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Role of phytohormones in the interaction of plant pathogens *Pseudomonas syringae* and *Leptosphaeria maculans* with their hosts

Role fytohomonů v interakci patogenů *Pseudomonas syringae* a *Leptosphaeria maculans* s hostitelskými rostlinami

DISSERTATION THESIS

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Abstract

Phytohormones are small molecules that regulate almost all aspects of plant life including defence reactions. Plant defence and immunity are mainly regulated by two hormones – salicylic acid (SA) and jasmonic acid (JA). Other hormones such as auxins, cytokinins brassinosteroids or gibberellins modulate plant immunity to lesser extent. It has been described that plant pathogens are able to interfere with plant hormone signalling to overcome plant defence. Some pathogens are able to produce plant hormones themselves. This thesis is focused on plant hormone signalling involved in plant immunity both from the plant side and pathogen side and possible hormonal crosstalk in this interaction.

The first part is focused on salicylic acid signalling connected with plant actin cytoskeleton roles in plant immunity. It has been described that desintegration of actin cytoskeleton leads to increased plant susceptibility to bacteria. However, it has been also shown that pharmacological desintegration of actin filaments induces transcription of salicylic acid responsive genes *PR1* (*Pathogenesis related 1*) and *ICS1* (*Isochorismate synthase 1*). In this thesis we have investigated this inconsistency using actin depolymerizing drugs latrunculin B, cytochalasin E and jasplakinolide and two different pathosystems: *Arabidopsis thaliana* x *Pseudomonas syringae* pv. *tomato DC 3000* and *Brassica napus* x *Leptosphaeria maculans*. We treated the *A. thaliana* plants with the cytoskeletal drugs and first analyzed phytohormone profile and defence gene transcription. Specific induction of salicylic acid production and salicylic acid marker genes (*ICS1*, *ICS2* (*Isochorismate synthase 2*), *PR1*) was observed. Subsequently we infected the drug-pretreated *A. thaliana* or *B. napus* plants with corresponding pathogens which eventually resulted in increased resistance in both pathosystems. This phenomenon is salicylic acid dependent. It also depends on treatment timing, infection duration and specific pathosystem. Since actin dynamics is vital for correct cellular trafficking and membrane formation, we investigated deeper into this mechanism and focused on the role of phospholipids. We used *A. thaliana* mutant in phosphatidylinositol-4-kinase β1 and β2 (PI4Kβ1β2), which is known to be an SA overaccumulator, and a set of mutants affected in salicylic acid signalling. First, we tested callose deposition which is a defence mechanism requiring functional trafficking machinery. We observed that treatment with cytoskeletal drugs triggers callose deposition via the activity of callose synthase 12 and is SA independent since it was observed even in mutants with blocked SA accumulation. Defence gene transcription and SA accumulation were blocked in the SA-signalling impaired mutants and reverted or partly reverted in triple mutants impaired in SA-signalling and

pi4kβ1/β2. Altogether the results show that relationship between the actin cytoskeleton and plant immunity is more complex than generally assumed. Salicylic acid seems to be a major regulator of the onset of actin-depolymerization- triggered defence. Correct phospholipid signalling also seems to be important in this process.

Since we have focused on the role of salicylic acid we have established a collection of *A. thaliana* mutants that are affected in SA production, accumulation or signalling. Several of these mutants show affected resistance to pathogens. We have extensively chracaterized this mutant collection in terms of growth, cultivation condition dependancy and SA production to create a tool for future studies dealing with plant immunity. Our characterization clearly shows correlation between SA overaccumulation and rosette growth retardation.

Second part of the thesis is focused on plant pathogens infection strategies affecting hormone signalling in plants. Pathogens secrete a variety of molecules that manipulate host hormone signalling. *Leptosphaeria maculans* is an important fungal pathogen of the brassica crops. We investigated the impact *of L. maculans* effector AvrLm4-7 on virulence and host defence. We performed inoculation assay with *L. maculans* isolates possesing functional and non-functional alelle of *AvrLm4-7* that revealed that effector AvrLm4-7 contributes significantly to *L. maculans* virulence. Further we analyzed host defence reactions – defence gene transcription, phytohormone profile and ROS burst. Infection with *AvrLm4-7* containing isolate reduced SA-dependent defence response in *B. napus* plants. ROS burst was also supressed. The results show that effector AvrLm4-7 increases virulence of *L. maculans* by suppressing SA related defence mechanisms.

Since there is increasing evidence that pathogens are able to produce phytohormones to manipulate host plant defence, we tested whether *L. maculans* posseses such activity. We tested phytohormone production in *L. maculans* and identified a variety of auxins, particularly the bioactive form indole-3-acetic acid (IAA). The IAA production can be stimulated by supplementing *L. maculans* culture with biosynthetic precursors tryptophan and tryptamine. There are orthologues of several known biosynthetic genes in *L. maculans* genome. The precursors induce transcription of several of those genes; mainly *LmTAM1*, *LmIPDC2* and *LmNIT1*. Transcription of *LmIPDC1*, *LmIaaM3* and *LmIaaM5* was only slightly induced. Exogenous addition of highly concentrated auxin inhibited growth of *L. maculans* while no stimulatory effect was observed even upon low concentration of IAA. Auxin profile of infected plant showed only minor changes; ednogenous concentration of indole-3-acetonitrile increased upon infection with *L. maculans*. The results show that *L. maculans* is able to produce high

concentration of bioactive auxin but with no significant role in virulence. Auxin might function as a regulator in *L. maculans* itself.

This thesis focuses on particular aspects of plant signalling mainly connected with salicylic acid and other hormones to lesser extent and provides new insight into phytohormone signalling during infection process.

Abstrakt

Fytohormony jsou malé molekuly podílející se na řízení téměř všech životních procesů v rostlinném organismu včetně obranných reakcí. Hlavními fytohormony, které regulují rostlinné obranné reakce, jsou kyselina salicylová (SA) a kyselina jasmonová (JA). Další hormony jako auxiny, cytokininy, brassinosteroidy nebo gibberelliny ovlivňují rostlinnou imunitu zpravidla nepřímo. Rostlinné patogeny jsou schopny narušovat hormonální signalizaci hostitele, díky čemuž úspěšně překonávají rostlinné obranné mechanismy a způsobují infekci. Některé patogeny samy produkují fytohormony. Tato práce se soustředí na rostlinnou hormonální signalizaci hrající roli v imunitní odpovědi z pohledu hostitelské rostliny i z pohledu rostlinného patogenu.

První část se zabývá rolí signální dráhy keseliny salicylové a aktinového cytoskeletu v obranné signalizaci. Bylo popsáno, že porušení integrity aktinového cytoskeletu vede ke snížení odolnosti rostlin k bakteriální infekci. Dále je také známo, že farmakologické porušení aktinového cytoskeletu indukuje transkripci markerových genů dráhy kyseliny salicylové (*ICS1*, *PR1*). Tato práce se zabývá tímto rozporuplným fenoménem. K experimentům byly použity cytoskeletární drogy cytochalasin E, latrunculin B a jasplakinolid a dva patosystémy: *Arabidopsis thaliana* x *Pseudomonas syringae* pv. *tomato DC3000* a *Brassica napus* x *Leptosphaeria maculans*. Nejprve byly rostliny *A. thaliana* ošetřeny cytoskeletárními drogami a byl analyzován hormonální profil a transkripce obranných genů. Došlo ke specifickému zvýšení produkce kyseliny salicylové a transkripce markerových genů dráhy kyseliny salicylové. Dále byly ošetřené rostliny *A. thaliana* a *B. napus* infikovány příslušnými patogeny a překvapivě v obou patosystémech došlo ke zvýšení odolnosti ošetřených rostlin. Tento jev je regulovaný kyselinou salicylovou a jeho navození je závislé na režimu ošetření, čase infekce a konkrétním patosystému.

Neporušená dynamika aktinového cytoskeletu je nezbytná pro vnitrobuněčný transport a syntézu membrán, proto jsme se dále zabývali rolí fosfolipidů během indukce resistence rozrušením aktinového cytoskeletu. Pro tyto experimenty byly použity rostliny *A. thaliana* s mutací v genech pro fosfatidylinositol-4-kinasu β1 a β2 (PI4Kβ1β2), u nichž byla popsána zvýšená akumulace kyseliny salicylové, a několik dalších mutantů *A. thaliana* s mutacemi v signální dráze SA. Nejprve bylo otestováno ukládání kalosy, což je obranná reakce vyžadující funkční buněčný transport. Ošetření cytoskeletárními drogami spouští ukládání kalosy díky aktivitě kalosasynthasy 12 a je nezávislé na SA, neboť bylo pozorováno i u mutantů se zablokovanou indukcí SA. Transkripce obranných genů a akumulace SA nebyly pozorovány u mutantů defektních v signální dráze SA a byly úplně nebo částečně revertovány u trojitých mutantů defektních v SA sigální dráze a zároveň v genech pro PI-4-

kinasu β1 a β2. Tyto výsledky naznačují, že role aktinového cytoskeletu v rostlinné imunitě je komplexnější než se dosud předpokládalo. Kyselina salicylová hraje významnou roli při vyvolání obranné reakce spuštěné dezintegrací aktinových vláken a zároveň je v tomto procesu významná fosfolipidová signalizace.

Jelikož se tato práce významně zabývá kyselinou salicylovou, v další části byla vytvořena kolekce mutantů *A. thaliana* ovlivněných v produkci, akumulaci nebo signalizaci kyseliny salicylové. U některých těchto mutantů byly popsány změny v odolnosti vůči patogenům. Všichni mutanti kolekce byli pěstováni v několika různých kultivačních režimech a následně byl charakterizován růst růžic a kořenů, intenzita fotosyntézy, koncentrace SA a transkripce SA markerových genů. Tato kolekce představuje užitečný nástroj pro další studie zabývající se rostlinnou imunitou. Z výsledků charakterizace vyplývá jasná korelace mezi retardací růstu růžic a zvýšenou akumulací SA.

Druhá část práce se zabývá infekčními strategiemi rostlinných patogenů ovlivňujícími hormonální signalizaci hostitele.

Patogeny sekretují také další molekuly, kterými manipulují hormonální signalizaci hostitele. Malé sekretované proteiny, tzv. efektory, jsou příkladem takových molekul. Tato práce se zabývá efektorem AvrLm4-7 a jeho významem pro virulenci a tlumení obranných reakcí hostitele. Inokulační test rostlin *B. napus* izoláty *L. maculans* exprimujícími funkční a nefunkční alelu *AvrLm4-7* ukázal, že přítomnost funkčního efektoru AvrLm4-7 výrazně přispívá k virulenci *L. maculans*. Dále byly analyzovány obranné reakce – transkripce obranných genů, hormonální profil a produkce reaktivních forem kyslíku. Infekce izolátem s funkčním AvrLm4-7 vedla ke snížené indukce SA a nižší transkripci SA markerových genů. Produkce reaktivních forem kyslíku byla rovněž snížena. Efektor AvrLm4-7 přispívá k virulenci *L. maculans* potlačením SA-dependentních obranných reakcí hostitele.

Některé patogeny samy produkují rostlinné hormony. V rámci této práce bylo zjištěno, že houbový patogen řepky olejky (*B. napus) L. maculans* také produkuje řadu rostlinných hormonů. Tato práce se soustředí především na auxiny. V myceliu *L. maculans* bylo nalezeno několik forem auxinů, v nejvyšší koncentraci se vyskytovala bioaktivní forma, indol-3-octová kyselina (IAA). Produkce IAA může být zvýšena přidáním biosyntetických prekurzorů tryptofanu a tryptaminu k tekuté kultuře. V genomu *L. maculans* byly nalezeny orthology několika známých genů auxinových biosyntetických drah. Biosyntetické prekurzory indukovaly transkripci několika těchto genů; především *LmTAM1*, *LmIPDC2* a *LmNIT1*. Transkripce genů *LmIPDC1*, *LmIaaM3* a *LmIaaM5* byla zvýšena pouze mírně. Exogenní aplikace auxinu ve vysoké koncentraci inhibovala růst *L. maculans* a žádná z použitých koncentrací neměla stimulační efekt. Auxinový profil infikovaných rostlin *B. napus* se liší pouze

minimálně; v rostlinách infikovaných *L. maculans* byla pozorována zvýšená koncentrace indol-3-acetonitrilu. Tyto výsledky ukazují, že *L. maculans* je schopna produkovat vysokou koncentraci bioaktivního auxinu IAA, který ale nemá výrazný vliv na průběh infekce. Mohl by ale plnit regulační funkci v samotném patogenu.

Tato práce se zabývá specifickými aspekty rostlinné signalizace, především ve spojení s kyselinou salicylovou a dalšími rostlinnými hormony.

Table of contents

List of abbreviations

12,13-EOT	12,13(S)-epoxy-octadecatrienoic acid
AAO	Abscisic aldehyde oxidase
ABA2	Xanthoxin dehydrogenase
ABA4	ABA deficient 4
Abm	Antibiotic biosynthesis monooxygenase
ACC	1-aminocyclopropane-1 carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AFB	Auxin signalling F-box protein 1
AHK2	Arabidopsis histidin kinase 2
AHK3	Arabidopsis histidin kinase 3
AMP	Adenosinmonophosphate
ANAC019	NAC domain containing protein 19
AOS	Allene oxide synthase
ARF3	Auxin responsive factor 3
ARFs	Auxin responsive factors
BA2H	Benzoic acid 2-hydroxylase
BAK1	BRI1-associated receptor kinase 1
BES1	BRI1-EMS-suppressor 1
BIK1	Botrytis induced kinase 1
BIN2	Brassinosteroid insensitive 2
BKI1	BRI1 kinase inhibitor 1
BRI1	Brassinosteroid insensitive 1
BRL1	BRI1-like 1
BSMT	BA/SA carboxyl methylatransferase 1
BZR1	Brassinazole resistant 1
CAT1	Catalase
CBPg60	Calmodulin-binding protein 60-like g
CDG1	Serine/threonine-protein kinase CDG1

CERK1	Chitin elicitor receptor kinase 1
CHASE	Cyclases/histidine kinases-associated sensing extracellular
COI1	Coronatine insensitive 1
CPS	Ent-copalyl-diphosphate-synthase
CTR1	Constitutive triple response 1
CWAS	Cell wall appositions
DAG	Diacylglycerol
DAMPs	Damage associated molecular patterns
DELLA	Transcription factors containing DELLA domain
DGK	DAG-kinase
DMAPP	Dimethylallylpyrophosphate
EAR	Ethylene responsive element binding factor-associated Amphiphilic Repression
EIL1	Ethylene insensitive 3-like 1
EIN3	Ethylene-insensitive3
ER	Endoplasmic reticulum
ERFs	Ethylene responsive transcription factors
ESP1	Pyruvoyl-glutamate lyase
ETI	Effector trigerred immunity
ETS	Effector triggered susceptibility
ETT	Auxin response transcription factor 3
FLS2	Flagellin insensitive 2
GAI	Gibberellic acid insensitive
GGPP	Geranyl geranyl diphosphate
GGPPS	Geranyl geranyl diphosphate synthase
GH3	IAA-amido synthase
GID1	GA insensitive dwarf 1
GID2	GA insensitive dwarf 2
HKs	Histidine kinase receptors

HMBDP	4-hydroxy-3-methyl-but-2-enyl diphosphate
HR	Hypersensitive response
IAA	Indole-3-acetic acid
IAN	Indole-3-acetonitrile
ICS1	Isochorismate synthase 1
ICS2	Isochorismate synthase 2
IND	Transcription factor IND
iPRDP	Isopentenyladenosine-5'-diphosphate
iPRTP	Isopentenyladenosine-5'-triphosphate
IPT	Isopentenyltransferase
JA	Jasmonic acid
Jai1	Jasmonate insensitive 1
Ja-Ile	Jasmonoyl-isoleucine
JAZ	Jasmonate ZIM-domain
KS	Ent-kaurene synthase
LOG	LONELY GUY
LORE	Lipooligosascharide-specific reduced elicitation
LOX2	Lipoxygenase 2
LRR	Leucine-rich repeat
LYM1/3	LysM domain-containing GPI-anchored protein 1/3
MED25	Mediator 25
MeJA	Methyljasmonate
MEP	Methylerythritol phosphate
MeSA	Methyl salicylate
MPK4	Mitogen activated protein kinase 4
NCED	9-cis-epoxycarotenoid dioxygenase
NIE	Nitro-2-indolyl-ethane
NINJA	Novel interactor of JAZ

NLPs	Necrosis and ethylene-inducing peptide 1-like proteins
NPC	Non-specific phospholipase C
NPR1	Nonexpressor of pathogenesis related 1
OPDA	Oxo-phytodienoic acid
OPR3	12-oxophyto-dienoate reductase
PA	Phosphatidic acid
PAMP	Pathogen associated molecular patterns
PBS3	Amidotransferase avrPphB SUSCEPTIBLE 3
PC	Phosphatidylcholine
PCRK1	Pattern-triggered immunity compromised receptor-like cytoplasmic kinase 1
PDF1.2	Plant defensin 1.2
PE	Phosphatidylethanolamine
PEPR1/2	Perception of the *Arabidopsis* danger signal peptide
PG	Phosphatidylglycerol
PHPs	Pseudo-histidine phosphotransfer proteins
PI(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
PI4Kβ1/β2	Phosphatidylinositol-4-kinase β1 and β2
PI4P	Phosphatidylinositol-4-phosphate
PI-PLC	Phosphoinositie-specific phospholipase C
PLA	Phospholipase A
PLD	Phospholipase D
PR1	Pathogenesis related 1
PR2	Pathogenesis related 2
PR-proteins	Pathogenesis-related proteins
PRRs	Pathogenesis related receptors
PRXR1	Peroxidase with putative IPL activity

PTI	PAMP-triggered immunity
PYR/PYL/RCAR	Pyrabactin resistant/Pyrabactin resistant-like/Regulatory component of ABA response
R proteins	Resistance proteins
RGA	DELLA protein RGA
RGL1	RGA-like 1
RGL2	RGA-like 2
RGL3	RGA-like 3
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor-like kinase
RLP23	Receptor like protein 23
RLPs	Receptor-like proteins
ROS	Reactive oxygen species
SA	Salicylic acid
SAG	SA-glucoside
SAR	Systemic acquired resistance
SARD1	SAR deficient 1
SDR1	Short chain dehydrogenase/reductase-like 1
SERK3	Somatic embryogenesis receptor-like kinase 3
SLR1	Slender rice 1
SLY1	SLEEPY1
SnRK2	SNF1-Related protein kinase 2
SOBIR1	SUPPRESSOR OF BIR1-1
TAAs	tryptophan aminotransferases
TGA	TGACG-binding factors
TIR1	Transport inhibitor response 1
TPL	TOPLESS repressor
WRKY	Transcription factors containing WRKY domain
YUC	Flavin-containing monooxygenase

ZEP	Zeaxanthin epoxidase

1. Introduction

1.1. Plant immunity and phytohormone signalling

1.1.1. Pathogen life strategies

Plants live in complex environment where they are constantly threatened by a wide range of pathogens including viruses, bacteria, fungi, oomycetes, nematodes or herbivores. Plant pathogens can be divided into three main categories based on their lifestyles – biotrophic, necrotrophic and haemibiotrophic. Necrotrophic pathogens produce toxins to kill plant tissues and feed on the dead remnants afterwards. Biotrophic pathogens manipulate metabolism of hosts living cells to get nutrients. For this purpose they use special structures such as haustoria. Many pathogens can switch from one strategy to the other based on their life cycle stage and thereby they are classified as haemibiotrophs. Plants have evolved complex defence strategies against these pathogens including mechanic barriers, toxin production and sophisticated signalling mechanisms. Many pathogens although are succesful in overcoming these defence mechanisms. Plants for this reason further possess mechanisms for specific recognition of the pathogen[1].

1.1.2. PAMP triggered immunity

The plant first recognizes conserved microbial structures such as flagellin, chitin, glycoproteins or lipopolysaccharides. These structures are collectivelly known as microbe associated molecular patterns – MAMPs, or when specified to pathogens only – PAMPs (Pathogen associated molecular patterns). The PAMPs are recognized by so-called pattern recognition receptors – PRRs that subsequently trigger defence signalling (Fig. 1). Host molecules that are degradation products occuring during wounding or infection are reffered to as damage associated molecular patterns (DAMPs) and their perception is also part of the innate immunity[2]. The PRR receptors are located on the plasma membrane and are considered the first layer of plant defence is often called PAMP-triggered immunity – PTI [3,4].

Throughout evolution pathogens have evolved molecules that are delivered directly into the host cells to supress PTI. These molecules are collectivelly called effectors and the reaction they cause is reffered to as effector triggered susceptibility – ETS. As a response to effectors plants have evolved receptors generally located in cytosol that recognize specific effectors and trigger secondary immune response called effector trigerred imunity – ETI [5,6]. Many defence and signalling components that participate in PTI and ETI have been identified in recent years including callose and lignin biosynthesis and their

deposition into the cell wall, synthesis of antimicrobial secondary metabolites such as phytoalexins and PR proteins (Pathogenesis-related proteins). PR proteins include glucanases and chitinases which degrade fungal and oomycetal cell wall. Effector recognition during ETI is followed by rapid induction of reactive oxygen species (ROS) which leads to hypersensitive response (HR) and apoptosis (Fig. 1). The aim of this reaction is to prevent the pathogen from spreading. This strategy is predominantly used against biotrophic pathogens since necrotrophs would use it for their advantage[1]. Immune signalling against necrotrophs can be triggered by recognition of toxins or DAMPs such as host cell wall fragments. The signalling principles of PTI and ETI are similar, ETI usually leads to faster and more dramatic immune response and often results in programmed cell death. PTI functions as immune mechanism against wide range of microorganisms in a process called non-host resistance. ETI components require specific cytoplasmic receptors. Many of those have been identified in recent years. Structurally and functionally similar receptors involved in immune signalling occur in both plants and animals[1].

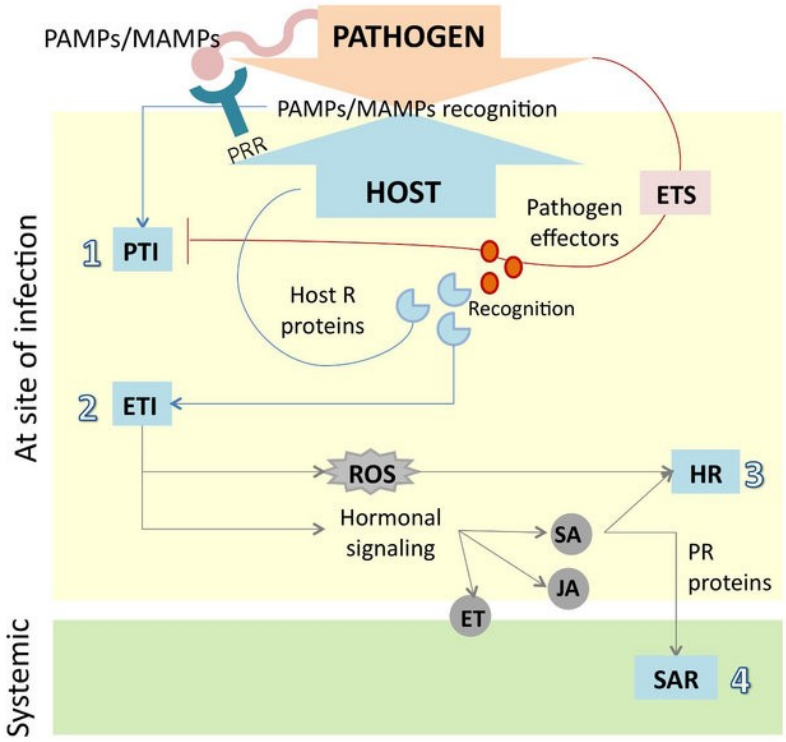


Figure 1: Layers of plant innate immunity and its connection with hormone signalling pathways. Local infection site is recognized and signal is transduced via plant hormone pathways to trigger sytemic acquired resistance (SAR). PAMPs - Pathogen-associated molecular patterns, PRR - Pattern recognition receptor; PTI - PAMP-triggered immunity, R proteins - Resistance proteins, ETS - Effector triggered susceptibility, ETI - Effector triggered immunity, ROS - Reactive oxygen species, HR - Hypersensitive response, PR proteins - Pathogen related proteins. Modified from[7].

1.1.3. Pattern Recognition Receptors and downstream signalling

The PRRs are plasma membrane located multimeric protein complexes able to bind certain PAMPs and recruit different cytoplasmic kinases to process downstream signalling. The receptors are composed of an ectodomain responsible for ligand binding, a single transmembrane domain and an intracellular kinase domain (Fig. 2)[8].

There are several types of ectodomains binding different kind of MAMPs – leucin-rich repeat (LRR) domains that predominantly bind peptides such as flagellin or bacterial elongation factor Ef-Tu[5], ectodomains containing more lysine motifs binding carbohydrates such as chitin or bacterial peptidoglycan[9], lectin-type PRRs binding lipopolysaccharides or extracellular ATP and receptors with epidermal-growth factor-like ectodomains binding oligogaracturonides derived from the cell wall[10]. Ligand binding results in phosphopylation within the receptor complex that leads to further activation of ROS burst, calcium burst, activation of cytoplasmic kinase cascades, actin reorganization and transcriptional reprogramming[10]. For example, the common PAMP flagellin or its flg22, which is conserved motif of flagellin consisting of 22 amino acids and is often used in research, is recognized in plants via plasma membrane receptor complex FLS2 (Flagellin insensitive 2) and induces immune responses. Flagellin peptide flg22 enters the cell together with the FLS2 receptor complex and further on is transported to distant organs via closest vascular connections. This is in contrast with transport mechanisms for other molecules that require membrane transporters[11].

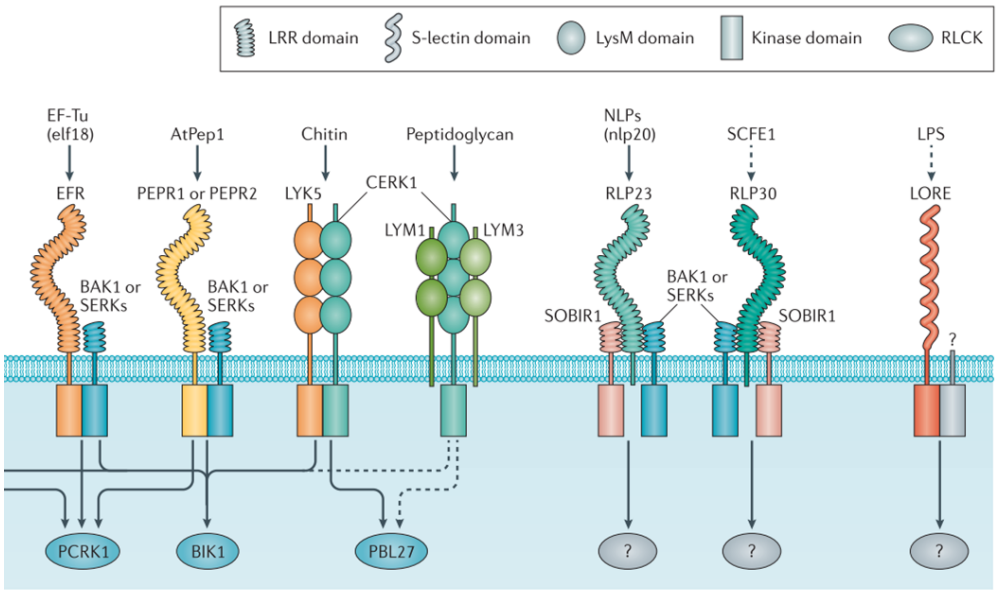


Figure 2: Domain structure of the most important up to date known PRR receptors in *A. thaliana*. EFR – EF-Tu receptor, PCRK1 – Pattern-Triggered Immunity Compromised Receptor-like Cytoplasmic Kinase1, PEPR1/2 – Perception of the Arabidopsis Danger Signal Peptide 1/2, LYK5 – LysM domain receptor-like kinase 5, PBL27 – PBS1-like protein 27, LYM1/3 – LysM domain-containing GPI-anchored protein 1, NLPs – necrosis and ethylene-inducing peptide 1-like proteins, RLP23/30 – Receptor like protein 23, LPS – lipopolysachcaride, LORE – lipooligosascharide-specific reduced elicitation, BAK1 – BRI1-associated receptor kinase 1, also reffered to as SERK3, CERK1 – chitin elicitor receptor kinase 1, LRR – leucin-rich repeat, RLPs – receptor-like protein, SOBIR1 – supressor of BIR1-1, BIK1 – Botrytis induced kinase 1, RLCK – Receptor-like cytoplasmic kinase. Modified from[10].

1.1.4. Phytohormones involved in stress signalling

Phytohormones are small molecules with vital regulatory functions in all aspects of plant life processes. Similarly to animal hormones they act in low concentrations but are less specific and no special glands or tissues are responsible for their production. The same compound causes different effects and final reaction depends on concentration ratio of several different phytohormones. They participate in growth regulation and development, reproduction and reactions to biotic and abiotic stresses[12].

Plant immune signalling involves several hormones. The main defence hormones are salicylic acid (SA) and jasmonic acid (JA). Other hormones such as ethylene (ET), abscisic acid (ABA), gibberellins (GA), auxins, cytokinins (CKs) and brassinosteroids (BRS) modulate defence signalling indirectly or to a lesser extent (Fig. 3). Regulation of concentration ratio of different hormones impacts stress

adaptation. The final ratio and timing is crucial for the resulting resistance or susceptibility of the infected tissue. The onset of immune response usually slows down plant growth. Antagonistic and synergistic action of hormones allows plants to balance effectively their nutrient sources and regulate defence reactions on various levels at the same time[13].

The SA pathway is predominantly activated as a response to biotrophic pathogens whilst the JA pathway is triggered by necrotrophs. The two pathways are generally known to act antagonistically. This antagonistic effect was documented in *Arabidopsis thaliana* inoculated with avirulent bacterial strain of *Pseudomonas syringae*. The activation of SA pathway supressed JA signalling which subsequently resulted in increased susceptibility to necrotrophic fungus *Alternaria brassicicola*[12]. Another study mentioned induction of SA pathway by a biotrophic pathogen *Hyaloperonospora arabidopsidis* which led to increased vulnerability to *Pieris rapae* catterpillars[14]. The JA pathway is also able to supress the SA pathway. The haemibiotroph *P. syringae* produces phytotoxin coronatine which is structurally similar to bioactive derivate of JA, jasmonoyle-isoleucine. This phytotoxin is able to suppress SA signalling and thus increase hosts susceptibility to *P. syringae* [15].

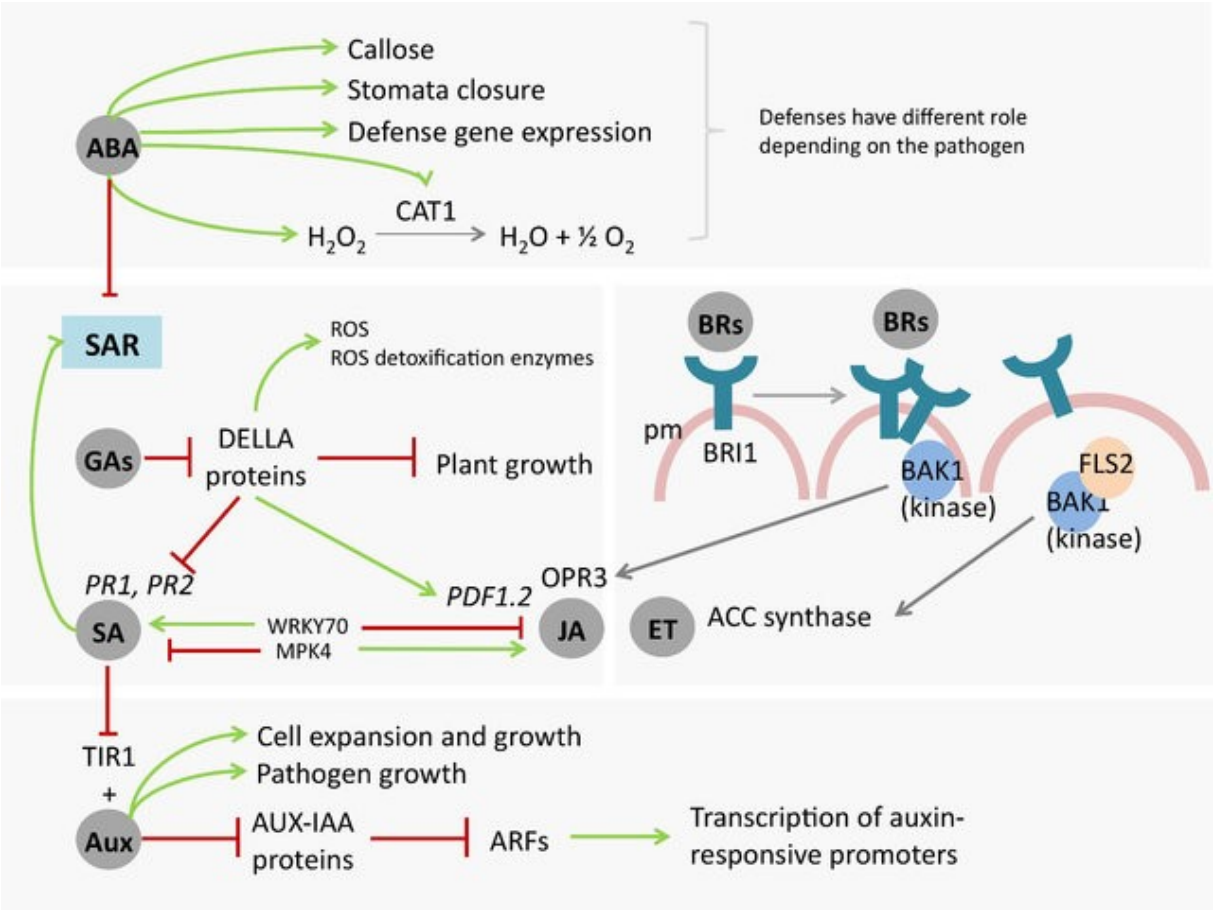


Figure 3: Involvement of main groups of phytohormones in stress signalling ABA – absciscic acid, CAT1 – catalase, SAR – systemic acquired resistance, GAs – gibberellins, ROS – reactive oxygen species, PR1, PR2 – pathogenesis releted genes 1 and 2, SA – salicylic acid, WRKY70 – transcription factor WRKY DNA-binding protein 70, MPK4 – mitogen activated protein kinase 4, PDF1.2 – plant defensin 1.2, BRs – brassinosteroids, BRI1 – brassinosteroid-insensitive 1 receptor, FLS2 – flagellin-sensitive receptor, BAK1 – BRI1-associated receptor kinase, OPR3 – 12-oxophyto-dienoate reductase, ACC synthase - 1-aminopropane-1-carboxylic acid synthase, JA – jasmonic acid, ET – ethylene, Aux – auxin, IAA – indole-3-acetic acid, TIR1 – Transport inhibitor response 1, ARFs – auxin responsive factors, pm – plasmatic membrane. Modified from[7].

Apart from the antagonistic effect of the SA and JA pathways there is also evidence of their synergistic actions. Treatment of tobacco plant*s* with low concentration of SA and JA simultaneously led to transcription of defence genes responding to JA (*PDF1.2*) and SA (*PR1*) simultaneously. When higher concentrations were applied antagonistic action was observed which suggests that resulting effect relies on the concentration ratio[16].

Other hormones contributing to the immune response are auxins which act synergistically with the JA pathway. Auxin treament led to expression of the JA-regulated genes, SA supresses expression of the genes that respond to auxin. Final result of treatment with auxin is usually increased resistance to

biotrophs[17]. Abscisic acid (ABA) mediates mainly abiotic stress signalling. In biotic stress signalling it plays a role of negative regulator since it supresses induction of SA- and JA- responsive genes [17].

1.1.5. Salicylic acid biosynthesis and signalling

Salicylic acid is a phenolic compound biosynthesized in plants via two major pathways – the isochorismate pathway and phenylalanine pathway. Both biosynthetic pathways originate from shikimate that is converted to chorismate (Fig. 4). Then chorismate can be either converted to phenylalanine or isochorismate. In the first case, phenylalanine is transformed into trans-cinnamic acid by phenylalanine amonium lyase (PAL). Trans-cinnamic acid is then converted to benzoic acid. The enzyme responsible for the catalysis of this reaction has not been described up to date. Benzoic acid is afterwards hydroxylated to bioactive SA by benzoic acid 2-hydroxylase (BA2H)[18]. The other biosynthetic pathway continues with conversion of isochorismate by the isochorismate synthase (ICS) and subsequently to SA by isochorismate pyruvate lyase (IPL) (Fig. 4). There are two isoforms of the ICS gene in *A. thaliana* but the IPL gene has not been characterized in plants yet. This pathway is responsible for synthesizing the vast majority of stress-related SA in *A. thaliana*[19]. Recent study showed that *A. thaliana* peroxidase PRXR1 exhibits IPL-like activity when expressed in *E. coli*[20]. Isochorismate produced by ICS enzymes can be also processed by cytosolic amidotransferase avrPphB SUSCEPTIBLE 3 (PBS3) yielding isochorismoyl-glutamate A which can either spontaneously degrade into SA and N-pyruvoylglutamate or this reaction can be catalyzed by ESP1, a pyruvoyl-glutamate lyase[21,22].

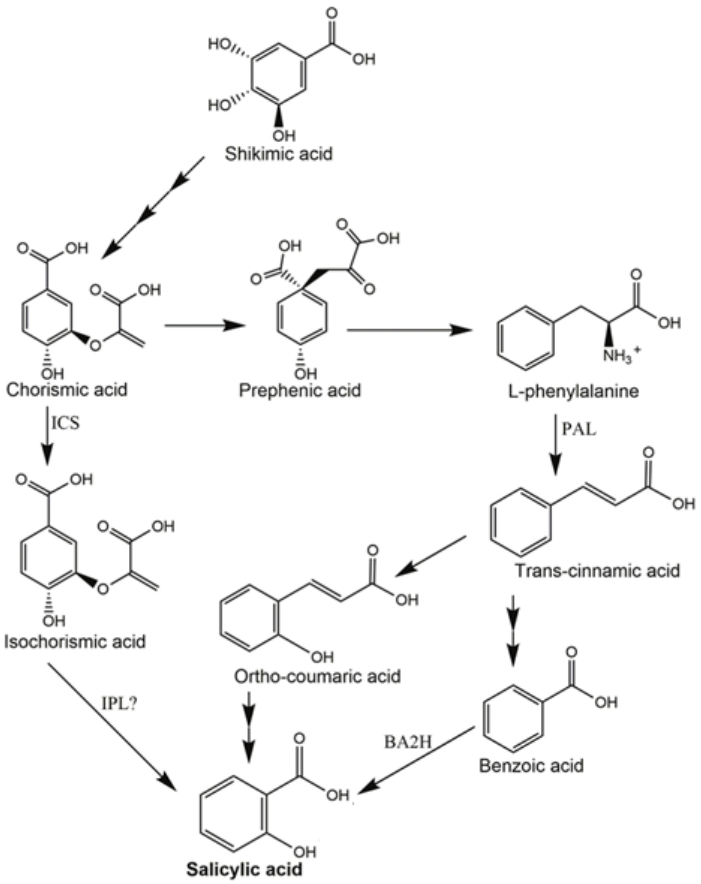


Figure 4: Biosynthetic pathways of salicylic acid. SA in plants is biosynthesized via two main pathways: Isochorismate pathway comprising the isochorismate synthase (ICS) and phenylalanine pathay comprising the phenylalanine ammonia-lyase (PAL). BA2H – benzoicacid-2-hydroxylase, IPL – isochorismate pyruvate-lyase. Modified from[23].

The main up to date known component of SA signalling is nuclear transcription regulator NPR1 (Nonexpressor of pathogenesis related 1). SA mediates NPR1 action via controlling its protein level. NPR1 interacts with transcription factors of the TGA family[24]. Recently, proteins NPR3 and NPR4 were identified as SA receptors with different binding affinity. The NPR3 and NPR4 target NPR1 for ubiquitination and degradation upon high (NPR3) or low (NPR4) SA level[25]. NPR1 is functional in intermediate SA concentration suggesting that SA gradient is crucial for correct signalling [26] (Fig. 5).

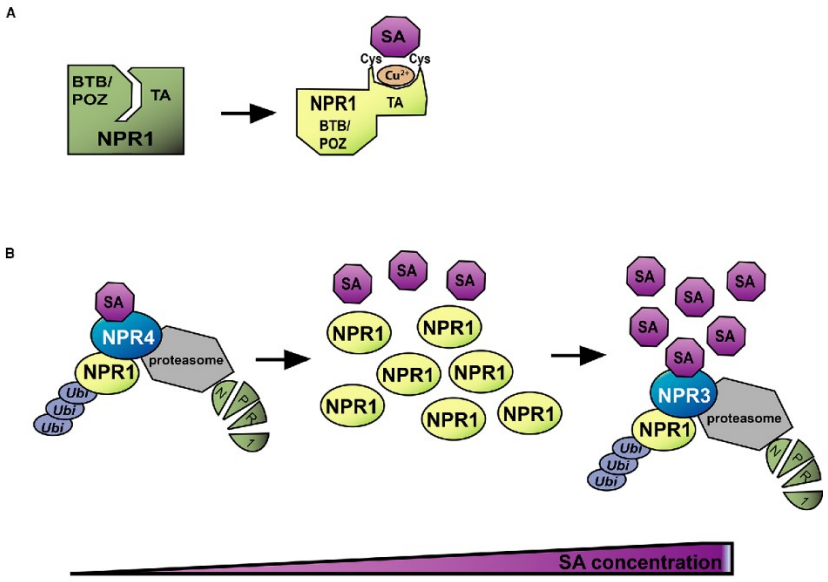


Figure 5: Salicylic acid signal transduction. (A) Under low SA concetnration the C-terminal transactivation domain of NPR1 (Nonexpresser of PR genes 1) is inhibited by the the N-terminal BTB/POZ domain thus keeping NPR1 inactive. SA binds to NPR1 via $Cys^{521/529}$ and copper ion that initiates conformation change of NPR1 resulting in its activation. (B) NPR1 accumulation is regulated by SA via receptors NPR3 (Nonexpresser of PR genes 3) and NPR4 Nonexpresser of PR genes 4) in SA concentration dependent manner. NPR3 and NPR4 directly bind SA. In case of low SA, NPR4 directs NPR1 for degradation through the 26S proteasome. When SA levels are intermediate, the NPR1 protein accumulates and regulates transcription of SA-dependent genes. High SA concentration triggers the NPR3-mediated NPR1 degradation. Intermediate SA levels are required for transcriptional reprogramming. Modified from[27].

ICS expression is induced locally and systemically by pathogen infection in *A. thaliana*[19]. Several transcription factors that regulate the ICS transcription have been identified up to date: SARD1, CBPg60, WRKY28, EIN3 (Ethylene insensitive 3) or ANAC019 [28,29,25]. Increased expression of ICS1 leads to SA accumulation which causes elevated expression of NPR1 which is responsible for further SA-dependent downstream signalling. NPR1 also negatively regulates expression of ICS1[30]. The *npr-1* mutants accumulated higher levels of the *ICS1* transcript and SA levels itself than wild type plants. The mechanism of action of NPR1 has ot been fully elucidated yet, members of the WRKY family might be involved[30].

Levels of bioactive SA must be tightly regulated throughout the plant organism. The SA molecule can be modified by glycosylation, methylation and amino acid conjugation. Two main SA metabolites are SA-glucoside (SAG) and methyl salicylate (MeSA). SAG is formed by SA glucosyltransferase and

allows the inactive product of SA to be stored in vacuole[18]. The methylation is catalyzed by BA/SA carboxyl methyltransferase 1 (BSMT) to form MeSA which is suggested signal for systemic acquired resistance (SAR)[31].

1.1.6. Jasmonic acid biosynthesis and signalling

Jasmonic acid, its methylester (MeJA) and isoleucine conjugate (Ja-Ile) are collectively known as jasmonates and have been identified as stress regulators. Apart from this main function they also participate in processes as regulation of stomatal opening, Rubisco biosynthesis inhibition or uptake of nitrogen and phosphorus[32].

Majority of JA is synthesized via the octadecene pathway starting from α-linolenic acid and the hexadecane pathway that begins with hexadecatrienoic acid[33] (Fig. 6). The biosynthesis takes place in three different compartments: the chloroplast, peroxisome and cytoplasm. The oxo-phytodienoic acid (OPDA) is synthesized in chloroplast and subsequently converted to JA in peroxisome. JA is metabolized into MeJA, Ja-Ile, cis-jasmone or 12-hydroxyjasmonic acid in cytoplasm[34].

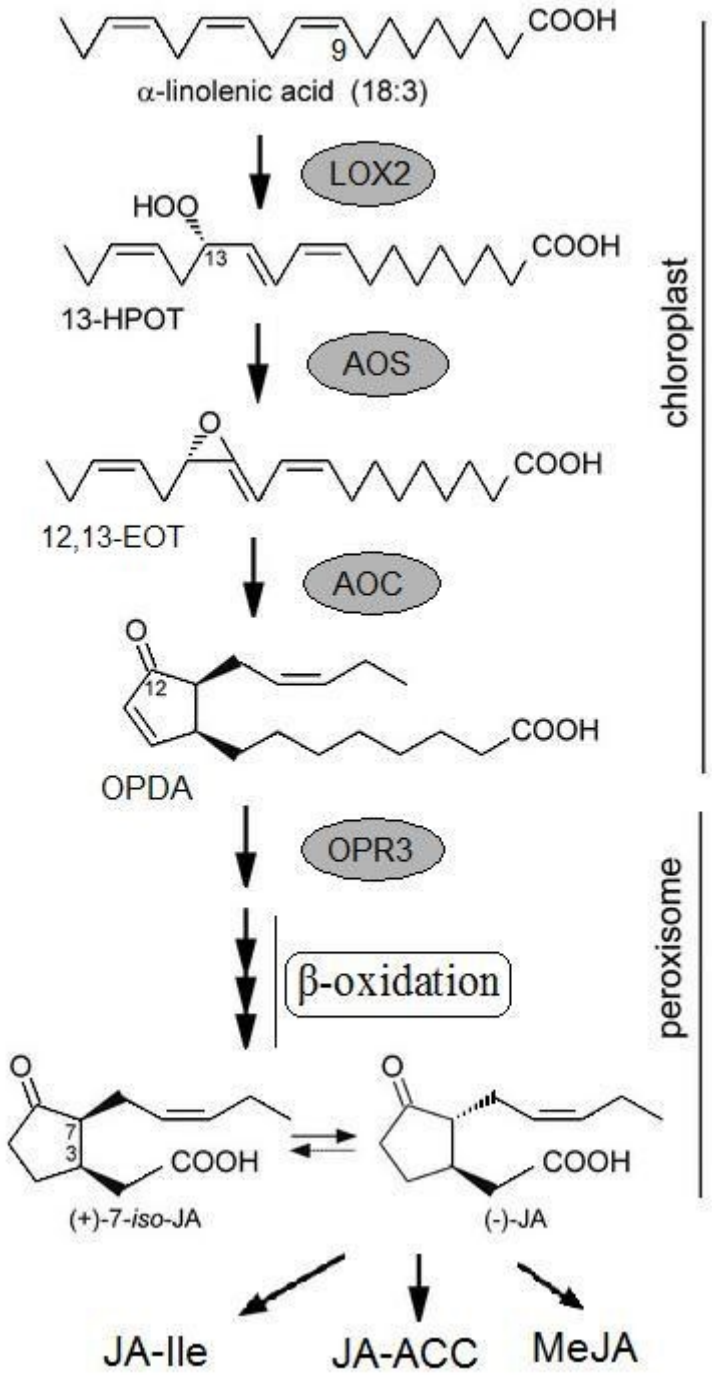


Figure 6: Jasmonic acid biosynthesis starting from α-linolenic acid. The biosynthetic pathway begins with α-linolenic acid oxygenation by lipoxygenase 2 (LOX2) forming a 13S-hydroperoxy linolenic acid (13-HPOT) that is further processed by allene oxide synthase (AOS) producing 12,13(S)-epoxy-octadecatrienoic acid (12,13-EOT). The oxo-phytodienoic acid (OPDA) is produced from 12,13-EOT by 12-oxophyto-dienoate reductase (OPR3). OPDA undergoes β-oxidation into JA. JA can be further metabolized into jasmonylisoleucin (JA-Ile), methyljasmonate (MeJA) or jasmonyl-aminocyclopropane carboxylic acid (JA-ACC). Modified from[35].

JA signalling pathway is composed of several components. The F-box protein COI1 (Coronatine insensitive 1) is assembled into a complex with the SCF E3 ubiquitin ligase[36]. Upon JA signalling the F-box protein forms a complex with target proteins that are subsequently degraded by 26S proteasome[37].

The JAZ (Jasmonate ZIM-domain) proteins are major component of the JA signalling mechanism. They contain two main conserved domains: the ZIM domain and the Jas domain[38]. The ZIM domain mediates dimerization of the JAZ proteins and its interaction with NINJA (Novel interactor of JAZ) proteins. NINJA recruits transcriptional co-supressor TPL (TOPLESS) via the EAR (Ethylene responsive element binding factor-associated Amphiphilic Repression) domain. It also competes with MED25 (Mediator25) for the interaction with MYCs transcription factors[39]. The Jas domain mediates interaction between JAZ proteins and COI1 or other transcription factors[40] (Fig. 7).

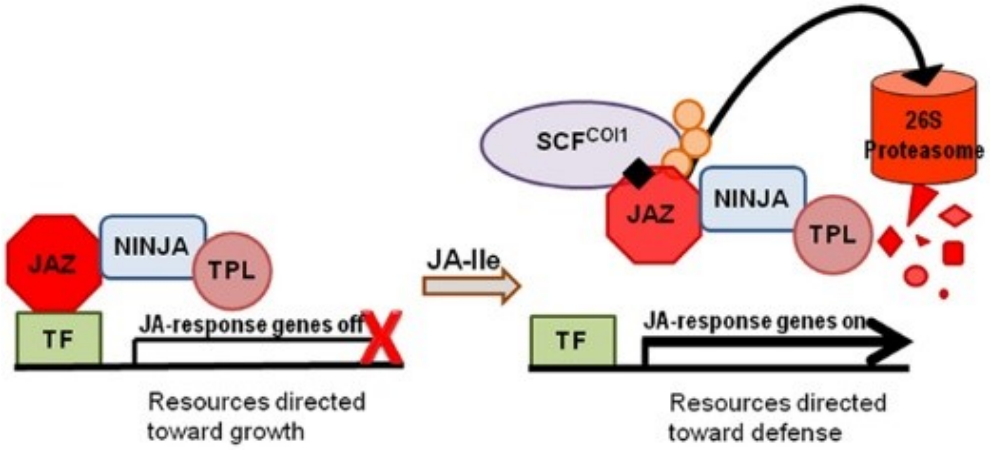


Figure 7: JA signal transduction. JA-responsive transcription factors are repressed by JAZ proteins and co-repressors NINJA and TOPLESS (TPL). In the presence of jasmonyl-isoleucine (Ja-Ile) the repressor complex is directed for degradation by 26S-proteasome. Modified from[41].

COI1, JAZ and MYC are the core components of JA signalling. The JAZ form complexes with specific transcription factors and regulate multiple downstream responses[42]. The bioactive form of JA is the jasmonyl-isoleucine (JA-Ile). JA-Ile concentration regulates formation of the COI-JAZ complexes[43]. The concentration of JA-Ile increases upon wounding, insect feeding or nectrotrophic pathogen attack. The signal is percieved by the COI1 protein in complex with SCF. This complex directs the JAZ proteins for ubiquitination and degradation. Downstream signalling transcription factors such as MYCs can be released afterwards[40].

The MYC transcription factors participate in negative regulation of gene expression in cell cycle and contribute to plant growth inhibition. They form a complex with the JAZ proteins in JA signal transduction process [42]. Complex of JAZ-MYC regulates concentration of defence compounds[41].

1.1.7. Cytokinin biosynthesis and signalling

Cytokinin biosynthesis begins with conversion of AMP and dimethylallylpyrophosphate (DMAPP) to active cytokinin molecule (N^6-(δ^2-isopentenyl)adenosine-5'-monophosphate). The reaction is catalyzed by isopentenyltransferase IPT[44]. The genome of *A. thaliana* contains 9 isoforms of the IPT gene with particular importance of isoforms AtIpt2 and AtIpt9 for cis-zeatin biosynthesis. Disruption of these two isoforms led to decrease in cis-zeatin production while disruption of the other seven genes resulted in decreased level of trans-zeatin and its metabolites[45]. Tissue specific expression of several AtIPT isoforms was reported which specifies the most likely sites of cytokinin production[46,47].

The isoprenoid chain of isopentenyl and trans-zeatin (tZ) is mainly acquired from the DMAPP produced from methylerythritol phosphate (MEP) pathway in plastids of *A. thaliana*. The product is called the isopentenyl ribotide and is further hydroxylated to trans-zeatin[48] (Fig. 8). The initial reaction produces iPRTP (Isopentenyladenosine-5'-triphosphate) and iPRDP (Isopentenyladenosine-5'-diphosphate) that are hydroxylated to trans-zeatin by cytochrome P450 enzymes CYP735A1 and CYP735A2[47]. The active forms of cytokinines are the free bases produced from the cytokinin ribotides. The release is catalyzed by the LONELY GUY (LOG) enzyme family. There are seven LOG isoforms in *A. thaliana* genome[49]. LOG7 and LOG4 are mainly active in the shoot apical meristem and LOG3 and LOG4 in roots[50]. On the other hand, the isoprenoid chain of cis-zeatin (cZ) is mainly synthesized by the mevalonate (MVA) pathway. Mevalonate is converted to isopentenyl pyrophosphate (IPP) by cytokinin synthetase[51].

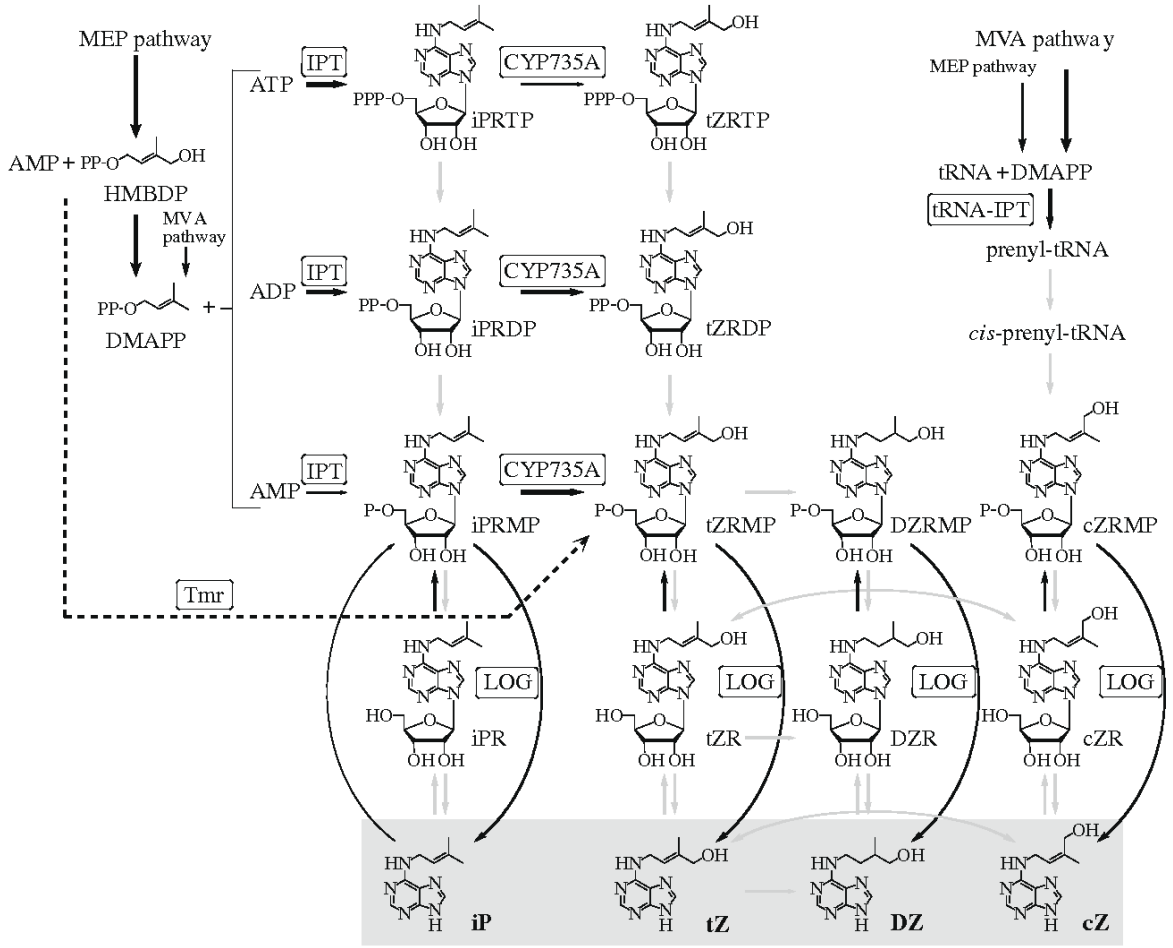


Figure 8: Cytokinin biosynthesis. The isoprenoid side chain of iP and tZ is mainly synthesized via the MEP pathway, whereas in cZ it mostly comes from the MVA pathway. Adenosine phosphate-isopentenyltransferases (IPTs) utilize ATP and ADP and dimethylallylpyrophosphate (DMAPP), producing isopentenylriboside-triphospate (iPRTP) or -diphosphate (iPRDP). The CK-nucleotides are converted into the corresponding tZ-nucleotides by CYP735A. Dephosphorylation by phosphatase may occur in those di- or tri-phosphorylated CK-nucleosides. In *A. tumefaciens* infected cells Tmr encoded by the bacterium mediates N-prenylation of AMP with HMBDP (4-hydroxy-3-methyl-but-2-enyl diphosphate), producing tZRMP (dashed arrow line). The tRNA-IPTs catalyze prenylation of tRNA that leads to the production of cZRMP. The CK-nucleoside 50 -monophosphates are directly catalyzed to active form (free-base form) CKs by LOG. cZ and tZ may be enzymatically interconverted by zeatin cis–trans isomerase. The genes of enzymes involved in phosphorylation of iPR and conjugation of phosphoribosyl moieties to iP are adenosine kinase (AK) and adenine phosphoribosyltransferase (APRT), respectively. APRT utilizes not only iP but also other CK nucleobases. Modified from[51].

Cytokinin signalling mechanism involves two main components: a membrane localized sensor kinase and a response regulator that can directly regulate transcription of the target genes. The signal

transduction occurs via transphosphorylation of a His residue of the sensor kinase and an Asp residue of the regulator element[52]. Plant histidine kinase receptors (HKs) contain extracytosolic region with a conserved hormone-binding CHASE domain (cyclases/histidine kinases-associated sensing extracellular), at least two transmembrane domains and a cytosolic region with a histidine kinase domain, a canonical receiver domain, and a diverged receiver domain unlikely to function in phosphotransfer[53,54,55]. The receptors are predominantly localized in ER with the CHASE domain oriented inside the ER membrane. The site of cytokinin binding is the ER lumen[56,57,58]. Lower number of the receptors can be also found on the plasma membrane[59].

After binding of the cytokinin the His residue of the CHASE domain transfers phosphate signal to the Asp residue of the receiver domain and downstream to AHP and type-B RR proteins (described further)[60] (Fig. 9). The downstream AHPs show no enzymatic activity but they act as phospho-donors for efficient RR protein phosphorylation[61]. They have a conserved Cys residue that can be S-nitrosylated by nitric oxide which inhibits the signalling. The nitrosylation inhibits the phosphotransfer activity resulting in lower ability to receive a phosphate from the AHK and transfer the phosphate to the RRs[62]. Pseudo-histidine phosphotransfer proteins (PHPs) have been also identified in plants as negative regulators of the cytokinin signalling[60].

The phosphate signal is transfered by the kinases to the RR proteins which are of two types in plants: type-A RRs and type-B RRs. The Type-B RRs are vital for the first transcription response to cytokinin signal[63,64]. They have a receiver domain and a large C-terminal extesion that includes a Myb-like DNA-binding domain. The rate of turnover is partly regulated by an E3-ubiquitin ligase complex including the KISS ME DEADLY (KMD) F-box proteins[65]. Type-A RRs act as negative regulators. Their trascription is induced in response to cytokinin by the type-B RRs[66,67,68]. Cytokinins also stabilizes many type-A RRs in a phosphorylation-dependent manner[69]. They are responsive to other hormonal stimuli as well. The regulation of their expression by interefering of other signalling pathways can modulate cytokinin sensitivity in different tissues. The mechanism of regulation is not well described up to date[60].

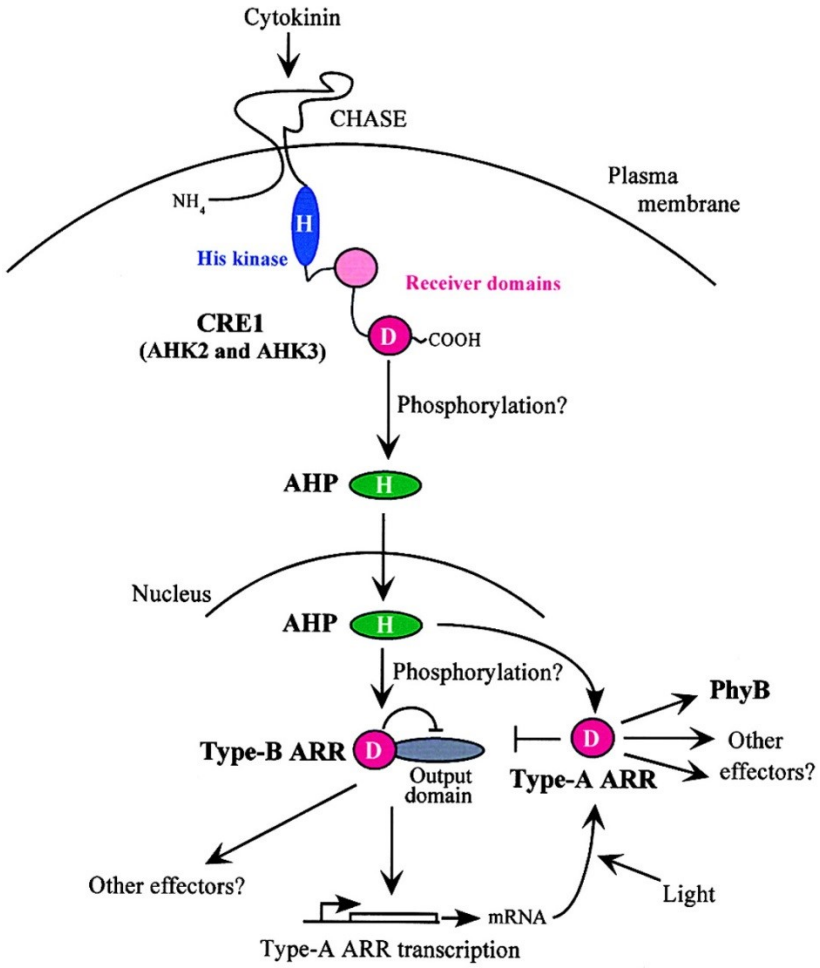


Figure 9: Cytokinin signal transduction. Cytokinin binds to CRE1 (Cytokinin response 1), and possibly other His kinase–like proteins such as AHK2 and AHK3 (*Arabidopsis* histidin kinase 2/3), within the CHASE domain. The binding of cytokinin causes autophosphorylation on a histidine activates the transmitter domain (blue), which autophosphorylates on a His (H). With a series of transphosphorylations the signal is transferred to an AHP (*Arabidopsis* histidine phosphotransfer) protein, which translocates to the nucleus, where it activates type-B ARR receptors. The activated type-B ARRs induce transcription of the type-A ARRs, which feed back to inhibit their own transcription. PhyB – Phytochrome B, D – Aspartate residue. Modified from[70].

Exogenous treatment with cytokinins reduces susceptibility of *A. thaliana* to biotrophic pathogen *H. arabidopsidis* and haemibiotrophic *P. syringae*[71,72]. Mutants in cytokinin receptors AHK2 and AHK3 were no longer resistant to the infection. Plants overexpressing biosynthetic IPT gene show increased levels of endogenous cytokinins and reduced *P. syringae* infection rate[72]. Enhaned defence gene expression and callose deposition was observed in the IPT-overexpressing mutants[72,71]. Cytokinins alone did not induce defence reactions so in this context their role in immunity can be observed as priming agents[73]. Similar effect was observed in other pathosystems: *N. benthamiana* x *P. syringae*,

Oryza sativa x *Magnaporthe oryzae*[74,75]. Increased resistance to viruses and nematodes was also reported[76,77].

Cytokinins contribute to enhancing immune reactions against biotrophic pathogens. The cytokinin-induced immunity is mainly connected with SA signalling[78], although there is evidence of SA-independent immunity activation: SA hydroxylase NahG expressed in tobacco did not alter the protectvie effect of cytokinin against *P. syringae*. Production of antimicrobial phytoalexins in plants is activated by cytokinin treatment[74]. Specific SA-pathway signalling components are involved in the cytokinin-dependent immunity. Regulators of cytokinin signalling ARR2 type-B directly interact with SA-responsive transcription factor TGA3 and the NPR1 receptor. This leads to *PR1* expression. Negative regulators of cytokinin signalling, the type-A ARRs, suppress SA-dependent defence gene expression[71]. ROS production also plays a role in the regulation of immunity induction by cytokinins[78]. There is also evidence of so-called cytokinin-induced susceptibility which is usually associated with low levels of cytokinin content in plants[71]. Exogenous application of low concentration of cytokinins led to induced susceptibility to *H. arabidopsidis* or powdery mildew[71,79].

1.1.8. Auxin biosynthesis and signalling

The most important biosynthetic pathway in plants is the indolepyruvate (IPyA, IPA) pathway. It involves two-step conversion of tryptophan into IAA (Fig. 10). The first step is catalyzed by tryptophan aminotransferases (TAAs). There are three TAA isoforms in *A. thaliana*. An unstable intermediate indole-3-pyruvate (IPyA) is produced from tryptophan in the reaction catalyzed by TAAs[80]. Inactivation of these enzymes leads to massive reduction of endogenous auxin levels accompanied by developmental defects[81]. The IPyA is subsequently converted into IAA by the YUC monooxygenase genes (YUC, YUCCA). There are 11 YUC genes in *A. thaliana* genome suggesting high redundancy in function or tissue-dependent regulation possibility[82,83]. Overexpression of the YUC genes led to auxin overproduction[80,84]. The YUC genes are also involved in the tryptamine (TAM) pathway where they convert tryptamine to N-hydroxytryptamine. Another plant biosynthetic pathway converts tryptophan into indole-3-acetonitrile (IAN). The pathway begins with conversion of tryptophan into indole-3-acetaldoxime (IAOx). The reaction is catalyzed by the cytochrome P450 enzymes CYP79B2 and CYP79B3. IAOx is converted to IAN which is processed by nitrilases into IAA[85].

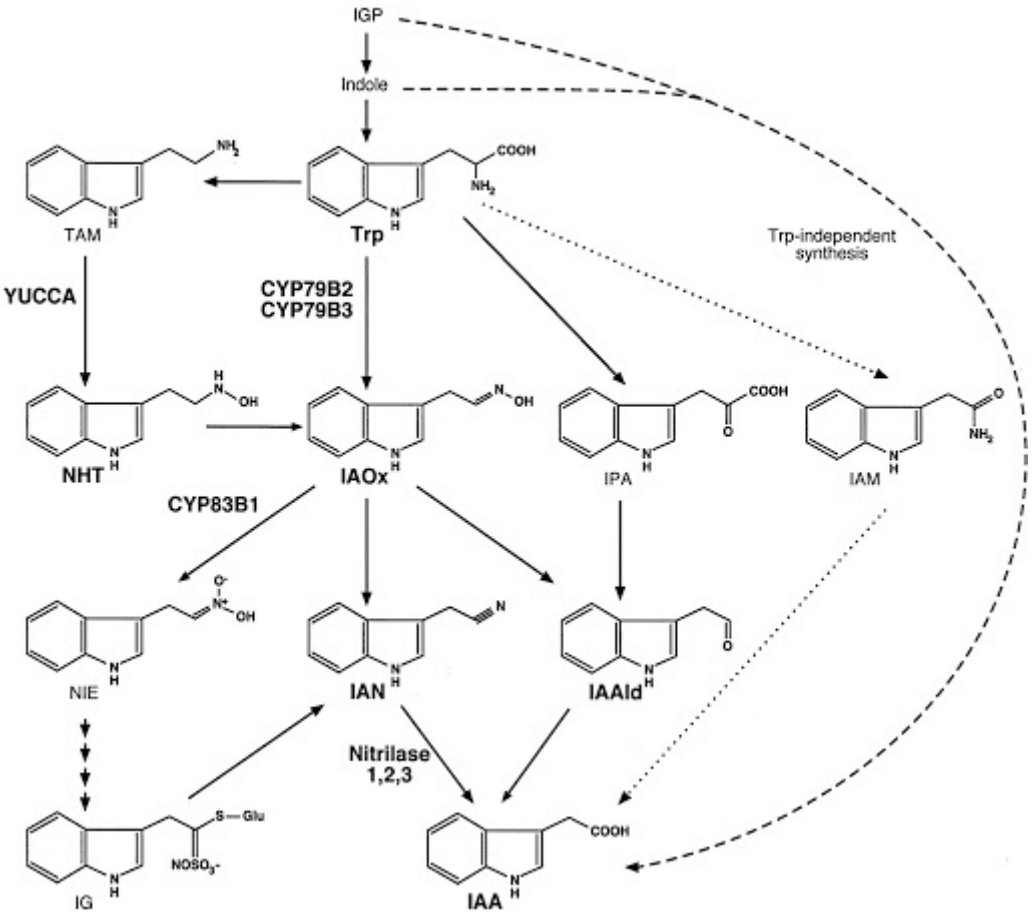


Figure 10: Auxin biosynthesis in plants. Auxin biosynthesis in plants begins with tryptophan synthesized from indole. The tryptamine pathway (TAM) begins with conversion of tryptophan by TAAs and subsequently hydroxylation by the YUCCA enzymes. The IAOx (Indole-3-acetaldoxime) pathway comprises cytochrome P450 enzymes CYP79B2/B3 producing IAOx which is converted to IAN (Indole-3-acetonitrile). The IAN is converted into IAA by nitrilases. The IPA (Indole-3-pyruvate) pathway in plants involves TAAs, YUCCAs and IADs (Indole-3-acetaldehyde dehydrogenases). IAA might be also synthesized by tryptophan independent pathways originating either from indole or indole-glycerolphosphate. Glucosinolate (IG) synthesis is derived from the IAOx pathway. NHT – N-hydroxytryptamine; NIE – nitro-2-indolyl-ethane. Modified from[85].

Auxin is transported into the cell via the AUX1 transporters. In the nucleus auxin binds to TIR1/AFB receptors and activates transcription via ubiquitination of Aux/IAA transcription repressors. Auxin also releases repressors ETT (Auxin response transcription factor 3) from ARF3 and other transcription factors such as IND. This leads to transcription reprogramming and developmental changes. Receptor complex of TIR1/AFB-Aux/IAA can also reside in cytoplasm where it regulates Ca^{2+} influx and contributes to root growth inhibition[86] (Fig. 11). The endocytosis of the PIN auxin transporters is another level of regulation of auxin signalling[87].

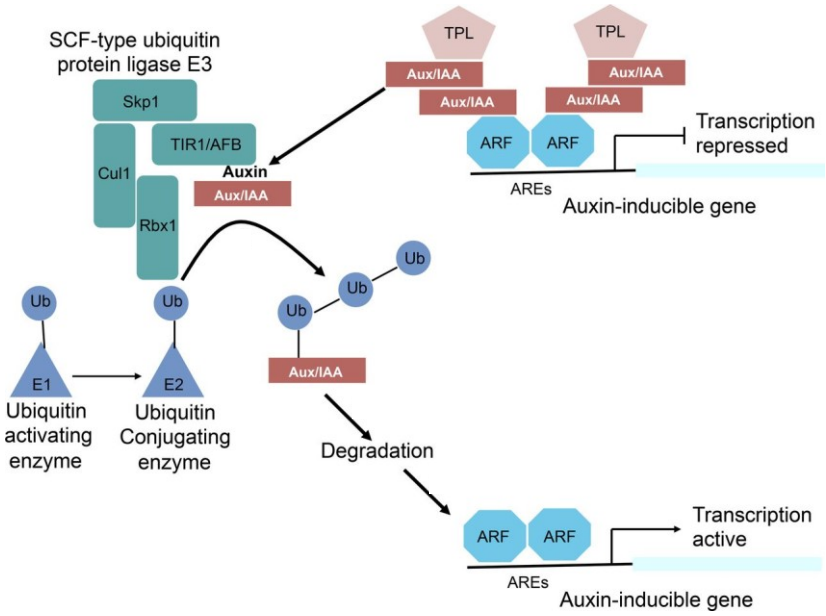


Figure 11: Auxin signal transduction. Auxin responsive elements (AREs) of auxin-responsive genes are bound to auxin responsive factors (ARFs). The Aux/IAAs transcription repressors interact with ARFs and TPLs to prevent gene transcription. Auxin facilitates the interactions between Aux/IAAs and F-box proteins of the TIR1/AFB family. These F-box proteins are part of an SCF-type E3 ubiquitin protein ligase complex that transfers activated ubiquitin (Ub) from an E1/E2 enzyme system. Polyubiquitination of the Aux/IAAs results in their degradation which releases the repression of ARFs. Modified from[88].

Pathogen infection affects auxin homeostatis and auxin-responsive gene expression. Downregulation of most auxin-responsive genes was reported upon infection with *Botrytis cinerea*[89]. Similar case was reported in cotton infected with *Fusarium oxysporum*[90]. Repression of auxin signalling leads to *A. thaliana* resistance to *P. syringae*[91]. SA treatment represses auxin signalling pathways as a part of disease resistance mechanism. Increased level of Aux/IAA was observed after SA analog treatment[92]. This may point to repression of auxin-responsive genes. JA biosynthetic genes are also downregulated upon IAA treatment[93]. Auxin responsive genes *GH3* play a role in plant defence. Overexpresison of gene *GH3.5* results in elevated SA accumulation and *PR1* expression. Mutant *gh3.5* showed compromised resistance and overexpresison of *GH3.8* enhanced resistance of rice to *Xanthomonas oryzae*[94,95].

1.1.9. Ethylene biosynthesis and signalling

Ethylene levels increase in stressed plants. The increase leads to growth inhibition and delayed flowering. Ethylene is biosynthesized from ACC (1-amino1-aminocyclopropane-1 carboxylic acid). The ACC is formed by the ACC synthase (ACS). ACC is then oxidised by ACC oxidase (ACO) (Fig.

12). Both of these enzymes occur in large families in plants and different isoforms are responsive to different kinds of internal and external stimuli[96].

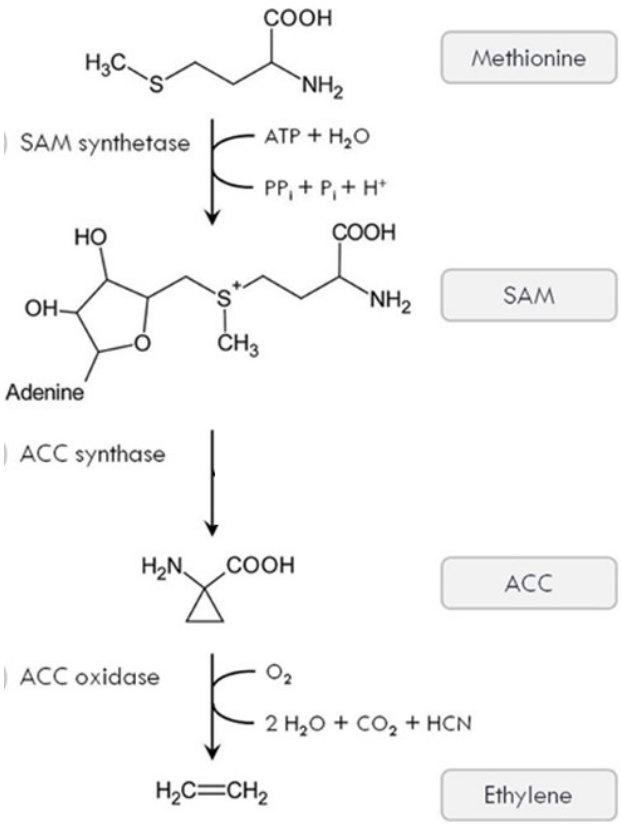


Figure 12: Ethylene biosynthesis. Methionine is converted to S-adenosyl-methionine (SAM) by SAM synthetase using ATP. SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). The reaction also releases 5′-methylthioadenosine (MTA), which is recycled back to methionine. ACC is oxidized by ACC oxidase (ACO) to produce ethylene, CO2 and HCN. Modified from[96].

The key molecule of ethylene signalling is a negative regulator CTR1 (Constitutive triple response 1). CTR1 inhibits gene expression in the absence of ethylene. Binding of ethylene to its receptor that is localized in the ER and Golgi apparatus inactivates the receptor EIN2 and CTR1[97]. When CTR1 is inhibited, EIN2 is released and acts as positive regulator of ethylene signalling. EIN3 and EIL1 (Ethylene insensitive 3-like 1) are transcription factors further downstream in the signalling pathway. They act as positive regulators of expression of the ethylene responsive transcription factors (ERFs) that results in ethyelene-mediated stress response. ERF-activity is involved in the immune signalling (Fig. 13)[98].

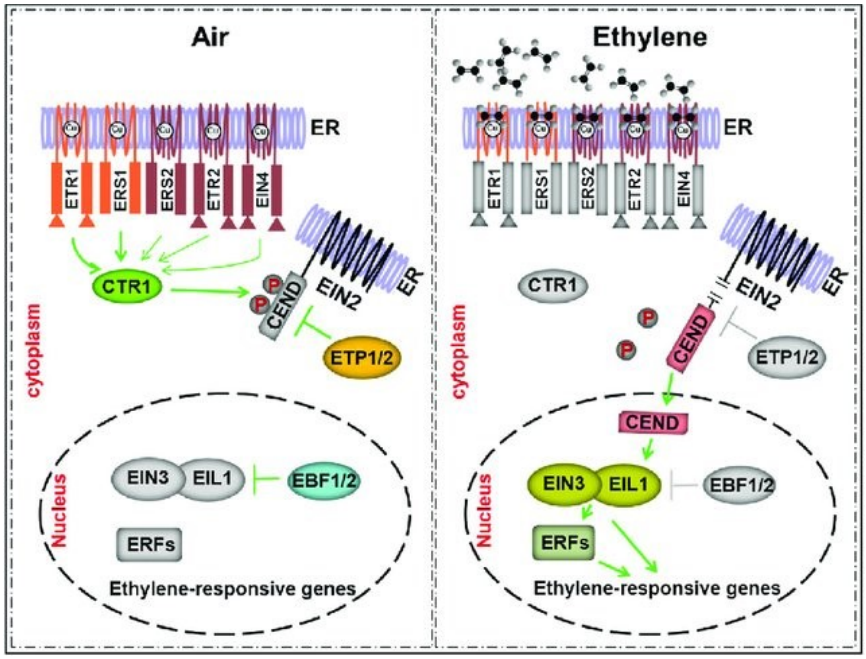


Figure 13: Ethylene signal transduction. The ethylene receptors ETR1, ETR2, ERS1, ERS2, EIN4, are located in the membrane of endoplasmic reticulum (ER). The receptors activate kinase domain of downstream signalling component CTR1 which phosphorylates the EIN2 C-terminal domain (CEND). Phosphorylated EIN2 is targeted by ETP1/2 (Ethylene insensitive 2 targeting protein 1/2) for proteosomal degradation and thus does not transduce signal. In the nucleus, the Ethylene insensitive 3 binding F-box1 (EBF1) and EBF2 target the EIN3/EIL1 transcription factors for proteasomal degradation, preventing induction of gene transcription. Ethylene binding inactivates ethylene receptor signalling. The levels of ERS1 and other ethylene receptor isoforms increase, CTR1 levels increase and protect the ETR1 receptor from proteolysis. The EIN2 is no longer phosphorylated and the CEND moves to the where it regulates proteosomal degradation of EBF1/2. Subsequently it causes stabilization and accumulation of master transcription factors EIN3/EIL1 which activate the downstream ERF1 transcription factor gene. Modified from[99].

1.1.10. Abscisic acid biosynthesis and signalling

ABA biosynthesis draws out from the mevalonate pathway in plastids[100]. Carotenoids are produced in the beginning: sequence of condensation reactions catalyzed by geranyl geranyl diphosphate synthase (GGPPS) adds one isoprene unit at a time yielding C10, C15 and C20 molecules – geranyl geranyl diphosphate (GGPP). Two GGPP molecules condense head to head producing C40 molecule phytoene. The reaction is catalyzed by phytoene synthase. Phytoene is desaturated and lycopene is formed; two enzymes are involved in this process: phytoene desaturase and carotene desaturase. Lycopene can be further processed either into α-carotene or β-carotene. Only β-carotene is further metabolized into ABA. β-carotene hydroxylases metabolize low amount of β-carotene into zeaxanthin. Zeaxanthin is

converted into violaxanthin by zeaxanthin epoxidase (ZEP). ZEP regulation is dependent on drought in roots but not in leaves in *A. thaliana*[101]. The transcript levels fluctuate diurnally but protein levels do not change massively. The ZEP transcript levels correlate with ABA concentration in seeds suggesting its regulatory role. Overexpression of ZEP led to increased tolerance to salt and drought stress[102]. Trans-neoxanthin (C40 compound) is produced and both compounds – violaxanthin and neoxanthin are isomerised to cis isomers and cleaved by 9-*cis*-epoxycarotenoid dioxygenase (NCED). This reaction yields xanthoxin (C15 compound). NCED expression is responsive to stress, developmental signals and circadian rhythms. NCEDs occur in large families with different plastid localization and expression patterns. After this reaction, xanthoxin is released from plastid to cytoplasm. Xanthoxin is subsequently oxidised to ABA via abscisic aldehyde intermediate. The reaction chain is catalyzed by short chain dehydrogenase/reductase-like (SDR1) and abscisic aldehyde oxidase (AAO)[103] (Fig. 13). The expression of ABA biosynthetic enzymes differs in different tissues. For instance, SDR1/ABA2 and AAO3 are only expressed in vascular parenchymes. NCED3 is mainly expressed in vascular parenchyma[104]. NCED3 promoter is active also in guard cells[105]. ABA is primarily synthesized in vascular tissues and transported to target tissues via both xylem and phloem. Upon drought stress apoplastic pH increases which results in increased retention of ABA. This causes reduced transpiration in leaves[105]. Local production of ABA in leaves can be also induced by changes in water potential and root drying before the transport from roots occurs[106,107].

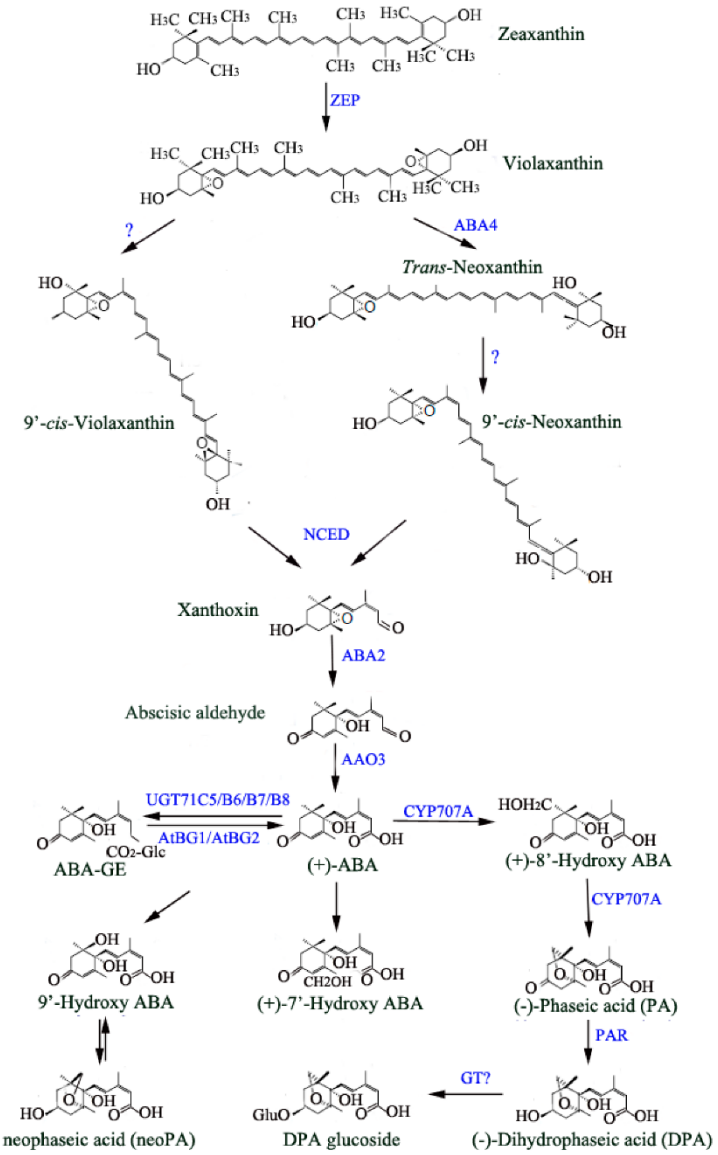


Figure 13: Abscisic acid biosynthesis. ABA de novo biosynthesis is catalyzed by a series of enzymes including zeaxanthin epoxidase (ZEP), ABA deficient 4 (ABA4), 9-cis-epoxycarotenoid dioxygenase (NCED), Xanthoxin dehydrogenase (ABA2) and Abscisic-aldehyde oxidase AAO3, while ABA degradation is mainly catalyzed by CYP707A and the products are phaseic acid (PA) and and dihydrophaseic acid (DPA). The UDP-glucosyltransferase UGT71C5/B6/B7/B8 modify ABA to ABA-glucose ester, while the glycosyl hydrolase AtBG1/2 convert ABA-glucose ester to ABA. Modified from[108].

Abscisic acid signalling pathway involves receptors described as „Pyrabactin Resistant/Pyrabactin Resistant-Like/Regulatory Component of ABA Response" (PYR/PYL/RCAR), protein phosphatases 2C and SNF1-Related protein kinase 2 (SnRK2)[109](Fig. 14). In the process of stress signalling ABA interacts with JA and SA signalling pathways. Stomatal closure upon flg22 perception involves ABA-mediated response. At the same time SA-defence pathway is triggered. Bacterial pathogen *P. syringae*

further on is able to secrete structural analogue of JA – coronatine – that causes stomatal reopening. Due to its effects coronatine can be denoted as an effector. It stimulates JA biosyntesis via Jai1 (Jasmonate insensitive 1) which downregulates the expression of SA-related *PR1* gene and induces expression of the MYC2 transcription factors related to wounding[110]. Protein NOG1-2 stimulates stomatal closure during abiotic stress. *N. bethamiana nog1-2* mutant is more susceptible to bacterial infection since it is unable to close the stomata.

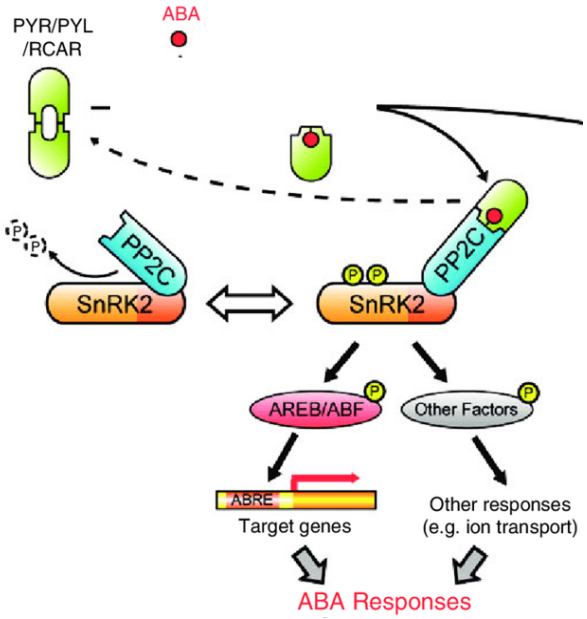


Figure 14: Abscisic acid signal transduction. PYR/PYL/RCAR, PP2C and SnRK2 form a complex. In unstressed plant, PP2C negatively regulates SnRK2 by direct interactions and dephosphorylation of of SnRK2. ABA binds PYR/PYL/RCAR and interacts with PP2C to inhibit its phosphatase activity. Subsequently SnRK2 is released from PP2C-dependent regulation and phosphorylates transcription factors AREB/ABF or membrane proteins involving ion channels. Modified from[111].

The expression of plant defensin *PDF1.2* gene, that is a typical marker of wounding and induction of the JA pathway, is upregulated by NOG1-2 in the presence of ABA, thereby it is pressumed that ABA signalling closely interacts mainly with the JA signalling pathway[110].

1.1.11. Brassinosteroid biosynthesis and signalling

Brassinosteroids (BRS) are steroidal hormones and play essential role in plant growth. At least 69 different molecules with typical BRS structure and activity have been identified up to date in various plant and algal species. The most biologically active compounds are brassinolide and castasterone [112]. Brassinosteroid biosynthetic pathways originate from triterpenoid molecules. Different plant sterols

such as cholesterol, campesterol and sitosterol can be processed as precurosrs for 28-norbrassinolide, brassinolide and 28-homobrassinolide. First, squalene is cyclized into cycloartenol (Fig. 15)[113], then BRS biosynthesis is catalyzed by various members of the cytochrome P450 group. The main pathways are so-called early C-6 and late C-6 oxidation pathways, however different modifications and inter-pathway interactions are possible due to the activity of DWF4 enzyme[113].

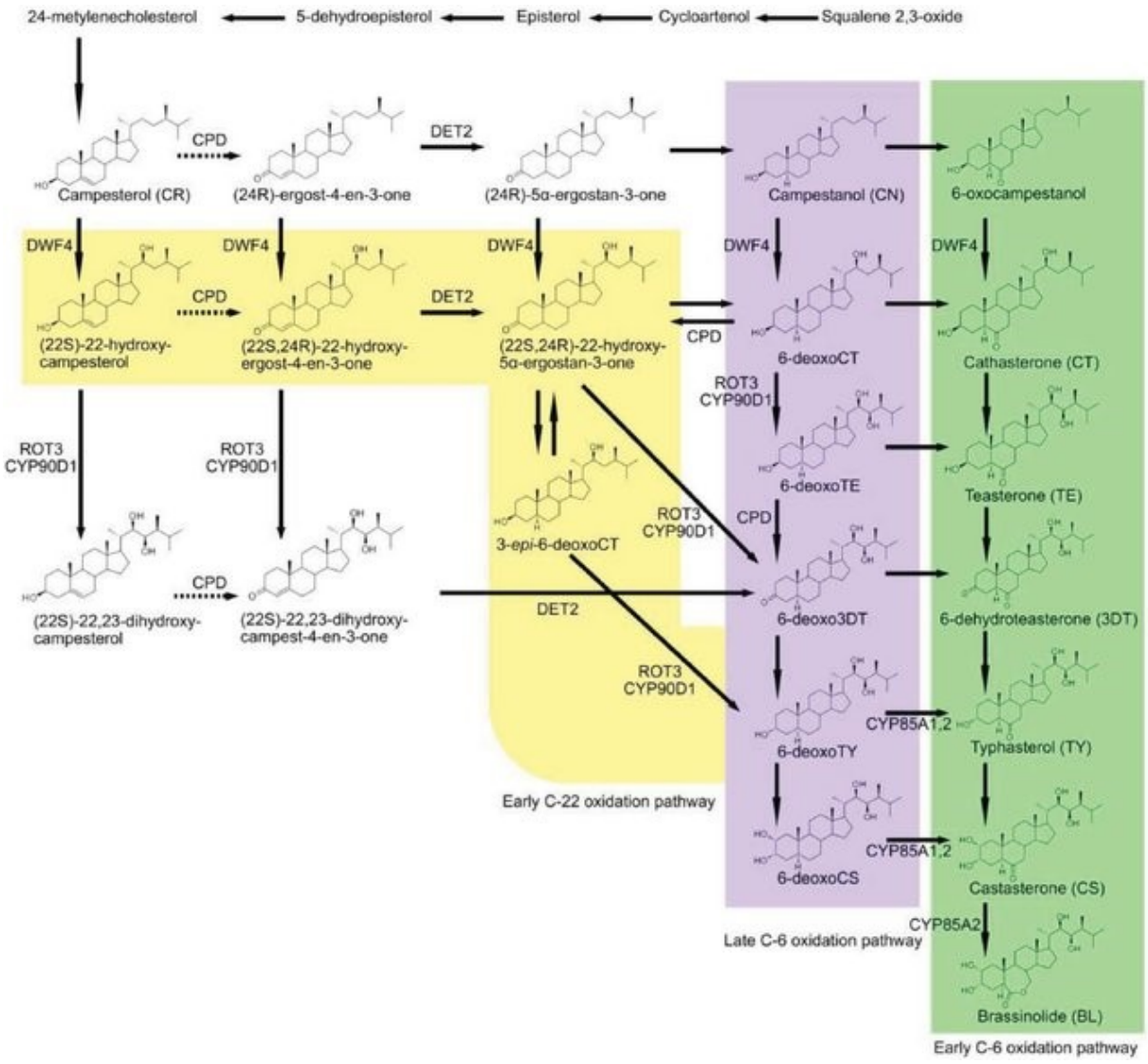


Figure 15: Brassinosteroid biosynthesis The three major biosynthetic pathways starting from campesterol are shown. The early C-22 oxidation pathway (yellow) hydroxylates C-22 by DWF4 and is campestanol-independent. There are two campestanol (CN)-dependent pathways: the early and late C-6 oxidation routes. The early C-6 oxidation route (green) undergoes C-6 oxidation ahead of C-22 oxidation. The late C-6 oxidation pathway hydroxylates C-22 ahead of C-6 oxidation (purple)[113].

Brassinosteroid signalling pathway in *A. thaliana* involves membrane receptor BRI1 (Brassinosteroid insensitive 1) that directly binds BRS. BRI1 is a LRR-RLK protein[114]. A molecule of brassinosteroid binds to the extracellular domain of BRI1 that triggers formation of receptor complex BRI1/BAK1 (Brassinosteroid insensitive 1-associated receptor kinase 1, also known as SERK3). This triggers downstream phosphorylation cascade[115] that results in activation of transcription factors BZR1 (Brassinazole resistant 1) and BES1 (BRI1-EMS-suppressor 1)[116]. The BZR1/BES1 are phosphorylated and inactivated by BIN2 kinase (Brassinosteroid insensitive 2) in absence of a BRS molecule. There are also homologues of the main functional BRI1 receptor named BRL1, BRL2 and BRL3 (BRI1-like 1,2 and 3). BRL1 and BRL3 can bind steroid molecules, BRL2 does not seem to have such activity[117]. Unlike BRI1 which is expressed ubiquitiously in roots, the BRLs are expressed only in some specific tissues. BRL1 and BRL3 were found in vascular stem cells[117,118,119]. Both BRL1 and BRL3 can form a complex with BAK1[118](Fig. 16). This suggests that BRI1 and BRLs are able to form different receptor complex in order to maintain specific downstream signalling cascades, that in case of the BRLs remain largely unknown[120].

The role of BRs in plant defence is less well understood, however there is increasing evidence of BRs signalling being important for plant immunity. A positive but variable effect of exogenous BL application on tobacco and rice on resistence against distinct leaf pathogens was observed[121]. BR application also protects barley from several *Fusarium* species. In contrast to these studies, there is evidence of negative regulation of disease progress in plants. Exogenous BL did not alter resistance of *A. thaliana* against *P. syringae* or *A. brassicicola*[122]. BL pretreatment resulted in susceptibility of rice to root pathogens *Pythium graminicola* and *Meloidogyne graminicola*[123,124]. It has been also suggested that *P. graminicola* hijacks plant BR signalling to promote infection[123]. BRs signalling pathways seem to work as an innate regulator of the trade-off [125].

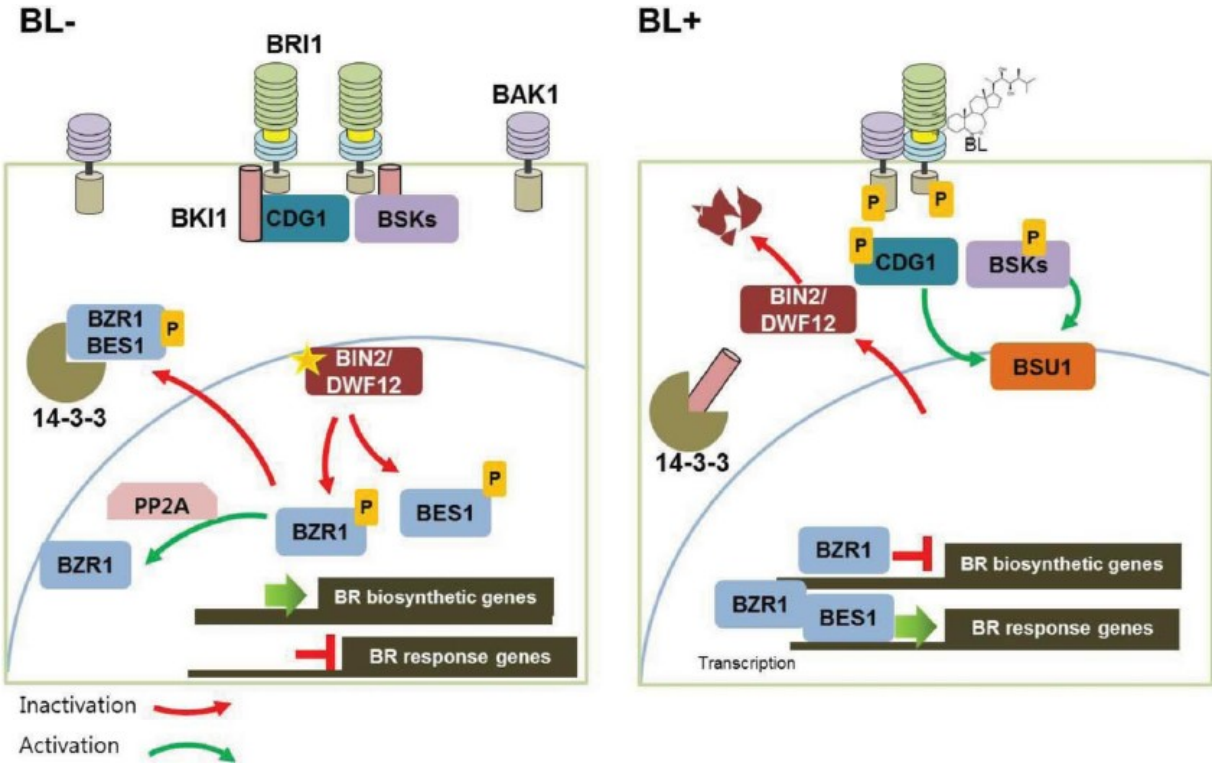


Figure 16: Brassinosteroid signal transduction. In the absence of brassinosteroids BKI1 (BRI1 kinase inhibitor 1) associated with serine/threonine protein kinases CDG1 and BSKs interacts with BRI1 (Brassinosteroid insensitive 1) preventing it from forming a complex with BAK1 (BRI1-associated receptor kinase 1). Transcription factors BZR1 (Brassinazole resistant 1) and BES1 (BRI1-EMS-suppressor 1) are phosphorylated by BIN2 (Brassinosteroid insensitive 2) kinase and expelled from the nucleus. Brassinosteroid molecule binds to BRI1 receptor kinase which leads to auto- and trans-phosphorylation of BKI1 and BAK1. BKI1 dissociates from BRI1, which forms dimeric complexes with BAK1. The heterodimeric complex transduce signal via CDG1 and BSKs. CDG1 and BSKs activate protein phosphatase BSU1 which inhibits BIN2. Transcription factors BZR1 and BES are no longer inhibited by BIN2 and regulate gene transcription. Modified from[113].

1.1.12. Gibberellin biosynthesis and signalling

Gibberellins (GAs) were originally identified in fungus *Gibberella fujikuroi* and later on even in many plant and bacterial species[125]. GA biosynthesis begins with geranyl geranyl diphosphate (GGDP) that is synthesized from isopentenyl diphosphate[126]. Isoprenoid molecules are synthesized either via the mevalonate pathway or themethyl erythritol (MEP) pathway. GGDP is cyclized via ent-copalyl diphosphate intermediate. Resulting coumpound, ent-kaurene, is then oxidised at C-19 to form kaurenoic acid. This compound is further oxidised to the ent-7α-hydroxykaurenoic acid. Final oxidation yields GA12-aldehyde. This pathway in plants involves ent-copalyl-diphosphate-synthase

(CPS) and ent-kaurene synthase (KS)[127]. GA12-aldehyde is converted to GA12, which is hydroxylated on C-13, forming GA53. Both the compounds are substrates for oxidation on C20. Resulting compounds are GA9 and GA20[128]. The final step to generate bioactive GAs in plants is hydroxylation at 3b position[129] (Fig. 17).

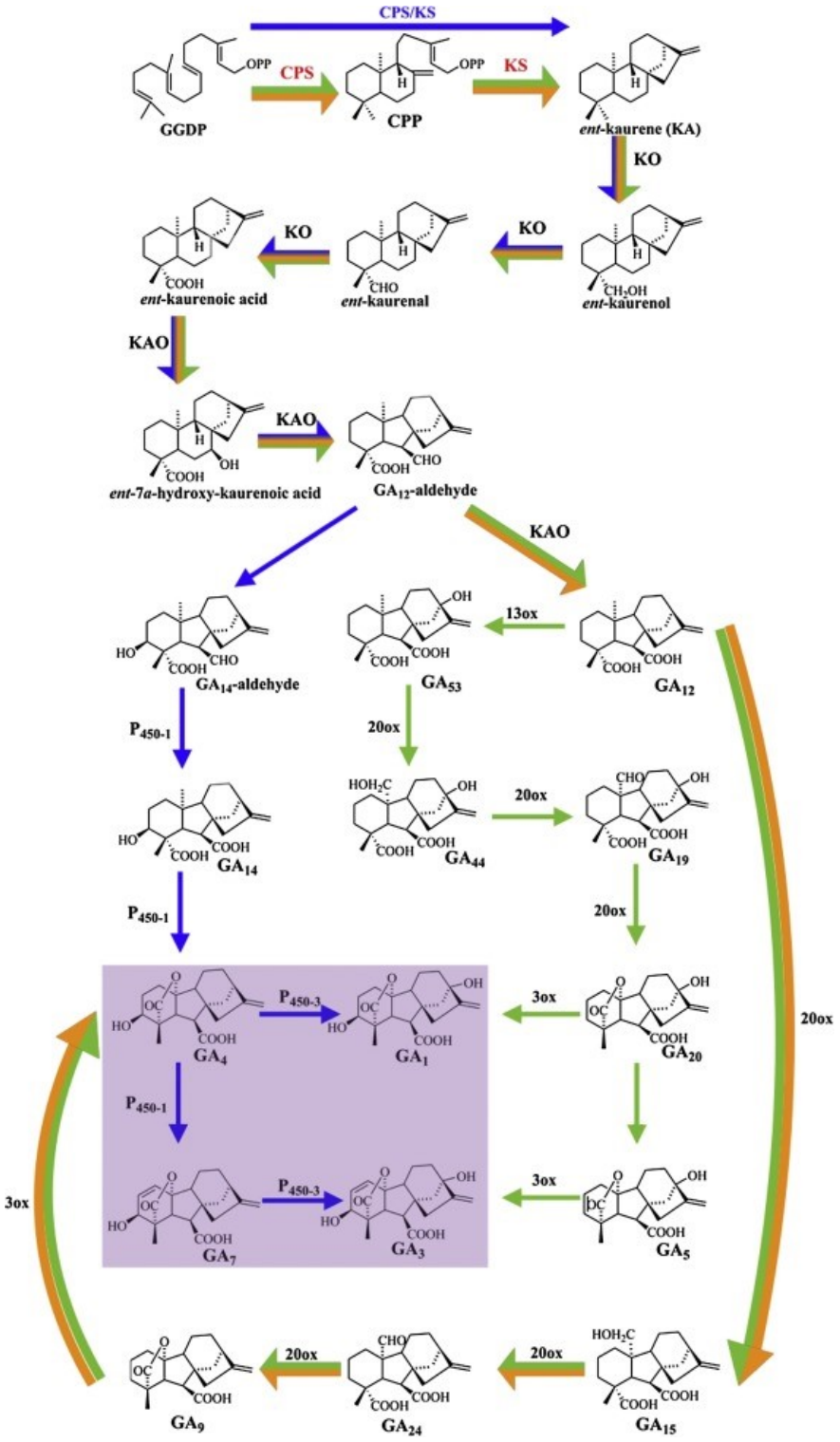


Figure 17: Gibberellin biosynthesis. Biosynthetic pathways described in fungi (blue), plants (green) and bacteria (yellow). Bioactive GAs are shown in a purple box. GGDP – geranylgeranyl diphosphate, CPS – ent-copalyl diphosphate synthase, CPP – ent-copalyl diphosphate, KS – ent-kaurene synthase, CPS/KS – bifunctional terpene cyclase, KA – ent-kaurene, KO – ent-kaurene oxidase, KAO – ent-kaurenoic acid oxidase, 13ox - 13 oxidase, 20ox – 20-oxoglutarate-dependent dioxygenase, 3ox – 3 oxidase, P450-1 – cytochrome P450 monooxygenase 1, P450-3 – cytochrome P450 monooxygenase 3. Modified from[129].

GAs stimulate plant growth and development by causing degradation of DELLA stranscription factors. The pathway was first described in rice. GA receptor GID1 (GA insensitive dwarf 1) binds GAs and its N-terminal lid domain interacts with a specific DELLA protein, SLENDER RICE 1 (SLR1) [130,131]. The formed complex is polyubiquitinylated by GID2 (GA insensitive dwarf 2) and degraded[132]. *A. thaliana* posseses similar pathway: there are three receptors GID1a, GID1b and GID1c, five DELLA proteins RGA, GAI, RGL1, RGL2, and RGL3 and F-box protein SLY1[133] (Fig. 18).

The evidence of pathogens interfering with plant GA signalling was first reported in 2005. Rice dwarf virus capsid protein P2 was shown to interact with ent-kaurene oxidases which resulted in decreased GA production[134]. *A. thaliana* knock-out in four of the five DELLA proteins showed high induction of SA-dependent defence mechanisms upon haemibiotrophic pathogen attack, decreased JA-dependent marker gene *PDF1.2* and increased susceptibility to necrotrophic fungus *A. brassicicola* [135]. These findings suggest that GA signalling rather promotes JA-related defence and suppresses SA-pathway. Similar case was reported in wheat. Exogenous GA treatment of rice lowered resistance to haemibiotrophs *M. oryzae* and *X. oryzae* [136,137]. Rice plants with deactivated GA accumulation also showed decreased level of SA and enhanced resistance to *M. oryzae* and *X. oryaze*[136], suggesting that compromised GA signalling affects resistance to biotrophs in rice[137]. Defence against necrotrophic pathogens can be increased by GAs in rice also[123]. Collectively the up to date literature shows that GA signalling contributes to JA-mediated defence and rather represses SA-mediated defence. DELLA proteins have been reported to affect immunity via controlling cytoskeleton dynamics. DELLAs physically interact with prefoldin complex, co-chaperone required for tubulin folding. In the absence of GA DELLAs compromise tubulin heterodimer availabitity[137,125].

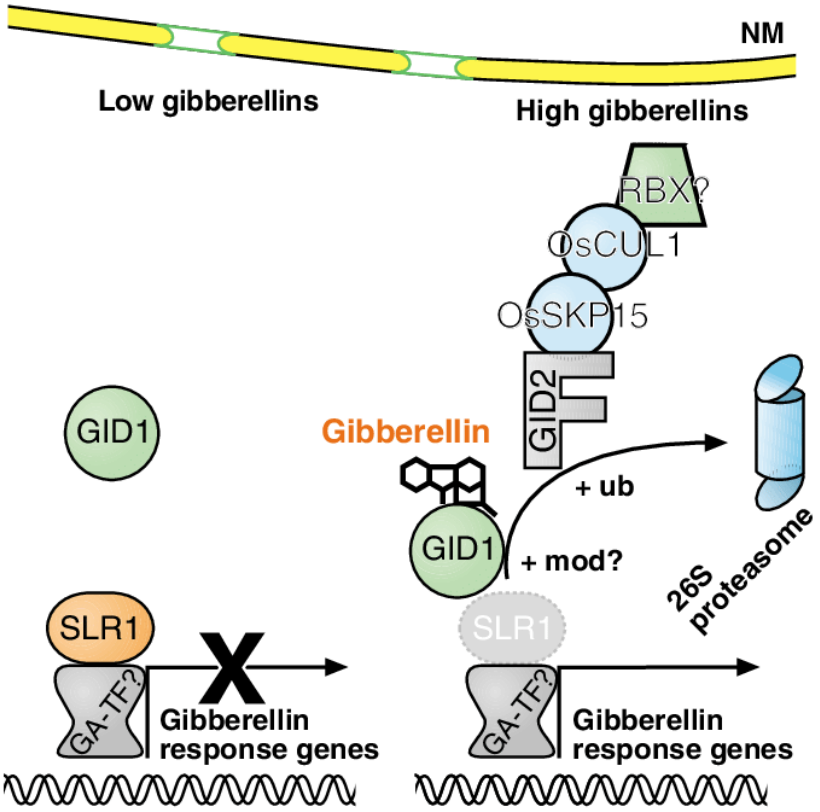


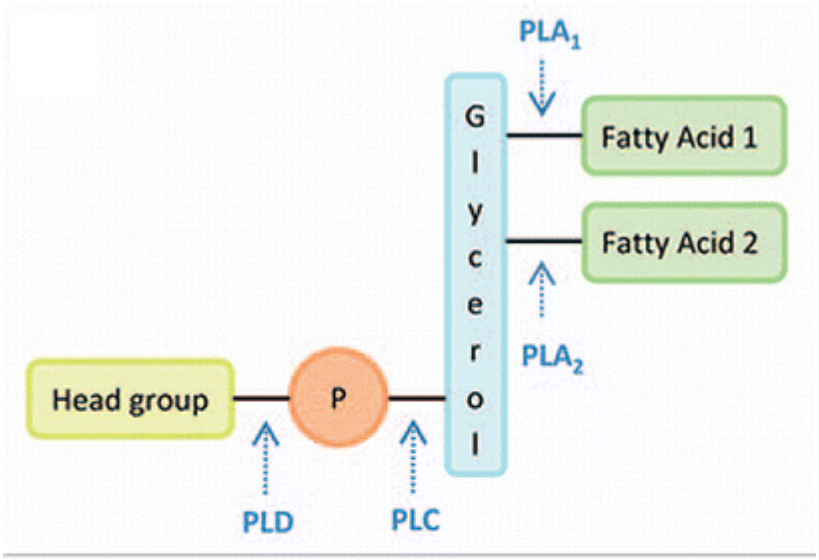
Figure 18: Gibberellin signalling pathway. In absence of gibberellin SLR1 (Slender rice 1) inhibits gene transcription. Gibberellin binds to the GID1 (GA insensitive dwarf 1) protein that interacts with SLR1. The SCF forms complex with GID2 (GA insensitive dwarf 2)to ubiquitinylate SLR1 which is subsequently degraded by proteasome and gene transcription is allowed. Modified from[138].

1.2. Phospholipid signalling in plant immunity

Phospholipids are found in cell membranes and at the same time they serve as a source of signal molecules. Their concentration is regulated by lipidkinases and phospholipases which are activated upon certain stimuli. Phospholipid molecules consist of central molecule of glycerol with two fatty acid chains and a phosphate group with attached polar group. The polar group can be formed from choline, inositol, serin and others (Fig. 19). Phospholipids are often localized with membrane receptors. Receptor activation induces enzyme that cleaves the signal molecule from the phospholipid[139].

1.2.1. Phospholipases

Phospholipases are enzymes that hydrolyze membrane phospholipids and generate molecules that are used to transduce stress signal. These molecules include phosphatidic acid, oxylipins, jasmonates or lysophospholipids[140].



Head group	Phospholipid
- Inositol	- PI
- Inositolmonophosphate	- PIP
- Inositolbisphosphate	- PIP2
- Choline	- PC
- Ethanolamine	- PE
- OH	- PA

Figure 19: Cleavage sites of different phospholipases. Modified from[140].

1.2.2. Phospholipase D

Phospholipase D (PLD) hydrolyses membrane phospholipids into phosphatidic acid (PA) and a headgroup of several types. In plants the PLDs occur in large families[141]. PLDs are mainly membrane associated enzymes and require Ca^{2+} for their activity. Upon stress condition they are allocated to membranes in concentration-dependent manner and activated[142]. PLDs are considered to be the main players in PA signalling along with PLC/DGKs[143].

1.2.2. Phospholipase C

1.2.2.1. Phosphoinositie-specific phospholipase C

Phosphoinositie-specific phospholipase C (PI-PLC) hydrolyses phosphoinositides such as phosphatidylinositol-4-phosphate (PI4P) or phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) producing diacylglycerol (DAG) and phosphorylated myo-inositol. DAG si subsequently phosphorylated to PA and contributes to PA signalling[144]. The changes in the phosphoinositide levels may have a signalling function per se[145].

1.2.2.2. Non-specific phospholipase C

Non-specific phospholipase C (NPC) hydrolyzes several phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylglycerol (PG). Products of the reaction are DAG and certain phoshorylaclohol. The NPCs are not structurally related to PI-PLCs and probably do not significantly contribute to plant signalling apart from the DAG production[146].

1.2.3. Phospholipase A

Phospholipases A (PLAs) cleave many membrane phospholipids producing free fatty acid chain and and a lysophospholipid. The products can be metabolically active[147]. The PLAs are divided into two groups depending on the cleavage site of the fatty acid chain – PLA_1 and PLA_2. The function of PLA_2 has been observed in terms of plant defence and reaction to auxin. Activity of PLA_2 can be induced by yeast elicitors accompanied by other defence reactions such as phytoalexin biosynthesis and lysophosphatidylcholine productin. The lysophosphatidylcholine production further leads to signal transduction via pH changes[148]. No important function in signalling has been reported for PLA_1 up to date[149].

1.2.4. Diacylglycerol kinase

Diacylglycerolkinases (DGKs) catalyze phosphorylation of DAG producing PA. DGKs play a key role in regulation of plant cell processes[144] such as cold stress signal transduction[150]. Inhibition of DGK activity resulted in growth retardation[151] and defence responses[152].

1.2.5. PA kinase

PA can be phosphorylated into GDPP by a PA kinase, an enzyme that has not been thoroughly characterized yet. GDPP plays a role in signal transduction during abiotic stress mediated by ABA[139,153].

1.2.6. PI- and PIP- kinases

The most important enzymes of this class are PI4-kinases and PIP5-kinases producing PI4P and PI(4,5)P2. They participate in many plant signalling events including stress signalling[145]. Plant PI4-

kinases are classified into type IIIa, type III band type II. Particular role of PI4Kβs in plant immunity connceted with SA and cytoskeleton would be discussed further in this thesis[154].

1.2.7. Lipid phosphatases

Plant lipid phosphatases act together with kinases to maintain equilibrium. Suppressor of actin (SAC) phosphoinositide phosphatases were shown to play a role in trafficking and vacuolar development. Lipid phopshate phosphatase dephophorylates PA which leads to signal attenuation. This mechanism is important in drought stress signalling[155].

1.3. Phospholipid signalling in biotic stress

Plant phospholipases play essential role in immunity, particularly PTI. PA levels increase fast after treatment with chitosan elicitor which implicates participation of PLDs, PLCs and DGKs[156]. Accumulation of lysophosphatidylcholine after elicitation with yeast glycoprotein suggests PLA_2 involvement[148]. Other elicitors were shown to inhibit the activity of NPC[147]. PA accumulates in *A. thaliana* upon effector AvrRpm1 and AvrRpt2 sensing[157]. PLD-mediated accumulation of PA occurs after wounding suggesting its role in herbivore attack signalling[158]. Silencing of PLC4 in tomato weakened HR upon Avr4 recognition. Silencing of PLC6 did not show similar effect, but was required for signalling mediated by Ve1 and Pto/Prf proteins[159].

Quantitative proteomics experiments revealed that during immune reactions certain members of lipid signalling are induced: DGK5, PLDα1, PLDγ1, PLP2α/pPLA-IIa and SOBER1[160]. PA seems to act as an active signalling molecule in the immune response. Exogenous PA or DAG treatment triggers ROS production and expression of defence-related genes[161],[162]. PA induces phytoalexin accumulation in tobacco[163]. Phospholipases were also described to participate during the onset of immunity. PLDγ accumulates near the infection sites of the powdery mildew fungus[164]. PLDγ-defficient mutants show lower resistence to fungal spore penetration. Mutants in *pldγ* mutants showed lower expression of chitin-inducible defence genes[164]. Silencing of *PLP1* gene in pepper lowered resistence to *Xanthomonas campestris*. Expression of this gene in *A. thaliana* enhanced resistance, induced ROS burst and expression of certain defence genes[165]. Other phospholipase isoforms seem to have different functions: Suppression of PLDβ1 in rice activated defence-like reactions like ROS and phytoalexin accumulation and defence-gene expression. This suggest PLDβ1 constitutively represses plant defence[166]. Stress hormone signalling is also tightly connected with phospholipid signalling. PLDβ1 enhances JA-mediated defence response to *B. cinerea*[167] while is supresses SA-mediated defence against *P. syringae*.

Phospholipase D-derived PA promotes root hair development under phosphorus deficiency by suppressing vacuolar degradation of auxin transporter PIN2[168].

1.4. Role of salicylic acid in phospholipid signalling

SA stress signalling is tightly connected with various aspects of lipid signalling. SA treatment leads to increased concentration of phosphoinositides and decrease in PA. SA also inhibits activity of PI-PLC[146,169]. *A. thaliana* plants treated with *n*-butanol, a specific modulator of PLD activity, showed supressed transcription of SA pathway marker genes (*PR1* and *WRKY 38*). The *n*-butanol treatment also resulted in decreased nuclear localization of NPR1[170]. SA activated PLD 45 min after incubation with *A. thaliana* cells[171]. This event happens upstream of *PR1* induction since diversion of PLD activity with primary alcohol inhibited expression of *PR1*[171]. PI-PLC is negatively regulated by SA[172].

Expression of AtPLDβ1 was supresed by SA and knock-out of *atpldβ1* gene resulted in increased SA and ROS and enhanced resistence to *P. syringae*[173]. PA induces ROS production via activation of NADPH oxidase RBOHD[174], ROS induction can lead to SA concentration induction[175]. PI4Kβ1 and β2 and the product of their catalytic activity PI4P negatively regulate SA signalling via modulating homeostasis of FLS2[176]. Mutant in *pi4kβ1β2* showed induced SA accumulation, ROS production, constitutive expression of *PR1*, dwarf phenotype and enhanced resistence to *P. syringae*[177]. PI4P can also indirectly act as negative regulator of SA signalling. PI4P can be converted into PI(4,5)P2 that activated PLDβ, a negative regulator of SA pathway[173].

1.5. Role of actin cytoskeleton in plant immunity

Plant cytoskeleton is an essential component for maintaining plant cell life processes including stress responses. It is comprised of two main structures – actin filaments and microtubules. This thesis is focused on the role of actin filaments during immune responses.

The actin cytoskeleton is formed by polymerization of globular (G) actin into filamentous (F) actin. This polymerization required a large number of different cofactors reffered to as actin-binding proteins[178]. Generally the cellular function of actin is to control organelle movement, cell division, trafficking and endocytosis and thus tight regulation of very rapid actin dynamics and reorganization is vital[179]. There are two main roles of plant actin cytoskeleton during immune responses: a) maintainig of correct signalling events; b) cellular trafficking.

As far as the signalling is concerned there is evidence of several processes that depend on actin. Disruption of actin leads to enhanced ROS burst which is a result of activation of the FLS2 receptor by its flagellin derived ligand flg22[180]. During both PTI and ETI cytoplasmic receptor turnover needs to be precisely tuned to maintain correct intensity of defence reactions. This process is regulated by clathrin-dependent endocytosis which relies on functional actin[181]. Clathrin-dependent endocytosis is required also for other important signalling receptors such as EFR or PEPR1/2[182,183].

As for the trafficking role plant immunity involves synthesis and transport of various defence molecules. For instance flg22 treatment induces transcription of FLS2, EFR, BAK1 and RBOHD[184]. These components need to be translocated to their sites of action at the plasma membrane which requires actin cytoskeleton. Another example is the deposition of callose at the cell wall upon pathogen attack. Callose is a polysaccharide synthesized by callose synthases which need to be transported from Golgi apparatus to the pathogen-attacked site. This transport event again involves the cytoskeleton. Disruption of actin cytoskeleton leads to dysfunction of the callose synthases[185]. However in our laboratory we have observed contradictive results showing enhanced callose deposition in *A. thaliana* seedlings after pharmacological actin disruption[154]. This work would be later discussed in this thesis. The deposition of callose is a stress marker which suggest that plant reacts to disrupted actin dynamics. Another example is relocalization of organelles such as ER and Golgi apparatus to the site of infection by a biotrophic pathogen *H. arabidopsidis* simultaneously with rapid remodelling of actin[186,187]. Plant cytoskeleton does not only react to the presence of a pathogen but presence of MAMPs was shown to be sufficient to elicit similar alterations in actin architecture[188,189].

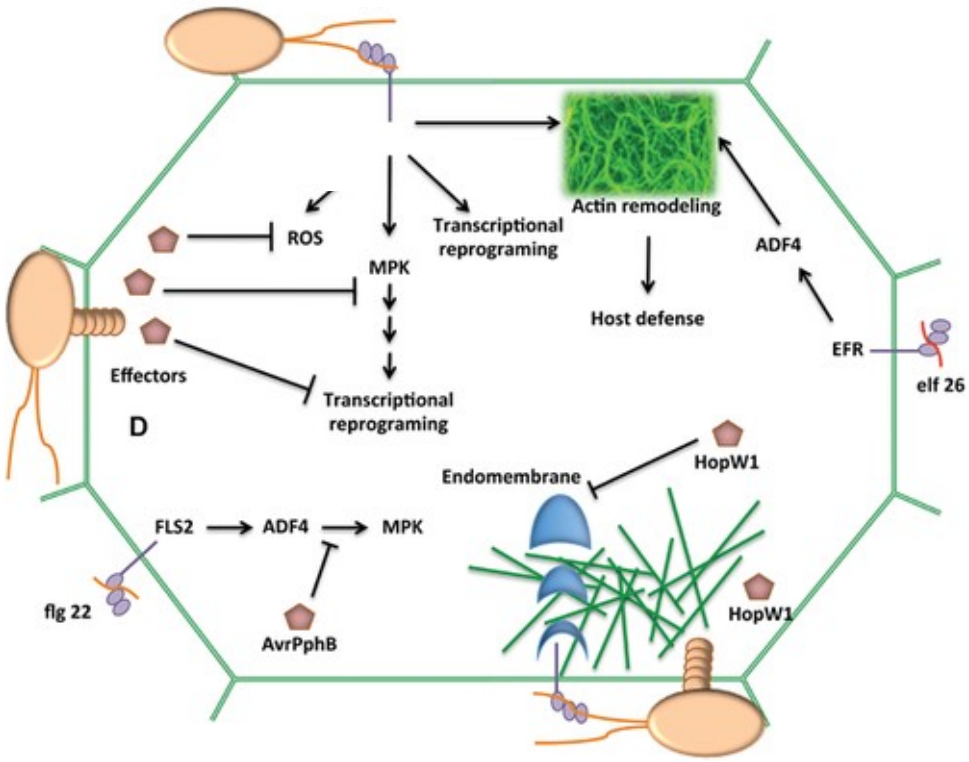


Figure 20: Targeting of actin cytoskeleton by plant pathogens. PAMPs recognition leads to several cellular signalling events including actin remodelling. The ADF4 protein is involved in actin remodelling associated with PTI response upon stimulation with elf26 recognized by the EFR receptor and MPK activation induec by flg22 recognized by the FLS2 receptor. The bacterial effector HopW1 targets actin and alters the endomembrane trafficking associated with resistance through the actions of both actin and myosin. Modified from[190].

Pathogens have also evolved more sophisticated means to target actin cytoskeleton in order to supress plant immunity (Fig. 20). Several proteins targeting actin were identified in *P. syringae*: type III secretion system helper protein HrpZ causes remodeling of actin and microtubules[191]. *P. syrinage* effector *HopG1* induces cytoskeletal reorganization and infection-associated chlorosis. The chlorosis induction can be partially reduced by co-infiltration with actin stabilizing cytoskeletal drug jasplakinolide, while Cytochalasin D treatment led to more severe chlorotic symptoms. Infiltration of either of the drugs only did not cause such effect. The *HopG1* gene forms a complex with mitochondria localized kinesin motor protein and thus indirectly interacts with actin filaments[192]. Another *P. syringae* effector, HopW1 directly disrupts actin cytoskeleton and compromises cellular trafficking. HopW1 interacts with most of 8 isoforms of plant actin, including ACT7 that is the only isoform regulated by phytohormones and environmental factors. Dynamic changes of ACT7 may be involved in plant immunity and related hormone signalling[193,194].

There is also evidence of actin targeting effectors produced by animal pathogens, for example VipA from *Legionella* or YopE from *Yersinia*. These effectors disrupt intracellular trafficking to avoid immune response and phagocytosis. The mechanisms of action of these effectors may vary: VipA is an actin nucleator, YopE has Rho GAP activity that disrupts actin filaments[195,196].

Other group of plant proteins involved in immune response connected with cytoskeleton are the actin depolymerizing factors important for actin filament turnover. Several of those proteins play a role in immunity. The *adf4* (Actin Depolymerizing Factor 4) *A. thaliana* knock-out mutant has reduced resistance to *P. syringae* expressing the AvrPphB effector. This effector is recognized by the RSP5 receptor. The expression of RSP5 is mediated by ADF4. The actin cytoskeleton of uninfected *adf4* mutant however is similar to WT[197]. Another study mentioned ADF from wheat contributing to plant resistance. The expression of TaADF3 was induced by ABA, drought, cold and virulent *Puccinia striiformis*. Gene silencing caused decrease in haustoria formation and penetration rate in both virulent and avirulent *P. striiformis*[198]. Actin filament distribution in epidermal cells was compromised in TaADF3-knockdown which possibly contributes to attenuating fungal penetration. Thus, these findings suggest that TaADF3 positively regulates wheat tolerance to abiotic stresses and negatively regulates wheat resistance to *P. syringae* in an ROS-dependent manner, possibly underlying the mechanism of impeding fungal penetration dependent on the actin architecture dynamics[199]. Contradictive evidence of the role of actin depolymerizing factors was documented also in wheat. Virus-induced gene silencing of *TaADF3* led to enhanced resistence to avirulent *P. striiformis*, reduced ROS burst and hypersensitive response.

Proteins involved in actin assembly were reported to play a role in immunity. Profilin binds some variants of membrane phospholipids (phosphatidylinositol (4,5)-bisphosphate and inositol trisphosphate). The function of this interaction is the sequestration of profilin in an "inactive" form, from where it can be released by action of the phospholipase C enzyme. Arabidopsis profilin isoform AtPRF3 is involed in actin assembly. Upon treatment with elicitor flg22 root growth of *prf3* mutant was more reduced that in WT (intact *prf3* mutant grows longer roots than WT). ROS burst was more pronounced in prf3 mutants after flg22 or elf26 treatment. Similar results were obtained when using elf26. Transcription level of *AtPRF3* in WT reduced 2,5 times upon flg22 treatment while other isoforms *AtPRF1* and *AtPRF2* showed slight increase. Simultaneous treatment with flg22 and Latrunculin B also enhanced ROS burst in *prf3* mutants. The *prf3* mutants were less resistant to *P. syringae* infection while mutants in other isoform, *prf1*, showed resistance. Opposite functions of *AtPRF3* and other *AtPRF* isoform seem to provide senstitive regulation of actin assembly in processes of plant defence[180].

Actin mediated transport of larger structures was also described to be involved in immune response. Stromules are tubular extensions from chloroplasts with proposed function during innate immunity. Actin filaments provide anchor points for stromules and the stromules establish conncetion between chloroplasts and nucleus during infection[200] Upon stimulation with plant virus, larger chloroplast movement around nucleus was observed suggesting another important role of actin cytoskeleton during immunity[201] Protein FORMIN4 contributes to local cytoskeletal dynamics in the process of forming the cell wall appositions. Cell wall appositions (CWAS) are structures such as papillae that form first line of plant defence. These complex structures are formed by callose, proteins, various phenolic compounds and inorganic compounds, especially opal silica and, at least transiently, ROS[202]. They are formed to arrest microbial invasion through the local inversion of plant cell growth. Actin–mediated transport of the structural components is essential for this formation. Deactivation of FORMIN4 partially alters subsequent defence and F-actin distribution at mature CWAS. FORMIN4 works as a spatial feedback element in cytoskeletal response when CWAS are formed[203].

Various cytoskeletal drugs are often used to manipulate the plant cytoskeleton. These commonly used drugs inlude latrunculins, cytochalasins, oryzaline or jasplakinolide (Fig. 21). Several studies report effects on plant immunity upon treatment with such compounds. Cytochalasin E increased the penetration of *A. thaliana* plants by *Colletotrichum* species[204] and the rate of entry to barley by *Blumeria graminis* f. sp. *hordei*[205]. Non-host resistance to *Erysiphe pisi* decreased after treatment with cytochalasins in barley, wheat, cucumber and tobacco[206] as did resistance to *B. graminis* f. sp. *tritici* after cytochalasin E treatment of *A. thaliana*. Cytochalasin E and latrunculin B induce transcription of SA-related defence gene *PR1 in A. thaliana* and *N. benthamiana*[207]. Latrunculin B contributes to flg22-induced ROS production[184]. Actin remodelling occurs after elicitation with different MAMPs (chitin, flg22) and DAMPs (Pep1, oligogalacturonides). Another MAMP elf26 did not induce such response. The MAMP-induced remodelling requires ROS generated by RBOHD, defence-associated NADPH oxidase. Perception of flg22 by FLS2 triggers actin remodelling via the activation of RBOHD ROS production. Treatment with latrunculin B only did not trigger ROS production. Latrunculin B treatment of flg22-induced capping protein mutants did not elevate the ROS burst as in WT[184].

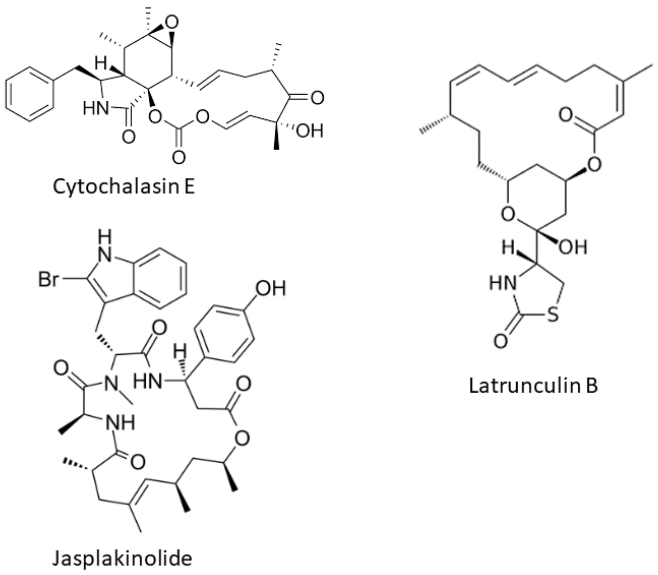


Figure 21: Structure of cytoskeletal drugs used in this thesis.

Another gene recently connected with the role of actin in plant immunity is the *ARPC4*. *ARPC4* disruption impairs actin dynamics during early stage of *S. sclerotiorum* infection and callose deposition induced by wounding. Expression of *PR1* is constitutively downregulated in *arpc4* mutants. Infection with *S. sclerotiorum* strongly induces its expression. *A. thaliana arpc4* mutants show susceptibility to *S. sclerotiorum*, defects in trichomes, epidermal pavement cell morphology and stomatal closure upon hydrogen peroxide treatment. Also other defence-related genes showed altered expression: *PDF1.2* was downregulated in intact mutants with expression further lowered upon infection, *PR4* was also downregulated. This suggests JA pathway is impaired in the *arpc4* mutants[208]. The actin cytoskeleton is being extensively studied in terms of connection with plant immunity.

1.6. Phytohormones produced by fungi

Phytohormones are small molecules with various regulatory functions in plants. Molecules with similar structures are also produced by a large variety of other organisms than plants.

1.6.1. Auxins

Auxin molecules generally consist of an aromatic ring and a carboxylic acid group. Although they were the first plant hormones to be identified, they have been later found to be produced also by a variety of microorganisms including plant pathogens[209]. The most common auxin is the indole-3-acetic acid (IAA) and up to date several microbial biosynthetic pathways were described[210]. The most common plant biosynthetic pathways were discussed above.

Similar to plants, the most known microbial biosynthetic pathways begin with tryptophan. The pathways are further referred to according to the first major metabolite downstream of tryptophan. The fungal indole-3-pyruvic acid (IPyA) pathway was first described in *Ustilago maydis* and it concerns aminotransferases UmTAM1 and UmTAM2[211]. They process tryptophan into IPyA. IPyA is further processed into indole-3-acetaldehyde (IAAld). The enzyme involved in this reaction was not described in *U. maydis*, however the conversion can happen spontaneously. IAAld is further processed by UmIAD1 and UmIAD2 into IAA. IAD genes were found functional also in ectomycorrhizal fungus *Tricholoma vaccinum*[212]. IPyA can be processed by the indole pyruvate decarboxylases (IPDC), enzymes that were functionally described in bacteria and symbiotic fungus *Neurospora crassa*[213,214]. Another known biosynthetic pathway originates with indole-3-acetamide (IAM) downstream of tryptophan. Originally it was described in bacterium *Pseudomonas savastanoi* concerning two-step conversion of tryptohan by tryptophan monooxygenase (iaaM) and indoleacetamide hydrolase (iaaH)[215]. Later this pathway was described as functional in fungi, namely the *Fusarium* species and the wheat rust fungus *Puccinia graminis*[216].

There are also pathways converting tryptophan into IAA via tryptamine. Tryptophan decarboxylase MrTDC from inscet-pathogenic fungus *Metarhiziium robertsii* converts tryptophan into tryptamine[216]. *F. graminearum* is also able to process tryptamine into IAA. The same fungus also can convert IAN into IAA[216]. There is also evidence of a tryptophan independent pathway. *Saccharomyces cerevisiae* with deleted aldehyde dehydrogenase genes did not process tryptophan but still was able to produce IAA[217].

The effects of auxins on fungal physiology and signalling may be variable. High exogenous auxin inhibited growth of pathogenic fungus *Harpophora maydis*[218] but in contrast it stimulated growth in *S. cerevisiae* and *C. albicans*[217]. *Colletotrichum gleosporioides* showed altered colony morphology and increased spore production upon auxin treatment[219].

1.6.2. Cytokinins

Cytokinin biosynthesis has been extensively studied in plants and several publications revealed recently that also fungi are capable of cytokinin production. The first evidence was reported in *M. oryzae* and *Claviceps purpurea*[220,221,222]. Biosynthetic genes identified in *C. purpurea* show isopentenyl transferase, cytochrome P450 and LOG activity. *L. maculans* is able to produce a variety of cytokinins, mainly free cytokinin bases unlike plants. *L. maculans* posseses functional IPT involved in cis-zeatin production, adenosine kinase (AK) involved in phosphorylation of ribosides to nucleotides and a degradation enzyme CKX – cytokinin oxidase/dehydrogenase[223]. *Fusarium*

pseudograminearum produces a novel group of cytokinins which are biologically active in the host[78]. Production of cytokinins by *M. oryzae* alters host plant metabolism near the infection sites leading to elevated concentration of sugars and amino acids which might serve the pathogen as an infection strategy[220]. *L. maculans* AK-silenced mutants exhibit altered colony morhphology and fastened mycelial senescence[223].

1.6.3. Abscisic acid

The only evidence of fungal ABA production was up to date reported in *L. maculans* that is able to produce ABA in axenic culture. As far as the biosynthetic pathway is concerned, two main components have been identified: polyketide synthase pks5 and six adjacent genes, including transcription factor abscisid-acid-like 7 gene (*Abl7*) form a gene cluster whose activity correlates with production of ABA in axenic culture. Overexpression of abl7 led to induction of ABA synthesis. Mutation of two genes in the cluster did not affect pathogenicity of *L. maculans*[224].

1.6.4. Salicylic acid

The ability to biosynthesize SA has not been documented in fungi up to date, however some microorganisms are able to degrade this compound[224]. The ability to sense SA was described in corn pathogen *U. maydis*. *U. maydis* senses SA via receptor Rss1 that has dual function as SA receptor and transcription activator of genes important for SA and tryptophan degradation. However, Rss1 does not have an impact on virulence which suggest that there might be redundancy in SA sensing in *U. maydis*[224].

1.6.5. Jasmonic acid

Microbial production of jasmonic acid was recently reported in fungus *Lasiodiploidia theobromae*. Metabolite characterization suggests that *L. theobromae* uses similar biosynthetic pathway as plants, but no biological function of jasmonic acid was observed[225]. *M. oryzae* is able to produce JA and posseses biosynthetic apparatus to further metabolize either endogenous or host-derived JA into effector molecule. The Antibiotic biosynthesis monooxygenase (Abm) converts JA into 12-hydroxyjasmonic acid (12OH-JA) during infection. 12-OH-JA interferes with host JA signalling. In the absence of Abm, *M. oryzae* accumulates MeJA which stimulates host defence. *F. oxysporum* and *Aspergillus flavus* produce oxilipins that are structurally analogous to JA[226].

1.6.6. Gibberellins

Gibberellins (GA) were at first identified in a fungus *G. fujikuroi*[227] and later on in plants. GA biosynthesis begins with GA12-aldehyde hydroxylation that produces GA14-aldehyde that is oxidised to GA14[127]. This compound is further oxidised to form GA4, the first bioactive gibberellin. Then

desaturation to GA7 and conversion to GA3 occurs on C13. GA1 is synthesized as a side product. The main difference in GA biosynthesis in fungi and plants is the stage of 3β-and the 13-hydroxylations. In fungi the GA12-aldehyde is 3b-hydroxylated do GA14-aldehyde and C13-hydroxylation happens at the very last stage to form GA3[129]. In plants on the other hand the final reaction is 3b-hydroxylation of GA9and GA20 to GA4 and GA1[128,127].

1.7. Pathogens studied in this thesis

1.7.1. *Pseudomonas syringae*

Pseudomonas syringae is a Gram-negative bacterium able to infect wide range of plant hosts including model plants *A. thaliana*, *N. benthamiana* or tomato. There are more than 60 pathovars of *P. syringae*, in this thesis strain *P. syringae* pv. tomato DC3000 was used. *P. syringae* is a gram-negative bacterium belonging to *Pseudomonadaceae*. This strain has been widely used and the molecular basis of its interaction with host have been well characterized[228]. The virulence factors possessed by *P. syringae* include toxins, extracellular proteins and polysaccharides or type III secretion system for effector transport, cell wall degrading enzymes, plant hormones or molecules that mimic plant hormones. *P. syringae* colonizes plant surface as an epiphyte and later enters the plant via wounds or stomata and multiplies in apoplast. Plants have evolved defence mechanism of induced stomatal closure upon perception of bacterial MAMPs. Stomatal closure is also regulated by humidity which can affect the rate of bacterial infection[229].

1.7.2. *Leptopshaeria maculans*

Leptosphaeria maculans in a haemibiotrophic fungal pathogen of the *Brassica* crops. Nowadays its main host is oilseed rape (*Brassica napus*) on which it causes the so called phoma stem canker and thus it is responsible for severe crop losses every year, mainly in Australia, Canada and Europe[230]. The infection cycle starts with ascospore germination on leaf surface and subsequent invasion into cotyledons and young leaves via stomata or wounds. The hyphae grow down the petiole into stem cortex and cause black/brown blackleg necrotic lesions[231]. During the first phase of infection, the fungus act as a biotroph and colonizes the leaf mesophyll with no visible symptoms. The pathogens then switches to necrotrophic lifestyle and lesions with pycnidia appear on the infected leaves. During this period the fungus grows asymptomatically through petiole to the stem base, where it causes severe necrosis. The most effective way to control *L. maculans* is to breed resistant cultivars. The sequencing of the *L. maculans* genome uncovered hundreds of putative effectors. Several other virulence factors such as toxins have been identified in *L. maculans*. Sirodesmin PL is a major non-host-specific toxin

and phomalide is a host-selective toxin[232]. Mechanisms of action of particular effectors are being studied. Resistance against L. maculans can be induced by the SA analogue benzothiadiazole (BTH) and the infection is associated with SA marker gene induction. *L. maculans* is also able to produce phytohormones that have been suspected to be used as virulence factors[223,224,233,234].

2. Research aims

Aim 1

The first aim of the thesis was to analyze more precisely the defence-related events connected with hormone signalling observed in plants treated with cytoskeletal drugs in previous publication of our laboratory[207].

- to characterize conditions that are required for the onset of resistance induced by cytoskeletal drugs
- to assess whether the resistance phenomenon is more generally valid among plant species
- to assess which phytohormone pathways are activated upon actin cytoskeleton degradation

Aim 2

The second aim of the thesis was to assess the role of phospholipids involved in plant immunity in connection with SA-signalling and actin stability. For this purpose we use a set of *A. thaliana* mutants impaired both in SA- and phospholipid signalling.

Aim 3

Previous results of our laboratory revealed that plant pathogen *L. maculans* is able to produce phytohormones[223]. The second part of the thesis was focused on the ability of *L. maculans* to produce molecules that may possibly alter host phytohormone signalling. We focused either on small secreted proteins – effectors or phytohormone-like molecules. The main aims were:

- to characterize impact of effector AvrLm4-7 on host hormone signalling, virulence and internal fungal hormone content
- to characterize the ability of *L. maculans* to produce auxins
- to characterize role of the fungal produced auxins in the interaction of *L. maculans* with its plant host

3. Publications included in the dissertation thesis

Results of this thesis are summarized in six impacted publications

1. **Leontovyčová, H**., Kalachova, T., Trdá, L., Pospíchalová, R., Lamparová L., Dobrev, P. I., Malínská, K., Burketová, L., Valentová, O. and Janda, M.: Actin depolymerization is able to increase plant resistance against pathogens via activation of salicylic acid signalling pathway. *Scientific Reports* **9**, 10397 (2019; IF 3,998) https://doi.org/10.1038/s41598-019-46465-5

2. Kalachova, T., **Leontovyčová, H.**, Iakovenko, O., Pospíchalová, R., Maršík, P., Klouček, P., Janda, M., Valentová, O., Kocourková, D., Martinec, J., Burketová, L. and Ruelland, E.: Interplay between phosphoinositides and actin cytoskeleton in the regulation of immunity related responses in *Arabidopsis thaliana* seedlings. *Environmental and Experimental Botany* **167**,103867 (2019; IF 4,010); doi: 10.1016/j.envexpbot.2019.103867

3. Pluhařová, K., **Leontovyčová, H**., Stoudková, V., Pospíchalová, R., Maršík, P., Klouček, P., Starodubtseva, A., Iakovenko, O., Krčková, Z., Valentová, O., Burketová, L., Janda, M. a Kalachova, T.: Salicylic acid mutant collection" as a tool to explore the role of salicylic acid in regulation of plant growth under a changing environment. *International Journal of Molecular Sciences* **20**, 6365 (2019, IF 4,183); doi:10.3390/ijms20246365

4. Nováková M., Šašek, V., Trdá, L., **Krutinová H.**, Mongin, T., Valentová, O., Balesdent, M.-H., Rouxel, T. and Burketová, L.: *Leptosphaeria maculans* effector AvrLm4-7 affects salicylic acid (SA) and ethylene (ET) signalling and hydrogen peroxide (H_2O_2) accumulation in *Brassica napus*. *Molecular Plant Pathology* (2015; IF 4,335); doi: 10.1111/mpp.12332

5. **Leontovyčová, H.,** Trdá, L., Dobrev, P. I., Šašek, V., Gay, E., Balesdent M.-H., and Burketová, L.: Auxin biosynthesis in the phytopathogenic fungus *Leptosphaeria maculans* involves indole-3-pyruvate decarboxylase LmIPDC2 and tryptophan aminotransferase LmTAM1. *Research in Microbiology* (2020; IF 3,217); doi: https://doi.org/10.1016/j.resmic.2020.05.001

6. **Leontovyčová, H.,** Kalachova, T. and Janda, M.: Disrupted actin: a novel player in pathogen attack sensing? *New Phytologist* (2020, IF 7,690); doi: https://doi.org/10.1111/nph.16584

3.1. Publication 1

Actin depolymerization is able to increase plant resistance against pathogens via activation of salicylic acid signalling pathway.

3.1.1. Summary of results

Actin cytoskeleton integrity is necessary for immune response upon pathogen attack, both by providing physical barrier and by its involvement in transport of callose synthases, antimicrobial compounds and cell wall components to the infection site[178,179]. Regarding these facts it has been generally assumed that desintegration of actin cytoskeleton results in enhanced plant susceptibility to pathogens. This fact has been experimentally proved by many studies [189,192,188,179].

However previous study of our laboratory showed that treatment of *A. thaliana* seedlings with cytoskeletal drugs latrunculin B and cytochalasin E induces transcription of defence related genes[207]. We attempted to investigate this apparent inconsistency and show that the relationship between actin depolymerization and plant resistance is more complex than previously thought. We screened phytohormone profile of *A. thaliana* seedlings after cytoskeletal drug treatment to prove that the induction of defence genes is SA-dependent. The major induced hormone in our set was indeed SA. The ICS pathway is responsible for this SA induction since transcription of *ICS1* and *ICS2* genes was induced and no changes were observed in *PAL1-4* genes. We were interested if these effects might actually lead to resistance, so we pretreated *A. thaliana* seedlings with latrunculin B for 24h.

We developed two different setups for testing seedling resistance *in vitro* against bacterial pathogen *P. syringae* after cytoskeletal drug treatment. Furthermore we performed similar screening in different pathosystem: *B. napus* and its fungal pathogen *L. maculans* to prove that our results might be more general among plant species.

Upon seedling treatment with cytoskeletal drugs we observed high induction of SA whilst only minor changes were observed in other hormone levels. Latrunculin B induced transcription of *PR1*, *PR2* and *ICS1* genes which are specifically connected with SA-defence pathway. Subsequently, we have observed increased resistance of the treated seedlings *in vitro*. This effect was also proved in 4-week-old *A. thaliana* plants. We observed increased defence gene transcription and lowered susceptibility also in a completely different pathosystem *B. napus* x *L. maculans* which suggests that the mechanism triggered by actin depolymerization leading to induced resistance is more general among plant species. This study opens a new field of research of the role of actin dynamics and its regulation of plant immunity. Further research is needed to discover the mechanism of perception and signalling of the degraded actin that eventually leads to specific onset of the SA-mediated defence.

3.1.2. My contribution

I performed extraction of phytohormones in all experiments. I quantified gene transcription in most of the experiments. I designed and performed the flood inoculation assays. I contributed to validation of the data and writing the manuscript.

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Actin depolymerization is able to increase plant resistance against pathogens via activation of salicylic acid signalling pathway

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The integrity of the actin cytoskeleton is essential for plant immune signalling. Consequently, it is generally assumed that actin disruption reduces plant resistance to pathogen attack. Here, we demonstrate that actin depolymerization induced a dramatic increase in salicylic acid (SA) levels in *Arabidopsis thaliana*. Transcriptomic analysis showed that the SA pathway was activated due to the action of isochorismate synthase (ICS). The effect was also confirmed in *Brassica napus*. This raises the question of whether actin depolymerization could, under particular conditions, lead to increased resistance to pathogens. Thus, we explored the effect of pretreatment with actin-depolymerizing drugs on the resistance of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*, and on the resistance of an important crop *Brassica napus* to its natural fungal pathogen *Leptosphaeria maculans*. In both pathosystems, actin depolymerization activated the SA pathway, leading to increased plant resistance. To our best knowledge, we herein provide the first direct evidence that disruption of the actin cytoskeleton can actually lead to increased plant resistance to pathogens, and that SA is crucial to this process.

The actin cytoskeleton plays a key role in plant immunity[1,2], both by providing a physical barrier and by its involvement in the transport of callose, antimicrobial compounds and cell wall components to an infection site[3]. Additionally, actin filament reorganization is a very fast response to treatment with conserved microbial compounds, MAMPs (microbe-associated molecular patterns), such as flg22, elf26 and chitin. The recognition of MAMPs triggers a specific set of immune responses, including cytoskeleton reorganization. It underpins the important role of actin cytoskeleton in plant defense[4,5]. Several studies have shown that when drugs, such as cytochalasins or latrunculin B, depolymerize the actin cytoskeleton, different plant species become more susceptible to pathogens. For example, treatment of *A. thaliana* with latrunculin B resulted in higher susceptibility to infection by *Pseudomonas syringae*[5–7]. In plants, actin depolymerizing factors serve to sever filamentous actin. The *adf4* (Actin Depolymerizing Factor 4) *A. thaliana* knock out mutant had reduced resistance to *Pseudomonas syringae* pv *tomato DC 3000* (*Pst* DC3000) expressing the AvrPphB effector[8]. This is because ADF4 is necessary for the expression of RPS5, the resistance protein that recognises AvrPphB[9]. However, in the intact *adf4* mutant, the density and skewness of actin filaments were the same as in control plants, implying that the actin cytoskeleton is not modified before infection[10]. ADF4 plays an indispensable role in the actin reorganisation upon elf26, but not in response to chitin[4]. The importance of actin cytoskeleton is also highlighted by the fact that *Pst DC3000* secretes at least two effectors modulating actin cytoskeleton. The effector HopW1 disrupts the

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actin cytoskeleton[6,7]. Another effector, HopG1, was shown to affect the remodelling of the actin cytoskeleton in *Pst* DC3000-infected *A. thaliana*[11]. Furthermore, treatment with cytochalasin E increased the penetration of *A. thaliana* plants by *Colletotrichum* species[12] and the rate of entry to barley by *Blumeria graminis* f. sp. *hordei*[13]. Non-host resistance to *Erysiphe pisi* decreased after treatment with cytochalasins in barley, wheat, cucumber and tobacco[14], as did resistance to *Blumeria graminis* f. sp. tritici after cytochalasin E treatment of *A. thaliana*. Moreover, treatment with cytochalasin E in the absence of EDS1 (enhanced diseased resistance 1), an upstream component of the salicylic acid (SA) signalling pathway, strongly enhanced the inhibitory effect on non-host resistance[15]. However, in tobacco, cytochalasin E induced the transcription of *NtPR-1* (pathogenesis-related 1), a defence-related SA marker gene, and is able to prime cells to HR-like cell death in response to *Erysiphe cichoracearum*[16]. Furthermore, both cytochalasin E and latrunculin B induced the transcription of several SA marker genes (*AtPR-1*, *AtPR-2* and *AtWRKY38*) in *A. thaliana* seedlings[17]. This suggests that while such drugs do indeed cause actin depolymerization, the effects of such depolymerization may not always be adverse. Could it be that drug-induced actin depolymerization actually triggers processes that induce the SA pathway and thereby increase plant resistance to pathogens?

Results

Actin depolymerization induce salicylic acid biosynthesis via ICS1 dependent pathway. To establish that SA levels can increase upon actin depolymerization, we measured phytohormone content in *A. thaliana* seedlings treated with just 200 nM latrunculin B. Such a low concentration of latrunculin B proved sufficient to depolymerize actin filaments in the seedlings within 24 h (Fig. S1). Additionally, we showed that 24 h treatment with latrunculin B does not induce plant cell death (Fig. S2). Significantly, by that time there was a sevenfold increase in the free SA level of the treated seedlings compared with the control ones. The only other phytohormone to display an increase (twofold) was jasmonic acid (JA). Apart from Indole-3-acetamide (IAM), which showed a threefold decrease, the other tested phytohormones remained largely unaltered (Fig. 1a; Table S1).

Having shown this dramatic rise in SA level in *A. thaliana*, we wondered which of its two SA biosynthetic pathways was responsible for this increase or whether they both contributed to it. One pathway involves phenylalanine ammonia-lyase (PAL, EC 4.3.1.24), which exists in four isoforms, while the other involves isochorismate synthase (ICS; EC 5.4.4.2), which occurs in two isoforms[18]. Analysis of the transcription of all *AtPAL* and *AtICS* genes in the seedlings revealed that only the *AtICS* genes were induced by latruculin B (Fig. 1b). This shows that drug-induced actin depolymerization activates the ICS-dependent pathway and that this pathway alone is responsible for SA biosynthesis under these conditions.

Actin depolymerization leads to induced resistance of *A. thaliana* against *Pst* DC3000. Given that increased resistance to pathogens in *A. thaliana* is associated with SA biosynthesis through the ICS pathway[19,20], is it possible that activation of the same pathway invoked by drug-induced actin depolymerization also results in increased resistance? To investigate this, we used Ishiga *et al.*[21] protocol as a basis for performing two *in vitro A. thaliana-Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) flood-inoculation assays in liquid and solid media[21]. We treated the seedlings with latrunculin B 24 h before inoculation with *Pst* DC3000. Remarkably, under both conditions, the latrunculin B-pretreated seedlings were more resistant than the control ones (Fig. 1c,d,e).

To ensure that this phenomenon is not just associated with *in vitro* conditions, we also performed experiments using four-week-old *A. thaliana* plants cultivated in soil, such plants typically being used for studies of *A. thaliana* resistance to *Pst* DC3000[22]. Unlike in the seedlings, 24 h treatment with 200 nM latrunculin B did not activate the SA pathway in the adult plants and, thus, no increased resistance was observed (Fig. 2a,b). However, the transcription of SA marker genes (*AtPR-1*, *AtICS1*) was induced after 24 h treatment with 1 µM latrunculin B (Fig. 2a), leading to increased resistance to *Pst* DC3000 (Fig. 2b). This suggests that plant resistance is strongly dependent on latrunculin B concentration, probably due to differences between the efficiency of latrunculin B-induced actin depolymerization in seedlings and in adult plants (Figs S1 and S3). Similar to latrunculin B, pretreatment with cytochalasin E led to both SA-induced gene transcription (Fig. S4a) and increased plant resistance to *Pst* DC3000 (Fig. S4b), thereby strengthening the notion that such resistance is due to the depolymerizing activity of cytoskeletal drugs. It should be noted that we exclude the antibacterial effect of latrunculin B because *Pst* DC3000 grew *in vitro* in the presence of latrunculin B at a similar rate as in the control medium (Fig. S5).

The induced resistance caused by actin depolymerization is dependent on salicylic acid. To further demonstrate the dependence of such resistance on the SA pathway, we performed assays using mutants known to have an impaired SA pathway and thus be more susceptible to *Pst* DC3000: *nahG*, which induces low endogenous SA levels through the expression of SA-hydroxylase[23], and *sid2*, a knock-out mutant of the *AtICS1* gene[24]. As expected, latrunculin B did not induce resistance in the *nahG* plants (Fig. 2d,e). *Sid2* plants treated with latrunculin B were more resistant compared to *sid2* controls. However, latrunculin B treated *sid2* plants were still more susceptible than WT controls (Fig. 2e). The SA level is not induced in *sid2* plants (Fig. 2f) which correlates with the fact that none of SA biosynthetic genes does have induced transcription (Fig. S6b). Contrarily in seedlings, *ICS2* transcription is induced by latB (Fig. S6a). Altogether these results clearly confirm the crucial role of SA for actin depolymerization-induced resistance. However, increased resistance of latB treated *sid2* mutants uncover a new possible unknown SA independent mechanism triggering immunity.

Actin depolymerization induce SA pathway in *B. napus* and enhance its resistance against *L. maculans*. To show that this phenomenon is neither species-specific nor pathogen-specific, we investigated the effect of latrunculin B on an important crop, oilseed rape (*Brassica napus*). As in the case of *A. thaliana* in *B. napus*, latrunculin B upregulated the transcription of SA marker genes (*BnPR-1*, *BnICS1*) (Fig. 3a). Furthermore,

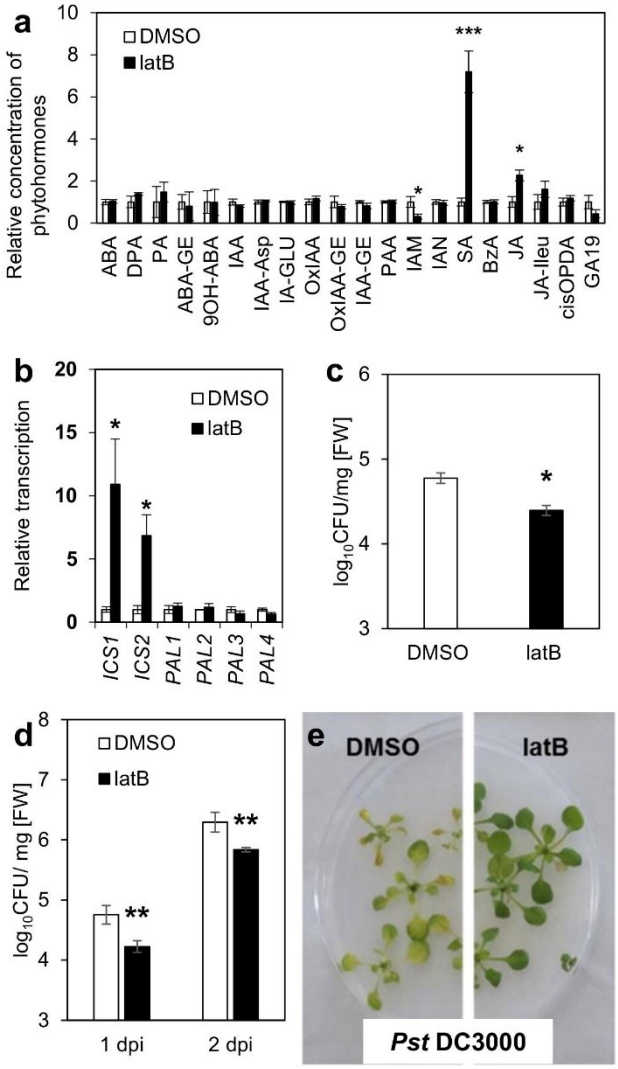

Figure 1. Seedlings of *A. thaliana*: Latrunculin B triggers SA biosynthesis and resistance to *Pst* DC3000. Seedlings were grown *in vitro* in liquid MS medium (**a**–**c**) and seedlings were grown *in vitro* in solid MS/2 medium (**d**,**e**). (**a**) Phytohormone analysis. Seedlings were treated for 24 h with 200 nM latrunculin B (latB) or 0.01% DMSO (control). For abbreviations of analyzed phytohormones, see Table S1. (**b**) Transcription of SA biosynthetic genes *ICS1*, *ICS2*, *PAL1*, *PAL2*, *PAL3* and *PAL4*. Seedlings were treated for 24 h with 200 nM latB or 0.01% DMSO. The transcription level was normalized to the reference gene, *SAND*. (**c**) Bacterial titres (liquid medium). Seedlings were pretreated for 24 h with 200 nM latB or 0.01% DMSO before inoculation with *Pst* DC3000. Tissue was harvested 1 day after inoculation with bacteria. (**d**) Bacterial titres (solid medium). Seedlings were pretreated for 24 h with 200 nM latB or 0.01% DMSO before inoculation with *Pst* DC3000. Tissue was harvested 1 and 2 days after inoculation with bacteria. (**e**) Representative photographs of seedlings grown on solid medium 2 days after inoculation with *Pst* DC3000. The values represent mean and error bars (SEM) from four (**a**,**c**), three to four (**b**) and five (**d**) independent samples. The asterisks represent statistically significant changes in latB-treated samples compared with controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; two tailed Student's t-test).

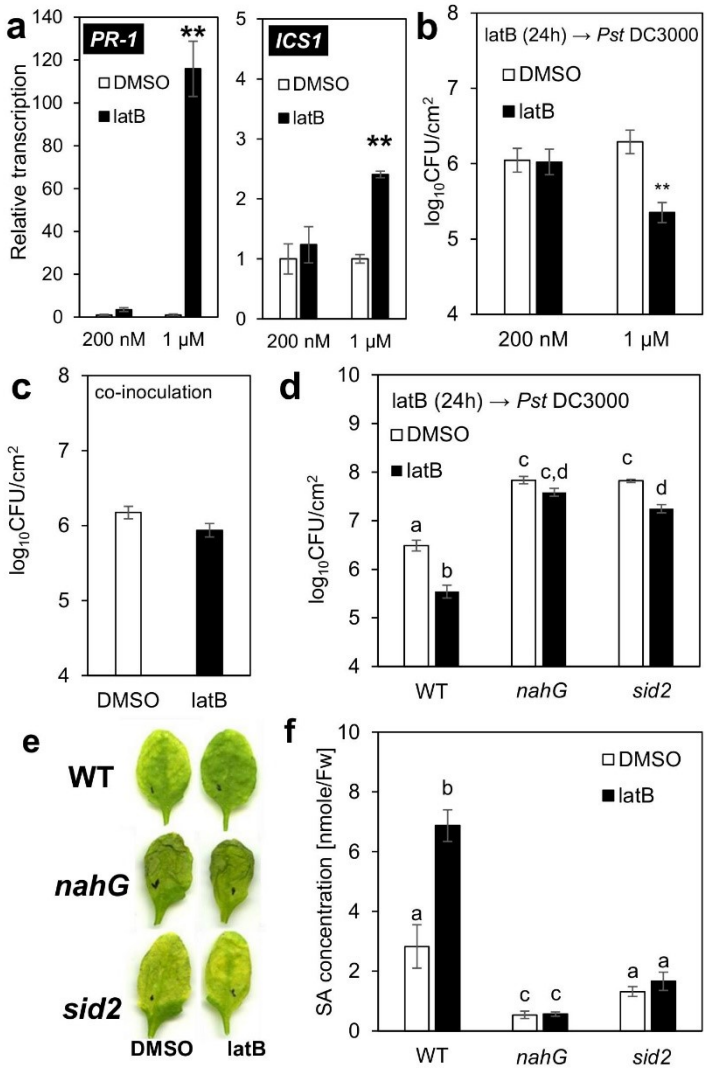

Figure 2. Four-week-old *A. thaliana*: Latruculin B-triggered SA pathway is necessary for higher resistance to *Pst* DC3000 (**a**). Transcription of SA marker genes *PR-1* and *ICS1* in four-week-old *A. thaliana* plants. Plants were treated for 24 h with 200 nM or 1 µM latrunculin B (latB). The transcription level was normalized to the reference gene, *TIP41*. (**b,c**) Bacterial titres in four-week-old plants. (**b**) Plants were pretreated with 200 nM or 1 µM latB for 24 h before inoculation with *Pst* DC3000. Control plants were pretreated with 0.01 or 0.05% DMSO. (**c**) Plants were treated with 1 µM latB or 0.05% DMSO, each in a solution containing *Pst* DC3000. (**d**) Bacterial titres in four-week-old plants. (**e**) Representative photographs of adult *A. thaliana* leaves infected with *Pst* DC3000 3 days after inoculation. (**f**) Salicylic acid (SA) concentration after 24 h 1 µM latB treatment. Plants were treated for 24 h with 1 µM latB or 0.05% DMSO before inoculation with *Pst* DC3000. *A. thaliana* WT plants (col-0) and mutants with impaired SA pathways (*nahG* and *sid2*) were used (d, e, f). Tissue was harvested 3 days after inoculation with *Pst* DC3000. The values represent mean and error bars (SEM) from four (**a,f**) and six (**b,c,e**) independent samples. The asterisks represent statistically significant changes in latB-treated samples compared with controls (**$P < 0.01$; two tailed Student's t-test) and statistical differences between the samples (**d,f**) were assessed using a one-way ANOVA, with a Tukey honestly significant difference (HSD) multiple mean comparison post hoc test. Different letters indicate a significant difference, Tukey HSD, $P < 0.01$, $n = 6$.

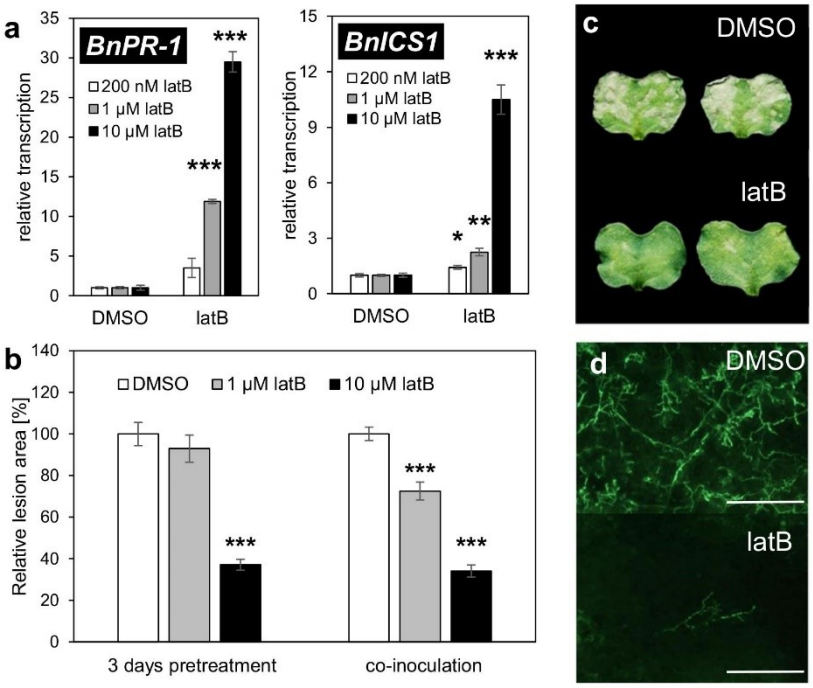

Figure 3. *B. napus* cotyledons: Latrunculin B triggers SA pathway and resistance to *L. maculans* (**a**). Transcription of SA marker genes *BnPR-1* and *BnICS1* in *B. napus* cotyledons. Cotyledons were treated for 24 h with infiltrations of 0.2, 1 or 10 µM latrunculin B (latB). Control cotyledons were treated for 24 h with a corresponding concentration of DMSO (0.01, 0.05 or 0.5%). The transcription level was normalized to the reference gene, *BnTIP41*. (**b**) *B. napus* susceptibility to *L. maculans* was evaluated as the relative lesion area (ratio of lesion area to whole leaf area) on the cotyledons. Cotyledons were treated with latrunculin B (latB; 1 µM or 10 µM) or DMSO control (0.05 or 0.5%), either 3 days before inoculation or simultaneously to inoculation by *L. maculans*. Lesions of DMSO controls in each treatment conditions were set as 100%. (**c**) Representative images of *L. maculans*-infected cotyledons. (**d**) Representative microscopy images of *L. maculans* hyphae proliferation in *B. napus* cotyledons in response to 10 µM latB or 0.5% DMSO. The bars correspond to 500 µM. The values represent mean and error bars (SEM) from three to four (**a**) and 60 -142 (**b**) independent samples. The asterisks represent statistically significant changes in latB-treated samples compared with controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; two tailed Student's t-test).

as with adult *A. thaliana*, the effect of latrunculin B on *B. napus* was concentration dependent (Fig. 3a). The increased transcription of *BnPR-1* also occurred 72 h after latrunculin B treatment. On the other hand, *BnICS1* was not induced, indicating a transient effect of actin depolymerisation on *BnICS1* transcription (Fig. S7). The treatment of *B. napus* with 10 µM latrunculin B 3 days before inoculation with a hemibiotrophic fungal pathogen, *L. maculans*, efficiently inhibited hyphal colonisation and necrosis formation in the infected cotyledons (Fig. 3b,c,d). Treatment with 1 µM latrunculin B led to much weaker and variable resistance against *L. maculans* (Fig. 3b), corresponding to the weaker transcription of defence-related genes (Fig. 3a). These data are in accordance with our previous study characterizing the importance of SA in the defence of *B. napus* against *L. maculans*[25]. In addition, we observed significant cytochalasin E-induced resistance to *L. maculans* in *B. napus* (Fig. S8), which suggests that the effect is not compound-specific. Furthermore, neither latrunculin B nor cytochalasin E displayed antifungal activity on *L. maculans* growth *in vitro* (Fig. S9). Interestingly, the co-inoculation of *B. napus* cotyledons with a joint solution of 1 and 10 µM latrunculin B and *L. maculans* conidia also induced resistance (Fig. 3b).

Discussion

Our results indicate that depolymerized actin can trigger resistance to bacterial or fungal pathogens. Thus, we have shown that plant immunity is strongly activated by depolymerised actin and that this phenomenon appears to be generally valid; namely, it seems not to be species specific, pathogen-type specific or drug-type specific. These findings do not negate those of previous studies that showed the susceptibility of plants treated

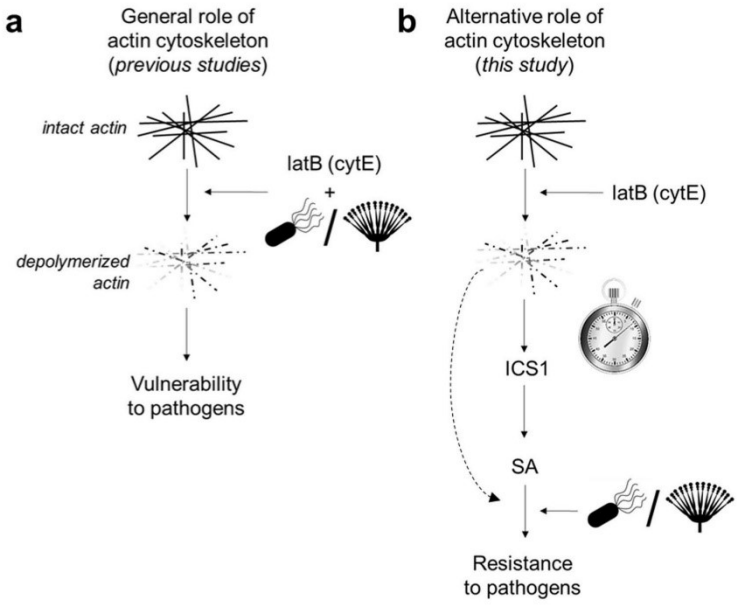

Figure 4. Possible dual role of actin cytoskeleton in plant response to pathogens. (**a**) The widely-published scenario in which depolymerization of the actin cytoskeleton by treatment with latrunculin B or cytochalasin E leads to increased plant vulnerability to pathogens. Studies showing this phenomenon co-inoculated plants with a drug and pathogen. (**b**) The new alternative scenario for the role of the actin cytoskeleton proposed in this manuscript. Plants pretreated with latrunculin B or cytochalasin E before inoculation with a pathogen have time to activate the salicylic acid signalling pathway, resulting in increased resistance to the subsequently inoculated pathogens. latB = latrunculin B; cytE = cytochalasin E; SA = salicylic acid; ICS1 = isochorismate synthase 1; = fungi; = bacteria.

with cytoskeletal drugs to pathogens[5–7,12,13,15]. Rather, they reveal that the plant disease resistance is strongly dependent on whether the plant has sufficient time to activate SA-mediated immunity (Fig. 4). This was clearly shown by our experiments with *Pst* DC3000, in which pre-infection treatment with cytoskeletal drugs resulted in resistance whilst co-inoculation did not (Fig. 2b,c,e). The co-inoculation of cytochalasin D and *Pst* DC3000 also had no effect on resistance according to Shimono *et al.*[11]. Other previous studies using actin-depolymerizing drugs showed higher susceptibility to *Pst* DC3000 when co-inoculation was used[5–7](Fig. 4). It is also important to mention that actin filaments response to plant immunity is strongly dependent on conditions used in the study. A good example are effects of different MAMPs (flg22 and elf26) on actin reorganization. Using 24 day-old plants infiltrated with MAMPs, Henty-Ridilla *et al.*[5] showed that treatment with flg22 induces actin reorganization, while elf26 does not[5]. Contradictorily to that, in epidermal cells of hypocotyl grown in the dark, Henty-Ridilla *et al.*[4] showed that elf26 induces reorganisation and flg22 does not[4] (an explanation could be that under these conditions, FLS2 receptor of flg22 is not expressed). In this study, we excluded the effect of different conditions on induced resistance of *A. thaliana* against *Pst DC3000* by testing three different setups (Figs 1c,d and 2b,e). The result was in all cases similar, whereby pretreatment with latrunculin B induced resistance of *A. thaliana* against *Pst DC3000*.

Interestingly, treatment with latrunculin B resulted in increased resistance in both *L. maculans* setups: pre-treatment (Fig. 3b) and co-inoculation (Fig. 3b). This suggests that the rapidity of pathogen growth is a crucial factor. In contrast to *Pst* DC3000, which strongly damaged the inoculated leaves within three days, almost no multiplication of *L. maculans* occurred during the same period[25]. Thus, it appears that the slow growth of *L. maculans* enabled *B. napus* to establish the SA pathway, which was induced within 24 hours of cytoskeletal drug treatment (Fig. 3a). Overall then, while it is true that plant resistance to pathogens is decreased by a disrupted actin cytoskeleton, our results show that, given sufficient time, plants are able to trigger SA-based defence mechanisms to overcome such threats. This could be due to SA antimicrobial activity, accompanied by the SA-induced production of antimicrobial compounds. These powerful SA properties have been nicely demonstrated in relationship to so-called age-related resistance[26–28]. Our study shows that SA pathway, specifically induced by actin depolymerization, is more powerful despite the missing actin dynamics.

Up to date, some other works suggest the possible positive effect of depolymerization of actin cytoskeleton on plant immunity. Kobayashi and Kobayashi[16] showed that treatment with cytochalins induce *NtPR1* transcription in tobacco. Additionally, cytochalasin E primed tobacco cells to induce HR-like cell death in presence of *Erysiphe cichoracearum*. We can speculate that it could lead to higher resistance against this biotrophic pathogen but it was not explicitly tested[16]. We confirmed the induction of *AtPR-1* gene upon treatment with cytoskeletal depolymerizing drugs in *A. thaliana*[17,29]. Recently it was shown that overexpression of *At*PRF3, which leads to depolymerization of actin filaments, increased ROS production upon flg22 treatment[30]. However to our best knowledge, we herein provide the first direct evidence that disruption of the actin cytoskeleton can actually lead to increased plant resistance to pathogens, and that SA is crucial to this process. We strongly believe that our work opens a new and important direction for further research. Is the influence of the actin cytoskeleton on vesicle trafficking involved in SA biosynthesis? For example, when PRRs (pattern recognising receptors) on the plasma membrane recognize MAMPs, it triggers PRRs endocytosis which, in turn, might activate the SA pathway[31,32]. A well characterised example is the internalization of FLS2, which is dependent on the actin-myosin complex[33]. Thus, could an imbalance in PRRs result in constitutively activated immunity and, thereby, induce SA biosynthesis? A hint in support of such a hypothesis is provided by a double mutant with impaired phosphatidylinositol-4-kinase β1 and β2 (*pi4kβ1β2*), which has been shown to alter vesicle trafficking and constitutively increase SA concentration[34,35]. It is also possible that plants have evolved a system for detecting actin cytoskeleton disruption and that the activation of such a system triggers SA-specific immune responses. However, as yet, we are not able to determine if chemically-depolymerised actin is really the triggering event for immune signalling or whether a pleiotrophic event, such as endoplasmic reticulum stress, results in SA induction. For this reason, further research should be focused on deciphering the specific mechanism by which actin depolymerization triggers SA biosynthesis and the ensuing increased plant resistance to pathogens.

Materials and Methods

Plant material. For the *A. thaliana* experiments, the following genotypes were used: Columbia-0 (WT); *sid2-3* (SALK_042603)[24]; *nahG*[23] and *pUBC*::Lifeact-GFP[36]. *A. thaliana* seedlings were grown either in liquid MS medium or on solid MS/2 medium. Per litre, the liquid MS medium contained the following: 4.41 g Murashige and Skoog medium including vitamins (Duchefa, Netherlands), 5 g sucrose, 5 g MES monohydrate (Duchefa, Netherlands). Per litre, the solid MS/2 medium contained 2.2 g Murashige and Skoog medium (Duchefa, Netherlands) with 10 g sucrose and 8 g Plant agar (Duchefa, Netherlands). Both media were adjusted to pH 5.7 using 1 M KOH. For cultivation in the liquid, surface-sterilized seeds were sown in 24-well plates containing 400 μL of liquid MS medium per well. The plants were cultivated for 10 days under a short-day photoperiod (10 h/14 h light/dark regime) at 100–130 $\mu E\ m^{-2}\ s^{-1}$ and 22 C. On the 7th day, the medium in the wells was exchanged for a fresh one. For cultivation on the solid MS/2 medium, seedlings were grown in Petri dishes for 12 days under a long-day photoperiod (16 h/8 h light/dark regime) at 100–130 $\mu E\ m^{-2}\ s^{-1}$ and 22 °C. For *A. thaliana* plants grown for 4 weeks in soil, surface-sterilized seeds were sown in Jiffy 7 peat pellets and the plants cultivated under a short-day photoperiod (10 h/14 h light/dark regime) at 100–130 $\mu E\ m^{-2}\ s^{-1}$, 22 °C and 70% relative humidity. They were watered with fertilizer-free distilled water as necessary

For the *B. napus* experiments, plants of the Eurol cultivar were grown hydroponically in perlite in Steiner's nutrient solution (Steiner, 1984) under a 14 h/10 h light/dark regime (25 °C/22 °C) at 150 $\mu E\ m^{-2}\ s^{-1}$ and 30–50% relative humidity. True leaves were removed from 14-day-old plantlets to avoid cotyledon senescence.

Treatment with chemical compounds. As actin depolymerizing drugs, latrunculin B (Sigma-Aldrich, USA) and cytochalasin E (Sigma-Aldrich, USA) were used. Latrunculin B and cytochalasin E were both dissolved in DMSO; the concentration of the stock solutions were 2 mM and 4 mM, respectively.

For the *Pst* DC3000 resistance assay, the seedlings grown in 24-well plates were treated by replacing the pure liquid MS medium in the plate wells with medium containing 200 nM latrunculin B or 0.01% DMSO control. The seedlings cultivated on the solid medium were treated 24 h by flooding with 10 mL of MS/2 medium containing 200 nM latrunculin B or 0.01% DMSO control. Fully-developed leaves from four-week-old *A. thaliana* grown in soil were infiltrated either with 200 nM or 1 μM latrunculin B (0.01% or 0.05% DMSO as respective controls) or with 1 μM or 10 μM cytochalasin E (0.025% or 0.25% DMSO as respective controls) 24 h before *Pst* DC3000 infection using a needleless syringe.

For the transcriptomic assay, the seedlings *of A. thaliana* grown in 24-well plates were treated 24 h with 200 nM latrunculin B (0.01% DMSO control) or 10 μM cytochalasin E (0.25% DMSO control). Four-week old *A. thaliana* were infiltrated with 200 nM (0.01% DMSO) or 1 μM latrunculin B (0.05% DMSO) for 24 h. The 10-day-old cotyledons of *B. napus* were infiltrated either with 1 μM or 10 μM latrunculin B or with 10 μM cytochalasin E (in all cases with corresponding DMSO controls) using a needleless syringe. For infection assay 3 days before infection with *L. maculans*, for transcriptomic assay 24 and 72 h before harvesting tissue.

Inoculation of *A. thaliana* seedlings with *Pst* DC3000. After the *A. thaliana* seedlings had been cultivated in 24-well plates in the liquid MS medium for 10 days, the cultivation medium was exchanged for one containing latrunculin B or cytochalasin E, and incubated for 24 h. On day 11, the medium was replaced with a bacterial suspension of *Pst* DC3000 in 10 mM $MgCl_2$ ($OD_{600} = 0.01$). The seedlings were incubated in this bacterial suspension for 1 min. After incubation, the suspension was replaced with the liquid MS medium. On day 12, the seedlings were harvested, each sample taken containing all of the seedlings from three wells. The seedlings were then homogenized in tubes with 1 g of 1.3 mm silica beads using a FastPrep-24 instrument (MP Biomedicals, USA). The resulting homogenate was serially diluted and pipetted onto King B plates. The colonies were counted after 1–2 days of incubation at 28 °C.

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Author Contributions

H.L., L.T., T.K., M.J. designed the experiments; H.L., L.T., T.K., L.L., R.P., P.I.D., K.M., M.J. performed experiments; H.L., L.T., T.K., L.B., O.V., M.J. analysed the data; H.L., M.J. wrote the manuscript. All the authors discussed the results and commented on the manuscript.

Additional Information

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3.2. Publication 2

Interplay between phosphoinositides and actin cytoskeleton in the regulation of immunity related responses in *Arabidopsis thaliana* seedlings.

3.2.1. Summary of results

This study focused on further characterization of the effects triggered by actin depolymerization drugs that were observed in the previous study. Precise regulation of actin dynamics is vital for correct cellular trafficking that is a necessary component of plant response to pathogens. The exact mechanisms of the regulation have not yet been described but phospholipids were shown to play a certain role[235]. Using *A. thaliana* mutant in *PI4Kβ1/β2* genes we attempted to broaden the knowledge about actin dynamics and its role in plant immunity.

Callose deposition is a well described defence mechanism that is regulated by trafficking. We attempted to characterize changes in callose deposition upon cytoskeletal drug treatment in set of SA-pathway affected mutants combined with *pi4kβ1/β2* mutants. The used cytoskeletal drugs latrunculin B and cytochalasin E triggered callose deposition via the activity of callose synthase 12 using *A. thaliana* mutant *pmr4*. This effect is SA-independent since we still observed callose accumulation in *NahG* transformed plants and *pad4* mutants impaired in SA-signalling. Further we demonstrated that specific gene transcription corresponding to certain defence pathways is triggered by actin degradation suggesting that the observed effect is not a general stressor causing broad transcriptomic deregulation.

Further on we investigated the role of phospholipids in this phenomenon. We analyzed the cytoskeleton degradation caused by Latrunculin B in Lifeact tagged *pi4kβ1/β2* mutants. After 24h treatment with latrunculin B the actin filament density and occupancy was much lower in *pi4kβ1/β2* suggesting that its actin filaments are more sensitive to cytoskeletal drugs. Further testing was focused on the connection of impaired SA signalling with the role of PI4Kβ1/β2 enzymes in the process introducing triple mutants affected in both SA signalling and phospholipid signalling: *sid2/ pi4kβ1/β2*, *NahG/pi4kβ1/β2* and *pad4/pi4kβ1/β2*. Single mutants in *sid2*, *pad4*, *NahG* transformed plants and double mutant in *PI4Kβ1/β2* were used as control. First the control lines were tested for SA accumulation and callose deposition after 24h latrunculin B treatment. SA accumulation in *pi4kβ1/β2* was similar to WT while in *NahG* and *pad4* it only reached 3times lower level. No accumulation was observed in *sid2* mutant. Significant induction in callose deposition was observed in all the treated lines.

We analyzed related gene transcription in the similar treatment setup using the *Nah*G and *pad4* plants. The transcription of SA biosynthetic gene *ICS1* was induced in both mutants. The SA-responsive genes

PR1 and *WRKY38* that were induced by latrunculin B in WT were no longer induced in *NahG* and *pad4*. *PR2* induction remained in *NahG* and *pad4*. Induction of *BAP1* was weakly significant in *NahG* but not significant in *pad4*. The transcription of the *PAD4* gene was not affected in *NahG* plants.

Further we tested the same set of mutants affected in PI4Kβ1/β2 enzymes (*sid2/pi4kβ1/β2*, *NahG/pi4kβ1/β2* and *pad4/pi4kβ1/β2*) in similar setup for SA accumulation, callose deposition and defence gene transcription. SA accumulation was partly restored in *NahG/pi4kβ1/β2* and *pad4/pi4kβ1/β2* when compared to *NahG* resp. *pad4*. No SA accumulation was observed in *sid2/pi4kβ1/β2*. Callose accumulation remained induced independently of SA accumulation.

Gene transcription analysis correlated with the SA accumulation levels: *ICS1* transcription was induced in *NahG/pi4kβ1/β2* but not in *pad4/pi4kβ1/β2*. SA-responsive genes *PR1*, *PR2* and *WRKY 38* were induced in all tested lines. *PAD4* transcription was also induced in *NahG/pi4kβ1/β2* and *sid2/pi4kβ1/β2*. Wounding marker *BAP1* was induced in *pad4/pi4kβ1/β2* but not in *NahG/pi4kβ1/β2*.

This study provided deeper insight into defence-related effects connected with actin cytoskeleton integrity, particularly focusing on the role of phospholipids. We propose a model suggesting the role of PI4Kβ1/β2 enzymes in the process and distiguishing between SA dependent and independent effects caused by actin disruption.

3.2.2. My contribution

I performed extraction of phytohormones for all the experiments and quantified gene transcription in the majority of experiments. I contributed to validation of the data and writing the manuscript.

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Interplay between phosphoinositides and actin cytoskeleton in the regulation of immunity related responses in *Arabidopsis thaliana* seedlings

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ABSTRACT

Actin cytoskeleton is indispensable for plant cell integrity. Besides, increasing evidences illustrate its crucial role in plant responses to environment, including defence against pathogens. Recently, we have demonstrated that pre-treatment with actin disrupting drugs latrunculin B (latB) and cytochalasin E can enhance plant resistance against bacterial and fungal pathogens via activation of salicylic acid (SA) pathway. Here, we show that actin depolymerization in *Arabidopsis thaliana* seedlings not only triggers SA biosynthesis by ICS1, but also induces callose deposition via callose synthase PMR4. This effect is SA-independent since still present in mutants that do not accumulate SA. LatB also triggers the expression of several defence related genes. We could distinguish genes, induced in a SA-dependent manner (*PR1, WRKY38, ICS1*) and those that are SA-independent (*PR2, PAD4, BAP1*). As actin cytoskeleton is tightly connected with membrane trafficking, we assayed the effect of latB on mutant plants invalidated in phosphatidylinositol 4-kinase beta1 and beta2 (PI4Kβ1β2). Deficiency in PI4Kβ1β2 enhanced latB-triggered actin filaments depolymerisation. Yet, it did not lead to a stronger callose deposition or SA biosynthesis in response to latB. Surprisingly, introduction of *NahG* construct or *pad4* mutation in *pi4kβ1β2* background had much lower effect on SA induction and SA-dependent gene expression changes than it has in wild type. We can thus conclude that actin disruption itself triggers immune-like responses: there is an induction of SA *via* PAD4 coupled to ICS1; it leads to the induction of *PR1* and *WRKY38*, and this requires a functional PI4Kβ1β2 to be properly regulated. However, an alternative, SA-independent pathway also exists that leads to the enhanced expression of *PR2* and to callose accumulation.

1. Introduction

Plants are always confronted to changes in their environment. This can concern physical parameters such as light intensity and/or quality, temperature or water availability, or interactions with other organisms, including bacteria or fungi. Some of the interactions can lead to diseases. Nowadays, plant responses to the environmental challenges, either abiotic or biotic, are an object of an intense research. A novel aspect of such a research is the investigation of the roles of processes that were so far mainly (or uniquely) considered as important for the cell structure. Such a process is the dynamics of cytoskeleton. Cytoskeleton is comprised of microfilaments and microtubules. Actin filaments (F-actin) are highly dynamic structures formed from monomers (G-actin) (Drobak et al., 2004). Even in basal conditions, filaments constantly grow at one end and depolymerize at the opposite one (Li et al., 2017). The filaments are indispensable for cell shaping, growth

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and polarity (Andreeva et al., 2010), cytokinesis (Brill et al., 2000), but also for intracellular trafficking of vesicles (Geitmann and Nebenführ, 2015). Yet, the direct mechanisms regulating actin dynamics in regards to vesicle trafficking are still waiting to be revealed. A particular role has been proposed for phospholipids, especially phosphoinositides (Ebine and Ueda, 2015). Phosphoinositides are phosphorylated forms of phosphatidylinositol (PI). From the position and number of the phosphate groups on the inositol ring, we can distinguish PI-3-phosphate (PI3P), PI-4-phosphate (PI4P) and PI-4,5-bisphosphate (PIP_2). Phosphoinositides play a key role in vesicle trafficking (Delage et al., 2013) and also interact with cytoskeleton: for instance, F-actin capping protein interaction with actin monomers is regulated by PIP_2 (Pleskot et al., 2012). This makes phosphoinositides potential central players in the regulation of cytoskeleton dynamics versus vesicle trafficking. Phosphatidylinositol-4 kinases (PI4Ks) are the first enzymes committing PI into phosphoinositides phosphorylated at position 4 of inositol. The study of these enzymes and of their corresponding mutants can be useful to investigate the regulation of the vesicle trafficking together with cytoskeleton dynamics. In *Arabidopsis thaliana,* the isoforms At-PI4Kβ1 and AtPI4Kβ2 appear to be located in the endomembrane system (Antignani et al., 2015; Kang et al., 2011). They participate in cytokinesis in *A.thaliana* roots by targeting actin filaments to the cell plate (Lin et al., 2019).

Regarding cytoskeleton and the responses to pathogens, it must be noted that soon after contact between a plant cell and a pathogen, actin filaments get rapidly reordered to support transport of the stress-related compounds. For instance, in the case of fungal infection, actin is needed for the transport of antimicrobial compounds and callose to the penetration site (Hardham, 2007). This results in forming cell wall appositions that arrest pathogen growth at the invaded cell (Sassmann et al., 2018). This is part of plant immunity response. In case of bacterial infection, the reaction at the level of cytoskeleton is more diffuse along the leaf tissue but an increase of actin filament density and bundling can nevertheless be measured (Lu and Day, 2017). Various components of pathogen cells can trigger actin polymerization in plants. The most described are the so-called microbe associated molecular patterns (MAMPs), like flagellin flg22, elf18, chitin, oligogalacturonides (Li et al., 2017), or secreted effectors (*e.g.* HopG1 of *Pseudomonas syringae*, (Shimono et al., 2016). Those compounds not only affect cytoskeleton, but induce other immune responses, especially reactive oxygen species formation, defence genes expression, biosynthesis of salicylic acid (SA) (Bigeard et al., 2015).

A well described response to pathogens is callose accumulation (Schneider et al., 2016). Callose is a polysaccharide, β-1,3-glucan, synthetized in the apoplast and involved in various processes in plants including cytokinesis (Chen et al., 2009; Thiele et al., 2009), pollen development (Záveská Drábková and Honys, 2017), cell-to-cell communication through plasmodesmata (Cui and Lee, 2016), and various stress responses. Arabidopsis genome encodes twelve callose synthases, each of a specific function (Chen and Kim, 2009). Callose synthase 12 (also known as GLUCAN-SYNTHASE LIKE 5, GSL5, of POWDERY MILDEW RESISTANT 4, PMR4) is the one responsive to wounding and pathogen responses (Ellinger et al., 2014; Keppler et al., 2018). Callose biosynthesis in the apoplast and its accumulation highly depend on the transport of the substrates outside of the cells. Notably, distinct signalling pathways may result in callose formation. For instance, wounding-induced and MAMP-triggered callose might represent different branches of responses going through PMR4 (Keppler et al., 2018). We therefore wanted to investigate the influence of actin depolymerization on callose accumulation. An elegant way to investigate the role of cytoskeleton is the use of drugs that alter cytoskeletal dynamics. Latrunculin B (latB) is such a drug. It originates from a Red Sea sponge *Latrunculia magnificans*. LatB reversely binds actin monomers preventing their polymerization (Morton et al., 2000). It is often used in plant biology to assess the role of actin in endo- and exocytosis (Moscatelli et al., 2012), pollen tube elongation (Gibbon et al., 1999) and growth (Baluška et al., 2001). Concerning the response to pathogens, it has been shown that when applied simultaneously with pathogens, latB negatively affects plant resistance to them (Henty-Ridilla et al., 2013). However, latB is able to activate SA-associated gene expression in *Arabidopsis thaliana* (Matoušková et al., 2014). In *Brassica napus* and *Nicotiana benthamiana,* the pre-treatment of plant leaves with low concentrations of latB - increased SA levels in tissues, activated defence gene expression and was sufficient to increase resistance to further infection. Similar results were obtained with another actin disrupting drug, cytochalasin E (Leontovyčova et al., 2019).

We firstly show that actin disruption by latB triggers callose accumulation in *A. thaliana* seedlings through the callose synthase PMR4. Using mutants altered in SA signalling (*pad4*), biosynthesis (*sid2*) and accumulation (*NahG*), we demonstrate that this callose deposition is SA-independent. Furthermore, we found that latB effect on gene expression does not correspond to a general transcriptome deregulation, but that specific pathways related to the response to pathogens are the ones triggered by latB. Amongst the gene responses, we were also able to reveal SA-dependent and SA-independent effects of latB. Interestingly, we show that deficiency in PI4Kβ1β2 enhanced the susceptibility of actin filaments to latB while latB does not appear to impact phosphoinositides levels on plasma membrane. To study the role of PI4Kβ1β2 in SA-independent responses to actin disruption, we have introduced *NahG* construct as well as *sid2* and *pad4* mutations into *pi4kβ1β2* background. Surprisingly, the latB treatment still strongly induced SA in *pad4pi4kβ1β2* and *NahGpi4kβ1β2* triple mutants, while SA accumulation was prevented in *pad4* and*NahG* mutant plants. To conclude, here we characterize the immunity-like responses induced by latB, showing that actin depolymerisation acts through both SA-dependent and SA-independent pathways. Moreover, we reveal the existence of an unexpected cross-talk between actin filaments and phosphoinositides in the regulation of those defence-related responses.

2. Materials and methods

2.1. Plant material

The following *Arabidopsis thaliana* genotypes were used: Columbia-0 (WT); *NahG* (Delaney et al., 1994); *NahGpi4kβ1β2* (Šašek et al., 2014); *pad4.1* (NASC stock N3806); *pad4pi4kβ1β2* (this study); *sid2, pi4kβ1β2, sid2pi4kβ1β2* (Kalachova et al., 2019); *pmr4*; pUBQ::PMR4-GFP in WT background; pUBQ::PMR4-GFP in *pmr4* background (Kulich et al., 2018); Lifeact-GFP (Cvrčková and Oulehlová, 2017); pUBQ::mCitrine-2xFAPP1 (Simon et al., 2014).

2.2. Plant cultivation and treatment

Seedlings were grown in MS medium, containing 4.41 g Murashige and Skoog medium including vitamins (Duchefa, Netherlands) and 5 g sucrose per litre, pH adjusted to 5.7 using 1 M KOH. Surface-sterilized seeds were sown in 24-well plates, 4–5 seeds per well containing 400 µL of medium and cultivated for 10 days before treatment under a short-day photoperiod (10 h/14 h light/dark regime) at 100–130 µE m^{-2} s^{-1} and 22 °C. On the 7th day, 200 µL of fresh medium was added to the wells.

Latrunculin B (latB, Sigma-Aldrich, USA) was dissolved in DMSO at 2 mM stock solution. The seedlings were treated 24 h with 200 nM latB and 0.01% DMSO as mock control prior to sampling.

2.3. Gene expression analysis

The whole seedlings (approximatively 300 µg fresh weight) from three wells were immediately frozen in liquid nitrogen. The tissue was homogenized in plastic Eppendorf tubes with 1 g of 1.3 mm silica beads using a FastPrep-24 instrument (MP Biomedicals, USA). Total RNA was isolated using Spectrum Plant Total RNA kit (Sigma-Aldrich, USA) and

treated with a DNA-free kit (Ambion, USA). Subsequently, 1 µg of RNA was converted into cDNA with M-MLV RNase H– Point Mutant reverse transcriptase (Promega Corp., USA) and an anchored oligo dT21 primer (Metabion, Germany). Gene expression was quantified by qPCR using a LightCycler 480 SYBR Green I Master kit and LightCycler 480 (Roche, Switzerland). The PCR conditions were 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s. Melting curve analysis was then conducted. Relative expression was normalized to the housekeeping gene *AtTIP41*. A list of the analysed genes and primers is available in Supplemental table ST1.

2.4. Trypan blue and anilin blue staining

Seedlings were immersed into the staining solution (10 mL lactic acid (85%, w:w), 10 mL phenol, 10 mL glycerol, 10 mL distilled H_2O, 40 mg trypan blue) for 2 min (Fernández-Bautista et al., 2016). Seedlings were then washed twice in ethanol:glacial acetic acid (1:3, v:v) until leaves were fully decoloured. Seedlings were rehydrated by incubating in ethanol solutions of decreasing concentrations (70%, 50%, 30%, v:v) for at least 30 min each. They were then stained in 0.01% aniline blue in 150 mM K_2HPO_4, pH 9.5 for 4 h and kept in water for the microscopy observation.

2.5. Callose imaging and quantification

Callose deposition was observed by fluorescence microscope using a Zeiss AxioImager ApoTome2 (objective 5x). Callose accumulation was calculated using FIJI software (https://fiji.sc/) (Schindelin et al., 2012) as the percent occupancy of aniline blue signal (spots) per region of interest (ROI). One round-shaped ROI (d = 1500 µm) was taken from one cotyledon, at least 18 independent cotyledons were analysed per variant.

2.6. Confocal microscopy

For *in vivo* microscopy, a Zeiss LSM 880 inverted confocal laser scanning microscope (Carl Zeiss AG, Germany) was used with either a 40 × C-Apochromat objective (NA = 1.2 W) or a 20x Plan-Apochromat objective (NA = 0.8). Fluorescence associated with actin filaments (LifeAct-GFP) or phosphoinositides (mCitrine-2xFAPP1) was acquired at excitation 488 nm emission 490–540 nm for GFP and excitation 516 nm emission 520–552 nm for mCitrine. Images were acquired at in z-stacks 20 µm thickness). Actin filaments density analysis was calculated by Fiji software as the percent occupancy of GFP/mCitrine signal in each Maximum intensity projection. For Lifeact-GFP, image threshold was set to include all actin filaments and area fraction was measured. For each variant, fluorescent intensity of at least 30 ROI (50 x 50 µm) from 10 to 14 leaves was analysed.

2.7. Salicylic acid measurement

All seedlings from 3 wells were sampled as 1 sample, frozen in nitrogen and stored at −80 °C until the analysis. Samples were homogenized in tubes with 1.3 mm silica beads using a FastPrep-24 instrument (MP Biomedicals, CA, United States) with 2 × 500 µl of extraction reagent methanol/water/formic acid (15:4:1, v:v:v) supplemented with stable isotope-labelled 13C-SA internal standards, each at 100 ng per sample, to check the recovery during the purification and to validate the quantification. Extract was subjected to solid phase extraction using Oasis MCX cartridges (Waters Co., Milford, MA, United States) and eluted with methanol. The eluate was evaporated to dryness and dissolved in 30 µl of 15% acetonitrile/water (v/v) immediately before the analysis. immediately before the analysis. Quantification was performed on an Ultimate 3000 high-performance liquid chromatograph (UHPLC, Dionex; Thermo Fisher Scientific, Waltham, MA, United States) coupled to IMPACT II Q-TOF ultra-high resolution and high mass accuracy mass spectrometer (HRAM-MS; Bruker Daltonik, Bremen, Germany). Separation was carried out using column Acclaim RSLC 120 C18 (2.2 µm, 2.1 x 100 mm; Thermo Fisher Scientific, Waltham, MA, United States) tempered at 30 °C and mobile phase consisting of 0.1% formic acid (A) and methanol (B) by gradient elution. The gradient started at 1%B and going to 39% B in 3 min, 60% B in 7 min, 100% B in 8 min, followed by 3 min of washing by 100% B and 5 min equilibration at initial conditions (1%B). Flow rate was changing during separation from 200 µl/min to 300 µl/min in 7 min and then kept at this rate to 16 min. Injection volume was 5 µL. The full-scan data were recorded in negative electrospray ionization (ESI) mode and processed using Data Analysis 4.3 and TASQ 1.4 software (both Bruker Daltonik, Bremen, Germany). SA and labelled internal standard ($^{13}C_6$-SA) were monitored as ions of their deprotonated molecules [M − H]- (137.0239 m/z and 143.0440 m/z, respectively). SA content was expressed in ng/g fresh weight (FW).

3. Results

3.1. Latrunculin B induces callose deposition via PMR4 callose synthase activity

Ten-day-old *A. thaliana* seedlings were treated for 24 h with 200 nM latB. As expected, latB treatment caused actin disruption, measured as the decrease of filament abundancy (Fig. S1). The concentration used for the drug did not lead to cell death (Fig. S1). More unexpectedly, the treatment also caused callose accumulation (measured as the area occupied with callose spots) (Fig. 1A,B).

Among twelve known callose synthases in *A. thaliana,* PMR4 was shown to be a major one involved to biotic stress responses (Nishimura et al., 2003; Kulich et al., 2018). We therefore tested its contribution to latB-triggered callose. In the *pmr4* mutant seedlings, the callose level detected after latB treatment was nearly equal to that in mock-treated control. When a pUBQ::PMR4-GFP construct was expressed in WT or *pmr4* backgrounds, basal callose level in cotyledons remained low. Yet, when latB was added, callose accumulation was restored (Fig. 1B). Besides, such an induction did not rely on transcriptional changes, as soon as the expression of *PMR4* was not affected (Fig. 1C).

3.2. Latrunculin B effects on callose accumulation is SA-independent

The fact that latB treatment causes callose accumulation through PMR4 strongly suggests that other defence-related responses should be studied. We therefore decided to study the role of SA content and signalling pathway in the observed callose deposition. To do so, we first measured SA levels in seedlings after latB treatment. A 20-fold increase of SA was observed in WT seedlings. Notably, *pmr4* plants behaved similarly to WT, suggesting callose to be either downstream, either independent on SA (Fig. 1D, Supplementary table ST3). To address this, we analyzed SA induction and callose deposition in plants with altered SA pathway: *NahG* mutant plants, expressing the bacterial salicylate hydroxylase that degrades SA to inactive catechol and therefore unable to accumulate SA; *sid2* plants, deficient in ISOCHORISMATE SYNTHASE 1 (ICS1); and *pad4* plants, with a mutation in PHYTOALEXIN DEFICIENT 4 (PAD4) (Fig. 2, Supplementary table ST3). PAD4 protein has been shown to regulate the synthesis of SA *via* the ICS1 pathway (Cui et al., 2017). As expected, both *NahG* and *pad4* plants had lower basal content of SA, and smaller (approx. 5-fold) increase in SA after latB comparing to WT. *Sid2* plants showed no increase in SA, pointing out that the detected SA was produced by ICS1. On the contrary, callose production was not affected any of the studied mutants, confirming SA-independent nature of the process (Fig. 2B,C).

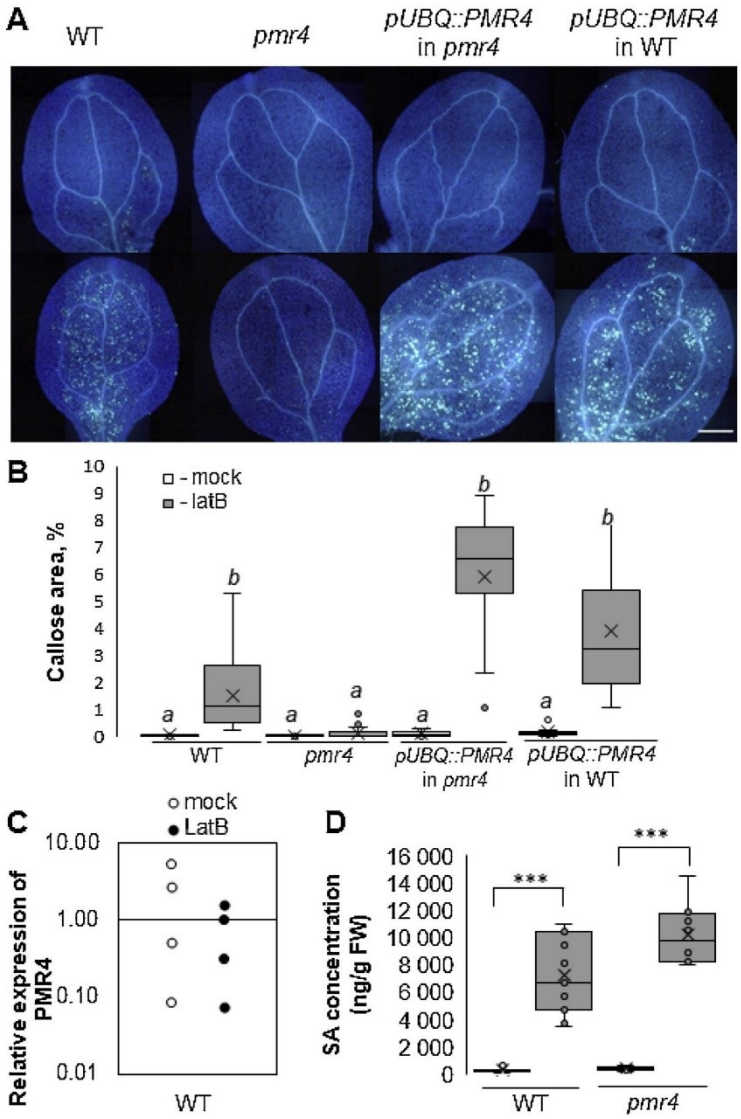

Fig. 1. Latrunculin B (latB) induces PMR4-dependent callose accumulation. Ten days old *A. thaliana* seedlings were treated for 24 h with 0.05% DMSO (mock) of 200 nM latB. A. Representative images of aniline blue stained cotyledons of WT, *pmr4*, pUBQ::PMR4-GFP in *pmr4* and pUBQ::PMR4-GFP in WT, epi-fluorescent microscopy, scale bar = 500 µm. B. Quantification of the relative area occupied with callose particles, n = 18. Values for the mock-treated plants are presented in white boxes, latB treated - in dark grey boxes. In the plots, central line represents the median occupancy, cross represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the ends of whiskers are set at 1.5 times the interquartile range. Values outside this range are shown as outliers. Different letters indicate variants that are significantly different, one-way ANOVA with Tukey-HSD test, $p < 0.05$. C. Relative expression of *PMR4*. Relative expression of *PMR4* in WT plants. Values were normalized for the untreated WT. *TIP41* was used as the reference gene. D. Salicylic acid content. *** - variants are significantly different, two-tailed *t*-test, $p < 0.001$ n = 8.

3.3. Latrunculin B affects gene expression in both SA-dependent and -independent manner

We had already shown that latB could induce the expression of some genes related to plant defence (Leontovyčova et al., 2019; Matoušková et al., 2014). Here we studied whether intracellular level of SA or an intact SA-signalling pathway is needed for latB to trigger gene expression changes (Fig. 3, Supplementary Fig. S2, Supplementary table ST2). To do so, we analysed the expression of several defence marker genes in *NahG* and *pad4* mutants. Eleven-day-old *in vitro* grown plants (whole seedlings) of WT and mutant genotypes were harvested for gene expression studies 24 hpt by 200 nM latB. Concerning the gene response in WT plants, we show here that the defence-associated *PR1, PR2, WRKY38, ICS1* and *PAD4* were induced by latB (Fig. 3). We also detected an activation of the wounding marker *BAP1*, even though no cell death was yet detected by trypan blue staining (Supplemental Fig. S1). On the contrary, no significant changes were observed in the expression of SA biosynthetic genes other than *ICS1* (*ICS2, PAL1, PAL2, PAL3*). To be noted, ICS2 expression seems to be very dependent on the growth conditions, as we previously detected its induction after latB treatment (Leontovyčova et al., 2019). In addition, neither jasmonate biosynthesis marker genes (*LOX2, AOS1*), nor jasmonate responsive genes (*VSP2* and *PDF1.2*), nor ethylene marker gene (*ERF1*) were significantly affected by the treatment (Supplemental Fig. S2, Supplemental Table ST2).

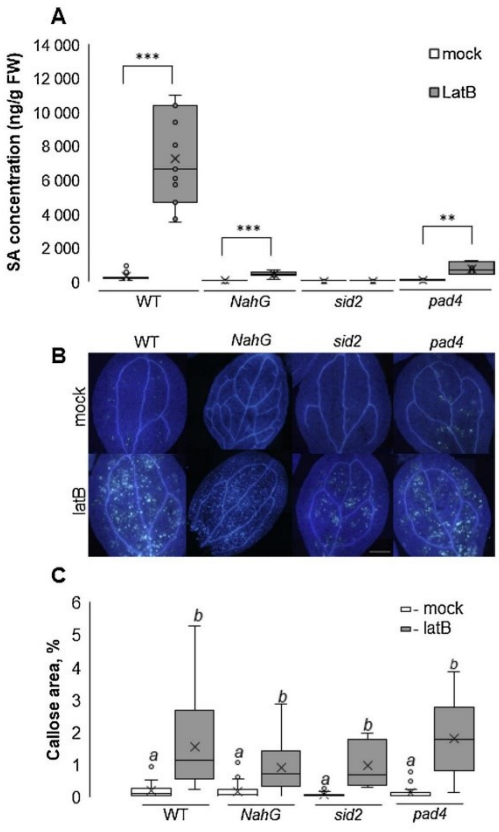

Fig. 2. SA-independent induction of callose accumulation by latB treatment. Ten-day-old seedlings of WT, *NahG*, *sid2* and *pad4* were treated by 200 nM latB or 0.01% DMSO as the control. Seedlings were harvested 24 h after the treatment A. SA content. *** - samples are different, $p < 0.001$, two-tailed *t*-test. B. Representative images of aniline blue stained cotyledons, scale bar 500 µm. C. Relative area of callose particles. Central line of the boxplot represents the median occupancy, cross represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the ends of whiskers are set at 1.5 times the interquartile range. Values outside this range are shown as outliers. Different letters indicate variants that are significantly different, one-way ANOVA with Tukey-HSD test, $p < 0.05$. At least 30 independent cotyledons were analysed for each variant.

However, we detected that *PR1* and *WRKY38* inductions by latB were abolished in *NahG* and *pad4* mutants, strongly suggesting these inductions rely on a SA-dependent pathway (fig. 3). This also involves that SA is accumulated in response to latB (Fig. 2A). On the contrary, the expression of *PR2* in response to latB appears not to be dependent on SA since it was still present in *NahG* and *pad4* mutants. Similarly, the expression of the *PAD4* by latB was still present in *NahG* plants: it does not rely on SA accumulation. The expression of *ICS1* in response to latB does not appear to be controlled by SA level in the tissues, as seen in *NahG* mutant. Yet, PAD4 protein regulates *ICS1* expression, at least partly. As for the induction of the expression of *BAP1* by latB, it was diminished in *NahG* and *pad4* mutants (though still present), and so can be considered as partly SA-dependent.

We can thus conclude that latB induces several defence-like responses: there is an induction of SA; it leads to the induction of *PR1* and *WRKY38*. However, an alternative, SA-independent, pathway also exists. It controls the expression of *PR2*, *ICS1*, *PAD4* and callose accumulation in response to latB.

3.4. Effects of latB on cytoskeleton are enhanced in pi4kß1ß2 double mutant

Phosphoinositides, that are the phosphorylated forms of phosphatidylinositol (PI), are involved in vesicular trafficking. This process requires correct actin cytoskeleton organization (Pleskot et al., 2014). There is therefore a link between actin cytoskeleton and phosphoinositides. We wanted to investigate whether the effect of latB could be impacted by a modification in phosphoinositide metabolism. To do so, we used the *pi4kß1ß2* double mutant. This mutant is invalidated in the two beta isoforms of phosphatidylinositol-4-kinases. This double mutant was shown to have impaired vesicle trafficking, dwarf phenotype and increased SA level (Kang et al., 2011; Šašek et al., 2014).

We firstly introduced LifeAct-GFP construct into *pi4kß1ß2* background by crossing. Four independent lines were tested. No major defects in actin filaments organization in epidermal cells were observed while compared to WT (Fig. 4). After 24 h of latB treatment, the density of the filaments in *pi4kß1ß2* plants was 20-times lower than that in non-treated double mutant, while in the WT background this decrease was 5-fold. Consequently, in *pi4kß1ß2* plants the actin filaments were almost lost while in WT their occupancy was still 7% (Fig. 4B). The actin cytoskeleton of *pi4kß1ß2* mutants thus appear to be more sensitive to latB. Meanwhile, we wanted to evaluate the impact of latB directly on the PI4P content using genetically encoded biosensor mCitrine-2xFAPP1 for PI4P (Simon et al., 2014). The sensor mostly labels PI4P localized in plasma membrane. In our setup, the content of PI4P remained stable in response to latB (Fig. S3A, C).

3.5. Effects of latB on callose accumulation in pi4kß1ß2 double mutant is still SA-independent

Because it seems there was a positive (enhancing) interaction between latB and PI4Kß mutations on actin filaments, we studied SA levels, callose accumulation and gene expression responses to latB in *pi4kß1ß2* plants. Indeed, latB triggered SA increase in *pi4kß1ß2* plants (Fig. 5A). Surprisingly, introduction of *NahG* construct or *pad4* mutation into *pi4kß1ß2* background did not fully prevent SA increase as it did in WT background (Fig. 2A). However, *sid2* mutation was sufficient to block SA production in both WT and *pi4kß1ß2*, confirming that the entire SA pool induced by latB treatment is synthetized due to ICS1 activity in both backgrounds. As for callose, its basal levels in 8-, 10- and 14-day-old *pi4kß1ß2* seedlings were similar to that of WT (Supplemental Fig. S4). To study the involvement of SA, the callose accumulation was also tested in triple mutants *NahGpi4kß1ß2* (Šašek et al., 2014), *sid2pi4kß1ß2* (Kalachova et al., 2019) and *pad4pi4kß1ß2*, obtained by crossing *pi4kß1ß2* to *pad4* (homozygous F3 plants were used). After latB treatment, accumulation of callose in all studied mutants was similar to that in WT (Fig. 5B), suggesting that callose response to latB in *pi4kß1ß2* is still not SA-dependent (Fig. 5B). This is consistent with our findings in WT plants.

3.6. Effects of latrunculin B on defence genes expression in pi4kß1ß2 double mutant

As for *PR1* and *WRKY38*, after latB treatment their induction in *pi4kß1ß2* was comparable to that in WT. Yet, contrary to what was observed in WT background, their induction was also observed in *NahGpi4kß1ß2* and *pad4pi4kß1ß2* triple mutants (Fig. 6). This is in agreement with the fact that SA is still accumulated in these triple mutants. *BAP1* expression in PI4K deficient mutants after latB treatment was induced less than in WT and was partly SA-dependent

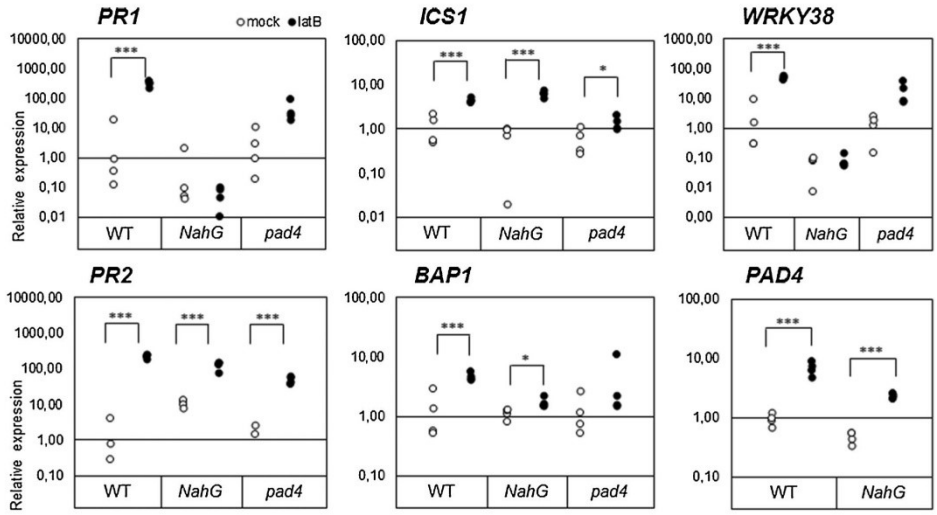

Fig. 3. SA-independent expression of defence-related genes by latB treatment. Ten-day-old seedlings of WT, *NahG* and *pad4* were treated by 200 nM latB or 0.01% DMSO as the control. Seedlings were harvested 24 h after the treatment. Data from the representative experiment are shown. Values were normalized for mock treated WT, note that data are plotted at the $\log_{10}$ scale. Asterisks indicate values, different from respective controls for each genotype, * - $p < 0.05$; *** - $p < 0.001$, two-tailed Student *t*-test). Reference gene *TIP41*.

(Fig. 6). Besides, neither induction of SA biosynthetic enzymes other than *ICS1* was detected, nor of JA-, ABA-, or ethylene markers (Supplemental Fig. S5, supplemental table ST2).

4. Discussion

We show here that latrunculin B induces callose accumulation in seedlings. It is also true for cytochalasin E, another actin filament disruptor (data not shown). Therefore, it is very likely that this effect of latB is due to actin depolymerization. Callose deposition was reported in responses to various stresses, like wounding or pathogen infection (Jacobs et al., 2003). We had previously shown that chemical disruption of actin led to some immunity-related responses such as activation of *PR1* expression (Matoušková et al., 2014). In this regard, callose is particularly important, as another biotic-stress-like response, partly connected with SA. Arabidopsis genome encodes twelve callose synthases (Voigt, 2014). PMR4 (POWLDERY MILDEW RESISTANT 4, also called GLUCAN SYNTHASE LIKE 5, GLS5 or CALS12) is precisely the one activated during infection (Nishimura et al., 2003). Here we first show that callose accumulation induced by latB fully relies on PMR4. It indicates its connection with a biotic stress. Interestingly, using *NahG* and *pad4* mutants, we show that latrunculin B effects on callose accumulation is SA-independent and not downstream of PAD4. To be noted, *pmr4* mutant itself was reported to have a constitutively activated SA pathway in adult plants and contribute to most of callose accumulations in studies connected with biotics stres (Nishimura et al., 2003). Nevertheless, SA level in *pmr4* seedlings did not differ from that in WT (Supplementary table ST3). It was also true for *pi4kß1ß2* plants, known

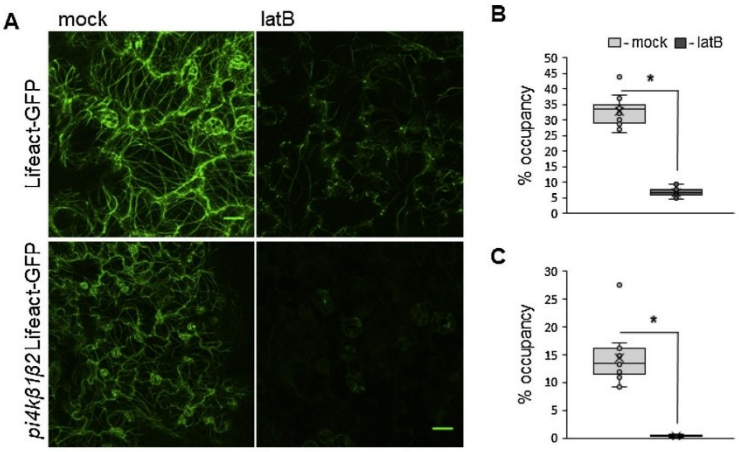

Fig. 4. Effect of latB on actin filaments organization of *pi4kß1ß2* mutants. A. Representative images of maximum intensity projections of 10 µm abaxial epidermis of LifeAct-GFP expressing WT and *pi4kß1ß2* plants. Confocal microscopy, scale bar = 20 µm. Quantification of relative area occupied with fluorescent filament in WT (B) and *pi4kß1ß2* (C). For each variant at least 15 independent seedlings were imaged, n = 12. For quantification, maximµm intensity projections of 10 µm Z-stacks of abaxial sides of the leaves were used. At least 30 regions of interest (ROI) measured for each treatment. In the plots, central line represents the median occupancy, cross represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the ends of whiskers are set at 1.5 times the interquartile range. Values outside this range are shown as outliers. Asterisks indicate statistically significant differences with the mock treatment, Student *t*-test, $p < 0.05$.

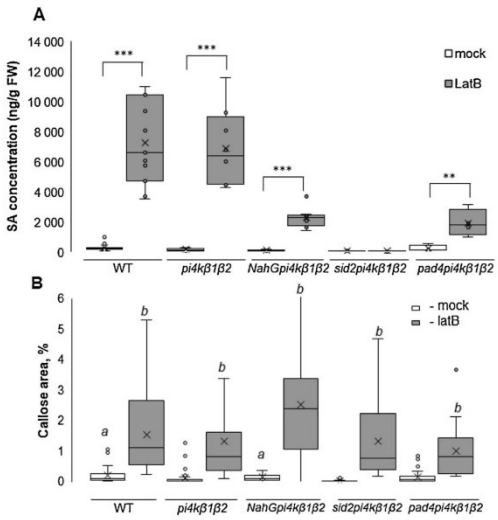

Fig. 5. LatB induced SA and callose accumulation in *pi4kβ1β2* mutants. Ten-days-old seedlings were treated by 200 nM latB or 0.01% DMSO as mock control. Seedlings were harvested 24 h after the treatment. A. SA content. Data from two independent experiments are plotted. B. Relative area of callose particles in the cotyledons. At least 30 independent cotyledons were analysed for each variant. Experiment was repeated three times with similar results, the data from a representative repetition are shown. In boxplots, central line represents the median occupancy, cross represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the ends of whiskers are set at 1.5 times the interquartile range. Values outside this range are shown as outliers. Different letters mark significantly different values, $p < 0.05$; Student *t*-test, unequal variance.

as SA and callose accumulators while being grown up to 4-weeks (Kalachova et al., 2019). However, neither SA nor callose levels were increased in non-stimulated seedlings (Fig. 5). A negative interaction between actin polymerisation, callose and SA levels has been suggested recently: natural polymorphism of genetic disruption of Actin Related Protein Complex 4 (ARPC4) affected pathogen- and wounding-induced callose deposition, as well as affected expression of *PR1* in response to *Sclerotinia sclerotiorum* (Badet et al., 2019).

As latB action on callose appeared as SA-independent, we wanted to check whether the effect of latB on gene expression was also independent on SA. We first better detailed the changes in gene expression triggered by latB. We show here that *PR1, PR2, WRKY38, ICS1* and *PAD4* were induced by latB in WT. It is also the case for a marker of cell death in pathogen and wounding responses, *BAP1* (Yang et al., 2007). *BAP1* was described as wounding marker, but also as a SA-associated gene: it negatively regulates cell death in response to *Pseudomonas syringae* pv *tomato* and *Hyaloperonospora parasitica* acting in the same pathway with *BON1*, *SNC1* and *PAD4* (Yang et al., 2006) *BAP1* expression can be induced by high temperature or ROS formation, however this induction was abolished in mutants with altered SA pathways (*NahG*, *pad4*) (Hedtmann et al., 2017; Zhu et al., 2011). In our setup, the expression of BAP1 was increased independently of the SA content in plants, indicating a new role of this gene in plant stress responses. Yet, no significant changes were observed in the expression of SA biosynthetic genes other than ICS1 (*ICS2, PAL1, PAL2, PAL3*), nor in the expression of *LOX2* and *AOS*, a JA-biosynthesis marker genes, nor to the jasmonate responsive genes *VSP2* and *PDF1.2*. Genes involved in ethylene biosynthesis (*ACS2*) (Denoux et al., 2008), ethylene response (*ERF1*, *LEA4-1*)(Lorenzo et al., 2003) or to the response of abscisic acid (*ABI1*)(Leung et al., 1997) were not induced by latB. Therefore, it seems that latB action is not "universal", that it does not lead to a deregulation of the whole transcriptome. Its action seems to be directed mainly towards the SA signaling pathway or toward response to biotrophic pathogens.

Clearly, a SA dependent pathway is activated by latB. Expression of *PR1*, a classical representative of defence genes, is usually induced during pathogen attack, but also as a reaction to any SA increase. Besides, *ICS1*, that is responsible for most of the SA accumulation in response to bacteria (Li et al., 2019; Wildermuth et al., 2001; Zheng et al., 2015), was strongly induced. Therefore, the question was the following: did the changes in gene expression, that target SA responsive genes, occur through a SA-dependent pathway. We could show that the expression of *PR1* and *WRKY38* by latB relied on SA accumulation and a functional PAD4 protein. The fact that *ICS1* expression is controlled by PAD4 in response to pathogen was established (Cui et al., 2017). Yet, the expression of *PR2* and *ICS1* is independent on SA, as seen in the

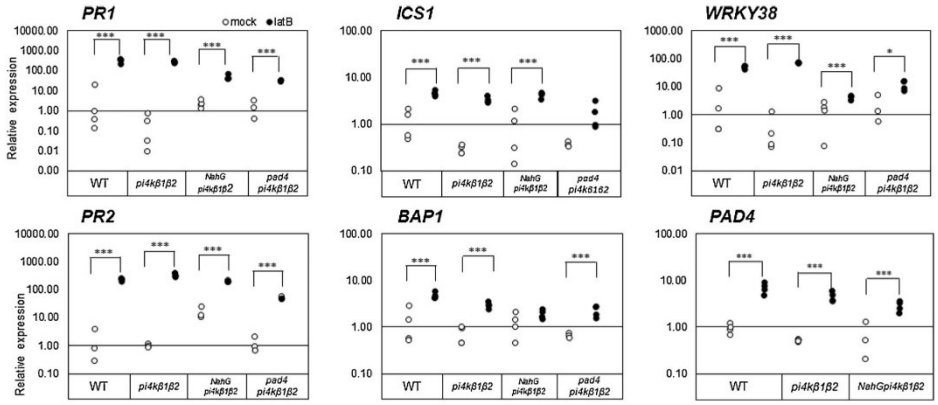

Fig. 6. LatB–induced gene expression in *pi4kβ1β2* mutants. Ten-days-old seedlings of WT, *pi4kβ1β2, NahGpi4kβ1β2* and *pad4pi4kβ1β2* were treated by 200 nM latB or 0.01% DMSO as mock control. Seedlings were harvested 24 h after the treatment. Data are normalized to mock-treated WT, note that data are plotted at the $\log_{10}$ scale. Asterisks indicate values, different from respective controls for each genotype, * - $p < 0.05$; *** - $p < 0.001$, two-tailed Student *t*-test). Reference gene *TIP41*.

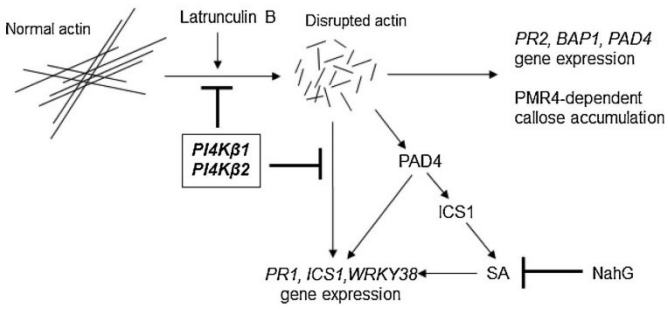

Fig. 7. Graphical summary of the latrunculin B effects in seedlings. The disruption of actin polymers by latB activates two kinds of signalling pathways. A first pathway is independent on SA; it leads to the expression of genes such as *PR2*, but also to callose accumulation. The second pathway involves SA synthesis *via* PAD4 and ICS1; it leads to the expression of genes such as *PR1* and *WRKY38*. The double mutation in PI4K beta1 and beta2 makes the effect of latB on filaments more pronounced. Interestingly, the PR1 expression in response to latB is only partly SA-dependent in the *pi4kß1ß2* background: a SA independent pathway leading to PR1 expression is thus suggested.

NahGpi4kß1ß2. *PR2* is one of the early defence genes, usually co-expressed with *PR1* and *PR5* and used as a marker of SA pathway (Hamamouch et al., 2011). Nevertheless, here we describe its clear SA-independent induction. It might seem contradictory, unless we take into account that PR2 protein is β-1,3-glucanase, particularly important for antifungal defences (Ali et al., 2018). PR2 is crucial for callose degradation and contributes to the SA increase; its overexpression enhances resistance both to fungal and bacterial pathogens (Oide et al., 2013).

It thus appears that latB (likely through its action on actin filaments) activates a signaling pathway typical of the response to pathogens. It activates PAD4. Some of the PAD4 action leads to *ICS1* expression, to SA accumulation and to the expression of SA responsive genes. Yet, a part of PAD4 action is not dependent on SA, as seen by *ICS1* expression. LatB also triggers a pathway that does not involve PAD4 nor SA, and that leads to callose accumulation and *PR2* expression.

The action of latB is likely to occur through actin filament depolymerization. Actin filaments are involved in the control of vesicle trafficking. Phosphoinositides are also involved in vesicular trafficking. There is indeed an interaction/interplay between actin filaments and phosphoinositides (Pleskot et al., 2014). F-actin capping protein interaction with actin monomers is regulated by phosphatidic acid and phosphatidylinositol-4,5-bisphosphate, a phosphoinositide (Pleskot et al., 2012). In pollen tube, cytoskeletal disruption by latB or jasplakinolide not only affects actin and arrested tube growth, but also causes relocalization and even dissociation of RabA4a and RabA4b-tagged vesicles (Zhang et al., 2010). Interestingly, PI4Kβ1 and PI4Kβ2 were shown to be recruited by RabA4b. Due to this interaction between actin and phosphoinositides in relation to vesicle trafficking, we were interested to investigate whether the action of latB could be impacted by alteration in phosphoinositide metabolism. To address this, *pi4kß1ß2* mutant was a tool of choice. As already mentioned, PI4Ks are the first enzymes that commit PI toward the synthesis of phosphoinositides, and the beta isoforms PI4Kβ1 and PI4Kβ2 were shown to be recruited by RabA4b. PI4Kbeta proteins participate in cytokinesis in Arabidopsis roots by targeting actin filaments to the cell plate (Lin et al., 2019). Nevertheless, it is important to consider the fact that *pi4kß1ß2* plants exhibit constitutive activation of SA-related genes and high basal levels of callose. Yet it is true for mature 4 week-old soil-grown plants (Antignani et al., 2015; Kalachova et al., 2019; Šašek et al., 2014). In younger plants, no *PR1* overexpression was detected (Sasek et al., 2014). In the present study we used 10-day-old seedlings grown *in vitro*. In these conditions, neither SA accumulation (fig. 5A, Supplementary table ST3), nor constitutively high expression of *PR1* and *ICS1* was observed (Fig. 6).

The actin cytoskeleton of *pi4kß1ß2* mutants appear to be more sensitive to latB (fig. 4). This is consistent with the above mentioned references which show F-actin capping protein interaction with actin monomers is regulated by phosphatidic acid and phosphatidylinositol-4,5-bisphosphate, a phosphoinositide (Pleskot et al., 2012). In response to latB, *pi4kß1ß2* seedlings accumulated callose, in a SA- and PAD4-independent way, as observed in WT plants. Yet, when we assay the SA increase in response to latB, a process not observed in WT is then detected: the SA induction in *pi4kß1ß2* background still occurs, despite the mutation in PAD4 or expression of NahG. The *pi4kß1ß2* double mutation allows to reveal a signalling pathway, triggered here by latB, that leads to SA biosynthesis and *PR1* expression independently on PAD4. Yet, the *PR1* expression in response to latB was not only observed in *NahGpi4kß1ß2* and *pad4pi4kß1ß2* triple mutants, but also in *sid2pi4kß1ß2* (data not shown). Therefore, our data on the existence of such a SA independent pathway are strong. Is this pathway usually actively inhibited by functional PI4Ks? Is it linked to the effect of *pi4kß1ß2* double mutation on trafficking? Or is it due to a role of phosphoinositides on signalling? An important contribution may come from immune-related MAPKs, whose transient or sustained activation works as a switch between pattern-triggered immunity and effector triggered immunity (Tsuda et al., 2013). Indeed, *Tsuda et al.* described expression of *PR1* in *sid2* background, induced by constitutive activation of MPK3 and MPK6. Another enzyme of the cascade, MPK4, was shown to interact with PI4Kβ1 during cell plate formation in cytokinesis (Lin et al., 2019). More research is necessary to understand and characterize this pathway.

To summarize, we here report the defence-like consequences of actin cytoskeleton disruption by latB (Fig. 7). Two kinds of signalling pathways were activated by latB treatment: a first pathway is independent on SA; it leads to the expression of genes such as *PR2*, but also to callose accumulation through PMR4. The second pathway involves SA synthesis *via* PAD4 and ICS1; it leads to the expression of genes such as *PR1* and *WRKY38*. The double mutation in PI4Kß1ß2 makes the effect of latB on filaments more pronounced. Interestingly, the SA increase and *PR1* expression in response to latB in the *pi4kß1ß2* background is also triggered in an unconventional, PAD4-independent pathway. Either this pathway is actively inhibited in WT background by PI4Kß1ß2 (and thus not seen), or it is activated only when the PI4Kß1ß2 are missing.

Still several open questions remain: what makes actin depolymerization trigger these pathways? Is it the disrupted actin *per se* that act? Or are these pathways activated as a response to a process altered due to lack of good filaments? Such a process could be a modified vesicle trafficking due the missing actin filaments. In this regard, it would be interesting to monitor the processing of receptors of MAMPs in seedlings submitted to artificial cytoskeleton depolymerization agents.

We believe this work sheds light on the connection between cytoskeleton dynamics, vesicular trafficking and immunity at the cellular and subcellular level and thus will contribute to better understanding of plant responses to changing environment.

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CRediT authorship contribution statement

Tetiana Kalachova: Conceptualization, Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Hana Leontovyčová:** Data curation, Formal analysis, Methodology, Writing - original draft. **Oksana Iakovenko:** Data curation, Formal analysis, Methodology. **Romana Pospíchalová:** Data curation. **Petr Maršík:** Data curation, Methodology. **Pavel Klouček:** Data curation. **Martin Janda:** Conceptualization, Writing - review & editing. **Olga Valentová:** Funding acquisition, Resources, Project administration, Writing - review & editing. **Daniela Kocourková:** Methodology. **Jan Martinec:** Funding acquisition, Resources, Project administration, Writing - review & editing. **Lenka Burketová:** Funding acquisition, Resources, Project administration, Writing - review & editing. **Eric Ruelland:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

Authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.103867.

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3.3. Publication 3

"Salicylic acid mutant collection" as a tool to explore the role of salicylic acid in regulation of plant growth under a changing environment

3.3.1. Summary of results

Salicylic acid is one of the most important phytohormones mediating plant defence. Level of SA rapidly increases upon pathogen attack and leads to typical „SA-related defence responses". However, some mutations and variations can lead to SA accumulation even in basal conditions. It has been described that SA accumulation leads to growth inhibition. In this study we characterized a collection of *A. thaliana* mutants impaired in SA signalling and accumulation in terms of growth under different cultivation conditions. The main aim of this study was to provide a variable tool for studying the role of SA connected with plant growth and stress tolerance.

We have established a collection of mutants impaired in the SA signalling pathway or were identified as SA-overaccumulators: two SA signalling mutants deficient in SA accumulation (*sid2*, *NahG*), eight suspected SA-overaccumulators (*cpr5-1*, *acd6-1*, *pi4kβ1/β2*, *fah1fah2*, *bon1-1*, *exo70B1-2*, *pmr4-1*, *edr2-6*) and four SA-overaccumulators crossed with SA-defficient mutants (*sid2/pi4kβ1/β2*, and *NahG/pi4kβ1/β2*, *NahG/edr2-6*, *bon1-1/snc1-11*).

First we analyzed growth of four-week-old plants under different light regimes, short day nad long day. Under long day conditions all plants except SA-defficent lines exhibited retarded rosette growth. Under short day conditions, the differences in growth retardation were less pronounced. We have simultaneously analyzed the SA content that confirmed overaccumulation of SA is linked with retarded rosette size. Surprisingly, the difference was more pronounced in short day conditions. This can be due to higher basal SA accumulation in long day. We further analyzed transcription of typical SA-pathway marker genes – *PR1* and *ICS1*. The gene transcription data revealed that higher level of SA mainly correlates with induced basal expression of *PR1* and *ICS1*. Next we measured photosynthesis intensity which revealed no significant differences.

In the second part of this study, we characterized *in vitro* setup of 11-day-old seedlings which are often used for studies of developmental changes. This system is highly artificial because of the exposure of roots to continuous light, so we hypothesized that also SA content and growth might differ from the 4-week-old plant system. The seedlings were grown under two light intensities: 450 $E.m^{-2}.s^{-1}$ and 170 $E.m^{-2}.s^{-1}$. Growth of WT plants was not affected but mutants with high SA content grew better under higher light intensity. The "reverted" mutants did not show such effect which suggests that SA plays a role in this phenomenon.

To summarize all the outcomes we performed correlation analysis regarding all tested parameters: rosette size, SA content, transcription of *ICS1* and *PR1* in 4-week-old plants grown in short day and long day; rosette weight and primary root length in *in vitro* grown seedlings cultivated in two light intensities. The correlation showed several clear outcomes: rosette size negatively correlates with SA content and SA-gene transcription, root growth was SA-independent. The study provides extensive characterization of 14 mutants affected in SA signalling which can be used as a tool for future immunity related studies.

3.3.2. My contribution

I optimized the method for salicylic acid extraction and performed the phytohormone extraction. I analyzed the data and contributed to writing of the manuscript.

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Article

"Salicylic Acid Mutant Collection" as a Tool to Explore the Role of Salicylic Acid in Regulation of Plant Growth under a Changing Environment

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Abstract: The phytohormone salicylic acid (SA) has a crucial role in plant physiology. Its role is best described in the context of plant response to pathogen attack. During infection, SA is rapidly accumulated throughout the green tissues and is important for both local and systemic defences. However, some genetic/metabolic variations can also result in SA overaccumulation in plants, even in basal conditions. To date, more than forty *Arabidopsis thaliana* mutants have been described as having enhanced endogenous SA levels or constitutively activated SA signalling pathways. In this study, we established a collection of mutants containing different SA levels due to diverse genetic modifications and distinct gene functions. We chose prototypic SA-overaccumulators (SA-OAs), such as *bon1-1*, but also "non-typical" ones such as *exo70b1-1*; the selection of OA is accompanied by their crosses with SA-deficient lines. Here, we extensively studied the plant development and SA level/signalling under various growth conditions in soil and in vitro, and showed a strong negative correlation between rosette size, SA content and *PR1*/*ICS1* transcript signature. SA-OAs (namely *cpr5*, *acd6*, *bon1-1*, *fah1*/*fah2* and *pi4kβ1β2*) had bigger rosettes under high light conditions, whereas WT plants did not. Our data provide new insights clarifying a link between SA and plant behaviour under environmental stresses. The presented SA mutant collection is thus a suitable tool to shed light on the mechanisms underlying trade-offs between growth and defence in plants.

Keywords: Salicylic acid; Arabidopsis mutants; light; growth; gene transcription

1. Introduction

Salicylic acid (SA; 2-hydroxybenzoic acid) is a plant hormone (phytohormone) which plays a role in numerous plant physiological processes. It influences plant development, seed germination [1], cell cycle [2], flowering and responses to stresses [3]. In particular, the importance of SA has been reported in various abiotic stresses: response to high and low temperatures, humidity and drought, salt and osmotic stress [4] or responses to UV light [5]. However, since the 1990s, SA research has mainly focused on its role in immunity [6,7].

The role of SA in plant responses to stresses is generally studied using two approaches: a pharmacological approach using SA treatment on plants and a genetic approach that relies on mutants with modulated endogenous SA concentrations or SA-related signalling. These mutants can be either deficient in SA accumulation, or accumulating high levels of SA (in basal conditions and/or upon stimulation). The widely used SA-deficient lines are *sid2*, carrying a T-DNA insertion in *ISOCHORISMATE SYNTHASE 1* and thus showing lower production of SA upon pathogen attack [8]; or *NahG*, expressing bacterial SA hydroxylase that degrades SA to inactive catechol [9,10]. These mutants are generally more susceptible to pathogen attack, especially by biotrophs [11]. On the other hand, mutants synthesising higher basal levels of SA (SA-overaccumulating mutants; SA-OAs) tend to be more resistant to pathogens. The boom of using SA-OA mutants emerged with forward genetics approach, where EMS mutants were screened for spontaneous lesions and small sizes, which often coincided with high levels of SA and enhanced resistance to pathogens [12,13]. However, such resistance often correlates with general dwarfism [14]. This remains a critical disadvantage for potential use of SA pathway modification in agriculture, where the high yield is needed. Hence, the modulation of SA in terms of possible use in agriculture has to be carefully prepared. However, until now, the molecular mechanism of the trade-off is not fully understood yet.

The increased SA level in mutants could be triggered by distinct events (mutations). The SA-OA phenotype can be caused by gain-of-function mutation (typically activation of immune receptor) or by loss-of-function mutation (typically negative regulation of SA pathway). For example, *bon1-1* shows enhanced immunity and SA levels due to the mutation in the negative regulator of SNC1. This phenotype is thus reversed by introducing an *snc1-11* point mutation [15]. Particular interest has been devoted to mutants with altered phospholipid turnover/signalling and vesicular trafficking that were reported to have pleiotropic effects, often connected with SA accumulation. In particular, *pi4kβ1β2* mutation in phosphatidylinositol-4-kinases β1 and β2 was recently reported as an SA overaccumulator [16,17], or *exo70b1-1* [18]. *fah1/fah2* is deficient in fatty acid hydroxylase genes and also showed enhanced immune responses and a modulated sphingolipid profile [19]. Further characterisation of those lines will thus help in mechanistic understanding of the connections between phospholipid metabolism, vesicular trafficking and immunity in plants.

Here, we present a collection of *Arabidopsis thaliana* (hereinafter Arabidopsis) mutants with SA content altered in various ways: affected immunity-related signalling, modified vesicular trafficking and a directly altered SA biosynthesis/accumulation. As controls, we included crosses of SA-OAs with SA-deficient lines. We propose this collection as a tool to investigate the role of SA in plant growth regulation and stress tolerance.

2. Results

2.1. Cultivation Conditions Influence the Phenotype of the SA Collection Mutants

Our aim was to establish a collection from available sources of Arabidopsis mutants with alterations in the SA pathway with special attention to creating a group of highly diverse SA-OA mutants, and not only prototypic SA-OA ones. Thus, we selected two SA-deficient mutants (*NahG*, *sid2*), eight known or putative SA-OA mutants (*cpr5-1*, *acd6-1*, *pi4kβ1β2*, *fah1fah2*, *bon1-1*, *exo70B1-2*, *pmr4-1*, *edr2-6*), and four "reverted" mutants: SA-OA mutants crossed with the SA-deficient ones (*sid2pi4kβ1β2*, *NahGpi4kβ1β2*, *NahGedr2-6*, *bon1-1snc1-11*) (see Table 1).

Table 1. Selected Arabidopsis mutants with potentially affected salicylic acid (SA) signatures.

Mutant Name		Targeted Gene	Targeted Process	Reference
cpr5	*CPR5*	Constitutive Expression of Pathogenesis-related genes 5	Constitutive expression of pathogenesis-related genes 5	Yoshida et el. 2002 [20]
bon1-1	*BON1*	BONZAI 1	Negative regulator of cell death, defence responses and several R genes	Li et al. 2007 [15]
acd6	*ACD6*	Accelerated Cell Death 6	Dose-dependent activation of defence signalling, accelerated cell death observed	Rate et al. 1999 [21]
pi4kβ1β2	*PI4Kβ1*, *PI4Kβ2*	Phosphatidylinositol-4-kinase β1 and β2	Second messenger, phosphatidyl inositol-4-phosphate production	Preuss et al. 2006 [22]
fah1fah2	*FAH1*, *FAH2*	Fatty acid5-hydroxylase 1 and 2	Fatty acid hydroxylation	Konig et al. 2012 [19]
edr2-6	*EDR2*	Enhanced Disease Resistance 2	Negative regulation of cell death	Vorwerk et al. 2008 [23]
exo70B1-1	*EXO70B1*	Exocyst Complex Component EXO70B1	Endomembrane trafficking	Kulich et al. 2013 [18]
pmr4-1	*CALS12*	Callose Synthase 12	Pathogen-induced callose synthesis	Nishimura et al. 2003 [24]
sid2 pi4kβ1β2	*ICS1*, *PI4Kβ1*, *PI4Kβ2*	Isochorismate synthase 1, phosphatidylinositol-4-kinase β1 and β2	SA biosynthesis, second messenger inositol-1,4,5-trisphosphate production	Sasek et al. 2014 [25]
NahG pi4kβ1β2	*NahG*, *PI4Kβ1*, *PI4Kβ2*	SA hydroxylase, phosphatidylinositol-4-kinase β1 and β2	SA degradation, second messenger inositol-1,4,5-trisphosphate production	Sasek et al. 2014 [25]
NahG edr2-6	*NahG*, *EDR2*	SA hydroxylase, enhanced disease resistance 2	SA degradation, negative regulation of cell death	Vorwerk et al. 2008 [23]
bon1-1 snc1-11	*BON1*, *SNC1*	BONZAI 1, Suppresssor npr1-1, constitutive 1	*bon1-1* crossed to the *snc1-11*, loss-of-function point mutation of the *SNC1*	Li et al. 2007 [15]
sid2	*ICS1*	Isochorismate synthase 1	SA biosynthesis	Wildermuth et al. 2001 [8]
NahG	*NahG*	SA hydroxylase	SA degradation	Nawrath and Metraux 1999 [26]

First we analysed the growth of selected mutants under long day (LD) and short day (SD) conditions. We initially focused on the 4 week old plants and analysed their rosette size and SA content (Figure 1; Supplementary Figure S1, Supplementary Table S2). Except for SA-deficient lines (*NahG* and *sid2*) and *exo70B1*, all others responded to LD condition with retarded growth. Due to their distinctive dwarf phenotypes (with an 85–50% reduction of rosette area compared to WT), we could clearly identify several SA-OA mutants: *cpr5*, *pi4kβ1β2*, *acd6* and *bon1-1* (Figure 1A,B). In SD conditions, the differences between mutants in growth were comparable to those under LD, although less important by absolute values (Figure S1A,B). In contrast, the differences in SA content were more pronounced at SD conditions. This could be due to a higher basal level of SA in the LD condition connected with a developmental stage; in LD conditions the plants started bolting at 3–3.5 weeks. In the "reverted mutants", the SA level was decreased to the level of WT, which correlated with the WT-like rosette size (Figure 1 and Figure S1).

For better description of the effect of the growth conditions on dwarf phenotypes of SA-OAs, we focused on a subset of four mutants: WT, *sid2*, *pi4kβ1β2* and *sid2pi4kβ1β2*, previously used for studies of SA-related effects [17]. We compared the growth dynamics of this subset in several cultivation conditions often used in stress-related studies: SD, LD and greenhouse (Supplementary Figure S2).

The *pi4kβ1β2* mutant appeared dwarfed under all conditions. Rosettes of *pi4kβ1β2* were smaller than in WT during the early developmental stages (2 week old seedlings), and the difference increased with time. Notably, the smaller rosettes did not result in a delay in development, since all plants started flowering simultaneously. Unexpectedly, *sid2pi4kβ1β2* grew bigger than *pi4kβ1β2* under all conditions but never reached the size of WT plants. This finding was surprising considering the previously published full reversal of growth in *sid2pi4kβ1β2* [25]. This finding indicates a high sensitivity of SA-related phenotypes to cultivation conditions.

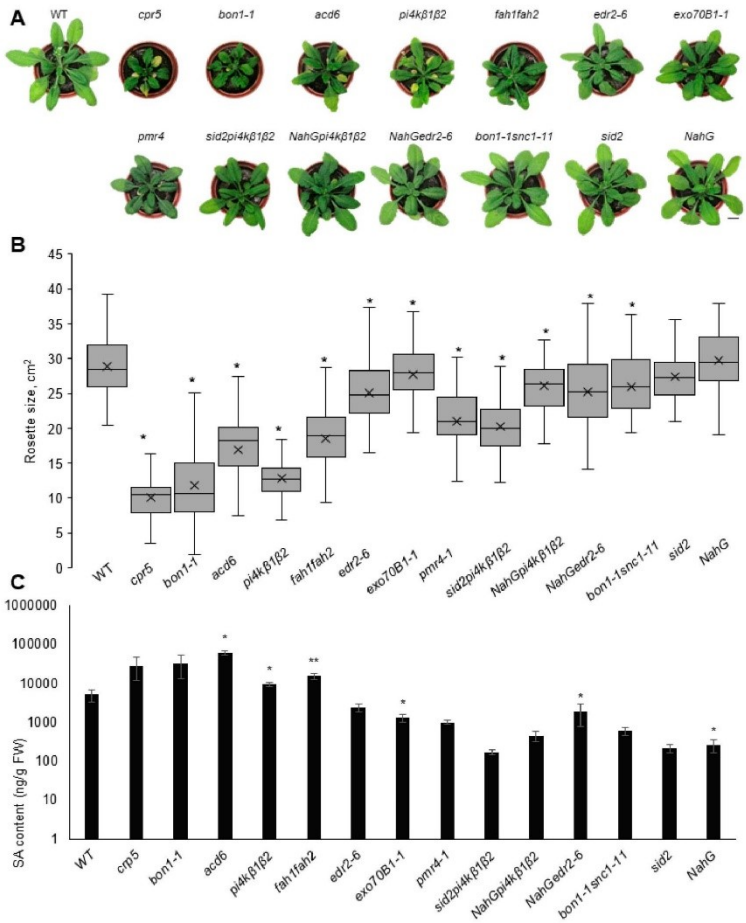

Figure 1. Rosette size and SA content of plants cultivated under long-day conditions. (**A**) Representative images of 4 week old plants cultivated at 22 °C, 16 h light/ 8 h dark. (**B**) Rosette size (area). Data are from three biological replicates, $n \geq 70$. Central line of the boxplot represents the median occupancy, cross represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the ends of whiskers are set at 1.5 times the interquartile range. (**C**) SA content in the leaves, $n = 4$. Data represent means + SEM, asterisks indicate variants different from WT, one-way ANOVA with Tukey's HSD post hoc test, * $p < 0.05$, ** $p < 0.01$.

Particular attention was directed to the *pmr4* mutant, deficient in callose synthase CALS12 and first described as POWDERY MILDEW RESISTANT 4 [24]. Under both SD and LD, the plants appeared slightly smaller than WT; however, no increase of SA was detected (Figure 1, Supplementary Figure S1, Supplementary Table S2). To the best of our knowledge, this is the first report on the (comparative) determination of the SA content in *pmr4* mutants, as the previous studies claimed a constitutively activated SA pathway based on SA-related gene transcription and resistance to pathogens [24].

2.2. SA-Related Gene Transcription Varies in Different Growth Conditions

We analysed the SA marker genes' (*PR1* and *ICS1*) transcription in soil-grown plants under SD and LD conditions. In most cases, the expression of the *PR1* gene coincided with small rosettes and a higher level of SA (Figure 2, Supplementary Figure S3). Generally, our results confirmed those from the studies wherein the mutants were first described. Under SD conditions, the differences between mutants were more pronounced both in *PR1*/*ICS1* transcription and in SA content. As gene transcription data were normalized to WT in both conditions, and WT at LD had almost 5 times higher SA content than in SD, that might have strongly affected basal *PR1* level.

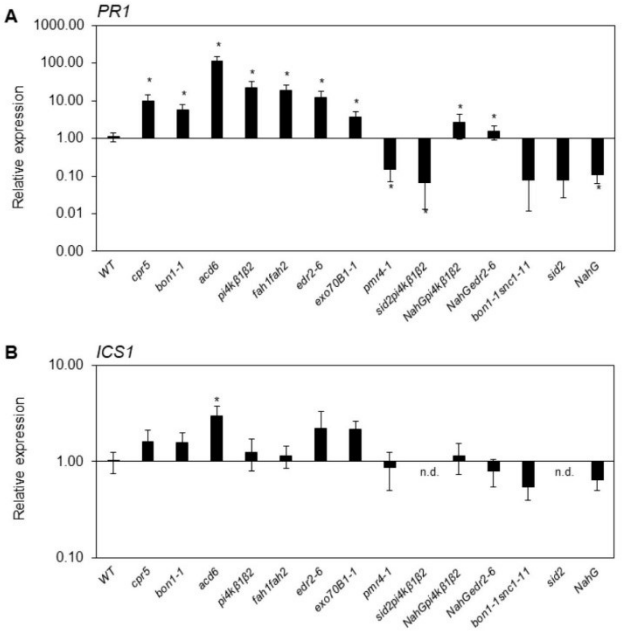

Figure 2. Transcription of *ICS1* and *PR1* in soil-grown plants cultivated under LD conditions. Samples were collected from four 4 week old plants. Values were normalized to WT at the respective conditions. *TIP41* was used as a reference gene. Data represent means + SEM, asterisks indicate values different from WT, *t*-test, * $p < 0.05$, $n = 4$.

To link SA-related signatures to the physiological state of plants, we measured photosynthetic activity. As reliable parameters of photosynthetic state, we chose quantum yield of PSII photochemistry (QY) and non-photochemical quenching (NPQ) [27]. Increase of NPQ can occur as a result either of the processes that protect the leaf from damage or of the damage itself [28]. The changes in NPQ were observed in several studied mutants (Supplementary Figure S4). NPQ at steady state (NPQ_Lss) was decreased in *snc1-1* compared to WT, but increased in several other mutants from the

collection. These mutants were mainly "immune-related" mutants *cpr5* and *bon1-1* and "trafficking" mutants *pi4kβ1β2* and *exo70B1-1*. Notably, the values were similar to those in "reverted" mutants *sid2pi4kβ1β2*, *NahGpi4kβ1β2* and *bon1-1snc1-11*, suggesting SA-independent origin of NPQ_Lss increase. The SA-deficient mutants also showed slight (by 10%) increase in NPQ_Lss, indicating SA independency. Maximum quantum efficiency of PSII photochemistry (QY_max) was stable among all studied mutants. Generally, the analysis of photosynthetic parameters did not reveal any drastic differences between the selected mutants in basal conditions.

2.3. Overaccumulation of SA Increases High Light Sensitivity in In-Vitro-Grown Seedlings

To investigate the behaviour of the SA mutant collection under in vitro conditions, we switched to the in vitro setup often used for the study of developmental defects. Continuous illumination of the roots, though widely used in research, can cause diverse effects on the phenotype, including spontaneous production of reactive oxygen species [29]. To study the reliability of our collection for root phenotyping, we studied seedling growth in vitro upon different light conditions. Seedlings were grown in vertically placed Petri dishes under LD light regime and at two light intensities, 450 $\mu E.m^{-2}.s^{-1}$ and 170 $\mu E.m^{-2}.s^{-1}$. Rosette weight and primary root length were evaluated at 11 dpg (Figure 3). While the rosette weight of WT plants did not change in response to light intensity, the mutants exhibiting high changes in SA content showed more intensive growth under 450 $\mu E.m^{-2}.s^{-1}$ light. Interestingly, such an effect was not evident within the majority of the reverted group, except for *bon1-1snc1-11*. SA-deficient genotypes and *pmr4-1* grew similarly at both light intensities, thus highlighting the role of SA in this effect (Figure 3A).

The growth of the primary roots was moderately inhibited by high light intensity in WT and also in both genotypes carrying the *NahG* transgene; however, the roots of other mutants were almost insensitive to different light conditions (Figure 3B). On the other hand, some mutants with pronounced dwarf rosette phenotypes had roots of the same size as WT plants (*bon1-1, acd6*). In contrast, in the *pi4kβ1β2* mutants, which had both small rosettes and short roots in all studied setups, the mechanisms regulating root and rosette growth inhibition seemed to be distinct. Indeed, while the rosette sizes were particularly restored by preventing SA accumulation (*sid2pi4kβ1β2*, *NahGpi4kβ1β2*), the roots remained small, indicating the SA-independent character of the phenotype (Figure 3B). To quantify this in time-course and to further investigate the effect of light regime on root growth, we focused on the phosphatidylinositol-4-kinase- related subset (WT, *sid2*, *pi4kβ1β2* and *sid2pi4kβ1β2*). First, we measured root elongation in kinetics at the light intensity corresponding to 170 $\mu E.m^{-2}.s^{-1}$ (Supplementary Figure S5A). The root defects caused by *pi4kβ1β2* mutations appeared at the very early germination stages and this effect was enhanced in time. To confirm the light independency of the phenotype, we also analysed root length in a semi-dark setup, modified from Silva-Navas et al., 2015 [30]. The Petri dishes were placed in dark plastic covers to shadow roots (Supplementary Figure S5B). In both setups, the growth kinetics were comparable: in the dark setup, *pi4kβ1β2* roots were about 3 times shorter than WT at 4 dpg, and about 5 times shorter at 8 dpg. Furthermore, *sid2pi4kβ1β2* roots were about 20% longer than *pi4kβ1β2* at 4 dpg and 50 % longer at 8 dpg. However, while the difference between *pi4kβ1β2* and WT remained stable in the light setup (up to 6 times at 8dpg), the difference between *pi4kβ1β2* and *sid2pi4kβ1β2* was more pronounced, up to 30% at 4 dpg and up to 200% at 8 dpg. This confirmed the SA-dependent sensitivity to light in in vitro growth conditions, and it also means that the light regime should be seriously considered in various types of experiments, especially those connected with SA.

With the SA collection, we were able to show that the regulation of the rosettes and root size is independent: the SA content mostly influenced the aboveground plant part, while the root length corresponded to SA-independent phenotype. Indeed, while *bon1-1* and *pi4kβ1β2* mutants were similar in terms of rosette growth, the roots of *bon1-1* were twice longer than that of *pi4kβ1β2* at both light intensities (Figures 1B and 3A,B).

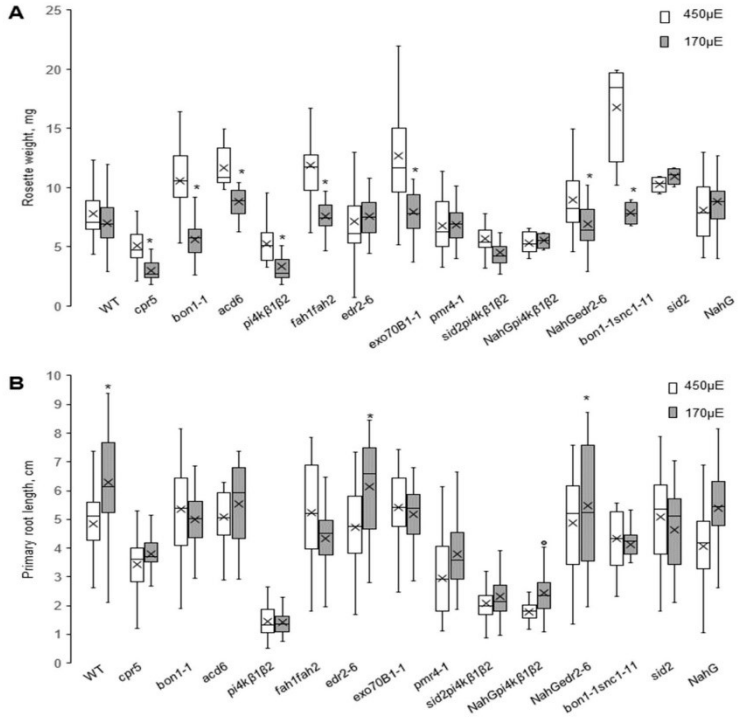

Figure 3. In vitro growth of SA collection mutants under different light intensities. Two week old seedlings were cultivated on $\frac{1}{2}$ MS medium under 450 $\mu E.m^{-2}.s^{-1}$ or 170 $\mu E.m^{-2}.s^{-1}$ under 12 h light /12 h dark photoperiod. (**A**) Rosette weight. (**B**) Primary root length. Data represent four biological repetitions; at least 10 seedlings were measured for each variant in each biological repetition. Central line of the boxplot represents the median occupancy, cross represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the ends of whiskers are set at 1.5 times the interquartile range, asterisks indicates variants different from those for the 450 $\mu E.m^{-2}.s^{-1}$ intensity the same genotype, * $p < 0.01$, *t*-test.

2.4. Salicylic Acid's Effect on the Root Growth and Shoot Growth is Distinct

To evaluate the behaviour of the presented SA collection in various growth setups, we performed a correlation analysis among all studied parameters: rosette size, SA content and expression of *ICS1* and *PR1* genes in soil-grown plants under two light regimes; and rosette weight and primary root length of seedlings cultivated in vitro under two light intensities. Putting together data of three biological repeats of all 15 genotypes in the collection, we quantified Pearson correlations (Figure 4).

The correlation table provided several clear outcomes: the rosette size of plants grown in soil negatively correlated with SA content accompanied with *PR1*/*ICS1* upregulation, which has been abundantly shown in previous studies [31]. Rosette growth correlated positively in all conditions. In contrast, the root growth in in vitro conditions was SA-independent (Figure 4). Generally, the rosette growth correlated with root growth, despite the above-mentioned difference between *bon1-1* and *pi4kβ1β2*. Interestingly, only seedlings grown under the 170 $\mu E.m^{-2}.s^{-1}$ intensity strongly correlated with rosette growth of soil-grown plants, suggesting that particular attention needs to be paid to light intensity while comparing data obtained in different growth conditions.

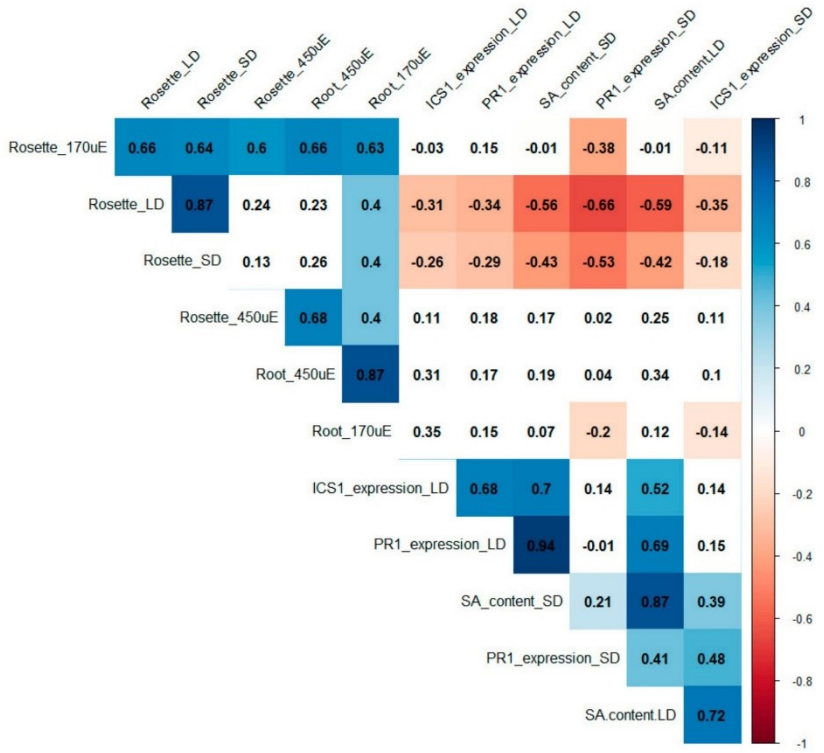

Figure 4. Correlation table of SA effects on growth. The matrix was built using the Pearson correlation for 12 parameters (rosette size, SA content and SA-related gene expression (*ICS1* and *PR1*) for soil-grown plants under short-day (SD) and long-day (LD) conditions; and rosette weight and primary root length for in vitro grown plants grown under an LD photoperiod at 450 uE or 170 uE light intensity). Measurements were taken for 15 genotypes (listed in Table 1). Data are from three biological repetitions for each variant. Positive correlations are displayed in blue and negative correlations in red. Correlation coefficients are indicated. Only results that passed the 0.05 threshold for significance are displayed in colour.

3. Discussion

SA plays a role in many fundamental processes in plants. Nowadays, it is probably the best characterised phytohormone in connection with plant immunity. A great tool which have provided insight into SA signalling pathways and their roles, especially in Arabidopsis, is SA-OA mutants. Interestingly, some of the initially described immunity-related mutants later appeared to have altered SA metabolism/signalling [31]. Changes in SA levels have a very strong impact on plant growth, and the majority of known SA-OA mutants are dwarfs. Because of their clearly distinguishable growth phenotypes, SA-OA mutants have been successfully used to find new components of plant immunity in forward genetic screening [32], in evolutionary studies [33,34] and in studies of ubiquitination cascades [35]. Growth inhibition of SA-OA has been used as a marker of an activated immune state in heat stress experiments: SA-OA mutants exhibit dwarf phenotypes under 22 °C, but have WT-like phenotype under 28 °C [36]. Although they have been studied for more than 30 years, SA-OA mutants still display many features that lack mechanistic explanation. One of them is the impact of cultivation conditions on SA-regulated growth, which has never been extensively studied.

To gain a complex understanding of the connection between growth and SA, we created a collection of 14 SA-modulated Arabidopsis mutants in a Col-0 background. We collected mutants from already published studies, including prototypic SA-deficient mutants *sid2* and *NahG* and prototypic SA-OA mutants *bon1-1*, *cpr5-1* and *acd6-1*. Additionally, we included recently described SA-OAs connected with lipid signalling, *pi4kβ1β2* and *fah1fah2*, and mutants associated with SA signalling based on gene expression analysis and pathogen assays, *edr2-6*, *pmr4-1*, *exo70b1* (Table 1). To complete the picture, we included three SA-OA "reverted lines", in which SA-OA mutants were prevented from accumulating a high SA level by affected biosynthesis (*sid2pi4kβ1β2*) or accumulation (*NahGpi4kβ1β2* and *NahGedr2-6*). All the selected mutants have been reported as having altered resistance to pathogens [31].

While analysing mutant phenotypes under various conditions, it is often difficult to distinguish between "typical" immune response and "just pleotropic" effects caused by mutation. A good example is the *pi4kβ1β2* mutant with impaired vesicle trafficking, which is a ubiquitous process that affects almost everything in plant cells [22]. We studied the SA-(in) dependent effects in *pi4kβ1β2*, showing that resistance to adapted pathogens is strictly SA-dependent, but callose production is SA-independent [17]. By creating this type of collection, we wanted to be able to compare more mutants with modulated SA patterns under exactly the same experimental conditions. For this purpose, we started with characterisation of the plant growth under short-day (SD) and long-day (LD) conditions. In general, our data confirmed previously published data that SA-OA mutants exhibit dwarf phenotypes (Figure 1A,B and Figure S1A,B). The SA content negatively correlated with rosette size (Figure 1 and Supplementary Figure S1,S4) which has been previously shown in literature [31]. However, the SA measurement also revealed that *edr2* and *exo70b1* are SA-OAs only under short-day conditions and *pmr4* is not SA-OA at all. This is particularly important as *edr2* and *pmr4* mutants have previously been described to accumulate high SA under biotic stress conditions, which thus suggests enhanced SA pathways at basal conditions as well. As expected, higher SA content in basal conditions was shown for *edr2* [23], but, surprisingly, we were unable to find in the literature any SA measurement for the *pmr4* mutant, although it is generally referred to as the one with constitutively induced SA pathways [24]. Again, SA marker genes are highly enhanced in *pmr4* under biotic stress conditions [37]. This statement is based on *PR1* gene expression, but not on SA level itself. In our setup, *PR1* expression was not highly induced even in basal conditions.

First, we characterised the SA collection's growth, *PR1*/*ICS1* transcription and SA content in plants cultivated in soil under LD and SD conditions (Figure 1 and Figure S1). Interestingly, in WT plants PR1 transcription was 5 times higher under LD than SD. That coincides with the fact that under LD conditions, plants tended to bolt at the age of 4 weeks. The induction of flowering is also associated with an increase in SA content and vice versa—SA treatment can trigger flowering [38]. No drastic changes in photosynthesis efficiency were detected (Supplementary Figure S3). For the full set of analysed mutants, we observed a negative correlation between rosette size and SA content under both LD and SD conditions. However, our data suggest that growth phenotype related to SA content would be better investigated under LD conditions. On the other hand, differences in SA content and gene transcription of SA marker genes were more pronounced under SD conditions. In comparison with the literature, our data showed that the mutants with modulated SA pathways were very sensitive to growth conditions. In terms of growth size, this could be clearly seen in the WT, *sid2*, *pi4kβ1β2* and *sid2pi4kβ1β2* subsets. This has been previously used to distinguish between SA-dependent and SA-independent effects of *pi4kβ1β2* deficiency [17,25]. Interestingly, in Šašek et al. (2014) [25], we showed that crossing of *pi4kβ1β2* with *sid2* led to a fully reverted phenotype when plants were grown in soil for 4 weeks. In our current cultivation conditions, we were not able to fully revert the growth (Figure 1A). We studied this in more detail under three distinct growth conditions. Two were in climate chambers with 8 h/16 h (light/dark) (short day) or 16 h/8 h (light/dark) (long day) and one was in greenhouse conditions. In all setups, *sid2pi4kβ1β2* was smaller than WT. In SD, *sid2pi4kβ1β2* had a size comparable even to *pi4kβ1β2* (Figure S2). The data of *ICS1* expression showed that the *ICS1* mutation was functional in both *sid2* and *sid2pi4kβ1β2* lines (Figure 2 and Figure S2).

Such behaviour can affect data interpretation and highlights the importance of checking SA levels while studying pleiotropic phenotypes, especially in a newly discovered mutant lines.

Early studies of SA mutants were mostly done on the rosettes (leaves) of soil-grown plants, while in recent years, the usage of in-vitro-grown seedlings as a model system has been rapidly increasing. The induction of the SA pathway has been shown during infection with root pathogen *Trichoderma* [39]. The sensitivity of Arabidopsis roots to SA treatment was recently demonstrated by a proteomics and metabolomics approach using SA-altered mutants [40]. Furthermore, the role of SA in root morphogenesis was recently shown by Pasternak et al. 2019 [41]. These authors reported SA treatment to modulate root meristem patterning by affecting auxin signalling in a concentration-dependent manner. However, no mutants with modulated SA were used in the study and the usage of only a pharmacological approach often raises questions about appropriate controls. We believe that the SA collection could be a helpful tool to continue studies of hormonal cross-talk in Arabidopsis roots. Here, we showed that root growth in the SA mutant collection is highly variable (Figure 3), and is not correlated with SA levels or SA marker gene expression in the rosettes of soil-grown plants (Figure 4). A clear example is the comparison of *bon1-1* phenotype (small rosette and almost WT-size roots) to *pi4kβ1β2*, which also had small rosettes but impaired root growth (Figures 1 and 3). Our data confirmed the critical role of PI4Kβ1β2 for root growth (Figure 3) [16,25]. To precisely analyse the SA role in seedlings' sensitivity to light, we used the subset of WT, *sid2*, *pi4kβ1β2* and *sid2pi4kβ1β2*. We grew plants in a light growth setup (roots were exposed to light) and dark growth setup (roots were shadowed by placing in dark chambers). The SA-deficient mutant *sid2* grew similarly to WT under dark conditions, but slower in the light setup, and both *pi4kβ1β2* and *sid2pi4kβ1β2* roots grow slower, while the difference between them was more pronounced in a light setup (Figure S4).

As mentioned above, SA-OA mutants are indispensable in studies of SA-related immunity. Additionally, SA's role in biotic stress was also shown via SA-OA involvement in response to abiotic stresses. In particular, the role of SA in cold stress was shown using *acd6* [21], *cpr1* and *pi4kβ1β2* [42]; in potassium stress by using *cpr5* [43], in response to drought and ABA treatment by using *cpr5* and *acd6* [44,45], and in sugar sensing by using *acd6* and *cpr1* [46]. Here, we tested the behaviour of these mutants under distinct growth conditions and under moderate abiotic stress in vitro (distinct light intensities). Surprisingly, the SA-OA mutants *cpr5*, *acd6*, *bon1-1*, *fah1/fah2* and *pi4kβ1β2* tended to form bigger rosettes under higher light intensities, while size of the WT rosettes was not affected (Figure 3). This indicates that SA makes plant more sensitive to high light conditions. In contrast, WT root growth was inhibited by high light but the roots of the above-mentioned SA-OA mutants were not affected (Figure 3B). These findings suggest an opposite effect of light on rosette and root growth.

The trade-off between immunity and growth has been widely discussed [14,47–49]. Our data present a robust quantitative background for this. We have shown strong negative correlation between SA levels and *PR1/ICS1* transcript signature with rosette size, but no correlation with root growth. This is important to take into account while planning phenotyping of mutants on different scales, and also confirms the suitability of putative SA-OA mutants for studies of root growth without impact of SA itself.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

Arabidopsis thaliana Col-0 was used as a wild type (WT), and the collection consisted of following mutants (see Table 1): *crp5 (SALK_071947)*, *bon1-1 (SALK_123132)*, *acd6 (SALK_059132)*, *pi4kβ1β2* (SALK_040479/SALK_09069), *fah1fah2* (SALK_094443, SALK_033090), *exo70B1-1 (CS410875)*, *edr2-6 (CS66944)*; *NahG* [9], *sid2-3* (*SALK_042603*); *bon1-1snc1-11* (SALK_047058, SALK_123132), *NahGpi4kβ1β2*, *sid2pi4kβ1β2* [25] and *NahGedr2-6 (CS66944)*. Prior to experiments, all seeds were propagated for one generation under the same conditions and genotyped as described in the literature (see Table 1).

Plants were grown in two main setups: in a cultivation substrate (soil) (a), and in vitro (b). For both setups, seeds were sterilized in 1.6% sodium hypochloride (30% of SAVO®, Unilever) solution with 0.02%TWEEN20 (Sigma Aldrich, St. Louis, Missouri, USA). Stratification for 2 days at 4 °C in dark conditionswas applied to break dormancy. (a) In soil: seeds were transferred to pots with substrate tablets (Jiffy, Kristiansand, Norway and grown in cultivation chambers (Snijders, Drogenbos, Belgium at 22 °C day temperature, 65–70% humidity and 16 h light/ 8 h dark (LD) or 12 h light/ 12 h dark (SD). After one week, the seedlings were replanted to one plant per pot. Four week old plants were used for analysis. (b) In vitro: seeds were germinated for 3 days in Petri dishes containing a half-strength Mirashige–Skoog medium ($\frac{1}{2}$ Murashige–Skoog basal salts (Duchefa), pH = 5.7) supplemented with 1% sucrose and 0.8% plant agar (Duchefa, Haarlem, Netherlands. At 4 days, seedlings were aseptically transferred to new plates and cultivated in a vertical position in cultivation chambers (Snijders) at 22 °C under long-day light conditions. After one week (11 days after germination), the Petri dishes were scanned (Epson Perfection V700 Photo, Suwa, Japanc), the root length was measured and the rosettes were cut and weighted. Root length was measured by FiJi software [50].

To investigate the effect of light on root development, the seedlings were grown under continuous exposure to light at different intensities, 450 $\mu E.m^{-2}.s^{-1}$ or 170 $\mu E.m^{-2}.s^{-1}$, or in the dark (plates were put in black chambers to shadow roots, Supplementary Figure S5B). To investigate the kinetics of root growth, the primary root length was monitored daily from 4 dpg to 8 dpg and measured using FiJi [50]. At least 10 roots were analysed for each condition.

4.2. Plant Phenotyping

Rosette size of soil-grown plants and primary root length of seedlings were measured by FiJi (area tool) [50]. Rosette weight of 11 day old seedlings was determined using analytical scales.

4.3. SA Concentration Measurements

Leaf tissue was collected from three plants (approximately 100 mg, three 6 mm discs from three leaves) in Eppendorf tubes with 1 g ceramic beads and frozen in liquid nitrogen. Hormone extraction procedure and salicylic acid content measurement were done as in [51]. Briefly, frozen samples were homogenized in tubes with silica beads using a FastPrep-24 instrument (MP Biomedicals, CA, United States) with extraction reagent methanol/water/formic acid (15:4:1, *v/v/v*) supplemented with stable-isotope-labelled 13C-SA internal standards. Extracts were subjected to solid phase extraction using Oasis MCX cartridges (Waters Co., Milford, MA, United States) and eluted with methanol. The eluate was evaporated to dryness and dissolved in 15% acetonitrile/water (*v/v*) immediately before the analysis. Quantification was performed on an Ultimate 3000 high-performance liquid chromatograph (UHPLC, Dionex; Thermo Fisher Scientific, Waltham, MA, United States) coupled to a IMPACT II Q-TOF ultra-high resolution and high-mass-accuracy mass spectrometer (HRAM-MS; Bruker Daltonik, Bremen, Germany). Separation was carried out using an Acclaim RSLC 120 C18 column (2.2 m, 2.1 × 100 mm; Thermo Fisher Scientific, Waltham, MA, United States) mobile phase consisting of 0.1% formic acid (A) and methanol (B) by gradient elution. The full-scan data were recorded in negative electrospray ionization (ESI) mode.

4.4. Gene Transcription Analysis

Total RNA was extracted as in [51]. Briefly, plant tissue was frozen in liquid nitrogen. The tissue was homogenized in plastic Eppendorf tubes with silica beads using a FastPrep-24 instrument (MP Biomedicals, USA). Total RNA was isolated using Spectrum Plant Total RNA kit (Sigma-Aldrich, St Louis, Missouri, USA) and treated with a DNA-free kit (Ambion, Austin, Texas, USA). Subsequently, 1 µg of RNA was converted into cDNA with M-MLV RNase H– Point Mutant reverse transcriptase (Promega Corp., Madison, Wisconsin, USA) and an anchored oligo dT21 primer (Metabion, Planegg, Germany). Transcription of *PR-1* and *ICS1* genes was determined using real-time

qPCR. Gene transcription values were normalized to *TIP41*. The primers used are listed in the Supplementary Table S1.

4.5. Photosynthetic Parameter Analysis

Plants were put in the dark for 15 min, and then the photosynthetic parameters were measured using FluorCam Handy FC 1000-H (PSI, Drasov, Czech Republic). Images of whole plants were taken. Chlorophyll fluorescence images were analysed using FluorCam 7.0 (PSI) software. Non-photochemical quenching (NPQ) was calculated as (Fm − Fm')/Fm' and maximum quantum efficiency of PS II photochemistry (QY) was calculated as Fv/Fm. Fm and Fm´ are the maximal fluorescence level from the dark-adapted and light-adapted leaf, respectively, and Fv is variable fluorescence from the dark-adapted leaf [52].

4.6. Statistical Analysis

All experiments were done in three biological repetitions. For soil-grown plants, $n = 24$; for in vitro grown plants, $n \geq 10$ for each genotype. Graphs display analysis of all values together, unless stated otherwise. Student's *t*-test and one-way ANOVA with Tukey's post hoc test were applied for the comparisons, $p < 0.05$. Correlation analysis was done using R software, *Corrplot* package [53]. Pearson coefficients were quantified, and only the values that passed the $p < 0.05$ threshold are displayed.

5. Conclusions

In this study, we introduced a new tool for studying the role of SA role in plants, the Arabidopsis "SA collection". It provides a robust tool benefitting from the distinct origin of the modulated SA pathway in *Arabidopsis thaliana*. The effective usage of the SA collection was demonstrated by phenotyping under different growing conditions, in soil and in vitro, using several light regimes. First, our data confirmed the correlation of SA content and expression of SA-related genes in different cultivation setups. Second, we clearly showed that SA is responsible for the regulation of rosettes, but not growth. Additionally, the SA collection revealed that a high basal SA content makes rosettes more sensitive to light. Surprisingly, we reassessed that *pmr4* mutant is not SA-OA under basal growing conditions. The presented SA collection is a starting point for future research trying to determine the roles of SA in response to environmental changes and to shed light on the complexity of SA-triggered signalling.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/24/6365/s1.

Author Contributions: Conceptualization, M.J. and T.K.; methodology, M.J., T.K., Z.K., P.M., H.L.; formal analysis, K.P., V.S., H.L., A.S., O.I., T.K.; resources, L.B., O.V.; data curation, K.P., V.S., R.P., H.L., P.M., P.K., O.I., A.S., Z.K.; writing—original draft preparation, M.J., T.K., K.P.; writing—review and editing, M.J., T.K.; project administration, L.B.; funding acquisition, O.V., L.B. All the authors commented and approved the text.

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Conflicts of Interest: The authors declare that there is no conflict of interest.

Abbreviations

SA	Salicylic acid
SA-OA	Salicylic acid overaccumulating mutants
ICS1	Isochorismate synthase 1
PR1	Pathogenesis related protein 1
SD	Short day conditions
LD	Long day conditions

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3.4. Publication 4

***Leptosphaeria maculans* effector AvrLm4-7 affects salicylic acid (SA) and ethylene (ET) signalling and hydrogen peroxide (H_2O_2) accumulation in *Brassica napus*.**

3.4.1. Summary of results

Pathogens have throughout evolution evolved sophisticated means to overcome host defence mechanisms. One of such mechanisms is production of small secreted proteins that have different functions in the host organism, these proteins are collectively called effectors[236]. Plants have evolved specific receptors to recognize effectors, the so-called Avr genes.

In this study we investigated the effect of *L. maculans* effector AvrLm4-7 on plant defence responses. First we tested 26 isogenic *L. maculans* isolates possessing either functional or non-functional allele of AvrLm4-7 on two susceptible *B. napus* cultivars lacking resistance genes *rlm4* and *rlm7*. Seven isolates with both non-functional alleles (a4a7), eight isolates containing alleles a4A7 and eleven isolates with both functional alleles (A4A7) were screened. The necrotic area observed on infected *B. napus* cotyledons was significantly higher for the isolates possessing functional alleles of AvrLm4-7.

Two isogenic isolates differing in the presence of AvrLm4-7 (further reffered to as a4a7 and A4A7 respectively) transfromed with β-glucuronidase (GUS)[237] were used for virulence tests. Isolate A4A7 exhibited about 40% higher lesion area on *B. napus* cv. Columbus cotyledons. Next step was monitoring of *in vitro* and *in planta* growth of both isolates. *In planta* growth was assessed on *B. napus* cv. Columbus cotyledons inoculated by puncture. No necrotic symptoms were visible until 8 dpi. Fluorescence measurement revealed that at 10 dpi growth of isolate A4A7 was 3times higher that a4a7. We assessed relative transcription of AvrLm4-7 *in vitro* and *in planta* by RT-qPCR showing that AvrLm4-7 is expressed in planta from 6 dpi about 1000fold higher than *in vitro*. The expression continued to be high till 10 dpi.

Further we assessed the phytohormone profile of infected host tissue. We focused mainly on the profile of stress related hormones – SA, JA and ABA. SA level was significantly suppressed in the A4A7 infected cotyledons at 8 dpi, but the levels reached similar values at 10 dpi in both isolates. ABA level was also reduced in A4A7 infected leaves at 8dpi, then increased to similar level as samples infected with the a4a7 isolate at 10 dpi. No significant changes were observed in JA concentration or any other screened phytohormones.

We wanted to correlate the phytohormone profile with the plant defence signalling. To adress this we screened transcription of defence marker genes of *B. napus* defined previously in our lab[234]. The

transcription of SA-related genes (*ICS1*, *PR1*) was lowered at 8 dpi in leaves infected with A4A7 which corresponds with the SA level. No significant changes were observed in JA- or ABA- markers. Surprisingly ethylene marker genes (*ACS2*, *HEL*) were also lowered in A4A7 infected leaves. Another defence reaction that was screened was the ROS production, which was lower in A4A7 infected leaves. Tratment with antioxidant agent ascorbic acid increased virulence of the a4a7 isolate.

We observed that presence of AvrLm4-7 suppressed salicylic acid and ethylene dependent signalling, which was identified as effective defence againts *L. maculans*[234]. ROS accumulation decreased in plants infected with AvrLm4-7 possessing isolate. Treatment with ascorbic acid led to increased virulence of the isolate lacking AvrLm4-7 while no effect was observed on agresivity of the isolate with AvrLm4-7. The results suggest that AvrLm4-7 affects ROS production as an important defence mechanism linked to suppresed SA and ET defence signalling.

3.4.2. My contribution

I performed phytohormone extraction from the mycelia. I evaluated the phytohormone content data and contributed to writing the manuscript.

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Leptosphaeria maculans effector AvrLm4-7 affects salicylic acid (SA) and ethylene (ET) signalling and hydrogen peroxide (H_2O_2) accumulation in *Brassica napus*

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SUMMARY

To achieve host colonization, successful pathogens need to overcome plant basal defences. For this, (hemi)biotrophic pathogens secrete effectors that interfere with a range of physiological processes of the host plant. AvrLm4-7 is one of the cloned effectors from the hemibiotrophic fungus *Leptosphaeria maculans* 'brassicaceae' infecting mainly oilseed rape (*Brassica napus*). Although its mode of action is still unknown, AvrLm4-7 is strongly involved in *L. maculans* virulence. Here, we investigated the effect of AvrLm4-7 on plant defence responses in a susceptible cultivar of *B. napus*. Using two isogenic *L. maculans* isolates differing in the presence of a functional *AvrLm4-7* allele [absence ('*a4a7*') and presence ('*A4A7*') of the allele], the plant hormone concentrations, defence-related gene transcription and reactive oxygen species (ROS) accumulation were analysed in infected *B. napus* cotyledons. Various components of the plant immune system were affected. Infection with the '*A4A7*' isolate caused suppression of salicylic acid- and ethylene-dependent signalling, the pathways regulating an effective defence against *L. maculans* infection. Furthermore, ROS accumulation was decreased in cotyledons infected with the '*A4A7*' isolate. Treatment with an antioxidant agent, ascorbic acid, increased the aggressiveness of the '*a4a7*' *L. maculans* isolate, but not that of the '*A4A7*' isolate. Together, our results suggest that the increased aggressiveness of the '*A4A7*' *L. maculans* isolate could be caused by defects in ROS-dependent defence and/or linked to suppressed SA and ET signalling. This is the first study to provide insights into the manipulation of *B. napus* defence responses by an effector of *L. maculans*.

Keywords: AvrLm4-7, *Brassica napus*, effector, ethylene, *Leptosphaeria*, ROS, salicylic acid.

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INTRODUCTION

Plant immunity consists of multi-layered defence responses (Chisholm *et al.*, 2006) as represented by the 'zig-zag' model designed by Jones and Dangl (2006). The first layer is based on the recognition of pathogen- (or microbe-) associated molecular patterns (PAMPs or MAMPs), components common to whole classes of microbes, by surface transmembrane pattern recognition receptors (PRRs). The perception activates PAMP-triggered immunity (PTI), also referred to as basal innate immunity. However, adapted pathogens have acquired effector proteins that overcome PTI. This phase in the plant–pathogen interaction is called effector-triggered susceptibility (ETS) and leads to a compatible interaction (i.e. susceptibility of the plant). In the co-evolutionary arms race, plants have struck back by the recognition of these effectors through additional intracellular receptors. This second layer of plant immunity is called effector-triggered immunity (ETI) (Dodds and Rathjen, 2010) and usually provides an incompatible interaction (i.e. resistance of the plant and avirulence of the pathogen). As the effectors are recognized by plant immunity on ETI, they are termed avirulence (*Avr*) effectors encoded by *Avr* genes. A plethora of cellular events follows the activation of both PTI and ETI: a rapid influx of calcium ions, an oxidative burst characterized by the production of reactive oxygen species (ROS), hormonal changes and transcriptional reprogramming. Often, ETI is followed by localized cell death in the form of a hypersensitive response (HR) (Dodds and Rathjen, 2010; Tsuda and Katagiri, 2010).

The key components of the plant immune system are the plant hormones that operate signal transduction after the perception of a pathogen (Pieterse *et al.*, 2009). Although salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the principal defence hormones in plants, other phytohormones, such as abscisic acid (ABA), auxin (indole-3-acetic acid, IAA), cytokinins (CKs), brassinosteroids (BRs) and gibberellins (GAs), also intervene in plant defences. Hormonal homeostasis and crosstalk between signalling pathways are crucial for the fine regulation of plant immunity (Pieterse *et al.*, 2012; Robert-Seilaniantz *et al.*, 2011). The changes in phytohormone

levels (or sensing) lead to transcriptional reprogramming to favour defence over other cellular processes, such as growth and development (Buscaill and Rivas, 2014; Denancé *et al.*, 2013; Robert-Seilaniantz *et al.*, 2011). SA, JA and ET signalling can all be activated during some cases of PTI and ETI (Tsuda and Katagiri, 2010). Although there are exceptions, SA signalling is generally implicated in defence against biotrophs, the types of pathogen that grow and retrieve nutrients from living plant tissue, whereas JA/ET signalling mediates defence against necrotrophic pathogens that kill plant cells and feed on dead tissue (Glazebrook, 2005). Naturally, plant pathogens have evolved a variety of strategies to overcome plant hormone-mediated immunity or to induce host susceptibility by interfering with various hormonal processes (Dou and Zhou, 2012).

Typically, effectors are defined as small secreted proteins (*c.* 300 amino acids) that are highly expressed during host infection, do not include known conserved domains or motifs and are often enriched in cysteine residues engaged in disulphide bridges for protein stability after secretion (Doehlemann and Hemetsberger, 2013). A more general definition describes effectors as 'small secreted proteins or molecules that alter host-cell structure or function' (Hogenhout *et al.*, 2009). The recent advances in fungal genomics have allowed the prediction of hundreds of effector genes in the genomes of different fungal pathogens (Schmidt and Panstruga, 2011).

Although more is known about the interference of bacterial effectors with the plant immune system (Block and Alfano, 2011; Deslandes and Rivas, 2012), notably in the *Pseudomonas–Arabidopsis* pathosystem, recent progress has also been made in deciphering the roles of fungal effectors (Giraldo and Valent, 2013; Rafiqi *et al.*, 2012). Despite the diversity of fungal effectors and the differences between fungal and bacterial effectors, it seems that they impair the host plant immune system at similar key steps, such as the perception of PAMPs and downstream signalling, hormonal homeostasis and crosstalk, plant cell death or the production of antimicrobial compounds (Doehlemann and Hemetsberger, 2013; Dou and Zhou, 2012).

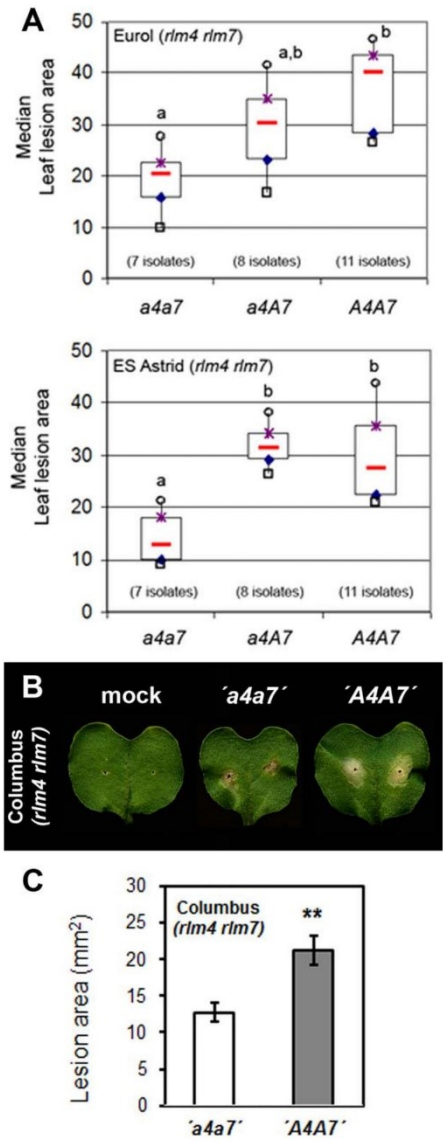
Leptosphaeria maculans is a fungal plant extracellular pathogen belonging to the *Dothideomycetes*. The fungus infects mainly *Brassica* crops. In oilseed rape (*Brassica napus*), *L. maculans* causes blackleg (or phoma stem canker), the most damaging disease of this crop in Australia, Canada and Europe (Howlett, 2004; West *et al.*, 2001). The infection cycle begins with the germination of ascospores on the leaf surface, which penetrate cotyledons and younger leaves via stomata or wounds. The fungus grows in the extracellular space (apoplast) and initially colonizes the tissue as a biotroph, but, behind the hyphal front, the fungus becomes necrotrophic and kills plant cells. Concomitantly, hyphae spread down the petiole in an endophytic manner, eventually reaching the stem cortex and causing black/brown blackleg necrotic lesions (Hammond and Lewis, 1987; West *et al.*, 2001). In *L. maculans*, the genomic location of 11 *Avr* genes, designated *AvrLm1*, *2*, *3*, *4*, *5*, *6*, *7*, *8*, *9*, *11*, *AvrLepR1* and *AvrLmJ1*, has been identified to date (Balesdent *et al.*, 2002, 2005; Ghanbarnia *et al.*, 2012; Van de Wouw *et al.*, 2014). Among these, six have been cloned: *AvrLm1* (Gout *et al.*, 2006), *AvrLm2* (Ghanbarnia *et al.*, 2015), *AvrLm4-7* (Parlange *et al.*, 2009), *AvrLm6* (Fudal *et al.*, 2007), *AvrLm11* (Balesdent *et al.*, 2013) and *AvrLmJ1* (Van de Wouw *et al.*, 2014). These are involved in PTI with a series of *Brassica* hosts, such as *B. napus*, *B. rapa* and/or *B. juncea*. Although some of the *L. maculans* effectors have been shown to be involved in fungal aggressiveness (Huang *et al.*, 2006, 2010), we currently have no mechanistic understanding of their function and how they may interfere with plant defences or other metabolic pathways.

One of the most interesting candidates for such an interaction is the Avr protein AvrLm4-7, recognized by two resistance proteins coded by *Rlm4* and *Rlm7*. During adaptation to resistance, a single non-synonymous base mutation in *AvrLm4-7* led to the escape of effector recognition by Rlm4, whereas the recognition by Rlm7 was conserved (Parlange *et al.*, 2009). Moreover, comparison of two near-isogenic *L. maculans* isolates differing in the presence of an *AvrLm4-7* allele showed that the virulent *avrLm4* allele was associated with a decrease in fungal aggressiveness (Huang *et al.*, 2006, 2010). The importance of the effector is underlined by the fact that, in nature and (before the use of *Rlm7*) in agronomic practice, all the examined strains virulent on susceptible genotypes of *B. napus* (*rlm4 rlm7*) possessed AvrLm4-7 (Balesdent *et al.*, 2006). The AvrLm4-7 effector was thus postulated to be implicated as a virulence factor of *L. maculans* (Rouxel and de Wit, 2012). Although AvrLm4-7 has been studied mainly as an Avr protein, we focus here on its activity as an effector and investigate how the AvrLm4-7 effector alters the *B. napus* immune system during ETS. For this objective, we investigated the effect of two isogenic isolates of *L. maculans*, harbouring or not the *AvrLm4-7* allele, following inoculation on a susceptible *B. napus* cultivar (*rlm4 rlm7*). Different defence responses were monitored, including the production of plant hormones, expression of defence-related genes and ROS accumulation. Our additional scope was to characterize the defence responses triggered after the recognition of AvrLm4-7 by Rlm4 in *B. napus* in the incompatible interaction and compare them with previously reported AvrLm1-triggered defence responses (Šašek *et al.*, 2012).

RESULTS

The presence of a functional allele of AvrLm4-7 promotes the aggressiveness of *L. maculans* during a compatible interaction

It has been reported previously that the AvrLm4-7 effector contributes to *L. maculans* fitness and increases its virulence, and that even differences in alleles (i.e. *AvrLm4-AvrLm7* versus *avrLm4-AvrLm7* alleles) could lead to fitness differences, with the virulent allele being responsible for a fitness deficit (Huang *et al.*, 2006, 2010). Here, we expanded the experiment by comparing 26



isogenic *L. maculans* isolates differing at the *AvrLm4-7* locus for aggressiveness on two susceptible *B. napus* cultivars, Eurol and ES Astrid, both lacking the corresponding resistance genes (*rlm4 rlm7*) (Fig. 1A). The wild-type (WT) isolate Nzt-4 (*a4a7*) contains a highly

Fig. 1 Introduction of a functional allele of *AvrLm4-7* increases *Leptosphaeria maculans* aggressiveness on susceptible *Brassica napus* cultivars lacking corresponding resistance genes (*rlm4* and *rlm7*). (A) Isogenic isolates of Nzt-4, with the *a4a7*, *a4A7* or *A4A7* allele of *AvrLm4-7*, were puncture inoculated on cv. 'Eurol' and cv. 'ES Astrid'. At 12 days after inoculation (dai), the cotyledons were scanned. The box-plots represent the median lesion area obtained for each isolate/plant genotype combination. For a given plant genotype, groups with the same letter do not induce significantly different median leaf lesions (red trait) (Kruskall–Wallis test, 5% threshold). (B) Disease symptoms at 13 dai caused by two isogenic *β*-glucuronidase (GUS)-tagged Nzt-4 isolates lacking (*a4a7*) or complemented (*A4A7*) with the *AvrLm4-7* gene. (C) Quantification of disease symptoms caused by the two isogenic GUS-tagged Nzt-4 isolates. Lesion areas were measured using image analysis. Values represent means ± standard error (SE) ($n > 22$). Asterisks indicate statistically significant differences between '*a4a7*' and '*A4A7*' isolates (**$P < 0.01$, Student's *t*-test).The experiment is a representative from three independent biological experiments.

degenerated non-functional allele of *AvrLm4-7*. A series of Nzt-4 isolates complemented with either the *AvrLm4-AvrLm7* (*A4A7*, 11 isolates) or *avrLm4-AvrLm7* (*a4A7*, eight isolates) allele of the gene (Parlange *et al.*, 2009) was compared with control WT Nzt-4 isolates that underwent the transformation process but did not integrate the construct (*a4a7*, seven isolates). In both *B. napus* cultivars, the group of *L. maculans* isolates complemented with the *A4A7* allele caused significantly larger cotyledon lesions than the control group of isolates lacking *AvrLm4-7* (Fig. 1A). Cotyledons of cv. ES Astrid infected with isolates complemented with the *a4A7* allele exhibited more severe symptoms in comparison with the control group, whereas, on cv. Eurol, the *L. maculans* infection with isolates carrying the *a4A7* allele increased the lesion size only slightly, but without statistical significance (Fig. 1A).

In further experiments, a pair of isogenic *L. maculans* Nzt-4 isolates differing only in the presence of the *AvrLm4-7* gene (Parlange *et al.*, 2009) were chosen, transformed by the *β-glucuronidase* (*GUS*) gene from *Escherichia coli* using *Agrobacterium tumefaciens* (Gardiner and Howlett, 2004), and referred to as '*a4a7*' and '*A4A7*' hereafter. Although the GUS transformation increased slightly the aggressiveness of the '*a4a7*' isolate compared with the WT (Fig. S2, see Supporting Information), the difference between the transformed isolates '*a4a7*' and '*A4A7*' was conserved. On susceptible *B. napus* cv. Columbus, the '*A4A7*' isolate exhibited about 40% larger lesions than the '*a4a7*' isolate at 13 days after inoculation (dai) (Fig. 1B,C).

In vitro and *in planta* growth of isogenic *L. maculans* Nzt-4 isolates and expression of *AvrLm4-7*

We first monitored the *in vitro* growth of the '*a4a7*' and '*A4A7*' isolates to check whether any growth difference could be observed that might have an impact on symptom severity. Both *L. maculans* isolates differing in *AvrLm4-7* grew similarly on V8 juice agar *in vitro* (Fig. S1 and Table S1, see Supporting Information).

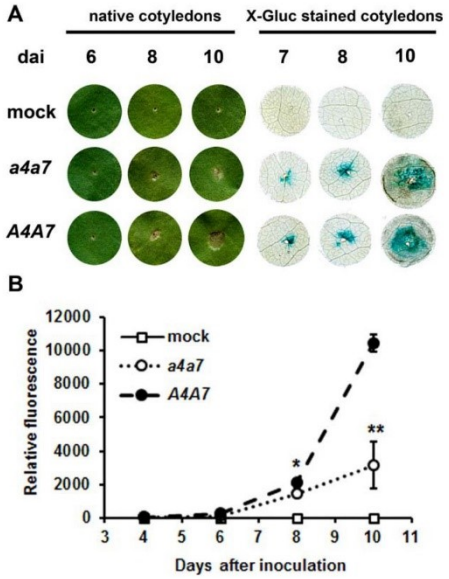


Fig. 2 *In planta* growth of *Leptosphaeria maculans* isolates differing in the presence of the *AvrLm4-7* gene on susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*), visualized and quantified using β-glucuronidase (GUS) activity. Plants (14 days old) were puncture inoculated by either water (mock) or a spore suspension of GUS-tagged *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented (*A4A7*) with the AvrLm4-7 effector. (A) Disease symptoms and histochemical visualization of fungal mycelia by GUS staining at 6 or 7, 8 and 10 days after inoculation (dai). (B) GUS activity in inoculated cotyledons measured by a 4-methylumbilliferone glucuronide (MUG) assay at 4, 6, 8 and 10 dai. Relative fluorescence is reported to fungal mass at the infection site. Values represent means ± standard error (SE) from three biological replicates. Asterisks indicate statistically significant differences between '*a4a7*' and '*A4A7*' isolates (*$P < 0.05$ and **$P < 0.01$; Student's *t*-test). The experiment was repeated three times with similar results.

The spread of infection *in planta* was then monitored in susceptible *B. napus* Columbus (*rlm4 rlm7*) cotyledons infected by puncture with either an *L. maculans* '*a4a7*' or '*A4A7*' isolate starting from 4 dai. None of the isolates developed macroscopically visible symptoms until 8 dai. To precisely monitor the growth of *L. maculans* isolates in infected plant tissue, we adopted a fluorescence measurement method of GUS activity using 4-methylumbilliferone glucuronide (MUG). As prerequisite, the GUS activity in *in vitro*-grown mycelia was shown to be similar (Fig. S3, see Supporting Information). Both isolates colonized the plant tissue similarly until 8 dai (Fig. 2A,B). Later, at 10 dai, the colonization by the '*A4A7*' isolate was more intense and its mycelium quantity reached three times that of the '*a4a7*' isolate (Fig. 2B).

Typically, the expression of *L. maculans* effectors is linked to the biotrophic stage of infection. To confirm this, we analysed the expression of *AvrLm4-7* in *L. maculans* isolate Nzt-4 complemented with an *AvrLm4-7* allele originating from JN3 ('*A4A7*' isolate) during infection of susceptible *B. napus* cv. Columbus and after *in vitro* growth. The expression of *AvrLm4-7* in *L. maculans* isolate JN3 was also determined *in vitro*. Consistent with previous observations (Parlange *et al.* 2009; Soyer *et al.*, 2014), the expression of *AvrLm4-7* was strongly repressed *in vitro* (Fig. 3). During infection, almost 10 000 times more *AvrLm4-7* transcripts were detected in the complemented '*A4A7*' isolate compared with *in vitro* growth (Fig. 3). The peak occurred during the asymptomatic stage of infection until 8 dai.

AvrLm4-7 decreases SA and ABA levels during compatible interactions

We hypothesized that the presence of *AvrLm4-7* could subvert plant immunity. First, in infected *B. napus* cv. Columbus cotyledons, we checked the levels of the principal plant hormones by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

The SA concentration increased strongly with infection. Differences between isolates were observed at 6 dai, but reached a maximum at 8 dai. Being surprisingly low, the level of SA in '*A4A7*'-infected cotyledons then increased strongly, reaching a comparable level to that of cotyledons infected with the '*a4a7*' isolate at 10 dai (Fig. 4).

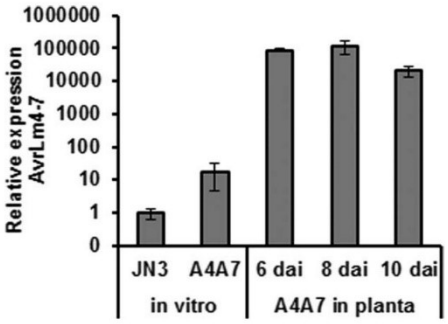


Fig. 3 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) relative expression of *AvrLm4-7* during the growth of *Leptosphaeria maculans* isolates JN3 and Nzt-4 '*A4A7*' *in vitro* and during infection on susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*) infected with isolate Nzt-4 '*A4A7*'. *In vitro* spore suspensions of JN3 and Nzt-4 were grown in Gamborg B5 medium for 10 days. Plants (14 days old) were inoculated by puncture inoculation with a spore suspension of *L. maculans* Nzt-4 isolate complemented with (*A4A7*) the *AvrLm4-7* gene. Expression of *AvrLm4-7* is normalized to *LmITS1* and relative to *AvrLm4-7* expression in JN3 *in vitro*. Values represent means ± standard error from three biological replicates.

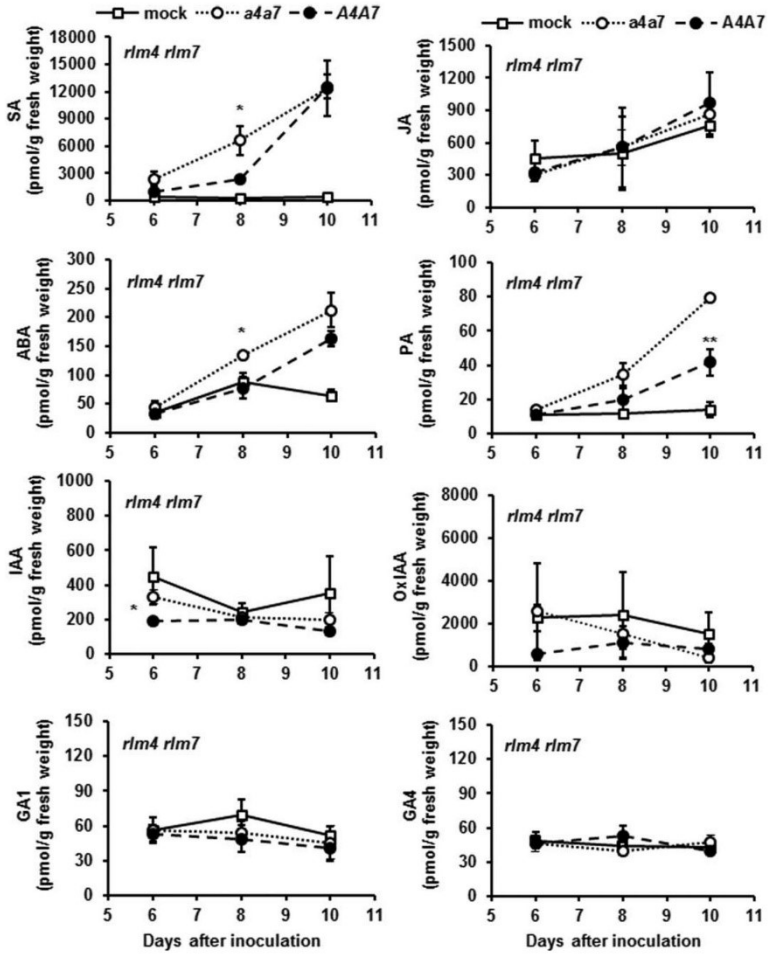


Fig. 4 Liquid chromatography–mass spectrometry analysis of plant hormone levels in susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*) infected with *Leptosphaeria maculans* isolates differing in the presence of the *AvrLm4-7* gene. Salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA) and its catabolic product phaseic acid (PA), indole-3-acetic acid (IAA) and its oxidized product 2-oxo-IAA (OxIAA), gibberellin 1 (GA1) and gibberellin 4 (GA4) were determined in extracts from cotyledons. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the *AvrLm4-7* gene. Values represent means ± standard error (SE) from three independent experiments. Asterisks indicate statistically significant differences between cotyledons infected with '*a4a7*' and '*A4A7*' isolates (* $P < 0.05$ and ** $P < 0.01$; one-tailed Student's *t*-test).

Interestingly, the ABA content was significantly reduced in cotyledons infected with the '*A4A7*' isolate compared with '*a4a7*'-infected cotyledons at 8 dai (Fig. 4). The level of the ABA degradation product, phaseic acid (PA), increased more slowly in '*A4A7*'-infected plants and, at 10 dai, the content of PA was significantly reduced in comparison with plants infected with the '*a4a7*' isolate.

The contents of gibberellins, auxin and its degradation product 2-oxo-IAA (OxIAA) exhibited no change in comparison with

either the mock-treated cotyledon or between the two isolates (Fig. 4).

The JA concentration increased similarly in the mock-treated and infected cotyledons (Fig. 4). We explain the slight JA increase in mock-treated and infected cotyledons between 6 and 10 dai as being the effect of puncture inoculation, as JA responds to wounding (Bell *et al.*, 1995).

SA- and ET-dependent signalling is lowered in susceptible *B. napus* cotyledons infected with the '*A4A7*' isolate

To confirm our findings, we examined whether decreased concentrations of plant hormones are also reflected at the transcript level. For this, we used a set of *B. napus* defence signalling marker genes that were identified in our previous study (Šašek *et al.*, 2012).

The expression induction of a gene coding for the key enzyme in the SA biosynthetic pathway, *ISOCHORISMATE SYNTHASE 1* (*ICS1*), was lower in cotyledons infected with the '*A4A7*' isolate compared with the virulent recipient at 8 dai (Fig. 5). Moreover, we observed a delayed expression induction of the widely used SA marker *PATHOGENESIS-RELATED GENE 1* (*PR1*) in cotyledons infected with the '*A4A7*' isolate. In cotyledons infected with the '*a4a7*' isolate, the transcription of marker genes increased almost linearly from 6 to 10 dai, whereas, in cotyledons infected with the '*A4A7*' isolate, the increase in expression was only seen from 8 dai (Fig. 5).

In addition, we also analysed the transcription of genes related to ET signalling. The transcription rate of a gene involved in the biosynthesis of 1-aminocyclopropane (ET precursor), *1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2* (*ACS2*), was always lower in cotyledons infected with the '*A4A7*' isolate compared with those infected with '*a4a7*'. Similarly, *hevein-like protein* (*HEL*; also classified as *PR4*), responding to ET and JA signalling pathways concomitantly, was induced to a lesser degree in cotyledons infected with the '*A4A7*' isolate (Fig. 5). The transcription of the ABA-related marker genes *NCED3*, a gene coding for the key enzyme of ABA biosynthesis, and *RD26*, another ABA-inducible gene, was not significantly affected (Fig. 5).

Hydrogen peroxide (H_2O_2) accumulation is decreased in cotyledons infected with the '*A4A7*' isolate at late stages of infection

Reactive oxygen species (ROS) are components of importance to the plant immune system, acting either as signalling molecules or exerting a direct toxic effect on invading microbes (Apel and Hirt, 2004; Foyer and Noctor, 2013). As several plant pathogens subvert ROS production (Doehlemann and Hemetsberger, 2013), we examined the possibility that AvrLm4-7 also affects ROS levels. The production of H_2O_2 was monitored from 6 to 10 dai by diaminobenzidine (DAB) staining. At 6 dai, no cotyledon showed brown staining (Fig. 6A). At this stage of the infection, only a little *L. maculans* mycelium is detected at the site of infection (Fig. 2B). In infected cotyledons, H_2O_2 stained by DAB appeared from 8 dai, showing more intense accumulation at 10 dai. We detected significantly less DAB-stained area in cotyledons infected with the '*A4A7*' isolate than in those infected with the '*a4a7*' isolate at both time points (Fig. 6). Furthermore, we examined the expression of NADPH oxidase genes during the response of cv. Columbus to '*a4a7*' and '*A4A7*' *L. maculans* isolates. NADPH oxidases are involved in the production of superoxide, an important precursor of several ROS. Amongst these, the transcription of *rbohF* was slightly induced in cotyledons when infected with the '*a4a7*' isolate only (Fig. S4, see Supporting Information).

Treatment with an antioxidant agent increases '*a4a7*' aggressiveness

Ascorbic acid belongs to the natural antioxidants in plants and, together with other antioxidants, determines the lifetime of ROS (Foyer and Noctor, 2013). Infected cotyledons were treated with water or 5 mM ascorbic acid at 3 and 6 dai, and disease symptoms were quantified at 13 dai. Although the necrotic lesion area caused by the '*A4A7*' isolate was unaffected by treatment, the lesions produced by the '*a4a7*' isolate increased after ascorbic acid treatment to reach the level obtained following '*A4A7*' infection (Fig. 7). This result indicates that H_2O_2 is involved in the defence response of *B. napus* to *L. maculans* at late stages of the primary infection, and that its accumulation is affected by the action of AvrLm4-7.

Recognition of AvrLm4-7 by Rlm4 induces SA and ET signalling

In addition to the effect of AvrLm4-7 during a compatible interaction, we also aimed to characterize the defence pathways triggered by the recognition of AvrLm4-7 in *B. napus*. For this purpose, the same two isogenic isolates were also inoculated onto an *Rlm4 B. napus* genotype (cv. Pixel). In the incompatible interaction, the recognition of AvrLm4-7 by Rlm4 led to the inhibition of infection spread. Indeed, only small greyish lesions with sharp margins developed on cotyledons and were restricted to a small area surrounding the site of inoculation. In contrast, the '*a4a7*' isolate lacking the AvrLm4-7 effector (compatible interaction) caused large greyish necrotic lesions that spread up to the cotyledon margin at 10 dai (Fig. 8A).

Compared with the isolate lacking AvrLm4-7, the '*A4A7*' isolate induced higher transcription of the SA marker genes *ICS1* and *PR1*. The transcription of *WRKY 70*, an SA-responsive transcription factor, was also induced on AvrLm4-7 recognition, but without statistical significance (Fig. 8B). Similar to *PR1* and *ICS1*, the

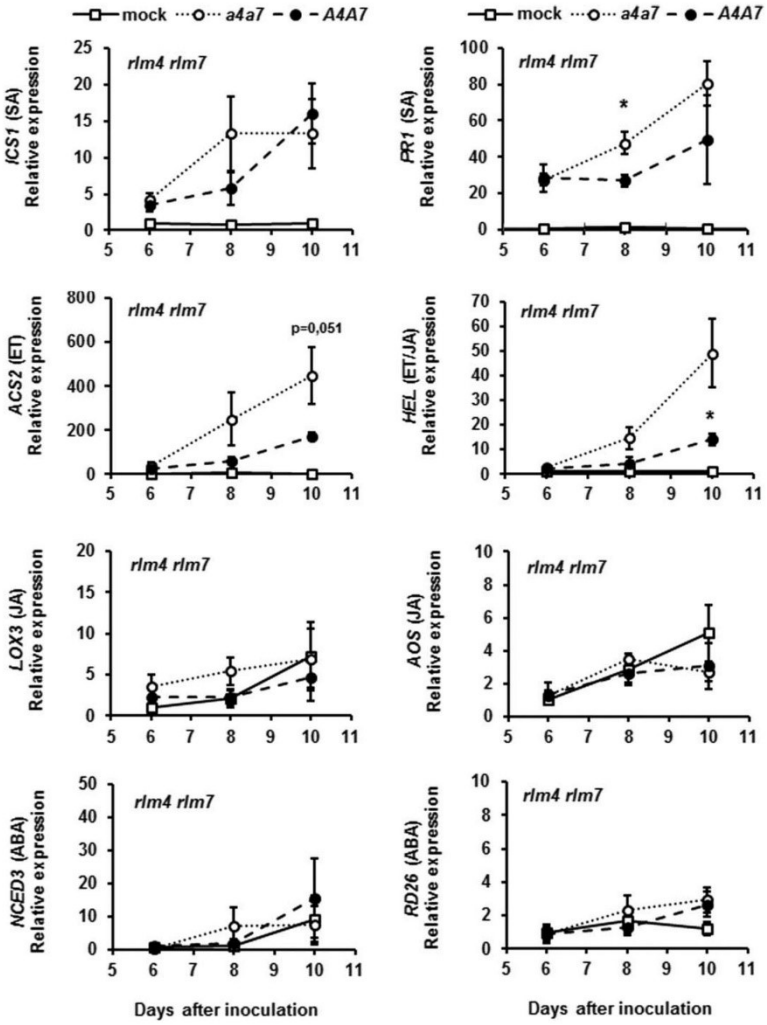


Fig. 5 Relative expression of salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) marker genes in susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*) infected with *Leptosphaeria maculans* isolates differing in the presence of the *AvrLm4-7* gene. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the *AvrLm4-7* gene. The expression of *B. napus* signalling marker genes was determined by reverse transcription-quantitative polymerase chain reaction and normalized to *B. napus Actin*. Values represent means ± standard error (SE) from three independent experiments. Asterisks indicate statistically significant differences between cotyledons infected with '*a4a7*' and '*A4A7*' isolates (* $P < 0.05$; one-tailed Student's *t*-test).

ET-related marker gene *ACS2* was also strongly induced in cotyledons infected with the '*A4A7*' isolate. In addition, *HEL* and chitinase (*CHI*), which respond concomitantly to ET and JA, were induced. Here, we attribute the increase to the effect of ET, as the JA-dependent marker genes *AOS* and *LOX3* revealed no difference in transcription (Fig. 8B). Interestingly, the recognition of AvrLm4-

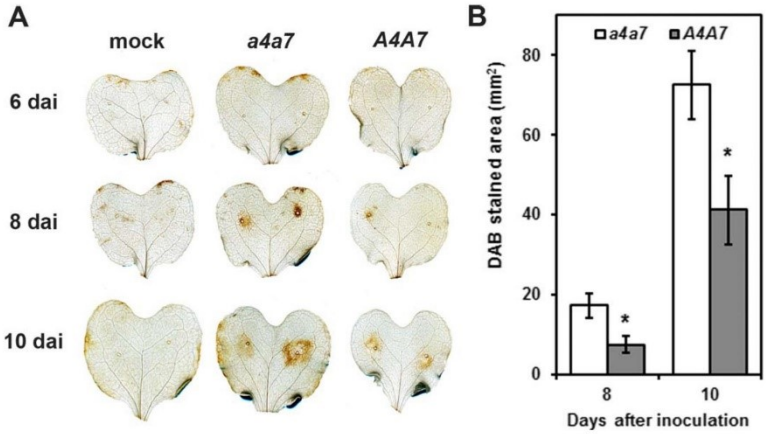


Fig. 6 Detection of hydrogen peroxide in susceptible *Brassica napus* 'Columbus' cotyledons infected with *Leptosphaeria maculans* isolates differing in the presence of the AvrLm4-7 effector using diaminobenzidine (DAB) staining. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the *AvrLm4-7* gene. (A) DAB-stained cotyledons at 6, 8 and 10 days after inoculation (dai). (B) DAB-stained area was measured using image analysis at 8 and 10 dai. Values represent means ± standard error from 22 cotyledons. Asterisks indicate statistically significant differences between *L. maculans* isolates (*$P < 0.05$; Student's *t*-test). The experiment was performed twice with similar results.

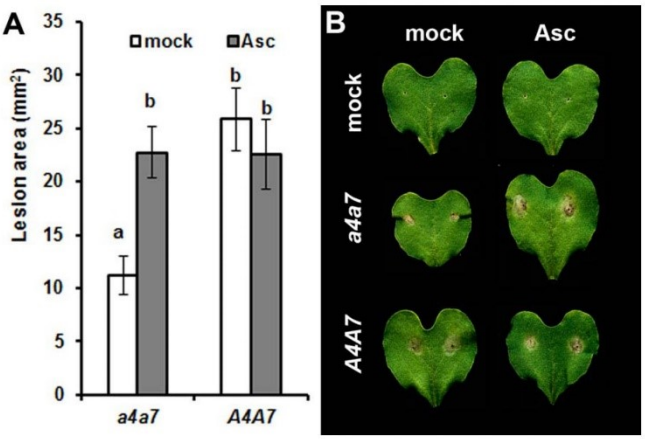


Fig. 7 Treatment with ascorbic acid increases the aggressiveness of *Leptosphaeria maculans* isolate Nzt-4 '*a4a7*' on susceptible *Brassica napus* 'Columbus'. Plants (14 days old) were inoculated by puncture inoculation by water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the *AvrLm4-7* gene. At 3 and 6 days after inoculation (dai), inoculation sites were treated with either water (mock) or 5 mM ascorbic acid (Asc). (A) Quantification of disease symptoms at 13 dai. The necrotic lesion area was measured using image analysis. Values represent means ± standard error (SE) ($n \geq 40$). Different letters above the columns indicate statistically significant differences according to Student's *t*-test ($P < 0.05$). (B) Representative cotyledons at 13 dai.

7 induced a slight but statistically significant increase in the transcription of the ABA-dependent genes *NCED3* and *RD26* (Fig. 8B).

In summary, the recognition of *L. maculans* AvrLm4-7 by Rlm4 triggered strong activation of SA- and ET-dependent signalling pathways, whereas ABA signalling was only slightly induced and

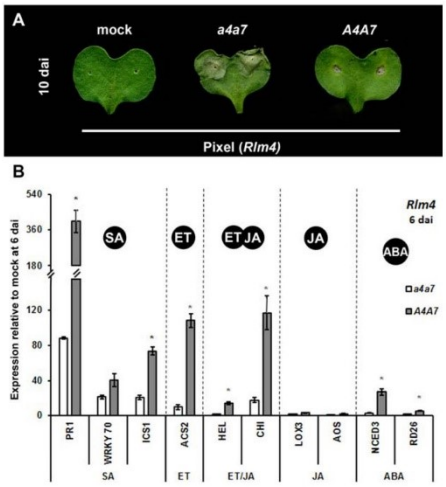


Fig. 8 Compatible and incompatible interaction of *Leptosphaeria maculans* isolates differing in the presence of the AvrLm4-7 effector with *Brassica napus* 'Pixel' carrying the resistance gene (*Rlm4*). Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the *AvrLm4-7* gene. (A) Disease symptoms developed at 10 days after inoculation (dai). (B) Expression of salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) marker genes relative to mock at 6 dai determined by reverse transcription-quantitative polymerase chain reaction. Values represent means ± standard error (SE) from three biological replicates. Asterisks indicate statistically significant differences between *L. maculans* isolates (* $P < 0.01$; Student's *t*-test).

JA-dependent signalling exhibited no activation. The successful elimination of *L. maculans* growth by the defence triggered by the SA and ET signalling pathways also illustrated the expression decrease of activated marker genes at 10 dai (Fig. S5, see Supporting Information). In '*A4A7*'-infected cotyledons, SA and ET signalling marker genes exhibited a lower transcription rate at 10 dai in comparison with 6 or 8 dai, whereas the expression increased steadily from 6 to 10 dai in cotyledons infected with the virulent isolate '*a4a7*' (Fig. S5).

DISCUSSION

This study aimed to confirm and find a cause for the increased aggressiveness of *L. maculans* isolates harbouring the AvrLm4-7 effector by investigating its effect on *B. napus* defence responses. This work is the first attempt so far to investigate the role of *AvrLm4-7* as an effector during compatible interactions with *B. napus*, with the working hypothesis that it may interfere with plant defence signalling.

First, using a set of *L. maculans* isolates differing at the *AvrLm4-7* locus, we showed that the absence of a functional allele of *AvrLm4-7* was responsible for the reduced aggressiveness on cotyledons, as measured by the size of the lesions in control conditions. In contrast with *AvrLm1*, which has also been shown to contribute to pathogen aggressiveness to some extent (Huang *et al.*, 2006, 2010), the increase in aggressiveness caused by the presence of the *AvrLm4-7* gene was much higher and cultivar independent, as three different susceptible cultivars of *B. napus*, namely Eurol, Columbus and ES Astrid, revealed greater symptom development during primary infection when infected with isolates complemented with the *AvrLm4-7* gene. Huang *et al.* (2006) have reported previously decreased aggressiveness associated with the loss of *AvrLm4* specificity as a result of a single base mutation in the *AvrLm4-7* gene, but did not analyse the effect of the loss of the gene. In our study, we found decreased aggressiveness following the loss of the gene, but no significant effect of the single amino acid change. The reasons for these different findings are unclear, but we stress the very different starting material and inoculation protocols between the two studies. In particular, Huang *et al.* (2006) departed from two field isolates that showed, or not, *AvrLm4* specificity, and used the progeny of isolates obtained following a series of backcrosses to generate batches of progeny harbouring, or not, the *AvrLm4* allele in a related, but not isogenic, genetic background. In this study, genetic complementation allowed us to compare the different alleles of *AvrLm4-7* (including the virulent degenerated allele) in the same genetic background. Moreover, Huang *et al.* (2006) used inoculations with ascospores without wounding the cotyledons; this is a tedious procedure, more closely resembling what happens in the field than the usual cotyledon inoculation test involving the inoculation of conidia on wounded cotyledons. It is possible that the inoculation with ascospores is more sensitive and may reveal fitness differences as a result of a single amino acid change that cannot be seen using the cotyledon inoculation test; for example, the ascospore inoculation procedure may reveal a fitness deficit linked to the penetration step that is not accessible when wounding the tissues prior to inoculation. All in all, the importance of the *a4A7* allele in fungal aggressiveness strongly suggests that the single mutation in the *AvrLm4-7* gene, allowing it to escape *Rlm4* resistance only slightly (if at all), compromises the effector function of the gene.

Further, we demonstrated that the two selected isogenic isolates '*a4a7*' and '*A4A7*', differing by the presence of *AvrLm4-7*, grew similarly *in vitro*, in accordance with the findings of Huang *et al.* (2006), substantiating the fact that AvrLm4-7, as a regular effector, does not have an intrinsic function for the fungus, but rather exerts its effect on the plant during the interaction. However, it must be remembered that the expression of *AvrLm4-7*, similar to that of other effector genes located in AT isochores of *L. maculans*, is strongly repressed during vegetative growth (Soyer *et al.*, 2014).

Once in the plant, the repression of effector gene expression was relieved, and a sharp increase in expression was observed between 3 and 6–8 dai, followed by reduced levels of expression at later stages (Parlange *et al.*, 2009; this study). Both isolates '*a4a7*' and '*A4A7*' colonized the plant tissue comparably and grew asymptomatically on susceptible *B. napus* cotyledons until 8 dai. We assume that the effect of *AvrLm4-7* is linked to the biotrophic stage of the primary infection, as the wave of effector expression to which that of *AvrLm4-7* belongs peaked during the asymptomatic stage of leaf colonization prior to the development of leaf lesions. This is a commonly found feature for many biotrophic and hemibiotropic filamentous phytopathogens for which waves of expression of effectors associated with the biotrophic stage of infection are common (Gan *et al.*, 2013; Lee and Rose, 2010). In other cases, however, such as that of *Zymoseptoria tritici*, waves of expression of putative effectors seem to be linked rather to the necrotic stage of hemibiotrophic behaviour (Rudd *et al.*, 2015). In later stages of infection, AvrLm4-7 had a significant impact on the development of necrotic lesions. Similarly, the presence of the *AvrLm4-AvrLm7* allele of *AvrLm4-7* increased the hyphal growth towards the petiole to reach the stem (Huang *et al.*, 2006).

Analogy in signalling after perception of AvrLm1 and AvrLm4-7

The recognition of AvrLm4-7 by Rlm4 examined in this study revealed a strong induction of SA- and ET-dependent signalling pathways early in the incompatible interaction. The same signalling pathways mediated defence in our previous study (Šašek *et al.*, 2012), in which the incompatible interaction based on the recognition of AvrLm1 by Rlm1 was examined. AvrLm1 is recognized by the *LepR3* resistance gene, which is an RLP (Larkan *et al.*, 2013), and by *Rlm1* (not cloned to date), but there is still debate on whether or not the two R genes may be the same (Rouxel and Balesdent, 2013). Nevertheless, one difference between the defence signalling mediated by AvrLm1 and AvrLm4-7 recognition still exists. The perception of the AvrLm4-7 effector by Rlm4 in *B. napus* cv. Pixel also slightly induced marker genes related to ABA signalling. Moreover, all compatible interactions in the two studies revealed elevated ABA levels, but we were unable to detect the up-regulation of the ABA-dependent marker genes *NCED3* and *RD26* (Šašek *et al.*, 2012). Considering our previous finding that the induction of ABA signalling prior to infection decreased symptoms caused by a virulent isolate (Šašek *et al.*, 2012), the role of ABA in the *L. maculans–B. napus* interaction remains obscure and requires further investigation.

Increased aggressiveness is linked to the suppression of signalling pathways by AvrLm4-7

In *B. napus*, SA and ET signalling pathways mount an effective defence response to *L. maculans* (Šašek *et al.*, 2012), as was also shown here during the course of the *AvrLm4–Rlm4* interaction. Our study suggests that, in a compatible interaction, these signalling pathways may be the primary targets of the AvrLm4-7 effector. Our results indicate that the presence of AvrLm4-7 in infected cotyledons causes suppression of SA signalling as a whole, affecting biosynthesis, the SA level and the response of the SA-related marker gene *PR1*. Moreover, ET signalling seems to be lowered in infected cotyledons when AvrLm4-7 is present: the transcription of the ET-responsive genes *ACS2* and *HEL* was repressed from 6 to 10 dai.

To our knowledge, this is the first study to indicate the manipulation of SA pathways by an effector from hemibiotrophic fungi. A strategy of other (hemi)biotrophic fungi involves effectors targeting JA signalling to promote susceptibility in a host (Kazan and Lyons, 2014). For example, *Fusarium oxysporum* secretes the Fo-SIX4 effector that induces JA signalling in *Arabidopsis thaliana* and contributes to disease development (Thatcher *et al.*, 2009). The SA pathway is also manipulated by bacterial effectors (Kazan and Lyons, 2014). The type III effector XopD from *Xanthomonas campestris* pv *vesicatori* represses SA-dependent gene expression and SA production in tomato plants (Kim *et al.*, 2008). Recently, Kim *et al.* (2013) have reported that XopD interacts directly with the tomato ET response factor ERF4 in subnuclear foci and also suppresses ET-stimulated immunity late in infection. In this aspect, the effect of AvrLm4-7 on *B. napus* defence signalling resembles that exerted by XopD in tomato.

H_2O_2 as the key player in the playground?

Previously, Jindřichová *et al.* (2011) have shown that H_2O_2 accumulates during *L. maculans* infection. In this study, we demonstrated that the removal of H_2O_2 by ascorbic acid during the biotrophic stage of the infection increased the lesion development of the less aggressive isolate '*a4a7*' lacking the AvrLm4-7 effector. This illustrates the importance of H_2O_2 in the defence response to *L. maculans* infection. Interestingly, the presence of AvrLm4-7 in the interaction led to a decreased accumulation of H_2O_2 during the later stages of the primary infection. Moreover, the transcription of *rbohF*, a gene coding for one of the NADPH oxidases, which are a source of ROS in the apoplast, was reduced when cotyledons were infected with the '*A4A7*' isolate harbouring the AvrLm4-7 effector. These results suggest a role for AvrLm4-7 in affecting the accumulation of H_2O_2. Among fungal effectors known to interfere with ROS production in plants, the pep1 effector of the biotrophic pathogen *Ustilago maydis* has been shown to inhibit the maize peroxidase POX12 in the apoplast (Hemetsberger *et al.*, 2012). Restricted colonization of the plant tissue by *U. maydis* is associated with the accumulation of ROS when pep1 is absent in the interaction. Until the effect of purified AvrLm4-7 protein on ROS-producing enzymes is examined, the possibility that AvrLm4-7 also acts in the apoplast by direct inhibition of ROS

production in a pep1-like manner cannot be excluded. However, alternative explanations of the H_2O_2 decrease are possible. ROS have been proposed to act synergistically in a signal amplification loop with SA to drive the HR and the establishment of systemic defences (Draper, 1997; Herrera-Vásquez *et al.*, 2015). Possibly, the modest ROS accumulation at the infection site of cotyledons infected by isolates harbouring AvrLm4-7 could reflect the decreased SA level and SA signalling. Nevertheless, the difference in SA level between the isolates disappeared at 10 dai, whereas the difference in ROS accumulation increased. A noticeable role in the regulation of H_2O_2 accumulation could also be played by ET signalling. ET can induce programmed cell death and senescence, processes that are also associated with the action of ROS (de Jong *et al.*, 2002). Therefore, in the absence of AvrLm4-7, the accumulation of ROS at 10 dai could be stimulated by increased ET signalling.

Taken together, we speculate that the increased aggressiveness of *L. maculans* isolates harbouring the functional *AvrLm4-7* allele could be caused by defects in ROS accumulation or by the complex effects (involving ROS accumulation) exerted by AvrLm4-7 on the *B. napus* defence system through the suppression of SA and ET signalling. Recent findings of Blondeau *et al.* (2015), showing that AvrLm4-7 is translocated into the plant cell, rather suggest that SA and/or ET signalling is the primary target of AvrLm4-7. The identification of the interacting partner of AvrLm4-7 upstream of these processes represents the next step in our understanding of the molecular mechanisms of this unique *L. maculans* effector.

EXPERIMENTAL PROCEDURES

Plant and pathogen cultivation

Brassica napus plants were grown in soil mixture Atami BioGrowmix (GROWMAN PLAINS, Prague, Czech Republic) in a cycle of 14 h of daylight (150 μE/m/s, 24°C) and 10 h of night (19°C) at 70% relative humidity in a cultivation chamber (Snijders Labs, Tilburg, the Netherlands). *Leptosphaeria maculans* isolates Nzt-4 AvrLm4_6 and Nzt-4 AvrLm4_3 (Parlange *et al.*, 2009), here referred to as '*a4a7*' and '*A4A7*', respectively, were cultivated on V8 juice agar medium at 26°C in the dark. Sporulation was obtained as described by Ansan-Melayah *et al.* (1995). Spores were washed once with distilled water after harvesting, diluted to 10^8 spores/mL and stored at −20°C. For aggressiveness studies following inoculation on compatible *B. napus* varieties, additional isogenic isolates were used: (i) a total of seven single-conidia isolates of Nzt-4, lacking AvrLm4-7; (ii) a total of eight Nzt-4 isogenic isolates having integrated the *avrLm4-AvrLm7* allele of AvrLm4-7 (a4A7); and (iii) a total of 11 isogenic isolates having integrated the *AvrLm4-AvrLm7* allele of AvrLm4-7 (A4A7) (Parlange *et al.*, 2009).

Plant inoculation and treatments

Plants were inoculated by puncture inoculation, i.e. by placing 10 μL of spore suspension (10^6 spores/mL) on a cotyledon that was punctured by a sterile needle.

For experiments with antioxidant agent, 10 μL of water (mock) or 5 mM ascorbic acid (Farmakon, Olomouc, Czech Republic) were placed on the puncture at 3 and 6 dai.

For aggressiveness studies, isolates were inoculated with the puncture method on the two compatible varieties, i.e. devoid of *Rlm4* and *Rlm7*, Eurol and ES-Astrid, using 10–12 inoculation points per isolate per plant variety. At 12 dai, cotyledons were scanned to measure lesion areas. The experiment was repeated twice. Median lesion areas were calculated and the data were subjected to non-parametric tests (Kruskall–Wallis) to test the significance of the differences observed between the three groups of isolates.

Transformation of *L. maculans*

Leptosphaeria maculans isolates Nzt-4 AvrLm4_6 and Nzt-4 AvrLm4_3 were transformed with a pSO1 construct (Persson *et al.*, 2009) carrying the gene for GUS from *E. coli*. The transformation was performed using *A. tumefaciens* LBA4404 according to Gardiner and Howlett (2004). GUS-transformed isolates were assessed for GUS activity *in vitro* and compared with WT (recipient) isolates for phenotype changes and virulence to exclude possible alterations caused by transformation. Unless stated otherwise, all inoculations were performed using GUS-tagged isolates.

GUS staining

Discs (diameter, 12 mm) surrounding the inoculation sites were cut from *B. napus* cotyledons and stained as described previously (Šašek *et al.*, 2012). In addition, cotyledon discs were submerged in 10% (w/w) NaOH (Lachema, Brno, Czech Republic) and incubated for 1 h at 37°C with shaking at 130 rpm. Rehydrated cotyledon discs were scanned in a thin layer of water under transmission light.

MUG assay

Fluorescence measurements of GUS activity were performed according to a method described by Jefferson *et al.* (1987) with several modifications.

For the determination of GUS activity in mycelia of GUS-tagged *L. maculans* isolates, 15 mg of mycelia filtered from the cultivation medium Gamborg B5-MES were homogenized in a 2-mL screw cap filled with 0.5 g of 1.3-mm-diameter silica beads with 500 μL of GUS extraction buffer (Jefferson *et al.*, 1987) using a FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA, USA), centrifuged and 50 μL of supernatants were pipetted to a 96-well plate maintained at 37°C. To each well, 200 μL of 1 mM MUG (Duchefa Biochemie, Haarlem, the Netherlands), preheated to 37°C, was added. Immediately, 50 μL were removed to 300 μL of STOP buffer (0.2 M Na_2CO_3) in a black Nunc™ F96 Microwell Polystyrene plate (Thermo Fisher Scientific, Waltham, MA, USA) for blank measurement. The mixture was incubated for 15 min at 37°C and stopped as described above. The fluorescence of 4-methylumbelliferone (MU) formed by GUS activity was measured in a Tecan Infinite F200 plate reader (Tecan, Männedorf, Switzerland) equipped with a 360/20-nm excitation filter and 465/25-nm emission filter.

For the determination of GUS activity in *B. napus* cotyledons infected with GUS-tagged *L. maculans* isolates, four discs (approximately 50 mg fresh weight) surrounding the inoculation sites were cut by Harris Uni-Core® (diameter, 6 mm) from four plants. The extraction of GUS, the

reaction with MUG and the detection of fluorescence proceeded similarly to that with *L. maculans* mycelium, except that the reaction time was 30 min.

Analysis of plant hormones

Plant hormones were extracted from approximately 200 mg of fresh tissue and determined as described previously (Dobrev and Kamínek, 2002; Dobrev and Vankova, 2012). The addition of appropriate internal standards to the samples preceded the analyses. Quantification was performed on an Ultimate 3000 high-performance liquid chromatograph (Dionex, Bannockburn, IL, USA) coupled to a 3200 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA).

Gene expression analysis

Discs surrounding the inoculation sites were cut by Harris Uni-Core® (diameter, 6 mm) from at least six plants. Homogenization, total RNA extraction, treatment with DNA-Free kit (Ambion, Austin, TX, USA) and reverse transcription of RNA to cDNA were performed as described previously (Šašek *et al.*, 2014). The reverse transcription-quantitative polymerase chain reactions (RT-qPCRs) were performed as described previously (Janda *et al.*, 2015). For *ACS2a*, the annealing conditions were modified to 55°C for 20 s in all reactions. The relative expression of *B. napus* and *L. maculans* genes was calculated with efficiency correction and normalization to *B. napus Actin* and *L. maculans ITS1* (Persson *et al.*, 2009), respectively. Primer sets for *B. napus* signalling marker genes were characterized, designed and verified in our previous study, where a complete list is given (Šašek *et al.*, 2012). Primers for *LmITS1* (FJ172239) and *AvrLm4-7* were designed by Persson *et al.* (2009) and Parlange *et al.* (2009), respectively.

H_2O_2 detection using DAB

Detection of H_2O_2 in *B. napus* cotyledons using staining with DAB (Sigma-Aldrich) was performed as described previously (Šašek *et al.*, 2012).

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SUPPORTING INFORMATION

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

Figure S1 Phenotypes of aerial colonies of *Leptosphaeria maculans* wild-type Nzt-4 and *β*-glucuronidase (GUS)-tagged Nzt-4 isolates harbouring (*A4A7*) or lacking (*a4a7*) the AvrLm4-7 effector. The transformed and wild-type (WT) isolates were grown on V8 juice agar medium and the photographs were taken 7 days after subculture.

Table S1 *In vitro* growth rate (mm/day) of wild-type *Leptosphaeria maculans* and *β*-glucuronidase (GUS)-tagged isolates

Figure S2 Aggressiveness of *Leptosphaeria maculans* wild-type (recipient) Nzt-4 and *β*-glucuronidase (GUS)-tagged Nzt-4 isolates harbouring (*A4A7*) or lacking (*a4a7*) the AvrLm4-7 effector following inoculation on the susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*). Plants (14 days old) were inoculated by infiltration of a spore suspension of wild-type *L. maculans* (WT) or GUS-tagged (GUS) Nzt-4 isolates (*a4a7* and

A4A7). The area of the necrotic lesion relative to the total cotyledon area was measured using image analysis at 10 days after inoculation. Values represent means ± standard error (SE) ($n > 21$). Different letters above the columns indicate statistically significant differences according to Student's *t*-test ($P < 0.05$).

Figure S3 β-Glucuronidase (GUS) activity in mycelia of GUS-tagged *Leptosphaeria maculans* isolates Nzt-4 differing in the presence of the AvrLm4-7 effector. *Leptosphaeria maculans* mycelium was harvested from axenic culture in Gamborg B5 by filtration at 10 days after inoculation of the medium. GUS activity was measured by a 4-methylumbilliferone glucuronide (MUG) assay. Relative fluorescence indicates the fluorescence of 4-methylumbelliferone formed by GUS activity. Values represent means ± standard errors from three biological replicates. No statistically significant difference was observed between '*a4a7*' and '*A4A7*' isolates ($P > 0.05$, Student's *t*-test).

Figure S4 Relative expression of *rboh* genes in susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*) infected with *Leptosphaeria maculans* isolates differing in the AvrLm4-7 effector, determined by reverse transcription-quantitative polymerase chain reaction. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or harbouring (*A4A7*) the AvrLm4-7 effector. Expression was normalized to *B. napus Actin*. Values represent means ± standard error (SE) from three independent experiments. Asterisks indicate statistically significant differences between cotyledons infected with 'a4a7' and 'A4A7' isolates ($*P < 0.05$; one-tailed Student's *t*-test).

Figure S5 Relative expression of salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) marker genes in resistant *Brassica napus* 'Pixel' (*Rlm4*) infected with *Leptosphaeria maculans* virulent (*a4a7*) and avirulent (*A4A7*) Nzt-4 isolates, determined by reverse transcription-quantitative polymerase chain reaction. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or harbouring (*A4A7*) the AvrLm4-7 effector. Values represent means ± standard error from three biological replicates. Asterisks indicate statistically significant differences between *L. maculans* isolates ($*P < 0.05$ and $**P < 0.01$; Student's *t*-test).

3.5. Publication 5

Auxin biosynthesis in the phytopathogenic fungus *Leptosphaeria maculans* involves indole-3-pyruvate decarboxylase LmIPDC2 and tryptophan aminotransferase LmTAM1

3.5.1. Summary of results

Auxins are hormones that regulate many vital processes in plants. Besides plants, also various microorganisms produce auxins, including pathogenic and symbiotic fungi. This study is focused on auxin production characterization in *L. maculans* and its potential role in virulence on *B. napus* plants.

Several auxin biosynthetic pathways were identified in microorganisms up to date. For example *U. maydis* uses tryptophan aminotransferases UmTAM1 and UmTAM2 and indole-3-acetaldehyde dehydrogenases UmIAD1 and UmIAD2 for auxin biosynthesis via indole-3-acetic acid intermediate[211]. Symbiotic fungus *N. crassa* uses indole-3-pyruvate decarboxylase IPDC for auxin production[214]. This gene for auxin production was identified in bacteria as well[238].

We screened two sister isolates JN2 and JN3 *in vitro* for auxin production. This experiment revealed both isolates produce bioactive form of auxin, IAA, but in very different concentration range. JN2 produced auxin in concentrations about 50 pmol/g FW whilst production in JN3 was around 5000 pmol/g FW. The screening also revealed minor concentration of other auxin precursors (indole-3-acetonitrile (IAN) or inactive metabolites (oxindole-3-acetid acid (OxIAA) and conjugated forms with amino acids (IAA-Asp, IAA-Glu). Structurally different molecule with weaker auxin activity phenylacetic acid (PAA) was observed as well.

We were further interested if it is possible to manipulate the auxin production. We supplemented *L. maculans* culture of both isolates with auxin biosynthetic precursors tryptophan and tryptamine[210] and analyzed the auxin production. Surprisingly, the isolates differed in reaction to supplementation: JN2 isolate produced about 10x more IAA already 1h after treatment with tryptophan and about 100x more after tryptamine treatment. JN3 did not show altered auxin production. No major changes in other metabolites were observed. Furthermore we were interested in which genes participate in the biosynthesis. We performed bioinformatic analysis of *L. maculans* genome and identified orthologues of previously characterized auxin biosynthetic genes. The analysis revealed orthologues that belong to several up to date identified pathways. Transcription of the candidate genes was analyzed in JN2 culture supplemented with precursors to reveal transcriptomic changes. Upon treatment with

tryptophan induced transcription of genes *LmTAM1*, *LmIPDC1*, *LmIPDC2*, *LmNIT1, LmIaam3* and *LmIaaM5* was observed. Tryptamine slightly induced transcription of *LmNIT1* and *LmIaaM3*.

Furthermore we analyzed phytohormone profile of *B. napus* cotyledons infected with *L. maculans* JN3. The only significant change was increase in OxIAA upon *L. maculans* infection. To test if *L. maculans* might produce auxins for its own regulation we cultivated GFP tagged *L. maculans* in the presence of auxin and observed luminiscence that reflects growth rate. No stimulatory effect of observed in either of the used isolates. High concentration of auxin ihibited growth of *L. maculans*.

This study proposes a model of auxin metabolism in *L. maculans*. It is the first evidence of an indole-3-pyruvate decarboxylase gene involved in auxin biosynthesis in a pathogenic fungus. We did not reveal any impact of auxin production on *L. maculans* virulence nor did we observe it would be importnant for *L. maculans* physiology.

3.5.2. My contribution

I designed majority of the experiments. I performed phytohormone extraction, gene transcription analysis, growth inhibition tests, inoculation tests, bioinformatic analyses concerning the orthologue identification and phylogenetics. I validated the data and contributed to writing the manuscript.

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Original Article

Auxin biosynthesis in the phytopathogenic fungus *Leptosphaeria maculans* is associated with enhanced transcription of indole-3-pyruvate decarboxylase *LmIPDC2* and tryptophan aminotransferase *LmTAM1*

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ABSTRACT

Auxins are hormones that regulate growth and development in plants. Besides plants, various microorganisms also produce auxins. Here we investigate whether and how the phytopathogenic fungus *Leptosphaeria maculans* biosynthesizes auxins. We characterized the auxin profile of *in vitro* grown *L. maculans*. The culture was further supplied with the auxin biosynthetic-precursors tryptophan and tryptamine and gene expression and phytohormone content was analyzed. *L. maculans in vitro* produced IAA (indole-3-acetic acid) as the predominant auxin metabolite. IAA production could be further stimulated by supplying precursors. Expression of indole-3-pyruvate decarboxylase *LmIPDC2*, tryptophan aminotransferase *LmTAM1* and nitrilase *LmNIT1* genes was mainly upregulated after adding tryptophan and correlated with IAA production, suggesting that these genes are the key components of auxin biosynthesis in *L. maculans*. Tryptamine acted as a potent inducer of IAA production, though a pathway independent of *LmIPDC2/LmTAM1* may be involved. Despite *L. maculans* being a rich source of bioactive IAA, the auxin metabolic profile of host plant *Brassica napus* was not altered upon infection. Exogenous IAA inhibited the growth of *L. maculans in vitro* when supplied in high concentration. Altogether, we showed that *L. maculans* is capable of IAA production and we have identified biosynthetic genes that were responsive to tryptophan treatment.

1. Introduction

Auxins are important phytohormones that play a decisive role in many aspects of plant development. An auxin molecule generally possesses an aromatic ring and a carboxylic acid group. The most common auxin is the indole-3-acetic acid (IAA) and its function has been predominantly studied in plants, covering such areas as: growth, development, phototropism, gravitropism and hydrotropism [1]. Auxin is also responsible for shoot apical dominance, initiation of root hair formation and promotion of cell elongation, wound response, and plant immunity [2–4]. It has also been shown that other organisms, such as bacteria, yeasts or filamentous fungi, are also able to produce auxin [5,6]. Auxin can induce

Abbreviations: TAM, tryptophan aminotransferase; IPDC, indole-3-pyruvate decarboxylase; IAD, indole-3-acetaldehyde dehydrogenase; IAN, indole-3-acetonitrile; IAM, indole-3-acetamide; IPyA, indole-3-pyruvate; IAA, indole-3-acetic acid; iaaM, tryptophan monooxygenase; IaaH, indole-3-acetamide hydrolase; OxIAA, oxindole-3-acetic acid; NIT, nitrilase; IAOx, indol-3-acetaldoxime; Trp, tryptophan; Trm, tryptamine.

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morphological changes in hyphae or conidia germination in fungi. Some virulence traits of yeast *Candida albicans* towards its human host have been attributed to the production of auxin [7]. Several different auxin biosynthetic pathways have been described in plants as well as microorganisms to date [7].

In most of the known auxin biosynthetic pathways the first substrate is tryptophan. The pathways are further referred to according to the major metabolite produced downstream of tryptophan. The indole-3-pyruvic acid (IPyA) pathway has been described in plants and several microorganisms, including fungi. In *Arabidopsis thaliana,* tryptophan is processed by a family of tryptophan aminotransferases into IPyA. IPyA is converted to IAA by YUCCA enzymes [8]. A similar pathway has been described in the fungus *Ustilago maydis*, where aminotransferases UmTAM1 and UmTAM2 participate in the conversion of tryptophan into IPyA. Downstream of IPyA in *U. maydis,* the aldehyde dehydrogenases UmIAD1 and UmIAD2 convert indole-3-acetaldehyde (IAAld) into IAA [9]. The function of the IAD genes for auxin biosynthesis was also confirmed in the ectomycorrhizal fungus *Tricholoma vaccinum* [10]. In bacterium *Erwinia herbicola*, IPyA is processed by decarboxylase IPDC into IAA [11,12].

Another biosynthetic pathway of indole-3-acetamide (IAM) has been described predominantly in the bacterium *Pseudomonas savastanoi*. Tryptophan is converted to IAM in a reaction catalyzed by tryptophan monooxygenase (iaaM) and subsequently into IAA by indoleacetamide hydrolase (iaaH) [13]. Later this pathway was discovered in the fungal genus *Fusarium*, where iaaM and iaaH participate in IAA biosynthesis [14]. In the wheat rust fungus *Puccinia graminis,* Ptg-IafaM is a key enzyme for IAA biosynthesis [15].

There are also pathways converting tryptophan into IAA via tryptamine. In the insect-pathogenic fungus *Metarhiziium robertsii,* tryptophan decarboxylase MrTDC has been described [16]. *Fusarium graminearum* is also able to convert tryptamine into IAA [16]. In plants, this conversion involves mainly flavin-containing monooxygenases from the YUCCA family that have not been described other than in plant species up to now [7].

Plants also possess the indole-3-acetonitrile pathway (IAN). The complete pathway has only been described in plants, while its components have also been found in microorganisms. In *A. thaliana*, the cytochrome P450 enzymes CYP79B2 and CYP79B3 process tryptophan into indole-3-acetaldoxime (IAOx), which is subsequently converted into IAN [17]. Subsequently, IAN is metabolized by nitrilases (NIT) into IAA [7]. It is interesting to consider, also, that fungus *F. graminearum* is able to convert IAN into IAA [16].

Apart from tryptophan-dependent biosynthetic pathways, there is evidence that IAA can be synthesized in other ways. *Saccharomyces cerevisiae,* with deleted aldehyde dehydrogenase ALD, was not able to metabolize radiolabeled Trp, but still produced IAA - suggesting that a tryptophan-independent pathway probably exists in *S. cerevisiae*. However, no tryptophan-independent biosynthetic genes have been reported to date [18].

Several studies have explored the role of IAA in fungal physiology. The effects may be adverse. High exogenous IAA inhibited growth in the maize fungal pathogen *Harpophora maydis* [19]. In contrast, exogenous IAA stimulated pseudohyphal growth in *S. cerevisiae* and hyphal growth in *C. albicans* [18]. IAA also increased spore production and caused changes in colony morphology in the fungus *Colletotrichum gloeosporioides* [20].

For this study we have focused on the phytopathogenic fungus *Leptosphaeria maculans* (anamorph *Phoma lingam* and its ability to produce auxins and related metabolites). *L. maculans* is classified within the *Dothideomycetes* and is an important pathogen of *Brassica* crops. In oilseed rape (*Brassica napus*), *L. maculans* causes phoma stem canker (so-called blackleg), the most damaging disease of this crop in Australia, Canada, and Europe [21,22]. The infection cycle begins when ascospores germinate on the leaf surface, invading the cotyledons and younger leaves via the stomata or wounds. The hyphae spread down the petiole, finally reaching the stem cortex and causing black/brown, blackleg necrotic lesions [21,23]. *L. maculans* produces different molecules, such as effectors and phytohormones that can affect the host [24–27]. The purpose of this study is to assess whether *L. maculans* can produce auxins and to investigate the genetics basis of their biosynthesis.

2. Materials and methods

2.1. Cultivation of plants and fungi

B. napus plants of cultivar cv. Eurol were used and previously characterized in [27]. Plants were cultivated hydroponically in perlite supplied with Steiner nutrient solution [28] with a 14 h of day (150 μE/m^2 s, 22 °C) and 10 h of night (19 °C) cycle at 50% relative humidity in a growth room. Cotyledons of 14-day-old plants were used for experiments. True leaves were removed before treatment and inoculation assays to prevent senescence of used cotyledons.

L. maculans strains JN2 and JN3 were characterized previously [25,29]. *L. maculans* liquid culture was prepared by inoculation of Gamborg medium with aqueous conidial suspension to final concentration 10^5/ml conidia. 7-day-old mycelium was used for experiments. Medium Gamborg B5 (Duchefa, G0210, Haarlem, The Netherlands), supplemented with 3% w/v sucrose and buffered with 10 mM MES (final pH 6.8) was used for cultivation. Conidia were prepared according to [27]. After harvesting, the conidia were washed with distilled water, diluted to concentration 10^8/ml and stored in −20 °C.

2.2. Pathogen infection

For phytohormone analysis, 14-day-old plants were inoculated by placing 10 μl of aqueous conidia suspension (concentration 10^6/ml) on a cotyledon puncture created by a sterile syringe needle. Inoculated plants were kept in the same conditions as mentioned above (2.1.) as uninfected plants. At 10 days post inoculation, a 1-mm band surrounding necrotic lesions was sampled, stored in liquid nitrogen, and the phytohormone profile analyzed. At least 12 plants were assessed for each condition.

For the RNA sequencing experiments, cotyledon inoculations were performed on 10 days-old seedlings that were punctured and inoculated with 10 μl of inoculum (10^7/ml pycnidiospores), as described in [29]. A 5-mm diameter surface was punched around the inoculation site at different time points post-inoculation (2, 5, 7, 9, 12, 15 days) on two independent, biological replicates.

2.3. Antifungal activity assay

L. maculans isolates JN3 and JN2 transformed with a pCAMBgfp construct [30] carrying the sGFP gene [24,31] were used. Conidia of JN3-GFP and JN2-GFP were grown under axenic conditions in Gamborg B5 medium (Duchefa) supplemented with 0.3% (w/v) sucrose and 10 mM MES (pH 6.8) at the final concentration of 2500 conidia per well of black 96-well plate (Nunc®). IAA (Sigma-Aldrich, St. Louis, MO, United States) dissolved in EtOH (100 mM stock solution) was applied to wells in concentrations ranging from 0.1 μM to 1000 μM. 1% EtOH was used as the control treatment. Plates covered with lids and sealed with 3 M tape were incubated in darkness at 26 °C. Fluorescence was measured using a Tecan F200 fluorescence reader (Tecan, Männedorf, Switzerland) equipped

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with a 485/20 nm excitation filter and 535/25 nm emission filter. Twelve wells for each treatment were measured.

2.4. Chemical treatment

7-day-old *L. maculans* liquid culture in Gamborg B5 medium was treated with auxin biosynthetic precursors tryptophan or tryptamine (Sigma-Aldrich, St. Louis, MO, United States). Both tryptophan and tryptamine were prepared as 100 mM stock solution dissolved in 0.1 M HCl and 1 mM final concentration was used for the culture treatment. An equivalent amount of 0.1 M HCl was used as the control treatment.

2.5. Phytohormone analysis

150 mg of freshly-filtered mycelium or 2 ml of culture medium were frozen in liquid nitrogen and stored at −80 °C until the analysis. *B. napus* cotyledons were cut with a Harris UniCore punch and 150 mg of tissue was frozen in nitrogen and stored at −80 °C. Extraction was performed as described previously [32]. Briefly, samples were homogenized in tubes with 1.3 mm silica beads using a FastPrep-24 instrument (MP Biomedicals, CA, United States) with extraction reagent methanol/H_2O/formic acid (15:4:1, v:v:v) supplemented with stable isotope-labeled auxin internal standards, each at 10 pmol per sample, to check the recovery during the purification and to validate the quantification. Supernatant was subjected to solid phase extraction using Oasis MCX cartridges (Waters Co., Milford, MA, United States). The eluate was evaporated completely to leave a dry pellet which was dissolved in 30 µl of 15% (v/v) acetonitrile in water. Quantification was performed on an Ultimate 3000 high-performance liquid chromatograph (HPLC; Dionex, Bannockburn, IL, United States) coupled to a 3200 Q TRAP hybrid triple quadrupole/linear, ion-trap, mass spectrometer (MS; Applied Biosystems, Foster City, CA, United States) as described in [33]. Metabolite levels were expressed in pmol/g fresh weight (FW) for mycelium or plant tissue, or in pmol/ml for cultivation media.

2.6. Gene expression analysis

100–150 mg of filtered mycelium or fresh plant tissue was frozen in liquid nitrogen and homogenized using FastPrep-24 (MP Biomedicals). Total RNA was extracted using RNA Spectrum Plant Total RNA Kit (Sigma-Aldrich) according to manufacturer's instructions. RNA was treated with DNAFree Kit (Ambion) to eliminate DNA contamination. Concentration of RNA was determined by Nanodrop ND-1000 (ThermoFisher Scientific). Equivalent of 1 µg RNA was converted into cDNA using oligo dT_{21} primer (Metabion, Martinsried, Germany) and M-MLV Rnase H(−)Point Mutant reverse transcriptase (Promega Corp., Fitchburg, WI, United States). The cDNA was diluted 20x and the equivalent of 6.25 ng of original RNA of diluted cDNA was used for quantitative PCR. Light Cycler® 480 SYBR Green I Master mix and appropriate primers were used for qPCR analysis in LightCycler® 480 Instrument (Roche, Basel, Switzerland). The PCR program was set as follows: 95 °C for 10 min; followed by 45 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s; followed by melting curve analysis. Threshold cycles and melting curves were calculated using LightCycler Software 4.1 (Roche). Relative transcription was calculated with efficiency correction and normalization to transcription of *LmTubulin (LmTUB)*. Primers were designed using software PerlPrimer version 1.1.21 [34]. Accession numbers of *L. maculans* genes of interest and primers are listed in Supp. Tables 2 and 4.

2.7. Homology and phylogenetic analyses

Protein homology searches were performed using BLASTp implemented in NCBI. For *L. maculans*, orthologues were searched for in the genome of isolate JN3 [35]. For phylogenetic tree construction, the protein sequence of PaIPDC of *Pantoea ananatis* [11] was used as a query. Protein sequences were aligned with the MUSCLE program as implemented in www.phylogeny.fr [36]. The Maximum-likelihood phylogenetic tree was generated with SeaView version 4 software [37] using a LG substitution model and bootstrapping with 100 replications. The tree was displayed with MEGA 5.2.2. software [38].

2.8. RNA sequencing and analysis

RNA sequencing data used in this work are part of a large scale sequencing project (http://www.genoscope.cns.fr/externe/leptolife/index.html). cDNA library and 150 bp paired read sequencing were done by the Genoscope (Evry, France) using Illumina HiSeq2500. Reads were mapped on the new version of the reference genome of *L. maculans* (JN3) [35,39] to obtain raw data count expression. Statistical analyses were done with the EdgeR package [40]. Raw counts of *L. maculans* from 2 days post-inoculation to 15 days post-inoculation were normalized by the TMM method. A general linearized model was applied with the glmQLFit function and then the glmQLFTest was used to make the statistical comparisons. Gene expression at each time point was compared to gene expression of all other time points resulting in 21 comparisons. For each gene of interest, the resulting *p*-value corrected for multiple testing and the log2(fold change) for *L. maculans* auxin pathways genes are indicated in the Supp. Table 3. RNAseq data are available at the EMBL-EBI nucleotide Archive under the project #PRJEB21682 (https://www.ebi.ac.uk/ena/data/view/PRJEB21682) containing 24 RNAseq samples including data for 5 dpi and 9 dpi, and under the project #PRJEB34899 for the remaining time points. Accessions number for RNAseq samples are as follows: in BioProject Id PRJEB34899, accessions ERX3592526 and ERX3592531 for infected cotyledons at 2 dpi; ERR3596732 for 5 dpi; ERR3596733 and ERR3596738 for 7 dpi; ERR3596734 for 9 dpi; ERR3596735 and ERR3596739 for 12 dpi; ERR3596740 and ERR3596736 for 15 dpi, along with accessions ERR2027131 and ERR2027132 in PRJEB21682 for additional RNAseq data for 5 and 9 dpi, respectively, previously characterized in [39].

2.9. Statistics in other experiments

If not stated otherwise, all experiments were repeated independently three times, with at least three independent samples (from independent biological material, cultivated under the same conditions). Using Statistica 12 software, statistical analyses were performed by paired two-tailed Student's *t*-test.

3. Results

3.1. *Mycelium of* L. maculans *produces auxin* in vitro

Several microorganisms, including fungi, have been reported to produce auxin [41,42]. We investigated whether the phytopathogenic fungus *L. maculans* is also capable of auxin production. For this purpose, the sequenced isolate v23.1.3 [35], referred to as JN3, was used and compared to its sister isolate v23.1.2, further referred to as JN2. Both isolates were issued from the same parent cross [29]. Isolates JN3 and JN2 were grown *in vitro* in liquid culture in chemically-defined Gamborg medium and the presence of auxins was analyzed both in mycelium and medium by liquid

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chromatography coupled to mass spectrometry. We have shown that *L. maculans* produces several forms of auxins, out of which IAA is the most abundant (Table 1). We observed that mycelium of isolate JN3 contained IAA at a concentration in thousands of pmol per gram fresh weight (FW) of mycelium. The oxidised form of IAA (OxIAA), lacking biological activity in plants [43], was the second most common auxin metabolite. Besides these two auxin forms, the IAA biosynthetic intermediate IAN was also present. IAA conjugates, such as IAA-Asp and IAA-Glu, were also detected, but at only very minor concentrations with less than reproducible detection. IAA and OxIAA were also detected in cultivation medium in both isolates (Table 1). The mycelium and medium of the JN2 isolate contained the same auxin metabolites as JN3; however, surprisingly, the IAA levels in JN2 mycelium were substantially lower than in the JN3 isolate, not exceeding 100 pmol IAA/g FW (Table 1). Correspondingly, production of IAA derivatives was about 100 fold lower than in JN3. In addition, both *L. maculans* isolates produced and secreted into the medium substantial levels of phenylacetic acid (PAA), another naturally-occurring auxin with a lower biological activity than IAA in plants [44]. Altogether, we have characterized the auxin profile of *in vitro* grown *L. maculans* and have shown that *L. maculans* is capable of IAA production and secretion into medium.

3.2. *IAA production in* L. maculans *can be stimulated by biosynthetic precursors*

Knowing that *L. maculans* is equipped with an active IAA biosynthetic apparatus, we aimed to characterize orthologs of biosynthetic enzymes in conditions stimulating IAA production. We therefore first evaluated whether the IAA content in *L. maculans* can be further enhanced by the exogenous addition of IAA biosynthetic precursors. Tryptophan (Trp) has been previously shown to stimulate IAA production in other fungi [7,14,45]. The structurally-similar molecule tryptamine was used for comparison [7]. IAA levels in mock-treated mycelium or the medium of both isolates remained constant during 6 h of experiment (data not shown), therefore mock-treated samples at 1 h were chosen as a control treatment (Fig. 1, Supp. Fig. 1). Adding tryptophan or tryptamine at 1 mM final concentration into the mycelial culture did not show any significant induction of IAA production in JN3 isolate (Fig. 1A and B); however, we were able to induce IAA levels in its sister isolate JN2 (Fig. 1C and D, Supp. Table 1). Indeed, mock-treated mycelium contained IAA level of about 50 pmol/g FW (Fig. 1C and D), while IAA levels in precursor-treated JN2 mycelium were gradually increasing with time as could already be observed 1 h after treatment (Fig. 1C and 1D). After 6 h, tryptophan-induced IAA level reached 600 pmol/g FW in JN2 (Fig. 1C). Interestingly, tryptamine-induced IAA level reached up to 18,000 pmol/g FW (Fig. 1D), suggesting that tryptamine acts as a very potent IAA inducer in *L. maculans*. In correlation with the data in mycelium, overproduction of IAA following precursor treatment in JN2 isolate was also detectable in the culture medium (Supp. Fig. 1). Besides IAA, we observed that the treatment with precursors did not significantly enhance levels of most other auxin forms in JN2 mycelium or medium. The exception was observed for oxIAA and PAA being enhanced after 6 h of tryptamine treatment (Supp. Table 1).

3.3. *Analysis of biosynthetic pathways present in* L. maculans

Given that *L. maculans* possesses a functional and tryptophan/tryptamine-reactive auxin biosynthetic apparatus, we screened the genome of *L. maculans* [35] for orthologs of previously-characterized, auxin biosynthetic enzymes. Based on the BLAST analysis, we revealed that the *L. maculans* genome encodes putative orthologs of all of the yet-known biosynthetic pathways (Fig. 2; see Supp. Table 2 for accession codes). Concerning the IPyA pathway, we found two clear orthologs of *U. maydis* tryptophan aminotransferase UmTAM1 (E-values of $3e^{-49}$ and $1e^{-27}$ for LmTAM1 and LmTAM2, respectively) and two clear orthologs for indole-3-acetaldehyde dehydrogenase UmIAD1 (E-values of $1e^{-178}$ and $1e^{-177}$ for LmIAD1 and LmIAD2, respectively) out of a 15-member family. Besides, *L. maculans* possesses two orthologs of indole-3-pyruvate decarboxylase IPDC (E-values of $8e^{-119}$ and $4e^{-95}$ for LmIPDC1 and LmIPDC2, respectively) associated with the IPyA pathway in bacteria [12]. We also found orthologs of the flavin-containing monooxygenase AtYUCCA1 enzyme of *A. thaliana* that can be either assigned to the IPyA pathway or tryptamine pathway [46]. Concerning the IAN pathway, 2 orthologs of nitrilase AtNIT were detected (with a best E-value match of $4e^{-92}$). The orthology for CYP79B2/B3 was difficult to evaluate due to the vastness of the cytochrome families and estimation of enzyme specificity based on protein similarity. Interestingly, *L. maculans* further possessed *iaaM*- and *iaaH*-like genes from the IAM pathway described mainly in bacteria. The detailed results of the orthology analysis based on protein similarity are shown in Supp. Table 2. The best matching orthologs were retrieved for further functional characterization.

3.4. *Candidate biosynthetic genes of IPyA pathway are upregulated upon IAA production in* L. maculans

To investigate the involvement of the retrieved candidate orthologs from different pathways in the IAA biosynthesis, their expression upon IAA production was analyzed. For such functional characterization we took advantage of the previously-characterized setup, the mycelium of JN2 isolate exogenously treated with tryptophan or tryptamine, which stimulates IAA production. At 1 h after

Table 1
Auxin form content in mycelium and medium of *L. maculans* isolates JN3 and JN2. *L. maculans* was grown for 7 days in liquid culture. Data represent mean concentrations of detected auxin forms (pmol/g fresh weight, FW, of mycelium or pmol/ml of medium) ± SE from 4 independent experiments, each in triplicates (n = 12). Only values above 0.2 pmol/g FW or 0.2 pmol/ml are shown, otherwise mentioned as "Nd", not detected. IAN – indole-3-acetonitrile, IAA – indole-3-acetic acid, IAA-Asp – IAA-Aspartate, IAA-Glu – IAA-Glutamate, OxIAA – oxindole-3-acetic acid, PAA – phenylacetic acid.

Auxin form	JN3		JN2	
	Mycelium (pmol/g FW)	Medium (pmol/ml)	Mycelium (pmol/g FW)	Medium (pmol/ml)
IAN	4.6 ± 2.3	Nd	1.1 ± 0.6	Nd
IAA	5882.7 ± 1064.0	2245.2 ± 1188.3	52.5 ± 10.1	26.6 ± 1.7
IAA-Asp	0.4 ± 0.3	Nd	Nd	Nd
IAA-Glu	0.7 ± 0.2	Nd	0.5 ± 0.2	Nd
OxIAA	105.6 ± 46.0	19.6 ± 6.3	25.2 ± 4.0	37.7 ± 11.1
Total IAA derivatives	5994.1 ± 1112.8	2264.8 ± 1194.6	79.3 ± 14.8	64.3 ± 12.8
PAA	2635.7 ± 565.2	1897.8 ± 249.5	1699.2 ± 457.6	1569.8 ± 369.0

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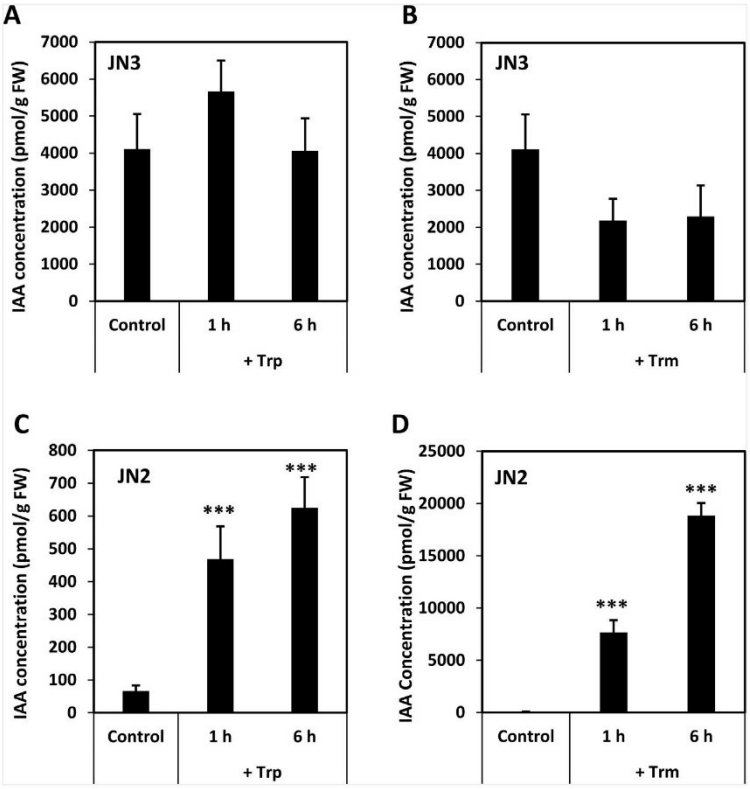

Fig. 1. Stimulation of IAA production by auxin biosynthetic precursors in *L. maculans in vitro*. 7-day old mycelium of *L. maculans* cultivated *in vitro* in liquid medium was treated with 1 mM tryptophan (+Trp; A, C) or tryptamine (+Trm; B, D). Mycelium samples were collected 1 h and 6 h after treatment and reported to mock treatment at 1 h (Control). Data represent mean values (pmol/g fresh weight, FW) ± SE of 4 biological replicates. The experiment was repeated three times. Two isolates were analyzed in this experiment – JN3 (A, B) and JN2 (C, D). Asterisks indicate significant differences between the precursor-treated sample and the corresponding control according to two-tailed Student's t-test (***$P < 0.001$).

tryptophan treatment, we observed significantly induced expression of *LmTAM1*, *LmIPDC2* and *LmNIT1* genes compared to the control treatment (Fig. 3A, 3B, 3C). Furthermore, *LmIPDC1*, *LmiaaM3* and *LmiaaM5* genes were weakly upregulated (Fig. 3A and 3C) with about 2-fold induction. The expression of other genes was not altered (Fig. 3). Interestingly, tryptamine treatment, which was shown to be a very strong inducer of IAA production, led only to mild stimulation of gene expression in the investigated time points. We observed induction of *LmNIT1*, and *LmiaaM3*, but it was, however, to a lesser extent (Fig. 3B and 3C). To sum up, these data suggest that *L. maculans* can produce IAA via the IPyA pathway from tryptophan, using *LmTAM1* and *LmIPDC2/1* genes. In parallel, *LmNIT1* from the IAN pathway and *LmiaaM5* and *LmiaaM3* from the IAM pathway may be also active enzymes participating in auxin production in *L. maculans*, converting tryptophan to IAA.

3.5. IPDC family in fungi

Little is known about the IPDC family in fungi. We have carried out a phylogenetic analysis of putative IPDC proteins for the most common phytopathogenic fungi (Fig. 4) based on the orthology with PaIPDC of *P. ananatis* bacterium. Other organisms, such as non-pathogenic fungi, yeast and bacteria, were used for comparison. The *L. maculans* genome harbors two copies of genes encoding IPDC-like proteins, similarly to other fungi classified in the *Dothideomycetes* (Fig. 4). We found the closest orthologs of LmIPDC proteins in *Parastagonospora nodorum*, *Alternaria alternata*, *Bipolaris maydis* and *Pyrenophora tritici-repentis* (Fig. 4). IPDC1 and IPDC2 putative proteins form two distinct groups and do not cluster with IPDC proteins of yeast species, such as *S. cerevisiae* or *C. albicans*. Interestingly, the strictly biotrophic fungi *Blumeria graminis* and *P. graminis* IPDCs form a separate branch very distinct from other fungal species. Only one orthologue of IPDC was found in *Magnaporthe oryzae*, *Neurospora crassa* and *U. maydis* (Fig. 4).

3.6. Auxin profile of an infected host plant

Since we have shown that the *L. maculans* isolate JN3 contains a high basal level of IAA and excretes auxin out of the mycelium (Table 1), we investigated auxin metabolic changes in the infected host-plant tissue of *B. napus* cotyledons (Table 2) 9 days post infection. The main auxin metabolites in the sampled mock-infected plant tissue were the auxin intermediates IAN and IAM, then the IAA oxidation product oxIAA and its glucose ester oxIAA-GE and IAA. Upon *L. maculans* infection, the profiles of auxin metabolites in JN3-infected and mock-treated cotyledons did not differ

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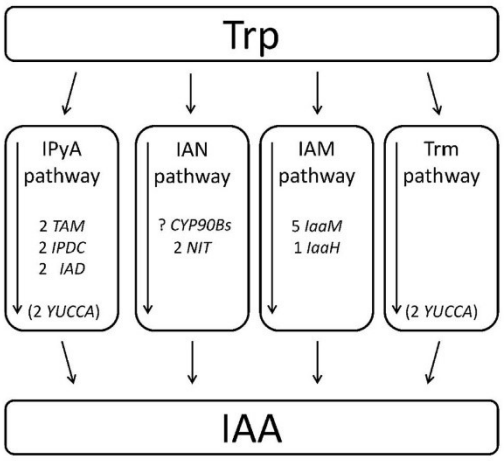

Fig. 2. Number of orthologues of known tryptophan dependent IAA biosynthesis genes in *L. maculans* genome. Orthologues of tryptophan (Trp)-dependent IAA biosynthetic enzymes were identified in *L. maculans* genome: IPyA pathway consists of orthologues of TAM (tryptophan aminotransferase), IPDC (indole-3-pyruvate decarboxylase), IAD (indole-3-acetaldehyde dehydrogenase) and YUC (flavin-containing monooxygenase). IAN pathway consists of orthologues of cytochrome P450, CYP79B2/3 and NIT (nitrilase). IAM pathway consists of orthologues of iaaM (tryptophan monooxygenase) and iaaH (indoleacetamide hydrolase). Tryptamine (Trm) pathway consists of orthologues of YUC. Accession codes and parameters of protein orthology are listed in Supp. Table 2.

significantly for most metabolites (Table 2). The only exception was the OxIAA metabolite which increased more than 4 times upon infection. IAA levels increased in JN3-infected tissue but not significantly. The total level of auxin showed an almost 60% decrease, caused mainly by decrease in IAN.

In addition, the expression of orthologous auxin biosynthetic genes was analyzed using a RNAseq dataset corresponding to a time-course series of infected plant tissue. The 6 genes which have been detected upregulated upon IAA production are shown in Supp. Fig. 2. Expression of *LmIPDC2*, *LmIPDC1*, *LmTAM1* and *LmNIT1* decreased in the initial phase of infection with the lowest expression at 7 dpi. Later on the expression of these genes increased slightly. Statistical analyses of the other auxin candidate genes are shown in Supp. Table 3.

3.7. *Externally-added auxin inhibits growth of* L. maculans

We further assessed whether IAA might be important for *L. maculans* physiology and growth regulation, similarly to plants. Growth-inhibition tests were therefore carried out using GFP-labeled *L. maculans* isolates, enabling sensitive detection by measuring fluorescence in 96-well-plates. The presence of exogenous IAA during growth in Gamborg medium caused a concentration-dependent inhibition of growth on both the studied isolates, JN3 and JN2 (Fig. 5). Exogenous IAA inhibited growth in a concentration- and strain-dependent manner. A concentration of 500 μM and higher reduced growth by more than 90% in both isolates. 1 mM IAA totally blocked conidial germination. No beneficial effect of IAA on *L. maculans* growth was observed (Fig. 5).

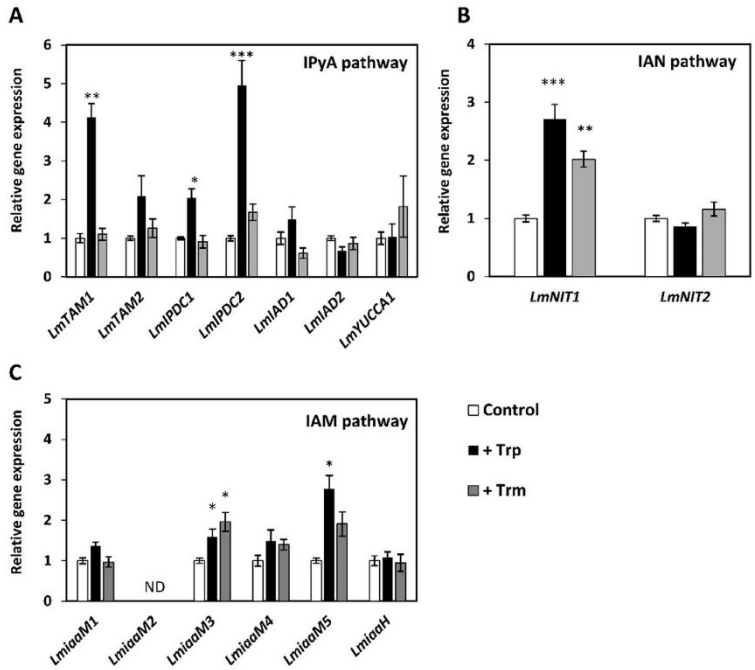

Fig. 3. Expression of auxin biosynthesis genes can be stimulated by biosynthetic precursors in *L. maculans*. 7-day-old *in vitro* culture of *L. maculans* isolate JN2 was supplemented with 1 mM tryptophan (+Trp), tryptamine (+Trm) or a control treatment. Samples were collected 1 h after treatment. Gene expression was analyzed by qPCR, normalized to *LmTUB* and data were reported to control (set as 1). Genes belonging to IPyA (A), IAN (B) and IAM (C) pathway are presented. Data represent means ± SE from 8 biological replicates from three independent experiments. Asterisks indicate statistically significant differences when compared to control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; two-tailed Student's *t*-test).

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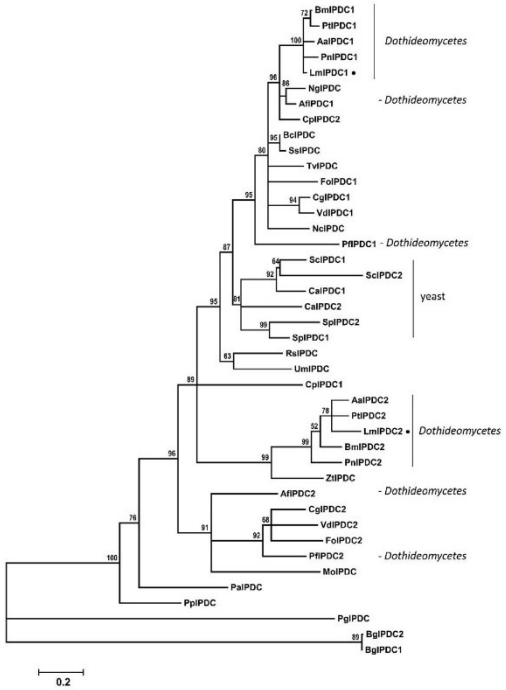

Fig. 4. IPDC phylogeny in fungi. Maximum-likelihood phylogenetic tree showing the relationship between protein sequences (GenBank) of indolepyruvate decarboxylases (IPDCs) from bacteria *Pantoea ananatis* (PaIPDC) and *Pseudomonas putida* (PpIPDC), and putative orthologues from fungus *L. maculans* (LmIPDC1, LmIPDC2; highlighted with ●) and other fungi. Branches with bootstraps lower than 50 (out of 100) were merged. Aa - *Alternaria alternata*, Af - *Aspergillus fumigatus*, Bc - *Botrytis cinerea*, Bg - *Blumeria graminis*, Bm - *Bipolaris maydis*, Ca - *Candida albicans*, Cg - *Colletotrichum graminicola*, Cp - *Coccidioides posadasii*, Fo - *Fusarium oxysporum*, Mo - *Magnaporthe oryzae*, Nc - *Neurospora crassa*, Ng - *Nannizzia gypsea*, Pa - *Pantoea ananatis*, Pf - *Pseudocercospora fijiensis*, Pg - *Puccinia graminis*, Pn - *Parastagonospora nodorum*, Pp - *Pseudomonas putida*, Pt - *Pyrenophora tritici-repentis*, Rs - *Rhizoctonia solani*, Sc - *Saccharomyces cerevisiae*, Sp - *Schizosaccharomyces pombe*, Ss - *Sclerotinia sclerotiorum*, Tv - *Trichoderma virens*, Um - *Ustilago maydis*, Vd - *Verticillium dahliae*, Zt - *Zymoseptoria tritici*.

4. Discussion

4.1. Auxin biosynthesis in fungi

In addition to plants, there are various microorganisms that produce auxins. Among these are both phytopathogenic fungi and symbiotic fungi [9,41,47]. Here we have characterized auxin levels in two isolates of the phytopathogenic fungus *L. maculans*. We have clearly shown that *L. maculans* can produce and secrete IAA *in vitro*, which we detected both in the mycelium and cultivation medium (Table 1). We also showed that the basal level of auxin production among these isolates differed (Table 1), as did the inducibility of IAA production upon exogenous treatment with biosynthetic precursors, such as tryptophan and tryptamine. We demonstrated that the *L. maculans* isolate JN3 produces high amounts of IAA, even without precursor stimulation. Interestingly, the precursor treatment did not further enhance IAA levels in the JN3 isolate (Fig. 1A and B). On the other hand, the JN2 isolate with low basal IAA production could be stimulated, either by tryptophan or tryptamine, to produce substantial amounts of IAA (Fig. 1C and D). Similarly, rice blast fungus *M. oryzae* [41], or several white rot fungi

Table 2
Auxin profile in cotyledons of *B. napus* infected with *L. maculans*. 14-day-old *B. napus* cv. Eurol cotyledons were inoculated with conidia of *L. maculans* JN3 or mock-inoculated. The tissue area around lesions was sampled 10 days after inoculation. Auxin content was analyzed by LCMS. Data represent mean concentrations (pmol/g fresh weight, FW of plant tissue) ± SE from 4 biological replicates. The experiment was repeated three times. Asterisks represent statistically significant difference (*$P < 0.05$, two-tailed Student's *t*-test) between JN3-infected and mock-infected cotyledons. IAN – indole-3-acetonitrile, IAM – indole-3-acetamide, IAA – indole-3-acetic acid, IAA-Asp – IAA-aspartate, IAA-Glu – IAA-glutamate, IAA-Leu – IAA-leucine, IAA-GE – IAA-glucose ester, OxIAA – oxindole-3-acetic acid, OxIAA-GE – OxIAA-glucose ester, PAA – phenylacetic acid derivatives.

Auxin form	Concentration (pmol/g FW)	
	mock-infected	JN3-infected
IAN	36251.8 ± 18895.8	14120.1 ± 5212.8
IAM	315.1 ± 124.0	77.5 ± 17.3
IAA	107.4 ± 11.4	172.8 ± 93.8
IAA-Asp	2.9 ± 1.2	5.3 ± 2.0
IAA-Glu	1.5 ± 0.5	1.6 ± 0.3
IAA-Leu	0.6 ± 0.1	0.2 ± 0.0
IAA-GE	1.1 ± 0.2	2.9 ± 0.5
OxIAA	214.5 ± 29.0	906.1 ± 214.4*
OxIAA-GE	423.8 ± 185.5	452.7 ± 131.4
Total IAA derivatives	37245.4 ± 11584.0	15453.8 ± 3581.9
PAA	353.9 ± 150.0	781.1 ± 347.9

[42], can produce IAA without exogenous stimulants. Other fungi require supplementation with appropriate biosynthetic precursors to produce IAA; this has been reported in *Colletotrichum gloeosporioides*, *U. maydis*, *N. crassa* and *Fusarium oxysporum* [9,41,48,49]. The genetic basis for this differential behaviour between JN2 and JN3 needs to be further investigated.

Based on the *L. maculans* genome analysis, our study also identified genes encoding putative enzymes of different IAA biosynthetic pathways (Fig. 2, Supp. Table 2), orthologous to those previously characterized in plants, fungi and bacteria. We found conserved genes for the IPyA, IAN and IAM pathway. Our data suggest that *L. maculans* mainly biosynthesizes IAA via the IPyA pathway. We show that among the analyzed biosynthetic orthologs, those predominantly induced were *LmTAM1*, encoding putative tryptophan aminotransferase, and *LmIPDC2* and *LmIPDC1* genes, encoding putative indole-3-pyruvate decarboxylases (Fig. 3A). LmTAM1 may convert tryptophan into IPyA which is subsequently metabolized into IAA by IPDC. The aldehyde dehydrogenase IAD has also been reported to participate in IPyA pathway-mediated IAA biosynthesis in fungi [9], although no induction of IAD genes has been observed in *L. maculans* upon IAA production. Neither IPyA nor IAAld intermediates could be detected, possibly due to the instability of the metabolites. The involvement of IPDC genes have been previously reported in several plant-associated bacteria, for example, *Azospirillum brasilense*, *Pseudomonas fluorescens* and *P. ananatis*, and the plant-associated trypanosomatid *Phytomonas serpens* [11,50–52]. We have identified two clear orthologs of the *P. ananatis* IPDC in the *L. maculans* genome (Fig. 4). Both *LmIPDC* genes were induced upon tryptophan treatment (Fig. 3). Recently, IPDC involvement in IAA biosynthesis has also been described in the non-pathogenic fungus *N. crassa* [49].

Besides the IpyA pathway, our data suggest that *L. maculans* probably also uses the IAN pathway to biosynthesize IAA. Indeed, *LmNIT1*, encoding a putative nitrilase orthologous to *A. thaliana* nitrilase able to convert IAN into IAA [53], was upregulated following tryptophan treatment (Fig. 2B). Furthermore, the IAN metabolite was detected in *L. maculans* mycelium, though only in minor amounts (Table 1). Concerning the IAM pathway, specific intermediates were not detected (Table 1); however, a mild but

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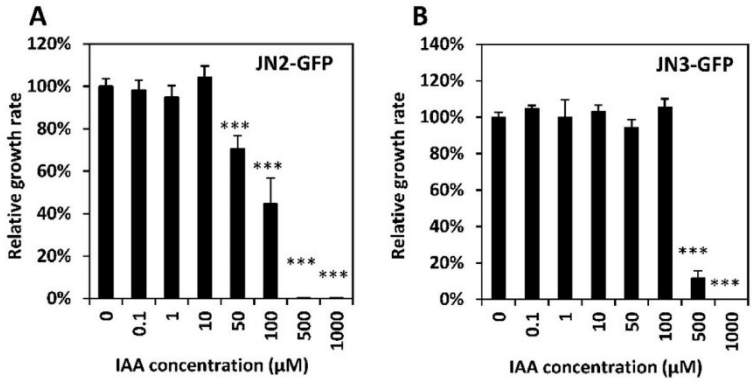

Fig. 5. Exogenous IAA inhibits growth of *L. maculans* JN2-GFP and JN3-GFP. *L. maculans*, strains JN2-GFP (A) and JN3-GFP (B) was cultivated *in vitro* in 96-well-plate in medium containing IAA. Fluorescence was measured at 4 dpi and reported to mock (IAA 0) treatment. Data represent mean ± SE from 12 biological replicates. The experiment was repeated three times. Asterisks indicate significant differences between precursor treated and mock (0) treated samples (**$P < 0.01$, ***$P < 0.001$; two-tailed Student's *t*-test).

significant overexpression of *LmiaaM3* and *LmiaaM5* genes was observed upon IAA production (Fig. 3C), suggesting that this pathway, at least partly, may also be functional in *L. maculans* cultivated *in vitro*. IAM induced IAA production in the non-pathogenic fungus *N. crassa,* although this fungus is not able to produce IAM itself [49].

4.2. IAA metabolism in fungi

IAA is the major auxin metabolite of *L. maculans*. Similarly, IAA was also the principal metabolite to be enhanced after precursor treatment (Fig. 1C and D, Supp. Table 1). In other fungi, IAA is often the most abundant auxin; however, other auxin forms can be detected in high concentration as well. *Fusarium* species also produce indole-3-acetamide and tryptophol, besides IAA at a high concentration [14]. Further, IAA and tryptophol were the major detected auxins in *M. oryzae* [41]. *N. crassa* produces indole-3-lactic acid (ILA) and tryptophol besides IAA [49]. Amino acid or glucose conjugates of IAA occurred only very weakly, both in the mycelium and cultivation medium treated or not with precursors (Table 1, Supp. Table 1). Among these conjugates only IAA-Asp, IAA-Glu, and a glucose ester of oxIAA (oxIAA-GE) were detected; however, at very low concentrations and not always in a reproducible manner, showing levels close to the detection limit of the method. These data suggest only a very weak metabolization in comparison to plants, where GE conjugates are highly present (Table 2). OxIAA is the second most important auxin metabolite in *L. maculans* (Table 1). The massive increase in IAA levels after tryptamine feeding at 6 h also led to an increase in oxIAA (Supp. Table 1). Its presence suggests that IAA is being oxidized in *L. maculans*. In plants, this oxidation is catalyzed by dioxygenase DAO1, identified in rice [46] and *A. thaliana* [54].

4.3. Evidence for PAA synthesis by L. maculans

Besides IAA and IAA derivatives, substantial amounts of PAA (Table 1, Supp. Table 1) were detected in both *L. maculans* isolates. PAA has been identified as an active auxin in plants and also in bacterial *Streptomyces* species [55] and the growth-promoting bacterium *A. brasilense* [51]. Furthermore, PAA produced by *Streptomyces humidus* acts as an antifungal agent against various fungi and oomycetes [56]. However, its biosynthesis and activity in fungi is mainly unexplored. Indole-3-pyruvate decarboxylase participates in the biosynthesis of PAA in *A. brasilense* [51], and tryptophan aminotransferases TAA and YUCCA enzymes have also been suspected to participate in the biosynthesis of PAA in plants [57,58].

4.4. Tryptamine-induced IAA biosynthetic route

Tryptophan is a precursor of IAA biosynthesis in different organisms and is commonly used to induce IAA. We show that tryptamine, a tryptophan derivative, is also able to induce IAA in *L. maculans,* leading to massive IAA levels some 30-fold higher than upon tryptophan treatment (Fig. 1). Accordingly, tryptamine has led to IAA production in *U. maydis* and *N. crassa* [9,49]. In *U. maydis*, tryptamine behaved as a potent IAA inducer causing a 10-fold higher IAA production than tryptophan [9], whilst in *N. crassa* it led only to a minor induction of IAA [49]. Interestingly, despite this massive tryptamine-induced IAA overproduction in *L. maculans*, the expression of the candidate genes was mostly unaltered. We could observe only a slight induction of *LmNIT1* and *LmiaaM3*, while the expression of *LmTAM1* and *LmIPDC2,* actively over-expressed upon tryptophan-induced IAA production, remained constant. These data suggest a separate tryptamine-induced IAA biosynthetic route in *L. maculans,* independent of a LmTAM1/IPDC-mediated pathway. Interestingly, neither the *UmTAM1* nor *UmTAM2* genes of *U. maydis* were involved in the conversion of tryptamine into IAA [9]; however, the genes *UmIAD1* and *UmIAD2* did participate in this conversion, unlike in our study. For *N. crassa*, it remains unclear which biosynthetic genes participate in this conversion [49]. In fungus *Metarhizium robertsii*, however, a tryptophan decarboxylase MrTDC was involved in the conversion of tryptamine into IAA [16].

4.5. IAA in the infection process

Despite the high levels of IAA produced by *L. maculans* JN3 *in vitro*, the levels of IAA in infected cotyledons were not significantly altered (Table 2). On the other hand, the infection with *L. maculans* was associated with an increase in the OxIAA metabolite. Assuming that *L. maculans* behaves as a source rich in IAA (Table 1), a plant may aim to regulate its endogenous IAA levels by converting/oxidizing the bioactive IAA molecule. In our hands, the *L. maculans* infection did not impact the plant's auxin metabolic profile. In some plant-microbial systems, IAA has been shown to act as an important virulence factor that impairs the defense-

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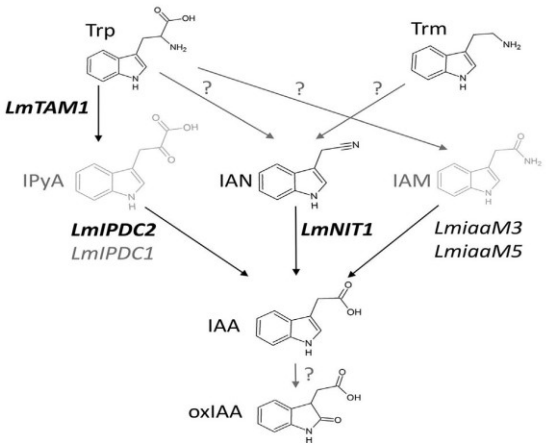

Fig. 6. Proposed model illustrating possible auxin metabolism in *L. maculans*. The model summarizes our experimental data based on gene expression and phytohormone content analyses upon feeding with precursors and integrates them into known biosynthetic pathways [7,64]. Supplying either tryptophan (Trp) or tryptamine (Trm) leads to IAA production in *L. maculans*. IAA is oxidized to oxindole-3-acetic acid (oxIAA). Conversion of Trp into IAA involves mainly genes encoding tryptophan aminotransferase *LmTAM1* and indolepyruvate decarboxylase *LmIPDC2* of the IPyA pathway and nitrilase *LmNIT1* of the IAN pathway. Trm is converted to IAA independently of the IPyA pathway and involves *LmNIT1* gene. Genes *iaaM3* and *iaam5* encoding indole-3-hydrolases might also participate in conversion of both Trp and Trm into IAA. Black arrows represent suggested conversions supported by the data from gene expression assays and the main overexpressed genes are shown in bold. Gray arrows represent putative conversions. Detected metabolites are shown in black. The intermediate metabolites (in gray) were not detected. IPyA: indole-3-pyruvate; IAN: indole-3-acetonitrile; IAM: indole-3-acetamide.

associated, phytohormone signaling of the host [15,59] in a similar way as effectors do [25,27,60]. For some of the IAA-producing fungi, such as the symbiotic *Piriformospora indica* or pathogenic *C. gloeosporioides* and *M. oryzae*, auxins are required for a proper colonization and infection process [41,48,61]. Externally-added IAA increased the virulence of *M. robertsii* spores on the insect *Beauveria bassiana* [16]. Loss of the ability to produce auxin led to a lowered virulence of *M. robertsii* against the pathogenic insect *B. bassiana* [16]. Mutants of *N. crassa* with blocked IAA biosynthesis produced less conidiospores compared to the WT [49].

4.6. *IAA in* L. maculans *physiology*

We have observed that exogenous IAA inhibits the growth of both the *L. maculans* isolates. Conidial germination was totally blocked by 1 mM IAA. For some fungi, IAA can be stimulatory at low concentrations and inhibitory at high concentration [62,63]. A similar effect was observed in the pathogen *F. graminearum*, which produced 50% less biomass when cultured in 1 mM IAA [62]. Loss of the ability to produce IAA resulted in developmental changes in *C. gloeosporioides* [20]. The effective concentration range is dependent on the species, strain, and cultivation conditions [62]. Growth of *L. maculans* was not affected by IAA unless it was used at a 1 mM concentration. Other fungi react to exogenous IAA differently, IAA ranging from 0.1 μM to 10 μM stimulated growth in *Moniliophtora perniciosa* in a concentration-dependent manner [63].

5. Conclusion

To sum up, this work shows that *L. maculans* can basally produce IAA in mycelium and into the medium. Production of IAA can be stimulated by using the biosynthetic precursors tryptophan and tryptamine. Tryptophan-induced IAA biosynthesis relies mainly on genes that belong to the IPyA and IAN pathways: aminotransferase *LmTAM1*, indole-3-pyruvate decarboxylase *LmIPDC2* and nitrilase *LmNIT1*. LmIPDC2 represents a new component of IAA biosynthesis in pathogenic fungi. The metabolic scheme in Fig. 6 has been proposed for IAA production in *L. maculans*. Altogether, this work characterizes auxin production in the fungus *L. maculans*, an important pathogen of oilseed rape. Deciphering the genetic basis for biochemical links in fungal auxin biosynthesis has set the basis for further functional genomic studies using knock-out mutants to assess the role of auxin in the life strategy of *L. maculans*, such as virulence and fitness. Broadening our knowledge of its infection strategies vis à vis its host, oilseed rape, can possibly be exploited by the biotechnology sector.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2020.05.001.

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3.6. Publication 6

Disrupted actin: a novel player in pathogen attack sensing?

3.6.1. Summary of results

The last included viewpoint article summarizes outcomes of recent studies dealing with the involvement of actin cytoskeleton in plant immunity. The majority of published studies points out that impaired function of actin cytoskeleton promotes plant susceptibility to pathogen infection since actin is a major cellular signalling platform. However, there is also indirect evidence that the effect of actin degradation on infection process may not always be adverse. Pharmacological treatment leading to actin disruption induced transcription of defence related genes[207]. The direct evidence that actin disruption indeed leads to plant resistance was shown in two studies included in this thesis [177], . The onset of resistance is specifically connected with the enhancement of SA-related signalling. Also some effects that are induced independently of SA were observed. These include callose deposition and transcription of defence genes. In the review article we hypothesize what is the mechanism that triggers immune signalling upon actin disruption and whether this effect is more generally valid. Why is this effect so specific in signalling only via the SA-pathway. We propose an updated model and potential directions for future research. Our main hypotheses are: 1) there is a specific receptor sensing depolymerized actin filaments. 2) Depolymerized actin affects connected processes such as ornagelle movement, which affects the SA-pathway. 3) Increased SA concentration is maintained by the effect of actin on SA-binding protein.

3.6.2. My contribution

I contributed to the viewpoint conceptualization, literature review and writing the manuscript.

New Phytologist

Forum

Viewpoint

Disrupted actin: a novel player in pathogen attack sensing?

Summary

The actin cytoskeleton is widely involved in plant immune responses. The majority of studies show that chemical disruption of the actin cytoskeleton increases plant susceptibility to pathogen infection. Similarly, several pathogens have adopted this as a virulence strategy and produce effectors that affect cytoskeleton integrity. Such effectors either exhibit actin-depolymerizing activity themselves or prevent actin polymerization. Is it thus possible for plants to recognize the actin's status and launch a counterattack? Recently we showed that chemical depolymerization of actin filaments can trigger resistance to further infection via the specific activation of salicylic acid (SA) signalling. This is accompanied by several defence-related, but SA-independent, effects (e.g. callose deposition, gene expression), relying on vesicular trafficking and phospholipid metabolism. These data suggest that the role of actin in plant–pathogen interactions is more complex than previously believed. It raises the question of whether plants have evolved a mechanism of sensing pathological actin disruption that eventually triggers defence responses. If so, what is the molecular basis of it? Otherwise, why does actin depolymerization specifically influence SA content but not any other phytohormone? Here we propose an updated model of actin's role in plant–microbe interactions and suggest some future directions of research to be conducted in this area.

Actin's involvement in plant immunity is being broadly studied (Li & Day, 2019). Plant immunity consists of two layers, separated temporally and spatially. The first one is associated with the plasma membrane and is dependent on the recognition of conserved molecules called microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) by pattern-recognizing receptors (PRRs). This immune layer is entitled pattern-triggered immunity (PTI). The second layer, effector triggered immunity (ETI), is based on the intracellular recognition of effectors, molecules secreted by pathogens inside cells to inhibit immune responses (Jones & Dangl, 2006).

The actin cytoskeleton is a highly dynamic structure maintained by a balance between monomeric G-actin and polymerized filamentous F-actin. The filaments are constantly growing from one barbed end and shortening from the other one (Porter & Day, 2016). The speed and direction of their growth are regulated by numerous factors (e.g. actin depolymerizing factors (ADFs)). In their resting state, actin filaments maintain cellular metabolism, providing a 'signalling friendly' environment, that is, by the correct recycling of the PRRs. Shortly after sensing a pathogen, actin is reorganized and its density increases at the infection site (Porter & Day, 2016). In this case, actin filaments serve as a delivery pathway for compounds restricting microbial spread (e.g., callose) and sending DAMPs (e.g. Pep1, oligogalacturonides) to the neighbouring cells (Choi & Klessig, 2016) (Fig. 1a). Actin reorganization can be triggered not only by living pathogens, but also by treatment with MAMPs (e.g. flg22, elf18, chitin) or DAMPs. The MAMP-induced remodelling requires reactive oxygen species (ROS) generated by RBOHD, defence-associated NADPH oxidase (Li *et al.*, 2017). If such polymerization is prevented/blocked in the presence of drugs (such as latrunculin B or cytochalasin E) or by genetically affected ADFs, several downstream events fail, thus leading to a higher susceptibility to pathogens (Henty-Ridilla *et al.*, 2013; Badet *et al.*, 2017; Li & Day, 2019). This brings the general conclusion that actin depolymerization causes susceptibility (Fig. 1b).

Several actin remodelling factors were also described in the context of immunity. For example, ADF4 in *Arabidopsis thaliana* is connected with the R-protein RPS5 recognizing the *Pseudomonas syringae* effector AvrPphB (Henty-Ridilla *et al.*, 2013, 2014; Li *et al.*, 2017). In wheat, ADF3 negatively regulates resistance to *Puccinia striiformis* f. sp. *tritici* in ROS-dependent manner (Tang *et al.*, 2015). Profilins form a complex with several other proteins to facilitate actin assembly and also binding membrane phospholipids (Pernier *et al.*, 2016). The transcription level of *AtPRF3* in *A. thaliana* decreased upon flg22 treatment, but the *A. thaliana prf3* mutant shows a stronger response to flg22 in actin density, ROS burst and root growth assay. Interestingly, this is followed by increased susceptibility to *P. syringae* (Sun *et al.*, 2018). Formins, particularly FORMIN4, contribute to local actin dynamics during interaction between *A. thaliana* and the nonhost pathogen *Blumeria graminis*, especially to the formation of cell wall appositions, the first line of plant defence composed of callose, proteins and phenolic compounds (Sassmann *et al.*, 2018). Capping protein (CP) is a major regulator of actin dynamics. For actin polymerization to occur, its expression needs to be downregulated, while its constitutive deficiency in the *A. thaliana cpb1* mutant results in a high density of filaments (Li *et al.*, 2015). However, the *cpb1* mutant supported higher bacterial growth, while CP-overexpressing plants were more resistant to *P. syringae* infection compared with the wild-type (WT) (Li *et al.*, 2017). Interestingly, to induce actin polymerization, CP needs to be downregulated after binding the phosphatidic acid (PA) that is derived from the activity of phospholipase D (Pleskot *et al.*, 2010; Li *et al.*, 2015). This launches a positive feedback loop, as F-actin activates phospholipase D of *Nicotiana tabacum*, while G-actin

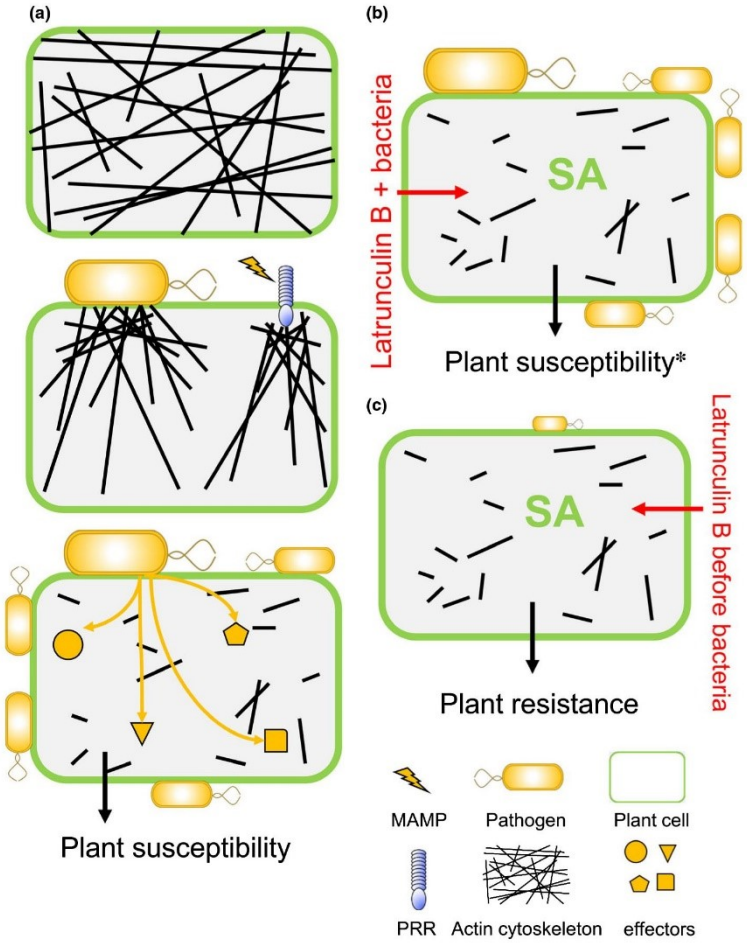

Fig. 1 (a) Model of the role of actin dynamics in plant–pathogen interactions showing the stages from normal actin cytoskeleton through reorganization after pathogen recognition to effects of secreted actin-depolymerizing effectors that lead to plant susceptibility. (b, c) Two distinct scenarios upon actin depolymerization after treatment with latrunculin B: (b) latrunculin B treatment leads to plant susceptibility; (c) latrunculin B pretreatment leads to induced resistance. MAMP, microbe-associated molecular pattern; PRR, pattern-recognizing receptor; SA, salicylic acid pathway. *, or no effect.

inhibits it *in vitro* (Pleskot *et al.*, 2010). This fits together with the fact that a PA increase follows the recognition of MAMPs and DAMPs, but also the application of salicylic acid (SA) (Kalachova *et al.*, 2013; Cacas *et al.*, 2017; Gully *et al.*, 2019). At the same time, chemical disruption of actin also triggers SA accumulation and SA-responsive genes (Matouskova *et al.*, 2014; Kalachova *et al.*, 2019; Leontovycova *et al.*, 2019). An activated SA pathway by actin disruption could even lead to increased plant resistance to pathogens (Leontovycova *et al.*, 2019) (Fig. 1c). This places actin remodelling at a crossroads of different signalling cascades. Another important connection of actin filaments with immunity lies in organelle movement coordination during pathogen attack. For example, actin is required for the anchoring of stromules, connecting the chloroplast and nucleus during infection (Kumar *et al.*, 2018). Chloroplasts are the major site of SA biosynthesis, so it is no surprise that pathogens may target chloroplasts to suppress SA-dependent immunity. This was recently described for bacterial and viral effectors (Medina-Puche *et al.*, 2019) and suggests an existing pathway, directly linking the plasma membrane to chloroplasts and activating plant defence. We believe that the actin network could be such a pathway.

Indeed, several pathogen effectors target the actin cytoskeleton's integrity to suppress plant immune responses. These effectors exhibit either actin-depolymerizing activity themselves or prevent actin polymerization. The *P. syringae*-secreted effector HopW1 disrupts the actin cytoskeleton and interacts with isoform 7 of vegetative actin (ACT7) (Jelenska *et al.*, 2014; Kang *et al.*, 2014). The Columbia ecotype of *A. thaliana* shows susceptibility when infected with *P. syringae* possessing this effector. However, in the Wassilewskija ecotype (Ws), HopW1 is recognized by WIN2 and WIN3 proteins with the subsequent onset of defence pathways triggering resistance against bacteria in Ws (Jelenska *et al.*, 2014). Another effector, HopG1, is responsible for the induction of cytoskeletal reorganization and infection-associated chlorosis. HopG1 interacts indirectly with actin filaments via forming a complex with mitochondria-localized kinesin protein. Moreover, the T3SS-deficient *P. syringae* strain *ΔhrpH* is both avirulent and unable to trigger the second phase of actin remodelling during PTI

in *A. thaliana* (Shimono *et al.*, 2016). It is worth noting that microtubules (MTs), the second component of the plant cytoskeleton, are also affected by secreted effectors, HopE1 (Cheong *et al.*, 2014) and XopL (Erickson *et al.*, 2018). MT disruption causes an increase in susceptibility to pathogens, as was shown for actin disruption (Schmidt & Panstruga, 2007; Lee *et al.*, 2012). However, treatment of *A. thaliana* with oryzalin, an MT-depolymerizing drug, does not trigger a strong immune response connected to SA (Matoušková *et al.*, 2014).

As actin polymerization occurs during PTI, and pathogens try to overcome it by secreting effectors leading to actin depolymerization, is it possible that plants have evolved another mechanism to sense a 'disrupted actin state' and relaunch immunity? If so, what mechanism lies beyond, and what molecule(s) is perceived? Is it based on a receptor-like signalling (e.g. free G-actin, short F-actin or ADFs interact with a receptor and this launches plant immunity) or is it an indirect connection (e.g. through the disturbed vesicular trafficking or phytohormones)? For example, actin was already shown to be a DAMP for mammalian (Srinivasan *et al.*, 2016) and insect (Ahrens *et al.*, 2012) cells.

We believe that future research can benefit hugely from natural variation studies. Indeed, a genome-wide associated study (GWAS) based on the screening of susceptibility to *Sclerotinia sclerotiorum* has already highlighted ARPC4, a new player involved in quantitative disease resistance and an actin organization regulator, both in the resting state and in response to infection (Badet *et al.*, 2019). Forward genetics can also be fruitful. In this case, it might be of interest to perform the ethyl methanesulfonate mutagenesis of plants with labelled actin filaments or with proteins responding to actin disruption (e.g. *PR2*). *PR2* expression could be a good marker, as it is induced after the chemical disruption of actin independently of SA (Kalachova *et al.*, 2019), so an impaired SA pathway in mutants will not influence the result. Reverse genetics is useful to understand signalling, but it needs to be carefully applied while working with immunity-related mutants. Indeed, many plants with altered immune pathways exhibit strong pleiotropic phenotypes, including growth retardation (Rate *et al.*, 1999; Yi & Richards, 2008). The behaviour of those plants is thus significantly affected by hormonal misregulation, especially SA increase (Pluharova *et al.*, 2019). The crossing of those mutants will bring new biases that need to be carefully interpreted. All this significantly limits actin-remodelling research, as we cannot fully exclude the pleiotropic effects of actin disruption caused by a pharmacological approach, while null mutants make time-shift studies impossible. The creation of transgenic plants with inducible actin depolymerization would therefore be very useful. One such line was recently prepared for animal studies (Harterink *et al.*, 2017) and has already been applied in plants as part of research into actin remodelling in lateral root formation (Vilches Barro *et al.*, 2019).

The specificity of triggering an SA pathway by actin disruption encourages us to believe that there is a unique signalling pathway involved. This pathway will probably overlap with other cell responses that coincide with cytoskeleton degradation (such as autophagy, senescence of induced cell death), but will include strict feedback regulation to prevent irreversible effects. Much still remains to be described. What is responsible for triggering the SA pathway? We can hypothesize about the earlier-mentioned receptor-like recognition, but it could also be that some SA-binding proteins exhibit actin-binding properties. In such a case, this double binding (or its interruption) could lead to further induction of an SA pathway. In human cells, actin ACTN1 and actin-binding filamin B (FLNB), the proteins directly involved in cytoskeletal rearrangements, were found to be SA-binding proteins (Choi *et al.*, 2019). In plants, the SA-binding proteins are mostly associated with respiration, photosynthesis, or signalling; no direct interactions with the cytoskeleton have yet been reported (Manohar *et al.*, 2014; Pokotylo *et al.*, 2019).

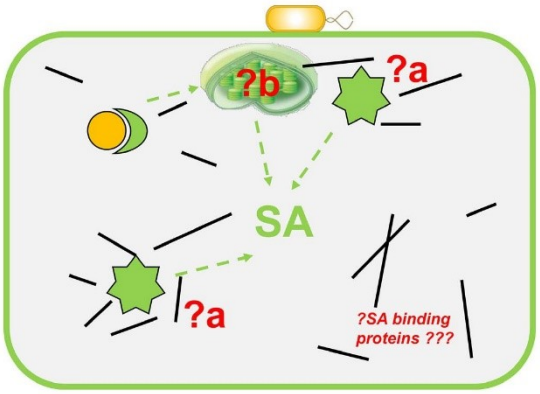

Fig. 2 How does actin depolymerization activate a salicylic acid (SA) pathway? In plants there is a receptor for depolymerized actin filaments (direct effect) (a). Depolymerized actin affects any of the processes such as organelle movements that subsequently activate an SA pathway (indirect effect) (b). Increased SA concentration is mediated through the effects of actin on SA-binding proteins.

Is the triggering of plant immunity (the SA pathway, in particular) caused by the disruption of actin filaments conserved during the evolutionary process? The SA pathway is conserved in land plants (Wang *et al.*, 2015). Two actin genes have already been seen in algae, while *A. thaliana* plants possess 12 isoforms of actin genes, expressed specifically in different tissue types and specific developmental stages (Slajcherova *et al.*, 2012). Similarly, CP-regulating actin polymerization has conserved sequencing found among all eucaryots (Cooper & Sept, 2008). This suggests that the mechanism of actin disruption sensing will be conserved across all land plants too. This may be investigated in parallel in diverse model systems.

Recent studies have found some answers, but they have also added more complexity to the current model of the role of the actin cytoskeleton in plant–microbe interactions (Fig. 2). Here we propose that a disrupted actin state be considered a plant cell component, which is sensed by a specific mechanism and thus triggers immune-like responses.

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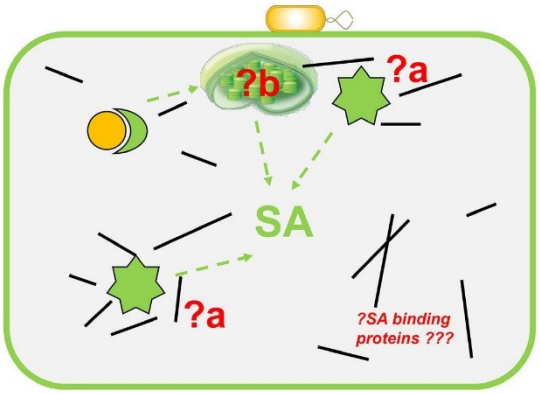
in *A. thaliana* (Shimono *et al.*, 2016). It is worth noting that microtubules (MTs), the second component of the plant cytoskeleton, are also affected by secreted effectors, HopE1 (Cheong *et al.*, 2014) and XopL (Erickson *et al.*, 2018). MT disruption causes an increase in susceptibility to pathogens, as was shown for actin disruption (Schmidt & Panstruga, 2007; Lee *et al.*, 2012). However, treatment of *A. thaliana* with oryzalin, an MT-depolymerizing drug, does not trigger a strong immune response connected to SA (Matoušková *et al.*, 2014).

As actin polymerization occurs during PTI, and pathogens try to overcome it by secreting effectors leading to actin depolymerization, is it possible that plants have evolved another mechanism to sense a 'disrupted actin state' and relaunch immunity? If so, what mechanism lies beyond, and what molecule(s) is perceived? Is it based on a receptor-like signalling (e.g. free G-actin, short F-actin or ADFs interact with a receptor and this launches plant immunity) or is it an indirect connection (e.g. through the disturbed vesicular trafficking or phytohormones)? For example, actin was already shown to be a DAMP for mammalian (Srinivasan *et al.*, 2016) and insect (Ahrens *et al.*, 2012) cells.

We believe that future research can benefit hugely from natural variation studies. Indeed, a genome-wide associated study (GWAS) based on the screening of susceptibility to *Sclerotinia sclerotiorum* has already highlighted ARPC4, a new player involved in quantitative disease resistance and an actin organization regulator, both in the resting state and in response to infection (Badet *et al.*, 2019). Forward genetics can also be fruitful. In this case, it might be of interest to perform the ethyl methanesulfonate mutagenesis of plants with labelled actin filaments or with proteins responding to actin disruption (e.g. *PR2*). *PR2* expression could be a good marker, as it is induced after the chemical disruption of actin independently of SA (Kalachova *et al.*, 2019), so an impaired SA pathway in mutants will not influence the result. Reverse genetics is useful to understand signalling, but it needs to be carefully applied while working with immunity-related mutants. Indeed, many plants with altered immune pathways exhibit strong pleiotropic phenotypes, including growth retardation (Rate *et al.*, 1999; Yi & Richards, 2008). The behaviour of those plants is thus significantly affected by hormonal misregulation, especially SA increase (Pluharova *et al.*, 2019). The crossing of those mutants will bring new biases that need to be carefully interpreted. All this significantly limits actin-remodelling research, as we cannot fully exclude the pleiotropic effects of actin disruption caused by a pharmacological approach, while null mutants make time-shift studies impossible. The creation of transgenic plants with inducible actin depolymerization would therefore be very useful. One such line was recently prepared for animal studies (Harterink *et al.*, 2017) and has already been applied in plants as part of research into actin remodelling in lateral root formation (Vilches Barro *et al.*, 2019).

The specificity of triggering an SA pathway by actin disruption encourages us to believe that there is a unique signalling pathway involved. This pathway will probably overlap with other cell responses that coincide with cytoskeleton degradation (such as autophagy, senescence of induced cell death), but will include strict feedback regulation to prevent irreversible effects. Much still remains to be described. What is responsible for triggering the SA pathway? We can hypothesize about the earlier-mentioned receptor-like recognition, but it could also be that some SA-binding proteins exhibit actin-binding properties. In such a case, this double binding (or its interruption) could lead to further induction of an SA pathway. In human cells, actin ACTN1 and actin-binding filamin B (FLNB), the proteins directly involved in cytoskeletal rearrangements, were found to be SA-binding proteins (Choi *et al.*, 2019). In plants, the SA-binding proteins are mostly associated with respiration, photosynthesis, or signalling; no direct interactions with the cytoskeleton have yet been reported (Manohar *et al.*, 2014; Pokotylo *et al.*, 2019).



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Author contributions

HL, TK and MJ conceived the study; HL, TK and MJ wrote the manuscript; MJ drew the figures; and all authors commented and approved the text.

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Key words: actin cytoskeleton, latrunculin B, plant immunity, plant–microbe interaction, salicylic acid (SA).

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4. Discussion

Actin cytoskleton disruption may have distinct effects in plant immunity

Plant actin cytoskeleton is highly dynamic structure that consists of monomeric G-actin and filamentous F-actin. The filaments are constantly being assemled at one barbed end and degraded at the other end[190] providing stable environment for cellular trafficking, metabolism and signalling. The actin cytoskeleton is involved in a range of plant defence reactions. Its involvement has been described in processes triggered by MAMP treatment or infection that include delivery of antimicrobial compounds or callose synthases to the infection site or trafficking of immune receptors[179]. Actin is also involved in the reorganization of chloroplasts during virus infection[239].

Treatment with MAMPs or DAMPs (chitin, flg22, elf26, Pep1, oligogalacturonides) leads to actin reorganization[184,199,201,203]. The MAMP-induced remodelling requires ROS generated by RBOHD, the defence-associated NADPH oxidase. Actin disruption enhances MAMP-induced ROS production, but itself does not trigger ROS production[180].

There is evidence that pathogens evolved an infection strategy aimed to prevent the MAMP triggered actin dynamics. They have evolved effectors able to target actin cytoskeleton specifically. These effectors have several modes of action: either they depolymerize actin themselves[193] or prevent actin from polymerization[194,192]. The *P. syringae* effector HopW1 targets ACT7 isoform of actin and causes actin disruption and reduced vesicular movement to fasten progress of infection. The expression of ACT7 isoform responds to phytohormone treatment (auxin, ABA) and environmental factors which suggests its role in plant immunity[193]. In the Columbia-0 ecotype the HopW1 presence indeed results in enhanced susceptibility, in another ecotype, Wassilewskija, which posseses receptors WIN2 and WIN3, that recognize this effector, defence reactions are triggered. However, the effector caused similar compromised trafficking in both these ecotypes as latrunculin B treatment[193].

Evidence of effectors targeting actin is not only limited to plant pathogens. For example animal pathogens of the geni *Legionella* or *Yersinia* posess effectors that disrupt intracellular trafficking to avoid immune responses and phagocytosis. The *Legionella* VipA effector is an actin nucleator, the YopE from *Yersinia* has Rho GAP activity that disrupts actin filaments[195,196].

These findings point to general conclusion that disruption of actin cytoskeleton dynamics leads to plant susceptibility. However, our results obtained in the included studies 1[240] and 2[154] show that the desintegration of actin cytoskeleton can at certain conditions result in plant resistance in at least two tested pathosystems: model plant *A. thaliana* x model bacteria *P. syringae* pv. *tomato DC3000* and *B.*

napus x *L. maculans*. These findings suggest that onset of the actin-derived immunity is more generally valid: it is not species specific or pathogen type specific. Furthermore, we obtained similar results when using different cytoskeletal drugs. The data revealed that the resistance occurs when the plant has sufficient time to activate the immunity which was shown in the inoculation experiment using *P. syringae* pv. *tomato DC3000*. When the plants were first treated with cytoskeletal drugs and infected after 24 h, the infection rate was lower than in control plants. When co-inoculation was used, no resistance was observed. Similar trend was previously observed by Henty-Ridilla et al.[189], who documented susceptibility triggered by cytoskeletal drugs used simultaneously with the infection agens. Shimono et al. did not observe any effect on virulence of different strains of *P. syringae* with simultaneous treatment with cytochalasin D or jasplakinolide[192]. The onset of resistance also seems to be different in every pathosystem. Surprisingly, pre-treatment and co-treatment of *B. napus* with latrunculin B and *L. maculans* resulted in resistance in both cases. This suggests that the rapidity of pathogen growth is also an important factor, since *L. maculans* grows assyptomatically for at least 5 days in our cultivation conditions. In contrast, bacterial pathogen *P. syringae* pv. *tomato DC3000* strongly infected leaves within three days. Host *B. napus* plants were probably provided with sufficient time to establish resistance even upon co-inoculation. It would though be interesting to investigate this phenomenon also in monocots and their pathogens. Cytoskeletal drug cytochalasin E induced transcription of orthologue of defence gene *NtPR1* in *Nicotiana tabaccum*[206], which is similar case as we have documented in *A. thaliana* plants and *B. napus*[207]. Also both our pathogens are classified as haemibiotrophs, it would be interesting to use necrotrophic pathogens in simiar setup as well.

We have shown that latrunculin B and cytochalasin E induce callose deposition in seedlings via the activity of PMR4 (Callose sythnase 12). Callose deposition is a well documented stress response[241]. There are 12 callose synthases in *A. thaliana* genome but callose synthase 12 (CALS12 or more often used abbreviation PMR4) has been atributed to synthesize biotic stress-induced callose[242]. We clearly show, using *A. thaliana* knock-out mutant *pmr4-1*, that latrunculin B trigerred callose accumulation fully relies on PMR4 which points to connection with "biotic stress-like response".

The majority of published studies points towards the presumption that compromising actin integrity would result in increased plant vulnerability, but there are also studies that indirectly point towards the fact that immune responses are triggered when actin stability is compromised. Disruption of actin leads to enhanced ROS burst in *A. thaliana* which is a result of activation of the FLS2 receptor by its flagellin derived ligand flg22[180]. Previous study done by our team on *A. thaliana* seedlings reported induction of SA-related genes[207]. Since we used similar setup as Matoušková et al. we further investigated whether indeed the observed resistance is based on induced SA signalling.

Immunity signalling triggered by actin depolymerisation is tightly connected with salicylic acid signalling

Since Matoušková et al. [207] documented that treatment with cytoskeletal drugs induces transcription of SA-related defence genes, we tested whether SA concentration does actually increase. In the included study 1 we analyzed phytohormonal profile of *A. thaliana* Col-0 (WT) plants treated with latrunculin B. Latrunculin B specifically induced SA increase. The only other significant changes were 2 times induction of JA and 2 times reduction of IAN. Levels of all other tested phytohormones remained unchanged by latrunculin B. The SA concentration increased also in *pmr4-1* mutant[154]. For further confirmation of involvement of SA signalling we analyzed SA concentration in SA pathway impaired plants: *NahG* transformed plants and *sid2* and *pad4* mutants. SA concentration did not increase in *sid2*, which suggests that biosynthetic gene ICS1 is responsible for the SA induction. The ICS pathway is responsible for the majority of stress-induced SA in *A. thaliana* infected by *P. syringae* pv. *tomato DC3000*[19,18,243].The *NahG* plant degraded most of its induced SA since it posses a SA hydroxylase[244]. SA concentration in *pad4* siginificantly increased although to much lesser extent than in WT which suggests signalling role of the *PAD4* gene upon latrunculin B treatment.

To verify whether indeed the *ICS1* biosynthetic gene is responsible for the SA increase, we tested transcription of all SA biosynthetic genes in *A. thaliana*. In WT we indeed observed induction of the *ICS1* gene. Other tested biosynthetic genes (*ICS2, PAL1, PAL2, PAL3*) showed no differences from the mock treated control. *ICS1* was induced also in *NahG* and *pad4*, which confirmed that the SA increase triggered by cytoskeletal drugs occurs via induction of the ICS pathway[240].

To complete the phytohormone profile, we also analyzed JA biosynthetic gene *LOX2* and observed no induction in WT or in any of the mutants. Since the stress induced SA can be synthesized also by the PAL pathway in other systems[245], we analyzed transcription of defence genes also in *B. napus* treated with latrunculin B. We again observed induced transcription of *ICS1*.

Since we observed induced resistance and onset of defence events such as callose accumulation upon bacterial and fungal infection, we analyzed defence gene transcription. Latrunculin B induced transcription of SA-related defence genes *PR1, WRKY38, PAD4* and *PR2* in WT. *PR1* is a typical SA pathway marker gene usually induced upon pathogen attack but generally reacts to any SA increase. The transcription of *PR1* and *WRKY38* by latrunculin B relied on SA accumulation and a functional PAD4 protein, which is a typical pathogen-triggered pathway. *PAD4* gene was induced also in *NahG* plants, since it is a regulation element that functions upstream of *ICS1*[246].

Surprisingly, *PR2* was induced also in both tested mutants independently of functional SA signalling. *PR2* is considered SA-related defence response usually co-expressed with *PR1* and *PR5*[247], but our results show that its induction can be also stimulated in mutants impaired in SA-signalling. Similar case was reported by our previous study using different cytoskeletal drug cytochalasin E[207]. The *PR2* protein coding β-1-3-glucanase important for antifungal defences[248]. The PR2 enzyme is involved in callose degradation and contributes to SA accumulation and resistance to fungi and bacteria[249]. Nevertheless, here we describe its clear SA-independent induction, that might be triggered by another pathway associated with callose-triggered signalling.

Wounding marker *BAP1* was also induced. Transcription of *BAP1* can be induced by high temperature or ROS and it was described as associated with SA[250]. It negatively regulates cell death in response to *P. syringae* and *Hyaloperonospora parasitica*[251]. The transcription induction was abolished in mutants with impaired SA pathway which strenghtens its role in the SA signalling. Transcription of other tested genes associated with JA or ABA pathways remained largely unchanged which suggests that latrunculin B is likely to mimic typical response triggered by biotrophic pathogens.

Consistently with our results, affected transcription of defence related genes was observed in actin-dynamics-compromised mutant *arpc4*, which was identified as a locus associated with quantitative disease resitance against *S. sclerotiorum*. The *ICS1* transcription was slightly induced in these mutants and surprisingly *PR1* transcription was constitutively reduced. JA related genes *PDF1.2* and *PR4* were downregulated in *arpc4*[208]. We thus suggest that the actin depolymerization is not percieved as a general stressor but it triggers specific immune responses, specifically the SA signalling and callose accumulation. For instance, we did not observe ROS accumulation. The resulting resistance might be partly due to antimicrobial activity of the SA molecule, partly due to SA-induced synthesis of antimicrobial compounds[252]. Testing other defence mechanisms might also give a hint of the specific mechanism that is triggered by latrunculin B.

Interestingly we show that callose accumulation after latrunculin B treatment can still be observed in *NahG* and *pad4* mutants which suggests that the underlying signalling is at least partly SA independent. The untreated *pmr4* mutans show similar SA level as WT. They are known to have generally stronger and faster SA-dependent response[242]. This suggests that certain "biotic stress-like" reponses can be triggered alternatively independent of the SA molecule. Negative correlation between actin dynamics and callose deposition was reported recently in mutants *arpc4*. These mutants show impaired actin dynamics and reduction of wounding and infection associated callose deposition[208].

The SA accumulation activated by degraded actin is connected with phospholipid signalling

Since callose accumulation relies on vesicular trafficking which depends on functional actin dynamics and also on phospholipid signalling, we further investigated the role of phospholipids in the latrunculin B induced resistance. Vesicular trafficking is therefore considered an important component of plant defence[179]. Signalling phospholipids interact with plant cytoskeleton[253].

To investigate whether latrunculin B treatment might cause different effects when phospholipid signalling is compromised we tested *A. thaliana* double mutant in *PI4Kβ1/β2* genes. This mutant shows altered growth phenotype: smaller rosettes at 4 weeks of cultivation while no growth retardation occurs in roots[177]. It also accumulates more callose and has constitutively elevated SA level which led to increased resistance against broad spectrum of pathogens[177,154,254].

In the included study no. 2 we performed similar set of tests in latrunculin B treated *pi4kβ1/β2* mutant. Untreated *pi4kβ1/β2* seedlings did not show elevated SA level or callose accumulation in comparison to WT, which is in accordance with previously observed data[177]. However, the actin cytoskeleton of *pi4kβ1/β2* mutants displayed higher level of degradation upon latrunculin B treatment. This fact suggests that *pi4kβ1/β2* double mutation compromises actin stability[154].

To adress potential involvement of phospholipids in the latrunculin B triggered SA pathway we used a set of triple mutants affected in SA signalling in the *pi4kβ1/β2* mutant background: *NahG/pi4kβ1/β2, pad4/pi4kβ1/β2* and *sid2/pi4kβ1/β2*. The SA induction in *pi4kβ1/β2* background still occurs despite the mutation in *PAD4* or expression of NahG. No SA accumulation was observed in *sid2/pi4kβ1/β2* which suggests that no other SA biosynthetic enzymes than ICS1 are activated by latrunculin B. Yet, the *PR1* transcription after latrunculin B treatment was not only observed in *NahG/pi4kβ1/β2* and triple *pad4/pi4kβ1/β2* mutants, but also in *sid2/pi4kβ1/β2* (data not shown). The *pi4kβ1/β2* background allows to reveal a signalling pathway triggered by latrunculin B leading to SA accumulation and *PR1* transcription independently of PAD4. Do functional PI4-kinases always inhibit this pathway? Is it linked to the effect of *pi4kβ1/β2* double mutation on trafficking? Or is it due to a role of phosphoinositide in signalling? Transcription of *PR1* in *sid2* background is induced by constitutive activation of MAPK3 and MPK6[255]. MPK4 interacts with PI4Kβ1 during cell plate formation during cytokinesis[168]. Therefore testing the activity of MAPKs might provide some insight into the induction of *PR1* here. The SA increase and *PR1* transcription in response to latrunculin B in the *pi4kβ1/β2* background is triggered in an unconventional, PAD4-independent pathway. Either this pathway is actively inhibited in WT by PI4Kβ1/β2 or it is activated only when the PI4Kβ1/β2 are missing. Further research is needed to characterize the involvement of phospholipids in the degraded actin

triggered defence. Here we have only adressed the phenomenon in seedlings and the effects surely may be variable in adult plants since the *pi4kβ1/β2* mutant differs in SA accumulation in different life stages[154,254,177]. The effect of *pi4kβ1/β2* double mutation on cytoskeleton might be broader than investigated here since the double mutants also show ectopic overstabilization of phragmoplast microtubules, which guide membrane trafficking at the cell plate[168].

Cultivation conditions highly contribute to SA-dependent growth phenotype

We observed that the *pi4kβ1/β2* mutant shows different level of basal SA accumulation compared to WT in seedlings and adult plants and this fact may affect results of further studies. For more complex characterization of the connection between SA and growth we created a collection of 14 mutants in Col-0 background having alterations in the SA pathway. The mutants were divided into several categories according to previously described phenotypes: SA-overaccumulators connected with lipid signalling (*pi4kβ1/β2*, *fah1/fah2*), suspected SA-overaccumulators (*cpr5-1*, *acd6-1*, *pi4kβ1/β2*, *fah1fah2*, *bon1-1*, *exo70B1-2*, *pmr4-1*, *edr2-6*), mutants associated with SA signalling based on gene transcription and pathogenicity assays (*edr2-6*, *pmr4-1*, *exo70B1-2*) and mutants with prevented SA accumulation (*sid2/pi4kβ1/β2*, *NahG/pi4kβ1/β2* and *NahG/edr2-6*). All the selected mutants exhibit altered resistance to pathogens.

First we studied plant growth under short day (SD) and long day (LD) conditions. Proposed SA overaccumulators generally displayed dwarf phenotype, which has been documented before[177,175]. Next we analyzed the SA content which mainly confirmed negative correlation with rosette size. However, some mutants exhibited different phenotypes: *edr2-6* and *exo70B1-2* are SA overaccumulators only under short-day conditions and pmr4 do not overaccumulate SA at all. These mutants have been previously described as SA overaccumulators under biotic stress which suggests also enhanced SA signalling in basal conditions [256]. The pmr4 though is generally reffered to as having constitutively induced SA pathways, but this statement is based solely on slight induction of transcription of the *PR1* gene[242,241]. In our setup basal level of *PR1* was not highly induced[254].

We analyzed also transcription of SA-related genes *PR1* and *ICS1*. First interesting result was that cultivation conditions affect transcription of SA-marker gene *PR1*: it was 5 times higher under LD than in SD in WT. This coincides with the fact that plants under LD started bolting already at 3.5 weeks of age. SA treatment can trigger flowering and *vice versa*, which we have confirmed in our setup[257]. Photosynthesis efficiency remained largely unchanged. Our analysis suggests that growth phenotype related to SA content would be better investigated when plants would be grown in LD

conditions, while differences in SA content itself or gene transcription would be better pronounced in SD.

Mutants with affected SA pathway were very sensitive to growth conditions as seen in set consisting of WT, *sid2*, *pi4kβ1/β2* and *sid2/pi4kβ1/β2 plants.* Rosette size has been previously used to divide SA-dependent and SA-independent effects of *pi4kβ1/β2* defficiency[177]. Šašek et al. 2014[177] showed that crossing *pi4kβ1/β2* with *sid2* fully reverted growth phenotype in 4-week-old soil grown plants. In our setup we did not see full reveration even when several different sets of conditions were applied.

The importance of *in-vitro*-grown seedlings and particularly *A. thaliana* roots as a model system is increasing. The induction of SA pathway by root pathogen of the genus *Trichoderma* has been recently shown[258]. The roots of *A. thaliana* are sensitive to SA treatment as has been demonstrated on SA-altered mutants by Pasternak et al. [259]. We showed that, contradictory to the rosette size, the root growth in the SA mutant collection is highly variable and it is not connected with SA content or SA-marker gene transcription level as observed in soil-grown plants. The comparison of *bon1-1* phenotype (small rosette and almost WT-size roots) to *pi4kβ1/β2,* which also had small rosettes but impaired root clearly demonstrated this. Due to material and time limitation we did not perform SA content measurement in seedlings.

The PI4Kβ1/β2 enzymes are critical for root growth. The PI4P biosynthesis regulated by PI4Kβ1/β2 is essential for lateral root formation regulated by endocytic trafficking to the vacuole[260]. For more detailed analysis of the SA role in seedlings' sensitivity to light we grew set of WT, *sid2*, *pi4k/β1/β2* and *sid2/pi4kβ1/β2* mutants in different light regimes: either the roots were exposed to light or shadowed by placing in dark chambers. The *sid2* mutant grew slower in the light setup and roots of both mutants in *pi4kβ1/β2* grew slower. This suggests a potential new role of the PI4Kβ1/β2 enzymes in root growth regulation in terms of abiotic conditions.

Overall, by creating and characterizing the collection of SA-affected mutants we adressed the importance of precise characterization of cultivation conditions since some phenotypes might be only pronounced in specific environment. These findings add complexity to whether these phenotypes are also valid in complex natural environment? These questions have been already partially addressed by several studies using mutants from our collection: the role of SA in cold stress was shown using *acd6*[261] , *cpr1* and *pi4kβ1/β2*[262]; in potassium stress by using *cpr5*[263], in response to drought and ABA treatment by using *cpr5* and *acd6*[264, 265], and in sugar sensing by using *acd6* and *cpr1*[266].

Miccrobial Effectors affect plant-hormone signalling

The second part of the thesis is focused rather on pathogens weapons to overcome host plant defence, particularly those dealing with phytohormone signalling.

In the included study no. 4[233] we focused on the effect of effectors on host plant phytohormone signalling. For this purpose we used two isolates of *L. maculans* differing in the presence of the AvrLm4-7 effector. Phytohormone content and defence gene screening revealed that effector AvrLm4-7 impacts hosts phytohormone defence signalling. Phytohormonal content of *L. maculans* was not affected by the presence or absence of AvrLm4-7[233]. This is not a general feature of presence or absence of effectors since the results of presented study no. 5 revealed that sister isolates JN2 and JN3 that differ in the presence of the AvrLm1 effector differ dramatically in terms of auxin production [267].

The AvrLm4-7 effector is recognized by the RLM4 receptor. This interaction is accompanied by strong induciton of SA and ET signalling in the host. These pathways were documented to mediate defence during incompatible interaction in *B. napus* x *L. maculans* system[234]. However, the recognition of the AvrLm4-7 effector also induced ABA marker genes in *B. napus* cultivar Pixel. Moreover, the ABA concentration during compatible interaction increased but no induced transcription of ABA marker genes *NCED3* and *RD26* was observed[234,233]. The role of ABA during the infection process remains elusive since it has also been documented that induction of ABA pathway before infection decreased symptoms caused by virulent strain of *L. maculans*[234]. In the compatible interaction the SA and ET signalling pathways might be primary targets of the AvrLm4-7 effector.

AvrLm4-7 seems to suppress SA signalling both on the level of SA biosynthesis and transcription of SA-responsive genes (*BnPR1*). The ET signalling is attenuated in AvrLm4-7 infected cotyledons; transcription of ET-responsive genes *ACS2* and *HEL* decreased in time during infection. We reported the first evidence of manipulation of SA signalling pathway by a haemibiotrophic fungus. Manipulation of the JA signalling has been already described[268]. *Fusarium oxysporum* effector FoSIX4 induces JA signalling and contributes to disease development[269]. Since SA and JA signalling often act antagonistically, this can be seen as an indirect manipulation of the SA pathway. Several bacterial effectors targeting the SA pathway have been identified too: The type III effector XopD from *X. campestris* pv. *vesicatory* suppresses transcription of SA-dependent genes and SA biosynthesis in tomato[270]. The XopD effector also interacts with ERF4 and thus affects ET signalling[271]. This is a similar effect as observed in AvrLm4-7.

Hydrogen peroxide (H_2O_2) accumulates during *L. maculans* infection[272]. Our results revealed that removal of the H_2O_2 by ascorbic acid during the biotrophic phase of infection increased lesion area

caused by isolate lacking the AvrLm4-7 effector. The presence of AvrLm4-7 on the other hand led to decreased hydrogen peroxide accumulation during later stage of infection and reduced transcription of NADPH oxidase *RbohF* that contributes to ROS production. This contributes to the fact that H_2O_2 is an important part of *B. napus* defence against *L. maculans* and that the AvrLm4-7 effector affects ROS accumulation. Several fungal effectors are known to interfere with ROS production in plants, for example the PEP1 effector from *U. maydis* inhibits maize peroxidase POX12 in the apoplast[272]. ROS accumulation accompanied with reduced colonization was observed in the absence of PEP1. The AvrLm4-7 may also directly inhibit ROS production in the apoplast similarly but further research is needed. Alternative explanation of the H_2O_2 decrease might be possible. The ROS act synergistically with SA signalling to trigger HR and systemic resistance[273]. The low ROS accumulation at the infection site might reflect the decreased SA level and SA signalling.

The ROS accumulation can be also associated with altered ET signalling. ET can induce PCD and senescence which are also associated with ROS[274]. Therefore, in the absence of AvrLm4-7, the accumulation of ROS at 10 dpi could be stimulated by increased ET signalling. The included study no. 4 aimed to confirm and find a cause for the increased aggressiveness of *L. maculans* isolates harbouring the AvrLm4-7 effector by investigating its effect on *B. napus* defence responses. Taken together, we speculate that the increased aggressiveness of *L. maculans* isolates harbouring the functional AvrLm4-7 allele could be caused by defects in ROS accumulation or by the complex effects (involving ROS accumulation) exerted by AvrLm4-7 on the *B. napus* defence system via the suppression of SA and ET signalling. Recent findings of Blondeau et al. [274], showing that AvrLm4-7 is translocated into the host cell, rather suggest that SA and/or ET signalling is the primary target of AvrLm4-7. The identification of the interacting partner of AvrLm4-7 upstream of these processes would be the next step in our understanding of the molecular mechanisms of this particular *L. maculans* effector.

Since the effectors largely affect plant hormone signalling and we have observed that the fungus itself is able to synthesize a variety of phytohormone-like structures we speculated whether these compounds might function as effectors themselves?

Role of auxins during growth and infection process of *L. maculans*

Various microorganisms were documented to produce phytohormone-like molecules [220,275,211,276,276]. In the included study no. 5[267] we focused particularly on the ability of *L. maculans* to produce auxins. We hypothesized that the pathogen might produce auxins as an infection strategy similarly as the effectors.

L. maculans is able to synthesize a variety of phytohormones including cytokinins[223], abscisic acid [224], salicylic acid (preliminary data) and auxins. The main auxin produced by *L. maculans in vitro* is the bioactive molecule IAA. Since it is produced in high concentration and can be also excreted from the mycelium into cultivation medium, we hypothesized that it might function as an infection strategy. The hormone profile of infected plants though did not differ dramatically, although an increase in OxIAA was obsereved. OxIAA is a degradation product of IAA which is no longer bioactive. This fact might suggest that the plant is trying to maintain constant levels of bioactive IAA. No genes that participate in the conversion of IAA into OxIAA were indentified in *B. napus* up to date, although they were (functionally) identified in *A. thaliana*[277,278].

There is evidence that auxin might function in effector-like way in other plant-mirobial systems[279,268,233,234,216]. For *Piriformospora indica, C. gloeosporioides* and *M. oryzae* production of auxin is required for proper colonization and infection process [216,219,280]. The virulence of *M. robertsii* on insect *B. bassiana* increased upon auxin treatment and loss of the ability to produce auxin lowered the virulence of *M. robertsii* [276]. Loss of the ability to produce IAA led to lowered spore production in *N. crassa*[214].

Our results show that exogenous IAA inhibits growth of both *L. maculans* isolates in high concentration. Conidial germination was totally blocked by 1 mM IAA. Inhibitory effect was observed in fungus *F. graminerarum* that produced 50% less biomass when cultured in 1 mM IAA[281]. On the other hand growth of *Moniliophtora perniciosa* was stimulated by low concentration of IAA [282]. We have observed no stimulatory effect in *L. maculans*. In the presented thesis we have not confirmed that auxins function as a virulence trait in this fungus in a similar way effectors do, but it might serve as internal regulation molecule for the fungus itself.

***Leptosphaeria maculans* produces a variety of auxins using certain biosynthetic genes**

Since we did not observe any clear role of fungal produced auxins as an infection strategy, we further investigated its internal biosynthesis, metabolism and putative signalling function.

We have tested two sister isolates (entitled JN2 and JN3) obtained in the same parent cross[283] and observed that both isolates are able to produce bioactive form of auxin, IAA, and a variety of other auxin forms in minor concenration. We have also revealed that the production of auxin is very strain dependent since JN3 produced about 100 times more auxin than JN2. This is not a unique feature of *L. maculans*, similar case was reported in fungi and bacteria [211,284]. The strain specificity was observed in another set of experiments. JN2 IAA production increased upon stimulation with biosynthetic precursors tryptophan and tryptmaine whilst JN3 did not react.

Genome analysis of *L. maculans* revealed presence of orthologues of genes previously identified as auxin biosynthetic in other microorganisms and plants. Genes participating in IPyA, IAN and IAM biosynthetic pathways were identified. Precursor feeding experiments on JN2 isolate revealed that mainly genes from the IPyA pathway are active in converting Trp to IAA. Predominantly induced genes were *LmTAM1* (tryptophan aminotransferase), *LmIPDC1* and *LmIPDC2* (indole-3-pyruvate decarboxylases). *LmTAM1* might directly metabolize tryptophan into IPyA which would be subsequently converted to IAA by IPDC. The aldehyde dehydrogenase IAD that has also been reporrted to particiapte in IPyA pathway mediated biosynthesis in fungi was also found in *L. maculans* genome. No induction of the IAD orthologs was observed in our setup. Intermediates predicted in the IPyA pathway were not detected possibly due to their unstability.

Our study is the first to report involvement of an IPDC gene in auxin biosynthesis in a pathogenic fungus. Recent study confirmed its activity in a symbiotic fungus *N. crassa*[224]. Functional IPDCs were previously described in several plant-associated bacteria such as *Azospirillum brasilense, Pseudomonas fluorescens* or *Pantoea ananatis*[285,286,286,238,213].

Auxins were identified in various fungi, both symbiotic and pathogenic[211,275,287]. We have characterized auxin production of two different isolates of *L. maculans*. Our results show that *L. maculans* produced and secretes IAA *in vitro* as it was detected both in mycelium and cultivation medium. The data also show that basal production of auxin differs among the used isolates and so did the inducibility of IAA production when in vitro culture was supplemented with biosynthetic precursors. The production in JN3 isolate was high even without precursor stimulation and precursor feeding had no effect on the production. On the other hand, the JN2 isolate synthesized only low level of IAA, but precursor supplementation stimulated it to produce substantial amount of auxin. The rice blast fungus *M. oryzae* or the white rot fungi[275,288] produce auxin without any stimulation. Many more fungi are able to produce auxin when appropriate biosynthetic precursors are provided: *C. gloeosporioides*, *U. maydis*, *N. crassa* and *F. oxysporum*[211,275,219,214]. The genetic basis for this differential behaviour between JN2 and JN3 needs to be further investigated. Sequencing and comparison of genomes of both isolates might contribute to understanding such behaviour.

Further we investigated the genetic basis of the biosynthetic apparatus. Based on genome analysis we identified orthologous genes of those previously identified in other fungi, bacteria or plants. We found conserved genes belonging to IPyA, IAN and IAM pathways. *L. maculans* mainly uses the IPyA pathway for IAA biosynthesis. Predominantly induced genes were *LmTAM1* encoding putative tryptophan aminotransferase and *LmIPDC2* and *LmIPDC1* encoding putative indole-3-pyruvate

decarboxylases. *LmTAM1* may convert tryptophan into IPyA which is subsequently metabolized into IAA by IPDC. The aldehyde dehydrogenase IAD can participate in IPyA pathway in fungi[211]. There are orthologues of the IAD genes in *L. maculans* genome, but no induction of transcription was observed in our experiments. We were not able to detect any biosynthetic intermediates predicted (IPyA nor IAAld). This might be due to their instability. The involvement of IPDC genes have been previously reported in several plant-associated bacteria, for example, *A. brasilense, P. fluorescens* and *P. ananatis*, and the plant-associated trypanosomatid *Phytomonas serpens*[285,286,238,289].

There are two clear orthologs of the *P. ananatis* IPDC in the *L. maculans* genome, both were induced upon tryptophan treatment. Recently, the involvement of IPDC in IAA biosynthesis has been documented in a non-pathogenic fungus *N. crassa*[214]. We are the first to report the activity of this gene also in a pathogenic fungus.

Besides the involvement of *LmIPDC*s, we have observed induced transcription of *LmNIT1*, encoding putative nitrilase orthologous to plant nitrilases[256], upon tryptophan treatment. We also detected the IAN metabolite in the *in vitro* culture. This suggests that also the IAN pathway is active in *L. maculans*. Concerning the IAM pathway we observed slight transcription induction of *LmIaaM3* and *LmIaaM5*, but the IAM metabolite was not detected. This pathway might be at least partially functional in *L. maculans* cultivated in vitro. IAM induced IAA production in the non-pathogenic fungus *N. crassa,* although this fungus is not able to produce IAM itself [214].

Among all auxin metabolites we detected the bioactive from, IAA, in highest concentration. After precursor treatment the IAA was predominantly enhanced. Other fungi also predominantly synthesize IAA, however other forms can be detected in high concentration as well. The *Fusarium* species produce IAM and tryptophol besides IAA[290]. Tryptophol was also found in *M. oryzae*[275]. *N. crassa* synthesizes indole-3-lactic acid (ILA) a tryptophol besides IAA[214]. Tryptophol seems to be highly abundant metabolite among fungi. In *L. maculans* we were not able to detect it.

Non-active IAA metabolites such as conjugates with amino acids or glucose were detected only in low concentrations both in mycelium and cultivation medium and both in basal conditions and upon precursor treatment. Among these conjugates only IAA-Asp, IAA-Glu, and a glucose ester of oxIAA (oxIAA-GE) were detected; but not in reproducible manner due to technical limits of the detection method. This suggests *L. maculans* metabolizes IAA only weakly in comparison to plants where the conjugates are predominantly present.

Another non-active form, the OxIAA, is the second most abundant auxin in *L. maculans*; its concentration also increased after tryptamine feeding. This suggests that IAA being oxidized in *L.*

maculans. In plants, this oxidation is catalyzed by dioxygenase DAO1, identified in rice[291] and *A. thaliana* [292].

The phenylacetic acid (PAA) is another molecule with documented auxin activity in plants and bacterial species *Streptomyces* or *A. brasilense*[286,256] . Biosynthetic pathways of PAA remain largely unexplored though. In *A. brasilense* the indolepyruvate decarboxylase is involved and aminotransferases TAA and the YUCCA enxymes are suspected to participate in plants[293,294]. Even less evidence is available as far as its function in fungi is concerned. Medium used for cultivation of *Streptomyces malachitofuscus* has antifungal activity against *Mucor miehei* and *Candida albicans* and PAA was later identified as one of putative antifungal agents in the medium[294].

Besides tryptophan, a direct IAA precursor used to induce IAA synthesis, we used tryptamine for this purpose. We show that tryptamine induced massive IAA production in the JN2 isolate, about 30-fold higher that tryptophan treatment. Tryptamine induced IAA production also in *U. maydis* and *N. crassa*[211,214]. In *U. maydis* tryptamine acted as a very potent inducer whilst in *N. crassa* only low IAA synthesis was observed. Interestingly, despite this massive tryptamine-induced IAA overproduction, the transcription of the candidate genes did not change much. We observed only minor of *LmNIT1* and *LmIaaM*3 transcription, while the transcription of *LmTAM1* and *LmIPDC2* that were stimulated upon tryptophan treatment remained constant. These data suggest existence of a separate tryptamine-induced IAA biosynthetic pathway in *L. maculans*. Similarly, neither the *UmTAM1* nor *UmTAM2* genes of *U. maydis* were involved in the conversion of tryptamine into IAA[211]. On the other hand, the *UmIAD1* and *UmIAD2* genes did participate in this conversion, unlike in our study. For *N. crassa*, it remains unclear which biosynthetic genes participate in this conversion[214]. In fungus *M. robertsii*, however, a tryptophan decarboxylase MrTDC was involved in the conversion of tryptamine into IAA[276].

The levels of IAA in host plant did not significantly change upon infection with the JN3 isolate which produced high amount of IAA *in vitro*. The infection with *L. maculans* though as associated with an increase in the OxIAA metabolite in the infected tissue. Thereby the plant may aim to maintain stable levels of its endogenous IAA by converting/oxidizing the bioactive IAA. The host auxin profile remained otherwise largely the same. In some plant-microbial systems, IAA has been shown to act as an important virulence factor that impairs the defense-associated, phytohormone signalling of the host[279,216] in a similar way as effectors do[233,234,268]. Some fungi require auxin for proper colonization and infection process as documented in pathogens *C. gloeosporioides* and *M. oryzae* or symbiotic fungus *Piriformospora indica*[280,219,275]. Externally-added IAA increased the virulence of

insect-pathogenic *M. robertsii* spores on *B. bassiana*[276] and loss of auxin production lowered virulence of *M. robertsii* against the pathogenic insect *B. bassiana*[276]. *N. crassa* with blocked IAA biosynthesis produces less conidiospores[214].

Growth of both used *L. maculans* isolates was inhibited by exogenously added IAA. 1mM IAA completely blocked conidial germination. Some fungi show enhanced growth when auxin is supplemented in low concentration and growth inhibition upon highly concentrated auxin treatment [281,282]. Such effect was observed in *F. graminearum,* which produced 50% less biomass when cultured in 1 mM IAA[281]. Loss of the ability to synthesize IAA resulted in developmental changes in *C. gloeosporioides* [295]. On the other hand, IAA ranging from 0.1 µM to 10 µM stimulated growth in *Moniliophthora perniciosa* in a concentration-dependent manner[282].

Overall this thesis deals with the role of phytohormones within plant defence. We demonstrated that actin-depolymerization triggered defence pathway that might eventually result in plant resistance due to SA signalling. Apart from SA, phospholipids are involved in the correct onset of this particular cytoskeleton-connected immunity. Following research would be dedicated to understanding what is the molecule being sensed when the actin degradation occurs. Further, we are providing evidence that auxins are produced by *L. maculans* with specific strain-dependent manner. We did not prove that auxins produced by *L. maculans* are produced as virulence factors neither we have found a clear clue they work as internal regulators in fungi. This would be adressed by future research.

5. Conclusion

This thesis focuses on several aspects of plant immunity involving phytohormone signalling. The included publications deal with stress hormone signalling both from the plant side and the pathogen side. The main findings presented in this thesis are:

- Actin cytoskeleton disruption leads at certain conditions to plant resistance
- The onset of resistance triggered by actin desintegration is mediated by salicylic acid signalling
- There are also immunity effects triggered by actin depolymerization independetly of SA signalling
- Functional phospholipid signalling is important in the process of actin-depolymerization-triggered immunity response
- Mutants impaired in salicylic acid signalling pathway provide a useful tool for future studies dealing with growth and immunity
- Effector AvrLm4-7 affects SA and ET signalling pathways and ROS burst in *B. napus*
- *L. maculans* produces bioactive auxin
- *L. maculans* genes *LmTAM1* and *LmIPDC2* are associated with the auxin production

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