Phytohormone-Related Crosstalk in Pathogen and Stomatal Responses in *Arabidopsis thaliana*

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ACADEMIC DISSERTATION

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Cover image: Photographs of Col-0, *d14-1*, *d14-seto5*, *max2-1*, *max2-4*, *max3-9*, *max3-11*, *max4-1* and *max4-7* plants.

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“Shine like thunder,
Cry like rain”
Temple of Love
The Sisters Of Mercy

To Aapo, Eemil and $F1_2$

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List of Original Publications

I *Hu Z., *Keçeli M.A., **Piisilä M.**, Li$_F$ J., Survila M., Heino P., Brader G., Palva E.T. and Li J. 2012. F-box protein AFB4 plays a crucial role in plant growth, development and innate immunity. Cell Research 22:777-781. doi:10.1038/cr.2012.12. *The first authorship is shared.

II **Piisilä M.**, Keçeli M.A., Brader G., Jakobson L., Jõesaar I., Sipari N., Kollist H., Palva E.T. and Kariola T. 2015. The F-box protein MAX2 contributes to resistance to bacterial phytopathogens in *Arabidopsis thaliana*. BMC Plant Biology 15:53. doi:10.1186/s12870-015-0434-4.

III **Kalliola, M.**, Jakobson L., Davidsson, P., Pennanen, V., Waszczak, C., Yarmolinsky, D. Zamora, O., Palva E.T., Kariola T., Kollist H., Brosché, M. 2019. The role of strigolactones in regulation of stomatal conductance and plant-pathogen interactions in *Arabidopsis thaliana*. Preprint BIORXIV/2019/573873. https://doi.org/10.1101/573873

Author's contribution

I **MK** carried out the mutant screen analysis and pathogen experiments with MAK.

II **MK** did the mutant screen analyses with MAK. **MK** performed all the porometer measurements, water-loss experiments, ion leakage measurements and the qPCR analysis after ozone and pathogen experiments. MAK performed all the pathogen calculations, and statistical analysis of all the experiments. Writing was carried out by **MK** and TK and contributed by MAK.

III **MK** did all the bacterial calculations, the qPCR after bacterial infection and porometer measurements with PD's contribution. MB did the qPCR analysis after strigolactone treatment. **MK** did the ozone experiments and the stomatal aperture measurements after strigolactone spray. **MK** and LJ did together 50 % of the gas-exchange experiments, the other 50 % were done by LJ alone. VP did the SA analysis. CW made the double mutants and their stomatal conductance was measured by OZ. Writing was carried out by MB and **MK**.

Abbreviations

ABA	abscisic acid
ABFs	ABRE-binding factors
ABREs	ABA-responsive elements
AFB	auxin signaling F-box
ARF	auxin response factor
Aux/IAA	auxin/indole-3-acetic acid
BAK	bri1-associated-kinase
BL	brassinolide
BR	brassinosteroid
BRI	brassinosteroid insensitive
Ca^{2+}	calcium
CDK	calcium-dependent protein kinases
CERK	chitin elicitor receptor kinase
CO_2	carbon dioxide
COR	coronatine
DAB	3,3' –diaminobenzidine
DAMP	danger-(damage-)associated molecular pattern
EMS	ethylmethylsulfonate
EFR	elongation factor-Tu receptor
ERF	ethylene response factor
ET	ethylene
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
FLS2	flagellin sensing 2
GA	gibberellins
H_2O_2	hydrogen peroxide
HR	hypersensitive response
IAA	indole-3-acetic acid
JA	jasmonic acid
JAZ	jasmonate-zim domain
K^+	potassium
LRR	leucine-rich repeat
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
MPK	mitogen-activated protein (MAP) kinase
MAX	more axillary growth
MeJA	methyl jasmonate
MeSA	methyl salicylate
NADPH	nicotinamide adenine dinucleotide phosphate
NB-LRR	nucleotide-binding site leucine-rich repeat
NCED	nine-cis epoxycarotenoid dioxygenase
NO	nitric oxide
NPR	non-expressor of PR proteins
$^1O_2$	singlet oxygen
$O_2^{\bullet-}$	superoxide radical
O_3	ozone
$HO^{\bullet}$	hydroxyl radical
PAMP	pathogen-associated molecular pattern
PAL	phenylalanine ammonia-lyase
PCD	programmed cell death
PDF	plant defensin
PR	pathogenesis related
PRRs	pattern recognition receptors
PTI	PAMP-triggered immunity /pattern-triggered immunity
R	resistance
RBOH	respiratory burst oxidase homolog
RCD	radical-induced cell death
RLK	receptor-like kinase
RLP	receptor-like protein
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
SCF	SKP1-CULLIN1-F-box protein
SKP	s-phase kinase-associated protein
SLs	strigolactones
SMXL	suppressor of more axillary growth2-like
SOD	superoxide dismutase
TIR	transport inhibitor response
TF	transcription factor
T3SS	type-III secretion system
X/XO	xanthine/xanthine oxidase

Abstract

Phytohormones affect all the developmental stages of plant from germination to flowering but also plant responses to biotic (e.g. pathogens) and abiotic stresses (e.g. drought and cold) and furthermore, acclimation to environmental changes. Phytohormones form a signalling network affecting both directly and indirectly many functions in plants; for example stomatal openness is affected by phytohormones. Stomata allow gas-exchange between air and leaf, thus optimizing between CO_2 intake i.e. effectiveness of photosynthesis, and the inevitable evaporation of water through the open stomata. Stomata furthermore provide an entrance for pathogens but also for example air pollutants, like tropospheric ozone that causes oxidative stress in plants, employing stomata to enter the plant. Phytohormones also have a central role in pathogen responses and innate immunity in plants. Thus, it can be concluded that stress and pathogen responses, innate immunity, oxidative stress tolerance and stomatal responses are all tied together via the phytohormone signalling network.

This thesis concentrates mainly on effects of two phytohormones; auxin and the rather newly discovered class of phytohormones, strigolactones. Auxin is historically known for its role in plant growth and development, but it also affects stress and defence responses by interacting with other hormones; auxin and the most important hormone in bacterial pathogen responses, salicylic acid, act in a mutually antagonistic manner and many pathogenic micro-organisms produce auxin within their interactions with plants. Interestingly, auxin is also the target of strigolactone pathway and strigolactones act by dampening auxin transport. Thus, both strigolactones and auxin affect lateral branching in plants – auxin by classical polarity of auxin transport and strigolactones via affecting auxin. Strigolactone and auxin furthermore share other commonalities since also strigolactone was recently discovered to affect stress and pathogen responses in plants.

Strigolactone signalling is complex and all the details are still not known. In strigolactone perception the F-box protein MAX2 functions together with the strigolactone receptor, D14 protein. Other MAX proteins (MAX1, 3 and 4) function in strigolactone synthesis. In this thesis first MAX2 and later also all the other strigolactone-related proteins were discovered to have a role in susceptibility to pathogens. However, MAX2 was found to have an exceptional role compared to the other strigolactone-related proteins; only *max2* plants have higher stomatal conductance than the wild-type plants and only *max2* was found to contribute to sensitivity to oxidative stress. MAX2 also affects hormonal signalling; ABA levels in *max2* were particularly high in excised leaves that were left to dry. Interestingly, it was concluded that MAX2 acts in a parallel signal pathway to the well characterized guard cell ABA signalling pathway, which was discovered by crossing *max2* with the well-known guard cells affecting mutants (ABA biosynthesis mutant *aba2*, guard cell ABA signalling mutant *ost1*, and *ghr1* required to regulate ion channel activity).

1. Introduction

Arabidopsis thaliana, the thale cress, is widely used as a model organism in plant molecular biology and genetics. Arabidopsis is a small dicotyledenous species which belongs to the *Brassicaceae* or mustard family and is closely related to many important crop plants, even though Arabidopsis itself is not economically important. Arabidopsis is a convenient model as it has a relatively small genome that has been fully sequenced and it is easily manipulated. The genetic and biochemical mechanisms in the plant kingdom are widely overlapping and thus the results gained with Arabidopsis will be applicable also to economically important species. The life cycle of Arabidopsis is rapid; generation from germination to mature seeds takes approximately 6 weeks which makes it an efficient tool for research (Koornneef and Meinke, 2010).

In the natural environment plants are exposed to both biotic and abiotic stress factors which have a major impact in the yield in agriculture and forestry. The biotic stress factors include living organisms: bacteria, fungi, oomycetes, viruses and herbivores. The abiotic stress factors include drought, high and low temperatures, light, ozone, osmotic stress and salinity. In the field both abiotic and biotic stressors often occur simultaneously and can have a combinatory positive or negative impact on a plant. Arabidopsis shares overlapping functions between development, growth and stress responses which form a molecular signalling network inside a plant.

Phytohormones are chemicals that are produced inside the plant and are essential in transmitting and executing various plant responses; they regulate plant growth and function as signal molecules that affect almost all the vital developmental, growth and stress related functions in the plant. They can work also independently but mostly interconnected to other phytohormone responses. Plants exposed to different kind of stresses display severe growth retardation and reduced productivity (Kazan and Manners, 2009, Panchal et al., 2016b). The plant immune signalling network response should be adjustable and robust at the same time; unnecessary induction of immunity consumes resources and results in fitness costs in the plants (Heidel et al., 2004, Kim et al., 2014). Moreover, “false alarms” may be caused by some harmless trigger; for example the same microbe-associated molecular patterns (MAMPs) can be produced by both pathogenic and non-pathogenic microbes, and if non-pathogenic MAMPs led to a strong defence induction, it would incur a fitness cost (Kim et al., 2014).

1.1 F-box proteins in plant stress signalling

Selective protein degradation is essential in post-translational control of regulatory proteins; approximately 10 % of all intracellular proteins are short-lived and most of them are subjected to proteosomal degradation (Stefanowicz et al., 2015). The ubiquitin proteasome system regulates many biological processes and functions as part of a tightly-regulated proteolytic pathway, which functions in the nucleus and cytoplasm of cells. Ubiquitin has been connected to several signalling pathways related to e.g. development, phytohormones, defence and stress responses by targeting proteins for degradation (Dreher and Callis, 2007, Lechner et al., 2006).

In the ubiquitin proteasome system, the proteins to be eliminated are recognised and tagged with ubiquitin molecules by covalently ligating ubiquitin into the protein i.e. polyubiquitylated. Ubiquitin is a small 8,5 kDA protein containing 76 amino acid residues. The proteins are tagged with ubiquitin molecule chains via a sequential action of E1, E2 and E3 enzymes, and are subsequently recognized and degraded by 26S proteasome. The specificity of ubiquitination and thus substrate specificity is mainly controlled by E3 ubiquitin-protein ligases that catalyse the attachment of polyubiquitin chains to target proteins. Two other enzymes are also involved in conjugation of ubiquitin to the protein target; the E1 ubiquitin-activating enzyme forms a thioester intermediate (E1-S~Ubi), which is further trans-esterified to E2 ubiquitin-conjugating enzymes. The E3 ligases are classified into four major types of which the best characterised are SCF (SKP1-CUL1-F-box) complexes, which are a small subgroup belonging to the large family of E3 ligases called Cullin-RING Ligases (CRL). The SCF complex consists four subunits: SUPPRESSOR OF KINOTOCHORE PROTEIN 1 (SKP1), Cullin1 (Cul1), RING-BOX1 (RBX1)/REGULATOR OF CULLINS 1 (ROC1) and an F-box protein (Dreher and Callis, 2007, Lechner et al., 2006, Somers and Fujiwara, 2009, Stefanowicz et al., 2015).

During functional characterization of hormone or defence pathways the activity of an E3 ligase can be altered through a mutation or transcriptional silencing which results in down-regulation of the E3 ligase. If an E3 ligase targeting a transcriptional activator for degradation is down-regulated, it should lead to increased levels of the activator and thus increased expression of downstream genes. In the case that E3 targets a transcriptional repressor for degradation, plants lacking the E3 ligase would have elevated levels of the repressor and reduced transcription of downstream genes (Dreher and Callis, 2007, Lechner et al., 2006, Stefanowicz et al., 2015).

The F-box protein family is the largest known protein superfamily in plants; nearly 700 putative F-box proteins have been predicted in Arabidopsis. Many well-known F-box proteins function in phytohormone signalling and numerous F-box proteins with different C-terminal motifs have been identified as SCF components (Gagne et al., 2002, Somers and Fujiwara, 2009, Stefanowicz et al., 2015). For example, the F-box protein COI1 (CORONATINE INSENSITIVE1) serves as a primary receptor for jasmonic acid. COI1 forms the SCF^{COI1} complex, which furthermore targets degradation of the JAZ (jasmonate ZIM domain) transcriptional repressor proteins via 26S proteasome pathway (Yan et al., 2009, Yan et al., 2018).

1.2 Stomata

Stomata play a central role in stress responses since they regulate the gas flow in and out of the plant. Stomata are present in the leaf epidermis but also on other aerial parts of the plant. The stomatal pores are formed by two surrounding guard cells dynamically regulating the size of stomatal apertures. As a main function, the stomata allow sufficient atmospheric carbon dioxide (CO_2) to enter via open stomatal aperture in order to acquire a level of optimal photosynthesis. However, through the open stomata water will be inevitably lost and thus the width of stomatal aperture is adjusted to ensure enough water remaining as required by the plant. Moreover, stomata respond to various endogenous and environmental stimuli by opening or closing. The stomata provide a main entrance for air pollutants e.g. ozone gas and some bacterial plant pathogens. Thus, stomatal closure is an

integral part of the plant defence and innate immune response (Melotto et al., 2006, Merilo et al., 2013, Vahisalu et al., 2008).

1.2.1 Stomatal opening and closure

The stomatal aperture is regulated by transporting osmotically active ions and metabolites including potassium (K^+), chloride (Cl^-) and malate across guard cell membranes which causes changes in osmotic potential and thus osmotic swelling or shrinking of guard cells respectively. Guard cells respond to changes in air humidity, light and CO_2 concentration. Rapid stomatal closure is induced by environmental changes such as CO_2 elevation, air humidity reduction, darkness and pulses of ozone. Thus, guard cells have a vital role in controlling plant water loss, ozone sensitivity and CO_2 supply. Bacteria and PAMPs are known to trigger stomatal closure and also air pollutants such as ozone (O_3) cause stomatal closure (Acharya and Assmann, 2009, Melotto et al., 2006, Merilo et al., 2013, Merilo et al., 2014, Vahisalu et al., 2008).

As the situation is typically in nature, plants are exposed simultaneously to several environmental factors and the stomatal responses are altered compared to a situation in which plants are exposed just to a single environmental factor. In Arabidopsis simultaneous application of known stomatal closing and opening factors always resulted in a slight or considerable stomatal opening (Merilo et al., 2014). The stomatal properties also affect the immunity, and high humidity was concluded to suppress stomatal defence (Melotto et al., 2006, Panchal et al., 2016a). High humidity promotes disease-development because high humidity can compromise *P. syringae* triggered stomatal closure in Arabidopsis, followed by early activation of JA signalling pathway, and simultaneous suppression of the SA signalling pathway in guard cells (Panchal et al., 2016a). Coronatine (COR) secreted by *P.syringae*, a mimic of bioactive jasmonic acid (JA-Ile), prevents stomatal closure to allow entry of bacteria (Geng et al., 2014). Moreover, production of coronatine was found to support the bacterial infection at night by forcing the stomata to open under darkness and thus allowing the entry of bacteria via stomata (Panchal et al., 2016b).

Stomatal regulation involves several phytohormones; ABA, JAs, BRs and SA are positive regulators of stomatal closure while auxins and cytokinin are generally positive regulators of stomatal opening (Acharya and Assmann, 2009). However, very few regulators of guard cell signalling independent of ABA have been found (Assmann and Jegla, 2016, Engineer et al., 2016). ABA signalling is initiated through binding of the hormone to PYR/RCAR receptors which leads to inhibition of PP2C protein phosphatases (e.g. ABSCISIC ACID-INSENSITIVE1 and 2 [ABI1, ABI2], HYPERSENSITIVE TO ABA1 [HAB1], PP2CA), followed by activation of Snf-related protein kinases such as OST1 (Acharya and Assman, 2009, Jalakas et al., 2018, Merilo et al., 2013, Merilo et al., 2015, Vahisalu et al., 2010). In turn OST1 activates the central ion channel SLOW ANION CHANNEL1 (SLAC1) (Geiger et al., 2009, Vahisalu et al., 2010). Several Ca^{2+} dependent protein kinases also regulate SLAC1 activation (Brandt et al., 2015). GHR1 (GUARD CELL HYDROGEN PEROXIDE-RESISTANT1) is involved in ABA- and hydrogen peroxide-induced activation of SLAC1, and it is proposed to act as a scaffold to bring together various proteins needed to initiate stomatal closure (Hua et al., 2012, Merilo et al., 2013, Sierla et al., 2018). ABA signalling through PYR/RCAR has an essential role in rapid stomatal closure to darkness, reduced air humidity and O_3 and moreover, was involved in stomatal responses to elevated CO_2. ABA signalling through PYR/RCAR is also a fundamental

element in whole-plant steady-state stomatal conductance and guard cell ABA signalling is important in regulating basal stomatal openness and rapid stomatal responses to environmental stimuli (Merilo et al., 2013, Merilo et al., 2015).

Strigolactone has been proven to affect the stomata and strigolactone-biosynthesis mutants (*max1-1*, *max3-9*, *max4-1*) and strigolactone-signalling mutants (*d14-5* and *max2-1*) exhibit larger stomatal apertures compared to Col-0, indicating that endogenous strigolactones positively control stomatal closure (Lv et al., 2017). Furthermore, exogenous strigolactones induce stomatal closure in wild-type and strigolactone-synthesis mutant plants but not in *d14* and *max2* plant, which indicates that D14 and MAX2 are required for strigolactone-triggered stomatal closure (Lv et al., 2017). Strigolactone functions independently of ABA and ABA signalling - disruption of ABA genes or genes functioning in guard cell ABA signalling resulted in WT-like stomatal closure in response to application of strigolactone analog GR24 (Lv et al., 2017). However, exogenous application of the strigolactone synthesis inhibitor, TIS108, exerted no effect on stomatal apertures (Zhang et al., 2018).

1.3 Reactive oxygen species (ROS)

1.3.1 Role of ROS molecules in plants

ROS is a collective term for highly reactive forms of molecular oxygen including singlet oxygen $^1O_2$, superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) which are able to react with a broad range of biomolecules including lipids, proteins and nucleic acids. Because of their harmful properties, ROS molecules are normally quenched quickly to protect various biomolecules from oxidation. The antioxidant system availability and composition thus determine concentration and longevity of ROS; for the most reactive forms of ROS ($HO^{\bullet}$ and $^1O_2$) the estimated lifetime is nanoseconds or microseconds; but for the most stable forms of ROS (H_2O_2 and $O_2^{\bullet -}$) the estimated lifetime is from milliseconds to seconds (Waszczak et al., 2018). $O_2^{\bullet -}$ is dismutated by superoxide dismutase (SOD) into H_2O_2 that is furthermore catalysed to water and oxygen by catalase (CAT) enzyme. ROS also function as critical signalling intermediates in versatile biological processes including stress responses, stomatal regulation, development, growth and cell expansion. Other signalling pathways, especially plant hormones such as SA, JA, ABA and ethylene, typically function in association with ROS (Overmyer et al., 2018, Vaahtera et al., 2014, Wrzaczek et al., 2013, Xu and Brosché, 2014).

ROS are produced in response to both abiotic (e.g. drought, salinity, extreme temperatures and excess light) and biotic stresses (pathogens, herbivores) in plants. Also in response to wounding ROS (H_2O_2 and $O_2^{\bullet -}$) are produced within minutes (L'Haridon et al., 2011). SA and ROS signalling can form a positive feedback loop that amplifies signal leading to defence or cell death (Xu et al., 2015b). Moreover, SA signalling has been shown to inhibit apoplastic ROS signalling and furthermore, JA signalling was concluded to restrict lesion formation (Xu and Brosché, 2014). Apoplastic ROS also have been shown to transiently suppress auxin signalling; in response to ozone the transcript levels of TIR1, AFB1, AFB3 and AFB5 were decreased (Blomster et al., 2011). Moreover, apoplastic ROS were shown to cause stress-induced morphogenic response seen as changes in leaf morphology such as epinastic curling and relative growth rate (Blomster et al., 2011).

In plants $O_2^{\bullet-}$, H_2O_2, and $HO^{\bullet}$ can be produced in nearly every subcellular compartment and thus compartmentalization of production and scavenging determines the biological function of ROS (Waszczak et al., 2018). In plants the largest ROS producers are chloroplasts and peroxisomes. Intracellular ROS are produced in chloroplasts during photosynthesis and in the mitochondria and peroxisomes in metabolic reactions. Unique to chloroplasts is production of the nonradical highly reactive $^1O_2$ at PSII during the energy transfer from the triplet state of the primary PSII electron donor ($^3P_{680}$) to the ground state triplet oxygen (Waszczak et al., 2018). Moreover, $O_2^{\bullet-}$ is produced at PSI, which can be further enhanced by treatments with methyl viologen that disrupts the electron transport in PSI which leads to $O_2^{\bullet-}$ production. The choroplastic ROS is required for intercellular ROS signalling. In peroxisomes oxidation of the photorespiratory glycolate and β-oxidation of fatty acids produces H_2O_2 (Overmyer et al., 2018, Shapiguzov et al., 2012, Vaahtera et al., 2014, Waszczak et al., 2018, Xu and Brosché, 2014). In plants the role of mitochondria in ROS production is smaller than in animals, and under normal growth conditions the contribution of mitochondria to total ROS pool is rather small. However, $O_2^{\bullet-}$ is produced in mitochondrial electron transport chain located in the inner mitochondrial membrane and $O_2^{\bullet-}$ is further dismutated to H_2O_2 by the mitochondrial superoxide dismutase (SOD) (Shapiguzov et al., 2012, Vaahtera et al., 2014, Wrzaczek et al., 2013).

ROS arise also from metabolic imbalances after changes in environmental conditions. Extracellular ROS is produced in the apoplast, which is the intercellular space outside the plasmamembrane and also the first contact surface for substances entering the leaf. Thus, many environmental perturbations first affect the apoplast, and diffusion through the apoplast is much faster than through cytosol which facilitates rapid cell-cell communication. The main source of ROS in the apoplast are the plasma membrane-localized NAPDPH oxidases (RESPIRATORY BURST OXIDASE HOMOLOGs, RBOHs) producing superoxide, and cell wall peroxidases producing H_2O_2 (Overmyer et al., 2018, Shapiguzov et al., 2012, Vaahtera et al., 2014, Wrzaczek et al., 2013, Xu and Brosché, 2014). In addition to a role in signalling, ROS in the apoplast are also necessary for formation of lignin polymers forming the major component of the plant cell wall (Kärkönen and Kuchitsu, 2015). The RBOHs are transmembrane flavoproteins oxidizing cytoplasmic NADPH, translocating electrons across the plasma membrane and reducing extracellular triplet oxygen to yield superoxide in the cell wall. Moreover, apoplastic ROS produced by RBOH proteins is regulated by Ca^{2+} and phosphorylation (Shapiguzov et al., 2012, Vaahtera et al., 2014, Wrzaczek et al., 2013).

In the apoplast the superoxide is rapidly dismutated into hydrogen peroxide either spontaneously or catalysed by SOD. H_2O_2 can be transported across the plasma membrane into the cytosol via aquaporin channels that mainly function in facilitating rapid transport of water between cells (Overmyer et al., 2018, Shapiguzov et al., 2012, Wrzaczek et al., 2013). The NADPH oxidase RBOHD (RESPIRATORY BURST OXIDASE HOMOLOG D) mediates systemic signalling by self-propagating mechanism for cell-to-cell signalling in response to several stimuli such as wounding, heat, cold, high light or salinity (Miller et al., 2009). ROS participates also in cell death regulation but not primarily due to toxicity of ROS but rather because ROS are connected to signalling events that are linked to programmed cell death (PCD) (Wrzaczek et al., 2013).

1.3.2 Other ROS-related responses to stress

In addition to a ROS burst, common stress responses include altered cytoplasmic and chloroplastic Ca^{2+} transients; ROS activates Ca^{2+} channels in plant membranes and ROS induces Ca^{2+} influx into the cell. Calcium is an important second messenger in plant stress and developmental signalling (Mori and Schroeder, 2004, Vainonen and Kangasjärvi, 2015, Wrzaczek et al., 2013). Transcriptional reprogramming is also a common response to stress and transcription factors often act in signal integration between different signalling pathways such as wounding, immunity and cell death. Transcriptional regulation also involves co-transcriptional regulators such as NPR1 and MPKs (mitogen-activated protein [MAP] kinases) that act in regulating location and activity of TFs. MAP kinase cascades consist of MAPKKK (MAPK kinase kinase), MAPKK (MAPK kinase) and a MAPK kinase. Activated MAP kinases phosphorylate and regulate the target protein activity of which several are transcription factors such as WRKY33 (WRKY DNA-BINDING PROTEIN 33), ERF6 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR6), HSF4 (HEAT SHOCK FACTOR4), which implies that MPK signalling regulates transcriptional responses to stress in plants. In response to most stresses the MPK activity is increased which indicates that their function is required for plant defences (Overmyer et al., 2018, Wrzaczek et al., 2013).

The effect of a combination of several stresses has been found to lead to a substantially different transcriptional response compared to the response elicited by a single stress factor (Rasmussen et al., 2013). Furthermore, Vaahtera et al. (2014) compared the gene expression patterns of commonly used defence and ROS marker genes in response to several different abiotic and biotic stress treatments. They concluded that many marker genes, proposed to be specific for a single stress, instead respond with altered expression in response to many stresses. Thus, selecting a specific marker gene responding only to a single treatment is not trivial and should take advantage of publicly available microarray and RNA-seq data (Overmyer et al., 2018, Rasmussen et al., 2013, Vaahtera et al., 2014).

Changes in the cellular reductive and oxidative states are mediated by glutaredoxins (GRXs) and thioredoxins (TRXs) via their ability to catalyze disulfide transitions and thus affect redox regulation of protein activity. Also the phytohormones have a role in redox regulation via affecting the cellular redox buffer glutathione; SA increases both the cellular amount and the ratio of oxidized and reduced glutathione while JA decreases the glutathione pool. GRX480 is used as an early marker for oxidative stress. GRX480 interacts with TGA transcription factors and suppresses transcription of the JA marker PDF1.2. Transcription of GRX480 is induced and regulated by SA and ROS, and moreover requires NPR1 (Blanco et al., 2009, Koornneef and Pieterse, 2008, Pieterse et al., 2012, Xu et al., 2015a).

In response to drought and heat stress DREB2A (DEHYDRATION-RESPONSIVE ELEMENT BINDING 2A) functions as a major regulator which is regulated via protein stability. DREB2A interacts with RCD1 (RADICAL-INDUCED CELL DEATH), a transcriptional co-regulator (Jaspers et al., 2009). The *rcd1* mutant is highly pleiotropic and stress related phenotypes include sensitivity to apoplastic ROS and ozone (Ahlfors et al., 2004, Overmyer et al., 2000, Vainonen et al., 2012).

1.3.3 ROS in regulation of stomata

ROS are involved in the regulation of stomatal signalling via several phytohormones such as ABA, MeJa and SA (Vahisalu et al., 2010). ROS are generated in guard cells in response to ABA which is mediated by the NADPH oxidase catalytic subunits RBOHD and RBOHF. Moreover, the stomatal response to high CO_2 concentration highly overlaps with ABA response; ROS production and thus subunits RBOHD and RBOHF, and furthermore, PYR/RCAR family of ABA receptors and ABA itself are required for high CO_2-induced stomatal closure (Chater et al., 2015, Kwak et al., 2003, Waszczak et al., 2018). Similarly, the response to ozone involves components from ABA-dependent stomatal responses; OST1 and SLAC1 are mediating ozone-initiated stomatal closure (Vainonen and Kangasjärvi, 2015). ABA-regulated stomatal movement requires H_2O_2 and this abscisic acid- and hydrogen peroxide-regulated stomatal movement is regulated by GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1) that was also shown to be required for stomatal closure (Hua et al., 2012, Sierla et al., 2018).

Ozone (O_3) triggers a rapid transient decrease in stomatal conductance, which was induced within a few minutes of ozone exposure. However, the stomata reopen despite the continuous presence of ozone, which implies that stomatal closure results from ROS formed in apoplast and not the physical ozone damage. Ozone-triggered rapid transient decrease in stomatal conductance involves a rapid burst of ROS. The ozone-triggered rapid transient decrease in stomatal conductance was also shown to require functional SLAC1, OST1, ABI1 and ABI2 proteins (Vahisalu et al., 2010).

1.3.4 Ozone as a research tool

Ozone (O_3) is a major air pollutant impacting negatively on crop yields, carbon fixation and thus climate change. Ozone enters the plant through stomata and immediately degrades to ROS ($O_2^{\bullet-}$ and H_2O_2) in the apolastic space of plant cells. Other early events after O_3 treatment include activation of MPK signalling and altered gene expression. O_3 also initiates cell death signalling, which results in visible lesions of collapsed dead tissue, but even without detectable damage ozone exposure initiates changes in gene expression, enzyme activities and metabolic profiles. The ROS burst after ozone exposure is similar to the ROS burst observed after pathogen infection, and activation of cell wall peroxidases and NADPH oxidases. O_3 is a convenient tool to screen stomatal and ROS sensitive mutant plant lines and has been used extensively to study the role of apoplastic ROS (Overmyer et al., 2018, Vaahtera et al., 2014, Vainonen and Kangasjärvi, 2015, Vahisalu et al., 2010, Xu et al., 2015b).

ROS can be measured with several methods either directly by using stains that visualize ROS accumulation (DAB, 3,3'-diaminobenzidine**,** stain for H_2O_2 and NBT, nitro blue tetrazolium, for $O_2^{\bullet-}$), trypan blue stain that visually mark dead or dying cells or indirectly by measuring ROS-induced cell death as the amount of electrolyte leakage i.e. ion leakage. ROS also elicits other responses than cell death such as priming of plant defences and stomatal closure. The changes in gene expression (i.e. transcript abundance) caused by ROS can be measured by using suitable marker genes (Vaahtera et al., 2014).

1.4 Phytohormone signalling

Phytohormones are organic substances naturally present in plants and influence physiological processes at low concentrations. Phytohormones play pivotal roles in growth, development and responses both to biotic and abiotic stresses. Cross-communication between hormonal signalling pathways enables the plant to adjust the stress and defence responses according to the type of stress or pathogen. In general auxin and salicylic acid (SA) pathways act in a mutually antagonistic manner during plant defence, whereas auxin and jasmonic acid (JA) signalling share many commonalities. SA and JA are known to generally antagonize each other and the antagonistic cross-talk is mediated by cytosolic NPR1 (Dempsey et al., 2011, Pieterse et al., 2009).

1.4.1 Abscisic acid (ABA)

ABA plays an important role in plant developmental processes e.g. seed development and dormancy and responses to environmental stresses, especially abiotic stress. Under normal growth conditions, ABA levels in plants is maintained low but a rapid synthesis is triggered in response to several stress factors; especially water deficit promotes ABA biosynthesis, accumulation and transport of ABA from roots to the shoots via xylem. Moreover, ABA promotes stomatal closure in order to limit transpirational water loss (Acharya and Assmann, 2009, Kim et al., 2010). Osmotic stress also activates ABA biosynthesis (Wang et al., 2011) and ABA signalling is known to be triggered by salt stress to enhance stress tolerance (Chung et al., 2014) and cold (Acharya and Assmann, 2009). ABA signalling has been linked to mediating JA-biosynthesis affecting pathogen (the oomycete *Pythium irregulare*) and wounding responses (Adie et al., 2007, L'Haridon et al., 2011). ABA-impaired biosynthesis and signalling mutants were shown to overexpress defensive-signalling pathways, which resulted in enhanced resistance to many pathogens such as *B. cinerea* and *P. syringae* (Denancé et al., 2013). ABA affects stomatal development, and density independent of OST1; in ABA-deficient mutants stomatal density is increased (Jalakas et al., 2018, Merilo et al., 2018). ABA furthermore affects stomatal conductance; all ABA-deficient and insensitive lines had higher stomatal steady-state conductance compared with wild-type (Merilo et al., 2018). Shoots are the main source of ABA in plants but also guard cells and phloem companion cells can synthesize ABA. The guard and phloem companion cells were found to be functionally redundant, since restoring ABA synthesis in either of them restored leaf ABA levels, visual phenotype and stomatal conductance in an ABA deficient mutant, *aba2-11* (Merilo et al., 2018).

ABA is a terpenoid which is synthesized from carotenoid precursors; the major step in ABA biosynthesis is the carotenoid cleavage that is catalysed by the NCED protein (Nine-cis epoxycarotenoid dioxygenase), which is considered to be the step limiting the rate of ABA biosynthesis (Fan et al., 2009, Tan et al., 2003, Wang et al., 2011). ABA binds to soluble receptors of the PYR1 (PYRABACTIN RESISTANCE 1)/PYL (PYR1-LIKE)/RCARs (REGULATORY COMPONENTS OF ABA RECEPTORS) family proteins, which induces biosynthesis of ABA via NCED as a positive feedback mechanism. PYR/PYLs are ABA receptors, and binding of ABA to PYR1 inhibits PP2Cs (type 2C protein phosphatases) ABI1 and ABI2 that act by negatively regulating ABA responses (Acharya and Assmann, 2009, Chung et al., 2014, Ma et al., 2009, Park et al., 2009). NCEDs belong to the carotenoid cleavage dioxygenase enzymes which also include the enzymes CCD7 and CCD8, shown to be MAX3 and MAX4 respectively in

Arabidopsis (Booker et al., 2004, Sorefan et al., 2003). CCDs specifically cleave double bonds in carotenoid molecules to form apocarotenoids that are carbonyl compounds (Saeed et al., 2017). The tomato orthologs LeCCD7 and LeCCD8 had reduced expression in ABA deficient mutants, while expression of other carotenoid cleavage genes was not affected (López-Ráez et al., 2010).

ABA strongly affects transcription of the downstream target genes; ABREs (ABA-responsive elements) are present within promoters of many ABA-upregulated genes. The bZIP transcription factors called ABRE-binding factors (ABFs) bind to the ABREs, and may be the major downstream target of ABA signalling responses (Kim et al., 2010, Yoshida et al., 2015). Moreover, MYBR (MYB-recognition site) and MYCR (MYC-recognition site) are cis-elements in the promoters of ABA-regulated genes and have a major role in ABA-related stomatal regulation associated to e.g. light and abiotic stresses (Kim et al., 2010). Another pathway to transmit water-deficiency signals through vascular tissues use the CLAVATA3/EMBRYO-SURROUNDING REGION-RELATED 25 (CLE25) peptide. CLE25 is a member of a protein family of which CLAVATA3 (CLV3) is well characterized for involvement in shoot apical meristem formation. CLE25 moves from the roots to the leaves, and affects abscisic acid biosynthesis and furthermore stomatal closure, thus enhancing resistance to dehydration stress. The BARELY ANY MERISTEM (BAM) receptors in leaves are required for the CLE5 peptide-induced dehydration response, and CLE5 movement from roots to leaves modulates NCED3 expression in leaves in association with the receptor-like kinases BAM1 and BAM3 (Takahashi et al., 2018).

ABA interacts with many other phytohormones and is suggested to inhibit the action of growth promoting hormones e.g. brassinosteroids (BR), however the regulatory mechanism coordinating ABA and BR activity is unknown (Chung et al., 2014). ABA was suggested to have a role in the regulation of strigolactone biosynthesis (López-Ráez et al., 2010). ABA attenuates JA/ET-dependent gene expression, and affects JA biosynthesis and resistance against JA-inducing nectrotrophic pathogens (Pieterse et al., 2009). ABA and SA have been shown to function antagonistically in resistance to some pathogens and trigger stomatal closure to avoid penetration of *P. syringae* in Arabidopsis (Denancé et al., 2013, Melotto et al., 2006). ABA seems to function as a repressor of innate immune response (Wasilewska et al., 2008) and there are many possible connections between pathogen resistance and ABA signalling; e.g. both pathogen attack and ABA trigger formation of the H_2O_2 as a second messenger (Kwak et al., 2003, Torres and Dangl, 2005, Wasilewska et al., 2008). The ABA signalling pathway in stomatal closure and the immune response pathway dependent on FLS2 and affecting stomatal opening are interconnected (Wasilewska et al., 2008).

1.4.2 Auxins

Auxins are a class of phytohormones which include indole-3-acetic acid (IAA), one of the predominant naturally occurring forms of auxin in plants. Auxins have important role in plant growth, development and stress responses; generally auxins promote both cell division and elongation, and are classically known for their role in apical dominance and being involved in tropic responses (Acharya and Assman, 2009, Denancé et al., 2013). In addition, many plant pathogenic microorganisms produce auxin during their interactions with plants. For example, the flg22-triggered suppression of auxin signalling leads to increased resistance to *Pseudomonas syringae* pv. tomato (Denancé et al., 2013, Kazan and

Manners, 2009). Furthermore, it was found that the salicylic acid (SA) inhibits pathogen growth through repressing the auxin signalling pathway (Wang et al., 2007). However, auxin has been proven to promote susceptibility to *P. syringae* independent of suppression of SA-mediated defences (Mutka et al., 2013). The modulation of host auxin signalling is not restricted to bacterial pathogens but also infection by *Botrytis cinerea* leads to altered expression of key genes involved in auxin signalling. In plant defence, auxin interacts with other phytohormones; auxin and SA pathways act in a mutually antagonistic manner, but JA and auxin share many commonalities (Denancé et al., 2013, Kazan and Manners, 2009, Pieterse et al., 2009).

Auxins are perceived by a family of F-box-protein TIR1 (TRANSPORT INHIBITOR RESPONSE 1) /AFBs (AUXIN SIGNALING F-BOX) as intracellular receptors (Prigge et al., 2016, Shimizu-Mitao and Kakimoto, 2014, Xu et al., 2014). AUXIN-BINDING PROTEIN1 (ABP1), previously assumed to function as an extracellular receptor in auxin perception, has been proven not to be involved either in auxin signalling or plant development (Gao et al., 2015). In Arabidopsis auxin responsive genes are under control of transcription factors called auxin response factors (ARFs), which are negatively regulated by interaction with the complex of transcriptional repressors Aux/IAA (AUXIN/INDOLE-3-ACETIC ACID) and TOPLESS (TPL) co-repressor proteins; in the absence of auxin, the transcriptional repressors Aux/IAA (AUXIN/INDOLE-3-ACETIC ACID) proteins bind to the transcription factors AUXIN RESPONSE FACTORs (ARFs). At higher auxin concentration Aux/IAA proteins are polyubiquitinated and degraded by $SCF^{TIR1/AFB1-5}$ (Li et al., 2016, Stefanowicz et al., 2015). Calderon Villabolos et al. (2012) showed that assembly of an auxin co-receptor complex consisting of TIR1 and an Aux/IAA protein is crucial for efficient auxin binding. Thus, protein complexes containing $SCF^{TIR1/AFB}$ and Aux/IAA are referred to as a co-receptor of auxin (Calderon Villalobos et al., 2012, Shimizu-Mitao and Kakimoto, 2014). In Arabidopsis there are 29 Aux/IAA proteins and five TIR1 homologs, AFB1-AFB5 proteins, which interact with Aux/IAAs in an IAA-dependent manner (Shimizu-Mitao and Kakimoto, 2014).

1.4.3 Strigolactones (SLs)

Strigolactones are endogenous butenolide hormones historically known as germination stimulants of parasitic plants of the genera *Striga* and *Orobanche* that are root parasites affecting many important crop plants (Cardoso et al., 2011). SLs are produced in plant roots and transported to shoots but the route is unclear; Kohlen et al. (2011) proposed that strigolactone is transported through the xylem which was questioned by results from Xie et al. (2015). SLs function as rhizosphere signalling molecules; the primary function of SLs is in development and interacting with auxin, which dominates in SL regulated developmental processes (Hayward et al., 2009, Janssen and Snowden, 2012, Mishra et al., 2017, Waldie et al., 2014) - SLs were shown to affect branching by dampening auxin transport (Bennett et al., 2006, Crawford et al., 2010, Hayward et al., 2009). Results by Brewer et al. (2015) suggest that strigolactones could inhibit branching also independent of auxin but the putative alternative inhibition mechanism remains unknown. Moreover, SLs not only regulate shoot and root architecture in plants but also stimulate hyphal branching and growth in arbuscular mycorrhizal (AM) fungi (Cardoso et al., 2011, Janssen and Snowden, 2012, López-Ráez et al., 2010, Waters et al., 2012).

Strigolactones have been shown to affect several functions in the plants in addition to branching; e.g. senescence, drought, salinity, and light stress (Bu et al., 2014, Gomez-Roldan et al., 2008, Ha et al., 2014, Umehara et al., 2008). *max2* was also shown to promote photomorphogenesis (Shen et al., 2007, Shen et al., 2012). *max2* and SL-deficient mutants have been shown to have reduced drought resistance (Bu et al., 2014, Ha et al., 2014) and strigolactones were recently concluded to be common regulators in induction of stomatal closure *in planta* (Zhang et al., 2018). Moreover, Stes et al. (2015) showed that strigolactones contributed to leafy gall syndrome (caused by *Rhodococcus fascians*) tolerance, which indicates that strigolactone might be involved in the sensitivity to plant pathogens.

In the root exudates of several plant species more than 20 SL and SL-like compound have been identified (Saeed et al., 2017). All the strigolactones discovered so far seem to derive from the same biosynthetic pathway (López-Ráez et al., 2010). Strigolactone biosynthesis (Figure 1) starts in plastid in which all-trans-β-carotene is modified by the enzymes D27, CCD7 and CCD8 in order to be converted to carlactone. Carlactone moves to cytosol where it is further oxidised by cytochrome P450 monooxygenase (MAX1) and putatively several yet unidentified enzymes to be finally converted into strigolactones (Abe et al., 2014, Booker et al., 2005, Goulet and Klee, 2010, Mishra et al., 2017). Biosynthesis of strigolactone and ABA both begin from carotenoids, and are potentially linked via NCED enzyme that is known to function in ABA biosynthesis. It has been shown in NCED mutants of tomato and maize that a reduction in germination stimulatory activity correlates with a reduction in the strigolactone production (López-Ráez et al., 2010). Moreover, expression of strigolactone biosynthesis genes was clearly reduced in ABA-deficient mutants. These findings indicate that NCED enzymes might be involved in strigolactone biosynthesis in addition to ABA biosynthesis (López-Ráez et al., 2010). Two genes are essential in strigolactone perception, *DWARF14* (*D14*) and *MORE AXILLARY GROWTH2* (*MAX2*). D14 encodes an alpha/beta hydrolase that is located in the cytoplasm (Chevalier et al., 2014, Waldie et al., 2014). MAX2 is identical to ORE9 which functions as a positive regulator of leaf senescence (Woo et al., 2001). MAX2 is also identical to PPS (pleiotropic photosignaling): *pps* has longer hypocotyls and slightly smaller cotyledons under continuous R, FR, and B light (Shen et al., 2007). MAX2 has been shown to localize to nucleus, and as an F-box protein it functions as part of SCF complex (E3 ligase) marking proteins for degradation (Stirnberg et al., 2007). Targets of MAX2 include SUPPRESSOR OF MORE AXILLARY GROWTH2-LIKE6 (SMXL6), SMXL7 and SMXL8, which were shown to be required for SL-dependent regulation of shoot branching in Arabidopsis. SMXL proteins can form a complex with the transcriptional corepressor TOPLESS-RELATED PROTEIN2 (TPR2), which function together as transcriptional repressors of unknown transcription factors (TFs) and furthermore, repress the downstream target genes eventually leading to inhibition of SL signalling (Wang et al., 2015).

According to the current hypothesis, strigolactones are first hydrolyzed by D14, which results in a conformational change in D14. This facilitates D14 to interact with SCF^{MAX2} to trigger ubiquitination and degradation of downstream signalling components (Lv et al., 2017). D14 confers responses to karrikins that are bioactive compounds, butelinoids, derived from burnt vegetation. The karrikins are structurally similar to strigolactones but physiologically distinct plant growth regulators. Karrikins function as germination stimulants and enhance seedling responses to light (Li et al., 2017, Nelson et al., 2011, Waters et al., 2012). However, despite the different origins of SL and karrikin, their signalling pathways

converge upon *MAX2* (Waters et al., 2012). Karrikins and SLs induce similar effects at the germination and seedling stages and their signalling requires MAX2. The signal transduction pathway for both SLs and karrikins require MAX2 protein and closely related α/β hydrolase fold proteins DAD2 and KAI2 (KARRIKIN INSENSITIVE2). KAI2 encodes the proposed karrikin receptor and was found to promote drought in Arabidopsis (Li et al., 2017). Furthermore, KAI2 is an ancestral D14 paralogue that is specifically required for karrikin responses but not for strigolactones which suggests that karrikins have evolved as adaptive responses to smoke (Janssen and Snowden, 2012, Li et al., 2017, Nelson et al., 2011, Waters et al., 2012).

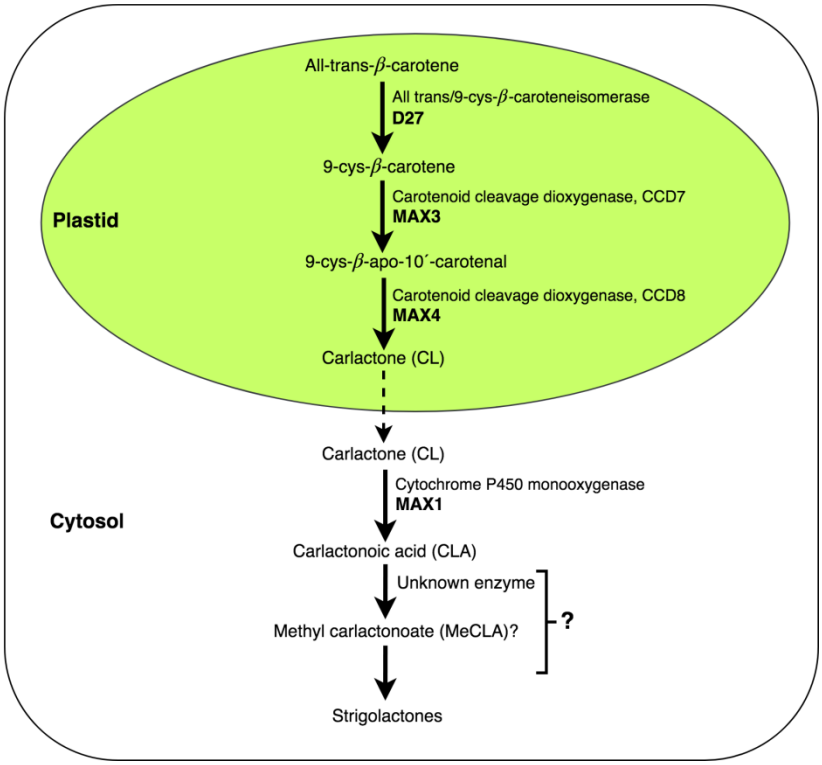

Figure 1. Strigolactone synthesis route, based on Abe et al., 2014, Mishra et al., 2017 and Saeed et al., 2017. Biosynthesis starts and proceeds in plastid until carlactone is produced, which moves to the cytosol where synthesis continues. After carclactonoic acid is produced, the last steps of the synthesis are still putative and details are unknown.

1.4.4 Brassinosteroids (BRs)

Brassinosteroids are polyhydroxylated steroidal phytohormones promoting plant growth via stem elongation and vascular differentiation, but also function in senescence and stress responses. Moreover, BRs regulate development and function of stomata, and thus cross-talks with ABA in regulation of stomatal development (Acharya and Assmann, 2009, Chung et al., 2014). ABA is proposed to inhibit the action of BRs, and an antagonistic interaction between ABA and BRs was proposed particularly when stress and growth responses compete for resources (Chung et al., 2014).

The most active form of brassinosteroids is brassinolide (BL). BRs are perceived at the cell surface by BRI1 (BRASSINOSTEROID INSENSITIVE1), causing release of the inhibitory protein BKI1 (BRI1 KINASE INHIBITOR 1) that is bound to BR1 in the absence of BL. After binding of

BL, BRI1 furthermore forms a heterodimer with BAK1 (BRI1-ASSOCIATED-KINASE1) that phosphorylates BRI1 to initiate down-stream signalling. BAK1 is an essential co-receptor for multiple other receptors and acts as an essential component in PTI (PAMP triggered immunity) by interacting with FLS2 and EFR and phosphorylating a down-stream kinase BIK1 (BOTRYTIS INDUCED KINASE1) (Chung et al., 2014, Overmyer et al., 2018).

Brassinosteroids and strigolactones have been linked in several ways e.g. via BES1 (bri-EMS-suppressor 1) which is a positive regulator in BR signalling pathway and acts as a downstream transcription factor to directly regulate BR-responsive gene expression (Wang et al., 2013). BES1 acts as a substrate of MAX2 protein and furthermore, BES1 interacts with MAX2 to regulate SL-responsive gene expression. Moreover, strigolactone/MAX2-induced degradation of BES1 regulates shoot branching and furthermore, the SL receptor D14 can promote BES1 degradation (Wang et al., 2013).

1.4.5 Gibberellins (GAs)

Gibberellins are growth regulators that control seed development, germination and also are involved in organ elongation and flowering time. Similar to auxin and JA signalling, GA-responsive genes are activated by degradation of DELLA transcriptional repressors. Under low GA concentration DELLA proteins inhibit transcription of GA responsive genes by binding to PIF (PHYTOCHROME INTERACTING FACTORS) transcription factors. DELLA proteins are targeted for degradation by the SCF associated F-box-proteins SLEEPY1 (SLY1) and SNEEZY (SNE) (Stefanowicz et al., 2015).

1.4.6 Salicylic acid (SA)

Salicylic acid (SA) regulates many aspects in plant growth and development but especially plays a major role in disease resistance signalling. SA-mediated defences are generally effective against biotrophic pathogens and moreover, SA is known to be necessary and sufficient to induce a plant-wide defence mechanism called systemic acquired resistance (SAR). SA treatment has also been shown to induce PR gene expression and enhanced resistance in many plant species (Dempsey et al., 2011, Fu and Dong, 2013). Plants overexpressing the *nahG* encoding SA-degrading enzyme salicylate hydroxylase and SA-synthesis mutant *ics1* (*isochorismate synthase 1*) are defective in SAR. Furthermore, Arabidopsis *NahG* plants were found to be defective in non-host resistance to *Pseudomonas syringae* pv. *phaseolicola* due to degradation of SA (Fu and Dong, 2013, Overmyer et al., 2018, Wees van and Glazebrook, 2003).

The Arabidopsis NPR1 (non-expressor of PR1 genes) protein is a master regulator of SAR and serves as a receptor for SA (Wu et al., 2012). Exogenous application of SA induces a change in cellular redox state, which can be sensed by NPR1 as it switches between monomer and oligomer. Under normal growth conditions NPR1 is kept inactive as an oxidized multimer in the cytosol, but after biotic or abiotic stress NPR1 is reduced by a thioredoxin which results in the release of monomeric forms subsequently entering nucleus and acting as a co-regulator of gene expression (Fu and Dong, 2013, Vaahtera et al., 2014). NPR3 and NPR4 also function as SA receptors but independently of NPR1 and compared to NPR1, they play opposite roles in transcriptional regulation of SA-induced defence gene expression (Ding et al., 2018). Ding et al. (2018) proposed a model in which NPR proteins associate with TGA

transcription factors, and the NPR-TGA protein complexes compete for binding to SA-responsive defence gene promoters. NPR3 and NPR4 were concluded to function as SA-sensitive co-repressors that repress the defence gene promoters under conditions of low SA. Under high SA-levels, NPR1 complexes are activated and SA releases the repression by blocking C-terminal domains of NPR3 and NPR4, which leads to high levels of defence gene expression (Ding et al., 2018, Innes 2018).

SA is a phenolic compound that can be synthesized from chorismate either via PAL (phenylalanine ammonia lyase) or isochorismate ICS1/SID2 (ISOCHORISMATE SYNTHASE 1) pathway, of which PAL pathway has a minor role in SAR-associated SA synthesis. A stressed Arabidopsis appears to synthesize SA primarily via the isochorismate pathway in the chloroplast, in which ICS1 and ICS2 proteins catalyze chorismate into isochorismate. Level of SA is normally tightly regulated in plants and for example after pathogen infection most of the SA is glucosylated by the SA glucosyltransferase (SAGT) to form inactive SA glucosidase (SAG) (Dempsey et al., 2011, Dempsey and Klessig, 2017, Fu and Dong, 2013, Pieterse et al., 2009). ICS1 has been shown to be involved in PTI, ETI and SAR since in *ics1* mutant e.g. susceptibility to virulent pathogens is increased, and moreover they fail to develop SAR in the systemic leaves of pathogen or elicitor-treated plants. After being synthetized SA undergoes a number of modifications including glucosylation, methylation and amino acid conjugation. Glucosylation inactivates SA in order to allow vacuolar storage in a non-toxic storage form, SAG (SA 2-O-β-D-glucoside), that can furthermore be hydrolysed to release free SA in response to pathogen attack. Methylation of SA generates methyl salicylate (MeSa) to allow more effective long-distance transport of the mobile defence signal via phloem. The AA conjugation is not well characterised but is possibly linked in SA catabolism (Dempsey et al., 2011, Dempsey and Klessig, 2017).

1.4.7 Jasmonic acid (JA)

Jasmonates include jasmonic acid (JA) and its derivatives, and are lipid-derived signalling molecules that are involved in plant growth and development, but also are crucial in mediating plant responses to abiotic stresses (UV light and ozone), insects and necrotrophic pathogens (Pieterse et al., 2009). Jasmonate signalling perception and transduction mechanism resembles auxin mechanism; the jasmonate ZIM domain (JAZ) proteins together with other co-repressors including TPL-protein block JA-mediated transcription by interacting with MYB/MYC transcription factors. Most JA-responses are mediated through the F-box protein COI1 (CORONATINE-INSENSITIVE1) that functions as part of the SCF^{COI1} complex; COI1 specifically recognizes the JAZ proteins and directs them for degradation which results in release of MYB/MYC transcription factors and induction of JA-responsive gene transcription. The COI1 and JAZ protein function as co-receptors for JA-Ile (JA conjugated with amino acid isoleucine). The Ja-Ile is the physiologically active form of jasmonate which stabilizes the COI1- JAZ interaction (Acharya and Assmann, 2009, Pieterse et al., 2012, Robert-Seilaniantz et al., 2011, Stefanowicz et al., 2015).

The transcription factor MYC2 (also named JIN1 for JASMONATE INSENSITIVE1) not only plays important role regulating JA-responsive genes; MYC2 positively regulates JA-responsive genes such as VSP2 (VEGETATIVE STORAGE PROTEIN 2) and LOX2 (ARABIDOPSIS THALIANA LIPOXYGENASE 2) but furthermore, negatively regulates JA/ET responsive genes such as *PDF1.2 (PLANT DEFENSIN 1.2*). When the JA response is activated together with ET,

the ERF (ETHYLENE RESPONSE FACTOR) branch of the JA response is activated, whereas the in the absence of ET the MYC2 branch of the JA response is activated (Pieterse et al., 2009, Pieterse et al., 2012).

1.4.8 Ethylene (ET)

Ethylene (ET) is a gaseous hormone that regulates abscission, senescence, seed germination and responses to both abiotic and biotic stresses. SCF-associated F-box proteins are involved at several levels in ethylene signalling; ET-response genes are activated by two positive transcriptional regulators EIN3 (ETHYLENE INSENSITIVE 3) and EIN3-Like (EIL1), which are regulated via interaction with two F-box proteins EIN3-Binding F-box1 (EBF1) and EBF2. In the absence of ethylene $SCF^{EBF1/2}$ mediates ubiquitination and proteosomal degradation of EIN3/EIL3. However, at increased ET levels EBF1/2 proteins are proteosomally degraded. EIN3/EIL1 are stabilized by ETHYLENE INSENSITIVE2 (EIN2). EIN2 is tightly regulated by multiple mechanisms including SCF-mediated protein degradation, and in response to ET signal EIN2 is cleaved, and the EIN2 C-terminal domain is translocated to the nucleus. ET is perceived via the membrane-bound receptors ETR1, ERS1, ETR2, ERS2 and EIN4 located in the ER which are acting as negative regulators of ET signalling (Stefanowicz et al., 2015).

ERFs belong to the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factor superfamily. ERF1 and ORA59 belonging to this superfamily function as integrators of JA and ET signalling pathways. ET interacts with other phytohormones; ET is especially known to synergistically interact with JA, and for example regulation of *PDF1.2*, requires concomitant activation of JA and ET response pathways. ET is also essential for insect and pathogen related defence responses. ET was found to be essential for onset of SA-dependent SAR upon tobacco mosaic virus infection and moreover, ET was shown to enhance the response to SA in Arabidopsis (Pieterse et al., 2009).

1.5 Plant defence systems

1.5.1 Defence responses

The ability of pathogens to infect a host and cause disease is called virulence, and a successful infection causing disease formation is defined as compatibility. There are several ways to classify plant defence responses: the classical way is to divide the resistance phenomena based on the origin of resistance i.e. innate or acquired resistance. The innate resistance can further be divided based on a non-specific general resistance which is effective against several pathogenic species and a specific resistance in which one plant cultivar can resist infection of one or a few pathogenic strains (Kiraly et al., 2007, Pieterse et al., 2009).

The non-host resistance is the most durable form of resistance meaning that all of the individual members or lines of a species are resistant to all of the races of a given pathogenic species. A non-host plant species is unable to support the life-strategy requirements of the pathogen, which will cause the incompatible plant-pathogen interaction. Moreover, basal resistance including PAMP, ROS, preformed structural barriers or antimicrobial compounds can confine successful infection to specialized pathogen

species. RLKs (receptor-like kinases) often mediate the initial stress perception in both biotic and abiotic stress; the best characterized RLK is probably the immunoreceptor FLS2 (FLAGELLIN-SENSITIVE 2) detecting bacterial flagellin (Jones and Dangl, 2006, Kiraly et al., 2007, Pieterse et al., 2009, Overmyer et al., 2018).

The aim of the activated specific resistance mechanisms is to keep the infection localized; especially the interaction between avirulence protein and R protein causing hypersensitive response (HR) involving ROS burst. In addition to ROS there are several proteins and small molecules participating in defence signalling including mitogen-activated protein (MAP) kinases (MPKs), transcription factors (TFs), nitric oxide (NO) and Ca^{2+}. Also many plant hormones have a central role, especially abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) (Hofius et al., 2007, Kiraly et al., 2007, Overmyer et al., 2018, Pieterse et al., 2009).

1.5.2 Plant immune system: Pathogen triggered immunity (PTI) and effector-triggered immunity (ETI)

Plant immunity includes several mechanisms by which plants recognize pathogen attack, and transduce the information through signalling networks within the cell, to adjacent cells and distant tissues. Immunity can be differentiated into basal and resistant(R)-gene-mediated defences in a two-phase immune system. In the first phase the early (10-30 min after pathogen attack) defence responses in plant-pathogen interaction are based on basal defences in which extracellularly elicited microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) are recognised by transmembrane pattern recognition receptors (PRRs). The PAMPs are components of pathogens such as bacterial flagellin which is a structural component of bacterial flagellum, a bacterial elongation factor Ef-Tu and fungal chitin, recognised by FLS2, EFR and CERK, respectively. One way to measure the output of flagellin activated signalling is to follow the flagellin-induced gene, *FRK1* (*FLG22-INDUCED RECEPTOR-LIKE KINASE 1*) that is an early flg22 response marker gene. It is also an efficient early marker in pathogen responses (Asai et al., 2002, Yeh et al., 2015).

In general, the recognition of PAMPs/MAMPs by PRRs results in pathogen triggered immunity (PTI). Plants can also detect damage-associated molecular patterns (DAMPs), degradation products and endogenous signals generated upon cellular disintegration due to a pathogen attack e.g. oligogalacturonides derived from the pectin by pectinases, and recognition of DAMPs triggers a similar immune response as PTI (Bigeard et al., 2015, Saijo et al., 2018). PTI functions efficiently against a broad range of microbes and activation of PTI causes production of reactive oxygen and nitrogen species, and a rapid Ca^{2+} influx. PTI signalling is propagated via mitogen-activated protein kinase cascades (MAPKKK, MAPKK, MAPK), which results in phosphorylation and activation of transcription factors and furthermore, expression of defence-related genes. Also callose deposition at the cell periphery is associated with basal defence, and is believed to limit pathogen virulence. The activated PTI leads to growth retardation as trade-off between growth and defence physiology (Abramovitch et al., 2006, Hofius et al., 2007, Withers and Dong, 2017).

The so far identified PRRs fall into two classes; receptor-like proteins (RLP) and transmembrane receptor-like kinases (RLK). The RLPs do not have an intracellular kinase domain, whereas the RLK structure includes an extracellular domain, a transmembrane domain and an intracellular kinase domain (Couto and Zipfel, 2016, Saijo et al., 2018). In

order to function, most known PRRs require BAK1 (BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1) and activation of PRR by PAMP perception stimulates also recruitment of BIK1 (BOTRYTIS INDUCED KINASE1) (Dodds and Rathjen, 2010, Pieterse et al., 2009, Withers and Dong, 2017). BAK1 is a leucin-rich repeat (LRR) receptor-like kinase (RLK) that is also involved in brassinosteroid-dependent development, plant immunity and cell-death control by interacting with the brassinosteroid receptor BRI1, immune receptors e.g. FLS2 and EFR, and BAK1-interacting receptor-like kinase named BIR1 that has been shown to negatively regulate plant immunity. All BIRs (BIR1-BIR4) were shown to interact with BAK1 and both *bir1* and *bir2* mutants displayed enhanced SA-dependent cell-death. BIR2 also has a critical role in negative regulation of flg22-induced responses by preventing the association of FLS2 and BAK1 (Halter et al., 2014, Ma et al., 2017). Thus, BAK1 can be considered a central regulator in plant immunity and the target of several pathogen virulence effector molecules, with an exception the fungal chitin receptor CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) that is known to respond to an unknown bacterial PAMP (Dodds and Rathjen, 2010). Activation of PRRs leads to ROS burst by RBOHD which is activated via phosphorylation by BIK1 and calcium-dependent protein kinases (CDKs) (Overmyer et al., 2018, Withers and Dong, 2017). RBOHD is involved in immunity by existing in complexes with EFR and FLS2 and moreover, BIK1, interacts with RBOHD and phosphorylates it upon PAMP perception (Kadota et al., 2014).

Effectors are small protein molecules secreted by pathogens that can interfere with PTI, which results in effector-triggered susceptibility (ETS). The second phase of immunity will follow later, typically 2-3 hours after infection, in which intracellular resistance (R) proteins recognise specific effectors in the cytoplasm. The R-gene mediated defences are activated after the delivery of type III effectors into the host cytoplasm. The effectors are also called avirulence (Avr) proteins, because they elicit immunity reaction in the plants expressing a specific avirulence gene. The detection of Avr protein by R-protein induces effector-triggered immunity (ETI). The effector recognition can happen either through direct ligand-receptor interactions, or through indirect detection in which the effector modifies an accessory protein, and the accessory protein is then recognised by the NB-LRR receptor. The natural selection drives pathogens to avoid recognition by the immune system, e.g. by diversifying the recognised effector gene, or by acquiring additional effectors to suppress ETI. However, natural selection furthermore results in new *R* gene specificities for recognition and thus, triggering ETI (Dodds and Rathjen, 2010, Giraldo and Valent, 2013, Hofius et al., 2007, Jones and Dangl, 2006). PTI and ETI often launch somewhat similar responses; however, ETI is qualitatively stronger, faster and often involves a hypersensitive response (HR) at the infection site. ETI commonly leads to generation of ROS and nitrogen species, calcium accumulation, transcriptional reprogramming, hypersensitive response leading to localized programmed cell death (PCD) to limit pathogen spread and moreover, SA biosynthesis, expression of pathogenesis-related (PR) proteins and finally establishment of systemic acquired resistance (SAR). In brief, PTI contributes to basal resistance to diverse adapted and non-adapted microbes, whereas ETI is central in defending against race-specific and host-adapted pathogens (Abramovitch et al., 2006, Hofius et al., 2007, Jones and Dangl, 2006, Withers and Dong, 2017).

ETI is often based on intracellular recognition by R proteins with a nucleotide-binding domains and leucine-rich repeats, NB-LRR proteins. A direct interaction between R-protein and Avr protein has been identified only in few cases, and therefore, also a guard hypothesis

has been suggested in which R proteins recognize the pathogen through an accessory protein. Most of the characterized plant R proteins belong to the class of nucleotide binding-leucine-rich repeat (NB-LRR), which can be further classified into two categories based on the N terminus containing either a toll-interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain. In their signalling pathways most CC-type R proteins generally use NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE 1) and TIR-type NB-LRR proteins use EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) (Dempsey et al., 2011, Hofius et al., 2007). EDS1 functions both at transcriptional regulation in the nucleus and at regulating cell death in the cytosol; EDS1 has been found to be a prominent regulator of cell death together with SA. Moreover, EDS1 forms alternate complexes with a defence regulator PAD4 (PHYTOALEXIN DEFICIENT4) or SAG101 (SENESCENCE-ASSOCIATED GENE101) (Overmyer et al., 2018).

In the classical gene for gene model of disease resistance R genes are only effective in the presence of *avr* gene in a pathogen. One of the best-studied bacterial effectors is AvrRpt2, a type III effector produced by *P. syringae*. If plants are unable to recognize AvrRpt2 due to the lack of corresponding disease resistance gene *RPS2* (*RESISTANT TO P. SYRINGAE2*), the disease will occur. In addition to AvrRpt2, also the type III effector AvrRpm1 inhibits flg22-induced defences in Arabidopsis. Moreover, AvrRpt2, AvrRpm1 and AvrB interact each with RIN4 (RPM-1 INTERACTING PROTEIN 4), a negative regulator of both PTI and ETI in Arabidopsis. Another type III effector, AvrPto, suppresses basal defences in Arabidopsis and contributes to the virulence of *P. syringae* in tomato (Abramovich et al., 2006, Dodds and Rathjen, 2010, Jones and Dangl, 2006, Kazan and Manners, 2009).

Most plant pathogens are highly specialized and have a narrow host range and thus, adapted pathogens are able to suppress the basal defence of their host plants but not in non-host plant species. PTI is often called a basal defence and can generally be considered a non-host resistance, which is effective against non-adapted pathogens and detection of broadly conserved molecular features of PAMPs, whereas ETI functions against adapted pathogens and detection of specific avirulence effectors (AVR effectors) (Denancé et al., 2013, Dodds and Rathjen, 2010).

1.5.3 Hypersensitive response and systemic acquired resistance

Hypersensitive response (HR) is generated in response to ETI and includes oxidative burst i.e. RBOH-dependent ROS production. Characteristic to HR are necrotic areas where infected cells have undergone programmed cell death. In addition to ROS burst HR involves increased transmembrane ion flux (Ca^{2+}, K^{+}, H^{+}), production of nitric oxide (NO), production of antimicrobial secondary metabolites (phytoalexins) and antimicrobial proteins (defensins), and upregulation of stress-related genes (Fu and Dong, 2013, Hofius et al., 2007, Kiraly et al., 2007, Marino et al., 2012, Overmyer et al., 2003, Overmyer et al., 2018, Pieterse et al., 2009). The local induction of immunity upon infection can trigger a response also in distal tissues leading to systemic acquired resistance (SAR), which will protect against secondary pathogen infections for a period of weeks to months. SAR-associated immune memory functions through priming and induction of SAR itself can be considered a process of priming. SAR can even be passed to progeny through epigenetic regulation e.g. changes in DNA methylation or histone modifications. In plants SAR is a broad spectrum with no specificity to initial infection and an avirulent pathogen causing local programmed cell death can induce SAR (Fu and Dong, 2013, Shan and He, 2018).

SA synthesis is induced both locally and systemically upon SAR induction and ETI can trigger SAR trough both local and systemic synthesis of SA. Execution of SAR involves transcriptional reprogramming regulated by transcriptional events initiated by NPR1 (Fu and Dong, 2013). Induction of SAR is generated through mobile signals within 4-6 hours of primary infection, followed by accumulation of the plant hormone salicylic acid (SA) and secretion of the antimicobial PR (pathogenesis related) proteins); the expression of PR1 (function unknown) (Uknes et al., 1992), PR2 (encoding β-1,3-glucanase) and PR5 (encoding a thaumatin-like protein) is induced by SA and used as a readout for SAR (Fu and Dong, 2013). SA is considered the primary trigger for the systemic upregulation of defence-related genes and the SA receptor, NPR1, is a master regulator of SAR (Shan and He, 2018). However, even though accumulation of SA in uninoculated tissues is required for SAR; SA itself is not the critical mobile signal in SAR (Klessig et al., 2018).

Several plant hormones and proteins have been suggested to function as mobile signals of SAR; the methylated derivative of SA, methyl salicylate (MeSA), serves as a critical phloem-mobile SAR signal (Dempsey et al., 2011, Klessig et al., 2018). Several other molecules have been discovered to function as mobile inducers of SAR; a nine-carbon dicarboxylic acid azelaic acid (AzA), glycerol-3-phosphate (G3P) or a G3P-dependent factor, the abietane diterpenoid dehydroabietinal (DA), and the lysine (Lys) derivative pipecolic acid (Pip) (Klessig et al., 2018, Shine et al., 2019). Pip is further converted to N-hydroxypipecolic acid (NHP) that functions as a critical metabolic regulator of SAR (Shan and He, 2018). It has been suggested that SA putatively with DA and Pip regulate one branch of SAR, whereas AzA and G3P together with NO and ROS regulate the other branch (Shine et al., 2019).

1.5.4 Phytopathogen life strategies

Pathogens are divided according to their lifestyles into biotrophs and necrotrophs. The necrotrophs destroy host cells often via phytotoxin production and then feed on the cell contents. Biotrophs derive nutrients from living host tissues by invaginating cell using specialized feeding structures without disrupting the cell. Many of the plant pathogens are hemibiotrophs displaying both life styles. Plants have physiological barriers e.g. waxy cuticle on the leaf surfaces, the cell wall and plasma membrane which plant utilize to prevent access of pathogens into plants (L'Haridon et al., 2011, Pieterse et al., 2009).

Pathogens landing on a plant must first penetrate the wax covered cutin surface. Bacteria use either wounds or natural openings such as stomata to enter the plant and occupy either the intercellular space i.e. apoplast or xylem in the plant. The virulence strategies of plant-pathogenic bacteria are either specialized to plant tissues or are broadly conserved among pathogens. Bacteria can attack the plant cell wall by extracting extracellular virulence factors such as cell wall degrading enzymes and cell wall-permeable toxins. Bacteria enter plant tissues either by wounds or through natural openings such as stomata, hydathodes or lenticels. The fungal pathogens such as *Botrytis cinerea* grow enzyme-secreting appressoria using inner turgor to breach the plant cuticula, penetrate the surface and host cell wall. Both bacterial and fungal phytopathogens will try to defeat the physical protecting barriers of plants by secreting cuticle and cell-wall degrading enzymes; lipase will degrade plant waxes, cutinase will degrade cuticle and cellulase, pectinase and xylanase are hydrolyzing the plant cell wall. The physiological barriers of plants stop many plant pathogens, furthermore plants can produce a variety of chemicals e.g. saponins that are glycosylated

triterpenoids on surfaces of many plant species and function especially against fungal pathogens (Abramovitch et al., 2006, Fu and Dong, 2013, Kan van, 2006).

Plant-pathogen bacteria have virulence strategies that are either specialized to plant tissues or broadly conserved among both plant and animal pathogens. The virulence can be denoted as increases in the growth rate or final population size and enhanced disease symptoms. Bacteria multiply in the apoplast, and there are several distinct protein secretion pathways to deliver effector proteins through the plasma membrane into intracellular compartments. Probably the most effective virulence strategy is type III secretion system (T3SS), which consists of more than 20 proteins. TT3S is a bacterial membrane-spanning protein complex with a pilus which functions like a syringe to inject the bacterial proteins called effectors into the host cell cytoplasm. After being injected into the plant, the effectors modulate the plant physiology to benefit the pathogen; the effectors function to manipulate host cell processes to shut down critical processes required for pathogen infection. The effector proteins promote pathogenicity, enhance infection and pathogen proliferation. The T3SS is used by many bacteria of different lifestyles e.g. biotrophic, soft-rotting bacterial pathogens and some symbiotic bacteria (Abramovitch et al., 2006, Hofius et al., 2007, Knepper and Day, 2010).

For example, *Pseudomonas syringae* pv. tomato uses TT3S to deliver directly into the host cell over 30 effector proteins with diverse enzymatic activities e.g. such as cysteine protease, E3 ubiquitin ligase and protein phosphatase activity. *P. syringae* is a gram-negative pathogenic bacterium that causes bacterial speck disease in tomato. In the natural environment *P. syringae* uses standard mechanisms of dispersal e.g. rain, insects, animals etc. in order to establish itself on the plant surface as an epiphyte and gaining entry into the intercellular space (Knepper and Day, 2010).

The other relevant secretion system is the type II secretion system (T2SS) that is used by many microbes with a soft-rotting lifestyle and is especially characteristic to *Pectobacterium*, e.g. *Pectobacterium carotovorum* causing blackleg. The soft-rot causes rotting and macerating phenotypes in plants, which is caused by several cell wall degrading enzymes e.g. pectinases, endoglucanases and cellulases exported by T2SS. The type IV secretion system (T4SS) is used by *Agrobacterium tumefaciens* (causing Crown gall) and mediates trafficking of bacterial proteins and DNA into the plant cell (Abramovitch et al., 2006, Knepper and Day, 2010).

Botrytis cinerea, commonly called grey mould, is a wide host-spectrum fungal phytopathogen and a good model organism for studying nectrotrophs. Moist conditions are necessary for *Botrytis* infection since they help adhesion of conidia on plant surface. In wounded plants, there is a strong resistance to *B. cinerea*, which is explained by the broken cuticle barrier inducing ROS and resistance in the plants. However, this effect can be abolished by ABA. Under normal circumstances the cuticle prevents access of any elicitors and no response is induced (Kan van, 2006, L'Haridon et al., 2011).

1.5.5 The role of plant hormones in defence

Plants do not have specialized immune cells and thus balancing growth and defence is critical for survival of the plants, which is achieved through crosstalk between different phytohormones and defence responses (Fu and Dong, 2013). The antagonism between JA

and SA allows plants to prioritize the defence between biotrophic or necrotrophic pathogens and insects. SA antagonism of JA signalling is observed both when plants are infected with different pathogens and when plants are directly treated with hormones. In general, the defence against biotrophic pathogens is SA-dependent and JA-mediated signalling functions in defence against necrotrophic pathogens. An infection by a biotrophic pathogen will lead to activation of SA-mediated defence and repression of JA-mediated pathway both at the gene expression level and plant immunity. However, the interaction between JA and SA is concentration-dependent, and application of low concentrations of SA and JA resulted in synergistic expression of both the SA target gene *PR1* and JA marker genes e.g. *PDF1.2* (*PLANT DEFENSIN 1.2*), but in higher concentrations of SA and JA have antagonistic effects on expression of these genes. The co-transcriptional regulator NPR1 is regulated by the cellular redox balance and post-translational modifications including S-nitrosylation and phosphorylation (Withers and Dong, 2016). Moreover, the phytohormone ABA promotes proteasome-mediated degradation of NPR1 and the phytohormone ABA and SA antagonistically influence cellular NPR1 protein levels (Ding et al., 2016). However, also a positive interaction between SA and JA has been described by Liu et al. (2016) in which JA was found to function as a positive regulator of RPS2-mediated ETI and production of both JA and SA followed after infection with *P. syringae* pv maculicola ES4326/avrRpt2 (Fu and Dong, 2013, Liu et al., 2016, Overmyer et al., 2018, Withers and Dong, 2016).

Bacteria use several small molecules e.g. toxins, plant hormones, auto inducers and exopolysaccharides (EPS) to promote disease by interfering or sometimes killing plant cells. Many pathogens have evolved mechanims to promote virulence of which the bacterial toxin coronatine (COR) is a prime example. Coronatine is well-known to be produced by several strains of *Pseudomonas syringae* and functions as a mimic of bioactive jasmonic acid conjugated with isoleucine (JA-Ile) and has been shown to bind to the Ja-Ile coreceptors COI1-JAZ with high affinity. Thus, coronatine targets the JA-receptor COR insensitive 1 (COI1). COR stimulates JA-signalling, and thus also suppresses SA-dependent defence, prevents stomatal closure to allow bacterial entry through stomata and causes chlorotic symptoms in infected plants. Coronatine also stimulates stomata to re-open and promotes bacterial growth both locally and systemically. MeJa treatment inhibits ABA induced stomatal closure indicating antagonistic signalling in defence responses. Also many strains of *Pseudomonas* produce the phytohormone auxin and it has been shown that plants gain enhanced disease resistance by down-regulating auxin levels in response to pathogen infection (Abramovitch et al., 2006, Fu and Dong, 2013, Geng et al., 2014, Kim et al., 2010). In general, phytohormones regulate many functions in plants, and also the hormones that have historically been considered to be important in development and growth like auxin, have in later research been shown to also affect immunity and other functions in plants.

2. Materials and methods

Method	Publication
DNA/RNA extraction and purification	1, 2, 3
Hormonal assays	1, 2, 3
Infection of Arabidopsis with *P. syringae*	2, 3
Infection of Arabidopsis with *P. carotovorum*	1, 2
Infection of Arabidopsis with *B. cinerea*	1, 2
Ion leakage (ozone/ xanthine/xanthine oxidase)	2, 3
Methyl viologen assay	2
Mutant screen	1, 2
Ozone treatment	2, 3
PCR	1, 2, 3
Quantitative RT-PCR	2, 3
Stomatal aperture measurement	2, 3
Stomatal conductance measurements (porometer)	2, 3
Stomatal conductance measurements (a gas-exchange device in Uni. Tartu)	2, 3
Water-loss measurement	2, 3

Organism	Publication
Arabidopsis thaliana accession Col-0	1, 2, 3
Botrytis cinerea B05.10	1, 2
Pseudomonas syringae pv. tomato DC3000	2, 3
Pectobacterium carovotorum subsp. *carotovorum* SCC1	1, 2

Plant line	Reference	Source of the seeds	Publication
Col-0		Nottingham Arabidopsis Stock Centre (NASC)	1, 2, 3
afb4-1	GK_068E01	NASC	1
max2-1	G→A substitution which causes Asp → Asn, Stirnberg et al., 2002.	NASC	2, 3
max2-4	SALK_028336	NASC	2, 3
max3-9	EMS mutation: a deletion of 16 nucleotides in exon 2, replaced by 42 nucleotides of unknown origin. Leads to a predicted protein of 255 amino acids plus one new amino acid compared to wild-type. Booker et al., 2004.	NASC	3
max3-11	SALK_023975	NASC	3
max4-1	transposon insertion, Sorefan et al., 2003.	NASC	3
max4-7	SALK_082552	NASC	3
d14-seto5	C→T transition, which causes a Pro→Leu substitution at position 169 of the encoded protein, Chevalier et al., 2014.	Pilar Cubas lab	3
d14-1	Wisconsin DsLox T-DNA insertion, CS913109, Waters et al., 2012.	NASC	3
max2-1 d14-seto5	Chevalier et al., 2014.	Pilar Cubas lab	3
aba2-11	González-Guzmán et al., 2002.	NASC	3
ghr1-3	GK_760C07, Sierla et al., 2018.	Jaakko Kangasjärvi	3
ost1-3	*srk2e*, SALK_008068, Yoshida et al., 2002.	NASC	3

3. Aims of the study

The aim of the present study was to find out about the phytohormone-related crosstalk in pathogen and stomatal responses in *Arabidopsis thaliana.* The large overlap in pathogen and ROS-induced signalling is known to involve many phytohormones such as salicylic acid, jasmonic acid, abscisic acid and ethylene and furthermore, is linked to development and growth via auxin and strigolactones. Especially we wanted to examine two phytohormones affecting development, growth and stress responses in Arabidopsis; auxin and strigolactone.

The specific aims of this study are:

Characterization of the F-box protein AFB4 in plant abiotic/biotic stress tolerance.
Characterization of the F-box protein MAX2 in susceptibility to pathogens and responses to apoplastic ROS.
Characterizing the role of the plant hormone strigolactone in stomatal regulation and susceptibility to pathogens in *Arabidopsis thaliana.*

4. Results and Discussion

4.1 F-box protein mutant screen (Publications I and II)

We conducted a reverse genetic screen on 60 T-DNA insertion lines representing putative F-box knockouts to identify novel F-box genes involved in plant responses to environmental stresses. The F-box protein superfamily is one of the largest known in plants and consists of nearly 700 identified potential F-box genes in Arabidopsis. The lines were selected based on the classification by Gagne et al. 2002 in which the F-box proteins were divided into five families (A-E) and then further into subfamilies. The F-box protein families C1, C2, C3 and C4 contain not only many known phytohormone-signalling proteins such as TIR1, COI1 and EBF1 and EBF2 (EIN3 BINDING F-BOX1 and 2) but also a lot of proteins with unknown function. We screened the selected lines for their sensitivity to ozone, pathogens (*P. carotovorum*, *P. syringae* and *B. cinerea*), cold and several phytohormones (ABA, SA and MeJa). The mutants with clearly altered phenotype(s) were chosen for further analysis; e.g. *afb4-1* was found in the pathogen screens and *max2* was found in the ozone screen.

4.2 F-box protein AFB4 plays a crucial role in plant growth, development and innate immunity (Publication I)

In the mutant screen, a plant line carrying a mutation in *AFB4* (At4g24390) showed altered growth phenotype and pathogen responses. This GABI-Kat insertion mutation, *afb4-1*, was characterized, and the T-DNA position and homozygosity of the mutant were confirmed using gene specific primers for sequencing (Figure 2A). Also RT-PCR analysis was used to confirm that there were no AFB4 transcripts in the homozygous *afb4-1* line (Figure 2B).

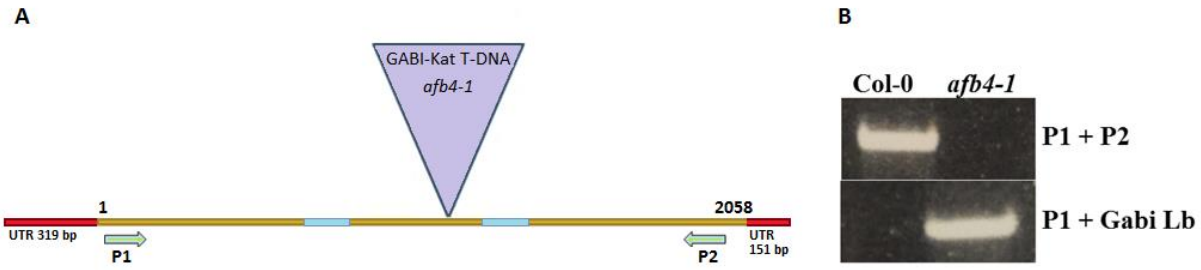

Figure 2. Characterization of the T-DNA knockout line, *afb4-1*. A) The triangle-pointed location of the T-DNA insertion in *ABF4*. Numbers represent genomic sequence, and the yellow color in the sequence represents exons and the blue introns. B) Characterization of the T-DNA insert by the gene specific primers (P1 and P2) in combination with the T-DNA left-border primer.

The loss of function in *afb4-1* results in impaired growth phenotype compared to Col-0; *afb4-1* has a small rosette with small distorted leaves and short petioles (Figure 3), which is typical to auxin response mutants (Parry et al., 2009). In order to determine the role of *afb4-1* in disease resistance, we determined the disease resistance to both to necrotrophic bacterial pathogen (*P. carotovorum* subsp. *carotovorum* SCC1) and fungal pathogen (*Botrytis cinerea*). In response to infection, *afb4-1* showed enhanced resistance to *B. cinerea*; after five days of infection *afb4-1* plants had developed HR symptoms around the infection area, whereas Col-0 plants showed high susceptibility (Figure 4A). Moreover, enhanced resistance to *P. carotovorum* was visible both as macerated area 48 h post inoculation, and also as growth in planta calculated as cfu/cm^2 leaf tissue in several time points (Figure 4B). On the other hand, infection with *P. syringae* did not show statistically significant difference in resistance between *afb4-1* and Col-0 (result not shown). Furthermore, Prigge et al. (2016) got contradictory results showing that e.g. rosette growth phenotype and susceptibility to bacterial pathogens of another *AFB4* allele, *afb4-8*, are similar to wild-type. The allele *afb4-1* was found to be rather unstable and other factors might contribute to the behaviour of this line (Prigge et al., 2016). However, differences in growth conditions between different laboratories can also lead to different mutant phenotypes. This has for example been observed in the defense and cell death mutant *dnd1* (Clough et al., 2000).

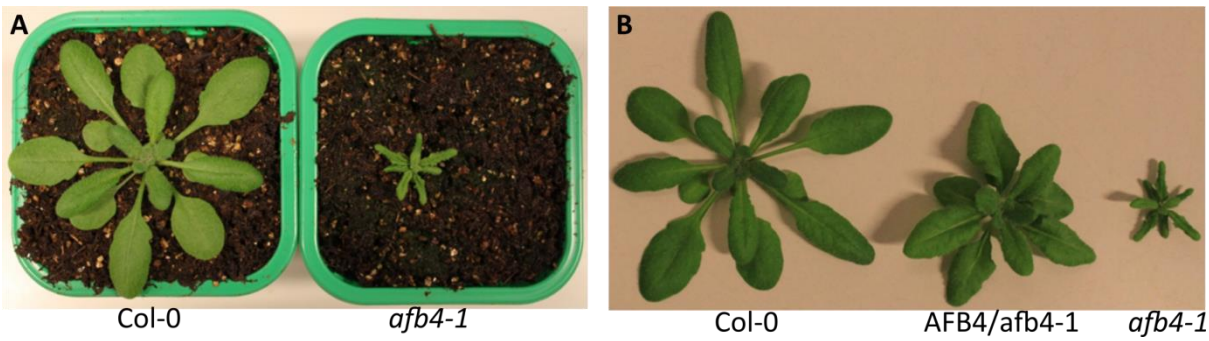

Figure 3. Growth phenotype of soil grown three weeks old wild-type Col-0, *afb4-1* (A) and heterozygous AFB/afb4-1 plants (B).

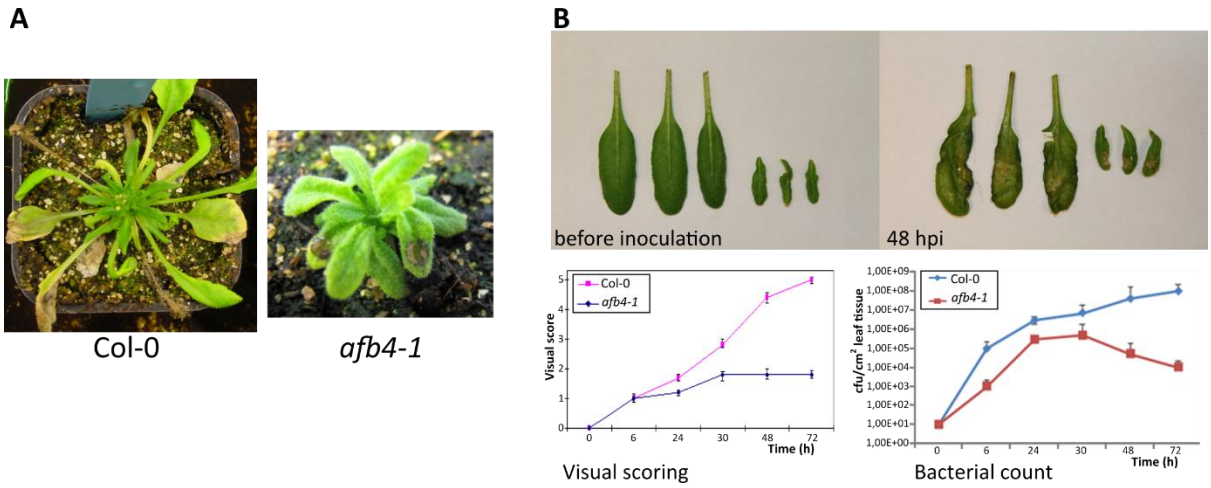

Figure 4. Pathogen response of *afb4-1*. Plants (Col-0 and *afb4-1*) were inoculated with *B. cinerea* and *P. carotovorum,* and disease development was evaluated. Plants were photographed 5 days post inoculation with *B. cinerea* (A) and 48 h post inoculation (1×10^6 cfu/ml, inoculated by pipetting) with *P. carotovorum* (B). Upon *P. carotovorum* infection, amount of bacteria in planta was calculated at indicated time points. Disease development was also measured by scoring visually (0: no disease symptoms 5: whole plants macerated).

4.3 The F-box protein MAX2 contributes to resistance to bacterial phytopathogens in *Arabidopsis thaliana* (Publication II)

4.3.1 F-box protein MAX2 is required for ozone tolerance and provides tolerance to apoplastic $O_2^{\bullet-}$

In the mutant screen another mutant with a distinguishable growth phenotype i.e. bushy rosette and moreover, high sensitivity to ozone was identified. This mutant, *max2-4*, harboured T-DNA insertion in the *MAX2* gene. In order to confirm the role of *max2* in ozone sensitivity, we included another *max2* mutant line, *max2-1,* carrying a point mutation in *MAX2* (At2g42620) gene (Figure 5).

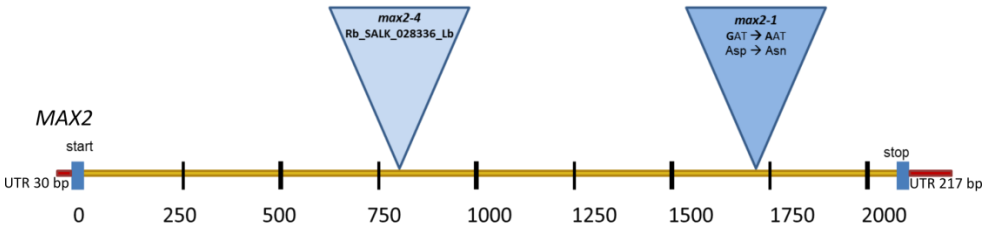

Figure 5. Position of *max2-1* and *max2-4*. *MAX2* gene consists of only exons.

Both *max2* mutants showed increased sensitivity to ozone compared to wild-type plants, which was analysed both visually and as ion leakage in several time points from the beginning of ozone exposure. Like many other environmental stresses, ozone is known to trigger superoxide production in the apoplastic space of plant cells, which causes formation of visible lesions in sensitive plants and also increased ion leakage due to the cell damage. After 6 hours of exposure to 300 ppb of ozone the damage was clear in the *max2* mutant

plants that had developed visible lesions (Figure 6A) and their ion leakage (Figure 6B) was increased, whereas wild-type plants showed no damage. As a positive control we included *rcd1-4* (*radical-induced cell death*) plants that are well-characterized for sensitivity to ozone and apoplastic ROS (Overmyer et al., 2000).

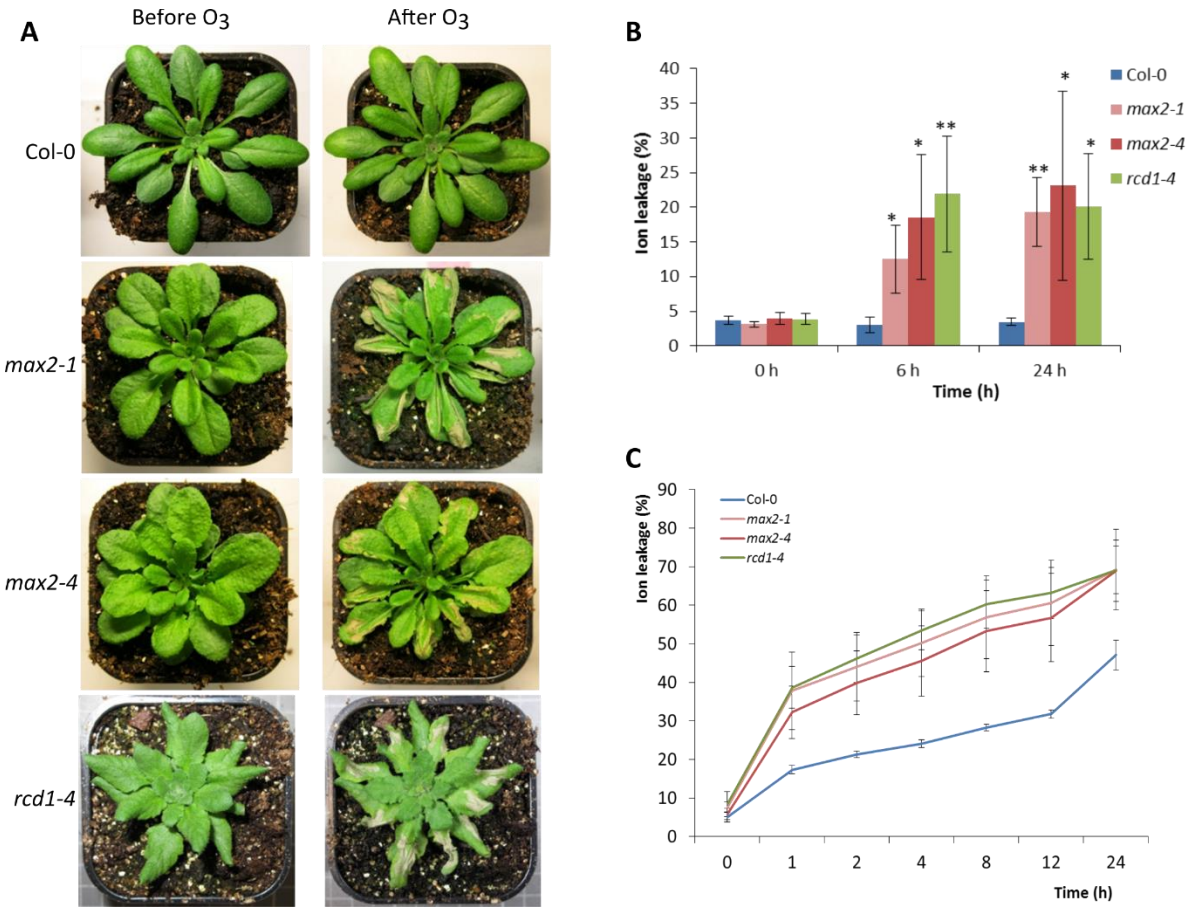

Figure 6. *max2* plants are sensitive to apoplastic ROS which was measured both as visible lesions (A) and ion leakage (B) after ozone exposure and furthermore, as increased ion leakage in response to superoxide and H_2O_2 production induced by xanthine/xanthine oxidase system (C). These figures are also presented in Publ. II as Fig. 1 and Fig 2. Methodological and statistical information: B) The result is presented as ratio of ion leakage of total ion concentration. Data represents the means ± SE of 3 independent experiments with 5 plants/line in every time point in each experiment **P < 0.01; two-tailed t test. C) Cell death was measured as relative ion leakage for 24 h. Data are means ± SE from 3 independent experiments with >20 leaves/line in each experiment. The result is presented as ratio of ion leakage of total ion concentration.

To further investigate the role of ROS in ozone sensitivity of *max2* plants, we employed extracellular ROS generating system using xanthine (X)/xanthine oxidase (XO), in which XO reduces oxygen to generate superoxide and H_2O_2 (Sawa et al., 2000). The leaves of Col-0 and *max2* plants were infiltrated with X/XO and cell death was measured as a relative ion leakage for 24 h. Already during the first hour, the increase in ion leakage was 25 % in *max2* and 15 % Col-0, but even more distinct during the following 12 hours (Figure 6C). To further characterize if MAX2 also has a role in ROS responses inside the cell, we analysed the sensitivity to methyl viologen, which causes ROS production inside the chloroplasts. However, no difference between *max2* and wild-type plants was observed (Publ. II; Figure

S1). The expression of oxidative stress marker genes was upregulated in *max2*; *GRX480* was upregulated both in response to ozone exposure and *P. syringae* infection and *GST1* was upregulated in response to *P. syringae* infection (Publ. II; Figure 7A, 7B and 7C). Thus, it can be concluded that MAX2 contributes specifically to apoplastic ROS tolerance. In ozone exposure the role of stomata is essential, while X/XO infiltration is independent of stomata and as a conclusion it seems that MAX2 influences plant sensitivity to ROS beyond the stomatal level in Arabidopsis.

4.3.2 MAX2 influences stomatal properties in Arabidopsis

Ozone and pathogens enter the plant via natural openings as stomata and thus, stomata have a central role in sensitivity to apoplastic ROS. Porometer measurements were used to show that *max2* plants had a higher stomatal conductance than wild-type plants (Publ. II; Figure 3A). Furthermore, the *max2* mutants had increased fresh weight loss in excised *max2* leaves (figure 7A), and increased ABA hormone levels (Figure 7B) after the leaves were cut and left to dry.

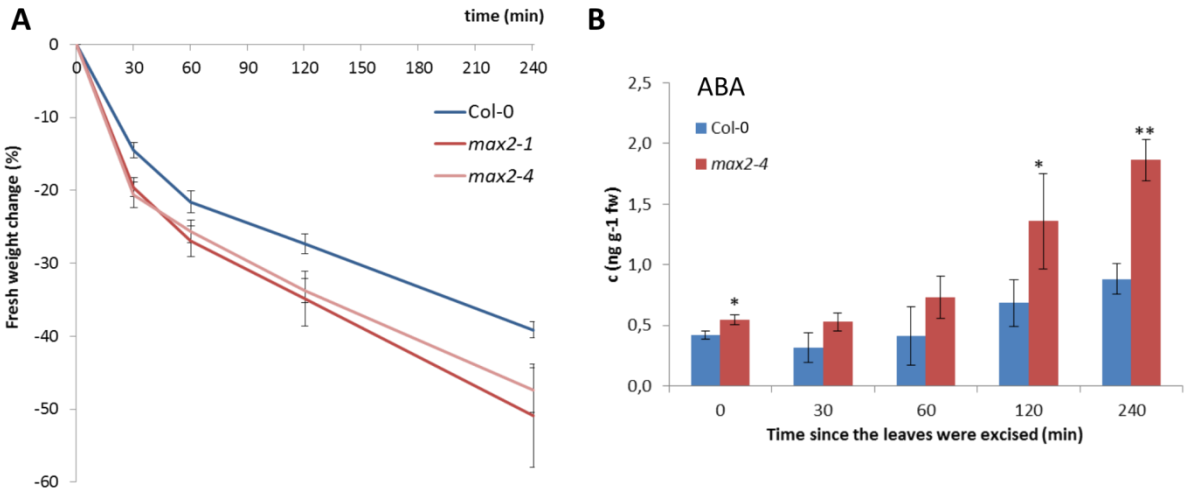

Figure 7. *max2* plants have enhanced response to increased water loss measured as a fresh weight change and concentration of ABA after the leaves were cut and left to dry. These figures are also presented in Publ. II as Fig. 3B and Fig 9A. Methodological and statistical information: A) For each line 5 plants were used in each experiment and the results are shown as means ± SE. Experiments were repeated 5 times with similar results. B) The values are means ± SE. The experiment was repeated twice with 3 biological repeats in each experiment. Asterisks indicate significant differences, as determined by Student's t-test (*P < 0.05; **P < 0.01; two-tailed t test).

The enhanced stomatal conductance of *max2* mutants was further confirmed by measuring stomatal conductance using a custom-made device using whole rosettes. The basal level of stomatal conductance is approximately two times higher in *max2* mutants than in Col-0 plants (Figure 8A). The stomatal phenotype of *max2* is also supported by similar results from Bu et al., 2014 and Ha et al., 2014.

A rapid stomatal closure in response to application of 350 ppb ozone gas was wild-type like in *max2* mutants. The slight recovery of stomatal conductance was observed in Col-0 plants but not in *max2* plants (Figure 8A) due to the ozone-induced cell death in *max2* mutants,

which is also recognized as the quick decrease in general photosynthesis (CO_2 uptake, figure 8B). Even if the ozone-induced stomatal closure was as rapid in *max2* mutants as in Col-0, the intake of ozone was still higher in *max2* mutants (Figure 8C). Also the stomatal O_3 uptake rate was higher in *max2* mutants than Col-0 (Figure 8D). These are explained probably by higher stomatal conductance at the beginning of ozone exposure and more open stomata.

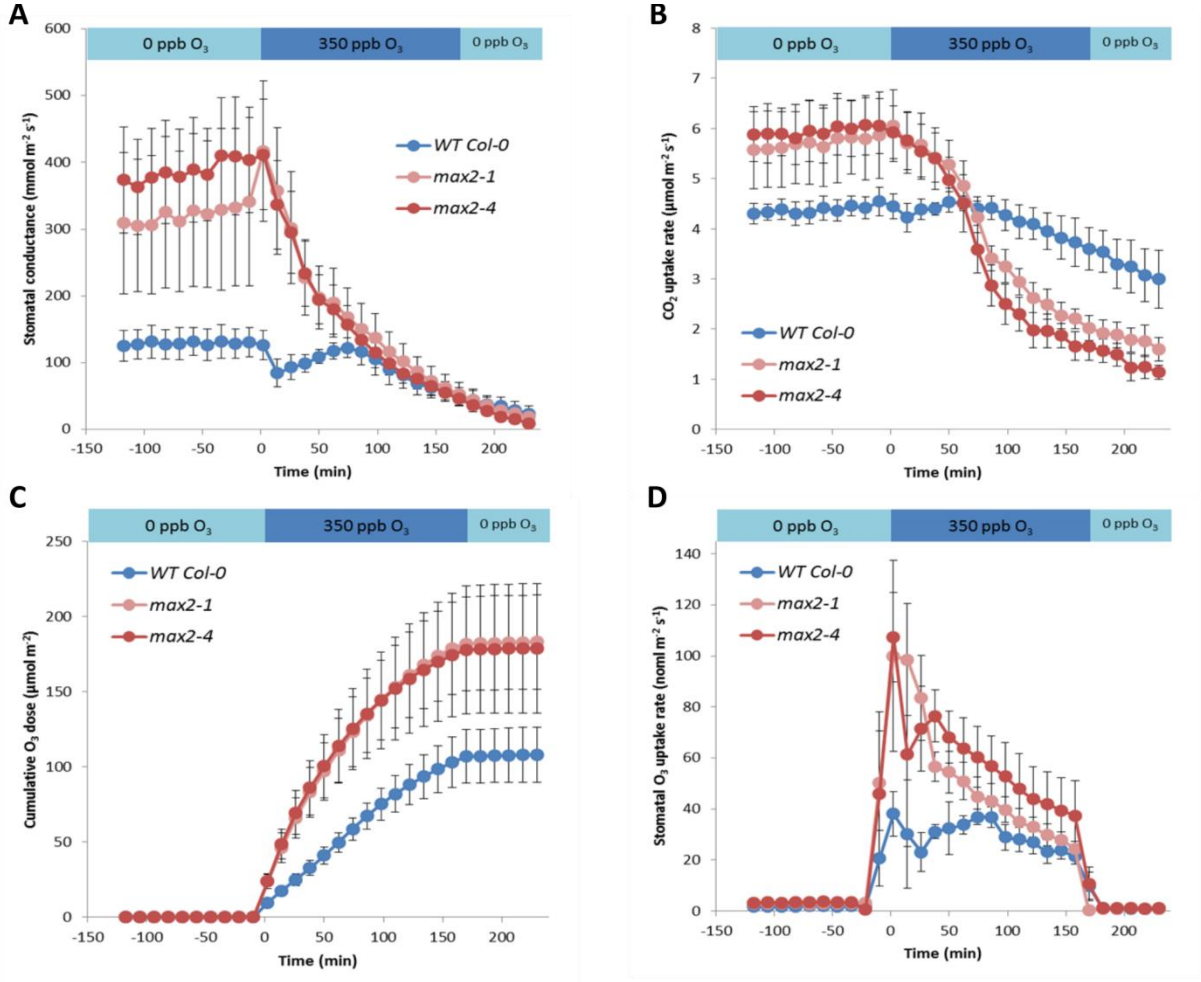

Figure 8. MAX2 has normal stomatal closure in response to ozone. The response of stomatal conductance to ozone gas (350 nmol/mol for 3 hours) was measured with a custom made whole-rosette gas exchange measurement device. A) Stomatal conductance before, during and after 3 h O_3 exposure in Col-0 and *max2* plants. B) CO_2 uptake rate of *max2* mutants and Col-0 before, during and after 3 h O_3 exposure. C) Cumulative dose of O_3 absorbed by *max2* and Col-0 plants before, during and after 3 h O_3 exposure. D) Stomatal O_3 uptake rate of *max2* and Col-0. These figures are also presented in Publ. II as Fig. 4. Methodological and statistical information: For each line 4 plants were used in the experiment and the results are shown as means ± SE. Experiments were repeated twice with similar results.

4.3.3 MAX2 contributes to resistance to bacterial pathogens

The F-box protein MAX2 had not previously been characterized for sensitivity to biotic stress. The *max2* mutant lines were analysed and they were more susceptible to spray inoculation with bacterial hemibiotroph *P. syringae* pv. tomato DC3000, which was evaluated both as a visual phenotype and by calculating the bacteria.

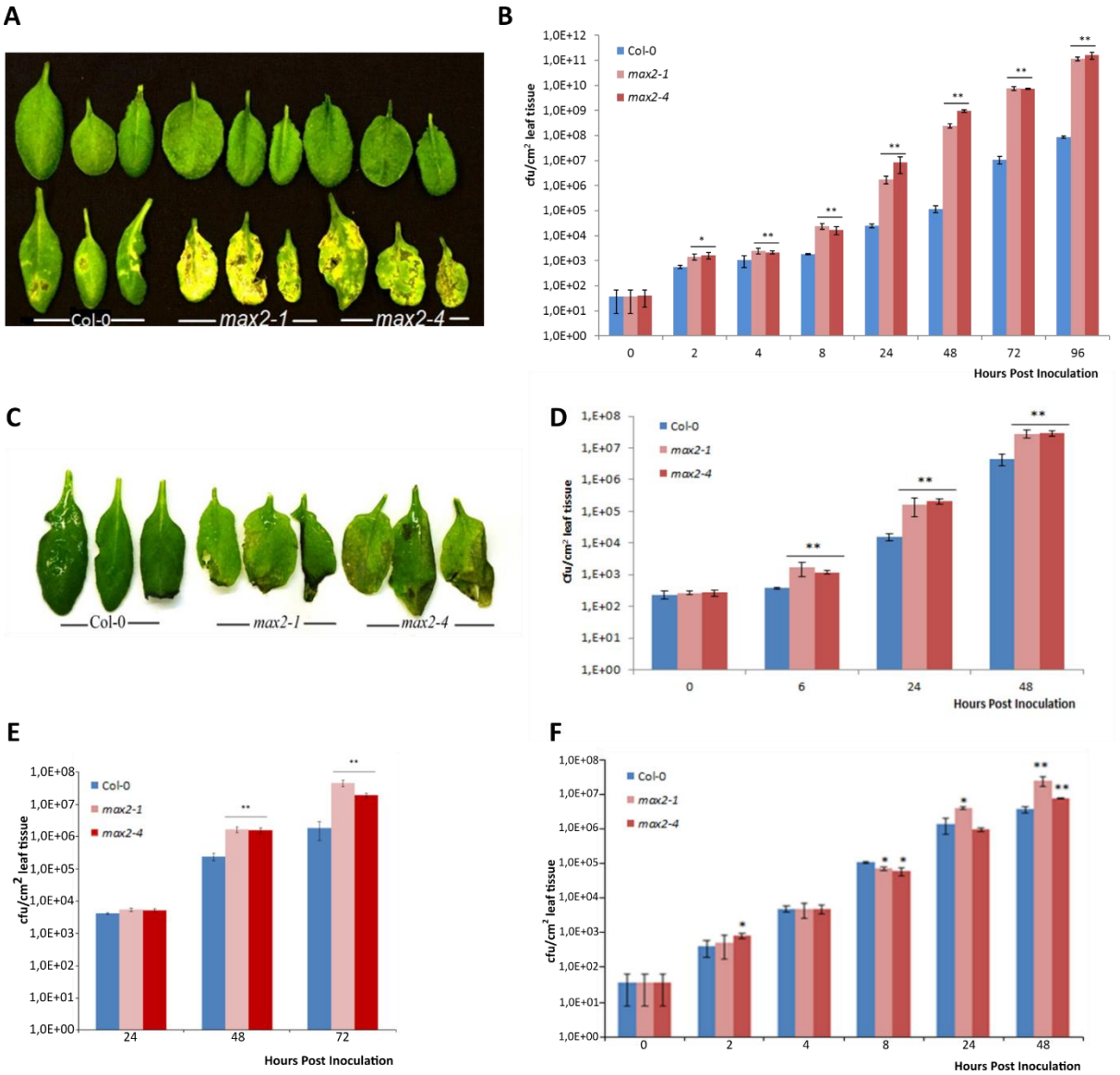

Figure 9. *max2* has decreased resistance to *P. syringae* and *P. carotovorum*. A) Leaves of Col-0 and *max2* before infection (upper row) and 5 days post spray inoculation (lower row) with *P. syringae* ($1x10^7$ cfu/ml). B) Growth of *P. syringae* in planta was calculated at 0, 2, 4, 8, 24, 48, 72 and 96 h post spray inoculation C) Col-0 and *max2* leaves 2 days post spray inoculation with *P. carotovorum* ($1x10^6$ cfu/ml). D) Growth of *P. carotovorum* in planta 0, 6, 24 and 48 h post spray inoculation. E) Infiltration inoculation with *P. syringae* (10 µl, 10^6cfu/ml was used for infiltration) and bacteria was calculated at indicated time points. F) Inoculation by pipetting *P. carotovorum* on leaves; a small wound was made on a plant leaf and 10 µl of bacterial solution ($1x10^5$ cfu/ml) applied by pipetting followed by bacterial calculation at indicated time points. These figures are also presented in Publ. II as Fig. 5 and Fig S3.

Methodological and statistical information: In the experiments A-D soil-grown 4-week old plants were used; in each experiment, 3 plants/line and 3 leaves/plant were used to check the phenotype and to measure the bacterial concentration. All the experiments were repeated at least 4 times with similar results. The results are shown as means ± SE. (*P < 0.05; **P < 0.01; two-tailed t test). E) At the indicated times, 0.5 cm^2 leaf disc at the site of infection were harvested and the number of viable bacteria in each disc was determined. The results are shown as means ± SE (**P < 0.01; two-tailed t test). F) Asterisks indicate significant differences, as determined by Student's t test (*P < 0.05; ** P < 0.01; two-tailed t test).

The coronatine produced by *P. syringae* causes the heavy yellowing in the infected leaves, which is recognisably stronger in susceptible plants. Moreover, the susceptibility to a bacterial necrotroph *P. carotovorum* SCC1 was also analysed by spray infection and the result was evaluated both as a visual phenotype and calculating the bacteria. As a nectrotrophic bacteria, *P. carotovorum* causes masceration in infected plants. The high susceptibility of *max2* plants to both *P. syringae* and *P. carotovorum* was clear from visual phenotype as extensive yellowness (Figure 9A) and tissue maceration (Figure 9C) respectively, and also from increased growth of the bacteria *in planta* (9B and 9D). The effect of infection method was assessed by also doing an infiltration with *P. syringae* (9E), and pipetting of *P. carotovorum* on a leaf wounded slightly with a pipette tip (9F). By both infection methods a statistically significant increase in susceptibility was observed in *max2,* even though the difference was smaller than by spray inoculation method. This suggests that the stomatal phenotype of *max2* has a central impact on the outcome of the infection but importantly, *max2* affects the susceptibility to bacterial pathogens also independent of stomata.

Pathogen triggered stomatal closure was analysed with a method by Chitrakar and Melotto (2010) in which the stomata are dyed with propidium iodide and bacterial solution is pipetted onto the leaf. In *max2* plants stomatal closure was impaired in response to *P. syringae* infection (Figure 10A). This clearly indicates that the observed susceptibility to *P. syringae* is not only due to more open stomata at the basal level but also inability to close the stomata normally in order to restrict the pathogen infection.

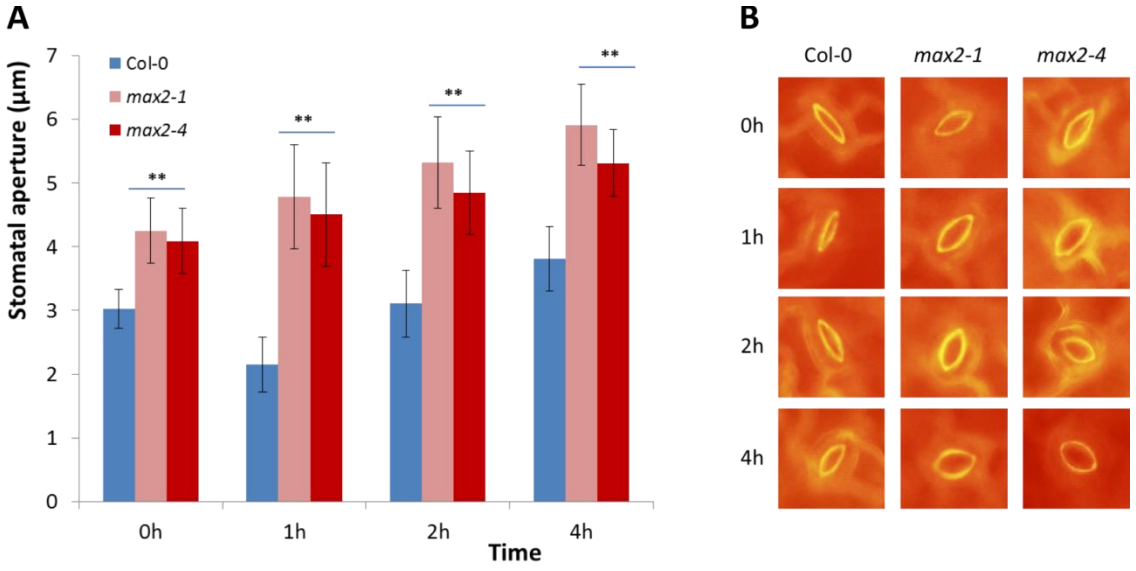

Figure 10. *max2* has impaired pathogen-triggered stomatal closure in response to *P. syringae* pv. tomato DC3000. A) Stomatal aperture of Col-0 and *max2* in response to *P.*

syringae. Leaves were first stained with 20 µM propidium iodide (PI) solution and then inoculated with 300 µl of bacterial solution (10^8 cfu/ml). B) Representative pictures of stomatal response of Col-0 and *max2* lines under fluorescent microscope using 20x objective 0, 1, 2 and 4 h post inoculation with the bacteria. These figures are also presented in Publ. II as Fig. 6. Methodological and statistical information: Four-week old wild-type Col-0 and *max2* were used in the analysis. Results are shown as the mean (n = 80-100) ± SE. **P < 0.01; two-tailed t test. The experiments were repeated three times with similar results.

4.4. The role of the plant hormone strigolactone in stomatal conductance and susceptibility to bacterial pathogens in *Arabidopsis thaliana* (Publication III)

Since the previous results (publication II) suggested a larger role for strigolactone in stomatal regulation and susceptibility to pathogens in Arabidopsis, I wished to find additional evidence for the role of strigolactone in plant defence response by including both biosynthesis (MAX3, MAX4) and the receptor (D14) mutants in the research (Figure 11). Moreover, *max2* was crossed to well known mutants with defective guard cell signalling to assess the possible interaction between ABA and strigolactone signalling.

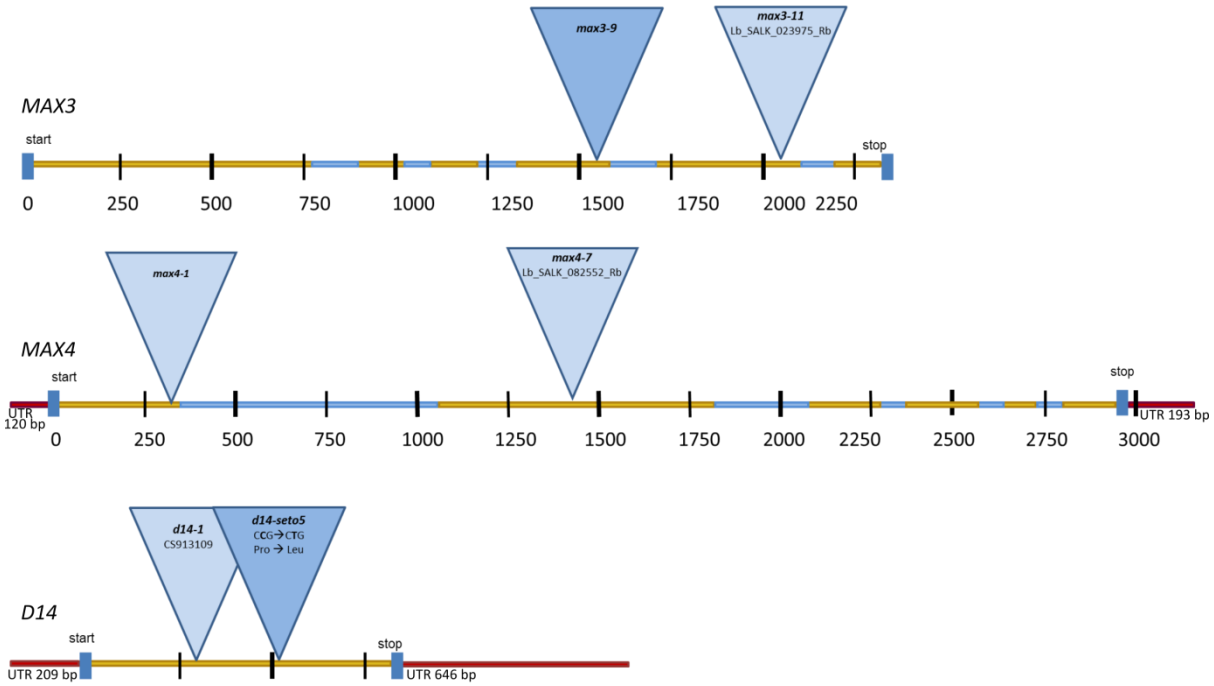

Figure 11. Position of *max3* (At2G44990) mutants (*max3-9* and *max3-11), max4* (At4g32810) mutants (*max4-1* and *max4-7)* and *d14* (AT3G03990) mutants (*d14-1* and *d14-seto5).*

4.4.1 Strigolactone affects sensitivity to pathogens in Arabidopsis but only strigolactone perception contributes to the ozone sensitivity

Both the strigolactones signalling and synthesis mutants were more sensitive to spray infection with *P. syringae* DC3000 at a late timepoint 48 hpi (Figure 12). In addition, both *max2* mutants were more sensitive already at 1,5 hpi. Thus, it seems that strigolactone affects sensitivity to pathogens, and either stomatal or other stress-affecting properties of *max2* makes it susceptible already in early infection. The mechanism of pathogen sensitivity

remains unclear but as auxin is the target of strigolactone pathway, strigolactone could putatively alter sensitivity by affecting auxin signalling, and/or stomatal properties.

Despite the general higher pathogen susceptibility of strigolactone-related mutants, the phytohormone strigolactone seems not to be a major regulator in defence gene expression. The gene expression in response to *P. syringae* spray infection were analysed, and the changes in gene expression between the mutant and the wild type were subtle with a rather high variation between biological repeats (Publ. III; Figure 3b-d). Furthermore, as a technical point it should be noted that when repeating a pathogen experiment for a gene expression analysis, the timing of biological sampling might cause some variation in results. The infection spread speed varies each time even the conditions are carefully adjusted and the gene expression in response to pathogen-caused apoplastic ROS is time-sensitive. Thus, obtaining representative results would require multiple time-points with quite small intervals until minimum 48 hours.

Previous results from ozone exposure and X/XO experiments (publication II) implied that strigolactone might have a role in sensitivity to ozone and furthermore to apoplastic ROS. However, only strigolactone perception (*max2* plants) had increased ion leakage in response to ozone exposure (Publ. III; Figure 4). Since ozone enters the plants through stomata, also stomatal phenotype strongly affects the result. Therefore, X/XO infiltration was tried several times with *d14* plants resulting in slightly higher ion leakage than Col-0 (Figure 13), but due to the high variation between the repeated experiments, no conclusion could be drawn. However, it cannot be excluded that strigolactone might affect sensitivity to apoplastic ROS independent of stomata.

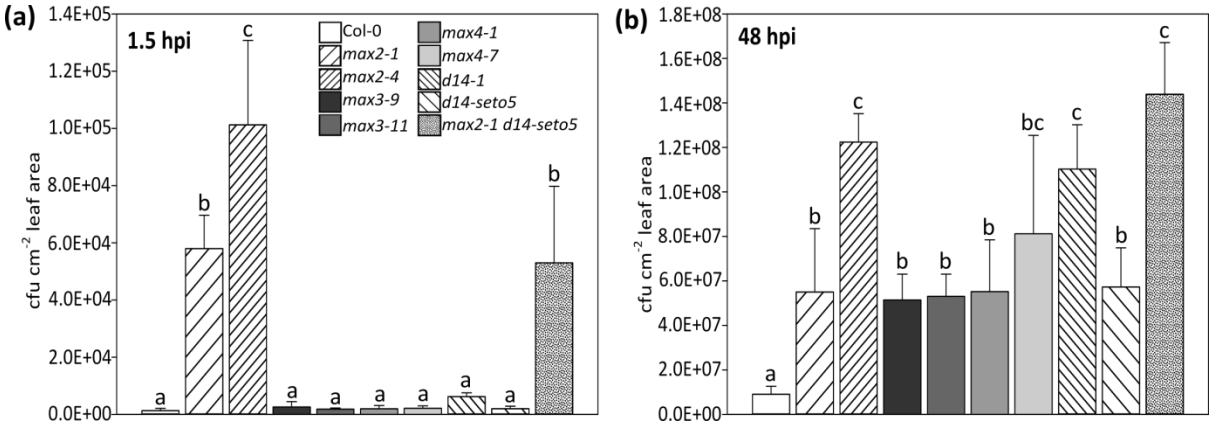

Figure 12. Strigolactone affects sensitivity to pathogens in Arabidopsis. The infection was done by spraying ($1x10^7$ cfu/ml) followed by calculation of bacteria at (a) 1,5 hours and (b) 48 hours post inoculation. In each experiment four plants/line and three leaves/plant were used to measure the bacterial concentration. These figures are also presented in Publ. III as Fig. 1. Methodological and statistical information: The experiment was repeated three times with similar results. The results are shown as means ± SE. In statistical analysis the data has a logarithmic transformation combined to the univariate analysis of variance and to Hochberg post hoc test.

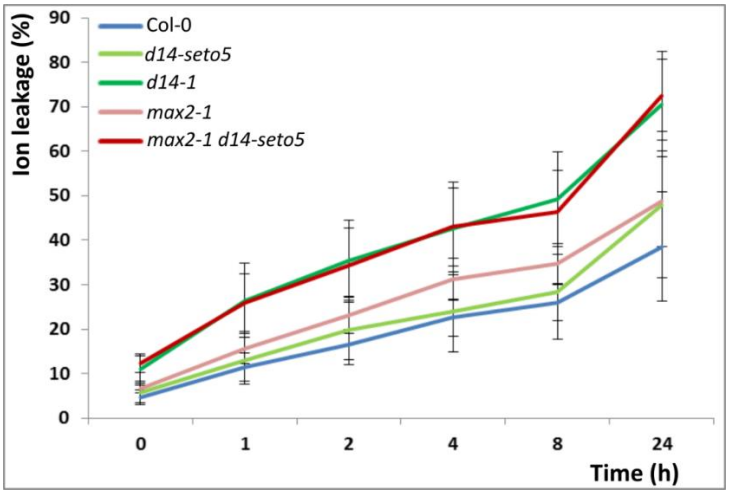

Figure 13. Ion leakage in strigolactone perception mutants in response to superoxide and H_2O_2 production induced by xanthine/xanthine oxidase system. The figure represents one experiment based on 5 plants/a plant line, and data are presented as means ± SE.

4.4.2 Strigolactone affects stomatal properties but strigolactone spray does not directly affect the stomatal aperture or gene expression

Stomatal conductance was analysed in all the strigolactone-related (signalling and synthesis) mutants, both from single leaves using a porometer, and from whole plants using a custom made gas-exchange device in University of Tartu. With a porometer stomatal conductance was measured from 5-6-week old plants from different developmental stages 1-2, 3-4 and 5 as defined by Boyes et al., 2001, which roughly corresponds to old, middle aged and young leaves. The measurement was done only from abaxial side of the leaves. In these porometer measurements both strigolactone perception and biosynthesis mutants had higher stomatal conductance at all leaf developmental stages (Publ. III; Figure 6). To complement the porometer data also the whole-plant stomatal conductance was measured, but with this method only *max2* mutants had clearly higher basal level of stomatal conductance (Figures 14a and 14b). This might be explained by methodological differences, since the porometer sensor head is clipped onto a single leaf which required touching the plant and moreover, the sensor head covers only a small area of the leaf when measuring. Thus, methodologically the porometer provides a possibility to measure a specific area independent from the rest of the plant. The microclimate might also affect the result since the strigolactone-related mutants are bushy and their leaves overlap and cover each other, which might explain the differences in these two methodologically different analysis.

The response of the mutants to different stimuli was measured using whole plants. All the measured stimuli (darkness, high CO_2 and ABA spray) induce stomatal closure in wild-type plants. However, in darkness both strigolactone biosynthesis (*max3*, *max4*) and perception (*max2*, *d14*) mutants had significantly slower rate of stomatal closure than Col-0 (Figures 14c and 14d) and also response to high CO_2 was impaired in *max2* and *d14* (Figures 14e and 14f). The response to ABA spray was not impaired, but instead stomatal closure rate was enhanced in *max2-1*, possibly due to more open stomata before ABA treatment (Figures 14g and 14h). The impaired responses to darkness and CO_2 suggest that strigolactone possibly affects guard cell signalling in Arabidopsis.

We further examined if strigolactone directly affects the stomatal aperture. Ha et al. (2014) rescued the drought phenotype of the strigolactone synthesis (*max3* and *max4*) mutants with strigolactone spray, which strongly implied that strigolactone would have a direct impact on the stomatal aperture. However, according to our results, strigolactone spray provides no differences in stomatal conductance nor in stomatal aperture (Publ. III; Figure 5). Effect of the strigolactone foliar spray on stomatal conductance was analysed by spraying Col-0 plants with GR24 dissolved in DMSO and the stock diluted in water with 0, 012% Silwet L-77. Before the spray the plants were pre-incubated in the measuring cuvettes, then sprayed with 10 µM of GR24, and after spraying stomatal conductance was monitored starting from 8 min after spraying for 56 minutes using the the gas-exchange system described by Kollist et al., 2007. However, we found no change in stomatal conductance in response to GR24 spray (Publ. III; Figure 5A). To confirm this result, we measured also the long-term effect of GR24 spray on stomatal apertures using the method by Chitrakar and Melotto (2010). One challenge in using GR24 is that for dissolving the GR24 chemical no standard method is available, and the solvent might possibly affect the results. Thus, we tried to dissolve GR24 both in DMSO and acetone, diluted the stock to 5 µM in water with 0,02 % Silwet L-77 and sprayed on Col-0 plants 24 hours before the measurements. The spray with either of the solvent did not result in differences in the stomatal aperture size (Publ. III; Figure 5B). Furthermore, different concentrations of GR24 spray and GR24 watering were tried to pretreat the plants both before stomatal conductance measurements and also before *P. syringae* spray infection. However, no difference between control plants and GR24-treated plants was observed in stomatal conductance or pathogen tolerance (results not shown). Interestingly, Lv et al., (2017) showed by using epidermal peels that the stomata close in response to GR24 and strigolactones were concluded to be common regulators in stomatal closure *in planta* (Zhang et al., 2018). We were not able to obtain the same result by using strigolactone spray on intact plants, which might be explained by the methodological difference.

Furthermore, the gene expression was also analysed in response to strigolactone spray but in general the fold changes in gene expression were rather small (Publ. III; Figure 3A). The available microarray data is consistent with our results, which supports that impact of strigolactone in gene expression is rather small both as the amount of affected genes and the magnitude i.e. fold change of altered gene expression (Mashiguchi et al., 2009).

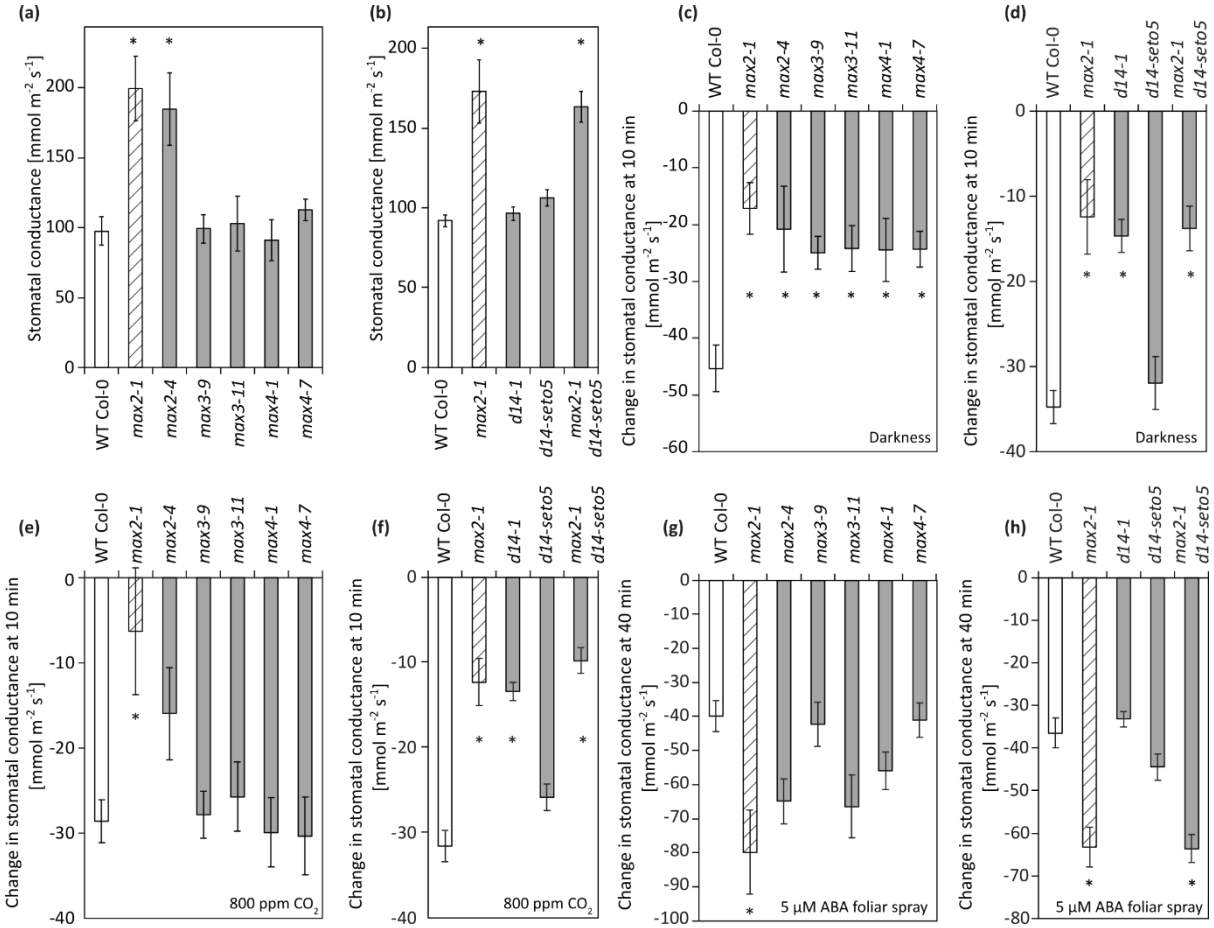

Figure 14. The basal level of stomatal conductance and responses to darkness, 800 ppm CO2 and ABA foliar spray (5 µM) were analysed in strigolactone perception (*max2 and d14)* and synthesis (*max3* and *max4*) mutants. (a) Stomatal conductance of *max2*, *max3* and *max4* mutants (n= 4-12). (b) Stomatal conductance of *max2* and *d14* mutants (n= 10-23). (c) Darkness-induced stomatal closure of *max2*, *max3* and *max4* mutants (10 min after induction; n = 7-11). (d) Darkness-induced stomatal closure of *max2* and *d14* mutants (10 min after induction; n = 10-20). (e) High CO_2-induced stomatal closure of *max2*, *max3* and *max4* mutants (10 min after induction; n = 6-12). (f) High CO_2-induced stomatal closure of *max2* and *d14* mutants (10 min after induction; n = 10-23). (g) ABA-induced stomatal closure of *max2*, *max3* and *max4* mutants (40 min after induction; n = 6-12). (h) ABA-induced stomatal closure of *max2* and *d14* mutants (40 min after induction; n = 13-23).

Darkness-induced stomatal closure is reduced in both the perception (*d14, max2*) and biosynthesis *(max3* and *max4)* mutants. High CO_2-induced stomatal closure is WT-like in *max3* and *max4* mutants but impaired in *max2* and *d14* mutants. Furthermore, ABA-induced stomatal closure is enhanced in *max2-1*, but WT-like in all the other measured mutants (*max2-4* and all *d14*, *max3* and *max4* mutants). These figures are also presented in Publ. III as Fig. 7. All graphs present mean ± SEM. Asterisks denote statistically significant differences according to one-way ANOVA with Tukey HSD post hoc test.

4.4.3 MAX2 regulates stomatal function independent of ABA signalling

Since there were many implications that strigolactones regulate stomatal properties, even though the mechanim is unknown, we wished to further study the relationship between

strigolactones and ABA signalling. Thus, to examine if ABA signalling and *MAX2* share the same elements in guard cell signalling, the *max2* mutant was crossed with the guard cell signalling mutants (*ost1*, *ghr1)* and the ABA biosynthesis mutant *aba2.* All the crosses (*max2 ghr1*, *max2 ost1* and *max2 aba2*) had significantly higher stomatal conductance than the corresponding single mutants. Thus, it appears that MAX2 functions on a signalling pathway that is parallel to ABA signalling pathway. Combining this with the results of the impaired CO_2 and darkness response in *max2,* the results strongly implicate the MAX2 F-Box protein has a crucial role in guard cell regulation, putatively by targeting some guard cell signalling-related protein for degradation.

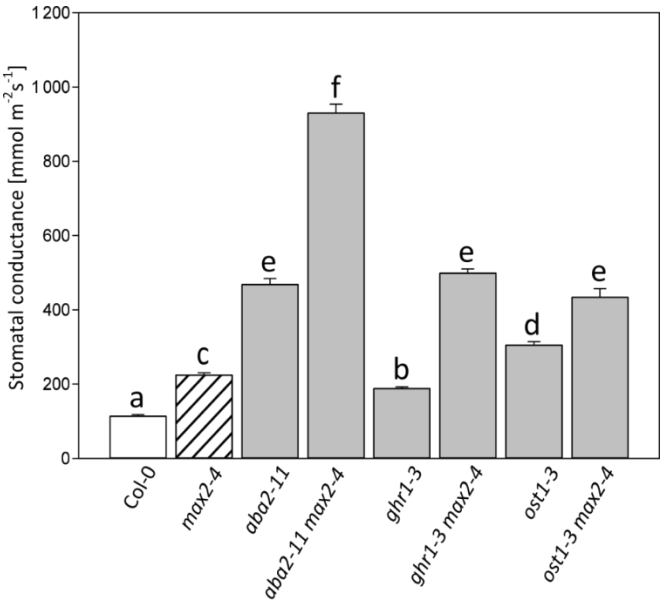

Figure 15. The basal level of stomatal conductance of the *max2* crossed with *aba2-11*, *ghr1-3*, *ost1-3* measured with gas-exchange device using intact plants. This figure is also presented in Publ. III as Fig. 8. Data is presented as the mean ± SEM. In statistical analysis we conducted a logarithmic transformation on the data, then univariate analysis of variance combined to Tukey HSD post hoc test.

5. Conclusion and future prospects

This thesis combines effects of two phytohormones, auxin and strigolactones, on plant stress tolerance via their effect on stomata, oxidative stress and other phytohormones. Auxin is classically known for affecting plant growth and development but it has much larger role by indirectly affecting stress and defence responses via other phytohormones. Auxin signalling is also connected to the phytohormone strigolactone since auxin is the target of the strigolactone pathway - the bushy "more axillary branching" phenotype is caused by the dampened auxin transport in the strigolactone-related mutants.

Strigolactones are a rather newly discovered class of phytohormones and new research results broaden their role continuously. Recently strigolactones were discovered to affect stress and pathogen responses in plants. In strigolactone perception MAX2 functions together with the strigolactone receptor D14 and the other MAX proteins (MAX1, 3 and 4) function in strigolactone synthesis. Interestingly, compounds derived from burnt vegetation, karrikins, were found to induce similar effects in germination and seedling as SLs, and furthermore, both of their signalling requires MAX2, and they both affect drought tolerance in Arabidopsis.

The F-box protein MAX2 was found to have an exceptional role compared to the other proteins involved in strigolactone pathway. All the other strigolactone-related proteins have a role in susceptibility to pathogens but unlike the other strigolactone-related mutants, *max2* mutants were already sensitive in very early time point of the infection and were found to be more sensitive to pathogens also independent of stomata (pipetting and infiltration of bacteria). Moreover, compared to the other strigolactone-related mutants, only *max2* had higher stomatal conductance and *max2* was found to contribute to oxidative stress sensitivity. Interestingly, MAX2 was concluded to act on a parallel signal pathway to the ABA signalling pathway – most of the knowledge of guard cell signalling is based on ABA, which suggests an independent role for strigolactone in guard cell signalling. Putatively, the differential response of *max2* mutants might be explained by being linked both to strigolactone and karrikin signalling or MAX2 could target some guard cell signalling-related protein for degradation. In the future research, it would be intriguing to find out the mechanism that strigolactone affects pathogen susceptibility - putatively via affecting auxin signalling and stomatal properties or via other mechanism, for example by modulating oxidative stress tolerance. Thus, a pathogen screen with both stomata-dependent and independent infection methods for the *max2* double mutants (crossed with guard cell affecting mutants), could give useful insights into the role of guard cell signalling putatively connected to strigolactone in the infection. Moreover, protein interaction studies using MAX2 from isolated guard cells might further provide information of the putative novel role for MAX2 in guard cell signalling.

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