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Określenie roli modułu sygnalizacyjnego MAPKKK17/18/ABI1 PP2C w regulacji odpowiedzi komórkowej na kwas abscysynowy

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Determining the role of the MAPKKK17/18–ABI1 PP2C signalling module in regulating the cellular response to abscisic acid

PhD thesis

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To my daughter, wife, and father

CONTENT

List of abbreviations

ABA	Abscisic Acid
ABI1	ABA Insensitive 1
ABRE	ABA-Responsive Element
ABF	ABRE-Binding Factor
AIK1	ABA and Immunity Kinase 1
BAM	Binary Alignment Map
BBDuk	BBMap Deduplicator/Trimmer
cDNA	Complementary DNA
CP	Core Particle (of 20S proteasome)
CRL	Cullin-RING Ligase
DEGs	Differentially expressed genes
DUB	Deubiquitylating Enzyme
E1	Ubiquitin-Activating Enzyme
E2	Ubiquitin-Conjugating Enzyme
E3	Ubiquitin Ligase Enzyme
FDR	False Discovery Rate
FPKM	Fragments Per Kilobase of Transcript per Million Mapped Reads
GTF	Gene Transfer Format
GO	Gene Ontology
HECT	Homologous to the E6-AP Carboxyl Terminus
MAPK	Mitogen-Activated Protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MAPKKKK	MAPK kinase kinase kinase
MEKK	MAPK/ERK kinase kinase
MKK	MAP kinase kinase (Arabidopsis Nomenclature)
MPK	Mitogen-Activated Protein kinase
MS	Murashige and Skoog (plant growth media)
NGS	Next Generation Sequencing
PCA	Principal Component Analysis
PE	Paired-End (Sequencing)
PP2C	Protein Phosphatase 2C
PIR	Protein Interacting with RING
PYL	PYR1-like
PYR	Pyrabactin Resistance
RACK1B	Receptor for Activated C Kinase 1B
RIN	RNA Integrity Number
RP	Regulatory Particle (of 26S proteasome)
RSL1	RING-type E3 Ligase 1
SAUR	Small Auxin Up RNA
SCF	SKP1-CUL1-F-box Protein Complex
SnRK2	SNF1-related Kinase 2
STAR	Spliced Transcripts Alignment to a Reference
TAIR	The Arabidopsis Information Resource
TPM	Transcripts Per Million
UBC	Ubiquitin-Conjugating Enzyme (E2)
UPS	Ubiquitin-Proteasome System
UPL	Ubiquitin Protein Ligase
WT	Wild Type
ZIK	Zipper-containing Kinase-like

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Streszczenie

Rośliny opierają się na złożonych sieciach sygnalizacyjnych, które umożliwiają im adaptację do stresu abiotycznego przy jednoczesnym zachowaniu elastyczności rozwojowej. Kwas abscysynowy (ABA) jest kluczowym regulatorem reakcji na stres, a jego współdziałanie z kaskadami kinaz MAPK oraz układem regulacji proteasomalnej zapewnia swoistość odpowiedzi i umożliwia adaptację do warunków środowiska. Niniejsza rozprawa dotyczy roli *MAPKKK17*, *MAPKKK18*, fosfatazy *ABI1* oraz ligaz ubikwityny typu HECT: *UPL4* i *UPL6* u *Arabidopsis thaliana* w odpowiedzi na traktowanie ABA. Analizy przeprowadzono z wykorzystaniem sekwencjonowania RNA, badań różnicowej ekspresji genów oraz testów fenotypowych linii mutantów i roślin z nadekspresją wybranych genów.

Uzyskane wyniki ujawniły odmienne, choć częściowo nakładające się funkcje *MAPKKK17* i *MAPKKK18* w odpowiedzi na ABA. *MAPKKK17* pełnił głównie rolę w regulacji metabolizmu RNA i procesów potranskrypcyjnych w warunkach traktowania ABA, natomiast *MAPKKK18* kontroluje rozwój organów i aparatów szparkowych, łącząc sygnalizację ABA z plastycznością morfogenezy. Obie kinazy regulowały wspólne moduły transkrypcyjne związane z transportem jonów, sygnalizacją kwasu jasmonowego oraz wapniową regulacją aparatów szparkowych, co wskazuje na dwupoziomową architekturę kontroli sygnałowej ABA-MAPK. Analizy transkrypcyjne wykazały ponadto, że *ABI1* oraz *MAPKKK18* wpływają na ekspresję genów odpowiedzi na auksynę (SAUR, Aux/IAA, PIN), wspierając model antagonizmu ABA–auksyna poprzez wspólne sieci transkrypcyjne. W oparciu o obserwacje profili transkrypcyjnych postawiliśmy następnie pytanie, czy regulacja zależna od ABA w sieci ABI1–MAPKKK18 ma także związek z degradacją proteasomalną, która odgrywa kluczową rolę w regulacji (resetowaniu) ścieżek sygnałowych. Część rozprawy poświęcona regulacji transkrypcyjnej czynników proteolitycznych obejmowała charakterystykę ligaz ubikwityny: *UPL4* i *UPL6*. Stwierdzono, że *UPL4* działa jako pozytywny regulator wzrostu korzeni zależnego od ABA oraz jako negatywny regulator rozwoju aparatów szparkowych, natomiast *UPL6* hamuje proliferację komórek epidermy, wpływając na gęstość aparatów szparkowych pod wpływem ABA. Ekspresja obu ligaz była podwyższona w mutantach *abi1td* i *mkkk17*, co sugeruje ich powiązanie z kluczowymi elementami sygnalizacji ABA. Podobieństwo fenotypów linii z modyfikowaną aktywnością UPL do mutantów *MAPKKK*18 dodatkowo potwierdza ich rolę w procesach sygnalizacyjnych zależnych od ABA i MAPK.

Podsumowując, przedstawione wyniki definiują sieć regulacyjną zależną od ABA, w której kaskady kinaz, fosfatazy i ligazy ubikwitynowe wspólnie modulują profile (programy) transkrypcyjne oraz procesy rozwojowe. Integracja regulacji transkrypcyjnej oraz potranskrypcyjnej, obejmującej kontrolę proteolityczną, stanowi mechanistyczne wyjaśnienie sposobu, w jaki rośliny równoważą adaptację do stresu z utrzymaniem wzrostu

Abstract

Plant responses rely on complex signalling networks to adapt to abiotic stress while maintaining developmental flexibility. Abscisic acid is a key regulator of stress responses, and its interaction with mitogen-activated protein kinase signalling and proteasome-mediated regulation ensures both specificity and adaptability. This thesis investigated the functional roles of *Arabidopsis thaliana MAPKKK17*, *MAPKKK18*, *ABI1*, and E3 ubiquitin ligases *UPL4* and *UPL6*, using RNA sequencing, differential gene expression analyses, and phenotypic assays in mutant and overexpression lines. The results revealed distinct yet overlapping roles for *MAPKKK17* and *MAPKKK18* in mediating ABA responses. *MAPKKK17* preferentially may modulate RNA metabolism and post-transcriptional regulation during stress, whereas *MAPKKK18* has been linked to organ development and stomatal patterning, thereby linking ABA signalling to morphogenetic plasticity. Both kinases share transcriptional targets involved in ion transport, jasmonic acid signalling, and calcium-mediated stomatal regulation, revealing a dual-layered regulatory architecture. Transcriptional analyses further indicated that *ABI1* and *MAPKKK18* influence the expression of auxin-responsive gene families (SAUR, Aux/IAA, PIN), supporting a role for ABA-auxin antagonism through shared transcriptional modules. These findings shed light on how plants balance stress acclimation with growth by integrating hormonal pathways through common regulatory circuits. Based on observed transcriptional profiles, we next examined whether ABA-responsive regulation within the ABI1–MAPKKK18 signalling network also extends to the proteasomal layer, which plays a critical role in shaping (regulation and resetting) of signalling outputs. To examine transcriptional components involved in ubiquitin-mediated proteolysis within this circuit, we analysed the HECT-type E3 ligases UPL4 and UPL6. UPL4 exhibits a tissue-specific dual role: it acts as a positive regulator of ABA-dependent primary root elongation and as a negative regulator of stomatal development. UPL6 shows transcriptional and phenotypic patterns consistent with a role in ABA-dependent developmental regulation.

Together, these findings suggest a previously underexplored ABA-responsive regulatory framework where *MAPKKK17*/18, *ABI1*, and *UPL4*/6 converge. By integrating kinase cascades, phosphatase activity, and ubiquitin-mediated proteolysis, this work reveals an additional layer of ABA–MAPK regulatory control, filling a key knowledge gap in how hormonal and proteolytic networks jointly fine-tune plant stress adaptation and development.

1. Introduction

1.1. Overview of plant abiotic stress signalling

Abiotic stress signalling in plants constitutes an integrated network combining hormonal, transcriptional, and post-translational regulation to optimize survival under fluctuating environments. This section outlines key signalling mechanisms, particularly those involving ABA, which orchestrate plant responses to stress.

Plants regularly encounter various environmental stresses, including drought, salinity, and extreme temperatures, which can severely compromise growth, development, and productivity. To adapt, plants have evolved complex signalling networks that perceive stress cues and orchestrate appropriate physiological and transcriptional responses (Nishimura et al., 2007; Zhu, 2016). Among stress-responsive mechanisms, hormonal regulation plays a significant role in modulating plant stress responses. Abscisic acid (ABA) is a key phytohormone that mediates adaptation to abiotic stress by regulating processes such as stomatal closure, osmotic adjustment, seed dormancy, and transcriptional reprogramming of stress-responsive genes (Finkelstein, 2013). ABA levels rapidly increase in response to drought and salinity, leading to the activation of core components that transmit ABA signals to downstream targets (Fujita et al., 2009; Geiger et al., 2009; Ludwików et al., 2009; Umezawa et al., 2009; Yoshida et al., 2016). The spatiotemporal dynamics of ABA biosynthesis and signalling are tightly regulated, highlighting its dual role in stress protection and developmental control. Transcriptomic studies have revealed that ABA signalling triggers extensive changes in gene expression, affecting hundreds to over a thousand genes depending on the tissue, developmental stage, and intensity of stress (Song et al., 2016; Zhu et al., 2017). These transcriptional responses are coordinated by a network of protein kinases, phosphatases, and transcription factors, which act together to fine-tune the plant's physiological state during stress. Although core ABA components are well characterised, the upstream integration of ABA with parallel pathways like MAPK remains poorly understood.

1.2. ABA signalling pathway

The core ABA receptor signalling pathway is composed of three main components: *PYR/PYL/RCAR* receptors, type 2C protein phosphatases (PP2Cs), and SNF1-related protein kinases (*SnRK2s*) (Fujita et al., 2009; Park et al., 2009; Umezawa et al., 2009). This conserved pathway forms the basis of ABA-mediated responses (Park et al., 2009). In *Arabidopsis thaliana*, PP2C gene family contains 76 members, with clade A PP2Cs being central participants in ABA signalling (Santiago et al., 2009; Schweighofer et al., 2004). Key members of clade A PP2C include *ABI1*, *ABI2*, *HAB1*, and *PP2CA*, which are involved in the core ABA signalling module (Fujita et al., 2009; Nishimura et al., 2007; Rubio et al., 2009; Umezawa et al., 2009). Their conserved N-terminal domain interacts with PYR/RCAR receptors. The C-terminal domain dephosphorylates SnRK2 kinases, suppressing ABA responses (Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). The first step in the ABA signalling pathway is the perception of ABA by receptors (Hou et al., 2016). Upon ABA binding, PYR/PYL receptors undergo a conformational change, enabling them to interact with and inhibit type 2C protein phosphatases (PP2Cs), such as *ABI1* and homologous phosphatases (Han et al., 2022; Soon et al., 2012; Umezawa et al., 2009; Vlad et al., 2009). The inhibition of PP2Cs leads to the activation of SnRK2s (SNF1-related protein kinases 2). SnRK2s (such as *SnRK2.2, 2.3*, and *OST1/SnRK2.6*) are protein kinases that phosphorylate and activate downstream targets including transcription factors and ion channels involved in ABA signalling (Yan et al., 2017). These protein kinases play a key role in transducing the ABA signal, coordinating the plant's physiological and transcriptional responses to stress (Imes et al., 2013; Lee et al., 2009; Vahisalu et al., 2010).

In the absence of ABA, PP2Cs maintain SnRK2s in an inactive state by direct dephosphorylation, thereby suppressing ABA responses. Upon ABA binding, PYR/PYL receptors undergo a conformational change that enables them to inhibit PP2C activity, releasing SnRK2s from repression (Fujita et al., 2009; Krzywińska et al., 2016; Maszkowska et al., 2021). Activated SnRK2s, in turn, phosphorylate a variety of downstream effectors, including AREB/ABF-type transcription factors, ion channels, and other regulatory proteins, leading to widespread transcriptional reprogramming and physiological responses (Fujita et al., 2013; Yoshida et al., 2010). This signal transduction cascade ensures a rapid and robust shift in the cellular machinery in response to abiotic stress conditions.

This core signalling module ensures rapid and dynamic modulation of ABA-responsive genes. Notably, *SnRK2*.2, *SnRK2*.3, and *SnRK2*.6 (*OST1*) are the primary kinases responsible for activating ABA-responsive transcription factors such as ABF2/AREB1 (ABRE-Binding Factors), *ABF3*, and *ABF4*, which bind to ABA-responsive elements (ABREs) in gene promoters (Yoshida et al., 2015). This interaction drives the expression of hallmark stress-inducible genes such as *RD29A, RAB18,* and *COR15A*, which play crucial roles in osmoprotection, maintaining cellular homeostasis, and dehydration tolerance (Brocard-Gifford et al., 2004; Yamaguchi-Shinozaki & Shinozaki, 2006). In addition to transcriptional control, ABA signalling also modulates rapid responses through ion flux regulation and stomatal closure, processes that are particularly critical for water conservation during drought. Here, SnRK2-mediated phosphorylation of ion channels such as *SLAC1* and *KAT1* mediate ABA-induced ion flux changes in guard cells (Brandt et al., 2015; Geiger et al., 2009). These rapid, non-transcriptional responses complement the transcriptional regulation to ensure plant survival under fluctuating environmental conditions. Although the *PYR/PYL/RCAR-ABI1-SnRK2* cascade has been well-characterised, accumulating evidence suggests that it is a part of a larger, more complex signalling network (Wang et al., 2018). Crosstalk with other signalling pathways and the involvement of additional kinases, phosphatases, and E3 ubiquitin ligases suggest the presence of multiple regulatory layers that modulate ABA sensitivity and specificity (Bueso et al., 2014; Soon et al., 2012). This complexity underscores the need for further research into how ABA signalling is integrated and fine-tuned in different cellular contexts. In this context, determining how kinases such as MAPKKKs modulate ABA responses and how they are regulated themselves has become an emerging research priority.

In summary, plants deploy intricate signalling networks, with hormonal regulation, particularly via ABA, playing a pivotal role in orchestrating adaptive responses to abiotic stress.

1.3.MAPK cascades in plant signalling

Beyond core hormonal signalling, plants use MAPK cascades to rapidly relay stress cues and translate them into gene expression or physiological responses. MAPK cascades exhibit significant crosstalk with ABA signalling pathways at upstream and downstream regulatory levels, offering opportunities to decode crosstalk mechanisms.

Mitogen-activated protein kinase (MAPK) cascades constitute highly conserved intracellular signalling modules that mediate plant responses to a broad spectrum of stimuli, ranging from

developmental cues to environmental stress. These cascades are composed of three main tiers of kinases: MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MAPKKs), and MAP kinases (MAPKs), that act sequentially through phosphorylation events to regulate target proteins (Figure 1A). MAPKs are serine/threonine kinases that regulate processes such as proliferation, differentiation, apoptosis, drought, salinity, and cold stress responses (Colcombet & Hirt, 2008; Danquah et al.,2014, 2015; Komis et al., 2018; Sinha et al., 2011).

Despite being conserved in structure and function, MAPK cascades display high versatility and specificity. Activation of the cascade begins at the level of MAPKKKs, which may themselves be activated by upstream kinases (such as MAPKKKKs), small G-proteins, or phosphorylation at proline-rich motifs, leucine zippers, and G-protein binding domains (Colcombet & Hirt, 2008; Jagodzik et al., 2018; Matsuoka et al., 2015). MAPK cascades typically proceed through a canonical three-tiered phosphorylation hierarchy (Komis et al., 2018). The activated MAPKs then phosphorylate diverse cellular targets including transcription factors, metabolic enzymes, and regulatory proteins, allowing the plant to mount precise physiological responses (Meng et al., 2013; Raghuram et al., 2015; Zhang et al., 2022). The complexity of this signalling network in Arabidopsis thaliana is reflected by the presence of approximately 80 MAPKKKs, 10 MAPKKs, and 23 MAPKs, forming a highly redundant yet fine-tuned signalling system (Danquah et al., 2014, 2015; Forzani et al., 2011; Jonak et al., 2002; Teige et al., 2004; Wengier et al., 2018).The specificity of kinase interactions is guided by conserved docking motifs and spatiotemporal expression patterns (Bai & Matton, 2018; Benhamman et al., 2017). Moreover, differential subcellular localization and stimulus-dependent assembly of signalling complexes further enhance the precision of MAPK-mediated responses.

Overall, MAPK cascades provide a versatile and finely tuned signalling framework that allows plants to translate diverse external and internal stimuli into precise cellular responses.

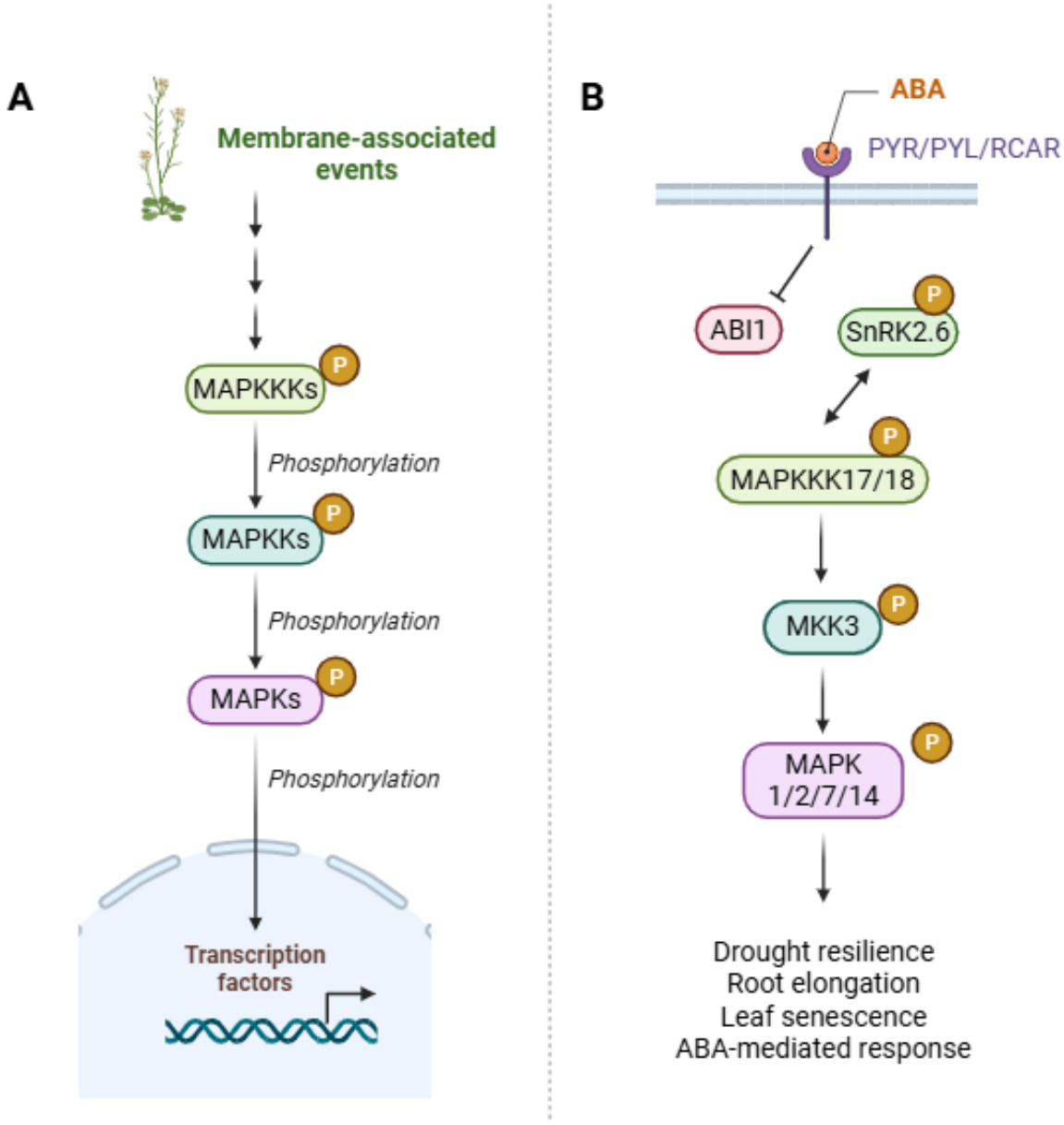

Figure 1. MAPK cascade and ABA-regulated *MAPKKK17*/18 signalling pathway in plants. (A) General MAPK signalling cascade comprising MAPKKK, MAPKK, and MAPK modules activated by developmental cues or environmental stress. Sequential phosphorylation events lead to substrate activation and downstream responses. (B) ABA-induced signalling pathway involving *PYR/PYL/RCAR* receptors and inhibition of *ABI1*, resulting in activation of *MAPKKK17/18, MKK3,* and *MPK1/2/7/14*. This cascade regulates drought resilience, root elongation, leaf senescence, and ABA-mediated responses. Figure redrawn in BioRender based on Danquah et al., 2015; Jagodzik et al., 2018.

1.3.1. MAP kinase kinase kinases

MAPKKKs initiate the MAPK cascade and are the most diverse component, with members grouped into three major subfamilies: MEKK-like, Raf-like, and ZIK-like kinases (Colcombet & Hirt, 2008; Danquah et al., 2014; Meng et al., 2013; Mitula et al., 2015). These subfamilies are distinguished by their domain architecture and conserved motifs.

The Arabidopsis MEKK-like kinase group consists of 22 members and contains the G(T/S) PX(W/Y/F) MAPEV motif which is located at the N- or C-terminus of the protein or in the central part of the protein. These kinases are primarily initiate MAPK cascades in response to external cues (Colcombet & Hirt, 2008; Jagodzik et al., 2018; Jonak et al., 2002; Nakagami et al., 2005). The MEKK-like kinases are the best characterised among the MAPKKK classes. In *Arabidopsis*, these are further divided into four subgroups (A1-A4) (Ichimura et al., 2002; Jagodzik et al., 2018). Subgroup A1 includes *MEKK1* to *MEKK4*, where *MEKK1* is a major player in both abiotic and biotic stress responses. Subgroup A2 contains kinases like *MAPKKK3*, *MAPKKK4* (*YODA*), and *MAPKKK5*, which are involved in the patterning of stomata (Ichimura et al., 2002; Jagodzik et al., 2018; Sun et al., 2018). The A3 subgroup (ANP1-3) is implicated in cytokinesis and oxidative stress response (Banerjee & Roychoudhury, 2017; Ichimura et al., 2002; Krysan et al., 2002), such as H_2O_2-induced activation of *MPK3* and *MPK6*, ultimately leading to repression of auxin-related gene expression (Colcombet & Hirt, 2008; Li et al., 2017; Raghuram et al., 2015). Subgroup A4 includes *MAPKKK6*, *MAPKKK7*, and *MAPKKK13-21*. *MAPKKK6* and *MAPKKK7* play key roles in pollen development and function (Chaiwongsar et al., 2012). Plants overexpressing *MAP3K16* show insensitivity to ABA during seed germination and cotyledon greening, while being hypersensitive to ABA during root development. Further studies have shown that *MAPKKK14-18* kinases are also involved in ABA responses (Choi et al., 2017).

The Raf-like group, with 46 members, has a characteristic C-terminal kinase domain with the GTXX(W/Y) MAPE motif and a long N-terminal regulatory domain. ZIK-like kinases (21 members) are characterised by the presence of a GTPEFMAPE(L/V/M) (Y/F/L) motif and an extended N-terminal domain that may regulate protein-protein interactions (Nakagami et al., 2005; Popescu et al., 2009; Wang et al., 2015; Zhang et al., 2018). Raf kinases are divided into two groups: group B, which is further divided into four subgroups (B1-B4), and group C (Lin et al., 2021; Saruhashi et al., 2015). To date, most members of group C have not been characterised.

Group B includes the following four subgroups (B1-B4), among which *EDR1* (Enhanced Disease Resistance 1) and *CTR1* (Constitutive Triple Response 1) are functionally significant. EDR1 plays a role in the developing resistance to pathogens (Christiansen et al., 2011; Frye et al., 2001; Neubauer et al., 2020). *Raf10* and *Raf11*, also in this group, participate in ABA signalling pathways (Lee et al., 2015). Furthermore, the *EDR1-MKK4*/*MKK5* module participates in regulating plant innate immunity mechanisms (Zhao et al., 2014). *CTR1* is a negative regulator of ethylene signalling (Ju et al., 2012) and influences stomatal closure via salicylic acid signalling (Wang et al., 2020). Recent studies have highlighted the role of Raf B2 and B3 kinases in mediating ABA-induced osmotic stress responses through activation of *SnRK2*.2/3/6 kinases, essential components in ABA signalling (Katsuta et al., 2020; Lin et al., 2021). Raf kinases thus play a significant role in plant tolerance to osmotic stress, ABA-coordinated responses, as well as in plant growth and development (Lin et al., 2020). Raf kinases such as *MAPKKKδ1*, *δ6*, and *δ7* are crucial for full activation of *SnRK2*.6 and downstream elements like the SLAC channel, with mutants exhibiting ABA insensitivity (Katsuta et al., 2020; Lin et al., 2021; Lin et al., 2020). Additionally, *Raf36* and *Raf22*, from group C, act as negative regulators of ABA signalling through interactions with *SnRK2*.6 (Kamiyama, Katagiri, et al., 2021). A newly identified Raf kinase, *MAPKKKδ*-1 (*MKD1*), has been linked to defence responses against bacterial and fungal pathogens (Asano et al., 2020).
ZIK-like kinases are the least characterised and possess unique sequence motifs such as GTPEFMAPE(L/V/M) (Y/F/L). Their regulatory roles are emerging, particularly in hormonal signalling and pathogen defence (Nakagami et al., 2005; Wang et al., 2015).
Collectively, MAPKKs serve as critical intermediates that translate upstream signals from MAPKKKs to MAPKs, thereby orchestrating complex plant responses to environmental and developmental cues.

1.3.2. MAP kinase kinases

MAPK kinases (MAPKKs), also known as dual-specificity kinases, are central to the MAPK cascade, linking MAPKKKs and MAPKs. They phosphorylate MAPKs at both threonine and tyrosine residues within the conserved T-X-Y motif, which is essential for full activation. Based on sequence similarity and domain architecture, the Arabidopsis MAPKKs are classified into four groups: A, B, C, and D (Banerjee et al., 2020; Hamel et al., 2006).

Group A MAPKK includes *MKK1, MKK2,* and *MKK6*, which participate in stress responses and innate immunity by interacting with *MPK4, MPK6,* and *MPK11* (Cristina et al., 2010; Jagodzik et al., 2018; Kong et al., 2013; Mészáros et al., 2006). In addition, *MKK1* activates *MPK3* and *MPK12* (Lee et al., 2008; Mészáros et al., 2006), and *MKK2* interacts with *MPK5*, *MPK10*, and *MPK13* (Gao et al., 2008; Lee et al., 2008; Teige et al., 2004). *MKK6* activates *MPK13* and interacts with *MPK12* in yeast cells (Jagodzik et al., 2018; Lee et al., 2008; Melikant et al., 2004).

Group B is represented solely by *MKK3*, a kinase primarily involved in ABA and osmotic stress signalling. *MKK3* activates *MPK1*, *MPK2*, *MPK7*, and *MPK14*, contributing to ABA-mediated gene expression and physiological adaptation (Dóczi et al., 2007).

Group C consists of *MKK4* and *MKK5*, both of which are integral to the *YODA-MKK4/5-MPK3/6* cascade. This pathway regulates stomatal development and cell fate determination (Meng et al., 2012; Meng & Zhang, 2013). Additionally, *MKK4* and *MKK5* are directly activated by receptor-like kinases such as *TMK1* and *TMK4* in response to auxin, representing a direct link between hormonal perception and MAPK signalling (He & Meng, 2020; Huang et al., 2019).

Group D includes *MKK7, MKK8, MKK9,* and *MKK10*, which mediate response to hormone and abiotic stress (Dóczi et al., 2007; Ichimura et al., 2002; Jagodzik et al., 2018; Teige et al., 2004). *MKK7* and *MKK9* kinases involved in the crosstalk of stress-induced signalling pathways with plant growth regulatory pathways (Dóczi et al., 2007). *MKK7* interacts with several MPKs, including *MPK2, MPK6, MPK12,* and *MPK15*, and is involved in defence, cell death, and auxin transport regulation (Dai et al., 2006; Jia et al., 2016; Lee et al., 2009; Lee et al., 2008; Shen et al., 2019). *MKK9* activates *MPK6, MPK10, MPK12, MPK17,* and *MPK20*, playing a role in ethylene biosynthesis and signalling (Lee et al., 2009; Lee et al., 2008; Shen et al., 2019; Xu et al., 2008; Yoo et al., 2008). In addition, *MKK7* promotes plant protection against pathogens and participates in programmed cell death (Dai et al., 2006; Jia et al., 2016; Popescu et al., 2009; Zhang et al., 2007). *MKK9* is functionally related to both ethylene biosynthesis, as well as signalling (Xu et al., 2008; Yoo et al., 2008). *MKK7* and *MKK9* are both associated with salt stress responses and stomatal development (Alzwiy & Morris, 2007; Lampard et al., 2009; Shen et al., 2019; Xu et al., 2008). Recently, the role of the *MKK10-MPK6* module in photomorphogenesis, which is controlled by red light has also been described (Xin et al., 2018).

Overall, MAPKKs function as pivotal integrators within MAPK cascades, linking upstream MAPKKKs to downstream MAPKs and modulating responses to diverse signals.

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1.3.3. MAP kinases

MAP kinases (MAPKs), also known as MPKs in plants, constitute the terminal components in the canonical MAPK signalling cascades. They translate upstream signals into cellular responses via the phosphorylation of various substrates. These serine/threonine protein kinases are activated by the dual phosphorylation of conserved threonine (T) and tyrosine (Y) residues within the T-X-Y motif, which is located in the activation loop of the kinase domain. This phosphorylation is mediated by upstream MAPK kinases (MAPKKs), which confer signal specificity and enable diverse functional outputs (Ichimura et al., 2002). In *Arabidopsis thaliana*, a total of 20 MAPKs has been identified and functionally classified into four major groups (A-D), based on sequence homology, domain organization, and activation loop motifs (Bigeard & Hirt, 2018; Ichimura et al., 2002; Jagodzik et al., 2018; Wang et al., 2015). These groups exhibit variations in their T-X-Y motifs, with groups A, B, and C (12 members) containing the canonical T-E-Y (threonine-glutamic acid-tyrosine) sequence, whereas group D kinases (8 members) possess the T-D-Y (threonine-aspartic acid-tyrosine) motif, potentially imparting functional divergence in signalling dynamics (Ali et al., 2021; Bigeard & Hirt, 2018).

Group A MAPKs include *MPK3, MPK6,* and *MPK10*. Among these, *MPK3* and *MPK6* are the most characterised and functionally versatile MAPKs in plants. They are implicated in a broad spectrum of signalling pathways, including the regulation of innate immune responses (Asai et al., 2002; Su et al., 2018), abiotic stress responses such as oxidative and osmotic stress (Kovtun et al., 1999; Zhao et al., 2017), and hormone-mediated pathways involving ethylene and jasmonic acid (Guan et al., 2015; Han et al., 2010; Han et al., 2019). Furthermore, *MPK3* and *MPK6* play crucial roles in developmental processes such as stomatal patterning and differentiation, where they act downstream of the *YODA-MKK4*/5 module to regulate asymmetric cell division (Bergmann et al., 2004; Wang et al., 2022; Wang & Gou, 2020).

MPK3 and *MPK6*, due to their extensive involvement in various signalling contexts, exemplify the functional redundancy and complexity inherent to the MAPK family. *MPK3*/*MPK6* act as master regulators of stress-induced transcriptional programs in response to environmental and developmental signals (Lee et al., 2019). Regulation of GDSL-like lipase genes (*GLIP1*, *GLIP3*, *GLIP4*) are induced by *MPK3*/*MPK6* in response to biotic stress and hormone treatment (Han et al., 2019). The transcription factor *WRKY33* has been identified as a key downstream target of

MPK3/*MPK6* in this pathway, and it is required for the expression of GLIP1 and other pathogen defence-related genes (Han et al., 2019; Zhao et al., 2020)

Group B encompasses *MPK4*, *MPK5*, and *MPK11*-13. *MPK4*, in particular, has received attention due to its dual role as both a negative regulator of salicylic acid (SA)-dependent defence responses under normal conditions and as a positive regulator of stress tolerance upon activation (Gao et al., 2008; Ichimura et al., 2002). *MPK11* to *MPK13* function in redundant or partially overlapping signalling circuits with *MPK3*/6, often contributing to stress-related and developmental responses under specific contexts (Ichimura et al., 2002; Wang et al., 2015).

Group C includes MAPKs such as *MPK1*, *MPK2*, *MPK7*, and *MPK14*, which are known to act downstream of *MKK3*. These kinases have been associated with stress-related signalling, including ABA-mediated responses and osmotic stress adaptation (Liang et al., 2013; Wang et al., 2015). The involvement of these MAPKs in ABA signalling indicates a convergence between the MAPK cascade and the core ABA components, highlighting the versatility of MAPK modules in hormonal crosstalk (Danquah et al., 2015).

Group D represents the least characterised class of MAPKs, comprising *MPK8*, *MPK9*, and *MPK15*-20 (Liang et al., 2013; Mohanta et al., 2015; Wankhede et al., 2013). These kinases contain the T-D-Y motif and are believed to be involved in more specialized or tissue-specific signalling events. Although data on their precise biological functions remain limited, emerging studies suggest that members of this group may regulate ion channel activity, participate in guard cell signalling, and contribute to responses to mechanical stimuli and stress-induced calcium fluxes (Mohanta et al., 2015; Wankhede et al., 2013).

In summary, MAPKs are critical signalling hubs that integrate diverse cues into tailored physiological responses. While *MPK3*, *MPK4*, and *MPK6* are well characterised, further investigation into groups C and D MAPKs is essential for a comprehensive understanding of the MAPK network's role in plant plasticity and resilience.

1.3.4. MAP kinase cascades

Despite extensive research, only a limited number of fully characterised three-tiered MAPK signalling cascades have been identified in Arabidopsis thaliana. Several major cascades have been studied in detail, including the *MEKK1-MKK1/2/4/5-MPK3/6* pathway, the *YODA-MKK4/5/7/9-MPK3/6* developmental cascade, the immune-responsive *MAPKKK3/5-MKK4/5-MPK3/6*

cascade, and the ABA-responsive *MAPKKK17/18-MKK3-MPK1/2/7/14* cascade. The first and most comprehensively defined cascade is the *MEKK1-MKK4/5-MPK3/6* pathway, which is activated upon perception of bacterial pathogen-associated molecular patterns (PAMPs) by the flagellin receptor *FLS2* (Asai et al., 2002). *MEKK1* phosphorylates *MKK4* and *MKK5*, which in turn activate *MPK3* and *MPK6*. *MPK3* and *MPK6* subsequently phosphorylate the *WRKY22* and *WRKY29* transcription factors, initiating defence gene expression against bacterial and fungal pathogens (Liang et al., 2013). In addition, *MEKK1* activates two further MAPK modules: *MEKK1-MKK1/2-MPK4/6*, which mediates responses to abiotic stresses, including those caused by cold and salinity (Teige et al., 2004); and *MEKK1-MKK1/2-MPK4*, which is primarily involved in basal immune signalling and the suppression of autoimmunity (Gao et al., 2008; Takagi et al., 2019). Furthermore, in the *MEKK1-MKK1/2-MPK4* cascade, *MEKK1* phosphorylates and stabilises the transcription factor *STOP1*, thereby enhancing aluminium resistance (Zhou et al., 2023). *MEKK1* also modulates Agrobacterium-mediated transformation efficiency via the *MEKK1-MKK4/5-MPK3/6* module (Asai et al., 2002). Taken together, these findings establish *MEKK1* as a pivotal regulatory hub in plant MAPK signalling, highlighting its functional diversity (Matsuoka et al., 2018).

The second well-characterised signalling cascade is the *YODA-MKK4/5/7/9-MPK3/6* module, which plays a critical role in plant development, particularly stomatal patterning (Wang et al., 2022). This cascade regulates asymmetric cell divisions in the epidermis, thereby controlling stomatal density and distribution. Beyond stomatal development, YODA signalling influences organogenesis and inflorescence architecture through downstream activation of *MPK3* and *MPK6* (Meng et al., 2012). Intriguingly, the kinases *MKK7* and *MKK9*, also phosphorylated by *YODA*, can activate *MPK3/6* and substitute for *MKK4/5* in stomatal regulation (Lampard et al., 2009). Moreover, *MAPKKK3* and *MAPKKK5*, homologous to *YODA*, activate the same *MKK4*/5-*MPK3/6* module but are triggered by receptor-like kinases (RLKs) upon PAMP detection, thereby functioning in immune responses (Sun et al., 2018). These two modules-*YODA* and *MAPKKK3/5*-initiated cascades-exert mutually antagonistic regulation, whereby *YODA* suppresses *MPK3/6* activation by PAMPs, and conversely, *MAPKKK3/5* signalling dampens YODA-mediated developmental processes (Sun et al., 2018; Wang et al., 2022).

A prominent MAPK module is the ABA-responsive *MAPKKK17/18-MKK3-MPK1/2/7/14* cascade. That cascade is activated under drought and osmotic stress conditions and functions in

parallel with canonical ABA signalling pathways (Danquah et al., 2015; Matsuoka et al., 2015). A related cascade, the *MAPKKK20* (also known as *AIK1*)-*MKK5*-*MPK6* pathway, contributes to ABA-induced stomatal closure and root development. AIK1 mutants exhibit impaired ABA responses, including defective root growth inhibition and stomatal function under ABA treatment (Li et al., 2017). *AIK1* participates in non-canonical MAPK modules, such as one involving *AIK1*, *MKK3*, and an unidentified MPK, with the *AIK1* C-terminal domain mediating interaction with *MKK3* via a DEF docking site (Bai & Matton, 2018; Benhamman et al., 2017). Remarkably, *AIK1* can also bypass MAPKKs and directly phosphorylate *MPK18* (Benhamman et al., 2017).

Recently, the *MAPKKKδ*-1 kinase (*MKD1*) has been identified as an initiator of an immune-responsive MAPK cascade essential for resistance against bacterial (*Pseudomonas syringae*) and the fungal (*Fusarium sporotrichioides*) pathogens. *MKD1* phosphorylates *MKK1* and *MKK5* in vitro and interacts with in vivo. Functional analyses reveal that mutants lacking *MKK1* or with suppressed *MKK5* expression exhibit increased susceptibility to Fusarium infection. *MKD1* is crucial for full activation of *MPK3* and *MPK6*, implicating the *MAPKKKδ*-1-*MKK1/MKK5*-*MPK3/MPK6* cascade as a key component of plant immunity (Asano et al., 2020). *Despite these advances, many stress-responsive MAPKKKs remain poorly characterised. The detailed upstream activators, downstream effectors, and regulatory dynamics of these kinases remain unknown. In particular, the mechanisms by which MAPKKK17 and MAPKKK18, two closely related MEKK-like kinases, integrate ABA signalling with developmental processes remain to be elucidated. Additionally, the extent to which these MAPKKKs engage in hormonal crosstalk, particularly with auxin signalling pathways, is a key area for future research.*

In summary, the elucidated MAPK cascades in Arabidopsis demonstrate complex regulatory networks that integrate environmental and developmental signals. The *MEKK1-MKK1/2/4/5-MPK3/6, YODA-MKK4/5-MPK3/6,* and ABA-responsive *MAPKKK17/18-MKK3-MPK1/2/7/14* modules exemplify how plants utilize specific MAPK modules to orchestrate defence, development, and stress adaptation. Nonetheless, a substantial number of MAPKKKs await functional characterization to fully comprehend the MAPK signalling architecture and its role in plant plasticity and resilience. Whether and how these kinases contribute to hormonal crosstalk remains unclear and requires further investigation.

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1.3.5. Role of *MAPKKK*17/18 cascade in ABA signalling and response

As mentioned above, only a few MAPKKKs have been clearly characterised as specific activators of downstream MAPKKs. These include *MEKK1*, *YODA* (*MAPKKK4*), *ANP*, and *MAPKKK18* (Mitula et al., 2015; Sun et al., 2018; Wang & Gou, 2020; Xie et al., 2023). Within the extensive MAPK signalling network, *MAPKKK17* and *MAPKKK18* function as key regulators of ABA responses and abiotic stress adaptation (Zhao et al., 2023). These two closely related Raf-like MAPKKKs constitute the upper tier of an ABA-responsive MAPK module comprised of *MAPKKK17/18, MKK3*, and the C-group *MAPKs MPK1, MPK2, MPK7,* and *MPK14* (Danquah et al., 2015; Figure 1B; Table 1). Genetic and molecular evidence indicates that *MAPKKK17* and *MAPKKK18* are co-expressed and functionally redundant in mediating ABA signalling, senescence, and responses to drought and salt stress (Choi et al., 2017; Eckstein et al., 2021). *MAPKKK18* is the smallest MAPKKK in Arabidopsis, consisting of 339 amino acids (~38 kDa), and structurally belongs to the MEKK subfamily. Uniquely, it lacks the canonical N-terminal regulatory domain but contains a noncanonical C-terminal domain with three predicted phosphorylation sites of unknown function (Mitula et al., 2015). *MAPKKK18* expression is tightly regulated at multiple levels and is strongly induced by ABA and osmotic stress. Transcriptionally, ABA-responsive element-binding factors (ABFs), including *ABF2*, *ABF3*, and *ABF4*, directly bind the *MAPKKK18* promoter to transactivate its expression in response to ABA (Lin et al., 2017; Yu et al., 2021). Beyond ABA, *MAPKKK18* is also upregulated by various environmental and biotic stressors such as wounding, salt (NaCl), polyethylene glycol (PEG), mannitol, light stress, ozone, jasmonic acid, and pathogen-associated signals (Danquah et al., 2015; De Torres-Zabala et al., 2007; Hoth et al., 2002; Leonhardt et al., 2004; Ramel et al., 2012; Taki et al., 2005). Promoter::GUS analyses demonstrate ABA-dependent activation of *MAPKKK18* expression in guard cells, root tips, hydathodes, and flowers (Mitula et al., 2015; Okamoto et al., 2006). This induction requires intact core ABA signalling, as evidenced by reduced *MAPKKK18* expression in mutant's defective in ABA receptors and signalling components such as *abi1*, *pyr1pyl1pyl2pyr4*, *hab1*, and *pyr1pyl1* (Danquah et al., 2015; Matsuoka et al., 2015, 2018; Vaidya et al., 2019). This broad range of inducers underscores its role as an integrative signalling node in plant stress responses.

Post-translationally, *MAPKKK18* is activated by *SnRK2*.6-mediated phosphorylation and inactivated by *ABI1*-mediated dephosphorylation. *ABI1*, a protein phosphatase type 2C promotes degradation of *MAPKKK18* via the 26S proteasome (Matsuoka et al., 2015; Tajdel et al., 2016). Proteomic studies have identified lysine residues K154 and K237 as key ubiquitination sites targeted by E3 ligases such as *KEG*, *UPL1*, and *UPL4*, which fine-tune *MAPKKK18* protein levels under ABA stress (Tajdel-Zielińska et al., 2024). In *abi1td* mutants, *MAPKKK18* degradation is slowed, and ABA treatment stabilizes *MAPKKK18* by suppressing proteasomal turnover. Additionally, persulfidation of *ABI4* by hydrogen sulfide (H_2S) indirectly enhances *MAPKKK18* transcription, highlighting complex regulation at multiple layers (Zhou et al., 2021). *MAPKKK18* localizes to both the cytoplasm and nucleus, consistent with its dual role in transmitting stress signals and regulating gene expression (Mitula et al., 2015; Zhao et al., 2023).

Functionally, *mapkkk18* loss-of-function mutants show increased drought sensitivity and delayed ABA-induced senescence, whereas overexpression lines exhibit accelerated senescence and heightened ABA responsiveness. *MAPKKK18* promotes stomatal closure under ABA treatment, with overexpression lines demonstrating faster closure and improved water retention, while mutants exhibit impaired stomatal control (Li et al., 2017). Interestingly, *MAPKKK18* overexpression increases stomatal density, while knockout mutants show reduced density, suggesting a developmental role in stomatal patterning (Mitula et al., 2015). MAPKKK18 also modulates seed sensitivity to ABA, as overexpression reduces ABA sensitivity during germination, whereas mutants display enhanced ABA-induced inhibition of germination. Physiologically, *MAPKKK18* overexpression enhances drought resistance, reduces water loss, and accelerates leaf senescence and chlorophyll degradation, especially following ABA exposure (Matsuoka et al., 2015, 2018)

MAPKKK18 activate the C-group MAPK cascade through *MKK3*, leading to phosphorylation of *MPK1, MPK2, MPK7,* and *MPK14* (Danquah et al., 2015). Physical interactions between *MAPKKK18* and *MKK3* have been confirmed by yeast two-hybrid and BiFC assays, and ABA-induced activation of *MPK7* is abolished in *mapkkk17*/*mapkkk18* and *mkk3* mutants (Benhamman et al., 2017; Table 1). This demonstrates *MAPKKK*18's upstream role in the ABA-specific MKK3-MPK module. The double mutant *mapkkk17mapkkk18* exhibits hypersensitivity to ABA compared to wild type, indicating overlapping regulatory functions (Choi et al., 2017; Danquah et al., 2015; Table 1). Similarly, *MAPKKK17* is activated by drought, salt, and ABA treatments, sharing

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overlapping roles with *MAPKKK18* in promoting ABA responses. *MAPKKK17* overexpression reduces ABA sensitivity, whereas double mutants with *MAPKKK18* exhibit hypersensitivity (Danquah et al., 2015; Matsuoka et al., 2018). Both kinases contribute to stomatal closure and senescence regulation, while *MAPKKK17* also influences flowering transitions and may regulate *MPK18*-mediated microtubule organization in the root cortex (Benhamman et al., 2017).

Table 1. Summary of major MAPK cascades in Arabidopsis

No	MAPKKK	MAPKK	MAPK	Function	Stimulus
1	*MEKK1*	*MKK4/5*	*MPK3/6*	Innate immunity	PAMPs
2	*YODA*	*MKK4/5*	*MPK3/6*	Stomatal development	developmental
3	*MAPKKK17/18*	*MKK3*	*MPK1/2/7/14*	ABA signalling, drought response	ABA, salt
4	*AIK1*	*MKK3/5*	*MPK6* or *MPK18*	Stomatal closure, root growth	ABA
5	*MKD1*	*MKK1/5*	*MPK3/6*	Pathogen resistance	bacterial/fungal infection

Taken together, *MAPKKK17* and *MAPKKK18* function as positive regulators of ABA signalling by modulating the C-group MAPK module and coordinating stress- and development-related responses (Dai et al., 2006; Danquah et al., 2015). Their activity is tightly controlled at transcriptional, post-translational, and proteolytic levels, positioning them as critical nodes for signal amplification and specificity within the ABA network. Beyond ABA responses, these kinases contribute to leaf senescence and microtubule organization in the root cortex, where *MAPKKK18* interacts with both *MKK3* and *MPK18* in parallel signalling branches (Benhamman et al., 2017). Their expression is strongly induced by salt stress, indicating additional roles in salt tolerance and redundant regulation of ABA responses (Choi et al., 2017).

Despite considerable progress, important knowledge gaps remain. The specific downstream transcriptional targets of MAPKKK17 and MAPKKK18 have yet to be identified, limiting understanding of their direct regulatory roles within ABA signalling. Additionally, their involvement in ABA-auxin crosstalk, a pivotal aspect of plant developmental and stress response integration, remains unresolved. It also remains unclear whether MAPKKK17 and MAPKKK18 confer distinct signalling specificities or function redundantly, an issue essential for dissecting their individual contributions. Addressing these questions through comparative transcriptomic and phosphoproteomic analyses of respective mutants under ABA and auxin treatments will be crucial to unravel their precise mechanistic roles.

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1.4. Ubiquitin-Proteasome System

As both ABA and MAPK signalling rely on rapid turnover of regulatory proteins, the role of the ubiquitin-proteasome system (UPS) becomes crucial in maintaining signalling fidelity. UPS controls key steps in ABA perception, kinase activation, and transcription factor stability (Wang et al., 2022). UPS is central to regulation of protein degradation in plants, playing a vital role in development, cellular homeostasis, and adaptive responses to stress (Dou et al., 2021; Li et al., 2013). Environmental or intracellular stress often leads to the accumulation of damaged or misfolded proteins, which are selectively removed through proteolysis. While organellar proteases contribute to bulk degradation, the UPS operates in the cytoplasm and nucleus, targeting specific substrates via polyubiquitination for degradation by the 26S proteasome (Dubiella & Serrano, 2021; X. Wu & Rapoport, 2018). In *Arabidopsis thaliana*, the UPS is remarkably expanded, with over 1300 genes, about 5% of the genome, encoding components related to ubiquitin-mediated functions (Mazzucotelli et al., 2006; Vierstra, 2009). Notably, 90% of these genes encode E3 ubiquitin ligases, reflecting the system's high substrate specificity and complexity. The UPS modulates various biological processes by selectively degrading regulatory proteins involved in hormone signalling, immune responses, cell cycle progression, and developmental transitions (Dreher & Callis, 2007; Hershko, 2005; Stone et al., 2006; Vierstra, 2009). This tightly controlled proteolytic system is essential for maintaining proteome integrity and ensuring plant viability, particularly under abiotic and biotic stress conditions.

1.4.1. Mechanism of ubiquitination

Ubiquitination is a central and highly conserved post-translational modification unique to eukaryotic cells (Hershko & Ciechanover, 1998; Zhao et al., 2017). It involves the covalent attachment of ubiquitin, a 76-amino-acid, 8.6 kDa polypeptide, to target proteins, typically via the ε-amino group of a lysine residue, although linkages through the hydroxyl group of serine or the thiol group of cysteine have also been reported (He et al., 2020; Müller & Hoppe, 2024). This modification functions not only as a signal for proteasomal degradation but also regulates processes such as signal transduction, endocytosis, DNA repair, and environmental stress responses (He et al., 2019).

The ubiquitination process proceeds via a well-defined three-step enzymatic cascade. First, ubiquitin is activated in an ATP-dependent reaction by an E1 ubiquitin-activating enzyme. The activated ubiquitin is then transferred to a cysteine residue on an E2 ubiquitin-conjugating enzyme through transesterification. Finally, the E3 ubiquitin ligase catalyses the transfer of ubiquitin from E2 to the target protein. This final step is critical, as the E3 enzyme confers substrate specificity by selectively recognizing and binding target proteins (He et al., 2019; Lee et al., 2011; Smalle & Vierstra, 2004; Figure 2A). In *Arabidopsis thaliana*, two E1 enzymes, at least 37 E2 enzymes, and over 1,400 E3 ligases have been identified, highlighting the system's remarkable substrate selectivity and regulatory potential (Callis, 2014; Du & Zhang, 2025; Smalle & Vierstra, 2004).

Ubiquitin can be attached to substrates as a single moiety (monoubiquitination), at multiple lysines (multi-monoubiquitination), or as polyubiquitin chains on a single lysine residue (Peng et al., 2003). The formation of polyubiquitin chains is highly versatile, as ubiquitin itself contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and an N-terminal methionine, allowing for diverse linkage types and even branched chain formation (Kirisako et al., 2006; Müller & Hoppe, 2024; Pickart & Fushman, 2004; Romero-Barrios et al., 2020). Among these, K48-linked chains serve as the canonical signal for proteasomal degradation and are the most prevalent in cells (Hershko & Ciechanover, 1998; C. Huang et al., 2020; Kerscher et al., 2006). K11-, K27-, K29-, and K33-linked chains have also been implicated in protein degradation (Blount et al., 2020; French et al., 2021), while K63-linked chains are mainly associated with non-degradative processes such as signal activation, development, and plant responses to environmental stress (Jiménez-López et al., 2018; Romero-Barrios et al., 2020).

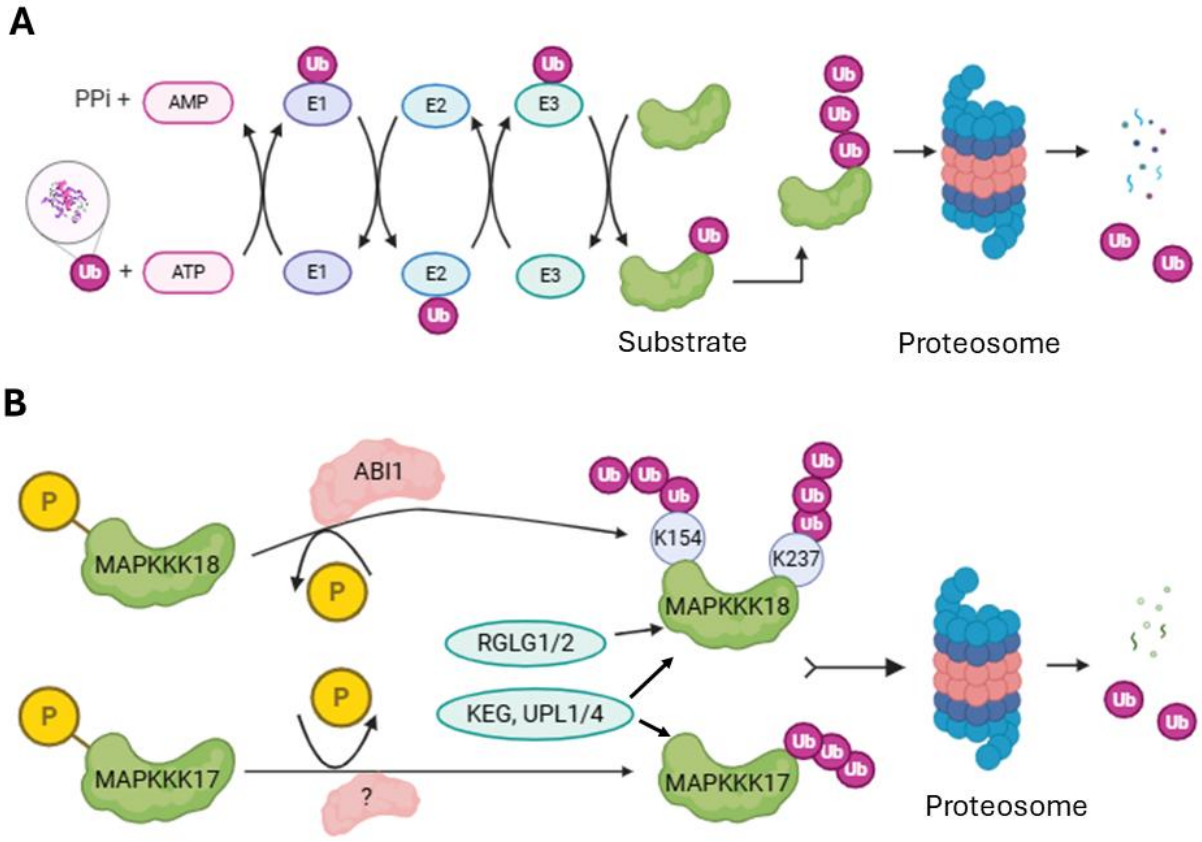


Figure 2. Ubiquitin-Proteasome System and UPS-mediated degradation of *MAPKKK17*/18.

(A) Schematic representation of the ubiquitin-proteasome system (UPS), where substrates are tagged with ubiquitin through a cascade involving E1 (activating), E2 (conjugating), and E3 (ligase) enzymes, followed by proteasomal degradation. (B) UPS-mediated regulation of *MAPKKK17* and *MAPKKK18* stability. *MAPKKK17*/18 are phosphorylated and targeted for ubiquitination and degradation by E3 ligases including *KEG*, *UPL1*/4, and *RGLG1*/2. *ABI1* modulates *MAPKKK18* stability via dephosphorylation, while the regulatory mechanism for *MAPKKK17* remains unclear. Figure redrawn in BioRender based on (Callis, 2014; Tajdel-Zielińska et al., 2024).

Once polyubiquitinated, target proteins are directed to the 26S proteasome, a large ATP-dependent protease complex that degrades unwanted or damaged proteins, thereby maintaining protein homeostasis (Bard et al., 2018). The 26S proteasome consists of two functionally distinct subcomplexes: the 20S catalytic particle (CP) and the 19S regulatory particle (RP). The 20S CP possesses proteolytic activity and executes substrate degradation, while the 19S RP mediates recognition, unfolding, and translocation of ubiquitinated substrates into the 20S core (Bhattacharyya et al., 2014; Greene et al., 2020; Marshall & Vierstra, 2022; Shang & Taylor, 2011).

The RP itself contains two subdomains: the lid, which recognizes and binds ubiquitinated substrates, and the base, which interacts with the 20S CP to facilitate substrate entry.

Proteasomal degradation proceeds through several coordinated steps. Initially, ubiquitinated proteins are recognized by ubiquitin receptors in the regulatory subunit of the proteasome, including intrinsic receptors such as *Rpn1*, *Rpn10*, and *Rpn13*, and shuttle factors like *Rad23* and *Dsk2* (Bard et al., 2018; Kim et al., 2011; Riedinger et al., 2010; Shi et al., 2016; Tomko & Hochstrasser, 2011). Following recognition, ATPases in the 19S RP unfold the substrate, while deubiquitylating enzymes (DUBs) remove the ubiquitin chains for recycling (Collins & Goldberg, 2017; Finley & Prado, 2020; Marshall & Vierstra, 2019). The unfolded polypeptide is then translocated into the narrow catalytic chamber of the 20S CP, which comprise four stacked heptameric rings-two outer rings of α-subunits and two inner rings of β-subunits harbouring the protease active sites (Tanaka, 2009; Zhang et al., 2024)

Although K48-linked chains were long considered the primary degradation signal, recent studies demonstrate that the proteasome can recognize a broader array of polyubiquitin linkages. Each linkage type may interact with distinct ubiquitin receptors, expanding the functional diversity of the UPS and challenging earlier paradigms. Interestingly, while branched polyubiquitin chains are common in other eukaryotes, only linear, unbranched forms have been identified in plants to date (He et al., 2020; Tracz & Bialek, 2021)

1.4.2. E3 ubiquitin ligases

E3 ubiquitin ligases constitute the largest and most functionally diverse group of enzymes in the ubiquitination pathway. They play a significant role in regulating protein turnover in eukaryotic cells, primarily due to their substrate specificity and regulatory complexity (Moon et al., 2004; Smalle & Vierstra, 2004). Unlike the limited number of E1 and E2 enzymes, the *Arabidopsis thaliana* genome encodes over 1,400 putative E3 ligases, underscoring their critical roles in plant growth, development, and stress responses (Liu et al., 2021; Rotin & Kumar, 2009). A defining feature of E3 ligases is their versatility: a single E3 can ubiquitinate multiple substrates and interact with various E2 enzymes. Conversely, different E2-E3 pairings can ubiquitinate the same substrate, resulting in distinct ubiquitin chain types and functional consequences (Deshaies & Joazeiro, 2009; Freemont, 2000; Liu et al., 2021; Moon et al., 2004; Pickart & Fushman, 2004).

Therefore, understanding the structural and biochemical diversity of E3s is vital for decoding the ubiquitin-proteasome system (UPS).

E3 ligases are broadly classified into three main structural types based on their E2-binding domains and mechanisms of ubiquitin transfer: RING (Really Interesting New Gene), U-box, and HECT (Homologous to the E6-AP C-Terminus) domain-containing ligases (Deshaies & Joazeiro, 2009; Freemont, 2000; Liu et al., 2021; Moon et al., 2004; Pickart & Fushman, 2004). Among these, RING-type ligases are the most abundant, with approximately 465 members in *Arabidopsis*. They contain a ~70 amino acid zinc-binding RING finger domain that facilitates direct ubiquitin transfer from the E2 to the substrate, without forming a covalent intermediate. RING ligases can function as single-subunit enzymes or as components of multi subunit complexes (Freemont, 2000; Moon et al., 2004). U-box E3 ligases, of which ~64 is predicted in *Arabidopsis*, are structurally similar to RING domains but lack coordinated zinc atoms. Instead, they maintain their fold through hydrogen bonding and hydrophobic interactions (Yee & Goring, 2009). Like RING-type E3s, they mediate direct ubiquitin transfer. Despite their lower abundance, U-box ligases are involved in key biological processes such as hormone signalling and plant immune responses.

A prominent subgroup of RING ligases forms Cullin-RING Ligase (CRL) complexes, including SCF (SKP1-Cullin-F-box), CUL3-BTB (BTB/POZ domain-containing), and CUL4-DDB1 (Damaged DNA Binding Protein 1) assemblies. These complexes share a modular structure composed of a RING protein (RBX1), a cullin scaffold, and a variable substrate recognition module (*F-box, BTB/POZ,* or *WD40/DCAF*), which defines substrate specificity depending on the cullin type (Bernhardt et al., 2006; Figueroa et al., 2005; Gray et al., 1999; Tan et al., 2019). In *Arabidopsis*, five canonical cullins (*CUL1, CUL2, CUL3A, CUL3B,* and *CUL4*) serve as scaffolds for these complexes, while F-box and BTB proteins provide the substrate-adapting modules (Mazzucotelli et al., 2006). This modularity allows plants to rapidly and flexibly adjust protein degradation in response to developmental or environmental signals. Another essential multisubunit RING-type complex is the Anaphase-Promoting Complex/Cyclosome (APC/C), which includes at least eleven subunits. Among them, *APC2* (a cullin-like scaffold) and *APC11* (a RING-type E3) are central to its function. APC/C temporally regulates the cell cycle by targeting specific regulatory proteins for degradation (Capron et al., 2003). Together, these RING-based ligases control diverse aspects of plant growth and stress responses. In contrast to RING and U-box ligases, HECT-type E3 ligases (~20 predicted in *Arabidopsis*) use a two-step mechanism. First,

ubiquitin is transferred from E2 to a conserved cysteine residue in the E3, forming a thioester intermediate. Then, the ubiquitin is transferred to the substrate (Pickart & Fushman, 2004; N. Zheng & Shabek, 2025). The ~350-amino acid HECT domain includes distinct regions for E2 binding and ubiquitin transfer, allowing these ligases to exert precise control over polyubiquitin chain formation and linkage types (Sharma et al., 2021; Weber et al., 2019). Though less abundant, HECT ligases are gaining attention for their emerging regulatory roles in plants.

In summary, E3 ubiquitin ligases, through their structural and mechanistic diversity, orchestrate highly regulated protein ubiquitination events. Their classification into RING, U-box, HECT, and CRL families reflects a sophisticated, modular system essential for proteostasis, signalling, and stress adaptation in plants.

1.4.3. UPL ligases

Arabidopsis thaliana encodes seven HECT-type E3 ubiquitin ligases known as UPLs (Ubiquitin Protein Ligases), which are divided into five subfamilies: *UPL3* and *UPL4* (Subfamily I), *UPL7* (II), *UPL6* (III), *UPL1/2* (V), and *UPL5* (VI) (Downes et al., 2003; Marín, 2013; Meng et al., 2015). All UPLs contain a conserved C-terminal HECT domain responsible for ubiquitin transfer, while their divergent N-terminal regions determine substrate specificity and subcellular localization (Lan & Miao, 2019; Sluimer & Distel, 2018). For example, *UPL1* contains UBA, UIM, and ankyrin repeats, while *UPL4* includes only ankyrin motifs-domains that facilitate protein-protein interactions and are implicated in abiotic stress responses (Zhao et al., 2020).

Individual UPLs exhibit specialized biological functions. *UPL3* is involved in trichome development, genome endoreduplication, and seed size regulation. It interacts with *GL3* and *EGL3* transcription factors, which are key regulators of trichome morphogenesis and flavonoid biosynthesis (Patra et al., 2013). *UPL5* contributes to leaf senescence by targeting the transcription factor *WRKY53* for degradation (Miao & Zentgraf, 2010). *UPL1*, *UPL3*, and *UPL5* have also been linked to plant immunity (El Refy et al., 2004; Furniss et al., 2018; Miller et al., 2019). Additionally, *UPL3* has roles in gibberellin signalling and modulates salicylic acid (SA)-responsive gene expression, potentially through its interactions with the 19S proteasome and other UPS components (Furniss et al., 2018; Wang et al., 2022)

UPL4, which shares 53% sequence identity with *UPL3* but lacks a 225-residue segment in the C-terminal region, co-regulates processes such as root growth, hypocotyl elongation, and apical hook

formation. However, unlike *UPL3*, it does not influence trichome development, indicating a functional divergence despite structural similarity (Wang & Spoel, 2022). The physiological roles of *UPL1, UPL4, UPL6,* and *UPL7* remain less well-characterised, but domain structure and inducible expression patterns suggest they are involved in abiotic stress and hormone signalling (Lan & Miao, 2019; Shu & Yang, 2017).

Recent studies have highlighted the relevance of UPL ligases in ABA signalling, particularly in the context of drought response. UPLs may regulate the abundance of key components in the MAP kinase cascade, a major transduction pathway downstream of ABA signalling. For example, *MAPKKK18*, a positive regulator of ABA-induced stomatal closure, undergoes proteasomal degradation mediated by E3 ligases such as *RGLG1*, *RGLG2*, and members of the UPL family (Yu et al., 2021). *MAPKKK18* is activated by ABA and regulated post-translationally by the phosphatase *ABI1* but is subsequently targeted for degradation through the UPS (Mitula et al., 2015).

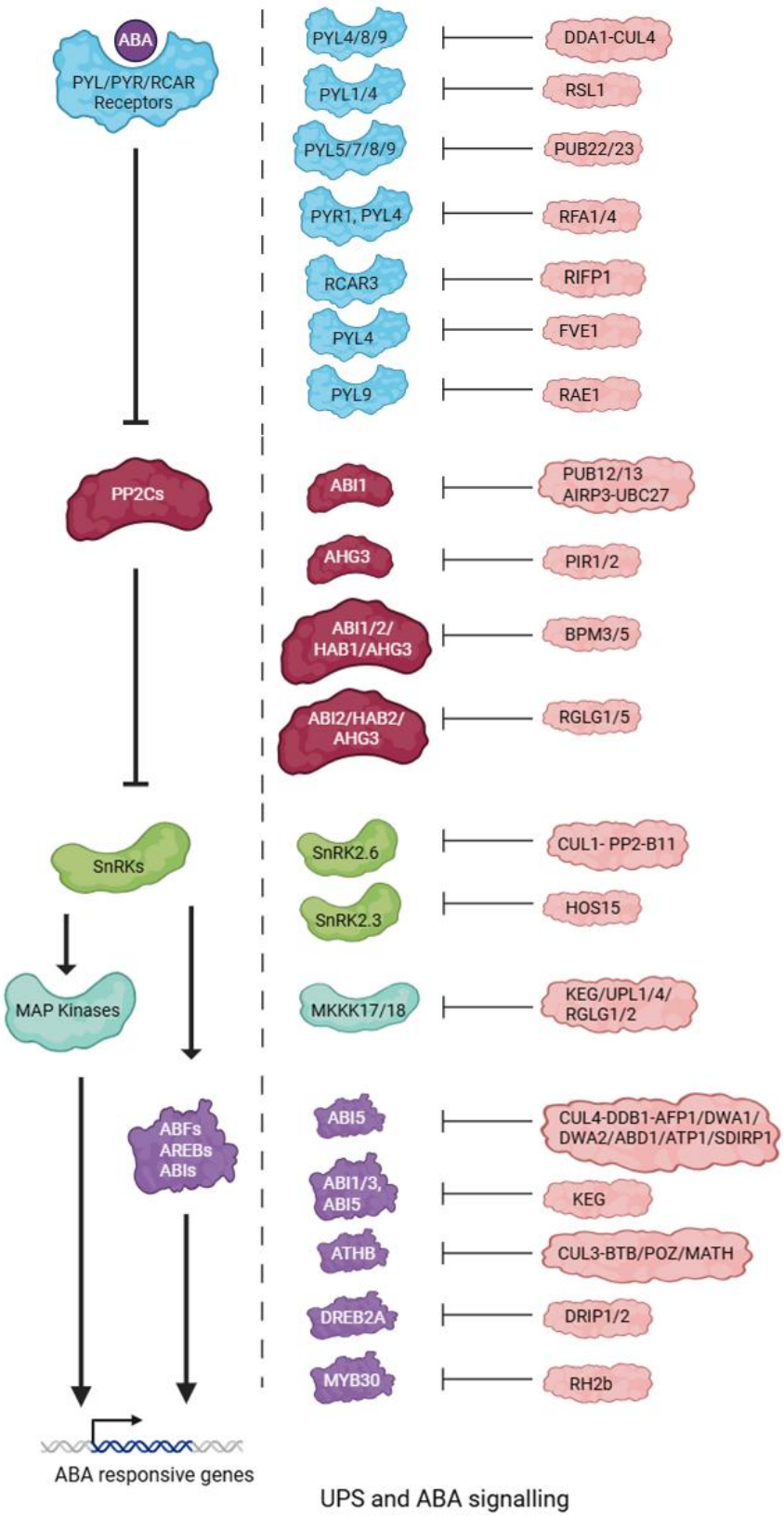
Proteomic studies have identified lysine residues K154 and K237 on *MAPKKK18* as key ubiquitination sites. E3 ligases including *KEG, UPL1,* and *UPL4* mediate this modification, leading to *MAPKKK18* turnover and modulation of ABA signalling outputs (Tajdel-Zielińska et al., 2024; Figure 2B). These findings support a direct link between E3 ligase activity and MAPK cascade regulation in ABA-mediated drought responses.

Although *UPL4* and *UPL6* have been implicated in *MAPKKK18* degradation and ABA signalling, their broader physiological roles remain to be fully defined. While the current study emphasizes transcriptomic and kinase-level regulatory mechanisms, the contribution of E3 ligases such as UPLs to fine-tuning ABA responses represents a promising direction for future research.

1.4.4. UPS-mediated regulation of ABA signalling components

The ubiquitin-proteasome system (UPS) plays a vital role in the ABA signalling pathway by mediating the degradation of key signalling components. This regulated proteolysis maintains cellular protein homeostasis and fine-tunes hormonal responses, particularly under in response to abiotic stress (Smalle & Vierstra, 2004; Stone et al., 2006). E3 ubiquitin ligases, as substrate-specific mediators of ubiquitin transfer, are especially critical in modulating the abundance of ABA pathway components including receptors, phosphatases, kinases, and transcription factors (Meng et al., 2020; Sharma et al., 2021; Yoshida et al., 2019; Figure 3)

ABA receptors of the PYR/PYL/RCAR family are subjected to ubiquitin-mediated degradation by several E3 ligases. The RING-type CUL4 E3 ligase complex (*COP10-DET1-DDB1-DDA1-CUL4*) targets *PYL4, PYL8,* and *PYL9* via its *DDA1* adaptor (Irigoyen et al., 2014). Interestingly, ABA inhibits DDA1-mediated degradation of *PYL8*, highlighting a feedback mechanism wherein the hormone stabilizes its own receptors under stress. *PYL9* also interacts with *RAE1*, a substrate of the CUL4 ligase, suggesting additional regulatory inputs into receptor turnover (Li et al., 2018). RING Finger ABA-Related1 (*RFA1*) and *RFA4* also regulate ABA signalling by targeting *PYR1* and *PYL4* for degradation through either the *RSL1*-dependent endosomal/vacuolar pathway or the 26S proteasome (Bueso et al., 2014; Figure 3). Another RING E3 ligase, *RSL1*, facilitates the degradation of *PYL4* and *PYR1* at the plasma membrane. *RSL1* overexpression decreases ABA sensitivity, whereas its suppression enhances it (Bueso et al., 2014). Additionally, *RSL1* interacts with *FVE1/FREE1*, a component of the endosomal sorting complex, which recruits *PYL4* into endosomal compartments for degradation, further refining receptor availability (Belda-Palazon et al., 2016). U-box E3 ligases *PUB22* and *PUB23* also contribute to ABA signalling by targeting *PYL9* and interacting with *PYL5, PYL7,* and *PYL8* (Zhao et al., 2017). The F-box E3 ligase *RIFP1* negatively regulates ABA signalling by facilitating the degradation of ABA receptor *RCAR3* (Li et al., 2016).



UPS and ABA signalling

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Figure 3. Integration of the Ubiquitin-Proteasome System with ABA signalling pathways.
Schematic overview of ABA signalling and its regulation by the ubiquitin-proteasome system. ABA perception via PYR/PYL/RCAR receptors inhibits PP2Cs, leading to activation of SnRK2s, MAP kinases, and ABA-responsive transcription factors (ABFs/AREBs). Multiple E3 ligases target the ABA core signalling components, including receptors, phosphatases (e.g., *ABI1 and AHG3*), kinases (e.g., *SnRK2*.6 and *MAPKKK17*/*18*), and transcription factors (e.g., *ABI5* and *DREB2A*), highlighting the UPS as a critical modulator of ABA-mediated stress responses. The figure was drawn using BioRender.

Beyond receptors, type A PP2Cs, such as *ABI1, ABI2, HAB1,* and *PP2CA*, function as negative regulators of ABA signalling and are also controlled by E3 ligase-mediated degradation. *PUB12* and *PUB13* specifically promote the degradation of *ABI1* in an ABA-enhanced manner through receptor interaction, but do not affect *ABI2* (Kong et al., 2015; Figure 3). Multimeric RING-CUL3 E3 ligase complexes, which use *BPM3* and *BPM5* adaptors mediate the degradation of *ABI1*, *ABI2*, *HAB1,* and *AHG3*, promoting ABA-induced stomatal closure (Julian et al., 2019). The E2 enzyme *UBC27*, in cooperation with RING E3 ligase *AIRP3*, also targets ABI1 for degradation, forming a positive feedback loop enhanced by ABA (Pan et al., 2020). Furthermore, *RGLG1* and *RGLG5* promote the degradation of *ABI2, HAB2,* and *AHG3* in response to ABA (Wu et al., 2016), while *PIR1* and *PIR2* specifically degrade *AHG3*, with loss-of-function mutants showing increased ABA sensitivity (Baek et al., 2019).

ABA-activated SnRK2 kinases, including *SnRK2*.2, *SnRK2*.3, and *SnRK2*.6 (OST1), are also subject to UPS-mediated regulation. The F-box protein PP2-B11, which is part of the SCF E3 ligase complex, targets *SnRK2*.3 for degradation and is itself transcriptionally upregulated by ABA, indicating a feedback control mechanism (Cheng et al., 2017; Figure 3). The *RING-CUL4-DDB1* complex also degrades *SnRK2*.6, utilizing *HOS15* as a substrate adaptor. *HOS15* preferentially interacts with the inactive (dephosphorylated) form of *SnRK2*.6. Notably, ABA disrupts this interaction, allowing *SnRK2*.6 autophosphorylation and subsequent activation of downstream signalling (Ali et al., 2019; Ali & Yun, 2020)

Besides SnRKs, the UPS also regulates *MAPKKK17* and *MAPKKK18*, which are kinases involved in drought stress tolerance. *RGLG1* and *RGLG2* have been shown to negatively modulate *MAPKKK18*-mediated responses (Yu et al., 2021). Additionally, the RING-type E3 ligase *KEG* and UPL-type ligases *UPL1* and *UPL4* can ubiquitinate *MAPKKK17*/18 at lysine residues K154 and K237, promoting their proteasomal degradation (Tajdel-Zielińska et al., 2024; Figure 3).

Finally, the stability of ABA-responsive transcription factors is tightly regulated by E3 ligases. *ABI5*, a bZIP transcription factor crucial for ABA-induced gene expression, is targeted by multiple

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CUL4-DDB1-based complexes. Substrate adaptors such as *AFP1*, *DWA1*/2, and *ABD1* mediate its degradation to fine-tune ABA responses (Chen & Hellmann, 2013; Lopez-Molina et al., 2003; Seo et al., 2014). The RING-type E3 ligase *KEG* plays a broader regulatory role by targeting not only *ABI5* but also its close homologs *ABF1* and *ABF3*, affecting various transcriptional outputs in the ABA signalling cascade (Liu & Stone, 2014; Stone, 2014). RING E3 ligases *ATP1* and *SDIRP1* act upstream of *ABI5*, serving as negative regulators of its accumulation and thus modulating ABA-mediated gene expression (Oh et al., 2017). Beyond core ABA components, *RGLG1* and *RGLG2* interact with the ERF transcription factor *AtERF53* to negatively regulate plant drought stress responses, linking UPS activity to broader abiotic stress adaptation (Wu et al., 2016; Yu et al., 2021). *DRIP1* and *DRIP2*, two RING E3 ligase homologs, mediate ubiquitination, and subsequent degradation of *DREB2A*, serving as negative regulators of drought-responsive gene expression (Qin et al., 2008). Additionally, CUL3-based *BTB/POZ/MATH* E3 ligases modulate ABA signalling by targeting the transcription factor *ATHB6* for proteasomal degradation (Lechner et al., 2011). Under water-deficit conditions, the RING E3 ligase *RHA2b* targets *MYB30* for degradation via the 26S proteasome; the *rha2a rha2b* double mutant displays enhanced ABA insensitivity, implicating both ligases as positive regulators of ABA responses (Zheng et al., 2018).

Collectively, these studies reveal that E3 ligases are key modulators of ABA signalling, dynamically regulating both positive and negative components of the pathway. We are beginning to gain important insights into the mechanisms by which the ubiquitin–proteasome system (UPS) regulates ABA responses. Through targeted proteolysis of receptors, phosphatases, kinases, and transcription factors, E3 ligases enable precise fine-tuning of ABA signalling, allowing plants to adapt to fluctuating environmental conditions. The UPS regulation of ABA pathway is essential for maintaining resilience during drought and other abiotic stresses. Therefore, the identification and functional characterization of UPS components involved in ABA regulation is crucial to fully understand how plants achieve such stress adaptability.

1.5. ABA and Auxin crosstalk

In addition to ABA-MAPK integration, the interplay between ABA and other hormones, particularly auxin, is vital for coordinating stress responses with developmental plasticity.

Plant hormones regulate every aspect of plant growth and development, often through complex interactions. Among them, ABA and auxin (indole-3-acetic acid, IAA) frequently exhibit antagonistic roles. ABA is known primarily as a stress hormone, regulating seed dormancy, stomatal closure, and growth inhibition, particularly under abiotic stress conditions like drought. Auxin, conversely, promotes cell elongation, root development, and organ patterning, acting as a central driver of plant morphogenesis. Although their functions appear opposing, increasing evidence shows that ABA and auxin often interact closely at the physiological, transcriptional, and signalling levels to regulate developmental processes such as seed germination, root elongation, lateral root formation, cotyledon expansion, and stomatal regulation (Ortiz-García et al., 2023).

This crosstalk occurs through multiple mechanisms: shared or overlapping transcriptional targets, hormone-responsive cis-elements, chromatin remodelling, and direct or indirect interactions between hormone-specific transcription factors (Zhang et al., 2023). Such interaction can be synergistic or antagonistic depending on spatial, temporal, and environmental contexts.

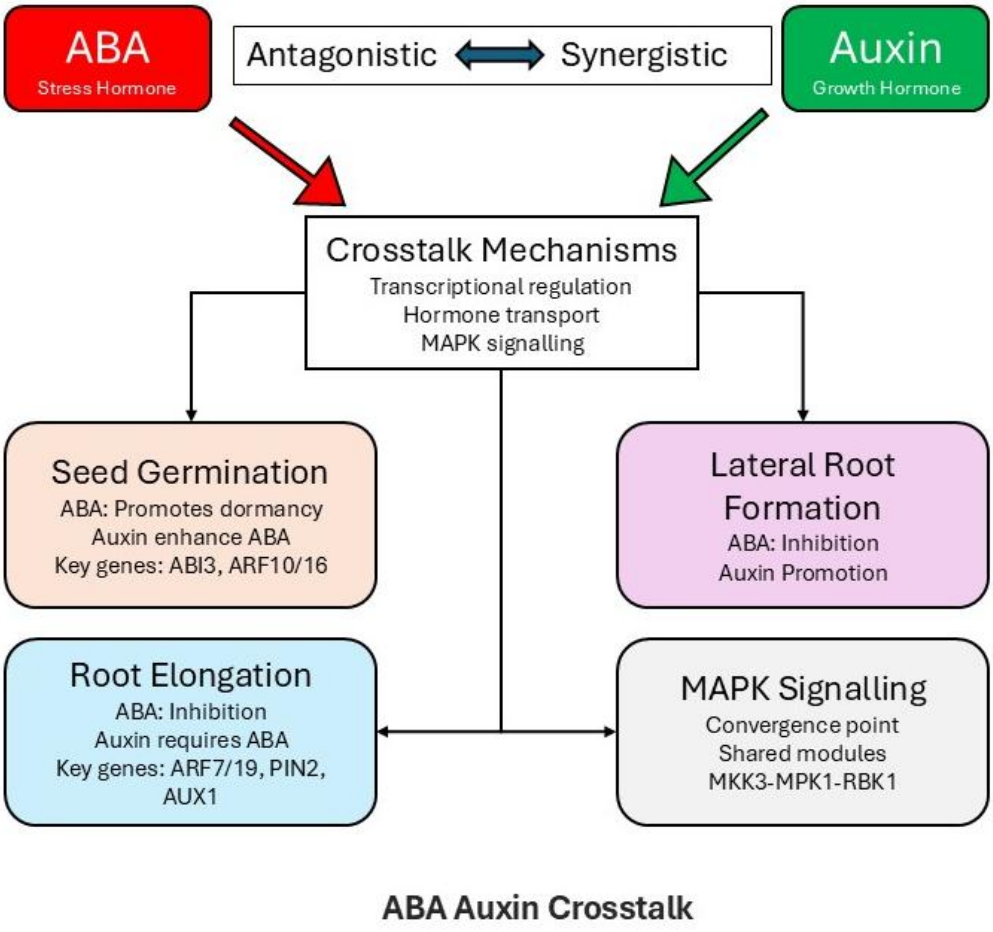


Figure 4. Crosstalk between ABA and Auxin signalling pathways in plant development
ABA and auxin interact through shared signalling pathways to regulate seed germination, root elongation, and lateral root formation. Their effects can be antagonistic or synergistic, with MAPK signalling acting as a common integration point.

1.5.1. Auxin-ABA interactions in seed germination

Seed germination is tightly regulated by the antagonism between ABA, which enforces dormancy, and gibberellins (GA), which promote germination (Emenecker & Strader, 2020; Salehin et al., 2019; Shuai et al., 2017; Shani et al., 2017). Auxin, while typically a growth promoter, can act in synergy with ABA to suppress seed germination under certain conditions. ABA exerts its inhibitory effects via key transcription factors such as *ABI3*, *ABI4*, and *ABI5* (Bentsink & Koornneef, 2008; Emenecker & Strader, 2020). These ABI transcription factors mediate ABA's suppression of germination by controlling the expression of ABA-responsive genes.

Auxin enhances ABA sensitivity in seeds by regulating *ABI3* expression. Specifically, *ARF10* and *ARF16*, two auxin response factors, positively regulate *ABI3* expression and thereby seed dormancy (Li et al., 2017; Liu et al., 2017; Shuai et al., 2017; Figure 4). Although the *ABI3* promoter contains an auxin-responsive element (AuxRE), *ARF10* and *ARF16* do not directly bind this element, implying that the regulation is indirect-through repression of a negative regulator of *ABI3* (Wang et al., 2005). Additionally, *IAA8*, a member of the Aux/IAA family, is involved in regulating *ABI3*. Loss-of-function mutants of *iaa8-1* show delayed germination, and *IAA8* is known to bind an AuxRE in the *ABI3* promoter (Hussain et al., 2020). This implies that *IAA8* may work in conjunction with ARFs to regulate ABI3 expression. Previous studies have shown that *ARF16* interact with *IAA8* (Piya et al., 2014). Furthermore, ABI3 can repress *MIR160B* transcription, and since miR160 targets *ARF10* and *ARF16*, this may form a positive feedback loop that reinforces auxin's promotion of ABA-mediated seed dormancy (Tian et al., 2004).

ABI4 also plays a pivotal role in this process. It activates the ABA biosynthetic gene *NCED6*, thereby enhancing ABA levels and reinforcing dormancy (Mazzoni-Putman et al., 2021; Munguía-Rodríguez et al., 2020; Shu et al., 2013; Shu, Liu, et al., 2016). Mutants lacking *ABI4* are partially insensitive to auxin-induced inhibition of germination, indicating that ABI4 is necessary for auxin to exert its ABA-like effects (Mazzoni-Putman et al., 2021; Munguía-Rodríguez et al., 2020). Similarly, *ABI5*, a bZIP transcription factor that operates downstream of SnRK2 kinases in ABA signalling, is critical for mediating ABA responses via binding to ABRE elements in gene promoters (Finkelstein, 2013; Liu & Stone, 2014; Yu et al., 2017; Zhou et al., 2021). Moreover, auxin biosynthesis and transport are important for ABA-induced inhibition of seed germination. Mutants deficient in *YUCCA1* and *YUCCA6* (auxin biosynthetic genes) are less sensitive to ABA, whereas plants overproducing auxin show increased ABA sensitivity (Cheng et al., 2006; Kamiyama, Hirotani, et al., 2021). However, exogenous auxin alone does not significantly suppress germination, suggesting that auxin potentiates ABA signalling rather than acting independently. ABA may control auxin biosynthesis, PIN-mediated transport, or Aux/IAA stability to fine-tune seed germination (Emenecker & Strader, 2020).

1.5.2. Auxin-ABA interactions in root elongation

Root elongation is a critical developmental process at which ABA and auxin signalling pathways interact. ABA inhibits primary root (PR) elongation, especially under stress conditions (Qin et al.,

2023). This inhibition requires a functional auxin signalling pathway (Slovak et al., 2016). Mutants such as *tir1, ibr5, axr1,* and gain-of-function Aux/IAA lines exhibit reduced sensitivity to ABA, indicating that auxin perception and signalling are prerequisites for ABA's inhibitory effect (Monroe-Augustus et al., 2003; Strader et al., 2008). Notably, ABA-insensitive mutants (*abi1*-1, *abi2*-1, and *abi3*-1) still respond to exogenous auxin, suggesting complex regulation between these two pathways (Thole et al., 2014; Figure 4).At the transcriptional level, *ARF7* and *ARF19* activate the expression of ABA biosynthesis genes, while *ARF2* suppresses ABA signalling by downregulating *HB33*, a gene involved in the regulation of ABA-mediated root growth (Wang et al., 2011). Overexpression of *HB33* increases sensitivity to ABA, further linking ARFs to ABA responsiveness (Huang et al., 2020; Lu & Huang, 2008; Tu et al., 2022; Meng et al., 2015; Wang et al., 2011; Zhong et al., 2009). Auxin transport is also essential for ABA responses in roots. *AUX1* (an auxin influx carrier) and *PIN2* (an efflux carrier) are key components of this regulation. Mutants lacking either gene exhibit altered responses to ABA during root elongation (Li et al., 2017; Xie et al., 2021) . Notably, *AUX1* and *AXR4* mutants show reduced expression of ABA-responsive genes such as *ProDc3:GUS*, highlighting a direct impact of auxin transport on ABA-induced transcriptional responses (Jagodzik et al., 2018). This indicates that auxin transport does not just influence hormone gradients but also modulates ABA-mediated gene expression. Furthermore, AUX/IAA protein-transcriptional repressors in the auxin pathway have been shown to interact with ABA-responsive elements (ABREs), suggesting direct crosstalk between ABA and auxin signalling at the promoter level (Emenecker & Strader, 2020). These findings reveal that auxin transport and transcriptional machinery are integral to ABA's action on root growth. Intriguingly, the effect of ABA on root growth is concentration dependent. At low ABA levels (∼0.1 μM), root elongation is promoted via enhanced auxin efflux, while at high levels (∼10 μM), root elongation is inhibited, requiring both auxin influx and efflux (Belin et al., 2009; Liu et al., 2018; Thole et al., 2014; Wang et al., 2015). This evidence suggests a model in which ABA modulates auxin transport and signalling in a concentration- and context-dependent manner, using auxin as a downstream effector to control root elongation.

Collectively, these hormone-hormone and hormone-kinase interactions highlight the need to map signalling convergence points, such as *MAPKKK17*/*18*, which may serve as integrators of ABA, MAPK, and auxin pathways.

1.5.3. Auxin-ABA interactions in lateral root formation

Lateral root (LR) formation is another developmental process where ABA and auxin interactions are evident, often antagonistically. Auxin is known to promote LR initiation and outgrowth, while ABA typically inhibits these processes (Casimiro et al., 2003; De Smet et al., 2003). However, recent studies indicate more nuanced interactions. For instance, ABA receptors *PYL8* and *PYL9* interact with the MYB transcription factors *MYB77* and *MYB44*, which are responsive to auxin, to promote LR outgrowth under specific conditions (Xing et al., 2016, Mazzoni-Putman et al., 2021; Figure 4). This suggests a synergistic interaction under certain environmental contexts. Furthermore, *ABI1* is required for normal auxin sensitivity in LR development. Mutations in *ABI1* result in reduced responsiveness to auxin, indicating that ABA signalling components modulate auxin perception (Li et al., 2018). *ABI4* inhibits LR development by repressing PIN1 expression, thus impairing polar auxin transport (Maymon et al., 2022; Shkolnik-Inbar & Bar-Zvi, 2010). *ABI3* negatively regulates LR emergence by activating *ERF1*, creating a transcriptional module (*ABI3-ERF1*) that integrates ABA and auxin responses (Zhang et al., 2023). *ABI5* also modulates LR development by regulating the accumulation and localization of PIN proteins (Waidmann et al., 2020). In the context of osmotic/salt stress, the *WRKY46* transcription factor binds to the promoters of auxin-conjugating enzymes and ABA-related genes such as *ABI4*, modulating LR formation through coordinated hormone regulation (Ding et al., 2016). In summary, auxin, and ABA act in a balanced manner during lateral root formation, where auxin generally promotes initiation and emergence, while ABA imposes context-dependent inhibition that integrates environmental stress signals. This crosstalk ensures that lateral root development is tightly regulated to optimize growth under varying conditions.

1.5.4. Auxin-ABA interactions in MAP kinase cascades

Beyond transcriptional and transport-level interactions, ABA and auxin also converge on MAPK signalling pathways. The *MKK3-MPK1-RBK1* module is activated by both ABA and auxin, modulating auxin-dependent growth through the small GTPases *ROP4* and *ROP6* (Enders et al., 2017; Jagodzik et al., 2018). Upstream components such as *MAPKKK17* and *MAPKKK18*, which are activated by ABA, stimulate *MKK3*, suggesting a direct ABA input into auxin-regulated MAPK signalling (Matsuoka et al., 2015; Figure 4). Additionally, the *MKK5-MPK6* cascade is regulated

by ABA through the kinase AIK1, further reinforcing hormonal control of MAPK activity (Li et al., 2017). On the auxin side, *MPK12*, which is activated by auxin, serves as a negative regulator of auxin signalling, introducing a negative feedback loop that modulates auxin responses (Aerts et al., 2021; Jagodzik et al., 2018).

In conclusion, ABA-auxin crosstalk represents a dynamic and multilayered network encompassing transcriptional, post-transcriptional, transport-mediated, and signalling interactions. Despite substantial progress, significant knowledge gaps remain. For example, it is still unclear how key ABA components such as ABI1 or MAPKKK18 orchestrate large-scale transcriptional changes in concert with auxin. A comprehensive system-level understanding, incorporating transcriptomics, proteomics, and interactomics, will be essential to unravel the full complexity of ABA-auxin interactions. Although this study centres on ABA-induced transcriptional responses mediated by MAPKKK17/18 and ABI1, it considers hormone crosstalk in the broader context of ABA signalling regulation.

2. Hypothesis and aims

Abscisic acid (ABA) is a key phytohormone that regulates plant responses to abiotic stress, particularly drought, by reprogramming stress-responsive gene expression. While the core components of the ABA signalling cascade such as PYR/PYL receptors, the PP2C phosphatase *ABI1*, and SnRK2 kinases are well established, emerging studies suggest additional upstream regulatory layers, including MAP kinase kinase kinases (MAPKKKs) and ubiquitin E3 ligases, are essential for fine-tuning ABA responses. Among these regulators, *MAPKKK17* and *MAPKKK18* have been implicated in ABA signalling and drought tolerance, yet their downstream transcriptional networks and specificity remain undefined. In parallel, recent findings highlight a potential role for UBIQUITIN PROTEIN LIGASEs (UPLs), particularly *UPL4* and *UPL6*, in modulating ABA sensitivity via proteasomal degradation pathways. Furthermore, increasing evidence points to crosstalk between ABA and the UPS system as a critical determinant of stress adaptation and developmental plasticity, yet the molecular integration of these pathways especially through shared regulators like *ABI1* and MAPKKKs remains unresolved.

2.1. Hypothesis

We hypothesize that (1) *MAPKKK17*, *MAPKKK18*, *UPL4*/6, and *ABI1* co-ordinately regulate ABA-induced transcriptional networks, and (2) these modules integrate stress and developmental signalling

2.2. Specific aims

- SA1: To characterize the role of *MAPKKK17* in ABA signalling.
- SA2: To investigate the transcriptional responses mediated by *MAPKKK18*.
- SA3: To identify transcriptional programs that are uniquely or redundantly regulated by *MAPKKK17* and *MAPKKK18* through comparative DEG and GO enrichment analysis.
- SA4: To examine auxin-responsive gene expression patterns in *abi1td* and *mkkk18-1* mutants and assess the functional implications of ABA-auxin antagonism.
- SA5: To evaluate the contribution of *UPL4* and *UPL6* to ABA-mediated root and stomatal development and determine their impact on transcriptional regulation.

3. Materials and Methods

3.1. List of enzymes

- Platinum™ SuperFi™ DNA Polymerase (Invitrogen, Thermo Fisher Scientific)
- RNase-free DNase I (Maxima First Strand cDNA Synthesis Kit) Cat. No. K1642

3.2. List of molecular markers

- Abscisic acid (ABA), stock: 1 mM in methanol (Sigma-Aldrich, Cat. No. A1049)

3.3. List of reagents and kits

- Qiagen RNeasy Plant Mini Kit (Qiagen, Cat. No. 74904)
- Direct-zol RNA Miniprep Kit (Zymo Research, Cat. No. R2050)
- Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific, Cat. No. K1672)
- RNase Decontamination Solution (in-house prep)
- DEPC-treated water (in-house prep)
- SYBR Green Real-Time PCR Master Mix

3.4. List of equipment

- Agilent 2100 Bioanalyzer (Agilent Technologies)
- Bioanalyzer RNA Nano Chips
- Chip Priming Station and Gel Dye Mix (Agilent)
- Bioanalyzer Chip Vortexer (IKA MS 3)
- NanoDrop™ 2000/2000c Spectrophotometer
- Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR System

3.5. Media and solutions

- Murashige and Skoog (MS) Basal Medium with vitamins (Sigma-Aldrich, Cat. No. M5519)

- Sucrose (1%, w/v), Plant Agar (0.8%, w/v), KOH (0.1 M)

3.6.Plant materials

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 (WT Col-0) and T-DNA insertion lines *abi1td* (SALK_076309), *mkkk17-1* (SALK_080309C), *mkkk18-1* (SALK_087047), *upl4*.1 (SALK_091246C), *upl4*.2 (SALK_105288C), *upl6* (SALK_055609C) were used in this study. WT Col-0 was used as a control in all experiments. The *abi1td* line was previously characterised by (Ludwików et al., 2009), while *mapkkk18-1* was described by (Mitula et al. 2015), and the *upl4td* lines were described. Seeds of the *mapkkk17-1* knockout line were kindly provided by Dr. Jean Colcombert (INRAE, France). All mutant lines were confirmed for homozygosity via genotyping and grown under identical controlled conditions to ensure experimental comparability.

3.7.Seed sterilization

Seed sterilization was carried out using a vapor phase method (3% HCl). The 5 ml solution was transferred to a desiccator and pre-labelled centrifuge tubes containing the seeds were placed inside. The desiccator was sealed, and the seeds were exposed to sterilizing vapours for 5 hours. This method was chosen for its effectiveness in eliminating surface-borne contaminants without compromising seed viability. After sterilization, seeds were allowed to equilibrate in a laminar flow hood for 15 minutes before plating.

3.8.MS medium

To prepare 200 ml of ½ Murashige and Skoog (MS) media (Murashige T. and Skoog F., 1962), 0.44 g of MS powder and 1% (w/v) sucrose were dissolved in distilled water with constant stirring. The pH of the solution was adjusted to 5.7 with 0.1 M KOH, followed by the addition of 0.8% (w/v) plant agar. The medium was sterilized by autoclaving at 121°C for 20 minutes. After sterilization, the medium was cooled to below 50°C and poured into 90 mm sterile petri dishes (~20 mL per plate) under aseptic conditions.

3.9. Plant growth conditions

Sterilized seeds were stratified at 4°C for 2 days in the dark by placing them in Jiffy-7 peat pellets (42 mm; Jiffy Products International AS, Norway). The seeds were transferred to a growth chamber maintained at a constant temperature of 22°C under cool fluorescent light at ~100-120 µmol m^{-2} s^{-1} and a 16-hour light / 8-hour dark photoperiod (Conviron). The relative humidity in the growth chamber was maintained at 60-70% , and CO_2 was ambient. Plants were grown for two weeks with regular monitoring to confirm sufficient moisture levels as previously described by (Ludwików et al., 2009). For plate-grown seedling experiments, sterilized seeds were sown on MS plates (approximately 80 seeds per 10 cm^2 plate) under sterile conditions and maintained in the same growth chamber. Growth chamber calibration and environmental uniformity were verified weekly to minimize experimental variation.

3.10. Plant treatment

To assess transcriptional changes induced by ABA in key signalling mutants, a standardized treatment protocol was employed. To prepare samples for total RNA sequencing, seedlings of WT Col-0, *abi1td*, *mkkk17-1* or *mkkk18-1* were grown under sterile conditions on ½ MS plates for 2 weeks as described in Materials & Methods 1.1. 2-week-old plants were treated with 100 µM abscisic acid (ABA; Sigma-Aldrich, Cat. No. A1049), prepared with 1 mM methanol, or mock-treated with 1 mM methanol alone. Treatments were applied directly to the media surface. After 4 hours of treatment, samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. This concentration and time point were selected based on previous studies demonstrating robust ABA transcriptional responses (Nelson et al., 2007). Three independent biological replicates were performed per treatment. Mock treatments (1 mM methanol) were included for all genotypes to control for solvent effects. Untreated WT Col-0 seedlings served as baseline controls to confirm the specificity of ABA-induced responses.

3.11. Total RNA isolation

Total RNA was extracted from plant samples for RNA sequencing using the Qiagen RNeasy Plant Mini Kit according to the manufacturer's instructions. Approximately 100 mg of frozen plant tissue was ground using a pre-chilled mortar and pestle in liquid nitrogen. Lysates were centrifuged at

12,000 × g for 5 minutes to remove debris before transferring to spin columns. All centrifugation steps were performed at 4°C to preserve RNA integrity. The purified RNA was eluted in 30 µL of nuclease-free water. RNA concentration and purity were measured using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific), and purity was assessed based on the 260/280 nm absorbance ratio, with values between 2.0 and 2.1 considered acceptable.

3.12. RNA integrity

The quality and integrity of the extracted RNA were assessed using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA). Two microliters of each RNA sample were loaded onto Agilent RNA Nano Chips according to the manufacturer's instructions. The RNA Integrity Number (RIN) scores were determined using the 2100 Expert software. Samples with RIN ≥ 8.0 were considered suitable for RNA-seq library preparation. All reagents, including gel dye mix, RNA marker, RNA ladder, and DEPC-treated water, were prepared according to the manufacturer's guidelines. RNase decontamination was ensured throughout using RNase Zap (Thermo Fisher Scientific).

3.13. RNA-seq library construction and sequencing

RNA sequencing and library generation was performed at Macrogen Ltd. (Seoul, South Korea) using the TruSeq Stranded mRNA Sample Preparation protocol (Illumina, Cat. No. 15031047 Rev. E). For each library, 1 µg of total RNA was used, and poly(A)+ mRNA was enriched using oligo-dT magnetic beads. Prior to library construction, RNA samples were treated with RNase-free DNase I to eliminate residual genomic DNA. Stranded mRNA libraries were constructed for all samples, with three biological replicates for both mock and ABA-treated conditions. High-throughput sequencing was performed on the Illumina NovaSeq 6000 platform, generating 100 bp paired-end reads with a sequencing depth of ~60 million reads per sample. Strand-specificity and poly(A) selection ensured high-confidence detection of mRNA species relevant to ABA signalling.

3.14. Read alignment and preprocessing

The bioinformatics pipeline was optimized (Figure 5) in collaboration with Dr. Michal Wojciech Szczesniak from the Laboratory of Functional and Evolutionary Genomics, Institute of Human

Biology and Evolution, Faculty of Biology, Adam Mickiewicz University in Poznan. Initial quality control was conducted using FastQC (v0.11.8) to assess metrics such as per-base sequence quality, GC content, and duplication levels. Adapter and quality trimming were performed using BBDuk with the following parameters: k=23; mink=11; hdist=1; tbo; tpe; minlength=50; removeifeitherbad=t; overwrite=t (Bushnell et al., 2017). To identify and exclude contaminating ribosomal RNA (rRNA) sequences, a genome index was built using Bowtie2 (v2.4.2) (Langmead & Salzberg, 2012)from Arabidopsis thaliana rRNA sequences downloaded from Ensembl Plants (release 41) (Kersey et al., 2018). The eads were aligned to this index and filtered prior to genome mapping. Reads were then aligned to the *Arabidopsis thaliana* TAIR10 reference genome using the STAR aligner (version 2.7.10a) (Dobin et al., 2013). GTF annotations were performed using *Arabidopsis.thaliana*. TAIR10.gtf (version 42) (Lamesch et al., 2012). A genome index was built using STAR, and alignment was performed using default parameters. Paired-end reads were aligned to generate BAM files. Post-alignment quality metrics were reviewed, including mapping rate (target >95%), duplication rate, and read distribution across genomic features. Only reads with Phred scores ≥33 was retained for downstream analyses.

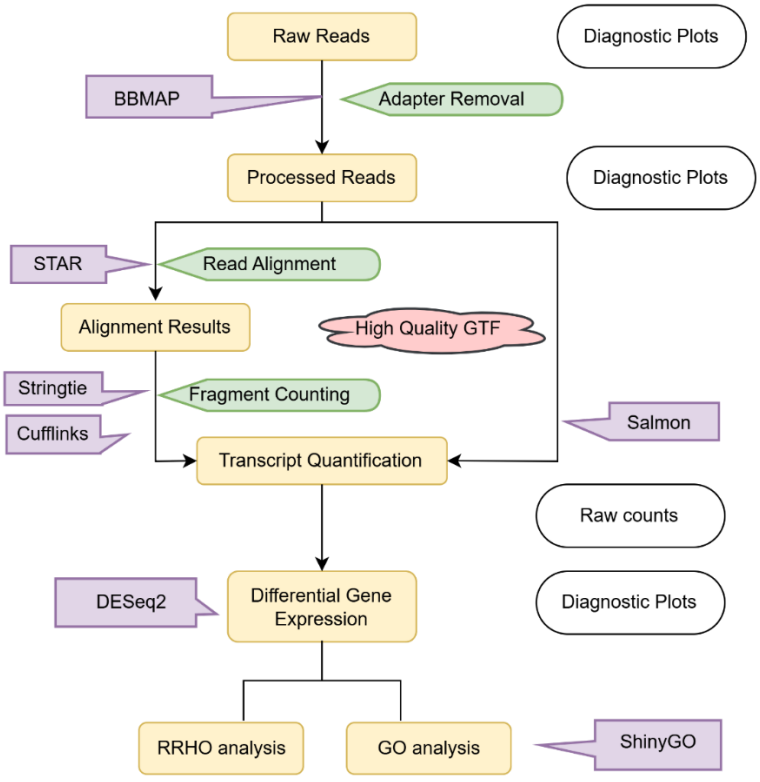


Figure 5. Bioinformatics pipeline for RNA sequencing analysis.

Schematic diagram summarizing the main steps of the RNA-seq data processing workflow, including raw read quality control, adapter trimming, rRNA filtering, genome alignment, transcript assembly, quantification, Differential gene expression analysis, and functional enrichment.

3.15. Transcript assembly (*ab initio*)

Transcriptome assembly was performed using StringTie on cleaned BAM files to construct a reference-guided transcriptome (Pertea et al., 2015). Individual transcript assemblies (GTF files) were created for each sample and subsequently merged into a single transcriptome using StringTie's merge function. The resulting file, merged_transcriptomes.gtf, was refined by removing unstranded transcripts to yield transcriptome_pre.gtf. The merged transcriptome was validated against the reference genome annotation using cuffcompare, to produce the comparison file all.combined.gtf. To ensure only novel or accurate transcript models were retained, transcripts with class codes 'c', 'e', 'p', and 's' were excluded using a custom in-house Python script, and the resulting output was saved as transcriptome.gtf. To confirm that all transcripts from the refined file were captured, the -C flag was used in cuffcompare, and filtering was repeated. The final transcriptome was converted into FASTA format using gffread, generating both transcriptome.fasta and a high-quality transcriptome.gtf. Known issues such as polymerase run-on fragments, overlapping single-exon transcripts, and incomplete models were manually reviewed and excluded. Transcript annotation completeness was validated by comparison with ENSEMBL's TAIR10 annotation. Across all RNA-seq libraries, over 660 million paired-end reads per genotype were processed. Using the final transcriptome annotation, Transcript Per Million (TPM) values were calculated for 52,544 transcript isoforms, corresponding to 23,007 genes. Novel isoforms were excluded from quantification to focus on genotype-specific expression of annotated transcripts.

3.16. Transcript quantification

Transcript-level abundance estimation was performed using Salmon (v1.10) (Patro et al., 2017). First, a transcriptome index was built using the transcriptome.fasta file, to enable quasi-mapping mode with a k-mer size of 31 (-k 31). To improve quantification accuracy, bias correction options were activated during mapping: --seqBias and --gcBias. Quantification outputs were generated in quant.sf format for each sample, capturing TPM and read counts for each transcript. Transcript-to-gene mapping was performed using the GTF annotation file to associate transcript-level expression

values with gene identifiers. Salmon outputs were imported using the tximport package in R to generate both transcript- and gene-level abundance matrices (Soneson et al., 2019). Low-abundance genes (TPM < 1 across all samples) were excluded to improve differential expression reliability. The final gene-level count matrices were used for downstream analysis including normalization, filtering, and statistical testing.

3.17. Differential gene expression analysis

Differential gene expression analysis was performed using a generalized linear model (GLM) implemented in the DESeq2 package (version 1.22) from R/Bioconductor (Love et al., 2014). The analysis was conducted on "count data" derived from the high-quality ab initio transcriptome GTF as described in Section 3.10. The GLM enabled the identification of genes with statistically significant changes in expression between mock and ABA-treated conditions. Genes with a Benjamini-Hochberg-adjusted p-value < 0.05 and an absolute log2 fold change ≥ 1 (i.e., fold change ≥ 2) were considered significantly differentially expressed. This dual threshold ensured both statistical and biological significance of the identified DEGs. Independent validation using RT-qPCR was performed on select genes to confirm RNA-seq findings (Section 3.16). Normalization of raw counts was performed using the DESeq2 default method, which adjusts for variations in library size and sequencing depth. Genes with extremely low expression across all samples were excluded during pre-filtering to increase detection sensitivity and reduce noise. Genes with adjusted p-value < 0.05 and fold change > 2 were classified as upregulated, while those with adjusted p-value < 0.05 and fold change < 0.5 were classified as downregulated. To assess sample distribution and experimental reproducibility, principal component analysis (PCA) was conducted using variance-stabilizing transformed (VST) counts. The PCA analysis helped visualize sample clustering across genotypes (WT Col-0, *abi1td*, *mkkk17-1*, and *mkkk18-1*) and treatment conditions (mock and ABA).

3.18. Gene annotation

Gene annotation was conducted to link DEGs with functional, positional, and structural features. Gene and transcript-level metadata were obtained from Ensembl Plants (release 42) via the BioMart interface (Smedley et al., 2009). The retrieved annotations included are Gene and transcript stable IDs, Gene name and biotype, Chromosomal location (chromosome number,

strand, start, and end), Functional description and Associated Gene Ontology (GO) terms. An in-house Python script was used to match transcript IDs with DEGs and integrate functional metadata for downstream analyses.

3.19. Enrichment analysis

To gain insight into the biological functions of the DEGs, Gene Ontology (GO) enrichment analysis was performed using the ShinyGO web platform (version 0.80) (Ge et al., 2020). Only genes that passed the DESeq2 statistical threshold (adjusted p-value < 0.05 and fold change ≥ 2) were used for enrichment analysis. The hypergeometric test was applied to determine overrepresented GO categories across three domains: biological process, molecular function, and cellular component. The background gene universe was defined based on the annotated Arabidopsis reference genome. Enrichment significance was adjusted for multiple testing using FDR, and enriched terms were visualized based on fold enrichment and p-value. These GO terms provided insight into cellular and molecular processes most affected by ABA treatment across different genotypes.

3.20. Rank-Rank Hypergeometric Overlap analysis

To assess transcriptional similarity and co-regulation between genotypes, Rank-Rank Hypergeometric Overlap (RRHO2) analysis was performed using the RRHO2 R package (Cahill et al., 2018; Plaisier et al., 2010). This threshold-free method compares ranked gene lists based on their differential expression. Gene lists from *abi1td*, *mkkk17-1*, and *mkkk18-1* were ordered by $\log_2$fold-change and compared with WT Col-0 as the reference. The RRHO2 function with adaptive p-value thresholding was used to identify areas of maximal gene overlap between two ranked lists. Overlap matrices and heatmaps were generated to visualize the degree and direction of transcriptional similarity. The number of overlapping upregulated and downregulated genes was quantified, and their biological significance was assessed based on known ABA and stress-responsive gene sets. Statistical significance of overlaps was determined using RRHO2's built-in permutation tests. All analyses were conducted in R, and results were visualized using RRHO2's default plotting functions.

3.21. Quantitative RT-qPCR

Total RNA was extracted from plant tissues using the Qiagen RNeasy Plant Mini Kit, following the manufacturer's protocol. To eliminate genomic DNA contamination, RNA samples were treated with DNase (Maxima First Strand cDNA Synthesis Kit for RT-qPCR, Thermo Scientific). cDNA was synthesized from 2 µg of DNase-treated RNA using oligo-dT primers and diluted 1:10 before use in quantitative PCR (qPCR) reactions. Negative controls lacking reverse transcriptase (-RT) were included to confirm the absence of genomic DNA. Gene-specific primers were used for validation, as previously reported in (Ludwików et al., 2009), and are listed in Table 2. 18S rRNA and ACT2 were used as internal reference genes for normalization of gene expression.

Table 2. List of primer sequences

Gene Name	Orientation (5'-3')	Primer sequence
18S	Forward	GGTCTGTGATGCCCTTAGATGTT
18S	Reverse	GGCAAGGTGTGAACTCGTTGA
act2	Forward	GAGAGATTCAGATGCCCAGAAGTC
act2	Reverse	TGGATTCCAGCAGCTTCCA
rab18	Forward	GGAGAAGTTGCCAGGTCATCATG
rab18	Reverse	CACCGTAGCCACCAGCATCATA
upl4	Forward	CATTGCCTGGCTACACGGATTATGATC
upl4	Reverse	TTGGATCCCATTACATACTGTGGCATTG
upl6	Forward	TTTGTGACAGGCTGCTCACGAGGAC
upl6	Reverse	GGCGTACATCAATTTGGTCTCAAGTAGC

qPCR reactions were performed in triplicate using SYBR Green Master Mix (Thermo Fisher Scientific) on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). Melting curve analysis was performed after each run to verify primer specificity. Relative gene expression was calculated using the $2^{\wedge-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Data visualization and statistical analysis were performed using in-house R and Python scripts, and Student's t-test was used to assess significance ($p < 0.05$). Each gene was assessed with three biological replicates, ensuring reproducibility.

3.22. Generation of transgenic lines

To generate transgenic Arabidopsis lines for functional analysis of *UPL*4 and *UPL6*, full-length cDNAs of both genes were amplified using Platinum™ SuperFi™ DNA Polymerase (Invitrogen) according to manufacturer's protocol. The PCR products were first cloned into the

pENTR™/SD/D-TOPO® entry vector, and positive clones were verified Sanger sequencing to confirm insert identity and orientation. The confirmed entry clones were transferred into pEarleyGate103 destination vector to generate 35S:UPL4-GFP and 35S:UPL6-GFP constructs using LR Gateway™ recombination as previously described in Earley et al., 2006; Tajdel et al., 2016.

Recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by electroporation and confirmed using colony PCR. Stable Arabidopsis transgenic lines were generated using the floral dip transformation method. Transformed seeds (T1 generation) were selected on MS medium supplemented with the appropriate plant selection marker (e.g., Basta®/glufosinate), and resistant seedlings were transferred to soil.

For each construct, multiple independent T1 lines were screened, and homozygous T3 lines were generated through consecutive selection and segregation analysis. Two independent homozygous lines for each gene were chosen for experiments to avoid position-effect bias:

Two independent transgenic lines for each gene were selected and assessed:

- *UPL*4 : 35S: *UPL4-GFP-HIS/upl4.1*, *35S:UPL4-GFP-HIS/WT-4*
- *UPL6* : *35S: UPL6-GFP-HIS/upl6-1*, *35S:UPL6-GFP-HIS/WT-3*

The validated lines were subsequently used for phenotypic, molecular, and ABA-response assays.

3.23. Primary root growth assay

Arabidopsis seeds were grown as described in Section 3.4. Five-day-old seedlings were transferred to fresh MS plates supplemented with or without 5 μM ABA and grown vertically for 3-4 additional days. Primary root lengths were measured post-treatment. At least 15-18 seedlings per genotype per condition were analysed per replicate. Measurements were performed using ImageJ (NIH), calibrated to a reference scale. Relative root growth was calculated by comparing treated vs. mock conditions. Statistical significance was assessed using an unpaired, two-tailed Student's t-test ($p < 0.05$), and experiments were conducted in at least three biological replicates to ensure reproducibility.

3.24. Stomatal development assays

Stomatal patterning was analysed using cotyledons from 6- or 10-day-old seedlings. Fully expanded cotyledons were mounted abaxial side up in water and imaged using a ZEISS microscope. For each genotype, images from ≥10 leaves, with multiple random fields per leaf, were collected. Guard cells (GCs) and pavement cells (PCs) were counted manually using ImageJ.

Stomatal index (SI) was calculated using the formula:

$$\mathrm{SI} = \frac{\text{Number of Stomata}}{\text{Number of pavement cells} + \text{number of stomata}}$$

At least 150 cells were counted per sample, and all experiments were independently repeated three times. Statistical analysis was performed using a student's t-test, with significance thresholds of $p < 0.05$, $p < 0.005$, and $p < 0.0005$.

4. Results

4.1. Global transcriptional changes in *mkkk17*, *mkkk18* and *abi1td* mutants

It is known that ABA signalling drives broad transcriptional changes through MAPK cascades. While *MAPKKK17*, *MAPKKK18* and the phosphatase *ABI1* are recognised as upstream regulators, the distinct transcriptional footprints of these regulators under ABA stress remain unclear. This is especially pertinent in the context of the ABA signalling pathway, since *ABI1* is known to work upstream of *MAPKKK18*. Here, RNA sequencing was performed to characterise the global gene expression profiles of knockout mutants (*mkkk17-1* and *mkkk18*-1) and compared to WT Col-0 and *abi1td* under ABA treatment. This revealed their distinct and overlapping roles in ABA-induced gene regulation (*specific aims 1-2*).

4.1.1. Transcriptional responses of *MAPKKK*17, *MAPKKK*18, *ABI1* in response to ABA treatment

To dissect the contribution of *MAPKKK17*, *MAPKKK18*, and *ABI1* to ABA-regulated gene expression, RNA-seq was performed on Arabidopsis knockout mutants (*abi1td*, *mkkk17-1*, *mkkk18-1*,) and WT Col-0, following ABA and mock treatment (see Methods 3.4-3.7). Previously, plants were cultivated under standardized growth conditions, and exogenous ABA was applied as described in Sections 3.4 and 3.5. Samples from both mock- and ABA-treated plants were harvested for total RNA extraction, followed by quality assessment and library preparation as outlined in Sections 3.6 and 3.7. High-throughput sequencing was performed to generate transcriptomic profiles reflecting genotype- and treatment-specific responses.

Differential gene expression analysis was performed by comparing mock- and ABA-treated samples within each genotype. This approach enabled the identification of ABA-responsive genes directly or indirectly regulated by *MAPKKK17*, *MAPKKK18*, and *ABI1*. Notably, canonical ABA marker genes such as *RAB18*, *RD29A*, and *COR15A* were consistently upregulated across backgrounds, validating the experimental design and treatment efficacy. This data establishes that while all three regulators modulate ABA responses, they do so via both overlapping and gene-specific pathways. These results establish a foundation for exploring genotype-specific ABA response mechanisms.

4.1.2. Principal component analysis of DEGs

To evaluate the major sources of transcriptional variance across genotypes and treatments, principal component analysis (PCA) was performed. This helps visualize global expression shifts and potential clustering patterns reflective of genotype-specific or treatment-induced transcriptional responses. Results revealed that the first principal component (PC1) accounted for 86% of the total variance and clearly separated ABA-treated samples from mock-treated controls in all genotypes, confirming a dominant effect of ABA on gene expression (Figure 6).

The second principal component (PC2), which explained 5% of the variance, distinguished mutant lines (*abi1td*, *mkkk17-1*, *mkkk18-1*) from the WT Col-0, indicating genotype-specific transcriptomic signatures in response to ABA.

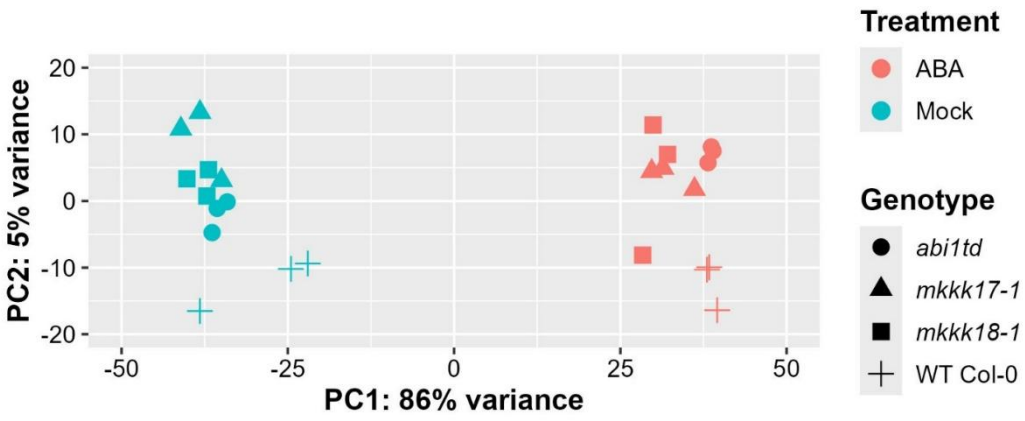


Figure 6. Principal component analysis of gene expression levels in response to ABA treatment.
Principal component analysis (PCA) of gene expression in WT Col-0 and mutant lines under mock and ABA treatment. Points represent individual biological replicates. ABA-treated samples (red) are clearly separated from mock-treated samples (green) along PC1. Genotypes are distinguished by shape: WT Col-0 (plus), *abi1td* (circle), *mkkk17-1* (triangle), and *mkkk18-1* (square). PC1 and PC2 account for 86% and 5% of variance, respectively.

Importantly, all biological replicates clustered tightly, supporting the reproducibility of the dataset and the robustness of the treatment effects. The PCA clustering highlights that *ABI1*, *MAPKKK17*, and *MAPKKK18* confer distinct transcriptional identities under ABA stress, reinforcing their non-redundant roles. These results highlight that treatment-driven separation along PC1 and genotype-driven variation along PC2 (Figure 6).

4.1.3. Differentially expressed genes in response to ABA treatment

Differential gene expression analysis was conducted to identify ABA-responsive genes within each genotype. Using a threshold of adjusted p-value < 0.05 and absolute fold change ≥ 2, Differentially expressed genes (DEGs) were identified upon ABA treatment across wild-type and mutant lines (Table 3).

In the WT Col-0, 4,022 DEGs were identified, comprising 2,231 upregulated and 1,791 downregulated. The *abi1td* mutant exhibited the most extensive transcriptional changes, with 5,259 DEGs (2,613 upregulated, 2,646 downregulated), suggesting a broad, bidirectional regulatory role for *ABI1* (Table 3). In contrast, the *mkkk17-1* and *mkkk18-1* mutants showed fewer DEGs (3,884 and 3,816, respectively), with a slightly higher proportion of upregulated genes, suggesting a more specific role for these kinases in ABA-regulated transcription. In conclusion, ABA treatment induces distinct transcriptional changes, with several genes upregulated to enhance stress tolerance and others downregulated to conserve resources. These differential expression patterns underscore the pivotal role of ABA as a central regulator that fine-tunes molecular-level responses.

Table 3. Differentially expressed genes in WT Col-0 and knockouts (*abi1td*, *mkkk17-1*, *mkkk18-1*) in response to ABA treatment.

Sample	Effect	Comparison	Differential expressed genes	Upregulated genes	Downregulated genes
WT Col-0	Treatment	ABA vs Mock	4022	2231	1791
abi1td	Treatment	ABA vs Mock	5259	2613	2646
mkkk17-1	Treatment	ABA vs Mock	3884	2229	1655
mkkk18-1	Treatment	ABA vs Mock	3816	2170	1646

In addition, these findings indicate that *MAPKKK*17 and *MAPKKK18* contribute to ABA-responsive transcription, but the broader range of DEGs observed in the *abi1td* mutant underscores *ABI1*'s vital role as a regulator of ABA responses. The loss of *ABI1* leads to extensive transcriptomic reprogramming, highlighting its dominant position in controlling ABA-mediated gene expression.

4.1.4. Cross comparison of DEGs across genotypes

To examine the overlap and divergence in ABA-responsive gene expression across genotypes, Venn diagram was generated to compare DEGs in WT Col-0, *abi1td*, *mkkk17-1*, and *mkkk18-1* (Figure 7). A core set of 2,092 genes was commonly regulated across all four genotypes, indicating a conserved ABA response network. However, substantial genotype-specific differences were also observed.

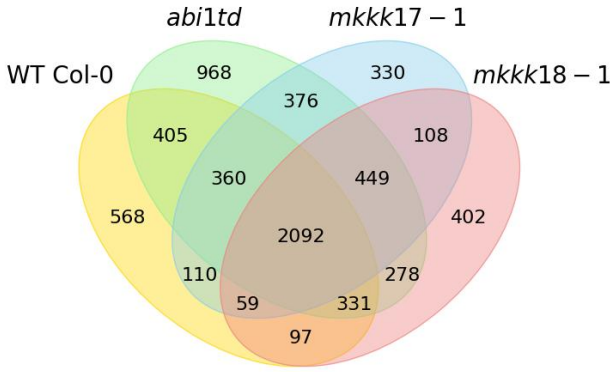


Figure 7. Comparison of DEGs in WT Col-0 and knockouts after ABA treatment.
Venn diagram shows the shared and unique ABA-responsive Differentially expressed genes (DEGs) across WT Col-0, *abi1td*, *mkkk17-1*, and *mkkk18-1*. DEGs were defined as those with $|\log_2 FC| \geq 1$ and an adjusted $p < 0.05$ relative to mock controls. A core set of 2,092 genes is commonly regulated, while genotype-specific subsets highlight distinct regulatory roles.

The *abi1td* mutant exhibited the largest number of unique DEGs (968), reinforcing its extensive regulatory influence on ABA signalling. *mkkk18-1* and *mkkk17-1* showed 402 and 330 unique DEGs, respectively, while WT Col-0 had 568 unique DEGs not detected in the mutants. These distinct expression profiles suggest that *MAPKKK17*, *MAPKKK18*, and *ABI1* each regulate both shared and exclusive subsets of the ABA transcriptome.

4.1.5. Hierarchical clustering of DEGs

To explore expression patterns of genes that are strongly responsive to ABA, hierarchical clustering was performed on the top 100 most highly regulated DEGs across all genotypes (Figure 8). The heatmap revealed genotype-specific clustering and consistent upregulation of well-characterised ABA marker genes, including *RAB18*, *RD29A*, *XERO2*, *COR15A*, *SAG113*, *PP2CA*, and *PUB19*.

Compared to mock controls, ABA-treated samples across all genotypes displayed clear expression shifts, supporting the transcriptional responsiveness of the experimental system. Distinct

expression signatures within each genotype further emphasize the differential regulatory input of *ABI1, MAPKKK17,* and *MAPKKK18* in modulating ABA-induced gene networks (Figure 8).

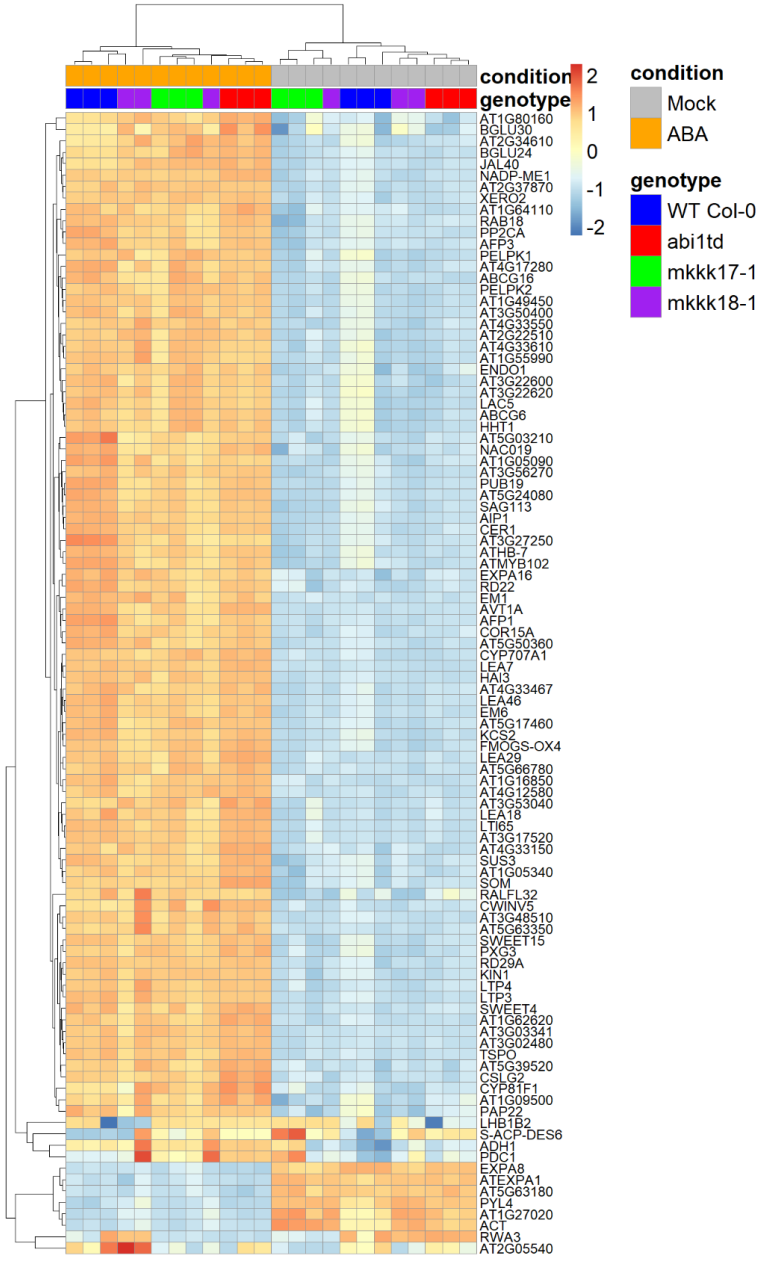


Figure 8. Heatmap of the Top 100 Differentially expressed genes.
Heatmap of the top 100 Differentially expressed genes (DEGs) across genotypes and treatments. Rows represent genes and columns represent individual samples. Colours indicate fold change (red = upregulated, blue = downregulated). Mock samples are in grey, ABA-treated samples in orange. Genotypes are color-coded: WT Col-0 (blue), *abi1td* (red), *mkkk17-1* (green), *mkkk18-1* (purple).

4.1.6. Rank-Rank Hypergeometric Overlap analysis of DEGs

To quantify the transcriptional similarity between genotypes, Rank-Rank Hypergeometric Overlap (RRHO) analysis was conducted by comparing $\log_2$ fold-change-ranked gene lists for each mutant against WT Col-0 under ABA treatment (Figure 9). Strong overlaps were observed in both the upregulated and downregulated quadrants between WT Col-0 and each mutant, indicating a conserved core of ABA-responsive genes. Among the mutants, *abi1td* showed the greatest divergence from WT, consistent with its expanded set of DEGs (Figure 9). The overlap between *mkkk17-1* and *mkkk18-1* was especially pronounced (Figure 10), suggesting functional redundancy or convergence in their downstream transcriptional targets.

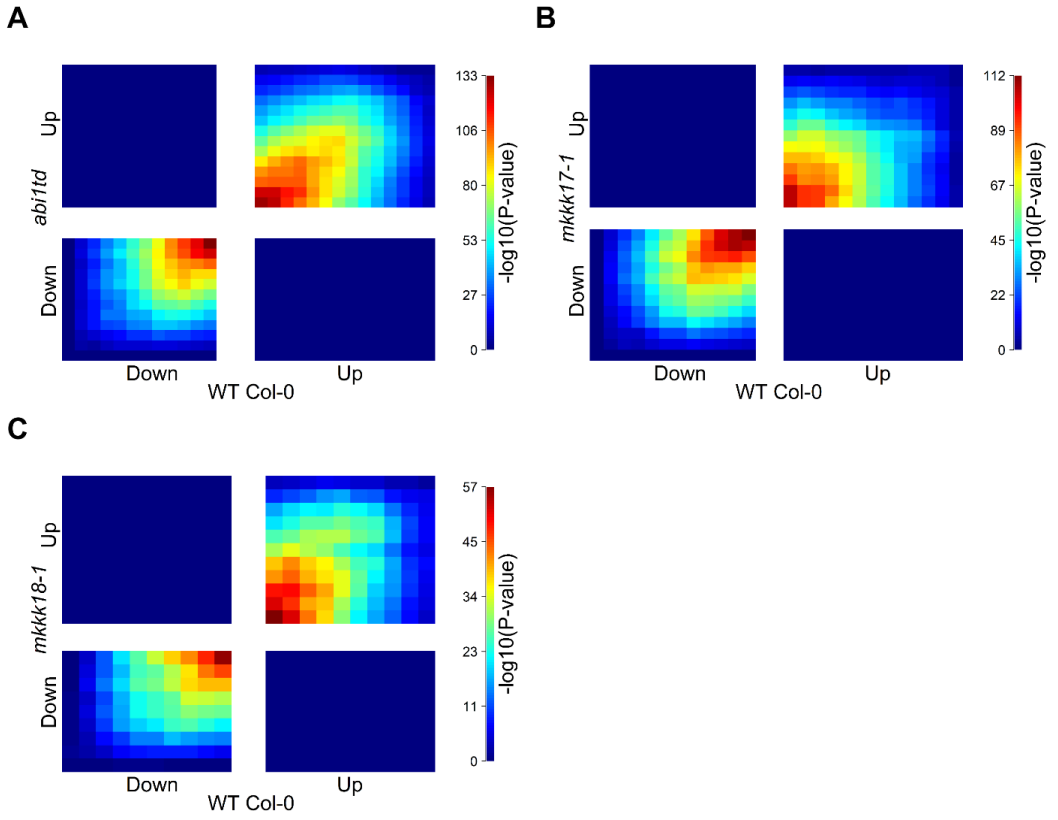


Figure 9. Rank-Rank Hypergeometric Overlap analysis of Differential gene expression between WT Col-0 and knockouts.
Rank-Rank Hypergeometric Overlap (RRHO) analysis comparing WT Col-0 and mutant genotypes (*abi1td*, *mkkk17-1*, *mkkk18-1*). Each heatmap shows statistically significant overlaps of DEGs ranked by $\log_2$ fold-change. Bright red indicates high enrichment $-\log_{10}$(p-value), between ranked gene lists ($\log_2$FC > 1). Overlapping up/downregulated gene sets occupy diagonal quadrants.

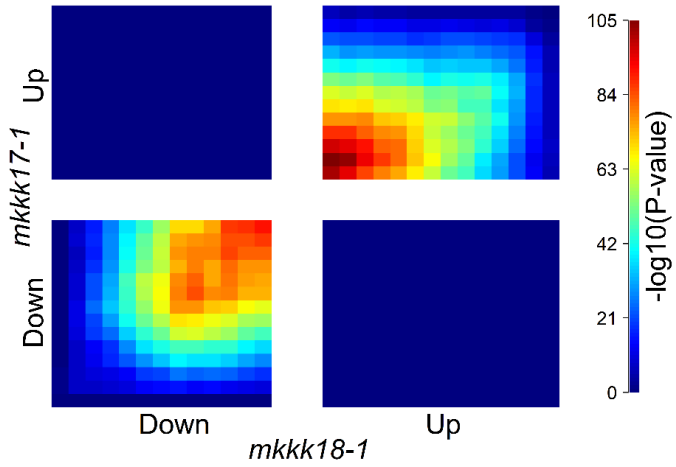


Figure 10. Rank-Rank Hypergeometric Overlap analysis of Differential gene expression between MAP kinases. Rank-Rank Hypergeometric Overlap (RRHO) analysis comparing mutant genotypes (*mkkk17-1*, *mkkk18-1*). Each heatmap shows statistically significant overlaps of DEGs ranked by $\log_2$ fold-change. Bright red indicates high enrichment $-\log_{10}$(P-value), between ranked gene lists ($\log_2$FC > 1). Overlapping up/downregulated gene sets occupy diagonal quadrants.

Finally, RRHO analysis comparing the transcriptional responses of the *abi1td* and *mkkk18-1* mutants (Figure 11), revealed a similar distribution of upregulated and downregulated genes in both mutant lines, further indicating that *ABI1* and *MAPKKK18* share overlapping roles in regulating the ABA transcriptional response. Previous studies demonstrate that ABI1 dephosphorylate *MAPKKK18* (Mituła et al., 2015). This finding therefore further suggests that *ABI1* and *MAPKKK18* work together to coordinate ABA-induced gene expression.

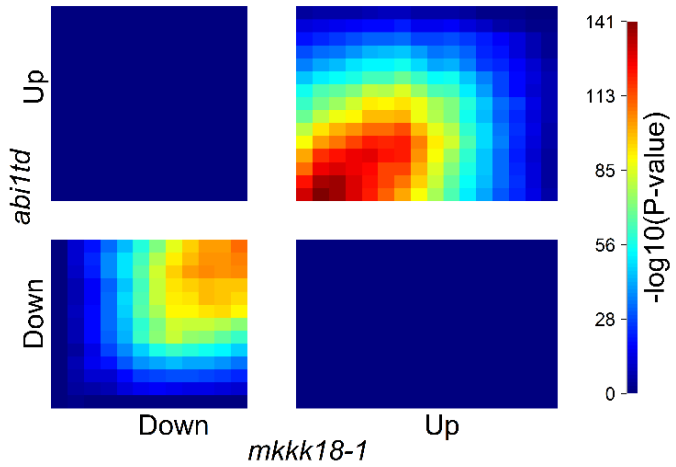


Figure 11. Rank-Rank Hypergeometric Overlap analysis of Differential gene expression between *abi1td* and *mkkk18-1*. Rank-Rank Hypergeometric Overlap (RRHO) analysis comparing mutant genotypes (*abi1td*, *mkkk18-1*). Each heatmap shows statistically significant overlaps of DEGs ranked by $\log_2$ fold-change. Bright red indicates high enrichment $-\log_{10}$(P-value), between ranked gene lists ($\log_2$FC > 1). Overlapping up/downregulated gene sets occupy diagonal quadrants.

4.2. Differential gene expression analysis of *mkkk17-1* reveals altered ABA response

MAPKKK17 is a critical upstream component in the ABA-responsive MAPK signalling cascade, yet its genome-wide transcriptional effects remain underexplored. To characterize its contribution in ABA signalling (Specific Aim 1), we analysed RNA-seq data from the *mkkk17-1* mutant and WT Col-0 following ABA treatment. Transcriptomic profiling identified 3884 DEGs in *mkkk17-1* and 4022 in WT Col-0 in response to ABA (Table 3). To further dissect the transcriptional alterations attributed to the *MAPKKK17* mutation, DEGs from *mkkk17-1* were compared with those from WT Col-0 (Figure 12). This comparison revealed 1263 genes that were uniquely expressed in *mkkk17-1* (562 upregulated and 701 downregulated), and 2621 genes were commonly regulated in both genotypes.

Within this overlapping group, 249 genes were more strongly upregulated (≥2-fold) and 139 were more strongly downregulated (≥2-fold) in *mkkk17-1* than in *WT*, indicating enhanced transcriptional responses in the mutant for specific ABA-regulated genes. This distinction suggests that although *mkkk17-1* retains some ABA-responsive pathways, it exhibits altered transcriptional dynamics indicative of *MAPKKK17*-specific regulatory functions. These unique and overlapping gene sets were subjected to Gene Ontology (GO) enrichment and pathway analyses to elucidate the biological significance of *MAPKKK17* in ABA signalling.

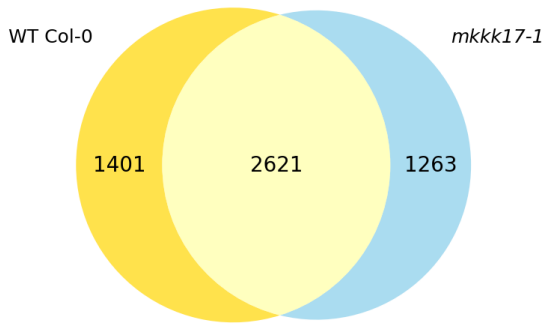


Figure 12. Genotype-specific and shared transcriptional responses to ABA in WT Col-0 and *mkkk17-1*.
The Venn diagram shows unique and overlapping DEGs between WT Col-0 and *mkkk17-1* under ABA treatment. Genes were classified based on significant expression changes ($log_2FC \geq 1$, adjusted $p < 0.05$). The diagram highlights 1,263 *mkkk17-1*-specific genes and 2,621 overlapping DEGs.

The results demonstrate that *MAPKKK17* contributes to the fine-tuning of ABA-mediated transcription, influencing both shared and genotype-specific gene expression changes. These findings establish *MAPKKK17* as an important regulator within ABA signalling and provide a basis for future studies to identify its downstream targets and signalling modules.

4.2.1. Functional characterization of unique DEGs in *mkkk17-1*

To explore the functional significance of genes uniquely regulated in *mkkk17-1* following ABA treatment, Gene Ontology (GO) enrichment analysis was performed. This analysis aims to identify biological processes that are differentially influenced by the absence of *MAPKKK17*. By examining the 1263 *mkkk17-1*-specific DEGs identified in response to ABA treatment, we sought to uncover ABA-induced transcriptional regulation of *MAPKKK17*. The GO enrichment analysis was conducted as detailed in Section 3.14, with a focus on identifying significant KEGG pathways and biological processes that were specifically enriched in *mkkk17-1* (Figure 13 and Figure 14).

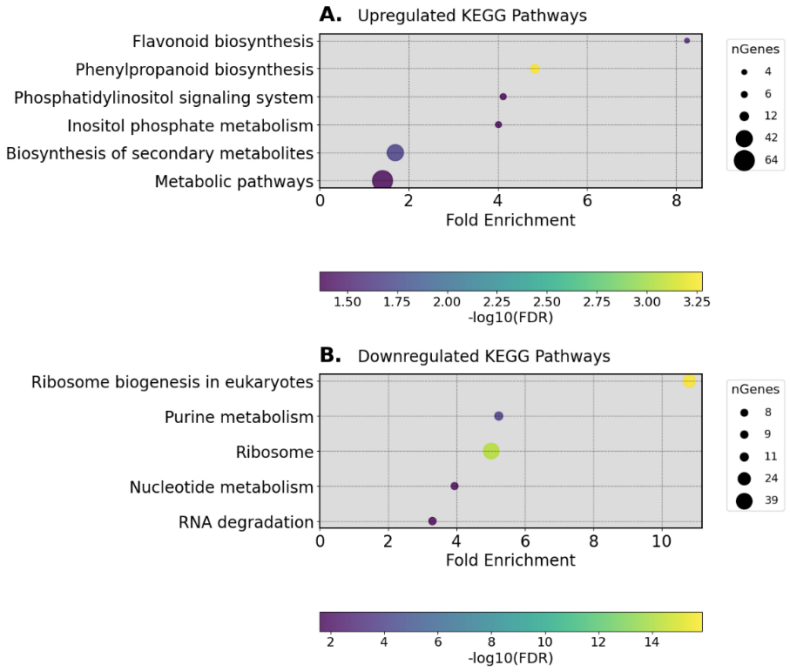
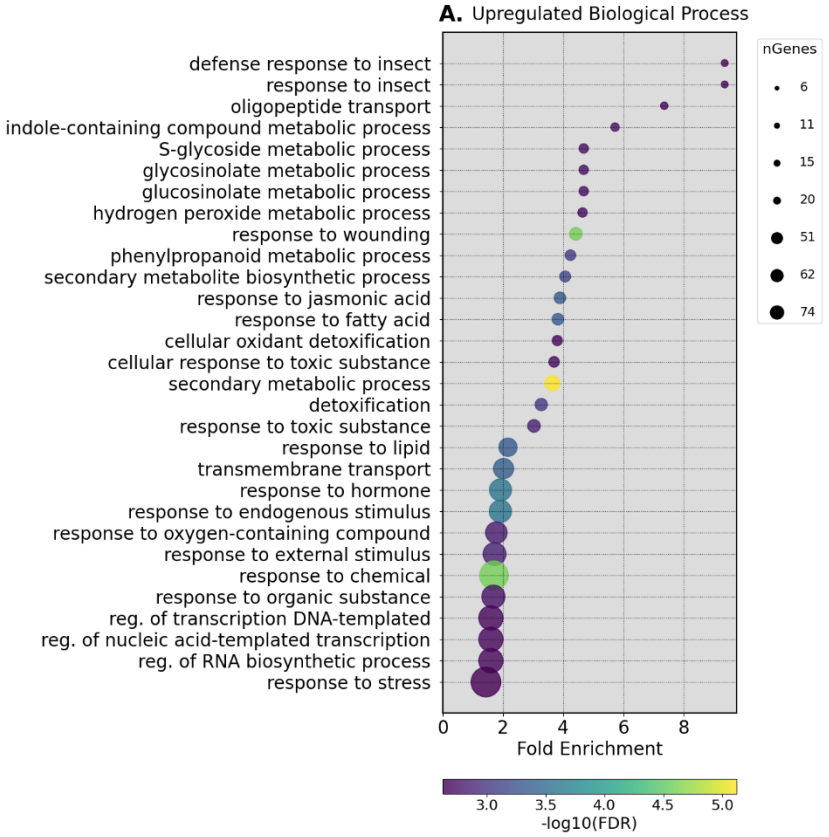


Figure 13. KEGG pathway enrichment from Gene Ontology analysis of unique DEGs in *mkkk17-1*.
KEGG pathway enrichment of uniquely regulated DEGs in *mkkk17-1*. (A) Pathways enriched among 562 upregulated genes. (B) Pathways enriched among 701 downregulated genes. Dot size indicates gene count, and colour intensity represents statistical significance ($-\log_{10}$ FDR). Pathways are ranked by fold enrichment.


A. Upregulated Biological Process
defense response to insect
response to insect
oligopeptide transport
indole-containing compound metabolic process
S-glycoside metabolic process
glycosinolate metabolic process
glucosinolate metabolic process
hydrogen peroxide metabolic process
response to wounding
phenylpropanoid metabolic process
secondary metabolite biosynthetic process
response to jasmonic acid
response to fatty acid
cellular oxidant detoxification
cellular response to toxic substance
secondary metabolic process
detoxification
response to toxic substance
response to lipid
transmembrane transport
response to hormone
response to endogenous stimulus
response to oxygen-containing compound
response to external stimulus
response to chemical
response to organic substance
reg. of transcription DNA-templated
reg. of nucleic acid-templated transcription
reg. of RNA biosynthetic process
response to stress
0
2
4
6
8
Fold Enrichment
3.0
3.5
4.0
4.5
5.0
-log10(FDR)
nGenes
6
11
15
20
51
62
74

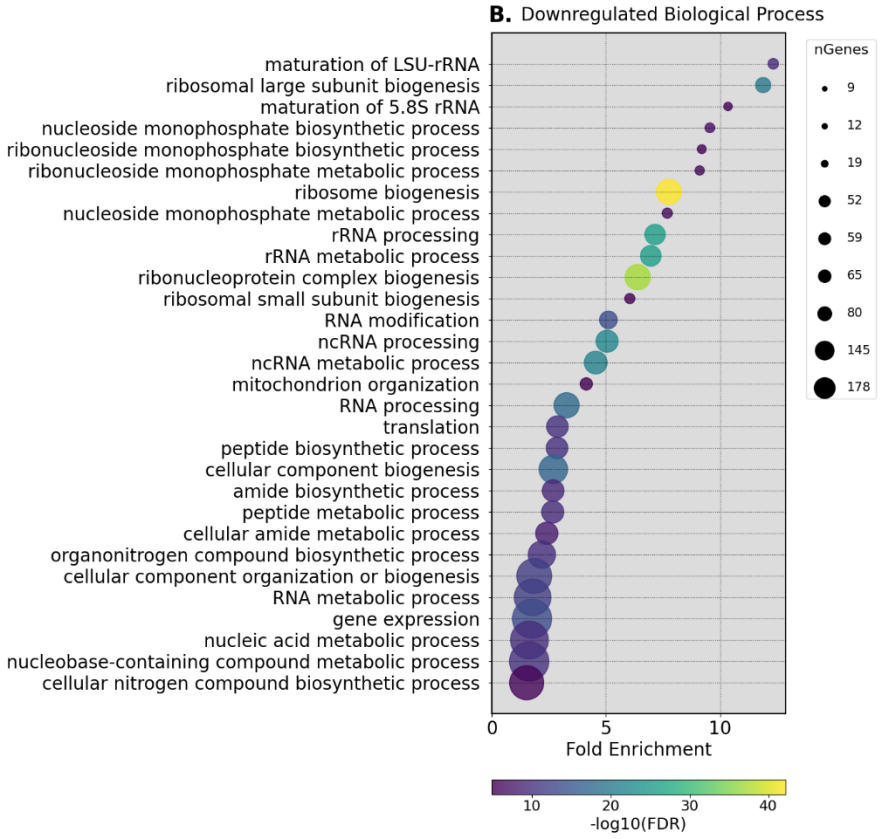

B. Downregulated Biological Process
maturation of LSU-rRNA
ribosomal large subunit biogenesis
maturation of 5.8S rRNA
nucleoside monophosphate biosynthetic process
ribonucleoside monophosphate biosynthetic process
ribonucleoside monophosphate metabolic process
ribosome biogenesis
nucleoside monophosphate metabolic process
rRNA processing
rRNA metabolic process
ribonucleoprotein complex biogenesis
ribosomal small subunit biogenesis
RNA modification
ncRNA processing
ncRNA metabolic process
mitochondrion organization
RNA processing
translation
peptide biosynthetic process
cellular component biogenesis
amide biosynthetic process
peptide metabolic process
cellular amide metabolic process
organonitrogen compound biosynthetic process
cellular component organization or biogenesis
RNA metabolic process
gene expression
nucleic acid metabolic process
nucleobase-containing compound metabolic process
cellular nitrogen compound biosynthetic process
0
5
10
Fold Enrichment
10
20
30
40
-log10(FDR)
nGenes
9
12
19
52
59
65
80
145
178

Figure 14. Biological Process enrichment from Gene Ontology analysis of unique DEGs in *mkkk17-1*.

Biological Process enrichment of uniquely regulated DEGs in *mkkk17-1*. (A) Pathways enriched among 562 upregulated genes. (B) Pathways enriched among 701 downregulated genes. Dot size indicates gene count, and colour intensity represents statistical significance ($-\log_{10}$ FDR). Pathways are ranked by fold enrichment.

The results of the GO enrichment analysis revealed distinct patterns in the upregulated and downregulated genes in *mkkk17-1*. The upregulated genes were primarily associated with metabolic processes, including "flavonoid and phenylpropanoid biosynthesis", and "secondary metabolite biosynthesis" (Figure 13A, Figure 14A). These pathways are critical for various stress responses and metabolic adaptations. Genes such as *4CL2, LOX3, BGLU18, DOX1, NPC5, TPPI, FLS2,* and *MYB3* were significantly upregulated in these processes , with the upregulation being stronger in *mkkk17-1* compared to the wild type plants. suggesting a potential role of *MAPKKK*17 in regulating secondary metabolism during ABA-induced stress (Table 4). The S-glycoside metabolic process was also enriched in the upregulated gene set, with genes such as *BGLU18*, *WRKY70*, *MYB34*, and *MYB73* showing known ABA-related functions.

Additionally, pathways related to transmembrane transport were enriched in the upregulated gene set, with genes such as *ZIP1, ALMT9, ALMT12, ZIFL1, CCX2, CLC-A, KEA5, CNGC4* and *SULTR3;1* , with the upregulation being stronger in *mkkk17*-1 compared to the WT Col-0 showing differential expression. These findings suggest that *MAPKKK*17 might be involved in regulating transport processes that are crucial for maintaining cellular function under ABA stress conditions.

Table 4. Selected GO Terms from 562 upregulated unique genes of *mkkk17-1*

Category	GO Terms	Genes
KEGG	Phenylpropanoid biosynthesis	*AT2G18150, AT2G35380, AT2G41480, 4CL2, CAD6, ELI3-2, PRX52, PA2, AT5G06730, AT5G15180, AT5G19880, AT5G47000*
KEGG	Biosynthesis of secondary metabolites	*APS2, PGL1, AT1G14240, LOX3, GA2OX4, BGLU18, BCDH BETA1, BGLU46, CDS1, ATTPS6, GA2, GA3OX2, AT2G18150, AAS, AT2G35380, AT2G41480, DOX1, NPC5, 4CL2, AT3G47040, BGLU16, ACS6, EDA36, AT4G14090, NCED4/CCD4, AT4G19860, LDOX, KCS16, CAD6, ELI3-2, CYP79B2, PRX52, PA2, AT5G06730, TPPI, AT5G15180, AT5G19880, AT5G28237, DFR, AT5G47000, FLS2, FLS3*

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Biological process	S-glycoside metabolic processes	*BGLU18, BGLU16, ESP, NSP4, CYP81F4, AT1G68360, IGMT5, AT3G11580, WRKY70, MYB73, MYB34*
Biological process	phenylpropanoid metabolic processes	*AT3G59940, CAD6, ELI3-2, 4CL2, AT4G11190, AT4G14090, LAC11, LAC17, GXMT1, BGLU46, LDOX, PRX52, CYP86A1, MYB3*
Biological process	transmembrane transport	*AT1G78720, NHX6, ABCC4, AT3G03700, AT3G09450, ZIP1, AT3G30390, HA4, ABCB9, HA3, UMAMIT25, NAT8, OZS1, UMAMIT19, AT1G33440, CAT2, AT1G59740, AT1G72140, AT2G04080, AT2G04100, AT2G34350, AT2G37900, AT2G40460, AT2G44280, MHX, ALMT9, ALMT12, YSL6, UMAMIT46, ABCA8, GTR1, SULTR3;1, AT3G53960, AAT1, KT2/3, GLUR2, AT5G01990, UMAMIT9, ZIFL1, AT5G17700, CCX2, SWEET12, YSL2, AT5G38030, UMAMIT42, CLC-A, AT5G49990, KEA5, CNGC4, AT5G55950, AT1G53110*

In contrast, downregulated genes in *mkkk17-1* were primarily associated with "ribosome biogenesis", "purine metabolism", and "RNA degradation" (Figure 13B, Figure 14B). The downregulation of these genes indicates potential disruptions in fundamental cellular processes such as protein synthesis and RNA stability. Genes such as *PWP2, TLP6, GHS40, ABO6, ATRH9, EBP2, RACK1B* and *GHS40*, which are involved in "ribosome biogenesis", were significantly downregulated in the *mkkk17-1* mutant compared to the wild type (Table 5). These disruptions in ribosome-related processes could impair cellular function, contributing to the altered stress response observed in the absence of *MAPKKK17*. Moreover, the downregulation of genes involved in RNA degradation processes further suggests that *MAPKKK17* may play a role in maintaining RNA integrity under ABA stress.

Overall, the GO enrichment analysis highlights the critical role of *MAPKKK17* in regulating various processes in response to ABA. The upregulation of genes involved in secondary metabolism and transport, along with the downregulation of genes associated with ribosome biogenesis and RNA stability, underscores the importance of *MAPKKK17* in modulating both metabolic and fundamental cellular processes during ABA-induced stress responses. These findings suggest that *MAPKKK17* may contribute to stress resilience by maintaining cellular homeostasis and supporting adaptive metabolic pathways.

Table 5. Selected GO Terms from 701 downregulated unique genes of *mkkk17-1.*

Category	GO Terms	Genes
KEGG Pathway	Ribosome biogenesis in eukaryotes	*PWP2, TLP6, NOP56, AT1G63810, AT2G18900, SWA1, AT3G03920, AT3G05060, AT3G12860, AT3G21540, AT3G23860, NAP57, AT3G57940, AT4G04940, AT4G22380, AT5G08600, GHS40, AT5G14050, TOZ, AT5G20160, AT5G22100, AT5G27120, FIB1, AT5G66540*
KEGG pathway	RNA degradation	*RRP4, PAB4, AT2G25355, HSP60-2, AT3G07750, HSP60, AT3G46210, mtHsc70-1, ABO6*
Biological Process	ribosome biogenesis	*AT1G01080, RRP4, NAF1, LIP2, AT1G07070, PWP2, AT1G18540, AT1G23280, AT1G29320, AT1G30960, AT1G31660, AT1G52930, RPL3B, AT1G63810, AT1G74270, AT2G20450, AT2G25355, AT2G34357, AT2G40010, AT2G42710, EMB2777, AT2G44120, AT2G44860, SWA1, AT3G03920, AT3G07750, AT3G10530, AT3G12930, AT3G18600, AT3G21540, PMH1/ATRH9, AT3G22660/ EBP2, AT3G23860, RID3, CP33, RBL, EDA7, NAP57, AT3G57940, AT3G58660, AT3G62870, AT4G04940, AT4G15770, AT4G22380, AT4G23540, AT4G25730, AT4G28450, AT5G12220, AT5G14520, TOZ, AT5G20160, AT5G22100, AT5G26180, RPL5B, AT5G48240, FIB1, OLI2, RID2, AT5G61170, AT5G67510, AT1G07615, NRPA2, NUC-L1, NOP56, AT1G69210, AT1G79150, AT3G05060, AT3G12860, AT3G46210, AT5G08600, AT5G14050, AT5G15550, AT5G27120, AT5G66540, RACK1B, AT1G29250, AT1G18850, GHS40*

4.2.2. Functional insights from common DEGs in *mkkk17-1* and WT Col-0

To further investigate the functional implications of the overlapping genes expressed in both *mkkk17-1* and WT Col-0 in response to ABA treatment, Gene Ontology (GO) enrichment analysis was performed on the 2,621 overlapping DEGs. These genes represent a core ABA-responsive transcriptome that is preserved despite the loss of *MAPKKK17*. Among these, 249 genes were more strongly upregulated (≥2-fold), and 139 were more strongly downregulated (≥2-fold) in *mkkk17-1* compared to WT Col-0. This analysis aimed to identify the biological processes and pathways that are similarly regulated across both genotypes (Figure 15, Figure 16).

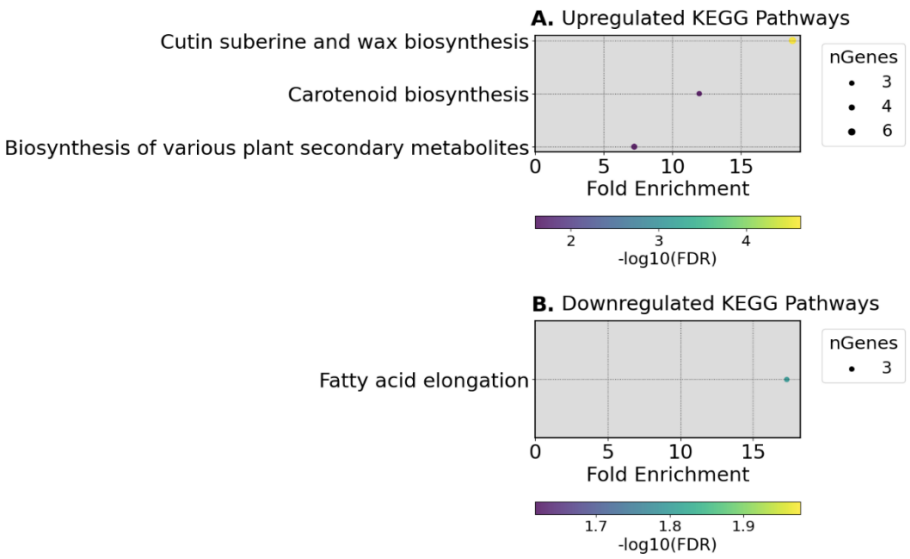

Figure 15. KEGG pathway enrichment from Gene Ontology analysis of overlapping DEGs in *mkkk17-1* and WT Col-0.

KEGG pathway enrichment of overlapping DEGs between *mkkk17-1* and WT Col-0 after ABA Treatment. (A) Pathways enriched among 249 upregulated genes, (B) Pathways enriched among 139 downregulated genes. Dot size indicates gene count, and colour intensity represents statistical significance ($-\log_{10}$ FDR). Pathways are ranked by fold enrichment.

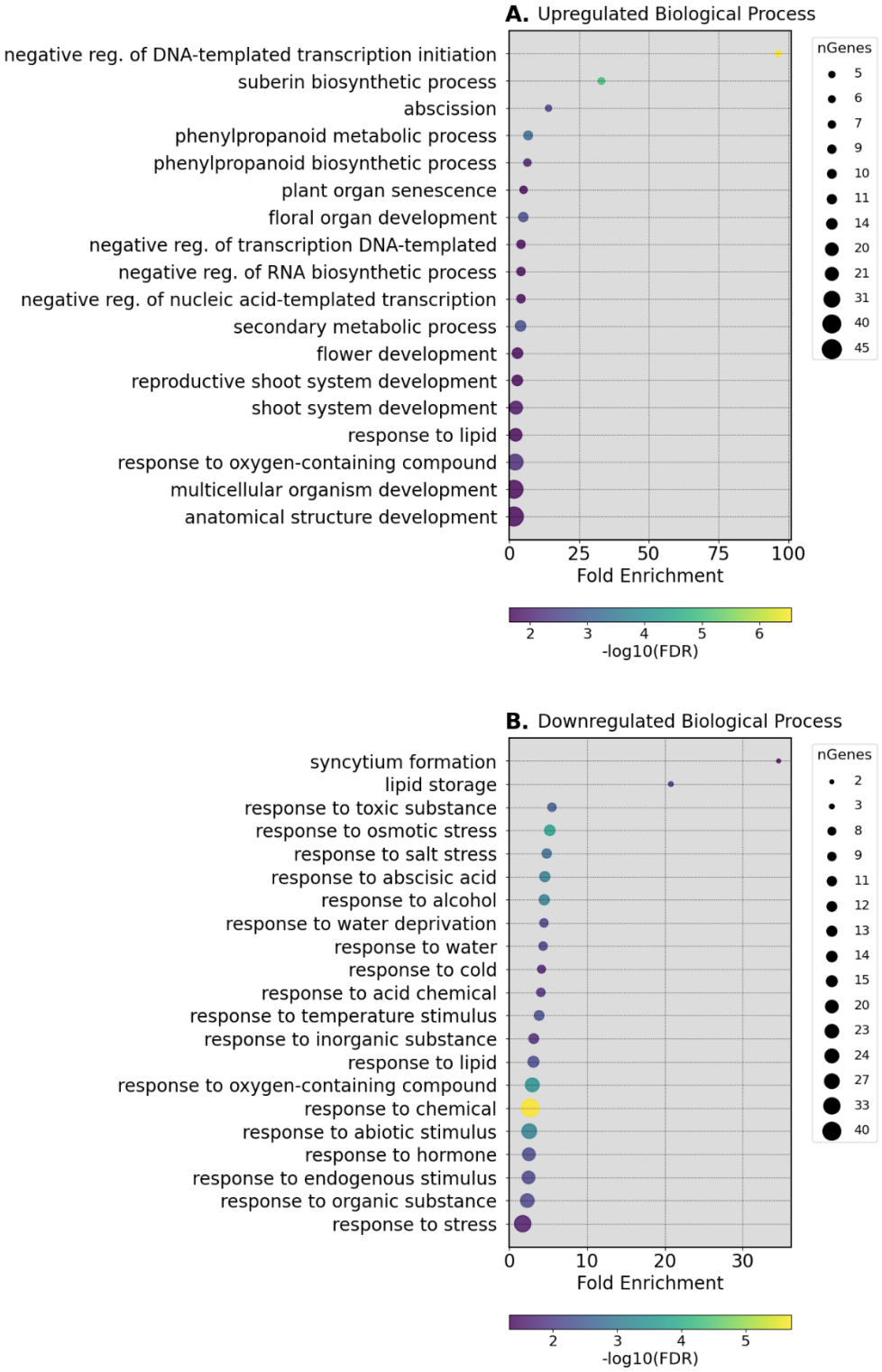

Figure 16. Biological Process enrichment from Gene Ontology analysis of overlapping DEGs in *mkkk17-1* and WT Col-0.

Biological Process enrichment of overlapping DEGs between *mkkk17-1* and WT Col-0 after ABA Treatment. (A) Pathways enriched among 249 upregulated genes, (B) Pathways enriched among 139

downregulated genes. Dot size indicates gene count, and colour intensity represents statistical significance ($-\log_{10}$ FDR). Pathways are ranked by fold enrichment.

The upregulated overlapping genes, that were significantly enriched in metabolic and regulatory pathways were associated with stress adaptation (Figure 15A, Figure 16A). Key biological processes that were enriched in this gene set included the "suberin biosynthetic process," "negative regulation of RNA biosynthetic process," and "secondary metabolic processes." Specific genes such as *RD20, FAR4, FAR5, CYP86B1, NCED3*, and *DIN2* showed significant expression and are known to play important roles in ABA-related stress responses. Notably, suberin biosynthesis was particularly enriched, with genes including *FAR4, FAR5, ABCG6, GPAT5, CYP86B1*, and *RWP1* playing key roles in ABA signalling and stress tolerance. Additionally, genes associated with negative regulation of RNA biosynthesis and secondary metabolic processes, including *AITR3, AITR4, AITR5, MYB41, DIN2, ABCG6, GPAT5*, and *CYP86B1*, were also differentially expressed (Table 6). These findings suggest that *mkkk17-1* may influence RNA metabolic regulation as part of the ABA-mediated stress response.

Table 6. Selected GO Terms from 249 upregulated overlapping genes of *mkkk17-1*

Category	**GO Terms**	**Genes**
KEGG Pathway	Cutin suberine and wax biosynthesis	*RD20, FAR4, FAR5, AT5G08250, CYP86B1, WP1*
KEGG Pathway	Carotenoid biosynthesis	*CYP707A2, NCED3, CYP707A4*
KEGG Pathway	Biosynthesis of various plant secondary metabolites	*NAS4, DIN2, BGLU7, BGLU24*
Biological Process	suberin biosynthetic process	*FAR4, FAR5, ABCG6, GPAT5, CYP86B1, RWP1*
Biological Process	negative regulation of RNA biosynthetic process	*AT3G07255, ROXY2, RAV2, AT3G27250, AT3G48510, AITR3, AITR4, AITR5, MYB41*
Biological Process	secondary metabolic process	*AT2G44130, FAR4, FAR5, DIN2, SCPL19, BGLU24, LAC5, LAC12, ABCG6, GPAT5, CYP86B1, RWP1, FACT, GSTF12*

In contrast, the downregulated overlapping gene set in *mkkk17-1* showed enrichment in pathways associated with fatty acid elongation and the response to lipid (Figure 15B, Figure 16B). Several genes, including *KCS3, LEA, PYL6, EM1, RAS1, IOS1, LTI65, RAP2.6, PP2C5,* and *AHG1* (Table 7), were significantly involved in these pathways. Many of these genes have previously been linked to ABA signalling, lipid metabolism, and stress adaptation mechanisms. The

downregulation of these genes suggests that *mkkk17-1* may exhibit altered lipid and fatty acid metabolism in response to ABA, potentially affecting stress response mechanisms in this genotype.

Table 7. Selected GO Terms from 139 downregulated overlapping genes of *mkkk17-1.*

Category	GO Terms	Genes
KEGG Pathway	Fatty acid elongation	*KCS3, KCR2, AT5G47330*
Biological Process	response to lipid	*LEA, PYL6, EM1, AT4G39130, RAS1, IOS1, GASA3, GASA2, AT4G25580, LTI65, AT1G09310, RAP2.6, HVA22B, PP2C5, AHG1*

These findings suggest that while *mkkk17-1* shares core ABA-responsive mechanisms with WT Col-0, it also exhibits distinct modulation of metabolic and lipid-related processes. This differential regulation may contribute to the altered stress adaptation observed in *mkkk17-1* under ABA treatment.

4.2.3. Identification of transcription factors regulated by *MAPKKK17* in ABA signalling

To investigate the potential involvement of transcriptional regulators downstream of *MAPKKK17*, Gene Ontology (GO) enrichment analysis was performed on the 1,263 uniquely upregulated genes in the *mkkk17-1* mutant under ABA treatment. The analysis focused on identifying molecular functions associated with transcriptional activity. GO molecular function analysis revealed significant enrichment in categories such as DNA-binding transcription factor activity, sequence-specific DNA binding, and cis-regulatory region binding (Figure 17), highlighting a potential role for *MAPKKK17* in modulating transcription factor activity during ABA responses.

The enriched categories included a diverse set of transcription factors, among them *SCL3, PHE1, AT1G68360, NF-YA3, bZIP44, LRL1, BLH5, TCP10, AIB, bHLH38, AT4G19520, AT4G20970, WRKY53, AT4G29930, HSF4, MYB73, bZIP7, NF-YA10, NIG1, AT5G50915, SCL8, AGL8, AGL42, MYB93, ARR11, WRKY70, NAC6, LFY, WIP3, AT1G12890, bZIP58, NAC010, WOX4, ABF1, DOF1, AT1G77640, HB17, bZIP48, AT2G22200, WRKY23, AT3G11580, SMZ, NGA2, NAC075, RRTF1, WRKY48, MYB78, MP, MYB3, NAP, MYB95, AT2G18490, NAC038, MYB106, NAC046, MYB67, SPL5, NAC058, MYB84, AT5G10970, SPL7, MYB37, MYB34, NAC100, MYB68,* and *NAC105*. The wide representation of transcription factor families such as MYB,

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WRKY, NAC, bZIP, and MADS-box suggests that ABA responses in *mkkk17-1* may involve in potential coordination of multiple transcriptional networks.

Several of these transcription factors, including *LFY, NAP, MYB78, FYF, WRKY70*, and *ABF1*, have previously been implicated in ABA-related signalling pathways. Their presence within the uniquely upregulated gene set supports the hypothesis that *MAPKKK17* influences ABA signalling through modulation of transcriptional regulators. Notably, *ABF1* and *WRKY70* are known components of ABA-responsive gene expression, further suggesting that these may act as key downstream targets of *MAPKKK17*-mediated signalling.

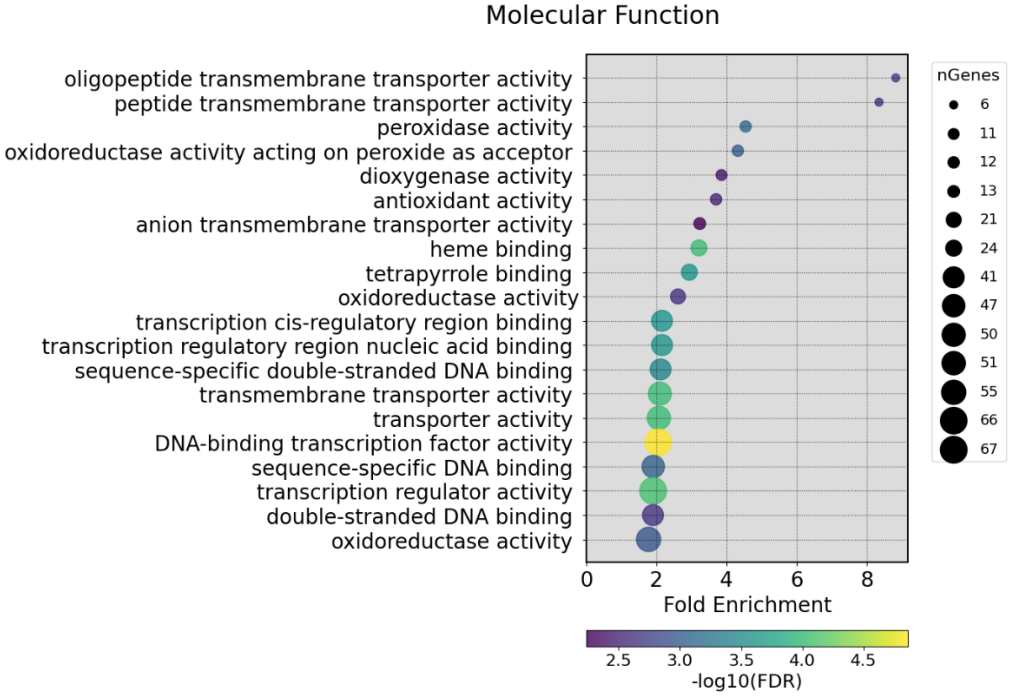

Figure 17. Molecular Function enrichment from Gene Ontology analysis of unique upregulated genes in *mkkk17-1*.

Gene Ontology (GO) enrichment of molecular function terms from 1,263 upregulated genes in *mkkk17-1*. Highlighted terms include transcription factor activity and cis-regulatory DNA binding. Dot plots show fold enrichment, gene counts, and statistical significance ($-\log_{10}$FDR).

These findings collectively suggest that *MAPKKK*17 affects ABA signalling not only at the level of metabolic and stress-related genes but also through transcription factors that potentially regulate broader gene expression programs associated with stress adaptation and hormonal responses.

4.3. Differential gene expression analysis of *mkkk18-1* in ABA-mediated regulation

MAPKKK18 has been reported to be induced by osmotic stress and ABA and act upstream in MAPK cascades to regulate guard cell signalling and stomatal dynamics (Mitula et al., 2015). While earlier studies have implicated *MAPKKK18* in drought tolerance and root development (Li et al., 2017), its genome-wide transcriptional effects have not yet been systematically characterised under ABA treatment. To investigate the role of *MAPKKK18* in ABA-mediated transcriptional regulation, differential expression analysis was performed. A total of 3,816 genes were differentially expressed in the *mkkk18-1* mutant following ABA treatment (Table 3). To better understand genotype-specific transcriptional responses, DEGs from *mkkk18-1* were compared with those from WT Col-0 (Figure 18).

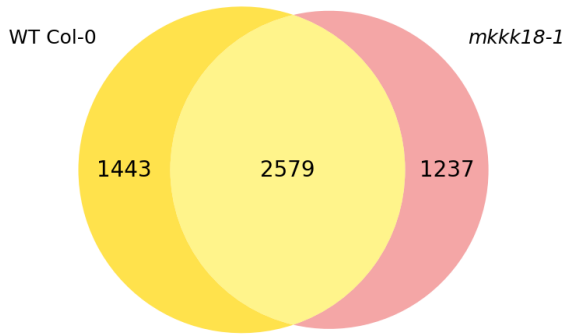

Figure 18. Genotype-specific and shared transcriptional responses to ABA in WT Col-0 and *mkkk18-1*.
The Venn diagram shows unique and overlapping DEGs between WT Col-0 and *mkkk18-1* following ABA treatment. Genes were classified based on significant expression changes ($log_2FC \geq 1$, adjusted $p < 0.05$). The diagram highlights 2,579 *mkkk18-1*-specific genes and 1,237 overlapping DEGs.

We identified 1,237 genes that were uniquely differentially expressed in *mkkk18-1*, not observed in WT Col-0, with 593 genes upregulated and 644 downregulated. Additionally, 2,579 genes were commonly differentially expressed in both genotypes. Within this overlapping gene set, 271 genes were significantly more upregulated in *mkkk18-1* (≥2-fold), while 152 genes were more strongly downregulated (≥2-fold) compared to WT Col-0. These genotype-specific and overlapping DEGs were subjected to functional annotation and enrichment analysis to uncover distinct biological roles.

The following sections examine the unique and overlapping gene sets to uncover ABA-specific signalling pathways modulated by *MAPKKK*18 and compare them with those regulated by *MAPKKK17* and other key ABA signalling components.

4.3.1. Functional characterization of unique DEGs in *mkkk18-1*

To elucidate the biological processes specifically influenced by *MAPKKK18*, GO and KEGG enrichment analyses were performed on the 1,237 DEGs unique to *mkkk18-1*. These genes represent pathways that are either selectively activated or repressed in the mutant and not in WT Col-0 under identical ABA treatment conditions (Figure 19 and Figure 20).

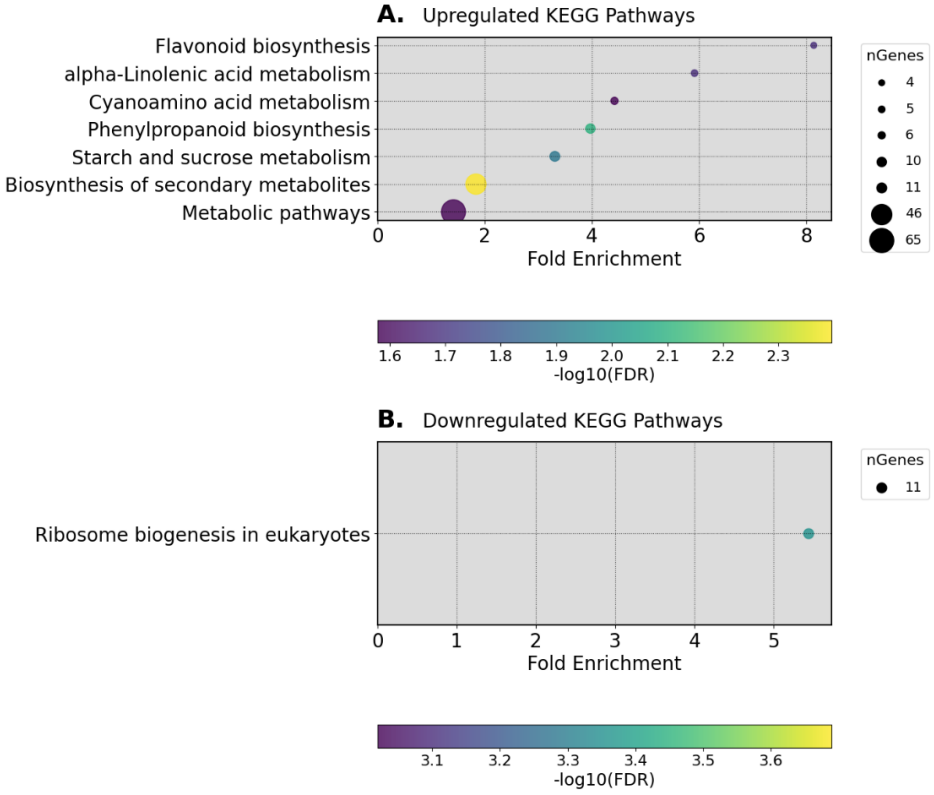

Figure 19. KEGG pathway enrichment from Gene Ontology analysis of unique DEGs in *mkkk18-1*.
KEGG pathway enrichment of uniquely regulated DEGs in *mkkk18-1*. (A) Pathways enriched among 593 upregulated genes. (B) Pathways enriched among 644 downregulated genes. Dot size indicates gene count, and colour intensity represents statistical significance (-$\log_{10}$ FDR). Pathways are ranked by fold enrichment.

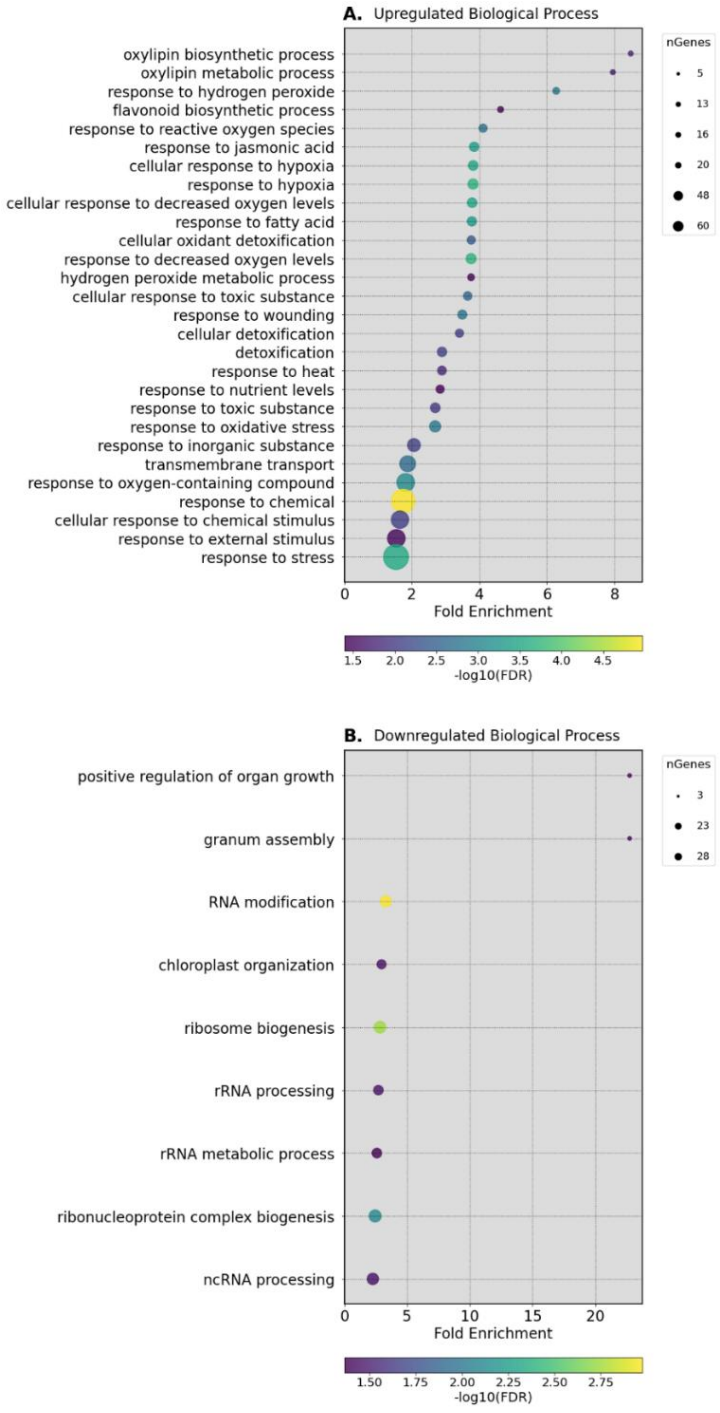

Figure 20. Biological Process enrichment from Gene Ontology analysis of unique DEGs in *mkkk18-1*.

Biological Process enrichment of uniquely regulated DEGs in *mkkk18-1*. (A) Pathways enriched among 593 upregulated genes. (B) Pathways enriched among 644 downregulated genes. Dot size indicates gene count, and colour intensity represents statistical significance ($-\log_{10}$ FDR). Pathways are ranked by fold enrichment.

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Among the 593 uniquely upregulated genes, significant enrichment was observed in several ABA- and stress-associated metabolic pathways, including “flavonoid biosynthesis”, “phenylpropanoid biosynthesis”, “cyanoamino acid metabolism”, “oxylipin biosynthesis”, and “response to jasmonic acid” (Figure 19A). Many of these enriched pathways were linked to ABA signalling mechanisms. Several of these genes, including *LDOX, CCoAOMT1, DFR, FLS3,* and *MYB12*, are well-established participants in secondary metabolism and plant defence. For example, *LDOX* and *CCoAOMT1* are involved in flavonoid biosynthesis, *LOX3* and *LOX4* participated in alpha-“linolenic acid metabolism”, *NIT2* contributed to “cyanoamino acid metabolism”, and *TPS1*, *TPPI* are associated with “starch and sucrose metabolism” within the KEGG pathways (Figure 20A). Top enriched biological processes included oxylipin and flavonoid biosynthesis, as well as responses to jasmonic acid, with notable representation of genes such as *MYB12, LOX3, LOX4, JAZ5, VSP1*, and *WRKY53* (Table 8). These results suggest that loss of *MAPKKK18* may enhance ABA-mediated activation of defence-related secondary metabolic pathways.

Table 8. Selected GO Terms from 593 upregulated unique genes of *mkkk18-1.*

Category	**GO Terms**	**Genes**
KEGG Pathway	Flavonoid biosynthesis	*LDOX, CCoAOMT1, DFR, FLS3*
KEGG Pathway	alpha-Linolenic acid metabolism	*LOX3, LOX4, JMT, DOX1, AT4G19860*
KEGG Pathway	Cyanoamino acid metabolism	*BGLU40, BGLU6, NIT2, NIT3, AT3G47040, BGLU16*
KEGG Pathway	Phenylpropanoid biosynthesis	*AT2G18150, AT2G35380, AT2G38390, AT2G39420, AT2G41480, CCoAOMT1, CAD6, ELI3-2, PRX52, AT5G15180*
KEGG Pathway	Starch and sucrose metabolism	*BGLU11, APS2, BGLU40, BGLU6, TPS1, SBE2.1, SUS4, AT3G47040, BGLU16, ISA3, TPPI*
Biological process	oxylipin biosynthetic process	*LOX3, JMT, LOX4, MES3, DOX1*
Biological process	flavonoid biosynthetic process	*DFR, AT5MAT, MYB12, AT4G14090, LDOX, FLS3, CYP711A1*
Biological process	response to jasmonic acid	*JAZ5, OBP2, LOX3, NATA1, JR1, FAMT, YLS2, AT3G51450, LDOX, VSP1, PROPEP1, JRG21, AT5G05600, ESP, WRKY53, RGLG3, VSP2*

In contrast, GO and KEGG analysis of the 644 uniquely downregulated genes in *mkkk18-1* revealed enrichment for pathways and processes involved in “ribosome biogenesis”, “RNA

metabolism", "RNA modification", "ribonucleoprotein complex biogenesis" and "positive regulation of organ growth" (Figure 19B and Figure 20B).

Key downregulated genes such as *ARL, ARGOS,* and *CYP78A5* have been implicated in growth regulation, while others including *SLO2, AHG11, ABO8,* and *PSRP2*, are known to participate in ABA-related RNA processing and signalling mechanisms (Table 9). These findings imply that *MAPKKK18* may play a role in sustaining essential growth and RNA metabolic functions during ABA responses, and its disruption may lead to growth repression and altered gene regulation at the post-transcriptional level.

Table 9. Selected GO Terms from 644 downregulated unique genes of *mkkk18-1*

Category	**GO Terms**	**Genes**
KEGG Pathway	Ribosome biogenesis in eukaryotes	*AT1G26530, NOP56, AT2G18900, SDN1, AT3G57940, AT4G04940, AT4G22380, AT5G08600, AT5G20160, AT5G22100, AT5G27120*
Biological process	positive regulation of organ growth	*ARL, ARGOS, CYP78A5*
Biological process	RNA modification	*AT1G03510, pde194, OTP87, AT1G77010, SLO2, AT2G27610, AT2G33760, AT2G37320, AT2G40720, AHG11, AT3G03580, AT3G13770, AT3G21470, AT3G47840, AT3G57940, AT4G19191, AT4G40000, IPT9, AT5G27110, AT5G52850, TRM82, AT4G11690/ABO8, DOT4*
Biological process	ribonucleoprotein complex biogenesis	*LIP2, AT1G30960, AT1G31660, AT1G52930, UGE3, AT2G25355, AT3G07750, AT3G12930, RID3, PSRP2, CP33, RBL, AT3G57940, AT4G04940, AT4G15770, AT4G22380, AT4G30330, AT5G20160, AT5G22100, rpl22, AT1G26530, NOP56, AT1G79150, EIF3G2, AT5G08600, AT5G27120, AT1G29250, AT1G18850*

These results demonstrate that *MAPKKK18* modulates a broad range of ABA-responsive gene expression, encompassing metabolic, stress-related, and developmental pathways. This distinct transcriptional landscape sets the stage for further investigation into overlapping and unique targets of other MAPKKKs in ABA signalling.

4.3.2. Functional insights from common DEGs in *mkkk18-1* and WT Col-0

To gain insight into the broader transcriptional programs affected by *MAPKKK*18 during ABA responses, 2579 DEGs overlapped between *mkkk18-1,* and WT Col-0 were examined. These common genes represent a core ABA-responsive transcriptome, with a subset showing genotype-specific expression magnitudes. Analyses were focused on the 271 genes that were more highly upregulated (≥2-fold) and the 152 genes that were more strongly downregulated (≥2-fold) in *mkkk18-1* relative to WT Col-0. GO and KEGG pathway enrichment analyses were performed separately on these gene subsets to determine functional differences in ABA responsiveness between the two genotypes (Figure 21 and Figure 22).

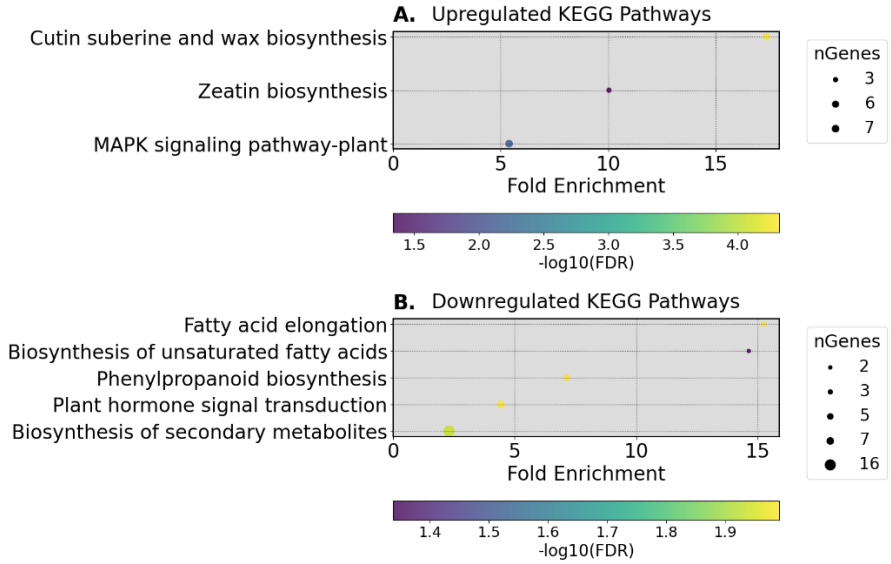

Figure 21. KEGG pathway enrichment from Gene Ontology analysis of overlapping DEGs in *mkkk18-1* and WT Col-0.

KEGG pathway enrichment of overlapping DEGs between *mkkk17-1* and WT Col-0 after ABA Treatment. (A) Pathways enriched among 271 upregulated genes, (B) Pathways enriched among 152 downregulated genes. Dot size indicates gene count, and colour intensity represents statistical significance ($-\log_{10}$ FDR). Pathways are ranked by fold enrichment.

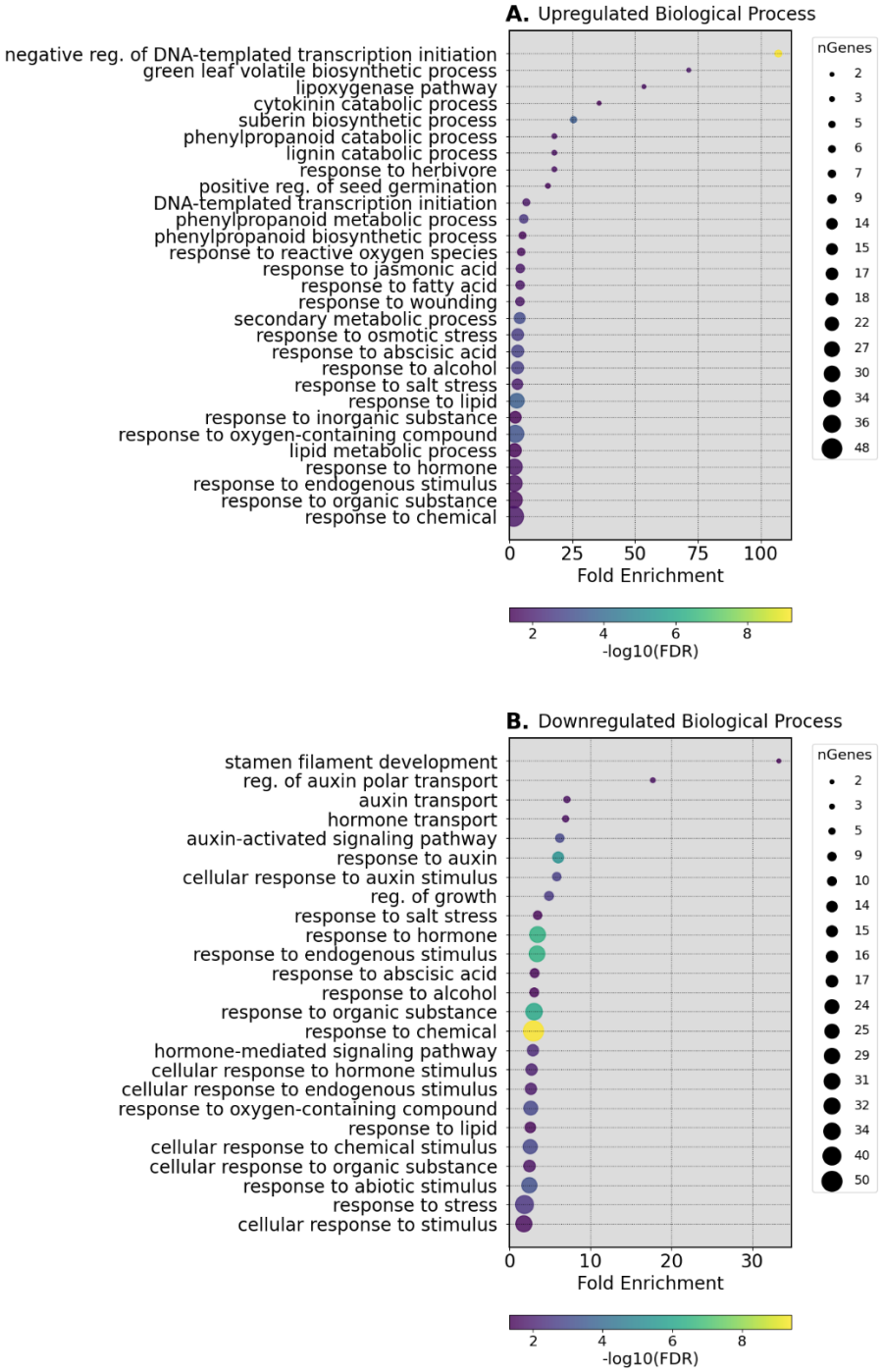

Figure 22. Biological Process enrichment from Gene Ontology analysis of overlapping DEGs in *mkkk18-1* and WT Col-0.

Biological Process enrichment of overlapping DEGs between *mkkk18-1* and WT Col-0 after ABA Treatment. (A) Pathways enriched among 271 upregulated genes, (B) Pathways enriched among 152 downregulated genes. Dot size indicates gene count, and colour intensity represents statistical significance ($-\log_{10}$ FDR). Pathways are ranked by fold enrichment.

Among the 271 genes that were upregulated, significant enrichment was observed for pathways related to “cutin, suberin, and wax biosynthesis”, “phenylpropanoid metabolism”, and “responses to jasmonic acid and wounding” (Figure 21A and Figure 22A). Genes such as *RD20, FAR4, FAR5, RWP1,* and *CYP86B1* were enriched in “cuticle-related biosynthetic processes”, highlighting an enhanced protective response under ABA treatment in *mkkk18-1*. Similarly, genes including *5PTASE11, LOX2, FAR4, FAR5,* and *CYP94B3*, were enriched in the biological processes related to “jasmonate signalling” and mechanical stress responses (Table 10). The overrepresentation of these stress-responsive pathways suggests that *MAPKKK18* may act as a modulator of transcriptional intensity, fine-tuning the amplitude of ABA-induced defence and cuticle associated responses.

Table 10. Selected GO Terms from 271 upregulated overlapping genes of *mkkk18-1*

Category	**GO Terms**	**Genes**
KEGG Pathway	Cutin suberine and wax biosynthesis	*RD20, FAR4, FAR5, AT5G08250, CYP86B1, RWP1*
Biological process	phenylpropanoid metabolic process	*FAR4, FAR5, ELI3-1, LAC5, LAC12, LAC13, ABCG6, CYP86B1, RWP1*
Biological process	response to jasmonic acid	*5PTASE11, MDHAR, LOX2, TPS03, CORI3, UF3GT, SBT4.12, TPS04, PAP1/IAA26*
Biological process	response to wounding	*AT5G43570, TPS04, FAR5, LOX2, CYP94B3, TPS03, MYB102, CORI3, FAR4*

In contrast, the 152 genes more strongly (≥2 fold) downregulated in *mkkk18-1* compared to WT Col-0 were significantly enriched for functions associated with “auxin metabolism”, “secondary metabolite biosynthesis”, and “auxin signalling and transport” (Figure 21B and Figure 22B). Downregulation of genes such as *ACS7, CYP707A3, TYRDC,* and *PORA* indicates potential interference in hormone crosstalk mechanisms. Several auxin-responsive genes, including *SAUR54, SAUR19*, and *IAA19*, were significantly repressed in *mkkk18-1* relative to WT Col-0, pointing to a potential negative interaction between ABA signalling and auxin-mediated growth responses in the absence of *MAPKKK18* function (Table 11). This may reflect a broader hormonal reprogramming, where *MAPKKK18* contributes to balancing growth and stress responses during ABA exposure.

Table 11. Selected GO Terms from 152 downregulated overlapping genes of *mkkk18-1*

Category	GO Terms	Genes
KEGG Pathway	Biosynthesis of secondary metabolites	*GA2OX6, KCS3, KCR2, CCOAMT, ALD1, AT2G29370, AGT3, PRXCA, AT4G17690, ACS7, TYRDC, PAS2, AT5G19890, AT5G39580, CYP707A3, PORA*
Biological process	auxin transport	*AT1G29430, AT3G27025, AT5G65980, RGF6, SAUR19*
Biological process	response to auxin	*AT1G19830/SAUR54, AT1G29420, AT1G29430, AT1G29490, SAUR29, IAA19, AT4G12410, AT4G38825, SAUR15, SAUR19, SAUR20, AT5G65980, AT3G60650, RGF6, BT1*

Collectively, these findings suggest that *MAPKKK18* contributes to ABA-specific signalling while modulating the amplitude and polarity of cross-hormonal transcriptional responses, particularly involving jasmonate and auxin pathways. These observations provide a foundation for further dissecting downstream effectors of *MAPKKK18*, particularly transcription factors and interacting signalling modules.

4.4. Distinct and shared transcriptional roles of *MAPKKK17* and *MAPKKK18* in ABA response

Despite previous evidence linking these two homologous *MAPKKK17* and *MAPKKK18* to ABA signalling and drought responses, the extent to which their transcriptional outputs overlap or diverge remains unclear. To address this, we directly compared the transcriptomic profiles of *mkkk17-1* and *mkkk18-1* mutants to WT Col-0 under ABA treatment. A total of 706 genes were uniquely differentially expressed in *mkkk17-1*, while 680 were unique to *mkkk18-1*. In addition, 557 DEGs were commonly expressed in both mutants but not in WT Col-0 (Figure 23). These results highlight a dual landscape of MAPKKK-mediated gene regulation, involving both unique and overlapping sets of ABA-responsive genes.

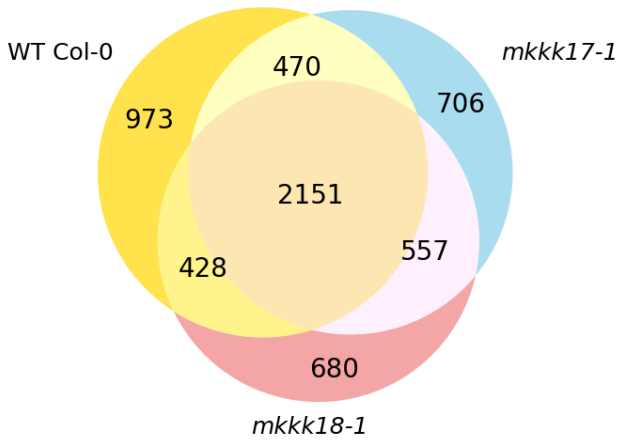

Figure 23. Genotype-specific and common transcriptional responses in WT Col-0, *mkkk17-1*, and *mkkk18-1*.
The Venn diagram shows unique and overlapping DEGs in WT Col-0, *mkkk17-1*, and *mkkk18-1* following ABA treatment. Genes were classified based on significant expression changes ($log_2FC \geq 1$, adjusted $p < 0.05$).

To further dissect the nature of this overlap, Rank-Rank Hypergeometric Overlap (RRHO) analysis was performed (Figure 10). The result suggests that *MAPKKK17* and *MAPKKK18* co-regulate a substantial portion of ABA-responsive transcriptional activity. These data suggest a model in which *MAPKKK17* and *MAPKKK18* function partially redundantly yet also retain distinct roles in mediating specific gene networks within the ABA signalling framework. To further explore this, both unique and overlapping gene expression programs and their biological significance.

4.4.1. Functional analysis of unique DEGs in *mkkk17-1* and *mkkk18-1*

To investigate the unique biological roles of *MAPKKK*17 and *MAPKKK18*, the DEGs specific to each mutant were functionally categorized. In *mkkk17-1*, the GO enrichment analysis revealed a strong enrichment for RNA metabolism-related processes, including “ribosome biogenesis”, “RNA processing”, “RNA modification”, and general “RNA metabolic activity” (Figure 24). These processes were represented by a group of genes such as *PWP2, PMH1, EBP2, RACK1B, GHS40,* and *MORF8*, many of which have known or predicted roles in ABA-mediated responses (Table 12). This strong transcriptional signature suggests that *MAPKKK*17 may regulate ABA responses by modulating RNA maturation and translation.

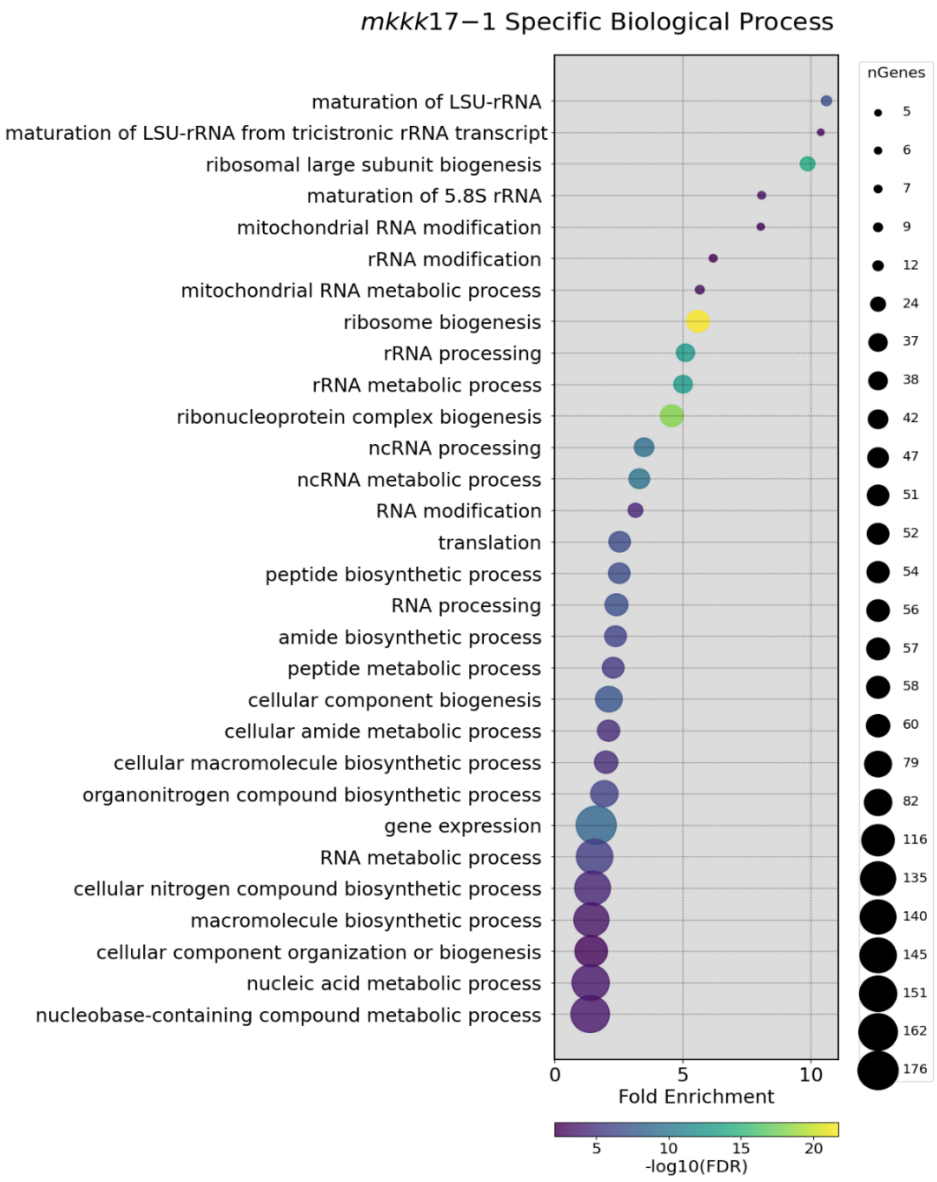

Figure 24. Biological Process enrichment from Gene Ontology analysis of *mkkk17-1* specific DEGs following ABA treatment.

Biological Process enrichment of uniquely regulated 706 DEGs in *mkkk17-1* specific DEGs, not present in WT Col-0 and *mkkk18-1*. Dot size indicates gene count, and colour intensity represents statistical significance (-$\log_{10}$ FDR). Pathways are ranked by fold enrichment.

Table 12. Selected Biological Process from 706 unique DEGs of *mkkk17-1*.

Biological process	**Genes**
ribosome biogenesis	*AT1G01080, RRP4, NAF1, AT1G07070, PWP2, AT1G18540, AT1G23280, AT1G29320, RPL3B, AT1G63810, AT1G74270, AT2G20450, AT2G34357, AT2G40010, AT2G42710, EMB2777, AT2G44120, AT2G44860, SWA1, AT3G03920, AT3G10530, AT3G18600, AT3G21540, PMH1, AT3G22660/EBP2, AT3G23860,*

	EDA7, NAP57, AT3G58660, AT3G62870, AT4G23540, AT4G25730, AT4G28450, AT5G12220, AT5G14520, TOZ, AT5G26180, RPL5B, AT5G48240, FIB1, OLI2, RID2, AT5G61170, AT5G67510, AT1G07615, NRPA2, NUC-L1, AT1G69210, AT3G05060, AT3G12860, AT3G46210, AT5G14050, AT5G15550, AT5G66540, RACK1B, AT5G11240/ GHS40
RNA modification	*AT1G31790, PGN, MORF6, AT3G03920, MORF8/ RIP1, AT3G28660, AT3G47530, NAP57, AT4G04670, MORF1, AT4G25730, AT4G27340, AT5G16860, AT5G26180, AGL26, MORF4, FIB1, OLI2, RID2, AT5G66500, NAF1, AT3G27180, AT1G64310, GR-RBP3*

In contrast, the 680 DEGs unique to *mkkk18-1* were enriched in biological processes linked to growth and developmental regulation, including "positive regulation of organ growth", "cell population proliferation", and "response to hypoxia" (Figure 25). Notable genes in these categories included *ARL, ARGOS, CYP78A5, RGF3,* and *SDD1*, which are associated with cell expansion, proliferation, and stomatal development (Table 13). These results imply that *MAPKKK18* coordinate ABA-dependent developmental response as compared to *MAPKKK17*.

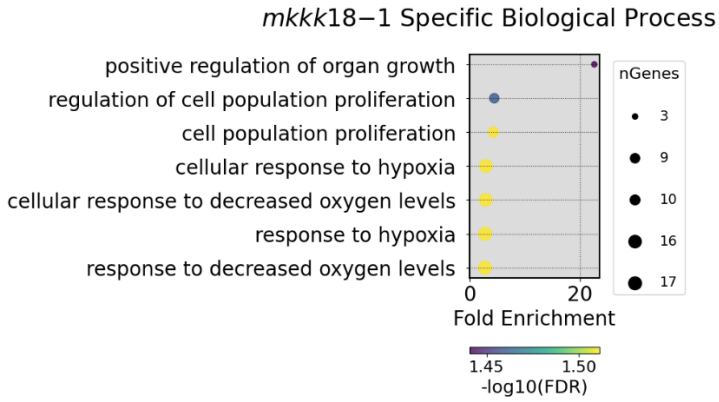

Figure 25. Biological Process enrichment from Gene Ontology analysis of *mkkk18-1* specific DEGs following ABA treatment.
Biological Process enrichment of uniquely regulated 680 DEGs in *mkkk18-1* specific DEGs, not present in WT Col-0 and *mkkk17-1*. Dot size indicates gene count, and colour intensity represents statistical significance (-$\log_{10}$ FDR). Pathways are ranked by fold enrichment.

Table 13. Selected Biological Process from 680 unique DEGs of *mkkk18-1*.

Biological Process	**Genes**
positive regulation of organ growth	*ARL, ARGOS, CYP78A5*
regulation of cell population proliferation	*RGF3, RTFL3, RTFL19, RTFL13, RTFL18, SDD1, CYP78A5, SMP1, ARGOS*

In summary, this analysis suggests that *MAPKKK17* and *MAPKKK18* independently orchestrate distinct transcriptional responses under ABA treatment, reflecting their non-overlapping roles in targeting different layers of stress and growth regulation.

4.4.2. Shared transcription profile suggests redundant regulatory roles for *MAPKKK17/18*

To explore the redundancy between *MAPKKK17* and *MAPKKK18*, GO enrichment analysis was performed on the 557 DEGs overlapping between the two mutants but not present in WT Col-0 following ABA treatment (Figure 23). This analysis sheds light on both common downstream targets and diverging regulatory roles. This analysis revealed enrichment in diverse biological processes, including “transmembrane transport”, “response to fatty acid”, “pigment biosynthesis”, “ribosome biogenesis”, and “jasmonic acid signalling” (Figure 26). These processes involved genes linked to “pigment biosynthesis” (e.g., *LDOX, CHLI2*), “jasmonic acid signalling” (*LOX3, WRKY53*), and “membrane transport” (*NHX6, ABCC4, ZIP1, STP4, SULTR3;1*), reflecting convergence on core stress-responsive pathways (Table 14).

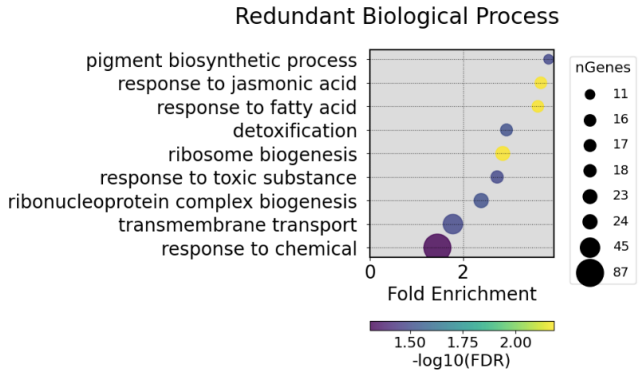

Figure 26. Biological Process enrichment from Gene Ontology analysis of overlapping DEGs between *mkkk17-1* and *mkkk18-1* but not WT Col-0
Biological Process enrichment of overlapping DEGs between *mkkk17-1* and *mkkk18-1*, but not in WT Col-0 after ABA Treatment. Dot size indicates gene count, and colour intensity represents statistical significance ($-\log_{10}$ FDR). Pathways are ranked by fold enrichment.

The functional enrichment among these overlapping DEGs suggests a core subset of ABA-responsive genes that are redundantly regulated by both *MAPKKK17* and *MAPKKK18*. The joint regulation of jasmonate-responsive genes and membrane transporters highlights the potential for these kinases to coordinate adaptive responses that intersect hormone crosstalk and metabolic reprogramming.

Table 14. Selected GO Terms from 557 overlapping DEGs in *mkkk17-1* and *mkkk18-1*

Biological Process	**Genes**
pigment biosynthetic process	*UPM1, DFR, CHLI2, AT5MAT, HEME2, GSA2, AT4G14090, LDOX, FLS3, LIL3:1, CYP711A1*
response to jasmonic acid	*BOP1, AT4G26120, LOX3, NATA1, AT3G15356, JR1, IAA7, YLS2, LDOX, PROPEP1, JRG21, AT5G05600, ESP, WRKY53, RGLG3, VSP2*
response to fatty acid	*BOP1, AT4G26120, LOX3, NATA1, AT3G15356, JR1, IAA7, YLS2, LDOX, PROPEP1, JRG21, AT5G05600, ESP, WRKY53, RGLG3, VSP2*
transmembrane transport	*NHX6, ABCC4, AT3G03700, AT3G09450, ZIP1, AMT1;3, AHA4, AR192, HA3, EMB3144, UMAMIT25, NAT8, UMAMIT19, AT1G33440, UMAMIT36, WAT1, DTX1, AT2G04080, AT2G34350, NIP2;1, AT2G37900, AT2G44280, MHX, MEE67, STP4, ABCA8, GTR1, SULTR3;1, AT3G53940, ACA12, ALMT12, KT2/3, AT4G23030, ABCG4, AT5G01990, UMAMIT9, ZIFL1, ABCI11, YSL2, AT5G38030, UMAMIT42, CNGC4, HP22, AT5G55950, AT1G53110*

These findings support a model in which *MAPKKK17* and *MAPKKK18* act both independently and cooperatively to fine-tune gene expression in response to ABA. *MAPKKK17* mainly influences RNA processing and ribosome biogenesis, while *MAPKKK18* governs developmental and proliferative signals. However, their overlapping regulation of key stress-responsive pathways highlights a layered MAPKKK interact strategy that integrates specificity with robustness in hormone signalling.

4.5. Transcriptional crosstalk between ABA and Auxin via *ABI1* and *MAPKKK18*

Hormonal crosstalk between ABA and auxin is a well-established mechanism that coordinates plant growth and stress responses. While ABA primarily mediates adaptive responses to abiotic stress, auxin governs developmental processes such as cell division, elongation, and differentiation. Under stress conditions, ABA often acts antagonistically to auxin, repressing growth to prioritize survival. Despite the recognition of this antagonism, the transcriptional mechanisms and signalling components mediating this interplay remain incompletely understood. To explore the ABA-auxin interface at the transcriptomic level, we focused on two key signalling regulators: the clade A protein phosphatase *ABI1*, a core negative regulator of ABA signalling, and *MAPKKK18*, a stress-responsive kinase implicated in both ABA and developmental pathways. Using RNA-seq data from ABA-treated *abi1td* and *mkkk18-1* mutants, we examined changes in

the expression of auxin-related genes. In earlier analyses (Section 5.3.2), the enrichment of auxin-related biological processes among downregulated genes in the *mkkk18-1* mutant suggested a possible regulatory link between *MAPKKK18* and auxin signalling. Given the known antagonistic relationship between ABA and auxin, particularly during stress adaptation, we hypothesized that *ABI1* might similarly participate in repressing auxin-responsive gene networks under ABA treatment.

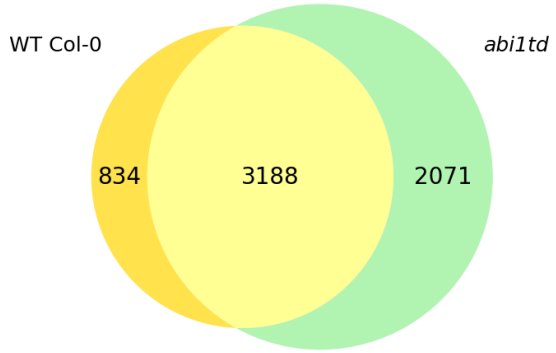

Figure 27. Genotype-specific and common transcriptional responses in WT Col-0 and *abi1td*.
The Venn diagram shows unique and overlapping DEGs in WT Col-0 and abi1dt following ABA treatment. Genes were classified based on significant expression changes ($log_2FC \geq 1$, adjusted $p < 0.05$).

To test it further, results of transcriptional profiles from RNA-seq analysis on *abi1td* and WT Col-0 seedlings treated with ABA were compared. As mentioned earlier in the section 5.1.3, a total of 5259 DEGs were identified in *abi1td*, while 4022 DEGs were detected in WT Col-0. Of these, 3188 DEGs overlapped between the two genotypes (Figure 27), representing a core set of ABA-responsive genes. Within this overlapped gene set, 260 genes were more highly upregulated (≥2-fold) in *abi1td*, and 164 genes were more strongly downregulated (≥2-fold) in *abi1td* than in WT Col-0. These 164 downregulated genes were selected for further functional characterization to determine their involvement in auxin-related processes.

Gene Ontology (GO) enrichment analysis of this downregulated subset revealed a significant overrepresentation of auxin-related biological processes, including “response to auxin,” “cellular response to auxin stimulus,” and “auxin-activated signalling pathway” (Figure 28). The genes contributing to these enrichments included several Small Auxin Up RNA (SAUR) family members, such as *SAUR19, SAUR22, SAUR61, SAUR63, and SAUR29*, as well as known auxin-responsive transcription factors *IAA19*, and *IAA29*, and the auxin transporter gene *PIN5*. *IAA19* and *IAA29* are particularly noteworthy because of their previously reported roles in ABA-related

responses and leaf senescence, suggesting a possible functional convergence between ABA and auxin regulatory modules.

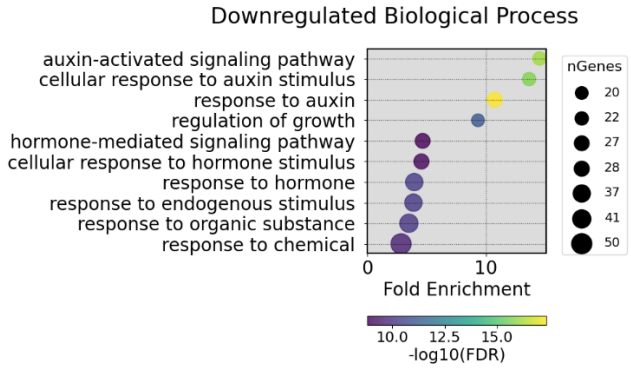

Figure 28. Biological Process enrichment from Gene Ontology analysis of overlapping DEGs in a*bi1td* and WT Col-0. Biological Process enrichment of 164 downregulated genes (≥2-fold) in *abi1td* compared to WT Col-0 following ABA treatment. Dot size indicates gene count, and colour intensity represents statistical significance (-$\log_{10}$ FDR). Pathways are ranked by fold enrichment.

This pattern suggests that *ABI1* may help redirect transcriptional programs away from growth-promoting pathways like auxin signalling and toward stress-responsive gene expression, reinforcing its role in adaptive stress responses. When considered alongside the GO term enrichments observed in *mkkk18-1* (Section 5.3.2), these findings suggest that both *ABI1* and *MAPKKK18* suppress auxin-related gene expression as part of the ABA response.

4.6. Role of ubiquitin-proteasome system components in ABA-dependent transcription and development

Given the post-translational regulation of *MAPKKK*18 by the ubiquitin-proteasome system (UPS) (Mitula; Tajdel-Zielińska et al.), the extensive RNA-seq analysis presents an opportunity to investigate whether UPS-related genes show altered transcriptional profiles, particularly in *mkkk17* or *mkkk18* mutants in response to ABA treatment.

We systematically screened ABA-responsive DEGs annotated as UPS components, focusing on E3 ubiquitin ligases due to their substrate specificity in ubiquitination cascades. Across all genotypes, numerous genes encoding proteins with predicted ubiquitin–protein transferase activity were differentially expressed following ABA exposure, including members of the UBC (E2 conjugating enzyme) family, RING- and HECT-type E3 ligases, and several plant U-box (PUB) proteins (Table 15). While WT Col-0 plants displayed a broad but largely non-significant transcriptional response of these genes to ABA, the *abi1td*, *mkkk17-1*, and *mkkk18-1* backgrounds

showed more extensive and statistically significant changes, suggesting an enhanced transcriptional engagement of the UPS under perturbed ABA signalling.

Table 15. List of Differentially expressed genes with ubiquitin–protein transferase activity

Genotype	ABA-responsive ubiquitin-protein transferase genes
WT Col-0	*AT3G19950, UBC19, AT3G24760, AT3G30460, UBC13, RDUF1, AT3G47550, AT3G48070, AT3G54360, AT3G55070, AT3G60080, AT4G00305, RHA1A, RHF1A, AT4G19670, AIRP1, RIN2, UBC9, AT4G31450, MBR2, AT4G37880, LUL1, AT5G09630, AT5G10650, AT5G15820, RBX1, RHF2A, PEX4, UBC4, AT5G41350, AT5G41400, UBC18, BRG1, UBC33, UBC27, UBC30, ATCRT1, UBC7, RDUF2, AT5G62460, UBC3, AT5G62800, PUB18, SKP2A, PUB45, AT1G61620, DAL1, AT1G78420, AT2G24330, UBA1, DRIP2, RHC1A, RFI2, RGLG1, AT3G05545, PUB9, PUB25, AT3G29270, AT3G56580, ATL4, AT4G22820, AT4G28370, AT4G28890, XBAT33, RGLG2, AT5G42200, AT5G49665, XBAT32, AT5G66070, PUB54, PUB19, PUB43, AT3G10815, AT4G23860, AT5G09800, AT5G65920, AT5G67340, RHA3B, AT3G19910*
abi1td	*AT1G10650, AT1G13195, AT1G14200, UBC1, RHA2A, UBC36, UBC34, AT1G18660, AT1G24440, AT1G26800, UPL3, NHL8, AT1G32740, AT1G33480, ATUBC2-1, SBP1, AT1G47570, AT1G53025, AT1G53190, HUB2, UPL1, AT1G60610, UBC5, AT1G63840, UBC28, Hrd1B, ARI8, AT1G71980, AT1G74770, AT1G75340, UBC16, UBC35, BRG2, AT2G01275, AT2G02960, VSR4, VSR3, MBR1, UBC29, UBC23, RKP, AT2G22690, AT2G23780, VSR2, ARI7, AT2G37150, RHC2A, AT2G42030, BRIZ1, AT2G44410, UBC6, AT3G02290, AT3G02340, CHIP, UBC11, LOG2, SAP5, AT3G13430, FUS9, AT3G15070, UBC25, Hrd1A, UBC32, UPL6, BTS, AT3G19950, UBC19, AT3G24760, PRT1, AT3G45630, UBC13, RDUF1, AT3G47160, AT3G47550, AT3G48070, VSR1, UPL7, LUL2, AT3G54360, AT3G55070, AT3G58030, SINAT2, AT3G58720, AT3G60080, AT3G61790, AT3G62240, AT4G00305, PIT1, ORTHL, RHA1A, UPL5, RHF1A, AT4G17410, AT4G19670, RING, VSR7, AIRP1, RIN2, RMA3, AT4G27880, UBC9, AT4G31450, MBR2, ARI1, AT4G37880, KAK, AT5G01960, PRT6, UPL4, LUL1, UBC22, AT5G09630, AT5G10650, AT5G15710, AT5G15820, RBX1, RHF2A, AT5G24870, PEX4, AT5G38070, AT5G38895, UBC4, AT5G41350, AT5G42940, UBC18, BRG1, AT5G47050, AT5G47430, UBC33, UBC27, UBC10, UBC30, ATCRT1, UBC7, AT5G60170, AT5G60820, AT5G62460, UBC3, AT5G62800, CUL4, PUB18, SKP2A, PUB45, PUB17, AT1G61620, DAL1, AT1G78420, TED3, WAVH1, PUB4, AT2G24330, PEX10, UBA1, DRIP2, RHC1A, RFI2, RGLG1, AT3G05545, AT3G06330, PUB9, PUB24, XBAT35, AT3G29270, PUB13, SIS3, PUB14, SDIR1, AT3G56580, ATL4, AT4G22820, AT4G28370, AT4G28890, AT4G31080, XBAT33, RGLG2, AT5G18340, AT5G42200, AT5G49665, XBAT32, RGLG3, AT5G66070, PUB54, AT1G24330, SPL2, PUB19, CMPG1, AT1G72175, PUB43, AT2G45920, APC11, AT3G10815, AT3G49060, AT4G23860, SAUR21, AT5G05230, AT5G57035, AT5G65920, AT5G67340, NLA, RHA3B, AT2G20650, AT3G19910, VFB2, SKIP2, CER9*

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mkkk17-1	*AT1G13195, UBC1, UBC36, UBC34, AT1G17970, AT1G18660, AT1G24440, AT1G26800, NHL8, AT1G32740, AT1G33480, SBP1, AT1G53025, UPL1, AT1G60610, AT1G63840, Hrd1B, ARI8, AT1G71980, AT1G73760, UBC16, UBC35, AT2G01275, AT2G02960, VSR4, VSR3, MBR1, RKP, AT2G22690, AT2G23780, VSR2, ARI7, AT2G37150, RHC2A, AT2G44410, UBC6, AT3G02290, CHIP, UBC11, LOG2, SAP5, Hrd1A, UBC32, UPL6, BTS, AT3G19950, AT3G24760, PRT1, UBC13, RDUF1, AT3G47550, AT3G48070, VSR1, UPL7, LUL2, AT3G54360, AT3G55070, AT3G60080, AT4G00305, PIT1, RHA1A, AT4G17410, AT4G19670, RING, AIRP1, RIN2, AT4G31450, MBR2, ARI1, AT4G35070, AT4G37880, AT5G01960, PRT6, UPL4, LUL1, UBC22, AT5G10650, AT5G15710, RBX1, RHF2A, AT5G24870, PEX4, AT5G38070, AT5G38895, UBC4, AT5G41350, AT5G42940, UBC18, BRG1, AT5G47430, UBC33, UBC27, UBC10, UBC30, ATCRT1, UBC7, RDUF2, AT5G62460, UBC3, AT5G62800, TIR1, PUB18, SKP2A, PUB45, AT1G61620, DAL1, AT1G78420, WAVH1, PEX10, UBA1, DRIP2, RHC1A, RFI2, RGLG1, AT3G05545, AT3G06330, PUB9, PUB25, XBAT35, AT3G29270, SIS3, SDIR1, AT3G56580, AT4G22820, AT4G28370, XBAT33, RGLG2, AT5G42200, AT5G49665, XBAT32, RGLG3, AT5G66070, AT1G24330, PUB19, PUB43, AT3G10815, AT5G05230, AT5G11620, AT5G67340, NLA, RHA3B, AT3G19910, VFB2, SKIP2***
mkkk18-1	*AT1G13195, UBC1, RHA2A, UBC36, UBC34, NHL8, AT1G32740, AT1G33480, ATUBC2-1, SBP1, UPL1, AT1G60610, AT1G63840, UBC28, Hrd1B, ARI8, UPL2, AT1G73760, AT1G75340, BRG2, AT2G01275, AT2G02960, VSR4, VSR3, MBR1, UBC29, AT2G22690, VSR2, ARI7, AT2G37150, UBC6, AT3G02290, UBC11, LOG2, UBC32, AT3G19950, AT3G24760, AT3G30460, UBC13, RDUF1, AT3G47180, AT3G47550, AT3G48070, VSR1, AT3G54360, AT3G55070, AT3G58720, AT3G60080, AT4G00305, ORTHL, RHF1A, AT4G19670, VSR7, AIRP1, RIN2, UBC9, AT4G31450, MBR2, UPL4, AT5G10650, RHF2A, AT5G24870, PEX4, AT5G38895, UBC4, AT5G41350, UBC18, BRG1, UBC33, UBC10, UBC30, ATCRT1, AT5G62460, UBC3, AT5G62800, PUB18, SKP2A, PUB45, AT1G61620, AT1G78420, RGLG4, WAVH1, UBA1, RHC1A, RFI2, RGLG1, PUB9, XBAT35, AT3G29270, AT3G56580, AT4G22820, AT4G28370, AT4G28890, XBAT33, RGLG2, AT5G42200, AT5G49665, XBAT32, RGLG3, AT1G24330, PUB19, AT1G60360, PUB43, AT2G45920, AT3G10815, AT5G05230, AT5G11620, AT5G57035, AT5G67340, RHA3B, AT3G19910, VFB2*

We identified several DEGs show ubiquitin–protein transferase activity and UPS related transcripts (see Table 15) that may be involved in the regulation of the ABA signalling pathway, including potential regulators of *MAPKKK*17/18 protein turnover.

In the context of ABA signalling, the UPS not only regulates the stability of *MAPKKK17*/18 but also modulates other core components such as *ABI1* and SnRK2 kinases. E3 ligases like *KEG*, *RGLG1*/2, and UPLs have been shown to fine-tune ABA sensitivity by targeting these proteins for

degradation, thereby ensuring dynamic adjustment of signalling strength (Yu et al., 2021). Such regulation illustrates the multilayered control observed in MAPK cascades, reinforcing the role of the UPS as an integral component of ABA-responsive networks

Furthermore, we focused on the UPL (Ubiquitin Protein Ligase) family of E3 ligases, as previous studies have shown that *UPL1* and *UPL4* participate in the degradation of *MAPKKK18* (Tajdel-Zielińska et al., 2024).

MAPKKK18 is known to play a role in stress responses and developmental events such as stomatal formation. Recent proteomic studies have further illuminated its post-translational regulation. Mass spectrometry analysis of *MAPKKK18* immunoprecipitates revealed several UPLs as potential interacting partners. Notably, both HECT-type ligases (*UPL1* and *UPL4*) and the RING-type ligase KEG were identified as regulators of *MAPKKK18* stability through ubiquitin-mediated proteasomal degradation (Tajdel-Zielińska et al., 2024). Therefore, it was explored whether these or related UPL ligases also contribute to ABA-dependent transcriptional regulation and developmental processes.

Previous studies showed that UPL3 is involved in trichome morphology and is necessary to repress excess branching and endoreplication of Arabidopsis trichomes (Downes et al., 2003). Among UPL family members, *UPL4* and *UPL6* are structurally related to *UPL3* and share features such as Armadillo repeat motifs and a HECT domain, suggesting roles in substrate recognition and ubiquitin transfer. While *UPL3* has been implicated in developmental regulation, the specific roles of *UPL4* and *UPL6* in ABA signalling and stomatal development remain poorly understood.

To gain insight into the transcriptional and physiological roles of *UPL4* and *UPL6* in ABA responses, we analysed gene expression profiles, root growth phenotypes, and stomatal patterning in *upl4* and *upl6* T-DNA insertion mutants.

4.6.1. Expression of *UPL4* and *UPL6* is ABA-responsive and genotype-dependent

To investigate the role of post-translational regulation in ABA signalling, we explored the function of two HECT-type E3 ligases, *UPL4* and *UPL6*, which are hypothesized to modulate MAPK pathway components via targeted protein degradation.

To determine whether UPLs are transcriptionally regulated by ABA , transcript abundance of seven UPL genes between ABA-treated and mock-treated samples across WT Col-0 and three ABA-

pathway mutants (*abi1td*, *mkkk17-1*, and *mkkk18-1*) were compared (Table 16). In WT Col-0, all UPL genes showed fold changes close to 1 and no statistically significant differences (adjusted $p > 0.65$), indicating that under our treatment conditions, ABA does not substantially alter UPL expression in the WT Col-0.

In contrast, multiple UPL genes exhibited significant induction in the mutants, particularly in *abi1td* and *mkkk17-1*. Among them, *UPL4* and *UPL6* displayed consistent and robust upregulation across *abi1td* (fold change 1.60 and 1.43 respectively) and *mkkk17-1* (1.37 and 1.28 respectively). *UPL4* also remained significantly induced in *mkkk18-1* (1.30), whereas *UPL6* showed a smaller, non-significant change (1.18) in this genotype.

Other UPLs, including *UPL1, UPL3,* and *UPL5*, were significantly upregulated in specific mutants, but their induction was less consistent across all three genotypes. For example, *UPL1* was strongly induced in *abi1td* and *mkkk18-1* but showed a smaller effect in *mkkk17-1*; *UPL5* was significantly induced only in *abi1td*. *UPL2* and *UPL7* displayed modest fold changes, with statistical significance limited to one or two mutants.

Taken together, the expression profiles highlight *UPL4* and *UPL6* as the most consistently and significantly ABA-responsive UPL ligases in ABA-pathway mutant backgrounds, suggesting a possible common role in the transcriptional response associated with altered ABA signalling.

Table 16. Differential expression of UPL Ligases in response to ABA treatment

Ligase	Genotype	Comparison	Fold change	adj p.value
***UPL*1**	WT Col-0	ABA vs Mock	0.97	0.938
	abi1td	ABA vs Mock	1.75	4.14E-06
	mkkk17-1	ABA vs Mock	1.36	0.000381
	mkkk18-1	ABA vs Mock	1.64	0.000624
***UPL*2**	WT Col-0	ABA vs Mock	0.93	0.821
	abi1td	ABA vs Mock	1.45	0.064
	mkkk17-1	ABA vs Mock	1.11	0.323
	mkkk18-1	ABA vs Mock	1.38	0.011
***UPL*3**	WT Col-0	ABA vs Mock	0.94	0.831
	abi1td	ABA vs Mock	1.38	0.000741
	mkkk17-1	ABA vs Mock	1.16	0.051
	mkkk18-1	ABA vs Mock	1.21	0.059
***UPL*4**	WT Col-0	ABA vs Mock	0.95	0.864
	abi1td	ABA vs Mock	1.60	1.87E-12
	mkkk17-1	ABA vs Mock	1.37	2.51E-08
	mkkk18-1	ABA vs Mock	1.30	0.043
***UPL*5**	WT Col-0	ABA vs Mock	0.92	0.678

	abi1td	ABA vs Mock	1.36	6.74E-09
	mkkk17-1	ABA vs Mock	1.14	0.248
	mkkk18-1	ABA vs Mock	0.90	0.603
UPL6	WT Col-0	ABA vs Mock	1.10	0.655
	abi1td	ABA vs Mock	1.43	7.84E-12
	mkkk17-1	ABA vs Mock	1.28	2.73E-07
	mkkk18-1	ABA vs Mock	1.18	0.294
UPL7	WT Col-0	ABA vs Mock	0.89	0.575
	abi1td	ABA vs Mock	1.33	5.91E-06
	mkkk17-1	ABA vs Mock	1.20	0.045
	mkkk18-1	ABA vs Mock	1.25	0.059

In addition to the ABA treatment analysis, we examined publicly available transcriptomic data (https://bar.utoronto.ca/eplant/) to further characterize the expression patterns of *UPL4* and *UPL6* across different tissues. *UPL4* was observed to be expressed in diverse tissues, with higher expression levels observed in senescent leaves and root tissues. *UPL6* showed moderate expression across several tissues, with particularly higher levels in seedlings, adult leaves, flowers, and roots. These findings indicate that both *UPL4* and *UPL6* are expressed in various tissues, suggesting they may play a role in regulating developmental processes. The fact that their expression is also responsive to ABA treatment in certain genotypes further supports the idea that they may participate in ABA-mediated stress responses and developmental regulation, potentially interacting with *MAPKKK18/17* signalling.

Overall, the expression patterns of *UPL4* and *UPL6* in response to ABA treatment suggest that they may be involved in fine-tuning ABA signalling, particularly in the context of developmental processes and stress responses. Their regulation in mutants with altered ABA signalling suggests that *UPL4* and *UPL6* may act upstream of or in conjunction with *MAPKKK18/17* to regulate ABA-mediated responses in plants.

4.6.2. Generation and molecular characterization of UPL lines

Potential involvement of *UPL4* and *UPL6* in ABA-mediated signalling and their regulatory interaction with upstream signalling components were analysed using knockout and overexpression lines for both *UPL4* and *UPL6*. The generation of overexpression lines for *UPL4* and *UPL6* is described in the methods section 3.17. Plants were cultivated under specific growth

conditions, and tissue samples were collected according to the protocols outlined in Sections 3.4 and 3.5.

To examine the transcript expression levels of *UPL4* and *UPL6*, primers spanning the intron-exon junctions of the T-DNA insertion lines (*upl4*.1, *upl4*.2, and *upl6*) were designed, as shown in Figure 29A and Figure 29B. RT-qPCR analysis confirmed the disruption of gene expression in these knockout lines, as evidenced by the absence of 18S rRNA transcripts (Figure 29C and Figure 29D). In addition, UPL overexpression lines (35S:UPL4/upl4.1, 35S:UPL4/WT-4, 35S:UPL6/*upl6-1* and 35S:UPL6/WT-3) were selected for further experiments to assess their physiological responses and roles in ABA-related processes.

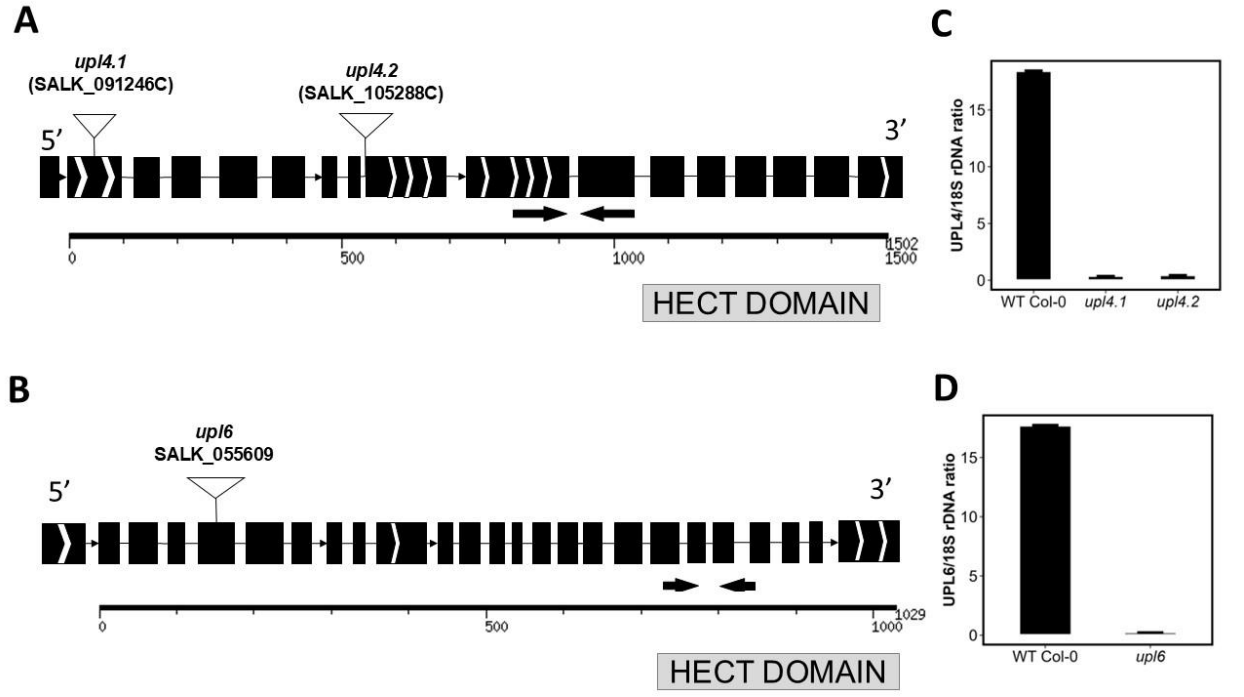

Figure 29. T-DNA insertion sites and transcript expression analysis of *UPL4* and *UPL6*
(A) Schematic representation of the UPL4 gene indicating T-DNA insertion sites in *upl4*.1 and *upl4*.2 lines.
(B) Schematic of the *UPL6* gene showing the T-DNA insertion site in the *upl6* line. Triangles represent T-DNA insertions; closed boxes denote exons; connecting lines indicate introns. Arrows mark the locations of primers used for qPCR validation.
(C) qPCR analysis of *UPL4* transcript levels in homozygous knockout lines (*upl4*.1, *upl4*.2), showing near-complete loss of expression relative to the 18S rRNA internal control.
(D) qPCR analysis of *UPL6* transcript levels in the *upl6* knockout line, confirming absence of expression.

These validated genetic lines provided the necessary tools for subsequent functional analyses of *UPL*4 and *UPL*6 in ABA-regulated physiological and developmental processes, including root growth inhibition and stomatal patterning.

4.6.3. *UPL4* and *UPL6* influence primary root elongation under ABA treatment

Root elongation is a well-established phenotypic output of ABA signalling, where exogenous ABA treatment leads to significant suppression of primary root growth (Qin et al., 2023). Previous studies have established that *MAPKKK18*-overexpressing lines show ABA-related phenotypes at the level of germination and root growth. *MAPKKK18* modulates ABA-induced inhibition of root elongation by controlling the length of the elongation zone, thereby affecting overall root growth. To evaluate whether *UPL4* and *UPL6* also contribute to this phenotype, we analysed the root elongation phenotypes of knockout and overexpression lines under ABA stress conditions.

Seeds were grown under standard conditions as described in Methods 3.1 to 3.3, and ABA treatment and mock treatments were applied as outlined in Section 3.5. Primary root growth assays were performed following the protocol in Section 3.18, and root lengths were measured using ImageJ. Root length measurements were analysed using two-way ANOVA (genotype and treatment) to test overall effects. For pairwise comparisons, Student's t-test was applied.

Under control (mock) conditions, no significant differences in primary root elongation were observed among WT Col-0, knockout lines (*upl4*.1, *upl4*.2, *upl6*), and overexpression lines (35S:UPL4/upl4.1, 35S:UPL4/WT-4, 35S:UPL6/upl6.1, 35S:UPL6/WT-3). This is consistent with the basal expression profiles of *UPL4* and UPL6, which showed minimal changes in the absence of ABA (Section 5.6.1).

Upon ABA treatment, root growth was inhibited across all genotypes, supporting the expected ABA-induced suppression of root elongation. However, seedlings overexpressing *UPL4* (35S:UPL4/*upl4.1* and 35S:UPL4/WT-4) showed a significant increase in primary root elongation relative to ABA-treated WT Col-0 ($n = 50$; $P < 0.0001$) (Figure 30A). In contrast, *upl4*.1 and *upl4*.2 knockout lines exhibited significantly reduced primary root growth compared to WT Col-0 under the same ABA conditions ($n = 50$; $P < 0.0001$). Together, these results indicate that *UPL4* functions as a positive regulator of ABA-dependent primary root elongation.

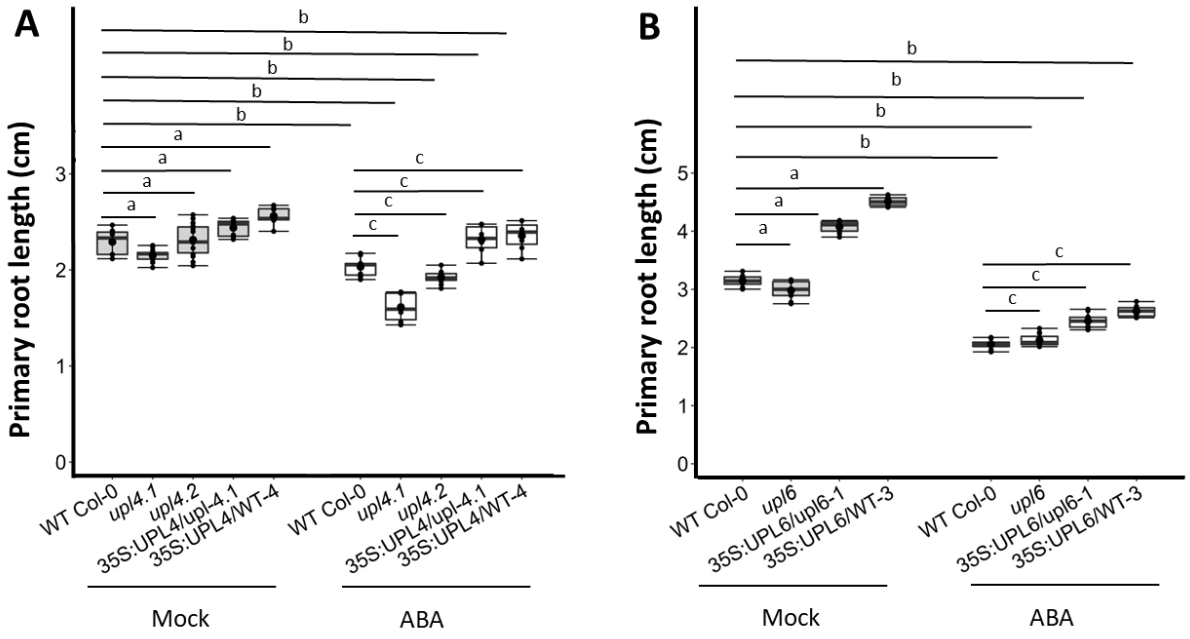

Figure 30. Primary root lengths of *UPL4, UPL6* mutants and overexpression lines under mock and ABA treatment.
(A) Root growth phenotypes of *UPL4* knockout (*upl4*.1, *upl4*.2) and overexpression lines (35S:UPL4/upl4.1, 35S:UPL4/WT-4) compared to WT Col-0.
(B) Root growth phenotypes of UPL6 knockout (*upl6*) and overexpression lines (35S:UPL6/upl6.1, 35S:UPL6/WT-3) compared to Col-0.
Measurements were taken 7 days after treatment with 5 µM ABA and measured using ImageJ. Data represent mean ± standard deviation (SD) (n = 50). Statistical significance between genotypes and treatments was determined using Student's t-test ($p < 0.0005$). Statistical analysis was performed using two-way ANOVA (genotype and treatment) and denoted as b. For pairwise comparisons, Student's t-test ($p < 0.0005$) was applied to determine significance between genotypes (denoted as a) and treatments (denoted as c)

In the case of *UPL6*, overexpression lines (35S:UPL6/*upl6.1* and 35S:UPL6/WT-3) show longer roots compared to WT Col-0 under mock conditions. Under ABA treatment, all tested genotypes exhibit reduced primary root length ($n = 50$; $P < 0.0001$). However, root length of ABA-treated UPL6 overexpressing lines was longer that the WT Col-0, grown in the same conditions. These findings indicate that, *UPL6* is very likely involved in regulation of ABA-dependent root growth.

These findings were consistent across three independent experimental replicates, further supporting the conclusion that both *UPL*4 and *UPL6* contribute to ABA-induced changes in

primary root elongation. Notably, *UPL*4 appears to play a more prominent role in modulating plant sensitivity to ABA, highlighting its potential significance in ABA signalling pathways.

4.6.4. *UPL4* is involved in stomatal development under Abscisic Acid

Stomatal development and function are critical for plant adaptation to environmental stress and are tightly regulated by hormonal signals, notably ABA (Tanaka et al., 2013). ABA serves as a key modulator of both stomatal aperture and formation, particularly under abiotic stress conditions such as drought. It regulates guard cell differentiation and restricts excessive stomatal proliferation through a multilayered signalling cascade that involves MAP kinase modules and transcriptional regulators.

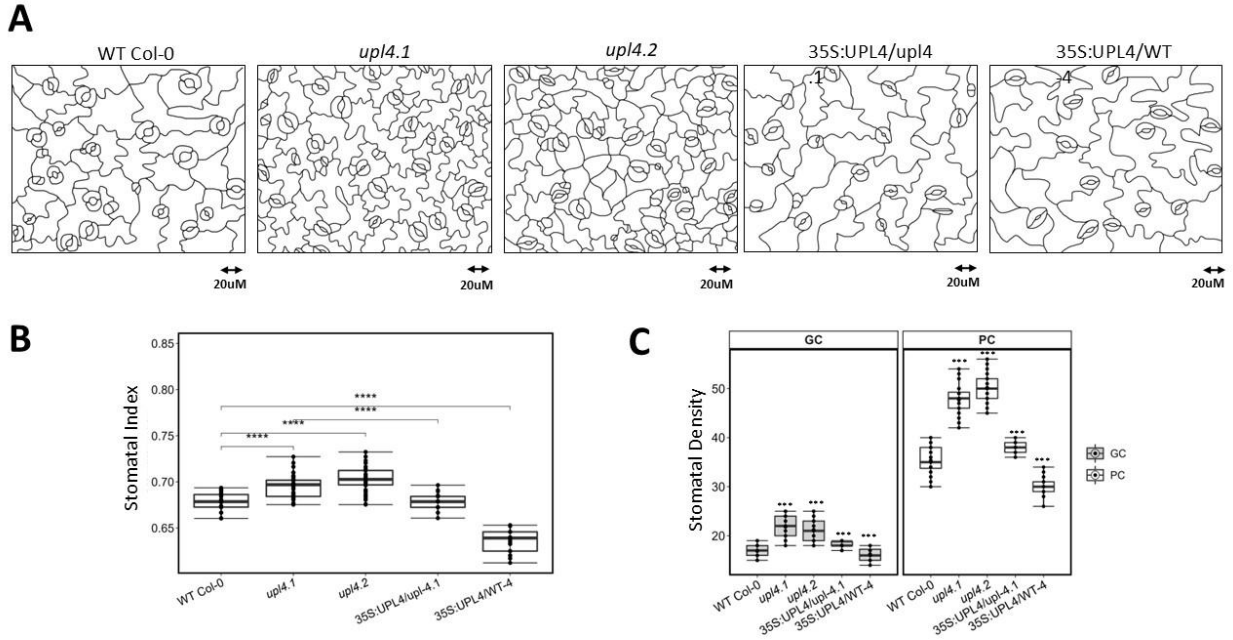

Figure 31. *UPL4* modulates stomatal development in Arabidopsis cotyledons.
(A) Representative schema of the abaxial epidermal surface of 6-day-old leaves illustrating stomatal patterning.
(B) Stomatal development is enhanced in *upl4* knockout lines relative to WT Col-0. Quantification was performed using 100-150 stomata per genotype across three independent biological replicates.
(C) Quantitative analysis of guard cells (GCs) and pavement cells (PCs) per 250 µm segment in WT Col-0, *upl4* knockout, and *UPL4* overexpression lines. Data are presented as mean ± standard deviation (n = 200) from three independent experiments. Statistical significance was assessed using Student's *t*-test (*p < 0.05; **p < 0.005; ***p < 0.0005).

Previous studies have identified *MAPKKK18* as a component of the ABA-responsive MAPKKK18–MKK3–MPK1/2/7/14 cascade, influencing ABA-driven stomatal dynamics via activation of downstream MAPK modules (Zhao et al., 2023). In Section 5.6.2, we demonstrated the involvement of *UPL4* and *UPL6* in ABA-mediated root growth. In addition, *UPL*4 regulates stability of *MAPKKK*18 (Tajdel-Zielinska et al., 2023). These observations raised the possibility that *UPL4* may also extend its regulatory function beyond root development and participate in additional ABA-regulated processes, such as stomatal patterning.

To investigate the potential role of *UPL4* in stomatal development, stomatal density, and patterning in *upl4* knockout and *UPL4* overexpression lines were quantified. Plants were grown under standardized conditions described in Methods Sections 3.4 and 3.5. Fully expanded cotyledons were harvested from six-day-old seedlings, and epidermal impressions were generated as outlined in Section 3.19. Epidermal cells were visualized using contrast-enhanced microscopy on a ZEISS imaging system, and digital images were acquired from at least ten independent leaves per genotype. Guard cells (GCs) and pavement cells (PCs) were manually counted, and the stomatal index (SI) defined as the proportion of stomata among total epidermal cells (Tanaka et al., 2013) was calculated. All SI measurements were derived from 10 biological replicates per genotype, each with three technical replicates.

Compared to WT Col-0, both *upl4.1* and *upl4.2* mutants exhibited a significant increase in stomatal index (SI) ($p < 0.0001$), indicating enhanced stomatal formation. In contrast, *UPL4* overexpression lines displayed significantly lower SI values: *35S:UPL4/upl4.1* ($p < 0.001$) and *35S:UPL4/WT-4* ($p < 0.0001$) both showed reduced stomatal densities relative to WT Col-0 (Figure 31). These findings suggest that *UPL*4 negatively regulates stomatal formation, potentially by restricting the entry of epidermal cells into the stomatal lineage or by modulating ABA-responsive transcriptional regulators of stomatal development.

To determine whether these SI differences were driven by altered cell proliferation or by changes in stomatal lineage commitment, absolute numbers of GCs and PCs were also evaluated. In *UPL4* overexpression lines, GC and PC counts were not significantly different from WT Col-0, indicating that reduced SI was not due to decreased total cell numbers. Conversely, both GC and PC counts were significantly increased in *upl4* knockout lines, suggesting that loss of *UPL*4 enhances epidermal cell division or increases the frequency of stomatal lineage specification.

These findings suggest that *UPL4* plays a role in regulating stomatal development, contributing to control of stomatal patterning. Given the established role of *MAPKKK18* in ABA-mediated stomatal function and its connection to root elongation phenotypes described previously, it is likely that *UPL4* and *MAPKKK18* operate within intersecting or parallel signalling modules to fine-tune ABA responses across different tissues. This expands the functional relevance of *UPL4* as a regulatory node in the broader ABA signalling network governing both developmental and stress-responsive processes.

4.6.5. *UPL6* impacts stomatal development due to Abscisic Acid

Previous analyses established a functional role for *UPL6* in regulating primary root growth under ABA treatment (Section 5.6.2), where UPL6 overexpression lines show a complex phenotype and can retain slightly longer roots under ABA, indicating balanced UPL6 activity is required for normal ABA response. Moreover, transcriptome data presented in Section 5.6.1 revealed that *UPL6* is transcriptionally upregulated in response to ABA in a manner dependent on upstream signalling components such as *ABI1* and *MAPKKK17*. These findings support a role for *UPL6* as a component of the ABA signalling pathway, influencing developmental and stress-responsive processes.

To determine whether *UPL6* also contributes to ABA-mediated development regulation in aerial tissues, we investigated its potential involvement in stomatal development. Stomatal density and epidermal patterning were analysed in the *upl6* knockout line and two independent overexpression lines (*35S:UPL6/WT-3* and *35S:UPL6/WT-4*). Plants were grown under the controlled conditions as described in Methods Sections 3.4 and 3.5. Fully expanded cotyledons and true leaves were collected from 6-day- and 10-day-old seedlings, and stomatal traits were assessed using the protocol detailed in Section 3.19, with high-resolution imaging used to quantify guard cells (GCs) and pavement cells (PCs). The stomatal index (SI) was calculated as mentioned in section 5.6.4, to determine genotype-dependent differences in stomatal development (Figure 32).

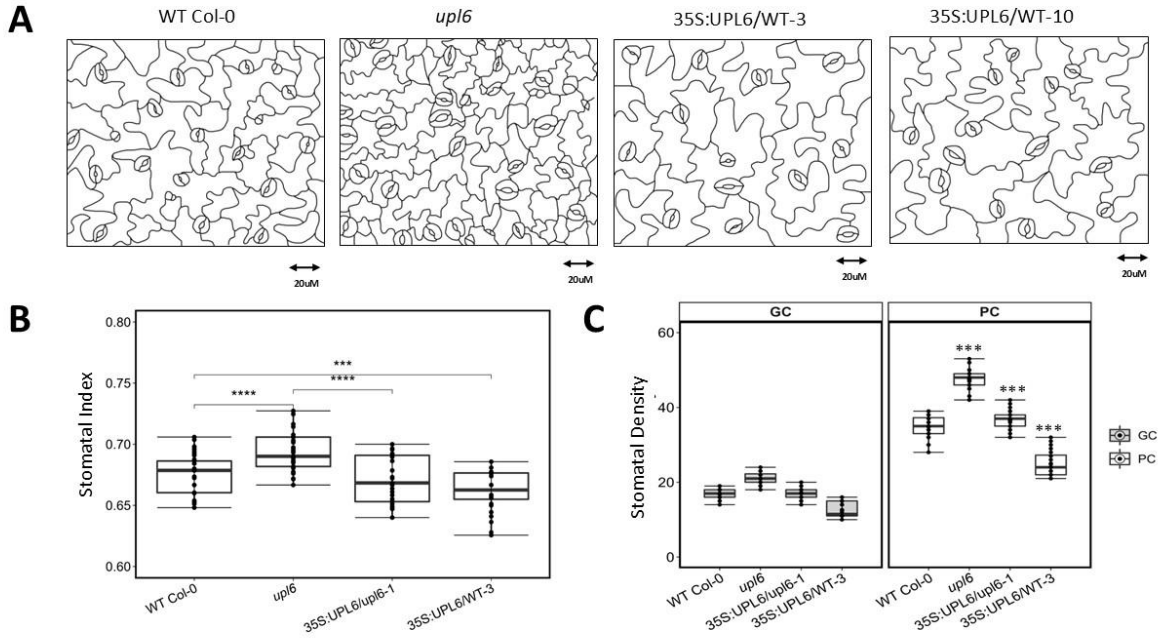

Figure 32. Stomatal development and epidermal cell analysis in *UPL6* Lines
(A) Representative schematic illustration showing the abaxial leaf epidermis on day six.
(B) Stomatal development is enhanced in *upl6* knockout lines compared to WT Col-0. Data were collected from 100-150 stomata per genotype across three independent biological replicates.
(C) Quantification of guard cells (GCs) and pavement cells (PCs) per 250 µm epidermal field in WT Col-0, *upl6* knockout, and *UPL6* overexpression (35S:UPL6) lines. Data represent mean ± SD (n = 200) from three independent experiments. Statistical significance was determined using Student's t-test ($p < 0.05$, $p < 0.005$, $p < 0.0005$).

In comparison to WT Col-0, the *upl6* mutant slightly increased in SI ($p < 0.0001$), indicating enhanced stomatal production. In contrast, both *UPL6* overexpression lines exhibited a significant decrease in SI ($p < 0.0001$), reflecting a suppression of stomatal development. These effects were observed consistently across both developmental stages analysed, reinforcing the conclusion that *UPL6* acts to restrict stomatal formation.

To investigate whether changes in SI developed from altered cell proliferation or shifts in stomatal lineage specification, absolute counts of GCs and PCs were performed. In the *upl6* knockout line, both guard cell and pavement cell numbers were significantly elevated compared to WT, suggesting increased epidermal cell division and/or a bias toward stomatal lineage fate in the absence of *UPL6* function. In contrast, overexpression lines showed significant reductions in both

GC and PC counts, supporting the notion that elevated *UPL6* levels suppress epidermal cell proliferation or inhibit the recruitment of precursor cells into the stomatal lineage.

These results demonstrate that *UPL6* functions as a negative regulator of stomatal development by modulating epidermal cell proliferation and differentiation under ABA influence. Together with its ABA-inducible expression and inhibitory effects on root elongation, *UPL6* emerges as a key component of the ABA signalling network, coordinating developmental responses across multiple tissues.

Collectively, these findings establish a multi-layered regulatory network where *MAPKKK17/18, ABI1,* and *UPL4/6* co-ordinately shape ABA-induced transcriptional responses, impacting both stress adaptation and developmental processes. These regulators integrate upstream hormonal signals with downstream gene expression and developmental reprogramming.

5. Discussion

The aim of this study was to elucidate how *MAPKKK17*, *MAPKKK18*, *ABI1*, and *UPL4*/6 contribute to ABA-mediated transcriptional regulation and developmental responses. Through transcriptomic profiling and phenotypic analyses, *MAPKKK17* and *MAPKKK18* were found to differentially influence ABA-responsive gene expression, with *MAPKKK17* predominantly may modulate RNA metabolism and *MAPKKK18* may regulate organ development (Table 12, Table 13). *ABI1* emerged as a central negative regulator, whose loss led to widespread transcriptional reprogramming, while *UPL4* and *UPL6* were shown to fine-tune ABA-dependent root elongation and stomatal development through proteolytic control. Together, these findings reveal a layered regulatory framework in which kinase cascades, phosphatases, and ubiquitin ligases converge to balance stress adaptation with growth.

5.1. Unique *MAPKKK*17-regulated genes reveal possible roles in stress adaptation

The transcriptional profiling of unique genes in the *mkkk17-1* mutant under ABA treatment reveals a distinctive expression landscape that highlights the role of *MAPKKK*17 in mediating plant responses to abiotic stress. Loss of *MAPKKK*17 showed upregulation of genes involved in secondary metabolism, ion transport, and hormone signalling. Conversely, genes associated with ribosome biogenesis and cellular maintenance were repressed, suggesting a dual role of *MAPKKK*17 in modulating both stress activation and homeostatic balance during environmental challenges. Among the upregulated genes, those associated with the phenylpropanoid and flavonoid biosynthesis pathways, such as *4CL2*, *MYB3*, *WRKY70*, *WRKY54*, *MYB34*, and *LOX3*, are involved in key processes related to secondary metabolism, stomatal regulation, and ABA signalling (Chun et al., 2019; Kim et al., 2022; Besseau, et al., 2013; Ding et al., 2016; Frerigmann & Gigolashvili, 2014; Kim et al., 2013; Yu et al., 2021). The elevated expression of *4CL2*, previously shown to be induced in salt-adapted root cells, suggests that its role in lignin and flavonoid biosynthesis may contribute to cell wall fortification under salt stress conditions (Chun et al., 2019). Similarly, *MYB3* functions as a transcriptional repressor regulating anthocyanin and lignin accumulation, processes often associated with salt stress adaptation (Kim et al., 2022). The coordinated upregulation of transcription factors *WRKY70* and *WRKY54*, which are known to have

negative regulators of stomatal closure and osmotic stress tolerance, is particularly intriguing (Besseau, et al., 2013). Their elevated expression may reflect a compensatory mechanism in *mkkk17-1* to fine-tune stomatal responses and water loss. Additionally, the loss of the R2R3-MYB transcription factor *MYB73* results in the hyper-induction of salt overly sensitive genes *SOS1* and *SOS3* under high salinity conditions, indicating a disruption of ion homeostasis mechanisms (Frerigmann & Gigolashvili, 2014; Kim et al., 2013). These findings suggest that *MAPKKK*17 indirectly influences ionic stress signalling through transcriptional regulation of ion transport pathways. Additionally, the increased expression of *LOX3*, a gene involved in lipid-derived signalling and ABA responses, is consistent with its known role in salt stress tolerance. *LOX3* expression is reduced in ABA receptor mutants but elevated in receptor overexpression lines, and *lox3* mutants are hypersensitive to salt stress (Ding et al., 2016; Yu et al., 2021), emphasizing its critical function in hormone signalling and stress adaptation.

Several other genes that are upregulated in the *mkkk17-1* mutant reinforce the centrality of ABA homeostasis and signalling. *BGLU18* (also known as AtBG1), *DOX1*, and *NPC5* are involved in the hydrolysis of ABA-glucose conjugates, modulation of ABA signalling, and root development under salt stress, respectively (Jiao et al., 2020). Notably, *AtBG1*-dependent regulation of ABA levels has been associated to improved drought tolerance and controlled stomatal development (Allen et al., 2019). The upregulation of *DOX1*, an oxylipin biosynthetic enzyme that influences ABA homeostasis (Vicente et al., 2012), supports the view that *MAPKKK*17 may regulate key enzymes involved in hormonal balance under stress. *NPC5*, known to influence lateral root development in response to salt (Peters et al., 2014), further ties developmental plasticity to environmental adaptation in the mutant. *TPPI*, which enhances drought tolerance by regulating stomatal aperture (Lin et al., 2020), and *FLS2*, a receptor-like kinase involved in MAP kinase signalling for innate immunity, may link stress response pathways (Orosa et al., 2018).

In addition to signalling and metabolic genes, a group of transport-related genes were found to be upregulated, pointing toward enhanced ionic and osmotic regulation. For instance, *ZIP1*, a zinc transporter repressed by ABA-responsive transcription factors such as *ABI4*, may modulate stomatal regulation under stress (Meng et al., 2022). Similarly, *ALMT9* and *ALMT12*, both involved in stomatal movement via anion transport, likely contribute to improved stomatal dynamics under ABA conditions (De Angeli et al., 2013; Jaślan et al., 2023). *ZIFL1*, essential for efficient response to both dark- and ABA-induced stomatal closure (Remy et al., 2013), and *CCX2*,

a calcium exchanger that contributes to salt and osmotic tolerance, indicates a broader remodeling of ionic flux mechanisms in *mkkk17-1* (Corso et al., 2018). The upregulation of *KEA5*, which helps maintain pH homeostasis in endosomal compartments (Zhu et al., 2018), and *CLC*a, a chloride channel involved in stomatal regulation (Yang et al., 2023), further supports this interpretation. In support of this, the *sultr3;1* mutant show hypersensitivity to exogenous ABA, underlining the importance of sulfate transporters in maintaining ABA-mediated stress tolerance (Cao et al., 2014). Finally, *CNGC4*, a cyclic nucleotide-gated channel, helps raise cytosolic Ca^{2+} in response to environmental cues like high humidity and is associated with stress acclimation (Hussain et al., 2024). Together, these findings suggest that the loss of *MAPKKK*17 leads to the induction of genes that collectively improve resilience through altered transport, signalling, and metabolic pathways. In contrast, several genes involved in ribosome biogenesis and translational control were significantly downregulated in the *mkkk17-1* mutant, indicating a suppression of growth-related processes under ABA treatment. Notably, *PWP2*, a WD40-repeat protein upregulated by sucrose and involved in stomatal signalling, was repressed (Bates et al., 2012). This may signify a reduction in cellular signalling efficiency as part of a broader metabolic reprioritization under stress. *TLP6* interacts with *ASK11*, which likely functions as an SCF-type E3 ligase complex (Bao et al., 2014) , suggesting that *TLP6* may participate in targeted protein degradation pathways relevant to ABA signalling. *GHS40*, a negative modulator of ABA degradation and signalling under high glucose conditions, were also downregulated (Hsiao et al., 2016). This repression may reflect a shift in hormonal balance favouring stress response overgrowth.

Additional downregulated genes such as *ABO6*, *EBP2*, and *RACK1B* suggest a dampening of mitochondrial, ribosomal, and ABA co-regulatory processes (He et al., 2012; Jeon et al., 2015; Guo & Sun, 2017). ABA overly sensitive 6 (*ABO6*), a mitochondrial RNA helicase, is associated with ABA sensitivity and improved drought tolerance when mutated, and its reduced expression may imply disruption in mitochondrial-nuclear communication under stress (He et al., 2012). EBNA1 binding protein 2 (*EBP2*), a nucleolar protein involved in ribosome biogenesis and senescence regulation (Jeon et al., 2015), and *AtRH9*, a DEAD-box RNA helicase implicated in seed germination and freezing tolerance, both contribute to maintaining cellular vitality under stress (Kim et al., 2008). The downregulation of Receptor for Activated C Kinase 1B (*RACK1B*), a protein that interacts with ABA-related transcription factors and is stabilized by sumoylation

during stress (Guo & Sun, 2017), further indicates potential attenuation of signalling crosstalk involving *MAPKKK*17.

Taken together, the unique gene expression patterns in *mkkk17-1* reveal a complex regulatory network in which *MAPKKK17* acts as a modulator balancing growth and stress adaptation via ABA pathway. Its loss results in the activation of stress-protective genes, many of which are tightly linked to ABA signalling and stomatal regulation, while concurrently suppressing genes involved in growth, protein biosynthesis, and cell stability. These findings suggest that *MAPKKK17* may function as a checkpoint kinase, restraining excessive activation of stress programs under basal conditions while allowing a flexible response under ABA or salt stress. The interplay between metabolic shifts, hormonal signalling, and transport regulation observed in *mkkk17-1* offers valuable insights into the mechanisms by which plants optimize survival under adverse environmental conditions.

5.2. Functional insights of overlapping genes in *MAPKKK17* and WT Col-0

To determine the functional significance of the shared transcriptomic responses to ABA in *mkkk17-1* and WT Col-0, we investigated DEGs common to both genotypes following ABA treatment. These overlapping genes, comprising 249 upregulated and 139 downregulated DEGs in *mkkk17-1*, reflect a conserved ABA-responsive core transcriptome. Their differential modulation offers insights into how *mkkk17-1* alters stress-responsive and developmental signalling pathways, despite retaining substantial ABA responsiveness.

The upregulated shared DEGs in *mkkk17-1* were significantly enriched in pathways related to suberin biosynthesis, secondary metabolism, and transcriptional regulation, suggesting an enhanced or restructured ABA-mediated protective program.

Among the prominent genes*, RD20/CLO3* (caleosin), which is highly inducible by ABA in roots, negatively regulates lateral root development under ABA stress (Brunetti et al., 2023). Its sustained induction suggests a retained mechanism for developmental modulation under osmotic stress. Similarly, *FAR4* and *FAR5*, key contributors to suberin monomer synthesis, were also upregulated. These genes are known to be responsive to salt stress and are instrumental in reinforcing the root's protective barrier (Choi et al., 2023)

Further substantiating suberin regulation, *WRKY9*, a transcription factor implicated in the activation of *CYP94B3* and *CYP86B1*, was part of this transcriptional landscape (Krishnamurthy

et al., 2021). *CYP86B1*, in particular, plays a crucial role in the biosynthesis of aliphatic suberin, and increases salt tolerance linking ABA signalling to structural reinforcement of root tissues.

The presence of *AtNCED3*, a pivotal ABA biosynthetic gene that modulates endogenous ABA levels under drought, also underscores the core role of hormonal feedback in drought stress adaptation (Iuchi et al., 2001). Upregulation of *DIN2/BGLU30*, typically induced under various abiotic stresses including salinity, drought stress and dehydration, further points to a coordinated stress-responsive gene expression program (Seok et al., 2020).

Notably, suberin-related genes such as *FAR4, FAR5, ABCG6, GPAT5*, and *RWP1*, all involved in suberin monomer transport and polymerization, were also upregulated. Mutants deficient in *RWP1* exhibit increased permeability and salt sensitivity, reinforcing the idea that suberin-associated modifications are essential for ABA-mediated stress tolerance (Gou et al., 2009). The previously reported downregulation of these genes in *aba1* mutants further emphasizes the tight linkage between ABA signalling and suberin pathway gene expression (Choi et al., 2023)

Beyond structural adaptations, transcriptional regulators involved in RNA biosynthesis repression such as *AITR3, AITR4*, and *AITR5*, were also differentially expressed. These genes, when mutated, confer ABA hyposensitivity and enhanced drought/salt tolerance, suggesting that their induction in *mkkk17-1* may reflect a feedback modulation mechanism to balance ABA responsiveness (Chen et al., 2021; Tian et al., 2017). Additionally, the *MYB41-BRAHMA* regulatory module, implicated in ABA-mediated chromatin remodelling and drought tolerance, further supports the presence of a sophisticated ABA-governed transcriptional network (Gao et al., 2024).

In contrast, the downregulated DEGs were significantly enriched for processes related to fatty acid elongation and lipid response pathways, suggesting a selective attenuation of ABA-responsive lipid metabolism in *mkkk17-1*. 3-Ketoacyl-CoA synthase 3 (*KCS3*), a key enzyme in the elongation of very long chain fatty acids (VLCFAs), was among the downregulated genes. Mutants in *KCS3* exhibit hypersensitivity to drought, highlighting the importance of cuticular lipid biosynthesis in water retention and abiotic stress tolerance (Huang et al., 2023).

Several canonical ABA signalling genes, including *LEA, PYL6, EM1*, and *IOS1*, were also suppressed. LEA proteins are essential in early seed germination under salt (Aleman et al., 2016), while *PYL6* functions as a high-affinity ABA receptor required for effective hormonal signalling and growth regulation (Aleman et al., 2016). *EM1* is transcriptionally regulated by *ABI5*, which binds to ABA-responsive elements (ABREs) in target promoters during stress (Carles et al., 2002).

The downregulation of these genes may indicate altered ABA perception or signal transduction in *mkkk17-1*. Similarly, *IOS1,* a negative modulator of ABA sensitivity, was also downregulated. Previous study indicates that loss-of-function *ios1* mutants exhibit hypersensitivity to ABA and enhanced ABA-responsive gene expression (Giordano et al., 2022). This observation, along with the downregulation of *RAS1* (a repressor of salt tolerance), *LTI65* (an ABRE-regulated dehydration-responsive gene), and *RAP2.6*, suggest that a subset of ABA signalling outputs may be selectively inhibited in *mkkk17-1* (Nakashima et al., 2006). Apart from those post-translational regulators were also affected. *RACK1B*, whose sumoylation enhances interaction with *RAP2*.6 in ABA responses, and *PP2C5*, a phosphatase that modulates ABA-induced MAPK activation, both showed transcriptional reduction (Guo & Sun, 2017; Hann et al., 2024). Moreover, *AHG1*, a clade A PP2C, was also downregulated. Loss-of-function *ahg1* mutants exhibit ABA hypersensitivity during seed germination and heightened susceptibility to osmotic stress (Nishimura et al., 2007). Collectively, these findings reveal that *mkkk17-1* retains core ABA-responsive elements while selectively modulating transcriptional and metabolic responses in response to ABA. Enhanced expression of suberin biosynthesis genes and transcriptional repressors suggest an effort to reinforce physical and molecular barriers. This may represent a compensatory response to the absence of *MAPKKK*17. Meanwhile, the downregulation of key lipid and ABA signalling components highlights a divergence in metabolic fine-tuning and receptor-mediated responses. This complex interplay of conserved and reprogrammed pathways likely underpins the altered physiological phenotypes observed in *mkkk17-1* under ABA treatment.

5.3. Transcription Factors downstream of *MAPKKK*17 in ABA signalling and stress response regulation

To elucidate the transcriptional dynamics downstream of *MAPKKK*17, Gene Ontology (GO) enrichment analysis was performed on the 1,263 genes uniquely upregulated in the *mkkk17-1* mutant under ABA treatment. GO molecular function terms revealed significant enrichment in DNA-binding transcription factor activity, sequence-specific DNA binding, and cis-regulatory region binding (Figure 13). These findings suggest that *MAPKKK*17 is intricately involved in modulating the expression of key transcriptional regulators that shape the ABA-responsive transcriptome under stress conditions. The uniquely upregulated genes encompass a diverse array of TFs, notably from the MYB, WRKY, NAC, bZIP, and MADS-box families. The variety of

transcription factors (TF) families suggest that *MAPKKK*17 modulates ABA responses through a multifaceted regulatory network involving both hierarchical control and combinatorial gene activation.

Notably, transcription factors (TFs) such as *LFY* (LEAFY), *NAP*, *MYB78*, *FYF* (FOREVER YOUNG FLOWER), and *ABF1* emerged as key regulators with known roles in ABA signalling and stress responses, supporting the notion that *MAPKKK17* impacts both developmental and abiotic stress-related processes via transcriptional control mechanisms. The transcription factor *LFY* (LEAFY), a master regulator of floral meristem identity, exhibits ABA-responsive expression changes mediated by ABI4: LFY transcription is elevated in *abi4* mutants but suppressed in *ABI4*-overexpressing lines (Shu et al., 2016). The upregulation of *LFY* in *mkkk17-1* suggests a possible regulatory connection between *MAPKKK17* and ABI4-mediated repression, potentially impacting flowering or floral transition during stress.

NAP (NAC-Like, activated by AP3/PI) is a well-characterised transcription factor that promotes chlorophyll degradation and senescence by inducing expression of the ABA biosynthesis gene *AAO3* (Yang et al., 2014). Its elevated expression in *mkkk17-1* points to a compensatory mechanism aimed at sustaining ABA levels during abiotic stress, further reinforcing *MAPKKK17's* involvement in ABA homeostasis.

MYB78, which is transcriptionally regulated by *NAC103*, also plays a role in ABA signalling modulation (Sun et al., 2020). The MYB–NAC regulatory axis is frequently implicated in abiotic stress responses, and its induction in *mkkk17-1* may reflect a stress-activated backup mechanism compensating for disrupted kinase signalling.

FYF, a MADS-box transcription factor, has been shown to delay floral senescence and suppress abscission when ectopically expressed (Chen et al., 2011). Upregulation of *FYF* in the *mkkk17-1* may reflect altered ABA-responsive developmental regulation, potentially linked to delayed organ detachment or prolonged flower viability under stress.

ABF1, a member of the bZIP family, acts downstream of ABA-activated SnRK2 kinases (e.g., *SRK2D*/E/I), functioning as a central transcriptional regulator of ABA-responsive genes (Yoshida et al., 2015). The presence of *ABF1* in the uniquely upregulated gene set implicates a convergence point between *MAPKKK*17-dependent signalling and the canonical ABA signalling cascade mediated by SnRK2 kinases.

In addition to these key regulators, numerous other TFs including members of the WRKY (e.g., *WRKY23*, *WRKY48*, *WRKY70*), NAC (e.g., *NAC010*, *NAC075*, *NAC100*), and MYB families (e.g., *MYB93*, *MYB95*, *MYB106*) were significantly enriched among the upregulated genes. This highlights a broader reprogramming of transcriptional networks in *mkkk17-1*, possibly reflecting a compensatory activation of stress-responsive pathways to maintain ABA sensitivity and resilience in the absence of *MAPKKK17* function.

These transcription factors are likely to regulate overlapping sets of downstream genes involved in stress protection, suberin biosynthesis, hormonal crosstalk, and developmental timing, reinforcing the concept that *MAPKKK17* serves as a important node in integrating environmental cues into gene regulatory responses.

Collectively, these findings suggest that *MAPKKK17* influences ABA signalling not only through direct regulation of metabolic or stress-protective genes but also by modulating a complex hierarchy of transcription factors. The upregulation of TFs such as *LFY, NAP, MYB78, FYF*, and *ABF1* in *mkkk17-1* underscores the kinase's upstream role in fine-tuning ABA-responsive gene expression. Through its regulatory control over diverse TF families, *MAPKKK*17 likely coordinates transcriptional networks that balance developmental progression and stress adaptation under ABA-induced osmotic and drought stress.

5.4. *MAPKKK*18 is implicated in ABA-linked stress and developmental responses

Differential gene expression analysis of the *mkkk18-1* mutant under ABA treatment revealed distinct transcriptional profiles implicating *MAPKKK*18 as a important modulator of ABA-mediated metabolic reprogramming, hormonal crosstalk, and growth adaptation. The 1,237 genes uniquely differentially expressed in *mkkk18-1*, including 593 upregulated and 644 downregulated genes, represent pathways that are selectively responsive to ABA in the absence of functional *MAPKKK*18. These findings reveal how ABA responses in *mkkk18-1* balance stress resilience with developmental plasticity. The upregulated genes in *mkkk18-1* were enriched for ABA-associated secondary metabolism and defence-related biosynthetic pathways, including flavonoid and phenylpropanoid biosynthesis, oxylipin production, and responses to jasmonic acid. Several of these genes are key nodes in known ABA-stress signalling cascades. For instance, *CCoAOMT1*, previously shown to promote drought tolerance through ABA-mediated hydrogen peroxide

regulation, was among the upregulated genes, suggesting a compensatory enhancement of defence-related lignin biosynthesis in the mutant (Chun et al., 2021). Similarly, *LDOX, DFR, FLS3*, and *MYB12*, all involved in flavonoid biosynthesis, were upregulated and pointed to increased synthesis of protective secondary metabolites under ABA stress. Notably, *MYB12* has been linked to elevated flavonoid and ABA levels under salt and drought conditions, highlighting its dual role in metabolic and hormonal regulation (Wang et al., 2016). Upregulation of *LOX3* and *LOX4*, key enzymes in alpha-linolenic acid and oxylipin biosynthesis, supports the involvement of jasmonate-ABA crosstalk in *mkkk18-1*. These genes are known to be positively regulated by ABA receptors, reinforcing their integration into ABA-induced stress signalling (Yu et al., 2021). Upregulation of *JAZ5* and *VSP1*, core components of JA signalling, reinforces the active crosstalk between jasmonate and ABA pathways in *mkkk18-1*. *JAZ5* is particularly notable for its interaction with ABI5 in coordinating ABA and JA signalling during stress (Ju et al., 2019), while *VSP1* expression in *aba2-2* mutants links its expression directly to ABA function (Lin et al., 2020; Van Houtte et al., 2013).

Genes involved in carbohydrate metabolism and osmotic regulation were also prominent. TPS1 and *TPPI*, both associated with starch and sucrose metabolism, have established roles in stomatal regulation and drought adaptation (Lin et al., 2020; Van Houtte et al., 2013) . Additionally, *NIT2*, a contributor to cyanoamino acid metabolism, has been implicated in enhancing salinity tolerance and maintaining ionic homeostasis under stress (Cackett et al., 2022). Upregulation of *WRKY53*, an early senescence regulator, points to *MAPKKK18's* involvement in ABA-dependent modulation of growth-defence trade-offs (Miao et al., 2004). Similarly, *MYB3* acts as a negative regulator of *LDOX* to control anthocyanin accumulation during salt stress, indicating a fine-tuning of flavonoid metabolism under stress (Kim et al., 2022).

In contrast, genes uniquely downregulated in *mkkk18-1* revealed an opposing trend, with significant enrichment for processes related to ribosome biogenesis, RNA modification, and organ growth regulation. These findings suggest that *MAPKKK*18 helps sustain growth and translational capacity under ABA stress, and its loss triggers a shift toward stress survival at the expense of cellular proliferation and biosynthesis. Key growth regulators such as *ARGOS*, *ARL*, and *CYP78A5*/*KLU* were downregulated, indicating a shift away from growth-promoting programs in favour of stress adaptation. *CYP78A5*, in particular, is known to enhance tolerance to multiple abiotic stresses, and its suppression may reflect a controlled reduction in cell proliferation to

conserve energy under ABA signalling (Kajino et al., 2022; Kuluev et al., 2019). Several RNA-related genes affected in *mkkk18-1*, including *AHG11*, *ABO8*, and *SLO2*, are recognized for their roles in ABA hypersensitivity and RNA processing during seedling development (Murayama et al., 2012; Yang et al., 2014; Zhu et al., 2014). Their downregulation may influence post-transcriptional control of ABA responses, potentially contributing to developmental delays or altered germination phenotypes. Finally, the observed downregulation of *PSRP2*, a gene previously reported to repress seedling growth under salt stress, suggests that its reduced expression could act to counterbalance excessive growth inhibition during prolonged ABA exposure (Xu et al., 2013).

Together, these findings highlight the dual regulatory role of *MAPKKK*18 in promoting ABA-induced defence and metabolic responses while simultaneously preserving RNA processing and growth-related functions. Loss of *MAPKKK18* results in an ABA-dependent shift toward enhanced secondary metabolism, jasmonate signalling, and stress adaptation at the expense of growth maintenance and ribosomal activity. This transcriptional reprogramming highlights *MAPKKK18's* distinct role among MAPKKKs as a critical integrator of developmental and stress-responsive ABA signalling pathways.

5.5. Functional insights from shared Differentially expressed genes in *MAPKKK18* and WT Col-0 under ABA treatment.

The functional characterization of commonly DEGs in *mkkk18-1* and WT Col-0 provides valuable insight into the molecular pathways modulated by ABA treatment, revealing how *MAPKKK*18 influences both shared and genotype-specific transcriptional responses. GO enrichment analyses revealed strong overrepresentation of pathways associated with "cutin, suberin, and wax biosynthesis," "phenylpropanoid metabolism," and "responses to jasmonic acid and wounding." Notable genes in these categories included *RD20, FAR4, FAR5, RWP1*, and *CYP86B1*, all of which are involved in protective cuticle development. Additionally, *5PTASE11, LOX2*, and *CYP94B3* were enriched in stress-responsive processes such as jasmonate signalling and mechanical wounding. For instance, *5PTASE11* encodes a phosphatase that hydrolyzes IP_3, serving as a negative regulator of ABA signalling by modulating stomatal closure and stress-responsive gene expression (Burnette et al., 2003). Similarly, *MYB102* is rapidly induced by ABA and osmotic stress and enhances abiotic stress tolerance by regulating ABA-responsive transcriptional networks

(Denekamp & Smeekens, 2003). These results suggest that ABA-induced upregulation of protective genes in both *mkkk18-1* and WT Col-0 may be developed in the mutant, reflecting a compensatory response to the absence of *MAPKKK18*-mediated modulation. This supports the role of *MAPKKK18* as a modulator that fine-tunes the intensity of ABA-triggered transcriptional activity.

In contrast, 152 shared DEGs were enriched in processes including auxin metabolism, transport, and secondary metabolite biosynthesis. The repression of genes such as *ACS7*, *CYP707A3*, *TYRDC*, and *PORA* points to altered hormonal crosstalk and stress adaptation in *mkkk18-1*. In contrast, *ACS7* is involved in ethylene biosynthesis, undergoes N-terminal degradation regulated by senescence signalling and interacts with ABA phosphatases such as *ABI1*, *ABI2*, and HAB1 (Sun et al., 2017), indicating ABA-ethylene interaction. Repression of *CYP707A3*, a central ABA-catabolizing enzyme, implies enhanced ABA retention and heightened responsiveness, consistent with previous reports showing improved drought tolerance in *cyp707a3* (Umezawa et al., 2006). *TyrDC*, which is responsive to drought but suppressed under salt stress, further underscores hormonal complexity in stress responses (Lehmann & Pollmann, 2009). The repression of *PORA*, a gene involved in chlorophyll biosynthesis and indirectly linked to stomatal development via *SPEECHLESS* (Kim & Apel, 2012), suggests broader impacts on photosynthesis and transpiration under ABA stress. Downregulation of auxin-responsive genes such as *IAA19*, *SAUR19*, and *SAUR54* highlights ABA's antagonistic effect on auxin-mediated growth pathways. Notably, *IAA19* is required for optimal drought tolerance, and its repression could represent a mechanism by which *MAPKKK18* indirectly regulates growth suppression under stress (Salehin et al., 2019; Shani et al., 2017). Additionally, the downregulation of *SAUR54* upon ABA treatment (van Mourik et al., 2017) further supports the model wherein ABA acts to inhibit auxin-mediated growth processes during stress.

Overall, *MAPKKK18* fine-tunes ABA output through promotion of protective pathways (cuticle biosynthesis, JA response) and Repression of growth-favouring hormonal pathways (auxin, ethylene). Together, these data suggest *MAPKKK*18 as a key regulatory node that calibrates both the strength and polarity of ABA-induced transcriptional responses, promoting stress resilience while restraining growth, in part through targeted suppression of auxin and ethylene signalling.

5.6. Functional divergence and redundancy of *MAPKKK*17 and *MAPKKK*18 in ABA-mediated stress signalling and developmental regulation

Transcriptomic comparisons between *mkkk17-1* and *mkkk18-1* mutants revealed both functional divergence and partial redundancy in ABA signalling, underscoring their specialized yet overlapping regulatory roles. The unique and shared Differentially expressed gene sets highlight how these MAPKKKs selectively regulate distinct biological processes while also converging on core stress signalling pathways.

The transcriptional landscape of *mkkk17-1* mutants was characterised by a strong enrichment of genes involved in RNA-related metabolic processes. Gene Ontology (GO) analysis identified categories such as “ribosome biogenesis,” “RNA processing,” and “RNA modification” as prominently enriched. These categories included multiple RNA-binding and processing factors, such *as PWP2, PMH1, EBP2, MORF8*, and *RACK1B*, which have been implicated in post-transcriptional control mechanisms responsive to abiotic stress. Notably, *MORF8*, recently reported to undergo chloroplast-localized phase separation under heat stress (Wu et al., 2025), further substantiates the role of *MAPKKK*17 in RNA maturation pathways that underpin ABA-driven stress adaptation. *MAPKKK*17-specific DEGs were enriched in RNA processing and ribosome biogenesis categories, suggesting that this kinase modulates ABA signalling partly through post-transcriptional regulatory mechanisms, a dimension not shared with *MAPKKK*18.

In contrast, *mkkk18-1* mutants exhibited enrichment for genes regulating growth and development, including processes such as “positive regulation of organ growth,” “regulation of cell population proliferation,” and “response to hypoxia.” Key genes in these categories, such as *CYP78A5/KLU, ARGOS, RGF3*, and *SDD1*, reflect a signalling architecture that links *MAPKKK*18 to developmental plasticity under stress. For example, *CYP78A5* has been shown to improve tolerance to diverse abiotic stresses through its role in organ size regulation (Kajino et al., 2022), and *RGF3* is central to root meristem maintenance via peptide-receptor mediated signalling (Ou et al., 2022). The involvement of *SDD1*, a negative regulator of stomatal density, underscores *MAPKKK*18’s role in ABA-mediated stomatal control and transpiration regulation, which are critical under drought stress (Schluter, 2003; Von Groll et al., 2002).Together, these findings suggest that *MAPKKK*17 predominantly modulates stress responses at the post-transcriptional level, whereas *MAPKKK*18 primarily acts through developmental and morphogenetic pathways.

Despite their distinct transcriptional signatures, *MAPKKK*17 and *MAPKKK*18 mutants shared a core set of 557 DEGs not observed in WT Col-0 under ABA treatment. GO enrichment among these shared DEGs revealed convergence on processes including "transmembrane transport," "response to fatty acid," "jasmonic acid signalling," and "pigment biosynthesis." These categories encompass key stress-responsive pathways and suggest overlapping functions of the two MAPKKKs in orchestrating adaptive responses to ABA. Genes such as *LOX3* and *WRKY53*, known for their involvement in jasmonate biosynthesis and stress-induced senescence, respectively, were co-regulated in both mutants, aligning with previous findings of hormone crosstalk between ABA and jasmonic acid signalling (Chapters 6.1–6.4). *LDOX*, implicated in flavonoid biosynthesis and salt tolerance, further reinforces this overlap in stress signalling. Moreover, several shared DEGs were associated with ion and metabolite transport, such as *NHX6, ABCC4, STP4*, and *DTX1*. The vacuolar trafficking roles of *NHX5*/6 are well-documented in salt stress responses (Bassil et al., 2011; Dragwidge et al., 2018), while *ABCC4* is crucial for root development and ion homeostasis under osmotic stress (Uragami et al., 2024). *STP4*, a hexose transporter highly expressed in guard cells, is required for efficient ABA-induced stomatal closure (Flütsch et al., 2020), highlighting a shared mechanism for water conservation and gas exchange under drought.

Further convergence is evidenced by shared regulation of stress-responsive transporters, whose mutant phenotypes highlight overlapping functions in ion homeostasis and stress resilience. For example, the *aha4-1* mutant, which exerts a dominant-negative effect, impairs ion homeostasis and nutrient translocation in the root endodermis, ultimately weakening stress barrier functions in this tissue (Vitart et al., 2001). *DTX1*, a shared DEG in both *mkkk17-1* and *mkkk18-1*, also reflects this axis of stress regulation. Notably, *dtx1* mutants show reduced germination under oxidative stress but enhanced seedling survival under salinity and other abiotic conditions (Burke et al., 2023), suggesting that regulation of transporter activity is context-dependent and linked to broader ABA-mediated signalling circuits. Moreover, calcium signalling, central to ABA responses, appears to be a point of integration between both MAPKKKs. Elevated expression of *ACA12*, a plasma membrane-localized Ca^{2+}-ATPase enriched in guard cells, was observed among the shared DEGs. Given its role in modulating cytosolic calcium levels during stomatal movement (Yu et al., 2018), *ACA12* likely acts downstream of convergent signalling from both *MAPKKK17* and *MAPKKK18*, coupling MAPK signalling with calcium-mediated control of stress physiology.

Other co-regulated genes such as *HP22*, which accelerates chloroplast protein degradation during senescence and thus contributes to stress-induced remobilization of nutrients (Rossig et al., 2021), and *YSL2*, a yellow stripe-like (YSL) metal transporter involved in micronutrient (e.g., iron) mobilization and linked to ABA-responsive iron homeostasis (Lei et al., 2014), underscore further functional overlap in nutrient mobilization and long-term stress adaptation.

Altogether, these findings support a two-tiered regulatory architecture in which *MAPKKK17* specializes in post-transcriptional control of stress responses, while *MAPKKK18* governs developmental plasticity and morphogenetic adaptation under ABA signalling. Despite these distinct roles, both kinases converge on a shared core transcriptional program involved in ion transport, jasmonate signalling, and stomatal function. The co-regulation of transporters such as *STP4*, *DTX1*, and *NHX6*, as well as calcium signalling genes like *ACA12*, underscores a mechanistic intersection point where MAPK and calcium signalling jointly orchestrate drought resilience. By integrating hormone crosstalk, transporter regulation, and metabolic reprogramming, this combinatorial model illustrates how specialization and redundancy in MAPKKK signalling together ensures a robust and flexible ABA stress response across multiple physiological domains.

5.7. ABA-Auxin antagonism: transcriptional patterns involving *ABI1* and *MAPKKK18*

ABA-auxin crosstalk is crucial for integrating growth and stress signals, yet the transcriptional mechanisms governing this antagonism remain incompletely understood. RNA-seq analysis revealed that ABA treatment induces strong downregulation of auxin-responsive genes in both *abi1td* and *mkkk18-1* mutants, indicating that *ABI1* and *MAPKKK18* converge on a shared transcriptional program that represses auxin signalling during stress. GO enrichment analysis of the downregulated genes in *abi1td* mutants showed a marked overrepresentation of auxin-related processes. This pattern mirrors that observed in *mkkk18-1* (Section 5.5), supporting a model in which ABA suppresses growth-promoting auxin pathways through both signalling components.

Among the most significantly downregulated genes were several members of the Small Auxin Up RNA (SAUR) gene family (e.g., *SAUR19, SAUR22, SAUR29, SAUR61, SAUR63*), the Aux/IAA transcriptional repressors *IAA19* and *IAA29*, and the auxin transporter gene *PIN5*. The repression of *SAUR19* and *SAUR63* is particularly noteworthy, as these genes are well-characterised positive

regulators of cell expansion and elongation. While *SAUR19* and *SAUR63* are not highly expressed in guard cells under normal conditions, overexpression studies (SAUR19OX, SAUR63OX) show impaired ABA-induced stomatal closure and reduced inward-rectifying K^+ channel activity, suggesting that SAUR proteins can antagonize ABA signalling, possibly through indirect or systemic mechanisms (Cañibano et al., 2025; Chae et al., 2012; Spartz et al., 2014; Wong et al., 2021). Mechanistically, *SAUR19* has been shown to inhibit PP2C.D family phosphatases, key negative regulators in the ABA signalling pathway, highlighting a potential feedback loop in which auxin signalling components modulate ABA responsiveness (Spartz et al., 2014; Wong et al., 2019, 2021). The transcriptional repression of *IAA19* and *IAA29* adds another layer of regulatory complexity. *IAA19*, a well-studied Aux/IAA protein, integrates auxin and ABA signalling pathways, particularly in response to drought and other environmental stresses. It contributes to both hypocotyl elongation and root development and is directly regulated by *DREB2A*/*DREB2B*, transcription factors central to abiotic stress responses (Nam et al., 2023; Salehin et al., 2019; Shani et al., 2017). Meanwhile, *IAA29* has been implicated in heat-induced leaf senescence through its regulation by *PIF4* and *PIF5*, highlighting its role in stress-adaptive developmental processes (Li et al., 2021). Although *ABI1* is canonically described as a negative regulator of ABA signalling, our data suggest that under prolonged stress, it contributes to transcriptional repression of auxin-responsive genes, potentially through indirect feedback loops or context-specific transcriptional co-regulators.

Another key finding is the repression of *PIN5*, an auxin efflux carrier localized to the endoplasmic reticulum that regulates intracellular auxin homeostasis. Given that *PIN5* overexpression inhibits primary root elongation and that loss-of-function *pin5-5* mutants display reduced root and hypocotyl growth, the repression of *PIN5* under ABA treatment may represent an additional mechanism to restrict growth and preserve energy during stress (Seifu et al., 2024). This intracellular regulation of auxin distribution by ABA further illustrates the depth of integration between hormonal pathways at the cellular level.

Collectively, these findings support a regulatory model in which ABA suppresses auxin-responsive transcriptional programs such as SAURs, Aux/IAA genes, and PIN transporters via signalling nodes like *ABI1* and *MAPKKK18*. This repression likely serves to shift plant priorities from growth to stress adaptation. By establishing a mechanistic basis for ABA-auxin antagonism, the study highlights how hormonal networks integrate environmental cues to modulate development.

Further investigation is required to determine whether the observed transcriptional repression of auxin-responsive genes is mediated directly by *ABI1* and *MAPKKK18*, for instance through the recruitment of chromatin remodelers or transcriptional repressors to target gene promoters, or whether it occurs indirectly via broader ABA-induced hormonal imbalances and signalling cascades. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) or assay for transposase-accessible chromatin using sequencing (ATAC-seq) in *ABI1*- and *MAPKKK18* – overexpressing or nuclear-localised tagged lines, together with *abi1td* and *mkkk18-1* mutants as controls, could provide critical insights into whether these regulators physically associate with auxin-responsive loci and influence chromatin accessibility during ABA treatment.

5.8. *UPL4* and *UPL6* may contribute to ABA-dependent regulation of root and stomatal development

The distinct subfamilies of HECT-type UPL E3 ubiquitin ligases in *Arabidopsis thaliana* exhibit diverse biological functions, ranging from developmental regulation to stress responses. For example, *UPL3* plays a role in trichome morphogenesis and *UPL5* is involvement in leaf senescence and immunity (Furniss et al., 2018; Lan et al., 2022). Recent studies highlighted the involvement of UPL ligases, particularly *UPL1*, and *UPL6*, in ABA signalling via ubiquitination of key MAP kinase components such as *MAPKKK18* (Tajdel-Zielińska et al., 2024). This suggests that UPL family members modulate ABA-dependent processes through targeted proteolysis, a critical mechanism in plant stress adaptation. Building on this framework, the present study investigates the roles of *UPL4* and *UPL6* in fine-tuning ABA-mediated developmental responses, including root elongation and stomatal patterning.

This study identifies *UPL4* and *UPL6* as playing distinct yet overlapping roles in mediating ABA-dependent developmental processes. Through combined phenotypic, genetic, and transcriptomic analyses, we show that these HECT-type ligases fine-tune ABA responses in both root and shoot tissues, influencing growth regulation and epidermal patterning.

The induction of *UPL4* in the *abi1*, *mkkk17*, and *mkkk18* mutants emphasizes its significant role as a regulatory node for convergence of multiple ABA-signalling branches. *UPL4* may act as a proteolytic integrator fine-tuning developmental responses across tissues.

Both *UPL4* and *UPL6* are required for fine-tuning primary root elongation in response to ABA. *UPL4* overexpression confers partial ABA insensitivity (**Figure 30**A), resulting in longer roots,

whereas *upl4* mutants are ABA-hypersensitive (**Figure 30**A), These phenotypes indicate that *UPL4* is a positive regulator of primary root elongation under ABA. These observations align with reports that identify the elongation zone as a primary site of ABA action and suggest that *UPL*4 may attenuate ABA signalling through targeted degradation of inhibitory components.

In contrast, *UPL*6 overexpression lines did not show stronger inhibition of root elongation compared to WT Col-0. Instead, they retained root lengths slightly greater than WT Col-0 under ABA treatment, suggesting that UPL6 overexpression confers partial resistance to ABA-mediated inhibition of root growth. These results indicate that both loss and overexpression of *UPL*6 impair normal ABA responses, pointing to a requirement for balanced *UPL*6 activity to maintain proper root growth regulation. Importantly, these effects were specific to ABA treatment, as no significant differences in root elongation were detected under mock conditions. Taken together, the data suggest that *UPL*4 and *UPL*6 act through distinct but complementary mechanisms to regulate ABA-mediated root growth, potentially by modulating the stability of transcription factors or signalling intermediates within the pathway.

In addition to regulation, *UPL*4 also represses stomatal development in an ABA-dependent manner. The *upl4* mutants displayed an increased stomatal index (SI), whereas overexpression lines showed reduced SI. These differences arose from altered stomatal density, while the total number of epidermal cells remained unchanged, indicating that *UPL*4 specifically affects the proportion of stomatal cells rather than overall epidermal cell proliferation. This suggests that *UPL*4 primarily affects lineage entry or stomatal fate commitment, rather than global cell proliferation. These findings support the established role of ABA as a developmental signal that restricts stomatal formation during drought or stress conditions. Given *UPL*4's dual functions in roots and leaves, it likely acts at a convergence point of ABA signalling, potentially regulating transcriptional modules or MAPK effectors through ubiquitin-mediated proteolysis.

Similarly, *UPL6* overexpression suppressed stomatal formation, while *upl6* mutants exhibited increased SI and epidermal cell numbers. This indicates that *UPL6* functions as a broader suppressor of cell proliferation and differentiation in the epidermis. These effects were consistent across cotyledons and true leaves, and at multiple developmental stages, reinforcing *UPL*6 as a robust regulator of epidermal patterning. Unlike *UPL*4, which alters stomatal index with minimal changes in overall epidermal cell numbers, *UPL*6 exerts broader control over epidermal cell proliferation, pointing to a distinct mode of regulation within the ABA-mediated network.

RNA-seq analysis revealed that *UPL4* and *UPL6* expressions are not significantly altered by ABA in WT Col-0 (Table 16). This indicates that their basal transcription is ABA-independent, and that dynamic regulation likely occurs post-transcriptionally or under specific genetic perturbations. However, their expression was significantly elevated in *abi1td* and *mkkk17-1* mutants under ABA treatment. These patterns suggest that *ABI1* and *MAPKKK17* suppress *UPL4* and *UPL6* expression during ABA signalling. The de-repression observed in mutants indicate that both ligases are regulated by upstream ABA components and may function downstream to reinforce ABA outputs. The additional induction of *UPL4*, but not *UPL6*, in *mkkk18-1* suggests a broader regulatory integration for *UPL4* within ABA-MAPK paths. This broader responsiveness implies that *UPL4* may act as a convergence node within the ABA-MAPK network, integrating inputs from multiple upstream regulators to modulate developmental outcomes.

The similar root and stomatal phenotypic results of *UPL4* and MAPKKK18, along with the findings of Tajdel-Zielińska et al., 2024 , suggest that *UPL*4 function upstream of *MAPKKK18* in MAPK signalling. Given that MAPK cascades are often prime factors for proteasomal degradation through phosphorylation, these E3 ligases may confer substrate specificity, targeting phosphorylated repressors or modulators for ubiquitination. In this way, *UPL4* and *UPL6* could help sharpen or reset ABA signalling outputs. *UPL4's* induction by all three mutants (*abi1td*, *mkkk17-1*, and *mkkk18-1*) and its involvement in both root and shoot development position as a key regulatory node for ABA-dependent developmental plasticity. This convergence suggests *UPL4* may serve as a signal integration point, responding to diverse upstream cues to modulate organ development under stress. Future work should determine whether *UPL4* directly targets MAPK cascade repressors for proteasomal degradation.

Despite phenotypic and transcriptional evidence, the direct substrates of *UPL4* and *UPL6* remain unknown. Identifying these substrates will be essential for deciphering how UPL ligases confer selectivity within ABA-MAPK pathways and how proteolysis reshapes developmental plasticity under stress. Future studies using ubiquitin remnant profiling, co-IP mass spectrometry, or targeted interactome screens will be necessary to identify their targets and confirm their integration with ABA-MAPK modules. Furthermore, ABA responses often operate within broader hormonal and environmental networks. Investigating *UPL*4/6 functions under combined stress conditions (e.g., drought, salinity) or interactions with other hormones (e.g., auxin, ethylene) could reveal additional layers of cross-regulation and potential agronomic relevance

The regulation of primary root elongation by UPL4 and UPL6 is reminiscent of other E3 ubiquitin ligases and ABA signalling regulators that fine-tune root growth under stress. For example, the RING-type ligase KEG controls ABI5 stability, and keg mutants exhibit hypersensitive root growth inhibition under ABA (Stone et al., 2006;. Similarly, RGLG1/2 and AIRP3 have been implicated in ABA-mediated growth regulation through modulation of signalling intermediates Pan., et al 2020; Wu et al., 2016; Yu et al., 2021;. Beyond the ubiquitin pathway, transcription factors such as ABI3/ABI5 and kinases like SnRK2s and MAPKKK18 have also been shown to suppress root elongation in response to ABA Carles et al., 2002; Lopez-Molina et al., 2003; Nakashima et al., 2006; Zhao et al., 2023). The similarity of phenotypes between *upl* mutants and these known ABA regulators suggests that *UPL4* and *UPL6* participate in a broader network of proteolytic and transcriptional control mechanisms that integrate hormonal and stress signals to modulate root development.

Overall, the results establish *UPL4* and *UPL6* as modulators of ABA-responsive development, with *UPL4* promotes ABA-dependent root growth but suppresses stomatal formation. In contrast, *UPL6* overexpression lines retained root lengths are greater than WT Col-0 under ABA Treatment, indicating partial resistance to ABA inhibition, whereas *upl6* mutant lines displayed hypersensitivity. Together, these findings identify UPL6 as a regulator of ABA sensitivity in roots and stomatal development , but their precise mechanistic roles remain to be studied.

Transcriptomic evidence further supports their regulation downstream of key ABA pathway components (*ABI1*, *MAPKKK17*, and *MAPKKK18* for *UPL4*). These findings highlight a novel proteolytic dimension to ABA signalling and provide potential targets for manipulating plant stress tolerance and developmental outcomes. By linking ubiquitin-mediated turnover to hormonal signal integration, this study opens new avenues for optimizing plant growth-defence balances through targeted manipulation of UPL ligase activity. Moreover, the use of ubiquitin profiling and interactome mapping could identify context-specific substrates of *UPL4*/6 under different hormonal combinations, providing tools for precision breeding or genetic engineering of stress-resilient crops.

6. Summary

Mitogen-activated protein kinase kinase kinases (MAPKKKs) play pivotal roles in integrating ABA signalling to regulate plant stress responses and developmental processes. Their functional specialization and interplay contribute to the dynamic balance between growth and adaptation under abiotic stress conditions.

- **Functional divergence and redundancy of *MAPKKK17* and *MAPKKK18*:**
  - *MAPKKK17* may contribute to post-transcriptional regulation by influencing RNA processing and metabolism pathways during ABA-mediated stress responses.
  - *MAPKKK18* may govern developmental plasticity, affecting organ growth, cell proliferation, and stomatal density, thereby linking stress signalling with morphogenetic adaptation.
  - Both kinases share a subset of differentially expressed genes involved in ion transport, jasmonic acid signalling, and calcium-mediated stomatal regulation, demonstrating overlapping roles within the ABA signalling network. This overlap reflects a built-in redundancy that likely enhances the robustness of ABA-mediated stress adaptation, while their divergence enables modular regulation of distinct physiological processes.
- **ABA-auxin crosstalk mediated by *ABI1* and *MAPKKK18*:**
  - *ABI1* and *MAPKKK18* coordinate to repress auxin-induced growth signals under ABA stress. Transcriptomic data indicates suppression of auxin-responsive genes including SAURs, IAAs, and PIN transporters. This transcriptional antagonism provides mechanistic insight into hormone prioritization under stress, where ABA suppresses cell expansion and division pathways to conserve resources.
- **Role of *UPL4* and *UPL6* E3 ubiquitin ligases in ABA-dependent development:**
  - *UPL4* functions as an important regulator of ABA sensitivity, controlling primary root elongation and stomatal development through ubiquitin-mediated proteolysis of signalling components.

- *UPL6* acts as a key regulator of stomatal development by constraining epidermal cell proliferation, while also contributing to ABA-mediated regulation of root growth.
- Both *UPL4* and *UPL6* are transcriptionally regulated in *abi1* and *mkkk17* mutants, indicating that their activity is integrated with core ABA signalling modules to fine-tune developmental and stress responses.

- **Integration of hormonal and signalling networks:**
  - The findings highlight a multi-layered regulatory architecture wherein MAP kinase signalling, hormonal antagonism, and ubiquitin-dependent proteolysis converge to balance growth and stress adaptation in plants.

Together, these findings propose a multilayered model in which *MAPKKK17*/18 and *ABI1* define transcriptional responses, while *UPL4*/6 enforce proteasome-level control to refine output dynamics. This hierarchy enables ABA to fine-tune growth-stress balances through spatially and temporally distributed modules. Targeting these nodes could allow the development of crop varieties with optimized resilience and adaptability under abiotic stress conditions.

7. Conclusions

1. MAPKKK17 and MAPKKK18 exhibit both overlapping and redundant roles in the regulation of cellular processes. *MAPKKK*17 primarily influences post-transcriptional regulation, particularly RNA processing and metabolism, during ABA-mediated stress responses. In contrast, *MAPKKK*18 governs developmental plasticity, affecting organ growth, cell proliferation, and stomatal density. Both kinases co-regulate genes involved in ion transport, jasmonic acid signalling, and calcium-dependent stomatal control, highlighting their shared functions within the ABA signalling network.

2. UPL4 and UPL6 fine-tune ABA-mediated developmental and stress responses by acting downstream of core signalling elements and modulating root growth and stomatal development through targeted protein degradation.

8. List of figures

9. List of tables

10. References

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