Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Ragnhild Sødal Gjennestad

A study of PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6) in regulation of plant immunity

Master's thesis in Cell and Molecular Biology
Supervisor: Atle M. Bones
Co-supervisor: Javad Najafi
May 2021

NTNU
Norwegian University of
Science and Technology

Ragnhild Sødal Gjennestad

A study of PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6) in regulation of plant immunity

Master's thesis in Cell and Molecular Biology
Supervisor: Atle M. Bones
Co-supervisor: Javad Najafi
May 2021

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

NTNU
Kunnskap for en bedre verden

Abstract

As sessile organisms, plants have evolved defence mechanisms against a variety of pathogens. The plant immune system consists of constitutively expressed outer barriers and inducible defence mechanisms. One of the inducible defence mechanisms include recognition of pathogen- and damage-associated molecular patterns (PAMPs/DAMPs) by pattern-recognition receptors (PRRs). Perception of such a signal lead to pattern-triggered immunity (PTI) that normally includes a calcium influx, production of reactive oxygen species (ROS), stomatal enclosure, callose deposition, production of antimicrobial compounds and phytohormones as well as transcriptional reprogramming to inhibit growth of the pathogen. Transcriptional reprogramming include genes for small secreted post-translationally modified peptides that works as signalling agents in cell-to-cell communication. This project aims to contribute to the investigation of PIPL6 in regulation of plant immunity and the molecular mechanisms behind it. Expression of *PIPL6* was induced upon receptor recognition of plant elicitors, possibly by activated WRKY transcription factors. Treatment with synthetic PIPL6 peptide induced transcriptional reprogramming of many immune related genes that are involved in induction and repression of jasmonic acid, genes involved in production of callose and camalexin and ethylene (ET) biosynthesis. The perception of PIPL6 downstream signalling is likely to be mediated by the receptors RLK7 and/or SRR1. Transcriptional reprogramming probably involve transcription and activation of WRKY and other transcription factors. Both Knock-out and overexpression of *PIPL6* possibly led to higher susceptibility of the hemi-biotrophic pathogen *Pseudomonas syringae.* This might be a result of disruption of the carefully regulated fine-tuned response. Knock-out of *PIPL6* lead to higher susceptibility to the necrotrophic pathogen *Botrytis cinerea* compared to wild-type as well reduced or absent induction of camalexin and jasmonic acid. *PIPL6* overexpression lines showed enhanced growth inhibition as well as an increased ROS production in response to flg22 comparted to wild-type. *PIPL6* overexpression lines might be more resistant to *B. cinerea*, possibly by a heightened immune response. Altogether, the above mentioned results are a strong indicator that PIPL6 may play an important role in plant immunity. Increased knowledge about this peptide as well other plant signalling peptides could be important for future applications to enhance disease resistance in plants.

Sammendrag

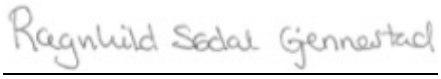
Planter har utviklet et robust forsvarssystem mot en rekke organismer som kan forårsake sykdom hos planten. Planteimmunforsvaret består av både induserbare og generelt uttrykte forsvarsmekanismer. En av de induserbare mekanismene iverksettes når patogen- og skadeassosierte molekylære strukturer (PAMPs/DAMPs) gjenkjennes av sine respektive reseptorer (PRRs). Gjenkjenning av et slikt signal fører til en PAMP-indusert immunitet kalt PTI. PTI inkluderer vanligvis en rekke forandringer intracellulært og ekstracellulært som innstrømming av kalsiumioner, produksjon av reaktive oksygen arter (ROS), lukking av stomataåpninger, kalloseavsetning ved infeksjonsstedet, produksjon av antimikrobielle molekyler og plantehormoner og en omregulering av genuttrykk. Alle disse mekanismene bidrar til å hemme vekst av de patogene mikroorganismene som iverksatte immunresponsen. Omregulering av genuttrykk inkluderer gener som koder for små peptider som produseres og skilles ut i rommet utenfor cellemembranen. Disse peptidene kan fungere som signalmolekyler for nabocellene. Dette masterprosjektet har som mål å bidra til å undersøke rollen peptidet PIPL6 har i forsvarsmekanismen til planter og de molekylære mekanismene bak dette. Behandling av spirer med diverse PAMPs førte til førte til en rask og kortvarig økt transkripsjon av *PIPL6*. Denne økningen skyldes trolig aktivering av blant annet WRKY transkripsjonsfaktorer. Syntetisk PIPL6 peptid ble påført på spirer og denne behandlingen førte til økt genuttrykk av mange immunrelaterte gener. Blant de induserte genene var gener involvert i aktivering og hemming av plantehormonet jasmoninsyre (JA), gener involvert i produksjon av kallose og syntese av etylen (ET) og det antimikrobielle molekylet camalexin. PIPL6 bruker muligens RLK7 og/eller SRR1 som reseptor(er) for videre signalisering. En slik signalisering inkluderer sannsynligvis intracellulær aktivering og transkripsjon av WRKY og andre transkripsjonsfaktorer. «Knock-out» og overuttrykk av *PIPL6* fører muligens til bedre vekst av den biotopiske bakterien *Pseudomonas syringae*. Dette kan være et resultat av forstyrrelse av en finjustert reguleringsmekanisme av plantens immunforsvar mot denne spesifikke mikroorganismen. «Knock-out» av *PIPL6* førte også til lavere resistens mot infeksjon av den nekrotrofe soppen *Botrytis cinerea*, samt redusert eller fraværende indusert camalexin og JA i respons til infeksjonen. Overuttrykk av *PIPL6* viste seg å føre til økt vekstinhibering og større ROS produksjon i respons til flg22 sammenliknet med villtype. Dette tyder på en forhøyet immunrespons og at overuttrykk av *PIPL6* muligens kan gi mer motstandsdyktighet mot *B. cinerea.* Resultatene fra dette masterprosjektet tyder på at PIPL6 kan spille en viktig rolle i immunforsvaret til planter. Økt kunnskap om PIPL6 og andre signalpeptider kan muligens brukes i fremtidige formål for å øke sykdomsresistens hos planter.

Preface

This Master´s project was kindly provided by professor Atle M. Bones and Dr. Javad Najafi at the Cell, Molecular Biology and Genomics group (CMBG) at The Department of Biology at The Norwegian University of Science and Technology (NTNU). I would like to give a warm thank you to my supervisor Professor Atle M. Bones and my co-supervisor Dr. Javad Najafi for all help with academic work as well as motivational and inspirational enthusiasm throughout the last two years. I would also like to thank all the members of the CMBG group for their help and support through long days in the lab. A special mention to senior engineer Torfinn Sparstad and associate professor Per Winge for their help with calculations and data processing and to Dr. Javad Najafi and staff engineer Zdenka Bartosova for performing hormonal analysis. Also thank you to Dr. Javad Najafi for providing me with previous cDNAs for flagellin timeseries experiment and WRKY transcription factor knock-outs and for always being available for advice and support. This project would not have been possible without you.

Working with this project have further encouraged me to pursue an academic career. I look forward to the future and to contribute to NTNU´s vision "Knowledge for a better world".

Signed May 2021, Trondheim:



Ragnhild Sødal Gjennestad

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABA	ABSCISIC ACID
ACS	AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE
ANOVA	ANALYSIS OF VARIANCE
ASR3	ARABIDOPSIS SH4-RELATED 3
Avr factors	AVIRULENCE FACTORS
BAK1	BRI1-ASSOCIATED RECEPTOR KINASE 1
BES1	BRI1-EMS-SUPRESSOR 1
BIK1	BOTRYTIS-INDUCED KINASE 1
CaM	CALMODULIN
CDKC	CYCLIN-DEPENDENT KINASE
cDNA	COMPLIMENTARY DNA
CDPK	CALCIUM-DEPENDENT PROTEIN KINASE
CERK1	CHITIN ELICITOR RECEPTOR KINASE 1
CMBG	CELL, MOLECULAR BIOLOGY AND GENOMICS GROUP
Ct	CYCLE THRESHOLD
CYP	CYTOCHROME P
DAMP	DAMAGE-ASSOCIATED MOLECULAR PATTERN
EDS1	ENHANCED DISEASE SUSCEPTIBILITY 1
ERF	ETHYLENE RESPONSIVE TRANSCRIPTION FACTOR
ET	ETHYLENE
ETI	EFFECTOR-TRIGGERED IMMUNITY
ETR gene	ETHYLENE RESPONSE GENE
Flg22	FLAGELLIN22
FLS2	FLAGELLIN-SENSITIVE 2
FRK1	FLG22-INDUCED RECEPTOR-LIKE KINASE 1
GST1	GLUTATHIONE S-TRANSFERASE 1
HAESA	RECEPTOR-LIKE KINASE 5
HR	HYPERSENSITIVE RESPONSE
HRP	HORSERADISH PEROXIDASE
ICS1	ISOCHORISMATE SYNTHASE 1
IDA	INFLORESCENCE DEFICIENT IN ABSCISSION
IDL	IDA-LIKE
IGS	INDOLIC GLUCOSINOLATES
JA	JASMONIC ACID
JAZ	JASMONATE-ZIM-DOMAIN PROTEINS
LRR	LEUCINE-RICH REPEAT
LYK	LYSM RECEPTOR-LIKE KINASE
LysM	LYSIN MOTIFS
MAMP	MICROBE-ASSOCIATED MOLECULAR PATTERN
MAPK	MITOGEN-ACTIVATED PROTEIN KINASE
MEKK	MAP KINASE KINASE KINASE

MKK	MAP KINASE KINASE
MPK	MAP KINASE
MQ	AUTOCLAVED DOUBLE DISTILLED WATER
MYB51	MYB DOMAIN PROTEIN 51
MYC2	MYC2 TRANSCRIPTION FACTOR
NB-LRR	NUCLEOTIDE BINDING PROTEINS WITH LEUCINE-RICH REPEAT DOMAINS
NO	NITRIC OXIDE
NOS	NITRIC OXIDE SYNTHASE
NPR	NONEXPRESSER OF RP GENES
NTNU	NORWEGIAN UNIVERSITY OF SCIENCE AND TECHNOLOGY
OD_{600}	OPTICAL DENSITY AT 600NM
ORF	OPEN READING FRAME
OX	OVEREXPRESSION
PAD	PHYTOALEXIN DEFICIENT
PAL	PHENYLALANINE AMMONIA LYASE
PAMP	PATHOGEN-ASSOCIATED MOLECULAR PATTERN
PBL	AVRPPHB SUSCEPTIBLE 1 LIKE
PCR	POLYMERASE CHAIN REACTION
PDF	PLANT DEFENSIN
PEP	PLANT ELICITOR PEPTIDE
PEPR	PEP RECEPTOR
PIP	PAMP-INDUCED SECRETED PEPTIDE
PIPL	PIP-LIKE
PR	PATHOGENESIS-RELATED GENES/PROTEINS
PROPEP	ELICITOR PEPTIDE PRECURSOR
PRR	PATTERN-RECOGNITION RECEPTOR
PTI	PATTERN- OR PATHOGEN-TRIGGERED IMMUNITY
qPCR	QUANTITATIVE PCR
RBOHD	RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D
RLK	RECEPTOR-LIKE KINASE
RLU	RELATIVE LIGHT UNIT
ROS	REACTIVE OXYGEN SPECIES
Rpm	REVOLUTIONS PER MINUTE
R proteins	RESISTANCE PROTEINS
RRTF1	REDOX RESPONSIVE TRANSCRIPTION FACTOR 1
RT	REVERSE TRANSCRIPTASE
SA	SALICYLIC ACID
SAR	SYSTEMIC ACQUIRED RESISTANCE
SIGnAL	SALK INSTITUTE GENOMIC ANALYSIS LABORATORY
SRR1	SUGAR RESPONSIVE RLK 1
TAIR	THE ARABIDOPSIS INFORMATION RESOURCE
TAT3	TYROSINE AMINO TRANSFERASE 3
T-DNA	TRANSGENIC DNA

TIP41	INTERACTING PROTEIN OF 41 KDA
VQP	VQ MOTIF-CONTAINING PROTEIN
WRKYs	WRKY TRANSCRIPTION FACTORS
Wt	WILD-TYPE
ZAT12	ZINC FINGER PROTEIN 12
1/2MS	HALF-STRENGTH MURASHIGE-SKOOG

1 Introduction

1.1 Plant immunity; a brief overview

As sessile organisms, plants have evolved sophisticated defence mechanisms against a variety of microbial pathogens and herbivores. Some defence mechanisms are constitutively active, while other parts of the defence system are induced upon pathogen attack. Constitutive expression of a defence system is an energy demanding process and have negative impacts on normal plant growth and development. In order to bypass this issue, plants have gained rapid inducible general and specific responses to pathogen attack. There are two branches of the plant immune system. The first active defence system rely on transmembrane pattern-recognition receptors (PRRs) that trigger a general immune response against pathogens, while the second uses intracellular receptor proteins. The intracellular receptors are so called plant resistance (R) proteins that function as a species specific defence response through recognition of effectors produced by some pathogens species (Jones and Dangl 2006). PRRs recognise microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) and trigger a general defence response called pattern- or pathogen-triggered immunity (PTI). PTI can include Ca^{2+} influx, production of reactive oxygen species (ROS), production of ethylene (ET) and salicylic acid (SA), transcription of immune related genes, callose deposition, camalexin production and stomatal enclosure (Nicaise et al. 2009).

Many phytopathogens have developed effector proteins that inhibit critical steps of PTI or other cellular functions. Such inhibition results in repression of defence related pathways and consequently increase the disease susceptibility towards the pathogen (Jones and Dangl 2006). As a result of coevolution, some plant species have evolved plant R proteins to protect the PTI pathways from the effect of the effector proteins. This phenomenon is called the gene-for-gene hypothesis, where susceptible plant species have evolved R genes that recognise strain specific effector proteins (Nürnberger et al. 2004). R proteins are mostly NB-LRRs that consist of poly-morphic nucleotide-binding (NB) proteins with leucine-rich repeat (LRR) domains. Effectors recognised by R proteins are called avirulence (Avr) factors. An infection with an avirulent pathogen will trigger effector-triggered immunity (ETI). ETI is an enhanced form of the PTI response and result in a type of programmed cell death called the hypersensitive response (HR). It is thought that PTI is a preparation for further defence by boosting the responsiveness to other microbial patterns (Jones and Dangl 2006; Wu et al. 2014). This elicitation of the PTI immune response include production of ROS, biosynthesis and accumulation of SA, HR and increased expression of pathogenesis related (PR) genes (Wu et al. 2014). According to the zigzag model explained by Jones and Dangl (2006), the PTI defence response is not sufficient to induce HR without the amplification of the immune response by ETI. ETI and HR also result in a type of immunity against future pathogens called systemic acquired resistance (SAR). SAR is an enduring, heightened state of resistance against secondary attack by phytopathogens. SAR is generated by SA biosynthesis at the infected site and transport through the phloem to uninfected parts of the plant. Next, SA lead to transcriptional reprogramming and possibly immune memory in the uninfected tissue by chromosomal changes (epigenetics) that prime target genes for enhanced transcription (Spoel and Dong 2012).

1.2 PAMP-triggered immunity – the first line of inducible defence

Upon pathogen attack, the phytopathogen has to pass through the outer barriers to access nutrients from the host plant. The outer barriers are constitutively expressed and are composed of wax layers, a rigid cell wall, anti-microbial enzymes and secondary metabolites. If a pathogen overcome the outer barriers, they are perceived by receptor proteins localized at the plasma membrane of the cell (Nürnberger et al. 2004). Microbes enter the interior of the cell through stomatal openings or wounds in the plant tissue. At the plasma membrane, the pathogen is sensed by PRRs that recognize PAMPs/MAMPs. Upon binding of PAMPs to PRRs, PTI is induced. PAMPs acts as elicitors and are usually a highly conserved and functionally important part of the microbe and these structures are normally not produced by the host itself. Common PAMPs are for example flagellin and chitin (Chisholm et al. 2006; Nürnberger et al. 2004).

Recognition of PAMPs by their corresponding PRRs activate a mitogen-activated protein kinase (MAPK) cascade followed by phosphorylation of downstream target proteins including WRKY transcription factors. WRKY transcription factors binds to and facilitate transcription of a wide range of immune related genes that further activate and transcribe more genes to enhance the immune response (Li et al. 2016). Some of the responsive genes induce synthesis and production of the SA phytohormone that in turn leads to expression of genes used for SA synthesis and PATHOGENESIS RELATED (PR) proteins (Glazebrook 2005). WRKY transcription factors also transcribe genes involved in biosynthesis of ET and the antimicrobial compound camalexin (Mao et al. 2011; Li et al. 2016; Devendrakumar et al. 2018). Recognition of PAMPs by PRRs and activation of MAPK cascade also phosphorylate proteins that activate the respiratory burst oxidase homolog protein D (RBOHD) that start to produce ROS at the outside of the plasma membrane (Li et al. 2016). ROS auto-propagates as a wave, rapidly traveling through the apoplast of neighbouring cells and activate a systemic response (Zandalinas et al. 2019). Plants also have elicitor responsive Ca^{2+} permeable ion channels that lead to a rapid influx of Ca^{2+} in the presence of an elicitor. Ca^{2+} give a conformational change to calmodulin (CaM) that leads to expression of immune related genes that induce SA biosynthesis, and genes involved synthesis of cell wall components resulting in callose deposition at the site of infection (Lecourieux et al. 2006; Li et al. 2016). CaM also activate nitric oxide synthase (NOS) that produce nitric oxide (NO) required for stomatal enclosure (Guo et al. 2003). The increase of Ca^{2+} also activate calcium-dependent protein kinases (CDPKs) that activate RBOHD to increase the ROS production (Tsuda and Somssich 2015). ROS can inhibit pathogen growth by creating a oxidative stress environment in the apoplast, ROS also cause oxidative cross-linkage of glycoproteins in the cell wall, strengthening it to inhibit pathogen entry to the cytoplasm (Ghozlan et al. 2020; Torres et al. 2006).

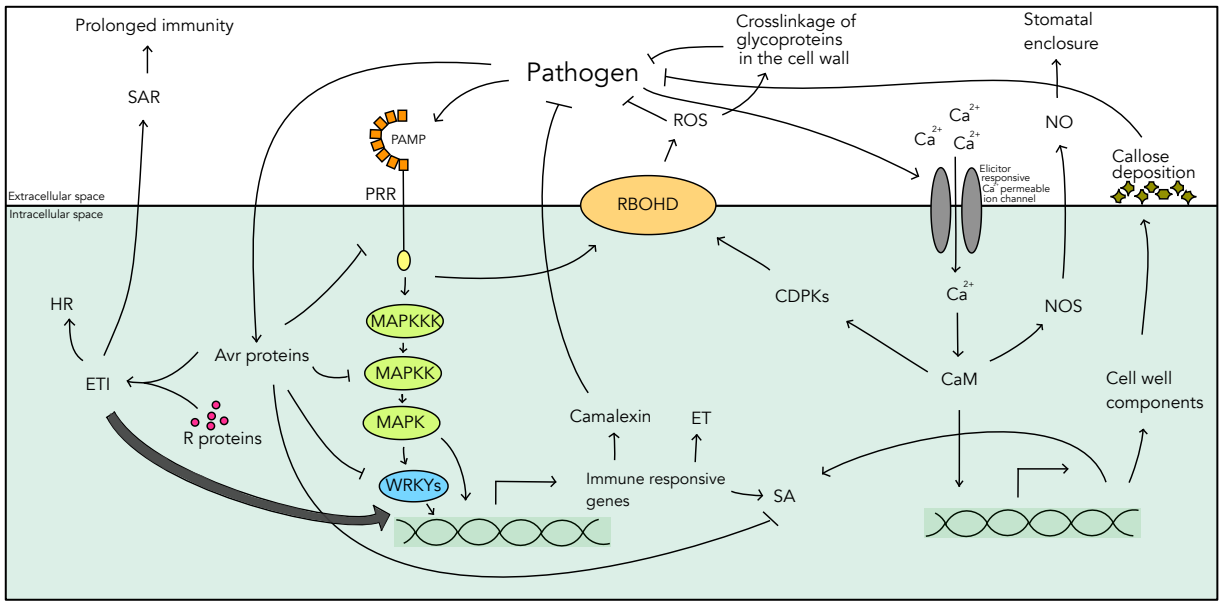


Figure 1: A schematic illustration of the inducible plant immune responses towards pathogens.
Pathogens can be recognized as pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) on the host cell and induce pathogen-triggered immunity (PTI). Recognition of a pathogen lead to several defence mechanisms including transcriptional reprogramming, burst of reactive oxygen species (ROS), calcium influx, callose deposition, stomatal enclosure, production of salicylic acid (SA), ethylene (ET) and camalexin. Some pathogens also produce avirulence (Avr) proteins that are recognised by resistance (R) proteins inducing effector triggered immunity (ETI) in the host cell. ETI is an enhanced PTI response that lead to a programmed cell death called the hypersensitive response (HR) in the infected tissue and systemic acquired resistance (SAR) in the uninfected parts of the plant. Further details about the pathways are outlined in the text. Abbreviations are as followed; MAP kinase (MAPK), MAP kinase kinase (MAPKK), MAP kinase kinase kinase (MAPKKK), WRKY transcription factors (WRKYs), calmodulin (CaM), respiratory burst oxidase homolog protein D (RBOHD), calcium-dependent protein kinases (CDPKs), nitric oxide (NO) and NO synthase (NOS).

PTI induced factors and pathways as well as ETI is illustrated in **Figure 1**. Plant immunity is a highly complex molecular regulatory system which consist of many overlapping functions, pathways and feedback loops. For the sake of simplicity, not all interactions between different signalling pathways and components are indicated in the figure.

1.2.1 Flagellin induced PTI

Flagellin is a subunit of the bacterial flagellum, a whip-like appendage that facilitate bacterial motility. A twenty-two amino acid peptide, flagellin22 (flg22), from the highly conserved terminal of flagellin subunits is sufficient to activate its receptor FLAGELLIN-SENSITIVE 2 (FLS2) and induce PTI. FLS2 is a membrane bound receptor-like kinase (RLK) with an extracellular LRR domain and an intracellular serine/threonine kinase domain. When FLS2 recognize flg22, it forms a heterodimer with its coreceptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1). Heterodimerization leads to phosphorylation and dissociation of the constitutively associated proteins BOTRYTIS-INDUCED KINASE 1 (BIK1) and AVRPPHB SUSCEPTIBLE 1 LIKE (PBL) 1 (PBL1) and the activation of two separate MAPK cascades. BIK1 phosphorylate and activate the plasma membrane bound RBOHD that produce ROS at the outside of the plasma membrane (Li et al. 2016; Chinchilla et al. 2007; Spoel and Dong 2012; Chisholm et al. 2006). Release

of BIK1 also phosphorylate and activate a calcium channel leading to calcium influx (Tian et al. 2019).

One MAPK cascade include phosphorylation and activation of the MAPKKK 1 (MEKK1) followed by MAPKK 4 and 5 (MKK4 and MKK5) followed by MAPK 3 and 6 (MPK3 and MPK6) that phosphorylate CYCLIN-DEPENDENT KINASEs (CDKCs), WRKY33, ETHYLENE RESPONSIVE TRANSCRIPTION FACTOR (ERF) 6 and 104 (ERF6 and ERF104) and BRI1-EMS-SUPRESSOR 1 (BES1) (Li et al. 2016; Asai et al. 2002). The second MAPK cascade include phosphorylation of MEKK1 and activation of MKK1 and MKK2 that activate MPK4 that further phosphorylate and activate ARABIDOPSIS SH4-RELATED 3 (ASR3) and release of WRKY33 (Li et al. 2016; Qiu et al. 2008). ASR3 is a negative regulator of PTI and works to downregulate the signalling response. WRKY transcription factors regulate a variety of genes related to plant immunity. MPK3 and MPK6 are shown to directly phosphorylate and activate WRKY33, WRKY22 and WRKY29. It is also suggested that the MAPKs phosphorylate an inhibitor of WRKY proteins which release them upon phosphorylation. A good candidate for this is the VQ motif-containing proteins (VQPs) (Asai et al. 2002; Li et al. 2016). WRKY proteins have at least one DNA-binding WRKY-domain. The WRKY-domain binds to the W-box element (5´-(C/T)TGAC(T/C)-3´) in the promoter region of target genes. Many of the WRKY proteins also have W-box elements in their promoter regions and their expression often lead to a positive feedback-loop and amplification of the immune signal (Pandey and Somssich 2009; Carr et al. 2010). Many of the early expressed immune response genes are genes involved in SA biosynthesis and signalling as well as receptor proteins and their targets. For example, WRKY33 both activate and binds to the promoter of *AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE (ACS) 2 (ACS2)* and *ACS6* that lead to ET production. ET binds to and activate ETHYLENE RESPONSE (ETR) 1 (ETR1) that further activate ETR2 and ETR3 that binds to and promote the expression of the signal-perception protein genes *FLS2* and *BIK1* and *PLANT ELICITOR PEPTIDE PRECURSORS (PROPEPs)* (Li et al. 2016). The role of PROPEPs and other damage-associated molecular patterns (DAMPs) will be further explained in section 1.3.

Many genes are upregulated in response to the perception of flg22 by FLS2. Some of them are directly transcribed by MAPK activated WRKYs like *ISOCHORISMATE SYNTHASE 1* (*ICS1), RBOHD, WRKY transcription factors (WRKYs), PLANT DEFENSIN (PDF) 1.2 (PDF1.2), PHYTOALEXIN DEFICIENT (PAD) 3 (PAD3), ERF59, ACS2, ACS6* and *NONEXPRESSER OF PR GENES 1* (*NPR1)* (An and Mou 2011; Li et al. 2016; Qiu et al. 2008). Other genes are induced by proteins transcribed downstream of other responsive genes like *FLG22-INDUCED RLK 1* (*FRK1), GLUTATHIONE S-TRANSFERASE 1 (GST1), PATHOGENESIS-RELATED GENE 1* and *5 (PR1* and *PR5), PAD4, ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), FLS2, BIK1, PROPEP*s and other *WRKYs* (Asai et al. 2002; Glazebrook 2005; An and Mou 2011; Li et al. 2016). The early response gene *ICS1* (which transcription is also induced by Ca^{2+} mediated CaM activation upon pathogen recognition) encodes a key enzyme in the SA biosynthesis pathway (An and Mou 2011; Li et al. 2016). SA is recognized by NPR3 and NPR4 that mediate the cleavage of oligomerized NPR1 to monomeric NPR1 and translocation to the nucleus where it binds additional transcription factors and activate transcription of genes. The transcribed genes include further activation of the SA biosynthesis responsive genes (*EDS1, PAD4*), production of pathogenesis related genes (*PR1*), and proteins involved in repression of jasmonic acid (JA)-dependent gene expression (Yan and Dong 2014; Fu et al. 2012; Glazebrook 2005; An and Mou 2011). The PTI in plants is a highly complex and detailed system of interactions, pathways and cascades where feedback-loops, activation and

inhibition of proteins and gene transcription are regulated by interaction of different external and internal signals and other pathogen repressive processes like generation of ROS, calcium influx and phytohormone production. Although some pathways are well studied, the complexity and signalling pathways of PTI is still poorly studied and not well understood. **Figure 2** illustrate a simplified picture of molecular events and pathways included in the flagellin induced immune response.

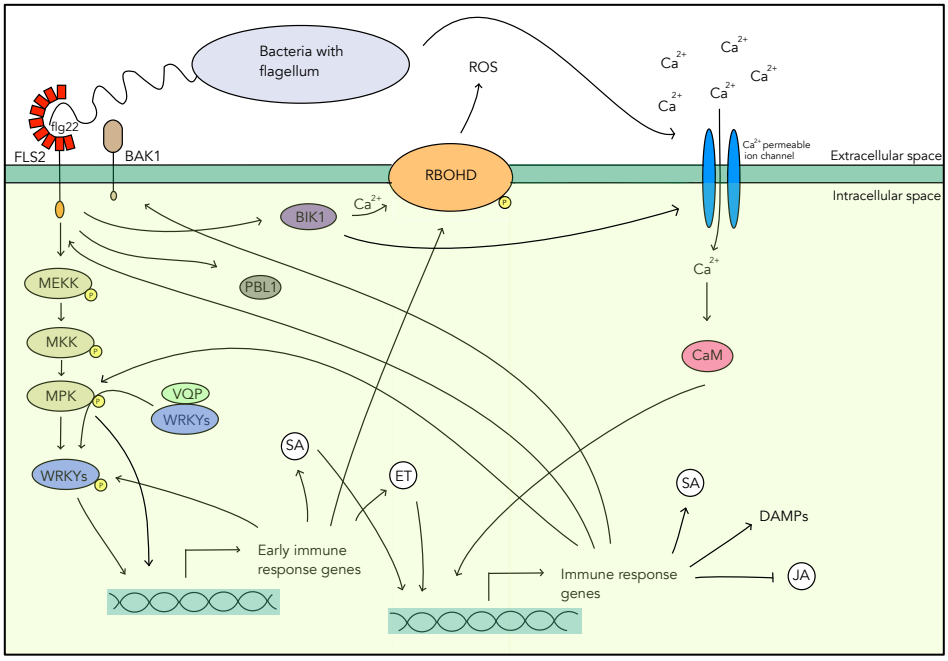


Figure 2: Flagellin induced pattern-triggered immunity (PTI).
The subunit flagellin (flg22) is recognised by FLAGELLIN-SENSITIVE 2 (FLS2) that lead to release of BOTRYTIS-INDUCED KINASE 1 (BIK1) and AVRPPHB SUSCEPTIBLE 1 LIKE 1 (PBL1) and activation of a MAP kinase (MAPK) cascade. This further induce production of reactive oxygen species (ROS), calcium influx and transcriptional reprogramming that include production of salicylic acid (SA), ethylene (ET) and damage-associated molecular patterns (DAMPs) and reduced production of jasmonic acid (JA). Further details about the pathways are outlined in the text. Abbreviations are as followed; BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), MAP kinase kinase kinase (MEKK), MAP kinase kinase (MKK), MAP kinase (MPK), WRKY transcription factors (WRKYs), VQ motif-containing protein (VQP), calmodulin (CaM) and respiratory burst oxidase homolog protein D (RBOHD).

1.2.2 Chitin induced PTI

Chitin is a highly conserved structural component of fungal cell walls and function as a PAMP to PRRs. Chitin oligomers are released from the fugal cell wall upon exposure to plant-produced chitinases (Gong et al. 2020). Chitin monomers are not very functional, but chitin oligomers with polymerization degrees of 6 to 8 are strong inducers of PTI (Pusztahelyi 2018). Chitin is recognized by RLKs with multiple lysin motifs (LysM). A chitin elicitor receptor kinase 1 (CERK1) also named LysM RLK (LYK) 1 (LYK1) is identified as a receptor for chitin and require LYK4 and LYK5 for its signalling. Recognition of chitin leads to interaction between LYK5 and CERK1 that activate CERK1 by phosphorylation. The receptors can form hetero and homodimers and dimerization is suggested to be a critical step in the binding of chitin oligomers (Cao et al. 2014; Gong et al. 2020). Phosphorylation of CERK1 lead to release of BIK1 and PBL19 and PBL27. PBL19 and PBL27 both induce MAPK cascades. PBL19 mobilize MEKK1 that activate MKK1 and MKK2 that activate MPK4. PBL27 phosphorylate MEKK5 that activate MKK4 and MKK5 that further activate MPK3 and MPK6 (Gong et al. 2020; Yamada et al. 2016). The

downstream MAPK cascade activation and release of BIK1 result in similar events as described in the previous section about the flagellin induced PTI (illustrated in **Figure 2**). Plant response to chitin treatment include activation of RBOHD and ROS production, stomatal enclosure, callose deposition, MAPK cascade activation, and transcriptional activation of immune related genes. The transcriptional upregulated genes include *PR* genes, *PDF1.2, RBOHD*, genes encoding chitinases*, phenylalanine ammonia lyase (PAL), MPKs, WRKYs, MYB transcription factors* and *WRKY* target genes (Cheval et al. 2020; Kohari et al. 2016; Miya et al. 2007; Ramonell et al. 2002; Libault et al. 2007; Luna et al. 2011; Albert et al. 2006). PAL is the minor of two SA-biosynthesis pathways that lead to increased SA biosynthesis (An and Mou 2011). The ROS production in response to chitin and flagellin differ. The ROS response to flagellin is more transient than the response to chitin, but the callose deposition occur much earlier upon chitin exposure than flagellin. It is suggested that callose deposition is ROS dependent for flagellin, but ROS independent for chitin signal perception (Luna et al. 2011). This suggest many similar pathways for flagellin and chitin perception. Regulatory differences in the PTI induction likely leads to an individual and customized response to pathogen perception. Chitin perception and signalling is important in response to fungal necrotrophic pathogens. Activation of the MAPK cascade and WRKY33 is crucial to induce ET and camalexin biosynthesis (Lai and Mengiste 2013).

1.3 Plant peptides signals as damage-associated molecular patterns

DAMPs are endogenous molecular patterns produced by the host that are able to elicit an immune response. Some DAMPs are a result of damage caused by pathogens, like oligogalacturonides and cutin monomers released from the cell wall and cuticle by fungal enzyme activity. Others are produced by the host as a part of the PTI, like systemin and plant elicitor peptides (PEPs) (Bartels and Boller 2015). PEPs are also shown to be involved in other plant processes like abiotic stress response, plant development and plant reproduction (Bartels et al. 2013).

PAMP recognition by PRRs leads to production of ET and synthesis of PROPEPs as described earlier in section 1.2.1. Both *PROPEPs* and *PEP receptors (PEPRs*) are transcriptionally induced by elicitor induced PRR activation (Bartels and Boller 2015). The promoters of *PROPEP2* and *PROPEP3* include several W-box elements and are shown to be transcriptionally regulated by the WRKY33 transcription factor (Logemann et al. 2013). Two LRR RLKs called PEPR1 and PEPR2 are known to recognize PEPs. PEPR1 functions as a receptor for Pep1-6 and PEPR2 functions as a receptor for PEP1 and PEP2 (Yamaguchi et al. 2010; Yamaguchi et al. 2006). PEPR1 forms a heterodimer with BAK1 upon binding of PEP1 (Schulze et al. 2010). PEPR1 is also shown to interact with and phosphorylate BIK1 and PBL1 upon recognition of PEPs and is required for ET- triggered defence responses (Liu et al. 2013). Phosphorylation of BIK1 could explain its function to activate ROS production reported by Huffaker et al. (2006). PEPR activation pathways are similar to FLS2 receptor signalling. Activation leads to Ca2+ influx, callose deposition, ROS production, activation of MAPK cascade and WRKY transcription factors that lead to production of antimicrobial proteins and secondary metabolites (Bartels and Boller 2015). PEPs are shown to be included in co-activation of both SA- and JA-mediated immunity and induce the expression of the SA marker *PR1* and the JA and ET-marker *PDF1.2* (Ross et al. 2014). PEP1 was discovered by Huffaker et al. (2006) and was the first plant-produced elicitor identified in *Arabidopsis*. A 23 amino acids PEP1 peptide is shown to be

sufficient to activate ROS production, transcription activation of *PDF1.2* and its own precursor gene *PROPEP1* (Huffaker et al. 2006).

Small secreted peptides are classified into three groups based on their biosynthetic pathways; small post-translationally modified peptides, cysteine-rich peptides and intermediate-type peptides (Matsubayashi 2011). PEPs are likely in the category of post-translationally modified peptides as PEP1 is derived from the C-terminal precursor protein PROPEP1. PROPEP1 is also shown to be proteolytically processed by a Ca^{2+}-dependent protease (Shen et al. 2019; Huffaker et al. 2006). The genes for the small post- translationally modified peptides are translated as pre-propeptides and are processed to propeptides by cleavage of the N-terminal signal peptide by signal peptidase (Matsubayashi 2011). Propetides are then translocated to the apoplast and proteolytically processed into mature functional peptides where they function as signalling molecules for their respective receptors (Tabata and Sawa 2014; Murphy et al. 2012).

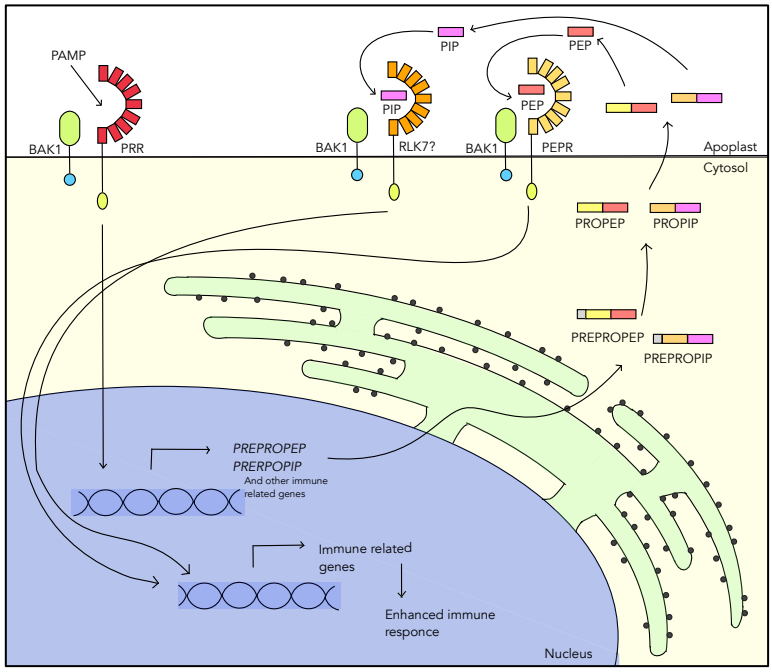


Figure 3: Pathogen recognition induce production of small secreted peptides to enhance the immune response.
Recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) induce expression of *PREPROPEP*, *PREPROPIP* and other immune related genes. *PREPROPEP* and *PREPROPIP* are translated as pre-propeptides and cleaved to form propeptides (PROPEP and PROPIP). The propeptides are translocated to the apoplast and proteolytically cleaved into the mature peptides PEP and PIP. Recognition of PEP and PIP by their receptors induce expression of immune related genes to further enhance the immune response. Further details are outlined in the text. Abbreviations are as followed; PLANT ELICITOR PEPTIDE (PEP), PAMP-INDUCED SECRETED PEPTIDE (PIP), BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), RECEPTOR-LIKE KINASE 7 (RLK7), PEP receptor (PEPR).

Another class of small post-translationally modified peptides have recently been discovered. The PAMP-INDUCED SECRETED PEPTIDES (PIPs) and PIPL-LIKE (PIPLs) families consist of PIP1-3 and PIPL1-8 (Vie et al. 2015; Hou et al. 2014). Members of this family are recognized by the presence of a N-terminal signal peptide region followed by a variable region and one or two SGPS core motifs with a C-terminal GxGH motif. PIP/PIPL families are present in both eudicotyledons and monocotyledons and are suggested to have evolved from retrotransposable elements (Vie et al. 2015). During translation, the

prepropeptides are secreted by the endoplasmic reticulum/Golgi apparatus and the N-terminal is cleaved resulting in a propeptide that is secreted to the apoplast and proteolytically processed to a mature C-terminal peptide. The secretion to the apoplast and proteolytical processing is confirmed in PIP1 (Hou et al. 2014). The same study revealed that RLK7 functions as a receptor for PIP1. PIP1 triggered signalling through RLK7 is suggested to act as a PTI amplifier and enhancer of the immune response in cooperation with PEP1 signalling via PEPR1. The suggested model include the recognition of PAMPs by FLS2 leading to expression of immune marker genes as well as *PIP1*, *PEP1* and their receptors *RLK7* and *PEPR1* that further amplify the immune signal and give a combined effect against the pathogen (Hou et al. 2014). PIP1 and PIP2 have been shown to induce the transcription of the immune responsive genes *FRK1*, *WRKY30, WRKY33, WRKY53* and *PR1* additional to stomatal enclosure, ROS production, callose deposition and MAPK phosphorylation (Hou et al. 2014; Shen et al. 2020; Hou et al. 2019). PIP3 have been shown to play an opposite role in regulation of the *Arabidopsis* immune response. *PIP3* overexpression lines exhibited reduced ROS production and callose deposition and increased susceptibility to *Pseudomonas syringae* and *Botrytis cinerea* (Najafi et al. 2020). **Figure 3** illustrate the pathways of PEP and PIP peptides in their function to elevate the initiated immune response trough their corresponding receptors.

1.4 Plant immune response to pathogens with different lifestyles

Plant pathogens are divided into biotrophs and necrotrophs based on their lifestyles. Biotrophs obtain nutrients from living host tissue and necrotrophs gain nutrients from dead or dying cells. Some pathogens, called hemi-biotrophs, can behave both as biotrophs and necrotrophs depending on external factors and the stage of life cycle. Recognition of Avr proteins by R proteins mediate ETI which enhance the PTI response and induce HR. HR restricts nutrient source for the biotrophic pathogen but at the same time also provide decreased resistance to necrotrophs. SA increase resistance towards biotrophs, while JA and ET dependent responses increase resistance towards necrotrophs (Glazebrook 2005; Kliebenstein and Rowe 2008).

Gene-for-gene resistance appear to be the most important factor for resistance against biotrophs because it is able to induce HR and limit pathogen growth (Barna et al. 2012). Gene-for-gene resistance is usually not associated with necrotrophs and the immunity against necrotrophs are mostly dependent on the balance between phytohormones (Ghozlan et al. 2020). The relationship between SA and JA appears to be antagonistic. Elevated biotrophic resistance correlate with increased necrotrophic susceptibility and elevated necrotrophic resistance correlate with enhanced biotrophic susceptibility (Robert-Seilaniantz et al. 2011). SA also supress the JA biosynthesis pathway, but induce ET which increase biosynthesis of JA. Early stages of infection are similar in biotrophic and necrotrophic pathogen infections, while the later stages of response are different in the two pathogen infections (Ghozlan et al. 2020). The relationship between SA, JA and ET in immune responses are complicated and their regulation have still not been fully understood. The interaction and overlap also involve fine-tuners which help with the balance between pathogens with different lifestyles (Kliebenstein and Rowe 2008; Veronese et al. 2006; Roux et al. 2011; Lai and Mengiste 2013; Robert-Seilaniantz et al. 2011; Ghozlan et al. 2020). The DELLA proteins have been identified to modulate the balance between JA and SA signalling. Gibberellic acid positively regulate SA and degrade DELLA proteins leading to release of jasmonate-zim-domain proteins (JAZs) that inhibit JA signalling (Navarro et al. 2008; Robert-Seilaniantz

et al. 2011). *Pseudomonas syringae* is an example of a biotrophic pathogen infecting plants, but the pathogen can also be considered a hemi-biotroph. The bacteria enter the host tissue via stomata or wounds and produce type III secretion Avr proteins. Recognition of Avr proteins by plant R proteins mediate ETI and HR leading to programmed cell death. The bacteria multiply in the intracellular space. Host cell death does not occur in the early stages of infection, but later stages are associated with chlorosis and necrosis. The virulent strain *P. syringae* pv. *tomato* DC3000 produce toxins that structurally resemble JA and precursor of ET that repress SA-signalling leading to enhanced growth of the pathogen (Glazebrook 2005). On the other hand, *Botrytis cinerea* is a necrotrophic pathogen and kill host cell in the early stages of infection and cause substantial damage to the host tissue. *B. cinerea* produce phytotoxins that promote ROS production and induce cell death to increase susceptibility. JA and camalexin production are important to support further growth of this pathogen (Lai and Mengiste 2013; Glazebrook 2005; Barna et al. 2012).

1.5 *Arabidopsis thaliana* as a model plant

Traditionally, plant research was built on investigation of genetics, molecular biology and physiology of a wide range of plant species. The research was not directed to plant fundamental aspects that resulted in limited understanding of plant growth and development. *Arabidopsis* was first accepted as a model plant in the early 1980s and the interest further increased significantly over the years (Meinke et al. 1998). The whole *Arabidopsis* genome was sequenced in the year of 2000 and *Arabidopsis* have obtained a crucial role as a model organism for plant research (Somerville and Koornneef 2002). *Arabidopsis* has many advantageous properties for laboratory experiments, such as a small size, simple growth requirements, self-fertilization, small genome, short life cycle and it is easily transformed. *Arabidopsis* is also closely related to several hundred plant species in the Brassicaceae (Somerville and Koornneef 2002).

Forward genetic have traditionally been used, where genetic mutations or allelic variants are discovered by alteration of the phenotype. Resent research often use reverse genetics were a mutant gene is obtained and alteration in phenotype is studied (Krysan et al. 1999). To perform genetic analysis, both chemical and insertional mutagenesis have been used (Meinke et al. 1998). Insertional mutagenesis can be based on the use of transposable elements or transgenic DNA (T-DNA). T-DNA is transferred DNA from a disarmed tumour-inducing plasmid from *Agrobacerium tumefaciens*. The T-DNA is randomly inserted to the plant genome and may lead to disruption of the gene it is inserted into. Thousands of transgenic T-DNA insertions are available from public stock centres and are used to find potential knock-out mutant lines for a desired gene (Krysan et al. 1999; Meinke et al. 1998). Disruption of gene expression by knock-out or knock-down of a gene, provide investigation opportunities for the *in-situ* function of the respective gene which is highly important in current research methods. A T-DNA insertion within a gene can be detected by using a combination of gene specific and T-DNA specific primers (Krysan et al. 1999). Recently, clustered regulatory interspaced short palindromic repeats (CRISPR) technology in combination with CRISPR-associated protein 9 (Cas9) based genome editing systems have also been developed to generate targeted modifications in the genome as an alternative approach for reverse genetics (Jiang et al. 2013).

1.6 Methods for investigating the immune system in plants

Plant immune responses are accompanied by stomatal enclosure, production of antimicrobial compounds, ROS production, calcium influx, callose deposition, transcriptional reprogramming and production of phytohormones (Bigeard et al. 2015). Most research on plant immunity aims to detect and quantify the above mentioned physiological and molecular responses. Stomatal enclosure can be studied by gas exchange analysis of stomatal conductance or measurement of the stomatal aperture in response to different treatments (Ceciliato et al. 2019; Pei et al. 1997). ROS production can be measured based on different assays including 3,3´-diaminobenzidine (DAB) staining or a luminol chemiluminescence based quantification (Desaki et al. 2019). Calcium influx can for example be measured by fluorescent staining or an aequorin-bioluminescence based quantification (Qu et al. 2012; Mithöfer and Mazars 2002). Callose deposition can either be visualized and quantified with aniline or methyl blue staining and the stained callose can also by extracted and quantified by fluorescence spectroscopy (Scalschi et al. 2015; Kohari et al. 2016; Kohler et al. 2000). Some phytohormones and antimicrobial compounds can for example be measured by targeted metabolite analysis employing liquid- or gas- chromatography combined with tandem mass spectrometric analysis (Šimura et al. 2018; Savatin et al. 2015). Transcriptional reprogramming as a result of immune response can be detected by the change in gene expression. Reverse transcriptase (RT) polymerase chain reaction (PCR) can be used to detect changes in expression of specific genes, either quantitatively by a quantitative PCR (qPCR) or semi-quantitatively with a regular PCR followed by visualization by gel electrophoresis. Changes in gene expression can also be analysed by the use of microarrays or next-generation sequencing (NGS) technologies (Aharoni and Vorst 2002; Garg and Jain 2013). Investigation of the plant immune response is often based on differences between treatments and control. Functions of genes are often investigated by analysis of response-differences between knock-out and overexpression of one or more genes compared to the wild-type.

1.7 Background and aim of study

As described in the previous sections, PTI is a complex and still not well understood phenomenon of the plant immune response. PTI is the primary response to pathogen attack and is shown to be highly conserved between plant species in contrast to the ETI that is more species specific and a result of the gene-for-gene evolutional concept. A deeper insight to the carefully regulated primary response to pathogens could be useful to identify methods for increasing immunity and survival in plants used for crop production. Increased yield of food production is important to feed the constantly growing human population. The recently described family of PIPs and PIPLs have been investigated to some extent by the Cell, Molecular Biology and Genomics group (CMBG) at the Norwegian University of Science and Technology (NTNU). They have investigated the role of PIP1, PIP2 and PIP3 in plant immunity (Najafi et al. 2020). To better understand these peptides involvement in the regulation of plant immunity, further studies are essential. DAMPs have been suggested to function as the main resistance enchasing compounds in plants to increase phytopathogen resistance, and is believed to provide the plant vaccine for the future (Quintana-Rodriguez et al. 2018). PIPs and PIPLs role in plant immunity will give us a better insight into possible utilization of these peptides in the control of phytopathogens.

The PIP and PIPL family of peptides are composed of 13 members. Based on previous studies conducted by the CMBG group, members of this family can be divided into two categories based on their expression patterns and responses to different biotic and abiotic stimuli. Among the highly responsive genes in this family is *PIPL6*. *PIPL6* possess one SGPS motif at the C-terminal and is expressed at very low levels under normal conditions. It has been shown that *PIPL6* expression is highly responsive to treatment with the elicitor chitin, the generalist aphid *Brevicoryne brassicae* and the two protein synthesis inhibitors cycloheximide and anisomycin (Vie et al. 2015). These results are strong indicators that PIPL6 may play a role in plant immune signalling. This study aims to contribute to the investigation of PIPL6 in regulation of plant immunity and the molecular mechanisms behind it.

2 Method

2.1 Plant material and growth conditions

All experiments were conducted with *Arabidopsis thaliana* ecotype Columbia-0 (N1092) wild-type (Wt) obtained from the European Arabidopsis Stock Centre (NASC, Nottingham, UK).Mutant lines were selected in Col-0 background. The lines used were *rlk7* (SALK_094492H), *srr1-1* (GAB_179_E06, described by (Najafi 2015)), *fls2* (SALK_026801), *wrky33* (GABI_324B11), *wrky18* (Salk_093916), *wrky60* (Salk_120706), *wrky40* and double mutants *wrky18/40*, *wrky40/60*, *wrky18/60* and triple mutant *wrky18/40/60* earlier described by Xu et al. (2006). Two possible knock-out lines for *PIPL6* were screened by searching The Arabidopsis Information Resource (TAIR) webpage and Salk Institute Genomic Analysis Laboratory (SIGnAL) genome browser. According to the SIGnAL T-DNA express database, *pipl6-1* (SALK_106769) had a T-DNA inserted in the promotor region, and *pipl6-2* (Wiscseq_DsLoxHs144_04E.1) had a transposon inserted in the coding region of the *PIPL6* coding sequence. *PIPL6* overexpression (PIPL6:OX) lines were previously generated by (Najafi 2015). The coding sequence of *PIPL6* from wild-type was amplified by PCR, and cloned into the destination vector pEG100 under control of the 35S promoter using Gateway technology. Transformation were performed using *Agrobacterium tumefaciens* and the floral dip method.

All seeds used in experiments were surface sterilized with chlorine gas (100mL of Chlorine and 3 mL 37,5% HCl in a closed box) for 3 hours, then suspended in 0,1% phytoagar (P1003.5000, Duchefa Biochemie B.V) and stratified for 2 days at 4°C to establish homogenous germination. The plants were either grown in autoclaved soil or in growth medium. Unless stated otherwise, the medium used for growing seedlings were half-strength Murashige-Skoog (1/2MS) basal Salt mixture (M5524, Sigma-Aldrich) with 1% sucrose and 0,6% phytoagar (P1003.5000, Duchefa Biochemie B.V), pH was adjusted to 5,75. The liquid medium contained no phytoagar. To avoid contamination and still maintain aeration, plates were sealed with Micropore™ tape (3M). The plants were grown in long days (16 hours light (70μmol $m^{-2}s^{-1}$), 8 hours dark at 22°C 20-25% relative humidity), short days (8 hours light, 16 hours dark 20°C 80% relative humidity) or constant light (150μmol $m^{-2}s^{-1}$, 22°C, 50% relative humidity).

2.2 Screening of *PIPL6* knock-out lines and generation of overexpression lines

Homozygous plants of *pipl6-1* and *pipl6-2* were selected based on screening by growth on selection medium (Kanamycin or BASTA) and PCR on genomic DNA based on gene specific and T-DNA specific primers (Appendix 1). Tissues were harvested in a 1,5mL Eppendorf tube and grinded with a blue pestle and DNA extraction and PCR reaction were performed according to Appendix 3. 5μL of PCR products were applied to a 1% agarose gel at 65 volt for 75 minutes using a 1kb ladder (GeneRule™1kb Plus DNA Ladder, Thermo Scientific) and the gel was post stained with a 1:3300 dilution of GelRed (20000x) in autoclaved double distilled water (MQ) for 20 minutes prior to visualization in G:Box (Syngene). For confirmation of homozygous lines, a combination of the gene

specific (LP and RP) and T-DNA specific primers (LBN and JL-202) were used. Appendix 1 includes all primers used in this project. PCR products for *pipl6-1* and *pipl6-2* were sent for external sanger sequencing (GATC Biotech AG, Cologne, Germany). The insertion site for *pipl6-2* was also confirmed using TOPO PCR cloning explained in Appendix 2, and plasmids were sequenced externally by GATC Biotech AG (Cologne, Germany).

Transgenic overexpression lines were screened for a single copy of T-DNA insertion by growth on selection medium (BASTA). Confirmation of constitutive overexpression of *PIPL6* were performed by RNA expression analysis. Two overexpression lines, PIPL6:OX3 and PIPL6:OX5, were used in further analysis.

Confirmation of knock-out and overexpression of *PIPL6* were performed by RT-qPCR. 7 days old seedlings were treated with or without the elicitor flg22 in 1/2MS liquid medium for 1 hour according to the method described in section 2.4 (only liquid medium was used as a control). Additional RT-PCR was performed on the same samples using RT-PIPL6 F and qPIPL6 R primers (Appendix 1) and *Actin2* as a reference gene according to Appendix 3. PCR products were applied to a 1% agarose gel at 65 volt for 75 minutes with a 1kb ladder (GeneRule™1kb Plus DNA Ladder, Thermo Scientific). The gel was post stained with a 1:3300 dilution of GelRed (20000x) in MQ water for 20 minutes prior to visualization in G:Box (Syngene).

2.3 Gene expression analysis

Unless otherwise is stated, tissues for gene expression analysis were collected in a 2mL Eppendorf tube with a steal bead, directly frozen in liquid nitrogen (-196°C) and stored at -80°C prior to RNA isolation. All RNA samples were isolated using the Spectrum™ Plant Total RNA kit (Sigma life Science) by the method descried in Appendix 2. DNA was eliminated using an on-column DNase digestion by the Rnase-free Dnase set (Qiagen) as described in Appendix 2. The concentration of the eluted RNAs were measured on Nanodrop One (Thermo Scientific) and complementary DNAs (cDNAs) were synthesised using the reverse transcription kit 2000 (Qiagen) according to Appendix 3. The final products were diluted by 5X. Unless otherwise is stated, RT-qPCR was performed using 10μL PCR Master MIX (LightCycler®480SYBR®Green I Master, Roche), 5μL cDNA template and 500nM of each primer in a 20μL total volume reaction with PCR grade water. qPCR was performed according to Appendix 3. Primer efficiency was calculated using LinRegPCR (Version 2015.4). Relative expression of genes were calculated using qBase+ (Version 3.2, Biogazelle) and quantified relative to the reference gene *Interacting protein of 41 kDa* (*TIP41*).

2.4 Elicitor treatments

Expression of *PIPL6* in response to the elicitors flagellin, chitin and PEP1 and the herbicide paraquat were analysed by RT-qPCR. Flg22 (QRLSTGSRINSAKDDAAGLQIA) and PEP1 (ATKVKAKQRGKEKVSSGRPGQHN) were ordered from Biomatik (Cambridge, Ontario, Canada), chitin hexamer was kindly provided by Prof. Kjell Morten Vårum (department of Biotechnology, NTNU) and Paraquat, also known as Methyl Viologen (SIGMA) was obtained.

Wild-type seedlings were grown with a density of 10-12 seedlings per well in 6-well culture plates containing 1,5 mL 1/2MS liquid medium per well. Three biological replicates (3 wells) for each timepoint, including control, were implemented. The seedlings were grown for 7 days (PEP1) or 10 days (chitin and paraquat) under long day

conditions ahead of treatment. The treatments were performed by replacing the liquid medium with new liquid medium containing PEP1 (100nM), chitin hexamer (500nM) or paraquat (5μM). For the control, only fresh liquid medium was added. Tissues were harvested at the following timepoints; 15 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 24 hours and 48 hours after treatment. The control was only harvested after 15 minutes. Tissues from each well were harvested by quickly dipping the seedlings in double distilled water followed by drying on paper towel prior to snap freezing in liquid nitrogen.

Treatment with flg22 was performed previously by Najafi et al. (2020) and cDNAs were obtained from this experiment. Unlike the described treatments, seedlings were grown on petri dishes and sprayed with 100nM flg22 prior to vacuum infiltration as described for peptide treatments in section 2.5. Tissues were harvested at the timepoints 1 minute, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 60 minutes, 6 hours and 24 hours after treatment.

2.5 Peptide treatments using synthetic PIPL6 peptide

The effects of synthetic PIPL6 peptide on the expression of immune marker genes were performed in both wild-type, *rlk7* and *srr1-1* seedlings. Gene expression was analysed using RT-qPCR. The peptide sequence was selected previously in relation to the study by Najafi et al. (2020). The selected sequence was in the C-terminus of the PIPL6 propeptide containing the conserved SGPS motif with the following sequence: H-AFRLASGPSRKGRGH-OH. Peptides were synthesized with a purity of more than 95% by Biomatik (Cambridge, Ontario, Canada).

Seedlings were grown under long day conditions for 14 days in petri dishes containing 1/2MS medium with a density of 10-15 seedlings per plate. After 14 days, the plates were sprayed with 1μM PIPL6 peptide 0,02% (w/v) Silwet L-77 (Lehle Seeds, Round Rock, TX, USA) in MQ water with the spray placed approximately 10 cm from the plate. The control was only sprayed with 0,02% (w/v) Silwet L-77 in MQ water. Peptides were infiltrated to the tissues by the use of the vacuum chamber PDS-1000/HE Biolistics® Particle Delivery System (BIORAD) at 25Hg for 60 seconds. After treatment, the plates were transferred to the growth room and aerial tissues were harvested for gene expression analysis after 3 hours. Treatment of wild-type was also performed with 100nM concentration of synthetic PIPL6 peptide.

2.6 RNA sequencing

Three replicates for control and PIPL6 peptide treatment of wild-type seedlings were sent for RNA sequencing. The quality of the RNAs were first examined on Aligent 2100 Bioanalyzer (Aligient Technologies) using the Aligent RNA 6000 Nano kit (Aligient Technologies), performed as described by the manufacturer. RNA sequencing was performed externally (GENEWIZ, Leipzig, Germany) using an Illumina DNA sequencing platform. The following sequencing data processing was performed by associate professor Per Winge (Department of Biology, NTNU). The raw sequence reads in fastq format was mapped against Arabidopsis TAIR10 gene models with the Bowtie2 sequence mapper in a very-sensitive-local mode (Langmead and Salzberg 2012). The sequence Alignment/Map file generated by the Bowtie2 program was used to produce a "count-table" were reads mapping specific *Arabidopsis* genes were registered for each of the individual biological replicates. The edgeR software package was used for statistical analysis of the mapped sequence reads in the count table (Robinson et al. 2010). Low expressed genes were identified and filtered out, according to the following criteria; a gene cut-off at 1 hit pr. 1

million reads, keeping only genes that are above the cut-off for all 3 of the biological replicas. The data was analysed using statistical methods based on generalized linear models, and a likelihood ratio test was used to identify differentially expressed genes. Genes with a false discovery rate below 0.05 and log2 value > ± 0.5 were defined as significantly differentially expressed genes.

2.7 Phenotype assays

Functional studies of PIPL6 were performed by testing phenotypical changes of knock-out and overexpression lines compared to wild-type. Both growth and pathogen assays were performed as described below.

2.7.1 Root growth and growth inhibition assay

Root growth of *PIPL6* knock-out and overexpression lines was measured and compared to the wild-type. Seedlings were grown on square plates containing 1/2MS medium supplemented by 1% sucrose and 1% phytoagar kept in vertical positions during growth. Two independent experiments were performed either under long or short day conditions. Root growth was measured every day from day 6 to day 16. The measurements were performed by photographing the plates and root length was calculated in the image processing program ImageJ (Version 1.53a).

Growth inhibition in response to the elicitor flagellin was performed. Seedlings were grown on 1/2MS agar plates under long day conditions for 5 days. Treatment was performed by transferring 5 days old seedlings to a 48-well-plate with 1/2MS liquid medium (one seedling per well with 600μL medium) containing flg22 (100nM) or only liquid medium as a control. Each genotype had 24 replicates for control treatment and 48 replicates for flagellin treatment. The plates were incubated in long day conditions for the next 10 days and fresh biomass was measured on Mettler AE 100. The flagellin sensitive *fls2* line was used as a control in the assay.

2.7.2 Reactive oxygen species (ROS) production assay

The production of ROS in response to the elicitor flagellin was analysed using a Luminol/peroxidase-based ROS generation detection method described by Bisceglia et al. (2015). Plants were grown in short day conditions for 4-5 weeks prior to experiments. Leaf disks from fully developed leaves were made along the mid rib using a cork borer (0,125cm^2) and immersed in MQ water. The MQ water was replaced two times every hour before leaf disks were transferred to a 96-well luminometer plate (Thermo Scientific) containing 200μL MQ water per well. 12 replicates were performed for each genotype. The plates were wrapped in aluminium foil and kept in dark overnight. The MQ water was replaced with 200μL MQ water containing luminol (Sigma-Aldrich) and horseradish peroxidase (HRP) (Sigma-Aldrich) using a multichannel pipettor. 50μL flg22 was added and the final concentrations were 100nM flg22, 200nM luminol and 550nM HRP. The plates were directly placed in the instrument for the detection of luminescence. For detection of ROS production, Cytation 5 Cell Imaging Multi-Mode Reader instrument (BioTek) and Gen5™ Microplate reader and imager software (version 305, BioTek) was used with the following settings; Shake Orbital 2 milli seconds 237cpm (4mm) slow speed, kinetic interval 120-210 seconds with 21-26 reads, run time 50-70 minutes, read height 1,00 mm, Integration time 100-140 milli seconds, Gain 200. The raw data as relative light units (RLU) were exported to Excel (Microsoft, version 16.49) and the kinetic of ROS production was mapped. The flagellin sensitive *fls2* line was used as a control in the assay.

2.7.3 Infection with *Pseudomonas syringae*

The phenotype of *PIPL6* knock-out and overexpression lines in response to the hemi-biotroph *Pseudomonas syringae* pv. *tomato* DC3000 was assessed according to the method described by (Lee et al. 2011) with some modifications. Seedlings were grown in a 6-well-plate (one well per genotype) containing 1,8mL liquid 1/2MS medium with the confluency of 15-18 seedlings per well. The seedlings were grown under constant light for 7 days prior to inoculation. The inoculum was prepared by growing *Pseudomonas syringae* pv. *tomato* DC3000 on LB agar (recipe in Appendix 4) containing Rifampicin (25μg/mL) and incubation in 28°C for 2 days. One colony was transferred to a culture tube with 4-5mL Kings B liquid medium (recipe in Appendix 4) containing Rifampicin (25μg/mL) and left in a shaker incubator at 28°C 220 revolutions per minute (rpm) for 8 hours. 50-100μL was transferred to a culture flask with 50mL Kings B liquid medium containing Rifampicin (25μg/mL) and incubated in a shaker incubator at 28°C 220rpm overnight. The culture was transferred to a 50mL Falcon tube and centrifuged at 4000rpm for 10 minutes. Supernatant medium was discarded, and the pellet was washed twice in $MgCl_2$ (10mM) by vortex followed by centrifugation (4000rpm at 4°C) for 5 minutes. After, the supernatant was discarded and the pellet was resuspended in $MgCl_2$ (10mM). An optical density at 600nm (OD_{600}) was measured and adjusted to 0,02 ($=1*10^7$CFUs/mL) by the use of NanoDrop 2000c Spectrophotometer (Thermo Scientific) and the software NanoDrop 2000/2000c (version 1.4.1, Thermo Scientific). 50μL inoculum (OD_{600}=0,02) per mL 1/2MS liquid medium without sucrose was prepared for inoculation of seedlings. The seedlings were first washed by replacing the medium with 1/2MS liquid medium without sucrose to wash away additional carbon source for the bacterium. The medium was then removed and replaced with 1mL liquid medium with inoculum. Seedlings were then incubated under constant light on a shaker at 50rpm for 3 days. After 3 days, the seedlings were washed twice in 70% ethanol for 30 seconds, dried on paper towel before they were washed twice in distilled water for 30 seconds and dried on paper towel. 3 seedlings were placed in each Eppendorf tube (5 replicates per genotype) containing 100μL $MgCl_2$ (10mM) and kept on ice. The tissue from the seedlings were homogenized using a blue pestle and EUROSTAR 20 digital (IKA®). The residues left on the pestle were washed of inside the Eppendorf tube with 200μL of $MgCl_2$ (10mM). The pestle was disinfected with 70% ethanol and dried with paper towel between each sample. Serial dilutions of the samples were prepared in a 96-well plate in $MgCl_2$ (10mM). 10μL of dilution 1:1 000 to 1:100 000 000 series were plated on LB agar with Rifampicin (25μg/mL). The droplets dispensed on the agar plates were spread by tilting the plate until droplets made a smear on the agar. The plates were incubated at 28°C for about 36 hours prior to colony count. The number of colony forming units (CFUs) per seedling were calculated and the mean value for the genotypes were compared. The whole experiment was repeated 10 times.

2.7.4 Infection with *Botrytis cinerea*

The phenotype of *PIPL6* knock-out and overexpression lines in response to the necrotrophic pathogen *Botrytis cinerea* isolate 2100 (CECT2; Spanish type) were assessed according to the method described by Birkenbihl et al. (2012) with modifications. Plants were grown for 5 weeks under short day conditions prior to treatment. Spores of *Botrytis cinerea* isolate 2100 (CECT2; Spanish type) were diluted in Vogel buffer (43.86mM sucrose, 11.63mM Na-citrate, 24.98mM K_2HPO_4, 0.81mM $MgSO_4*7H_2O$, 0.9mM $CaCl_2*2H_2O$, 24.98mM NH_4NO_3) to a density of $2,5*10^5$ spores per mL^{-1}. Droplets of 2μL were applied to each side of fully developed leaves for each

genotype (only buffer was used as a control). Both attached leaves and detached leaves were used. Detached leaves were carefully fixed in an agar plate containing only 0,6% phytoagar (P1003.5000, Duchefa Biochemie B.V) and pictures were taken 7 days after inoculation. Attached leaves were harvested after 3 days for genomic DNA qPCR analysis of *Arabidopsis* and *B. cinerea* DNA described by Gachon and Saindrenan (2004). Inoculated leaves were cut from the plants and the spores on the surface of the leaves were removed by a paper towel. The tissues were frozen in 2mL Eppendorf tubes with a steal bead in liquid nitrogen. The Eppendorf tubes were placed in a precooled (-18◦C) Tissuelyser module and tissues were crushed by the use of TissueLyserII (Qiagen) 25Hz for 2 minutes prior to DNA extraction performed according to Appendix 3. About 50ng of genomic DNA were used as templates in a 10μL total volume PCR reaction with 5μL PCR Master MIX (LightCycler®480SYBR®Green I Master, Roche), 500nM primers and PCR grade water. qPCR was performed according to Appendix 3. Relative amounts of plant specific genes for *B. cinerea* (*Bc*CutA) and *Arabidopsis* (*At*SKII) were calculated based on cycle threshold (Ct)-values from LightCycler® 96 (version 1.1, Roche). Three biological replicates were performed per genotype, and two technical replicates for each sample were implemented. The average of the technical replicates was used to quantify and calculate the *Bc*CutA/*At*SKII ratio. *Botrytis cinerea* spores were also sprayed onto intact plants with a density of $5*10^5$ spores per mL^{-1}. Leaf tissues were harvested after 48 hours in liquid nitrogen. Extraction and measurements of phytohormones (Abscisic acid, Jasmonic acid, Camalexin and Salicylic acid) were conducted according to the protocol by Salem et al. (2020) and performed by Dr. Javad Najafi and staff engineer Zdenka Bartosova. *Wrky33* was used as a susceptible control to *B. cinerea* infection.

2.8 Statistial analysis

All statistical analysis of RT-qPCR data were performed by using the qBase+ software (Version 3.2, Biogazelle) and the statistical outputs were graphed using Prism 9 for macOS (version 9.1.0, GraphPad software). All other statistical data were analysed and graphed in Prism 9 for macOS (version 9.1.0, GraphPad software), except RNA sequencing data which calculations are outlined in section 2.6. Statistical details are further explained for each experiment.

3 Results

This project aimed to contribute to the investigation of PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6) in regulation of plant immunity and the molecular mechanisms behind it. This included studies of expression of *PIPL6* in response to different elicitors, transcriptional responses of synthetic PIPL6 peptide on the regulation of plant immune marker genes and phenotype assessment of *PIPL6* knock-out and overexpression lines challenged by phytopathogens with different life-styles.

3.1 Screening and confirmation of knock-out and overexpression lines

Possible knock-out lines for *PIPL6* were obtained from the Nottingham Arabidopsis Stock Centre. Two independent lines were identified from SALK T-DNA and Wisconsin DS-LOX transposon insertion collections. Seedlings were grown on a selective medium and genomic DNAs and RNAs were isolated to confirm the homozygosity of lines at DNA and mRNA levels.

3.1.1 *PIPL6* knock-out lines screened based on DNA

Pipl6-1 (SALK_106769) and *pipl6-2* (Wiscseq_DsLoxHs144_04E.1) were T-DNA and transposon insertion lines respectively. Homozygosity of mutation was confirmed by genomic analysis using gene specific (LP and RP) and T-DNA specific primers (LBN and JL-202). Homozygosity of the knock-out lines were determined based on the presence of the T-DNA and absence of gene specific PCR products detected for the wild-type (**Figure 4**). A number of faded bands were present in the gel picture as a result of unspecific binding, only the abundantly expressed bands were considered as a positive amplification of the desired product. Identified homozygous lines were grown in soil and seeds were harvested for further analysis.

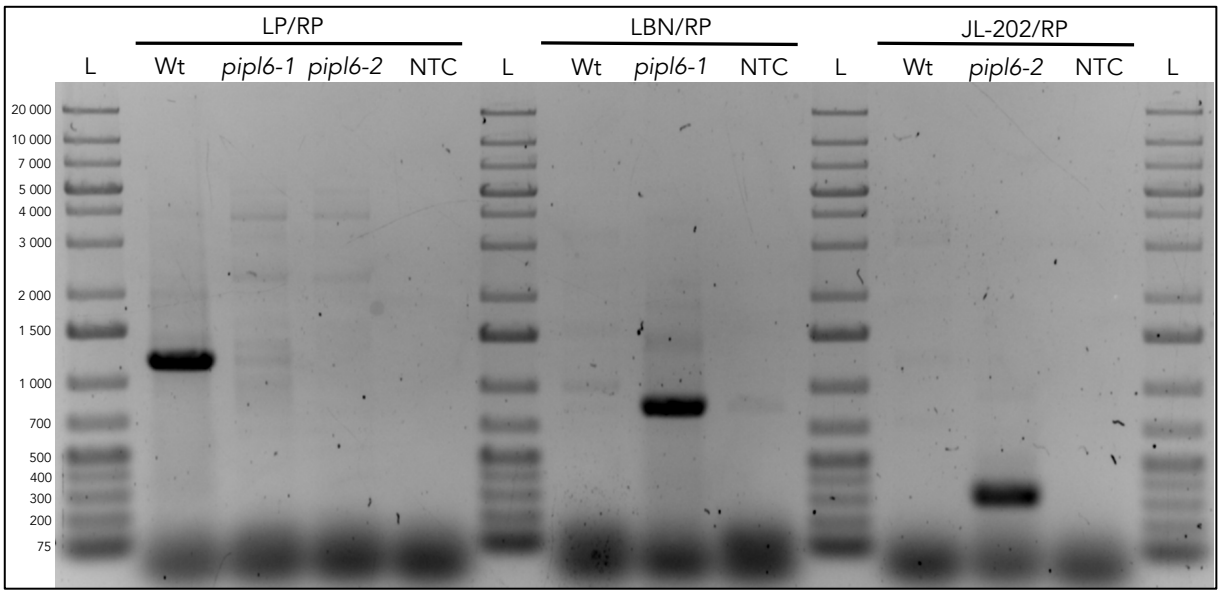


Figure 4: Conformation of homozygosity of *PIPL6* knock-out lines.
Genomic DNA was isolated from wild-type (Columbia-0), *pipl6-1* and *pipl6-2* and PCR was performed for amplification of the *PAMP-INDUCED SECRETED PEPTIDE LIKE 6* (*PIPL6)* coding sequence using the primers indicated above each sample. Gene specific primers (LP and RP) and transgenic DNA (T-DNA) specific primers (LBN and JL-202) was used. Negative control (NTC) and 1kb plus ladder (L) were indicated and the number of base pairs for the respective bands were listed.

Confirmation of exact T-DNA insertion site was performed by sanger sequencing of PCR products from **Figure 4** and sequences were aligned to the wild-type genomic region (**Figure 5**). The T-DNA insertion site for SALK_106769 (*pipl6-1)* was aligned approximately where it was expected to be located, 180 base pairs upstream of the coding sequence of *PIPL6*. However, the insertion site of Wiscseq_DsLoxHs144_04E.1 (*pipl6-2)* was expected to be located inside the coding region, but the sequenced results showed that the T-DNA was inserted 8 base pairs downstream of the coding region of *PIPL6* at the 3´UTR region. TOPO-cloning was also performed to confirm the location for *pipl6-2* T-DNA insertion site.

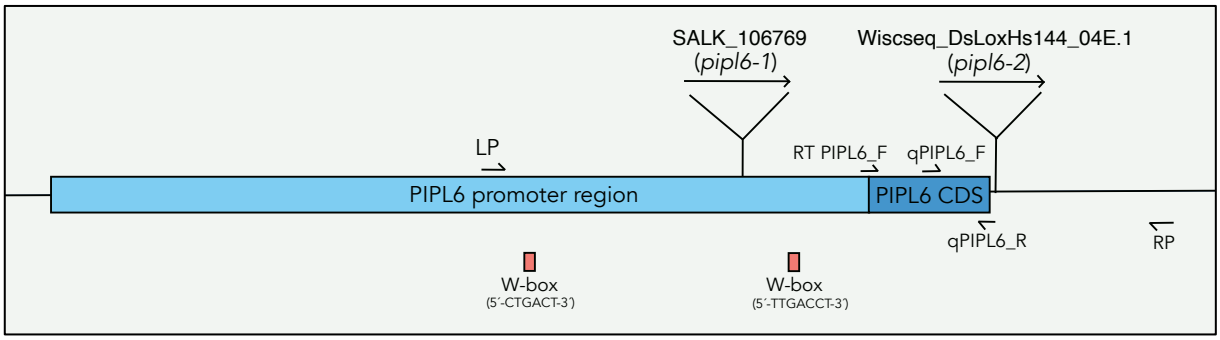


Figure 5: *PIPL6* genomic region including T-DNA insertion site, primer binding sites and W-box elements.
PAMP-INDUCED SECRETED PEPTIDE-LIKE 6 (PIPL6) promoter region and coding sequence (CDS) illustrated aligned with primers used in the project. Insertion sites of knock-out lines SALK_106769 (*pipl6-1*) and Wiscseq_DsLoxHs144_04E.1 (*pipl6-2*) were placed 180 base pair upstream of the *PIPL6* CDS and 8 base pairs downstream for the *PIPL6* CDS respectively. WRKY transcription factor elements (W-box elements) aligned to the promotor region are indicated in red. Two W-box elements (5´-(C/T)TGAC(T/C)-3´) were found at -155 and -744 positions upstream of the *PIPL6* CDS.

3.1.2 Confirmation of *PIPL6* knock-out and overexpression at mRNA level

Gene expression analysis of both knock-out and overexpression lines were performed. Since *PIPL6* was normally expressed low levels, gene expression was measured before and after 1 hour of flagellin treatment (**Figure 6**). Expression of *PIPL6* in mutant lines were scaled relative to the wild-type control. *Pipl6-1* had downregulation, but not knock-out of *PIPL6* expression. The expression was increased with flagellin treatment to levels of the wild-type control. Interestingly, no expression of *PIPL6 was detected in pipl6-2*, and treatment with flagellin did not induce the expression. The qPCR primers were outside of the transposon insertion site of *pipl6-2* with its reverse primer starting 10 base pairs downstream of the coding sequence (stop codon) while the forward primer starting 126 base pairs downstream of the start codon (**Figure 5**). As expected, *PIPL6* expression was strongly increased in overexpression lines. PIPL6:OX3 and PIPL6:OX5 lines expressed about 2 000- and 6 000-fold change of *PIPL6* expression respectively compared to wild-type control. Both overexpression lines showed increased expression of *PIPL6* in response to treatment with flagellin.

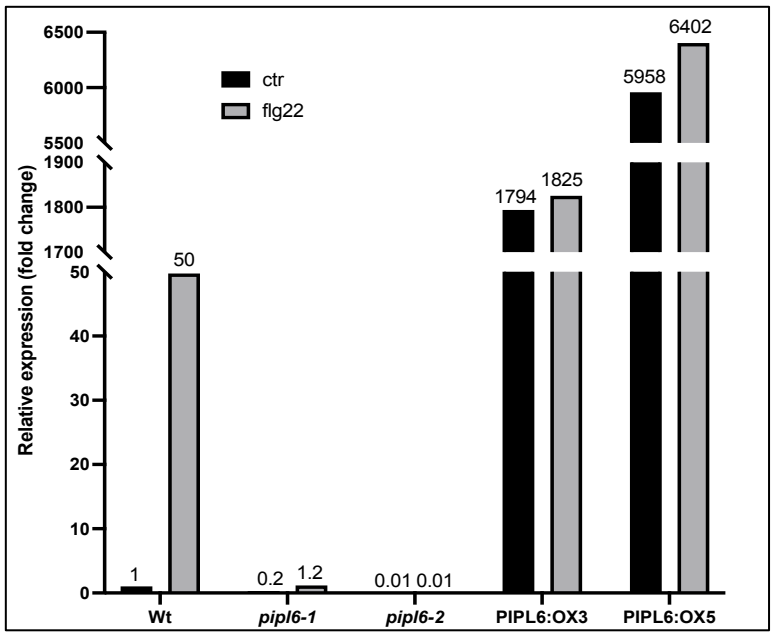


Figure 6: qPCR: Expression of *PIPL6* in knock-out and overexpression lines.
Seedlings were gown under long day conditions for 7 days prior to treatment with (flg22) or without (ctr) 100nM flagellin22 for 1 hour. Relative expression of *PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6*) was quantified by RT-qPCR in relation to the reference gene (*TIP41*). Calculations were performed using qBase+ and the relative expression was scaled to wild-type (Columbia-0) (Wt). Numbers indicated relative expression for the respective treatment with one biological replicate.

Expression of *PIPL6* was also visualized by RT-PCR (**Figure 7**). The primers were designed to amplify the whole coding sequence from 12 base pairs upstream to 10 base pairs downstream of the coding region of *PIPL6*. *Actin2* was used as a reference gene. The RT-PCR confirmed the results from RT-qPCR. The product for *pipl6-1* treated with flagellin was about the same intensity as wild-type control. No product was detected for *PIPL6* in *pipl6-2*. Overexpression lines showed high intensity bands for *PIPL6.* No obvious differences in expression of *Actin2* were detected except for PIPL6:OX5 which the PCR product was slightly fader than the rest of the samples.

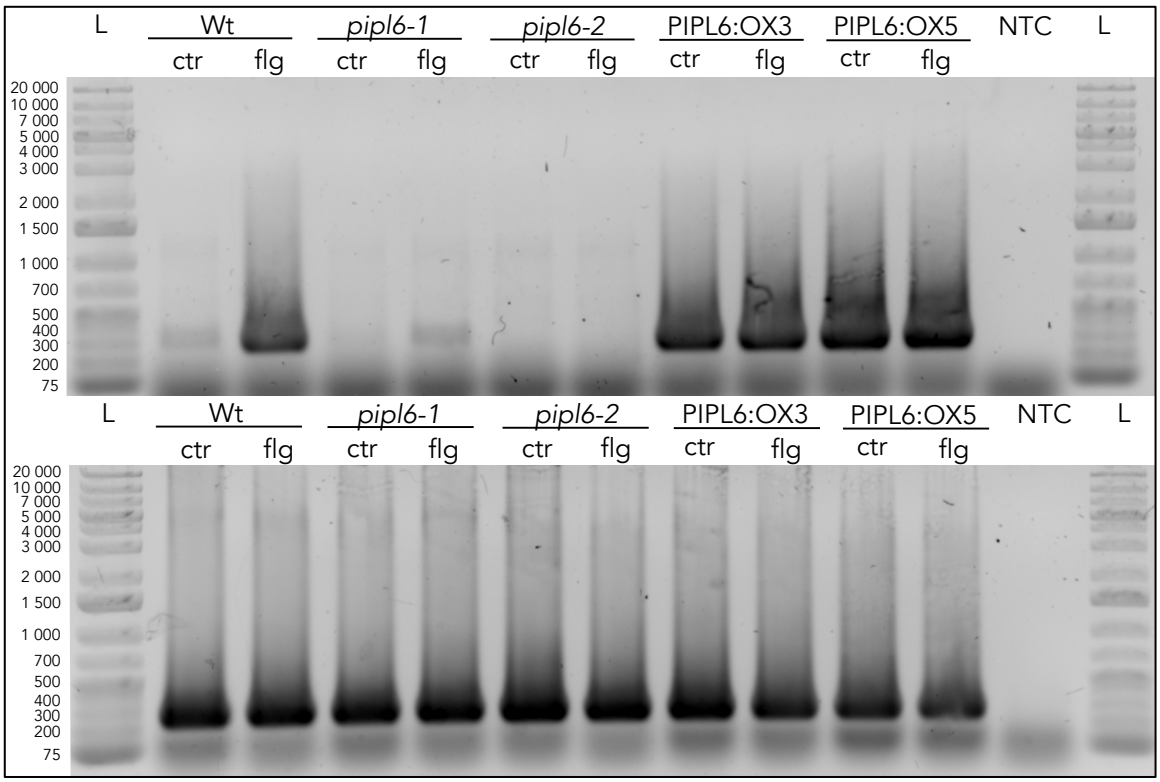


Figure 7: RT-PCR: Expression of *PIPL6* in knock-out and overexpression lines. Seedlings were gown under long day conditions for 7 days prior to treatment with (flg22) or without (ctr) 100nM flagellin22 for 1 hour. RT-PCR was performed and expression of *PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6)* (top) or the reference gene *Actin2* (bottom) were visualized. Negative control (NTC) and 1kb plus ladder (L) were indicated and number of base pairs for the respective bands were listed.

3.2 *PIPL6* was highly and transiently induced by elicitors (PAMPs and DAMPs)

In order to assess whether PIPL6 were involved in regulation of the plant immune response, comprehensive time-series experiments were performed to study transcriptional kinetics of *PIPL6* in response to exogenous application of flg22, chitin, PEP1 and paraquat in wild-type seedlings. The relative expression of *PIPL6* at different timepoints after treatments were quantified by RT-qPCR analysis (**Figure 8**). Flagellin, chitin and PEP1 caused a rapid increase of *PIPL6* expression. The expression were recovered to basal levels after 3 hours for chitin, while the induced expression was prolonged to 6 hours and after 24 hours for PEP1 and flagellin respectively. Both flagellin and chitin induced a peak of expression 1 hour after treatment, while PEP1 caused culmination of *PIPL6* expression faster with a peak at 30 minutes to 1 hour after treatment. The relative expression of *PIPL6* in response to paraquat was significantly decreased at 30 minutes and increased at 48 hours after treatment (**Figure 8D**).

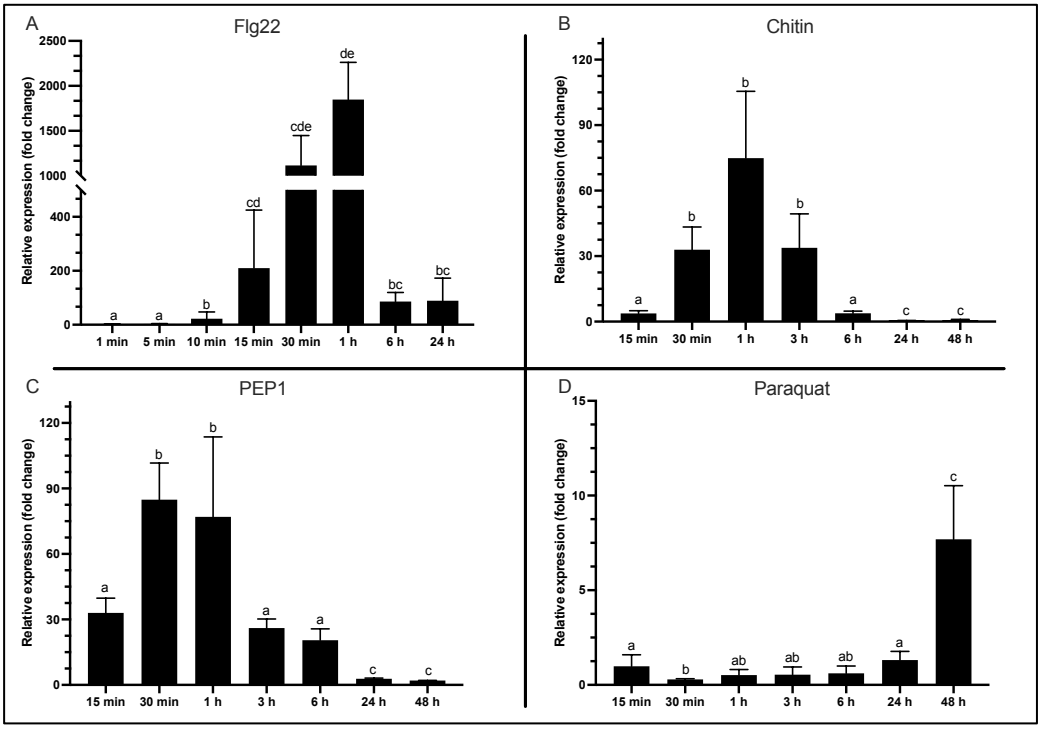


Figure 8: Timeseries of *PIPL6* expression in response to elicitors.
Relative expression of *PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6)* in wild-type (Columbia-0) seedlings treated with 100nM flagellin (flg22) (A), 500nM chitin hexamer (B), 100nM PLANT ELICITOR PEPTIDE 1 (PEP1) (C) and 5μM paraquat (D). The horizontal axis state minutes (min) and hours (h) after treatment was initiated. Transcriptional levels of *PIPL6* was quantified by RT-qPCR and relative expression was calculated based on Ct-values in relation to the reference gene (*TIP41*). Calculations was performed using qBase+ and one-way ANOVA analysis. The relative expression was scaled to the control which was harvested at the first timepoint for all treatments. Bars and error bars represented mean and standard error respectively calculated from 3 biological replicates. Letters indicated significant difference at (P<0,0001).

3.3 Exogenous application of synthetic PIPL6 peptide differentially regulated expression of immune marker genes

The observations from **Figure 8** promoted further evaluation of the effect of PIPL6 peptide on the expression of well-known immune marker genes. The transcriptional effect of PIPL6 was analysed by application of synthetic PIPL6 peptide on wild-type seedlings. The peptide was designed from the conserved C-terminal of PIPL6 prepropeptide. Three hours after PIPL6 peptide treatment, expression of immune marker genes were calculated relative to the control treatment (**Figure 9**). The treatment showed that many immune related genes were significantly induced upon PIPL6 treatment including the JA marker gene *PDF1.2* among with the receptors *FRK1* and *RLK7* and several *WRKYs*, *PIPs* and *JAZs*. Interestingly, genes involved in SA biosynthesis (*ICS1*) and responsive marker gene (*PR1*) were not significantly regulated.

The treatment was also performed using 100nM concentrations of the synthetic PIPL6 peptide (data not shown). Only few genes were tested at both 100nM and 1μM concentrations. The data showed that 1μM concentration had significant effect on the transcription of many marker genes. Therefore, 1μM was used as a standard for the rest of the analysis.

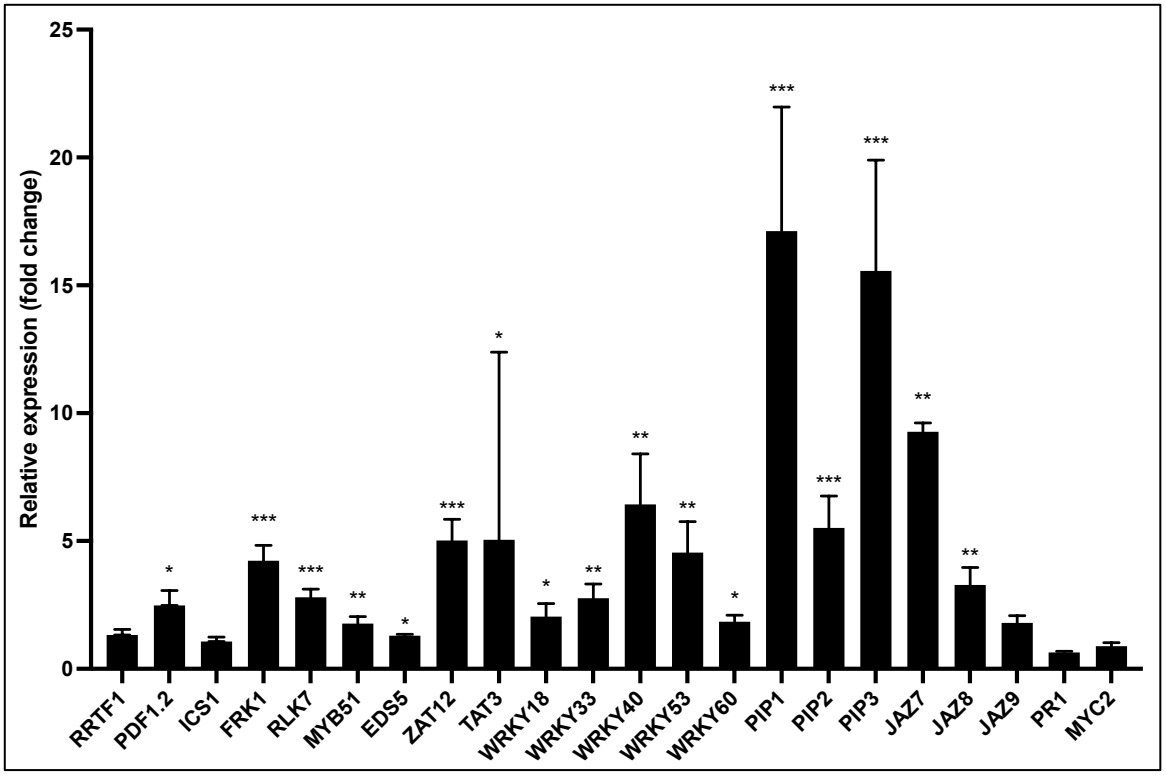


Figure 9: Exogenous application of synthetic PIPL6 peptide regulated expression of immune related marker genes.
Wild-type (Columbia-0) seedlings were grown under long day conditions for 14 days prior to treatment with 1μM PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6) synthetic peptide (or water as control) for 3 hours. Relative expression was quantified by RT-qPCR in relation to the reference gene (*TIP41*). Calculations were performed using qBase+ and unpaired t-test. Bars and error bars represented mean and standard error respectively calculated from four biological replicates. Expression of immune marker genes (horizontal axis) was scaled to control treatment and significant difference was indicated by *(P≤0,05), **(P≤0,01) and ***(P≤0,001). Abbreviations were; *REDOX RESPONSIVE TRANSCRIPTION FACTOR 1 (RRTF1), PLANT DEFENSIN 1.2 (PDF1.2), ISOCHORISMATE SYNTHASE 1 (ICS1), FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1), RECEPTOR-LIKE KINASE 7 (RLK7), MYB DOMAIN PROTEIN 51 (MYB51), ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5), ZINC FINGER PROTEIN 12 (ZAT12), TYROSINE AMINO TRANSFERASE 3 (TAT3), WRKY transcription factor (WRKY), PAMP-INDUCED SECRETED PEPTIDE (PIP), JASMONATE-ZIM-DOMAIN PROTEIN (JAZ), PATHOGENESIS-RELATED PROTEIN 1 (PR1)* and *MYC2 transcription factor (MYC2).*

The RNAs from three biological replicates of both control and treatment with synthetic PIPL6 peptide were sequenced by illumina sequencing. The performed RNA sequencing gave a total number of ~254 million pair-end reads of 150 base pairs in length. The three treated samples contained between 27 – 35 million pair-end reads, while the three control samples contained between 41 -63 million pair-end reads. Mapping resulted in an average of 90.6 % of the reads (~ 80 million) from the treated samples mapped to the TAIR10 reference genome assembly, while the control samples had a somewhat lower mapping rate of 61,9 % (~90 million reads). The lower mapping rate was mainly due to contamination during the library production process and did not affect the further analysis. A total 55.1 % of the genes registered in TAIR10 were included in the analysis, while 44.9 % were filtered out due to insufficient read counts. The results showed upregulation of 1300 genes and downregulation of 530 genes (data not shown). A selection of differently expressed genes were presented in **Table 1**. The data showed that PIPL6 were involved in regulation of expression of 20 different *WRKYs*, 4 *MAP-kinase* protein genes and 3 *JAZs*. Only the genes known to be involved in plant

immunity are shown in **Table 1**. The data also showed upregulation of *PAD3, FRK1, PLANT PDF1.3, RLK7, MYB DOMAIN PROTEIN 51 (MYB51), BIK1, SUGAR RESPONSIVE RLK 1 (SRR1), RLK5 (HAESA), RBOHD, ACS2* and *ACS6*. Downregulation of *PAL4* was also detected. The data also indicated regulation of several *ERFs*, *cytochrome P (CYP)* genes and *VQPs* (data not shown).

Table 1: A selection of differently regulated genes after treatment with PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6)

Locus	Description	Fold change	P-value
WRKY transcription factors			
AT4G31800.1	WRKY18 (WRKY transcription factor 18)	2,13	1,37E-16
AT4G01250.1	WRKY22 (WRKY transcription factor 22)	1,69	0,00017032
AT4G18170.1	WRKY28 (WRKY transcription factor 28)	2,22	3,50E-06
AT5G24110.1	WRKY30 (WRKY transcription factor 30)	10,73	2,02E-21
AT2G38470.1	WRKY33 (WRKY transcription factor 33)	2,84	3,42E-42
AT1G80840.1	WRKY40 (WRKY transcription factor 40)	7,87	4,29E-37
AT3G01970.1	WRKY45 (WRKY transcription factor 45)	3,38	4,59E-11
AT5G49520.1	WRKY48 (WRKY transcription factor 48)	2,44	2,30E-05
AT4G23810.1	WRKY53 (WRKY transcription factor 53)	3,59	1,36E-36
AT2G25000.1	WRKY60 (WRKY transcription factor 60)	2,51	4,57E-09
AT3G56400.1	WRKY70 (WRKY transcription factor 70)	1,49	8,21E-08
AT5G13080.1	WRKY75 (WRKY transcription factor 75)	5,91	1,52E-05
MAP-kinase			
AT1G01560.1	MPK11 (MAP kinase 11)	2,82	0,000239703
JAZ-protein			
AT1G19180.2	JAZ1 (jasmonate-zim-domain protein 1)	2,67	1,38E-12
AT2G34600.1	JAZ7 (jasmonate-zim-domain protein 7)	9,27	4,18E-09
AT1G70700.2	JAZ9 (jasmonate-zim-domain protein 9)	1,70	9,92E-06
Other immune related genes			
AT3G26830.1	PAD3 (PHYTOALEXIN DEFICIENT 3)	22,64	1,55E-47
AT2G19190.1	FRK1 (FLG22-induced receptor-like kinase 1)	3,56	1,02E-20
AT2G26010.1	PDF1.3 (plant defensin 1.3)	3,72	1,79E-05
AT1G09970.2	RLK7 (receptor-like kinase 7)	2,63	1,17E-35
AT1G18570.1	MYB51 (myb domain protein 51)	2,50	2,51E-24
AT2G39660.1	BIK1 (botrytis-induced kinase1)	1,86	6,45E-17
AT1G74360.1	SRR1 (Sugar Responsive RLK 1)	3,85	2,01E-41
AT4G28490.1	HAESA (receptor-like kinase 5)	1,71	7,84E-13
AT5G47910.1	RBOHD (respiratory burst oxidase homologue D)	1,47	8,20E-07
AT3G10340.1	PAL4 (phenylalanine ammonia-lyase 4)	0,65	3,72E-06
AT1G01480.2	ACS2 (1-aminocyclopropane-1-carboxylic acid (acc) synthase 2)	8,42	5,71E-07
AT4G11280.1	ACS6 (1-aminocyclopropane-1-carboxylic acid (acc) synthase 6)	2,46	9,49E-18

3.4 WRKY transcription factors as positive regulators of *PIPL6*

Several WRKY transcription factors showed elevated mRNA levels in response to exogenous application of synthetic PIPL6 peptide (**Figure 9** and **Table 1**). Sequence analysis of the promoter region of *PIPL6* revealed presence of several WRKY transcription factor binding elements (W-box elements). Two such W-box elements (5´-(C/T)TGAC(T/C)-3´) were found at positions -155 and -744 base pairs upstream of the coding sequence for *PIPL6* (**Figure 5**). This observation led to analysis of potential involvement of WRKYs in regulation of *PIPL6*.

Possible regulation by WRKY transcription factors were analysed by investigating the expression of *PIPL6* in single, double and triple knock-out mutants of WRKY18, 40 and 60 and the WRKY33 knock-out line. cDNAs were obtained from previous experiment by Najafi et al. (2020). Seedlings were grown on agar plates and tissue was harvested after 2 weeks for expression analysis (**Figure 10**). Interestingly, the data showed that *PIPL6* expression dropped about 90% in *wrky33*. *PIPL6* expression was found significantly downregulated in double mutants *wrky18/40,* but not in any of the other single, double or triple mutants of WRKY18, 40 and 60. Downregulation of *PIPL6* expression in knock-out mutants indicated positive regulation by WRKY transcription factors.

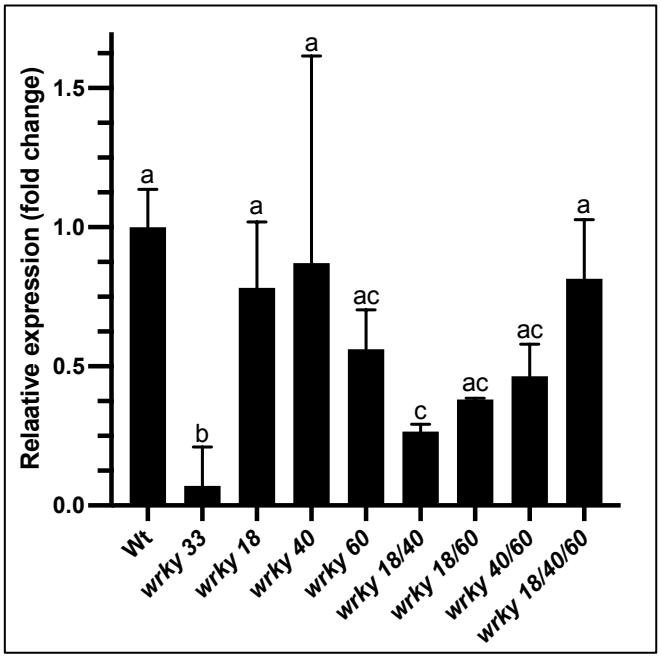


Figure 10: WRKY transcription factors functioned as regulators of *PIPL6*.
Relative expression of *PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6)* in WRKY transcription factor knock-out lines (horizontal axis), both single, double and triple mutant lines scaled to wild-type (Wt) (Columbia-0) expression. Seedlings were grown on agar plates for 2 weeks before shoot tissue was harvested. Relative expression of *PIPL6* was quantified by RT-qPCR and calculated based on Ct-values in relation to the reference gene (*TIP41*). Calculations were performed in qBase+ using a one-way ANOVA analysis. Bars and error bars represented mean and standard error respectively calculated from 3 biological replicates. Letters indicated significant difference at (P<0,0001).

3.5 RLK7 functioned as a major receptor for PIPL6

Previous studies have shown that RLK7 functioned as a major receptor for PIP1 in regulation of plant immunity (Hou et al. 2014). Because of this, RLK7 was viewed as a potential receptor for PIPL6. This hypothesis was strengthened by the fact that *RLK7* expression was significantly induced three hours after PIPL6 treatment (**Figure 9** and **Table 1**). In addition to the *RLK7*, expression of the receptor *SRR1* was found significantly induced (**Table 1**). To test whether signalling initiated by PIPL6 requires RLK7 and/or SRR1, PIPL6 peptide treatment was repeated on wild-type and *rlk7* and *srr1* lines followed by gene expression analysis of marker genes induced by the PIPL6 peptide (**Figure 11**). The difference in gene expression between treatment and control for both lines were displayed together with the wild-type response in the same experiment (**Figure 11**). *Rlk7* had reduced induction of *JAZ7* and almost absent expression of *PAD3* and *WRKY40* compared to wild-type response (**Figure 11A**). The results also revealed that in *srr1*, the expression of *JAZ7* was not upregulated in response to PIPL6 peptide treatment and the elevated expression of *WRKY40* was abolished compared to wild-type response (**Figure 11B**). The reduced response in *srr1* and rlk7 lines to synthetic PIPL6 peptide indicated a potential function for these proteins as receptors of PIPL6.

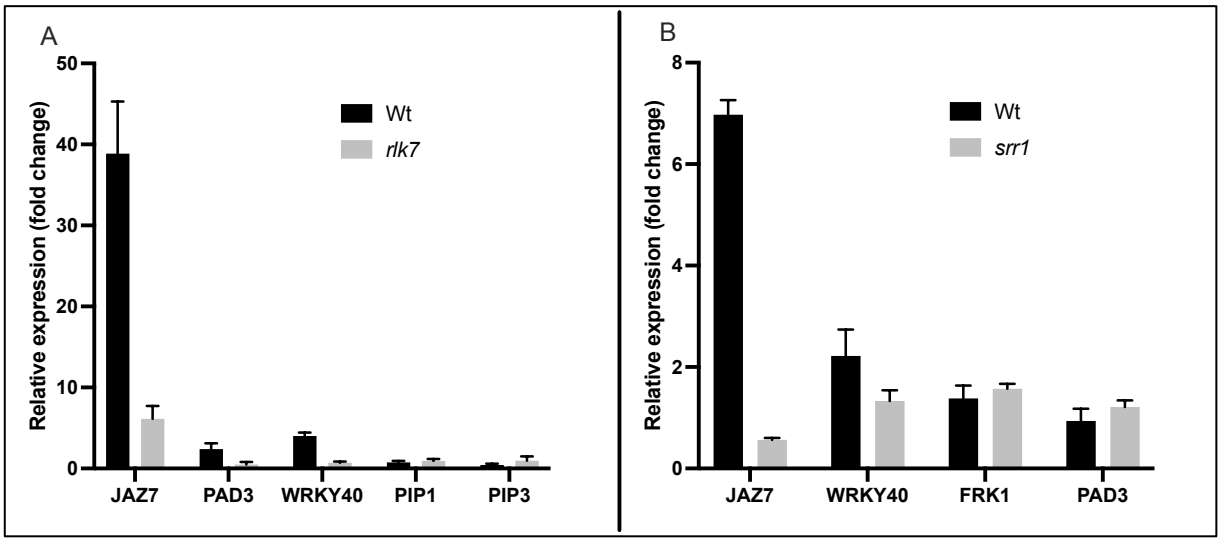


Figure 11: Signalling response by PIPL6 was altered in *rlk7* and *srr1* knock-out lines. Seedlings were grown under long day conditions for 14 days prior to treatment with 1μM PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6) synthetic peptide (or water for control) for 3 hours. Relative expression was quantified by RT-qPCR in relation to the reference gene (*TIP41*). Calculations were performed using qBase+ and Mann-Witney analysis. Bars and error bars represented mean and standard error respectively calculated from three biological replicates at 95% confidence interval. Expression of immune marker genes (horizontal axis) was scaled to control treatment for each genotype. Wild-type (Wt) expression compared to RECEPTOR-LIKE PROTEIN KINASE 7 mutant *rlk7* (A) and SUGAR RESPONSIVE RECEPTOR-LIKE KINASE 1 mutant *srr1* (B) was shown. Abbreviations were; *JASMONATE-ZIM-DOMAIN PROTEIN 7 (JAZ7), PHYTOALEXIN DEFICIENT 3 (PAD3), WRKY transcription factor 40 (WRKY40)*, *PAMP-INDUCED SECRETED PEPTIDE (PIP)* and *FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)*.

3.6 PIPL6 altered immune related phenotypes

The functionality of PIPL6 was investigated by analysing if knock-out or overexpression of the *PIPL6* gene were sufficient to cause any altered macroscopic growth or developmental phenotypes compared to wild-type. To answer this question, both growth and pathogen assays were performed.

3.6.1 Root growth and growth inhibition in *PIPL6* knock-out and overexpression lines

Previous studies with PIP1 and PIP2 have revealed that overexpression lines of these two peptide genes negatively affected root growth (Hou et al. 2014). This promoted the idea to analyse whether knock-out or overexpression of *PIPL6* alter the root growth. Root growth for knock-out and overexpression lines were measured under both long and short day conditions (**Figure 12A-B**). The results showed that *pipl6-2* had significantly shorter roots under long day conditions for day 6 to 10, but not after 14 days (**Figure 12A**). *Pipl6-1* had significantly longer roots under short day conditions (**Figure 12B**). No differences were detected in overexpression lines compared to wild-type seedlings later than day 6.

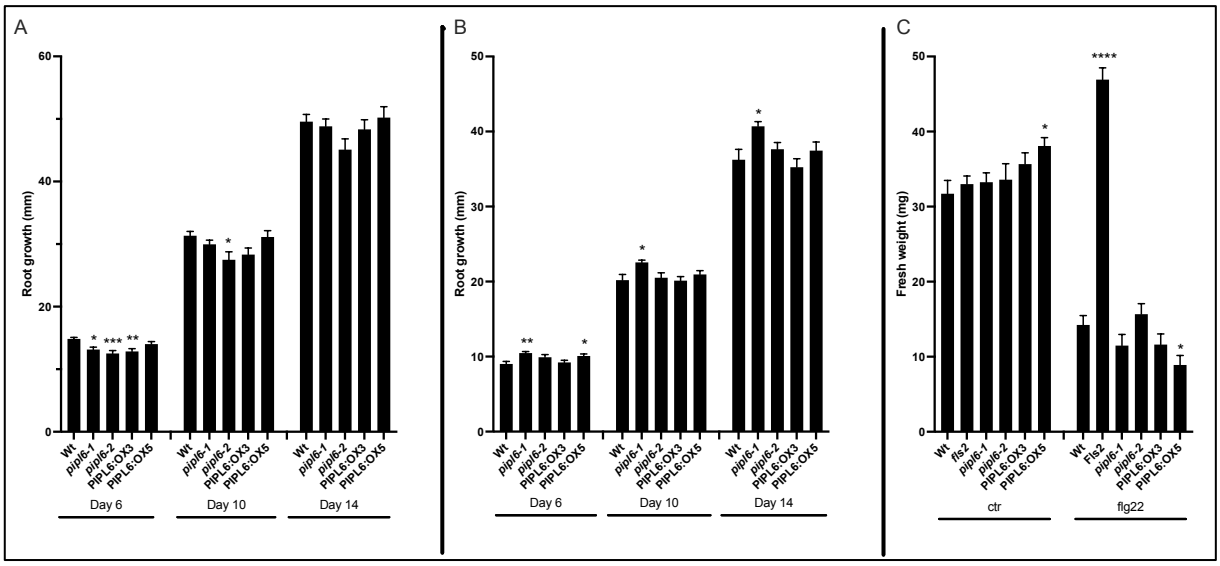


Figure 12: Root growth and growth inhibition by flagellin in *PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6)* knock-out and overexpression lines.
Seedlings were grown on vertically positioned square plates in long day (A) or short day (B) conditions. Root length was calculated in ImageJ after 6, 10 and 14 days. Growth inhibition by flagellin (C) was performed by placing 5 days old seedlings in liquid medium with (flg22) or without (ctr) 100nM flagellin22 for 10 days before fresh weight was measured. The flagellin insensitive line *fls2* was used as a negative control for growth inhibition. Bars and error bars indicated mean and standard error respectively. The experiments included twenty-five replicates for long day conditions (A), eighteen replicates for short day conditions (B), and twenty-four (ctr) and forty-eight (flg22) replicates for growth inhibition detection (C). One-way ANOVA analysis was performed for all experiments and significant difference to wild-type was indicated by *(P≤0,05), **(P≤0,01), ***(P≤0,001) and ****(P≤0,0001).

Studies have shown that growth of *Arabidopsis* seedling was negatively affected by the presence of different classes of elicitors including flg22 (Gómez-Gómez and Boller 2000). Growth inhibition by the elicitor flg22 was evaluated by measurement of fresh weight of seedlings in the presence of flagellin. Few changes in fresh weight were present in the knock-out and overexpression lines compared to the wild-type (**Figure 12C**). PIPL6:OX5 had significant increased fresh weight in control conditions while significantly lower fresh weight compared to the wild-type after treatment. This established a larger effect of the growth inhibition for PIPL6:OX5 to its respective control treatment than compared to wild-type with flagellin. Similar effects were seen in PIPL6:OX3, but no significant differences were detected compared to wild-type for either control or treatment. No significant difference was detected for the knock-out lines *pipl6-1* and

pipl6-2. The flagellin insensitive line *fls2* was used as a control for growth inhibition and did not have growth inhibition by flagellin.

3.6.2 Altered production of reactive oxygen species in *PIPL6* knock-out and overexpression lines

Perception of the elicitor flg22 is normally accompanied by a rapid and transient ROS production. This production were completely diminished in the flagellin insensitive line *fls2* (Felix et al. 1999; Gómez-Gómez and Boller 2000). ROS production has been shown to be altered in *PIP3* overexpression lines (Najafi et al. 2020). This influenced the idea to analyse alteration in ROS production for *PIPL6* knock-out and overexpression lines. A luminol-based assay was performed to evaluate ROS production in *PIPL6* knock-out and overexpression lines compared to the wild-type response (**Figure 13**). *Fls2* was used as a negative control. The assay was repeated 14 times with inconsistent results (Appendix 5). **Figure 13** represents the assay that best captured the trends in ROS production for the different genotypes. The assay showed a decreased level of ROS production for *pipl6-1* and *pipl6-2* compared to wild-type and increased ROS production for PIPL6:OX5. *Fls2* showed no elevated ROS production confirming the flagellin induced ROS production.

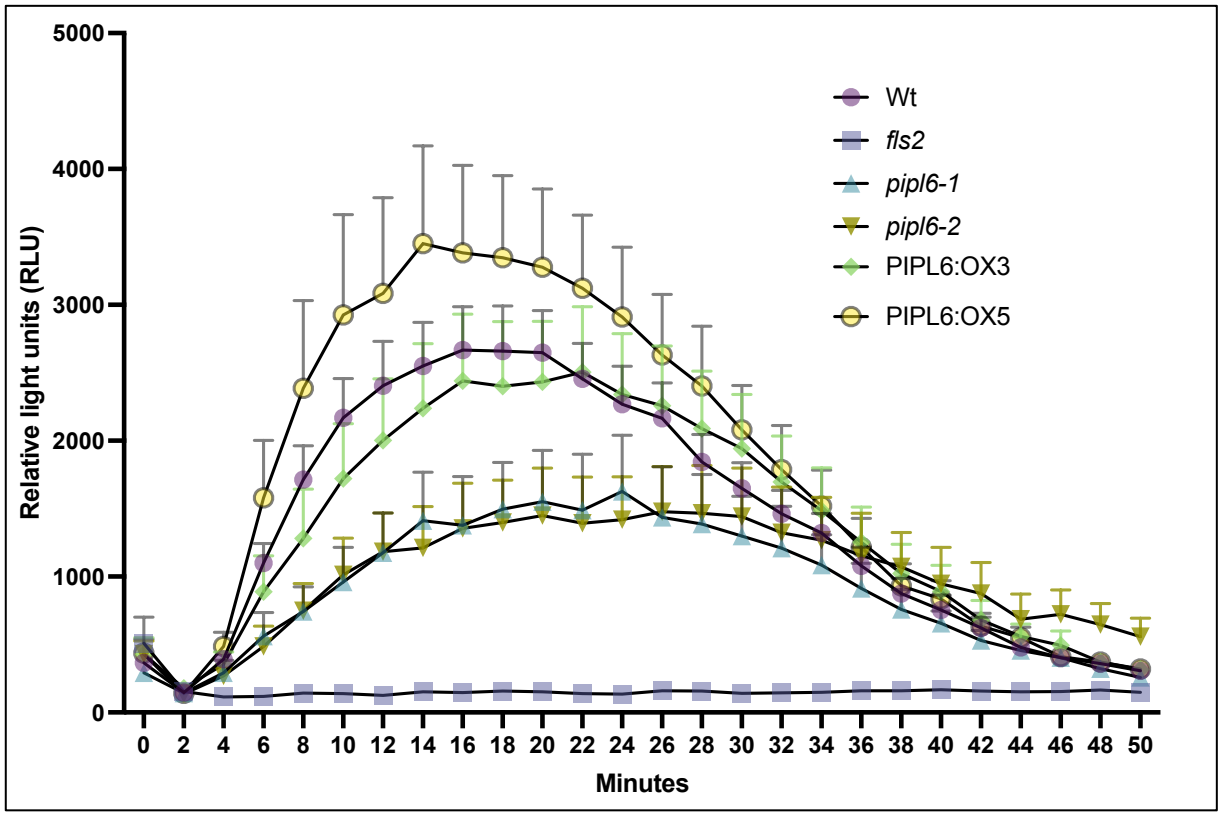


Figure 13: Knock-out and overexpression of *PIPL6* caused changes to the ROS production in response to flg22 treatment.
Production of reactive oxygen species (ROS) induced by flagellin in *PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6*) knock-out and overexpression lines compared to wild-type (Wt) (Columbia-0). Leaf disks of 4-5 weeks old plants were exposed to 100nM flagellin. ROS production was measured as luminescence recorded over time as relative light units (RLU). The curves represented trend lines between mean RLU for each timepoint and error bars indicated standard error calculated from twelve biological replicates. The flagellin insensitive line *fls2* was used as a negative control.

3.6.3 Infection with *Pseudomonas syringae*

Presented results from gene expression analysis on PIPL6 treated seedlings, growth inhibitory assay and ROS detection led to interest in examining if knock-out and/or overexpression of *PIPL6* could alter the response to pathogens with different lifestyles. To approach this, *PIPL6* knock-out and overexpression lines were infected with *Pseudomonas syringae* pv. *tomato* DC3000 and susceptibility was quantified as colony forming units (CFUs) after 3 days of cocultivation (**Figure 14**). The experiment was repeated ten times with highly variable results (**Figure 14A**). To look at the overall trend of the data, the ratio of different genotypes to wild-type was calculated. The data showed a significant difference for *pipl6-2* and PIPL6:OX5 compared to wild-type (**Figure 14B**). The trend for *pipl6-1* and PIPL6:OX3 was difficult to observe, this corresponds with the lack of significant increase in genotype/Wt ratio for these lines, but the trend was still an increase in susceptibility. *Pipl6-2* and PIPL6:OX5 had a significant genotype/Wt ratio of about 1.15. In case of a wild-type measurement of 32 460 000 CFUs per seedling (corresponding to a log10 of 7.5), a genotype/Wt ratio of 1.15 would give an increase in 4 869 000 CFUs per seedling.

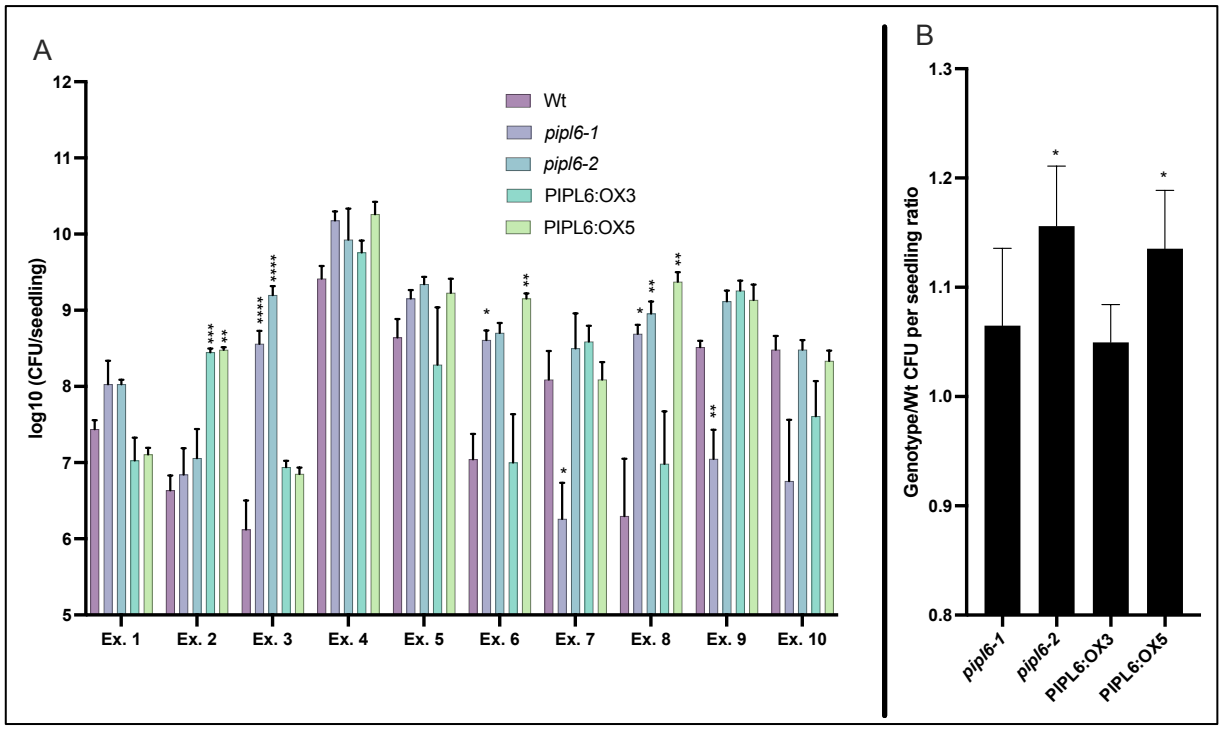


Figure 14: Susceptibility to infection by *Pseudomonas syringae* in *PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6)* knock-out and overexpression lines.
Seedlings were grown for 7 days prior to co-cultivation with *Pseudomonas syringae* pv. *tomato* (DC3000) for 3 days. Susceptibility was measured and calculated as colony forming units (CFUs) per seedling with five biological replicates per experiment. Log10 CFU per seedling for ten individual experiments (Ex.) were performed (A) and the ratios of different genotypes to wild-type (Wt) were calculated for the ten experiments (B). Bars and error bars represented mean and standard error respectively. One-way ANOVA analysis was performed for each experiment and significant difference to wild-type was indicated by *(P≤0,05), **(P≤0,01), ***(P≤0,001) and ****(P≤0,0001). Significant difference in Genotype/Wt ratio was calculated by a two-tailed t-test with P<0,05.

3.6.4 Infection with *Botrytis cinerea*

Botrytis cinerea isolate 2100 (Spanish type) was used as a necrotrophic pathogen to evaluate the effect of knock-out and overexpression of *PIPL6*. Wild-type plants have been reported to be resistant against this strain (Birkenbihl et al. 2012). *PIPL6* knock-out and overexpression lines were infected with spores collected from *B. cinerea* and susceptibility was quantified by qPCR (**Figure 15A**), hormone extraction (**Figure 15B**) and phenotypic imaging (**Figure 15C**). For the quantification of the susceptibility towards *B. cinerea*, the amount of genomic DNA ratio between the pathogen and *Arabidopsis* was measured by qPCR (**Figure 15A**). High degree of variations were observed in the data as illustrated by the error bars. No significant differences were calculated between the genotypes, including *wrky33*. PIPL6:OX3 appeared to have lower *Bc*CutA/*At*SKII ratio compared to Wild-type, but this difference was non-significant. The knock-out line *wrky33* was used as a susceptible control and exhibited the highest ratio of *Botrytis* DNA to plant DNA, however still not significantly different from wild-type based on the one-way ANOVA (Analysis of variance).

The Plant´s responses to pathogens are driven by a hormonal balance and production of phytoalexins. Among phytohormones, SA, JA and abscisic acid (ABA) plays major roles in the regulation of plant immunity. Hence, the levels of these three hormones and camalexin the major antifungal phytoalexin was tested 48 hours after *Botrytis* infection (**Figure 15B**). Only wild-type was tested in control conditions. The results showed that in response to *Botrytis* infection, PIPL6:OX5 produced similar levels of SA, JA, ABA and camalexin compared to the wild-type after treatment. On the other hand, *pipl6-1* had significantly lower expression of JA and camalexin, but same level as SA and ABA compared to wild-type plants after treatment. *Wrky33* had lower levels of camalexin but higher levels of ABA, SA and JA compared to wild-type after treatment. For ABA, only *wrky33* showed significant elevated levels compared to the other genotypes. For camalexin, increased levels of about 3000-5000ng/g fresh weight were observed in *pipl6-1* and *wrky33*, and even higher levels of camalexin induction in wild-type and PIPL6:OX5 (>9000ng/g fresh weight) were recorded. In addition, quantification of JA in wild-type and PIPL6:OX5 revealed induced levels of around 600-1000ng/g fresh weight, while *wrky33* exhibited very high levels of JA induction with 2000ng/g fresh weight. *Pipl6-1* did not have an induced production of JA.

For the phenotype assay, leaves of all genotypes were inoculated by *B. cinerea* spores (**Figure 15C**). The assay revealed that both knock-out lines were more susceptible to infection compared to the wild-type. Furthermore, four days after inoculation, wild-type showed necrosis symptoms at the infection site but the lesion size did not expand further. Both overexpression lines showed a tendency to be more resistant towards this strain of *Botrytis* (smaller lesions). Since wild-type has been recorded to be resistant for this strain, it was not possible to evaluate if overexpression lines were more resistant.

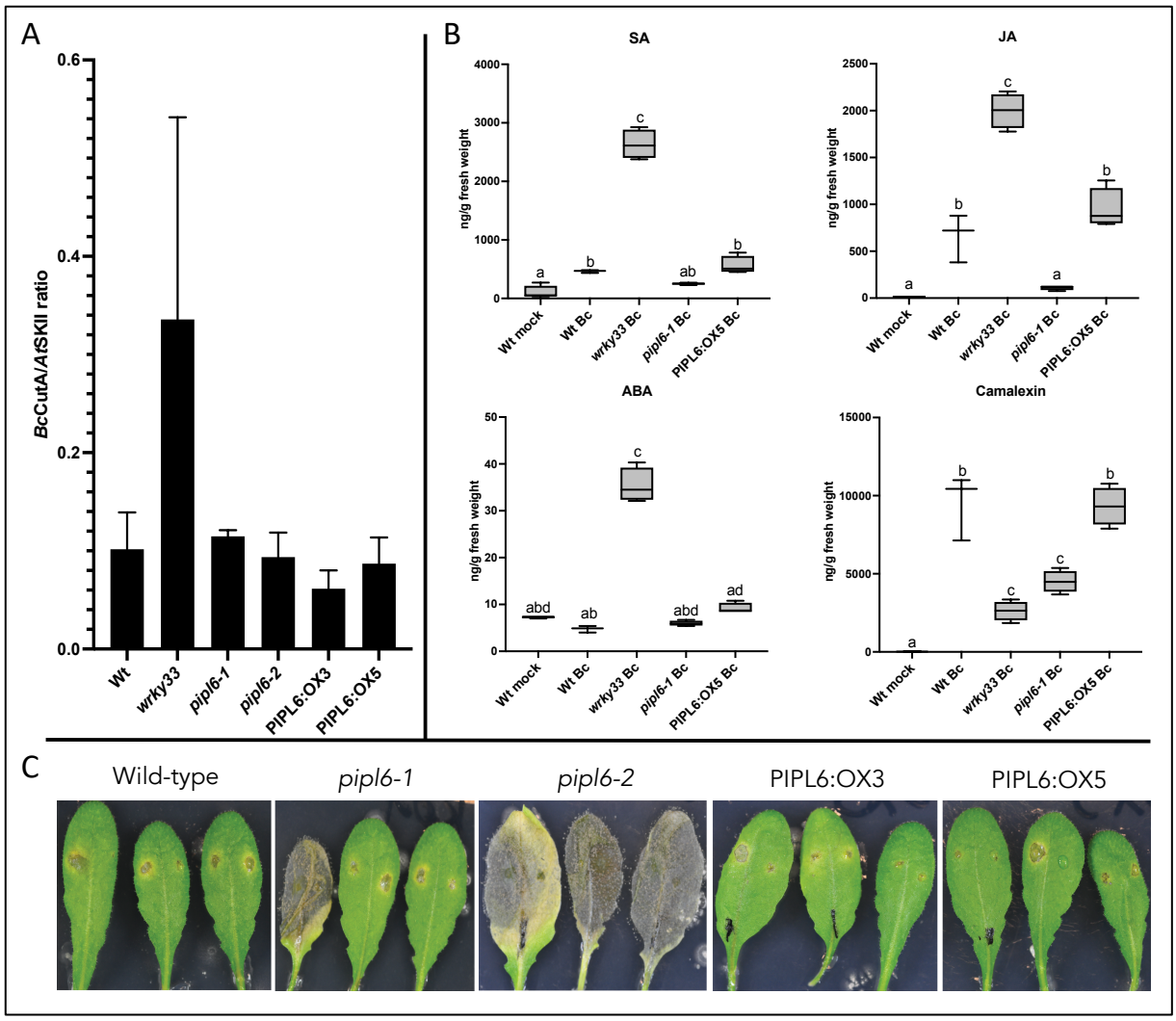


Figure 15: Effect of *Botrytis cinerea* isolate 2100 in *PAMP-INDUCED SECRETED PEPTIDE-LIKE 6 (PIPL6)* knock-out and overexpression lines.
(A) 5 weeks old plants were inoculated with droplets of *B. cinerea* spores in 3 days. The relative abundance of *B. cinerea* and *Arabidopsis* DNA was determined by qPCR using pathogen specific (*Bc*CutA) and plant-specific (*At*SKII) primers. Bars and error bars represented mean and standard error respectively with three biological replicates. Statistical difference was calculated by a one-way ANOVA analysis and no significant difference was detected between the different genotypes. (B) 5 weeks old plants were sprayed with (Bc) or without (mock) *B. cinerea* spores and the hormones abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) and the antifungal phytoalexin camalexin was extracted and quantified in ng/g fresh weight with four biological replicates (only three for wild-type (Wt) treatment). Statistical analysis was performed by one-way ANOVA analysis and significant difference (P<0,05) was indicated by letters. (C) 5 weeks old plants were inoculated with droplets of *B. cinerea* spores and pictures were taken 7 days later.

4 Discussion

Traditionally, phytohormones were considered as essential in the regulation of plant intercellular signalling. However, many studies during the last two decades have revealed the importance of secreted peptide hormones as signalling agents in cell-to-cell communication. Peptide hormones have been shown to be involved in many key developmental processes, such as maintenance of stem cell niches at shoot and root meristem, organ abscission, cell separation, cell elongation, cell proliferation and differentiation, gravitropism and defence (Matsubayashi 2011). About a 1000 open reading frames (ORFs) with potential to produce secreted signalling peptides have been identified in the *Arabidopsis* genome (Matsubayashi 2011). Among different classes of identified/known signalling peptides, are the PIP and PIPL family with eleven members (Vie et al. 2015). Analysis of expression patterns and *in-silico* data showed that some members of this family have the potential to act as DAMPs and ability to elicit the immune response in *Arabidopsis thaliana*. Despite their proposed functional importance, many genes of the *PIP-LIKE (PIPL)* family and peptide products thereof have not yet been characterized (Vie et al. 2015). One of the members in this family was *PIPL6*. Previous experiments showed that *PIPL6* was highly induced in response to the elicitor chitin and the aphid *Brevicoryne brassicae* as well as the protein synthesis inhibitors cycloheximide and anisomycin (Vie et al. 2015). This was considered a strong indicator that PIPL6 might play an important role in the plant immune response. The presented study was directed towards contributing to the investigation of PIPL6 in regulation of plant immunity utilizing methods from physiology, genetics, transcriptomics, metabolomics and phenomics.

4.1 Screening and confirmation of knock-out and overexpression lines

To be able to analyse the functional role of PIPL6, phenotypical changes due to knock-out and overexpression of the gene were analysed. Two independent T-DNA and transposon insertion lines were obtained that were expected to have a T-DNA insertion in the promoter region (SALK_106769) and in the coding sequence (Wiscseq_DsLoxHs144_04E.1). A T-DNA insertion can be thousands of base pairs long (Krysan et al. 1999) and could possibly disrupt the gene function. An insertion in the promoter region would generate the opportunity to alter the expression of the target gene by inhibiting or enhancing transcription factor binding activity. An insertion in the coding sequence would possibly disrupt the gene function and alter the reading frame of the gene or simply produce a non-functional protein.

In order to investigate the function of any target genes, homozygous lines were required. Both *pipl6-1* (SALK_106769) and *pipl6-2* (Wiscseq_DsLoxHs144_04E.1) homozygous lines were screened using a PCR-based method (**Figure 4**). Homozygous lines were selected based on the presence of PCR products generated by the combination of gene specific and T-DNA specific primers. The combination of gene specific LB and RP primers did not give a PCR product for *pipl6-1* and *pipl6-2*, indicating that the T-DNA was disrupting both alleles of *PIPL6.* Confirmation of insertion site by sanger sequencing revealed that the T-DNA in the *pipl6-2* line (Wiscseq_DsLoxHs144_04E.1) was not

inserted in the coding sequence for the *PIPL6* gene (**Figure 5**). The T-DNA was mapped 8 base pairs downstream of the coding sequence. However, mRNA amplification by both RT-qPCR (**Figure 6**) and RT-PCR (**Figure 7**) showed no expression of *PIPL6* for this genotype. The T-DNA and gene specific primers used to map the insertion site for *pipl6-2* amplified the region from the end of the T-DNA insertion to the PR primer (placed 178 base pairs downstream of the *PIPL6* coding sequence). There was however a possibility that the T-DNA insertion caused a small deletion in the Arabidopsis DNA of the *PIPL6* gene and that the start of the insertion site was inside the *PIPL6* coding sequence. This was consistent with the RT-qPCR and RT-PCR data, since the reverse primer used for amplification started 10 base pairs downstream of the coding sequence. A T-DNA insertion starting 8 base pairs downstream of the coding sequence would only give a 2 base pairs miss match at the 3´end of the primer used for RT-qPCR and RT-PCR. The mismatch could alter binding activity but probably not exclude binding. This indicated that the T-DNA insertion had also deleted some of the genomic Arabidopsis DNA possibly disrupting the *PIPL6* gene. The *pipl6-2* line was considered as a knock-out line in the analyses performed in the present study.

Insertion site was confirmed to be placed in the promoter region of *PIPL6* for the *pipl6-1* (SALK_106769) line.(**Figure 5**). The insertion caused a downregulation, but not knock-out of the *PIPL6* gene (**Figure 6** and **Figure 7**). The levels of *PIPL6* expression was very low and the level induced by flagellin only reached to wild-type control levels, where as the wild-type had 50 folds elevated expression in response to flagellin treatment. The result suggested that although T-DNA insertion in *pipl6-1* only knocked-down the *PIPL6* expression, the *PIPL6* response would possibly not be sufficient to trigger PIPL6 downstream events when induced by a pathogen or elicitor.

Analysis of *PIPL6* overexpression lines showed very high expression levels of *PIPL6* compared to wild-type (**Figure 6** and **Figure 7**). PIPL6:OX3 and PIPL6:OX5 had about 2 000- and 6 000-fold expression respectively scaled to wild-type control. These lines highly qualified as overexpression lines for *PIPL6*.

4.2 *PIPL6* was highly and transiently induced by elicitors (PAMPs and DAMPs)

The plant´s response to pathogens is triggered by recognition of PAMPs and DAMPs at the plasma membrane by receptors. Upon perception of PAMPs and DAMPs, a set of biochemical reactions are initiated that leads to transcriptional reprogramming and a proper immune response based on the nature of the invader. Among the regulated genes are genes encoding for propeptides of signalling peptides such as PEP1 and PIP1 which in turn functions as amplifiers to activate local and systemic defence mechanisms (Hou et al. 2014). To investigate the function of the PIPL6 peptide in plant defence, gene expression of *PIPL6* elicited by PAMPs and DAMPs was recorded in timeseries for individual elicitors. Flagellin and chitin are derived from bacterial and fungal cells respectively and are recognized as PAMPs by PRRs. Recognition by such a signal lead to to change in gene expression in response to the pathogen infection. *PIPL6* showed rapidly increased expression induced by both flagellin and chitin (**Figure 8A-B**). A similar response to flagellin was reported for *PIP1-3* (Najafi et al. 2020). PEPs are also produced as a response to PAMPs by PRRs and binds to its corresponding membrane bound receptors (PEPR1 and PEPR2) to induce a immune response similar to the flagellin induced response (Bartels et al. 2013; Bartels and Boller 2015). *PIPL6* expression was shown to be rapidly induced by PEP1 (**Figure 8C**) with a sharp and transient response

similar to the response induced by flagellin and chitin. The rapid response of *PIPL6* to PEP1 corresponded with other PEP1 responsive genes (Yamaguchi et al. 2010). These observations suggested that PIPL6 functioned downstream of flagellin and chitin signalling likely leading to an enhanced immune response. The induction of *PIPL6* expression could be even further elicited by the flagellin induced *PEP1* expression.

Paraquat is a common herbicide and is used to induce oxidative stress by promoting a rapid formation of ROS (Vicente et al. 2001). The expression of *PIPL6* slightly decreased 15 minutes after treatment and slightly increased at 24 hours after treatment with paraquat compared to the control. The result indicated that increased *PIPL6* expression in response to elicitors is not likely mediated by the oxidative burst following perception of PAMPs and DAMPs. *PIPL6* expression 24 hours after treatment was not significantly different from the control and the increase at 48 hours had a high variance, but still showed a significant difference to control treatment. It was hard to determine the cause of this increase, but it could result from cross talk between oxidative stress and immune signalling pathways involving elevation of transcription factors that regulated the expression of *PIPL6*.

4.3 Exogenous application of synthetic PIPL6 peptide differentially regulated expression of immune marker genes

Exogenous application of synthetic PIPL6 peptide on the wild-type seedlings followed by RNA sequencing profiling reviled significant regulation of 1830 genes. Among these genes, 22 genes were tested by RT-qPCR. *FRK1* have been accepted as a marker gene for the early response to MAMPs/PAMPs. Three hours after PIPL6 peptide treatment, *FRK1* expression was significantly induced (**Figure 9** and **Table 1**). This finding was consistent with results reported by Najafi et al. (2020) showing that exogenous applications of PIP1, PIP2 and PIP3 synthetic peptides resulted in induction of *FRK1* expression. *FRK1* expression has been shown to be the result of activated WRKY transcription factors in response to a flagellin induced MAPK cascade pathway (Asai et al. 2002). This suggested that PIPL6 initiated a signalling pathway that share similarities to the MAPK cascade downstream of flagellin signalling. Among the significantly upregulated genes were also the plant defensins *PDF1.2* and *PDF1.3*. Plant defensins have been shown to have antifungal activity and *PDF1.2* is considered a strong marker gene for activation of JA signalling and is transcriptionally activated by WRKY33 (Thomma et al. 2002; Glazebrook 2005; Li et al. 2016). Classification of differentially regulated genes by synthetic PIPL6 peptide also showed significant induction of many RLKs including *RLK7, HAESA* and *SRR1*. RLK7 have been shown to be the major receptor for PIP1 (Hou et al. 2014). On the other hand, HAESA have been recognized as a receptor for the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and IDA-LIKE (IDL) peptides which are closely related to PIP and PIPL peptides (Stenvik et al. 2008; Vie et al. 2015). Considering the same concepts and the fact that closely related peptide ligands are likely to interact with members closely related RLKs families (Butenko et al. 2009), RLK7 could be hypothesized as a potential receptor for the other members of PIP/PIPL family of peptides including PIPL6. Flagellin treatment have been shown to induce the expression of its own receptor *FLS2*, *BIK1* and *PROPEP*s through ET responsive transcription factors (Li et al. 2016). Additional to induction of RLKs, synthetic PIPL6 peptide also induced transcription of *BIK1* (**Table 1**) and *PIP1-3* (**Figure 9**).These results suggested that PIPL6 might induce the expression of its own receptor as well as coreceptor *BIK1* and other signalling peptides. The expression of *RLK7*, *HAESA, SRR1* and *PIP1-3* may also be

induced by similar mechanisms as with flagellin signalling, but the transcription activators for these genes were not examined. The expression of *PROPEP*s were not tested by qPCR, but none of the *PROPEP* genes were identified as differentially regulated genes in the RNA sequencing data. The induced expression of *PIP1-3* by PIPL6 indicated that PIP and PIPL proteins may induce expression of each other through positive and negative feedback-loops to fine-tune the immune response.

Analysis of the RNA sequencing data of the PIPL6 treated wild-type seedlings, showed that a number of 20 WRKY transcription factors were differentially regulated. The WRKY family of transcription factors have been comprehensively studied for their critical roles in the regulation of plant defence responses. WRKY18, WRKY40, WRKY60, WRKY75, WRKY33 and others have been shown to actively regulate the expression of numerous genes in response to pathogens and flg22 treatment (Pandey and Somssich 2009). Loss of WRKY33 function led to enhanced activation in the SA-related host response as well as reduced activation in the JA-associated responses when infected with *B. cinerea* (Birkenbihl et al. 2012). WRKY33 have been shown to be essential for defence towards necrotrophic pathogens and to induce expression of many immune related genes including *PAD3, PDF1.2, ACS2* and *ACS6* (Birkenbihl et al. 2012; Lai and Mengiste 2013; Li et al. 2016; Chen et al. 2020). PAD3 is a key component of biosynthesis of the antimicrobial compound camalexin (Schuhegger et al. 2006). *PAD3* was detected as one of the highly upregulated genes by synthetic PIPL6 peptide (**Table 1**). This suggested that synthetic PIPL6 peptide triggered a pathway that is involved in the induction of camalexin biosynthesis, likely through activation and increased transcription of WRKY33. PIPL6 treatment also resulted in upregulation of *ACS2* and *ACS6* (**Table 1**). ACS2 and ACS6 are rate-limiting enzymes in ET biosynthesis (Li et al. 2016). This suggested that PIPL6 may induce ET biosynthesis by activation and/or transcription of the WRKY33 transcription factor. An increased ET biosynthesis in response to PIPL6 could explain the induction of many *ERFs* present in the RNA sequencing data as well as the increased expression of *BIK1* that is normally induced in response to ET. The results also showed that several *JAZ* genes were induced upon PIPL6 exogenous application. JAZ proteins repress JA signalling (Robert-Seilaniantz et al. 2011). JAZ8 has also been reported to repress WRKY75, a positive regulator of JA-mediated plant defence towards necrotrophs (Chen et al. 2020). Both *JAZ8* and *WRKY75* were induced by synthetic PIPL6 peptide. The regulatory effect of JAZs as well as JAZ8 on the function of WRKY75, indicated PIPL6 as a good candidate for fine-tuning of the plant immune response. Plants carefully regulate their response to pathogens, meaning that a disruption in gene expression could potentially interfere with the balance of responses that make the plants resistant towards certain pathogens.

The plant´s response to pathogens and insects are also regulated by the production of secondary metabolites such as glucosinolates. Biosynthesis of different glucosinolates are under control of MYB transcription factors (Seo and Kim 2017). *MYB51* is shown to be upregulated in response to treatment with synthetic PIPL6 peptide (**Figure 9** and **Table 1**). MYB51 is a transcription activator that activate *CYP*-genes involved in indolic glucosinolates (IGS) biosynthesis and modification that cause callose deposition at the infection site (Li et al. 2016; Gigolashvili et al. 2007). Application of synthetic PIPL6 peptide also showed upregulation of many *CYP*-genes, this might be a result of induction of *MYB51* transcription. This indicated that synthetic PIPL6 peptide possibly led to increased glucosinolates biosynthesis and callose deposition.

Synthetic PIPL6 peptide also showed an increased expression of *RBOHD* (**Table 1**) that have been shown to be responsible for the oxidative burst upon infection (Li et al. 2016). *RBOHD* transcription has been reported to be induced by chitin and WRKY transcription factors (Albert et al. 2006; Li et al. 2016). The increased expression of *RBOHD* could be a result of WRKY transcriptional regulation initiated by PIPL6.

PIPL6 also induced transcription of several *VQP* encoding genes. VQPs may inhibit WRKY transcription factors but are degraded when MPK3 and MPK6 are activated upon elicitor perception (Li et al. 2016). This indicated that PIPL6 may be involved in first elevation of the immune response and also attenuation of the response by induced transcription of inhibitors. The PTI response is transient and regulatory factors that decrease the response could be important to not prolong the expression and activity of related genes and proteins. Many of the responses are also composed of positive feedback-loops, as well as priming of further immune elevating signalling. Negative regulation was seen in FLS2 that was degraded within 1 hour upon flagellin elicitation (Li et al. 2016) and could also be present in PIPL6 regulatory mechanisms.

There are two enzymatic SA biosynthesis pathways that include either isochorismate synthase (ICS) or phenylalanine ammonia lyase (PAL) (An and Mou 2011). Expression of *PAL4* was shown to be downregulated (**Table 1**) and *ICS1* expression was slightly but not significantly downregulated by the PIPL6 peptide treatment (**Figure 9**). Considering ICS1 as the major pathway in SA production, it seemed that synthetic PIPL6 peptide application did not affect the SA synthesis three hours after treatment. However, the possible effect of PIPL6 of SA production and signalling at later time points was not ruled out.

Expression of genes encoding several MAP-kinases were also induced by synthetic PIPL6 peptide, including *MPK11* (**Table 1**). A MAPK cascade is an important part of PTI signalling and is also induced transcriptionally by PRRs recognition of PAMPs and DAMPs (Li et al. 2016). An induction of MAP-kinases along with other immune responsive genes support the theory that PIPL6 signals similar to the flagellin response and induction of *PIPL6* by flagellin may lead to further elevation of the initial PTI against pathogens.

Although expression of mRNA is a good indicator that a protein is more abundant in response to treatment, there are not always corelations between levels of mRNAs and their corresponding protein levels. This have to be carefully considered in the interpretation of the data obtained from transcriptomic analysis.

Synthetic peptides are not processed *in-vivo* and might lack processing event like proteolytic processing, sulfidation, hydroxylation and glycosylation that can influence the activity of the mature native peptide (Stührwohldt and Schaller 2019). Hydroxylation of prolines have been shown to be crucial for the function of IDA, the closely related family of PIP and PIPL peptides (Butenko et al. 2014). Glycosylation of the hydroxyprolines further increased IDA´s receptor affinity (Ohyama et al. 2009). Proline hydroxylation have also been shown to contribute to the activity of PIP1 (Hou et al. 2014). Hence, post-translational modifications may also be important for the activity of PIPL6.

4.4 WRKY transcription factors as positive regulators of *PIPL6*

Several WRKY transcription factors are activated upon PAMP recognition by PRRs and the following MAPK cascade. WRKY transcription factors bind to W-box elements in the promotor region of their targets to either enhance or inhibit expression of the genes (Li et al. 2016; Pandey and Somssich 2009). WRKY transcription factors often work in positive feedback-loops where expression lead to amplification of the immune signal (Pandey and Somssich 2009). Several *WRKY transcription factor* genes was expressed upon PIPL6 perception (**Figure 9** and **Table 1**). **Figure 5** showed that two W-box elements were placed in the promoter region of *PIPL6*. The W-box elements were located at -155 and -744 base pairs upstream of the coding sequence. These binding sites were strong indicators that WRKY transcription factors could be involved in the regulation of *PIPL6* expression. WRKY transcription factors have been shown to be major transcription activators of *PEPs*, but repressors for *PIP1, PIP2* and *PIP3* expression (Logemann et al. 2013; Najafi et al. 2020). To investigate the role of WRKY transcription factors in the regulation of *PIPL6*, the gene expression in *WRKY* knock-out lines were analysed (**Figure 10**). The results showed that the expression of *PIPL6* is downregulated and almost diminished in the *wrky33* mutant. This indicated that WRKY33 plays a positive role in the regulation of *PIPL6*. The expression of *WRKY33* was also induced in response to exogenous application of PIPL6 (**Figure 9**). These observations suggest that PIPL6 is involved in a positive feedback-loop for *WRKY33* transcription and protein activity. In addition, gene expression analysis showed that in single knock-outs of *wrky18*, *wrky40* and *wrky60, PIPL6* expression was not significantly reduced compared to wild-type. However, double knock-out *wrky18/40* had significantly lower expression than wild-type, indicating a possible cooperation between these proteins for regulation of *PIPL6*. This corresponds with the fact that WRKY18, 40 and 60 have been shown to cooperate and form homo and hetero-dimers (Xu et al. 2006; Chen et al. 2010). Interestingly, the triple mutants *wrky18/40/60,* as well as the double mutants *wrky18/60* and *wrky40/60* did not show significant difference to wild-type. However, the double mutants appeared to be decreased. Since *PIPL6* is normally expressed at very low levels, an analysis of induced expression in the mutants could give a more certain indicator of their possible role in regulation of *PIPL6*. The non-regulative effect of the triple mutant was hard to explain. According to Xu et al. (2006), the triple mutant gave an enhanced effect of regulation. It is possible that these WRKY transcription factors are regulatory elements of other genes that also effect the *PIPL6* expression, and a knock-out of these genes reverse the regulatory effect on *PIPL6*. The results suggested that *PIPL6* was positively regulated by WRKY33 and possibly a cooperative induction by WRKY18 and WRKY40, but further analysis were needed to conclude on their effect on *PIPL6* expression.

4.5 RLK7 functioned as a major receptor for PIPL6

LRR-RLKs belong to the super family of RLKs consisting of about 600 members in the *Arabidopsis* genome. RLKs functions as antennas to monitor internal and external changes and to control fine-tuned physiological responses of plants to internal and external stimuli. Based on the structural diversity of extracellular domains, RLKs are classified into different subgroups. LRR-RLKs represents the largest group of RLKs with approximately 220 members in *Arabidopsis*. RLK7 belongs to that LRR-RLK superfamily XI. This subfamily contains many well-known receptors (Shiu and Bleecker 2001). PIPL6 belongs to a class of post-translationally modified peptides (Vie et al. 2015). Post-translationally modified peptides are secreted to the apoplast to function as a signalling molecules by binding to transmembrane receptors (Tabata and Sawa 2014). PIP1 have

been shown to initiate its function by binding to RLK7 and induce immune responses (Hou et al. 2014). For this reason, RLK7 was considered as a potential receptor for PIPL6. RLK7´s role as a potential receptor was examined by exogenous application of synthetic PIPL6 peptide in *rlk7* knock-out line and comparison of expression marker genes relative the wild-type response. Analysis of gene expression revealed that mutation in *RLK7* repressed the induction of *JAZ7, PAD3* and *WRKY40* that were present in the wild-type (**Figure 11**). This suggested that RLK7 plays a role as a receptor in PIPL6 triggered signal transduction. However, induction of marker genes were not eliminated completely, suggesting that RLK7 was not the only receptor that mediated PIPL6 signalling. Najafi et al. (2020) determined that RLK7 is not likely a major receptor for PIP3. This suggested that multiple RLKs might function as receptors for the PIP and PIPL family of peptides.

Among the upregulated genes in response to synthetic PIPL6 peptide treatment was the receptor *SRR1.* SRR1 have been characterized for its possible function in extracellular sugar sensing and signalling (Najafi 2015). A potential function of SRR1 as a receptor for PIPL6 was examined using the same method as for RLK7. The results showed that mutation in *SRR1* resulted in abolished *JAZ7* induction in response to PIPL6 exogenous application as well as *WRKY40* that exhibited lower induction compared to the wild-type (**Figure 11**).

Decreased or eliminated effect of PIPL6 signalling was confirmed in both lines, suggesting that both RLK7 and SRR1 are potential candidates as receptor proteins for PIPL6 signalling. To confirm RLK7 and SRR1 as PIPL6 receptors, further studies are necessary. Direct binding of PIPL6 to RLK7 and SRR1 should be confirmed by analysis of protein interaction and binding activity analyses.

4.6 PIPL6 altered immune related phenotypes

4.6.1 Root growth and growth inhibition in *PIPL6* knock-out and overexpression lines

Root growth analysis of *PIP1* and *PIP2* overexpression lines, as well as effects of synthetic PIP1 and PIP2 peptides have been shown to inhibit root growth (Hou et al. 2014; Ghorbani et al. 2015). Root growth of *PIPL6* knock-out and overexpression lines revealed only a minor change under both long and short day conditions (**Figure 12A-B**). This indicated that unlike PIP1 and PIP2, PIPL6 had no effect on root growth in *Arabidopsis*.

Previous studies have shown that presence of elicitors in the growth medium could affect normal growth and development of *Arabidopsis (Gómez-Gómez and Boller 2000).* Growth inhibition by flagellin was examined in *PIPL6* knock-out and overexpression lines by measuring the fresh weight after 10 days of exposure to flagellin (**Figure 12C**). PIPL6:OX5 exhibited an increased growth inhibition by flagellin. PIPL6:OX3 also had the same trend, but the difference was not significant. Since immune responses are highly energy demanding, activation can alter the growth of the plant. An increased growth inhibition in *PIPL6* overexpression lines suggested a heightened immune response compared to wild-type. No significant differences were seen for *PIPL6* knock-out lines. It has been shown that overexpression of *PIP3* had increased growth inhibition, while knock-out line of *PIP3* had no alteration in phenotype compared to wild-type (Najafi et al. 2020), this was consistent with the results obtained for PIPL6.

Overexpression of a gene do not directly reflect on the level of active proteins present in the mutant lines. Protein abundance and activity are also influenced by regulation of post-transcription, translation and degradation as well as protein processing, transportation and presence of cofactors (Vogel and Marcotte 2012). Protein overexpression have also been shown to cause resource overload as well as stoichiometric imbalance that both lead to cellular defects (Moriya 2015).This have to be carefully considered in the interpretation of the results and the effect of overexpression lines.

4.6.2 Altered production of reactive oxygen species in *PIPL6* knock-out and overexpression lines

ROS production is one of the early responses to a variety of pathogens and elicitors. Production of ROS in *PIPL6* knock-out and overexpression lines were measured in response to flagellin. High degrees of variations in the data were observed from experiment to experiment, indicating low repeatability of the result. The calculations were performed by taking the average of twelve replicates for each time point, this meant that one or two very high measurements could increase the average to a large extent. The experiment was performed multiple times with extra precaution to leaf and leaf disk treatment. The order of the leaf disks in the 96-well plate was also changed randomly to avoid positional effects. Leaf disks for multiple assays prepared at the same time would give different outputs, indicating that the observed data was not consistent. Interestingly, the high variability was mostly observed in the wild-type. This made the evaluation and comparisons for the other lines challenging. The high ROS measurements for wild-type were seen when the plants were getting older, at the end of the fifth week of growth under short day conditions. There was also inconsistency of the ROS production for the other genotypes. ROS production is a general plant response to many signals including growth, development and biotic- and abiotic stress (Baxter et al. 2014). The variation in the data therefore may be influenced by many factors that can account for the low repeatability of the results.

Although the data was not very consistent for all genotypes, trends in ROS production indicated that knock-out lines produced less ROS than wild-type and overexpression lines generated more or equal ROS production comparted to wild-type. **Figure 13** showed the ROS assay performed that best represented the trend in the data. Due to lower ROS production in *PIPL6* knock-out lines and equal or higher ROS production in overexpression lines, one could conclude that PIPL6 might be involved in ROS production. It has been shown that PEP1 enhances ROS production and PIP1 have been showed to have similar effect (Flury et al. 2013; Hou et al. 2014). However, a study reported by Najafi et al. (2020) showed no elevation or higher ROS production in *pip3* knock-out lines and lower ROS production in *PIP3* overexpression lines compared to wild-type. The observed discrepancy between PIP3 and PIPL6 may be explained by their differential response to activity of WRKY transcription factors. While WRKY33, WRKY18 and WRKY40 were suggested to be positive regulators of *PIPL6* they have been shown to be negative regulators of *PIP3* (Najafi et al. 2020). The possible increased ROS production in *PIPL6* overexpression lines could be related to their increased growth inhibition by flagellin. Production of ROS could be a result of a heightened immune response and give altered energy demands (Flury et al. 2013).

4.6.3 Infection with *Pseudomonas syringae*

The results obtained from molecular studies led to examination of phenotypical changes in *PIPL6* knock-out and overexpression lines by pathogens with different life styles. The hemi-biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 was co-cultivated with 7 days old seedlings for 3 days. The results showed that both *PIPL6* knock-out and overexpression lines were more susceptible to infection than wild-type. The results from *pipl6-2* and PIPL6:OX5 was most consistent and these lines were highly and significantly more susceptible to infection compared to wild-type. Higher variation was observed for *pipl6-1* and PIPL6:OX3, and they were not significantly different from the wild-type response. This difference between *pipl6-1* and *pipl6-2* could be a result of downregulation, but not knock-down of *pipl6-1.* Differences between the overexpression lines could be a result of a positional effect of the T-DNA insertion in the host genome. Previous studies showed that PIP1 and PIP2 peptides decreased susceptibility to *P. syringae* (Hou et al. 2014) while *PIP3* overexpression lines had increased susceptibility to *P. syringae* (Najafi et al. 2020). The increased susceptibility as a result of both knock-out and overexpression of *PIPL6* could be explained by the role of PIPL6 peptide as a fine-tuner of the immune response towards *P. syringae*. The plant´s immune response is highly regulated and adapted to the invading pathogen. A disruption or interference of such a response could result in interruption of defence mechanisms against the pathogen. The high variance in the data could also indicate that PIPL6 was not involved in the plant´s response towards *P. syringae*. Since PIPL6 might repress *PR1* and *PAL4*, it might have no or little involvement in SA biosynthesis, a key component for defence against this pathogen.

The applied method for infection with *P. syringae* was highly dependent on the seedling size. More tissue would give more surface and carbon source for the pathogen and would result in more pathogen growth. No visible size differences were observed between the genotypes while performing the assay, but the fact that seedling size could influence the results was still present. A method described by Katagiri et al. (2002) perform inoculation on 5 weeks old plants instead of seedlings and could possibly eliminate the size effect as a potential error. Additionally, there might be differences in infection responses in seedlings and older mature plants. A method performed on fully grown plants could be used to further investigate and confirm the role of PIPL6 in the regulation of plant immunity against *P. syringae.*

4.6.4 Infection with *Botrytis cinerea*

B. cinerea is a common necrotrophic pathogen and attack various plant species. *B. cinerea* isolate 2100 is an avirulent strain for *A. thaliana* and the resistance against this strain is highly dependent of the function of WRKY33 (Liu et al. 2017). The phenotype against this strain of *Botrytis* was investigated in *PIPL6* knock-out and overexpression lines.

The quantification of susceptibility by qPCR showed no significant difference between the different genotypes (**Figure 15A**). Some inconsistency was observed between the wild-type and the other genotypes as the wild-type appeared to be susceptible. The non-significant difference between the susceptible control *wrky33* and wild-type indicated that the assay was not performed correctly. Using the same strain, significant differences between wild-type and *wrky33* have been reported (Najafi et al. 2020). The lack of significant susceptibility between the genotypes could be a result of high humidity or different light conditions prior to inoculation. The resistant phenotype of

wild-type was obtained in later experiments, and differences between the genotypes were more visible (**Figure 15C**). Because of the high susceptibility for the wild-type in the experiment and lack of symptoms for *wrky33*, the results of the *PIPL6* knock-out and overexpression lines were most likely not valid. This suggested that a quantitative difference between genotypes of *PIPL6* were still possible. The assay should be repeated to establish the effect between genotypes seen in the phenotype assay (**Figure 14C**).

Hormonal levels of *pipl6-1* and PIPL6:OX5 was measured 48 hours after spraying with *B. cinerea* spores (**Figure 15B**). Wild-type plants were also treated with only buffer as a control to measure hormonal responses. PIPL6:OX5 had similar hormonal and camalexin levels to wild-type in response to treatment, this could be an explanation of the resistant phenotype observed by droplet inoculation (**Figure 15C**). It has been shown that infection by *Botrytis* resulted in JA and SA induction 24 hours after inoculation (Rossi et al. 2011). Analysis of free JA and SA levels showed that the JA induction is completely abolished in *pipl6-1* compared to wild-type plants. The level of SA was lower in *pipl6-1* than wild-type, but not significantly lower. Although the difference was non-significant, one cannot rule out that the lower SA level was a result of the lack of SA signalling pathway genes seen in response to PIPL6 peptide treatment. The levels of camalexin in *pipl6-1* was at the same levels as *wrky33*, indicating that the low levels of camalexin in *PIPL6* knock-out lines could be linked to the function of the WRKY33 transcription factor. This was consistent with the suggested positive regulatory effect of WRKY33 on *PIPL6* (**Figure 10**). PAD3 is the critical enzyme in the biosynthesis of camalexin and it has been shown that *PAD3* expression is driven by WRKY33 (Schuhegger et al. 2007; Zhou et al. 2020; Qiu et al. 2008). Increased *PAD3* expression by exogenous application of PIPL6 peptide (**Table 1**), indicated that PIPL6 played a role in camalexin biosynthesis induction through activation of WRKY33. This was consistent with the repressed camalexin induction for *pipl6-1* in response to *B. cinerea* infection. ABA have been shown to be negatively regulated by WRKY33, and *wrky33* exhibited elevated ABA levels (Liu et al. 2015). This negative regulation was present in *wrky33* but not present in *pipl6-1* (**Figure 15B**) indicating that PIPL6 is not involved negative regulation of ABA. Although the data for the hormonal analysis were very promising for determining the effect of PIPL6 in regulation of different hormone levels, the control treatment for all genotypes are necessary to conclude that if the observed differences are caused by *B. cinerea* or is simply the result of constitutive expression of *PIPL6*.

After 7 days of *B. cinerea* inoculation, prominent differences was visible between the *PIPL6* lines (**Figure 15C**). *Pipl6-1* and *pipl6-2* showed higher susceptibility to *B. cinerea.* The differences between *pipl6-1* and *pipl6-2* could be explained by the low inducible expression of *PIPL6* in *pipl6-1* as the line was only a knock-down line and not knock-out line. The phenotype of *pipl6-2* was similar to the *wrky33*. Susceptibility to *B. cinerea* in knock-out/down lines was consistent with the disruption of the hormonal balance seen for *pipl6-1* and *wrky33* (**Figure 15B**). *PIPL6* overexpression lines may possess more resistance towards the pathogen, but it was not possible to detect using the *B. cinerea* isolate 2100 as the wild-type was resistant against this strain. Inoculation with a more virulent strain could be helpful in determining an elevated resistance by *PIPL6* over-expression. It was possible that also differences between genotypes would be detected by repetition of quantification of the *Bc*CutA/*At*SKII ratio in successful droplet assays. The difference in susceptibility visualized in **Figure 15C** were not detected by quantification of the *Bc*CutA/*At*SKII ratio (**Figure 15A**), another indicator that the quantification assay was not successful and should be repeated under different environmental conditions.

4.7 A suggested model for PIPL6 involvement in plant immunity

Based on the overall results obtained in this project, the function of PIPL6 in the regulation of plant immunity can be outlined (**Figure 16**). *PIPL6* expression is induced upon perception by PAMPs and DAMPs and is possibly regulated by WRKY33 and a combination of WRKY18 and WRKY40. *PIPL6* is likely translated as a prepropeptide and is processed and cleaved to the mature PIPL6 peptide in the apoplast where is can bind to its receptor. RLK7 and SRR1 are possible receptors for PIPL6. Perception of PIPL6 by its corresponding receptor/s (RLK7 and/or SRR1) triggers a new signalling cascade that reprogram expression of a high number of genes including many *WRKY*s, *JAZs*, *PAD3, MYB51, PDF1.2, ASC2, ACS6, BIK1, CYP*-genes and others. *WRKY33* is induced by PIPL6 and is possibly also the cause of increased levels of *PAD3, MYB51* and *ACS2/ACS6*. Induction of *PAD3* lead to increased camalexin biosynthesis and resistance towards the necrotrophic fungi *B. cinerea.* MYB51 increase the expression of *CYP*-genes that are involved in IGS biosynthesis and modification, and might cause an alteration in callose deposition. ACS2 and ACS6 induce ET synthesis that most likely further activate and induce ET-responsive genes including *BIK1*. PIPL6 might repress *PR1* and *PAL4* that indicate no or little involvement of PIPL6 in SA biosynthesis. JA biosynthesis is likely induced by PIPL6 signalling by an unknown mechanism. PIPL6 also increase transcription of *JAZ* genes that works as a negative feedback to control the JA induced response. The suggested mechanism of PIPL6 in immune signalling is illustrated in **Figure 16**. The pathways, genes and proteins investigated in this master´s thesis are indicated in blue while the suggested and possible pathways/mechanisms involved are indicated in red. The red lines and outcomes are based on already known mechanisms in similar signalling events. It remains to further investigate the red parts of the figure.

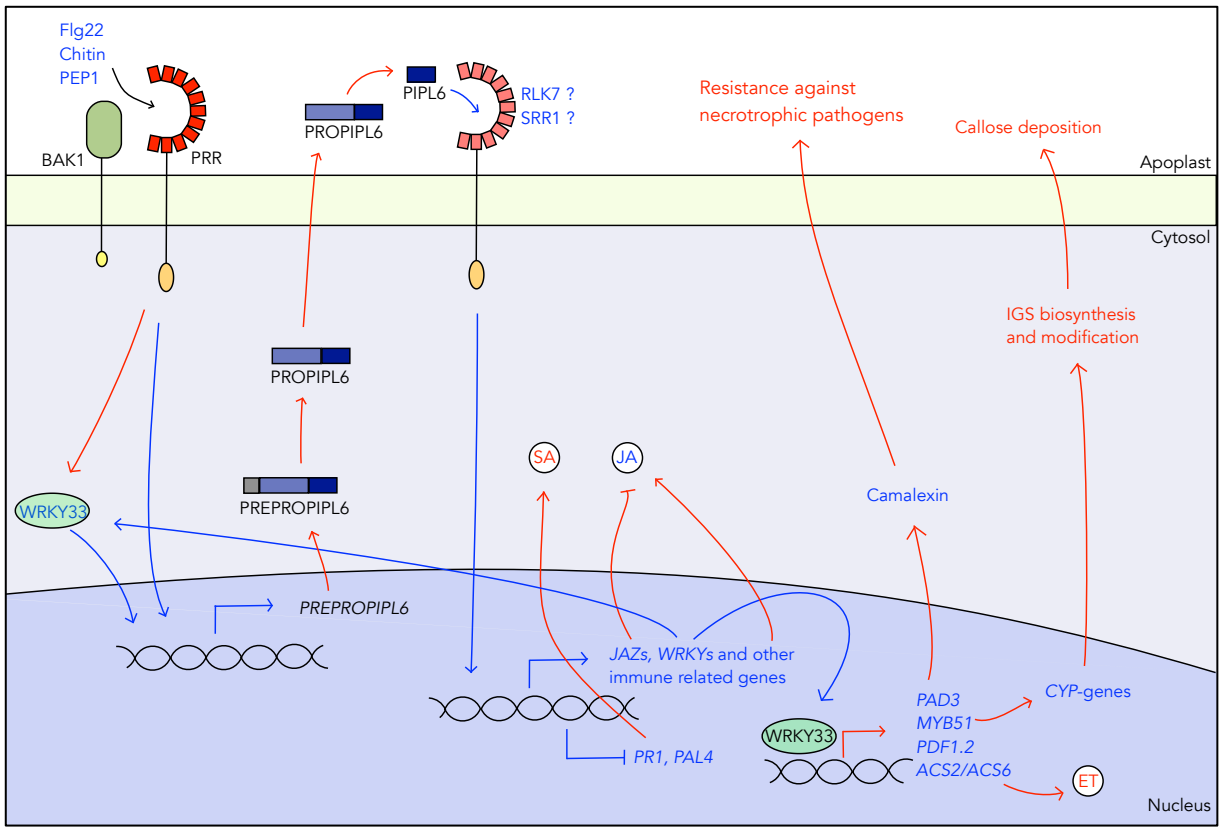


Figure 16: A suggested model for PIPL6 involvement in plant immunity.
Expression of *PAMP-INDUCED SECRETED PEPTIDE LIKE 6 (PIPL6)* is induced by the elicitors flagellin22 (flg22), chitin and the PLANT ELICITOR PEPTIDE 1 (PEP1). The elicitors binds to their respective pattern-recognition receptors (PRRs) and induce expression of *PIPL6*, possibly trough activation of the WRKY33 transcription factor. *PIPL6* is translated as a prepropeptide that is post-translationally cleaved, transported to the apoplast and proteolytically processed to the mature PIPL6 peptide that bind to its receptor RLK7 and/or SRR1. Perception of PIPL6 initiate transcriptional reprogramming and induce expression of many immune related genes that are involved in induction and repression of jasmonic acid (JA), genes involved in callose deposition and camalexin and ethylene (ET) biosynthesis, possibly by transcription and activation of WRKY and other transcription factors. PIPL6 also repress genes involved in salicylic acid (SA) biosynthesis. Known and suggested/possible pathways of the signalling are indicated in blue and red respectively. Abbreviations are as followed; *BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), JASMONATE-ZIM-DOMAIN PROTEINs (JAZs), PHYTOALEXIN DEFICIENT 3 (PAD3), PLANT DEFENSIN 1.2 (PDF1.2), RECEPTOR-LIKE KINASE 7 (RLK7), MYB DOMAIN PROTEIN 51 (MYB51), SUGAR RESPONSIVE RLK 1 (SRR1), PHENYLALANINE AMMONIA-LYASE 4 (PAL4), 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE (ACS)* and indolic glucosinolates (IGS)

5 Conclusion

This project aims to contribute to the investigation of PIPL6 in regulation of plant immunity and the molecular mechanisms behind it. A model for its function and signalling is outlined in **Figure 16**. The results suggest that PIPL6 plays a role in plant immune responses. The gene is expressed at low levels under normal conditions, but is highly and transiently induced by treatment with different plant elicitors. The results propose that *PIPL6* is positively regulated by WRKY transcription factors, and is likely to be a part of positive feed-back loops for enhancing their effect. PIPL6 is suggested to signal through the transmembrane receptors RLK7 and/or SRR1. Treatment with PIPL6 peptide induce expression of many immune related genes, and PIPL6 is suggested to function by further enhancing the immune response initiated by elicitors. Knock-out and overexpression of *PIPL6* might lead to higher susceptibility to infection by a virulent strain *(*pv. *tomato* DC3000) of the hemi-biotrophic pathogen *Pseudomonas syringae* that might be a result of disruption of a response carefully regulated by fine-tuners. However, the method used have some limitations and the results should be confirmed in fully grown plants. Knock-out of *PIPL6* lead to higher susceptibility to a avirulent strain (isolate 2100, CECT2; Spanish type) of the necrotrophic pathogen *Botrytis cinerea* compared to wild-type as well as disturbance of the balance of JA and camalexin production in response to infection. *PIPL6* overexpression lines might be more resistant to *B. cinerea*, but this effect remains to be investigated. *PIPL6* overexpression lines possess stronger growth inhibition and possibly a higher ROS production in response to flagellin compared to wild-type. This indicates that *PIPL6* overexpression result in a stronger immune response. Altogether, the results suggest that PIPL6 works as a plant-produced damage-associated molecular pattern to elicit and elevate plant defence responses. PIPL6 may also be involved in fine-tuning of the hormonal signalling pathways in response to pathogens with different lifestyles. It remains to be investigated whether application of PIPL6 would function as resistance enhancers in wild-type plants towards pathogen infections. In the future, it would be interesting to determine and further investigate the pathways for PIPL6 signalling (**Figure 16**) and the role of PIPL6 in the production of glucosinolates and callose deposition as well as ethylene production. Knowledge about peptide signalling might be utilized for breeding of crop plants with stronger pathogen/pest tolerance. Hence, increased insight about PIPL6 could be an important foundation for future applications to enhance disease resistance in plants.

References

Aharoni A, Vorst O (2002) DNA microarrays for functional plant genomics. *Plant Molecular Biology* 48 (1):99-118. doi:10.1023/A:1013734019946

Albert P, Miya A, Hiratsuka K, Kawakami N, Shibuya N (2006) A high-throughput evaluation system for Arabidopsis mutants for defense signaling. *Plant Biotechnology* 23 (5):459-466. doi:10.5511/plantbiotechnology.23.459

An C, Mou Z (2011) Salicylic acid and its function in plant immunity. *Journal of Integrative Plant Biology* 53 (6):412-428. doi:10.1111/j.1744-7909.2011.01043.x

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu W-L, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* 415 (6875):977-983. doi:10.1038/415977a

Barna B, Fodor J, Harrach B, Pogány M, Király Z (2012) The Janus face of reactive oxygen species in resistance and susceptibility of plants to necrotrophic and biotrophic pathogens. *Plant Physiology and Biochemistry* 59:37-43. doi:10.1016/j.plaphy.2012.01.014

Bartels S, Boller T (2015) Quo vadis, Pep? Plant elicitor peptides at the crossroads of immunity, stress, and development. *Journal of Experimental Botany* 66 (17):5183-5193. doi:10.1093/jxb/erv180

Bartels S, Lori M, Mbengue M, van Verk M, Klauser D, Hander T, Böni R, Robatzek S, Boller T (2013) The family of Peps and their precursors in Arabidopsis: differential expression and localization but similar induction of pattern-triggered immune responses. *Journal of Experimental Botany* 64 (17):5309-5321. doi:10.1093/jxb/ert330

Baxter A, Mittler R, Suzuki N (2014) ROS as key players in plant stress signalling. *Journal of Experimental Botany* 65 (5):1229-1240. doi:10.1093/jxb/ert375

Bigeard J, Colcombet J, Hirt H (2015) Signaling mechanisms in pattern-triggered immunity (PTI). *Molecular Plant* 8 (4):521-539. doi:10.1016/j.molp.2014.12.022

Birkenbihl RP, Diezel C, Somssich IE (2012) Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward Botrytis cinerea infection. *Plant Physiology* 159 (1):266-285. doi:10.1104/pp.111.192641

Bisceglia NG, Gravino M, Savatin DV (2015) Luminol-based assay for detection of immunity elicitor-induced hydrogen peroxide production in Arabidopsis thaliana leaves. *Bio-Protocol* 5:e1685. doi:10.21769/BioProtoc.1685

Butenko MA, Vie AK, Brembu T, Aalen RB, Bones AM (2009) Plant peptides in signalling: looking for new partners. *Trends in Plant Science* 14 (5):255-263. doi:10.1016/j.tplants.2009.02.002

Butenko MA, Wildhagen M, Albert M, Jehle A, Kalbacher H, Aalen RB, Felix G (2014) Tools and strategies to match peptide-ligand receptor pairs. *The Plant Cell* 26 (5):1838-1847. doi:10.1105/tpc.113.120071

Cao Y, Liang Y, Tanaka K, Nguyen CT, Jedrzejczak RP, Joachimiak A, Stacey G (2014) The kinase LYK5 is a major chitin receptor in Arabidopsis and forms a chitin-induced complex with related kinase CERK1. *eLife* 3:e03766. doi:10.7554/eLife.03766

Carr JP, Lewsey MG, Palukaitis P (2010) Signaling in induced resistance. *Advances in Virus Research* 76:57-121. doi:10.1016/S0065-3527(10)76003-6

Ceciliato PH, Zhang J, Liu Q, Shen X, Hu H, Liu C, Schäffner AR, Schroeder JI (2019) Intact leaf gas exchange provides a robust method for measuring the kinetics of stomatal conductance responses to abscisic acid and other small molecules in Arabidopsis and grasses. *Plant Methods* 15 (1):1-10. doi:10.1186/s13007-019-0423-y

Chen H, Lai Z, Shi J, Xiao Y, Chen Z, Xu X (2010) Roles of Arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. *BMC Plant Biology* 10 (1):1-15. doi:10.1186/1471-2229-10-281

Chen L, Zhang L, Xiang S, Chen Y, Zhang H, Yu D (2020) AtWRKY75 positively regulates jasmonate-mediated plant defense to necrotrophic fungal pathogens. *Journal of Experimental Botany* 72 (4). doi:10.1093/jxb/eraa529

Cheval C, Samwald S, Johnston MG, de Keijzer J, Breakspear A, Liu X, Bellandi A, Kadota Y, Zipfel C, Faulkner C (2020) Chitin perception in plasmodesmata characterizes submembrane immune-signaling specificity in plants. *Proceedings of the National Academy of Sciences* 117 (17):9621-9629. doi:10.1073/pnas.1907799117

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JD, Felix G, Boller T (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448 (7152):497-500. doi:10.1038/nature05999

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* 124 (4):803-814. doi:10.1016/j.cell.2006.02.008

Desaki Y, Shimada H, Takahashi S, Sakurayama C, Kawai M, Kaku H, Shibuya N (2019) Handmade leaf cutter for efficient and reliable ROS assay. *Plant Biotechnology*:19.0921. doi:10.5511/plantbiotechnology.19.0921a

Devendrakumar KT, Li X, Zhang Y (2018) MAP kinase signalling: interplays between plant PAMP-and effector-triggered immunity. *Cellular and Molecular Life Sciences* 75 (16):2981-2989. doi:10.1007/s00018-018-2839-3

Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *The Plant Journal* 18 (3):265-276. doi:10.1046/j.1365-313X.1999.00265.x

Flury P, Klauser D, Schulze B, Boller T, Bartels S (2013) The anticipation of danger: microbe-associated molecular pattern perception enhances AtPep-triggered oxidative burst. *Plant Physiology* 161 (4):2023-2035. doi:10.1104/pp.113.216077

Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486 (7402):228-232. doi:10.1038/nature11162

Gachon C, Saindrenan P (2004) Real-time PCR monitoring of fungal development in Arabidopsis thaliana infected by Alternaria brassicicola and Botrytis cinerea. *Plant Physiology and Biochemistry* 42 (5):367-371. doi:10.1016/j.plaphy.2004.04.001

Garg R, Jain M (2013) RNA-Seq for transcriptome analysis in non-model plants. In: *Legume Genomics*. Humania Press, Totowa, pp 43-58. doi:10.1007/978-1-62703-613-9_4

Ghorbani S, Lin Y-C, Parizot B, Fernandez A, Njo MF, Van de Peer Y, Beeckman T, Hilson P (2015) Expanding the repertoire of secretory peptides controlling root development with comparative genome analysis and functional assays. *Journal of Experimental Botany* 66 (17):5257-5269. doi:10.1093/jxb/erv346

Ghozlan MH, Eman E-A, Tokgöz S, Lakshman DK, Mitra A (2020) Plant Defense against Necrotrophic Pathogens. *American Journal of Plant Sciences* 11 (12):2122-2138. doi:10.4236/ajps.2020.1112149

Gigolashvili T, Berger B, Mock HP, Müller C, Weisshaar B, Flügge UI (2007) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in Arabidopsis thaliana. *The Plant Journal* 50 (5):886-901. doi:10.1111/j.1365-313X.2007.03099.x

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* 43:205-227. doi:10.1146/annurev.phyto.43.040204.135923

Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor–like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular Cell* 5 (6):1003-1011. doi:10.1016/S1097-2765(00)80265-8

Gong B-Q, Wang F-Z, Li J-F (2020) Hide-and-seek: chitin-triggered plant immunity and fungal counterstrategies. *Trends in Plant Science* 25 (8):805-816. doi:10.1016/j.tplants.2020.03.006

Guo F-Q, Okamoto M, Crawford NM (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* 302 (5642):100-103. doi:10.1126/science.1086770

Hou S, Shen H, Shao H (2019) PAMP-induced peptide 1 cooperates with salicylic acid to regulate stomatal immunity in Arabidopsis thaliana. *Plant Signaling & Behavior* 14 (11):1666657. doi:10.1080/15592324.2019.1666657

Hou S, Wang X, Chen D, Yang X, Wang M, Turrà D, Di Pietro A, Zhang W (2014) The secreted peptide PIP1 amplifies immunity through receptor-like kinase 7. *PLOS Pathogens* 10 (9):e1004331. doi:10.1371/journal.ppat.1004331

Huffaker A, Pearce G, Ryan CA (2006) An endogenous peptide signal in Arabidopsis activates components of the innate immune response. *Proceedings of the National Academy of Sciences* 103 (26):10098-10103. doi:10.1073/pnas.0603727103

Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Research* 41 (20):e188-e188. doi:10.1093/nar/gkt780

Jones JD, Dangl JL (2006) The plant immune system. *Nature* 444 (7117):323-329. doi:10.1038/nature05286

Katagiri F, Thilmony R, He SY (2002) The Arabidopsis thaliana-Pseudomonas syringae interaction. *The Arabidopsis Book* 1. doi:10.1199/tab.0039

Kliebenstein DJ, Rowe HC (2008) Ecological costs of biotrophic versus necrotrophic pathogen resistance, the hypersensitive response and signal transduction. *Plant Science* 174 (6):551-556. doi:10.1016/j.plantsci.2008.03.005

Kohari M, Yashima K, Desaki Y, Shibuya N (2016) Quantification of stimulus-induced callose spots on plant materials. *Plant Biotechnology* 33 (1):11-17. doi:10.5511/plantbiotechnology.15.1120a

Kohler A, Schwindling S, Conrath U (2000) Extraction and quantitative determination of callose from Arabidopsis leaves. *Biotechniques* 28 (6):1084-1086. doi:10.2144/00286bm06

Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in Arabidopsis. *The Plant Cell* 11 (12):2283-2290. doi:10.1105/tpc.11.12.2283

Lai Z, Mengiste T (2013) Genetic and cellular mechanisms regulating plant responses to necrotrophic pathogens. *Current Opinion in Plant Biology* 16 (4):505-512. doi:10.1016/j.pbi.2013.06.014

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9 (4):357. doi:10.1038/nmeth.1923

Lecourieux D, Ranjeva R, Pugin A (2006) Calcium in plant defence-signalling pathways. *New Phytologist* 171 (2):249-269. doi:10.1111/j.1469-8137.2006.01777.x

Lee H, Chah O-K, Sheen J (2011) Stem-cell-triggered immunity through CLV3p–FLS2 signalling. *Nature* 473 (7347):376-379. doi:10.1038/nature09958

Li B, Meng X, Shan L, He P (2016) Transcriptional regulation of pattern-triggered immunity in plants. *Cell Host & Microbe* 19 (5):641-650. doi:10.1016/j.chom.2016.04.011

Libault M, Wan J, Czechowski T, Udvardi M, Stacey G (2007) Identification of 118 Arabidopsis transcription factor and 30 ubiquitin-ligase genes responding to chitin, a plant-defense elicitor. *Molecular Plant-Microbe Interactions* 20 (8):900-911. doi:10.1094/MPMI-20-8-0900

Liu S, Kracher B, Ziegler J, Birkenbihl RP, Somssich IE (2015) Negative regulation of ABA signaling by WRKY33 is critical for Arabidopsis immunity towards Botrytis cinerea 2100. *elife* 4:e07295. doi:10.7554/eLife.07295

Liu S, Ziegler J, Zeier J, Birkenbihl RP, Somssich IE (2017) Botrytis cinerea B05. 10 promotes disease development in Arabidopsis by suppressing WRKY33-mediated host immunity. *Plant, Cell & Environment* 40 (10):2189-2206. doi:10.1111/pce.13022

Liu Z, Wu Y, Yang F, Zhang Y, Chen S, Xie Q, Tian X, Zhou J-M (2013) BIK1 interacts with PEPRs to mediate ethylene-induced immunity. *Proceedings of the National Academy of Sciences* 110 (15):6205-6210. doi:10.1073/pnas.1215543110

Logemann E, Birkenbihl RP, Rawat V, Schneeberger K, Schmelzer E, Somssich IE (2013) Functional dissection of the PROPEP2 and PROPEP3 promoters reveals the importance of WRKY factors in mediating microbe-associated molecular pattern-induced expression. *New Phytologist* 198 (4):1165-1177. doi:10.1111/nph.12233

Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J (2011) Callose deposition: a multifaceted plant defense response. *Molecular Plant-Microbe Interactions* 24 (2):183-193. doi:10.1094/MPMI-07-10-0149

Mao G, Meng X, Liu Y, Zheng Z, Chen Z, Zhang S (2011) Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. *The Plant Cell* 23 (4):1639-1653. doi:10.1105/tpc.111.084996

Matsubayashi Y (2011) Post-translational modifications in secreted peptide hormones in plants. *Plant and Cell Physiology* 52 (1):5-13. doi:10.1093/pcp/pcq169

Meinke DW, Cherry JM, Dean C, Rounsley SD, Koornneef M (1998) Arabidopsis thaliana: a model plant for genome analysis. *Science* 282 (5389):662-682. doi:10.1126/science.282.5389.662

Mithöfer A, Mazars C (2002) Aequorin-based measurements of intracellular Calcium-signatures in plant cells. *Biological Procedures Online* 4 (1):105-118. doi:10.1251/bpo40

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proceedings of the National Academy of Sciences* 104 (49):19613-19618. doi:10.1073/pnas.0705147104

Moriya H (2015) Quantitative nature of overexpression experiments. *Molecular Biology of The Cell* 26 (22):3932-3939. doi:10.1091/mbc.E15-07-0512

Murphy E, Smith S, De Smet I (2012) Small signaling peptides in Arabidopsis development: how cells communicate over a short distance. *The Plant Cell* 24 (8):3198-3217. doi:10.1105/tpc.112.099010

Najafi J (2015) *Molecular investigation of signalling components in sugar sensing and defence in Arabidopsis thaliana*. Doctoral dissertation. Norwegian University of Science and Technology, Department of Biology

Najafi J, Brembu T, Vie AK, Viste R, Winge P, Somssich IE, Bones AM (2020) PAMP-INDUCED SECRETED PEPTIDE 3 modulates immunity in Arabidopsis. *Journal of Experimental Botany* 71 (3):850-864. doi:10.1093/jxb/erz482

Navarro L, Bari R, Achard P, Lisón P, Nemri A, Harberd NP, Jones JD (2008) DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. *Current Biology* 18 (9):650-655. doi:10.1016/j.cub.2008.03.060

Nicaise V, Roux M, Zipfel C (2009) Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiology* 150 (4):1638-1647. doi:10.1104/pp.109.139709

Nürnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological Reviews* 198 (1):249-266. doi:10.1111/j.0105-2896.2004.0119.x

Ohyama K, Shinohara H, Ogawa-Ohnishi M, Matsubayashi Y (2009) A glycopeptide regulating stem cell fate in Arabidopsis thaliana. *Nature Chemical Biology* 5 (8):578-580. doi:10.1038/nchembio.182

Pandey SP, Somssich IE (2009) The role of WRKY transcription factors in plant immunity. *Plant Physiology* 150 (4):1648-1655. doi:10.1104/pp.109.138990

Pei Z-M, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI (1997) Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and abi1 and abi2 mutants. *The Plant Cell* 9 (3):409-423. doi:10.1105/tpc.9.3.409

Pusztahelyi T (2018) Chitin and chitin-related compounds in plant–fungal interactions. *Mycology* 9 (3):189-201. doi:10.1080/21501203.2018.1473299

Qiu JL, Fiil BK, Petersen K, Nielsen HB, Botanga CJ, Thorgrimsen S, Palma K, Suarez-Rodriguez MC, Sandbech-Clausen S, Lichota J (2008) Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *The EMBO Journal* 27 (16):2214-2221. doi:10.1038/emboj.2008.147

Qu H, Jiang X, Shi Z, Liu L, Zhang S (2012) Fast loading ester fluorescent Calcium and pH indicators into pollen of Pyrus pyrifolia. *Journal of Plant Research* 125 (1):185-195. doi:10.1007/s10265-011-0440-z

Quintana-Rodriguez E, Duran-Flores D, Heil M, Camacho-Coronel X (2018) Damage-associated molecular patterns (DAMPs) as future plant vaccines that protect crops from pests. *Scientia Horticulturae* 237:207-220. doi:10.1016/j.scienta.2018.03.026

Ramonell KM, Zhang B, Ewing RM, Chen Y, Xu D, Stacey G, Somerville S (2002) Microarray analysis of chitin elicitation in Arabidopsis thaliana. *Molecular Plant Pathology* 3 (5):301-311. doi:10.1046/j.1364-3703.2002.00123.x

Robert-Seilaniantz A, Grant M, Jones JD (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology* 49:317-343. doi:10.1146/annurev-phyto-073009-114447

Robinson MD, McCarthy DJ, Smyth GK (2010) EdgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26 (1):139-140. doi:10.1093/bioinformatics/btp616

Ross A, Yamada K, Hiruma K, Yamashita-Yamada M, Lu X, Takano Y, Tsuda K, Saijo Y (2014) The Arabidopsis PEPR pathway couples local and systemic plant immunity. *The EMBO Journal* 33 (1):62-75. doi:10.1002/embj.201284303

Rossi FR, Gárriz A, Marina M, Romero FM, Gonzalez ME, Collado IG, Pieckenstain FL (2011) The sesquiterpene botrydial produced by Botrytis cinerea induces the hypersensitive response on plant tissues and its action is modulated by salicylic acid and jasmonic acid signaling. *Molecular Plant-Microbe Interactions* 24 (8):888-896. doi:10.1094/MPMI-10-10-0248

Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, Holton N, Malinovsky FG, Tör M, de Vries S, Zipfel C (2011) The Arabidopsis leucine-rich repeat receptor–like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *The Plant Cell* 23 (6):2440-2455. doi:10.1105/tpc.111.084301

Salem MA, Yoshida T, Perez de Souza L, Alseekh S, Bajdzienko K, Fernie AR, Giavalisco P (2020) An improved extraction method enables the comprehensive analysis of lipids, proteins, metabolites and phytohormones from a single sample of leaf tissue under water-deficit stress. *The Plant Journal* 103 (4):1614-1632. doi:10.1111/tpj.14800

Savatin DV, Bisceglia NG, Gravino M, Fabbri C, Pontiggia D, Mattei B (2015) Camalexin quantification in Arabidopsis thaliana leaves infected with Botrytis cinerea. *Bio-Protocol* 5 (2):e1379-e1379. doi:10.21769/BioProtoc.1379

Scalschi L, Llorens E, Camañes G, Pastor V, Fernández Crespo E, Flors V, García Agustín P, Vicedo B (2015) Quantification of callose deposition in plant leaves. *Bio-Protocol* 5 (19). doi:10.21769/BioProtoc.1610

Schuhegger R, Nafisi M, Mansourova M, Petersen BL, Olsen CE, Svatoš A, Halkier BA, Glawischnig E (2006) CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. *Plant Physiology* 141 (4):1248-1254. doi:10.1104/pp.106.082024

Schuhegger R, Rauhut T, Glawischnig E (2007) Regulatory variability of camalexin biosynthesis. *Journal of Plant Physiology* 164 (5):636-644. doi:10.1016/j.jplph.2006.04.012

Schulze B, Mentzel T, Jehle AK, Mueller K, Beeler S, Boller T, Felix G, Chinchilla D (2010) Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *Journal of Biological Chemistry* 285 (13):9444-9451. doi:10.1074/jbc.M109.096842

Seo M-S, Kim JS (2017) Understanding of MYB transcription factors involved in glucosinolate biosynthesis in Brassicaceae. *Molecules* 22 (9):1549. doi:10.3390/molecules22091549

Shen J, Diao W, Zhang L, Acharya BR, Wang M, Zhao X, Chen D, Zhang W (2020) Secreted peptide PIP1 induces stomatal closure by activation of guard cell anion channels in Arabidopsis. *Frontiers in Plant Science* 11:1029. doi:10.3389/fpls.2020.01029

Shen W, Liu J, Li J-F (2019) Type-II metacaspases mediate the processing of plant elicitor peptides in Arabidopsis. *Molecular Plant* 12 (11):1524-1533. doi:10.1016/j.molp.2019.08.003

Shiu S-H, Bleecker AB (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Proceedings of the National Academy of Sciences* 98 (19):10763-10768. doi:10.1073/pnas.181141598

Šimura J, Antoniadi I, Široká J, Tarkowská D, Strnad M, Ljung K, Novák O (2018) Plant hormonomics: multiple phytohormone profiling by targeted metabolomics. *Plant Physiology* 177 (2):476-489. doi:10.1104/pp.18.00293

Somerville C, Koornneef M (2002) A fortunate choice: the history of Arabidopsis as a model plant. *Nature Reviews Genetics* 3 (11):883-889. doi:10.1038/nrg927

Spoel SH, Dong X (2012) How do plants achieve immunity? Defence without specialized immune cells. *Nature Reviews Immunology* 12 (2):89-100. doi:10.1038/nri3141

Stenvik G-E, Tandstad NM, Guo Y, Shi C-L, Kristiansen W, Holmgren A, Clark SE, Aalen RB, Butenko MA (2008) The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in Arabidopsis through the receptor-like kinases HAESA and HAESA-LIKE2. *The Plant Cell* 20 (7):1805-1817. doi:10.1105/tpc.108.059139

Stührwohldt N, Schaller A (2019) Regulation of plant peptide hormones and growth factors by post-translational modification. *Plant Biology* 21:49-63. doi:10.1111/plb.12881

Tabata R, Sawa S (2014) Maturation processes and structures of small secreted peptides in plants. *Frontiers in Plant Science* 5:311. doi:10.3389/fpls.2014.00311

Thomma BP, Cammue BP, Thevissen K (2002) Plant defensins. *Planta* 216 (2):193-202. doi:10.1007/s00425-002-0902-6

Tian W, Hou C, Ren Z, Wang C, Zhao F, Dahlbeck D, Hu S, Zhang L, Niu Q, Li L (2019) A calmodulin-gated calcium channel links pathogen patterns to plant immunity. *Nature* 572 (7767):131-135. doi:10.1038/s41586-019-1413-y

Torres MA, Jones JD, Dangl JL (2006) Reactive oxygen species signaling in response to pathogens. *Plant Physiology* 141 (2):373-378. doi:10.1104/pp.106.079467

Tsuda K, Somssich IE (2015) Transcriptional networks in plant immunity. *New Phytologist* 206 (3):932-947. doi:10.1111/nph.13286

Veronese P, Nakagami H, Bluhm B, AbuQamar S, Chen X, Salmeron J, Dietrich RA, Hirt H, Mengiste T (2006) The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens. *The Plant Cell* 18 (1):257-273. doi:10.1105/tpc.105.035576

Vicente JA, Peixoto F, Lopes ML, Madeira VM (2001) Differential sensitivities of plant and animal mitochondria to the herbicide paraquat. *Journal of Biochemical and Molecular Toxicology* 15 (6):322-330. doi:10.1002/jbt.10010

Vie AK, Najafi J, Liu B, Winge P, Butenko MA, Hornslien KS, Kumpf R, Aalen RB, Bones AM, Brembu T (2015) The IDA/IDA-LIKE and PIP/PIP-LIKE gene families in Arabidopsis: phylogenetic relationship, expression patterns, and transcriptional effect of the PIPL3 peptide. *Journal of Experimental Botany* 66 (17):5351-5365. doi:10.1093/jxb/erv285

Vogel C, Marcotte EM (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics* 13 (4):227-232. doi:10.1038/nrg3185

Wu L, Chen H, Curtis C, Fu ZQ (2014) Go in for the kill: How plants deploy effector-triggered immunity to combat pathogens. *Virulence* 5 (7):710-721. doi:10.4161/viru.29755

Xu X, Chen C, Fan B, Chen Z (2006) Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. *The Plant Cell* 18 (5):1310-1326. doi:10.1105/tpc.105.037523

Yamada K, Yamaguchi K, Shirakawa T, Nakagami H, Mine A, Ishikawa K, Fujiwara M, Narusaka M, Narusaka Y, Ichimura K (2016) The Arabidopsis CERK 1-associated kinase PBL 27 connects chitin perception to MAPK activation. *The EMBO Journal* 35 (22):2468-2483. doi:10.15252/embj.201694248

Yamaguchi Y, Huffaker A, Bryan AC, Tax FE, Ryan CA (2010) PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis. *The Plant Cell* 22 (2):508-522. doi:10.1105/tpc.109.068874

Yamaguchi Y, Pearce G, Ryan CA (2006) The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in Arabidopsis, is functional in transgenic tobacco cells. *Proceedings of the National Academy of Sciences* 103 (26):10104-10109. doi:10.1073/pnas.0603729103

Yan S, Dong X (2014) Perception of the plant immune signal salicylic acid. *Current Opinion in Plant Biology* 20:64-68. doi:10.1016/j.pbi.2014.04.006

Zandalinas SI, Sengupta S, Burks D, Azad RK, Mittler R (2019) Identification and characterization of a core set of ROS wave-associated transcripts involved in the systemic acquired acclimation response of Arabidopsis to excess light. *The Plant Journal* 98 (1):126-141. doi:10.1111/tpj.14205

Zhou J, Wang X, He Y, Sang T, Wang P, Dai S, Zhang S, Meng X (2020) Differential phosphorylation of the transcription factor WRKY33 by the protein kinases CPK5/CPK6 and MPK3/MPK6 cooperatively regulates camalexin biosynthesis in Arabidopsis. *The Plant Cell* 32 (8):2621-2638. doi:10.1105/tpc.19.00971

Appendixes

Appendix 1 List of primers

Appendix 2 RNA isolation and TOPO-cloning

Appendix 3 cDNA synthesis, DNA extraction and PCR details

Appendix 4 Medium recipes

Appendix 5 Additional ROS

Appendix 1 List of primers

Genotyping primers	Locus	Forward	Reverse
*LBN (*SALK_106769)	AT1G47178[a]	CGGAACCACCATCAAACAGGAT	
*JL-202 (*Wiscseq_DsLoxHs144_04E.1)	AT1G47178[a]	CATTTTATAATAACGCTGCGGACATCTAC	
RP (gene specific primer)	AT1G47178[a]		AGCCAGTAACTAAGCCTCTGATTAGTG
LP (gene spesific primer)	AT1G47178[a]	GAAAACACGAAATCCAAATCAAC	

In-planta pathogen assay	Locus	Forward	Reverse
*At*SKII	AT5G26751	CTTATCGGATTTCTCTATGTTTGGC	GAGCTCCTGTTTATTTAACTTGTACATACC
*Bc*CutA	Z69264	AGCCTTATGTCCCTTCCCTTG	GAAGAGAAATGGAAAATGGTGAG

RT-PCR primers	Locus	Forward	Reverse
RT-PIPL6	AT1G47178[a]	AGTCAAGGATCGATGGAGAAGAAG	qPIPL6 reverse primer
Actin2	AT3G18780	TCCCTCAGCACATTCCAGCAGAT	AACGATTCCTGGACCTGCCTCATC

qPCR primers:

Gene name	Locus	Forward	Reverse
PIPL6	AT1T47178[a]	CTAATCAGAGGCTTAGTTACTG	ATGTATAACGACTTGTGCAAAC
TIP41	AT4G34270	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
RRTF1	AT4G34410	AGTTGAAGCAGAGCAATGG	TCATCCACTCCTCCATATTGC
PDF1.2	AT5G44420	GCAAGAATCAGTGCATTAACCT	TACACACGATTTAGCACCAAAG
ICS1	AT1G74710	ATTGATCTATGCGGGGACAG	TGGACAAAAGCTCGTACCTGAG
FRK1	AT2G19190	CTGGATCCATCGGTTACCTTGA	CTGGATCCATCGGTTACCTTGA
RLK7	AT1G09970	ACCGATAGAGGCAGAGTTTGG	TGTCCACAATCTCCATCACACT
MYB51	AT1G18570	TGATGTTGGAGGAGTCTTGTG	AAGTCTTGACGTTCATAGACCG
EDS5	AT4G39030	GCGTCGTTCGGTTACAGG	GCGTCGTTCGGTTACAGG
ZAT12	AT5G59820	AAGCAGTTTCATTCGTTCCAAG	TTCTTCATCAATCCAGACGACA
TAT3	AT2G24850	TGATTTCTGCACGAAGCTAGT	TTGTACCACTGATTCGTCGGTT
WRKY18	AT4G31800	AATCCTTTCTCCGCAAAAC	CTCACTTGCGCTCTCGTCTT
WRKY33	AT2G38470	GACATTCTTGACGACGGTTACA	CGATGGTTGTGCACTTGTAGTA
WRKY40	AT1G80840	GCGAGTTGAAGAAGATCCA	TGTCACACATCAAGGTTAGCATC
WRKY53	AT4G23810	GAGTCATCATCGCCAAGATTAC	TTCTCCAGCTAAAGACATCATC
WRKY60	AT2G25000	ACTCAGTCTCGGACCAATCG	CATTGATATCCATCTTTCACAGTCA
PIP1	AT4G28460	GATGAGAAGAGTTAGTTGGTC	GGAACCACCACGTGTTCTACGA
PIP2	AT4G37290	TGTTAAGCACTCAGGTCCAAG	TTTCTCGACCTAGATGGGTATG
PIP3	AT2G23270	ACTGAGACGCTTGAATATGGT	AAGCAGCCACATTAACTGGTTC
JAZ7	AT2G34600	AACTGCGACAAGCCTTTACTCA	TTGATTCGTCCAACGAGCTATG
JAZ8	AT1G30135	AGATGTTACCCATCTTCAGGC	ACCCGTTTGAGGATGACTTCAGGC
JAZ9	AT1G70700	TCAATGCAGCTCCTCGTAACA	TTTGCGCTTCTCCAAGAACCGA
PR1	AT2G14610	TCTTCCCTCGAAAGCTCAA	TCTTCCCTCGAAAGCTCAA
MYC2	AT1G32640	CCGAGTCCGGTTCATTCT	TCTCGGGAGAAAGTGTTATTGAA
PAD3	AT3G26830	GGTTTCTCGACAGTTCCGTTGA	TTCAACAATGCCATCTCAACAAGTA

Appendix 2 RNA isolation and TOPO-cloning

RNA isolation and DNase treatment:

RNA isolation using the Spectrum™ Plant Total RNA kit (Sigma life Science) and DNase treatment by RNase-free DNase set (QIAGEN) was performed as described below. The Eppendorf tubes containing frozen plant tissue were placed in a precooled (-18°C) tissuelyser module and tissues were crushed by the use of in TissueLyserII (Qiagen) 25Hz for 2 minutes. The module was flipped over vertically after 1 minute to ensure equal crushing of the tissue. 500μL Lysis solution mix were added to the samples and the tissuelyzer step was repeated at room temperate. The samples were incubated at 56°C for 3-5 minutes and centrifuged (13 000rpm) for 3 minutes. The supernatants were transferred to filtration columns and centrifuged (13 000rpm) for 1 minute and the filters were discarded. 500μL binding solution was added and mixed by pipetting before transferring 500μL of the solution to binding columns prior to centrifugation (13 000rpm) for 1 min. Flow-through was discarded and rest of the sample mix was transferred to the column, centrifuged and flow through was discarded. 300μL wash solution 1 was added and centrifuged (13 000rpm) for 1 min and flow through was removed. In order to eliminate DNA from the columns, treatment with the RNase-free DNase set (QIAGEN) was performed by applying 80μL of DNase mix (70μL RDD buffer and 10μL DNAse) to the column followed by incubation for 15 minutes at room temperature. 500μL wash solution 1 were added and the samples were centrifuged (13 000rpm) for 1 min and flow through was removed. 500μL wash solution 2 was added followed by centrifugation (13 000rpm) for 30 seconds. Flow through was removed and wash with wash solution 2 was repeated. The filters were dried by centrifugation (13 000rpm) for 1 minute and new collection tubes were used when 50μL elution buffer was dispensed in the middle of each filter, incubated at room temperature for 1 minute and centrifuged (13 000rpm) for 1 minute. RNA concentration was measured using Nanodrop One (Thermo Scientific).

TOPO-cloning:

PCR gel products were recovered from the gel and purified using Wizard®SV Gel and PCR Clean up system (Promega) according to the protocol provided by the manufacturer. The PCR product was inserted into a TOPO vector using TOPO TA cloning kit for sequencing (Invitrogen) according to protocol by the manufacturer. The TOPO reaction was transformed to the super competent *Escherichia coli* cells (Top10 strain) and incubated 30 minutes on ice before heat shock at 42°C for 30 seconds followed by 1 minute on ice. 500μL LB liquid medium was added to the Eppendorf tube and put in shaker incubation at 220 rpm for 1 hour. After incubation, 25μL of the sample was transferred to a LB agar plate containing ampicillin (50μg/mL) and incubated in 37°C over-night. Colonies were picked (8 replicates) and resuspended in 4mL liquid LB medium with ampicillin (50μg/mL) in a culture tube and incubated in 37°C for 5 hours before 2mL was transferred to an Eppendorf tube and centrifuged (13 000rpm 4°C) for 2 minutes. Supernatant was discarded and cell pellet was used for plasmid isolation. Plasmid isolation was performed by the following method. Bacterial overnight liquid cultures were centrifuged (13 000rpm at 4°C) and supernatant medium was discarded. The bacterial cell pellets were homogenized with 300μL solution I (10mM EDTA and 10μg/mL RNase) and vortexed. 300μL solution II (100mM Natrium Hydroxide, 1%W/V SDS) was added. The tubes were inverted gently 5-10 times and 300μL of solution III (3M Acetate potassium) was added and mixed by inverting 5-10 times. The samples were centrifuged (13 000rpm at 21°C) for 10 minutes and the supernatants were transferred to new tubes. 1mL 96% ethanol (precooled at -20°C) was added and samples were centrifuged (13 000rpm at 4°C) for 4 minutes. The supernatants were discarded and pellets were washed in 300μL 70% ethanol followed by an additional centrifugation (13 000rpm at 4°C) for 4 minutes. The supernatants were discarded and the samples were dried at 37°C for 10 minutes and eluted in 50-100μL nuclease free water (Promega). The plasmid concentration was measured using Nanodrop One (Thermo Scientific).

Appendix 3 cDNA synthesis, DNA extraction and PCR details

cDNA synthesis:

cDNA synthesis was performed using the reverse transcription kit 2000 (Qiagen) with the following method; 1μg of total RNAs were used for cDNA synthesis as described by the manufacturer. Briefly, the template RNAs were mixed with 2μL genomic DNA wipe-out buffer and diluted with RNAse free water up to 14μL. A mix containing 1μL Quantiscript Reverse transctiptase (RT), 4μL RT Buffer and 1μL RT-primer mix was added followed by incubation at 42°C for 15 minutes (enzymatic reaction) and 95°C for 3 minutes (deactivation of enzyme).

DNA extraction:

DNA was extracted as previously described by Edwards et al. (1991)[1]. Tissue was homogenized with 400μL extraction buffer (200mM Tris HCl pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) and centrifuged (13 000rpm) for 3 minutes. Supernatant (300μL) was transferred to a new Eppendorf tube, mixed with 300μL isopropanol and incubated 2 minutes at room temperature. The solution was centrifuged (13 000rpm) for 5 minutes, the supernatant was removed, and the pellet was dried in 2-5 minutes at 37°C before the DNA was dissolved in 200μL elution buffer (OMEGA Bio-tek). DNA concentration was measured using Nanodrop One (Thermo Scientific).

qPCR:

The light cycler® 96 (Roche) was used with the software LightCycler® 96 (version 1.1, Roche) with the following thermoprofile; preincubation at 95°C for 10 minutes, amplification in 45 cycles with denaturation at 95°C for 10 seconds, annealing at 55°C for 10 seconds and elongation at 72°C for 10 seconds. After each run, melding point was determined by increased temperature from 65°C to 97°C.

PCR for screening of knock-out lines:

About 50ng of genomic DNA was used in a PCR reaction with 2μL 10x DreamTaq Green Buffer (Thermo Scientific), 0,125mM dNTPs, 0,5μM forward and reverse primer, 0,5μL DreamTaq DNA polymerase (Thermo Scientific), and nuclease free water (Promega) up to 20μL. The thermocycler profile used was initial denaturation for 3 minutes at 95°C and 30 cycles of 95°C 30 seconds denaturation, 55°C 30 seconds annealing and 72°C 60 seconds elongation followed by final elongation of 72°C for 5 minutes.

RT-PCR on knock-out and overexpression lines:

5μL cDNA was used in a 20μL reaction with 2μL 10X Dream Taq Green Buffer (Thermo Scientific), 0,125mM dNTPs, 0,25μM forward and reverse primer and 0,5μL Dream Taq DNA Polymerase (Thermo Scientific). The thermocycler profile included initial denaturation at 95°C for 1 minute followed by 30 cycles of 95°C denaturation for 15 seconds, 60°C annealing for 15 seconds and 72°C elongation for 30 seconds.

---

[1] Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* 19 (6):1349. doi:10.1093/nar/19.6.1349

Appendix 4 Medium recipes

Luria-Bertani (LB) medium:

10 g/L Pancreatic Peptone (VWR BDH Chemicals)

5g/L Bacto™ Yeast Extract (Becton, Dickinson and Company)

5g/L Sodium Cloride (NaCl)

*14g/L Bacteriological agar (VWR BDH Chemicals)

*Bacteriological agar is not added to liquid LB medium.

Kings B medium:

10 g/L Pancreatic Peptone (VWR BDH Chemicals)

1,5g/L Dipotassium phosphate (K_2HPO_4)

15g/L Glycerine (VWR BDH Chemicals

**10mM Magnesium sulphate ($MgSO_4$)

pH=7

**Added after autoclavation

1/2MS:

2,2 g/L Murashige-Skoog basal Salt mixture (M5524, Sigma-Aldrich)

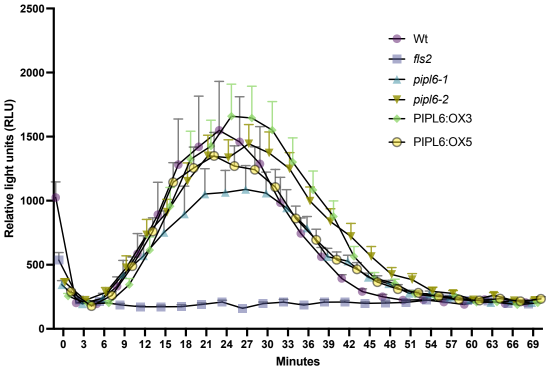
10g/L Sucrose

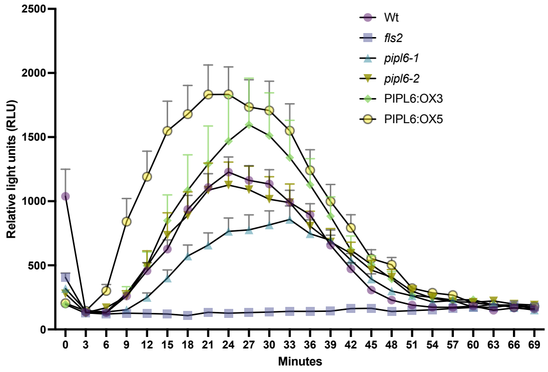
***3g/L Phytoagar (P1003.5000, Duchefa Biochemie B.V)

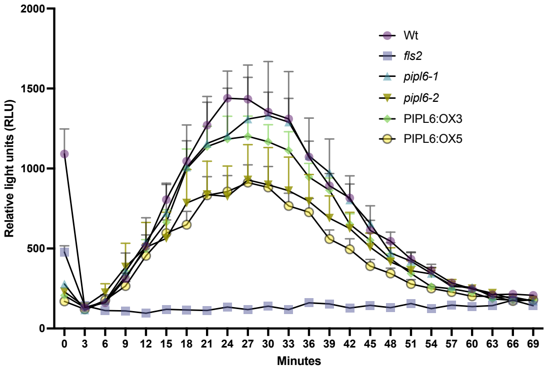
pH=5,75

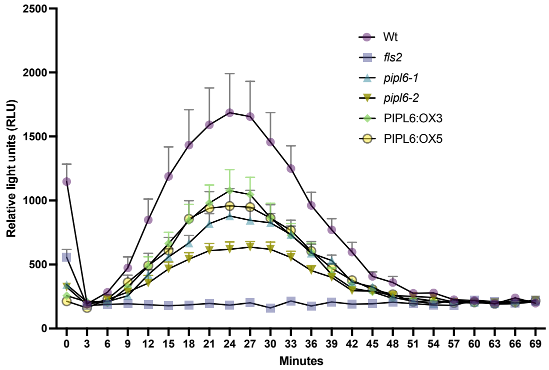
***Added after pH adjustment and not added in liquid medium

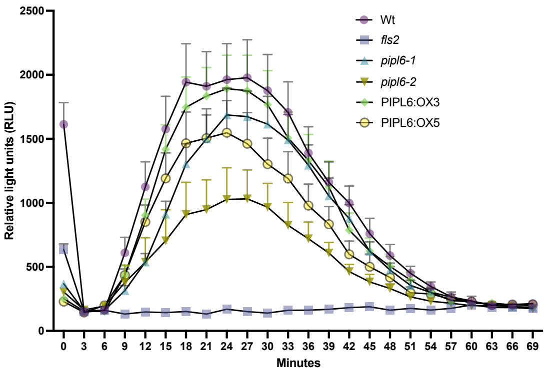
Appendix 5 Additional ROS analyzes

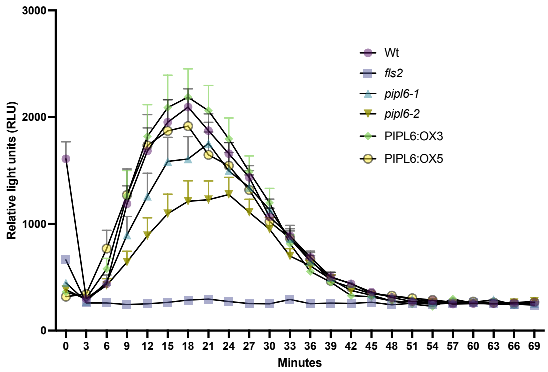


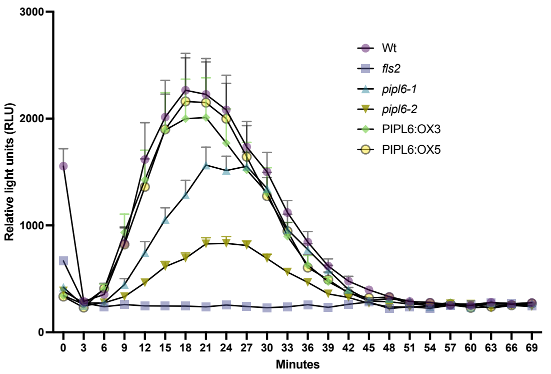


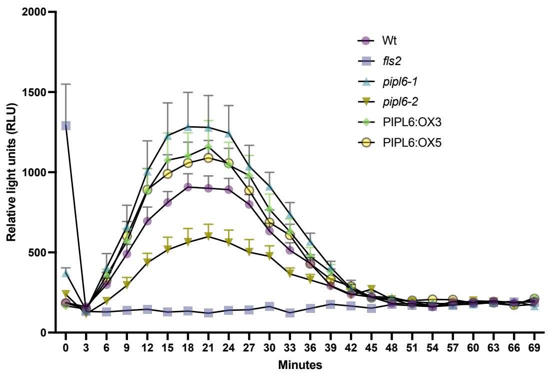


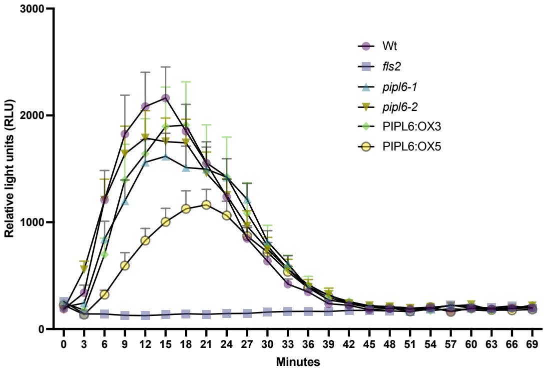


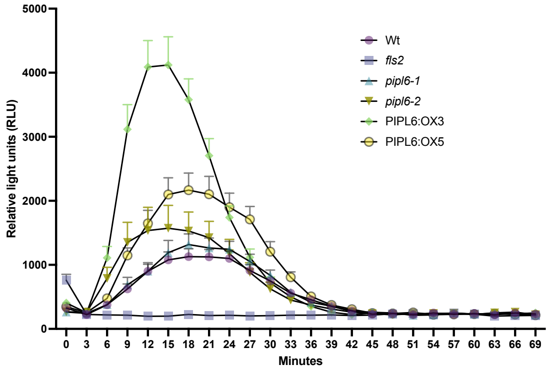


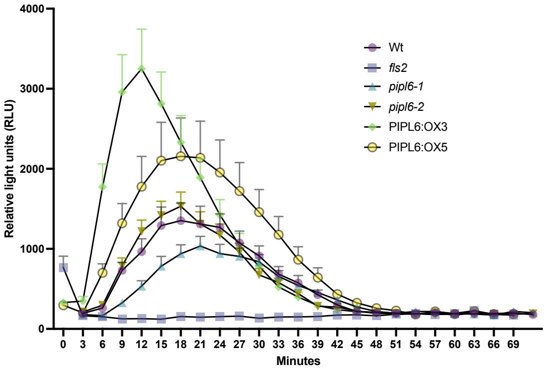


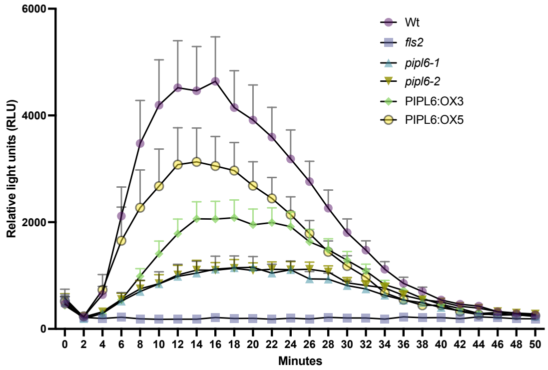


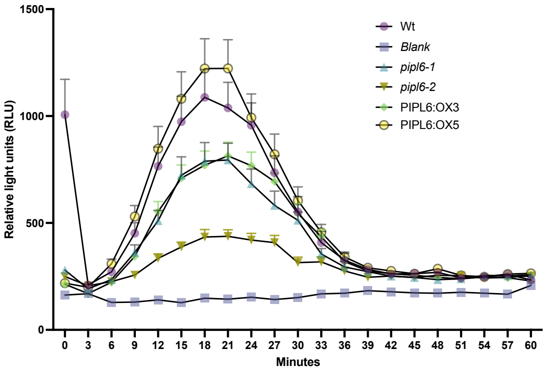


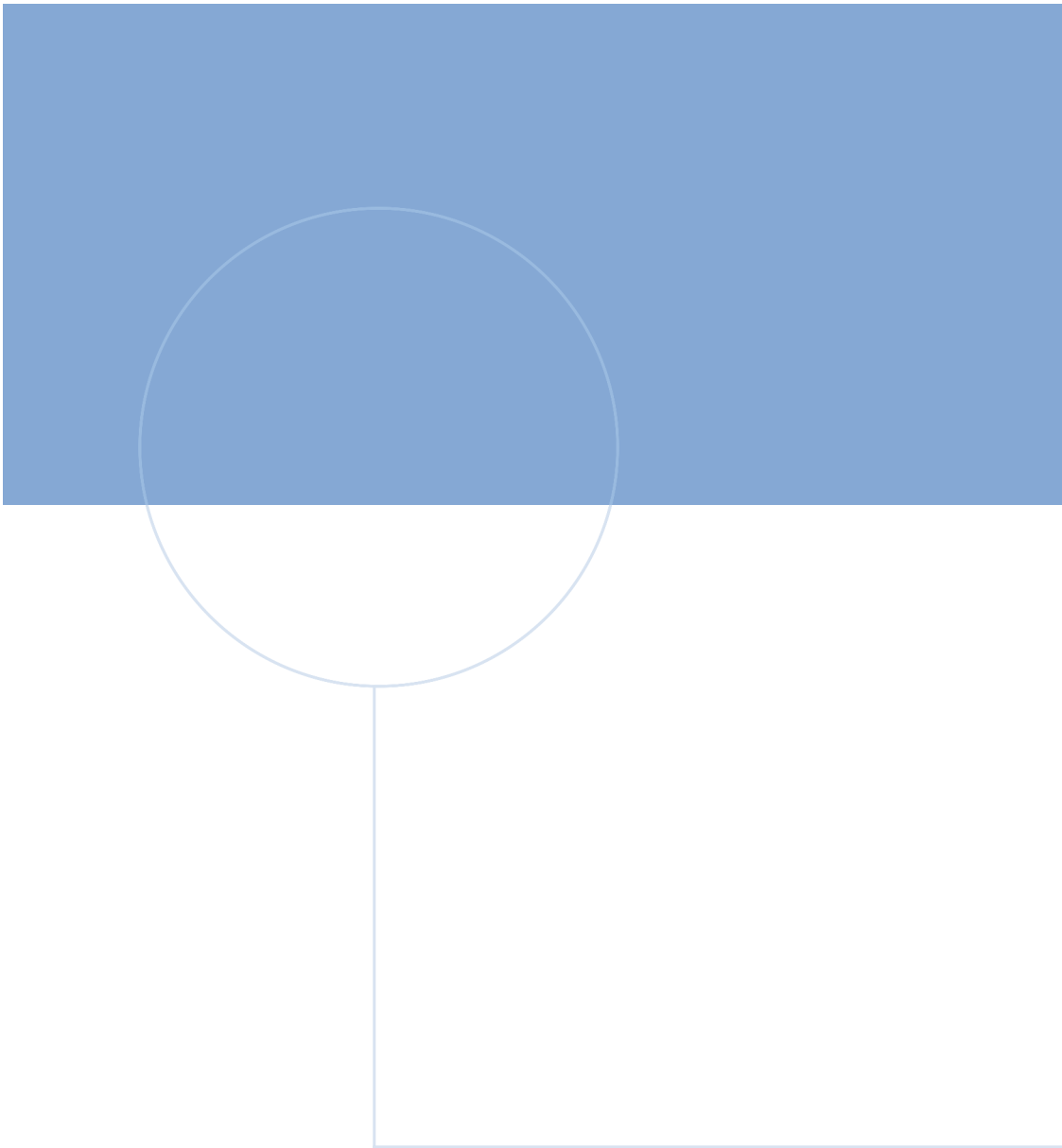

Relative light units (RLU)
Minutes
Wt
fls2
pipl6-1
pipl6-2
PIPL6:OX3
PIPL6:OX5


Relative light units (RLU)
Minutes
Wt
fls2
pipl6-1
pipl6-2
PIPL6:OX3
PIPL6:OX5


Relative light units (RLU)
Minutes
Wt
fls2
pipl6-1
pipl6-2
PIPL6:OX3
PIPL6:OX5


Relative light units (RLU)
Minutes
Wt
fls2
pipl6-1
pipl6-2
PIPL6:OX3
PIPL6:OX5


Relative light units (RLU)
Minutes
Wt
Blank
pipl6-1
pipl6-2
PIPL6:OX3
PIPL6:OX5



NTNU

Norwegian University of
Science and Technology