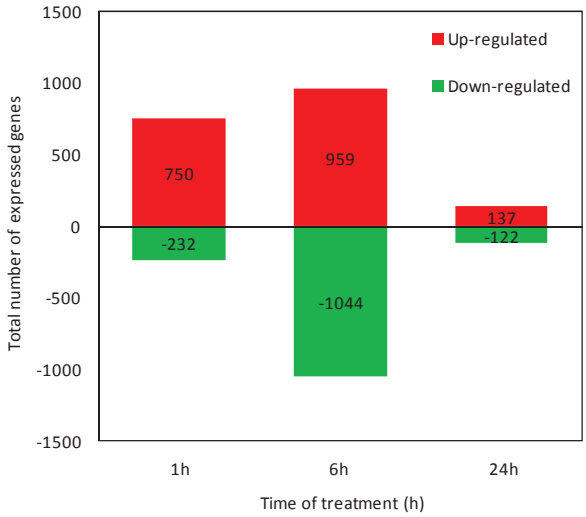


Figure 7 : Réponse transcriptomique aux OGs régulée par NO.

Nous avons ensuite réalisé une analyse fonctionnelle de ces gènes régulés par le NO grâce aux outils «GO annotation» (www.arabidopsis.org) et MapMan. On observe une sur-représentation de gènes liés à la réponse aux stress biotiques parmi les gènes cibles du NO à 1 h, 6 h et 24 h de traitement. On remarque également une sur-représentation des gènes liés aux processus de transcription et à la reconnaissance des pathogènes.

7. Recherche de sites de liaison aux facteurs de transcription

Nous avons recherché, parmi les gènes cibles du NO, la présence de sites de liaison de facteurs de transcription en amont de la séquence codante du gène. Les analyses obtenues avec la

base de données Athena (*Arabidopsis thaliana* Expression Network Analysis ; http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) mettent en évidence un grand nombre de sites de liaison de facteurs de transcription sur les séquences promotrices des gènes. Il apparait que 79% et 75% des gènes régulés positivement respectivement à 1 h et 6 h de traitement possèdent un nombre significativement élevé de motifs W-box connus pour être des sites de liaison pour les facteurs de transcription de type WRKY. Ces motifs W-box ne sont pas significativement sur-représentés à 24 h.

8. Etude fonctionnelle des gènes cibles du NO

8.1. Choix des gènes candidats

Parmi les gènes cibles du NO, sept gènes, présentant des taux d'induction élevés à 1h et 6 h de traitement ont été sélectionnés pour une analyse fonctionnelle (Tableau 1). Quatre de ces gènes codent pour des facteurs de transcription (TFs) : WRKY75 (At5g13080), WRKY41 (At4g11070), CRF3 (At5g53290) et un facteur de transcription non caractérisé contenant un motif de type bHLH (At1g10585). Deux gènes codent pour des récepteurs potentiels impliqués dans la reconnaissance des pathogènes : TIR (At5g52900) et RLP7 (At1g47890). Enfin le dernier gène a été choisi car il était modulé tout au cours de l'expérience (1h, 6h et 24h). Il s'agit de SRP /GEX3 (At5g16020).

Les différentes lignées mutantes, invalidées dans l'expression des gènes sélectionnés, ont tout d'abord été génotypées afin de vérifier si elles étaient homozygotes. Ce génotypage nous a permis de choisir une lignée homozygote pour chaque mutant.

Tableau 1 : Liste et niveau d'expression des gènes régulés par NO sélectionnés pour l'étude fonctionnelle.

Gene ID	Name of gene	1 h	6 h	24 h
AT1G52900.1	TIR	71.25	11.058	x
AT1G47890.1	AtRLP7	25.84	x	x
AT5G16020.1	SRP (stress reelated protein)/GEX3	8.56	-6.096	9.34
AT5G13080.1	WRKY 75	57.7	x	x
AT4G11070.1	WRKY 41	19.93	x	x
AT1G10585.1	Transcription factor similar to bHLH	x	17.13	x
AT5G53290.1	CRF3 (Cytokinin response factor 3)	x	10.444	x

8.2. Rôle des gènes candidats dans l'interaction *A. thaliana* / *B. cinerea*

Afin de comprendre le rôle des gènes sélectionnés par l'analyse transcriptomique, les plantes mutantes invalidées dans l'expression de ces gènes et le génotype sauvage Col0 ont été inoculés avec le champignon *B. cinerea*. Six des sept gènes étudies semblent impliqués dans l'interaction *A. thaliana/ B. cinerea* (Tableau 2). En effet, trois des quatre lignées mutantes correspondant aux gènes codant pour les TFs ont montré des symptômes significativement différents de ceux observés chez Col0. Les gènes *CRF3* et *BHLH* sont considérés comme régulateurs positifs de la résistance à *B. cinerea.* Au contraire, le gène *WRKY41* semble un régulateur négatif (les plantes mutantes sont plus résistantes). Nous n'avons pas observé de différence significative entre la plante mutante *wrky75* et le génotype Col0. Enfin, les mutants *srp*, *tir et rlp7* présentent des lésions plus importantes par rapport à Col0 et semblent plus sensibles à *B. cinerea* que Col0.

Tableau 2 : Résistance basale à *B. cinerea* des lignées d'*A. thaliana* affectée dans l'expression de gènes régulés par le NO. S : sensibles, R : résistantes, ND : non déterminé

Gene ID	Gene name	Number of performed Experiments	Tendency	Statistically Significant experiments	Conclusions
At1g52900	TIR	3	S	1	S
At1g47890	RLP7	3	S	1	S
At5g16020	Stress related protein	3	S	2	S
At5g13080	WRKY 75	5	ND	0	ND
At4g11070	WRKY 41	5	R	3	R
At1g10585	Transcription factor similar to bHLH	3	S	1	S
At5g53290	CRF3 (Cytokinin response factor 3)	5	S	3	S

V. Conclusions - Perspectives

Ce travail avait comme objectif de mieux comprendre le rôle du NO dans les mécanismes de signalisation cellulaire activés chez les plantes en réponse aux stress biotiques et plus particulièrement en réponse aux OGs, un éliciteur des réactions de défense.

Premièrement nous avons tenté d'élucider les mécanismes responsables de la production et de régulation du NO induites par les OGs. Nous avons pu démontrer l'implication de deux voies enzymatiques étroitement liées, la voie NR et la voie L-arginine dépendante, dans la biosynthèse du NO. Néanmoins il semble qu'au moins une autre source, encore inconnue, soit également impliquée. Nous avons également observé que la production de NO était reliée à d'autres événements de signalisation comme l'influx calcique, les activités CDPK et la production de ROS.

Deuxièmement, grâce à la mise en évidence de la régulation par le NO de gènes candidats induits par les OGs, et de l'implication de deux de ces gènes et du piègeur de NO, cPTIO, dans l'interaction *A. thaliana /Botrytis cinerea*, nous avons pu montrer que le NO participe à la réponse d'*A. thaliana* au stress biotique.

Troisièmement, l'analyse du transcriptome *d'A. thaliana,* grâce aux puces à ADN en présence ou non d'un piégeur de NO, le cPTIO, nous a permis de dresser une liste des gènes cibles du NO produit en réponse aux OGs. Cette étude globale a permis de démontrer que la réponse transcriptomique aux OGs régulée par le NO était principalement orientée vers des gènes connus pour être impliqués dans la réponse au stress biotique (et plus particulièrement des gènes codant pour des récepteurs putatif et des facteurs de transcription de la famille WRKY). De plus, nous avons mis en évidence une surreprésentation de site de liaison aux facteurs de transcription de type WRKY dans les régions régulatrices de ces gènes cibles de NO. Enfin, il apparait qu'au moins trois gènes régulés par NO codant des facteurs de transcription soit impliqués dans la résistance d'*A. thaliana* à *B. cinerea*. Ces éléments suggèrent que le NO peut induire ces effets de manière indirecte en régulant l'expression de gènes par la régulation transcriptionnelle de facteurs de transcription spécifiques. Nous ne pouvons pas exclure que le NO puisse également réguler l'expression des gènes par des modifications post traductionnelle de certains facteurs de transcription.

Considérés dans leur ensemble, nos résultats ont permis de décrypter une partie des mécanismes liant la production de NO, les réponses de défense et la résistance basale d'*A. thaliana* à *B. cinerea*. Nos données renforcent le concept que NO est un médiateur clé des réponses de défense des plantes.

En termes de perspectives, plusieurs directions pourront être poursuivies. D'une part, nous tenterons de mieux comprendre les mécanismes de synthèse du NO et plus particulièrement le lien entre la voies L-arg et Nitrite dépendante. Il est possible que les effets de NO sur la stimulation de l'activité NR soient dus par une interaction directe entre NO et la protéine par l'intermédiaire d modifications post traductionelles. D'autre part, nous tenterons de confirmer le rôle de facteurs de transcription spécifique dans la régulation transcriptionelle des gènes cibles du NO par l'étude des interactions physiques entre les facteurs de transcription et les régions promotrices des gènes.

ANNEXES

ANNEX 1

Supplemental Data

CD contains

- List of common genes induced by OGs between 1h, 6h and 24h post-treatment
- List of NO-responsive genes after 1h, 6h and 24h treatment
- List of common genes between OGs 2010 vs. OGs 2009

ANNEX 2

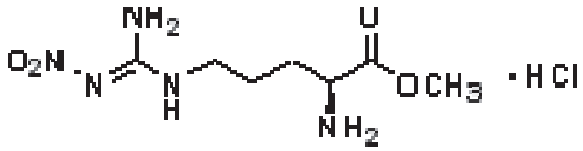
Properties of Chemicals used for pharmacological approach

L- NAME (N_ω-Nitro-L-arginine methyl ester hydrochloride)

L-NAME hydrochloride

Empirical Formula : $C_7H_{15}N_5O_4 \cdot HCl$

Molecular Weight: 269.69



An analog of arginine that inhibits NO production. It has multiple effects.

Sodium tungstate

Tungstic acid sodium salt dihydrate

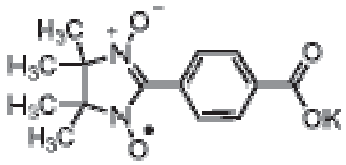
Linear Formula: $Na_2WO_4 \cdot 2H_2O$

Molecular Weight: 329.85

cPTIO (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt, 2-(4-Carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide potassium salt)

Empirical Formula : $C_{14}H_{16}KN_2O_4$

Molecular Weight: 315.39

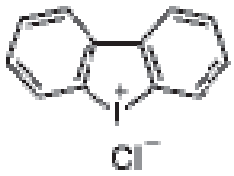


Reacts with nitric oxide to form carboxy-PTI derivatives which in turn inhibits nitric oxide synthase

DPI (Dibenziodolium chloride)

Formula $C_{12}H_8ClI$

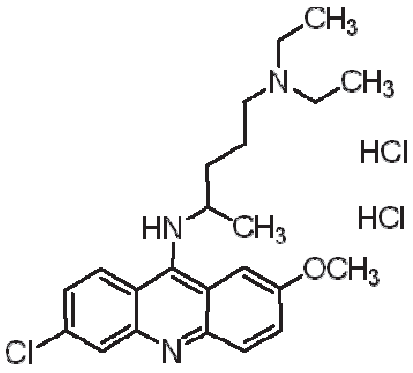
Molecular Weight: 314.55



A potent inhibitor of flavoenzymes and also used to inhibit NADPH oxidase.

Quinacrine dihydrochloride

6-Chloro-9-(4-diethylamino-1-methylbutylamino)-2-methoxyacridine dihydrochloride, Atebrin dihydrochloride, Mepacrine dihydrochloride. Non-selective MAO-A/B inhibitor



Empirical Formula : $C_{23}H_{30}ClN_3O \cdot HCl$

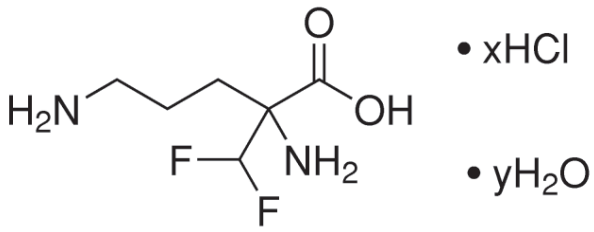
Molecular Weight: 472.88

DFMO

2-(Difluoromethyl)ornithine hydrochloride hydrate, DFMO hydrochloride hydrate, Eflornithine hydrochloride hydrate

Empirical Formula : $C_6H_{12}F_2N_2O_2 \cdot xHCl \cdot yH_2O$

Molecular Weight: 182.17 (anhydrous free base basis)



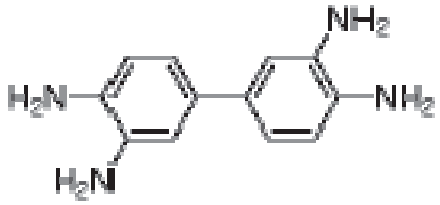
Difluoromethylornithine (Eflornithine) inhibits polyamine biosynthesis by the selective, irreversible inhibition of ornithine decarboxylase (ODC). A chemoprotective agent that blocks angiogenesis.

DAB (3,3′-Diaminobenzidine)

3,3′,4,4′-Biphenyltetramine, 3,3′,4,4′-Tetraaminobiphenyl, DAB

Linear Formula: $(NH_2)_2C_6H_3C_6H_3(NH_2)_2$

Molecular Weight: 214.27

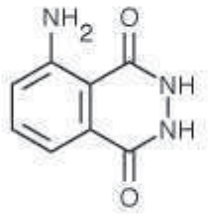


Luminol is a chemiluminescent horseradish peroixdiase (HRP) substrate.

Linear Formula: $C_8H_7N_3O_2$

Molecular Weight: 177.16

1,4-Phthalazinedione, 5-amino-2,3-dihydro/ 521-31-3



Horseradish peroxidase (hydrogen-peroxide oxidoreductase)

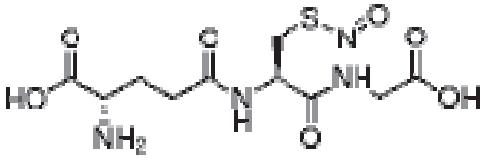
Enzyme Commission (EC) Number: 1.11.1.7

Horseradish peroxidase is isolated from horseradish roots (*Amoracia rusticana*) and belongs to the ferroprotoporphyrin group of peroxidases. HRP is a single chain polypeptide containing four disulfide bridges. It is a glycoprotein containing 18% carbohydrate. The carbohydrate composition consists of galactose, arabinose, xylose, fucose, mannose, mannosamine, and galactosamine depending upon the specific isozyme. Its molecular weight (~44 kDa) includes the polypeptide chain (33,890 Daltons), hemin plus Ca^{2+} (~700 Daltons), and carbohydrate (~9,400 Daltons). At least seven isozymes of HRP exist. The isoelectric point for horseradish peroxidase isozymes ranges from 3.0 - 9.0.

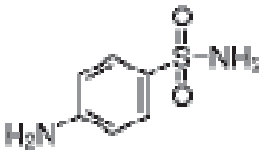
GSNO, SNOG (S-Nitrosoglutathione)

Formula $C_{10}H_{16}N_4O_7S$

Molecular Weight: 336.32



Sulfanilamide



Synonym: *p*-Aminobenzenesulfonamide

Linear Formula: $H_2NC_6H_4SO_2NH_2$

Molecular Weight: 172.20

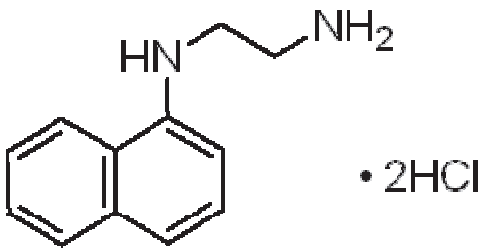
Sulfonamide antibiotic that blocks the synthesis of dihydrofolic acid by inhibiting the enzyme dihydropteroate synthase.

NED (*N*-(1-Naphthyl) ethylenediamine dihydrochloride)

Synonym: 2-(1-Naphthylamino)ethylamine dihydrochloride

Linear Formula: $C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$

Molecular weight ; 259.17



ANNEX 3

PUBLICATION / COMMUNICATION

PUBLICATIONS

Besson-Bard A., Astier J., **Rasul S**., Wawera I., Dubreuil-Maurizi C., Jeandroz S. and Wendehenne D. (2009). Current view of nitric oxide-responsive genes in *Arabidopsis thaliana* *Plant Sci*. 177: 302-309.

Astier J., Besson-Bard A., Wawer I., Parent C., **Rasul S**., Jeandroz S., Dat J. and Wendehenne D. (2010). Nitric oxide signaling in plants: cross-talk with Ca^{2+}, protein kinases and reactive oxygen species. Annual Plant Reviews Volume 42: Nitrogen Metabolism in Plants in the Post-Genomic Era. Page 147-170. Foyer C. and Zhang H. Eds, Blackwell Publishing Ltd. (Book chapter).

Astier J., **Rasul S**., Koen E ., Manzoor H., Besson-Bard A, Lamotte O., Jeandroz S., Durner J., Lindermayr C. and Wendehenne D. (2011). S-nitrosylation: An emerging post-translational protein modification in plants. *Plant Sci*. 181: 527-533.

Rasul S., Dubreuil C., Poinssot B., Lamotte O., Wendehenne D., Jeandroz S. (2011) NO production mediates OGs-triggered immunity and resistance to *B. cinerea* in *Arabidopsis thaliana* (re-submitted "Plant Cell and Environment").

Rasul S., Wendehenne D. and Jeandroz S. (2012) Nitric oxide-regulated transcriptomic response to oligogalacturonides in *Arabidopsis thaliana* (In preparations).

Rasul S., Bourque S., Wendehenne D. and Jeandroz S. (2012) Relationships between MAPKs/CDPKs activities and OGs-induced NO production (In preparations).

COMMUNICATIONS (POSTERS / PRESENTATIONS)

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ABSTRACT

Characterization and role of Nitric oxide production in *Arabidopsis thaliana* defense response induced by oligoglacturonides

Nitric oxide (NO) regulates a wide range of plant processes from development to environmental adaptation. In this study, NO production and its effects were investigated in a plant-pathogen context. The production of NO following Arabidopsis treatment with oligogalacturonides (OGs), an endogenous elicitor of plant defense, was assessed using the NO sensitive probe 4, 5-diamino fluorescein diacetate. Pharmacological and genetic approaches were used to analyze NO enzymatic sources and its role in the *Arabidopsis thaliana* /*Botrytis cinerea* interaction. We showed that NO production involves both a L-arginine- and a nitrate reductase (NR)-pathways. OGs-induced NO production was Ca^{2+}-dependent and modulated RBOHD-mediated ROS production. NO production was also regulated by CDPKs activities, but worked independently of the MAPKs pathway. Using a transcriptomic approach, we further demonstrated that NO participates to the regulation of genes induced by OGs such as genes encoding disease-related proteins and transcription factors. The over-representation of certain regulatory elements (e.g. W-BOX) in promoter sequences of target genes also suggests the involvement of specific transcription factors in the NO response. Mutant plants impaired in several selected NO-responsive genes, as well as Col-0 plants treated with the NO scavenger cPTIO, were more susceptible to *B. cinerea.* Taken together, our investigation deciphers part of the mechanisms linking NO production, NO-induced effects and basal resistance to *Botrytis cinerea*. More generally, our data reinforce the concept that NO is a key mediator of plant defense responses.

Keywords: nitric oxide, oligogalacturonides, nitrate reductase, plant defense, *Arabidopsis thaliana*, *Botrytis cinerea*, calcium, reactive oxygen species, transcriptome.

RÉSUMÉ

Caractérisation et rôle de la production du monoxyde d'azote en réponse aux oligogalacturonidase chez *Arabidopsis thaliana*

Le monoxyde d'azote (NO) régule un grand nombre de processus physiologiques tel que le développement ou les réponses aux modifications des conditions environnementales. Dans ce travail, la production de NO et ses effets ont été étudiés dans le contexte des interactions plante – pathogène. La production de NO générée chez *Arabidopsis thaliana* par les oligogalacturonides (OGs), eliciteur endogène des réactions de défense, a été mesurée par la sonde fluorescente 4, 5-diamino fluoresceine diacetate. L'utilisation d'approches pharmacologiques et génétiques ont permis d'étudier les sources enzymatiques de la production de NO et son rôle dans l'interaction *A. thaliana*/*Botrytis cinerea.* Nous avons montré que le NO est produit par une voie dépendante de la L-arginine ainsi que d'une voie impliquant la Nitrate Réductase. La production de NO induite par les OGs est dépendante du Ca^{2+} et modulée par les formes activées de l'oxygène (produites par AtRBOHD). La production de NO est également régulée par les CDPKs mais est indépendante des activités MAPKs. A l'aide d'une approche transcriptomique nous avons ensuite démontré que le NO participe à la régulation de l'expression de gènes induits par les OGs tels que des gènes codant pour des protéines PR ou des facteurs de transcription. La sur-représentation de certains éléments régulateurs (par exemple de type W-box) dans les régions promotrices des gènes cibles du NO suggère également l'implication de facteurs de transcription spécifiques dans la réponse au NO. Enfin, des plantes mutantes, affectées dans l'expression de gènes cibles de NO, ainsi que des plantes de type sauvage (Col-0) traitées par le piégeur de NO, cPTIO, sont plus sensibles à *B. cinerea*. L'ensemble de ces résultats nous a permis de mieux comprendre les mécanismes liant la production de NO, ses effets et la résistance d'*A. thaliana* à *B. cinerea,* confirmant que le NO est un élément-clé des réactions de défense des plantes.

Mots clés : monoxyde d'azote, oligogalacturonides, nitrate réductase, réactions de défenses des plantes, *Arabidopsis thaliana*, *Botrytis cinerea*, calcium, formes activées de l'oxygène, transcriptome.