Cross-Regulation of Hormone Signalling by Transcription Factors in *Arabidopsis thaliana*

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LIST OF ABBREVIATIONS

ABA	Abscisic Acid
ABF	ABRE binding factor
ABI1	ABA INSENTIVITY 1
ABI2	ABA INSENTIVITY 2
ABI5	ABA INSENTIVITY 5
ABRE	ABA responsive element
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AOC3	ALLENE OXIDE CYCLASE 3
AP2/ERF	APETALA 2/ETHYLENE RESPONSE FACTOR
ARF	AUXIN RESPONSE FACTOR
AtD14	Arabidopsis DWARF14
AUX/IAA	Auxin/indole-3-acetic acid
BAK1	BRI1-ASSOCIATED RECEPTOR KINASE 1
BES1	BRI1-EMS-SUPPRESSOR 1
bHLH	Basichelix-loop-helix
BIN2	BRASSINOSTEROID-INSENSITIVE 2
BR	Brassinosteroid
BRI1	BRASSINOSTEROID INSENSITIVE 1
BRL	BRI1 LIKE
BSK	BR-SIGNALLING KINASE
BSU1	BRI1 SUPPRESSOR 1
bZIP	Basic leucine zipper
BZR1	BRASSINAZOLE RESISTANT 1
ChIP-seq	Chromatin immunoprecipitation sequencing
CK	Cytokinin
COI1	CORONATINE INSENSITIVE 1

CTR1	CONSTITUTIVE TRIPLE RESPONSE 1
DAP-seq	DNA affinity purification sequencing
DREM	Dynamic Regulatory Events Miner
EAR	Ethylene-responsive element binding factor amphiphilic repression
EBF	EIN3-BINDING F-BOX PROTEIN
EDF	ETHYLENE RESPONSE DNA-BINDING FACTOR
EIL	EIN3 LIKE
EIN2	ETHYLENE INSENSITIVE 2
EIN2-CEND	EIN2 C-terminal domain
EIN3	ETHYLENE INSENSITIVE 3
EIN4	ETHYLENE INSENSITIVE 4
ER	Endoplasmic reticulum
ERF	ETHYLENE RESPONSE FACTOR
ERS1	ETHYLENE RESPONSE SENSOR 1
ET	Ethylene
ETR1	ETHYLENE RECEPTOR 1
FUF1	FYF UP-REGULATING 321 FACTOR 1
GA	Gibberellin
GA20OX2	GIBBERELLIN 20 OXIDASE 2
GA3OX1	GIBBERELLIN 3-OXIDASE 1
GRP23	GLYCINE-RICH PROTEIN 23
GRN	Gene regulatory network
HLS1	HOOKLESS1
JA	Jasmonic acid
JA-Ile	Jasmonoyl-L-isoleucine
JAs	Jasmonates
JAM1	JA-ASSOCIATED MYC2-LIKE 1
JAZ	JASMONATE-ZIM DOMAIN
KAI2	KARRIKIN INSENSITIVE 2

KAR	Karrikin
LOX2	LIPOXYGENASE 2
LRR	Leucine-rich repeat
MAX2	MORE AXILLARY BRANCHES 2
MeJA	Methyl Jasmonate
MKK	MAP KINASE KINASE
MPK	MITOGEN-ACTIVATED PROTEIN KINASE
MPKKK	MAP KINASE KINASE KINASE
MYC	Myelocytomatosis oncogenes homolog
NINJA	NOVEL INTERACTOR OF JAZ
NLP7	NIN-LIKE PROTEIN 7
NPR1	NONEXPRESSER OF PR GENES 1
ORA59	OCTADECANOID RESPONSIVE ARABIDOPSIS 59
PDF1.2	PLANT DEFENSIN 1.2
PIF3	PHYTOCHROME INTERACTING FACTOR 3
PP2Cs	Group-A protein phosphatases of the type 2C
PR	Pathogenesis-related
PYL	PYR1-LIKE
PYR1	PYRABACTIN RESISTANCE 1
RAV	RELATED TO ABI3/VP 1
RCAR	Regulatory component of ABA receptor
RLK	Receptor-like kinases
RNA-seq	RNA sequencing
RVE8	REVEILLE 8
SA	Salicylic Acid
SAR	Systemic-acquired resistance
SCF	Skp–Cullin–F-box
SDREM	Signalling and Dynamic Regulatory Events Miner
SL	Strigolactone

SMXL	SUPPRESSOR OF MAX2-LIKE
SnRK2s	SNF1-RELATED PROTEIN KINASE 2
STZ	SALT TOLERANCE ZINC FINGER
SUMO	SMALL UBIQUITIN-LIKE MODIFIER
TF	Transcription factor
TGA	TGACG (TGA) motif-binding
TPL	TOPLESS
TPR	TOPLESS-RELATED
TREE1	Transcriptional Repressor of EIN3-dependent Ethylene-response 1
TRX	Thioredoxin
VSP	VEGETATIVE STORAGE PROTEIN

ABSTRACT

Cross-regulation between hormone signalling pathways is essential for plants to initiate appropriate responses to diverse environmental stimuli. Broad gene expression reprogramming occurs during hormone responses and is an important component of cross-regulation. However, the molecular mechanisms of hormonal cross-regulation of gene expression are under-explored. This thesis describes my investigation into transcriptomic reprogramming induced by six hormones and the cross-regulation of six hormone signals in etiolated Arabidopsis seedlings by focusing on hormone transcription factors (TFs). Each hormone recruits different combinations of TFs that target and regulate transcription of genes. Hub target genes bound by multiple TFs existed within hormone transcriptional networks. Differential expression of hub targets was stronger in response to hormones and they were primarily TFs themselves. A cross-regulation network was generated to explain how hormone signals are integrated from multiple pathways to dynamically cross-regulate gene expression. A group of mitogen-activated protein kinases that might be key points of hormonal cross-regulation were identified from this. I also conducted a focused analysis of the functions of a family of TFs involved in responses to the hormone ethylene, the ETHYLENE RESPONSE DNA-BINDING FACTORS (EDFs) 1, 2, 3. I combined analysis of chromatin immunoprecipitation sequencing and transcriptomics data to enable dynamic gene regulatory network modelling. This illustrated the potential involvement of EDFs in cross-regulation between ethylene and other hormones. The transcriptional repressor activity of the EDFs was determined and their functions compared to the master TF ETHYLENE INSENSITIVE3 were identified during the ethylene response. Taken together, my thesis provides a comprehensive understanding of how hormonal cross-regulation regulates gene expression during plant hormone responses. I determined the fundamental principles underlying this process. This thesis also increased our understanding of how TFs function downstream of the master regulatory TFs to coordinate the hormone-induced transcriptional cascade.

STATEMENT OF AUTHORSHIP

This thesis includes work by the author that has been published or accepted for publication as described in the text. Except where reference is made in the text of the thesis, this thesis contains no other material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma.

No other person's work has been used without due acknowledgment in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

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1. CHAPTER 1. Introduction

Plants are exposed to a wide variety of environmental stimuli during their life cycles. Upon experiencing stress conditions, rapid and precise responses are essential for plant survival in nature. Plant hormones are small but crucial signalling molecules involved in growth, development and stress resistance. To date, ten classes of hormones have been identified and characterized in the plant kingdom: abscisic acid (ABA), auxins, brassinosteroids (BRs), cytokinins (CKs), ethylene (ET), gibberellins (GAs), jasmonates (JAs), salicylic acid (SA), strigolactones (SLs) and the more recently identified karrikin (KAR) (Richards et al., 2001; Rogg and Bartel, 2001; Wang et al., 2002a; Akiyama and Hayashi, 2006; Dixon et al., 2009; Cutler et al., 2010; Keshishian and Rashotte, 2015; Ahmad et al., 2016; Ding and Ding, 2020; Nolan et al., 2020). Plant hormones enable effective responses in part by broad-scale regulation of gene expression, which is achieved by hormone perception and transduction by cellular receptor proteins, downstream signalling cascades, and ultimately changes in the transcription and translation of responsive genes.

Transcriptional regulation is a fundamental mechanism in the regulation of plant growth, development and environmental stress responses (Kaufmann et al., 2010; Mercer and Mattick, 2013). TFs are proteins that play an important role in transcriptional regulation either by promoting or supressing gene expression by binding to a specific DNA sequence of their target genes (Franco-Zorrilla and Solano, 2017). About 5% of genes in the Arabidopsis genome encode TFs (Riechmann et al., 2000). Arabidopsis TFs are grouped into 58 protein families according to their DNA binding domains (Hong, 2016; Lehti-Shiu et al., 2017; Igler et al., 2018). The small subset of the genome encoding TFs indicates the combinatorial control mechanisms and complexity of transcriptional regulation by TFs. Firstly, TFs can be activators or repressors. While activators activate gene expression by helping general TFs and/or RNA polymerase assemble, repressors result in transcriptional repression by predominantly recruiting co-repressor complexes and/or blocks general TFs and/or RNA polymerase assemble (Payankaulam et al., 2010; Mercer and Mattick, 2013). Secondly, the same TF may be necessary for different protein complexes to target different genes at different developmental stages and in response to different stimuli (Brkljacic and Grotewold, 2017). Finally, a single TF may target hundreds of genes and possibly hundreds of TFs are involved in the regulation of single biological process, such as hormone and low-temperature responses (Chang et al., 2013; Park et al., 2015; Song et al., 2016; Zander et al., 2020).

TFs are indispensable elements in hormone responses (Choi et al., 2000; Chang et al., 2013; Kazan and Manners, 2013; Song et al., 2016; Xie et al., 2018; Zander et al., 2020). TFs that regulate hormone responses belong to different families and can be involved in the regulation of multiple hormone responses. For example, Myelocytomatosis oncogenes homolog (MYC) TFs, especially MYC2 and MYC3, which come from the basic helix-loop-helix (bHLH) TF family, are master TFs of JA response because they activate the expression of a large proportion of JA-responsive genes and loss function of these TFs results in a clear decrease in JA sensitivity (Kazan and Manners, 2013; Zander et al., 2020). MYC2 is also involved in the ABA, ET and SA signalling pathways (Abe et al.,

2003; Zheng et al., 2012; Zhang et al., 2014). ABA signalling pathway requires the functions of many ABA-responsive elements (ABREs) binding factors and several WRKY family TFs (Choi et al., 2000; Furihata et al., 2006; Rushton et al., 2012; Fujita et al., 2013). Understanding transcriptional regulation of multiple hormone responses by TFs is more complex than focusing on a single hormone response, which results in the substantial challenge to determine how TFs influence dynamic expression patterns during hormone cross-regulation.

Plant responses to stimuli are not activated only by a single mechanism but instead result from a complex network of interactions between different signalling pathways (Alazem and Lin, 2015; Hu et al., 2017; Vishal and Kumar, 2018; Stepanova and Alonso, 2019; Altmann et al., 2020; Khan et al., 2020; Pan et al., 2020). This occurs because plants are simultaneously exposed to multiple environmental stimuli, which may create competing or complementary demands that must be reconciled, such as demonstrated in the growth and defence trade-off (Karasov et al., 2017; Figueroa-Macías et al., 2021). In this process, signalling by the defence hormone JA interferes with the activity of the growth-promoting hormone GA to prioritize defence over growth (Hou et al., 2010; Yang et al., 2012). Cross-regulation between hormone signalling pathways allows plant to process internal and environmental signals, then, decide upon appropriate responses in the context of limited resources. Many examples of hormone cross-regulation have been characterised but they only described interactions between pairs or small numbers of hormones (Rowe et al., 2016; Hickman et al., 2019). Cross-regulation in fact occurs at many points between multiple hormones (Altmann et al., 2020; Zander et al., 2020). To fundamentally understand the hormone cross-regulation mechanisms for plant to grow, development and response to stress conditions, and potentially to modify it rationally, it is crucial to identify the key TFs and signalling molecules involved in this process and elucidate how their activity is coordinated across the complex network of hormone interactions. This would help to deliver deeper insight into hormone mediated responses which help plants to adapt to diverse conditions.

1.1 Six hormone signalling pathways in plants

1.1.1 ABA signalling pathway

ABA is one of the best studied plant hormones. ABA regulated processes include bud dormancy, seed dormancy and germination, seedling growth, leaf abscission, fruit ripening and primary root growth, as well as adaptive responses to various stresses. Such stress conditions include drought stress, salt tolerance, UV-B radiation, pathogen attack and heavy metal tolerance in response to cadmium (Cd) (Cutler et al., 2010; Leng et al., 2014; Osakabe et al., 2014; Yoshida et al., 2014; Lievens et al., 2017; Vishwakarma et al., 2017; Vos et al., 2019; Chen et al., 2020).

ABA is perceived by a family of 14 intracellular receptors (Ma et al., 2009; Park et al., 2009). Park and colleagues named the receptors as PYRABACTIN RESISTANCE 1 (PYR) / PYR1-LIKE1-13 (PYLs) because they performed the genetic dissection experiments using pyrabactin as a selective ABA agonist (Park et al., 2009). Ma and colleagues identified these receptors as interactors with ABA

INSENTIVITY 2 (ABI2), a member of Group-A protein phosphatases of the type 2C (PP2Cs), through yeast two-hybrid experiments. Thus the receptors were named as regulatory component of ABA receptor 1- 14 (RCARs) (Ma et al., 2009). The functions of ABA receptors are redundant (Chen et al., 2020).

In Arabidopsis, several components in the ABA signalling pathway were discovered earlier than the ABA receptors. The PP2Cs, which include ABA INSENTIVITY 1 (ABI1) and its homologs ABI2, function as negative regulators to repress downstream kinases and block ABA signalling (Koornneef et al., 1984; Leung et al., 1994; Meyer et al., 1994; Sheen, 1998). SNF1-RELATED PROTEIN KINASE 2 (SnRK2s) are positive regulators in ABA signalling (Fujii et al., 2007). They are plant specific protein kinases, which have 10 members in Arabidopsis (Hrabak et al., 2003). Many SnRK2s are involved in ABA responses, such as SnRK2.6 which functions in ABA-mediated stomatal closure, and SnRK2.2 and SnRK2.3 which play roles in seed germination, dormancy and seedling growth inhibition (Mustilli et al., 2002; Yoshida et al., 2006). ABREs are found in the promoter regions of many ABA-induced genes (Uno et al., 2000). The first ABRE binding factor (ABF), EmBP-1, was identified based on its interaction with an ABRE in the 5' regulatory region of *Em* gene in wheat (Guiltinan et al., 1990). ABFs, including ABF1, ABF2, ABF3, ABF4, and ABA INSENTIVITY 5 (ABI5), are key TFs which activate transcription during ABA responses (Choi et al., 2000; Uno et al., 2000; Furihata et al., 2006; Skubacz et al., 2016).

Protein phosphorylation and dephosphorylation are central characteristics of ABA signalling (Figure 1). The core components, ABA receptors-PP2Cs-SnRK2s, phosphorylate downstream factors to transmit the ABA signal (Umezawa et al., 2013). In the absence of an ABA signal, the negative regulators, PP2Cs, interact with and inhibit the activity of positive regulators SnRK2s. When the cell perceives ABA, signalling starts from the PYR/PYL/RCARs intracellular receptor family. After ABA binding, PYR/PYL/RCARs recruit PP2Cs and form complexes with the PP2Cs, allowing the activation of SnRK2s (Furihata et al., 2006; Fujii et al., 2007; Fujii et al., 2009; Ma et al., 2009; Park et al., 2009). SnRK2s phosphorylate their target substrates, which include many TFs, such as AREB/ABF-type basic leucine zipper (bZIP) TFs. As a result, the ABA-induced transcriptional cascade is initiated (Kobayashi et al., 2005; Fujita et al., 2013; Umezawa et al., 2013).

1.1.2 BR signalling pathway

BRs, which include brassinolide and its related steroids, are growth-promoting plant hormones that cause cell elongation and division, dwarfism, reduced fertility, delayed senescence and photomorphogenic defects in various plant species (Mandava, 1988; Yin et al., 2002; Lee et al., 2015; Ibanez et al., 2018; Planas-Riverola et al., 2019; Nolan et al., 2020). BRs (termed brassins) were first extracted by Mitchell and colleagues from the rape pollen (*Brassica napus* L.) (Mitchell et al., 1970). Grove and associates successfully determined brassinolide structure and validated its biological activity with the observation that crystalline brassinolide produced significant increase in bean internode elongation (Grove et al., 1979). Since then, analogues of brassinolide have been isolated

and synthesized. Subsequently the components of BR signalling pathways were identified through multiple genetic screens of BR-deficient and BR-insensitive mutants in Arabidopsis.

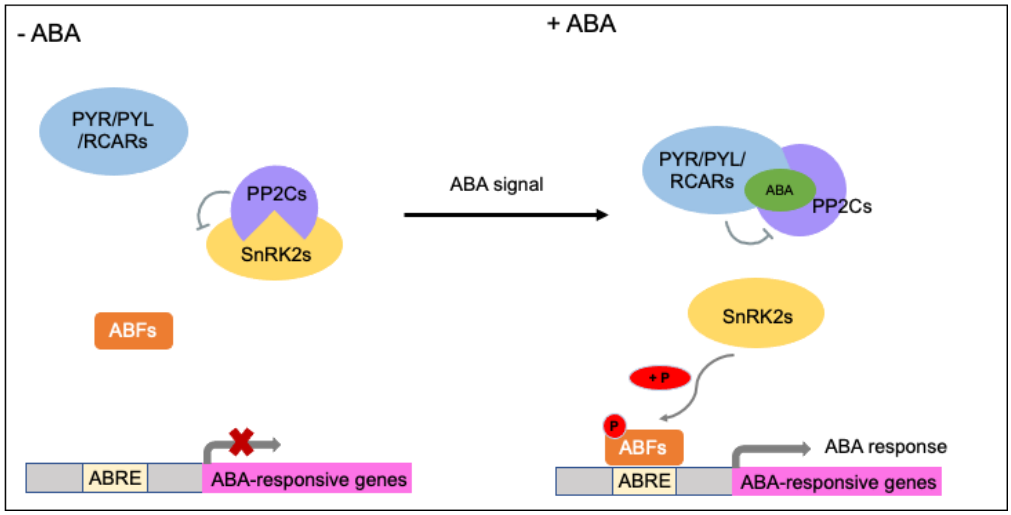

Figure 1. A simplified diagram of the ABA signalling cascade with and without ABA.
In the absence of ABA signals, PP2Cs interact with and repress the activity of SnRK2s, which block ABA signalling. In the presence of ABA signals, ABA binding triggers PYR/PYL/RCARs to recruit PP2Cs and form an ABA-receptors-PP2Cs complex. This leads to the activation of SnRK2s. SnRK2s then phosphorylate ABF TFs. As a result, activated ABFs bind to the ABRE promoter regions of ABA-responsive genes and initiate ABA responses. Adapted from Park et al. (2009), Cutler et al. (2010) and Chen et al. (2020).

BRASSINOSTEROID INSENSITIVE 1 (BRI1) is the primary receptor in the BR signalling pathway. It belongs to a large family of leucine-rich repeat (LRR) receptor-like kinases (RLK) in Arabidopsis. BRI1 is located in the plasma membrane and its cytoplasmic kinase domain contains multiple phosphorylation sites (Kim and Wang, 2010; Nolan et al., 2020). BRI1 can bind to brassinolide, the most biologically active of BRs, *via* its extracellular domain (Wang et al., 2001). There are three close homologs of BRI1 – BRI1 LIKE 1 (BRL1), BRL2 and BRL3, in the Arabidopsis genome. BRL1 and BRL3 were found to bind brassinolide with high affinity and are putative BR receptors which function specifically in provascular differentiation (Cano-Delgado et al., 2004). However, the expression patterns of these homologs differ from BRI1. BRL1/BRL3 are expressed specifically in some tissues, such as root vascular stem cells and vascular tissues in the primary root, whereas BRI1 is expressed ubiquitously in the root (Cano-Delgado et al., 2004; Salazar-Henao et al., 2016; Fabregas et al., 2018).

The BR mediated signalling pathway is an intracellular phosphorylation relay cascade. BR is perceived by and binds directly to the extracellular domain of BRI1. Then BR signals are transduced *via* the phosphorylation and activation of the cytoplasmic kinase domain of BRI1. A second LRR RLK protein - BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) - is a cofactor that forms a heterodimer with BRI1 by reciprocal phosphorylation during BR binding. This transphosphorylation enhances the signal capacity of BRI1 and in turn initiates the BR transcriptional cascade (Li et al., 2002; Russinova et al., 2004). BRASSINOSTEROID-INSENSITIVE 2 (BIN2) functions as a negative regulator in the

BR signalling pathway because loss of function of BIN2 partially suppressed *bri1* mutation while overexpression of BIN2 inhibited BR signalling (He et al., 2002). BR-SIGNALLING KINASES (BSKs) are plasma membrane proteins associated with BRI1 in an inactive state in the absence of BR signals which function upstream of BIN2 (Tang et al., 2008). A protein phosphatase, BRI1 SUPPRESSOR 1 (BSU1) also functions upstream of BIN2. The activity of BSU1 might be enhanced by BSKs binding (Kim et al., 2011). BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1) are phosphorated by and function downstream of BIN2 (He et al., 2002; Yin et al., 2002). These two TFs are key components in BR signalling which regulate downstream BR-responsive genes and ultimately confer plant growth and developmental responses (Wang et al., 2002b; Yin et al., 2002; Li and Deng, 2005).

When BR levels are low, BZR1 and BES1 are phosphorylated by BIN2 and targeted for degradation by the proteasome (Figure 2). Upon BR perception, BR binding to BRI1 activates BRI1 kinase and promotes the interaction between BRI1 and BAK1, which result in the phosphorylation of BSKs and their dissociation from BRI1. Then the phosphorylated BSKs phosphorylate BSU1. As a result, the activated BSU1 inhibits BIN2 by dephosphorylating the phospho-tyrosine residue of BIN2 (Kim and Wang, 2010; Kim et al., 2011), which leads to the dephosphorylation and nuclear accumulation of BZR1 and BES1 proteins (Kim and Wang, 2010; Clouse, 2011; Nolan et al., 2020). Secondary suppression of BR signalling occurs through BZR1, in which BZR1 confers to feedback regulation by repressing the expression of BR biosynthesis gene, and ultimately to optimal BR levels (He et al., 2005).

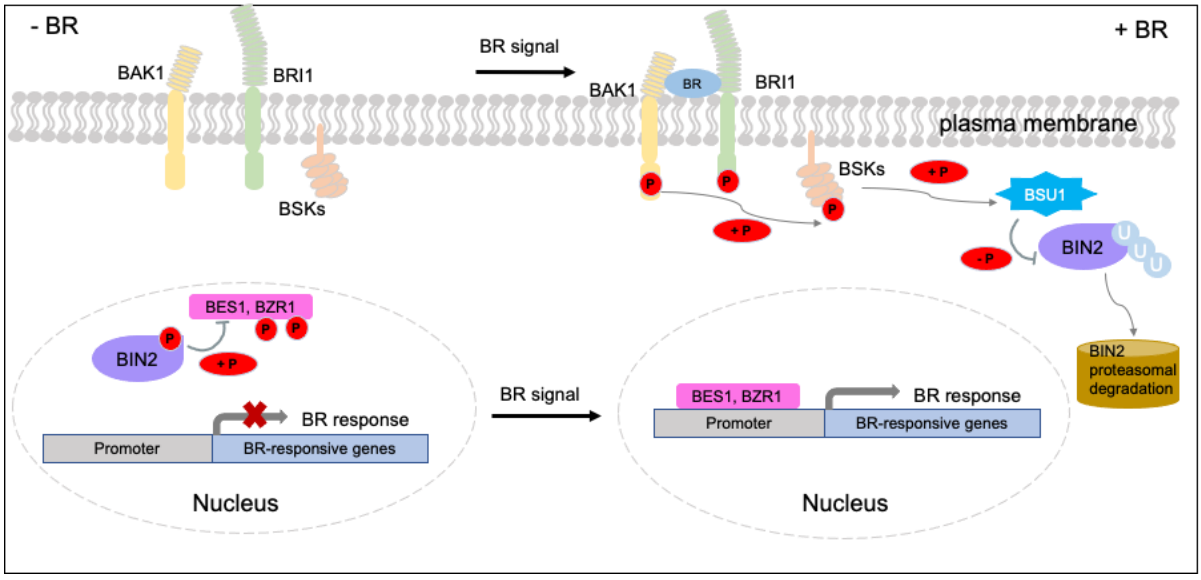

Figure 2. A simplified BR signalling cascade.

In the absence of BR signals, BIN2 phosphorylates BES1 and BZR1 TFs and inhibit their activity. In the presence of BR signals, BR binds to the receptor BRI1, activates BRI1 kinase and promotes the interaction between BRI1 and BAK1. This results in the phosphorylation of BSKs. The phosphorylated BSKs activate BSU1. The activated BSU1, in turn, dephosphorylates BIN2 and inhibits BIN2 activity. As a result, dephosphorylation of BES1 and BZR1 leads to the promotion of BR-induced gene expression. Adapted from Nolan et al. (2020).

1.1.3 ET signalling pathway

ET is the only gaseous phytohormone in the plant kingdom. It regulates seed germination, leaf and floral senescence, fruit ripening and abscission. Moreover, ET can mediate a wide variety of abiotic and biotic stress responses, such as nutrient deficiency, flooding and drought responses, salt stress and pathogen attack resistance (Bleecker and Kende, 2000; Iqbal et al., 2017; Harkey et al., 2019; Binder, 2020).

The basic ET signalling pathway has been elucidated through elegant molecular genetic screens (Bleecker et al., 1988; Ecker, 1995; Roman et al., 1995; Johnson and Ecker, 1998; Wang et al., 2002a). ET is perceived by a family of five receptors which are predominantly located at the endoplasmic reticulum (ER) (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998; Shakeel et al., 2013). The receptors are ETHYLENE RECEPTOR 1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR 1 (ERS1), ERS2 and ETHYLENE INSENSITIVE 4 (EIN4) (Mount and Chang, 2002; Wang et al., 2006). These five ET receptors are negative regulators with overlapping and nonoverlapping functions in the signalling pathway (Shakeel et al., 2013).

In the absence of ET, the receptor complex is tightly associated with and activates the inhibitory protein CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) (Kieber et al., 1993; Roman et al., 1995) (Figure 3). CTR1 phosphorylates ETHYLENE INSENSITIVE 2 (EIN2) which promotes the proteolytic degradation of EIN2. This prevents signal transduction through EIN2, thus suppressing the ET response (Alonso et al., 1999). Upon ET binding, the receptors are inactivated, then the inhibition of CTR1 activity results in the cleavage, migration and nuclear localization of EIN2 C-terminal domain (EIN2-CEND) (Ju et al., 2012; Qiao et al., 2012). In the nucleus, EIN2-CEND increases the activity of master TFs ETHYLENE INSENSITIVE 3 (EIN3) / EIN3 LIKE protein 1 (EIL1) *via* ENAP1, which leads to primary transcriptional changes (Ju et al., 2012; Binder, 2020). EIN3 and EILs are required for a large proportion of ethylene responses (Chao et al., 1997; Solano et al., 1998; Chang et al., 2013). EIN3 binds promoters of ET-regulated genes, including genes encoding TFs, such as ETHYLENE RESPONSE FACTORS (ERFs) and ETHYLENE RESPONSE DNA-BINDING FACTORS (EDFs), stimulating their expression and thus triggering broad downstream transcriptome changes in response to ET (Solano et al., 1998; Alonso et al., 2003; Alonso and Stepanova, 2004).

Other elements of the ET response beyond the core ET signalling pathway have been identified as well. EIN3-BINDING F-BOX PROTEIN 1 (EBF1) and EBF2 are two F-box proteins. They can physically interact with EIN3/EIL1 and trigger the degradation of EIN3/EIL1 through a ubiquitin/proteasome pathway in the absence of ET (Guo and Ecker, 2003; Potuschak et al., 2003). When a plant perceives ET signals, EIN2-CEND supresses the EBFs-mediated EIN3 degradation (Li et al., 2015; Merchante et al., 2015). A suppression response following the nuclear translocation of EIN2-CEND, in which CTR1 relocates from the ER to the nucleus and promotes degradation of EIN3 by directly interacting with EBF proteins. This suppression is necessary to optimize ET responses under various environmental conditions (Lee et al., 2020).

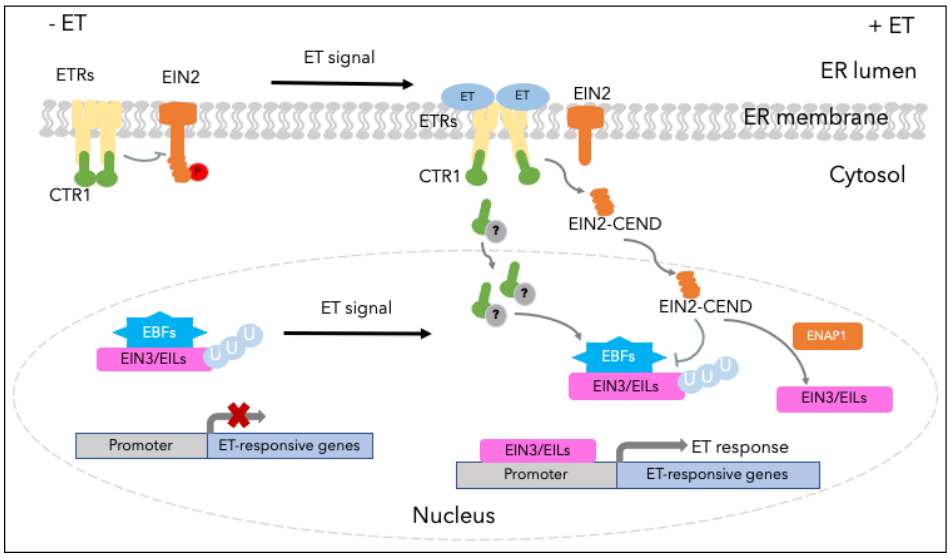

Figure 3. A simplified model of ET signalling cascade with and without ET signal.

In the absence of ET, the receptor ETRs interact with and activate CTR1. The ETRs-activated CTR1 then phosphorylates EIN2, which leads to the degradation of EIN2 and repression of ET signalling. In the presence of ET, the receptors became inactive, which results in the inhibition of CTR1 activity. As a result, EIN2 is no longer phosphorylated and the EIN2-CEND is proteolytically cleaved and translocates to the nucleus. There it inhibits the EBFs-mediated EIN3/EILs degradation and increases the activity of the TFs EIN3/EILs. This ultimately leads to the initiation of a primary ET response. After EIN2-CEND translocates to the nucleus, CTR1 is released from the receptor complex and relocates to the nucleus. The nuclear-localized CTR1 can promote the degradation of EIN3 *via* EBFs, which fine-tunes ET responses. Adapted from Binder (2020) and Lee et al. (2020).

1.1.4 JA signalling pathway

JAs, which include jasmonic acid (JA) and its derivatives, such as jasmonoyl-L-isoleucine (JA-Ile) and methyl jasmonate (MeJA), are plant specific signalling molecules. They have roles in response to various environmental stresses, such as microbial pathogen attack, mechanical/biotic wounding, water deficit, high salinity and low temperature (Dar et al., 2015; Riemann et al., 2015; Ahmad et al., 2016; Per et al., 2018). Moreover, JAs are implicated in the regulation of developmental processes in plants, such as root growth inhibition, fruit ripening, senescence, male fertility and pollen development (Santino et al., 2013; Zhai et al., 2015; Hu et al., 2017; Huang et al., 2017).

The primary elements in the JA signalling pathway have been elucidated (Figure 4). JA-Ile is the receptor-active form of JAs in plants (Huang et al., 2017). In the absence of JA signals, JASMONATE-ZIM DOMAIN (JAZ) proteins repress the activity of MYC TFs, which are the master TFs regulating JA responsive genes (Lorenzo et al., 2004; Kazan and Manners, 2013; Zhang et al., 2015; Zander et al., 2020). This repression might be achieved by direct repression of transcription at target genes by JAZs or the recruitment of co-repressors such as TOPLESS (TPL) by JAZs (Chung et al., 2009; Pauwels et al., 2010; Kazan and Manners, 2012; Zhang et al., 2015). In the presence of JA-Ile, the JA receptor protein CORONATINE INSENSITIVE 1(COI1), which is also a part of a Skp–Cullin–F-box (SCF) E3 ubiquitin ligase complex (SCF^{COI1}), binds JAZ proteins, resulting in

ubiquitination of JAZs by the 26S proteasome followed by their degradation (Xie et al., 1998; Chini et al., 2007; Thines et al., 2007). Simultaneously, MYC TFs are released to activate the expression of downstream target genes (Wasternack and Hause, 2013; Hickman et al., 2017). These JA-responsive target genes are involved in the growth and development of plants, stress responses and adaptation to the environment (Pauwels et al., 2008; Santino et al., 2013; Howe et al., 2018; Yang et al., 2019). Furthermore, many other JAZ-interacting TFs, such as EIN3 and ABI5, contribute to the complexity and specificity of signalling outputs as well (Zhu et al., 2011; Kazan and Manners, 2012; Zhang et al., 2015; Ju et al., 2019). The function of JAZ proteins linking JA-Ile perception to the activity changes of key TFs from diverse hormone pathways, make JAZ proteins a hub for cross-regulation (Chini et al., 2007; Kazan and Manners, 2012).

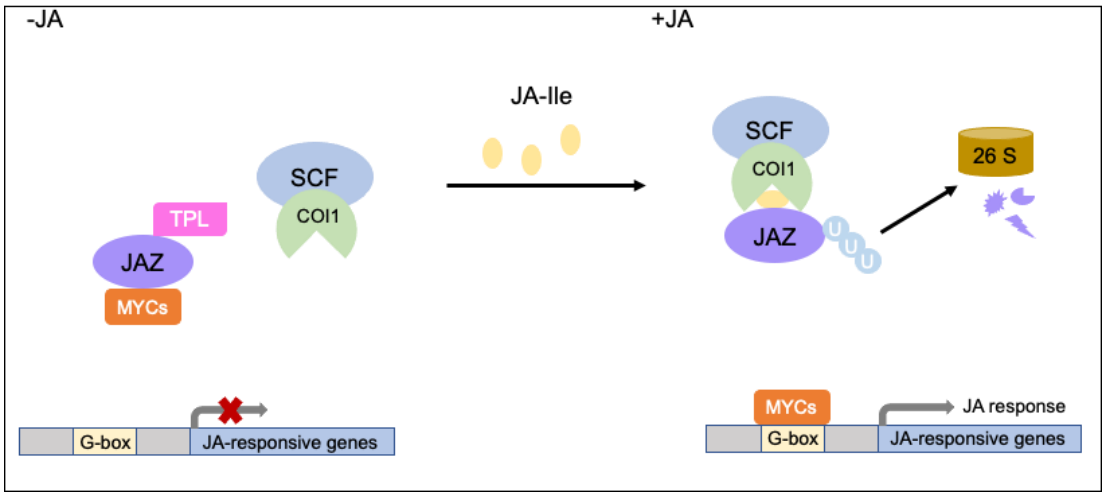

Figure 4. A simplified diagram of JA signalling cascade with and without bioactive JA.
In the absence of JA signals, the expression of JA-responsive genes is restrained by JAZ transcriptional repressors. JAZs bind and inhibit the MYCs through direct inhibition or recruiting TPL co-repressors. In the presence of JA signals, the bioactive JA-Ile facilitates the formation of a co-receptor complex with JAZs and SCF^{COI1}. This formation leads to the ubiquitination of JAZs by the 26S proteasome followed by their degradation. As a result, downstream MYC TFs are released to activate JA-responsive genes and JA responses. Adapted from Song et al. (2014) and Huang et al. (2017).

1.1.5 SA signalling pathway

SA is an endogenous signal in plants and well known as a plant immunity-related hormone, which plays a central role in mediating local and systemic-acquired resistance (SAR) against biotrophic pathogens (An and Mou, 2011; Vicente and Plasencia, 2011; Miura and Tada, 2014; Koo et al., 2020). SA was first reported to be involved in plant defence responses with the observations that exogenous application of acetylsalicylic acid (aspirin) can induce pathogenesis-related (PR) protein accumulation and resistance against tobacco mosaic virus in *Nicotiana tabacum* (White et al., 1983). Subsequently SA was found to act as a protective hormone signal regulating hypersensitive response-associated cell death and leading to SAR after recognition of pathogens (Yalpani et al., 1991; Delaney et al., 1994; Radojičić et al., 2018).

The major downstream components involved in the SA-triggered immune signalling pathway were identified using genetic screens and molecular approaches. NONEXPRESSER OF PR GENES 1 (NPR1) was demonstrated to be an SA receptor with the observations that NPR1 protein can bind to SA directly and specifically (Wu et al., 2012). NPR1 was also identified as an SA binding protein in a high throughput screen (Manohar et al., 2015). However, NPR3 and NPR4, not NPR1 were proposed to act as SA receptors by functioning as adaptors of Cullin 3 E3 ligase and mediating NPR1 degradation, with the evidence that NPR3 and NPR4 bind to SA whilst NPR1 does not and that *npr3npr4* double mutant plants have increased NPR1 accumulation and are insensitive to SAR induction (Fu et al., 2012). Another study demonstrated that NPR1, NPR3 and NPR4 are all SA receptors with opposite functions, NPR1 is transcriptional co-activator whereas NPR3 and NPR4 are transcriptional repressors, in the SA signalling pathway (Ding et al., 2018). It has also been proposed that NPR2 has a role in SA perception in a functionally redundant manner with NPR1 (Castelló et al., 2018).

The nuclear localization of NPR1 is crucial for SA-mediated gene expression (Cao et al., 1994). NPR1 is a central transcriptional coregulator of SA perception, mediating *in vivo* recruitment of the bZIP TGACG motif-binding (TGA) TFs to the SA-responsive elements and triggering the expression of *PR* genes (Johnson et al., 2003). There are at least two forms of NPR1 in plant cells; the inactive oligomeric and active monomeric forms (Figure 5). Their conformations are sensitive to cell redox state (Mou et al., 2003; Tada et al., 2008). In normal cells or in the absence of SA inducers, e.g., pathogen attacks, NPR1 exists in the cytoplasm as an oligomer through an intermolecular disulphide bond (Tada et al., 2008). However, when there is pathogen infection and the SA level is elevated, the changed cell redox state triggers the monomerization of NPR1 *via* the thioredoxin (TRX)-h3 and TRX-h5 activity (Tada et al., 2008). The monomeric NPR1 is released and translocated to the nucleus where it interacts with TGA TFs and activates the expression of *PR* genes which ultimately initiate the SA-associated transcriptional cascade (Spoel and Dong, 2012; Ding and Ding, 2020).

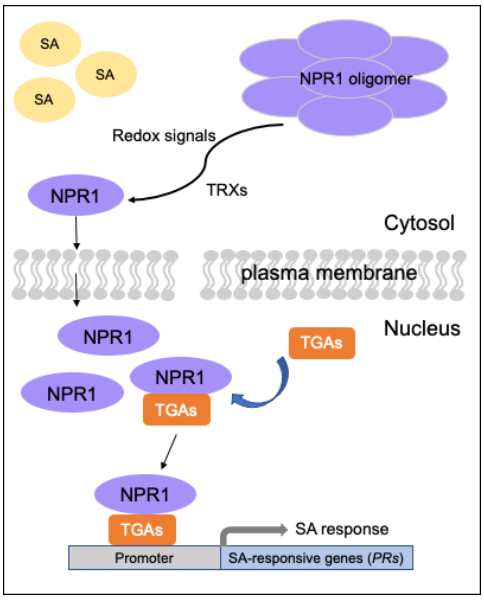

Figure 5. A simplified model for the NPR1-mediated SA signalling pathway.

In normal conditions, NPR1 exists in the cytoplasm as an oligomer. In the presence of SA signals, cell redox signals trigger the monomerization of NPR1 *via* the activity of TRXs. Monomeric NPR1 is released from the cytosol and is translocated to the nucleus. Then it recruits TGA TFs to form a complex which can bind to the promoters of and activate the expression SA-responsive genes, such as *PR* genes. This process ultimately initiates the SA-associated transcriptional cascade. Adapted from Pajerowska-Mukhtar et al. (2013) and Huang et al. (2020).

1.1.6 SL and KAR signalling pathways

SLs are a class of carotenoid derivatives that act as plant hormones with roles in regulating shoot branching repression, internode elongation promotion, root architecture regulation and host-symbiont interactions (Jia et al., 2017; Waters et al., 2017; Bürger and Chory, 2020). Several lines of evidence indicate that SLs also function in plant abiotic stresses responses, such as drought, salt stresses and phosphate starvation (Ha et al., 2014; Decker et al., 2017). SLs were first identified as rhizosphere signalling molecules stimulating seed germination for the parasitic weeds *Striga* and *Orobanche* (Cook et al., 1966; Bouwmeester et al., 2003). Much later, SLs were reported as a novel type of plant hormones controlling plant above-ground architecture with the observation that application of a synthetic analogue of SLs, GR24, can affect shoot branching in pea and inhibit tillering in rice (Gomez-Roldan et al., 2008; Umehara et al., 2008). Since then, enormous progress has been made in elucidating the signal perception and transduction of SL signals.

KARs were initially identified as seed germination stimulants isolated from smoke derived from burning plant and cellulose material (Flematti et al., 2004; Long et al., 2010; Flematti et al., 2013). Unlike the other hormone signalling pathways, which have been studied for many decades, KARs have been identified relatively recently. As a family of small, structurally-related butanolide molecules discovered in wildfire smoke, KARs play a key role in various biological processes, including seed dormancy release, germination regulation, seedling establishment and root development (Dixon et al., 2009; Nelson et al., 2009; Villaécija-Aguilar et al., 2019; Yao and Waters, 2020).

SLs and KARs have high similarity in both chemical structure and signal transduction pathways but have different functions (Waters et al., 2012; Morffy et al., 2016). Three conserved protein groups for SL and KAR signalling have been identified in Arabidopsis. AtD14 (also known as Arabidopsis DWARF14) is the core receptor protein of SL in Arabidopsis, and is conserved in rice, petunia and poplar (Arite et al., 2009; Hamiaux et al., 2012; Waters et al., 2012; Zheng et al., 2016). The KAR receptor is a paralogous protein of AtD14, KARRIKIN INSENSITIVE 2 (KAI2) (Waters et al., 2012). Although KAI2 and AtD14 are receptor proteins for the KAR and SL signalling pathways respectively, they are both involved in signalling triggered by a synthetic analogue of SLs, GR24 (Waters et al., 2012; Scaffidi et al., 2014). Thus, GR24 treatment can initiate both SL and KAR responses. In addition, SL and KAR signalling both depend on the activity of MORE AXILLARY BRANCHES 2 (MAX2), which is an F-box leucine-rich protein and acts as a part of the SCF E3 ubiquitin ligases that ubiquitinate target proteins for degradation by the 26S proteasome (Nelson et al., 2011). The third signalling protein group comprises members from SUPPRESSOR OF MAX2-LIKE (SMXL) protein family. SMAX1 and SMXL2 act in downstream of KAI2-MAX2-mediated development processes, whereas SMXL6, SMXL7 and SMAXL8 mediate SL-dependent responses (Soundappan et al., 2015; Wang et al., 2015; Waters et al., 2017; Villaécija-Aguilar et al., 2019). SMXLs are putative MAX2 targets and might function in a transcriptional corepressor complex by interacting with TPL and TOPLESS-RELATED (TPR) proteins through ethylene-responsive element binding factor amphiphilic repression (EAR) motifs (Soundappan et al., 2015). Although SL and KAR signalling pathways share

components, the usage of different receptors and SMXL members distinguishes these two hormone signals and brings about their different functions (Wang et al., 2020; Yao and Waters, 2020).

The structure of the SL signalling pathway is becoming increasingly well understood (Figure 6). In the absence of SL signals, SMXL6, 7, 8 work with TPL/TPR corepressors and unknown TFs to repress the expression of SL-responsive genes (Soundappan et al., 2015; Wang et al., 2020). Upon perception of SL signals, AtD14 bind and hydrolyse SL, triggering the formation of a receptor-SCF^{MAX2}-SMXLs complex. This complex functions in the ubiquitination and proteolytic degradation of SMXL repressive proteins, which relieves the repression of SL-responsive genes and ultimately brings about transcriptome changes (Wang et al., 2015; Wang et al., 2020; Yao and Waters, 2020). Secondary suppression of SL signalling occurs through SMXL6, in which SMXL6 can directly bind to the promoters of itself, and the promoters of SMXL7 and SMAXL8 to form a negative feedback loop and ultimately maintain SL signalling homeostasis (Wang et al., 2020). The molecular mechanisms for KAR and SL signalling are similar in Arabidopsis, with components shared (Yao and Waters, 2020). However, there are some differences. For example, the degradation of KAI2 is independent of MAX2 in KAR signalling, while AtD14 was likely degraded in a MAX2-dependent manner (Waters et al., 2015). Moreover, genetic evidence explaining how KAR activates the KAI2 receptor and how SMAX1, SMXL2 are degraded is lacking.

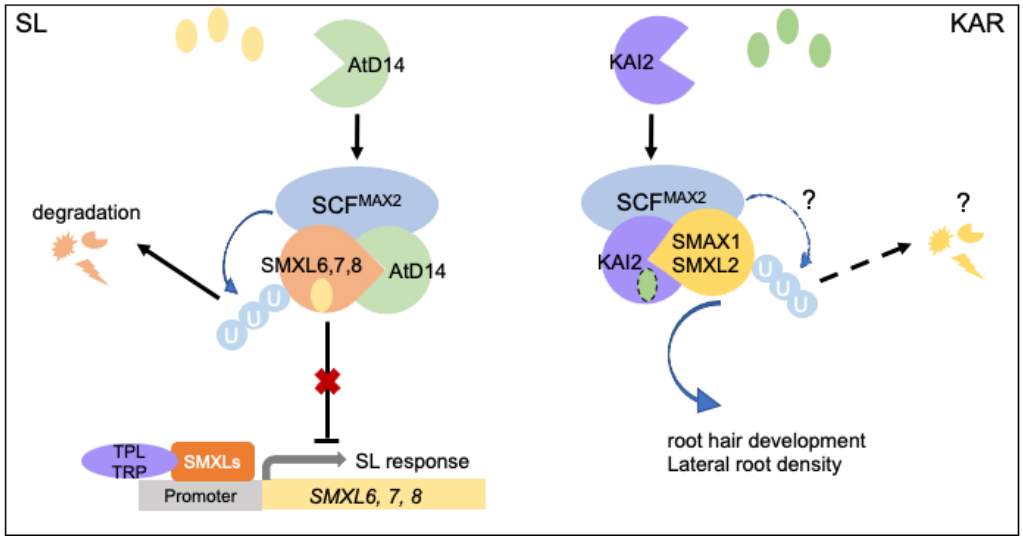

Figure 6. A simplified model for SL and KAR signalling in Arabidopsis.

(Left) In normal conditions, SMXLs work with TPL/TPR corepressors to repress the expression of SL-responsive genes. Upon perception of SL, AtD14 binds and hydrolyses the SL, triggering the formation of a receptor-SCF^{MAX2}-SMXLs complex. Then the SMXL repressive proteins are targeted for ubiquitination and degradation, which relieves the repression of SL-responsive genes and initiates the SL-associated transcriptional cascade. (Right) The receptor, SCF^{MAX2} and SMXL proteins are three functionally conserved groups for SL and KAR signalling. The connections, which are inferred from published evidence (or for KAR signalling from SL signalling) are shown by dashed lines. Adapted from Villaécija-Aguilar et al. (2019), Yao and Waters (2020) and Wang et al. (2020).

1.2 Hormone cross-regulation in plants

Each hormone has its canonical and distinct signalling pathway as describe in section 1.1. However, environmental signals usually result in responses regulated simultaneously by multiple signalling pathways. The regulation of a single biological process might involve multiple hormones, and each hormone pathway influences other hormone pathways, forming a complex network mixing of synergistic, antagonistic interactions. ABA, ET, JA and SA are four main hormones in plant defence responses. JA and ET co-regulate plant defence to necrotrophic pathogen infection (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003; Berrocal-Lobo and Molina, 2004), while JA and ABA work cooperatively to mediate chewing insect resistance (Vos et al., 2019). However, in many cases, SA and JA function antagonistically in plant defence pathways. For example, SA application can supress the expression of JA-induced defence gene *LIPOXYGENASE 2 (LOX2)*, *VEGETATIVE STORAGE PROTEIN* (*VSP)* and *PLANT DEFENSIN 1.2* (*PDF1.2)* in response to pathogen infections (Spoel et al., 2003). The SA accumulation induced by *Pseudomonas syringae* pv tomato DC3000, is associated with the suppression of JA accumulation (Ndamukong et al., 2007; Robert-Seilaniantz et al., 2011). Moreover, the action of hormones may differ according to environmental condition changes or responses to different stimuli. For example, auxin, BR, ET and GA all stimulate hypocotyl development of light-grown Arabidopsis seedlings, whereas BR and ET, inhibit hypocotyl growth when seedlings are grown in the dark but, auxin and GA continue to stimulate it (Van De Poel et al., 2015).

The integration of hormone signalling pathways often occurs by one hormone regulating the signalling pathway elements or the biosynthetic genes of a second hormone (Nemhauser et al., 2006; Jaillais and Chory, 2010). The regulations of the signalling pathway elements can occur, affecting hormone receptors, core signalling proteins and TFs in a signalling cascade. Furthermore, hormone signalling pathways may interact with other signalling mechanisms, such as MITOGEN-ACTIVATED PROTEIN KINASES (MPKs, also known as MAPKs) cascade (Raja et al., 2017; Bigeard and Hirt, 2018; Jagodzik et al., 2018).

1.2.1 Signalling pathway elements in the integration of hormone signalling pathways

1.2.1.1 Hormone receptor proteins mediate hormone cross-regulation

The roles of ABA receptors in hormone cross-regulation have been demonstrated by several studies (Lackman et al., 2011; Aleman et al., 2016). For example, expression of *PYL4* and *PYL5* was regulated by MeJA treatment. JA responses were altered in *pyl4* and *pyl5* knockout mutants (Lackman et al., 2011). The ABA receptor PYL6, which belongs to the same subgroup as PYL4 and PYL5, interacts directly with the JA signalling master TF MYC2 and can modulate MYC2 transcriptional activity. This interaction between PYL6 and MYC2 forms a possible link between ABA and JA signalling (Aleman et al., 2016). Moreover, the expression of *PYL4/PYL5/PYL6* can be modulated by many other elicitors, not only MeJA, suggesting the regulation ABA receptors through transcription might be an important feature in ABA-other hormone cross-regulation (Lackman et al., 2011).

The SA receptor NPR1 is also involved in hormone cross-regulation. NPR1 is required for the SA-mediated suppression of expression of three JA-responsive genes *LOX2*, *VSP* and *PDF1.2* in response to infection by *Pseudomonas syringae* pv *tomato* DC3000 (Spoel et al., 2003). Exogenous SA works antagonistically with ET to regulate etiolated seedling growth *via* NPR1 as well. NPR1 interacts with EIN3 and disrupt the interaction between EIN3 and its target gene *HOOKLESS1 (HLS1)*, which functions downstream of EIN3 in regulation of apical hook development, to reduce the apical hook angle of etiolated Arabidopsis seedlings (Lehman et al., 1996; Jin and Zhu, 2019; Lyu et al., 2019; Huang et al., 2020). Moreover, NPR1 might be involved in cross-regulation between BR and SA, because the exogenous application of BR is unable to confer salt stress tolerance or thermotolerance during seed germination in the *npr1-1* mutant seedlings (Divi et al., 2010).

1.2.1.2 JAZ and DELLA proteins mediate hormone cross-regulation

JAZ proteins, which play linking roles in the JAZ-MYC module of JA signalling pathway, are able to physically interact with numerous TFs to modulate cross-regulation between JA and other hormones (Qi et al., 2011; Zhu et al., 2011; Jiang et al., 2014; Ju et al., 2019; Pan et al., 2020). JAZ repressors are involved in ABA responses by interacting with ABI TFs, such as ABI3 and ABI5 (Ju et al., 2019; Pan et al., 2020). ABA treatment induces expression of JA biosynthesis genes and promotes the accumulation of JA. As a result, JAZ repressors are degraded which then de-represses activity of ABI5, promoting ABA-induced gene expression (Ju et al., 2019). In addition, JAZ proteins mediate cross-regulation between JA and ET in response to necrotrophic fungi infections. They do so by recruiting an RPD3-type histone deacetylase corepressor and then inhibiting the activity of key TFs, EIN3 and EIL1, in ET signalling (Zhu et al., 2011).

DELLA proteins play prominent roles in regulation of plant growth and development. They do so by acting as negative nuclear regulators of GA signalling pathway as well as modulating the balance between GA with other hormones, such as ABA, auxin, ET, JA and SA (Achard et al., 2003; Fu and Harberd, 2003; Navarro et al., 2008; Hou et al., 2010; Yang et al., 2012; Lim et al., 2013). Auxin enhances the GA-mediated degradation of DELLA proteins, which promotes the growth of Arabidopsis roots, whereas ET delays GA-mediated degradation of DELLAs to maintain the apical hook structure of etiolated Arabidopsis seedlings (Achard et al., 2003; Fu and Harberd, 2003). DELLA proteins might function as integrative regulators to fine-tune plant growth-defence dynamics by modulating the JA and GA signalling pathways (Navarro et al., 2008; Hou et al., 2010; Yang et al., 2012; Li et al., 2019). For example, in response to JA, but in the absence of GA, DELLAs promote MYC2 release from JAZ/MYC2 complexes by competing with MYC2 to bind to JAZs. On the contrary, GA promotes DELLA degradation which allows JAZ-MYC2 binding and thus represses JA signalling (Hou et al., 2010). Conversely, JAZ9 inhibits the interaction of DELLAs and a target protein, the growth-promoting TF PHYTOCHROME INTERACTING FACTOR 3 (PIF3), which supresses GA responses (Yang et al., 2012).

Given the existence of transcriptional repression mechanisms in several hormone signalling pathways, such as DELLA proteins in GA, JAZ proteins in JA, and auxin/indole-3-acetic acid

(AUX/IAA) proteins for auxin (Gray et al., 2001; Rogg and Bartel, 2001; Freire-Rios et al., 2020), it is interesting to investigate novel ways in which repressors may function as points of hormone cross-regulation.

1.2.1.3 TFs are involved in hormone cross-regulation

The master TFs MYC2, in the JA signalling pathway, and EIN3/EILs, in the ET signalling pathway, act as switches in hormone responses and can modulate multiple environmental response signals. For example, MYC2 and EIN3 act antagonistically in JA/ET regulation of Arabidopsis apical hook development. This mechanism works at two levels. Firstly, JA-activated MYC2 physically interacts with EIN3 and represses its transcriptional activator activity. Secondly, MYC2 directly binds to the promoter of EBF1 and induces its expression, which promotes the degradation of EIN3 (Zhang et al., 2014). EIN3/EIL1 are an integration point mediating JA and ET synergy in the regulation of root development and necrotrophic pathogen defence. During these processes, EIN3/EIL1 are targeted and repressed by JAZs under normal conditions, JA application results in the degradation of JAZs and thus releases the activity of EIN3/EIL1, which promote the synergy in ET/JA gene expression (Zhu et al., 2011). MYC2 may function in cross-regulation between the ABA and JA signalling pathways, because it is a transcriptional activator in the ABA signalling pathway and positively regulates the ABA-induced gene *RD22* (Abe et al., 1997; Abe et al., 2003; Lorenzo et al., 2004).

Many other TFs are involved in hormone cross-regulation. SA-mediated suppression of JA signalling can be achieved *via* several TGA and WRKY TFs (Li et al., 2004; Caarls et al., 2015). Two TFs from a subgroup of the plant-specific APETALA 2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily, ERF1 and OCTADECANOID RESPONSIVE ARABIDOPSIS 59 (ORA59, also known as ERF59), are involved in hormone cross-regulation during plant defence responses (Lorenzo et al., 2003; Pré et al., 2008). Evidence indicates ERF1 is a common element in ET and JA signalling pathways in response to necrotrophic pathogens (Pré et al., 2008). Similarly, ORA59 is an integrator TF in JA and ET cross-regulation that mediates expression of JA and ET responsive genes, such as *PDF1.2,* during infection by the fungus *Botrytis cinerea* (Pré et al., 2008). In addition, the accumulation of ORA59 is significantly reduced by SA which suppresses the JA signalling pathway where it acts as a transcriptional activator (Van der Does et al., 2013). All these discoveries indicate TFs are key points of modulation between multiple hormone signals.

1.2.2 Hormone biosynthetic genes in the integration of hormone signalling pathways

Hormone cross-regulation can be achieved by influencing hormone biosynthesis. ET biosynthesis is one such case. Two major enzyme families are required for ET biosynthesis: 1-aminocyclopropane-1-carboxylic acid synthases (ACSs) and 1-aminocyclopropane-1-carboxylic acid oxidases (ACOs). ACSs and ACOs are responsible for the conversion of S-adenyl-L-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC), then ACC to ET, respectively (Chae and Kieber, 2005; Vanderstraeten and Van Der Straeten, 2017). ACSs are the rate limiting enzymes and might be convergence points for integrating ET response with other hormones because their stability is

regulated by other hormones (Han et al., 2010; Ludwików et al., 2014; Lee et al., 2017; Seo and Yoon, 2019; Marczak et al., 2020). For example, under oxidative stress conditions, the ABA signalling component ABI1 destabilizes ACS6 by interacting with and dephosphorylating the C-terminal fragment of ACS6 (Ludwików et al., 2014). Degradation of ACS7 is hampered in PP2Cs knockout plants, further suggesting the role of ABA signalling elements in the regulation of ET biosynthesis process (Marczak et al., 2020). BR application can affect the biosynthesis of ET by promoting transcription of *ACS5* and increasing the stability of the ACS5 protein in etiolated Arabidopsis seedlings (Hansen et al., 2009). In addition, SL application can promote ET biosynthesis in etiolated Arabidopsis seedlings by regulating the expression of *ACO* genes (Lee and Yoon, 2020).

Additional examples demonstrate the links between hormone biosynthesis and cross-regulation. In Arabidopsis, during infection by *Pseudomonas syringae* pv tomato DC3000, endogenously synthesized SA exerts an antagonistic effect on JA biosynthesis by inhibiting the expression of *LOX2*, which encodes a key enzyme in JA biosynthesis (Spoel et al., 2003). ABA application promotes the expression of two genes, *PLASTID LIPASE 2* and *3*, which encode plastid phospholipases that are involved in JA biosynthesis (Wang et al., 2018). Application of BR to maize leave induces tolerance to oxidative damage by promoting the expression of *VP14*, which encodes an ABA biosynthetic enzyme (Zhang et al., 2011). Overall, these data indicate hormone cross-regulation through hormone biosynthesis is common in plants.

1.2.3 The link between MPKs cascade and hormone cross-regulation

Protein phosphorylation cascades *via* MPKs are involved in intracellular signalling of several stress responses (Cristina et al., 2010). A MPK cascade is minimally composed of three protein kinase groups: MAP KINASE KINASE KINASES (MPKKKs), MAP KINASE KINASES (MKKs) and MPKs, which activate each other sequentially *via* phosphorylation (Cristina et al., 2010; Jagodzik et al., 2018). In Arabidopsis, 20 MPKs have been identified (Bigeard and Hirt, 2018).

Several MPKs function in hormone signalling pathways (Figure 7). The kinase activity of MPK1 and 2 can be induced by wounding, JA and ABA signals and they contribute to SA-mediated leaf senescence (Ortiz-Masia et al., 2007; Zhang et al., 2020). MPK3 negatively regulates defence gene expression and SA accumulation induced by flg22 , which is a 22-amino acid peptide present in bacterial flagellin that triggers plant defence-related responses (Gómez-Gómez et al., 1999; Frei Dit Frey et al., 2014). The activity of MPK3 is regulated by SA under ozone stress conditions (Šamajová et al., 2013). In addition, MPK3 acts in ABA-mediated early seedling development arrest (Lu et al., 2002). MPK6 can be activated by JA and acts in the JA-induced root growth inhibition (Takahashi et al., 2007). MPK6 is a positive regulator of SA-induced leaf senescence, regulating the expression of *NPR1 via* TF WRKY9 (Chai et al., 2014). MPK3 and MPK6 together work downstream of MKK9 to positively regulate EIN3-mediated transcription in response to ET (Yoo et al., 2008). MPK4 is a key factor in regulating ET, JA and SA defence signals (Petersen et al., 2000; Brodersen et al., 2006). MPK9 and 12 are required for the ROS-mediated ABA signalling in guard cells and JA and SA induced stomatal closure (Jammes et al., 2009; Khokon et al., 2015; Khokon et al., 2017). ABA activates a

MPK cascade which includes MPK7 and MPK14 (Danquah et al., 2015). MPK10 plays an essential role in the regulation of auxin transport and auxin-mediated leaf vascular development (Heberle-Bors et al., 2014).

Several MPKs function in multiple hormone signalling pathways. This, and the broad regulatory activity of MPKs, indicate that they might be convergence points integrating multiple hormone signals and driving downstream responses. For example, loss of *mpk4* function results in constitutive expression of three SA-inducible *PR* genes and repressed expression of the JA-responsive and ET-responsive gene, *PDF1.2* (Petersen et al., 2000; Brodersen et al., 2006). This indicates that MPK4 functions as a modulator to balance the antagonism between SA and JA/ET signalling pathways. Another example is MPK6, which phosphorylates ACS2 and ACS6 to promote ET biosynthesis in Arabidopsis and positively regulates the ET response (Liu and Zhang, 2004; Yoo et al., 2008). On the other hand, the negative regulator of ABA signalling ABI1, can inhibit MPK6 activity which ultimately affects the ACS6 phosphorylation level (Leung et al., 2006; Ludwików et al., 2014). This implicates MPK6 in cross-regulation between ET and ABA signalling pathways. However, the mechanisms by which MPKs coordinate with their substrates and other signalling elements to fine-tune the complex multiple hormonal signalling networks are not fully described.

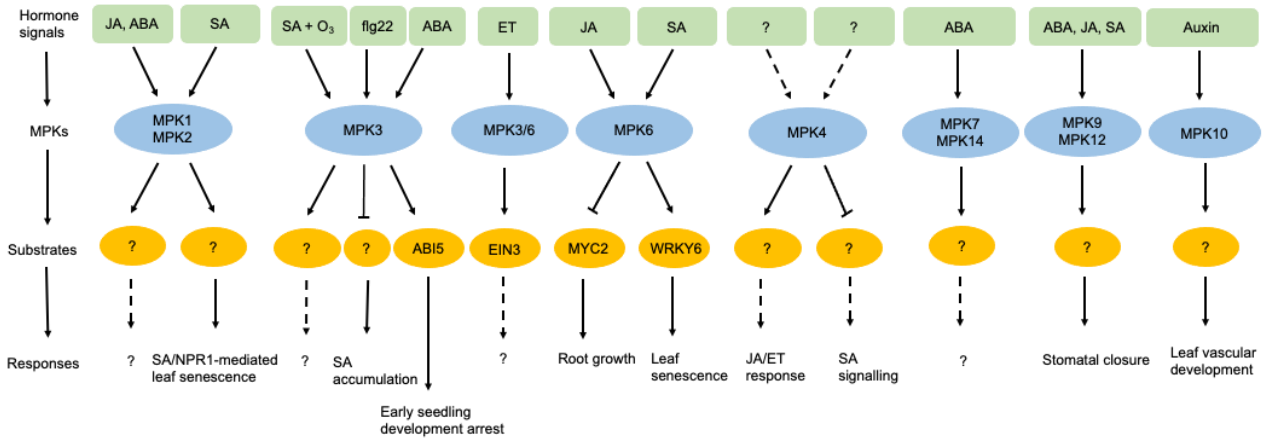

Figure 7. A summary of the links between MPKs and hormone signalling pathways.

Hormones can activate the kinase activity of MPKs. MPKs then phosphorylate their substrates, which further drives plant signalling responses. The dashed lines indicate hypothesised relationships that require further investigation.

1.3 Transcriptional regulatory network studies in plants

1.3.1 The development of transcriptional regulatory network studies in plant science

TFs control gene expression by binding to and regulating transcription of target genes. This occurs throughout plant development and during environmental responses. The interactions between TFs and their target genes form gene regulatory networks (GRNs). Transcriptional regulation during hormone or stress responses involves tens to hundreds of TFs and involves combinatorial actions of these TFs (Park et al., 2015; Song et al., 2016; Zander et al., 2020). Over the past two decades, the

roles of TFs and their target genes within hormone responses have been characterized in order to understand how hormone signals, environmental cues and physiological status dynamically regulate transcription. Application of several methods has contributed to these discoveries. Yeast one-hybrid assays are used to identify the interactions between TFs and a DNA sequence of interest and to map GRNs under specific conditions (Ouwerkerk and Meijer, 2001). For example, a set of transcriptional repressor TFs modulating auxin signalling were identified by using a semiautomated enhanced yeast one-hybrid assay. The repressors function as a network in regulating the expression of AUXIN RESPONSE FACTORs (ARFs), which are master transcriptional activators of auxin-responsive genes (Truskina et al., 2021). More recently, sequencing technologies have accelerated transcriptional regulatory network studies. Chromatin immunoprecipitation sequencing (ChIP-seq) and DNA affinity purification sequencing (DAP-seq) have allowed researchers to identify the binding sites and targets of TFs genome-wide *in vivo* and *in vitro* (Kaufmann et al., 2010; O'Malley et al., 2016). Time series RNA sequencing (RNA-seq) is a useful tool for quantification and monitoring dynamic changes of the transcriptome (Wang et al., 2009). These tools are useful to investigate dynamic transcriptional regulation by TFs in hormone responses.

Computational and machine learning methods have been developed to integrate high-throughput data and to train models to predict and construct larger scale transcriptional regulatory networks. Computational inference approaches, such as ExRANGES, can improve the identification of TF targets by combining expression levels and the rate of expression changes from time series RNA-seq data, which leading to improvement of GRN construction (Desai et al., 2017). The Dynamic Regulatory Events Miner (DREM) is a program for mapping the dynamics of transcriptional gene regulation through combined analysis of TF-gene interactions and time series gene expression data (Ernst et al., 2007; Schulz et al., 2012). This was applied to construct the transcriptional regulatory networks of ABA and ET responses (Chang et al., 2013; Song et al., 2016). The ET transcriptional response occurred in four waves with distinct functions and was driven by the master TF EIN3 (Chang et al., 2013). Eleven transcriptional waves were observed during 8 hours treatment with ABA and the dynamic binding of multiple TFs was responsible for rapid transcriptome changes (Song et al., 2016). DREM modelling was also used to model the transcriptional dynamics during seed germination and the DNA damage response and identify the TFs involved (Narsai et al., 2017; Bourbousse et al., 2018). A GRN model of the JA signalling pathway, which I contributed to during my PhD, was generated using similar time-resolved data but a different GRN modelling method (Zander et al., 2020). It was found that the master TFs MYC2 and MYC3 activate hundreds of downstream JA-responsive TFs, thereby forming a large TF regulatory network to drive the JA response. Machine-learning was applied to construct a maize leaf GRN by using ChIP-seq data from 104 TFs to train the models (Tu et al., 2020). TFs were observed as the hub of this transcriptional regulatory network. Similar GRNs have been studied for salt stress responses in *Marchantia polymorpha* and Arabidopsis (Wu et al., 2021). These studies demonstrate the utility of computational analyses to gain insight into regulation of transcription.

Few GRN studies to date have attempted to characterise integration of multiple hormone responses. The interplay between two or a small number of hormones has been investigated. The combination treatment of JA and SA resulted in dynamic transcriptional changes, with a large suite of JA-induced genes suppressed by SA treatment within the first 3 hours (Hickman et al., 2019). An interaction network involving ABA, auxin, CK and ET was generated based on experimental results to explaining how hormones cross-regulate Arabidopsis root growth upon osmotic stress conditions (Rowe et al., 2016). However, hormone cross-regulation occurs between many hormones at many points and affects expression of a large number of genes (Altmann et al., 2020; Zander et al., 2020). This creates a substantial challenge in characterising hormone GRNs and determining the activity of TFs and signalling components across the network of hormone interactions. The Signalling and Dynamic Regulatory Events Miner (SDREM) is a computational method which can link transcriptional regulatory networks to upstream signalling pathways (Gitter and Bar-Joseph, 2013; Gitter et al., 2013; Gitter and Bar-Joseph, 2016). This is achieved by using regulatory TFs as junction points. SDREM first identifies key TFs that control dynamic gene expression through combined analysis of TF-gene interactions and time series transcriptome data. Next, it searches for paths and signalling components which are responsible for activating these TFs from the proteins that initiate the signalling through protein-protein interactions, and iteratively refines the network by penalizing TFs that are not supported by the discovered paths. SDREM has been successfully applied to explore the signalling pathways of yeast stress response, human and Arabidopsis immune responses (Gitter and Bar-Joseph, 2013; Gitter et al., 2013). The integration of dynamic transcriptomes with TF-gene interactions and protein-proteins interactions by SDREM modelling provides an approach to generate a multi-hormone model explaining how hormones integrate signals from many pathways to dynamically cross-regulate gene expression.

1.3.2 Secondary TFs in hormone transcriptional regulatory networks

Secondary TFs are TFs that are directly targeted by and function downstream of master TFs, and they are an important component of hormone signalling pathways. For example, ERF1 is a direct target of the master ET TF EIN3 and consequently a secondary TF of the ET signalling pathway (Solano et al., 1998). ERF1 confers resistance to a wide range of pathogens, such as *Botrytis cinerea*, *Plectosphaerella cucumerina,* and the soilborne fungi *Fusarium oxysporum sp. conglutinans* and *F. oxysporum f. sp. Lycopersici* (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003; Berrocal-Lobo and Molina, 2004). It also regulates abiotic stress responses, such as to salt, water and heat stresses (Cheng et al., 2013). Moreover, ERF1 act as a convergence point between ET and JA signalling pathways to activate the expression of defence genes that confer pathogen resistance (Lorenzo et al., 2003).

Most hormone transcriptional regulatory network studies have focused on master TFs (Chang et al., 2013; Zander et al., 2020). These master TFs, such as MYCs and EIN3, target a large suite of components in their respective pathway and activate the hormone response through a large TF network as described in section 1.3.1. However, the secondary TFs, and other TFs further

downstream, are also important regulators of gene expression and plant biological processes. For example, early nitrogen-responsive genes include a set of secondary TFs that are targets of the master TF NIN-LIKE PROTEIN 7 (NLP7) (Alvarez et al., 2020). These secondary TFs amplify the NLP7-initiated transcriptional cascade by regulating late nitrogen-responsive genes in Arabidopsis roots. However, the roles of secondary TFs in GRNs that mediate hormone responses have been explored relatively less. However, there are examples of the importance of secondary TFs in hormone GRNs. One such example is the TF SALT TOLERANCE ZINC FINGER (STZ). This is a target of master TF MYC2 in the JA GRN and therefore a secondary TF of the JA response. STZ appears to function as a transcriptional repressor that acts to prioritize the JA response by repressing other hormones (Zander et al., 2020). Deeper study of secondary hormone response TFs will assist us in better understanding hormone GRNs, expanding our currently limited knowledge.

1.3.3 EDFs are secondary TFs in the ET signalling pathway

The master TF EIN3 induces the expression of a large suite of ET-responsive genes by binding to the ET responsive element motif in promoters (Chao et al., 1997; Chang et al., 2013). The transcriptional regulatory network around EIN3 has been investigated and a considerable number of EIN3's target genes are TFs themselves (Chang et al., 2013). EDF1, 2, 3, 4 are a group of secondary TFs directly downstream of EIN3 that operate in addition to ERF1 (Alonso et al., 2003). They belong to a plant specific RELATED TO ABI3/VP1 (RAV)/EDF TF family, which comprises six members in Arabidopsis: EDF1 (also known as TEMPRANILLO1/TEM1 and AT1G25560), EDF2 (also known as RAV2, TEM2 and AT1G68840), EDF3 (also known as RAV1-Like, RAV3 and AT3G25730), EDF4 (also known as RAV1 and AT1G13260), and two other proteins encoded by genes *AT1G50680* and *AT1G51120*.

EDF1, 2, 3, 4 have several characteristics which make them worthy of deeper investigation. The EDFs have two plant specific DNA binding domains, termed AP2 and B3 (Kagaya et al., 1999; Alonso et al., 2003). This is unusual because most plant TFs only have one DNA-binding domain. The B3 domain was identified as a transcriptional repression domain by possessing a conserved repressive R/KLFGV amino acid motif, and EDFs function as transcriptional repressors controlling the flower senescence and abscission in Arabidopsis (Ikeda and Ohme-Takagi, 2009; Chen et al., 2015). Few repressive TFs have been characterized within the ET GRN. Investigation of the transcriptional activity of EDFs in the GRN during an ET response might extend our understanding of mechanisms of transcriptional regulation in the ET signalling pathway. Moreover, EDFs are involved in a wide range of developmental processes, abiotic stress and hormone responses. These include flowering time, leaf senescence, responses to drought and salt stresses, responses to ABA and BR (Hu et al., 2004; Castillejo and Pelaz, 2008; Woo et al., 2010; Fu et al., 2014; Osnato et al., 2020; Sengupta et al., 2020). Consistent with this, expression of *EDFs* is regulated by several hormones and abiotic stresses (Hu et al., 2004; Licausi et al., 2013; Fu et al., 2014; Matias-Hernandez et al., 2014; Duan et al., 2016; Zhao et al., 2017; Xie et al., 2019; Osnato et al., 2020). This may indicate that EDFs are hormone cross-regulation points. However, the investigation of EDF functions is limited because only a small

number of EDF target genes have been identified. The few known targets include the flowering regulator and GA biosynthetic genes *GIBBERELLIN 3-OXIDASE 1 (GA3OX1) and GA3OX2* (Castillejo and Pelaz, 2008; Osnato et al., 2012). Determining the complete population of EDF target genes will extend our ability to understand the functions and regulatory roles of these secondary TFs in a transcriptional cascade during ET responses and hormone cross-regulation.

1.4 The aims of this study

This study will use a combination of functional genomics and computational modelling approaches to address the following aims:

Aim 1: Evaluation of transcriptome changes and transcriptional regulation during responses to multiple hormones

This study aims to determine transcriptome dynamics and the key regulatory TFs and signalling proteins in responses of etiolated Arabidopsis seedlings to the hormones ABA, BR, ET, JA, SA, and SL/KAR.

Aim 2: Identification of convergence points in hormone cross-regulation network

This thesis will reconstruct a hormone cross-regulation network model which describes how multiple hormones signals are integrated and influence the gene expression of etiolated Arabidopsis seedlings. The proteins associated with multiple hormone pathways will be identified.

Aim 3: Investigation the role of EDF TFs in the ET response

This study will characterize the functions of EDF TFs at genome scale by identifying their target genes and analysing the effects of the EDFs on these genes. This work will also examine the roles of EDFs in hormone cross-regulation.

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2. CHAPTER 2. Transcription factor activity in cross-regulation between seedling hormone responses

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2.1 Abstract

Cross-regulation between hormone signalling pathways is indispensable for plant growth and development. However, the molecular mechanisms by which multiple hormones interact and co-ordinate activity are poorly understood. Here, we generated a cross-regulation network explaining how hormone signals are integrated from multiple pathways in etiolated Arabidopsis (*Arabidopsis thaliana*) seedlings. To do so we comprehensively characterized transcription factor activity during plant hormone responses and reconstructed dynamic transcriptional regulatory models for six hormones; abscisic acid, brassinosteroid, ethylene, jasmonic acid, salicylic acid and strigolactone/karrikin. These models incorporated target data for hundreds of transcription factors coupled with thousands of protein-protein interactions. Each hormone recruited different combinations of transcription factors, a subset of which were shared between hormones. Hub target genes existed within hormone transcriptional networks, exhibiting transcription factor activity themselves. A group of MITOGEN-ACTIVATED PROTEIN KINASES were also identified as potential key points of cross-regulation between multiple hormones. Finally, we determined that all hormones drive substantial alternative splicing that has distinct effects on the transcriptome compared with differential gene expression, acting in early hormone responses. These results provide a comprehensive understanding of the common features of plant transcriptional regulatory pathways and how cross-regulation between hormones acts upon gene expression.

2.2 Introduction

Cross-regulation between hormone signalling pathways is fundamental to plant growth and development. It allows plants to monitor a multitude of external environmental and internal cellular signals, process and integrate this information, then initiate appropriate responses. This enables plants to exhibit plastic development, adapting to their local environment, optimizing resource usage and responding to stresses (Jaillais and Chory, 2010; Vanstraelen and Benková, 2012; Aerts et al., 2020; Khan et al., 2020). The growth-defence trade-off is a well-known example, whereby plants experiencing pathogen attack will prioritize resource allocation to defence at the expense of growth (Karasov et al., 2017; Figueroa-Macías et al., 2021). This trade-off can be condition-dependent, with plants growing in nutrient-rich conditions not necessarily needing to prioritize one response over the other (Figueroa-Macías et al., 2021).

Each plant hormone has a recognized, distinct signalling pathway (Huang et al., 2017; Binder, 2020; Bürger and Chory, 2020; Chen et al., 2020; Ding and Ding, 2020; Nolan et al., 2020; Yao and Waters, 2020). Cross-regulation between these pathways occurs during signal transduction and regulation of transcription (Jaillais and Chory, 2010). Cross-regulation of transcription can occur through regulation of shared transcription factors (TFs) between pathways and by regulation of shared target genes by independent TFs. The latter is considered less common because a minority of genes is shared between the transcriptional responses to different hormones (Nemhauser et al., 2006). The DELLA and JASMONATE-ZIM DOMAIN (JAZ) proteins and NONEXPRESSER OF PR GENES 1 (NPR1) are classic examples of hormone cross-regulation. In each case, these proteins are primarily regulated by one hormone and are involved in the regulation of that hormone's pathway, but they also influence other hormone signalling pathways (Achard et al., 2003; Fu and Harberd, 2003; Hou et al., 2010; Yang et al., 2012). Recent research demonstrates that there may be multiple points of contact between most plant signalling pathways, indicating hormone signalling pathways likely operate as a highly connected network that permits complex exchange and processing of information (Altmann et al., 2020).

The expression of thousands of genes changes in response to a hormone stimulus (Nemhauser et al., 2006). These expression changes are dynamic over time, with great diversity between the expression patterns of genes (Chang et al., 2013; Song et al., 2016; Xie et al., 2018; Zander et al., 2020). Different TFs act at different times during responses to regulate genes in this dynamic manner. Expression of tens to hundreds of TFs is regulated by the hormones abscisic acid (ABA), ethylene (ET) and jasmonic acid (JA) and it is likely all hormones do similarly (Chang et al., 2013; Song et al., 2016; Zander et al., 2020). Individual TFs often target hundreds to thousands of genes and individual genes may be targeted by multiple TFs. This enables dynamic and complex expression patterns to be achieved but presents a substantial problem in determining which TFs regulate these patterns (Chang et al., 2013; Song et al., 2016).

The extent of alternative splicing in hormone responses is not fully understood (Zander et al., 2020). Alternative splicing and variant isoform usage diversify the proteome by permitting individual

genes to encode multiple proteins that may vary in structure and function (Syed et al., 2012; Filichkin et al., 2015; Hartmann et al., 2016; Calixto et al., 2018). Variant isoforms of the JAZ repressor JAZ10, one encoding an active form of the protein and one a dominant negative form, have an important role in regulating the core JA signalling pathway (Yan et al., 2007; Chung et al., 2009; Moreno et al., 2013). More recently, greater than 100 genes were determined to switch dominant isoforms during a JA response in etiolated Arabidopsis seedlings (Zander et al., 2020). However, whether or not variant isoform usage is a core feature of hormone signalling pathways is unknown.

In this study we set out to understand how cross-regulation of dynamic transcriptional responses occurs in the etiolated Arabidopsis seedling, a well-characterized model for plant hormonal signalling and development. We did so by analysing transcriptome dynamics following stimulation of the ABA, brassinosteroid (BR), ET, JA, salicylic acid (SA) and strigolactone/karrikin (SL/KAR) signalling pathways. We determined the *in vivo* target genes of key TFs then developed models of hormone transcriptional responses that integrated target data for hundreds of other TFs and thousands of protein-protein interactions. The multi-hormone transcriptional model we have developed helps explain how hormones integrate signal from multiple pathways to dynamically cross-regulate gene expression. We also identified genes that undergo differential alternative splicing during hormone responses, which extends our understanding of hormone signalling complexity.

2.3 Results

2.3.1 Reconstruction of dynamic hormone transcriptional regulatory pathways

The major aims of our study were to determine the extent to which cross-regulation of transcription occurs between hormone signalling pathways and to identify components responsible for this cross-regulation. To do so we first reconstructed the dynamic hormone transcriptional regulatory pathways for ABA, BR, ET, JA, SA and SL/KAR from hormone receptors, through signal transduction to TF-gene binding and differential gene expression (Fig. 1).

We generated a model of each hormone which described regulation of the transcriptome over time following treatment with that hormone (Fig. 1a). This was achieved by analysing time-series transcriptomes from our own and published data (BR, ET, JA, SA, SL/KAR, 0-24 h after treatment; ABA, 0-60 h; data sources detailed in Methods) (Extended Data Fig. 1, 2; Supplementary Table 1). We applied Signalling and Dynamic Regulatory Events Miner (SDREM) modelling to reconstruct individual hormone pathways (Fig. 1a) (Gitter and Bar-Joseph, 2013; Gitter et al., 2013; Gitter and Bar-Joseph, 2016). SDREM first identifies TFs that bind groups of co-regulated genes during the hormone transcriptional response. Next, it searches for paths from the receptor(s) of that hormone through protein-protein interactions to these TFs, inferring that the paths are mechanisms that may activate the TF during the hormone response. SDREM then iteratively refines the network by penalizing TFs that are not supported by signalling pathways from the receptors.

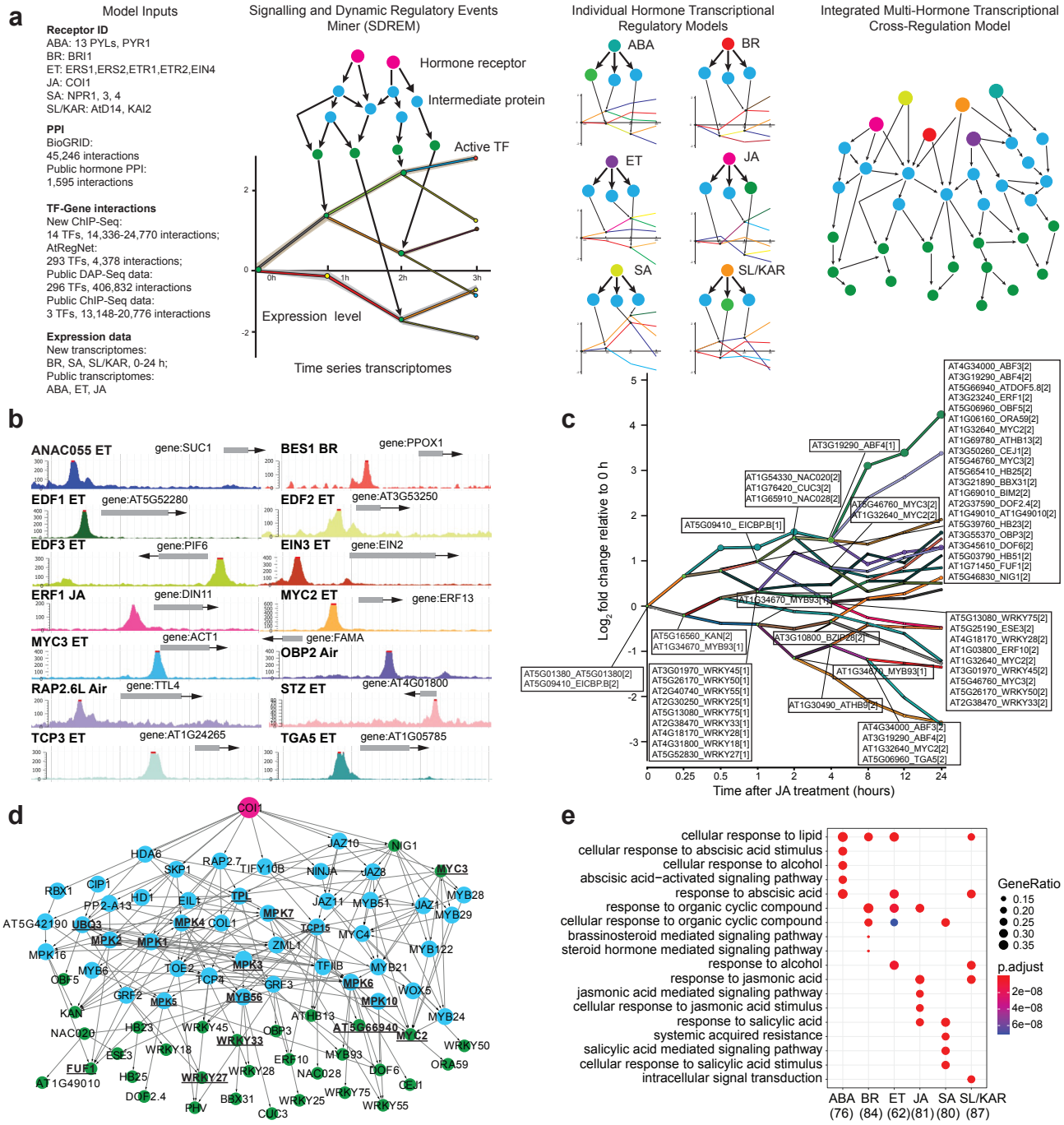

Figure 1. Overview of hormone transcriptional regulatory models reconstructed using the SDREM modelling framework. a, The modelling approach underlying our hormone cross-regulation network. Model inputs lists data generated by our lab or from published studies used in the models. SDREM integrates TF-gene interactions and PPIs with time series expression data to build models in an iterative manner. It first identifies active TFs that bind cohorts of co-regulated genes, then searches for paths from hormone receptor(s) to these TFs. Individual models were generated for each hormone of ABA, BR, ET, JA, SA and SL/KAR. These were combined to give the integrated model. **b**, Genome browser screen shot visualizing representative target genes from ChIP-seq samples of 14 TFs. **c**, The regulatory network of the JA model. The network displays all predicted active TFs at each branch point (node) and the bars indicate co-expressed and co-regulated genes. [1] indicates the TF primarily controls the lower path out of the split and [2] is for the higher path. The y-axis is the log2 fold change in expression relative to expression at 0 h. **d**, The JA signalling pathway reconstructed by SDREM. The JA receptor, intermediate proteins and active TFs are indicated by magenta, blue and green nodes respectively. The proteins shared by at least 4 hormone pathways, are in black bold text and have underlined names. **e**, Top five significantly enriched (p.adjust < 0.05) gene ontology biological process terms amongst the predicted nodes of the reconstructed signalling pathway for each hormone.

SDREM modelling requires extensive data about TF-target gene interactions and protein-protein interactions to reconstruct signalling pathways. To enable this we determined the *in vivo* target genes of 14 hormone TFs by chromatin immunoprecipitation sequencing (ChIP-seq; Fig. 1b; Supplementary Table 2, 3) to use in model construction. These were combined with public TF-target gene data for a further 516 TFs (Yilmaz et al., 2010; Song et al., 2016; Narsai et al., 2017; Zander et al., 2020). The known receptors for each hormone and extensive public Arabidopsis protein-protein interaction data (46,841 interactions) were used to build signalling pathways (Stark et al., 2006). We successfully reconstructed the transcriptional regulatory pathways for all six hormones (ABA, BR, ET, JA, SA, SL/KAR) using this approach, demonstrated by each model being enriched for known components of the relevant hormone signalling pathway (Fig. 1c, d, e; Extended Data Fig. 3, 4, 5, 6; Supplementary Table 4, 5). The models illustrate that each hormone remodels the transcriptome rapidly - within 15 minutes (BR, ET, JA, SA, SL/KAR) or 1 hour (ABA) - of perception of that hormone. Remodeling is extensive and dynamic, affecting thousands of genes over 24 h (Extended Data Fig. 2).

2.3.2 The populations of TFs employed by each hormone differ but share some individual components

We examined the extent to which individual hormones used different TFs to control gene expression. We did so by first identifying shared and unique regulatory TFs within each hormone model. A minority of unique TFs was present in the models of every hormone (19.5% to 34.7% of TFs per model, Fig. 2a; Supplementary Table 6). This is consistent with the prior observation that most genes differentially expressed in response to individual hormones are not shared between different hormones (Nemhauser et al., 2006) (Supplementary Table 7). However, there were also TFs shared between multiple hormone models (Fig. 2a). Fourteen TFs were shared between the models of 4 or more hormones. For example, MYC2, MYC3 and WRKY33 were all predicted regulators in the BR, JA, SA and SL/KAR models (Fig. 2a). Overall, we observed that each hormone utilized distinct combinations of TF families to regulate transcription. This was demonstrated by the relative enrichment of TF families between hormone models (Fig. 2b; Supplementary Table 6). These results demonstrate that although the responses to ABA, BR, ET, JA, SA and SL/KAR do share some TFs, their unique transcriptional regulatory pathways are established by recruiting different combinations of TFs.

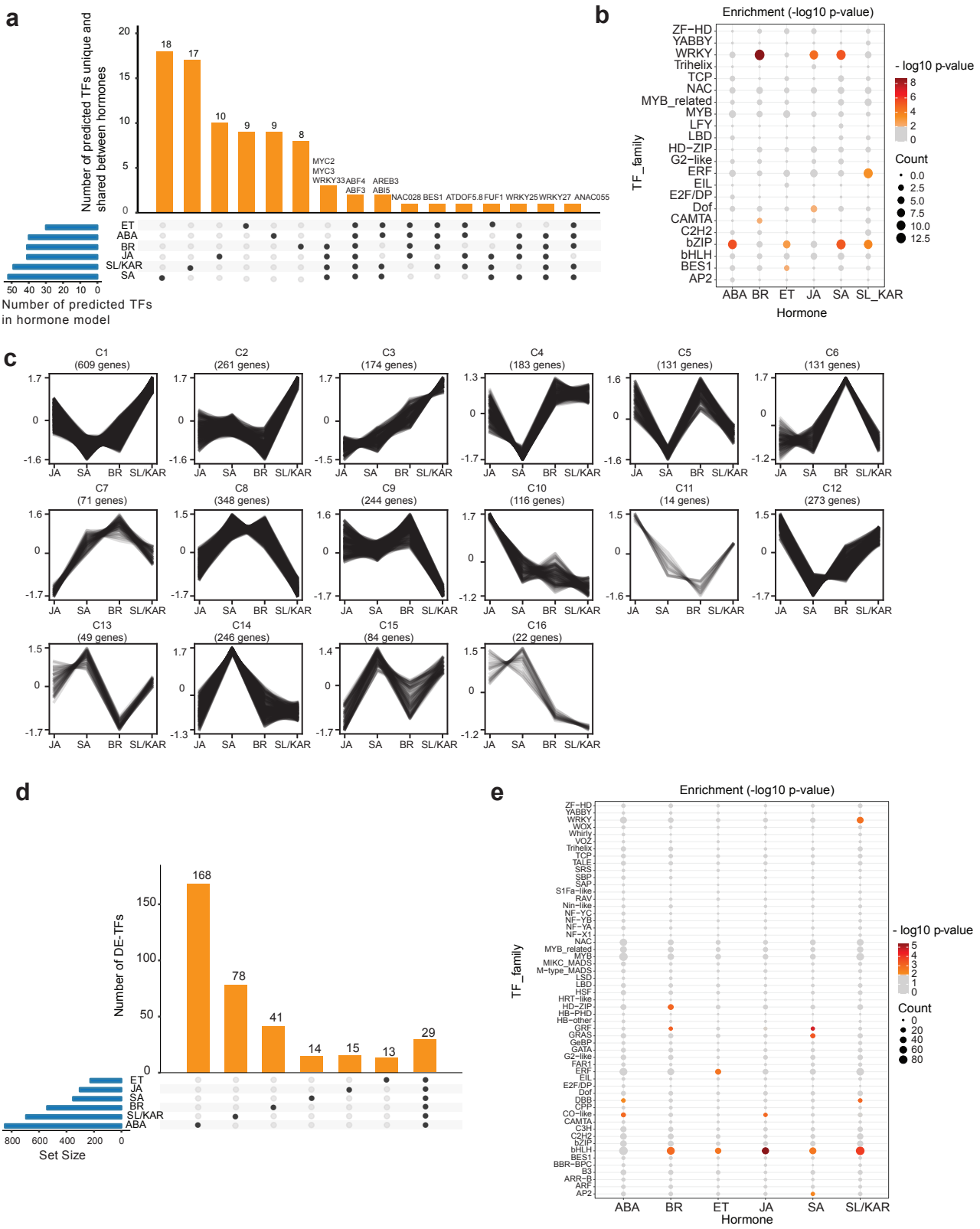

Figure 2. Different TFs regulate the response to each hormone. a, The number of active TFs unique to and shared between all six hormone models. Names of TFs shared between 4 or more hormone models are labelled at the top of respective columns. **b**, Significantly enriched TF families found within each hormone model (p-value < 0.01; hypergeometric test). The size and colour of each circle represents per-family TF count and enrichment p-value range respectively. **c**, K-means clustering of expression of MYC2 target genes during JA, SA, BR and SL/KAR hormone responses. Expression is given as normalized transcripts per million (TPM). **d**, The number of unique and shared differentially expressed TFs (DE-TFs) between six hormones. **e**, Significantly enriched TF families amongst DE-TFs for each hormone (p-value < 0.01; hypergeometric test). The size and colour of each circle represents per-family TF count and enrichment p-value range respectively.

TFs shared between multiple hormones may perform the same function for each hormone, meaning that they regulate one set of genes in the same manner in all conditions. However, we observed that shared TFs were predicted to regulate gene expression at different times post-treatment in each hormone response and were associated with both up and down-regulated genes (Extended Data Fig. 7a). This suggested the former proposal was unlikely. Alternatively, shared TFs might regulate a common set of genes in different manners - promoting expression for one hormone, repressing expression for another - or regulate different sets of genes for each hormone. We investigated these possibilities by examining expression of target genes of the three TFs shared between the JA, SA. BR and SL/KAR models: MYC2, MYC3 and WRKY33. The expression of many target genes of these TFs differed between hormone treatments (Fig. 2c; Extended Data Fig. 7b, c, d). For example, different clusters of MYC2 target genes were more highly expressed after JA treatment (cluster 11), SA and BR treatment (cluster 7) and SL/KAR treatment (cluster 1). This indicates that the functions or activity of shared TFs may differ between hormone regulatory pathways. Alternative possibilities are that as-yet competitor TFs regulate these same target genes, that different partner proteins may be recruited, or that the TFs themselves may be modified differently, under certain hormone conditions.

TFs themselves can be differentially expressed in response to hormones, influencing TF abundance and activity (Chang et al., 2013; Zander et al., 2020). We determined that large and unique suites of TFs were differentially expressed in response to each hormone. In total, 849 (ABA), 542 (BR), 227 (ET), 304 (JA), 353 (SA) and 695 (SL/KAR) TFs were differentially expressed during the response to each hormone (Fig. 2d; Supplementary Table 7). A subset of these differentially expressed TFs were exclusive to one hormone, while only 29 TFs were shared between all hormones (Fig. 2d). In addition, a distinct pattern of enriched TF families was observed between the differentially expressed genes for each hormone (Fig. 2e; Supplementary Table 7). This, combined with the observations from the models of hormone transcriptional regulatory pathways, indicates that dynamic remodeling of the transcriptome by ABA, BR, ET, JA, SA and SL/KAR involves large, hormone-specific suites of TFs despite the fact that hormones influence many overlapping growth and developmental processes. Each hormone recruits different combinations of TFs and activity of shared TFs may differ between hormones.

2.3.3 Hub target genes are more highly responsive to hormones and are enriched in TFs

We investigated whether hub target genes exist within hormone transcriptional regulatory pathways and what their properties are. Hub targets are genes bound by many TFs (Heyndrickx et al., 2014). In plant transcriptional networks, unlike in animals, this high degree of binding is thought to be regulatory. Hub target genes may also exhibit different expression characteristics than non-hubs, being expressed under a wider range of conditions, likely as a result of being bound by many TFs (Heyndrickx et al., 2014). We identified the hub target genes in networks of 17 hormone TFs for which ChIP-seq data was available, focusing on this data type alone because it provides the most comprehensive map of TF-target interactions (Fig. 3; Supplementary Table 8). A TF-target network

was generated for each hormone because multiple hormone-specific ChIP-seq datasets were available for some TFs. Most genes (63.2 to 71.8% per hormone) were bound by more than one TF. Binding of target genes by multiple TFs was observed for all hormones, with some bound by as many as 12 TFs (Fig. 3a). We consequently defined hub target genes as genes bound by at least 7 TFs (Supplementary Table 8). By this threshold we identified 1,103 hub target genes across all hormones, compared with 15,203 non-hub target genes.

The difference in the number of TFs that hub and non-hub target genes are bound by indicates that expression of these two classes of genes may be regulated differently. The expression responses of hub and non-hub target genes to hormones differed, in accordance with this (Fig. 3b). Target genes were divided into three categories; low, moderate and hubs, bound by 1-3, 4-6 and 7 or more TFs, respectively. Differential expression for each target gene in the three categories was calculated, plotted in density plots, then differences between distributions assessed (Supplementary Table 8; Kolmogorov-Smirnov test, p-value < 0.05). Hub target genes were more highly differentially expressed in response to 5 of the 6 hormones than non-hub target genes (Fig. 3b). This indicates that the regulation of hub and non-hub target genes indeed differs. The increased differential expression may be occur due to the additive action of the bound TFs bound at any gene. However, we highlight that TFs can have activator or repressive activity, leading to potentially conflicting influence upon the expression of bound genes.

Hub and non-hub target genes also differed in their annotated functional roles (Fig. 3c; Supplementary Table 8). Both hub and non-hub target genes were enriched in genes from hormone signalling pathways and genes with TF activity. However, enrichment for TF activity was greater amongst the hub target genes (Fig. 3c; low, 8.7%; moderate 13.6%; and hubs 54.6% of terms per group). Accordingly, more hub target genes encoded TFs than non-hub target genes.

Considered together, our findings indicate that a small proportion of genes in hormone transcriptional regulatory pathways are hub target genes, bound by many TFs. The existence of hub target genes allows regulation to converge at certain genes, which presumably permits information from different signalling pathways to be integrated. Hub target genes are more strongly differentially expressed in response to hormones than non-hubs and are enriched for genes with TF activity. The hub target genes were similarly enriched for TFs in a network examining the expression of target genes of known flowering, circadian rhythm, and light response TFs (Heyndrickx et al., 2014). Given these similar features of the hormonal and flowering networks, it remains to be examined whether the TF activity of hub target genes is a more general principle of plant transcriptional networks.

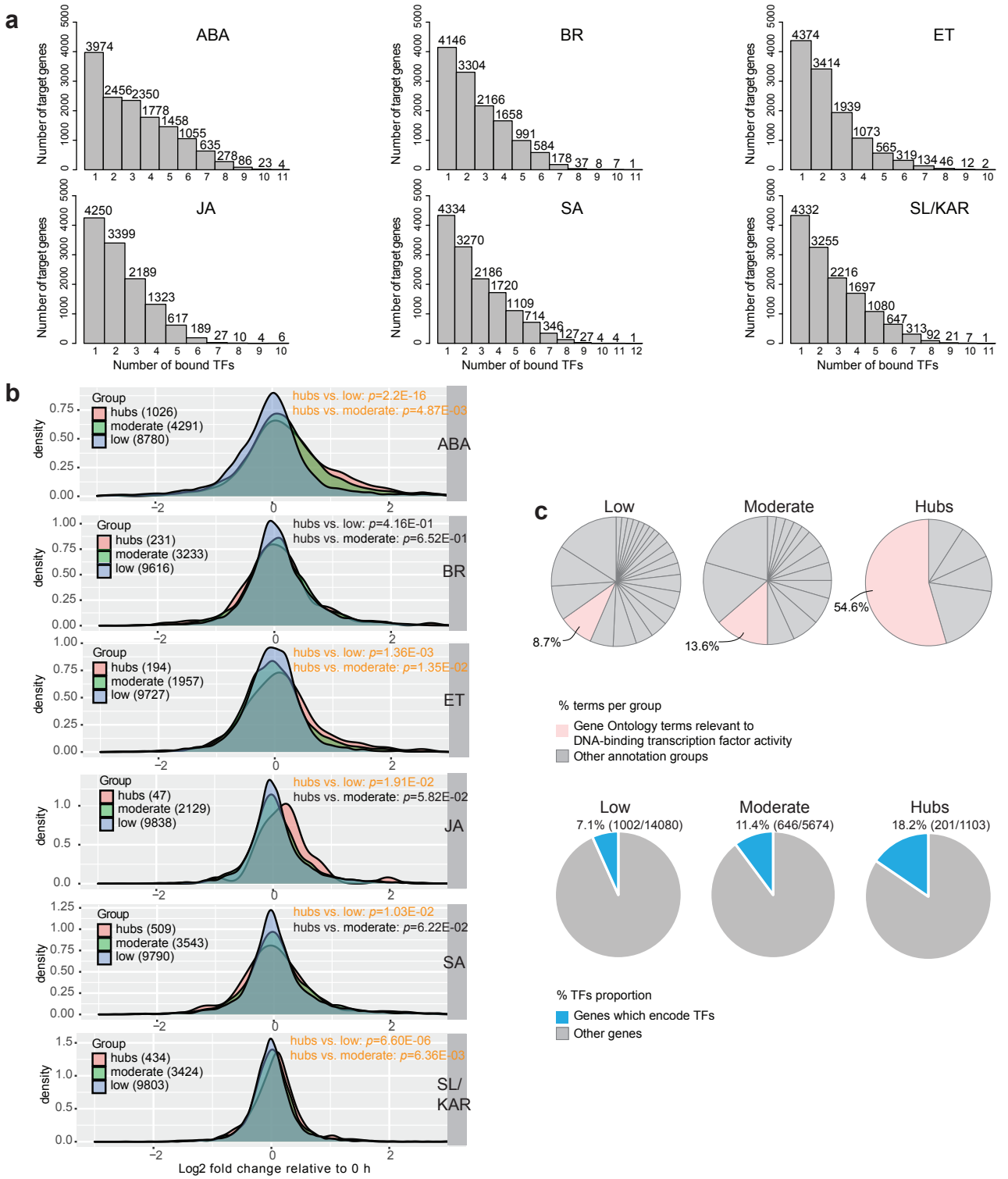

Figure 3. Hub target genes are more highly responsive to hormones and are enriched in TFs in hormone transcriptional networks. a, Plots show the number of TFs (x-axis) binding each target gene. Hub target genes were defined as genes bound by at least 7 TFs. **b**, Density plots show the differential expression for each target gene in three groups (low, genes bound by 1-3 TFs; moderate, genes bound by 4-6 TFs; hubs, genes bound by at least 7 TFs). The number of targets in each group are listed in parentheses. The x-axis is log2 fold change relative to 0 h. Each individual plot reports target gene expression for one of the six hormones. Significant difference in distribution is indicated by orange text (p-value < 0.05; two-sample Kolmogorov-Smirnov test). **c**, Pie charts in the upper panel show the percentage of DNA-binding transcription factor activity terms amongst all enriched gene ontology terms of the target genes. Individual pie charts present data for each group (low, moderate and hubs). The pie charts in the bottom panel give the proportion of genes encoding TFs in each group. The percentage and the numbers of TFs (in parentheses) in each group are listed at the top of corresponding pie chart.

2.3.4 MAP kinases conduct cross-regulation between multiple hormone transcriptional regulatory pathways

Plant hormone signalling pathways do not operate in isolation from one-another. Multiple points of contact exist between different pathways, facilitating hormone cross-regulation (Altmann et al., 2020). We examined how cross-regulation between plant hormone signalling pathways influences TF activity and identified network components that may be responsible for cross-regulation. The most comprehensive current analysis of plant hormone signalling cross-regulation is a large-scale protein-protein interaction network (Altmann et al., 2020). We extended upon this by connecting hormone signalling protein-protein interactions to TF-gene interactions and gene expression. To do so we generated an integrated transcriptional cross-regulation model by overlaying the individual hormone transcriptional regulatory models (Fig. 1a; 4a; Supplementary Table 5). The integrated model was composed of 291 individual genes, 23 of which were shared by at least 4 hormones (Fig. 4a, b; Supplementary Table 5). These 23 shared genes were 13 TFs from different families, 8 MITOGEN-ACTIVATED PROTEIN KINASES (MPKs) and the genes TOPLESS (TPL, AT1G15750) and POLYUBIQUITIN 3 (UBQ3, AT5G03240). The 23 genes were significantly enriched for signal transduction functions (Fig. 4c; p-value < 0.01). We infer that these genes are likely nodes of cross-regulation in a broad, multi-hormone regulatory network.

The 23 predicted multi-hormone cross-regulation genes included TFs directly and indirectly associated with hormone signalling. Seven of thirteen TFs (53.8%) were known regulators of hormone responses (Supplementary Table 5). These were ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 3 (ABF3), ABF4, ABA INSENSITIVE 5 (ABI5), MYC2, MYC3, BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1), associated with the ABA, JA and BR signalling pathways (Choi et al., 2000; He et al., 2005; Li and Deng, 2005; Fujita et al., 2013; Kazan and Manners, 2013; Salazar-Henao et al., 2016; Skubacz et al., 2016; Hickman et al., 2017; Ibanez et al., 2018; Ju et al., 2019; Chen et al., 2020; Zander et al., 2020). Two of these TFs have defined roles in cross-regulation between a small number of hormones (ABI5 - ABA and JA, ET; MYC2 - JA and ET, ABA, SA) (Abe et al., 2003; Wild et al., 2012; Zhang et al., 2014; Ju et al., 2019). The remaining TFs were not characterized as directly involved in hormone signalling but had roles in processes associated with hormones, such as plant defence, abiotic stress responses and flowering (Zheng et al., 2006; Mukhtar et al., 2008; Pandey and Somssich, 2009; Chen et al., 2015; He et al., 2015). These shared TFs provide a potential mechanism for cross-regulation of gene expression between multiple hormone signalling pathways.

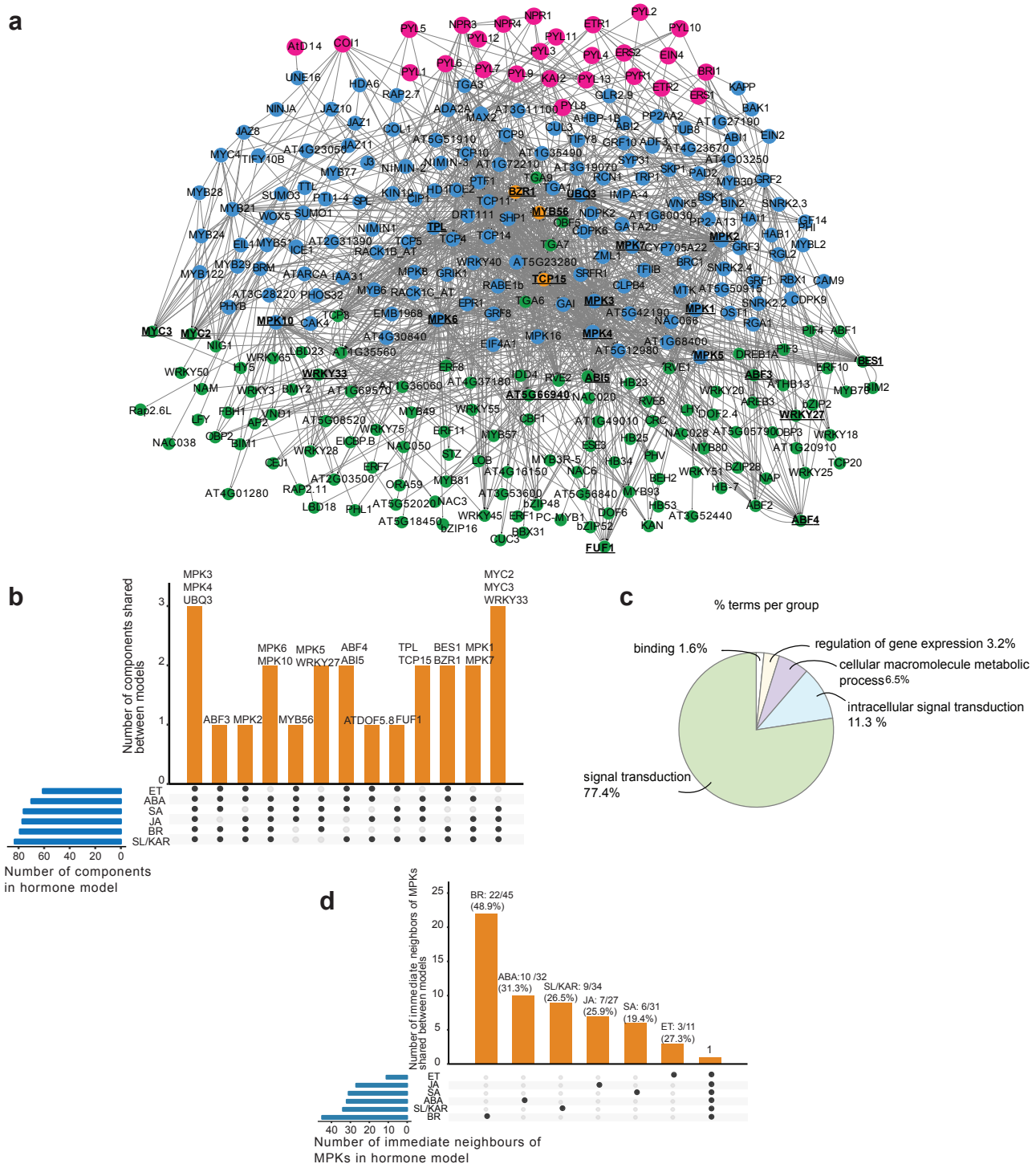

Figure 4. MPKs are convergence nodes in the integrated multi-hormone cross-regulation model. a, The multi-hormone cross-regulation network was built by integrating models of each hormone. Magenta nodes: upstream proteins given as hormone receptors. Blue nodes: predicted signalling proteins. Green nodes: active TFs responsible for transcriptional changes. Orange nodes: proteins that have both signalling and active TFs roles. The proteins shared by at least 4 hormone pathways are in black bold text and have underlined names. **b**, Twenty-three proteins are shared by at least 4 hormone signalling pathways and are putative hormone cross-regulation nodes. These proteins were enriched in MPKs (8/23). **c**, Pie chart shows the functional groups of enriched (p-value < 0.05) gene ontology terms of 23 proteins. The listed group name is the term has highest significance in its functional group. The percentages of the enriched terms in each groups amongst all enriched gene ontology terms of 23 proteins are listed following the group names. **d**, Unique and shared immediate neighbours of MPKs in each individual hormone model. The numbers and percentages at the top of each column indicate what proportion the unique immediate neighbours are of the total immediate neighbours.

The large number of MPKs present amongst the 23 predicted multi-hormone cross-regulation genes was notable (8/23 genes, Fig. 4b; Supplementary Table 5). Protein phosphorylation cascades transduce developmental and environmental signals and regulate cell functions *via* MPKs (Cristina et al., 2010; Bigeard and Hirt, 2018; Jagodzik et al., 2018). MPKs often occupy core positions in signal transduction pathways, receiving information from several upstream inputs. They then phosphorylate downstream proteins, frequently including TFs, thereby regulating their activity. These properties would make them extremely suitable as central components for multi-hormone cross-regulation. TFs are a large proportion of the immediate (first-degree) neighbours of MPKs in our model (73.5%, 75/102; Supplementary Table 5). This is consistent with the previous results of a MPK target network (Popescu et al., 2009). Many of these immediate neighbours were unique to individual hormones (ABA, 10/32, 31.3%; BR, 22/45, 48.9%; ET, 3/11, 27.3%; JA, 7/27, 25.9%; SA, 6/31, 19.4%; SL/KAR, 9/34, 26.5%; Fig.4d; Supplementary Table 5). This indicates that, despite being shared between hormone pathways, the MPKs are likely to target different downstream proteins dependent upon the hormone they respond to.

2.3.5 Alternative splicing is a core component of hormone responses

Alternative splicing contributes to reprogramming of gene expression, changing the functional composition of proteins expressed from individual genes (Narsai et al., 2017; Calixto et al., 2018). JA responses include alternative splicing, but the influence of alternative splicing on broader hormone responses is not understood (Chung et al., 2010; Moreno et al., 2013; Zander et al., 2020). To examine this we identified genes whose transcripts were differentially alternatively spliced following each hormone treatment. We analysed the time-series RNA-seq data at transcript-level for all hormones except ET; the ET sequence read length was too short for this type of analysis. There were 1155 (ABA), 1016 (BR), 415 (JA), 613 (SA), 797 (SL/KAR) genes whose transcripts were differentially alternatively spliced during the response to each hormone (Fig. 5a; Supplementary Table 9). Amongst these, 818 (ABA), 376 (BR), 96 (JA), 153 (SA) and 411 (SL/KAR) were also differentially expressed at gene-level, which indicates that many genes are regulated by both transcription and alternative splicing. However, a notable amount of genes was not differentially expressed, and consequently regulated only by alternative splicing (337, 29.2%, ABA; 640, 63.0%, BR; 319, 76.9%, JA; 460, 75.0%, SA; 386, 48.4%, SL/KAR; Fig. 5b). The three most abundant types of events amongst hormone responsive differentially alternative spliced transcripts were intron retention (36.7-38.7%), alternative 3’ splice sites (28.0-29.1%) and alternative 5’ splice sites (21.4-21.7%) (Extended Data Fig. 8a; Supplementary Table 9). This was common across all hormones and is comparable with alternative splicing during cold responses (Calixto et al., 2018). A large proportion of differentially alternatively spliced genes were unique to individual hormones (Extended Data Fig. 8b). These results demonstrate that alternative splicing is a general component of plant hormone signalling.

Isoform switching is a phenomenon whereby the relative abundance of two transcript isoforms from a single gene reverse following a stimulus (Guo et al., 2017). Such events change the dominant form of the transcript present and the structure of the subsequent mature protein, which can influence

cellular processes (Chung et al., 2009). Isoform switching occurs during the JA response in etiolated Arabidopsis seedlings, but its contribution to other hormone responses is not known (Zander et al., 2020). We found 350 (ABA), 185 (BR), 120 (JA), 190 (SA) and 169 (SL/KAR) genes underwent isoform switching (Supplementary Table 10). Almost all of these isoform switching events involved at least one protein-coding transcript isoform (456, 96.6%, ABA; 229, 97.0%, BR; 132, 96.4%, JA; 241, 98.0%, SA; 190, 95.0%, SL/KAR; Supplementary Table 10). This indicates that the events have the potential to change the function of the proteins expressed in a hormone response. The majority of isoform switching genes differed between hormones (Fig. 5c). Transcripts of two genes did undergo isoform switching in response to all 5 hormones (*AT3G09600*, also known as *REVEILLE 8* and *RVE8*; *AT2G32690*, also known as *GLYCINE-RICH PROTEIN 23* and *GRP23*). However, different pairs of *GRP23* isoforms were affected across the five hormones. *RVE8* isoform switching was dominated by one pair across four hormones, but the switch time point differed (Fig. 5d). These results indicate isoform switching is a common feature of hormone responses.

The functional influence of alternative splicing on plant hormone responses is also unknown. Two molecular characteristics were notable amongst the hormone-responsive differentially alternatively spliced genes. First, mRNA splicing-related functions were enriched for 4 of 5 hormone (Fig. 5e; Supplementary Table 9). Accordingly, 4.3-6.9% of differentially alternatively spliced genes were RNA binding proteins, splicing factors (SF-RBPs) or spliceosome proteins. Second, transcripts encoding many TFs were differentially alternative spliced during hormone responses (Fig. 5a; Supplementary Table 9). The majority of these were alternatively spliced uniquely in response to a single hormone, except for JA-responsive TFs (unique splicing events; ABA, 71.0%; BR, 50.0%; JA, 26.3%; SA, 53.5%; SL/KAR, 68.0%; Fig. 5f; Supplementary Table 9), indicating that individual hormones regulate distinct sets of TFs through alternative splicing. A small number of common alternatively spliced TFs did exist, with eight TFs shared by at least three hormones. Amongst these, published data connected to hormone signalling existed only for ELONGATED HYPOCOTYL5 (HY5) (Hamasaki et al., 2020; Ortigosa et al., 2020). These findings indicate that hormone responses use alternative splicing to further diversify the transcriptome by influencing TFs and the alternative splicing machinery itself.

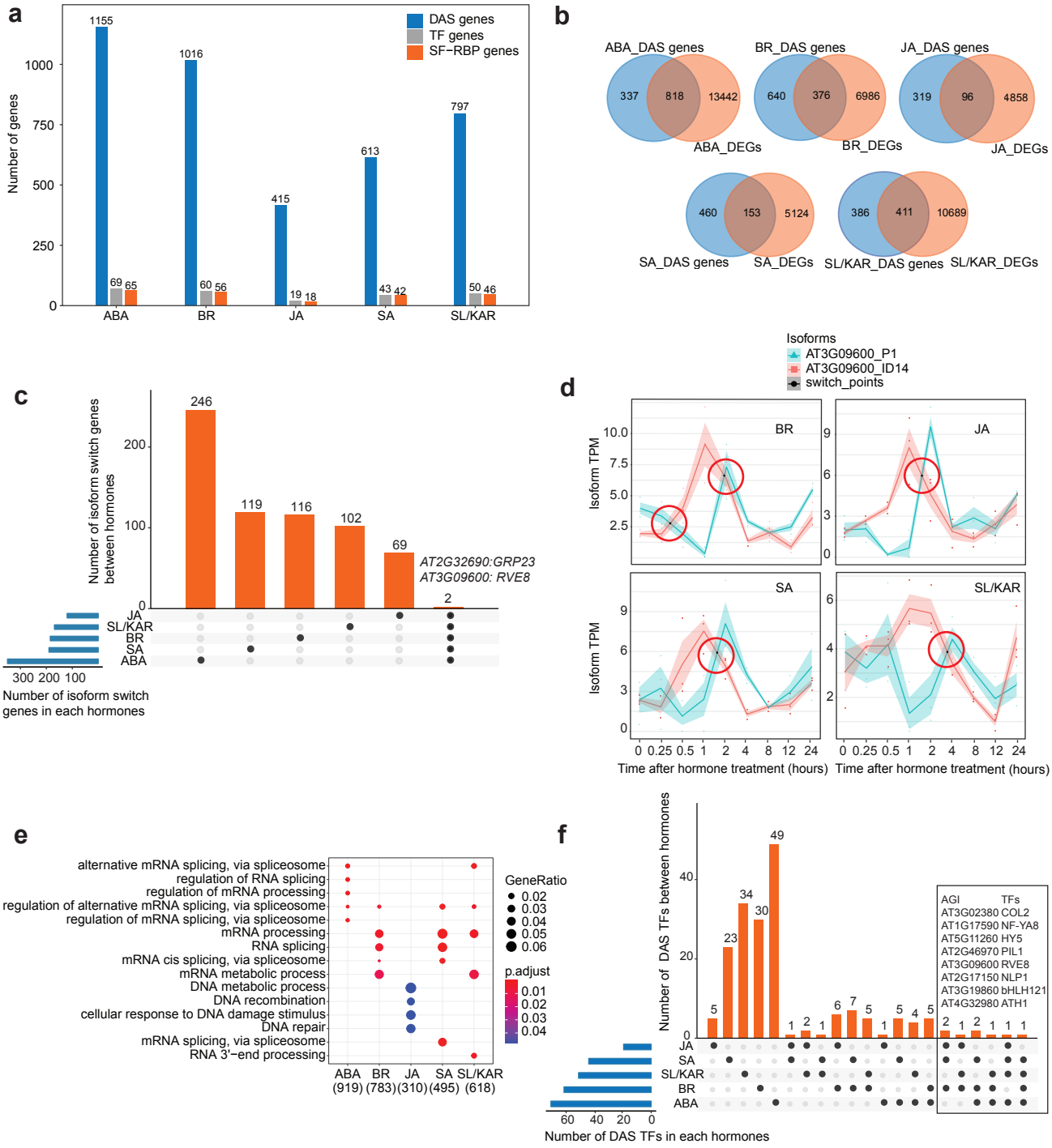

Figure 5. Alternative splicing is a core component of hormone responses. a, The numbers of significant differentially alternative spliced (DAS) genes (FDR < 0.05), and the number of genes encoding TFs, and splicing factors and RNA binding proteins (SF-RBPs) amongst the DAS genes following each hormone treatment. **b**, Overlap between DAS genes and differentially expressed genes (DEGs) for each hormone. **c**, Number of genes that exhibit isoform switching between hormones. Isoform switch event describes the splicing phenomenon whereby the relative abundance of two transcript isoforms from a single gene reverse following hormone treatment. The plot shows how many are unique to a hormone and how many are shared between all five hormones analysed. Two genes are shared by 5 hormones, whose gene ID and names are labelled at the top of respective columns. **d**, Example isoform switch events for *RVE8* (two different isoforms, P1 vs. ID14) for four hormone responses. The isoform switch points detected by TSIS are indicated with red circles. **e**, Top five significantly enriched (p.adjust < 0.05) gene ontology terms amongst the DAS genes upon each hormone treatment. **f**, DAS TFs that are unique and shared between the five hormone responses analysed.

2.3.6 Alternative splicing contributes to early hormone signalling responses independent of differential gene expression

Alternative splicing is co-transcriptional but can occur without differential gene expression (Marquez et al., 2012). We examined the relative dynamics of these two processes during hormone responses, to better understand their relative contributions to transcriptome reprogramming. We determined the time point at which each gene or transcript was first significantly differentially expressed or alternatively spliced relative to 0 h (Fig. 6a). The temporal dynamics of differential expression and alternative splicing differed, with alternative splicing appearing to be more frequent at early timepoints than differential expression. To examine this more closely we plotted the relative proportion of alternatively spliced and differentially expressed genes across all time points (Fig. 6b). The proportional contribution of alternative splicing to transcriptome remodelling was greatest at early timepoints for all hormones except BR. These responses occurred with the first 15 mins to 1 h after hormone treatment. Consequently, it is likely that alternative splicing has an important role in rapid responses to hormone signalling, acting independently of differential gene expression.

2.4 Discussion

Plant hormones do not operate in isolation; rather, they form a large network to optimize plant growth and development (Altmann et al., 2020). In this study, we aimed to determine the extent of cross-regulation in a network of 6 hormones and to understand how this was related to gene regulation. We reconstructed a dynamic signalling gene regulatory network model of hormone cross-regulation in etiolated Arabidopsis seedlings. This was achieved by integrating time-series transcriptomics following ABA, ET, JA, SA, BR or SL/KAR treatment with genome wide target maps for hundreds of TFs and large-scale protein-protein interaction maps. Our major finding from these analyses is that hormone cross-regulation occurs at multiple levels, spanning signal transduction, TF activity and gene expression. This integrated view extends our knowledge of the scale and mechanisms of hormone cross-regulation. It also increases our understanding of the fundamental principles of plant transcriptional regulation during hormone responses. The framework we have developed here to characterize regulatory networks in environmental responses and development can be applied broadly to plant biology and beyond.

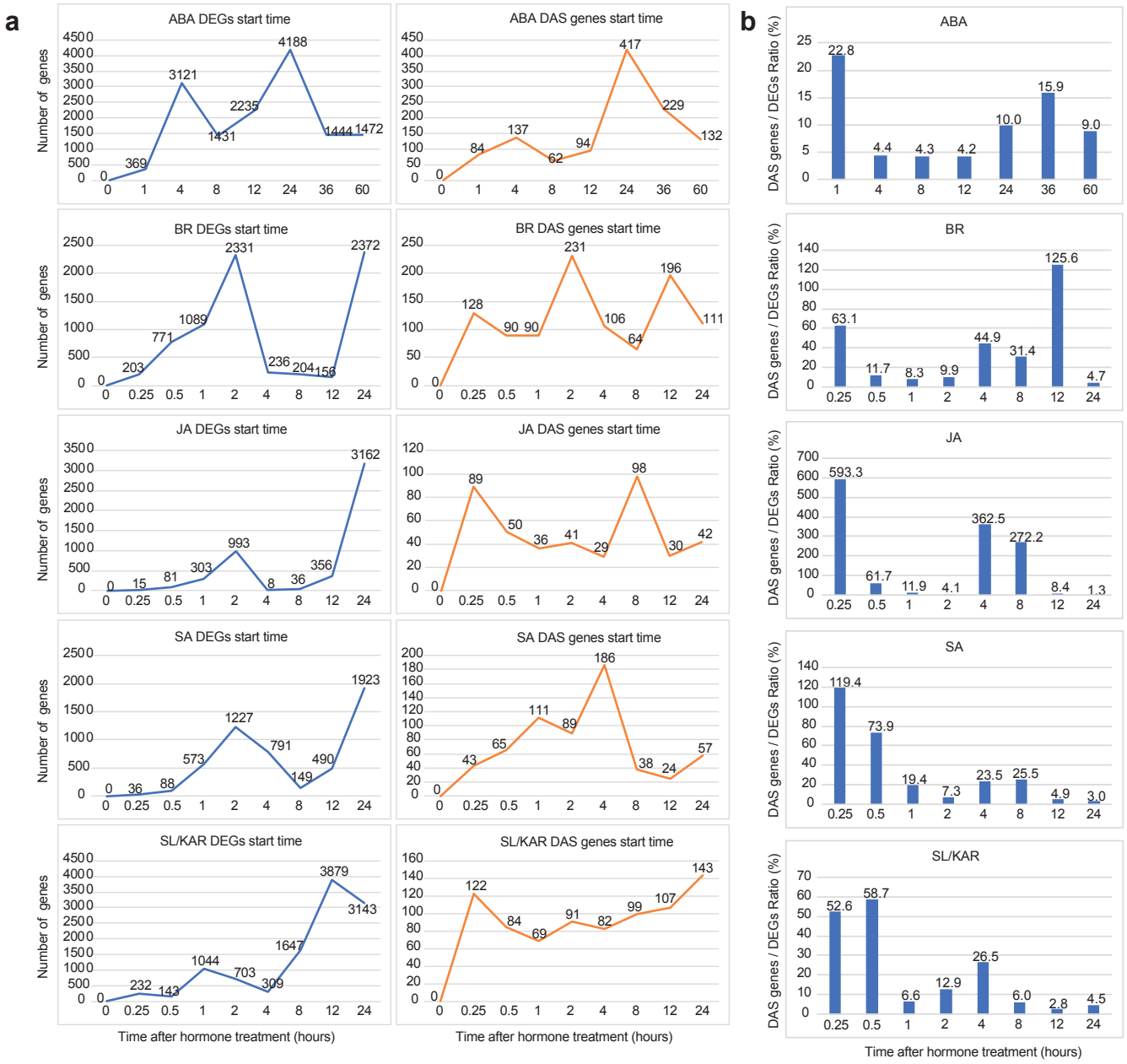

Figure 6. The relative dynamics of differential expression and alternative splicing in hormone responses. a, The number of genes or transcripts first significantly differentially expressed or alternatively spliced relative to 0 h at each time point. **b**, Plots show the relative proportion of differentially alternative spliced (DAS) genes and differentially expressed genes (DEGs) across all time points in each hormone dataset.

Our study illustrates that different hormones employ different combinations of TFs for transcriptional regulation, but a small number of TFs are shared between hormones. The functions of shared TFs may differ between hormone regulatory pathways as their target genes may differ, or they have differential function as an activator or repressor. We identified that hub target genes bound by multiple TFs exist, allowing regulation to converge at certain genes. Hub target genes exhibit stronger differential expression in response to hormones and greater enrichment for TF activity than non-hubs. These properties are consistent with the properties reported of hub target genes in a network of Arabidopsis flowering and light regulation, as well as a transcriptional regulatory network of maize leaf (Heyndrickx et al., 2014; Tu et al., 2020). Considering these together, our findings demonstrate that the regulatory activity of TFs during hormone responses is complex. The findings also provide evidence that the TF activity of hub target genes may be a general principle of plant regulatory networks.

In this study we predict that a group of MPKs have a central role in hormone cross-regulation. Their biochemical properties mean they are very well-suited to this. MPK cascade is a highly conserved feature of the signalling pathways that integrate environmental signals into rapid cellular responses (Cristina et al., 2010; Raja et al., 2017; Bigeard and Hirt, 2018; Jagodzik et al., 2018). The roles of single or pairs of MPKs in hormone signalling pathways have been well studied (Jagodzik et al., 2018). Here we demonstrate that MPKs act as convergence points for cross-regulation within a network of multiple hormones. In addition, we observed that MPKs have many immediate neighbours that differ between specific hormone responses, which may indicate that they drive different responses between hormones. However, the mechanisms of how MPKs select and phosphorylate different downstream substrates between hormone signalling pathways is not known. This may be programmed through modifiers such as in the example of the SMALL UBIQUITIN-LIKE MODIFIER (SUMO) interaction motif of MPK3/6, which enables MPK3/6 to differentially select and phosphorylate substrates (Verma et al., 2021). Moving forward it will be important to functionally validate the predicted roles of MPKs in hormone responses by comprehensively identifying the interactors and targets of these MPKs. This will allow us to better understand how plants process multiple hormone signals through MPKs to adapt to diverse environmental conditions.

Alternative splicing occurred within the early timepoints of hormone responses and made its largest contribution within this time window. This suggests alternative splicing acts independent of differential gene expression to some extent, which differs from plant responses to cold where alternative splicing accompanies the major transcriptional changes (Calixto et al., 2018). Hundreds of TFs, splicing factors and RNA binding proteins were alternatively spliced, all of which would act to further diversify the transcriptome. These features were common across all hormones. I speculate that this may indicate RNA splicing is an important component of hormone signalling mechanisms. Moving forward, validating the role of alternative splicing in hormone signalling will give us greater insight into the hormone responses mechanisms.

Our study provides a broad view of how multiple hormone signals cross-regulate dynamic gene expression and a framework to construct the regulatory network responsible for this. However, it does

not consider the impact of spatial regulation because the models in this study were constructed using data from whole seedlings. Spatial regulation is important for plant to reengineer behaviour during development and in response to environmental cues. Hormone interactions vary between plant tissues and cells (Moore et al., 2015; Topham et al., 2017). Further studies might focus on cell/tissue specific hormone cross-regulation networks to give greater insight into transcriptome dynamics during hormone responses, which would not be captured by our global view. They might also apply the network discoveries to modify growth, development and environmental responses. Moreover, ChIP-seq is limited by the fact that it is difficult to capture short-lived or transient TF-target binding. The cellular signal propagation happens on a shorter timescale might not be resolved in this study. Application of other techniques which can capture transient TF-target interactions genome-wide might lead to the improvement of dynamic GRN construction, such as the cell-based TARGET system (Alvarez et al., 2020).

2.5 Methods

2.5.1 Plant materials, growth conditions and hormone treatments

Three day old etiolated Arabidopsis seedlings of Col-0 background were used for RNA-seq and ChIP-seq experiments. The transgenic lines Col-0 ANAC055::ANAC055-YPet, Col-0 BES1::BES1-YPet, Col-0 EDF1::EDF1-YPet, Col-0 EDF2::EDF2-YPet, Col-0 EDF3::EDF3-YPet, Col-0 EIN3::EIN3-YPet, Col-0 ERF1::ERF1-YPet, Col-0 MYC2::MYC2-YPet, Col-0 MYC3::MYC3-YPet, Col-0 OBP2::OBP2-YPet, Col-0 RAP2.6L::RAP2.6L-YPet, Col-0 STZ::STZ-YPet, Col-0 TCP3::TCP3-YPet and Col-0 TGA5::TGA5-YPet, were generated by recombineering, as previously described (Zander et al., 2020).

Seeds were sterilized with bleach and sown on Murashige and Skoog (cat#LSP03, Caisson) media pH5.7, containing 1% sucrose and 1.8% agar. After stratification (three days dark at 4°C), seeds were exposed to light at room temperature for 2 hours to induce germination, then grown in the dark at 22°C for three days. Etiolated seedlings were subsequently treated with hormones.

For RNA-seq experiments, BR, SA and SL/KAR treatments were applied by spraying the plants until run-off occurred then samples were harvested at each time point. BR treatment was conducted using 10 nM epibrassinolide (Sigma, E1641), SA treatment using 0.5 mM SA (Sigma, S7401), and SL/KAR treatment using 5 µM rac-GR24 (Chiralix, Nijmegen, The Netherlands). For ChIP-seq experiments, BR and SA treatments were as described above. JA and ET treatments were performed as previously described (Chang et al., 2013; Zander et al., 2020).

2.5.2 ChIP-seq data generation

Three day old etiolated seedlings were used for ChIP-seq experiments. All ChIP-seq sequencing data was generated using biologically independent replicate experiments: ANAC055 (air, n=4; ET, n=4), ANAC055 (air, n=3; JA, n=4), BES1 (air, n=4; ET, n=4), BES1 (BR, n=2), EDF1 (air, n=3; ET, n = 3), EDF2 (air, n=3; ET, n=4), EDF2 (JA, n=2), EDF3 (air, n = 3; ET, n = 3), EIN3 (air, n = 3; ET, n= 4), EIN3 (JA, n=2), ERF1 (JA, n=3), MYC2 (air, n=3; ET, n = 3), MYC2 (air, n=3; JA, n=3),

MYC3 (air, n=3; ET, n=3), MYC3 (air, n=4; JA, n=4), OBP2 (air, n=2; ET, n=2), OBP2 (air, n=2; JA, n=3), RAP2.6L (air, n=3; ET, n=3), RAP2.6L (air, n=2; JA, n=2), STZ (air, n=3; JA, n=4), STZ (air, n=2; ET, n=1), TCP3 (air, n=4; ET, n=4), TCP3 (air, n=3; JA, n=3), TCP3 (BR, n=1), TGA5 (air, n=2; ET, n=2), TGA5 (SA, n=1). The ChIP-seq data for JA-treated ANAC055, MYC2, MYC3 and STZ, and the ChIP-seq data for air-treated STZ has been reported in our previous publication (Zander et al., 2020). The remaining ChIP-seq data was generated by this study. All ChIP-seq data were analysed using the workflow described here.

Seedlings were treated with BR, ET, JA, SA or air for 2 hours then collected and snap frozen in liquid nitrogen. Chromatin preparation and immunoprecipitation were performed as previously described (Zander et al., 2020). A goat anti-GFP antibody (supplied by D. Dreschel, Max Planck Institute of Molecular Cell Biology and Genetics) was used and mock immunoprecipitations conducted using whole goat IgG (005–000–003, Jackson ImmunoResearch). Immunoprecipitated DNA was used to prepare sequencing libraries. Libraries were sequenced on an Illumina HiSeq 2500 per manufacturer's instructions (Illumina).

The raw ChIP-seq data for three ABFs (ABF1, ABF3, ABF4) under air and ABA treatments was download from (Song et al., 2016). They were re-analysed using the uniform workflow described in the following ChIP-seq analyses section.

2.5.3 ChIP-seq data analyses

We developed an analysis workflow to process all raw fastq data in a uniform and standardized manner to enable integration and comparison. Fastq files were trimmed using Trimglore V0.4.4 then trimmed reads were mapped to the Arabidopsis TAIR10 genome using Bowtie2 V2.2.9 (Langmead and Salzberg, 2012). The mapped reads were filtered with MAPQ > 10 using samtools V1.3.1 to restrict the number of reads mapping to multiple locations in the genome (Li et al., 2009). Filtered reads were used for all the subsequent analysis. PhantomPeakQualTools v.2.0 was used to assess ChIP-seq experiment quality after read mapping by determining the normalized strand cross correlation (NSC) and relative strand cross correlation (RSC) of each alignment bam files.

MACS V2.1.0 was used to identify the peaks for all replicates by comparison with mock IP of wild-type Col-0 (default parameters except –g 1.19e8 and –q 0.05) (Zhang et al., 2008). Mapped reads and peak locations were visualized using JBrowse (Buels et al., 2016). Only peaks with a q-value >= 1^{e-15} were used in following analyses. Furthermore, only replicates with more than 50 peaks were retained. The total numbers of biological replicates retained for peak annotation were: ANAC055 (air, n=3; ET, n=3), ANAC055 (air, n=2; JA, n=3), BES1 (air, n=2; ET, n=1), BES1 (BR, n=1), EDF1 (air, n=1; ET, n =2), EDF2 (air, n=2; ET, n=3), EDF2 (JA, n=2), EDF3 (air, n =1; ET, n =3), EIN3 (air, n =2; ET, n=3), EIN3 (JA, n=2), ERF1 (JA, n=3), MYC2 (air, n=3; ET, n =2), MYC2 (air, n=3; JA, n=3), MYC3 (air, n=3; ET, n=3), MYC3 (air, n=3; JA, n=3), OBP2 (air, n=2; ET, n=2), OBP2 (air, n=1; JA, n=2), RAP2.6L (air, n=3; ET, n=2), RAP2.6L (air, n=1; JA, n=1), STZ (air, n=1; JA, n=2), STZ (ET, n=1), TCP3 (air, n=3; ET, n=3), TCP3 (air, n=2; JA, n=2), TCP3 (BR, n=1), TGA5 (air, n=2; ET, n=2),

TGA5 (SA, n=1). In general, there were at least two biological replicates for each ChIP-seq sample (35/45, 77.8%).

For each TF, peaks that had at least 50% intersection in at least two independent biological replicates were merged using bedtools V2.26.0 and retained, with all other peaks eliminated (Quinlan and Hall, 2010). Peaks were associated to their nearest genes as annotated in the TAIR10 using R package ChIPpeakAnno with default parameters (Zhu et al., 2010).

2.5.4 RNA isolation and library preparation

Total RNA was isolated from liquid nitrogen ground whole etiolated seedlings using the RNeasy Plant Kit (Qiagen, CA, USA). cDNA libraries were constructed using the Illumina TruSeq Total RNA Sample Prep Kit (Illumina, CA, USA) as per manufacturer's' instructions. Single-end reads were generated by the HiSeq 2500 Sequencing System (Illumina).

2.5.5 RNA-seq analyses

FastQC V0.11.5 was used to perform quality control. Trimglore V0.4.4 (https://www.bioinformatics.babraham.ac.uk/projects/) was used to remove low-quality reads and adapters from raw RNA-seq reads. Trimmed reads of the ET transcriptome data were mapped onto the Arabidopsis genome with the Araport11 annotation using HiSat2 V2.0.5 (Kim et al., 2015). Read counting in genome features was performed using Htseq V0.8.0 (Anders et al., 2015). This different process was necessary because the ET RNA-seq data were from color-space sequencing.

For the other five hormone transcriptome datasets (ABA, BR, JA, SA, SL/KAR), quantification of transcripts was performed using Salmon v0.8.1 in conjunction with AtRTD2-QUASI reference transcriptome (Zhang et al., 2017). A quasi mapping-based index was built using an auxiliary k-mer hash over k-mers of length 31 (k=31). Salmon parameters were kept default for quantification except that fragment-level GC biases ("–gcBias") correction was turned on. The Tximport pipeline was used to summarize transcript-level abundance to gene-level abundance.

Differentially expressed genes in time-series RNA-seq were identified using edgeR 3.28.1 with quasi-likelihood (QL) F-test (using the functions glmQLFit and glmQLFTest). First, lowly expressed genes were filtered using filterByExpr function and then batch correction was performed using the additive model formulas in edgeR. Significantly differentially expressed genes were those having an FDR < 0.01 for BR, ET, JA and SA RNA-seq datasets or FDR < 0.05 with no batch effect correction for the ABA and SL/KAR datasets (Robinson et al., 2010).

For TF family enrichment analysis, the hypergeometric distribution was performed using phyper function in R. The distributions with p-value < 0.01 were considered significant. Known Arabidopsis TF information was obtained from PlantTFDB 5.0 (Jin et al., 2016). To estimate the significance of overlap between any two hormone treatments, a 2*2 table was generated as described in Nemhauser et al. (2006), and the Chi-square test (using chisq.test function in R) was performed based on the table. Clust analysis was performed according to Abu-Jamous and Kelly (2018). Heatmap analyses were performed by pheatmap with default parameters (https://github.com/raivokolde/pheatmap). The

expression data 4 hours after hormone treatments were used for plotting the heatmaps because 2 hours data for ABA and ET treatments were not available.

2.5.6 Hub target gene identification

Hub target genes were identified from networks of 17 hormone relevant TFs built from ChIP-seq data generated by ourselves and two published studies (Song et al., 2016; Zander et al., 2020). All the ChIP-seq data used for constructing each hormone transcriptional network is described in detail in Supplementary Table 8 (ST. 8_1). Target genes in these networks were binned by number of TFs that bind them. Target genes bound by more than 7 TFs were defined as hub target genes. The remaining genes were non-hub genes and were divided into the other two groups (group low, genes that are targeted by 1-3 TFs; group moderate, genes that are targeted by 4-6 TFs.).

For differential expression density plots, log2 fold changes in gene expression relative to 0 h were calculated. The expression data 2 hours after BR, JA, SA and SL/KAR treatment were used for consistency with ChIP-seq data, but 4 hours after ABA and ET treatments because 2 hours data were not available. The p-value was calculated by two-sample K-S test to indicate the distribution difference (p-value < 0.05).

2.5.7 Signalling and dynamic regulatory events modelling

Regulatory networks were modelled using the SDREM framework (Gitter and Bar-Joseph, 2016). SDREM modelling needs time series transcriptomes, hormone receptors identities, TF-gene interactions and protein-protein interactions (PPIs) as input data.

For the time series transcriptomes, Log2 fold changes relative to 0 h for all expressed genes were calculated at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h post-hormone treatment.

TF-target gene interactions came from several sources. First, the TF-gene interactions identified from ChIP-seq data for 17 TFs. The detailed information of which data was used for constructing SDREM models for each hormone is described in Supplementary Table 8 (ST. 8_1). Second, 406,832 TF-gene interactions for 296 TFs were included from published DAP-seq studies (O'Malley et al., 2016; Narsai et al., 2017; Zander et al., 2020). Third, confirmed and direct 4,378 interactions for 293 TFs were obtained from the Arabidopsis Gene Regulatory Information Server repository (Yilmaz et al., 2010).

PPIs were obtained from BioGRID and the combined phytohormone interactome network (Stark et al., 2006; Altmann et al., 2020). The PPI weight score was applied according to SDREM methods (Gitter and Bar-Joseph, 2016).

The identities of hormone receptors for each hormone are listed in Supplementary Data 2 (file *hormone*_source.txt). The receptors for ABA were 14 PYR/PYL/RCARs (Ma et al., 2009; Park et al., 2009), for ET were ETR1, 2, ERS1, 2 and EIN4 (Bleecker et al., 1988; Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998), for SA were NPR1, 2, 3 (Castelló et al., 2018; Ding et al., 2018), and for SL/KAR were AtD14 and KAI2 (Waters et al., 2012). The receptor for BR was BRI1 (Clouse et al., 1996; Wang et al., 2001), and for JA was COI1 (Xie et al., 1998).

When running SDREM the Minimum_Absolute_Log_Ratio_Expression parameter was used at default parameter 1, which retains only the genes whose largest absolute log2 fold changes across all time points is greater than 1. The maximum path length parameter was set to 5 and only binary splits were allowed in the regulatory paths. SDREM was run for 10 iterations for ABA, BR,JA, SA and SL/KAR models. We extended extra 2 iterations for ET SDREM model as the TFs and signalling proteins predicted in each iteration did not substantially converge across iterations when only running 10 iterations. The parameters used in modified DREM and SDREM modelling are given in Supplementary Data 1, 2. SDREM reconstructed signalling pathway results were visualized in Cytoscape v3.8.0. Intersection analysis was conducted using Intervene (Khan and Mathelier, 2017). The integrated transcriptional cross-regulation model was generated by overlaying the individual hormone transcriptional regulatory models.

2.5.8 Functional enrichment analyses

Gene ontology (GO) enrichment analysis of predicted nodes in each hormone model was conducted using the compareCluster function in clusterProfiler with default parameters (Yu et al., 2012).

The functional grouped network of hub genes (group; hubs) and non-hub genes (groups; low and moderate) was performed using ClueGO v2.5.7 in Cytoscape v3.8.0 with ontologies were updated as GO_MolecularFunction-Custom-GOA-ACAP-ARAP_28.08.2020 (Bindea et al., 2009). Benjamini-Hochberg was used to correct the p-values for multiple testing. Functional groups with a p-value < 0.01 were considered statistically significantly enriched. The network specificity was set to 'Global'. GO term fusion was selected to reduce terms redundance. The kappa score was set as ≥ 0.4 to connect the terms in the network. All the settings above were kept same for the three groups.

ClueGO V2.5.7 was used for GO enrichment analysis of the 23 proteins shared by at least four hormone signalling pathways. The ontologies were updated as GO_MolecularFunction-Custom-GOA-ACAP-ARAP_28.08.2020; GO_CellularComponent-Custom-GOA-ACAP-ARAP_28.08.2020 and GO_BiologicalProcess-Custom-GOA-ACAP-ARAP_28.08.2020. Benjamini-Hochberg was selected to correct the p-values for multiple testing corrections. Functional groups with a p-value < 0.05 were considered statistically significantly enriched. The network specificity was set to 'Global' with minimum 5 genes/term. GO term fusion was selected to reduce term redundancy. The other settings were kept as default.

2.5.9 Identification of differentially alternatively spliced genes and isoform switch events

To detect differentially alternatively spliced genes, the union pipeline was used (Guo et al., 2020). Only expressed transcripts that had ≥ 1 counts per million (CPM) in one or more samples were retained. Read counts were normalized by the Trimmed Mean of M-values (TMM) method using edgeR (Robinson et al., 2010). Batch effects were estimated and removed using RUVSeq R package with the remove unwanted variations (RUVs) approach (Risso et al., 2014). Then the voom-weight

function in limma and DiffSplice functions were used for differentially alternatively spliced analysis (Ritchie et al., 2015).

Significantly differentially alternatively spliced genes were determined by using the criteria following. Firstly, at least one of the transcripts differed significantly in log2 fold changes from the corresponding gene with an adjusted p-value of < 0.05, and secondly at least one of the transcripts of the gene exhibited Δ percent spliced (Δ PS) ≥ 0.1. The PS value was estimated as the ratio of a transcript's average abundance divided by the average of its corresponding gene abundance. The SF-RBPs list was obtained from Calixto et al. (2018).

For detection of alternatively spliced isoform-switch events, the TSIS R package was used with time-series transcriptome data as described previously (Zander et al., 2020). Transcripts with average TPM across all time points > 1 were included in the TSIS analysis. The mean expression approach was used to search for interaction points. Statistically significant switch events were identified using the following filtering parameters: (1) probability cut-off value of > 0.5; (2) differences cut-off value of > 1; (3) *p* cut-off value of < 0.05; (4) minimum time in interval of > 1. The protein coding transcripts information was obtained from Zhang et al. (2017).

2.6 Data availability

All described lines can be requested from the corresponding authors. The PPIs with applied scores, the TF-target interaction inputs, the parameters and the output models for recreating models for each hormone in this study can be found in Supplementary Data 1, 2.

Sequence data for new ChIP-seq data decribed in this study can be downloaded from the Gene Expression Omnibus repository (GEO, https://www.ncbi.nlm.nih.gov/geo/). RNA-seq data for BR, SA, SL/KAR can be downloaded from GEO with accession number GSE18261 and reviewer token qrunyeymtpurngt.

ET RNA-seq data raw reads were downloaded from Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) with accession number SRA063695. ABA RNA-seq raw reads and ChIP-seq raw reads for ABF1, 3, 4 were downloaded from GEO with accession number GSE80568. JA RNA-seq raw reads were downloaded from GEO with accession number GSE133408.

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2.8 Contributions

MGL, JRE and LY designed the study. MGL, MZ, MX, LS, JPSG, EH, RCS and BJJ generated the transgenic constructs and carried out the RNA-seq and ChIP-seq lab experiments. SCH, SJ, AW and ZB-J provided new analytical tools. LY analysed and integrated all data, interpreted results and prepared figures. LY wrote the manuscript and MGL provided feedback. All authors read and approved the final manuscript.

2.9 Supplementary Table Legends

Supplementary Table 1. Differentially expressed genes for each hormone response time series, determined by RNA-seq.

Supplementary Table 2. Overview of quality metrics of generated ChIP-seq datasets by this study.

Supplementary Table 3. Target genes and binding sites of 17 TFs under air or hormone treatments, determined by ChIP-seq.

Supplementary Table 4. The regulatory network determined by modified DREM models for all hormones.

Supplementary Table 5. Overview of six hormone signalling pathways reconstructed by SDREM modelling.

Supplementary Table 6. The predicted active TFs and their family distributions for each hormone.

Supplementary Table 7. List of number of differentially expressed genes shared between any two hormone response transcriptomes and overview of the differentially expressed TFs and their family distributions in each hormone datasets.

Supplementary Table 8. Overview of hub target genes and non-hub genes bound by 17 hormone TFs in hormone transcriptional networks.

Supplementary Table 9. Overview of the differentially alternatively spliced genes for each hormone response time series analysed, determined by transcript-level time series RNA-seq.

Supplementary Table 10. Overview of the isoform switch events in the time series RNA-seq for each hormone analysed.

2.10 Supplementary Data Legends

Supplementary Data 1. The inputs, parameters and output models for recreating the regulatory networks for each hormones.

Supplementary Data 2. The inputs, parameters for recreating the signalling pathways for each hormones.

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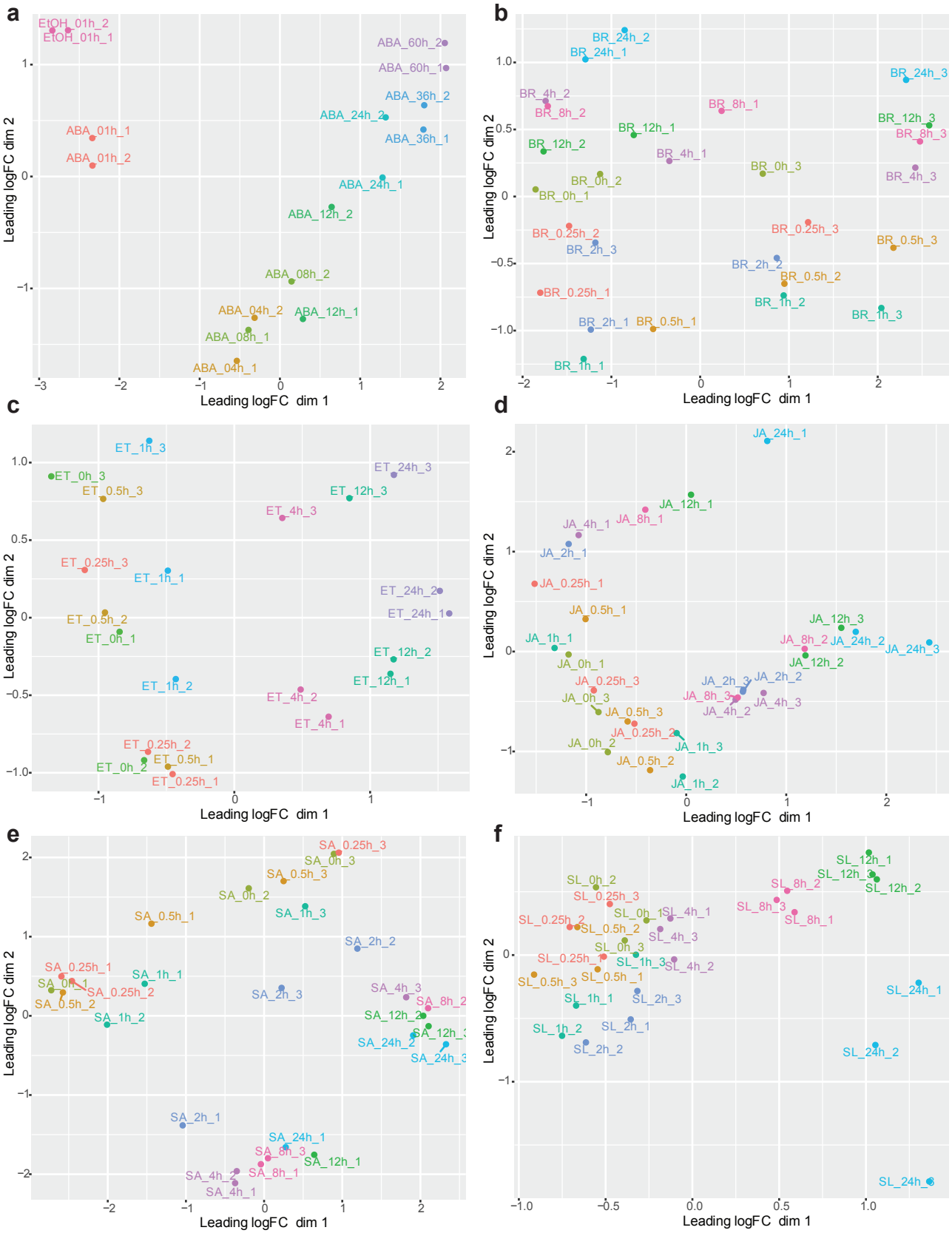
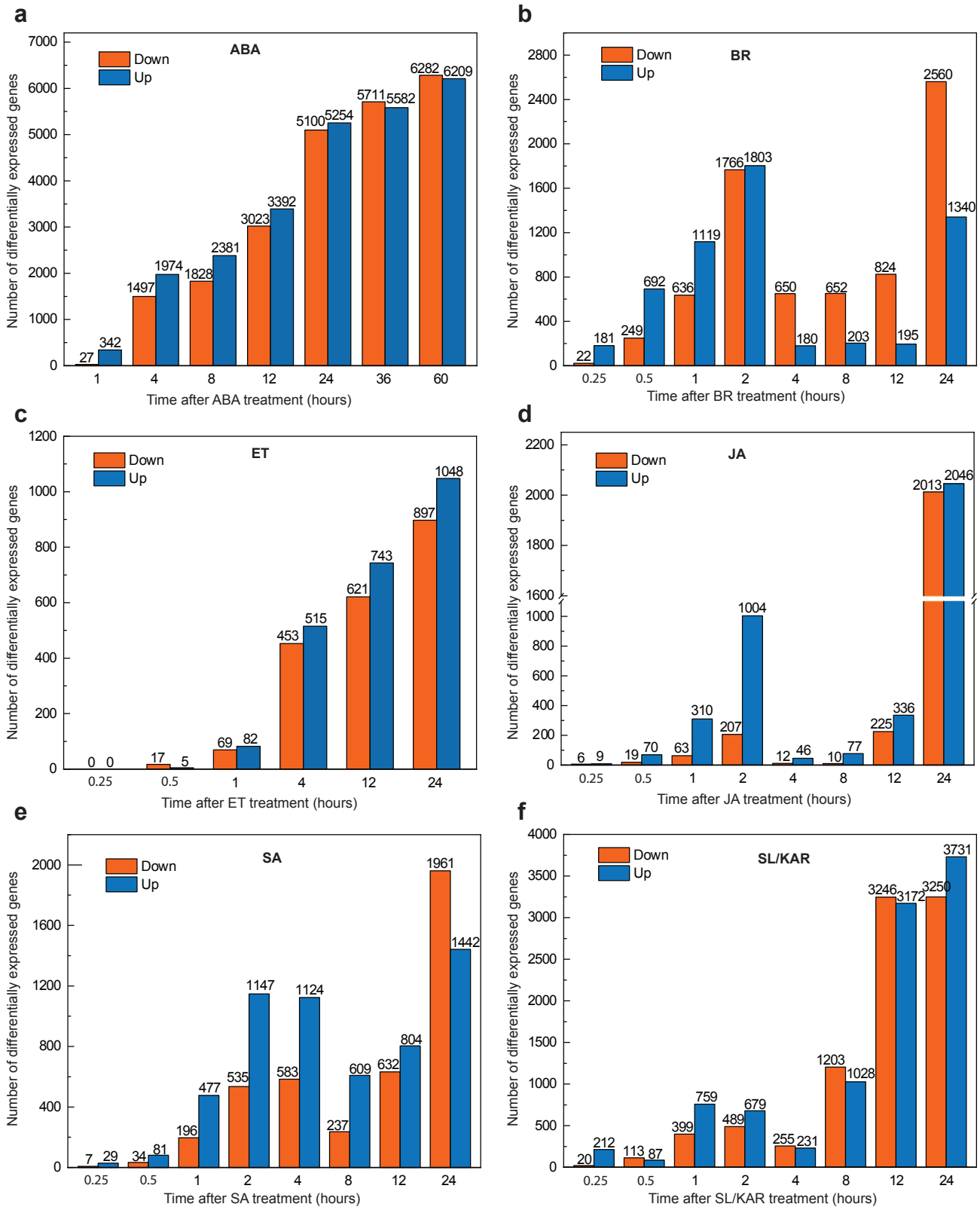
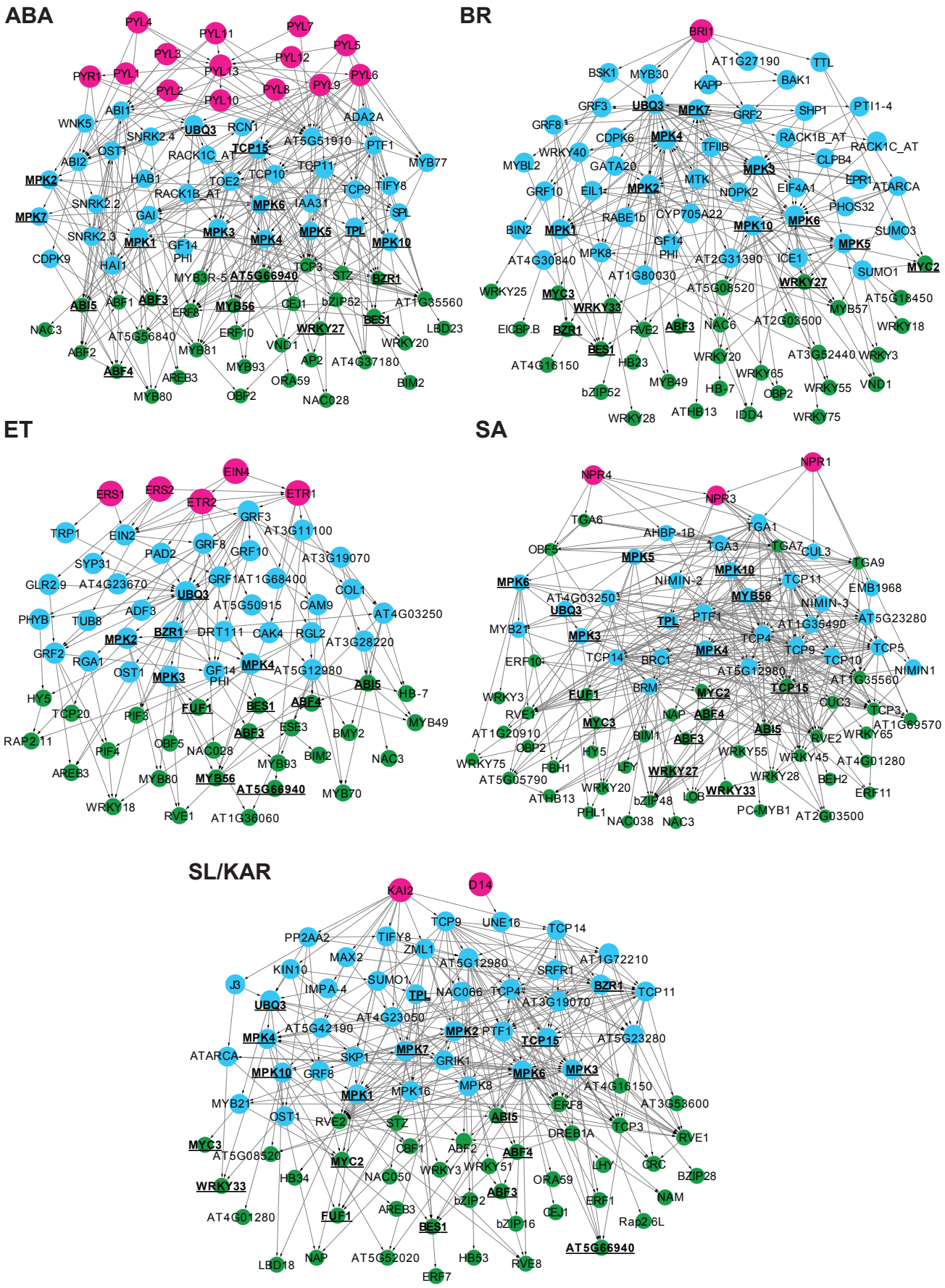


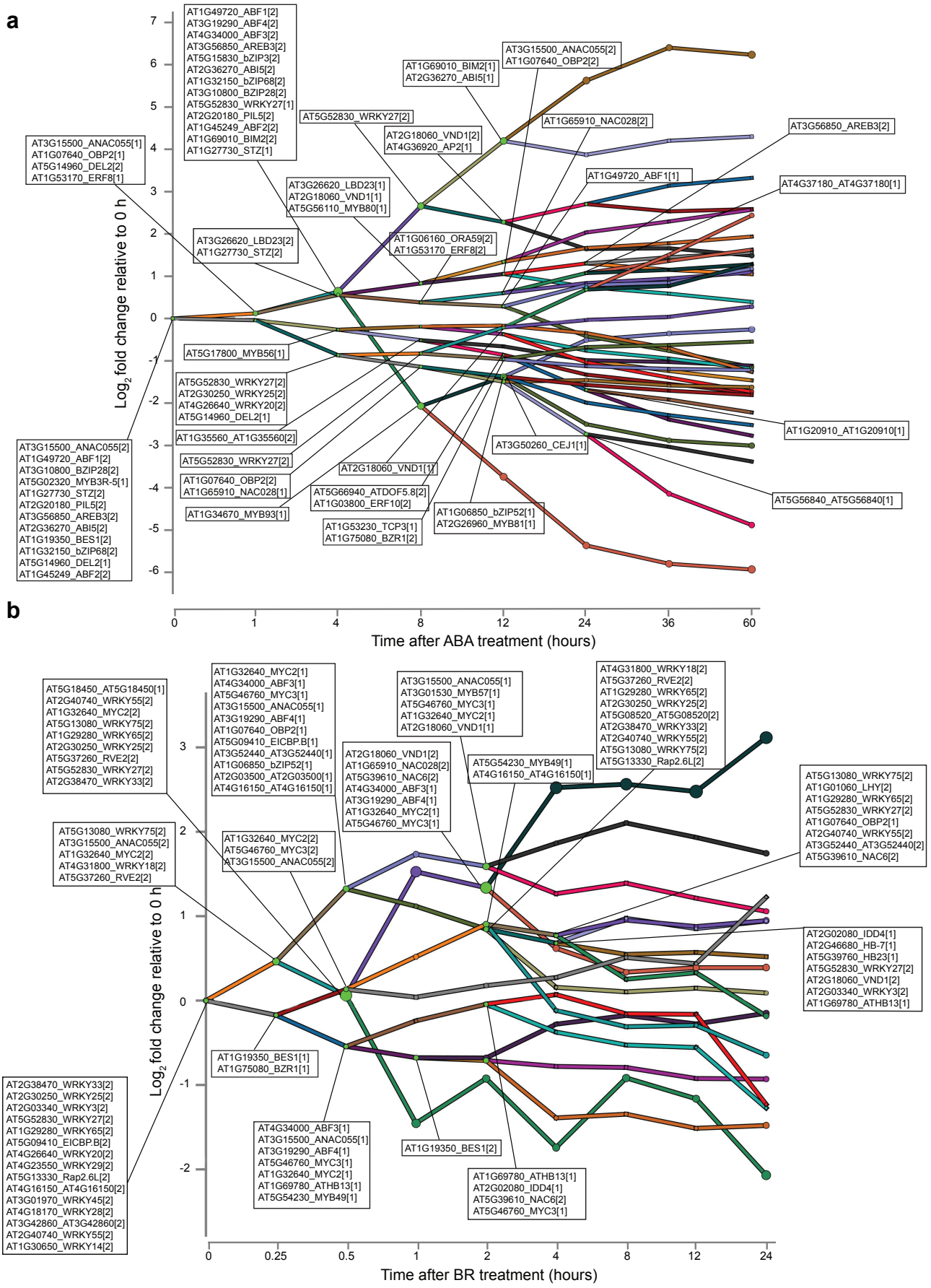
Extended Data Figure 1. Overview of quality metrics of RNA-seq data. a-f, Multidimensional scaling (MDS) plots of replicate samples of the ABA, BR, ET, JA, SA and SL/KAR (labelled here as SL) treatment RNA-seq time-series in WT. BR, ET, JA, SA and SL/KAR treatment time series consist of three independent samples (n = 3) for each time point. ABA treatment time series consist of two independent samples (n = 2) for each time point. The ethanol treated 1 hour sample (EtOH_01h) was used as the mock control sample for ABA treated samples when performing the differentially expressed gene analysis, because no 0 hour sample was collected during the experiment.



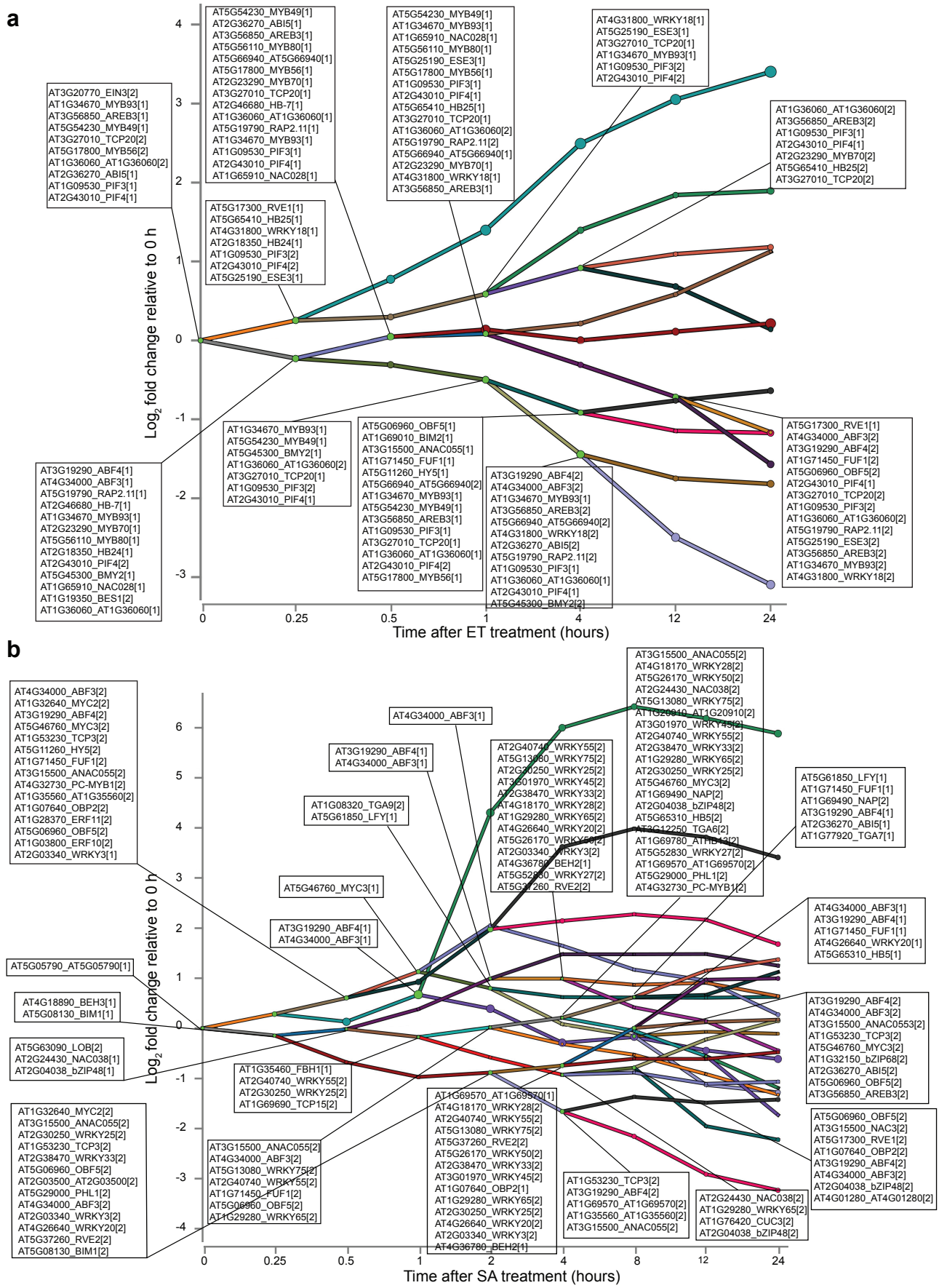
Extended Data Figure 2. Time-series transcriptome analysis. a-f, Plots show the numbers of significantly differentially expressed genes (FDR < 0.01 for BR, ET, JA and SA datasets; FDR < 0.05 for ABA and SL/KAR datasets) relative to 0 h upon hormone treatment. The x-axis represents time after hormone treatment (hours). The y-axis represents the numbers of significantly down-regulated and up-regulated genes, which are represented by orange and blue bars respectively.



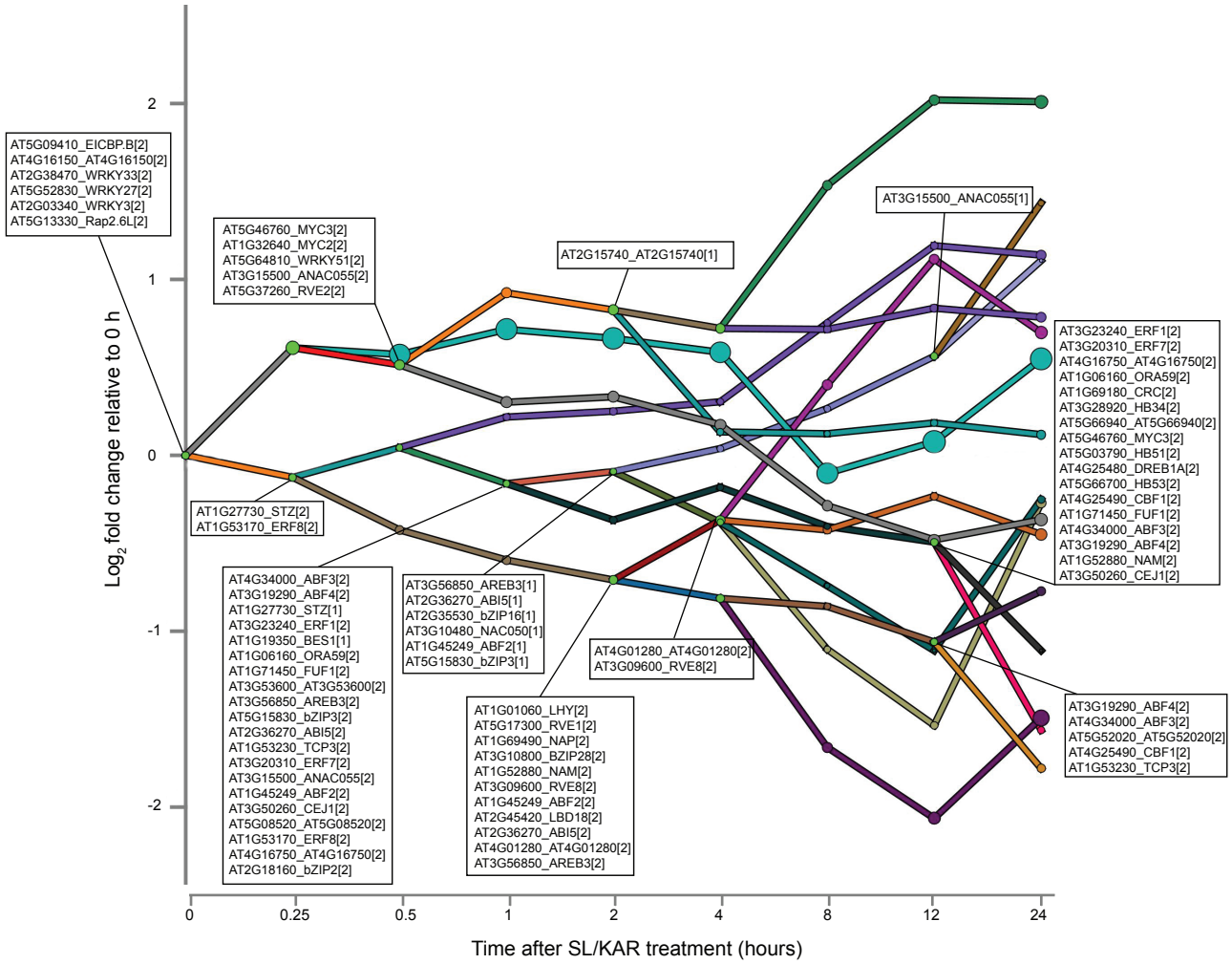
Extended Data Figure 3. The reconstructed transcriptional regulatory models for ABA, BR, ET, SA and SL/KAR. The hormone receptor(s), intermediate proteins and active TFs are represented by magenta, blue and green nodes respectively. The proteins shared by at least 4 hormone pathways are in black bold text and have underlined names.



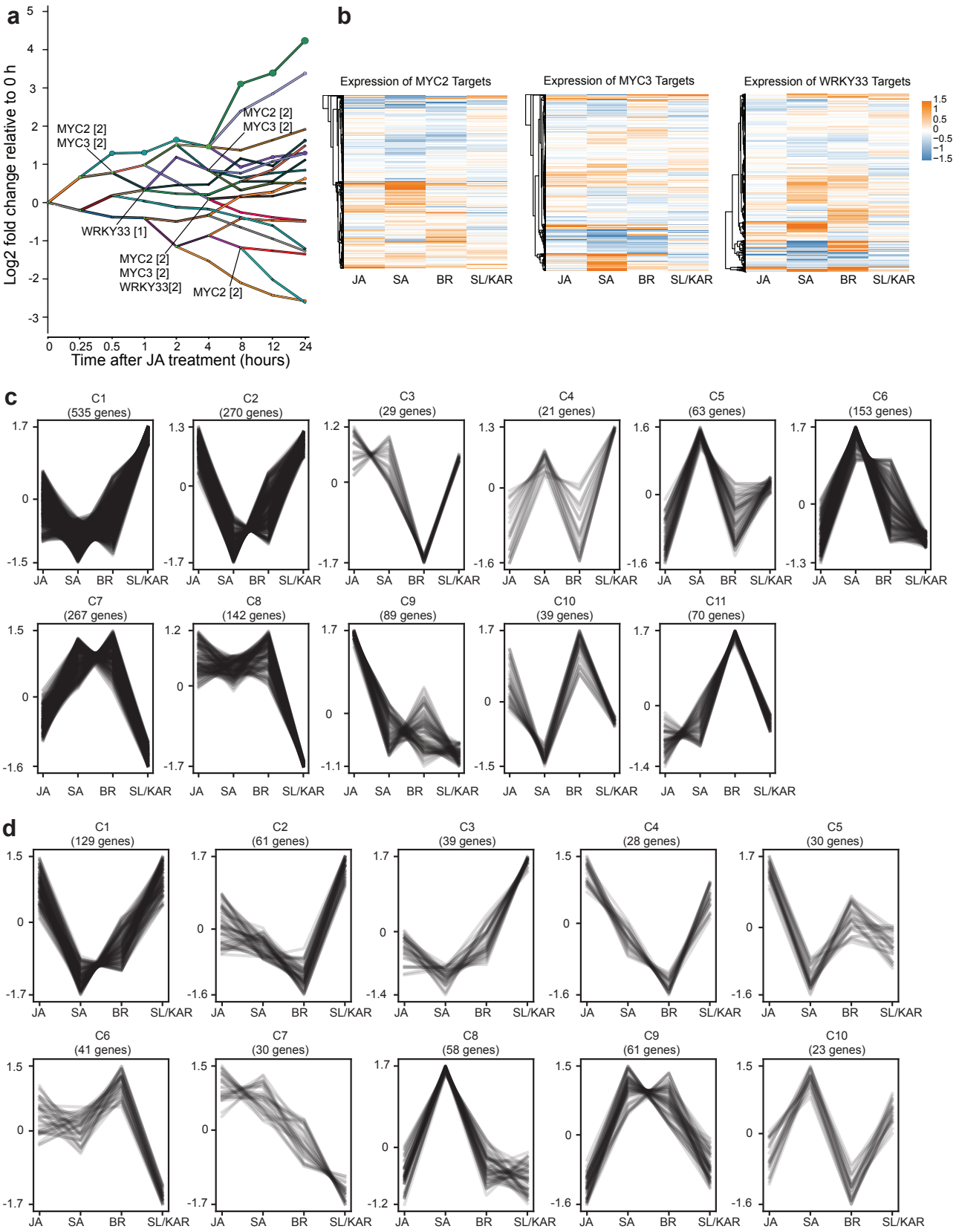
Extended Data Figure 4. The transcriptional regulatory network component of the ABA (a) and BR (b) models. The networks display all predicted active TFs at each branch point (node) and the bars indicate co-expressed and co-regulated genes for each hormone. [1] indicates the TF primarily controls the lower path out of the split and [2] is for the higher path. The y-axis is the log2 fold change in expression relative to expression at 0 h.



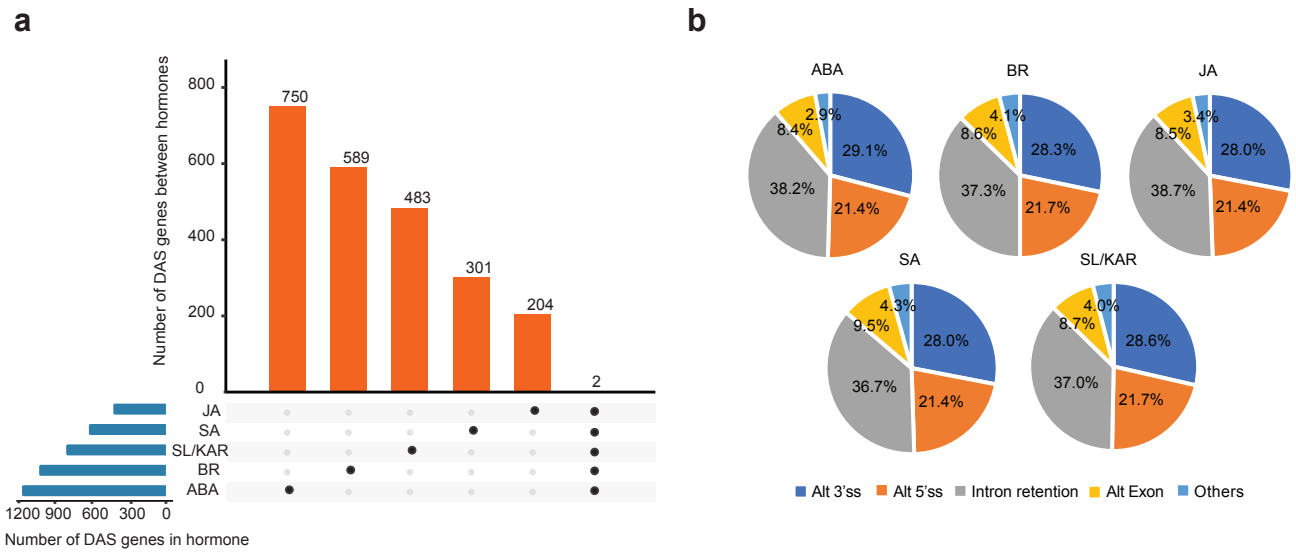
Extended Data Figure 5. The transcriptional regulatory network component of the ET (a) and SA (b) models. The networks display all predicted active TFs at each branch point (node) and the bars indicate co-expressed and co-regulated genes for each hormone. [1] indicates the TF primarily controls the lower path out of the split and [2] is for the higher path. The y-axis is the log2 fold change in expression relative to expression at 0 h.



Extended Data Figure 6. The transcriptional regulatory network component of the SL/KAR model. The network display all predicted active TFs at each branch point (node) and the bars indicate co-expressed and co-regulated genes. [1] indicates the TF primarily controls the lower path out of the split and [2] is for the higher path. The y-axis is the log2 fold change in expression relative to expression at 0 h.



Extended Data Figure 7. Different activity of shared TFs between hormone models. a, Simplified transcriptional regulatory network component for JA response highlighting the association of MYC2, MYC3 and WRKY33 with up and down regulated genes. **b**, Heatmap of expression of targets for MYC2, MYC3 and WRKY33 during JA, SA, BR and SL/KAR hormone responses. Hormone models are indicated in the x-axis, expression is given as log2 fold change relative to 0 h. **c**, **d**, K-means clustering of expression of MYC3 (**c**) and WRKY33 (**d**) target genes during JA, SA, BR and SL/KAR hormone responses. Expression is given as normalized transcripts per million (TPM).



Extended Data Figure 8. The number and alternative splicing types of the differentially alternative spliced genes in response to hormone. a, The number of differentially alternative spliced (DAS) genes unique to and shared between between all five hormones analysed. **b**, The major alternative splicing types of DAS genes in each hormone. Alt 3'ss: Alternative 3' (A3) splice sites, Alt 5'ss: Alternative 5' (A5) splice sites, Intron retention, Alt Exon: Skipping exon (SE) and Mutually Exclusive (MX) exons, Others: Alternative First (AF) and Last (AL) exons.

3. CHAPTER 3. ETHYLENE RESPONSE DNA-BINDING FACTORs are transcriptional repressors responsible for hormone cross-regulation during the ethylene response

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Supplementary tables and source data are available from this link:
https://figshare.com/s/da0976d939001ba33f12

3.1 Abstract

Ethylene is a gaseous plant hormone that regulates plant growth and development. Broad reprogramming of gene expression is required for ethylene responses. The master ethylene transcription factor (TF) ETHYLENE INSENSITIVE3 (EIN3) drives expression of secondary TFs including the ETHYLENE RESPONSE DNA-BINDING FACTORS (EDFs), but the role of the EDFs within the ethylene genome regulatory network is not understood. Here, we describe an investigation into the function of the EDFs in ethylene signalling and hormonal cross-regulation. We determined the target genes and binding dynamics of EDF1, 2, 3 during an ethylene response and the effects of *edf1234* quadruple mutation on gene expression. The EDFs and EIN3 shared a large proportion of their target genes but had different functions. The EDFs were associated with repression of target genes, but this was superseded by activation when EIN3 bound the same genes. Genes important in other hormone signalling pathways, in particular abscisic acid (ABA), were targets of the EDFs. This demonstrates how ethylene engages hormonal cross-regulation to repress genes in competing signalling pathways and prioritize itself.

3.2 Introduction

Ethylene is a plant hormone that regulates plant growth and development including seed development, germination and early seedling growth, fruit ripening and disease resistance (Iqbal et al., 2017; Harkey et al., 2019; Binder, 2020). The etiolated Arabidopsis (*Arabidopsis thaliana*) seedling is the classic model for study of ethylene-mediated regulation of development. In this system, ethylene controls apical hook formation, hypocotyl and root growth (Guzmán and Ecker, 1990). Ethylene is perceived by a family of five receptors - ETHYLENE RECEPTOR 1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR 1 (ERS1), ERS2 and EIN4 - which are localized in the endoplasmic reticulum and are negative regulators of the ethylene signalling pathway (Bleecker et al., 1988; Chang et al., 1993; Hua et al., 1995; Hua et al., 1998). In the absence of ethylene, a Raf-like serine/threonine kinase, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) is activated and tightly associated with the receptor complex, blocking signal transduction by phosphorylating EIN2 (Kieber et al., 1993; Alonso et al., 1999). When plants perceive an ethylene signal, the inactivated CTR1 no longer phosphorylates EIN2, allowing EIN2 to translocate to the nucleus and activate TF EIN3 (Johnson and Ecker, 1998). Master TFs activate the expression of a large proportion of hormone-responsive genes and loss function of these TFs results in a clear decrease in hormone sensitivity. EIN3 is the master TF which activates transcription of the genes it binds directly, binding to the ethylene responsive element in the promoters of hundreds of genes and inducing ethylene responsive gene expression in ethylene signalling pathway (Solano et al., 1998; Chang et al., 2013). Loss of function of EIN3 causes ethylene insensitivity (Chao et al., 1997). EIN3 also has indirect, tissue-specific, transcriptional repressive activity in etiolated seedling shoots (Wang et al., 2020). This occurs by the interaction of EIN3 with the protein Transcriptional Repressor EIN3-dependent Ethylene-response 1 (TREE1). A considerable number of EIN3's targets that are also regulated by ethylene are TFs themselves (180/1314, 13.7%; Hypergeometric distribution test, p-value = 3.32E-34), termed secondary TFs which are TFs that are directly targeted by and function downstream of master TFs (Chang et al., 2013). This forms an ethylene-dependent gene regulatory network that regulates broad reprogramming of gene expression, affecting thousands of genes, and driving the cellular response to ethylene. However, little is known about the genome-wide targets and roles of the secondary TFs that act immediately downstream of EIN3.

The four EDF TFs (EDF1/TEM1, AT1G25560; EDF2/RAV2/TEM2, AT1G68840; EDF3/RAV1-Like/RAV3, AT3G25730; EDF4/ RAV1, AT1G13260) are a group of secondary, ethylene-inducible TFs immediately downstream of EIN3 (Alonso et al., 2003; Alonso and Stepanova, 2004). They belong to a subfamily of the RAV/EDF TFs that have dual AP2/B3 DNA binding domains, which bind CAACA and CACCTG motifs respectively (Kagaya et al., 1999; Alonso et al., 2003; Matias-Hernandez et al., 2014). The EDFs are transcriptional repressors, carrying a conserved repressive R/KLFGV amino acid motif in their B3 domain (Ikeda and Ohme-Takagi, 2009; Chen et al., 2015). They are involved in diverse plant developmental processes and abiotic stress responses, encompassing flowering time, flower and leaf senescence, flower abscission, salt and drought stresses in

Arabidopsis (Hu et al., 2004; Castillejo and Pelaz, 2008; Woo et al., 2010; Osnato et al., 2012; Fu et al., 2014; Matias-Hernandez et al., 2014; Chen et al., 2015; Osnato et al., 2020; Sengupta et al., 2020; Verhage, 2021). The EDF homologs in soybean, pepper and rice are similarly implicated in responses to a range of biotic and abiotic stresses (Sohn et al., 2006; Duan et al., 2016; Zhao et al., 2017).

The EDFs are functionally connected to several hormone signalling pathways, consistent with their broad observed functions. Expression of *EDF1, 2, 3* and *4* is induced by ethylene and, whilst no distinct phenotypes are observed in single *edf* mutants, *edf1234* quadruple mutant plants are mildly ethylene insensitive (Alonso et al., 2003). This indicates functional redundancy exists amongst the family. EDF1 directly regulates the expression of two gibberellin (GA) biosynthetic genes, *GIBBERELLIN 3-OXIDASE 1* (*GA3OX1*) and *GA3OX2*, to control Arabidopsis flowering time (Osnato et al., 2012). EDF 1 and 2 may upregulate the expression of jasmonic acid biosynthesis genes to promote leaf senescence under salt stress (Osnato et al., 2020). *EDF4* is down-regulated upon brassinosteroid (24-epibrassinolide; epiBL) treatment (Hu et al., 2004). It is also repressed by the ABA signalling pathway through ABA INSENTIVITY 3 (ABI3) during dehydration stress and mutation of *EDF4* results in ABA hypersensitivity (Feng et al., 2014; Fu et al., 2014; Sengupta et al., 2020). The expression of *ABI3*, *ABI4*, *ABI5* can be repressed by EDF4, which further suggests EDFs might have a role in ethylene-ABA cross-regulation (Feng et al., 2014).

The effects of TFs are a result of transcriptional activation or repression of their target genes. A small number of EDF target genes have been identified through low-throughput and site-specific assays (Castillejo and Pelaz, 2008; Osnato et al., 2012). However, TFs typically target hundreds to thousands of genes *in vivo* (Chang et al., 2013; Nagel et al., 2015; Song et al., 2016; Zander et al., 2020; Varala et al., 2018; Brooks et al., 2019; Alvarez et al., 2020). The complete population of EDF target genes has not been determined, which limits our ability to understand the functions of EDFs within the ethylene gene regulatory network and signalling pathways that they influence. Here we investigate the functions of the EDFs in etiolated Arabidopsis seedlings by mapping the effects of ethylene signalling on binding of EDF1, 2, 3 genome-wide. We find that the ethylene-responsive binding dynamics of EDFs differ from one-another and that the EDFs have both common and unique target genes. Transcriptomic analyses of wild-type and *edf1234* quadruple mutant plants support the proposal that EDFs are transcriptional repressors *in planta*. Lastly, genome regulatory network modelling indicates that the EDFs have a role in cross-regulation of other hormones by ethylene.

3.3 Results

3.3.1 Binding of EDF1, 2, 3 to their target genes is ethylene-responsive and may act to cross-regulate other signalling pathways

Our primary aim was to determine the functions of secondary ethylene TFs EDF1, 2, 3 in the ethylene signalling pathway. EDF4 was excluded from analysis because it is only very weakly ethylene inducible (Alonso et al., 2003). To do so we first identified their target genes genome-wide using chromatin immunoprecipitation sequencing (ChIP-seq). Experiments were performed in

etiolated Arabidopsis seedlings 2 hours after ethylene gas or air treatment and the master ethylene TF EIN3 was analysed for comparison (Extended Data Fig. 1; Supplementary Table 1). We observed that ethylene treatment substantially increased the total number of EDF target genes, similar to the response of EIN3 (Fig. 1b, Supplementary Table 2). The ethylene-responsive binding of EIN3 has previously been analysed by ChIP-seq with a different antibody strategy (Chang et al., 2013). The ethylene-responsive binding of EIN3 observed in our study is consistent with Chang's finding where they observed that EIN3 binding was ethylene-induced and increased with ethylene treatment to a maximum at 4 hours of ethylene treatment. The magnitude of change in EDF binding was from between 23 and 168 target genes (air-treatment) to between 1527 and 2701 target genes (ethylene-treatment) (Fig. 1b). This demonstrates that in the absence of ethylene the EDFs bind very few of their target genes in the absence of ethylene and that ethylene signalling promotes EDF DNA-binding activity.

Between them, EIN3 and EDF1, 2, 3 bind 38.3% (128/334) of genes that have an ethylene pathway-related annotation ,which indicates that the ethylene treated ChIP-seq experiments of EIN3 and EDF1, 2, 3 in this study were successful as we observed a considerable number of their targets do have previous ethylene annotations (Fig. 1c; Supplementary Table 3). However, EIN3 shared only a subset of its target genes with EDF1, 2, 3 (647 of 1146 EIN3 target genes) (Fig. 1c). Additionally, the target genes of the EDFs and EIN3 had different functional annotations (Fig. 1d; Supplementary Table 4). The target genes of EDF1, 2, 3 were not significantly enriched for ethylene annotations, but were enriched for karrikin (EDF1), flowering (EDF2), ABA (EDF3) and auxin (EDF1, 2, 3) annotations (p.adjust < 0.05). These data indicate that EDF1, 2, 3 may have roles in cross-regulation of other hormonal signalling pathways by ethylene. They also indicate that the EDFs may have distinct functions from one-another.

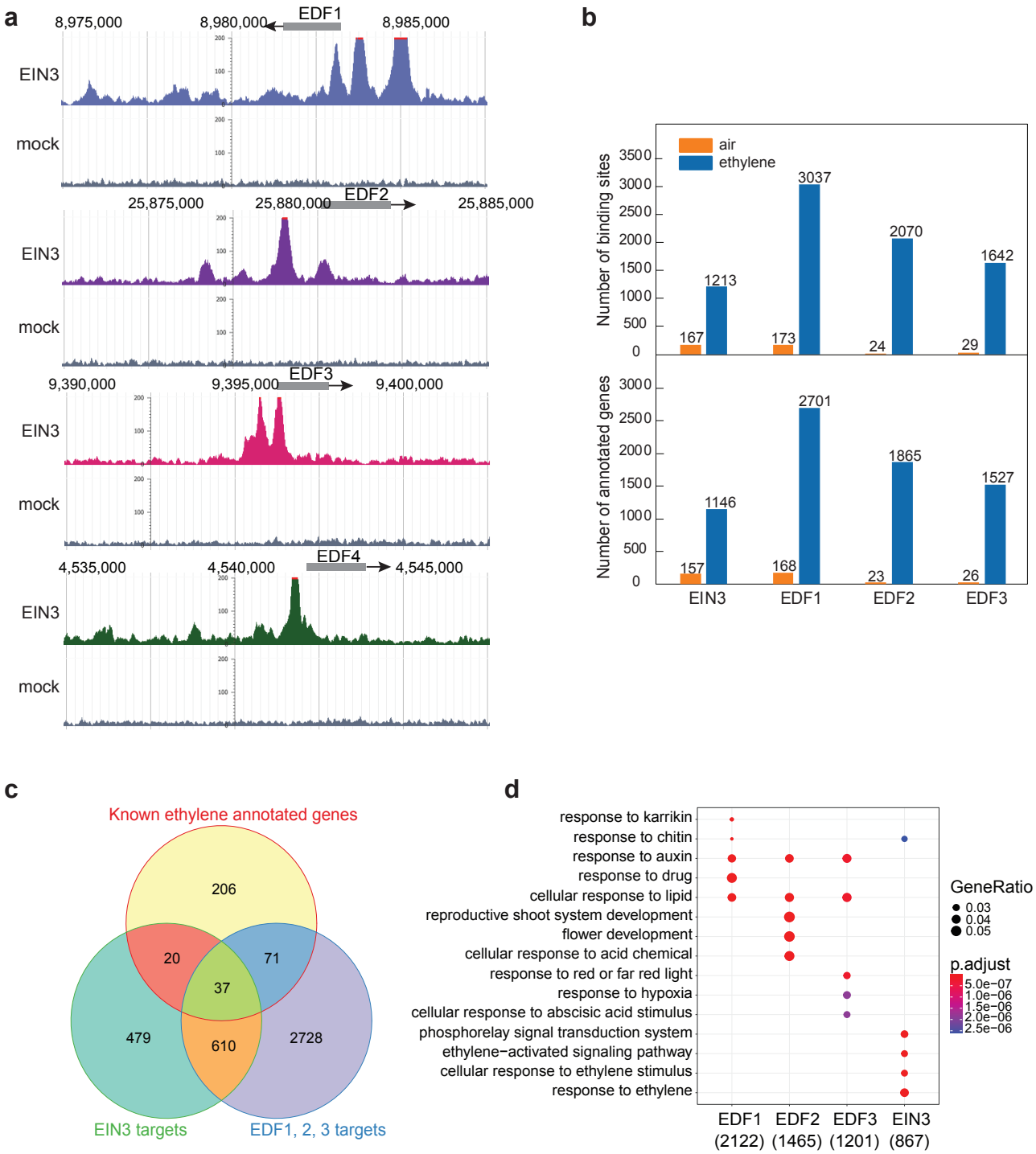

Figure 1. Overview of predicted targets and functions of EDF1, 2, 3 and EIN3. a, Genome browser screen shot visualizing the binding of EIN3 to the promoter regions of EDF1, 2, 3, 4 following 2 hours ethylene treatment, from ChIP-seq experiments. Mock indicates negative control immunoprecipitation with goat whole IgG. **b**, Binding of EDF1, 2, 3 and EIN3 to their target genes is ethylene-responsive. Plots show the total number of binding sites (upper panel) and target genes (lower panel) upon air (orange bars) and ethylene (dark blue bars) treatments. A minimum of two biological replicates were used for each treatment/condition combination. **c**, Overlap between EDF1, 2, 3 and EIN3 targets with known ethylene annotated genes. **d**, Top five significantly enriched (p.adjust < 0.05) gene ontology biological process terms amongst the targets of EDF1, 2, 3 and EIN3 under ethylene treatment.

3.3.2 EDF1, 2, 3 are transcriptional repressors but are superseded by the activator EIN3

In vitro and protein fusion assays indicate that the EDFs are transcriptional repressors, whereas EIN3 is primarily a transcriptional activator *in planta* (Solano et al., 1998; Ikeda and Ohme-Takagi, 2009; Chang et al., 2013; Chen et al., 2015). We investigated how the EDFs may interact with EIN3 at shared target genes to assess the effects of this potentially contrasting activity. Following an ethylene stimulus EDF1, 2, 3 and EIN3 bind to 29.9% (1543/5162) of ethylene-responsive genes (Fig. 2a; Supplementary Table 5). This was assessed by comparing the targets of each TF with genes differentially expressed in three day old etiolated seedlings treated with ethylene for 2 hours (relative to air-treated controls). Amongst the ethylene-responsive genes, some were bound only by the EDFs (999/5162, 19.4%), some only by EIN3 (226/5162, 4.4%), and some by both EIN3 and the EDFs (318/5162, 6.2%) (Fig. 2a, b; Supplementary Table 5).

We next examined whether the EDFs acted as repressors *in planta*. To do so we further analysed those of their target genes which were significantly differentially expressed following the 2 hours ethylene treatment. We categorized these differentially expressed target genes as genes only bound by EIN3, only bound by EDF1, 2, 3 or bound by both EIN3 and EDF1, 2, 3. We then assessed whether the genes in each category were up- or down-regulated in response to ethylene treatment (Fig. 2c; Supplementary Table 3 and 5). Expression of the majority (89.8%) of EIN3-specific target genes was up-regulated, indicating that EIN3 predominantly function as a transcriptional activator. This is consistent with the observation in a published time series ethylene response study (Chang et al., 2013). However, over two thirds (79.2%) of EDF1, 2, 3-specific target genes were down-regulated, indicating that EDFs may predominantly function as transcriptional repressors in the ethylene response. The remaining 20.8% of EDF1, 2, 3-specific target genes were up-regulated in response to ethylene. One potential explanation might be these genes are targeted by other TFs whose activity supersedes that of EDFs at those genes. Furthermore, the majority (83.6%) of genes targeted by both EIN3 and EDF1, 2, 3 were up-regulated. This result would be consistent with a model whereby, when EIN3 and EDFs bind to the same genes the activator function of EIN3 may supersede the repressor function of EDFs. However, this conclusion is confounded by the fact that the ChIP-seq data used in this study comes from heterogenous tissue samples, and in order to test this hypothesis it would be necessary to perform tissue specific analyses in the future.

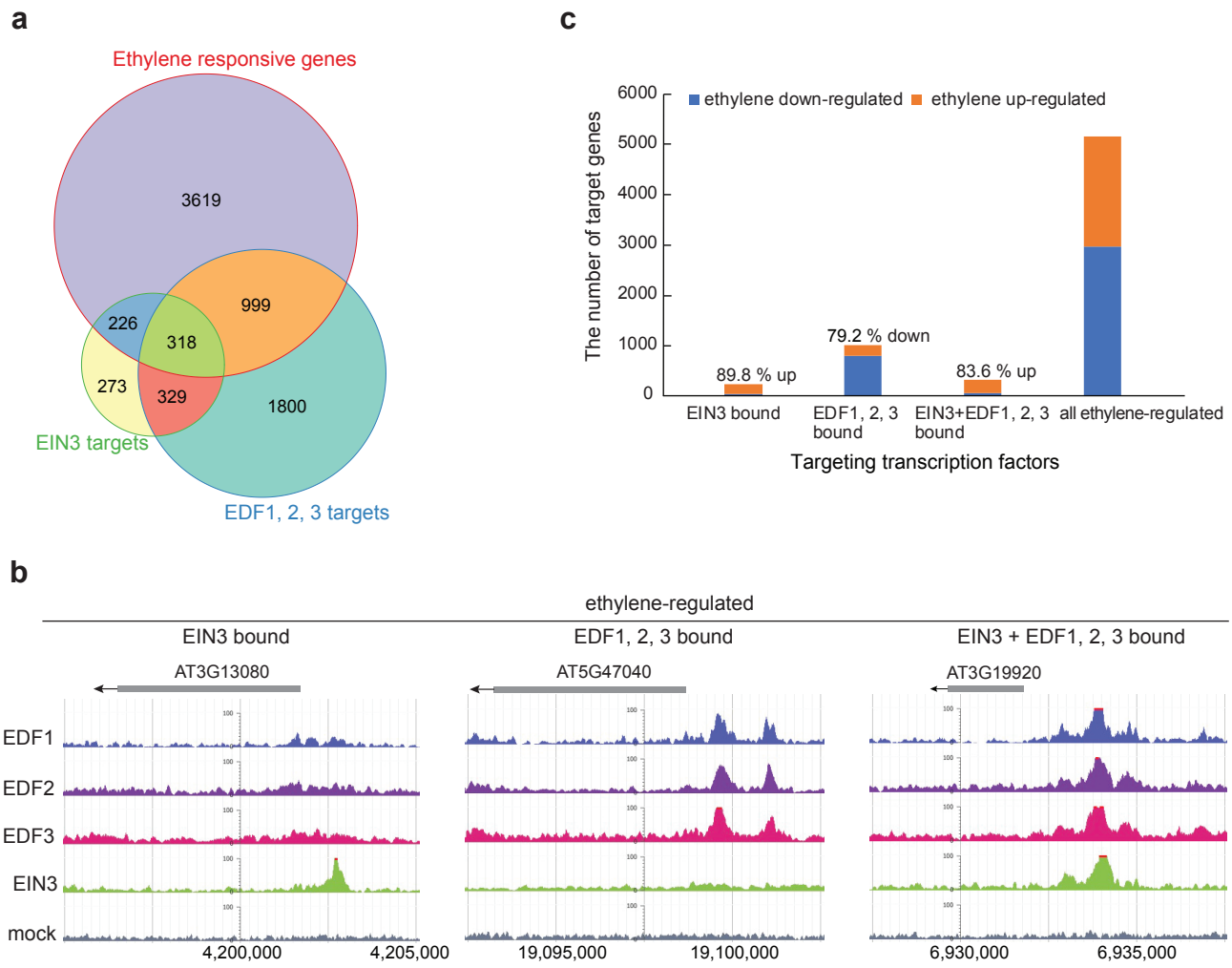

Figure 2. EDF1, 2, 3 are transcriptional repressors but are superseded by the activator EIN3. **a**, Overlap of EDF1, 2, 3 and EIN3 target genes with genes differentially expressed in response to ethylene (ethylene responsive genes). Differentially expressed genes were determined from transcriptomic analysis of seedlings 2 hours after ethylene treatment. **b**, Genome browser screen shot visualizing the binding of EDF1, 2, 3 and EIN3 to their unique and common sites in response to ethylene. **c**, Proportion of EDF1, 2, 3 and EIN3 targets that are up- or down-regulated following ethylene treatment. Groups are the differentially expressed genes only bound by EIN3 (EIN3 bound), genes only bound by EDF1, 2, 3 (EDF1, 2, 3 bound), genes both bound by EIN3 and EDF1, 2, 3 (EIN3+EDF1, 2, 3), and the total number of genes that are ethylene responsive (all ethylene-regulated).

3.3.3 EDF1, 2, 3 and EIN3 have different roles in the dynamic regulation of ethylene-response genes

Transcriptome regulation during environmental responses and development is dynamic, changing over time as the response progresses (Chang et al., 2013; Song et al., 2016; Varala et al., 2018; Brooks et al., 2019; Alvarez et al., 2020; Zander et al., 2020). We investigated the roles of EDF1, 2, 3 and EIN3 in regulating the dynamics of the ethylene response transcriptome. We first assessed expression of the target genes of the EDFs and EIN3 in a public ethylene time-series transcriptome dataset (three day old etiolated seedlings, 0-24 h, 6 time points) (Chang et al., 2013). A considerable proportion of EIN3 and EDF1, 2, 3 target genes were differentially expressed during the 24 hours of the ethylene response (31.8% - 61.6%; Fig. 3a; Supplementary Table 6). We next applied Dynamic Regulatory Events Miner (DREM) modelling to determine the structure of the ethylene-response transcriptome dynamics over the 24 h period (Fig. 3b; Extended Data Fig. 2) (Schulz et al., 2012). This approach identifies groups/cohorts of co-expressed genes and predicts the regulatory roles of individual TFs. It does so by integrating gene expression data with TF-gene binding data, then searching for associations between gene expression changes and the TFs that bind those genes. DREM can use both condition-specific and general binding data. Thus, in addition to the ChIP-seq targets we identified for EIN3 and the EDFs following ethylene treatment, the inputs for DREM modelling included predicted targets for 519 additional TFs from published studies (Source Data). The inclusion of a large number of TFs allows robust transcriptional models to be constructed. The resulting model identified 15 gene expression paths over 24 h within the ethylene response (Fig. 3b; Extended Data Fig. 2; Supplementary Table 7). Each path represents a group of genes that are co-expressed and co-regulated and their regulators.

The DREM model identified a total 85 TFs as regulators of ethylene treatment response, including EIN3, EDF2 and EDF3 (Supplementary Table 7). EIN3 was associated with groups of early and late ethylene up-regulated genes (within 30 mins and after 12 h), consistent with previous analysis of EIN3's role in the dynamic response to ethylene (Chang et al., 2013). EDF2 was also associated with a subset of these early EIN3-bound groups, further indicating that the regulatory roles of EIN3 and EDF2 within the ethylene response overlap. The groups of genes EDF2 was associated with were more modestly and slowly upregulated than the EIN3 early upregulated genes. EDF3 was associated with down-regulated groups of genes at intermediate timepoints (between 1 h and 4 h). EDF1 was not significantly associated with any dynamic expression paths. The observations for EDF2 and EDF3 are consistent with them acting *in planta* as transcriptional repressors, and with EDF2 potentially moderating transcriptional activation by EIN3 at shared target genes.

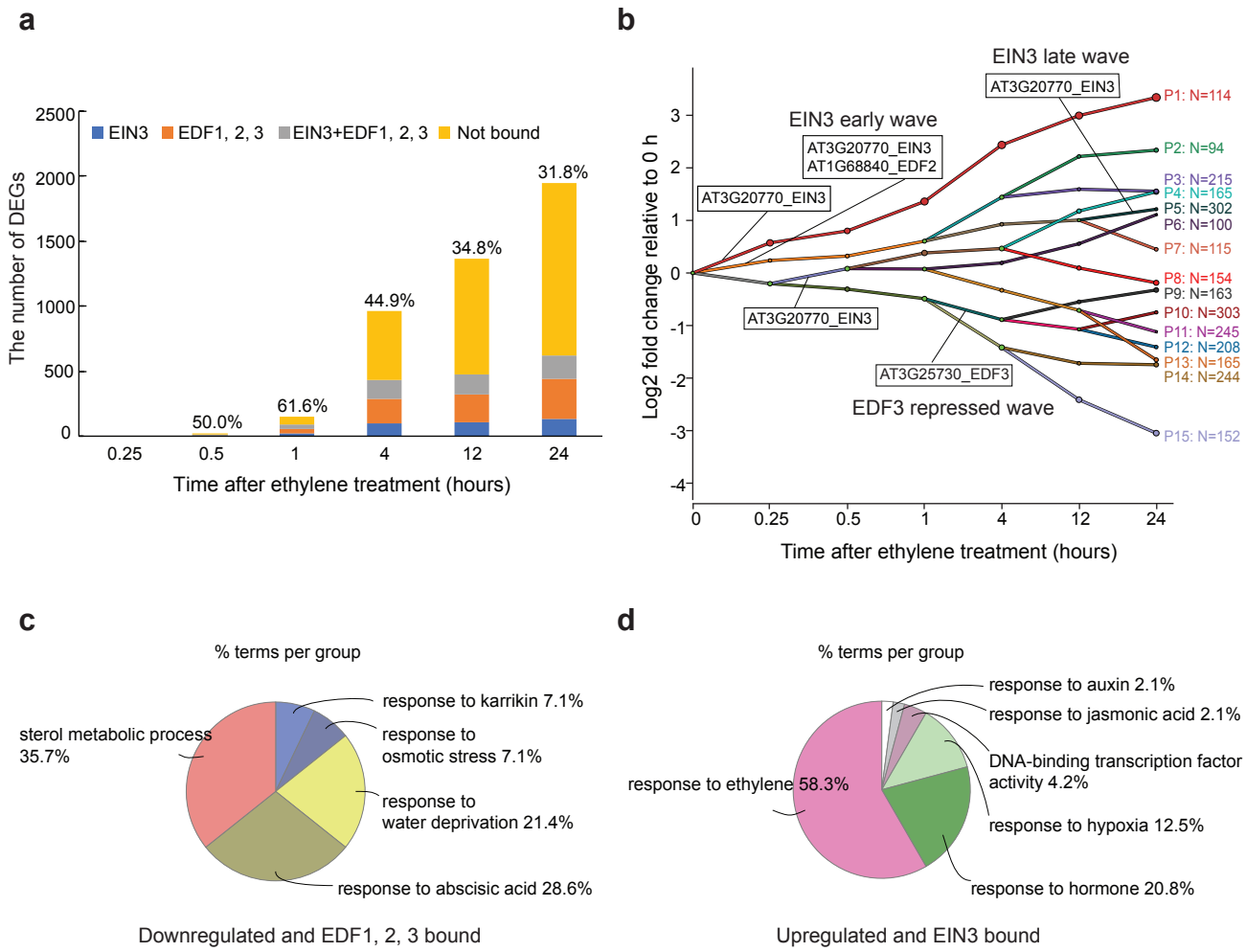

Figure 3. The regulatory roles of EDF1, 2, 3 and EIN3 in the dynamic transcriptional response to ethylene. a, The number of EDF1, 2 ,3 and EIN3 bound genes that are differentially expressed (DEGs) over a 24 h time series following ethylene treatment. The total percentage of bound genes is labelled at the top of each column. **b**, Reconstructed dynamic network using DREM for ethylene response transcriptome dynamics. The network displays the expression of groups of genes (paths) and TFs predicted to regulate them. The y-axis is log2 fold change relative to expression at 0 h. EIN3 and EDFs are labelled on the expression branches they were predicted to regulate. At right, P1-15, N indicates the number of genes on that expression path. **c-d**, Functionally grouped, significantly enriched (p-value < 0.01) gene ontology terms of genes down-regulated by ethylene and EDF1, 2, 3 bound, and genes up-regulated by ethylene and EIN3 bound.

We next assessed the functions of the target genes of EIN3 and EDF1, 2, 3 within the dynamic transcriptional response to ethylene. Our earlier analyses indicate that EDF1, 2, 3 predominantly function as transcriptional repressors in the ethylene response (Fig. 2). Consequently, we assessed the functions of the EIN3 and EDF1, 2, 3 target genes by dividing all those within the DREM dynamic ethylene response model into two relevant sets (Fig. 3c, d; Supplementary Table 8). The first set contained all genes upregulated by ethylene over 24 h and bound by EIN3. The second set contained all downregulated, EDF1, 2, 3-bound genes. EIN3-bound upregulated genes were significantly enriched in ethylene related annotations (58.3% had ethylene-relevant annotations, corrected p-value = 2.39E-13). Contrastingly, EDF1, 2, 3-bound downregulated genes were significantly enriched in annotations associated with other hormones, such as ABA (50.0% of all terms, corrected p-value = 7.80E-6), karrikin (7.1%, corrected p-value = 9.46E-4) and sterol/brassinosteroid (35.7%, corrected p-value = 1.15E-3). EDFs regulate ABA and abiotic stress responses (Fu et al., 2014; Osnato et al., 2020; Sengupta et al., 2020). Our observation that EDF1, 2, 3-bound, ethylene-downregulated genes were strongly associated with the hormone ABA provides a mechanism through which ethylene could repress ABA signalling. More broadly these results indicate that the regulatory role of the EDFs within the ethylene response may be to cross-regulate gene expression in other hormone signalling pathways.

3.3.4 Different members of the EDF TF family have redundant and specific functions

EDF TF family members may function redundantly in ethylene responses, drought stress and salt-induced seed germination inhibition (Alonso et al., 2003; Fu et al., 2014; Osnato et al., 2020). To examine potential mechanisms for this we determined the proportions of common and unique target genes between EDF1, 2, 3, reasoning that shared target genes would enable redundant EDF function (Fig. 4a, b, c; Supplementary Table 9). All three EDFs shared a large proportion of their target genes (873 shared between all, 25.3%). The target genes shared by all three EDFs were involved in multiple hormone responses, including ABA, gibberellin, auxin and karrikin (gene ontology enrichment analysis, significantly enriched terms, p.adjust < 0.05) (Fig. 4d; Supplementary Table 9). These shared target genes were also enriched for annotations to several plant growth and developmental processes, such as circadian rhythms and meristem development. The shared targets provide a potential mechanism for redundant EDF function in cross-regulation of hormone signalling. The diversity of functions of shared EDF target genes may explain why EDFs have been previously implicated in a range of plant growth, developmental, stress and hormonal processes (Hu et al., 2004; Castillejo and Pelaz, 2008; Woo et al., 2010; Fu et al., 2014; Matias-Hernandez et al., 2014; Xie et al., 2019; Osnato et al., 2020; Verhage, 2021).

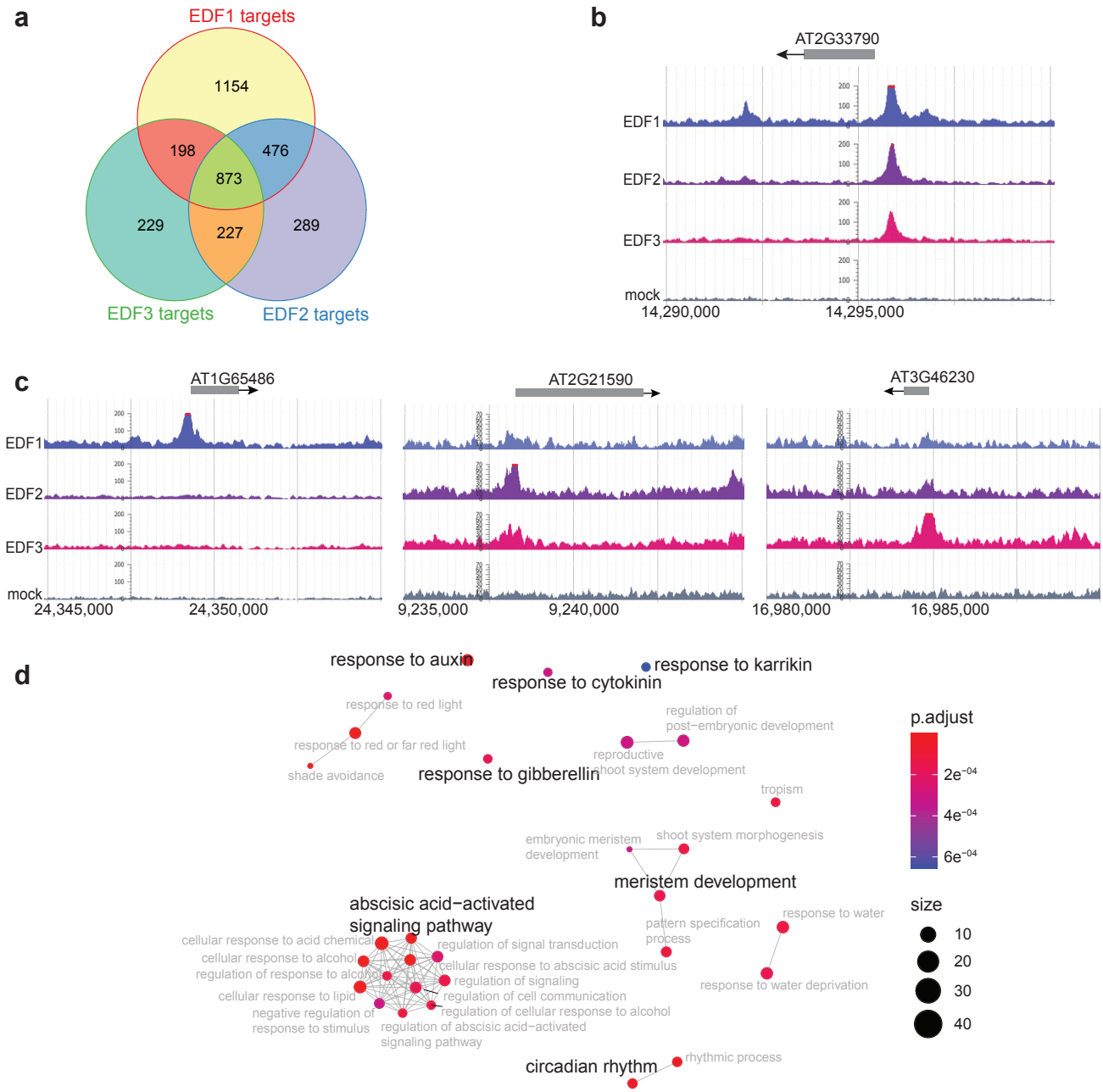

Figure 4. Overview of the redundant and specific functions of EDF1, 2, 3. **a**, Common target and unique targets of EDF1, 2, 3 after ethylene treatment. **b**, **c**, Genome browser screen shot visualizing the binding of EDF1, 2, 3 to their common (b) and unique sites (c, from left to right: EDF1 unique binding site, EDF2 unique binding site, EDF3 unique binding site) in response to ethylene. **d**, Map of the top 30 significantly enriched (p.adjust < 0.05) gene ontology (GO) annotations of 873 shared target genes of EDF1, 2, 3. The map groups related terms into a network. **e-g**, Visualization of the reduced, simplified functional annotations associated with target genes specific to EDF1, EDF2 and EDF3. Boxes labelled "main node" contain summarized high-level GO terms. All other boxes contain lower-level, detailed annotations enriched within the main node terms. EDF3 does not contain a main node box because few terms were enriched.

Individual EDFs also have specific, unique functions (Hu et al., 2004; Fu et al., 2014; Sengupta et al., 2020). For example, the responses of EDF 2 and 3 to abiotic stress and the extent to which they negatively regulate stress responses differ (Fu et al., 2014). To determine the basis for functions specific to individual EDFs, we examined the target genes unique to each EDF. A considerable proportion of EDF target genes were unique to a single EDF (Fig. 4a, c, EDF1: 1154/2701, 42.7%; EDF2: 289/1865, 15.5%; EDF3: 229/1527, 15.0%). We reduced and visualized functional annotations associated with EDF-specific target genes, which allowed us to focus on the high-level pathways identified and how they differed between EDFs (Fig. 4e, f, g; Supplementary Table 9). The functional annotations differed between EDFs 1, 2 and 3-specific target genes. EDF1-specific target genes were enriched in annotations relevant to regulation of hormone levels, stress responses and secondary metabolism, whilst EDF2-specific targets were enriched in annotations relevant to plant development, such as meristem development and regulation of the vegetative/reproductive transition. EDF3-specific targets were enriched in terms related to transcriptional activation activity which indicates that EDF3 may target and regulate TFs further downstream in ethylene response network. These analyses demonstrate how the EDFs may enact their specific functions, through targeting of unique sets of genes. They also illustrate that TFs within a single family may have both shared core functions and individual functions.

3.3.5 Binding of the EDFs *in planta* is through their AP2 motif, not their B3 motif

The EDFs bind to CAACA and CACCTG motifs *in vitro* through their AP2 and B3 domains, respectively (Kagaya et al., 1999). Possession of two DNA binding motifs is unusual in plant TFs. The relative roles of the AP2 and B3 motifs in binding genes *in planta* is not known, nor are their contributions to ethylene-responsive gene regulation. To investigate this, we analysed the motifs present in binding sites at EDF target genes after ethylene treatment. The majority of EDF1, 2, 3 binding sites contained a CAACA motif, with some variation in the bases flanking this core sequence (EDF1, 3666 of 6848 targets, 53.5%; EDF2, 2283 of 4341 targets, 52.6%; EDF3, 2759 of 4248 targets, 64.9%; Fig. 5a, b, c; Supplementary Table 10). The motifs we detected were comparable to the CACAA motif bound *in vitro* by the EDF AP2 domain (Kagaya et al., 1999). Contrastingly, the B3 motif was not enriched in the binding sites of any of the EDFs. These findings indicate that the EDF AP2 motif is the major regulatory motif in response to ethylene. The slight differences in the motifs observed between EDF1 and EDF2, EDF3 binding sites may relate to their differing target genes.

3.3.6 EDF expression and binding affinity responds to ethylene and other hormones

The increase in number of genes targeted by the EDFs following ethylene treatment indicates that EDFs are regulated by ethylene. This might occur through changes in EDF abundance stimulated by ethylene and/or changes in EDF activity. To examine this, we first determined the expression of *EDF1, 2, 3, 4* following ethylene treatment, across the time-series transcriptome dataset (Fig. 5d, Supplementary Table 11). *EDF1, 2, 3,* but not *EDF4,* were rapidly and significantly induced in response to ethylene gas, their mRNA abundance increasing within 15 mins (log2 values, *EDF1* 1.82

fold up-regulated, *EDF2* 1.05 fold up-regulated, *EDF3* 1.37 fold up-regulated). The increased expression of *EDF1, 2, 3, 4* correlates with an ethylene-responsive increase in EDF1, 2, 3 binding affinity (Fig. 5e; Supplementary Table 12). This was determined by quantitative analysis of the EDF 1, 2, 3 ChIP-seq data, comparing the extent of binding at individual sites before and after ethylene treatment. EDF1 had a greater number of sites with significantly different binding affinities than EDF2 or EDF3 between air and ethylene (FDR < 0.05). The differing ethylene responsiveness of the EDFs supports the finding that they have differing targets and functions. Considered together, these results indicate that ethylene signalling drives a rapid increase in *EDF* expression as well as increased binding of EDFs to target genes.

Hormones affect the expression of *EDFs* at various Arabidopsis developmental stages (Hu et al., 2004; Magome et al., 2008; Fu et al., 2014; Osnato et al., 2020). Regulation of *EDF* expression by other hormones in addition to ethylene may contribute to the EDFs ability to function in such a wide range of processes. However, the response of *EDF* expression to a range of hormones has not been examined consistently in a single developmental stage, such as etiolated Arabidopsis seedlings. We determined the expression changes of *EDF1, 2, 3, 4* in etiolated Arabidopsis seedlings treated with the hormones ABA, brassinosteroid, jasmonic acid, salicylic acid and GR24 (stimulating strigolactone/karrikin signalling) using time-series transcriptome datasets. Brassinosteroid, salicylic acid and GR24 transcriptomes were generated for this study, whilst ABA and jasmonic acid treatments were retrieved from public resources (Song et al., 2016; Zander et al., 2020). At least two *EDFs* were significantly differentially expressed in response to each hormone, but none of the hormones induced a response as strongly as ethylene (Fig. 5d; Supplementary Table 11). *EDF1* and *EDF4* were both significantly differentially up-regulated 4 hours after ABA treatment and remained up-regulated throughout the time-series. The other hormones caused relatively lesser differential expression of *EDFs* at a small number of timepoints, unlike the strong and consistent expression changes caused by ABA and ethylene. *EDF2* was up-regulated in response to jasmonic acid relatively rapidly, but only statistically significantly so after 24 hours. Overall, expression of each individual *EDF* responded differently when considered across all six hormones. This analysis indicates that the expression responses of *EDFs* are hormone-specific, with primary responses driven by ethylene and ABA.

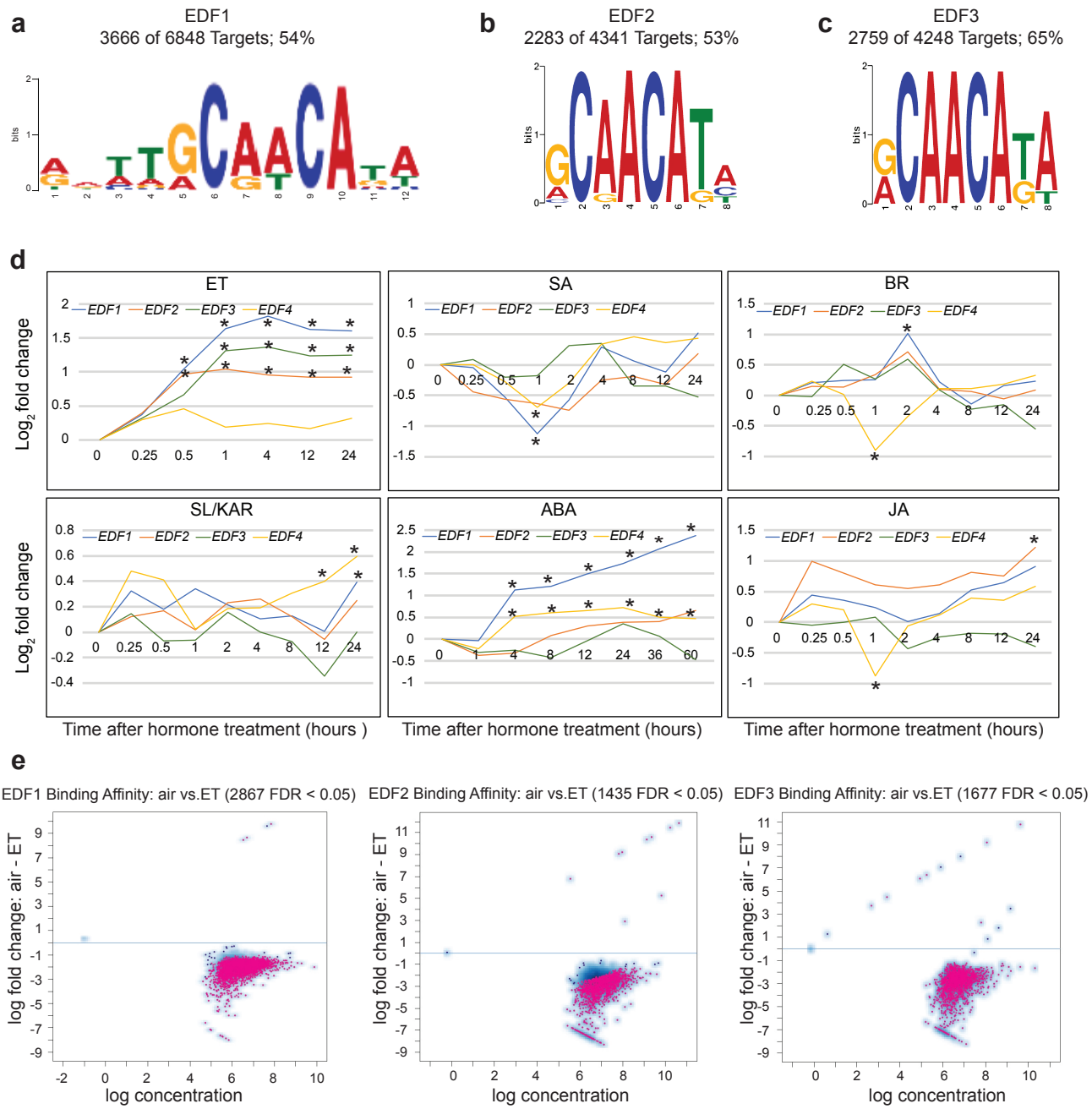

Figure 5. EDF expression and binding affinity responds to ethylene and other hormones. a-c, The top-ranked motif in EDF1 (**a**), EDF2 (**b**) and EDF3 (**c**) ChIP-seq data was the CAACA motif. Motifs were determined by MEME analysis. **d**, Overview of hormonal effects on the expression of EDF1, 2, 3, 4, assayed by RNA-seq. Hormone treatments were ABA, abscisic acid; BR, brassinosteroid; ET, ethylene; JA, jasmonic acid; SA, salicylic acid; SL/KAR: strigolactone/karrikin (GR24). The y-axis is log2FC relative to 0h. * indicates that the gene was significantly differentially expressed at that time point (edgeR; FDR < 0.01 or 0.05). **e**, Differential binding analysis of EDF1, 2, 3 using ChIP-seq data analysed by DiffBind. Datapoints represent an individual binding site of the TF, with smoothing applied where many datapoints are closely located. The y-axis indicates binding affinity in air *vs.* ethylene treatment, with a positive value indicating the TF has greater binding affinity in air and a negative value indicating greater binding affinity in ethylene. The x-axis represents mean binding affinity for all samples at that binding site. Pink datapoints indicated sites significantly differentially bound (FDR < 0.05) by the TF between air and ethylene treatments, blue datapoints indicated sites bound non-differentially between treatments. Total number of differentially bound sites is given at the top of each plot.

3.3.7 RNA-seq profiling of an *edf1234* quadruple mutant supports a role for EDF1, 2, 3, 4 in hormone cross-regulation.

We next investigated the effect of genetic disruption of the EDFs on expression of their target genes. We analysed the transcriptome of three day old etiolated *edf1234* quadruple mutant seedlings during air and ethylene treatments, compared with wild-type control seedlings (Fig. 6; Extended Data Fig. 3; Supplementary Table 13). First, we confirmed that expression of the four target genes was disrupted, which had been observed for each in the corresponding single mutant lines (Alonso et al., 2003). The expression of all four *EDFs* was reduced in *edf1234* quadruple mutant seedlings compared to wild-type control seedlings when grown in air (Fig. 6a). However, these reductions were greater for *EDFs 1, 2* and *3* and, amongst those, *EDF3* was most strongly affected (Supplementary Table 13). *EDF4* expression was affected only modestly, in contrast to the near total loss of the *EDF4* transcript in the corresponding single mutant (Alonso et al., 2003). This discrepancy may have occurred because the *EDF4* promoter is targeted by EDF1, 2, 3 (Supplementary Table 2). Consequently, the reduced expression of transcriptional repressors EDF1, 2, 3 may have resulted in some compensatory increase in expression of *EDF4*. The *edf4* mutation used here is in the gene's promoter rather than the coding region, so may permit this (Alonso et al., 2003). Similarly, ethylene-induced expression of *EDF1, 2, 3* was inhibited in *edf1234* mutant seedlings, but *EDF4* expression was not.

Reduced expression of the *EDFs* would be expected to cause misexpression of EDF target genes. Typically, not all genes targeted by a TF are regulated by that TF, however (Farnham, 2009; Macquarrie et al., 2011; Chang et al., 2013; Lehti-Shiu et al., 2017; Zander et al., 2020). For example, only 30% of binding sites of the master regulator EIN3 in the ethylene signalling pathway were associated with transcriptional changes (Chang et al., 2013). We examined the genes that were targeted and regulated by the EDFs by comparing gene expression and TF binding data. To do so we first identified all significantly differentially expressed genes between *edf1234* and wild-type seedlings (Fig. 6b; Supplementary Table 13). Few genes were differentially expressed in *edf1234* mutant seedlings relative to wild-type controls under air treatment (31 up-regulated in *edf1234*, 7 down-regulated). However, a larger number were differentially expressed following ethylene treatment, again supporting that ethylene drives EDF activity (174 up-regulated in *edf1234*, 154 down-regulated). Of these, 50% (87/174) of up-regulated genes and 22.1% (34/154) of down-regulated genes were EDF1, 2, 3 targets (Extended Data. Fig. 3b, Supplementary Table 13). The observation that a larger proportion of genes up-regulated in *edf1234* are EDF targets is consistent with the EDFs being transcriptional repressors.

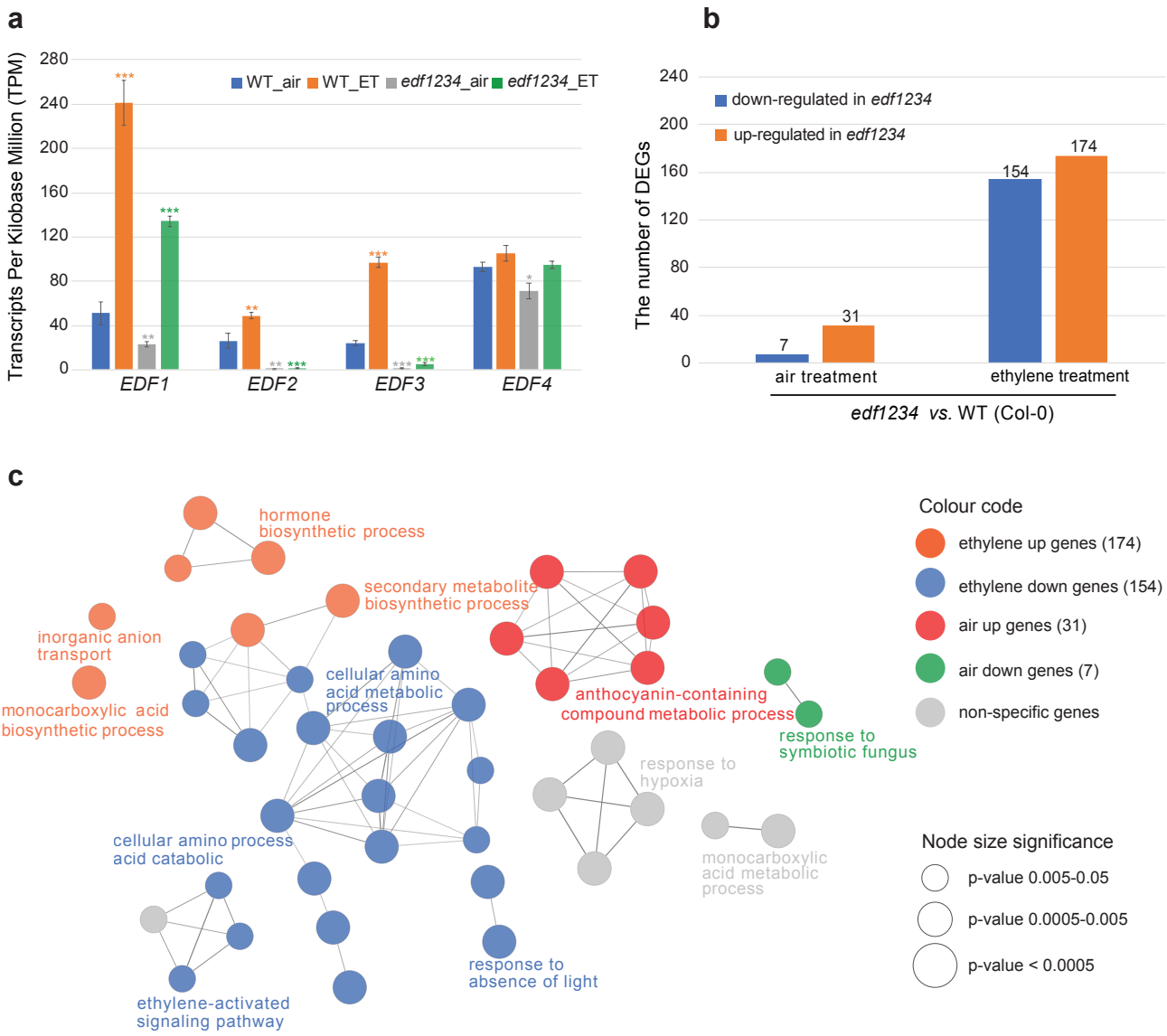

Figure 6. Expression profiles of *edf1234* quadruple mutant seedlings in response to air and ethylene. **a**, EDF transcript abundance in wild-type and *edf1234* mutant seedlings for 2 hours air or ethylene (ET) treatments in TPM (Transcripts Per Kilobase Million) from RNA-seq analyses. Differential expression was calculated using Student's t-test (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$). Three comparisons were made: orange *, ethylene treated compared to air treated wild-type seedlings; grey *, air treated *edf1234* seedlings compared to air treated wild-type seedlings; green *, ethylene treated *edf1234* seedlings compared to ethylene treated wild-type seedlings. **b**, Numbers of up- and down-regulated differentially expressed genes (DEGs) in *edf1234* seedlings compared with wild-type, after air or ethylene treatment. **c**, Map showing connections between the significantly enriched gene ontology annotations (generated by ClueGO v2.5.7 in Cytoscape v3.8.0 with ontologies updated as GO_BiologicalProcess-Custom-GOA-ACAP-ARAP_04.03.2021, p-value < 0.05, biological process) of four differentially expressed gene categories in Fig. 6b (Methods section 3.5.5).

To further understand the biological processes directly regulated by the EDFs, we performed gene ontology enrichment analysis of the significantly differentially expressed genes in *edf1234* compared to WT under air and ethylene treatments. Analysis was performed on four gene categories; up-regulated in *edf1234 versus* WT in air, down-regulated in *edf1234 versus* WT in air, up-regulated in *edf1234 versus* WT in ethylene, down-regulated in *edf1234* versus WT ethylene (Fig. 6b, c; Supplementary Table 13). The genes up-regulated in *edf1234 versus* WT in ethylene were enriched for annotations that included hormone biosynthesis process, hormone metabolic process and regulation of hormone levels (Fig. 6c; Supplementary Table 13). Moreover, among the genes in this category, there were two jasmonic acid biosynthetic genes (*ALLENE OXIDE CYCLASE 3*, *AOC3*, *AT3G25780* and *ARABIDOPSIS LIPOXYGENASE 1, LOX1*, *AT1G55020*), one GA biosynthetic gene (*GIBBERELLIN 20 OXIDASE 2*, *GA20OX2*, *AT5G51810*), and two ethylene biosynthetic genes (*ACC SYNTHASE 5*, *ACS5, AT5G65800* and *ACS8*, *AT4G37770*). We determined that *LOX1*, *ACS5* and *ACS8* are also direct targets of the EDFs by examination of the ChIP-seq data. This suggests that during an ethylene response loss of function of the EDFs may relieve repression of expression of hormone biosynthetic genes. These results further indicate that the EDFs play a role in cross-regulation by ethylene to other hormone signalling pathways through direct targeting of hormone biosynthetic genes.

3.4 Discussion

How hormones regulate large gene networks that lead to distinct and diverse transcriptional responses is a fundamental question in plant biology. Previous studies of transcriptional regulation by plant hormones have often focused on master TFs that have broad effects across response networks (Chang et al., 2013; Zander et al., 2020). The role of secondary TFs in regulating specific components within hormone response networks is less well investigated. In this study, we identified the likely functions of secondary ethylene TFs EDF1, 2, 3 during an ethylene response. We combined genome-wide TF target maps, time-series transcriptomics and dynamic gene regulatory network modelling to demonstrate the transcriptional repressor roles of the EDFs *in planta*, identify their distinct functions within the ethylene response compared to function of the master TF EIN3, and determine their potential roles in cross-regulation of other hormones. We also determined the core and unique properties of the EDFs and how the ethylene response dynamics of individual family members differ. These discoveries increase our knowledge of how secondary TFs act within the transcriptional cascade downstream of primary TFs.

Our analyses illustrated how the EDF TFs can act as a mechanism of hormone cross-regulation. The EDFs are known negative regulators of the ABA response (Feng et al., 2014; Fu et al., 2014). Our findings demonstrate that, due to their activation by ethylene, the EDFs can act as a component of the ethylene response to repress ABA signalling. This increases our mechanistic understanding of ethylene/ABA antagonism (Cheng et al., 2009). However, the genes targeted and/or regulated by the EDFs were also enriched in karrikin, auxin and sterol/brassinosteroid signalling components. Furthermore, the jasmonic acid biosynthetic gene *LOX1* and two ethylene biosynthetic genes, *ACS5*

and *ACS8*, were all bound and repressed by the EDFs. These indicate that the EDFs may also cross-regulate other hormone signalling pathways and play feedback roles in ethylene responses. EDF1 was found previously to repress expression of two GA biosynthetic genes *GA3OX1* and *GA3OX2*, decreasing GA concentration and influencing flowering time, and the effects of the EDFs on jasmonic acid pathway genes have been observed in previous studies (Osnato et al., 2012; Osnato et al., 2020). Our results are consistent with that EDFs having a broad role in regulating hormone signalling pathways, which would provide an explanation for the EDFs' effects on such a wide range of plant growth and developmental processes (Castillejo and Pelaz, 2008; Fu et al., 2014; Matias-Hernandez et al., 2014; Chen et al., 2015; Osnato et al., 2020). Hence, we conclude that ethylene signalling induced cross-regulation of other hormones might be achieved *via* the EDFs.

Our study determined that the EDFs are transcriptional repressors *in planta*. More than two thirds of EDF-specific bound genes were down-regulated in response to ethylene and a larger proportion of EDF targets are up-regulated in *edf1234* quadruple mutant seedlings. This *in vivo* evidence is consistent with published evidence that EDF members exhibit repressive activity *in vitro* through their R/KLFGV repressor domain (Kagaya et al., 1999; Ikeda and Ohme-Takagi, 2009). Transcriptional repressors account for about 10% of TFs in Arabidopsis (Ikeda and Ohme-Takagi, 2009). It is likely that they contribute to the regulation of transcriptional networks by repressing pathways and processes that compete for cellular resources, that lead to non-optimal environmental responses, or that themselves exert repressive activity over the activated pathway. They may also modulate the activated pathway and prevent run-away signalling (Zander et al., 2020). Investigation of the roles of repressive TFs in plant signalling networks has been restricted to a small number of examples, but repressive TF activity appears to be a general feature of core hormone responses (He et al., 2005, Truskina et al., 2021). Moving forward, it is important to define the roles of repressor TFs broadly in plant biological networks, to provide the tools and parameters that allow us to rationally re-engineer plant responses to their environments.

3.5 Methods

3.5.1 Plant materials and growth conditions

Three day old etiolated seedlings of Arabidopsis in the Col-0 background were used for all experiments. Seed sterilization was performed as described previously (Zander et al., 2020). Seeds were stratified at 4°C for three days, exposed to daylight for 2 hours to induce germination, then returned to the dark and cultivated at 22°C for three days. For ethylene response experiments, seedlings were treated with ethylene gas for 2 hours as previously described (Chang et al., 2013). We passed 10 parts per million ethylene gas across the seedlings in a sealed chamber. The control was hydrocarbon free air. Transcriptomic responses of wild-type Col-0 seedlings to rac-GR24 (Chiralix, Nijmegen, The Netherlands)., salicylic acid (Sigma, S7401) and brassinosteroid (Sigma, E1641) were assayed by applying hormone solutions in 0.5x Linsmaier and Skoog basal medium (with macronutrients, micronutrients and vitamins, Caisson Labs) using a spray bottle to run-off. The

transgenic lines Col-0 EDF1::EDF1-YPet, Col-0 EDF2::EDF2-YPet, Col-0 EDF3:: EDF3-YPet, Col-0 EIN3::EIN3-YPet were generated by recombineering as previously described (Zander et al., 2020). The *edf1234* (*edf1_2 edf2_2 edf3_1 edf4_1*) mutant was previously described (Alonso et al., 2003).

3.5.2 ChIP-seq

Three day old etiolated seedlings were used for ChIP-seq experiments (Col-0 EDF1::EDF1-YPet, Col-0 EDF2::EDF2-YPet, Col-0 EDF3::EDF3-YPet, Col-0 EIN3::EIN3-YPet). All ChIP-seq sequencing data were generated with biologically independent replicate experiments: EDF1 (air, n=3; ET, n = 3), EDF2 (air, n=3; ET, n=4), EDF3 (air, n = 3; ET, n = 3), EIN3 (air, n = 3; ET, n= 4). Seedlings were collected and snap frozen in liquid nitrogen after ethylene gas or air treatment for 2 hours. Chromatin preparation and immunoprecipitation was performed as previously described (Zander et al., 2020). A goat anti-GFP antibody (supplied by D. Dreschel, Max Planck Institute of Molecular Cell Biology and Genetics) was used and mock immunoprecipitations conducted using whole goat IgG (005–000–003, Jackson ImmunoResearch). Immunoprecipitated DNA was used to prepare sequencing libraries (Zander et al., 2020). Libraries were sequenced on an Illumina HiSeq 2500 according to manufacturer's instructions (Illumina).

3.5.3 ChIP-seq analyses

Fastq files were trimmed using Trimglore V0.4.4 and trimmed reads were mapped to the Arabidopsis TAIR10 genome using Bowtie2 V2.2.9 (Langmead and Salzberg, 2012). To restrict the number of reads mapping to multiple locations in the genome, the mapped reads were filtered with MAPQ > 10 using samtools V1.3.1 (Li et al., 2009). Filtered reads were used for all the subsequent analysis. PhantomPeakQualTools v.2.0 was used to assess the ChIP-seq experiment quality after read mapping, determining the normalized strand cross correlation (NSC), relative strand cross correlation (RSC) and shift size of each alignment bam file. ChIP-seq experiments need to pass the quality control with NSC and RSC no less than 1.0 and 0.8, respectively. Moreover, the biological replicates outliners determined by principal component analysis were excluded from the following analysis. So only the following ChIP-seq replicates were retained for each TF and treatment combination and used for subsequent analysis: EDF1 (air, n=3; ET, n = 2), EDF2 (air, n=2; ET, n=2), EDF3 (air, n =3; ET, n = 3), EIN3 (air, n = 3; ET, n= 3)

MACS V2.1.0 was used to identify enriched binding sites for in ChIP samples by comparison with mock IP of wild-type Col-0 (parameters options–nomodel –shiftsize –g 1.19e8 –q 0.05) (Zhang et al., 2008). Mapped reads and peak locations were visualized using JBrowse (Buels et al., 2016). A set of high-quality TF-binding sites were obtained for each ChIP-seq sample of EDF 1, 2, 3 and EIN3, respectively. Only narrowPeaks with a q-value < 1^{e-16} were considered for the differential enrichment analyses and annotation analyses. Differential enrichment analysis between ChIP-seq air and ethylene treatment sample groups was performed using the DiffBind Bioconductor package in R (Stark and Brown, 2011). The significantly differentially bound sites were identified by DESeq2 with the FDR threshold < 0.05. To annotate enriched binding sites, narrowPeaks with 50% intersection in

at least two independent biological replicates were integrated using bedtools V2.26.0 (Quinlan and Hall, 2010). Sites were associated to their nearest genes in the TAIR10 using R package ChIPseeker with default parameters (Yu et al., 2015).

For motif enrichment analysis summits with a q-value < 1^{e-16} were expanded from single nucleotides to 500 nt. Only summits with at least 50% nucleotide overlap between at least two biological replicates were retained to give the final set of high-confidence binding sites, and their sequences extracted, using bedtools V2.26.0. Next, enriched binding motifs were determined using the MEME-ChIP in MEME Suite 5.3.1 website server with default parameters and known motif information used Arabidopsis motifs downloaded from PlantTFDB v5.0 (Bailey et al., 2009; Machanick and Bailey, 2011; Jin et al., 2016).

3.5.4 RNA isolation, library preparation and RNA-seq analyses

The Qiagen RNeasy Plant kit was used for RNA isolation according to manufacturer's instructions. RNA-seq library preparation was conducted with the Illumina TruSeq Total RNA sample prep kit. All sequencing protocols were carried out per manufacturer's instructions using the Illumina HiSeq 2500.

FastQC V0.11.5 and Trimglore V0.4.4 (https://www.bioinformatics.babraham.ac.uk/projects/) were used to perform quality control and remove low-quality reads and adapters from raw RNA-seq reads. Trimmed RNA-seq reads for ethylene RNA-seq time-series data were mapped onto the Arabidopsis genome with the Araport11 annotation using HiSat2 V2.0.5 (Kim et al., 2015). Read counting in genome features was performed using Htseq V0.8.0 (Anders et al., 2015). This different process was necessary because the data are color-space sequencing.

For all other transcriptome data, quantification of transcripts was performed using Salmon v0.8.1 in conjunction with AtRTD2-QUASI reference transcriptome (Patro et al., 2017; Zhang et al., 2017). A quasi mapping-based index was built using an auxiliary k-mer hash over k-mers of length 31 (k=31). Salmon parameters were default for quantification except that fragment-level GC biases ("–gcBias") correction was turned on. Tximport pipeline was used to summarize transcript-level abundance to gene-level abundance (Soneson et al., 2015).

Differentially expressed genes were identified using the edgeR 3.28.1 package in R with quasi-likelihood (QL) F-test (using the functions glmQLFit and glmQLFTest) (Robinson et al., 2010). Lowly expressed genes were filtered using filterByExpr function and then batch correction was performed using the additive model formulas in edgeR. Significant differentially expressed genes were those having FDR < 0.01 or FDR < 0.05 for hormone treatment time-series data (FDR < 0.01 for BR, ET, JA and SA RNA-seq datasets, FDR < 0.05 with no batch effect correction for the ABA and SL/KAR datasets) and FDR < 0.05 for *edf1234* quadruple mutant RNA-seq data, respectively.

Known Arabidopsis ethylene annotated genes used for Fig. 1c Venn diagram analysis were extracted from TAIR gene ontology annotations downloaded June 2020 using 'ethylene' as keyword.

3.5.5 Functional enrichment analyses

Gene ontology and KEGG enrichment analysis was conducted using clusterProfiler with default parameters (Yu et al., 2012; Wu et al., 2021). The functionally grouped network analyses of down-regulated and EDF 1, 2, 3 targeted and up-regulated and EIN3 targeted gene sets was generated by ClueGO v2.5.7 in Cytoscape v3.8.0 with ontologies updated as GO_MolecularFunction-Custom-GOA-ACAP-ARAP_28.08.2020 (Bindea et al., 2009). Benjamini-Hochberg was selected to correct the p-values for multiple testing corrections. Gene ontology terms with a p-value < 0.01 were considered statistically significantly enriched. The network specificity was set as 'Global', kappa score as ≥ 0.4, and all other settings kept as default. The functionally grouped network map of four differentially expressed gene categories in Fig. 6b was generated by ClueGO v2.5.7 in Cytoscape v3.8.0 with ontologies updated as GO_BiologicalProcess-Custom-GOA-ACAP-ARAP_04.03.2021 (Bindea et al., 2009). Gene ontology terms with a p-value < 0.05 were considered statistically significantly enriched. Kappa score as ≥ 0.1, Merge redundant groups with > 50.0% overlap, and all other settings kept as default.

3.5.6 Modelling the ethylene regulatory network

Log2 fold changes relative to 0 h for all expressed genes across all time points were used to project TFs onto the transcriptional ethylene response using DREM (Schulz et al., 2012). DREM modelling was performed as previously, with the following modifications (Narsai et al., 2017). TF-target gene interactions were updated to include those from the ChIP-seq data in this study, the confirmed and direct interactions for 293 TFs from the Arabidopsis Gene Regulatory Information Server repository, and interactions form two published studies (Yilmaz et al., 2010; O'Malley et al., 2016; Song et al., 2016; Zander et al., 2020). A probabilistic logistic regression classification model is used to learn the branching model and to split gene expression into paths (DREM parameters: the "Path Significance Conditional on Split" option). Following model learning, the significance of TFs association with paths and splits can be determined using the hypergeometric distribution, Only those TFs with a split score of less than < 0.001 are shown.

3.6 Data availability

All plant lines described here can be requested from the corresponding authors. The inputs, parameters and output model for recreating DREM model in this study can be found in Source Data folder from this link: https://figshare.com/s/da0976d939001ba33f12

New sequencing data can be downloaded from the Gene Expression Omnibus repository (GEO, https://www.ncbi.nlm.nih.gov/geo/) with accession number GSE182617 and the reviewer token qrunyeymtpurngt.

Visualized ChIP-Seq data can be found at JBrowse link: https://jbrowse.latrobe.edu.au/EDFs-network/.

Ethylene, ABA and jasmonic acid RNA-seq raw reads were downloaded from Sequence Read Archive (SRA , https://www.ncbi.nlm.nih.gov/sra) or GEO with accession numbers SRA063695, GSE80568 and GSE133408.

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3.8 Contributions

MGL, JRE and LY designed the study. MGL, EH, JPSG, BJJ and ANS generated the transgenic constructs and carried out the RNA-seq and ChIP-seq experiments. JRN conducted sequencing operations. SJ and ZB-J provided new analytical tools. LY analysed and integrated all data, interpreted results and prepared figures. LY wrote the manuscript and MGL provided feedback. All authors read and approved the final manuscript.

3.9 Supplementary Table Legends

Supplementary Table 1. Overview of quality metrics of ChIP-seq datasets.

Supplementary Table 2. Target genes and binding sites of EDF1, 2, 3 and EIN3 determined by ChIP-seq analysis.

Supplementary Table 3. Overview of target genes of EDF1, 2, 3 and EIN3 in response to ethylene which have ethylene associated annotations.

Supplementary Table 4. Gene ontology term enrichment analysis of EDF1, 2, 3 and EIN3 target genes.

Supplementary Table 5. Overview of the overlap between EIN3 and EDF1, 2, 3 target genes with differentially expressed genes in response to ethylene (ethylene responsive genes).

Supplementary Table 6. Overview of the numbers of EDF1, 2, 3 and EIN3 target genes which are also up- or down-regulated differentially expressed genes under ethylene treatment over 24 h period.

Supplementary Table 7. Complete DREM model results including the genes in each path and the predicted regulatory TFs.

Supplementary Table 8. EDF1, 2, 3 and EIN3 target genes from DREM modelling and their functional annotations.

Supplementary Table 9. Overview of genes specific to or shared by EDF1, 2, 3 and their functional annotations.

Supplementary Table 10. Motifs detected de novo within EDF1, 2, 3 binding sites.

Supplementary Table 11. The transcript abundance of EDF1, 2, 3, 4 relative to 0 hr (log2FC, FC - fold change) following six hormone treatments.

Supplementary Table 12. List of significantly differential binding sites between air and ethylene treated ChIP-seq samples for EDF1, 2, 3 using Diffbind analysis.

Supplementary Table 13. Expression values and functional analysis of genes differentially expressed in edf1234 quadruple mutant seedlings relative to wild-type after air or ethylene treatment.

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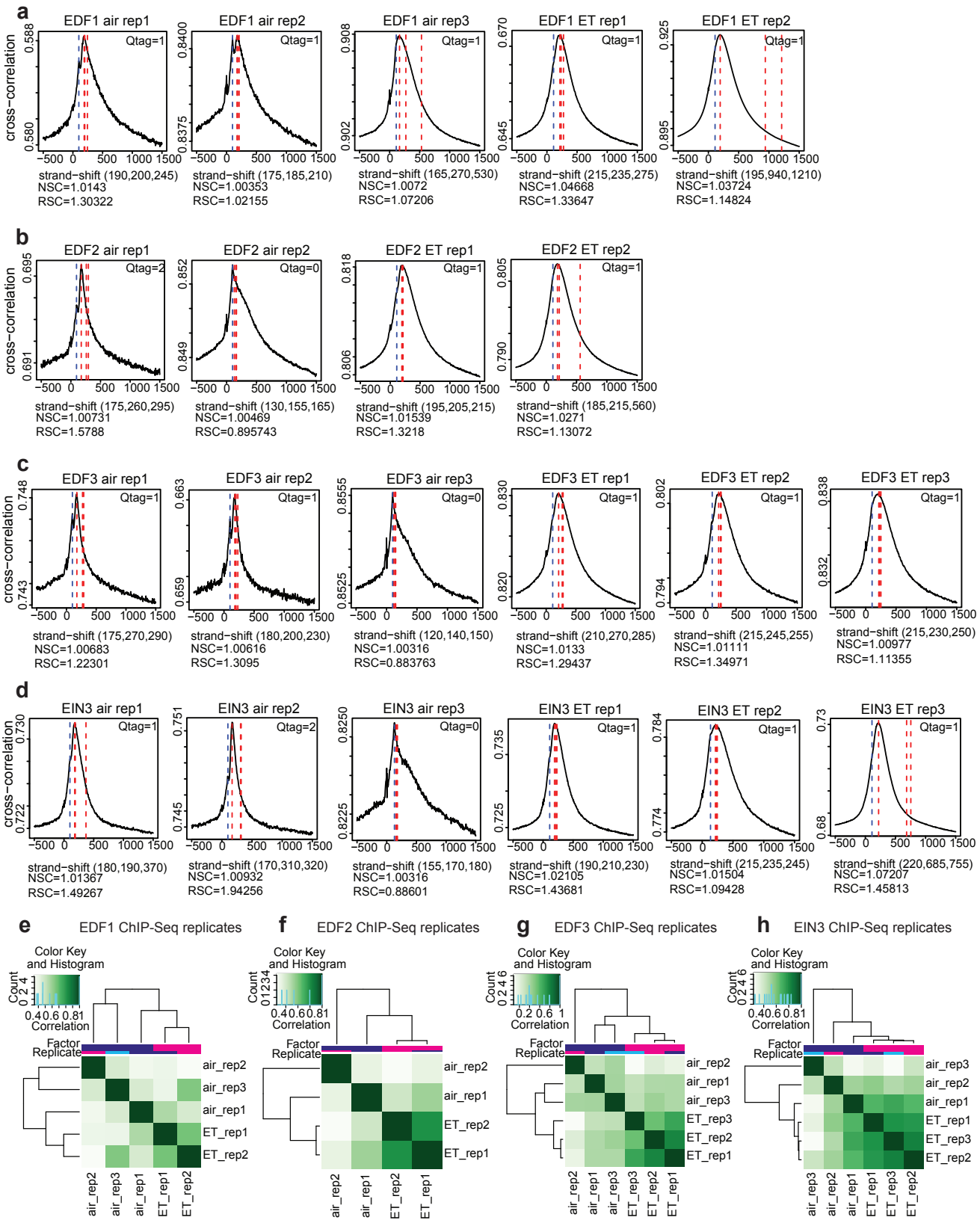
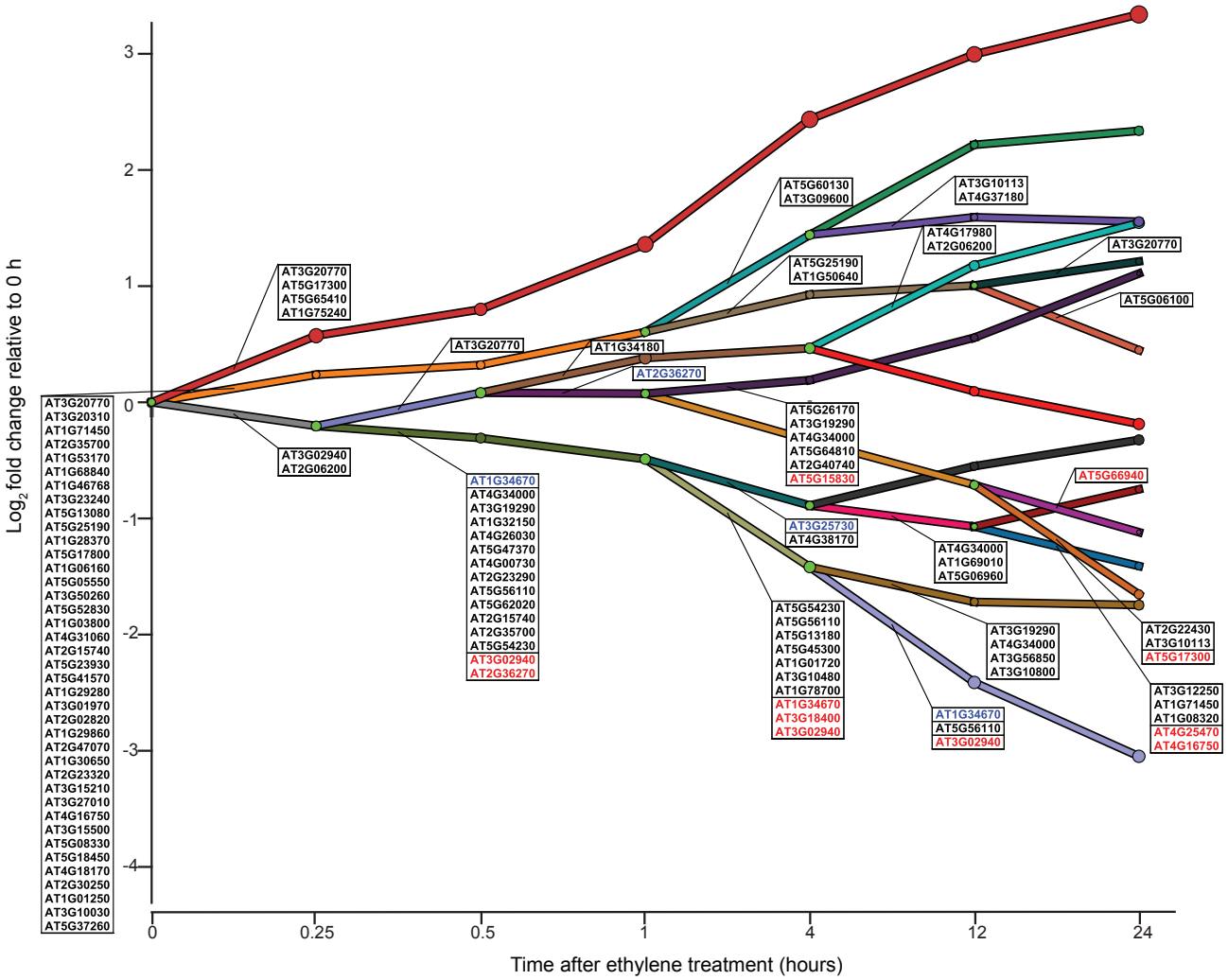
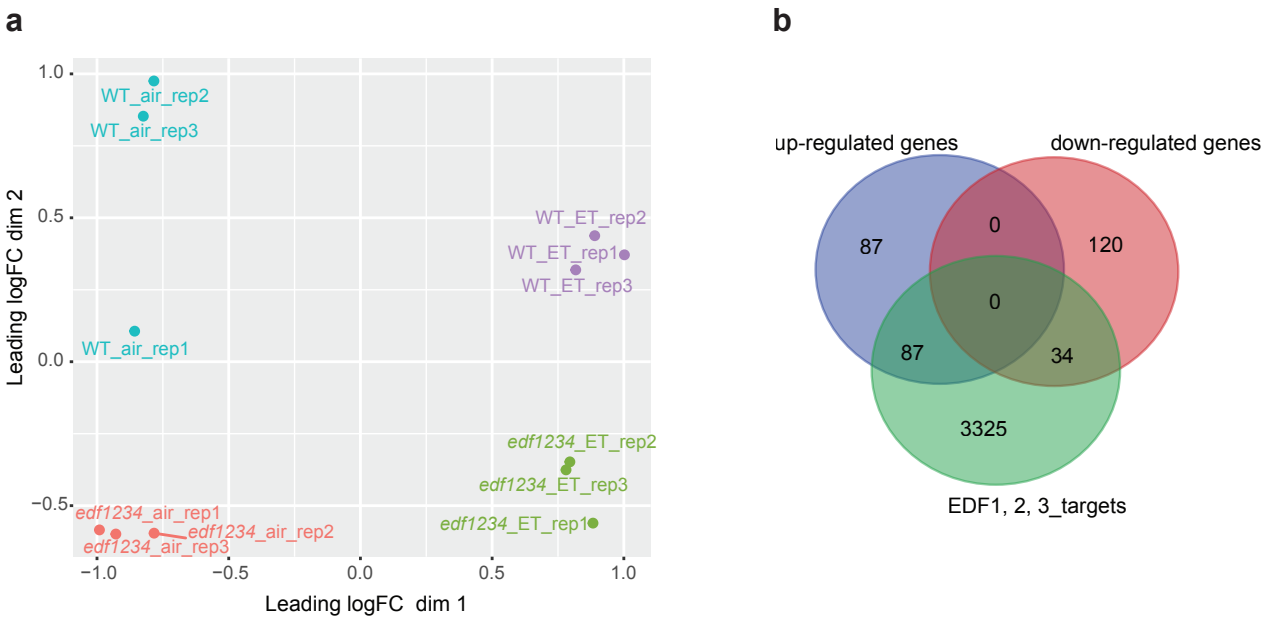


Extended Data Figure 1. Overview of quality metrics of ChIP-seq datasets. **a–d**, Correlation plot of TF ChIP-seq samples generated in this study. Clustering is determined by the degree of correlation (Pearson correlation). NSC means normalized strand cross-correlation coefficient and RSC means relative strand cross-correlation coefficient. Qtag means quality tag based on threshold RSC (codes = −2: very low, −1: low, 0: medium, 1: high, 2: very high). **e-h**, For each TF, clustering of replicate ChIP-seq samples after air and ethylene (ET) treatment, using Pearson correlation. All ChIP-seq replicates are derived from biologically independent experiments: EDF1 (air, n=3. ethylene (ET) , n = 2), EDF2 (air, n=2. ET, n = 2), EDF3 (air, n = 3. ET, n = 3), EIN3 (air, n = 3. ET, n = 3).



Extended Data Figure 2. Modelling transcriptional regulation in response to ethylene. DREM model showing predicted TFs assigned to each path. TFs are ranked high to low association, top to bottom. The y-axis indicates the log2 fold change in expression in response to ethylene. Transcriptionally up-regulated TFs are colored in blue, transcriptionally down-regulated transcription factors are shown in red.



Extended Data Figure 3. Quality metrics of *edf1234* quadruple mutant RNA-seq data and the combined analyses with EDF1, 2, 3 ChIP-seq data. **a**, Multidimensional scaling plots of independent biological replicate samples of air and ethylene (ET) treatment RNA-seq in wild-type and *edf1234* mutant seedlings. **b**, Overlap between EDF1, 2, 3 target genes with differentially expressed genes. up-regulated genes: up-regulated genes in ethylene-treated *edf1234* mutant seedlings compared to wild-type controls. down-regulated genes: down-regulated genes in ethylene-treated *edf1234* mutant seedlings compared to wild-type controls.

4. CHAPTER 4. General Discussion

4.1 The importance of characterising cross-regulation between plant hormones

Plants continuously face abiotic and biotic stresses during their lifecycle. They must process fluctuating environmental signals rapidly and correctly to survive in nature while avoiding growth arrest, initiating responses or developmental transitions appropriately (Kaufmann et al., 2010). Plant responses to stimuli result from dynamic modulation of molecular events, which involves a complex network of interactions between multiple plant hormones and signalling molecules. Hormone cross-regulation is an essential mechanism for plants to achieve optimal growth and development. Broad-scale regulation of gene expression is an important component of this process, and needs to be coordinated to initiate appropriate cellular responses. Attempts have been made to understand the mechanisms of hormone cross-regulation networks that monitor external or developmental cues (Moore et al., 2015; Rowe et al., 2016). However, interactions have primarily been examined for only relatively small numbers of hormones. There are nine major plant hormones known in the plant kingdom and cross-regulation might exist between them at multiple points (Chang et al., 2013; Altmann et al., 2020; Zander et al., 2020). The most comprehensive plant hormone signalling cross-regulation network prior to my research was developed in a large-scale protein-protein interaction study (Altmann et al., 2020). Whilst this approach did incorporate the interactions of TFs with other proteins, it did not directly relate hormone signalling to gene expression changes, because gene expression data was not a component of the study.

In my study, I developed a framework to analyse the structure and function of hormone cross-regulation networks (Chapter 2, Fig.1a). Using this I identified the signalling proteins and key TFs involved in hormone-regulated gene expression (Chapter 2, Fig.1c, d; Extended Data Fig. 3, 4, 5, 6). The activity of these TFs was comprehensively characterized for six plant hormone responses (Chapter 2, Fig. 2). I also assessed how hormones integrate signals from multiple pathways to dynamically cross-regulate gene expression. I identified key convergence points that integrate information from six hormones and drive downstream outputs, which included signal transduction proteins and TFs (Chapter 2, Fig. 4a, b; Supplementary Table 5). Moreover, the contribution of alternative splicing to hormone responses was identified (Chapter 2, Fig. 5, 6). Overall, this research provides a broad view of the mechanisms of hormone cross-regulation, which can occur through signal transduction, TF activity and gene expression. A limitation of my approach is that my models were constructed using data from whole seedlings. Hormone interactions must vary between plant tissues and cells in order for developmental patterning to occur (Moore et al., 2015; Topham et al., 2017). Future investigation could therefore consider the cell-specific structure of hormone cross-regulation networks, for example analysing variation in hormone signalling pathways using tissue-specific and single-cell transcriptome profiling methods (Iacono et al., 2019; Liew et al., 2020).

The third chapter of this thesis describes my investigation of a family of TFs, EDF1, 2, 3. I determined that the activity of the EDFs was ET responsive and EDFs may act as components of the

ET response to cross-regulate gene expression with other hormone signalling pathways. The target genes of EDFs during an ET response were largely repressed and were associated with other hormone signalling components, such as ABA, KAR and sterol/BR (Chapter 3, Fig. 2b, 3c). Several biosynthetic genes of ET and JA were bound and repressed by the EDFs (Chapter 3, Fig. 6c; Supplementary Table 13). Collectively, these results suggest EDFs are nodes through which ET signalling is prioritized over other hormone pathways. Moreover, EDFs targeting and repressing the ET biosynthetic genes suggests feedback-regulation of the ET signalling pathway. EDFs are involved in many individual hormone signalling pathways, such as ABA, BR, GA and JA (Hu et al., 2004; Osnato et al., 2012; Feng et al., 2014; Fu et al., 2014; Matías-Hernández et al., 2016; Osnato et al., 2020; Sengupta et al., 2020). However, the roles of EDFs in hormone cross-regulation were largely unknown prior to my study. EDF1 and EDF2 were previously identified as convergence points integrating GA and CK signals during trichome development in Arabidopsis (Matías-Hernández et al., 2016). Further studies could focus on the phenotypes of *edf1234* seedlings under different hormone treatments, which may help explain the roles of EDFs in mediating hormone cross-regulation.

FYF UP-REGULATING 321 FACTOR 1 (FUF1) was predicted to be a regulatory TF in the ET, JA, SA and SL/KAR signalling pathways and was a convergence point in the hormone cross-regulation network (Chapter 2, Fig. 2a, 4b; Supplementary Table 5). Interestingly, FUF1 functions upstream of the EDFs and negatively regulates their expression to control Arabidopsis flower senescence and abscission (Chen et al., 2015). The target genes of EDFs were enriched in KAR signalling components (EDF1) and flower development (EDF2) (Chapter 3, Fig. 1d). This might suggest that the interaction between FUF1 and EDFs acts in the cross-regulation between JA, SA and SL/KAR with ET because these signals are converged at FUF1. It will be intriguing to investigate this possibility to validate the SDREM-based framework prediction results and to better understand the mechanism of FUF1 and EDFs mediated hormone cross-regulation. All the above findings shed light on how plants utilize multiple hormone signals to initiate appropriate cellular responses to adapt to diverse environmental conditions.

4.2 MPKs in hormone cross-regulation

MPK cascades have strong links with hormone signalling pathways. Hormones can induce the activity of MPKs which phosphorylate target proteins, changing properties such as their stability, localization or function. Ultimately this can affect the expression of genes, especially when those targets are TFs. MPKs phosphorylate diverse targets, many of which are components of hormone responses (Liu and Zhang, 2004; Brodersen et al., 2006; Ortiz-Masia et al., 2007; Takahashi et al., 2007; Yoo et al., 2008; Chai et al., 2014; Danquah et al., 2015; Zhang et al., 2020). MPKs are themselves modified by various upstream MKKs. These characteristics place MPKs centrally in many signal transduction pathways and make MPKs well-suited to being convergence points mediating hormone cross-regulation. The functions of MPK4 and MPK6 in hormone responses are the most prominent examples. MPK6 is implicated in cross-regulation between the ABA and ET signalling pathways (Liu and Zhang, 2004; Leung et al., 2006; Ludwików et al., 2014). Notably, MPK6 and MPK3

are partially redundant in their activities coordinating ET and immune responses (Yoo et al., 2008; Galletti et al., 2011; Sun et al., 2018). MPK4 functions as a key factor to balance the antagonism between SA with JA/ET defence signalling pathways (Petersen et al., 2000; Brodersen et al., 2006). However, no previous studies have tested the hypothesis that MPKs act as convergence points for cross-regulation between multiple hormone signals.

In this study, I identified a group of MPK proteins (8 MPKs including MPK3, MPK4 and MPK6) as convergence points in the hormone cross-regulation network (Chapter 2, Fig. 4b; Supplementary Table 5). To functionally validate the prediction results, phenotypic screening experiments were performed by using T-DNA insertion mutant lines to characterise the impact of individual *mpk* genes on regulation of multiple hormone responses (See Appendix). However, behaviour varied between different mutant alleles of individual *MPKs*, so deeper genetic characterization will be necessary in order to draw robust conclusions. There was not sufficient time to complete this work, so I include the data here as an Appendix only. These observations are not unexpected as the functional diversity and redundancy of MPK proteins likely result in difficulties defining their specific functions (Cristina et al., 2010; Bigeard and Hirt, 2018; Jagodzik et al., 2018). In the future, a comprehensive study that assesses the functional effects of MPKs on etiolated seedling development and their functions across several hormone signalling pathways is required.

TFs from many hormones are regulated by MPK phosphorylation, such as EIN3, ERF6, MYC2, STZ and WRKY33 (Yoo et al., 2008; Mao et al., 2011; Nguyen et al., 2012; Meng et al., 2013; Sethi et al., 2014). In my study, TFs are a large proportion of the direct interactors of MPKs in each hormone model (Chapter 2, Supplementary Table 5). This is consistent with a previous study of MPK phosphorylation substrates in a MPK target network (Popescu et al., 2009). I demonstrated that MPKs to a large extent interact with different proteins between the six hormone responses (Chapter 2, Fig. 4d; Supplementary Table 5). This further demonstrates that MPKs can integrate hormone signals and deliver these signals to cellular response by modifying downstream substrates. However, how MPKs identify their specific substrates is still a substantial question. SMALL UBIQUITIN-LIKE MODIFIER (SUMO) may act as a mechanism assisting MPK3 and MPK6 to selectively phosphorylate WRKY33 in plant immune response (Verma et al., 2021). The process of SUMO conjugate with its substrates (SUMOylation) is emerging as important posttranslational modification for plants to rapidly reprogram cellular events in response to diverse stresses (Elrouby and Coupland, 2010; Elrouby, 2015; Ghimire et al., 2020). This modification is also involved in ABA, BR and SA signalling pathways (Lois et al., 2003; van Den Burg et al., 2010; Srivastava et al., 2020). It will be intriguing to investigate the roles of SUMO and their interplay with MPKs during hormone responses.

Research in animal systems demonstrated that phosphorylation affects the activity and localization of splicing factors (Blaustein et al., 2007; Stamm, 2008; Razanau and Xie, 2013). In Arabidopsis, splicing factors are common substrates of MPKs (Feilner et al., 2005; Van Bentem et al., 2006; de La Fuente Van Bentem et al., 2008). In this study, I determined that alternative splicing made a larger contribution at earlier time points in hormone responses than at late time points (Chapter 2, Fig. 6b). This could potentially result from rapid phosphorylation to activate/deactivate splicing factors,

conducted by the MPKs. Considering the potential for phosphorylation of TFs and splicing factors, my work may suggest a model where MPKs function as convergence points which integrate multiple hormone signals and specifically regulate TFs and splicing factors, and that these regulatory factors in turn regulate downstream genes, thereby resulting in dynamic transcriptome changes during hormone responses.

I propose that a comprehensive assessment of the functions of the MPKs across several hormone signalling pathways would be beneficial in the future. The targets and interactors of MPK proteins during hormone responses should be confirmed *in planta*. This could be conducted using methods such as TurboID (a biotin ligase-based proximity labelling method) and shotgun phosphoproteomics. TurboID would enable identification of proteins that interact with MPKs following stimulation of each hormone signalling pathway, and thereby understanding of how the substrates differ depending upon the stimulus (Branon et al., 2018). These analyses could be supported by shotgun phosphoproteomics of genetic mutants in candidate MPKs. Together this will provide knowledge of the proteins that the MPKs physically interact with and how they influence phosphorylation *in vivo*, in response to each hormone. A quantitative assessment of the affect of each *MPK* on activity of each hormone signalling pathway could also be performed. This could be achieved by assaying genome-wide hormone-responsive gene expression in non-treated and hormone-treated of *mpk* mutant seedlings and wild-type controls. The phenotypic screening experiments, MPKs interactors and targets investigation and transcriptome data could then be integrated to better understand the roles of individual MPKs and to mechanically position them in the hormone cross-regulation networks.

4.3 Transcriptional repression within hormone responses

Plants have evolved complex transcriptional regulatory mechanisms that include transcriptional repression to ensure correct gene expression patterns (Thiel et al., 2004; Payankaulam et al., 2010; Mercer and Mattick, 2013; Brkljacic and Grotewold, 2017; Igler et al., 2018). It is essential to control hormone responses at an appropriate level during plant growth and reproduction. Plants are continually exposed to stress stimuli in their environments, for example through pathogen attack, temperature extremes and water deficiency. Continuous activation of a defence hormone response, such as JA, can result in growth inhibition (Hou et al., 2010; Yang et al., 2012). Thus, negative regulation of hormone responses is necessary for plants to mitigate the inhibitory effect of hormone signalling under stress conditions and promote growth recovery after the stress elimination. Another function of transcriptional repression is to favour one hormone signalling pathway over another. Transcriptional repression in hormone responses may occur through multiple mechanisms, which include regulating the activity or stability of TFs both activator and repressor TFs, as well as by recruitment of corepressors such as TPL (Long et al., 2006; Causier et al., 2012; Martin-Arevalillo et al., 2017; Plant et al., 2021).

In this study, I identified POLYUBIQUITIN 3 (UBQ3) as a convergence node of hormone cross-regulation (Chapter 2, Fig. 4b; Supplementary Table 5). UBQ3 encodes ubiquitin, which is a

proteasome-targeting signal in the ubiquitin-proteasome system. The ubiquitin-dependent protein degradation machinery is involved in transcriptional repression by indirectly mediating the activity of master TFs of several hormone signalling pathways (Thiel et al., 2004; Payankaulam et al., 2010; Mercer and Mattick, 2013; Brkljacic and Grotewold, 2017; Igler et al., 2018). The AUX/IAA proteins, DELLA proteins and JAZ proteins in auxin, GA and JA signalling pathways respectively are targets for ubiquitination. These labile repressor proteins negatively regulate hormone responses by inhibiting the activity of master regulator hormone TFs (ARFs, MYCs and PIFs, respectively) in the absence of hormone signals. Following a hormone stimulus these repressor proteins are ubiquitinated and targeted for 26S proteosome-mediated degradation by SCF E3 ubiquitin ligase complexes. This degradation subsequently allows expression of genes governed by the master TFs (Krogan and Long, 2009). My analyses indicate that UBQ3 is regulated by multiple hormone pathways, but its downstream effects following hormone stimulation remain to be examined *in vivo.*

TPL was a predicted convergence point between ABA, JA, SA and SL/KAR signalling pathways in the hormone cross-regulation network (Chapter 2, Fig. 4b; Supplementary Table 5). TPL is a well-studied co-repressor protein in Arabidopsis, with roles in various biological processes (Fujimoto et al., 2000; Ohta et al., 2001). TPL cannot bind DNA directly, but instead interacts with a wide range of TFs. This makes it well suited to act as a point of integration across multiple signals, and previous studies support this (Causier et al., 2012). TPL mediates auxin-dependent transcriptional repression by directly interacting with AUX/IAAs to inhibit the expression of auxin-responsive gene (Szemenyei et al., 2008). Similarly, TPL is recruited by the adapter protein NOVEL INTERACTOR OF JAZ (NINJA) to repress the activity of MYC2, thereby suppress downstream gene expression in the absence of JA (Pauwels et al., 2010). In SL signalling pathway, TPL is recruited by SMXL6 to bind directly to the promoters of SMXL6, 7, 8 and repress their expression, which form a negative feedback loop to maintain SL signalling homeostasis (Zander et al., 2020). This demonstrates that TPL is a general co-repressor in plant hormone signalling pathways recruited by specific proteins within each hormone response.

Repression of the ET signalling pathway by degradation of the master TF EIN3 is well described (Guo and Ecker, 2003; Potuschak et al., 2003; Lee et al., 2020). However, how other TFs within ET responses repress transcription is still largely unknown. In this study, I identified that EDF1, 2, 3 are important transcriptional repressors in ET responses of etiolated Arabidopsis seedlings (Chapter 3). The EDFs are targeted by EIN3 and their expression is upregulated following an ET signal (Chapter 3, Fig. 1a, 5d). More than two thirds of EDF target genes during an ET response were down-regulated (Chapter 3, Fig. 2c). Furthermore, a large proportion of EDF target genes were up-regulated when EDF1, 2, 3, 4 function was lost (Chapter 3, Extended Data Fig. 3b). These findings provide new insight into the transcriptional repression mechanisms of ET signalling and extend our understanding how plants fine-tune ET responses.

A considerable number of TFs possess repressive activity in plants as they contain repression domains, namely EAR motifs or B3 domains (Ohta et al., 2001; Hiratsu et al., 2003; Ikeda and Ohme-Takagi, 2009). EDF1, 2, 3, 4 possess the RLFGV repression motif in their B3 DNA-binding domain

and the isolated B3 domains have repressive activity *in vitro* (Ikeda and Ohme-Takagi, 2009). Repressor TFs have important roles in modulating transcriptional repression in hormone responses. ERF3 and ERF4 possess the repressive EAR motifs and act as repressors in Arabidopsis leaves (Ohta et al., 2001; Hiratsu et al., 2003; Ikeda and Ohme-Takagi, 2009). JA-ASSOCIATED MYC2-LIKE 1 (JAM1) competes with MYC2 for binding to target sequence thereby negatively regulate JA signalling (Nakata et al., 2013). A set of transcriptional repressors modulate auxin signalling and these repressor TFs function as a network to regulate the gene repression of *ARF*s (Truskina et al., 2021). These studies indicate that transcriptional repression by TFs is a common theme in regulating hormone-responsive gene expression. Efforts in the future should be made to isolate novel transcriptional repressors and clarify their possible roles in plant biological processes. This would allow us to characterize the finely tuned molecular mechanisms of transcription regulation in plants.

I observed that EIN3 itself predominantly functions as a transcriptional activator because the expression of the majority (89.8%) of EIN3-specific target genes was induced by ET (Chapter 3, Fig. 2c). This result is consistent with the activator role of EIN3 in a published transcriptome study (Chang et al., 2013). However, a recent study demonstrated that EIN3 can repress transcription by directly interacting with a repressor protein, Transcriptional Repressor of EIN3-dependent Ethylene-response 1 (TREE1), in etiolated Arabidopsis shoot tissue (Wang et al., 2020). The conflicting findings of my study with Wang's might have occurred because Wang analysed tissue-specific transcriptomes, whereas I analysed whole seedling transcriptomes. Wang found there was only a 40% overlap between the ET-regulated differentially expressed genes identified from whole seedlings and those from their tissue-specific (shoot and root) samples. EIN3 repressed transcription in shoots only. However, for the shared differentially expressed genes in both tissues (shoot and root), EIN3 targets are largely up-regulated under ET treatment (Wang et al., 2020). Other TFs are known to have dual-roles. BZR1 is an example, which can activate and repress genes following a BR stimulus (He et al., 2005). This is thought to enable formation of a negative feedback loop that optimizes cellular BR abundance. Thus, the investigation of the functions of TFs in a tissue specific level will be of future interest.

4.4 Summary

This study examined hormone cross-regulation mechanisms controlling gene expression in etiolated Arabidopsis seedlings. Regulatory TFs and signalling proteins were identified by analysis of the transcriptional response to six hormones and integrative dynamic modelling. Candidate cross-regulation points, a set of MPKs, were identified using a hormone cross-regulation network model. This prediction was further investigated using T-DNA mutants, determining that MPK3 and MPK6 might affect etiolated seedling growth. Furthermore, the functions and regulatory roles of a group of TFs, EDFs, in the ET gene regulatory network were characterized. The EDFs function as transcriptional repressors during an ET response. Analyses indicated that EDFs may mediate cross-regulation of other hormones by ET through repressing hormone biosynthetic genes. This extends our understanding of the transcriptional repression mechanisms of the ET response. This work

uncovered new aspects of transcriptional regulatory networks and plant hormone cross-regulation mechanisms which can be used to improve our understanding of how plants successfully adapt to ever changing environments.

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5. Appendix

5.1 Introduction

Twenty-three proteins shared by four or more hormone signalling pathways were identified within the integrated hormone cross-regulation network (Chapter 2, Fig. 4; Supplementary Table 5). I propose that these integrate signals from multiple hormones to influence the development of etiolated Arabidopsis seedlings. These 23 proteins were enriched in MPKs (MPK 1, 2, 3, 4, 5, 6, 7, 10). MPKs have roles in individual hormone responses and, in many cases, in multiple hormone responses (Introduction, Figure 7). Functional validation experiments were subsequently designed to further examine the potential roles of the MPKs. Reverse-genetic screening was performed using *mpk* loss-of-function lines to characterize the impact of individual *mpk* genes on hormone responses. Etiolated seedlings establish an elongated hypocotyl and form an apical hook with unexpanded cotyledons to minimize mechanical damage caused by pushing through the soil (Boron and Vissenberg, 2014; Mazzella et al., 2014). Root growth occurs at the same time, as the plant establishes itself in the soil. Thus, root and hypocotyl growth were chosen as the phenotypic parameters to assess. Ethylene (ET) is a well characterized regulator of etiolated seedling development and treatment with ET results in phenotypic changes, including inhibition of hypocotyl and root elongation (Goeschl et al., 1966; Goeschl et al., 1967; Guzmán and Ecker, 1990; Ecker, 1995). However, the phenotypic effects of treatment with other hormones at this growth stage are poorly defined.

5.2 Phenotypic screening results

The functional consequences of disrupting hormone cross-regulation mediated by MPKs on development of etiolated Arabidopsis seedlings were analysed. To do so it was necessary first to identify mutants in each of the *MPK* genes. Characterised mutant alleles were available for some *mpks* from published studies, but these were supplemented by additional alleles from the SALK, GABI-KAT, SAIL and WiscDsLox collections (Sessions et al., 2002; Alonso et al., 2003; Rosso et al., 2003; Woody et al., 2007). At least two T-DNA insertion alleles were obtained for each *MPK* gene, except *MPK5* (Table 1). The genotypes of the mutants were determined by PCR using two pairs of primers that detected the wild-type copy of the gene and the T-DNA/genomic DNA junction sequence (Fig. 1, 2) (O'Malley et al., 2015).

Etiolated *mpk* mutant seedlings (*mpk1-1, mpk1-2, mpk2-1, mpk2-2, mpk3-1, mpk3-2, mpk4-1, mpk4-2, mpk5, mpk6-1, mpk6-2, mpk7-1, mpk7-2, mpk10-1, mpk10-2*) were grown on Murashige and Skoog (MS) medium supplemented with or without hormones for 7 days. Root and hypocotyl growth of the seedlings were then assessed and compared to growth of wild-type seedlings and a panel of known hormone mutants grown under the same conditions (ET, *ein3-1*; jasmonic acid (JA), *myc2* (*jin1-8*); salicylic acid (SA), *npr1-1*) (Fig. 3). To the date of this thesis, the phenotypic screening of responses to three hormones (ET, using the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) treatment, which is stimulates ET signalling; JA; SA) has been completed. Visual observation

indicated that growth of most *mpk* mutants did not differ from wild-type (*mpk1-1, mpk1-2, mpk2-1, mpk2-2, mpk4-1, mpk4-2, mpk5, mpk7-1, mpk7-2, mpk10-1, mpk10-2*). Only the mutants with visually-obvious altered phenotypes were measured and statistically analysed (*mpk3-1, mpk3-2, mpk6-1, mpk6-2*). The measurement data is available in Supplementary Tables 2-7.

Gene	Name	Allele name	Stock number	Collection	Publications
AT1G10210	MPK1	*mpk1-1*	SALK_063847C	SALK	N/A
		mpk1-2	SALK_102175	SALK	N/A
AT1G59580	MPK2	*mpk2-1*	SALK_019507C	SALK	N/A
		mpk2-2	SALK_047422C	SALK	N/A
AT3G45640	MPK3	*mpk3-1*	GK-734C01	GABI-KAT	N/A
		mpk3-2	SALK_209371C	SALK	N/A
AT4G01370	MPK4	*mpk4-1*	SALK_032449	SALK	N/A
		mpk4-2	SAIL_750_A10	SAIL	McElver et al., 2001
AT4G11330	MPK5	*mpk5*	WiscDsLox430A12	WiscDsLox	N/A
AT2G43790	MPK6	*mpk6-1*	SALK_004221C	SALK	N/A
		mpk6-2	SALK_062471C	SALK	Li et al., 2017
AT2G18170	MPK7	*mpk7-1*	SALKseq_033098.2	SALK	N/A
		mpk7-2	SALK_113631.2	SALK	N/A
AT3G59790	MPK10	*mpk10-1*	SALK_039102C	SALK	Stanko et al., 2014
		mpk10-2	SALK_136149C	SALK	N/A

Table 1. Details of the T-DNA insertion alleles for each *MPK* genes used in the phenotypic screening.

Loss of *mpk3* function affected hormone responses, but the results differed between alleles. Firstly, the development of *mpk3-1* etiolated seedlings differed from those of *mpk3-2* in non-hormone treated conditions. Roots and hypocotyls of *mpk3-1* seedlings were longer than wild-type seedlings, whilst roots and hypocotyls of *mpk3-2* seedlings were shorter (Fig. 4a, b, e, f, I, j; Supplementary Table 2, 3, 4). ACC repressed root and hypocotyl growth of wild-type seedlings (Fig. 4a, b). Root and hypocotyl growth of *ein3-1* was less repressed by ACC (Fig. 4c, d). Hypocotyl growth of *mpk3-2* was less repressed by ACC than hypocotyl growth of wild-type seedlings, similar to the ET insensitivity of *ein3-1* mutants, but root growth repression of *mpk3-2* was unchanged compared with wild-type seedlings (Fig. 4c, d; Supplementary Table 2). The responses of *mpk3-2* to JA were different from the responses to ACC. JA repressed root and hypocotyl growth of wild-type seedlings, whilst root and hypocotyl growth of the JA insensitive *myc2* mutants was less repressed by JA (Fig. 4e, f, g, h). Root growth of *mpk3-2* behaved similarly to *myc2*, being less repressed by JA than wild-type seedlings, but repression of *mpk3-2* hypocotyl elongation by JA was not significantly different from that of wild-type seedlings (Fig. 5g, h; Supplementary Table 3). SA repressed root growth, but not hypocotyl length, of wild-type seedlings (Fig. 4i, j). Root growth of *npr1-1* was insensitive to SA (Fig. 4i). Root and hypocotyl growth of *mpk3-2* were repressed by SA (Fig. 4i, j), but there was no statistically significant difference in the extent of SA repression between *mpk3-2* and wild-type seedlings (Fig. 4k, l; Supplementary Table 4). ACC repressed hypocotyl growth of the second *mpk3* allele, *mpk3-1*, less

than it repressed hypocotyl growth of wild-type seedlings, consistent with the behaviour of *mpk3-2* (Fig. 4d; Supplementary Table 2). However, the responses of *mpk3-1* and *mpk3-2* etiolated seedlings to JA and SA were different from each other. Root growth of *mpk3-1* was repressed by JA to the same extent as wild-type seedlings (Fig. 4g). Contrastingly, root growth of *mpk3-2* was less repressed by JA than wild-type seedlings (Fig. 4g; Supplementary Table 3). SA treatment increased hypocotyl growth of *mpk3-1* seedlings compared with the effect on wild-type seedlings, whilst growth was unchanged in *mpk3-2* mutants (Fig. 4l; Supplementary Table 4). These observations indicate that MPK3 may regulate root and hypocotyl growth during hormone responses, but the conflicting data between alleles indicate that further genetic characterisation of the alleles is necessary to draw robust conclusions. I subsequently determined that *mpk3-1* carries a second T-DNA insertion in the 5'-UTR of gene *AT3G45645* which encodes a transmembrane protein using the TAIR database. It is possible this extra insertion may have affected the responses of the etiolated seedlings to hormone treatments.

Loss of *mpk6* function affected ACC responses, but the results were not consistent between two independent alleles. Under non-hormone treated conditions, *mpk6-2* seedlings appeared to be stunted, with shorter root and hypocotyl length compared to wild-type controls, whilst *mpk6-1* seedlings were of similar size to wild-type controls in most cases (Fig. 5a, b, e, f, I, j; Supplementary Table 5, 6, 7). Roots and hypocotyls of *mpk6-2* seedlings were shorter than wild-type seedlings in non-hormone treated conditions (Fig. 5a, b, e, f, I, j). Root growth of *mpk6-2* behaved in the opposite manner to *ein3-1* mutants, being more repressed by ACC compared with wild-type seedlings (Fig. 5c; Supplementary Table 5). However, the repression of *mpk6-2* hypocotyl growth was not statistically significantly different compared with wild-type seedlings (Fig. 5d; Supplementary Table 5). JA repressed root and hypocotyl growth of wild-type and *mpk6-2* mutants (Fig. 5e, f). However, the extent of repression was unchanged in *mpk6-2* seedlings compared with wild-type controls (Fig. 5g, h; Supplementary Table 6). Similarly, SA repressed root growth of wild-type and *mpk6-2* mutants to the same extent (Fig. 5i, Fig. 5k; Supplementary Table 7). The response of the second *mpk6* allele, *mpk6-1*, to ACC was different from *mpk6-2*. ACC induced root growth inhibition of *mpk6-1* was not significantly different from the response of wild-type seedlings (Fig. 5c; Supplementary Table 2). These observations indicate that MPK6 may play a role in the regulation of root growth during ET responses, but further investigation and genetic characterisation is required because the phenotypes were inconsistent between the two alleles.

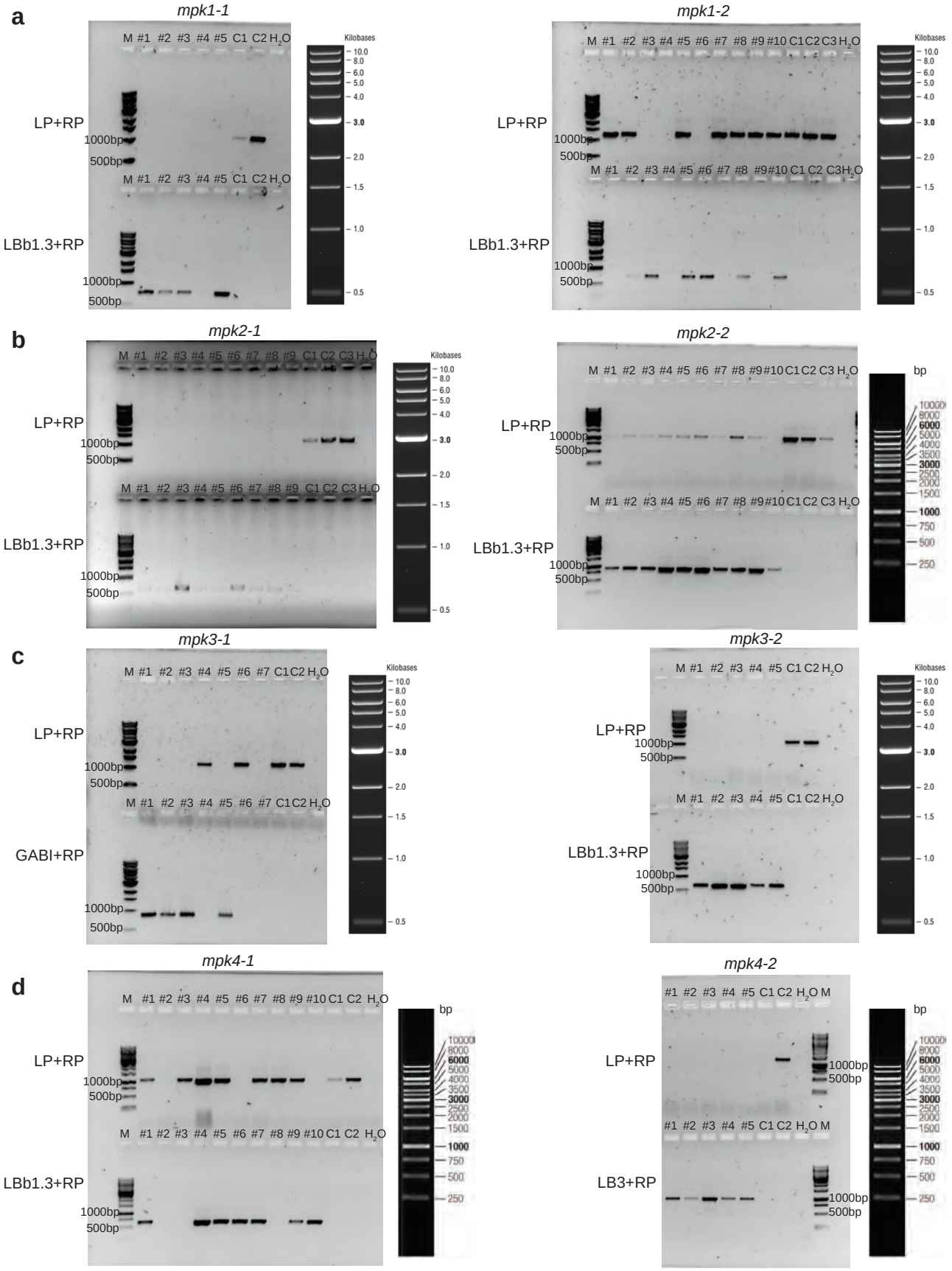

Figure 1. Gel electrophoresis image of PCR products from *mpk1* (a), *mpk2* (b), *mpk3* (c), *mpk4* (d) genotyping. LP, left genomic primer. RP, right genomic primer. LBb1.3, left border primer of the T-DNA insertion for SALK lines. GABI, left border primer of the T-DNA insertion for GABI-KAT lines. LB3, left border primer of the T-DNA insertion for SAIL lines. M, DNA ladder. #1 to #10, individuals of *mpk* mutant plants. C1, 2, 3, individuals of wild-type plants (Col-0). H_2O, negative control.

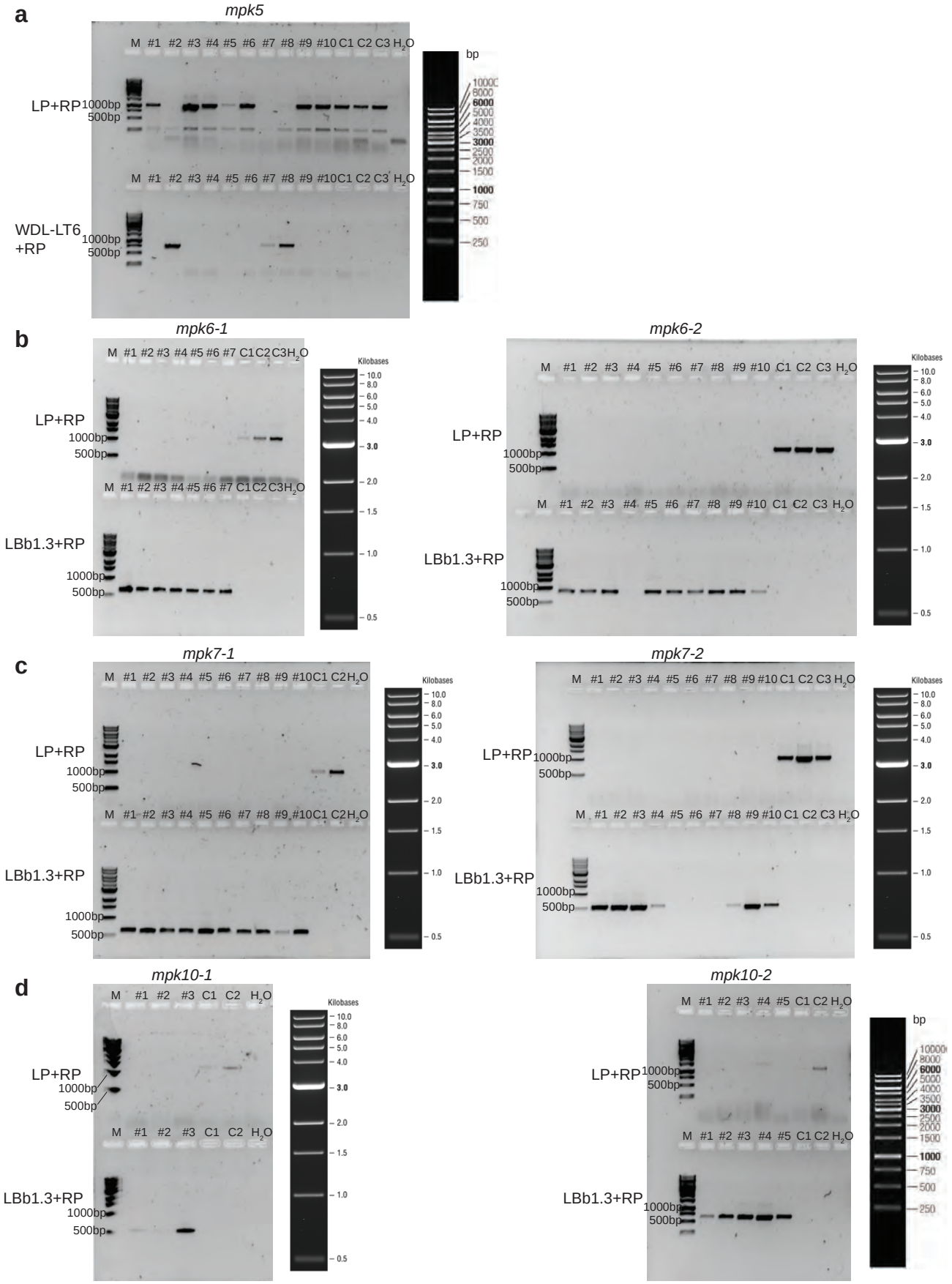

Figure 2. Gel electrophoresis image of PCR products from *mpk5* (a), *mpk6* (b), *mpk7* (c), *mpk10* (d) genotyping. LP, left genomic primer. RP, right genomic primer. LBb1.3, left border primer of the T-DNA insertion for SALK lines. WDL-LT6, left border primer of the T-DNA insertion for WiscDsLox lines. M, DNA ladder. #1 to #10, individuals of *mpk* mutant plants. C1, 2, 3, individuals of wild-type plants (Col-0). H_2O, negative control.

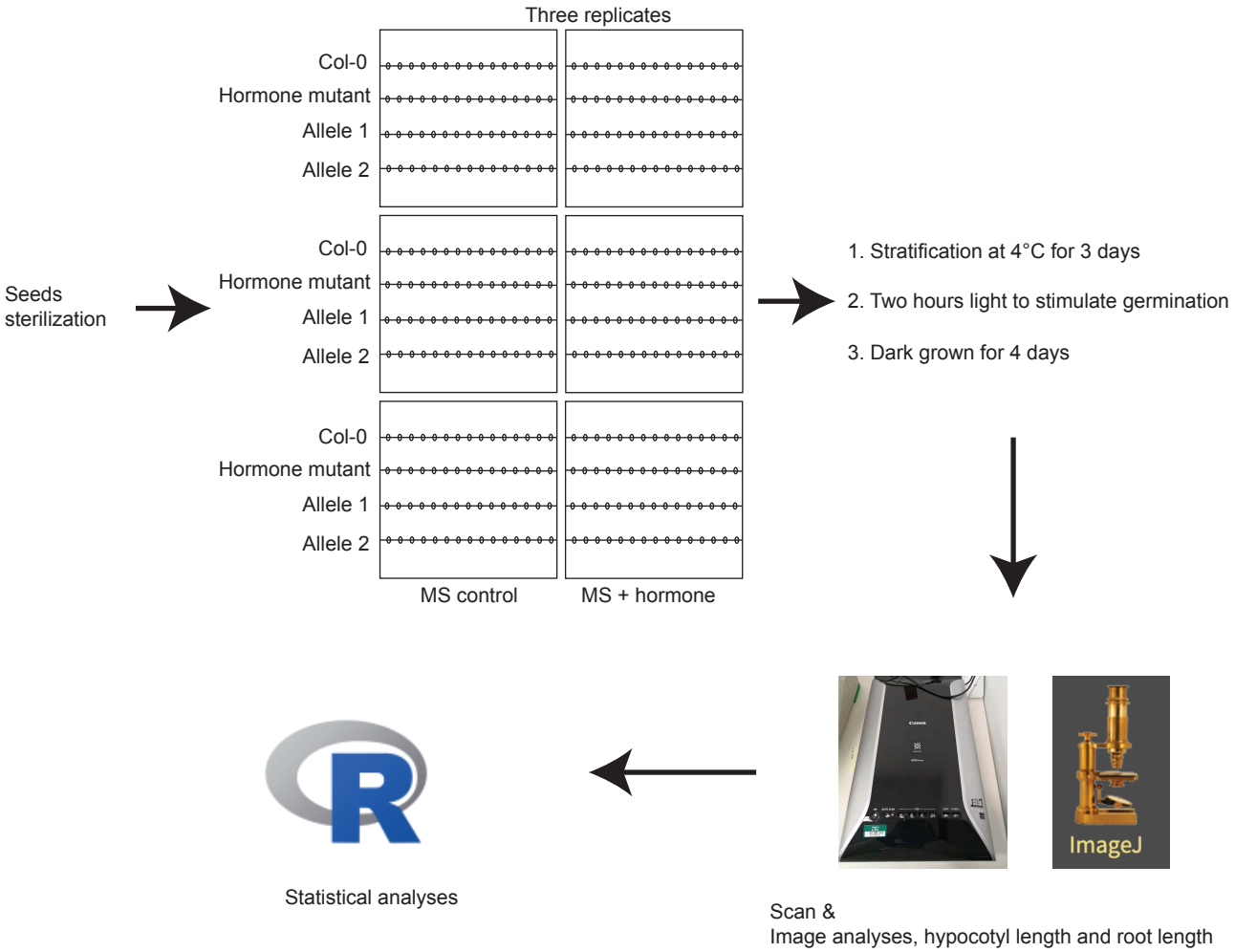

Figure 3. Overview of the experimental design for phenotypic screening. Seeds were sterilized using chlorine gas then sown on individual agar plates containing Murashige and Skoog medium (MS) supplemented with or without 10 µM ACC (Sigma: A-3903, dissolved in double distilled water) or 20 µM Me-JA (Methyl jasmonate, Sigma: 392707, dissolved in 0.1 % (v/v) ethanol) or 20 µM SA (Sigma: S7401, dissolved in 0.05 % (w/v) ethanol). At least 30 seedlings in total were used for each treatment. After stratification for 3 days in the dark at 4°C, seeds were given 2 hours light to stimulate germination. Then seeds were grown in the dark at 21°C for 4 days. Plates were scanned afterwards by using Canon 9000F MarkII and root and hypocotyl lengths were measured using ImageJ. The statistical analyses were performed in R (version 3.5.3).

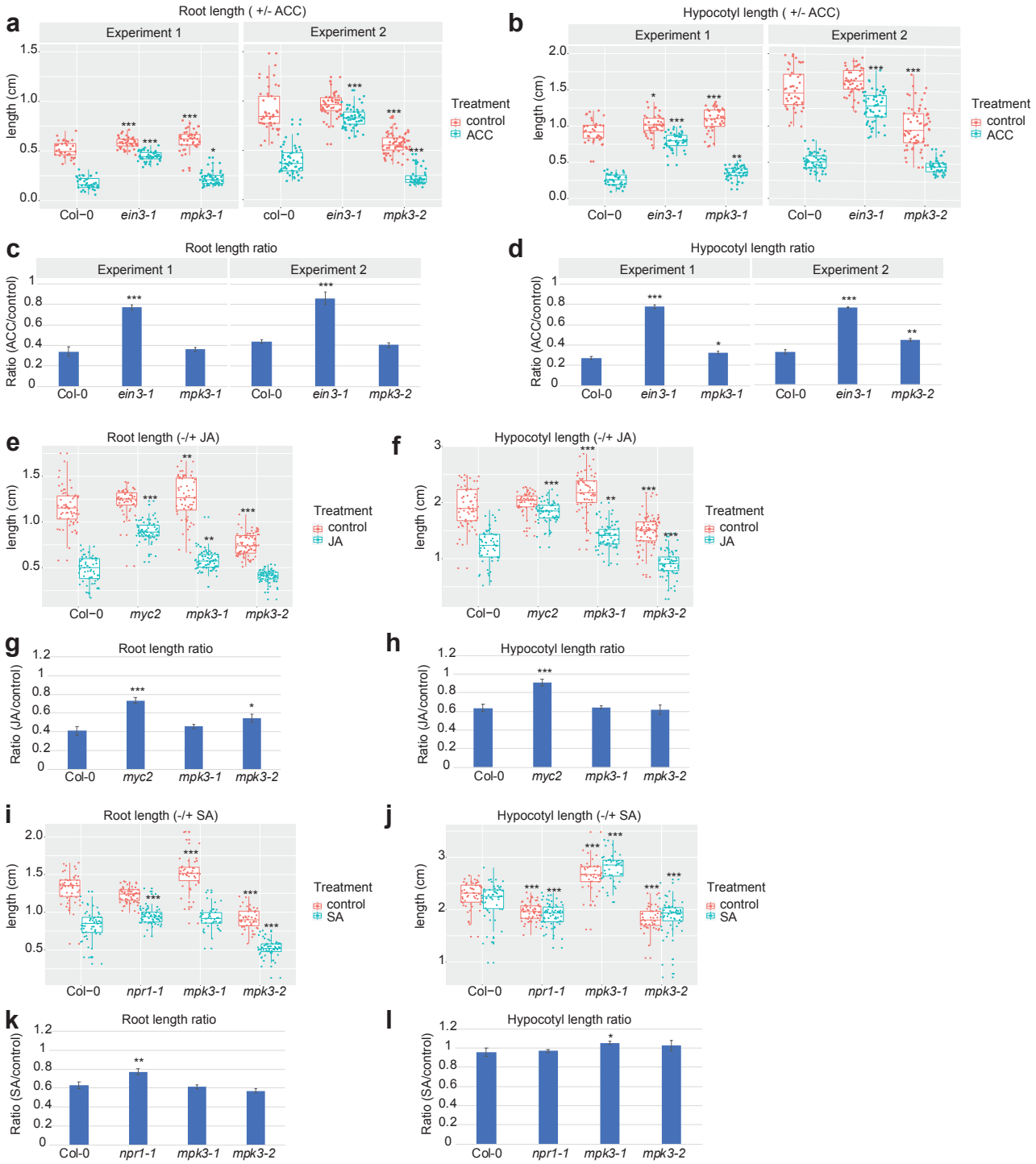

Figure 4. The effect of ACC, JA and SA on root and hypocotyl length of *mpk3* mutant seedlings compared with non-hormone control conditions. a, **b**, **e**, **f**, **i**, **j**, Box plots show the root length and hypocotyl length of two alleles of *mpk3* seedlings grown on control plates and seedlings grown on plates containing hormones ACC (**a**, **b**) JA (**e**, **f**) or SA (**i**, **j**). The ACC assay of *mpk3-1* and *mpk3-2* was performed in two individual experiments (experiment 1 and 2) because root and hypocotyl length of *mpk3-2* was too short for precise measurement in experiment 1. Statistical analyses were performed with R (version 3.5.3) using analysis of variance (ANOVA), followed by Tukey's pairwise multiple comparison of means. The comparison were made as: mutant:control - Col-0: control and mutant:hormone - Col-0:hormone, separately. Differences were considered significant at $p < 0.05$. The significance code is: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. **c**, **d**, **g**, **h**, **k**, **l**, Bar plots show the ratio of root length and hypocotyl length of *mpk3-1* and *mpk3-2* seedlings grown on plates containing hormones ACC (**c**, **d**), JA (**g, h**) or SA (**k, l**) compared with seedlings grown on control plates. The x-axis indicates different genotypes. Statistical analyses for hormone/-control ratio were performed using Student's Test. Differences were considered significant at $p < 0.05$. The significance code is: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

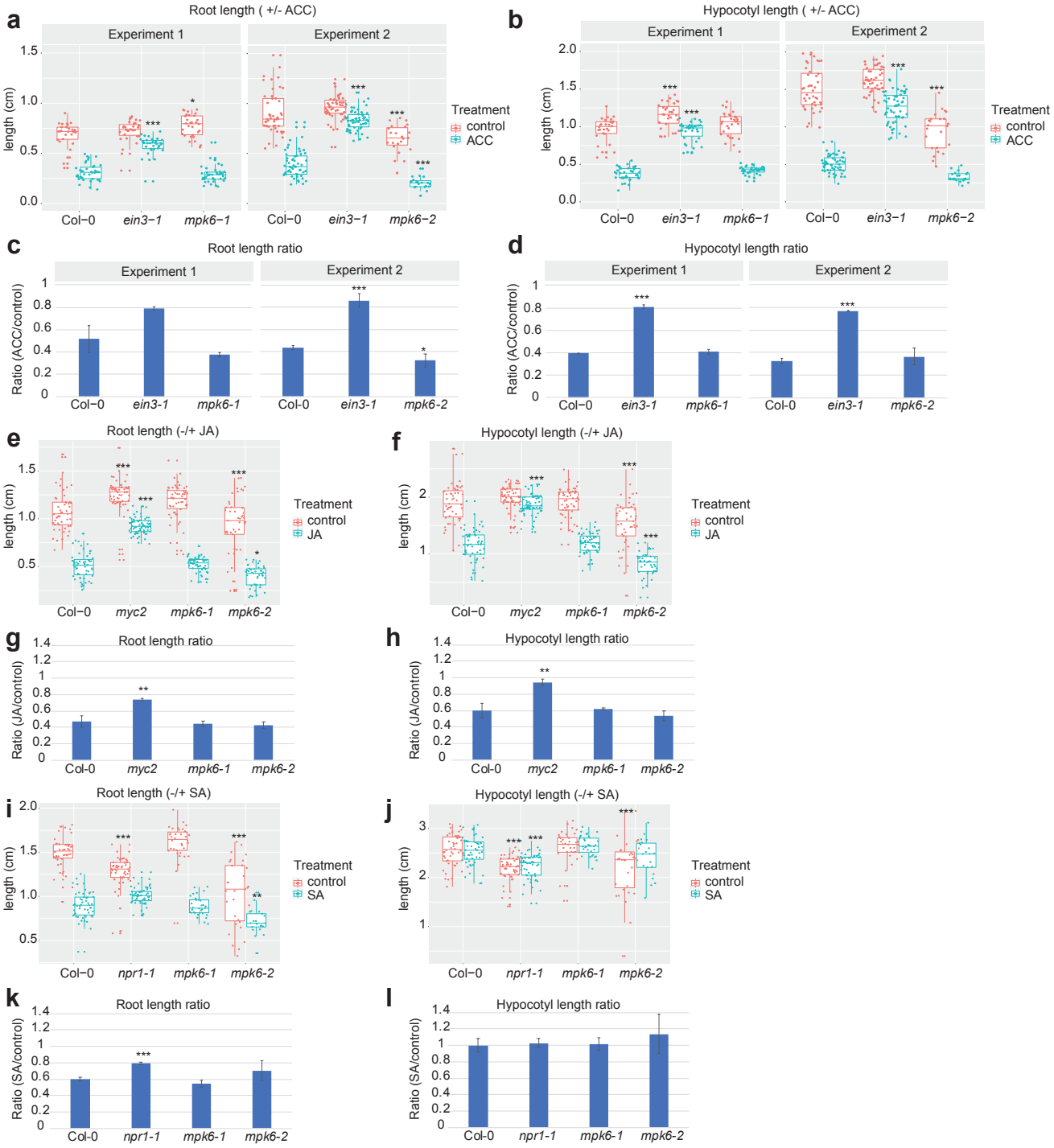

Figure 5. The effect of ACC, JA and SA on root and hypocotyl length of *mpk6* mutant seedlings compared with non-hormone control conditions. **a**, **b**, **e**, **f**, **i**, **j**, Box plots show the root length and hypocotyl length of two alleles of *mpk6* seedlings grown on control plates and seedlings grown on plates containing hormones ACC (**a**, **b**) JA (**e**, **f**) or SA (**i**, **j**). The ACC assay of *mpk6-1* and *mpk6-2* was performed in two individual experiments (experiment 1 and 2) because root and hypocotyl length of *mpk6-2* was too short for precise measurement in experiment 1. Statistical analyses were performed with R (version 3.5.3) using analysis of variance (ANOVA), followed by Tukey's pairwise multiple comparison of means. The comparison were made as: mutant:control - Col-0: control and mutant:hormone - Col-0:hormone, separately. Differences were considered significant at $p < 0.05$. The significance code is: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. **c**, **d**, **g**, **h**, **k**, **l**, Bar plots show the ratio of root length and hypocotyl length of *mpk6-1* and *mpk6-2* seedlings grown on plates containing hormones ACC (**c**, **d**), JA (**g, h**) or SA (**k, l**) compared with seedlings grown on control plates. The x-axis indicates different genotypes. Statistical analyses for hormone/-control ratio were performed using Student's Test. Differences were considered significant at $p < 0.05$. The significance code is: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

5.3 Discussion and future work

The phenotypic screening results above indicate that MPK3 and MPK6 might play roles in the hormone responses of etiolated Arabidopsis seedlings. Hypocotyl and/or root developmental phenotypes of two alleles of *mpk3* (*mpk3-1* and *mpk3-2*) and one allele of *mpk6* (*mpk6-2*) were altered by at least one hormone treatment compared with wide-type seedlings. However, the phenotypic screening studies did not permit solid conclusions. Firstly, most *mpk* mutations did not result in visually observable differences of root or hypocotyl growth relative to wild-type seedlings following hormone treatment. This is perhaps to be expected considering that MPK proteins are functionally redundant (Bigeard and Hirt, 2018; Jagodzik et al., 2018). Secondly, the responses to hormones differed between *mpk3* and *mpk6* alleles in most cases. For example, the response of *mpk6-2* to ACC differed from wild-type, whilst *mpk6-1* did not. Root and hypocotyl responses of the two *mpk3* alleles to JA and SA differed, though in both alleles hypocotyl growth was less repressed by ACC than wild-type controls. The growth of non-hormone treated *mpk3-1* and *mpk3-2*, *mpk6-1* and *mpk6-2* also differed. These discrepancies must be understood to interpret the data. The *mpk3-1* allele contains an extra gene insertion which may potentially affect the development of etiolated seedlings and hormone responses and explain the observed results. Obtaining other homozygous T-DNA insertion lines and *mpk* double mutants, or generating precise *mpk* knock out alleles using CRISPR-Cas9 techniques, would be useful to obtain a clear understanding of the roles of MPKs in hormone cross-regulation.

5.4 Materials and Methods

5.4.1 Plant materials and mutants

mpk mutants, the *MYC2* mutant *jin1-8* (SALK_061267), and the *EIN3* mutant *ein3-1* were obtained from the Arabidopsis Biological Resource Centre (ABRC) and Nottingham Arabidopsis Stock Centre (NASC) (Chao et al., 1997; Zander et al., 2020). The *npr1-1* (N3726) EMS mutant was kindly provided by Prof. James Whelan in La Trobe University (Cao et al., 1997). Detailed mutant information is provided in Supplementary Table 1.

5.4.2 Genotyping

Seeds were stratified in water for 3 days at 4°C in the dark then sown on soil (standard potting mix, Van Schaik's BioGro, Australia). The plants were grown in a controlled environment room with following environmental settings: 16/8-hour light/dark cycle, 22°C/19°C (light/dark), 55% relative humidity and 120 µmol m−2 s−1 light intensity to maturity.

Leaf samples were harvested by snap-freezing in liquid N_2 and stored at -80°C. Then frozen samples were ground using the TissueLyser II (QIAGEN) and extracted with a fast genomic DNA extraction protocol. Extraction buffer was prepared freshly with following solutions: 1 M Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride) Buffer (pH 8.0), 100mM EDTA (Ethylenediaminetetraacetic acid) (pH 8.0), 2.5M KCL (Potassium chloride). For 10 mL extraction

buffer, the above solutions were mixed according to the ratio Tris-HCL: EDTA: KCL: H_2O, 1:1:4:4 (v:v:v:v). DNA concentrations were determined using a Nano photometer (BMG Labtech).

Primer pairs for genotyping were designed using http://signal.salk.edu/tdnaprimers.2.html. Two paired reactions were set up for the following PCR reactions; left genomic primer (LP) plus right genomic primer (RP) for detecting the presence of a wild-type copy of the gene (WT), and left border primer of the T-DNA insertion (LBb1.3 / GABI / LB3 / WDL-LT6, for SALK, GABI-KAT, SAIL and WiscDsLox collections respectively) plus RP for detecting the T-DNA/genomic DNA junction sequence (TDNA) (Sessions et al., 2002; Alonso et al., 2003; Rosso et al., 2003; Woody et al., 2007; O'Malley et al., 2015). Homozygosity of a line was confirmed if it exhibited the pattern of no product for WT and but positive for the TDNA product. The primers used for genotyping are described in Supplementary Table 1.

PCR was performed in a total reaction volume of 10µL containing 1µL genomic DNA, 0.5µL primer mix (primer concentrations 10µM), and 5µL PCR master mix (Thermo Fisher Scientific, M0486L). PCR was conducted on the T100 Thermal Cycler PCR system (BIO-RAD) with PCR conditions as follows: 94 °C for 30 sec, and 30 cycles at 94 °C for 30 sec / 55 °C for 30 sec, 68 °C for 1 min 10 sec, then 68°C for 5 min. PCR products were loaded on 1% Agarose gel. Gel electrophoresis was carried out in 1× TBE stock buffer at 120V for 30 min. The Gel Doc™ XR+ imaging system (BIO-RAD) was used for gel visualization.

5.4.3 Phenotypic screening of mutant lines

For ET (ACC), JA and SA phenotypic assays, seeds were sterilized using chlorine gas by mixing 100:3 (v/v) Sodium hypochlorite (125 g/L) and Hydrochloric acid (HCl, 38 % w/v). Seeds were then suspended with 0.1% agarose and sown on control Murashige and Skoog (MS, PhytoTech LABS M524) media pH 5.7, containing 1% sucrose and 0.9% agar or the same MS media with 10 µM ACC (Sigma: A-3903, dissolved in double distilled water) or 20 µM Me-JA (Sigma: 392707, Methyl jasmonate, dissolved in 0.1 % (v/v) ethanol) or 20 µM SA (Sigma: S7401, dissolved in 0.05 % (w/v) ethanol). After stratification for 3 days in the dark at 4°C, seeds were given 2 hours light at 21°C to stimulate germination. Seeds were then grown in the dark at 21°C for 4 days before plates were scanned. A Canon 9000F MarkII flatbed scanner was used for scanning all the experimental plates. The ACC assay of *mpk3-1* and *mpk3-2* was performed in two individual experiments (experiment 1 and 2 respectively) because root and hypocotyl length of *mpk3-2* was too short for precise measurement in experiment 1. Similarly, the ACC assay of *mpk6-1* and *mpk6-2* was performed in two individual experiments (experiment 1 and 2 respectively).

5.4.4 Primary root and hypocotyl length measurement

Image analysis of scanned plates was performed using the SmartRoot plugin in ImageJ to assess seedling hypocotyl length and root length (Lobet et al., 2011; Schneider et al., 2012). Statistical analyses were performed with R (version 3.5.3) using analysis of variance (ANOVA), followed by Tukey's pairwise multiple comparison of means (Team, 2020). Statistical analyses for

hormone/control ratio were performed using Student's Test. Differences were considered significant at $p < 0.05$. The significant code is: 0 '***' 0.001 '**' 0.01 '*' 0.05.

5.5 Supplementary Table legends

Supplementary tables can be found at: https://figshare.com/s/8a653ce1f0e581e61b7f

Supplementary Table 1. The details of T-DNA insertion mutant lines and primer sequences used in genotyping PCR.

Supplementary Table 2. Measurement data and statistics of the phenotypic screening for *mpk3-1* and *mpk3-2* mutant seedlings grown on plates containing ACC compared with seedlings grown on non-hormone control plates.

Supplementary Table 3. Measurement data and statistics of the phenotypic screening for *mpk3-1* and *mpk3-2* mutant seedlings grown on plates containing hormone JA compared with seedlings grown on non-hormone control plates.

Supplementary Table 4. Measurement data and statistics of the phenotypic screening for *mpk3-1* and *mpk3-2* mutant seedlings grown on plates containing hormone SA compared with seedlings grown on non-hormone control plates.

Supplementary Table 5. Measurement data and statistics of the phenotypic screening for *mpk6-1* and *mpk6-2* mutant seedlings grown on plates containing ACC compared with seedlings grown on non-hormone control plates.

Supplementary Table 6. Measurement data and statistics of the phenotypic screening for *mpk6-1* and *mpk6-2* mutant seedlings grown on plates containing hormone JA compared with seedlings grown on non-hormone control plates.

Supplementary Table 7. Measurement data and statistics of the phenotypic screening for *mpk6-1* and *mpk6-2* mutant seedlings grown on plates containing hormone SA compared with seedlings grown on non-hormone control plates.

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