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van Aubel, Géraldine

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University of Namur
Faculty of Sciences - Department of Biology
Research Unit in Plant Cellular and Molecular Biology

Study of the mode of action of COS-OGA, a new class of elicitors of plant innate immunity

Géraldine van Aubel

Thesis submitted in partial fulfillment of the requirements
for the degree of doctor (PhD) in Sciences

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Abstract

There is an urgent need to reduce our dependence on chemical pesticides for plant protection and the stimulation of the plant innate immunity constitutes a promising alternative. The aim of this work was to study the mode of action and the activity spectrum of a novel biological elicitor of plant defenses, COS-OGA. This elicitor combines fungal-derived chitooligosaccharides (COS) and plant cell wall-derived oligogalacturonides (OGA) which mimic plant interaction with fungi and inform plant cells on both cell wall degradation and pathogen presence.

Preventive sprayings of FytoSave® containing 12.5 g/l COS-OGA were shown to be highly efficient against powdery mildew on grapevine, cucumber and tomato. FytoSave® effect on tomato plants in absence of pathogen showed that upon repeated COS-OGA sprayings, foliar content of the plant hormone salicylic acid (SA) increased. COS-OGA applications also led to overexpression of SA-related genes and proteins while genes linked to jasmonic acid and ethylene were not regulated. These results suggest that FytoSave® cumulatively stimulates SA-dependent systemic acquired resistance.

FytoSave® was also tested against potato late blight provoked by *Phytophthora infestans* and partly reduced the disease severity. But FytoSol, another COS-OGA composition, completely protected potato against late blight under controlled conditions after repeated applications short before pathogen infection. Both products induced the expression of defense-related genes but both FytoSave® and the necrotrophic stage of *P. infestans* induced accumulation of SA in potato leaves while FytoSol decreased SA level and seemed to act through other hormonal pathways.

RNA-seq performed on leaves revealed a massive downregulation of potato genes by *P. infestans* in its biotrophic stage as well as hormonal hijacking while leaves pretreated with FytoSol and inoculated with *P. infestans* underwent upregulation of many genes encoding peroxidases, glutathione S-transferases, pathogenesis-related proteins, enzymes involved in cell wall turnover and receptor-like kinases, among which several wall-associated kinases. FytoSol enhanced up to variable levels abscisic acid, ethylene and oxylipin pathways but not SA. FytoSol appears to be a promising elicitor that blocks SA-related potato gene regulation by *P. infestans* and triggers a still unknown defense pathway.

List of abbreviations

2D-DIGE	Two-dimensional difference in-gel electrophoresis
ABA	Abscisic acid
AI	Active ingredient
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AUDPC	Area under the disease progression curve
Avr	Avirulence
BABA	β-amino-butyric acid
BBCH	Biologische Bundesanstalt Bundessortenamt und CHemische Industrie
BCA	Biological control agent
BLAST	Basic local alignment tool
BR	Brassinosteroid
BTH	Benzothiadiazole
CA2	Carbonic anhydrase
CESA	Cellulose synthase
CK	Cytokinin
COS	Chitooligosaccharides
CV	Cultivar
CWDE	Cell wall-degrading enzyme
CWI	Cell wall integrity
DA	Degree of acetylation
DAA	Days after application
DAMP	Damage-associated molecular pattern
DBH	Days before harvest
DBI	Days before inoculation
DE	Differentially expressed
DHA	Dehydroascrobate
DHAR	DHA reductase
DOX	Dioxygenase
DP	Degree of polymerization
EFR	EF-Tu RECEPTOR
ET	Ethylene
EFSA	European Food Safety Agency
EPPO	European and Mediterranean Plant Protection Organization
ETI	Effector-triggered immunity
FC	Fold change
FDR	False discovery rate

GA	Gibberellin
Flg22	Flagellin 22
FLS2	Flagellin sensing 2
fw	Fresh weight
G3P	Glycerol 3-phosphate
GlcNac	N-acetylglucosamine
GR	GSH reductase
GSH	Glutathione
GSSH	Oxidized glutathione
GST	Glutathione S-transferase
HDS	1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase
HG	Homogalacturonan
hL	Hectoliter
HPI	Hour post-inoculation
HR	Hypersensitive response
HSD	Honestly significant difference
HSP	Heat shock protein
HPI	Hour post-inoculation
HPT	Hour post treatment
IPM	Integrated Pest Management
ISR	Induced systemic resistance
JA	Jasmonic acid
JA–Ile	Jasmonoyl-isoleucine
JAZ	Jasmonate zim repressor
LRR	Leucine-rich repeat
LSD	Fisher's least significant difference
LWA	Leaf wall area
MAPK	Mitogen-activated protein kinase
MEcDP	Methylerythritolcyclodiphosphate
MDHA	Monodehydroascorbate
MDHAR	MDHA reductase
MDS	Multidimensional scaling
NO	Nitric oxide
MRL	Maximal residue level
NGS	Next Generation Sequencing
NLP	Nep1-like protein
NPR1	Non expressor of PR genes 1
NBS	Nucleotide binding-site
OGA	Oligogalacturonides
OPDA	2-oxo-phytodienoic acid
PAE	Pectin acetylesterase

PCD	Programmed cell death
PCR	Polymerase chain reaction
PGPR	Plant growth-promoting rhizobacteria
PG	Polygalacturonase
PGIP	PG-inhibiting protein
PGN	Peptidoglycan
PHI	Pre-harvest interval
PME	Pectin methylesterase
PMEI	PME inhibitor
PPO	Polyphenol oxidase
PR	Pathogenesis-related
PRR	Pattern recognition receptors
PTI	PAMP-triggered immunity
PUFA	Polyunsaturated fatty acids
FA	Fatty acid
R	Resistance
RBH	Reciprocal best BLAST hit
RBOH	Respiratory burst oxidase homolog
RH	Relative humidity
RLCK	Receptor-like cytoplasmic kinases
RLK	Receptor-like kinase
RLP	Receptor-like protein
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SA	Salicylic acid
SAG	Glycosyl-Salicylic acid
SAR	Systemic acquired resistance
SD	Standard deviation
SL	Soluble liquid concentrate
SNK	Student-Newman-Keuls
T3SS	Type-III secretion system
TF	Transcription factor
TL5	Toll-like receptor 5
WAK	Wall-associated kinase

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CHAPTER 1

LITERATURE REVIEW

1. Introduction Research background and thesis outline

After the Second World War, industrialized countries experienced an extraordinary period of tremendous crop productivity increase. That period of time called the Green Revolution started in the fifties and lasted for about fifty years. The yield increase was more the result of intensification than surface area extension (Pingali, 2012). Several factors explain this strong productivity increase among which mechanization but first of all, the key factor was the development of high-yielding crop varieties. These varieties allowed an increase in cropping intensity thanks to a decreased time to maturity and were also very responsive to improved inputs such as irrigation and fertilizers. The benefits of The Green Revolution were uneven because poverty and food insecurity still persist for millions of people. But at least in developed countries, it brought significant socioeconomic profits contributing to decrease poverty and global hunger and allowing people to access a larger variety of food products at a cheaper price.

The environmental impact of the Green Revolution was mixed. It prevented the conversion of thousands of hectares of land to cultivated area but unintended effects were also observed. Soil and water quality were degraded due to excessive exploitation and especially the overuse of inputs induced a large chemical pollution. Indeed, crop improvement focused essentially on yield while traits such as pest and disease resistance and stress tolerance were long neglected (Pingali, 2012). Most varieties required an extensive use of conventional pesticides to keep crops healthy and to secure yields. As a result, our agricultural ecosystems mostly rely on monoculture or on limited rotations that consist in uniform plant populations grown at a high density that favors disease emergence and pathogen dissemination (McDonald and Stukenbrock, 2016).

Rachel Carson and her famous book “The Silent Spring” in 1962 was one of the first to raise the alarm on the adverse effects of pesticide use in agriculture. She set the basics for the development of an environmental policy, helping to raise the awareness of possible contamination of ecosystems by pesticides and its concomitant undesirable health effects (DeMarco, 2017; Gay, 2012).

Concerning environmental policy, the European Union has set up a first legislative framework which became effective in 1993 under the form of the Pesticide Authorization Directive 91/414/EEC. Its main objectives were to harmonize regulation between Member States but foremost to decrease environmental contamination and to increase safety of plant protection products for farmers and consumers (Hillocks, 2012). This legislation required each active ingredient (AI) to undergo risk assessment to calculate user and consumer exposure. If the level of exposure was below the no observable adverse effect dose, the product was granted. Out of almost 1,000 AI approved for use as pesticides in the EU before 1993, the program led to the withdrawal of 74% of them (Lamichhane *et al.*, 2015). The legislative framework also comprises a definition of maximal residue levels (MRL) compatible with an appropriate use of each AI. The European Food Safety Agency (EFSA) in charge of the control of the maximum pesticide residue levels in food recently reported that most controls fell below regulatory limits but public opinion in European countries is now very concerned about long-term or chronic effects from exposure to pesticides, even at low doses (Fantke *et al.*, 2012; Lamichhane *et al.*, 2015).

However the Directive has reached its limits because exposure was calculated according to chemical risk assessment based on appropriate use, which cannot always be taken for granted. Moreover, years of systematic use of some products resulted in their accumulation in soil or water, negatively affecting the risk/benefit balance of pesticide use. This led to the recognition that zero risk was unreachable and caused a switch from risk-based to hazard-based evaluation (Fantke *et al.*, 2012). Directive 91/414/EEC was therefore replaced by Regulation 1107/2009/EC. This means that any new AI must comply with this new regulation to be approved and all remaining AIs (circa 250) need to be reevaluated. Pesticide authorization now relies on hazard-based criteria which imposes the removal from the market of products falling into these categories: persistent organic pollutants, persistent - bio accumulative - toxic, very persistent - very bio accumulative, endocrine disruptors, and carcinogenic - mutagenic - repro-toxic. It is estimated that the implementation of the new regulation will lead to the removal of 20% to 50% of the presently available AI, even if part of them is seen as essential for plant protection (Hillocks, 2012; Lamichhane et al., 2015).

Pesticide efficacy is also increasingly compromised as many cases of pest resistance against herbicides, fungicides and insecticides are discovered. For example, among fungicides (Table l.1), number of highly selective and systemic modern fungicides with a single biochemical target have been rendered inefficient by a single mutation in the pathogen's

genome. This is the simple consequence of natural selection when a pathogen population is under strong selection pressure that favors the emergence of variants with mutated targets or possessing detoxifying mechanisms (Cools and Hammond-Kosack, 2013; Frenkel *et al.*, 2014; McGrath, 2001). This problem observed with weeds, insects and pathogens could worsen with the reduction in number of AI that shall arise from the entry into force of the Regulation 1107/2009/EC.

Table 1.1: Examples of reported cases of fungicide resistances for globally important plant pathogenic fungi and oomycetes.
Adapted from Cools and Hammond-Kosack (2013), McGrath (2001) and Frenkel *et al.* (2014).

Species	Crop	Fungicide family with reported resistance
Blumeria graminis f. sp. *hordei*	Barley	Methylbenzimidazole carbamates
		Demethylation inhibitors
		Azanaphtalenes
		Quinone outside inhibitors
Botrytis cinerea	Various crop plants, especially grapevine	Methylbenzimidazole carbamates
		Demethylation inhibitors
		Succinate dehydrogenase inhibitors
		Quinone outside inhibitors
Erysiphe necator	Grapevine	Demethylation inhibitors
Fusarium spp.	Various crop plants, tomato, cereals	Methylbenzimidazole carbamates
		Demethylation inhibitors
		Phenylpyrroles
Magnaporte oryzae	Rice	Quinone outside inhibitors
		Melanin biosynthesis reductase inhibitor s
		Melanin biosynthesis dehydratase inhibitors
Mycosphaerella fijiensis	Banana	Methylbenzimidazole carbamates
		Demethylation inhibitors
		Quinone outside inhibitors
Mycosphaerella graminicola	Wheat	Methylbenzimidazole carbamates
		Demethylation inhibitors
		Quinone outside inhibitors
		Succinate dehydrogenase inhibitors
Phytophthora infestans	Potato and tomato	Phenylamides
		Carboxylic acid amides
Puccinia graminis f. sp. *Tritici*	Wheat	Demethylation inhibitors
Sphaerotheca fuliginea	Cucumber	2-Amino-pyrimidines
		Benzimidazoles
		Quinone outside inhibitors
		Demethylation inhibitors

There is therefore an urgent need to develop integrated pest management (IPM) methods that reduce the dependence on conventional pesticides and keep intact crop yield, quality and profitability (Hillocks, 2012; Lamichhane *et al.*, 2015). IPM is a system approach that integrates all available preventive mechanical, physical and biological control methods combined with a possible limited recourse to conventional pesticides. IPM aims at controlling plant pests and diseases with the least possible disruption of agro-ecosystems and encourages natural pest control mechanisms. The overall approach is the reliance on a diversity of solutions to ensure the long-term success of the control measures and finally their sustainability. IPM is not only an elegant option for crop protection because its implementation is mandatory in European Member States: as a complement to the new regulation, an additional Directive 2009/128/EC for sustainable use of pesticides has also been adopted. More specifically, this directive imposes the suppression of aerial spraying, a regular control of professional spraying equipment, the creation of pesticide user training programs, the enhancement of aquatic environment protection and the establishment of National Action Plans (NAP) to reduce hazards, risks and dependence on chemical control for plant protection (Hillocks, 2012). In Belgium and more specifically in the Walloon Region, the Walloon Program for Pesticides Reduction includes several measures to reduce dependence on conventional pesticides and notably financial support to IPM.

It is within this framework that this thesis has been performed thanks to a research project granted by the Walloon Region to two research partners: the Laboratory of Phytopathology of the Earth and Life Institute of the Université Catholique de Louvain (UCL) and the Unit of Research in Plant Cellular and Molecular Biology (URBV) at the University of Namur. The project aimed at studying the mode of action and the possibility to use COS-OGA in integrated potato protection. Potato is indeed one of the most cultivated crops in Belgium (Statistics Belgium, 2016). Most varieties used are very susceptible to the late blight agent *Phytophthora infestans* but remain widely used because of their processing qualities. Controlling the disease generally requires weekly fungicide sprayings, making potato culture the largest pesticide-consuming crop in Belgium with more than 15 kg AI/ha/year (ELI - UCL, 2016). Moreover, characterization of Belgian population of *P. infestans* strains revealed that they constantly evolve and become increasingly aggressive (César *et al.*, 2011).

COS-OGA is an innovative patented new AI based on renewable materials from biological origin approved for use as plant protection product under the European Regulation 1107/2009/EC (European Commission, 2015). Its innovative mode of action is based on the stimulation of plant immunity, meaning that it is an interesting AI in the framework of IPM. Even more, COS-OGA use has been allowed in Organic Farming since October 2016 (European Commission - EGTOP, 2016).

As a consequence, part of the research performed concerns the development of a strategy for the use of COS-OGA for potato protection against late blight, but the work has been widened to the study of the mode of action of COS-OGA. At the beginning of this work, the proof of concept on potato was still necessary since most of the available data on COS-OGA effect on plant defense mechanisms had been obtained on *Arabidopsis thaliana* cell suspensions (Cabrera *et al.*, 2010).

The first chapter summarizes the present knowledge on plant immunity and its functioning with a special focus on hormonal modulation. The literature review also highlights the importance of plant cell wall in plant defense as well as its link to the COS-OGA composition. The second chapter discusses the first field efficacy trials of COS-OGA against powdery mildews on grapevine and on cucumber that were the first targets for COS-OGA registration. In this part, the conditions necessary to obtain a successful plant protection with COS-OGA will be discussed. The third chapter is about the mode of action of COS-OGA on tomato, a solanaceous model, first without pathogen. These chapters two and three focus on FytoSave®, the first commercial COS-OGA formulation produced and commercialized by FytoFend SA (Isnes, Belgium). The fourth chapter presents results on a second COS-OGA composition, FytoSol, evaluated side by side with FytoSave®. Data are presented on protection and on the mode of action of these elicitors against potato late blight in controlled conditions. As FytoSol was very effective in controlled conditions and its mode of action was still obscure, the fifth chapter presents an overview of RNA-seq results obtained on the *P. infestans* - *S. tuberosum* interaction w/without elicitor pre-treatment.

2. The lifestyle of a plant pathogen: From camouflage to hijacking

The causal agents of plant diseases belong to various classes including virus, viroids, phytoplasmas, bacteria, oomycetes, protozoa, fungi and nematodes (Strange and Scott, 2005). Plant-microbe interactions range between three trophic spaces from mutualism to pathogenesis and even parasitism. The microbial organisms developing the relation linked to the two last terms are often grouped together under the generic term of plant pathogens. The boundary between these different behaviors is not always clear and can be described by a continuum of variation. In the case of a mutualistic interaction, both plants and microbes benefit from the interaction. Symbiotic bacteria like *Rhizobium* sp which fix gaseous nitrogen for the plant and plant growth-promoting rhizobacteria (PGPRs) that enhance nutrient uptake and host resistance against pathogens fall in this category (Goh *et al.*, 2013; Newton *et al.*, 2010).

Beyond mutualism, commensalism is a relation in which one species gets advantage from the interaction while the other is no affected. When it is at the expense of the plant host, the relationship evolves toward parasitism. A biotrophic interaction can be considered a symbiotic relationship with nutrient diversion but the host is kept alive. At the other end of the pathogenesis gradient, necrotrophic microbes obtain nutrients from dead host cells (Fig. 1.1). Between these two feeding strategies, hemibiotrophic pathogens show a first symptomless or so-called latent phase of development and then evolve into necrotrophy. The dynamics of the relationship depends on the life cycle of both plant and microbe, but environmental stresses also play a role in determining the trophic stage (Newton *et al.*, 2010).

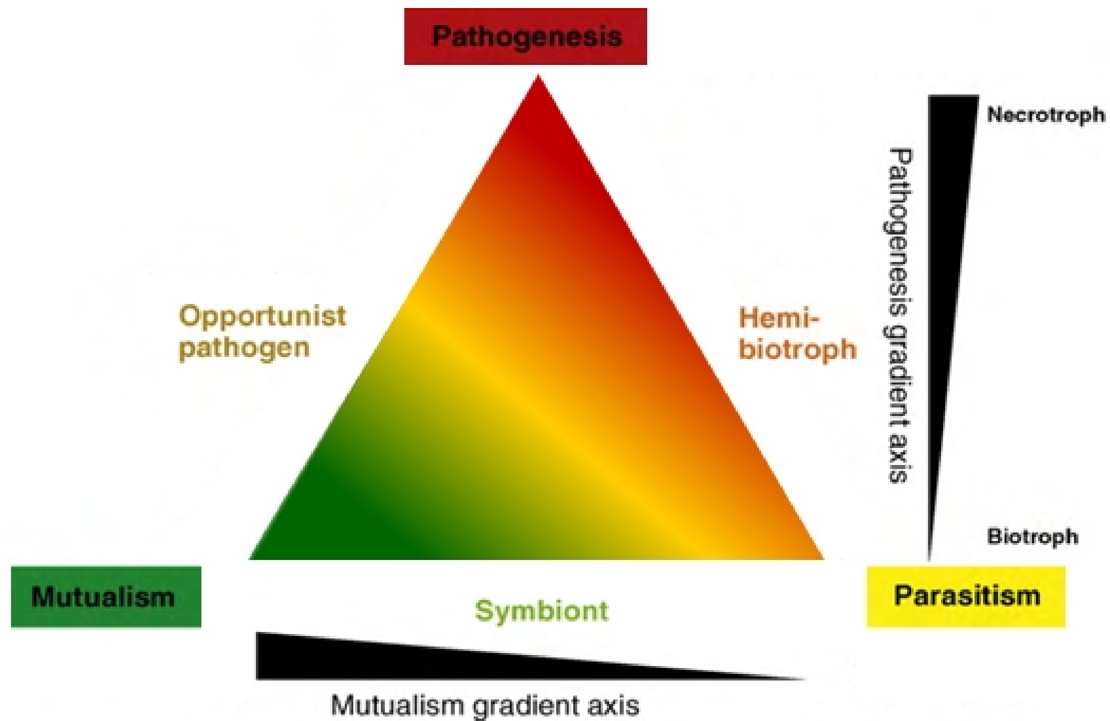

Fig. 1.1: Different trophic stages of interaction between plants and their host microbes. The relationship ranges between the extremes at the corners of the triangle. For symbiotic relationships, the X-axis presents a gradient from mutualism to parasitism and for pathogenesis, the Y-axis presents a gradient from biotroph to necrotroph. Adapted from Newton *et al.* (2010).

Infection is limited to a certain number of adapted pathogens which evolved specific strategies to colonize plants. Indeed the start of a successful pathogenic interaction requires entry into a plant containing naturally resistant cell walls forming a pectocellulosic barrier and covered by a hydrophobic layer of cuticule. Viruses and bacteria do not possess active mechanisms of penetration into their plant hosts. They rely on openings such as stomata, hydathodes or wounds to penetrate or require a third party such as insects to overcome plant barriers. Fungi and oomycetes also penetrate plants using natural wounds or stomata but they have evolved appressoria or infection cushions. Appressoria are specialized infection tools that adhere to plant surfaces and exert physical pressure to penetrate cell walls. Pressure can reach 8 MPa with the help of melanin that stiffens fungal cell walls. The infection peg at the tip of appressoria also secretes enzymes that hydrolyze cell walls and cuticles as well as effectors that help bypass plant defenses (Ryder and Talbot, 2015). Nematodes and particularly cyst and root-knot nematodes which are obligate plant endoparasites possess a stylet to subtract food from plant cells and induce the formation of cells syncytia, or a few discrete giant cells at their feeding sites (Molinari, 2011).

The colonization strategy of biotrophic pathogens is significantly different from the one evolved in a necrotrophic interaction. The germinating conidia of a biotrophic pathogen differentiate an appressorium that allows penetration and hyphal growth between mesophyll cells. Once settled intercellularly, hyphae develop specialized feeding structures named haustoria that remain separated from host cells by the plasma membrane (Glazebrook, 2005). Diverted nutrients are then transported to the epiphytic hyphae that proliferate and start producing conidiophores several days after inoculation. They emerge from stomata to release conidia, the asexual form of multiplication that reinfects appropriate hosts. During their full lifecycle, plant biotrophs maintain their plant partner alive and create a complex interaction with their host. The relation can thus also be described as parasitism (Newton et al., 2010; Spanu, 2012). The genome of this class of pathogens generally encodes lots of effectors defined as virulence factors that facilitate evasion or suppression of plant defense and manipulation of the host metabolism (Asai and Shirasu, 2015). Effectors restrict host defense below a certain threshold to avoid accumulation of toxic secondary metabolites as well as programmed cell death (PCD).

Generally speaking, most biotrophic pathogens are highly specialized and restricted to a single host genus and part of them are obligate pathogens. Powdery mildews belong to the Erysiphales order of ascomycetes and are the perfect example of obligate biotrophs that develop exclusively on aerial plant parts (Glazebrook, 2005; Spanu, 2012). Powdery mildews multiply asexually with conidia but as heterothallic fungi they can also multiply sexually. Powdery mildew can sporulate in five days at optimal temperatures between 23 - 30°C and 85% relative humidity (RH) (Gadoury *et al.*, 2012). Powdery mildews are responsible for the largest share of fungicide sales in Europe and different species isolates target a diverse range of important crops such as cereals, grapevine, numerous vegetables and fruits as well as ornamentals such as roses (Kiss, 2003). For what concerns *Erysiphe necator* on grapevine (Fig. 1.2), more than seven fungicide treatments per season are necessary for an efficient control (American Phytopathology Society, 2006; Kast and Bleyer, 2011; Schumann, 1991).

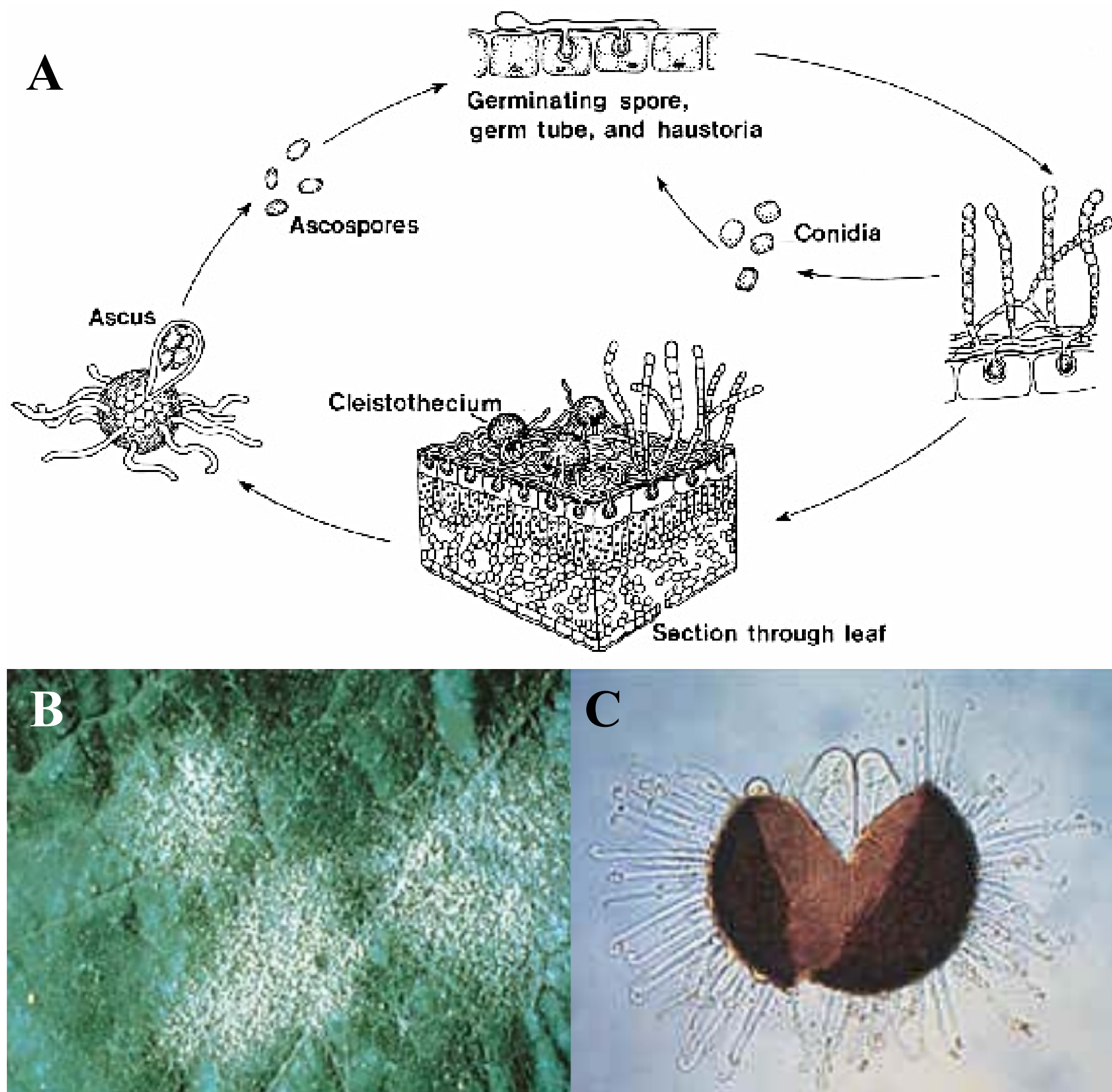

Fig. 1.2: Focus on powdery mildews
A. General disease cycle of powdery mildew on plant leaves (Schumann, 1991).
B. Picture of *E. necator* sporulation on a grapevine leaf (American Phytopathology Society, 2006)
C. Micrography of a cleistothecium, the sexual multiplication structure of *E. necator* (American Phytopathology Society, 2006).

Other known biotrophs that cause downy mildew generally belong to oomycetes such as *Hyaloperonospora arabidopsidis* (Fig. 1.3) that causes downy mildew on *Arabidopsis* or the grapevine downy mildew *Plasmopara viticola*. Oomycetes are highly specialized pathogens and phylogenetic analysis reveals that they are related to algae. They also differ from fungi by having cellulose in their cell walls instead of chitin (Gessler *et al.*, 2011; Spanu, 2012).

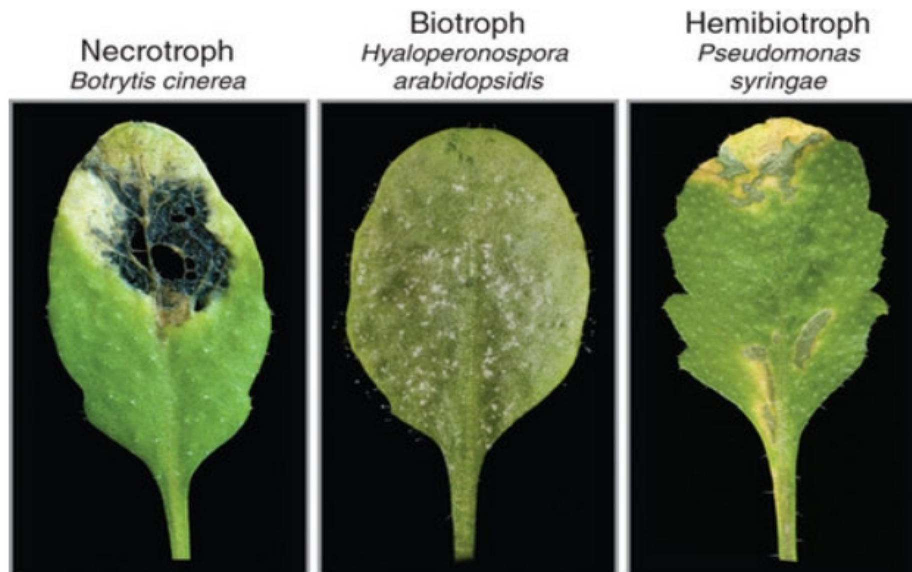

Fig. 1.3: ***A. thaliana*** **leaves infected with pathogens possessing various feeding strategies.**
The necrotroph *B. cinerea*, an ascomycete fungus, the causal agent of grey mold disease, induces extensive necrosis. The hemibiotroph oomycete *H. arabidopsidis* causes powdery mildew and covers plant leaves with white sporulation but keeps plant cells alive. The hemibiotroph *P. syringae* is a gram negative bacteria that first colonizes living tissues before inducing necrosis (Pieterse et al., 2009).

The opposite strategy, necrotrophy, is characteristic of microbial pathogens that kill and feed on dead plant tissues (Spanu, 2012). The genome of several necrotrophic pathogens encodes numerous genes linked to their necrotic lifestyle including the production of toxic compounds or cell wall-degrading enzymes (CWDE) necessary for cell necrosis and nutrient leakage (Mengiste, 2011; Schmidt and Panstruga, 2011). Some necrotrophs produce host-specific toxins required for virulence on their host, but the class mainly contains broad-host range pathogens. The two ascomycetes *Sclerotinia sclerotiorum* and *B. cinerea* (Fig. 1.3) that cause white and grey mold diseases respectively are typical necrotrophic pathogens with broad host ranges causing pre-harvest as well as post-harvest diseases in various fruits and vegetables. Both release oxalic acid that promotes disease development by complexing calcium ions, thereby destabilizing pectin and ensuring ideal acidic conditions for their CWDE. *Pectobacterium carotovorum*, a gram-negative bacteria is also a necrotrophic pathogen that produces various enzymes such as cellulases, proteases and phospholipases that break down plant cell walls and membranes, leading to soft rot disease (Laluk and Mengiste, 2010; Mbengue *et al.*, 2016).

As shown on the trophic space diagram (Fig. 1.1), there is no clear boundary between these two pathogen lifestyles. The hemibiotrophs display both forms of nutrient acquisition: they start interaction with their plant host with an early asymptomatic biotrophic phase and evolve to necrotrophy at later stages of the disease. Biotrophic and necrotrophic phase duration varies significantly among hemibiotrophic pathogens. The gram negative bacteria *Pseudomonas syringae* (Fig. 1.3) is an hemibiotroph that first multiply on leaf surfaces and then colonizes the apoplasm. Once the bacterial population reaches a certain level, it triggers necrotic symptoms typical of the bacterial speckle disease. *P. syringae* uses a type-III-secretion system (T3SS) which directly delivers effectors in the cytoplasm of the host to reprogram its metabolism (Fatima and Senthil-Kumar, 2015; Newton et al., 2010).

Finally, *Phytophthora infestans* is a hemibiotroph pathogen of historical importance that provokes late blight in several *Solanaceae* species and more particularly potato late blight. *P. infestans* became infamous at its arrival in Europe for being responsible for the Irish Potato Famine of 1845 that lasted for several years. Potato was central to European agriculture at this period because tubers were providing twice as many calories per hectare as wheat (Kamoun *et al.*, 2015). *P. infestans* is an heterothallic oomycete although it multiplies essentially asexually through asexual spores named sporangia easily detached by wind or rain from sporulating potato leaves or tubers (Fig. 1.4). The sporangia are able to germinate directly at 20-25°C or to release six to eight biflagellated zoospores at lower temperatures (10-15°C) that can swim in a liquid film for several hours and then encyst and germinate.

A biotrophic asymptomatic development phase lasts for 36 to 48 h and comprises hyphal growth in intercellular spaces and haustoria invagination in plant plasma membranes before turning necrotrophic. After 72 to 96 h *P. infestans* becomes fully necrotrophic and starts producing sporangiophores that emerge through stomata and bear new sporangia ready for a new cycle of infection (Fig. 1.4). A single lesion can produce up to 300,000 sporangia and in favorable conditions, the complete cycle from infection to sporulation can be completed in four days, which makes this polycyclic disease particularly devastating. The disease evolves so rapidly in the field (Fig. 1.5) that it was reported that *P. infestans* could kill a plant “in a matter of hours” (Avrova *et al.*, 2008; Fry, 2008; Fry *et al.*, 2015; Schumann and D'Arcy, 2012).

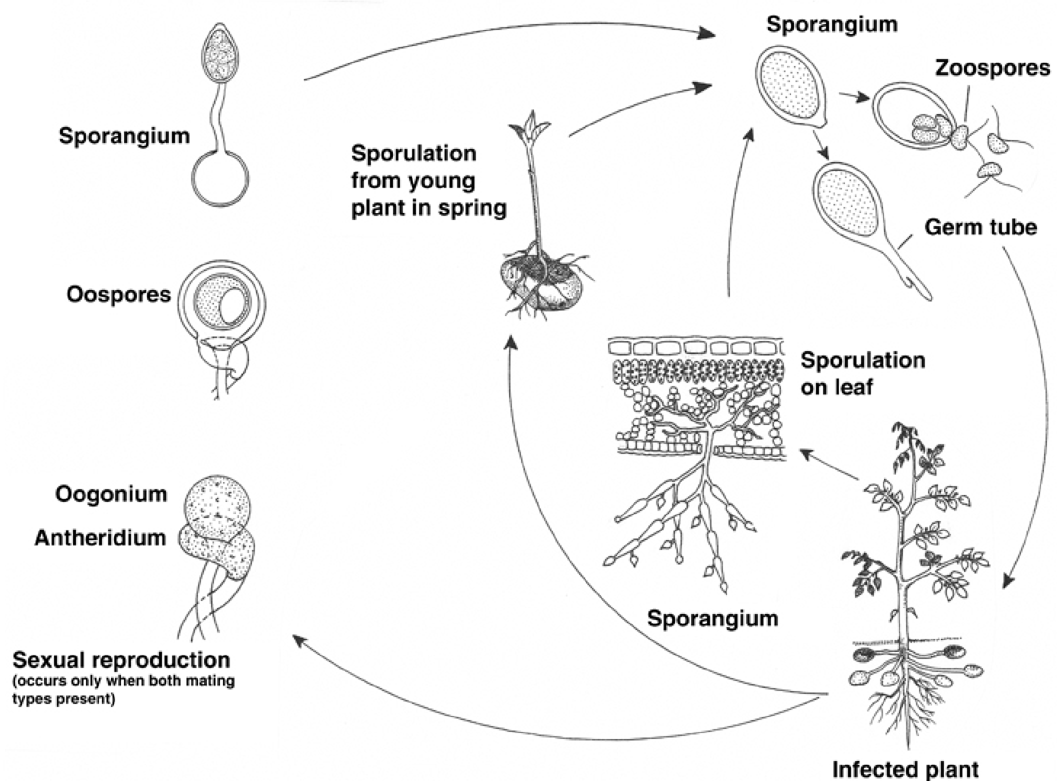

Fig. 1.4: Disease cycle of *P. infestans*, the causal agent of late blight (Schumann and D'Arcy, 2012).

Fig. 1.5: Late blight symptoms on potato cultivar Bintje caused by *P. infestans* in a field trial (Gembloux, Belgium).

A. First symptoms on whole potato plants are discrete spots of disease that correspond to brownish necrosis surrounded by a white to silvery margin of sporulation.

B. After complete colonization, leaves become dry with a silvery-brown color and only upper leaves and stems are still green.

A. At the last stage of late blight, the whole plant turns silvery-brown and dry (C).

3. Plant immunity, the art of counter-attack: current concepts and mechanisms

Plant immunity is first and foremost made of preformed constitutive barriers that evolution has endowed to sessile land plants. The first physical barrier is cuticle which, besides protecting against water loss and irradiation, limits pathogen establishment. Cuticle is constituted by diverse compounds such as cutin formed by hydroxy and epoxy fatty acids and waxes comprising very long chain fatty acids and their derivatives. These compounds form a hydrophobic coating on the epidermal cells of leaves (Serrano et al., 2014). Cell walls mainly comprised of cellulose, hemicellulose and pectin provide rigidity and shape to plant cells and are a very tick protective physical barrier against potential invaders (Kubicek et al., 2014).

Other constitutive defenses include the chemical barrier formed by the broad diversity of plant secondary metabolites (Osbourn, 1996). Beside these constitutive barriers, plants possess an array of inducible mechanisms that allow detection of enemies. Plants lack adaptive immunity so they rely on innate immune responses for defense. Plants cells individually distinguish between self and non-self-molecules and respond autonomously first, then emit chemical signals to orchestrate a complex collective answer from the whole organism against the potential invader in which plant hormones play a crucial role (Zipfel, 2014).

3.1. Pathogen perception: military intelligence at the service of invader recognition

3.1.1. The zigzag and the co-evolutionary models

The adequate sensing of environmental cues and the ability to respond in appropriate ways is essential for sessile organisms permanently confronted to potential pathogens. Pathogen detection takes place at two locations: in the apoplasm and inside the cell (Win et al., 2012). This dual perception system at plasmalemma surface and in the cytoplasm results from plant-pathogen coevolution and has been summarized into the so-called “zigzag model” (Fig. 1.6) proposed by Jones and Dangl (2006).

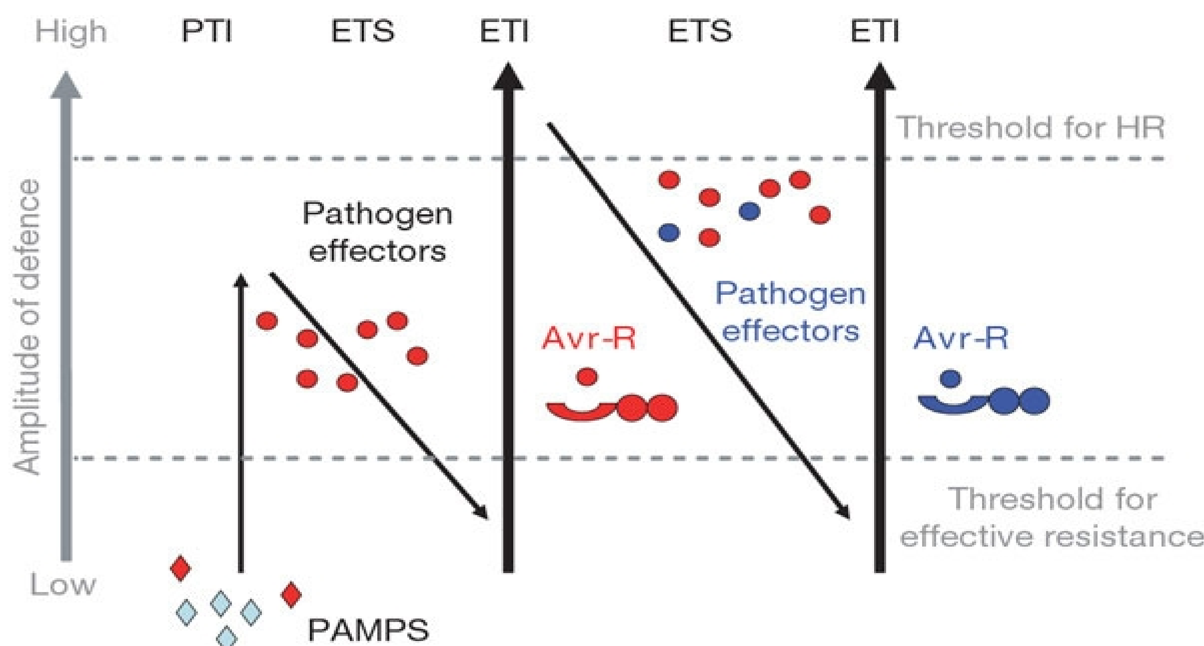

Fig. 1.6: The "zigzag model" from Jones and Dangl (2006).
The first layer of plant immunity is the recognition of conserved microbial signatures known as pathogen-associated molecular patterns (PAMPs) by pattern-recognition-receptor (PRRs). Plants start a basal immune response called PAMP-triggered immunity (PTI) that restricts pathogen development. The selective pressure driven by PAMP recognition favors the emergence of pathogens possessing effectors, products of avirulence (Avr in red) genes able to bypass the plant immune response, leading to effector-triggered susceptibility (ETS). Pathogen-mediated ETS induces plants to evolve and acquire resistance genes (R, in red), leading to effector-triggered immunity (ETI). The run can further continue with ETS exerting a new selective pressure on the pathogen population that acquires other Avr (in blue) that will again downregulate plant defense and further favor emergence of new alleles of R genes (in blue) resulting in a new specific resistance.

Plants are indeed able to perceive conserved microbial signatures termed pathogen-associated molecular pattern or PAMPs. This perception occurs extracellularly and triggers a first general immune response of the plant called PAMP-triggered immunity or PTI. Pathogens under this selection pressure have circumvented this first layer of immunity by injecting effectors directly inside host cells. Effectors are virulence factors that help pathogens suppress PTI, and are products of avirulence genes (Avr). The result is an increased susceptibility of the plant towards the pathogen resulting in the Effector-Triggered Susceptibility (ETS). During evolution, plants gained a monitoring system of effectors based on proteins encoded by resistance (R) gene. Specific effector recognition by R proteins induces effector-triggered immunity (ETI) which is a faster form of PTI often characterized by a programmed cell death (PCD) at the site of pathogen penetration called hypersensitive response (HR). Plant-pathogen coevolution forces invaders to acquire new effectors to trigger ETS, which subsequently induces hosts to evolve new R proteins and recover ETI response (Jones and Dangl, 2006).

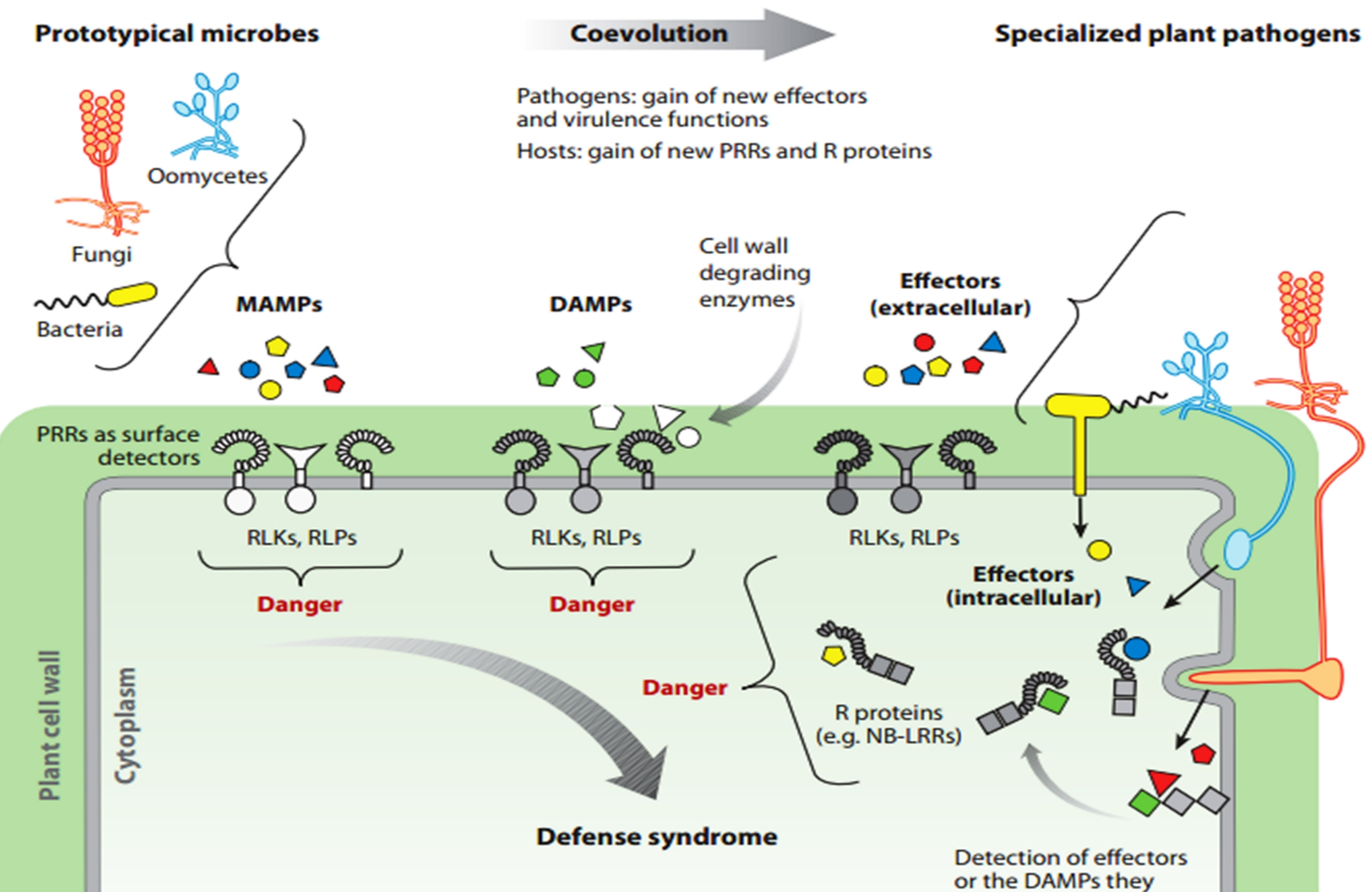

Fig. 1.7: The plant-pathogen coevolution model from Boller and Felix (2009).
Bacteria, fungi and oomycetes release microbe-associated molecular patterns (MAMPs) or degradation products called damage-associated molecular patterns (DAMPs), both produced outside the cell. Effectors can be directly injected by T3SS of bacteria or released by haustoria inside the cytoplasm. Extracellular MAMPs and DAMPs are recognized through pattern recognition receptors (PRRs) belonging to the class of receptor-like kinases (RLKs) or receptor-like proteins (RLPs). In the course of coevolution, pathogen-acquired effectors became detected by plant Resistance (R) proteins mostly related to nucleotide binding-site–leucine-rich repeat (NBS-LRR) family. Sensing of these danger signals triggers a defense syndrome in the plant.

PTI events related to perception occur in the apoplasm (Fig. 1.7) which includes the cell wall and extracellular spaces outside the plasma membrane. Pattern recognition receptors (PRRs) localized to the plasma membrane are the sentinels of the apoplasm, dedicated to PAMP sensing. PRRs comprise a ligand-binding ectodomain and are separated between two main classes: the receptor-like kinases (RLKs) possessing an intracellular kinase domain and the receptor-like proteins (RLPs) that lack the kinase domain and do not possess known intracellular signaling domain (Macho and Zipfel, 2014). PRRs also differ in their ectodomains which are ligand-binding domains that recognize small conserved epitopes.

The leucine-rich repeat (LRR) domain is involved in protein and peptide recognition. The first extensively described PRR was FLAGELLIN SENSING 2 (FLS2). It specifically recognizes flg22, a 22 amino-acids sequence originating from flagellin (Fig. 1.8), an essential component of the flagellum propeller. Flagellin is also recognized by the mammalian TOLL-LIKE RECEPTOR 5 (TLR5) which initiates an inflammatory response. EFR (EF-Tu RECEPTOR) specifically detects elf18, a conserved peptide of the bacterial elongation factor EF-Tu (Fig. 1.8 and Fig. 1.9) and the most abundant protein in bacteria.

The extracellular Lysine-motif (LysM) is involved in the perception of two structurally close compounds: the bacterial peptidoglycan (PGN) and chitin (Fig. 1.8). Chitin is a common PAMP because this polymer of N-acetylglucosamine (GlcNAc) is the main component of fungal cell walls but also of insect exoskeletons. PGN is found bacterial cell wall and consists in an alternation between GlcNAc and N-acetylmuramic acid and forms a carbohydrate backbone on which short polypeptide chains are linked. Two orthologue LysM receptors have been in *Arabidopsis*: CERK1 and more recently the LYSM-CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5) exhibiting a higher chitin affinity than CERK1 (Fig. 9). PGN is also the ligand of the LYSM-domain RLPs LYM1 and LYM3 (Couto and Zipfel, 2016).

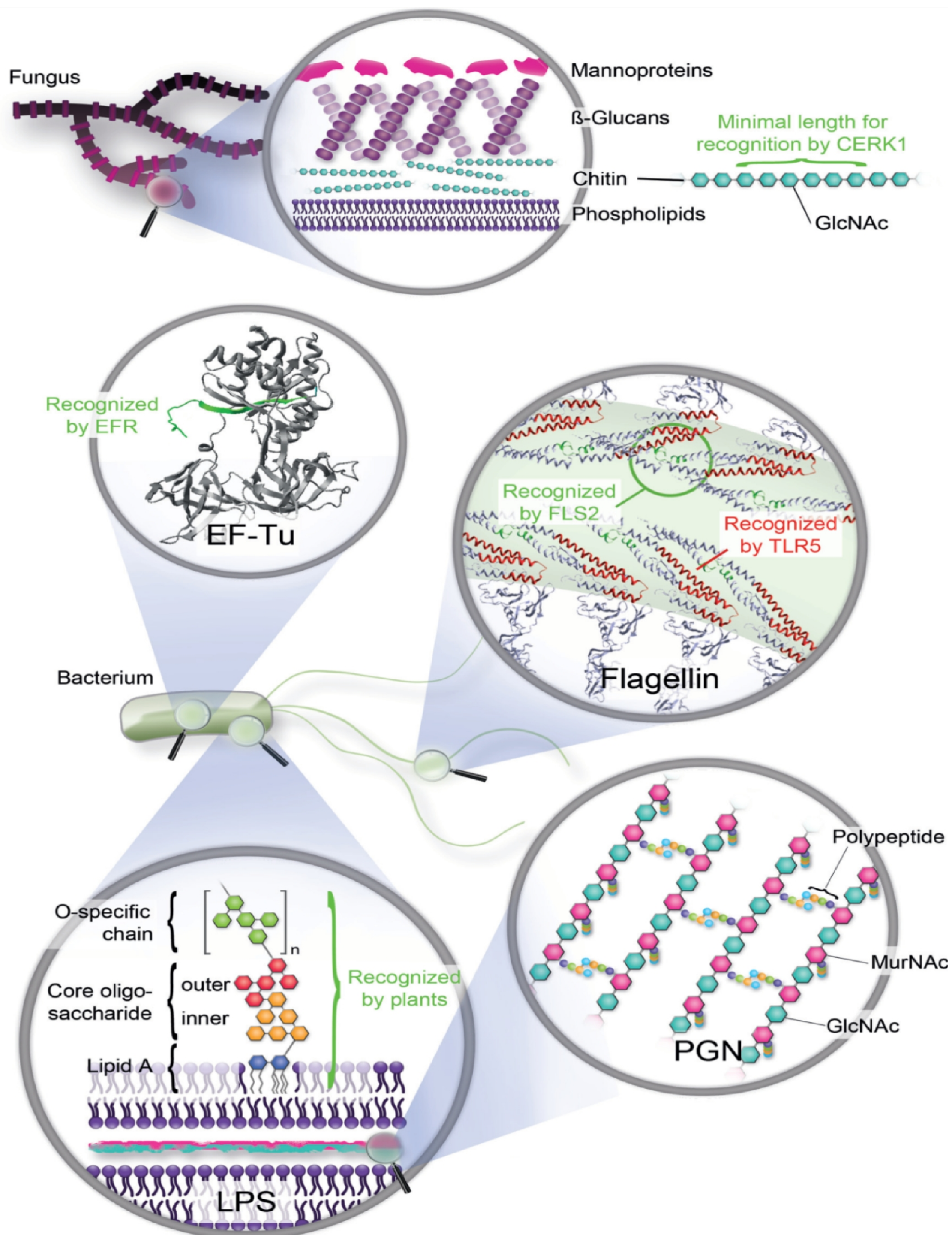

Fig. 1.8: Commons MAMPs sensed by extracellular PRRs.
Chitin is a polymer of N-acetylglucosamine (GlcNac) from fungal cell walls. Studies of its receptor CERK1 showed that at least six GlcNac units are necessary for proper binding of the molecule. Various compounds from bacteria are known to be recognized by plants. The elongation factor thermo unstable (EF-Tu) is perceived by EF-Tu RECEPTOR (EFR) while flg22 is sensed by FLAGELLIN SENSING 2 (FLS2). The mammalian TOLL-LIKE RECEPTOR 5 (TLR5) also binds flagellin but through another immunogenic epitope. The three parts of lipopolysaccharides (LPS), lipid A, the core oligosaccharides and the O-specific chain were all demonstrated to be PAMPs in plants. Peptidoglycan (PGN), another component of bacterial cell wall is also able to trigger defense responses in plants. PGN is a polymer composed of alternate monomers of GlcNAc and N-acetylmuramic acid (MurNAc) derivatized by small polypeptidic chains (Pel and Pieterse, 2012).

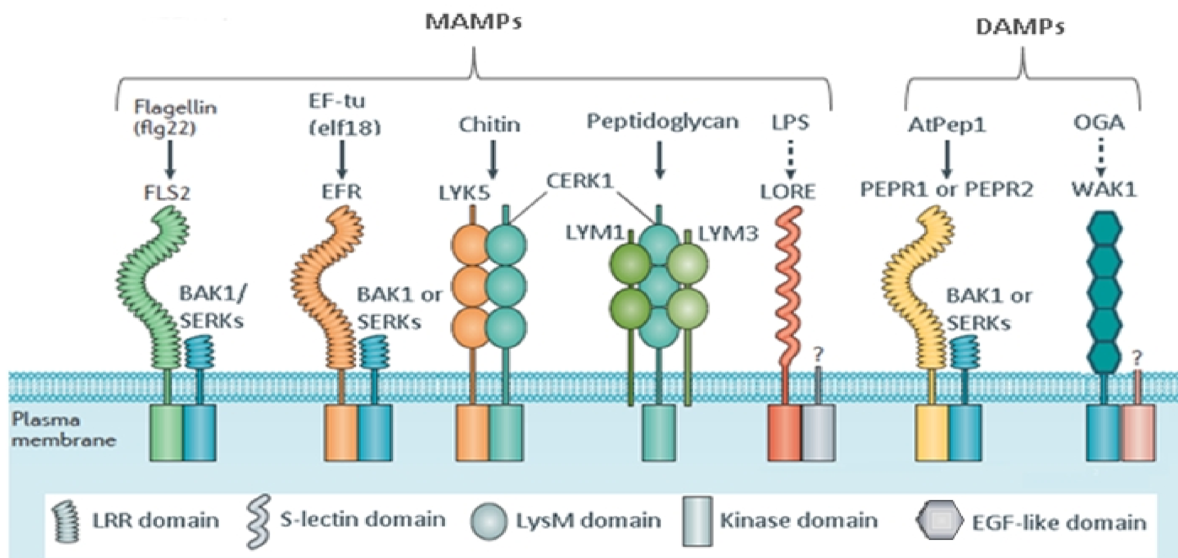

Fig. 1.9: Commons PRRs involved in PAMP sensing.
Microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) released by microbes at the cell surface are sensed by plasma membrane-bound RLPs and RLKs. The extracellular leucine-rich repeat (LRR) receptors such as FLAGELLIN SENSING 2 (FLS2), EF-TU RECEPTOR (EFR) and PEP RECEPTOR (PEPR) recognize peptides such as flg22, elf18 and the plant endogenous peptide AtPeps. LysM motif receptors such as LYSM-CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5), LYSM-CONTAINING receptors LYM1 and LYM2 bind peptidoglycan and chitin. Epidermal growth factor-like (EGF-like) domain from WALL-ASSOCIATED KINASE 1 (WAK1) recognizes oligogalacturonides (OGA) and S-lectin domains from LORE receptor binds carbohydrates-containing molecules such as lipopolysaccharides (LPS). Immediately after perception, PRRs form receptor complexes with regulatory receptor kinases such as BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) aka SOMATIC EMBRYOGENESIS KINASES (SERK) and CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1). Adapted from Couto and Zipfel (2016).

S-lectin domain motifs are involved in the recognition of carbohydrate-containing molecules. The lectin S-domain-1 receptor-like kinase, LORE (Fig. 1.9) has recently been shown to bind lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria such as *Pseudomonas* and *Xanthomonas*. LPS (Fig. 1.8) consists of a polysaccharide grafted on an oligosaccharide core linked to the membrane through lipid A, a phospholipid anchor, the most conserved domain of LPS and the ligand of LORE (Fig. 1.9) (Pel and Pieterse, 2012; Ranf *et al.*, 2015).

Other PAMPs recognized by PRRs include endogenous DAMPs such as systemin and AtPeps, these being 20 amino-acid-long derivatives from the C-terminal end of larger precursor proteins PROPEPs. AtPeps bind two LRR-RLKs: PEP-Receptor 1 (PEPR1) and PEPR2 (Fig. 1.9) (Klauser et al., 2015). WALL-ASSOCIATED KINASE 1 (WAK1) (Fig. 1.9) is a RLK with an epidermal growth factor-like (EGF-like) domain possessing an extracellular domain that recognizes oligogalaturonides (OGA), pectin fragments acting as DAMPs released from the plant cell wall (Couto and Zipfel, 2016; Decreux and Messiaen, 2005; Macho and Zipfel, 2014; Pel and Pieterse, 2012).

ETI, the second branch op plant immunity (Fig. 1.7), is activated by effectors that help the pathogen colonize its host by manipulating plant defenses (Win *et al.*, 2012). These effectors also termed *Avr* gene products or Avr proteins often have an active role in the pathogenicity and mostly have an immune-inhibitory activity. They were initially described as molecules injected in the host cytosol by bacterial T3SS and perceived intracellularly by nucleotide binding-site-leucine-rich repeat (NBS-LRR). The use of this terminology has been widened because fungal hyphae and oomycete haustoria also secrete effectors inside plant cells using transport vesicles and target them to different subcellular compartments (Stuart et al., 2013). Another contribution of the model of Boller and Felix (2009) is that effectors are not only perceived intracellularly but also extracellularly (Fig. 1.7). Indeed the *Cladosporium fulvum* effectors Avr2, Avr4 and Avr9 that disturb the normal course of PTI are detected in the apoplasm by membrane-bound LRR-RLPs called Cf2, Cf4 and Cf9, respectively (Boller and Felix, 2009). Effectors are now considered as a central class of compounds in plant–microbe interactions and they are considered as “molecules secreted by plant-associated organisms that alter host-cell structure and function” (Hogenhout *et al.*, 2009; Win *et al.*, 2012). Effectors have mainly two kinds of behaviors inside their host cell: they can either be enzymes having direct biological activity or divert host proteins activity for the benefit of the pathogen. Effectors often possess a functional redundancy meaning that a pathogen can secrete several of them which target the same host pathway. Effectors from unrelated pathogens often converge to the same target because they need to modify important plant processes in order to establish themselves on their host. Bacteria are continuously betrayed by their conserved PAMPs EF-Tu and flg22 so the effectors they inject through T3SS mostly inhibit responses downstream to this perception. Fungal pathogens similarly target molecular events downstream of chitin perception. (Win *et al.*, 2012).

ETI also called vertical resistance is an enlarged concept of the “gene-for-gene resistance”, a model first described by Flor at the beginning of the 1950s in which the product of a plant R gene is able to recognize its counterpart derived from a pathogen Avr gene (Flor, 1956; Stuart *et al.*, 2013). Later on, Avr gene products were associated to pathogen virulence factors and finally called effectors, leading to the ETI model. ETI is generally associated with a complete resistance against a specific strain of a pathogen but is also seen as a short-lived immunity because a single mutation in the effector or the R gene can completely abolish recognition. Most *R* genes encode intracellular NBS-LRR proteins (Fig. 1.7) dedicated to effector sensing and the induction of strain- or race-specific defense reactions culminates in

HR. The central domain composed by the nucleotide-binding site is flanked by an ARC domain which binds and hydrolyzes ATP and seem required for downstream signal transduction. In most cases, the binding of the effector relieves receptor inhibition by inducing a conformational change mediated by ATP hydrolysis at the NBS domain. The receptor is "turned on", giving the NBS-LRR receptor the name "molecular switch". (Rodewald and Trognitz, 2013; Zhang *et al.*, 2016).

3.1.2. The limit of the current models

The "zigzag model" and the coevolution model are essentially expository models to clarify the principles of plant-pathogen interaction but lots of important parameters are not included. The models are essentially valid for biotrophic plant pathogens but not for the necrotrophic ones. This last class of microbes does not develop complex interactions but promotes necrosis to ensure their nutrition. But what may appear as a primitive strategy is in fact much more subtle: host-specific toxin are secreted by certain necrotrophs to manipulate plant immunity. For example, the necrotrophs *Pyrenophora tritici-repentis* and *Stagonospora nodorum* produce a proteinaceous host-specific toxins called ToxA that targets the wheat R gene product TSN1, a NBS-LRR R gene necessary for resistance against stem rust, *Puccinia graminis* a biotrophic pathogen. There is thus growing evidence that these host-specific toxins help the pathogen to subverted ETI, rendering necrotrophic pathogen hypervirulent (Keller et al., 2016; Pritchard and Birch, 2014).

PAMPs are often distinguished from effectors by the fact that they are necessary for survival but not directly involved in virulence. However, change in LPS composition as well as reduced motility in flagellin mutants of *P. syringae* both affect bacterial virulence. The best conserved property of PAMPs is that they are perceived extracellularly in a receptor-ligand way with their receptors (Thomma *et al.*, 2011).

The time-scale and the ordering of the events in the "zigzag model" are also very simplified and likely to be false. The molecular processes described occur essentially at the population level such for *R* and *Avr* genes acquisition. In this model effectors appear to completely repress the PTI. However they are perceived at the same moment and the answer of the plant is a combinatory event that comprises both PAMP and effector perception. It also describes PTI and ETI as differential processes but they are likely component of the same pathway as the events downstream to pathogen recognitions are similar (Pritchard and Birch, 2014).

It appears that the PTI-ETI dichotomy leads to several misconceptions because both models are governed by the same forces. In the last presented models, ETI is not anymore distinguished from PTI and PAMPs and effectors are shown to operate on an equal footing. (Cook *et al.*, 2015). Moreover it seems that PTI and ETI share the same signaling components downstream of perception, even if PTI is often seen as a weak form of ETI. But this vision seems more and more blurred as both PTI and ETI can be weak or robust, according to the type of ligand-receptor considered. It was proposed that ETI occurs quickly, culminating in HR with a more lasting effect marked by the establishment of Systemic Acquired Resistance (SAR). But HR has been also observed in *Arabidopsis* following the sole PAMPs perception such as flg22. SAR has also been identified during PTI in *Arabidopsis* without absolutely requiring the occurrence of HR (Thomma *et al.*, 2011; Zipfel, 2009).

3.2. Signal transduction: military cooperation between cellular messengers

3.2.1. Receptor complex formation and kinase recruitment

After perception of a potential invader, the plant cell initiates intracellular responses activated thanks to a signal transduction cascade. Receptor-ligand binding generally induces a conformational change leading to receptor homo- or hetero-dimerization in about two minutes. Even if RLKs possess a kinase domain sufficient for intracellular signalization, they form dynamic complexes. For example, FLS2, EFR and PEPR 1 and 2, all associate with BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) or SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERKs) (Fig. 1.9) upon binding to their cognate ligand. In *Arabidopsis* CERK1 homodimerizes upon binding chitin but CERK1 acts also as co-receptor for LYK5 and the RLP LYM1 and LYM3 while the formal co-receptor of LORE and of WAK1 still need to be identified (Fig. 1.9). The interaction of a PRR with its co-receptor brings the kinase domains of the receptors in close contact and initiates signal transduction by intracellular protein transphosphorylation. Receptor dimerization is a frequently observed feature of PAMP perception. Then the PRR complexes associate temporarily with receptor-like cytoplasmic kinases (RLCK) before dissociating and activating downstream intracellular signaling.

RLPs that miss an intracellular kinase domain function in a similar way. Following ligand perception, they bind to the suppressor of BIR1-1 (SOBIR1) or to SOBIR1-like LRR-receptor kinases to form a bimolecular complexes. Then the RLP-SOBIR1 complexes recruit then the traditional co-receptors BAK1 or SERKs. (Couto and Zipfel, 2016; Seybold *et al.*, 2014). Similarly to PRR it seems that plants have evolved a large repertoire of RLCKs which vary in their levels of affinity for the diverse PRRs and in their capacity to stimulate different PTI pathways. RCLKs also demonstrate some plasticity as one RCLK is able to interact with different receptors complexes, explaining that signaling of different PAMPs converges to the same internal transduction pathway (Couto and Zipfel, 2016; Wu *et al.*, 2014).

3.2.2. Modification of ions fluxes

After receptor dimerization and phosphorylation events due to PAMP perception, the next signaling steps are modifications of membrane permeability, ions fluxes and ionic composition of the cytoplasm (Fig. 1.10). Usually plants maintain an electrochemical gradient of proton thanks to H^+-ATPases but within minutes after addition of PAMPs such as flg22, Pep-13, EF-Tu, ion flux alteration leads to fast acidification of the cytoplasm and extracellular alcalinization. This phenomenon is thought to be mediated by the activation of K^+/H^+ antiporters but also by the inhibition of the plasma membrane-localized H^+-ATPases probably ensured by phosphorylation or by calcium. Inhibition of these proton pumps causes membrane depolarization and movement of Cl^-, K^+ and NO^{3-} across the membrane.

Beside pH modifications, a transient rise of cytosolic calcium concentration is usually observed. For *Arabidopsis* leaves infiltrated with *Pseudomonas syringae* the Ca^{2+} concentration peaks in about 10 minutes. Observations performed with different type of PAMPs showed that the calcium oscillation amplitude and lasting time depend on the type of elicitor used. These variations are also induced by effector detection. Up to now how PRRs control calcium channel activity is not clearly understood. The increase in cytosolic Ca^{2+} level acts as a signal amplifier detected by calcium sensors containing Ca^{2+}-binding domains such as EF-hand motifs or C2 domains. Calmodulin (CaM) with four EF-hands regulates many of its interacting partners such as transcription factors (TFs) in a calcium dependent manner. (Cheval *et al.*, 2013; Elmore and Coaker, 2011; Seybold *et al.*, 2014; Wu *et al.*, 2014).

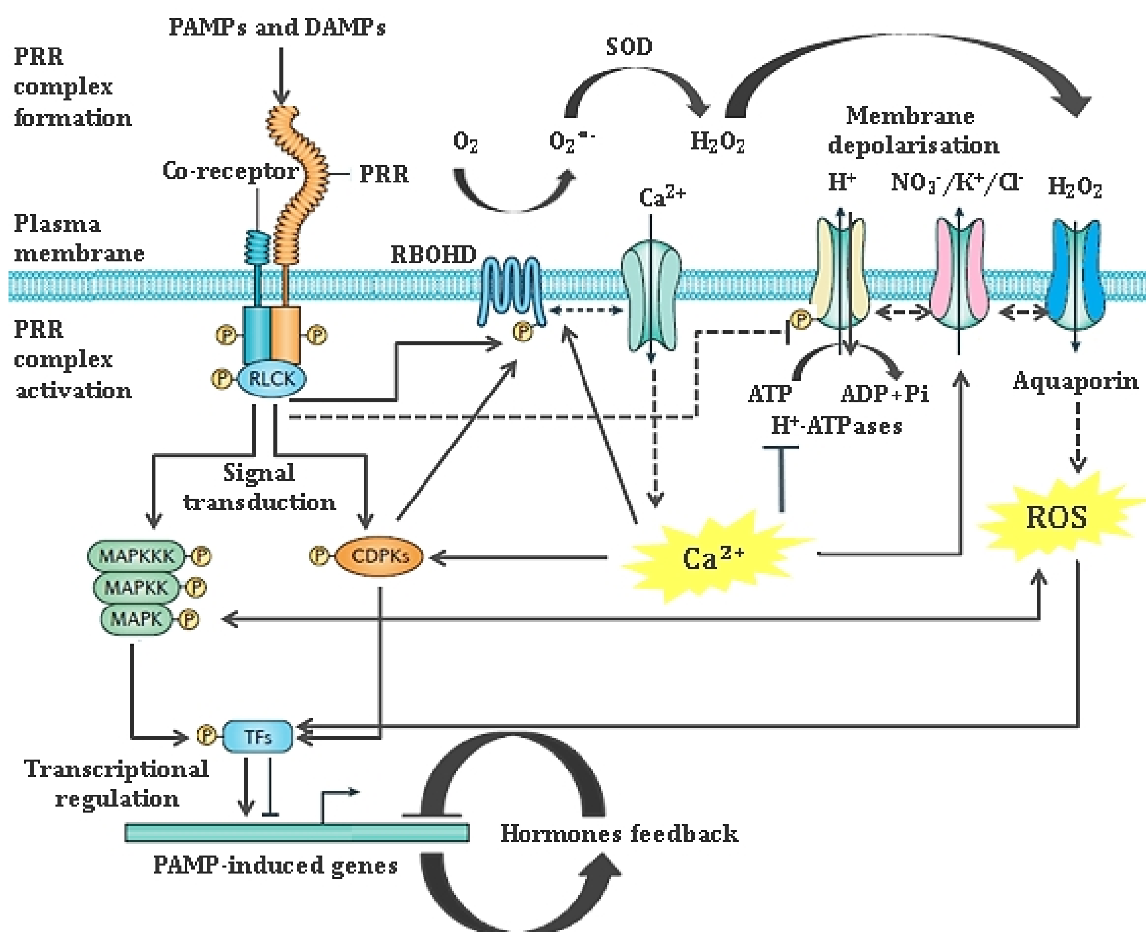

Fig. 1.10: Simplified view of early molecular events in PTI signal transduction. Upon PAMP perception, a PRR dimerizes with its co-receptor and recruits receptor-like cytoplasmic kinase (RLCK). Phosphorylation events contribute to the activation of many signaling components in PTI such as MAPK cascades, calcium-dependent protein kinases (CDPKs), respiratory burst homologs (RBOH) like RBOHD, transcription factors (TFs) and inhibition of proton pumps (H^+-ATPases). PAMP perception also induces an influx of calcium which is necessary for the activity of CDPKs and takes part in the activation of RBOHD as well as in the inhibition of certain types of proton pumps leading to extracellular alkalinization. This proton pump inhibition provokes membrane depolarization, contributing to the opening of ion channels such as potassium channels. The activation of RBOH ensures production of $O_2^{\bullet -}$ rapidly dismutated in H_2O_2 by superoxide dismutases. H_2O_2 can enter the cytosol using aquaporins and contributes to the pool of intracellular ROS also involved in the activation of MAPKs and TFs. TFs activation notably by MAPKs and CDPKs allows the regulation of downstream PAMP-induced genes which in turn regulate the blend of plant hormones involved in a feedback loop. Adapted from Couto and Zipfel (2016).

3.2.3. Protein kinases: CDPKs and MAPK cascades

Protein kinases phosphorylate other targets, affecting important properties such as enzyme activity, subcellular localization and stability. Phosphorylation is the most common post-translational modification found in eukaryotes and is an essential component of PTI and ETI signaling and it is a common way to activate TF (Fig. 1.10) (Bigeard *et al.*, 2015).

As mentioned above, plants possess calcium sensors among which the calcium-dependent protein kinases (CDPKs) which are protein kinases activated by Ca^{2+} rising levels. CDPKs influence the plant immune response by activating proteins by phosphorylation like the respiratory burst homologs (RBOHs) involved in ROS production (Bigeard *et al.*, 2015; Wu *et al.*, 2014).

Besides CDPK, MAPKs are a distinctive conserved family within the protein kinases that generally involves three protein kinases acting in series, a MAP kinase (MAPK), a MAPK kinase (MAPKK/MEK/MKK), and a MAPK kinase kinase (MAPKKK/MEKK). The mechanism of signal transduction is based on serial phosphorylation: MAPKKK acts upstream of MAPKK which in turn regulates MAPK. These MAPK cascades transmit and amplify extracellular and intracellular signals. MAPKs act upstream and downstream of ROS production and downstream of receptor signaling as they can be activated by RLCK. There is a tight interplay as well as a feedback loop between Ca^{2+} signaling, activation of TFs and MAPK cascades as well as ROS production by RBOHs (Fig. 1.10) (Couto and Zipfel, 2016; Meng and Zhang, 2013; Wu *et al.*, 2014).

The MAPK cascades appear as a convergence point in PTI signaling for different PAMPs since flg22 and elf18 activate the same MAPK chains. (Bigeard *et al.*, 2015; Meng and Zhang, 2013; Wu *et al.*, 2014). ETI is also able to trigger MAPK cascades but with a prolonged and sustained effect compared to PTI which only induces a transient activation. The difference in amplitude between the activation of MAPK cascades is susceptible to affect differently the set of genes regulated (Tsuda *et al.*, 2013). MAPK cascades are active in the initiation of several important responses of plant immunity such as hormone and phytoalexin biosynthesis, cell wall reinforcement, activation of defense-related genes, stomatal closure, ROS generation and HR. jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are three important plant hormones for the establishment of plant immunity. It probably explains why MAPK cascades are the target of numerous pathogen effectors such as the protein HopAI1 from *P. syringae* which deactivates several MAPKs. (Meng and Zhang, 2013; Wu *et al.*, 2014).

3.2.4. Reactive oxygen species and redox balance

Following pathogen infection, or shortly after treatment with PAMPs, a rapid oxidative burst is often induced in plant cells. The two major reactive oxygen species (ROS) formed are superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) but hydroxyl radical ($OH^{\bullet}$) and hydroperoxyl radical ($HO^{2\bullet}$) are also observed (Lehmann *et al.*, 2015). In case of R protein activation, the oxidative burst is biphasic: after a first small transient ROS accumulation, a second phase with higher amplitude and prolonged accumulation is observed and seemingly involved in HR (Wu *et al.*, 2014). ROS are common in living cells because they play an essential role in signaling and development but uncontrolled accumulation can cause great damage. ROS waves work as a systemic warning of localized (a)biotic stresses. ROS production in cells can occur following stress perception or in response to early signaling such as MAPK activation, protein phosphorylation and Ca^{2+} flux modification.

In plants, the major ROS producers are the RBOHs which are membrane-localized enzyme complexes that produce $O_2^{\bullet -}$ by electron transfer from the cytosolic NADPH or NADH to apoplastic oxygen. $O_2^{\bullet -}$ is then rapidly dismutated to H_2O_2 either spontaneously or by the action of a superoxide dismutase. H_2O_2 is a membrane-permeable signaling molecule that mainly diffuses to neighboring cells via aquaporins (Kadota *et al.*, 2014; Tian *et al.*, 2016). The *Arabidopsis* genome encodes ten different RBOHs (A-J) but RBOHD seems involved in the largest share of ROS production in the context of biotic stress (Torres and Dangl, 2005). RBOHD is directly regulated by PRR as upon PAMP perception RBOHD is directly phosphorylated (Fig. 1.10). RBOHD is regulated by two main mechanisms that probably act synergistically: phosphorylation mediated by RLCK and/or CDPKs and Ca^{2+} binding to its EF-hand motifs. RBOHs are not the only source of ROS in plants because they can also be by-products of several oxidizing activities in peroxisomes and in the electron transfer chains of chloroplasts and mitochondria (Fig. 1.11) (Kadota *et al.*, 2014; Lehmann *et al.*, 2015). There are also other enzymes involved in significant ROS production such as glycolate oxidase, oxalate oxidase, xanthine oxidase, amine oxidase and especially peroxidases (Fig. 1.11) (Gupta and Igamberdiev, 2015).

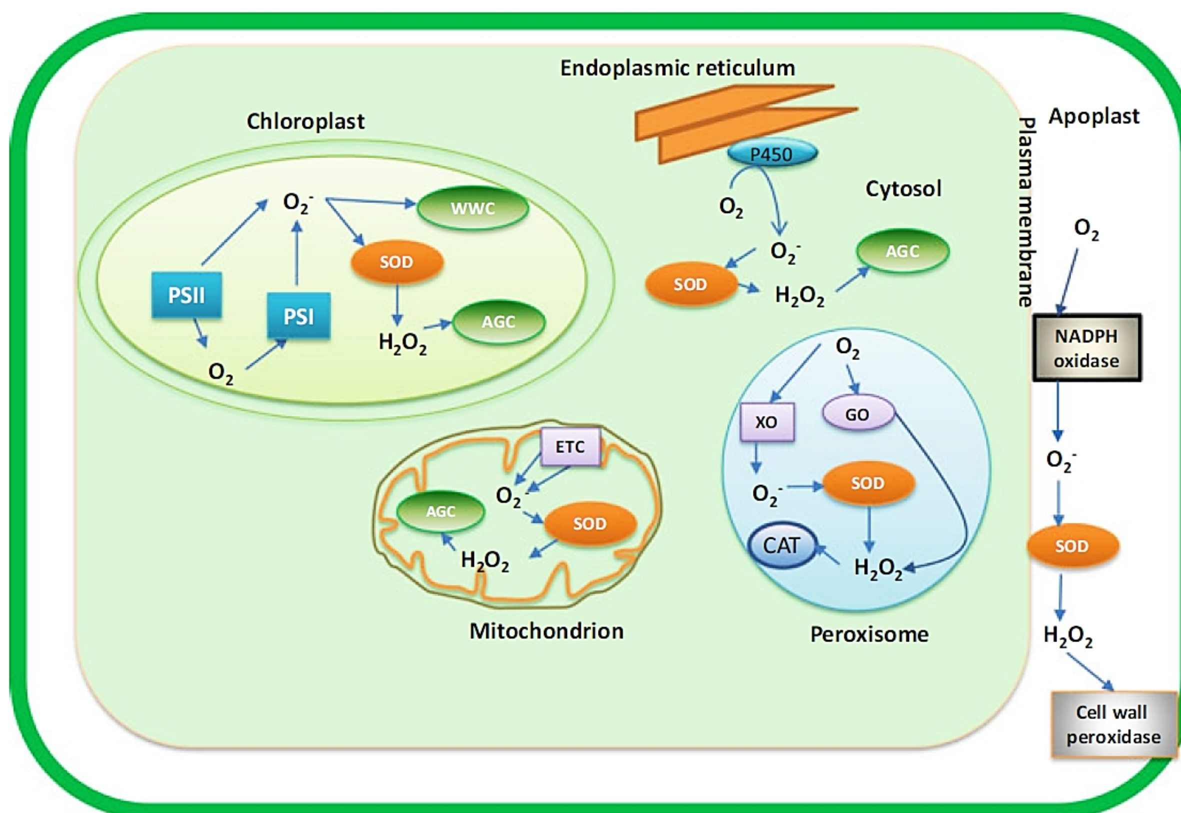

Fig. 1.11: Cellular compartments where ROS generation takes place.
Membrane-bound NADPH oxidases aka RBOH generate $O_2^{\bullet-}$ which is spontaneously converted into H_2O_2 in the apoplast or with the help of superoxide dismutase (SOD). Cell wall peroxidases are also able to generate or to metabolize H_2O_2. In chloroplasts and mitochondria, the photosystems I and II and the complexes I and III, respectively are the sites of ROS production. $O_2^{\bullet-}$ generated there is dismutated to H_2O_2 by SOD and follows the ascorbate-glutathione cycle (AGC) or the water-water cycle (WWC). The cytochrome P450 in the endoplasmic reticulum generates superoxide dismutated by cytosolic SOD and AGC. In peroxisomes the glycolate oxidase (GO) and the xanthine oxidase (XO) are the ROS producers while SOD and catalases (CAT) are the scavengers (Gupta and Igamberdiev, 2015).

Cell wall peroxidases also called class III peroxidases are considered as the second source of ROS and seem to produce up to 50% of H_2O_2 during the oxidative burst. Peroxidases 33 and 34 have been observed to be the major ROS providers in *Arabidopsis* following contact with fungal cell wall elicitors and bacterial pathogens. The production of the plant polysaccharide callose that blocks fungal appresoria depends on ROS formed by peroxidases. ROS originating from peroxidases also seem important for regulation of defense-related genes as well as for the PAMP-induced stomatal closure, a plant defense mechanism mediated by ROS (Baxter *et al.*, 2013; Daudi *et al.*, 2012; Kadota *et al.*, 2015).

When challenged by fungal elicitors, peroxidase mutants (*prx33/34*) show reduced expression of defense genes and callose deposition, compared to *rbohD* mutants. Therefore, ROS generated by RBOHs may not be functionally equivalent to ROS produced by class III peroxidases (Baxter et al., 2013). Similar studies with mutants show that RBOHD is necessary for the rapid ROS generation following PAMP treatment while peroxidases are probably involved in the secondary late peak of ROS production (Kadota *et al.*, 2015). Next to peroxidases, the cell wall contains other enzymes involved in minor apoplastic ROS production such as polyamine oxidases, germin-like oxalate oxidases, both cell wall-linked oxidases which mostly produce H_2O_2. There are also cell wall-bound lipoxygenases that mediate hydroperoxidation of polyunsaturated fatty acids (PUFA), an important component of plasma membrane that generates several types of ROS such as $O_2 \bullet^-$, H_2O_2 and OH• (Das and Roychoudhury, 2014).

ROS play also a role in the signaling during PTI. Indeed their high reactivity allows them to quickly propagate signals within different cell compartments as well as from cell to cell. ROS-propagated signals can be dynamically controlled because each cell possesses the machinery to scavenge excess ROS. ROS act synergistically with Ca^{2+} and they are both co-produced and co-regulated. ROS are also an important actor of post-translational modifications in proteins. H_2O_2 can oxidize the cysteine residues to form disulfide bridges. ROS can regulate the activity of several TFs and the best example is the induction of SA-dependent responses by oxidation events (Fig. 1.12). At the resting state, NON EXPRESSOR OF PR GENE 1 (NPR1) is present in the cytoplasm as an oligomer trough *S*-nitrosoglutathione (GSNO)-mediated oxidation of the disulfide bridge. Rising levels of SA are sensed by the SA receptor NPR1 which simultaneously requires redox modification. This occurs by the reduction of the intramolecular disulfide bounds present in NPR1 with the help of thioredoxins (TRXs). This results in monomerization of NPR1 that allows it to move to the nucleus where it activates the expression of SA-responsive genes in concert with TGA TFs that also need oxidation for successful interaction (Lehmann *et al.*, 2015).

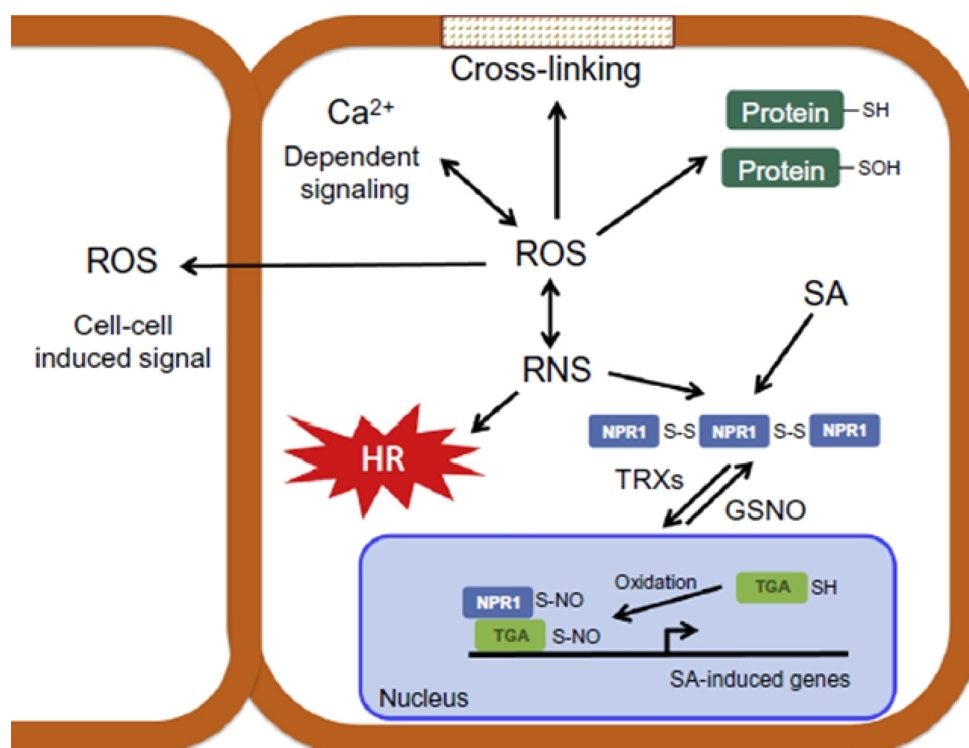

Fig. 1.12: ROS signaling in the plant cell during plant innate immunity.
ROS can either move to neighboring cells, interact synergistically with calcium signaling or with reactive nitrogen species (RNS) to induce HR. ROS can also perform crosslinking in the cell wall or modify proteins by oxidative cysteine modifications. ROS, together with salicylic acid (SA) and reactive nitrogen species (RNS) contribute the monomerization of NPR1 with the help of S-nitrosoglutathione (GSNO) and thioredoxins (TRXs) (Lehmann et al., 2015).

ROS can also act in concert with nitric oxide (NO) and NO-derived reactive nitrogen species (RNS). There is a correlation between RNS and ROS accumulation: NO accumulation occurs concomitantly with the oxidative burst. Both ROS and RNS have been involved in HR and the balance between these two compounds seems to be a key component in its outcome (Lehmann *et al.*, 2015; Spoel and Loake, 2011; Wang *et al.*, 2013).

ROS generated during plant-pathogen interaction can originate from both partners. Fungi, especially the filamentous necrotrophic ones, generate their own set of ROS to promote disease development. During infection, *B. cinerea* seems to produce H_2O_2 and $O_2\bullet^-$ through NADPH oxidases and NADPH-dependent oxidase complexes (NOX). Mutations in the genes coding for these enzymes impair *B. cinerea* penetration. Similarly, deletion of the NOX complex in *Magnaporthe oryzae* causes apathogenicity, demonstrating the importance of fungal ROS generation for penetration (Heller and Tudzynski, 2011).

Plants have therefore evolved multiple systems to protect against ROS and to contain oxidative burst below a certain level. If ROS level exceeds the signaling threshold it can damage DNA, lipids and proteins (Das and Roychoudhury, 2014). Two ROS-scavenging machineries exist in plants. The first is based on multiple enzymes such as superoxide dismutase, ascorbate peroxidase (APX), guaiacol peroxidase, glutathione-S-transferase (GST) and catalase. The second consists of antioxidants such as reduced glutathione (GSH), ascorbic acid, carotenoids, phenolics, α-tocopherol, flavonoids and proline (Das and Roychoudhury, 2014).

Superoxide dismutase is present in several cellular compartments and dismutates $O_2^{\bullet-}$ into H_2O_2 to suppress $OH^{\bullet-}$ formation. Catalase is mostly found in peroxisomes where it converts H_2O_2 into O_2 and water. But the main plant redox hub (Fig. 1.13) is the ascorbate/glutathione cycle whose function is to keep ROS under control. First APX reduces H_2O_2 to water with electrons obtained from ascorbate oxidation into monodehydroascorbate (MDHA). MDHA reductase (MDHAR) reduces MDHA back into ascorbate using NADPH. But the part of MDHA which is not directly reduced, disproportionates into ascorbate and dehydrooascorbate (DHA). The DHA reductase (DHAR) regenerates ascorbate at the expense of GSH, forming oxidized glutathione (GSSG). GSH, the reduced form of GSSH is regenerated by GSH reductase (GR). Ascorbate regeneration can be independent of GSH because some glutathione peroxidases use thioredoxins as electron donors to reduce H_2O_2. GSH can also be oxidized independently of DHA as glutathione S-transferases have GSH-dependent peroxidase activity using H_2O_2. Similarly glutaredoxins or peroxiredoxins can also use GSH to scavenge ROS (Fig. 1.13). By controlling ROS, ascorbate and GSH are components of the cell redox balance. The reduced to oxidized ratio of these compounds is sensed by the cell and plays a signaling role in combination with ROS levels. For example, a decrease in GSH pool induces important modifications of transcript level of genes coding for proteins involved in oxidative defenses. (Foyer and Noctor, 2011; Kovacs *et al.*, 2015). Recently, another element of the ascorbate-glutathione cycle, the pool of NAD and its phosphate derivative NADP, mainly present at the oxidized state emerged as other important components of cell redox balance signaling for plant defense. When NAD concentration increases, it triggers ROS production, modification of hormone levels and participates in the induction of callose deposition (Pétriacq *et al.*, 2016).

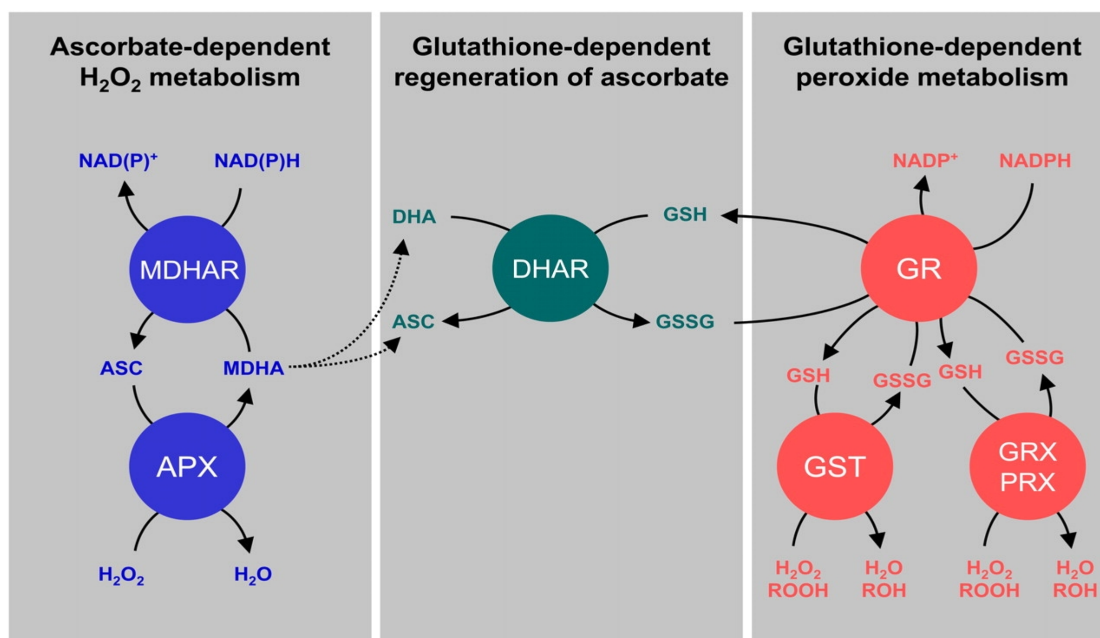

Fig. 1.13: The ascorbate-glutathione cycle.
The ROS scavenging processes depend mainly on ascorbate (ASC) and glutathione (GSH). Abbreviations are as follows: MDA, monodehydroascorbate; MDHAR, MDHA reductase; DHA, dehydroascorbate; GSSG, oxidized glutathione; GR, GSH reductase; GST, glutathione S-transferases; GRX, glutaredoxin; PRX, peroxiredoxins (Foyer and Noctor, 2011).

3.2.5. Transcription factors

TFs orchestrate the plant defense regulatory network directly or indirectly by recruiting or releasing RNA Polymerase II. TFs are not only DNA-binding molecules with a direct transcriptional activator or repressor activity but also co-activators and co-repressors that interact with other TFs to modify gene transcription. The activity of these TFs influences the blend of phytohormones produced, which in turn affects TF activity, forming a regulatory loop (Fig. 1.10). TFs must rapidly react to upstream cues originating from PRR activation such as Ca^{2+} signals, redox changes, MAPK cascades and hormone level modifications. TFs must also rapidly localize to their targets, mostly nuclear DNA, and cooperate within a complex regulatory network of other TFs. In plants, TFs involved in regulation of plant immunity belong mainly to the families of the APETALA2/ETHYLENE-RESPONSE ELEMENT BINDING FACTOR (AP2/ERF), basic-helix-loop-helix (bHLH), basic domain leucine zipper (bZIP), MYBs and WRKY (Moore *et al.*, 2011; Tsuda and Somssich, 2015).

The AP2/ERF family notably includes the ethylene-responsive factors (ERFs) involved in the regulation of ET- and JA-related genes such as OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59 (ORA59), ERF1, ERF6 and ERF104 in *Arabidopsis*. Most ERFs are positive regulators of plant immunity by binding to specific GCC *cis*-elements of pathogenesis-related (PR) genes but some of them are also negative regulators such as StERF3 from *S. tuberosum* as its silencing induces transcription of defense-related genes as well as enhanced resistance towards *P. infestans* (Pré *et al.*, 2008; Tian *et al.*, 2015; Tsuda and Somssich, 2015).

WRKY TFs are one of the most important TF families linked to defense regulation and are exclusively found in plants. WRKY are major targets of the MAPK cascades and they are also involved in the regulation of the crosstalk between SA- and JA-mediated signaling WRKYs. Numerous WRKYs are directly targeted by NPR1 in order to amplify SA-related defense responses. Among them, WRKY70 is an activator of SA-responsive genes but a potent repressor of JA-responsive genes. On the contrary WRKY33 in *Arabidopsis*, is linked to JA and ET signaling and is activated by MAPK3 and MAPK6. WRKY33 promotes resistance to necrotroph *B. cinerea* but not to the hemibiotroph *P. syringae*. WRKY33 positively regulates the expression of *PAD3* involved in biosynthesis of the phytoalexin camalexin as well as of ACC synthase involved in ET production. In potato, StWRKY1 related to the SA pathway was shown to increase resistance towards *P. infestans* by inducing the transcription of genes related to phenylpropanoid synthesis involved in cell wall reinforcements (Huang *et al.*, 2016; Lai *et al.*, 2011; Moore *et al.*, 2011; Yogendra *et al.*, 2015).

The MYB TFs is one of the largest family with more than 160 members in rice and in *Arabidopsis*. MYBs possess the R2R3 domain that binds specific DNA sequence elements and are involved in the regulation of the pathways of several phytohormones such as SA, JA, abscisic acid (ABA) and gibberellins (GA). The most studied AtMYB30 is involved in HR initiation in *Arabidopsis* as well as in regulation of phenylpropanoid metabolism in numerous plant species (Ambawat *et al.*, 2013; Liu *et al.*, 2015a; Tsuda and Somssich, 2015).

Only few members of the bHLH family have been identified as actors of plant immunity but this family contains the MYC2, MYC3, and MYC4 TFs which are related to JA-mediated defenses responses and take part to crosstalk with other phytohormones like SA. (Pireyre and Burow, 2015; Tsuda and Somssich, 2015).

The TGA members of the bZIP family are involved in plant immunity, especially in the control of SA-signaling pathways. TGAs generally bind DNA as homo- or hetero-dimers. In *Arabidopsis*, Class II TGAs co-regulate SA-responsive genes together with NPR1 but it was recently observed that these TFs are also involved in SA suppression of ET-induced genes. Indeed the ORA59 TF possesses a binding site for class II TGAs, making them an essential hub of SA/ET crosstalk (Zander *et al.*, 2014).

3.2.6. Ubiquitin-mediated regulation

Ubiquitination is a common post-translational modification of proteins that involves three main enzymes: UBIQUITIN-ACTIVATING ENZYME (E) 1, E2 and E3, this last one being important for specificity. This mechanism known for its role in protein turnover is also important for plant defense regulation and autoimmunity avoidance, a phenomenon by which plants show disease symptoms such as necrosis in absence of pathogen. The autoimmune phenotype is often observed in mutants of negative regulators of immunity and/or cell death (Rodriguez *et al.*, 2016; Wu *et al.*, 2014). This regulation already takes place at the level of receptor endocytosis and degradation following ligand activation. PUB12 and PUB13, two plant U-box E3 ubiquitin ligases are associated to BAK1 and FLS2 degradation while PUB22, PUB23 and PUB24 also downregulate FLS2 signaling (Wu *et al.*, 2014).
The degradation of ligand-bound receptors required for receptor turnover, generally causes a refractory period called receptor desensitization during which the plant becomes resistant to subsequent stimulation. The phenomenon has been observed for flg22 and chitin oligomers and lasts for a few hours until new receptor synthesis and exposition at the cell surface (Felix *et al.*, 1998; Smith *et al.*, 2014).

Beside PRR and NBS-LRR, TFs are also targets of ubiquitination. NPR1 monomers that escape oligomerization are rapidly targeted to the proteasome by a Cullin 3–based ubiquitin ligase. This phenomenon termed as nuclear clearance allows immunity regulation. Hence, clearing nuclear (co)activators like NPR1 and EIN3 are of major importance for silencing specific transcription programs (Moore et al., 2011). Ubiquitin can also alter TF activity in a positive manner: addition of a first ubiquitin unit is sometimes required to favor TF binding to DNA while poly-ubiquitination leads to TF degradation (Pireyre and Burow, 2015).

3.3. Hormonal modulation of plant defense: fight back mission program

Plant hormones (Fig. 1.14) are signaling molecules that regulate a significant part of plant immunity. They are low molecular compounds able to travel to distant targets and to bind receptors that transduce the signal to transcriptional complexes, allowing a coordinated answer of the whole plant. These messengers are able to interact together by crosstalk mechanism mainly regulated at TF level. SA, JA and ET are the three core phytohormones, termed here as "The Big Three" that regulate plant defenses. Generally speaking and deduced from observations performed mainly on *Arabidopsis*, SA-associated plant defenses are linked to resistance against biotrophic and hemibiotrophic plant pathogens while JA and ET positively regulate defense against necrotrophic plant pathogens. There is an extensive crosstalk between these three pathways which will be presented briefly. Other less important players which sparked growing interest in the recent years are abscisic acid which mainly helps the plant coping with abiotic stresses and hormones primarily studied for their involvement in plant growth regulation namely auxin, cytokinin (CK), gibberellin (GA) and brassinosteroids (BRs) (Tsuda and Somssich, 2015).

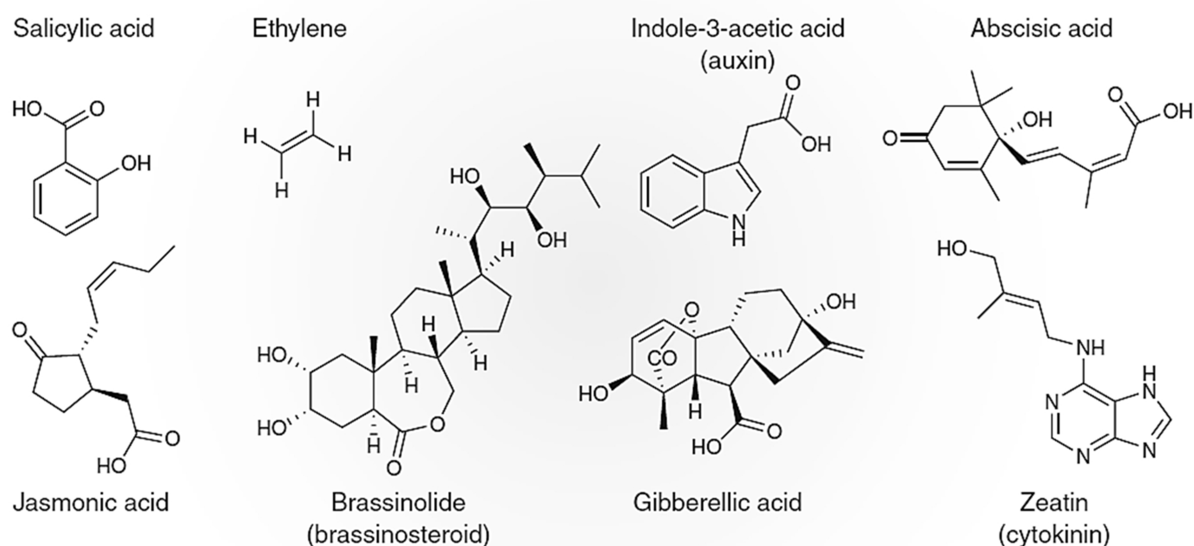

Fig. 1.14: Plants hormones involved in modulation of plant immunity.
SA, JA, ET and ABA contribute mainly to plant adaptation to (a)biotic stresses while auxin, BR, CK and GA are mainly related to developmental regulation (Pieterse et al., 2009).

3.3.1. Salicylic acid (SA)

The plant hormone SA plays a pivotal role in the regulation of both local defense and systemic acquired resistance (SAR) activated by PTI or by ETI. SAR occurs when plant defenses are activated locally by PAMP application or by a primary infection possibly accompanied by a HR, and then a signal is propagated across the whole plant to protect the undamaged tissues. SAR protects against subsequent infections by a broad range of pathogens and its onset is often characterized by SA accumulation (Pieterse *et al.*, 2014).

SA is a phenolic compound originating from chorismate and synthetized in the chloroplast by two distinct pathways (Fig. 1.15). The first route of synthesis relies on phenylalanine ammonia-lyase (PAL) which converts chorismate in cinnamic acid leading to SA via either a benzoate or O-coumarate intermediates after several enzymatic reactions. The second route depends on isochorismate synthase (ICS/SID2) which converts chorismate in SA. In *Arabidopsis*, SA synthesis triggered by biotic stresses derives mainly from ICS1 while in potato PAL is the main source of SA following elicitation with arachidonic acid (Coquoz et al., 1998; Vlot et al., 2009). ICS1 was shown to play a critical role in ETI, PTI, and SAR but appeared dispensable for HR. The biological activity of SA is regulated by conjugation to various compounds. The main form of SA storage is glycosyl-SA or SA obtained through the action of SA glucosyltransferase (SAGT). Other minor forms of storage are salicyloyl ester (SGE) but SA can be subjected to methylation and amino acid conjugation. This phenomenon allows to finely tune SA levels in plant cells and also allows storage of large amounts of SA avoiding its toxicity seeing its role in HR (Dempsey et al., 2011).

Studies in Arabidopsis demonstrated that MAPK cascades, redox and calcium signaling triggered by PRR or NBS-LRR activation are important regulators and act upstream of SA biosynthesis. TFs that bind to the promotor of *ISC1* to activate its transcription are activated by Ca^{2+} trough Ca^{2+} sensors proteins. Calcium signaling participates in the accumulation of the protein EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1), an activator of SA accumulation which acts in combination with PAD4 (PHYTOALEXIN-DEFICIENT 4) to maintain a positive feedback loop in SA synthesis. EDS1 is also positively regulated by the FLAVIN-DEPENDENT MONOXOYGENASE 1 (FMO1) which responds to $O2\bullet^-$ but not to H_2O_2 accumulation. It is also suggested that EDS1 and PAD4 are directly sensitive towards redox modifications and may have a fundamental role in transducing redox signals. (Pieterse *et al.*, 2012; Seyfferth and Tsuda, 2014; Vidhyasekaran, 2015b).

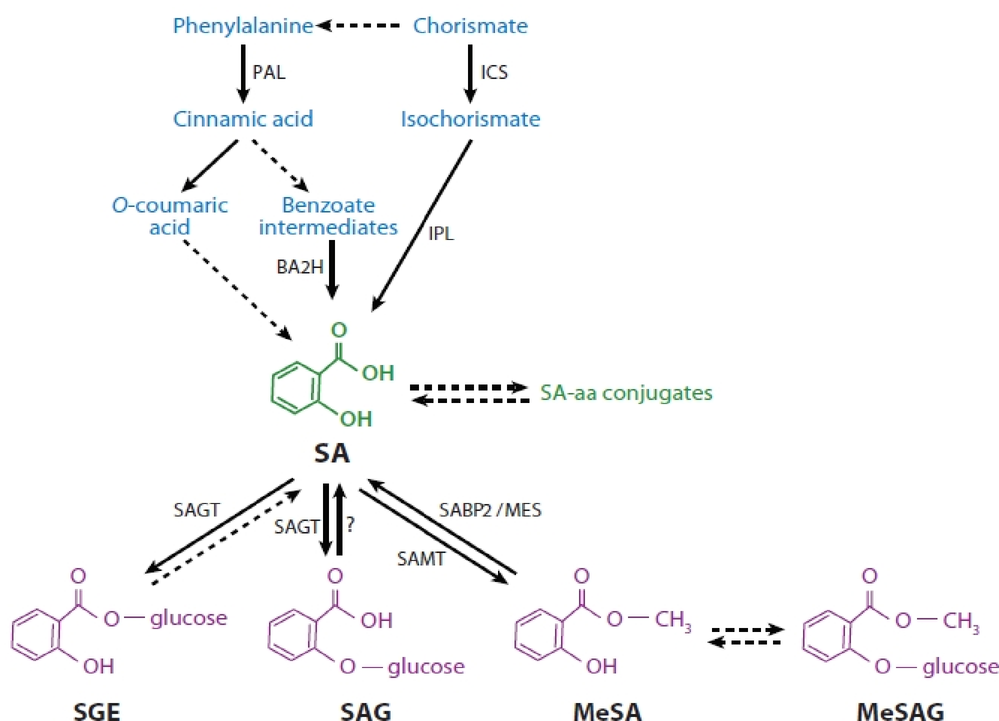

Fig. 1.15 : Simplified representation of the two major routes of SA synthesis.
BA2H, benzoic acid-2-hydroxylase; ICS, isochorismate synthase; IPL, isochorismatepyruvate lyase; MES, methyl esterase; MeSA, methyl salicylate; MeSAG, methyl salicylate O-β-glucoside; PAL, phenylalanine ammonia lyase; SA, salicylic acid; SABP2, SA-binding protein 2; SAGT, SA glucosyltransferase; aa, amino acid; SAMT, SA methyltransferase; SGE, salicyloyl glucose ester; SAG, SA *O-β*-glucoside (Vlot et al., 2009).

SA accumulation in the cell needs to be perceived to trigger transcriptional reprogramming. NPR1 (aka NIM1), the master regulator of SA signaling, acts downstream of SA accumulation and needs to be monomerized to enter the nucleus in order to activate genes transcription. This monomerization was first thought to be caused only by modification of the cytoplasmic redox status generated by SA accumulation (Lindermayr *et al.*, 2010). But Wu *et al.* (2012) demonstrated that specific cysteine residues of NPR1 bind directly SA via Cu^{2+}, triggering a conformational change that releases the C-terminal transactivation domain from the N-terminal autoinhibitory BTB/POZ domain leading to NPR1 monomerization (Fig. 1.16). SA concentration also regulates NPR1 accumulation with the help of the SA receptors NPR3 and NPR4 which are both adaptors for Cullin 3 ubiquitin E3 ligases: upon binding to NPR1, they target it to the proteasome degradation pathway. SA increases the binding between NPR1 and NPR3 but disrupts the interaction between NPR1 and NPR4 meaning that NPR1 activation occurs only at intermediate SA concentration (Fig. 1.16). NPR1 is notably degraded trough NPR3 during ETI which triggers high levels of SA at the local site of infection. The degradation of NPR1 combined to high SA levels are required to initiate HR following NBS-LRR activation (Liu *et al.*, 2016a; Seyfferth and Tsuda, 2014).

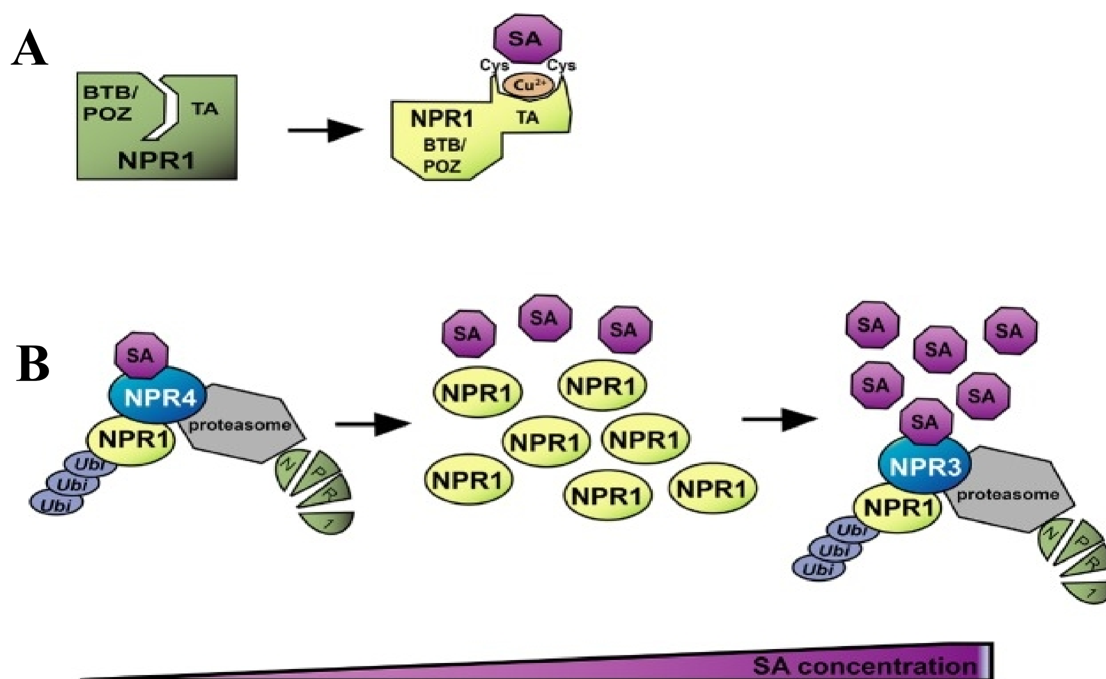

Fig. 1.16 : Model of SA perception by its receptors NPR1, NPR3, NPR4.
A. SA binding to its receptor NPR1 relieves the inhibition of its transactivation domain (TA) by the BTB/POZ domain thanks to conformational changes.
B. At low SA concentration, NPR4 binds to NPR1 and induces its degradation through the ubiquitin-mediated proteasome degradation. At intermediate SA concentration, NPR1 is stabilized and accumulates but at high SA concentration NPR3 interacts with NPR1 to trigger its degradation (Seyfferth and Tsuda, 2014)**.**

At the resisting state, cytoplasmic NPR1 is an oligomer maintained by intermolecular disulfide bridges reinforced by S-nitrosoglutathione. Rising SA levels after biotic stress induces redox changes and reduction of the cysteine residues by thioredoxins. The breakdown of disulfide bridges results in NPR1 monomerization and translocation to the nucleus through the nuclear pore (Pieterse *et al.*, 2012). Once in the nucleus, NPR1 binds TGA TFs and interacts with the promoter of SA-responsive genes to start transcription. NPR1 then becomes phosphorylated and targeted to the proteasome for degradation by an E3 ubiquitin-ligase with high affinity for phosphorylated NPR1. NPR1 turnover seems important for successful expression of SA-responsive genes enabling a new round of transcription by other NPR1 monomers (Fig. 1.17). (Pieterse *et al.*, 2012). SA perception induces a massive transcriptional reprogramming leading to the expression of defense-related genes mostly comprising *WRKYs*, *ERF*s, genes encoding proteins with antimicrobial activity and *PR* genes among which the well-studied SA-marker, *PR1*. The role of TFs such as ERFs and WRKYs is to maintain and increase the diversity of PR genes expressed that directly contribute to the resistance of the plant towards subsequent pathogen challenges (Fesel and Zuccaro, 2016; Fu and Dong, 2013; Vidhyasekaran, 2015b).

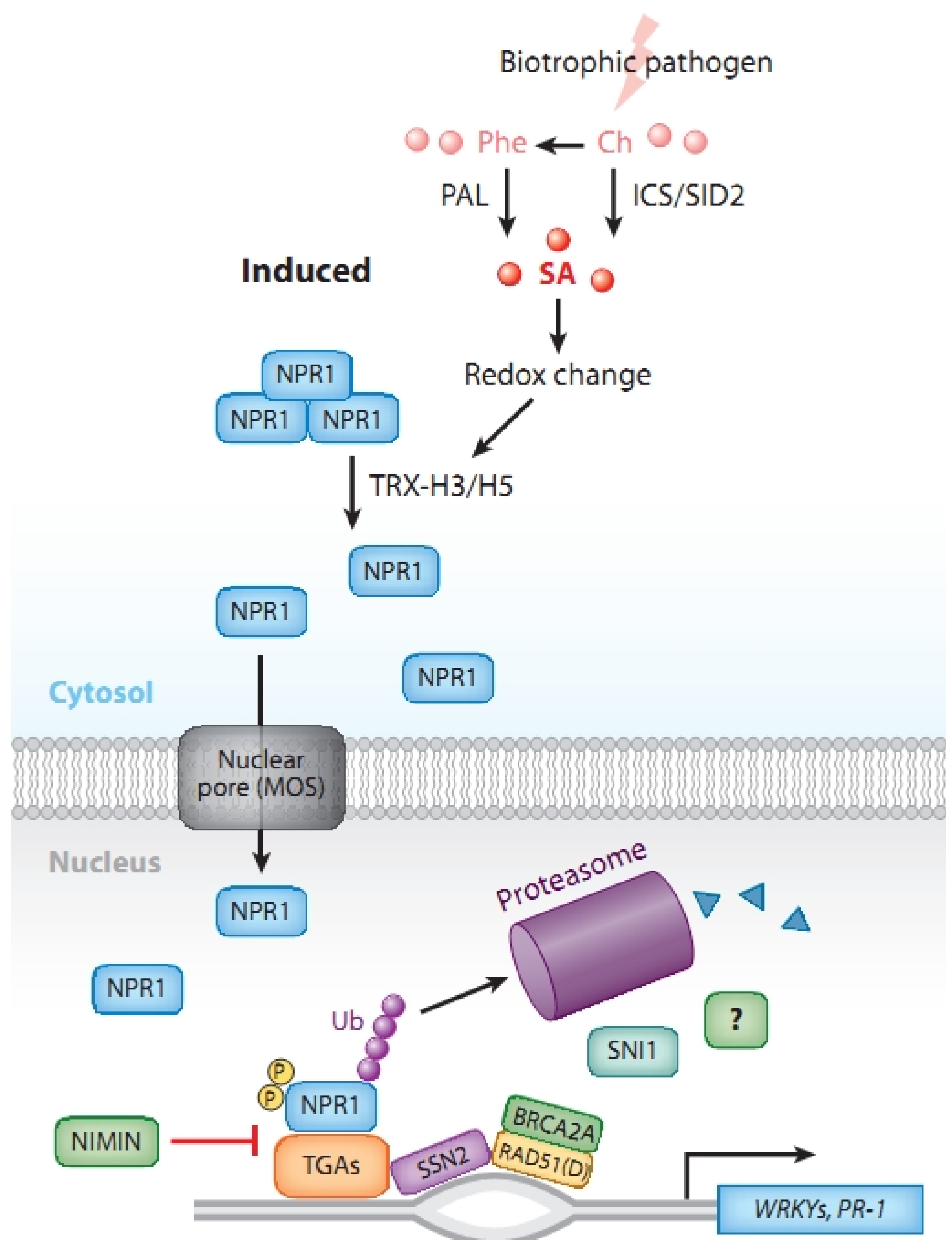

Fig. 1.17 : NPR1 regulation of SA-responsive genes.
Biotic stress perception triggers salicylic acid (SA) synthesis, either from phenylalanine (Phe) by phenylalanine ammonia lyase (PAL) or from chorismate (Ch) by isochorismate synthase (ICS/SID2). SA accumulation triggers redox changes that together with SA and thioredoxins TRX-H3 and TRX-H5 leads to NONEXPRESSOR OF PR GENES 1 (NPR1) monomerization which further moves to the nucleus through the nuclear pore MODIFIER OF snc1 (MOS). In the nucleus, NPR1 associates to TGA transcription factors and binds to the promoter of SA-responsive genes to activate transcription. Then NPR1 becomes phosphorylated and is targeted to the proteasome for degradation by an E3 ubiquitin-ligase. At resting state, NPR1-regulated genes are repressed by NIM1-INTERACTING (NIMIN) and the SUPPRESSOR OF NPR1 INDUCIBLE1 (SNI1) which inhibit TGA transcription factors or gene promotors to prevent fortuitous activation. Genes under NPR1-transcriptional control also require the displacement of the inhibitor SNI1 from the promotor thanks to an interaction with a DNA repair complex comprising: RAS ASSOCIATED WITH DIABETES 51D (RAD51), SUPPRESSOR OF SNI2 2 (SSN2) and BREAST CANCER 2A (BRCA2A) which renders DNA sequence accessible for transcription (Pieterse et al., 2012).

Reviews on the role SA-dependent plant defense are almost unanimous on the prominent role of SA against biotic plant pathogens, notably because high SA levels promote HR (Glazebrook, 2005). In 17 species including *Arabidopsis*, tomato, tobacco but not potato, an antagonism exists between SA and JA/ET pathways (Halim *et al.*, 2009; Thaler *et al.*, 2012). This last pathway takes part in resistance against herbivorous insects and necrotrophic pathogens and numerous plant enemies take advantage of this antagonism by manipulating phytohormones to stimulate their development. It was indeed shown that *B. cinerea* in tomato favor the SA-signaling pathway to promote disease (Rahman *et al.*, 2012). But conflictual information exists in the literature, even in closely-related species. For example, *nahG* mutants show that SA defense is involved in resistance against the biotrophic powdery mildew *Oidium neolycopersici* in tobacco but not in tomato. Benzothiadiazole (BTH) treatment also induced resistance against the necrotroph *B. cinerea* in tomato but not in tobacco (Achuo et al., 2004), showing that SA-related defense can be sometimes efficient against necrotroph. This discordance mainly originates from the use *A. thaliana* whose results are not always transferable to other species: in particular, potato plants contain a higher basal SA level than *Arabidopsis*. Exogenous application of SA does not directly induce SAR in potato, no more than treatment by arachidonic acid or infiltration with *P. syringae* that do not lead to systemic SA accumulation (Halim *et al.*, 2007). But synergies between SA and JA have also been reported, mainly at low concentration in *Arabidopsis* and in tobacco (Mur *et al.*, 2006; Pieterse *et al.*, 2009; Sanchez *et al.*, 2012).

In the general model, SA pathway is typically prioritized over JA and the molecular basis of the SA repression of JA/ET-responsive genes begins to be well characterized in *Arabidopsis* (Thaler et al., 2012). The control of SA on JA pathway occurs essentially downstream of JA biosynthesis and perception and mainly takes place at transcriptional level (Caarls *et al.*, 2015). The first main element of SA control over JA is redox signaling. SA accumulation enhances GSH levels in plant cells while JA tends to increase the oxidized form GSSH and the timing of GSH accumulation following SA application coincides with SA-suppression of JA-responsive genes. When both hormones are applied simultaneously, only GSH levels rise which is compatible with SA prioritization over JA (Koornneef *et al.*, 2008).

The second component of the SA-JA antagonism consists of TFs. SA is able to promote sequestration or degradation of key TFs of the JA and ET-responsive genes. SA was shown to lead to the degradation of ORA59, a positive regulator in the ERF branch of the JA/ET pathway and also more recently from MYC2, the master regulator of the MYC branch in the JA pathway (Fig. 1.20) (Schmiesing *et al.*, 2016).

3.3.2. Jasmonic acid and oxylipins

The plant hormone JA and more largely jasmonates are important regulators of responses against (a)biotic stresses. JA signaling is triggered by wounding but also by associated DAMPs such as OGAs or endogenous peptides. MAMPs such as EF-Tu, chitin and flg22 stimulate multiple immune signaling cascades including JA-associated defense responses. Mutants in JA synthesis or signaling are more susceptible to herbivorous insects and to necrotrophic pathogens. JA and jasmonates are members of a larger family of compounds, the oxylipins that also play an important role in plant defense taking part in signal transduction or possessing direct effect against pathogens (Kachroo and Kachroo, 2012). PUFAs at the origin of oxylipins derive from the action of lipid hydrolyzing enzymes on membrane lipids which release linoleic acid (C18:2,), linolenic acid (C18:3) and hexadecatrienoic acid (C16:3). JA synthesis starts in the chloroplast from LnA oxidized by three successive enzymes:13-lipoxygenase (13-LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) giving rise to oxo-phytodienoic acid (OPDA).

OPDA crosses the cytoplasm to the peroxisome where it is again enzymatically converted to form JA (Fig. 1.18). JA can undergo multiple types of conjugation such as the conversion in the volatile form methyl jasmonate by JA CARBOXY METHYLTRANSFERASE (JMT). But the biologically active form of JA is JA-isoleucine (JA-Ile) formed in the cytoplasm thanks to JA INSENSITIVE 1 protein, JAR1 (Dave and Graham, 2012; Riken, 2010; Vidhyasekaran, 2015a; Wasternack and Hause, 2013).

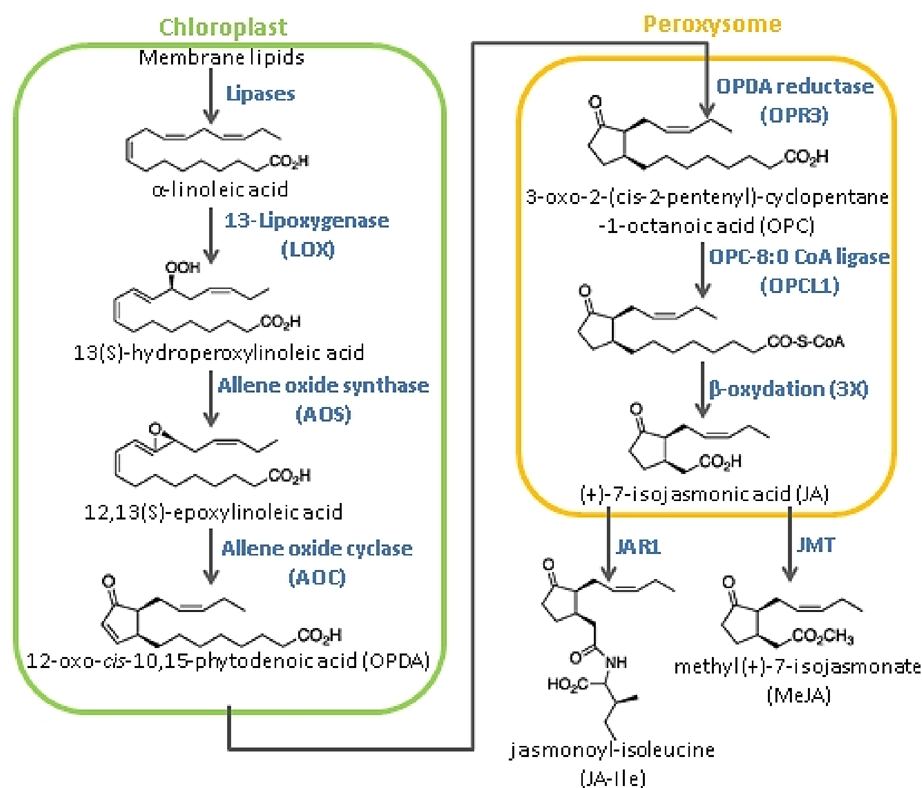

Fig. 1.18 : Simplified view of jasmonic acid (JA) synthesis.
JA synthesis begins in the chloroplast and finishes in the peroxisome. In the cytoplasm, JA can be converted in its biologically active form jasmonoyl-isoleucine (JA-Ile) by JASMONIC ACID-AMINO ACID SYNTHASE (JAR1) or in the volatile methyl jasmonate (Me-JA) by JASMONIC ACID CARBOXYL METHYLTRANSFERASE (JMT). Modified from Dave and Graham (2012) and Riken (2010).

JA-Ile is not the only bioactive oxylipin because OPDA is more than an intermediate, as it plays a signaling role even if it is not yet fully elucidated (Wasternack and Hause, 2013). Beside their direct activity in plant defense, oxylipins compete for the substrate LnA used for JA synthesis. (Eckardt, 2008). Many other relevant oxylipin compounds (Fig. 1.19) derive from the action of 9-LOXs or 13-LOXs that insert one oxygen molecule on carbon 9 or carbon 13 from PUFA, respectively. The results are two kinds of 9- or 13-hydroperoxides which can be further converted into other compounds. The peroxygenase and the epoxy alcohol synthase (EAS) catalyze the formation of epoxy and hydroxyl fatty acids. Vinyl ether-containing PUFAs such as colnele(n)ic acids are formed under the action of divinyl ether synthase (DES). The oxo fatty acids and aldehydes which are volatiles and non-volatiles oxylipins are formed by hydroperoxyde lyase (HPL) (Robinson and Bostock, 2014; Yan *et al.*, 2013). HPL in rice was shown to be involved in the formation of green leafy volatiles but downregulated the synthesis of JA following substrate competition. Another round of action of LOX leads to synthesis of keto PUFAs (Göbel et al., 2001; Wasternack and Hause, 2013).

The α-dioxygenase catalyzes the conversion of the PUFAs to other fatty acid hydroperoxides that are substrates for alternative metabolic pathways to the one of LOXs. But ROS production can also be directly responsible for non-enzymatic membrane-derived lipid peroxidation which also serve as signal molecules (Mosblech *et al.*, 2009). The product of the 9-LOX and the α-dioxygenase have been shown to be involved in resistance against the hemibiotrophic *P. syringae* in *Arabidopsis* (Vicente *et al.*, 2012). In potato, resistance to late blight has been correlated to the accumulation of the product of the 9-LOX, colnelenic and colneleic acid because of their possible antimicrobial effect (Royo et al., 1996). The transcription of 13-LOX has been observed in rice and in wheat following pathogen attack but in *Solanaceae* such as potato and tobacco, only the 9-LOX accumulates following oomycete or oomycete-derived elicitor treatment (Göbel *et al*., 2001).

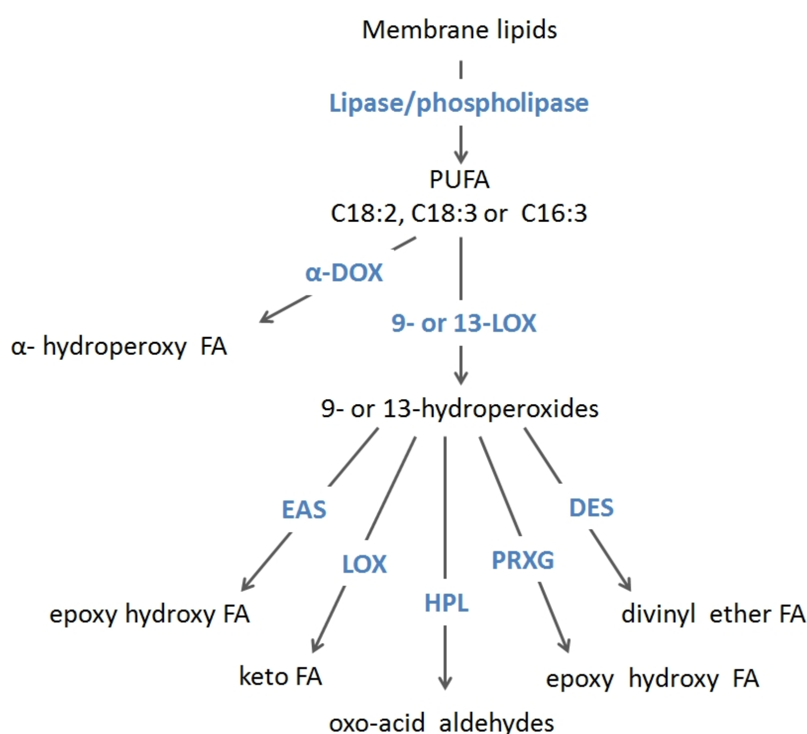

Fig. 1.19: Schematic view of a part of the oxylipins synthesis pathway.
The action of lipases or phospholipases on membrane lipids releases three polyunsaturated fatty acids (PUFAs): hexadecatrienoic acid (C16:3), linoleic acid (C18:2) and linolenic acid (C18:3). These PUFAs can directly undergo the action of α-dioxygenase (α-DOX) leading to α-hydroperoxy fatty acids (FA). PUFAs can also be oxidized by 9-lipoxygenase (LOX) or 13-LOX generating 9- and 13-hydroperoxydes which can again be modified by several other enzymes including epoxy alcohol synthase (EAS), LOX, hydroperoxyde lyase (HPL), peroxygenase (PRXG), divinyl ether synthase (DES). Modified from Yan *et al.* (2013) and Robinson and Bostock (2014).

Beside competition with the synthesis of other oxylipins, JA synthesis is also regulated by MAPK cascades and calcium signaling but the precise sequence of events is still poorly understood. JA synthesis is subjected to a positive feed-back regulation loop because JA-Ile synthesis leads to the degradation of the repressors of JA-responsive genes, the jasmonate ZIM-domain proteins (JAZs). JAZ repressors (Fig. 1.20) maintain repression of JA-responsive genes and bind many positive regulator of JA-responsive genes (Wasternack and Hause, 2013). The perception of JA-Ile (Fig. 1.20) by the protein CORONATINE INSENSITIVE1 (COI1) leads to the formation of an ubiquitin complex interacting with JAZ which is then targeted to the proteasome for degradation (Pieterse *et al.*, 2012). JAZ degradation releases many positive TFs of JA-responsive genes. Indeed, JAZ acts as repressor of the positive transcriptional regulators MYC2, MYC3, and MYC4. MYCs control the expression of the MYC branch (Fig. 1.20) of JA-related defenses associated to the wound responses and the resistance against herbivorous insects which is characterized by the expression of the downstream marker gene VEGETATIVE STORAGE PROTEIN 2 (VSP2) (Fig. 1.20). MYC2 also represses the second branch of the JA-signaling cascade termed ERF branch regulated by TFs such as ORA59 and ERF1 whose transcription is increased by both ET and JA. The ERF branch is mainly efficient in the control of necrotrophic pathogens and that part of the pathway is characterized by several marker genes directly involved in pathogen growth restriction encoding the PLANT DEFENSIN 1.2 (PDF1.2) but also the PATHOGENESIS-RELATED PROTEINS 3 and 4 (PR3 and PR4) (Campos et al., 2014; Kazan and Manners, 2013; Pieterse et al., 2012; Wasternack and Hause, 2013). Beside the possible synergy with ET and JA, JA pathway is also able to antagonize SA upstream of NPR1 notably through TF phosphorylation by MAPK4. Indeed, *Arabidopsis mpk4* mutants exhibit constitutive activation of SA-related defense genes but are impaired in their response to JA and ET (Brodersen *et al.*, 2006; Caarls *et al.*, 2015).

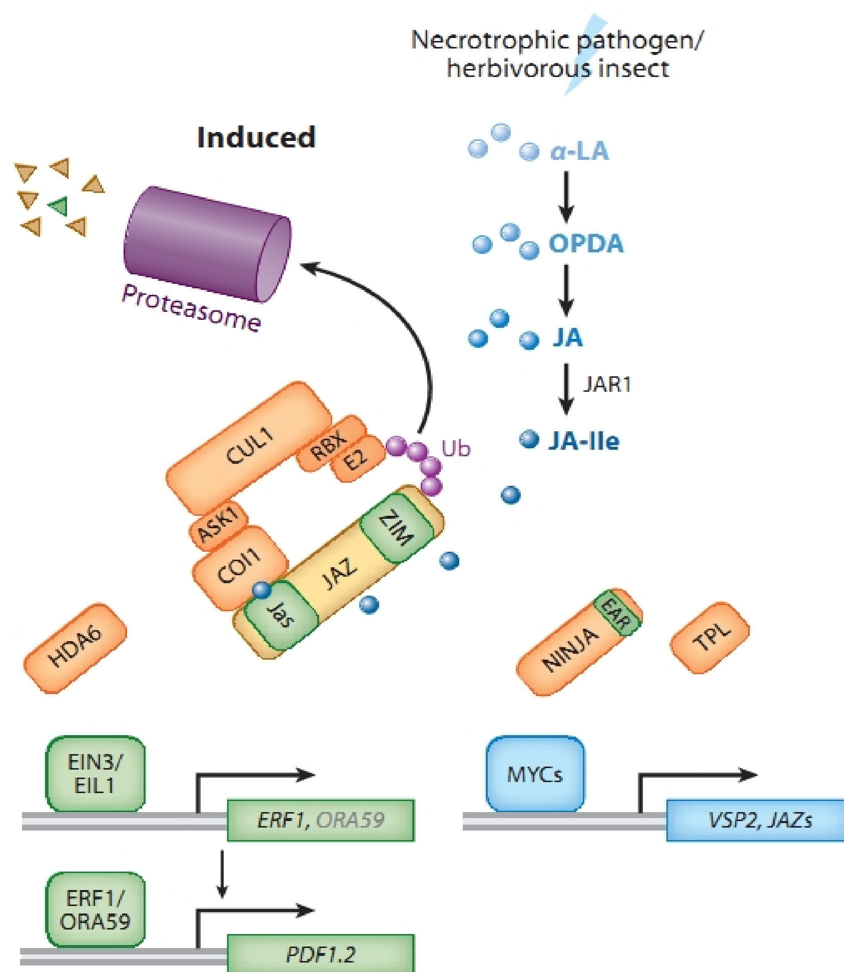

Fig. 1.20: JA perception via the COI1-JAZ co-receptor complex and the associated signaling cascade in *Arabidopsis*.

The perception of biotic stimuli such as herbivorous insects or necrotrophic pathogens induces the synthesis of jasmonic acid (JA) starting from α-linolenic acid (α-LA) which is converted to the JA precursor, oxo-phytodienoic acid (OPDA). JA is converted in the biologically active jasmonoyl-isoleucine (JA-Ile) by JA INSENSITIVE 1 (JAR1) protein. JASMONATE ZIM (JAZ) repressors maintain repression of JA-responsive genes notably by interaction of their ZIM-domain with the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA). NINJA recruits the corepressor TOPLESS through its ERF-ASSOCIATED AMPHIPHILIC REPRESSION (EAR) motif. TOPLESS notably contributes to repression of gene transcription via HISTONE DEACETYLASE 6 (HDA6) and HAD19. In absence of JA-Ile, JAZ also physically interacts with positive regulators of JA-responsive genes such as ETHYLENE INSENSITIVE 3 (EIN3), EIN3-LIKE 1 (EIL1) and various MYC transcription factors. But the perception of JA-Ile by the protein CORONATINE INSENSITIVE1 (COI1) which forms an E3 ubiquitin-ligase SKP1-Cullin-F-box complex SCF^{COI1}, allows its interaction with the repressor JAZ. JAZ is then ubiquitinylated and degraded via the proteasome (Pieterse *et al.*, 2012). JAZ degradation relieves the JA-related transcription factors, allowing the expression of JA-responsive genes such as *ETHYLENE RESPONSE FACTOR 1 (ERF1), PLANT DEFENSIN1.2 (PDF1.2); OCTADECANOID-RESPONSIVE ARABIDOPSIS 59 (ORA59)* and *VEGETATIVE STORAGE PROTEIN 2 (VSP2)* (Pieterse *et al.*, 2012)

3.3.3. Ethylene (ET)

ET (C_2H_4) is involved in the regulation of a broad range of physiological plant processes including growth, germination, senescence, mechanosensing and especially ripening which nicknamed it the ripening hormone. ET is produced in the plant in response to life-cycle events or (a)bitoic stresses including diseases. ET is part of the “Big Three” involved in plant defense and mainly acts as an arbiter between SA and JA. Mutant studies generally demonstrated that ET promotes disease resistance against necrotrophic but not against (hemi)biotrophic pathogens (van Loon *et al.*, 2006a). ET is synthetized (Fig. 1.21) from *S*-adenosyl-methionine (*S*AM) that derives from methionine. SAM is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase, the rate limiting enzyme in ET synthesis. ET is finally obtained by the oxidation of ACC by ACC oxidase (Agarwal *et al.*, 2012; Arc *et al.*, 2013; Pieterse *et al.*, 2012).

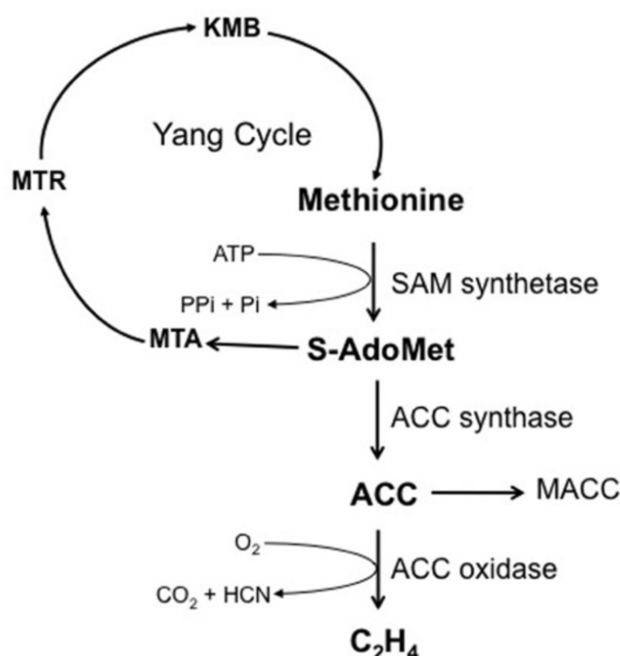

Fig. 1.21: Simplified diagram of the ET biosynthesis pathway.
The ET precursor *S*-adenosyl-methionine (*S*AM) originates from the Yang Cycle. SAM is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. ET is generated by the action of ACC oxidase using oxygen and releasing carbon dioxide and hydrogen cyanide. During the Yang Cycle, SAM is produced by the action of SAM synthase on methionine which also releases 5′-methylthioadenosine (MTA). MTA is recycled to methionine by successive enzymatic reactions which pass through several intermediates (MTR, 5-methylthioribose; KMB, 2-keto-4-methylthiobutyrate). Adapted from Arc *et al.* (2013)**.**

In *Arabidopsis*, in absence of ET, ET INSENSITIVE 2 (EIN2) an essential positive regulator in the ET signaling pathway is targeted the proteasome pathway. But when ET is perceived by the receptors, EIN2 is relieved as well as its downstream TFs such as EIN3 and

EIN3-like proteins (EILs) which are able to bind to the promoter of ET-RESPONSIVE FACTORS (ERFs). ERFs are plant-specific downstream TFs of ET-signaling pathway that promote the transcription of ethylene-regulated *PR* genes (Wang *et al.*, 2002). In particular EIN3 promotes the expression of *ORA59* and *ERF1* which are both key elements of ERF branch from JA pathway. JA and ET act synergistically in the activation of the ERF branch of the JA pathway while the other branch, regulated by MYC2 is a negative regulator of JA/ET responses. (Pieterse *et al.*, 2009; Robert-Seilaniantz *et al.*, 2011). But ET was also demonstrated to act synergistically with SA. In tobacco, ET was essential for the initiation of a SAR and in *Arabidopsis* the ET-mediated increase of the SA-induced *PR1* expression was impaired in *ein2* mutants. ET also reverts the prioritization of SA pathway in *Arabidopsis*. If JA and ET signaling are activated prior to the onset of the SA-signaling, the plant the antagonistic effect of SA is completely abrogated (Leon-Reyes *et al.*, 2010; Pieterse *et al.*, 2009).

3.3.4. The emerging players and their crosstalks with the "Big Three"

3.3.4.1. Abscisic acid

ABA is primarily known for its involvement in abiotic stress tolerance and allows plants to finely tune their metabolism in order to withstand the adverse effect of drought, heat and salinity. Beside its role in abiotic stress, ABA takes part in the modulation of plant growth and development (Sah *et al.*, 2016) but ABA also interferes with plant defense responses. Many observations performed on ABA deficient mutants as well as on exogenous application of ABA led to the assumption that increased levels of this plant hormone correlated with increased susceptibility (Mauch-Mani and Mauch, 2005). But studies performed in various pathosystems yield mixed results: ABA contribution to disease susceptibility depends on the infected tissue, the stage of development and the pathogen feeding strategy (Mengiste, 2011). It seems that there are antagonisms between ABA and SA-mediated plant defenses. ABA application on *sitiens*, a tomato mutant more resistant to *B. cinerea*, restores its susceptibility to the necrotroph. Indeed, as previously explained the resistance of tomato against *B. cinerea* seems to be related to SA (Audenaert et al., 2002). But ABA promotes stomatal closure and callose deposition which are efficient pre-invasive and early post-invasive defenses, respectively. β-amino-butyric acid (BABA) and chitosan both prime the plant for enhanced callose deposition in a process that requires ABA. At later stages, ABA seems to suppress ROS, SA-related defense and the ERF branch of JA-related defenses associated with late

post-invasive defense (Ton *et al.*, 2009). However the MYC branch of the JA-pathway is synergistically activated by ABA (Pieterse *et al.*, 2014). On one hand the antagonism between SA and ABA is probably regulated at NPR1 level as ABA was shown to promote NPR1 degradation in complex with NPR3 and NPR4. But on the other hand the role of ABA in NPR1 turnover seems important for the activation of NPR1-dependent gene expression (Ding et al., 2016).

3.3.4.2. Auxins

Auxins and more particularly indole-3-acetic acid are especially known for their activity in mediating differential growth in response to gravitropism and light stimuli as well as being master regulators of root development. For this last phenomenon, auxins interplay with lots of other hormones including JA, ET, BRs, GA and ABA (Saini *et al.*, 2013). But the first indication of auxins crosstalk with plant defense came from observations of *P. syringae* pv tomato interaction with its host plant. The hemibiotrophic bacteria promote accumulation of auxin and transcription of auxin-responsive genes. These genes promote accumulation of JA, camalexins and lower glucosinolates and inhibit SA signaling, which favors bacterial speck development. *Arabidopsis* treatment with the SA analogue, BTH downregulates auxin-responsive genes. High SA levels lead to indole-3-acetic acid depletion, growth restriction and probably allocation of resources for defense (Naseem *et al.*, 2015).

3.3.4.3. Gibberellins

GAs are diterpene phytohormones whose responses rely on their DELLA repressors degradation through proteasome, a pathway very similar to JA regulation of JAZ repressors. The precise mechanism in *Arabidopsis* shows that GA interacts first with its receptor (GID1a, GID1b, and GID1c), then with one of the five DELLA proteins (RGA, GAI, RGL1, RGL2, and RGL3) and recruits the F-box protein SLY. This last event targets the complex to the proteasome degradation pathway. DELLAs mutants generated in *Arabidopsis* show high SA levels coupled to enhanced resistance to the hemibiotrophic bacteria *P. syringae* and increased susceptibility toward the necrotrophic fungus *A. brassicola* (De Bruyne *et al.*, 2014). DELLA proteins compete with MYC2 for binding JAZ repressors (Fig. 1.22) which relieves MYC2 repression by JAZ and allows expression of JA-responsive gene (Wasternack and Hause, 2013). In a nutshell, high GA levels promote SA pathway and defense against (hemi)biotrophic pathogens while low GA levels promote JA pathway and defense against necrotrophic pathogens.

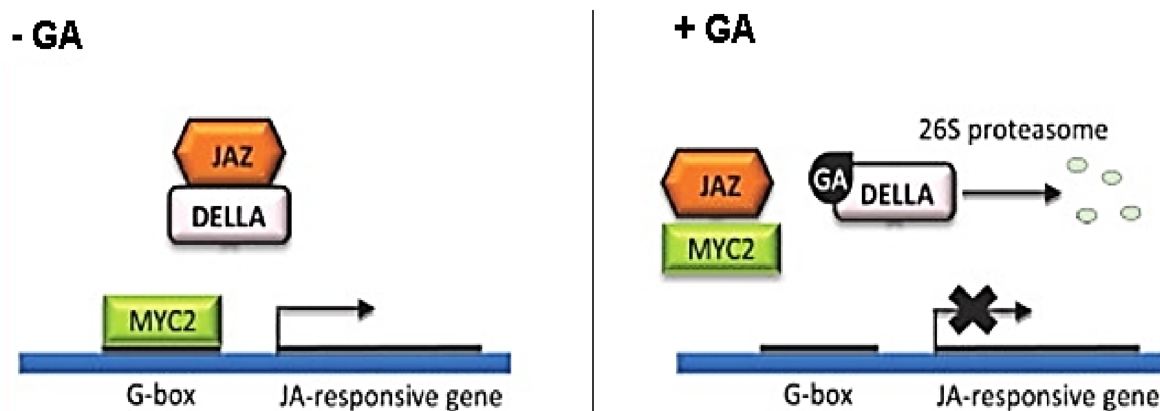

Fig. 1.22: DELLA-JAZ interaction model.
In the absence of GA (-GA), DELLA forms a repressor complex with JAZ. MYC2 is free to interact with the promotor of JA-responsive genes leading to their transcription. When GA levels rise (+GA), DELLA is degraded and JAZ binds to MYC2 TF preventing JA-responsive gene transcription (De Bruyne *et al.*, 2014).

3.3.4.4. Brassinosteroids

BRs are a group of steroid hormones involved the regulation of growth and development. Mutants of BR synthesis or signaling show severe growth defects mainly linked to dwarfism because BRs take part in the regulation of cell elongation. BRs bind to the extracellular domain of a cell-surface receptor kinase called BRASSINO-STEROID INSENSITIVE 1 (BRI1) that involves the co-receptor BAK1. BAK1 is also the co-receptor of several PRRs such as FLS and EFR, which creates competition between BR signaling and PAMP perception. An antagonism was thus first imagined but studies showed that BRs treatment enhances resistance against biotrophic pathogen, increases SA levels and *PR1* expression (Robert-Seilaniantz *et al.*, 2011; Zhu *et al.*, 2013).

3.3.4.5. Cytokinins

CKs are mainly substituted adenine-derived compounds involved in a broad range of growth and development processes. But recently, *Arabidopsis* treated with CK revealed a positive effect on plant defense activation. CKs seem to promote SA pathway in particular via WRKYs but it appears that the JA pathway is also positively regulated. The basic isoform of PR1, LOX3 as well as a gibberellin oxidase involved in GA synthesis appeared as three densely connected nodes in the *Arabidopsis* proteome following CK treatment (Naseem *et al.*, 2014). Up to now, only few studies have been performed with pathogens but Choi *et al.* (2010) demonstrated that CKs enhanced SA accumulation, *PR1* transcription and resistance against the hemibiotrophic *P. syringae* in *Arabidopsis*.

3.4. Activated defense mechanisms: Weapons of massive destruction

PAMP signaling followed by hormonal reprogramming of the whole plant leads to a disease resistance state for which several mechanisms have been characterized such as SAR, induced systemic resistance (ISR), priming and even sometimes HR. Plants being in this induced state of defense will already exhibit or start more rapidly metabolic modifications such as cell wall reinforcement, phytoalexin synthesis, production of antimicrobial peptides and PR proteins that will directly stop or dampen the development of novel invaders.

3.4.1. Systemic acquired resistance and induced systemic resistance

SAR is a SA-dependent state of defense effective against a broad spectrum of pathogens activated across the whole plant following local stimulation of ETI and PTI (Shah and Zeier, 2013). Local accumulation of SA seems required for SAR induction but grafting experiments on *nahG*, the mutant expressing the SA-degrading enzyme salicylate hydroxylase show that SA is not the mobile signal. Other molecules are potent SAR transmitters (Fig. 1.23). The first candidate is methyl-salicylate, the volatile methyl-conjugated form of SA. In *Arabidopsis* and potato, the distal tissues perceive methyl-salicylate via SABP2, a lipid protein with esterase activity and strong affinity for SA that cleaves inactive methyl-salicylate back into active SA. Other studies have demonstrated the involvement of the lipid transfer protein DIR1 that acts in cooperation with the lipid-derived compound glycerol 3-phosphate as mobile signal. Mutants unable to produce glycerol 3-phosphate are defective in SAR. The phloem of SAR-induced plants was also found to be enriched with azelaic acid and dehydroabietinal, both suspected partners of the mobile SAR signal. Dehydroabietinal is a diterpene that originates from geranylgeranyl pyrophosphate and exogenous application of this compound was shown to be sufficient for inducing *ICS1-, FMO1*- and *NPR1*-dependent SAR. Finally pipecolic acid, a non-protein amino acid deriving from lysine catabolism is associated with various stresses in plants and animals. It is also involved in SAR amplification and its increase precedes SA in systemic leaves of SAR-induced plants. SAR induction by pipecolic acid also requires *ICS1, FMO1* and *NPR1*, as observed for dehydroabietinal. Redundancy of SAR mobile signals probably gives flexibility to the plant which might be helpful in a context where pathogen effectors continuously try to hijack plant defense signaling (Fu and Dong, 2013; Shah *et al.*, 2014).

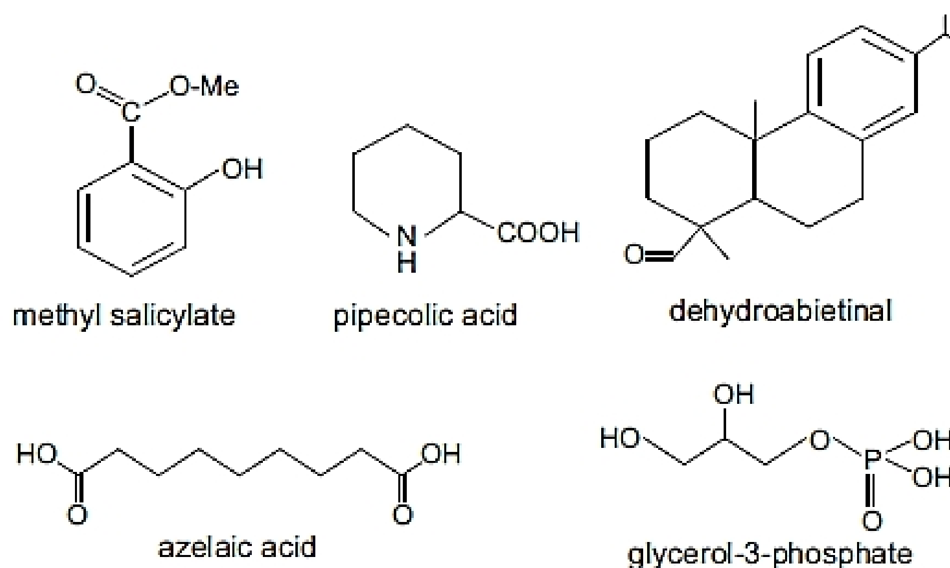

Fig. 1.23: Small molecules involved in the long distance transmission of the SAR signal (Shah *et al.*, 2014).

Another mechanism of systemic resistance is the induced systemic resistance (ISR) mainly triggered by above-ground beneficial microbes such as PGPRs. ISR is similar to SAR but it engages other mechanisms as it is mainly regulated by ET and JA. Indeed mutants in the JA and the ET signaling pathways are unable to mount a correct ISR following PGPRs inoculation. This observation was not only done on *Arabidopsis* but also on rice and tomato. Surprisingly, NPR1 was shown to be required for JA/ET-mediated responses triggered by several PGPRs (Pieterse et al., 2014). Moreover, studies performed by Pieterse *et al.* (1998) with mutants revealed that ET acts downstream of JA-pathway during ISR. (Fig. 1.24). SAR and ISR are not only triggered by microbe perception but also by chemical and natural compounds such as the SA analogue BTH, the synthetic amino acid BABA, lipid-derived compounds such as hexanoic acid, linoleic acid, inorganic salts like phosphites, compounds from fungal origin as chitosan, β-glucan, ergosterol, compounds from bacterial origin such as lipopeptides and plant-derived polysaccharides such as OGA, laminarin and ulvan. Only a few of this product have reached the field mainly because of their low efficacy. As mentioned above, for successful use of elicitors in agriculture it is important to understand their effects not only on plant defense but also on other aspects of plant development and environmental responses (Alexandersson *et al.*, 2016; Ongena and Jacques, 2008; Wiesel *et al.*, 2014).

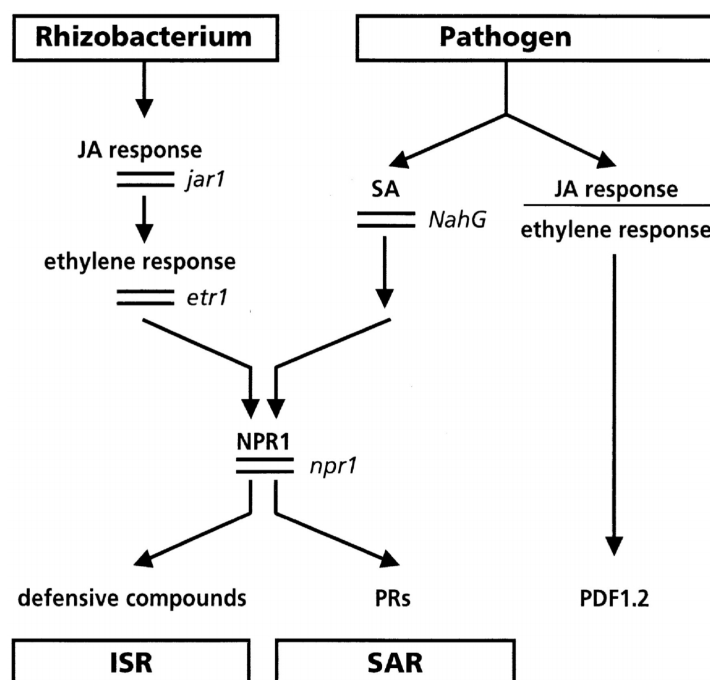

Fig. 1.24: Synergy between ethylene (ET) and salicylic acid (SA) and between jasmonic acid (JA) and ET in systemic acquired resistance (SAR) and induced systemic resistance (ISR).
This model was deduced from studies with *Arabidopsis* mutants. The accumulation of the *plant defensin PDF1.2* is independent of SA and requires both ET and JA signaling components. The *NahG* mutant unable to accumulate SA failed to induce SAR and showed no expression of *pathogenesis-related protein 1* (*PR1*) marker gene. The mutants *jar1*, deficient in methyl-jasmonate response and *etr1* deficient in ET response succeed in inducing SAR in response to pathogen but are unable to start ISR following contact with rhizobacteria. In *npr1* mutants both SAR and ISR responses are impaired (Pieterse *et al.*, 1998).

3.4.2. Priming

Priming allows plants to start a more efficient defense response after a first stimulation by keeping among others defense-response genes in a poised state. Defense gene priming is observed in several situations, following SA-dependent SAR as well as following stimulation of JA- and ET-linked ISR. Priming can be triggered by chemical compounds such as the SA analogue BTH and the β-amino butyric acid (BABA), but also by necrotizing pathogens and beneficial microbes among which arbuscular mycorrhizal fungi. The effect is observed in tissues directly in contact with the PAMPs or the chemical inducers as well as systemically in untreated parts of the plants (Conrath *et al.*, 2015).

First observations of priming were performed on parsley cells suspensions which showed enhanced transcription of *PAL* genes when challenged with *Phytophthora sojae* following SA pretreatment at low dose, whereas nothing happened with SA alone (Conrath *et al.*, 2006). Priming probably relies on dormant MAPKs as in *A. thaliana*, plants primed with BTH accumulate inactive MPK3 and MPK6. Following pathogen challenge, more MPK3/MPK6 proteins are activated in primed than unprimed plants (Reimer-Michalski and Conrath, 2016). Another key component of priming is the overexpression of PRR receptors at the cell surface, which probably allows faster answer to the pathogen arrival. For example, BTH increases the levels of FLS2, BAK1 and CERK1 in *Arabidopsis* (Conrath *et al.*, 2015).

But the most likely molecular explanation of priming is DNA architecture modification. DNA is packed around histone proteins into condensed chromatin. Histone modifications, especially acetylation of lysine residues loosen the histone-DNA interaction which renders DNA more accessible for transcription. Acetylation of histones H3 and H4 are thought to enhance transcriptional activity following plant defense priming. Histones can also be methylated and the trimethylation of lysine 4 in histone H3 (H3K4me3) upon BTH treatment has been observed on the *Arabidopsis WRKY29* gene as well as acetylation of certain associated histones. However, these modifications do not activate the *WRKY* genes unless the plant is challenged by a biotic stress. Similarly, local *Pseudomonas* infection in *Arabidopsis* induces increased acetylation and methylation marks in histones associated with *WRKY* promoters in distal leaves (Conrath et al., 2015).

It is currently considered in models of plant innate immunity that plants do not possess any immune memory because they lack specialized cells such as mammalian memory B cells. But the existence of priming raises questions, especially because inheritance of the primed state has been observed: acetylation marks H3K4me3 on *PR1*, *WRKY6*, and *WRKY53* promotors were found in the offspring of *Arabidopsis* challenged with *Pseudomonas*, pointing to a memory of previous infection encountered by parents (Reimer-Michalski and Conrath, 2016).

3.4.3. Hypersensitive response

HR is a form of PCD that surrounds infection sites and aims at limiting pathogen growth by severing access to nutrients. Cells in HR process undergo vacuolization, chloroplast disruption, cytoplasmic shrinkage and release compounds playing a signaling role for surrounding tissues. HR is often considered as the hallmark of an incompatible interaction leading to disease resistance. HR has first been connected with ETI activation and occurs downstream of NADPH oxidases-driven ROS production and SA accumulation. PTI can also lead to HR initiation but is often slower than ETI, although upstream events leading to HR are likely similar (Coll *et al.*, 2011). ROS and more particularly H_2O_2 play an active role in HR initiation but they seem to require the synergistic action of NO. In *Arabidopsis* and tobacco, even a massive burst of H_2O_2 triggers a weak HR in absence of NO. Similarly, NO accumulation does not lead to HR in absence of H_2O_2 accumulation. Chloroplasts produce both RNS and ROS but also take part in JA and SA synthesis involved in HR initiation (Coll et al., 2011; Frederickson Matika and Loake, 2014).

Other important components of HR that act upstream of ROS and RNS are the modifications of calcium fluxes and the MAPK cascades. Perturbation of one or several of these components can impair plant cell HR (Wang *et al.*, 2013). In mammals, the PCD that mainly relies on ROS accumulation is called necroptosis while the other form of PCD depends on specific proteases named caspases. Instead of close caspase homologs, plants possess vacuolar processing enzymes with caspase-like protease activities. Recent studies on tomato demonstrate that accumulation of hydrolases such as vacuolar processing enzymes and subtilases precedes HR initiation although their exact roles in plant HR is still poorly understood (Sueldo *et al.*, 2014). There is a general consensus on the effectiveness of HR against biotrophic pathogens while cell death is believed to enhance susceptibility towards necrotrophs. Interaction studies between *Brassica napus* and *Sclerotinia sclerotiorum* revealed that proteins involved in resistance against this stem rot necrotroph are mainly APX, CAT, SOD and peroxidases that lower ROS levels and delay HR onset. Concerning hemibiotrophs which turn necrotrophs at later stages of infection, HR is still seen as effective. Indeed, a rapid HR was associated with effective resistance against *P. infestans* in potato and strongly correlated with resistance levels (Wen, 2013). HR occurs very rapidly after inoculation of completely resistant cultivars (CVs) and takes place later in partially resistant ones, allowing certain hyphae to start biotrophic interactions (Vleeshouwers *et al.*, 2000b).

But it is not exclusively HR that prevents pathogen proliferation as for example the resistance gene Rx protects potato against late blight without apparent HR (Coll *et al.*, 2011).

3.4.4. Cell wall reinforcement

At the site of pathogen attempt of penetration, plants can form a thickened structure called papilla to block pathogen entry. The papilla is composed of callose, phenolic compounds and cell wall proteins including peroxidases and thionins and contains ROS. Callose is a β-(1,3)-glucan polymer with some β-(1,6)- branching produced between the outer plasma membrane and the plant cell wall. Under normal conditions, callose is present in the pollen as well as in sieve elements from the phloem, but it can be synthetized after mechanical damage or biotic stress. Several callose synthase genes have been characterized in *Arabidopsis*. They are encoded by *GSL (GLUCAN SYNTHASE-LIKE)* genes among which *GSL5* is specifically involved in callose deposition during papilla formation. Callose deposition and papilla formation are pre-invasive defense barriers, classified as late PTI responses because they occur hours after pathogen attack (Ellinger and Voigt, 2014). The plant defense inducer BABA protects several species against fungi and oomycetes by priming callose accumulation at papilla formation sites or by encasement of haustoria with callose. Mutant studies revealed that BABA biological activity necessitates an intact ABA pathway (Cohen *et al.*, 2016). *Arabidopsis* treatment with PAMPs also enhances callose deposition that requires H_2O_2 from RBOHD for flg22 but not for chitosan (Luna *et al.*, 2010).

However, efficacy of callose deposition is questionable since enhanced callose deposition alone is not sufficient to increase resistance to bacterial pathogens (Ellinger and Voigt, 2014). Similarly, a mutant of *A. thaliana pmr4* (*POWDERY MILDEW RESISTANT 4*) is impaired in GSL5 activity and shows enhanced resistance against several powdery mildew species. There is papilla formation at the attempted fungal penetration site but without callose production. Additional studies with other mutants revealed that *pmr4* constitutively activated the SA pathway responsible for the high resistance (Nishimura *et al.*, 2003). Surprisingly, plants overexpressing *PMR4* also showed an increased resistance against the same set of pathogens thanks to enhanced callose deposition. It is thus hypothesized that callose deposition contributes to penetration resistance and PTI-associated phenomena but *pmr4* acts also as susceptibility gene by suppressing SA signaling (Ellinger *et al.*, 2013).

Next to the barrier formed around the infection peg, the cell walls surrounding the attack site undergo molecular reinforcement to prevent penetration and isolate pathogens from the rest of the plant. Lignin is one of the most abundant organic compounds in plants and is deposited in secondary cell walls. This highly branched aromatic polymer originates from the phenylpropanoid pathway that forms monolignols polymerized in the plant cell wall by laccases and peroxidases. Lignin forms a hydrophobic barrier of great chemical resistance against pathogen cell wall-degrading enzymes (CWDEs). The polysaccharide matrix of plant cell walls can also be strengthened by oxidative cross-linking of phenolic compounds, hydroxyproline-rich glycoproteins, arabinogalactan proteins, extensins and lectins. All this contributes to the formation of a resistant barrier against the pathogen (Bellincampi *et al.*, 2014; Deepak *et al.*, 2010; Malinovsky *et al.*, 2014).

3.4.5. Antimicrobial peptides and pathogenesis-related proteins

Both JA and SA pathways can lead to the accumulation of different blends of antimicrobial peptides among which thionins, defensins, lipid transfer proteins, knottins, cyclotides, hevein-like proteins and snakins. These are usually cationic peptides ranging from 2 to 10 kDa, stabilized by disulfide bridges and toxic for fungi, oomycetes, bacteria and sometimes herbivorous insects. Produced as propeptides, they are mostly targeted to the extracellular spaces where they overwhelm invading pathogens. Both thionins and defensins are thought to destabilize membranes through amphipathic properties and phospholipid binding activities. Lipid transfer proteins are abundant in epidermal cells and participate to phospholipid transfer between membrane compartments which probably contributes to the toxicity of other antimicrobial compounds. Hevein-like peptides possess a strong affinity for chitin, are able to inhibit fungal growth and are also toxic for oomycetes. Knottins also possess a chitin-binding activity but differ from hevein-like peptides by the number of disulfide bridges. Knottins are toxic for fungal pathogens and Gram-positive bacteria. Cyclotides are found as well in monocots as in dictos and present antimicrobial as well as insecticidal properties. Finally, snakins which are more specific to *Solanaceae* are antifungal and antibacterial compounds associated to the gibberellin pathway (Stotz *et al.*, 2013).

PRs are small proteins ranging from 5 to 75 kDA secreted in the apoplastic spaces or targeted to the vacuole. The size overlap explains that some antimicrobial peptides are also classified as PR proteins (Table 1.2). They were first discovered by their overrepresentation in infected plants but contribute to disease resistance. Studies with transgenic plants showed that PR proteins are more efficient in disease control when they are collectively overexpressed rather than individually (Fu and Dong, 2013). PR proteins are considered as "the executioners of plant immunity" thanks to their hydrolase and antimicrobial activities. PR proteins are mainly induced by SAR and were classified into 17 families (Table 1.2), according to their biological activity (van Loon *et al.*, 2006b). Nowadays, it is widely accepted that PR accumulation is not only regulated by SAR and SA but also by JA, ET and ABA (Jiang *et al.*, 2015). More precisely, SA generally triggers accumulation of PR1, PR2 (β-1,3-glucanase) and PR5 (thaumatin) (van Loon *et al.*, 2006b).

Table 1.2: Pathogenesis-related (PR) protein families.
PR proteins were classified according to their serological relatedness and/or biological activity, adapted from van Loon *et al.* (2006).

Family	Type member	Properties
PR1	Tobacco PR-1a	Sterol-binding and -sequestration
PR2	Tobacco PR-2	β-1,3-glucanase
PR3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR4	Tobacco "R"	Chitinase type I, II
PR5	Tobacco S	Thaumatin-like
PR6	Tomato inhibitor I	Proteinase-inhibitor
PR7	Tomato P69	Endoproteinase
PR8	Cucumber chitinase	Chitinase type III
PR9	Tobaccoo "lignin forming peroxidase"	Peroxidase
PR10	Parsley "PR1"	Ribonuclease-like
PR11	Tobacco "class V" chitinase	Chitinase, type I
PR12	Radish Rs-AFP3	Defensin
PR13	Arabidopsis THI2.1	Thionin
PR14	Barley LTP4	Lipid-transfer protein
PR15	Barley OxOa (germin)	Oxalate oxidase
PR16	Barley Oxo	Oxalate oxidase
PR17	Tobacco PRp27	Unknown

As a consequence, *PR1* expression has been used for years as a marker of SA-related defense despite ignoring its exact biological function. However in rice which has particularly high levels of SA, PR1 is not always specifically related to SA. In that species, the constitutively high level of SA induces synergy between SA and JA pathways rather than antagonism and SA, JA and ET all synergistically contribute to the overexpression of *OsPR1* (Mitsuhara et al., 2008). Recently Gamir et al. (2017) studied the tobacco acidic PR-1a and the tomato basic PR1 named P14c and showed that both proteins have an antimicrobial activity, especially against oomycetes. It appears that both plant proteins have a strong sterol binding capacity which completely prevents pathogens from obtaining sterols. Sterols are indeed essential components of pathogen membranes and hormonal signaling. Oomycetes that belong to the Peronosporales such as *Phytophthora* sp. cannot synthesize sterols but must divert them from their plant hosts (Gaulin *et al.*, 2010).

Other PRs such as PR2, PR3 and PR8 showing glucanase and chitinase activities are likely toxic to fungal and oomycete pathogens as they are able to hydrolyze cell walls and induce osmotic burst. PR6 and chitinases are expected to be effective against nematodes and PR8 is effective against bacteria because it has a lysozyme activity. PR7 are more specific to *Solanaceae* and are proteinases suspected of disrupting microbial cell walls and recently shown to accumulate just before HR. PR9 participate in plant cell wall reinforcement with lignin while PR10 have a ribonuclease activity that likely protects against viruses. PR14 and PR15 are specific to monocots and are able to mediate ROS production in plant cell walls (Sueldo *et al.*, 2014; van Loon *et al.*, 2006b). Even if enzymes such as phenylalanine ammonia-lyases (PALs), peroxidases, and polyphenoloxidases contribute to the production of antimicrobial secondary metabolites, they are not considered as PR proteins because they are constitutively expressed in healthy plant tissues.

3.4.6. Phytoalexins

Phytoalexins are plant secondary metabolites of low molecular weight that can be either constitutively present or induced by pathogens or environmental stresses. The type of phytoalexins produced essentially depends on the plant family. Phytoalexins possess antimicrobial properties and their toxicity depends on the pathogen ability to efficiently metabolize them. Phytoalexin accumulation is regulated by JA, SA, ET, ABA, auxins, cytokinins and to a lesser extent by GA. MAPK cascades, especially MAPK3/6 play an important role in regulation of their synthesis (Jeandet *et al.*, 2014; Pusztahelyi *et al.*, 2015).

Phytoalexins are mainly produced through three main metabolic routes. Phytoalexins that derive from phenylpropanoids originate from PAL activity and lead to flavonoid molecules. The most prominent is resveratrol, a flavonoid compound essentially found in grapevine and precursor of stilbene derivatives. These compounds were shown to be toxic to *B. cinerea* conidia, leading to disorganization of its plasma membrane. Terpene phytoalexins are obtained by the non-mevalonate pathway based on the methylerythritol phosphate or by the mevalonate pathway originating from geranyl-geranyl diphosphate (Jeandet *et al.*, 2014). Jasmonates treatments enhance the production of this class of phytoalexins as they lead to terpene synthase overexpression. Terpene phytoalexins participate in plant defense by attracting insect predators but they also have adverse effects against fungal pathogens. For example, rice produces at least 14 diterpene phytoalexins directly toxic to *M. grisea* (Singh and Sharma, 2015). The last important pathway is the indole phytoalexin pathway that notably produces camalexin, the main phytoalexin of *A. thaliana*. Indole phytoalexins require several cytochrome P450 enzymes such as CYP71B15 which corresponds to the knock-out gene *PHYTOALEXIN DEFICIENT 3* in *pad3* mutant. Camalexin production enhances *Arabidopsis* resistance to a broad spectrum of pathogens including *B. cinerea*, *A. brassicola*, and *Phytophthora brassicae* (Liu *et al.*, 2016b).

3.5. Defense budget: Tradeoffs associated with plant defense

As previously detailed, the interaction between growth and defense is mainly regulated by hormonal crosstalk. SA and BTH suppress auxin signaling by downregulating auxin transporters. JA inhibits growth by disrupting normal auxin distribution while auxin induces the expression of the JAZ repressor. There is also an antagonism between JA and GA regulated by the interaction among MYC2 and DELLA. That is why in some situations, PTI and ETI have been shown to reduce plant fitness, even in absence of pathogen. Plants genetically modified to express high SA level have a dwarf phenotype and repeated BTH applications reduce plant biomass. Similarly most R-genes are expressed constitutively, even in absence of pathogen which may represent a metabolic cost or be toxic for the plant.

But the tight control of the timing and the duration of plant defense induction may reduce its metabolic load and priming which involves a boosted plant answer after pathogen arrival is often seen as less expensive. There are thus possible tradeoffs between plant development and immunity because the energy diverted for defense is not available for growth especially in cases of limited nutrients availability where the metabolic prioritization is a survival issue (Brown and Rant, 2013; Huot *et al.*, 2014; Karasov *et al.*, 2017). But the systematic yield penalty for plant defence induction must not be generalized because it is carbon reduction that matters which is often limited by sink capacity for photosynthates and not by photosynthesis. The excess energy produced by photosynthesis must often be dissipated by photoprotection to limit ROS production, in which case energy expenditure for priming would come at no cost to the plant. In some instances, plant defense inducers could also be beneficial as for example hexanoic acid primes redox-related genes to increase antioxidants which limit ROS such as H_2O_2, a potent inhibitor of photosynthesis (Aranega-Bou *et al.*, 2014; Foyer and Shigeoka, 2011; Zhu *et al.*, 2010).

Another component of tradeoffs associated with plant defense is linked to the resistance towards pathogens with different lifestyles. Consistent with the antagonism between JA and GA pathways, semi-dwarf wheat genotypes encoding GA-insensitive DELLA proteins are more sensitive to the biotroph *Blumeria graminis* but show higher resistance against the necrotroph *Oculimacula spp* provoking the eyespot disease. PTI and ETI transfer in the field should thus be accompanied by a study of their fitness costs to evaluate their real benefits (Brown and Rant, 2013).

4. Plant cell wall: More than a passive frontier

Cell walls are the main elements beside chloroplasts that distinguish plant cells from animal cells. The cell wall plays essential roles for plant growth and development. First, it is a physical barrier that acts as an exoskeleton providing shape and rigidity to withstand mechanical stresses such as variations of cell water content. Plant growth and architecture are dependent on the regulation of cell wall properties. Cell walls are an essential component of water flow in the xylem that also act as a diffusion barrier for nutrients, ions and other charged macromolecules. But the most important wall property that will be discussed in this work, is its role of active barrier against pathogen invasion (Taiz and Zeiger, 2010).

4.1 The Great Wall architecture: Composition of plant cell walls

The plant cell wall is comprised of heteropolysaccharides intermeshed in a dense molecular network. The primary cell wall is synthetized during cell growth while the secondary cell wall is formed later between the membrane and the primary wall once cell enlargement has stopped and is possibly strengthened and waterproofed by lignin. The middle lamella deriving from the phragmoplast is found between walls of neighboring cells (Fig. 1.25) (Pogorelko *et al.*, 2013b; Taiz and Zeiger, 2010).

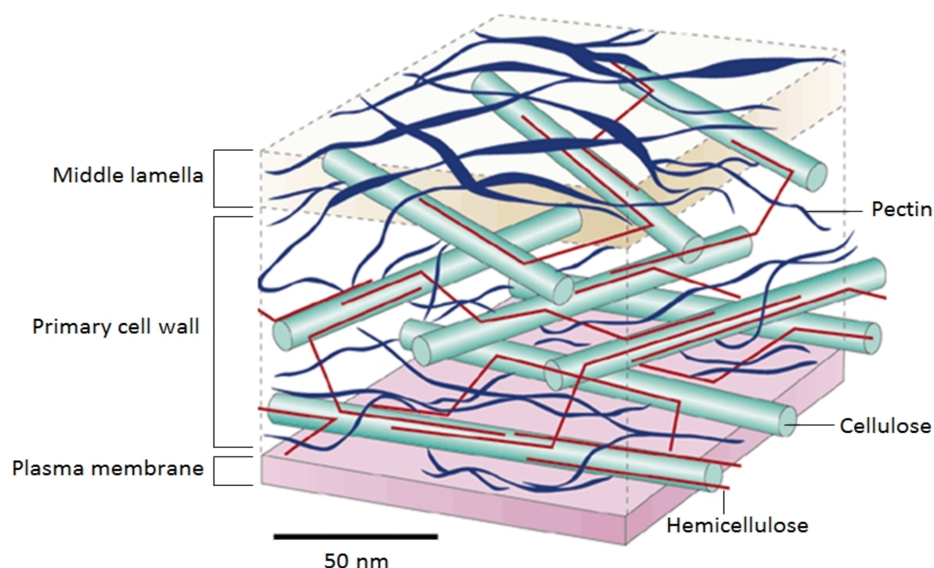

Fig. 1.25: The architecture of the primary plant cell wall.
Primary wall is localized outside the plasma membrane and is mainly made of cellulose arranged in microfibrils or branched with various glucans giving hemicellulose. The second most abundant component is pectin (Smith, 2001).

The main constituent of primary cell walls is cellulose, a polymer of β-(1,4)-glucose units hydrogen-bound into insoluble microfibrils. The second component are matrix polysaccharides comprising hemicelluloses and pectin. Hemicellulose is also a polymer of β-(1,4)-glucose with branchings that include xylan, arabinoxylan, glucuronoxylan, glucomannan and xyloglucan, also insoluble in water (Fig. 1.25). The matrix also contains 2 to 10% soluble proteins such as hydroxyproline-rich glycoprotein, proline-rich proteins, glycine rich-proteins and arabinogalactan proteins. They participate in the mechanical strength of the cell wall and are completed by other proteins with enzymatic activities mediating polysaccharide turnover within the cell wall. The other matrix polymer is pectin, a gel-forming polysaccharide in which cellulose and hemicelluloses are embedded (Fig. 1.25).

Pectin (Fig. 1.26) represents around 35% of the dry mass of the dicot cell wall and is mainly formed of α-(1,4)-galacturonic acid units composing a uniform polymer of homogalacturonan (HG) or mixed with other sugar units giving xylogalacturonan, apiogalacturonan, rhamnogalacturonan II, and rhamnogalacturonan I, depending on their main sugar residues (Albuquerque *et al.*, 2016; Cao, 2012; de Souza *et al.*, 2014; Taiz and Zeiger, 2010).

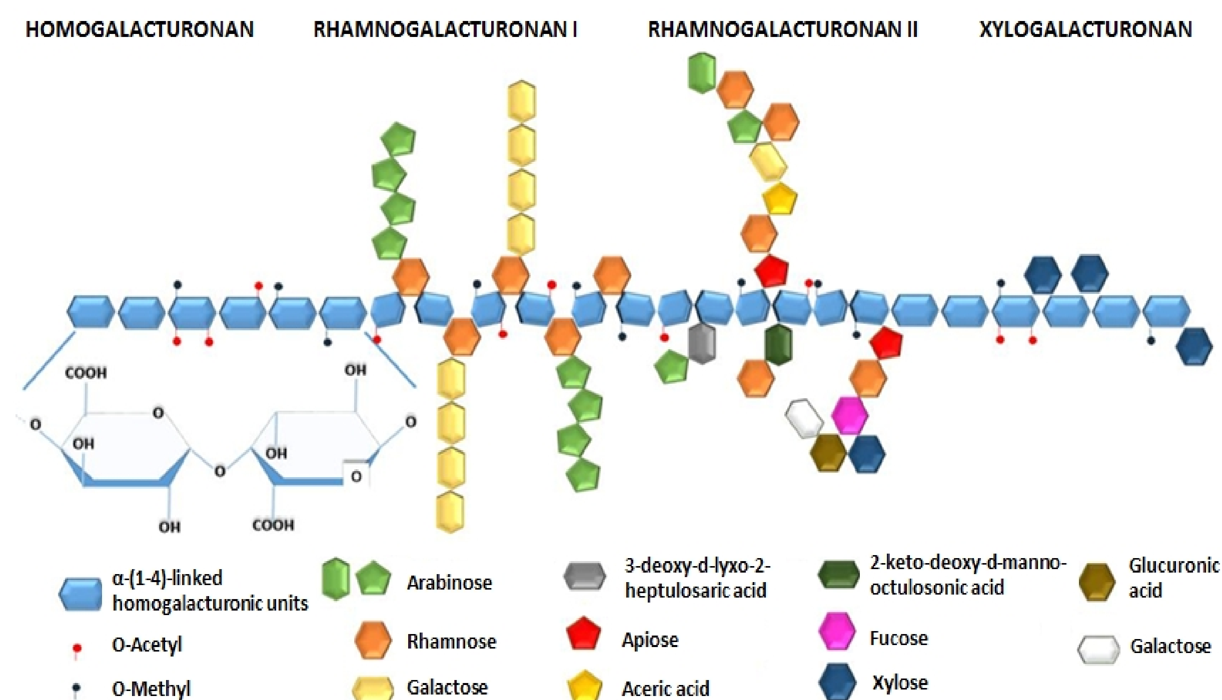

Fig. 1.26: Structure of pectin.
The most abundant polymer is homogalacturonan followed by rhamnogalacturonan I while xylogalacturonan and rhamnogalacturonan II are less abundant (Albuquerque *et al.*, 2016).

Galacturonic acid residues inside the HG chains can be methylesterified on carbon six and/or acetylesterified on carbon atoms two and three (Thonar *et al.*, 2006). It is thus possible to distinguish three types of HG modification in plant cell walls: acidic, methylesterified and sometimes acetylesterified galacturonic acid residues (Van Cutsem and Messiaen, 1996). Pectin is synthesized under a methylesterified form essentially found in primary cell walls, middle lamellae and more particularly in meristematic areas. Methylesterified HG shows gelation properties under acidic conditions in aqueous media of high sugar content (Dickinson, 2003; Van Cutsem and Messiaen, 1993, 1996).

Unsubstituted HG or acidic pectin is negatively charged by deprotonation of its carboxylic acid function which allows pectin cross-linking by calcium bridges and gel formation. Antiparallel chains of HG stack together thanks to Ca^{2+} that form a particular network called egg boxes (Fig. 1.27) (Kirtil *et al.*, 2014; Van Cutsem and Messiaen, 1993). These structures were first thought to give rigidity and cohesiveness to cell walls but *in situ* studies suggest that egg boxes are quickly subject to enzymatic degradation and are associated with a decrease in cell wall stiffness. Material expanded at cell junctions and extracellular boundaries are enriched in this form that is almost absent from the rest of primary cell walls (Hocq et al., 2017; Kirtil et al., 2014; Van Cutsem and Messiaen, 1993, 1996). On the contrary, acetylesterification of acidic pectin strongly lowers its affinity for bivalent cations and prevents the formation of gel structures (Draye and Van Cutsem, 2008).

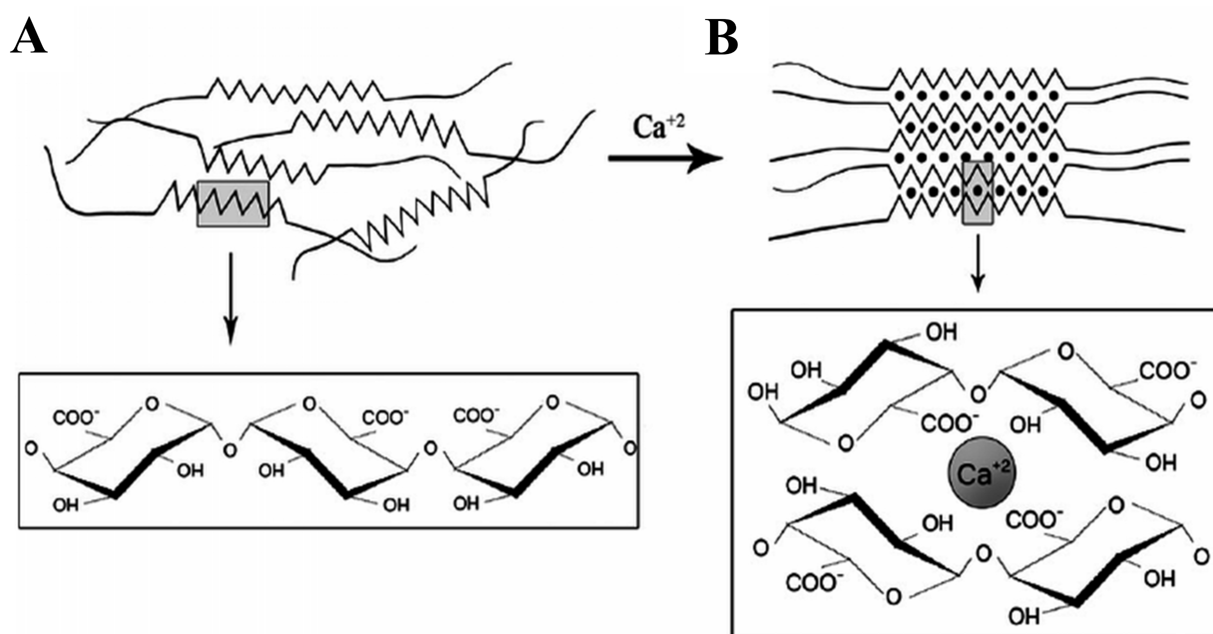

Fig. 1.27: Gelation process of demethylesterified homogalacturonan (HG) in presence of calcium.
A. Demethylesterified HG chains bear negative charges.
B. Two antiparallel HG chains bearing negative charges on both sides associate thanks to Ca^{2+} to form a so-called egg box structure. The stacking of multiple egg box structures forms a dense gel network (Kirtil *et al.*, 2014).

4.2 Insights from the front line: Cell wall turnover and disease susceptibility

The cell wall is a dynamic assembly under constant modification to allow cell expansion and differentiation. Under normal conditions, many enzymes mediate plant cell wall remodeling. Cellulose is synthesized by membrane-bound enzymatic complexes encoded by cellulose synthases (CESAs) while matrix polymers are produced in the Golgi complex and secreted via vesicles (Taiz and Zeiger, 2010; Van Cutsem and Messiaen, 1993). Pectin is synthesized under a methylesterified form but cell growth and expansion require modulation of its mechanical properties by cell wall degrading enzymes (CWDE) (Hocq *et al.*, 2017; Van Cutsem and Messiaen, 1994). Pectin acetylesterase (PAE) and methylesterase (PME) are responsible for the removal of acetyl and methyl residues respectively. PMEs impact drastically HG properties by methylester removal, allowing egg box formation and modifying the cell wall rheology (Liners *et al.*, 1994).

The spatiotemporal regulation of PME activities is an important component of plant growth and development but it also intervenes in response to pathogen (Mravec *et al.*, 2014). As a consequence, plants tightly control their PME activities by a vast array of PME inhibitors (PMEIs) (Pogorelko *et al.*, 2013a). Acidic pectin is indeed more susceptible to degradation as it becomes accessible for CWDEs which cleave unmethylesterified HGs into oligogalacturonides or OGAs. The loosening of the pectin network is necessary for cell expansion but pathogens also rely on a similar set of CWDEs to breach into their hosts (Hocq *et al.*, 2017; Pogorelko *et al.*, 2013a). CWDEs are assigned to several classes according to their main enzymatic function. Pectate lyases and pectin lyases all perform β-elimination to fragment pectate and pectin chains respectively while polygalacturonase (PG) degrades HG by hydrolyzing the glycosidic bonds between galacturonic acid residues. Together with expansins, PGs participate in the natural processes of cell elongation and fruit ripening by mediating cell wall loosening (Cantu *et al.*, 2008; Draye and Van Cutsem, 2008). Similarly to PMEs, PGs are modulated by PG-inhibiting proteins (PGIPs) which also take part in the control of CWDEs from pathogen origin. PGIPs possess a LRR structure largely conserved between monocot and dicot plants. Moreover the interaction between PGs and PGIPs promotes the releases of long chain OGAs which are DAMPs that elicit plant defense responses (Kalunke *et al.*, 2015).

The regulation of this arsenal of CWDEs and their respective inhibitors relies on cell wall integrity (CWI) sensing which allows tight regulation of growth and development but that also respond to cell wall modifications in the context of (a)biotic stresses. CWI sensing relies on sensors that are mostly plasma membrane RLKs and some of them have been identified, mostly in *Arabidopsis*. HERCULES1 is involved in control of shoot growth and FERONIA participates in brassinosteroid-induced cell elongation while *Catharanthus roseus* RLK-like 1 (CrRLKL1) acts negatively on cell extensibility. THESEUS1 has been identified as a putative sensor for cellulose damage while wall-associated kinases (WAKs) sense pectin integrity. WAK1 has long been known as the receptor of OGAs in egg box conformation, well-known pectin-derived DAMPs (Brutus *et al.*, 2010; Cantu *et al.*, 2008; Decreux and Messiaen, 2005; Hématy *et al.*, 2007; Höfte, 2015; Pogorelko *et al.*, 2013a).

The connection between growth and defense has never been more evident than with plant cell walls because wall modifications directly impact disease resistance and susceptibility. Cell walls are a nutrient source but also a barrier that limits pathogen progression inside its host. One of the main targets of pathogens in the cell walls is pectin which is more susceptible to enzymatic degradation than cellulose and hemicellulose. Necrotrophs degrade cell walls more aggressively than biotrophs. For example, *B. cinerea* secrete a large array of CWDEs including cellulases, hemicellulases, PGs and PMEs (Fig. 1.28).

Acidic pectin is more susceptible to enzymatic degradation than methylesterified pectin, as discussed above. It is therefore not surprising that a first line of plant defense consists in secretion of PMEIs (Fig. 1.28) that prevent acidic HG formation and further degradation by PGs, and of PG inhibitors (PGIs) whose action restricts pectin depolymerization and results in the accumulation of OGAs long enough to be elicitor-active. WAK1 binding of OGAs triggers a whole array of plant defense responses including the secretion of chitinases and glucanases that target fungal cell walls for degradation (Fig. 1.28). Necrotrophs not only secrete CWDEs, they are also able to divert plant CWDEs at their own benefit to foster host cell wall degradation. In tomato, a PG (*LePG*) and an expansin (*LeExp1*) are overexpressed following *B. cinerea* infection. Similarly in *Arabidopsis*, Botrytis also induces the overexpression of *AtPME3* (Bellincampi *et al.*, 2014; Buchanan *et al.*, 2015; Cantu *et al.*, 2008).

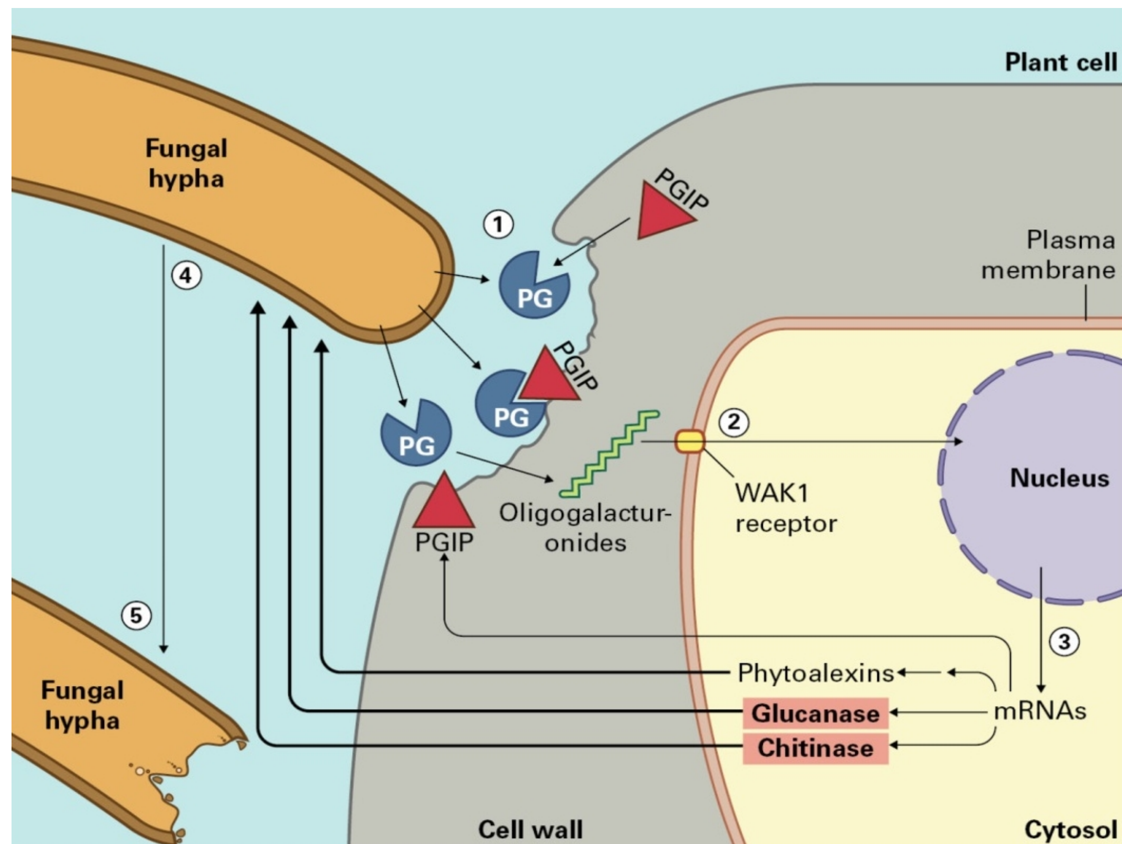

Fig. 1.28: Model of plant-pathogen interaction at the cell wall.
Fungal polygalacturonases (PGs) degrade plant cell walls. The plant first line of defense includes polygalacturonase inhibiting proteins (PGIPs). The interaction between PGs and PGIPs slows down the formation of oligogalacturonides (OGAs) which are then long enough to adopt the egg box conformation detected by plasmalemma receptors. OGAs are sensed by the cell wall receptor WAK1 (2). Signal transduction from OGA perception leads to the secretion of defense compounds including novel PGIPs, chitinases, glucanases and phytoalexins (3). Plant enzymes attack fungal hyphae to restrict pathogen growth and release conserved fungal motifs (5) (Buchanan *et al.*, 2015).

The action of biotrophic pathogens on plant cell walls does not aim at completely destroying the walls to avoid betraying their presence. They use mechanical pressure combined to the secretion of a limited amount of CWDEs to create a local loosening of the wall allowing their haustoria to reach host plasma membranes. The plant reacts by formation of papillae and ROS production. But the biotrophic pathogen can escape this first line of defense by inducing the expression of susceptibility factors such as *PMR4* encoding a callose synthase, *PM5* encoding a polysaccharide O-acetyltransferase and *PMR6* encoding a putative pectate lyase (Bellincampi *et al.*, 2014).

Those differences in pathogen-triggered cell wall modifications probably explain why plant cell wall turnover can influence disease resistance or susceptibility, depending on the pathogen's trophic strategy. The overexpression of PMEI in *Arabidopsis* enhances resistance towards the necrotrophs *B. cinerea* and *Pectobacterium carotovorum* but increases susceptibility to the biotroph *Hyaloperonospora parasitica*. Decreasing the degree of acetylation (DA) of pectin seems to enhance resistance against both types of pathogens. Indeed, *A. thaliana* and *Brachypodium dystachion* which overexpress *PAE* are more resistant to *B. cinerea* while *PMR5* expression which increases the DA is an enhancer of disease susceptibility towards powdery mildews. Mutation on cellulose synthase CESA3 involved in primary cell wall synthesis leads to constitutive expression of JA and ET-related defense and enhanced resistance to powdery mildews in *A. thaliana*. Similarly, an *Arabidopsis* mutant impaired in the expression of CESA4 involved in secondary cell wall assembly is more resistant to the hemibiotrophic bacteria *Ralstonia solanacearum* and the necrotrophic fungus *Plectosphaerella cucumerina*. Cell wall integrity plays an important role in plant defense: CWI sensors trigger signaling cascades involving phytohormones that finally activate defense genes expression (Bellincampi *et al.*, 2014; Cantu *et al.*, 2008; de Souza *et al.*, 2014; Nafisi *et al.*, 2015).

4.3 COS-OGA, a new weapon inspired from the cell wall

The Research Unit in Plant Cellular and Molecular Biology from the University of Namur has a long research history concerning plant cell walls and more specifically pectin. Several articles from the lab deal with pectin structure, conformation degradation and especially perception of its specific DAMPs: OGAs (Cabrera *et al.*, 2008; Decreux and Messiaen, 2005; Decreux *et al.*, 2006; Liners *et al.*, 1989; Liners *et al.*, 1992; Liners and Van Cutsem, 1992; Messiaen *et al.*, 1993).

Pectinases secreted by bacteria and fungi degrade pectin into OGAs recognizable by plants and able to trigger signal transduction pathways (Messiaen et al., 1993; Messiaen and Van Cutsem, 1994). OGAs trigger responses very rapidly, including protein phosphorylation, membrane depolarization and H_2O_2 accumulation. Late responses induced by OGAs include phytoalexin synthesis, production of proteinase inhibitors and cell wall strengthening. OGAs also induce H_2O_2-mediated stomatal closure in tomato. OGA treatment increases resistance of *A. thaliana* to *B. cinerea* independently of JA, ET and SA.

Simultaneous overexpression of PG and PG inhibiting protein in transgenic *Arabidopsis* leads to OGA accumulation and subsequent enhanced resistance against the necrotrophic pathogen *B. cinerea* and the hemibiotrophic *P. syringae* (Benedetti *et al.*, 2015; Galletti *et al.*, 2009; Trouvelot *et al.*, 2014). The major contribution of Namur University is related to the calcium-induced conformation of OGAs and its involvement in biological activity. It was shown that OGAs adopt the calcium-induced conformation under a specific Ca^{2+}/Na^{+} ratio (Fig. 1.29) and the ideal size of OGAs to obtain egg boxes consists in a degree of polymerization (DP) between 9 to 20 monomers of galacturonic acid residues and a total degree of methylation of the individual molecules lower than 30%. These data have been obtained thanks to the 2F4 monoclonal antibody, specific for OGAs in egg box conformation (Fig. 1.29, Liners et al. 1989, 1992) and later confirmed thanks to circular dichroism (Cabrera *et al.*, 2008; Van Cutsem and Messiaen, 1996). The most potent OGAs for inducing defence responses have a size comprised between DP 9 and 15 (Van Cutsem and Messiaen, 1996). The CWI sensor WAK1 has been shown to be the receptor of OGAs (Brutus *et al.*, 2010) and the team from the University of Namur showed that egg box conformation of OGAs was of paramount importance for the perception and the signal transduction by WAK1 receptor (Decreux and Messiaen, 2005).

In 2002, funding for a research project was granted by the Walloon Region to our laboratory to study a possible synergy between chitosan oligomers and pectin oligomers and to evaluate their biological effect on *A. thaliana* cell suspensions and on *Nicotiana tabacum* (Cabrera *et al.*, 2010). Indeed chitosan has a polycationic structure similar to the polyanionic structure of OGAs. Chitosan is a polymer of β-1,4-D-glucosamine containing β-1,4-N-acetyl-D-glucosamine that derives from chitin deacetylation (Fig. 1.30). Chitosan differs from the hydrophobic chitin by a degree of acetylation (DA) lower than 50% thereby making chitosan soluble in water. Chitin is the most abundant polymer in nature after cellulose and is found in fungal and certain oomycetes cell walls as well as in insect and crustacean exoskeleton (Beaulieu, 2007). Chitin is a well-known MAMP and its PRR receptor has been extensively described in rice and in *Arabidopsis* (Couto and Zipfel, 2016). During plant infection, several fungal pathogens deacetylate their cell wall chitin into chitosan to escape plant chitin receptors. This strategy has already been demonstrated for some fungi including *Colletotrichum graminicola*, *Puccinia graminis* and *M. oryzae* (Geoghegan and Gurr, 2016).

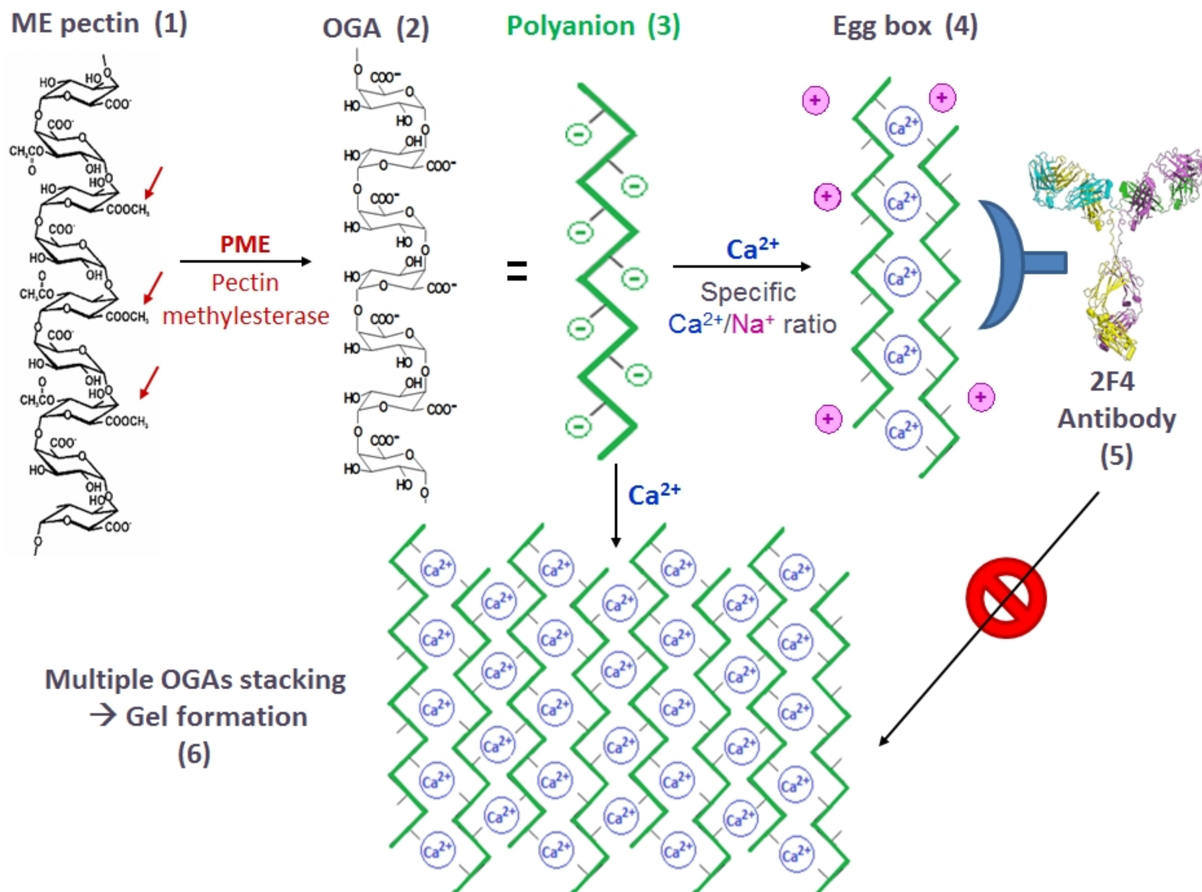

Fig. 1.29: The monoclonal antibody 2F4 specifically recognizes OGAs in egg box dimers. Pectin is synthetized under a methylesterified form (ME pectin, 1). Plant- or pathogen-derived PMEs demethylesterify pectin giving oligogalacturonides (OGAs, 2) which are polyanions (3) able to adopt a so-called egg box conformation under a specific Ca^{2+}/Na^{+} ratio (4). The dimeric egg box conformation of pectin is detected by the 2F4 antibody (5). In presence of excess Ca^{2+} in the medium, OGAs multimerize and form a gel not anymore recognized by 2F4 antibodies (6).

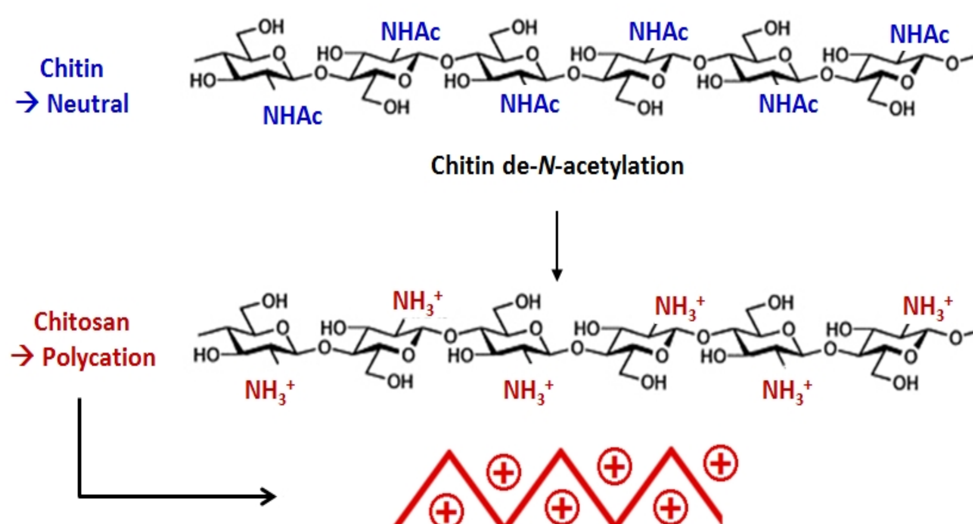

Fig. 1.30: Molecular structure of chitin and chitosan.
Chitin is a polymer consisting of β-1,4-N-acetylglucosamine residues. Chitin deacetylation leads to chitosan with DA<50%, a polycation soluble in water.

Chitosan has antimicrobial properties due to its polycationic structure that allows direct interaction with bacterial cell wall components and plasma membrane phospholipids, as well as chelation of metal elements and even binding with histone proteins (Malerba and Cerana, 2016). Chitosan is also able to trigger plant defense responses but its formal PRR has not yet been discovered (Iriti and Faoro, 2009). Chitosan molecules induce ROS accumulation such as NO but especially H_2O_2. Similarly to OGAs, chitosan also induces H_2O_2-mediated stomatal closure. Other molecular changes observed as well on plant cell culture than on whole plants include membrane depolarization, cytosolic Ca^{2+} increase, activation of MAPKs, callose deposition, synthesis of alkaloids and of the phytohormones JA and ABA (Malerba and Cerana, 2016; Trouvelot *et al.*, 2014). As for OGAs, DP of chitosan oligomers (chitooligosaccharides, COS), degree of acetylation (DA) and pattern of acetylation are of great importance for their biological activity. Cabrera *et al.* (2006) studied the effect of COS of low DPs (3-6) and high DPs (5-9) on *Arabidopsis* cell suspensions. Higher DPs increased more efficiently PAL activity and H_2O_2 production than the low DPs. The decrease in DA of COS induced higher H_2O_2 production but decreased the concentration at which COS became toxic for cells (Cabrera et al., 2006). Similarly, size effect of chitosan on resistance of potato tubers against *P. infestans* was tested and the lower molecular weight (5 kDa) conferred a better resistance in comparison with higher molecular weight polymers (24 kDa and 500 kDa) (Vasyukova *et al.*, 2001).

Cabrera *et al.* (2010) studied the interaction between polyanionic OGAs (DP 9 to 20) and polycationic COS with various DPs and DAs. With the help of the 2F4 antibody, they showed that the partially deacetylated COS (DA~25%) with high DPs (5-9) under a specific COS/OGA ratio strongly stabilized the biologically critical egg box structure of OGAs. The result is a trimeric complex which will be referred as COS-OGA in the rest of the manuscript (Fig. 1.31). COS with too high DA (50%) do not possess enough charges to interact with OGAs while COS with too low DA (0%) completely displace Ca^{2+} and thereby destroy the egg box conformation (Fig. 1.31).

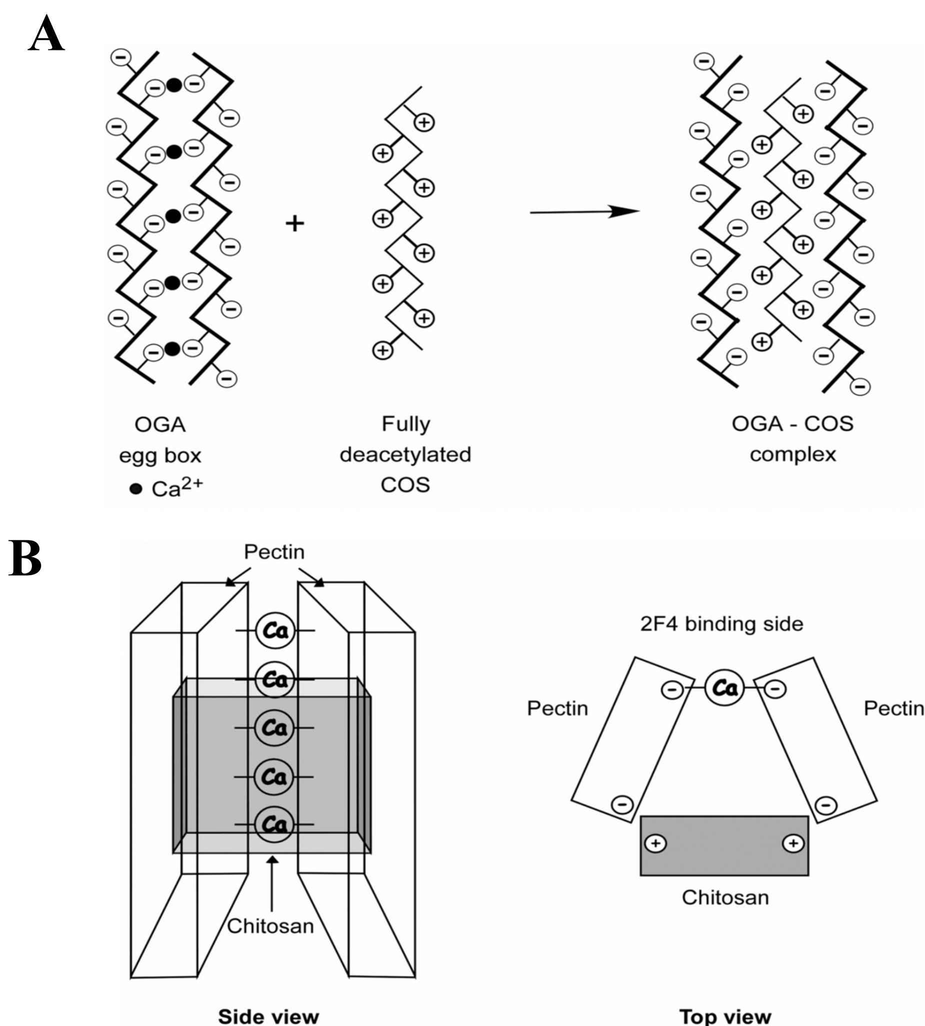

Fig. 1.31: Model of interaction between COS and OGAs.
A. Completely deacetylated COS destroy the egg box structure because COS displace all calcium ions.

B. Partially deacetylated COS associated with OGAs strongly stabilize the dimeric egg box conformation of OGAs. The result is a trimeric complex in which positively charged, partly acetylated COS bind external negative charges of calcium-bound OGAs. The putative model is a trimeric complex presented from side and top view (Cabrera *et al.*, 2010).

The COS-OGA complex conformation has probably a biological significance because a synergistic effect is observed between COS and OGA, for example on extracellular medium alkalinisation and K^+ efflux in *A. thaliana* cell suspension, two early markers of elicitation (Cabrera *et al.*, 2010). A synergy between COS and OGA was also observed at transcriptomic and proteomic levels on cell suspensions. The COS-OGA mix significantly regulated an important number of genes and proteins linked to plant defense responses that were not regulated by COS or OGAs applied individually (Cabrera Pino, 2008). The observation of these synergistic effects led to the filing of a patent in 2006 (WO/2008/065151) to protect COS-OGA compositions for use in crop protection (Van Cutsem and Cabrera Pino, 2006).

Indeed, next to an elegant electrostatic interaction, the associaton of COS, a MAMP and plant non-self molecule with OGA, a DAMP and a plant self molecule into the PAMP complex COS-OGA perfectly mimicks plant interaction with fungi (Fig. 1.32). COS-OGA could constitute a combined signal that informs plant cells on both cell wall degradation and pathogen presence. FytoFend was created in 2009 to produce and commercialize the elicitor after preliminary trials on whole plants had demonstrated significant disease reduction of apple scab (*Venturia inaequalis*) on apple trees and downy mildew (*Plasmopara viticola*) on grapevines.

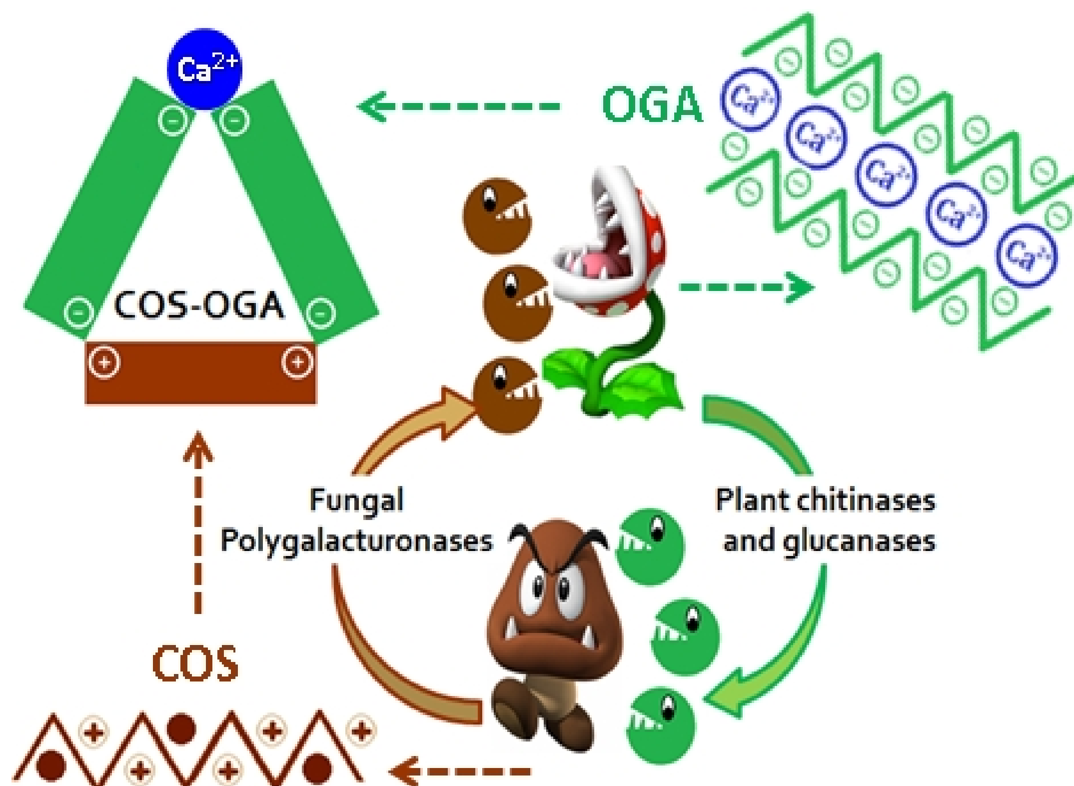

Fig. 1.32: COS-OGA mimics a classical plant-fungus interaction.
Fungi deacetylate their cell wall chitin into chitosan to escape plant chitin receptors. The fungus secretes fungal polygalacturonases to degrade plant cell walls and releases OGAs that adopt the egg box conformation in presence of calcium. OGA perception by plant receptors stimulates plant defenses and activates secretion of plant chitinases and glucanases. The action of plant enzymes on chitosan yields polycationic COS. Both compounds self-associate into a complex called COS-OGA (top view).