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Calcium signaling in plant defense: involvement of subcellular compartments and glutamate receptors

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DEDICATED

TO

MY SWEETEST MOTHER

WHO IS HEAVEN FOR ME
WHOSE HANDS ALWAYS
RAISED FOR MY WELL-BEING
EVEN AT THIS MOMENT OF TIME

MY DEAREST FATHER

WHOSE LOVE IS MORE PRECIOUS
THAN PEARLS AND DIAMONDS
BY THE VIRTUE OF WHO' S PRAYS,
I HAVE BEEN ABLE TO REACH
AT THIS HIGH POSITION

MY BROTHERS AND SISTER

WHO ARE THE WORLD FOR ME
WHOSE LOVE ENCOURAGED ME
AT EVERY STEP

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RÉSUMÉ

Les plantes présentent une forme d'immunité innée face à des agents potentiellement pathogènes qui se traduit par l'induction de réponses de défense. Les réponses immunes des plantes sont induites après détection de motifs moléculaires associés à des pathogènes ou à des micro-organismes par des récepteurs reconnaissant spécifiquement ces motifs et/ou des molécules dérivées des agents pathogènes ou de la plante, appelés éliciteurs de réaction de défense. La cryptogéine (Cry) et les oligogalacturonates (OGs) sont des éliciteurs établis de réactions de défense et leur reconnaissance induit une signalisation Ca^{2+}-dépendante : un influx calcique et une variation de la concentration cytosolique en Ca^{2+} libre ($[Ca]_{cyt}$) sont des événements précoces induisant une voie de signalisation de défense. Nous avons démontré que chez le tabac, les éliciteurs induisent une signalisation calcique dans les mitochondries et les chloroplastes. Des études pharmacologiques indiquent que des canaux IP_3-dépendants régulent la signalisation calcique induite par la Cry dans les mitochondries et les chloroplastes. La respiration mitochondriale et les mécanismes de dissipation de l'énergie dans les chloroplastes sont régulés en partie par la $[Ca^{2+}]$ dans ces organites. De plus, nous montrons par des approches pharmacologiques et génétiques, que des homologues aux récepteurs du glutamate (GLRs) participent à la signalisation calcique induite par les OGs dans *Arabidopsis*. Les GLRs contrôlent en partie la production d'oxyde nitrique (NO) et d'espèces réactives de l'oxygène (ROS), ainsi que l'expression de gènes de défense. Par ailleurs, les plantes traitées par des antagonistes des GLRs, présentent une moindre résistance au pathogène fongique nécrotrophique, *Botrytis cinerea* et à l'oomycète biotrophique, *Hyaloperonospora arabidopsidis*. L'analyse de mutants *Atglr* révèle l'importante contribution de AtGLR3.3 dans la résistance envers *H. arabidopsidis*. De plus, de frappantes similarités dans l'expression de gènes sont observées après traitement par les OGs ou après infection par *H. arabidopsidis*. Enfin, une analyse transcriptomique montre qu'environ 60 % des gènes modulés par les OGs ont une expression qui dépend de GLRs. Ces gènes dépendants de GLRs appartiennent à diverses familles fonctionnelles dont celle répondant aux stress biotiques. En conclusion, ces études montrent 1) que les mitochondries et les chloroplastes présentent aussi une signalisation calcique induite par des éliciteurs de réaction de défense chez le tabac et 2) l'implication de GLRs dans la signalisation calcique induite par des éliciteurs ou des agents pathogènes et la résistance envers des agents pathogènes chez *Arabidopsis*.

Mots clés: signalisation calcique, Cryptogéine, oligogalacturonates, mitochondries, chloroplastes, récepteurs au glutamate, *Hyaloperonospora arabidopsidis, Arabidopsis thaliana, Nicotiana tabacum*

ABSTRACT

Plants do not display an adaptive immune system but express an efficient innate immune system defending them by inducing sophisticated multilevel defense responses against different potential pathogens. Indeed, plant immune responses are triggered upon the detection of many common pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) through specific pattern-recognition receptors (PRRs) and/or pathogen- or plant-derived signal molecules called elicitors. Cryptogein (Cry) and oligogalacturonides (OGs) are well known elicitors of defense reactions and their recognition induce a Ca^{2+}-dependent signaling pathway: Ca^{2+} influx and subsequent free cytosolic [Ca^{2+}] ($[Ca^{2+}]_{cyt}$) variations are earliest steps to trigger downstream plant defense signaling. Here we have demonstrated that elicitor-induced Ca^{2+} signaling in tobacco also takes place in mitochondria and chloroplasts. Pharmacological studies indicated that IP_3-channels play an important role in the regulation of Ca^{2+} signaling in mitochondria and chloroplasts. Mitochondrial respiration and energy dissipation mechanisms in chloroplasts are partly controlled by [Ca^{2+}] in these organelles. Moreover, using pharmacological and genetic approaches, our data demonstrated that glutamate receptors homologs (GLRs) participate in OGs-mediated Ca^{2+} signaling in *Arabidopsis.* GLRs partly control OGs-induced nitric oxide (NO) production, reactive oxygen species (ROS) production and expression of defense-related genes. Importantly, plants treated with GLRs antagonists exhibited compromised resistance to necrotrophic fungal pathogen, *Botrytis cinerea* and biotrophic oomycete, *Hyaloperonospora arabidopsidis*. Analysis of *Atglr* single mutants revealed the important contribution of AtGLR3.3 in resistance against *H. arabidopsidis*. Moreover, striking similarities in gene expression levels were observed after OGs elicitation/*H. arabidopsidis* infection. Finally, transcriptomic analysis demonstrated that about 60 % of the total OGs-modulated genes modified their expression in GLRs-dependent manner. These GLRs-dependent genes belong to different functional categories including the category “responses to biotic stresses”. Taken together, these data provide strong evidences of 1) elicitor-induced Ca^{2+} signaling in mitochondria and chloroplasts in tobacco and 2) the regulation of elicitor/pathogen mediated plant defense signaling pathways through GLRs in *Arabidopsis thaliana*.

Key words: Calcium signaling, Cryptogein, oligogalacturonides, mitochondria, chloroplasts, glutamate receptors, *Hyaloperonospora arabidopsidis, Arabidopsis thaliana, Nicotiana tabacum*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS……………………………………………………………...viii

CHAPTER 1…………………………………………………1

BIBLOGRAPHIC CONTEXT……………………………………………………………1

1. Immune systems in plants……………………………………………………………1

1.1. Background and active resistance……………………………………………………1

1.2. A contemporary view of plant immunity…………………………………………….1

1.2.1. PTI (PAMP-triggered immunity)……………………………………………..3

1.2.2. ETI (effector-triggered immunity)……………………………………………6

2. Signal transduction during plant defense………………………………………...9

2.1. Ion fluxes……………………………………………………………………………10

2.2. Reactive oxygen species production………………………………………………..12

2.3. Nitric oxide production……………………………………………………………..13

2.4. Phosphorylation of mitogen-activated protein kinases……………………………..14

2.5. The role of phytohormones…………………………………………………………15

3. Plant defense responses: a final outcome……………………………………………..17

3.1. Modification of the cell wall………………………………………………………..17

3.2. Synthesis of antimicrobial compounds……………………………………………..18

3.3. Hypersensitive response……………………………………………………………19

3.4. Systemic acquired resistance………………………………………………………….20

3.5. Synthesis of pathogenesis-related proteins (PR proteins)…………………………..21

4. Ca^{2+} signaling in plants……………………………………………………………...23

4.1. Ca^{2+} signatures specificity……………………………………………………………..24

4.2. Cellular functions of Ca^{2+} signals in plants……………………………………………25

4.2.1. Ca^{2+} signaling at the single cell level……………………………………………..25

4.2.1.1. Regulation of stomatal guard cells signaling………………………………..26

4.2.1.2. The establishment of symbiosis: signaling in root hairs……………………..27

4.2.1.3. Signaling for tip growth in pollen tubes and root hair cells…………………29

4.2.2.1. Ca^{2+} signaling during plant-pathogen interactions…………………………30

4.3. Ca^{2+} homeostasis……………………………………………………………………32

4.3.1. Ca^{2+} signal modulation by the organelles………………………………………...33

4.3.1.1. Ca^{2+} signaling in the nucleus………………………………………………...34

4.3.1.2. Ca^{2+} signaling in the mitochondria……………………………………………..35

4.3.1.3. Ca^{2+} signaling in the chloroplasts………………………………………………37

5. Ca^{2+} transport systems…………………………………………………………………40

5.1. Ca^{2+} channels……………………………………………………………………………40

5.1.1. Voltage-dependent Ca^{2+}-permeable channels in the plasma membrane…………40

5.1.2. Ligand-gated Ca^{2+}-permeable channels……………………………………………..41

5.1.2.1. Cyclic nucleotide-gated channels……..………………………………………41

5.1.2.2. Glutamate-receptor-like channels………..……………………………………42

5.1.3. Voltage-dependent channels at vacuolar membranes……………………………43

5.1.4. Ligand-gated channels in the endomembranes…………………………………...44

5.2. Ca^{2+} pumps and transporters (exchangers)……………………………………………45

5.2.1. Ca^{2+}-ATPases…………………………………………………………………………..46

5.2.2. Ca^{2+} exchangers………………………………………………………………………..47

6. Ca^{2+} sensing and signaling…………………………………………………………..50

6.1. Sensor relays………..………………………..……………………………….…………....50

6.1.1. Calmodulin and calmodulin-like sensor proteins…………………………………50

6.1.2. Calcineurin B-like sensors………………………………………………………..54

6.2. Sensor responder proteins………………………………………………………………..56

6.2.1. Ca^{2+}-dependent protein kinases………………………………………………...56

6.2.2. Ca^{2+} and Ca^{2+}/CaM-dependent protein kinases…………………………………..58

7. Glutamate receptors……………………………………………………………………...59

7.1. Glutamate: a magical amino acid………………………………………………………...59

7.1.1. Glutamate metabolism in plants…………………………………………………..59

7.1.2. Glutamate homeostasis in plants…………………………………………………60

7.1.3. Glutamate signaling………………………………………………………………61

7.2. Glutamate receptors in animals…………………………………………………………..61

7.2.1. Ionotropic glutamate receptors……………………………………………………62

7.2.2. Metabotropic glutamate receptors………………………………………………...63

7.3. Plants glutamate receptor-like homologs………………………………………………...63

7.3.1. Expression patterns of GLRs in plants…………………………………………….64

7.3.2. Role of GLRs in plants………………………………………………………………65

7.3.2.1. Role in Ca^{2+} signaling……………………………………………………………..65

7.3.2.2. Role in light-dependent signaling and growth processes……………………66

7.3.2.3. Role in abiotic stresses…………………………………………………………67

7.3.2.4. Role in biotic stresses…………………………………………………………...67

8. Working models (plants/pathogens/elicitors)……………………………………………...69

8.1. Tobacco-cryptogein model……………………………………………………………69

8.1.1. Phytophthora……………………………………………………………………...69

8.1.2. Elicitins……………………………………………………………………………...70

8.1.3. Cry, an elicitor of defense reactions in tobacco……………………………………..71

8.1.4. Cry-induced signaling in tobacco……………………………………………......71

8.2. Oligogalacturonides/*Hyaloperonospora arabidopsidis*/*Arabidopsis thaliana* model...72

8.2.1. The oligogalacturonides (OGs)…………………………………………………...72

8.2.2. OGs and plant defense responses…………………………………………………72

8.3.1. Hyaloperonospora arabidopsidis…………………………………………………...74

CHAPTER 2…………………………………………………………..78

MATERIALS AND METHODS……………………………………………………...78

1. Materials…………………………………………………………………………...78

1. 1. Biological material related to tobacco…………………………………………...78

1.1.1. Cell Suspensions…………………………………………………………………78

1.2. Biological material related to *Arabidopsis*……………………………………………79

1.2.1. Plants…………………………………………………………………………...79

1.2.2. Pathogens………………………………………………………………………79

1.2.2.1. *Botrytis cinerea*…………………………………………………………………79

1.2.2.2. *Hyaloperonospora arabidopsidis*……………………………………………80

1.3. Elicitors……………………………………………………………………………..80

1.4. Chemicals…………………………………………………………………………...80

2. Methods…………………………………………………………………………………81

2.1. Mutant Genotyping…………………………………………………………………..81

2.1.1. Primer designing…………………………………………………………………81

2.1.2. Isolation of DNA…………………………………………………………………81

2.1.3. PCR………………………………………………………………………………82

2.1.4. Agarose gel electrophoresis……………………………………………………82

2.2. Measurement of free calcium concentration………………………………………83

2.2.1. In *Arabidopsis* plants…………………………………………………………..83

2.2.1.1. *Aequorin reconstitution and luminescence measurements*……………………..83

2.2.2. In tobacco subcellular compartments……………………………………………..83

2.2.2.1. In vivo aequorin reconstitution……………………………………………83

2.2.2.2. Measurement of the intracellular free calcium variation………………..….84

2.3. Reactive oxygen species production measurement in *Arabidopsis*………………..84

2.3.1. Luminol-dependent assay……………………………………………………...84

2.3.2. Diaminobenzidine (DAB) staining……………………………………………85

2.4. Nitric oxide production measurement……………………………………………85

2.4.1. In *Arabidopsis* leaf disks………..……………………………………...85

2.4.2. Measurement of nitric oxide production in tobacco cell suspensions……………86

2.5. Western Blotting………………………………………………………………..86

2.5.1. For activated MAPK detection in *Arabidopsis*…………………………………...86

2.5.2. Enhanced chemiluminescence (ECL) detection………………………………..87

2.5.3. Cell fractioning and western blotting…………………………………………..87

2.6. Mitochondrial O_2 uptake…………………………………………………………88

2.7. Measurement of Chl fluorescence yield…………………………………………88

2.8. *Botrytis cinerea* infection………………………………………………………..89

2.9. *Hyaloperonospora arabidopsidis* pathogenicity test………………………………...89

2.10. Gene expression by Real Time qPCR…………………………………………..90

2.10.1. RNA isolation……………………………………………………………...90

2.10.2. Synthesis of cDNA…………………………………………………………91

2.10.2.1. DNase treatment…………………...……………………………………91

2.10.2.2. First strand cDNA synthesis……………………………………………..91

2.10.2.3. Transcript accumulation analysis by Real Time qPCR……………………92

2.11. Transcriptome analysis…………..……………………………………………93

2.11.1. RNA extraction, cDNA synthesis and labeling…………….……………………...93

2.11.2. Array hybridization and scanning………………………………………………93

2.11.3. Microarray data analysis……………………………………………………...94

CHAPTER 3……………………………………………………..95

CALCIUM SIGNATURES AND SIGNALING IN CYTOSOL AND ORGANELLES OF TOBACCO CELLS INDUCED BY PLANT DEFENSE ELICITORS…………………95

ABSTRACT………………………………………………………………………..96

1. Introduction………………………………………………...………………..97

2.Materials and Methods…………………………………………………………99

2.1. Plant material and transformation……………………………………………..99

2.2. Cell fractioning and Western blotting……………………………………………………99

2.3. Cell preparation and treatment…………………………………………………………100

2.4. Measurement of [Ca^{2+}] variations………………………………………………………100

2.5. Measurement of nitric oxide……………………………………………………………100

2.6. Mitochondrial O_2 uptake…………………………………………………………..101

2.7. Measurement of Chl fluorescence yield……………………………………………...101

3. Results...……………………………………………………………………………………101

3.1. Cryptogein and OGs induced specific subcellular Ca^{2+} signatures in cytosol, chloroplasts and mitochondria…………………………………………………………………101

3.2. Cryptogein-induced [Ca^{2+}] signaling in organelles…………………………………..104

3.3. Impact of Cry-induced Ca^{2+} signaling on mitochondrial O_2 consumption…………..106

3.4. Impact of Cry-induced Ca^{2+} signaling on Chl fluorescence yield……………………107

4. Discussion……………………………………………………………………………...109

4.1. Spatiotemporal [Ca^{2+}] variations induced by elicitors of defense reactions in organelles………………………………………………………………………………………109

4.2. Cry-induced [Ca^{2+}] signaling in mitochondria and chloroplasts……………………..112

4.3. Ca^{2+} signaling and mitochondrial respiration in cryptogein-treated cells……………113

4.4. Ca^{2+} signaling and excess energy dissipation in cryptogein-treated cells……………114

5. Conclusions...…………………………………………………………………………117

CHAPTER 4……………………………………………………119

GLUTAMATE RECEPTORS ARE INVOLVED IN CA^{2+}-DEPENDENT PLANT DEFENSE SIGNALING AND RESISTANCE TO PATHOGENS……………………119

ABSTRACT………..………………………………………………………………………..120

1. Introduction……...…………………………………………………………………121

2. Materials and Methods…...……………………………………………………...………123

2.1. Biological materials……………………………………………………………………..123

2.2. Growth conditions and treatments……………………………………………………123

2.3. Mutant Genotyping……………………………………………………………………...123

2.4. [Ca^{2+}]$_{cyt}$ variation……………………………………………………………………..123

2.5. H_2O_2 detection…………………………………………………………………………..124

2.6. Nitric oxide detection…………………………………………………………………...124

2.7. *Botrytis cinerea* infection test………………………………………………………...125

2.8. *Hyaloperonospora arabidopsidis* infection test……………………………………...125

2.9. Gene expression analysis………………………………………………………………..125

3.Results……………………………………………………………………………………126

3.1. OGs signaling pathway is dependent on glutamate receptors…………………...126

3.1.1. OGs-induced [Ca^{2+}]cyt variation in *A. thaliana* are modulated by GLRs……….126

3.1.2. GLRs modulate OGs-triggered ROS production………………………………...127

3.1.3. GLRs participate in OGs-triggered NO production……………………………...127

3.1.4. GLRs-dependent defense gene expression………………………………………128

3.1.5. GLRs and basal resistance against necrotrophic and biotrophic pathogens……..128

3.2. Genetic evidences for *Arabidopsis* clade 3 GLRs involvement in OGs-induced.plant defense signaling and resistance against *H. arabidopsidis*…………….129

3.2.1. OGs-induced ROS and NO production in *Atglr* mutant plants…………………129

3.2.2. Resistance to *B. cinerea* and *H. arabidopsidis* in *Atglr* mutant plants…………..130

3.2.3. *AtGLR3.3* involvement in *H. arabidopsidis* resistance and plant defense signaling……………………………………………………………………………...130

4.Discussion……….……………………………………………………………………...131

CHAPTER 5…………………………………………………....137

GLUTAMATE RECEPTOR REGULATED GENE EXPRESSION IN GLU- AND OGs-TREATED PLANT TISSUES………………………………………………………….137

1. What is microarray?...137

1.1. Types of Microarrays…………………………………………………………...138

1.2. Principle………………………………………………………………………… 138

1.3. Applications……………………………………………………………………..139

2. Transcriptomic analysis to identify the modulated genes in response to Glu and OGs treatments in *Arabidopsis*………………………………………………………………140

2.1. Background Context……………………………………………………………140

2.2. Glu-responsive genes…………………………………………………………...142

2.3. OGs-responsive genes…………………………………………………………..142

2.4. GO annotation of Glu- and OGs-modulated genes in *Arabidopsis thaliana*………...143

2.4.1. Glu-responsive genes…………………………………………………………143

2.4.2. OGs-responsive genes………………………………………………………...144

2.5. MapMan analysis of Glu- and OGs-responsive genes in *Arabidopsis thaliana*……..144

2.5.1. MapMan biotic stress pathway of Glu-responsive genes……………………..145

2.5.2. MapMan biotic stress pathway of OGs-responsive genes…………………….145

3. Comparative analysis of OGs transcriptomics responses in *Arabidopsis thaliana*…..147

4. Identification and characterization of GLRs-responsive genes……………………148

4.1. GO annotation of GLRs-responsive genes in *Arabidopsis thaliana*…………………149
4.2. GLRs-responsive genes by MapMan……………………………………………...149
4.3. Identification of common genes regulated by Glu and GLRs………………………..150
5. Functional characterization of candidate genes……………………………………….151
5.1. Expression of candidate genes by RT-qPCR to validate microarray data…………....151
6. Discussion………………………………………………………………………………...152
7. Conclusions……………………………………………………………………………….164
CHAPTER 6……………………………………………………166
DISCUSSION AND PERSPECTIVES…………………………………………………...166
1. Discussion………...………………………………………………………………………166
2. Global perspectives………………………………………………………………………173
CHAPTER 7……………………………………………………177
LITERATURE CITED……………………………………………………………………177
PUBLICATIONS AND COMMUNICATIONS…………………………………………..

LIST OF ABBREVIATIONS

$[Ca^{2+}]_{chlo}$: Free chloroplastic calcium concentrations
$[Ca^{2+}]_{cyt}$: Free cytosolic calcium concentration
$[Ca^{2+}]_{ext}$: Free extracellular calcium concentrations
$[Ca^{2+}]_{mito}$: Free mitochondrial calcium concentrations
$[Ca^{2+}]_{nuc}$: Free nuclear calcium concentrations
$^{\bullet}OH$: Hydroxyl radical
***A. brassicicola*:** *Alternaria brassicicola*
***A. thaliana*:** *Arabidopsis thaliana*
***A. tumefaciens*:** *Agrobacterium tumefaciens*
ABA: Abscisic acid
Abs: Absorbance
ACA: Aut-oinhibited Ca^{2+}-ATPase
ADP: Adenosine diphosphate
AGP: Arabinogalactan protein
AM: Arbuscular micorrhiza
AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AOS: Active oxygen species
AOX: Alternative oxidase
AP-5: D-2-amino-5-phosphono pentanoic acid
AP-7: D-2-amino-7-phosphono pentanoic acid
APG: Arabinogalactane family
APX: Ascorbate peroxidase
Asp: Aspartate
AtCNGC1: *Arabidopsis thaliana* cyclic nucleotide-gated ion channel
ATP: Adenosine triphosphate
AtTPC1: *Arabidopsis thaliana* two pore channel
Avr: Avirulent genes
***B. cinerea*:** *Botrytis cinerea*
BABA: β-aminobutyric acid
BABA-IR: BABA-induced resistance
BAK1: Brassinosteroid Receptor1-Associated Kinase 1
BAPTA: 1,2-bis(aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BcPG1: *Botrytis cinerea* endopolygalacturonase
BMAA: [(S(+)-β-methylalpha,β-diaminopropionic acid]
BR: Brassinosteroid
BSA: Bovine serum-albumine
BTH: Benzo(1,2,3)thiadiazole-7-carbothioate
***C. fulvum*:** *Cladosporium fulvum*
C. marginiventris: *Cotesia marginiventris*
cADPR: Cyclic ADP ribose
CaM: Calmoduline
CaMBD: CaM-binding domain
CaMK: CaM-dependent protein kinase
cAMP: Cyclic adenosine monophosphate
CaMs: Calmodulins: Calcium-Modulated Protein

CAS: Calcium sensing receptor
CAX: Cation exchanger
CAX1: Calcium exchanger 1
CBL: Calcineurin B-like protein
CCaMKs: Ca^{2+}/CaM-dependent protein kinases
CC-NBS-LRR: coiled-coil nucleotide-binding leucine-rich repeat
CCX: Calcium cation exchanger
cDNA: Complementary deoxyribonucleic acid
CDPK: Ca^{2+} dependent protein kinase
CEBiP: Chitin elicitor binding protein
CERK1: Chitin elicitor receptor kinase1
cGMP: Cyclic guanosine monophosphate
CICR: Ca^{2+}-induced Ca^{2+}-release
CIPK: CBL-interacting protein kinase
CKs: Cytokinins
CML: Calmodulin-Like Protein
CNGC: Cyclic nucleotide gated channels
CNQX: 6-cyano-7-nitroquinoxaline- 2,3-dione
CNS: Central nervous system
Col-0: Columbia-0
cPTIO: Carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CRK: CDPK-related kinase
Cry: Cryptogein
CSP: Cold-shock proteins
cv.: Cultivar
CWE: Cell wall extract
Cy3: Cyanine 3
Cy5: Cyanine 5
Cyt c: Cytochrome c
DAB: 3,3′-diaminobenzidine
DACCs: Depolarization-activated Ca^{2+} channels
DAF-2DA: 4,5-diaminofluorescein diacetate
DAF-2T: Diaminofluorescein 2-Triazole
DAMP: Damage associated molecular pattern
DHAR: Dehydroascorbate reductase
DHS: D-erythro-sphinganine
dmi1: Does not make infection 1
DMSO: Dimethyl sulfoxide
dnd 1: Defence no death 1
DNQX: 6,7-dinitroquinoxaline-2,3-dione
DP: Degree of polymerization
dpi: Days post-inoculation
DPI: Diphenylene iodonium
DTT: Dithiothreitol
***E. coli*:** *Escherichia coli*
ECA: ER-type Ca^{2+}-ATPase
EDS1: Enhanced disease susceptibility 1
EDTA: Ethylene diamine tetra-acetic acid
EF: Elongation factor
EF1α: Elongation factor1α
EFR: Elongation factor receptor
EGTA: Ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N,- tetraacetic acid
EIN2: Ethylene insensitive 2
EIX1: Ethylene-inducing xylanase
elf18: Elongation factor 18
ENOD11: Early nodulation 11
ER: Endoplasmic reticulum
ET: Ethylene

ETC: Electron transport chain
ETI: Effector-triggered immunity
ETS: Effector-triggered susceptibility
FAD: flavin adenine dinucleotide
Flg22: Flagellin 22
FLS2: Flagellin sensing 2
FW: Fresh weight
FY: Fluorescence yield
GA: Gibberellic acid
GABA: γ-aminobutyric acid
GAD: Glutamate decarboxylase
GBP: Glucan-binding protein
Gln: Glutamine
GLR: Glutamate receptor-like genes
Glu: Glutamate
GluR: Glutamate receptor
Gly: Glycine
GOGAT: 2-oxoglutarate amidotransferase
GPCRs: G-protein coupled receptors
GS: Glutamine synthetase
GSTs: Glutathione transferases
H. arabidopsidis: *Hyaloperonospora arabidopsidis*
H. parasitica: *Hyaloperonospora parasitica*
H_2O_2: Hydrogen peroxide
HACCs: Hyperpolarization-activated Ca^{2+} channels
HDACs: Histone deacetylases
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hpa: *Hyaloparonospora araabidopsidis*
HR: Hypersensitive response
Hrp: Harpin
IAA: Auxin
ICS1: Isochorismate synthase 1
Idh: Isocitrate dehydrogenase
iGluR: Ionotropic glutamate receptor
IP_3: Inositol-1,4,5-triphosphate
ISR: Induced systemic resistance
JA: Jasmonic Acid
KA: Kainate
KCN: Potassium cyanide
La^{3+}: Lanthanum
LB: Lysogeny broth
LOX: Lipoxygenase
LPS: Lipopolysaccharide
LRR-(RK): Leucine-rich repeat (receptor kinase)
LRR: Leucine-rich repeat
LRR-RLKs: leucine-rich repeat receptor like kinase
LZ-CC: Leucine-zipper/coiled-coil domain
M. grisea: *Magnaporthe grisea*
M. truncatula: *Medicago truncatula*
MAMP: Microbe associated molecular pattern
MAPK: Mitogen-activated protein kinase
MBP: Myelin basic protein
MCU: Mitochondrial Ca^{2+} uptake (MCU)
MES: 2-(N-Morpholino)ethanesulfonic acid
MESA: Methyl salicylate
mGluR: Metabotropic glutamate receptor
MK-801: 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine

MNQX: 5,7-Dinitro-1,4-dihydro-2,3-quinoxalinedione
mPTP: Mitochondrial permeability transition pore
mRNA: Messenger ribonucleic acid
MS: Murashige and Skoog
***N. plumbaginifolia*:** *Nicotiana. plumbaginifolia*
***N. tabacum*:** *Nicotiana tabacum*
NAADP: Nicotinic acid adenine dinucleotide phosphate
NAD: Nicotinamide-adenine dinucleotide
NADPH oxidase: Nicotinamide-adenine dinucleotide phosphate-oxidase
NADPH: Nicotinamide-adenine dinucleotide phosphate
NASC: Nottingham *Arabidopsis* stock centre
NB: Nucleotide binding
NBS: Nucleotide-binding site
NCS: Neuronal calcium sensors
NCX: Sodium calcium exchanger
NMDA: N-methyl-D-aspartate
NMR: Nuclear magnetic resonance
NO: Nitric oxide
NOS: Nitric oxide synthase
NPQ: Non photochemical quenching
NPR1: Non-expressor of pathogenesis-related genes 1
NSCC: Non selective calcium channel
NTP: Nucleoside triphosphate
$O_2^{\cdot-}$: Superoxide Anion
O_2H^-: Perhydroxyl radical
OEC: Oxygen-evolving complex
OGs: Oligogalacturonides
$ONOO^-$: Peroxynitrite
***P. brassicae*:** *Phytophthora brassicae*
***P. cryptogea*:** *Phytophthora cryptogea*
***P. cucumerina*:** *Plectosphaerella cucumerina*
***P. sojae*:** *Phytophthora sojae*
P. syringae: *Pseudomonas syringae*
***Pad*:** *Phytoalexin deficient*
PAL: Phenylalanine ammonia lyase
PAM: Pulse amplitude modulation
PAMP: Pathogen associated molecular pattern
PCD: Programmed cell death
PCR: Polymerase chain reaction
PDA: Potato dextrose agar
PDB: Potato dextrose Broth
PDF1.2: Plant defensin protein 1.2
Pep13: Peptide 13
PER4: Anionic peroxidase 4
PG: Polygalacturonase
PGN: Peptidoglycan
Pi: Inorganic phosphate
PIP2: Phosphatidyl-inositol-4,5-bisphosphate
PIs: Proteinase inhibitors
PK: Protein kinase
PL: Phospholipase
PLA2: PhospholipaseA2
PLC: Phospholipase C
PM: Plasma membrane
PMCA: Plasma membrane Ca^{2+}-ATPase
PMSF: Phenylmethanesulfonyl fluoride
PP: Protein phosphatase

PPI: Phosphoinositide
PR: Pathogenesis-related protein
PRRs: Pattern recognition receptors
PS3: Sulphated laminarine
PSII: Photosystem II
PTI: PAMP-triggered immunity
PTP: Permeability transition pore
R: Resistance gene
rax1: *Regulator of APX2*
RbcL: RuBisCO large subunit
Rboh: Respiratory burst oxydase homologue
Real Time qPCR: Realtime quantitative polymerase chain reaction
RLKs: Receptor-like kinases
RLP: Receptor like protein
RLU: Relative luminescence unit
RNAi: RNA interference
ROS: Reactive oxygen species
rpm: Rotation per minute
RPP: Recognition of *Peronospora parasitica*
RR: Ruthenium red
R-SO_3H: Sulfonic acid
RTKs: Receptor tyrosine kinases
RuBisCO: Ribulose-1,5-bisphosphate carboxylase oxygenase
RYR: Ryanodine receptor
***S. littoralis*:** *Spodoptera littoralis*
***S. lycopersicum*:** *Solanum lycopersicum*
***S. meliloti*:** *Sinorhizobium meliloti*
SA: Salicylic acid
SABP2: SA binding protein 2
SAR: Sytemic acquited resistance
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser: Serine
SERCA: Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SHAM: Salicylhydroxamic acid
SIPK: Salicylic acid-induced protein kinase
SnRK2: SNF1-related protein kinase 2
SOD: Superoxide dismutase
STK: Ser/Thr kinase
SV: Slow vacuolar type
TAIR: The *Arabidopsis* Information Resource
TCA: Tricarboxylic acid
TFs: Transcription factors
TIR: Toll/interleukin-1 receptor
TMDs: Transmembrane domains
TMV: Tobacco mosaic virus
TPC: Two Pore Channel
TTSS: Type-III secretion system
***UBQ10*:** *Polyubiquitin*
***V. vinifera*:** *Vitis vinifera*
VOCs: Volatile organic compounds
VSP: Vegetative storage protein
WAK: Wall-associated kinase
WAKL: Wall associated kinase like
WIPK: Wound-induced protein kinase
Xanthi-Aeq-chloro: Xanthi aequorin chloroplastic cells
Xanthi-Aeq-cyto: Xanthi aequorin cytosolic cells
Xanthi-Aeq-mito: Xanthi aequorin mitochondrial cells

CHAPTER 1

CHAPTER 1

“Bibliographic context”

CHAPTER 1

BIBLOGRAPHIC CONTEXT

1. Immune systems in plants

1.1. Background and active resistance

During their life cycle, plants have to face a constant challenge of different environmental stresses that might pose adverse effects on their growth and development. Globally, these stresses can be classified into abiotic and biotic. In contrast to non living components of abiotic stresses (temperature, light, drought, wind *etc*.) living organisms like viruses, bacteria, fungi, nematodes, oomycetes and insects constitute biotic stresses components of the environment. Together, both these environmental factors are responsible for a significant loss in the crop productivity worldwide. In turn, this reduced crop yield is estimated to result in a hundreds of billions of dollars loss in farmers' income every year (Dhlamini *et al.*, 2005). Simultaneously, this situation is creating a big challenge to feed ever-increasing world population.

1.2. A contemporary view of plant immunity

In the absence of adaptive immune system, plants have to rely on their innate immune system by inducing sophisticated multilevel defense responses against these potential pathogens. During the evolution process, plants have enabled themselves to compete against these changing environmental factors by 1) developing particular physiological structures and 2) establishing specific cellular mechanisms. Plants have evolved a complex array of defense reactions to better combat these invading pathogens.

First line of plant defense is the formation of physical and chemical barriers by the plants (Garcia-Brugger *et al.*, 2006; Hückelhoven, 2007; Bhuiyan *et al.*, 2009). Among physical barriers, plant cuticles and cell walls are important. Plant cuticles are mainly composed of cutin and/or cutan impregnated with wax and are produced by the epidermal cells of leaves, young shoots and other aerial plant organs. These not only minimize water loss by coverings of aerial plant organs but also function to protect the plant against pathogen by reducing their entry through stomata. Plant cell wall is present around each cell and is composed of cellulose and pectin. Constitutive production of antimicrobial compounds such as glucosides and saponins, and secondary metabolites play a role of plants chemical barriers

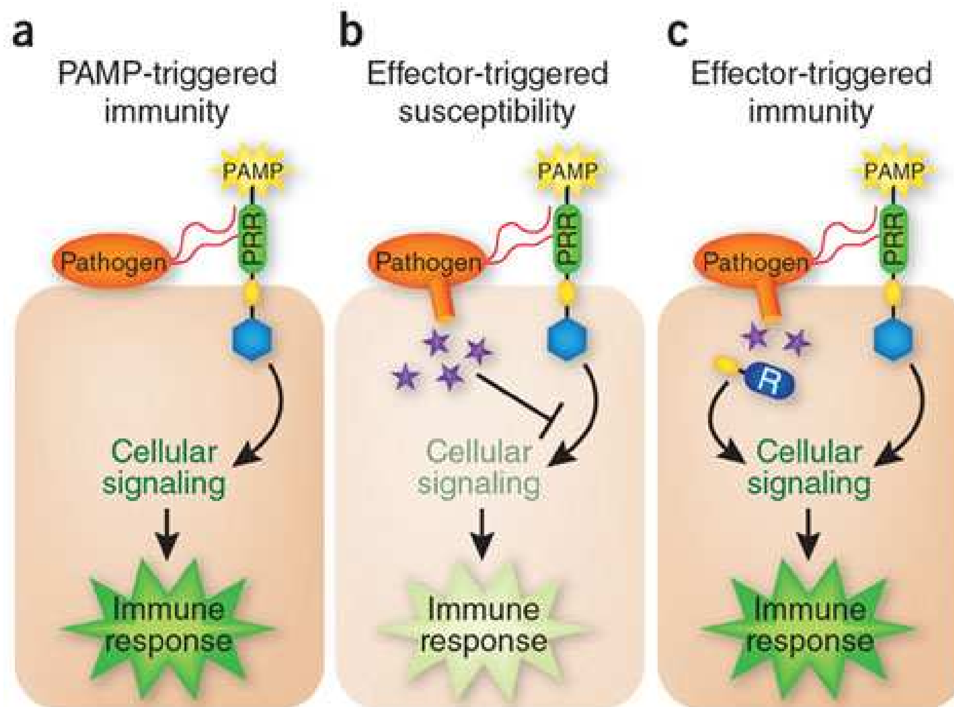


Figure 1.1: Plant immune response. A) After the pathogen attack, pathogen-associated molecular patterns (PAMPs) activate pattern-recognition receptors (PRRs) in the host, a consequence of this activation initiate the downstream signal transduction that ultimately leads to PAMP-triggered immunity (PTI). (**B**) Virulent pathogens have acquired effectors that suppress PTI, resulting in effector-triggered susceptibility. (**C**) **I**n turn, plants have acquired resistance (R) proteins that recognize these pathogen-specific effectors; outcome of this recognition is a secondary immune response called effector-triggered immunity (ETI; Pieterse *et al.,* 2009).

either by their toxicity to pathogen or by inactivating the enzymes secreted by the pathogen (Heath, 2000; Zhao *et al.*, 2005). Mostly, these barriers are efficient enough to protect the plant against the invading pathogens and are described as nonhost interactions. However, under certain conditions, these preformed structures and compounds fail to defend plants against attacking pathogens that may infect the plant through natural openings such as stomata or injury, or through the action of hydrolytic enzymes that degrade cuticle or cell wall. This condition is known as host interaction. At this stage, plant-pathogen interactions could be incompatible or compatible. During incompatible interactions, plants are able to recognize and check the pathogen growth by rapidly inducing defense signaling cascade and behave as resistant plants. In contrast, during compatible interaction they are unable to identify the pathogen and respond very slowly to behave as susceptible plants. In fact, plant defense responses are characterized by the recognition of the pathogen-derived molecules, known as pathogen associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), through specific pattern recognition receptors (PRR) mainly present on the plasma membrane (Bent and Mackey, 2007; Zipfel, 2009).

Plants demonstrate a great similarity to animal innate immune system at receptors level and highlight the presence of a conserved basic signal transduction mechanism to evoke defense responses (Nürnberger *et al.*, 2004; Garcia-Brugger *et al.*, 2006). During evolution, plants have developed two types of resistance in response to pathogens: non-specific resistance and specific resistance (Iriti and Faoro, 2007; Dodds and Rathjen, 2010). Non-specific resistance is the outcome of interactions between a large number of plant species and microorganisms. Hence, specific resistance can be induced by pathogen-or plant-derived signal molecules, called elicitors, on a limited or large variety of plants. Elicitors are grouped under the term PAMP and are recognized by PRRs (Boller and Felix, 2009). The second type of resistance is known as race-specific or specific resistance is based on "gene for gene" interaction in which specific avirulent (Avr) genes or effectors from the pathogen side are recognized by corresponding dominant resistance (R) gene in the host plant to confer resistance (Flor, 1971; Jones and Takemoto, 2004). Collectively, both types of resistances (non-specific and specific) are termed as innate immunity in plants and work in an efficient manner during plant protection against pathogens. Moreover, immunity based on the recognition of PAMPs and effectors are called PAMP-triggered immunity (PTI) and Effector-triggered immunity (ETI), respectively (Chisholm *et al.*, 2006; Jones and Dangl, 2006). In plant-pathogen context, an effector is a protein secreted by a pathogen and targets PTI actors to suppress the host plants' immune system capabilities thus leading to a condition known as

Table 1.1: An overview of different classes of plant defense elicitors. Brief description of each elicitor is given in table (Source, functions *etc*). More details of some of these elicitors are present in the text (table adapted from Mishra *et al.,* 2011).

Class	Elicitor	Sources	Type	Functions	Reference
Oligosaccharide Elicitors	Chito oligosaccharide Elicitors (Chitin)	Higher Fungi	General	Induced several defense-related genes, transient depolarization of membranes, extracellular alkalinization and ion efflux, changes in protein phosphorylation, generation of ROS	Ning *et al.*, 2004
	Glucans β-1,3-glucanase	Oomycetes cell wall, *Phytophthora sojae*	General		Fliegmann *et al.*, 2004
	Other Carbohydrate Elicitors Glucomannans	Oomycetes cell wall, *Phytophthora sojae; Blumeria graminis*		Induced the thaumatin-like proteins in barley, oat, rye, rice and maize	Keen *et al.*, 1983; Schweizer *et al.,* 2000
	LPS	*Burkholderia cepacia Escherichia coli,*		Induced ET, PR Proteins	Coventry and Dubery, 2001, Zeilder *et al.*, 2004; Silipo *et al.,* 2005
Protein / Peptide Elicitors	Elicitins (Cryptogein)	*Phytophthora* and *Pythium spp.*	Narrow	Induced an HR-like response, defence gene expression and systemic acquired resistance (SAR) to and the black shank causing agent *P. parasitica* var. Nicotianae in tobacco	Keller *et al.*, 1999
	AVR Elicitor Proteins (AVR4,AVR9))	*Cladosporium fulvum*	Race specific	Electrolyte leakage and lipoxygenase activity, induction of acidic forms of β-glucanase and chitinase, and production of activated oxygen species. Oxidative burst, H^+ -ATPase activation and HR	Joosten *et al.*, 1994; Hammond-Kosack et al. 1995; Wubben *et al.*, 1996; Vera-Estrella *et al.*, 1992, 1994; Westerlink *et al.*, 2002)
	Xylanase Elicitor (Endoxylase)	*Fusarium oxysporum, Macrophomina*	Race specific	The elicitor induces ET, PR Proteins, phytoalexin production, tissue	Dean and Anderson, 1991; Lotan

effector-triggered susceptibility (ETS) (Nicaise *et al.*, 2009; Pieterse *et al.*, 2009). Jones and Dangl (2006) propose a "zig zag" model of plant immunity in which they demonstrated that the ultimate amplitude of disease resistance or susceptibility in plants is proportional to [PTI – ETS + ETI]. This model was further improved by Pieterse *et al.* (2009). According to this model, in the first step, plants detect MAMPs/PAMPs via PRRs to trigger PTI. Under certain conditions, ETS occurs by the suppression of PTI by microbial effectors and finally, these pathogen effectors are recognized by protein encoded by resistance (R) genes present in plants that have been generated during evolution to activate ETI (Figure 1.1).

1.2.1. PTI (PAMP-triggered immunity)

The existence of a highly conserved system for the recognition of invading pathogens has been reported among higher eukaryotes (Nürnberger and Brunner, 2002; Nürnberger *et al.*, 2004). However, there also exist differences with respect to the nature of the receptors involved and the exact molecular patterns recognized (Zipfel and Felix, 2005). In plants, PAMPs and/or elicitors are either produced directly by pathogens or are released from the plant or pathogen cell wall by hydrolytic enzymes from the pathogen or the plant. They have a diverse chemical nature *e.g.* (glycol) proteins, lipids and oligosaccharides. An overview of different plant defense elicitors is presented in table 1.1 (Nürnberger *et al.*, 2004; Garcia-Brugger *et al.*, 2006; Boller and Felix, 2009). 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 among the most commonly studied elicitors in plants and confer resistance in *Arabidopsis thaliana* (Felix *et al.*, 1999; Kunze *et al.*, 2004; Zipfel and Felix, 2005). RNP-1 is a highly conserved RNA binding N-terminal peptide motif of gram-positive and negative bacterial cold-shock proteins (CSP) and is responsible for resistance in the Solanaceae (Felix and Boller, 2003). In addition, the peptide 13, derived from a cell wall localized-transglutaminase, is a highly conserved peptide from *Phytophthora* species. It is an efficient elicitor to induce defense responses in parsley and potato (Brunner *et al.*, 2002). Chitin and glucan are the important components of fungal and oomycete cell wall and function as defense elicitors in plants (Boller, 1995; Kaku *et al.*, 2006; Erbs *et al.*, 2008). Peptidoglycan (PGN) is a polymer of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc) residues in β-1–4 linkage which are cross-linked by short peptides, and is an essential and unique component of the gram-positive and gram-negative bacteria and provides rigidity and structure to the bacterial cell (Glauner *et al.*, 1988; Gust *et al.*, 2007). PGN function as

		phaseolina and *Trichoderma viride*		necrosis, lipid peroxidation, electrolyte leakage and cell death	and Fluhr 1990; Farmer and Helgeson 1987; Bailey *et al*., 1990; Ishii 1988; Bailey *et al*., 1990; Elbaz *et al*., 2002
	PaNie213 Elicitor PaNie(25 kDa)	*Pythium aphanidermatum*	General	Cell death and de novo formation of 4-hydroxybenzoic acid in cultured cells of carrot, callus formation on the cell walls of leaves of Arabidopsis, and necrosis in tobacco and tomato leaves	Veit *et al*., 2001
	Viral proteins *e.g.* viral coat protein Harpins (kDa)		Race specific	HR in Tobacco and tomato	
	NEP1 Elicitor	*Fusarium oxysporum*		Induces necrosis and ET production in leaves of many dicot plant species. Nepl induced extracellular alkalinization, ROS production and cell death	Steiner-Lange *et al*., 2003; Jennings *et al*., 2001
	NIP1 Elicitor	*Rhynchosporium Secalis*		Induced necrosis , accumulation of of pathogenesis-related (PR) proteins PR-1, PR-5, PR-9 and PR-10 in resistant barley varieties	
	PB90 Elicitor	*Pyhtophthora boehmeriae*		Triggered HR, H_2O_2 production, activate peroxidase and PAL activities	Wang *et al*., 2003; Zhang *et al*., 2004
	RNP-1	Bacteria	General	Resistance in Solanaceae	Felix and Boller, 2003
	Flagellin	Bacteria	General	Including medium alkalinization, oxidative burst, and increased biosynthesis of ET	
	EF-Tu	Bacteria		Triggered MAMP responses in Arabidopsis, innovation in the Brassicaceae	Kunze *et al*., 2004
Glycoprotein Elicitors	Carbohydrate moiety confer elicitor activity	*Colletotrichum Lagenarium*	General		Toppan and Exquerré-Tugayé, 1984
	Protein moiety confer elicitor	*Verticillium dahliae, Pythium*	General	Phytoalexin formation elicited oxalate oxidase-	Davis *et al*., 1998;

MAMP to activate different plant responses, such as medium alkalinization, elevation of cytoplasmic calcium concentrations ($[Ca^{2+}]_{cyt}$), camalexin and nitric oxide (NO) production, mitogen-associated protein kinases (MAPK) activation and genes expression in *Arabidopsis* (Gust *et al.*, 2007). Lipopolysaccharides (LPS) are the principle components of the outer membrane of gram-negative bacteria and activate plant defense responses (Zeidler *et al.*, 2004; Silipo *et al.*, 2005). Among other famous classes of elicitors, elicitins, sterols and binding proteins secreted by most *Phytophthora* species, cause defense responses including localized cell death and systemic acquired resistance in tobacco (Ricci *et al.*, 1989; Yu, 1995; Garcia-Brugger *et al.*, 2006). Cryptogein (Cry) is a well-known plant defense protein secreted by *Phytophthora cryptogea* and has been extensively studied during the generation of tobacco defense responses where it is able to induce hypersensitive responses (HR) and systemic acquired resistance (SAR; Ricci, 1997; Garcia-Brugger *et al.*, 2006). Ergosterol, the main sterol of fungi, has also been described to induce defense responses in different plant species (Granado *et al.*, 1995; Kasparovsky *et al.*, 2003, 2004; Laquitaine *et al.*, 2006; Lochman and Mikes, 2006). A new term, damage associated molecular patterns (DAMPs) has been attributed to elicitors class that are degraded products from pathogen or plant cell wall due to action of hydrolytic enzymes (Lotze *et al.*, 2007). A classic example of DAMPs is oligogalacturonates (OGs), a polymer of α-1,4-galacturonic acid which are formed either 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) In grapevine, BcPG1, an endopolygalacturonase from *B. cinerea*, acts as an elicitor and trigger early defense responses (Poinssot *et al.*, 2003). Ample data from the literature have demonstrated that OGs actively participate in the induction of signal transduction cascade that activates sophisticated multilevel defense responses in plants including variation in $[Ca^{2+}]_{cyt}$, production of reactive oxygen species (ROS) and NO, activation of MAPKs, membrane polarization, defense genes transcripts accumulation and phytoalexin production in *Arabidopsis* (Hu *et al.*, 2004; Lecourieux *et al.*, 2005; Ferrari *et al.*, 2007; Denoux *et al.*, 2008; Galletti *et al.*, 2008, 2011; Rasul *et al.*, 2012). In the past, it has been demonstrated that OGs treatments of *Vitis vinifera* and *Arabidopsis thaliana* leaves enhanced basal resistance against *Botrytis cinerea* (Aziz *et al.*, 2004; Ferrari *et al.*, 2007).

During PTI, these plant defense elicitors (PAMPs/DAMPs) are perceived by PRR receptors located on the surface of the cell. PRRs are a family of transmembrane proteins containing an extra-cytosolic leucin-rich repeat (LRR) and a C-terminal cytosolic Ser/Thr protein kinase region. Plants possess two types of PRRs: receptor-like kinases (RLKs;

	activity	*oligandrum*		like germin (OxOLG), glutathione S-transferase (GST), 5-enolpyruvylshikimate -phosphate synthase, PAL and aspartate amino transferase production in suger beet and wheet	Takenaka *et al*., 2006
	Glycoprotein (Pep13)	*Phytophthora* species, including *P. infestans, P. sojae*	General	Activation of defense-related genes in parsley and potato	Nürnberger *et al*., 1994; Halim *et al*., 2004
Lipid Elicitors	Sphingolipids (Cerebrosides)	*Cochliobolus miyabeanus, Cercospora solani-melongenae*, and *Mycosphaerella pinodes , M. grisea, Pythium m graminicola* and diverse strains of *Fusarium oxysporum*	General	Phytoalexin- inducing activity, expression of PR proteins in rice	Umemura *et al*., 2000
	Arachidonic and Eicosapentaenoic Acids	*Phytophthora infestans*	General	Elicitation of defense responses.	Creamer and Bostock, 1988
	Ergosterols		General	Induces changes in membrane potential, modifications of H^+ fluxes, production of active oxygen species and, in some cases, synthesis of phytoalexins	Corvone *et al*., 1997; Rossard *et al*., 2006; Kasparovsky *et al*., 2003

proteins with an intracellular kinase domain), and receptor-like proteins (RLPs) without cytoplasmic or intracellular domain (Nürnberger and Brunner, 2002; Pålsson-McDermott and O'Neill, 2007; Zipfel, 2009). The majority of these receptors are grouped in the class of LRR-RLKs (leucine-rich repeat receptor like kinase) and share a common domain organization with receptor tyrosine kinases (RTKs) found in animals (Jorissen *et al.*, 2003; Citri and Yarden, 2006). LRR-RLKs are highly sensitive and specific receptors and have been reported to be involved in the perception of pathogen factors. Previous reports indicate the identification of several members of the PRRs in various plants such as *Arabidopsis*, tomato and rice (Boller and Felix, 2009; Nürnberger and Kemmerling, 2009). In *Arabidopsis*, the perception of bacterial flagellin occurs through Flagellin-Sensing 2 (FLS2) receptor kinase where the conserved part of the flagellin polypeptide is recognized as PAMP by FLS2 (Zipfel and Felix, 2005). Previous studies have shown that *Arabidopsis* FLS2 directly binds the 22 amino acid flagellin epitope, and *flg22* and *fls2* mutant plants exhibited enhanced susceptibility to bacterial infection (Zipfel *et al.*, 2004). Recently, Zeng and He (2010) have reported a vital role of FLS2 in mediating stomatal response to *Pseudomonas syringae* in *Arabidopsis*. Moreover, MAPK and WRKY signaling pathways were found to function downstream of flagellin perception (Asai *et al.*, 2002). Elongation Factor Tu-Receptor (ERF) that recognizes EF-Tu/elf18 in *A. thaliana* is another well-characterized example of PRR (Kunze *et al.*, 2004; Zipfel *et al.*, 2006). EF-Tu receptor is highly conserved in all bacterial species and is known to be N-acetylated in *Escherichia coli*. *Arabidopsis* plants are able to specifically recognize the N terminus of the protein, and an N-acetylated peptide comprising the first 18 amino acids, termed elf18, is fully active as inducer of defense responses (Kunze *et al.*, 2004). In this regard, it has been reported that the expression of *Arabidopsis* EFR is able to confer responsiveness to EF-Tu (elf18) in *Nicotiana benthamiana* and *Solanum lycopersicum* and makes these plants more resistant against a broad spectrum of pathogens (Lacombe *et al.*, 2010). Moreover, high-affinity sites as Glucan-Binding Protein (GBP) and Chitin Elicitor Binding Proteins (CEBiP) are involved in the determination of oligosaccharides such as heptaglucanes of *Phytophthora sojae* and chitin (polymer of β-1,4 N acetylglucosamine) in soybean and rice, respectively (Kaku *et al.*, 2006). Ethylene-Inducing Xylanase (EIX1) is another type of RLP which recognizes the EIX1 xylanase in tomato (Ron and Avni, 2004; Göhre and Robatzek, 2008). In some cases, Brassinosteroid Receptor 1-Associated Kinase 1 (BAK1) and Chitin Elicitor Receptor Kinase 1 (CERK1) are implicated in elicitor recognition and behave as signaling adapter to initiate defense responses (Zipfel, 2009). Various studies have shown that *Bak1* null mutants are compromised in their

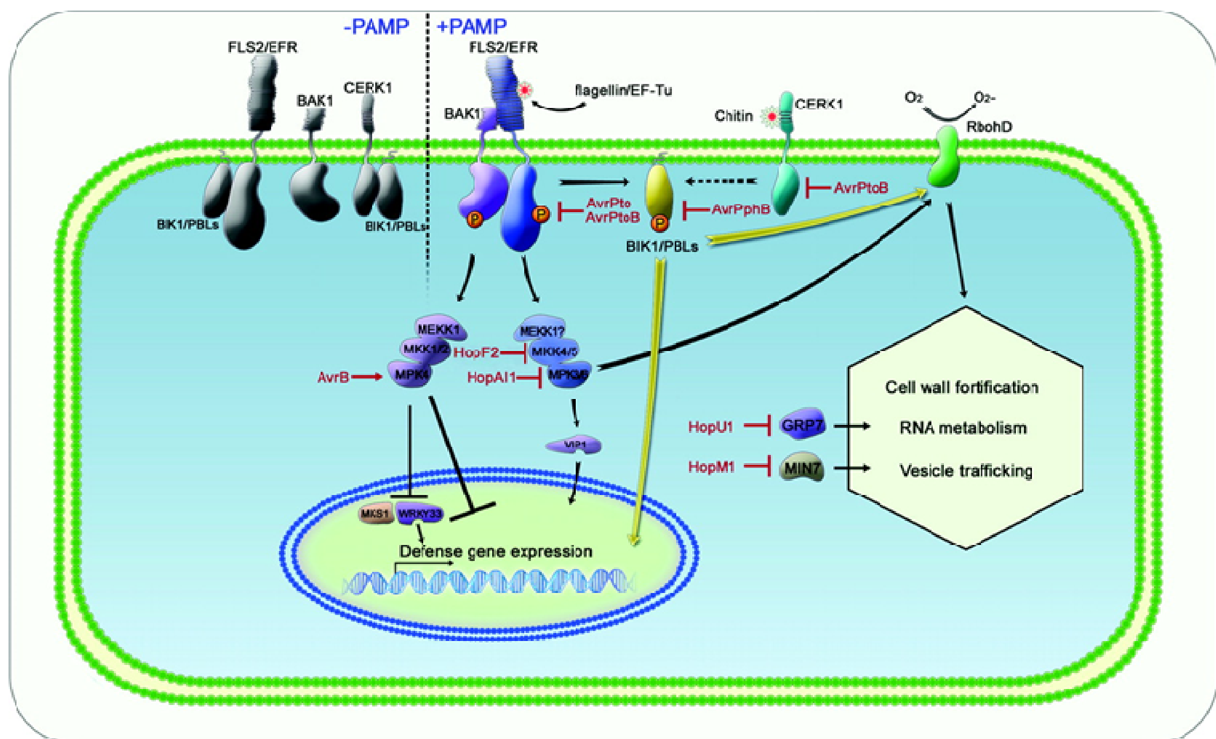


Figure 1.2: PAMP-triggered signal transduction pathways. In the first step when PAMPs are absent, the PRRs form a complex with BIK1 and PBLs. In contrast to this, sensing of PAMPs, such as flg22, EF-Tu, and chitin, stimulates an interaction between BAK1 and PRRs such as FLS2 and EFR. As a consequence of this interaction, cross-phosphorylation of PRRs and BAK1 takes place which ultimately activates PRR complex. PTI signaling pathways diverge downstream of PRRs [BIK1 and other PBLs associate with unactivated PRRs (gray) and activation of PRRs (color) by PAMPs]. *P. syrinage* effector (in red) proteins that inhibit or activate various PTI signaling components are also indicated (Zhang and Zhou, 2010).

responsiveness to several PAMPs including flg22, elf18, HrpZ, LPS, peptidoglycans, and DAMPs, such as AtPep1 (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Shan *et al.*, 2008; Krol *et al.*, 2010). Recently, it has been reported that BAK1 regulates the containment of microbial infection-induced cell death as *bak1* mutant plants exhibited necrotic symptoms upon bacterial infection (Kemmerling *et al.*, 2007). Moreover, BAK1 controls the phosphorylation-dependent differential regulation of cell death and innate immunity (Schwessinger *et al.*, 2011). CERK1 which contains an intracellular Ser/Thr kinase domain with an autophosphorylation/myelin basic protein (MBP) kinase activity has been shown to actively participate in plant immune responses. CERK1 is able to recognize an unknown MAMP from *P. syringae* (Gimenez-Ibanez *et al.*, 2009a,b).

Finally, the perceptions of PAMPs/MAMPs by PRRs lead to several physiological and molecular changes in plant with the ultimate activation of defense response against a variety of pathogens (Zipfel *et al.*, 2004; Hann and Rathjen, 2007; Jeworutzki *et al.*, 2010). Ca^{2+} fluxes, $[Ca^{2+}]_{cyt}$ variation, ROS and NO production, activation of MAPKs, defense genes transcripts accumulation and phytoalexin production are also amongst the important physiological and molecular events that are altered after PAMPs/MAMPs recognition in plants (Lecourieux *et al.*, 2005; Ferrari *et al.*, 2007; Galletti *et al.*, 2008; Tsuda and Katagiri, 2010; Galletti *et al.*, 2011). Moreover, PAMPs/MAMPs-induced cell wall thickening, callose deposition and stomatal closure results in plant protection against pathogens by the activating SAR and/or HR (Schwessinger and Zipfel, 2008). Figure 1.2 represents a general scheme of PAMP triggered signaling pathway in plants (Zang and Zhou, 2010).

1.2.2. ETI (effector-triggered immunity)

The evolutionary arms race has led to various interesting developments on both plants and pathogens sides with plant being on more beneficial side of the competition. Plants have developed highly sophisticated defense system that recognizes pathogen molecules and initiates specific defense signaling pathways against their attackers. During evolution, plants established defense responses in the form of PTI to ward off the invaders. In turn, virulent pathogens developed new strategies by acquiring effectors molecules to suppress PTI and ultimately led to ETS. Following to the “survival of the fittest” rule, plant responded by producing special resistance (R) proteins that are able to recognize these specific effectors and resulted in the evolution of secondary immune response called ETI. In plants, "gene for gene" hypothesis was proposed which suggested that a specific R gene in plant is present for each specific effector molecule from pathogen (Flor, 1971).

Table 1.2: List of effectors from different pathogens. Biochemical function and the effector targets are known for a subset of these effectors. Various methods have been used to characterize their role in PTI suppression (Göhre and Robatzek, 2008).

Name/ Alternative name	**Organism**	**Function**	**Target**	**Role in PTI suppression**	**References**
AvrB1/ AvrB	*P. syringae*		RIN4	Suppression of RAP2.6 induction	He *et al.*, 2004 ; Desveaux *et al.*, 2007; Ong and Innes, 2006
AvrE1/ AvrE	*P. syringae*			Suppression of callose deposition	DebRoy *et al.*, 2004
				Reduction of vascular flow	Oh and Collmer, 2005
AvrPto1/ AvrPto	*P. syringae*		Pto kinase/Pfr	Suppression of callose deposition	Hauck and Thilmony, 2003
				Reduction of vascular flow	Oh and Collmer, 2005
				Suppression of flg22 dep. NHO1 induction	Li *et al.*, 2005
				Suppression of FRK1/SIRK induction	He *et al.*, 2005
				Suppression of nonhost HR	Hann and Rathjen, 2007
				Interaction with Rab-GTPases	Bogdanove and Martin, 2000
AvrRpm1	*P. syringae*		RIN4		Lu *et al.*, 2001; Mackey et al., 2002
AvrRpt2	*P. syringae*	Cysteine protease	RIN4	Suppression of RAP2.6 induction	Gürlebeck *et al.*, 2006; He *et al.*, 2004; Kim *et al.*, 2005
HopA1/ HopPsyA	*P. syringae*				
HopAA1–1 / HopPtoA1	*P. syringae*			Suppression of flg22 dep. NHO1 induction	Li *et al.*, 2005
HopAB2/ AvrPtoB	*P. syringae*	Ubiquitin E3 ligase	Fen kinase	Suppression of non host HR	Hann and Rathjen, 2007
				Suppression of cell death (HopPsyA)	Jimer *et al.*, 2004; Rosebrock *et al.*, 2007
				Suppression of FRK1/SIRK induction	He *et al.*, 2005

HopAF1	*P. syringae*			Suppression of flg22 dep. NHO1 induction	Li *et al.*, 2005
HopAI-1	*P. syringae*	Phosphothreonine lyase	MPK3, MPK6	Suppression of flg22 dep. NHO1 induction	Li *et al.*, 2005; Zhang *et al.*, 2007
HopAM1/ AvrPpiB	*P. syringae*			Suppression of cell death (HopPsyA)	Jamir *et al.*, 2004
HopAO1/ HopPtoD2	*P. syringae*	Protein tyrosine phosphatase	Downstream of MAPK in PTI signaling	Suppression of PCD	Bretz *et al.*, 2003; Underwood *et al.*, 2007
HopAR1/ AvrPphB	*P. syringae*	Papain-like Cys Protease, YopT	PBS1	Suppression of RAP2.6 induction	Gürlebeck *et al.*, 2006; He *et al.*, 2004
HopC1/ AvrPpiC2	*P. syringae*	Papain-like Cys Protease, YopT		Suppression of flg22 dep. NHO1 induction	Li *et al.*, 2005
HopD1/ HopPtoD1	*P. syringae*				
HopE1 HopPtoE/ HopE	*P. syringae*			Suppression of cell death (HopPsyA)	Jamir *et al.*, 2004
HopF2/ AvrPphF	*P. syringae*			Reduction of vascular flow	Oh and Collmer, 2005
				Suppression of cell death (HopPsyA)	Jamir *et al.*, 2004
HopG1/ HopG	*P. syringae*			Reduction of vascular flow	Oh and Collmer, 2005
HopK1/ HopPtoK	*P. syringae*			Suppression of RAP2.6 induction	He *et al.*, 2004
HopM1/ HopPtoM	*P. syringae*	Adaptor for Ubiquitination machinery	MIN7	Suppression of callose deposition	DebRoy *et al.*, 2004
				Reduction of vascular flow	Oh and Collmer, 2005; Nomura *et al.*, 2006
HopN1/ HopPtoN	*P. syringae*	Papain-like Cys Protease, YopT			Lopez-Solanilla *et al.*, 2004
HopO1–1/ HopPtoS1	*P. syringae*	mono-ADP-ribosyltransferase	Chloroplast protein		Fu *et al.*, 2007
HopO1–2/ HopPtoS3	*P. syringae*	mono-ADP-ribosyltransferase	Chloroplast protein		Fu *et al.*, 2007
HopS1/ HolPtoZ	*P. syringae*			Suppression of flg22 dep. NHO1 induction	Li *et al.*, 2005
HopT1–1/ HolPtoU1	*P. syringae*			Suppression of flg22 dep. NHO1 induction	Li *et al.*, 2005
HopT1–2/ HolPtoU2	*P. syringae*			Suppression of flg22 dep. NHO1 induction	Li *et al.*, 2005

HopU1/ HopPtoS2	*P. syringae*	mono-ADP-ribosyltransferase	RNA-binding proteins (AtGrp7, AtGrp8)		Fu *et al.*, 2007
HopX1/ AvrPphE	*P. syringae*	Cysteine protease		Suppression of cell death (HopPsyA)	Jamir *et al.*, 2004
				Suppression of RAP2.6 induction	He *et al.*, 2004; Nimchuk *et al.*, 2007
Coronatine	*P. syringae*	JA mimic		Promotes reopening of stomata	Melotto *et al.*, 2006
AvrBsT	*X. campestris pv. Vesicatoria*	YopJ-like SUMO protease			Cunnac *et al.*, 2007
AvrRxv	*X. campestris pv. Vesicatoria*	YopJ-like SUMO protease	Cytoplasmic target?		Bonshtien *et al.*, 2005
AvrXv4	*X. campestris pv. Vesicatoria*	YopJ-like SUMO protease	Cytoplasmic target		Roden *et al.*,
XopD	*X. campestris pv. Vesicatoria*	SUMO protease	Transcription factors?	Structural determinants for substrates	Chosed *et al.*, 2008 ; Hosten and Mudgett, 2004
AvrBs3/ PthA	*Xanthomonas*	Transcription factor	Upa-box		Kay *et al.*, 2007
AvrBs2	*Xanthomonas*	Glycerophosphoryl diester phosphor-diesterase			Swords *et al.*, 1996
PopP1	*R. solanacearum*	YopJ-like SUMO protease			Lewis *et al.*, 2008
PopP2	*R. solanacearum*	YopJ-like SUMO protease			Lewis *et al.*, 2008
GALA	*R. solanacearum*	F-box proteins, ubiquitinaion (2)			Angot *et al.*, 2006
DspA/E	*E. amylovora*			Interaction with LRR-RLK	Meng *et al.*, 2006
				Suppression of callose deposition, delay of defense gene expression	Boureau *et al.*, 2006
AVRa10	*B. graminis*	Penetration			Ridout *et al.*, 2006
AVRk1	*B. graminis*	Penetration			Ridout *et al.*, 2006
Avr2	*C. fulvum*	Cysteine protease inhibitor	Rcr3		Rooney *et al.*, 2005
Avr4	*C. fulvum*	Chitin binding lectin	Chitinase		Van *et al.*, 2006

Effectors, also termed as toxins and effector proteins, are the pathogen-derived molecules intended to promote pathogen virulence during plant-pathogen interaction. Bacteria, fungi and oomycetes are able to secrete effectors which efficiently suppress plant immune responses (Chisholm *et al.*, 2006; Göhre and Robatzek, 2008; Stergiopoulos and De Wit, 2009; Dodds and Rathjen, 2010). The best characterized effectors belong to bacteria. In plants, *P. syringae* strains encode approximately 20 to 30 effectors molecules during infection (Chang *et al.*, 2005). Bacterial effectors are highly regulated and secreted directly into the host cytoplasm by a dedicated needle structure, the type-III secretion system (TTSS). They interfere directly with PTI responses, either by inactivating a target protein of host involved in PTI or by regulating host plants signaling cascade crucial for the plant development (Chisholm *et al.*, 2006; Jones and Dangl, 2006; Cunnac *et al.*, 2009). Interestingly, the absence of TTSS system in bacterial mutants leads to non-pathogenic phenotypes and the transgenic overexpression of an individual type-III effector in the host plant restores the ability of such single bacterial mutants to grow (Hauck *et al.*, 2003; Kim *et al.*, 2005). This suggests that bacterial pathogenicity only requires the suppression of PTI. In comparison to bacteria, very little is known about eukaryotic effectors and their functions. Both fungi and oomycetes are able to produce effectors that are released through the endomembrane system and are subsequently delivered into host cells by unknown mechanisms (Kamoun, 2007; Panstruga and Dodds, 2009). In *P. syringae,* two secreted effectors, AvrPto and AvrPtoB have been reported to physically interact with the kinase domains of FLS2 and EFR and results in the inhibition of the kinase activity of both PRRs (Xiang *et al.,* 2008). In another study, it was shown that AvrPto acts upstream of the MAPK signaling cascade to inhibit the PTI (He *et al.*, 2006). It should also be noted that all *P. syringae* strains are not able to express AvrPto and AvrPtoB. This suggests the presence of some other effector proteins able to block PRR signaling. HopAI1, another effector protein present in several *P. syringae* strains, is a phosphothreonine lyase that leads to dephosphorylation of MPK3 and MPK6 to inhibit PRR signaling (Zhang *et al.*, 2007). In addition to direct inhibition of PRR signaling, the effectors are also able to regulate downstream components of PRR signaling *e.g.* the *P. syringae* effector HopU1 modulates the expression of GRP7, an *Arabidopsis* RNA-binding proteins, by ADP-(adenosine diphosphate) ribosylation (Fu *et al.*, 2007; Jeong *et al.*, 2011). A list of effector molecules along with their origin, functions, target proteins and some of their functions in suppression of defense has been given in table 1.2 (adapted from Göhre and Robatzek, 2008).

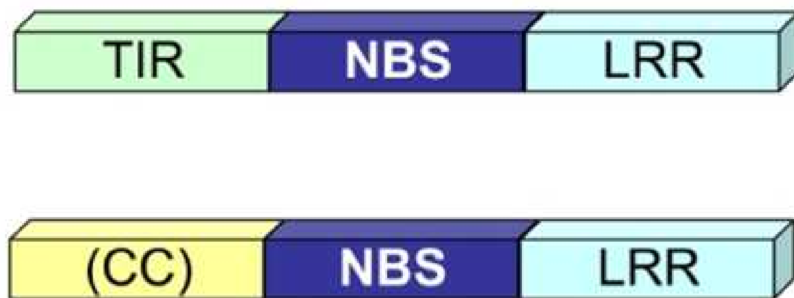


Figure 1.3: Types of plant NBS-LRR proteins. The two classes of NBS-LRR protein are differentiated by the N-terminal domain. TIR-NBS-LRR proteins have a Toll-interleukin-like receptor (TIR) domain, The N-terminal region of non-TIR-NBS-LRR proteins is less defined, but often contains a coiled-coil (CC) domain. In *R* genes, the NBS domain plays a role in intramolecular interactions with the LRR and N-terminal domains. The N-terminal domain influences the signaling pathway that could be activated upon effector recognition, and may also be involved in pathogen recognition and interactions with targets of pathogen effector (Tarr and Alexander, 2009).

Presence of *avr* genes and its corresponding R gene in pathogen and host plant, respectively, leads towards resistance while disease prevails when either they are absent or inactive (Flor, 1971). Pathogen *avr* (avirulence) genes are able to encode effectors proteins which interact with the corresponding plant disease resistance (R) gene products. Analyses of the R-genes products have demonstrated the existence of several conserved protein motifs among different plant species (Dangl and Jones, 2001). Despite a wide range of potential pathogen classes and their presumed pathogenicity effector molecules, only five classes of R genes are known in plants (Dangl and Jones, 2001). The presence of carboxy-term leucine-rich repeat (LRR) and conserved nucleotide-binding (NB) site are the main characteristics of these NB-LRR proteins. The LRR domains have been identified in diverse proteins and function as sites of protein-protein interaction, protein-carbohydrate interaction and peptide-ligand binding (Jones and Jones, 1996). The NBS domain is critical for the binding and hydrolysis of ATP, allows conformational changes of the protein and subsequent activation of signaling events necessary for the establishment of defense responses (Takken and Tameling, 2009). In *A. thaliana*, the NBS-LRR class of R proteins is predicted to be intracellular and could be subdivided into two distinct classes on the basis of deduced N-terminal structural features: CC-NBS-LRR and TIR-NBS-LRR (Figure 1.3; Tarr and Alexander, 2009). The CC-NBS-LRR class contains putative coiled-coil (CC) domains that is found in a variety of proteins and is involved in mediating protein-protein interactions. CC-NBS-LRR proteins have further classification based on the location of the CC domain. TIR-NBS-LRR class has a Toll and interleukin receptor (TIR) domain that shows resemblance with *Drosophila* Toll and mammalian interleukin (IL)-1 receptors. Moreover, these two sub-classes have different mechanisms of action in *Arabidopsis*: TIR-NB-LRR-mediated resistance is achieved through enhanced disease susceptibility 1 (EDS1; Parker *et al.,* 1996), while the CC-NB-LRR subclass signals through non-race specific disease resistance 1 (NDR1; Century *et al.*, 1997).

There exist several models for the recognition of effectors by R proteins in different pathosystems (Chisholm *et al.*, 2006; Jones and Dangl, 2006). One model suggests a direct interaction between the effector and the R protein and has been observed in bacteria, fungi and oomycetes. The first evidence in favor of this model came from the interaction between *P. syringae* effector AvrPto with the tomato R protein Pto (Tang *et al.*, 1996). In another study conducted by Jia *et al.* (2000), this type of interaction was demonstrated between a NBS-LRR protein *Pi-ta* from rice and the effector protein AVR-Pita from the fungus *Magnaporthe grisea*. However, in many pathosystems, no direct interaction between the effector and the R protein was reported (DeYoung and Innes, 2006; Figure 1.4). A protein of

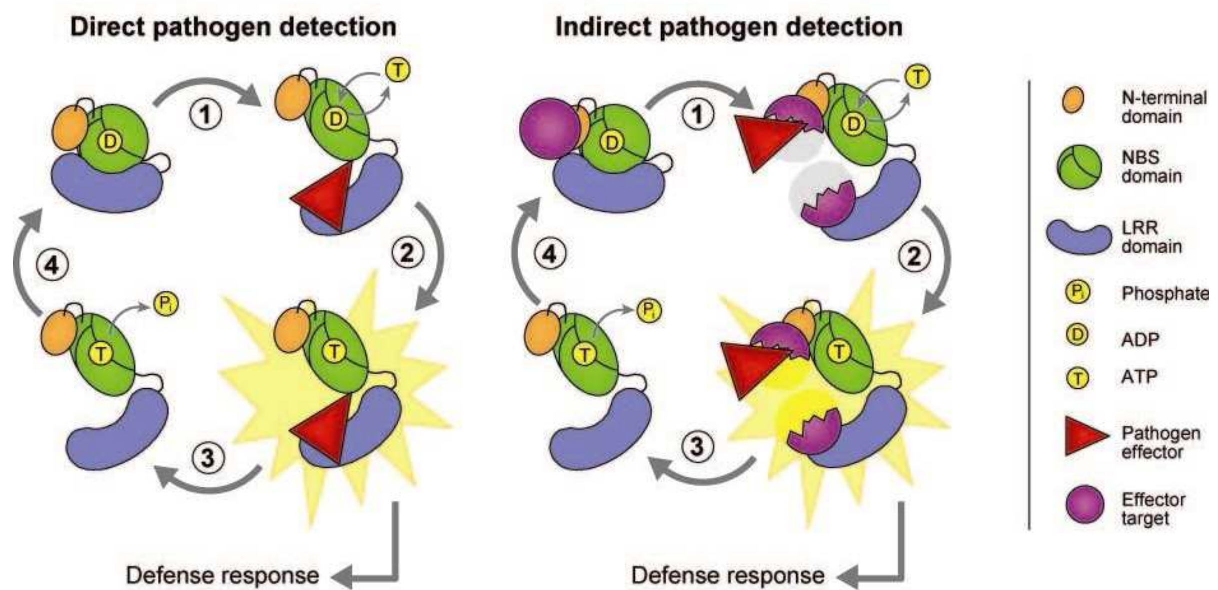


Figure 1.4: Models for plant NBS-LRR activation. Signaling is activated in a similar way for both modes of pathogen detection. Presence of the effector **(1)** changes the structure of NBS-LRR protein through direct binding (left) or alteration of additional plant proteins (right), allowing the formation or exchange 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 **(3)** along with hydrolysis of ATP **(4)** return the NBS-LRR protein to its inactive state (DeYoung and Innes, 2006).

the host then recognizes the complex formed between the effector and its target protein or target protein modified following its interaction with the effector (guard hypothesis; Dangl and Jones, 2001). It was reported that two *P. syringae* type III effectors, AvrRpt2 and AvrRpm1 inhibited PAMP-induced signaling and resulted in loss of host's basal defense system in *Araidopsis*. RPM1 interacting 4 (RIN4), an *Arabidopsis* protein, is targeted by AvrRpt2 and AvrRpm1 for degradation and phosphorylation, respectively. This modification of RIN4 in both cases is detected by R proteins, RPS2 and RPM1, respectively and R proteins guard the plant against these effectors and provide a mechanistic link between the plant basal defense and R-gene mediated defense systems (Kim *et al.*, 2005). Similarly, the *P. syringae* effector AvrPphB cleaves the PBS1, a protein kinase in *A. thaliana* and this cleavage is subsequently detected by the R protein RPS5 to mediate ETI (Shao *et al.*, 2003). RIN4 is a protein that interacts with two *P. syringae* effectors AvrB and AvrRpm1, the R protein RPM1 and represents is a good example of indirect interaction (Mackey *et al.*, 2002).

Many early signaling components of PTI and ETI have been identified in recent years (Göhre and Robatzek, 2008; Nürnberger and Kemmerling, 2009). Downstream of these early signaling events, plants respond by activating a large number of integrated defense responses to ward off the invaders (Figure 1.5; Göhre and Robatzek, 2008). As our research was focused on PAMPs signaling during plant defense responses, in the following section we will discuss in detail the signaling events that are mainly activated during plant basal defense *i.e.* PTI.

2. Signal transduction during plant defense

During plant defense responses, recognition of the pathogen is the foremost step to initiate an intracellular signaling cascade that contributes to the activation of an adaptive response of the plant (Jones and Dangl, 2006; Boller and Felix, 2009). Ample data from the literature is available to prove that elicitors are excellent tool to investigate the defense-related signal transduction pathways in plants. Interestingly, there exist great similarities between animals and plants for the recognition of these elicitor molecules thus indicating the conservation of a defense-related signaling system during evolution process in both life kingdoms (Dangl and Jones, 2001; Nürnberger *et al.*, 2004; Garcia-Brugger *et al.*, 2006). In contrast, effectors-mediating signaling in plants has not been thoroughly studied in plants and further information are needed to firmly establish effector-based signal transduction pathways (Zhao *et al.*, 2005; Hofius *et al.*, 2007). After elicitor recognition, an influx of Ca^{2+} and efflux of K^+ and some anions especially Cl^- or NO_3^- occur as an early step. These fluxes results in

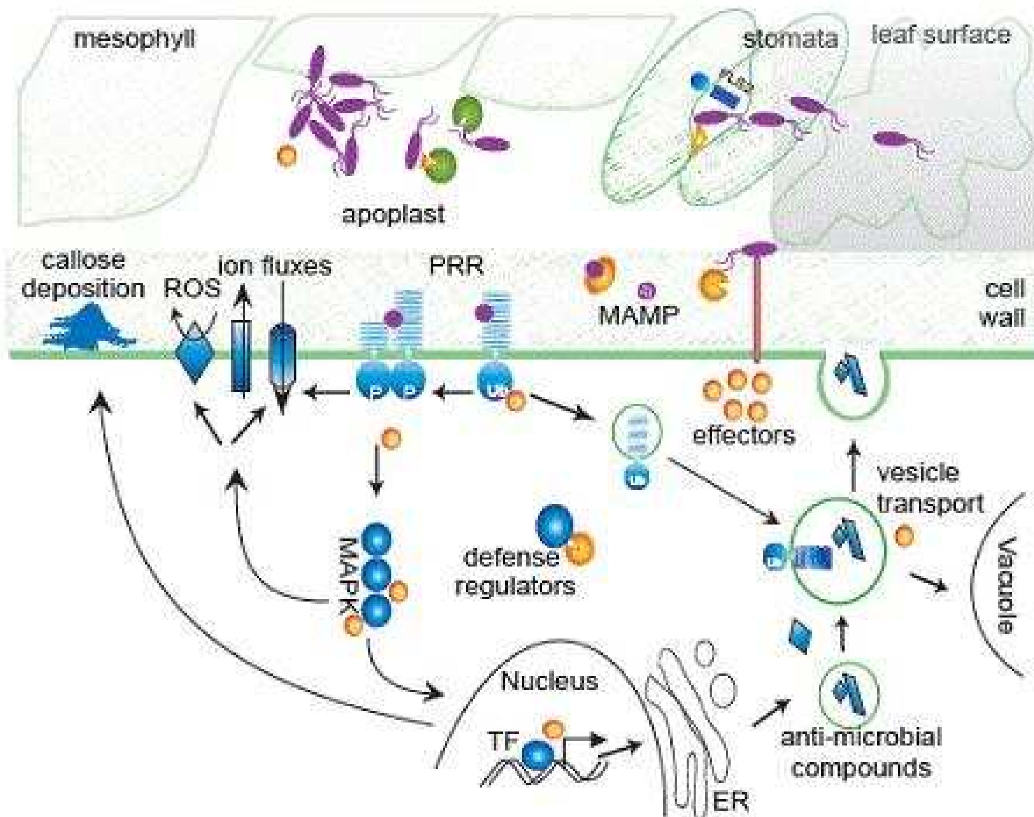


Figure 1.5: Defense mechanisms modulated by bacterial effectors and PTI signaling. Bacteria (*purple*) move toward openings such as stomata that are closed upon PAMP recognition. However, effectors (*orange*) *e.g.* coronatine lead to reopening. PAMPs (*purple circles*) are released during pathogen growth. Recognition of PAMPs leads to activation of defense responses (*blue*) such as ion fluxes (Ca^{2+} fluxes), ROS production, activation of MAPKs, callose deposition, and synthesis of antimicrobial compounds. Effectors like AvrPto and AvrPtoB interfere with receptor activation or early PAMP signaling. Effectors like HopAI1 suppressed signaling that takes place through MAPKs pathway. HopAO1 acts downstream of the MAPK cascade. Many defense reactions involve transcriptional changes, thus, effectors control defense transcriptome (*e.g.* HopU1 hinders with RNA binding proteins, XopD inactivates plant transcription factors and AvrBs3 family members are transcriptional activators) (Göhre and Robatzek, 2008).

membrane depolarization whose amplitude and duration mainly depends upon the nature of the elicitor (Garcia-Brugger *et al.*, 2006). It has been demonstrated that an early influx of Ca^{2+} across the plasma membrane is a prerequisite to initiate different downstream events of elicitor-dependent defense signaling (Lecourieux *et al.*, 2006; Dodds and Rathjen, 2010). Downstream of these fluxes, a cascade of phosphorylation of different protein kinases including MAPKs and CDPKs (Ca^{2+}-dependent protein kinases) is activated (Rodriguez *et al.*, 2010). Moreover, elicitor recognition also leads to the NO and ROS production (a detailed description of these signaling events will be presented in the following section). The mobilization of these different components of cell signaling results in the activation of various kinds of transcription factors (TFs) that contribute to genome reprogramming and subsequently leads to plant defense responses. Different plants and elicitors models have been studied in detail to explore these signal transduction mechanism during plants defense responses. Examples of these models include Cry/tobacco (Garcia-Brugger *et al.*, 2006), OGs/*A. thaliana* (Ferrari *et al.*, 2007; Galletti *et al.*, 2008; Rasul *et al.*, 2012), Flg/*A. thaliana* (Gómez-Gómez and Boller, 2002), *Pseudomonas* LPS and siderophores/tobacco (Van Loon *et al.*, 2006), OGs, laminarin, polymer of β-1,3-glucane or *Botrytis cinerea* endopolygalacturonase (BcPG1)/grapevine (Aziz *et al.*, 2003, 2004; Vandelle *et al.*, 2006). The respective perception of the two effector proteins from *Cladosporium fulvum* (Avr4 and Avr9) by two tomato R proteins (Cf-4 and Cf-9) have also been reported to initiate such type of signal transduction pathways (Stergiopoulos and De Wit, 2009). These findings have been further strengthened by the studies providing interesting informations that the nature of the defense responses that are activated during PTI and ETI shows substantial overlap despite these two types of defense responses clearly demonstrate differentially regulated mechanisms (Tsuda *et al.*, 2008; Tsuda and Katagiri, 2010). An exemplary systematic illustration of different signaling events in an elicitor mediated signaling pathway (tobacco-Cry model) is presented in figure 1.6 (adapted from Garcia-Brugger *et al.*, 2006).

2.1. Ion fluxes

Following the recognition of PAMPs/ elicitors or effectors molecules by host plant, the ion fluxes through the plasma membrane are among the earliest events in the plant-defense related signal transduction pathways. These fluxes have been observed after a very short time of elicitation *i.e.* during the first five min of elicitor recognition process and produced rapid changes in the concentration of different ions inside the cell. Transport of different cations such as Ca^{2+}, H^+ and K^+ and anions especially Cl^- and NO_3^- have been

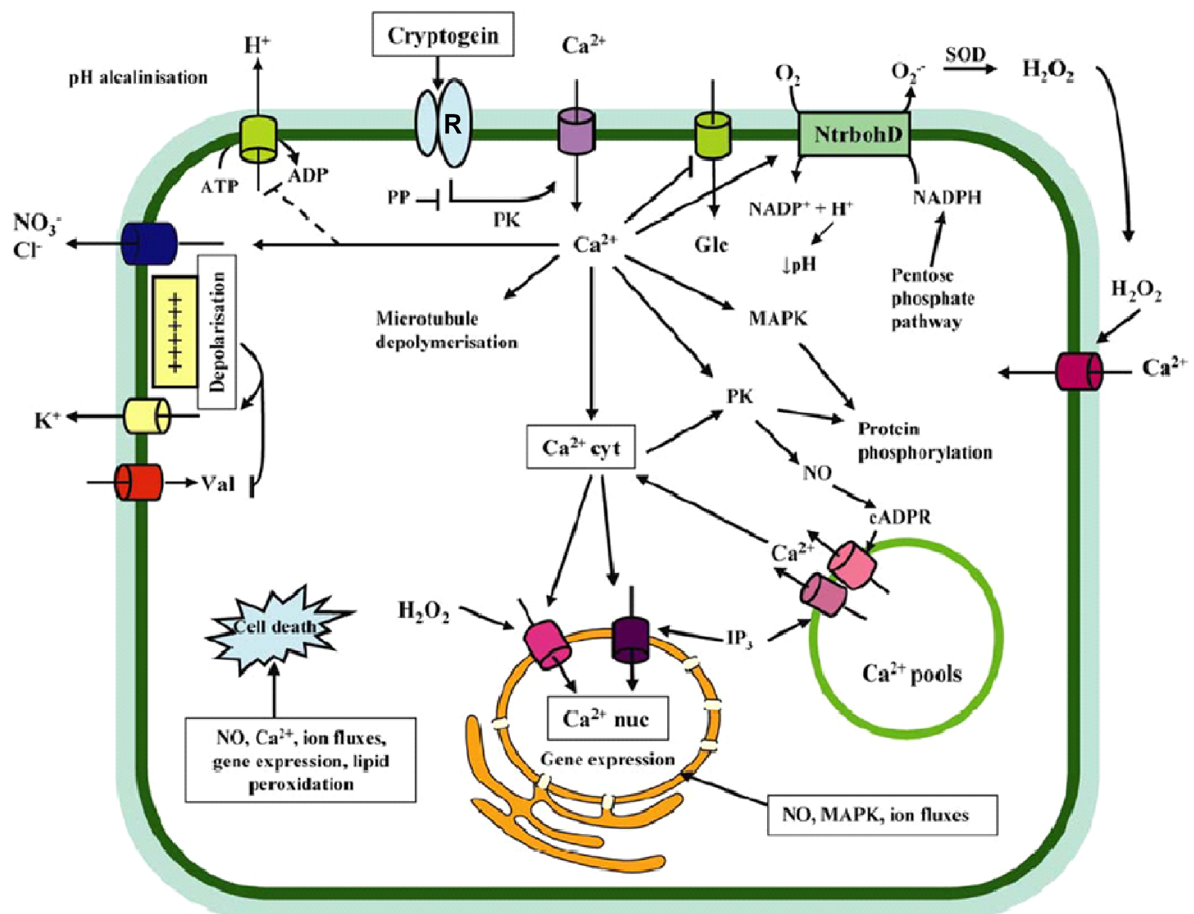


Figure 1.6: Cryptogein-induced signal transduction in plants. Recognition of plant defense elicitor, cryptogein (Cry) through specific receptor on plasma membrane initiates signaling cascade that ultimately leads to plant defense response. R: Receptor; NtrbohD: NADPH /Respiratory burst oxidase protein; SOD: Superoxide dismutase (Garcia-Brugger *et al.*, 2006).

reported after elicitor treatments in different plant species such as parsley, tobacco or *A. thaliana* (Nürnberger *et al.*, 1994; Jabs *et al.*, 1997; Pugin *et al.*, 1997; Yang *et al.*, 1997; Wendehenne *et al.*, 2002) and could trigger defense responses (Jabs *et al.*, 1997; Nürnberger *et al.*, 2004). These ion fluxes trigger plasma membrane depolarization of the excited cells (Garcia-Brugger *et al.*, 2006; Gauthier *et al.*, 2007). A decrease of apoplastic H^+ and a decrease in cytoplasmic pH were observed (Hückelhoven, 2007). Previous studies have reported that Ca^{2+} is the most important molecule among these ions, as its role as a second messenger in the activation of many downstream signaling events especially related to defense responses has been firmly established (for more details see the reviews; Garcia-Brugger *et al.*, 2006; Lecourieux *et al.*, 2006; Boudsocq *et al.*, 2010; Dodds and Rathjen, 2010; Kudla *et al.*, 2010). Activation of anion channels and plasma membrane NADPH-oxidase along with inhibition of plasma membrane H^+/ATPase was shown to be dependent on Ca^{2+} influx (Tavernier *et al.*, 1995; Scheel, 1998). In another study, it was shown that blocking of ion fluxes with Ca^{2+} chelators or Ca^{2+} channel blockers inhibited the induction of defense responses, supporting the essential role of Ca^{2+} and ion fluxes in defense (Dixon *et al.*, 1994).

Indeed, elicitor-induced Ca^{2+} influx leads to a subsequent increase in free cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) and participates in different downstream defense signaling pathways. After elicitation, a rapid increase in $[Ca^{2+}]_{cyt}$ has been reported in different plant species (Navazio *et al.*, 2002; Poinssot *et al.*, 2003; Hu *et al.*, 2004; Lamotte *et al.*, 2004; Lecourieux *et al.*, 2006; Qi *et al.*, 2010; Kwaaitaal *et al.*, 2011; Ranf *et al.*, 2011; Vatsa *et al.*, 2011). Moreover, increased $[Ca^{2+}]_{cyt}$ promotes the opening of other membrane channels (Blume *et al.*, 2000; Brunner *et al.*, 2002; Lecourieux *et al.*, 2002; Ranf *et al.*, 2008). Movement of Ca^{2+} across the plasma membrane takes place with help of plasma membrane channels. These include members of the cyclic nucleotide gated channels (CNGC) and the ionotropic glutamate receptors (iGluRs) family (Lacombe *et al.*, 2001; Mäser *et al.*, 2001). Two pore channel 1 (TPC1) were either located in the plasma membrane or vacuolar membrane may also contribute to Ca^{+2} influx (Kadota *et al.*, 2004; Kurusu *et al.*, 2005; Peiter *et al.*, 2005; Hamada *et al.*, 2012). Interestingly, all three types of channels provide putative pathways for Ca^{2+} signaling leading to plant defense, although TPC1 involvement is now a subject of debate (Ranf *et al.*, 2008).

Figure 1.7: Inter-conversion of reactive oxygen species (ROS). Molecular oxygen (O_2) can be activated by excess energy to form singlet oxygen ($^1O_2$). By another route, one electron reduction leads to the formation of superoxide radical which exists in equilibrium with its conjugate acid, hydroperoxyl radical. Subsequent reduction steps then form hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and water (H_2O). Metal ions that are mainly present in cells in the oxidized form (Fe^{3+}) are reduced in the presence of e^- and, consequently, may catalyse the conversion of H_2O_2 to $OH^{\cdot}$ by the Fenton or Haber–Weiss reactions (Vranová *et al.*, 2002).

2.2. Reactive oxygen species production

Reactive oxygen species (ROS) production is a common early response of plant cells to pathogen attack and elicitor treatment. A rapid and transient increase in ROS accumulation is known as "oxidative burst". They play important role in plant resistance by different following means: by limiting the pathogen development, by helping to strengthen the cell wall and/or by participating in the cell signaling cascade leading to resistance (Lamb and Dixon, 1997). ROS exist in various forms such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), perhydroxyl radical ($O_2H^{\cdot}$) and hydrogen peroxide (H_2O_2) (Apel and Hirt, 2004). Inter-conversion of ROS has been elaborated in figure 1.7 (Vranová *et al.*, 2002). Many studies focused on identifying the enzymatic source of H_2O_2 production in plant defense mechanisms have shown the involvement of NADPH oxidase activity (Pugin *et al.*, 1997; Simon-Plas *et al.*, 2002; Torres *et al.*, 2002; Torres and Dangl, 2005). In mammals, the NADPH oxidase was originally described as a plasma membrane bounded enzyme and is composed of two subunits gp91^{phox} and p22^{phox} (Lambeth, 2004). In plants, identification of the gene encoding the NADPH oxidase revealed that the protein corresponded to the catalytic subunit gp91-phox of mammalian (Torres, 1998). Plant NADPH oxidase has six transmembrane domains, two C-terminal binding domains for FAD and NADPH and two N-terminal EF-hands motifs (Keller *et al.*, 1998; Torres and Dangl, 2005; Sagi and Fluhr, 2006). The presence of EF-hands motifs in the N-terminal part suggest the Ca^{2+}-dependent regulation of this enzyme (Sagi and Fluhr, 2001; Kobayashi *et al.*, 2007). In addition, CDPK-dependent phosphorylation at the N-terminal domain was also found important for the activation of this protein (Kobayashi *et al.*, 2007). Respiratory burst oxidase homolog (RboH), that represents NADPH oxidase, has been identified in different plant species (Pugin *et al.*, 1997; Torres *et al.*, 2002; Torres and Dangl, 2005). Ten different isoforms of RboH (A to J) are present in *A. thaliana* (Torres, 1998; Sagi and Fluhr, 2006). However, previous investigations have shown that *Arabidopsis* RbohD and RbohF are the two major contributors in ROS accumulation during plant resistance against *Hyaloperonospora arabidopsidis* and *P. syringae* (Torres *et al.*, 2002).

In tobacco, plasma membrane bound NADPH oxidase has also been reported to participate in Cry-induced ROS production where the apoplastic O_2^- is immediately converted to H_2O_2 and both these exhibit antimicrobial activities (Pugin *et al.*, 1997; Simon-Plas *et al.*, 1997). In *Arabidopsis*, production of O_2^- and H_2O_2 has been observed after pathogen infections and elicitor treatments (Lamb and Dixon, 1997; Torres *et al.*, 2006; Van Loon *et al.*, 2008). Previous studies have demonstrated that OGs is able to induce H_2O_2 accumulation in different plant species (Aziz *et al.*, 2004; Hu *et al.*, 2004; Romani *et al.*, 2004; Galletti *et*

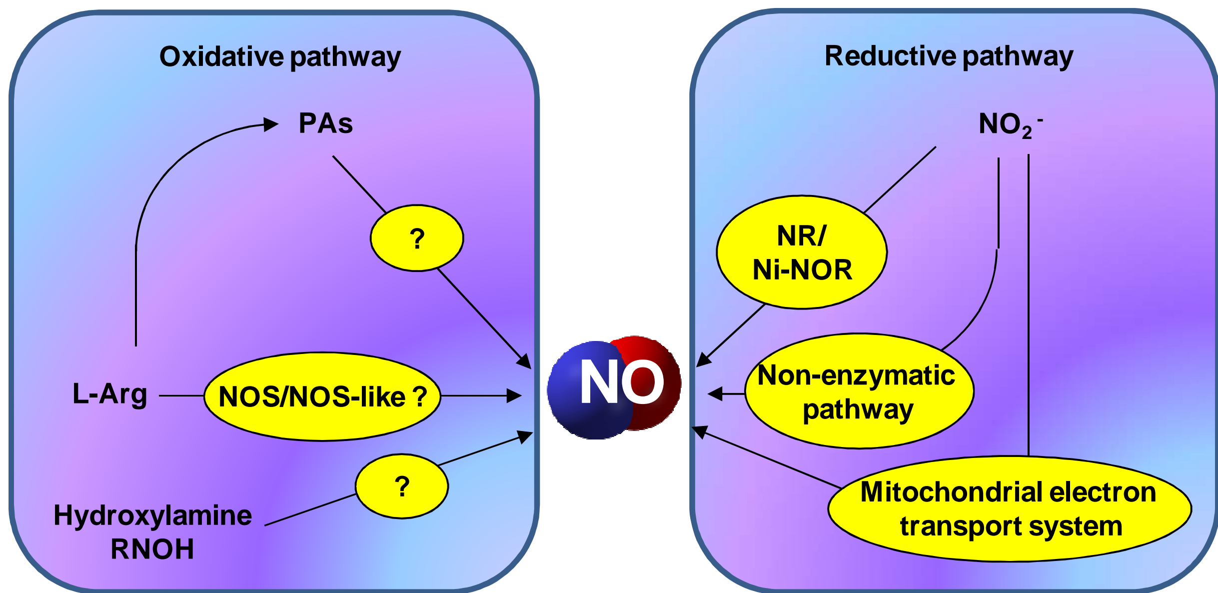


Figure 1.8: **Nitric oxide synthesis in plants**. Nitric oxide (NO) production in plants is mainly dependent on two pathways: the oxidative and the reductive pathway. In oxidative pathway, L-arginine is a source of NO by NOS-like activities in several land plant tissues as well as in cell suspensions. Moreover, L-Arg is also the source of PAs, and PAs like spermine and spermidine, trigger a fast NO production by action of one or several polyamine oxidase (PAOX). Hydroxylamine also produces NO by unknown or unidentified enzymes. The molecular basis of these pathways is poorly known. The nitrite-dependent NO synthesis involved mainly nitrate reductase (NR), NR catalyzes the reduction of nitrate (NO_3^-) into nitrite (NO_2^-) which reduce to NO both *in vitro* and *in vivo*. NO_2^- is also converted to NO by non enzymatic pathway in acidic pH. NOS: Nitric oxide synthase; L-Arg: L-Arginine; PAs: Polyamines; NO_2^-: Nitrite; NR: Nitrate reductase; Ni-NOR: nitrite-NO reductase (Adapted from Besson-bard *et al.*, 2008; Moreau *et al.*, 2010 and Gupta *et al.*, 2011).

al., 2008; Aslam *et al.*, 2009). Moreover, OGs-induced H_2O_2 could be inhibited by using lanthanum (La^{3+}) suggesting that Ca^{2+} influx is important for H_2O_2 production (Hu *et al.*, 2004). As signaling molecules, ROS actively participate in various defense signaling pathways to confer plant resistance against a variety of abiotic and biotic stresses (Apel and Hirt, 2004; Laloi *et al.*, 2004). They can participate in the regulation of MAPKs, $[Ca^{2+}]_{cyt}$ elevations, and cellular redox state (Lecourieux *et al.*, 2002; Rentel and Knight, 2004; Vandelle *et al.*, 2006).

2.3. Nitric oxide production

On the earth, nitric oxide (NO) exists as a gaseous free radical and participates in a variety of physiological processes in both plants and animals (Besson-Bard *et al.*, 2008b). In animals, NO is synthesized from L-arginine and oxygen by nitric oxide synthase (NOS) that oxidises L-arginine to form L-citrulline and NO (Figure 1.8; Besson-Bard *et al.*, 2008b; Moreau *et al.*, 2010; Gupta *et al.*, 2011) Although plants have also demonstrated NOS-like activities that are sensitive to mammalian NOS inhibitors yet molecular identifications of these homologs are still lacking in plant (Corpas *et al.*, 2009). In plants, different enzymatic pathways have been proposed for the production of NO. They include: Nitrate dependent and L-Arginine dependent. Nitrate reductase (NR) is the key enzyme in nitrate dependent NO synthesis and several reports have suggested the absence of animal NOS homolog in plants however, NOS-like activities, sensitive to mammalian NOS enzyme inhibitor in plant cell culture as well as in plant tissues, have been reported (Yamasaki and Sakihama, 2000; Besson-Bard *et al.*, 2008b). In the recent past, NO has gain much attention regarding its role as a second messenger and continuous efforts are being made to understand its involvement in physio-pathological context (Delledonne *et al.*, 1998; Wilson *et al.*, 2008; Leitner *et al.*, 2009). It has been firmly established as an intermediate signaling molecule in PAMPs/elicitor-mediated signaling processes and plays a crucial role in the plant adaptive response to pathogen attack (Besson-Bard *et al.*, 2008a; Asai and Yoshioka, 2009). Pharmacological studies in tobacco and grapevine have indicated that in response to plant defense elicitors, NO is produced (Lamotte *et al.*, 2004; Vandelle *et al.*, 2006; Courtois *et al.*, 2008). PAMPs- and/or elicitors-induced NO production has been reported as a Ca^{2+}- and phosphorylation-dependent event in plants (Lamotte *et al.*, 2004). LPS is able to induce a strong and quick burst of NO in cells suspension as well as in plant leaves (Zeidler *et al.*, 2004). Ali *et al.* (2007) have provided genetic evidences in this favor. They have shown that LPS-induced NO synthesis, is controlled by an upstream Ca^{2+} influx mediated by the plasma membrane Ca^{2+}-

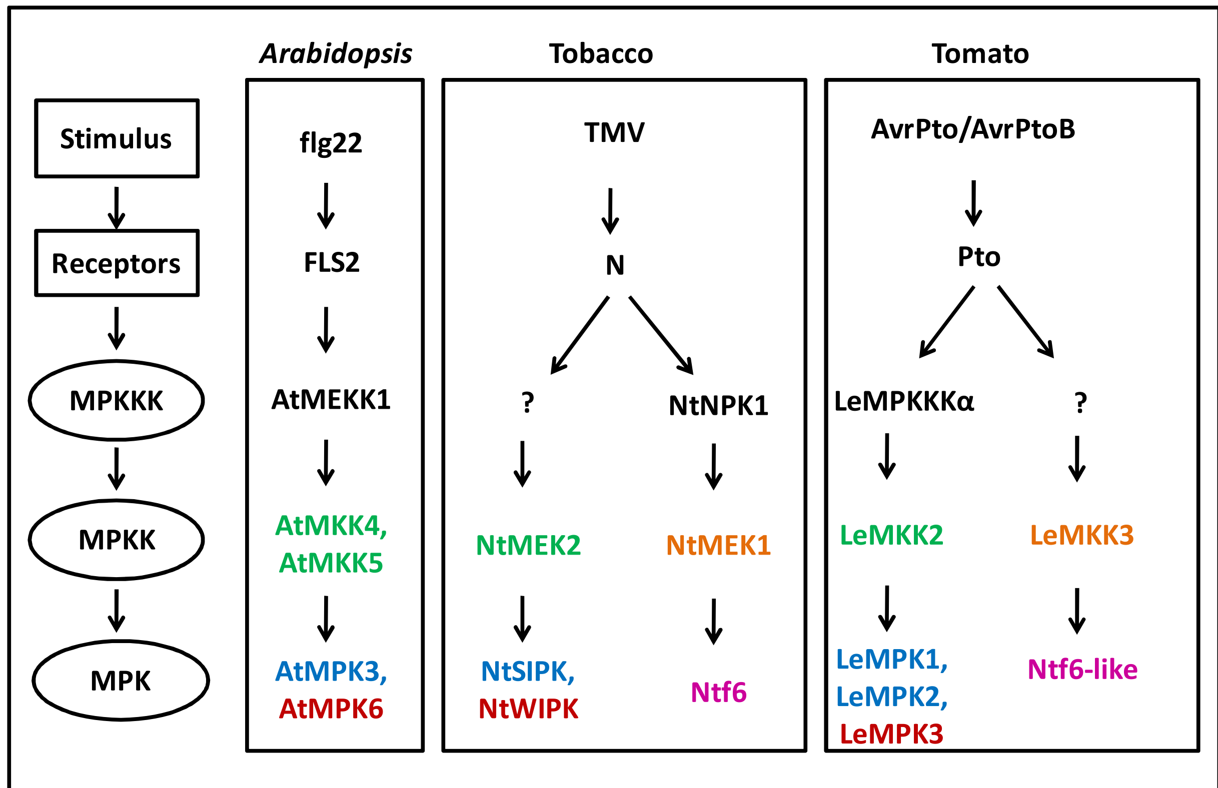


Figure 1.9: MAPK cascade in *Arabidopsis,* tobacco and tomato involved in defense-mediated signal transduction. In *Arabidopsis,* sensing of flagellin by the FLS2 receptor stimulates AtMEKK1, which in turn activates AtMKK4 and AtMKK5, and finally leads to the activation of two MAPKs, AtMPK3 and AtMPK6. In tobacco and tomato, two parallel pathways have been demonstrated that are activated downstream of the R proteins N and Pto, respectively. In tobacco, the first pathway involves sequentially NtNPK1, NtMEK1 and Ntf6. The second cascade leads to activation of unknown MAPKKK then NtMEK2 and after NtSIPK and NtWIPK. In tomato, a first cascade is represented by the MAPKKKα, MKK2, and MPK1/MPK2/MPK 3. The other involves an unknown MAPKKK, MKK3 and Ntf6-like (Adapted from Pedley and Martin, 2005).

permeable CNGC2. More recently, Vatsa *et al.* (2011) have shown that elicitor-induced NO production is regulated by a putative GLR homolog in tobacco. On the other hand, some studies have shown that NO contributes to $[Ca^{2+}]_{cyt}$ elevations by mobilizing Ca^{2+} from internal pools, probably via phosphorylation events (Lamotte *et al.*, 2006). Recently, it was reported that SNF1-related protein kinase 2 (SnRK2) was potentially involved in this process in tobacco (Wawer *et al.*, 2010). NO treatment leads to the induction of some stress- and disease-related signal transduction component genes. Some studies have shown that NO is involved in production of secondary metabolites. Elicitor-induced NO biosynthesis promotes the synthesis of H_2O_2 and transcription of some genes involved in defense, in oxidative stress and secondary metabolism (Grün *et al.*, 2006; Vandelle *et al.*, 2006). Moreover, OGs-mediated NO production partially stimulated saponin production in ginseng cell cultures (Hu *et al.*, 2003). Different Avr factors stimulate NO production, and NO by interacting with ROS during the oxidative burst, contributes in plant disease resistance (Delledonne *et al.*, 1998).

2.4. Phosphorylation of mitogen-activated protein kinases

In eukaryotes, MAPK pathways are ubiquitous signal transduction components and are highly conserved regulators of growth, differentiation, proliferation and stress responses (Nakagami *et al.*, 2005; Fiil *et al.*, 2009; Dodds and Rathjen, 2010). All MAPKs have a characteristic motif (TXY) that in involved in the phosphorylation of these protein kinases. This motif is conserved between insects, mammals and plants (Nürnberger *et al*., 2004). Typically, this cascade consists of a modular complex of three interlinking proteins: MAPK kinase kinase (MAPKKK), which phosphorylates a serine or threonine residue on a MAPKK, which in turn, phosphorylates a MAPK by dual phosphorylation of a threonine and tyrosine residue (Garcia-Brugger *et al.*, 2006; Vlot *et al.*, 2009; Rodriguez *et al.*, 2010). These activated MAPKs finally lead to the phosphorylation of TFs and other signaling components that are involved in the regulation of the expression of downstream genes (Sinha *et al*., 2011). The MAPKs cascade is one of the most studied features of plant defense signaling (Zhang and Klessig, 2001; Pitzschke *et al.*, 2009). Studies have shown that MAPK cascades are implicated in both PTI and ETI and activate similar gene expression signatures (Tao *et al.*, 2003; Pitzschke *et al.*, 2009). In response to plant defense elicitors, MAPKs have been identified in different plant species (Ortiz-Masia *et al.*, 2007). In tobacco, two MAPKs namely WIPK (wound-induced protein kinase) and SIPK (salicylic acid-induced protein kinase) were found to be activated after tobacco mosaic virus (TMV) infection and are suggested to be involved in the regulation of defense genes expression and the HR triggered

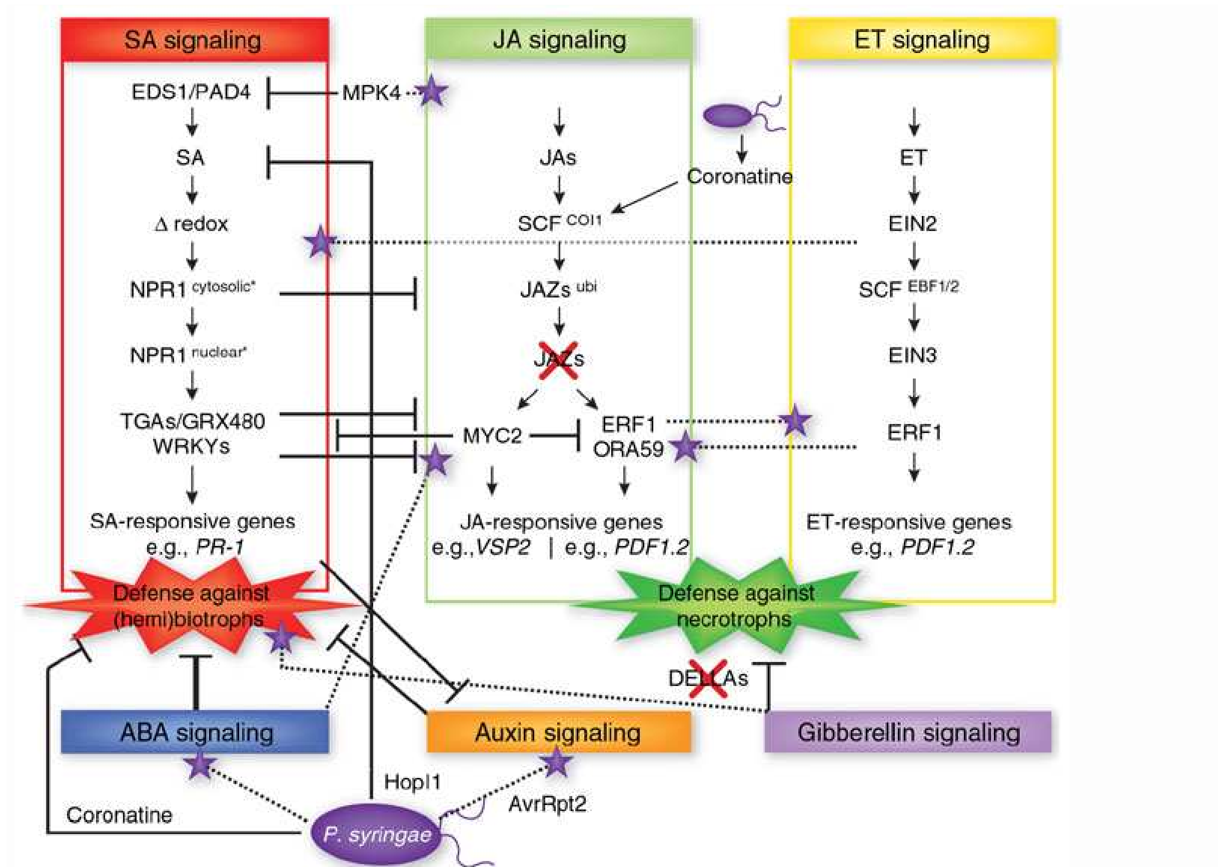


Figure 1.10: Networking by phyto-hormones in plant immune response. Pathogens such as *P. syringae* produce effector proteins (*e.g.* coronatine, HopI1 and AvrRpt2) that manipulate the signaling network to suppress host immune responses and promote virulence. The SA, JA and ET signaling pathways are the backbone of the defense signaling network, with other hormonal signaling pathways feeding into it. Briefly, the cellular redox potential activates NPR1 monomeric form during SA signaling pathway. Monomeric NPR1 is then translocated into the nucleus where it serves as a transcriptional co-activator of SA-responsive genes, such as *PR-1*. In the JA signaling cascade, the E3 ubiquitin ligase SCF^{COI1} complex and jasmonate ZIM-domain (JAZ) proteins form a complex that represses transcription of JA-responsive genes. Upon accumulation of JA, JA-isoleucine (JA-Ile) binds to the F-box protein COI1 in the SCF^{COI1} complex, which ultimately leads to the activation of JA-responsive genes through the action of transcription factors. In the ET signaling cascade, the gaseous hormone ET is perceived by plasma membrane receptors such as ETR1. Upon perception of ET, the repression of ET signaling by CTR1 is relieved, allowing downstream signaling through EIN2. Subsequently, critical positive regulators of ET-responsive gene expression, such as EIN3 are activated. EIN3-like transcription factors activate transcription factors such as ERF1, resulting in the expression of downstream ET-responsive genes. ⊥: negative effect; purple stars: positive effect (Pieterse *et al.,* 2009).

by Cry treatment in tobacco (Zhang *et al.*, 1998; Zhang and Klessig, 2001). Similarly, treatment of tobacco cell suspensions with Cry reveals the phosphorylation of these two MAPKs (Lebrun-Garcia *et al.*, 1998; Dahan *et al.*, 2009). A total of twenty MAPKs have been identified in *A. thaliana* genome but only few of them have been identified to participate in plant defense responses (Jonak *et al.*, 2002; Nakagami *et al.*, 2005). Two *Arabidopsis* MAPKs (MPK3 and MPK6) have been demonstrated to participate in flg22-mediated signaling and in the regulation of phytoalexins synthesis during *B. cinerea* attack (Asai *et al.*, 2002; Ren *et al.*, 2008). Phosphorylation and activation of MPK4 in response to flgellin or harpin as well as different abiotic stresses has been reported in the past (Ichimura *et al.*, 2000; Desikan *et al.*, 2001; Droillard *et al.*, 2004; Teige *et al.*, 2004). Recently, it has been reported that OGs- and flg22-induced defense responses effective against *B. cinerea* are mainly dependent on MAPKs, and the role of MAPK6 is very important in these responses (Galletti *et al.*, 2011). A generalized flow sheet of MAPK signaling cascade in different plant species is presented (Figure 1.9; adapted from Pedley and Martin, 2005).

2.5. The role of phytohormones

Hormone signaling is another important component during the activation of plant defense responses. Numerous studies have highlighted that phytohormones like salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play a key role in resistance to pathogens (Glazebrook, 2005; Lorenzo and Solano, 2005; Broekaert *et al.*, 2006; Grant and Lamb, 2006; Balbi and Devoto, 2008). In addition, other phytohormones such as auxins, Gibberellins, (GAs), Cytokinins (CKs), Brassinosteroids (BR) and Abscisic acid (ABA) which are mainly known as mediators of growth and development have also been reported to participate in defense mechanisms through unidentified mechanisms (Robert-Seilaniantz *et al.*, 2007; Pieterse *et al.*, 2009; Truman *et al.*, 2010).

SA, ET and JA are important regulators of defense responses in plants playing a crucial role in the activation of defense-related genes (Bari and Jones, 2009). SA is involved in resistance to biotrophic and hemi-biotrophic pathogens and JA and ET participate in resistance to necrotrophic pathogens and chewing insects (Figure 1.10; Pieterse *et al.*, 2009). Although, substantial differences in the gene expression outputs of these pathways have been observed yet there also exists considerable overlap between them (Vlot *et al.*, 2009; Dodds and Rathjen, 2010). Indeed, these pathways act agonistically, as it has been reported that in PTI, SA and JA–ET showed a synergistic effect to amplify defense response (Tsuda *et al.*, 2009). However, JA and ET are able to maintain a substantial level of pathogen resistance

even in the absence of SA signaling. These observations highlight that these compensatory interactions could be the result of higher signal flux in ETI, and their cumulative effect might robust the defense responses against pathogens (Dodds and Rathjen, 2010). *NahG* transgenic plants unable to accumulate SA because they express a bacterial hydroxylase that converts SA to catechol, *NahG* showed a loss of resistance to biotrophic oomycete such as *H. arabidopsidis*. However blocking the pathway of SA had no effect on the response to necrotrophic pathogens like *Alternaria brassicicola* and *B. cinerea* (Thomma *et al.*, 1998). Consistent with these data, the mutant non-expressor of PR gene 1 (*npr-1*), in which the SA signaling pathway is blocked, showed susceptible symptoms to *H. arabidopsidis* (Thomma *et al.*, 1998). Some studies have reported that the absence of the ethylene signaling pathway is ethylene insensitive 2 (*ein2*) mutant plants displayed a decreased resistance only to necrotrophic pathogens *B. cinerea* with no altered resistance to biotrophic *P. syringae* (Bent *et al.*, 1992; Thomma *et al.*, 1999). Several genes act as specific markers for the activation of SA, JA and ET pathways. *Isochrismate synthase 1* (*ICS1*) and *pathogenesis-related protein 1* (*PR-1*) genes are the specific marker genes of SA and are involved in SAR in plants (Maleck *et al.*, 2000; Lu, 2009). Similarly, vegetative storage protein 2 (VSP2) is specifically activated during JA pathway (Pieterse *et al.*, 2009). On the other hand, *plant defensin 1.2* (*PDF 1.2*) gene expression has been observed in both JA- and ET-dependent pathways (Guo and Ecker, 2004; Kazan and Manners, 2008).

3. Plant defense responses: a final outcome

Following the recognition of pathogens or elicitors, the ultimate outcome of the activated signaling cascades is the establishment of plant defense responses. This is mainly achieved by transcriptional reprogramming of certain genes especially related to defense responses (Caplan *et al.*, 2008). This modulation of genes expression is partly common in PTI and ETI (Tsuda and Katagiri, 2010) could lead to the up- or down-regulation of hundreds of genes. These genes encode the defense proteins which can be categorized into different functional groups: PR proteins, proteins associated with cell wall modification(s) and proteins involved in secondary metabolism (Stintzi *et al.*, 1993). In addition, biosynthesis of antimicrobial compounds and development of local and systemic resistance also contribute to limit the growth of pathogen. In the following section, we will briefly discuss these elements of plant defense.

3.1. Modification of the cell wall

Plant cell wall present around each cell is composed of cellulose and pectin and plays a significant role during plant pathogen interactions. During evolution, most pathogens have evolved new enzymes capable of degrading the cell wall. These include pectinases, cellulases and polygalacturonases that make the access of plants nutrients possible for pathogens (Hückelhoven, 2007). However, several modifications at the infection site have been observed in plant cell walls *e.g.* an accumulation of proteins responsible for inhibiting the pathogen cell wall degrading enzymes and the accumulation of toxic phenolic compounds to kill the pathogens (De Lorenzo and Ferrari, 2002; Lherminier *et al.*, 2003). In addition, callose deposition is often observed during pathogen attack that also leads to plant defense as has been suggested by previous studies (Ahn *et al.*, 2007; Trouvelot *et al.*, 2008). Lignin accumulation is another important cell wall modification during plant defense. In fact, cell wall lignification helps the plant by: providing better resistance to mechanical pressure, inhibiting cell wall-degrading enzymes and finally limiting the pathogen growth during infection process (Bechinger *et al.*, 1999; Hückelhoven, 2007; Bhuiyan *et al.*, 2009; Hamann *et al.*, 2009).

3.2. Synthesis of antimicrobial compounds

Another characteristic feature of plants to fight against pathogens is the production of antimicrobial compounds. Studies have demonstrated their production after pathogen attacks and elicitor treatments. In plants, they are known as phytoalexins and over 200 phytoalexins have been isolated and identified in plants (Coxon, 1982; Ingham, 1982; Kuc, 1982; Hammerschmidt, 1999). Phytoalexins belong to different structural including pterocarpans (*e.g.* glyceollin), isoflavans, prenylated isoflavonoids (*e.g.* kievitone), stilbenes, psoralens, coumarins, 3-deoxyanthocyanidins, flavonols (*e.g.* quercetin, kaempferol), and aurones (Dixon *et al.*, 1995). Camalexin is an example of well characterized phytoalexin in *Araidopsis* that belongs to tryptophan, a class of sulfur-rich compounds (Rauhut and Glawischnig, 2009). *Phytoalexin deficient 3* (*PAD3*) gene encodes the last enzyme, cytochrome P450, involved in the biosynthesis of camalexin and *pad3* mutants exhibited undetectable level of camalexin (Nafisi *et al.*, 2007; Zhou *et al.*, 1999). Genetic evidences have been provided for the role of phytoalexins in plant defense through the use of PAD3 knockout plants. *Pad3* mutant displayed increased susceptibility to *A. brassicicola* and *B. cinerea* (Thomma *et al.*, 1999; Ferrari *et al.*, 2003). Moreover, Ferrari *et al.* (2007) showed that PAD3 is required for OGs-induced resistance to *B. cinerea* in *A. thaliana.* Moreover, phytoalexin deficient mutants (*pad1*, *pad2* and *pad4*) were found susceptible to virulent and avirulent strains of *P. syringae* while *pad3* surprisingly remained resistant, showing different regulation of phytoalexin synthesis during defense responses against different pathogens (Glazebrook and Ausubel, 1994). Some other studies have reported the role of *A. thaliana phytoalexin deficient 2* (*pad2*) mutant in resistance to 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). Glucosinolates in Brassicaceae are another class of secondary metabolites that are rich in sulfur and play a crucial role in defense against herbivores (Rask *et al.*, 2000; Halkier and Gershenzon, 2006). Further studies have demonstrated that they are also produced after a pathogen attack and probably have an antimicrobial function (Clay *et al.*, 2009). Finally, *P. brassicae* has been reported to stimulate glucosinolates biosynthesis and the hydrolysis products of glucosinolates accompanied by camalexin played a vital role in resistance against this pathogen (Schlaeppi *et al.*, 2010).

3.3. Hypersensitive response

Cell death has been considered to play a central role in innate immune responses in both animals and plants. The term programmed cell death (PCD) represents a sequence of (potentially interruptible) events that lead to the controlled and organized destruction of the cell (Lockshin and Zakeri, 2004). In plants, PCD is essential not only for the development mechanisms but also enables the plant to restrict the spread of pathogens after infection (Lam, 2004; Coll *et al.*, 2011). In animals, cell death can be divided into apoptosis, autophagic cell death and necrosis on the basis of morphological observations (Reape and McCabe, 2008). HR is a form of programmed cell death that is similar to apoptosis in animals (Greenberg and Yao, 2004). It is observed in many interactions between plants/microorganisms and is characterized by localized cell death at the infection site of the pathogen, resulting in the emergence of macro- or microscopic lesions several hours after the onset of infection (Heath, 2000). The HR is characterized by several cellular events including condensation of cytoplasm and chromatin, the release of cytochrome c from the mitochondria or the activation of cysteine proteases (Wall *et al.*, 2008). It is generally accepted that the HR would be aimed at the containment of the pathogen in its site of infection by reducing access to available nutrients (Greenberg and Yao, 2004). Moreover, some people consider HR as the final step in the development of resistance (Mur *et al.*, 2008). This is an appropriate strategy during infection by a biotrophic or hemibiotrophic pathogen which requires living cells of the host to develop (Glazebrook, 2005). In contrast, HR supports the development of necrotrophic pathogens like *B. cinerea* that feed exclusively on dead tissue (Govrin and Levine, 2000). HR is mainly triggered following recognition of an effector molecule. Not all but some PAMPs, like harpin and Cry can induce HR responses (Garcia-Brugger *et al.*, 2006; Jones and Dangl, 2006). It is considered an important downstream event in elicitor-mediated signal transduction cascade and a link between HR and Ca^{2+} variations in the cell has been suggested by using *A. thaliana* mutant *defense no death 1* (*dnd1*) to avirulent pathogens, since the mutated gene code CNGC2, a nonselective channel permeable to Ca^{2+} (Clough *et al.*, 2000; Ma and Berkowitz, 2007). Moreover, in response to Cry treatment in tobacco cells, changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ have been reported to trigger HR responses (Wendehenne *et al.*, 2002; Lecourieux *et al.*, 2006). Previous reports have shown that ROS, together with NO, plays an important role in orchestrating the HR (Levine *et al.*, 1994; Zaninotto *et al.*, 2006). Indeed, a fine regulation of the balance ROS/NO is necessary to initiate cell death, knowing that NO alone is incapable of inducing the HR (Delledonne *et al.*, 2001; De Pinto *et al.*, 2002). Finally,

ROS play an important role in establishing the HR via lipid peroxidation that is dependent on lipoxygenase (LOX), this will disrupt the membranes (Wall *et al.*, 2008).

3.4. Systemic acquired resistance

In plants, the concept of SAR exists since the beginning of the 20th century. Most often but not necessarily, SAR is induced by necrotizing pathogens and is effective against viruses, bacteria, fungi, and oomycetes. Due to resistance against a wide range of pathogens, it is sometimes described as "broad spectrum" resistance. Generally speaking, SAR is induced following the primary pathogen infection and subsequently results in the development of increased resistance to secondary infection even in the unaffected distal plant tissues. This type of enhanced resistance is referred to as SAR. Some plant defense elicitors like polysaccharides or elicitins are also known to induce SAR. In fact, in the SAR state, plants are primed (sensitized) more quickly and more effectively activate defense responses the second time they encounter pathogen attack (Conrath, 2006). Since SAR depends on the ability to access past experience, acquired disease resistance is an outstanding example for the existence of “plant memory” (Conrath, 2006). The resistance conferred is long-lasting, sometimes for the lifetime of the plant (Ryals *et al.*, 1996). On molecular basis, SAR is characterized by the induction of a large number of PR genes and the activation of SAR requires the accumulation of endogenous SA in both local and systemic tissues (Métraux *et al.*, 2002; Durrant and Dong, 2004; Gautam and Stein, 2011). In *Arabidopsis*, PR-1, PR-2, and PR-5 are known SAR marker genes that have been successfully cloned and characterized. These genes have been used extensively to evaluate the onset of SAR (Ward *et al.*, 1991; Uknes *et al.*, 1992). PR1 and ICS1 are well known example to study SAR responses in plants (Maleck *et al.*, 2000). It has been reported that exogenous application of SA is able to induce defense gene expression, whereas the expression of the salicylate hydroxylase *nahG* (gene which inactivates SA by converting it to catechol) suppresses SAR (Ryals *et al.*, 1996; Rowland and Jones, 2001). Methyl salicylate (MESA), an inactive derivative SA, is an actor in the establishment of the SAR (Park *et al.*, 2007). It is carried by the vascular system and allows production of SA in distal sites of infection through the action of proteins as SA binding protein 2 (SABP2), which possesses an esterase MESA activity (Vlot *et al.*, 2008). SAR responses have been observed in different plant species including tobacco, tomato, pepper, grapevine and *Arabidopsis*. According to Grant and Lamb, (2006), acquired resistance can be classified into two broad categories, SAR and induced systemic resistance (ISR). There exist some characteristic differences between SAR and ISR. Unlike SAR, ISR is the result of beneficial interactions

Table 1.3: Pathogenesis-related protein (PRs) families and their putative functions in plants (Van Loon *et al*., 2006; Sels *et al*., 2008).

Family	Type member	Properties	Reporter Protein activity	Target pathogen site or Components	Gene symbols
PR-1	Tobacco PR-1a	Unknown	Pathogenesis - related protein 1 Precursor	Fungal membrane	Ypr1
PR-2	Tobacco PR-2	β-1,3-glucanase	β-1,3-glucanase	Cell wall glucanase	Ypr2, [Gns2 ('Glb')]
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	Endochitinase	Cell wall chitin	Ypr3, Chia
PR-4	Tobacco 'R'	Chitinase type I, II	Endochitinase	Cell wall chitin	Ypr4, Chid
PR-5	Tobacco S	Thaumatin-like	Osmotin	Fungal membrane	Ypr5
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	Protease inhibitor	Proteinase	Ypr6, Pis ('Pin')
PR-7	Tomato P69	Endoproteinase	Endoprotease	Endoproteinase	Ypr7
PR-8	Cucumber chitinase	Chitinase type III	Endo chitinase	Cell wall chitin	Ypr8, Chib
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase	Peroxidase	Plant cell wall lignifications	Ypr9, Prx
PR-10	Parsley "PR1"	Ribonuclease-like	Ribonucleases	Pathogen RNA	Ypr10
PR-11	Tobacco "class V" chitinase	Chitinase, type I	Endo chitinase	cell wall chitin	Ypr11, Chic
PR-12	Radish Rs-AFP3	Defensin	Defensin	Fungal membrane	Ypr12
PR-13	*Arabidopsis* THI2.1	Thionin	Thionin	Fungal membrane	Ypr13, Thi
PR-14	Barley LTP4	Lipid-transfer protein	Lipid transfer protein	Non specific lipid transfer protein	Ypr14, Ltp
PR-15	Barley OxOa (germin)	Oxalate oxidase	Oxalate oxidase	Superoxidase dismutase activity	Ypr15
PR-16	Barley OxOLP	Oxalate-oxidase-like	Oxalate oxidase- like	Superoxidase dismutase activity	Ypr16
PR-17	Tobacco PRp27	Unknown	Unknown	Unknown	Ypr17

between plants and some rhizobacteria known as plant growth-promoting rhizobacteria (PGPR) or plant-mycorrhizae (Van Loon *et al.*, 1998; Pozo and Azcon-Aguilar, 2007). Moreover, ISR is regulated by the JA and ET, and is independent of SA (Van Der Ent *et al.*, 2009). Furthermore, ISR induced modulation of gene expression, mainly involved in the defense or the regulation of transcription in the roots and deposition of callose and pectin in the leaves (Cordier *et al.*, 1998; Verhagen *et al.*, 2004). Finally, ISR is associated with priming rather than direct activation of defense (Conrath, 2006; Pozo *et al.*, 2008; Van Wees *et al.*, 2008).

Under some conditions, attack of herbivorous insects results in the induction of SAR in plants that is dependent on the production of JA that alters the development of the herbivores (Schilmiller and Howe, 2005). In addition, the production of volatile organic compounds (VOCs) activates inter-priming mechanisms among neighboring plants that participate in plant resistance (Engelberth *et al.*, 2004; Ferry *et al.*, 2004). Ton *et al.* (2007) have reported that when a healthy corn plant was exposed to VOCs produced by an infected plant attacked by cotton worm, priming effects for the defense genes induction were observed in the healthy plant. These priming effects were correlated with the lower production of larvae and a higher attraction of its respective parasite, *Cotesia marginiventris*.

3.5. Synthesis of pathogenesis-related proteins (PR proteins)

The term PR proteins has been defined as “those proteins that are not or only at basal concentrations detectable in healthy tissues, but for which accumulation at the protein level has been demonstrated upon pathological conditions and related situations in at least two or more plant–pathogen combinations (Van Loon and Van Strien, 1999; Van Loon *et al.*, 2006)”. Based on their biochemical properties, 17 well recognized families of PRs (named as PR-1, -2, -3 and so on) have been identified in different crop species to date (Table 1.3; Van Loon *et al.*, 2006; Sels *et al.*, 2008).

PR families are known as β-1,3-endoglucanases (PR-2), endochitinases (PR-3, -4, -8, and -11), thaumatin-like (PR-5), proteinase inhibitors (PR-6), endoproteases (PR-7), peroxidases (PR-9), ribonuclease-like protein (PR-10), defensins (PR-12), thionins (PR-13) and lipid transfer proteins (PR-14), oxalate oxidase (PR-15), oxalate oxidase-like (PR-16), and unknown (PR-17; Edreva, 2005; Van Loon *et al.*, 2006; Sels *et al.*, 2008). Most PR proteins have antimicrobial properties and act through hydrolytic activities, resulting in degradation of the wall of the pathogen. These include PR-2, -3, -4, -7 -8, -11, -12, -13 and -14 (Stec, 2006). PR-1 has antifungal properties, and along with PR-5, preferentially expressed

during oomycetes infection (Wildermuth *et al.*, 2001). On the other hand, PR-10 has viral targets for its induction. Class 6 belongs to serine proteinase inhibitors (PIs) and target nematodes and herbivorous insects. PR-9 is a member of lignin forming peroxidases and contributes to strengthen plant cell walls. Finally, PR-15 and PR-16 have superoxide dismutase activities and take part in H_2O_2 production and could participate in plant defense signaling (Van Loon *et al.*, 2006). PR proteins are induced through the action of SA, JA or ET and are thought to play a role in active defense by restricting pathogen growth and spread in the plants (Van Loon *et al.*, 2006). The expression of PR genes essentially coding acidic PR proteins is induced by SA and are normally active during SAR responses while JA and ET activate the transcription of genes coding inhibitors of proteases, the defensins, thionins and basic PR proteins (Ward *et al.*, 1991; Epple *et al.*, 1995). Other way round, these proteins are used as molecular markers in hormone signaling *i.e.* induction of PR-1 is under SA signaling and it is a well known SA marker gene in SAR while PR-12 (PDF1.2) is usually taken as a molecular marker for the induction of the JA- and ET-dependent defense-signaling pathway (Lay and Anderson, 2005).

Table 1.4: Examples of the developmental processes and responses to abiotic and biotic stresses initiated by $[Ca^{2+}]_{cyt}$ variations (White and Broadley, 2003).

Developmental process or environmental challenge	**Characteristic $[Ca^{2+}]_{cyt}$ perturbation**	**Stores releasing Ca^{2+} to cytosol**	**References**
Pollen tube elongation	Oscillation of high apical $[Ca^{2+}]_{cyt}$	Apoplast and internal	Malhó and Trewavas, 1996; Holdaway-Clarke *et al.*, 1997; Malhó *et al.*, 1998; 2000; Messerli *et al.*, 2000; Rudd and Franklin-Tong, 2001
Pollen tube self-incompatibility response	Intracellular $[Ca^{2+}]_{cyt}$ wave in shank	Apoplast Internal (IP_3-dependent)	Rudd and Franklin-Tong, 2001; Straatman *et al.*, 2001; Franklin-Tong *et al.*, 2002
Cell polarity after fertilization	Intracellular $[Ca^{2+}]_{cyt}$ wave from sperm fusion site leading to sustained $[Ca^{2+}]_{cyt}$ elevation	Apoplast	Antoine *et al.*, 2001
Cell division	Elevated $[Ca^{2+}]_{cyt}$		Bush, 1995
Seed germination (giberellins)	Slow rise in $[Ca^{2+}]_{cyt}$		Bush, 1995; Anil and Sankara Rao, 2001
Apoptosis	Slow, sustained $[Ca^{2+}]_{cyt}$ elevation		Levine *et al.*, 1996
Red light	Elevated $[Ca^{2+}]_{cyt}$	Apoplast	Shacklock *et al.* 1992; Malhó *et al.*, 1998
Blue light	Brief spike in $[Ca^{2+}]_{cyt}$ (seconds)	Apoplast	Malhó *et al.*, 1998; Baum *et al.*, 1999
Circadian rhythms	Circadian $[Ca^{2+}]_{cyt}$ oscillation		Johnson *et al.*, 1995; Wood *et al.*, 2001
Stomatal closure (ABA, sphingosine-1-phosphate)	(1) Elevated $[Ca^{2+}]_{cyt}$ at cell periphery (2) Elevated $Ca^{2+}]_{cyt}$ around vacuole (3) Oscillations in $[Ca^{2+}]_{cyt}$	(1) Apoplast (2) Vacuole (3) Apoplast and internal	McAinsh *et al.*, 1992; Allen *et al.*, 1999, 2000; Blatt, 2000*a*, *b*; White, 2000; Anil and Sankara Rao, 2001; Evans *et al.*. 2001; Ng *et al.*, 2001*a*, *b*; Schroeder *et al.*, 2001; Klüsener *et al.*, 2002
CO_2	Elevated $[Ca^{2+}]_{cyt}$ in guard cells	Apoplast	Webb *et al.*, 1996
Increasing apoplastic Ca^{2+}	Oscillations in $[Ca^{2+}]_{cyt}$ of guard cells	Apoplast	McAinsh *et al.*, 1995; Allen *et al.*, 1999, 2000
Auxin responses	(1) Slow, prolonged $[Ca^{2+}]_{cyt}$ increase (2) Oscillations in $[Ca^{2+}]_{cyt}$		Felle, 1988; Malhó *et al.*, 1998; Ng *et al.*, 2001*b*; Plieth, 2001; Plieth and Trewavas, 2002
Xylem K^+ loading	Elevated $[Ca^{2+}]_{cyt}$		De Boer, 1999
Exocytosis	Elevated $[Ca^{2+}]_{cyt}$		Battey *et al.*, 1999; Camacho and Malhó, 2003

4. Ca^{2+} signaling in plants

In eukaryotes, Ca^{2+} is a versatile second messenger and plays a pivotal role in a variety of physiological and developmental processes by participating in related signal transduction pathways. In plants, diverse arrays of environmental stimuli lead to the generation of Ca^{2+} signals by the regulated movement of Ca^{2+} ions between subcellular compartments and between the cell and its extracellular environment. These signals take the form of a strong, rapid and transient increase in free cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) whose frequency, amplitude and shape is determined by the nature of the stimulus (McAinsh and Pittman, 2009; Boudsocq and Sheen, 2010; Dodd *et al.*, 2010). At this stage, different Ca^{2+} binding proteins (such as CaM, CDPK and EF-hand containing proteins), capable of decoding and relaying the information encoded within stimulus-specific Ca^{2+} signatures, further define the specificity of these $[Ca^{2+}]_{cyt}$ changes within the cell to initiate a signal transduction pathway (Luan *et al.*, 2002; Sanders *et al.*, 2002; Batistič and Kudla, 2004; Dodd *et al.*, 2010; Kudla *et al.*, 2010). In addition to a variety of cellular transporters, these Ca^{2+} binding proteins mainly target different enzymatic and signaling proteins such as transcription factors and protein kinases (Reddy and Reddy, 2004; Finkler *et al.*, 2007; Kudla *et al.*, 2010). Thus, interplay between Ca^{2+} signatures and Ca^{2+} sensing contributes to the stimulus specificity of Ca^{2+} signaling (Dodd *et al.*, 2010). However, elevated levels of $[Ca^{2+}]_{cyt}$ can chelate negatively charged molecules in the cell and hence can cause cytotoxity (DeFalco *et al.*, 2010; Reddy *et al.*, 2011). Therefore, level of $[Ca^{2+}]_{cyt}$ is strictly maintained between 100-200 nM, a concentration that is 10^4 times less than in the apoplastic fluid and 10^4 to 10^5 less than that in cellular organelles (Bush, 1995; Stael *et al.*, 2012). A large variety of channels/pumps participates to regulate the Ca^{2+} balance, and excessive $[Ca^{2+}]_{cyt}$ immediately moves either to extracellular medium or is stored in the intracellular compartments (Sanders *et al.*, 2002; Clapham, 2007; McAinsh and Pittman, 2009; Dodd *et al.*, 2010). This consequently leads to the establishment of a steep concentration gradient between the cytosol and Ca^{2+} stores. In conclusion, a very sophisticated mechanism of Ca^{2+} regulation exists in plants and the input of information from biotic and abiotic sources leads to the activation of a network of signaling pathways. In turn, these output information plays a vital role in plant survival and development by posing a significant impact on gene expression and other physiological processes in plants. Table 1.4 highlights the role of Ca^{2+} in different development processes and responses to different environmental cues.

Root cell elongation	Sustained $[Ca^{2+}]_{cyt}$ elevation	Apoplast	Cramer and Jones, 1996; Demidchik *et al.*, 2002
Root hair elongation	Sustained high apical $[Ca^{2+}]_{cyt}$	Apoplast	Wymer *et al.*, 1997; White, 1998; Bibikova *et al.*, 1999
Inhibition of cyclosis	Elevated $[Ca^{2+}]_{cyt}$		Ayling and Clarkson, 1996
Nodulation (nod factors)	Initial $[Ca^{2+}]_{cyt}$ rise then oscillations in $[Ca^{2+}]_{cyt}$	Apoplast	Cárdenas *et al.*, 2000; Wais *et al.*, 2000; Walker *et al.*, 2000; Lhuissier *et al.*, 2001; Shaw and Long, 2003
Senescence	Sustained $[Ca^{2+}]_{cyt}$ elevation		Huang *et al.*, 1997
UV-B	Slow $[Ca^{2+}]_{cyt}$ rise, elevated $[Ca^{2+}]_{cyt}$ sustained for several minutes	Apoplast	Frohnmeyer *et al.*, 1999
Heat-shock	Elevated $[Ca^{2+}]_{cyt}$ sustained for 15–30 min	Apoplast and internal (IP_3-dependent)	Gong *et al.*, 1998; Malhó *et al.*, 1998
Cold-shock	(1) Single brief $[Ca^{2+}]_{cyt}$ spike (seconds) (2) Oscillations in $[Ca^{2+}]_{cyt}$	(1) Apoplast	Knight *et al.*, 1991; Malhó *et al.*, 1998; White, 1998; Plieth *et al.*, 1999; van der Luit, 1999; Allen *et al.*, 2000; Knight, 2000; Cessna*et al.*, 2001; Plieth, 2001
Slow cooling	Biphasic (1) Brief $[Ca^{2+}]_{cyt}$ spike (seconds) (2) Slow $[Ca^{2+}]_{cyt}$ elevation (minutes)	(1) Apoplast (2) Apoplast and internal (IP_3-dependent)	Knight *et al.*, 1996; Plieth *et al.*, 1999; Knight, 2000; Knight and Knight, 2000; Moore *et al.*, 2002
Oxidative stress (paraquat, superoxide, H_2O_2, ozone)	(1) Brief $[Ca^{2+}]_{cyt}$ spike (2) Sustained $[Ca^{2+}]_{cyt}$ elevation (3) Oscillations in $[Ca^{2+}]_{cyt}$	(1) Apoplast (2) Apoplast and internal (IP_3-dependent)	Price *et al.*, 1994; Levine *et al.*, 1996; McAinsh *et al.*, 1996; Knight *et al.*, 1998; Malhó *et al.*, 1998; Clayton *et al.*, 1999; Allen *et al.*, 2000; Kawano and Muto, 2000; Knight, 2000; Klüsener *et al.*, 2002; Lecourieux *et al.*, 2002
Anoxia	Biphasic (1) Slow spike (duration of minutes) (2) Sustained $[Ca^{2+}]_{cyt}$ elevation (hours)	(1) Apoplast(2) Internal including mitochondria	Subbaiah *et al.*, 1994, 1998; Sedbrook *et al.*, 1996; Malhó *et al.*, 1998; Plieth, 2001
Drought/hyper-osmotic stress (mannitol)	Biphasic (1) Slow spike (duration of minutes) (2) Sustained $[Ca^{2+}]_{cyt}$ elevation (hours)	Apoplast and vacuole	Knight *et al.*, 1997, 1998; Malhó *et al.*, 1998; Cessna *et al.*, 2001; Pauly *et al.*, 2001; Plieth, 2001

4.1. Ca^{2+} signatures specificity

As it is mentioned above, prolonged elevated $[Ca^{2+}]_{cyt}$ is lethal for the cell and is implicated in apoptosis both during normal plant development and during HR to pathogens (Levine *et al.*, 1996). $[Ca^{2+}]_{cyt}$ perturbations of low amplitude or transients are sufficient to respond to a diverse range of developmental cues and environmental challenges (White, 2000; Sanders *et al.*, 2002). The transient increases in $[Ca^{2+}]_{cyt}$ could be of different shape. This $[Ca^{2+}]_{cyt}$ change could be in the form of a Ca^{2+} spike (a single wave), a biphasic Ca^{2+} response (a double wave) or it could adopt the form of Ca^{2+} oscillations (multiple waves) depending upon the nature of the stimuli. These stimulus-specific Ca^{2+} signals, in term of the spatial and temporal dynamics of the changes in $[Ca^{2+}]_{cyt}$ have been referred to as 'Ca^{2+} signature' (Figure 1.11; Ng and McAinsh, 2003; McAinsh and Pittman, 2009). Stimulus-induced changes in plant $[Ca^{2+}]_{cyt}$ are observed in many different cell types in response to a diverse range of abiotic and biotic stimuli, examples of which include: osmotic, salt and drought signals (Knight *et al.*, 1997; Ranf *et al.*, 2008), oxidative stress (Evans *et al.*, 2005), cold (Knight *et al.*, 1991, 1996), gaseous pollutants (Evans *et al.*, 2005), light (Shacklock *et al.*, 1992), plant hormones (McAinsh *et al.*, 1990; Allen *et al.*, 2001), pathogens (elicitors; Lecourieux *et al.*, 2006) and bacterial and fungal signals (Ehrhardt *et al.*, 1996; Kosuta *et al.*, 2008). The characteristic changes in $[Ca^{2+}]_{cyt}$ leads to stimulus specific responses through the induction of various signal transduction pathways in plants (McAinsh and Pittman, 2009). The generation of Ca^{2+} signatures has also been reported in non-cytosolic locations such as nucleus, chloroplasts and mitochondria. Moreover, the organelles Ca^{2+} signatures have been shown to possess their own specificity not superimposed with $[Ca^{2+}]_{cyt}$ signals (Johnson *et al.*, 1995; Pauly *et al.*, 2000; Logan and Knight, 2003). Beside the stimulus specificity, Ca^{2+} signature also depends on the specific cell type in a tissue that is under investigation (Kiegle *et al.*, 2000; White and Broadley, 2003). This is strengthened by the fact that plant tissues contain a populations of heterogeneous cells with contrasting abilities to generate $[Ca^{2+}]_{cyt}$ signatures. There exist characteristic differences in $[Ca^{2+}]_{cyt}$ variations in the root cells, in response to different mechanical stimuli *e.g.* osmotic stress, cold shock and salinity (Kiegle *et al.*, 2000; Moore *et al.*, 2002). Moreover, during anoxia conditions, a biphasic $[Ca^{2+}]_{cyt}$ response has been reported in shoot cells in contrast to a very slow $[Ca^{2+}]_{cyt}$ increase in root cells (Sedbrook *et al.*, 1996; Plieth, 2001).

These $[Ca^{2+}]_{cyt}$ variations are very efficiently regulated by the presence of Ca^{2+} transporters in the plasma membrane and endomembrane systems. Each signature is specified by its magnitude, shape, temporal and spatial parameters, which ultimately control the

Salinity (NaCl)	Biphasic tissue $[Ca^{2+}]_{cyt}$ wave (1) Slow spike (duration of minutes) (2) Sustained $[Ca^{2+}]_{cyt}$ elevation (hours) (3) Reduced $[Ca^{2+}]_{cyt}$ (days)	Apoplast and vacuole (IP_3-dependent)	Knight *et al.*, 1997; Kiegle *et al.*, 2000; Knight, 2000; DeWald *et al.*, 2001; Pauly *et al.*, 2001; Moore *et al.*, 2002; Halperin *et al.*, 2003
Hypo-osmotic stress	Biphasic (1) Small $[Ca^{2+}]_{cyt}$ elevation (2) Large $[Ca^{2+}]_{cyt}$ elevation	(1) Apoplast (2) Apoplast and internal (IP_3-dependent)	Takahashi *et al.*, 1997; Malhó *et al.*, 1998; Knight, 2000; Cessna and Low, 2001; Cessna *et al.*, 2001; Pauly *et al.*, 2001; Plieth, 2001
Mechanical stimulation (motion, touch, wind)	Single brief $[Ca^{2+}]_{cyt}$ spike (seconds) $[Ca^{2+}]_{cyt}$ wave	Internal	Knight *et al.*, 1991, 1992; Haley *et al.*, 1995; Legue *et al.*, 1997; Malhó *et al.*, 1998; van der Luit, 1999; Plieth, 2001; Fasano *et al.*, 2002
Aluminium stress	Elevated $[Ca^{2+}]_{cyt}$		Zhang and Rengel, 1999
Pathogens (elicitors)	Biphasic (1) Slow spike (duration of minutes) (2) Sustained $[Ca^{2+}]_{cyt}$ elevation (hours) (3) Oscillations in $[Ca^{2+}]_{cyt}$ (the relative magnitude of different phases varies with elicitor identity)	(1) Apoplast (2) Apoplast and internal (IP_3-dependent)	Knight *et al.*, 1991; Malhó *et al.*, 1998; Mithöfer *et al.*, 1999; Blume *et al.*, 2000; Fellbrich *et al.*, 2000; Grant *et al.*, 2000; Cessna and Low 2001; Cessna *et al.,* 2001; Rudd and Franklin-Tong, 2001; Klüsener *et al.*, 2002; Lecourieux *et al.*, 2002 ; Lecourieux *et al.*, 2005

reversible binding of Ca^{2+} to specific protein sensors before passing the decoded information onto targets. As a result, specific Ca^{2+} signatures trigger altered protein phosphorylation, gene expression patterns and the subsequent responses in plant cells (Luan *et al.*, 2002; Sanders *et al.*, 2002; Finkler *et al.*, 2007).

4.2. Cellular functions of Ca^{2+} signals in plants

Plants growth and development is dependent on their surrounding environment that includes different types of (a) biotic stresses, availability of plant nutrients *etc.* Plants respond to these external cues by modification in their biochemical, physiological, and/or morphological attitude that ensure plant survival. The perception of external stimuli is relayed by secondary messengers such as Ca^{2+} ions, cyclic nucleotides, inositol polyphosphates (InsP), NO, ROS and lipids (Sanders *et al.*, 2002; Reddy *et al.*, 2011). Signals generated by these molecules are decoded by different proteins like Calmodulin (CaM) and CaM like proteins, protein kinase (PK), protein phosphatase (PP), phospholipase (PL) and NO synthase. Among these, Ca^{2+} appears to be an important nutrient and most used messenger in plants and animals. As a nutrient molecule, role of Ca^{2+} has been reported in maintaining the structural rigidity of the cell walls and in membrane structure and function (Hepler, 2005). The role of Ca^{2+} as a secondary messenger is supported by the fact that a large variety of environmental factors are able to modify the levels of not only the $[Ca^{2+}]_{cyt}$ but also mitochondrial Ca^{2+} ($[Ca^{2+}]_{mito}$), nuclear Ca^{2+} ($[Ca^{2+}]_{nuc}$) and chloroplastic Ca^{2+} ($[Ca^{2+}]_{chlo}$) under certain conditions and that Ca^{2+} participates in different steps of cell signaling (Lecourieux *et al.*, 2006; Mazars *et al.*, 2009; McAinsh and Pittman, 2009; DeFalco *et al.*, 2010). Indeed, it acts as a convergence point, linking a range of highly diverse stimuli to specific responses (Sanders *et al.,* 1999; Sanders *et al.*, 2002; McAinsh and Pittman, 2009). Moreover, it also interacts with other second messengers to deliver characteristics responses to different stimuli (Besson-Bard *et al.*, 2008a).

4.2.1. Ca^{2+} signaling at the single cell level

One of the most intriguing aspects of stimulus-specific Ca^{2+} signaling is that it occurs both at single cell and whole tissue/organ level. The nature of response could be completely different depending upon the type of stimuli or cell/organ. In plants, guard cells, growing pollen tubes and root hairs represent the excellent models to study primary and autonomous Ca^{2+} responses at single cell level. This investigation is even more interesting to explore the specificity of $[Ca^{2+}]_{cyt}$ response as studies have demonstrated that even two guard cells of a

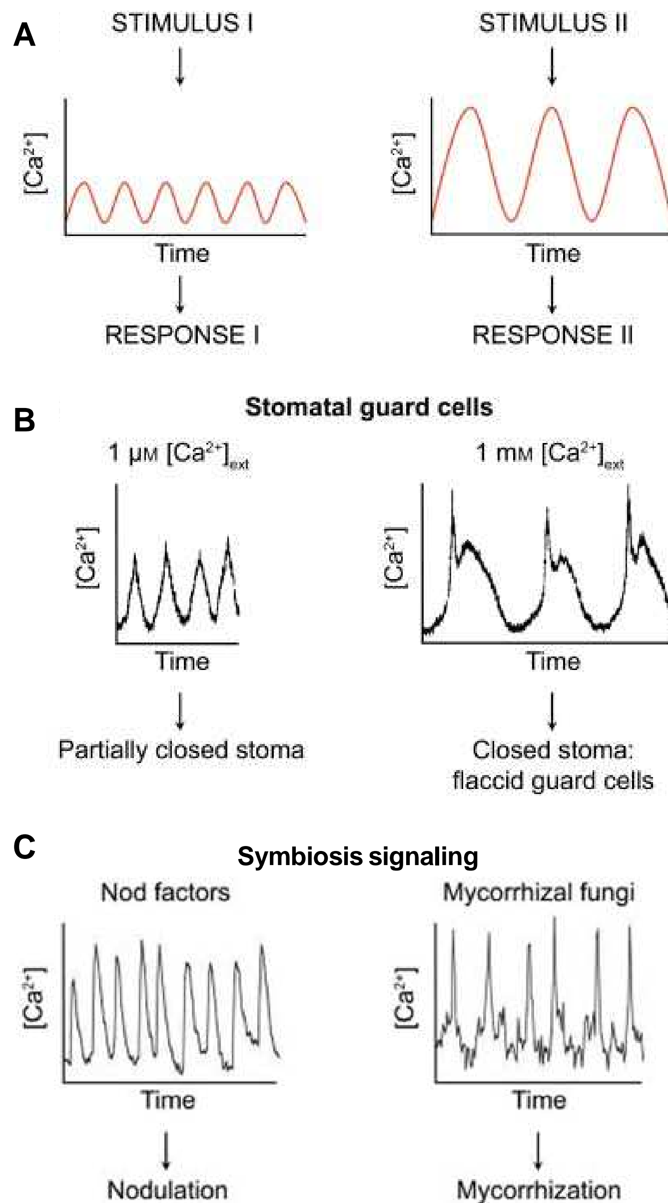


Figure 1.11: Stimuli-specific Ca^{2+} signature in plants. A) A schematic representation of the encryption of signaling information in the temporal dynamics of Ca^{2+} oscillations. **B)** In *Commelina communis* guard cells, the strength of the external Ca^{2+} ($[Ca^{2+}]_{ext}$) stimulus has been correlated directly with the pattern of Ca^{2+} oscillations (i.e. the period, frequency and amplitude), which in turn dictates the resultant steady-state stomatal aperture . **C)** Nod factors and the mycorrhizal fungi produce Ca^{2+} oscillations in *Medicago truncatula*, which differ in their period and amplitude; this may provide a mechanism for the observed differences in the physiological response to rhizobial bacteria and mycorrhizal fungi (Adapted from McAinsh and Pittman, 2009).

stomata behave differently and seldom display similar $[Ca^{2+}]_{cyt}$ change in response to a defined stimulus (Allen *et al.*, 1999).

4.2.1.1. Regulation of stomatal guard cells signaling

In plants, Ca^{2+} signaling in stomatal guard cells represents the most compelling evidence that signaling information can be encoded in the spatiotemporal dynamics of plant Ca^{2+} signatures (Ng and McAinsh, 2003; McAinsh, 2007). Different stimuli such as cold, elevation of external Ca^{2+}, abscisic acid (ABA), atmospheric CO_2 and H_2O_2 are able to induce $[Ca^{2+}]_{cyt}$ oscillations in *Arabidopsis* stomatal guard cells and only the oscillations within a defined window of frequency, transient number, duration and amplitude result in steady-state stomatal closure (Allen *et al.*, 2000, 2001; Li *et al.*, 2006; Young *et al.*, 2006). These changes in guard cell $[Ca^{2+}]_{cyt}$ include localized increases and oscillations (Evans *et al.*, 2001; McAinsh, 2007). Depending upon the signatures, two differentially regulated mechanisms have been proposed for stomatal closure in *Arabidopsis*: short-term Ca^{2+}-reactive closure and long-term Ca^{2+}-programmed closure (Kudla *et al.*, 2010). As for as the short-term Ca^{2+}-reactive closure is concerned, it is a rapid response to increasing $[Ca^{2+}]_{cyt}$ elevation and does not depend on different parameters of $[Ca^{2+}]_{cyt}$ oscillations whereas long-term Ca^{2+}-programmed closure is strictly dependent on the pattern of $[Ca^{2+}]_{cyt}$, having a defined range of frequency, transient number, duration and amplitude. Various studies have shown that the *Arabidopsis* Ca^{2+}-dependent protein kinase (CDPK) double mutant *cpk3cpk6* is defective in short-term closure, but not in long-term closure in response to oscillations in guard cell $[Ca^{2+}]_{cyt}$ that are also responsible for the activation of S-type anion channels (Mori *et al.*, 2006). This clearly highlights that these two processes have separate regulation mechanisms. Through a hyperpolarization-activated Ca^{2+}-permeable channel, H_2O_2 mediated Ca^{2+} influx in the protoplast and an increased $[Ca^{2+}]_{cyt}$ level in intact *Arabidopsis* guard cells which are responsible for the closure of stomata (Pei *et al.*, 2000). Moreover, ABA-insensitive mutant *gca2* is impaired in the activation of Ca^{2+} channels by H_2O_2 and ABA- and H_2O_2-induced stomatal closure. ABA pretreatment of guard cells is responsible for the increase in the magnitude of S-type anion efflux currents and the down-regulation of K^+ currents in response to Ca^{2+}, thus suggesting the sensitivity of different Ca^{2+} sensors to ABA in guard cell signaling (Siegel *et al.*, 2009). Moreover, it has been reported that *Arabidopsis* plant mutated in Slow Anion Channel-Associated 1 (SLAC1), a guard cell anion efflux channel which plays a central role in Ca^{2+}-reactive stomatal closure, had abolished Ca^{2+}-reactive stomatal closure and displayed compromised stomatal responses to different stimuli including ABA, Ca^{2+} ions,

CO_2, NO, H_2O_2, light/dark transitions and humidity change (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). Recently, Cho *et al.* (2009) have reported that the glutamate receptor homolog AtGLR3.1 which is preferentially expressed in guard cells plays a vital role in Ca^{2+}-induced stomatal closure. However, they demonstrated a contrasting mechanism for the regulation of stomatal closure as compared to CDPK double mutant, *cpk3cpk6.* Over-expression of AtGLR3.1 resulted in impaired external Ca^{2+}-induced stomatal closure despite that S-type anion channel activity was normal in the AtGLR3.1 over-expressing plants. These over-expressing plants were only defective in long-term programmed stomatal closure without having any effect on short-term Ca^{2+}-reactive closure. Additionally, the wild-type plants mimicked the guard cell behavior of the AtGLR3.1 over-expressing plants in the presence of cyclohexamide, a translational inhibitor, demonstrating that *de novo* protein synthesis contributes to the maintenance of long-term Ca^{2+}-programmed stomatal closure. Taken together, these data strongly support a role for $[Ca^{2+}]_{cyt}$ oscillations in the signaling pathway associated with stomatal closure by activating preexisting proteins and inducing the expression of some required genes. At the same time, different Ca^{2+} sensor proteins and ion channels also actively participate in this process. However, stomatal closure have also been observed in the absence of guard cell $[Ca^{2+}]_{cyt}$ oscillations, and spontaneous Ca^{2+} transitions do not always lead to stomatal closure (Hetherington and Brownlee, 2004; Levchenko *et al.*, 2005; Young *et al.*, 2006). This not only raises the question of how the Ca^{2+} decoding system is able to decode these variable oscillations into defined downstream responses but it also adds further complexity in guard cell signaling network.

4.2.1.2. The establishment of symbiosis: signaling in root hairs

Another important model to study the Ca^{2+} oscillations at single cell level is the symbiosis signaling in legumes. The pivotal role of Ca^{2+} signaling in plant-symbiosis interactions has been known since long time. Rhizobial-derived nodulation (Nod) factors are secreted by nitrogen fixing bacteria present in the proximity to legumes roots and lead to establish a symbiosis interaction between the bacteria and plants. These Nod factors are able to induce a biphasic $[Ca^{2+}]_{cyt}$ change in legume root hair cells. This biphasic $[Ca^{2+}]_{cyt}$ response actually comprises an initial Ca^{2+} influx and a subsequent long-term Ca^{2+} oscillation in the perinucleus (Shaw and Long, 2003). But this is not the case all the times as studies with different *Medicago truncatula does not make infection* (*dmi*) mutants have shown a different behaviour for $[Ca^{2+}]_{cyt}$ responses under different conditions. For example, *dmi1* and *dmi2* mutants are defective in the Ca^{2+}spiking but retain the initial Ca^{2+} influx. In

contrast, low concentration of Nod factor (10^{-11} to 10^{-12} M) induced Ca^{2+} spiking but failed to induce Ca^{2+} influx, suggesting that they are separable responses (Shaw and Long, 2003). *M. truncatula Early Nodulation 11* (*MtENOD11*) is one of the earliest genes expressed in the root epidermis of *M. truncatula* following the initial contact with *Sinorhizobium meliloti* and is a widely used marker gene for endosymbiotic associations involving both rhizobia and arbuscular mycorrhizal fungi (Journet *et al.*, 2001; Charron *et al.*, 2004). Studies by using blockers for Ca^{2+} channels and Ca^{2+} pumps have shown an inhibition of both Ca^{2+} spiking and transcript accumulation of *ENOD11* (Engstrom *et al.*, 2002; Charron *et al.*, 2004) and suggested that Ca^{2+} spiking and a subsequent gene expression is essential for the regulation of nodulation. This was further proved by Miwa *et al.* (2006) who showed that *ENOD11* inductions were observed only when the Ca^{2+} spiking lasted for at least 60 min. At the same time, this also indicated that a strong correlation exist between the number of Ca^{2+} spikes and *ENOD11* expression levels. Another study conducted by using *dmi1* and *dmi2* mutants plus *dmi3* mutant, defective in encoding Ca^{2+} calmodulin-dependent kinase (CCaMK) gene, reported a decreased induction of *ENOD11* (Gleason *et al.*, 2006). This study demonstrated the essential function of this CCaMK in the regulation of nodule development (Gleason *et al.*, 2006).

$[Ca^{2+}]_{cyt}$ transients have also been reported during symbiotic interactions between various legumes and arbuscular micorrhizal (AM) fungi. Rapid and transient elevations in $[Ca^{2+}]_{cyt}$ were recorded in *Glycine max* cell cultures treated with *Gigaspora margarita* spores, thus indicating that diffusible molecules released by the mycorrhizal fungus were perceived by host plant cells through a Ca^{2+}-mediated signaling (Navazio *et al.*, 2007). These responses were AM symbiosis specific as an up-regulation of *M. truncatula* genes, *DMI1*, *DMI2* and *DMI3*, essential for the establishment of the AM symbiosis was observed in *Glycine max* cell cultures. Moreover, non host culture cells of *Arabidopsis thaliana* did not induces these $[Ca^{2+}]_{cyt}$ changes (Navazio *et al.*, 2007). Similarly, $[Ca^{2+}]_{cyt}$ transients were also observed when *M. truncatula* root hair cells were exposed to AM fungi, *Glomus intraradices*. Moreover, AM-induced $[Ca^{2+}]_{cyt}$ transients were abrogated in *dmi1* and *dmi2* mutant plants, suggesting the existence of common signaling components during nodulation and mycorrhizal infection (Kosuta *et al.*, 2008; Parniske, 2008). However, Ca^{2+} spiking with a shorter period and smaller amplitudes was identified in response to AM fungi compared to Nod factors (Kosuta *et al.*, 2008). This response is in complete accordance with the need to transduce two different signals, one from rhizobial bacteria and one from mycorrhizal fungi, by using common components of a single signaling pathway (Kosuta *et al.*, 2008). Finally, the cell wall

extract (CWE) from the growth-promoting fungus *Piriformospora indica* is able to induce a $[Ca^{2+}]_{cyt}$ elevation in the roots of *Arabidopsis* and tobacco plants (Vadassery *et al.*, 2009). Interestingly, CWE was involved in the phosphorylation of MAPKs in a Ca^{2+}-dependent manner without having any effect on H_2O_2 production, and both CWE and MAMPs increase expression of MAPK6, a defense-related gene. Moreover, CWE was responsible for the transcript induction of CNGC10, CNGC13, Calmodulin-Like Protein 42 (CML42), and CML38. These data demonstrate that Ca^{2+} signaling is a common feature of plant-microbe interactions.

4.2.1.3. Signaling for tip growth in pollen tubes and root hair cells

The first indication of the interaction between Ca^{2+} and pollen tube growth was reported about fifty years ago when Brewbaker and Kwack (1963) showed that Ca^{2+} is essential for *in vitro* pollen tube cultures. Since then, a lot of progress has been made to explore the relationship between the Ca^{2+} concentration $[Ca^{2+}]$ and pollen tube growth. Pollen tubes are one of the most extensively studied tip-growing model systems in plants. *In vitro* growing pollen tubes have displayed regular oscillations in many parameters such as apical ion flux, cytosolic pH, and $[Ca^{2+}]_{cyt}$ (Moreno *et al.*, 2007). The tip-specific $[Ca^{2+}]_{cyt}$ gradient plays a significant role in controlling pollen tube elongation (Malho *et al.*, 1995; Franklin-Tong, 1999; Iwano *et al.*, 2009). Various studies using a Ca^{2+}-sensitive vibrating electrode have revealed that extracellular Ca^{2+} influx is involved in the maintenance of the Ca^{2+} gradient in the tip region of the pollen tube (Malho *et al.*, 1995; Holdaway-Clarke *et al.*, 1997; Franklin-Tong *et al.*, 2002; Cheung and Wu, 2008). Stretch-activated Ca^{2+} channels have been identified in the plasma membrane using patch-clamp electrophysiology and pharmacological inhibition of these channel activities resulted in the disruption of the Ca^{2+} influx at the apex and terminates pollen tube elongation (Picton and Steer, 1985; Kühtreiber and Jaffe, 1990). In accordance with an essential function of stretch-activated channels, Dutta and Robinson (2004) have suggested the involvement of these channels in the maintenance of the tip-focused Ca^{2+} gradient. Plasma membrane Ca^{2+} channel activity in pollen has been studied by electrophysiology (Shang *et al.*, 2005; Qu *et al.*, 2007; Wu *et al.*, 2010) or by genetic analysis of CNGCs (Frietsch *et al.*, 2007). CNGC18, a Ca^{2+}-permeable channel in the plasma membrane, has been demonstrated to be an essential component for pollen tube growth (Frietsch *et al.*, 2007). In a recent study, by pharmacology and loss-of-function mutants, GLR channels, another class of Ca^{2+}-permeable channels in the plasma membrane, have been reported to modulate the apical $[Ca^{2+}]_{cyt}$ gradient in tobacco and

Arabidopsis. Consequently, this $[Ca^{2+}]_{cyt}$ gradient affect pollen tube growth (Michard *et al.*, 2011).

Study of root hair cells is another interesting example for the Ca^{2+} signaling at signal cell level. Although, not too much data is available, yet different recent studies have demonstrated the presence of an active Ca^{2+} signaling mechanism during root hair extensions.

A tip-focused Ca^{2+} gradient with a Ca^{2+} oscillation were detected in root hair cells of *Arabidopsis* and similar dynamic in tip-focused Ca^{2+} gradient and root hair elongation was observed in these studies (Monshausen *et al.*, 2008). In another study, Takeda *et al.* (2008) demonstrated that Root Hair Defective 2 (RDH2; also known as RBOH C) is present in the plasma membrane of growing tips of root hair cells of A*rabidopsis thaliana* and participates in the appropriate growth of root hairs. RDH2-dependent ROS leads to a Ca^{2+} influx that, in turn, activates the RHD2 to produce ROS in the root tip growing regions. This demonstrates the existence of a positive feedback mechanism to sustain root hair cell growth (Takeda *et al.*, 2008). Previous studies have shown that activation of Rboh oxidase is dependent both on Ca^{2+} binding to EF-hand domains and CDPK-dependent phosphorylation at the N-terminal domain (Sagi and Fluhr, 2001; Kobayashi *et al*., 2007). In agreement to these findings, activation of RHD2 was observed after the Ca^{2+} binding to two EF-hands and Ca^{2+}-dependent phosphorylation of two serine residues on RHD2 (Takeda *et al.*, 2008).

4.2.2.1. Ca^{2+} signaling during plant-pathogen interactions

Although studies have reported the significance of Ca^{2+} oscillations at single cell level in plants by their implication in plant response to external stimuli yet the final response is demonstrated by the regulation of complex growth processes in distinct tissues and organs. This is especially important in case of plant systemic response. Therefore, studies to elucidate Ca^{2+} signaling in the tissue context and in the whole organism have a major significance. Ca^{2+} signaling is involved in almost all kind of plant response to (a)biotic stress responses (Garcia-Brugger *et al.*, 2006; Lecourieux *et al.*, 2006; McAinsh and Pitmann, 2009; Dodd *et al.*, 2010; Kudla *et al.*, 2010). In the following section we will discuss in detail the role of Ca^{2+} dynamics during plant-pathogen interaction.

Apart from its role in growth and development, it has become evident that Ca^{2+} is one of the most important second messengers involved in different signal transduction pathways leading to defense responses in plants (Lecourieux *et al.*, 2006). The key role of Ca^{2+} in the signaling pathway received particular attention in the area of plant defense against pathogens (Nürnberger and Scheel, 2001). A variety of PAMPS/MAMPs and elicitors have been

reported to participate in Ca^{2+}-dependent defense signaling in plants (Garcia-Brugger *et al.*, 2006; Boller and Felix, 2009). Indeed, different elicitors are able to induce a Ca^{2+} influx that leads to a subsequent increase in $[Ca^{2+}]_{cyt}$ and participates in different downstream defense signaling pathways. After treatments with a variety of elicitors, a rapid increase in $[Ca^{2+}]_{cyt}$ has been reported in different plant species (Poinssot *et al.*, 2003; Hu *et al.*, 2004; Zhao *et al.*, 2005; Lecourieux *et al.*, 2006; Ma *et al.*, 2009). We have authentic confirmations about stimulus-specific patterns of $[Ca^{2+}]_{cyt}$ increase in plants where each stimulus gives its own characteristic Ca^{2+} signature. In case of elicitor treatments, the nature of $[Ca^{2+}]_{cyt}$ signatures could be different in term of intensity, kinetics and duration (Lecourieux *et al.*, 2002). However, an interesting aspect of elicitors-induced Ca^{2+} signaling lies in the fact that these molecules do not encode elicitor-specific information primarily because similar prolonged $[Ca^{2+}]_{cyt}$ increases induce similar general pattern of defense responses irrespective of their nature (Ma and Berkowitz, 2007). In response to plant defense elicitors, Ca^{2+} is mobilized not only from extracellular medium but also from the intracellular Ca^{2+} stores and actively participates in plant defense signal transduction pathways. Here we are presenting a well known model of elicitor-induced Ca^{2+} signaling in plants. Figure 1.6 summarizes the cryptogein signaling pathway and clearly shows the importance of Ca^{2+} in activating various types of signaling events since all the downstream events are dependent on Ca^{2+} influx (adapted from Garcia-Brugger *et al.*, 2006).

Different pharmacological and $^{45}Ca^{2+}$-based approaches have reported that Ca^{2+} fluxes and $[Ca^{2+}]_{cyt}$ variations take an active part in elicitor-mediated plant defense responses (Conrath *et al.*, 1991; Mathieu *et al.*, 1991; Nürnberger *et al.*, 1994; Tavernier *et al.*, 1995; Romani *et al.*, 2004; Vatsa *et al.*, 2011). Changes in $[Ca^{2+}]_{cyt}$ have been reported in tobacco cells after treatments with Cry or OGs (Lecourieux *et al.*, 2002) and were amplified by H_2O_2 generated during the elicitation process (Klüsener *et al.*, 2002; Lecourieux *et al.*, 2002). Pep-13, the *Phytophthora sojae*–derived oligopeptide elicitor has shown to induce Ca^{2+} influx in parsley cells. Moreover, Pep-13 was also found essential after receptor binding, for $[Ca^{2+}]_{cyt}$ variations and activation of defense-associated responses. These data indicate the involvement of elicitor-induced $[Ca^{2+}]_{cyt}$ changes in pathogen defense signaling in plants (Blume *et al.*, 2000). In another study, Poinssot *et al.* (2003) reported that BcPG1 (*Botrytis cinerea* endopolygalacturonase 1), a potent elicitor of defense response in grapevine, resulted a biphasic and sustained $[Ca^{2+}]_{cyt}$ elevation in grapevine cells that leads to the production of NO and ROS, the two important components of plant defense. Similarly, OGs is able to induce a rapid, substantial and transient $[Ca^{2+}]_{cyt}$ elevation in *A. thaliana* (Hu *et al.*, 2004; Galletti *et*

al., 2008). Romani *et al.* (2004) have demonstrated the role of OGs-mediated Ca^{2+} influx and $[Ca^{2+}]_{cyt}$ in the production of ROS in *Arabidopsis,* and further elucidated the role of Ca^{2+} signaling in defense responses. Flg22, OGs and elf18 are able to induce characteristic Ca^{2+} influx signatures in *Arabidopsis* plants (Aslam *et al.*, 2009). Combination of different elicitors showed additive, synergistic and interference effects under certain conditions. Moreover, these elicitor-induced Ca^{2+} changes were involved in the induction of different defense-related genes (Aslam *et al.*, 2009).

4.3. Ca^{2+} homeostasis

Calcium's role as a second messenger and an essential nutrient has been firmly established in plants. In the soil, Ca^{2+} is taken up by plant roots and is transported to the shoot areas via the xylem vessels, either through the spaces between cells (the apoplast) or through the cytoplasm of cells linked by plasmodesmata (the symplast; White, 2001). Cellular Ca^{2+} levels are regulated within very strictly defined limits and this homeostasis is very important for normal cell life. In order to maintain a fine balance of Ca^{2+}, the rate of Ca^{2+}delivery to the xylem must be appropriately controlled and a system to prevent the accumulation of toxic cations in the shoot must be present to keep the Ca^{2+} at a constant level. Under normal conditions, plants have shoot Ca^{2+}concentrations between 0.1 and 5 % of their dry weight (Marschner, 2011). Ca^{2+} deficiency leads to several disorders in plants like: poor root development, blossom end rot, leaf necrosis and curling, poor fruit storage *etc*. A relatively brief Ca^{2+} starvation leads to the death of apical meristem cells and cessation of growth (White and Broadley, 2003). In contrast, excessive Ca^{2+} concentrations are cytotoxic for plants and could lead to the reduced germination of seeds and plant growth rates. In plants, the total Ca^{2+} is of the mM order in comparison to their cytosolic requirements that are in submicromolar range under normal conditions (Hetherington and Brownlee, 2004; Hepler, 2005). A rapid increase in $[Ca^{2+}]_{cyt}$ has been observed in response to developmental cues or environmental challenges (McAinsh and Pittman, 2009; Dodd *et al.*, 2010). However, this increased $[Ca^{2+}]_{cyt}$ is regulated to normal levels by Ca^{2+}-ATPases and H^{+}/Ca^{2+}-antiporters (Sze *et al.*, 2000; Hirschi, 2001). These enzymes transport Ca^{2+} to either the apoplast or the subcellular organelles. Under resting conditions, the $[Ca^{2+}]_{cyt}$ is maintained between 100-200 nM (Bush, 1995), 10^4 times less than that in the apoplastic fluid (where it is in mM concentrations range) and 10^4 to 10^5 less than that in cellular organelles, providing the potential for the ready import of Ca^{2+} into the cytosol. Plant vacuole, endoplasmic reticulum

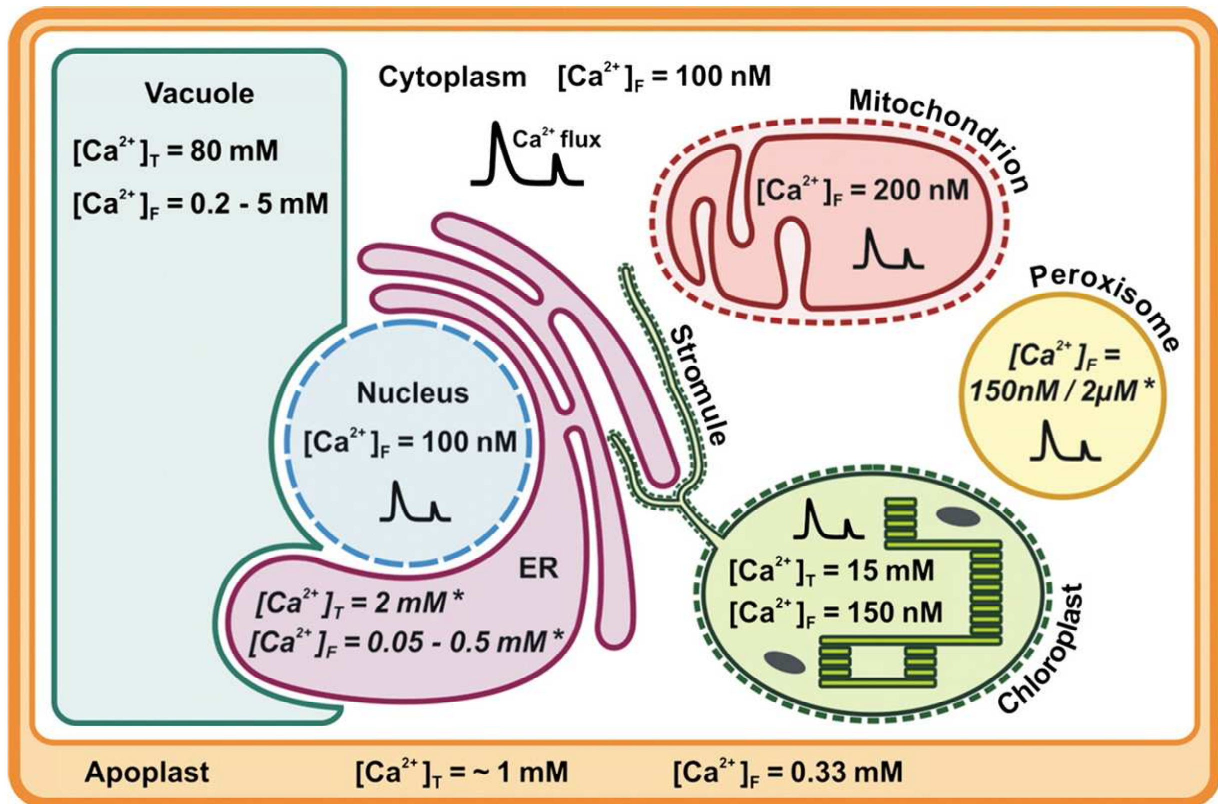


Figure 1.12: Ca^{2+} concentrations in the plant cell organelles. Values for reported total ($[Ca^{2+}]_T$) and free resting ($[Ca^{2+}]_F$) Ca^{2+} concentrations in organelles (apoplast, cytoplasm, vacuole, nucleus, ER, chloroplast, mitochondrion, and peroxisome). The values are approximate values and probably vary depending on the tissue or plant species, but nevertheless they provide a general impression of Ca^{2+} levels across the cell. For ER and peroxisomes, no data on Ca^{2+} concentration in plants are available. (*): means that these values come from animal system. Double peak-shaped symbol: $[Ca^{2+}]$ fluxes (adapted from Stael *et al.,* 2012).

(ER), mitochondria and chloroplasts are the main internal Ca^{2+} stores in plant cells. These organelles are able to exchange Ca^{2+} with the cytosol to maintain a balance not only in the $[Ca^{2+}]_{cyt}$ but also within the organelles. Plant vacuole which covers almost 90 % of the total cell volume contains 1-10 mM free calcium and ER also has Ca^{2+} in mM range. The overall Ca^{2+} contents have been estimated in mM range for mitochondria (200-300 nM in the matrix) and between 4-23 mM for the chloroplast (200-300 nM in the stroma; Portis and Heldt, 1976; Sai and Johnson, 2002; Logan and Knight, 2003). This diverse distribution of Ca^{2+} in different subcellular compartments clearly indicates their role in maintaining the Ca^{2+} homeostasis inside the cell. Figure 1.12 represents the Ca^{2+} variation in cytosol and different subcellular compartments (adapted from Stael *et al.*, 2012).

4.3.1. Ca^{2+} signal modulation by the organelles

Among different cellular organelles, the concentration of free calcium varies considerably. Plant nucleus, mitochondria and chloroplasts also have considerable amount of Ca^{2+} and can also function as stores for Ca^{2+} release (McAinsh and Pittman, 2009). Interestingly, various previous studies have shown that these subcellular organelles respond to various stimuli by changes in their free calcium concentrations (Johnson *et al.*, 1995; Logan and Knight, 2003; Lecourieux *et al.*, 2005) but the physiological importance of this Ca^{2+} concentration has not been very extensively studied to date.

During elicitor-mediated plant defense signaling, involvement of Ca^{2+} from the internal stores has also been suggested. For example, pretreatments of parsley cells with neomycin (a phospholipase C antagonist that inhibits IP_3-mediated Ca^{2+} release) before Pep-13 challenge were followed by a significant reduction in the first transient $[Ca^{2+}]_{cyt}$ elevation but this pretreatment with neomycin did not affect the sustained $[Ca^{2+}]_{cyt}$ increase (Blume *et al.*, 2000). In another study, neomycin preincubations of tobacco cells before Cry elicitation yielded similar results (Lecourieux *et al.*, 2002). These data suggest the contribution of IP_3-dependent internal Ca^{2+} release to the transient $[Ca^{2+}]_{cyt}$ peak. On the other hand, in soybean and tobacco cells treated with β-glucans and OGs, respectively, neomycin resulted in a very strong inhibition of the second transient $[Ca^{2+}]_{cyt}$ peak without affecting the first one (Mithöfer *et al.*, 1999; Lecourieux *et al.*, 2002). Vandelle *et al.* (2006) have demonstrated the possibility of involvement of internal Ca^{2+} stores during the first transient increase in $[Ca^{2+}]_{cyt}$ in BcPG1-treated grapevine cells. Treatment with different inhibitors impacting the activity of Ca^{2+} permeable channels *e.g.* neomycine, U73122 (a specific phospholipase inhibitor), and ruthenium red (which blocks the intracellular cADPR-dependent Ca^{2+}

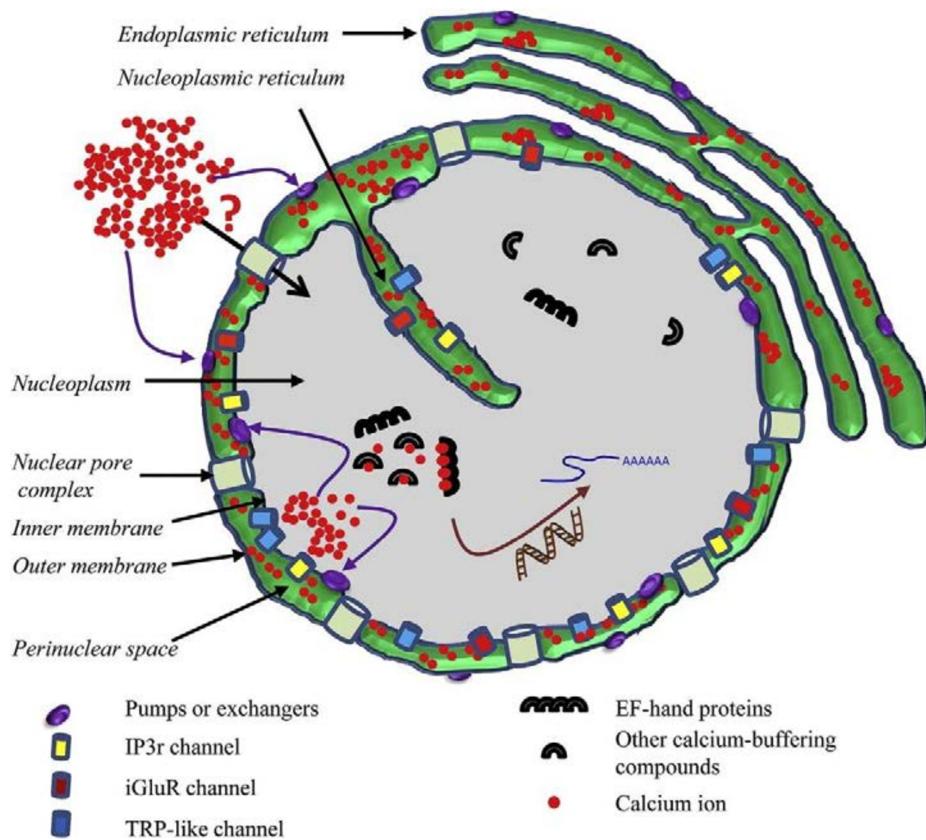


Figure 1.13: Calcium homeostasis in the nucleus of tobacco cells. Briefly, upon a stimulus, calcium ions coming from the perinuclear space enter into the nucleoplasm through different types of calcium channels. Calcium ions penetrate into the nuclear envelope through different calcium transporters *e.g.* ATPases or exchangers. Calcium ions also interact with calcium binding proteins to activate downstream nuclear events such as enzyme activation or transcriptional regulation processes (Mazars *et al.*, 2011).

permeable channels ryanodine-receptor like (RYR-like) limited the first transient increase in $[Ca^{2+}]_{cyt}$ to a sharp peak whereas second sustained peak was not affected. This suggested that the first BcPG1-induced $[Ca^{2+}]_{cyt}$ peak is the combined effect of an influx of Ca^{2+} from the extracellular medium plus the Ca^{2+} subsequently mobilized from the internal stores via the activation of IP_3 dependent, RYR-type of Ca^{2+} permeable channels, or both located in the membrane of internal stores, including endoplasmic reticulum and vacuoles (Vandelle *et al.*, 2006). Same conclusions were drawn using tobacco cell suspensions treated by Cry (Lecourieux *et al.*, 2002; Lamotte *et al.*, 2004). These findings clearly suggest the involvement of different Ca^{2+} stores in the elicitor-induced $[Ca^{2+}]_{cyt}$ elevations.

4.3.1.1. Ca^{2+} signaling in the nucleus

Plant nucleus is the place of transcriptional regulation of thousand of genes important in plant growth and developments. Ca^{2+} signals in the nucleus enable the cell to respond to environmental changes by alteration of gene expression in animals and plants (Ikura *et al.*, 2002; Kim *et al.*, 2009; Mazars *et al.*, 2009; Galon *et al.*, 2010; Reddy *et al.*, 2011). Studies have revealed that nuclear-induced Ca^{2+} signature is independent of $[Ca^{2+}]_{cyt}$ thus suggesting independent regulation mechanisms for $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ variations (McAinsh and Pittman, 2009; Mazars *et al.*, 2010). However, the mechanisms and the channels involved in signal-induced changes in $[Ca^{2+}]_{nuc}$ have not been identified. Recently, Mazars *et al.* (2011) have proposed a model for Ca^{2+} homeostasis in the nucleus of tobacco cells. This model is based on the data available from the literature and demonstrate the existence of different types of channels and transporters that are involved in the regulation of $[Ca^{2+}]_{nuc}$ (Figure 1.13).

A variety of (a)biotic stimuli and symbiotic signals are able to generate nuclear Ca^{2+} fluxes (Pauly *et al.*, 2000; Lecourieux *et al.*, 2005; Oldroyd and Downie, 2006; Sieberer *et al.*, 2009). Although, stimulus-induced Ca^{2+} variations have been reported in plant nuclei yet not too much work has been carried out on this interesting subject (Van Der Luit *et al.*, 1999; Mazars *et al.*, 2010). Pauly *et al.* (2000) have demonstrated in their work that when tobacco protoplasts was treated with mastoparan (a toxin peptide from wasp venom), nuclei from plant cells were capable of generating their own calcium signals independently of changes in calcium ion concentration in the cytosol. In response to several biotic and abiotic stimuli, different signatures of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ were observed in tobacco cells *e.g.* a hypo-osmotic shock resulted in a bimodal and monophasic response for $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ elevations, respectively (Mithofer and Mazars, 2002). In another study, Lecourieux *et al.* (2005) have shown the involvement of $[Ca^{2+}]_{nuc}$ variations in plant defense responses. Upon

treatment with different elicitors, pronounced and sustained $[Ca^{2+}]_{nuc}$ elevations were observed in tobacco cells. However, elicitin induced-$[Ca^{2+}]_{nuc}$ variation was more pronounced than that of OGs or laminarin. Moreover, elicitin-induced $[Ca^{2+}]_{nuc}$ elevations were found to be dependent on Ca^{2+},influx, IP_3-regulated Ca^{2+} channels, and active oxygen species (AOS) but independent of NO production (Lecourieux *et al.*, 2005). Furthermore, treatment of tobacco cells with a jasmonate derivative, jasmonate-isoleucine, led to generate nuclear Ca^{2+} fluxes without any measurable cytosolic Ca^{2+} responses (Walter *et al.*, 2007). In addition, studies with isolated nuclei have also demonstrated the autonomous regulation of nuclear activities in tobacco cells (Xiong *et al.*, 2004, 2008). These data suggest the independent regulation of nuclear Ca^{2+} that may involve P-ATPases and nucleotide gated channels located at the inner membrane of the nucleus (Mazars *et al.*, 2009). Nuclear Ca^{2+} has also been found to be involved in the controls the apoptotic-like cell death (Lachaud *et al.*, 2010). When tobacco cells were challenged with D-erythro-sphinganine (DHS), an apoptotic-like cells death was observed. Treatments with DL-2-amino-5-phosphopentanoic acid (AP5) and (+)-dizocilpine (MK-801), two inhibitors of animals and plants ionotropic glutamate receptors, suppress DHS-induced cell death symptoms by selectively inhibiting the variations in $[Ca^{2+}]_{nuc}$ (Lachaud *et al.*, 2010). DHS also activated the expression of defense-related genes but this effect was independent of $[Ca^{2+}]_{nuc}$ (Lachaud *et al.*, 2010). Castor and Pollux represent two nuclear ion channels permeable to K^+. Originally, they were considered as chloroplastic-localized channels (Imaizumi-Anraku *et al.*, 2005) but a recent study have shown that both Castor and Pollux were located in the nuclear envelope (Charpentier *et al.*, 2008). In Loss-of-function *Castor* and *Polux* mutants, perinuclear Ca^{2+} spiking was greatly affected and was followed by a failure of mutant plants to establish a symbiotic relationship with AM fungi and rhizobial bacteria in leguminous and non-leguminous crop species. It was suggested that Castor and Pollux modulated nuclear envelope membrane potential, triggering the opening of Ca^{2+} channels or compensating the charge release during Ca^{2+} efflux (Charpentier *et al.*, 2008; Chen *et al.*, 2009).

4.3.1.2. Ca^{2+} signaling in the mitochondria

Mitochondria is an important Ca^{2+} storing compartment in both animals and plants and is able to accumulate high level of Ca^{2+} (Putney and Thomas, 2006). In the past, extensive studies have been made in animals to explore the mechanism involved in the transport of Ca^{2+} in the mitochondria and the underlying mechanisms of free matrix $[Ca^{2+}]$ ($[Ca^{2+}]_{mit}$) signaling. Studies have demonstrated a very well defined role of mitochondria in animals where they

work as transient Ca^{2+} stores in regions of close interacting with the ER or the PM in which high [Ca^{2+}] can be formed (termed Ca^{2+} microdomains), thereby modulating Ca^{2+} signatures (Clapham, 2007; Laude and Simpson, 2009). In animals, studies have shown that $[Ca^{2+}]_{mito}$ plays an important role in modulating $[Ca^{2+}]_{cyt}$ and in the regulation of apoptotic like cell death (Giacomello *et al.*, 2007). Higher $[Ca^{2+}]_{mit}$ leads to the induction of apoptosis by opening of the mitochondrial permeability transition pore (mPTP) and the subsequent release of mitochondrial apoptosis markers, such as cytochrome *c* (Giacomello *et al.*, 2007; Szabadkai and Duchen, 2008). In contrast, not too much data are available about mitochondria Ca^{2+} signaling in plants. The resting $[Ca^{2+}]_{mit}$ in plants has been estimated to be ~200 nM (Logan and Knight, 2003) and most of this Ca^{2+} is probably bound in the form of a ready-releasable amorphous phosphate precipitate (Chalmers and Nicholls, 2003; Starkov, 2010). Previous work of Logan and Knight (2003) showed that *Arabidopsis* cells respond to different stimuli (cold, osmotic, mechanical and oxidative stress) by an elevation of both $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ in the matrix. They also suggested an independent regulation pathway for $[Ca^{2+}]_{mit}$ based on their distinct nature of the signals from $[Ca^{2+}]_{cyt}$. It is not clear whether mitochondria are a store that releases Ca^{2+} to the cytosol or if mitochondria contribute to the pumping of cytosolic Ca^{2+}. $[Ca^{2+}]_{cyt}$ regulation by mitochondria is possible as studies have shown that $[Ca^{2+}]_{mit}$ contribute to the increase in the $[Ca^{2+}]_{cyt}$ in maize suspension-cultured cells under anoxia (Subbaiah *et al.*, 1998).

Apoptotic-like cell death has also been reported in plants through a mechanism that resemble to animals (Arpagaus *et al.*, 2002; Tiwari *et al.*, 2002; Virolainen *et al.*, 2002). In plants, the initiation of cell death leads to the loss of mitochondrial transmembrane potential and the release of cytochrome *c* (Cyt c) from mitochondria into the cytoplasm, which results in cell death (Yao *et al.*, 2004). Scott and Logan (2008) have demonstrated the importance of $[Ca^{2+}]_{mit}$ in the process of programmed cell death (PCD). They reported that a mild heat shock, or treatment with strong oxidants to the leaves or protoplasts of *Arabidopsis thaliana,* induced a very rapid transition in mitochondrial morphology, which preceded subsequent cell death. Disruption of cellular calcium flux with La^{3+} abolished these events, showing the importance of Ca^{2+} efflux in PCD process. When mitochondria isolated from potato are incubated in the presence of Ca^{2+} and inorganic phosphate (Pi), it follows swelling of mitochondria and a release of Cytc (Arpagaus *et al.*, 2002). Treatment with high concentrations of Ca^{2+} (0.5-2.5 mM) caused swelling of mitochondria isolated from wheat roots (Virolainen *et al.*, 2002). In the same study, different Ca^{2+} treatments under anoxic conditions resulted in the swelling of mitochondria and the release of Cyt c.

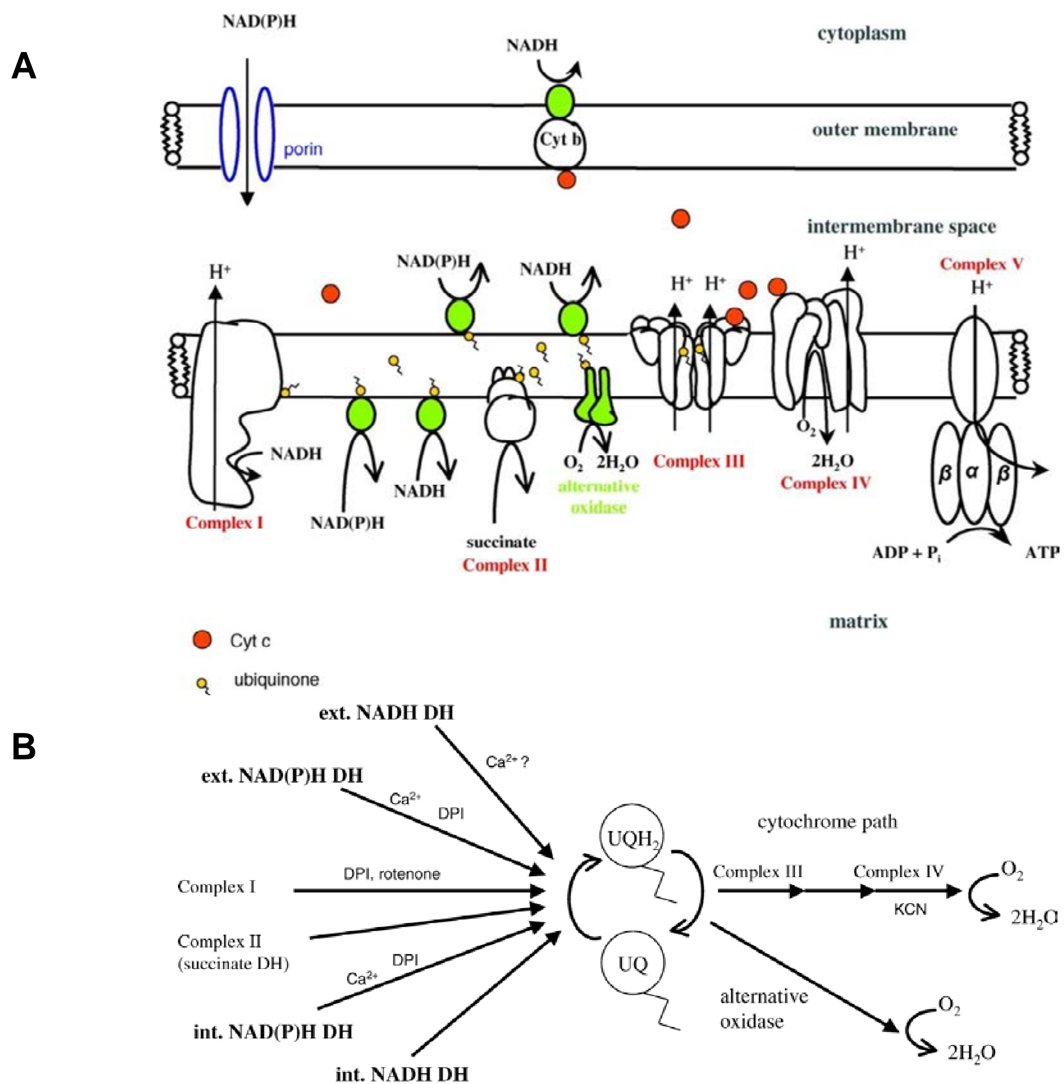


Figure 1.14: **Alternative oxidase in plants mitochondrial electron transport chain. A)** Membrane model of the plant mitochondrial electron transport chain. Alternative NAD(P)H dehydrogenases and the alternative oxidase are shown in green. **B)** Schematic view of the plant mitochondrial electron transport chain. Multiple dehydrogenases reduce a common pool of ubiquinone, which is then oxidized by either the traditional cytochrome pathway or the alternative oxidase (Rasmusson *et al.*, 2004).

Another important aspect of mitochondria is their association with energy transduction processes in animals and plants. Mitochondria are able to sense Ca^{2+} changes and activate tricarboxylic acid (TCA) cycle dehydrogenases leading to energy production (Denton and McCormack, 1980; Rasmusson *et al.*, 2004). In addition to electron transport chain (ETC), plant also possess mitochondrial alternative NAD(P)H dehydrogenases and an alternative oxidase (AOX) that took part in O_2 consumption (Figure 1.14; Rasmusson *et al.*, 2004). Moreover, activity of both NAD(P)H dehydrogenases and AOX is dependent on Ca^{2+} elevation (Vanlerberghe *et al*., 2002; Rasmusson *et al.*, 2004). AOX provides an alternative route for electrons passing through ETC to reduce oxygen. During alternative pathway, as several proton-pumping steps are bypassed, activation of the AOX results in reduced ATP synthesis. AOX has been reported to take part in SA-induced cell death processes (Robson and Vanlerberghe, 2002; Noctor *et al.*, 2007). Harpin, an inducer of cell death in tobacco has been demonstrated to increased AOX activity and subsequent cell death in tobacco cells (Xie and Chen, 2000). Moreover, treatments of tobacco cells with same elicitor leads to $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ variations (Lecourieux *et al.*, 2005), thus suggesting a link between Ca^{2+} signaling, elicitor induced cell death and AOX activity. Although not demonstrated, Ca^{2+} changes in the mitochondria, that might take place after the increase in $[Ca^{2+}]_{cyt,}$ may also be involved in this cell death process.

All these above mentioned studies clearly suggest the role and the importance of mitochondria not only in the regulation of cellular Ca^{2+} levels but also contributing to important physiological processes such as cell death. However, clear information about Ca^{2+} transporters of plant mitochondria are still missing. In *Arabidopsis*, 6 genes have been identified that encode proteins showing homology with human mitochondrial Ca^{2+} uptake (MCU) protein and share the pore-forming domain with two transmembrane helices connected by a conserved DVME motif (Stael *et al.*, 2012). MCU imports Ca^{2+} from microdomains with highly elevated $[Ca^{2+}]_{cyt}$ (Baughman *et al*., 2011; De Stefani *et al*., 2011). It has been reported that different *Arabidopsis* MCU isoforms have possible localization in mitochondria, except for At5g66650, which is also predicted to co-localize in chloroplast (Schwacke *et al*., 2003).

4.3.1.3. Ca^{2+} signaling in the chloroplasts

Chloroplasts are the place of photosynthetic activities in plant and other photosynthetic organisms. However, they are also implicated in various metabolic and regulation pathways that are important for plant survival. Although chloroplasts have been reported to contain high

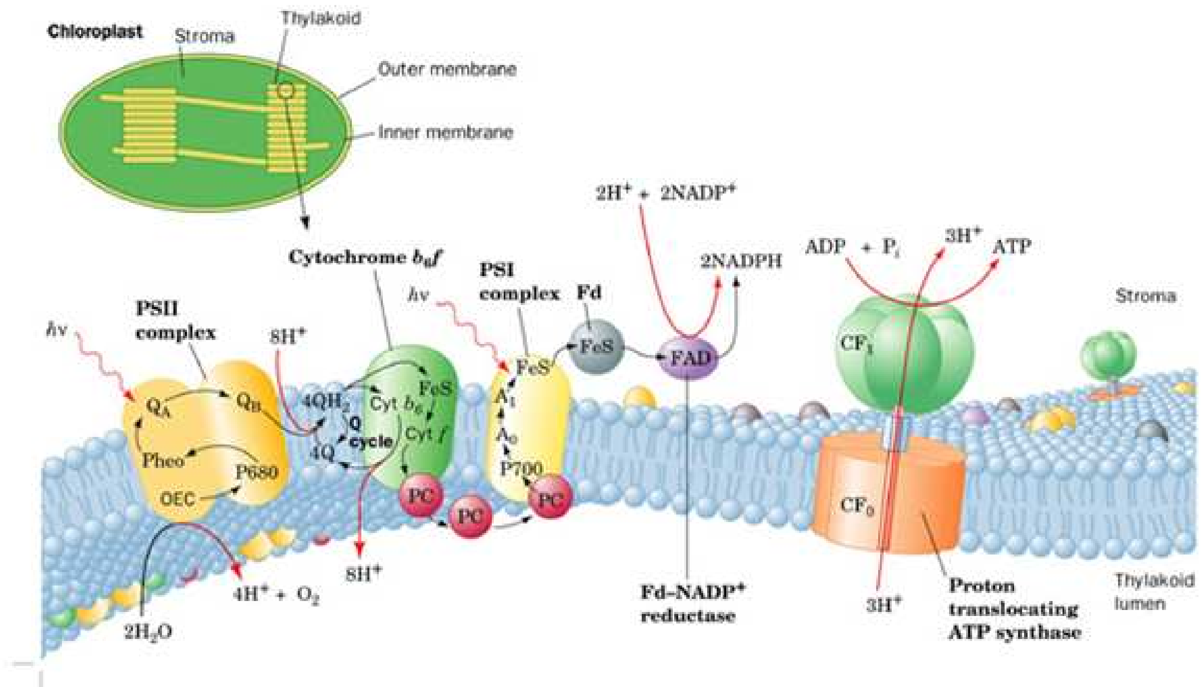


Figure 1.15: **Chloroplast and structure of a thylakoid membrane and its components important for the photochemical electron transport pathway**. OEC: oxygen evolving complex; Pheo: pheophytine; Q_A: quinone A; Q_B: quinone B; QH_2: hydroxyplastoquinone; FeS: Rieske apparatus; Fd: ferredoxin; PC: plastocyanine; CF_0; CF_1: coupling factors of ATP synthase (Roháček *et al.*, 2008).

concentrations of Ca^{2+}, their potential role in cellular Ca^{2+} homeostasis and signaling has remained largely unexplored (Johnson *et al.*, 2006). The average Ca^{2+} concentration in the chloroplast varies between 4-23 mM (Portis and Heldt, 1976). During day light, an external Ca^{2+} uptake leads to a significant change in total chloroplastic Ca^{2+} concentrations (Kreimer *et al.*, 1985; Roh *et al.*, 1998). At the same time, higher chloroplastic Ca^{2+} concentrations are harmful for cell as they could forms insoluble precipitates with chloroplastic phosphate and could hinder photosynthetic activities. Therefore, it is believed that most of the chloroplastic Ca^{2+} is bound either to stromal proteins or in thylakoid lumen (Gross and Hess, 1974; Davis and Gross, 1975; Brand and Becker, 1984; Kreimer *et al.*, 1987). This also highlights that chloroplasts might also play a role in the homeostasis of other ions besides Ca^{2+} (Portis and Heldt, 1976). Johnson *et al*. (1995) have demonstrated that the level the resting stromal Ca^{2+} could be 200-300 nM.

In the past, different studied were conducted to investigate the role of $[Ca^{2+}]_{chlo}$ variation in the chloroplasts. One study examining Ca^{2+} movement into intact wheat chloroplasts (Muto *et al.*, 1982) indicated that the Ca^{2+} uptake occurs via an H^{+}/Ca^{2+}-antiport mechanism, and that the K_m was only slightly higher than $[Ca^{2+}]_{cyt}$. The light induces a Ca^{2+} influx across the envelope of intact chloroplasts isolated from spinach that is mediated by a uniport-type carrier linked to photosynthetic electron transport but this do not significantly change Ca^{2+} concentrations in the stroma (Kreimer *et al.*, 1985). A huge $[Ca^{2+}]_{chlo}$ variation in the stroma was observed with some circadian chloroplast Ca^{2+} oscillation (Johnson *et al.*, 1995). Similarly dark-induced increases in stroma Ca^{2+} concentrations precede the generation of elevations of $[Ca^{2+}]_{cyt}$ in tobacco (*Nicotiana plumbaginifolia*) leaves (Sai and Johnson, 2002). Light stimulates uptake of Ca^{2+} into the chloroplast but the Ca^{2+} concentrations in the stroma do not change significantly during illumination (Kreimer *et al.*, 1988; Sai and Johnson, 2002). Moreover, Ca^{2+} is also required for the proper functioning of photosystem II located in thylakoid membrane (Figure 1.15; Homann, 2002; Loll *et al.*, 2005). With regards to its role in photosynthesis, Ca^{2+} is an essential component of the oxygen-evolving complex (OEC) being part of the catalytic centre with n=1 to 3 depending on the redox state and number of polypeptids (Johnson *et al.*, 2006). Ca^{2+} after binding to ATP synthase subunit, also regulates the ATP synthesis and photosynthetic proton flow (Zakharov *et al.*, 1993; Ifuku *et al.*, 2010). These results show although Ca^{2+} is needed to carry on chloroplastic activities yet its concentration should not exceed beyond the optimum limits.

Interestingly, chloroplasts could also serve the role of an alternative source of Ca^{2+} release uptake for $[Ca^{2+}]_{cyt}$ signaling. The overexpression of a pea chloroplast protein, PPF1, a

putative Ca^{2+} channel, in *Arabidopsis* guard cells significantly reduced $[Ca^{2+}]_{cyt}$ transients, as Ca^{2+} was retained in the chloroplast (Wang *et al.*, 2003; Li *et al.*, 2004). Another important aspect of the chloroplasts Ca^{2+} signaling is the presence of Ca^{2+}-sensing receptor (CAS) protein exhibiting low-affinity/high-capacity Ca^{2+} binding through an N-terminal domain of the protein. CAS was originally thought to be plasma membrane-localized protein that regulates stomatal closure in responses to elevation of extracellular Ca^{2+} concentrations (Han *et al.*, 2003) but recent studies have clearly demonstrated that it is present specifically at the thylakoid membrane (Peltier *et al.*, 2004; Nomura *et al.*, 2008; Vainonen *et al.*, 2008; Weinl *et al.*, 2008). Various studies have reported the involvement of CAS in external Ca^{2+} ($[Ca^{2+}]_{ext}$)-induced stomatal closure in *Arabidopsis thaliana*. Through reverse genetic approach, Weinl *et al.* (2008) demonstrated that different *cas* mutant lines were impaired in stomatal closure in response to $[Ca^{2+}]_{ext.}$ However, ABA treatments did not reveal any differences between wild type and *cas* mutant plants thus suggesting the specificity of CAS in the regulation of $[Ca^{2+}]_{ext}$-induced stomatal closure. In continuity to these results, Nomura *et al.* (2008) also demonstrated by using knockout mutants, cDNA mutant-complemented plants and CAS overexpressor plants, that CAS is essential in stomatal closure induced by $[Ca^{2+}]_{ext}$. Moreover, $[Ca^{2+}]_{ext}$-induced $[Ca^{2+}]_{cyt}$ transients were significantly compromised in CAS knockout mutants. Again, these data suggested that CAS regulates $[Ca^{2+}]_{ext}$-induced $[Ca^{2+}]_{cyt}$ transients and stomatal closure. In a recent study, Wang *et al.* (2012) provided evidences for the role of CAS in the regulation of stomatal closure through NO and H_2O_2 accumulations in the guard cells. NO and ROS result in Ca^{2+} transients that lead to stomatal closure. From these data, we could assume that chloroplasts are important Ca^{2+} storing compartments and play a vital role in the generation of stimulus-mediated $[Ca^{2+}]_{cyt}$ signals.

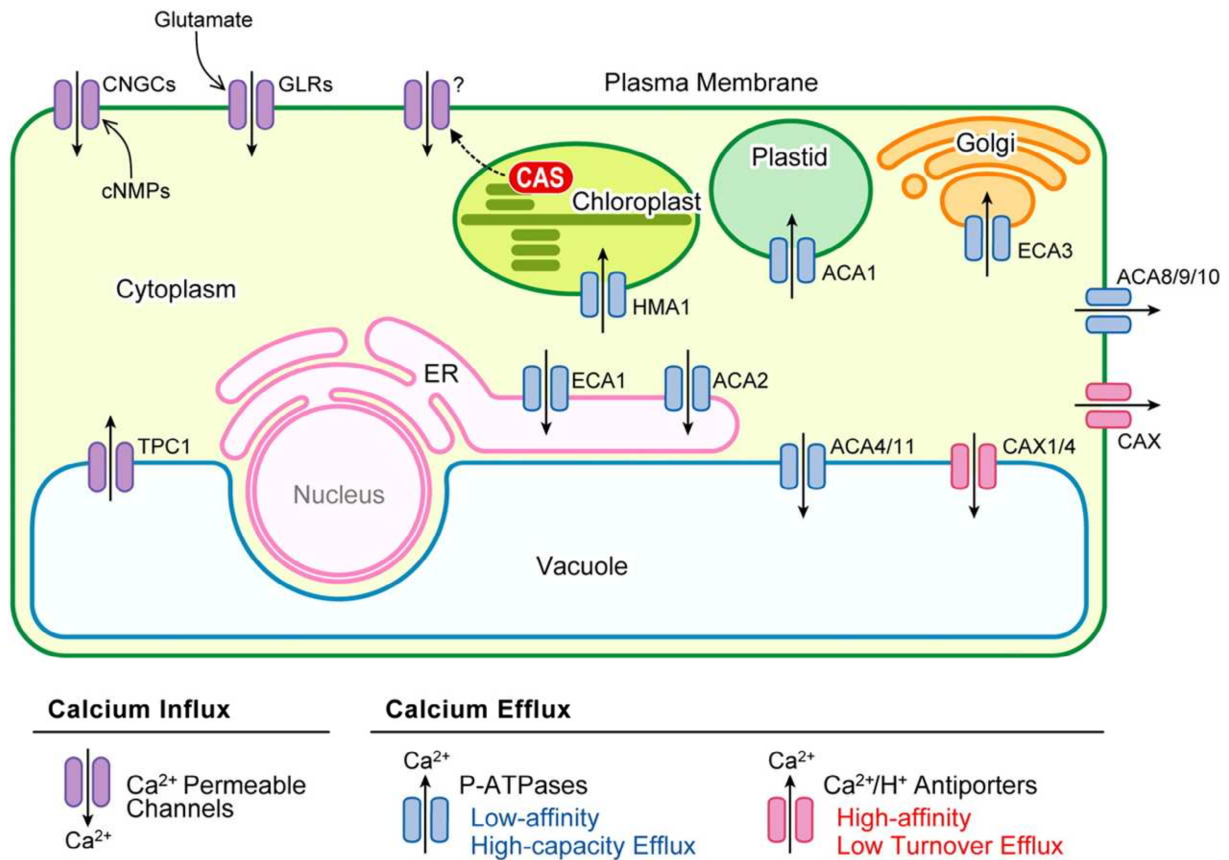


Figure 1.16: *Arabidopsis* Ca^{2+} transport systems. Ca^{2+} influx/efflux pathways have been demonstrated at molecular level. Complete description of different influx and efflux channels and transporters is present in ''Ca^{2+} transport system" section. CNGC, cyclic nucleotide channel; GLR, glutamate receptor; TPC1: two-pore channel 1; CAS: Ca^{2+}-sensing receptor; ACA: autoinhibited calcium ATPase; ECA: ER type calcium ATPase; HMA1: heavy metal ATPase1; CAX: cation exchanger (adapted from Kudla *et al.,* 2010).

5. Ca^{2+} transport systems

In order to achieve Ca^{2+} homeostasis and to respond to stimulus-specific Ca^{2+} variations, plants have developed a complex transport system that is composed of a large variety of different types of channels, pumps and exchangers present on the plasma membrane and endomembranes. These proteins finely regulate the intracellular and intercellular influx and efflux activities of the plant cells. They have been identified through biochemical, electrophysiological and molecular studies and have been excellently reviewed by different research groups around the globe (Sanders *et al.*, 2002; White and Broadley, 2003; Hetherington and Brownlee, 2004; Shigaki and Hirschi, 2006; Demidchik and Maathuis, 2007; McAinsh and Pittman, 2009; Ward *et al.*, 2009; Boudsocq and Sheen, 2010; DeFalco *et al.*, 2010; Dietrich *et al.*, 2010; Dodd *et al.*, 2010; Kudla *et al.*, 2010; Barbier-Brygoo *et al.*, 2011; Jammes *et al.*, 2011). An overview of these channels, pumps and transporters is presented (Figure 1.16 and Table 1.5)

5.1. Ca^{2+} channels

Among the different pathways to regulate the $[Ca^{2+}]_{cyt}$, Ca^{2+} channels in the plasma membrane and endomembranes which mediate Ca^{2+} release into the cytosol have been extensively studied in plants (for review: McAinsh and Pittman, 2009; Boudsocq and Sheen, 2010; Dodd *et al.*, 2010; Kudla *et al.*, 2010). Based on their activation mechanisms, Ca^{2+} channels can be divided into following categories: voltage-dependent channels, ligand-dependent channels and stretch-activated channels (Sanders *et al.*, 2002; White *et al.*, 2002; White and Broadley, 2003; Dutta and Robinson, 2004; Nakagawa *et al.*, 2007; McAinsh and Pittman, 2009; Kudla *et al.*, 2010). The presence of various types of channels clearly reflects the existence of different pathways and in response to different stimuli these channels could generate specific Ca^{2+}signals. Moreover, due to large variability in cell types and their requirements, a wide range of channel types definitely helps cells to meet up their needs.

5.1.1. Voltage-dependent Ca^{2+}-permeable channels in the plasma membrane

In plants, several types of voltage-dependent Ca^{2+}-permeable channels are present on different cellular membranes and based on their voltage dependence, these channels can be categorized into two main classes: depolarization-activated Ca^{2+} channels (DACCs) and hyperpolarization-activated Ca^{2+} channels (HACCs; White, 2000; Miedema *et al.*, 2001; Sanders *et al.*, 2002). Although DACCs and HACCs channels have been very well

Table 1.5: Ca^{2+} transport pathways and Ca^{2+} fluxes in plant cell. ABA: abscisic acid; ACA: auto-inhibited Ca^{2+}-ATPase activated by calmodulin (CaM); $[Ca^{2+}]_{cyt}$: cytosolic Ca^{2+}; $[Ca^{2+}]_{vac}$: vacuolar lumen Ca^{2+}; CAX: H^+/Ca^{2+} exchanger; CNGC: cyclic nucleotide-gated channel; DACC: depolarization-activated Ca^{2+} channel; ΔpH: pH gradient; ECA: ER-type Ca^{2+}-ATPase; GLR: glutamate receptor-like channel; HACC: hyperpolarization-activated Ca^{2+}channel; $InsP_3R$-like: inositol 1,4,5-trisphosphate receptor-like channel; MCC: mechano-sensitive Ca^{2+} channel; NAADPR: nicotinic acid adenine dinucleotide phosphate receptor-like channel; NSCC: nonselective cation channel; ROS, reactive oxygen species; RyR-like: cyclic ADP-ribose (cADPR)-activated ryanodine receptor-like channel; SV channel: slow-activating vacuolar Ca^{2+} channel; VVCa channel: vacuolar voltage-gated Ca^{2+} channel. ? (Question mark) indicates that the evidence is not clear-cut or is inferred from the animal literature (McAinsh and Pittman, 2009).

Membrane Ca^{2+} flux	Direction of Ca^{2+} flux	Transporter type	Main physiological regulators
Plasma membrane	Into the cytosol	CNGC	cAMP, cGMP, CaM
		DACC	Voltage
		GLR	Amino acids
		HACC	Voltage, ROS, $[Ca^{2+}]_{cyt}$, ABA
		MCC	Mechanosensitive
	Out of the cell	ACA	$[Ca^{2+}]_{cyt}$, CaM
		ECA?	$[Ca^{2+}]_{cyt}$?
Tonoplast	Into the cytosol	$InsP_3R$-like	$InsP_3$
		RyR-like	cADPR
		SV channel (AtTPC1)	Voltage, $[Ca^{2+}]_{cyt}$, pH, CaM
		VVCa channel	Voltage, $[Ca^{2+}]_{cyt}$, $[Ca^{2+}]_{vac}$
	Into the vacuole	ACA	CaM
		CAX	ΔpH, $[Ca^{2+}]_{cyt}$
Endoplasmic reticulum (ER)	Into the cytosol	$InsP_3R$-like	$InsP_3$
		NAADPR-like	NAADP
		NSCC	Voltage
		RyR-like	cADPR
	Into the ER lumen	ACA	$[Ca^{2+}]_{cyt}$, CaM
		ECA	$[Ca^{2+}]_{cyt}$?
Mitochondria (inner membrane)	Into the cytosol	Ca^{2+} exchanger?	ΔpH?, Ca^{2+}?
	Into the matrix	Ca^{2+} uniporter?	Ca^{2+}?, voltage?
Chloroplast (inner envelope)	Into the cytosol	ACA?	$[Ca^{2+}]_{cyt}$, CaM
	Into the stroma	Ca^{2+} uniporter Ca^{2+}/H^+ exchanger	ΔpH, voltage
Golgi	Into the cytosol	Unknown	
	Into the Golgi	ECA	$[Ca^{2+}]_{cyt}$?
Nuclear envelope	Into the envelope	ECA?	$[Ca^{2+}]_{cyt}$?
	Into the nucleus	NSCC	Voltage

investigated through electrophysiology yet very few examples are described at molecular level (Thion *et al.*, 1998; Hamilton *et al.*, 2000; Pei *et al.*, 2000; Klüsener *et al.*, 2002; White *et al.*, 2002; White and Broadley, 2003; Demidchik and Maathuis, 2007). Both these types of channels are activated in response to different environmental stimuli. For example, DACCs activations have been reported during chilling and plant-microbe interactions (Thion *et al.*, 1998) and induce a short transient Ca^{2+} influx. Different types of DACCs have been identified in plants (White *et al.*, 2002). Although having unique electrophysiological and pharmacological properties, they are all permeable to both mono- and divalent cations including Ca^{2+}. On the other hand, HACCs are activated in response to Ca^{2+} nutrition, ABA and blue light and contribute to a sustained influx of Ca^{2+} (Hamilton *et al.*, 2000; Pei *et al.*, 2000; Miedema *et al.*, 2001; Miedema *et al.*, 2008; Harada and Shimazaki, 2009). Plant annexins, that are able to bind to plasma membrane and endomembranes in a Ca^{2+}-dependent or Ca^{2+}-independent manner, have been reported to activate HACCs (Demidchik and Maathuis, 2007; Mortimer *et al.*, 2008; Laohavisit *et al.*, 2009). Molecular characterization of voltage-dependent Ca^{2+}-permeable channels will really help to understand their contributions during Ca^{2+} signaling pathways.

5.1.2. Ligand-gated Ca^{2+}-permeable channels

In plants, glutamate receptors-like (GLRs) and cyclic nucleotide gated channels (CNGCs) are the two potential candidate gene families for plasma membrane Ca^{2+}-permeable channels and each channel family encompass 20 members (Lacombe *et al.*, 2001; Mäser *et al.*, 2001). In fact, these channels belong to non-selective cation channels (NSCCs) category and are also known as voltage-independent Ca^{2+}-permeable channels (McAinsh and Pittman, 2009; Kudla *et al.*, 2010) but have higher permeability for Ca^{2+}. In plants, molecular identification of both GLRs and CNGCs has been made and functional characterization of these channels is under investigations. Recently, two excellent reviews have been made by Dietrich *et al.* (2010) and Jammes *et al.* (2011) to demonstrate their functions in response to different environmental challenges and to provide evidences for their involvement in Ca^{2+}-dependent signaling.

5.1.2.1. Cyclic nucleotide–gated channels

CNGCs are ligand-gated plasma membrane cation permeable channels that are activated by cyclic nucleotides, cAMP and cGMP. The very first report for the identification of CNGCs came from barley (Schuurink *et al.*, 1998). Then, *Arabidopsis thaliana* genome

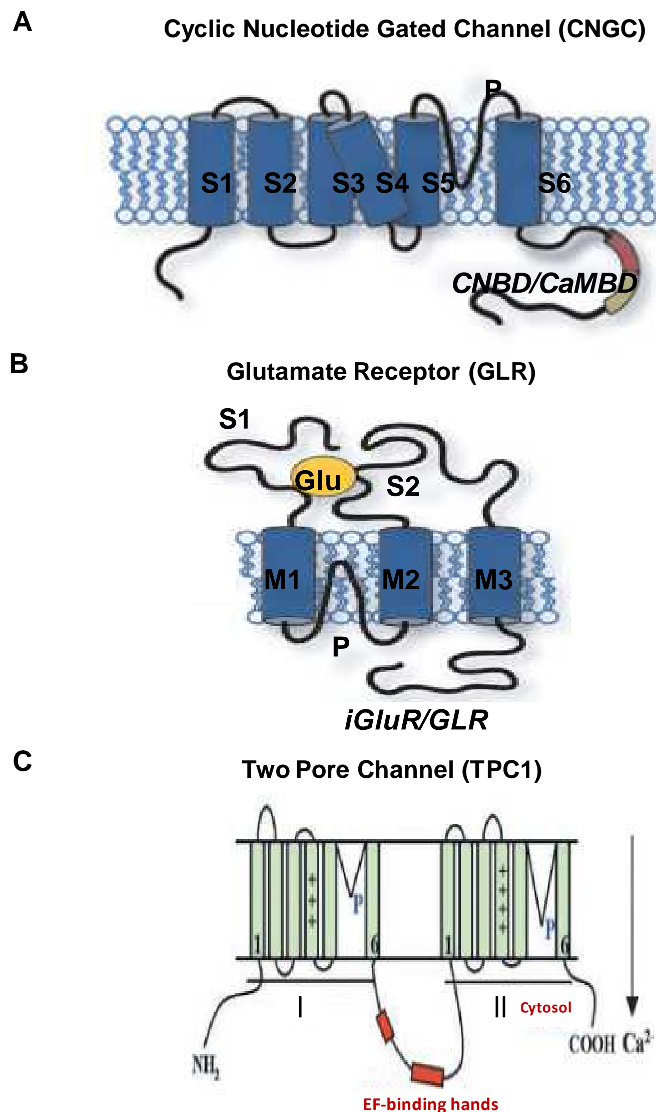


Figure 1.17: Topology models of putative plasma membrane proteins involved in calcium influx in the cytosol. A) CNGCs structure also contains a P loop and, unlike counterparts in animals, overlapping of the calmodulin and cyclic nucleotide binding domains at the C terminus of the protein. **B)** GLR structure is similar to that of animal ionotropic glutamate receptors and is composed of four membrane-localized domains among which M2 is predicted not to span the membrane, with a pore loop (P) interlocalized in the membrane. Two glutamate binding domains are localized on the outside of the membrane. **(C)** The two-pore channel (TPC1) is composed of two EF calcium binding hands, which could be involved in the feedback control of the channel activity via cytosolic calcium concentration. The pore loop (P) is localized between the 5th and 6th transmembrane domains of each repeat. The 4th transmembrane domain in each repeat is enriched in basic residues, which might suggest that the channel is voltage gated (adapted from Dietrich *et al.*, 2010; Sander *et al.*, 2002).

sequence completion revealed the presence of 20 CNGC genes (Mäser *et al.*, 2001) and based on their sequence homology, they were subdivided into four groups (Dietrich *et al.*, 2010). Plasma membrane localization has been attributed to different CNGCs studied so far (Arazi *et al.*, 2000; Gobert *et al.*, 2006; Ali *et al.*, 2007; Urquhart *et al.*, 2007). These channels contain six transmembrane domains (TMDs; S1–S6) and a pore loop between S5 and S6, and are supposed to form a tetrameric pore channel by assembly of four subunits (Figure 1.17A). Moreover, plants CNGCs specifically have a characteristic calmodulin-binding domain (CaMBD) that is present within the alpha C helix of the putative cyclic nucleotide-binding domain (CNBD) in the C-terminal part (Köhler *et al.*, 1999; Arazi *et al.*, 2000). Several studies have demonstrated the involvement of CNGCs in plant responses to pathogens (Balagué *et al.*, 2003; Yoshioka *et al.*, 2006; Ali *et al.*, 2007; Urquhart *et al.*, 2007; Rasul *et al.*, 2012). CNGC2 was first identified as Defense No Death1 (DND1) gene in *Arabidopsis* and loss of gene leads to enhanced resistance and plant failure to trigger Ca^{2+}-mediated HR when exposed to *Pseudomonas syringae* (Yu *et al.*, 1998; Clough *et al.*, 2000). Ali *et al.* (2007) demonstrated that *cngc2* mutants were deficient in LPS-induced NO production that is essential for the induction of HR. Moreover, Ca^{2+} influx was dependent on CNGC2 channel activity. Recently, Rasul *et al.* (2012) showed that *cngc2* mutant plants were deficient in NO production in response to OGs. Loss-of-function of CNGC4 plants also showed lack of HR and enhanced resistance during *Pseudomonas syringae* attack (Balagué *et al.*, 2003; Jurkowski *et al.*, 2004). In the same manner, mutation in the chimeric CNGC-encoding gene, CNGC11⁄12, constitutively activated defense responses in *Arabidopsis* and resulted in enhanced resistance to *Hyaloperonospora parasitica Emco5* (Yoshioka *et al.*, 2006). CNGC18 is localized preferentially to the tip region of pollen tubes and could be involved in the regulation of pollen tube growth by establishing a tip-focused Ca^{2+} gradient (Frietsch *et al.*, 2007). CNGC3 and CNGC10 are important in Na^+/K^+ homeostasis during salt stress conditions (Gobert *et al.*, 2006; Guo *et al.*, 2008).

5.1.2.2. Glutamate-receptor-like channels

GLRs are the second important class of ligand-gated plasma membrane Ca^{2+}-permeable channels. In animals, they work as non-selective cation channels at postsynaptic membranes and are activated by glutamate (Glu) and glycine (Gly; Dingledine *et al.*, 1999). In plants, GLRs homologues have also been identified in different plant species including radish, tobacco, poplar, rice, tomato and *Arabidopsis* (Ward *et al.*, 2009; Aouini *et al.*, 2012). The *Arabidopsis* genome encodes 20 members of AtGLRs which can be grouped into three

clades and contain all the signature domains of animal ionotropic GluRs (Figure 1.17B; Lacombe *et al.*, 2001; Chiu *et al.*, 2002; Davenport, 2002). Each GLR channel contains three TMDs (S1-S3), a pore loop and two putative ligand binding motifs. Tetrameric/pentameric configurations have been proposed for functional channel activity (Lam *et al.*, 1998; Dietrich *et al.*, 2010). The existence of Glu-activated ion channels in plants and their potential relationship to a gene family that is homologous to iGluRs indicates that the well known signaling properties of the amino acid Glu in animals may also extend to the plant kingdom (Forde and Lea, 2007). Further details on GLRs will be explained in the section “GLRs signaling in plants”.

5.1.3. Voltage-dependent channels at vacuolar membranes

Besides plama membrane, the activity of ligand-gated Ca^{2+}-permeable channels has also been reported in vacuolar membrane. Slow vacuolar type (SV) channel represents an example of this type of channels in plants (Johannes *et al.*, 1992; Allen and Sanders, 1994). Dual working behavior of SV channel in Ca^{2+} signaling has been shown in the past. For example, Hedrich and Neher, (1987) demonstrated that SV channels activity is controlled by changes in $[Ca^{2+}]_{cyt}$ but Allen and Sanders, (1994) proposed the more important contribution of SV in Ca^{2+} release. Recently, it has been shown that SV activity is regulated not only by the increase in the $[Ca^{2+}]_{cyt}$ but also through the Ca^{2+} concentrations inside the vacuolar cavity (Pottosin and Schönknecht, 2007; Beyhl *et al.*, 2009). Two-Pore Channel 1 (TPC1) is localized on the vacuolar membrane and represents the only member of TPC family in *Arabidopsis* that has been reported to have ligand-gated activity (Peiter *et al.*, 2005). TPC1 gene encodes a protein with 12 TMDs and two pore domains hence the protein in known as TPC. Moreover, two putative Ca^{2+}-binding EF-hands and a 14-3-3 binding domain form a cytosolic loop between TMDs 6 and 7. Positively charged residues are present on TMDs 4 and 10 that contribute to the voltage-gated property of TPC1 (Figure 1.17C; Peiter *et al.*, 2005). TPC1exhibited a ubiquitous expression pattern and genetic studies have showed the inability of the *tpc1* mutants to generate SV channel activities in *Arabidopsis* plants (Hedrich *et al.*, 1988). Moreover, electrophysiological investigations have clearly demonstrated the Ca^{2+} permeable nature of TPC1 that participates in Ca^{2+}-induced Ca^{+} release activity (Pottosin *et al.*, 2009). Activation of TPC1 has been reported to be stimulus-specific and *tpc1* mutants were found defective in Ca^{2+}-induced stomatal closure and ABA-dependent germination inhibition (Peiter *et al.*, 2005). In contrast, the involvement of this channel was neither observed in response to both Ca^{2+}- and ABA-induced stomatal closure nor in response to

other biotic and abiotic stresses that are potentially involved in stimulating Ca^{2+} signals in plants (Ranf *et al.*, 2008). However, in tobacco and rice, suppression of NtTPC1A and B caused suppression of Cry-induced cell death and defense related gene expression (Kadota *et al.*, 2004) and suppression of OsTPC1 suppressed cell death and MAPK activation induced by *Trichoderma viride* xylanase TvX (Kurusu *et al.,* 2005). In another study, loss-of-function of TPC1 revealed the incapability of mutant plants compared to wild type (WT) plant to accumulate transcripts of JA biosynthesis genes, *Plant Defensin 1.2A* (*PDF1.2A*) and *Thionin 2.1* (*THI2.1*) after *Botrytis cinerea* challenge (Bonaventure *et al.*, 2007b). However, mutation in Fatty Acid Oxygenation Upregulated 2 (FOU2) which is a gain-of-function allele of *TPC1*, resulted in enhanced synthesis of JA thereby increased expression of *Lipoxygenase* (*LOX*) genes, and enhanced resistance to *Botrytis cinerea* (Bonaventure *et al.*, 2007a). In accordance with these studies, Beyhl *et al.* (2009) further demonstrated the role of FOU2 in the induction of JA and suggested that wounding *fou2* plants could lead to the strong generation of vacuole-dependent $[Ca^{2+}]_{cyt}$ signals with a subsequent JA overproduction.

Despite of all these informations, ambiguities still exist about the localization of TPC1 in plants. TPC1 localization studies in species other than *Arabidopsis* have suggested that it might encode a putative plasma membrane Ca^{2+}-permeable channels in rice, wheat and tobacco (Demidchik and Maathuis, 2007; Pottosin and Schönknecht, 2007). Interestingly, this also leads to the hypothesis of species-specific targeting of TPC1 to different cell membranes. Very recently, it was reported that OsTPC1, as a plasma membrane Ca^{2+} channel in rice, participates to *Trichoderma viride* xylanase protein TvX-induced Ca^{2+} influx which was followed by phytoalexin biosynthesis. The *Ostpc1* knockouts showed impairment not only in TvX-induced Ca^{2+} variations but were also unable to regulate the expression of many diterpene cyclase genes that are needed for phytoalexin biosynthesis (Hamada *et al.*, 2012).

5.1.4. Ligand-gated channels in the endomembranes

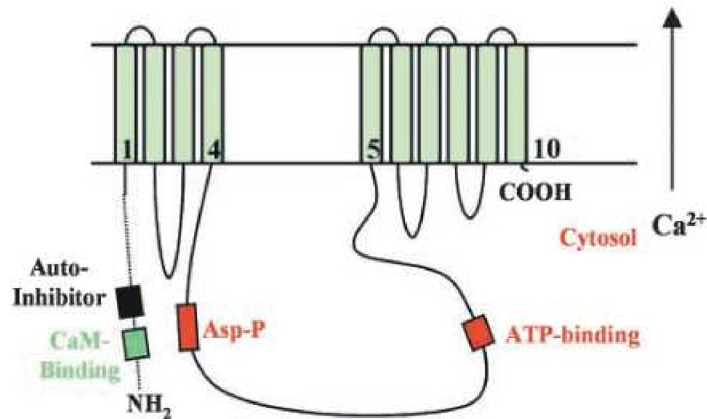
The existence of ligand-gated Ca^{2+}-channels in plant endomembranes especially ER and vacuoles have been reported in literature (Sanders *et al.*, 2002; White and Broadley, 2003; Hetherington and Brownlee, 2004; Demidchik and Maathuis, 2007; Pottosin and Schönknecht, 2007). Unlike ligand-gated channels present on the plasma membrane, molecular characterization of these endomembrane-specific ligand-gated channels have not yet been made and only electrophysiological approaches revealed their presence on different endomembranes. The important endomembrane ligand-gated channels in plants include: inositol 1,4,5-trisphosphate (IP_3)-gated channels, cyclic ADP-ribose (cADPR)-gated channels

and nicotinic acid adenine dinucleotide phosphate (NAADP)-gated channels (reviewed by McAinsh and Pittman, 2009; Dodd *et al.*, 2010; Kudla *et al.*, 2010). So far, the genes encoding an ADP ribosyl cyclase enzyme that is essential for cADPR production have not been identified in plants. Moreover, presence of ryanodine receptors (RYR) that are potential targets of animal cADPR is also debatable. In the same way, no indications are available for animal IP_3 receptors homologues presence in higher plants although some algae like *Volvox* and *Chlamydomonas* seem to have IP_3 receptor channels (Wheeler and Brownlee, 2008; Berridge, 2009). Consequently, the absence of molecular evidences of their existence in plants have really checked the progress in understanding their roles in plant Ca^{2+} signaling pathways and have added further challenges to assign these channel activities to specific Ca^{2+} signatures generated in response to different stimuli. Despite these controversies about their presence in plants, all the three types have been proposed to release Ca^{2+} from ER while IP_3 and cADPR have been described for the release of vacuolar Ca^{2+} (Allen and Sanders, 1995; Muir and Sanders, 1996; Navazio *et al.*, 2000; Navazio *et al.*, 2001). Various stress response-related functions of Ca^{2+} signaling have been attributed to these channels in plants (Knight *et al.*, 1996; Lamotte *et al.*, 2004; Lecourieux *et al.*, 2006; Vandelle *et al.*, 2006). For example, IP_3 have been reported to participate in gravitropism, salt and hyperosmotic stress signaling, ABA signaling and especially in elicitor-mediated defense signaling processes (Sanders *et al.*, 2002; Lecourieux *et al.*, 2006). On the other hand, cADPR participates in ABA signaling and regulates the activation of some defense-related genes (Sanders *et al.*, 2002; Dodd *et al.*, 2007). Navazio *et al.* (2000) have suggested the presence of NAADP-gated channels localized on ER membrane which should participate to Ca^{2+} release. These data are in favour of ligand-gated endomembrane Ca^{2+}-permeable channels in plants like in their animal counterparts. At the same time, these studies provide strong evidences for their potential role in Ca^{2+} signaling during plant stress responses.

5.2. Ca^{2+} pumps and transporters (exchangers)

Ca^{2+} homeostasis in the cell is very important for the proper growth and development because high concentration of Ca^{2+} is toxic to cell. It has been already demonstrated that cellular Ca^{2+} homeostasis is achieved by combined action of different channels and transporters proteins present on plasma membrane and other endomembranes (McAinsh and Pittman, 2009; Dodd *et al.*, 2010). According to Kudla *et al.* (2010), "A Ca^{2+} signal is defined by the balanced activation of Ca^{2+} channels at different cellular membranes, which is followed by the subsequent inactivation of channels and activation of efflux transporters to

A ***Arabidopsis* Autoinhibited Calcium ATPase (ACA type ATPase)**



B ***Arabidopsis* Calcium-Proton Exchanger (CAX1)**

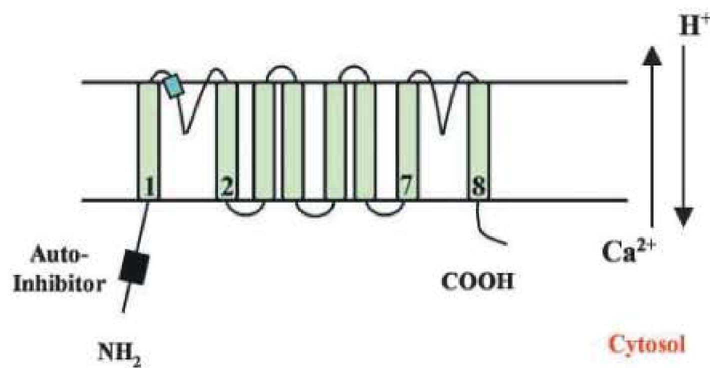


Figure 1.18: Topology models of putative Ca^{2+}-ATPase and Ca^{2+}/H^+ antiporters involved in Ca^{2+}-transport in *Arabidopsis*. A) Ca^{2+}-ATPase (ACA type) are ECA-type Ca^{2+} pumps that do not have unique feature of an N-terminal auto-inhibitor domain and calmodulin binding site. **B)** Ca^{2+}/H^+ antiporters. The topology of CAX1, The number of transmembrane domains predicted varies from eight to eleven. CAX1 has an N-terminal auto-inhibitor domain, blue portion indicates the position of an amino-acid sequence that is specific for cation transport (Sanders *et al.*, 2002).

terminate Ca^{2+} influx and to rebalance the cellular Ca^{2+} homeostasis". This clearly suggests the importance of Ca^{2+} transporters in controlling Ca^{2+} homeostasis. In plants, two main classes of proteins fulfill this role: Ca^{2+}-ATPases and Ca^{2+}/proton antiporters. Ca^{2+}-ATPases generate a low-affinity high-capacity ATP-driven Ca^{2+} efflux and are thought to maintain the low resting [Ca^{2+}]. In contrast, Ca^{2+}/H^{+} antiporters generate a high-affinity low turnover efflux and are postulated to maintain the stimulus induced Ca^{2+} influx to its basal value (Hirschi, 1999). The sequences of these proteins are highly conserved among different life kingdoms and genetic basis are known. Consequently, their regulation, membrane localization, kinetic characteristics, expression pattern, and physiological functions have been well identified (details can be find in the following reviews: Shigaki and Hirschi, 2006; Boursiac and Harper, 2007; McAinsh and Pittman, 2009; Kudla *et al.*, 2010).

5.2.1. Ca^{2+}-ATPases

During past years, many Ca^{2+}-ATPases have been identified and characterized in plants and animals. ATP-driven Ca^{2+} pumps (Ca^{2+}-ATPases) are a subgroup of the P-type ATPases (the P_2-ATPases; Baxter *et al.*, 2003). Ca^{2+}-ATPases are further divided into P_{2A}-ATPases and P_{2B}-ATPases. In animals, P_{2A}-type ATPases contain the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and P_{2B}- type ATPases, include the animal CaM-regulated plasma membrane Ca^{2+}-ATPases (PMCA). PMCA functions in a similar manner to other P-type ion pumps: ATP shift a phosphate to the PMCA, whose configuration is changed or altered into a phosphorylated form. Further activation takes place when Ca^{2+}/calmodulin bind to PMCA. At normal [Ca^{2+}] levels, PMCA is suitable for the regulation of [Ca^{2+}] as it is effective even when [Ca^{2+}] within the cell is very low.

In higher plants, Ca^{2+}-ATPases are grouped as type P_{2A} or P_{2B} based on their homology with animal counterparts. Type P_{2A}- ATPase consists of the ER-type Ca^{2+}-ATPase (ECA), and P_{2B}-ATPase, corresponds to auto-inhibited Ca^{2+}-ATPase (ACA; Figure 1.18A). Two major features distinguish ECAs from ACAs (Boursiac and Harper, 2007). First, an N-terminal cytosolic domain present only in ACAs binds calmodulin that is bound to Ca^{2+}, and this interaction activates Ca^{2+} pumping (Bækgaard *et al.*, 2005). Second, there are differences in membrane-located residues that are thought to be involved in Ca^{2+} binding.

In *Arabidopsis*, 12 Ca^{2+}-ATPases have been identified (4 ECAs and 8 ACAs). Plant isoforms of both ECAs and ACAs have been observed at the ER, plasma membrane and tonoplast (Ferrol and Bennett, 1996; Liang *et al.*, 1997; Hong *et al.*, 1999; Bonza *et al.*, 2000; Lee *et al.*, 2007). More precisely, P_{2A}-type ATPases are localized at the ER (ECA1; Liang *et*

al., 1997), the Golgi (ECA3; Mills *et al.*, 2008) and endosomes (also ECA3; Li *et al.*, 2008). P_{2B}-types ATPases are localized at the ER (ACA2; Harper *et al.*, 1998), vacuole (ACA4 and ACA11; Geisler *et al.*, 2000; Lee *et al.*, 2007) , plasma membrane (ACA8, ACA9 and ACA10; Bonza *et al.*, 2000; Schiott *et al.*, 2004; George *et al.*, 2008) and at the plastid envelope (ACA1; Huang *et al.*, 1993). There are also some evidences that regulation by CaM is not solely a characteristic of ACAs, as CaM binding by ECAs has been observed (Subbaiah and Sachs, 2000; Navarro-Aviñó and Bennett, 2003). In plants, no direct regulation of a type P_{2A} ATPase has been demonstrated which may suggest a constitutive role in maintaining resting cytosolic $[Ca^{2+}]_{cyt}$ levels.

Studies with knock-out Ca^{2+}-ATPase plants indicated that *Arabidopsis* Ca^{2+}-ATPases are not vital as single pumps (Boursiac and Harper, 2007). However, some studies highlight the importance of ACAs type pumps in specific Ca^{2+} pathways. For example, *ACA12* and *ACA13* transcripts are dramatically upregulated by pathogen stress (Boursiac and Harper, 2007). Furthermore, transcript abundance of the closely-related *ACA8* and *ACA10* is differentially regulated by cold (Schiøtt and Palmgren, 2005), whereas *ACA8* and *ACA9* gene expression are both acutely upregulated by ABA (Cerana *et al.*, 2006). In response to cold stress, *AtACA8* and *AtACA10* are differentially regulated (Schiøtt and Palmgren, 2005). On the other hand, there are only indirect evidences that plant Ca^{2+}-ATPases play a role in abiotic stress-induced genes expression levels (Qudeimat and Frank, 2009). At the same time mutants studies of *AtACA10* signify a role in vegetative development (George *et al.*, 2008). Ca^{2+} signaling pathway is very important during pollen tube growth (Holdaway-Clarke and Hepler, 2003). A plasma membrane bounded pump, AtACA9 is vital for pollen tube growth and fertilization (Schiott *et al.*, 2004). Moreover, loss-of-function studies demonstrated that both *Ataca9* and *Ataca10* mutants caused specific deficits in plant development (*e.g.* partial sterile male, abnormal vegetative growth (George *et al.*, 2008).

5.2.2. Ca^{2+} exchangers

In animal cells, Ca^{2+} efflux by Ca^{2+}/Na^{+} exchangers is coupled to Na^{+} flux. However, plants possess a structurally related family of cation exchanger (*CAX*) genes that encode Ca^{2+}/H^{+} exchangers (Cai and Lytton, 2004; Shigaki and Hirschi, 2006). In *Arabidopsis*, 6 members in the Ca^{2+}/cation antiporter (CaCA) superfamily proteins, referred as cation exchangers (CAX; Mäser *et al.*, 2001; Shigaki *et al.*, 2006), monitor the homeostasis of Ca^{2+} and other divalent cations, in the cells (Catalá *et al.*, 2003; Cheng *et al.*, 2003; Korenkov *et al.*, 2007; Zhao *et al.*, 2008). *Arabidopsis* contains five *CAX*-related genes, designated

cation/Ca^{2+} exchanger (*CCX*) (originally named *AtCAX7-AtCAX11*) that are more similar to an animal Ca^{2+}/Na^{+} exchanger (Cai and Lytton, 2004; Shigaki *et al.*, 2006). Structurally, CAX is an of integral membrane proteins with 10 to 11 transmembrane (TM) domains that transports Ca^{2+} or other cations using the gradient of H^{+} or Na^{+} generated by energy-coupled primary transporters (Figure 1.18B) (Busch and Saier, 2002; Cai and Lytton, 2004; Shigaki *et al.*, 2006). Additionally, four putative antiporters encoded in the *Arabidopsis* genome contain EF-hand Ca^{2+} binding motifs, implicating that these transporters are directly regulated by Ca^{2+} (Shigaki *et al.*, 2006). *CAX* genes encoding tonoplast H^{+}/Ca^{2+} exchangers have been subsequently identified from various plant species *e.g.* carrot, oat, sugar beet, and maize (Hirschi *et al.*, 1996; Ueoka-Nakanishi *et al.*, 2000; Kamiya *et al.*, 2006). Moreover, antiporter activity has also been demonstrated from cytosol to the vacuole (Schumaker and Sze, 1985; Blumwald and Poole, 1986) and to other membranes including the plasma membrane (Kasai and Muto, 1990).

Ca^{2+}/H^{+} antiporters, such as the *Arabidopsis* Ca^{2+}/H^{+} antiporter CAX1 (Ca^{2+} exchanger 1; Hirschi *et al.*, 1996) is able to remove Ca^{2+} from the cytosol. CAXs proteins participate in a multitude of cellular responses in plants. They are thought to have an impact on Ca^{2+} and other heavy metal signaling events (Shigaki and Hirschi, 2006; McAinsh and Pittman, 2009). Members of the *Arabidopsis CAX* gene family have been well characterized at both the molecular and whole-plant level. AtCAX1 is a negative regulator of the cold-acclimation response (Catalá *et al.*, 2003). AtCAX1 activity may be regulated by phosphorylation (Pittman *et al.*, 2002) or via various CAX interacting proteins (CXIP), including CXIP4 (Cheng *et al.*, 2004a,b; Cheng and Hirschi, 2003). In *Arabidopsis*, CAX1 is highly expressed in leaf tissue, and modestly expressed in roots, stems, and flowers and show the highest reduction in expression in the Ca^{2+}-stressed mutant, while CAX3 is most abundant in roots and its expression increases upon overnight exposure to exogenous Ca^{2+} (Cheng *et al.*, 2003; Cheng *et al.*, 2005; Chan *et al.*, 2008). Moreover, *cax1/cax3* double mutant plants display more severe Ca^{2+} sensitivity than either of the single mutants (Cheng *et al.*, 2005). CAX3 is not only involved in salt stress as *cax3* mutant lines showed an altered response to Na^{+} and Li^{+} but also exhibited sensitivities to low pH conditions. In addition, *cax3* mutant lines also displayed reduced plasma membrane H^{+}-ATPase activity (Zhao *et al.*, 2008). Different *cax* mutants demonstrate specific responses to the individual stresses. Moreover, abiotic stress phenotypes of *cax1* and *cax3* knockout mutants demonstrate the function of these transporters in responses to stresses. Both *cax1* and *cax3* mutant plants are hypersensitive to ABA during germination (Zhao *et al.*, 2008). It has also been reported that

cax3 has high sensitivity to salt stress (Zhao *et al.*, 2008). In *Arabidopsis*, cold and salt stresses are able to induced $[Ca^{2+}]_{cyt}$ oscillations which ultimately lead to specific response and release of Ca^{2+} from vacuole (Knight *et al.*, 1996, 1997; Evans *et al.*, 2001). CAX4 is involved in root growth and development under metal (Ni^{2+} or Mn^{2+}) stress. Moreover, CAX4 cation/H^+ antiport activity is necessary for auxin-mediated root growth and development (Mei *et al.*, 2009).

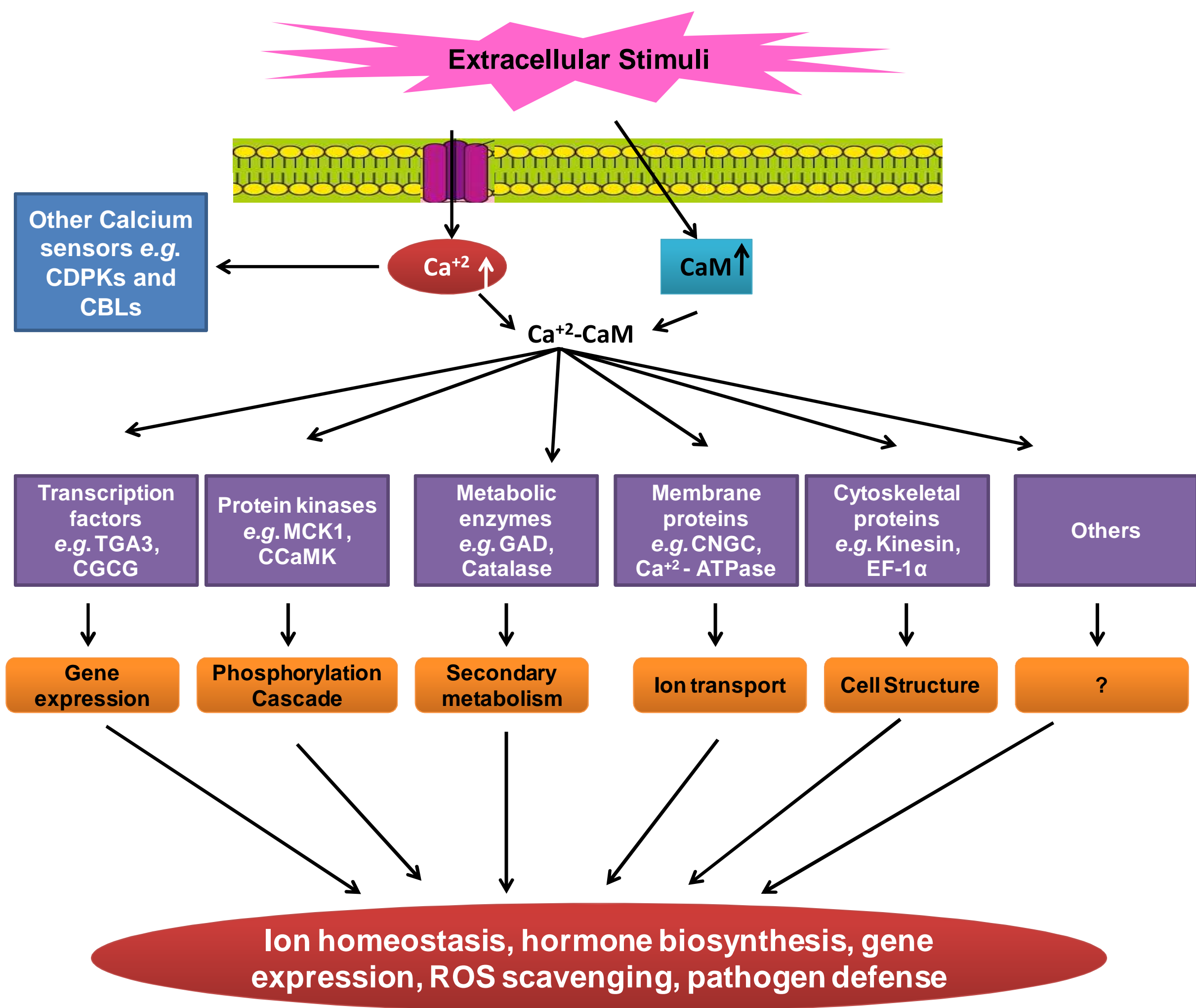


Figure 1.19: Ca^{2+}/CaM-mediated network in plants. Ca^{2+} signal changes are triggered by environmental, hormonal or developmental stimuli. The Ca^{2+} signatures are decoded by Ca^{2+} sensors, such as calmodulin (CaM), Ca^{2+} -dependent protein kinase (CDPK) and calcineurin-B like protein (CBL). Expressions of some CaM genes are also induced by these stimuli. The activated Ca^{2+}/CaM complex binds to numerous target proteins and modulates their activities. Target proteins include transcription factors, protein kinases, metabolic enzymes, ion channels and transporters, and cytoskeleton proteins. Finally, the Ca^{2+}/CaM-mediated signal network results in physiological responses such as cell growth, differentiation, stress tolerance and cell death (Yang and Poovaviah, 2003; Kudla *et al.,* 2010).

6. Ca^{2+} sensing and signaling

In response to different developmental cues and environmental challenges, a rapid Ca^{2+} influx occurs that leads to the generation of $[Ca^{2+}]_{cyt}$ perturbations (White, 2000; Sanders *et al.*, 2002). These Ca^{2+} variations are ultimately translocated into various biological responses through the involvement of different Ca^{2+}-binding proteins (CBPs) known as "Ca^{2+} sensor proteins" (Batistič and Kudla, 2004; Weinl and Kudla, 2009). These Ca^{2+} sensors proteins undergo conformational changes and also modify their catalytic activity upon Ca^{2+} binding and interact with downstream effectors (Clapham, 2007; Gifford *et al.*, 2007). The most common Ca^{2+}-binding structural motif in proteins is the EF-hand present in pairs that facilitate high-affinity cooperative binding of Ca^{2+}. This helix-loop-helix structure is found in more than 250 proteins encoded in the *Arabidopsis* genome (Day *et al.*, 2002). It has been extensively studied (Kawasaki *et al.*, 1998; Ikura and Ames, 2006; Gifford *et al.*, 2007), but the majority of EF-hand Ca^{2+} sensors remain unstudied in plants. The three largest categories of EF-hand proteins in plants include: CaMs (calmodulins: Calcium-Modulated Protein) and CMLs (CaM-like proteins), the CDPKs (Ca^{2+}-dependent protein kinases) and the CBLs (calcineurin B-like proteins). Although CaM is found in all eukaryotes, CMLs, CDPKs and CBLs are restricted to plants and some protists. Ca^{2+} sensor proteins in plants have been divided into sensor relays and sensor responders (Luan *et al.*, 2002; Sanders *et al.*, 2002). Figure 1.19 shows various Ca^{2+} sensors of stimulus-induced $[Ca^{2+}]_{cyt}$ variations along with their putative target proteins (Adapted from Yang and poovaviah, 2003; Kudla *et al.*, 2010).

6.1. Sensor relays

This class is composed of CaM, CMLs and CBLs that effectively bind Ca^{2+} ions and undergo conformational changes upon Ca^{2+} binding but do not contains other effector domains. These sensor relay proteins are dependent on other target proteins to transmit the Ca^{2+} signal.

6.1.1. Calmodulin and calmodulin-like sensor proteins

CaM and CML related proteins are well recognized classes of Ca^{2+} sensors. In spite of the fact that number and organization of *CaM* genes varies among different organisms, CaM is one of the most conserved proteins in eukaryotes. CaMs superfamily encompasses proteins having EF hands that bind to Ca^{2+} (Snedden and Fromm, 1998; Zielinski, 1998; Snedden and Fromm, 2001).

In higher plants, CaM is a small (~ 150 residues), acidic protein of 15-17 KDa, extremely conserved and having large affinity for Ca^{2+} and composed of two pairs of Ca^{2+} binding EF-hands domains (Figure 1.21; Snedden and Fromm, 2001; Luan *et al.*, 2002). Upon Ca^{2+} binding, CaM globular structure changes into an open conformation that allows interaction with proteins (Yamniuk and Vogel, 2005). This interaction subsequently activates (Lee *et al.*, 2000) or inhibits (Choi *et al.*, 2005; Yoo *et al.*, 2005) CaM targets, that ultimately translate Ca^{2+} signal into a biochemical or physiological response.

Animal genomes typically contain only a few *CaM* genes (*e.g.* three in humans), whereas plant genomes have multiple *CaM* genes that encode identical CaMs or highly similar isoforms. In *Arabidopsis* genome, three main group are defined, CaMs, CaM-like proteins, and CaM-related proteins. One group contains seven different *CaM* genes (CaM1 to CaM7). These are highly similar to each other and also to animal CaM (>95% identical in amino acid sequence; 89% identical with human CaM). Two sets encode identical isoforms (CaM1 and CaM4; CaM2, CaM3 and CaM5) that differ by only one to four amino acids. Moreover, two CaM genes (CaM6 and CaM7) are closely related and have ~ 99% identity with CaM2 (McCormack and Braam, 2003).

Investigations have started to explore and understand the mechanisms of CaM target interaction and their specificity (Ishida and Vogel, 2006; Rainaldi *et al.*, 2007; Ishida *et al.*, 2008). The ability of CaM to bind and regulate a vast array of targets probably derives from three important features (Ishida and Vogel, 2006). First, the linker region of CaM imparts it with substantial flexibility. Secondly, CaM can bind certain targets in the Ca^{2+}-free (apo) state. Thirdly, the hydrophobic surface of CaM, through which it typically interacts with targets, is flexible due to a large number of methionine residues.

CaMs have multiple sub-cellular localizations (Yang and Poovaiah, 2003). CaMs have been identified in the cytosol, nucleus and fixed to the plasma membrane. In tobacco, a CaM binding ER-localized Ca^{2+}-ATPase plays an important role in MAMP/PAMP–induced Ca^{2+} changes. Silencing of this ATPase altered the MAMP-induced $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ signature and stimulated pathogen- and elicitor-induced cell death (Zhu *et al.*, 2010).

In spite of higher sequence similarities, CaMs have been shown to modulate a large number of cellular activities by interacting with a variety of proteins (Figure 1.19). CaMs target proteins have been identified through cDNA libraries screening by labeled CaMs (Fromm and Chua, 1992). In plants, a large number of CaM binding proteins have been identified. CaM modulated target proteins including protein kinases such as CDPK-related kinases (CRKs), CBL-interacting protein kinases (CIPKs), CDPK and CCaMK. The CDPK

and CCaMK can directly bind Ca^{2+} through their EF-hand motifs (Harper *et al.*, 2004). Glu decarboxylase (GAD) is one of the best studied examples of protein target (Baum *et al.*, 1993, 1996; Snedden *et al.*, 1996; Zik *et al.*, 1998). GAD catalyzes the conversion GABA and is activated rapidly during several stress responses (Snedden and Fromm, 1998, 2001). In response to developmental and environmental signals, plants showed a differential expression of specific CaM proteins (Bouché *et al.*, 2005; McCormack *et al.*, 2005). AtCAM7 plays a role in the regulation of light-induced gene expression by CaM (Kushwaha *et al.*, 2008). Specifically, the CAM7 but not other isoforms CAM2/3/5, is the transcriptional regulator that directly bind with promoters of several light-inducible genes. Previously, the microinjection of phytochrome A into hypocotyl cells of tomato *aurea* mutant (phytochrome deficient) have suggested a vital role of Ca^{2+} and CaM for the regulation of light-responsive gene expression (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994).

CaM proteins have an important function in pathogenesis and wounding (Bergey and Ryan, 1999; Heo *et al.*, 1999; Yamakawa *et al.*, 2001) and in the HR (Levine *et al.*, 1996; Harding *et al.*, 1997; Harding and Roberts, 1998; Heo *et al.*, 1999; Blume *et al.*, 2000). Expression of CaMs is induced by touch, cold, heat shock or salinity (Jang *et al.*, 1998; Luan *et al.*, 2002; Yang and Poovaiah, 2003). Moreover, CaMs express in a developmentally regulated and tissue-specific manner (Yang *et al.*, 1996, 1998). Role for various CaM isoforms has been demonstrated in plant defense using different plants such as soybean, *Arabidopsis*, and tobacco (Harding *et al.*, 1997; Heo *et al.*, 1999; Chiasson *et al.*, 2005; Takabatake *et al.*, 2007; Zhu *et al.*, 2010). Direct evidence for the CaM implications in plant defense responses comes by overexpression studies with soybean CaMs (ScaMs). The expression of SCaM4 and SCaM5 in transgenic tobacco and *Arabidopsis* led to spontaneous lesions, higher expression of PR genes and increase resistance to bacteria, fungi, and viruses (Heo *et al.*, 1999; Park *et al.*, 2004). It was reported that the defense associated Ca^{2+} signal is specific and only SCaM4 and SCaM5, but not other CaMs, were induced in response to pathogens. In response to cellulase, harpin, incompatible bacteria, and mechanical stress, transgenic tobacco cells having a CaM mutation (VU-3) showed higher ROS production (Harding *et al.*, 1997) giving to an indirect evidence of CaM implication in plant defense responses. Cell death was shown to be accelerated in transgenic tobacco plants inoculated with incompatible *Pseudomonas syringae* pv syringae (Harding and Roberts, 1998). Moreover, in tomato (*Solanum lycopersicum*), silencing of specific pathogen-induced CaM isoforms led to increase susceptibility to virulent necrotrophic bacteria and fungi (Takabatake *et al.*, 2007). These results highlight the implication of specific CaM isoforms in basal defense

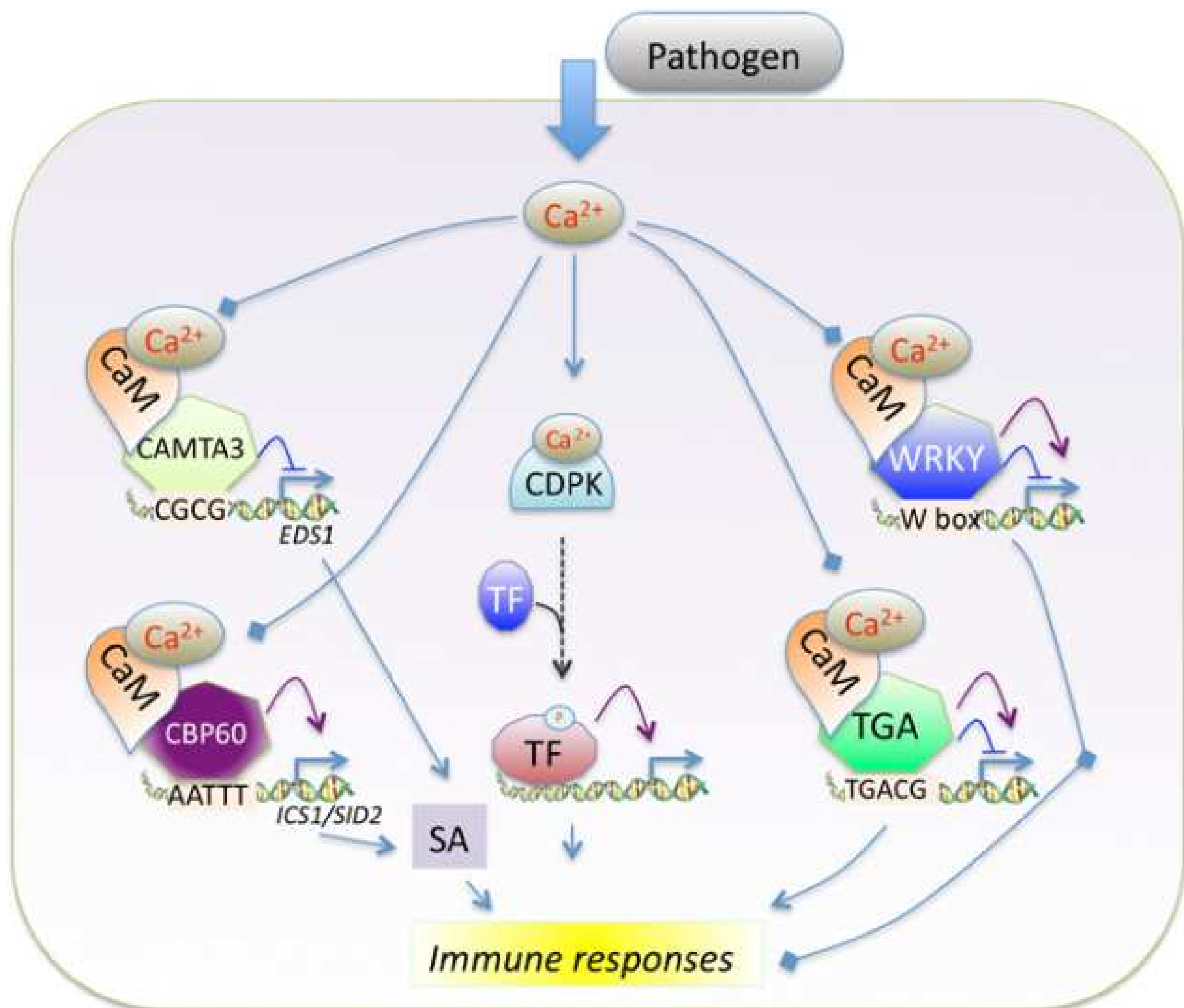


Figure 1.20: Roles of Ca^{2+} and Ca^{2+}/CaM Binding Transcription factors (TFs) in regulating gene expression involved in plant immunity. Purple arrows indicate activation of gene expression; blue lines with a horizontal line indicate repression. Pathways lacking evidence are draw by doted arrows. Diamonds at the lines' end indicate that the effect of Ca^{2+}/CaM binding on TFs function is not known (source: Reddy *et al*., 2011)

against necrotrophic pathogens. Moreover, CaM involvement in plant defense reactions exhibits specificity to different pathogens (Zhu *et al.*, 2010). For example, silencing of a tobacco CaM, Nb-CaM1, suppressed the TMV p50-induced HR in tobacco cells but not the Cf9-Avr9 or Pto-AvrPto and *Pst* DC3000-induced cell death (Zhu *et al.*, 2010). Collectively, these data provide sufficient evidences that CaMs are key players in transducing the pathogen-induced Ca^{2+} increase to downstream components of defense signaling.

CaM-interacting TF families includes: members of the CaM binding transcription activators (CAMTAs), TFs with a WRKY domain (WRKY TFs), TFs with a conserved MYB domain (MYB TF), TGACG motif-binding factor (TGA) which is a member of TFs with a basic leucine zipper domain (bZIP) and members of CaM binding protein 60 (CBP60s). Interaction between CaM and these TF families plays a crucial role in biotic stresses by modulating the expression of defense genes (Figure 1.20).

Arabidopsis genome contains six members of CAMTA TFs that have a conserved structure domain (Finkler *et al.*, 2007). In plants, CAMTA TFs display a role in controlling the C-repeat binding factor (CBF)-regulated cold-responsive gene expression. This regulation of gene expression may occur through direct interaction. In *Arabidopsis*, Ca^{2+}-dependent interaction of CAMTAs with members of CaM Ca^{2+} sensors family has been shown (McCormack *et al.*, 2005). Both CAMTA1 and CAMTA3 are the regulators of cold tolerance because these CAMTA proteins bind to regulatory elements in the promoter region of the *DREB1c/CBF2* gene (Doherty *et al.*, 2009). Moreover, CAMTA3 is a negative regulator of fungal resistance. Loss-of-function mutants showed increased resistance to *Botrytis cinerea* (Galon *et al.*, 2008).

CaM not only interacts with CAMTAs but also interacts with MYB and WRKY family TFs (Park *et al.*, 2005; Yoo *et al.*, 2005). For example, WRKY TFs family (WRKY11 and WRKY17; Park *et al.*, 2005) was enhanced by chitin treatment (Libault *et al.*, 2007): as chitin is fungal cell wall component so it is assumed that these TFs play a role in fungal defense. Another class of plant-specific CaM binding proteins, CBP60s, was first isolated from maize (*Zea mays*; Reddy *et al.*, 1993). CBP60s were also isolated from tobacco, *Arabidopsis* and *Phaseolus vulgaris* (Lu and Harrington, 1994; Reddy *et al.*, 2002; Ali *et al.*, 2003). In *Arabidopsis,* seven out of eight members in this family can bind CaM (Reddy *et al.*, 2002; Wang *et al.*, 2009; Zhang *et al.*, 2010). They have their CaM binding domain at the C and N terminus (Reddy *et al.*, 1993; Lu and Harrington, 1994; Reddy *et al.*, 2002; Wang *et al.*, 2009; Zhang *et al.*, 2010). They exhibit differential expression in response to biotic stresses and elicitors (Ali *et al.*, 2003; Wang *et al.*, 2009). Recently, SAR deficient 1

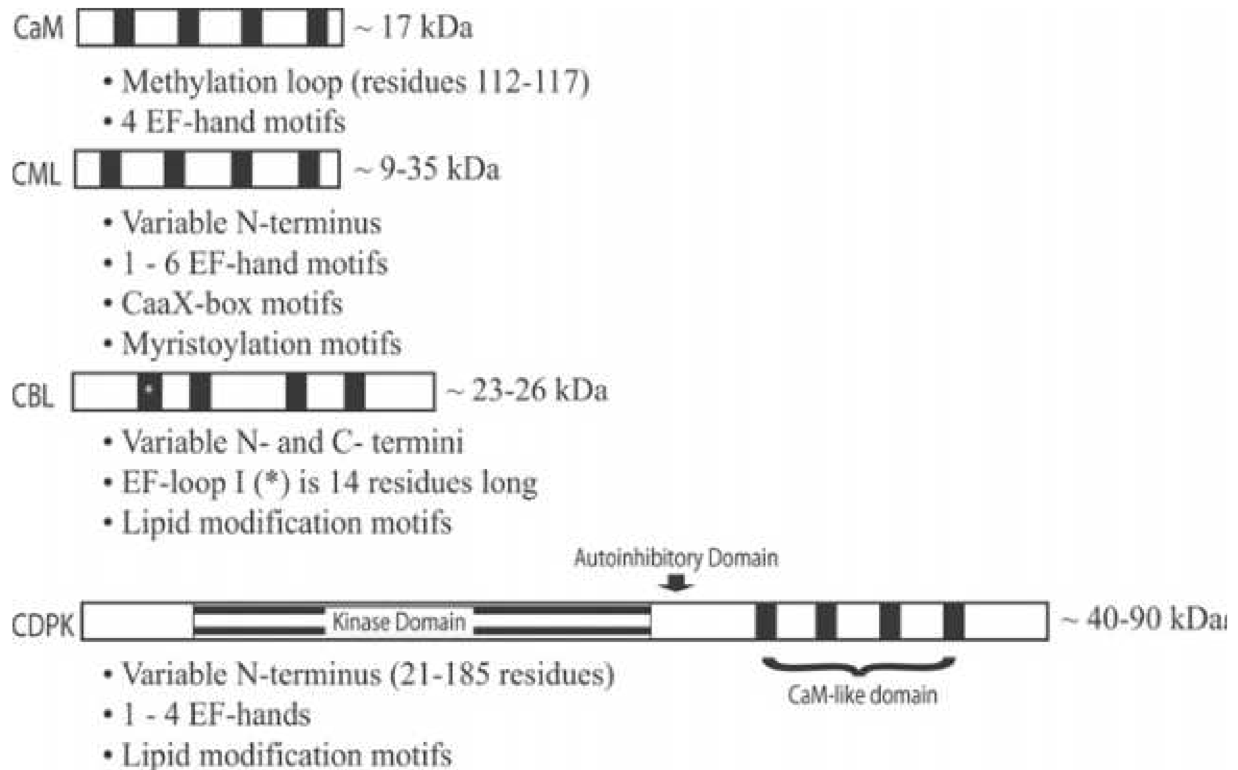


Figure 1.21: Classes of Ca^{2+} sensors in plants. All classes have four EF-hands (indicated as black boxes) although the number may vary in CDPKs and CMLs. Many CMLs and CDPKs possess N- or C-terminal extensions of unknown function. Lipid modification motifs are predicted for most CDPKs, and some CBLs and CMLs. CaM: Calmodulin; CMLs: Calmodulin Like; CBLs: Calcineurin-B-like protein; CDPKs: calcium-dependent protein kinase; ROS: reactive oxygen species (DeFalco *et al.*, 2010).

(SARD1) and $CBP60_g$, two members of CBP60 family in *Arabidopsis* were reported to bind DNA and regulate expression of specific genes of SA pathway (Zhang *et al.*, 2010).

In addition to CaMs protein, plants have another family of protein which is named as CMLs. *Arabidopsis* genomes encode 50 CMLs. Most CMLs have four EF-hands, although this number may varies from 1 to 6 (Figure 1.21; McCormack and Braam, 2003). Sequence divergence within the EF-hands of CMLs probably enables differential response among family members to Ca^{2+} signals (Snedden and Fromm, 2001; McCormack *et al.*, 2005). In plants, the implication of CMLs proteins as regulators of different developmental and stress responses have been reported (Popescu *et al.*, 2007; Magnan *et al.*, 2008). Expression of CMLs is induced by touch, cold, heat shock or salinity (Luan *et al.*, 2002; Yang and Poovaiah, 2003). In *Arabidopsis*, a CML (CML43) is induced by pathogens and its overexpression accelerated HR (Chiasson *et al.*, 2005). Additionally, a tomato CML (APR134) is also induced by pathogens, and silencing of this gene compromised immune response (Chiasson *et al.*, 2005). These studies highlight the important contribution of CMLs in plant defense responses. However, a direct effect on defense genes expression (*e.g.* PR) and on the resistance was not demonstrated.

In conclusion, CaMs and CMLs indirectly regulate different cellular processes by interacting with other Ca^{2+}-dependent proteins and modulating their activity.

6.1.2. Calcineurin B-like sensors

Calcineurin is a Ca^{2+}-CaM-dependent protein phosphatase that is highly conserved in eukaryotes from yeast to mammals. Calcineurin B-like proteins (CBLs) represent a plant specific form of Ca^{2+}-binding proteins. CBLs range in predicted size from approx. 23 to 26 KDa, making them slightly larger than CaM (~ 17 KDa) and comparable in size with many CMLs. CBL proteins are closely related to regulatory B subunit of calcineurin and to the family of Neuronal Calcium Sensors (NCS), from animals and yeast (Kudla *et al.*, 1999). All CBL proteins share a rather conserved core region encompass with four Ca^{2+} binding sites (EF hand) that are arranged in completely invariant spacing within the protein (Kolukisaoglu *et al.*, 2004). Sequence similarity of CBLs to CaM is restricted to the EF-hands (Figure 1.21).

CBLs are encoded by a multigene family: the genome of the model plant *Arabidopsis* encodes 10 CBLs which have similar structural domains with small variations in the length of the coding regions (Kudla *et al.*, 1999; Kim *et al.*, 2000; Albrecht *et al.*, 2001; Guo *et al.*, 2001). Their amino acid sequence identity from 20 to 90 % is sufficient for functional redundancy among the closely related members while allowing for functional specificity

among more diverged members. CBL proteins have multiple sub-cellular localizations. For example, four CBLs are localized each to the plasma membrane and vacuolar membrane and two CBLs are detected in the cytoplasm and nucleus (Batistič *et al.*, 2010). *Arabidopsis CBL* gene exhibits specific expression patterns in response to different stimulus. *CBL1* expression is induced strongly by wounding, drought, high salt, cold, and ABA (Kudla *et al.*, 1999; Piao *et al.*, 2001; Luan *et al.*, 2002). Both *CBL1* and *CBL2* respond to light, but *CBL2* lacks the other responses of *CBL1* (Nozawa *et al.*, 2001). This expression pattern highlights that *CBL1* and *CBL2* have specific functions in certain signal transduction pathways.

CBLs specifically interact with a family of serine-threonine protein kinases designated as CBL-interacting protein kinases (CIPKs; Kudla *et al.*, 1999). CIPKs belong to the superfamily of SNF-like kinases (Batistič and Kudla, 2004) and there are 25 kinases of the CIPK-type present in *Arabidopsis* (Kolukisaoglu *et al.*, 2004). All CIPK-type kinases are composed of a conserved N-terminal kinase domain and a C-terminal regulatory domain, which are separated by a variable junction domain. The conserved NAF domain that is necessary for CBL interaction is present within divergent regulatory domains (Albrecht *et al.*, 2001). Binding of CBL to the NAF domain of CIPKs change or transform kinase in active phase (Guo *et al.*, 2001; Gong *et al.*, 2002). Moreover, phosphorylation of CBL proteins by their interacting CIPKs enhances the CBL-CIPK interaction (Mahajan *et al.*, 2006; Lin *et al.*, 2009). Most CIPKs are localized in cytoplasme and nucleoplasme (D'Angelo *et al.*, 2006; Batistič *et al.*, 2010). Different reports highlight the specific interaction among various members of the CBL and CIPK families. It was studied that some CBLs interact with more than one CIPK and or vice versa (Kim *et al.*, 2000; Albrecht *et al.*, 2001; Guo *et al.*, 2001). For example, CIPK1 is targeted to the plasma membrane by CBL1 or CBL9 (Cheong *et al.*, 2007; Waadt *et al.*, 2008). However, upon interaction with CBL2, the resulting CBL2/CIPK1 complexes are localized exclusively to the tonoplast (Batistič *et al.*, 2010). Similarly, CIPK14/CBL2 complexes have been detected at the tonoplast, while the same kinase is targeted to the plasma membrane upon interaction with CBL8 (Batistič *et al.*, 2010). The increasing number of available full-genome sequences has facilitated the study of the evolution of the CBL/CIPK signaling system. Single CBL and CIPK genes have been identified in green alga species, such as *Ostreococcus tauri* and *Chlorella spp*, whereas the moss *Physcomitrella patens* contains four CBLs and seven CIPKs, and the genome of the fern *Selaginella moellendorfii* possesses a complement of five CBL and five CIPK genes (Batistič and Kudla, 2009; Weinl and Kudla, 2009; Batistič *et al.*, 2010). CBL1 interacts with

CIPKs through the C-terminal non-kinase domain that contains a conserved region among different CIPK members (Shi *et al.*, 1999). Interestingly, interaction between CBL1 and CIPK1 requires micromolar levels of Ca^{2+}.

6.2. Sensor responder proteins

Unlike sensor relay, sensor responder proteins exhibit two functions: the sensing function during which these proteins bind Ca^{2+} and undergo Ca^{2+}-induced conformational changes and a response activity (*e.g.* protein kinase activity). CDPKs are best example of this type of proteins.

6.2.1. Ca^{2+}-dependent protein kinases

Ca^{2+}-dependent protein kinases (CDPKs) have been identified throughout the plant kingdom from green algae to angiosperms (Hrabak, 2000; Harmon *et al.*, 2001). CDPKs, also named as CPKs, are unique to plants and play a role in the majority of Ca^{2+}-responsive kinase activity in plants (Zhao *et al.*, 1993; Cheng *et al.*, 2002). The *Arabidopsis* genome encodes 34 CDPKs (*e.g. Arabidopsis* AtCPK1-AtCPK34) which are subdivided in four groups (Boudsocq *et al.*, 2010) and eight additional CDPK-related kinases (Hrabak *et al.*, 2003). Similar CDPK families have also been identified in rice (31 members; Asano *et al.*, 2005; Ray *et al.*, 2007) poplar (30 members) and wheat (26 members; Li *et al.*, 2008).

Arabidopsis 34 CDPKs are highly homologous to each other. Protein sequences analyses indicate that the overall identities (39 % to 95 %) and similarities (56 % to 96 %). CDPKs range from ~ 40 to 90 KDa, and are composed of five domains. Much of the difference in CDPK size between isoforms can be attributed to the variable domain at the N-terminus, which ranges from 21 to 185 amino acids in length among *Arabidopsis* CDPKs, and shows little sequence conservation (Cheng *et al.*, 2002). These proteins possess a C-terminal CaM like regulatory domain with four Ca^{2+}-binding EF-hands, following a protein kinase catalytic domain (Figure 1.22; Harper and Harmon, 2005). Conversely, the catalytic region is a highly conserved serine/threonine kinase domain (Klimecka and Muszyńska, 2007). Binding of Ca^{2+} to the C-terminal EF hand–containing regulatory domain leads to conformational changes, resulting in activation of the respective CDPK. This process is enhanced by autophosphorylation of the CDPKs that contributes to full activation of the kinases (Ludwig *et al.*, 2004). CDPKs demonstrate multiple localization including the cytosol, nucleus, cytoskeleton and are associated to membranes (Sanders *et al.*, 2002). The

identification of potential CDPK targets has been identified via protein-protein interactions (Patharkar and Cushman, 2000).

CDPKs play important roles in regulation of plant growth and development, responses to biotic and abiotic stresses (Cheng *et al.*, 2002; Ludwig *et al.*, 2004; Klimecka and Muszyńska, 2007; DeFalco *et al.*, 2010). ABA-induced CDPK activity has been reported in both tobacco and rice (Yoon *et al.*, 1999; Li and Komatsu, 2000a; Li and Komatsu, 2000b). The expression of many CDPKs is induced by stress-response and regulates plant abiotic stress responses (Milla *et al.*, 2006; 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 various plants, CDPKs modulate the gene expression in response to environmental stress stimuli and in some cases, CDPKs become rapidly activated upon stress exposure of plants or cell cultures (Böhmer *et al.*, 2006). CDPK enzymatic activity has been correlated with osmotic stress and elicitation (Takahashi *et al.*, 1997; Allwood *et al.*, 2002). In potato, two CDPKs (StCDPK4 and StCDPK5) phosphorylate and activate an elicitation-dependent NADPH-oxidase thereby positively regulating the production of ROS (Kobayashi *et al.*, 2007). A 68/70 KDa CDPK was identified in tobacco that was activated in response to Avr9 elicitation in Cf-9 tobacco (Romeis *et al.*, 2000). In tobacco, CDPK1 regulates the repression of shoot growth (RSG) transcription factor in response to gibberellins (Ishida *et al.*, 2008). AtCPK10 is linked with the induction of environmental stress-related promoters after ABA treatment (Sheen, 1996). Upon ABA treatment, *Arabidopsis* CPK3 and CPK6 play role in the regulation of stomatal closure and external Ca^{2+} elevation (Mori *et al.*, 2006). Also, *Arabidopsis* CPK4 and CPK11 are important for ABA responsiveness of guard cells and phosphorylate the ABA-responsive transcription factors ABF1and ABF4 in vitro (Zhu *et al.*, 2007).

In *Arabidopsis*, CDPKs are the convergence point of MAMPs-triggered signaling. Boudsocq 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 CDPK activities, displayed gradual decline in oxidative burst and were more susceptible to pathogens. OsCPK7/OsCDPK13 are activated by a 3 h cold treatment (Abbasi *et al.*, 2004) and over-expression of either *OsCPK7/OsCDPK13* or *OsCPK13/OsCDPK7* confers cold tolerance in transgenic rice (Saijo *et al.*, 2000; Abbasi *et al.*, 2004).

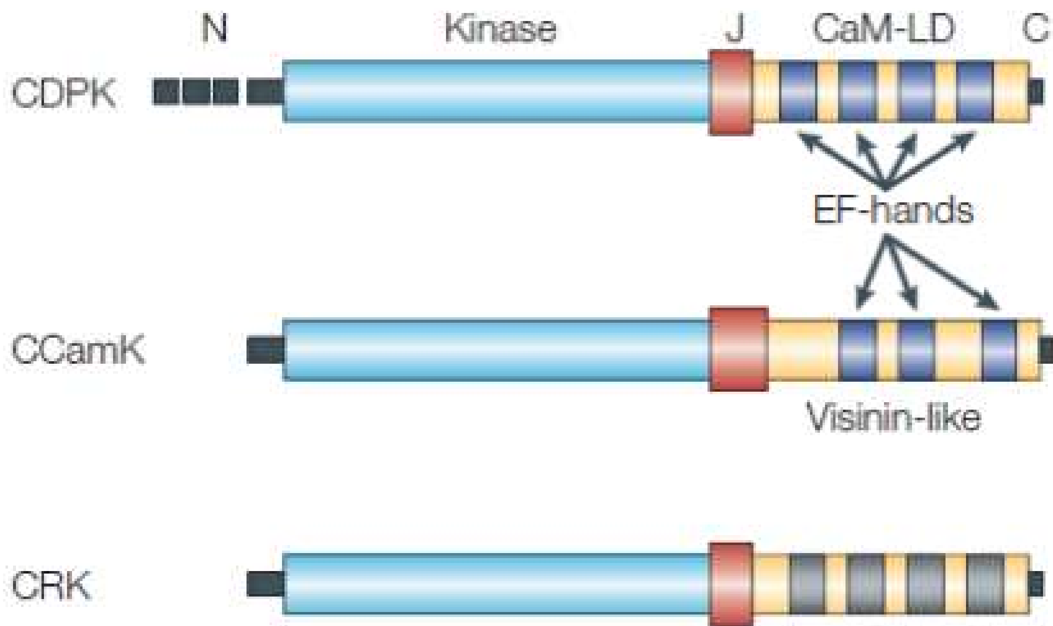


Figure 1.22: Structural differences between CDPK, CRK and CCaMK. All three kinases have five domains. The kinase catalytic domains of CDPKs and CDPK-related kinases (CRKs) are more closely related to each other. The junction (J) domain contains an auto-inhibitory pseudosubstrate sequence and a binding site for either intermolecular interaction with CaM in CCaMKs and some CRKs, or intramolecular interaction with the CaM-like domain in CDPKs or the visinin-like domain in CCaMKs. The main distinguishing feature of the three kinases is the domain that is adjacent to the junction domain. CDPK has a CaM-like domain, in which most isoforms have four predicted EF-hand Ca^{2+}-binding sites. The domain of CRK is related in sequence to that of CDPK, but has apparently degenerated and non-functional EF-hands. CCaMK has a visinin-like domain with three EF-hands. The C-terminal domain (C) of each kinase is short, and its function is unknown. CaM-LD: calmodulin-like domain (Harper and Harmon, 2005).

6.2.2. Ca^{2+} and Ca^{2+}/CaM-dependent protein kinases

Another classs of protein kinases are the CCaMKs (Ca^{2+}- and Ca^{2+}/CaM-dependent protein kinases) that are structurally very similar to CDPKs: their structure consist of N-terminal kinase domain of variable length, a conserved Ser/Thr kinase domain and a Ca^{2+}-binding domain at the C-terminus. However, unlike CDPKs, CCaMKs have a CaM-binding domain (CaMBD) adjacent to the kinase domain and visinin-like domain with three EF-hands (Figure 1.22). Additionally, CCaMKs encompass an autoinhibitory domain which overlaps the CaMBD (Ramachandiran *et al.*, 1997; Sathyanarayanan and Poovaiah, 2004). This leads to a complex regulatory mechanism involving Ca^{2+} and Ca^{2+}/CaM binding for the activation of CCaMK. Ca^{2+} binding to the visinin-like domain activates/accerlates autophosphorylation and increases CaM affinity which ultimately leads to autoinhibition and stimulates the activity of kinase (Sathyanarayanan and Poovaiah, 2004). CCaMKs have been reported in plant species and are present as a single gene in a number of species including tobacco, maize, *Lotus japonicus* and *Medicago trunculata*. But interestingly, there is no evidence for their presence in *Arabidopsis* (Harper *et al.*, 2004; DeFalco *et al.*, 2010). The majorities of studies on CCaMKs have been performed in legumes and suggest that CCaMKs play important roles in mediating symbiotic relationships with bacteria and fungi. In legumes, CCaMKs play a vital role in nodule morphogenesis through transmission of Nod factor-induced Ca^{2+} transients and are involved in the gene regulation essential for N_2 fixation (Gleason *et al.*, 2006; Tirichine *et al.*, 2006). So far, only one report showed the implication of CCaMKs in stress responses. In pea roots, nucleus-localized PsCCaMK exhibits high protein level after cold and salt stress (Pandey *et al.*, 2002).

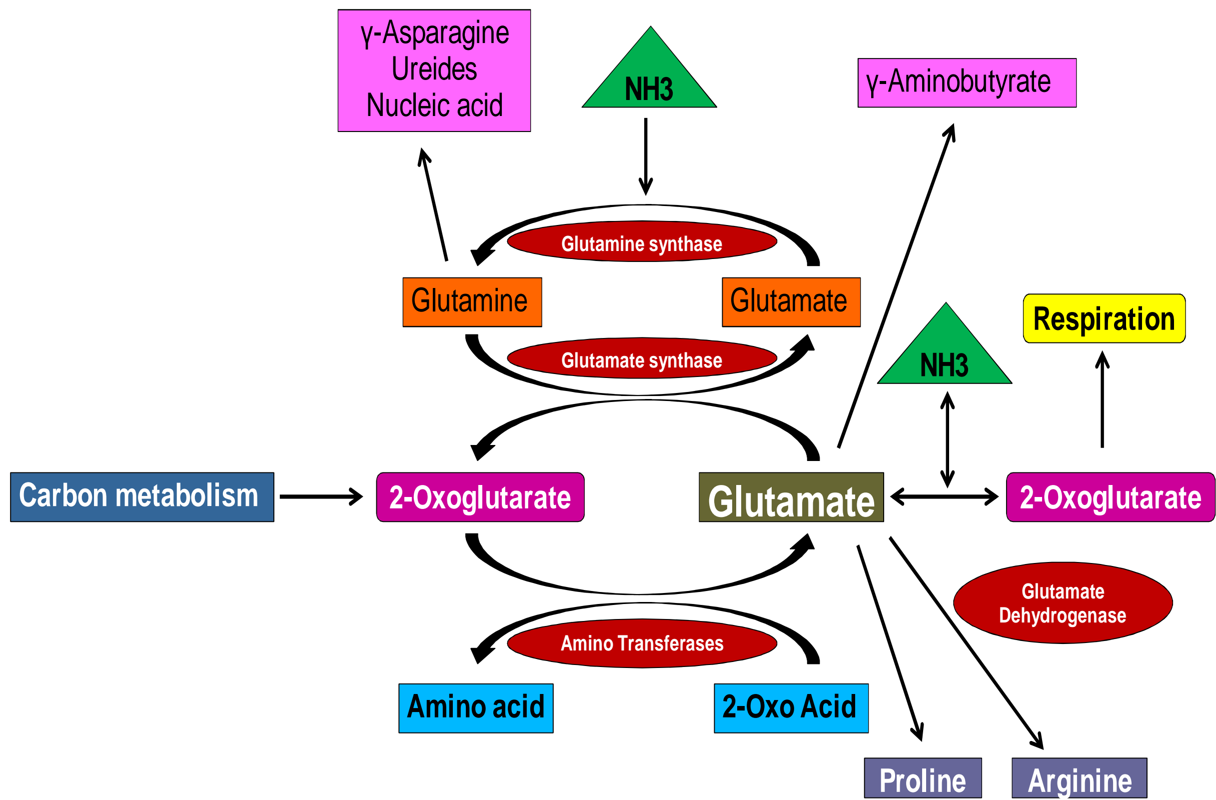


Figure 1.23: Glutamate biosynthesis and metabolism in plants. Glutamate is synthesized by glutamate synthase from glutamine. Glutamate is also converted to other amino acids (Arginine, Proline, Asparagine, Aminobutyrate and 2-oxoglutarate). Source: Forde and Lea (2007).

7. Glutamate receptors

7.1. Glutamate: a magical amino acid

There are 20 proteinogenic amino acids present in nature and glutamate (Glu), because of its unique chemical and biochemical properties, is one of the most exciting amino acid among them. Its chemical and biochemical properties make it suitable to participate in a wide range of biological processes both in animals and plants. It may serve the role of a metabolite, a nutrient, an energy source, a determinator of protein structures and even a signaling compound (Young and Ajami, 2000; Forde and Lea, 2007). The role of this magical amino acid in animals has been established for a long time, although its real importance in plant systems has been discovered only in recent past. Deciphering its role seems an exciting area of future research for plant biologists.

7.1.1. Glutamate metabolism in plants

In plants, Glu has a central position in amino acids metabolism in higher plants. *De novo* synthesis of Glu occurs via glutamine and 2-oxo-glutarate and the α-amino group of Glu plays a direct role in both in the assimilation and dissimilation of ammonia (NH_4), and through the action of various amino tranferases, amino group can be transferred to other amino acids (Dietrich *et al.*, 2010). During N assimilation in plants, Glu and glutamine (Gln) are the two important metabolites that are formed and they further lead to the production of other amino acids including aspartate (Asp) and asparagine (Asn; Stitt *et al.*, 2002; Walch-Liu *et al.*, 2006). Moreover, the α-amino group and the carbon skeleton both are involved in the synthesis of γ-aminobutyric acid (GABA), arginine and proline (Figure 1.23; Forde and Lea, 2007). On these basis, Glu has a vital role in plant N metabolism and is an essential precursor of chlorophyll biosynthesis in developing leaves (Young and Ajami, 2000; Lea and Miflin, 2003; Yaronskaya *et al.*, 2006).

Different enzymes participate in *de novo* synthesis of Glu. Among these, glutamate synthase that is also known as glutamine or 2-oxoglutarate amidotransferase (GOGAT) is a main participant in Glu synthesis. In this reaction, glutamine is transferred to 2-oxoglutarate to finally yield two Glu molecules. Two isoforms of this enzyme exists in higher plant: Fd-GOGAT that uses reduced ferredoxin (Fd) as the electron donor and NADH-GOGAT that uses that uses NADH as the electron donor. Fd-GOGAT is a 130-l80 KDa iron-sulphur flavoprotein and generally functions as a monomer. This enzyme is thought to be involved in the photosynthetic activities in the chloroplasts where it uses the light energy directly as a

supply of reductant. In *Arabidopsis*, GLU1 and GLU2 are the two genes encoding functional Fd-GOGAT and are preferentially expressed in leaves and root tissues, respectively, suggesting the presence of two distinct isoforms of Fd-GOGAT in plants (Forde and Lea, 2007). On the other hand, NADH-GOGAT is also reported to be present in chlorpolasts and is preferentially expressed in non-photosynthesizing cells, where reductant is supplied by the pentose phosphate pathway (Bowsher *et al.*, 2007).

In addition, a third enzyme known as Glu dehydrogenase (GDH) also takes part in Glu synthesis. GDH catalyses a reversible amination/deamination reaction and could leads to either the synthesis or the catabolism of Glu. In plants, GDH role in Glu metabolism is a subject of debate since long time (Dubois *et al.*, 2003; Tercé-Laforgue *et al.*, 2004). However, the combine use of different techniques like overexpression and antisense strategies, gas chromatography– mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) led to the establishment of a clearer function of this enzyme during Glu metabolism. It has been reported that GDH participates in the synthesis of Glu in tobacco leaves (Masclaux-Daubresse *et al.*, 2006). Indeed, Glu is synthesized via the combined action of Gln synthetase (GS) and Glu synthase while GDH is implicated in the deamination of Glu. Moreover, in response to biotic or abiotic stresses, GAD used Glu for the synthesis of GABA (Shelp *et al.*, 1999; Bouché and Fromm, 2004; Bown *et al.*, 2006).

7.1.2. Glutamate homeostasis in plants

Various previous studies have stated the low [Glu] variations in different plant species during changing growth conditions. For example, in tobacco leaves under changing growth conditions, [Glu] were found between 3 to 4 µmol g^{-1} FW throughout the day, whilst [Gln] fluctuated between 5 to 15 µmol g^{-1} FW (Geiger *et al.*, 1998). In another study with tobacco plants grown on 2 mM nitrate, Glu contents were observed constant at 4 µmol g^{-1} FW during the light/dark cycle (Matt *et al.*, 2001). In contrast, Gln contents varied between 5 to 15 µmol g^{-1} FW under same conditions. In the same way, Masclaux-Daubresse *et al.* (2002) showed a slight change in [Glu] during light and dark periods. In the older tobacco source leaves, Glu was present in the range of 1100–1400 nmol mg^{-1} chlorophyll whereas in younger sink leaves of tobacco, the [Glu] were observed 1600 nmol mg^{-1} chlorophyll and 900 nmol mg^{-1} chlorophyll during day and night conditions, respectively. In potato leaves, again a stable concentration of Glu was detected (1.0–1.3 µmol g^{-1} FW) whilst Gln, Asp, alanine (Ala) and glycine (Gly) showed higher variations (1–3 µmol g^{-1} FW; Urbanczyk-Wochniak *et al.*, 2005). Finally, in a general study, 137 different metabolites in *Arabidopsis* rosettes were

verified for their contents, during a 12/12 h light/ dark cycle. Most of these metabolites including the amino acids showed significant diurnal changes while Glu exhibited very small oscillations (Gibon *et al.*, 2006). These data clearly suggest that Glu homeostasis is efficiently regulated in plants possibly through the combine action of different regulating pathways. In favour of this concept, Masclaux-Daubresse *et al.* (2006) have suggested that GS and GDH are the two major enzymes that regulate Glu homeostasis in plants.

7.1.3. Glutamate signaling

Glu has been firmly established as a major signaling molecule in the mammalian central nervous system (CNS) where it is involved in the excitory transmission (Watkins and Jane, 2006). As far as the plants are concerned, different evidences suggest that Glu signaling also occurs in plants (Chiu *et al.*, 1999; Davenport, 2002; White *et al.*, 2002; Lam *et al.*, 2006). The most important impact of Glu both in plants and animals is the activation of glutamate receptor proteins that function as plasma membrane ligand-gated Ca^{2+} permeable channels and are involved in a series of biological responses.

In the following sections, an overview of these channel proteins along with their classification and putative involvement into different physiological processes in animals and plants will be presented.

7.2. Glutamate receptors in animals

In animals, study of the central nervous system (CNS) has led to the characterization of two types of glutamate receptors (GluR): ionotropic type of glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). These two types have some common features *e.g*. both are able to bind Glu and this binding influences the ion channel permeability. At the same time, some differences also exist between two types (Howe, 1999). In addition to their presence in CNS, both types of GluRs have also been reported in many non-excitable cells, particularly in the immune cells like dendritic cells, neutrophils, macrophages, stem cells, lymphocytes and T-cells (Nedergaard *et al.*, 2002; Pacheco *et al.*, 2007; Yawata *et al.*, 2008). In addition to brain tissues, GluRs are widely distributed in many other tissues like blood vessels, oocytes, pancreatic islets, lungs and spleen (Gill and Pulido, 2001).

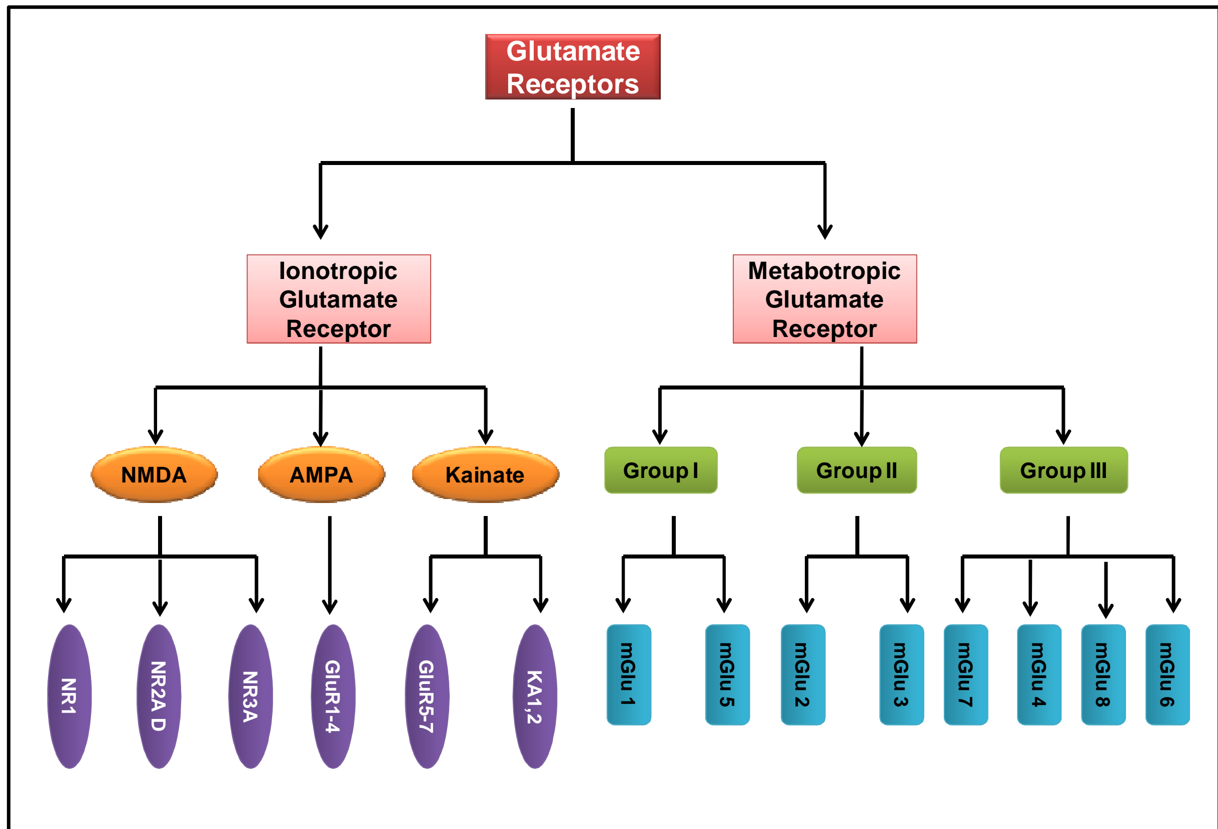


Figure 1.24: Classification of glutamate receptors. Ionotropic receptors also referred to as ligand-gated ion channels (LGICs) that are involved in the movement of Na^{+} and Ca^{2+} across the post synaptic plasma membrane. These are multi-meric assembly of 4-5 subunits. Ionotropic receptors are subdivided into 3 groups; each group has 1 to 3 subgroups and contain 1-4 members. Metabotropic receptors or G protein-coupled receptors are indirectly linked with ion-channels on the plasma membrane of the cell. They are divided in 3 groups and each group contains 2 or 4 members.

7.2.1. Ionotropic glutamate receptors

In animals, as a signaling molecule, Glu is able to activate iGluRs which are non selective cation channels (NSCC) and are involved in the movement of Ca^{2+} across the post-synaptic plasma membrane. They have been extensively studied from their structural and functional aspects and their role in neurotransmission and immunotransmission has been firmly established (Gill and Pulido, 2001; Skerry and Genever, 2001; Boldyrev *et al.*, 2005; Pacheco *et al.*, 2007; Rousseaux, 2008). The iGluRs are essential components of cell-cell communication in nervous systems (Dingledine *et al.*, 1999). In the past, different pharmacological and electrophysiological approaches were adapted to investigate the specific types of iGluRs. Based on their sequence similarities, electrophysiological properties and their affinity to bind with specific Glu agonists, iGluR were divided into three major subtypes: *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate (KA; Dingledine *et al.*, 1999; Rousseaux, 2008). Different classes of GluR antagonist are available: they can be classified as competitive and non-competitive or specific and non specific. Following are some examples of iGluRs antagonists. DNQX (6,7-dinitriquinoxaline-2,3 dione) and CNQX (6-cyano-7-nitroquinoxaline- 2,3-dione) are competitive AMPA/KA receptor antagonists while MNQX (5,7-Dinitro-1,4-dihydro-2,3-quinoxalinedione) is a competitive antagonists for NMDA receptors. AP-7 (2-amino-7-phosphonoheptanoic acid) and AP-5 (D-2-amino-5-phosphono pentanoic acid) are selective NMDA receptor antagonists. Moreover, MK-801 (5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) and Memantine (1-amine-3,5-dimethyladamantan) are non-competitive antagonists targeting NMDA receptors (Dingledine *et al.*, 1999; Bräuner-Osborne *et al.*, 2000). These iGluRs antagonists have been used to characterize different GluR channels in both plants and animals.

Animal GluRs exist as homo- or hetero-tetramers by joining same or different subunits with specific physiological and pharmacological properties (Rousseaux, 2008). AMPA and NMDA form heterotetramer assemblies of different subunits (Mansour *et al.*, 2001). The AMPA and KA receptors have ubiquitous expression throughout the CNS at varying levels whereas the expression of NMDA receptors has been observed in the forebrain. NMDA receptors are able to mediate excitatory neurotransmission in the CNS. NMDA receptors are more permeable to Ca^{2+} than AMPA and KA that exhibit more preference to Na^{+} and K^{+} (Hollmann and Heinemann, 1994; Dingledine *et al.*, 1999). NMDA receptors contain a number of distinct binding sites including a binding site for Gly or D-serine (Ser) along with

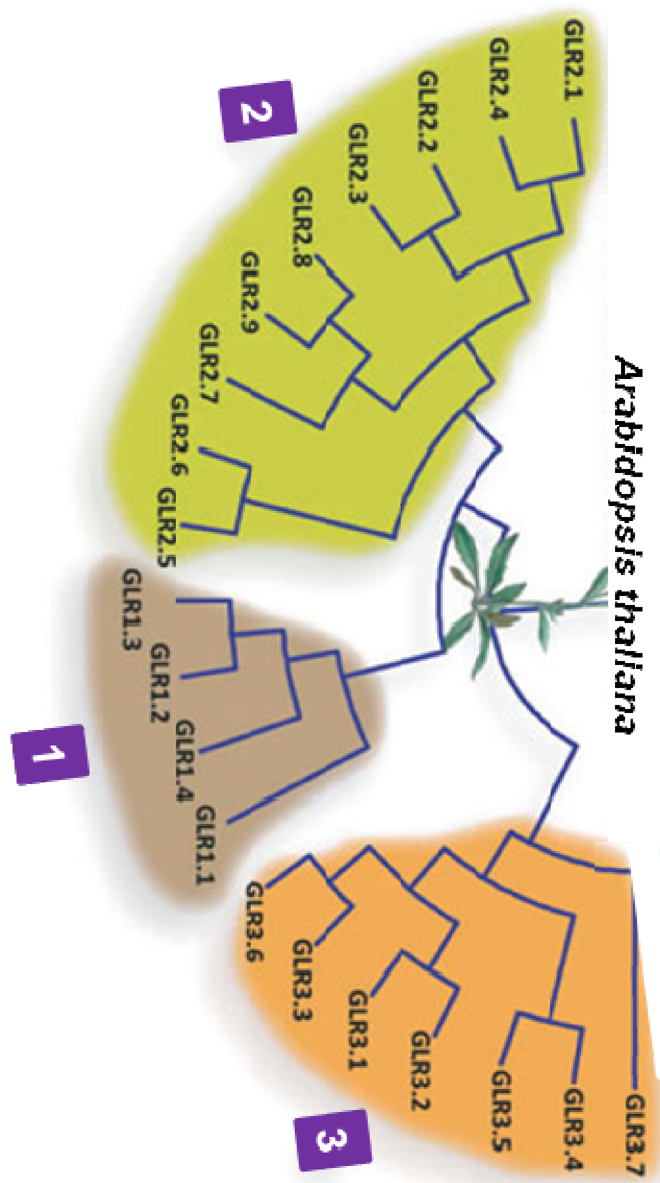


Figure 1.25: Phylogenetic relationship of glutamate receptor (GLR) family of *Arabidopsis*. GLRs family is subdivided into three distinct classes on the basis of their sequence similarity (adapted from Dietrich *et al*., 2010).

the recognition site for Glu (Hara and Snyder, 2007). The iGluRs are further subdivided into different subgroups (Figure 1.24).

7.2.2. Metabotropic glutamate receptors

The second type of glutamate receptors named as mGluRs has also been reported in the CNS. The activity of mGluRs was coupled to small GTP-binding proteins and mGluRs were assigned modulatory role in synaptic activity. In contrast to iGluRs, they were suggested to impose slower and longer effects (Nakanishi, 1992; Watkins, 2000). Along with $GABA_B$ receptors, mGluRs are member of G-protein coupled receptors (GPCRs) that are implicated in the activation of Ca^{2+} and K^+ channels (Chen *et al.*, 1995; Hara and Snyder, 2007). These receptors are divided into three different groups on the basis of their sequence homology and G-protein coupling (Hara and Snyder, 2007). Group I is composed of mGluR1 and mGluR5 and both these are postsynaptic. Group I receptors are coupled to the excitatory G_q protein and act by stimulating phospholipase C (PLC) to produce IP_3, along with Ca^{2+} release. Moreover, mGluR5 receptors are physically linked to NMDA receptors by a chain of anchoring proteins, including PSD-95 (postsynaptic density 95). Group II include mGluR2 and mGluR3 receptors that are linked to the inhibitory G_i/G_o proteins (Pin and Acher, 2002). Both mGluR2 and mGluR3 have been reported to locate on glutamatergic neurons terminals and glial cells, respectively. Finally, group III receptors such as mGluR4, mGluR6, mGluR7, and mGluR8 are coupled to G_i/G_o proteins showing a resemblance with group II. Both the Group II and Group III receptors lead to the inhibition of adenylyl cyclase (Hara and Snyder, 2007).

7.3. Plants glutamate receptor-like homologs

In plants, GLRs have been implicated in signaling of various physiological processes (Forde and Lea, 2007). GLRs have been identified in different monocotyledon and dicotyledon species of higher plants and showed amino acid sequence similarities related to animal iGluRs (Lam *et al.*, 1998; Ward *et al.*, 2009; Aouini *et al.*, 2012). These receptors genes encode proteins that are considered to be involved in plasma membrane non-selective cation fluxes especially Ca^{2+}. The *Arabidopsis* genome encodes 20 members of GLRs (AtGLRs) which are grouped into three clades and contain all the signature domains of animal ionotropic GluRs (NMDA type) including: three TMDs (S1-S3), a pore loop and two putative ligand binding motifs (Lam *et al.*, 1998; Lacombe *et al.*, 2001; Chiu *et al.*, 2002; Davenport, 2002). A phylogenetic tree of different classes of GLRs is presented in figure 1.25 (Adapted from Dietrich *et al.*, 2010). Gilliham *et al.* (2006) reported that all 20 AtGLR genes were

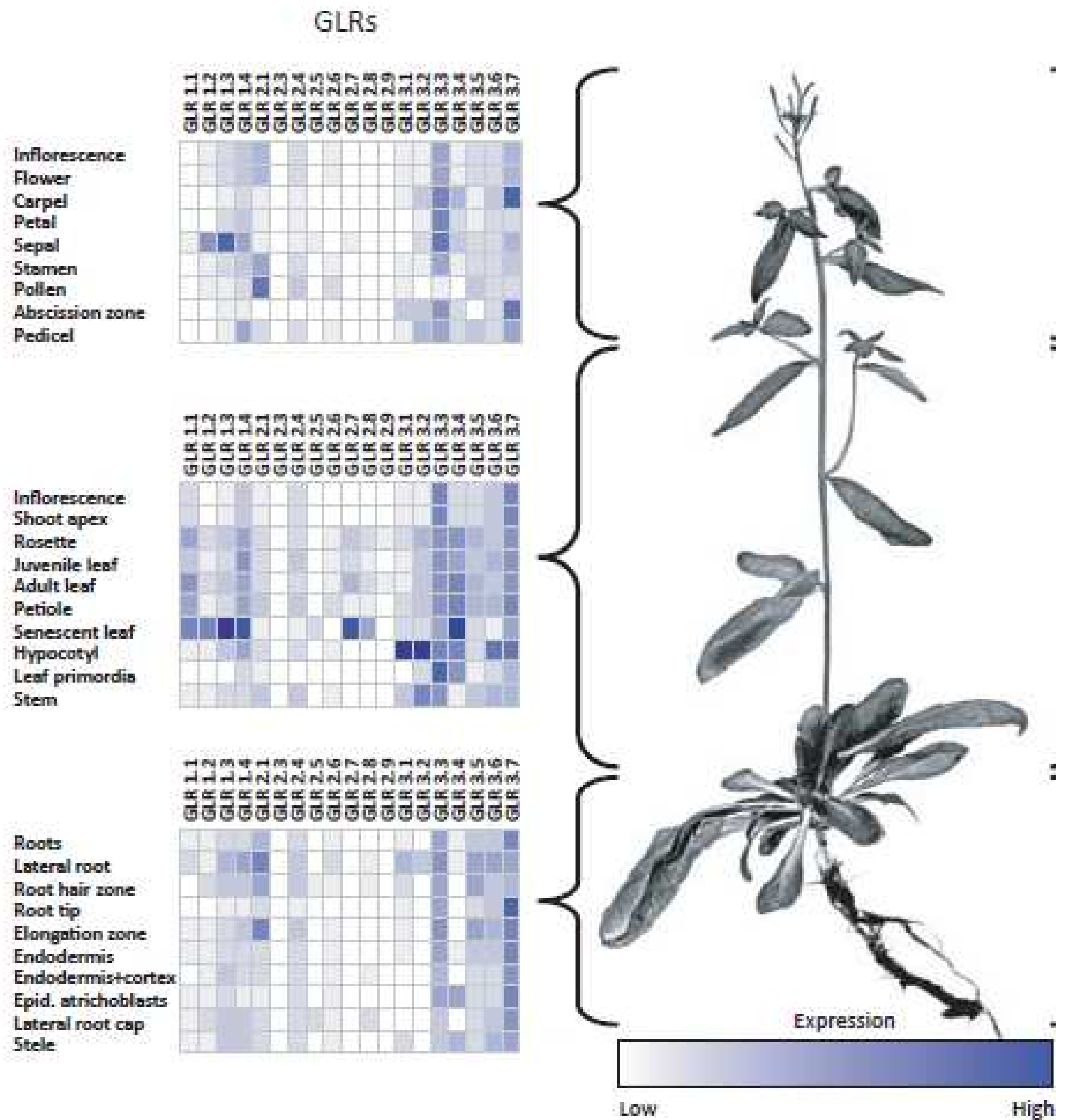


Figure 1.26: Expression patterns of 20 members of GLRs family channel in *Arabidopsis*. Expression tables are adopted from Genevestigator database (adapted from Dietrich *et al.*, 2010).

transcribed and some splice variants were also detected. This leads to a further addition to the total number of potential AtGLR gene products in *Arabidopsis.* Besides *Arabidopsis*, 13, 61 and 13 GLRs genes have been identified in rice, poplar and tomato, respectively (Ward *et al.*, 2009; Aouini *et al.*, 2012). The phylogenetics studies have suggested that the divergence of AtGLRs occured long ago from the divergence of GluRs into different classes (NMDA, APMA, KA) (Lacombe *et al.*, 2001). This could be further explained by different investigations made in the past. For example, it has also been reported that different GluRs antagonist like DNQX, CNQX, MK-801 and AP-5 are more or less equally efficient to block channel activities in plants (Dubos *et al.*, 2003; Qi *et al.*, 2006; Kwaaitaal *et al*., 2011; Michard *et al.*, 2011; Vatsa *et al.*, 2011) but at comparatively higher concentrations than those used in animals. In the same way, difference also exists concerning the ligand needed to activate these receptors. In animals, Gly is compulsory for the activation of NMDA receptors while plant does not really showed this type of obligation (Hardingham and Bading, 2003; Stephens *et al.*, 2008). Moreover, differences in the putative pore region have been observed between plant GLRs and animal GluRs: This can modify GLRs ion channel activity and selectivity for the transportations of new ions (Davenport, 2002).

The existence of Glu-activated ion channels in plants and their similarity to animal iGluRs gene family indicate that Glu-mediated signaling pathways could also be extended to plants (Forde and Lea, 2007). In the same way, the large diversity of plant GLRs *e.g.* 20 in *Arabidopsi*s, 13 in rice and tomato each, and 61 in poplar clearly suggest their possible implication in a wide range of plant physiological processes.

7.3.1. Expression patterns of GLRs in plants

In order to investigate the expression patterns of GLRs in plants, two comprehensive studies were conducted by Chiu *et al.* (2002) and Roy *et al.* (2008). In these studies, RT-PCR analyses of all the *Arabidopsis* GLRs were conducted and no clade-specific expression in organs was detected (Chiu *et al.*, 2002), although clade 2 genes were mostly expressed in roots, suggesting a possible involvement of clade 2 GLRs in ion transport and uptake (Chiu *et al.*, 2002). It should be noted that uptake of Ca^{2+} occurs through plant roots and then Ca^{2+} is translocated to other plant parts (Yang and Jie, 2005). All the 20 genes were detected in roots (Chiu *et al.*, 2002), however, microarray analyses indicated a low level of most of the genes in different organs (Figure 1.26). Using a single cell sampling technique, no consistent pattern of co-expression or cell-type specific expression was detected (Roy *et al.*, 2008). However, AtGLR3.7 was detected in every cell type of the leaves (Roy *et al.*, 2008).

Table 1.6: Role of glutamate receptors in plants. A brief description of different GLRs is presented in the following table. R: root; Rt: root tip; St: stem; P: petiole; L: leaf; H: hypocotyl; C: cotyledon; Fl: flower; Inf: inflorescence; S: seed; Sh: shoot; Sil: silique; Vsc: vascular tissue; GC: guard cell; Pol: pollen; nd: not determined (Modified according to Dietrich *et al.,* 2010 and Jammes *et al.,* 2010).

Gene name	Expression pattern by RTqPCR	Other ion permeability	Physiological role	References
AtGLR1.1 At3g04110	R, St, P, L, S	Na^+, K^+	Carbon/nitrogen sensing; ABA biosynthesis, signaling and metabolism	Chiu *et al.*, 2002; Tapken and Hollmann, 2008; Kang and Turano, 2003; Kang *et al.*, 2004
AtGLR1.2 At5g48400	R, St, P, L, Fl, S	Nd	Pollen tube growth	Michard *et al.*, 2011
AtGLR1.4 At3g07520	R, St, P, L, Fl, S	Na^+, K^+	Have functional Na^+ and K^+ and Ca^{2+}-pereable ion pore domains	Chiu *et al.*, 2002;Tapken and Hollmann, 2008
AtGLR2.1 At5g27110	R, St, P, L	Nd	Nd	Chiu *et al.*, 2002
AtGLR2.4	Ubiquitous			Mustroph *et al.*, 2009
AtGLR3.1 At2g17260	R, St, P, L, Fl, S, GC	Nd	Regulation of cytosolic Ca^{2+} oscillations during Ca^{2+}-induced stomatal closure	Chiu *et al.*, 2002; Cho *et al.*, 2009
AtGLR3.2 At4g35290	R, St, P, L, Fl, S, Vsc	Nd	Calcium utilization/ion stress	Kim *et al.*, 2001; Chiu *et al.*, 2002
AtGLR3.3 At1g42540	R, St, P, L, Fl, S	Nd	Root gravitropism	Roy *et al.*, 2008; Qi *et al.*, 2006; Stephens *et al.*, 2008; Miller *et al.*, 2010
AtGLR3.4 At1g05200	R, St, P, L, Fl, S	Nd	Touch and cold responses	Meyerhoff *et al.*, 2005; Stephens *et al.*, 2008
AtGLR3.7 At2g32400	R, St, P, L, Fl, S	Na^+, Ba^+	Pollen tube growth	Roy *et al.*, 2008; Michard *et al.*, 2011
OsGLR3.1 Os04g49570	R, L	Nd	Cell division and survival	Li *et al.,* 2006
RsGLR	Nd	Nd	Ca^{2+} influx and resistance to fungi	Kang *et al.*, 2006

7.3.2. Role of GLRs in plants

In plants, GLRS have gained much intention after the discovery of ionotropic type GLR homologs in *Arabidopsis* (AtGLRs; Lam *et al.*, 1998; Lacombe *et al.*, 2001). Plant GLRs have been reported to be implicated in many different physiological processes including signal transduction, ion transport, growth processes, and adaptation to biotic and abiotic stresses (for review; Dietrich *et al.*, 2010; Jammes *et al.*, 2011).

Following section will be oriented towards different aspects of GLRs roles in plant processes. An overview of different functions of plant GLRs is presented in table 1.6.

7.3.2.1. Role in Ca^{2+} signaling

Various studies have demonstrated that Glu is able to activate GLRs-dependent Ca^{2+} signaling in plants. GLRs activation triggered [Ca^{2+}] variations with a subsequent regulation of other Ca^{2+} dependent processes (Dennison and Spalding, 2000; Dubos *et al.*, 2003; Demidchik *et al.*, 2004; Meyerhoff *et al.*, 2005; Kang *et al.*, 2006; Qi *et al.*, 2006; Stephens *et al.*, 2008; Michard *et al.*, 2011). In *Arabidopsis* plants, Glu is able to induce a very large and fast change in $[Ca^{2+}]_{cyt}$ that is subsequently followed by a large transient membrane depolarization (Dennison and Spalding, 2000). Moreover, Glu-induced $[Ca^{2+}]_{cyt}$ variations were strongly suppressed by DNQX and CNQX treatments (Meyerhoff *et al.*, 2005). Similar results were obtained by Dubos *et al.* (2003) who also proposed synergistic effects of Gly with Glu to control ligand-mediated Ca^{2+} signaling. Through genetic approach, Qi *et al.* (2006) reported that loss-of-function mutant *glr3.3* in *Arabidopsis* was unable to produce Glu-dependent $[Ca^{2+}]_{cyt}$ changes in root cells. It was demonstrated that GLRs could be organized in different channel subtypes with at least one AtGLR3.3 subunit required for Ca^{2+} flux and membrane depolarization in *Arabidopsis* (Stephens *et al.,* 2008). In another study, overexpression of *AtGLR3.1* resulted in impaired external Ca^{2+}-induced stomatal closure, suggesting a role of GLRs in stomatal guard cells Ca^{2+} signaling (Cho *et al.*, 2009). In a recent work, Vatsa *et al.* (2011) demonstrated the generation of Glu-induced Ca^{2+} influx and $[Ca^{2+}]_{cyt}$ elevations that were significantly inhibited by GluRs antagonist treatments. More recently, it was proved that GLRs are involved in the generation of Ca^{2+} influx oscillations in pollen tubes induced by D-Ser that were inhibited by CNQX and DNQX applications in tobacco and *Arabidopsis* (Michard *et al.*, 2011).

7.3.2.2. Role in light-dependent signaling and growth processes

It has been reported by Walch-Liu and colleagues (2006) that even micromolar concentrations of exogenous Glu are able to trigger important changes in the morphology of *Arabidopsis* roots. A prominent inhibition of primary root growth and stimulation in the root branching near the root apex region was observed after Glu treatment even when applied at low concentrations. This effect is thought to be the result of the inhibition of meristematic activity at the primary root tip and an early activation of lateral root branching at the root apex region of the primary roots. More interestingly, these effects were found to be genotype specific in *Arabidopsis*, as C24 (Columbia-24) was observed most sensitive while RLD1 was found least sensitive (Walch-Liu *et al.*, 2006). This specificity of Glu suggested that the changes in the apoplastic L-glutamate concentration could be evaluated by the root tip. Later on, other studies have proved that root tip region is the place of Glu sensing and signaling where it is antagonized by nitrate (Walch-Liu and Forde, 2008). Various studies have shown the involvement of GLRs in light-dependent signaling and root morphology (Lam *et al.*, 1998; Brenner *et al.*, 2000; Dubos *et al.*, 2003; Dubos *et al.*, 2005; Li *et al.*, 2006; Walch-Liu *et al.*, 2006). In 1998, Lam *et al.* demonstrated that DNQX application of *Arabidopsis* plants resulted in the impairment of light signal transduction in two ways. First, *Arabidopsis* long-hypocotyl (*hy*) mutants were defective in light-induced hypocotyl growth inhibition. Secondly, chlorophyll synthesis was also impaired in plant grown under light conditions. Interestingly, Glu and/or Gly treatments reversed this phenotype. Moreover, treatments of *Arabidopsis* plants with BMAA [(S(+)-β-methylalpha,β-diaminopropionic acid], a cycad-derived iGluR agonist, also exhibited light-specific effects on hypocotyl elongation. An increase in hypocotyl elongation and inhibition of cotyledon opening were the outcomes of BMMA treatments (Brenner *et al.*, 2000). Interestingly, kanamycin and polyamines which are inhibitors of NMDA type iGluRs, agonized the AtGLRs and rescued the *de-etiolated3* (*det3*) mutant phenotype. Similar effect was observed when Glu and Gly were applied together (Dubos *et al.*, 2005). Mutation in a rice *OsGLR3.1* revealed a short root phenotype with distorted meristematic activity and enhanced programmed cell death, thus predicting the involvement of OsGLR3.1 in cell division and processes (Li *et al.*, 2006). Recently, Miller *et al.* (2010) reported the slow root turning of *atglr3.3* mutant plants thus exhibiting defects in gravitropism response. GLRs implications in carbon (C) and nitrogen (N) metabolism have been reported. When *Arabidopsis* seeds expressing an antisense construct of AtGLR1.1 were grown in the presence of sucrose, no germination was observed but this germination was

rescued with nitrate treatment, suggesting the role of ATGLRA.1 in C and N balancing in *Arabidopsis* (Kang and Turano, 2003).

7.3.2.3. Role in abiotic stresses

GLRs have been reported to participate in different abiotic stress responses in plants. *AtGLR1.1* antisense plants showed ABA hypersensitivity symptoms such as reduced stomatal apertures and resulted in the down-regulation of the type 2C protein phosphatises ABI1 and ABI2, two negative regulators of ABA signaling (Kang *et al.*, 2004). Work from Kim *et al.* (2001) have provided evidences that constitutive overexpression of *AtGLR3.2* altered Ca^{2+} homeostasis in transgenic plants and showed hypersensitivity responses to Na^+ and K^+ ionic stresses. Meyerhoff and colleagues (2005) showed that *AtGLR3.4* expression is stimulated in response to touch, osmotic stress and cold stimuli in a Ca^{2+}-dependent manner and ABA-independent manner.

7.3.2.4. Role in biotic stresses

In the recent past, different investigations using pharmacological and genetic approaches have strongly suggesting the role of GLRs in plant defense responses, an aspect that has firmly established for animal GluRs.

Studies with knock-out rice mutant have shown the enhanced programmed cell death in the apical meristem area of root cells in *Osglr3.1* mutant plants, suggesting a role of OsGLR3.1 in cell survival (Li *et al.*, 2006). Similarly, overexpression in *Arabidopsis* of a radish GLR, homologue to *AtGLR3.2*, exhibited Ca^{2+} deficiency symptoms such as leaf tip and margin necrosis, dwarf size with multiple secondary inflorescences, retarded growth and more importantly, these overexpressing plants showed an enhanced resistance to *B. cinerea* (Kang *et al.*, 2006). It was recently reported that GLRs were involved in elicitor-induced plant defense signaling in tobacco and *Arabidopsis* (Kwaaitaal *et al.*, 2011; Vatsa *et al.*, 2011). In tobacco cells, Cry-induced Ca^{2+} influx and $[Ca^{2+}]_{cyt}$ variation was inhibited by GluRs antagonists and NO production, a downstream event in elicitor-dependent signaling was also affected (Vatsa *et al.*, 2011). Many previous reports have suggested the involvement of elicitor-mediated NO production in plant responses to stress (Besson-Bard *et al.*, 2008a). Similarly, partial inhibition of $[Ca^{2+}]_{cyt}$ variations induced by the flg22, elf18 or chitin elicitors were observed in *Arabidopsis* plants treated with GluRs antagonists (Kwaaitaal *et al.*, 2011). Moreover, MAPK activation and the accumulation of defense gene transcripts, two

important elements of plant defense signaling, were found to be regulated by GLRs (Kwaaitaal *et al*., 2011).

8. Working models (plants/pathogens/elicitors)

In order to study the role of Ca^{2+} in plant defense signaling pathways, appropriate selection of plant biological material and patho-systems/elicitors is really important. During this thesis work, two well-established models were used. Following section will explain in more detail the reason behind their selection.

8.1. Tobacco-cryptogein model

8.1.1. *Phytophthora*

Phytophthora is a very important genus of microorganisms that has approximately 500 species and belongs to order Peronosporales of the class oomycetes (water molds). It is a Greek word that means 'plant destruction'. Although *Phytophthora* have many morphological similarities with true fungi yet they have very distinct evolutionary history and have been placed in a separate kingdom Chromalveolata. Many species of *Phytophthora* are potential pathogens of different agricultural crops, ornamentals and native plants and are responsible for a huge loss in crop productivity every year. In addition to damage the crops and economy, they also cause the environmental destruction in natural ecosystems. The *Phytophthora* species (*spp.*) are considered as the most devastating pathogenic microorganisms of dicotyledonous plants such as potato, tomato, soybean *etc* (Erwin and Ribeiro, 1996; Kamoun, 2003). Following are some examples of diseases in different plants that are caused by *Phytophthora spp*. *P. infestans* is a very well known destructive pathogenic oomycete in this class. It is a causal agent of potato late blight disease and led to famous potato blight famine in the mid-nineteenth century in Europe, Ireland (1845) and Highland (1846). During 1990s, a new strain of *P. infestans*, which was more aggressive and was fungicide-insensitive, appeared in Europe and North America thus resulted in a severe loss of potato and tomato crops (Kamoun, 2001; Garelik, 2002). *P. parasitica* is known to cause Blackfoot disease of tobacco plants (Csinos and Hendrix, 1977; Ricci *et al.*, 1989). Moreover, *P. sojae* is a harmful pathogen for soybean where it causes root and stem rot disease (Schmitthenner, 1985). Recently, *P. ramorum,* another *Phytophthora spp.* that is responsible for the spread of Sudden Oak Death diseases, has been reported to infect and kill tens of thousands of oak trees and ornamental plants in the southern United States, primarily in California and Oregon (Grünwald *et al.*, 2008). It is very difficult to overcome these diseases by chemical measures and the creation of disease resistance cultivars seems an appropriate solution against *Phytophthora.*

Interestingly, some *Phytophthora spp.* such as *P. cryptogea* did not result in any disease symptoms rather led to dwarfism and the appearance of localized cell death in tobacco plants, thus suggesting the implication of this *Phytophthora* strain in plant resistance (Csinos and Hendrix, 1977). More remarkably, pretreatment of tobacco plants with *P. cryptogea* and a subsequent infection by *P. parasitica* showed very interesting results. Tobacco plants exhibited resistance symptoms by the induction of HR and SAR (Csinos and Hendrix, 1977). Later studies have demonstrated the important role of *P. cryptogea* in the establishment of plant defense response (Ricci *et al.*, 1989). Indeed, *P. cryptogea* produce some proteins, known as elicitins, which are responsible for the induction of defense responses in tobacco (Bonnet and Rousse, 1988). Due to numerous species and wide host range, *Phytophthora spp.* are outstanding tool for the study of host pathogen interactions.

8.1.2. Elicitins

Many studies have disclosed that *Phytophthora* secrete a family of structurally related proteins called elicitins (Baillieul *et al.*, 2003). They are 10 KDa globular proteins comprising 98 amino acids. They have 70 % sequence similarities with other unrelated forms. Structurally, six conserved cysteine residue at position 3, 27, 51, 56, 71, and 95 form a disulfide bond that is essential for their activity (Boissy *et al.*, 1996; Fefeu *et al.*, 1997; Baillieul *et al.*, 2003; Figure 2.2B). The elicitins can be grouped into five classes based on their primary structure (Baillieul *et al.*, 2003). Based on their isoelectric point (pI), elicitins can be divided into two groups: 1) α-elicitins, with a pI below 5, which have few or no necrotizing properties and 2) β-elicitins that have the pI greater than 7.5, and are very necrotic and induce better protection in host plant (Ricci *et al.*, 1989). All *Phytophthora spp.* contain genes encoding elicitins. Specifically, α-elicitins are produced by all *Phytophthora* species, however, β-elicitins are secreted by a less number of species (Ponchet *et al.*, 1999).

The three-dimensional structure of elicitins was demonstrated from Cry through X-ray diffraction (Boissy *et al.*, 1996) and nuclear magnetic resonance (Fefeu *et al.*, 1997). It is comprised of five α-helices, one β sheet and one ω-loop (Boissy *et al.*, 1996). Interestingly, ω-loop structure is highly conserved and defines a hydrophobic site. The hydrophobic site of elicitins has the ability to bind plant sterols with rather high affinity (Mikes *et al.*, 1997). Furthermore, it was studied that Cry catalyzes the transfer of sterols between biological membranes (Mikes *et al.*, 1998; Vauthrin *et al.*, 1999), indicating the role of elicitins to seize/capture the sterols from host plant membranes to supply the micro-organism (Ponchet *et al.*, 1999). As it was reported, the *Phytophthora* are unable to synthesize sterols necessary for

their reproduction. This function supports the conservation of genes encoding different elicitins in *Phytophthora Spp.* during evolution.

In the past, role of elicitins has been tested on different plant families. On elicitin treatment, only a few species of the Brassicaceae family, such as radish or rapeseed varieties develop HR type responses (Kamoun *et al.*, 1993; Ponchet *et al.*, 1999). Moreover, species of the genus *Nicotiana* are able to develop HR and SAR. All elicitins secreted by *Phytophthora* are capable to induce defense reactions in tobacco. These responses include HR when elicitins (basic) are infiltrated in leaves (Kamoun *et al.*, 1993) and the SAR when applied to the stem of decapitated tobacco (Bonnet *et al.*, 1996).

8.1.3. Cry, an elicitor of defense reactions in tobacco

Cry is a 10 KDa elicitin of β class produced by *P. cryptogea* which confers protection to tobacco plants against subsequent infection by other pathogenic strains (Ricci *et al.*, 1989). It displays only the elicitin domain of 98 amino acids (Figure 2.2 in Materials and Methods Chapter 2). Previous studies have demonstrated that Cry is one of the most reactive elicitins in terms of induction of defense responses in tobacco (Bourque *et al.*, 1998; Lecourieux *et al.*, 2002). It is able to induce HR and SAR responses in tobacco. Previous studied have shown that the establishment of SAR requires SA pathway and an accumulation of *PR* genes in both local and systemic tissues (Keller *et al.*, 1996a, b). Moreover, some other defense mechanisms *e.g*. peroxidation of membrane lipids, ET production and synthesis of phytoalexins have been reported in the developing areas of HR after treatment with Cry (Milat *et al.*, 1991; Rustérucci *et al.*, 1996). In addition to these defense responses, cellulose deposition at the infection site, to limit the intracellular penetration of the microorganism was demonstrated after pretreatment of tobacco plants with Cry and infection by *P. parasitica* (Lherminier *et al.*, 2003).

8.1.4. Cry-induced signaling in tobacco

The tobacco cell suspensions are a biological model of great interest to understand the cellular signaling events underlying the activation of defense responses triggered by Cry. These events are summarized in figure 1.6 (Garcia-Brugger *et al.*, 2006). In tobacco, Cry, in its mode of action, is recognized by high affinity binding site located on the plasma membrane (Bourque *et al.*, 1999). This results in a large calcium influx which is necessary for the induction of many downstream events such as protein phosphorylation (Lecourieux-Ouaked *et al.*, 2000), MAPK activation (Lebrun-Garcia *et al.*, 1998), anion effluxes and plasma membrane depolarization (Pugin *et al.*, 1997; Wendehenne *et al.*, 2002), microtubule

depolymerization (Binet *et al.*, 2001), NADPH oxidase activation (Simon-Plas *et al.*, 2002), ROS production (Tavernier *et al.*, 1995), anion channel activation (Wendehenne *et al.*, 2002), inhibition of glucose transporter(s) (Bourque *et al.*, 2002), NO production (Lamotte *et al.*, 2004) and [Ca^{2+}] elevations in the cytosol and the nucleus (Lecourieux *et al.*, 2002; Lecourieux *et al.*, 2005). These events are followed by gene expression and HR (cell death).

8.2. Oligogalacturonides/*Hyaloperonospora arabidopsidis*/*Arabidopsis thaliana* model

8.2.1. The oligogalacturonides (OGs)

In the past, elicitors belonging to different classes have been extensively studied in order to explore the mechanisms by which different biological signals are perceived and transduced by plant cells with an ultimate outcome of the induction of plant defense. During different plant-pathogen interactions, cell wall-degrading enzymes help the pathogen to obtain nutrients from plant. At the same time, the activity of these enzymes also leads to the production of pectic fragments that also act as potential elicitors of defense reactions in plants (Shibuya and Minami, 2001). OGs (α-1,4-galacturonic acid) are the best known examples of these types of elicitors which are the polysaccharides derived from pectin after the hydrolysis of plant cell wall during the interaction with pathogens. They are released by the action of polygalacturonases (PGs) and act as endogenous elicitors (Figure 2.1 in Materials and Methods Chapter 2) (Shibuya and Minami, 2001; Boller, 2005). They are non-specific elicitors and cannot be classified as PAMPs because these are not derived from the pathogen. However, they are known as 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). OGs are perceived by specific plasma membrane receptor and trigger defense signaling cascade with a subsequent activation of different cellular targets by the transcriptional regulation process.

8.2.2. OGs and plant defense responses

The OGs are characterized by a degree of polymerization (DP) and this DP is an important element of OGs signaling. OGs with a DP (10-15) have been reported as appropriate oligomers to induce defense responses although smaller oligomers can also activate plant defense responses. In a previous investigation, Hahn *et al.* (1981) reported that OGs with a DP between 10 and 15 were accumulated when fungal polygalacturonases degrade the homogalacturonan component of plant pectin. In a study conducted on soybean

cells, it was shown that OGs with a DP between 9 and 18 were the most effective in increasing the intracellular [Ca^{2+}]. However, a weak Ca^{2+} have also indicated that OGs with a DP < 8 were also able to trigger cell death in potato plants during soft rot disease induced by *Erwinia carotovora* (Weber *et al.*, 1996), to activate genes involved in JA synthesis in *Arabidopsis* (Norman *et al.*, 1999) and to accumulate protease inhibitors in tomato (Moloshok *et al.*, 1992). At the same time, OGs with a DP of 2 to 4 have been demonstrated to trigger defense responses in potato against *E. carotorova* (Wegener *et al.*, 1996). In tomato plants, OGs with a DP range of 4–6 resulted in the upregulation of aminocyclopropane-1-carboxylic acid oxidase (*AOC*) mRNA levels, an important gene that encodes the ET forming enzyme, thereby leading to ET production (Simpson *et al.*, 1998).

The involvement of OGs in the regulation of Ca^{2+} levels inside the cells have been reported in numerous studies conducted in different plant species (Chandra and Low, 1997; Navazio *et al.*, 2002; Hu *et al.*, 2004). In addition, studies have also shown that OGs participate to trigger various early events of defense signaling including protein phosphorylation and activation of MAPKs, synthesis and accumulation of phytoalexins, glucanase, chitinase, activation of ion fluxes and membrane depolarization with H^+ influx and K^+ efflux, production of active oxygen species (H_2O_2, and O_2^-), NO production and transcriptional activation of defense genes (Davis *et al.*, 1986; Davis and Hahlbrock, 1987; Broekaert and Peumans, 1988; De Lorenzo *et al.*, 1997; Rouet-Mayer *et al.*, 1997; Binet *et al.*, 1998; Droillard *et al.*, 2000; Galletti *et al.*, 2008; Rasul *et al.*, 2012). Recently, Wall Associate Kinase 1 (WAK1) has been reported as receptor of OGs perception in *Arabidopsis* (Brutus *et al.*, 2010). OGs-induced defense responses have been demonstrated in different plant species including, parsley, soybean, tobacco, grapevine and *Arabidopsis*. For example, in *Arabidopsis* or grapevine (*Vitis vinifera*), OGs stimulates a variety of defense responses including accumulation of phytoalexins, β-1,3-glucanase and chitinase, and NO production (Aziz *et al.*, 2003; Hu *et al.*, 2004). 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). Moreover, OGs have been reported to participate in increased resistance to *B. cinerea* that is independent of JA, ET and SA signaling pathways (Aziz *et al.*, 2004; Ferrari *et al.*, 2007). In the past, different transcriptome studies carried out in *Arabidopsis* have revealed that OGs are able to modulate the expression of a large number of genes that are involved in different physiological pathways especially related to defense responses in plants (Moscatiello *et al.*, 2006; Ferrari *et al.*, 2007).

These above data clearly demonstrate that OGs have emerged as a powerful tool to analyze defense signaling pathways during plant pathogen interactions. Therefore, we decided to use OGs in our working model.

8.3.1. Hyaloperonospora arabidopsidis

Hyaloperonospora arabidopsidis (*Hpa*) is a pathogenic microorganism that belongs to the family Peronosporaceae in the order Peronosporales of class oomycetes. It is one of the few obligate biotrophic pathogens that specifically infects model plant *Arabidopsis thaliana* (Koch and Slusarenko, 1990; Schlaich and Slusarenko, 2009; Coates and Beynon, 2010). It is the casual agent of downy mildew disease in *Arabidopsis*. Previously, it was known as *Hyaloperonospora parasitica* (*Peronospora parasitica*) and was named due to its specificity to infect *Arabidopsis* (Göker *et al.*, 2004). Different isolates of *H. arabidopsidis* have been identified and were named according to naming system proposed by Eric Holub (see Dangl *et al.*, 1992). In this system, each *H. arabidopsidis* isolate name is designated on the basis of the pathogen origin and their respective host cultivar. For example, one isolate found in the East Malling is able to establish a compatible interaction with Wassilewskija accession of *Arabidopsis*, was named as EMWA by taking two words each from East Malling and Wassilewskija. Similarly, NOCO was named on the basis of its identified location Norwich and its susceptible host cultivar Columbia (Col-0) (Slusarenko and Schlaich, 2003). Moreover, the compatible interaction between a specific *Arabidopsis* accession and its corresponding isolate of *H. arabidopsidis* is an excellent example of gene to gene interaction that has established between plants and pathogens during the course of evolution. In different *Arabidopsis* cultivars, different resistance genes known as *RPP* (recognition of *Peronospora parasitica*) genes have been assigned to specific *H. arabidopsidis* isolates (Crute *et al.*, 1993). Up till now, in the 5 chromosomes of *Arabidopsis,* 27 *RPP* genes have been postulated depending on differential interactions among host and pathogen (Tör *et al.*, 1994; Slusarenko and Schlaich, 2003). Some of these *RPP* genes have already been cloned and *RPP5* is the first gene that was cloned from *Arabidopsis* accession Landsberg erecta (Ler), and confers resistance to *H. arabidopsidis Noco* isolate (Parker *et al.*, 1996). *Arabidopsis* accession Col-0 is susceptible to *Noco* isolate as *RPP5* is absent in Col-0. Under some conditions, some *RPP* genes have allelic variants and confer resistance to different pathogens *e.g. RPP8*, which is responsible for resistance in Ler against EMCO, also participates in resistance to cucumber mosaic virus and turnip crinkle virus (Cooley *et al.*, 2000; Takahashi *et al.*, 2002). Although *Hpa* does not cause diseases of economic importance in plants, it has been established as a

pathogen model on the basis of the involvement of *RPP* genes in different signaling pathways in plants. Moreover, as we know that *Arabidopsis* belongs to family Brassicacae, it is expected that understanding this model pathosystem will help to extend its applications to other economically important crops of Brassicacae family.

THESIS OBJECTIVES

During Ph.D. training, my research was focused on the understanding of the following aspects of Ca^{2+} signaling in plants:

- Study of elicitor-induced Ca^{2+} signaling in different subcellular compartments (*e.g*. chloroplasts and mitochondria) and its physiological importance.
- Characterization of *Arabidopsis* GLRs in the OGs-mediated plant defense signaling.
- Identification of genes regulated after GLRs activation in OGs-induced defense signaling pathways.

We wanted to understand how Ca^{2+} signaling propagates into the cell and to which extent it might affect the function of different Ca^{2+} storing compartments? During our work, we tried **1)** To investigate the variations in [Ca^{2+}] in these cell compartments after treatment with different plant defense elicitors (Cry and OGs), **2)** To know the characteristic features of Ca^{2+} signatures in these compartments and their regulation mechanisms, **3)** To pharmacologically characterize the channels, pumps and transporters present on the membranes of organelles and which are involved in these Ca^{2+} variations and **4)** To evaluate the impact of these Ca^{2+} changes on the different physiological events taking place in chloroplasts and mitochondria. For this purpose, aequorin technology based on bioluminescence in transformed *N. tabacum* var. Xanthi cell cultures was used to compare [Ca^{2+}] variations in cytosol, nucleus, chloroplasts, and mitochondria.

In the second part of the study, the main objectives were to investigate the involvement of AtGLRs in OGs-induced Ca^{2+} variations, to demonstrate which downstream events of plant defense Ca^{2+} signaling are influenced by GLRs and finally to characterize specific GLRs that could be implicated in resistance against biotrophic and necrotrophic pathogens. To meet these objectives, both the pharmacological and genetic investigations were made. For pharmacological studies, GLRs antagonists belonging to different classes were used while genetic approach was based on the use of *Arabidopsis thaliana* T-DNA insertion single mutants. After confirmation that OGs induced $[Ca^{2+}]_{cyt}$ variations in a GLRs-dependent manner, we verified the effect of GLRs on other signaling events like ROS and NO production, activation of MAPKs, and the accumulation of defense-related genes. Meanwhile, we also studied the role of GLRs in *Arabidopsis* basal resistance against *B. cinerea* and *H. arabidopsidis* and characterized the GLR3.3 as an important gene contributing in basal resistance against *H. arabidopsidis*.

Although, the third and final part of my thesis work was mainly focused on the identification of GLRs target genes in *Arabidopsis* yet many additional investigations were also made during this work. For example, we were interested to evaluate the contributions of Glu (a ligand to activate animal iGluRs) and OGs (a well known plant defense elicitor) in the transcriptional regulation of different physiological pathways especially related to plant defense responses. At the same time we tried to obtain a general idea about the similarities and the differences that exist in the transcriptional responses between these two different signaling molecules. Moreover, we wanted to identify the specific genes classes that could be regulated during Glu and OGs signaling. Finally, we were interested to know how GLRs regulate OGs-mediated signaling by inducing the expression of GLRs-dependent genes. To answer these different questions, a NimbleGen microarray analysis was performed in *Arabidopsis* plants and the obtained data were analyzed through different bioinformatics tools already developed by different laboratories.

Collectively, this thesis work will help us to better understand the elicitor-mediated Ca^{2+} signaling in plants by providing us an overview of the global applications of this Ca^{2+} signaling in diverse biological functions in plants. We will also be able to define the role of GLRs during plant-pathogen interactions and finally the molecular basis of this defense regulation processes.

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CHAPTER 2

CHAPTER 2

“Materials and Methods”

Table 2.1: List of *Arabidopsis thaliana* glutamate receptors and other T-DNA mutant lines used in this study.

Gene ID	*Mutant name*	Description	Mutant lines	Seed Obtained
At3g04110	*Atglr1.1*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 1.1	SALK_057748C	NASC
At5g48400	*Atglr1.2*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 1.2	SALK_053535C	NASC
At5g48410	*Atglr1.3*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 1.3	GK_030F04	NASC
At3g07520	*Atglr1.4*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 1.4	SALK_129955C	NASC
			SALK_021986C	NASC
At5g27100	*Atglr2.1*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 2.1	GK_897G01	NASC
At2g24720	*Atglr2.2*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 2.2	SALK_036453	NASC
At2g24710	*Atglr2.3*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 2.3	SALK_113206	NASC
At4g31710	*Atglr2.4*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 2.4	SALK_010571C	NASC
At5g11210	*Atglr2.5*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 2.5	SALK_078407C	NASC
At5g11180	*Atglr2.6*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 2.6	SALK_132296C	NASC
At2g29120	*Atglr2.7*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 2.7	SALK_121990C	NASC
At2g29110	*Atglr2.8*	Mutant impaired in the gene	SALK_111659	NASC

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1. 1. Biological material related to tobacco

1.1.1. Cell Suspensions

The transgenic *Nicotiana Tabacum* var. Xanthi cell cultures expressing apoaequorin in chloroplasts, cytosol and mitochondria (named as Xanthi-Aeq-chloro, Xanthi-Aeq-cyt and Xanthi-Aeq-mito) were obtained after transformation of wild type *N. Tabacum* leaves with *Agrobacterium tumefaciens* carrying plasmids pMAQ6 (Johnson *et al.*, 1995), pRTL2 (Knight *et al.*, 1991) and pBIN AGA#2 (Logan and Knight, 2003) for chloroplasts, cytosol and mitochondria, respectively. Following is a brief description of the three plasmide constructions:

- pRTL2 plasmid containing the cDNA for the expression of apoaequorin in the cytosol (furnished by Prof. Marc R. Knight, Durham University, UK).
- pMAQ6 plasmid containing the cDNA for the expression of apoaequorin in the chloroplast (furnished by Prof. Marc R. Knight, Durham University, UK). The plasmid pMAQ6 for the chloroplastic targeting contains the apoaequorin structural gene fused to the constitutive cauliflower mosaic virus (CaMV) 35S promoter. It includes the coding sequence of the chloroplast-targeting transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (pea *rbc*S) fused to the 5' end of the apoaequorin coding region.
- pBIN AGA#2 plasmid targeting apoaequorin in the mitochondria (furnished by Prof. David C. Logan, University of St. Andrews, UK). The plasmid pBIN AGA#2 comprises the first 87 aa sequence of mitochondria *N.plumbaginifolia* ATPase β subunit fused with the mGreen fluorescent sequence and downstream the apoaequorin cDNA, to express a GFP-aequorin fusion protein in the matrix of mitochondria.

For complete details of transformation, see Ph.D. manuscript of Vatsa (2010).

Screening of kanamycin resistant transformed plants for apoaequorin expression was carried out by conventional Western-blotting and transformed *N. tabacum* var Xanthi plantlets were used to generate cell suspensions as described by Lecourieux *et al.* (2005). Verification of cytosolic, mitochondrial and chloroplastic targeting of apo-aequorin was made by Western

		encoding a Putative ligand-gated ion channel 2.8		
At2g29100	*Atglr2.9*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 2.9	SALK_125496	NASC
At2g17260	*Atglr3.1*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 3.1	SALK_063873C	NASC
At4g35290	*Atglr3.2*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 3.2	SALK_150710	NASC
At1g42540	*Atglr3.3-1* *Atglr3.3-2* *Atglr3.3-3*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 3.3	SALK_066021	NASC
			SALK_066009	Dr. E.P. Spalding (University of Wisconsin, USA)
			SALK_040458	Dr. E.P. Spalding (University of Wisconsin, USA)
At1g05200	*Atglr3.4*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 3.4	SALK_079842	NASC
At2g32390	*Atglr3.5*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 3.5	SALK_035264C	NASC
At3g51480	*Atglr3.6*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 3.6	SALK_091801C	NASC
At2g32400	*Atglr3.7*	Mutant impaired in the gene encoding a Putative ligand-gated ion channel 3.7	SALK_022757	NASC
At5g47910	*AtrbohD*	Mutant impaired in the gene encoding NADPH/respiratory burst oxidase protein D (RbohD).		Dr. Torres (University of North Carolina, USA).
	Arabidopsis WT plants expressing Aequorin in the cytosol			Dr. M. Knight (Durham, UK)

blotting of cytosolic fractions, or mitochondrial and chloroplastic fractions obtained after organelles isolation. Transgenic tobacco cell suspensions have the same growth kinetic and the same morphology when compared to untransformed tobacco cell grown under same conditions.

Transformed cell cultures were maintained in Chandler's medium (Chandler *et al.*, 1972). They were kept under constant agitation (125 rpm) in an air conditioned culture room (25 °C) in continuous light (2000 $ergs.cm^{-2}$). At every 7th day, when they reached at exponential growth phase, cell suspensions were sub-cultured by adding 7 mL of cells in 100 mL of new culture medium. For biological assays, saturated cell suspensions (6 days) were diluted to half by the addition of new culture medium, 24 h before use.

1.2. Biological material related to *Arabidopsis*

1.2.1. Plants

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) and T-DNA insertion mutants lines used in this study were either obtained directly from NASC (Nottingham *Arabidopsis* Stock Center; http://www.arabidopsis.org) or were a gift from different laboratories (Table 2.1). All plant lines used in this study are in the Col-0 background.

Plants were grown in commercial soil (Jiffy-7, Puteaux, France; http://www.puteaux-sa.fr). Prior to germination, seeds were vernalized at 4 °C in the dark for 48 h and then were shifted to a climatic growth chamber (KBW 720, BINDER, Germany; http://www.binder-world.com) with 10 h light:14 h dark (short day) conditions with the following settings: 20 °C light, 18 °C dark; 70 % relative humidity light / 95 % dark; light intensity 175 $\mu E.s^{-1}$.

1.2.2. Pathogens

Following is the brief description of the pathogens used in this study.

1.2.2.1. Botrytis cinerea

Botrytis cinerea isolate BMM, kindly provided by Dr. Laurent Zimmerli (University of Fribourg, Switzerland; Zimmerli *et al.*, 2000), was grown for 10-12 days (light 10 h, 20 °C; dark 14 h, 18 °C) on 39 $g.L^{-1}$ PDA (potato dextrose agar, DIFCO; BD Biosciences; http://www.bdbiosciences.com). Spores were harvested in water and filtrated through glass wool to remove hyphae. Spores concentration was determined by microscope and they were stored at 4 °C.

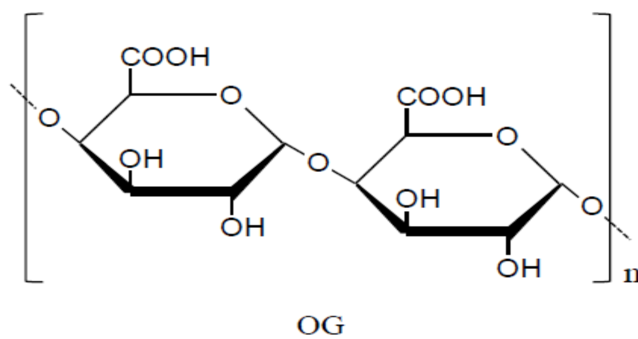

Figure 2.1: Chemical structure of oligogalacturonides (OGs). OGs (α-1,4-galacturonic acid) is a polysaccharide derived from pectin after the hydrolysis of plant cell wall and acts as endogenous elicitor. OGs with a degree of polymerization (DP) of 25 was used in this study.

1.2.2.2. Hyaloperonospora arabidopsidis

H. arabidopsidis (*Hpa*) isolate *Noco2* was provided by Dr. Patrick Saindrenan (University Paris Sud XI, France). The *Hpa* strain was maintained by weekly sub-culturing on genetically susceptible *Arabidopsis* Col-0 plants (as *RPP5* gene responsible for resistance against *Noco2* is absent in Col-0). Ten days old seedlings were spray-inoculated to saturation with a spore suspension of 4.10^4 spore.mL^{-1} in distilled water. Seedlings were kept in a growth chamber at 16 °C for 7 days with a 16 h photoperiod under 80-100 % relative humidity.

1.3. Elicitors

In this study, cryptogein (Cry) and oligogalacturonide**s** (OGs), two well known plant defense elicitors, were used. In work with tobacco, both Cry and OGs were tested whereas only OGs were used in *Arabidopsis* studies. A brief description of these elicitors is as given below:

- OGs (α-1,4-galacturonic acid) are polysaccharides derived from pectin after the hydrolysis of plant cell wall by PGs enzymes and act as endogenous elicitors (Figure 2.1; Shibuya and Minami, 2001; Boller, 2005). OGs were provided by GOEMAR SA (Roscoff, France) with an approximate degree of polymerization (DP) of 25. A stock solution of 50 mg.mL^{-1} of OGs was prepared in ultrapure water and stored at -20 °C and was used at a working concentration of 2.5 mg.mL^{-1} for different biological assays except where mentioned.
- Several *Phytophthora* secrete a family of structurally related proteins called elicitins (Baillieul *et al.*, 2003). All the elicitins share a conserved elicitin domain from amino acids 1 to 98. The elicitins can be grouped into five classes based on their primary structure (Baillieul *et al.*, 2003). Cry is a 10 kDa elicitin obtained from the oomycet, *Phytophthora cryptogea* and displays only the elicitin domain of 98 amino acids. (Figure 2.2). Cry was purified in the lab from the filtrate of the *Phytophthora cryptogea* culture according to (Bonnet *et al.*, 1996). The concentration of Cry was determined by the absorbance at 280, nm E_s = 0.605 g^{-1}.L.cm^{-1}. It is prepared in ultrapure water at the concentration of 100 µM and stored at -20 °C.

1.4. Chemicals

For different experiments, OGs (oligogalacturonides), cPTIO (4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide), La^{3+} (Lanthanum chloride), BAPTA (1,2-

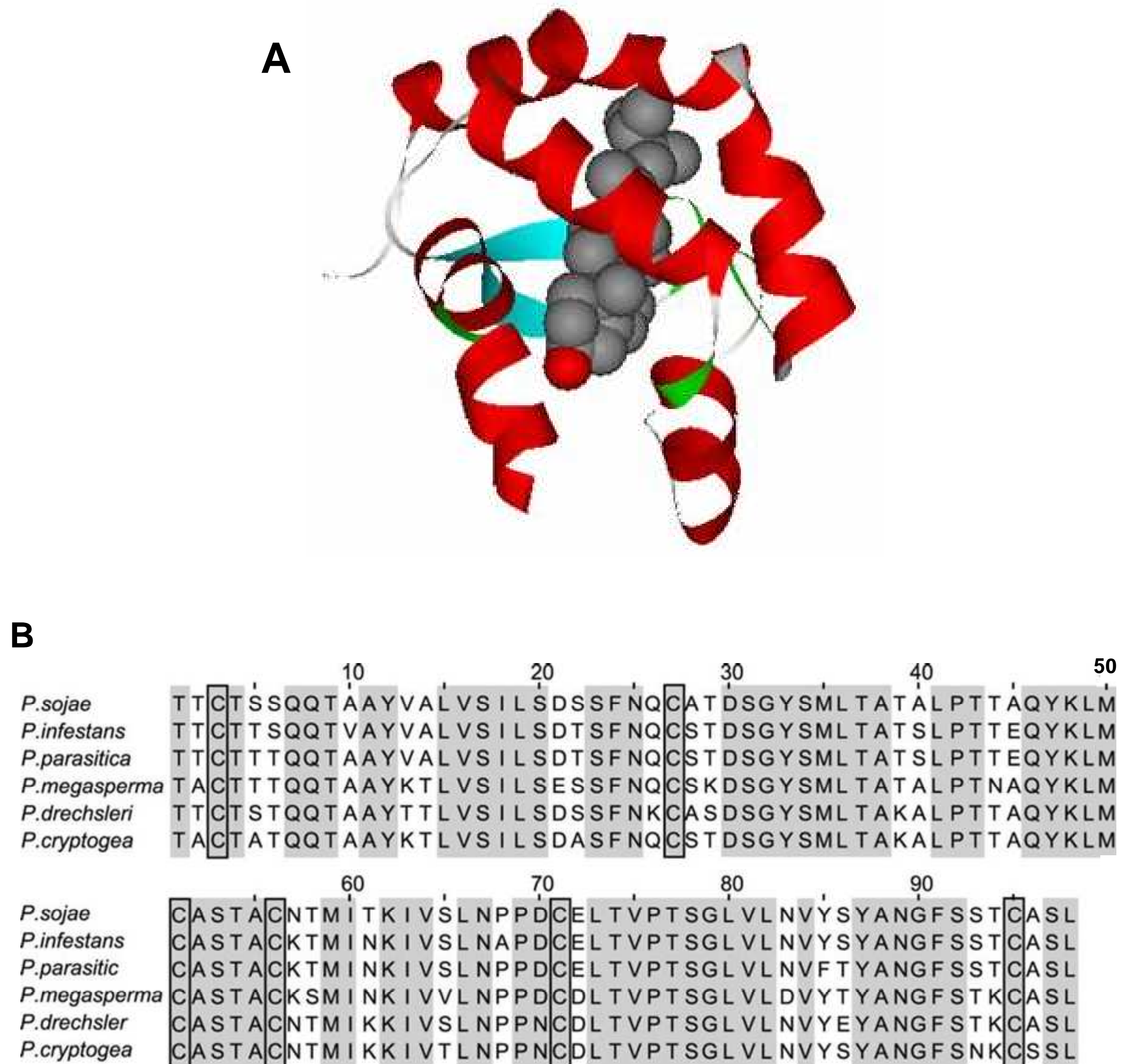

Figure 2.2: Cryptogein structure. A) β-cryptogein-Ergosterol complex. The structure of ß-Cryptogein reveals a protein fold with five helices and double stranded ß-sheath and Ω-loop (Adapted from http://mason.gmu.edu). **B)** Sequence alignment of elicitins secreted by different *Phytophthora* species. *P. soja, P. infestens* and *P. parasitica* have α-elicitins while *P. megasperma, P. drechsler* and *P. cryptogea* secrete β-elicitins. Amino acids having sequence identities are represented in gray background. The six conserved cystein residues involved in the formations of disulfide bonds are enclosed in black boxes (Astier, 2011).

bis(aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), ruthenium red, neomycine, KCN (potassium cyanide), SHAM (salicylhydroxamic acid) and MK-801 (5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) were dissolved in water whereas DNQX (6,7-dinitriquinoxaline-2,3 dione), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), luminol (5 Amino-2,3-dihydro-1,4-phthalazinedione), DAF-2DA (4,5-diaminofluorescein diacetate) and DPI (diphenylene iodonium) were dissolved in DMSO. DAB (3, 3-diaminobenzidine) was dissolved in HCl (Hydrochloric acid) and U73122 in ethanol. All the above mentioned chemicals were purchased from SIGMA-ALDRICH (http://www.sigmaaldrich.com) except OGs which was obtained from GOEMAR, France (http://www.goemar.com). Coelenterazine was prepared in ethanol and was obtained from UPTIMA, France (http://www.interchim.com). For all performed experiments, 4-5 weeks old *Arabidopsis* plants were used except where mentioned and final working concentrations for OGs, DNQX, CNQX and MK-801 were 2.5 $mg.mL^{-1}$, 500 µM, 500 µM and 400 µM, respectively, in all experiments.

2. Methods

2.1. Mutant Genotyping

2.1.1. Primer designing

Genotyping of the *Arabidopsis* mutant plants was carried out by PCR with genomic DNA using PCR primers designed by SIGnal T-DNA Express (http://signal.salk.edu/tdnaprimers.2.html) or by AmplifX 1.1 software (http://ifrjr.nord.univ-mrs.fr/AmplifX) and following the recommended combinations of primers. A complete list of PCR primers is shown in Table 2.2.

2.1.2. Isolation of DNA

To sort out homozygous mutant individuals, genomic DNA was isolated from leaf tissues using standard protocol. Briefly, leaf disks were excised and ground manually in 400 µL of DNA extraction buffer (1 M NaCl; 1 M Tris-HCl, pH 7.5; 0.5 M EDTA, pH 8.0 and 10 % SDS; Edwards *et al.*, 1991), and debris was pelleted by a 5 min centrifugation at 13,000 rpm at room temperature. A 50 % isopropanol precipitation followed by a washing step with 70 % ethanol was made to recover the DNA pellet. After drying, DNA was re-suspended in 50 µL ultrapure water. To check the quality of DNA, quantification was made through UV spectrophotometry.

Table 2.2: Primers used for GLRs T-DNA mutants genotyping in *Arabidopsis.*

Accession No.	Gene name	Mutant lines	Primers sequence (5' =====> 3')	Annealing Temp. (℃)
At3g04110	*AtGLR1.1*	SALK_057748C	**LP**: CTTGTGGCTAGCAAGTGGAGCTTT	54
			RP: ACGGAAGGTGAATCGCTTAGATGC	
At5g48400	*AtGLR1.2*	SALK_053535C	**LP**: ACGTTGCCATACACTGAAATGGGT	58
			RP: GCTTGTCCTCAGCTTCGCAATCT	
At5g48410	*AtGLR1.3*	GK_030F04	**LP**: GGGTTTGTTTGGATCCTGACTGCT	59
			RP: CCAAGACCCATTTCAGTGAATGGC	
At3g07520	*AtGLR1.4*	SALK_129955C	**LP**: TATATTTGGCCAAGCTCAACG	54
			RP: CTTATAGTGCGGGCTTTGTTG	
		SALK_021986C	**LP**: AGGCAAGCTTAAGACCCTGAG	55
			RP: TTGGTGGTTCCAGAGACAAAC	
At5g27100	*AtGLR2.1*	GK_897G01	**LP**: AATGGGAAAAGGCTGCAGATAGGC	59
			RP: CCCAGAAGCTAAGCACTCGTTCTC	
At2g24720	*AtGLR2.2*	SALK_036453	**LP**: CTTTGATCTGGCTTGCGTGAGTGA	60
			RP: CATGGCAACATCCGGGTATGAAGT	
At2g24710	*AtGLR2.3*	SALK_113206	**LP**: CACACTCGAAGGTTGTCATGCTCT	59
			RP: CAGTGGCATTGATAGCGATAACGG	
At4g31710	*AtGLR2.4*	SALK_010571C	**LP**: AGGGAAAACATGTGATTGTGC	54
			RP: TCCAATAATGCCCTTGTCAAG	
At5g11210	*AtGLR2.5*	SALK_078407C	**LP**: GACCAAAGCTGTGTCGACTTC	59
			RP: CAAGCAGATGAGGAGTTCAGG	
At5g11180	*AtGLR2.6*	SALK_132296C	**LP**: TCTACGGTGAACCAAAGTTGG	54
			RP: TTTTCACAAGGGTTCTTGTGG	
At2g29120	*AtGLR2.7*	SALK_121990C	**LP**: GGAAATCTTGCCGGTTAAAAG	54
			RP: ACAAATTTGGGGACATTAGGG	
At2g29110	*AtGLR2.8*	SALK_111659	**LP**: CGCCATAGACATCTTTGAAGC	54
			RP: ACAATGGCATATTTGGAGCAG	
At2g29100	*AtGLR2.9*	SALK_125496	**LP**: TGACAAGGTGCTCCCATTATC	54
			RP: AGAAATTCATGGTGACGGTTG	
At2g17260	*AtGLR3.1*	SALK_119230C	**LP**: CACTTGGTCGTATGGTGCTTCTGA	57
			RP: GTCTTTGCAGAAGTCGCGGATT	
At4g35290	*AtGLR3.2*	SALK_150710	**LP**: TTTTGGATCCAGCATTAGTCG	52
			RP: TTTTGCGGTTTTGTTTGTAGG	
At1g42540	*AtGLR3.3*	SALK_066021	**LP**: GAAGCACCAGACATCTTACGC	56
			RP: TGAAGCAACTCTGGACTTTCTTC	
At1g05200	*AtGLR3.4*	SALK_079842	**LP**: GGGTTAATCCGGCTTATGAAG	56
			RP: GAAGTGAGACTGGCCGTGTAG	
At2g32390	*AtGLR3.5*	SALK_035264C	**LP**: TGAAGTTGCTGCAAATGTGAG	54

2.1.3. PCR

For each mutant line, two PCRs were performed to verify the wild type gene and mutant gene products. In the first PCR reaction, LP+RP (left primer and right primer) combination was used. This primer pair specifically amplified a fragment of the gene of interest only in wild type allele (in the wild type homozygous and the heterozygote genotypes) as LP and RP are located upstream and downstream of the T-DNA insertion but in case of mutated allele, no PCR fragment was amplified as T-DNA fragment prevents amplification in these conditions (Figure 2.3A). A second PCR reaction was carried out with RP primer in combination with a left-border T-DNA primer (LB: ACTGGCCGTCGTTTTACAACG), a universal complementary primer for the T-DNA insertion. In this case, amplification was only possible in homozygous and heterozygous plants for the mutated allele but no PCR fragment was amplified in wild type plants (Figure 2.3B).

PCR were performed in a thermocycler (MyCycler, BIORAD, USA; http://www.bio-rad.com) with a final volume of 25 µL containing 2 µL of genomic DNA in the following PCR mix: 1 µM of dNTP, 200 nM each of the primers (RP/LP or RP/LB, as the case was); 2.5 µL of 10 X *Taq* polymerase buffer and 0.75 U *Taq* Polymerase (GO *Taq,* Promega).

Following settings were used for the qPCR reaction: first denaturation step of 2 min at 95 °C, followed by 40 cycles, each consisting of a denaturation step of 30 s at 95 °C, an annealing step of 30 s at 50-60 °C (depending upon primers Tm) and an extension step of 45 s at 72 °C. A final extension step of 10 min at 72 °C was carried out to ensure that primer extension reactions were well completed.

2.1.4. Agarose gel electrophoresis

The final step in mutant genotyping was the confirmation of the homozygous status by running the PCR samples on the gel. Depending upon the size of the PCR product, samples were run on agarose gel electrophoresis at 100 V for 30 min. Gels were stained with ethidium bromide and evaluated using UV imaging system (Molecular imager GelDoc XR system, BIORAD; http://www.bio-rad.com).

			RP: TGTCGACATGTCCACAGCTAG	
At3g51480	*AtGLR3.6*	SALK_091801C	**LP**: TTCGTTCAAAGGTGGCATAAC	54
			RP: CGACTATGAGGAAAGACGCAG	
At2g32400	*AtGLR3.7*	SALK_022757	**LP**: TCTTCTGTCCGGATGAGTTTG	54
			RP: CGAAGAAAGAAGGGAAATTGG	

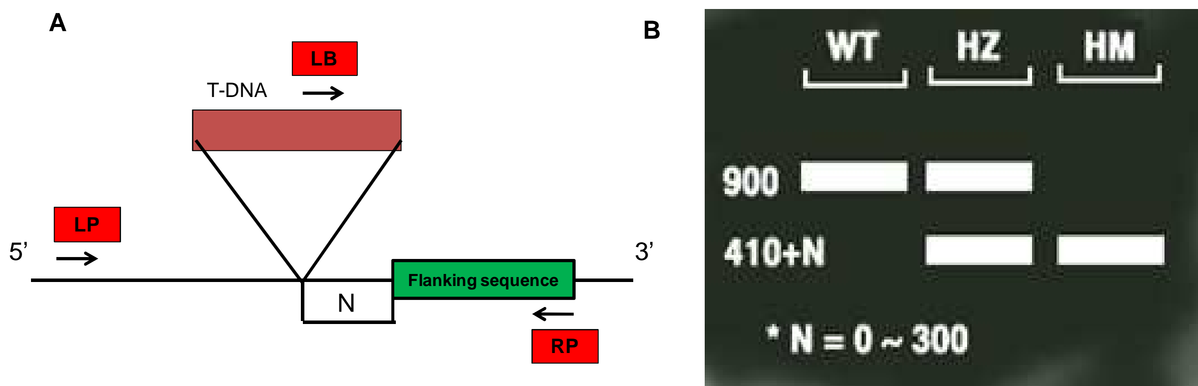

Figure 2.3: Diagrammatic illustration of T-DNA mutants lines genotyping through PCR. A) Position of primers used for genotyping**.** LP and RP: Left and Right genomic primers, LB: T-DNA border primer, N: Difference of the actual insertion site and the flanking sequence position, usually 0 - 300 bases. **B)** Possible PCR products for WT (Wild Type), HM (Homozygous lines) and HZ (Heterozygous lines) visualized through agarose gel electrophoresis. WT (no insertion) should get a product of about 900-1100 bps (LP-RP; depending upon the size of product); HM (insertions in both chromosomes) would 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 would get both bands.

2.2. Measurement of free calcium concentration

2.2.1. In *Arabidopsis* plants

2.2.1.1. *Aequorin reconstitution and luminescence measurements*

Arabidopsis transformed plants expressing apoprotein aequorin in the cytosol were a kind gift from Prof. Marc R. Knight (Durham University, UK). For $[Ca^{2+}]_{cyt}$ measurement, 7 mm diameter leaf disks were prepared from 4–5 weeks old plants and put individually in luminometer assay tubes containing 200 µL of H_2O. Overnight reconstitution of aequorin was made in darkness with 10 µM native coelenterazine (Figure 2.4). Luminescence was measured using a single-tube luminometer (Lumat LB 9507, BERTHOLD TECHNOLOGIES, Germany; http://www.berthold.com). Assay tubes were placed individually into the luminometer, and luminescence recorded at 1 s integration intervals. After a lapse of 1 min, elicitor treatments were applied manually and measurements were continued for 4-5 min. The remaining aequorin was discharged by automatic injection of 1 volume of 2 M $CaCl_2$, 20 % ethanol, and luminescence were recorded for another 8–10 min until values were within 1 % of the highest discharge value. Controls were performed by addition of an equal volume of water or DMSO as it was required. La^{3+} and GLRs inhibitors treatments were made 10 min prior to elicitor treatment. To calculate Ca^{2+} concentrations, relative luminescence values were converted by using the following calibration equation developed by Rentel and Knight (2004):

$$\mathbf{pCa = 0.332588(-\log k) + 5.5593}$$

Where k is the luminescence counts per sec/total luminescence counts remaining.

2.2.2. In tobacco subcellular compartments

2.2.2.1. In vivo aequorin reconstitution

Transformed tobacco cells expressing apoaequorin in cytosol, mitochondria and chloroplasts, were collected in the exponential phase of growth and diluted to half 24 h before experiment. Cells were washed 3 times with M-10 buffer (10 mM MES; 175 mM D-mannitol; 0.5 mM K_2SO_4; $CaCl_2$ 0.5 mM, pH 5.75). Then, cells were adjusted to 0.1 g $FW.mL^{-1}$ of suspension buffer. *In vivo* reconstitution of aequorin was initiated by addition of 1 µM native coelenterazine to cells for at least 2 h in the dark (130 rpm, 25 °C). As coelenterazine is photo-sensitive, cells were maintained in obscurity until the end of the experiment.

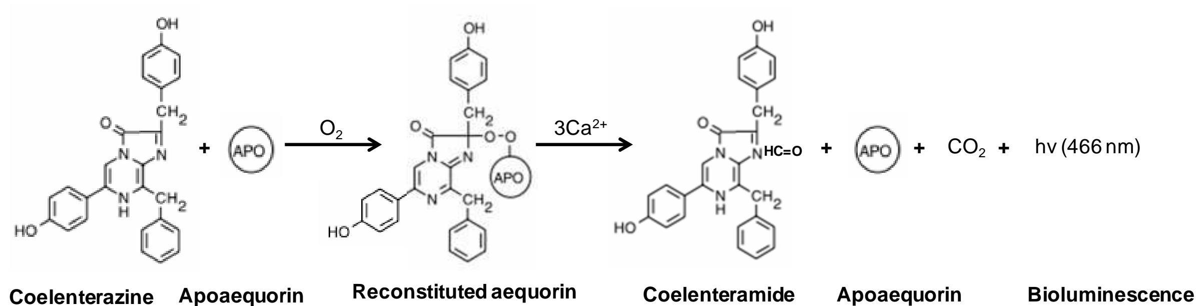

Figure 2.4: In vivo functional reconstitution of aequorine with coelenterazine. Apoaequorin reaction with coelenterazine in the presence of molecular oxygen results in the formation of an aequorine complex (reconstituted aequorine). Upon binding of Ca^{2+} ions with aequorine complex, a conformational change occurs that leads to the oxidation of coelenterazine into coelenteramide along with the generation of luminescence equivalent to free Ca^{2+} concentration. This emitted luminescence can be detected at 466 nm. Similarly, by addressing aequorine to different cellular compartments (cytosol, chloroplast, mitochondria and nucleus), Ca^{2+} variations can be successfully measured in these compartments.

2.2.2.2. Measurement of the intracellular free calcium variation

The measurement of the luminescence was recorded using a digital luminometer (Lumat LB 9507, BERTHOLD TECHNOLOGIES, Germany; http://www.berthold.com). After gentle mixing, 250 µL of cell suspension were removed and transferred to a luminometer assay tube. For each measurement, assay tubes were placed individually into the luminometer, and the relative luminescence was recorded at 1 s integration intervals. All the inhibitors and control solvents were added 10 min before elicitor treatments. Remaining aequorin was discharged by automatic injection of 300 µL of lysis buffer {$CaCl_2$ 10 mM, Nonidet P40 2 % (v / v), ethanol 20 % (v / v)} and luminescence recorded for another 8–10 min until values return to basal level. Controls were performed by addition of an equal volume of water or other solvents as it was required. The luminescence data were converted into Ca^{2+} concentrations as described by Allen *et al.* (1977), according to following equation:

$$[Ca^{2+}] = [(L_0/L_{max})^{1/3} + 55\ (L_0/L_{max})^{1/3} - 1] / [2.10^6 - 2.10^6\ (L_0/L_{max})^{1/3}]$$

Where $[Ca^{2+}]$ is the Ca^{2+} concentration, $L_{max} = \Sigma L_0 + \Sigma L_r$, $\mathbf{L_0}$ is the luminescence intensity per second, $\mathbf{L_{max}}$ is the total amount of luminescence present in the entire sample over the course of the experiment, $\mathbf{L_r}$ is the total amount of luminescence obtained during the discharge of residual aequorin remaining in the cells.

2.3. Reactive oxygen species production measurement in *Arabidopsis*

2.3.1. Luminol-dependent assay

Reactive oxygen species (ROS) released by leaf tissues were measured by a luminol-dependent assay (Keppler *et al.*, 1989) with minor modifications (Figure 2.5). For each condition, two leaf disks (7 mm in diameter) from 4-5 weeks-old *Arabidopsis* wild type (Col-0) and mutant plants were overnight incubated in assay tubes containing 200 µL H_2O at room temperature (25 °C). Luminol (60 µM) was added and luminescence was measured immediately with a single-tube luminometer (Lumat LB 9507, BERTHOLD TECHNOLOGIES, Germany; http://www.berthold.com). In inhibitor experiments, La^{3+}, DNQX, CNQX and MK-801 were added 10 min prior to Luminol treatment. OGs treatment or an equal volume of water/DMSO (control) was made at 5 min of luminal treatment when luminescence reached back to basal level. Measurement was made with 1 s integration time at 10 s intervals for a total time of 40 min.

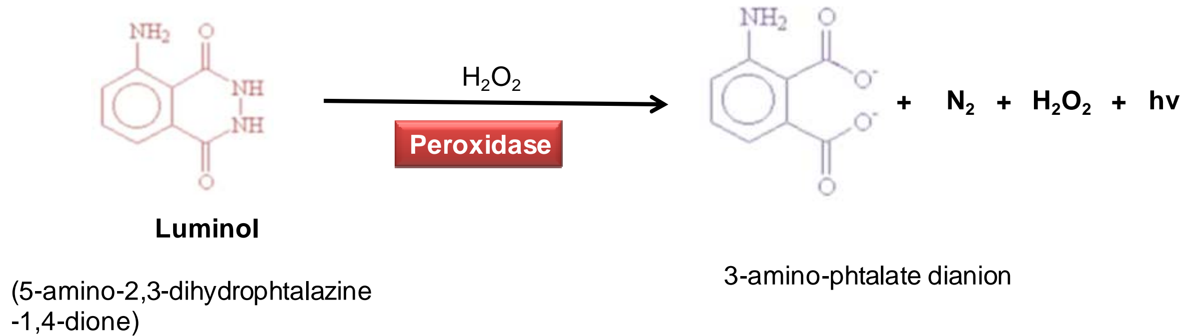

Figure 2.5: Luminol (5-amino-2,3-dihydrophtalazine-1,4-dione) based chemiluminescence measurement for the production of H_2O_2 in *Arabidopsis thaliana* leaf disks in the presence of peroxidase. Chemiluminescence is a redox reaction of luminol during which luminol loses electrons in the presence of H_2O_2 (which act as an oxidizing agent). Final outcome of his reaction is the production of aminophtalates ions, nitrogen, H_2O_2 and a characteristic blue glow called chemiluminescence.

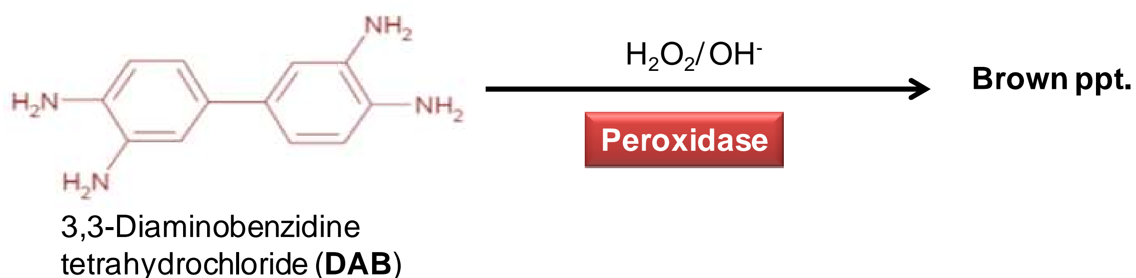

Figure 2.7: DAB-based H_2O_2 detection in *Arabidopsis thaliana* leaves in the presence of peroxidase. In the presence of H_2O_2, DAB is converted to an insoluble brown precipitate and water by the enzyme horse radish peroxidase (HRP). This can be visualized clearly as brown spots on leaf tissues.

2.3.2. Diaminobenzidine (DAB) staining

To visualize H_2O_2 *in situ*, 3, 3-diaminobenzidine (DAB) staining was performed on mature leaves from 4-5 weeks old *Arabidopsis* plants (Col-0 or mutants). For each treatment, 6-8 leaves from different plants were used in the assay. All the treatments (OGs and/or GluRs inhibitors) were vacuum-infiltrated for 3 min with the DAB solution. After washing, keeping the adaxial side up, leaves were placed in plastic boxes under high humidity until brown precipitate was observed (Figure 2.6). A direct application of H_2O_2 to the leaves was used as a positive control. Time kinetics between 4-8 h was made and leaves were de-stained with methanol to remove the chlorophyll.

2.4. Nitric oxide production measurement

2.4.1. In *Arabidopsis* leaf disks

Intracellular nitric oxide (NO) accumulation in *A. thaliana* (Col-0) and mutant plants was monitored by using DAF-2DA (4,5-diaminofluorescein diacetate), a membrane-permeable derivative of the NO-sensitive fluorescent probe that enters the cell through its two acetate groups. Following the cleavage of these acetate groups by intracellular esterases, liberated DAF-2 react with NO and its derivatives (especially N_2O_3 and NO^+) and ultimately yield DAF-2T (DAF-2 triazole) fluorescence (Jourd'heuil, 2002; Figure 2.7). This detection method has been successfully used in plants for NO detection.

For NO production measurement, 7 mm leaf disks from *Arabidopsis* plants (wild type and mutants) were prepared and first infiltration was done under vacuum for 3 min with an aqueous solution of 20 µM DAF-2DA in Tris-HCl 50 mM, pH 7.5. The disks were incubated 1 h in obscurity and washed three times with Tris-HCl 50 mM, pH 7.5 to remove excessive fluorophore. A second infiltration for 3 min was made with OGs or water in the same Tris-HCl buffer. La^{3+} and GLRs inhibitors (DNQX, CNQX and MK-801) were added in both infiltrations. For fluorescent measurements, eight leaf disks per treatment/genotype were put separately in a 96 wells plate (Microtest ^{Tm} flatbottom, BD, Europe; http://www.bdbiosciences.com) with 200 µL of the infiltration solution in each well. The increase in fluorescence that reflects the accumulation of DAF-2T, and thus NO production, was measured after every 30 min for a maximum time duration of 16 h with a spectrofluorometer (Mithras L 940, BERTHOLD TECHNOLOGIES, Germany; http://www.berthold.com) under following settings; λ excitation 485 nm, λ emission of 515 nm at 25 °C and a 10 min agitation after each recording. Fluorescence was expressed as relative fluorescence units (RFU).

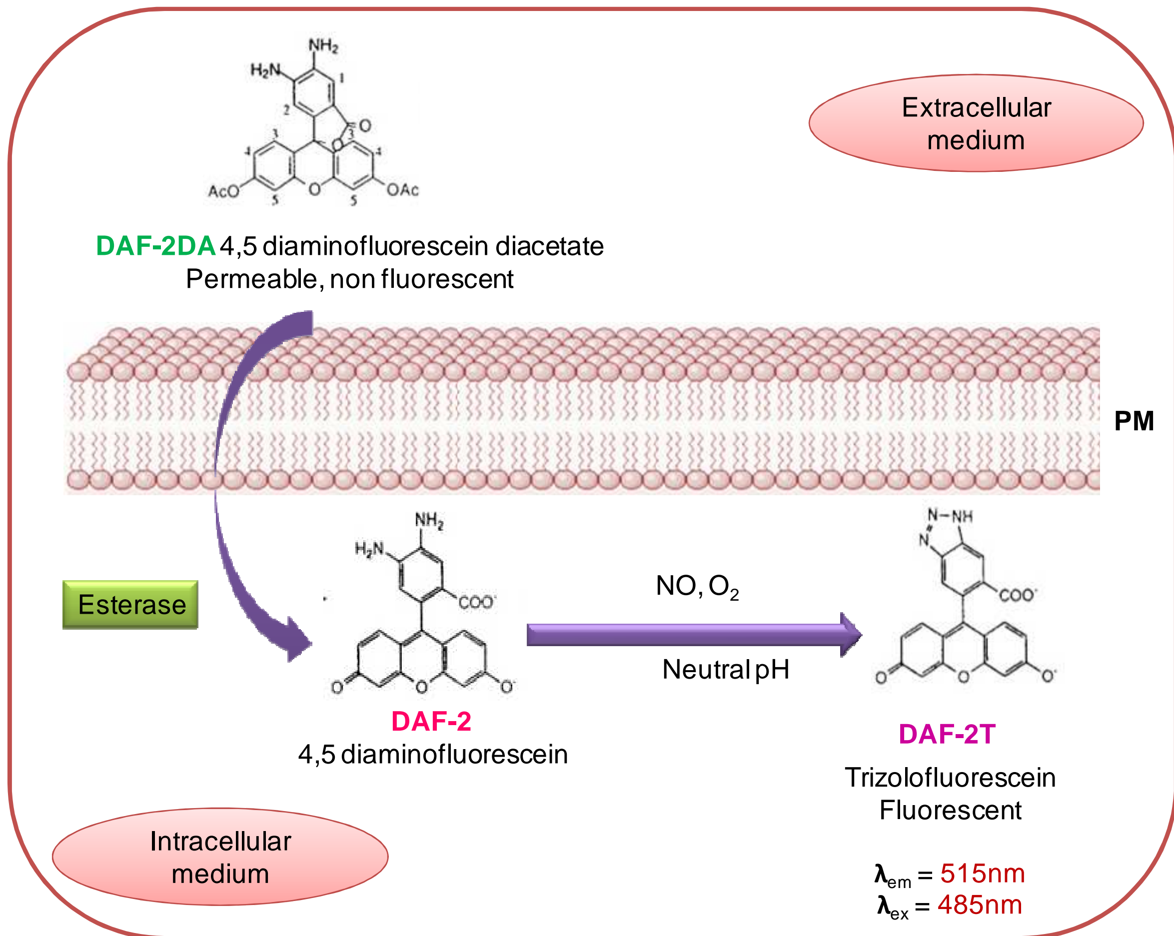

Figure 2.7: Intracellular NO accumulation with DAF-2DA. DAF-2DA is a membrane permeable fluorophore. In the cytosol, it is hydrolyzed by esterase and is converted into DAF-2. In the presence of oxygen, at neutral pH, it reacts with NO to yield fluorescent complex, triazolofluorescein (DAF-2T). The fluorescent product can be quantified by spectrofluorimetry using emission and excitation filter of 485 nm and 515 nm, respectively.

2.4.2. Measurement of nitric oxide production in tobacco cell suspensions

Nitric oxide (NO) accumulation was monitored using the fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich) as described by Lamotte *et al.* (2004). Briefly, 7 days old transformed tobacco cells were diluted to half 24 h before experiment. Cells were washed twice with M-10 buffer and were adjusted to 0.1 g $FW.mL^{-1}$ of suspension buffer. Cells were then incubated in the dark for 1 h with DAF-2DA (20 µM) at 24 °C on a rotary shaker (130 rpm) and were rinsed three times with fresh equilibration buffer to remove excessive fluorescent probe. These cells were transferred into 24-well plates (Costar, Corning, USA; http://www.corning.com) containing 1 mL of cells per well and stirred on a rotary shaker (130 rpm) during the whole period of experiment. Cells were pre-incubated for 10 min with cPTIO (500 µM) before Cry treatments. NO production was measured using a 24-well reader fluorometer (Mithras L 940, BERTHOLD TECHNOLOGIES, Germany; http://www.berthold.com) with 485 nm excitation and 515 nm emission filters. Fluorescence was expressed as RFU (relative fluorescence units). For each treatment, time kinetics for NO measurement was made on the same batch of cells. As DAF-2DA is light-sensitive, cells were maintained in obscurity until the end of the experiment.

2.5. Western Blotting

2.5.1. For activated MAPK detection in *Arabidopsis*

Arabidopsis thaliana (Col-0) leaf tissues were infiltrated with water or OGs and co-treated with DNQX to investigate the effect of GLRs inhibitor on MAPK activation. In mutant study, leaf tissues from WT (Col-0) and *Atglr3.3* were treated with OGs or water (control). Leaf tissues (100 mg) were homogenized by grinding in the automatic tissue grinder (Fisher Scientific; http://www.fishersci.com). Two hundred 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 at 14,000 *g* for 15 min at 4 °C.

Protein quantification was done using Bradford method (Bradford, 1976). For protein estimation, BSA was used as standard protein (0-10 $\mu g.\mu L^{-1}$).

Total protein extracts were diluted in a modified Laemmli buffer (Laemmli, 1970) and then heated at 95 °C for 5 min for gel electrophoresis. Gel plates were placed in a vertical gel electrophoresis system containing 10 % resolving and 4 % stacking gel. Twenty µg protein was loaded on a 10 % polyacylamide gel electrophoresis. Running buffer (25 mM Tris

Base; 190 mM Glycine; 0.1 % SDS) was added in gel electrophoresis reservoir and gel was run at 80 V for 30 min and then at 120 V for 1 h 30 min.

After electrophoresis, proteins were 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.

To check the equal amount of protein in each sample gel was 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). After de-staining, gel was washed {5 % glycerol in water (v/v)} and dried under vacuum drier.

To verify the equal and good transfer of proteins in each sample, nitrocellulose membrane was stained with ponceau red. Nitrocellulose membrane was put in TBST-1 % BSA at 4 °C overnight. After three successive rinsings with TBST for a total time of 30 min (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. http://www.cellsignal.com)] in 10 mL TBST-1% BSA for 2 h at room temperature. Membrane was rinsed 3 times, 10 min each time, with TBST. Nitrocellulose membrane was soaked with secondary antibody {dilution 1/60,000; Horseradish peroxidase antirabbit (BIORAD; http://www.bio-rad.com)}, in 20 mL TBST-1% BSA for 1 h at room temperature. Again nitrocellulose membrane was rinsed three time 15 min each time with TBST.

2.5.2. Enhanced chemiluminescence (ECL) detection

Twenty X LumiGLO^{TM} reagent and peroxidase (Cell Signaling Technomogy, Inc. http://www.cellsignal.com) was diluted to 1 X in water. The nitrocellulose membrane was incubated with diluted solution for 1 min and expose to X-ray film for 10-15 min. The film was soaked in revelation solution and then in fixation solution (Kodak).

2.5.3. Cell fractioning and western blotting

Cytosolic extracts from non-transformed *N. Tabacum* var Xanthi and Aeq-Cyt cells were obtained as previously described by Lebrun-Garcia *et al.* (1998).

Chloroplasts and mitochondria fractions were isolated according to Rödiger *et al.* (2010), except they were isolated from cells instead of plants: approximately 0.5 g of non-transformed Xanthi, Aeq-Cyt, Aeq-Chlo or Aeq-Mit cells were filtered on a GF-A glass filter and ground in a mortar in the presence of 1.5 mL extraction buffer (0.45 M sucrose; 15 mM MOPS; 1.5 mM EGTA; 0.6 % PVP; 10 mM DTT and 0.2 mM PMSF). Residual cell debris

were removed by filtering the homogenate through two layers of Miracloth (100 µM, pore size) and one layer of nylon mesh (25 µm, pore size). The filtered homogenate was centrifuged for 5 min at 2,000 *g* to obtain the crude chloroplast fraction. The mitochondria-containing supernatant of this first centrifugation step was cleared from most residual plastids by an additional centrifugation for 5 min at 6,000 *g*. The crude mitochondria fraction was pelleted by centrifugation at 16,000 *g* for 10 min. The pellets comprising crude chloroplasts or mitochondria were washed twice with the extraction buffer and recovered under Laemmli buffer conditions. All the operations described above were performed at 4° C. Gel electrophoresis of protein under denaturing conditions and immunoblot analysis were performed as described above. A primary rabbit polyclonal to aequorin antibody (dilution 1:2,000: Abcam, UK: http://www.abcam.com) and a secondary Horseradish peroxidase antirabbit antibody (dilution 1:10,000: BIORAD, USA; http://www.bio-rad.com) were used to perform the ECL detection.

2.6. Mitochondrial O_2 uptake

O_2 uptake rate of Aeq-mit cells (0.1 g FW.mL^{-1} in the suspension buffer) was measured at 25 °C with a Clark-type oxygen electrode system purchased from Hansatech Instrument (Hansatech Instruments Ltd. UK; http://www.hansatech-instruments.com). Inhibitors were added 10 min before Cry treatment to 3 mL of cell suspension in small Erlenmeyers. Thereafter, 1 mL was withdrawn, transferred into the electrode cuvette and treated with 100 nM Cry. Slope of O_2 consumption was measured after 2 min of equilibration. The O_2 concentration in air-saturated medium was taken as 237 nmol.mL^{-1} at 25 °C.

2.7. Measurement of Chl fluorescence yield

Chl fluorescence of Aeq-chlo cells was measured using a PAM fluorometer (PAM-100, WALZ, Germany; http://www.walz.com) using the following parameters in slow kinetic mode: the minimal fluorescence (F_0) and the maximal fluorescence (F_M) were obtained using a 36 µE modulated light and one saturation pulse (SP) of 1000 µE respectively. Variable fluorescence (F_V) was measured applying a non-saturating and non-modulated continuous actinic light (AL) with an intensity of 216 µE, and SP every 10 s. For recording, 1 mL of cell suspension treated or not with Cry was withdrawn and placed in a stirring cuvette, illuminated with far red light to measure F_0 before fluorescence measurements and F_M after the first SP.

F_V was then measured for 3 to 10 min depending on the experiments. La^{3+} was added 10 min prior to Cry treatment

2.8. *Botrytis cinerea* infection

For basal resistance studies against necrotrophic pathogen, *Botrytis cinerea*, GLRs inhibitors *e.g.* DNQX, CNQX and MK-801 or DMSO/water (as a control) were syringe infiltrated in 5-6 intact leaves of *Arabidopsis* (Col-0) 1 h before pathogen infection. About 30 min before inoculation, harvested spores of BMM isolate of *Botrytis cinerea* were diluted in ¼ PDB (potato dextrose broth, 6 $g.L^{-1}$ DIFCO; BD Biosciences; http://www.bdbiosciences.com) to obtain a final concentration of 5.10^4 $spores.mL^{-1}$. Droplets of 6 µL of spore suspension were deposited on treated leaves. The inoculated plants were kept in growth chamber under high humidity to provide the better growth condition to pathogen. To check the development of pathogen, quantification of outgrowth lesions was made at 3 days post infection (dpi) with the help of an electrical vernier calliper. In different biological replicates, 10-12 plants for each treatment (5 to 6 inoculations per plant) were used. Data was statistically analyzed by by one-way ANOVA on ranks followed by Dunnett's test ($p<0.05$) using the SigmaPlot software.

To further link the involvement of AtGLRs in basal resistance against *Botrytis cinerea*, infection tests were performed on GLRs mutant plants in the same way as mentioned above.

Microscopic observations were also made to verify the effect of inhibitors treatments on *in vitro* hyphal growth of *Botrytis cinerea* at different time points. To achieve this objectice, spores were diluted in PDB medium to obtain a final concentration of 5.10^4 $spore.mL^{-1}$ containing either the GLR inhibitors or the corresponding volume of DMSO or water (depending on the solvent used to dissolve the GLR inhibitor). Then, 12 µL droplet of spore suspension were placed on glass slides and were let to grow under high humidity for 10-12 h. Finally, the growth pattern (germination of hyphae) of the pathogen was observed under the microscope.

2.9. *Hyaloperonospora arabidopsidis* pathogenicity test

To demonstrate the role of GLRs in resistance against biotrophic pathogen, *Hyaloperonospora arabidopsidis* (*Hpa*), both pharmacological and genetic approaches were used. In pharmacological study, 12-16 seedlings of 3 weeks old *Arabidopsis* (Col-0) were

sprayed with DNQX, CNQX and MK-801 and were kept in the growth chamber for 48 h before inoculation with freshly harvested *Hpa* suspension having a spore concentration of 4.10^4 spore.mL^{-1}. Seedlings were shifted to growth chamber and were kept under high humidity for 7 days. At 7th day, aerial parts of the plants were harvested, pooled for each treatment and weighed. Spores were extracted in water with 10 min vortex and filtered through glass wool. The liberated spores were counted under microscope and infection intensity was calculated as number of spores.g^{-1} FW.

In case of GLRs mutants, inoculation was made in the same way as described above and susceptibility was compared to wild type Col-0 plants. Statistical significance of the date were analysed by one-way ANOVA on ranks, followed by Dunnett's test ($p<0.05$) using the SigmaPlot software.

The effects of DNQX, CNQX and MK801 on pathogen development were determined in *planta*. Briefly, leaf disks from Col-0 plants were treated for 48 h with GLRs inhibitors or the corresponding controls, DMSO or water, depending on the solvent used to dissolve the GLR inhibitors. Then, the leaf disks were inoculated with *HpaNoco2* strain (4.10^4 spores mL^{-1}) and pathogen was allowed to grown under high humidity for 7 days. Aniline blue staining of the treated disks was made and *Hpa* development was investigated through microscopy.

2.10. Gene expression by Real Time qPCR

To know whether GLRs are able to modulate the expression of different defense-related genes, expression pattern of some plant defense-related genes were tested in response to OGs/*Hap* in *Atglr3.3* (SALK_066021) mutants or OGs/*Hpa* co-treatment with DNQX in wild type (Col0) plants. For OGs treatments, 5 weeks old Col-0 plants were syringe infiltrated with OGs (2.5 mg.mL^{-1}) in the presence or absence of DNQX (500 µM). On the other hand, 3 weeks old plants were used to test the effect of DNQX on *H. arabidopsidis* infection. For mutant study, Col-0 and *Atglr3.3* plants were treated with OGs/*Hpa*. At defined time points, for all above mentioned experiments, samples were collected in liquid nitrogen at different time intervals and were stored at -80 °C before RNA extraction.

2.10.1. RNA isolation

To isolate RNA, leaf tissues were ground to fine powder by using automatic tissue grinder system (Fisher Scientific). Samples were homogenized by adding 1 mL of TRIzol reagent (Molecular Research Centre Inc. USA; http://www.mrcgene.com) per 50-100 mg of leaf tissue and were incubated for 5 min at room temperature. Then, 200 µL of chloroform

Table 2.3: List of GLRs-dependent genes with their T-DNA mutant lines.

Gene ID	Mutant name	Description	Salk lines	Seed Obtained
At1g57560	*atmyb50*	Mutant impaired in the gene encoding member of MYB Transcription Factor	SALK_035416C	NASC
At5g01490	*cax4*	Mutant impaired in the gene encoding a CATION EXCHANGER 4 of CAX 2 family	SALK_119863	NASC
At5g01900	*wrky62*	Mutant impaired in the gene encoding member of WRKY Transcription Factor	016H10	NASC
At1g66600	*wrky63*	Mutant impaired in the gene encoding member of Group III WRKY Transcription Factor	SALK_075986C	NASC
			SALK_068280C	NASC
At3g29000	*Calcium-binding EF hand family protein*	Mutant impaired in the gene encoding member of Calcium-binding EF-hand family protein	SALK_110088	NASC
At2g15760	*Calmodulin-binding protein*	Protein of unknown function	SALK_120829C	NASC
			SALK_114734	NASC
At3g25600	*Calcium-binding EF-hand family protein*	Mutant impaired in the gene encoding member of Calcium-binding EF-hand family protein	SALK_026276C	NASC
			SALK_086408C	NASC
At4g08780	*Peroxidase*	Mutant impaired in the gene encoding member of Peroxidase superfamily protein	SALK_142205C	NASC
			SALK_002964C	NASC

was added in each sample tube followed by a vigorous shaking for about 15 s and samples were centrifuged at 14,000 *g* for 15 min at 4 °C. This leads to the separation of mixture into three layers. The upper aqueous phase, containing RNA (approx. 500 µL), was removed cautiously in a new tube and an addition of 500 µL of isopropanol followed by a 10 min incubation at at 4 °C was carried out to precipitate the RNA. In the next step, samples were centrifuged at 14,000 *g* for 10 min at 4 °C. The supernatant was removed from the tubes and 1 mL of 75 % diethyl pyrocarbonate (DEPC)-treated ethanol was added to each tube to wash the RNA pellet. Samples were centrifuged at 10,000 *g* for 10 min at 4 °C. The ethanol was discarded and the pellet was allowed to dry for 5-10 min. Finally, the RNA pellet was resuspended in 20 µL of DEPC-treated H_2O and was then heated for 10 min at 55 °C. RNA yield was quantified by measuring nucleic acid absorbance at 260 nm using Biophotometer EPPENDORF; http://www.eppendorf.com) and the concentration of RNA was determined as follows:

$$\textbf{[RNA] (µg/mL)} = \mathbf{A_{260} \times Dilution \times 40}$$

RNA quality was analysed by running the samples on 1.5 % agarose gel electrophoresis (50 V, 45 min). Gels were stained in ethidium bromide for 10 min and pictures were taken by using Molecular imager GelDoc XR imaging system (BIORAD; http://www.bio-rad.com).

2.10.2. Synthesis of cDNA

2.10.2.1. DNase treatment

DNase treatments were performed according to manufacturer's advice by using Deoxyribonuclease I amplication grade (DNase I) (SIGMA-ALDRICH; http://www.sigmaaldrich.com). Briefly, 1 µL of Amplification Grade DNase I (1 unit/µL) and 1 µL of 10 X reaction buffer was added to 2 µg of total RNA in 8 µL water, mixed gently and was incubated for 15 min at room temperature. Then, 1 µL of stop solution was added and the mixture was heated in water bath at 70 °C for 10 min to denature DNase I and RNA and was followed by a subsequent chilling on ice.

2.10.2.2. First strand cDNA synthesis

First strand synthesis of cDNA was carried out using a cDNA synthesis kit (ImProm-II^{TM} Reverse Transcriptase, Promega; http://www.promega.com), according to the manufacturer's protocol. One µg of DNase-treated RNA template and 1 µL of oligo dT

Table 2.4: List of primer used for genotyping of T-DNA mutant lines of GLRs-dependent genes.

Accession No.	Gene name	Salk lines ID	Primers sequence (5' =====> 3')	Tm(℃)
At1g57560	*AtMYB50*	SALK_035416C	**LP**: CAGAAAAGTAGGAATAATGTGATTGG	59.40
			RP: AACATCGACGATGGTTCTGTC	59.99
At5g01490	*CAX4*	SALK_119863	**LP**: ACAGACGCAAAAACATTGACC	60.03
			RP: TTATCATCTCCGTTGCGTTTC	60.09
At5g01900	*WRKY62*	016H10	**LP**: TCTTGCCAACAAAAGGCTATG	60.25
			RP: ATAAATGTCCGCTGATGGTTG	59.84
At1g66600	*WRKY63*	SALK_075986C	**LP**: TCAGTGTTTCAAGGAACCACC	60.00
			RP: AAAAACAGAGCAAATGGCATG	60.12
		SALK_068280C	**LP**: GAAGAATTTAGTGAAAAGACTGAATCG	60.07
			RP: TTACAGTTGTCGAGGACCGTC	60.16
At3g29000	*Ca^{2+}-binding EF hand family protein*	SALK_110088	**LP**: TTTTCCATCTTTGTTCCCATC	58.87
			RP: TGACCATGAAATCATGTTGTTC	58.33
At2g15760	*Calmodulin-binding protein*	SALK_120829C	**LP**: TGGAAGGAGTAGTAATCTTGGC	57.52
			RP: TCAACAAAAATGGTTGCTTCC	59.97
		SALK_114734	**LP**: AGTTGTTGCCAAATCGAGTTG	60.16
			RP: CTCCTCATTTCGAAGATTTCG	58.91
At3g25600	*Ca^{2+}-binding EF-hand family protein*	SALK_026276C	**LP**: AAGCTTCTGGGGACAAGAGAG	60.00
			RP: TTACGGTCGATTTGGTTCAAC	59.85
		SALK_086408C	**LP**: AAGCTTCTGGGGACAAGAGAG	60.00
			RP: TTACGGTCGATTTGGTTCAAC	59.85
At4g08780	*Peroxidase*	SALK_142205C	**LP**: TGGTTTTGAAATTTTGGCTTTC	60.32
			RP: TTTTGCCAAAAGTGTGACCAC	60.95
		SALK_002964C	**LP**: TTGTGGCGTAGTATAGTAGCGC	59.49
			RP: GGCAAATTCAAGAGCATCATC	59.66

primer (13 µM) were added in a PCR tube and a final volume of 5 µL was obtained by adding nuclease free water. Mixture was incubated at 70 °C for 5 min and a quick chilling was done at 4 °C for 5 min. In each tube, 15 µL of reverse transcription mix {containing 4 µL of 5X reaction buffer; 2.4 µL of $MgCl_2$ 25 mM; 1 µL of 10 mM dNTP and 1 µL of reverse transcriptase (ImProm-II™ RT; 200 units/µL, in 6.6 µL nuclease free water)} was added. cDNA synthesis was performed under following conditions: annealing at 25 °C for 5 min, extension step at 42 °C for 60 min and final heating at 70 °C for 15 min. Synthesized cDNA were stored at -20 ^{o}C.

2.10.2.3. Transcript accumulation analysis by Real Time qPCR

Gene-specific primers corresponding to different genes of defense signaling pathway and GLRs-dependent genes (obtained after microarray analysis) selected either from CATMA (http://www.catma.org/database/simple.htmL) or were designed using AmplifX 1.1 (http://ifrjr.nord.univ-mrs.fr/AmplifX; Table 2.5) for the gene expression analysis through qRT–PCR. Before gene expression analysis, optimum Tm of these primers was verified by conventional PCR.

Gene expression was analysed by Real Time qPCR using ubiquitin *UBQ10* (At4g05320), 5'-ctatatgctcgctgctgagc-3'and 5'-aagccaggcagagacaactc-3' as internal standard. Amplification of the cDNA was performed with a light cycler 480 (Prime detection system, Roche, France; https://www.roche-applied-science.com) in 384-well plate (Optical reaction plate with Bar code, Applied Biosystem) as recommended by the manufacturer. Reactions were performed in a final volume of 5 µL containing 2 µL cDNA (dilution 1/10), 200 nM 0.25 µL each of forward and reverse primers and 2.5 µL Absolute ™ QPCR SYBR© Green ROX Mix (Thermo Fisher Scientific, USA; http://www.thermofisher.com) containing the fluorophore, polymerase, dNTPs and reaction buffer of the enzyme. Following settings were used for the qPCR reaction: first denaturation step of 15 min at 95 °C, followed by 40 cycles, each consisting of three further steps: 1) 30 s at 95 °C (denaturation step), 2) 30 s at 50-60 °C (hybridization step, Tm depending on primers), 3) 45 s at 72 °C (amplification of the complementary strand) and a final step consisting of 15 s at 95 °C, 15 s at 72 °C and 15 s at 95 °C. Fluorescence was measured using the SDS software (Applied Biosystems).

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: $\Delta Ct = \Delta Ct_{gene\ of\ interest} - \Delta Ct_{HK}$. The $2^{-\Delta\Delta CT}$ method

Table 2.5: List of gene specific primer used for RT-qPCR.

Accession No.	Gene name	Primer sequence (5' =====> 3')	Annealing Temp. (℃)
At3g29000	*Ca^{2+}-binding EF hand family protein*	**LP**: CATCAACGCGTTTTCGACA	53
		RP: CTTCCAAACTCGCTTCTTTCTC	
At3g22910	*Ca^{2+}-transporting ATPase (ACA13)*	**LP**: GAGAAACGTATCTGATCATGC	50
		RP: TGAACGTCTACGTTGAATCT	
At1g69930	*GSTU11*	**LP**: AGGAGAAAACATCGGGTT	50
		RP: GAGCCTAGCGAACTGGAC	
At1g57560	*MYB50*	**LP**: TTCCACAAGTTTTTCCTCAG	50
		RP: AATCGAGGGTTTCACGCA	
At5g01490	*CAX4*	**LP**: GACAGGGAGATATGAACAGCA	53
		RP: AGAGGTGAAAGATGAGAAAAGCC	
At1g12663	*PR13*	**LP**: TCTGGCTCAGACAGCGGCTCA	53
		RP: AGTTGCACATCCGAGTTTGCAGA	
At5g22570	*WRKY38*	**LP**: AACCGAACATAATCTAGATGCC	53
		RP: TCTTGACAATCTTGGTCATTACTGG	
At5g01900	*WRKY62*	**LP**: TGAACTCTTGCCAACAAAAGGC	53
		RP: TTCCCCTCTTGTGAAGTGGTTT	
At1g66600	*WRKY63*	**LP**: TCCCCGCCTTGATGACGGCT	60
		RP: CCCCCAAAAGACTTCATCATCCACC	
At1g43160	*RAP2.6*	**LP**: AACTCAGACGATTCAACGA	50
		RP: GTATTGATCATATTCGGTCC	
At5g47910	*RBOHD*	**LP**: GACGATGAGTACGTGGAG	53
		RP: AAAACTTGGCAGAGAGTAAG	
At1g74710	*ICS1*	**LP**: GGGATAAGGGGTTCTCAC	53
		RP: AACAATCATAACAGCTAGGC	
At1g14540	*PER4*	**LP**: CACTGGTTCAGATGGACAAA	53
		RP: AACAAACGAATTATCGCTGC	
At2g14610	*PR1*	**LP**: CACTACACTCAAGTTGTTTGG	53
		RP: TGATAAATATTGATACATCCTGC	
At4g05320	*UBQ10*	**LP**: GAGATAACAGGAACGGAAACATAG	55
		RP: GGCCTTGTATAATCCCTGATG	

was used to analyze the relative changes in gene expression from real-time quantitative PCR experiments (Livak and Schmittgen, 2001).

2.11. Transcriptome analysis

Arabidopsis thaliana (Col-0) plants were grown under same conditions as described earlier in biological material section. Four weeks old plant leaves were syringe infiltrated with water/DMSO, OGs + DMSO, OGs + DNQX, DNQX and Glu. OGs, DMSO, DNQX and Glu were used at a working concentration of (2.5mg.mL^{-1}, 500 µM, 500 µM and 10 mM, respectively. Samples were collected at different time intervals (T0, 1 h and 6 h). Three independent biological replicates containing three plants for each condition and time point were used for NimbleGen Array.

2.11.1. RNA extraction, cDNA synthesis and labeling

Total RNA from frozen leave tissues of *Arabidopsis thaliana* was extracted with RNeasy Plant mini kit (QIAGEN, Germany; http://www.qiagen.com) according to manufacturer's protocols. RNA was quantified by nanodrop 100 (Thermo scientific, USA; http://www.nanodrop.com) and total RNA quality was checked by microchips on Agilent bio-analyzer 2100 (Agilent Technologies, USA; http://www.chem.agilent.com). 10 µg of total RNA was reverse transcribed in double stranded cDNA using SuperScript double-stranded cDNA synthesis kit (Invitrogen, USA; http://www.invitrogen.com) according to manufacturer's protocols. One color DNA labeling kit (Roche NimbleGen Inc. http://www.nimblegen.com) was used to label double-stranded cDNA. Briefly, 1 µg of double stranded cDNA was incubated with Cy3-random nonamers primers at 98 °C for 10 min (denaturation) and then was incubated with dNTP and Klenow enzyme at 37 °C for 2 h. Purified Cy3 labeled cDNA was quantified with nanodrop 100.

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). In each replicate, each gene was targeted by 4 probes. Briefly, 4 µg of Cy3-labeled cDNA was mixed with alignment oligonucleotides, 2X hybridization buffer and samples tracking controls (STC) provided in the Nimblegen Hybridization kit and heated at 95 °C for 5 min. Using NimbleGen hybridization system, the mixture was hybridized at 42 °C for 20 h and slides were rinsed by

NimbleGen wash buffer kit (Roche NimbleGen, Inc. http://www.nimblegen.com) according to manufacturer's instructions.

The slides were scanned with a GenePix 4000B scanner (Axon, Union City, USA; http://www.axon.com) and GenePix software fitted with the laser set at 532 nm, laser power 100 %, and the photomultiplier tube voltage (PMT) was at 540V. 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.

2.11.3. Microarray data analysis

Relative intensity of the raw hybridization signal on arrays varies in different replicates. To determine differentially expressed genes in treated condition as compared to control, Quantile normalization (Bolstad *et al.*, 2003) and Robust Multi-array average (RMA; Irizarry *et al*., 2003) were performed. A background correction was also performed. Based on these statistical analysis, genes were considered significantly up- or down-regulated in response to treatment if the fold change between treated and control plants is ≥ 2.0, with P value ≤ 0.01.

Up- or down-regulated genes that showed a greater than two fold change (FC) in the expression ratio (treated leaf tissue versus control leaf tissue) in all arrays were identified as OGs-responsive, Glu-dependent and GLRs-dependent gene. Further analyses to investigate the involvement of these genes were performed through GO annotation and MapMan software. To validate the microarray data, expression levels of some selected genes were verified through RT-qPCR as described in above section.

CHAPTER 3-5

“Results”

CHAPTER 3

CHAPTER 3

"Calcium signatures and signaling in cytosol and organelles of tobacco cells induced by plant defense elicitors"

CHAPTER 3

CALCIUM SIGNATURES AND SIGNALING IN CYTOSOL AND ORGANELLES OF TOBACCO CELLS INDUCED BY PLANT DEFENSE ELICITORS

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ABSTRACT

Calcium signatures induced by two elicitors of plant defense reactions, namely cryptogein and oligogalacturonides, were monitored at the subcellular level, using apoaequorin-transformed *Nicotiana tabacum* var Xanthi cells, in which the apoaequorin calcium sensor was targeted either to cytosol, mitochondria or chloroplasts. Our study showed that both elicitors induced specific Ca^{2+} signatures in each compartment, with the most striking difference relying on duration. Common properties also emerged from the analysis of Ca^{2+} signatures: both elicitors induced a biphasic cytosolic [Ca^{2+}] elevation together with a single mitochondrial [Ca^{2+}] elevation concomitant with the first cytosolic [Ca^{2+}] peak. In addition, both elicitors induced a chloroplastic [Ca^{2+}] elevation peaking later in comparison to cytosolic [Ca^{2+}] elevation. In cryptogein-treated cells, pharmacological studies indicated that IP_3 should play an important role in Ca^{2+} signaling contrarily to cADPR or nitric oxide, which have limited or no effect on [Ca^{2+}] variations. Our data also showed that, depending on [Ca^{2+}] fluxes at the plasma membrane, cryptogein triggered a mitochondrial respiration increase and affected excess energy dissipation mechanisms in chloroplasts. Altogether, the results indicate that cryptogein profoundly impacted cell functions at many levels, including organelles.

1. Introduction

Plants possess an innate immune system that allows fighting against potential pathogens. Recognition of potential pathogens is achieved through pattern recognition receptors (PRRs) interacting with conserved Pathogen- or Microbe-Associated Molecular Patterns (PAMPs or MAMPs) and initiates a complex signaling pathway leading to host defense. Early signaling steps include Ca^{2+} fluxes at the plasma membrane (PM) level, due to the activities of Ca^{2+} channels with cyclic nucleotide gated channels (CNGCs) and glutamate receptors (GluRs) as potential candidates. Activation of PM Ca^{2+} channels is relayed by calcium sensors that rapidly convert the signal to complex arrays of responses including protein phosphorylations and production of second messengers such as inositol-1,4,5-triphosphate (IP_3), reactive oxygen species (ROS), nitric oxide (NO) and cyclic nucleotides. The second messengers are able to trigger subsequent elevations in free cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) to µM values by activating of PM and/or endomembrane Ca^{2+} channels. In plants, internal stores of calcium include the vacuole, which occupies upto 90 % of the cell volume, endoplasmic reticulum (ER), mitochondria and chloroplasts. Ca^{2+} concentrations in these organelles are in the mM range (McAinsh and Pittman, 2009). However, free $[Ca^{2+}]$ in mitochondrial matrix or chloroplast stroma is finely controlled and maintained below µM range in resting cells (Johnson *et al.*, 1995; Sai and Johnson, 2002; Logan and Knight, 2003; McAinsh and Pittman, 2009). In matrix, free mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_{mit}$) elevations are observed upon treatment of *Arabidopsis thaliana* seedlings by different stimuli, such as cold, touch, osmotic stress or H_2O_2 treatment (Logan and Knight, 2003). It was also shown that isolated mitochondria from potatoes or wheat are able to uptake Ca^{2+} (Arpagaus *et al.*, 2002; Virolainen *et al.*, 2002) and to release it under anoxia (Subbaiah *et al.*, 1998; Virolainen *et al.*, 2002). Furthermore, isolated wheat mitochondria undergo a swelling after Ca^{2+} treatment, indicative of an opening of permeability transition pore (PTP) as in animal cells, and a release of cytochrome c, which might precede cell death (Virolainen *et al.*, 2002). Chloroplasts accumulate calcium in thylakoid lumen through the activity of a Ca^{2+}/H^{+} antiport in a light-dependent process that generates an H^{+} gradient inside the thylakoid lumen (Ettinger *et al.*, 1999). In the stroma, free chloroplastic $[Ca^{2+}]$ ($[Ca^{2+}]_{chlo}$) stays rather stable during illumination (Johnson *et al.*, 1995), but after light-to-dark transition, a $[Ca^{2+}]_{chlo}$ burst occurs peaking at 20-30 min after the onset of darkness (Johnson *et al.*, 1995; Sai and Johnson, 2002). This stromal free Ca^{2+} burst could originate from the thylakoid

lumen store or from a non identified stromal store, and might produce a moderate $[Ca^{2+}]_{cyt}$ elevation that returns to basal level within 2 hours (Sai and Johnson, 2002).

As calcium is a major second messenger in plant defense signaling and because chloroplasts and mitochondria are also involved in plant defense reactions (Abbink *et al.*, 2002; Matsumura *et al.*, 2003; Scharte *et al.*, 2005; Bonfig *et al.*, 2006; Jelenska *et al.*, 2007; Liu *et al.*, 2007; Belhaj *et al.*, 2009; Pandelova *et al.*, 2009; Dodd *et al.*, 2010; Gleason *et al.*, 2011), it was of high interest to study whether PAMPs are able to promote $[Ca^{2+}]$ variations in these compartments and whether $[Ca^{2+}]$ variations could affect important physiological processes taking place in organelles, such as photosynthesis and mitochondrial respiration. Two PAMPs, namely cryptogein (Cry) and oligogalacturonides (OGs) were used in this study. Cry, a 10 kDa protein secreted by the oomycete *Phytophthora cryptogea*, induces hypersensitive response (HR) and systemic acquired resistance (SAR) in tobacco. When recognized by high affinity binding sites located on the PM, Cry promotes a large calcium influx necessary for the induction of many signaling events (Garcia-Brugger *et al.*, 2006), and triggers $[Ca^{2+}]_{cyt}$ and free nuclear $[Ca^{2+}]$ ($[Ca^{2+}]_{nuc}$) elevations (Lecourieux *et al.*, 2002, 2005). The huge calcium influx and the disruption of Ca^{2+} homeostasis induce cell death in tobacco cell suspensions measurable within the first 24 h of treatment (Binet *et al.*, 2001; Lecourieux *et al.*, 2002). OGs are breakdown fragments of plant cell wall pectin hydrolyzed by fungal endopolygalacturonases and behave as elicitors of plant defense reactions in different plant materials. In particular, OGs induce a rapid and transient $[Ca^{2+}]_{cyt}$ increase in tobacco cells (Chandra and Low, 1997; Lecourieux *et al.*, 2002) but a fairly small $[Ca^{2+}]_{nuc}$ elevation (Lecourieux *et al.*, 2005). Both elicitors efficiently promote ROS production, which was shown to be dependent on Ca^{2+} influx for Cry (Tavernier *et al.*, 1995; Chandra and Low, 1997). Contrarily to Cry, OGs are non necrotic elicitors, and this property might be correlated to their low Ca^{2+} influx induction (Binet *et al.*, 2001).

In this study, $[Ca^{2+}]$ variations induced by Cry and OGs were measured in cytosolic, chloroplastic and mitochondrial compartments, and also compared to $[Ca^{2+}]_{nuc}$ previously described (Lecourieux *et al.*, 2005). Interestingly, both elicitors induced significant $[Ca^{2+}]$ variations in all the mentioned compartments, except in the nucleus for OGs. The Ca^{2+} signature depended on the elicitor, and each compartment could be distinguished by a unique Ca^{2+} signature. Compared to the $[Ca^{2+}]_{cyt}$ elevation, the $[Ca^{2+}]_{chlo}$ elevation was peaking later, while in mitochondria the elicitor treatment led to a $[Ca^{2+}]_{mit}$ elevation concomitant with the first peak of the $[Ca^{2+}]_{cyt}$ increase. This suggested that organelles had different regulations depending on the channels, pumps, transporters and storage capacity. Using Cry, we showed

that Ca^{2+} signaling pathway relies mostly on an IP_3-related process. Furthermore, we showed that, depending on Ca^{2+} fluxes at the PM, Cry increased mitochondrial respiration and slowed down the dissipation of excess light energy.

2. Materials and Methods

2.1. Plant material and transformation

Wild type *N. tabacum* var Xanthi leaves were transformed using *A. tumefaciens* carrying either the plasmid pRTL2 for cytosol-targeted apoaequorin (Knight *et al.*, 1991), the pMAQ6 plasmid for chloroplast-targeted apoaequorin (Johnson *et al.*, 1995) or the pBIN AGA#2 plasmid for the mitochondria-targeted apoaequorin (Logan and Knight, 2003). Screening of kanamycin resistant transformed plantlets for apoaequorin expression was made by conventional Western-blotting, and transformed *N. tabacum* var Xanthi cell suspensions were generated as described in Lecourieux *et al.* (2005).

Transformed cell suspensions were subcultured every 7 days by addition of 7 mL cells to 100 mL fresh liquid Chandler's medium (Chandler *et al.*, 1972) and maintained in a culture chamber at 25°C, continuous shaking (120 rpm) and light (2000 $ergs.cm^{-2}$).

2.2. Cell fractioning and Western blotting

Cytosolic extracts from non transformed *N. Tabacum* var Xanthi and Aeq-Cyt cells were obtained as previously described (Lebrun-Garcia *et al.*, 1998).

Chloroplasts and mitochondria fractions were isolated according to Rödiger *et al.* (2010), except they were isolated from cells instead of plants: approximately 0.5 g of non transformed Xanthi, Aeq-Chlo or Aeq-Mit cells were filtered on a GF-A glass filter and ground in a mortar in the presence of 1.5 mL extraction buffer (0.45 M sucrose; 15 mM MOPS; 1.5 mM EGTA; 0.6 % PVP; 10 mM DTT and 0.2 mM PMSF). Residual cell debris were removed by filtering the homogenate through two layers of Miracloth (100 µM, pore size) and one layer of nylon mesh (25 µm, pore size). The filtered homogenate was centrifuged for 10 min at 1200 *g* to obtain the crude chloroplast fraction. The mitochondria-containing supernatant of this first centrifugation step was cleared from most residual plastids by an additional centrifugation for 10 min at 6000 *g*. The crude mitochondria fraction was pelleted by centrifugation at 16000 *g* for 10 min. The pellets comprising crude chloroplasts or mitochondria were washed twice with the extraction buffer and recovered under Laemmli buffer conditions. All the operations described above were performed at 4 °C.

Gel electrophoresis of protein under denaturing conditions and immunoblot analysis were performed as described in Lebrun-Garcia *et al.* (1998). Primary rabbit polyclonal antibodies were used against aequorin (Abcam, Cambridge UK), Idh or RbcL (Agrisera AB, Sweden). The secondary Horseradish peroxidase antirabbit antibody used to perform the ECL detection was from Biorad.

2.3. Cell preparation and treatment

Transgenic tobacco cells from 6-days old cultures corresponding to the exponential phase of growth were collected by filtration, washed twice and adjusted to 0.1 g fresh weight (FW).mL^{-1} with the suspension buffer (175 mM mannitol; 0.5 mM $CaCl_2$; 0.5 mM K_2SO_4 and 10 mM MES, pH 5.75). After 2 h incubation (130 rpm, 25 °C), cells were treated with Cry or OGs. Cry was purified from the filtrate of *P. cryptogea* culture according to Baillieul *et al.* (1995). OGs, with an average degree of polymerization of 25, were obtained from GOEMAR (Saint Malo, France). Inhibitors purchased from Sigma-Aldrich, were dissolved in water except U73122 and U73343 which were prepared in ethanol, so that the final concentration in cells did not exceed 1 %. The corresponding 1 % ethanol control showed no effect on cells. Inhibitors were added 10-20 min before elicitor treatment.

2.4. Measurement of [Ca^{2+}] variations

[Ca^{2+}] variations were measured using apoaequorin-transformed *N. tabacum* var Xanthi cells. *In vivo* reconstitution of aequorin was initiated by addition of 1 µM native coelenterazine (Uptima) to cells prepared in the suspension buffer as above for at least 2 h in the dark (130 rpm, 25 °C). Relative luminescence units were recorded with a digital luminometer (Lumat LB 9507, Berthold, Bad Wildbad, Germany) and were converted into Ca^{2+} concentrations (Lecourieux *et al.*, 2005). Because the N-terminal fusions of aequorin do not affect aequorin activity (Logan and Knight, 2003; Rizzuto *et al.*, 1992; Knight and Knight, 1995), the same [Ca^{2+}] calibration parameters were used.

2.5. Measurement of nitric oxide

NO accumulation was determined using the fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich) as described in Lamotte *et al.* (2004).

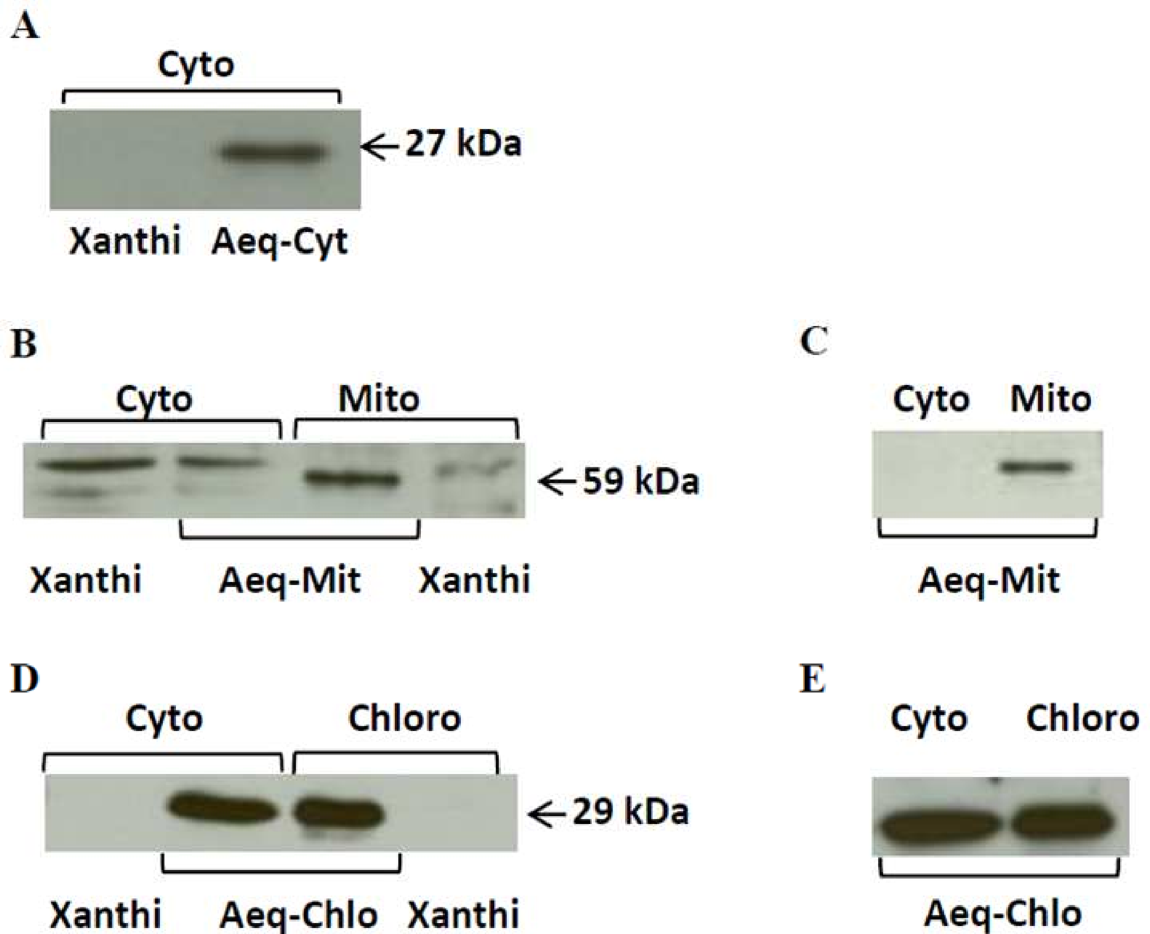

Figure 3.1: Targeting of apoaequorin in transgenic cell suspensions. Western-blots: against apoaequorin in cytosolic extracts of Xanthi or Aeq-Cyt cells **(A)** against apoaequorin in cytosolic or mitochondrial extracts of Xanthi or Aeq-Mit cells **(B)** against Idh in cytosolic or mitochondrial extracts of Aeq-Mit cells **(C)** against apoaequorin in cytosolic or chloroplastic extracts of Xanthi or Aeq-Chlo cells **(D)** against RbcL in cytosolic or chloroplastic extracts of Aeq-Chlo cells **(E)** The arrows indicate the size of the native or recombinant apoaequorin. Cyto: cytosol extract, Mito: mitochondrial extract, Chloro: chloroplasts extract.

2.6. Mitochondrial O_2 uptake

O_2 uptake rate of Aeq-mit cells (0.1 g FW.mL^{-1} in the suspension buffer) was measured at 25° C with a Clark-type oxygen electrode system purchased from Hansatech Instrument (Ltd. Norfolk, UK). Inhibitors were added 10 min before Cry treatment to 3 mL of cell suspension in small Erlenmeyers. Thereafter, 1 mL was withdrawn, transferred into the electrode cuvette and treated with 100 nM Cry. Slope of O_2 consumption was measured after 2 min of equilibration. The O_2 concentration in air-saturated medium was taken as 237 nmol.mL^{-1} at 25 °C.

2.7. Measurement of Chl fluorescence yield

Chl fluorescence yield of Aeq-chlo cells was measured using a PAM fluorimeter (PAM-100, Walz, Germany) using the following parameters in slow kinetic mode: the minimal fluorescence yield (F_0) and the maximal fluorescence yield (F_M) were obtained using a 36 µE modulated light and one saturation pulse (SP) of 1000 µE, respectively. Fluorescence yield (F_t) was measured applying a non-saturating and non-modulated continuous actinic light (AL) with an intensity of 216 µE, and SP every 10 sec. For recording, 1 mL of cell suspension treated or not with Cry was withdrawn and placed in a stirring cuvette, illuminated with far red light to measure F_0 before fluorescence measurements and F_M after the first SP. F_t was then measured for 3-10 min depending on the experiments and normalized to F_M. La^{3+} was added 10 min prior to Cry treatment.

3. Results

3.1. Cryptogein and OGs induced specific subcellular Ca^{2+} signatures in cytosol, chloroplasts and mitochondria

In order to compare the kinetics and amplitudes of [Ca^{2+}] variations induced by elicitors in different compartments, apoaequorin-transformed *N. tabacum* var Xanthi cells were generated from leaves of *N. tabacum* var Xanthi plants transformed by *Agrobacterium tumefaciens* carrying different constructs. Genes under the 35S promoter were used to target apoaequorin either in the cytosol, the chloroplast or the mitochondria: the gene products corresponded respectively to wild type apoaequorin, apoaequorin fused with the chloroplast-targeting transit peptide of the stromal small subunit of RuBisCO, and apoaequorin fused in its N-terminal part with the first 87 amino acid peptide of the mitochondrial matrix *N. plumbaginifolia* β-subunit of ATPase and the mGreen Fluorescent Protein. The corresponding

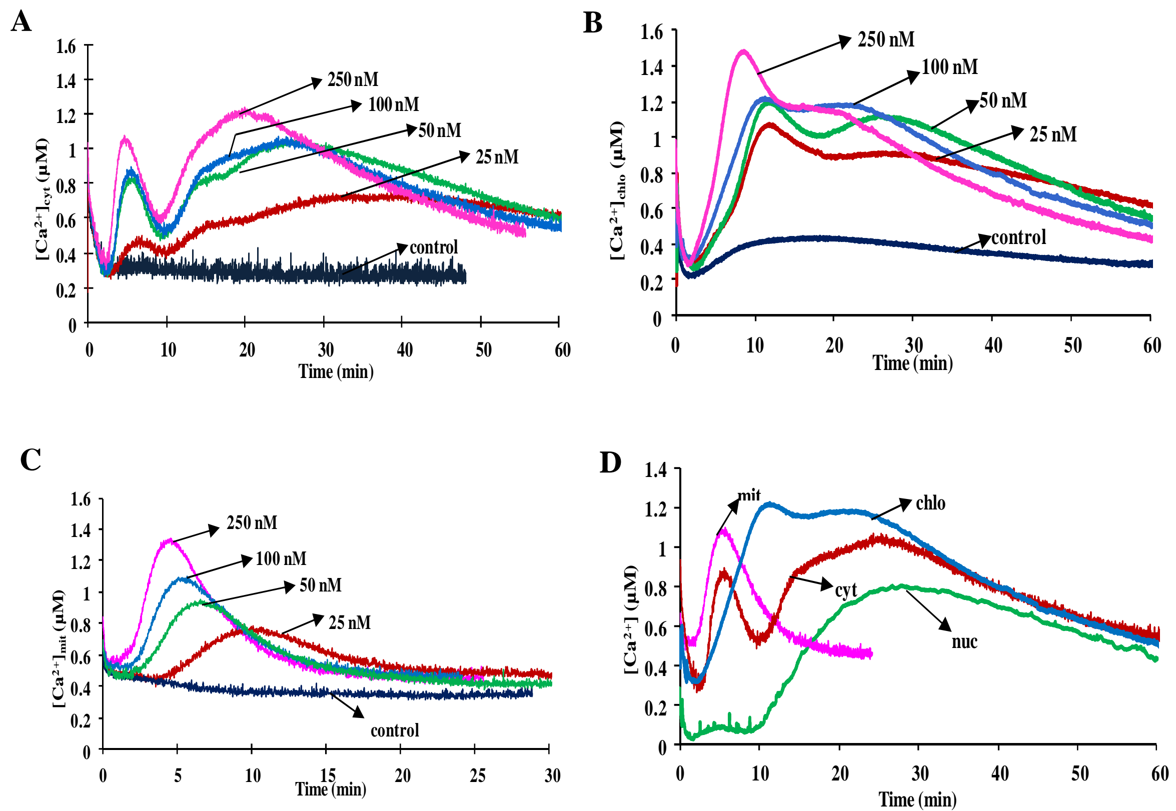

Figure 3.2: Dose-dependent effects of Cry on Aeq-Cyt cell suspensions. (A) Aeq-Chlo cell suspensions **(B)** and Aeq-Mit cell suspensions **(C)** Cry was used in a concentration range from 25 to 250 nM. **(D)** Overlay of $[Ca^{2+}]$ elevations induced by 100 nM Cry treatment in the cytosol (Cyt), chloroplasts (Chlo), mitochondria (Mit) and nucleus (Nuc). Basal level $[Ca^{2+}]$ varied from 0.05 to 0.4 µM depending on the compartment.

native or recombinant apoaequorins were very efficiently targeted to the expected compartment in *N. plumbaginifolia* or *A. thaliana* (Knight *et al.*, 1991; Sai and Johnson, 2002; Logan and Knight, 2003). It was also shown that N-terminal fusion to apoaequorin did not affect the aequorin activity (Rizzuto *et al.*, 1992; Knight and Knight, 1995). In our study, transgenic plants showing the highest amount of apoaequorin were used to generate cell suspensions, and cell suspensions with the most efficient *in vivo* aequorin reconstitution were selected. Selected stable transformed cell lines were named Aeq-Cyt, Aeq-Chlo and Aeq-Mit according to the subcellular localization of apoaequorin in the cytosol, chloroplast and mitochondria respectively, and were used to monitor [Ca^{2+}] variations in the subcellular compartments. The subcellular localization of apoaequorin was verified by cell fractioning and Western-blotting showing that apoaequorin was targeted in the cytosol (Figure 3.1A) or in the organelles (Figure 3.1B-E), depending on their respective targeting sequence. In Aeq-Cyt cells, the anti-apoaequorin antibody revealed one band with the expected size in cytosolic extracts (Figure 3.1A). In Aeq-Mit cells, the anti-apoaequorin antibody revealed one band with the expected size in isolated mitochondria, which was neither present in isolated mitochondria of non transformed Xanthi cells, nor in both the cytosolic protein extracts of Aeq-Mit cells and Xanthi cells (Figure 3.1B), thus showing that aequorin was properly targeted to the mitochondria in Aeq-Mit cells. Furthermore, an antibody raised against isocitrate dehydrogenase (Idh), a cellular compartment marker of mitochondrial matrix, revealed Idh only in the isolated mitochondria preparation confirming the localization of apoaequorin in the mitochondria (Figure 3.1C). The same Western-blottings were made using Aeq-Chlo cells with the anti-aequorin antibody and an antibody against the RuBisCO large subunit (RbcL), a cellular compartment marker of chloroplastic stroma. The anti-aequorin antibody revealed the apoaequorin in the cytosolic and chloroplastic preparations of Aeq-Chlo cells, but not in cytosolic and chloroplastic preparations of Xanthi cells (Figure 3.1D). Using the anti-RbcL antibody, a similar quantity of RbcL in the cytosolic and chloroplastic extracts was revealed, thus showing that cytosolic extracts were contaminated by chloroplastic proteins (Figure 3.1E). The percentage of RbcL and aequorin in the cytosolic extracts corresponded to 55.6 ± 2.6 % and 52 ± 1.7 % respectively, indicating consequently that aequorin was properly targeted to the chloroplasts in Aeq-Chlo cells. Other cell fractioning procedures also produced a strong contamination of chloroplastic proteins in cytosolic extracts (data not shown).

Dose-dependent treatments with Cry and OGs were performed in each type of transformed cell suspensions. Cry used at 25-250 nM induced a biphasic cytosolic $[Ca^{2+}]_{cyt}$

Table 3.1: Cry- and OGs-induced [Ca^{2+}] variations. The peak times and amplitudes of the peaks (mean values ± SD, n = 6 to 21) are given for the 100 nM Cry and 100 µg.mL^{-1} OGs concentrations. The peaks 1 and 2 corresponded to the first and second [Ca^{2+}] elevations.

Cry-induced [Ca^{2+}] variations

	Peak 1		Peak 2	
	Time (min)	Amplitude (µM)	Time (min)	Amplitude (µM)
[Ca^{2+}]$_{cyt}$	6.7 1.8	0.82 0.26	24.6 7.8	1.00 0.31
[Ca^{2+}]$_{chlo}$	12.4 1.8	1.07 0.32	26.9 5.2	1.19 0.25
[Ca^{2+}]$_{mit}$	6.4 1.4	1.14 0.30		

OGs-induced [Ca^{2+}] variations

	Peak 1		Peak 2	
	Time (min)	Amplitude (µM)	Time (min)	Amplitude (µM)
[Ca^{2+}]$_{cyt}$	2.2 0.6	1.14 0.44	2.8 0.7	1.17 0.29
[Ca^{2+}]$_{chlo}$	8.6 1.1	1.26 0.47		
[Ca^{2+}]$_{mit}$	2.5 0.4	1.36 0.26		

rise, which increased with the elicitor concentration (Figure 3.2A). The first elevation peaked at 5-7 min depending on the elicitor concentration. The second elevation, observed with a maximum in-between 20 and 40 min, was also depending on the Cry concentration and lasted for more than 1 hour. The higher the Cry concentration, the shorter is the lag between the two elevations. Chloroplastic signature induced by Cry was also biphasic, starting within the same time frame, but the first peak was delayed in comparison to the first $[Ca^{2+}]_{cyt}$ peak, reaching a maximum near 9-12 min depending on the Cry concentration; the second peak had a maximum in-between 22 and 30 min (Figure 3.2B). Control Aeq-Chlo cells also showed a weak $[Ca^{2+}]_{chlo}$ elevation at about 10 min that might be due to some sensitivity of cells to manipulate. Alternatively it might correspond to the light-to-dark transition peak, previously observed in tobacco plants (Sai and Johnson, 2002), and that might also be triggered when Aeq-Chlo cells were transferred to the luminometer. In mitochondria, Cry induced a single peak with a maximal $[Ca^{2+}]_{mit}$ obtained at 5-10 min depending on the elicitor concentration (Figure 3.2C). Cry-induced $[Ca^{2+}]_{nuc}$ showed a biphasic shape with two peaks concomitant with the $[Ca^{2+}]_{cyt}$ peaks, the first one being of very low amplitude compared to the latter (this study, Figure 3.2D; Lecourieux *et al.*, 2005).

Overlay of the $[Ca^{2+}]$ rises in each compartment generated by 100 nM Cry clearly showed that $[Ca^{2+}]_{cyt}$, $[Ca^{2+}]_{mit}$, $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{chlo}$ elevations started within the same time lag, but the first chloroplastic peak was clearly shifted. The second peaks of $[Ca^{2+}]_{cyt}$, $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{chlo}$ elevations were obtained within the same period (Figure 3.2D). The absence of a second $[Ca^{2+}]_{mit}$ peak but also the delayed $[Ca^{2+}]_{chlo}$ elevation indicated that Ca^{2+} cannot diffuse freely from one compartment into the mitochondria or into the chloroplasts.

The mean values obtained for the 100 nM Cry treatment corresponding to the maximal $[Ca^{2+}]$ variations at the peaks and peak times are reported in Table 3.1. Amplitude was in the 1 µM range, except for the first small $[Ca^{2+}]_{nuc}$ peak in the 0.1 µM range, and a slightly higher value was obtained for chloroplasts and mitochondria. $[Ca^{2+}]_{chlo}$ peaks 1 and 2 were delayed by approximately 6 and 2 min respectively in comparison to $[Ca^{2+}]_{cyt}$ peaks 1 and 2. Maximal $[Ca^{2+}]_{mit}$ elevation was obtained at the same time than $[Ca^{2+}]_{cyt}$ peak 1.

Compared to Cry-induced $[Ca^{2+}]$ elevations, apoaequorin-transformed cell lines treated with OGs in a concentration range of 25 to 500 $\mu g.mL^{-1}$ showed faster $[Ca^{2+}]$ elevations whatever the elicitor concentration that returned faster to basal level after 15-20 min (Figure 3.3). OGs-induced $[Ca^{2+}]_{cyt}$ rise started within the first min and had also a biphasic shape with two successive peaks at around 2 and 3-4 min depending on OGs concentration (Figure 3.3A). $[Ca^{2+}]_{chlo}$ elevation was delayed in comparison to OGs-induced

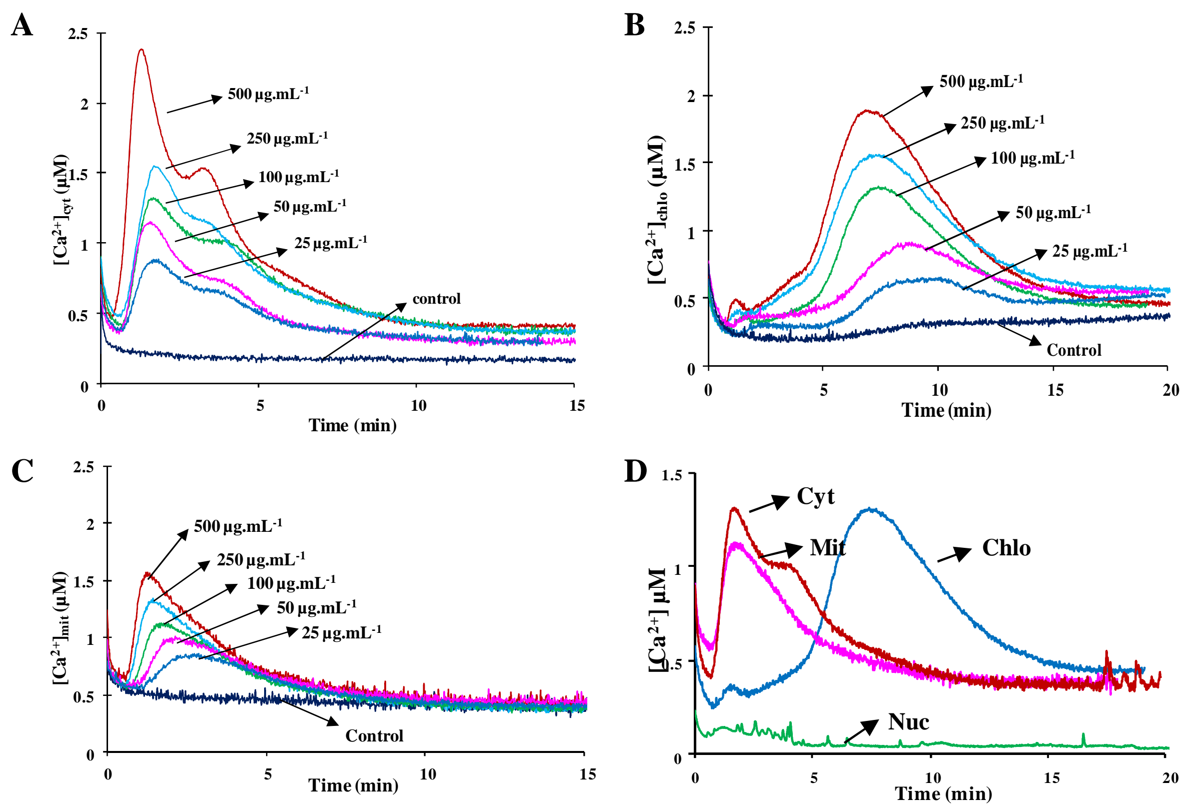

Figure 3.3: Dose-dependent effects of OGs on Aeq-Cyt cell suspensions. (A) Aeq-Chlo cell suspensions **(B)** and Aeq-Mit cell suspensions **(C)** OGs were used in a concentration range from 25 to 500 µg.mL^{-1}. **(D)** Overlay of [Ca^{2+}] elevations induced by 100 µg.mL^{-1} OGs treatment in the cytosol (Cyt), chloroplasts (Chlo), mitochondria (Mit) and nucleus (Nuc).

$[Ca^{2+}]_{cyt}$ rise with a maximum in between 7 and 9 min, and had a different shape (Figure 3.3B). At high OGs concentrations, a small shoulder was observed in the beginning of the $[Ca^{2+}]_{chlo}$ rise, fitting with $[Ca^{2+}]_{cyt}$ elevation. OGs-induced $[Ca^{2+}]_{mit}$ elevation corresponded to a single peak with a rapid rise, a slower decrease and a maximum around 2-3 min depending on the OGs concentration (Figure 3.3C). Superimposition of curves obtained with the 100 µg.mL^{-1} OGs treatment showed that although the OGs-induced Ca^{2+} signatures differed in shape and kinetics as compared to Cry-induced Ca^{2+} signatures, the lag phase in the $[Ca^{2+}]_{chlo}$ peak was also observed (Figure 3.3D), while the $[Ca^{2+}]_{mit}$ elevation was concomitant to $[Ca^{2+}]_{cyt}$. OGs induced a very faint $[Ca^{2+}]_{nuc}$ elevation concomitant with the first $[Ca^{2+}]_{cyt}$ elevation (this study and Lecourieux *et al.*, 2005).

The mean values obtained for the 100 µg.mL^{-1} OGs treatment corresponding to the maximal $[Ca^{2+}]$ variations at the peaks and peak times are reported in Table 3.1. Similar to Cry treatment, amplitude in the µM range was observed in all compartments, except for nucleus in the 0.1 µM range, and was also slightly more elevated in chloroplasts and mitochondria in comparison to cytosol. $[Ca^{2+}]_{chlo}$ peak was delayed by approximately 7 min, while $[Ca^{2+}]_{mit}$ peak was occurring within the same time frame as the peak 1 of $[Ca^{2+}]_{cyt}$ elevation.

3.2. Cryptogein-induced [Ca²⁺] signaling in organelles

Calcium influx at the PM might generate an increase in $[Ca^{2+}]_{cyt}$, which, together with the specific activation of second messengers, might amplify Ca^{2+} signaling leading to a stimulus-specific Ca^{2+} signature. We first verified whether Cry-induced $[Ca^{2+}]$ variations in the subcellular compartments were dependent on a Ca^{2+} influx at the PM level using lanthanum (La^{3+}), a Ca^{2+}channel blocker, or 1,2-bis(aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA), a membrane impermeable Ca^{2+} chelator, both used at 2 mM. Results are reported as a percentage of Cry-induced $[Ca^{2+}]$ variations in the presence of La^{3+} or BAPTA measured at the maximum value of the cytosolic, chloroplastic and mitochondrial $[Ca^{2+}]$ peaks compared to the corresponding peaks obtained from Cry-treated cells (Figure 3.4). When applied 10 min before Cry on the aequorin-expressing cell suspensions, La^{3+} produced a very strong to near complete inhibition of $[Ca^{2+}]$ elevation ranging from 86 % to 98 % depending on the subcellular $[Ca^{2+}]$ peak. A similar level of inhibition for $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{chlo}$ variations was obtained with BAPTA, while $[Ca^{2+}]_{mit}$ elevation was less efficiently inhibited by 66 %. Increasing BAPTA concentration produced a slightly higher inhibition of

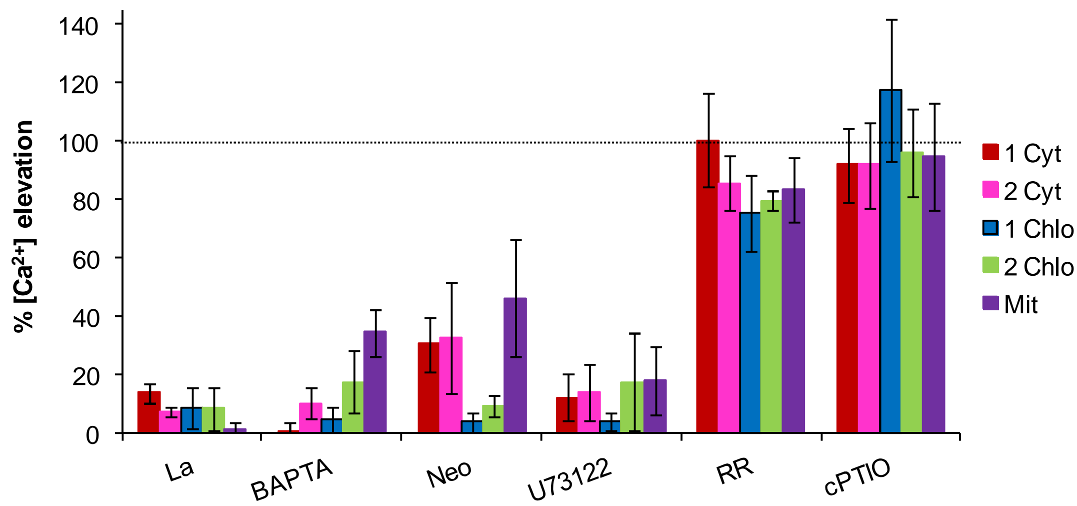

Figure 3.4: Effect of inhibitors on cryptogein-induced [Ca^{2+}] variations. Transgenic cell suspensions were pretreated with 2 mM La^{3+} (La), 2 mM BAPTA, 200 µM neomycin (Neo), 10 µM U73122, 20 µM RR or 500 µM cPTIO for 10-20 min before addition of 100 nM Cry. [Ca^{2+}] variations are expressed as a percentage of [Ca^{2+}] variations ± SD (n= 3 to 7) measured in presence of inhibitors and Cry compared to the variations in Cry-treated cells. These variations were measured at peaks 1 and 2 of the cytosolic and chloroplastic [Ca^{2+}] variations (1 Cyt, 2 Cyt, 1 Chlo, 2 Chlo respectively) and at the mitochondrial [Ca^{2+}] peak (Mit).

$[Ca^{2+}]_{mit}$ elevation (data not shown). Taken together, these results indicated that the intracellular $[Ca^{2+}]$ elevations are strongly depended on channel activities at the PM level.

Regulation of $[Ca^{2+}]$ variations in mitochondria and chloroplasts being unknown, we focus our efforts in determining which second messengers could regulate Cry-induced $[Ca^{2+}]_{cyt}$ and the $[Ca^{2+}]$ rises in organelles, and if these $[Ca^{2+}]$ variations were similarly regulated. Various inhibitors known to affect ligand-gated Ca^{2+} channels were tested on the three transgenic cell lines. U73122 and neomycin inhibit Ca^{2+} release through IP_3-regulated Ca^{2+} channels by acting on phospholipase C or by chelating phosphatidylinositol 4,5-bisphosphate (PIP_2) respectively, and ruthenium red (RR), a Ca^{2+} channel blocker, predominantly inhibits cADPR-activated ryanodine receptors (RYRs)-like intracellular channels (Allen *et al.*, 1995; Lecourieux *et al.*, 2005). Neomycin used at 200 µM had a partial inhibitory effect on Cry-induced $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ corresponding to 70 and 54 % inhibition respectively, but a 90-95 % strong inhibitory effect on $[Ca^{2+}]_{chlo}$ (Figure 3.4). U73122 used at 10 µM had a higher effect than neomycin on $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ variations with inhibition ranging from 88 to 96 %, and a similar inhibitory effect in comparison to neomycin on $[Ca^{2+}]_{chlo}$ variation (Figure 3.4). U73343, the inactive structural analog of U73122, did not affect Cry-induced $[Ca^{2+}]$ variations (data not shown). Neomycin and U73122 had also a partial inhibitory effect on Cry-induced $[Ca^{2+}]_{nuc}$ elevation (Lecourieux *et al.*, 2005). These data argued for a strong occurrence of IP_3-related calcium signaling pathway in Cry-treated cells that impacted Ca^{2+} homeostasis in cytosol and in organelles.

RR used at 20 µM had no effect or a slight effect on the first or the second Cry-induced $[Ca^{2+}]_{cyt}$ peaks, respectively. RR also slightly decreased the Cry-induced $[Ca^{2+}]$ elevations in the organelles by 20-25 % (Figure 3.4). As shown in (Lecourieux *et al.*, 2005), RR had no effect on Cry-induced $[Ca^{2+}]_{nuc}$ elevation. This indicated a limited involvement of cADPr-based Ca^{2+}-signaling although it cannot be excluded that RR in these experiments induced an inhibition of Ca^{2+}-ATPases, as RR is also known to act on animal mitochondria Ca^{2+}-uniporter (Moore, 1971).

ROS and NO are also known to modify the activity of Ca^{2+} channels in plants (Besson-Bard *et al.*, 2008a). In *N. plumbaginifolia* cells, where Cry induced a biphasic $[Ca^{2+}]_{cyt}$ variation, the first peak was reduced by scavenging H_2O_2 production by catalase, by inhibiting NADPH oxidase with diphenylene iodonium (DPI), or by scavenging NO production by 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yl-oxy-3-oxide (cPTIO) (Lecourieux *et al.*, 2002; Lamotte *et al.*, 2004). Unfortunately, ROS inhibitors could not be used in *N. tabacum* var Xanthi cells as they produced by themselves a significant

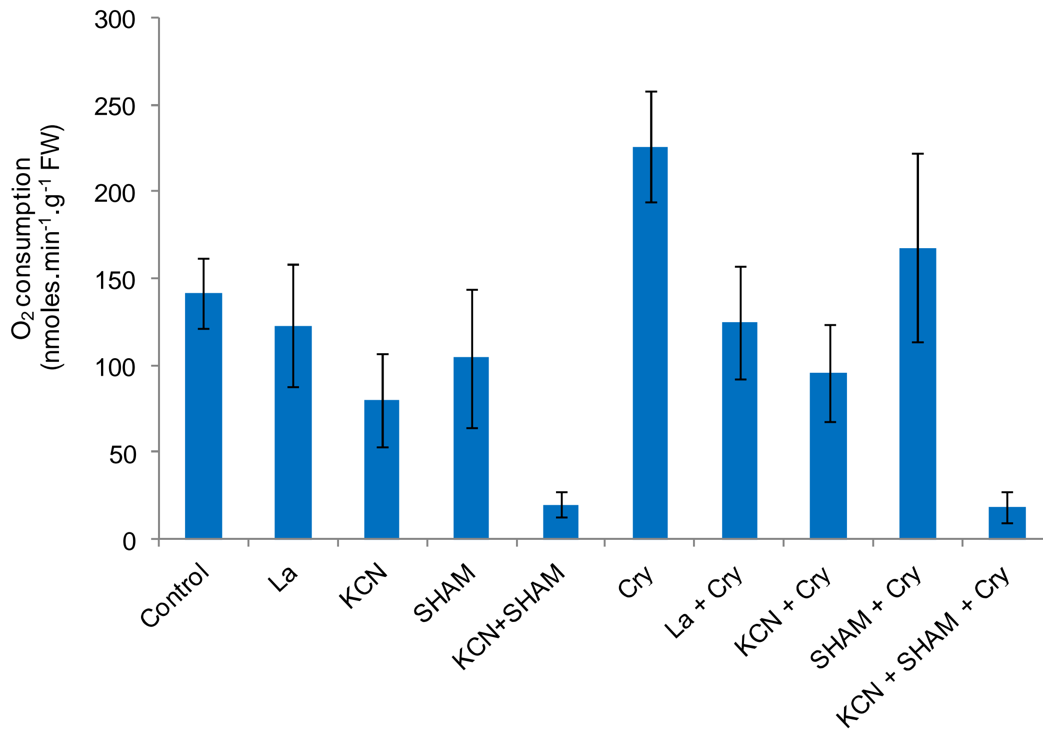

Figure 3.5: Mitochondrial O_2 consumption induced by Cry. Aeq-Mit cell suspensions were treated with 100 nM Cry and/or La^{3+} and inhibitors of mitochondrial respiration. Inhibitors were added 10 min before Cry treatment. Control corresponds to non-treated cells. Values correspond to the mean value ± SD of 5 biological repeats including each 2 to 3 experimental repeats. Lanthanum: La.

$[Ca^{2+}]_{cyt}$ elevation, however organelles responded to H_2O_2 treatment by $[Ca^{2+}]$ variations (data not shown). NO production analyzed after 2 h of Cry treatment in presence of 500 µM c-PTIO corresponded to 12 ± 2 %, 30 ± 12 %, and 3 ± 6 % (n =3) of the Cry-induced NO production in Aeq-Cyt, Aeq-Chlo and Aeq-Mit respectively. However, cPTIO did not significantly modify the Cry-induced $[Ca^{2+}]$ variations in the different subcellular compartments (Figure 3.4), contrarily to Cry-induced first $[Ca^{2+}]_{cyt}$ peak in *N. plumbaginifolia* (Lecourieux *et al.*, 2002). This might be explained by the difference in *N.* species. cPTIO used at the same concentration in Cry-treated *N. tabacum* var Xanthi cells expressing apoaequorin in the nucleus had also no effect on $[Ca^{2+}]_{nuc}$ variations (Lecourieux *et al.*, 2005).

3.3. Impact of Cry-induced Ca^{2+} signaling on mitochondrial O_2 consumption

Plant and animal mitochondria are sensitive to calcium environment and can uptake Ca^{2+} which stimulates tricarboxylic acid (TCA) cycle dehydrogenases leading to energy production (Denton and McCormack, 1980; Rasmusson *et al.*, 2004). Plants have in addition to the universal electron transport chain (ETC), mitochondrial alternative NAD(P)H dehydrogenases in the first part of the chain and an alternative oxidase (AOX), which are stimulated by calcium elevation (Rasmusson *et al.*, 2004). Although the alternative NAD(P)H dehydrogenases are not directly involved in proton pumping or ATP synthesis, they feed the ETC and their activities resulted in O_2 consumption. AOX catalyses the O_2-dependent oxidation of ubiquinol and avoid the saturation of ETC consequently lowering ROS formation. Thus, the activities of plant AOX and NAD(P)H dehydrogenases result in O_2 consumption. As Cry induced cytosolic and mitochondrial $[Ca^{2+}]$ rises, and according to the above rationale, the possibility that the $[Ca^{2+}]$ rises could impact the O_2 consumption was tested using a Clark-type electrode (Figure 3.5). O_2 consumption was measured after a short equilibration time of near 2 min after addition of Cry or inhibitors, to avoid biases due to O_2 consumption by PM-localized NADPH oxidase, whose activity, close to the basal level after 5 min of Cry treatment, peaks about 20-30 min of treatment (data not shown). Aeq-Mit control cells showed a regular O_2 consumption of 141.7 ± 20.1 $nmoles.min^{-1}.g^{-1}$ FW, which was inhibited by 43 % by addition of 1 mM potassium cyanide (KCN), an inhibitor of respiratory pathway acting on cytochrome c oxidase. Addition of 1 mM salicylhydroxamic acid (SHAM), an inhibitor of AOX (Schonbaum *et al.*, 1971), reduced O_2 consumption by 27 % in control cells. When added together to control cells, residual respiration accounted for 16 %, showing that most of the O_2 consumption was due to mitochondrial activity.

Cry-treated Aeq-Mito cells had an O_2 consumption of 226.0 ± 32.3 nmoles.min^{-1}.g^{-1} FW which corresponded to a 60 % increase in O_2 consumption in comparison to control cells. In presence of KCN, Cry-treated cell O_2 consumption was only slightly above the value in KCN-treated cells. This indicated that most of the O_2 consumption induced by Cry was due to an increase of the respiratory pathway through mitochondrial ETC at the onset of Cry treatment. In presence of SHAM, Cry-treated cells showed also a reduction in O_2 consumption by 26 %, pointing out a moderate involvement of AOX. In addition, in Cry-treated cells, KCN and SHAM abolished O_2 consumption to the same level than in control cells, indicating again a mitochondrial origin of O_2 consumption.

Addition of La^{3+}, which strongly or completely suppress Cry-induced $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ elevations respectively, slightly decreased by 13 % the O_2 consumption in control cells and annihilated the Cry-induced O_2 consumption rise, which was brought to the same level than in La^{3+}-treated cells, demonstrating the strong influence of PM-derived Ca^{2+} signals on mitochondrial activities. This process might involve changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$.

3.4. Impact of Cry-induced Ca^{2+} signaling on Chl fluorescence yield

In this study, Cry is shown to induce biphasic $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{chlo}$ elevations with the first chloroplastic peak delayed by near 6 min in comparison to the first $[Ca^{2+}]_{cyt}$ peak. The $[Ca^{2+}]_{chlo}$ increase, measured in the stroma, may come from uptake of cytosolic Ca^{2+} and/or release from the high Ca^{2+} content thylakoid lumen; Ca^{2+} should thereafter return to storage compartment to allow the cell to regain its resting state. Ca^{2+} could be transported in the thylakoid lumen through the activity of the Ca^{2+}/H^{+} antiporter characterized on the thylakoid membrane (Ettinger *et al.*, 1999). The transport is energized by the proton gradient formed during illumination, or by ATP consumption in the dark (Ettinger *et al.*, 1999). As a consequence of Ca^{2+} uptake in thylakoids, the thylakoid lumen pH should increase. It is noteworthy that modification of thylakoid lumen pH, and especially over-acidification naturally occurring under high light intensity is known to activate the xanthophyll cycle as a protective response, triggering the enzymatic conversion of violaxanthin to the de-epoxidized forms antheraxanthin and zeaxanthin (Gilmore, 1997; Lemoine and Schoefs, 2010). These pigments together with acidic pH conditions act to dissipate, by non photochemical quenching (NPQ), the excess of energy of some of the antennae Chl pigments in photosystem II (PSII), thereby resulting in a Chl fluorescence yield (FY) decrease (Gilmore, 1997). Thus, under Cry-treatment of tobacco cells, if Ca^{2+} is uptaked in the thylakoid lumen through a Ca^{2+}/H^{+} antiporter, alcalinization of the thylakoid lumen pH should occur and thus should restrict the

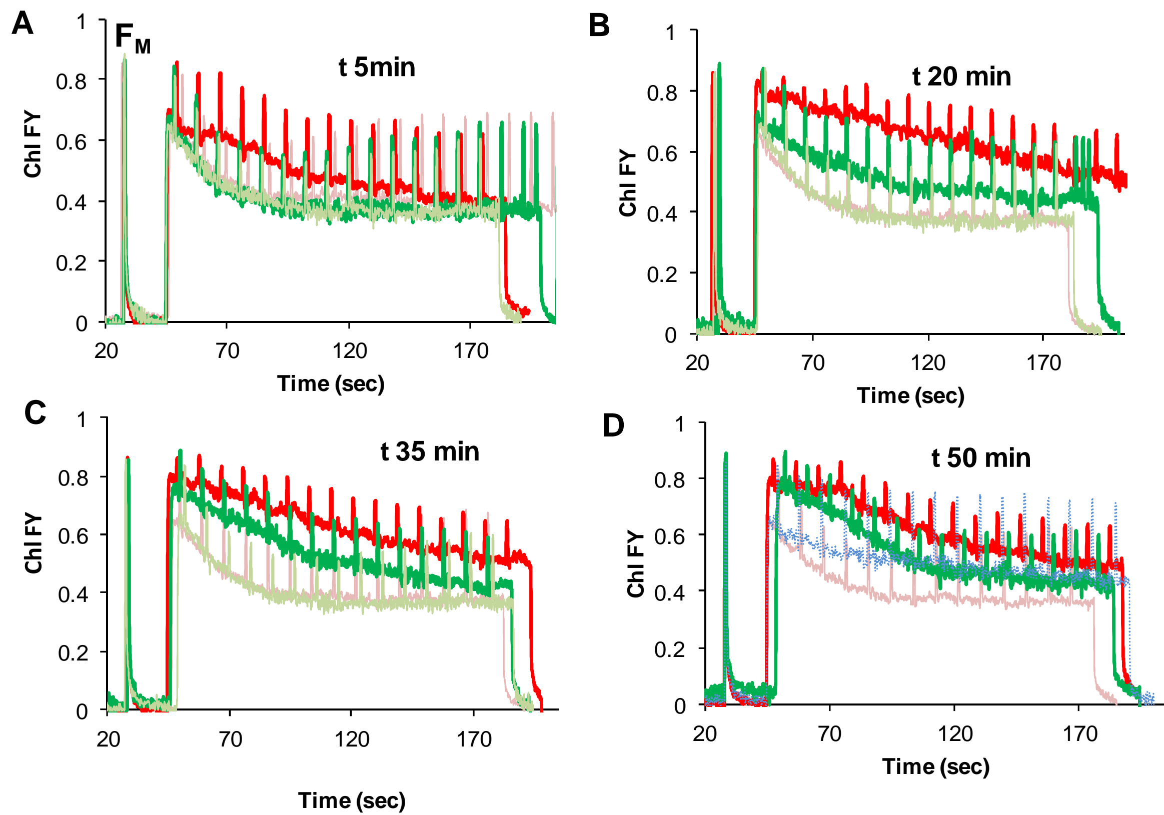

Figure 3.6: Chlorophyll fluorescence yield in presence of Cry. Aeq-Chlo cell suspensions were treated with 100 nM Cry (Red curve), Cry and 2 mM La^{3+} (Dark green curve), 2 mM La^{3+} (light green curve), 5 µM A23187 (dotted blue curve on Figure. 6D) or non-treated (pink curve, mostly superimposed with curves obtained with the La^{3+} treatment). Measurements were made after 5, 20, 35 and 50 min of Cry treatment (Figure **A-D,** respectively). For clarity, the curve obtained after La^{3+} treatment of tobacco cells was removed in D. The peaks corresponding to F_M were indicated in A.

Chl FY decrease. To test this hypothesis, Chl FY was measured in Aeq-Chlo cell suspensions using a pulse amplitude modulation (PAM) fluorimeter. Cell suspensions treated with Cry were withdrawn at different time points and Chl FY was then measured for 3-10 min and compared with that obtained in control cell suspensions. The maximal Chl FY denoted F_M, measured after a light saturating pulse (SP) was around 0.8, which indicated a proper functioning of PSII center in Aeq-Chlo cells (Figure 3.6; first peaks of Chl FY recordings). In control cells, Chl FY after illumination of cell suspension with actinic light and SPs, was decreasing rapidly and stabilized after near 1 min (Figure 3.6). In Cry-treated cells, the Chl FY decreased much slower reaching the control level after 2 min or more depending on the time point treatment (Figure 3.6). 5 min of cell treatment with Cry were sufficient to induce this effect (Figure 3.6A). The maximum effect of Cry on Chl FY was obtained at time 20 min, in accordance with the higher $[Ca^{2+}]_{chlo}$ at that time, and was observed until the end of the kinetics at time 50 min (Figure 3.6B-D). These data, which reflected the lower acidification of the thylakoid lumen in Cry-treated cells are consistent with an increase activity of a Ca^{2+}/H^{+} antiporter, indicating thereby that cells are probably rapidly storing Ca^{2+} in the thylakoids during Cry treatment.

Treatment of cell suspension by 5 µM A23187, a Ca^{2+} ionophore, induced in addition to a rapid increase in $[Ca^{2+}]_{chlo}$ (data not shown), a lower Chl FY decrease which intensity slowly returned to control levels (curves were similar whatever the time point and one is shown for the 50 min time point; Figure 3.6D). This data strengthened the connection between excess energy dissipation and $[Ca^{2+}]_{chlo}$ increase. Treatment of cells with 2 mM La^{3+} had no effect on Chl FY, curves being superimposed with these obtained in controls (Figure 3.6). However, La^{3+}, which suppressed Cry-induced $[Ca^{2+}]_{chlo}$ increase, inhibited totally the effect of Cry on Chl FY at time 5 min. At longer Cry treatment times, La^{3+} had an intermediate effect that might be either related to the small $[Ca^{2+}]$ variations still present with La^{3+} or to other Ca^{2+}-independent events also impacting Chl FY.

4. Discussion

4.1. Spatiotemporal [Ca^{2+}] variations induced by elicitors of defense reactions in organelles

[Ca^{2+}]$_{cyt}$ variations induced by elicitors of defense reactions or PAMPs have been characterized with many types of elicitors and using a variety of plant material (Lecourieux *et al.*, 2006). By using pharmacological tools, calcium influx through PM Ca^{2+} channels proved to be required in most studies but the molecular identification of these channels is still limited to a few cases (Demidchik and Maathuis, 2007; Ma and Berkowitz, 2011). In addition to external supply, [Ca^{2+}]$_{cyt}$ variations may be shaped by the influx and efflux of Ca^{2+} from different internal stores: up to now the contributions of internal stores have been under-evaluated and the Ca^{2+} signatures in these stores and organelles are unknown or poorly described, moreover in a plant defense context (McAinsh and Pittman, 2009; Dodd *et al.*, 2010). Effects of two well characterized elicitors of plant defense reactions, namely Cry and OGs have been studied on [Ca^{2+}]$_{cyt}$ in *N. plumbaginifolia* and on [Ca^{2+}]$_{nuc}$ in *N. tabacum* var Xanthi: both elicitors induce [Ca^{2+}]$_{cyt}$ rises and only Cry triggers a significant [Ca^{2+}]$_{nuc}$ elevation (Lecourieux *et al.*, 2002, 2005). Our aims were to investigate whether mitochondria and chloroplasts of tobacco cells also responded to these elicitors, and to compare the corresponding Ca^{2+} signatures to the cytosolic and nuclear Ca^{2+} signatures. Knowledge of the spatiotemporal [Ca^{2+}] variations in these organelles that accumulated Ca^{2+} may help to analyze the [Ca^{2+}]$_{cyt}$ signature and the subsequent induced-signaling events, and will also provide interesting data on the potential organelles' contributions, through [Ca^{2+}] variations, to plant defense signaling. For that purpose, we generated transgenic *N. tabacum* var Xanthi cells expressing the apoaequorin calcium sensor in the cytosol, mitochondrial matrix or chloroplastic stroma (Figure 3.1). The peptide sequences fused to aequorin efficiently addressed 90-95 % of the total aequorin into the proper subcellular compartment (Knight *et al.*, 1991; Johnson *et al.*, 1995; Logan and Knight, 2003; Lecourieux *et al.*, 2005), this efficient targeting was also confirmed in our study (Figure 3.1). Although not detected by Western-blotting, a slight amount of aequorin still not addressed might explain small [Ca^{2+}] elevations such as the first Cry-induced [Ca^{2+}]$_{nuc}$ peak or the small OGs-induced [Ca^{2+}]$_{chlo}$ shoulder which are concomitant with the corresponding first [Ca^{2+}]$_{cyt}$ peak. The transgenic cells have the same phenotypic appearance and growth rate. In addition, the same range of elicitor concentration could be used in the different transgenic lines to induce [Ca^{2+}]

variations (Figures 3.2 and 3.3; Table 3.1). These [Ca^{2+}] variations were elicitor- and subcellular compartment-specific as observed in our study.

Dose-dependence experiments and overlays of [Ca^{2+}] variations pointed out many striking properties of elicitor-induced variations: first of all, Cry and OGs induced a well-marked biphasic $[Ca^{2+}]_{cyt}$ signature at high elicitor concentrations, which suggested the onset of different Ca^{2+} fluxes at different times, thus potentially implicating different stores and/or Ca^{2+} channels and pumps. This is in accordance with previous studies showing that $[Ca^{2+}]_{cyt}$ elevation induced by Cry in *N. plumbaginifolia* cell suspensions, which is also biphasic, had at least two origins: continuous extracellular Ca^{2+} uptake and internal store Ca^{2+} release sensitive to neomycin and RR (Lecourieux *et al.*, 2002; Lamotte *et al.*, 2004). In Cry-treated *N. plumbaginifolia* cell suspensions, mostly the first rise was sensitive to neomycin and RR. Although it cannot be excluded that the biphasic shape of Ca^{2+} signature corresponded to distinct subpopulations of cells in the culture responding with different kinetics to cryptogein, the absence of a biphasic mitochondrial Ca^{2+} signature together with two very dissymmetric $[Ca^{2+}]_{nuc}$ elevations did not argued for a cellular process leading to distinct cell populations after cell generation from callus. In addition, it has always been observed a biphasic cytosolic signature either in *N. plumbaginifolia* (Lecourieux *et al.*, 2002) or in *N. tabacum* var Xanthi transgenic cell lines generated in this study.

Except for nucleus, which showed an IP_3-sensitive Ca^{2+} uptake, data is lacking concerning the elicitor-induced [Ca^{2+}] variations in organelles. Our studies revealed that in Cry-treated Xanthi cells, $[Ca^{2+}]_{mit}$ is increasing concomitantly with $[Ca^{2+}]_{cyt}$ rises, and $[Ca^{2+}]_{chlo}$ elevation, although starting within the same period, peaked at the valley between the first and second peak of $[Ca^{2+}]_{cyt}$ rise. Furthermore, a biphasic [Ca^{2+}] curve is obtained only in the cytosol, nucleus and chloroplasts, with a second elevation of $[Ca^{2+}]_{chlo}$ and $[Ca^{2+}]_{nuc}$ similar to the second $[Ca^{2+}]_{cyt}$ elevation and lasting at least 1h (Figure 3.2D). These data suggested the following hypothesis: mitochondria and chloroplasts promptly uptake cytosolic Ca^{2+}, but for some reason that might be related to mitochondria storage capacity, inactivation of Ca^{2+} pumps, or to the disruption of mitochondria functions, mitochondria apparently cease to import Ca^{2+} while chloroplasts, which are comparatively 10 times larger organelles than mitochondria and may have higher Ca^{2+} storage capacities, continue to accumulate cytosolic Ca^{2+}. Interestingly, it was shown in animals, that the mitochondrial Ca^{2+} uniporter could be inactivated in response to sustained $[Ca^{2+}]_{cyt}$ elevation, a process that prevents excessive mitochondrial Ca^{2+} accumulation (Putney and Thomas, 2006). However, as the measure of free [Ca^{2+}] reflects the balance between bound and released Ca^{2+}, it is not excluded that

mitochondria still incorporated Ca^{2+}, efficiently buffered by organic acids or phosphate. Consistent with this hypothesis, in animals, Ca^{2+} is rapidly stored in the matrix as an amorphous Ca^{2+}-phosphate precipitate appearing in form of large electron-dense granules in immediate proximity to the inner membrane (Starkov, 2010).

In our study, $[Ca^{2+}]$ elevations in the subcellular compartments depended on a Ca^{2+} influx through the PM: the Ca^{2+} load might increase first $[Ca^{2+}]_{cyt}$ and subsequently the $[Ca^{2+}]$ in organelles. However, it cannot be excluded that the matrix and stromal $[Ca^{2+}]$ elevations are due to liberation of Ca^{2+} from suborganellar compartments, such as intermembrane space and thylakoids. However, the outer envelope of chloroplasts and the outer membrane of mitochondria should be permeable to small molecules, and our data rather suggested that thylakoids uptake Ca^{2+} during Cry treatment of tobacco cells (see below and last discussion section 3).

The second peaks of Cry-induced cytosolic and chloroplastic $[Ca^{2+}]$ variations are occurring within equivalent time frame, with only a small shift for $[Ca^{2+}]_{chlo}$, being both brought forward when cells were treated with high Cry concentrations (Figure 3.2A and B). The major $[Ca^{2+}]_{nuc}$ elevation also fitted with the second cytosolic phase. From these data, it seems that chloroplasts could sense the $[Ca^{2+}]_{cyt}$ elevation all along the treatment, with similar total durations of $[Ca^{2+}]_{chlo}$ and $[Ca^{2+}]_{cyt}$ elevations. This would indicate that chloroplasts have the possibility to uptake Ca^{2+} continuously, which might be deleterious to chloroplast functions. This uptake did not correspond to diffusion process through membranes because the $[Ca^{2+}]_{chlo}$ peaks are delayed in time and the $[Ca^{2+}]$ in the cytosol and chloroplasts are not equivalent. The storage forms of chloroplastic Ca^{2+} are unknown, although it might be expected that thylakoids will concentrate Ca^{2+}, necessary for PSII formation and functioning (Homann, 2002; Loll *et al.*, 2005). Ca^{2+} fluxes from the cytosol to the stroma, and from stroma to thylakoid lumen can have different intensities during the treatment, the net balance would then specify stromal signature in comparison to cytosolic signature. In plant defense context, other compartments, such as vacuole and ER, are also potentially major regulators of $[Ca^{2+}]_{cyt}$ signature: indeed, it was recently shown that silencing an ER-localized Ca^{2+}-ATPase (nbCA1) leads to an increase of Cry-induced Ca^{2+}-regulated cell death (Zhu *et al.*, 2010) and that disruption of two *A. thaliana* vacuolar Ca^{2+} pumps (ACA4 and ACA11) potentiates the activation of a salicylic acid-dependent cell death pathway (Boursiac *et al.*, 2010).

OGs treatment of the transgenic *N. tabacum* cell suspensions induced a biphasic $[Ca^{2+}]_{cyt}$ elevation, one peak elevation in mitochondria concomitant with the first $[Ca^{2+}]_{cyt}$ phase, and an asymmetric chloroplastic peak with a small $[Ca^{2+}]$ elevation at the beginning of

the kinetics (concomitant with $[Ca^{2+}]_{cyt}$ elevation), later increasing and peaking near 6-7 min after the first $[Ca^{2+}]_{cyt}$ elevation (Figure 3.3; Table 3.1). Similarities of Ca^{2+} signatures in response to OGs with the Cry-induced Ca^{2+} signatures included the biphasic $[Ca^{2+}]_{cyt}$ elevation, the monophasic $[Ca^{2+}]_{mit}$ elevation superimposed with the first $[Ca^{2+}]_{cyt}$ elevation, the lag time in $[Ca^{2+}]_{chlo}$ peak (Figure 3.3; Table 3.1). Differences consisted of faster responses, lower durations of $[Ca^{2+}]$ elevations, a different shape of the second $[Ca^{2+}]_{cyt}$ phase and a Ca^{2+} signature in chloroplasts which could not be superimposed on cytosolic Ca^{2+} signature. Once again, these data suggested that mitochondria and chloroplasts have different ways to cope with $[Ca^{2+}]_{cyt}$ elevation. Taken as a whole, the overall $[Ca^{2+}]$ variations induced by OGs, including the very transient $[Ca^{2+}]_{cyt}$ and low $[Ca^{2+}]_{nuc}$ rise, indicated a restricted Ca^{2+} homeostasis disruption, which may explain the absence of necrotic effect induced by OGs in comparison to Cry-induced $[Ca^{2+}]$ perturbations leading to Ca^{2+}-dependent strong necrotic effects (Binet *et al.*, 2001; Lecourieux *et al.*, 2002, 2005). Interestingly, in addition to the above data, it was recently shown that NtHD2a/b, two tobacco type-2 nuclear histone deacetylases (HDACs), were negative regulators of elicitor-induced cell death and were negatively regulated in Cry-treated tobacco plants but not in OGs-treated plants (Bourque *et al.*, 2011), emphasizing the role of nuclear-derived signal transduction in cell death regulation.

4.2. Cry-induced $[Ca^{2+}]$ signaling in mitochondria and chloroplasts

The PAMP elicitor Cry induced an early calcium influx, regulated by phosphorylation processes, that is located upstream all known signaling events in this cascade including ROS and NO production (Tavernier *et al.*, 1995; Lamotte *et al.*, 2004; Garcia-Brugger *et al.*, 2006). The calcium influx is mounted through diverse non identified Ca^{2+} channels but that included members of the ionotropic family of glutamate receptors (GluRs) (Vatsa *et al.*, 2011). This influx, together with Ca^{2+} release from internal stores, contributed to $[Ca^{2+}]_{cyt}$ elevation (Lecourieux *et al.*, 2002; Vatsa *et al.*, 2011). Our results also showed that inhibiting PM-localized Ca^{2+} channels with La^{3+} or BAPTA, abrogated or strongly inhibited $[Ca^{2+}]_{chlo}$ and $[Ca^{2+}]_{mit}$ elevations, probably as a consequence of the absence of $[Ca^{2+}]_{cyt}$ elevation.

Potential known signaling molecules that could regulate Cry-induced $[Ca^{2+}]$ signaling included IP_3, cADPR, NO and H_2O_2 (Lecourieux *et al.*, 2002; Lamotte *et al.*, 2004): their contributions to mitochondrial and chloroplastic $[Ca^{2+}]$ variations were assayed using a pharmacological approach, but due to cell sensibility to scavengers, H_2O_2 implication could not be probed. Our results showed that neomycin and U73122 strongly reduced Cry-induced

[Ca^{2+}] variations in the cytosol or organelles suggesting a strong involvement of IP_3 in Ca^{2+} signaling. Interestingly, in guard cells stimulated by external [Ca^{2+}] increase, IP_3 was also linked to $[Ca^{2+}]_{cyt}$ elevations necessary for stomatal closure: this pathway was dependent on the presence of the calcium sensor CAS as evidenced by genetic studies in *A. thaliana* (Tang *et al.*, 2007). This process probably involved chloroplasts as CAS was mainly localized in the thylakoid membrane with the calcium binding site likely exposed to the stromal side (Nomura *et al.*, 2008; Weinl *et al.*, 2008). In addition, although the exact mechanism linking IP_3, CAS and $[Ca^{2+}]_{cyt}$ is unknown at the moment, it involves H_2O_2 and NO, with H_2O_2 probably generated by the chloroplasts in guard cells (Wang *et al.*, 2012).

The cADPR-related signaling pathway appeared to have a much more limited influence, with a slight inhibition by RR of the [Ca^{2+}] variations ranging from 0 to 25 % depending on the subcellular compartment (Figure 3.4).

Scavenging Cry-induced NO production by cPTIO in *N. plumbaginifolia* diminishes the first $[Ca^{2+}]_{cyt}$ peak (Lamotte *et al.* 2004), but neither affect $[Ca^{2+}]_{nuc}$ (Lecourieux *et al.*, 2005) nor has a feed-back effect on calcium influx in *N. tabacum* cells (Vatsa *et al.*, 2011). Our data also showed that although cPTIO efficiently scavenged NO production by 70-100 %, it did not modify significantly the calcium variations in cytosolic, mitochondrial and chloroplastic compartments (Figure 3.4), indicating that NO is not used as a major Ca^{2+} signal amplifier. This did not exclude a specific role of NO in mitochondria or chloroplasts that have been proposed as sources for NO production (Foissner *et al.*, 2000; Guo *et al.*, 2003; Planchet *et al.*, 2005; Jasid *et al.*, 2006).

In conclusion, our data argued for an IP_3-related Ca^{2+} signaling pathway induced by Cry in *N. tabacum* var Xanthi cells, that substantially modifies subcellular [Ca^{2+}], including in nucleus (Lecourieux *et al.*, 2005).

4.3. Ca^{2+} signaling and mitochondrial respiration in cryptogein-treated cells

In animal mitochondria, Ca^{2+} stimulates TCA cycle dehydrogenases and signals ATP consumptive processes in the cytosol (Denton and McCormack, 1980). However, excess of Ca^{2+} uptake can trigger the mitochondrial permeability transition leading to cell death. Plant mitochondria are also able to uptake Ca^{2+} undergoing swelling and release of cytochrome c, which are hallmarks of mitochondrial induced cell death (Virolainen *et al.*, 2002). In addition to TCA cycle dehydrogenases, Ca^{2+} stimulates AOX and alternative plant NAD(P)H dehydrogenases therefore leading to increased O_2 consumption (Rasmusson *et al.*, 2004). AOX stimulation has been shown to be involved in stress adaptation and attenuates cell death

induced by salicylic acid (SA), a hormone required for plant resistance to pathogens (Robson and Vanlerberghe, 2002; Noctor *et al.*, 2007). AOX is believed to protect plants by controlling the over-reduction of the ETC and thus limiting ROS production, while alternative NAD(P)H dehydrogenases will feed the ETC and could thereby saturate the reduced pool of ubiquinone. The literature highlighted the complex role of Ca^{2+} with either positive or deleterious effects on mitochondrial functions. In our study, we aimed to verify whether Cry-induced $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ elevations could impact mitochondrial functions, and thus we monitored O_2 uptake as a potentially Ca^{2+}-regulated process in mitochondria. Our data showed that Cry induced a 60 % increase in O_2 uptake, which could be almost abolished by a combined treatment with inhibitors of cytochrome c pathway and AOX, reflecting a mitochondrial O_2 uptake. The rapid O_2 consumption is in agreement with the $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ rising after 2 min of Cry treatment. The cytochrome c pathway was mostly responsible for O_2 consumption at the beginning of the Cry treatment, as addition of Cry to KCN-treated cells resulted only in a small increase of O_2 uptake in comparison to KCN treated cells. This O_2 uptake increase, not inhibited by KCN, could correspond to AOX-dependent O_2 uptake: indeed in presence of SHAM, an AOX inhibitor, Cry-induced O_2 uptake is reduced by 26 %. As a whole, our data indicated that both ETC and AOX activities are induced by Cry. Interestingly, in presence of La^{3+}, Cry-induced O_2 uptake is similar to control values, indicating that PM-derived Ca^{2+} signals controlling $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ rises, are upstream the enzymes involved in mitochondrial respiration. It was previously shown that Cry-induced cell death depends on Ca^{2+} influx (Binet *et al.*, 2001; Lecourieux *et al.*, 2002): although it is not known whether mitochondria participated to Cry-induced cell death, the increased O_2 uptake, which reflects a higher electron flow might favor superoxide production in mitochondria (Noctor *et al.*, 2007), and could participate in presence of high $[Ca^{2+}]$ to cell death. Interestingly, in Cry-treated *N. tabacum* cv. BY-2 cells, ROS generation in subcellular compartments showed an accumulation in endomembranes, cytoplasmic and nuclear compartments with a strong colocalization in mitochondria, indicating a potential role of mitochondria in ROS generation consistent with the respiration function of these organelles (Ashtamker *et al.*, 2007).

4.4. Ca^{2+} signaling and excess energy dissipation in cryptogein-treated cells

Up to date, *in vivo* $[Ca^{2+}]$ variations in chloroplasts have been described during an illumination and during the light to dark transitions (Johnson *et al.*, 1995; Logan and Knight, 2003), though it is expected that $[Ca^{2+}]_{chlo}$ could vary under many conditions that modify

$[Ca^{2+}]_{cyt}$. During the last few years, it was also demonstrated that increases in extracellular $[Ca^{2+}]$ induce $[Ca^{2+}]_{cyt}$ transients in guard cells that were dependent on the thylakoid-membrane CAS, leading to stomata closure (Han *et al.*, 2003; Tang *et al.*, 2007; Nomura *et al.*, 2008; Weinl *et al.*, 2008). In our study, we report $[Ca^{2+}]_{chlo}$ variations in response to plant elicitors whose peaks were delayed in comparison to $[Ca^{2+}]_{cyt}$, suggesting (i) that chloroplasts uptake cytosolic Ca^{2+} and (ii) that a chloroplast specific regulation is set up during this process. Elevation of $[Ca^{2+}]_{chlo}$ in the stroma is known to modify the activity of enzymes involved in photosynthesis CO_2 fixation (Portis and Heldt, 1976), and was suggested to act as a signal to inhibit photosynthesis-related process during the dark period (Sai and Johnson, 2002). Isolated chloroplasts can uptake Ca^{2+} upon illumination (Muto *et al.*, 1982), but as the stromal $[Ca^{2+}]_{chlo}$ stays rather stable during the light period, it is assumed that Ca^{2+} is rapidly bound or stored into the thylakoid lumen, partially through the activity of a Ca^{2+}/H^+ antiport that depends on light-generated proton gradient (Ettinger *et al.*, 1999; Sai and Johnson, 2002). Ca^{2+} in the thylakoid lumenal store is required for the proper assembly of PSII during initial assembly or during repair of photodamaged PSII reaction center (Mattoo *et al.*, 1989; Grove and Brudvig, 1998; Loll *et al.*, 2005), and is instrumental to the activity of the oxygen-evolving complex (OEC) as Ca^{2+} extraction greatly diminishes O_2 evolution (Van Der Meulen *et al.*, 2002). Stresses can modify photosynthetic performance with considerable changes in chemical and pigment composition of leaves: the established link between excess energy dissipation by NPQ and chlorophyll fluorescence can then be used for an early detection of stresses (Roháček *et al.*, 2008). In our study, elicitor perception, symptomatic to biotic stress, is signaled through $[Ca^{2+}]$ variations, and it was assumed that elicitor treatment would result in modification of Chl FY, which is sensitive to the thylakoid lumenal pH, if Ca^{2+} was stored in thylakoid lumen through the activity of the Ca^{2+}/H^+ antiport. Consistent with this hypothesis, our results showed that Cry maintains a high Chl FY slowly returning to control level (Figure 3.6). This higher FY is observed from the first time point of Cry treatment at 5 min, fitting with the beginning of $[Ca^{2+}]_{chlo}$ elevation. The effect on Chl FY was more pronounced at 20 min until the end of the experiment at time 50 min. In the presence of 2 mM La^{3+}, Cry-treated cells presented a decrease of Chl FY, similar to that of control cells at time 5 min. After a longer period of co-treatment, La^{3+} (even used at 4 mM, data not shown) was unable to completely suppress the sustained Cry-induced FY, suggesting that Ca^{2+}-dependent and independent events were controlling Chl fluorescence in Cry-treated cells. Results obtained with A23187, a Ca^{2+} ionophore, which induces an increase in Chl FY (and a $[Ca^{2+}]_{chlo}$ increase, data not shown), also argued for a strong effect of Ca^{2+} on Chl FY. Taken

as a whole, our data indicated that $[Ca^{2+}]_{chlo}$ elevations perturbated the thylakoid lumenal pH, and suggested that Ca^{2+} could be stored into the thylakoids through the activity of the Ca^{2+}/H^{+} antiport.

In the past few years, data accumulated showing a strong relationship between plant defense and chloroplastic functions: for example, pathogen attack or elicitor treatment causes suppression of photosynthetic gene expression (Matsumura *et al.*, 2003; Pandelova *et al.*, 2009) and a concomitant inhibition of photosynthesis (Scharte *et al.*, 2005; Bonfig *et al.*, 2006). Interestingly, RNA helicase domain of tobacco mosaic virus (TMV) replicase targeted the 33K subunit of OEC, and silencing the 33K subunit resulted in a 10-fold increase of TMV accumulation suggesting that TMV could use this strategy to suppress basal resistance (Abbink *et al.*, 2002). It was also shown that the *Pseudomonas. syringae*-specific Hop1 virulence effector is localized to chloroplasts (the site of SA synthesis, a hormone involved in plant defense) and is involved in thylakoid structure remodeling together with SA accumulation suppression. As a whole, data indicated that chloroplasts are targeted by pathogens to reduce plant resistance. However, as a consequence of disruption of photosynthetic machinery or in response to pathogens, chloroplasts will produce higher amounts of ROS, leading to HR and pathogen restriction to infected sites (Liu *et al.*, 2007; Pandelova *et al.*, 2009). Interestingly, it was observed that the activation of the MAPK pathway, leading to the activation of the MAPKs SIPK, Ntf4 and WIPK, is associated to increased ROS production in chloroplasts (Liu *et al.*, 2007), and Cry was also shown to activate SIPK and WIPK (Lebrun-Garcia *et al.*, 1998; Zhang *et al.*, 1998). These observations are consistent with the decrease of the excess energy dissipation observed in Cry-treated cells that should favor electron flow, potentially leading to ROS production in chloroplasts.

5. Conclusions

Our data indicated that Ca^{2+}-signaling pathway induced by two elicitors of a different nature showed common properties: (i) both Cry and OGs triggered [Ca^{2+}] variations in the cytosol, chloroplasts and mitochondria, (ii) Cry and OGs induced a biphasic $[Ca^{2+}]_{cyt}$ rise, (iii) the transient $[Ca^{2+}]_{mit}$ variation was concomitant with the first $[Ca^{2+}]_{cyt}$ elevation, (iv) $[Ca^{2+}]_{chlo}$ signature was delayed in comparison to $[Ca^{2+}]_{cyt}$ signature. However, OGs did not induce a significant $[Ca^{2+}]_{nuc}$ elevation contrarily to Cry, and Ca^{2+} signatures were stimulus-specific, particularly considering the duration of [Ca^{2+}] variations which resulted in a much more extended period of high free [Ca^{2+}] in Cry-treated cells, particularly in the cytosol, chloroplasts and nucleus. It is assumed that these high and prolonged [Ca^{2+}] variations will generate a Ca^{2+} pathway outcome quite different for Cry-treated cells in comparison to OGs-treated cells. In Cry-treated cells, all the [Ca^{2+}] variations depended on an initial Ca^{2+} influx and we had evidences that the second messenger IP_3 plays an important role in these variations. We also demonstrated that Cry, through Ca^{2+} signaling, causes perturbations in two important organelle functions, namely mitochondrial respiration and chloroplastic energy dissipation process, potentially adding Ca^{2+}-dependent ROS production sources to the PM NADPH oxidase. Thus, our data strengthened the idea that Ca^{2+} in organelles is not simply sequestered and buffered but contributes to plant defense signaling.

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CHAPTER 4

CHAPTER 4

“Glutamate receptors are involved in Ca^{2+}-dependent plant defense signaling and resistance to pathogens”

CHAPTER 4

GLUTAMATE RECEPTORS ARE INVOLVED IN CA^{2+}-DEPENDENT PLANT DEFENSE SIGNALING AND RESISTANCE TO PATHOGENS

Running title: Glutamate receptors are involved in plant defense

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ABSTRACT

Like their animal counterparts, plant glutamate receptors-like (GLRs) homologs are intimately associated with Ca^{2+} influx through plasma membrane and participate in various physiological processes. In pathogen associated molecular patterns (PAMP)-/elicitor-mediated resistance, Ca^{2+} influx and subsequent cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{cyt}$) variations are early important steps activating downstream signaling events related to plant defense. In this study, oligogalacturonides (OGs), which are endogenous elicitors derived from cell wall degradation, were used to investigate the role of GLRs in plant defense signaling. Using aequorin-transformed *Arabidopsis thaliana,* we demonstrated that OGs-induced $[Ca^{2+}]_{cyt}$ variations are sensitive to GLRs antagonists. Both pharmacological and genetic investigations indicated that OGs-induced nitric oxide (NO) production, reactive oxygen species (ROS) production and expression of defense-related genes are partly controlled by GLRs. In addition, wild type Col-0 plants treated with the glutamate receptor antagonist DNQX (6,7-dinitriquinoxaline-2,3 dione) had a compromised resistance to *Botrytis cinerea* and *Hyaloperonospora arabidopsidis*. Furthermore, we showed that analysis of defense genes expression triggered by OGs or *H. Arabidopsidis* in *Atglr3.3* or DNQX-pretreated Col-0 plants revealed striking similarities, and we demonstrated that AtGLR3.3 is a vital gene involved in resistance against *H. arabidopsidis*. Taken together, these data provide strong evidences for the regulation of elicitor/pathogen mediated plant defense signaling pathways through GLRs in *Arabidopsis thaliana*.

1. Introduction

Calcium (Ca^{2+}) is a versatile second messenger that plays a pivotal role in a variety of physiological and developmental processes in plants and animals. In plants, environmental stimuli including cold, light, osmotic stress, oxidative stress, hormones, symbiosis and plant defense elicitors are coupled with characteristic changes in free cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) and leads to stimulus specific responses through the induction of various signal transduction pathways (Garcia-Brugger *et al.*, 2006; Lecourieux *et al.*, 2006; Demidchik and Maathuis, 2007; Kosuta *et al.*, 2008; Ranf *et al.*, 2008; Dodds and Rathjen, 2010; Kudla *et al.*, 2010).

During plant pathogen interactions, pathogen- or plant-derived molecules (elicitors) are recognized by specific pattern recognition receptors (PRRs). Elicitors provide an excellent tool to elucidate the mechanisms of defense in plants. Oligogalacturonides (OGs) are well known plant defense elicitors which are produced following the hydrolysis of plant cell wall and are able to induce defense responses in plants (Hahn *et al.*, 1981; Ridley *et al.*, 2001). Recently, wall-associated kinase 1 (WAK1) has been identified as a receptor of OGs (Brutus *et al.*, 2010). OGs perception initiates a signal transduction cascade that activates sophisticated multilevel defense responses in plants including variation in $[Ca^{2+}]_{cyt}$, production of reactive oxygen species (ROS) and nitric oxide (NO), activation of mitogen activated protein kinases (MAPKs), membrane depolarization, defense gene transcripts accumulation and phytoalexin production (Hu *et al.*, 2004; Lecourieux *et al.*, 2005; Ferrari *et al.*, 2007; Denoux *et al.*, 2008; Galletti *et al.*, 2008; Galletti *et al.*, 2011; Rasul *et al.*, 2012). It has been demonstrated that OGs treatments of *Vitis vinifera* and *Arabidopsis thaliana* leaves enhance basal resistance against *Botrytis cinerea* (Aziz *et al.*, 2004; Ferrari *et al.*, 2007).

The elicitor-induced activation of pathways leading to plant defenses have been shown to be mediated by $[Ca^{2+}]_{cyt}$ elevations due first to Ca^{2+} entry from extracellular medium and Ca^{2+} mobilization from internal stores (Garcia-Brugger *et al.*, 2006; Lecourieux *et al.,* 2006). Plasma membrane Ca^{2+} permeable channels and particularly cyclic nucleotide gated channels (CNGC) and ionotropic glutamate receptors homologs (GLRs) which have been identified at the molecular level are potential candidates to mediate calcium influx (Lacombe *et al.*, 2001; Mäser *et al.*, 2001).

In *Arabidopsis*, GLRs encompass 20 members that are grouped into three clades based on their sequence homology and contain all the signature domains of animal ionotropic glutamate receptors (iGluRs; Lacombe *et al.*, 2001; Chiu *et al.*, 2002; Davenport, 2002). In

animals, iGluRs are non-selective cation channels (NSCC) and are involved in the movement of Ca^{2+} across the post-synaptic plasma membrane. They function as heterotetramer and are essential components of cell-cell communication in nervous system (Dingledine *et al.*, 1999). Moreover, iGluRs have been implicated in immunity system in vertebrates (Pacheco *et al.*, 2007). Plant GLRs are involved in ion transport, growth processes, signal transduction and adaptation to biotic and abiotic stresses (for details, see the reviews by Dietrich *et al.*, 2010; Jammes *et al.*, 2011). Electrophysiological studies have shown plant GLRs involvement in Ca^{2+} variations and other Ca^{2+}-dependent processes (Dennison and Spalding, 2000; Dubos *et al.*, 2003; Demidchik *et al.*, 2004; Meyerhoff *et al.*, 2005; Kang *et al.*, 2006; Qi *et al.*, 2006; Stephens *et al.*, 2008; Michard *et al.*, 2011). Recently, Vatsa *et al.* (2011) have demonstrated that GLRs participate in cryptogein (a proteinaceous elicitor of plant defense)-mediated Ca^{2+} influx and NO production in tobacco cell suspensions, suggesting a role of GLRs in plant defense signaling. Moreover, in response to bacterial peptides (flg22 and elf18) and the fungal carbohydrate chitin, a significant impact of GLRs was observed on Ca^{2+} influx, MAPK activation and on the accumulation of defense gene transcripts in *Arabidopsis* plants (Kwaaitaal *et al.*, 2011). However, none of the implicated GLRs have been identified at the molecular level. Interestingly, overexpression in *Arabidopsis* of a radish GLR, homologue to *AtGLR3.2*, a member of the GLR clade 3, exhibited Ca^{2+} deficiency symptoms such as necrosis and dwarf stature, and more importantly, these overexpressing plants resulted in enhanced resistance to *B. cinerea* (Kang *et al.*, 2006).

Based on these results, we investigated the possible role of GLRs in OGs-induced plant defense signaling through both pharmacological and genetic approaches using T-DNA insertion mutant lines of *Arabidopsis thaliana* clade 3 GLRs. GLRs-dependent $[Ca^{2+}]_{cyt}$ variations induced by OGs were measured in *A. thaliana* transgenic line expressing aequorin in presence of widely used GLRs antagonists. OGs-induced downstream responses such as NO production, ROS generation and defense genes expression were investigated in wild type (Col-0) or *glr* mutants to elucidate the role of GLRs in these signaling events. We further showed that AtGLRs inhibition, or disruption of some *AtGLR* genes, resulted in compromised resistance to the necrotrophic fungal pathogen, *B. cinerea* and to the biotrophic oomycete pathogen, *Hyaloperonospora arabidopsidis*. Especially, it was demonstrated that *AtGLR3.3* is an important gene involved in basal resistance against *H. arabidopsidis* and that both OGs- and *H. Arabidopsidis*-induced expression of some defense genes was regulated by AtGLR3.3. Altogether, data demonstrated an important role of GLRs in elicitor/pathogen mediated plant defense signaling pathways.

2. Materials and Methods

2.1. Biological materials

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) and T-DNA insertion mutant lines were either obtained directly from NASC (Nottingham *Arabidopsis* Stock Center; http://www.arabidopsis.org) or were a gift from different laboratories (Table S4.1). All mutant lines used in this study are in Col-0 background.

2.2. Growth conditions and treatments

Plants were grown in commercial soil (Jiffy-7, Puteaux, France; http://www.puteaux-sa.fr). Prior to germination, seeds were vernalized at 4 °C in the dark for 48 h and were then shifted to climatic growth chamber (KBW 720, BINDER, Germany; http://www.binder-world.com) with 10 h light:14 h dark (short day) conditions with the following settings: 20 °C light, 18 °C dark, 70 % relative humidity light / 95 % dark, light intensity 175 $\mu E.s^{-1}$.

For all experiments, 4-5 weeks old plants were used except where mentioned. In all experiments, final working concentrations for OGs, DNQX, CNQX and MK-801 were 2.5 $mg.mL^{-1}$, 500 µM, 500 µM and 400 µM, respectively. cPTIO, DPI and La^{3+} were used at 500 µM, 50 µM and 1 mM, respectively.

2.3. Mutant Genotyping

To screen homozygous mutant individuals, genomic DNA was isolated from leaf tissues using standard protocol (Edward *et al.*, 1991). PCR were performed to verify the presence of wild type gene or the T-DNA insertion in homozygous mutants plants by using a left-border T-DNA primer (ACTGGCCGTCGTTTTACAACG) in combination with gene-specific primers (Table S4.2). PCR products were evaluated through agarose gel electrophoresis.

2.4. $[Ca^{2+}]_{cyt}$ variation

For $[Ca^{2+}]_{cyt}$ measurements, 7 mm diameter leaf disks from *Arabidopsis* transformed plants expressing apoaequorin in the cytosol were overnight incubated in darkness with 10 µM native coelenterazine (Uptima; http://www.interchim.com) in luminometer assay tubes containing 200 µL of H_2O. Pretreatment with GLRs antagonists was made 10 min prior to OGs treatment and luminescence was measured at 1 s integration intervals using a single tube luminometer (Lumat LB 9507, BERTHOLD TECHNOLOGIES, Germany;

http://www.berthold.com). Remaining aequorin was discharged by automatic injection of 200 μL of 2 M $CaCl_2$ in 20 % ethanol, and luminescence was recorded until values were within 1 % of the highest discharge value. Relative luminescence values were converted into $[Ca^{2+}]$ by using the calibration equation developed by Rentel and Knight (2004).

2.5. H_2O_2 detection

H_2O_2 released by leaf tissues was measured by a luminol-dependent assay (Keppler *et al.*, 1989) with some modifications. For each condition, 2 leaf disks (7 mm in diameter) were overnight incubated in assay tubes containing 200 μL H_2O at 25 °C. Luminol (60 μM) was added and luminescence was measured immediately with a single-tube luminometer (Lumat LB 9507, BERTHOLD TECHNOLOGIES, Germany; http://www.berthold.com). OGs treatments were made at 5 min when luminescence stabilized to basal level. Measurement was made with 1 s integration time at 10 s intervals for a total time of 40 min. For pharmacological studies, a 10 min pretreatment with GLRs antagonists was made before luminol addition.

2.6. Nitric oxide detection

Intracellular NO accumulation was monitored using 4,5-diaminofluorescein diacetate (DAF-2DA). Leaf disks (7 mm in diameter) from *Arabidopsis* plants were vacuum infiltrated for 3 min in an aqueous solution of 20 μM DAF-2DA in Tris-HCl (50 mM, pH 7.5). The disks were incubated for 1 h in the dark and washed twice with Tris-HCl to remove excessive fluorophore. A second infiltration for 3 min was made with OGs or water. GLRs inhibitors were added in both infiltrations. For fluorescent measurements, eight leaf disks per treatment were put separately in a 96 well plate (Microtest™ flatbottom, BD Biosciences, Europe; http://www.bdbiosciences.com) containing 200 μL of the infiltration medium. Reactive nitrogen species (notably N_2O_3 and NO^+) derived from NO auto-oxidation nitrosate DAF-2 yielding to the highly fluorescent DAF-2 triazole (DAF-2T). Increase in DAF-2T fluorescence was measured with a spectrofluorometer (Mithras L 940, BERTHOLD TECHNOLOGIES, Germany; http://www.berthold.com) with 485 nm excitation 535 nm emission filters. Fluorescence was expressed as relative fluorescence units (RFU).

2.7. *Botrytis cinerea* infection test

B. cinerea isolate BMM, kindly provided by Dr. Laurent Zimmerli (University of Fribourg, Switzerland), was grown for 10-12 days (light 10 h, 20 °C; dark 14 h, 18 °C) on 39 $g.L^{-1}$ PDA (potato dextrose agar, DIFCO; BD Biosciences; http://www.bdbiosciences.com). Spores were harvested in water and $^{1}/_{2}$ h before inoculation, harvested spores were diluted in ¼ PDB (potato dextrose broth, 6 $g.L^{-1}$ DIFCO; BD Biosciences; http://www.bdbiosciences.com) to obtain a final concentration of 5.10^{4} $spores.mL^{-1}$. Droplets of 6 µL of *B. cinerea* spores suspension were deposited on intact leaves and plants were kept in growth chamber under high humidity. After 72 h, lesion spots were quantified to check the pathogen development. One hour before *B. cinerea* infection, GLRs antagonists were syringe infiltrated in 5-6 intact leaves.

2.8. *Hyaloperonospora arabidopsidis* infection test

H. arabidopsidis (*Hpa*) isolate NOCO2 was kindly provided by Dr. Patrick Saindrenan (University Paris Sud XI, France) and maintained by weekly sub-culturing on genetically susceptible Col-0 plants. For pathogen challenge, 3 weeks old plants were spray-inoculated to saturation with freshly harvested spores (4.10^{4} $spores.mL^{-1}$). Plants were kept in a growth chamber under high humidity. The 7th day, aerial parts of plants were harvested, pooled for each treatment/genotype and weighed. The liberated spores were counted under microscope and infection intensity was calculated as number of $spores.g^{-1}$ FW. Plants were sprayed with GLRs antagonists 48 h before *H. arabidopsidis infection.*

2.9. Gene expression analysis

Expression pattern analyses of defense-related genes were performed in response to OGs/*H. arabidopsidis* in *atglr3.3* (SALK_066021) mutants and after DNQX co-treatment with OGs/*H. arabidopsidis* with DNQX in wild type (Col0) plants. For OGs induced gene expression, 5 weeks old plants were syringe infiltrated with OGs (in the presence or absence of DNQX when inhibitors effects were tested). To investigate *H. arabidopsidis*-induced gene expression, 3 weeks old plants were infected with *H. arabidopsidis* (plants were sprayed 48 h before *H. arabidopsidis* infection to test inhibitors effects).

Treated leaf tissues were frozen in liquid nitrogen and total RNA was extracted with TRI reagent (Molecular Research Centre Inc. USA; http://www.mrcgene.com) according to the manufacturer's protocol. RNA yield was quantified at 260 nm and purity was estimated by

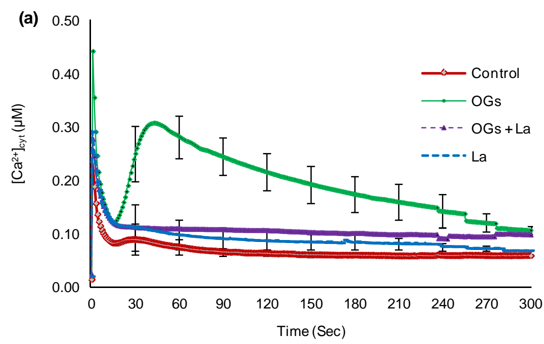
260/280 nm ratio. DNase treatments were performed with deoxyribonuclease I amplication grade (SIGMA-ALDRICH; http://www.sigmaaldrich.com) and first strand synthesis of cDNA was carried out using a cDNA synthesis kit (ImProm-II™ Reverse Transcriptase, Promega; http://www.promega.com), according to manufacturer's advice. Real-Time quantitative PCR analysis was performed with a light cycler 480 (Prime detection system, Roche, France; https://www.roche-applied-science.com) in 384-well plate. Reactions were performed in a final volume of 5 µL containing 2 µL cDNA (dilution 1/10), 0.25 µL each of forward and reverse primers (200 nM) and 2.5 µL Absolute ™ QPCR SYBR© Green ROX Mix (Thermo Fisher Scientific, USA; http://www.thermofisher.com). After normalization with *UBQ10*, levels of transcripts were calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Sequences of primers used are given in table S3.

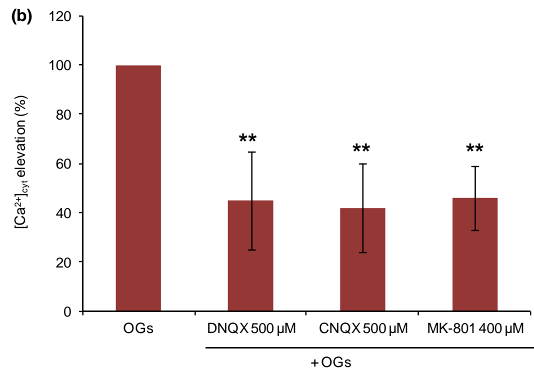
3. RESULTS

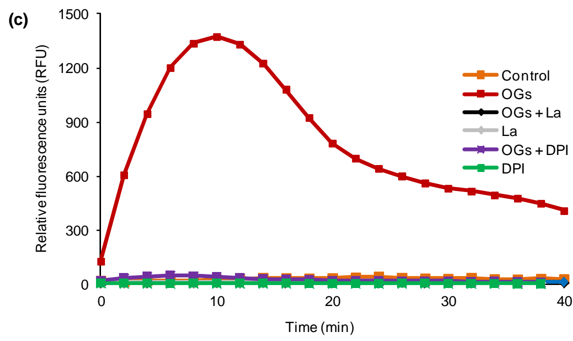
3.1. OGs signaling pathway is dependent on glutamate receptors

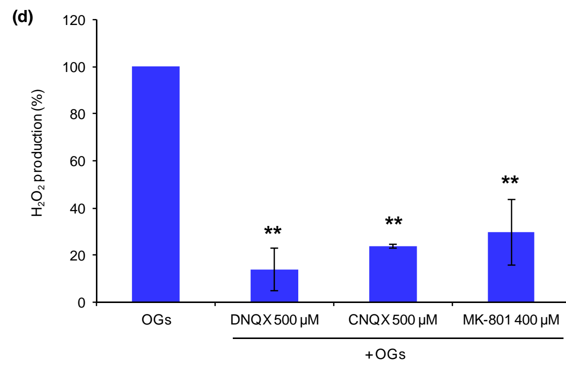
3.1.1. OGs-induced [Ca^{2+}]cyt variations in *A. thaliana* are modulated by GLRs

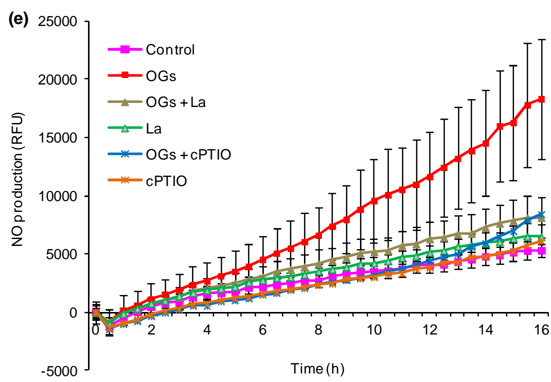
OGs induced a very rapid and transient $[Ca^{2+}]_{cyt}$ variation in *Arabidopsis* leaf disks (Figure 4.1a). $[Ca^{2+}]_{cyt}$ elevation started about 10 s after OGs treatment and reached to its maximum at 35-45 s before coming back to basal level at 300 s. A pretreatment with lanthanum chloride (La^{3+}), a calcium channel blocker, resulted in a strong inhibition of OGs-induced $[Ca^{2+}]_{cyt}$ elevation indicating that Ca^{2+} influx from the extracellular medium is required for $[Ca^{2+}]_{cyt}$ rise. In order to assess the involvement of GLRs in $[Ca^{2+}]_{cyt}$ variations, a pharmacological approach using the GLRs inhibitors DNQX (6,7-dinitriquinoxaline-2,3 dione), CNQX (6-cyano-7-nitroquinoxaline- 2,3-dione) and MK-801 (5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) was first applied. DNQX and CNQX inhibitors belong to competitive and non-NMDA ionotropic glutamate receptors (iGluR) channels category (Honore *et al.*, 1988; Armstrong and Gouaux, 2000) while MK-801 is a non-competitive inhibitor specifically targeting the NMDA channel domain of iGluR (Foster and Wong, 1987; Tuneva *et al.*, 2003). These inhibitors were shown to be efficient in plants, as elicitor- and glutamate (Glu)-induced Ca^{2+} influx and $[Ca^{2+}]_{cyt}$ variations were suppressed after their applications (Lam *et al.*, 1998; Dubos *et al.*, 2003; Sivaguru *et al.*, 2003; Kwaaitaal *et al.*, 2011; Michard *et al.*, 2011; Vatsa *et al.*, 2011). Our data confirmed that DNQX, CNQX and MK-801 were capable of reducing OGs-induced $[Ca^{2+}]_{cyt}$ variations by 55-60 % (Figure

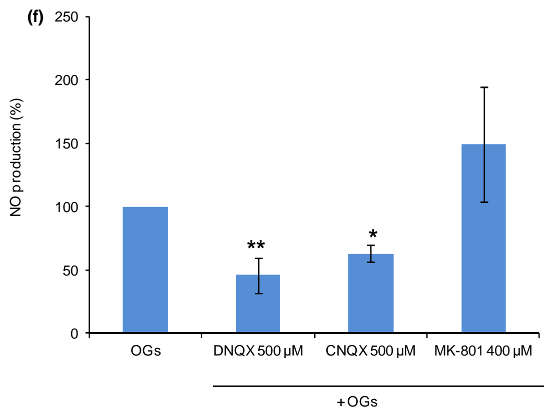

(a)
[Ca2+]cyt (μM)
Time (Sec)
Control
OGs
OGs + La
La


(b)
[Ca2+]cyt elevation (%)
**
OGs
DNQX 500 μM
CNQX 500 μM
MK-801 400 μM
+OGs


(c)
Relative fluorescence units (RFU)
Time (min)
Control
OGs
OGs + La
La
OGs + DPI
DPI


(d)
H2O2 production (%)
**
OGs
DNQX 500 μM
CNQX 500 μM
MK-801 400 μM
+OGs


(e)
NO production (RFU)
Time (h)
Control
OGs
OGs + La
La
OGs + cPTIO
cPTIO


(f)
NO production (%)
**
*
OGs
DNQX 500 μM
CNQX 500 μM
MK-801 400 μM
+OGs

4.1b), indicating the involvement of potential GLRs in OGs-induced Ca^{2+}-dependent signaling pathways.

3.1.2. GLRs modulate OGs-triggered ROS production

OGs-induced ROS production depends on $[Ca^{2+}]_{cyt}$ elevation (Hu *et al*., 2004; Rasul *et al*., 2012), however it is unknown whether GLRs are involved in this process. In leaf disks, OGs-induced ROS production could be monitored after 2 min of OGs treatment and was peaking at 10 min before reaching its background level after about 40 min (Figure 4.1c). The OGs-induced ROS production was completely abolished by diphenylene iodonium (DPI), an inhibitor of the plasma membrane NADPH oxidase, and by La^{3+}, confirming that extracellular Ca^{2+} influx is a prerequisite for OGs-induced ROS production (Figure 1c). DNQX, CNQX and MK-801 pretreatment resulted in a very strong inhibition of OGs-induced ROS production by 86 %, 76 % and 70 %, respectively (Figure 4.1d). *In situ* detection of OGs-induced H_2O_2 production by 3,3-diaminobenzidine (DAB) staining also showed a reduction of ROS production in DNQX-pre-treated leaves as compared to non pre-treated leaves (Supplementary Figure S1). Based on these observations, our data clearly demonstrated that GLRs, by controlling $[Ca^{2+}]_{cyt}$ elevation, play an important role in OGs-induced ROS generation.

3.1.3. GLRs participate in OGs-triggered NO production

Nitric oxide (NO) is an intermediate signaling molecule in elicitor-mediated defense responses and plays a crucial role in the plant adaptive response to pathogen attack (Besson-Bard *et al*., 2008; Gaupels *et al*., 2011). Moreover, elicitors-induced NO production is dependent on a Ca^{2+} influx (Lamotte *et al*., 2004; Vandelle *et al*., 2006). We therefore tested whether OGs-evoked NO generation could be controlled by Ca^{2+} influx through GLRs. OGs provoked a significant and sustained increase in NO production that started at 1 h of OGs treatment and continued during the whole period of observation (Figure 4.1e). Pretreatment with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), an NO scavenger, resulted in complete suppression of OGs-induced NO production. As expected, a very strong reduction in OGs-induced NO production was observed after pretreatment of leaf disks with La^{3+}, indicating that NO production depends on Ca^{2+} influx. Pre-incubation of leaf disks with DNQX and CNQX resulted in a partial but significant inhibition of OGs-induced NO production by 54 % and 37 %, respectively (Figure 4.1f). MK-801 pretreatment delayed OGs-induced NO production but at longer times NO production was not reduced. Lower NO

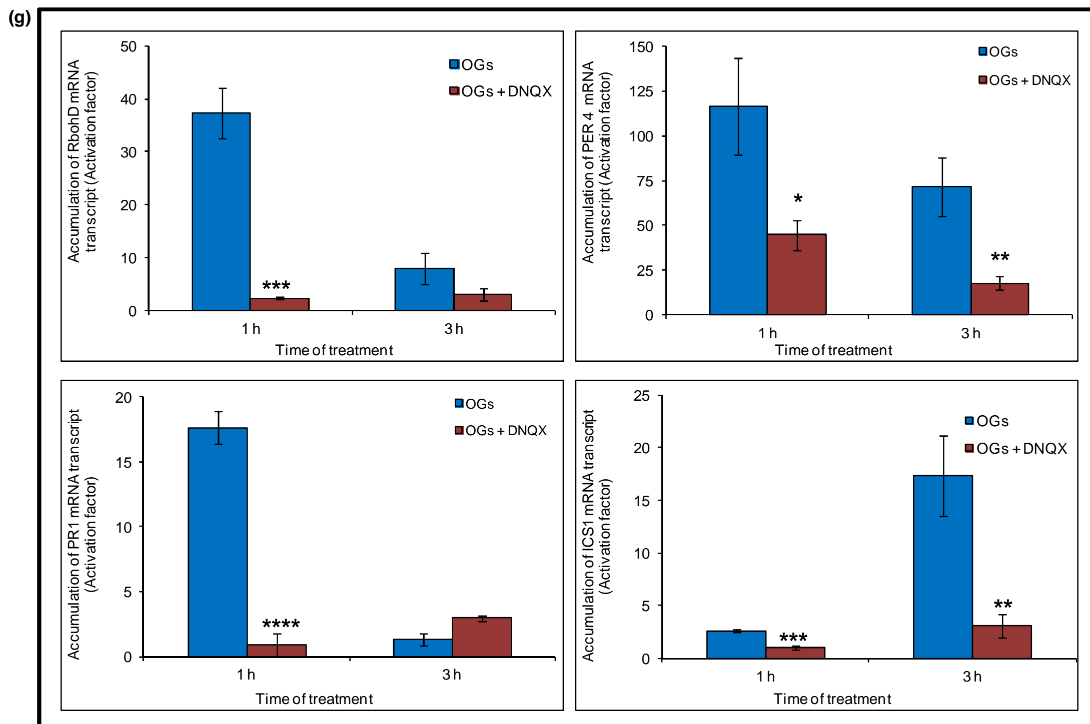


Figure 4.1: Effects of GLRs on OGs-induced signaling events in Col-0. a) $[Ca^{2+}]_{cyt}$ variations induced by OGs (n=6). **b)** OGs-induced $[Ca^{2+}]_{cyt}$ variation in presence of GLRs inhibitors, expressed as a percentage of the maximal peak response after subtracting background luminescence of corresponding controls. Data represent the mean ± SD (n=3). Inhibitor treatments were made 10 min prior to OGs treatment. **c)** OGs-induced H_2O_2 production in presence or not, of La^{3+} (1 mM) or DPI (50 µM), (n=5). **d)** OGs-induced H_2O_2 production with or without GLRs inhibitor treatment, expressed as a percentage of the maximal peak response observed after OGs-elicitation (100 %). Data are mean ± SD, (n=3). **e)** Time course of OGs-induced NO production monitored by measuring DAF-2T fluorescence in presence or not, of La^{3+} (1 mM) or cPTIO (0.5 mM). Each point represents the mean of 8 parallel measurements ± SD. Curves are 1 representative experiment out of 3. **f)** OGs-induced NO production determined at 16 h of treatment in presence or not of GLRs inhibitors, expressed as percentage of the response obtained in OGs treatment (100 %) after subtracting background fluorescence of corresponding control. Data are mean ± SD (n=5). Inhibitors treatments were made 1 h prior to OGs treatment. **g)** Quantitative RT-PCR of different genes of plant defense signaling pathway. Bar graphs indicate the mean ± SD of three technical replicates. Three biological replicates were performed with similar results. La: La^{3+}.

For $[Ca^{2+}]_{cyt}$, ROS and NO production data, statistical differences were determined by one-way ANOVA, followed by Dunnett's test using SigmaPlot software. Student's t test for pair-wise comparison was performed for gene expression analysis. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

production inhibition by MK-801 in comparison to DNQX was also observed in tobacco cells treated by cryptogein (Vatsa *et al*., 2011). The lower NO production in presence of GLRs antagonists (except MK-801), is in agreement with the corresponding inhibition of OGs-induced $[Ca^{2+}]_{cyt}$ variation (Figure 4.1b).

3.1.4. GLRs-dependent defense gene expression

Expression pattern of some plant defense-related genes was studied in *Arabidopsis* Col-0 plants after OGs treatment, in the presence or absence of DNQX. The selected genes encode following proteins: the respiratory burst oxidative hydrogenase D (RbohD), anionic peroxidase 4 (PER4), pathogenesis-related protein 1 (PR1) and isochrismate synthase (ICS1). *RbohD* encodes a plasma membrane NADPH oxidase enzyme that is involved in ROS generation (Pugin *et al*., 1997; Torres *et al.* 2002). *PER4* is reported to be induced by OGs (Ferrari *et al.,* 2007) and recently Rasul *et al*. (2012) have demonstrated that *PER4* is an NO-dependent gene in OGs signaling, and is involved in resistance against *B. cinerea* in *Arabidopsis.* ICS1 is an important enzyme of SA pathway and is induced in response to both necrotrophic and biotrophic pathogens (Wildermuth *et al*., 2001). *PR-1*, a SAR marker gene, is a well known studied SA-inducible gene and its expression is modulated by a variety of pathogens (Uknes *et al*., 1992; Rögers and Ausubel, 1997; Maleck *et al*., 2000; Navarro, 2008). PR1 is present downstream of ICS1 in SA pathway leading to plant defense (Maleck *et al*., 2000).

As shown in figure 4.1g, our data demonstrated that OGs treatment resulted in a significant accumulation of *RbohD, PER4, PR1* and *ICS1* transcripts at 1 h. *PER4, ICS1* and to a less extent *RbohD* also showed significant activation at 3 h of OGs treatment. The increase in the transcript level was significantly inhibited with DNQX treatment at 1 h and also at 3 h for *PER4* and *ICS1* genes (Figure 4.1g). Our data highlight that expression of these genes is well under the control of GLRs.

3.1.5. GLRs and basal resistance against necrotrophic and biotrophic pathogens

B. cinerea is a necrotrophic and polyphage fungus responsible for the grey mould disease on more than 200 host plants including *Arabidopsis* (Elad *et al*., 2004). *H. arabidopsidis (Hpa)*, an obligate biotrophic oomycete, is one of only a few pathogens that naturally infect *Arabidopsis* causing downy mildew infection (Holub *et al*., 1994; Coates and Beynon, 2010). Ecotype Col-0 is susceptible to *HpaNoco2* isolate as its corresponding R gene *RPP5* is absent in Col-0.

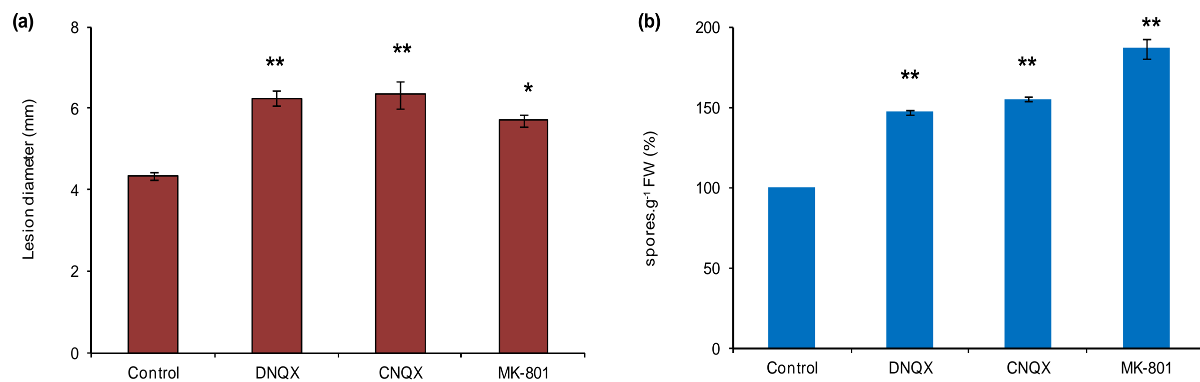


Figure 4.2: GLRs are involved in basal resistance in Col-0. a) Basal resistance to *B. cinerea*. One hour after DNQX, CNQX or MK-801 application, Col-0 plants were inoculated with *B. cinerea* spores (5.10^4 spores.mL^{-1}) and disease symptoms were measured 72 h post-inoculation. Bar graph represents the lesion diameter (mean ± SE) of pooled data of 5 different experiments. Each individual experiment was performed on 12 plants for each treatment (4-6 inoculations per plant). **b)** Basal resistance to *H. arabidopsidis*. Three weeks-old Col-0 plants were treated with GLRs inhibitors and 48 h later, plants were sprayed with *H. arabidopsidis* spores (4.10^4 spores.mL^{-1}). Bar graph represents the percentage of spores in GLRs inhibitors pretreated plants compared to mock-treated plants at 7 days post-inoculation. Data are means ± SE of at least 3 experiments (each with 12-16 pooled plants per treatment).

Data underwent statistical analysis by Dunnett's test using the SigmaPlot software. Asterisks indicate statistically significant differences after GLRs inhibitors treatments (*$p < 0.05$, **$p < 0.01$).

Taken into account that OGs-induced NO and ROS production in *Arabidopsis* was partially dependent on Ca^{2+} through GLRs (our data) and that both signaling compounds took part in plant defense against a variety of pathogens (Besson-Bard *et al*., 2008; Wendehenne *et al*., 2004; Delledonne, 2005; Zaninotto *et al*., 2006), one of our purposes was to establish a link between GLRs and basal resistance. Using *B. cinerea* as a plant challenger in plants pretreated with DNQX, CNQX and MK-801, we showed a statistically significant increase in the average area of necrotic lesions (Figure 4.2a). With *HpaNoco2*, a prominent increase of 55 %, 60 % and 80 % in the total number of spores.g^{-1} FW was observed after DNQX, CNQX and MK-801 treatments, respectively (Figure 4.2b). It was also verified that GLRs inhibitors had no effect on *in vitro* pathogen development (Supplementary figure S4.2 and S4.3).

Collectively, these findings indicate that GLRs are involved in basal defense against necrotrophic and biotrophic pathogens.

3.2. Genetic evidences for *Arabidopsis* clade 3 GLRs involvement in OGs-induced plant defense signaling and resistance against *H. arabidopsidis*

It was previously reported that overexpression of a radish GLR ortholog of a clade 3 AtGLR (AtGLR3.2) in *Arabidopsis* increased basal resistance to *B. cinerea* (Kang *et al.*, 2006). Thus, based on this indication and on our above results, single T-mutants of *Arabidopsis thaliana* clade 3 GLRs were screened for modified defense signaling events and resistance.

3.2.1. OGs-induced ROS and NO production in *Atglr* mutant plants

Our data demonstrated that *Atglr3.1*, *Atglr3.2*, *Atglr3.3, Atglr3.4* and *Atglr3.6* mutants showed a significant 52 %, 54 %, 63 %, 50 % and 42 % reduction of OGs-induced ROS production compared to Col-0 plants, respectively (Figure 4.3a). Thus, except for AtGLR3.5 and AtGLR3.7 gene products, it indicated that most of the clade 3 AtGLR gene products participated in OGs-induced signaling pathway leading to ROS production.

The OGs-induced NO production was slightly reduced in *Atglr3.1*, *Atglr3.2, Atglr3.3* and *Atglr3*.6 mutants by nearly 20 to 25 % as compared to Col-0 plants (Figure 4.3b), indicating a limited impact of one *AtGLR* gene disruption on NO production.

Based on agonist studies, Stephens *et al.* (2008) proposed the existence of different classes of GLR channels, formed by different combination of GLR subunits. This could be supported by our data, showing the involvement of different gene products. Interestingly, we

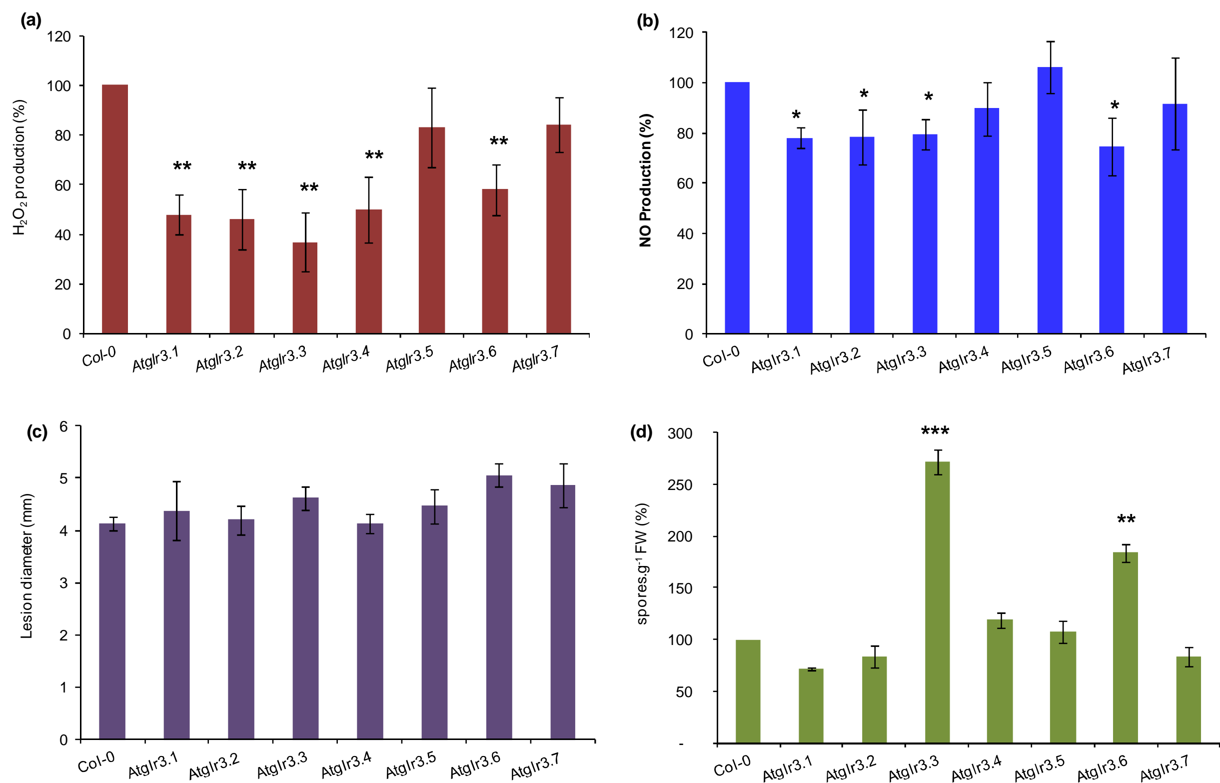


Figure 4.3: GLRs mutants are compromised in OGs-induced signaling and resistance to pathogens. a) OGs-induced H_2O_2 production expressed as the percentage of the maximal peak response in *Atglr* mutants compared to response in Col-0 (100 %). Each bar is the average ± SD of at least 5 independent experiments. **b)** OGs-induced NO production expressed as the percentage of fluorescence at 16 h post-treatment in *Atglr* mutants compared to response in Col-0 (100 %). Data are means ± SD from at least 3 experiments. **c)** Basal resistance to *B. cinerea*. Inoculation was made as described in figure 2a. Histogram indicates the means ± SE of the lesion diameter of pooled data from 3 experiments. **d)** Basal resistance to *H. arabidopsidis*. Infection tests were performed as described in figure 2b. Results are presented as the percentage of spores ± SE (n=6) in *Atglr* mutants compared to the spores in Col-0 (100 %) at 7 days post-inoculation.

Statistical differences were determined by one-way ANOVA, followed by Dunnett's test using the statistical software SigmaPlot. Asterisks indicate statistically significant differences (*p < 0.05, **p < 0.01, ***p < 0.001).

obtained a lower OGs-induced ROS and NO production in presence of GLR antagonists in comparison to the production in mutants, suggesting that GLR antagonists targeted GLR channels composed of different subunits and/or more than one homotetrameric GLR channels.

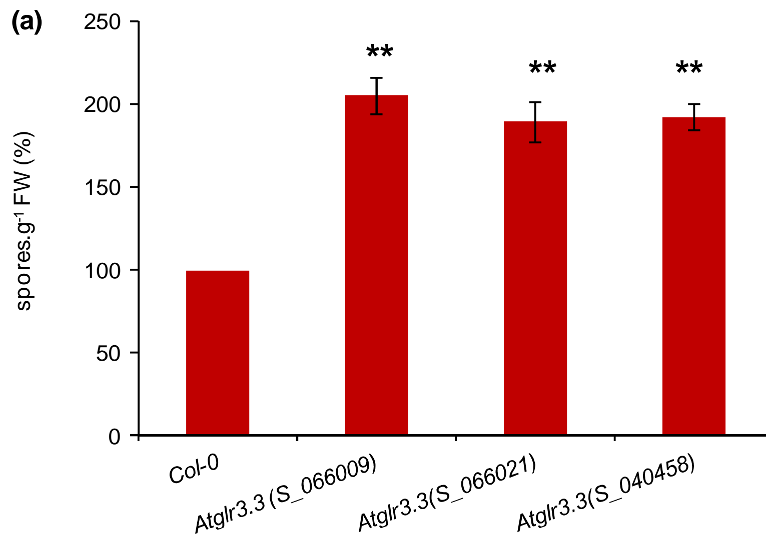
3.2.2. Resistance to *B. cinerea* and *H. arabidopsidis* in *Atglr* mutant plants

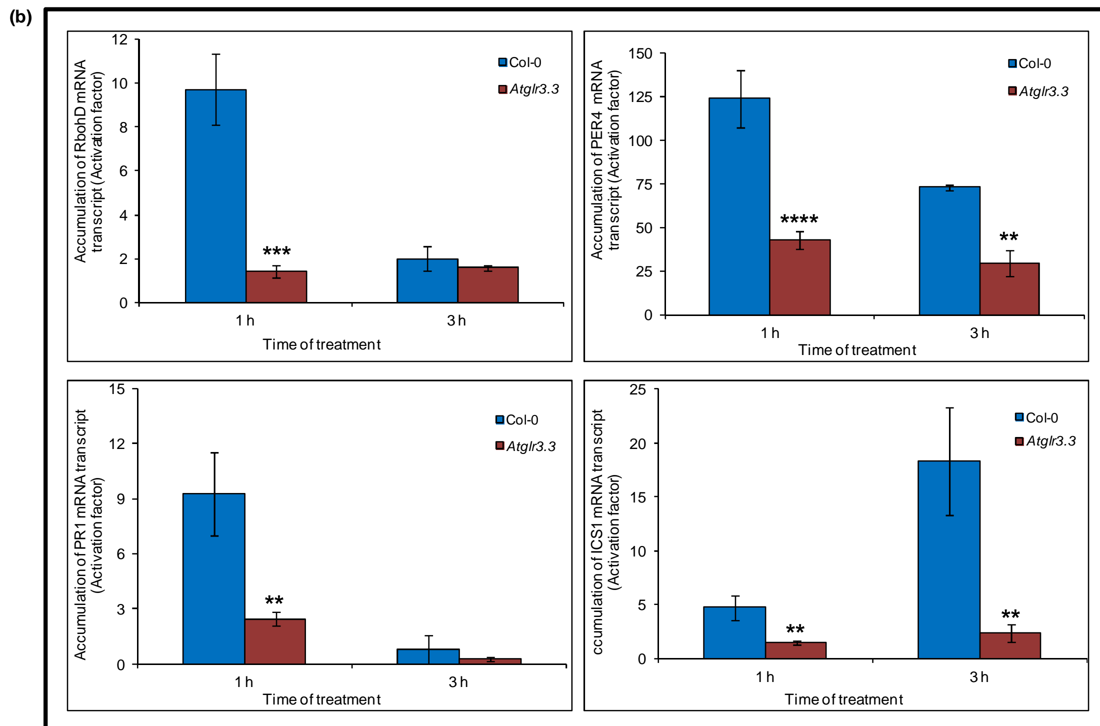
When challenged with *B. cinerea*, some mutants showed increased disease symptoms, yet none of the tested mutant was found to be statistically more susceptible against this necrotrophic fungal pathogen (Figure 4.3c). However, *Atglr3.3* and *Arglr3.6* mutant lines were significantly more susceptible to *H. arabidopsidis* as compared to Col-0 plants (Figure 4.3d).

3.2.3. *AtGLR3.3* involvement in *H. arabidopsidis* resistance and plant defense signaling

To further strengthen the involvement of AtGLR3.3 in plant defense signaling, two other *Atglr3.3* mutant lines (SALK_066009 and SALK_040458) were tested in addition to the *Atglr3.3* mutant line used before (SALK_066021). Similar to SALK 066021, SALK_066009 mutant line had a T-DNA insertion in the first exon, while SALK_040458 had a second exon T-DNA insertion. The 3 mutant lines showed a similar higher susceptibility to *H. arabidopsidis* compared to Col-0 (Figure 4.4a). OGs-induced ROS accumulation, NO production and *B. cinerea* infection response in all the 3 mutant lines were also similar, clearly demonstrating that our results were not an artefactual effect due to other mutations in the selected *Atglr3.3* lines (Supplementary Figures S4.4 – S4.6).

In parallel to OGs-induced signal transduction and *H. arabidopsidis* resistance process, defense gene expression was studied in OGs- and *H. arabidopsidis*-treated Col-0 and *Atglr3.3* (Salk_066021) plants. OGs-induced gene expression of *RbohD*, *PER4*, *PR1* and *ICS1* in *Atglr3.3* mutant was significantly decreased in comparison to the OGs-induced gene expression in Col-0 plants (Figure 4.4b). This decrease is in complete agreement with OGs-induced gene expression in Col-0 plants pre-treated with DNQX (Figure 4.1g), indicating the prominent role of AtGLR3.3 in defense gene expression. DNQX effect on *H. arabidopsidis*-induced gene expression in Col-0 plants was also investigated. First, our data showed that *H. arabidopsidis* induced the expression of the plant defense-related genes: *RbohD* expression was slightly increased at 48 h post inoculation whereas *PER4*, *PR1* and *ICS1* expression were observed at 96 h post inoculation in Col-0 plants (Figure 4.4c). Second, DNQX pretreatments


(a)
spores.g-1 FW (%)
250
200
150
100
50
0
**
**
**
Col-0
Atglr3.3 (S_066009)
Atglr3.3(S_066021)
Atglr3.3(S_040458)


(b)
Accumulation of RbohD mRNA transcript (Activation factor)
Col-0
Atglr3.3
***
1 h
3 h
Time of treatment
Accumulation of PER4 mRNA transcript (Activation factor)
****
**
Accumulation of PR1 mRNA transcript (Activation factor)
**
cumulation of ICS1 mRNA transcript (Activation factor)
**
**

significantly inhibited the expression of these genes (Figure 4.4c), indicating common signaling steps in OGs and *H. arabidopsidis* signaling pathways depending on GLRs activation. To further show the involvement of AtGLR3.3 in *H. arabidopsidis* resistance, the same gene expression experiments were conducted in the *Atglr3.3* mutant background. In the mutant line, *H. arabidopsidis*-induced gene expression was much lower than in Col-0 (Figure 4.4d). When the *H. arabidopsidis*-induced gene expression in DNQX-pretreated Col-0 and *Atglr3.3* were compared, the same pattern of reduction in gene expression was observed, with a somewhat stronger reduction in DNQX-pretreated plants compared to *Atglr3.3* mutant. Taken together, these data further concluded to the important role of GLRs, and particularly of AtGLR3.3, in elicitor/pathogen-induced plant defense signaling pathways.

4. Discussion

GLRs are ligand gated cation channels present across various kingdoms of life including bacteria, animals and plants. In animals, GLRs have been deeply studied from their structural and functional point of view and their role as neurotransmitter and immunotransmitter has been firmly established (Gill and Pulido, 2001; Boldyrev *et al.*, 2005; Pacheco *et al.*, 2007; Rousseaux, 2008). In plants, they have gained much attention after the discovery of ionotropic type GLRs homologs in *Arabidopsis* (Lam *et al.*, 1998; Lacombe *et al.*, 2001). Plant GLRs were shown to be implicated in many different physiological processes (Dodd *et al.*, 2010; Jammes *et al.*, 2011). It was recently reported that GLRs were involved in elicitor-induced plant defense signaling in tobacco and *Arabidopsis* (Kwaaitaal *et al.*, 2011; Vatsa *et al.*, 2011), but molecular characterization of the involved GLRs was not made. In this study, we brought pharmacological and genetic evidences of the involvement of *Arabidopsis* GLRs belonging to clade 3 in elicitor-induced plant defense signaling and in basal resistance against *B. cinerea* and *H. arabidopsidis*.

Consistent with previous studies (Hu *et al.*, 2004; Moscatiello *et al.*, 2006), our data illustrated that the OGs elicitor induced a very rapid and transient $[Ca^{2+}]_{cyt}$ variation in *Arabidopsis*, suppressed by a La^{3+} pretreatment (Figure 4.1a). Moreover, pretreatment with the iGluR antagonists DNQX, CNQX and MK-801 resulted in a strong inhibition (50-60 %) of OGs-triggered $[Ca^{2+}]_{cyt}$ variations, concluding to the existence of Ca^{2+} fluxes due to the activation of potential GLRs by OGs. Similar to our findings, by using the elicitor Cry and the amino acid glutamate (Glu), Vatsa *et al.* (2011) demonstrated that GLRs were involved in Ca^{2+} influx and $[Ca^{2+}]_{cyt}$ variation in tobacco cells, these processes being either completely or

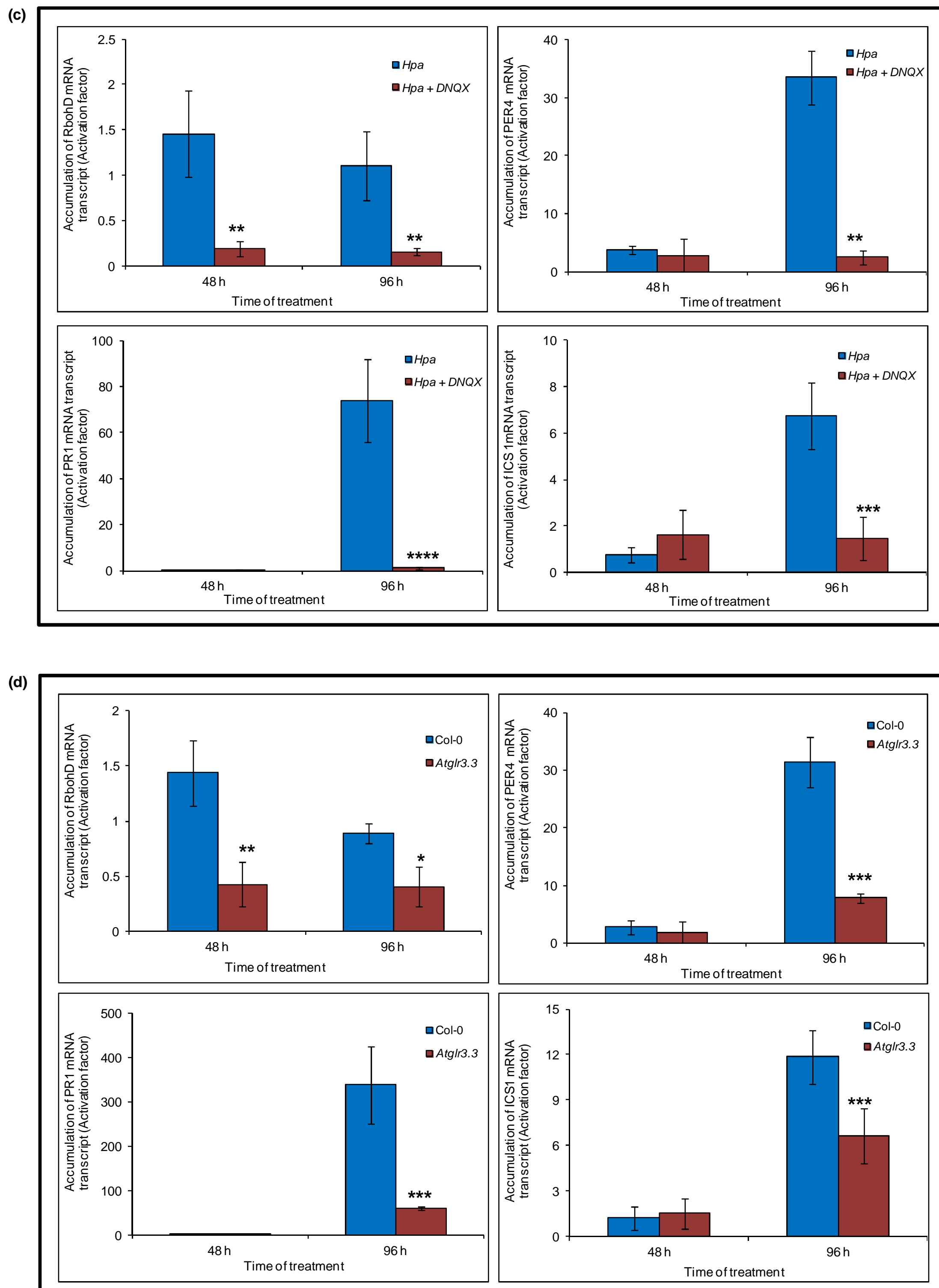


Figure 4.4: AtGLR3.3 is an important player in OGs and *H. arabidopsidis* signaling pathway. a) Sporulation of *H. arabidopsidis* in different *Atglr3.3* mutant lines. Results are presented as the percentage of spores growth in three *Atglr3.3* mutants lines compared to Col-0 (100 %) at 7 days post-inoculation.

partially inhibited depending on the GLRs antagonist used in the assay. $[Ca^{2+}]_{cyt}$ variations induced by flg22, elf18 or chitin elicitors were also partially inhibited in *Arabidopsis* with some set of GLRs antagonists (Kwaaitaal *et al.*, 2011). Downstream signaling events, such as NO production or MAPK activation, were also affected depending on the elicitor and the GLRs inhibitor (Kwaaitaal *et al.*, 2011; Vatsa *et al.*, 2011). Supporting our data, it has also been reported that Glu induced a very large and fast change in $[Ca^{2+}]_{cyt}$ followed by a large transient membrane depolarization in *Arabidopsis* partly due to Ca^{2+} influx (Dennison and Spalding, 2000), and Glu-induced $[Ca^{2+}]_{cyt}$ variations were strongly suppressed by DNQX and CNQX treatments (Meyerhoff *et al.*, 2005). In another study, Dubos *et al.* (2003) provided evidences for synergistic effects of glycine (Gly) with Glu to control ligand-mediated Ca^{2+} signals in plants, and DNQX or CNQX treatments significantly inhibited Glu- mediated $[Ca^{2+}]_{cyt}$ elevations in *Arabidopsis*. Recently, it has been proved that GLRs are involved in the generation of Ca^{2+} influx oscillations in pollen tubes induced by D-serine that were inhibited by CNQX and DNQX applications in tobacco and *Arabidopsis* (Michard *et al.*, 2011). In their investigations, Qi *et al.* (2006) indicated that *GLR3.3* participates in $[Ca^{2+}]_{cyt}$ variations in *Arabidopsis* root cells as mutant lines of *GLR3.3* failed to produce Glu-induced $[Ca^{2+}]_{cyt}$. They also demonstrated that six amino acids (glutamate, glycine, alanine, serine, asparagine and cysteine) and the tripeptide glutathione are GLRs agonists to trigger Ca^{2+} flux and membrane depolarization by a mechanism that relied on AtGLR3.3. Further studies in *Arabidopsis* hypocotyl cells highlighted that these six amino acids were not equally efficient agonists to induce transient Ca^{2+} influx and membrane depolarization (Stephens *et al.*, 2008). Based on desensitization assays in wild type (WT), *Atglr3.3* and *Atglr3.4* mutants, it was proposed that GLRs could be organized in different channel subtypes with at least one AtGLR3.3 subunit required for Ca^{2+} flux and membrane depolarization (Stephens *et al.*, 2008). Thus, the overall above data concluded to the existence of functional GLRs, promoting Ca^{2+} fluxes in a stimulus-specific fashion, potentially activated by different agonists and those activities are inhibited to different extent by diverse GLR antagonists depending on their specificity. It is expected that GLRs activities will depend on the number of GLRs types expressed in the cell and on the potential tetrameric channel structures that can be formed. Furthermore, as suggested by pharmacology and genetic studies, different channel subtypes may be co-activated with some having a prominent role in inducing a specific downstream signaling event {(Qi *et al.*, 2006; Stephens *et al.*, 2008; Kwaaitaal *et al.*, 2011; Vatsa *et al.*, 2011) and this study (see below)}. Vatsa *et al.* (2011) have also demonstrated that in elicitor-

Data are means ± SE (n=3). *Atglr3.3* (S_066021) mutant line corresponds to the one used in all our studies. **b)** OGs-induced gene expression in *Atglr3.3* mutant (S_066021). **c)** *H. arabidopsidis*-induced gene expresssion in the presence of DNQX in Col-0 plants. **d)** *H. arabidopsidis*-induced gene expression in *Atglr3.3* mutant (S_066021).

In figure b, c, and d, bar graphs indicate the mean ± SD of three technical replicates. Three biological replicates were performed with similar results. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

mediated signaling, extracellular Glu released following exocytosis could potentially activate GLRs.

In order to decipher part of the plant defense signaling pathway initiated downstream GLRs activation, OGs-induced signaling events were studied both through pharmacological and genetic approaches. OGs induced Ca^{2+}-dependent ROS and NO production, and defense gene expression in plants (Mathieu *et al.*, 1991; Ferrari *et al.*, 2007; Rasul *et al.,* 2012). Remarkably, ROS and NO production are also triggered by animal GluRs emphasizing the potential interest in these two defense events. Indeed, activation of NMDA type of iGluR resulted in $[Ca^{2+}]_{cyt}$ increase with a subsequent ROS accumulation that could participate either to necrotic or apoptotic cell death via activation of caspase-3 (Boldyrev *et al.*, 2005). Moreover, Kulikov and Boldyrev (2006) reported that both iGluR and metabotropic GluRs could increase ROS level in mice cells with iGluRs-activated ROS participating in cell death. Neuronal isoform of NO synthase (nNOS) is co-localized and activated by an NMDA receptor via a postsynaptic density protein (PSD95) through NMDA receptor-mediated $[Ca^{2+}]$ rise (Stamler *et al.*, 2001). In plants, elicitor-induced ROS accumulation mainly relies on RbohD activity, a membrane-localized NADPH oxidase, and Ca^{2+} influx is required for ROS production (Pugin *et al.*, 1997; Hu *et al.*, 2004; Torres *et al.*, 2006; Zhang *et al.*, 2007). As expected, different GLRs antagonists suppressed the OGs-induced ROS (H_2O_2) production, which is sensitive to DPI and La^{3+}, by 70 to 86 % in *Arabidopsis* (Figure 4.1c, 4.1d). Moreover, when AtGLR clade 3 was analyzed, selected because of its potential involvement in plant defense (Kang *et al.,* 2006), it appeared that 5 out of 7 *Atglr* mutants had lowered ROS production: *Atglr3.1*, *Atglr3.2*, *Atglr3.3, Atglr3.4* and *Atglr3.6* showed a 40 to 60 % reduced level of ROS accumulation compared to Col-0 plants (Figure 4.3a). Moreover, partial reduction of ROS production is in agreement with partial inhibition of $[Ca^{2+}]_{cyt}$ elevation and pointed out the potential involvement of other channels such as CNGCs in elicitor-mediated Ca^{2+} influx and cytosolic Ca^{2+} elevation (Ma *et al.*, 2009). When OGs-induced NO production was monitored in *Arabidopsis* plants, our data indicated that DNQX and CNQX reduced the NO production by 54 % and 37 %, respectively (Figure 4.1f). Analysis of clade 3 *Atglr* mutants indicated a reduction of NO production of 20 to 25 % in *Atglr3.1*, *Atglr3.2*, *Atglr3.3,* and *Atglr3.6* mutants as compared to NO production in Col-0 (Figure 4.3b). These results indicated a limited effect of single gene disruption of clade 3 GLR subunits on NO production. Due to the number of *glr* mutants affected in NO and ROS production, it is hypothesized that different GLR hetero/homotetrameric structures could exist, with potentially redundant functions in ROS and NO production favored by subunit replacement.

This hypothesis is further supported by the higher reduction of ROS and NO production in OGs-treated Col-0 plants antagonized by GLR inhibitors, which may target more than one class of GLR. As a whole, our genetic and pharmacologic studies confirmed the involvement of GLRs in NO production as previously observed in tobacco (Vatsa *et al.*, 2011) and showed the involvement of GLRs in ROS production. It was also reported that OGs and lipopolysaccharides treatments induced NO production which was reduced in *Arabidopsis cngc2* mutant (Ali *et al*., 2007; Rasul *et al*., 2012), indicating a complex Ca^{2+}-regulated pathway involving different Ca^{2+} channel classes in addition to GLRs. We also verified whether activation of GLRs could induce defense gene expression in accordance with our above results. As shown in figure 4.1d, our results also demonstrated that OGs treatment in Col-0 plants resulted in a significant accumulation of *RbohD*, *PER4*, *ICS1* and *PR-1* genes transcripts that are involved in plant defense responses; detail of their corresponding functions are present in results part (Uknes *et al.*, 1992; Rogers and Ausubel, 1997; Maleck *et al.*, 2000; Wildermuth *et al.*, 2001; Torres *et al.,* 2002; Ferrari *et al.,* 2007; Navarro, 2008; Rasul *et al.*, 2012). More interestingly, increase in the transcript level of these genes was significantly inhibited with DNQX treatment (Figure 4.1d). In addition, it was also reported that *PER4* expression was partly dependent on NO production (Rasul *et al.*, 2012): this is also in accordance with our results showing than NO production and *PER4* expression depended on GLR activation. These results clearly highlight that expression of these genes is well under the control of GLRs.

Further evidences for a strong link between GLRs and plant defense reactions were obtained when resistance to *B. cinerea* and *H. arabidopsidis* was tested in Col-0 plants pretreated with GLRs antagonists and *in Atglr* single mutants. *B. cinerea* infected Col-0 plants pretreated with DNQX, CNQX and MK-801 showed a statistically significant increase in the average area of necrotic lesions (Figure 4.2a). Although some *Atglr* mutants showed increased disease symptoms when infected with *B. cinerea*, yet none of them was found to be statistically more susceptible against this necrotrophic fungal pathogen (Figure 4.3c). It has to be pointed out that the GLR antagonist effects are in general stronger than the effect of a single mutation. In Col-0 plants pretreated with DNQX, CNQX or MK-801 and infected with *H. arabidopsidis*, a prominent increase of 55 %, 60 % and 80 % in the number of spores.g^{-1} FW was observed, respectively (Figure 4.2b). In accordance with these data, *Atglr3.3* and *Atglr3.*6 lines showed nearly 2-fold higher susceptibility to *H. arabidopsidis* as compared to Col-0 plants, the effect being more pronounced in *Atglr3.3* line (Figure 4.3d). This result is consistent with the fact that *Atglr3.3* and *Atglr3.*6 are the closest homologs in *Arabidopsis*

(Chiu *et al.*, 2002). Thus we focused our study on *Atglr3.3* mutant (Salk_066021) showing the highest susceptibility to *H. arabidopsidis*. Lowered ROS and NO production together with higher susceptibility to *H. arabidopsidis* were confirmed in two other *Atglr3.3* mutant lines (SALK_066009 and SALK _040458; Figure 4.4). Both OGs and *H. arabidopsidis* triggered SA-mediated defense mechanisms (Van Der Biezen *et al.*, 2002; Durrant and Dong, 2004) which can be typified by induced expression of *PR1* and *ICS1* genes. In addition, ROS and NO are known to increase the SA-mediated responses (Vlot *et al.*, 2009). Thus, we examined the expression of defense genes involved in SA-related pathway in both Col-0 plants infected with *H. arabidopsidis* and pretreated with DNQX, or in *Atglr3.3* infected plants. In parallel, gene expression in OGs-treated *Atglr3.3* mutant line was also monitored. Interestingly, OGs-induced gene expression was similarly reduced in DNQX-treated Col-0 plants and in *Atglr3*.3 mutant line (Figures 4.1g and 4.4b). *H. arabidopsidis* infection also up-regulated the expression of OGs-induced genes. In addition, a significantly lower expression of these genes was observed either in DNQX-pretreated Col-0 plants or in *Atglr 3.3* mutant plants infected by *H. arabidopsidis*, highlighting the similarity in OGs- and *H. Arabidopsis*-induced gene expression. Collectively, our results are consistent with a wealth of data linking SA-mediated defense to resistance to biotrophic pathogens (Glazebrook, 2005; Thatcher *et al.,* 2005; Wiermer *et al.*, 2005) and clearly identified AtGLR3.3 as a new player in plant defense against *H. arabidopsidis*.

ACKNOWLEDGEMENTS

We are grateful to Marc H. Knight (Durham, UK) for providing seeds of aequorin expressing *Arabidopsis* transformed line; to Edgar P. Spalding (Wisconsin, USA) for homozygous mutant lines of *glr3.3* (Salk_066009, Salk_040458); Miguel A. Torres for *AtrbohD* mutant line and Nottingham Arabidopsis Stock Center (NASC) for providing seeds of GLRs mutants. We would like to thank Siham Madani and Agnes Klinguer for technical assistance. We are grateful to Sumaira Rasul, Benoit Poinssot, Sylvain Jeandroz and Olivier Lamotte for helpful discussions and technical assistance. Hamid Manzoor was supported by a fellowship from Higher Education Commission (HEC), Pakistan. Research funding was provided by Conseil Régional de Bourgogne and Caphe ANR program.

CHAPTER 4

“Supporting information”

SUPPORTING INFORMATIONS

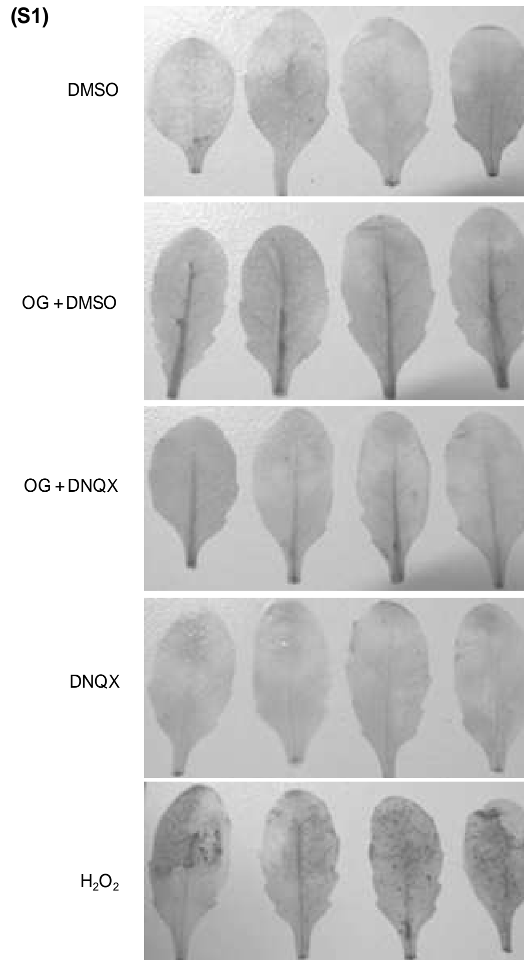


Figure S4.1. *In situ* detection of OGs-induced ROS accumulation after DNQX treatment in Col-0 using DAB staining method. To visualize H_2O_2 *in situ*, 3, 3-diaminobenzidine (DAB) staining of *Arabidopsis* leaves was made according to Torres *et al.*, 2002. DAB staining was performed on mature leaves from 4-5 weeks old *Arabidopsis* Col-0 plants. All the treatments (OGs and/or DNQX) were vacuum-infiltrated for 3 min with the DAB solution. After washing, keeping the adaxial side up, leaves were placed in plastic boxes under high humidity until brown precipitate was observed. A direct application of H_2O_2 to the leaves was used as a positive control. ROS induction is mainly located in the veins of the leaves.

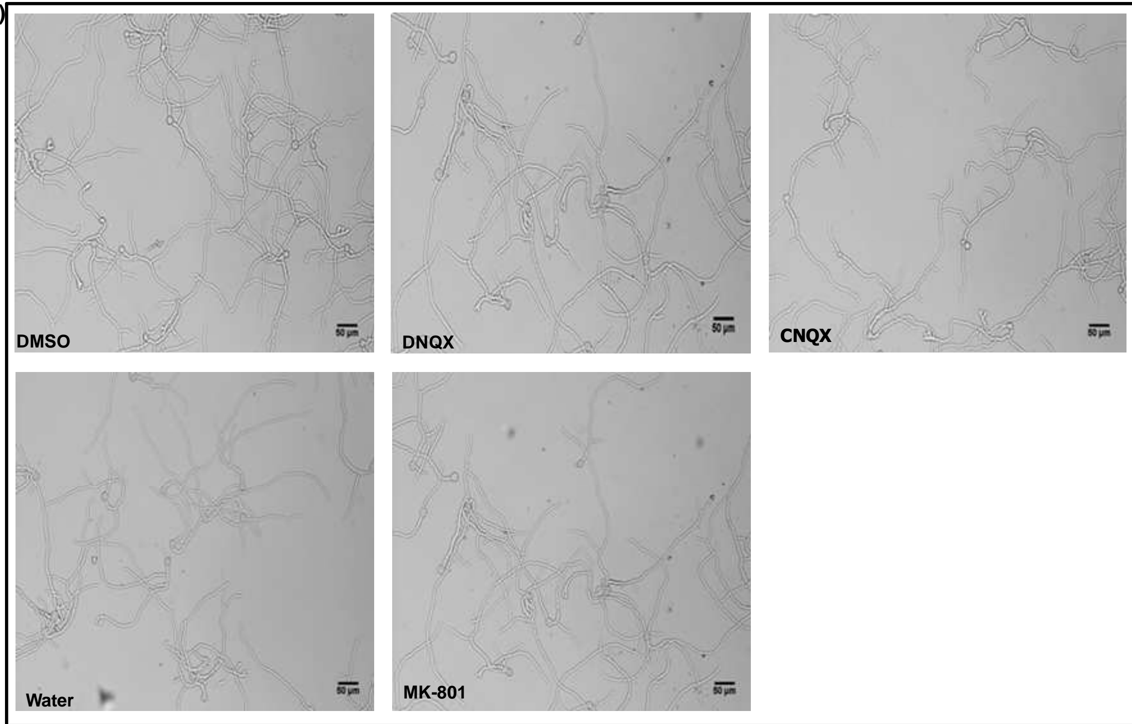


Figure S4.2: Effect of GLRs inhibitors on *Botrytis cinerea* growth. To verify the effect of different GLRs inhibitors (DNQX, CNQX, MK801) on *B. cinerea* hyphal growth, spores were diluted to obtain the final concentration of 5.10^4 spores.mL^{-1} in PDB medium containing either the GLR inhibitor (0.5 mM) or the corresponding volume of DMSO or water depending on the solvent used to dissolve the GLR inhibitor. 12 µL of spore suspension were placed on glass slides and *B. cinerea was* allowed to grow under high humidity. At 12 h, fungal growth was observed under microscope. Differences in *B. cinerea* hyphal growth were not observed whatever the treatment was.

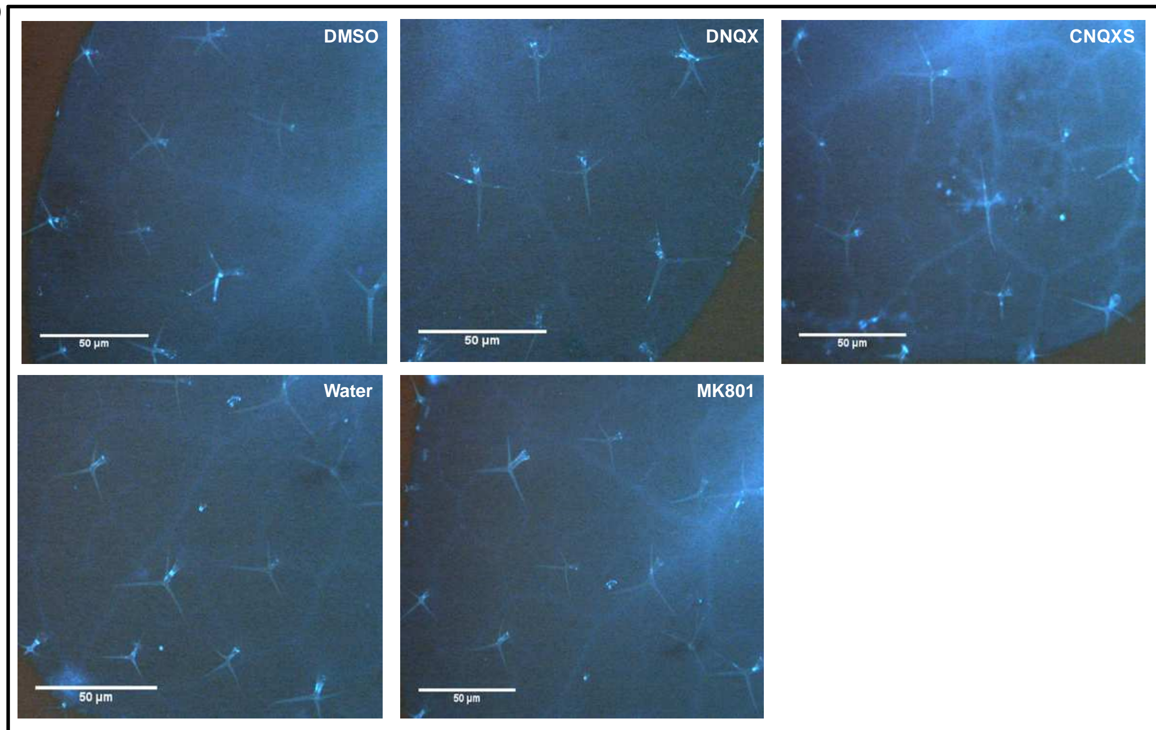


Figure S4.3: Effect of GLRs inhibitors on *Hyaloperonospora arabidopsidis* spore development. The effects of DNQX, CNQX and MK801 on pathogen development were determined in *planta*. Briefly, leaf disks from Col-0 plants were treated for 48 h with GLRs inhibitors or the corresponding controls, DMSO or water, depending on the solvent used to dissolve the GLR inhibitors. Then, the leaf disks were inoculated with *HpaNoco2* strain (4.10^4 spores mL^{-1}) and pathogen was allowed to grown under high humidity for 7 days. *Hpa* development was investigated by aniline blue staining in microscopy. Irrespective of the different treatments made, similar patterns in *Hpa* development were observed.

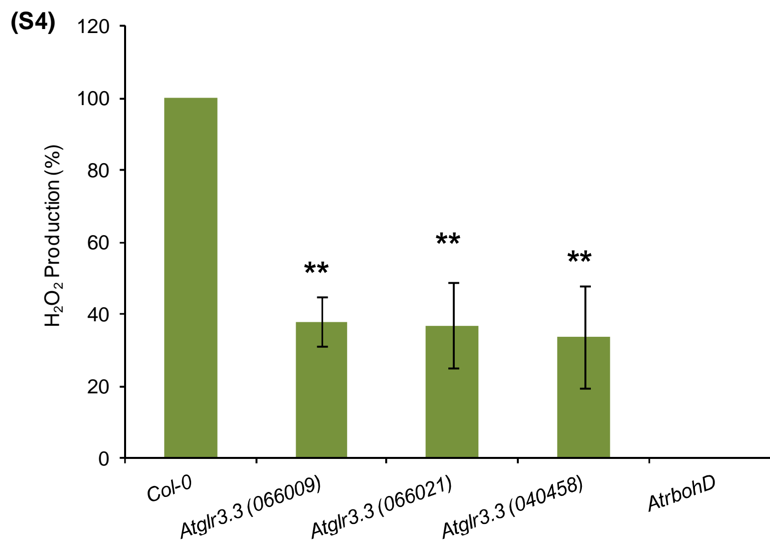


Figure S4.4. OGs-induced ROS (H_2O_2) generation in different SALK lines of *Atglr3.3* and in *AtrbohD* mutant. ROS production is expressed as a percentage of the maximal peak response observed after OGs elicitation in *Atglr3.3* and in *AtrbohD* mutant lines compared to Col-0 (100 %). Each bar is the average ± SD of at least 3 independent experiments.

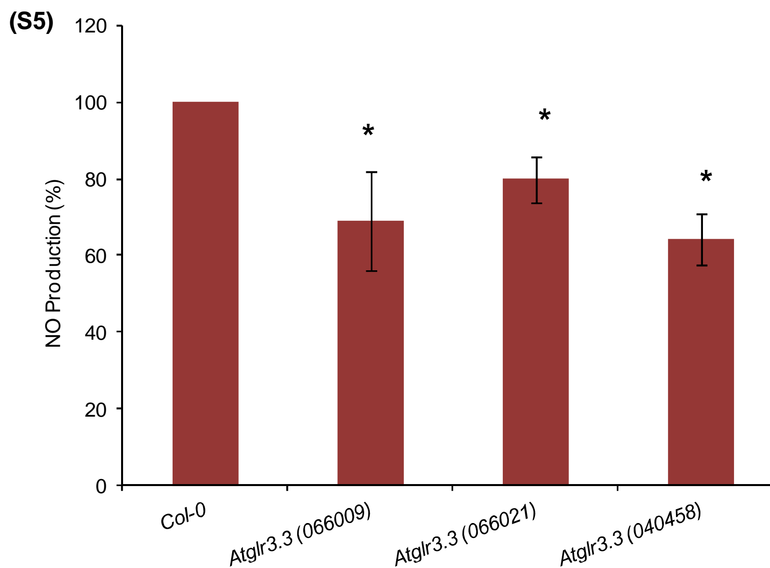


Figure S4.5. NO production induced by OGs in different *Atglr3.3* mutant SALK lines. Bar graph represents the OGs-induced NO production expressed as the percentage of fluorescence at 16 h post-treatment in *Atglr3.3* mutant lines compared to response in Col-0 (100 %). Data are mean ± SD (n=3).

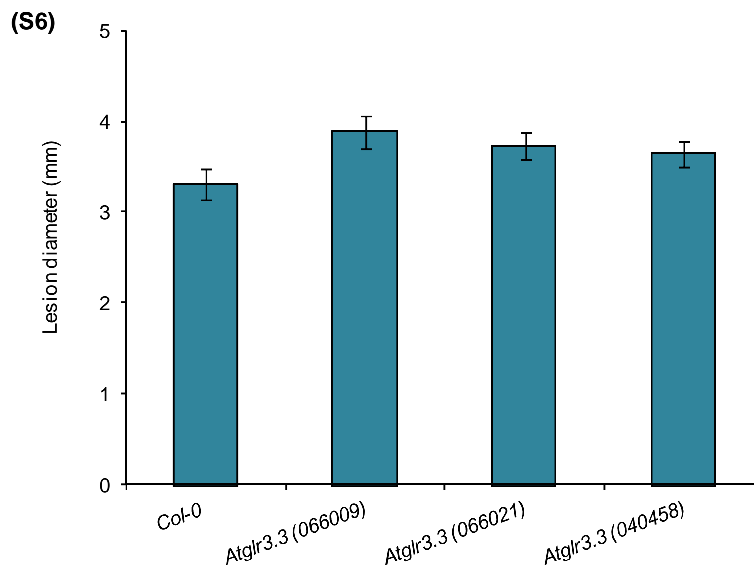


Figure S4.6. ***B. cinerea*** **infection assay in different** ***Atglr3.3*** **mutant SALK lines.** Pathogen inoculation was made as described in figure 2a. Histogram indicates the means ± SE of the lesion diameter of pooled data from 3 independent experiments.

In figures S4.4-S4.6, statistical differences were determined by one-way ANOVA, followed by Dunnett's test using the statistical software SigmaPlot. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table S4.1: List of T-DNA mutant lines of *Atglrs* and other genes used in this study.

Gene ID	Mutant name	Description	Mutant lines	Seed Obtained
At2g17260	*Atglr3.1*	Mutant impaired in the gene encoding a putative ligand-gated ion channel 3.1	SALK_063873C	NASC
At4g35290	*Atglr3.2*	Mutant impaired in the gene encoding a putative ligand-gated ion channel 3.2	SALK_150710	NASC
At1g42540	*Atglr3.3*	Mutant impaired in the gene encoding a putative ligand-gated ion channel 3.3	SALK_066021	NASC
			SALK_066009	Dr. E.P. Spalding (University of Wisconsin, USA)
			SALK_040458	Dr. E.P. Spalding (University of Wisconsin, USA)
At1g05200	*Atglr3.4*	Mutant impaired in the gene encoding a putative ligand-gated ion channel 3.4	SALK_079842	NASC
At2g32390	*Atglr3.5*	Mutant impaired in the gene encoding a putative ligand-gated ion channel 3.5	SALK_035264C	NASC
At3g51480	*Atglr3.6*	Mutant impaired in the gene encoding a putative ligand-gated ion channel 3.6	SALK_091801C	NASC
At2g32400	*Atglr3.7*	Mutant impaired in the gene encoding a putative ligand-gated ion channel 3.7	SALK_022757	NASC
At5g47910	*AtrbohD*	Mutant impaired in the gene encoding NADPH/respiratory burst oxidase protein D (RbohD).		Dr. Torres (University of North Carolina, USA).
	Arabidopsis WT plants expressing aequorin in the cytosol			Dr. M. Knight (Durham, UK)

Table S4.2: List of primers used for GLRs T-DNA mutants genotyping in *Arabidopsis.*

Accession No.	Gene name	Mutant lines	Primers sequence (5' =====> 3')	Annealing Temp. (℃)
At2g17260	*AtGLR3.1*	SALK_119230C	**LP**: CACTTGGTCGTATGGTGCTTCTGA	57
			RP: GTCTTTGCAGAAGTCGCGGATT	
At4g35290	*AtGLR3.2*	SALK_150710	**LP**: TTTTGGATCCAGCATTAGTCG	52
			RP: TTTTGCGGTTTTGTTTGTAGG	
At1g42540	*AtGLR3.3*	SALK_066021	**LP**: GAAGCACCAGACATCTTACGC	56
			RP: TGAAGCAACTCTGGACTTTCTTC	
At1g05200	*AtGLR3.4*	SALK_079842	**LP**: GGGTTAATCCGGCTTATGAAG	56
			RP: GAAGTGAGACTGGCCGTGTAG	
At2g32390	*AtGLR3.5*	SALK_035264C	**LP**: TGAAGTTGCTGCAAATGTGAG	54
			RP: TGTCGACATGTCCACAGCTAG	
At3g51480	*AtGLR3.6*	SALK_091801C	**LP**: TTCGTTCAAAGGTGGCATAAC	54
			RP: CGACTATGAGGAAAGACGCAG	
At2g32400	*AtGLR3.7*	SALK_022757	**LP**: TCTTCTGTCCGGATGAGTTTG	54
			RP: CGAAGAAAGAAGGGAAATTGG	

Primers were designed by SIGnal T-DNA Express (http://signal.salk.edu/tdnaprimers.2.html) or by AmplifX 1.1 software, http://ifrjr.nord.univ-mrs.fr/AmplifX).

Table S4.3: List of gene-specific primers used for RT-qPCR analysis.

Accession No.	Gene name	Primer sequence (5' =====> 3')	Annealing Temp. (℃)
At5g47910	*RBOHD*	**LP**: GACGATGAGTACGTGGAG	53
		RP: AAAACTTGGCAGAGAGTAAG	
At1g74710	*ICS1*	**LP**: GGGATAAGGGGTTCTCAC	53
		RP: AACAATCATAACAGCTAGGC	
At1g14540	*PER4*	**LP**: CACTGGTTCAGATGGACAAA	53
		RP: AACAAACGAATTATCGCTGC	
At2g14610	*PR1*	**LP**: CACTACACTCAAGTTGTTTGG	53
		RP: TGATAAATATTGATACATCCTGC	
At4g05320	*UBQ10*	**LP**: GAGATAACAGGAACGGAAACATAG	53
		RP: GGCCTTGTATAATCCCTGATG	

Gene-specific primers corresponding to different genes of defense signaling pathway were selected from CATMA (http://www.catma.org/database/simple.html).

ANNEXES

ANNEX 1

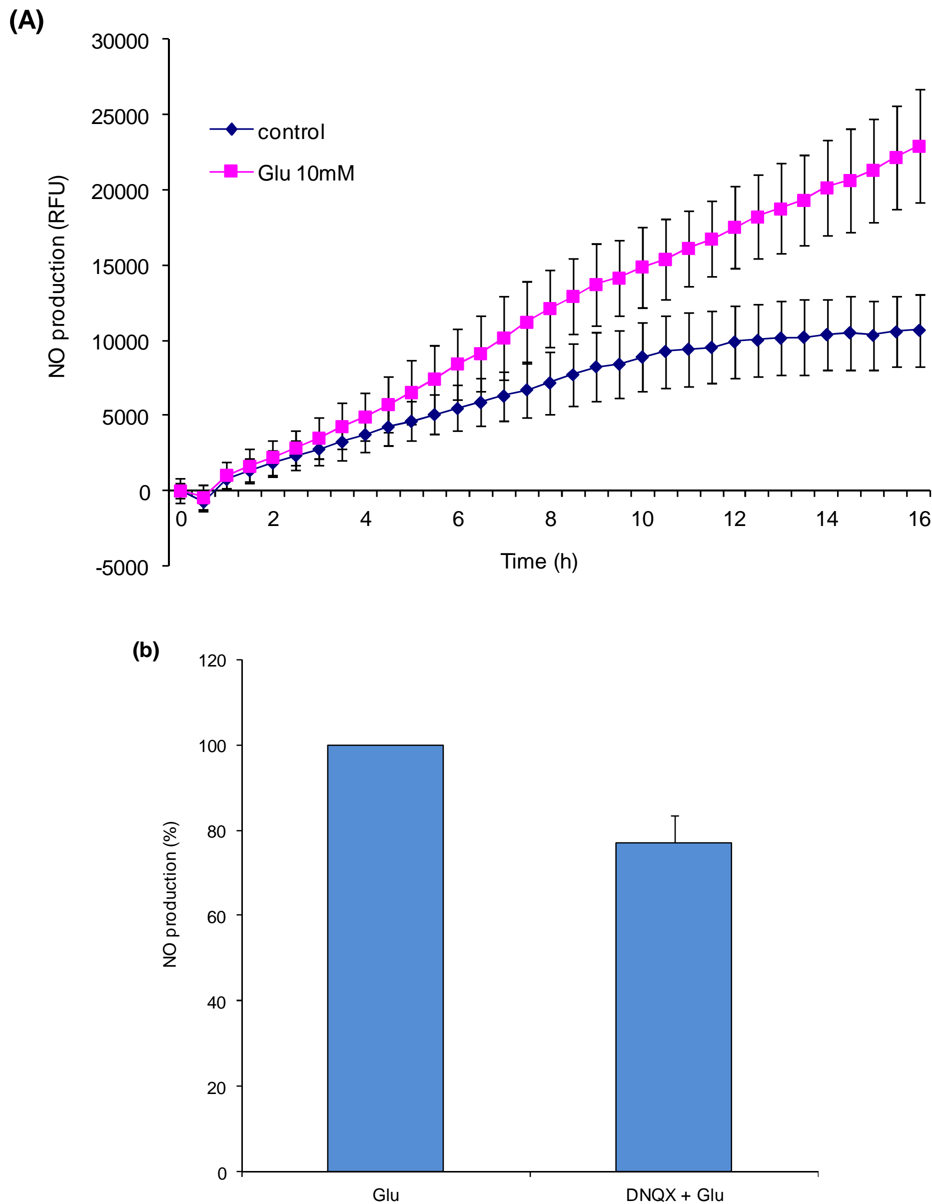


Figure 1: Glu-induced NO production in *Arabidopsis* leaf disks. A) NO production was monitored by measuring DAF-2T fluorescence in the absence (control) or presence of Glu (10 mM). Each point represents the mean of 8 parallel measurements ± SD. Curves are 1 representative experiment out of 3. **B)** Glu-induced NO production determined at 16 h of treatment in presence or not of DNQX, expressed as percentage of the response obtained in Glu treatment (100 %) after subtracting background fluorescence of corresponding control. Data are mean ± SD (n=3). DNQX treatment was made 1 h prior to Glu treatment.

Short conclusion: These results showed that Glu is able to induce NO production in *Arabidopsis* **(1A)** that was partially inhibited (about 25 %) by DNQX treatments **(1B).**

ANNEX 2

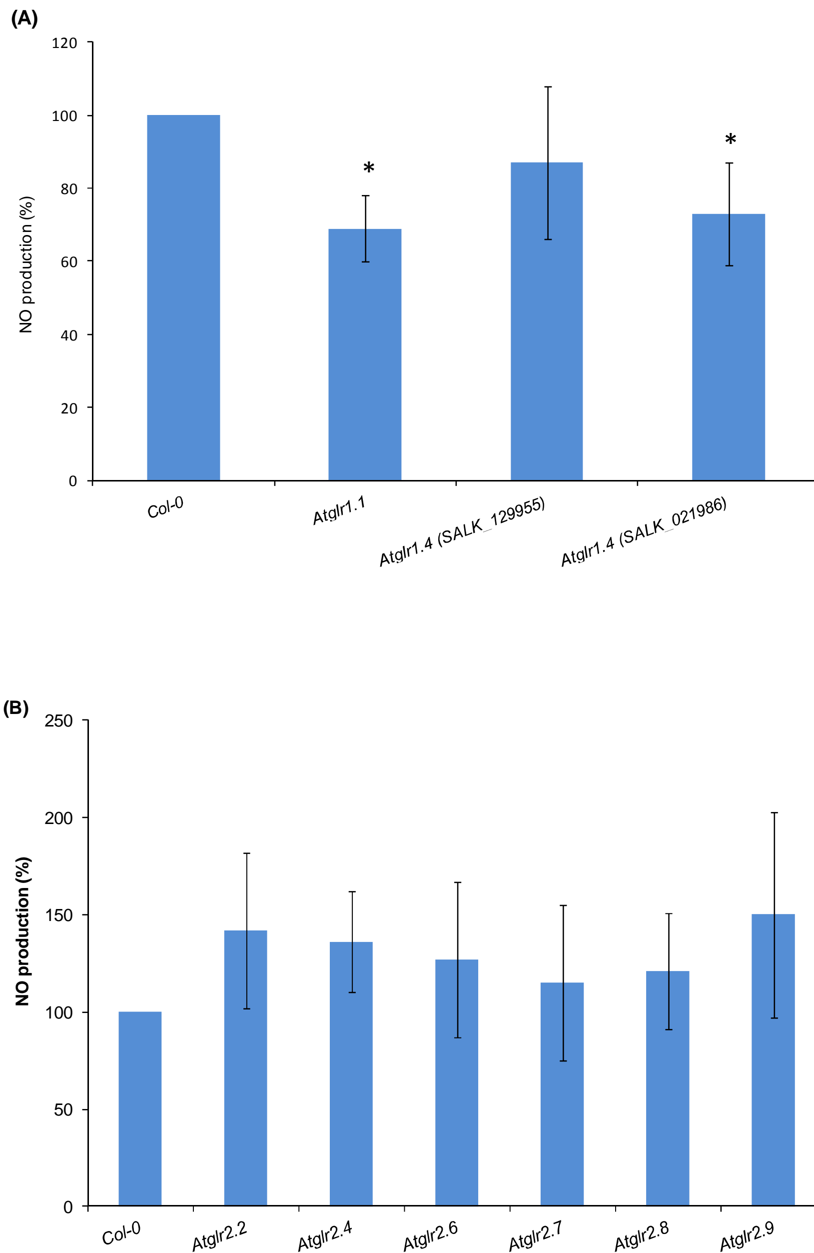


Figure 2: NO production in AtGLRs mutants. OGs-induced NO production determined at 16 h of OGs treatment in GLRs mutants of clade 1 **(A)** and 2 **(B)**, expressed as percentage of the response obtained in OGs treatment (100 %) after subtracting background fluorescence of corresponding control. Data are mean ± SD (n=3).

Short conclusion: Above results indicate that *Atglr1*.1 and *Atglr1*.4 mutants from clade 1 showed a partial but significant decrease in OGs-induced NO production as compared to Col-0 **(2A).** In clade 2, although most of the mutants showed increased level of NO production in comparison to Col-0 yet these differences were not significant on statistical basis **(2B).**

ANNEX 3

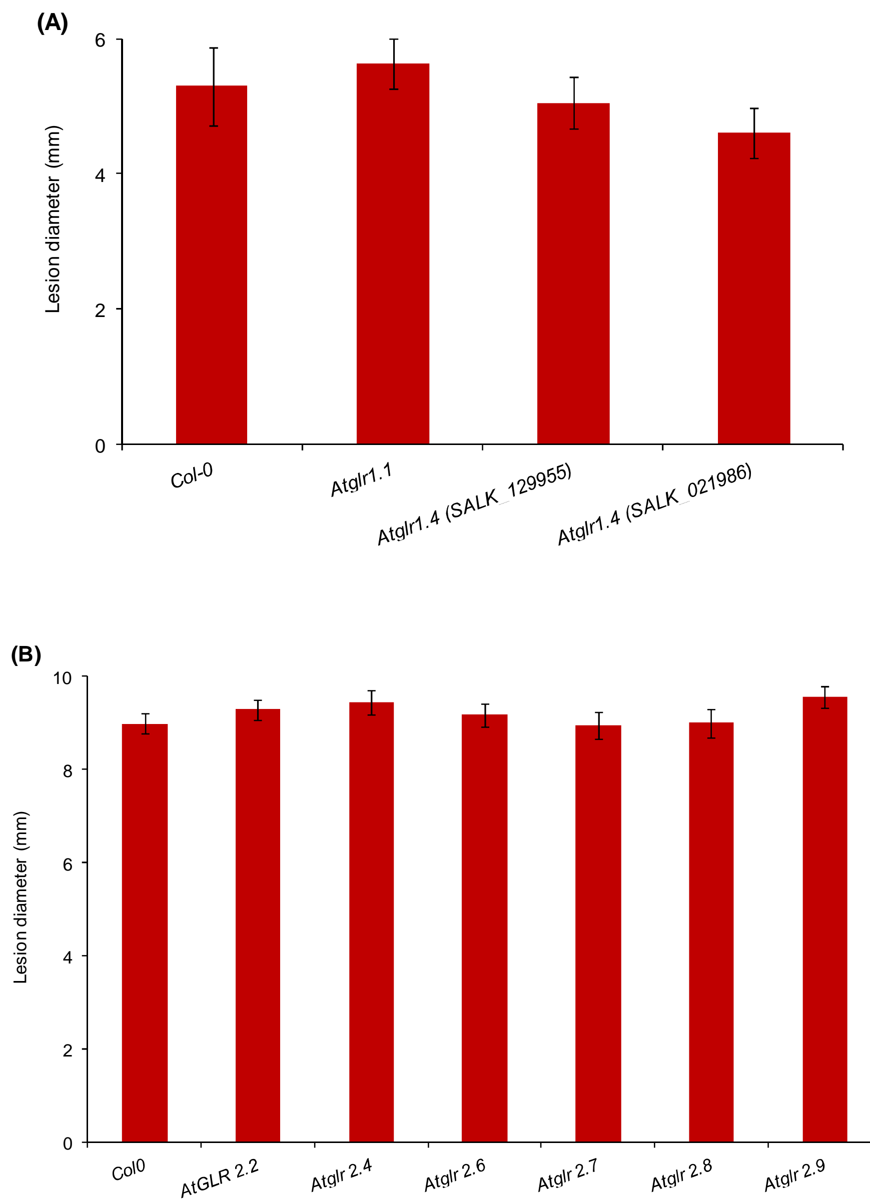


Figure 3: Analysis of basal resistance to *B. cinerea* in GLRs mutants. A) Col-0 and GLR mutants from Clade 1 **(A)** and 2 **(B)** were inoculated with *B. cinerea* spores (5.10^{4} spores.mL^{-1}) and disease symptoms were measured 72 h post-inoculation. Bar graph represents the lesion diameter (mean ± SE) of pooled data of 3 different experiments. Each individual experiment was performed on 12 plants for each treatment (4-6 inoculations per plant).

Short conclusion: When infected with necrotrophic fungal pathogen, *Botrytis cinerea*, the investigated GLRs mutants from clade 1 and 2 showed no differences in susceptibility as compared to Col-0 plants.

ANNEX 4

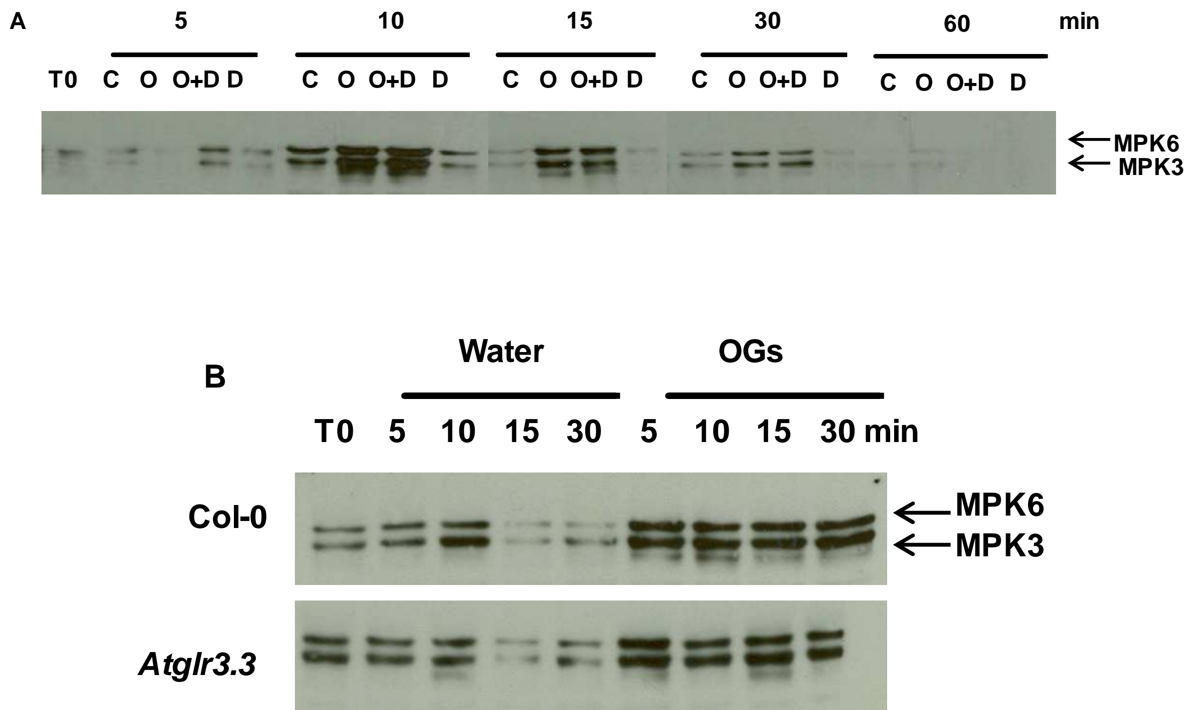


Figure 4: OGs-induced phosphorylation of MAPKs in *Arabidopsis*. A) Effect of DNQX on OGs-induced phosphorylation of MAPKs in Col-0 plants. Leaf discs were taken after different time intervals (T0, 5, 10, 15 and 30 min). Phosphorylated MAPK were immunodetected from total protein extract using specific antibodies. Leaf disks were infiltrated in the absence (control) or presence of OGs (2.5 $mg.mL^{-1}$) with or without inhibitor (DNQX, 500 µM). **B)** Phosphorylation of MAPKs in Col-0 and *Atglr3.3* mutants (SALK_066021). Leaf disks were infiltrated in the absence (control) or presence of OGs (2.5 $mg.mL^{-1}$). Both **A)** and **B)** are 1 representative experiment out of 3. **C**: Control, **O**: OGs, **O+D**: OGs + DNQX, **D**: DNQX.

Short conclusion: These results showed that both the GLRs inhibitor (DNQX) and mutation in *AtGLR3.3* did not lead to any decrease in OGs-induced activation of MPK3 and 6, thus suggesting that GLRs may not be involved in OGs-induced MAPKs activation.

CHAPTER 5

CHAPTER 5

“Glutamate receptor regulated gene expression in Glu- and OGs- treated plant tissues”

CHAPTER 5

GLUTAMATE RECEPTOR REGULATED GENE EXPRESSION IN GLU- AND OGS-TREATED PLANT TISSUES

The functional analyses of genes provide us information about their diverse role in living organisms. Several conventional and advanced techniques to study the expression patterns of genes are available these days. The techniques generally used to study the gene expression at transcriptional level include northern hybridization, serial analysis of gene expression (SAGE), differential display, dot-blot analysis and RT-qPCR (Alwine *et al.*, 1977; Lennon and Lehrach, 1991; Liang and Pardee, 1992; Yasui *et al.*, 2004; Hu and Polyak, 2006; Vanguilder *et al.*, 2008). However, using these gene expression techniques, it is not possible to study a large number of genes at a time. Microarray technology has empowered the scientific community to analyze the expression of thousands of genes in a single reaction and short time in an efficient manner. These reactions are highly sensitive to detect a change in the expression level by using smaller amounts of starting materials and with the advantage of fewer repetitions. With microarray data, it is possible to detect the significant differences at transcription levels, which is generally more than two-fold, of many genes between different samples/treatments. On the basis of the observed changes in the levels of accumulation of mRNA, microarray is a simple and quick method for determining the candidate genes for further analysis. Microarray has provided us a unique opportunity to identify candidate genes with important roles in defined physiological processes (Schena *et al.*, 1995). Conversely, based only on microarray data, it is difficult to claim that all the observed differences are authentic. Proper normalization of the data, use of proper controls and housekeeping genes showing constant expression levels are helpful to resolve this issue (Smyth and Speed, 2003). Microarray data, therefore, should be regarded as primary screening method and the use of northern hybridization or RT-qPCR for re-examining the transcription levels of selected genes is recommended.

1. What is microarray?

Microarray is a tool used to analyze the information contained within a genome. It consists of different nucleic acid probes orderly arranged on a solid substrate *i.e.* a microchip, a glass slide or a microsphere-sized bead. It is a 2D array that assays large amounts of biological material using high-throughput screening. Microarrays can differ in fabrication,

workings, accuracy, efficiency, and cost. Additional factors for microarray experiments are the experimental design and the methods of analyzing the data.

1.1. Types of Microarrays

There exist different criteria to classify microarrays into various categories. They can be classified based on the kinds of the samples used *e.g.* DNA microarrays, Protein microarrays, Antibody microarrays and Carbohydrate arrays (glycol-arrays) *etc.* These can be differentiated from each other with respect to the kind of immobilized DNA used to generate the array and, ultimately, the kind of information that is derived from the chip *e.g.* microarray for expression analysis, microarray for mutation and microarray for comparative genomic hybridization. In case of microarray for expression analysis, the cDNA (complementary DNA) derived from the mRNA of known genes is immobilized and sample contains genes from both healthy as well as the diseased tissues. On the other hand, gDNA (genomic DNA) is used in microarray for mutation analysis. The investigated genes might differ from each other by as less as a single nucleotide base. Finally, for comparative genomic hybridization, investigations are carried out to identify the increase or decrease of the important chromosomal fragments harboring genes involved in a disease.

There are three basic types of samples that can be used to construct DNA microarrays, two are genomic and the third one is **"transcriptomic",** that is, it measures mRNA levels. In a DNA microarray, thousands of spots are arrayed in orderly rows and columns on a glass surface and each DNA spot contains a unique DNA sequence (normally of 10^{-12} moles) and is called probe. The spots themselves can be DNA, cDNA, or oligonucleotides (Van Hal *et al.*, 2000). Each probe contains multiple identical strands of DNA and represents one gene. For data analysis, precise location and sequence of each spot is recorded in a computer database. DNA microarrays can be used to measure changes in expression levels, to detect single nucleotide polymorphisms (SNPs), or to genotype/resequence mutant genomes. DNA arrays are different from other types of microarray only in that they either measure DNA or use DNA as part of its detection system.

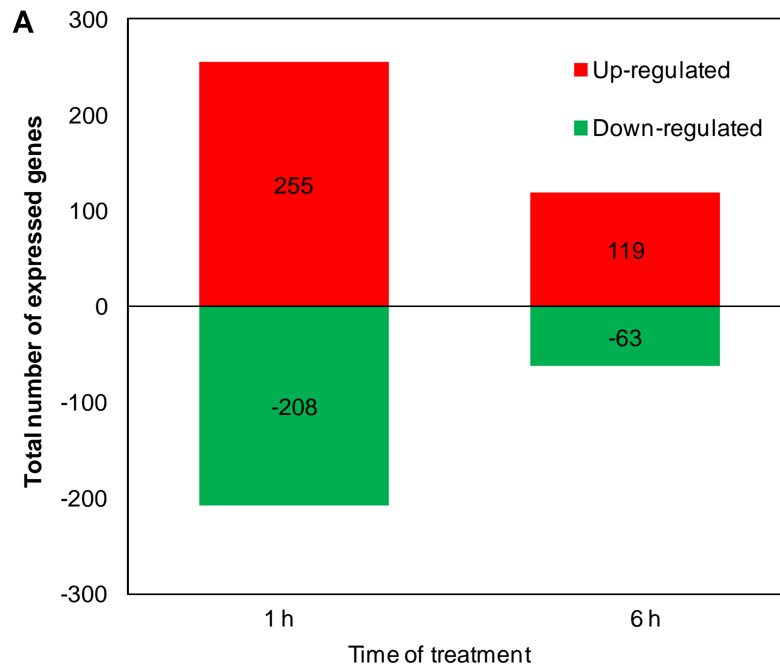
1.2. Principle

In standard microarrays, the probes are attached via surface engineering to a solid surface by a covalent bond with a chemical matrix (via epoxy-silane, amino-silane, lysine, polyacrylamide or others).

The core principle behind microarrays is hybridization between two DNA strands, a property of complementary nucleic acid sequences to specifically pair with each other through hydrogen bonding. A high number of complementary base pairs in a nucleotide sequence mean tighter non-covalent bonding between the two strands. After washing off of non-specific bonding sequences, only strongly paired strands will remain hybridized. So, fluorescently labeled target sequences that bind to a probe sequence would generate a signal depending upon the strength of the hybridization determined by the number of paired bases, the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal depends upon the amount of target sample binding to the probes present on that spot. These data are then stored in a computer, and a special program is used either to calculate the red-to-green fluorescence ratio or to subtract out background data for each microarray spot by analyzing the digital image of the array. If calculating ratios, the program then creates a table that contains the ratios of the intensity of red-to-green fluorescence for every spot on the array. Each spot on an array is associated with a particular gene. Depending on the type of array used, the location and intensity of a color will tell whether the gene, or mutation, is present in either the control and/or sample DNA. It will also provide an estimate of the expression level of the gene (s) in the sample and control DNA.

1.3. Applications

Microarrays are a significant breakthrough both because they may analyze very large number of genes and because of their small size. They help in the identification, functioning and expression level studies of new genes within a single sample or in two different cell types or tissue samples, such as in control and treated tissues or in healthy and diseased tissue. They have extensive applications in pharmacogenomics. Since, they can be used to examine the expression of hundreds or thousands of genes at once, they have revolutionize the way to examine gene expression in short period of time. Ultimately, they can be used to study the size of existing gene families, to reveal new patterns of coordinated gene expression across gene families and to uncover entirely new categories of genes. This technology will help to study the integration of gene expression and function at the cellular level, revealing how multiple gene products work together to produce physical and chemical responses to both static and changing cellular needs.



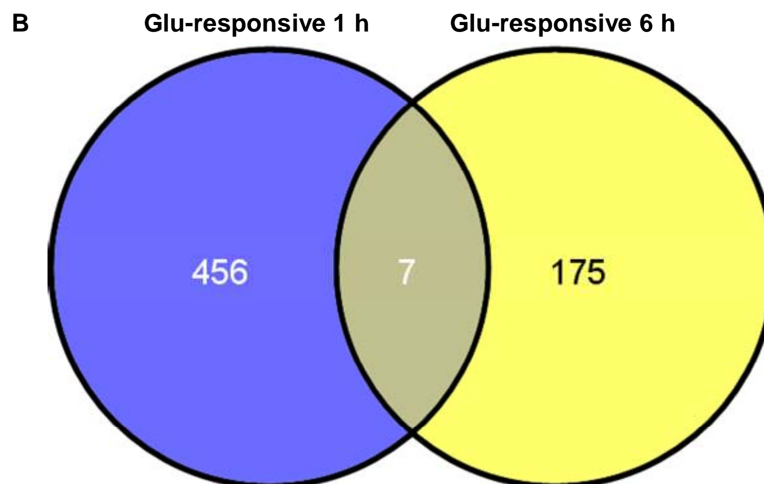


Figure 5.1: Glutamate (Glu)-responsive genes in *Arabidopsis thaliana* (ecotype Col-0). A) Differential expression of *Arabidopsis thaliana* genes in response to Glu. Up- and down-regulated genes are represented in red and green color bars, respectively. **B)** Overlap of significantly expressed genes after Glu treatment. Venn diagram of overlapped and non-overlapped *Arabidopsis* genes at different time points after Glu treatment. Leaves were infiltrated with Glu (10 mM) or water (control). 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. Messenger RNA was subjected to transcriptomic analysis (NimbleGen array). Three independent biological replicates were carried out.

2. Transcriptomic analysis to identify the modulated genes in response to Glu and OGs treatments in *Arabidopsis*

2.1. Background Context

Ca^{2+} serves as a second messenger for numerous signals and confers specific cellular responses in locally defined signaling events. Many reports have demonstrate the presence of Ca^{2+} transporting proteins activity in plant cells (McAinsh and Pittman, 2009; Dodd *et al.*, 2010). In plants, decoding of Ca^{2+} signatures is accomplished by Ca^{2+} binding proteins that function as Ca^{2+} sensors (Day *et al.*, 2002; Boonburapong and Buaboocha, 2007). These Ca^{2+} binding proteins are thought to sense changes in diverse subcellular compartments and regulate downstream signaling events, ultimately eliciting physiological responses which are specific for each signal. The key role of calcium in the signaling pathway has received particular attention in the area of plant defense against pathogens (Nürnberger and Scheel, 2001). Plant cells respond to environmental and developmental stimuli by a change in their free cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) and stimulus-induced changes in plant $[Ca^{2+}]_{cyt}$ are observed in many different cell types in response to pathogens/elicitors (Lecourieux *et al.*, 2006) bacterial and fungal signals (Ehrhardt *et al.*, 1996; Kosuta *et al.*, 2008). The elicitor-induced $[Ca^{2+}]_{cyt}$ variations result in the induction of a signaling pathway that ultimately leads to plant defense.

Previous studies have illustrated a strong link between Ca^{2+} and OGs-induced signal transduction (Chandra and Low, 1997; Navazio *et al.*, 2002). There are ample evidences from the literature that OGs invoked a Ca^{2+} influx and characteristic $[Ca^{2+}]_{cyt}$ elevations in different plant species (Lecourieux *et al.*, 2002; Navazio *et al.*, 2002; Hu *et al.*, 2004; Romani *et al.*, 2004; Aslam *et al.*, 2009). In fact, OGs perception by its plasma membrane receptor (WAK1) initiates a signal transduction cascade that activates sophisticated multilevel defense responses in plants including variation in $[Ca^{2+}]_{cyt}$, ROS and NO production, activation of MAPK, membrane polarization, defense genes transcripts accumulation and phytoalexin production (Hu *et al.*, 2004; Galletti *et al.*, 2008, 2011; Rasul *et al.*, 2012). Activation of defense-related genes is one of the important components of this signal transduction process. It has been demonstrated that Ca^{2+} elicitation to *Arabidopsis* seedlings regulate the expression of about 1000 genes (Kaplan *et al.*, 2006). A large number of downstream genes are regulated in multiple Ca^{2+}-signaling pathways in plants. Pretreatment of cells with Ca^{2+} channel blocker, La^{3+}, completely abolished the Ca^{2+} variation transiently induced by OGs (Moscatiello *et al.*, 2006) and transcripts accumulation of a large number of OGs-induced genes were

Table 5.1: List of common genes at 1 h and 6 h of Glu treatment in *Arabidopsis thaliana.*

Gene ID	SYMBOLS	DESCRIPTION	Fold change 1 h Glu	Fold change 6 h Glu
AT4G29030.1		Glycine-rich protein	4.69017198	-6.58384659
AT3G08500.1	MYB83, AtMYB83	MYB83 (myb domain protein 83); DNA binding / transcription factor	3.76986365	-4.66456968
AT3G48850.1		Mitochondrial phosphate transporter, putative	3.11307585	5.98058238
AT1G50060.1		Pathogenesis-related protein, putative	2.82102779	3.34337268
AT2G13431.1		Other RNA	-5.03975705	-3.52868666
AT5G44345.1		F-box family protein-related	-7.03556508	3.76576768
AT1G75717.1		Unknown protein	-11.678918	2.91020943

significantly modulated after La^{3+} pretreatment. This further suggests a very strong implication of Ca^{2+} in OGs signaling pathway.

Plasma membrane Ca^{2+}-permeable channels and transporters play a central role in transporting extracellular Ca^{2+} into the cell and ionotropic glutamate receptors homologs (GLRs) are among the potential candidates for such channels that underlie Ca^{2+} influx (Lacombe *et al.*, 2001). We also have the information that Glu, as a signaling molecule, can activate ionotropic type of glutamate receptors (iGluRs) in animals and in plants. Glu is able to induce a very quick and significant change in $[Ca^{2+}]$ and Glu-induced $[Ca^{2+}]_{cyt}$ variations were strongly suppressed by DNQX and CNQX treatments (Dennison and Spalding, 2000; Dubos *et al.*, 2003; Meyerhoff *et al.*, 2005; Vatsa *et al.*, 2011).

Based on this information, we were interested in analyzing the Ca^{2+}-regulated genes dependent on GLRs activations. Therefore, a whole genome transcript analysis was performed with OGs-treated *Arabidopsis* (Col-0) plants leaves in the presence of DNQX, and in parallel in the response to Glu treatment. Complete details of the experimental design and procedure are present in transcriptome analysis section of materials and methods. In order to exclude non significant genes, data was normalized and statistical analysis was performed (P value $\leq$ 0.01) and genes only with a fold-change ≥ 2 were considered as significant. These analyses yield different lists of modulated genes in response to different treatments. Further comparisons of the transcriptome response between control and treated plants were made using the program developed in Excel by FiRE (Beckers and Conrath, 2006; Garcion *et al.*, 2006). From these comparisons, lists of Glu-dependent genes, OGs-responsive genes and GLRs-dependent genes were obtained. As the lists of these differentially expressed genes were very long, so it was difficult to manually interpret the involvement of these genes into different physiological pathways. By using different bioinformatics tools (GO annotation, MapMan), we were able to study in detail the role of these genes in different cellular, biological and physiological processes. Transcriptomic analyses, to investigate the genes that are involved in OGs signaling, have already been performed in several laboratories (Moscatiello *et al.*, 2006; Ferrari *et al.*, 2007). These studies have indicated the differential expression of many genes belonging to different physiological pathways. These genes are putatively involved not only in defense and stress responses but also in other important processes like cellular transport, signal transduction, metabolism, and photosynthesis. Many of those identified genes belong to different families of transcription factors (TFs). Taking advantages of these previous studies, comparison was also made between the genes identified in our study and previous studies. This led us to identify the genes that were commonly

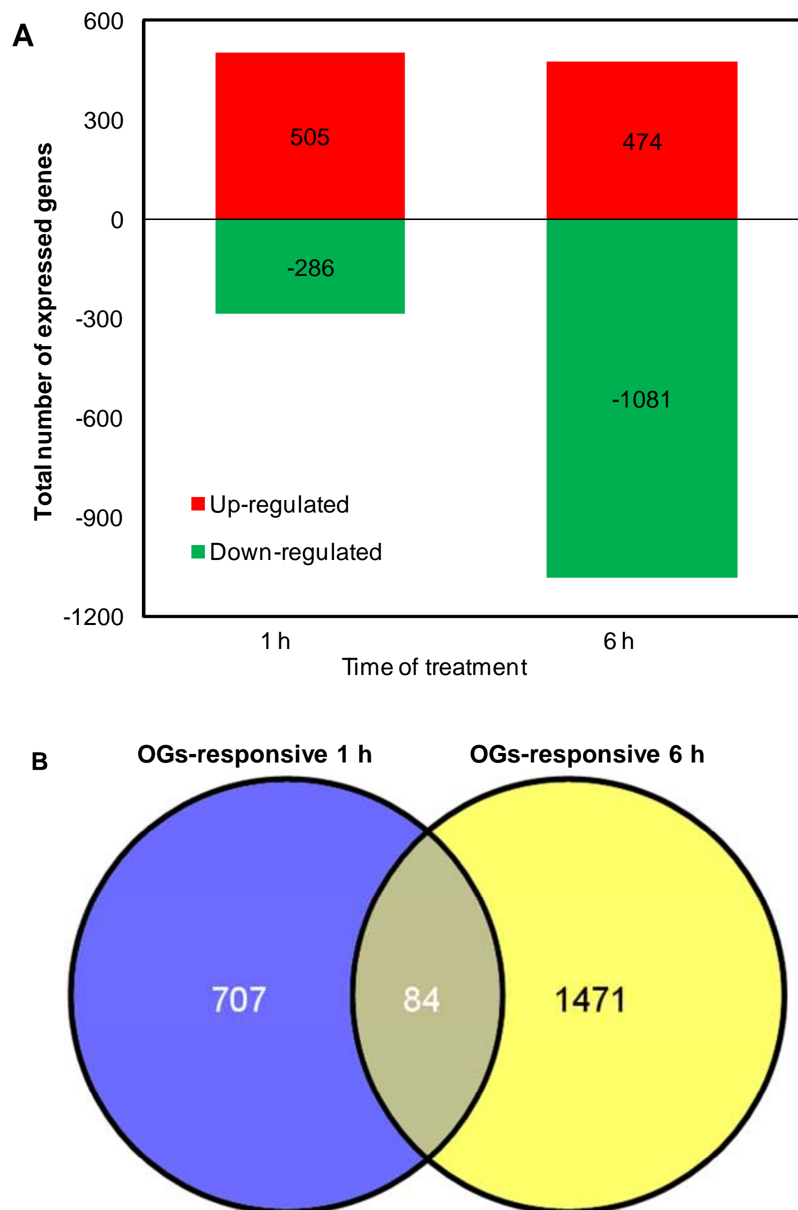


Figure 5.2: Oligogalacturonides (OGs)-responsive genes in *Arabidopsis thaliana* (Col-0). A) Differential expression of *Arabidopsis thaliana* genes in response to OGs. Up- and down-regulated genes are represented in red and green, respectively. **B)** Overlap of significant expressed genes after OGs treatment. Venn diagram of overlapped and non-overlapped genes after OGs treatment at different time points in Col-0 plants. Leaves were infiltrated with OGs (2.5 $mg.mL^{-1}$) or DMSO (control). Messenger RNA was subjected to transcriptomic analysis (NimbleGen array). 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. Three independent biological replicates were carried out.

expressed in different studies. In our investigations, we mainly focused on the genes related to stresses responses, especially biotic stresses, signaling pathways, and different TFs families that are important in defense responses. For further analysis, we have selected 10 genes belonging to above mentioned categories. In order to validate the expression of these selected genes at transcriptional levels, RT-qPCR analyses were performed to study their expression pattern. In future, mutant lines of these selected genes would be investigated for functional analyses especially related to plant resistance against pathogens (biotrophic and necrotrophic) to better understand their role in plant defense mechanisms during plant pathogen interaction.

In the following sections, we will explain the main features of our transcriptomic data. At the same time, we will try to further explore the genes modulated by Glu, OGs and GLRs by dividing these differentially expressed genes into different functional categories with the aid of bioinformatics tools (GO annotation, MapMan).

2.2. Glu-responsive genes

In order to identify the genes that are modulated by Glu, a comparison of the transcriptome between water (control) and Glu-treated plants (water vs Glu) was made using the program developed in Excel by FiRE (Beckers and Conrath, 2006; Garcion *et al.*, 2006). The genes whose expression was specifically modulated in response to Glu were termed as "Glu-responsive genes". At 1 h and 6 h after Glu treatment, a total of 645 genes were significantly modulated (fold-change ≥ 2; P value ≤ 0.01) with 374 up-regulated and 271 down-regulated genes. Out of 463 genes that specifically change their expression at 1 h, 255 genes were up-regulated while 208 were down-regulated. Similarly, from 182 genes that specifically change their expression at 6 h, 119 and 63 genes showed an up- and down-regulation of their expression, respectively (Figure 5.1A; list of genes in Supplemental Table S1 and S2). Moreover, 7 genes were found common at both time points, with 4 up-regulated and 3 down-regulated genes (List of genes in Table 5.1). Venn diagram of Glu-responsive genes at both times kinetics is shown in figure 5.1B.

2.3. OGs-responsive genes

In order to sort out the genes modulated by OGs treatment, a comparison was made between the transcriptome date obtained after DMSO (control, DMSO is the DNQX solvent) and OGs+DMSO treatment (DMSO vs OGs + DMSO) at 1 h and 6 h. "OGs-responsive genes" represent the genes uniquely expressed after OGs + DMSO treatment. A total of 2346

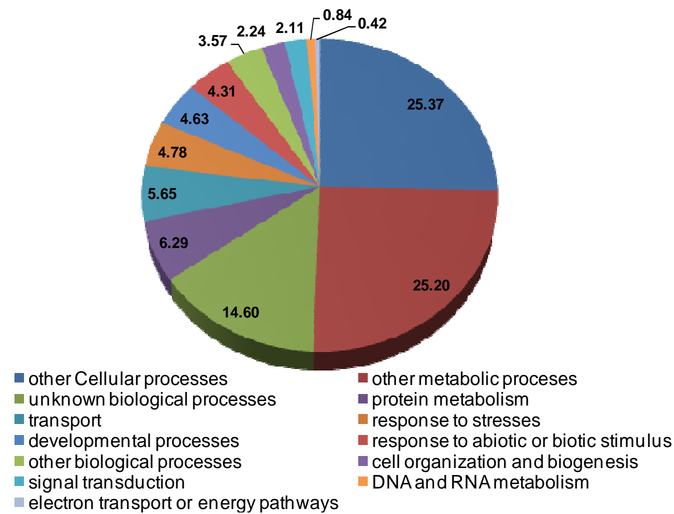


Figure 5.3: Distribution of *Arabidopsis thaliana* whole genome into putative functional classes assigned through Gene Ontology (GO). Pie chart represents the biological process classified into different functional classes. The percentages assigned to different classes indicate the abundance of each category within the whole dataset. Assignments are based on the data available at The *Arabidopsis* Information Resource (TAIR) and from the GO Annotation Database.

genes were specifically modulated at 1 h and 6 h of OGs treatment compared to control (fold-change ≥ 2; P value ≤ 0.01). Out of 791 genes (List of genes in Supplemental Table S3) that change their expression at 1 h, 505 genes were up-regulated while 286 were down-regulated. Similarly, from 1555 genes (List of genes in Supplemental Table S4) that change their expression at 6 h, 474 genes showed an up-regulation of expression and 1081 were down-regulated (Figure 5.2A). Our results showed that 6 h time point was more effective for OGs response as about $^{2}/_{3}$ of the total OGs-modulated genes expressed at 6 h were modified but at the same time the ratio of up-regulated to down-regulated genes was significantly lower at this time compared to the 1 h time point. Moreover, 84 genes were found to be common in both 1 h and 6 h OGs-treatments with 50 up-regulated and 34 down-regulated genes (List of genes in Supplemental Table S5). Venn diagram of the common and specific genes after OGs treatment at 1 h and 6 h is shown in figure 5.2B.

2.4. GO annotation of Glu- and OGs-modulated genes in *Arabidopsis thaliana*

From our transcriptomic data, genes with modified expression in response to different treatments were analyzed by GO annotation. GO annotation is web-based software which distributes genes into different functional categories (biological processes, molecular functions and cellular components) according to gene ontology (GO) classifications. We were particularly interested in 4 functional categories representative of the expected cellular changes: changes in the percentage of the Glu and OGs responsive genes within these categories are presented in table 5.2 Following is the detail of functional annotation of Glu-dependent genes and OGs-responsive genes.

2.4.1. Glu-responsive genes

In silico functional annotation of Glu-responsive genes was performed by GO annotation. Pie chart of Glu-dependent genes implicated in biological processes is presented in figure 5.4A.

In biological functions classification, out of total modulated genes at 1 h of Glu treatment, 1.68 % genes belong to signal transduction, 6.57 % genes are related to transport, 3.22 % and 2.66 % correspond to stresses and response to (a)biotic stimulus, respectively (Figure 5.4A; Table 5.2). At 6 h of Glu treatment, 9.24 % and 8.28 % of Glu-responsive genes are related to stresses and response to (a)biotic stimulus, respectively. This ratio is approximately two fold higher than the stress responsive genes (4.78 %) and genes in response to (a)biotic stimulus (4.31 %) in the whole *Arabidopsis* genome (Figure 5.3).

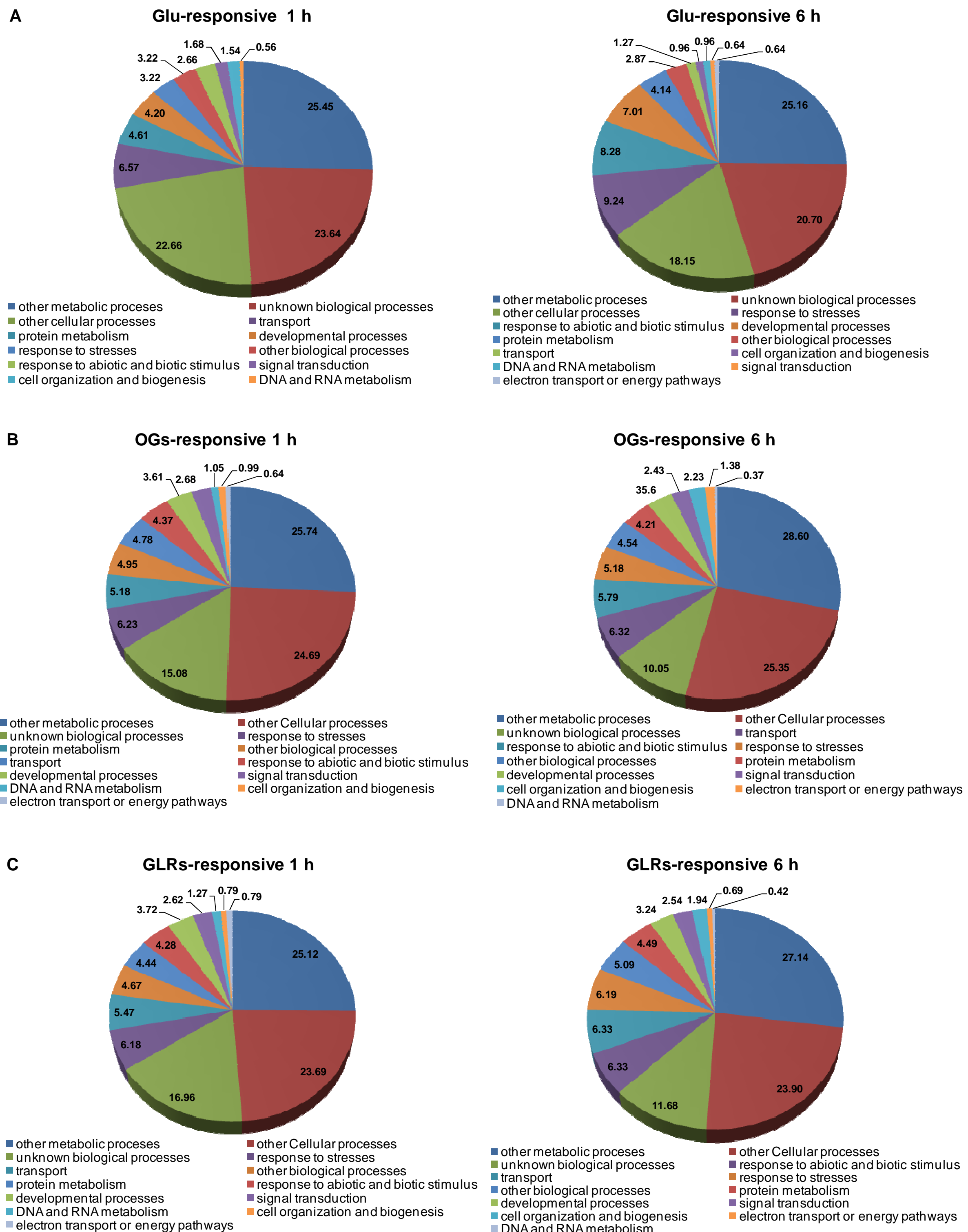


Figure 5.4: GO annotation of biological process of Glu-responsive, OGs-responsive and GLRs-responsive genes in *Arabidopsis thaliana*. Pie charts indicate the distribution of **A)** Glu-responsive **B)** OGs-responsive and **C)** GLRs-responsive genes into different functional categories at 1 h and 6 h. 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 (GO) Annotation Database.

However, 0.64 % and 1.27% Glu-responsive genes correspond to signal transduction and transport, respectively. This percentage is comparatively lower not only to 1 h Glu treatment but also from whole genome of *Arabidopsis* where 2.11 % and 5.65 % genes are related to signal transduction and transport, respectively (Figure 5.4A; Figure 5.3; Table 5.2). Interestingly, the higher percentage of transport genes in 1 h Glu treatment potentially indicate that Glu is actively transported and compensated through activities raised within the same time period. Moreover, higher percentage of signal transduction genes activation at 1 h as compared to 6 h Glu treatments suggests that signal transduction is an earlier response which ultimately leads to the activation of stress responses in *Arabidopsis*. This is further evident by the fact that genes related to stress and (a)biotic stimulus showed a pronounced higher percentage at 6 h of Glu treatment.

2.4.2. OGs-responsive genes

After 1 h of OGs treatment, responsive genes corresponded to 4.78 %, 2.68 %, 6.23 % and 4.37 % of the genes belonging to transport, signal transduction, response to stresses and response to (a)biotic stimulus categories, respectively (Figure 5.4B; Table 5.2). Among these mentioned categories, genes related to signal transduction and response to stresses showed a higher percentage of modulated genes compared to whole genome of *Arabidopsis* (Figure 5.3) thus clearly demonstrating the role of OGs in plant defense related signaling pathways. In response to OGs at 6 h, four categories have higher percentage of expressed genes than the whole *Arabidopsis* genome: signal transduction (2.43 %), response to stresses (5.18 %), response to (a)biotic stimulus (5.79 %) and transport (6.32 %) (Figure 5.3, Figure 5.4B and Table 5.2).

2.5. MapMan analysis of Glu- and OGs-responsive genes in *Arabidopsis thaliana*

To gain functional insight in the transcriptional profiles induced by different treatments (Glu and OGs) in *Arabidopsis*, genes were analyzed by MapMan software (Thimm *et al.*, 2004; Usadel *et al.*, 2005; Rotter *et al.*, 2009). This software allows the categorization of *Arabidopsis* genes into different functional groups. It also helps us to identify the set of genes or groups that are significantly different from other set of genes or groups within the data under analysis. Moreover, it can displayed data onto pictorial diagrams that represent a biological function. From these diagrams we can observe the pattern of transcriptional

Table 5.2: Summary of the percentages of total modulated genes in selected categories in response to Glu, OGs and GLRs in *Arabidopsis thaliana*. The percentages were derived from GO annotation analyses. The underlined values indicate significant higher percentages from whole *Arabidopsis* genome.

	***Arabidopsis* whole genome**	**Glu 1 h**	**Glu 6 h**	**OGs 1 h**	**OGs 6 h**	**GLRs 1 h**	**GLRs 6 h**
Signal transduction	2.11	1.68	0.64	2.68	2.43	2.62	2.54
Transport	5.65	6.57	1.27	4.78	6.32	5.47	6.33
Stresse	4.78	3.22	9.24	6.23	5.18	6.18	6.19
(a)biotic stimuli	4.31	2.66	8.28	4.37	5.79	4.28	6.33
Total genes	**24000**	**463**	**182**	**791**	**1555**	**632**	**926**

modulation within the same group and among different groups; this is not possible by analyzing individual genes.

2.5.1. MapMan biotic stress pathway of Glu-responsive genes

Through MapMan analysis, specific genes categories involved in different cellular pathways (regulation, metabolism, cell cycle and biotic stress *etc.*) can be identified but here we will only focus on biotic stress category: a diagram indicating biotic stress pathway is presented in figure 5.5. MapMan does not analyze any data set having splice variants so the first step is to obtain lists of genes without splice variants. At 1 h of Glu treatment, there were 8 splice variants out of 463 genes. Our results showed that 17.58 % of 1 h Glu-dependent genes were found to be putatively involved in biotic stress pathway. Pictorial diagram indicate that majority of these putative biotic stress pathway genes belonged to the following functional categories: signal transduction (12.5 %), PR proteins (15 %), TFs (10 %), cell wall related (12.5 %) and protein hydrolysis (30 %), (Figure 5.5A). Among the 10 genes annotated to signal transduction, GLR2.5 was significantly up-regulated in response to Glu. Moreover, the genes related to wall-associated kinases (At1g17910) and receptor-like protein kinases (At2g31620) were overexpressed at 1 h of Glu treatment. Transcription factors of WRKY class (WRKY43, WRKY55, and WRKY74) and MYB family (MYB83 and MYB78) were also among the Glu-modulated genes. In addition, polygalacturonase (At4g13760), pectin esterase family protein (At3g60730) and arabinogalactan proteins, AGP17 (At2g23130) and AGP6 (At5g14380), were also among several cell wall-associated proteins up-regulated with Glu. Among twelve *PR* genes with modified expression, disease resistance genes (At4g11170, At4g16930 and At4g16095) and defensin-like (DEFL) family genes (At5g46877, At2g03937, At3g04545 and At5g55132) were with enhanced expression. On the other hand, at 6 h of Glu treatment, 19.78 % (36 out of 182 genes) genes were putatively related to biotic stress pathway, (Figure 5.5B). These include genes from MYB TF family (MYB83 and MYB39), Ethylene-Responsive TF family (ERF1; At1g24590), defensin-like (DEFL) family (At1g63522) and Cell wall family (AGP; At2g47930). Most of these identified genes have been shown to be involved in response to biotic stresses, especially related to response to wounding or pathogen attacks.

2.5.2. MapMan biotic stress pathway of OGs-responsive genes

The information generated through MapMan highlights that about 26.76 % of OGs-modulated genes at 1 h post-treatment belong to biotic stress pathway. This is quite high

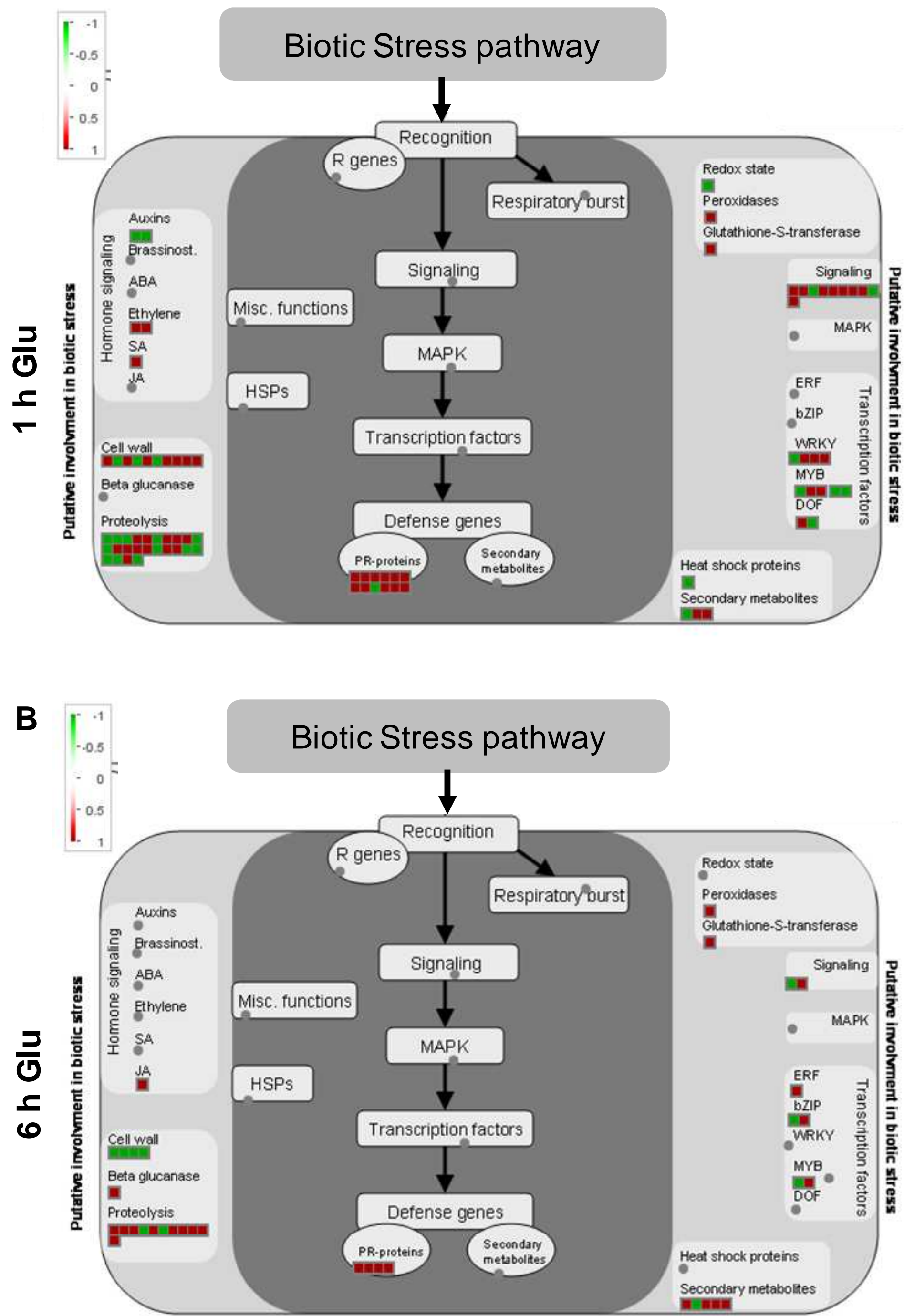


Figure 5.5: The MapMan's "Biotic stress pathway" display created with Glu-regulated genes in *Arabidopsis thaliana*. Each square represents a single gene and the direction of transcriptional change is indicated by square color. Up and down regulated genes are represented in red and green squares, respectively. The average fold change of the three biological replicates is displayed as illustrated in the fold change color bar and color intensity of the square indicates the fold change in the expression of Glu-responsive genes at 1 h **(A)** and at 6 h **(B).**

percentage of genes clearly demonstrating the importance of OGs-induced signaling in plant defense responses. Through this analysis, OGs-responsive genes were mapped into functional categories of genes that are putatively involved in biotic stress pathway in *Arabidopsis thaliana*. In figure 5.6A, six most affected categories in response to OGs in biotic stress signaling pathway are: “Proteolysis (28.49 % of total biotic stress genes)”, “signaling (17.20 % of total biotic stress genes)”, “PR proteins (6.99 % of total biotic stress genes)”, “Hormone signaling (12.35 % of total biotic stress genes)”, “Transcription factors (6.45 % of total biotic stress genes)” and “Cell wall (5.91 % of total biotic stress genes)”. These groups contained either significantly differently responding gene sets or relative large numbers of responding genes when compared to the other groups. In signaling class, GLR2.8, GLR2.9, wall associated kinase-like 4 (WAK-like 4; At1g16150), Ca^{2+} binding protein (At4g20780), Ca^{2+} binding EF-hand (At3g29000), and CPK27 (At4g04700) were among the significantly modulated genes. Polygalacturonase (At4g13760), pectate lyase family protein (At4g13710) and peptidoglycan-binding LysM domain-containing protein (At5g62150) with a fold change of 44.38 were important elements of cell wall. As for as PR genes are concerned, RLP22, disease resistance genes (At1g56540, At5g66890) and defensin-like family genes (DEFL; At2g04925, At1g54445, At1g35537) were significantly modulated in biotic stress pathway. Moreover, TFs genes related to WRKY (WRKY30 and WRKY62), MYB (MYB40, MYB98) and AtGSTU11 (member of glutathione S transferases family) were also overexpressed during OGs elicitation. Interestingly, at 1 h of OGs treatment, most of the modulated genes in the biotic stress pathway are up-regulated. For example, in signaling category, approximately 81 % genes are up-regulated.

Our transcriptomic data have shown that, with OGs elicitation, 6 h time point was most responsive with 1551 genes (1342 after removing splice variants) showing significant modulation in their expression. In biotic stress pictorial diagram, 25.78 % of the total modulated genes were involved and most affected categories that were significantly modulated at 6 h of treatment include: “Proteolysis”; 18.20 % of the total biotic stress modulated genes, “Signaling”; 18.20 % of the total biotic stress modulated genes, “Cell wall”; 14.16 % of the total biotic stress modulated genes, “Hormones”; 9.25 % of the total biotic stress modulated genes, “Transcription factors”; 6.94 % of total modulated genes and “PR proteins”; 3.76 % of the total biotic stress modulated genes (Figure 5.6B). In contrast to 1 h, most of the genes were down-regulated at this time point *e.g.* in cell wall and signaling categories, 73.46 % and 68.25 % of the genes were down-regulated, respectively. However, all the genes of WRKY family and ABA signaling are upregulated. Interestingly, the ethylene

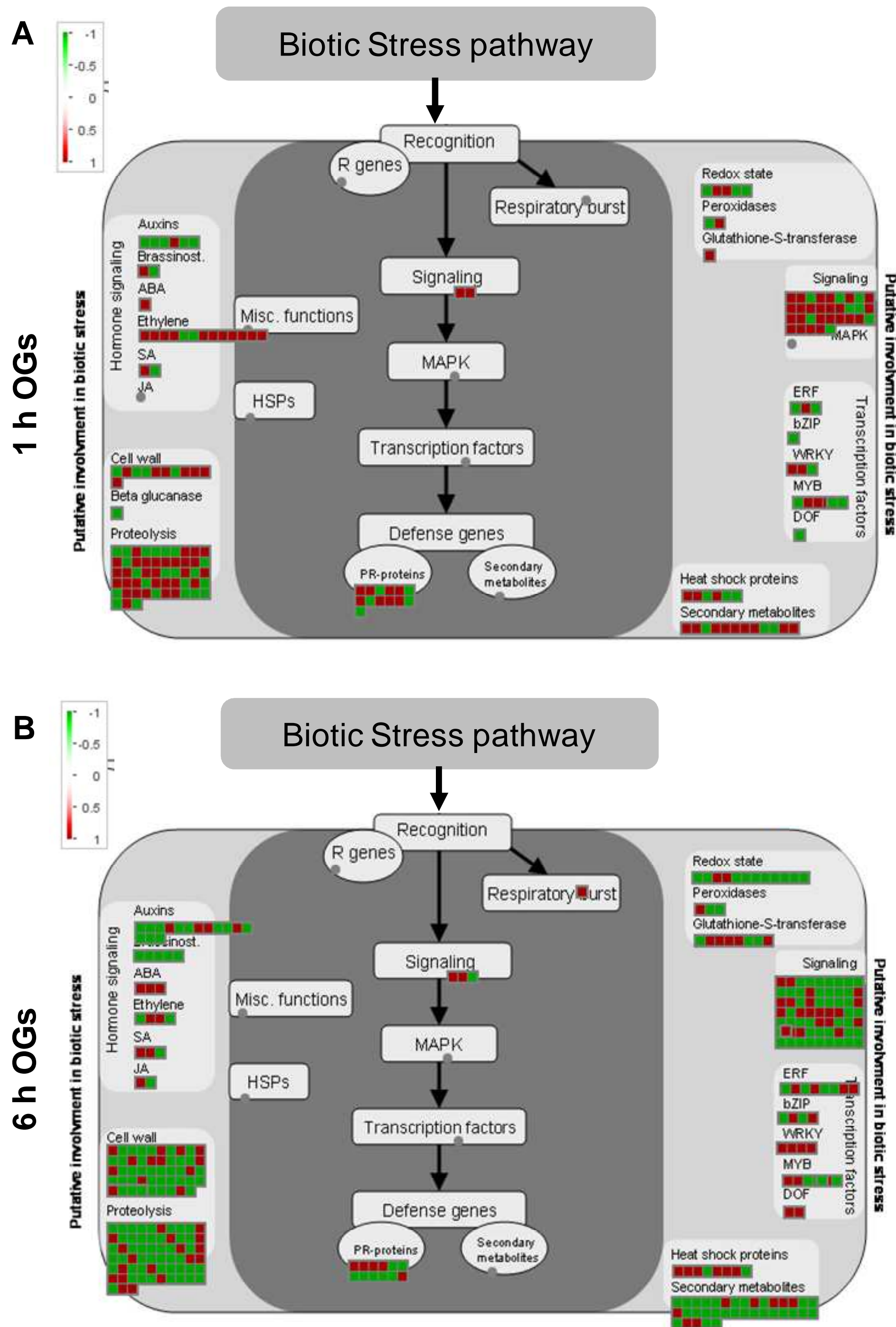


Figure 5.6: MapMan distribution of OGs-responsive genes in biotic stress pathway in *Arabidopsis thaliana*. Genes from different classes that are putatively involved in biotic stress showed modulation in their expression at 1 h **(A)** and at 6 h **(B)** of OGs treatment and are represented by colored squares that indicate the direction of transcriptional change color. Up and down regulated genes are represented in red and green squares, respectively. Color intensity indicates the average fold change in three biological replicates performed.

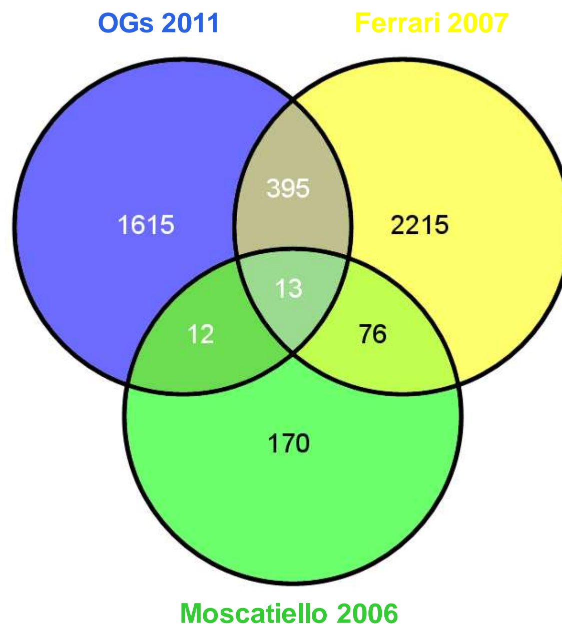


Figure 5.7: Overlap of significantly expressed genes after OGs treatment in different studies conducted on *Arabidopsis thaliana*. Venn diagram of overlapped and non-overlapped genes obtained after OGs treatment in OGs 2011 (My own data), Ferrari *et al.* (2007) and Moscatiello *et al.* (2006). For comparison, all the OGs modulated genes at all time points were pooled and compared.

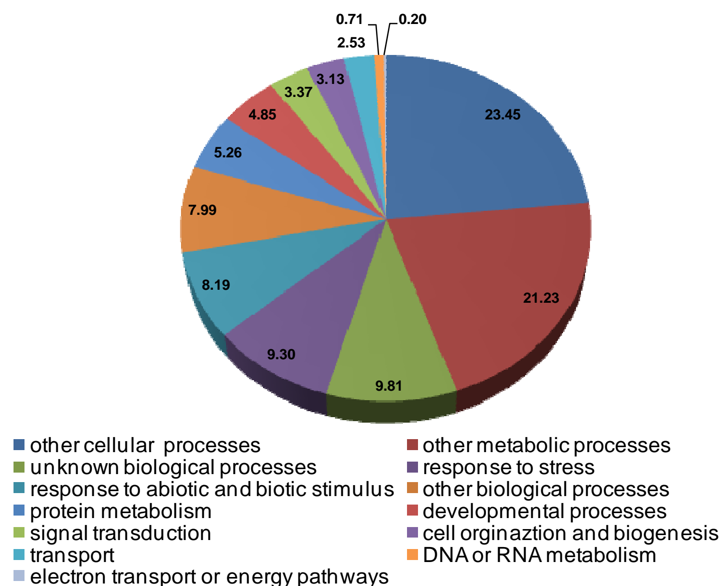


Figure 5.8: GO annotation of biological processes of OGs-responsive genes, commonly identified in "My own data" and "Ferrari *et al.*, 2007" in *Arabidopsis thaliana*. Pie chart indicates the distribution of commonly induced OGs-responsive genes into different functional categories. The percentages indicate the abundance of each category within the commonly identified genes; Assignments are based on the data available at the TAIR and from the GO Annotation Database.

Table 5.3: Summary of commonly modulated genes after OGs treatment in different studies in *Arabidopsis*. Number of genes modulated after OGs treatment in our study and previously published studies. Numbers of modulated genes represent significantly up-regulated or down-regulated genes with a fold change ≥ 2 after OGs treatment as compared to control.

Data source	**Biological model**	**Treatment mode**	**OGs conc. & treatment Time**	**Total number of OGs modulated genes**	**Common genes in OGs 2011 and OGs 2007**	**Common genes in OGs 2011 and OGs 2006**	**Common genes in OGs 2007 and OGs 2006**
OGs 2011 My own data	*Arabidopsis* plants (4 weeks old)	OGs syringe infiltration in leaves	2.5 $mg.mL^{-1}$ 1 h and 6 h	2348	**408**	**25**	**89**
OGs 2007 Ferrari *et al.*, *2007*	*Arabidopsis* seedlings (10 days old)	OGs in the culture medium	50 $\mu g.mL^{-1}$ 1 h and 3 h	2699			
OGs 2006 Moscatiello *et al.*, 2006.	*Arabidopsis* cell suspensions (10 days old)	Cell treated with OGs	200 $\mu g.mL^{-1}$ 2 h	271			

pathway which is highly induced at 1 h, seems to go down to resting conditions at 6 h. GLR2.5, GLR1.3, RLP24, MAPKKK18, Ca^{2+} transport ATPase (ACA13; At3g22910), CaM binding (At5g10680) and Mildew resistance locus O 6 (MLO6) are the important members of signaling category that were up-regulated. Other major category is related to cell wall, which contains most of the down-regulated members *e.g.* pectate lyase family genes (At5g48900, At5g63180, At3g53190, At3g09540, At1g67450) and arabinogalactan protein (AGP) gene family (AGP1, AGP2, AGP5, AGP7, AGP12, AGP24). PDF1.3, PDF1.2B, defensin-like protein gene (DEFL; At3g61185) and TFs genes from different classes (WRKY63, WRKY64, WRKY66, WRKY67, MYB50, MYB61, RAP2.6 and RAP2.6L) were among other interesting candidates that were up-regulated in response to OGs.

3. Comparative analysis of OGs transcriptomics responses in *Arabidopsis thaliana*

Changes in *Arabidopsis* gene expression in response to OGs have been investigated in many studies using microarray analyses. These studies were completed by full genome global transcripts profiling analyses. The gene expression was analyzed in *Arabidopsis* leaf tissues infiltrated by syringe, vacuum infiltration of leaf disks, *Arabidopsis* cells suspensions and seedlings using a genome array covering over 24,000 genes.

To determine the extent of overlap between transcriptional responses induced by OGs-treatment, analyses were performed with pooled transcriptomic data from different studies. For this purpose, we selected data from two recently conducted studies by Moscatiello *et al.* (2006) and Ferrari *et al.* (2007) on *Arabidopsis* cell suspensions and leaf tissues, respectively. These comparisons provided an insight how OGs treatment regulates expression of genes related to different physiological pathways. The number of total and common genes in response to OGs in each study is presented in table 5.3 and figure 5.7. Apart from genes encoding large number of unknown and hypothetical proteins after OGs treatment, genes related not only to stress- and disease-(defense) related protein family, signaling components and transcription factors but also enzymes implicated in primary and secondary metabolism, were identified in these comparisons.

Using a whole *Arabidopsis* genome microarray, 408 common genes were identified in plants and seedling exposed to OGs (between our data and Ferrari *et al.*, 2007). All the commonly modified genes behave in a similar fashion to show a similar expression profile (201 genes were up-regulated and 194 genes were down-regulated in both cases) except 21

Table 5.4: Common elements identified with OGs elicitation in "My own data", "Ferrari *et al.*, 2007" and "Moscatiello *et al.*, 2006". NA: fold change was not available.

Gene ID	SYMBOLS	DESCRIPTION	Fold change "OGs" My own data	Fold change "OGs" Mocatiello *et al.*, 2006	Fold change "OGs" Ferrari *et al.*, 2006
AT4G12400		Stress-inducible protein, putative	7.38	NA	3.74
AT5G67080	MAPKKK19	MAPKKK19; ATP binding / kinase/ protein kinase/ protein serine/threonine kinase	6.39	3.42	12.92
AT1G01480	ACS2, AT-ACC2	ACS2; 1-aminocyclopropane-1-carboxylate synthase	4.95	-2.00	2.17
AT2G37430		Zinc finger (C2H2 type) family protein (ZAT11)	3.98	2.28	58.66
AT4G19810		Glycosyl hydrolase family 18 protein	3.79	-2.08	2.43
AT4G04700	CPK27	CPK27; ATP binding / calcium ion binding / kinase/ protein kinase/ protein seri	3.79	-5.50	2.70
AT4G38540		Monooxygenase, putative (MO2)	2.82	NA	2.10
AT4G02130	GATL6, LGT10	GATL6; polygalacturonate 4-alpha-galacturonosyltransferase/ transferase, transf	-3.30	2.11	-2.37
AT4G29360		Glycosyl hydrolase family 17 protein	-4.93	NA	-2.31
AT1G56010	NAC1, anac021	NAC1; transcription factor	-5.11	-2.12	-2.97
AT1G64390	AtGH9C2	AtGH9C2 (Arabidopsis thaliana glycosyl hydrolase 9C2); carbohydrate binding / c	-5.76	-3.21	-2.54
AT4G22730		Leucine-rich repeat transmembrane protein kinase, putative	-8.85	-2.03	-6.69
AT1G21910		AP2 domain-containing transcription factor family protein	-11.03	6.03	-3.10

genes that behave differently in these two studies (Supplemental Table S6). We also analyzed the common genes obtained in both studies by GO annotation to get an overview of their putative involvement in different processes. Although ratio of activated genes in each functional category was varying yet the distribution in functional categories indicate the prominent genes involvement in signaling, stresses and (a)biotic stimuli (Figure 5.4B, Figure 5.8).

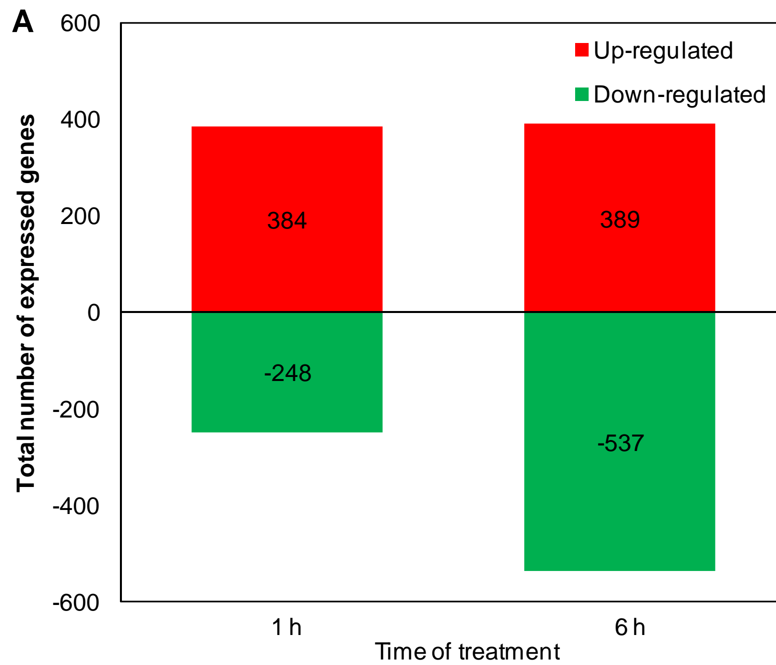
Similarly, in response to OGs, only 25 genes were found to be commonly modulated between our data and Moscatiello *et al*. (2006; list of genes with their putative functions are presented in Supplemental Table 7). Six genes showed similar expression pattern in both cases (two up-regulated and four down-regulated genes in both cases). MAPKKK19 (At5g67080), CPK27 (At4g04700) AtGSTU25 (At1g17180), NAC1 (At1g56010), BGLU18 (At1g52400) and GATL6 (At4g02130) were the commonly identified genes in these studies.

Overall, thirteen genes were common in our data, Mocateillo *et al*. (2006) and Ferrari *et al*., (2007) (list of genes with their putative functions are presented in Table 5.4). From these thirteen genes, only two genes, MAPKKK19 (At5g67080) and ZAT11 (At2g37430; C_2H_2 type zinc finger family protein), were overexpressed while three genes, AtGH9C2 (At1g64390), NAC1 (At1g56010) and LRR protein kinase (At4g22730), were suppressed in all the three studies.

The comparison of these three studies pointed out the similarity between our study and Ferrari *et al*. (2007) and high heterogeneity of results when comparison was made with the data from Moscatiello *et al*. (2006). This may not be surprising because not only the biological materials are different (plant/plantlets versus cell suspensions) but the time kinetics of OGs treatment varies too. This is an established fact that gene expression is very sensitive to time kinetics. Multiple time points in a gene expression analysis will be informative about the global tendency of OGs-induced signaling pathway in plants and cells.

4. Identification and characterization of GLRs-responsive genes

To further investigate the involvement of GLRs in OGs-mediated signaling processes, a two-step analysis of the transcriptomic data was carried out. In the first step, a comparison between DMSO and OGs + DMSO treatments was conducted to obtain the list of genes specifically modulated by OGs (the same comparison made above to obtain OGs responsive genes). Another comparison between transcriptomic data of DNQX and OGs + DNQX treatments was made to obtain the genes specifically expressed in OGs + DNQX treatment.



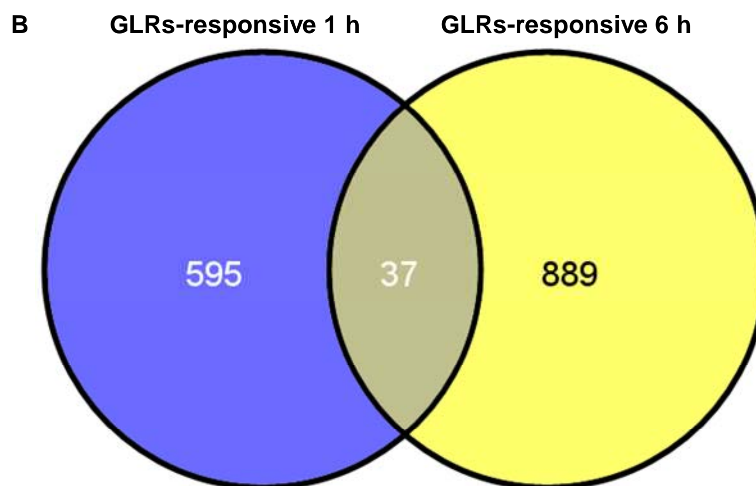


Figure 5.9: Glutamate receptors (GLRs)-responsive genes in *Arabidopsis thaliana* (Col-0). A) Differential expression of GLRs-responsive genes in OGs elicitation. Up- and down-regulated genes are represented in red and green respectively. **B)** Overlap of significantly expressed GLRs-responsive genes at 1 h and 6 h after DNQX treatment in the presence of OGs. Venn diagram of overlapped and non-overlapped GLRs-responsive genes at different time points in Col-0 plants. Leaves were infiltrated with OGs (2.5 $mg.mL^{-1}$) or DMSO (control) +/- DNQX (500 µM). 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. Three independent biological replicates were carried out.

These genes correspond to OGs-responsive genes in the presence of DNQX (independent of GLRs). In the second step, comparison was made between genes specifically expressed in OGs + DMSO and OGs + DNQX treatments. This analysis generated three different lists of genes: genes uniquely expressed after OGs + DMSO treatment, genes uniquely expressed after OGs + DNQX treatment and genes expressed commonly in both conditions. Genes that were uniquely expressed after OGs + DMSO treatment termed as "GLRs-dependent genes" and genes that were uniquely expressed after OGs + DNQX treatment termed as "GLRs-independent genes", in OGs-induced signaling pathway. Using a fold change of ≥ 2 and a cut off value of $P \leq 0.01$ and pooling the data at 1 h and 6 h, our data showed a total of 1558 GLRs-dependent genes, with 773 up-regulated and 785 down-regulated genes. Out of these 1558 genes, 632 (384 up-regulated and 248 down-regulated) and 926 (389 up-regulated and 537 down-regulated) genes specifically showed modified expression at 1 h and 6 h, respectively (Figure 5.9A; list of genes in Supplemental Table S8 and S9). More precisely, 595 (363 up-regulated and 232 down-regulated) and 889 (376 up-regulated and 533 down-regulated) genes were modulated uniquely at 1 h and 6 h, respectively. At both time points, 37 common genes (21 up-regulated and 16 down-regulated) were identified (fold-change ≥ 2; P value ≤ 0.01; list of genes in Table 5.5). Venn diagram of GLRs-responsive genes uniquely expressed at 1 h and 6 h is presented in Figure 5.9B.

4.1. GO annotation of GLRs-responsive genes in *Arabidopsis thaliana*

In parallel to Glu- and OGs-responsive genes, GLRs-responsive genes were also analyzed by GO annotation (Figure 5.4C). Similar higher upregulation of GLRs genes as compared to whole *Arabidopsis* genome under normal conditions were observed in signal transduction (at 1 h and 6 h), transport (at 6 h), stress response (at 1 h and 6 h) and (a)biotic stresses (at 6 h) (Figure 5.3 and 5.4C; Table 5.2). Elevated percentage of modulated genes belonging to above mentioned categories clearly demonstrates the involvement of GLRs in plants biological processes especially in the context of plant pathogen interactions.

4.2. GLRs-responsive genes by MapMan

After getting a broader overview through GO annotation, GLRs-dependent genes were further analyzed by MapMan software to investigate in detail which specific genes are related to which physiological pathway. As we are more interested to check the involvement of GLRs in plant defense, genes involved only in biotic stress pathway were explored. A pictorial

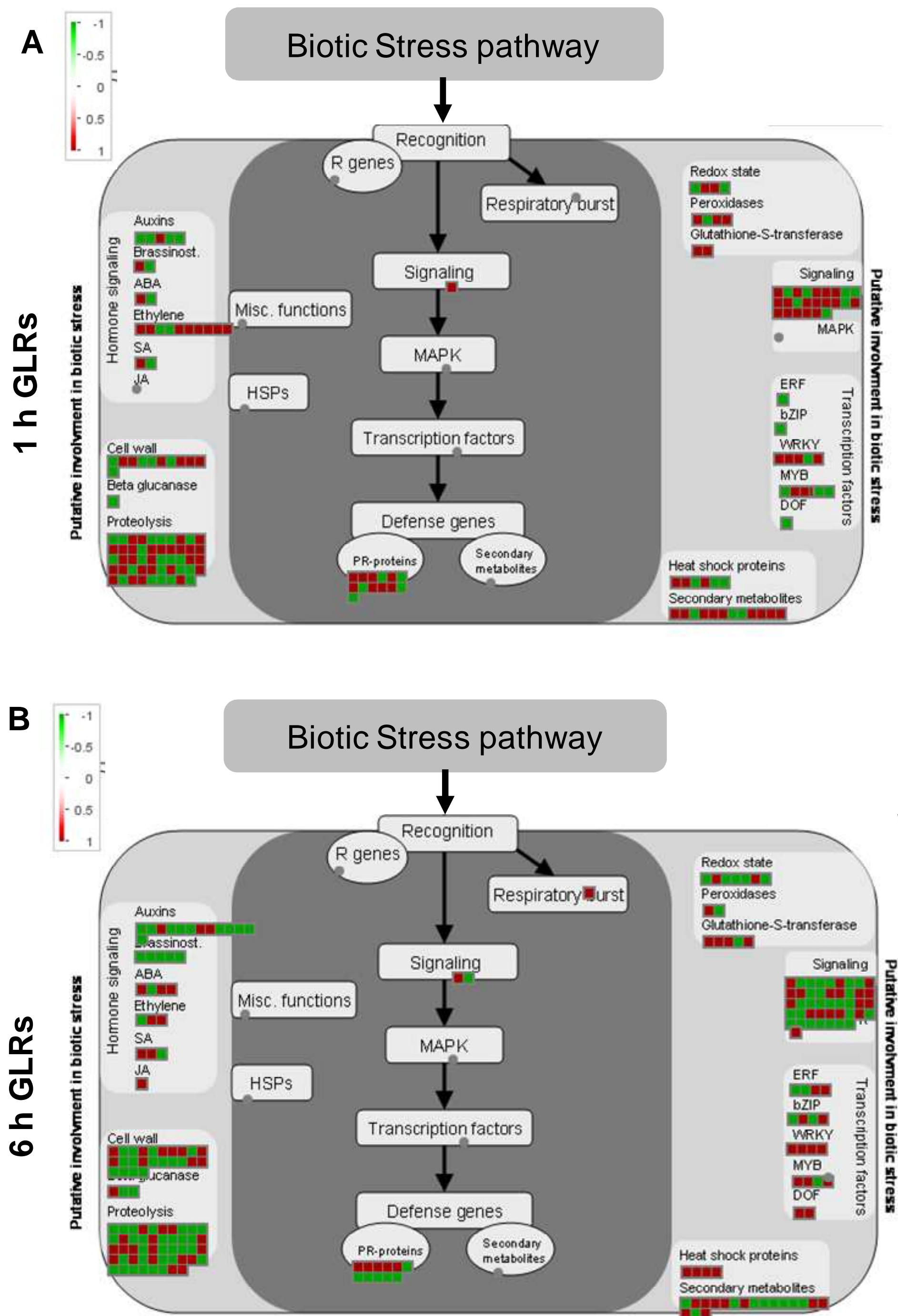


Figure 5.10: Biotic stress pathway pictorial diagram for *Arabidopsis thaliana* GLRs-responsive genes in response to OGs. A) and **B)** represent the modified GLRs genes at 1 h and 6 h of OGs treatment. Single genes are represented by a square while the color indicates the direction of transcriptional change (up and down regulated genes are represented in red and green squares, respectively). This analysis was performed by MapMan software.

diagram of the distribution of GLRs-responsive genes putatively involved in biotic stress is shown in figure 5.10. Focusing on Biotic stress diagram, the observed up- and down-regulated genes encoded proteins mainly involved in signaling, TFs, PR proteins, cell wall, hormone signaling and secondary metabolites (Figure 5.10A). Globally, 27.56 % of the total GLRs-dependent genes were found to be involved in biotic stress response. GLR2.8, CaM binding protein (At2g15760), Ca^{2+} binding EF hand (At3g29000), RLK (At3g22040), Ca^{2+} ion binding (At3g25600), PROPEP2 and putative peroxidase (At4g08780, At4g08780, At4g26010) were among the twenty four modulated genes related to signaling pathway (9.88 %). Cell wall group that constitutes 6.98 % of the total biotic stress related genes encodes proteins for polygalacturonase (At4g13760) and pectate lyase family protein (At4g13710) *etc*. Similarly, 13 TFs genes (7.56 % of total biotic stress pathway genes) including WRKY28, WRKY54, WRKY62, WRKY67, MYB40 and MYB98 were significantly overexpressed in GLRs-dependent manner. Moreover, PR proteins (7.56 %), proteolysis (28.49 %), hormones (12.21 %) and secondary metabolites (6.98 %) were among other important genes categories modulated by GLRs in biotic stress pathway. From figure 5.10A, it is also evident that most of the genes from signaling and PR proteins were up-regulated while cell wall, hormones and transcription factors related genes were equally up- and down-regulated. In comparison, at 6 h, 25.16 % of the total genes modulated by GLRs were involved in biotic stress pathway. Seven categories with significant number of modulated genes were: signaling (18.45 %), PR proteins (4.72 %), cell wall (10.30 %), hormones (13.73 %) and TFs (7.72 %), secondary metabolites (7.72 %) and proteolysis (20.60 %). In signaling category, GLR1.3, RLP24, MAPKKK18, Ca^{2+} transport ATPase (ACA13; At3g22910), CaM binding (At5g10680) and CBL10 were significantly up-regulated GLRs-dependent genes. Transcription factors of WRKY family (WRKY28, WRKY63, WRKY64 and WRKY67), MYB family (MYB50 and MYB61) and ERF family (RAP2.6) were also up-regulated. In contrast, pectate lyase family (At3g09540) and arabinogalactan protein (APG) family (AGP1, AGP2, AGP5, AGP7, AGP12 and AGP24) in cell wall category contains the down-regulated members. Overall, more GLRs-dependent genes in the biotic stress category were down-regulated at 6 h compared to 1 h.

4.3. Identification of common genes regulated by Glu and GLRs

GLRs are thought to be activated by Glu which acts as a ligand for these channels. From our transcriptome data, we have identified the genes under the control of Glu and GLRs-responsive genes induced by OGs treatment. Now, our next goal is to identify the

Table 5.5: List of commonly induced *Arabidopsis* GLRs-dependent genes at 1 h and 6 h of OGs elicitation.

Gene_ID	SYMBOLS	DESCRIPTION	Fold change GLRs-dependent 1 h	Fold change GLRs-dependent 6 h
AT2G28210.1	ATACA2	ATACA2 (ALPHA CARBONIC ANHYDRASE2); carbonate dehydratase/ zinc ion binding	8 .93464845	18.2573903
AT4G18430.1	AtRABA1e	AtRABA1e (Arabidopsis Rab GTPase homolog A1e); GTP binding	8.85263622	5.29078125
AT5G44990.2		FUNCTIONS IN: molecular_function unknown	7.93174546	6.3700367
AT2G15490.2	UGT73B4	UGT73B4 (UDP-GLYCOSYLTRANSFERASE 73B4); UDP-glucosyltransferase/ UDP-glycosyltr	7.70716366	4.70899798
AT2G07170.1		binding	7.54358142	-5.25503562
AT2G29750.1	UGT71C1	UGT71C1 (UDP-GLUCOSYL TRANSFERASE 71C1); UDP-glycosyltransferase/ quercetin 3'-	7.02408603	6.97708972
AT4G15120.1		VQ motif-containing protein	6.33994057	11.2345929
AT1G22370.1	AtUGT85A5	AtUGT85A5 (UDP-glucosyl transferase 85A5); glucuronosyltransferase/transferase	6.10910756	-3.47940653
AT2G40113.1		Unknown protein	6.00662883	5.56744951
AT5G02780.1		In2-1 protein, putative	5.06682188	9.36414206
AT2G26390.1		Serpin, putative/serine protease inhibitor	4.58431359	6.0229799
AT3G50140.1		Unknown protein	4.54774556	3.83391831
AT1G03850.2		Glutaredoxin family protein	4.50723756	6.19890021
AT4G22520.1		Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	4.50363699	6.75563122
AT1G33030.1		O-methyltransferase family 2 protein	4.30112462	4.55311019
AT1G11610.1	CYP71A18	CYP71A18; electron carrier/heme binding/ iron ion binding/monooxygenase	4.28451966	5.70809196
AT3G22510.1		Unknown protein	4.01502739	4.68371971
AT1G65690.1		Harpin-induced protein-related/HIN1-related /harpin-responsive protein-relat	3.85497486	4.05663262
AT2G32200.1		Unknown protein	3.22794367	4.44759734
AT3G05360.1	AtRLP30	AtRLP30 (Receptor Like Protein 30); kinase/protein binding	3.03823717	3.46033015
AT4G18170.1	WRKY28, ATWRKY28	WRKY28; transcription factor	2.39199671	6.02502319
AT4G12320.1	CYP706A6	CYP706A6; electron carrier/heme binding/ iron ion binding/monooxygenase	-2.66148332	-3.2638669
AT4G19170.1	NCED4	NCED4 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 4)	-3.31312506	-3.75383335
AT1G50390.1		Fructokinase-related	-3.32600831	4.17171289
AT4G30650.1		Hydrophobic protein, putative/low temperature and salt responsive protein, pu	-3.46956266	-2.31614013
AT1G16880.2		Uridylyltransferase-related	-3.48120253	-3.10827126
AT3G55240.1		Overexpression leads to PEL (Pseudo-Etiolation in Light) phenotype.	-3.78337688	-7.62033877
AT1G09350.1	AtGolS3	AtGolS3 (*Arabidopsis thaliana* galactinol synthase 3); transferase, transferring	-3.79006596	-7.99057256

AT5G15740.1		Unknown protein	-4.73164829	-3.09566153
AT2G34890.1		CTP synthase, putative/UTP-ammonia ligase, putative	-5.10266577	4.63664378
AT5G67390.2		Unknown protein	-5.21014792	-2.36437276
AT5G02890.1		Transferase family protein	-5.53805702	-6.11995864
AT5G23280.1		TCP family transcription factor, putative	-5.72382921	-3.46003427
AT4G03039.1	MIR826A	MIR826a; miRNA	-6.13725379	-4.40024331
AT5G29807.1		Unknown protein	-8.44331163	2.92799389
AT5G25550.1		Leucine-rich repeat family protein/extensin family protein	-8.57045043	-12.8394923
AT2G18300.1		Basic helix-loop-helix (bHLH) family protein	-22.4595568	-5.32119894

Table 5.6: List of *Arabidopsis thaliana* common genes in Glu-response and GLRs-dependent in response to OGs at 1 h of treatment.

Gene ID	SYMBOLS	DESCRIPTION	Fold change 1 h OGs	Fold change 1 h Glu
At1g69930.1	ATGSTU11	ATGSTU11 (GLUTATHIONE S-TRANSFERASE TAU 11); glutathione transferase	9.58508996	11.3188814
At4g36060.1		Basic helix-loop-helix (bHLH) family protein	8.5634732	-8.17987662
At5g01490.1	CAX4, ATCAX4	CAX4 (CATION EXCHANGER 4); calcium:cation antiporter/calcium:hydrogen antiport	7.78346053	2.96682506
At1g04550.1	IAA12, BDL	IAA12 (AUXIN-INDUCED PROTEIN 12); transcription factor/ transcription repressor	6.86046465	-12.2875195
At5g07322.1		Other RNA	6.85822072	-8.36191439
At3g32035.1		Transposable element gene	6.06863645	5.32677627
At3g01760.1		Lysine and histidine specific transporter, putative	5.43931916	3.77103457
At4g16520.2	ATG8F	ATG8F (autophagy 8f); microtubule binding	3.55007101	-2.77890425
At2g35710.1		Glycogenin glucosyltransferase (glycogenin)-related	3.15680531	4.52598722
At3g49580.1	LSU1	LSU1 (RESPONSE TO LOW SULFUR 1)	2.89931152	6.68365276
At3g01710.1		FUNCTIONS IN: molecular_function unknown	2.70140831	2.70327329
At4g13760.1		Polygalacturonase	-3.75439521	5.11567692
At2g45960.2	PIP1B, TMP-A, ATHH2,	PIP1B (NAMED PLASMA MEMBRANE INTRINSIC PROTEIN 1B); water channel	-3.88311272	-3.67558149
At3g10113.1		Myb family transcription factor	-4.44980892	-2.81413389
At1g16680.1		DNAJ heat shock N-terminal domain-containing protein/S-locus protein, putativ	-4.54440129	-2.79235342
At3g31993.1		Transposable element gene	-4.68991013	8.72611135
At4g14620.1		Unknown protein	-4.87769101	-4.37387245
At5g48700.1		Ubiquitin-related	-5.88649665	4.44523865
At3g61660.1		Unknown protein	-6.78027145	5.75105667
At1g18330.1	EPR1	EPR1 (EARLY-PHYTOCHROME-RESPONSIVE1); DNA binding/TF	-8.82088885	-4.04054813
At5g23970.1		Transferase family protein	-11.0975287	6.46499743
At5g19473.1		FUNCTIONS IN: molecular_function unknown	-12.0823193	10.6067829
At1g29430.1		Auxin-responsive family protein	-20.7053304	-2.6953927
At2g28760.1	UXS6	NAD-dependent epimerase/dehydratase family protein	-36.3686304	9.06243619

Table 5.7: List of *Arabidopsis thaliana* common genes in Glu-response and GLRs-dependent in response to OGs at 6 h of treatment.

Gene ID	SYMBOLS	DESCRIPTION	Fold change 6 h OGs	Fold change 6 h Glu
At3g13610.1		Oxidoreductase, 2OG-Fe(II) oxygenase family protein	10.9827811	8.21434372
At5g02780.1		In2-1 protein, putative	9.36414206	3.55458018
At5g38900.1		DSBA oxidoreductase family protein	7.10512303	3.78794291
At1g30850.1		Unknown protein	7.02078691	2.98146749
At5g18270.1	ANAC087	ANAC087; transcription factor	6.74884017	3.48044948
At1g32950.1		Subtilase family protein	5.84298265	3.18782418
At3g46090.1	ZAT7	ZAT7; nucleic acid binding/transcription factor/zinc ion binding	5.83886208	3.89978256
At1g74140.4		FUNCTIONS IN: molecular_function unknown	5.51809199	4.23142131
At2g39530.1		Integral membrane protein, putative	5.41966606	8.34247586
At5g15140.1		Aldose 1-epimerase family protein	5.16533994	5.11364845
At4g33860.1		Glycosyl hydrolase family 10 protein	4.99345255	2.92321317
At4g17800.1		DNA-binding protein-related	3.94601711	6.00987659
At1g32960.1	ATSBT3.3, SBT3.3	SBT3.3; identical protein binding/serine-type endopeptidase	3.29086026	3.02204426
At5g02670.1		FUNCTIONS IN: molecular_function unknown	-4.03204703	-2.50581249
At1g22170.1		Phosphoglycerate/bisphosphoglycerate mutase family protein	-4.4411825	-3.98547785
At3g50440.1	ATMES10, MES10	MES10 (METHYL ESTERASE10);hydrolase /hydrolase, acting on ester bonds/methy	-7.64291869	-2.69572436
At2g42170.1		actin, putative	-9.06173318	-4.75591342

common genes that are modulated by Glu and are GLRs dependent. Comparison of the gene lists of Glu-dependent and GLRs-dependent showed that the transcriptomic responses at different time points were very dissimilar in both cases.

Amongst the Glu- and GLRs- responsive genes, 24 and 17 genes were found common at 1 h and 6 h, respectively (List of genes in Table 5.6 and 5.7). At 1 h, only 7 genes were up-regulated in both treatments, whereas 13 out of 17 genes were up-regulated at 6 h time interval. These genes belong to different functional categories: Cation exchanger 4 (CAX4; At5g0149), glutathione s-transferase tau 11 (ATGSTU11; At1g69930) and response to low sulfur 1 (LSU1; At3g49580) encoding proteins were among the up-regulated elements in both cases.

5. Functional characterization of candidate genes

Our transcriptomic analysis identified several interesting genes related to different functional classes especially related to signaling and stress responses. As we were more interested in the identification of plant defense-related genes which were under the control of GLRs in OGs signaling pathway and the genes which were specifically modulated by Glu, a selection of genes involved in biotic stress pathway was made. Totally, ten genes were selected: 6 GLRs-dependent genes in OGs signaling, 2 Glu-dependent genes and 2 genes commonly modulated both by Glu and GLRs (Table 5.8). These genes encode proteins related to transport, calcium signaling, plant response to stresses and TFs.

5.1. Expression of candidate genes by RT-qPCR to validate microarray data

Selected genes were analyzed by RT-qPCR to validate transcriptomic analysis. Although these selected genes have varied expression values in one given biological repeat but the average fold-change of these genes was always ≥ 2 in the three biological replicates. The accumulation of gene transcripts in response to OGs and OGs +/- DNQX, and in parallel Glu was followed by RT-qPCR at 1 h, 3 h and 6 h of treatment. The RT-qPCR data was normalized using *UBQ10* expression. A comparison of the expression profiles (fold-change) of these selected candidate genes was made between RT-qPCR and transcriptomics data (Table 5.9, Table 5.10 and Figure 5.10).

In response to OGs and OGs +/- DNQX, transcriptomic data showed an upregulation of Ca^{2+} binding EF-hand (At3g29000) at 1 h while RT-qPCR analysis demonstrated a significant upregulation at 1 h, 3 h and 6 h of treatment. Significant differences in transcripts

Table 5.8: List of selected candidates from GLRs-dependent genes in OGs pathway and Glu-dependent genes in *Arabidopsis thaliana* for functional characterization. Red colour indicates the up-regulation of genes.

Gene ID	SYMBOLS	DESCRIPTION	Fold change GLRs-responsive 1 h	Fold change GLRs-responsive 6 h	Fold change Glu-responsive 1 h
AT3G29000		Calcium-binding EF-hand family protein; FUNCTIONS IN: calcium ion binding;	4.78358445	X	X
AT3G22910		Calcium transporting ATPase E1-E2 type family protein plasma membrane type	X	3.55756815	X
AT1G69930	ATGSTU11	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002).	9.58508996	X	11.3188814
AT1G57560	AtMYB50 | AtMYB50	Regulation of transcription, DNA-dependent, response to IAA, GA, JA and SA stimulus	X	16.881034	X
AT5G01490	CAX4, ATCAX4 | CAX4	Encodes a cation/proton antiporter, a member of low affinity calcium antiporter CAX2 family.	7.78346053	X	2.96682506
AT1G12663	PR (pathogenesis-related) protein	Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant thionin (PR-13) family	X	X	18.0201362
AT5G22570	WRKY38, ATWRKY38 | WRKY38; t	Defense response to bacterium, regulation of transcription, DNA-dependent, SA mediated signaling pathway	X	X	6.17997417
AT5G01900	WRKY62, ATWRKY62 | WRKY62;	Defense response to bacterium, regulation of transcription, DNA-dependent, SA mediated signaling pathway	6.9174811	X	X
AT1G66600	WRKY63, ATWRKY63 | WRKY63;	ABO3, WRKY63, T12I7.5, T12I7_5, ATWRKY63, WRKY DNA-BINDING PROTEIN 63, ABA OVERLY SENSITIVE MUTANT 3 A member of WRKY Transcription Factor; Group III. Regulation of plant responses to ABA and drought stress.	17.024163	X	X
AT1G43160	RAP2.6 | RAP2.6	Encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family (RAP2.6)	X	7.18584715	X

accumulation of Ca^{2+} ATPase (At3g22910) and ATMYB50 (At1g57560) were observed at 6 h of OGs treatment in both transcriptomic data and RT-qPCR analysis. WRKY63 (At1g66600) showed an upregulation at 6 h in transcriptomic data and at 1 h, 3 h and 6 h of OGs treatment in RT-qPCR analysis. Moreover, OGs treatments induced the upregulation of WRKY62 (At5g01900) at 1 h and, 1 h and 6 h in RT-qPCR and in transcriptomic analysis, respectively. However, RAP2.6 (At1g43160) was upregulated at 6 h in transcriptomic data and, at 3 h and 6 h in RT-qPCR studies. In response to OGs, increase in expression levels of ATGSTU11 (At1g69930) and ATCAX4 (At5g01490) was found at both 1 h and 6 h in transcriptomic data. In RT-qPCR analysis, ATGSTU11 and ATCAX4 showed higher expression at 1 h and 6 h, respectively (Table 5.9; Figure 10).

With Glu treatment, a prominent increase in the expression of ATGSTU11 was observed at 1 h and 3 h through trancriptome and RT-qPCR approach, respectively. On the other hand, ATCAX4 demonstrated an upregulation at 1 h and 6 h in transcriptomic analysis as compared to 1 h and 3 h expression with RT-qPCR. Among the two other genes that were specifically upregulated after Glu treatment, WRKY38 (At5g22570) was significantly overexpressed at 1 h and 6 h in transcriptomics data and at 1 h and 3 h in RT-qPCR analysis. PR13 (At1g12663) showed a significant increase in transcript level at 1 h and 6 h of Glu treatment in both cases (Table 5.10; Figure 10).

Globally, similar trends of changes in gene transcripts were observed regardless of experimental techniques used. However, the absolute extent of genes activation and the time kinetics varies depending on the method of analysis. This may not be unexpected since different methodologies are being used in transcriptomic analysis and qRT-PCR experiments. This fact is strengthen by the reported discrepancies between the results of these two different techniques with values ranging from 55 to 20-30% (Czechowski *et al.*, 2004; Salzman *et al.*, 2005; Svensson *et al.*, 2006). In conclusion, our data give good correlation between transcriptomic approach and real-time RT-PCR analyses and we could rely on the authenticity of our transcriptomic data.

6. Discussion

We examined the gene expression profile by microarray (transcriptomics) analysis. The comprehensive analysis revealed the characteristic gene expression profiles of all expressed genes after Glu and OGs treatments of *Arabidopsis* leaf tissues. These results demonstrated that transcript levels of many genes changed substantially, even after the

Table 5.9: Summary of Transcriptome and RT-qPCR gene expression comparisons of GLRs-dependent selected candidate genes in *Arabidopsis thaliana*.

Gene ID	Name of gene	Trancriptome analysis	RT-qPCR
AT3G29000	Ca^{2+} binding EF hand	1 h	1h, 3 h, 6 h
AT3G22910	Ca^{2+} ATPase (ACA13)	6 h	6 h
AT1G69930	ATGSTU11	1 h, 6 h	1 h
AT1G57560	ATMYB50	6 h	6 h
AT5G01490	CAX4, ATCAX4 | CAX4	1 h, 6 h	6 h
AT5G01900	WRKY62, ATWRKY62 | WRKY62;	1 h	1 h, 6 h
AT1G66600	WRKY63, ATWRKY63 | WRKY63;	6 h	1h, 3 h, 6 h
AT1G43160	RAP2.6 | RAP2.6	6 h	3 h, 6 h

Table 5.10: Summary of Transcriptome and RT-qPCR gene expression comparisons of Glu-dependent selected candidate genes in *Arabidopsis thaliana*.

Gene ID	Name of gene	Trancriptome analysis	RT-qPCR
AT1G69930	ATGSTU11	1 h	3 h
AT5G01490	CAX4, ATCAX4 | CAX4	1 h, 6 h	1 h, 3 h
AT1G12663	PR13	1 h, 6 h	1 h, 6 h
AT5G22570	WRKY38, ATWRKY38 | WRKY38; t	1 h, 6 h	1 h, 3 h

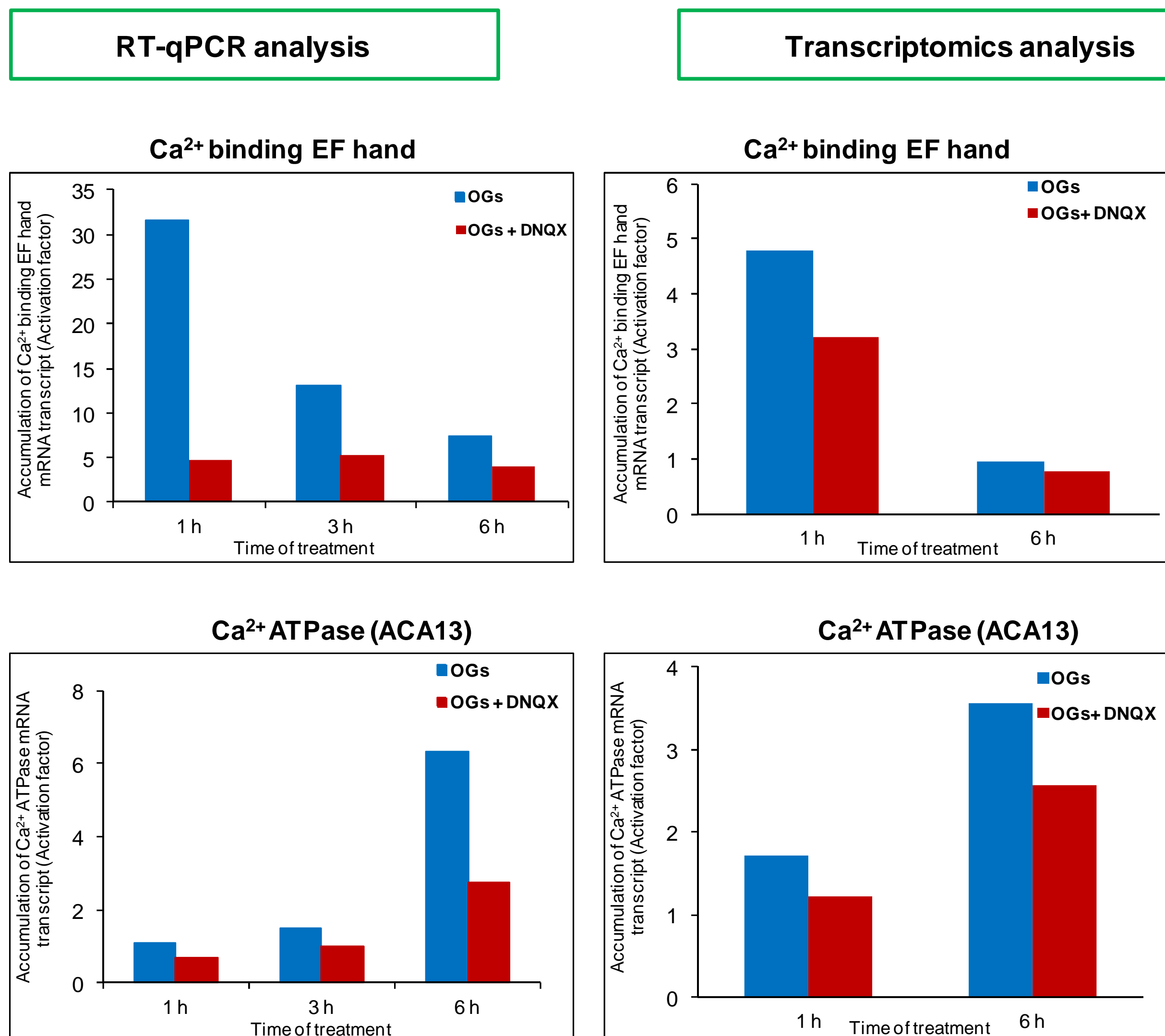
relatively short time to Glu and OGs exposure. Moreover, our transcriptome data revealed very interesting and informative results concerning the fascinating role of Glu, OGs and GLRs in plant defense responses.

Using our significance criteria (fold-change ≥ 2; *P* value ≤ 0.01), around 645 genes significantly changed their expression after Glu treatment. Among these, 463 genes (72 % of total Glu-responsive genes) were modulated within 1 h of Glu exposure. On the other hand, 182 transcripts (28 % of total OGs-responsive genes) modified their expression within 6 h of Glu treatment. This indicates that cells quickly modified the gene expression after perceiving Glu. Many of Glu-responsive genes belong to stress and (a)biotic stimuli categories and included PR proteins, transcription factors, and cell wall related proteins categories. These results clearly highlight the importance and role of Glu as a signaling molecule that participates in defense induction processes in plants.

By analyzing OGs-responsive genes through GO annotation, our data provide a general overview about the molecular mechanism modified by OGs treatments. Interestingly, about 2346 genes that constitute about 9 % of the total genes in *Arabidopsis* genome responded to OGs treatment, and about 10 % of the total OGs-responsive genes belong to signaling pathways. Among these, 791 genes (32 % of total OGs-responsive genes) were modulated within 1 h OGs exposure. In contrast, 1555 genes (63 % of total OGs-responsive genes) modified their expression within 6 h of OGs treatment. This indicates that the response to OGs treatment expended on longer period than the response to Glu treatment. Here, a large number of induced genes belongs to signal transduction, transport, stress and (a)biotic categories, and included disease resistance genes, transcription factor genes and genes coding for proteins involved in signaling, thus indicating that OGs are perceived as plant defense elicitor. Previous studies have demonstrated that the regulation of stress-related genes occurs primarily at transcriptional level, and plays a vital role in plant stress response (Rhouton and Eglume, 1998). In case of OGs, majority of genes were modulated at 6 h contrary to Glu treatment where most of the genes changed their expression at 1 h, suggesting that Glu signaling completed before than OGs signaling.

Recently, it has been shown that response to Glu treatment is much rapid than response to the elicitor Cry: Glu induced a more rapid $[Ca^{2+}]_{cyt}$ variation in tobacco cells. Moreover, elicitor treatment firstly resulted in Glu efflux through exocytosis and then increased concentration of Glu in the extracellular medium activated Glu signaling (Vatsa *et al.,* 2011). This may explain Glu and OGs transcriptome response in term of time kinetics.

Figure 5.10: Summary of comparison of expression pattern (profile) of selected GLRs- and Glu-responsive genes using transcriptomics and RT-qPCR analyses. For both transcriptomics and RT-qPCR, *Arabidopsis* (Col-0) plants were grown and treated under similar conditions as described in "Materials and Methods" section. Transcript accumulation was analyzed by real-time qPCR (left panel). After normalization with *UBQ10* gene expression, results were expressed as the fold changes in transcript level compared to control. The bar graph is the mean of the three technical repeats from one out of three biological replicates performed with similar results. On the right panel, the fold-change patterns from the transcriptome analysis. The bar graphs are the mean of three biological replicates.



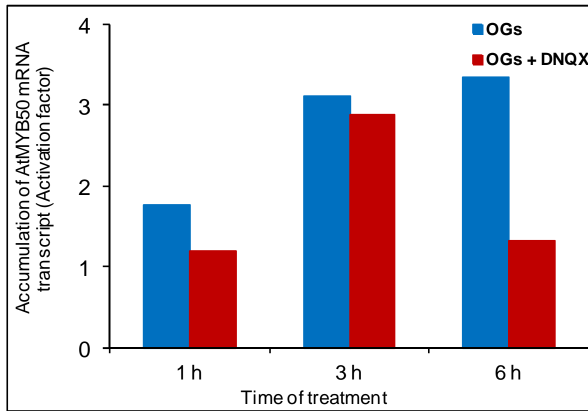
In *Arabidopsis*, various transcriptome analyses carried out with flg22, elf18, fungal MAMPs and hairpin treatment have displayed significant overlap among induced genes (Ramonell *et al.*, 2002; Zipfel *et al.*, 2004; Moscatiello *et al.*, 2006; Thilmony *et al.*, 2006). Moreover, it was reported that flg22 and OGs induced highly correlated early responses but the responses differed in late stages and kinetics (Denoux *et al.*, 2008). These data demonstrated that the transcriptional responses were predominantly elicitor-specific, but shared similarity in functions and processes (*e.g*. RNA regulation). This overlap between transcriptional changes and common gene expression suggests that all elicitors displayed a conserved basal response resulting from the convergence of a limited number of signaling pathways (Jones and Dangl, 2006). In conclusion, different elicitors induced changes in similar plant processes through largely conserved transcriptional modulations.

Comparative studies with already published microarray data, obtained after OGs treatments of *Arabidopsis* plants or cell suspensions (Ferrari *et al*., 2007 and Moscatiello *et al*., 2006), were also performed. These studies showed that an impressive number of commonly induced genes belong to transcription factors, signaling components, cell wall and PR Proteins categories. Surprisingly, we identified a low percentage of common genes in these comparisons (4-20 %). When we compared our data with that of Ferrari *et al*., (2007), 408 genes ware found to be commonly modulated. These genes were categorized in processes such as signaling, disease or defense, RNA regulation (TFs) and cell wall construction *etc.* In comparison to Moscatiello *et al*. (2006), only 25 genes were identified common. Finally, only 13 genes were identified common between these three studies. These common genes encode protein kinases, transcription factors and glycosyl hydrolase family protein *etc*. Overall, the number of identified common genes is really small despite the use of OGs as an elicitor compound in all these studies. These observations might not only be due to different OGs concentrations used, but may also result from either the use of different type of tissues or experimental systems.

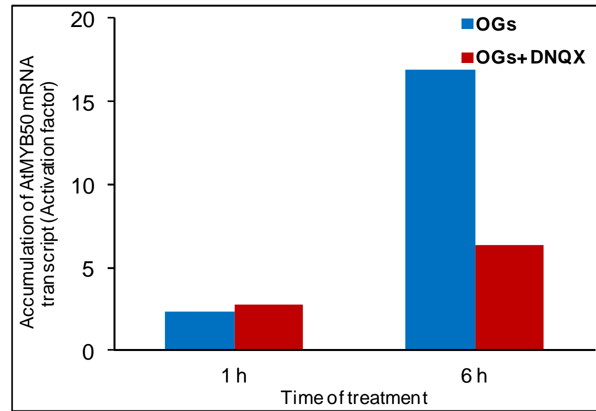
There also exist some similarities in transcriptome response between Glu signaling and GLRs-dependent genes in OGs signaling as some common genes were observed in both cases. There were 24 and 17 genes commonly modified in both cases at 1 h and 6 h, respectively (Table 5.6, Table 5.7). These genes belong to TFs, hormones, signaling and stresses. Some important genes identified were: CAX4 (At5g01490), ATGSTU11 (At1g69930), IAA12 (At1g04550) and LSU1 (At3g49580). These observations might be a clue about the important correlation between Glu signaling and GLRs activation in plants as it has already been documented by Vatsa *et al*. (2011). Common expression profiles were more

RT-qPCR analysis | **Transcriptomics analysis**

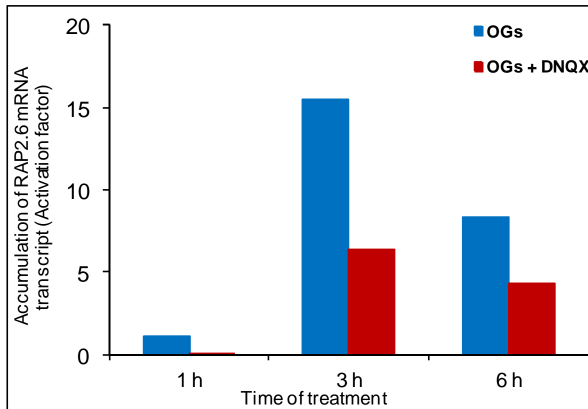
AtMYB50



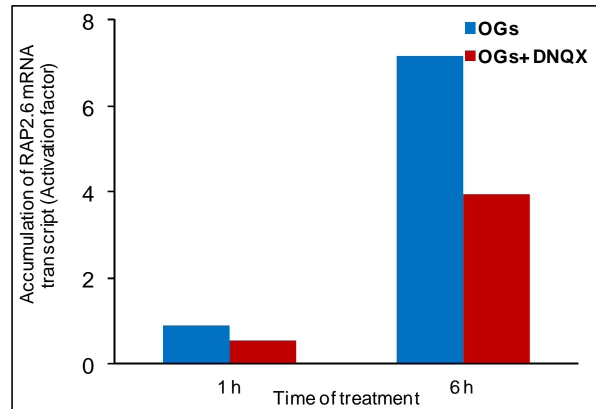
AtMYB50



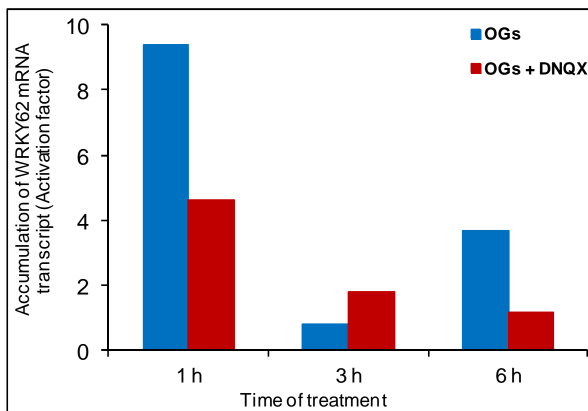
RAP2.6



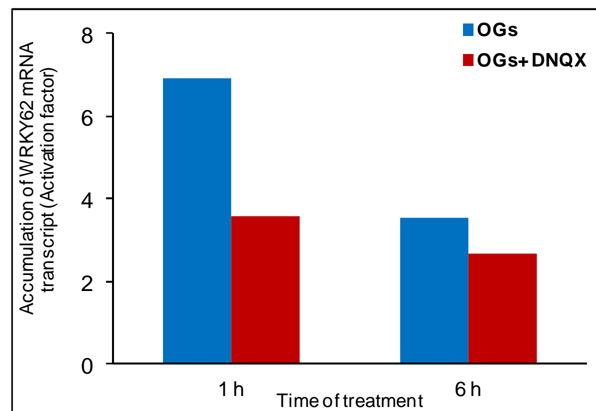
RAP2.6



WRKY62



WRKY62



coherent at 6 h treatment than at 1 h treatment, with genes being up- and down-regulated for both OGs and Glu treatments.

GLRs have been reported to play a role in Ca^{2+} influx and we found some of GLRs dependent genes that are known to be modulated by Ca^{2+} as reported by Moscatiello *et al.* (2006). These genes include At4g12400 (stress-inducible protein putative), At4g29360 (glycosyl hydrolase family 17 protein), At4g19810 (glycosyl hydrolase family 18 protein), At5g67080 (MAPKKK19), At1g21910 (AP2 domain-containing transcription factor family protein), At4g04700 (CPK27) and At4g39510 (CYP96A12). So, according to these results, we could assume that signaling through GLRs and overall Ca^{2+} signaling linked to unique sets of signaling, transcriptional and physiological pathways which ultimately share common downstream events that lead to biological response.

OGs treatment modulates about 9 % of the genes from *Arabidopsis* and about 66 % of these genes behaved in a GLRs-dependent manner. Most of the changes were observed at 6 h of treatment. MapMan biotic stress diagram showed 7 categories (including stress- and disease-related proteins, signaling components, cell wall and transcription factors) were significantly affected in GLRs-regulated transcriptome response at 1 h and 6 h of OGs treatment. GLRs-oriented genes encode Ca^{2+} transport proteins and transcription factors of WRKY, MYB and ERF families. Moreover, GLRs-responsive genes include some members of GLRs as well as receptor kinases. Another important feature of GLRs-responsive genes is the coordinated response of proteolysis and signaling related genes in the biotic stress pathway. The genes in these groups are mostly up-regulated at 1 h but at 6 h of treatment majority of genes were down regulated. These observations strengthen our hypothesis that co-expression of regulated genes is under the control of set of TFs that bind to common *cis*-regulatory elements in the promoter regions of regulated genes.

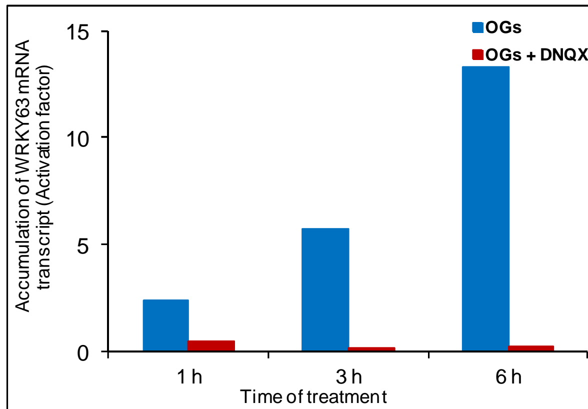
From our transcriptomic data, 10 genes were selected to validate their expression pattern through RT-qPCR. These genes were either under the control of GLRs in OGs signaling pathway (GLRs-dependent: 6 genes) or were specifically modulated by Glu treatment (Glu-dependent: 2 genes), and in some cases were controlled by both *i.e.* GLRs and Glu (details of these genes are present in Table 5.8). The selection of these genes was made on the basis of their reported role in plant stress responses. Good correlation between transcriptome data and RT-PCR analysis was observed in our investigations.

Downstream, a survey of the literature is provided concerning the involvement of these genes mainly during the biotic stress induced pathways.

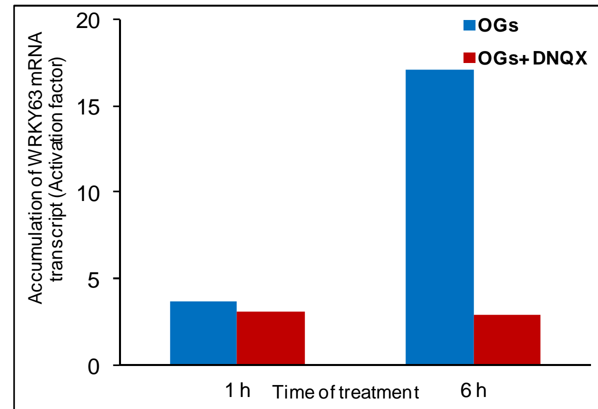
RT-qPCR analysis

Transcriptomics analysis

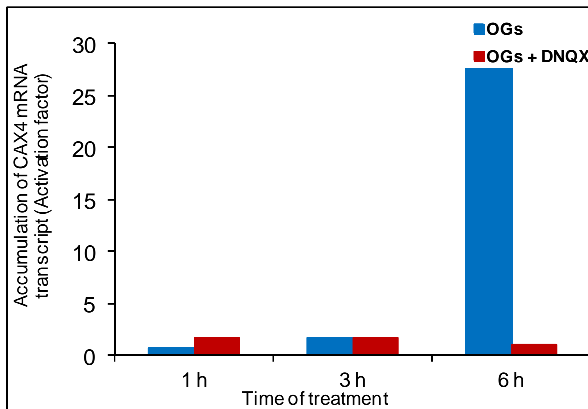
WRKY63



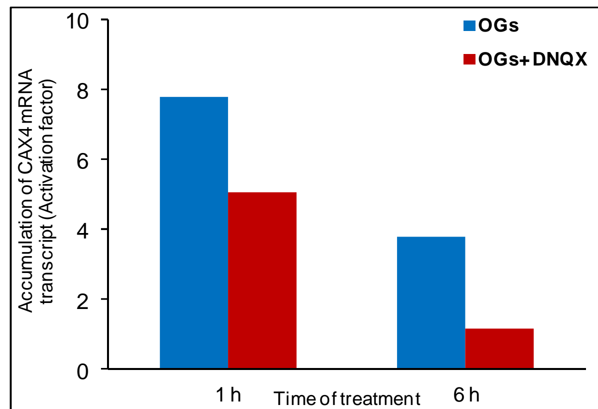
WRKY63



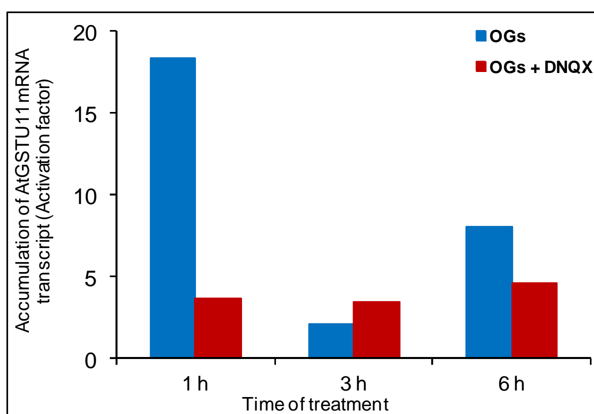
CAX4



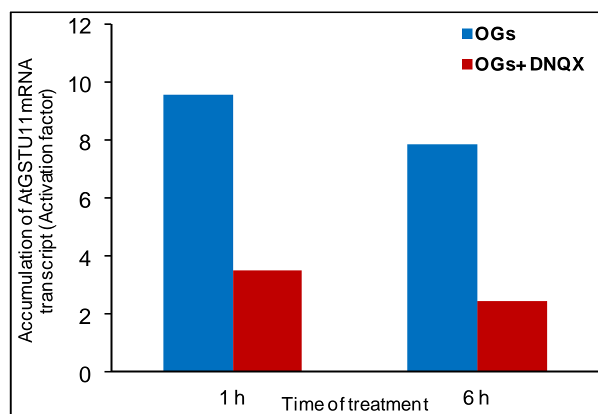
CAX4



ATGSTU11



ATGSTU11



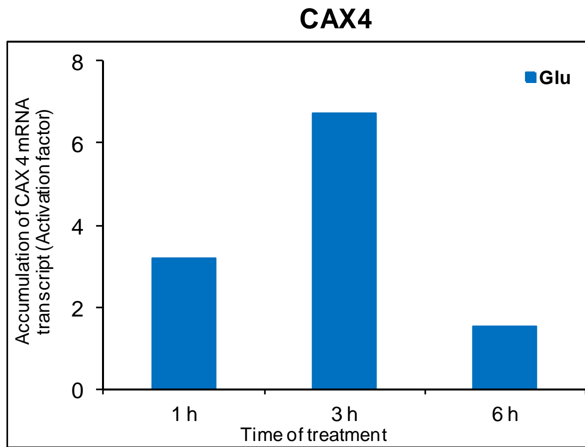
We know that Ca^{2+} participates in almost all kind of growth and developmental processes in plants and change in $[Ca^{2+}]_{cyt}$ is one of the determinant step to regulate these processes. The uptake of Ca^{2+} and its redistribution is important for homeostasis and to transduce endogenous and exogenous stimuli. These functions relied on the presence of proteins of different classes. Ca^{2+} channels are thermodynamically passive and are present in the plasma membrane and endomembranes *e.g*. GLRs, CNGCs and TPC. Through these channels, Ca^{2+} transportation is made possible into the cytosol. On the other hand, Ca^{2+} efflux transporters are thermodynamically active and rapidly remove excessive Ca^{2+} from the cytosol to maintain an optimum $[Ca^{2+}]_{cyt}$ inside the cell (McAinsh and Pittman, 2009). Removal of excessive Ca^{2+} is of utmost importance as higher Ca^{2+} is toxic for plant health. In plants, CAX antiporters and P-type ATPase pumps are the principal molecular entities. Both of these active transporters load Ca^{2+} into specific cell compartments. Moreover, CaM-activated Ca^{2+} pumps in endomembrane systems also play a significant role in maintaining Ca^{2+} homeostasis that could otherwise prove fatal to plant (Spalding and Harper, 2011).

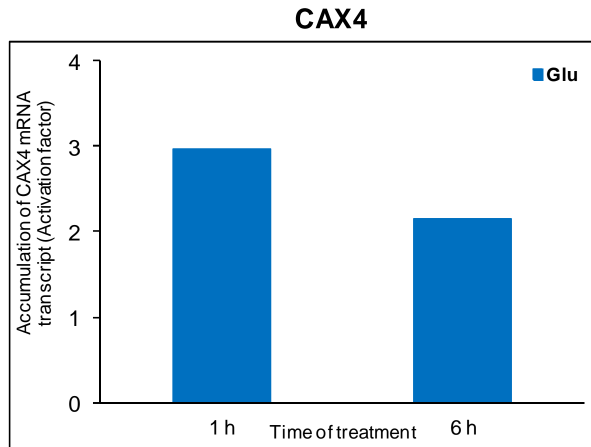
A significant number of genes related to Ca^{2+} transportation during plant stress responses were also identified in our study. Among these, genes encoding Ca^{2+} binding EF-hand protein (At3g29000), CaM binding (At3g25600 and At4g20780), Ca^{2+}-ATPase protein (At3g22910) and ATCAX4 (At5g01490) are related to Ca^{2+} transportation during plant stress responses. The *Arabidopsis* genome contains seven CAM and 50 CAM-like (CML) genes that encode potential calcium sensors (McCormack *et al.*, 2005). Moreover, the presence of about 232 / 250 EF hand-containing proteins has been demonstrated in *Arabidopsis* (Day *et al.*, 2002). Wang *et al.* (2008) indicated in their microarray analysis the up-regulation of At3g25600 (CML16) and At4g20780, (CML42) during pollen tube growth (PTG) process.

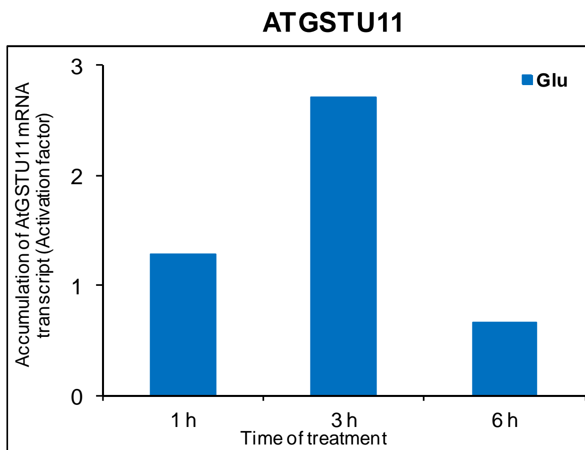
ATCAX4 (At5g01490) gene was commonly up-regulated during OGs and Glu treatment. It is member of CAXs (for CAtion eXchanger) family which is one of the 6 members in the Ca^{2+}/cation antiporter (CaCA) superfamily proteins, a type of integral membrane proteins with 10 to 11 transmembrane (TM) domains that transport Ca^{2+} or other cations using the gradient of H^+ or Na^+ generated by energy-coupled primary transporters (Busch and Saier, 2002; Cai and Lytton, 2004; Shigaki *et al.*, 2006). CAXs proteins participate in a multitude of cellular responses in plants. They are thought to have an impact on Ca^{2+} and other heavy metal signaling events (Shigaki and Hirschi, 2006; McAinsh and Pittman, 2009). Members of the *Arabidopsis* CAX gene family have been well characterized at both the molecular and whole-plant level. CAX4 is preferentially expressed in roots and has 53 % amino acid sequence similarity with CAX1, 42 % identical to CAX2, and 54 % identical

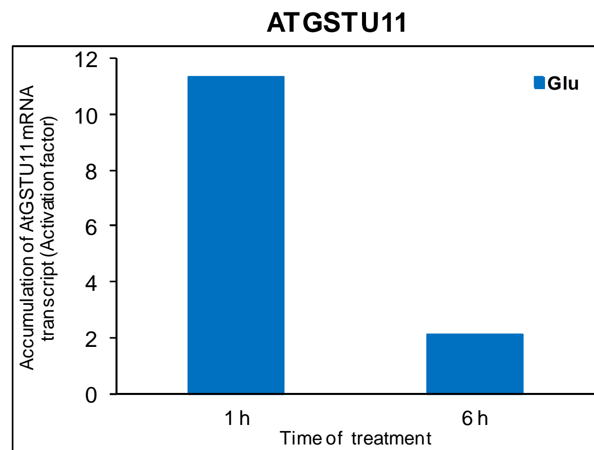
RT-qPCR analysis

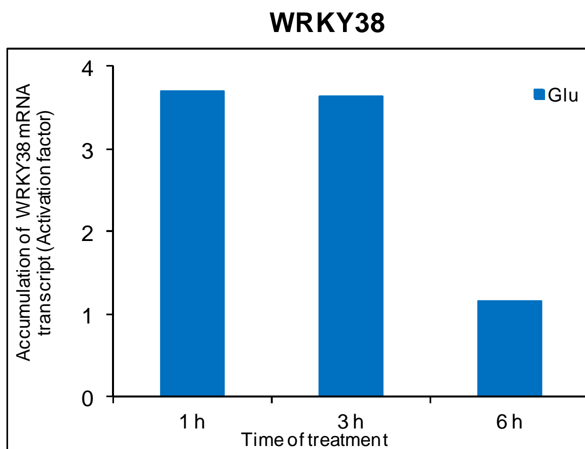
Transcriptomics analysis

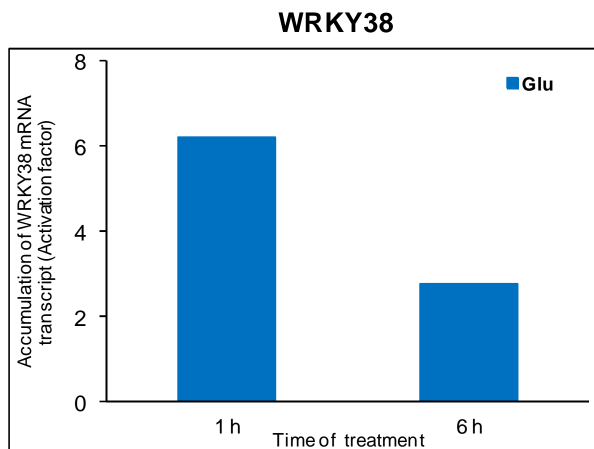












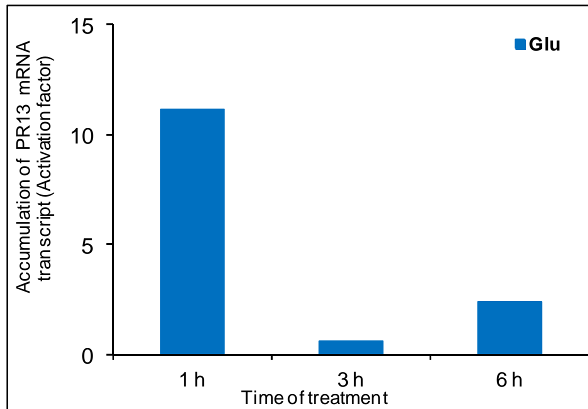
to CAX3 (Cheng *et al.*, 2002). In *Arabidopsis,* CAX4 is involved in root growth and development under metal (Ni^{2+} or Mn^{2+}) stress and is capable of transporting Cd^{2+} as well as Ca^{2+} into the vacuole. Moreover, CAX4 cation/H^+ antiport activity is necessary for auxin-mediated root growth and development in *Arabidopsis* (Mei *et al.*, 2009). Addition of amino acids to the N terminus of CAX4 and CAX3 caused both transporters to suppress the sensitivity of yeast strains deficient in vacuolar Ca^{2+} transport. These findings suggest that CAX transporters may modulate their ion transport properties through alterations at the N terminus (Cheng *et al.*, 2002). Among other members of CAX family, CAX1 is a low-affinity and high-capacity Ca^{2+}/H^+ antiporter and a higher expression of CAX4 has been observed in *cax1* knockout line in *Arabidopsis*. This increased expression of CAX4 resulted in a 29 % increase of Ca^{2+}/H^+ antiport activity in *cax1* mutant (Mei *et al.*, 2009). In *Arabidopsis*, CAX1 is highly expressed in leaf tissue, and modestly expressed in roots, stems, and flowers. CAX1 is the most regulated gene in Ca^{2+}-stressed mutant, while CAX3 is most abundant in roots and its expression increases upon overnight exposure to exogenous Ca^{2+} (Cheng *et al.*, 2003; Cheng *et al.*, 2005; Chan *et al.*, 2008). Moreover, *cax1/cax3* double mutant plants display more severe Ca^{2+} sensitivity than either of the single mutants (Cheng *et al.*, 2005). CAX3 is not only involved in salt stress as *cax3* mutant lines showed an altered response to Na^+ and Li^+ but also exhibited sensitivities to low pH conditions. In addition, *cax3* mutant lines also displayed reduced plasma membrane H^+-ATPase activity (Zhao *et al.*, 2008).

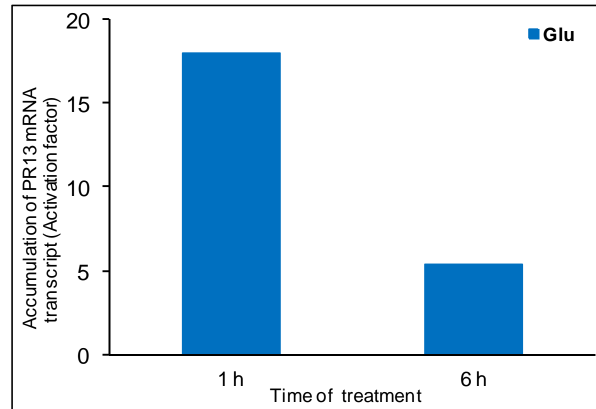
As endomembrane Ca^{2+} transporters are believed to play a significant role in specifying the duration and amplitude of cytosolic Ca^{2+} fluctuations (Sanders *et al.*, 2002). The identification of different Ca^{2+} transporting families in response to Glu and GLRs-dependent OGs signaling highlights that Glu and GLRs are not only important in mediating Ca^{2+} influxes and $[Ca^{2+}]_{cyt}$ variations but also consequently regulate the activities of efflux transporters in order to maintain a Ca^{2+} balance in plants and to respond under stress conditions in plants.

Role of different wall associated proteins families cannot be ignored in signaling processes, especially related to plant defense. A significant number of genes related to cell wall associated families were activated in our transcriptomic analysis. Shiu and Bleecker (2001) have identified a gene family similar to Wall associated kinases (WAKs) in *Arabidopsis* containing 22 members, and was named as WAK-like (WAKL). In our data, 1 h Glu and OGs treatments induced WAKL13 (At1g17910) and WAKL4 (At1g16150), respectively (Supplemental Table S8 and S9). WAKs represent a unique class of receptor-like kinase (RLK) genes that encodes a transmembrane protein with a cytoplasmic Ser/Thr kinase

RT-qPCR analysis

Transcriptomics analysis





(STK) domain and an extracellular region with similarity to vertebrate epidermal growth factor (EGF)-like domains (Verica and He, 2002). WAKs are considered to physically link the extracellular matrix and the cytoplasm and to serve a signaling function in these compartments (He *et al.*, 1996; Kohorn, 2000). In *Arabidopsis*, WAKs and WAKL are assigned to play important role during plant development, pathogen resistance and heavy-metal tolerance (Kanneganti and Gupta, 2008). Recently, WAK1 was identified as a receptor of OGs (Brutus *et al.*, 2010). Previous studies have shown that WAK1 is induced by SA in an NPR1-dependent manner (nonexpresser of pathogenesis-related genes) and its induction is required for plant resistance to *Pseudomonas syringae* and during SAR (Maleck *et al.*, 2000). Moreover, WAK1 is induced by the fungal pathogen *Alternaria brassicicola* and the defense related signaling molecules methyl JA and ET (Schenk *et al.*, 2000). Genetic studies showed that WAKL4 is involved in mineral nutrition responses in *Arabidopsis* where its expression is induced by Na^{+}, K^{+}, Cu^{2+}, Ni^{2+} and Zn^{2+}. Moreover, WAKL4 promoter impairment inhibited WAKL4-induced expression by all the metal ions except the Ni^{2+} (Hou *et al.*, 2005).

The polygalacturonase (PG) gene family is another wall associated family identified in our transcriptome analysis. It is one of the largest gene families in plants. PG is a pectin-digesting enzyme with a glycoside hydrolase 28 domain. These genes are involved in numerous plant developmental processes (Kwon *et al.*, 2008). With Glu and OGs elicitation at 1 h, At4g13760 was commonly activated. Similarly, At5g62150, a peptidoglycan-binding LysM domain-containing protein also showed a highly significant up-regulation at 1 h of OGs treatment. This gene has already been identified by Thilmony *et al.* (2006) in response to *P. syringae* and *E. coli*. Moreover, At5g62150 was also identified during transcriptome response of cabbage leaf curl virus (CaLCuV) infection in *Arabidopsis* (Ascencio-Ibáñez *et al.*, 2008). These results showed the important role of different cell wall associated proteins in defense responses and their activation in response to Glu and OGs clearly demonstrate the role of these signaling molecules in plant defense.

In our study, a large number of TFs belonging to different families were also identified. Generally, plants devote a large portion of their genome capacity to transcription, with the *Arabidopsis* genome coding about 1600 TFs that represents about 6 % of total genome (Riechmann *et al.*, 2000). A single TF can regulate the expression of numerous genes including its own gene and activates the adaptation process of organism to changed environment (Khong *et al.*, 2008). These TFs often belong to large gene families, which in some cases are unique to plants. It has been demonstrated that about 45 % of TFs are from families specific to plants (Riechmann *et al.*, 2000). It is evident from the literature that

reprogramming of the transcriptome is an important aspect of stress signaling and adaptation in plants. However, molecular mechanisms by which stresses modulate gene expression and the role of stress-regulated genes in stress adaptation are just beginning to be uncovered. Previous studies have shown that changes in the transcriptome are primarily established by changes in gene expression, which are regulated by TFs (Brivanlou and Darnell, 2002). The functional link between TFs and defense genes during plant stress responses has been shown for specific proteins (Pandey and Somssich, 2009; Galon *et al.*, 2010).

In our transcriptome data, many WRKY family genes (-28, -38, -43, -54, -55, -62, -63, -64, -67 and -74) found to be up-regulated in response to Glu and OGs treatment. A total of 74 WRKY genes are present in *Arabidopsis thaliana* genome (Eulgem and Somssich, 2007). Their DNA binding domain (WRKY domain) comprises 60 amino acids, but the overall structures of WRKY proteins are highly divergent and can be categorized into distinct groups, which might reflect their different functions (Euglem *et al.*, 2000). Multiple studies have demonstrated the ability of WRKYs to bind the W box element (TTGACC/T) (Yamasaki *et al.*, 2005; Rushton *et al.*, 2010), which is found in the promoters of many plant defense genes (Maleck *et al.*, 2000; Chen *et al.*, 2012). W box or W box-like sequences often occur in clusters within promoters, suggesting a possible synergistic action with other WRKY proteins and/or other classes of transcription factors (Maleck *et al.*, 2000).

As far as the function of WRKY is considered, they are involved in the regulation of various physiological processes and their expression is modified during wounding, pathogen infection, sugar signaling, senescence, trichome development, root growth and phosphate acquisition, drought, cold adaptation and heat-induced chilling tolerance (Euglem *et al.*, 2000; Chen *et al.*, 2012). The majority of the analyzed WRKY genes respond to pathogen attack and to the endogenous signal molecule SA (Eulgem and Somssich, 2007). WRKY62, WRKY63 and WRKY38 are involved in many processes related to plant defense against stresses (Rushton *et al.*, 2010). WRKY38 and WRKY62, two structurally related WRKY TFs of type III, are induced by both pathogen infection and SA treatment (Dong *et al.*, 2003; Kalde *et al.*, 2003; Mao *et al.*, 2007). Similarly, AtWRKY38 and AtWRKY62 contribute negatively to basal resistance towards *P. syringae* (Kim *et al.*, 2008). AtWRKY62 expression is induced by SA and JA in a NPR1-dependent manner. It has been demonstrated that loss of AtWRKY62 function resulted in up-regulation of JA-responsive gene (LOX2) and SA-response gene (PR1), whereas AtWRKY62 overexpressor lines led to suppression of JA- and SA-response genes (Mao *et al.*, 2007; Kim *et al.*, 2008). The single and double mutants of *wrky38* and *wrky62* have enhanced disease resistance to *PstDC3000* and WRKY38 and WRKY62

suppress the expression of defense and defense-related genes, including SA-regulated PR1. These results indicate that WRKY38 and WRKY62 function additively as negative regulators of plant basal defense. In another study in rice, Peng *et al*. (2008) reported that OsWRKY62 overexpressing plants were compromised in basal defense, Xa21-mediated resistance to *Xanthomonas oryzae* and resulted in the down-regulation of defense related genes. These results further suggest the role of WRKY62 as negative regulator in innate immunity and race-specific defense responses. WRKY63 (ABO3) has been reported to play an important role in plant responses to ABA and drought stress. Upon ABA perception, ABI5 (a bZIP transcription factor) is activated following phosphorylation by the SnRK2 kinase and this leads to the transcriptional activation of the AtWRKY63. In turn, AtWRKY63 activates downstream target genes of ABA signaling such as RD29A, ABF2 and COR47 (Rushton *et al.*, 2012). In a recent work, Ren *et al.* (2010) demonstrated that *abo3* mutant showed hypersensitive response to ABA during seedling growth and establishment stage. Moreover, *abo3* mutant was more sensitive to drought stress due to its lower sensitivity to ABA-induced stomatal closure. WRKY38, WRKY54 and WRKY66 are among eight WRKY genes (WRKY18, 38, 53, 54, 58, 59, 66, and 70) identified as direct targets of NPR1 thus suggesting their role in defense pathways (Wang *et al.*, 2006; Spoel *et al.*, 2009). At higher SA accumulation levels, WRKY54/70 act as negative regulators of SA biosynthesis probably by direct negative regulation of ICS1 (a marker gene of SA pathway). At the same time, they activate other SA-regulated genes thus indicating the dual role of these WRKY homologs in plant defense (Kalde *et al.*, 2003; Wang *et al.*, 2006). Flg22 treatments lead to the activation of MAPK pathway with a subsequent accumulation of SA. SA accumulation is under the strong control of *ICS1* and it is suggested that activation of *ICS1* gene expression is likely to occur *via* WRKY transcription factors. It has been observed that flg22 treatments rapidly results in the activation of WRKY28 (Navarro *et al.*, 2004). These results demonstrate the role of WRKY induced SA pathway that is also an important component of plant defense.

Another important class of TFs is MYB family proteins. MYB is diverse class of DNA-binding gene family in plants and is subdivided into groups depending on the sequence of the binding site. Usually, a MYB domain is composed of one to three imperfect repeats, each with about 52 amino acid residues that adopt a helix-turn-helix conformation that intercalates in the major groove of the DNA (Yanhui *et al.*, 2006). In *Arabidopsis*, MYB superfamily is the largest TFs family. *Arabidopsis* MYB proteins are classified into following different groups: R2R3-MYB, with two adjacent repeats (126 members); R1R2R3-MYB, with three adjacent repeats (5 members); 4R-MYB, the smallest class with four R1/R2-like

repeats (1 members) and IR-MYB or MYB-related proteins (64 members) which usually but not always, contain a single MYB repeat (Dubos *et al.*, 2010). Among these groups, R2R3-MYB was extensively studied in the past and has been reported to be involved in many physiological and biochemical processes, such as the regulation of primary and secondary walls construction, developmental processes, cell fate and identity, and responses to biotic and abiotic stresses (Yanhui *et al.*, 2006; Dubos *et al.*, 2010).

In our study, MYB39, MYB78 and MYB83 were overexpressed with Glu treatment, whereas MYB40, MYB50, MYB61 and MYB98 were significantly up-regulated with OGs treatment in GLRs-dependent manner. MYB50 and MYB61 belong to sub-group 13 of R2R3-MYB group. In *Arabidopsis*, MYB61 has been identified to play a role in mucilage production, pleiotropic effect by influencing lignin deposition, and stomatal aperture, suggesting that it might act upstream of different pathways perhaps by regulating carbon allocation (Penfield *et al.*, 2001; Newman *et al.*, 2004; Liang *et al.*, 2005; Zhao and Dixon, 2011). Yanhui *et al.* (2006) reported an increase in the expression level of MYB50 in *Arabidopsis* plants when treated with different hormones including SA, JA, IAA and GA. In the same study, they also showed that salt stress also enhanced the expression of MYB72. These data suggest the role of MYB50 and MYB78 in hormone signaling and plant stress responses (Yanhui *et al.*, 2006). Previous studies have demonstrated that MYB83 is another molecular switch in the SND1-mediated transcriptional network regulating secondary wall biosynthesis. In MYB83 overexpressing plants, a number of the biosynthetic genes induce ectopic secondary wall deposition. Moreover, double T-DNA knockout mutations of MYB83 and its homolog MYB46 cause a lack of secondary walls in vessels and an arrest in plant growth. These results demonstrate that MYB83 and MYB46, both of which are SND1 direct targets, function redundantly in the transcriptional regulatory cascade leading to secondary wall formation in fibers and vessels (McCarthy *et al.*, 2009). MYB98 has been shown to be localized in the synergid cells nuclei and is implication in the regulation of the expression of a certain set of genes that are needed for pollen tube guidance and filiform apparatus formation (Kasahara *et al.*, 2005; Punwani *et al.*, 2007). From other MYB TFs, MYB102 has been reported to contribute to plant resistance against insects and probably affects dehydration after wounding (De Vos *et al.*, 2006). Similarly, MYB72 is a key regulator required in *Arabidopsis* roots during early signaling steps of induced systemic resistance mediated by beneficial fungi and bacteria (Van Der Ent *et al.*, 2008; Segarra *et al.*, 2009). However, putative role of MYB39 and MYB40 has not been demonstrated yet.

In our transcriptome data, members from AP2/ERF family of TFs, were also identified. This family belongs to APETALA2 (AP2) gene family of TFs containing 145 members in *Arabidopsis* (Sakuma *et al.*, 2002). This family has a characteristic AP2 domain, which contains 68 amino acids and is also referred to as AP2/ethylene responsive element binding factor domain (AP2/ERF) (Hao *et al.*, 1998). AP2/ERF genes can be classified into two groups based on the number of AP2-DNA binding domains. The first group is AP2-like TFs, which encodes proteins with two AP2 domains (Gutterson and Reuber, 2004). The second group is ERF-like TFs which encodes proteins with only one AP2 domain and includes C-repeat/dehydration responsive element binding factors (CBFs/DREBs). Based on their DNA-binding regions, AP2/ERF genes are classified into five subfamilies: AP2, ERF, RAV, DREB, and others (Sakuma *et al.*, 2002). The ERF and DREB subfamily proteins regulate many stress responsive genes by binding to defined *cis*-regulatory sequence in their promoter regions (Guo *et al.*, 2005). In addition to their involvement in plant growth and development, AP2/ERF TFs have been implicated in the transcriptional regulation for increasing tolerance to biotic and abiotic stresses in plant (Chen *et al.*, 2008; Zhang *et al.*, 2009). RAP2.6 and RAP2.6L belong to ERF subfamily (Guo *et al.*, 2005). RAP2.6 is involved in plant stress and has been shown to code for protein that possesses transcription activator function (He *et al.*, 2004; Zhu *et al.*, 2010). RAP2.6 transcripts significantly increased following exposure to NaCl, heat, drought but was unaffected by freezing. RAP2.6 has been associated with signal transduction during infection of *Arabidopsis* with *Pseudomonas syringae* (He *et al.*, 2004). Moreover, RAP2.6 is responsive to both abiotic stresses and hormones JA, SA, ABA and ET. Phytohormone ABA is involved in abiotic stress signaling whereas hormones like JA, SA and ET are part of biotic stress response (Fujita *et al.*, 2006). It has been shown that among different TF families, ERF family is most responsive to JA and *Alternaria brassicola* (McGrath *et al.*, 2005). The gene ERF1, a member from the ERF subfamily has been suggested to integrate JA and ET signaling pathways in *Arabidopsis* and has also been demonstrated to confer resistance to necrotrophic fungi such as *B. cinerea* and *Plectosphaerella cucumerina* when overexpressed (Berrocal-Lobo et al., 2002). Moreover, it was studied that RAP2.6 over-expressing lines are hypersensitive to NaCl and ABA compared to WT (Zhu *et al.*, 2010) and have no difference in phenotype. In contrast, Krishnaswamy *et al.* (2011) did not observe sensitivity/ hypersensitivity of RAP2.6 transgenic lines to NaCl and ABA.

In *Arabidopsis* genome, 51-53 glutathione transferases (GSTs) belong to a group of soluble enzymes that can be divided into phi, tau, theta, zeta, lambda, dehydroascorbate

reductase (DHAR) and tetrachlorohydroquinone dehalogenase-like (TCHQD) classes (Sappl *et al.*, 2004; Dixon and Edwards, 2010). Plant GSTs are classified as enzymes of secondary metabolism, but while their roles in catalyzing the conjugation and detoxification of herbicides are well known, their endogenous functions are largely obscure. Proteomics data revealed that the GST transcripts are strongly up-regulated in response to stress, however these correlations did not lead to any major new insights into defining GST function. Individual studies focusing on a subset of GSTs have again shown the strong inducibility of many of the transcripts (Deridder *et al.*, 2002; Wagner *et al.*, 2002; Sappl *et al.*, 2009) and each GST shows a distinct pattern of stress responsiveness. Analysis of multiple microarray expression data showed a diverse regulation of GST transcripts (Dixon *et al.*, 2010), and links with flavonoid metabolism, glucosinolate and phytoalexin synthesis and defense response identified. The plant-specific tau GSTUs are the most numerous GST class in *Arabidopsis* and also in other plants examined and are named as "Type III" GSTs (Droog *et al.*, 1995). Many *Arabidopsis* GSTUs have been identified as auxin-responsive genes. Induction by auxin highlights that this subset of GSTs is particularly abundant in actively growing tissues. From microarray data, GSTU9 (At5g62480) is present in mature seeds, and its expression in roots is induced following exposure to saline conditions or to ABA. GSTU10 (At1g74590) is also salt-inducible, with its transcripts accumulating in senescing tissues. Both GSTU9 and GSTU10 bind free C18 fatty acid derivatives such as divinylethers in plants. The second tau clade comprises GSTs U11 to U18. GSTU11 (At1g69930) was inactive towards all GST substrates tested despite retaining the active-site serine (Dixon and Edwards, 2010). Recently, Lin *et al.* (2011) identified the up-regulation of ATGSTU11 in short phosphate (Pi) deficiency (1 h) conditions in their microarray analysis. On the other hand, At3g22910 (a CaM binding gene that is significantly overexpressed in our data) was repressed in Pi deficiency conditions in *Arabidopsis*.

Numerous pathogenesis-related proteins (PRs) belonging to different families (thionins and defensin-like) showed modified expression in our data. At1g12663 is one of the highly induced PRs gene identified in transcriptome data of Glu treated plants. It belongs to thionins (PR13) family of PRs in *Arabidopsis*. Previous studies have demonstrated that PRs are inducible proteins identified in many plant species in response to pathogen attack. So far, 17 well recognized families of PRs (named as PR-1, -2, -3 and so on) have been identified in different crop species (Van Loon *et al.*, 2006; Sels *et al.*, 2008). PRs families are also known as β-1,3-endoglucanases (PR-2), endochitinases (PR-3, -4, -8, and -11), thaumatin-like (PR-5), proteinase inhibitors (PR-6), defensins (PR-12) and thionins (PR-13) *etc.* (Edreva, 2005;

Van Loon *et al.*, 2006). PRs are induced through the action of SA, JA or ET and are thought to play a role in active defense by restricting pathogen growth and spread in the plants (Van Loon *et al.*, 2006). These proteins are used as molecular markers in hormone signaling *i.e.* induction of PR-1 gene is under SA signaling and it is a well known SA marker gene in SAR while PR-12 gene (PDF1.2) is usually taken as a molecular marker for the induction of the JA- and ET-dependent defense-signaling pathway (Lay and Anderson, 2005). PDF1.2b (At2g26020) and PDF1.3 (At2g26010) were among the up-regulated plant defensin genes in 6 h OGs treatment thus indicating the possible role of OGs in JA- and ET-dependent defense-signaling pathways.

Thionins (PR13) have broad antibacterial and antifungal activities and like plant defensins, their antimicrobial effects lead to the permeabilization of cell membranes (Stec, 2006). Thionins have a direct role in *A. thaliana* defense against *Fusarium oxysporum* (Epple *et al.*, 1995). It was reported that wounding and several JA related compounds like coronatine were also able to induce thionins and that this induction could not be observed in the *coi1* mutant background (Bohlmann *et al.*, 1998). Moreover, transcripts of *PR-1* and *PR13/thionin* were induced in tobacco after *P. syringae* challenge and PR13/ thionin demonstrated antibacterial activity and participated in plant resistance to herbivores (Rayapuram *et al.*, 2008). There are indications for a regulatory role as thionins have thioredoxin activity and hereby could act as secondary messengers in the redox regulation on enzymes (Johnson *et al.*, 1987). Thionins found in seeds, could also function as storage proteins, especially as a source of sulfur (Castro and Fontes, 2005). Overall, PR proteins participate not only in defense response but other in vivo functions have also been proposed for thionins in plants.

7. Conclusions

Finally, this study provides an overview into the molecular basis for the seemingly diverse biological functions in plants. From this above information, following general conclusions could be drawn:

- Glu and OGs are very important signaling components in plant stress responses. These molecules modulate the expression of a large number of gene transcripts that actively participate in plant physiological responses. With OGs elicitation, about 10 % of the whole *Arabidopsis* genome showed a modified expression.
- Glu signaling is more rapid than OGs signaling as most of the Glu-dependent genes transcripts altered their expression at 1 h contrary to 6 h time point with OGs elicitation.

- The modified genes belong to all important categories like signaling, cell wall associates, PR proteins, and TFs of different classes (MYB, WRKY and ER). These genes are involved in different regulation pathways in plants thus indicating the broader applications of Glu and OGs signaling in plants.
- Moreover, a strong link between GLRs and OGs signaling was observed as a large number of OGs modified genes (about 60 %) behaved in GLRs-dependent manner. These results clearly suggest that GLRs are potentially involved in OGs-mediating Ca^{2+} fluxes and downstream Ca^{2+} signaling.
- Interestingly, numerous OGs-mediated GLRs-dependent genes significantly altered their expression during biotic stress pathway. This highlights that GLRs are very important proteins in plants and the initiation of various signaling processes especially related to plant defense is dependent on the activation of these channels.
- Further functional characterization of these GLRs-dependent genes will definitely increase our understanding of the GLRs-dependent Ca^{2+} signaling mechanism during plant-pathogen interactions.

CHAPTER 5

“Supporting information”

ANNEX 5

Supplemental tables are present in the CD provided with this manuscript. CD contains:

Supplemental Table 1: List of Glu-responsive genes in *Arabidopsis thaliana* 1 h post Glu treatment.

Supplemental Table 2: List of *Arabidopsis thaliana* Glu-responsive genes at 6 h after Glu treatment.

Supplemental Table 3: List of OGs-responsive genes 1 h post OGs treatment in *Arabidopsis thaliana*.

Supplemental Table 4: List of OGs-responsive genes 6 h post OGs treatment in *Arabidopsis*.

Supplemental Table 5: List of OGs-responsive common genes at 1h and 6 h of OGs elicitation in *Arabidopsis.*

Supplemental Table 6: List of common genes in *Arabidopsis* treated with OGs (My own data vs Ferrari *et al*., 2007).

Supplemental Table 7: List of common genes in *Arabidopsis* treated with OGs (My own data vs Moscatiello *et al*., 2006).

Supplemental Table 8: List of GLRs-responsive genes at 1 h post OGs treatment in *Arabidopsis thaliana.*

Supplemental Table 9: List of GLRs-responsive genes at 6 h post OGs treatment in *Arabidopsis thaliana.*

CHAPTER 6

CHAPTER 6

“Discussion and Perspective”

CHAPTER 6

DISCUSSION AND PERSPECTIVES

1. Discussion

Ca^{2+} is the second most abundant metal on the earth and is an essential plant nutrient. As a second messenger, it participates in a variety of physiological and developmental processes in plants and change in $[Ca^{2+}]_{cyt}$ is one of the determinant step to regulate these processes. In plants, different developmental cues and environmental challenges lead to the generation of Ca^{2+} signals that take the form of a strong, rapid and transient increase in $[Ca^{2+}]_{cyt}$ whose frequency, amplitude and shape is determined by the nature of the stimulus (McAinsh and Pittman, 2009; Dodd *et al*., 2010; Boudsocq and Sheen, 2010). These $[Ca^{2+}]_{cyt}$ variations are resulted not only by the regulatory movement of Ca^{2+} ions between the cell and its extracellular environment but also through Ca^{2+} exchange between the cytosol and different cell organelles. As the higher Ca^{2+} levels inside the cells have cytotoxic effects so Ca^{2+} levels should be maintained at optimal level for the normal cell functioning. All this requires the presence of a very sophisticated regulation mechanism to efficiently fulfill these tasks: Ca^{2+} transport system comprising of different channels and transporters, and the Ca^{2+} sensors are the essential components of these stimulus-induced Ca^{2+} signaling pathways in plants. Plants possess a very complicated network of different types of channels, pumps and exchangers that are present on both the plasma membrane and endomembranes. This transport system leads to a very fine regulation of intra- and intercellular influx and efflux activities in plants. A large number of Ca^{2+} channels have been characterized through electrophysiological approaches however molecular identity of these channels is really limited (Demidchik and Maathuis, 2007; Ma and Berkowitz, 2010). These channels could be voltage dependent and sometimes need a ligand for their activations (Kudla *et al*., 2010). GLRs and CNGSs are example of ligand-gated channels in the plasma membranes whereas IP_3-dependent channels and RYR represent ligand-gated channels in the endomembrane system (McAinsh and Pittman, 2009; Jammes *et al*., 2011). Along with Ca^{2+} transport system, Ca^{2+} sensors that represent different types of Ca^{2+}-binding proteins (CBPs) play a major role in the transportation of Ca^{2+} across cell membranes. Upon binding to Ca^{2+}, CBPs undergo conformational changes and also modify their catalytic activity (Clapham, 2007; Weinl and Kudla, 2009). CaMs, CMLs, CDPKs and CBLs are the important examples of CBPs in plants. The EF-hand motif in CBPs helps them in binding to Ca^{2+} ions. Inside the cells, CBPs targets

different enzymatic and signaling proteins like TFs and PKs (Finkler *et al*., 2007). Finally, the activity of these Ca^{2+} sensor proteins lead to the transfer of stimulus-induced Ca^{2+} signals into ultimate biological response.

As mentioned above, constant higher $[Ca^{2+}]_{cyt}$ levels are lethal for cell so they should come back to basal level quickly. To meet this condition, Ca^{2+} is quickly transported either to other subcellular organelles which have higher reservation capacities compared to cytosol or moved out in the apoplast. At this stage, different types of Ca^{2+} channels and transporters (*e.g.* Ca^{2+}-ATPases and CAX) are actively involved to regulate this Ca^{2+} balance inside the cell (Dodd *et al*., 2010; Stael *et al*., 2012). Beside vacuole which is the biggest Ca^{2+} store inside the cell, plant mitochondria and chloroplasts also have a considerable amount of Ca^{2+} and possibly contribute to maintain a Ca^{2+} balance inside the cell (Sai and Johnson, 2002; Logan and Knight, 2003). These subcellular organelles are also able to respond to different stimuli by changing their free Ca^{2+} and concentrations (Johnson *et al*., 1995; Logan and Knight, 2003). This very interesting and important aspect of mitochondria and chloroplasts has been under-evaluated in the past and the exact nature of Ca^{2+} signals and their physiological importance are not completely known to date (McAinsh and Pittman, 2009; Dodd *et al*., 2010).

During PAMP-/elicitor-mediated resistance, Ca^{2+} plays an important role in the activation of different signaling pathways that ultimately leads to plant defense. In fact, plant defense responses are triggered upon the detection of many common PAMPs/MAMPs through specific PRRs otherwise by recognizing, through specific receptors, various pathogen- or plant-derived signal molecules called elicitors (Ebel and Cosio, 1994). Cry and OGs are excellent examples of two well characterized plant defense elicitors and have been extensively studied in the past. After elicitor recognition, an early Ca^{2+} influx and a subsequent $[Ca^{2+}]_{cyt}$ change are initial steps to trigger plant defense signaling pathways (Lecourieux *et al.,* 2002 Lecourieux *et al.,* 2005). Ultimately, the Ca^{2+} signals received at the plasma membrane are transduced into different biological processes that enable the plant to better compete unfavorable environment.

Interestingly, the involvement of Ca^{2+} from the internal stores has also been suggested during elicitor-mediated plant defense signaling (Blume *et al*., 2000; Lecourieux *et al*., 2002; Vandelle *et al*., 2006). In this regard, we decided to investigate whether Cry and OGs are able to induce $[Ca^{2+}]$ variations in cytosol, mitochondria and chloroplasts of tobacco cells. Have these subcellular compartments their own characteristic signature in comparison to cytosolic

and nuclear Ca^{2+} signatures? What are the consequences of these elicitor-induced $[Ca^{2+}]$ variations on physiological events taking place inside these organelles? We studied the effects of Cry-induced $[Ca^{2+}]$ variations on cellular oxygen consumption and PSII fluorescence quenching, two important physiological events taking place in mitochondria and chloroplasts, respectively. For this purpose, transformed *N. tabacum* var. Xanthi cell cultures expressing apoaequorin in mitochondria, chloroplast and cytosol were generated through stable transformation techniques in the laboratory.

Our results demonstrated that both OGs and Cry induced characteristic biphasic $[Ca^{2+}]_{cyt}$ variations in *N. tabacum* cells that highlight the presence of different Ca^{2+} fluxes at different times and at the same time suggest the involvement of different Ca^{2+} stores and/or Ca^{2+} channels and pumps to regulate these $[Ca^{2+}]_{cyt}$ changes. These results are in complete accordance with previous studies in *N. plumbaginifolia* cell suspensions that have demonstrated different origins to induce biphasic $[Ca^{2+}]_{cyt}$ elevations in response to Cry (Lecourieux *et al*., 2002; Lamotte *et al*., 2004). These studies have emphasized the involvement of Ca^{2+} from the extracellular medium through undefined Ca^{2+}-permeable channels as well as the Ca^{2+} release from internal stores possibly through IP_3- and RYR-mediated endomembrane channels. With the exception of nucleus, which showed an IP_3-sensitive Ca^{2+} uptake, the exact location of these elicitor-induced $[Ca^{2+}]$ variations has not been not identified so far. Both Cry and OGs induced elevations in $[Ca^{2+}]_{mito}$ and in $[Ca^{2+}]_{chlo}$ in transformed tobacco cells in a dose dependent manner. However, nature of Ca^{2+} transients is different in both organelles. In comparison to biphasic response of $[Ca^{2+}]_{cyt}$ change, mitochondria exhibited a single peak response starting at the same time as that $[Ca^{2+}]_{cyt}$ while $[Ca^{2+}]_{chlo}$ showed a biphasic response with a relatively delayed first peak without significant differences in the second $[Ca^{2+}]$ peak. These concomitant or delayed increases in $[Ca^{2+}]_{mito}$ and $[Ca^{2+}]_{chlo}$ with $[Ca^{2+}]_{cyt}$ suggest the role of these subcellular compartments in Ca^{2+} homeostasis by up taking the extra Ca^{2+} from the cytosol. Chloroplasts are almost 10 times bigger organelle than mitochondria and could have higher Ca^{2+} storage capacities that allow a continuous flow of Ca^{2+} into the chroroplasts. This hypothesis is supported by the fact that first $[Ca^{2+}]_{chlo}$ peak was followed by the generation of a second $[Ca^{2+}]_{chlo}$ peak that lasts for 1 h. In contrast, single peak response of mitochondria suggests that this organelle has to stop quickly to take up Ca^{2+} possibly due to its lower storage capacities, inactivation of Ca^{2+} pumps or through the disruption of mitochondria functions. These hypothesis could be strengthened by the facts that animals mitochondrial Ca^{2+} uniporter become inactive after a sustained $[Ca^{2+}]_{cyt}$ elevation (Putney and Thomas, 2006). In spite of general similarities

between Cry- and OGs- induced [Ca^{2+}] elevations in these compartment, we also observed some important differences like comparatively quicker response, smaller durations of [Ca^{2+}] elevations, a different shape of the second [Ca^{2+}]$_{cyt}$ phase and a Ca^{2+} signature in chloroplasts which could not be superimposed on cytosolic Ca^{2+} signature after OGs treatments: thus suggesting different regulation mechanisms and pathways for two different elicitors. In addition, [Ca^{2+}]$_{chlo}$ and [Ca^{2+}]$_{mit}$ variations were completely abolished by La^{3+} and BAPTA treatments, supporting that the extracellular Ca^{2+} influx through PM-localized Ca^{2+} channels is a prerequisite for [Ca^{2+}] elevations in these organelles.

We observed that neomycin and U73122 had a significant inhibitory effect on Cry-induced [Ca^{2+}] variations in cytosol and organelles while RR slightly decreased the Cry-induced [Ca^{2+}] elevations in these compartments. Moreover, cPTIO which efficiently scavenged NO production in the cytosol, mitochondria and chloroplasts did not significantly modify [Ca^{2+}]$_{cyt,}$ [Ca^{2+}]$_{mito}$ and [Ca^{2+}]$_{chlo}$ variations in response to Cry treatments. Collectively, these data demonstrated the occurrence of an IP_3-related calcium signaling pathway leading to [Ca^{2+}] variations in mitochondria and chloroplasts. In plants, the involvement of IP_3 channels in Ca^{2+} signaling especially during plant defense responses has been reported in the past. A pretreatment with neomycin before Pep-13 and Cry elicitations in parsley and tobacco cells, respectively, resulted in a significant inhibition in the first [Ca^{2+}]$_{cyt}$ elevation without affecting the sustained [Ca^{2+}]$_{cyt}$ increase (Blume *et al*., 2000; Lecourieux *et al*., 2002). On the other hand, in soybean and tobacco cells treated with β-glucans and OGs, respectively, neomycin pretreatments led to a very strong inhibition of the second transient [Ca^{2+}]$_{cyt}$ peak while had no effect on the first [Ca^{2+}]$_{cyt}$ rise (Mithöfer *et al*., 1999; Lecourieux *et al*., 2002). In our case, neomycin pretreatment of tobacco cells resulted in a significant inhibition of both the first and the second Cry-induced [Ca^{2+}] peaks. In the same manner, neomycin and U73122 also limited the first transient increase in [Ca^{2+}]$_{cyt}$ to a sharp peak in BcPG1-treated cells grapevine cells whereas second sustained peak was not affected (Vandelle *et al*., 2006). Recently, the role of IP_3 was also proposed in [Ca^{2+}]$_{cyt}$ elevations necessary for stomatal closure in *Arabidopsis* and this pathway was dependent on CAS present on the thylakoid membrane of the chloroplasts with the Ca^{2+} binding site likely exposed to the stromal side (Nomura *et al*., 2008; Tang *et al*., 2008; Weinl *et al*., 2008)

We further tried to investigate the physiological importance of these [Ca^{2+}] variations in mitochondria and chloroplasts. We investigated O_2 uptake as a potentially Ca^{2+}-regulated process in mitochondria. Aeq-Mit cells treated with Cry resulted in a significant increase in O_2 consumption that was almost completely abolished by a combined treatment with

inhibitors of cytochrome c pathway (KCN) and AOX (SHAM) thus indicating that both these pathways are involved in mitochondrial respiration process. Interestingly, when a pretreatment with La^{3+} was made, O_2 uptake response in Cry-treated cells was observed at the same level as that of control cells thus indicating the role of PM-derived Ca^{2+} signals in the regulation of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ elevations thereby controlling the enzymes involved in mitochondrial respiration.

Ca^{2+} in the thylakoid lumenal store is required for the proper assembly of PSII during initial assembly or during repair of photodamaged PSII reaction center (Grove and Brudvig, 1998; Mattoo *et al.*, 1989; Loll *et al.*, 2005) and is instrumental to the activity of the oxygen-evolving complex (OEC) as Ca^{2+} extraction greatly diminishes O_2 evolution (Van der Meulen, 2002). In Aeq-Chlo cell suspensions, Cry showed a significant effect on the FY with a slow return to the basal levels as compared to controls. These data, which reflects the lower acidification of the thylakoid lumen in Cry-treated cells, are in accordance with the higher activity of a Ca^{2+}/H^{+} antiporter, indicating thereby that cells are storing Ca^{2+} in the thylakoid lumen during Cry treatment. At initial time points, La^{3+} reduced the Cry-induced Chl FY that reached to the level of control cells however at longer time points La^{3+} was unable to completely suppress the sustained Cry-induced FY. This indicates that although fluorescence yield quenching is indeed sensitive to chloroplastic $[Ca^{2+}]$ variations, others Ca^{2+}-independent events also have an influence on PSII fluorescence. Overall, these results highlight the complexity of the Cry signaling pathway with chloroplasts and mitochondria participating actively to Ca^{2+} signaling upon elicitor treatment.

Another part of my thesis work was related to identify the role of GLRs homologs in plant defense signaling processes. GLRs are ligand-gated cation channels putatively involved in plasma membrane mediating non-selective cation fluxes especially Ca^{2+}. In animals, they have main function in neurotransmission in the CNS and play an additional role in immune responses (Gill and Pulido, 2001; Boldyrev *et al.*, 2005; Pacheco *et al.*, 2007; Rousseaux, 2008). In plants, GLRs are implicated in many different physiological processes (Dodd *et al.*, 2010; Jammes *et al.*, 2011) but no clear indications are available for their involvement in Ca^{2+}-mediated defense responses during plant-pathogen interactions. To explore this aspect of GLRs in plants, we made a combine use of both pharmacological and genetic approaches. During pharmacological approach, different well known GLRs antagonists were tested in *Arabidopsis thaliana* plants treated with OGs. On the other hand, T-DNA insertion mutant lines of AtGLRs were used for genetic studies. Our results showed that $[Ca^{2+}]_{cyt}$ variations induced by OGs are partly controlled by GLRs. These data are in complete agreement with

the recently published studies conducted through a pharmacological approach that indicated the involvement of GLRs in the regulation Ca^{2+} fluxes and $[Ca^{2+}]_{cyt}$ variations in response to different plant defense elicitors in tobacco and *Arabidopsis* (Kwaaitaal *et al.*, 2011; Vatsa *et al.*, 2011). We know that Ca^{2+} participates in many defense responses by controlling the activities of different downstream elements of defense signaling so we tested NO and ROS production, MAPK activation (annex 4) and the expression of defense related genes in wild type (Col-0) or *glr* mutants to elucidate the role of GLRs in these signaling events. Again we found prominent effects of GLRs on these above mentioned signaling events, except for MAPK activation. An important observation was made at this stage. Treatment with different GLRs antagonists induced a higher suppression in NO and ROS production, and gene expression as compared to single mutant plants. This indicated that GLRs antagonists could target more than one type of GLR composed potentially of different subunits at a time, so more pronounced effects are obvious. In the same way, use of number of inhibitors indicated that not all the same GLRs impacted NO and ROS generation. For example, MK-801 was able to strongly inhibit the ROS production but not the NO generation, thus suggesting that MK-801 targeted GLRs have a specific role in ROS signaling only.

However, direct evidences of GLRs involvement in plant defense were still missing. We tried to answer this question by investigating the *B. cinerea* and *H. arabidopsidis* infection responses in Col-0 plants treated with GLRs antagonists and *Atglr* mutant plants. Based on pharmacology, a compromised resistance to these pathogens was observed in our study. No single *Atglr* mutant was found more susceptible to *B. cinerea* (even in the GLR clades 1 and 2, annex 3). However, we demonstrated that *AtGLR3.3/3.6* are important genes involved in basal resistance against *H. arabidopsidis.* We also observed that *AtGLR3.3* regulates the expression of plant defense genes (especially some of SA pathway *e.g. ICS1* and *PR-1*) commonly induced by OGs- and *H. Arabidopsidis* thus suggesting respective similarities in OGs- and *H. Arabidopsidis* signaling in plants. Both *ICS1* and *PR-1* are specific marker genes of SA pathway, are involved in resistance to biotrophic pathogens in plants and participate in SAR responses (Maleck *et al*., 2000; Glazebrook, 2005; Lu, 2009). These data are also consistent with previous reports obtained with the mutant *npr-1* (nonexpressor of PR genes 1), in which the SA signaling pathway is blocked and a loss of resistance to *H. arabidopsidis* was reported (Thomma *et al*., 1998).

During pathogen and/or elicitor-mediated signaling pathways, the ultimate outcome is the establishment of defense responses in plants. Transcriptional reprogramming of a variety of genes that are directly or indirectly involved in these signaling processes is an essential step

to complete these tasks (Caplan *et al*., 2008). This transcriptional regulation and reprogramming could lead to the up- or down-regulation of hundreds of genes related to different genes families including: PR proteins, proteins associated with cell wall modifications, various classes of TFs and proteins involved in secondary metabolism *etc* (Stintzi *et al*., 1993). That is why a comprehensive analysis of expression profile of different GLRs-dependent genes was an ultimate requirement in order to have a better understanding of the GLRs in Ca^{2+} signaling pathways during plant pathogen interactions. We performed a whole genome transcript analysis to investigate the GLRs target genes in *Arabidopsis* after treatments with OGs in the presence of DNQX. In parallel, effect of Glu, which also induces NO production partly depending on GLRs (annex 1), was also analyzed on the transcriptional regulation in *Arabidopsis* plants. A large number of genes belonging to different functional categories, including signal transduction, transport, stress response, different classes of TFs and (a)biotic stresses, showed a modulated expression (up- or down-regulation) in GLRs- and Glu dependent manner. Interestingly, in OGs-mediated defense signaling, about 60 % of total modulated genes were GLRs-dependent indicating the role of GLRs in plants biological processes especially in the context of plant pathogen interactions.

Taken as a whole, in this thesis work, we tried to further improve our understanding about the important role of Ca^{2+} as a second messenger. Our data not only provided new insights into the under-studied function of mitochondria and chloroplasts as Ca^{2+} reservoirs that efficiently took part in Ca^{2+} homeostasis but also demonstrated the physiological role of this organelles Ca^{2+} in plants especially during plant defense. In addition, we provided strong pharmacological and genetic evidenced in favor of GLRs implications in elicitor/pathogen mediated plant defense signaling pathways.

2. Global perspectives

In the continuity of this thesis work, we would like to suggest different perspectives.

Concerning Cry-induced Ca^{2+} signaling in tobacco:

Our results showed Cry-induced free [Ca^{2+}] variations in mitochondria and chloroplasts. A lot of information is still needed to understand the mechanism of elicitors-dependent Ca^{2+} signaling in organelles:

- A comprehensive investigation to identify different channels, pumps and transporters that are involved in [Ca^{2+}] variations in these organelles is essentially needed. Electrophysiological studies could be helpful in this regard.
- It is really important to find out the total Ca^{2+} concentrations in mitochondria and chloroplasts (both in thylakoid lumen and stroma) under resting conditions and in response to different stimuli. Is there a real change in total Ca^{2+} concentrations in these organelles that would support their role as Ca^{2+} storing compartments?
- Do these organelles play a role in the constitution of Ca^{2+} microdomains, as in animals? Is there a specific spatial organization of chloroplasts and mitochondria with respect to other Ca^{2+} providing stores (vacuoles, ER, sub-plasma membrane ATPase microdomains)?
- Another important aspect of these organelles Ca^{2+} is the study of their implication in downstream signaling events of plant defense. Chloroplasts and mitochondria have been described as potential sites for NO and ROS generation. How the organelle [Ca^{2+}] variations control NO and ROS generation during plant defense responses is of real interest. In mitochondria, the increase in [Ca^{2+}] stimulates the activity of NAD(P)H deshydrogenases, thus increasing the pool of reduced ubiquinones, a situation that favours ROS production: this brings part of the response, but the exact mechanisms in mitochondria and chloroplasts for NO and ROS generation are not known.

Part of the answers would be provided by developing Ca^{2+} sensors such as cameleon Ca^{2+} reporting proteins that could be targeted to the proper subcellular compartments and will provide more precise information on spatially subcellular [Ca^{2+}] variations at the single cell level than aequorin-sensors and in real-time kinetics. But still the main effort will be to identify at the molecular level the proteins involved in these [Ca^{2+}] variations. Recently, the

animal Ca^{2+} uniporter was identified and the existence of homologs in *Arabidopsis* has provided some starting point to understand the Ca^{2+} fluxes in mitochondria. In chloroplasts, deciphering the mechanisms for CAS-induced changes in $[Ca^{2+}]_{cyt}$ variations will add an important link between chloroplasts and $[Ca^{2+}]_{cyt}$-induced signaling events.

Concerning GLR studies:

We have demonstrated that GLRs actively participate in elicitor-mediated signaling in plant defense. However, our investigation have opened the window for many important and interesting investigations that are essentially required to firmly establish their role in plant physiological processes including defense.

- Our results pointed out the important role of AtGLR3.3 in *H. arabidopsidis* resistance. It would be interesting to analyze in this background the expression pattern of the selected GLRs-dependent genes that would confirm the correlation between OGs-induced signaling and *H. arabidopsidis* resistance. Additionally, a whole transcriptomic approach comparing *H. arabidopsidis* resistance induced in Col-0 and in *Atglr3.3* mutant will be quite interesting to identify those genes important for *H. arabidopsidis* resistance.
- Our data also raised an important question about the subunit structure of efficient GLR channel in terms of composition and for the control of different downstream signaling event. Our results indicated that 4 to 5 GLR gene products could be implicated in NO (and potentially more involving clade 1 subunits, see annex 2) and ROS production. This is possibly due to heteromeric configuration of different GLRs as already reported in animals. So, the determination of the structural organization of the functional GLRs receptor would be important. Different GLRs could join to form a functional channel pore. In this regard, study of double, triple or quadruple mutant will be very interesting. Conventional crossing technique to obtain multiple genes mutation in a single plant is time-taking and is not very efficient in term of the possibility to obtain homozygous mutants plants. The advent of gene silencing through RNA interference (RNAi) could be a possible solution. Study of these multiple mutants would be interesting to check the combination of subunits involved in a specific signaling event, although keeping in mind that these signaling events are not necessary independent. For example, it is known that NO could regulate ROS production (Yun *et al*., 2011), also in OGs-induced signaling pathway (Rasul *et al*., 2012). In the case of

H. arabidopsidis basal resistance, the double mutant *Atglr3.3/Atglr3.6* would be particularly informative.

- Exploitation of transcriptomic results will lead to identity important genes whose products could play a role in defense signaling pathways downstream GLRs (GLRs-dependent genes). For that, after the screening to obtain homozygous mutant plants, T-DNA mutants of these selected GLRs-dependent genes could be analyzed for their effect on *H. arabidopsidis* or *B. cinerea* resistance. Some of these genes could also have an impact on NO and ROS production by feedback mechanisms leading to inhibition or amplification of the initial induced signaling event (*e.g.* NO/ROS production). Within the selected GLR-dependent genes, TF genes may have an important impact on plant defense signaling. These investigations will be helpful to better understand the relationships between GLRs and plant resistance.
- Although clade 2 GLR genes are in general poorly expressed in leaves yet clade 1 GLR genes are well-expressed in leaves. We had indications that some clade 1 GLR genes are involved in NO production (annex 2). It would be interesting to further study this clade and to specify its relationship to clade 3 genes.
- All our results were performed on the aerial part of *Arabidopsis,* but root colonizing pathogens may be stopped or limited in their growth by a signaling pathway involving potentially more *GLR* genes, as all *GLR* genes are expressed in roots. It would be interesting to develop a test by making infections in the soil to see whether clade 2 *GLR* genes could be involved in resistance.
- One interesting point raised by our study and equivalent studies performed with *cngc2* mutant lines indicated that both types of non selective channels (GLRs and CNGC) are involved in plant defense signaling and particularly concerning the regulation of NO production. In our group, it was shown that NO production was reduced in *cngc2* mutant and in some *glr* mutants. Relationships between GLR and CNGC channels are completely unknown, as well as relationships of these channels with the OGs receptor WAK1. This opens a wealth of interesting biochemical and genetic studies to specify the elements linking elicitor perception and Ca^{2+} signaling through the activation of these channels.
- Studies using tobacco cell suspensions and Cry indicated that Glu could activate GLR homologs after exocytosis of Glu. At the moment, nothing is known on *Arabidopsis* and mechanistic aspects are completely uncovered in plants. This raised the very interesting and important aspect of signaling through amino acids or amino acids-

derived molecules. Emerging data support a role for amino acid in signaling, as in animals. So it will be interesting to know how their concentrations can be locally modified, which are the sensors *etc.* Additionally, Glu is not the only amino acid able to activate GLRs, and investigations with other amino acid could add new elements on the regulation of GLRs-induced signaling pathway.

These different approaches definitely will make possible to understand the role of GLRs more comprehensively in different physiological processes in plants.

CHAPTER 7

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"Literature Cited"

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LITERATURE CITED

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“Publications and communications”

LIST OF PUBLICATIONS AND COMMUNICATIONS

Publications:

- **Manzoor, H**., Chiltz, A., Hichami, S., Vatsa, P., Wendehenne, D., Pugin, A. and Garcia-Brugger, A. **(2010)** Le calcium dans les mitochondries et les chloroplastes chez les végétaux. 14ème Atelier Théorique et Pratique *"Le Signal Calcium" SEIX, France:* pp. 30-35.

- 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.

- **Manzoor, H**., Chiltz, A., Madani, S., Vatsa, P., Schoefs, B., Pugin, A. and Garcia-Brugger, A. **(2012)** Calcium signatures and signaling in cytosol and organelles of tobacco cells induced by plant defense elicitors. *Cell Calcium.* doi:10.1016/j.ceca.2012.02.006.

- **Manzoor, H**., Chiltz, A., Wendehenne, D., Pugin, A. and Garcia-Brugger, A. **(2012)** Glutamate receptors are involved in Ca^{2+}-dependent plant defense signaling (Submitted in *Plant J.*).

- **Manzoor, H**. and Garcia-Brugger, A. **(2012)** Microarray investigation of OGs-induced glutamate receptors-dependent genes during plant defense signaling (In preparation).

Communications (posters/presentations)

- **Manzoor, H**., Chiltz, A., Wendehenne, D., Pugin, A. and Garcia-Brugger, A. Characterization of glutamate receptors in elicitor-mediated plant defense signaling. (Poster: 10th International Conference on Reactive Oxygen and Nitrogen Species in Plants, 5-8 July, **2011**, Budapest, Hungary).

- **Manzoor, H**., Chiltz, A., Madani, S., Schoefs, B., Wendehenne, D., Pugin, A. and Garcia-Brugger, A. Chloroplasts and mitochondria are involved in elicitor-induced calcium signaling in plants. (Poste : 17éme Forum des Jeunes Chercheurs, 16-17 June **2011**, Dijon, France).

- **Manzoor, H**., Chiltz, A., Wendehenne, D., Pugin, A. and Garcia-Brugger, A. Characterization of glutamate receptors in elicitor-induced plant

immune signaling. (Poster: 3ème Journées des doctorants SPE, 08-10 June 2011, Dijon, France).

- **Manzoor, H**., Chiltz, A., Hichami, S., Vatsa, P., Wendehenne, D., Pugin, A. and Garcia-Brugger, A. Le calcium dans les mitochondries et les chloroplastes chez les végétaux. (Oral communication: 14ème Atelier Théorique et Pratique "*Le Signal Calcium*" 18-21October **2010**, SEIX, France).

- **Manzoor, H**., Chiltz, A., Hichami, S., Wendehenne, D., Pugin, A. and Garcia-Brugger, A. Study of chloroplastic calcium variations in elicitor-induced plant defense signaling. (Poster: Plant Calcium Signalling meeting, 31 Aug - 04 Sept. **2010**, Munster, Germany).

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