

Cross-regulation of receptor signalling to maintain the balance between growth and immunity

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Abstract

Plant cells continuously perceive and respond to diverse extracellular signals. Among these signals are small peptides that are secreted by the plant and perceived by specialised cell-surface receptors. Plants secrete a vast diversity of these peptides which play critical roles in fine-tuning and facilitating cellular responses to biotic and abiotic stimuli and are therefore essential for environmental adaptation.

The receptor kinase PHYTOSULFOKINE RECEPTOR 1 (PSKR1) perceives the peptide PHYTOSULFOKINE (PSK) to modulate plant growth, immunity, and microbiome composition. To understand the molecular mechanisms downstream of PSKR1, I use Affinity Purification-Mass Spectrometry (AP-MS) to identify novel signalling components, including MITOGEN-ACTIVATED KINASE KINASE KINASE 7 (MKKK7). Functional analysis using *mkkk7* mutants generated by CRISPR-Cas9 reveals that MKKK7 regulates specific PSK-induced responses, such as protoplast expansion and nutrient-related gene expression, while growth promotion and suppression of immunity are unaffected.

MKKK7 was previously shown to act downstream of the immune receptor FLAGELLIN SENSITIVE 2 (FLS2), but its mode of action remained unexplored. Here, I show that MKKK7 likely acts partially redundantly with its closely related homologue MKKK6 in Arabidopsis, with double mutants showing severe developmental defects. I confirm that the perception of PATHOGEN-ASSOCIATED MOLECULAR PATTERN (PAMP) flg22 by FLS2 triggers MKKK7 phosphorylation and show in addition that the intracellular immune kinase BOTRYTIS INSENSITIVE 1 (BIK1) phosphorylates MKKK7. Lastly, I show that MKKK7 is an active kinase that triggers cell death when overexpressed in *Nicotiana benthamiana*, highlighting its role in plant growth and immunity.

When considered together, the complex relationship of MKKK7 with both the FLS2 immune- and PSKR1 growth-signalling pathways provides new insights into the mechanisms that balance plant growth and immunity. Understanding receptor kinase signalling at the molecular level will help to inform strategies to shift the balance in favour of plants that exhibit both robust growth and enhanced immunity.

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Abbreviations

AM	ARBUSCULAR MYCORRHIZAL
ANP	ARABIDOPSIS NPK1-LIKE PROTEIN KINASE
AP-MS	AFFINITY PURIFICATION MASS SPECTROMETRY
ARCK1	ABA- AND OSMOTIC-STRESS-INDUCIBLE RECEPTOR-LIKE CYTOSOLIC KINASE1
ARM	ARMADILLO
BAK1	BRI1 ASSOCIATED RECEPTOR KINASE 1
BAM	BARELY ANY MERISTEM
BIK1	BOTRYTIS INSENSITIVE 1
BIR2	BAK1-INTERACTING RECEPTOR-LIKE KINASE 2
BL	BRASSINOLIDE
BR	BRASSINOSTEROID
BRI1	BRASSINOSTEROID INSENSITIVE 1
BSK	BR-SIGNALING KINASE
CaM	CALMODULIN
CDG1	CONSTITUTIVE DIFFERENTIAL GROWTH 1
CDPK	CALCIUM-DEPENDENT CYTOPLASMIC KINASE
CEP	C-TERMINALLY ENCODED PEPTIDE
CEPR	CEP RECEPTOR
CIF	CASPARIAN STRIP INTEGRITY FACTORS
CLE	CLAVATA3/EMBRYO SURROUNDING REGION-RELATED
CLV	CLAVATA
CPK28	CALCIUM-DEPENDENT PROTEIN KINASE 28
CrRLK1L	CATHARANTHUS ROSEUS RLK1-LIKE
CRN	CORYNE
CST	CAST AWAY
CTR	CONSTITUTE TRIPLE RESPONSE
DAMP	DAMAGE-ASSOCIATED MOLECULAR PATTERN
ECD	EXTRACELLULAR LIGAND-BINDING DOMAIN/ECTODOMAIN
EDR	ENHANCED DISEASE RESISTANCE
EF-Tu	ELONGATION FACTOR-TU
EFR	ELONGATION FACTOR-TU RECEPTOR
EGF	EPIDERMAL GROWTH FACTOR
EPF	EPIDERMAL PATTERNING FACTOR
ERL	ERECTA-LIKE
ETI	EFFECTOR TRIGGERED IMMUNITY
FER	FERONIA
FLS2	FLAGELLIN SENSITIVE 2
HAE	HAESA
HPAT	HYP O-ARABINOSYLTRANSFERASE
HSL	HAESA-LIKE
Hyp	HYDROXYPROLINE
HypSys	HYDROXYPROLINE-RICH SYSTEMIN
IDA	INFLORESCENCE DEFICIENT IN ABSCISSION

IDAL ILK	IDA-LIKE INTEGRIN-LINKED KINASE
LIP LORE LRR LysM	LOST IN POLLEN TUBE GUIDANCE LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION LEUCINE-RICH REPEAT LYSIN MOTIF
MAMP MAPK MAP4K	MICROBE-ASSOCIATED MOLECULAR PATTERN MITOGEN-ACTIVATED PROTEIN KINASE MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE KINASE
MIK MKKK7	MALE DISCOVERER 1-INTERACTING RECEPTOR-LIKE KINASE MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 7
NLR NPK NUT	LEUCINE RICH REPEAT RECEPTOR PROTEIN NICOTIANA TABACUM PROTEIN KINASE PLANT SCREW UNRESPONSIVE RECEPTOR
OG OH-FA	OLIGOGALACTURONIDES HYDROXY FATTY ACIDS
PAMP PBL1 PEP PEPR PK PRR PSK PSKR1	PATHOGEN-ASSOCIATED MOLECULAR PATTERN AVRPPHB SUSCEPTIBLE 1-LIKE PLANT ELICITOR PEPTIDE PEP RECEPTOR PROTEIN KINASE PATTERN RECOGNITION RECEPTOR PHYTOSULFOKINE PHYTOSULFOKINE RECEPTOR 1
PSY PSYR	PLANT PEPTIDE CONTAINING SULFATED TYROSINE PLANT PEPTIDE CONTAINING SULFATED TYROSINE RECEPTOR
PTM PTI PUB PXY P4H	POST-TRANSLATIONAL MODIFICATION PRR-TRIGGERED IMMUNITY PLANT UBOX PROTEIN PHLOEM INTERCALATED WITH XYLEM PROLYL-4-HYDROXYLASE
RACK RAF RALF RBOHD RGFR RK RLK RLCK ROS RP RRA3	RECEPTOR FOR ACTIVATED KINASE C RAPIDLY ACCELERATED FIBROSARCOMA RAPID ALKALINIZATION FACTOR RESPIRATORY BURST OXIDASE HOMOLOG D ROOT GROWTH FACTOR RECEPTOR RECEPTOR KINASE RECEPTOR-LIKE KINASE RECEPTOR LIKE CYTOPLASMIC KINASE REACTIVE OXYGEN SPECIES RECEPTOR PROTEIN REDUCED RESIDUAL ARABINOSE 3
SBT SCOOP	SUBTILISIN-LIKE SERINE PROTEASE SERINE-RICH ENDOGENOUS PEPTIDE

SCREW	SMALL PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSS
SERK	SOMATIC EMBRYOGENESIS RECEPTOR KINASE
SIK1	SERINE/THREONINE KINASE1
SOBIR1	SUPPRESSOR OF BIR1
S1P	SITE-1 PROTEASE
TDIF	TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FAC
TDR	TDIF-RECEPTOR
TMM	TOO MANY MOUTHS
TPST	TYROSYLPROTEIN SULFOTRANSFERASE
WAK	WALL-ASSOCIATED KINASES
WUS	WUSHEL
XEG113	XYLOGLUCANASE 113
ZIK	ZIPPER INTERACTING PROTEIN KINASE
ZIP	Z. MAYS IMMUNE SIGNALLING PEPTIDE

General Introduction

1.1 Summary

The perception and transduction of extracellular signals across the plasma membrane is essential for living organisms. As multicellular and sessile organisms, plants have evolved an extensive suite of transmembrane receptor kinases to sense biotic and abiotic cues in their environment. Receptor kinases perceive exogenous and endogenous signals that result in cellular responses such as the activation of developmental programmes, reproduction and plant defence. Phosphorylation mediated by kinases is a key reversible posttranslational modification in these signalling processes. Mitogen-activated protein kinase (MAPK) cascades are evolutionary conserved intracellular signal transduction pathways that are activated by receptor kinases to regulate a wide range of cellular processes. Here I will provide an overview of signal perception by receptor kinases and focus on the role of MAPK cascades in the complex and often overlapping intracellular signalling pathways.

1.2 General characteristics of transmembrane receptor kinases

1.2.1 Domain Architecture of RKs and RPs

Plants have evolved extensive transmembrane receptor families divided into RECEPTOR KINASES (RKs) and RECEPTOR PROTEINS (RP). RKs are among the largest gene families in plants, with *Arabidopsis thaliana* encoding for over 600 RKs and RECEPTOR LIKE CYTOPLASMIC KINASES (RLCKs) and rice (*Oryza sativa*) more than 1000 (Shiu et al. 2004; Shiu and Bleecker 2001). During land plant evolution this receptor diversification has likely enhanced developmental stability across diverse environmental conditions.

Plant RKs can be structurally divided into three domains: an extracellular ligand-binding domain/ectodomain (ECD), a single pass transmembrane domain; and an intracellular protein kinase domain (PK) which is crucial for intracellular signal transduction (Shiu and Bleecker 2001). RECEPTOR PROTEINS (RPs) resemble the RK domain architecture but lack intracellular kinase domains (Figure 1). Extracellular domains of RKs and RPs are structurally diverse and capable of perceiving a wide range of self- and non-self-molecules including peptides, small proteins, lipids and polysaccharides (Dievart et al. 2020). Extracellular domain diversity enables the classification of RKs and RPs

in various subfamilies including leucine-rich repeat (LRR), Lysin motif (LysM), lectin domain, epidermal growth factor-like (EGF-like) and malectin-like receptors (Shiu and Bleecker 2001; Dievart et al. 2020). LRR is the most common extracellular motif found in at least 223 RKs (Gou et al. 2010) and 56 RPs in Arabidopsis (Fritz-Laylin et al. 2005) (Figure 1).

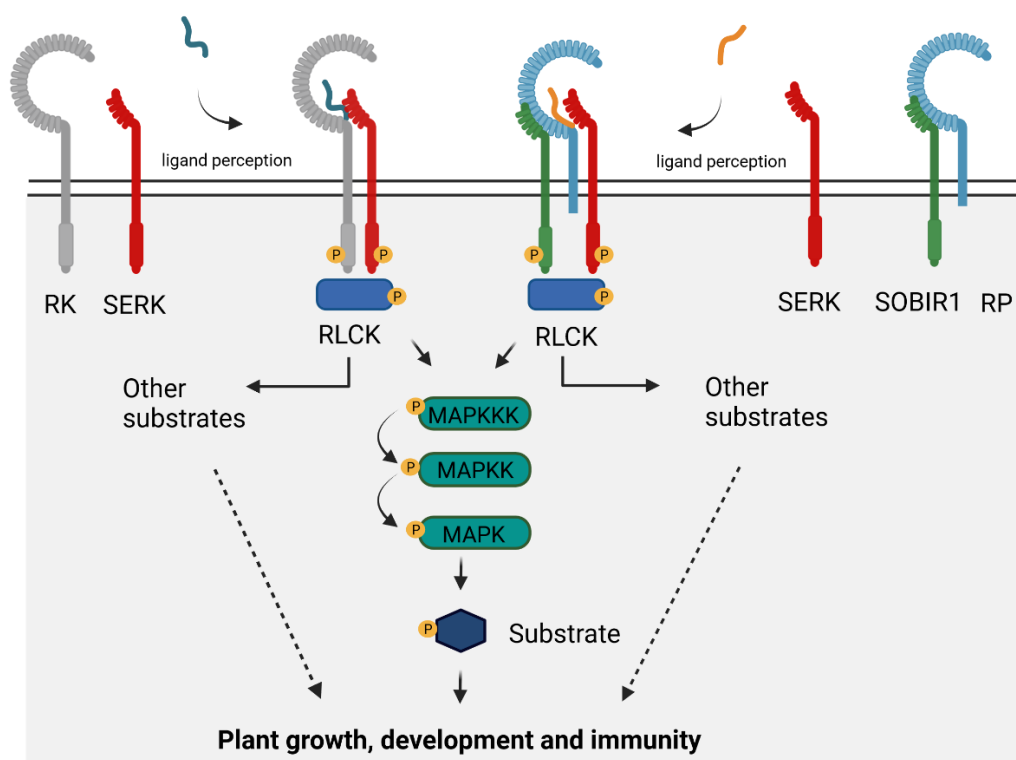


Figure 1. Overview of ligand induced Leucine-rich repeat receptor kinase (LRR-RK) and receptor protein (LRR-RP) activation and downstream signalling adapted from (He et al. 2018) and Rhodes 2019, PhD thesis. RKs and RPs are comprised of an extracellular domain, a transmembrane domain and RKs contain an additional intracellular kinase domain. Perception of endogenous and exogenous ligands via the extracellular domain often facilitates heterodimerization of the receptor with a co-receptor, frequently from the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family. RPs lack the intracellular kinase domain and rely on the interaction with SUPPRESSOR OF BIR1 (SOBIR1) and SERKs to transduce the signal. RECEPTOR-LIKE CYTOPLASMIC KINASES (RLCKs) function downstream of activated receptor complexes and activate several substrates including mitogen-activated protein kinases (MAPKs). MAPK cascade activation is one of the hallmarks of receptor signal transduction. MAPKs specifically phosphorylate a multitude of substrates which are involved in cellular responses such as growth, development, and immunity.

1.2.2 Activation of RK and RP signalling by complex formation

A common activation mechanism across RK families is the ligand-induced dimerization with shape complementary co-receptors of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family. SERKs are subfamily II LRR-RKs with relatively short ECDs that associate with several large-ECD RKs to modulate a variety of physiological processes (Aan den Toorn, Albrecht, and de Vries 2015). The most prominent SERK member is BRASSINOSTEROID INSENSITIVE 1 (BRI1) – ASSOCIATED RECEPTOR KINASE 1 (BAK1) also known as SERK3 which functions as a shared co-receptor for LRR-RKs in plant defence (Heese et al. 2007), but also for LRR-RKs that perceive endogenous signals such as brassinosteroids (BRs) (Li, Wen, et al. 2002; Nam and Li 2002). Ligand-dependent association brings the intracellular kinase domains of the receptor-co-receptor pair into proximity, which initiates a series of auto- and trans-phosphorylation events that ultimately amounts in the activation of downstream signalling components (Perraki et al. 2018) (Figure 1).

The ligand thereby generally acts as “molecular glue”, physically interacting with the receptor and the co-receptor (Moussu and Santiago 2019; Hohmann, Lau, and Hothorn 2017). The bacterial epitope flg22 binds to the receptor FLAGELLIN SENSITIVE 2 (FLS2) and contacts co-receptor BAK1 (SERK3). The steroid hormone Brassinolide (BL) promotes the association of BRI1 and SERK1 by facilitating the direct binding of the N-terminal part of SERK1 to the island domain of BRI1 (Santiago, Henzler, and Hothorn 2013). INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptides also contact both the LRR domain of HAESA and SERK1 (Santiago et al. 2016). An exception to the “molecular glue” model is the perception of PHYTOSULFOKINE (PSK) which binds and stabilises the island domain in LRR-RK PHYTOSULFOKINE RECEPTOR 1 (PSKR1) without making physical contact with the SERK co-receptor (Wang et al. 2015).

RPs, which lack intracellular kinase domains, require SUPPRESSOR OF BIR1-1 (SOBIR1) in addition to SERKs to function (Gust and Felix 2014). While RKs associate with SERKS upon ligand perception, RPs constitutively associate with SUPPRESSOR OF BIR1-1 (SOBIR1) and require SOBIR1 and BAK1 to function (Figure 1). For both RKs and RPs BAK1 acts as the complex activator by trans-phosphorylating the RK or the RP/SOBIR1 complex (Huang et al. 2024; van der Burgh et al. 2019).

The current paradigm of receptor activation suggests that ligand binding to the ECD of a receptor initiates the signalling process. However, Ogawa-Ohnishi et al. recently proposed a new model for PLANT PEPTIDE CONTAINING SULFATED TYROSINE RECEPTORS (PSYRs), termed “ligand deprivation-dependent activation” (Ogawa-Ohnishi et al. 2022). In this model, PSYRs are activated in response to depletion of their

peptide ligand PSY under biotic and abiotic stress, thereby triggering the plant's stress response.

1.2.3 Signalling downstream of membrane receptor complexes

Receptor-like cytoplasmic kinases

RECEPTOR LIKE CYTOPLASMIC KINASES (RLCKs) are among the first downstream targets of activated receptor complexes, bridging the gap between receptor activation and downstream signalling components (Lin et al. 2013; Liang and Zhou 2018) (Figure 1). RLCKs lack extracellular and transmembrane domains but nevertheless form a monophyletic group with RKs based on similar kinase domains (Shiu and Bleecker 2001). There are 149 RLCKs in Arabidopsis divided into 17 subfamilies that act both redundantly and specifically in the context of different RKs. RLCKs therefore act as regulatory nodes, connecting receptors to downstream modulators via a phosphorylation relay.

Around 49 RLCK subfamily VII (RLCK-VII) members, phylogenetically classified as RLCK-VII-1 to RLCK-VII-9, play distinct yet overlapping roles in immune signalling (Rao et al. 2018; Liang and Zhou 2018). BOTRYTIS-INDUCED KINASE1 (BIK1) and its close homologue AVRPPHB SUSCEPTIBLE 1-LIKE (PBL1) belong to RLCK-VII-8 and are some of the best-characterised RLCKs. BIK1 and PBL1 directly associate with immune receptors to potentiate immune signalling, thereby contributing to disease resistance against bacterial, fungal and aphid pathogens (Lu et al. 2010a; Kadota et al. 2014; Veronese et al. 2006; Lei et al. 2014; Li et al. 2014; Liu, Wu, et al. 2013; Zhang et al. 2010).

BIK1 is a crucial executor in RK-mediated immune signalling with its abundance being rate-limiting and tightly regulated. Heteromeric G proteins (Liang et al. 2016), mitogen-activated protein kinase kinase kinases (MAP4Ks), SERINE/THREONINE KINASE1 (SIK1) (Zhang, Chiang, et al. 2018) and MAP4K4 stabilize and positively regulate BIK1 abundance (Jiang et al. 2019). BIK1 abundance is negatively regulated by CALCIUM-DEPENDENT PROTEIN KINASE 28 (CPK28) (Monaghan et al. 2014), protein phosphatases PP2X38 (Couto and Zipfel 2016) and PP2C38 (Couto et al. 2016), E3-ligases PLANT UBOX PROTEIN 25 and 26 (PUB25/26) as well as RING-H2 FINGER A3A (RHA3A) and RHA3B (Wang et al. 2018; Yu et al. 2022), and PUB4 act as negative regulators of BIK1 abundance (Yu et al. 2022). In summary, these components collectively regulate BIK1 via phosphorylation, dephosphorylation, and ubiquitination and fine-tune its abundance in response to external stimuli (Mithoe and Menke 2018).

The function of RLCKs is not limited to immunity. RLCK-VII-6 and RLCK-VII-8 are involved in RALF23 and PHYTOSULFOKINE (PSK) signalling to regulate root growth

(Rao et al. 2018). BR-SIGNALING KINASEs (BSKs) play roles in brassinosteroid signalling (Tang et al. 2008) and immunity (Majhi, Sreeramulu, and Sessa 2019); CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) mediates brassinosteroid signalling (Kim, Guan, et al. 2011); CAST AWAY (CST) acts as a special inhibitor of cell separation to inhibit organ abscission (Burr et al. 2011); SHENG1 (SGN1/PBL15) is involved in casparian strip formation in the root endodermis (Alassimone et al. 2016; Fujita et al. 2020); LOST IN POLLEN TUBE GUIDANCE 1 and 2 (LIP1/2) in pollen tube guidance (Liu, Zhong, et al. 2013); ABA- AND OSMOTIC-STRESS-INDUCIBLE RECEPTOR-LIKE CYTOSOLIC KINASE1 (ARCK1) negatively regulates ABA and osmotic-mediated signal transduction (Tanaka et al. 2012). More recently, two RLCKs were shown to be required for arbuscular mycorrhizal (AM) symbiosis. AM-induced kinases AMK8 and AMK24 act together with RK KIN3 to promote AM symbiosis in *Lotus japonicus* and rice (Leng et al. 2023).

Mitogen-Activated Protein Kinases

Mitogen-activated protein kinase (MAPK) cascades are conserved signalling modules functioning downstream of multiple receptor complexes (Zhang and Zhang 2022). The typical MAPK cascade consists of three kinases that sequentially activate each other: MAPKKK - MAPKK – MAPK (Figure 1). Activation of MAPKKKs is essential for signalling through the cascade. RLCKs and feedback phosphorylation by MAPKs have been shown to link RK signalling to the activation of MAPK cascades (Rao et al. 2018; Bi et al. 2018a). The two most well-characterized MAPK cascades in plant immunity are MEKK1-MKK1/MKK2-MPK4/MPK11 and MKKK3/MKKK5-MKK4/MKK5-MPK3/MPK6. I will discuss the role of MAP kinases in signal transduction in detail in Section 1.4, “MAP kinases at the nexus of diverse signalling pathways.”

Signalling executors

RLCKs and MAPKs are core signalling modules that activate various downstream executors, often resulting in overlapping cellular responses regardless of the stressor (Figure 1). The NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD), responsible for ROS production, is activated by BIK1 phosphorylation in its N-terminus (Kadota et al. 2014; Benschop et al. 2007; Li et al. 2014). CNGC2 and CNGC4 form heteromeric calcium channels and BIK1 phosphorylation counteracts their inhibition by CALMODULIN (CaM) (Tian et al. 2019). OSCA1.3 mediates calcium influx and promotes stomatal closure upon BIK1/PBL1 phosphorylation (Thor et al. 2020). SHOU4 and SHOU4L, involved in cellulose synthesis, are N-terminally phosphorylated by BIK1. BIK1 phosphorylation of RBOHD, OSCA1.3 and SHOU4/4L has led to the identification

of a BIK1-specific phosphorylation motif, Ser-X-X-Leu. Compared to RLCKs, MAPK cascades are more often associated with triggering transcriptional reprogramming thorough the activation of downstream transcription factors (Menke et al. 2005; Ishihama et al. 2011; Andreasson et al. 2005).

1.3 Extracellular surveillance by cell-surface receptor kinases

Ectodomains of RKs perceive myriad molecular signals, both endogenous (self) and foreign (non-self). Foreign molecules, associated with pathogen infection, are known as pathogen/microbe-associated molecular patterns (PAMPs/MAMPs). Additional danger signals are damage-associated molecular patterns (DAMPs), which are modified-self molecules released upon cellular damage, allowing the plant to monitor a wider range of pathogens indirectly (Couto and Zipfel 2016). Many cell-surface receptors recognize PAMPs/DAMPs, while others with similar domain architecture recognize self-molecules such as phytohormones or small peptides to regulate growth and development. Their similar domain architecture suggests that immunity- and development-related receptors share a common evolutionary origin (Ngou et al. 2024). RKs can be divided into 60 subfamilies, with their diversity largely driven by subfamily expansion, but generally fall into two functional groups. The first group is involved in plant immunity and mainly perceives PAMPs, or DAMPs released during pathogen attacks (Macho and Zipfel 2014; Böhm et al. 2014). The second group perceives endogenous growth and developmental cues, such as the plant hormone BRs and signalling peptides like PSK (Tang, Wang, and Zhou 2017; Escocard de Azevedo Manhães et al. 2021). Some receptors, like the malectin-like receptor Feronia (FER), are involved in both immunity and development.

1.3.1 RKs in plant immunity – sensors of non-self

Central to the plant's survival is their ability to detect and ward off pathogen attacks (Boller and Felix 2009; Chisholm et al. 2006; Jones and Dangl 2006). Unlike animals, plants solely rely on innate immunity to do so. The core first layer of defence is the sensitive perception PAMPs/MAMPs and DAMPs, which can be carbohydrates, lipids, proteins and nucleic acids, by designated RKs called PATTERN RECOGNITION RECEPTORS (PRRs) (Boller and Felix 2009; Boutrot and Zipfel 2017). Ligand perception by PRRs leads to PAMP-, pattern-, - or PRR-triggered immunity (PTI). Hallmarks of PTI include the extracellular burst of reactive oxygen species (ROS), intracellular Ca^{2+} bursts and activation of Ca^{2+} dependent cytoplasmic kinases (CDPKs), activation of RLCKs and MAPK cascades and transcriptional and translational reprogramming (Yu et al. 2017; Couto and Zipfel 2016; Zhou and Zhang 2020).

Pattern recognition receptors (PRRs)

The LRR-RK FLS2 acts as the PRR for a specific 22 amino acid epitope (flg22) of the bacterial flagellin to induce resistance (Gómez-Gómez and Boller 2000; Zipfel et al. 2004; Felix et al. 1999; Meindl, Boller, and Felix 2000). First identified in *Arabidopsis thaliana*, FLS2 is critical for antibacterial immunity in most higher plant species (Boller and Felix 2009). The crystal structure of the ectodomain complex of FLS2 and BAK1 bound to flg22 revealed that flg22 binding by both FLS2 and BAK1 is crucial to stabilize the complex without causing conformational changes in the FLS2 receptor (Sun et al. 2013). Upon flg22 perception FLS2 rapidly recruits and forms a heterodimer with BAK1, which in turn triggers the rapid transphosphorylation of both FLS2 and BAK1 (Chinchilla et al. 2007; Schulze et al. 2010; Schwessinger et al. 2011). Binding to the elicitor flg22 also facilitates phosphorylation of the FLS2-BAK1 associated RLCK BIK1 (Benschop et al. 2007), which in turn dissociates and activates downstream signalling (Lu et al. 2010b; Zhang et al. 2010). Downstream immune responses include the production of reactive oxygen species (ROS), ion flux across the plasma membrane and triggering of mitogen-activated protein kinases (MAP) cascades (Chinchilla et al. 2007). To avoid detrimental growth defects through unwanted immune responses, FLS2 is actively maintained in a resting state by LRR-RK BAK1-INTERACTING RECEPTOR-LIKE KINASE 2 (BIR2), which acts as additional modulator. BIR2 contains a pseudo kinase domain and interacts with BAK1 in the absence of flg22, thereby preventing the formation of FLS2-BAK1 heterodimers (Halter et al. 2014).

ELONGATION FACTOR-TU (EF-Tu) RECEPTOR (EFR) perceives 18 amino acid epitope elf18 of the bacterial EF-Tu transcription factor (Zipfel et al. 2006). EFR is a non-RD type kinase that allosterically activates BAK1. Kinase inactive EFR alleles still retain their signalling function, making EFR an exception to the reciprocal transphosphorylation model (Bender et al. 2021; Mühlenbeck et al. 2024).

Endogenous plant elicitor peptides (Peps) are sensed as DAMPs by the PEP RECEPTORS (PEPR1 and PEPR2) (Krol et al. 2010; Yamaguchi, Pearce, and Ryan 2006). LysM-RKs perceive peptidoglycan and chitin from fungal and bacterial cell walls. LYK5 and CERK1 perceive fungal chitin while LYP4 and LYP6 sense both bacterial peptidoglycan and fungal chitin to trigger immune responses (Miya et al. 2007; Wan et al. 2008; Liu et al. 2012; Cao et al. 2014). WALL-ASSOCIATED KINASES (WAKs) harbour epidermal-growth factor (EGF) -like extracellular domains and were thought to recognise plant cell wall derived Oligogalacturonides (OGs), but a recent study in *Arabidopsis* shows that they are not genetically required for OG signalling (Herold et al. 2024). WAKs are expanded in monocots and are coming into focus as important players in plant

immunity and durable disease resistance (Stephens, Hammond-Kosack, and Kanyuka 2021). Lipids are perceived by G-type lectin PRRs (also known as S-domain RKs) such as LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE) which recognises bacterial medium-chain 3-hydroxy fatty acids (3-OH-FA)(Ranf et al. 2015; Kutschera et al. 2019).

Pathogens fight back

During evolution pathogens have evolved sophisticated strategies to circumvent the hostile environment created by PTI by secreting host specific effector proteins that target components of the PTI signalling machinery and other proteins, to compromise a cells immune response (Tang, Wang, and Zhou 2017; Couto and Zipfel 2016). *Pseudomonas syringae* AvrPphB and *Xanthomonas campestris* effector AvrAC both target subfamily VII RLCKs including BIK1 to inhibit PTI (Zhang et al. 2010; Feng et al. 2012). In an evolutionary arms race between the pathogen and the plant, plants have evolved intracellular nucleotide-binding leucine rich repeat receptor proteins (NLRs). NLRs perceive the presence of intracellular pathogen effector molecules and trigger the second layer of their immune response, called effector triggered immunity (ETI)(Förderer and Kourelis 2023). ETI and PTI are tightly connected and dependent on each other with PTI being the more general defence mechanism mounted on plasma membrane localized receptors (Ngou et al. 2020; Yuan et al. 2020).

1.3.2 RKs in in plant development – sensors of self

In addition to their role in immune signalling, RKs also regulate growth and developmental processes by facilitating cell-to-cell communication through autocrine and paracrine endogenous ligands. While most PRRs belong to subfamily XII, many receptors perceiving growth-promoting endogenous ligands belong to subfamily X and subfamily XI.

LRR-RK subfamily XI consists of 26-28 members, depending on whether full-length or ECD amino acid sequences are used (Furumizu and Aalen 2023; Wang, Chen, et al. 2022). Corresponding endogenous peptide ligands have been identified for all LRR-RK XI members, but HAIKU2 (IKU2), making it an exciting subfamily to study RK-peptide perception and evolution (Furumizu and Aalen 2023).

The CLAVATA (CLV) signalling pathway is essential for maintaining meristem homeostasis in plants by perceiving CLAVATA 3 (CLV3)/EMBRYO SURROUNDING REGION-related (CLE) peptides (Hirakawa 2021). The main components of this pathway are CLAVATA1 (CLV1)-type receptors including LRR-RK CLV1 and BARELY ANY

MERISTEM (BAM) 1/2/3 as well as LRR-RP CLAVATA2 (CLV2) and membrane-associated pseudokinase CORYNE (CRN) (DeYoung et al. 2006; Clark, Williams, and Meyerowitz 1997; Clark, Running, and Meyerowitz 1993). The CLV signalling module restricts the stem cell niche by limiting *WUSHEL* (*WUS*) expression which would otherwise promote stem cell identity and meristem growth (Somssich et al. 2016). CLV signalling plays important roles in meristem homeostasis, plant developmental adaptation to nutrient availability, abiotic stress and symbiosis. In the vasculature LRR-RK PXY/TDR (PHLOEM INTERCALATED WITH XYLEM/ TDIF-RECEPTOR) perceives another member of the CLE peptide family, TDIF (TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR) to control the expression of WUS-related HOMEODOMAIN transcription factors and thereby xylem differentiation and proliferation (Hirakawa, Kondo, and Fukuda 2010).

LRR-RKs *HAESA* (*HAE*) and its close homologue and *HAESA-like 2* (*HSL2*) function redundantly in regulating cell separation events such as floral organ abscission and root cap sloughing response to INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptides (Walker 1993; Jinn, Stone, and Walker 2000; Cho et al. 2008). Interestingly, the sequence-related LRR-RK *HAESA-like 1* (*HSL1*) binds IDA/IDL with high affinity and CLE peptides with lower affinity and thereby regulates leaf epidermal patterning independently of the BAM1/CLE signalling module (Roman et al. 2022).

HAESA LIKE3 (*HSL3*)/ PLANT SCREW UNRESPONSIVE RECEPTOR (NUT) is phylogenetically related to *HSL1/2* but perceives distinct CTNIP/ SMALL PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSS (SCREWS) peptides to regulate root development, stomatal re-opening and activate hallmarks of general stress response (ROS production, Ca²⁺ influx, MAPK activation) (Rhodes et al. 2022; Liu, Hou, et al. 2022).

C-TERMINALLY ENCODED PEPTIDES (CEPs) contribute to the regulation of nitrogen homeostasis by binding to their cognate LRR-RK CEP RECEPTOR1/2 (CEPR1/2). RECEPTOR-LIKE KINASE 7 (RLK7) was recently shown to be a novel CEP4-specific CEP receptor (Rzemieniewski et al. 2024). CEP signalling coordinates the nitrogen status of the plant and cell surface immunity, making it an important signalling module for biotic and abiotic stress integration (Rzemieniewski et al. 2024).

ROOT GROWTH FACTOR RECEPTORS 1/2/3 (RGFR1/2/3) recognizes RGFs to promote root meristem development (Shinohara et al. 2016; Song et al. 2016). Triple loss-of-function mutants *rgfr1/2/3* are insensitive to exogenous RGF application and

exhibit a short root phenotype with a strongly decreased number of meristematic cells (Shinohara et al. 2016).

Initial circumstantial evidence suggested that PSKR1 homologue At1g72300 could be the receptor for PLANT PEPTIDE-CONTAINING SULFATED TYROSINE 1 (PSY) and was therefore named PSY1R. However, PSY responses were not abolished in the *psy1r* mutant and recently three subfamily XI LRR-RKs *PSYR1* (At1g17230), *PSYR2* (At2g33170), and *PSYR3* (At5g63930) were identified as cognate receptors for PSY (Ogawa-Ohnishi et al. 2022).

LRR-RK SHENGGEN/GASSHO1 (SGN3/GSO1) perceive CASPARIAN STRIP INTEGRITY FACTORS (CIF) which activates Casparian strip formation (Okuda et al. 2020). GSO2, a close homologue of GSO1, acts together with GSO1/SGN3 in cuticle formation (Fujita 2021).

Members of **LRR-RK subfamily X** have also been linked to the perception of endogenous ligands. The perhaps best understood RK signalling process is mediated by LRR-RK BRASSINOSTEROID INSENSITIVE 1 (BRI1) which perceives brassinosteroids, a group of growth-modulating steroidal compounds (Li and Chory 1997). Brassinosteroid signalling is implicated in a wide range of cellular and developmental contexts such as flowering, root development, responses to light and temperature as well as cell elongation and expansion during etiolation of dark grown seedlings (Nolan et al. 2020).

LRR-RKs PSKR1 and PSKR2 recognise the plant peptide PHYTOSULFOKINE (PSK) (Matsubayashi et al. 2006; Amano et al. 2007). PSK signalling promotes cell proliferation, cell identity, growth, and cell expansion (Matsubayashi et al. 2006). PSK signalling also suppresses SA-mediated immunity which results in enhanced resistance to necrotrophic pathogens but susceptibility to biotrophic pathogens (Igarashi, Tsuda, and Katagiri 2012; Mosher and Kemmerling 2013). By modulating both growth and immunity signalling, PSK plays a major roles in plant microbiome homeostasis (Song et al. 2023).

Other receptor kinases involved in growth and development include *Malectin*-like domain-containing RK FERONIA (FER). FER is the best-characterised RK out of 17 CrRLK1L members in Arabidopsis (Franck, Westermann, and Boisson-Dernier 2018) and plays a pivotal role in the perception of RAPID ALKALIZATION FACTORS (RALFs) to control primary root growth (Haruta et al. 2014), female fertility and hormone- and pathogen signalling (Li, Wu, and Cheung 2016). ERECTA-family of LRR-RKs, ERECTA-LIKE1 (ERL1), and ERL2 perceive a family of secreted peptides EPIDERMAL PATTERNING FACTORS (EPFs)/EPF-LIKES (EPFLs) to control stomatal patterning

(Herrmann and Torii 2020; Torii 2012). The RLP TOO MANY MOUTHS (TMM) acts in concert with ERL1 and ERL2 to discriminate context-dependent EPF/EPFL signals (Herrmann and Torii 2020; Torii 2012).

1.3.3 Concluding Remarks

Upon ligand perception, RKs involved in growth and RKs involved in immunity often activate similar and overlapping downstream outputs (DeFalco et al. 2022). Key signalling components such as NADPH oxidase RBOHD appear to be utilised by multiple diverse signalling pathways (Ma et al. 2023). FLS2 activates RBOHD via BIK1 to trigger ROS production in plant defence (Kadota et al. 2014). SGN3 also activates NADPH oxidases RBOHD/F to stimulate lignification and Casparian strip formation (Fujita et al. 2020). The IDA/IDL-HAE/HSL2 signalling module induces cell separation and triggers ROS production and Ca²⁺ influx, signatures of immune responses, potentially to protect separating cells from pathogen attack (Lalun et al. 2024).

Understanding how plants achieve signalling specificity within these complex networks remains a major question in plant biology. Plant genomes encode thousands of small, often secreted, peptides that likely fine-tune RK signalling networks, contributing to the specificity required for diverse physiological responses. Next, I will provide an overview of the diversity of plant peptide ligands, their processing, and their potential roles in modulating immune and developmental signalling pathways.

1.4 Small signalling peptides modulate plant growth and immunity

Small signalling peptides coordinate and integrate cellular functions that are essential for many aspects of plant development (Matsubayashi and Sakagami 2006; Breiden and Simon 2016). With the advancements of genomic tools, many hundreds of small signalling peptides were predicted to be encoded in plant genomes, but relatively few have been characterised and their corresponding receptors identified (Olsson et al. 2019).

These small peptides show high sequence diversity across plant species and evidence suggests that they are highly active at low concentrations. Interestingly, no peptide-ligand homologues have been identified in algae, which may suggest that signalling through secreted peptides provides cell-to-cell communication benefits crucial for the establishment of multicellularity (Furumizu and Shinohara 2024). Peptide ligands can generally be categorized based on differences in their structure and biogenesis. Some are produced from small-peptide encoding genes, sORFs (sORFs; 30–100 amino acids) in 5'-UTRs of protein-coding genes in many eukaryotes (Kondo et al. 2010; Kastenmayer et al. 2006) or miRNAs (Tavormina et al. 2015). Other peptides are processed from

functional or non-functional precursor proteins through proteolytic cleavage and often require additional posttranslational modifications for their activity (Tavormina et al. 2015). These so-called precursor-derived peptides, their post-translational modifications, and their biological functions will be the focus of the next section of this chapter. Peptide ligands can be further divided into secreted and non-secreted peptides based on the presence or absence of a signal peptide in their prepropeptide (Matsubayashi 2014). Apart from a few exceptions, ENOD40, ROT4/DVL and POLARIS, all peptide signals studied to date act extracellularly (Tavormina et al. 2015).

1.4.1 Post-Translational Processing and Modification of peptide ligands

To become biologically active, most peptides undergo proteolytic cleavage and post-translational modifications (PTMs) within the secretory pathway and sometimes the extracellular space (Olsson et al. 2019; Matsubayashi 2014). The initial processing step in the conversion from an inactive prepropeptide to an active mature peptide is the removal of the sorting sequence by a peptidase in the endoplasmic reticulum. This cleavage results in the formation of the propeptide. Subsequent processing steps vary between different peptide families and even among peptides within the same family (Olsson et al. 2019; Matsubayashi 2014).

Disulfide bond formation is usually required in Cys-rich peptides to archive correct secondary folding and activity (Tavormina et al. 2015). In eukaryotes, disulfide isomerases are generally thought to catalyse disulfide bridge formation in the endoplasmic reticulum (Freedman, Hirst, and Tuite 1994), but little is known about how and which of the 22 disulfide isomerases in *Arabidopsis* contribute to disulfide formation in secreted peptide ligands (Houston et al. 2005).

PTMs are enzymatically added to peptides in the secretory pathway and are often necessary for their biological activity. Known types of PTMs on plant peptides include Tyrosine sulfation, Proline hydroxylation, and hydroxyl arabinosylation.

Tyrosine sulfation catalysed by the enzyme TYROSYLPROTEIN SULFOTRANSFERASE (TPST) is a common post-translational modification for proteins and secreted peptides in animals and plants (Moore 2003). Neither TPST orthologues nor TPST activity or tyrosine-sulfated proteins have been described in prokaryotes and yeast (Moore 2003; Kehoe and Bertozzi 2000). *Arabidopsis* encodes for a single *TPST* gene, and loss-of-function mutants exhibit severe phenotypes including dwarfism, stunted roots, early senescence and fewer flowers and siliques (Komori et al. 2009).

Proline hydroxylation is the most common PTM in humans and catalysed by PROLYL-4-HYDROXYLASES (P4Hs). In Arabidopsis, there are 13 P4H enzymes with high sequence similarity suggesting substantial functional redundancy which has impeded their precise functional characterisation. There is no consensus sequence for Pro hydroxylation, but some peptides are more efficiently hydroxylated than others suggesting context dependency.

Hydroxyproline arabinosylation, a plant-specific post-translational modification, involves the addition of O-linked L-triarabinose chains to hydroxyproline (Hyp) residues. This process occurs in two steps, catalyzed by different enzymes. First, HYP O-ARABINOSYLTRANSFERASE (HPAT) enzymes add an arabinose moiety to Hyp. Arabidopsis encodes three HPAT enzymes, and triple loss-of-function mutants exhibit pleiotropic phenotypes, including early flowering and senescence, cell wall thickening defects, pollen tube growth issues, and enhanced hypocotyl elongation, indicating Hyp O-arabinosylation's essential roles in vegetative and reproductive growth (Ogawa-Ohnishi, Matsushita, and Matsubayashi 2013). In a second enzymatic reaction catalysed by arabinosyltransferases REDUCED RESIDUAL ARABINOSE 3 (RRA3) and XYLOGLUCANASE 113 (XEG113), the arabinose chain is extended (Egelund et al. 2007; Gille et al. 2009). This extension is crucial for many membrane and cell wall-localized proteins, such as EXTENSIN proteins, which require arabinosylation for proper function.

Proteolytic cleavage is an essential mechanism for the release of bioactive peptides. The Arabidopsis genome encodes 56 subtilisin-like serine proteases (SBTs) making functional studies challenging (Rautengarten et al. 2005). Nevertheless, several SBTs were found to be involved in the processing of small, secreted peptide ligands. SBT4.12, SBT4.13, and SBT5.2 process IDA peptides to derive the 14-amino acid-long biologically active peptide (Schardon et al. 2016); SBT6.1 and SBT3.8 process tyrosine sulfated CLEL6 (GLV1/RGF6) and CLEL9 (GLV2/RGF9) (Ghorbani et al. 2016; Stührwohltdt et al. 2020); SBT3.8 C-terminally cleaves tyrosine sulfated proPSK1 peptide (Stührwohltdt et al. 2021); SBT1.1 N-terminally cleaves PSK4 in vitro (Srivastava, Liu, and Howell 2008). PROSCOOP12 and PROSCOOP20 are cleaved by subtilases SBT3.5 and SBT3.6, SBT3.8 and SBT3.9, respectively (Yang et al. 2023).

1.4.2 Biological function of peptide ligands

Non-secreted non-modified peptides

Systemin, plant elicitor peptide (PEP1) and *Z. mays* immune signalling peptide 1 (ZIP1) are passively released by cellular damage to amplify immune responses and are

therefore also considered DAMPs. Systemin, an 18-amino acid peptide first discovered in tomatoes, triggers systemic defence responses against herbivory and is found in most Solanaceae species (McGurl et al. 1992; Pearce et al. 1991; Zhang, Zhang, and Lin 2020; Ryan and Pearce 2003). The 23-aa peptide PEP1 from *Arabidopsis* triggers PTI responses and enhances resistance against *Pseudomonas syringae*, *Botrytis cinerea*, and *Phytophthora infestans*. Orthologues of PEP1 are found in many plant species, including rice, maize, potato, and soybean. ZIP1, a 17-aa peptide from *Zea mays*, enhances maize resistance to biotrophic *Ustilago maydis* and susceptibility to necrotrophic *B. cinerea* by inducing SA accumulation and activating SA-responsive genes.

Secreted non-modified peptides

SERINE-RICH ENDOGENOUS PEPTIDES (SCOOPs) are a large, Brassicaceae-specific peptide family perceived by the LRR-RK MALE DISCOVERER 1-INTERACTING RECEPTOR-LIKE KINASE 2 (MIK2) to trigger PAMP-like immune responses (Rhodes et al. 2021; Hou et al. 2021; Gully et al. 2019). Although SCOOP peptides are generally poorly conserved, they possess two conserved serines at positions 5 and 7, as well as a glycine-rich C-terminal region. This makes them distinct from other plant peptide families such as CLE, Pep, IDA, PIP, and CEP, which often contain arginine-histidine-asparagine motifs.

The 5-member CTNIP/SCREW (SMALL PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSS) peptide family forms an evolutionary ancient signalling module with LRR-RK HAESA-LIKE3 (HSL3)/ PLANT SCREW UNRESPONSIVE RECEPTOR (NUT) (Liu, Hou, et al. 2022; Rhodes et al. 2022). CTNIP/SCREW peptides are transcriptionally upregulated in response to biotic stress.

RAPID ALKALINIZATION FACTOR (RALF) peptides are cysteine-rich peptide hormones involved in various physiological and developmental processes and are unique to terrestrial plants. More than 30 RALF peptides are predicted to be encoded in the *Arabidopsis* genome and signal through *Catharanthus roseus* RLK1-LIKE (CrRLK1L) receptor kinases. RALF peptides trigger alkalisation and growth inhibition and the two signalling outputs are generally coupled. In most cases, cleavage of RALF pro-peptides by SITE-1 PROTEASE (S1P) results in the inhibition of immune responses by the mature peptide, whereas non-cleaved peptides play a positive role in immunity by potentiating PAMP-induced responses (Stegmann et al. 2017; Abarca, Franck, and Zipfel 2021).

Secreted post-translationally modified peptides

In the search for systemin-like peptides, actively secreted, hydroxyproline-rich systemin (HypSys) glycopeptides, were identified in most species of the Solanaceae family (Ryan and Pearce 2003).

Phytosulfokines (PSKs) are secreted, disulfated pentapeptides (Tyr(SO₃H)-Ile-Tyr(SO₃H)-Thr-Gln) that were first shown to promote cell proliferation of suspension-cultured mesophyll cells in *Asparagus officinalis* L (Matsubayashi and Sakagami 1996). PSK precursor genes are present in both monocotyledons and dicotyledons, which supports the fundamental importance of PSK signalling in plants (Yang et al. 2000; Sauter 2015). The YIYTQ pentapeptide is highly conserved and sulfation of both Y residues by TPST substantially increases its biological activity (Matsubayashi and Sakagami 1996; Kutschmar et al. 2009).

PLANT PEPTIDE CONTAINING SULPHATED TYROSINES (PSYs) are 15-18-aa peptides that contain a tyrosine sulfated moiety (Amano et al. 2007; Ogawa-Ohnishi et al. 2022). The *Arabidopsis* genome encodes 9 PSY peptides (Tost et al. 2021; Ogawa-Ohnishi et al. 2022). Some, like PSY1, contain additional hydroxyprolinated and arabinosylated residues, while others, such as PSY5/PSY6, and PSY8, lack these additional modifications (Ogawa-Ohnishi et al. 2022).

CLE peptides, named after CLAVATA 3 (CLV3)/EMBRYO SURROUNDING REGION-related, are a large family of peptides encoded by 32 genes in *Arabidopsis* (Oelkers et al. 2008; Cock and McCormick 2001). CLV3, the first identified member of this peptide family, is a Hyp O-arabinosylated 12-amino-acid glycopeptide. CLE peptides are thought to function as 12- or 13-amino-acid peptide that regulate meristem activity in shoots, roots and vascular tissue (Whitewoods 2021). They are classified into two groups: R-type and H-type CLEs, based on the first amino acid in the peptide sequence. R-type CLEs contain an arginine (R) and interact with the BAM/CLV1 receptor clade, while H-type CLEs contain a histidine (H) and interact with the PXY/TDR receptor clade (Furumizu and Shinohara 2024). R-type CLEs, such as CLV3, CLE6, CLE19 and CLE40, promote root and shoot apical meristem differentiation and are crucial for plant growth (Whitford et al. 2008). H-type CLEs, such as TDIF/CLE41/CLE44 and CLE42, promote procambial cell proliferation and suppress xylem differentiation and thereby ensure proper formation of vascular tissue (Hirakawa et al. 2008). Both R- and H-type CLEs can act synergistically in specific cellular contexts (Whitford et al. 2008).

INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and IDA-LIKE (IDL) proteins share a conserved C-terminal domain which is processed to a functional hydroxylated

12 amino acid peptide (Butenko et al. 2003; Butenko et al. 2014; Santiago et al. 2016). A recent study has elucidated how IDA/IDL rely on their conserved N-terminus and core regions for specific recognition by cognate receptor HSL1, whereas sequence related CLE9 and other CLE peptides predominantly rely on their N-terminus to target specific CLE receptors (Roman et al. 2022). Despite similarities, IDA/IDL and CLE/CLV3 peptides constitute two phylogenetically distinct gene families (Cock and McCormick 2001; Vie et al. 2015).

Three independent studies have identified the same peptide family named RGF (Matsuzaki et al. 2010) or GLV (Whitford et al. 2012) or CLEL (Meng, Buchanan, et al. 2012). The RGF family, with 9 members, was discovered using sequence information from PSK and PSY peptides, characterized by low cysteine residue abundance, a secretion signal, and an Asp-Tyr sequence essential for tyrosine sulfation (Matsuzaki et al. 2010). The 11 member GLV peptide family was identified through a reverse genetic screen followed by a homology search (Whitford et al. 2012). Lastly, 10 members of the CLEL family were identified using homology searches based on the unique CLE motif of CLE18 (Meng, Buchanan, et al. 2012). Henceforth, I will refer to this peptide family as RGFs. RGFs are tyrosine sulfated peptides involved in root growth and maintenance of the root stem cell niche and tyrosine sulfation is required for receptor binding (Matsuzaki et al. 2010; Song et al. 2016). Recently RGF9/GLV2/CLEL9 was shown to promote cell surface immune signalling by promoting FLS2 PRR abundance (Stegmann et al. 2022).

C-TERMINALLY ENCODED PEPTIDES (CEPs) play important roles in plant environmental adaptation as paracrine signals in short and long-distance signalling for nitrogen demand signalling and root development (Taleski, Imin, and Djordjevic 2018). Arabidopsis encodes 15 CEP encoding genes which are proteolytically processed to release one or more 15-aa peptides (Roberts et al. 2013; Delay, Imin, and Djordjevic 2013). CEP peptides are hydroxylated and sometimes glycosylated by tri-arabinylation at multiple proline residues (Mohd-Radzman et al. 2015) and perceived by CEP RECEPTOR 1.

CASPARIAN STRIP INTEGRITY FACTOR 1 (CIF1) and (CIF2) are involved in the regulation of Casparian strip formation in the root endodermis and are perceived by the receptor kinase SHENGEN 3 (Doblas et al. 2017; Nakayama et al. 2017).

1.4.3 Immune Modulation through Phytocytokines

With the characterisation of increasing numbers of plant peptides and their cognate receptors, it is becoming evident that small, secreted peptides often regulate both growth and immunity. In a manner similar to animal cytokines, which provide growth,

differentiation, and inflammatory or anti-inflammatory signals for cell-to-cell communication (Luo 2012; Turner et al. 2014), many plant peptides (PEPs, PIPs, SCOOPs, CTNIPs, CLEs, IDLs) are upregulated and/or released during biotic or abiotic stress and subsequently modulate immune and stress-response signalling. Due to their functional analogy to animal cytokines, these plant peptides are also referred to as phytocytokines. Mechanisms of immune modulation through phytocytokines often remain unclear due to the lack of knowledge of downstream signalling components. Some phytocytokines, such as SCOOPs, enhance plant immune signalling, while others, such as PSK, suppress immunity. Except for recently identified RLP RALR, all phytocytokine receptors are RKs (Rzemieniecki and Stegmann 2022; Lintz et al. 2023).

1.5 MAP Kinases at the nexus of diverse signalling pathways

The MAP kinase cascade is a universal eukaryotic signalling module that coordinates cellular growth, development, and environmental adaptation (Widmann et al. 1999; Cargnello and Roux 2011). Compared to other eukaryotes, plants have largely expanded kinase families, with 80 MAPKKKs, 10 MAPKKs and 20 MAPKs (Ichimura et al. 2002). This expansion likely goes hand in hand with expanded cell surface receptor repertoires and potentially the number of secreted signalling peptides discussed in previous chapters (Ma and Nicolet 2023). The “hourglass” pattern of the number of kinases on each tier of the cascade gives rise to thousands of potential cascade-forming units. Despite this diversity, only a few cascades have been characterized, likely due to the difficulties in studying MAPK kinases, which include lethal loss-of-function phenotypes, high degrees of convergence and redundancy, and transient interactions, all of which hinder the functional characterization of MAPK cascades (Zhang and Zhang 2022).

In this section, I will explore the functional diversity of MAP kinases in plants, emphasizing well-characterized MAPK cascades and regulatory mechanisms, with a particular focus on MAPKKKs of the MEKK family.

MAPKKKs

The MAPKKK family can be divided into MEKK, and RAPIDLY ACCELERATED FIBROSARCOMA (RAF) kinases, whereby RAF-like kinases are subdivided into B-RAFTs and C-RAFTS/ZIPPER INTERACTING PROTEIN KINASE (ZIKs) (Ichimura et al. 2002). Despite being the largest family of MAP kinases, the precise regulatory mechanisms of MKKKs in plants are not well understood. In mammals, two common mechanisms for activating MKKKs are phosphorylation and the binding of monomeric G proteins (Xu and Zhang 2015). Recent studies have shown phosphorylation by RLCKs

and complex feedback phosphorylation modulation within the MAP kinase cascade are crucial mechanism for MAP kinase signal transduction in plants (Bi et al. 2018b).

MEKKs and RAFs are either phylogenetically unrelated or only distantly related, and it has been suggested that RAFs have convergently evolved from distinct protein kinases to acquire biochemical functions similar to those of MEKKs (Jouannic et al. 1999). While substantial evidence shows that MEKKs activate downstream MKKs and subsequently MAPKs, there is little evidence that RAFs can activate MKK-MAPK modules in plants, further supporting their distant evolutionary and potentially distinct biochemical roles.

B-RAFTs fulfil multifaceted functions in hormone signalling and environmental responses, making them crucial for plant environmental adaptation (Wang 2024). In animals, B-RAFTs regulate cell division and growth and are classified as proto-oncogenes, making them a potential target for cancer therapy. Plant B-RAFTs are divided into functionally distinct subgroups B1-B4 and are implicated in ABA and osmotic stress signalling from which SnRK2s (serine/threonine kinases) have emerged as common downstream targets. Recently, B4 RAF-like kinases were identified as central signalling components in a conserved rapid auxin response pathway, relaying the auxin signal through SnRK2s (Kuhn et al. 2022). Well-characterized B-RAFTs ENHANCED DISEASE RESISTANCE 1 (EDR1) and CONSTITUTE TRIPLE RESPONSE 1 (CTR1) belong to the B3 family. EDR1 negatively regulates plant immunity in an SA-dependent manner (Frye, Tang, and Innes 2001) while CTR1 negatively regulates ethylene signalling (Huang et al. 2003).

Members of the C-RAF subfamily autophosphorylate *in vitro* and act together with CPK28 in plant immunity and stomatal opening (Dias et al. 2023). Subgroup 1 C-RAFTs, also known as Integrin-linked kinases (ILKs) based on their N-terminal Ankyrin repeat domains similar to animal ILKs, are thought to function in a wide range of cellular signalling pathways (Popescu et al. 2017). ILK5 acts downstream of extracellular ATP receptor P2K1 to trigger immune signalling via activation of MKK5. ILK5 is the only known plant RAF kinase to date that has been shown to activate a downstream MKK (Kim, Chen, et al. 2023). In general, RAFs may target diverse downstream effectors including protein kinases, ion channels or transcriptional regulators through phosphorylation or direct interaction and, compared to MEKKs, their functions remain much less understood.

MKKs of the MEKK family exhibit similarities to yeast Ste11 and are categorized into subclades A1-A4 with diverse roles in plant signalling and development (Ichimura et al.

2002). The A1 clade includes MEKK1, important for PRR signalling and development, along with MEKK2, MEKK3 and MEKK4, involved in stress response and growth regulation, but generally less well characterised. The A2 subclade contains YDA (MKKK4), regulating guard cell identity and stomatal pattern formation (Bergmann, Lukowitz, and Somerville 2004), and MKKK3 and MKKK5, which act redundantly in PRR-triggered immune signalling (Bi et al. 2018b). The A3 clade consists of *NICOTIANA TABACUM PROTEIN KINASE 1* (NPK1), which was the first MKKK characterised from plants (Banno et al. 1993), and its Arabidopsis homologues *ARABIDOPSIS NPK1-LIKE PROTEIN KINASE 1/2/3* (ANP1/2/3), all regulating cytokinesis (Krysan et al. 2002; Ishikawa et al. 2002). The A4 clade consists of MKKK6 and MKKK7 involved in male fertility and root growth (Chaiwongsar et al. 2006; Chaiwongsar et al. 2012). Many members of the MEKK family have been functionally linked to activate MAPK cascades which will be discussed further in sections 1.4.1 (MAPK cascades in plant stress response) and 1.4.2 (MAPK cascades in growth and development).

MAPKKs

The Arabidopsis genome encodes 10 conserved MAPKKs which are activated, like their mammalian counterparts, through dual phosphorylation in their characteristic activation loop sequence S/TxxxxxS/T by upstream MAPKKs (Cargnello and Roux 2011). Based on sequence similarity, MAPKKs are divided into four groups: Group A: MKK1, MKK2 and MKK6; Group B: MKK3; Group C: MKK4 and MKK5; Group D: MKK7, MKK8, MKK9, MKK10 (Ichimura et al. 2002). Upon activation, MAPKKs associate with downstream MAPKs through their N-terminal docking site [K/R][K/R][K/R]x(1–5)[L/I]x[L/I] (Jonak et al. 2002) and subsequently phosphorylate MAPKs in conserved Threonine (T) and Tyrosine (Y) residues, which trigger their activation.

MKK1 and MKK2 regulate abiotic and biotic stress responses by acting upstream of MPK4 and MPK6 (Gao et al. 2008; Qiu et al. 2008). MKK4 and MKK5 function redundantly to control immunity and development via MPK3 and MPK6 (Kim, Woo, et al. 2011; Asai et al. 2002; Lu et al. 2020). MKK3 is involved in JA signalling through MPK6. While group D MKKs are not well understood, MKK7 and MKK9 have been shown to act upstream of MPK3 and MPK6 (Wang et al. 2016).

MAPKs

MAPKs are proline-directed, serine/threonine kinases implicated in diverse processes including hormone signalling (Jia et al. 2016), cell division (Suzuki and Machida 2008) and immune responses (Asai et al. 2002; Meng and Zhang 2013; Menke et al. 2004). MAPKs are conserved across species and contain a TEY motif in their activation loop,

which requires phosphorylation by upstream MKKs for activation (Pearson et al. 2001). The common Phospho-p44/p42 antibody, which specifically reacts with phosphorylated TxY motifs in mammalian ERK1/ERK2 MAPKs, also recognises plant MPK3, MPK6, and MPK4/MPK11 phosphorylation.

MAPKs are grouped into two groups: Group A: MPK3, MPK6; Group B: MPK4, MPK5, MPK11, MPK12, MPK13. Plants also have a unique set of eight MAPKs with a TDY motif and no C-terminal docking site. These TDY or group D MAPKs include MPK8, MPK9, MPK15, MPK16, MPK17, MPK18, MPK19, and MPK20. MAP kinases constitute intracellular convergence hubs for many signalling pathways and thereby contribute to the cross-regulation between multiple hormones (Yin et al. 2023).

1.5.1 MAPK cascades in plant immunity

Two well-described MAPK cascades that function redundantly in plant immunity consist of MAPKKK5/3 - MKK4/5 - MPK3/6 and MEKK1 - MKK1/2 - MPK4/11 (Kong et al. 2012; Asai et al. 2002; Meng and Zhang 2013).

Nicotiana tabacum NtMEK2 acts upstream of SIPK and WIPK (Yang, Liu, and Zhang 2001). Arabidopsis homologues of NtMEK2 are MKK4 and MKK5 and homologues of SIPK and WIPK are MPK3 and MPK6. GOF and biochemical evidence suggested that MKK4/MKK5 act upstream of MPK3/MPK6 while double loss-of-function mutants in *mkk4/mkk5* or *mpk3/mpk6* are lethal (Zhang and Zhang 2022). Using analogue sensitive versions of MPK3/MPK6 with the inhibitor NA-PP1 revealed compromised PTI and ETI signalling (Su et al. 2017; Su et al. 2018). TILLING mutants with 90% reduced MKK4 activity (*mkkk4-18*) and a *mkkk5* null allele (*mkk5-18*) showed that the *mkk4-18mkk5-18* double mutants are impaired in MPK3/MPK6 activation and pathogen resistance (Su et al. 2017). MKKK3 (MKKK α) and MKKK5 are two highly homologous kinases that were identified to act upstream of MKK4/MKK5-MPK3/MPK6. *mkkk3mkkk5* LOF mutants show reduced MAPK cascade activation and enhanced susceptibility. Activated MPK3 and MPK6 phosphorylate and thereby enhance MKKK3 and MKKK5 activity, suggesting a positive feedback mechanism (Bi et al. 2018a).

The second kinase cascade in plant immunity consists of MEKK1-MKK1/MKK2-MPK4/MPK11. Initially, MEKK1 was found to activate MPK4 in response to flg22, but the severe dwarf and autoimmune phenotype of the *mekk1* T-DNA mutant led to MEKK1 being considered a negative regulator of immune signalling (Suarez-Rodriguez et al. 2007). MKK1 and MKK2 function redundantly downstream of MEKK1 to activate MPK4, with *mkk1/mkk2* mutants exhibiting seedling lethality (Gao et al. 2008; Qiu et al. 2008). The severe phenotypes of *mekk1* and *mkk1/mkk2* and *mpk4* are temperature-dependent and the integrity of the MEKK1-MKK1/MKK2-MPK4/MPK11 cascade is monitored by

diverse NLRs (Gao et al. 2008; Suarez-Rodriguez et al. 2007; Takagi et al. 2018; Zhang et al. 2012). The coiled-coil NLR SUMM2 guards the integrity of MPK4 by monitoring the CRCK3 substrate (Zhang et al. 2012), and the TIR NLR SMN1/RPS6 suppresses the dwarf, autoimmune phenotypes of *mekk1* and *mpk4* (Takagi et al. 2018).

1.5.2 MAPK cascades in growth and development

Arabidopsis NPK1-like protein 1/2/3 (ANP1/MKKK1, ANP2/MKKK2, ANP3/MKKK12) are closely related orthologues of *Nicotiana* protein kinase 1 (NPK1) shown to be essential for cell plate formation during cytokinesis (Krysan et al. 2002). ANPs act upstream of MKK6-MPK4/MPK11 in Arabidopsis cytokinesis and therefore reconstitute the homologous NPK1-NQK1-NRK1 kinase cascade from *Nicotiana benthamiana* (Krysan et al. 2002; Nishihama et al. 2001).

In primary root development and stomatal patterning, a complete MAPK cascade comprised of YDA (MKKK4)-MKK4/5-MPK3/6 has been reported to function downstream of the ERL1/ERK2-EPF/EPFL and RGF-RGI ligand-receptor pairs (Shao et al. 2020; Meng, Wang, et al. 2012). The YDA cascade regulates various biological functions including early embryogenesis, stomatal patterning and primary root growth. N-terminal deletions in YDA (*CA-YDA*) result in constitutive activation and embryo lethality, but in a heterozygous state result in broad-spectrum disease resistance, potentially due to altered cell wall composition and immune-related gene expression (Sopeña-Torres et al. 2018). In floral organ abscission, MKK4/MKK5, MPK3/MPK6 were the first MAPK cascade components linked to IDA-HAE/HSL1/HSL2 ligand-receptor pairs (Cho et al. 2008). The upstream MAPKKK in this pathway has not been identified yet.

1.5.3 Signalling specificity in MAPK cascades

In general, receptors perceive specific signals to activate cellular responses that are tailored to the nature of the signal. The downstream signalling components that mediate these responses are often shared and form highly interconnected networks. MAPK cascades are central signalling modules shared between many pathways. A key challenge in understanding kinase signalling is therefore determining how MAPK cascades maintain signalling specificity while preventing undesirable crosstalk. Several mechanisms contribute to the specific and selective activation of these cascades: Docking interactions, compartmentalisation, combinatorial signalling, cross-pathway inhibition and scaffold proteins (Ma and Nicolet 2023).

Docking interactions between MKKs and MAPKs as well as MAPKs and their downstream substrates ensure specific binding and increase the efficiency of the enzymatic reactions (Tanoue et al. 2000). Docking interactions occur outside of the

kinase active site and are well established in mammalian MKK-MPK and MPK-substrate interactions (Bardwell, Frankson, and Bardwell 2009). In plants, protein microarray data indicates that MKKs preferentially activate multiple MAPKs (Popescu et al. 2009). Compared to conserved docking motifs in mammalian MAPK substrates, these motifs are less common in plant MAPK substrates and the subgroup D of plant MAPKs, which also contains the TDY instead of the TEY motif, lacks the docking motif entirely (Ma and Nicolet 2023). Plants may have evolved alternative mechanisms for MAPK-substrate interactions beyond the canonical docking sites. For instance, the scaffold protein SCREAM requires both the conventional MAPK docking motif and a unique KRAAM motif to bind to MPK3/6 (Putarjunan et al. 2019).

Compartmentalization into specific cell-types, tissues or subcellular compartments ensures MAPK pathway signal insulation. However, plants are composed of fewer cell types than most metazoans and tissue-specific expression is rare in plants as most MAPKs are broadly expressed.

Combinatorial signalling is perhaps the most widespread mechanism to ensure the integration of various signals into specific cellular responses. In combinatorial signalling the MAPK cascade can act as a molecular “AND gate” which results in an output that is specified by the combination of two independent signalling pathways of which activation of one pathway alone cannot lead to the same output.

Cross-pathway inhibition is another mechanism for signalling specificity that has the opposite effect to combinatorial signalling. In cross-pathway inhibition, the input of one signalling pathway leads to the activation of MAPK downstream targets which then inhibit the activation of other downstream targets involved in a different signalling pathway that signals through the same cascade components. A conceptual model for cross-pathway inhibition and combinatorial signalling of MAPK cascades in the endodermis was recently proposed for FLS2 and SGN3 signalling (Ma et al. 2023).

Scaffold proteins bring two or more consecutively acting components together to enhance the efficiency of their interaction and accelerate their reaction rate. Yeast Ste5 is the scaffold recruited by activated Ste11^{MKKK} to provide a platform for the Ste11^{MKKK}-Ste7^{MKK}-Fus3^{MAPK} cascade that regulates mating. The same cascade utilises the MKK Pbs2 as a scaffold for osmosis signalling by recruiting Ste11^{MKKK} and Hog1^{MAPK} (Park, Zarrinpar, and Lim 2003). In plants RACK1 (RECEPTOR FOR ACTIVATED KINASE C) acts as a scaffold protein to connect heteromeric G-proteins to the MAPK cascade to initiate immune responses (Cheng et al. 2015). Scaffolding, especially the selective

activation of scaffold proteins, is mathematically the most efficient signal insulation mechanism. This efficiency stems from the ability of scaffold proteins to sequester signalling components, which effectively mimics the spatial separation seen in compartmentalisation, and their selective activation, which resembles combinatorial signalling (Bardwell et al. 2007).

Aims of this thesis

Plant genomes encode a vast diversity of small, secreted peptides that are recognised by cell-surface receptors. Increasing evidence shows that most receptors perceive specific ligands to activate unique sets of transcription factors, but intermediate components that facilitate the signal transduction, such as MAPK cascades, are shared among many different pathways. The signalling convergence of multiple receptor proteins through the same MAPK cascade enables signal integration and precise co-regulation (Ma et al. 2016), but inevitably raises the question of how signalling specificity is ensured (Ma and Nicolet 2023).

PHYTOSULFOKINE (PSK) signalling through PHYTOSULFOKINE RECEPTOR 1 (PSKR1) promotes plant growth and suppresses immunity, thereby modulating microbiome homeostasis. By understanding the downstream signalling processes in this pathway, we can develop strategies to enhance plant growth and vigour while maintaining strong immunity, ultimately boosting agricultural yield. In this thesis, I intend to dissect the PSK signalling pathway, with a particular emphasis on the identification of novel signalling components related to its immune-suppressive function by affinity purification mass spectrometry. In addition, I will functionally characterize the MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE 7 (MKKK7) in plant development and immune signalling. Previous studies have shown that MKKK7 attenuates FLS2 signalling and basal resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) and is associated with reduced root growth. By utilizing novel loss-of-function mutants and affinity purification-mass spectrometry, I aim to elucidate the roles of MKKK7 in growth and immune signalling processes. Together, this research seeks to enhance our understanding of the cross-regulation of receptor signalling components in the context of plant growth and immunity.

Material and Methods

2.1 Laboratory equipment and materials

2.1.1 Kits and enzymes

Listed below are the kits, enzymes, and their respective manufacturers used in this study.

Table 2.1. Kits and enzymes used in this study.

Kits/enzymes	Manufacturer
Phusion® High-Fidelity DNA Polymerase (2 U μL^{-1})	New England BioLabs® Inc.
Wizard® SV Gel and PCR Clean-Up System	Promega ¹
PureYield™ Plasmid Miniprep	Promega
In-Fusion® Snap Assembly Master mix	Takara ²
REDEExtractN-Amp Plant™ Plant PCR Kit Protocol	Merck ³
SapphireAmp® Fast PCR Master Mix	Takara
cellulase R10	Duchefa ⁴
macerozyme R10	Duchefa
GFP-Trap® Agarose	ChromoTek ⁵
ANTI-RFP mAb-Magnetic Beads	MBL ⁶

2.1.2 Working concentration of Antibiotics and Herbicides

All antibiotics listed in Table 2.2 were stored at -20°C.

Table 2.2. Antibiotics used in this study and their working concentrations.

Antibiotic	Working concentration
Carbenicillin	100 $\mu\text{g mL}^{-1}$
Chloramphenicol	34 $\mu\text{g mL}^{-1}$
Gentamycin	20 $\mu\text{g mL}^{-1}$
Kanamycin	50 $\mu\text{g mL}^{-1}$
Rifampicin	50 $\mu\text{g mL}^{-1}$

¹ www.promega.co.uk

² www.takarabio.com

³ www.merckmillipore.com

⁴ www.duchefa-biochemie.com

⁵ www.ptglab.com

⁶ www.mblintl.com

Antibodies and corresponding secondary antibodies are listed in Table 2.3.

Table 2.3. Antibodies used in this study and their working dilutions.

Antibody target	Dilution	Secondary antibody	Source
α -GFP-HRP	1:5000	-	Proteintech®
α -GFP (A-6455)	1:5000	Rabbit	ThermoFisher
α -p42/44	1:5000	Rabbit	Merck
α -BAK1	1:5000	Rabbit	(Roux et al. 2011)
α -rabbit-HRP (A-0545)	1:10000	-	Merck

2.1.3 Peptides used in this study

The table below lists all peptides used in this study along with their corresponding amino acid sequences and relevant publications. All peptides were diluted in water and stored at -20°C.

Table 2.4. Synthetic peptides used in this study.

Peptide	Amino Acid sequence	Source
flg22	QRLSTGSRINSAKDDAAGLQIA	(Felix et al. 1999)
elf18	acetyl-MSKEKFERTKPHVNVGTI	(Kunze et al. 2004)
Pep1	ATKVKAKQRGKEKVSSGRPGQHN	(Huffaker, Pearce, and Ryan 2006)
SCOOP12	PVRSSQSSQAGGR	(Gully et al. 2019)
PSK	Y[SO4]IY[SO4]TQ	(Matsubayashi and Sakagami 1996)

2.1.4 Oligonucleotides and sequencing

Oligonucleotides (Table S1) were synthesized by Merck at 100 μ M and diluted to 10 μ M in H₂O for working concentrations. Both stock and working solutions were stored at -20°C. Sanger sequencing was performed by GENEWIZ®.

2.1.5 Plant materials

All wild-type and mutant *Arabidopsis thaliana* lines used in this study are in Col-0 (Columbia-0) background. Details of mutant lines are shown in Table 2.5, and details of transgenic lines in Table 2.6. The t-DNA insertion mutant *mkkk7* was obtained from the European Arabidopsis Stock Centre (NASC) in Nottingham, UK, and initially characterized by Sharon Mithoe and colleagues (Mithoe et al. 2016); Mithoe 2013. PhD thesis). The t-DNA mutant *pskr1-3pskr2-1* was generated in Margret Sauter's Lab and kindly provided by Cyril Zipfel.

Table 2.5. Arabidopsis mutant alleles used in this study.

Mutant allele	Gene ID	Background	Identifier	Publication
<i>mkkk7-1</i>	At3g13530	Col-0	SALK_133360	(Mithoe et al. 2016)
<i>mkkk7-2</i>	At3g13530	Col-0	CRISPR-Cas9	this study
<i>mkkk7-3</i>	At3g13530	Col-0	CRISPR-Cas9	this study
<i>pskr1-3</i>	At2g02220	Col-0	SALK_008585	(Kutschmar et al. 2009)
<i>pskr2-1</i>	At5g53890	Col-0	SALK_024464	(Stührwohldt et al. 2011)
<i>pskr1-3pskr2-1</i>	At2g02220 At5g53890	Col-0	SALK_008585 SALK_024464	(Hartmann et al. 2013; Kaufmann, Motzkus, and Sauter 2017)

Table 2.6. Transgenic Arabidopsis lines used in this study.

Transgene	Background	Publication
<i>35S::PSKR1-GFP</i>	<i>pskr1-3 pskr2-1</i>	(Hartmann et al. 2013)
<i>pBRI1::BRI1-mCitrine</i>	<i>bri1</i>	(Geldner et al. 2007)
<i>pBAK1::BAK1-GFP</i>	<i>bak1-4</i>	(Ntoukakis et al. 2011)
<i>35S::Lti6B-GFP</i>	Col-0	
<i>35S::YFP-MKKK7</i>	<i>mkkk6mkkk7</i>	(Chaiwongsar et al. 2006)

2.1.6 Bacterial materials

Escherichia coli (*E. coli*) strain Dh5 α was used for cloning purposes and the *E. coli* strain Rosetta was used for protein purification purposes (Table 2.7).

Table 2.7. *Escherichia coli* strains used in this study.

Strain	Selectable Marker	Genotype
Dh5 α	Carbenicillin	F ⁻ ϕ 80/ <i>lacZ</i> Δ M15 Δ (<i>lacZ</i> YA- <i>argF</i>) U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ , m _K ⁺) <i>phoA supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>
Rosetta	Chloramphenicol, carbenicillin	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3) pRARE (Cam ^R)

Agrobacterium tumefaciens strain GV3101 was used for the transient transformation of *N. benthamiana* and the stable transformation of Arabidopsis. GV3101 carries the helper plasmid pM90 with Rifampicin and Gentamycin selectable markers.

Pseudomonas strains used in this study are shown in Table 2.8.

Table 2.8. *Pseudomonas* strains used in this study.

Strain	Selectable marker	Source
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	KAN, RIF	
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000 $\Delta\Delta$ avrPto/PtoB	KAN, RIF	(Lin and Martin 2005)
<i>Pseudomonas fluorescens</i> WCS365	none	(Song et al. 2023)

2.2 Plant methods

2.2.1 Growth conditions

Arabidopsis thaliana was grown on ½ MS agar plates (½ MS salts incl. vitamins, 0.8% [w/v] agar, 1% sucrose, pH 5.7). The seeds were surface sterilized in 5% bleach solution on a benchtop shaker at 100 rpm for 15 minutes, followed by at least five washes with dH₂O for 5 minutes each. After the final wash, 1 ml of dH₂O was added, and the seeds were stratified at 5-8°C for 48 hours. Seeds were then grown on square ½ MS agar plates under long-day photoperiod conditions (16 hours light/8 hours dark, 20°C) with a light intensity of 120-160 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in controlled environment rooms (CER), unless otherwise specified.

For soil growth, *Arabidopsis* seeds were either sown directly on soil and stratified for 48 hours at 4°C or used after the above stratification process. Plants were grown in controlled environment rooms (16 hours light/8 hours dark, 22°C) with a light intensity of 180-200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The plants were cultivated on a standardized soil mix (Levington F2: 600 L peat, 100 L 4mm grit, 196g Exemptor [Chloronicotinyl Insecticide]) provided by John Innes Horticultural Services.

Hypocotyl growth assays

For hypocotyl growth assays, *Arabidopsis thaliana* seedlings were grown on ½ MS agar plates in the dark for 9 days. Images were then taken with a Canon® reflex camera and hypocotyls were measured in ImageJ.

Microscopy

Arabidopsis thaliana seedlings were grown on ½ MS agar plates for 4-5 days. Seedlings were then placed on 1.2 mm Microscopy slides containing 40 μL of diluted PI solution. Roots were then covered with a cover slip and imaged on the Leica SP8 Confocal Microscope using a 10x air objective at 537 nm excitation and 600-650 nm emission. For

imaging mVenus, excitation and emission windows were set to ex:488 nm, em:519-559 nm.

2.2.2 Screening for transgenic Arabidopsis lines

Transgenic Arabidopsis seeds that contained the phosphinothricin (PPT)/BASTA resistance gene were selected on ½ MS agar medium (½ MS salts incl. vitamins, 0.8% [w/v] agar, 1% sucrose, pH 5.7) supplemented with 15 mg L⁻¹ PPT. Seeds containing the (FAST)-Red marker were selected as described by Shimada et al. (2010).

2.2.3 Arabidopsis root growth assays

Arabidopsis thaliana seedlings were grown for 4 days on ½ MS agar plates. Seedlings were then transferred and spaced out individually on ½ MS agar plates (mock) and ½ MS agar plates + 50 nM PSK. Photos of the seedlings were taken with a Canon® reflex camera and the root length was measured via ImageJ⁷ using a 30 cm ruler placed in each image to calibrate the measurements.

2.3 Bacterial methods

2.3.1 Transformation and growth conditions of *Escherichia coli*

50 – 100 µL chemically competent *Escherichia coli* (*E. coli*) Dh5α were incubated with 10-100 ng DNA on ice for 30 min before heat shocked at 42°C for 70 seconds in a water bath. Afterwards, the cells were recovered on ice for 2 min, diluted with 1 mL LB medium and incubated at 37°C at 200 rpm for 1-2 hours. If concentrated plasmids were transformed, 100 µL of the 1 mL suspension was plated on selective LB-agar. If ligation reactions were transformed, the 1 mL suspension was spun down and ~800-900 µL of the supernatant was removed before the pellet was resuspended in the remaining volume and plated on selective LB-agar plates. Plates were incubated at 37°C overnight.

2.3.2 *Pseudomonas syringae* infection assays

Pseudomonas syringae cultures were grown on KB agar plates (20 g L⁻¹ Peptone; 1.5 g L⁻¹ K₂HPO₄; 1.5 g L⁻¹ MgSO₄•7H₂O; 1% glycerol (v/v)) supplemented with 50 µg mL⁻¹ rifampicin. Single colonies were transferred to liquid KB medium containing 50 µg mL⁻¹ rifampicin and grown overnight at 28°C. The same day, 5-week-old plants were placed in a plastic tray with lid and well-watered to promote high cell turgor pressure and humidity. The following day, *Pseudomonas* cultures were centrifuged at 3000 g and the pellets were resuspended in 10 mM MgCl₂ solution. For spray infection the OD₆₀₀ was

⁷ <https://imagej.nih.gov/ij/download.html>

adjusted to 0.2; for infiltration, it was adjusted to 0.0002. Immediately before spray infection, 0.04% Silwet L-77 was added to the inoculum and the plants were homogenously sprayed and then covered with the clear lid for 3 days to maintain high humidity. For infiltration assays, three leaves per plant were marked with a permanent marker and fully infiltrated with the inoculum. On the third day, leaf discs were collected from three fully expanded leaves per plant (or three infiltrated leaves per plant) and the samples were homogenised in 10 mM MgCl₂. Homogenisation was performed using 2x3mm glass beads per sample in a Genogrinder at 1500 rpm for 1 minute. A dilution series from 10⁻¹ to 10⁻⁶ was prepared using 10 mM MgCl₂ and the samples were plated onto KB plates containing 50 µg mL⁻¹ rifampicin using a multichannel pipette.

2.3.3 *Pseudomonas fluorescens* root inoculation assays

Pseudomonas fluorescens was cultured overnight in 10 ml LB medium without antibiotics. The following day, 2 ml of the culture was centrifuged at 3500 g for 3:30 minutes and the pellet was resuspended in 1 ml sterile 10 mM MgSO₄. The OD₆₀₀ was adjusted to 0.001 before the roots of 9-day-old seedlings grown on ½ MS vertical plates without sucrose were inoculated with 10 µl of bacterial suspension. Arabidopsis seedlings were germinated and grown on ½ MS plates with 1% sucrose for seven days, then transferred to ½ MS plates without sucrose for two days before being inoculated with *P. fluorescens*. Pictures were taken five days after inoculation when seedlings were 16 days old.

2.4 Molecular Biology Methods

2.4.1 DNA extraction and genotyping PCR of *Arabidopsis thaliana*

DNA extractions and genotyping of *Arabidopsis thaliana* were conducted as described in the REExtractN-Amp Plant™ Plant PCR Kit Protocol. Half the recommended reaction volume (10 µL) was used and 1 µL of DNA was added per reaction.

2.4.2 Polymerase chain reaction

Polymerase chain reactions for cloning purposes were conducted according to the Phusion® high-fidelity DNA polymerase protocol provided by New England Biolabs. Colony PCRs were performed using the SapphireAmp® Fast PCR Master Mix at reduced reaction volumes of 20 µL.

2.4.3 Agarose gel electrophoresis

PCR reactions were separated on 1% (w/v) agarose melted in TAE buffer (Formedium™), containing 0.01% (w/v) ethidium bromide. The PCR samples were mixed

with loading buffer (15% Ficoll 400, a pinch of bromophenol blue) before loaded and run on the gel for 30-40 min at 100 V. Gel images were taken in Syngene's G:Box gel documentation system (www.syngene.com). As size standard, the 1 kb Plus DNA Ladder from New England BioLabs[®] Inc was used.

2.4.4 Plasmid and PCR product purification

PCR products of interest were cut out of the Agarose gel on a UV table (Uvitec, www.uvitec.co.uk) and purified according to the instructions provided in Wizard[®] SV Gel and PCR Clean-Up System. DNA was eluted in 30 µL dH₂O. Plasmids were purified from 5 mL cultures according to the plasmid purification protocol of the Wizard[®] SV Gel and PCR Clean-Up System. Plasmids were eluted in 50 µL of the provided elution buffer.

2.4.5 Restriction digest

DNA restriction digests were performed with FastDigest[™] restriction endonucleases obtained from Thermo Scientific[™] according to manufacturer specifications in a total volume of 20 µL. Reactions were incubated at 37°C for 1 h.

2.4.6 CRISPR-Cas9 mutagenesis

CRISPR-Cas9-induced mutagenesis was carried out as described (Castel et al. 2019). The Cas9 nuclease was driven by the *RPS5a* promoter, and FASTred selection was used to identify positive transformants. The guide RNA sequences used to generate the mutants in this study are shown in Table S2 and were predicted for specificity and efficiency from CHOPCHOP⁸.

2.5 Biochemical Methods

2.5.1 In-planta expression

Transgenic Arabidopsis lines were used for the stable expression of proteins of interest. For transient expression assays, *Agrobacterium tumefaciens* strains expressing the protein constructs of interest were infiltrated into *Nicotiana benthamiana* leaves and harvested for protein extraction after 3 days. For infiltration, *Agrobacterium tumefaciens* cultures were grown in LB medium at 28°C overnight before pelleted at 4000 g for 10 min down and resuspended in the same volume of 10 mM MgCl₂, 10 mM MES (pH 5.6). The OD₆₀₀ was adjusted to the desired value, typically 0.2-0.6, and 150 µM acetosyringone was added. The bacterial suspension was incubated on a benchtop shaker in the dark for 3-4 hours before infiltration.

⁸ <https://chopchop.cbu.uib.no/>

2.5.2 Protein extraction from *Arabidopsis* and *N. benthamiana*

Plant tissue was flash-frozen in liquid nitrogen and ground by hand using pre-chilled mortar and pestles. The resulting powder was weighed, and 2-2.5 times the volume of ice-cold protein extraction buffer was added. For *N. benthamiana* samples, the extraction buffer consisted of 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 2% PVPP, 10 mM DTT, 1x protease inhibitor cocktail (Sigma), 0.1% IGEPAL, 1 mM PMSF. For *Arabidopsis* samples, the extraction buffer contained 50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 10 mM DTT, 1x protease inhibitor cocktail (Sigma), 1% IGEPAL, 1 mM PMSF. Samples were incubated in the extraction buffer for 30 min at 4°C on a rotator and subsequently centrifuged at 10000 g for 30 min at 4°C. The supernatant was filtered using 0.45 µm syringe filters or decanted through Miracloth into a new falcon tube before further processing for affinity purification or SDS-PAGE.

2.5.3 Affinity Purification from *Arabidopsis*

Affinity purification experiments in this thesis were only conducted using *Arabidopsis thaliana* stably expressing proteins of interest tagged with GFP- or GFP-derivatives. Seedlings were grown on ½ MS 1% sucrose vertical plates for 5 days, then transferred (8 seedlings per well) to 6-well plates containing 5 ml of ½ MS 1% sucrose liquid medium. Plates were sealed with porous tape and seedlings were grown for further 12 days. On day 12, seedlings from 10 wells (1.5 plates/80 seedlings) per genotype were transferred into 250 mL beakers filled with 40 mL ½ MS 1% sucrose liquid medium and rested for 4 hours. Seedlings were then treated with 400 µl of 100 µM PSK to achieve a final concentration of 1 µM PSK for 20 min. Control samples were treated with water. All samples were vacuum infiltrated for 3 minutes before being incubated on a benchtop shaker at 120 rpm for 20 min. Afterwards, the seedlings were blotted dry and flash-frozen in liquid nitrogen. After protein extraction (see section 6.5.2), input samples were taken and 40 µL of GFP-TRAP AGAROSE BEADS (50% slurry, ChromoTek) was added to the samples. Before use, GFP-TRAP AGAROSE BEADS were washed with 1 ml protein extraction buffer. Samples were incubated with the beads for 4 hours at 4°C on a rotator. Beads were collected by centrifugation at 1500 g for 2 min and washed 4 times with extraction buffer. After the first wash, the IGEPAL concentration in the buffer was diluted from 1% to 0.5% to lower the stringency of the washes. Proteins were eluted from the beads by adding 2x Laemmli sample buffer and subsequent boiling at 75°C for 15 min.

2.5.4 SDS-PAGE and western blot

The separation of proteins by their molecular mass was conducted by the discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to

Laemmli (Laemmli 1970). BioRad Mini-PROTEAN® Precast Gels (www.bio-rad.com) were used. Resolved proteins were electrophoretically transferred from the SDS-PAGE to a PVDF membrane using the Trans-Blot Turbo Transfer System from BioRad. Afterwards, the membrane was incubated in Tris-buffered saline (TBS) blocking solution (50 mM Tris-HCL pH 7.5, 150 mM NaCl) containing 0.1% (v/v) Tween® 20 and 5% (w/v) dried skimmed milk powder for 1 hour at room temperature or 3-4 hours at 4°C. For immunolabelling the membranes were incubated with monoclonal primary antibodies overnight at 4°C at 40 rpm on a benchtop shaker. The following day membranes were washed 3 times 5-10 min with TBST buffer and, if required, incubated with a secondary HRP-conjugated antibody for 1 hour at room temperature. If a secondary antibody was added, the previous wash step was repeated before the excess liquid was removed by decantation and immunolabelling was visualized with the Pierce™ ECL Western Blotting Substrate in the ImageQuant LAS 4000 (GE Healthcare).

2.5.5 Sample preparation for mass spectrometry [In-gel digestion]

Affinity purified proteins were run approximately 1 cm into an SDS-PAGE gel. This portion of the gel was then excised and cut into smaller pieces. For all subsequent processing, LC-MS grade ingredients were used. Gel pieces were washed three times with 25 mM ammonium bicarbonate (ABC)/25% acetonitrile until completely destained. Then 100% acetonitrile was added until gel pieces appeared opaque. Samples were then incubated in 150 µl of 50 mM ABC/10 mM TCEP for 30 min while shaking at 800 rpm at 57°C. Samples were then cooled down to room temperature and 150 µl 55 mM Chloroacetamide/50 mM ABC were added and the samples incubated for an additional 30 min at room temperature in the dark. Subsequently, the liquid was discarded and the samples were washed 3 times with 1 ml 25 mM ABC/50% Acetonitrile before dehydration for 10 min in 100% Acetonitrile. After the removal of the Acetonitrile, 50 µl Trypsin solution in 25 mM ABC (2 ng/µl trypsin) was added and then an additional 100 µl 25 mM ABC were added before the samples were incubated at 37°C overnight. The next day, the supernatant was transferred to a new low-binding Eppendorf tube. Gel pieces were washed three times with 200 µl of 50% acetonitrile/5% formic acid, vortexed, and sonicated for 10 minutes after each wash. The liquid was combined with the earlier supernatant, flash-frozen, and dried in a vacuum concentrator.

2.6 Physiological assays

2.6.1 ROS measurements

Reactive oxygen species (ROS) measurements were conducted on leaf discs harvested from 4-week-old *Arabidopsis thaliana* plants grown under short day conditions using a

4 mm biopsy punch (Integra™ Miltex™). Leaf discs were collected into white 96-well-plates (Greiner Bio-One) filled with 100 µl of distilled water and rested overnight. Prior to ROS measurement, the water was removed and replaced with 100 µl ROS assay solution composed of 100 µM Luminol (Merck), 20 µg ml⁻¹ Horseradish peroxidase (Merck) with and without elicitors. After adding the ROS assay solution, the plates were immediately placed under a HIGH-RESOLUTION PHOTON COUNTING SYSTEM (HRPCS218, Photek) and light emission was recorded in complete darkness.

2.6.2 Cytoplasmic calcium burst measurements

For calcium burst assays *pUBQ10::Aequorin* lines were used (Ranf et al. 2014). Seedlings were grown for 3 days on ½ MS plates and then one seedling per well was transferred into 96-well-plates (655075, Greiner Bio-One) containing 100 µl of ½ MS liquid media. Seedlings were grown for another 5 days before the ½ MS media was replaced by 100 µl 20 µM coelenterazine (EC14031, Carbosynth) in the evening and the plate was incubated in the dark overnight. The next morning, the coelenterazine solution was replaced by 100 µl dH₂O and the plate was incubated for a further 30 minutes in the dark. Measurements were taken in a Spectramax iD5 microplate reader (Molecular Devices) for 5 minutes to determine the background RLU before 50 µl of 3x concentrated peptide solution was added manually. No further conversion of the data into cytoplasmic calcium concentration was performed.

2.6.3 MAP kinase activation assays

Arabidopsis seedlings were grown on ½ MS 1% sucrose vertical plates for 4 days and then two seedlings were transferred into each well of a 24-well plate (Greiner Bio-One) filled with 1 ml of ½ MS 1% sucrose liquid media. The plate was sealed with porous tape and seedlings were grown for another 10 days before being treated with 1 ml of 2x concentrated elicitor solution, blot-dried and flash-frozen at respective timepoints. Frozen plant tissue was ground using a Genogrinder with 2mm glass beads at 1400 rpm for 40 seconds. Afterwards, 2× Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris-HCl; 10 µL mg⁻¹ tissue) was added and samples were boiled at 95°C for 10 min. Samples were then spun down at 13000 rcf in a standard benchtop centrifuge and subsequently loaded on an SDS PAGE. After western blotting, PVDF membranes (BioRad) were blocked in 5% (w/v) milk powder (Tesco) prior to incubation with α-pMAPK (p44/42 MAPK [Erk1/2]) overnight. The following day the membranes were washed 5 times for 10 minutes with TBST buffer before the secondary α-rabbit-HRP (A-0545) antibody was added for 1 h. After 1 h the

membrane was again washed 3 times for 5 minutes with TBST before the blots were imaged.

2.6.4 Protoplast generation and cell expansion assays

This protocol for *Arabidopsis thaliana* protoplast isolation was adapted from (Yoo, Cho, and Sheen 2007). Only the enzyme solution and the W5 solution were used, as no protoplast transformation was carried out. For protoplast isolation 10-14-day old seedlings grown on vertical ½ MS plates were cut with fresh razor blades and left in the enzyme solution for 2-3 h at 40 rpm on a benchtop shaker. Enough protoplasts were generated from 5 rows of seedlings per plate. The enzyme solution contained 1% cellulase R10 (Duchefa) and 0.2% macerozyme R10 (Duchefa). No β-mercaptoethanol was added and the enzyme solution was not filtered through a 0.45 mm filter. After resuspending protoplasts in W5 solution, two additional wash steps with W5 solution were conducted to remove residual enzyme left from the digestion. After incubating the protoplasts on ice for 30 min they were resuspended in W5 solution containing double the amount of KCL (10 mM), as higher KCL concentrations have been linked to stronger protoplast expansion responses upon PSK treatment (Stührwohldt et al. 2011). Protoplasts were left overnight in a total volume of 5 ml in 20 mL Erlenmeyer flasks covered with foil on a benchtop shaker at 40 rpm. For expansion analysis 1 ml of the protoplast solution was transferred to a 1.5 mL Eppendorf tube with a cut pipette tip and mock and 2 nM PSK treatments were applied. 40 – 50 µL of the protoplast solution was then transferred to a microscope slide.

2.7 RNA sequencing and RT-qPCR

Arabidopsis seedlings were grown on ½ MS 1% sucrose vertical plates for 4 days and then two seedlings were transferred into each well of a 24-well plate (Greiner Bio-One) filled with 1 ml of ½ MS 1% sucrose liquid medium. The plate was sealed with porous tape and seedlings were grown for another 10 days. Total RNA was extracted from *Arabidopsis* tissue using the RNeasy kit from QIAGEN according to the manufacturer's protocol. DNase treatment using the RNase-free DNase set from QIAGEN was conducted according to the protocol. RNA sequencing was performed by Novogene. For RT-qPCR, RNA samples were first diluted with RNase-free water to 500 ng µL⁻¹. 500 ng of RNA (1µL) were then used for cDNA synthesis following the GoScript™ Reverse Transcriptase protocol from Promega with oligo(dT)₁₅ primers. The only alteration that was made to the protocol was that no optional Ribonuclease inhibitor was added. Final cDNA was diluted 5x to a final concentration of 100 ng µL⁻¹.

RNA sequencing data were analysed using FastQC, trimmed using trimmomatic (Bolger, Lohse, and Usadel 2014) and mapped to the Arabidopsis TAIR10 reference genome via HISAT2 (Kim et al. 2019). Mapped reads were assigned to genes using StringTie (Pertea et al. 2015) and read count tables from StringTie output were generated by prepDE.py. Differential gene expression analysis was performed using R packages edgeR and limma (Law et al. 2016). Output counts per million (CPM) values normalised to library size were visualised using Heatmapper⁹.

2.7.1 GO enrichment

Gene ontology (GO) term enrichment for biological processes and molecular functions was calculated in R using the package clusterProfiler (Yu et al. 2012) and Benjamini-Hochberg method for p-value adjustment.

2.8 LC-MS/MS analysis

Samples were prepared and analysed by LC-MS/MS by the TSL proteomics team. AP-MS samples (Replicate 1) were analysed using an Orbitrap Fusion trihybrid mass spectrometer (Thermo Scientific), YFP-MKKK7 pull-downs for phosphorylation analysis were analysed using an Orbitrap Eclipse trihybrid mass spectrometer (Thermo Scientific) equipped with a field asymmetric ion mobility spectrometry (FAIMS) interface. Both devices were coupled to a nanoflow UHPLC system U3000 (Thermo Scientific). Peptides were trapped in a reverse phase trap column (Acclaim PepMap, C18 5 µm, 100 µm x 2 cm, Thermo Scientific) connected to an analytical column (Waters, C18 3 µm, 75 µm x 50 cm, Thermo Scientific). Peptides were eluted in a gradient of 3-40% ACN in 0.1% formic acid (solvent B) over 70 min, followed by a gradient of 40-80% solvent B over 6 min at a 200 nL/min flow rate at 40°C. The mass spectrometer was operated in positive ion mode with a nano-electrospray ion source with ID 0.02 mm fused silica emitter (New Objective). A voltage of +2200 V was applied via platinum wire held in PEEK T-shaped coupling union with a transfer capillary temperature set to 275°C. The Orbitrap, MS scan resolution of 120,000 at 400 m/z, range 300 to 1800 m/z, was used, automatic gain control (AGC) was set at 2e5, and maximum inject time to 50 ms. In the linear ion trap, MS/MS spectra were triggered with a data-dependent acquisition method using 'top speed' and 'most intense ion' settings. The selected precursor ions were fragmented sequentially in the ion trap using the collision-induced dissociation (CID) and the higher-energy C-trap dissociation (HCD) cell. Dynamic exclusion was set to 15 s. Charge state allowed between +2 and +7 charge states to be selected for MS/MS fragmentation.

⁹ www.heatmapper.ca

Replicates 2-4 of the AP-MS experiment were run on the Bruker timsTOF Pro. LC-MS/MS was carried out using a nanoElute v2 LC system (Bruker Daltonics, Bremen, Germany) coupled to the timsTOF Pro, a TIMS Q-TOF mass spectrometer (Bruker Daltonics), using a CaptiveSpray nano-electrospray ion source (Bruker Daltonics). Peptide sample was loaded on a C18 RP trap column (PepMap™ Neo Trap Cartridge, i.d. 300 µm by 5 mm, 5 µm particle size, 100 Å pore size, Thermo Scientific™) and separated on an analytical C18 RP column (Aurora, i.d. 75 µm by 250 mm, 1.6 µm particle size, 120 Å pore size; IonOpticks, Fitzroy, Australia). Peptides were separated at 55 °C using a 120 min gradient at a flow rate of 200 nL/min (mobile phase A: 0.1% FA; B: 0.1% FA in acetonitrile). A stepwise gradient consisting of 2 to 14% B for 60 min, followed by a step 14 to 19 %B for 20 min, 19 to 34 %B for 35 min, 34 to 44 %B for 8 min, 44 to 54 %B for 2 min, 54 to 95% B for 0.5 min and finished with a wash at 95% B for 4.5 min in a total runtime of 130 min per analysis. The timsTOF Pro was operated in positive PASEF mode. Mass spectra for MS and MS/MS scans were acquired in the mass range of 100 to 1700 m/z. Ion mobility resolution was set to 0.65–1.45 V·s/cm over a ramp time of 166 ms. The data-dependent acquisition consisted of 10 PASEF MS/MS scans per cycle with a 100% duty cycle and a total cycle time of 1.89 s. A polygon filter was applied in the m/z and ion mobility ranges to exclude low m/z, singly charged precursor ions from detection. An intensity threshold and target intensity were 1000 and 25000, respectively, and the active exclusion time window of 0.4 min was used. The collisional energy was ramped as a function of ion mobility, 20 to 80 eV for 0.6 to 1.6 V·s/cm.

Peak lists in the form of Mascot generic files (.mgf files) were generated from raw data files using the MSConvert package (Matrix Science) or the Data analysis software from Bruker and sent to a peptide search on Mascot server v.2.7 using Mascot Daemon (Matrix Science) against an in-house constructs and contaminants database and the Araport11 database. Tryptic peptides with up to two possible mis-cleavages and charge states +2, +3 and +4 were allowed in the search. The following peptide modifications were included in the search: carbamidomethylated cysteine (fixed) and oxidized methionine (variable). Data were searched with a monoisotopic precursor and fragment ion mass tolerance 10 ppm and 0.8 Da respectively. The Decoy database was used to validate peptide sequence matches. Mascot results were combined in Scaffold v.5.1.2 (Proteome Software).

2.8.1 LC-MS/MS data analysis

MS2 spectral counts were exported from Scaffold v.5.1.2 (Proteome Software) as excel files and analysed in Rstudio with packages tidyverse, ggpubr, rstatix for statistical analysis and ggrepel for visualization. Scaffold settings for data export were set to 0.1% FDR peptide threshold, minimum of 1 peptide per protein, 95% protein confidence threshold, and total spectrum counts as the quantitative values. Missing spectral data were imputed with a value of 0.01 for logFC and -log10 p-value calculations.

Identifying Novel Signalling Components of The Phytosulfokine Pathway

3.1 Summary

Plant cells constantly need to adapt and respond to a variety of external stimuli. In the context of stress response, small peptides called phyto cytokines are released to modulate and fine tune the plant's physiological adaptation. Many peptide ligand-receptor pairs have been identified, but downstream signalling components in these pathways are often not known. The pentapeptide Phytosulfokine (PSK) promotes cell proliferation, longevity, and expansion, while simultaneously attenuating pattern-triggered immunity (PTI) (Sauter 2015). While several components of the PSK signalling pathway have been identified, the mechanisms by which PSK regulates these diverse cellular processes and integrates with other hormonal pathways remain unclear. Here, I utilized PSK-induced PSKR1-BAK1 complex formation as a proxy for PSK signalling to identify the optimal time point for affinity purification mass spectrometry (AP-MS). Subsequent analysis of PSKR1-GFP AP-MS revealed novel putative signalling components within the PSK pathway. Among these components, LRR-RK BAK1, auxin receptor TRANSMEMBRANE KINASE1 (TMK1), S-ACYLTRANSFERASE PAT09 and MILDEW RESISTANCE LOCUS O 1 (MLO1) are identified as PSK-dependent interactors of PSKR1. Interestingly, I identified MAP KINASE KINASE KINASE 7 (MKKK7) as a PSK-dependent interactor of PSKR1.

3.2 Introduction

Phytosulfokine (PSK) was one of the first plant signalling peptides identified, initially discovered in *Asparagus officinalis* cell culture in the 1990s for its growth-promoting activity (Matsubayashi and Sakagami 1996). PSK is recognised by leucine-rich repeat receptor kinases (LRR-RKs) PSKR1 and PSKR2 (Matsubayashi et al. 2002; Wang et al. 2015; Matsubayashi et al. 2006), which, in conjunction with auxin and cytokinin, promote cell proliferation (Matsubayashi et al. 1999).

The Arabidopsis genome encodes for *PSKR1* (At2g02220) and *PSKR2* (At5g53890) (Matsubayashi et al. 2006). PSKR1 is the main receptor for PSK signalling based on mutant analysis, but PSKR2 functions as an alternative PSK receptor, albeit with reduced

activity (Amano et al. 2007). Overexpression of both *PSKR1* and *PSKR2* can promote plant growth, suggesting their functionality (Hartmann et al. 2013). PSK binding to PSKR1 occurs with high affinity and both the abundance of the receptor and the ligand impact PSK signalling (Matsubayashi et al. 2002; Matsubayashi et al. 2006; Kaufmann, Stührwohldt, and Sauter 2021). Since its discovery and the subsequent identification of its receptors, several signalling components have been linked to PSK signalling, in *Arabidopsis* and tomato.

The current model for PSK signalling suggests that PSKR1 associates with members of the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES (SERK) co-receptor family upon PSK perception (Wang et al. 2015). Specifically, SERK1, SERK2 and BAK1 (SERK3) have been shown to associate with PSKR1, with co-crystallisation studies confirming the association of LRR-domains of PSKR1 and SERK1 (Wang et al. 2015). In addition, PSKR1 is thought to associate with plasma membrane-localized H⁺-ATPases AHA1 and AHA2 (Ladwig et al. 2015). CYCLIC NUCLEOTIDE-GATED CHANNEL 17 (CNGC17) is co-expressed with *PSKR1* and was shown to associate with AHA and SERKs to complete a heterooligomeric signalling complex consisting of PSKR1-AHA-CNGC17-BAK1-PSKR1 (Ladwig et al. 2015). However, evidence for this complex is inferred from 1:1 interaction studies between PSKR1-BAK1, PSKR1-AHA, AHA-CNGC17 and BAK1-CNGC17. Whether PSKR forms a heterooligomeric complex that is relevant for PSK signalling in vivo remains to be determined.

PSKR1 is classified as an RD-type kinase, and both its kinase activity and calmodulin (CaM) binding are essential for its signalling function (Hartmann et al. 2014). CaM binding does not affect the kinase activity of PSKR1 in vitro (Kaufmann, Motzkus, and Sauter 2017). Several phosphorylation sites were identified in PSKR1 in vitro, and mutations at key activation loop residues impair kinase activity and thereby receptor function (Kaufmann, Motzkus, and Sauter 2017; Hartmann et al. 2015). Additionally, PSKR possesses predicted guanylate cyclase activity which may contribute to its signalling function (Kwezi et al. 2011). The intracellular kinase domain of PSKR1 was recently shown to directly bind aluminium ions and modulate the plants' tolerance towards aluminium by directly interacting with and phosphorylating NADPH oxidase RBOHD to stimulate ROS production (Ding et al. 2024).

PSKR1 and BRASSINOSTEROID INSENSITIVE 1 (BRI1) are closely related subfamily X LRR-RKs (Xi et al. 2019). Both receptors associate with the co-receptor BAK1 to function and PSK signalling depends on brassinosteroid (BR) biosynthesis (Hartmann et al. 2013). PSK-induced primary root growth in the *tpst-1* (*tyrosylprotein sulfotransferase-1*) mutant is suppressed by the BR biosynthesis inhibitor brassinazole (BZ), but the root

phenotype is restored upon the addition of 24-epibrassinolide (BL) (Hartmann et al. 2013).

The small RECEPTOR-LIKE PROTEIN 44 (RLP44) is required for BR-signalling in response to perturbation of the cell wall (Wolf et al. 2014). RLP44 is also essential for PSK signalling, linking the two signalling pathways at the receptor level (Holzwart et al. 2018). The phosphorylation status of RLP44 determines its involvement in either BR or PSK signalling, thereby coordinating the two signalling processes to control vascular cell fate (Garnelo Gómez et al. 2021).

In the root quiescent centre (QC), BR and PSK synergistically promote cell division. BR prevents the degradation of the transcription factor ERF115 by inhibiting the ubiquitin ligase complex APC/C^{CCS52A2} (anaphase-promoting complex/cyclosomem (APC/C), CELL CYCLE SWITCH 52 A2 (CCS52A2)). Stabilized ERF115 then induces the expression of *PSK5*, which promotes cell division through the PSKR1 (Heyman et al. 2013).

In Arabidopsis PSK signalling suppresses plant immunity, although the specific signalling components involved in this pathway are not known (Igarashi, Tsuda, and Katagiri 2012). Loss of function mutants of *pskr1* show enhanced defence gene expression and resistance to *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 and *P. syringae* pv. *maculicola* (*Pma*) ES4326, but show increased susceptibility towards the necrotrophic fungal pathogen *Alternaria brassicicola* (Igarashi, Tsuda, and Katagiri 2012; Mosher et al. 2013)

In tomato, exogenous application of PSK triggers Ca²⁺ influx, leading to the activation of Calmodulins (CaMs). These CaMs then bind to flavin-containing monooxygenases YUCCAs (YUCs) to stimulate auxin biosynthesis. Subsequent activation of auxin-mediated pathways results in enhanced resistance to necrotrophic pathogen *Botrytis cinerea* (Zhang, Hu, et al. 2018). The tomato *PSKR1* homologue *SIPSKR1* associates with CPK28 to fine-tune the growth-defence response via phosphorylation of glutamine synthetase GS2 (Ding et al. 2022). Phosphorylation of GS2 at S334 promotes plant defence, while phosphorylation at S360 enhances growth by influencing nitrogen and carbon metabolism (Ding et al. 2022). *SIPSKR1* itself is regulated by the ubiquitin/proteasome degradation pathway through ubiquitination by PUB12 and PUB13 (Hu et al. 2023). PSK binding to *SIPSKR1* prevents ubiquitination and thereby stabilizes the receptor (Hu et al. 2023).

In summary, several components of the PSK signalling pathway have been identified, but the precise mechanisms by which PSK signalling influences cellular behaviours such as proliferation, expansion and immunity remain unclear. The limited understanding of downstream signalling components hinders our ability to determine how PSK signalling integrates with other hormonal pathways such as auxin, cytokinin and BR, and modulates the balance between growth and defence responses.

3.3 Results

3.3.1 PSKs and PSKR1 are transcriptionally upregulated in response to PAMPs but do not trigger PTI responses in *Arabidopsis thaliana*

To investigate the link between PSK signalling and plant immunity, I extracted publicly available expression data for PSKs and PSKR1. The expression data shows that out of seven PSK-encoding genes, PSK1-5 and PSKR1 are transcriptionally upregulated in response to several PAMPs including flg22, elf18 and Pep1 (Bjornson et al. 2021) (Figure 3.1 A). Transcriptional up-regulation in response to PAMPs/DAMPs is characteristic for Phytocytokines. PSKs are conserved tyrosine sulfated peptides with the amino acid (aa) sequence YIYTQ (Figure 3.1 B). Based on the transcriptional regulation and PSK's ability to induce Ca^{2+} influx in tomato, I tested whether exogenously applied PSK can trigger Ca^{2+} influx or other hallmarks of PTI such as ROS production and MAPK activation in Arabidopsis. Application of PSK did not trigger hallmarks of PTI including ROS production, MAP kinase cascade activation and Ca^{2+} influx in Arabidopsis (Figure 3.1 C-E).

3.3.2 PSKR1 is broadly expressed whereas PSKR2 show guard cell lineage specific expression

Both PSKR1 and PSKR2 function as receptors for PSK (Hartmann et al. 2013); however, only *pskr1* knockouts lead to noticeable growth defects (Kutschmar et al. 2009). To determine which receptor to focus on for AP-MS experiments and to investigate the underlying reasons for the distinct phenotypes observed in *pskr1* and *pskr2* mutants, I obtained single-cell expression data from Tatsuya Nobori. This data revealed that *PSKR2* expression is relatively higher and appears to be specific to guard cell (GC) lineage cells, while *PSKR1* shows a broader expression profile across different tissues and cell types. Among the PSK genes, *PSK2* and *PSK7* are relatively more expressed in the stem and siliques, respectively, whereas the other PSK genes are broadly expressed across different tissues and cell types (Figure 3.2). Together, *PSKR1* is the main receptor for PSK perception and I therefore selected 35S::*PSKR1-GFP* overexpression lines for the subsequent AP-MS experiments.

3.3.3 PSKR1-BAK1 complex formation increases over 30 minutes time course

Before conducting AP-MS experiments, I worked with students Clinton Asare and Michael Agbodo to investigate the temporal dynamics of PSKR1-BAK1 complex formation in response to PSK treatment, aiming to determine the optimal time point for pull-down assays. Previous studies in *Arabidopsis* seedlings demonstrated PSKR1-SERK1/SERK2/BAK1 complex formation after 12 hours of PSK treatment, while transient expression in *Arabidopsis* protoplasts showed complex formation after 15 minutes (Wang et al. 2015). A model of the PSKR1-SERK1 interaction is shown in Figure 3.3 A, based on the crystal structure of the PSKR1 and SERK1 ECDs and AlphaFold 3 predictions of their intracellular kinase domains. In our time-course PSKR1-GFP pull-down experiment over 30 minutes of PSK treatment, we observed a slight enhancement in PSKR1-BAK1 complex formation as early as 2.5 minutes post-treatment. This interaction progressively increased, becoming clearly detectable at 20 and 30 minutes (Figure 3.3 B). Based on these observations, I selected the 20-minute time point for the subsequent AP-MS experiments. We also observed a slight increase in the abundance of PSKR1-GFP during the 30-minute PSK treatment, although this finding requires further validation in future experiments (Figure 3.3 B).

3.3.4 PSKR1-GFP AP-MS results in 23 PSKR1-specific interactors that increase in abundance after PSK treatment

To identify putative PSKR-specific interactors, I utilized several transgenic *Arabidopsis thaliana* lines expressing GFP or GFP-derivates fusion proteins localised to the plasma membrane as negative controls. These include *pBRI1::BRI1-GFP*, because of the close relation of BRI1 and PSKR1 as LRR-RK subfamily X receptors. I included *pBAK1::BAK1-GFP* as a specific negative control and *35S::Lti6B-GFP* as well as *35S::YFP-MKKK7* as generic negative controls. All fusion proteins were successfully expressed and enriched following affinity purification (Figure 3.4).

Clinton Asare and Michael Agbodo conducted the experiment that yielded 292429 peptide spectra, leading to the identification of 2,544 proteins with at least two peptide spectra. Each sample + treatment was submitted in quadruplicates, except for the YFP-MKKK7+PSK condition, which had three replicates. In total 31 samples were submitted.

When comparing PSKR1-GFP mock samples to PSKR1-GFP PSK-treated samples, 37 proteins displayed increased spectral counts with a $\log_2$ -fold change ($\log_2FC$) ≥ 1 and a $-\log_{10}$ p-value ≥ 0.5 (Figure 3.5 A). The $-\log_{10}$ p-value threshold was chosen arbitrarily to serve as a reference for the consistency of specific peptide spectra identified across replicates. Higher values indicate more consistent detection of peptide spectra across multiple replicates, while lower values suggest greater variability and missing data.

Of the 37 proteins identified, 23 were exclusively detected in the PSKR1-GFP pull-down and were absent in all control samples (Figure 3.5 B). Consistent with our previous pull-down results (Figure 3.3 B), BAK1 was among these 23 enriched PSKR1 interactors, validating the PSK treatment and purification conditions used. The list of interactors also included S-acyltransferase PAT09, MAP KINASE KINASE KINASE 7 (MKKK7), MILDEW RESISTANCE LOCUS O 1 (MLO1) and the auxin receptor TRANSMEMBRANE KINASE1 (TMK1).

When I compared the AP-MS data with published PSKR1 interactors mentioned in the chapter introduction and summarized in Table 3.1, I confirmed that BAK1, RBOHD, AHA1 and AHA2 were present in our dataset. Except for BAK1, none of the published interactors showed clear PSK-treatment specific changes in spectral counts (Table 3.1). Interestingly, I noticed that 293 proteins detected in the PSKR1-GFP mock sample were absent in the PSK-treated pull-down (Figure 3.5 A). This striking observation prompted me to further analyse which proteins decreased in abundance by gene ontology (GO) term enrichment. Interestingly, all overrepresented GO-terms were related to RNA processing and stability, suggesting that PSK might impact global gene transcription and translation (Table S3, Figure S1).

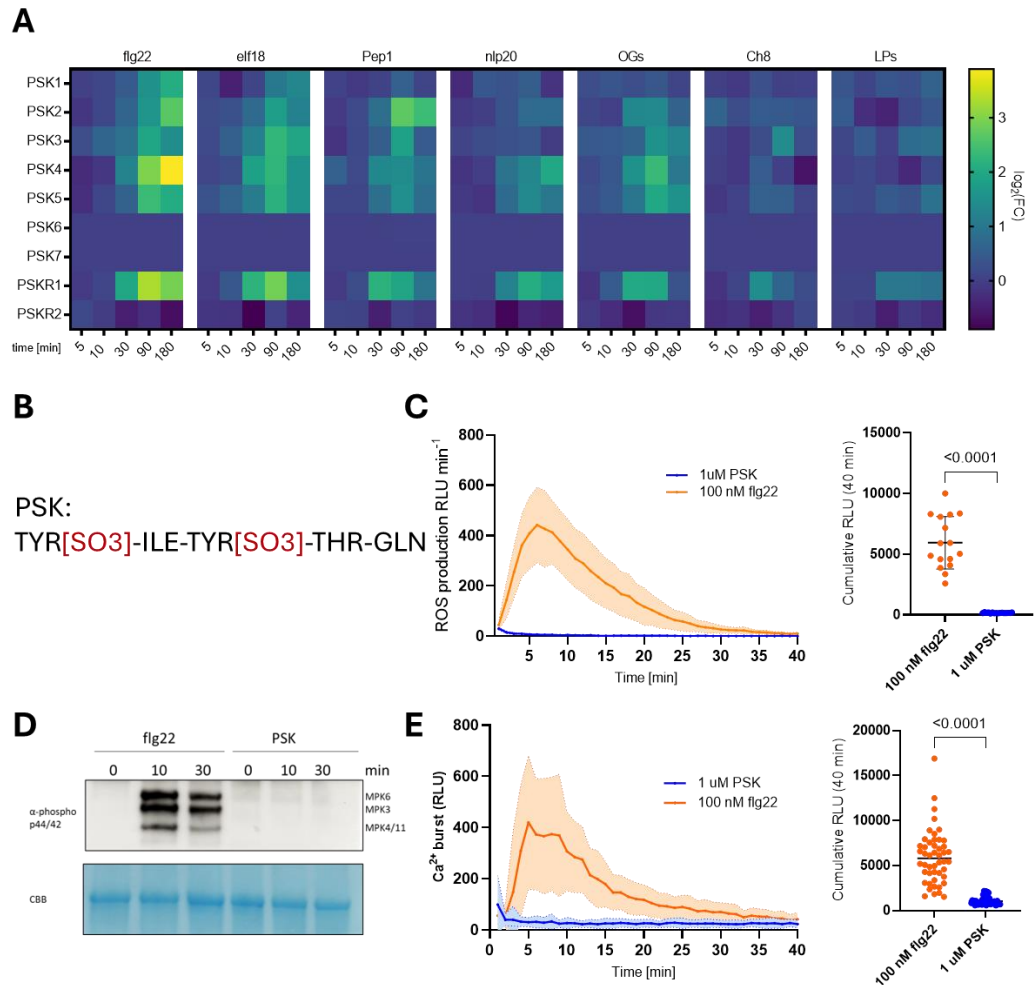


Figure 3.1. Phytosulfokine genes and their receptor PSKR1 are transcriptionally upregulated in response to PAMPs but do not trigger PTI signalling outputs. **A.** Heat map showing $\log_2(\text{FC})$ expression levels of *PSK1-7* and *PSKR1*, *PSKR2* in response to several PAMPs (data obtained from (Bjornson et al. 2021)). **B.** Amino acid sequence of PSK with tyrosine sulfation highlighted in red. **C.** ROS production in leaf disks collected from 4-week-old *Arabidopsis* plants induced by 100 nM flg22 or 1 μM PSK application (n =16). Line represent mean ± SEM. Cumulative ROS production over 40 min. Line represents mean; error bars represent SD (n =16). p-value based on one-way ANOVA followed by Tukey's post-hoc test. **D.** Western blot using α-p42/p44-ERK recognising phosphorylated MAP kinases in seedlings treated with 100 nM flg22 and 1 μM PSK for 10 and 30 minutes. 0-minute control was treated with water. CBB =Coomassie brilliant blue staining served as the loading control. **E.** Ca²⁺ burst in 1-week-old *Arabidopsis* seedlings induced by 100 nM flg22 or 1 μM PSK application (n =48). Line represent mean ± SEM. Cumulative Ca²⁺ burst over 40 min. Line represents mean; error bars represent SD (n =48). p-value based on one-way ANOVA followed by Tukey's post-hoc test. RLU = relative light units.

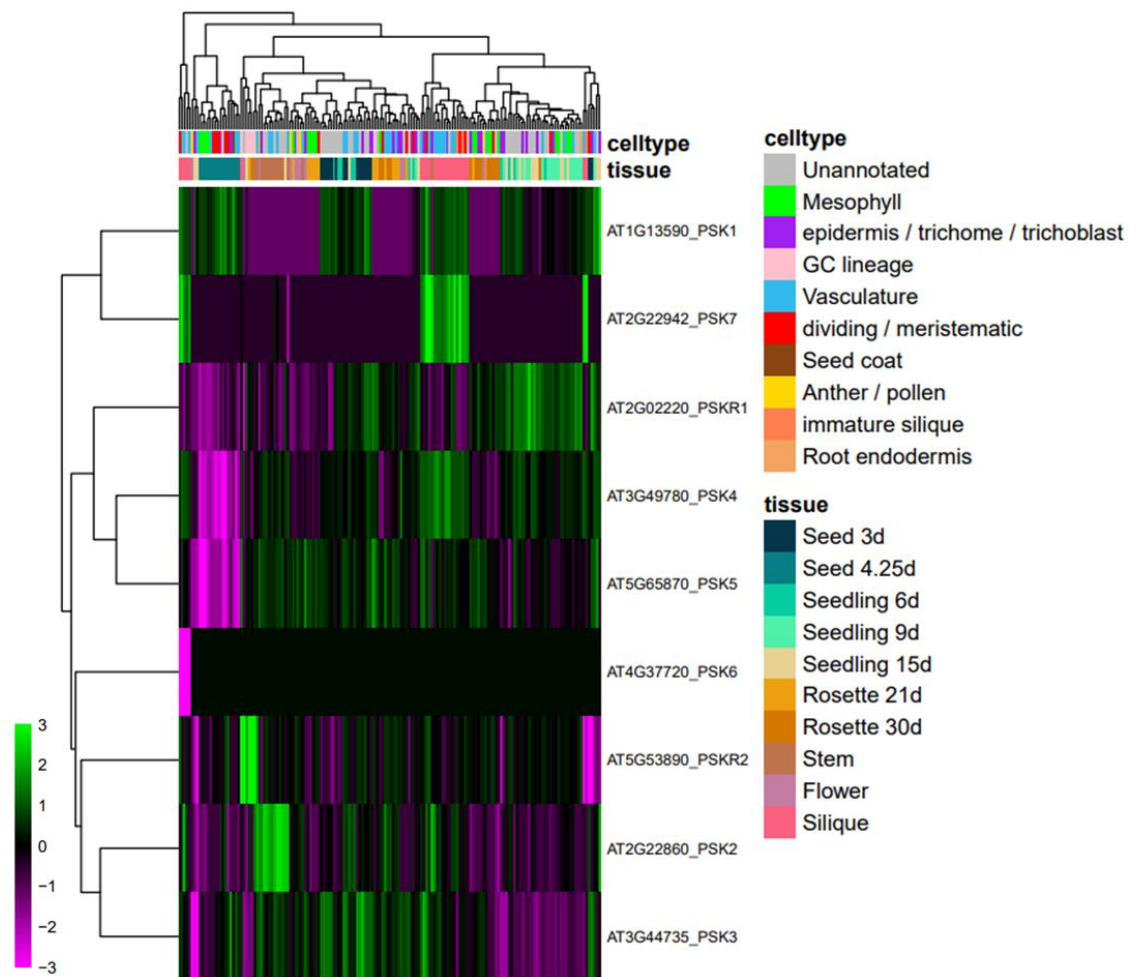


Figure 3.2. Cell- and tissue-specific expression profiles of *PSK* and *PSKR* genes. This data was kindly provided by Tatsuya Nobori. PSKs and PSKRs are annotated on the right of the heatmap. Horizontal clustering by expression, vertical clustering by cell type and tissue. Legend for cell type and tissue type is shown on the right.

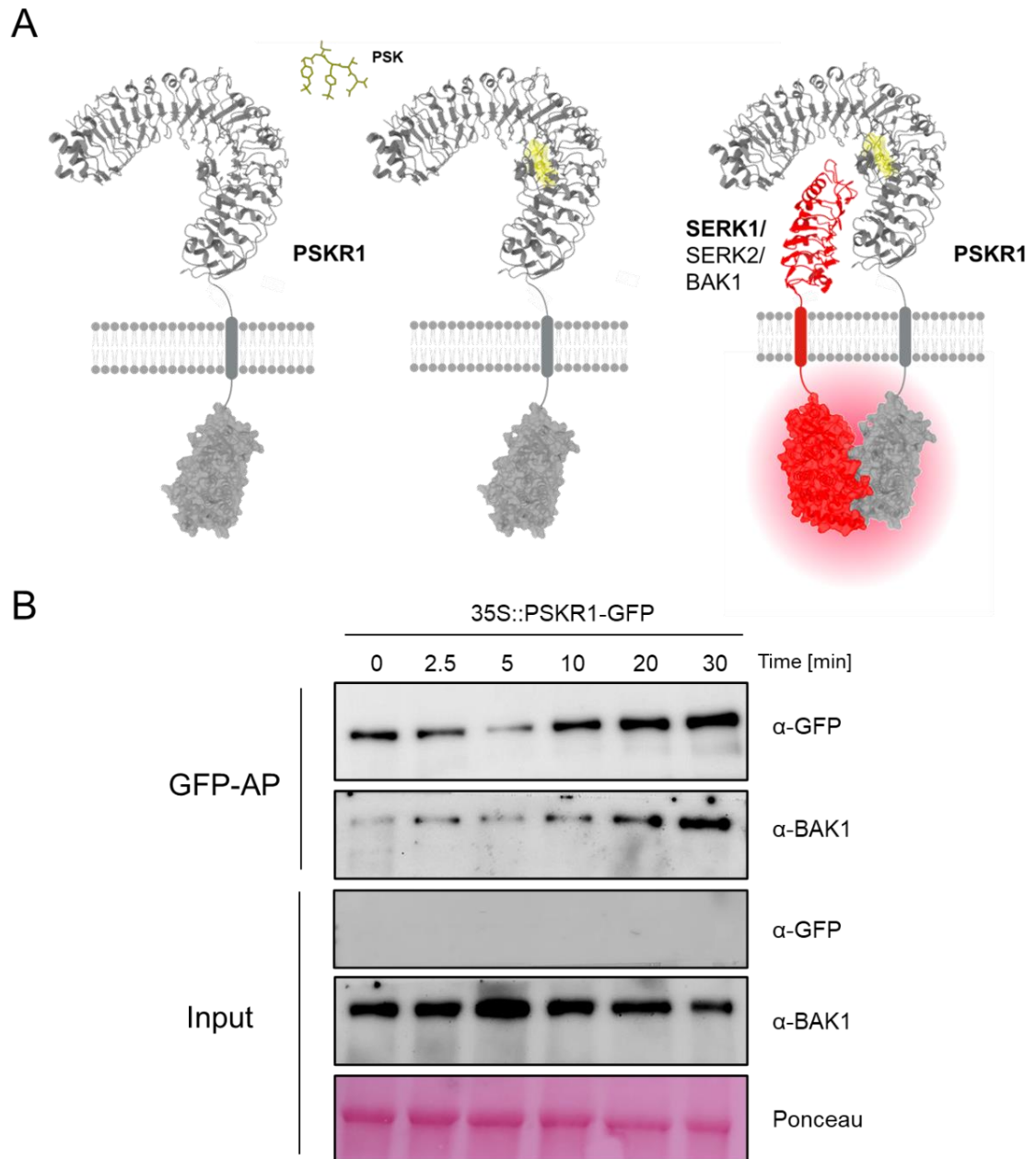


Figure 3.3. Complex formation between PSKR1 and BAK1 increases over 30 min.

A. Schematic of PSKR1 in grey showing the extracellular LRR domain, transmembrane domain and intracellular kinase domain. Tyrosine-sulfated PSK peptide is shown in yellow. SERK1 extracellular LRR domain, transmembrane domain and intracellular kinase domain are shown in red. Schematic was generated in Biorender and is based on PDB 4Z64 (extracellular domains of PSKR1 and SERK1) (Wang et al. 2015) and AlphaFold 3 predictions. **B.** Western blots showing affinity-purified (AP) PSKR1-GFP (α-GFP) from 35S::PSKR1-GFP transgenic lines and endogenous BAK1 (α-BAK1) pulled down with PSKR1 after 1 μM PSK treatment over 30 min.

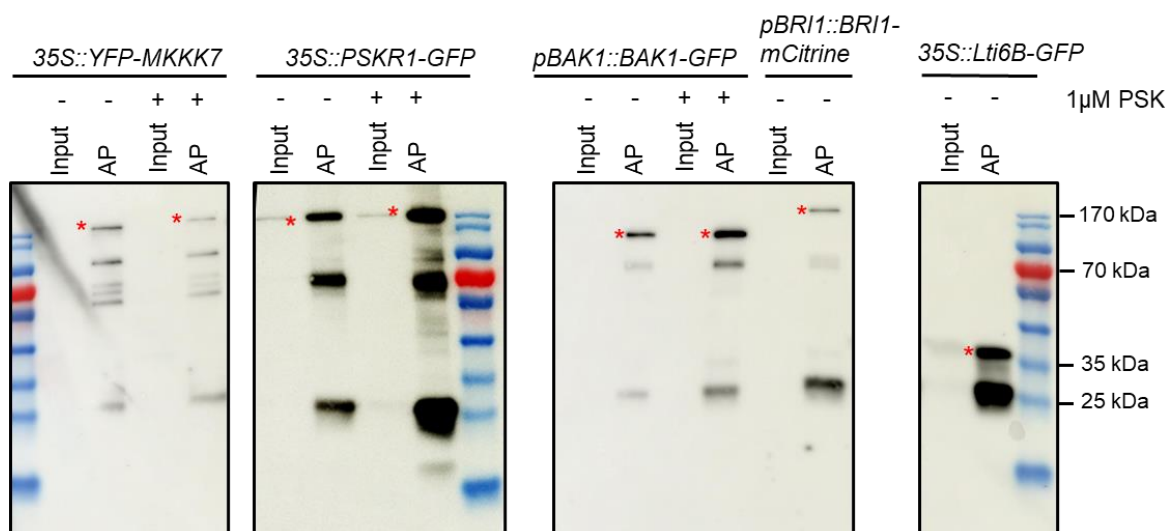
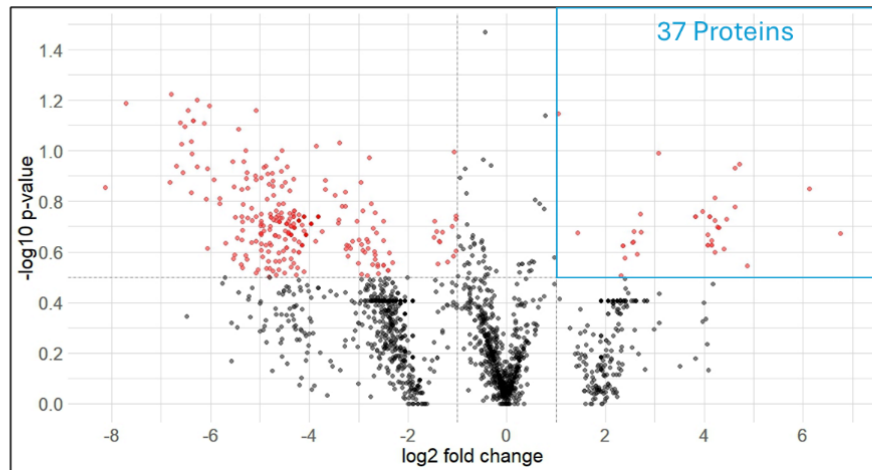


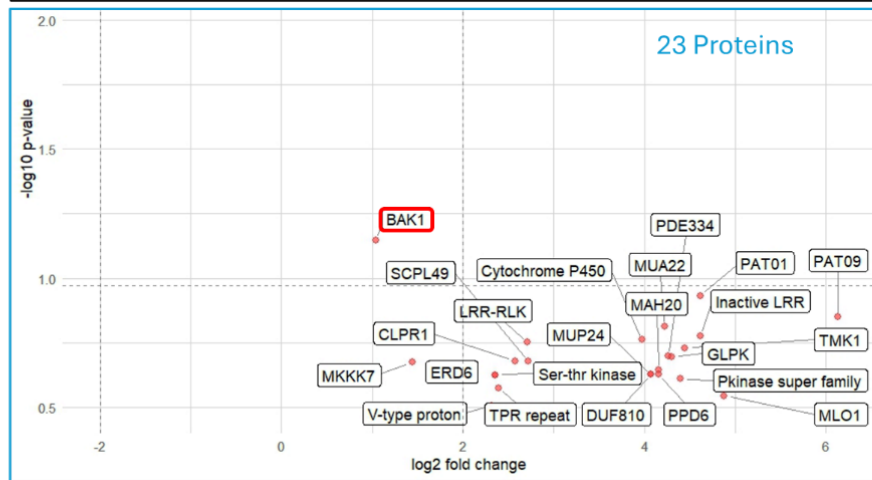
Figure 3.4. Western blot displays inputs and GFP-affinity purified constructs from stably expressing *Arabidopsis thaliana* lines, probed with α -GFP antibody. From left to right: 35S::YFP-MKKK7 (expected MW: 178 kDa); 35S::PSKR1-GFP (expected MW: 139 kDa); pBAK1::BAK1-GFP (expected MW: 100 kDa); pBRI1::BRI1-mCitrine (expected MW: 157 kDa); 35S::Lti6B-GFP (expected MW: 33 kDa).

A

PSKR-GFP mock vs. PSKR-GFP PSK



B



C

Proteins pulled down	Accession Number	log_fc	log_pval	Protein_name_short
DHHC-type zinc finger family protein	AT5G50020.2	6.12913277	0.848990731	PAT09
Seven transmembrane MLO family protein	AT4G02600.1 (+1)	4.87251471	0.544673974	MLO1
Leucine-rich receptor-like protein kinase family protein	AT3G28040.1	4.61440935	0.77739589	Inactive LRR
tryptophan biosynthesis 1	AT5G17990.1	4.61440935	0.931184035	PAT01
transmembrane kinase 1	AT1G66150.1	4.44489137	0.730418355	TMK1
Protein kinase superfamily protein	AT1G11390.1	4.39512353	0.610571146	Pkinase super family
Actin-like ATPase superfamily protein	AT1G80460.2	4.29865074	0.696652675	GLPK
ATPase%2C F0 complex%2C subunit B/B%2C bacterial/chloroplast	AT4G32260.1	4.26064997	0.698589056	PDE334
Major facilitator superfamily protein	AT5G14120.1	4.21816872	0.812820006	MUA22
thylakoid lumenal protein	AT3G56650.1	4.15241012	0.628158733	PPD6
ribosomal RNA small subunit methyltransferase J	AT5G08540.1	4.15241012	0.645200276	MAH20
Gls protein (DUF810)	AT2G20010.2	4.07192809	0.627155804	DUF810
Zinc finger (C3HC4-type RING finger) family protein	AT5G60710.1	4.07192809	0.627155804	MUP24
cytochrome P450%2C family 89%2C subfamily A%2C polypeptide 2	AT1G64900.1	3.96816872	0.761209523	Cytochrome P450
SERINE CARBOXYPEPTIDASE-LIKE 49	AT3G10410.1	2.71656215	0.678655846	SCPL49
Leucine-rich repeat protein kinase family protein	AT2G37050.3	2.7034453	0.75099878	LRR-RLK
CLP protease proteolytic subunit 1	AT1G49970.1	2.57192809	0.679761352	CLPR1
Calcium-binding tetrapeptide family protein	AT1G05150.1	2.3876867	0.57450228	TPR repeat
Major facilitator superfamily protein	AT1G08930.1 (+1)	2.34968592	0.625312713	ERD6
leucine-rich repeat transmembrane protein kinase family protein	AT5G10290.1 (+1)	2.34968592	0.625312713	Ser-thr kinase
vacuolar ATP synthase subunit H family protein	AT3G42050.1	2.3157586	0.506266764	V-type proton
mitogen-activated protein kinase kinase kinase 7	AT3G13530.1	1.4389365	0.675480764	MKKK7
BR1-associated receptor kinase	AT4G33430.1	1.04185454	1.146504905	BAK1

Figure 3.5. PSK-specific interactors identified by PSKR1-GFP AP-MS. Data is based on 4 biological replicates of PSKR1-GFP mock and PSK treatment for 20 min. **A.** Differential analysis of PSKR1-GFP interactors in mock vs. PSK-treated samples, based on MS2 spectral counts. Positive $\log_2$ fold changes indicate higher spectral counts in the PSK-treated samples compared to mock, negative $\log_2$ fold changes indicate a reduction. Red dots represent proteins with significant changes, determined by a $-\log_{10}$ p-value threshold of ≥ 0.5 and $\log_2$ fold changes ≥ 1 or ≤ -1 . Missing spectral data were imputed with a value of 0.01 for logFC and $-\log_{10}$ p-value calculations. **B.** Subset of 23 proteins enriched in PSK-treated samples (from A), filtered against all negative controls. **C.** Detailed list of 23 PSKR1-GFP interactors identified as PSK specific including their respective $\log_2$ fold changes and $-\log_{10}$ p-values (log_pval).

Table 3.1. Known PSKR1 interactors from *Arabidopsis thaliana* and *Solanum lycopersicum* (tomato) from literature analysis, along with their respective spectral counts as observed in the AP-MS data presented here. For tomato proteins homologues were identified in Arabidopsis: CPK28 AT5G66210; PUB12 AT2G28830; PUB13 AT3G46510.

Protein	Organism	Gene accession	Reference	Total Spectrum Count (PSKR1-GFP mock)	Total Spectrum Count (PSKR1-GFP PSK)
CaM2	<i>Arabidopsis thaliana</i>	AT2G41110	(Hartmann et al. 2014)	0	0
CaM4	<i>Arabidopsis thaliana</i>	AT1G66410	(Hartmann et al. 2014)	0	0
CaM6	<i>Arabidopsis thaliana</i>	AT5G21274	(Hartmann et al. 2014)	0	0
CaM7	<i>Arabidopsis thaliana</i>	AT3G43810	(Hartmann et al. 2014)	0	0
CML8	<i>Arabidopsis thaliana</i>	AT4G14640	(Hartmann et al. 2014)	0	0
AHA1	<i>Arabidopsis thaliana</i>	AT2G18960	(Ladwig et al. 2015)	541	454
AHA2	<i>Arabidopsis thaliana</i>	AT4G30190	(Ladwig et al. 2015)	437	365
SERK1	<i>Arabidopsis thaliana</i>	AT1G71830	(Wang et al. 2015)	0	0
SERK2	<i>Arabidopsis thaliana</i>	AT1G34210	(Wang et al. 2015)	0	0
SERK3 (BAK1)	<i>Arabidopsis thaliana</i>	AT4G33430	(Wang et al. 2015; Ladwig et al. 2015)	40	86
RBOHD	<i>Arabidopsis thaliana</i>	AT5G47910	(Ding et al. 2024)	43	41
RLP44	<i>Arabidopsis thaliana</i>	AT3G49750	(Holzwardt et al. 2018)	0	0
CPK28	<i>Solanum lycopersicum</i>	Solyc02g083850	(Ding et al. 2022)	0	0
PUB12	<i>Solanum lycopersicum</i>	Solyc11g066040	(Hu et al. 2023)	0	0
PUB13	<i>Solanum lycopersicum</i>	Solyc06g076040	(Hu et al. 2023)	0	0

3.4 Discussion

In this Chapter, I have demonstrated that although Arabidopsis PSKs and PSKR1 are transcriptionally upregulated in response to various PAMPs, the exogenous application of PSK does not trigger typical PTI signalling hallmarks. Through an extensive AP-MS experiment, incorporating multiple proteins as mutual negative controls, I have identified both well-established and novel interactors of PSKR1. Notably, I discovered that MAP KINASE KINASE KINASE 7 (MKKK7) co-purifies with PSKR1 upon PSK treatment, suggesting MKKK7 may be part of the PSK signalling network.

3.4.1 PSK-induced immune responses in Arabidopsis and tomato

Exogenous application of PSK does not trigger hallmarks of PTI signalling such as calcium influx, ROS production or MAPK activation under the tested conditions (Figure 3.1). This observation is consistent with PSK's role in suppressing PTI via modulation of salicylic acid (SA) signalling (Igarashi, Tsuda, and Katagiri 2012; Song et al. 2023; Mosher et al. 2013). However, this contrasts with findings in tomato, where PSK signalling induces a receptor-dependent calcium influx (Zhang, Hu, et al. 2018). It also differs from recent research that redefined PSKR1 as Aluminium receptor 1 (ALR1) because of its ability to perceive aluminium ions through its intracellular kinase domain. Aluminium binding activates PSKR1 which leads to the phosphorylation and activation of NADPH oxidase RBOHD and subsequent ROS production, visualised by 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) staining (Ding et al. 2024). In my experiments, using a luminol-based chemiluminescence assay to measure ROS, I found no evidence of PSK-induced ROS production.

In tomato, *SIPSKR1* turnover is regulated by the ubiquitin/proteasome degradation pathway via ubiquitination by PUB12 and PUB13 (Hu et al. 2023). After the addition of the PSK ligand, *SIPSKR1* ubiquitination is reduced and protein abundance increases (Hu et al. 2023). I observe a similar trend in our PSKR1 pull-down experiment, where PSK treatment results in a gradual increase in PSKR1 abundance (Figure 3.3B).

3.4.2 Can single-cell transcriptomics reveal the function of *At1g72300*?

The LRR-RK *At1g72300* is a closely related paralogue of *PSKR1/PSKR2* and was initially believed to recognise *PSY* and therefore named *PSY1R* (Amano et al. 2007). The recent definite identification of the three true PSYR receptors (Ogawa-Ohnishi et al. 2022) leaves *At1g72300* an orphan receptor with no confirmed ligand. Given the close phylogenetic relationship between *At1g72300* and *PSKR1/PSKR2*, I hypothesise that this receptor might recognise peptides similar to PSK. The single-cell transcriptomic data

from Tatsuya Nobori from the recently published Arabidopsis developmental atlas¹⁰ (Lee et al. 2023) reveals that *PSKR2* expression is relatively higher and specific to guard cell lineage cells, suggesting a cell-type-specific function for PSKR2 (Figure 3.2). It is therefore possible that *At1g72300* recognises PSK in a cell- or tissue-specific manner, potentially similar to *PSKR2*, which could explain why it has not yet been directly associated with PSK signalling. Supporting this, T-DNA triple mutants of *pskr1*, *pskr2*, and *At1g72300* exhibit a markedly more severe phenotype compared to *pskr1* single mutants or *pskr1pskr2* double mutants (Amano et al. 2007), suggesting a functional overlap or compensatory role among these receptors. It would be interesting to utilize single-cell transcriptomic analyses, which have the potential to significantly enhance our understanding of ligand-receptor interactions to address the role of *At1g72300*.

3.4.3 Identification of novel putative PSKR1 interactors

PSK's ability to promote cell proliferation depends on auxin and cytokinin, which are required for both PSK biosynthesis and PSK signalling (Matsubayashi et al. 1999). PSK-induced cell proliferation in asparagus cell cultures requires the continuous supply of both hormones (Matsubayashi et al. 1999), but the molecular mechanisms of this crosstalk remains unclear. In tomato, PSK-signalling triggers auxin-dependent immune signalling (Zhang, Hu, et al. 2018). Here, I find auxin receptor TMK1 as a putative interactor of PSKR1 after PSK treatment (Figure 3.5). TMK1, a member of LRR-RK subfamily IX, shares similarity with subfamily X receptors like PSKR1 and BRI1, including the presence of an island-domain/non-LRR region that separates the extracellular LRRs. TMK1 and its closely related homologues TMK2, TMK3 and TMK4 constitute a plasma-membrane localised auxin perception module that triggers global phosphorylation changes, activates H⁺-ATPases, and promotes apoplastic acidification and cell expansion in response to auxin (Friml et al. 2022b; Lin et al. 2021).

S-acyltransferase PAT09 plays a role in the negative feedback modulation of the immune receptor P2K1, an L-type Lectin receptor that perceives extracellular ATP (Chen et al. 2021). Upon ATP perception, P2K1 phosphorylates and activates PAT05 and PAT09, which subsequently S-acylate the receptor to dampen the immune response (Chen et al. 2021). Recently, a forward genetic screen for mutants with abnormal leaf microbiota identified S-acyltransferase *TIP GROWTH DEFECTIVE 1 (TIP1)* (Cheng et al. 2024). *tip1* mutants show leaf microbiome dysbiosis which results in autoimmunity, but when grown under axenic conditions, the autoimmune phenotype is markedly reduced (Cheng et al. 2024). PSK signalling through PSKR1 is important for plant microbiome

¹⁰ <http://neomorph.salk.edu:9000/>

homeostasis by preventing autoimmunity to rhizosphere microbiota (Song et al. 2023). It will therefore be interesting to investigate the link between PSKR1 and S-acetylation in the context of plant microbiome homeostasis.

MKKK7 also emerged as a putative interactor in our PSKR1 AP-MS dataset and was specifically enriched after PSK treatment. This finding suggests that MKKK7 might act downstream of PSKR1, potentially transducing the Phytosulfokine signal, a hypothesis that I will test in Chapter 5.

In summary, the AP-MS data presented here provides an excellent foundation for further dissection of the PSK-signalling pathway, particularly in exploring the potential interactions between PSKR1 and the core set of 23 identified interactors. It is important to note here that all analyses were done on fragment-ion (MS2) spectral counts. In the future, it would be desirable to compare and validate these findings with integrated peak intensities from the parent-ion masses (MS1).

Phenotypic Characterisation of *MKKK7* Reveals Roles in Shoot and Root Growth, With Severe Developmental Defects from Additional *MKKK6* Knockout

4.1 Summary

MAP kinases are essential eukaryotic signalling molecules and typically organised in three-tiered cascades involving a MAPKKK, MAPKK, and MAPK that sequentially activate each other through phosphorylation. Many members of the expanded plant MAPKKK gene family have not been functionally characterised. In this chapter, I describe the generation of novel CRISPR-Cas9 alleles in *mkkk7*, which recapitulate the reduced growth phenotypes observed in previously published T-DNA mutants. I show that three independent *mkkk7* mutants show reduced primary root growth, a phenotype that is similar to the phytosulfokine receptor mutant *pskr1-3pskr2-1*. MKKK6 is a closely related paralogue of MKKK7, and publicly available RNA sequencing data shows that both kinases have largely overlapping expression patterns. I therefore generated *mkkk6mkkk7* double mutants by CRISPR-Cas9 which exhibit severe developmental phenotypes, highlighting the importance of both kinases in Arabidopsis growth.

4.2 Introduction

The *Arabidopsis thaliana* genome encodes for 80 MAP KINASE KINASE KINASES (MAPKKKs), many of which remain functionally uncharacterised (Champion, Picaud, and Henry 2004). MAP KINASE KINASE KINASE 7 (*MKKK7*) belongs to the MEKK subfamily of MAPKKKs, specifically to the A4 subgroup, which also contains its closely related homologue, *MKKK6* (Ichimura et al. 2002). The domain architecture of MKKK6 and MKKK7 is unique within the MAPKKK family. Both kinases possess 2 ARMADILLO-repeat (ARM-repeat) domains in their C-termini alongside their N-terminal kinase domains (Ichimura et al. 2002). ARM-repeat containing proteins, like MKKK7, are functionally versatile and evolutionarily ancient. MKKK7-related proteins are found

across diverse eukaryotic lineages, including amoebae, fungi, green/red algae, plants, chromalveolates, and excavates, but are notably absent in animals (Tewari et al. 2010).

Phylogenetic analysis suggest that the closest non-plant homologues are *CDC7* from fission yeast *Shizosaccharomyces pombe* and *CDC15* from *Saccharomyces cerevisiae*, both of which are implicated in cell cycle exit and cytokinesis (Jouannic et al. 2001). *S. pombe* *cdc7p* is involved in the septum initiation network (SIN) pathway, which regulates septum formation and coordinates the transition from mitotic exit to cytokinesis (Fankhauser and Simanis 1994). Complementation assays have shown that *Brassica napus* *MKKK7* (*BnMKKK7*) can partially rescue the *cdc7* mutant phenotype in *S. pombe*, suggesting some functional similarity (Jouannic et al. 2001).

However, subsequent studies of *Arabidopsis* *MKKK7* found no evidence that would support a role in cell division. *MKKK7* predominantly localizes to the plasma membrane, and double T-DNA mutants in *mkkk6* and *mkkk7* exhibit plasma membrane irregularities leading to pollen lethality yet show no defects in pollen cell division (Chaiwongsar et al. 2006). Therefore, despite structural and functional parallels with yeast *cdc7p* suggested by complementation assays, *MKKK7* appears to function differently in *Arabidopsis*. This could imply that the SIN pathway has evolved distinct roles in plants, with ancient signalling components like *cdc7p*/*MKKK7* now fulfilling different functions (Chaiwongsar et al. 2006; Bedhomme et al. 2009; Bedhomme et al. 2008).

Genetic complementation of the pollen-lethal *mkkk6mkkk7* mutant using an ethanol-inducible *alc-YFP-MKKK7* construct rescues pollen lethality and revealed that *MKKK6* and *MKKK7* are important for sporophyte development (Chaiwongsar et al. 2012). Double mutants exhibit reduced shoot and root growth with smaller cell sizes, suggesting a role for these kinases in cell expansion (Chaiwongsar et al. 2012). While individual *mkkk7* T-DNA mutants show similarly reduced root growth and smaller root meristem size (Mithoe 2013, PhD thesis)(Chaiwongsar et al. 2012), *mkkk6* mutants do not display any apparent growth phenotype (Chaiwongsar et al. 2012). This led to the assumption that *MKKK7* is the predominantly active kinase out of these two paralogues in *Arabidopsis*. In *Nicotiana benthamiana* and *Solanum lycopersicum*, both of which have single orthologues of *MKKK7* known as *MKKKε*, silencing these genes similarly results in reduced cell expansion and stunted plant growth (King et al. 2014; Melech-Bonfil and Sessa 2010).

In summary, studies of *mkkk7* T-DNA mutants in *Arabidopsis*, combined with functional analyses of *MKKKε* in Solanaceae species and the ancient domain architecture of *MKKK7*, suggest that *MKKK7* is a key regulator of plant growth, cell expansion, and

development. In this chapter, I will discuss the generation and phenotypic characterization of novel *mkkk7* mutant alleles in Arabidopsis and compare their phenotypes to those observed in phytosulfokine receptor mutants, building on the AP-MS results presented in Chapter 3.

4.3 Results

4.3.1 Generating novel *mkkk7* mutants by CRISPR-Cas9

Previous studies of *MKKK7* have relied on T-DNA insertion mutants. While T-DNA insertion lines are a valuable resource for genetic studies, an increasing number of reports show that these lines often comprise a complex array of insertions that can cause extensive genomic rearrangements (Pucker, Kleinbölting, and Weisshaar 2021) or unwanted gene disruptions – such as those observed in AUXIN BINDING PROTEIN 1 (ABP1)(Dai et al. 2015). T-DNA insertions can also result in incomplete gene knockouts or the overexpression of truncated protein domains, as seen with *METACASPASE 2* (*MC2*)(Wu et al. 2024). T-DNA mutant *mc2-1* overexpresses the prodomain of *MC2* causing an autoimmune phenotype. In contrast, full T-DNA knockout alleles *mc2-2* and *mc2-3* do not exhibit autoimmune phenotypes (Wu et al. 2024). In my analysis, RT-qPCR revealed that the *MKKK7* transcript was still detectable upstream, but not downstream of the T-DNA insertion site in *mkkk7-1* (SALK_133360) (Figure 4.2 B, C). To establish a definite causal relationship between the *mkkk7* knockout genotype and phenotype, I used CRISPR-Cas9, to generate two additional *mkkk7* mutants: *mkkk7-2* and *mkkk7-3* (Figure 4.1).

mkkk7-2 contains a 194 bp deletion in exon 12 resulting in a frameshift and premature stop codon (Figure 4.1 A-C). Despite this deletion, the transcript was still detectable upstream and downstream of the deletion site (Figure 4.2 B, C). However, due to the resulting frameshift, the transcript is unlikely to be translated into a full-length protein. *mkkk7-3* is a heavily truncated version of *MKKK7*, generated by targeting the intron1-exon2 boundary and the 3'-UTR with gRNAs, leaving only the first exon intact (Figure 4.1 A, B, D). I confirmed the deletion by PCR and Sanger sequencing using a primer combination that binds to the UTR regions of the 6.8 kb *MKKK7* open reading frame. This primer pair could only amplify a fragment if a large proportion of *mkkk7* was deleted (Figure 4.1 B). I confirmed by RNA sequencing that no *MKKK7* transcript was detectable in the *mkkk7-3* mutant (Figure 4.2 D).

The large 5'-UTR of *MKKK7* encodes the *MYB5* transcription factor in reverse genetic orientation, which suggests that *MKKK7* and *MYB5* share the same promoter region. To further verify a causal relationship between the *mkkk7* mutant genotypes and

phenotypes, I performed RT-qPCR to show that the expression of *MYB5* was unaltered in *mkkk7-1* and *mkkk7-2* mutants (Figure 4.2 A), but I have not tested *MYB5* expression in *mkkk7-3*. Altogether, using CRISPR-Cas9 gene editing, I was able to successfully generate two additional *mkkk7* mutants.

4.3.2 *mkkk7* mutants show defects in root and shoot development

All three *mkkk7* mutants showed significantly shorter primary roots with shorter root meristems and fewer root meristem cortex cells compared to WT Col-0 (Figure 4.3). The root growth phenotype mimics to some extent the phytosulfokine receptor double mutant *pskr1-3pskr2-1* phenotype (Figure 4.3 A-B) (Kutschmar et al. 2009), but *mkkk7* and *pskr1-3pskr2-1* mutants show clear differences in root meristem cortex cell numbers (Figure 4.3 C-D).

To test whether the expression profile of *MKKK7* aligns with the observed root growth phenotype, I cloned the 1.5 kb promoter region of *MKKK7* and fused it to a nuclear localisation signal (NLS) and 3xVenus fluorescent tag. This construct allowed me to assess *pMKKK7* activity throughout the plant as a proxy for *MKKK7*'s site of action. *pMKKK7* was predominantly active in the root tip and elongation zone epidermal cells as well as emerging lateral roots, consistent with previous reports of *pMKKK7* GUS staining in the root tip (Figure 4.4) (Bedhomme et al. 2009). The *MKKK7* expression profile from the root cell atlas confirms its predominant expression in the root tip and elongation zone (Figure 4.6 B). *MYB5*, which is likely driven by the same promoter as *MKKK7*, has been previously detected in trichomes, epidermal cells on leaf margins, and the seed coat via promoter-driven GUS staining (Li et al. 1996). I have not yet comprehensively analysed the *pMKKK7* activity in the shoot. In addition to the short root phenotype, all *mkkk7* mutants showed slightly reduced rosette sizes, a phenotype that I also observed in the *pskr1-3pskr2-1* mutant (Figure 4.5 A) and that was independently recorded by our collaborator Edel Pérez-López in a different experiment (Figure 6.8). Overall, these results indicate that *MKKK7* function is implicated in root and shoot growth.

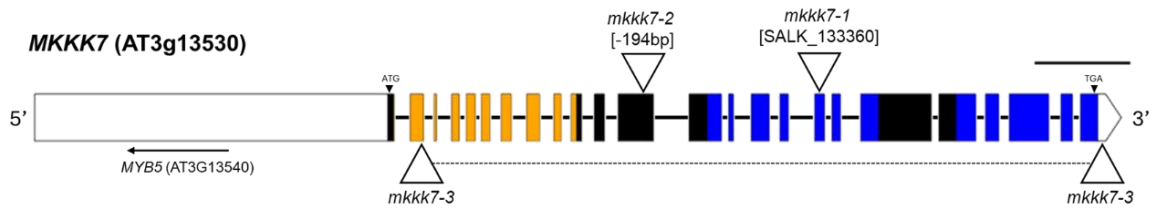
4.3.3 Functional redundancy between *MKKK6* and *MKKK7*

MKKK7 is one of two phylogenetically closely related kinases (*MKKK6*, *MKKK7*) that share 90.01% coding sequence (CDS) identity and 89.22% amino acid sequence identity. Publicly available RNA sequencing data suggests that both kinases exhibit overlapping expression patterns in the shoot but show more distinct expression patterns in the root, where *MKKK7* expression appears to be slightly higher (Figure 4.6 A-D). It is interesting to note that the expression of *MKKK6* is markedly higher in pollen than any

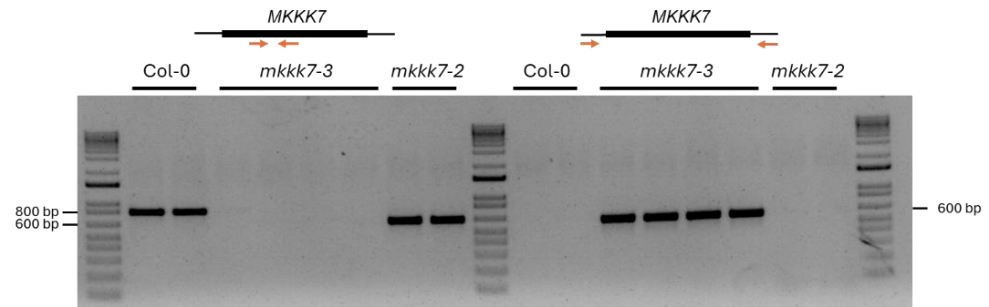
other tissue, whereas *MKKK7* does not appear to be expressed in pollen (Figure 4.6 C, D).

To further investigate the possibility of functional redundancy between *MKKK6* and *MKKK7*, I used CRISPR-Cas9 gene editing to generate *mkkk6* knockouts in the *mkkk7-3* background. After floral dip transformation, I selected positively transformed seeds using the fluorescence-accumulating seed technology (FAST)-Red selection marker (Shimada, Shimada, and Hara-Nishimura 2010) and plated them on ½ MS media containing 1% sucrose (Figure 4.7 A). Unexpectedly, many T0 seeds of *mkkk7-3* transformed with CRISPR-Cas9 and four guide RNAs targeting *MKKK6* did not germinate or exhibited severe developmental defects, which were ultimately lethal (Figure 4.7 B, C). Taken together, *MKKK6* and *MKKK7* exhibit similar expression patterns in Arabidopsis and their combined loss of function appears to be deleterious to the plant, suggesting a functional redundant role for these kinases.

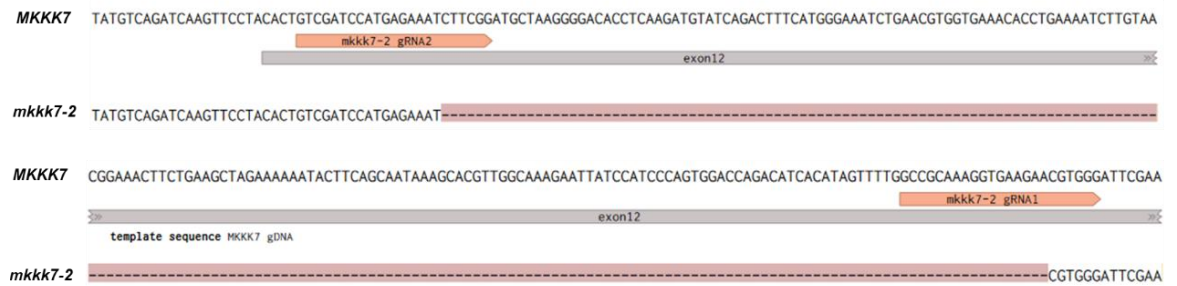
A



B



C



D



Figure 4.1. *mkkk7-2* and *mkkk7-3*, two novel CRISPR-Cas9-induced mutations in *MKKK7*. **A.** Intron-Exon Gene model of *MKKK7*. Exons are depicted as coloured boxes, introns are indicated by the black line, UTRs are depicted as white boxes. Gene model is overlaid with InterProScan predicted protein domains. Orange= Kinase domain, blue= Armadillo-type fold. Mutant alleles are indicated by triangles. The 5'-UTR of *MKKK7* encodes the *MYB5* transcription factor in reverse transcriptional direction. Black bar represents 1000 bp **B.** Agarose gel images of amplified fragments using two primer combinations: one binding within *MKKK7* (left) and the other binding in the UTR regions of *MKKK7* (right). **C.** Genomic sequence of *MKKK7* with guide RNA binding sites in exon 12, aligned with sanger sequencing results of *mkkk7-2*. **D.** Genomic sequence of *MKKK7* with guide RNA binding sites in intron 1/exon2 and 3' UTR. Sanger sequencing results of *mkkk7-3* show guide RNA cut site and fusion of Exon 2 and 3' UTR.

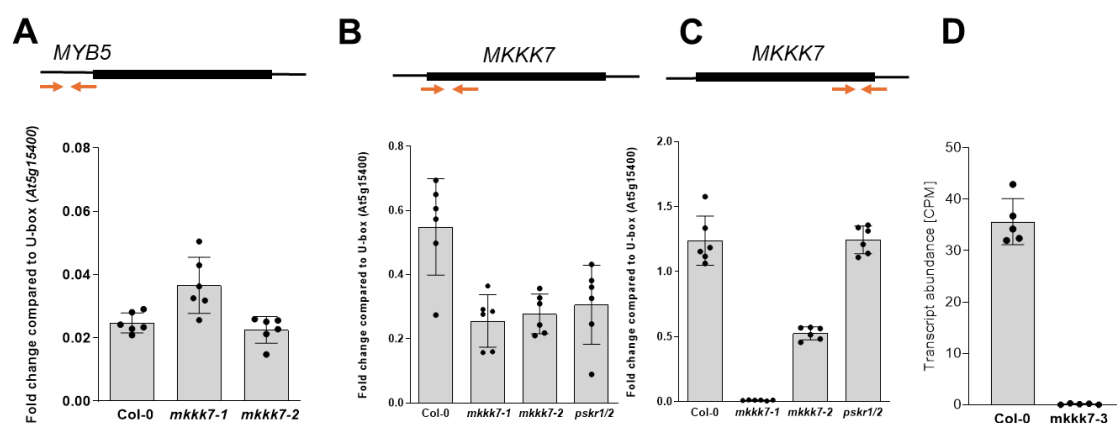


Figure 4.2. RT-qPCR and RNA sequencing analysis of *MKKK7* and *MYB5* expression in *mkkk7* mutants. **A.** RT-qPCR data of *MYB5* gene expression. **B-C.** RT-qPCR data of *MKKK7* expression using primer combination MD41-MD42 (Table S1) specific to the 5' end of *MKKK7* and primer combination MD39-MD40 (Table S1) specific to the 3' end of *MKKK7*. **D.** *MKKK7* transcript abundance in counts per million [cmp] in WT Col-0 and *mkkk7-3* plants.

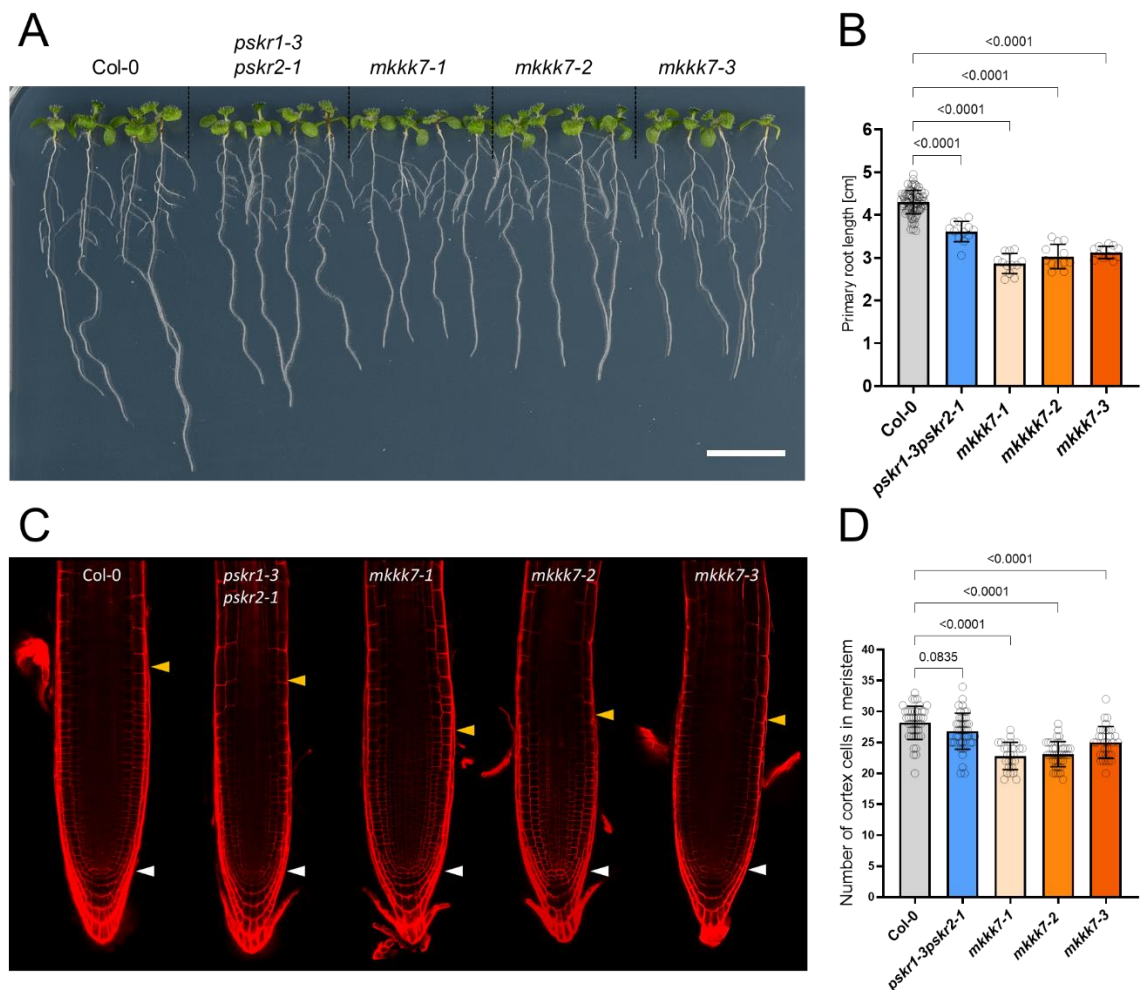


Figure 4.3. *mkkk7* mutants show reduced primary root growth with shorter root meristems. **A.** Representative image of 10-day-old *Arabidopsis thaliana* seedlings. Genotypes are indicated above. The white error bar represents 1 cm. **B.** Quantification of primary root length of at least 12 seedlings per genotype. p-values derived from one-way ANOVA followed by Tukey's post-hoc test are indicated above. Error bars represent standard deviation. **C.** Propidium iodide staining of 5-day-old primary roots. The white arrow indicates the quiescent centre, while the yellow arrow indicates the end of the root meristem detected as root cortex cells doubling in size. **D.** Quantification of root meristem cortex cell number of at least 25 seedlings per genotype. p-values derived from one-way ANOVA followed by Tukey's post-hoc test are indicated above.

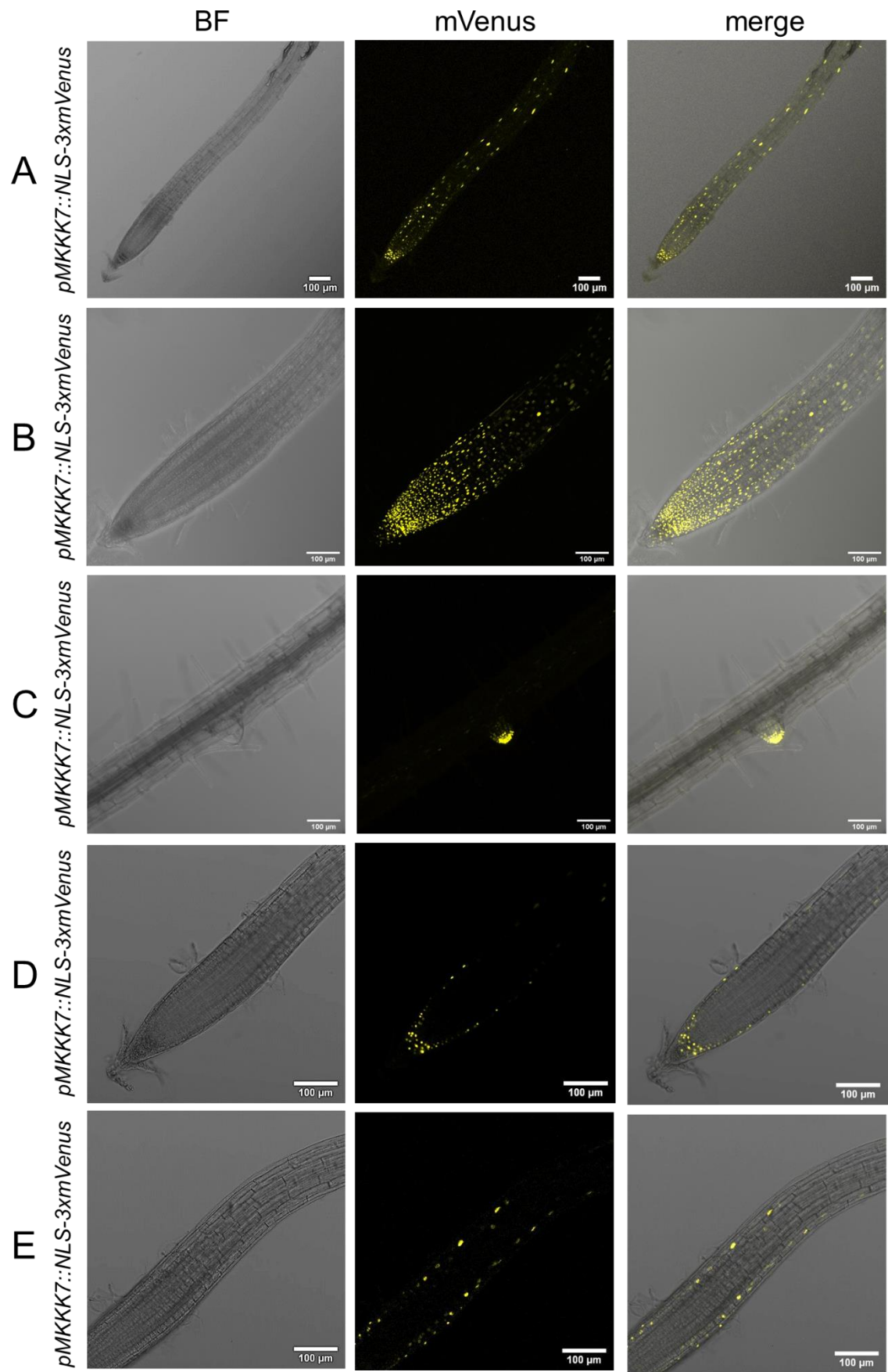


Figure 4.4. *pMKKK7* is predominantly active in root cap cells as well as meristem and elongation zone epidermal cells. Images of 5-day-old *Arabidopsis thaliana* primary roots stably expressing *pMKKK7::NLS-3xmVenus*. Panels on the left show bright field images (BF). Panels in the centre show the NLS-3xmVenus signal. Panels on the right are merged BF and mVenus images. The white bar represents 100 μm . **A.** 10x magnification maximum projection Z-stack showing the root meristem and elongation zone. **B.** 20x magnification maximum projection Z-stack. **C.** 20x magnification maximum projection Z-stack of an emerging lateral root. **D-E.** Longitudinal sections of the root meristem and elongation zone.

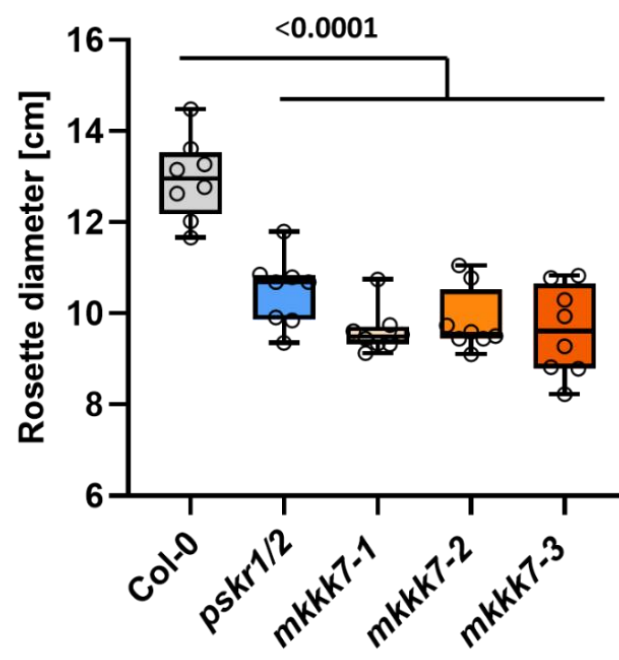
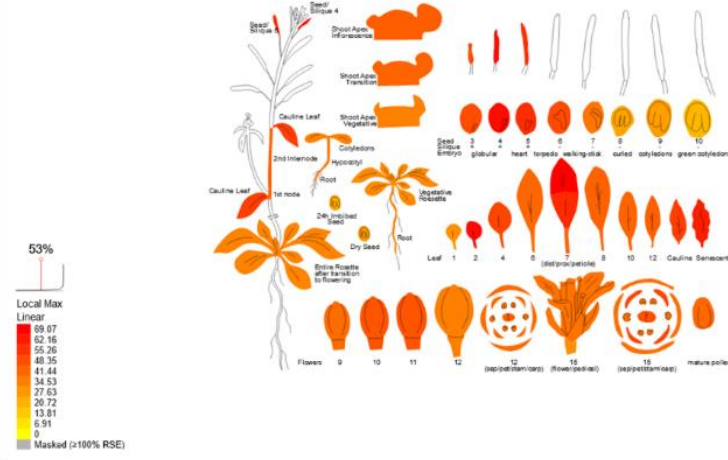
A**B**

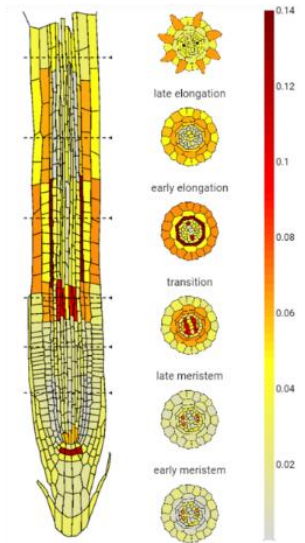
Figure 4.5. *mkkk7* and *pskr1/2* (*pskr1-3pskr2-1*) mutants show reduced rosette growth. **A.** 6-week-old soil-grown plants. The white bar represents 5 cm. **B.** Quantification of rosette diameter of 8 plants per genotype. One-way ANOVA multiple comparisons followed by Tukey's multiple comparison test. p-value is shown above. This experiment was repeated twice with similar results.

A

AtGenExpress eFP: AT3G07980 / MAP3KE2, MAPKKK6

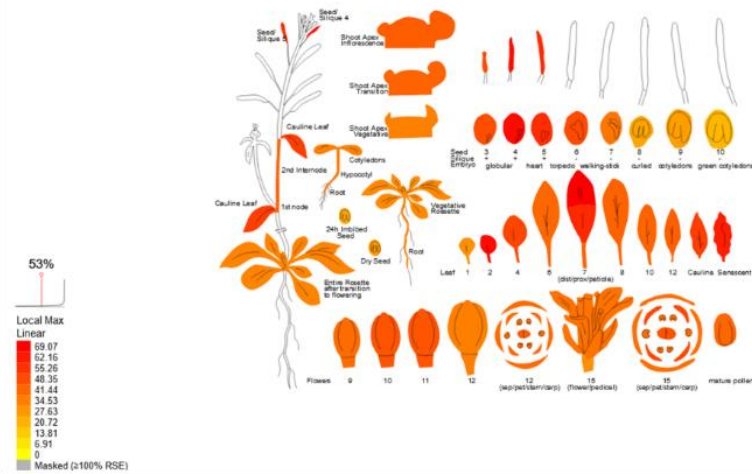


B

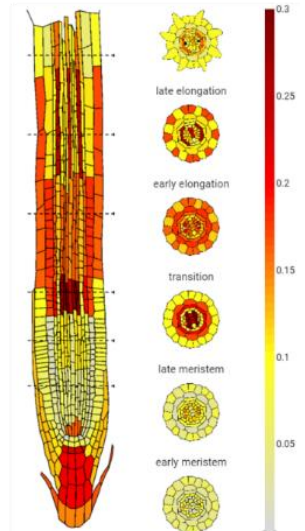


C

AtGenExpress eFP: AT3G13530 / MAP3KE1, MAPKKK7

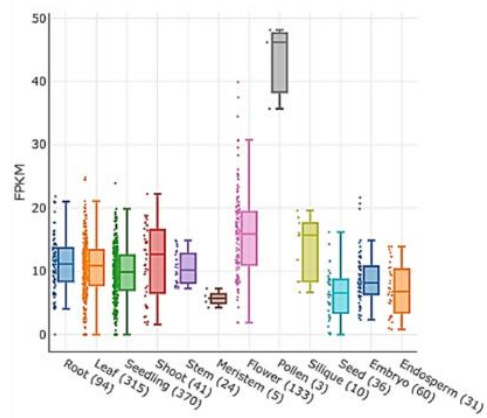


D



E

Expression levels of AT3G07980 among different tissues



F

Expression levels of AT3G13530 among different tissues

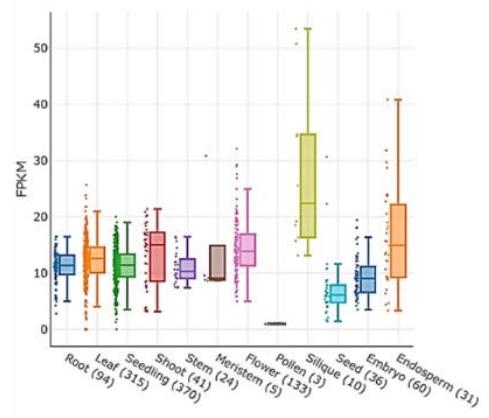


Figure 4.6. Publicly available expression data showing the overlapping expression profiles of *Arabidopsis thaliana* MKKK6 and MKKK7. **A-B.** Expression profile of MKKK6 (AT3G07980) in Arabidopsis shoot (Schmid et al. 2005) and root tissue¹¹. **C-D.** Expression profile of MKKK7 (AT3G13530) in Arabidopsis shoot (Schmid et al. 2005) and root tissue¹². **E-F.** Tissue-specific expression MKKK6 (AT3G07980) and MKKK7 (AT3G13530) based on integration of publicly available RNA-sequencing datasets¹³ (Zhang et al. 2020).

¹¹ <https://rootcellatlas.org/>

¹² <https://rootcellatlas.org/>

¹³ plantrnadb.com

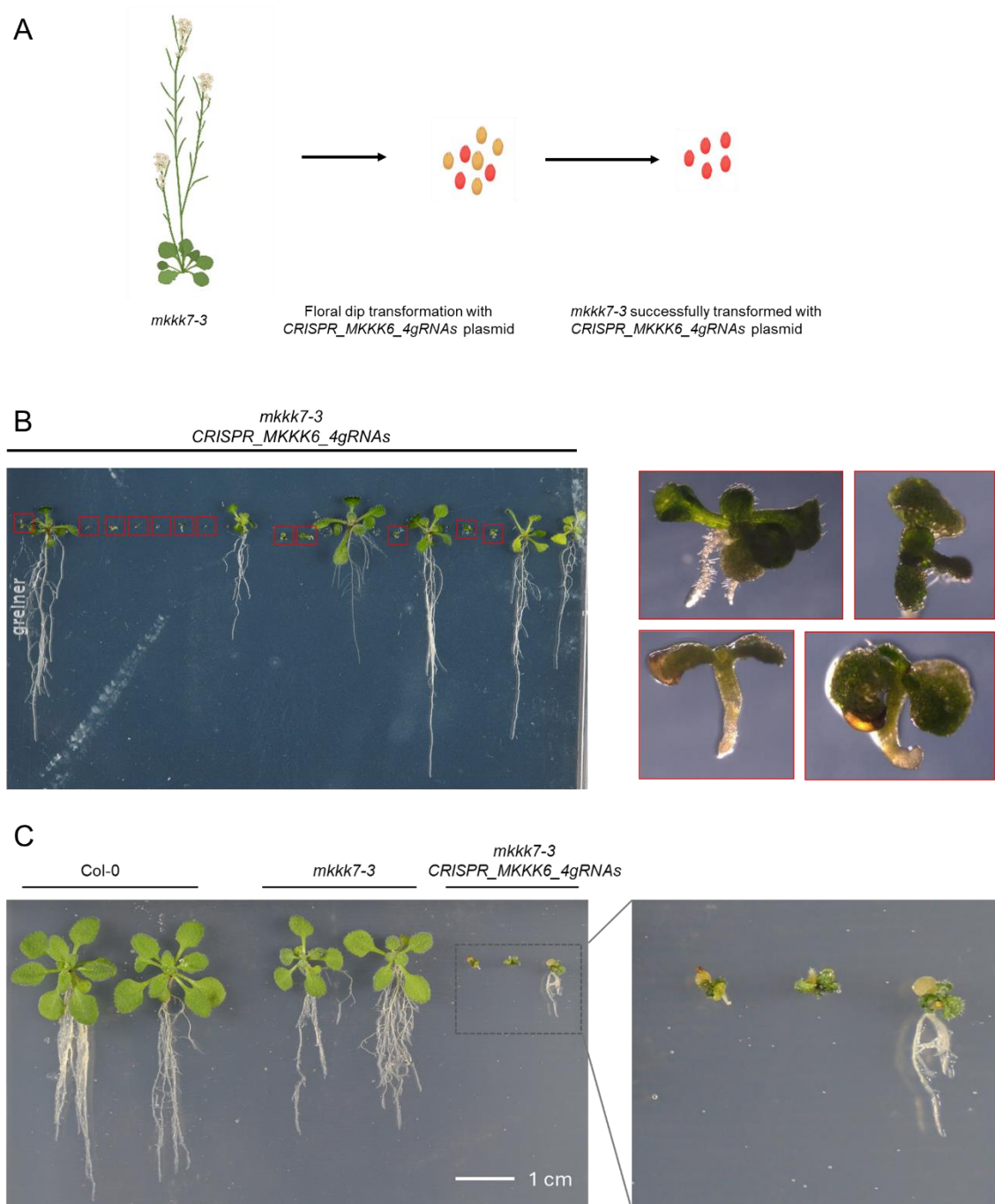


Figure 4.7. Phenotypic analysis of T0 *mkkk7-3* seedlings transformed with CRISPR-Cas9 constructs targeting *MKKK6* (*CRISPR_MKKK6_4gRNAs*). **A.** Schematic overview of transformation and FastRed selection process used to generate and identify successful transformants. **B.** Successfully transformed *mkkk7-3* T0 seeds grown on $\frac{1}{2}$ MS + 1% sucrose vertical plate for 4 weeks. Red boxes indicate germinated seedlings with developmental defects, with selected seedlings enlarged on the right. **C.** Comparison of 5-week-old Col-0, *mkkk7-3* and selected *mkkk7-3* transformed with *CRISPR_MKKK6_4gRNAs* seedlings grown on $\frac{1}{2}$ MS + 1% sucrose. Enlarged image on the right.

4.4 Discussion

In this chapter, I investigated the role of *MKKK7* in *Arabidopsis* growth by generating and phenotypically characterizing novel *mkkk7* mutant alleles. Three independent *mkkk7* mutant alleles show consistently shorter roots, a phenotype that is reminiscent of phytosulfokine receptor double mutants *pskr1-2pskr2-1*. The severe phenotypes of the novel putative *mkkk6mkkk7* double mutants provide substantial evidence supporting the functional redundancy between these two kinases.

4.4.1 Phenotypes of *mkkk7* and *pskr1-3pskr2-1* mutants

Phenotypic analysis of the previously published *mkkk7-1* T-DNA line (SALK_133360) and two independent *MKKK7* mutant lines (*mkkk7-2* and *mkkk7-3*) reveals a consistent reduction in primary root and shoot growth. Despite the detection of *MKKK7* transcript by RT-qPCR in *mkkk7-1* and *mkkk7-2* (Figure 4.1 C), these findings confirm published phenotypes (Chaiwongsar et al. 2012) (Mithoe 2013, PhD thesis). Comparing *mkkk7* and *pskr1-3pskr2-1* phenotypes reveals that both mutants exhibit reduced shoot and root growth, with the effect being more pronounced in *mkkk7* mutants (Figure 4.3, 4.5). This suggests that *MKKK7* may participate in additional signalling pathways beyond PSK signalling, contributing to broader aspects of plant growth.

4.4.2 Potential functional redundancy between *MKKK6* and *MKKK7*

Previous studies have shown that *mkkk6* T-DNA knockouts display no obvious growth defects (Chaiwongsar et al. 2012). Gene expression data suggest both *MKKK6* and *MKKK7* are expressed throughout the plant, with particularly high *MKKK6* expression in pollen (Figure 4.6 C, D). Crosses between *mkkk6* and *mkkk7* T-DNA mutants lead to pollen lethality, but their growth phenotypes, when complemented with an ethanol-inducible *alc-YFP-MKKK7* construct in the absence of ethanol, are similar to those of *mkkk7* mutants alone (Chaiwongsar et al. 2012). My attempt to generate an *MKKK6* knockout in the *mkkk7-3* background results in severe developmental abnormalities, providing insight into the potential functional redundancy and critical roles of these kinases in plant development. The unexpected severity of the putative *mkkk6mkkk7* mutant phenotype suggests that published T-DNA mutant crosses may not represent full knockouts.

Another explanation for the severe phenotypes could be that CRISPR-Cas9 gene editing of *MKKK6* results in a gain-of-function (GOF) allele, potentially creating an auto-active kinase that drives abnormal development. Additionally, *MKKK6*, *MKKK7* or other components in their signalling pathway may be guarded by immunity-related proteins such nucleotide-binding domain leucine-rich repeat containing proteins (NLRs) that

trigger immune responses upon disruption of the signalling process. This possibility is supported by parallels with another MAPKKK of the MEKK family, *MEKK1*. *mekk1* mutants exhibit severe dwarfism because components of the MEKK1-MKK1/MKK2-MPK4/MPK11 cascade are monitored by various NLRs (Gao et al. 2008; Suarez-Rodriguez et al. 2007; Takagi et al. 2018; Zhang et al. 2012). Lastly, off-target mutations from CRISPR-Cas9 editing could also account for the observed phenotypes, but the consistency and severity of developmental defects across multiple transformants make this explanation less likely.

Future studies will need to determine whether knocking out *MKKK6* in the *mkkk7-1* or *mkkk7-2* backgrounds results in similar phenotypes. Employing a conditional expression system, such as ethanol- or estradiol-inducible promoters for *MKKK7* or *MKKK6* expression in these double mutants could be particularly valuable for generating viable lines and identify the underlying cause of the observed phenotypes. An ethanol-inducible *mkkk6mkkk7alc-YFP-MKKK7* line was successfully developed by Patrick Krysans' research group (Chaiwongsar et al. 2012). However, upon inquiry, I was informed that this line does not exist anymore. Alternatively, crosses between independent *mkkk6* CRISPR-Cas9 mutants and *mkkk7-3* could result in viable heterozygous lines that may exhibit haploinsufficiency.

The severe *mkkk6mkkk7* mutant phenotype present an excellent opportunity for genetic suppressor screens, which have been proven successful in elucidating signalling components related to MAP kinase cascade signalling in the past. For example, an RNAi based screen in the *mekk1* dwarf mutant identified malectin-like RLK LETUM1 (LET1)(Liu et al. 2020), while a suppressor screen of the *mkk1mkk2* dwarf mutant identified the MAPKKK SUMM1 (SUPPRESSOR of *mkk1 mkk2* 1)(Kong et al. 2012), also known as *MEKK2*, along with the coiled-coil-type NLR SUMM2 (Zhang et al. 2012; Zhang et al. 2017). The abundance of *MEKK2* is regulated by the MEKK1-MKK1/MKK2-MPK4/MPK11 cascade; disruption of this cascade leads to an increase in *MEKK2* levels (Su et al. 2013). *MEKK2*, in turn, stabilises the complex of LET1 and SUMM2 which triggers autoimmune activation (Liu et al. 2020) and blocks MPK4 phosphorylation, thereby preventing its activation (Nitta et al. 2020). Two closely related MAPKKKs, *MEKK1* and *MEKK2*, have seemingly evolved opposing functions. It will therefore be important to re-assess published *mkkk6* T-DNA mutants by generating independent CRISPR-Cas9 mutants to determine whether *MKKK6* is functionally redundant with or distinct from *MKKK7*.

In conclusion, *mkkk7* and *pskr1-3pskr2-1* show reduced primary root and shoot growth. The severe developmental phenotypes observed in *mkkk6mkkk7* mutants, whether due to the complete knockout or truncation of *MKKK6*, strongly suggests that both kinases play essential roles in plant development. At the time of writing, viable *mkkk6mkkk7* mutants were not yet available, preventing further analysis of these mutants. As a result, subsequent investigation into their specific functions and interactions with the PSK signalling pathway focused solely on *mkkk7* mutants.

MKKK7 Acts on The Phytosulfokine Signalling Pathway to Modulate Cell Expansion and Gene Expression, Without Affecting PSK-Induced Growth or Immune Suppression

5.1 Summary

Phytosulfokine (PSK) is an important plant peptide hormone involved in growth promotion and modulation of immune signalling. MAP KINASE KINASE KINASE 7 (MKKK7) was identified as a potential downstream signalling component in the PSK signalling pathway. I show here that MKKK7 is involved in PSK-induced protoplast expansion and gene expression, but overall PSK-induced growth and immune suppression are not affected in *mkkk7* mutants. RNA sequencing reveals significant PSK-induced suppression of immune gene expression, independent of MKKK7. In addition, PSK suppresses DAMP/PAMP-induced ROS production independently of MKKK7. Genetic epistasis experiments show no clear interaction between MKKK7 and PSKR1 but reveal that PSKR1-induced root growth is slightly reduced in the *mkkk7-2* mutant background. These findings suggest that MKKK7 operates within a distinct regulatory module of the PSK signalling network, or that other signalling components, potentially other MAPKKs, perform redundant functions, thereby compensating for the loss of MKKK7.

5.2 Introduction

Phytosulfokine (PSK) peptide signalling is essential for various plant processes, including growth regulation, stress responses, and reproductive development, primarily by promoting cell expansion and proliferation (Sauter 2015). Additionally, PSK modulates plant immunity, with *pskr* receptor mutants showing increased resistance to biotrophic and hemibiotrophic pathogens but enhanced susceptibility to necrotrophic pathogens (Igarashi, Tsuda, and Katagiri 2012; Mosher et al. 2013).

Phytosulfokines are encoded by seven genes in *Arabidopsis* and, unlike other peptide families, all PSK precursor genes produce the same conserved, tyrosine-sulfated Y[SO₃]-I-Y[SO₃]-T-Q pentapeptide. At nanomolar concentrations, PSK is sufficient to enhance shoot and root growth in *Arabidopsis* (Matsubayashi et al. 2006; Kutschmar et al. 2009; Stührwohldt et al. 2011). Overexpression of PSK-signalling components such as *PSKR1*, *PSKR2* and *PSK4* promote root and shoot elongation (Stührwohldt et al. 2011; Matsubayashi et al. 2006). Conversely, *pskr1* mutants do not respond to external PSK and exhibit overall reduced root and hypocotyl growth (Igarashi, Tsuda, and Katagiri 2012; Amano et al. 2007). At the cellular level, PSK enhances cell proliferation in cultured cells and triggers plant protoplast expansion through the regulation of CYCLIC NUCLEOTIDE-GATED CHANNEL (CNGC17) and H⁺-ATPases AHA1 and AHA2 (Ladwig et al. 2015). Exogenous PSK application promotes pollen germination in culture (Chen, Matsubayashi, and Sakagami 2000) and stimulates pollen tube growth (Stührwohldt et al. 2015).

Beyond its role in growth regulation, PSK signalling is also implicated in biotic and abiotic stress responses. Upon wounding, several PSK genes, including *PSK3*, *PSK4*, *PSK5*, along with the *PSKR1* receptor, are transcriptionally upregulated, indicating a role for PSK signalling in wound responses (Matsubayashi et al. 2006; Loivamäki et al. 2010). Recent research in rice shows that *OsPSK3* and *OsPSK4* are not only upregulated following wounding but also in response to the DAMAGE-ASSOCIATED MOLECULAR PATTERN (DAMP) PLANT ELICITOR PEPTIDE 1 (PEP1). PSK signalling in rice antagonizes wound-induced defence responses, facilitating the transition from defence to growth (Harshith et al. 2024).

Agrobacterium tumefaciens-induced crown gall formation is significantly reduced in *pskr1-3* and *pskr1-3pskr2-1* mutants (Loivamäki et al. 2010). The development of crown galls requires the complex interplay of plant phytohormones auxin, cytokinin and abscisic acid (ABA) (Gelvin 2003) and virulence is controlled by ethylene and the defence hormone salicylic acid (SA) (Anand et al. 2008). Moreover, *pskr1-3pskr2-1* mutants exhibit enhanced PTI signalling, defence gene expression, and increased resistance to hemi-biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Igarashi, Tsuda, and Katagiri 2012). Conversely, disruption of PSK signalling results in increased susceptibility towards necrotrophic fungal pathogen *Alternaria brassicicola* (Mosher et al. 2013).

The *pskr1-3* mutant shows elevated SA levels following *Pto* DC3000 infection, suggesting the observed differences in resistance to biotrophic/hemibiotrophic versus necrotrophic pathogens, and reduced *Agrobacterium*-induced crown gall formation, may be linked to a hormonal shift towards SA signalling (Mosher et al. 2013). Recently, Song

et al. (2023) provided genetic evidence that PSK signalling suppresses SA-mediated defence responses in a *NPR1*- and *NPR3/NPR4*-dependent manner and thereby modulates microbiome homeostasis (Song et al. 2023).

In addition to modulating biotic stress response, PSK signalling also regulates abiotic stress responses. Exogenous application of PSK improves *Arabidopsis* seedling growth tolerance to high night-time temperatures (Yamakawa et al. 1999). In tomato, PSK signalling is implicated in drought-induced flower drop, a process of significant economic concern due to its impact on crop yield under environmental stress. In this process, Phytaspase 2 (*S/Phyt2*, Solyc04g078740) processes *proPSK* to release the active PSK pentapeptide Y[SO₃]IY[SO₃]TQ under drought conditions, which leads to pedicel abscission and subsequent flower drop (Reichardt et al. 2020). *proPSK* processing is thereby dependent on an aspartic acid (D) residue in the P1 position upstream of the mature peptide. The abscission process is driven by the expression of hydrolases that degrade the cell wall, possibly in combination with PSK-induced cell expansion, which may generate the shear forces necessary for organ detachment (Reichardt et al. 2020). Phytaspases including *S/Phyt2* form an expanded family of subtilases in the nightshade family but are absent in Brassicaceae (Reichardt et al. 2018).

In the Brassicaceae model species *Arabidopsis thaliana*, *PSK1*, *PSK3*, *PSK4*, *PSK5* and subtilases *SBT1.4*, *SBT3.7* and *SBT3.8* are up regulated in response to osmotic stress (Stührwohltdt et al. 2021). *SBT3.8* plays a key role in the maturation process of PSK by cleaving *proPSK1* at its C-terminus, a process that requires an aspartic acid (D) residue in the P1' position downstream of the mature peptide. Overexpression of either *proPSK1* or *SBT3.8* enhances fresh weight and root development under osmotic stress, indicative of improved drought tolerance (Stührwohltdt et al. 2021). Interestingly, among the seven PSKs in *Arabidopsis*, only *PSK1* contains the critical D residue in this position, suggesting that other proteases might be involved in processing the remaining PSKs. The proteases responsible for the N-terminal processing of *proPSKs* have yet to be identified in *Arabidopsis*.

In summary, PSK signalling is essential for promoting cell expansion and proliferation while modulating plant biotic and abiotic stress responses. Despite its importance, the downstream components of the PSK pathway, as with many peptide signalling pathways, remain largely unexplored. In Chapter 3, I identify *MKKK7* as a putative downstream component of *PSKR1* via AP-MS, and in Chapter 4, I demonstrated that *mkkk7* mutants partially mimic the *pskr1-3pskr2-1* double mutant phenotype. In this chapter, I will investigate PSK-induced signalling outputs in *mkkk7* mutants and address genetically whether *MKKK7* functions downstream of *PSKR1*.

5.3 Results

5.3.1 Reciprocal YFP-MKKK7 AP-MS data shows differences after PSK treatment

AP-MS analysis of the YFP-MKKK7 pull-downs with and without PSK treatment revealed 13 proteins that were more abundant in the PSK-treated sample compared to the mock-treated sample (Figure 5.1 A, B). However, these proteins were also present in other samples, regardless of the treatment, suggesting that they may not be specific interactors of MKKK7. PSKR1 was not identified using the filter parameters of $-\log_{10} p\text{-value} \geq 0.107$ and $\log_2 \text{fold changes} \geq 2$ or ≤ -2 . A closer look at PSKR1-specific spectral counts in YFP-MKKK7, BAK1-GFP, Lti6B-GFP and BRI1-mCitrine samples showed a slight increase in abundance in YFP-MKKK7 samples after PSK treatment (Figure S3). However, PSKR1 was detected across all samples, making it difficult to draw definitive conclusions based solely on spectral counts. Further investigation, including MS1-level quantification, is needed to determine if this increase confirms an enhanced association between PSKR1 and MKKK7 in response to PSK, as it was observed in the reciprocal PSKR-GFP pull-down (Figure 3.5). Additional experiments such as directed Co-IPs will be essential to confirm this interaction. In addition, I observed that PSK treatment resulted in a general reduction in peptide spectra detection, suggesting a decrease in protein abundance (Figure 5.1). A similar trend was observed in the PSKR1-GFP AP-MS data (Figure 3.5, Table S2, Figure S1), but the underlying cause for this reduction is not clear. GO-term enrichment analysis of these downregulated proteins showed that 'generation of precursor metabolites and energy' was the most overrepresented biological function (Table S3, Figure S2), which was different from RNA-related processes overrepresented in the PSKR1-GFP dataset.

5.3.2 PSK-signalling promotes primary root growth and hypocotyl elongation

To test whether PSK-induced signalling is compromised in *mkkk7* mutants I analysed primary root growth in response to PSK treatment as described previously (Kutschmar et al. 2009). Col-0 and *mkkk7-1*, *mkkk7-2*, *mkkk7-3* showed significantly enhanced primary root growth when grown in the presence of 50 nM PSK in the growth medium compared to the control medium without PSK (Figure 5.2). The *pskr1-3pskr2-1* mutant was insensitive to the treatment as would be expected (Kutschmar et al. 2009) (Figure 5.2).

Previous studies have shown that hypocotyls of etiolated *pskr1-3pskr2-1* seedlings are shorter than those of Col-0 (Hartmann et al. 2013). Expression analysis of *pMKKK7-NLS::3xmVenus* revealed promoter activity in growing hypocotyls (Figure 5.3 A). I therefore measured hypocotyl growth of *mkkk7* mutants but found no significant

differences compared to Col-0 (Figure 5.3 B). In contrast, *pskr1-3pskr2-1* mutants showed significantly reduced hypocotyl growth, in line with previous reports (Hartmann et al. 2013) (Figure 5.3 B). Together, these experiments suggest that *MKKK7* may not be required for endogenous or exogenous PSK-induced root and hypocotyl growth in Arabidopsis.

5.3.3 PSK-induced protoplast expansion and gene expression are attenuated in *mkkk7* mutants

In addition to promoting root and hypocotyl growth, PSK is known to induce protoplast expansion and modulate gene expression (Kaufmann, Stührwohldt, and Sauter 2021; Stührwohldt et al. 2011). To assess whether *mkkk7* mutants exhibited altered PSK-induced cellular responses, I conducted protoplast expansion assays using protoplasts isolated from *Col-0*, *mkkk7-1*, *mkkk7-2*, and *pskr1-3pskr2-1* seedlings. Protoplasts were treated with 2 nM PSK or water (mock) for 40 minutes. By measuring their circumference at time points $T_{0 \text{ min}}$ and $T_{40 \text{ min}}$, I calculated the volume change during the treatment, following previously described methods (Stührwohldt et al. 2011). *Col-0* protoplasts showed a 2-4% volume increase over 40 min, whereas *mkkk7-1* protoplasts showed an increase of ~1% and both *mkkk7-2* as well as *pskr1-3pskr2-1* showed no significant response to the PSK treatment (Figure 5.4).

To further confirm altered PSK-responses in the *mkkk7* mutants, I selected 4 PSK-responsive genes (Kaufmann, Stührwohldt, and Sauter 2021) and analysed their expression by RT-qPCR. In *Col-0*, PSK-induced gene expression peaked at 2 h, but this response was abolished in *mkkk7-1*, *mkkk7-2* and *pskr1-3pskr2-1* (Figure 5.5 A). I also tested whether auxin/IAA-induced gene expression is altered in *mkkk7* and *pskr1-3pskr2-1* mutants, but IAA-induced expression of *PIN1*, *IAA1*, and *SAUR19* remained unaltered in *mkkk7-1*, *mkkk7-2*, and *pskr1-3pskr2-1* mutants (Figure 5.6). These data suggested that *mkkk7* mutants are impaired in PSK-induced cell expansion and gene expression.

5.3.4 Transcriptomic perturbations in *mkkk7-3*

I next performed RNA sequencing to investigate transcriptomic changes in *mkkk7* mutants and to analyse global PSK-induced gene expression. The experiment was performed on fifteen-day-old seedlings grown in liquid MS media under the same conditions as the RT-qPCR experiments. There was a strong and significant downregulation of *MKKK7* transcript in *mkkk7-3* relative to *Col-0* as would be expected (Figure 5.7 A). To identify differentially expressed genes between *Col-0* and *mkkk7-3*, I applied cut-offs of $\log_2\text{FC} \geq 2$ and $\log_2\text{FC} \leq -3$, $p\text{-value} \leq 0.05$ and listed all identified

genes in Figure 5.7 B, C. Gene Ontology (GO) enrichment analysis under the category 'Biological process' (Mi et al. 2019) did not reveal any significantly enriched processes among the differentially expressed genes. It was interesting to note that several upregulated genes in *mkkk7-3* are linked to iron ion responses (Figure 5.7 B), while downregulated genes were predominantly linked to phosphate starvation (Figure 5.7 C).

5.3.5 Arabidopsis latent virus 1 contamination

It is important to note here that during my RNA sequencing analysis, I discovered that the *mkkk7-3* mutants were infected with Arabidopsis latent virus 1 (ArLV1), a comovirus widely spread across various Arabidopsis accessions and research institutions (Verhoeven et al. 2023). I first detected the virus because in several *mkkk7-3* samples only ~50% of RNA sequencing reads mapped to the *Arabidopsis thaliana* reference genome. I then confirmed the virus infection by RT-qPCR, following the protocol described by Verhoeven et al. 2023 (Figure 5.8). Through extensive testing over several generations, I successfully identified virus-free *mkkk7-3* mutants, which were subsequently used to validate earlier results and for all further experiments (Figure 5.8). Although the transcriptomic perturbations in *mkkk7-3* compared Col-0 appeared minimal, despite the viral infection, this experiment should be repeated with the virus-free *mkkk7-3* line. During the virus testing, I consistently observed phenotypic differences between virus contaminated and virus-free *mkkk7-3* mutants. Infected plants exhibited rounder, flatter leaves compared to the elongated and typically rounded leaves of healthy plants (Figure 5.8 B). This observation raised the potential for visual screenings of infected material based on these phenotypic traits. It would be valuable to share these findings with other research groups that have encountered virus-contaminated seed stocks and to determine whether this phenotype is consistent across different genotypes and growing conditions. It could potentially serve as a morphological indicator for ArLV1 infection.

5.3.6 Commonly upregulated and downregulated genes in *mkkk7-3* and *pskr1-3pskr2-1* mutants compared to WT Col-0

After I determined overall transcriptomic changes in *mkkk7-3*, I wanted to address if *mkkk7-3* and *pskr1-3pskr2-1* mutants show overlapping differences in upregulated and downregulated gene expression compared to WT Col-0. I compared RNA sequencing mock samples of Col-0/*mkkk7-3* with Col-0/*pskr1-3pskr2-1* and identified 42 genes (13.3%) that were commonly downregulated and 25 genes (10.7%) that were commonly upregulated in *mkkk7-3* and *pskr1-3pskr2-1* mutants (Figure 5.9 A, B). I selected the genes based on cut-offs of $\log_2$ fold change ($\log_2FC$) ≤ -1 and p-value ≤ 0.05 for downregulated genes and $\log_2FC \geq 1$ and p-value ≤ 0.05 for upregulated genes.

Visualisation of CPM values of up- and down-regulated genes shows clear differences between Col-0, *mkkk7-3*, and *pskr1-3pskr2-1* mutants (Figure 5.9 C, D). GO enrichment analysis of the 42 downregulated genes highlighted responses to external stimuli and nutrients as overrepresented biological processes (Figure 5.9 E, Table S5). Several downregulated genes were related to phosphate starvation responses (Table S5) and GO enrichment analysis for molecular functions suggests phosphate (P) transport and phosphatase activity are enriched functions (Figure 5.9 F). Downregulated genes include Arabidopsis high-affinity phosphate transporters PHOSPHATE TRANSPORTER 1;9 (PHT1;9) and PHOSPHATE TRANSPORTER 1;8 (PHT1;8), which facilitate inorganic phosphate (Pi) uptake under Pi starvation conditions (Remy et al. 2012) and PURPLE ACID PHOSPHATASEs 14 and 24 (PAP14 and PAP24) (Table S5). The Arabidopsis genome encodes 29 PAPs (Li, Zhu, et al. 2002), which play important roles in plant tolerance to P limitation (Wang et al. 2011).

On the other hand, no GO biological process was overrepresented among the upregulated genes. However, it was notable that three IRONMAN peptides *IRONMAN1* (*IMA1*) (AT1G47400), *IRONMAN2* (*IMA2*) (AT1G47395) and *IRONMAN3* (*IMA3*) (AT2G30766) were present in the list of upregulated genes (Table S6). While the precise molecular function of these peptides is still unclear, they have been implicated as mobile signals in iron ion transport, iron acquisition and immune signalling (Grillet et al. 2018; Cao et al. 2024).

Taken together, this data suggests that *mkkk7-3* and *pskr1-3pskr2-1* mutants show reduced expression of genes associated with nutrient sensing and starvation, particularly phosphate starvation, while several genes involved in iron uptake are upregulated.

5.3.7 RNA sequencing reveals PSK-induced downregulation of immunity-related genes independently of *MKKK7*

Lastly, I analysed the effect of PSK treatment on the transcriptional changes in Col-0, *mkkk7-3*, and *pskr1-3pskr2-1*. Previous transcriptional profiling of PSK-responsive genes in Col-0 and *tpst* mutants was conducted after 5 days of peptide treatment (Kaufmann, Stührwohldt, and Sauter 2021). Building on my RT-qPCR data (Figure 5.5 A), I performed RNA sequencing after 2 hours of PSK treatment. Differential gene expression analysis, using cut-offs of $FDR \leq 0.05$ and $\log_2FC \geq 1$, revealed no upregulated genes in response to PSK. However, there was a pronounced and significant downregulation of immunity-related genes (Figure 5.10 A-B).

GO enrichment analysis of the 203 downregulated genes highlighted 'response to salicylic acid' and 'response to molecule of bacterial origin' as the most significantly

overrepresented biological processes (Figure 5.10 B). The list encompasses several well characterised components of the PTI system, such as *BOTRYTIS-INDUCED KINASE 1* (*BIK1*), *MALE DISCOVERER 1-INTERACTING RECEPTOR LIKE KINASE 2* (*MIK2*) and *LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION* (*LORE*) as well as several *WRKY* transcription factors (*WRKY6,7,11, 15, 22, 27,29,49,53*) (Supplemental Table S7). In addition to *MIK2*, the expression of *PROSCOOP6* and *PROSCOOP12* was also significantly downregulated. Furthermore, PSK treatment significantly downregulated the expression of two E3 Ubiquitin ligases involved in PTI, *PLANT U-BOX TYPE E3 UBIQUITIN LIGASES 22* and *23* (*PUB22*) and (*PUB23*) (Supplemental Table S7). GO enrichment analysis based on molecular function revealed transmembrane transport activity as the most highly enriched category (Figure 5.10 C).

Genes such as *THAD1*, *MRN1*, and *DEFL*, which were upregulated in RT-qPCR assays, showed stronger expression in Col-0 compared to *mkkk7-3* and *pskr1-3pskr2-1*, though these differences were not statistically significant in the RNA sequencing data (Figure 5.5 C). Collectively, this data suggests that PSK treatment downregulates the expression of key immunity-related genes and transmembrane transport functions, independently of *MKKK7*.

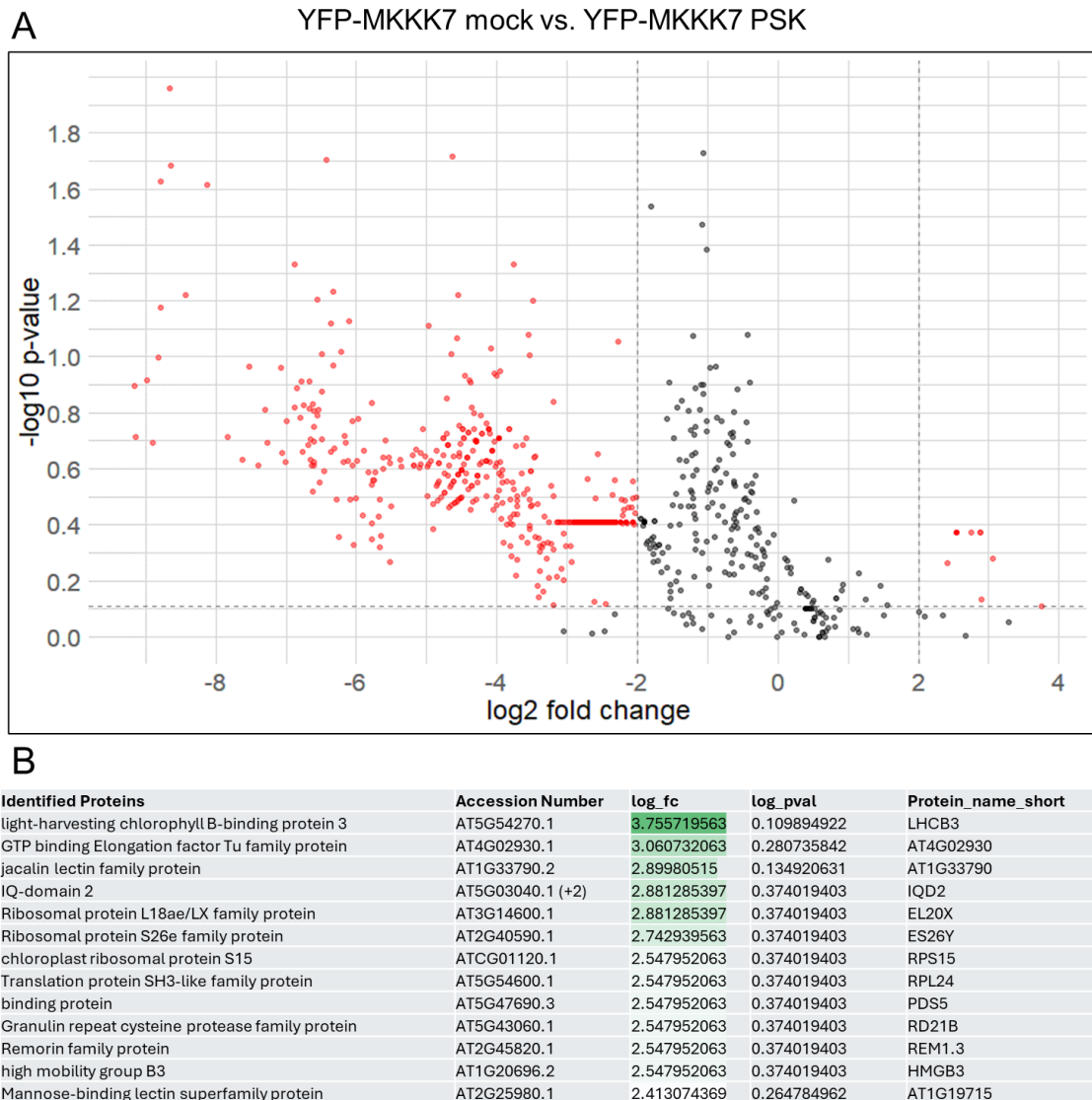


Figure 5.1. PSK-induced changes in peptide spectral counts in YFP-MKKK7 mock vs PSK samples. Data is based on 4 biological replicates of YFP-MKKK7 mock pull-downs and 3 biological replicates of YFP-MKKK7 treated with PSK for 20 minutes. **A.** Differential analysis of YFP-MKKK7 interactors in mock vs. PSK-treated samples, based on MS2 spectral counts. Positive $\log_2$ fold changes indicate higher spectral counts in the PSK-treated samples compared to mock, negative $\log_2$ fold changes indicate a reduction. Red dots represent proteins with significant changes, determined by a $-\log_{10}$ p-value threshold of ≥ 0.107 and $\log_2$ fold changes ≥ 2 or ≤ -2 . Missing spectral data were imputed with a value of 0.01 for logFC and $-\log_{10}$ p-value calculations. **B.** Detailed list of 13 proteins enriched in PSK-treated samples (from A). None of the 13 proteins was specific to YFP-MKKK7 pull-downs.

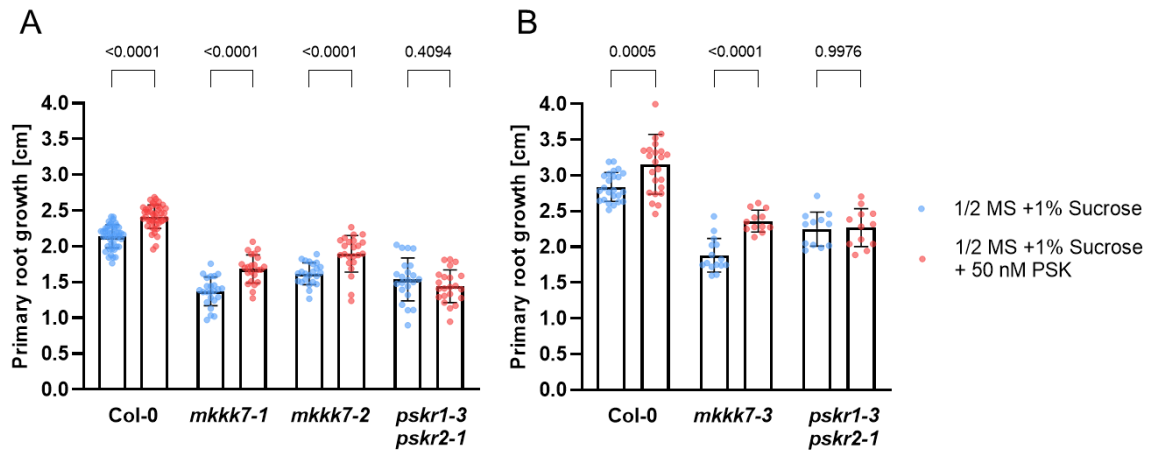


Figure 5.2. PSK-induced root growth is unaltered in *mkkk7* mutants. Seedlings were grown on ½ MS agar plates for 4 days and then transferred to ½ MS agar plates with and without 50 nM PSK for another 4 days before primary root length was measured. Data shows primary root length 8 days after germination. One-way ANOVA multiple comparisons followed by Tukey's multiple comparison test. p-value shown above. **A.** Comparison between Col-0, *mkkk7-1*, *mkkk7-2* and *pskr1-3pskr2-1*. **B.** Comparison between Col-0, *mkkk7-3* and *pskr1-3pskr2-1*. This experiment was repeated three times with similar results.

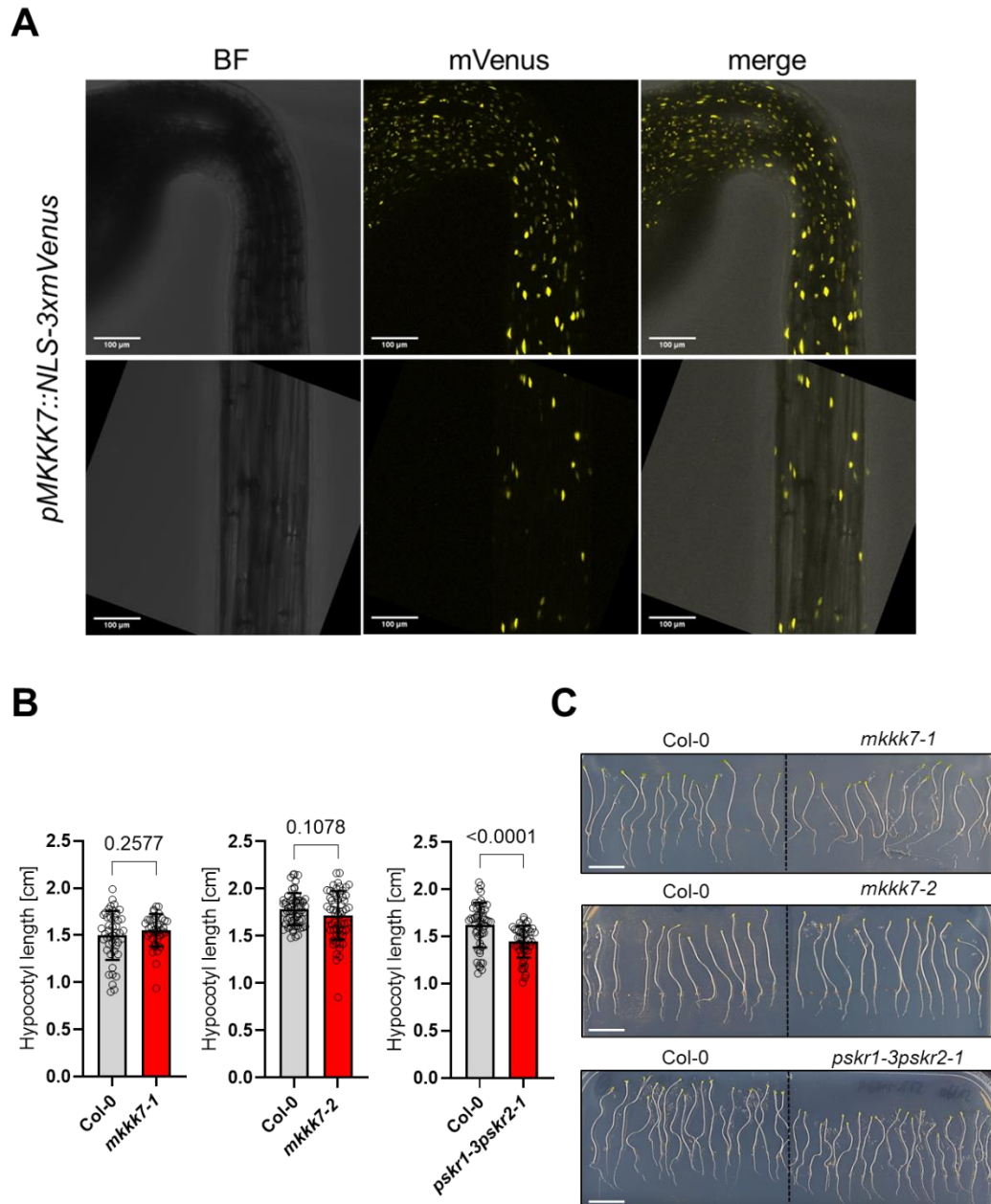


Figure 5.3. Hypocotyl elongation is unaltered in *mkkk7* mutants. *Arabidopsis thaliana* seedlings grown on ½ MS +1% sucrose vertical agar plates in the dark for 5 days. **A.** Confocal microscopy images of 5-day-old Col-0 seedlings stably expressing *pMKKK7::NLS-3xmVenus*. **B.** Quantification of hypocotyl length in cm of 5-day-old seedlings (n=30-60 per genotype per comparison). p-values derived from one-way ANOVA followed by Tukey's post-hoc test are indicated above. **C.** Representative image of 5-day-old, etiolated seedlings grown on plates in the dark.

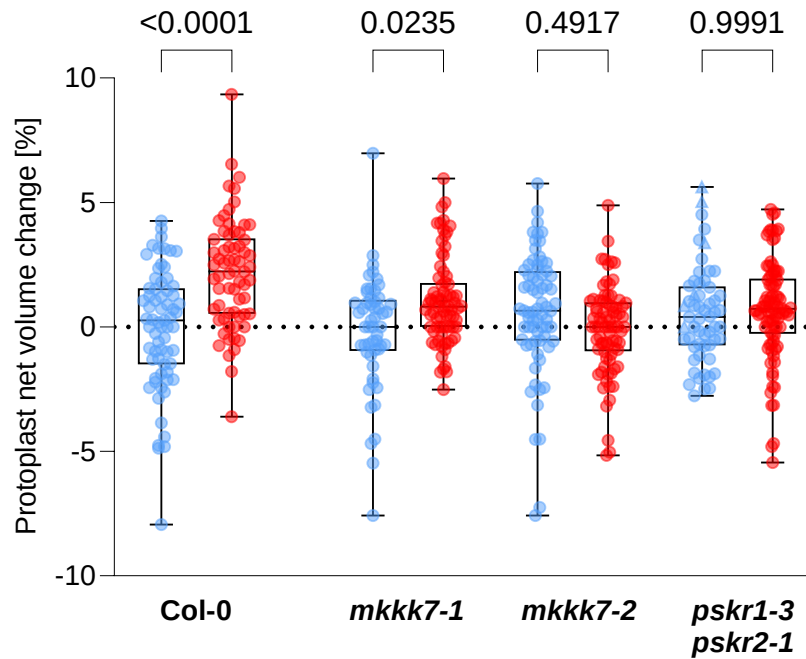
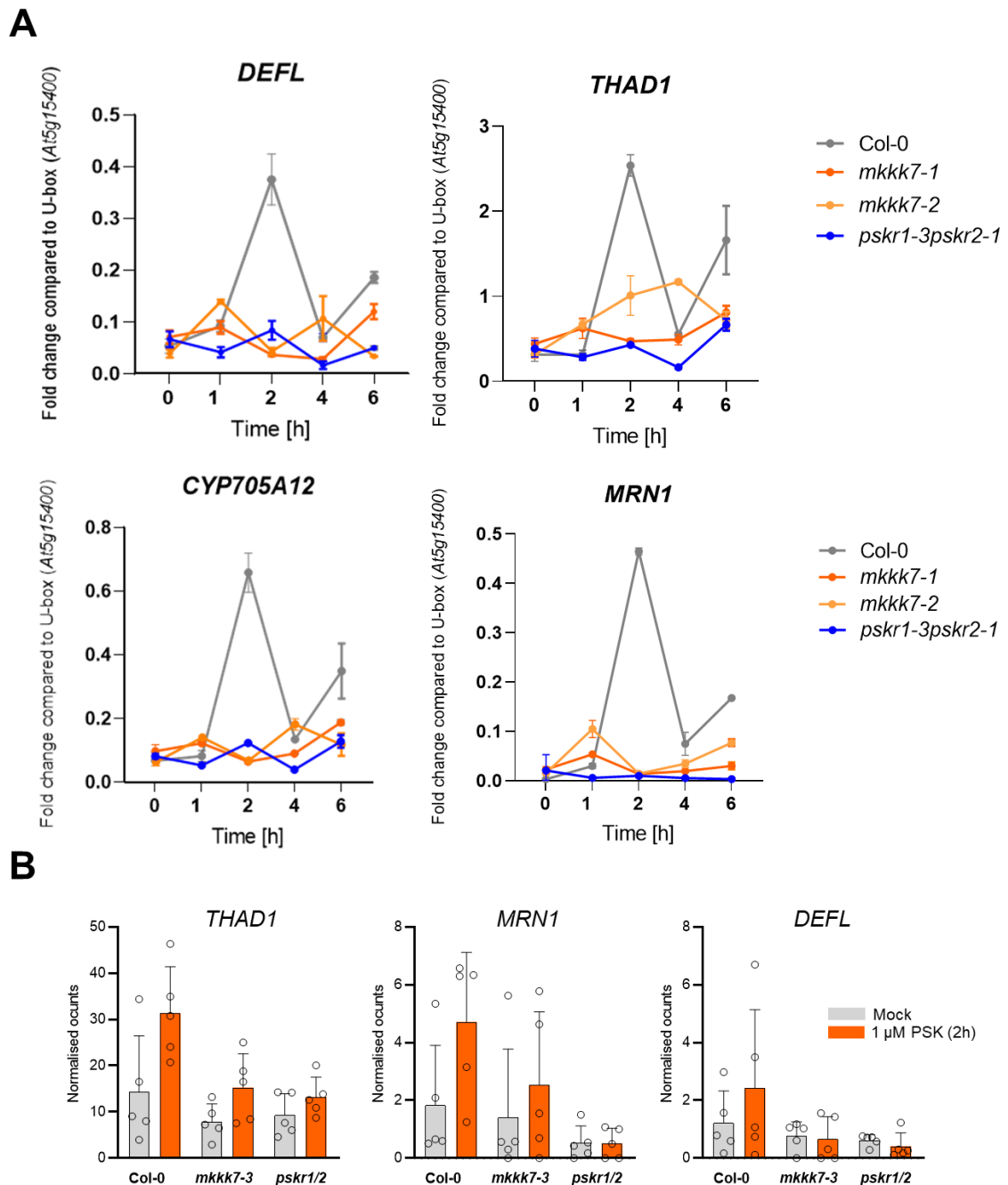


Figure 5.4. PSK-induced protoplast expansion is attenuated in *mkkk7* mutants. Protoplasts were isolated from shoots of 10–14-day old *Arabidopsis thaliana* seedlings grown on ½ MS +1% sucrose vertical agar plates. Net volume change [%] of the protoplasts in response to mock and 2 nM PSK was calculated from the difference in circumference from 3 min to 45 min. Data is represented as boxplots displaying the first and third quartiles, split by the median. Individual data points of 3 biological repeats are shown as dots. n= 67 (Col-0 mock), 66 (Col-0 2 nM PSK), 53 (*mkkk7-1* mock), 76 (*mkkk7-1* 2 nM PSK), 71 (*mkkk7-2* mock), 80 (*mkkk7-2* 2 nM PSK), 57 (*pskr1-3pskr2-1* mock), 89 (*pskr1-3pskr2-1* 2 nM PSK). p-values derived from one-way ANOVA followed by Tukey's post-hoc test are indicated above.



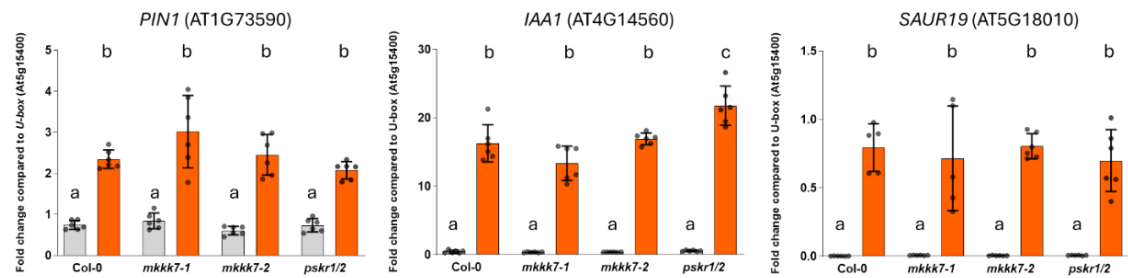
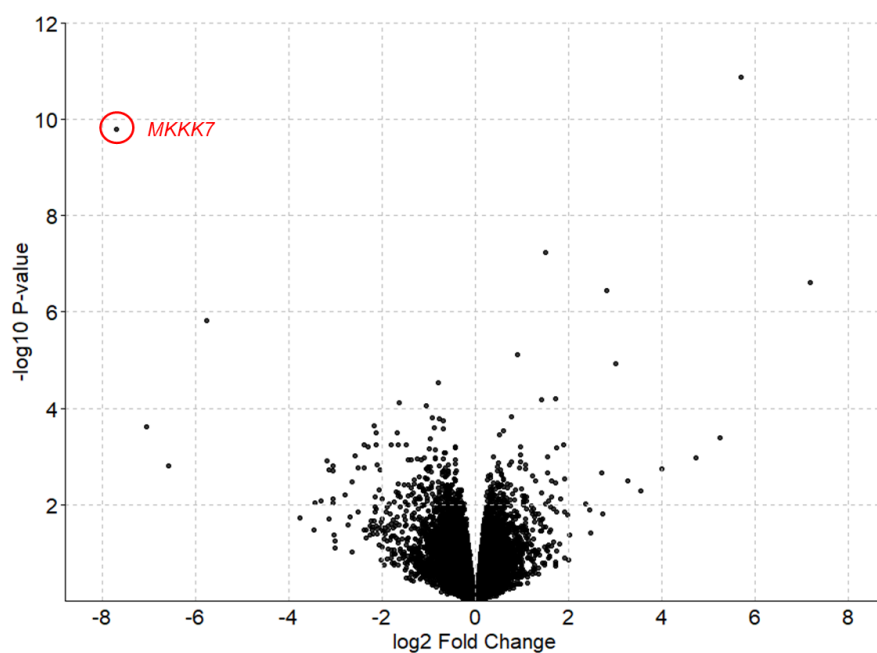


Figure 5.6. IAA-responsive gene expression is unaltered in *mkkk7* mutants. RT-qPCR analysis of auxin-responsive *PIN1* (AT1G73590), *IAA1* (AT4G14560) and *SAUR19* (AT5G18010) expression in 14-day-old *Arabidopsis thaliana* seedlings. Seedlings were treated with 10 μ M IAA for 60 minutes. Gene expression was normalised to *U-BOX* (At5g15400). Lowercase letters indicate significance groups based on one-way ANOVA followed by Tukey's post-hoc test. This experiment was repeated twice with three technical replicates each. Results from both independent experiments are shown in the graphs.

A



B

AGI	log ₂ FC	Other Name	GO Biological Process
AT4G08093	7.1896935	pseudogene	-
AT1G53480	5.6974661	MDR1	biological process
AT1G47400	5.238131	IMA1	positive regulation of iron ion transport, responst to iron ion starvation
AT2G01422	4.7326064	other RNA	-
AT1G47395	4.0115636	IMA2	positive regulation of iron ion transport, responst to iron ion starvation
AT5G04150	3.541438	BHLH101	cellular response to iron ion starvation, multicellular organismal-level iron ion homeostasis, regulation of DNA-templated transcription
AT2G30766	3.2633765	IAM3	positive regulation of iron ion transport, responst to iron ion starvation
AT5G65080	3.0200779	MAF5	negative regulation of flower development, vernalisation response
AT1G53490	2.8153368	HEI10	chiasma assembly, homologous recombination, meiotic cell cycle
AT2G15780	2.7287133	-	biological process
AT2G41260	2.7194986	M17	embryo development ending in seed dormancy
AT5G52390	2.4697384	PAR1	-
AT5G05250	2.4549593	-	-
AT5G59310	2.3700097	LTP4	lipod transport, response to abscisic acid, response to salt stress, response to water deprivation
AT3G05770	2.0323852	-	pollen tube development

C

AGI	log ₂ FC	Other Name	GO Biological Process
AT3G13530	-7.687166	MKKK7	plasma membrane organization, pollen development
AT5G39493	-7.0469228	SPH15	biological process
AT4G24890	-6.5801203	PAP24	dephosphorylation
AT1G01570	-5.7597434	DUF604	-
AT3G09922	-3.7574569	IPS1	cellular response to phosphate starvation
AT2G46880	-3.4599396	PAP14	biological process
AT3G28007	-3.4358102	SWEET4	carbohydrate transport, protein homooligomerization
AT1G50830	-3.3020227	Aminotransferase-like	meristem maintance
AT2G17850	-3.1812517	Cell cycle control phosphatase	biological process
AT2G21650	-3.1423043	MYB 1	embryo development ending in seed dormancy, gravitropism, response to light
AT4G38825	-3.1316544	SAUR13	response to auxin
AT3G47720	-3.0595764	SRO4	cellular response to hypoxia
AT2G11810	-3.0537189	MGD3	celular response to phosphate starvation, fatty acid metabolic process, galactolipid biosynthetic process, galactolipid metabolic process
AT4G24010	-3.0464649	CSLG1	cellulose biosynthetic process, plant-type primary cell wall biogenesis
AT5G28335	-3.0410854	transposable element	-
AT3G22231	-3.0227383	PCM4	response to bacterium, response to fungus

Figure 5.7. Transcriptomic perturbations in *mkkk7-3*. **A.** Volcano plot showing the transcriptomic perturbations in *mkkk7-3* compared to Col-0 in 14-day-old seedlings grown in MS media. Data is based on five biological replicates without treatment for *mkkk7-3* and Col-0. **B.** Genes significantly upregulated in *mkkk7-3* compared to Col-0 ($\log_2\text{FC} \geq 2$, $p\text{-value} \leq 0.05$). **C.** Genes significantly downregulated in *mkkk7-3* compared to Col-0 ($\log_2\text{FC} \leq -3$, $p\text{-value} \leq 0.05$).

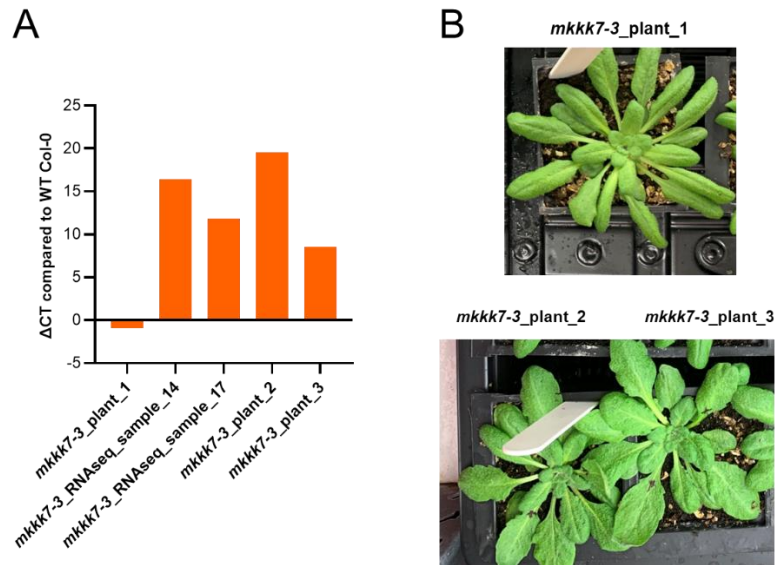


Figure 5.8. RT-qPCR results and phenotypes of Arabidopsis latent virus 1 (ArLV1) infected and uninfected mkkk7-3 plants. **A.** Plant material tested for ArLV1 RNA1. Δ CT values of calculated by subtraction from the average mock sample as described (Verhoeven et al. 2023). Δ CT values around 0 indicate that the sample is negative for ArLV1, positive values indicate the presence of ArLV1 RNA1. **B.** Representative images of tested plant material in A. *mkkk7-3_plant_1* is free from ArLV1, *mkkk7-3_plant_2* and *mkkk7-3_plant_3* are contaminated with the virus.

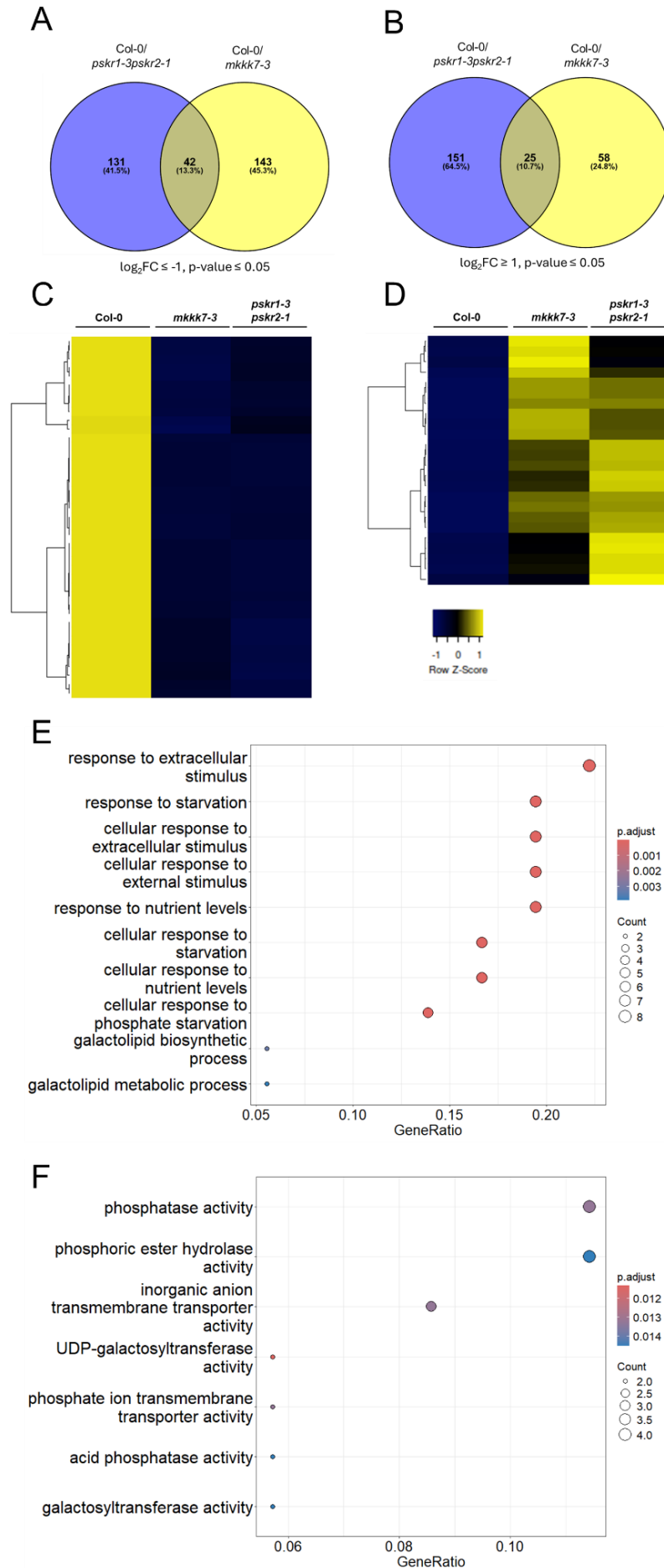


Figure 5.9. Commonly upregulated and downregulated genes in *mkkk7-3* and *pskr1-3pskr2-1* mutants compared to WT Col-0. **A.** Venn diagram showing commonly downregulated genes identified by comparing RNA sequencing mock samples of Col-0/*mkkk7-3* and Col-0/*pskr1-3pskr2-1*. Data is based on five biological replicates for each genotype without treatment. Genes were selected based on a $\log_2$ fold change ($\log_2FC$) ≤ -1 and $p\text{-value} \leq 0.05$. **B.** Venn diagram showing commonly upregulated genes identified by comparing RNA sequencing mock samples of Col-0/*mkkk7-3* and Col-0/*pskr1-3pskr2-1*. Genes were selected based on $\log_2FC \geq 1$ and $p\text{-value} \leq 0.05$. **C.** Heatmap showing average CPM values of the 42 commonly downregulated genes identified in panel A. The corresponding gene symbols and descriptions are detailed in Table S5. **D.** Heatmap showing CPM values of 25 commonly upregulated genes identified in panel B. The corresponding gene symbols and descriptions are detailed in Table S6. **E.** Biological function gene ontology (GO) enrichment analysis of 42 downregulated genes. **C.** Molecular function GO enrichment analysis of downregulated genes.

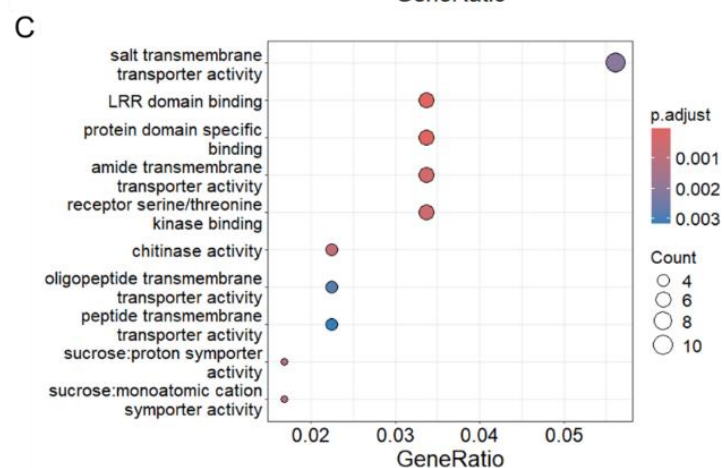
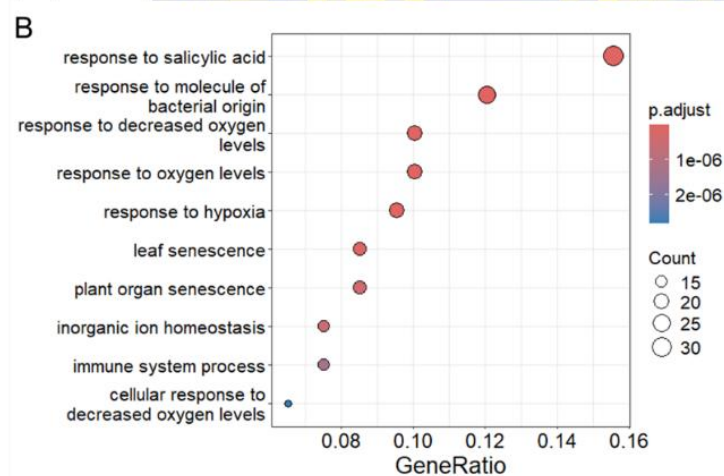
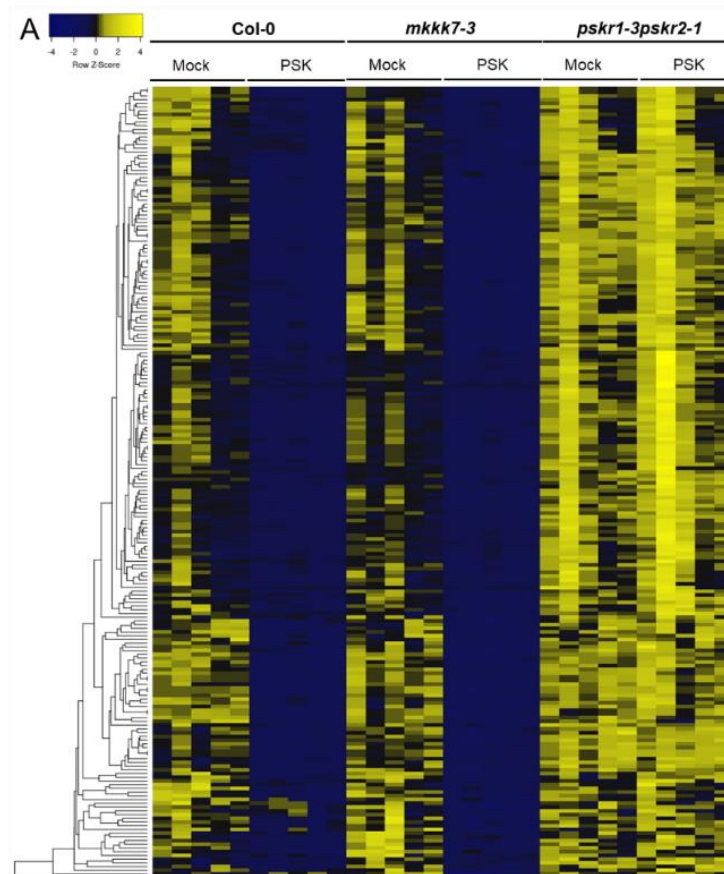


Figure 5.10. PSK treatment suppresses immunity-related gene expression in Col-0 and *mkkk7-3* but not *pskr1-3pskr2-1*. **A.** Heatmap showing CPM values of RNA samples from 14-day-old seedlings treated with water (mock) or 1 μ M PSK (PSK) for 2 hours. Genes were selected based on $\log_2FC \leq -1$ and $FDR \leq 0.05$ in comparison between Col-0 mock and PSK treatments (n=203). CPM values from all five biological replicates under mock and PSK-treated conditions are shown. **B.** Biological function gene ontology (GO) enrichment analysis of 203 identified genes, highlighting functional clusters significantly downregulated by PSK treatment. **C.** Molecular function gene ontology enrichment analysis for the same set of genes.

5.3.8 PSK-induced suppression of immunity is unaffected in *mkkk7* mutants

To corroborate the transcriptomic data, I investigated the impact of PSK on flg22-induced *WRKY22* and *FRK1* gene expression by RT-qPCR. I confirmed that the expression of both genes was significantly reduced in Col-0 and *mkkk7-2* seedlings co-treated with 1 μ M flg22 and 1 μ M PSK compared to those treated with 1 μ M flg22 alone, and this reduction was *PSKR* dependent (Figure 5.11 A).

To further validate whether PSK-induced suppression of immune responses operates independently of *MKKK7*, I examined the effects of PSK on PAMP/DAMP-induced ROS production. I showed previously that PSK alone does not trigger ROS production (Figure 3.1). Based on this observation and the transcriptomic data, I sought to investigate whether PSK suppresses ROS production in response to other stimuli. Given the quantitative nature and high-throughput potential of this assay, it provided a valuable approach to explore this aspect of PSK signalling further.

The results showed that PSK treatment significantly suppressed Pep1-induced ROS production in both Col-0 and *mkkk7-1* and *mkkk7-2* mutants, but not in *pskr1-3pskr2-1* (Figure 5.11 B-C). PSK also reduced flg22- and elf18-induced ROS production, albeit to a weaker extent, in a *MKKK7*-independent manner (Figure 5.11). These responses rely on the LRR-RKs PEPR1/2, FLS2, and EFR, which, like *PSKR1*, depend on *SERK* co-receptors (Shan et al. 2008; Schwessinger et al. 2011). I therefore tested whether PSK could suppress chitin-induced ROS production, which is *SERK*-independent (Shan et al. 2008), and observed a similar reduction (Figure 5.12). Interestingly, PSK did not affect *SCOOP12*-induced ROS production (Figure 5.12).

Together, these findings suggest that PSK-mediated suppression of elicitor-induced ROS, like PSK-induced transcriptional reprogramming, is independent of *MKKK7*.

5.3.9 *mkkk7* and *pskr1-3pskr2-1* mutants are sensitive to commensal *Pseudomonas fluorescens* WCS365

Song et al. (2023) reported the identification of *PSKR1* from a forward genetic screen based on altered levels of rhizosphere *Pseudomonas fluorescens*. They demonstrate that *pskr1-3* mutants are sensitive to *Pseudomonas fluorescens* WCS365 (*P.f.* WCS365) root inoculation, showing *P.f.* WCS365-induced growth inhibition, unlike Col-0.

I decided to investigate whether *pskr1-3pskr2-1* and *mkkk7* mutants exhibit similar sensitivities to commensal *P.f.* WCS365 root inoculation. We collaborated with Siyu Song and Cara Haney, who kindly provided the *P.f.* WCS365 strain used in their study. My findings revealed that *mkkk7* and *pskr1-3pskr2-1* mutants showed reduced root growth when inoculated with *P.f.* WCS365 compared to mock (MgSO₄) inoculation (Figure 5.13). In contrast, Col-0 showed no growth reduction when inoculated with *P.f.* WCS365 (Figure 5.13). It is important to consider here that *mkkk7* mutants exhibit a more pronounced reduction of root growth compared to *pskr1-3pskr2-1*, which could suggest that their inherently shorter roots and slower growth rates might contribute to *P.f.* WCS365 sensitivity. To fully assess this possibility, it would be important to include an unrelated mutant genotype with similar root growth reduction in future experiments.

Additionally, Song et al. (2023) reported that the activity of the *pPSKR1-GUS* fusion is enhanced upon *Pseudomonas* inoculation. To determine if *pMKKK7::NLS-3xmVenus* activity is similarly affected, I quantified nuclear fluorescence signals after *Pseudomonas* inoculation, but did not observe significant differences (Figure 5.14). This result aligns with publicly available transcriptomic data indicating that *MKKK7* (AT3G13530) expression is not influenced by abiotic and biotic stresses¹⁴.

In summary, this data shows that *mkkk7* and *pskr1-3pskr2-1* mutants are sensitive to commensal *P.f.* WCS365 root inoculations.

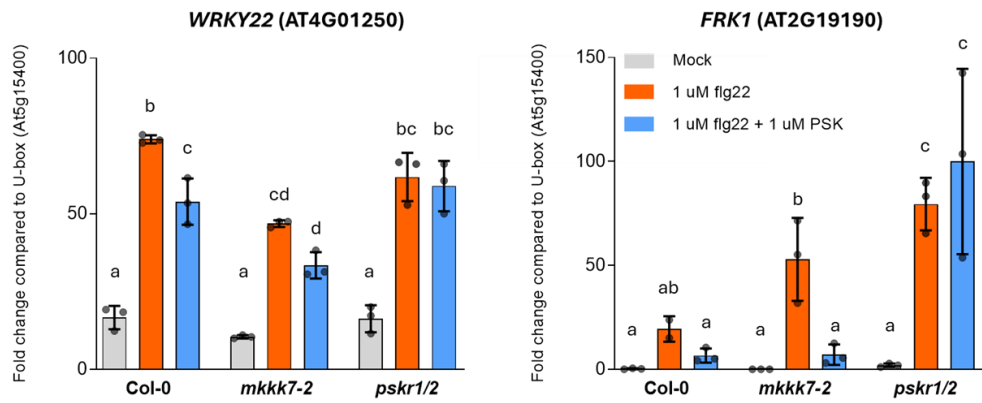
5.3.10 Genetic epistasis experiments reveal that *PSKR1*-induced growth is slightly reduced in the *mkkk7-2* mutant background

Finally, to investigate whether *MKKK7* acts genetically downstream of *PSKR1*, I conducted epistasis experiments using *mkkk7-2* mutants crossed with *35S::PSKR1-GFP* overexpression lines. I analysed the F3 progeny from three independent crosses for primary root growth. The results showed that *35S::PSKR1-GFP/mkkk7-2* crosses exhibited reduced root growth compared to *35S::PSKR1-GFP* lines (Figure 5.15). However, the roots were significantly longer than those of *mkkk7-2* mutants and, in two out of three lines, also significantly longer than those of Col-0 (Figure 5.15 A-C).

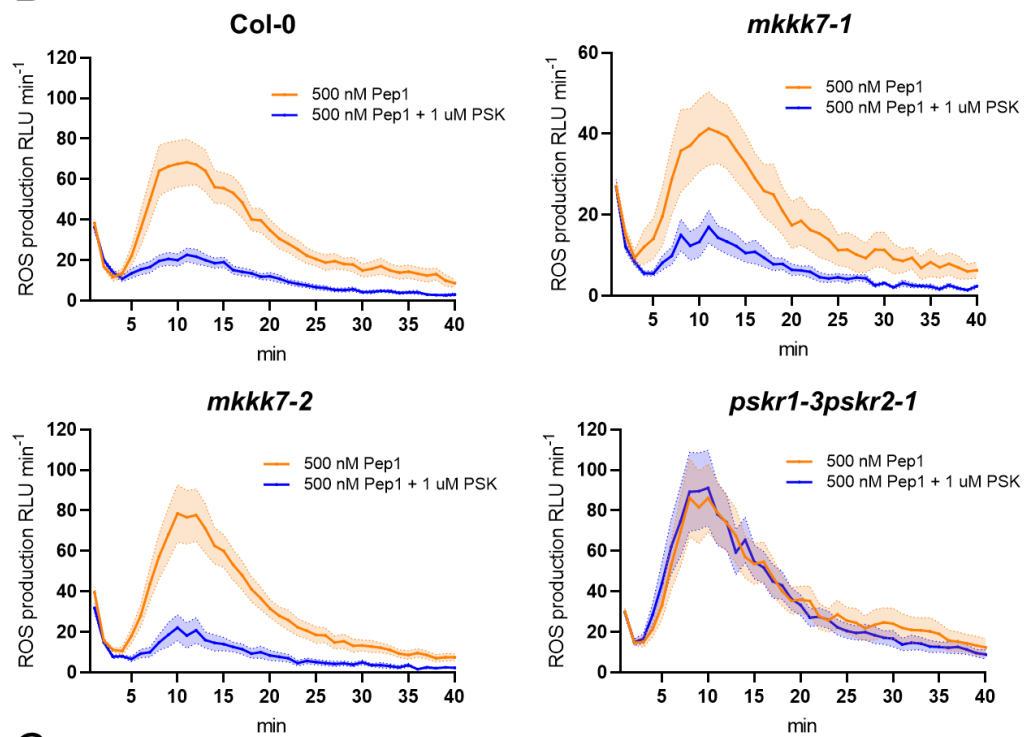
¹⁴ <https://bar.utoronto.ca/eplant/>

Interestingly, in two of the crosses (B5 and C9), I observed an increased signal in the western blot, indicating enhanced PSKR1-GFP protein abundance compared to *35S::PSKR1-GFP* (Figure 5.15 D). Together these findings suggest that *MKKK7* may function downstream of PSKR1, but redundant signalling components or pathways likely compensate for the loss of *mkkk7*.

A



B



C

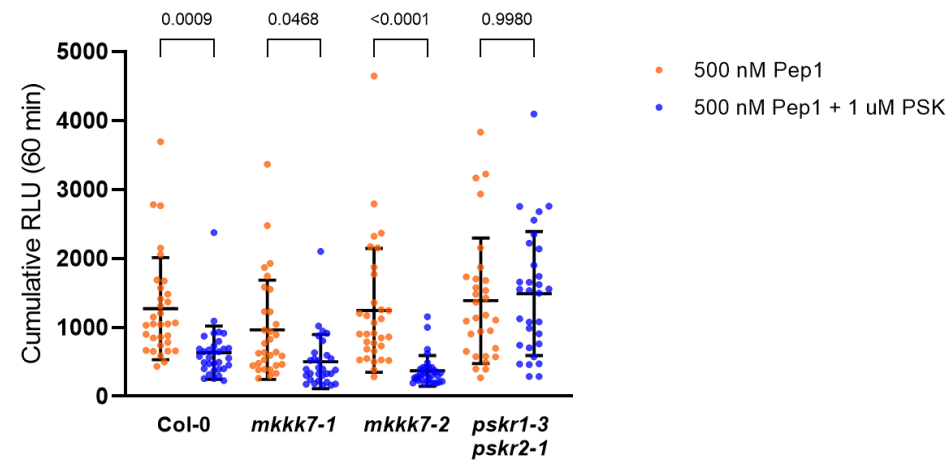


Figure 5.11. PSK-induced suppression of ROS production and defence gene expression is unaltered in *mkkk7* mutants. **A.** *WRKY22* and *FRK1* defence gene expression in response to 1 μ M flg22 and 1 μ M flg22 + 1 μ M PSK for 3 h. Expression was normalised to *U-BOX* (At5g15400). Lowercase letters indicate significance groups based on one-way ANOVA followed by Tukey's post-hoc test. This experiment was repeated twice with similar results. **B.** ROS production in leaf disks collected from 4-week-old *Arabidopsis* plants induced by 500 nM Pep1 or 500 nM Pep1 + 1 μ M PSK over 40 minutes (n =16). Line represents mean $\pm$ SEM. **C.** Cumulative ROS production over 40 min. Line represents mean; error bars represent SD (n =32). p-value based on one-way ANOVA followed by Tukey's post-hoc test.

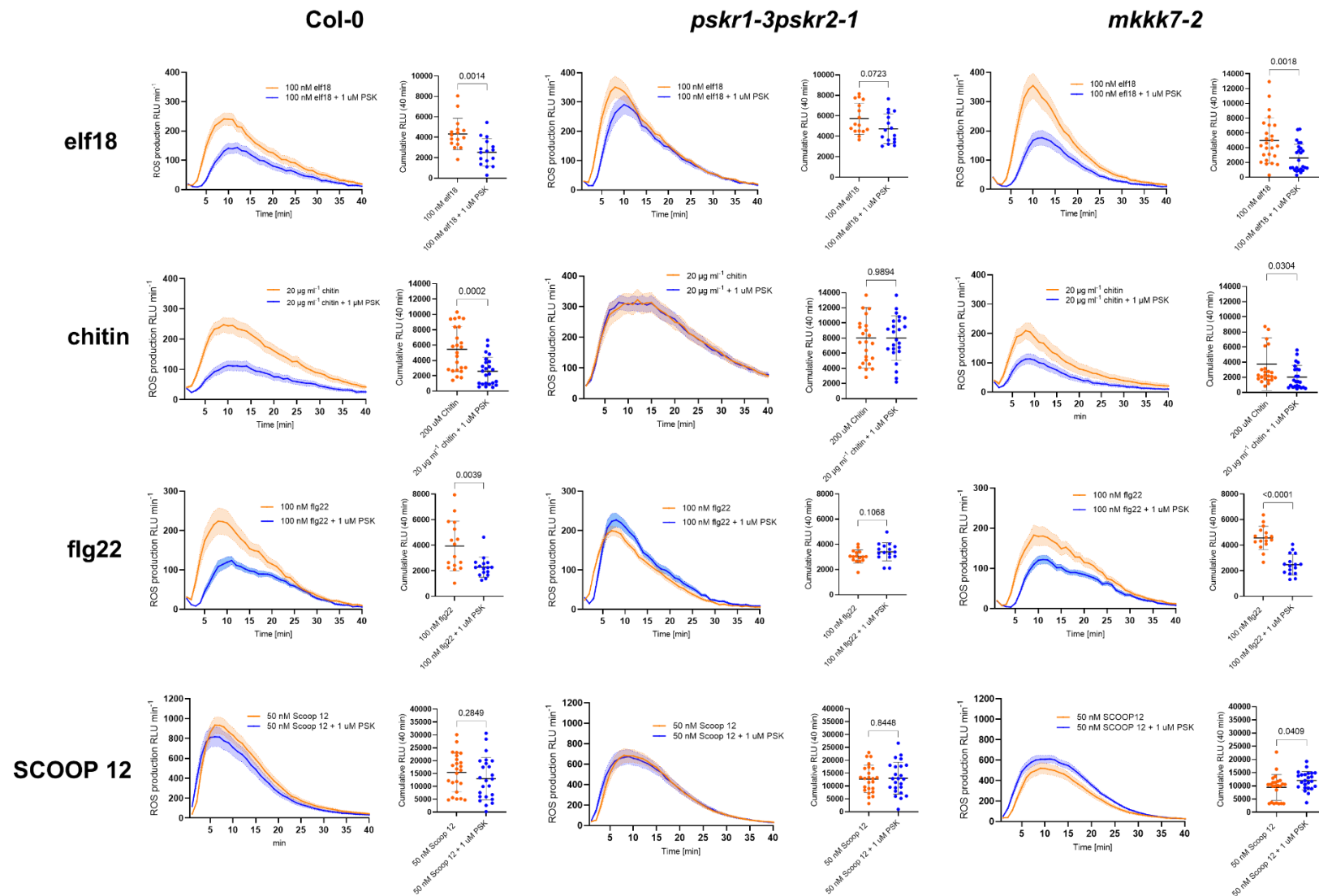


Figure 5.12. PSK suppresses PAMP-induced ROS production but does not affect SCOOP12-induced ROS production. ROS production in leaf disks collected from 4-week-old *Arabidopsis* plants induced by 100 nM elf18, 200 µg/ml chitin, 100 nM flg22 and 50 nM SCOOP12 over 40 min. In the ROS curves the line represents the mean $\pm$ SEM. In cumulative ROS production plot in relative light units (RLU) over 40 min, the line represents the mean; error bars represent SD (n =16-24). p-value based on one-way ANOVA followed by Tukey's post-hoc test. Each experiment was repeated at least three times with similar results and representative ROS production curves and cumulative RLU data are shown.

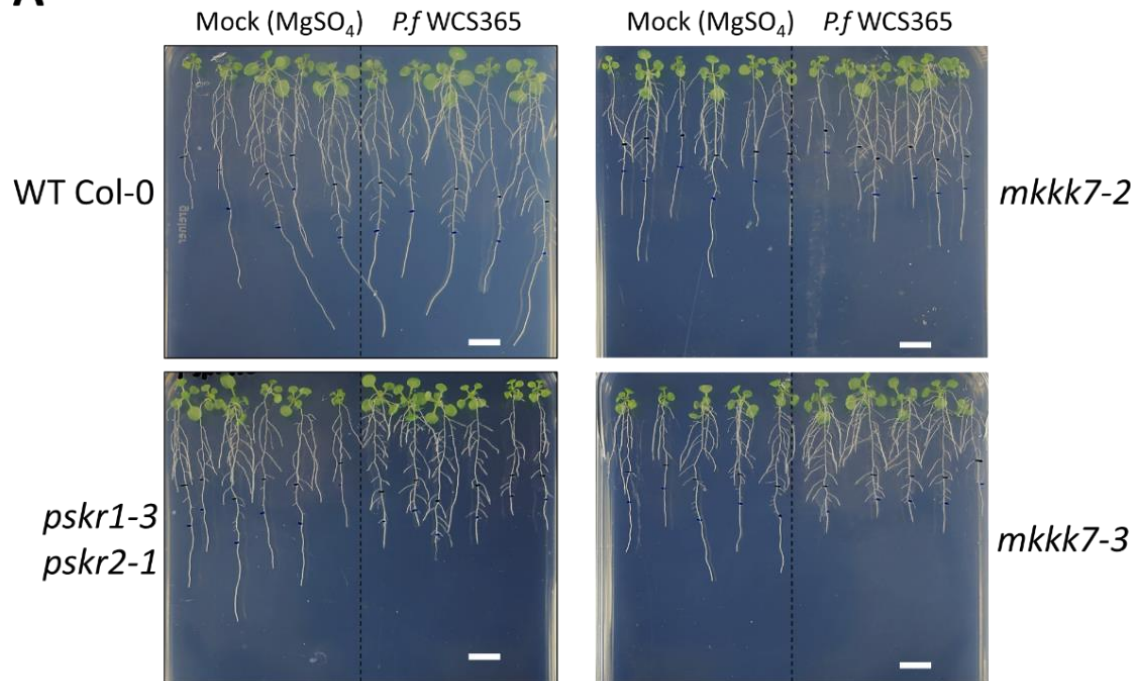
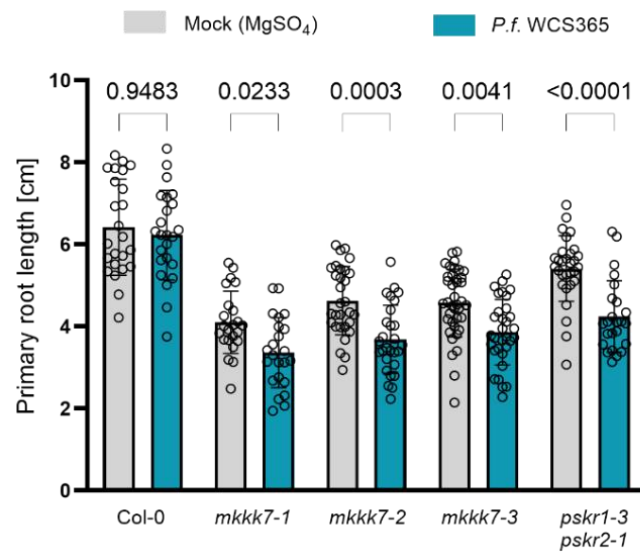
A**B**

Figure 5.13. *mkkk7* mutants are sensitive to *Pseudomonas fluorescens* WCS365 treatment. **A.** Seedlings germinated and grown on $\frac{1}{2}$ MS + 1% sucrose for 7 days. Then transferred to $\frac{1}{2}$ MS without sucrose and grown for an additional 2 days before inoculation with MgSO_4 (mock) and *P.f.* WCS365. Pictures were taken 5 days post inoculation (dpi). The seedlings in the picture are 14 days old. **B.** Quantification of primary root length 5 days after *P.fluorescens* WCS365 inoculation. The experiment was repeated three times on a total of n= 24 (Col-0), n=23 (*mkkk7-1*), n=30/28 (*mkkk7-2*), n=32/30 (*mkkk7-3*), n=30/24 (*pskr1-3pskr2-1*) seedlings and all data is represented in the graph. p-values derived from one-way ANOVA followed by Tukey's post-hoc test are indicated above.

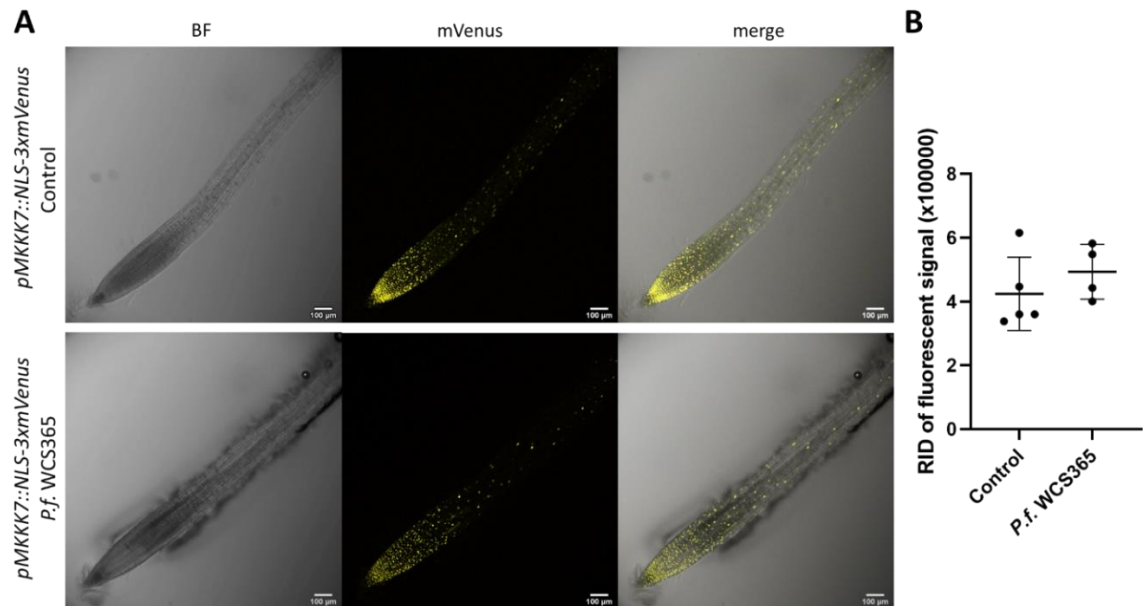


Figure 5.14. *pMKKK7::NLS-3xmVenus* signal shows no increase upon *P.f. WCS365* inoculation. Images of 14-day-old *Arabidopsis thaliana* primary roots stably expressing *pMKKK7::NLS-3xmVenus*. Panels on the left show bright field images (BF). Panels in the centre show the NLS-3xmVenus signal. The panels on the right are merged BF and mVenus images. The white bar represents 100 μ m. **A.** 10x magnification maximum projection Z-stack showing the root meristem and elongation zone. Representative images of the control root inoculated with water at the top and inoculated with *P.f. WCS365* at the bottom. **B.** RID, raw intensity density. RID of fluorescent signals is the sum of RID of each nuclear signal in the imaged area. n=5. This experiment was repeated twice with similar results.

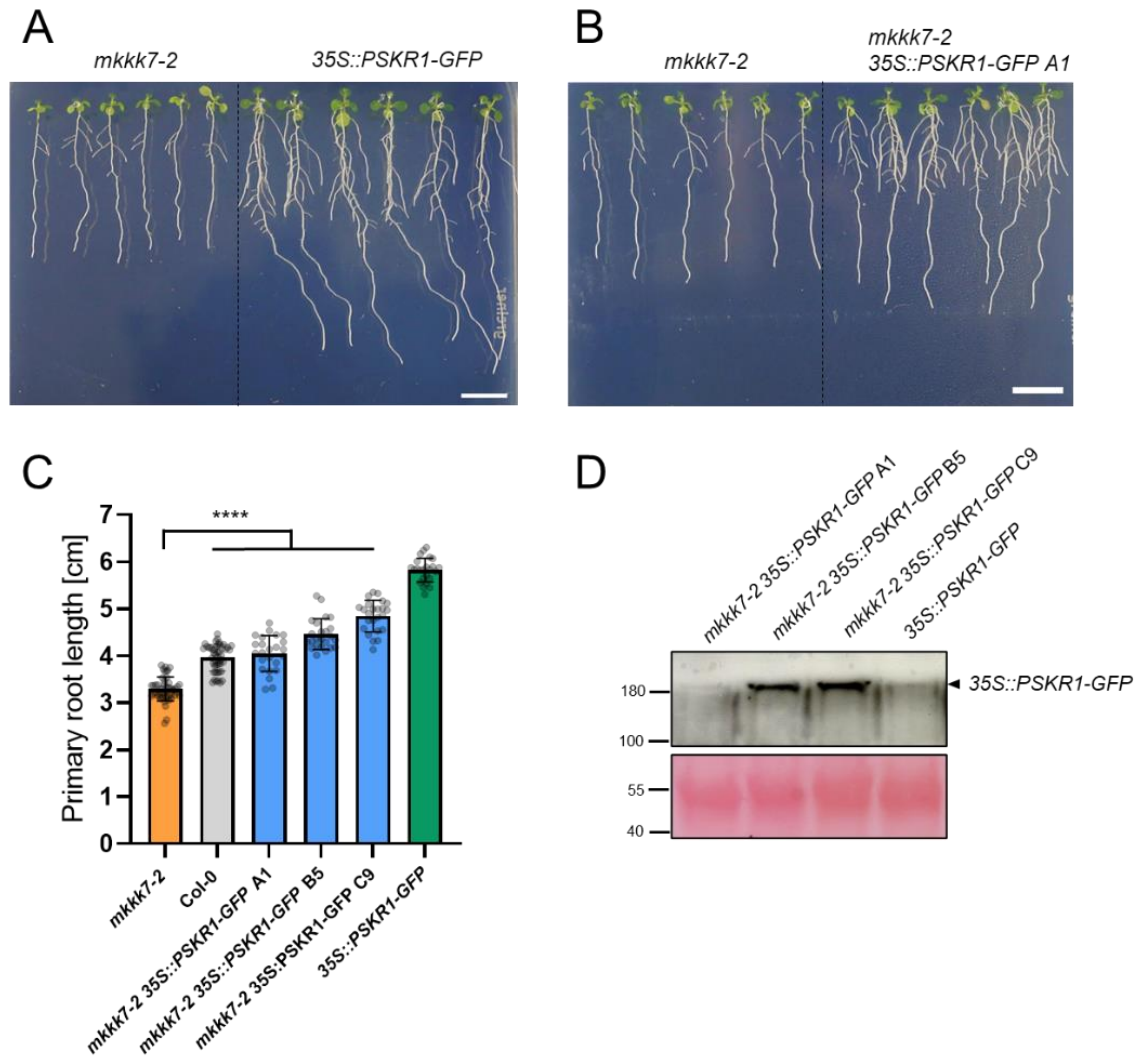


Figure 5.15. Genetic epistasis experiments show that PSKR1-induced growth is not strictly dependent on MKKK7. **A-B.** Representative images of *mkkk7-2*, *35S::PSKR1-GFP* and *mkkk7-2/35S::PSKR1-GFP* seedlings. **C.** Quantification of primary root growth of *mkkk7-2*, *Col-0*, *35S::PSKR1-GFP* and three independent *mkkk7-2/35S::PSKR1-GFP* crosses (A1, B5, C9) in F3 generation. One-way ANOVA followed by Tukey's multiple comparisons test **** p-value < 0.0001. **D.** α-GFP western blot indicating PSKR1-GFP expression. Ponceau staining to assess equal sample loading.

5.4 Discussion

5.4.1 Potential role of MKKK7 within the PSK-signalling pathway?

The experiments I conducted here demonstrate that both *mkkk7* and *pskr1-3pskr2-1* mutants are compromised in several PSK-induced responses, including protoplast expansion, gene expression, and sensitivity to *P.f* WCS365 root inoculation. RNA sequencing revealed that both mutants show reduced expression of nutrient-related genes, particularly those involved in phosphate metabolism, while iron uptake-related genes were upregulated. In contrast, PSK-induced responses such as growth and suppression of immunity were unaffected in *mkkk7* mutants. This, together with the genetic epistasis experiments, which showed that PSKR1-induced root growth was reduced in the *mkkk7-2* mutant background, could suggest that MKKK7 regulates a distinct branch of the PSK signalling pathway downstream of PSKR1.

A particularly interesting question is, why *mkkk7* mutants exhibit sensitivity to *P.f* WCS365 root inoculation? In *pskr1-3pskr2-1* mutants, this sensitivity is linked to overall enhanced immune responses in the absence of PSK-signalling (Song et al. 2023), a pattern that can also be seen in my RNA sequencing data, but it appears to be MKKK7 independent. One possible explanation for this is that the overall more pronounced reduction of root growth in *mkkk7* mutants contributes to their sensitivity to bacterial inoculation. This could be tested in the future by including additional mutants with a similar reduction in root growth such as *mekk1* (Shao et al. 2020) or *rgfr1,2,3* mutants (Shinohara et al. 2016). Alternatively, but not mutually exclusive to the root growth hypothesis, the gene expression differences observed in *mkkk7-3* may indirectly affect the plant's immune responses.

Both *mkkk7-3* and *pskr1-3pskr2-1* mutants show enhanced expression of *IRONMAN* peptide encoding genes *IMA1*, *IMA2* and *IMA3* (Figure 5.9 B, D. Table S6). *IMA* genes are usually upregulated in response to Fe deficiency and facilitate the accumulation of iron (Fe), Manganese (Mn) and Zinc (Zn) (Grillet et al. 2018). Microbial patterns such as *flg22* suppress *IMA1*, *IMA2* and *IMA3* expression and promote *IMA1* degradation, thereby negatively affecting the plant's iron deficiency response (Cao et al. 2024). Overexpression of *IMA1* not only enhances iron uptake but also increases resistance to *Pseudomonas syringae* pv. *tomato* DC3000 infection, suggesting that these peptides are part of a nutritional immune response that affects both iron uptake and immune signalling (Cao et al. 2024; Grillet et al. 2018).

On the other hand, both *mkkk7-3* and *pskr1-3pskr2-1* mutants reveal downregulation of genes related to the perception of extracellular stimuli and starvation, particularly phosphate starvation. Among the downregulated genes are phosphate transporters *PHT1;9* and *PHT1;8* which facilitate inorganic phosphate (Pi) uptake under Pi starvation conditions (Remy et al. 2012) as well as *PAP14* and *PAP24* which contribute to phosphate mobilisation (Table S5). Phosphate starvation response (PSR) represses plant immune signalling under P limiting conditions, thereby contributing to the assembly of a normal root microbiome (Castrillo et al. 2017). PSR is mainly facilitated by the master transcriptional regulator PHR1 and its paralogue PHL1 (Castrillo et al. 2017). PHR1 upregulates the expression of RALF peptides, which in turn suppress PRR immune receptor complex formation through Feronia (Tang et al. 2022). The suppression of phosphate starvation in *mkkk7* and *pskr1-3pskr2-1* mutants could lead to enhanced immune responses that result in sensitivity to commensal *P.f.* WCS365.

Together these findings suggest that MKKK7 might regulate a distinct signalling branch of the PSK pathway involved in the integration of the plant's nutritional status and immunity. Future growth and infection experiments under varying Fe and P conditions could help to better understand the links between micronutrient availability, immunity and plant microbiome assembly. If the perception of low P conditions and subsequent activation of PSR is impaired in the mutants, one would expect that *mkkk7-3* and *pskr1-3pskr2-1* mutants are more resistant to pathogen infection under P-limiting conditions because PSR which suppresses immunity is not activated. When phosphate is abundant, PSR might be equally reduced in Col-0 and *mkkk7-3* and *pskr1-3pskr2-1* mutants and therefore plants might show more similar infection phenotypes.

5.4.2 Redundancy between MKKK6 and MKKK7 could obscure clear link to PSK-signalling

Some of the experiments I discuss here support a connection between PSK signalling through PSKR1 and MKKK7, but I was unable to unequivocally prove their direct interaction. Previous studies have shown that MKKK7 associates with the LRR-RK FLS2 in Co-IPs (Mithoe et al. 2016). It would therefore be interesting to test whether MKKK7 associates with PSKR1 in Co-IPs using transient expression in *N. benthamiana*, in the presence and absence of PSK.

Potential redundancy between *MKKK6* and *MKKK7* may be the most likely explanation that currently obscures clear evidence of their involvement in PSK signalling and potentially other signalling pathways. Based on the severe phenotype of the putative *mkkk6mkkk7* double mutant shown in Chapter 4, which does not resemble *pskr* mutants,

it is possible that the integrity of a downstream signalling cascade involving MKKK6 and MKKK7 may be guarded by NLRs. Since the *pskr1-3pskr2-1* double mutant shows a mild phenotype, it suggests that both MKKK6 and MKKK7 could be involved in additional signalling processes including but not limited to PSK signalling.

Alternatively, MKKK6 and MKKK7 may function in an entirely different signalling process, and the observed effects of PSK-induced expansion and gene expression are pleiotropic outcomes rather than direct links to PSK signalling. These underlying processes could be brassinosteroid or auxin signalling, both of which are required for efficient PSK signalling (Hartmann et al. 2013; Matsubayashi et al. 1999) and both of which have been linked to MKKK7 (Kuhn et al. 2024; Kim, Park, et al. 2023). The addition of PSK to cell cultures only triggers proliferation when auxin and cytokinin are continuously supplied (Matsubayashi et al. 1999). If brassinosteroid biosynthesis is suppressed, PSK-induced root growth is abolished (Hartmann et al. 2013). Together this raises the possibility that compromised hormonal signalling in *mkkk7* mutants may result in partially altered PSK responses. The *mkkk6mkkk7* double mutant will be vital to test these ideas. If the double mutant still responds to PSK treatment, it suggests that these kinases act in a different signalling pathway. If the double mutant shows no response to PSK, it is likely that MKKK6 and MKKK7 function redundantly in PSK signalling and potentially other signalling pathways.

5.4.3 Phytosulfokine-induced immune suppression independent of MKKK7

Transcriptional reprogramming

I show in this chapter that PSK treatment has strong effects on immunity-related gene expression, a property of PSK not previously shown at this timepoint. Published microarray data from Arabidopsis seedlings treated with 1 μ M PSK for 5 days shows upregulation of genes associated with cell wall organisation and cell wall remodelling (Kaufmann, Stührwohltd, and Sauter 2021). The prolonged peptide treatment likely triggered distinct transcriptional reprogramming and morphological changes, which likely explain the differences from the short-term transcriptome data presented here. I decided to conduct a much shorter time-course experiment because long-term PSK-induced root growth is normal in *mkkk7* mutants. I was able to confirm by RT-qPCR that PSK treatment triggers the upregulation of genes identified in the previous microarray at 2 h, but I did not observe significant differences in my RNA sequencing data. Expression analysis by RT-qPCR is likely more sensitive and detects minor differences in gene expression compared to RNA sequencing.

The PSK-induced downregulation of gene expression is reminiscent of patterns observed for brassinosteroid (BR) signalling. In the BR pathway, master transcription factors BRZ-INSENSITIVE-LONG 1 (BIL1)/BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1) act as dual transcriptional activators and repressors for thousands of genes (Li et al. 2018). DNA Affinity Purification Sequencing (DAP-seq) experiments show that BZR1 recognises a preferred 10 bp DNA sequence motif that is enriched in BR downregulated genes (Nosaki et al. 2022). A similar motif was found for BES1 through chromatin immunoprecipitation coupled with Arabidopsis tiling arrays (ChIP-chip), suggesting that BES1 recognises promoters of BR downregulated genes similarly to BZR1 (Liu et al. 2023). Upon DNA binding, BZR1 recruits co-repressor TOPLESS (TPL) through its EAR domain, which results in transcriptional repression of BR responsive genes (Oh et al. 2014; Nosaki et al. 2022). It will be interesting to investigate *cis*-regulatory elements in promoter regions of PSK-downregulated genes and determine whether similar transcriptional repression mechanisms might exist.

Short-term suppression of ROS

The suppression of immunity-related gene expression cannot temporally explain the rapid suppression of PAMP/DAMP-induced ROS production by PSK. Therefore, a distinct mechanism is likely involved in the observed ROS phenotype. PSK-signalling was recently shown to induce ROS production by phosphorylating NADPH oxidase RBOHD in response to aluminium ions (Ding et al. 2024). However, the results here indicate that PSK co-treatment suppresses ROS production induced by PAMPs/DAMPs but not by the phyto cytokine SCOOP12.

Tyrosine-sulfated ROOT GROWTH FACTOR (RGF) peptides, have an acidic pH optimum, and the sulfotyrosine group in its protonated form is essential in facilitating the interaction between RGF and RGFR (Liu, Song, et al. 2022). Under alkaline conditions, however, deprotonation of RGF significantly weakens this binding. In contrast, Pep1 binds more effectively to its receptor PEPR1 under alkaline conditions, where critical Asp and Glu residues are deprotonated, while acid-induced protonation disrupts the Pep1-PEPR1^{LRR} interaction (Liu, Song, et al. 2022). PTI is known to trigger apoplastic alkalization (Boller and Felix 2009), which therefore inhibits RGF-mediated growth responses while enhancing Pep1-induced immune responses (Liu, Song, et al. 2022). Rhizosphere-associated *Pseudomonas* secrete organic acids to acidify the extracellular pH to suppress immunity and promote plant colonisation, which may suggest that pH regulation is a common mechanism to counteract plant immune responses (Yu et al. 2019). It is conceivable that PSK triggers changes in ion fluxes across the membrane

that result in decreases in apoplastic pH which dampen plant defence responses such as ROS production (Figure 5.9, 5.10) and regulate the association with commensal microbes (Figure 5.11). ROS production via RBOHD suppresses type II secretion system (T2SS) genes in commensal *Xanthomonas* L148 (Entila et al. 2024). *rbohD* mutants, on the other hand, die when inoculated with *Xanthomonas* L148, suggesting that ROS production is needed to control commensal *Xanthomonas* in the microbiome (Entila et al. 2024; Pfeilmeier et al. 2021). PSK-induced acidification could also sensitize the root to enhance peptide signalling through other growth-promoting factors such as RGFs. Interestingly, in both *mkkk7* and *pskr1-3pskr2-1* mutants, *RGF9* gene expression is suppressed (Table S5).

The Arabidopsis genome encodes for 11 plasma membrane (PM) H⁺-ATPases (AHAs) that control many aspects of plant physiology and plant microbe interactions (Elmore and Coaker 2011). They facilitate proton fluxes across the membrane and maintain electrochemical gradients required for ion transport processes and regulation of intracellular and extracellular pH (Sondergaard, Schulz, and Palmgren 2004). PM H⁺-ATPases are dynamically regulated through phosphorylation in their N- and C-terminal domains. PTI triggers extracellular alkalisation likely through the inhibition of PM H⁺-ATPases and activation of anion channels. Both AHA1 and AHA2 are rapidly dephosphorylated after flg22 treatment at the penultimate threonine residue, required for their activation by binding of 14-3-3 proteins (Benschop et al. 2007; Nühse et al. 2007; Fuglsang et al. 1999). PSK signalling has been linked to the activation of AHA1 and AHA2 (Ladwig et al. 2015) and it is therefore possible that PSK-induced proton efflux results in extracellular acidification which in turn causes reduced PAMP/DAMP-induced ROS production. Indeed, expression of a constitutively active allele of AHA1 in Arabidopsis results in reduced flg22-induced ROS production, likely due to less efficient membrane depolarisation and perturbed ion fluxes (Keinath et al. 2010). Whether PSK-induced suppression of PAMP/DAMP-induced ROS production can be directly linked to the opposing regulation of AHAs (phosphorylation vs dephosphorylation) or whether the suppression is linked to overall changes in the apoplastic pH which might reduce overall PAMP/DAMP perception by PRRs remains to be investigated. The first idea could be tested by using the engineered rapamycin-inducible (RiD) receptor system, which allows bypassing the effects of apoplastic pH changes on elicitor-PRR binding by inducing intracellular complex formation between the kinase domains of RKs and co-receptors in a rapamycin-dependent manner (Kim et al. 2021). For instance, one could induce PEPR1/BAK1 complex formation with rapamycin, which would trigger ROS production. If additional PSK treatment suppresses ROS production in this system, it would suggest

that the opposing regulation of AHAs or other membrane signalling components is the underlying cause for the ROS suppression phenotype. The *aha1aha2* double mutant is lethal (Arango et al. 2003), but it would be interesting to test whether PSK-induced ROS suppression is quantitatively reduced in single *aha1* and *aha2* mutants, or whether constitutively active AHA1 in the *ost2-1D* and *ost2-2D* mutants could be tested for PAMP-induced ROS production (Merlot et al. 2007). If PSK is found to trigger the phosphorylation of AHAs, especially at the penultimate threonine residue, phosphomimic and phosphodead amino acid substitutions could be introduced to examine whether they lead to suppression or enhancement of PAMP-induced ROS, respectively.

The second idea, that overall pH changes are responsible for the ROS suppression phenotype, could be tested by conducting ROS assays under pH-buffered conditions. If PSK-induced ROS suppression is abolished when the pH is stabilised, it would suggest that apoplastic pH changes are the underlying cause of the observed ROS phenotype. Perception of SCOOP12 by MIK2 might not be affected by changes in the extracellular ionic environment, however little is known about the effect of pH on MIK2-SCOOP binding.

Functional characterization of MKKK7 and its role in plant immune signalling

6.1 Summary

Many signalling pathways utilize shared intermediate components, but different stimuli nevertheless trigger distinct cellular responses appropriate for the nature of the stimulus. The mechanisms underlying signalling specificity and regulation of shared signalling components are not well understood. *Arabidopsis thaliana* contains 80 MAP KINASE KINASE KINASES that are likely involved in the integration and transduction of various extracellular signals. Among these, MKKK7 has been identified as highly phosphorylated in response to multiple stimuli. Here I explore the complex phosphorylation pattern of MKKK7 and discuss potential functional implications. I further show that MKKK7 is likely an active kinase capable of triggering cell death in transient *N. benthamiana* expression assays. I present *Pseudomonas syringae* pv *tomato* DC3000 infection assay data indicating that the resistance previously reported for the *mkkk7-1* T-DNA mutant is not consistently observed in *mkkk7-2* and *mkkk7-3* mutants.

6.2 Introduction

The *Arabidopsis thaliana* MAP KINASE KINASE KINASE 7 (MKKK7) (At3g13530) was identified through a proteomics screen for differentially phosphorylated proteins after flg22 treatment (Benschop et al. 2007). MKKK7 is a serine/threonine (S/T) kinase that belongs to the MEKK subfamily of MAPKKs (Ichimura et al. 2002). It is predominantly plasma membrane-associated but also shows cytosolic localisation and is characterized by a comparatively long C-terminal region that contains predicted ARMADILLO (ARM)/HEAT repeat domains (Ichimura et al. 2002; Chaiwongsar et al. 2006). *Arabidopsis* T-DNA mutants of *mkkk7* (*mkkk7-1*) show increased MAPK activation, defence gene expression and resistance to *Pseudomonas syringae* pv. DC3000 infection (Mithoe et al. 2016). MKKK7 associates with FLS2 and is phosphorylated on S337, S452, S503, S776, S854 after flg22 treatment (Mithoe et al. 2016; Benschop et al. 2007). Phosphomimic mutations of S452 and S854 to aspartic acid (D) reduce flg22-induced defence gene expression, positioning MKKK7 as a modulator of FLS2 signalling to attenuate immune responses (Mithoe et al. 2016).

Contradictory to *MKKK7* in Arabidopsis, *Solanum lycopersicum* orthologue *SIMAPKKKε*, *Solanum tuberosum* *StMKKKε* and *Nicotiana benthamiana* *NbMAPKKKε* act as positive regulators of immunity (Melech-Bonfil and Sessa 2010; King et al. 2014). Silencing *SIMAPKKKε* compromises *Xanthomonas campestris* and *Pseudomonas syringae* resistance in tomato (Melech-Bonfil and Sessa 2010), while silencing *NbMKKKε* compromises resistance to *Phytophthora infestans* in *N.benthamiana* (King et al. 2014). Silencing *NbMKKKε* reduces the hypersensitive response (HR) and cell death caused by the co-expression of effector gene/R gene pairs. HR activated by the expression of *Avr4/Cf4* and *AvrPto/Pto* is reduced when *NbMKKKε* is silenced, indicating dependency on *NbMKKKε* (King 2013, PhD thesis)(Melech-Bonfil and Sessa 2010). Expression of the secreted oomycete elicitor *INF1*, RXLR effector *AVR3a^{KI}* and effector *CRN8* trigger cell death independently of *NbMKKKε* (King 2013, PhD thesis). Genetic epistasis and overexpression experiments show that *SIMAPKKKε* activates the MEK2-SIPK/WIPK kinase cascade, leading to hypersensitive response (HR) and cell death (Melech-Bonfil and Sessa 2010; King et al. 2014). Overexpression of *SIMMKKKε* induces cell death, which can be suppressed by the co-expression of *P. infestans* effector *PexRD2*, an effector that binds to the kinase domain of *SIMMKKKε* (King et al. 2014). Intriguingly, *PexRD2* also suppresses cell death induced by overexpressing *MKKK7* orthologues from Potato and Arabidopsis (King 2013, PhD thesis).

In addition to flg22-induced phosphorylation, *MKKK7* is differentially phosphorylated following brassinosteroid (BR) and auxin treatment (Kim, Park, et al. 2023; Kuhn et al. 2024). A thorough study of the GLYCOGEN SYNTHASE KINASE 3 (GSK3)-like kinase BRASSINOSTEROID-INSENSITIVE2 (BIN2) by TurboID proximity labelling and phosphoproteomics revealed that *MKKK7* is both in proximity of BIN2 and undergoes dephosphorylation after BIKININ treatment (Kim, Park, et al. 2023). BIKININ is a specific inhibitor for BIN2 and other GSK3-like kinases and inhibition of BIN2 is the primary mode of BR signalling activation (De Rybel et al. 2009). The data therefore suggests that *MKKK7* is phosphorylated by BIN2 within the BR signalling network.

Rapid auxin-induced phosphorylation changes are mediated by B4 RAF kinases, which belong to the MAPKKK family (Kuhn et al. 2024). After auxin treatment, *MKKK7* is phosphorylated on three residues, S452, S533 and S854, but this phosphorylation is abolished in a B4 RAF septuple mutant where the whole B4 RAF subgroup is knocked out (Kuhn et al. 2024). NTEGRIN-LINKED KINASE1 (ILK1), a subgroup 1 C-RAF, contributes to plant immune signalling and abiotic stress tolerance by modulating K⁺ homeostasis via the putative (H⁺)/K⁺ symporter HAK5 (Brauer et al. 2016; Popescu et al.

2017). A kinase-client (KiC) assay, using a synthetic peptide library, shows that ILK1 phosphorylates the peptide NEATRLASISGGLDGQAPR, corresponding to MKKK7 S776 in vitro (Brauer et al. 2022). These results suggest that MKKK7 is phosphorylated by diverse RAF kinases.

In summary, MKKK7 undergoes extensive phosphorylation in response to various stimuli and plays critical roles in immune signalling in Arabidopsis and Solanaceae species. Beyond the growth and developmental phenotypes discussed in Chapter 4 and 5, these results raise the intriguing possibility that MKKK7 integrates defence mechanisms with other cellular processes through specific phosphorylation events. This makes MKKK7 an interesting target for studying the cross-regulation of receptor signalling via downstream components. Here I address the function of MKKK7 in plant immunity by comparing publicly available phosphorylation data with data generated by myself and the Proteomics Team. I further aim to study PTI outputs and conduct infection assays in *mkkk7* mutants and begin to develop a mechanistic model for MKKK7 function based on transient expression assays in *N. benthamiana* and AP-MS data.

6.3 Results

6.3.1 YFP-MKKK7 AP-MS data shows MKKK6 as the only unique interactor

One objective of my project was to determine the molecular function of MKKK7. To achieve this, I analysed the YFP-MKKK7 AP-MS data (Chapter 3, Chapter 4) to identify specific, unique interactors that may help to further elucidate MKKK7's function. Surprisingly, only one protein, MKKK6, was specifically present in 5 out of 7 YFP-MKKK7 samples but absent in all other 24 pull-downs with other tagged proteins (Figure 6.1). MKKK6 shares 90% amino acid sequence identity with MKKK7, making its accurate identification challenging. We successfully identified exclusive spectra for MKKK6 in MKKK7 pull-downs (Figure 6.1). The peptides identified and mapped to MKKK6 were (K)QMEGETGHSQDGGDLFR(L) and (K)KTNGDLEFIMAEFAEVSGR(G)/(K)TNGDLEFIMAEFAEVSGR(G) which differ from the corresponding MKKK7 peptides (K)QMEGETGHSNDGGDLFR(L) and (K)KNNGDLDFLMAEFAEVSGR(G) by one and two amino acid substitutions respectively.

6.3.2 MKKK7 is a phosphoprotein

MKKK7 is a highly phosphorylated protein, with 44 phosphorylation sites identified from publicly available datasets (Plant PTM Viewer 2.0¹⁵). It undergoes phosphorylation in response to various signals, including flg22 (Mithoe et al. 2016; Benschop et al. 2007),

¹⁵ <https://www.psb.ugent.be/webtools/ptm-viewer/>

brassinosteroids (Kim, Park, et al. 2023), and auxin (Kuhn et al. 2024), with several phosphorylation sites shared across these signalling pathways (Figure 6.2 B). All identified phosphorylated residues are located in structurally less defined regions specifically between the kinase domain and the first ARM repeat, or between the first and second ARM repeats (Figure 6.2 A). This is consistent with the general observation that most phosphorylation sites are located in intrinsically disordered regions (Iakoucheva et al. 2004; Collins et al. 2008).

The functional significance of many of these phosphorylation sites remains unclear, as does the overall mechanism of MKKK7 activation. To further explore MKKK7 phosphorylation, pre-doc Clinton Asare conducted YFP-MKKK7 pull-downs from transgenic Arabidopsis lines after a time-course of flg22 treatment. The purified YFP-MKKK7 was excised from SDS-PAGE gels and subjected to mass spectrometry analysis (Figure 6.2 C). MS1 peak intensities were analysed in Skyline software (MacLean et al. 2010) for several phosphorylated residues by Paul Derbyshire.

Among the phosphorylation sites, T450 showed the most significant increase in phosphorylation following flg22 treatment (Figure 6.2 D). This residue is located near S452, a site previously reported to be phosphorylated (Mithoe et al. 2016). Additionally, S533, S776, and S788 exhibited increased phosphorylation after flg22 treatment (Figure 6.2 E). Other sites such as T883 show a decrease in phosphorylation after flg22 treatment, indicating that overall protein abundance changes are likely not the underlying cause for increased detection of phosphorylation (Figure 6.2 F). Collectively, these findings suggest that MKKK7 is phosphorylated in response to flg22 on multiple residues, with residues such as S452 and S533 playing potential regulatory roles across different signalling pathways.

6.3.3 RLCK mediated phosphorylation of MKKK7

Receptor-like cytoplasmic kinases (RLCKs) of subfamily VII link PRR signalling to the activation of the MAP kinase cascade (Bi et al. 2018b; Rao et al. 2018). Given that MKKK7 plays a role in plant immunity, I explored the possibility that RLCKs might be involved in the activation or modulation of its activity.

BIK1, one of the best-characterized RLCKs, typically phosphorylates substrates containing the SxxL motif (DeFalco et al. 2022; Thor et al. 2020; Kadota et al. 2014). In collaboration with the Cyril Zipfel lab, we utilized their BIK1 phosphosite prediction algorithm, which is based on in vitro peptide array data and considers five residues upstream and downstream of the core SxxL motif (unpublished). The analysis identified four potential phosphorylation sites in MKKK7, with prediction scores ranging from 1.3 to

3.5 (Figure 6.3 A-B). For comparison, the BIK1 substrate RBOHD, a well-known target, possesses a highest-scoring phosphorylation site of 3.4 (Kadota et al. 2014). All four predicted sites in MKKK7 exhibit the typical SxxL motif, are located between ARM domain 1 and ARM domain 2 and are highly conserved across plant species (Figure 6.3 A-B).

In earlier experiments we identified flg22-induced phosphorylation at S788 (Figure 6.2), but we were unable to detect phosphorylation at additional predicted BIK1 sites. I therefore tested whether BIK1 could phosphorylate MKKK7 in vitro. I was unable to purify full-length MKKK7, but I successfully fused and purified the variable region between the two ARM repeats, which contains three of the four BIK1 sites, to maltose-binding protein (MBP) (Figure 6.3 D). When incubated with BIK1 and a kinase-dead BIK1 variant (BIK1kd), followed by mass spectrometry analysis, I observed specific phosphorylation of MKKK7 at S922 in the peptide HYS[+80]GQLDYVK, confirming that BIK1, but not BIK1kd can phosphorylate MKKK7 at this site in vitro (Figure 6.3 E). Parallel reaction monitoring (PRM) data from Arabidopsis Col-0 and *bik1pbl1* mutants, generated by Paul Derbyshire, indicated that phosphorylation at S922 significantly increased after 2.5 minutes of flg22 treatment in Col-0, but not in the *bik1pbl1* mutant. This suggests that BIK1/PBL1 phosphorylate MKKK7 in vivo in response to flg22. The reason why this phosphorylation site was not detected in YFP-MKKK7 pull-downs remains unclear but is likely due to technical differences in the analysis, such as data-dependent acquisition (DDA) versus PRM. Another possibility is that the expression of MKKK7 under the 35S promoter, used in the pull-down assays, may lead to differences that are not observed with the endogenous expression of MKKK7.

6.3.4 PTI signalling outputs in *mkkk7* mutants

Previous research suggests that MKKK7 attenuates flg22-triggered immunity, because the *mkkk7-1* T-DNA mutant displayed enhanced MAP kinase activation and increased defence gene expression. To further investigate the role of MKKK7 in plant immunity, particularly in PTI signalling, I analysed hallmark PTI responses in the newly generated *mkkk7-2* and *mkkk7-3* mutant alleles and compared them to the original *mkkk7-1* mutant. I also included the *pskr1-3pskr2-1* receptor mutant which exhibits enhanced PAMP-induced defence gene expression and *Pseudomonas syringae* resistance (Igarashi, Tsuda, and Katagiri 2012).

I did not observe clear differences in flg22-induced MAPK cascade activation between Col-0 and *mkkk7* mutants in 5 independent repeats (Figure 6.4 A). ROS production, another hallmark of PTI, was similarly unaffected in *mkkk7* and *pskr1-3pskr2-1* mutants

when compared to Col-0 (Figure 6.4 B). Lastly, I assessed flg22-induced *FRK1* and *WRKY29* defence gene expression. Both *mkkk7-1* and *pskr1-3pskr2-1 mutants* exhibited significantly enhanced flg22-induced *FRK1* expression. The *mkkk7-1* mutant also showed elevated basal *WRKY29* expression, whereas no significant differences were observed in the *mkkk7-2* and *mkkk7-3* mutants. These findings align with previous observations in the *mkkk7-1* background but suggest that the *mkkk7-1* T-DNA insertion line may exhibit slightly different immune signalling characteristics compared to *mkkk7-2* and *mkkk7-3*.

6.3.5 MKKK7 is likely an active kinase and triggers cell-death in *Nicotiana benthamiana*

Transient expression of *SlMKKKε* and *StMKKKε* in *N. benthamiana* induces cell death in a kinase activity-dependent manner (King et al. 2014). To investigate whether MKKK7 functions similarly, I transiently expressed MKKK7 alongside a kinase-dead mutant (MKKK7_K49R), which I generated using AF3 structure prediction and existing literature (King et al. 2014). Full-length MKKK7 triggers cell death, but kinase-dead MKKK7_K49R did not. Expressing only the kinase domain MKKK7₁₋₃₀₀ and MKKK7₁₋₃₀₀K49R as a control caused even more pronounced cell death, dependent on MKKK7's kinase activity (Figure 6.5 A).

Next, together with MSc student Clinton Asare, we examined whether mutations in BIK1 phosphorylation sites to phospho-dead alanine (A) or phospho-mimic aspartic acid (D) would affect cell death. The results showed that all mutant alleles triggered similar cell death responses, suggesting that these specific phosphorylation sites either do not modulate MKKK7's activity or introduce more subtle changes in activity or protein stability that were not detectable in this assay (Figure 6.5 B). Mutants of the additional phosphorylation sites shown in Figure 6.2 have not yet been tested in this assay. Additionally, expressing only the ARM repeat domains (MKKK7₅₆₅₋₁₃₆₈) did not elicit HR or cell death (Figure 6.5 C). Western Blots show that all proteins were expressed (Figure 6.5 D). These results strongly suggest that MKKK7 is an active kinase that triggers cell death in *N. benthamiana*, and its kinase activity is essential for this function.

6.3.6 Infection assays of *mkkk7* mutants

Pseudomonas syringae

Lastly, extensive infection assays with *Pseudomonas syringae* pv *tomato* DC3000 (Pto DC3000) and *Pto* DC300 $\Delta\Delta$ AvrPto/AvrPtoB lacking AvrPto and AvrPtoB were carried out by Clinton Asare together with me. We showed that *mkkk7-1* T-DNA mutants were mildly resistant against *Pto* DC3000, which is in line with previously published data

(Figure 6.6 B) (Mithoe et al. 2016). *mkkk7-2* and *mkkk7-3* on the other hand showed no resistance to *Pto* DC3000 infection (Figure 6.6 B, C). PTI defects can be more sensitively detected with weakly virulent bacterial strains such as *Pto* DC300 Δ AvrPto Δ AvrPtoB lacking AvrPto and AvrPtoB, two effectors involved in PTI suppression (Roux et al. 2011). All three *mkkk7* mutants were slightly more susceptible to *Pto* DC300 Δ AvrPto Δ AvrPtoB compared to Col-0 while *pskr1-3pskr2-1* mutants are consistently more resistant to infection (Figure 6.6). Together these data suggest that *mkkk7* mutants show no clear immune phenotype, albeit *mkkk7-1* appears to be slightly more resistant in some assays.

Verticillium dahliae

To evaluate how the mutants respond to a soil-borne pathogen, we collaborated with Bart Thomma's laboratory, which conducted *Verticillium dahliae* (Vd) infection assays for us. Vd is a hemibiotrophic fungal pathogen and the causal agent of *Verticillium* wilt (Song et al. 2020). It was recently shown to secrete the small cysteine-containing effector VdSCP8 that suppresses PTI by stimulating PSK signalling through PSKR1-BAK1 complex formation (Hua et al. 2023). PSKR receptor mutants (*pskr1-3pskr2-1*) were shown to be resistant to Vd infection (Hua et al. 2023). The results we received showed that *mkkk7* and *pskr1-3pskr2-1* mutants did not show a visually different phenotype compared to WT Col-0 after Vd infection and therefore no further quantification of fungal biomass, typically done to assess the severity of infection, was done (Figure 6.7). This suggests that under the conditions tested, neither *mkkk7* nor *pskr1-3pskr2-1* mutants are visibly resistant to Vd infection.

Plasmodiophora brassicae

To test how the mutants respond to an obligate biotrophic protist pathogen that infects the root, Edel Perez Lopez's group conducted infection assays with the clubroot pathogen *Plasmodiophora brassicae*. Clubroot infections were categorized into eight severity levels, which were used to calculate an overall disease index (DI). The severity of infection was reduced in *mkkk7* and *pskr1-3pskr2-1* mutants, with several plants showing low infection levels (levels 0-3) compared to WT Col-0 (Figure 6.8). None of the mutants exhibited the highest infection level (level 8), characterised by heavy gall formation in both primary and lateral roots. It was evident that gall formation in lateral roots was absent in *mkkk7* and *pskr1-3pskr2-1* mutants but not in WT Col-0 (Figure 6.8). As a result, the calculated disease index (DI) indicated that all mutants were less susceptible to the clubroot pathogen, with a DI of around 52-53% compared to 86% in Col-0. I also observed that all mutants exhibited less complex root systems and overall smaller shoots compared to Col-0 even in the uninfected samples (Figure 6.8).

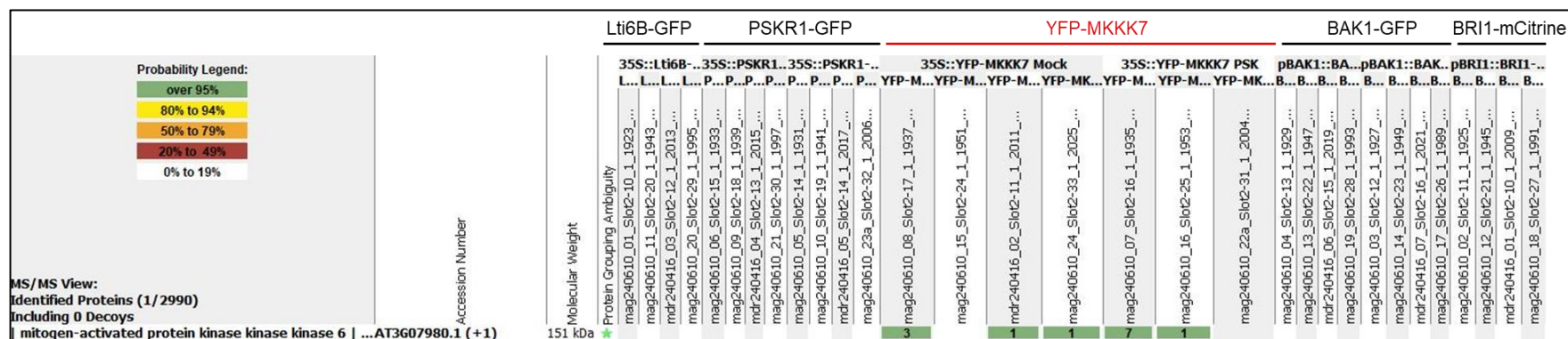


Figure 6.1. Exclusive spectral counts of MKKK6 identified across AP-MS samples. The analysis was performed in Scaffold with the following filter settings: 95% protein threshold, min # peptides 1, peptide threshold 1 % FDR, display options: Exclusive Spectrum Count. Samples are indicated above from left to right 35S::Lti6B-GFP (4 replicates), 35S::PSKR1-GFP (4 replicates mock, 4 replicates PSK), 35S::YFP-MKKK7 (4 replicates mock, 3 replicates PSK), pBAK1::BAK1-GFP (4 replicates mock, 4 replicates PSK), pBRI1::BRI1-mCitrine (4 replicates mock).

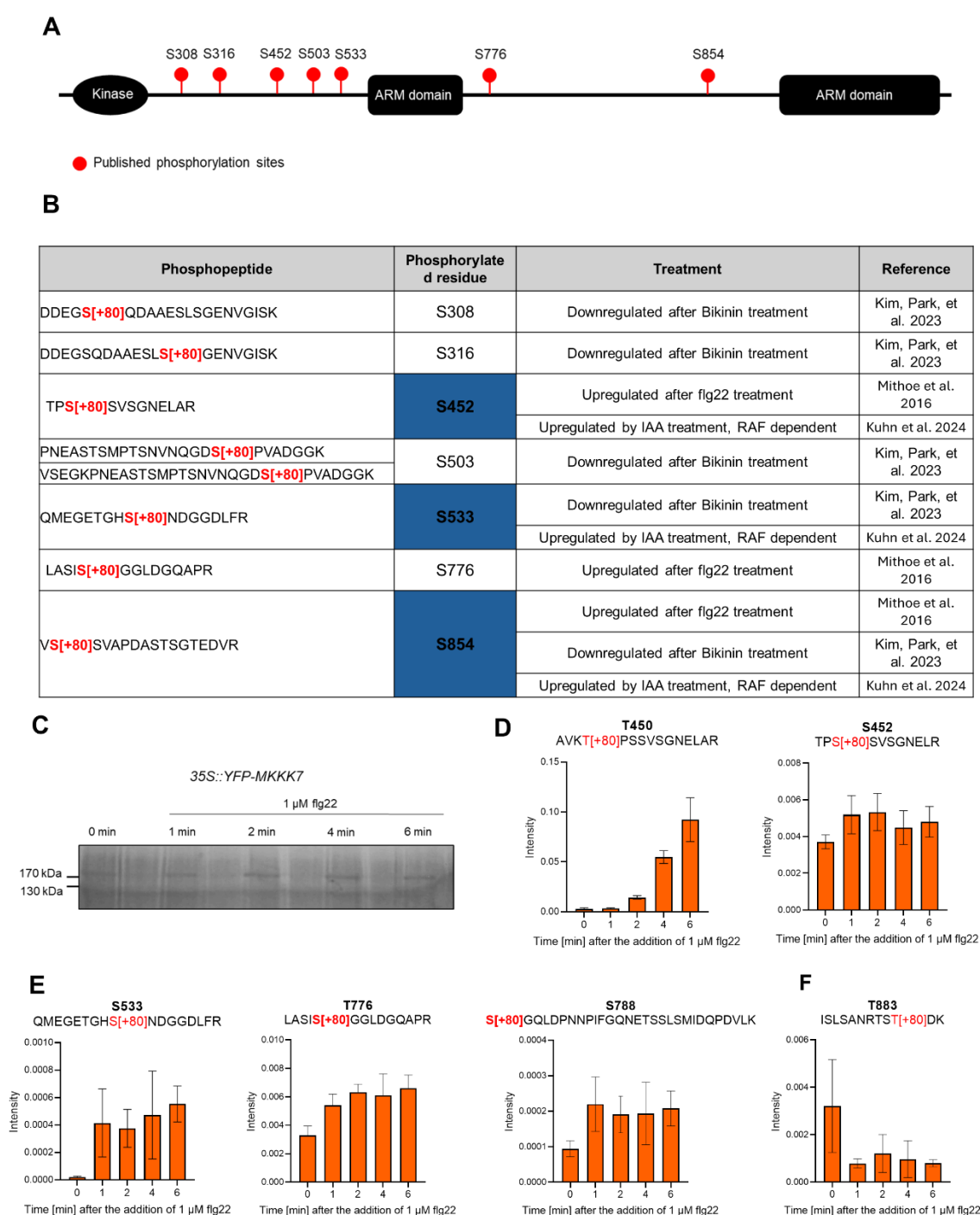


Figure 6.2. MKKK7 is phosphorylated on multiple residues. **A.** Schematic of the protein domain structure of MKKK7 with published phosphorylation sites indicated in red. **B.** Detailed information about the phosphorylated residues shown in A, including phosphopeptides, treatments and literature references. **C.** Representative image of SDS-PAGE showing YFP-MKMKK7 protein pulled-down from 14-day old *Arabidopsis thaliana* seedlings. **D-F.** MS1 quantification data of different selected phosphorylated residues found in MKKK7 over time-course flg22-treatment.



● Predicted RLCK (BIK1) phosphorylation sites

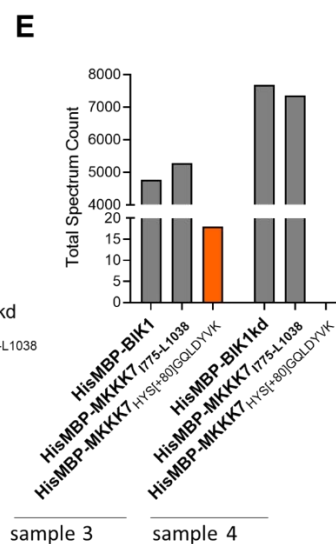
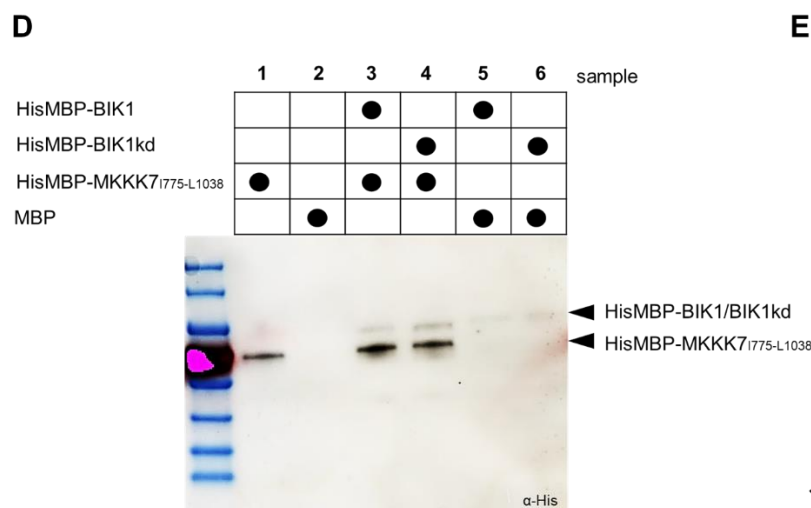
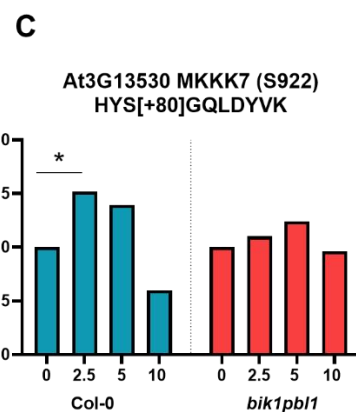
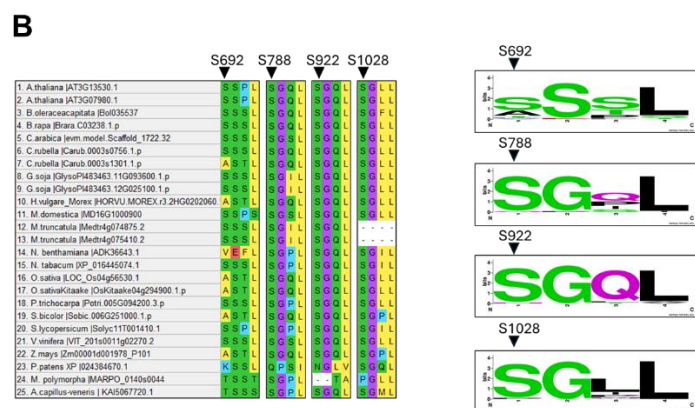


Figure 6.3. MKKK7 contains 4 conserved S/TxxL motifs and is phosphorylated by BIK on S922 in vivo and in vitro. **A.** Schematic of the protein domain structure of MKKK7 with predicted BIK1 phosphorylation sites in yellow. **B.** Multiple sequence alignment of MKKK7 residues from different plant species shows conserved BIK1 sites. **C.** Parallel reaction monitoring data (PRM) generated by Paul Derbyshire shows MKKK7 S922 phosphorylation. **D.** Western Blot incubated with α -His antibody shows presence of recombinant BIK1 and MKKK7 in in-vitro kinase assay. **E.** Total spectrum counts of recombinant MKKK7_{I775-L1038} incubated with either BIK1 or a kinase-dead version of BIK1 (BIK1kd). Samples correspond to western blot in panel D. The orange bar highlights phosphorylated S922 spectra detected in the presence of BIK1 (sample 3) but not BIK1kd (sample 4). This experiment was repeated twice and in both cases the phosphorylated peptide HYS[+80]GQLDYVK was detected in the BIK1 samples, but not in the BIK1kd samples.

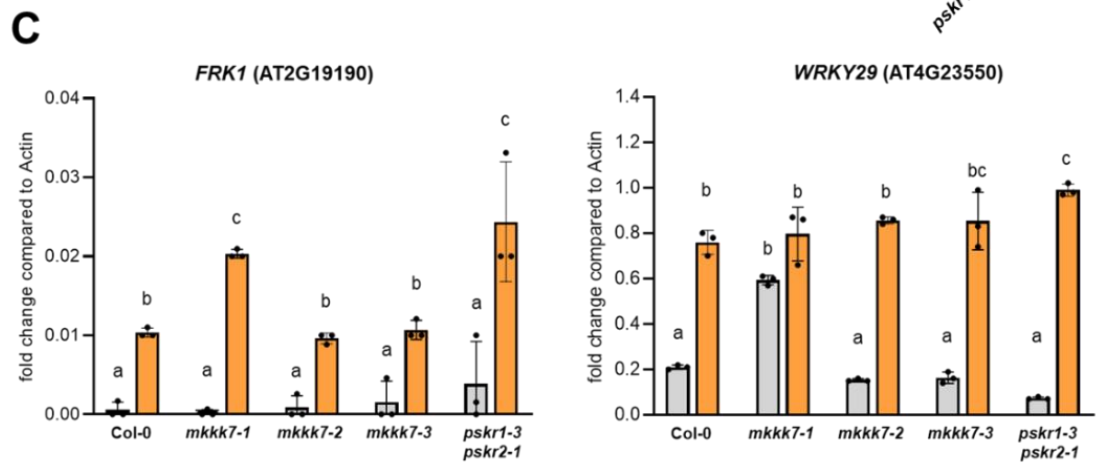
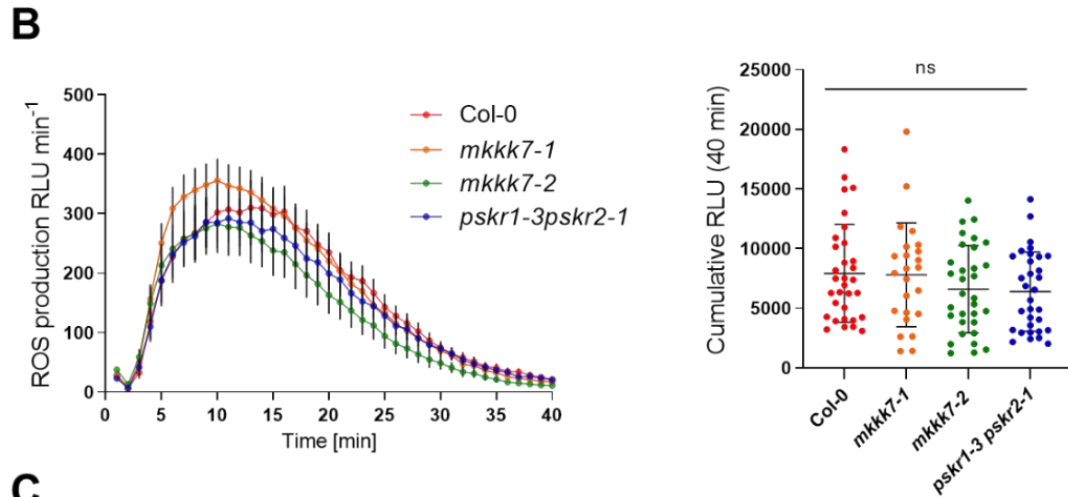
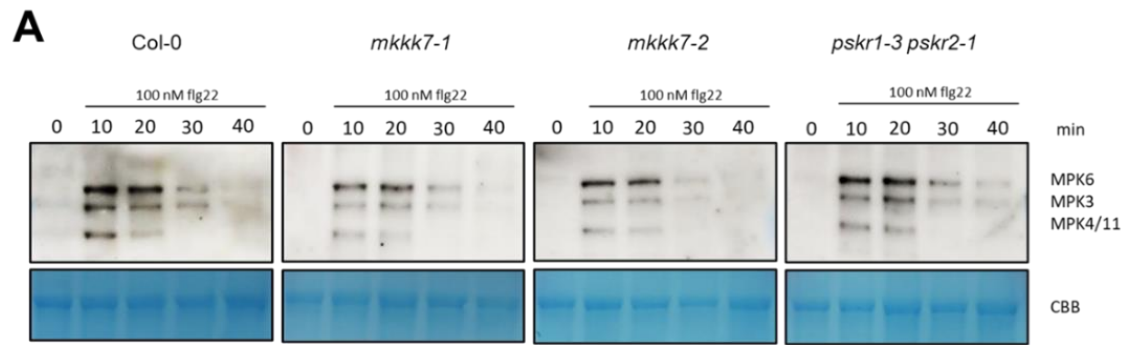
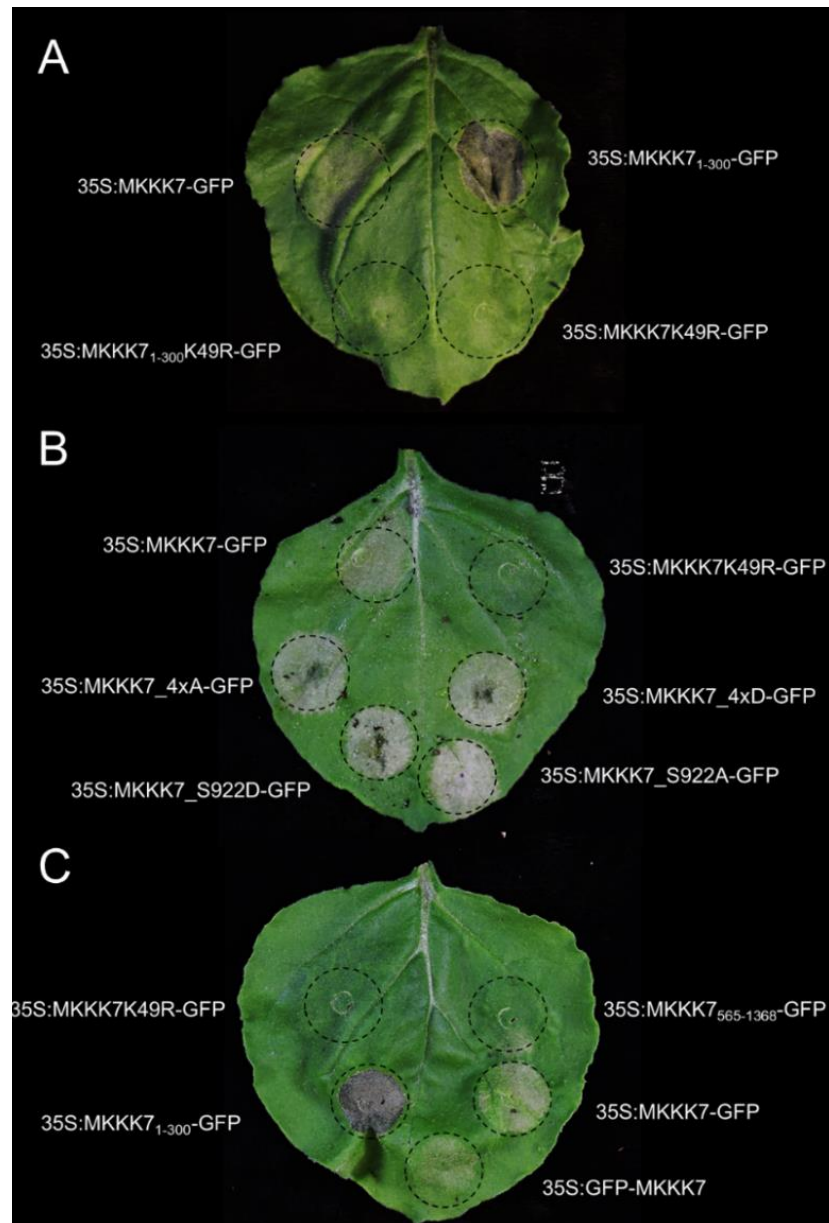


Figure 6.4. PTI signalling outputs MAPK activation, ROS production and defense gene expression in Col-0, *mkkk7-1*, *mkkk7-2*, *mkkk7-3*, *pskr1-3pskr2-1*. **A.** Western blot using α -p42/p44-ERK recognising phosphorylated MAP kinases in seedlings treated with 100 nM flg22 for 10 min, 20 min, 30 min and 40 min. 0 min (mock) was treated with water. This experiment was repeated 5 times and performed on seedlings and soil-grown plants. **B.** ROS production in leaf disks collected from 4-week-old *Arabidopsis* plants induced by 100 nM flg22 application ($n \geq 8$). Points represent mean; error bars represent SEM. Cumulative ROS production over 40 min shown on the right. Line represents mean; error bars represent SD ($n \geq 24$). ns= not significant based on one-way ANOVA followed by Tukey's post-hoc test. **C.** RT-qPCR analysis of *FRK1* and *WRKY29* gene expression in leaves of 4-week-old *Arabidopsis* plants infiltrated with 100 nM flg22 for 1 h. Graphs show Δ CT values of three technical replicates normalised to Actin. Error bars represent SD. Lowercase letters indicate significance groups based on one-way ANOVA followed by Tukey's post-hoc test.



D

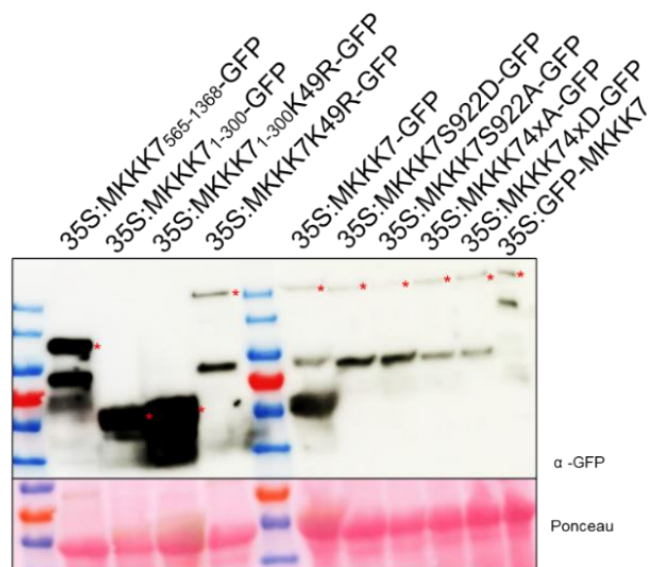


Figure 6.5. MKKK7 but not MKKK7K49R (kinase dead) triggers cell death in *Nicotiana benthamiana*. **A-C.** Cell death assay in *N. benthamiana* after transient expression of MKKK7 constructs. A kinase-dead version of MKKK7 (MKKK7K49R) was included as negative control. Expression of all constructs was driven by the 35S promoter, and all constructs were tagged with GFP. Lowercase numbers indicate the amino acid position of truncated MKKK7 protein domains. This experiment was repeated three times with similar results. **D.** Protein expression levels of all transiently expressed constructs in *N. benthamiana*. Red asterisks indicate the expected protein size, ponceau staining was used to confirm equal sample loading.

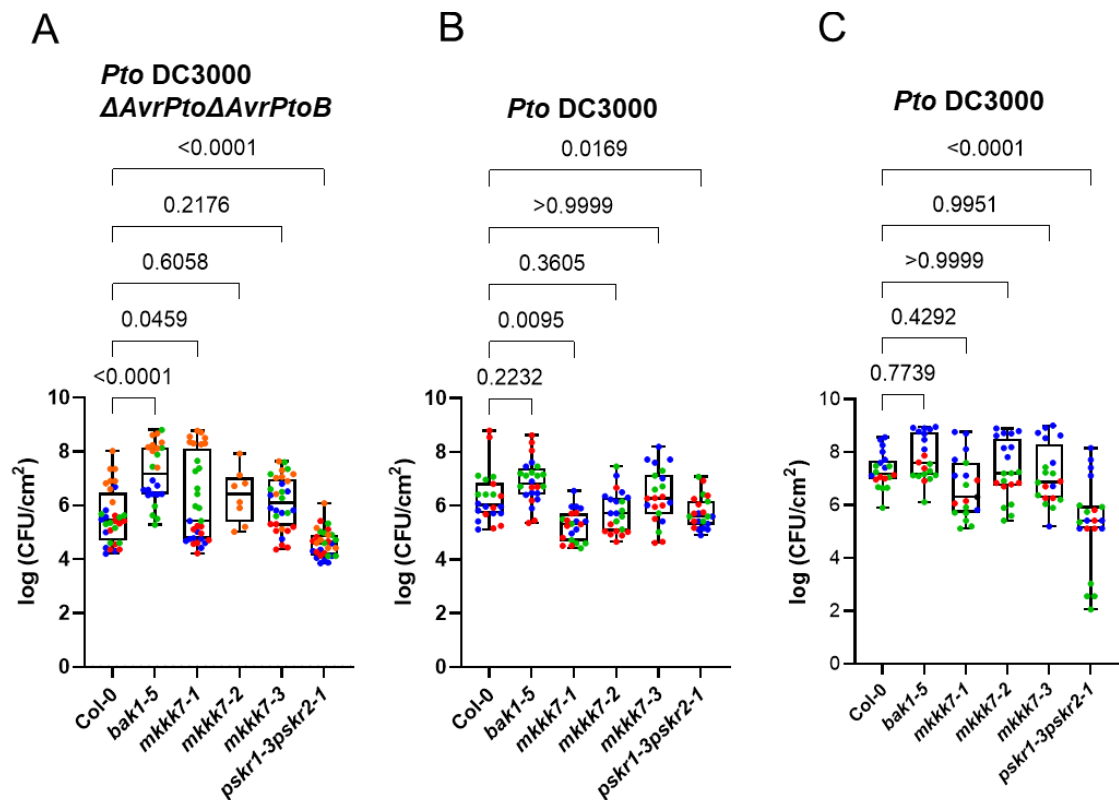


Figure 6.6. *Pseudomonas syringae* pv *tomato* DC3000 (*Pto* DC3000) infection assays **A.** Four-week-old plants (Col-0, *bak1-5*, *mkkk7-1*, *mkkk7-2*, *mkkk7-3*, *pskr1-3pskr2-1*) were spray inoculated with *Pto* DC3000 Δ AvrPto Δ AvrPtoB (OD₆₀₀=0.2). Repeated 4 times with n=8 plants per genotype. **B.** Four-week-old plants (Col-0, *bak1-5*, *mkkk7-1*, *mkkk7-2*, *mkkk7-3*, *pskr1-3pskr2-1*) were spray inoculated with *Pto* DC3000 (OD₆₀₀=0.2). Repeated 3 times with n=8 plants per genotype. **C.** Four-week-old plants (Col-0, *bak1-5*, *mkkk7-1*, *mkkk7-2*, *mkkk7-3*, *pskr1-3pskr2-1*) were syringe inoculated with *Pto* DC3000 (OD₆₀₀=0.0002). Repeated 3 times with n=4-8 plants per genotype. Bacterial counts were performed at 3 days after inoculation. CFU, colony-forming units. p-values derived from one-way ANOVA followed by Tukey's post-hoc test are indicated above. Colours indicate replicates.

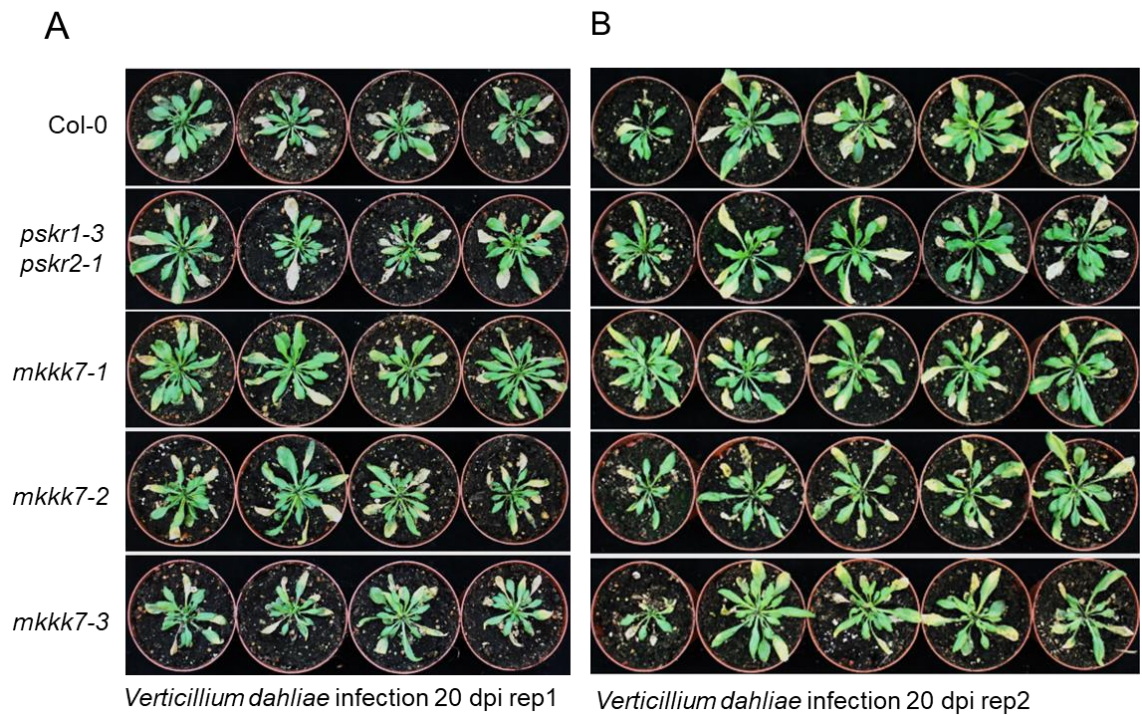


Figure 6.7. *Verticillium dahliae* (Vd) infection assays. A-B. Pictures of two replicates of *Verticillium dahliae* (Vd) infection assays of Col-0, *mkkk7-1*, *mkkk7-2*, *mkkk7-3*, *pskr1-3pskr2-1* plants 20 days post inoculation (dpi). Vd infection assays were performed by Hui Htian in collaboration with Bart Thomma.



Figure 6.8. *Plasmodiophora brassicae* (clubroot pathogen) infection assay on WT Col-0, *mkkk7-1*, *mkkk7-3* and *pskr1-3pskr2-1* (*pskr1/2*) mutants. This data was generated, analysed and provided by Edel Pérez-López Lab. The disease index (DI) was calculated based on the severity of disease symptoms classified in 8 levels with level 0 showing no signs of clubroot infection and level 8 showing severe infection with gall formation in both primary and lateral roots. Statistics: non-parametric Mann-Whitney U test between each mutant and Col-0, with Col-0 vs. *mkkk7-1* ($W = 743$, p -value = 0.001258051), Col-0 vs. *mkkk7-3* ($W = 637.5$, p -value = 0.0009828956), Col-0 vs. *pskr1/2* ($W = 745.5$, p -value = 0.002481599)

6.4 Discussion

In this Chapter, I have shown that MKKK7 is phosphorylated on multiple residues after flg22 treatment, corroborating previous findings (Mithoe et al. 2016; Benschop et al. 2007). A comparative analysis of these phosphorylation sites with those identified in other studies revealed that S533, S452 and S854 are commonly phosphorylated in response to flg22, brassinosteroids and auxin (Kuhn et al. 2024; Kim, Park, et al. 2023). In addition, I showed that MKKK7 harbours predicted high-scoring BIK1 phosphorylation sites and is phosphorylated by BIK1 in vitro and in vivo on S922. Infection assays revealed that *mkkk7* mutant alleles exhibit differences in *Pseudomonas syringae* resistance but show no visible resistance to *Verticillium dahliae*. On the other hand, *mkkk7* mutants appear to be resistant to *Plasmodiophora brassicae* root infection. Together, these findings underscore the potential significance of MKKK7 phosphorylation in plant immunity, but the functional relevance of these sites and MKKK7's precise function in plant immunity remain unclear. In this discussion, I will highlight the possible functions of the phosphorylation sites in MKKK7 as well as how to address its function in plant immunity.

6.4.1 MKKK7 phosphorylation by multiple upstream kinases

The *Arabidopsis thaliana* genome encodes a diverse array of 80 MAPKKKs (Ichimura et al. 2002). Kinases of the MEKK subfamily of MAPKKKs are generally considered to activate the canonical MAPK cascade by phosphorylating downstream MKKs (Champion, Picaud, and Henry 2004), but their activation mechanisms are poorly understood. RLCK VII-4 members phosphorylate MKKK5 at S599, which is required for its activation and subsequent immune activation (Bi et al. 2018a). MKKK5 activates MPK6 and is further phosphorylated by MPK6 at S682 and S692 in a positive feedback loop that amplifies the immune response (Bi et al. 2018a). A similar mechanism was found for MEKK1, which is phosphorylated by RLCK VII subfamily 4 members and MPK4 on S603 (Bi et al. 2018a).

MKKK7 contains high-scoring BIK1 phosphorylation sites, and BIK1 phosphorylates MKKK7 S922 in vivo and vitro, suggesting that RLCKs may play a role in modulating MKKK7 activity (Figure 6.3). BIK1 belongs to RLCK VII subfamily 8 and associates with both FLS2 and BAK1, while MKKK7 associates with FLS2, bringing these kinases in close subcellular proximity supporting their functional link (Lu et al. 2010a; Mithoe et al. 2016). In addition, RLCK VII subfamily 6 and 8 members, including BIK1, are likely required for PSK-induced growth as higher order *rlck vii-6* and *-8* mutants are less

sensitive to PSK treatment (Rao et al. 2018). It is therefore possible that, similar to MKKK5 and MEKK1, BIK1 and potentially other RLCKs act upstream of MKKK7.

In addition to its potential phosphorylation by upstream RLCKs, MKKK7 is phosphorylated at multiple residues in response to flg22, auxin and brassinosteroid treatment (Kuhn et al. 2024; Kim, Park, et al. 2023; Mithoe et al. 2016). This extensive phosphorylation suggests that several additional upstream kinases might regulate MKKK7, positioning it as a key convergence point for different signalling pathways.

The *Drosophila* protein Armadillo (ARM) was characterised as part of the Wntless/Wnt pathway. It is the homologue of vertebrate β -catenin which functions in cell adhesion by binding cadherins and gene expression regulation during development (MacDonald, Tamai, and He 2009). To avoid detrimental effects from the accumulation of β -catenin in the cell, it forms a preassembled β -catenin destruction complex. Within this complex, β -catenin is constantly phosphorylated by GLYCOGEN SYNTHASE KINASE 3 (GSK3) after priming phosphorylation by CASEIN KINASE 1 (CK1), which targets β -catenin for proteasomal degradation (Beurel, Grieco, and Jope 2015). The presence of the ligand, the secreted glycolipoprotein Wnt, triggers the disassembly of the complex and subsequent accumulation of β -catenin (MacDonald, Tamai, and He 2009). MKKK7 contains two ARM repeat domains, which are structurally related to β -catenin (Tewari et al. 2010). MKKK7 was also shown to be phosphorylated and in cellular proximity to GSK3-like kinase BIN2 in the brassinosteroid signalling pathway (Kim, Park, et al. 2023). It is therefore conceivable that some of the phosphorylation sites found in MKKK7 ARM domains are involved in the regulation of its stability and turnover.

MKKK7 is phosphorylated in response to IAA on S452, S533 and S854, but phosphorylation is abolished in B-RAF subfamily 4 higher order mutants (Kuhn et al. 2024). This suggests that RAF MAPKKs also play a role in MKKK7 regulation.

Studies of the yeast and human phosphoproteomes suggest that many phosphorylation events might be non-functional, occurring due to random encounters between kinases and their substrates. Functional phosphorylation sites are often located in disordered regions and tend to exhibit a higher degree of conservation, indicating their importance in cellular processes (Landry, Levy, and Michnick 2009). Analysing the evolutionary conservation of MKKK7 phosphorylation sites may therefore provide additional guidance for prioritisation for future functional studies.

6.4.2 Can MKKK7 oligomerise?

In addition to MKKK7 phosphorylation, I made the interesting observation that unique MKKK6 peptide spectra were detected in YFP-MKKK7 pull-downs, but no other pull-down. Overall spectral counts were low, and it is possible that PTMs such as methylation, which were not included in the mass-spectrometry database search, may have led to the misidentification of MKKK7 as MKKK6. On the other hand, this finding could also suggest a potential interaction between MKKK6 and MKKK7. This is surprising given that the *35S::YFP-MKKK7* Arabidopsis line, which was generated by Patrick Krysan's group, was previously described as a *mkkk6mkkk7* double T-DNA knockout mutant (Chaiwongsar et al. 2006). *MKKK6* (previously *MAP3Kε1* At3g07980; SALK_150512) and *MKKK7* (previously *MAP3Kε1* At3g13530; SALK_01724) (Chaiwongsar et al. 2006). The T-DNA in *MKKK6* is inserted in intron 6, raising the possibility of splicing out the insertion, leading to potentially minor changes in *MKKK6* expression. The expression level of *MKKK6* in this line has not been tested and future work should address this uncertainty. Despite this, the observation that MKKK7 might pull-down MKKK6 suggest the possibility that both kinases interact with each other. Tissue-specific expression of *MKKK6* and *MKKK7* likely plays a role in their function, but the use of the *35S::YFP-MKKK7* transgenic line for the pull-down assays may obscure this information. Under physiological conditions, it is possible that MKKK6 and MKKK7 function as homomeric protein complexes - a hypothesis that is currently being tested in the lab. Supporting this, yeast-two-hybrid data shows that *Solanum tuberosum* *StMAPKKKε* self-associates (King 2013, PhD thesis).

6.4.3 What are the downstream signalling components of MKKK7?

The transient expression experiments show that *MKKK7* triggers cellular responses that lead to HR and cell death in *N. benthamiana*. This is consistent with the results shown for *MKKK7*, *StMKKKε* and *SIMMKKKε* by Stuart King in his PhD thesis (King 2013, PhD thesis) and suggests that *MKKK7* is an active kinase. Genetic epistasis and overexpression experiments show that *StMKKKε* and *SIMMKKKε* activate immune signalling through the MEK2-SIPK/WIPK MAP kinase cascade (King et al. 2014; Melech-Bonfil and Sessa 2010). It is therefore possible that MKKK7 in Arabidopsis likewise activates MEK2 homologues MKK4 and MKK5, which in turn leads to the activation of downstream MPK3/MPK6, homologues of SIPK/WIPK. This could be tested with a gain-of-function approach by introducing the MKKK7 kinase domain into *mkk4-18mkk5-18* double mutants with only 10% MKK4 expression (Shao et al. 2020). Upon request, Shuqun Zhang has kindly provided this mutant line.

6.4.4 Functional redundancy of MKKK6 and MKKK7 might explain the Arabidopsis immune phenotypes and resolves conflicting literature in Solanaceae

MKKK7 was previously shown to attenuate flg22-induced immune responses in Arabidopsis with the *mkkk7-1* mutant showing enhanced disease resistance to *Pseudomonas syringae* infection (Mithoe et al. 2016). I show here that immune responses and *Pseudomonas syringae* resistance are not consistent between *mkkk7-1* and *mkkk7-2/mkkk7-3* mutants, despite similar growth phenotypes reported earlier (Chapter 4). *mkkk7-1* mutants show slightly enhanced resistance to *Pto* DC3000 infection and altered defence gene expression, which is in line with previous results, however *mkkk7-2* and *mkkk7-3* do not differ from WT Col-0 in their defence gene expression and resistance to *Pseudomonas* infection.

This could suggest that *mkkk7-1* is not a full knockout mutant with residual kinase domain, which might be crucial for immune signalling, still expressed and translated. Complementation experiments are currently underway in which I will introduce the kinase domain of MKKK7 (*pMKKK7::MKKK7¹⁻³⁰⁰-GFP*) into *mkkk7-3* to test for *Pseudomonas* resistance. Why *mkkk7-2* behaves similarly to *mkkk7-3* in immune assays, despite also having a CRISPR-Cas9-induced deletion downstream of the kinase domain and therefore theoretically able to translate a functional kinase domain remains unclear. Another possibility for the different resistance phenotypes is the presence of additional T-DNA insertions in the *mkkk7-1* line. Indeed, information from the Arabidopsis Biological Resource Centre¹⁶ (ABRC) shows that the original *mkkk7-1* SALK_133360 T-DNA line contains several additional T-DNA insertions which might be segregating in the background (Alonso et al. 2003). Among these, putative polymorphisms are present in CALCIUM PROTEIN KINASE 6 (CPK6), pectin methylesterase 3 and METHYL METHANE SULFONATE SENSITIVITY 21 (MMS21) which might interfere with the interpretation of the immunity data.

All *mkkk7* mutants appear resistant to a biotrophic pathogen *Plasmodiophora brassicae*. *Plasmodiophora brassicae* transforms the host root into a metabolic sink and triggers the formation galls. Different growth-promoting hormones play a key role in this process: auxin, cytokinins and brassinosteroids. Taking the growth phenotype of *mkkk7* and *pskr1-3pskr2-1* mutants described in Chapter 4 and Chapter 5 into account it is possible that the enhanced resistance towards *Plasmodiophora brassicae* infection is caused by the disruption of hormonal signalling networks that are also the cause for the reduced growth phenotype.

¹⁶ https://abrc.osu.edu/stocks/number/SALK_133360

At the time of writing, I assumed that MKKK6 plays a significant role in regulating immune signalling and preventing me from seeing a clear immune phenotype in *mkkk7* mutants, but neither *mkkk6* nor *mkkk6mkkk7* mutants were available to test. It will be necessary to test *mkkk6* and *mkkk6mkkk7* double mutants to proceed this avenue of research. These mutants will ultimately also help to understand the long-lasting question in the literature why Solanaceae species show increased susceptibility when *MKKKε* is silenced, but Arabidopsis *mkkk7* mutants show slightly increased resistance. Since only Arabidopsis harbours two closely related homologues *MKKK6* and *MKKK7*, individual knockouts of either kinase might not be sufficient to observe clear differences in immune signalling.

General Discussion

Understanding how signalling specificity is achieved downstream of membrane receptor complexes remains a major question in plant biology. Multiple factors and mechanisms likely contribute to ensuring signal accuracy and fidelity, with MAP kinases playing a central role in these processes. However, the molecular mechanisms and specific signalling components involved often remain poorly understood. In collaboration with my colleagues and MSc students, my thesis contributes to our understanding of these signalling processes. I have identified novel signalling components of the Phytosulfokine (PSK) pathway through AP-MS analysis (Chapter 3). Among these, I identified MKKK7 as a potential downstream kinase and I have provided both phenotypic and experimental evidence demonstrating that MKKK7 is involved in PSK-signalling (Chapter 4, Chapter 5). MKKK7 was previously linked to PAMP signalling through its established role in the FLS2 signalling pathway (Mithoe et al. 2016). I showed here that MKKK7 is an active kinase capable of triggering cell death in *Nicotiana benthamiana* and that it is phosphorylated on multiple residues in response to flg22 treatment (Chapter 6). Together, I show that MKKK7 acts in growth and immunity-related signalling processes and phosphorylation is likely involved in its regulation.

In this Chapter, I will recapitulate some of my results in the wider context, before proposing a hypothetical model for the crosstalk between PSK and immune signalling and the potential function of MKKK7.

7.1 New insights into PSK signalling through Phosphoproteomics

Signalling through small peptide ligand-receptor pairs is considered as a significant evolutionary advancement in land plants (Furumizu and Shinohara 2024). Phytosulfokine was the first, secreted and post-translationally modified peptide, identified from plants more than 25 years ago (Matsubayashi and Sakagami 1996). Since then, numerous signalling peptides and their corresponding receptors have been characterized, but downstream signalling mechanisms in many of these pathways remain poorly understood (Furumizu and Shinohara 2024; Olsson et al. 2019).

Phosphoproteomics has been instrumental in advancing our understanding of signalling events across various biological systems, including plants (Benschop et al. 2007; Kuhn

et al. 2022; Kim, Park, et al. 2023; Friml et al. 2022b) and fungi (Cruz-Mireles et al. 2024). For instance, the flg22-induced phosphoproteome has led to significant breakthroughs in plant immune signalling, including the identification of key downstream molecules such as RBOHD and BIK1 (Benschop et al. 2007; Nühse et al. 2007). Auxin-induced phosphoproteomics has led to the identification of a novel, deeply conserved RAF kinase-dependent auxin response mechanism downstream of ABP1 and TMK1 (Friml et al. 2022a; Kuhn et al. 2024). Bikinin-mediated inhibition of BIN2 has provided novel insights into the brassinosteroid phosphoproteome and identified associated cellular processes such as cytoskeleton organisation, endocytosis and regulation of transcription (Kim, Park, et al. 2023). A comparative phosphoproteomics analysis on the MAPK pathogenicity-mitogen-activated kinase 1 (Pmk1) has advanced our understanding of infection-related development in the rice blast fungus *Magnaporthe oryzae* (Cruz-Mireles et al. 2024). Despite these advancements, no comprehensive plant phosphoproteome has been generated in response to post-translationally modified peptide signals.

A key challenge for phosphoproteomics with endogenous ligands is their continuous presence in the plant, making receptor or peptide mutants essential controls. Phytosulfokine perception through PSKR1 is well characterised (Wang et al. 2015) and PSK is commercially available, which makes it an ideal candidate for phosphoproteomics studies. The *pskr1-3pskr2-1* receptor mutant is unresponsive to PSK and with only seven PSK encoding genes in Arabidopsis, higher order peptide mutants can be generated by CRISPR-Cas9. It will be particularly interesting to compare the PSK-induced phosphoproteome with published PAMP- and effector-triggered phosphoproteome datasets to identify potential molecular convergence points between plant growth and immune signalling pathways (Benschop et al. 2007; Nühse et al. 2007; Kadota et al. 2019).

Together with the work on PSK signalling presented in this thesis, a PSK-induced phosphoproteome study will generate novel insights into the downstream signalling processes and potentially help to understand the role of MKKK7 in this pathway. This work will further pave the way for similar studies in other peptide signalling pathways, generating novel insights beyond the transcriptomics-focused approaches that have been used in the field of plant peptide signalling thus far.

7.2 The role of tyrosine sulfation on small, secreted peptides

In the complex interplay between plants and microbes, proteinaceous patterns recognised by cell surface receptors are more abundant than non-proteinaceous ligands (Escocard de Azevedo Manhães et al. 2021). The plant apoplast, a critical battleground

in these interactions, is rich in proteases that help to protect the plant against pathogens by cleaving a wide array of proteins (Godson and van der Hoorn 2021). For plants, maintaining the stability of endogenous peptide ligands in the apoplastic space is crucial to preserve their activity and ensure effective signalling (Tavormina et al. 2015). It is interesting to speculate that energy-intensive PTMs such as tyrosine sulfation on PSK might not only contribute to receptor binding, but also provide a significant protection from apoplastic proteasomal degradation.

Tyrosine sulfation is catalysed by the enzyme TYROSYLPROTEIN SULFOTRANSFERASE (TPST) in both plants and animals, where structurally distinct TPST enzymes have evolved independently through convergent evolution (Komori et al. 2009). The minimum requirement for tyrosine sulfation is an Asp (D) residue immediately N-terminal to the Tyrosine (Y) in both animals and plants (Hanai et al. 2000; Niehrs et al. 1990), suggesting convergent substrate preferences (Komori et al. 2009). In addition, multiple acidic residues in proximity of Y significantly enhance sulfation by TPST (Niehrs et al. 1990; Hanai et al. 2000). Tyrosine sulfated peptides RGF, PSY and PSK have evolved in liverworts and mosses and are therefore evolutionary more recent compared to ancient IDA, CIF and CLE peptides (Furumizu et al. 2021). Tyrosine sulfation was thought to be unique to higher eukaryotes, but it was found in the gram-negative bacterium, *Xanthomonas oryzae* pv. *oryzae* which encodes the sulfotransferase RaxST (Han et al. 2012).

PSKR1 and BRI1, the respective receptors for PSK and brassinosteroids, harbour island domains of approximately 70 aa and 35 aa in their LRR regions. The BRI1 island domain forms a binding pocket for the steroid hormone BR, which is deeply inserted into the pocket with both the island domain and LRR domain contributing to hydrophobic interactions (Hothorn et al. 2011). The PSKR1 island domain is involved in PSK binding, but it does not form a binding pocket for PSK. PSK associates via hydrogen bonds and its sulfated residues only make a few contacts with the LRR and island domain (Hohmann, Lau, and Hothorn 2017; Wang et al. 2015). The dissociation constant for PSKR1-PSK measured by in vitro MST (MicroScale Thermophoresis) indicates that both sulfated and unsulfated PSK bind to PSKR1 with dissociation constants in the μM range of $K_d 1.55 \pm 0.12$ for sulfated PSK and $K_d 41.5 \pm 1.72$ for unsulfated PSK (Wang et al. 2015), though studies of microsomal fractions of carrot and Arabidopsis suggest that sulfated PSK binding occurs with dissociation constants in the nM range (4.2 and 7.7 nM) (Matsubayashi et al. 2006; Matsubayashi et al. 2002). Overexpression of PSKR1 in *tpst* loss-of-function mutants results in enhanced root growth (Kaufmann, Stührwohldt,

and Sauter 2021), suggesting PSK signalling in the absence of sulfated PSK. Alternatively, a high abundance of the receptor or clustering of the receptor at the plasma membrane might be sufficient to induce PSK signalling.

There is precedent for the importance of tyrosine sulfation in subtilase-mediated processing of small peptides, as demonstrated for the TWISTED SEED 1 (TWS1) peptide, an embryonic regulator of cuticle formation. Sulfotyrosine in TWS1 is essential for the accurate binding and processing of the precursor peptide by SBT1.8. The absence of tyrosine sulfation leads to miscleavage of the precursor, potentially resulting in an inactive mature peptide (Royek et al. 2022).

The proteases responsible for N-terminal cleavage of proPSK and the release of the mature peptide are currently not known in Arabidopsis. The ROS data I present here could provide a rapid and quantitative, albeit indirect, method to address the role of unsulfated PSK in PAMP-induced ROS production over time. If unsulfated PSK fails to suppress ROS, it suggests that sulfation is required for receptor binding and effective PSK signalling. If the lack of sulfation leads to miscleavage and inactivation of PSK, one would expect a gradual decline in ROS suppression by unsulfated PSK, whereas sulfated PSK would retain its signalling ability over time. This experimental approach could also be used to identify the subtilases involved in releasing the mature PSK peptide.

In summary, it could be interesting to investigate the role of tyrosine sulfation on PSK beyond its role receptor binding, especially in the context of peptide stability and potentially identify the proteases involved in its maturation process.

7.3 Activation and regulation of MAPKKKs through oligomerisation and liquid-liquid phase separation

Plant MAP kinase cascades form complex signalling networks, in contrast to the more linear pathways observed in yeast and mammals (Ma and Nicolet 2023). MAPKKKs are the top tier of the canonical cascade, but their activation and regulation mechanisms are mostly unknown. Phosphorylation is a key mechanism that contributes to the activation and regulation of MAPKKKs (Chadee, Yuasa, and Kyriakis 2002; Bi et al. 2018a). However, phosphorylation alone cannot fully explain how MAPKKKs are activated and then selectively and efficiently phosphorylate downstream substrates while limiting off-target phosphorylation. Additional mechanisms or regulatory factors must be involved in the activation and regulation of these kinases.

Oligomerisation and liquid-liquid phase separation (LLPS) may play important roles in kinase signalling by promoting compartmentalisation and thereby spatiotemporal control (López-Palacios and Andersen 2023). Out of 14 mammalian MAPKKKs (Zhang and Liu 2002) several have been shown to oligomerise. Rapidly accelerated fibrosarcoma (RAF) kinases ARAF, BRAF and CRAF are well-characterised components of the RAS-RAF-MEK-ERK signalling pathway, which regulates normal cell growth and development (Lavoie and Therrien 2015). Dysregulation of this pathway, particularly mutations in BRAF and CRAF, are frequently associated with cancer development, particularly in melanomas (McCubrey et al. 2007; Dunnett-Kane et al. 2020). These RAF kinases undergo complex regulatory processes including phosphorylation, localisation changes, intra- and intermolecular interactions and transition between various conformational states (Lavoie and Therrien 2015; Terrell et al. 2019). In their monomeric state, BRAF and CRAF are autoinhibited and dimerization is essential for their activation (Spencer-Smith and Morrison 2024). This model was confirmed in 2019 with the first cryo-EM structure of the BRAF kinase domain dimer in complex with dimeric 14-3-3 protein (Kondo et al. 2019), and the more recent structure of the CRAF kinase domain dimer in complex with dimeric 14-3-3 and MEK1 (Dedden et al. 2024). Similarly, mammalian MEKK2, a member of the MEKK/STE11 subgroup of MAPKKKs, was proposed to form homodimers that trigger self-phosphorylation and activation (Cheng et al. 2005).

LLPS might be another key mechanism for MAPKKKs to archive appropriately timed compartmentalisation which allows for spatiotemporal regulation in the absence of scaffold proteins (López-Palacios and Andersen 2023). While scaffold proteins are usually associated with these functions, so far only one scaffold protein was identified in plants (Cheng et al. 2015). Plant RAFs are an expanded subgroup of MAPKKKs that act as key regulators in various signalling pathways, including hormonal responses, environmental stress, and plant-pathogen interactions (Fàbregas, Yoshida, and Fernie 2020; Wang 2024). Despite limited knowledge about their precise functions, evidence shows that hyperosmolarity and auxin treatment trigger hyperphosphorylation of B-RAFTs in the moss *Physcomitrium patens* and *Arabidopsis* (Toriyama et al. 2022; Saruhashi et al. 2015; Kuhn et al. 2024). This phosphorylation is likely linked to receptor kinases like TMK1 (Kuhn et al. 2024) and upstream kinases such as MAP4Ks (Wang 2024) and histidine kinases (Toriyama et al. 2022). The exact functional role of these phosphosites warrants further investigation, but the hyperphosphorylation and intrinsically disordered regions typical of plant B-RAFTs may facilitate oligomerization and condensation, which are critical for B-RAF activation and function (Wang 2024). Phosphorylation sites are

usually enriched in intrinsically disordered regions which often have profound effects on LLPS and multivalent interaction surfaces (Landry, Levy, and Michnick 2009).

Kinase gene fusions that contain additional domains involved in oligomerisation may be particularly interesting for LLPS-mediated activation and regulation. Fusing protein scaffold or protein-protein interaction domains and catalytically active kinase domains may have provided an evolutionary benefit by increasing kinase signalling efficiency which often requires stable interaction with their target proteins (López-Palacios and Andersen 2023). B-RAFTs contain Phox and Bem1 (PB1) domains that could be implicated in their dimerization/oligomerisation (Wang 2024). MKKK7 is a kinase fused to two ARM scaffold domains, which are often implicated in protein-protein interactions (Ichimura et al. 2002; Tewari et al. 2010). The extensive phosphorylation of MKKK7 in its intrinsically disordered regions, as shown here and summarised from the literature, together with its ARM scaffold domains, suggests potential regulatory roles in oligomerisation and possibly LLPS. Studying the phosphorylation and oligomerisation behaviour of MAPKKs like MKKK7 using blue-native PAGE to visualise protein complexes, combined with Cryo-EM to resolve their structures, provides an exciting future research avenue.

In summary, oligomerisation and LLPS might be crucial mechanisms for MAPKK activation and function, and phosphorylation may hold part of the answer as to how LLPS and oligomerisation is initiated and regulated.

7.4 Does MKKK7 fit in the canonical MAPK signalling pathway?

Several MAPKKs are characterised in plants and linked to the activation of downstream MKs and MAPKs. Three kinases, YDA (MKKK4), MKKK3 and MKKK5 act upstream of the MKK4/MKK5-MPK3/MPK6 cascade (Liu, Leary, et al. 2022; Wang, Wu, et al. 2022; Sun et al. 2018) and I speculated in Chapter 6 that MKKK7 could also function upstream of this cascade, but is there room for another MAPKK?

YDA consists of a negative regulatory N-terminus and its removal renders the kinase constitutively active. Expression of N-terminal truncations of YDA results in phenotypes opposite to loss-of-function phenotypes of the *yda*-Y295 allele (Lukowitz et al. 2004; Bergmann, Lukowitz, and Somerville 2004; Sopeña-Torres et al. 2018). Silencing YDA with artificial microRNAs (amiRNAs) caused developmental defects that were largely suppressed in the *mkkk3mkkk5* mutant background (Sun et al. 2018). *yda*-silenced plants showed enhanced PAMP-induced MAP kinase activation, suggesting that YDA acts antagonistically to MKKK3MKKK5 upstream of the MKK4/MKK5-MPK3/MPK6

cascade (Sun et al. 2018). No obvious phenotype has been reported for *mkkk3mkkk5* mutants (Shao et al. 2020). However, triple mutants of *yda-11mkkk3mkkk5* exhibit a more severe growth phenotype than weak *yda-11* allele alone (Wang, Wu, et al. 2022). Additionally these triple mutants show greater attenuation of PAMP-induced MAP kinase activation, suggesting an opposing model, that both YDA and MKKK3/MKKK5 act redundantly in activating the MKK4/MKK5-MPK3/MPK6 cascade (Wang, Wu, et al. 2022; Liu, Leary, et al. 2022).

Recently, Herrmann et al. proposed that maintaining signalling specificity may depend on both the strength of the signalling input and the homeostasis of MAPKs that are yet to be activated (Herrmann et al. 2024). Both the ERECTA and FLS2 signalling pathways rely on MPK6 activity for stomatal and immune signalling, respectively. If ERECTA signalling is compromised, either due to suboptimal receptor levels or chemical inhibition of MPK6, FLS2 signalling can compensate by activating basal stomatal development. This compensation restores MAPK activation and nullifies the effects of chemical inhibition on MPK6 (Herrmann et al. 2024).

The MAPK homeostasis model proposed by Herrmann et al., 2024 might also provide an explanation for the discrepancy in the YDA, MKKK3/MKKK5 literature. It is possible that both YDA and MKKK3/MKKK5 act on the existing pool of MKK4/MKK5. If either MAPKKK is absent it may result in enhanced activation of the cascade through the other MAPKKK, hence the initial model which suggested antagonism between the two MAPKKKs. If however, both MAPKKKs are absent it leads to reduced MAP kinase cascade activation in response to the stimulus, in this case a PAMP, hence the proposed redundancy model. The redundancy model appears to be more plausible and, in sight of the results by Herrmann et al. 2024, also supports the previously postulated antagonism.

Interestingly, *yda-11mkkk3mkkk5* mutants still show residual MAPK activation after PAMP treatment, indicating the presence of yet unidentified MKKKs acting in parallel (Wang, Wu, et al. 2022). In this context, it is tempting to speculate that MKKK6/MKKK7 are the missing kinases acting upstream of MKK4/MKK5-MPK3/MPK6. Both kinases belong to the MEKK subfamily which has so far been operationally confirmed to act upstream of MKKs. To determine if Arabidopsis MKKK6 and MKKK7 act in this cascade, it will be necessary to test PAMP-induced kinase activation in a viable *mkkk6mkkk7* double mutant. Furthermore, generating a quintuple MAPKKK mutant in *yda-11mkkk3mkkk5mkkk6mkkk7* will be necessary, which may lack flg22-induced MAP kinase activation altogether. Such a mutant is likely not viable but following the idea of a pool of activatable MAP kinases in the cell, using any cell-type independent promoter

system such as a conditional or overexpression promoter with any of these MAPKKs might be able to rescue potentially severe phenotypes.

7.5 Concluding remarks and working model

The results here add to our understanding of the cross-regulation of receptor signalling pathways involved in growth and immunity. I have demonstrated that PSK signalling suppresses immunity-related gene expression (Figure 5.10, 5.11) and PAMP/DAMP-induced ROS production (Figure 5.11, 5.12). Since these two outputs are temporally distinct, it suggests that PSK-induced immune suppression occurs at multiple levels that merit further investigation. One possibility is that PSK counteracts PTI-induced dephosphorylation and inactivation of AHAs, thereby preventing apoplastic alkalization and disrupting ion fluxes that are critical for PTI (Figure 7.1). At the transcriptional level, PSK suppresses immune and SA-responsive genes, but the signalling components involved in this process are not known. I made the interesting observation that RNA-related proteins decrease in abundance in PSKR1-GFP AP-MS data (Figure 3.5, Figure S1) which may be linked to the suppression of immunity-related genes. My results suggest that MKKK7 is not involved in either of these processes but may regulate a distinct signalling branch of the PSK pathway involved in the regulation of growth and nutrient-responsive gene expression (Figure 7.1).

In addition to its role in PSK signalling, I confirmed that MKKK7 is strongly phosphorylated after flg22 treatment. Evidence from *Nicotiana benthamiana*, *Solanum lycopersicum* and *Solanum tuberosum* indicate that MKKK7 homologues function in the canonical MAPK cascade upstream of MEK2-SIPK/WIPK (Melech-Bonfil and Sessa 2010; King et al. 2014). Therefore, I propose that Arabidopsis MKKK7 also acts as an accessory kinase within the homologous MKK4/MKK5-MPK3/MPK6 signalling cascade, potentially regulating MKK4 and MKK5 to activate plant immune responses. This hypothetical model positions MKKK7 as a critical player not only in growth and nutrient signalling via PSK but also in the broader immune signalling network.

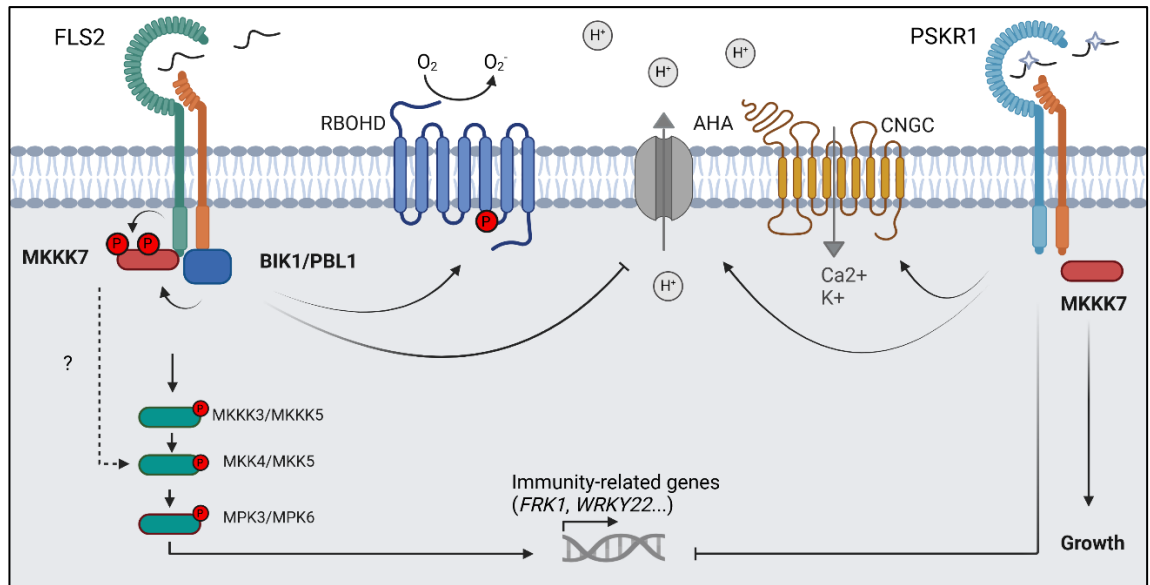


Figure 7.1. Hypothetical model for the immune suppression by PSK and the involvement of MKKK7. LRR-RKs FLS2 and PSKR1 associate with co-receptor BAK1 in the presence of their cognate ligands flg22 and PSK. FLS2 triggers the activation and phosphorylation of RLCKs BIK1 and its homologue PBL1 which in turn activate RBOHD. FLS2 signalling also triggers the dephosphorylation and inactivation of AHAs. The downstream MAP kinase cascade amplifies the FLS2 signal and results in transcriptional reprogramming. MKKK7 associates with FLS2 and is phosphorylated after flg22 perception (Mithoe et al. 2016). PSKR triggers the activation of AHAs and CNGC17 and suppresses immunity-related gene expression.

While the above model (Figure 7.1) provides information about the putative involvement of MKKK7 in growth and immunity-related pathways, it does not directly address the molecular function of MKKK7. Based on the AP-MS data I present here, and findings from Stuart King's PhD thesis (2013) on *Nicotiana benthamiana* *MKKKε*, I hypothesize that Arabidopsis MKKK6 and MKKK7 may form homo- or hetero-oligomers (Figure 7.2). MKKK7 is also highly phosphorylated and potentially involved in multiple hormone-signalling pathways (Kuhn et al. 2024; Kim, Park, et al. 2023). Oligomerisation and phosphorylation may be key regulatory mechanisms that modulate MKKK7's role in growth and immune signalling pathways. This model is not mutually exclusive to the previous one but provides a more detailed mechanism that potentially explains how MKKK7 is regulated.

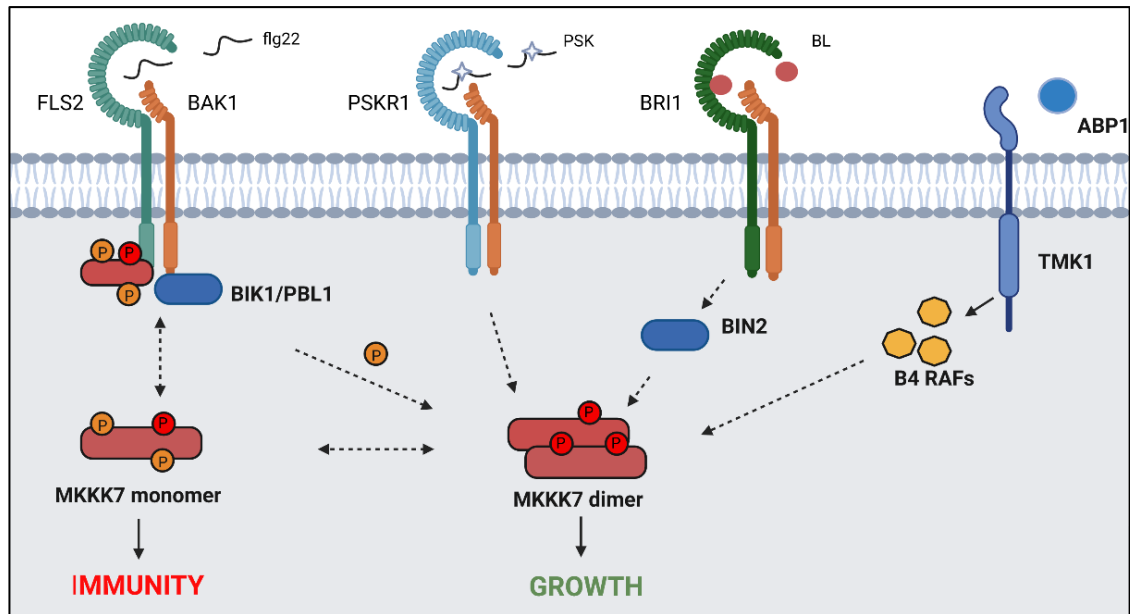


Figure 7.2. Hypothetical model for the molecular function of MKKK7 in plant growth, immunity, and its regulation by phosphorylation. MKKK7 has been identified in several phosphoproteomic datasets, including those in response to flg22 (Mithoe et al. 2016; Benschop et al. 2007) and this study, auxin (Kuhn et al. 2024), and BIKININ (Kim, Park, et al. 2023), suggesting its involvement in multiple signalling pathways. It is also potentially linked to PSK signalling, as demonstrated in this thesis. My AP-MS data indicates an association between MKKK6 and MKKK7 and Y2H data from King et al. (2013) shows that *StMKKKε* self-associates implying that these kinases may form homo/hetero-oligomers within the cell. MKKK7 associates with the FLS2 receptor to regulate immune signalling (Mithoe et al. 2016). This model proposes that MKKK7 may function in various oligomeric states, which could be dynamically regulated by phosphorylation, allowing it to modulate growth and immunity signalling.

Supplemental Data

Table S1. List of Primers used in this study. FW= forward primer, RV=reverse primer, LP=left primer, RP=right primer.

#Primer ID	Gene name and primer orientation	Primer sequence [5'-3']	Purpose
#MD241	MKKK7 SALK_133360_LP	AAC TTG TTGCCATGTTTCGTC	genotyping <i>mkkk7-1/T-DNA</i>
#MD242	MKKK7 SALK_133360_RP	AATCATTTCCTTGGGGTGGATC	genotyping <i>mkkk7-1</i>
#MD1	MKKK7 (AT3G13530) FW	TGTAAGATTCTGACTCAATCC	genotyping <i>mkkk7-2</i>
#MD11	MKKK7 (AT3G13530) RV	TTCCA ACTCAGTAAATAGAGCAC	genotyping <i>mkkk7-2</i>
#MD222	MKKK7 (AT3G13530) FW	ATTCTGTTTCCTTGAGGAGC	genotyping <i>mkkk7-3</i>
#MD224	MKKK7 (AT3G13530) RV	GCTAGGATTCATATAATCAATGGC	genotyping <i>mkkk7-3</i>
#MD239	PSKR1 SALK_008585.55.20 LP	CTCGCTTTCTGGTATGACGAG	genotyping <i>pskr1-3</i>
#MD240	PSKR1 SALK_008585.55.20 RP	TCCGAAACTATACACATCGCC	genotyping <i>pskr1-3/T-DNA</i>
#MD237	PSKR2 SALK_024464.53.15.x_LP	GAGAACTTGTTGGAGCTCACG	genotyping <i>pskr2-1</i>
#MD238	PSKR2 SALK_024464.53.15.x_RP	TTTTGGGATGTGAGCGTTTAG	genotyping <i>pskr2-1/T-DNA</i>
#Lba1	Lba1-left border primer for T-DNA	TGTTTCACGTAGTGGGCCATCG	T-DNA left border
#MD273	MKKK7 (AT3G13530) FW	TTGACCGAAGACTCAATGCGCGGCAAATGACGTCA	cloning MKKK7 1-300
#MD274	MKKK7 (AT3G13530) RV	TTGACCGAAGACTCCGA <u>ACTTG</u> CAGTGGCTTCCTTCA	cloning MKKK7 1-300
#MD289	MKKK7 (AT3G13530) FW	GCGAATGAAGACTCAATGGCGGAGAATCTTTTCCTC	cloning MKKK7 565-1367
#MD290	MKKK7 (AT3G13530) RV	GCGAATGAAGACTCCGAA <u>AATATTG</u> TGTTGATGTGAAGTGC	cloning MKKK7 565-1367
#MD129	MYB5 (AT3G13540) FW	ACGGTGGATGAACTATCTCC	MYB5 RT-qPCR
#MD130	MYB5 (AT3G13540) RV	TTCATTATCAGTCCTTCCCG	MYB5 RT-qPCR
#MD39	MKKK7 (AT3G13530) FW	ACCAGATGCCTCAACCTC	MKKK7 RT-qPCR
#MD40	MKKK7 (AT3G13530) RV	AGGAAAACCATTAGAGGCG	MKKK7 RT-qPCR
#MD41	MKKK7 (AT3G13530) FW	ATATGAAGGAAGCCACTG	MKKK7 RT-qPCR
#MD42	MKKK7 (AT3G13530) RV	TCGACACCCCTACCAG	MKKK7 RT-qPCR
#MD69	FRK1 (AT2G19190) FW	TAGATGCAGCGCAAGGACTA	immunity RT-qPCR
#MD70	FRK1 (AT2G19190) RV	CTTGCTCGAGGAACCATCT	immunity RT-qPCR

#MD113	<i>WRK22</i> (AT4G01250) FW	TCCTTCGGAGAGATTCGAGA	immunity RT-qPCR
#MD114	<i>WRK22</i> (AT4G01250) RV	CTGCTGCTACATGGCACACT	immunity RT-qPCR
#MD115	<i>WRK29</i> (AT4G23550) FW	CCCGGAGAAATTCACCATAA	immunity RT-qPCR
#MD116	<i>WRK29</i> (AT4G23550) RV	ATCAGCGGATGGGATCATAG	immunity RT-qPCR
#MD73	<i>IAA1</i> (AT4G14560) FW	CACCGACCAACATCCAATCTCC	auxin RT-qPCR
#MD74	<i>IAA1</i> (AT4G14560) RV	TGGACGGAGCTCCATATCTCC	auxin RT-qPCR
#MD97	<i>SAUR19</i> (AT5G18010) FW	CTTCAAGAGCTTCATAATAATTCAAACCTT	auxin RT-qPCR
#MD98	<i>SAUR19</i> (AT5G18010) RV	GAAGGAAAAAATGTTGGATCATCTT	auxin RT-qPCRt
#MD99	<i>PIN1</i> (AT1G73590) FW	ACAAAACGACGCAGGCTAAG	auxin RT-qPCR
#MD100	<i>PIN1</i> (AT1G73590) RV	AGCTGGCATTTCATGTTCC	auxin RT-qPCR
#MD83	<i>MRN1</i> (At5g42600) FW	TGCATACACTTCCACCGCAT	PSK RT-qPCR
#MD84	<i>MRN1</i> (At5g42600) RV	GCTCCCAAACCTGGCATACT	PSK RT-qPCR
#MD85	<i>THAD1</i> (At5g47990) FW	GAATGTCTCCTCTCGCCCTC	PSK RT-qPCR
#MD86	<i>THAD1</i> (At5g47990) RV	AGCAGCTTTTGGACCATGAAC	PSK RT-qPCR
#MD268	ArLV-RNA1-Fw	TCTGCCAGTACTGGAGAGG	virus test RT-qPCR
#MD269	ArLV1-RNA1-Rv	GTCATCCAACAAATAGGAAC	virus test RT-qPCR
#MD270	ArLV1-RNA2-Fw	CACCAATAACACCCCAAAA	virus test RT-qPCR
#MD271	ArLV1-RNA2-Rv (RNA2)	GCATTTCCACAGAGTCTCG	virus test RT-qPCR
#MD272	RNA1	TATCATTAAAAGGCGGCAGG	virus test RT-qPCR
#MD89	Actin FW	GCACCCTGTTCTTCTTACCG	Actin control
#MD90	Actin RV	AACCCTCGTAGATTGGCACA	actin control

Table S2. Guide RNAs used to generate the mutants described in this study. Sequences were predicted for specificity and efficiency by CHOPCHOP¹⁷ online tool.

Name	Gene	Locus	Target site	Efficiency	sgRNA sequence (PAM)	Generated
oNN0005	<i>MKKK7</i>	AT3G13530	exon 12	-	GCCGCAAAGGTGAAGAACGTGGG	Ana Lopez Vazquez
oNN0006	<i>MKKK7</i>	AT3G13530	exon 12	-	GTCGATCCATGAGAAATCTTCGG	Ana Lopez Vazeuqz
MDg1ex2	<i>MKKK7</i>	AT3G13530	exon 2	65.31	ATATGTTGAGGGTAGATGCTGGG	This study
MDg2ex1	<i>MKKK7</i>	AT3G13530	exon1	62	AGAAACGAGAAGAGATGGCGCGG	This study
MDg3_3'UTR	<i>MKKK7</i>	AT3G13530	3' UTR	72.00	AAAACGATAATATCCCTTGCGGG	This study
MDg4_3'UTR	<i>MKKK7</i>	AT3G13530	3' UTR	62.00	CAAGTGTTGGCATCTCTCGAGGG	This study
MDM3K6guide22	<i>MKKK6</i>	AT3G07980	exon 19	56.40	TCTAGTCTTGCAATGAGTAACGG	This study
MDM3K6guide2	<i>MKKK6</i>	AT3G07980	exon 22	71.00	GCAAGACGTTGACCAAAAGGTGG	This study
MDM3K6guide8	<i>MKKK6</i>	AT3G07980	intron 6	61.3	GTGTGTTTTTCTGTACCTTGTTGG	This study
MDM3K6guide15	<i>MKKK6</i>	AT3G07980	exon 1	59	AGTCTTTGATTTGTGGAACCTGGG	This study

¹⁷ <https://chopchop.cbu.uib.no/>

Table S3. Table of all PSK-specific interactors identified by PSKR1-GFP AP-MS based on log-fold change (logFC) ≥ 1 and logFC ≤ -1 with a $-\log_{10}$ p-value ≤ 0.5 . LogFC and $-\log_{10}$ p-value were calculated based on spectral counts. Proteins that increased in abundance after PSK treatment in PSKR1-GFP pull-downs are highlighted in blue, those that decreased are highlighted in orange. Missing spectral data were imputed with a value of 0.01 for logFC and $-\log_{10}$ p-value calculations. This table corresponds to Chapter 3, Figure 3.5.

Identified Proteins	Accession Number	logFC	log p-value
photosystem II subunit Q-2 Chr4:2672093-2673170 REVERSE LENGTH=230 201606	AT4G05180.1 (+1)	6.75209604	0.671760577
DHHC-type zinc finger family protein Chr5:20351210-20354124 FORWARD LENGTH=444 201606	AT5G50020.2	6.12913277	0.848990731
Seven transmembrane MLO family protein Chr4:1144141-1147156 FORWARD LENGTH=526 201606	AT4G02600.1 (+1)	4.87251472	0.544673974
3-oxo-5-alpha-steroid 4-dehydrogenase (DUF1295) Chr1:27688420-27689606 REVERSE LENGTH=219 201606	AT1G73650.4	4.70800822	0.946189672
Leucine-rich receptor-like protein kinase family protein Chr3:10435139-10438268 FORWARD LENGTH=1016 201606	AT3G28040.1	4.61440935	0.77739589
tryptophan biosynthesis 1 Chr5:5957330-5959681 FORWARD LENGTH=444 201606	AT5G17990.1	4.61440935	0.931184035
transmembrane kinase 1 Chr1:24631503-24634415 FORWARD LENGTH=942 201606	AT1G66150.1	4.44489137	0.730418355
Protein kinase superfamily protein Chr1:3834762-3837305 REVERSE LENGTH=624 201606	AT1G11390.1	4.39512353	0.610571146
Actin-like ATPase superfamily protein Chr1:30246960-30249055 REVERSE LENGTH=482 201606	AT1G80460.2	4.29865074	0.696652675
Transmembrane proteins 14C Chr2:16132178-16134229 FORWARD LENGTH=335 201606	AT2G38550.1	4.29865074	0.696652675
ATPase%2C F0 complex%2C subunit B/B%2C bacterial/chloroplast Chr4:15573859-15574586 REVERSE LENGTH=219 201606	AT4G32260.1	4.26064997	0.698589056
CLP protease proteolytic subunit 6 Chr1:3967609-3969535 FORWARD LENGTH=289 201606	AT1G11750.2	4.21816872	0.597689833
VPS54 Chr4:10616017-10622934 FORWARD LENGTH=1034 201606	AT4G19490.1 (+1)	4.21816872	0.725479782
Major facilitator superfamily protein Chr5:4556308-4558447 FORWARD LENGTH=579 201606	AT5G14120.1	4.21816872	0.812820006
Thioredoxin superfamily protein Chr3:9524807-9526123 FORWARD LENGTH=217 201606	AT3G26060.2	4.15241012	0.628158733
thylakoid lumenal protein (Mog1/PsbP/DUF1795-like photosystem II reaction center PsbP family protein) Chr3:20984807-20985913 FORWARD LENGTH=262 201606	AT3G56650.1	4.15241012	0.628158733
ribosomal RNA small subunit methyltransferase J Chr5:2763914-2765431 FORWARD LENGTH=346 201606	AT5G08540.1	4.15241012	0.645200276
alpha-amylase-like 3 Chr1:26288518-26293003 REVERSE LENGTH=887 201606	AT1G69830.1	4.11440935	0.740668703
photosystem I light harvesting complex protein Chr3:22745736-22747032 FORWARD LENGTH=257 201606	AT3G61470.1	4.11440935	0.740668703
Glc protein (DUF810) Chr2:8637977-8641184 REVERSE LENGTH=952 201606	AT2G20010.2	4.0719281	0.627155804
RNA binding Plectin/S10 domain-containing protein Chr5:16609377-16610583 REVERSE LENGTH=180 201606	AT5G41520.1	4.0719281	0.666790457
Zinc finger (C3HC4-type RING finger) family protein Chr5:24410953-24414849 REVERSE LENGTH=704 201606	AT5G60710.1	4.0719281	0.627155804
cytochrome P450%2C family 89%2C subfamily A%2C polypeptide 2 Chr1:24113283-24114803 FORWARD LENGTH=506 201606	AT1G64900.1	3.96816872	0.761209523

solanesyl diphosphate synthase 2 Chr1:5829289-5831215 FORWARD LENGTH=417 201606	AT1G17050.1	3.8219281	0.740668703
heat shock protein 70 (Hsp 70) family protein Chr4:9377153-9381232 FORWARD LENGTH=891 201606	AT4G16660.2	3.8219281	0.740668703
photosystem II reaction center protein H ChrC:74485-74706 FORWARD LENGTH=73 201606	ATCG00710.1	3.0719281	0.989248939
SERINE CARBOXYPEPTIDASE-LIKE 49 Chr3:3235518-3238063 REVERSE LENGTH=516 201606	AT3G10410.1	2.71656215	0.678655846
Leucine-rich repeat protein kinase family protein Chr2:15569290-15573477 FORWARD LENGTH=934 201606	AT2G37050.3	2.7034453	0.75099878
Plastid-lipid associated protein PAP / fibrillin family protein Chr3:9526904-9528199 FORWARD LENGTH=242 201606	AT3G26070.1	2.6376867	0.592271023
CLP protease proteolytic subunit 1 Chr1:18501936-18504462 REVERSE LENGTH=387 201606	AT1G49970.1	2.5719281	0.679761352
transmembrane protein%2C putative (DUF1118) Chr1:28078995-28079831 FORWARD LENGTH=198 201606	AT1G74730.1	2.55720467	0.640028803
protochlorophyllide oxidoreductase B Chr4:13725648-13727107 FORWARD LENGTH=401 201606	AT4G27440.1 (+1)	2.55070587	0.63692317
Calcium-binding tetratricopeptide family protein Chr1:1484280-1486706 REVERSE LENGTH=808 201606	AT1G05150.1	2.3876867	0.57450228
Major facilitator superfamily protein Chr1:2873604-2876979 FORWARD LENGTH=496 201606	AT1G08930.1 (+1)	2.34968592	0.625312713
leucine-rich repeat transmembrane protein kinase family protein Chr5:3235462-3238171 REVERSE LENGTH=613 201606	AT5G10290.1 (+1)	2.34968592	0.625312713
vacuolar ATP synthase subunit H family protein Chr3:14228846-14232228 REVERSE LENGTH=441 201606	AT3G42050.1	2.3157586	0.506266764
mitogen-activated protein kinase kinase kinase 7 Chr3:4411934-4419320 REVERSE LENGTH=1368 201606	AT3G13530.1	1.4389365	0.675480764
BRI1-associated receptor kinase Chr4:16086654-16090288 REVERSE LENGTH=615 201606	AT4G33430.1	1.04185454	1.146504905
subtilisin-like serine protease 2 Chr4:16656929-16659223 REVERSE LENGTH=764 201606	AT4G34980.1	-1.02219706	0.741616123
Major facilitator superfamily protein Chr3:17698126-17700771 REVERSE LENGTH=636 201606	AT3G47960.1	-1.02318929	0.60494373
GDSL-like lipase/acylhydrolase superfamily protein Chr3:4729886-4731562 FORWARD LENGTH=392 201606	AT3G14210.1	-1.03282017	0.729289384
ribosomal protein L34 Chr1:26189900-26191081 FORWARD LENGTH=119 201606	AT1G69620.1	-1.06382273	0.994448665
Phosphoglycerate mutase%2C 2%2C3-bisphosphoglycerate-independent Chr1:3165550-3167812 REVERSE LENGTH=557 201606	AT1G09780.1	-1.07746382	0.584236987
ribosomal protein L16 ChrC:81189-82652 REVERSE LENGTH=135 201606	ATCG00790.1	-1.10823985	0.700260046
beta galactosidase 1 Chr3:4511192-4515756 FORWARD LENGTH=847 201606	AT3G13750.1	-1.19469644	0.560653983
winged-helix DNA-binding transcription factor family protein Chr2:13045360-13046267 FORWARD LENGTH=273 201606	AT2G30620.1	-1.29951901	0.677187591
histone B2 Chr5:7652130-7652567 REVERSE LENGTH=145 201606	AT5G22880.1	-1.35258786	0.639441667
Mannose-binding lectin superfamily protein Chr3:5593029-5595522 FORWARD LENGTH=705 201606	AT3G16460.1 (+1)	-1.36623681	0.641931536
P-loop containing nucleoside triphosphate hydrolases superfamily protein Chr2:17705382-17708744 FORWARD LENGTH=633 201606	AT2G42520.1 (+2)	-1.39262751	0.553371659
transmembrane protein%2C putative (Protein of unknown function%2C DUF642) Chr5:8863430-8865394 FORWARD LENGTH=369 201606	AT5G25460.1	-1.42683978	0.679934454
Mannose-binding lectin superfamily protein Chr3:5588593-5589792 FORWARD LENGTH=300 201606	AT3G16450.1 (+2)	-1.46644338	0.722039644
glucoside glucohydrolase 2 Chr5:9072730-9075477 FORWARD LENGTH=547 201606	AT5G25980.2	-1.47239242	0.656788366
like AUXIN RESISTANT 1 Chr5:98228-101493 FORWARD LENGTH=488 201606	AT5G01240.1	-2.30720467	0.558610388

NADH:cytochrome B5 reductase 1 Chr5:5864543-5866495 REVERSE LENGTH=281 201606	AT5G17770.1	-2.37296327	0.595825093
DEA(D/H)-box RNA helicase family protein Chr1:11479921-11482707 FORWARD LENGTH=537 201606	AT1G31970.1	-2.41096405	0.526690781
flavodoxin-like quinone reductase 1 Chr5:22124674-22126256 FORWARD LENGTH=204 201606	AT5G54500.1	-2.41096405	0.526690781
secretory carrier 3 Chr1:22586035-22588664 FORWARD LENGTH=289 201606	AT1G61250.1	-2.49144607	0.547420236
FAD-binding Berberine family protein Chr2:14685292-14686914 FORWARD LENGTH=540 201606	AT2G34810.1	-2.49144607	0.547420236
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein Chr4:10012850-10015267 REVERSE LENGTH=621 201606	AT4G18030.1	-2.49144607	0.64587263
purin-rich alpha 1 Chr2:13642716-13644036 REVERSE LENGTH=296 201606	AT2G32080.1 (+1)	-2.49888291	0.72109154
TCP-1/cpn60 chaperonin family protein Chr3:3732734-3736156 FORWARD LENGTH=557 201606	AT3G11830.1	-2.59520545	0.515162861
choice-of-anchor C domain protein%2C putative (Protein of unknown function%2C DUF642) Chr4:15663036-15664859 REVERSE LENGTH=365 201606	AT4G32460.1 (+3)	-2.61280278	0.542540493
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein Chr5:22645742-22649383 REVERSE LENGTH=682 201606	AT5G55920.1	-2.61280278	0.549932603
chromosome-associated kinesin Chr4:13346760-13348791 FORWARD LENGTH=263 201606	AT4G26410.1	-2.6376867	0.67473471
Trimeric LpxA-like enzyme Chr2:14746340-14748532 FORWARD LENGTH=730 201606	AT2G34970.1	-2.66096405	0.754925322
2-oxoacid dehydrogenases acyltransferase family protein Chr3:9460632-9462585 FORWARD LENGTH=480 201606	AT3G25860.1	-2.66096405	0.570773182
chorismate synthase%2C putative / 5-enolpyruvylshikimate-3-phosphate phospholyase Chr1:18065154-18067956 REVERSE LENGTH=436 201606	AT1G48850.1	-2.66544452	0.587805927
ribosomal protein S17 Chr1:30041473-30041922 REVERSE LENGTH=149 201606	AT1G79850.1	-2.67856138	0.602020749
Pyridoxal phosphate (PLP)-dependent transferases superfamily protein Chr3:7835286-7838863 FORWARD LENGTH=504 201606	AT3G22200.1	-2.7034453	0.693535255
RNA-binding (RRM/RBD/RNP motifs) family protein Chr3:2492875-2495052 FORWARD LENGTH=494 201606	AT3G07810.1 (+1)	-2.72384874	0.549149506
ribosomal protein L14 ChrC:80696-81064 REVERSE LENGTH=122 201606	ATCG00780.1	-2.74144607	0.79029364
Class-II DAHP synthetase family protein Chr1:7912120-7914742 FORWARD LENGTH=527 201606	AT1G22410.1	-2.78692048	0.97357543
gamma carbonic anhydrase-like 2 Chr3:18035107-18036773 FORWARD LENGTH=256 201606	AT3G48680.1	-2.80720467	0.61458587
delta 1-pyrroline-5-carboxylate synthase 2 Chr3:20624278-20628989 REVERSE LENGTH=726 201606	AT3G55610.1	-2.80720467	0.638579573
eukaryotic translation initiation factor 3C Chr3:20833790-20836820 REVERSE LENGTH=900 201606	AT3G56150.1 (+1)	-2.81929543	0.507347197
ribosomal protein L9 Chr3:16386505-16387963 FORWARD LENGTH=197 201606	AT3G44890.1	-2.84158055	0.650625472
O-Glycosyl hydrolases family 17 protein Chr3:2332324-2333925 REVERSE LENGTH=460 201606	AT3G07320.1	-2.87958133	0.598728031
ribosomal protein S2 ChrC:15013-15723 REVERSE LENGTH=236 201606	ATCG00160.1	-2.9197129	0.607419087
Ribosomal L27e protein family Chr4:8571896-8572303 FORWARD LENGTH=135 201606	AT4G15000.1	-2.92206258	0.762439651
Plant L-ascorbate oxidase Chr5:7172727-7177409 FORWARD LENGTH=588 201606	AT5G21105.1	-2.9432848	0.628480678
phosphoenolpyruvate carboxykinase 1 Chr4:17802974-17806332 REVERSE LENGTH=671 201606	AT4G37870.1	-2.94377518	0.510488827
Ribosomal L22e protein family Chr5:9836166-9837113 FORWARD LENGTH=124 201606	AT5G27770.1	-2.95093188	0.874072594
chloroplast RNA-binding protein 29 Chr3:19819738-19821423 REVERSE LENGTH=363 201606	AT3G53460.4	-2.99144607	0.64617531
Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein Chr2:19529854-19531401 FORWARD LENGTH=257 201606	AT2G47610.1	-3.0056861	0.556477317

Peroxidase superfamily protein Chr5:25650824-25652062 REVERSE LENGTH=331 201606	AT5G64100.1	-3.03392732	0.720870972
ARM repeat superfamily protein Chr3:279171-283399 FORWARD LENGTH=1176 201606	AT3G01780.1	-3.12317194	0.642725729
Eukaryotic aspartyl protease family protein Chr3:19465644-19467053 REVERSE LENGTH=469 201606	AT3G52500.1	-3.16262998	0.614125916
poly(A) binding protein 8 Chr1:18416740-18419753 FORWARD LENGTH=671 201606	AT1G49760.1 (+1)	-3.2034453	0.623153316
polygalacturonase inhibiting protein 1 Chr5:2132373-2133434 FORWARD LENGTH=330 201606	AT5G06860.1	-3.21720564	0.583353788
Copper amine oxidase family protein Chr1:11343980-11347767 FORWARD LENGTH=677 201606	AT1G31690.1	-3.24144607	0.627653239
transmembrane protein%2C putative (Protein of unknown function%2C DUF642) Chr5:3644655-3646991 FORWARD LENGTH=366 201606	AT5G11420.1	-3.25858307	0.610347905
evolutionarily conserved C-terminal region 2 Chr3:4385274-4388220 REVERSE LENGTH=664 201606	AT3G13460.2	-3.27368094	0.780722258
SKU5-similar 6 Chr1:15603892-15607802 REVERSE LENGTH=542 201606	AT1G41830.1	-3.27582195	0.838058514
myosin family protein with Dil Chr5:6927064-6937380 REVERSE LENGTH=1531 201606	AT5G20490.2	-3.3294995	0.779309517
ribosomal protein S11 ChrC:78960-79376 REVERSE LENGTH=138 201606	ATCG00750.1	-3.39067986	1.031270821
fibrillarin 2 Chr4:13074239-13076205 FORWARD LENGTH=320 201606	AT4G25630.1	-3.39722048	0.726410825
dynamin-like protein 6 Chr1:3370774-3377120 FORWARD LENGTH=914 201606	AT1G10290.1	-3.40761849	0.714457274
S-adenosyl-L-homocysteine (SAH) hydrolase 2 Chr3:8588013-8589671 REVERSE LENGTH=485 201606	AT3G23810.1	-3.47637026	0.824546687
Peroxidase superfamily protein Chr1:26964359-26966557 FORWARD LENGTH=358 201606	AT1G71695.1	-3.67369829	0.882713265
transmembrane protein Chr3:8410483-8411841 FORWARD LENGTH=452 201606	AT3G23450.1 (+3)	-3.67943858	0.845910044
Glycosyl hydrolase family protein Chr5:7107609-7111385 REVERSE LENGTH=702 201606	AT5G20950.3	-3.73925352	0.681318839
periodic tryptophan protein 2 Chr1:5306159-5309460 REVERSE LENGTH=900 201606	AT1G15440.1	-3.8219281	0.740668703
Translation elongation factor EFG/EF2 protein Chr1:17172507-17176683 REVERSE LENGTH=754 201606	AT1G45332.1	-3.8219281	0.740668703
nucleotide transporter 1 Chr1:30191954-30194280 FORWARD LENGTH=624 201606	AT1G80300.1	-3.8219281	0.740668703
NAD-dependent malic enzyme 1 Chr2:5650089-5655103 FORWARD LENGTH=623 201606	AT2G13560.1	-3.8219281	0.740668703
hemoglobin 1 Chr2:6982782-6983522 REVERSE LENGTH=160 201606	AT2G16060.1	-3.8219281	0.740668703
SECY homolog 1 Chr2:8112231-8114452 REVERSE LENGTH=551 201606	AT2G18710.1	-3.8219281	0.740668703
AMP-dependent synthetase and ligase family protein Chr2:19393835-19397616 FORWARD LENGTH=660 201606	AT2G47240.1 (+2)	-3.8219281	0.740668703
Pyridoxal phosphate (PLP)-dependent transferases superfamily protein Chr3:39234-41865 REVERSE LENGTH=563 201606	AT3G01120.1	-3.8219281	0.740668703
cofactor assembly of complex C Chr3:9813550-9814606 FORWARD LENGTH=267 201606	AT3G26710.1	-3.8219281	0.740668703
pentatricopeptide (PPR) repeat-containing protein Chr4:9257985-9260093 FORWARD LENGTH=702 201606	AT4G16390.1	-3.8219281	0.740668703
mechanosensitive channel of small conductance-like 9 Chr5:6586079-6588771 FORWARD LENGTH=742 201606	AT5G19520.1 (+1)	-3.8219281	0.740668703
DEAD box RNA helicase (RH3) Chr5:9285540-9288871 REVERSE LENGTH=747 201606	AT5G26742.1 (+1)	-3.85902606	1.0173866
B-S glucosidase 44 Chr3:6191586-6194124 FORWARD LENGTH=512 201606	AT3G18080.1	-3.87792218	0.641738638
Stabilizer of iron transporter SufD / Polynucleotidyl transferase Chr1:17886285-17891892 REVERSE LENGTH=1048 201606	AT1G48410.1 (+2)	-3.96816872	0.711594731
mercaptopyruvate sulfurtransferase 1 Chr1:29800824-29803679 FORWARD LENGTH=379 201606	AT1G79230.1	-3.96816872	0.711594731

Transducin family protein / WD-40 repeat family protein Chr2:8461804-8464347 FORWARD LENGTH=469 201606	AT2G19540.1	-3.96816872	0.711594731
ARM repeat superfamily protein Chr2:14499236-14505555 FORWARD LENGTH=1280 201606	AT2G34357.1	-3.96816872	0.711594731
dihydroflavonol 4-reductase/flavanone protein Chr3:496179-498772 REVERSE LENGTH=348 201606	AT3G02420.1	-3.96816872	0.711594731
Eukaryotic aspartyl protease family protein Chr3:590561-593089 FORWARD LENGTH=488 201606	AT3G02740.1	-3.96816872	0.711594731
Plant invertase/pectin methylesterase inhibitor superfamily Chr3:3354639-3357581 REVERSE LENGTH=619 201606	AT3G10720.2	-3.96816872	0.711594731
dentin sialophosphoprotein%2C putative (DUF1296) Chr3:4625708-4630383 REVERSE LENGTH=848 201606	AT3G13990.1	-3.96816872	0.711594731
RNA-binding (RRM/RBD/RNP motifs) family protein Chr3:5052844-5054058 FORWARD LENGTH=404 201606	AT3G15010.1 (+1)	-3.96816872	0.711594731
ARID/BRIGHT DNA-binding domain-containing protein Chr3:15210032-15214440 REVERSE LENGTH=747 201606	AT3G43240.1	-3.96816872	0.711594731
Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family Chr4:578308-578991 FORWARD LENGTH=227 201606	AT4G01410.1	-3.96816872	0.711594731
Clathrin adaptor complexes medium subunit family protein Chr4:12675873-12678903 FORWARD LENGTH=451 201606	AT4G24550.2	-3.96816872	0.711594731
xyloglucan endotransglucosylase/hydrolase 14 Chr4:13130349-13131615 FORWARD LENGTH=287 201606	AT4G25820.1	-3.96816872	0.711594731
Pentatricopeptide repeat (PPR) superfamily protein Chr4:16599976-16605994 REVERSE LENGTH=1089 201606	AT4G34830.1	-3.96816872	0.711594731
SKU5 similar 15 Chr4:17494820-17497124 REVERSE LENGTH=541 201606	AT4G37160.1	-3.96816872	0.711594731
ARF GTPase-activating protein Chr5:4255923-4262018 REVERSE LENGTH=827 201606	AT5G13300.1	-3.96816872	0.711594731
embryo defective 3012 Chr5:16213361-16223982 FORWARD LENGTH=1923 201606	AT5G40480.1	-3.96816872	0.711594731
Major facilitator superfamily protein Chr5:25165430-25167822 REVERSE LENGTH=616 201606	AT5G62680.1	-3.96816872	0.711594731
Tetratricopeptide repeat (TPR)-like superfamily protein Chr2:15637177-15639450 REVERSE LENGTH=757 201606	AT2G37230.1	-4.0719281	0.666790457
Phosphoribosyltransferase family protein Chr2:17856396-17858394 FORWARD LENGTH=337 201606	AT2G42910.1	-4.0719281	0.666790457
GTP-binding family protein Chr3:2229602-2232279 REVERSE LENGTH=582 201606	AT3G07050.1	-4.0719281	0.666790457
DNA GYRASE A Chr3:3339612-3346243 REVERSE LENGTH=950 201606	AT3G10690.1	-4.0719281	0.666790457
pumilio 24 Chr3:5723436-5727539 REVERSE LENGTH=641 201606	AT3G16810.1	-4.0719281	0.666790457
expansin-like A1 Chr3:16896238-16897189 FORWARD LENGTH=265 201606	AT3G45970.1	-4.0719281	0.666790457
sugar transporter protein 7 Chr4:898387-900095 REVERSE LENGTH=513 201606	AT4G02050.1 (+1)	-4.0719281	0.666790457
inosine-uridine preferring nucleoside hydrolase family protein Chr5:6291023-6295581 FORWARD LENGTH=890 201606	AT5G18860.1 (+1)	-4.0719281	0.666790457
Disease resistance-responsive (dirigent-like protein) family protein Chr5:16998268-16998816 REVERSE LENGTH=182 201606	AT5G42510.1	-4.0719281	0.666790457
EXORDIUM like 3 Chr5:20939793-20940806 REVERSE LENGTH=337 201606	AT5G51550.1	-4.0719281	0.666790457
Ribosomal protein L31 Chr1:28272163-28272687 FORWARD LENGTH=144 201606	AT1G75350.1	-4.11440935	0.740668703
H/ACA ribonucleoprotein complex%2C subunit Gar1/Naf1 protein Chr3:1009123-1010379 REVERSE LENGTH=202 201606	AT3G03920.1	-4.11440935	0.740668703
Hydroxyproline-rich glycoprotein family protein Chr3:9354061-9357757 FORWARD LENGTH=1004 201606	AT3G25690.1 (+3)	-4.11440935	0.740668703
early nodulin-like protein 2 Chr4:13750668-13751819 REVERSE LENGTH=349 201606	AT4G27520.1	-4.11440935	0.740668703

1%2C2-alpha-L-fucosidase Chr4:16398130-16401591 FORWARD LENGTH=843 201606	AT4G34260.1	-4.11440935	0.521058235
xyloglucan endotransglucosylase/hydrolase 7 Chr4:17775703-17777372 REVERSE LENGTH=293 201606	AT4G37800.1	-4.11440935	0.740668703
Ribosomal protein S13/S18 family Chr5:4617839- 4618772 REVERSE LENGTH=169 201606	AT5G14320.1	-4.11440935	0.683964096
translation initiation factor 3 subunit H1 Chr1:3607885- 3610299 REVERSE LENGTH=337 201606	AT1G10840.1	-4.15241012	0.628158733
Pectin lyase-like superfamily protein Chr3:2212973- 2216306 REVERSE LENGTH=416 201606	AT3G07010.1	-4.15241012	0.628158733
xyloglucan endo-transglycosylase-related 8 Chr3:16447280-16448678 REVERSE LENGTH=293 201606	AT3G44990.1	-4.15241012	0.628158733
P-loop containing nucleoside triphosphate hydrolases superfamily protein Chr5:7391026-7394071 REVERSE LENGTH=458 201606	AT5G22330.1	-4.15241012	0.628158733
pyrimidine d Chr5:7847792-7850243 REVERSE LENGTH=460 201606	AT5G23300.1	-4.15241012	0.707377258
Phosphoinositide phosphatase family protein Chr1:7997889-8002787 REVERSE LENGTH=912 201606	AT1G22620.1	-4.21816872	0.725479782
sucrose-proton symporter 2 Chr1:8030911-8032970 REVERSE LENGTH=512 201606	AT1G22710.1	-4.21816872	0.725479782
nuclear RNA polymerase A2 Chr1:10479322-10486670 REVERSE LENGTH=1178 201606	AT1G29940.1	-4.21816872	0.725479782
NagB/RpiA/CoA transferase-like superfamily protein Chr2:2229759-2231839 FORWARD LENGTH=374 201606	AT2G05830.1	-4.21816872	0.597689833
MD-2-related lipid recognition domain-containing protein Chr2:6959347-6960135 REVERSE LENGTH=160 201606	AT2G16005.1	-4.21816872	0.725479782
histone H1-3 Chr2:7846095-7846670 FORWARD LENGTH=167 201606	AT2G18050.1	-4.21816872	0.725479782
AT hook motif DNA-binding family protein Chr3:1239245- 1241603 REVERSE LENGTH=411 201606	AT3G04590.2	-4.21816872	0.748524779
Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein Chr3:6453437-6453870 FORWARD LENGTH=112 201606	AT3G18740.1	-4.21816872	0.597689833
Eukaryotic aspartyl protease family protein Chr3:9358937-9360295 FORWARD LENGTH=452 201606	AT3G25700.1	-4.21816872	0.725479782
Nodulin MtN3 family protein Chr3:18052814-18054663 REVERSE LENGTH=289 201606	AT3G48740.1	-4.21816872	0.725479782
transcriptional regulator Chr4:15419435-15423939 REVERSE LENGTH=872 201606	AT4G31880.2	-4.21816872	0.638867115
Lipase/lipoxygenase%2C PLAT/LH2 family protein Chr4:18432950-18433581 FORWARD LENGTH=181 201606	AT4G39730.1	-4.21816872	0.725479782
SART-1 family Chr5:5517784-5521566 REVERSE LENGTH=820 201606	AT5G16780.1 (+2)	-4.21816872	0.725479782
chloride channel A Chr5:16381645-16384999 REVERSE LENGTH=775 201606	AT5G40890.1	-4.21816872	0.725479782
CLP protease proteolytic subunit 2 Chr1:4223099- 4224954 FORWARD LENGTH=279 201606	AT1G12410.1	-4.24592655	0.538506346
Plant invertase/pectin methylesterase inhibitor superfamily protein Chr5:25037504-25038112 FORWARD LENGTH=202 201606	AT5G62350.1	-4.26064997	0.509830181
Rho termination factor Chr1:1892589-1894078 REVERSE LENGTH=292 201606	AT1G06190.2	-4.29865074	0.696652675
pyruvate dehydrogenase E1 beta Chr1:10584350- 10586477 REVERSE LENGTH=406 201606	AT1G30120.1	-4.29865074	0.696652675
Alba DNA/RNA-binding protein Chr3:2223001-2225254 REVERSE LENGTH=406 201606	AT3G07030.2 (+2)	-4.29865074	0.763573772
strictosidine synthase-like 4 Chr3:19084082-19085451 FORWARD LENGTH=370 201606	AT3G51420.1	-4.29865074	0.696652675
Glutathione S-transferase family protein Chr4:10784691- 10786376 REVERSE LENGTH=382 201606	AT4G19880.2	-4.29865074	0.763573772
U4/U6.U5 small nuclear ribonucleoprotein Chr5:23244811-23245470 FORWARD LENGTH=219 201606	AT5G57370.1	-4.29865074	0.696652675
signal recognition particle-related / SRP-like protein Chr5:24888920-24893079 FORWARD LENGTH=605 201606	AT5G61970.1	-4.29865074	0.650828744
poly(A) binding protein 4 Chr2:9943209-9946041 FORWARD LENGTH=662 201606	AT2G23350.1	-4.3219281	0.554948533

SC35-like splicing factor 30A Chr3:4429564-4431602 REVERSE LENGTH=262 201606	AT3G13570.1	-4.3219281	0.604863704
Peroxidase superfamily protein Chr3:18524313-18525610 REVERSE LENGTH=329 201606	AT3G49960.1	-4.3219281	0.740668703
DEA(D/H)-box RNA helicase family protein Chr3:22666590-22669154 FORWARD LENGTH=498 201606	AT3G61240.1 (+1)	-4.3219281	0.740668703
Peroxidase superfamily protein Chr5:23599755-23601244 REVERSE LENGTH=316 201606	AT5G58390.1	-4.3219281	0.711097516
RNA polymerase subunit beta ChrC:23111-26329 REVERSE LENGTH=1072 201606	ATCG00190.1	-4.3219281	0.888045677
isopropyl malate isomerase large subunit 1 Chr4:7804194-7807789 REVERSE LENGTH=509 201606	AT4G13430.1	-4.33665152	0.748654492
20S proteasome beta subunit E1 Chr1:4452641-4454663 FORWARD LENGTH=274 201606	AT1G13060.1	-4.36440935	0.666933498
sucrose-proton symporter 1 Chr1:27054334-27056100 FORWARD LENGTH=513 201606	AT1G71880.1	-4.36440935	0.53972177
casein kinase II%2C alpha chain 2 Chr3:18534487-18536743 FORWARD LENGTH=403 201606	AT3G50000.1	-4.36440935	0.572086786
Remorin family protein Chr4:278434-279170 REVERSE LENGTH=123 201606	AT4G00670.1	-4.36440935	0.666933498
Cupredoxin superfamily protein Chr4:7349941-7352868 REVERSE LENGTH=587 201606	AT4G12420.1 (+1)	-4.36440935	0.702119055
flocculation protein Chr4:15248510-15251945 FORWARD LENGTH=485 201606	AT4G31430.1 (+1)	-4.36440935	0.666933498
ribosomal protein S12C ChrC:97999-98793 REVERSE LENGTH=85 201606	ATCG00905.1 (+1)	-4.36440935	0.666933498
chloroplast 30S ribosomal protein S20 Chr3:5116216-5117412 FORWARD LENGTH=202 201606	AT3G15190.1	-4.37913277	0.704190828
small subunit ribosomal protein 16 Chr4:16535084-16536092 REVERSE LENGTH=113 201606	AT4G34620.1	-4.40241012	0.84201419
cinnamyl alcohol dehydrogenase 9 Chr4:18291268-18292772 FORWARD LENGTH=360 201606	AT4G39330.1	-4.40241012	0.753566653
Protein kinase superfamily protein Chr5:359154-360867 FORWARD LENGTH=495 201606	AT5G01920.1 (+1)	-4.40241012	0.73139157
Class II aminoacyl-tRNA and biotin synthetases superfamily protein Chr4:15156696-15159362 FORWARD LENGTH=558 201606	AT4G31180.1 (+1)	-4.42000745	0.721970026
Glycosyl hydrolase family 35 protein Chr5:25530323-25535678 FORWARD LENGTH=718 201606	AT5G63800.1	-4.42000745	0.67704566
Nucleolar GTP-binding protein Chr1:18870555-18872570 FORWARD LENGTH=671 201606	AT1G50920.1	-4.42568747	0.672215956
SPFH/Band 7/PHB domain-containing membrane-associated protein family Chr4:13766984-13769832 REVERSE LENGTH=411 201606	AT4G27585.1	-4.42568747	0.672215956
ARM repeat superfamily protein Chr5:14273519-14276773 FORWARD LENGTH=411 201606	AT5G36230.1	-4.44489137	0.937610492
Ribosomal protein L1p/L10e family Chr3:21701574-21702914 FORWARD LENGTH=446 201606	AT3G58660.1	-4.46816872	0.711705103
Remorin family protein Chr3:22675403-22676701 REVERSE LENGTH=212 201606	AT3G61260.1	-4.46816872	0.711705103
RmIC-like cupins superfamily protein Chr5:15588699-15589526 FORWARD LENGTH=247 201606	AT5G38940.2	-4.46816872	0.617330832
protein kinase family protein Chr5:22077313-22079880 REVERSE LENGTH=855 201606	AT5G54380.1	-4.46816872	0.617330832
cellulose synthase 6 Chr5:25881555-25886333 FORWARD LENGTH=1084 201606	AT5G64740.1	-4.46816872	0.711705103
ubiquitin-specific protease 6 Chr1:19175805-19179894 REVERSE LENGTH=482 201606	AT1G51710.1	-4.48289214	0.875849911
hypothetical protein Chr4:17226139-17226507 REVERSE LENGTH=122 201606	AT4G36500.1	-4.48289214	0.683964096
IQ-domain 31 Chr1:28061498-28063924 REVERSE LENGTH=587 201606	AT1G74690.1	-4.48576605	0.64977315
Pyruvate kinase family protein Chr5:22820254-22822529 REVERSE LENGTH=498 201606	AT5G56350.1	-4.49703802	0.516544685
TGF-beta receptor interacting protein 1 Chr2:19003656-19005393 REVERSE LENGTH=328 201606	AT2G46280.1 (+1)	-4.51064997	0.569467145
Peroxidase superfamily protein Chr2:16079726-16081381 FORWARD LENGTH=349 201606	AT2G38390.1	-4.52376683	0.789569756
Cleavage/polyadenylation specificity factor%2C 25kDa subunit Chr4:13048519-13050873 FORWARD LENGTH=200 201606	AT4G25550.1	-4.52376683	0.6032409

RNI-like superfamily protein Chr1:3461771-3465590 FORWARD LENGTH=605 201606	AT1G10510.1	-4.54865074	0.860156961
SPFH/Band 7/PHB domain-containing membrane-associated protein family Chr2:1066717-1068934 FORWARD LENGTH=356 201606	AT2G03510.1	-4.54865074	1.000934901
sulfite oxidase Chr3:314919-317274 REVERSE LENGTH=393 201606	AT3G01910.1	-4.54865074	0.734428842
Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein Chr2:4311160-4312035 REVERSE LENGTH=291 201606	AT2G10940.1 (+1)	-4.5719281	0.919027904
GDSL-like Lipase/Acylhydrolase superfamily protein Chr3:5556928-5558351 FORWARD LENGTH=353 201606	AT3G16370.1	-4.5719281	0.755936716
Endomembrane protein 70 protein family Chr1:5037669-5040199 FORWARD LENGTH=592 201606	AT1G14670.1	-4.58952543	0.73407795
beta-galactosidase 2 Chr3:19581082-19586097 FORWARD LENGTH=781 201606	AT3G52840.2	-4.60424885	0.720243776
Subtilase family protein Chr4:11501314-11504656 REVERSE LENGTH=766 201606	AT4G21650.1	-4.60424885	0.720243776
Thiamine pyrophosphate dependent pyruvate decarboxylase family protein Chr4:15952519-15954676 REVERSE LENGTH=607 201606	AT4G33070.1	-4.60424885	0.720243776
DEAD box RNA helicase (PRH75) Chr5:24980542-24983879 REVERSE LENGTH=671 201606	AT5G62190.1	-4.61153544	0.563211921
Ribosomal protein S26e family protein Chr2:16945215-16946345 REVERSE LENGTH=131 201606	AT2G40590.1	-4.61440935	0.596463857
ATP binding cassette subfamily B19 Chr3:10870287-10877286 REVERSE LENGTH=1252 201606	AT3G28860.1	-4.61440935	0.659887975
methyl esterase 14 Chr1:12355909-12357894 FORWARD LENGTH=348 201606	AT1G33990.1	-4.62184618	0.528464721
Ribosomal L28 family Chr2:14173620-14174380 FORWARD LENGTH=143 201606	AT2G33450.1	-4.62184618	0.789430625
Polynucleotidyl transferase%2C ribonuclease H-like superfamily protein Chr3:3721490-3722092 REVERSE LENGTH=200 201606	AT3G11770.1	-4.6376867	0.744712065
RNA-binding (RRM/RBD/RNP motifs) family protein Chr4:16953211-16955127 REVERSE LENGTH=239 201606	AT4G35785.2	-4.65241012	0.970286681
Mannose-binding lectin superfamily protein Chr1:19383291-19385123 REVERSE LENGTH=437 201606	AT1G52100.1	-4.67000745	0.509013437
Subtilisin-like serine endopeptidase family protein Chr2:1401450-1407694 REVERSE LENGTH=772 201606	AT2G04160.1	-4.67000745	0.761983833
RNA-binding (RRM/RBD/RNP motifs) family protein Chr5:22544669-22546801 REVERSE LENGTH=710 201606	AT5G55670.1	-4.67000745	0.736189261
chloroplast ribosomal protein S14 ChrC:36938-37240 REVERSE LENGTH=100 201606	ATCG00330.1	-4.67000745	0.723159082
Alba DNA/RNA-binding protein Chr1:10223461-10224592 REVERSE LENGTH=130 201606	AT1G29250.1	-4.68473087	0.563370448
modifier of snc1 Chr4:12733425-12739737 FORWARD LENGTH=1427 201606	AT4G24680.1	-4.686786	0.666054157
rapid alkalinization factor 23 Chr3:5644748-5645164 FORWARD LENGTH=138 201606	AT3G16570.1 (+2)	-4.69489137	0.66525011
xyloglucan endotransglucosylase/hydrolase 32 Chr2:15472869-15474630 REVERSE LENGTH=299 201606	AT2G36870.1	-4.71816872	0.725522027
Xanthine/uracil permease family protein Chr5:25243723-25247075 FORWARD LENGTH=532 201606	AT5G62890.1 (+2)	-4.71816872	0.824893081
SH3 domain-containing protein Chr2:3047623-3057099 REVERSE LENGTH=1196 201606	AT2G07360.1 (+1)	-4.73289214	0.881591407
Alba DNA/RNA-binding protein Chr1:7005090-7007285 REVERSE LENGTH=315 201606	AT1G20220.1	-4.73576605	0.85877009
MIF4G domain-containing protein / MA3 domain-containing protein Chr1:30405774-30409499 REVERSE LENGTH=900 201606	AT1G80930.1	-4.73687753	0.524562633
Ribosomal protein L19 family protein Chr4:9780343-9781752 FORWARD LENGTH=225 201606	AT4G17560.1	-4.75616949	0.910854951
expansin A6 Chr2:12431840-12433482 REVERSE LENGTH=257 201606	AT2G28950.1	-4.77376683	0.737298732
Ribosomal protein L19e family protein Chr3:5708982-5710249 FORWARD LENGTH=209 201606	AT3G16780.1	-4.77376683	0.737298732
Peroxidase superfamily protein Chr5:25654575-25655946 REVERSE LENGTH=330 201606	AT5G64110.1	-4.77537339	0.62818098

DYNAMIN-like 1E Chr3:22244367-22247651 REVERSE LENGTH=624 201606	AT3G60190.1	-4.78553389	0.700050749
Cleavage and polyadenylation specificity factor (CPSF) A subunit protein Chr3:20460533-20464361 FORWARD LENGTH=1214 201606	AT3G55200.1 (+3)	-4.78849025	0.750857608
Leucine-rich repeat (LRR) family protein Chr5:4087782-4088897 FORWARD LENGTH=371 201606	AT5G12940.1	-4.79865074	0.535546448
LOW protein: zinc finger CCCH domain protein Chr5:21683679-21687970 REVERSE LENGTH=1181 201606	AT5G53440.1 (+1)	-4.79865074	0.688625121
Ribosomal protein L31e family protein Chr5:22944003-22944767 REVERSE LENGTH=119 201606	AT5G56710.1	-4.79865074	0.635507086
RNA polymerase I-associated factor PAF67 Chr5:8953564-8955511 FORWARD LENGTH=514 201606	AT5G25754.1 (+1)	-4.81624808	0.868131126
EXORDIUM like 4 Chr5:2938397-2939233 FORWARD LENGTH=278 201606	AT5G09440.1	-4.820902	0.500814752
Ribosomal protein S7e family protein Chr1:18059854-18060935 REVERSE LENGTH=191 201606	AT1G48830.1 (+1)	-4.82640857	0.562120726
beta-hexosaminidase 3 Chr1:24385996-24390989 FORWARD LENGTH=535 201606	AT1G65590.1	-4.83067695	0.600531061
serine/arginine-rich splicing factor-like protein%2C putative Chr5:25681849-25683553 REVERSE LENGTH=303 201606	AT5G64200.1 (+1)	-4.83952543	0.909118869
AAA-type ATPase family protein Chr5:5568578-5571565 FORWARD LENGTH=644 201606	AT5G16930.1	-4.85424885	0.808428102
DNA-directed RNA polymerase family protein Chr4:11535684-11542200 REVERSE LENGTH=1188 201606	AT4G21710.1	-4.86440935	0.535613593
ribosomal protein S15A D Chr3:16914891-16915529 FORWARD LENGTH=130 201606	AT3G46040.1	-4.87913277	0.938074529
SC35-like splicing factor 30 Chr3:20561024-20563502 FORWARD LENGTH=262 201606	AT3G55460.1	-4.87913277	0.6276238
homeobox-1 Chr1:9979936-9987460 FORWARD LENGTH=1705 201606	AT1G28420.1	-4.92000745	0.934232042
binding protein Chr5:19317899-19327014 FORWARD LENGTH=1607 201606	AT5G47690.3	-4.92729404	0.683493627
Ribosomal protein L13 family protein Chr3:2252092-2253332 FORWARD LENGTH=206 201606	AT3G07110.1 (+1)	-4.94041089	0.704761035
myosinase-binding protein 2 Chr1:19346090-19348282 REVERSE LENGTH=642 201606	AT1G52030.1 (+1)	-4.94122968	0.579797363
Alba DNA/RNA-binding protein Chr1:28528505-28530488 REVERSE LENGTH=350 201606	AT1G76010.1 (+1)	-4.96816872	0.720599752
Ribosomal L29 family protein Chr2:16450803-16451762 REVERSE LENGTH=123 201606	AT2G39390.1	-4.96816872	0.711760058
root hair specific 12 Chr3:3352289-3354237 FORWARD LENGTH=561 201606	AT3G10710.1	-4.96816872	0.632699319
poly(A) binding protein 2 Chr4:16336732-16339892 FORWARD LENGTH=629 201606	AT4G34110.1	-4.96816872	0.668543748
phospholipase D delta Chr4:16955774-16959875 REVERSE LENGTH=868 201606	AT4G35790.1	-4.96910675	0.89303886
PLC-like phosphodiesterases superfamily protein Chr1:27910396-27912785 FORWARD LENGTH=392 201606	AT1G74210.1	-4.9724371	0.672348119
arabinogalactan protein 31 Chr1:9889331-9890843 REVERSE LENGTH=359 201606	AT1G28290.1 (+1)	-4.97560556	0.607115465
GDSL-like Lipase/Acylhydrolase superfamily protein Chr4:14738603-14740548 REVERSE LENGTH=348 201606	AT4G30140.1	-4.97560556	0.748786739
Pathogenesis-related thaumatin superfamily protein Chr2:12354664-12355488 REVERSE LENGTH=274 201606	AT2G28790.2	-4.98289214	0.673152201
filaggrin-like protein Chr1:23888967-23889503 FORWARD LENGTH=178 201606	AT1G64370.1	-4.99305264	0.704325443
ribosomal protein S18 ChrC:67917-68222 FORWARD LENGTH=101 201606	ATCG00650.1	-5.03737877	0.529290571
U5 small nuclear ribonucleoprotein helicase Chr1:7302591-7309914 REVERSE LENGTH=2171 201606	AT1G20960.1 (+1)	-5.07389516	0.738957314
Ribosomal protein L35Ae family protein Chr1:15651585-15652427 REVERSE LENGTH=111 201606	AT1G41880.1 (+1)	-5.07752005	1.15821335
winged-helix DNA-binding transcription factor family protein Chr1:2076687-2077616 REVERSE LENGTH=274 201606	AT1G06760.1	-5.08003346	0.642723754

Peroxidase superfamily protein Chr2:15598225-15599734 REVERSE LENGTH=296 201606	AT2G37130.2	-5.0809715	0.515770989
IQ-domain 2 Chr5:710380-712406 REVERSE LENGTH=461 201606	AT5G03040.1 (+2)	-5.08302663	0.884833661
Galactose mutarotase-like superfamily protein Chr4:13161487-13163397 FORWARD LENGTH=318 201606	AT4G25900.1	-5.09681201	0.900128214
Ribosomal protein S25 family protein Chr2:9236629-9237510 FORWARD LENGTH=108 201606	AT2G21580.1 (+1)	-5.16051549	0.724924645
SKU5 similar 5 Chr1:28578211-28581020 REVERSE LENGTH=541 201606	AT1G76160.1	-5.17000745	0.772375121
beta glucosidase 25 Chr3:881028-884028 FORWARD LENGTH=531 201606	AT3G03640.1	-5.17264012	0.636044786
hypothetical protein Chr3:18676436-18683863 REVERSE LENGTH=2156 201606	AT3G50370.1	-5.24703802	0.852754868
SKU5 similar 4 Chr4:11663429-11666463 FORWARD LENGTH=541 201606	AT4G22010.1	-5.25239277	0.735071559
GDSL-like Lipase/Acylhydrolase superfamily protein Chr1:12267918-12269690 FORWARD LENGTH=370 201606	AT1G33811.1	-5.26624192	0.914883542
heat shock-like protein Chr5:21352557-21355147 FORWARD LENGTH=700 201606	AT5G52640.1	-5.26653865	0.571423102
Splicing factor%2C CC1-like protein Chr2:7342869-7347052 REVERSE LENGTH=599 201606	AT2G16940.3	-5.27376683	0.889703949
lipoxygenase 1 Chr1:20525798-20530143 FORWARD LENGTH=859 201606	AT1G55020.1	-5.28486536	1.001148764
Histone superfamily protein Chr4:18555840-18556827 REVERSE LENGTH=164 201606	AT4G40030.2	-5.28715419	0.585834761
glycine-rich protein Chr1:1348635-1349237 REVERSE LENGTH=200 201606	AT1G04800.1	-5.33302663	0.958400783
chromatin-remodeling protein 11 Chr3:1941066-1946700 FORWARD LENGTH=1055 201606	AT3G06400.1 (+2)	-5.33679021	0.787273721
Remorin family protein Chr2:18863147-18864576 REVERSE LENGTH=190 201606	AT2G45820.1	-5.34775005	0.686797929
pleiotropic drug resistance 4 Chr2:11481623-11487874 FORWARD LENGTH=1420 201606	AT2G26910.1	-5.35424885	0.717918593
nucleolin like 1 Chr1:18098186-18101422 FORWARD LENGTH=557 201606	AT1G48920.1	-5.3564414	0.537022169
Ribosomal protein L6 family protein Chr1:27847256-27848680 REVERSE LENGTH=233 201606	AT1G74050.1 (+1)	-5.36189593	0.740771005
beta glucosidase 34 Chr1:17491771-17494589 FORWARD LENGTH=511 201606	AT1G47600.1 (+1)	-5.36440935	0.648435133
NOP56-like pre RNA processing ribonucleoprotein Chr1:20984544-20986893 REVERSE LENGTH=522 201606	AT1G56110.1	-5.37314279	0.846687987
Leucine-rich repeat (LRR) family protein Chr4:7758610-7760892 FORWARD LENGTH=760 201606	AT4G13340.1	-5.37741117	0.549046157
Ribosomal protein L18ae/LX family protein Chr3:4910773-4911933 FORWARD LENGTH=178 201606	AT3G14600.1	-5.43127942	1.083996369
Subtilase family protein Chr2:2269831-2272207 REVERSE LENGTH=754 201606	AT2G05920.1	-5.4608781	0.655655129
Subtilase family protein Chr1:1061457-1063784 REVERSE LENGTH=775 201606	AT1G04110.1	-5.50048947	0.736061915
putative mitochondrial RNA helicase 2 Chr3:7892641-7895145 FORWARD LENGTH=616 201606	AT3G22330.1	-5.51445641	0.587913537
dynamin-like 3 Chr1:21893413-21900780 FORWARD LENGTH=920 201606	AT1G59610.1	-5.52612402	0.85739429
Ribosomal protein S3Ae Chr4:16548724-16550222 FORWARD LENGTH=262 201606	AT4G34670.1	-5.53072814	0.572302785
GDSL-like Lipase/Acylhydrolase superfamily protein Chr1:10375639-10377717 FORWARD LENGTH=431 201606	AT1G29670.2	-5.54865074	0.957348311
P-loop containing nucleoside triphosphate hydrolases superfamily protein Chr3:21657099-21660352 FORWARD LENGTH=646 201606	AT3G58570.1	-5.69210623	0.634657061
dynamin-like protein Chr5:16820661-16824536 REVERSE LENGTH=610 201606	AT5G42080.1 (+1)	-5.8090199	0.812421777
Mannose-binding lectin superfamily protein Chr1:19333352-19335700 REVERSE LENGTH=730 201606	AT1G52000.1 (+1)	-5.8140516	0.789582103
S-adenosylmethionine synthetase 1 Chr1:519037-520218 FORWARD LENGTH=393 201606	AT1G02500.1 (+1)	-5.93247737	0.885055772

eukaryotic translation initiation factor 3G1 Chr3:3578536-3580366 FORWARD LENGTH=321 201606	AT3G11400.2	-6.02537339	1.178221891
jacalin-related lectin 31 Chr3:5581830-5582959 FORWARD LENGTH=296 201606	AT3G16430.1 (+1)	-6.0607169	0.613258589
Ribosomal protein S10p/S20e family protein Chr3:4220310-4221526 REVERSE LENGTH=191 201606	AT3G13120.1 (+2)	-6.06337417	0.930270893
homologue of NAP57 Chr3:21154255-21155952 REVERSE LENGTH=565 201606	AT3G57150.1	-6.08868103	0.809279004
SAD1/UNC-84 domain protein 1 Chr5:1471698-1473770 REVERSE LENGTH=471 201606	AT5G04990.1	-6.12913277	1.107185839
Mov34/MPN/PAD-1 family protein Chr3:3528989-3531317 FORWARD LENGTH=310 201606	AT3G11270.1	-6.27537339	0.937524858
P-loop containing nucleoside triphosphate hydrolases superfamily protein Chr5:26967535-26969306 REVERSE LENGTH=469 201606	AT5G67630.1	-6.27537339	1.199191767
Transducin/WD40 repeat-like superfamily protein Chr3:18771292-18779220 FORWARD LENGTH=1606 201606	AT3G50590.2	-6.35585542	1.11961381
Ribosomal protein L17 family protein Chr3:20067672-20068385 REVERSE LENGTH=211 201606	AT3G54210.1	-6.35585542	1.11961381
chromatin protein family Chr1:28999791-29001632 REVERSE LENGTH=613 201606	AT1G77180.1 (+1)	-6.37913277	0.98738741
proline-tRNA ligase (DUF1680) Chr5:4093117-4096806 FORWARD LENGTH=861 201606	AT5G12950.1	-6.39385619	1.03673911
glyceraldehyde-3-phosphate dehydrogenase C subunit 1 Chr3:1081077-1083131 FORWARD LENGTH=338 201606	AT3G04120.1	-6.39421668	0.833222222
MOS4-associated complex 3B Chr2:14126584-14131000 REVERSE LENGTH=525 201606	AT2G33340.1 (+1)	-6.45961479	1.159929985
Endomembrane protein 70 protein family Chr5:8648374-8651015 REVERSE LENGTH=651 201606	AT5G25100.2	-6.52537339	1.09431261
dihydrolipoamide dehydrogenase Chr4:9153570-9157133 REVERSE LENGTH=567 201606	AT4G16155.1	-6.56337417	0.914473337
cytochrome oxidase 2 ChrM:40502-42628 REVERSE LENGTH=260 201606	ATMG00160.1	-6.5809715	1.027284734
pre-mRNA-processing protein 40A Chr1:16975930-16982818 FORWARD LENGTH=958 201606	AT1G44910.1	-6.60585542	1.111495285
long chain acyl-CoA synthetase 9 Chr1:29148501-29151776 REVERSE LENGTH=691 201606	AT1G77590.1	-6.68473087	0.938253955
FG-GAP repeat-containing protein Chr3:18954023-18957698 FORWARD LENGTH=698 201606	AT3G51050.1	-6.79009682	1.223077494
histone deacetylase 2B Chr5:7534120-7536054 FORWARD LENGTH=306 201606	AT5G22650.1	-6.8244727	0.874053015
prohibitin 4 Chr3:10076904-10078051 FORWARD LENGTH=279 201606	AT3G27280.1 (+1)	-7.71520674	1.187554945
glycine-rich protein 3 short isoform Chr2:1966803-1967820 FORWARD LENGTH=132 201606	AT2G05380.3	-8.12983652	0.854187578

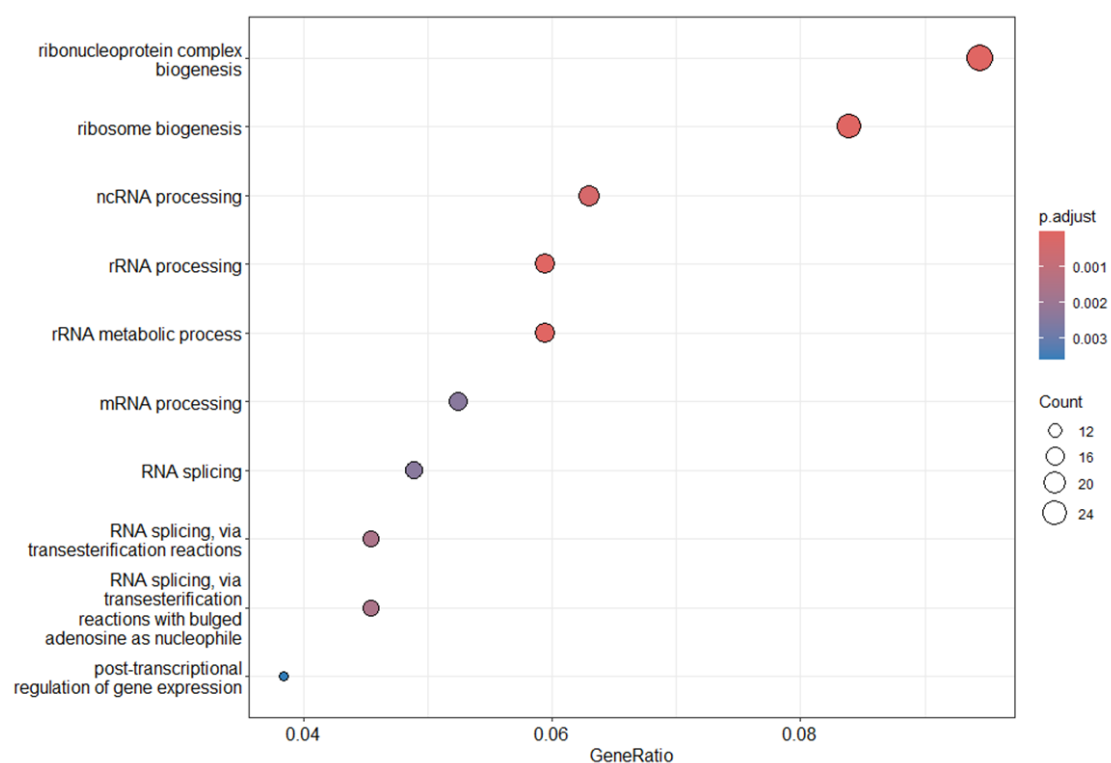


Figure S1. Gene ontology (GO) enrichment analysis of biological functions for 293 downregulated proteins identified in the PSKR1-GFP AP-MS data (Table S2).

Table S4. Table of all PSK-specific interactors identified by YFP-MKKK7 AP-MS based on log-fold change (logFC) ≥ 2 and logFC ≤ -2 with a $-\log_{10}$ p-value ≤ 0.5 . LogFC and $-\log_{10}$ p-value (log_pval) were calculated based on spectral counts. All proteins decreased in abundance after PSK treatment. Missing spectral data were imputed with a value of 0.01 for logFC and $-\log_{10}$ p-value calculations. This table corresponds to Chapter 3, Figure 3.5.

Identified Proteins	Accession Number	log_fc	log_pval
2-oxoglutarate dehydrogenase%2C E1 component Chr3:20541897-20545728 FORWARD LENGTH=1017 201606	AT3G55410.1 (+1)	-9.157676799	0.896973108
glutathione S-transferase PHI 9 Chr2:13139132-13140079 FORWARD LENGTH=166 201606	AT2G30860.2	-9.135302261	0.715160902
peroxidase CB Chr3:18207819-18210041 FORWARD LENGTH=353 201606	AT3G49120.1	-8.974338213	0.915215485
photosystem II subunit QA Chr4:11334446-11335587 FORWARD LENGTH=223 201606	AT4G21280.1	-8.90495472	0.692621056
alpha-glucan phosphorylase 2 Chr3:17301625-17306111 REVERSE LENGTH=841 201606	AT3G46970.1	-8.818966119	0.997900543
photosystem II light harvesting complex protein 2.3 Chr3:10255906-10256921 FORWARD LENGTH=298 201606	AT3G27690.2	-8.792502657	1.176500364
photosynthetic electron transfer D ChrC:76481-77672 FORWARD LENGTH=160 201606	ATCG00730.1	-8.790096815	1.628992708
P-loop containing nucleoside triphosphate hydrolases superfamily protein Chr1:27494344-27496844 REVERSE LENGTH=432 201606	AT1G73110.1	-8.663060089	1.960320454
Ion protease 2 Chr5:19093356-19098678 REVERSE LENGTH=888 201606	AT5G47040.1	-8.638176171	1.683182797
rubredoxin family protein Chr5:5649335-5650975 FORWARD LENGTH=271 201606	AT5G17170.1	-8.43633744	1.22055506
photosystem II reaction center protein H ChrC:74485-74706 FORWARD LENGTH=73 201606	ATCG00710.1	-8.120578839	1.61628191
tubulin beta chain 4 Chr5:17859442-17860994 REVERSE LENGTH=444 201606	AT5G44340.1	-7.833822808	0.714397012
Ribulose biphosphate carboxylase (small chain) family protein Chr5:15381203-15381978 REVERSE LENGTH=181 201606	AT5G38420.1	-7.625356044	0.630791308
formate dehydrogenase Chr5:4777627-4779190 FORWARD LENGTH=370 201606	AT5G14780.2	-7.53340386	0.963807767
Aldolase superfamily protein Chr2:95006-96491 REVERSE LENGTH=391 201606	AT2G01140.1	-7.39385619	0.612196435
high cyclic electron flow 1 Chr3:20016951-20018527 FORWARD LENGTH=417 201606	AT3G54050.1 (+1)	-7.294874021	0.810822489
photosystem I subunit E-2 Chr2:8736780-8737644 FORWARD LENGTH=145 201606	AT2G20260.1	-7.260023257	0.693476151
ATPase%2C V1 complex%2C subunit B protein Chr1:7016971-7020290 FORWARD LENGTH=487 201606	AT1G20260.1	-7.07873605	0.962700271
Pyridoxal phosphate (PLP)-dependent transferases superfamily protein Chr3:7835286-7838863 FORWARD LENGTH=504 201606	AT3G22200.1	-7.057694147	0.656270193
Aldolase superfamily protein Chr2:15296929-15298387 REVERSE LENGTH=358 201606	AT2G36460.1	-7.007291659	0.624909275
Translation elongation factor EF1B%2C gamma chain Chr1:3120162-3122152 FORWARD LENGTH=414 201606	AT1G09640.1	-6.99432239	0.769371842
ABC-2 and Plant PDR ABC-type transporter family protein Chr1:22034661-22039844 FORWARD LENGTH=1469 201606	AT1G59870.1	-6.878224881	1.331684926
phospholipase D alpha 1 Chr3:5330835-5333474 FORWARD LENGTH=810 201606	AT3G15730.1	-6.874787966	0.819156873
nitrogen fixation S (NIFS)-like 1 Chr5:26296349-26297710 FORWARD LENGTH=453 201606	AT5G65720.1 (+1)	-6.847750047	0.887527842
ATP citrate lyase (ACL) family protein Chr2:8805574-8807858 FORWARD LENGTH=421 201606	AT2G20420.1	-6.804242697	0.782730135
phospholipase C 2 Chr3:2582626-2585556 REVERSE LENGTH=552 201606	AT3G08510.3 (+1)	-6.790096815	0.913952729

2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein Chr2:10830286-10831563 REVERSE LENGTH=359 201606	AT2G25450.1	-6.762428978	0.659046665
G-box regulating factor 6 Chr5:3284452-3286261 REVERSE LENGTH=248 201606	AT5G10450.1	-6.743990672	0.827021018
Cytochrome C1 family Chr3:10056144-10058370 REVERSE LENGTH=307 201606	AT3G27240.1	-6.684730873	0.685017319
Ribosomal L22e protein family Chr3:1614641-1615204 FORWARD LENGTH=124 201606	AT3G05560.1 (+2)	-6.671614018	0.912568221
peroxisomal 3-ketoacyl-CoA thiolase 3 Chr2:14047814-14050983 REVERSE LENGTH=462 201606	AT2G33150.1	-6.670706131	0.816462724
glutathione synthetase 2 Chr5:9668211-9670912 REVERSE LENGTH=539 201606	AT5G27380.1	-6.658002072	0.706200014
glycine decarboxylase P-protein 1 Chr4:15926852-15931150 REVERSE LENGTH=1037 201606	AT4G33010.1	-6.647377111	0.631872399
cytosolic NADP+-dependent isocitrate dehydrogenase Chr1:24539088-24541861 FORWARD LENGTH=410 201606	AT1G65930.1	-6.62988339	0.611815796
Ribosomal protein L23/L15e family protein Chr4:9714407-9715545 REVERSE LENGTH=204 201606	AT4G17390.1	-6.618662956	0.8308625
hydroxymethylbilane synthase Chr5:2663763-2665596 REVERSE LENGTH=382 201606	AT5G08280.1	-6.613782631	0.518524312
aldehyde dehydrogenase 2B4 Chr3:17717082-17719843 REVERSE LENGTH=538 201606	AT3G48000.1	-6.607002656	0.750793292
DEAD box RNA helicase (RH3) Chr5:9285540-9288871 REVERSE LENGTH=747 201606	AT5G26742.1 (+1)	-6.605855416	0.624830763
2-oxoacid dehydrogenases acyltransferase family protein Chr3:9460632-9462585 FORWARD LENGTH=480 201606	AT3G25860.1	-6.605855416	0.806437363
Phosphoglycerate mutase%2C 2%2C3-bisphosphoglycerate-independent Chr1:3165550-3167812 REVERSE LENGTH=557 201606	AT1G09780.1	-6.604361924	0.696470767
cell division cycle 48 Chr3:3019494-3022832 FORWARD LENGTH=809 201606	AT3G09840.1	-6.600895592	0.612399521
ribosomal protein S2 ChrC:15013-15723 REVERSE LENGTH=236 201606	ATCG00160.1	-6.563374166	0.791809425
SPFH/Band 7/PHB domain-containing membrane-associated protein family Chr3:88252-89356 REVERSE LENGTH=285 201606	AT3G01290.1	-6.558255781	1.205269374
acetyl Co-enzyme a carboxylase carboxyltransferase alpha subunit Chr2:15917612-15920749 FORWARD LENGTH=769 201606	AT2G38040.1 (+1)	-6.538040317	0.811148057
NAD(P)-binding Rossmann-fold superfamily protein Chr2:2322876-2324867 FORWARD LENGTH=390 201606	AT2G05990.1 (+1)	-6.525373393	0.550642986
beta galactosidase 1 Chr3:4511192-4515756 FORWARD LENGTH=847 201606	AT3G13750.1	-6.493990672	0.706193855
Ribosomal protein S3Ae Chr3:1329751-1331418 FORWARD LENGTH=262 201606	AT3G04840.1	-6.487772467	0.875028419
ATP-citrate lyase B-1 Chr3:2079247-2082633 REVERSE LENGTH=608 201606	AT3G06650.1 (+1)	-6.485743452	1.009263899
monodehydroascorbate reductase 6 Chr1:23730095-23733534 FORWARD LENGTH=493 201606	AT1G63940.2	-6.458841208	0.590233444
signal peptide peptidase Chr1:27824465-27828807 FORWARD LENGTH=677 201606	AT1G73990.1	-6.420120526	1.70432797
Chaperone protein htpG family protein Chr2:1281983-1285909 FORWARD LENGTH=777 201606	AT2G04030.2	-6.366633941	0.65981085
ribosomal protein L16 ChrC:81189-82652 REVERSE LENGTH=135 201606	ATCG00790.1	-6.355855416	1.11961381
Transketolase family protein Chr5:20689671-20692976 FORWARD LENGTH=363 201606	AT5G50850.1	-6.329478006	0.967772762
3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 1 Chr4:18539654-18541832 FORWARD LENGTH=525 201606	AT4G39980.1	-6.322626775	1.234024434
Ribosomal L29 family protein Chr5:26061301-26062506 FORWARD LENGTH=173 201606	AT5G65220.1	-6.313374166	0.671394501
vacuolar ATP synthase subunit D (VATD) / V-ATPase D subunit / vacuolar proton pump D subunit (VATPD) Chr3:21718495-21719280 REVERSE LENGTH=261 201606	AT3G58730.1	-6.209614791	1.018034564
acetyl-CoA carboxylase carboxyl transferase subunit beta ChrC:57075-58541 FORWARD LENGTH=488 201606	ATCG00500.1	-6.179941214	0.623739775
glyceraldehyde 3-phosphate dehydrogenase A subunit 2 Chr1:4392634-4394409 REVERSE LENGTH=441 201606	AT1G12900.5	-6.161178618	0.718767759
tubulin beta-7 chain Chr2:12644258-12645932 REVERSE LENGTH=449 201606	AT2G29550.1	-6.159800947	0.692178613

sedoheptulose-bisphosphatase Chr3:20709640-20711421 FORWARD LENGTH=393 201606	AT3G55800.1	-6.113483932	0.627162217
Coatomer%2C alpha subunit Chr2:9152428-9156577 FORWARD LENGTH=1218 201606	AT2G21390.1	-6.097533294	1.125851135
sucrose synthase 1 Chr5:7050599-7054032 REVERSE LENGTH=808 201606	AT5G20830.1 (+2)	-6.061880674	0.611518291
polygalacturonase inhibiting protein 2 Chr5:2133941-2135016 FORWARD LENGTH=330 201606	AT5G06870.1	-6.055404472	0.769976546
Ribosomal protein L3 family protein Chr2:17894898-17895713 FORWARD LENGTH=271 201606	AT2G43030.1	-5.992144752	0.627918781
ribosomal protein L2 ChrC:84337-85843 REVERSE LENGTH=274 201606	ATCG00830.1 (+1)	-5.980161973	0.778384199
Eukaryotic aspartyl protease family protein Chr3:20140291-20142599 REVERSE LENGTH=425 201606	AT3G54400.1	-5.895236607	0.666100676
Phosphoglycerate kinase family protein Chr1:21028403-21030454 FORWARD LENGTH=478 201606	AT1G56190.1	-5.822631849	0.5895726
photosystem I subunit G Chr1:20802874-20803356 REVERSE LENGTH=160 201606	AT1G55670.1	-5.785174473	0.541826125
Ribosomal protein S24e family protein Chr3:1360989-1362065 FORWARD LENGTH=133 201606	AT3G04920.1	-5.781774692	0.8371004
Clp ATPase Chr3:18122363-18126008 REVERSE LENGTH=952 201606	AT3G48870.1 (+2)	-5.769311506	0.557675267
D-3-phosphoglycerate dehydrogenase Chr4:16374041-16376561 REVERSE LENGTH=603 201606	AT4G34200.1	-5.746144672	0.558185995
dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48kDa subunit family protein Chr5:26617840-26620581 REVERSE LENGTH=437 201606	AT5G66680.1	-5.740223929	0.588790611
S-adenosylmethionine synthetase 2 Chr4:796298-797479 REVERSE LENGTH=393 201606	AT4G01850.1 (+1)	-5.624291436	0.642079773
nitrile specifier protein 1 Chr3:5566516-5568330 FORWARD LENGTH=470 201606	AT3G16400.1 (+1)	-5.613154827	0.602055578
Clathrin%2C heavy chain Chr3:2587171-2595411 REVERSE LENGTH=1703 201606	AT3G08530.1	-5.578904594	0.701864701
phosphoglycerate kinase Chr1:29924347-29926295 REVERSE LENGTH=401 201606	AT1G79550.1 (+1)	-5.534865355	0.638755628
H[+]-ATPase 2 Chr4:14770820-14775920 REVERSE LENGTH=948 201606	AT4G30190.1	-5.518903735	0.620889751
methionine adenosyltransferase 3 Chr2:15479721-15480893 REVERSE LENGTH=390 201606	AT2G36880.1 (+1)	-5.375507875	0.63173801
ribulose biphosphate carboxylase small chain 1A Chr1:25048465-25049249 REVERSE LENGTH=180 201606	AT1G67090.1	-5.354248849	0.609408434
Phosphoglucosyltransferase/phosphomannosyltransferase family protein Chr1:26669020-26672726 REVERSE LENGTH=585 201606	AT1G70730.1	-5.194891369	0.648945939
cysteine synthase C1 Chr3:22735885-22737792 FORWARD LENGTH=368 201606	AT3G61440.1	-5.186786	0.612382351
UDP-GLUCOSE PYROPHOSPHORYLASE 1 Chr3:749761-754014 REVERSE LENGTH=469 201606	AT3G03250.1	-5.186786	0.612382351
malate dehydrogenase Chr3:17513657-17514868 FORWARD LENGTH=403 201606	AT3G47520.1	-5.156890596	0.667123605
O-acetylserine (thiol) lyase B Chr2:18129604-18132322 REVERSE LENGTH=392 201606	AT2G43750.1 (+1)	-5.104248849	0.606022979
Enoyl-CoA hydratase/isomerase family Chr4:14297312-14302016 REVERSE LENGTH=721 201606	AT4G29010.1	-5.093793805	0.656688481
glutathione S-transferase PHI 2 Chr4:1110673-1111531 REVERSE LENGTH=212 201606	AT4G02520.1	-5.085748704	0.614552587
glutathione S-transferase 6 Chr1:661363-662191 REVERSE LENGTH=208 201606	AT1G02930.1 (+1)	-5.051643904	0.740668703
Glycosyl hydrolase superfamily protein Chr4:9200180-9201245 REVERSE LENGTH=315 201606	AT4G16260.2	-5.023766825	0.644023121
glutathione S-transferase PHI 10 Chr2:13141490-13142392 FORWARD LENGTH=215 201606	AT2G30870.1	-4.99003443	0.607377557
aconitase 2 Chr4:13543077-13548427 FORWARD LENGTH=995 201606	AT4G26970.1	-4.982892142	0.504403253
light-harvesting chlorophyll-protein complex I subunit A4 Chr3:17493622-17494773 REVERSE LENGTH=251 201606	AT3G47470.1	-4.977421329	1.109860231
ascorbate peroxidase 1 Chr1:2438005-2439435 FORWARD LENGTH=250 201606	AT1G07890.1 (+7)	-4.96816872	0.617507176

Photosystem I reaction centre subunit IV / PsaE protein Chr4:14202951-14203888 REVERSE LENGTH=143 201606	AT4G28750.1	-4.944891369	0.62850352
succinate dehydrogenase 1-1 Chr5:26653776-26657224 FORWARD LENGTH=634 201606	AT5G66760.1	-4.864409345	0.539962975
phosphoserine aminotransferase Chr4:16904205-16905497 FORWARD LENGTH=430 201606	AT4G35630.1	-4.864409345	0.667076144
rotamase CYP 4 Chr3:22973708-22975139 FORWARD LENGTH=260 201606	AT3G62030.1	-4.843793805	0.547189356
nucleoside diphosphate kinase Chr4:5923484-5924366 FORWARD LENGTH=149 201606	AT4G09320.1	-4.827520048	0.621763331
GTP binding protein Chr1:10831953-10835454 REVERSE LENGTH=394 201606	AT1G30580.1	-4.827520048	0.621763331
Translation elongation factor EFG/EF2 protein Chr1:23233622-23236321 REVERSE LENGTH=783 201606	AT1G62750.1	-4.821928095	0.555202787
tripeptidyl peptidase ii Chr4:11160935-11169889 REVERSE LENGTH=1380 201606	AT4G20850.1	-4.767268023	0.650606244
RAB GTPase homolog 8A Chr3:16917908-16919740 FORWARD LENGTH=216 201606	AT3G46060.1 (+2)	-4.76064997	0.711741757
plasma membrane intrinsic protein 2 Chr2:15613624-15614791 REVERSE LENGTH=285 201606	AT2G37170.1	-4.76064997	0.711741757
Ribosomal protein L1p/L10e family Chr1:2636231-2637694 FORWARD LENGTH=216 201606	AT1G08360.1	-4.747038024	0.585319766
phosphoglucomutase Chr5:21063531-21067933 REVERSE LENGTH=623 201606	AT5G51820.1	-4.74003443	0.51391417
hypothetical protein Chr1:24655520-24657520 REVERSE LENGTH=356 201606	AT1G66200.1	-4.74003443	0.51391417
outer envelope membrane protein Chr3:23333773-23333982 REVERSE LENGTH=69 201606	AT3G63160.1	-4.722289781	0.85249612
allene oxide cyclase 2 Chr3:9406975-9407839 FORWARD LENGTH=253 201606	AT3G25770.1	-4.71816872	0.725522027
Succinyl-CoA ligase%2C alpha subunit Chr5:2667579-2669672 FORWARD LENGTH=347 201606	AT5G08300.1	-4.694891369	0.684632661
nitrilase 2 Chr3:15983351-15985172 FORWARD LENGTH=339 201606	AT3G44300.1	-4.694891369	0.684632661
ribosomal protein L4 Chr1:2249190-2250189 FORWARD LENGTH=282 201606	AT1G07320.1	-4.694891369	0.684632661
glutamate synthase 1 Chr5:1130031-1138186 FORWARD LENGTH=1648 201606	AT5G04140.2	-4.692587469	0.574455972
BCL-2-associated athanogene 7 Chr5:25052377-25054170 REVERSE LENGTH=446 201606	AT5G62390.1	-4.686786	0.612249183
rotamase CYP 1 Chr4:18083620-18084138 REVERSE LENGTH=172 201606	AT4G38740.1	-4.670007451	0.535451559
Glycine cleavage T-protein family Chr1:4001801-4003245 FORWARD LENGTH=408 201606	AT1G11860.1 (+2)	-4.648186457	1.010342945
Peptidase M1 family protein Chr1:23657791-23664243 REVERSE LENGTH=975 201606	AT1G63770.5 (+1)	-4.64327865	0.54461679
Lactate/malate dehydrogenase family protein Chr1:19854966-19856802 REVERSE LENGTH=341 201606	AT1G53240.1	-4.638560112	1.718467675
allene oxide synthase Chr5:17097803-17099359 REVERSE LENGTH=518 201606	AT5G42650.1	-4.614409345	0.64665455
Ribosomal protein S19 family protein Chr5:2955698-2956554 REVERSE LENGTH=152 201606	AT5G09510.1	-4.614409345	0.555118086
Inositol monophosphatase family protein Chr1:16468184-16470347 FORWARD LENGTH=341 201606	AT1G43670.1	-4.614409345	0.555118086
Aldolase-type TIM barrel family protein Chr3:4818667-4820748 FORWARD LENGTH=367 201606	AT3G14415.1 (+1)	-4.562953151	1.068951481
FtsH extracellular protease family Chr5:16902659-16905102 FORWARD LENGTH=704 201606	AT5G42270.1	-4.554678308	1.221921022
beta-D-xylosidase 4 Chr5:25810227-25813309 REVERSE LENGTH=784 201606	AT5G64570.1	-4.548650744	0.581345238
GroES-like zinc-binding dehydrogenase family protein Chr5:17684265-17686379 FORWARD LENGTH=379 201606	AT5G43940.1	-4.548650744	0.581345238
Ubiquinol-cytochrome C reductase iron-sulfur subunit Chr5:4308431-4310022 REVERSE LENGTH=274 201606	AT5G13440.1	-4.548650744	0.581345238
photosystem II subunit P-1 Chr1:2047940-2049186 FORWARD LENGTH=263 201606	AT1G06680.1	-4.539403208	0.642757774
annexin 1 Chr1:13225304-13226939 FORWARD LENGTH=317 201606	AT1G35720.1	-4.535315286	0.756346085

O-acetylserine (thiol) lyase (OAS-TL) isoform A1 Chr4:8518209-8520050 REVERSE LENGTH=322 201606	AT4G14880.1 (+4)	-4.532433829	0.656947525
plasma-membrane associated cation-binding protein 1 Chr4:10941593-10943227 FORWARD LENGTH=226 201606	AT4G20260.4	-4.523766825	0.689118839
acyl-CoA oxidase 4 Chr3:19225653-19229008 REVERSE LENGTH=436 201606	AT3G51840.1	-4.51064997	0.597869605
ribosomal protein L9 Chr3:16386505-16387963 FORWARD LENGTH=197 201606	AT3G44890.1	-4.51064997	0.597869605
multifunctional protein 2 Chr3:2161926-2166009 FORWARD LENGTH=725 201606	AT3G06860.1	-4.51064997	0.597869605
gamma carbonic anhydrase 2 Chr1:17321384-17323347 REVERSE LENGTH=278 201606	AT1G47260.1	-4.51064997	0.597869605
NADH-ubiquinone dehydrogenase Chr5:14897490-14900352 FORWARD LENGTH=745 201606	AT5G37510.1 (+1)	-4.497038024	0.500604844
Glucose-1-phosphate adenyltransferase family protein Chr2:16589401-16590741 FORWARD LENGTH=361 201606	AT2G39770.1 (+2)	-4.482892142	0.740668703
recA DNA recombination family protein Chr1:29737084-29740140 REVERSE LENGTH=439 201606	AT1G79050.1	-4.482892142	0.740668703
Rhodanese/Cell cycle control phosphatase superfamily protein Chr3:22086906-22090324 FORWARD LENGTH=686 201606	AT3G59780.1	-4.46816872	0.711705103
photosystem II subunit R Chr1:29736085-29736781 FORWARD LENGTH=140 201606	AT1G79040.1	-4.46816872	0.711705103
CLP protease proteolytic subunit 2 Chr1:4223099-4224954 FORWARD LENGTH=279 201606	AT1G12410.1	-4.46816872	0.617330832
carbonic anhydrase 2 Chr5:4760536-4762382 FORWARD LENGTH=259 201606	AT5G14740.2 (+4)	-4.463230048	0.933539821
10-formyltetrahydrofolate synthetase Chr1:18702064-18704687 FORWARD LENGTH=634 201606	AT1G50480.1	-4.46234262	0.554299728
6-phosphogluconate dehydrogenase family protein Chr1:23825549-23827012 REVERSE LENGTH=487 201606	AT1G64190.1	-4.435292507	0.582738623
pyruvate decarboxylase-2 Chr5:22310858-22312681 REVERSE LENGTH=607 201606	AT5G54960.1	-4.420007451	0.640255153
3-oxo-5-alpha-steroid 4-dehydrogenase family protein Chr5:5227982-5229012 FORWARD LENGTH=268 201606	AT5G16010.1	-4.420007451	0.640255153
Ribosomal protein S10p/S20e family protein Chr3:16471606-16472312 REVERSE LENGTH=124 201606	AT3G45030.1 (+2)	-4.420007451	0.640255153
proteasome alpha subunit F1 Chr5:17159270-17160975 REVERSE LENGTH=278 201606	AT5G42790.1	-4.402410119	0.73139157
glutathione reductase Chr3:20230356-20233100 REVERSE LENGTH=565 201606	AT3G54660.1	-4.402410119	0.527242702
arabinogalactan protein 31 Chr1:9889331-9890843 REVERSE LENGTH=359 201606	AT1G28290.1 (+1)	-4.402410119	0.73139157
hydroxypyruvate reductase Chr1:25493418-25495720 FORWARD LENGTH=386 201606	AT1G68010.1	-4.397571434	0.918257226
glutamate:glyoxylate aminotransferase Chr1:8268720-8271329 REVERSE LENGTH=481 201606	AT1G23310.1	-4.376134267	0.908803565
CLP protease R subunit 4 Chr4:9586740-9589297 REVERSE LENGTH=305 201606	AT4G17040.1	-4.364409345	0.53972177
pfkB-like carbohydrate kinase family protein Chr2:13383635-13386116 REVERSE LENGTH=325 201606	AT2G31390.1	-4.364409345	0.53972177
catalase 2 Chr4:16700937-16703215 REVERSE LENGTH=492 201606	AT4G35090.1	-4.357924964	0.820568124
serine transhydroxymethyltransferase 1 Chr4:17831891-17834742 REVERSE LENGTH=517 201606	AT4G37930.1	-4.344438308	0.665953044
Pyruvate kinase family protein Chr5:25490507-25492530 FORWARD LENGTH=510 201606	AT5G63680.1 (+2)	-4.321928095	0.740668703
Pyridine nucleotide-disulfide oxidoreductase family protein Chr1:27991248-27992845 FORWARD LENGTH=467 201606	AT1G74470.1	-4.320380779	0.798522836
chloroplast RNA binding protein Chr1:3015473-3018035 FORWARD LENGTH=378 201606	AT1G09340.1	-4.315340189	0.701502629
Leucine-rich repeat transmembrane protein kinase Chr3:4988271-4993891 FORWARD LENGTH=1020 201606	AT3G14840.2	-4.298650744	0.696652675
alanine-tRNA ligase Chr2:18584674-18586688 REVERSE LENGTH=272 201606	AT2G45060.1	-4.298650744	0.696652675

Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein Chr2:17590600-17591591 FORWARD LENGTH=173 201606	AT2G42210.2	-4.298650744	0.696652675
strictosidine synthase 2 Chr1:27835289-27837277 REVERSE LENGTH=335 201606	AT1G74020.1	-4.298650744	0.696652675
kunitz trypsin inhibitor 1 Chr1:27547410-27548057 REVERSE LENGTH=215 201606	AT1G73260.1	-4.298650744	0.696652675
photosystem II stability/assembly factor%2C chloroplast (HCF136) Chr5:7778154-7780463 FORWARD LENGTH=403 201606	AT5G23120.1	-4.289870665	0.700659519
glutamine synthetase 2 Chr5:13831220-13833239 FORWARD LENGTH=430 201606	AT5G35630.1 (+2)	-4.281867294	0.550033201
exocyst subunit exo70 family protein H7 Chr5:24064100-24066004 REVERSE LENGTH=634 201606	AT5G59730.1	-4.273766825	0.573843555
Plant invertase/pectin methylesterase inhibitor superfamily Chr2:18644281-18646394 REVERSE LENGTH=511 201606	AT2G45220.1	-4.273766825	0.573843555
aspartate aminotransferase 1 Chr2:13179012-13181686 FORWARD LENGTH=430 201606	AT2G30970.1 (+1)	-4.273766825	0.573843555
HAD superfamily%2C subfamily IIIB acid phosphatase Chr1:1042564-1043819 REVERSE LENGTH=271 201606	AT1G04040.1	-4.241581746	0.617230672
UDP-Glycosyltransferase superfamily protein Chr1:11920582-11923506 REVERSE LENGTH=610 201606	AT1G32900.1	-4.227421329	0.791582761
ADP-ribosylation factor A1E Chr3:23052287-23053545 FORWARD LENGTH=181 201606	AT3G62290.1 (+2)	-4.21816872	0.725479782
isovaleryl-CoA-dehydrogenase Chr3:16621659-16624848 REVERSE LENGTH=409 201606	AT3G45300.1	-4.21816872	0.725479782
disproportionating enzyme 2 Chr2:17045368-17050779 FORWARD LENGTH=955 201606	AT2G40840.1	-4.205451712	0.625319325
Glutathione S-transferase family protein Chr4:10784691-10786376 REVERSE LENGTH=382 201606	AT4G19880.2	-4.152410119	0.628158733
basic chitinase Chr3:3962501-3963984 REVERSE LENGTH=335 201606	AT3G12500.1	-4.152410119	0.628158733
Sucrose-6F-phosphate phosphohydrolase family protein Chr2:15053952-15055776 FORWARD LENGTH=422 201606	AT2G35840.1 (+3)	-4.152410119	0.628158733
vacuolar ATP synthase subunit E1 Chr4:6800091-6801696 FORWARD LENGTH=230 201606	AT4G11150.1	-4.150916626	0.763360578
AMP-dependent synthetase and ligase family protein Chr3:18159031-18161294 REVERSE LENGTH=514 201606	AT3G48990.1	-4.147448488	0.573385038
Phosphoglucosyltransferase/phosphomannosyltransferase family protein Chr1:8219946-8224186 FORWARD LENGTH=583 201606	AT1G23190.1	-4.144229847	0.501879158
Subtilase family protein Chr3:4741637-4743964 REVERSE LENGTH=775 201606	AT3G14240.1	-4.114409345	0.740668703
ARM repeat superfamily protein Chr3:279171-283399 FORWARD LENGTH=1176 201606	AT3G01780.1	-4.114409345	0.740668703
Ribosomal protein L13 family protein Chr1:29575997-29577406 FORWARD LENGTH=241 201606	AT1G78630.1	-4.114409345	0.740668703
Ribosomal protein L18e/L15 superfamily protein Chr1:26621168-26621608 REVERSE LENGTH=146 201606	AT1G70600.1	-4.114409345	0.740668703
Translation protein SH3-like family protein Chr3:18504311-18504751 FORWARD LENGTH=146 201606	AT3G49910.1	-4.107198699	0.624218919
photosystem II reaction center protein B ChrC:72371-73897 FORWARD LENGTH=508 201606	ATCG00680.1	-4.096514089	0.726179951
alanine:glyoxylate aminotransferase Chr2:5539417-5540902 REVERSE LENGTH=401 201606	AT2G13360.1 (+2)	-4.084730694	1.032062192
ribosomal protein S12C ChrC:97999-98793 REVERSE LENGTH=85 201606	ATCG00905.1 (+1)	-4.071928095	0.666790457
Plant invertase/pectin methylesterase inhibitor superfamily protein Chr3:23229782-23230366 FORWARD LENGTH=194 201606	AT3G62820.1	-4.071928095	0.666790457
allene oxide cyclase 1 Chr3:9403972-9405105 FORWARD LENGTH=254 201606	AT3G25760.1	-4.071928095	0.666790457
nuclear encoded CLP protease 5 Chr1:538000-539805 FORWARD LENGTH=298 201606	AT1G02560.1	-4.071928095	0.666790457
calcium sensing receptor Chr5:7736760-7738412 REVERSE LENGTH=387 201606	AT5G23060.1	-4.042220027	0.940939354
peroxisomal NAD-malate dehydrogenase 2 Chr5:2993645-2995551 REVERSE LENGTH=354 201606	AT5G09660.1	-4.032111548	0.69810309

phosphoenolpyruvate carboxylase 1 Chr1:19884261-19888070 REVERSE LENGTH=967 201606	AT1G53310.1 (+2)	-4.019012001	0.60767737
Chlorophyll A-B binding family protein Chr1:16871768-16873194 FORWARD LENGTH=265 201606	AT1G44575.1 (+1)	-4.010526489	0.621275194
ATPase%2C F0 complex%2C subunit B/B%2C bacterial/chloroplast ChrC:11529-12798 REVERSE LENGTH=184 201606	ATCG00130.1	-3.997314512	0.93383213
aconitase 3 Chr2:2141591-2146350 FORWARD LENGTH=990 201606	AT2G05710.1	-3.972820723	0.583326012
phosphofructokinase 7 Chr5:22924311-22926728 FORWARD LENGTH=485 201606	AT5G56630.1	-3.96816872	0.711594731
disease resistance protein (TIR-NBS class) Chr4:9560155-9565225 FORWARD LENGTH=801 201606	AT4G16990.12 (+1)	-3.96816872	0.711594731
Cupredoxin superfamily protein Chr4:7349941-7352868 REVERSE LENGTH=587 201606	AT4G12420.1 (+1)	-3.96816872	0.711594731
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein Chr3:23415816-23417002 REVERSE LENGTH=338 201606	AT3G63410.1	-3.96816872	0.711594731
long-chain acyl-CoA synthetase 6 Chr3:1786510-1791746 REVERSE LENGTH=701 201606	AT3G05970.1	-3.96816872	0.711594731
cellular apoptosis susceptibility protein%2C putative / importin-alpha re-exporter Chr2:19096867-19099785 FORWARD LENGTH=972 201606	AT2G46520.1	-3.96816872	0.711594731
gamma subunit of Mt ATP synthase Chr2:14018978-14021047 REVERSE LENGTH=325 201606	AT2G33040.1	-3.96816872	0.711594731
AT2G26780.1-DECOY	AT2G26780.1-DECOY (+1)	-3.96816872	0.711594731
NmrA-like negative transcriptional regulator family protein Chr1:28252030-28253355 FORWARD LENGTH=310 201606	AT1G75280.1	-3.96816872	0.711594731
TCP-1/cpn60 chaperonin family protein Chr3:1024432-1027604 FORWARD LENGTH=549 201606	AT3G03960.1	-3.966675228	0.523324298
annexin 2 Chr5:25973915-25975554 FORWARD LENGTH=317 201606	AT5G65020.1	-3.956050266	0.950797553
Ribosomal protein S5 family protein Chr2:17460016-17461398 REVERSE LENGTH=285 201606	AT2G41840.1	-3.953877702	0.625350117
sucrose synthase 4 Chr3:15179204-15182577 REVERSE LENGTH=808 201606	AT3G43190.1 (+1)	-3.946038609	0.577002777
lactate/malate dehydrogenase family protein Chr5:23580010-23582287 REVERSE LENGTH=442 201606	AT5G58330.2	-3.928674454	0.552720135
Clathrin%2C heavy chain Chr3:3482575-3491667 REVERSE LENGTH=1705 201606	AT3G11130.1	-3.926356642	0.59483567
Pyruvate kinase family protein Chr3:19649046-19652237 FORWARD LENGTH=527 201606	AT3G52990.1	-3.862705276	0.555340977
Thiamine pyrophosphate dependent pyruvate decarboxylase family protein Chr5:5724920-5726720 REVERSE LENGTH=572 201606	AT5G17380.1	-3.821928095	0.740668703
hypothetical protein Chr1:7530047-7530954 REVERSE LENGTH=126 201606	AT1G21500.1	-3.821928095	0.740668703
ABC transporter family protein Chr5:24453760-24455767 REVERSE LENGTH=595 201606	AT5G60790.1	-3.780168125	0.550310847
UDP-glucose 6-dehydrogenase family protein Chr5:15743254-15744696 FORWARD LENGTH=480 201606	AT5G39320.1	-3.765422103	0.527482912
Subtilase family protein Chr3:4658421-4660754 REVERSE LENGTH=777 201606	AT3G14067.1	-3.765347201	1.332383494
UvrABC system C protein%2C putative (DUF3411) Chr5:4044950-4047290 REVERSE LENGTH=386 201606	AT5G12470.1	-3.727421329	0.680148538
triosephosphate isomerase Chr3:20553794-20556078 FORWARD LENGTH=254 201606	AT3G55440.1	-3.715204335	0.501385051
homologue of NAP57 Chr3:21154255-21155952 REVERSE LENGTH=565 201606	AT3G57150.1	-3.650916626	0.582449243
chlorophyll a-b binding protein 6 Chr3:20339706-20340922 REVERSE LENGTH=241 201606	AT3G54890.1 (+1)	-3.634222298	0.685498475
cytosolic ribosomal protein S15 Chr1:1141852-1142960 REVERSE LENGTH=152 201606	AT1G04270.1 (+1)	-3.622731468	0.510295022
histone deacetylase 14 Chr4:16102774-16105439 REVERSE LENGTH=423 201606	AT4G33470.1	-3.583124127	0.711658227
Leucine-rich repeat (LRR) family protein Chr3:7280930-7282027 FORWARD LENGTH=365 201606	AT3G20820.1	-3.573421587	0.528810423
triacylglycerol lipase-like 1 Chr1:17123889-17128462 FORWARD LENGTH=479 201606	AT1G45201.1 (+1)	-3.546729921	1.078321999

DNAJ homologue 3 Chr3:15869115-15871059 REVERSE LENGTH=420 201606	AT3G44110.1	-3.541686439	1.006893773
FtsH extracellular protease family Chr2:13174680-13177064 FORWARD LENGTH=699 201606	AT2G30950.4	-3.515956254	0.592083561
hexokinase 1 Chr4:14352338-14354865 REVERSE LENGTH=496 201606	AT4G29130.1	-3.515422103	0.591729953
Serine/Threonine kinase domain protein Chr1:25872654-25875473 REVERSE LENGTH=562 201606	AT1G68830.1	-3.482779067	1.202842597
Albino or Glassy Yellow 1 Chr4:770926-776131 REVERSE LENGTH=1022 201606	AT4G01800.1 (+1)	-3.477421329	0.639897242
alpha-crystallin domain 32.1 Chr1:1967308-1969414 REVERSE LENGTH=285 201606	AT1G06460.1	-3.462142449	0.645317459
protochlorophyllide oxidoreductase B Chr4:13725648-13727107 FORWARD LENGTH=401 201606	AT4G27440.1 (+1)	-3.225814762	0.538442563
carbamoyl phosphate synthetase B Chr1:10468164-10471976 FORWARD LENGTH=1187 201606	AT1G29900.1	-3.200197653	0.502990946
ribosomal protein S15A Chr1:2408413-2409065 REVERSE LENGTH=130 201606	AT1G07770.1 (+3)	-3.190297817	0.837966274
NADP-malic enzyme 2 Chr5:3754456-3758040 FORWARD LENGTH=588 201606	AT5G11670.1	-2.712697907	0.563435838
Tyrosine transaminase family protein Chr4:12310657-12312885 FORWARD LENGTH=422 201606	AT4G23600.1	-2.566457282	0.654641422
PATELLIN 1 Chr1:27148558-27150652 FORWARD LENGTH=573 201606	AT1G72150.1	-2.354458055	0.504757856
GDSL-like Lipase/Acylhydrolase superfamily protein Chr1:10375639-10377717 FORWARD LENGTH=431 201606	AT1G29670.2	-2.316457282	0.558571485
lipxygenase 2 Chr3:16525437-16529233 FORWARD LENGTH=896 201606	AT3G45140.1	-2.278967516	1.055095853
ATP-dependent RNA helicase Chr4:14672947-14673219 FORWARD LENGTH=90 201606	AT4G30010.1	-2.066457282	0.55375046

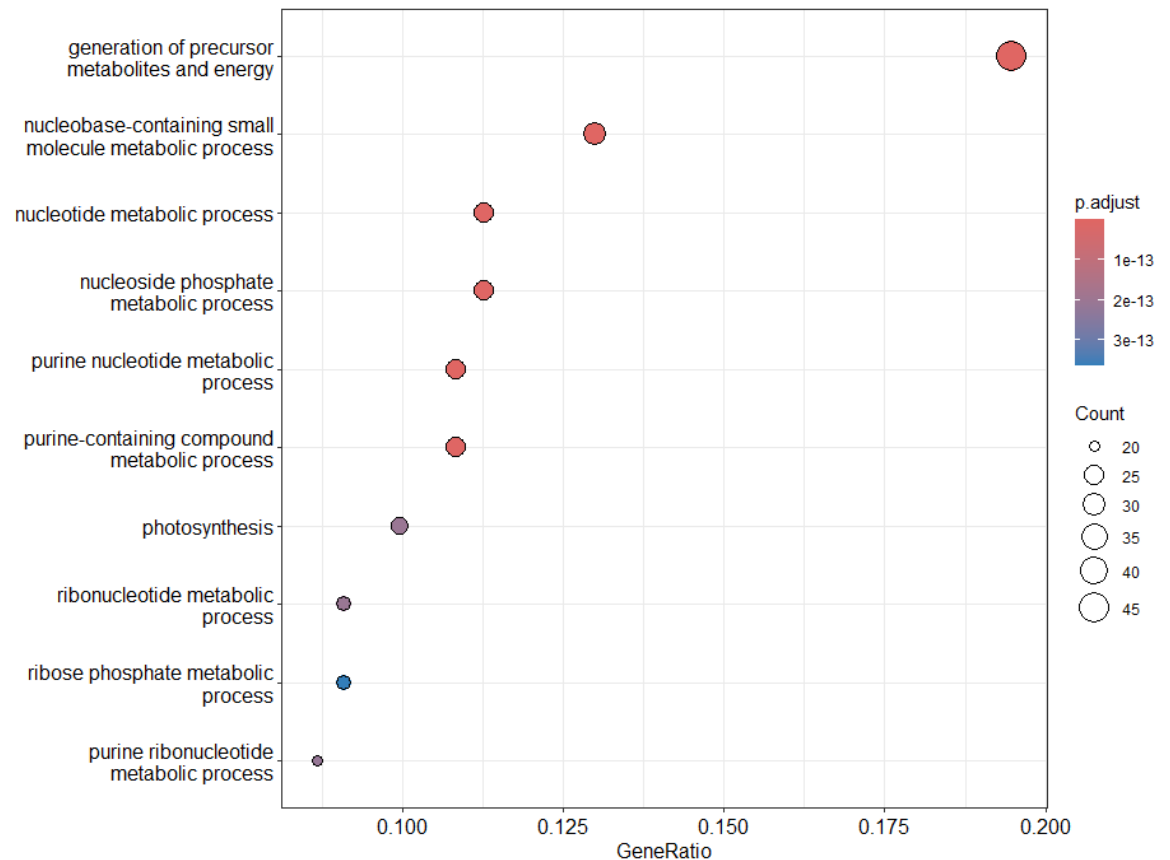


Figure S2. Gene ontology (GO) enrichment analysis of biological functions for 237 downregulated proteins identified in the YFP-MKKK7 AP-MS dataset. This figure is related to Table S3 and Figure 5.1.

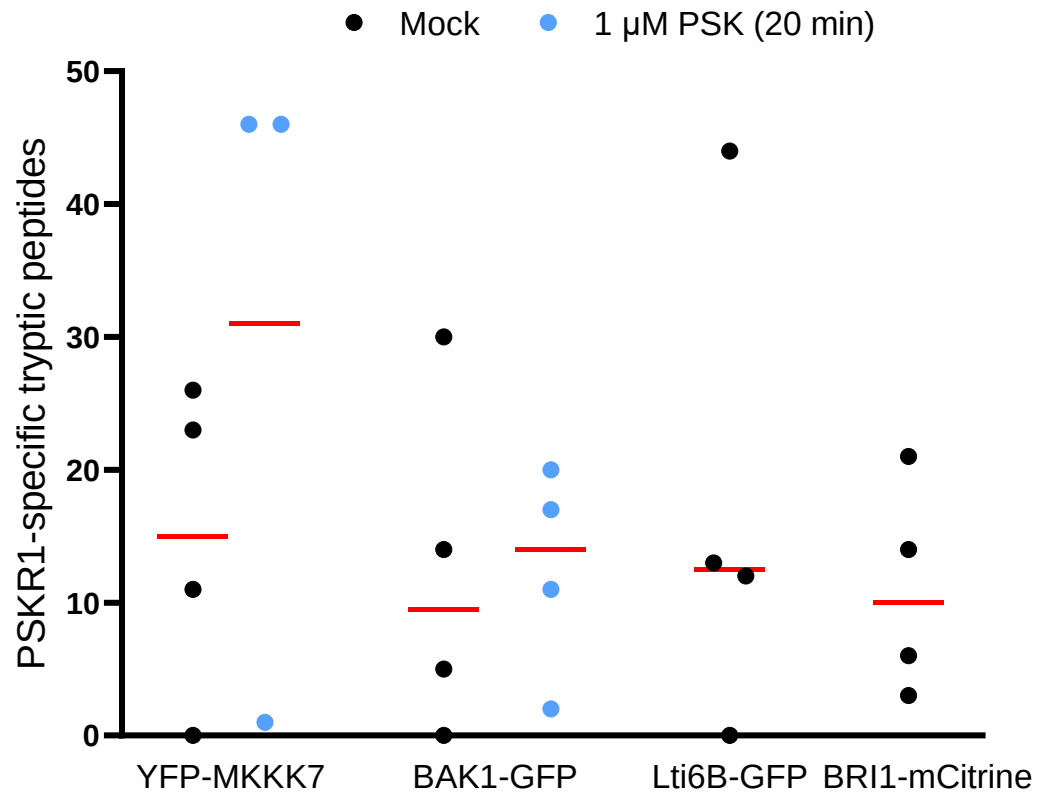


Figure S3. PSKR1-specific spectral counts in YFP-MKKK7, BAK1-GFP, Lti6B-GFP and BRI1-mCitrine pull-downs. PSK treatment was applied only to the YFP-MKKK7 and BAK1-GFP samples.

Table S5. Commonly downregulated genes in *mkkk7-3* and *pskr1-3pskr2-1* mutants. Table lists 42 genes that are downregulated in both *mkkk7-3* and *pskr1-3pskr2-1* mutants, identified through RNA-sequencing analysis. Primary gene symbols and gene description were extracted from the Araport 11 database via TAIR. Genes highlighted in blue are associated with the phosphate starvation response, while those highlighted in green encode secreted peptides. This table corresponds to Figure 5.9, C.

Locus Identifier	Primary Gene Symbol	Gene Description
AT5G39493	S-PROTEIN HOMOLOG 15 (SPH15)	SPH family protein containing 112 residues; eight beta strands and connecting loops.
AT3G09922	INDUCED BY PHOSPHATE STARVATION1 (IPS1)	Encodes a gene product whose expression is responsive to both phosphate (Pi) and phosphite (Phi) in both roots and shoots.
AT4G26255		other_RNA;(source:Araport11)
AT4G39795		hypothetical protein (DUF581);(source:Araport11)
AT5G34795		pseudogene of K-box region protein (DUF1985);(source:Araport11)
AT1G67105		other_RNA;(source:Araport11)
AT1G29395	COLD REGULATED 314 INNER MEMBRANE 1 (COR413IM1)	Integral membrane protein in the inner envelope of chloroplasts. Provide freezing tolerance. Expression is induced by short-term cold-treatment; water deprivation; and abscisic acid treatment. involved in response to salt tolerance
AT5G03545	AT4);INDUCED BY PI STARVATION 2 (ATIPS2)	Expressed in roots in response to phosphate starvation; this response is enhanced by the presence of IAA. Additionally; its expression is responsive to both phosphate (Pi) and phosphite (Phi) in shoots. The mRNA is cell-to-cell mobile.
AT5G64770	ROOT MERISTEM GROWTH FACTOR 9 (RGF9)	Encodes a root meristem growth factor (RGF). Belongs to a family of functionally redundant homologous peptides that are secreted; tyrosine-sulfated; and expressed mainly in the stem cell area and the innermost layer of central columella cells. RGFs are required for maintenance of the root stem cell niche and transit amplifying cell proliferation. Members of this family include: At5g60810 (RGF1); At1g13620 (RGF2); At2g04025 (RGF3); At3g30350 (RGF4); At5g51451 (RGF5); At4g16515 (RGF6); At3g02240 (RGF7); At2g03830 (RGF8) and At5g64770 (RGF9).
AT4G24890	PURPLE ACID PHOSPHATASE 24 (PAP24)	purple acid phosphatase 24;(source:Araport11)
AT2G40610	EXPANSIN A8 (EXPA8)	member of Alpha-Expansin Gene Family. Naming convention from the Expansin Working Group (Kende et al; 2004. Plant Mol Bio). Involved in the formation of nematode-induced syncytia in roots of Arabidopsis thaliana.
AT1G62981		transmembrane protein; putative (DUF1191);(source:Araport11)
AT1G76430	PHOSPHATE TRANSPORTER 1;9 (PHT1;9)	Encodes Pht1;9; a member of the Pht1 family of phosphate transporters which include: Pht1;1/At5g43350; Pht1;2/At5g43370; Pht1;3/At5g43360; Pht1;4/At2g38940; Pht1;5/At2g32830; Pht1;6/At5g43340; Pht1;7/At3g54700; Pht1;8/At1g20860; Pht1;9/At1g76430 (Plant Journal 2002; 31:341).
AT1G73010	PHOSPHATE STARVATION-INDUCED GENE 2 (PS2)	Encodes PPsPase1; a pyrophosphate-specific phosphatase catalyzing the specific cleavage of pyrophosphate (Km 38.8 uM) with an alkaline catalytic pH optimum. Expression is upregulated in the shoot of cax1/cax3 mutant.
AT5G20410	MONOGALACTOSYLDIACYLGLYCEROL SYNTHASE 2 (MGD2)	Encodes a type B monogalactosyldiacylglycerol (MGDG) synthase. Strongly induced by phosphate deprivation; and in non-photosynthetic tissues. Does not contribute to galactolipid synthesis under Pi-sufficient conditions but does under Pi starvation.
AT5G01600	FERRETIN 1 (FER1)	Encodes a ferretin protein that is targeted to the chloroplast. Member of a Ferritin gene family. Gene expression is induced in response to iron overload and by nitric oxide. Expression of the gene is downregulated in the presence of paraquat; an inducer of photoxidative stress.
AT5G63180	(ATPLL15)	Pectate lyase
AT5G22580		Stress responsive A/B Barrel Domain-containing protein;(source:Araport11)

AT5G24660	RESPONSE TO LOW SULFUR 2 (LSU2)	RESPONSE TO LOW SULFUR gene family member; expressed during sulfur deficiency. Part of an important plant defense mechanism that occurs in guard cells to prevent the entry of bacterial pathogens. Works as an integrator of light and chloroplast signaling.
AT3G46490	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein;(source:Araport11)
AT3G49160		Expression of the gene is downregulated in the presence of paraquat; an inducer of photooxidative stress.
AT3G43110		transmembrane protein;(source:Araport11)
AT3G16390	NITRILE SPECIFIER PROTEIN 3 (NSP3)	Encodes a nitrile-specifier protein NSP3. NSP3 is one out of five (At3g16400/NSP1; At2g33070/NSP2; At3g16390/NSP3; At3g16410/NSP4 and At5g48180/NSP5) A. thaliana epithiospecifier protein (ESP) homologues that promote simple nitrile; but not epithionitrile or thiocyanate formation. The mRNA is cell-to-cell mobile.
AT4G16540	Heat shock protein HSP20/alpha crystallin family	Heat shock protein HSP20/alpha crystallin family;(source:Araport11)
AT4G19380	Long-chain fatty alcohol dehydrogenase family protein	Long-chain fatty alcohol dehydrogenase family protein;(source:Araport11)
AT2G46880	PURPLE ACID PHOSPHATASE 14 (PAP14)	purple acid phosphatase 14;(source:Araport11)
AT1G64590	NAD(P)-binding Rossmann-fold superfamily protein	NAD(P)-binding Rossmann-fold superfamily protein;(source:Araport11)
AT1G53700	WAG 1 (WAG1)	The WAG1 and its homolog; WAG2 each encodes a protein-serine/threonine kinase that are nearly 70% identical to PsPK3 protein. All three together with CsPK3 belong to PsPK3-type kinases. At the N-terminus; all four possess a serine/threonine-rich domain. They are closely related to Arabidopsis kinases PINOID. wag1/wag2 double mutants exhibit a pronounced wavy root phenotype when grown vertically on agar plates (while wild-type plants develop wavy roots only on plates inclined to angles less than 90 degrees); indicating an overlapping role for WAG1 and WAG2 as suppressors of root waving. Simultaneous disruption of PID(AT2G34650) and its 3 closest homologs (PID2/AT2G26700; WAG1/AT1G53700; and WAG2/AT3G14370) abolishes the formation of cotyledons.
AT3G03260	HOMEODOMAIN GLABROUS 8 (HDG8)	Encodes a homeobox-leucine zipper family protein belonging to the HD-ZIP IV family.
AT3G15050	IQ-DOMAIN 10 (IQD10)	Member of IQ67 (CaM binding) domain containing family.
AT3G21950		SABATH family methyltransferase.
AT5G10230	ANNEXIN 7 (ANNAT7)	Encodes a calcium-binding protein annexin (AnnAt7).
AT2G22190	TREHALOSE-6-PHOSPHATE PHOSPHATASE E (TPPE)	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein;(source:Araport11)
AT2G11810	MONOGALACTOSYLDIACYLGLYCEROL SYNTHASE TYPE C (MGDC)	MGD3 is the major enzyme for galactolipid metabolism during phosphate starvation. Does not contribute to galactolipid synthesis under P1-sufficient conditions.
AT3G26200	CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 22 (CYP71B22)	putative cytochrome P450 The mRNA is cell-to-cell mobile.
AT1G20860	PHOSPHATE TRANSPORTER 1;8, PHT1;8	Encodes Pht1;8; a member of the Pht1 family of phosphate transporters which include: Pht1;1/At5g43350; Pht1;2/At5g43370; Pht1;3/At5g43360; Pht1;4/At2g38940; Pht1;5/At2g32830; Pht1;6/At5g43340; Pht1;7/At3g54700; Pht1;8/At1g20860; Pht1;9/At1g76430 (Plant Journal 2002; 31:341).
AT1G52190	NRT1/ PTR FAMILY 1.2 (NPF1.2)	Encodes a low affinity nitrate transporter that is expressed in the plasma membrane and found in the phloem of the major veins of leaves. It is responsible for nitrate redistribution to young leaves.
AT1G52290	PROLINE-RICH EXTENSIN-LIKE RECEPTOR KINASE 15 (PERK15)	Encodes a member of the proline-rich extensin-like receptor kinase (PERK) family. This family consists of 15 predicted receptor kinases (PMID: 15653807).
AT1G29720	RKF-LIKE 1 (RKFL1)	Encodes one of three RECEPTOR-LIKE KINASE IN FLOWERS 1 (RKF1) paralogues that is required in the stigmatic papillae and the female reproductive tract to promote compatible pollen grain hydration and pollen tube growth.
AT1G11460	USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 26 (UMAMIT26)	nodulin MtN21-like transporter family protein
AT1G23110		fold protein;(source:Araport11)
AT1G22160		senescence-associated family protein (DUF581);(source:Araport11)

Table S6. Commonly upregulated genes in mkkk7-3 and pskr1-3pskr2-1 mutants. Table lists 25 genes that are upregulated in both *mkkk7-3* and *pskr1-3pskr2-1* mutants, identified through RNA-sequencing analysis. Primary gene symbols and gene descriptions were extracted from the Araport 11 database via TAIR. This table corresponds to Figure 5.9, D. Genes highlighted in orange are secreted peptides involved in iron deficiency response.

Locus Identifier	Primary Gene Symbol	Gene Description
AT5G56795	METALLOTHIONEIN 1B (MT1B)	one of the five metallothioneins (MTs) genes identified in Arabidopsis. MTs are cysteine-rich proteins required for heavy metal tolerance. The MT1b gene; however, is indicated to be inactive.
AT3G02832		other_RNA;(source:Araport11)
AT5G41590		LURP-one-like protein (DUF567);(source:Araport11)
AT5G42445		pseudogene of R-protein L3 B;(source:Araport11)
AT5G59320	LIPID TRANSFER PROTEIN 3 (LTP3)	Predicted to encode a PR (pathogenesis-related) protein. Belongs to the lipid transfer protein (PR-14) family with the following members: At2g38540/LTP1; At2g38530/LTP2; At5g59320/LTP3; At5g59310/LTP4; At3g51600/LTP5; At3g08770/LTP6; At2g15050/LTP7; At2g18370/LTP8; At2g15325/LTP9; At5g01870/LTP10; At4g33355/LTP11; At3g51590/LTP12; At5g44265/LTP13; At5g62065/LTP14; At4g08530/LTP15. The mRNA is
AT5G57080		transmembrane protein;(source:Araport11)
AT1G47395	FE-UPTAKE-INDUCING PEPTIDE2 (FEP2);IRONMAN 2 (IMA2)	Involved in regulation of iron deficiency response genes.
AT1G70680	(CLO6)	Lipid droplet and endoplasmic reticulum-associated protein.
AT2G30766	FE-UPTAKE-INDUCING PEPTIDE1 (FEP1);IRONMAN 3 (IMA3)	Functions in iron homeostasis; activates iron deficiency response genes such as bHLH38; bHLH39; IRT1; and FRO2.
AT5G60630		transmembrane protein;(source:Araport11)
AT4G36700		RmlC-like cupins superfamily protein;(source:Araport11)
AT5G05250		hypothetical protein;(source:Araport11)
AT2G46830	(ATCCA1);CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)	Encodes a transcriptional repressor that performs overlapping functions with LHY in a regulatory feedback loop that is closely associated with the circadian oscillator of Arabidopsis. Binds to the evening element in the promoter of TOC1 and represses TOC1 transcription. CCA1 and LHY colocalize in the nucleus and form heterodimers in vivo. CCA1 and LHY function synergistically in regulating circadian rhythms of Arabidopsis. CCA1 binds the Gl
AT5G11330		FAD/NAD(P)-binding oxidoreductase family protein;(source:Araport11)
AT3G44300	NITRILASE 2 (NIT2);NITRILASE 2 (ANIT2)	Encodes an enzyme that catalyzes the hydrolysis of indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA) (nitrile aminohydrolase; EC 3.5.5.1) and IAN to indole-3-acetamide (IAM) at lower levels. Mutants have reduced sensitivity to IAN and are sensitive to IAA. This enzyme likely participates in other non-auxin-related metabolic pathways. The mRNA is cell-to-cell mobile.
AT3G13170	(ATSPO11-1);SPORULATION 11-1 (SPO11-1)	Encodes AtSPO11-1; one of the three Arabidopsis homologues of the archaeal DNA topoisomerase VIA subunit (topo VIA). Required for meiotic recombination. AtSPO11-1 and AtSPO11-2 have overlapping functions (i.e. both required for meiotic recombination) whereas AtSPO11-3 functions in DNA replication. AtSPO11-1 accumulates in foci in early G2. At 1 h post-S phase; no foci are observed; but by 3 h a majority (80%) of meiocytes at this time point contain >50 foci. However, by 5 h; AtSPO11-1 foci are no longer detectable. This suggests that the protein undergoes a rapid cycle of accumulation and disappearance in meiocytes over a period of between 1 and 5 h post-S phase.
AT3G45130		lanosterol synthase 1;(source:Araport11)
AT5G03550		MATH domain/coiled-coil protein;(source:Araport11)
AT2G02120	(PDF2.1);LOW-MOLECULAR-WEIGHT CYSTEINE-RICH 70 (LCR70)	Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant defensin (PDF) family with the following members: At1g75830/PDF1.1; At5g44420/PDF1.2a; At2g26020/PDF1.2b; At5g44430/PDF1.2c; At2g26010/PDF1.3; At1g19610/PDF1.4; At1g55010/PDF1.5; At2g02120/PDF2.1; At2g02100/PDF2.2; At2g02130/PDF2.3; At1g61070/PDF2.4; At5g63660/PDF2.5; At2g02140/PDF2.6; At5g38330/PDF3.1 and At4g30070/PDF3.2. Mediates ammonium metabolism by regulating glutamine synthetase activity.
AT1G71890	(SUC5);SUCROSE-PROTON SYMPORTER 5 (ATSUC5)	Encodes a sucrose transporter that is expressed in the endosperm. Mutants have delayed accumulation of fatty acids and embryo maturation.
AT1G67110	CYTOCHROME P450, FAMILY 735, SUBFAMILY A, POLYPEPTIDE 2 (CYP735A2)	cytochrome P450; family 735; subfamily A; polypeptide 2;(source:Araport11)
AT2G15780		Cupredoxin superfamily protein;(source:Araport11)
AT2G40200		basic helix-loop-helix (bHLH) DNA-binding superfamily protein;(source:Araport11)
AT1G47400	IRONMAN 1 (IMA1);FE-UPTAKE-INDUCING PEPTIDE3 (FEP3)	Involved in regulation of iron deficiency response genes. Overexpression results in hyperaccumulation of Fe and Mn.
AT1G60130		Mannose-binding lectin superfamily protein;(source:Araport11)

Table S7. Genes downregulated by PSK treatment. This table lists 203 genes that are downregulated in response to PSK treatment corresponding to Figure 5.10 A. Genes highlighted in blue are discussed in the main text of Chapter 5.

Locus Identifier	Primary Gene Symbol	Gene Description
AT2G39518	CASP-LIKE PROTEIN 4D2 (CASPL4D2)	Uncharacterized protein family (UPF0497);(source:Araport11)
AT2G25735		hypothetical protein;(source:Araport11)
AT1G65845		transmembrane protein;(source:Araport11)
AT1G36622		transmembrane protein;(source:Araport11)
AT1G69520		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein;(source:Araport11)
AT1G76560	CP12 DOMAIN-CONTAINING PROTEIN 3 (CP12-3)	CP12 domain-containing protein 3;(source:Araport11)
AT1G71880	SUCROSE-PROTON SYMPORTER 1 (SUC1)	Sucrose transporter gene induced in response to nematodes; member of Sucrose-proton symporter family. The mRNA is cell-to-cell mobile.
AT1G70520	CYSTEINE-RICH RLK2 (CRK2)	Encodes a cysteine-rich receptor-like protein kinase located to the plasma membrane. Involved in regulating microbe-associated molecular pattern-triggered ROS production and stress induced callose deposition at the plasmodesmata in roots. Required for MAMP-triggered responses and resistance to <i>Pseudomonas syringae</i> pv. tomato 118 DC3000 .
AT1G78000	SULFATE TRANSPORTER 1;2 (SULTR1;2)	Encodes a sulfate transporter that can restore sulfate uptake capacity of a yeast mutant lacking sulfate transporter genes.
AT2G22122		hypothetical protein;(source:Araport11)
AT2G29995		PSY3-like protein;(source:Araport11)
AT3G07195	(NOI11)	Member of the RIN4-like/NOI family; upregulated after spider mite feeding.
AT2G39530	CASP-LIKE PROTEIN 4D1 (CASPL4D1)	Uncharacterized protein family (UPF0497);(source:Araport11)
AT2G44490	PENETRATION 2 (PEN2)	Encodes a glycosyl hydrolase that localizes to peroxisomes and acts as a component of an inducible preinvasion resistance mechanism. Required for mlo resistance. The mRNA is cell-to-cell mobile.
AT1G72360	ETHYLENE RESPONSE FACTOR 73 (ERF73)	Encodes a member of the ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 5 members in this subfamily including RAP2.2 AND RAP2.12.
AT1G65486	(STMP4)	Secreted peptide which functions in plant growth and pathogen defense.
AT1G68840	RELATED TO ABI3/VP1 2 (RAV2)	Rav2 is part of a complex that has been named 'regulator of the (H ⁺)-ATPase of the vacuolar and endosomal membranes' (RAVE) The mRNA is cell-to-cell mobile.
AT1G74790		catalytics;(source:Araport11)
AT2G46430	CYCLIC NUCLEOTIDE GATED CHANNEL 3 (CNGC3)	Encodes a cyclic nucleotide gated channel; downstream component of the signaling pathways leading to hypersensitive response (HR) resistance.
AT2G44380		Cysteine/Histidine-rich C1 domain family protein;(source:Araport11)
AT2G37820		Cysteine/Histidine-rich C1 domain family protein;(source:Araport11)
AT2G35910	ARABIDOPSIS T??XICOS EN LEVADURA 70 (ATL70)	RING/U-box superfamily protein;(source:Araport11)
AT2G35930	PLANT U-BOX 23 (PUB23)	Encodes a cytoplasmically localized U-box domain containing E3 ubiquitin ligase that is involved in the response to water stress and acts as a negative regulator of PAMP-triggered immunity.
AT2G39660	BOTRYTIS-INDUCED KINASE1 (BIK1)	Encodes a plasma membrane-localized ser/thr protein kinase that is a crucial component of host response signaling required to activate the resistance responses to Botrytis and <i>A. brassicicola</i> infection. It is likely a negative regulator of salicylic acid accumulation and basal defense against virulent bacterial pathogens. Together with ER plays opposing roles in leaf morphogenesis and inflorescence architecture. Required to maintain appropriate auxin response during leaf margin morphogenesis. Interacts with ER-family proteins and directly phosphorylates ER.
AT2G32660		receptor like protein 22;(source:Araport11)
AT2G25680	MOLYBDATE TRANSPORTER 1 (MOT1)	Encodes a high-affinity molybdate transporter. Mutant has reduced concentrations of molybdate in roots and shoots; and reduced shoot and root length when growing on Mo-limited medium.
AT2G44370		Cysteine/Histidine-rich C1 domain family protein;(source:Araport11)
AT2G42360	ARABIDOPSIS T??XICOS EN LEVADURA 41 (ATL41)	RING/U-box superfamily protein;(source:Araport11)
AT2G39210	PICLORAM RESISTANT30 (PIC30)	Major facilitator superfamily transmembrane transporter responsible for the uptake of picolinate herbicides.
AT2G32530	CELLULOSE SYNTHASE-LIKE B3 (CSLB03)	encodes a gene similar to cellulose synthase

AT2G38870		Predicted to encode a PR (pathogenesis-related) peptide that belongs to the PR-6 proteinase inhibitor family. Six putative PR-6-type protein encoding genes are found in Arabidopsis: At2g38900; At2g38870; At5g43570; At5g43580; At3g50020 and At3g46860.
AT2G28960		Leucine-rich repeat protein kinase family protein;(source:Araport11)
AT1G69900		Actin cross-linking protein;(source:Araport11)
AT1G65390	PHLOEM PROTEIN 2 A5 (PP2-A5)	Phloem Protein2 family gene encoding a two-domain protein containing predicted lectin and Toll/Interleukin-1 receptor domains; which is induced upon spider mite attack and improves the ability to defend against T. urticae by participating in the tight regulation of hormonal cross talk upon mite feeding.
AT1G63860		Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)
AT1G70140	FORMIN 8 (FH8)	Encodes a group I formin. Binds to F-actin barbed ends. Has severing actin filaments activity. Binds profilin. Involved in the initiation and tip growth of root hairs through regulation of actin cytoskeleton.
AT1G64170	CATION/H ⁺ EXCHANGER 16 (CHX16)	member of Putative Na ⁺ /H ⁺ antiporter family
AT1G65500	(STMP6)	Secreted peptide which functions in plant growth and pathogen defense.
AT1G30755	PSK SIMULATOR 2 (PSI2)	elongation factor G; putative (DUF668);(source:Araport11)
AT3G04110	GLUTAMATE RECEPTOR 1.1 (GLR1.1)	putative glutamate receptor (GLR1.1). Contains a functional cation - permeable pore domain. Involved in cellular cation homeostasis.
AT2G17740	VACUOLELESS GAMETOPHYTES (VLG)	VACUOLELESS GAMETOPHYTES (VLG) as a DC1 domain containing protein that is found in the endomembrane system. It is essential for both female and male gametophyte development.
AT2G43620		Chitinase family protein;(source:Araport11)
AT2G32190	CYSTEINE-RICH TRANSMEMBRANE MODULE 4 (ATHCYSTM4)	cysteine-rich/transmembrane domain A-like protein;(source:Araport11)
AT2G23680		Cold acclimation protein WCOR413 family;(source:Araport11)
AT2G19190	FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)	Encodes a receptor-like protein kinase that is involved in early defense signaling. Expression of this gene is strongly induced during leaf senescence. It is a target of the transcription factor AtWRKY6.
AT2G29120	GLUTAMATE RECEPTOR 2.7 (GLR2.7)	member of Putative ligand-gated ion channel subunit family
AT1G51850	STRESS INDUCED FACTOR 2 (SIF2)	Malectin-like receptor-like kinase involved in MAMP mediated stomatal immunity. Interacts with BAK1/FLS2 signaling complex and subsequently phosphorylates and activates SLAC1.
AT1G33610		Leucine-rich repeat (LRR) family protein;(source:Araport11)
AT1G48500	JASMONATE-ZIM-DOMAIN PROTEIN 4 (JAZ4)	Jasmonate zim domain transcription factor family protein.Involved in freezing tolerance and JA induced leaf senescence.
AT1G71890	(SUC5)	Encodes a sucrose transporter that is expressed in the endosperm. Mutants have delayed accumulation of fatty acids and embryo maturation.
AT1G65730	YELLOW STRIPE LIKE 7 (YSL7)	Arabidopsis thaliana metal-nicotianamine transporter YSL4
AT1G21326		VQ motif-containing protein;(source:Araport11)
AT2G31880	SUPPRESSOR OF BIR1 1 (SOBIR1)	Encodes a putative leucine rich repeat transmembrane protein that is expressed in response to Pseudomonas syringae. Expression of SRRLK may be required for silencing via siRNAs. Regulates cell death and innate immunity.
AT2G24570	WRKY DNA-BINDING PROTEIN 17 (WRKY17)	member of WRKY Transcription Factor; Group II-d; negative regulator of basal resistance to Pseudomonas syringae.
AT2G28890	POLTERGEIST LIKE 4 (PLL4)	Encodes a protein phosphatase 2C like gene; similar to POL. Involved in leaf development. Knockout mutants have abnormally shaped leaves.
AT2G42350	ARABIDOPSIS T??XICOS EN LEVADURA 40 (ATL40)	RING/U-box superfamily protein;(source:Araport11)
AT2G02680		Cysteine/Histidine-rich C1 domain family protein;(source:Araport11)
AT2G35710	PLANT GLYCOGENIN-LIKE STARCH INITIATION PROTEIN 7 (PGSIP7)	Nucleotide-diphospho-sugar transferases superfamily protein;(source:Araport11)
AT3G01290	HYPERSENSITIVE INDUCED REACTION 2 (HIR2)	Hypersensitive response induced stomatin/prohibitin/flotillin/HflK/C (SPFH) domain containing protein that interacts with RPS2.
AT2G25470		receptor like protein 21;(source:Araport11)
AT2G03810		18S pre-ribosomal assembly protein gar2-like protein;(source:Araport11)
AT1G51860		Leucine-rich repeat protein kinase family protein;(source:Araport11)

AT1G51890		Leucine-rich repeat protein kinase family protein;(source:Araport11)
AT1G55390		Cysteine/Histidine-rich C1 domain family protein;(source:Araport11)
AT1G26420	(ATBBE7)	FAD-binding Berberine family protein;(source:Araport11)
AT1G49860	GLUTATHIONE S-TRANSFERASE (CLASS PHI) 14 (GSTF14)	Encodes glutathione transferase belonging to the phi class of GSTs. Naming convention according to Wagner et al. (2002). The mRNA is cell-to-cell mobile.
AT1G51620		Protein kinase superfamily protein;(source:Araport11)
AT1G52200		PLAC8 family protein;(source:Araport11)
AT2G27660		Cysteine/Histidine-rich C1 domain family protein;(source:Araport11)
AT2G22880	(VQ12)	VQ motif-containing protein;(source:Araport11)
AT2G28630	3-KETOACYL-COA SYNTHASE 12 (KCS12)	Encodes KCS12; a member of the 3-ketoacyl-CoA synthase family involved in the biosynthesis of VLCFA (very long chain fatty acids).
AT1G60110		Mannose-binding lectin superfamily protein;(source:Araport11)
AT1G61380	S-DOMAIN-1 29 (SD1-29)	Encodes a membrane localized S-domain receptor kinase that is involved in lipopolysaccharide (LPS) sensing. SD1-29 detected LPS of Pseudomonas and Xanthomonas species for which it serves as a microbe associated molecular pattern triggering innate immunity. Losses of function mutants are hyper susceptible to P.syringae.
AT1G30720	(ATBBE10)	FAD-binding Berberine family protein;(source:Araport11)
AT1G05300	ZINC TRANSPORTER 5 PRECURSOR (ZIP5)	member of Fe(II) transporter isolog family
AT1G33840		LURP-one-like protein (DUF567);(source:Araport11)
AT1G07630	POL-LIKE 5 (PLL5)	Encodes a protein phosphatase 2C like gene; similar to POL. Involved in leaf development. Knockout mutants have abnormally shaped leaves.
AT1G18200	RAB GTPASE HOMOLOG A6B (RABA6b)	Rab GTPase-like A11 protein;(source:Araport11)
AT1G22650	ALKALINE/NEUTRAL INVERTASE D (A/N-InvD)	Plant neutral invertase family protein;(source:Araport11)
AT1G16130	WALL ASSOCIATED KINASE-LIKE 2 (WAKL2)	Encodes a WAK-like receptor-like kinase with a cytoplasmic Ser/Thr protein kinase domain and an extracellular domain with EGF-like repeats.
AT1G23200	HIGHLY METHYL ESTERIFIED SEEDS (HMS)	ProPME pectin methyl esterase involved in embryo development; stomatal function; mucilage release.
AT1G02360		Chitinase family protein;(source:Araport11)
AT1G27020		plant/protein;(source:Araport11)
AT1G22400	(UGT85A1)	UDP-Glycosyltransferase superfamily protein;(source:Araport11)
AT1G28010	ATP-BINDING CASSETTE B14 (ABCB14)	Encodes an ATP-binding cassette (ABC) transporter. Expressed in the vascular tissue of primary stem.
AT1G18390	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR- LIKE PROTEIN KINASE-LIKE 1.2 (LRK10L1.2)	Serine/Threonine kinase family catalytic domain protein;(source:Araport11)
AT1G62300	(WRKY6)	Encodes a transcription factor WRKY6. Regulates Phosphate1 (Pho1) expression in response to low phosphate (Pi) stress.
AT1G05700		Leucine-rich repeat transmembrane protein kinase protein;(source:Araport11)
AT1G15890		Disease resistance protein (CC-NBS-LRR class) family;(source:Araport11)
AT1G27730	SALT TOLERANCE ZINC FINGER (STZ)	Related to Cys2/His2-type zinc-finger proteins found in higher plants. Compensated for a subset of calcineurin deficiency in yeast. Salt tolerance produced by ZAT10 appeared to be partially dependent on ENA1/PMR2; a P-type ATPase required for Li ⁺ and Na ⁺ efflux in yeast. The protein is localized to the nucleus; acts as a transcriptional repressor and is responsive to chitin oligomers. Also involved in response to photooxidative stress.
AT1G11540		Sulfite exporter TauE/SafE family protein;(source:Araport11)
AT1G16110	WALL ASSOCIATED KINASE-LIKE 6 (WAKL6)	Encodes a WAK-like receptor-like kinase with a cytoplasmic Ser/Thr protein kinase domain and an extracellular domain with EGF-like repeats. It has been shown to be localized to the cell wall.
AT1G22500	ARABIDOPSIS TOXICOS EN LEVADURA 15 (ATL15)	Gene encodes a putative C3HC4-type RING zinc finger factor. it is induced in response to light and ascorbate stimulus.
AT1G19380		sugar; putative (DUF1195);(source:Araport11)
AT1G21540	(AAE9)	Isobutyl-CoA synthetase involved in iso-branched wax biosynthesis.
AT1G13080	CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 2 (CYP71B2)	cytochrome P450 monooxygenase

AT1G15670	KISS ME DEADLY 2 (KMD2)	Encodes a member of a family of F-box proteins; called the KISS ME DEADLY (KMD) family; that targets type-B ARR proteins for degradation and is involved in the negative regulation of the cytokinin response. Also named as KFB1; a member of a group of Kelch repeat F-box proteins that negatively regulate phenylpropanoid biosynthesis by targeting the phenylpropanoid biosynthesis enzyme phenylalanine ammonia-lyase.
AT1G60130		Mannose-binding lectin superfamily protein;(source:Araport11)
AT1G30700	(ATBBE8)	FAD-binding Berberine family protein;(source:Araport11)
AT1G10340		Ankyrin repeat family protein;(source:Araport11)
AT5G44572	(PROSCOOP6)	SCOOP (SERINE RICH ENDOGENOUS PEPTIDE) family member; involved in immunity against herbivorous insects.
AT5G44585	PRECURSOR OF SERINE-RICH ENDOGENOUS PEPTIDE 12 (PROSCOOP12)	Precursor of serine-rich endogenous peptide which regulates defense response and root elongation. Has properties of phyto cytokines; activates the phospholipid signaling pathway; regulates reactive oxygen species response; and is perceived in a BAK1 co-receptor-dependent manner. SCOOP (SERINE RICH ENDOGENOUS PEPTIDE) family member; involved in immunity against herbivorous insects.
AT5G64100	(PRX69)	Class III peroxidase cell wall-targeted protein localized to the micropylar endosperm facing the radicle. Involved in seed germination.
AT5G40780	LYSINE HISTIDINE TRANSPORTER 1 (LHT1)	Encodes LHT1 (lysine histidine transporter); a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll.
AT5G44390	(ATBBE25)	FAD-binding Berberine family protein;(source:Araport11)
AT5G64120	PEROXIDASE 71 (PRX71)	Encodes a cell wall bound peroxidase that is induced by hypo-osmolarity and is involved in the lignification of cell walls. Class III peroxidase cell wall-targeted protein localized to the micropylar endosperm facing the radicle. Involved in seed germination.
AT5G57220	CYTOCHROME P450, FAMILY 81, SUBFAMILY F, POLYPEPTIDE 2 (CYP81F2)	member of CYP81F; involved in glucosinolate metabolism. Mutants had impaired resistance to fungi. The mRNA is cell-to-cell mobile.
AT5G43450		encodes a protein whose sequence is similar to ACC oxidase
AT3G29034		transmembrane protein;(source:Araport11)
AT4G35110	PHOSPHOLIPASE-LIKE PROTEIN 4 (PEARLI 4)	phospholipase-like protein (PEARLI 4) family protein;(source:Araport11)
AT4G32650	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 3 (KAT3)	Encodes KAT3; a member of the Shaker family of voltage-gated potassium channel subunits. Does not form functional potassium channel on its own. Involved in down-regulating AKT1 and KAT1 channel activity by forming heteromers with AKT1 or KAT1. The Shaker family K ⁺ ion channels include five groups based on phylogenetic analysis (FEBS Letters (2007) 581: 2357): I (inwardly rectifying conductance): AKT1 (AT2G26650); AKT5 (AT4G32500) and SPIK (also known as AKT6; AT2G25600); II (inward rectifying channel): KAT1 (AT5G46240) and KAT2 (AT4G18290); III (weakly inward rectifying channel): AKT2 (AT4G22200); IV (regulatory subunit involved in inwardly rectifying conductance formation): KAT3 (also known as AtKC1; AT4G32650); V (outward rectifying channel): SKOR (AT3G02850) and GORK (AT5G37500).
AT5G54040		Cysteine/Histidine-rich C1 domain family protein;(source:Araport11)
AT5G54720		Ankyrin repeat family protein;(source:Araport11)
AT5G42010		Transducin/WD40 repeat-like superfamily protein;(source:Araport11)
AT5G53550	YELLOW STRIPE LIKE 3 (YSL3)	YELLOW STRIPE like 3;(source:Araport11)
AT5G39020	MEDOS 3 (MDS3)	Involved in growth adaptation upon exposure to metal ions. Contributes together with the other MDS genes to the complex network of CrRLK1Ls that positively and negatively affect growth.
AT5G58660	GIBBERELLIN 2-OXIDASE 9 (GA2OX9)	Encodes a class III gibberellin 2-oxidase that oxidizes GA12 to GA110 and GA9 to GA40.
AT4G37520		Peroxidase superfamily protein;(source:Araport11)
AT4G38560		phospholipase-like protein (PEARLI 4) family protein;(source:Araport11)
AT4G38550		phospholipase-like protein (PEARLI 4) family protein;(source:Araport11)
AT4G20860	(ATBBE22)	involved in the generation of H ₂ O ₂ from reduced compounds

AT4G39830	CUPREDOXIN SUPERFAMILY PROTEIN (AO)	role in the degradation of ascorbate to (mono)dehydroascorbate; putative ascorbate oxidase with ferroxidase activity; candidate for the regulation of apoplastic iron excess.
AT4G21390	(B120)	S-locus lectin protein kinase family protein;(source:Araport11)
AT3G46330	MATERNAL EFFECT EMBRYO ARREST 39 (MEE39)	Leucine-rich repeat protein kinase family protein;(source:Araport11)
AT3G51340		Eukaryotic aspartyl protease family protein;(source:Araport11)
AT3G55980	TANDEM ZINC FINGER 11 (TZF11)	CCCH-type zinc finger protein involved in salt stress and immune responses.
AT3G54040		PAR1 protein;(source:Araport11)
AT3G54420	HOMOLOG OF CARROT EP3-3 CHITINASE (EP3)	encodes an EP3 chitinase that is expressed during somatic embryogenesis in 'nursing' cells surrounding the embryos but not in embryos themselves. The gene is also expressed in mature pollen and growing pollen tubes until they enter the receptive synergid; but not in endosperm and integuments as in carrot. Post-embryonically; expression is found in hydathodes; stipules; root epidermis and emerging root hairs.
AT5G36925	ARACIN1 (ARACIN1)	hypothetical protein;(source:Araport11)
AT5G44910		Toll-Interleukin-Resistance (TIR) domain family protein;(source:Araport11)
AT5G54710		Ankyrin repeat family protein;(source:Araport11)
AT5G46050	NRT1/ PTR FAMILY 5.2 (NPF5.2)	Encodes a di- and tri-peptide transporter involved in responses to wounding; virulent bacterial pathogens; and high NaCl concentrations. The protein is predicted to have 12 transmembrane helices.
AT5G39580	(PRX62)	Class III peroxidase cell wall-targeted protein localized to the micropylar endosperm facing the radicle. Involved in seed germination.
AT5G48430		Eukaryotic aspartyl protease family protein;(source:Araport11)
AT5G48540		receptor-like protein kinase-related family protein;(source:Araport11)
AT5G43170	ZINC-FINGER PROTEIN 3 (ZF3)	Encodes zinc finger protein. mRNA levels are elevated in response to high salinity and low temperature. The protein is localized to the nucleus and acts as a transcriptional repressor.
AT5G52830	WRKY DNA-BINDING PROTEIN 27 (WRKY27)	Encodes a WRKY transcription factor WRKY27. Mutation in Arabidopsis WRKY27 results in delayed symptom development in response to the bacterial wilt pathogen Ralstonia solanacearum.
AT5G55930	OLIGOPEPTIDE TRANSPORTER 1 (OPT1)	oligopeptide transporter
AT4G25110	METACASPASE 2 (MC2)	Encodes a type I metacaspase. Two Arabidopsis metacaspases; AT1G02170 (MC1) and AT4G25110 (MC2) antagonistically control programmed cell death in Arabidopsis. MC1 is a positive regulator of cell death and requires conserved caspase-like putative catalytic residues for its function. MC2 negatively regulates cell death. This function is independent of the putative catalytic residues. A third type I Arabidopsis metacaspase is MC3 (AT5g64240).
AT4G02330	(ATPMEPCRB)	Encodes a pectin methylesterase that is sensitive to chilling stress and brassinosteroid regulation; response to Botrytis cinerea.
AT4G02380	SENESCENCE-ASSOCIATED GENE 21 (SAG21)	Encodes AtLEA5 (late embryogenesis abundant like protein). Also known as SENESCENCE-ASSOCIATED GENE 21 (SAG21). Has a role on oxidative stress tolerance. mRNA levels are elevated in response to various stresses.
AT4G09420	TIR-NBS15 (TN15)	Disease resistance protein (TIR-NBS class);(source:Araport11)
AT4G12470	AZELAIC ACID INDUCED 1 (AZI1)	Encodes AZI1 (AZELAIC ACID INDUCED 1). Involved in the priming of salicylic acid induction and systemic immunity triggered by pathogen or azelaic acid. Targeting of AZI1 to chloroplasts is increased during SAR induction and that localization requires the PRR domain. It is involved in the uptake and movement of the azelaic acid signal. AZI1 uses a previously undescribed variant of the signal anchor proteins mechanism to target plastids. AZI1 uses a bipartite N-terminal signature: a non-cleavable TMD that anchors the protein to membranes; followed by a proline rich region with features that are shared with bona fide chloroplastic transit peptides. flg22 MAMP treatment strongly induces AZI1/EARL1 protein levels and increases their relative enrichment in the plastid fraction.
AT4G40070	ARABIDOPSIS T??XICOS EN LEVADURA 32 (ATL32)	RING/U-box superfamily protein;(source:Araport11)
AT1G66920		Protein kinase superfamily protein;(source:Araport11)
AT5G43300	GLYCEROPHOSPHODIESTER PHOSPHODIESTERASE 3 (GDPD3)	Encodes a member of the glycerophosphodiester phosphodiesterase (GDPD) family.
AT5G23510		hypothetical protein;(source:Araport11)

AT5G25930	HAESA-LIKE 3 (HSL3)	LRR transmembrane receptor kinase. Binds SCREW phyto cytokine signaling peptides during plant immune response.
AT5G16190		encodes a gene similar to cellulose synthase
AT5G19230		Glycoprotein membrane precursor GPI-anchored;(source:Araport11)
AT3G45650		Encodes a nitrate efflux transporter NAXT1 (for NITRATE EXCRETION TRANSPORTER1). Localized to the plasma membrane. NAXT1 belongs to a subclass of seven NAXT members from the large NITRATE TRANSPORTER1/PEPTIDE TRANSPORTER family and is mainly expressed in the cortex of mature roots.
AT3G59700	L-TYPE LECTIN RECEPTOR KINASE V.5 (LECRK-V.5)	Member of Receptor kinase-like protein family. Represses stomatal immunity induced by <i>Pseudomonas syringae</i> pv. tomato DC3000.
AT3G50480	HOMOLOG OF RPW8 4 (HR4)	Homolog of RPW8
AT3G46690		UDP-Glycosyltransferase superfamily protein;(source:Araport11)
AT4G23190	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 11 (CRK11)	Encodes putative receptor-like protein kinase that is induced by the soil-borne vascular bacteria; <i>Ralstonia solanacearum</i> . Naming convention from Chen et al 2003 (PMID 14756307)
AT4G22470	DOUBLE HYBRID PROLINE-RICH PROTEIN 1 (DHYPRP1)	Encodes a hybrid proline-rich protein that contains two tandem PRD-8CMs (proline-rich domain-eight cysteine motif). Its expression is induced by JA and certain pathogens. This pattern of induction; combined with overexpression and RNAi induced phenotypes; suggests involvement in defense response to pathogens.
AT4G21120	AMINO ACID TRANSPORTER 1 (AAT1)	Encodes a member of the cationic amino acid transporter (CAT) subfamily of amino acid polyamine choline transporters. Mediates efficient uptake of Lys; Arg and Glu in a yeast system. The mRNA is cell-to-cell mobile.
AT4G03460		Ankyrin repeat family protein;(source:Araport11)
AT4G14610		Probably not a pseudogene based on evidence for transcription (RNA-seq) and translation (Ribo-seq) described in PMID:27791167
AT4G01700		Chitinase family protein;(source:Araport11)
AT4G19370	MODIFYING WALL LIGNIN-2 (MWL-2)	chitin synthase; putative (DUF1218);(source:Araport11)
AT5G25440	SUPPRESSOR OF ZED1-D1 (SZE1)	Receptor like kinase involved in HopZ1a effector triggered immunity. Interacts with ZAR1. Localization to membrane is dependent on N-terminal myristoylation domain.
AT5G54140	IAA-LEUCINE-RESISTANT (ILR1)-LIKE 3 (ILL3)	encodes a protein similar to IAA amino acid conjugate hydrolase; member of ILR1-like (ILL) seven-gene family.
AT5G24230	(PRLIP6)	Lipase class 3-related protein;(source:Araport11)
AT3G26500	PLANT INTRACELLULAR RAS GROUP-RELATED LRR 2 (PIRL2)	Encodes PIRL2; a member of the Plant Intracellular Ras-group-related LRRs (Leucine rich repeat proteins). PIRLs are a distinct; plant-specific class of intracellular LRRs that likely mediate protein interactions; possibly in the context of signal transduction.
AT3G26220	CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 3 (CYP71B3)	cytochrome P450 monooxygenase
AT3G22800		Leucine-rich repeat (LRR) family protein;(source:Araport11)
AT4G23260	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 18 (CRK18)	Encodes a cysteine-rich receptor-like protein kinase.
AT4G23550	(WRKY29)	Encodes WRKY DNA-binding protein 29 (WRKY29). The mRNA is cell-to-cell mobile.
AT4G23570	(SGT1A)	Closely related to SGT1B; may function in SCF(TIR1) mediated protein degradation. AtSGT1a and AtSGT1b are functionally redundant in the resistance to pathogens. AtSGT1b was more highly expressed than AtSGT1. The N-terminal TPR domain of AtSGT1a reduces the steady-state level of Arabidopsis SGT1 proteins whereas the same domain from AtSGT1b enhances SGT1 accumulation. The TPR domain is dispensable for SGT1 resistance. AtSGT1a is induced upon pathogen infection and can function in R gene-mediated resistance.
AT4G15730		CW-type Zinc Finger;(source:Araport11)
AT4G01750	RHAMNOGALACTURONAN XYLOSYLTRANSFERASE 2 (RGXT2)	Encodes a protein with UDP-xylose-dependent xylosyltransferase activity; which transfers Xyl onto L-fucose and (albeit less efficiently) L-arabinose. The linkage to L-fucose was shown to be preferentially to the C-4 position. Analysis of mutant containing T-DNA insertion in this gene indicate that the RGXT2 protein might be involved in the synthesis of the α -D-Xyl-(1;3)- α -L-Fuc-(1;4)-L-Rha structure in pectic rhamnogalacturonan II. The mRNA is cell-to-cell mobile.

AT4G21410		Encodes a cysteine-rich receptor-like protein kinase.
AT5G45070	PHLOEM PROTEIN 2-A8 (PP2-A8)	phloem protein 2-A8;(source:Araport11)
AT5G50200	WOUND-RESPONSIVE 3 (WR3)	Wound-responsive gene 3 (WR3). Encodes a high-affinity nitrate transporter. Up-regulated by nitrate. Involved in jasmonic acid-independent wound signal transduction.
AT2G36690	GERMINATION INSENSITIVE TO ABA MUTANT 2 (GIM2)	Protein belonging to the Fe-dependent 2-oxoglutarate dioxygenase superfamily; catalyzes the stereospecific hydration of GA12 to produce DHGA12; negatively regulates ABA sensitivity during germination; phototrophic establishment and seedling development.
AT2G23320	WRKY DNA-BINDING PROTEIN 15 (WRKY15)	Encodes WRKY DNA-binding protein 15 (WRKY15).
AT3G20380		TRAF-like family protein;(source:Araport11)
AT3G19930	SUGAR TRANSPORTER 4 (STP4)	Encodes a sucrose hydrogen symporter that is induced by wounding. The mRNA is cell-to-cell mobile.
AT3G22060		contains Pfam profile: PF01657 Domain of unknown function that is usually associated with protein kinase domain Pfam:PF00069; however this protein does not have the protein kinase domain
AT4G07820		CAP (Cysteine-rich secretory proteins; Antigen 5; and Pathogenesis-related 1 protein) superfamily protein;(source:Araport11)
AT4G23810	(WRKY53)	member of WRKY Transcription Factor; Group III; t WRKY53 and CRK5 are antagonistic regulators of chlorophyll synthesis/degradation; senescence; and stomatal conductance.
AT3G19010	2-OXOGLUTARATE OXYGENASE (2-OXOGLUTARATE OXYGENASE)	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein;(source:Araport11)
AT3G26230	CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 24 (CYP71B24)	putative cytochrome P450
AT4G12480	EARLY ARABIDOPSIS ALUMINUM INDUCED 1 (EARL1)	Encodes a putative lipid transfer protein; vernalization-responsive and cold-induced. It is involved in priming the SAR and ISR responses; specifically in propagating the cell-to-cell mobile signal. The kinases MPK3 (AT3G45640) and MPK6 (AT2G43790) promote the accumulation of AZI1/EARL1 at plastids during defense priming induction. The kinases MPK3 (AT3G45640) and MPK6 (AT2G43790) promote the accumulation of AZI1/EARL1 at plastids during defense priming induction.
AT3G24300	AMMONIUM TRANSPORTER 1;3 (AMT1;3)	Encodes a plasma membrane localized ammonium transporter.
AT1G51800	IMPAIRED OOMYCETE SUSCEPTIBILITY 1 (IOS1)	The gene encodes a putative member of the LRR-RLK protein family. Expressin and mutant analysis revealed that it contributes to the interaction between Arabidopsis and Hyaloperonospora arabidopsidis. and The mRNA is cell-to-cell mobile.
AT1G51820	STRESS INDUCED FACTOR 4 (SIF4)	Leucine-rich repeat protein kinase family protein;(source:Araport11)
AT3G13610	(F6'H1)	Encodes a Fe(II)- and 2-oxoglutarate-dependent dioxygenase family gene F6'H1. Mutations in this gene compromise iron uptake and the production of fluorescent phenolics involved in Fe uptake. The mRNA is cell-to-cell mobile.
AT3G13790	(ATBFRUCT1)	Encodes a protein with invertase activity. Cell wall invertase; involved in pollen development and pollen tube growth.
AT1G30110	NUDIX HYDROLASE HOMOLOG 25 (NUDX25)	Encodes a ppGpp pyrophosphohydrolase.
AT3G50010		Cysteine/Histidine-rich C1 domain family protein;(source:Araport11)
AT5G40590		Cysteine/Histidine-rich C1 domain family protein;(source:Araport11)
AT4G31550	WRKY DNA-BINDING PROTEIN 11 (WRKY11)	member of WRKY Transcription Factor; Group II-d; negative regulator of basal resistance to Pseudomonas syringae.
AT5G43290	WRKY DNA-BINDING PROTEIN 49 (WRKY49)	member of WRKY Transcription Factor; Group II-c
AT3G52450	PLANT U-BOX 22 (PUB22)	Encodes a cytoplasmically localized U-box domain E3 ubiquitin ligase protein that is involved in the response to water stress and acts as a negative regulator of PAMP-triggered immunity.
AT4G24240	WRKY DNA-BINDING PROTEIN 7 (WRKY7)	Encodes a Ca-dependent calmodulin binding protein. Sequence similarity to the WRKY transcription factor gene family.
AT4G08850	MDIS1-INTERACTING RECEPTOR LIKE KINASE2 (MIK2)	MIK1 encodes a receptor kinase that forms a complex with MDIS1/MIK2 and binds LURE1; the female pollen guidance chemi-attractant. MIK1 phosphorylates MDIS1 and is autophosphorylated.
AT5G03700		D-mannose binding lectin protein with Apple-like carbohydrate-binding domain-containing protein;(source:Araport11)
AT3G26210	CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 23 (CYP71B23)	putative cytochrome P450 The mRNA is cell-to-cell mobile.

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