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Functional analysis of proteasome-associated ubiquitin ligases in plants

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Abstract

Degradation of intracellular proteins by the ubiquitin-proteasome system (UPS) is a sophisticated mechanism that begins with anchoring ubiquitin molecules to a substrate and ends with proteasome-dependent proteolysis. Initiation of ubiquitination by E3 ligases is a key step in this pathway that selectively labels unstable or damaged proteins. The ubiquitinated substrate is then recognised by proteasome-associated ubiquitin receptors and subsequently degraded by the proteasome. Recent studies have identified several E3 ligases that surprisingly associate with the proteasome as accessory proteins. As substrates are already modified by ubiquitin when they arrive at the proteasome, it is unclear what the role of these proteasome-associated ligases are. In this study, the role of proteasome-associated ubiquitin ligases in proteasomal substrate degradation was characterised and the functional significance of ubiquitin chain remodelling at the proteasome were explored *in planta*.

In *Arabidopsis thaliana*, HECT-type Ubiquitin Protein Ligases (UPLs) have been identified as proteasome-associated ubiquitin ligases that are required for salicylic acid (SA)-induced plant immunity. Accordingly, the mechanism behind regulation of plant immune response by UPLs is further studied in Chapter 3. Here, it is shown that UPLs control SA-dependent transcriptional reprogramming via regulating homeostasis of the SA-responsive coactivator NPR1. SA-induced accumulation of NPR1 was impaired in *upl* mutants, which resulted in diminished expression of immune genes. Additionally, proteasome-associated UPLs facilitated polyubiquitination of NPR1, and thereby promoted its proteasomal turnover. This process was indispensable for clearing inactive

NPR1 from chromatin. Thus, UPL-mediated remodelling of NPR1-attached ubiquitin chains at the proteasome is required for maximum transcriptional activity of NPR1.

In Chapter 4 I show that proteasome-associated UPLs also target other transcription activators, including the developmental and ethylene-responsive EIN3 activator. I demonstrate that by physically interacting with UPL3, the SCF^{EBF2} ubiquitin ligase complex directly escorted EIN3 to the proteasome. Subsequent 'eleventh-hour' ubiquitin chain remodelling by proteasome-associated UPL3/4 was required for processive degradation of EIN3 by the proteasome and was critical for removal of EIN3 from its target gene promoters.

Besides targeting substrates destined for the proteasome, I show in Chapter 5 that UPL3 and UPL4 are also involved in polyubiquitination of other E3 ligases. UPL3/4 catalysed ubiquitination of the immune-responsive U-box E3 ligase, PUB22, and controlled its proteasomal turnover. Mutation of *PUB22* and its homologues, *PUB23* and *PUB24,* supressed the disease susceptibility phenotype of the *upl3 upl4* mutant, indicating that UPL3/4 also regulate immunity via modulating homeostasis of PUB ligases.

Overall, my findings indicate that unstable hormone-responsive transcriptional activators are sequentially polyubiquitinated by relays of ubiquitin ligases in which HECT-type ligases prevent the stalling of proteasome-bound substrates. On the other hand, HECT-type ligases also target other E3 ligases for degradation, thereby indirectly influencing substrate levels of these E3 ligases. Thus, my findings demonstrate that proteasomes unexpectedly influence the ubiquitination and stability of both E3 ligases and their substrates to regulate transcriptional programmes in plants.

Lay summary

The ubiquitin-proteasome system (UPS) is the major proteolysis machinery in eukaryotic cells that controls homeostasis of 80-90% of cellular proteins, including tumour suppressors, cell-cycle regulators, signalling receptors, and transcription factors. Cells utilise the UPS to fine-tune multiple intracellular signalling networks and respond to various environmental cues. The activity of UPS is hence vital for the correct functioning of the cell and dysregulation of the ubiquitin-proteasome system usually leads to abnormal accumulation of substrate proteins. Accordingly, the UPS is implicated in multiple diseases and developmental defects. In plants, the diversity of UPS components is very high compared to other organisms, suggesting extensive specialization of the UPS in regulating cell signalling, including various developmental and stress-induced processes. In this thesis, the mechanism of substrate turnover by the UPS machinery was studied. My results show that proteasome-associated ubiquitin ligases remodel substrate-attached ubiquitin chains at the proteasome, thereby promoting substrate degradation. This work unveils a key step of the ubiquitin modification process, and also provides fundamental clues for comprehending the proteasome-mediated degradation pathway in both crops and animals.

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Declaration

I declare the thesis presented here is my own work, and has not been submitted in any form for any degree or professional qualification at University of Edinburgh or any other institute.

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List of abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ARM	Armadillo
as-1	Activation Sequence 1
ATP	Adenosine triphosphate
BiFC	Bimolecular fluorescence complementation
BTB/POZ	Broad complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger
CAB1	Chlorophyll A/B Binding Protein 1
CDC20	Cell Division Cycle 20
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide
Co-IP	Co-immunoprecipitation
COI1	Coronatine Insensitive 1
CP	20S core protease
CTR1	Constitutive Triple Response 1
CUL3	Cullin3-based E3 ubiquitin ligase
DMSO	Dimethyl Sulphoxide
DTT	Di-Thio-Threitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EBF1	EIN3-Binding F box protein 1
EDTA	Ethylenediaminetetraacetic acid
EF-Tu	Elongation Factor-Tu
EGL3	Enhancer of GL 3
EIN2	Ethylene Insensitive 2

EIN3	Ethylene-Insensitive 3
ER	Endoplasmic reticulum
ERF1	Ethylene Response Factor 1
ET	Ethylene
ETI	Effector-triggered immunity
ETP1	EIN2-Targeting Protein 1
Exo70B2	Exocyst Subunit Exo70 Family Protein B2
FLS2	Flagellin-Sensing 2
GA	Gibberellin
GFP	Green fluorescent protein
GL3	Glabrous 3
GO	Gene ontology
GST	Glutathione S-transferase
HECT	Homologous to the E6AP carboxyl terminus
HR	Hypersensitive response
HRP	Horseradish Peroxidase
HUL5	HECT Ubiquitin Ligase 5
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	Jasmonic acid
LEC2	Leafy Cotyledon 2
MPK3	Mitogen-Activated Protein Kinase 3
MS	Murashige and Skoog
NEM	N-ethylmaleimide
NLR	Nucleotide binding–leucine-rich repeat
NPR1	Non-expresser of Pathogenesis-Related genes 1
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death

PCR	Polymerase Chain Reaction
PD	Pull-down
PMSF	Phenylmethylsulfonyl fluoride
PR	Pathogenesis-related
PRR	Plant pattern recognition receptor
Psm	*Pseudomonas syringae* pv. *maculicola*
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP-triggered immunity
PUB	Plant U-box Type E3 Ligase
qPCR	Quantitative real-time PCR
RING	Really interesting new gene
ROS	Reactive oxygen species
RP	19S regulatory particle
RPN10	Regulatory Particle Non-ATPase 10
S2	Proteasome Non-ATPase Regulatory Subunit 2
SA	Salicylic acid
SAR	Systemic acquired resistance
SCF	Skp1/Cullin/F-box ubiquitin ligase complex
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SID2	Salicylic Acid Induction Deficient 2
TA	Transcriptional activator
TGA	TGACGTCA cis-element-binding protein
TIR1	Transport Inhibitor Response 1
TLCK	Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride
TPCK	N-p-Tosyl-L-phenylalanine chloromethyl ketone
TPR	Tetratricopeptide

TUBE	Tandem-repeated ubiquitin-binding entities
Ub	Ubiquitin
UBE4	Ubiquitin conjugation factor E4
UBQ5	Ubiquitin 5
UFD2	Ubiquitin Fusion Degradation 2
UPL	Ubiquitin Protein Ligase
UPS	Ubiquitin-proteasome system
WRKY70	WRKY DNA-Binding Protein 70
WT	Wild-type
YFP	Yellow Fluorescent Protein

Chapter 1:

Introduction

1.1 The ubiquitin-proteasome proteolytic pathway

As a major proteolytic machinery in eukaryotic cells, the ubiquitin-proteasome system (UPS) is responsible for removing most of the short-lived or damaged proteins in a cell. This proteasome-mediated degradation pathway is important for maintaining cellular homeostasis, as well as triggering responses to developmental and environmental cues. Degradation of proteins via the UPS is accomplished in two steps: (1) modification of target proteins by ubiquitin molecules and (2) proteolysis of ubiquitin-tagged substrates by the proteasome. Substrates of the UPS include myriad of essential proteins that regulate various cellular processes, such as transcription, cell cycle, signal perception and transduction, and immune responses (Geng et al., 2012, Teixeira and Reed, 2013, Hu and Sun, 2016, Haglund and Dikic, 2005). Although UPS components and their biological functions were already discovered several decades ago (Ciehanover et al., 1978, Hershko et al., 1980, Ciechanover et al., 1982, Ciechanover et al., 1984), the mechanistic functioning of this catabolic pathway is still not fully understood. In plants, the UPS is more elaborate than in mammals, reflected in the larger number of UPS component-coding genes identified in plant genomes (Smalle and Vierstra, 2004), and the UPS has been characterized to be involved in many plant developmental aspects (Vierstra, 2009).

1.1.1 The ubiquitination enzyme cascade

Ubiquitin is a highly conserved protein in eukaryotic cells that consists of only 76 amino acids (Pickart and Eddins, 2004). In stepwise catalytic reactions, the C-terminus of ubiquitin forms isopeptide bond with lysine residues on the target protein, which is then targeted for proteasome-mediated proteolysis. The process of labelling target proteins

with ubiquitin is known as ubiquitination, which is mediated by three types of enzymes. Briefly, ubiquitination starts with adenylation of ubiquitin that is catalysed in an ATP-dependent manner by a ubiquitin activating enzyme (E1), which generates a thioester bond with a ubiquitin C-terminal carboxyl group. The activated ubiquitin is then transferred and conjugated to a cysteine residue on a ubiquitin conjugating enzyme (E2). Lastly, a ubiquitin protein ligase (E3) interacts with the E2 and mediates transfer of ubiquitin to a lysine acceptor group on the substrate (Figure 1.1). The lysine residues of target proteins can be modified by a single ubiquitin (monoubiquitination), or by a chain consisting of multiple covalently linked ubiquitins, which is referred to as polyubiquitination.

The UPS is an intricate pathway for protein removal, and its complexity appears to be especially high in plants. In *Arabidopsis thaliana*, for example, the proteins identified as UPS components account for more than 5% of the whole proteome, including over 1,300 identified ubiquitin ligases, revealing that turnover of unstable proteins by the UPS is essential for regulating plant cellular functions (Vierstra, 2009). Contributions of the UPS to plant development include embryogenesis, organ growth, hormone signalling, and abiotic responses (Vierstra, 2009). Among all these pathways, developmental and stress-induced phytohormone signalling are typically regulated by UPS. Targets of E3 ligases include not only hormone biosynthesis enzymes and receptors, but also many hormone-induced transcriptional regulators (Kelley and Estelle, 2012). Therefore, the proteasome-mediated proteolysis of hormone-related proteins, especially short-lived transcription factors, often leads to the fine-tuning of transcriptional events (Spoel et al., 2010).

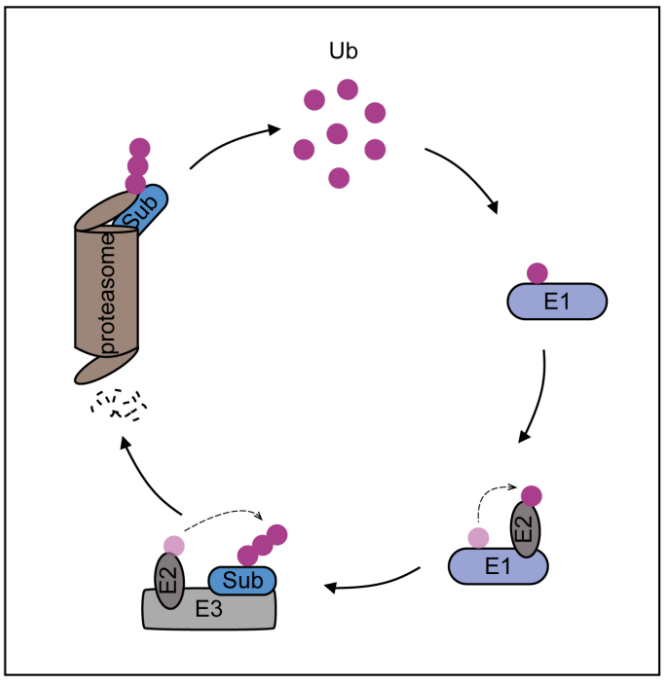

Figure 1. 1. The ubiquitin-proteasome system.

Ubiquitin (Ub) is activated by an E1 enzyme and then conjugated to an E2 enzyme. E3 ligases recognise the substrate and mediate transfer of ubiquitin from the E2 to the substrate. The ubiquitinated substrate is eventually detected and degraded by the proteasome.

1.1.2 Ubiquitin ligases

Unlike E1 and E2 enzymes, the E3 ligase family is highly diverse and hundreds of enzymes have evolved in eukaryotic cells. Selectively capturing a substrate largely relies on E3 ligases, which determine specificity of ubiquitination (Laney and Hochstrasser, 1999). Based on the structure of the core catalytic domain, ubiquitin ligases can be grouped into two classes: the RING (really interesting new gene) and U-box type, and the HECT (homologous to the E6AP carboxyl terminus) type ubiquitin ligases. The RING/U-box domain usually recruits ubiquitin-charged E2 to catalyse transfer of the active ubiquitin from the E2 directly onto the substrate (Deshaies and Joazeiro, 2009).

The one exception is a subgroup of ligases known as RBR (RING between RING) type ubiquitin ligases that contains two RING domains with a Cys/His region in between (Spratt et al., 2014). In contrast to conventional RING domains, the conserved cysteine residue in the second RING domain of RBR-type ligases receives ubiquitin from the E2 and then transfers it to the substrate. Likewise, ubiquitination mediated by HECT-type ubiquitin ligases also involves formation of a thioester-linked intermediate between the HECT domain and ubiquitin (Pickart, 2001). The N-terminus of each HECT-type ubiquitin ligase has a unique domain that is responsible for binding the substrate, whilst the HECT domain is normally located at the C-terminus (Rotin and Kumar, 2009). Structurally, the HECT domain is composed of a larger N-terminal lobe and a smaller C-terminal lobe. The N-terminal lobe is involved in binding of the E2 enzyme, while the active ubiquitin is transferred to the conserved catalytic cysteine residue in the C-terminal lobe, and is eventually attached to the substrate (Huang et al., 1999).

By cooperation between E1, E2 and E3 enzymes, only a limited number of ubiquitin molecules can be efficiently added as a chain onto a substrate. However, degradation of most proteins requires polyubiquitin chains that consist of 4 or more ubiquitins (Thrower et al., 2000). In recent years, it has become clear that elongation of longer ubiquitin chains often requires ubiquitin ligases that possess E4-like activity. The first known enzyme that catalyses long ubiquitin chain assembly, Ubiquitin Fusion Degradation 2 (UFD2), was identified in yeast (Koegl et al., 1999). Intriguingly, UFD2 was unable to substitute for E3 ligases to initiate assembly of the ubiquitin chain. Instead, UFD2 preferably bound to a ubiquitin chain on the substrate and elongated existing mono- or poly-ubiquitin chains (Koegl et al., 1999). UFD2-catalysed elongation of ubiquitin chains promotes delivery of substrates to the proteasome and stimulates proteasomal degradation of substrate

(Richly et al., 2005). The homolog of UFD2 in Arabidopsis, UBE4/MUSE3, was found to control the defence response against pathogens through regulating key plant immune regulators, including NLR (nucleotide binding–leucine-rich repeat)-type immune receptors and immune cofactors (Huang et al., 2014, Skelly et al., 2019). UBE4/MUSE3 directly binds to ubiquitinated substrates and catalyses their polyubiquitination, which is required for substrate turnover (Huang et al., 2014, Skelly et al., 2019). To date, several E4 ligases have been identified in different organisms with similar features, which indicates that this pathway is conserved across eukaryotes (Hoppe, 2005).

1.1.3 The 26S proteasome

Regulation of protein turnover by the UPS concludes with ATP-dependent proteolysis that is mediated by the proteasome. The 26S proteasome is a multiprotein complex that can be divided into two subcomplexes, i.e., the 20S core protease (CP) and the 19S regulatory particle (RP). The CP is composed of two outer rings which are each formed by seven related α subunits, and two inner rings that are each formed by seven related β subunits. The α-type rings mainly function as a physical barrel to govern the access of proteins into the CP and also provide a binding site for the RP (Groll et al., 2000). By contrast, the β-type rings, which contain three protease active sites, are responsible for cleavage of polypeptides (Groll et al., 1997). The RP consists of two subcomplexes, known as the Lid and the Base subcomplex. Subunits of the Base subcomplex include ubiquitin receptors that are involved in substrate recognition, whereas the Lid subcomplex is essential for removal of the ubiquitin chains from substrates (Husnjak et al., 2008, Elsasser et al., 2004, Verma et al., 2002). The unfolding of substrates into the CP and

the opening of the α ring gate are also processed with the assistance of the RP, followed by directing unfolded substrates into the CP for degradation (Finley, 2009).

Besides the conserved subunits that maintain the integrity of the proteasome, there are many proteasome-associated proteins that affect the proteolytic processivity of the proteasome. Among them, studies have identified the association of various types of ubiquitin ligase with the proteasome (Schmidt et al., 2005). Additionally, inhibiting the interaction between some E3 ligases and the proteasome has a limited effect on ubiquitination level of the substrate, however, it can prevent proteasomal degradation of the substrate (Xie and Varshavsky, 2002). This suggests that recruitment of ubiquitin ligases to the proteasome is somehow necessary for mediating substrate degradation.

Indeed, several studies have highlighted the significance of proteasome-associated ubiquitin ligases in promoting substrate degradation. One typical example is HUL5, which is a HECT-type E4-like ubiquitin ligase in yeast (Leggett et al., 2002). The association of HUL5 endows the proteasome with ubiquitin ligase activity, and allows remodelling of the ubiquitin chains on the substrate at the proteasome (Crosas et al., 2006). Moreover, proteasomes from the *hul5* mutant displayed reduced substrate degradation efficiency or partial substrate degradation, suggesting that HUL5-mediated ubiquitin chain remodelling is required for processive degradation of substrates (Crosas et al., 2006, Aviram and Kornitzer, 2010). Intriguingly, there are 7 HECT-type ubiquitin ligases were identified in Arabidopsis (Figure 1.2), known as Ubiquitin Protein Ligase (UPL), UPL3 has also been found to associate with the proteasome, and presence of UPL3 enhances proteasomal ubiquitin ligases activity (Furniss et al., 2018). It was shown that UPL3 is involved in various plant biological processes, including immune response and multiple developmental aspects (Miller et al., 2019, Downes et al., 2003, Bensussan et al., 2015).

In fact, loss of *UPL3* function remarkably suppressed global cellular ubiquitination level, indicating that UPL3 might control polyubiquitination of numerous substrates by remodelling anchored ubiquitin chains at the proteasome (Furniss et al., 2018).

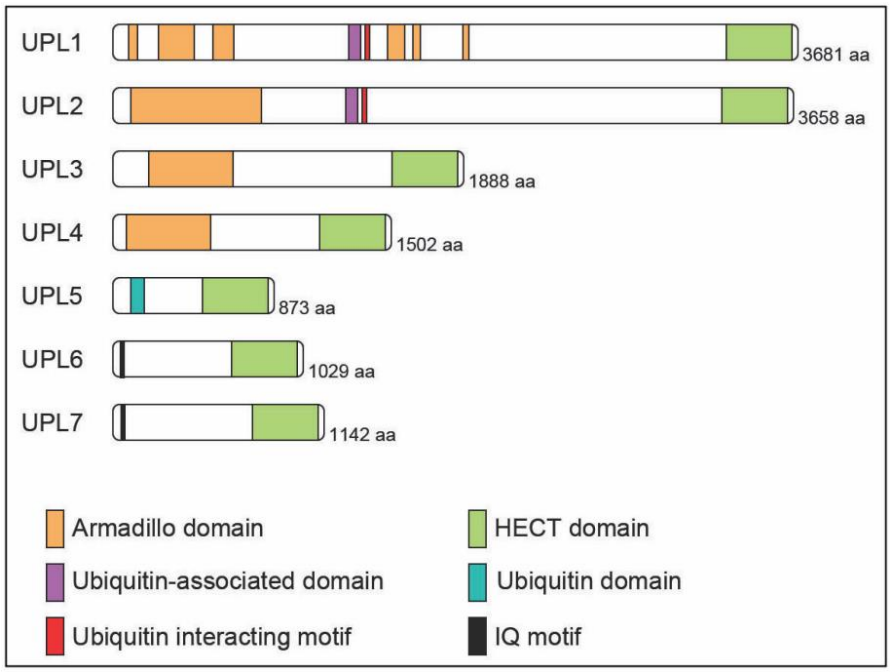

Figure 1. 2 Characteristic domains of UPL ubiquitin ligases.

UPL family proteins harbour a C-terminal HECT domain and a variable N-terminal region. HECT domain is conserved and responsible for substrate ubiquitination, whereas N-terminal regions might be involved in protein-protein interaction. Adapted from.

1.2 Regulation of SA/NPR1-mediated immunity by UPS

The UPS plays indispensable roles in many aspects of plant biology, including plant immune responses. Living in complex environments, plants are surrounded by a wide range of pathogenic organisms. Unlike mammals that have specialized immune cells, plants rely on the immune system of each individual cell to protect themselves from

pathogen threats. Salicylic acid (SA) is a plant defence hormone that activates immune responses to biotrophic pathogens. Accumulation of SA in plants launches defence responses or accelerates cell death in host tissues, thereby preventing the growth and spread of pathogens (Coll et al., 2011). Along with local responses to pathogen infection, multiple mobile immune signals are induced and transported to uninfected tissues to activate systemic acquired resistance (SAR), which can protect plants from secondary infection via inducing transcriptional reprogramming throughout the whole plant (Spoel and Dong, 2012).

1.2.1 SA-mediated plant defence response

As an effective elicitor of plant immunity, SA accumulates in both pathogen-infected and uninfected leaf tissues, indicating SA potentially participates in SAR induction (Malamy et al., 1990, Metraux et al., 1990). The central role of SA as an endogenous signal molecule in SAR was further revealed by using tobacco plants expressing the bacterial SA-degrading salicylate hydroxylase (*NahG*) gene (Gaffney et al., 1993). *NahG* plants failed to accumulate SA upon pathogen inoculation, which blocked establishment of SAR. Moreover, exogenous application of SA is sufficient to enhance plant resistance to pathogens through the activation of numerous immune-responsive genes (Ward et al., 1991).

By screening Arabidopsis mutants that displayed defective phenotypes in SA accumulation or transduction, the key components of SA biosynthesis and signalling pathways were subsequently identified (Wildermuth et al., 2001, Nawrath and Metraux, 1999, Cao et al., 1994, Delaney et al., 1995, Yang et al., 1997, Glazebrook et al., 1996). Among them, Non-expresser of Pathogenesis-Related genes 1 (NPR1) was found as a

central regulator of SAR (Cao et al., 1994). Loss of *NPR1* function blocks plant sensitivity to SA and prevents SA-induced transcriptional reprogramming and associated disease resistance (Wang et al., 2006, Cao et al., 1994). Impaired accumulation of NPR1 in SA-deficient mutants suggests accumulation of SA is crucial for maintaining homeostasis of NPR1 protein (Fu et al., 2012). No DNA binding domain can be identified in the NPR1 polypeptide, suggesting that NPR1 does not directly bind to gene promoters. Indeed, to activate immune-related genes, NPR1 was found to associate with members of the TGA family of transcription factors via its tandemly repeated ankyrin motifs (Zhou et al., 2000, Zhang et al., 1999, Despres et al., 2000). Presence of SAR inducers enhances formation of the NPR1-TGA complex in the nucleus, and thereby promotes DNA binding activity of NPR1 (Fan and Dong, 2002, Subramaniam et al., 2001). Moreover, the core element of the N-terminal BTB/POZ (Broad complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) domain and oxidation of the C-terminal cysteine residues are also required for transcription cofactor activity of NPR1 (Rochon et al., 2006).

1.2.2 UPS dynamically regulates transcriptional activity of NPR1

NPR1 proteins normally form oligomers in the cytoplasm through intermolecular disulphide bonds between cysteine residues, which are located in the BTB/POZ domain and the region between BTB/POZ and ankyrin domains (Mou et al., 2003). In the absence of pathogens, conversion of NPR1 oligomers to monomers in the cytoplasm is maintained at a low rate (Mou et al., 2003, Tada et al., 2008). Any monomeric NPR1 that escapes oligomerisation is thought to interact with NPR4, an adaptor of the Cullin 3 (CUL3) ubiquitin ligase complex (Fu et al., 2012). To avoid constitutive activation of immune-related genes, CUL3 targets NPR1 for ubiquitination and thereby promotes its

proteasomal degradation (Spoel et al., 2009). Upon pathogen infection or presence of SAR inducers, changes in cellular redox status induce formation and translocation of NPR1 monomer into the nucleus where it mediates transcriptional reprogramming via association with transcription factors (Kinkema et al., 2000, Mou et al., 2003, Tada et al., 2008). In the SAR-induced cells, NPR3 might act as an adaptor of CUL3 ubiquitin ligase complex instead of NPR4, which is thought to promote cell survival in regions adjacent to tissues undergoing SA-induced programmed cell death (Spoel et al., 2009, Fu et al., 2012).

UPS-mediated turnover of NPR1 has been found to be essential for controlling NPR1-induced transcriptional reprogramming. The significance of UPS in regulating NPR1 transcriptional activity was firstly established by analysing a *cul3a cul3b* loss-of-function mutant (Spoel et al., 2009). Knockdown of *CUL3* genes led to reduced NPR1 ubiquitination and thus accumulation of NPR1 protein. Unexpectedly, however, SA-induced activation of NPR1 target genes was attenuated in the *cul3a cul3b* mutant, suggesting ubiquitination and proteasomal turnover of NPR1 are required for triggering SAR (Spoel et al., 2009, Skelly et al., 2019). Indeed, CUL3-catalysed formation of short ubiquitin chains on NPR1 enhances its transcriptional activity (Skelly et al., 2019). Subsequently, the E4-like ubiquitin ligase, UBE4, further elongates these ubiquitin chains. Interestingly, UBE4-mediated polyubiquitination of NPR1 with long ubiquitin chains suppresses downstream gene expression. These findings imply that the E3/E4 ligase cassette works sequentially to first maximize activity of NPR1, followed by its deactivation and clearance of inactive NPR1 from gene promoters to allow reinitiation of the transcriptional cycle (Skelly et al., 2019).

1.3 SCF E3 ligase complex regulates developmental signalling

Involvement of the UPS in plant immune responses has been widely characterised, and its substrates include many defence signalling components, such as immune receptors and transcriptional regulators (Craig et al., 2009). A previous study reported that the homeostasis of intracellular NLR immune receptors is controlled by the SCF (Skp1/Cullin/F-box) ubiquitin ligase complex, and this links the function of SCF complexes to plant immunity (Cheng et al., 2011). Additionally, the SCF complex is also an essential regulator of plant developmental signalling, and many studies highlight that perception and signal transduction of plant hormones are tightly regulated by the SCF ubiquitin ligase complex (Sparks et al., 2013).

1.3.1 Architecture of the SCF ubiquitin ligase complex

The SCF ubiquitin ligase complex was originally identified in yeast and human, and consists of three primary subunits: Skp1, Cullin, and Rbx1/Roc1 (Deshaies, 1999, Feldman et al., 1997). Yeast cells carrying mutations in any of these subunits display similar phenotypes, indicating these subunits are all indispensable components of SCF complex (Bai et al., 1996, Schwob et al., 1994). Functional conservation of these subunits among different organisms suggests that the SCF complex is highly conserved throughout eukaryotes (Lyapina et al., 1998, Seol et al., 1999). The crystal structure of the SCF complex shows that the major scaffold structure of the SCF complex is formed by Cullin (Zheng et al., 2002). The C-terminus of Cullin directly interacts with Rbx1/Roc1, a small RING finger protein, to form a catalytic core that recruits E2 enzymes. On the other hand, the N-terminus of Cullin binds to Skp1, which functions as a connector to F-box proteins, which are responsible for selectively recruiting SCF substrates. Sequences

of the C-terminal regions of F-box proteins are highly variable but harbour a protein-protein interaction domain that is involved in capturing specific substrates. The F-box motif at the N-terminus is directly linked to the Cullin-Rbx1/Roc1 complex via association with Skp1 (Deshaies, 1999). SCF-mediated ubiquitination starts with substrate recognition that is commonly triggered by substrate phosphorylation (Skaar et al., 2013). The F-box protein first binds to the target protein independently, and then recruits the substrate to the SCF core scaffold, which interacts with the E2 and mediates transfer of ubiquitin onto the substrate. As a selectivity determinant, diversity of F-box proteins allows assembly of SCF complexes with distinct substrate specificities (Skowyra et al., 1997). In eukaryotic cells, many F-box proteins have been characterised, revealing they contribute to a broad spectrum of cellular activities.

1.3.2 Regulation of developmental hormones by SCF complex

Plants utilize multiple signalling mechanisms to coordinate processes of growth and development. Among these developmental strategies, plant hormones, which are multifunctional organic molecules, play a central part in directing diverse growth aspects. To elicit rapid communication among different organs, plant hormones are produced within the cell and transported throughout the entire plant (Gray, 2004). Developmental hormones such as ethylene, auxin, cytokinin, and abscisic acid, regulate a myriad of processes, ranging from seed germination and cell differentiation, to fruit ripening and organ senescence (Gray, 2004). Homeostasis of hormone-induced transcriptional regulators is tightly manipulated by the UPS (Smalle and Vierstra, 2004). Strikingly, SCF ligases play a particularly important role in orchestrating hormone-induced transcriptional reprogramming. For example, assembly of SCF complexes in response to jasmonic acid

and auxin differs only in terms of the utilised F-box protein. Coronatine Insensitive 1 (COI1) acts as a substrate adaptor in the SCF complex in response to jasmonic acid, whereas F-box protein TIR1 directly targets Aux/IAA transcription factors for ubiquitination to facilitate auxin response (Gray et al., 2001, Xu et al., 2002). In these cases, hormones function as a molecular glue to bring together co-receptors consisting of the F-box and its substrate, a transcriptional repressor.

The central role of the SCF complex is also well-established in ethylene signalling. Ethylene is a gaseous hormone that is involved in several plant developmental processes. The first step in the ethylene pathway is signal perception by receptors, leading to activation of an ER-membrane-bound positive regulator, EIN2 (Ethylene Insensitive 2) (Alonso et al., 1999). In the absence of ethylene, CTR1 (Constitutive Triple Response 1) kinase directly phosphorylates EIN2 to prevent transmission of the ethylene signal to the nucleus. Phosphorylation of EIN2 then induces $SCF^{ETP1/2}$-mediated ubiquitination that promotes proteasomal destruction of EIN2 (Alonso et al., 1999, Ju et al., 2012, Qiao et al., 2009). Conversely, accumulation of ethylene negatively regulates protein levels of ETP1/2 to induce accumulation of EIN2 (Qiao et al., 2009). The SCF complex also controls ethylene transcriptional responses in the nucleus. As master regulators of ethylene signalling, ethylene-induced transcriptional reprogramming is mainly regulated by EIN3 (Ethylene-Insensitive 3) family transcription factors (Dolgikh et al., 2019). EIN3 is unstable in the absence of ethylene, however, treatment with a proteasome inhibitor stabilized EIN3, suggesting it is degraded through a proteasome-dependent pathway (Guo and Ecker, 2003). It was later discovered that proteolysis of EIN3 largely depends on the $SCF^{EBF1/2}$ complex, which mediates ubiquitination and promotes proteasomal degradation of EIN3 (Potuschak et al., 2003, Guo and Ecker, 2003). Loss-of-function of

both *EBF1* and *EBF2* led to accumulation of EIN3, and plants exhibited constitutive ethylene-responsive phenotypes. Intriguingly, presence of ethylene promotes proteasomal proteolysis of EBF1/2, thereby stabilizing EIN3 and inducing downstream responsive signalling (An et al., 2010). Therefore, based on these studies it is suggested that the SCF complex modulates ethylene signalling at multiple tiers.

1.4 PUB ligases regulate PTI

Upon pathogen infection, plant pattern recognition receptors (PRRs) can detect pathogen-associated molecular patterns (PAMPs) to activate pattern-triggered immunity (PTI). PTI prevents further pathogen colonization in host tissues by inducing numerous cellular events, including changes in ion fluxes, activation of defence genes, and enhanced callose deposition around sites of infection (Boller and Felix, 2009). To achieve a precise and rapid immune response, PTI is tightly controlled by multiple regulators. Among them, U-box type E3 ligases have an important role in pathogen perception and immune signal transduction (Trujillo, 2018)

1.4.1 U-box type E3 ligases

The signature domain of U-box type E3 ligase is the highly conserved U-box motif that consists of ~70 amino acids. The U-box domain was originally identified in the yeast ubiquitin ligase UFD2 that contributes to stress tolerance (Koegl et al., 1999). Characterization of UFD2 homologs and other U-box proteins in various organisms, including humans, *Caenorhabditis elegans*, and *Drosophila melanogaster*, indicates that this class of E3 ligases is prevalent in eukaryotic cells (Azevedo et al., 2001). In humans and yeast, only eight and two U-box proteins have been identified, respectively. By

contrast, the diversity and abundance of plant U-box type E3 ligases (PUB) has been found to be much greater.

A total of 64 genes are predicted to encode U-box proteins in the Arabidopsis genome, 77 genes are annotated in the rice genome, and 125 genes have been identified in the soybean genome (Wiborg et al., 2008, Zeng et al., 2008, Wang et al., 2016). Sequence analysis of PUBs shows that in addition to the U-box motif, other protein domains are present, such as armadillo (ARM) repeats, WD40 repeats, and tetratricopeptide (TPR) domains. Classification of PUBs is usually based on the presence of these domains (Azevedo et al., 2001). Many *PUB* genes exhibit high sequence similarity, which directly relates to their functional redundancy (Sharma and Taganna, 2020, Zeng et al., 2008). Overall, expansion in the number of U-box type E3 ligases implies their importance in governing various biological aspects in plants.

1.4.2 The role of PUBs in the plant immune system

Genetic analysis of PUBs has illustrated their diverse functions in response to environmental and developmental cues, such as coping with biotic and abiotic stresses, and regulation of hormone signalling (Yee and Goring, 2009). The first predicted PUB protein, BnARC1, was identified in *Brassica napus* and is a positive regulator of self-incompatibility (Stone et al., 1999). BnARC1 was further characterized to possess U-box-dependent ubiquitin ligase activity, and BnARC1-mediated ubiquitination is required for the self-incompatibility response (Stone et al., 2003). The homolog of BnARC1 in Arabidopsis is AtPUB17. Although gene expression of *AtPUB17* can be detected in floral organs (Tung et al., 2005), no evidence has shown that it is involved in reproductive development. Instead, knock-out of AtPUB17 in Arabidopsis results in suppression of

pathogen effector-triggered immunity (ETI) (Yang et al., 2006). Moreover, the close homolog of AtPUB17 in *Nicotiana tabacum*, ACRE276, also functions as a positive regulator of ETI (Yang et al., 2006).

In addition to contributing to regulation of ETI, PUB E3 ligases have also been implicated in regulation of PTI. Flagellin-Sensing 2 (FLS2) is a plasma membrane-localized receptor in Arabidopsis that recognises the N-terminus of bacterial flagellin. In response to pathogen infection, FLS2 forms a receptor complex at the plasma membrane and induces immune signal transduction that leads to a burst of reactive oxygen species (ROS) and activation of defence genes (Henry et al., 2013). In turn, homeostasis of FLS2 is tightly controlled by proteasome-mediated proteolysis that provides negative feedback regulation of PTI via rapid reduction of the level of pathogen-activated FLS2 (Lu et al., 2011). Ubiquitination of FLS2 is directly catalysed by the E3 ligases PUB12/13, which are recruited to the activated FLS2 in the presence of flagellin. Loss of PUB12/13 activity induces accumulation of FLS2 and enhances the immune responses (Lu et al., 2011). Interestingly, cell death and SA accumulation are also negatively regulated by PUB13, indicating it has multiple functions in the plant immune system (Li et al., 2012). Taken together, PUBs play a key role in guarding pathogen perception and in triggering plant defence signalling.

1.5 Aims and objectives

UPS-mediated destruction of substrates has been established in eukaryotes as a crucial regulatory pathway to control protein homeostasis. With sequential reaction steps that involve E1, E2, and E3 enzymes, the target protein is labelled with ubiquitin chains and recognised by the proteasome for degradation. It remains unclear, however, how

ubiquitinated proteins are processed upon arrival at the proteasome. The recruitment of HECT-type ubiquitin ligases to the proteasome hints at the possibility that these proteasome-associated ubiquitin ligases might contribute to substrate degradation. Therefore, the aim of this study is to explore the mechanism of how proteasome-associated ligases are involved in substrate degradation, and what the functional significance of this process is *in planta*.

In Arabidopsis, members of the family of HECT-type ubiquitin ligases, known as UPLs, were found to be necessary for inducing SAR. Chapter 3 hence builds on this and aims to investigate how UPLs regulate SA-dependent immune responses. UPLs have been found to regulate the stability of several proteins (Patra et al., 2013, Miao and Zentgraf, 2010). Therefore, a potential substrate of UPLs that controls the SA signalling pathway is examined, and it is revealed how this contributes to plant immune response. In addition, the proteasome has been found to interact with various E3 ligases (Schmidt et al., 2005), however, the role of these E3 ligases at the proteasome is still unclear. We previously found that multiple E3 ligases interact with UPL3 in yeast two-hybrid assay. Therefore, Chapter 4 aims to test if E3 ligases are recruited to proteasomes via interaction with proteasome-associated UPL3 and how this affects substrate ubiquitination and turnover by the proteasome.

Because proteasome-associated UPL3 interacts with other E3 ligases, it is plausible that it does not only target their substrates, but that it also directly ubiquitinates these E3 ligases to control their activity. As UPL3 plays an essential positive role in plant immunity and interacted in yeast two-hybrid assays with specific PUBs that inhibit immunity (Furniss et al., 2018, Trujillo et al., 2008), Chapter 5 explores if UPL3 regulates immune signalling by modulating the proteolysis of PUB E3 ligases.

Overall, this thesis sheds new light on the role of the proteasome-associated HECT-type ubiquitin ligases in modifying the ubiquitination of substrates upon arrival at the proteasome and reveals this is necessary for their processing by the proteasome.

Chapter 2:

Materials and methods

2.1 Plant materials and growth conditions

Arabidopsis plant materials are all in the Col-0 ecotype. The *upl1*, *upl3*, *upl4*, *upl5*, *npr1-0* plants are SALK T-DNA insertion lines (Table 2.1). The *npr1-1*, *ein3-1*, *ebf1 ebf2*, *35S:YFP-UPL3* (*upl3*), *pub22/23/24*, *pUBQ10:GFP-PUB22* plants have been previously described (Furniss et al., 2018, Potuschak et al., 2003, Cao et al., 1994). The *pEIN3:EIN3-eGFP-3xFlag* construct was generated by the Genschik laboratory using the pART27 vector backbone (Gleave, 1992). Briefly, the 35S promoter of the expression vector pPILY was replaced with the genomic sequence of EIN3 using XhoI-NcoI restriction digest (Ferrando et al., 2000). After insertion of eGFP and Flag coding sequences, the fragment of EIN3 genomic fused with eGFP-3xFlag was inserted into binary vector pART27 using NotI restriction digest.

Table 2. 1 T-DNA insertion SALK lines.

Locus	Allele	SALK/SAIL line
AT1G55860	*upl1-1*	SALK_063972
AT4G38600	*upl3-4*	SALK_035524
AT5G02880	*upl4-2*	SALK_040984
AT4G12570	*upl5-1*	SALK_116446
AT1G64280	*npr1-0*	SALK_204100

Arabidopsis seeds were stratified for 2 days at 4°C. For experiments on adult plants, seeds were sown on soil and placed in a growth chamber that was maintained at 21°C and 100 µmol.m^{-2}.s^{-1} light on a 16-h-light/8-h-dark photoperiod at 65% day humidity and 55% night humidity. For experiments on seedlings, seeds were sterilized in 100% ethanol for 5 min with rotation followed by incubation with 10% bleach for 5 min with rotation. Seeds were washed 3 times with sterilized ddH_2O and then plated on Murashige and Skoog agar medium. Plates were incubated in the same conditions as mentioned above.

2.2 Generation of plasmid constructs and plant transformation

The N-terminal region of UPL1 (amino acids 1-2,130) and full length of UPL5 were cloned into pCR™8/GW/TOPO (Invitrogen) according to the manufacturer's instructions, and then recombined into pEarleyGate 103 and pEarleyGate 104, respectively, by using Gateway™ LR Clonase™ II Enzyme (Invitrogen). HECT domains of UPL1 (amino acids 3238-3681), UPL3 (amino acids 1403-1888), UPL5 (amino acids 444-873) were cloned into pENTR/D-TOPO (Invitrogen), and then recombined into pEarleyGate 104 by LR reaction. The cysteine residue in HECT domains of UPL1 (amino acid 3648), UPL3 (amino acid 1855), UPL5 (amino acid 839) were mutated into serine residues by using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). To generate the *pCAB1:NPR1-GFP* construct, the coding sequence of *NPR1* fused with *GFP* was cloned into pENTR/D-TOPO (Kinkema et al., 2000), and the entry clones were recombined into pCAB1:GW binary vector by LR reaction (Michniewicz et al., 2015).

Binary vectors were transferred into *Agrobacterium tumefaciens* strain GV3101 (pMP90) using a freeze-thaw method. Arabidopsis was transformed by using the floral dip method (Zhang et al., 2006) and transgenic plants were selected by spraying with 120 μg/ml glufosinate ammonium.

2.3 DNA extraction and genotyping

Plant tissues were homogenized in 200 μl of DNA extraction buffer (20 mM Tris-HCl [pH 7.5], 25 mM NaCl, 2.5 mM EDTA, and 0.05% SDS), and 0.5 μl of extract was used for PCR. For screening T-DNA knock-out lines, gene-specific primers that flank the T-DNA insertion site and a T-DNA left border primer (LBb1.3) were used for PCR reactions. For screening *npr1-1* and *ein3-1* mutant alleles, primers that flank mutation sites were used

for PCR reactions. PCR products of *NPR1* and *EIN3* were cut with restriction enzymes NlaIII and BstEII, respectively. Primers are listed in Table 2.2.

Table 2. 2 Primers used in genotyping *npr1-1* and *ein3-1* mutations.

Target allele	Primer sequence 5'-3'
upl1-1	F: GTATTCATGGTCGTACGCGAC R: GATAGTGCAAGTGCTTGAGCC
upl3-4	F: TCGGGCATAGTTTCCTACTTG R: CTTGTCTTTGAATGCTTTGGC
upl4-2	F: CAGGCTGATTGACGAGAAAAC R: AGTACTTGGACGTTGCTGAGC
upl5-1	F: TGATCGGTCCAATTTGCTATC R: ACTTGCTCCAAGATTGGTGTG
npr1-1	F: TGTCTCGAATGTACATAAGGCA R: GAGTGCGGTTCTACCTTCCA
ein3-1	F: ATGAATTGGAGAGGAGGAT R: TTGGCTTTCTCTTTCTCA

2.4 RNA extraction

Plant tissue was ground in liquid nitrogen, and mixed with warm 0.5 ml RNA extraction buffer (100 mM Tris [pH 8.0], 100 mM LiCl, 10 mM EDTA, 1% SDS). Then, 0.5 ml of phenol:chloroform:isoamylalcohol (25:24:1) was added and the mixture was vortexed thoroughly before being centrifuged at 13,000 rpm for 5 min at 4°C. The aqueous phase was transferred to a new tube, and mixed well with 0.5 ml of cold chloroform:isoamylalcohol (24:1). The mixture was centrifuged at 13,000 rpm for 5 min at 4°C, and the aqueous phase extracted again with cold chloroform:isoamylalcohol (24:1). The aqueous phase was then transferred to a new tube containing 1/3 volume of 8M LiCl, and incubated at 4°C for 1 h. Next, the solution was centrifuged at 13,000 rpm for 20 min at 4°C, followed by washing with cold 70% ethanol. The pellet was resuspended in 400 µl ddH_2O and incubated with 40 µl of 5 M NaAc (pH 5.2) and 1 ml

cold 96% ethanol overnight at -20°C. The next day the mixture was centrifuged at 13,000 rpm for 20 min at 4°C to pellet RNA, and the pellet washed with cold 70% ethanol. RNA was resuspended in ddH_2O, and the concentration was measured by using a Nanodrop spectrophotometer (Thermo Scientific).

2.5 cDNA synthesis

cDNA synthesis was performed by using SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The synthesized cDNA was diluted 20 times by ddH_2O, and stored at -20°C for further experiments.

2.6 Quantitative real-time PCR

Quantitative RT-PCR was performed by using Power SYBR™ Green PCR Master Mix (Applied Biosystems). Each reaction contained 2 µl of cDNA and gene-specific primers (Table 2.3), and the reaction was run on a StepOne Plus Real Time PCR system according to the manufacturer's instructions (Applied Biosystems).

Table 2. 3 Primers used for qPCR.

Name	Primer sequence 5'-3'	Source
PR1	F: CTAAGGGTTCACAACCAGGC R: AAGGCCCACCAGAGTGTATG	(Skelly et al., 2019)
PR2	F: CAGATTCCGGTACATCAACG R: AGTGGTGGTGTCAGTGGCTA	(Skelly et al., 2019)
NPR1	F: CTAAAACCGTGGAACTCGGG R: TCTCTTGTATTTCCATGTACCTTTGCT	(Skelly et al., 2019)
UBQ5	F: CCAAGCCGAAGAAGATCAAG R: ACTCCTTCCTCAAACGCTGA	
UPL3	F: CGTCTTCTTGTGCTGCTGTT R: GAGCACAGCCATAAGAGCAC	

UPL4	F: TCGCCCGTTCTTGTCCTATT R: GTTCATGCCCTCCAGACTCT	
ERF1	F: GAGGAAACACTCGATGAGACG R: GGAGCGGTGATCAAAGTCAC	(Yoo et al., 2008)
ERF2	F: CGAGCCAACTGAGAACTTTA R: CAAACCCTAGCTCCATTCTT	
ERF11	F: GTACTTTCGACACTCCTGAAG R: CAGATCCGAGGTTGAGATTAG	
ELF4a	F: TCATAGATCTGGTCCTTGAAAC R: GGCAGTCTCTTCGTGCTGAC	(Yoo et al., 2008)
EIN3	F: GGATAAGGGTAAAGAAGGTGTT R: GACCATTACGATCAAACCTAAC	
GFP	F: AAGCTGACCCTGAAGTTCATCTGC R: CTTGTAGTTGCCGTCGTCCTTGAA	
ACTIN	F: TCCTGATGGGCAAGTGATTAC R: TTGTATGTGGTCTCGTGGATTC	(Liu et al., 2012)

2.7 RNA-seq analysis

RNA extraction was performed as described in section **2.4**. RNA samples were submitted to Eurofins GATC Biotech (Constance, Germany) or Beijing Genomics Institute (Hong Kong, China) for RNA sequencing and bioinformatic analysis. Strand NGS software in RNA Seq workflow was used to quantify transcripts. Raw counts were normalised using DESeq with baseline transformation to the median of all samples. Data were then expressed as normalised signal values (i.e., log2[RPKM] where RPKM is read count per kilobase of exon model per million reads) for all statistical tests and plotting. RNA-Seq data have been deposited in Array Express at EMBL-EBI under accession codes E-MTAB-10963 and E-MTAB-10964.

2.8 Protein extraction

Frozen plant tissue was ground in protein extraction buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.2% Nonidet P-40, and protease inhibitor cocktail (50 μg/ml N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 50 μg/ml Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 0.6 mM phenylmethylsulfonyl fluoride (PMSF)) unless otherwise stated. Protein extracts were incubated with 1 × SDS sample buffer supplemented with 50 mM DTT at 80°C for 10 min, and then separated by SDS-PAGE. For detection of endogenous EIN3, frozen plant tissue was ground to a fine powder and homogenised in protein extraction buffer containing 62.5 mM Tris-Cl (pH 6.8), 3% SDS, 10% glycerol, 0.1% bromophenol blue, protease inhibitors cocktail (50 μg/ml TPCK, 50 μg/ml TLCK, 0.6 mM PMSF), and 3% 2-mercaptoethanol (Sigma-Aldrich), and the mixture was incubated at 95°C for 5 min. Endogenous EIN3 was detected by using an EIN3 antibody which has been described previously (Michaeli et al., 2019).

2.9 Cell-free protein synthesis

In vitro protein synthesis was performed according to the published protocol for wheat germ extracts (Nomoto and Tada, 2018). Primers are listed in Table 2.4, and the synthesized proteins were tested by running on an SDS-PAGE gel.

Table 2. 4 Primers used for in vitro protein synthesis.

Name	Primer sequence 5'-3'
FLAG-EBF2	F: CCAGCAGGGAGGTACTATGTCTGGAATCTTCAGA R: CCTTATGGCCGGATCCAAGAGCTCTTTTTTTTTTTTAGTAGAGTATATC GCA
PUB22-FLAG	F: CACAAAACATTTCCCTACATACAACTTTCAACTTCCTATTATGGATCAA GAGATAGAG

	R: AGTACCTCCCTGCTGGAGACCAGCAGGATACGAATC
YFP-PUB22	F: CACAAAACATTTCCCTACATACAACTTTCAACTTCCTATTATGGGCAAG GGCGAGGAG R: AGTACCTCCCTGCTGGAGACCAGCAGGATACGAATC

2.10 Recombinant protein purification

Ubiquitinated proteins were purified with previously described GST-TUBE (Skelly et al., 2019). GST-TUBE was expressed from the pGEX-6P-1 vector in BL21 (DE3) *E. coli* cells. Bacterial cultures were grown at 37°C overnight in Luria-Bertani (LB) broth medium. The following day cultures were diluted to an OD600 of 0.2 and grown at 37°C until OD600 reached 0.6 when 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for a further 4 h at 28°C. Cells were collected by centrifugation and extracted in a buffer containing 1X Bugbuster (Novagen), 1 X PBS, 1 mg/ml lysozyme, 25 U/ml Benzonase nuclease, 0.1% Triton-X-100 and a protease inhibitors cocktail (50 µg/ml TPCK, 50 µg/ml TLCK, 0.6 mM PMSF). Protein extracts were incubated at room temperature for 20 min with rotation and then centrifuged at 13,000 rpm for 20 min at 4°C. Supernatant that contained GST-TUBE proteins was purified on Glutathione Agarose 4B resin (GE Healthcare) by gravity flow. Columns were washed with 10 ml 1 X PBS supplemented with protease inhibitor cocktail, and proteins were eluted in a buffer containing 50 mM Tris-HCl (pH 8) and 10 mM reduced glutathione. Purified GST-TUBE was dialysed against 1X PBS using Slide-A-Lyzer dialysis cassettes (Thermo Scientific).

To generate recombinant His-TUBE proteins, the coding sequence of hHR23A was cloned into a pET-28a vector. The overnight bacterial cultures were diluted to OD600 of 0.2, and grown at 37°C until OD600 reached 0.6. IPTG was added to a final concentration

of 1 mM and the culture was incubated for a further 4 h at 37°C. Proteins were extracted in buffer containing 1X Bugbuster (Novagen), 50 mM potassium phosphate (pH 7.4), 300 mM NaCl, 10 mM imidazole, 1 µl/ml benzonase nuclease, 10 mM β-mercaptoethanol and a protease inhibitor cocktail. Protein extracts were incubated at room temperature for 20 min with rotation and then centrifuged at 13,000 rpm for 20 min at 4°C. Protein extracts were purified on HisPur™ Cobalt Resin (Thermo Scientific) by gravity flow. Columns were washed with wash buffer (50 mM potassium phosphate [pH 7.4], 300 mM NaCl, 10 mM imidazole, protease inhibitor cocktail (50 µg/ml TPCK, 50 µg/ml TLCK, 0.6 mM PMSF)), and proteins were eluted in a buffer containing 50 mM potassium phosphate (pH 7.4), 300 mM NaCl, and 500 mM imidazole. Purified His-TUBE was dialysed against 50 mM potassium phosphate (pH 7.4) using Slide-A-Lyzer dialysis cassettes (Thermo Scientific).

2.11 Transient protein expression in *Nicotiana benthamiana* leaves

Agrobacterium tumifaciens carrying indicated constructs were collected from the overnight cultures and resuspended in infiltration buffer containing 10 mM $MgCl_2$ and 10 µl/l 6-benzyladenine to OD_{600} = 0.5. *Nicotiana benthamiana* leaves were infiltrated with this Agrobacterium suspension and harvested after 3 days of infiltration. Proteins were extracted in buffer containing 125 mM Tris-HCl (pH 7.7), 0.25 mM EDTA, 2.5 mM $MgCl_2$, 5% glycerol, 5 mM ATP, and protease inhibitors.

2.12 TUBE purification of polyubiquitinated proteins

To assess polyubiquitination of NPR1-GFP, Arabidopsis plant tissues was ground in liquid nitrogen, and homogenised in extraction buffer (1x PBS, 1% Triton X-100, 10 mM NEM, 40 µM MG132, protease inhibitor cocktail, and 0.2 mg/ml GST-TUBE). Protein

extracts were centrifuged at 13,000 rpm for 20 min at 4°C and supernatant was incubated with Glutathione Agarose 4B resin overnight at 4°C with rotation. The agarose beads were washed 5 times with wash buffer (1X PBS, 1% Triton X-100, protease inhibitors cocktail (50 µg/ml TPCK, 50 µg/ml TLCK, 0.6 mM PMSF)) before boiling at 80°C for 10 min in 1 X SDS sample buffer containing 50 mM DTT.

To assess polyubiquitination of EIN3 and total cellular ubiquitination levels, extraction buffer contained 50 mM potassium phosphate (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10 mM NEM, 40 µM MG132, 1 X phosphatase inhibitor cocktail 3 (Sigma), protease inhibitor cocktail, and 0.2 mg/ml His-TUBE. Protein extracts were incubated with HisPur™ Cobalt Resin overnight at 4°C. Beads were washed 5 times with buffer containing 50 mM potassium phosphate (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10 mM imidazole, 40 µM MG132, and protease inhibitor cocktail (50 µg/ml TPCK, 50 µg/ml TLCK, 0.6 mM PMSF).

2.13 Protein-protein interaction assays

GFP-tagged proteins were purified by using GFP-Trap Agarose beads (ChromoTek) according to the manufacturer's instruction. For *in vivo* co-immunoprecipitation assay, agarose beads were boiled at 80°C for 10 min in 1 X SDS sample buffer containing 50 mM DTT. Proteins were run on an SDS-PAGE gel and interactors were detected by using an antibody as stated in the figure legends. For *in vitro* protein-protein interaction assays, proteins produced by cell-free protein synthesis system as described in section **2.9** were diluted in 500 µl of wash buffer (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, protease inhibitors cocktail) and incubated with GFP-Trap purified proteins for 1 h at 4°C.

Agarose beads were washed 3 times with wash buffer, and boiled at 80°C for 10 min in 1 X SDS sample buffer containing 50 mM DTT.

2.14 Proteasomal ubiquitin ligase activity assay

Four-week-old Arabidopsis plants were sprayed with H_2O or 0.5 mM SA, and plant tissues were collected 24 h after the treatment. Plant tissues were ground in liquid nitrogen, and proteins were extracted in 2 volumes of proteasome extraction buffer (125 mM Tris-HCl [pH7.7], 0.25 mM EDTA, 2.5 mM $MgCl_2$, 5% glycerol, 5 mM ATP, and protease inhibitor cocktail). Protein extracts were centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatant was incubated with anti-proteasome S2 antibody (Abcam, ab98865 at ratio 1:500) at 4°C overnight. The next day, protein extracts were centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was incubated with Protein A-agarose (Millipore) for 1 h at 4°C with rotation. Agarose beads were washed 3 times with proteasome extraction buffer, and incubated in 80 µl reaction buffer (125 mM Tris-HCl [pH7.7], 0.25 mM EDTA, 2.5 mM $MgCl_2$, 5 mM ATP, 1 mM DTT, 10 µM NSC632836 deubiquitinase inhibitor) supplemented with 0.2 µg human recombinant E1 enzyme (BioVision), 0.2 µg recombinant E2 enzyme UbcH5c (Ubiquigent), and 10 µg recombinant human Flag-ubiquitin (Boston Biochem) for 18 h at 30°C with shaking at 1,200 rpm. The reaction was terminated by incubation in 1 X SDS sample buffer supplemented with 50 mM DTT for 15 min at 70°C.

2.15 Measurement of protein degradation

For measuring stability of NPR1-GFP, 2-week-old seedlings were submerged in a solution containing 100 µM CHX (Sigma-Aldrich), and samples collected at indicated time

points. For testing stability of EIN3, 10-day-old seedlings were pre-treated with 50 μM ACC (1-aminocyclopropane-1-carboxylic acid) (Sigma-Aldrich) for 3 h. Seedlings were then transferred into MS liquid media containing 100 μM CHX and 100 μM $AgNO_3$ (Sigma-Aldrich), and samples collected at indicated time points.

For testing stability of PUB22-YFP, plant tissues from WT and *upl3 upl4* were ground in liquid nitrogen and protein extracted in modified proteasome reaction buffer (125 mM Tris-HCl [pH7.7], 0.25 mM EDTA, 2.5 mM $MgCl_2$, 5% glycerol, 5 mM ATP, 1 mM DTT, and protease inhibitors cocktail) before centrifuging at 13,000 rpm for 20 min at 4°C. Protein extracts were then incubated with PUB22-YFP that was synthesized using the above described cell-free protein synthesis system at 30°C, and samples collected at indicated time points for analyses by western blotting.

2.16 SA content measurement

SA content was determined according to previously described method with specified modifications (Matsuura et al., 2019). In brief, fresh leaves were ground in liquid nitrogen and 0.1 g of sample was suspended in 4 ml of extraction buffer (1% (v/v) acetic acid in acetonitrile/water (4:1)) with stable isotope-labelled internal standards. Suspended samples were extracted, centrifuged and concentrated as described before. Samples were purified by solid phase extraction using WAX cartridges (Waters Corp., Milford, MA, USA) to obtain SA fractions. SA was eluted in subsequent step from Oasis WAX cartridge by 3% (v/v) formic acid in acetonitrile. Following evaporation of each fraction, samples were analysed on Agilent 1260–6410 Triple Quad LC/MS system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a Capcell Pak ADME-HR S2 column (Osaka Soda Co. Ltd., Osaka, Japan).

2.17 ChIP-qPCR

Chromatin immunoprecipitation was performed as described but with minor modifications (Gendrel et al., 2005). A total of 500 mg tissue was crosslinked with 1% formaldehyde by vacuum infiltration for 15 mins at room temperature. Glycine was added to a final concentration of 125 mM and vacuum infiltrated for a further 5 mins to quench crosslinking. Crosslinked tissue was washed three times with ice-cold PBS before tissue was frozen in liquid nitrogen. Nuclei were isolated and lysed as described (Gendrel et al., 2005), while sonication was performed using a BioRuptor Plus (Diagenode) for 10 cycles of 30 s ON, 30 s OFF at high power. NPR1-GFP and YFP-UPL3 were immunoprecipitated using anti-GFP (Abcam, ab290), EIN3 using anti-EIN3 (Potuschak et al., 2003), and the proteasome using anti-RPN10 (Abcam). Enrichment at promoter binding sites was analysed by qPCR using primers listed in Table 2.5.

Table 2. 5 List of ChIP-qPCR primers.

Locus	Primer sequence 5'-3'	Source
PR1 promoter *as1* element	F: AGTGTATACAATGTCAATCGGTGATCTT R: GCCGCCACATCTATGACGTA	Skelly et al., 2019
ERF1 promoter	F: GGGGGCATGTATCTTGAATC R: TGCTGGATCAACTCAACAAAA	Chang et al., 2013

2.18 Pathogen infection

For detecting endogenous NPR1, *Psm* ES4326 was grown overnight in LB media supplemented with 10 mM $MgCl_2$ and 50 mg/ml streptomycin. The next day, the bacterial culture was diluted in LB media to an OD600 of 0.2 and grown further until OD600 reached 0.6. Cells were collected by centrifugation and diluted in 10 mM $MgCl_2$ to OD600 0.002. Plants were infiltrated with a syringe through the abaxial leaf surface. After 48

hours tissue was collected and protein extracted as described above. Proteins were then analysed by western blotting and endogenous NPR1 was detected by using an anti-NPR1 antibody (Agrisera).

For *Pst* DC3000 inoculation, bacteria were grown overnight in LB media supplemented with 50 mg/ml rifampicin overnight. The next day the overnight culture was diluted to an OD600 of 0.2, and grown until OD600 reached 0.6. Cells were collected by centrifugation, washed with ddH_2O, and resuspended in ddH_2O containing 0.04% Silwet L-77 to OD600 of 1. Four-week-old plants were sprayed with this bacterial solution and inoculated leaves collected 4 days later. Leaves were washed in 70% ethanol for 30 s, and washed again in H_2O for 30 s before being ground in 10 mM $MgCl_2$. The solution was plated onto LB agar plates and incubated at 30°C for 2 days.

2.19 ROS measurement

Leaf discs of 4-week-old plants floated on water in a 96-well plate overnight. The next day, elicitation solution containing 5 µg/ml Horseradish Peroxidase (HRP) and 0.2 µM Luminol with or without 1 µM flg22, was added to each well. Luminescence was measured every minute by using an Infinite M200 PRO Plate Reader (Tecan) with signal integration time of 0.3 s.

2.20 ACC treatment and plant phenotyping

For analysing gene expression and EIN3 protein levels in response to ACC, 10-day-old seedlings were floated on MS liquid medium supplemented with or without 50 µM ACC for 4h. RNA and protein extraction were performed as described in section **2.4** and **2.8**, respectively. For analysing ACC-responsive phenotypes, seeds were germinated on MS

agar medium supplemented with or without 10 µM ACC and left in dark for 4 days. Hypocotyl and root lengths were measured by using ImageJ.

2.21 Root growth assays

Seeds were germinated on MS agar medium and grown for 4 days. Seedlings were then transplanted onto MS agar medium supplemented with or without 1 µM flg22. Length of the main root was measured 6 days after transfer by using ImageJ software.

Chapter 3:

Proteasome-associated ubiquitin ligases control activity and stability of the transcription coactivator NPR1

3.1 Introduction

Plants utilize a sophisticated defence system to recognise and respond to pathogens. When encountering biotic stress, plants dynamically change the homeostasis of various hormones, thereby triggering plant immunity (Spoel and Dong, 2008). One of these hormones is SA, which is synthesized and accumulated in response to biotrophic and hemibiotrophic pathogen infections, followed by boosting SA-responsive cellular events (Spoel et al., 2003, Spoel et al., 2007). Besides fine-tuning immunity at the infected site, SA is also fundamental for the induction of SAR in uninfected tissues to protect plants from future pathogen attack (Spoel and Dong, 2012). Induction of SAR relies on accumulation of SA, which triggers transcriptional reprogramming to prioritise immune responses over normal cellular functions (Gaffney et al., 1993, Lawton et al., 1995, Durrant and Dong, 2004). The crucial link between SA accumulation and activation of immune-related genes is a transcription cofactor known as Nonexpresser of Pathogenesis-Related (PR) Genes 1 (NPR1). Genome-wide gene expression analysis revealed that transcriptional reprogramming by SA or its functional analogues predominantly depends on NPR1 (Wang et al., 2006, Skelly et al., 2019).

In resting conditions, NPR1 is maintained in an inactive state in the cytoplasm as a disulphide-linked oligomer (Mou et al., 2003). SAR induction triggers changes in the cellular redox homeostasis that result in oligomer-to-monomer conversion of NPR1 (Tada et al., 2008, Mou et al., 2003). Coupling between phosphorylation and SUMOylation further promotes the translocation of monomeric NPR1 from the cytoplasm into the nucleus for regulation of SA-dependent genes (Lee et al., 2015, Saleh et al., 2015, Kinkema et al., 2000). To activate immune-responsive genes in the nucleus, SA-induced NPR1 forms a complex with members of the TGA family of transcription factors and

enhances their DNA binding (Zhou et al., 2000, Johnson et al., 2003, Despres et al., 2000). Interestingly, the transcriptional activity of nucleus-localized NPR1 is coordinated by multiple post-translational modifications, including phosphorylation, SUMOylation, and ubiquitination (Withers and Dong, 2016, Saleh et al., 2015, Spoel et al., 2009). Among these modifications, ubiquitination regulates transcriptional activity of NPR1 and subsequently promotes its proteasomal turnover, allowing import of new NPR1 to reinitiate transcription (Spoel et al., 2009). Blocking the ubiquitination of NPR1 impairs SA-induced immunity, suggesting that ubiquitin-mediated proteasomal turnover is crucial for SAR activation (Spoel et al., 2009). In fact, formation and elongation of ubiquitin chains on NPR1 catalysed by an E3 and E4 ligase relay are associated with distinct functions (Skelly et al., 2019). Initially, NPR1 activity is enhanced by modification with short ubiquitin chains that are added by a Cullin3 (CUL3)-based E3 ubiquitin ligase, whereas further ubiquitin chain extension mediated by the E4 enzyme, UBE4 (Ubiquitin conjugation factor E4), inactivates NPR1 and ultimately leads to its proteasomal degradation (Skelly et al., 2019). This ubiquitin chain elongation on NPR1 can be reversed by the proteasome-associated deubiquitinases, UBP6 and UBP7, which rescue NPR1 from degradation and maintain its active state (Skelly et al., 2019). Accordingly, regulation of the ubiquitination state of NPR1 is essential for establishing SAR in *Arabidopsis thaliana*.

While the role of E3/E4-catalysed ubiquitination of NPR1 has been revealed, it is still unclear how ubiquitinated NPR1 is processed upon arrival at the proteasome. Generally, proteolysis of proteins by the proteasome is an intricate process that begins with recognition of a ubiquitin chain by proteasome-associated ubiquitin receptors (Husnjak et al., 2008, Deveraux et al., 1994, Verma et al., 2004, Paraskevopoulos et al.,

2014). Once the substrate is recognised, an additional level of regulatory substrate ubiquitination may be carried out at the proteasome by proteasome-associated ubiquitin ligases. Assembly of long ubiquitin chains on the substrate may ensure continuous high affinity for the proteasome and promotes substrate degradation (Thrower et al., 2000, Schmidt et al., 2005, Crosas et al., 2006). Intriguingly, ubiquitin chain remodelling at the proteasome largely relies on HECT type ubiquitin ligases (Schmidt et al., 2005). In Arabidopsis, members of UPLs (Ubiquitin Protein Ligase) family contain a conserved HECT domain in the C-terminal region, and UPLs are involved in SA-mediated immune responses (Furniss et al., 2018).

So what is the functional role of HECT-type ubiquitin ligases in the regulation of SA signalling? Previous results have shown that UPL3 is a proteasome-associated ubiquitin ligase and its activity regulates total cellular ubiquitination levels in response to SA, suggesting UPL3 is an essential regulator of SA-induced proteasome substrates (Furniss et al., 2018). Indeed, in the absence of functional *UPL1*, *UPL3*, or *UPL5*, Arabidopsis plants display impaired SA responses, including reduced induction of immune-related genes and abolished SA-induced resistance to pathogens (Furniss et al., 2018). Here we show that *upl* mutants are compromised in accumulation of endogenous NPR1 in response to SA and pathogens, which is likely due to decreased cellular SA levels. In addition, UPLs remodelled ubiquitin chains on NPR1 at the proteasome, which was essential for proteasomal degradation of NPR1. In particular UPL3, promoted the clearance of inactive NPR1 from SA-responsive promoters, providing the last activity checkpoint for this key immune transcriptional coactivator. Taken together, our results indicate multi-layered regulatory roles for UPLs in orchestrating SA-responsive transcriptional reprogramming and immunity.

3.2 Results

3.2.1 UPLs regulate SA-dependent total cellular ubiquitination

Ubiquitin Protein Ligase (UPL) family proteins have been identified in Arabidopsis that harbour a HECT domain at the C-terminal region (Furniss et al., 2018). HECT-type E3 ligases catalyse substrate ubiquitination in a two-step reaction, where the first step corresponds to transfer of the ubiquitin in a trans-thiolation reaction from the E2 to the conserved catalytic cysteine residue of the HECT domain. The second step involves transfer of the ubiquitin to the lysine residue of the substrate (Huang et al., 1999, Rotin and Kumar, 2009). To investigate if the HECT domains of UPL1 (amino acids 3238-3681), UPL3 (amino acids 1403-1888), and UPL5 (amino acids 444-873) possess catalytic activity, expression constructs containing *Yellow Fluorescent Protein* (*YFP*) fused with individual HECT domain coding sequences were expressed in *Nicotiana benthamiana*. When supplied with E1, E2, FLAG-ubiquitin, and ATP, the purified recombinant YFP-HECT proteins were able to build high mass ubiquitin conjugates *in vitro*. By contrast, omission of either E2 or YFP-HECT was largely insufficient to stimulate ubiquitin conjugate formation (Figure 3.1A). Compared with the intact HECT domains, mutation of the active-site cysteine residues (UPL1 amino acid 3648, UPL3 amino acid 1855, and UPL5 amino acid 839), which form a catalytic thioester intermediate with ubiquitin (Huang et al., 1999), dramatically reduced the catalytic activity of HECT domains (Figure 3.1A). Note that mutant HECT domains retained some ability to generate a small amount of ubiquitin conjugates as they can mediate the inefficient direct transfer of ubiquitin from the E2 to a substrate. Together these data indicate that UPL proteins have functional HECT domains with catalytic activity for assembling ubiquitin conjugates.

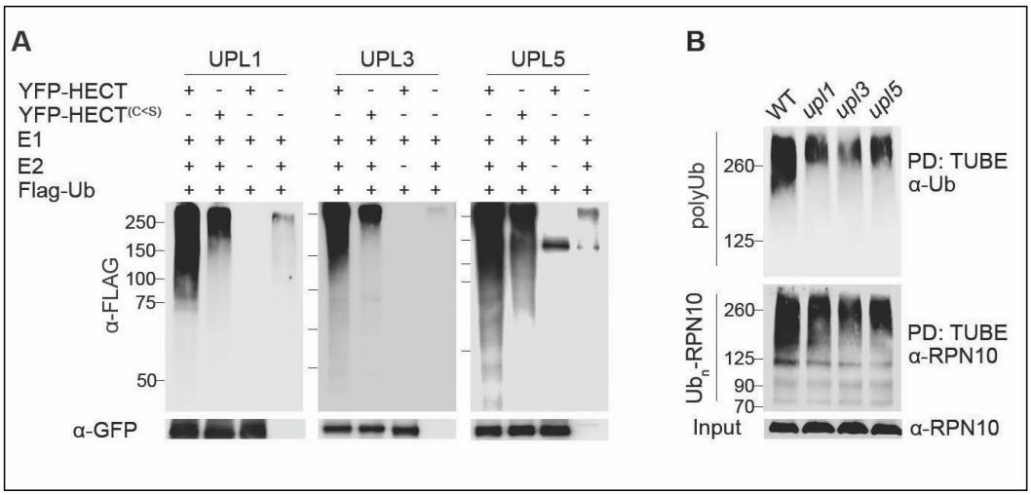

Figure 3. 1. UPLs are active HECT-type ligases that contribute to global cellular levels of ubiquitin conjugates.

(A) *35S:YFP-HECT* constructs were transiently expressed in tobacco leaves. Buffer-infiltrated leaves were used as a negative control. Purified proteins were incubated with E1 enzyme, E2 enzyme, FLAG-ubiquitin (FLAG-Ub) and ATP. Free ubiquitin conjugates that were formed by HECT domains were detected by western blotting using a FLAG antibody. Expression of YFP-HECT proteins was detected using a GFP antibody.

(B) Seedlings were treated with 0.5 mM SA and 0.1 mM MG132 for 6 h. Ubiquitinated substrates were pulled down using His-TUBE, and analysed by immunoblotting with a ubiquitin antibody. Polyubiquitinated and input RPN10 were detected using an antibody against RPN10.

It was previously reported that proteasome-associated UPL3 contributes to global cellular ubiquitination (Furniss et al., 2018). Therefore, we examined whether UPL1 and UPL5 also regulate total ubiquitination levels in response to SA *in vivo*. To this end, seedlings of wild-type plants (WT) and the *upl* mutants were treated with SA in the presence of the proteasome inhibitor MG132 to prevent degradation of polyubiquitinated substrates. Compared to WT, the SA-induced global cellular ubiquitination level was

strikingly lower in *upl1*, *upl3*, and *upl5* mutants, suggesting that UPLs broadly catalyse the polyubiquitination of myriad proteins in an immune context (Figure 3.1B). The role of UPLs in modulating cellular ubiquitin conjugate levels was further confirmed by assessing polyubiquitination of the universal E3 ligase substrate RPN10 (Uchiki et al., 2009) (Figure 3.1B).

The presence of UPLs in immunoprecipitated proteasome complexes suggests that UPLs might directly associate with the proteasome (Ustun et al., 2016). Indeed, we have previously reported that UPL3 associates with the proteasome via its N-terminal domain (Furniss et al., 2018). To test if UPL1 and UPL5 also associate with the proteasome, we generated transgenic plants that constitutively express *UPL1^{ARM}-GFP* or *YFP-UPL5*. Both the UPL1^{ARM}-GFP and YFP-UPL5 proteins could specifically pull down the proteasome *in vivo*, indicating that these UPLs also associate with the proteasome (Figure 3.2A). We then assessed if association with UPLs endows the proteasome with ubiquitin ligase activity. To this end, proteasomes isolated from *upl* mutants were used for *in vitro* ubiquitination assays. Consistent with a previous report (Furniss et al., 2018), we observed that compared to WT, proteasomal E3 ligase activity was dramatically reduced in the *upl3* mutant (Figure 3.2B). By contrast, proteasomes from the *upl1* and *upl5* mutants exhibited similar chain-elongating activity as proteasomes from WT plants (Figure 3.2B). This suggests that UPL3 is the predominant HECT ligase responsible for proteasome-associated E3 ligase activity.

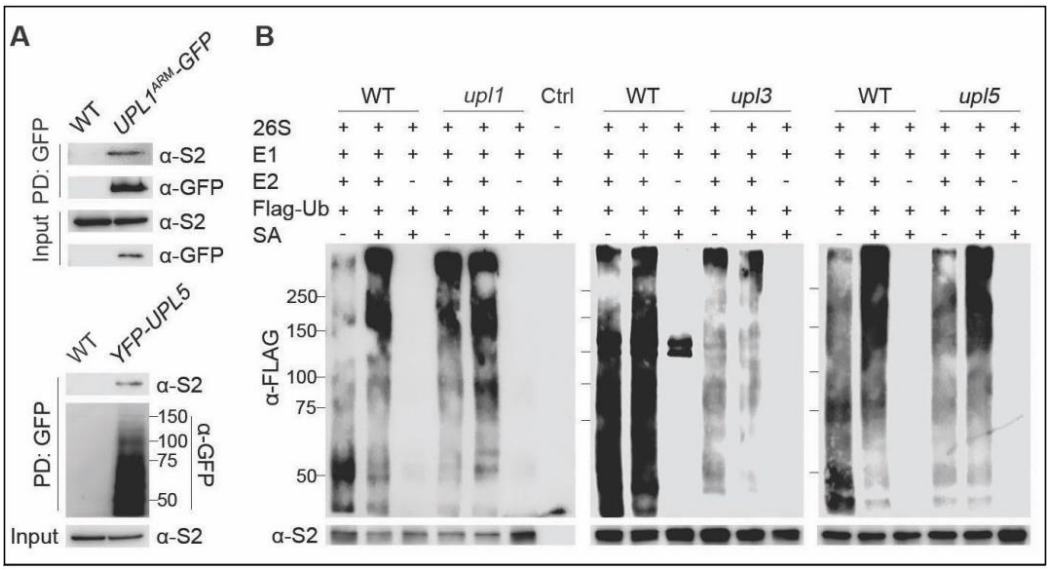

Figure 3. 2. UPL3 confers proteasome-associated ubiquitin ligase activity.

(A) UPL1 and UPL5 physically interact with the proteasome *in planta*. UPL1^{ARM}-GFP and YFP-UPL5 proteins were immunoprecipitated from seedlings expressing *35S:UPL1^{ARM} -GFP* and *35S:YFP-UPL5*, respectively, while WT plants were used as a negative control. Immunoprecipitated proteins and imputs were analysed by western blotting using antibodies against S2 and GFP.

(B) Adult plants were treated with (+) or without (-) 0.5 mM SA for 24 h. Proteasomes were then immunoprecipitated with an anti-S2 antibody, while mock immunoprecipitations without antibody were performed as a control (Ctrl). Purified proteins were incubated with E1 enzyme, E2 enzyme, FLAG-ubiquitin (FLAG-Ub) and ATP. FLAG-ubiquitin conjugates were detected by western blotting using anti-FLAG and the proteasomes were detected by using an anti-S2 antibody.

3.2.2 UPLs regulate immune responses by amplifying SA-dependent transcriptional reprograming

Ubiquitin signalling plays key roles in SA-responsive gene expression (Furniss and Spoel, 2015, Skelly et al., 2019). Given that SA-induced cellular ubiquitin conjugate levels are regulated by UPLs, we considered that each of them may target a wide range of substrates and that this might result in genome-wide transcriptional changes in response to SA. Indeed, we previously reported that the *upl3* mutant attenuated SA-induced transcriptional reprogramming, indicating that UPL3 acts as a genome-wide amplifier of SA-responsive transcriptional reprogramming (Furniss et al., 2018). To test if UPL1 and UPL5 also affect activation of immune-related genes, we investigated expression of the SA-dependent immune marker genes *PR1* and *PR2* in corresponding *upl* mutants. Upon SA treatment, *PR1* and *PR2* were markedly induced in WT plants, whereas mutation of the SA-responsive coactivator *NPR1* completely abolished their expression (Figure 3.3). Interestingly, SA-induced transcriptional activation of *PR1* and *PR2* was significantly reduced in all *upl* mutants tested, indicating that UPL1, UPL3 and UPL5 regulate SA-responsive gene expression (Figure 3.3).

To further explore the role of UPL1 and UPL5 in context of the entire SA-responsive transcriptome, we performed RNA-Seq on SA-treated WT and mutant *upl* plants. In response to SA, a total of 1,265 genes showed significant differences of at least 1.5-fold (p = 0.05) in WT, of which 589 were upregulated and 676 were downregulated (Figure 3.4A and B). In contrast, SA treatment had only a limited effect on the transcriptome of *npr1-1* mutants (Figure 3.4A), verifying the central role of NPR1 in regulating SA-dependent transcriptomic changes. Compared to WT, *upl* mutants displayed much less dramatic changes in SA-induced expression of many genes (Figure 3.4A and B),

indicating that UPLs are essential regulators of SA-mediated transcriptional reprogramming. Although some SA-responsive genes were specifically regulated by either UPL1 or UPL5, most genes were regulated by both (Figure 3.4C). Thus, the functions of UPL1 and UPL5 in facilitating the SA signalling pathway partially overlap.

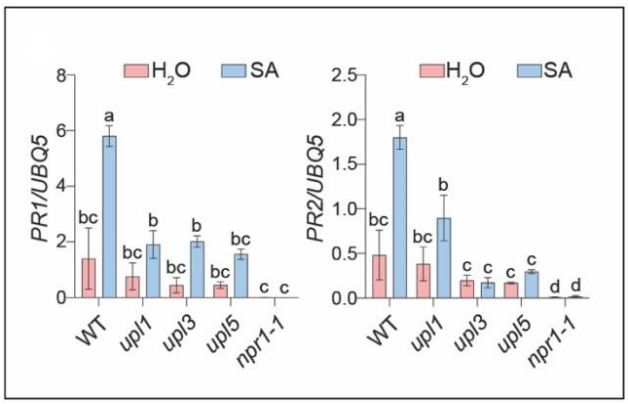

Figure 3. 3. Mutant *upl* plants exhibit reduced *PR* gene expression.

Adult plants were treated with 0.5 mM SA or H_2O for 24 h. Expression of *PR* genes was normalised to constitutively expressed *UBQ5*. Data represent mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; $\alpha = 0.05$, $n = 3$).

3.2.3 UPLs control NPR1-dependent transcriptional reprogramming

The expression of most SA-responsive genes is dependent on the activity of the master transcription coactivator NPR1 (Skelly et al., 2019) (Figure 3.4A and C). To further investigate the potential association between activities of UPLs and NPR1, we compared the expression of SA-responsive genes in the *upl* and *npr1* mutants. We found that among the 1,265 genes that were differentially expressed in WT plants in response to SA, about 75% were regulated by NPR1. Moreover, most of the SA-responsive genes that were regulated by UPL1 and UPL5 were also dependent on NPR1 (Figure 3.4C).

Since the majority of the UPL-regulated SA-responsive genes are NPR1-dependent, we investigated if UPLs regulate the NPR1 protein. First, we assessed accumulation of NPR1 protein in *upl* mutants in response to the bacterial pathogen *Pseudomonas syringae pv. maculicola* (*Psm*) ES4326, which induces strong SA signalling. Although the transcript levels of NPR1 displayed no significant differences between genotypes, pathogen-induced accumulation of NPR1 was compromised in the *upl1*, *upl3*, and *upl5* mutants (Figure 3.5A and B). Because NPR1 protein homeostasis is maintained by SA (Fu et al., 2012), we examined if pathogen-induced accumulation of SA is regulated by UPLs. Compared to WT plants, infection induced lower levels of SA in *upl1* and *upl5* mutants (Figure 3.5C) Moreover, accumulation of SA in the *upl3* single mutant was comparable to WT, whilst the level was significantly lower in the *upl3 upl4* double mutant, suggesting redundant roles of UPL3 and UPL4 in controlling SA production (Figure 3.5C). To further examine if low SA levels were responsible for reduced accumulation of NPR1, the *upl* mutants were treated with exogenous SA. Interestingly, SA treatment only restored some NPR1 protein accumulation (Figure 3.5D), suggesting the reduced accumulation of NPR1 in *upl* mutants was only partially due to

the defect in SA synthesis. Because NPR1 mRNA levels were comparable between WT and *upl* mutants (Figure 3.5A), it is likely that UPLs also affect translation of NPR1 mRNA.

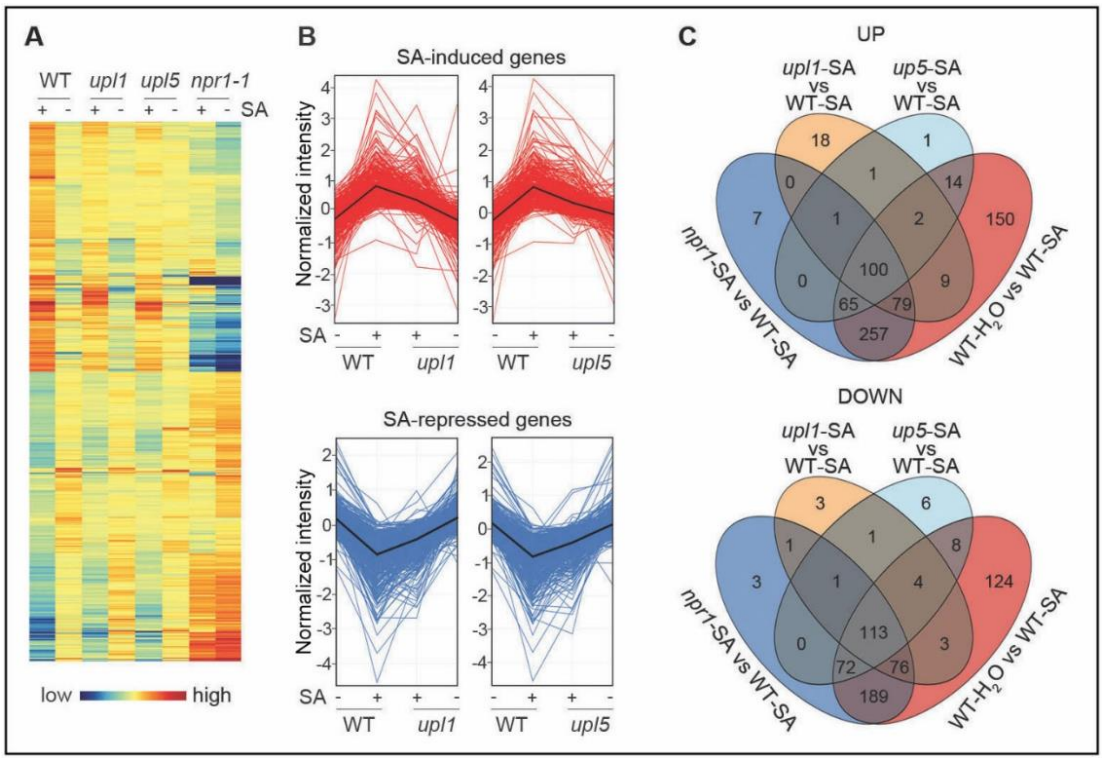

Figure 3. 4. Mutant *upl* plants are impaired in SA-induced transcriptional reprogramming. Adult plants were treated with 0.5 mM SA or H_2O for 24 h, mRNA was extracted and analysed by RNA-Seq. Genes with fold change of ≥ 1.5 (Benjamini Hochberg FDR, 2-way ANOVA $p \leq 0.05$, n = 3) in WT plants in response to SA are shown in a heat map (A) and profile plots (B). Black lines in (B) indicate the median expression level of all genes shown. Venn diagrams (C) illustrated the overlap between UPL1- and UPL5-regulated genes and NPR1-regulated genes.

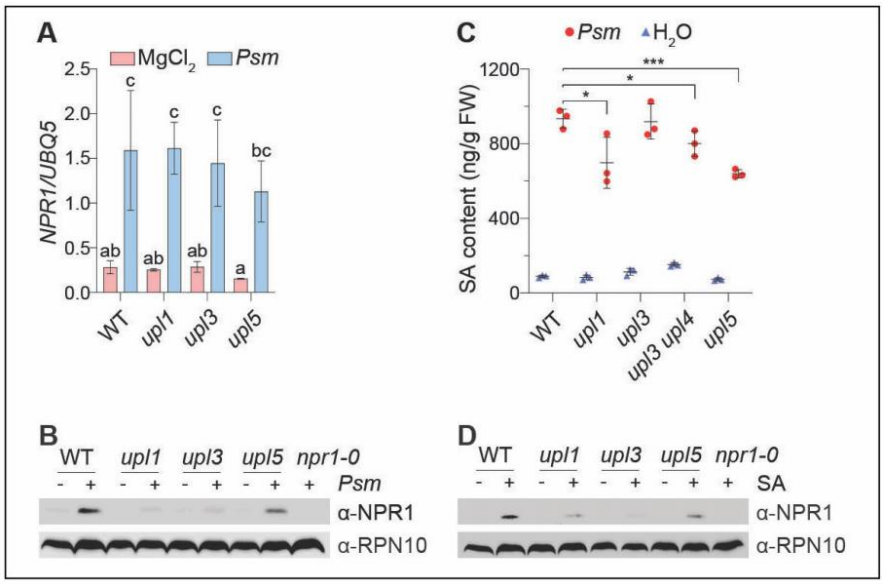

Figure 3. 5. Accumulation of endogenous NPR1 protein is controlled by UPLs.

(A-C) Plant leaves were inoculated with 10^6 cfu/ml *Psm* ES4326 or 10 mM $MgCl_2$, and leaves were collected at 48 h after infection. (A) Expression of *NPR1* genes was normalised to constitutively expressed *UBQ5*. Data represent the mean ± SD, lowercase letters (Tukey HSD ANOVA test; α = 0.05, n = 3) indicate statistically significant differences between samples. (B) Pathogen-induced accumulation of endogenous NPR1 protein was detected by immunoblot analysis, protein level of RPN10 was used as a loading control. (C) Pathogen-induced SA levels in different genotypes are shown, asterisk (two-tailed t test, *p ≤ 0.05, ***p ≤ 0.001, n = 3) indicate statistically significant differences between samples. (D) Adult plants were treated with 0.5 mM SA or H_2O for 24 h. NPR1 and RPN10 were detected as in (B).

3.2.4 UPLs control ubiquitin chain remodelling and stability of NPR1

To examine if UPLs have post-translational effects on NPR1 protein levels, we generated Arabidopsis transgenic plants carrying an *NPR1-GFP* construct without 5' or 3' untranslated regions (UTRs) driven by the constitutive *Chlorophyll A/B Binding Protein 1* (*CAB1*) promoter (Michniewicz et al., 2015). Seedlings with similar gene expression levels of the *NPR1-GFP* transgene were selected (Figure 3.6A). Accumulation of NPR1-GFP was remarkably higher in the *upl* mutants compared to plants carrying the wild-type *UPL* allele (Figure 3.6B). Although NPR1-GFP accumulated to higher levels in *upl* mutants, SA-induced expression of the NPR1 target gene *PR1* was comparable between WT, *upl1* and *upl5* genetic backgrounds (Figure 3.6C). Moreover, the *upl3* genetic background displayed significantly lower expression of *PR1* (Figure 3.6C). This indicates that the transcriptional activity of NPR1-GFP was impaired in all *upl* mutants and particularly *upl3*.

Because UPLs appear to affect NPR1 protein levels, we examined the ability of UPLs to physically bind to NPR1. While we were unable to express UPL1, both epitope-tagged UPL3 and UPL5 co-immunoprecipitated with NPR1-GFP, indicating that UPLs physically interact with NPR1 *in vivo* (Figure 3.7).

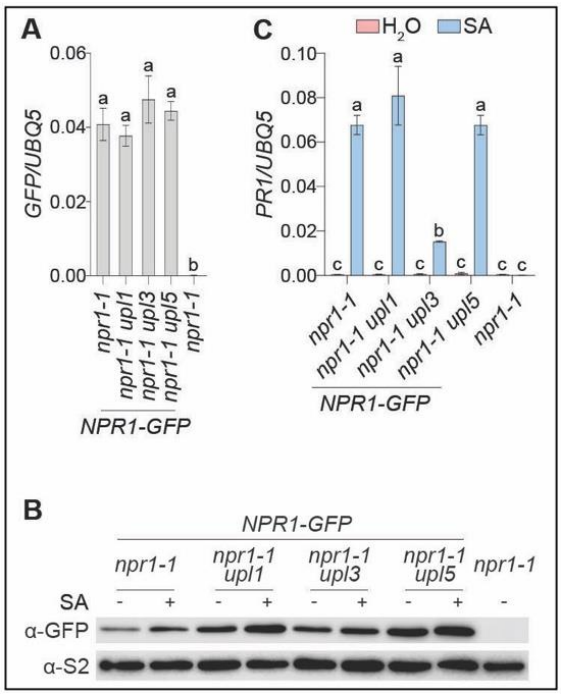

Figure 3. 6. Accumulation and transcriptional activity of NPR1 are control by UPLs.

(A) Expression of *NPR1-GFP* in the indicated genotypes was normalized to *UBQ5*. Data represent the mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 3).

(B) Seedlings were treated with 0.5 mM SA or H_2O for 6 h. The *npr1-1* mutant was used as a control. NPR1-GFP protein was detected by western blotting, protein level of S2 was used as a loading control.

(C) Seedlings constitutively expressing NPR1-GFP in the indicated genetic backgrounds were treated with 0.5 mM SA or H_2O for 6 h. *PR1* gene expression was normalised to *UBQ5*. Data represent the mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 3).

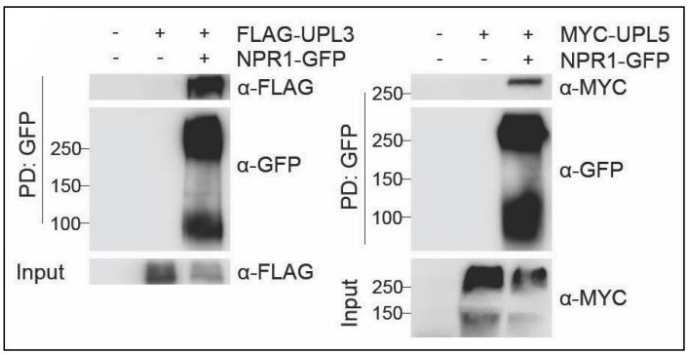

Figure 3. 7. UPLs physically interact with NPR1.

NPR1-GFP was transiently expressed with FLAG-UPL3 or MYC-UPL5 in *N. benthamiana*. Protein complexes were pulled down (PD) with GFP-Trap agarose and analysed by immunoblotting against GFP, FLAG and MYC.

Next, we explored whether UPL-mediated remodelling of ubiquitin chains affects stability of NPR1. When plants carrying WT *UPL* alleles were exposed to the protein synthesis inhibitor cycloheximide (CHX), NPR1-GFP was nearly completely degraded within 2 h (Figure 3.8A and B). By contrast, NPR1-GFP was much more stable in *upl* mutant genetic backgrounds (Figure 3.8A and B). Previous study has shown that the transcriptional activity of NPR1 is tightly governed by the length of NPR1-attached ubiquitin chains (Skelly et al., 2019). Formation of shorter ubiquitin chains on NPR1 promotes its transcriptional outputs, whereas UBE4-mediated elongation of ubiquitin chains leads to deactivation and proteasomal turnover of NPR1 (Skelly et al., 2019). Since stability of NPR1 was enhanced in the *upl* mutants, we investigated whether UPLs also polyubiquitinate NPR1. Seedlings were treated with SA and the proteasome inhibitor MG132, and total ubiquitinated proteins pulled down with tandem-repeated ubiquitin-binding entities (TUBE) (Hjerpe et al., 2009), followed by detection of NPR1-GFP. In plants carrying WT *UPL* alleles, SA-induced ubiquitination of NPR1 was readily detected

and ubiquitination levels were most intense at higher molecular weight (Figure 3.8C). However, mutation of *UPLs* markedly reduced polyubiquitination of NPR1-GFP, suggesting UPL ligase activity targets NPR1 (Figure 3.8C).

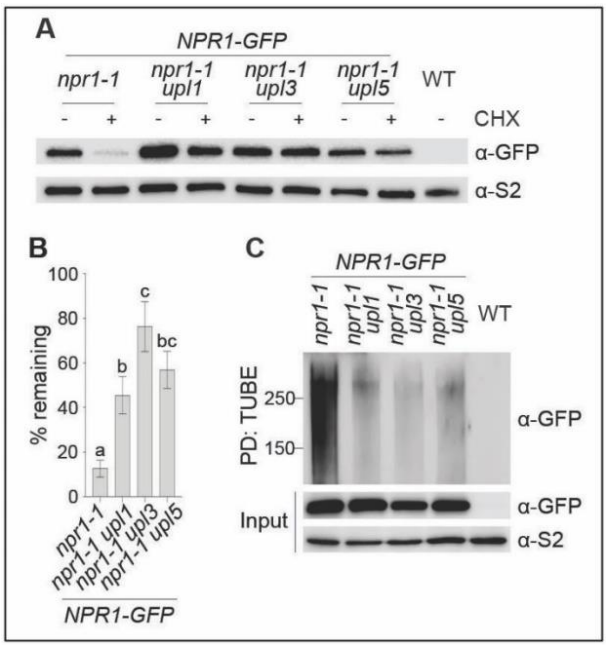

Figure 3. 8. UPLs affect stability and polyubiquitination of NPR1.

Seedlings were treated with 0.1 mM CHX or DMSO (vehicle) for 2 h. Stability of NPR1-GFP was analysed by western blotting (A) and NPR1-GFP protein levels were quantified and normalized to S2 protein levels (loading control). The percentage of remaining proteins was calculated relative to DMSO treated samples (B). Data represent the mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 3). (C) Seedlings expressing *NPR1-GFP* were treated with 0.5 mM SA and 0.1 mM MG132 for 6 h before total cellular ubiquitinated substrates were purified by using GST-TUBE. NPR1-GFP and S2 (loading control) were detected by using antibodies against GFP and S2, respectively.

Given that UPLs are associated with the proteasome, we reasoned that they might function sequentially after CUL3 and UBE4 ligases to modify NPR1 and promote its degradation. While CUL3-mediated ubiquitination promotes NPR1 activity, UBE4-mediated ubiquitin chain elongation deactivates NPR1. Consequently, *ube4* mutants show enhanced SA-induced activation of NPR1 target genes (Skelly et al., 2019). To test if UPL3 functions downstream of UBE4, we crossed *NPR1-GFP* (in *upl3*) plants with *ube4* mutant plants. Unfortunately, we were unable to obtain homozygous *upl3 ube4* double mutants expressing NPR1-GFP, suggesting this combination was lethal. However, in agreement with a ubiquitin ligase relay consisting of CUL3, UBE4 and ending with UPL3, heterozygous knockout of *UBE4* in *upl3* mutants restored NPR1's ability to activate *PR1* and *PR2* gene expression (Figure 3.9).

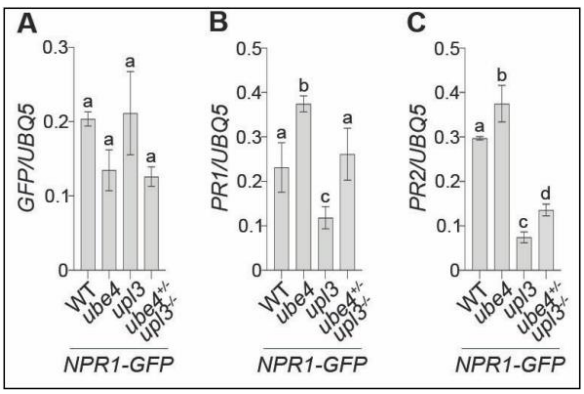

Figure 3. 9. Mutation of *UBE4* in the *upl3* background restores expression of *PR* genes.

Indicated genotypes expressing NPR1-GFP were treated with 0.5 mM SA for 24 h. Expression of *NPR1-GFP* (A), *PR1* (B), and *PR2* (C) genes was normalised to *UBQ5*. Data represent mean ± SD (Tukey HSD ANOVA test; α = 0.05, n = 3).

3.2.5 UPL3 is required for clearing inactive NPR1 from gene promoters

Taken together, our data suggests that in particular proteasome-associated UPL3 catalyses remodelling of NPR1-attached ubiquitin chains at the proteasome and promotes proteasomal degradation. Therefore, we performed chromatin immunoprecipitation (ChIP) experiments to evaluate if this process is required for clearing inactive NPR1 from its target promoter. Coinciding with the increased mRNA level of *PR1* (Figure 3.6C), more NPR1-GFP was detected at the *PR1* promoter after SA treatment of plants carrying the WT *UPL3* allele (Figure 3.10A). By contrast, loss of *UPL3* function resulted in significantly increased occupancy of NPR1-GFP at the *PR1* promoter already in untreated plants, and NPR1-GFP became even more enriched after SA treatment (Figure 3.10A). These results evidently show that in *upl3* mutant, inactive NPR1 is stalled at SA-responsive promoters and prevents further gene activation. As UPL3 directly catalyses ubiquitination of NPR1, we then assessed whether UPL3 might be also localized to the SA-responsive promoter. Notably, ChIP experiments demonstrated that YFP-UPL3 was constitutively associated with the *PR1* promoter, regardless of SA treatment (Figure 3.10B). Together, these data suggest that remodelling of NPR1-anchored ubiquitin chains at the chromatin by proteasome-associated UPL3 is essential for clearing inactive NPR1 from promoters to activate SA-responsive gene expression.

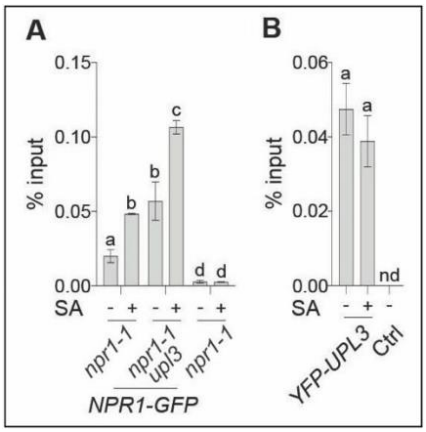

Figure 3. 10. UPL3 ligases are required to clear inactive ubiquitinated NPR1 from gene promoters.

Adult plants were treated with 0.5 mM SA or H_2O for 24 h before assessing NPR1-GFP (A) or YFP-UPL3 (B) binding to the *as-1* motif of the *PR1* promoter. WT plants were used as a negative control. Data represent mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 3); nd, not determined.

3.3 Discussion

The ubiquitin-proteasome system (UPS) is the major proteolysis machinery that controls homeostasis of transcriptional regulators in eukaryotic cells. Like many transcriptional activators (TAs), NPR1 is an unstable protein that is processively ubiquitinated and degraded by UPS-mediated proteolysis, a process that is necessary for appropriate control of the transcriptional activity of NPR1 (Skelly et al., 2019, Spoel et al., 2009). In this study we report that HECT-type ubiquitin ligases modulate SA-dependent transcriptional reprogramming by minimising accumulation of inactive NPR1 at its target gene promoters and promoting its proteasomal turnover.

We demonstrated that pathogen-induced accumulation of endogenous NPR1 is compromised in *upl* mutants (Figure 3.5B), which may in part be due to low amounts of endogenous SA in these mutants (Figure 3.5C). Indeed, it was previously shown that NPR1 protein homeostasis was impaired in mutant *ics1* and transgenic *NahG* plants that have very low SA content (Fu et al., 2012). Although significantly lower amounts of SA were produced in the *upl* mutants compared with WT plants, pathogen infection still induced SA synthesis (Figure 3.5C). Hence, the defect in NPR1 accumulation in the *upl* mutants might be only partially caused by suppression of SA production. Interestingly, expression of endogenous *NPR1* mRNA levels was comparable in *upl* mutants and WT plants (Figure 3.5A). Moreover, exogenous SA treatment did not fully rescue NPR1 protein levels (Figure 3.5D), suggesting that in addition to controlling accumulation of NPR1 by affecting SA synthesis, UPLs might also regulate translation of *NPR1* mRNA through its UTR regions. Indeed, this is further supported by expressing *NPR1-GFP* transgene without UTR regions in *upl* mutants, which displayed enhanced accumulation of NPR1-GFP protein compared to WT plants (Figure 3.6B).

To achieve precise transcriptional reprogramming, the activity of NPR1 is dynamically regulated by the ubiquitin-proteasome machinery. An E3/E4 regulatory cassette consisting of CUL3 and UBE4, respectively, manipulates SA-induced transcriptional activity of NPR1 by fine-tuning the length of its ubiquitin chains and eventually promotes its proteasomal degradation (Spoel et al., 2009, Skelly et al., 2019). We found that loss of function of *UPL*s leads to an increased level of NPR1 protein, and enhances binding of NPR1 to the *PR1* target promoter (Figure 3.6B and 3.10A). Taken together with the fact that loss of *UBE4* ligase function leads to enhanced occupancy of NPR1-GFP at the *PR1* promoter (Skelly et al., 2019), this suggests that long-chain polyubiquitination of NPR1 is indispensable for its degradation by the proteasome. Functionally, UBE4-mediated ubiquitin chain elongation triggers the switch from active chromatin-bound NPR1 to the inactive state (Skelly et al., 2019). Impaired proteasomal processivity in the *upl3* mutant, however, blunts expression of SA-responsive genes owing to inactive NPR1 stalled at the chromatin. This is consistent with the finding that preventing the proteolytic activity of the proteasome complex blocks *PR1* gene expression (Skelly et al., 2019). To remove inactive NPR1 from SA-responsive promoters, the proteasome complex targets polyubiquitinated NPR1 at the chromatin (Figure 3.10B), where UPLs further remodel NPR1-attached ubiquitin chains. Collectively these findings show that proteasome-associated UPL3 is the last in a relay of three ubiquitin ligases that polyubiquitinate NPR1 and ensures transcriptionally inactive NPR1 is cleared from target gene promoters by the proteasome (Figure 3.11).

Unlike E3 ligases that catalyse ubiquitination of specific substrates, UPLs are associated with the proteasome and therefore may have various cellular functions by targeting numerous proteins (Figure 3.1). Indeed, in different plant species, multiple

transcriptional regulators have been identified as substrates of UPLs, suggesting HECT-type ubiquitin ligases are novel regulators of cellular polyubiquitination in plants (Miller et al., 2019, Patra et al., 2013, Bensussan et al., 2015, Miao and Zentgraf, 2010). In fact, association of HECT-type ubiquitin ligases with the proteasome has been widely reported in other organisms (You and Pickart, 2001, Xie and Varshavsky, 2000, Crosas et al., 2006, Leggett et al., 2002). Recruitment of HUL5, a yeast HECT-type ubiquitin ligase, endows the proteasome with ubiquitin ligase activity, which allows remodelling of ubiquitin chains on proteasome-bound substrates (Crosas et al., 2006). Substrates could be only partially degraded in the *hul5* mutant, suggesting that remodelling of substrate-attached ubiquitin chains is indispensable for enhancing processive protein destruction (Crosas et al., 2006, Aviram and Kornitzer, 2010). Interestingly, mutant HECT-type ubiquitin ligases that lack the proteasome interaction domain retain the ability to ubiquitinate substrates, however, it impacts ubiquitin-dependent proteolysis (Xie and Varshavsky, 2002). This suggests that remodelling of ubiquitin chains by HECT-type ligases at the proteasome may be a general mechanism that is crucial for proteasomal proteolysis of substrates. However, it remains to be determined if HECT-type ubiquitin ligases only extend or branch existing ubiquitin chains, or whether they might also initiate new chains on their substrates.

In summary, our results show that UPLs modulate SA-induced plant immunity at multiple levels. UPLs are required for establishing SA-dependent immune signalling by controlling accumulation of NPR1. In addition, UPL-mediated remodelling of NPR1-attached ubiquitin chains at the proteasome promotes NPR1 degradation, and this process is essential for clearing of inactive NPR1 from the chromatin, thereby allowing ‘fresh’ active NPR1 proteins to reinitiate transcription. Ubiquitin chain remodelling by

HECT-type ubiquitin ligases at the proteasome may be a powerful process in eukaryotic cells in general for facilitating proteasomal turnover of unstable or misfolded proteins.

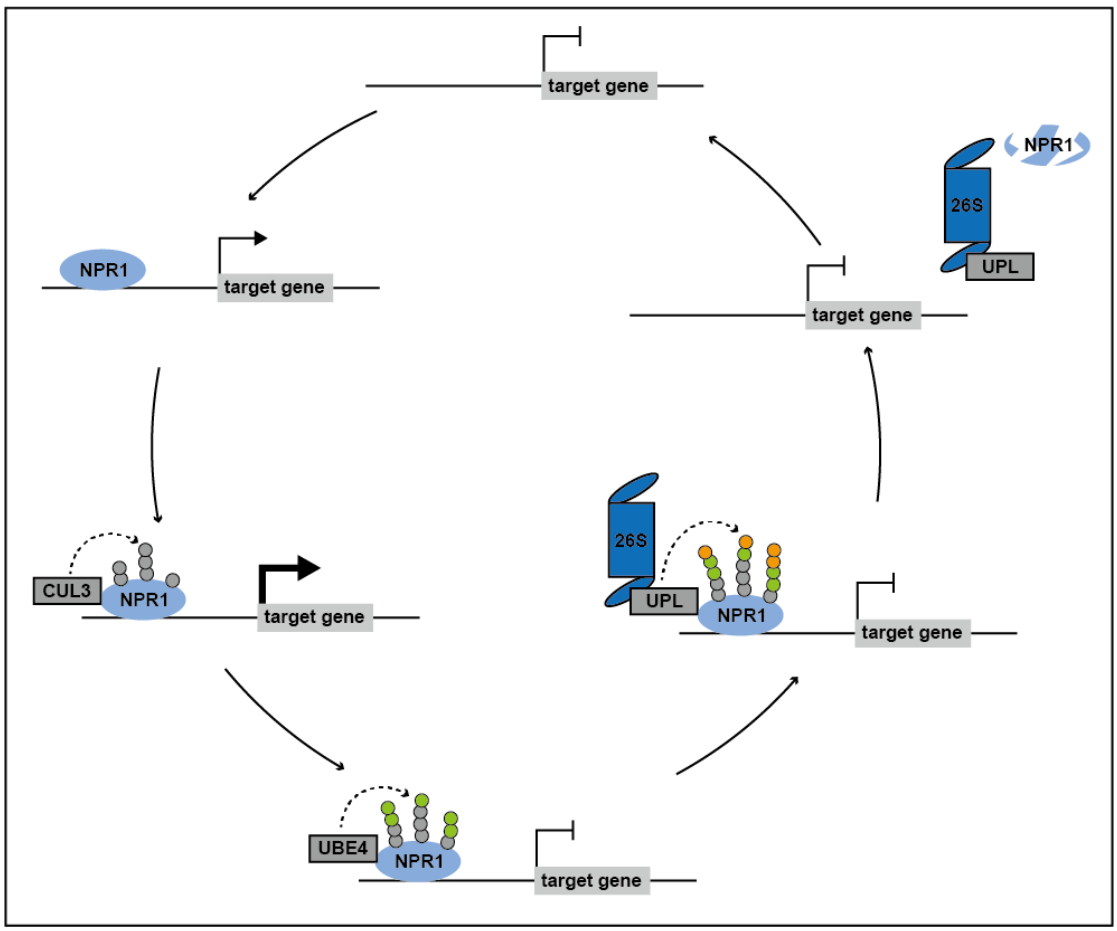

Figure 3. 11 Working model for how ubiquitin ligase relays modulate transcriptional activity of NPR1.

CUL3 catalyses initiation of ubiquitin chains (grey circles) on promoter-bound NPR1 that enhances its transcriptional output. However, UBE4-mediated elongation of ubiquitin chains (green circles) results in inactivation of target genes. The proteasome is then recruited and recognises inactive NPR1. Next, proteasome-associated UPL ligases further remodel ubiquitin chains (orange circles) and promote proteasomal degradation and clearance of inactive NPR1 from its target promoter.

Chapter 4:

A proteasome-associated ubiquitin ligase relay controls activity of the plant developmental regulator EIN3

4.1 Introduction

The ubiquitin-proteasome system (UPS) controls homeostasis of 80-90% of cellular proteins, including tumour suppressors, cell-cycle regulators, signalling receptors, and transcription factors (Rock et al., 1994, Voges et al., 1999). Activity of the UPS is vital for maintaining correct function of the cell, while dysregulation of the UPS usually leads to abnormal accumulation of substrate proteins and accordingly, is implicated in multiple diseases (Popovic et al., 2014). Proteasome-mediated protein degradation is initiated by covalent attachment of ubiquitin to the target protein, and this process involves three types of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3).

Due to the direct binding of E3 ligase to their substrates, protein-protein interaction screening is commonly used to identify E3 ligase target proteins. Intriguingly, the detected interactors of E3 ligase from these screenings include not only potential substrates, but also subunits of the proteasome (Lee et al., 2005, Verma et al., 2000). Although many *in vitro* and *in vivo* assays have confirmed that the interaction between E3 ligase and the proteasome exists in different organisms (Schmidt et al., 2005), the role of E3 ligases at the proteasome remains unclear. In Arabidopsis, the proteasome-associated HECT-type ubiquitin ligase, Ubiquitin Protein Ligase 3 (UPL3), has been found to interact with several E3 ligases (Furniss et al., 2018). This provides a clue that HECT-type ubiquitin ligases might build a link between E3 ligase-catalysed substrate ubiquitination and proteasomal degradation. However, how do HECT-type ubiquitin ligases contribute to proteasome-mediated degradation?

Proteasomal degradation of large proteins normally requires a chain of at least four ubiquitin molecules that are recognised by ubiquitin receptors of the proteasome

(Thrower et al., 2000). Formation of long ubiquitin chains often does not fully rely on E3 ligases, as these ligases become less efficient as the ubiquitin chain elongates (Pierce et al., 2009). Instead, ubiquitin chain elongation often relies on ubiquitin ligases that possess E4-like activity (Hoppe, 2005). E4-like ligases, especially HECT domain containing proteins, have been found to be essential for targeting substrates for proteasome-mediated degradation (Leggett et al., 2002). In yeast, proteasomal recruitment of the E4-like ligase, HUL5 (HECT Ubiquitin Ligase 5), endows the proteasome with ubiquitin ligase activity (Crosas et al., 2006). In the *hul5* mutant, target proteins can only be partially degraded, indicating that association of HUL5 and the proteasome is fundamental for processive protein degradation (Aviram and Kornitzer, 2010). This supports that remodelling of the ubiquitin chains by the proteasome-associated HECT-type ubiquitin ligase at the proteasome is crucial for proteasome-mediated proteolysis.

As a proteasome-associated HECT-type ubiquitin ligase, UPL3 is thought to act as an E4-like enzyme that is responsible for the majority of the proteasome's ubiquitin ligase activity (Furniss et al., 2018). UPL3's ability to form polyubiquitin chains at the proteasome and to govern total cellular ubiquitination levels suggests that UPL3 may have wide-ranging effects on cellular substrate degradation (Furniss et al., 2018). In Chapter 3, I demonstrated that UPL3 is the vital last component of a ubiquitin ligase relay that targets the immune coactivator NPR1 for degradation. So are ubiquitin ligase relays that end with proteasome-associated UPL3 a common mechanisms for the control of transcriptional activator activity in plants?

A previous yeast two-hybrid screening found that the armadillo repeat of UPL3 interacts with EBF2 (EIN3 Binding F-Box Protein 2), which is part of a $SCF^{EBF1/2}$ ubiquitin

ligase complex with ASKs (SKP1 homologs in Arabidopsis) and Cullin 1 (Gagne et al., 2004, Guo and Ecker, 2003, Potuschak et al., 2003). The $SCF^{EBF1/2}$ ubiquitin ligase complex physically interacts with Ethylene Insensitive 3 (EIN3) family transcriptional activators and catalyses their ubiquitination, thereby regulating ethylene-mediated transcriptional reprogramming (Wang et al., 2002, Chang et al., 2013, Guo and Ecker, 2003, Potuschak et al., 2003). However, how ubiquitinated EIN3 is delivered to the proteasome by $SCF^{EBF1/2}$ and how it is processed upon arrival is still unclear.

In this study, we demonstrate that the polyubiquitination of EIN3 by UPL3 and its closely related paralogue UPL4 is a key step in its proteasomal degradation. Intriguingly, UPL3 associated with both the $SCF^{EBF1/2}$ complex and EIN3, and the UPL3-EIN3 interaction was dependent on EBF1/2, suggesting a novel relay mechanism in which EIN3 is directly relayed from $SCF^{EBF1/2}$ to UPL3 at the proteasome. UPL3/4-mediated ubiquitination of EIN3 was required for its processive degradation by the proteasome and its clearance from ethylene-responsive promoters. Consequently, UPL3/4 were identified as key regulators of ethylene-mediated transcriptional events and associated plant morphological changes.

4.2 Results

4.2.1 UPL3 interacts with both $SCF^{EBF1/2}$ and its substrate EIN3

Association between EBF2 and UPL3 was previously detected by a yeast two-hybrid screening (Furniss et al., 2018). Therefore, we tested if this interaction also occurs *in planta*. To this end, we performed a co-immunoprecipitation (co-IP) assay by using YFP-UPL3 purified from transgenic *35S:YFP-UPL3* plants (Furniss et al., 2018) and incubated

it with recombinant FLAG-EBF2, which was produced by using a cell-free protein synthesis system (Nomoto and Tada, 2018). As shown in Figure 4.1A, FLAG-EBF2 was specifically pulled down with YFP-UPL3, confirming that UPL3 physically interacts with the SCF^{EBF2} complex (Furniss et al., 2018). Since interaction between the SCF^{EBF2} complex and UPL3 could be associated with the handover of cargo, we next investigated the possible interaction between UPL3 and EIN3, which is a direct target of the $SCF^{EBF1/2}$ complex. Thus, we performed co-immunoprecipitation experiments by trapping YFP-UPL3 and its associated proteins. We found that EIN3 specifically co-immunoprecipitated with YFP-UPL3 *in vivo* (Figure 4.1B). Strikingly, the co-immunoprecipitated protein complex also included the proteasome, as confirmed by detection of the proteasome subunit S2 (also known as RPN1) (Figure 4.1B), suggesting that interaction between EIN3 and UPL3 occurs at the proteasome. Next, to investigate whether interaction between UPL3 and EIN3 relies on the presence of the $SCF^{EBF1/2}$ complex, protoplasts from *35S:YFP-UPL3* (in *ein3-1*) and *35S:YFP-UPL3* (in *ebf1 ebf2 ein3-1*) plants were transfected with *35S:HA-EIN3*. YFP-UPL3 specifically pulled down HA-EIN3 in presence of functional EBF1/2, however, this interaction was completely abolished in their absence (Figure 4.1C), indicating that EBF1/2 are required for interaction between UPL3 and EIN3. Overall, these results indicate that the $SCF^{EBF1/2}$ complex directly relays its EIN3 cargo to proteasome-associated UPL3.

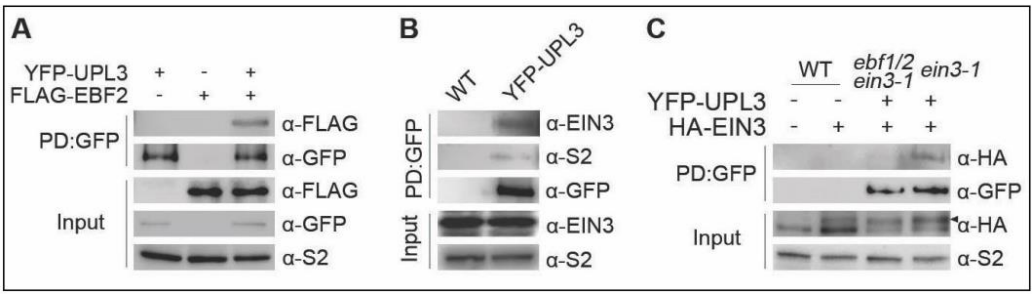

Figure 4. 1 UPL3 interacts with the SCFEBF1/2 complex and its cargo EIN3.

(A) YFP-UPL3 was purified with GFP-Trap from *35S:YFP-UPL3/upl3* plants and incubated with *in vitro* synthesised FLAG-EBF2. Inputs and immunoprecipitated proteins were analysed by western blotting with antibodies against GFP, FLAG and S2.

(B) YFP-UPL3 was pulled down with GFP-Trap from seedlings expressing *35S:YFP-UPL3*. Subsequently, EIN3, S2, and YFP-UPL3 were detected by immunoblotting.

(C) Protoplasts from *35S:YFP-UPL3/ein3-1* and *35S:YFP-UPL3/ebf1 ebf2 ein3-1* plants were transfected with plasmids expressing *35S:HA-EIN3*. YFP-UPL3 was pulled down with GFP-Trap. HA-EIN3 (arrowhead), YFP-UPL3, and S2 were detected by immunoblotting with HA, GFP, and S2 antibodies, respectively.

The interactions between key ethylene response regulators and UPL3 suggests that UPL3 is involved in the ethylene signalling pathway. To further test this, the mRNA and protein levels of UPL3 were evaluated in response to the ethylene precursor ACC. We found that gene expression of *UPL3*, but not of its close paralogue *UPL4*, was significantly induced after ACC treatment (Figure 4.2A). Moreover, the accumulation of YFP-UPL3 protein was induced by ACC, and this result is in line with the enhanced cellular abundance of YFP-UPL3 in root cells (Figure 4.2B and C). YFP-UPL3 was detected in the whole cell, but mainly localized to the nucleus, where $SCF^{EBF1/2}$ targets

EIN3 for ubiquitination (Figure 4.2C). These results suggest that *UPL3* is responsive to ethylene at both transcriptional and posttranslational levels.

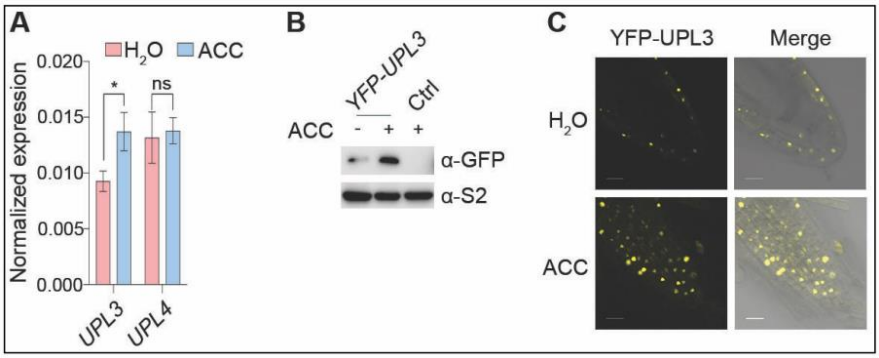

Figure 4. 2 Ethylene-responsiveness of UPL3 mRNA and protein.

(A) WT seedlings were treated with 50 μM ACC or H_2O for 3 h. Expression of *UPL3* and *UPL4* is normalized to constitutively expressed *ELF4a*. Data represent the mean ± SD, asterisk indicates statistically significant differences between samples (two-tailed t test, *p ≤ 0.05; ns, no significant difference; n = 3)

(B and C) Seedlings expressing *35S:YFP-UPL3* were treated with 50 μM ACC or H2O for 3 h. YFP-UPL3 and S2 were detected by immunoblotting with antibodies against GFP and S2, respectively (B). Subcellular localization was determined by confocal microscopy (C).

4.2.2 Remodelling of EIN3-attached ubiquitin chains by UPL3 promotes proteasomal degradation

Because EIN3 is relayed from $SCF^{EBF1/2}$ to UPL3, we investigated if EIN3 is further ubiquitinated by UPL3/4. To this end, total cellular ubiquitinated proteins were captured with tandem-repeated ubiquitin-binding entities (TUBE) (Hjerpe et al., 2009), followed by detection of endogenous EIN3. Interestingly, in WT plants EIN3 was ubiquitinated both in presence and absence of ACC, but polyubiquitination levels were markedly reduced in the *upl3 upl4* double mutant (Figure 4.3A). Together with the above protein-protein interaction data, this implies that the $SCF^{EBF1/2}$ complex initiates ubiquitination on EIN3 before handing it over to UPL3/4, which further remodels EIN3-attached ubiquitin chains at the proteasome.

We further investigated if UPL3/4-mediated ubiquitination of EIN3 affects its stability using the protein synthesis inhibitor, cycloheximide (CHX). However, this requires seedlings to be submerged, which induces ethylene production that inevitably stabilizes EIN3 through the degradation of $SCF^{EBF1/2}$ complex components (Guo and Ecker, 2003, An et al., 2010, Metraux and Kende, 1983). Therefore we co-treated seedlings with $AgNO_3$, a potent inhibitor of ethylene action, to eliminate the effect of ethylene on EIN3 stability (Beyer, 1976). After co-treatment with CHX and $AgNO_3$, EIN3 was a short-lived protein that was rapidly degraded within 20 min in the WT (Figure 4.3B and C). By contrast, turnover of EIN3 was much slower in the *upl3 upl4* double mutant, and a high level of EIN3 remained even 60 min after treatment (Figure 4.3B and C), indicating EIN3 was stabilized in the absence of both *UPL3* and *UPL4*.

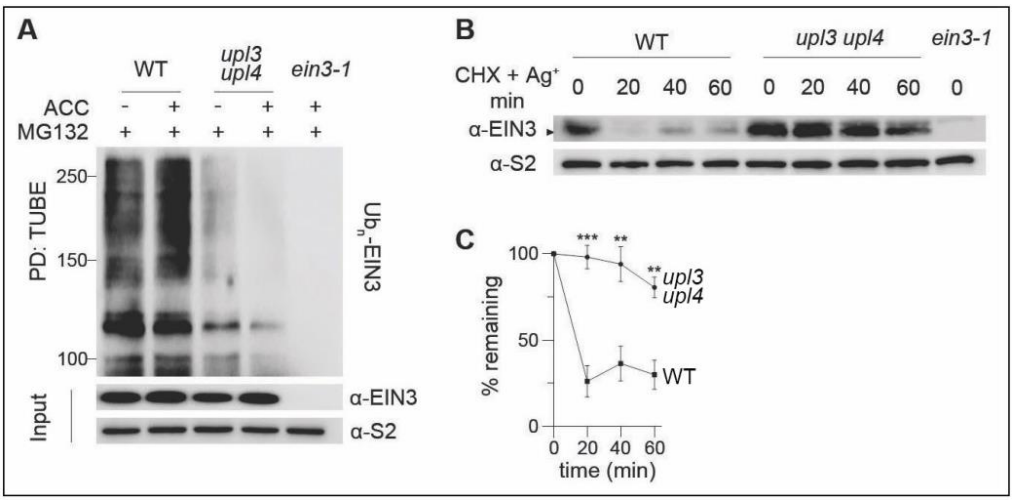

Figure 4. 3 UPL3/4-mediated polyubiquitination is required for proteasomal degradation of EIN3.

(A) UPL3/4 further polyubiquitinates EIN3. Seedlings were treated with 0.1 mM MG132 in the presence or absence of 50 μM ACC for 3 h before using His-TUBEs to pull down ubiquitinated proteins. Ubiquitinated and input EIN3, and S2 were detected by immunoblotting.

(B) Seedlings were pre-treated with 50 μM ACC for 3 h, following by co-treatment with 0.1 mM CHX and 0.1 mM AgNO3. Samples were collected at the indicated timepoints. The *ein3-1* mutant was used as a negative control.

(C) EIN3 protein levels were quantified from (B) and normalized to the levels of S2 protein. The percentage of the remaining proteins was calculated relative to the quantification at 0 min of the chase. Data represent the mean ± SD, asterisk indicates statistically significant differences between samples (two-tailed t test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$; n = 3)

Because UPL3 is a proteasome-associated ubiquitin ligase that interacts with both the proteasome and its substrates, we next investigated whether UPL3 affects residency time of EIN3 with the proteasome. To this end, EIN3-GFP-FLAG was expressed and purified from WT and *upl3 upl4* plants, and the amount of proteasomes that co-

precipitated was analysed. Although higher expression levels of *EIN3-GFP-FLAG* were detected in the WT background, its interaction with the proteasome was barely detectable (Figure 4.4). This is expected because upon arrival at the proteasome, ubiquitinated substrates rapidly undergo proteolytic degradation (Grice and Nathan, 2016). In contrast, EIN3-GFP-FLAG strongly interacted with proteasomes from *upl3 upl4* mutants (Figure 4.4), indicating that EIN3-GFP-FLAG was stalled on these proteasomes in the absence of UPL3 and UPL4. We conclude that UPL3/4-mediated ubiquitination ensures proteasomes processively degrade EIN3 and thus prevents substrate stalling.

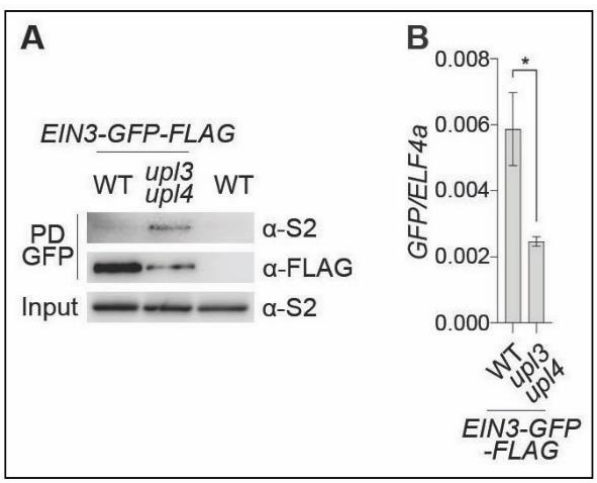

Figure 4. 4 EIN3 stalls at proteasomes of *upl3 upl4* mutants.

(A) Transgenic plants expressing *pEIN3:EIN3-GFP-FLAG* in the indicated genetic backgrounds were used for pull down assays with GFP-Trap agarose. Proteins were analysed by western blotting using antibodies against S2 and FLAG.

(B) Expression of GFP in the indicated genotypes was normalized to *ELF4a*. Data represent the mean ± SD, asterisk (two-tailed t test, *$p \leqslant 0.05$, n = 3) indicates statistically significant differences between samples.

4.2.3 Transcriptional levels of EIN3 target genes are regulated by UPL3 and UPL4

Previous research showed that inhibition of proteasome activity by MG132 induced accumulation of EIN3, and this accumulation was also observed in *EBF1* and *EBF2* loss-of-function mutants (Guo and Ecker, 2003). This indicates that the ubiquitin-proteasome system plays an important role in avoiding auto-ethylene responses under normal growth conditions by maintaining EIN3 at a low steady-state level. Since proteasomal degradation of EIN3 is affected in the *upl3 upl4* mutant, we tested whether UPL3/4-mediated turnover is required for clearing EIN3. As shown in Figure 4.5A, the level of endogenous EIN3 was relatively low without any stimulus in the WT plants. By contrast, compared to WT, higher levels of EIN3 were detected in both untreated *upl3* single and particularly *upl3 upl4* double mutants. Expression of *EIN3* mRNA was not significantly affected by mutation of *UPL3* and *UPL4* (Figure 4.5B), indicating that UPL3 and UPL4 are necessary for the turnover of EIN3 protein under steady-state conditions. In the presence of ACC, an increased amount of EIN3 was detected in WT plants, indicating that as expected ethylene promoted accumulation of EIN3 (Figure 4.5A). Moreover, ACC treatment enhanced accumulation of EIN3 in the *upl3 upl4* mutant but protein levels were much higher than in the ACC-treated WT (Figure 4.5A). The effects on EIN3 accumulation detected in the *upl3 upl4* double mutant was stronger than any of the single mutants, suggesting that UPL3 and UPL4 function additively in controlling EIN3 stability. Overall, these data show that UPL3/4 contribute substantially to maintaining appropriate levels of cellular EIN3 protein.

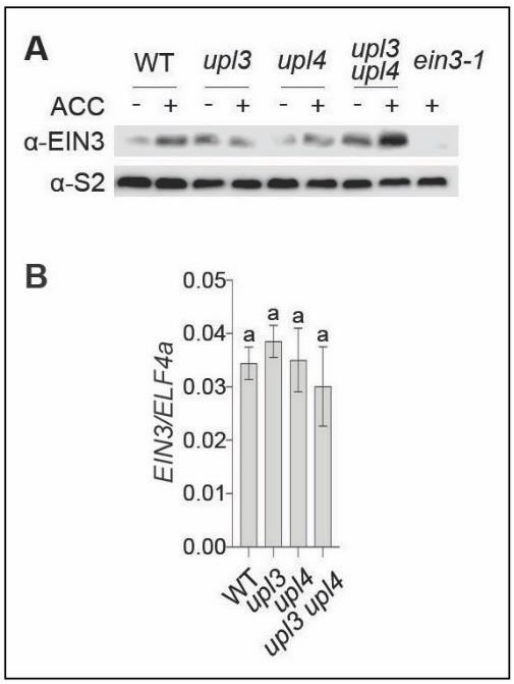

Figure 4. 5 Mutant *upl3 upl4* plants accumulate enhanced levels of EIN3.

(A) Seedlings of indicated genotypes were treated with 50 μM ACC or H_2O for 3 h. Proteins were analysed by western blotting using antibodies against EIN3 and S2. The *ein3-1* mutant was used as a negative control.

(B) Expression of *EIN3* was normalized to *ELF4a*. Data represent the mean ± SD, lowercase letters indicate no statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 3).

Since EIN3 is highly accumulated in the *upl3 upl4* mutant, we explored if this results in enhanced activation of downstream signalling. First, we tested the expression of ethylene-responsive genes in WT plants and *upl* single and double mutants. Compared to the WT, expression of EIN3 target genes was significantly higher in the *upl3 upl4* double mutant in absence of ethylene (Figure 4.6). This constitutive activation of ethylene-responsive genes in the *upl3 upl4* mutant was consistent with the enhanced accumulation of EIN3 observed above (Figure 4.5). Upon ACC treatment, EIN3 target genes were activated in the WT, but their expression was strikingly enhanced in *upl3 upl4* mutants (Figure 4.6). The expression of ethylene-responsive genes was less dramatically altered in *upl3* and *upl4* single mutants, confirming functional redundancy of UPL3 and UPL4 in regulating the ethylene signalling pathway.

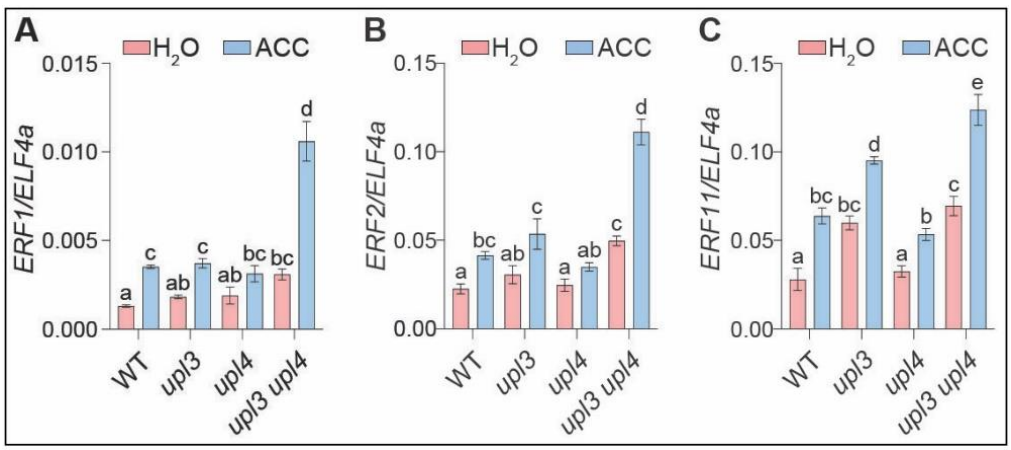

Figure 4. 6 Mutant *upl3 upl4* plants exhibit enhanced expression of EIN3 target gene.

Seedlings of indicated genotypes were treated with 50 μM ACC or H_2O for 3 h. Expression of *ERF1* (A), *ERF2* (B), and *ERF11* (C) was normalized to *ELF4a*. Data represent the mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; $\alpha = 0.05$, $n = 3$).

To test if the constitutive activation of ethylene-responsive genes in the *upl3 upl4* mutant was indeed due to enhanced EIN3 activity, we generated a *upl3 upl4 ein3-1* triple mutant. Consistent with the previous result, the *upl3 upl4* mutant displayed significantly higher expression of EIN3 target genes than WT under both mock- and ACC-induced conditions (Figure 4.7). Induction of ethylene-responsive genes by ACC was entirely abolished in *ein3-1* mutant, indicating the central regulatory role of EIN3 in the ethylene signalling pathway (Figure 4.7). Likewise, the expression of ethylene-responsive genes was insensitive to ACC treatment in the *upl3 upl4 ein3-1* triple mutant (Figure 4.7). Moreover, constitutive expression of EIN3 target genes observed in *upl3 upl4* mutants was largely abolished in this triple mutant (Figure 4.7). These data confirm that UPL3/4 regulate ethylene-responsive genes by modulating EIN3 protein levels.

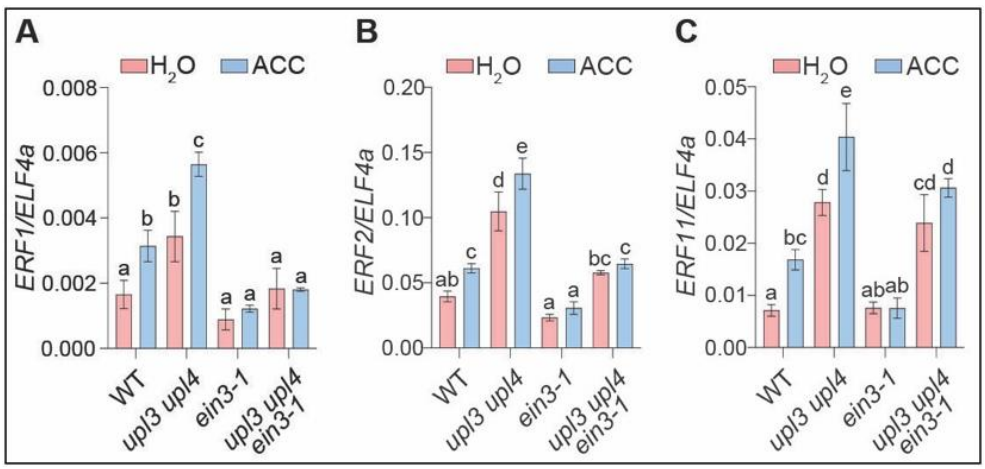

Figure 4. 7 Enhanced expression of EIN3 target genes in the *upl3 upl4* mutants is dependent on EIN3.

Seedlings of indicated genotypes were treated with 50 μM ACC or H_2O for 3 h. Expression of *ERF1* (A), *ERF2* (B), and *ERF11* (C) was normalized to *ELF4a*. Data represent the mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; $\alpha = 0.05$, $n = 3$).

To further explore how UPL3/4 regulate gene expression at a genome-wide level, we performed transcriptome analysis on WT plants, *ein3-1* single, *upl3 upl4* double and *upl3 upl4 ein3-1* triple mutants. ACC treatment caused differential expression of 1,165 genes in the WT plants (≥ 1.5 fold, $p \leq 0.05$), including 486 upregulated genes and 679 downregulated genes (Figure 4.8A). The majority of these ACC regulated genes in WT were dependent on EIN3 (Figure 4.8A). Strikingly, the *upl3 upl4* double mutant displayed a constitutive ethylene-responsive gene expression pattern (Figure 4.8B and C). By contrast, mutation of *EIN3* in the *upl3 upl4* double mutant dramatically supressed the constitutive transcriptional effects caused by knocking out of *UPL3* and *UPL4* (Figure 4.8B and C). These data show that UPL3 and UPL4 control genome-wide transcriptional reprogramming of ethylene-responsive genes by modulating the cellular levels of EIN3.

Gene ontology (GO) analysis of the genes regulated by both EIN3 and UPL3/4 showed enrichment particularly for transcription factor activity and the transcription process (Figure 4.9A). By contrast, EIN3-independent genes that are regulated by UPL3/4 were enriched for enzymatic activities, stress responses and signal transduction (Figure 4.9B). Moreover, there were many ACC-responsive genes regulated by UPL3/4 in an EIN3-independent manner (Figure 4.8A), suggesting that EIN3-like (EIL) proteins that share functional redundancy with EIN3 might also be targeted by UPL3 and UPL4 (Chao et al., 1997). Alternatively, other UPL3/4-regulated pathways might also contribute to the regulation of ethylene-responsive gene expression.

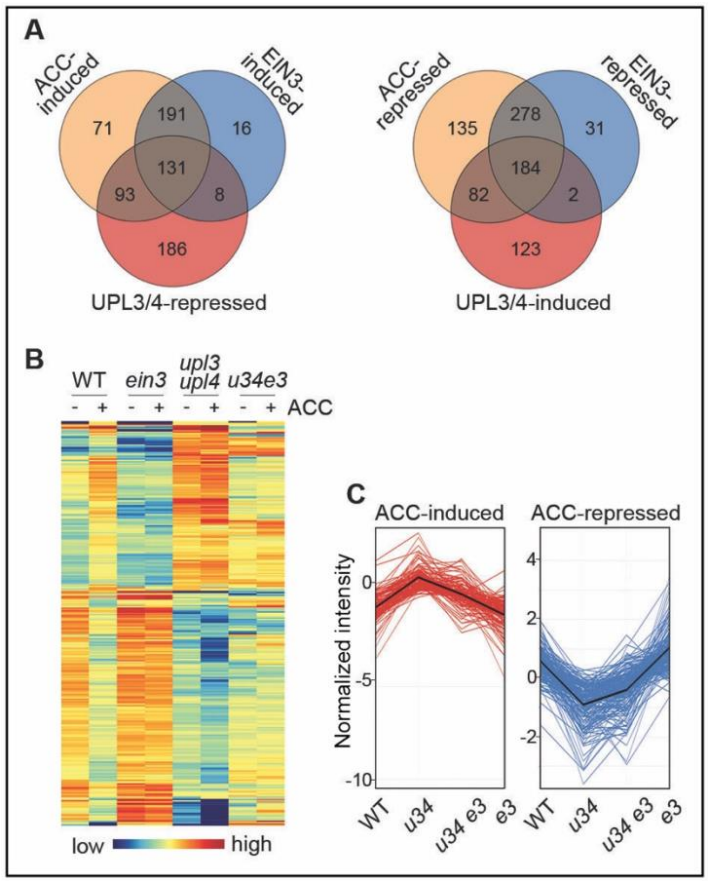

Figure 4. 8 Mutant *upl3 upl4* plants exhibit enhanced ET-responsive transcriptional reprogramming.

Seedlings were treated with 50 μM ACC or H_2O for 3 h, and mRNA was analysed by RNA-Seq. Venn diagrams (A) illustrated the overlaps of ACC-regulated genes, EIN3-regulated genes, and UPL3/4-regulated genes. ACC-responsive genes (fold change ≥ 1.5, Benjamini Hochberg FDR, 2-way ANOVA $p \leq 0.05$, n = 3) that were regulated by both UPL3/4 and EIN3 are shown in a Heat map (B) and profile plot (C).

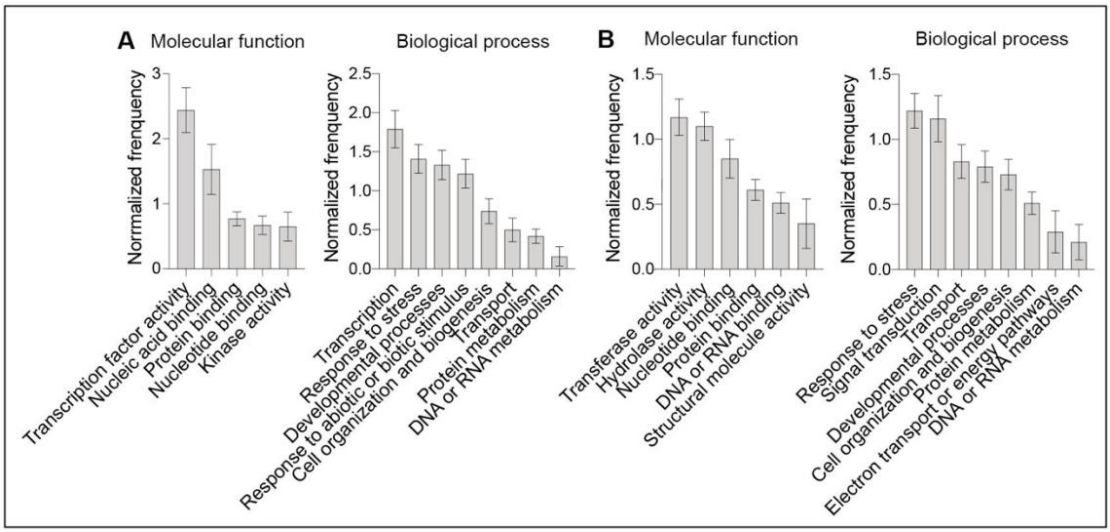

Figure 4. 9 UPL3/4-regulated genes are highly enriched in transcription factor activity.

GO term analysis was performed on EIN3-dependent and UPL3/4-regulated genes (A), or EIN3-independent and UPL3/4-regulated genes (B). Biological process and molecular function were analysed using Classification Superviewer on bar.utoronto.ca/. Normalized frequency, SD, and *P* values were determined as previously described (Provart et al., 2003), categories that have $p \leq 0.05$ are shown.

4.2.4 UPL3/4 control ethylene-responsive phenotypes by targeting EIN3

EIN3-triggered transcriptional reprograming in response to ethylene is associated with a series of phenotypic responses (Chao et al., 1997, Chang et al., 2013). Since the accumulation and activity of EIN3 was enhanced in the *upl3 upl4* mutant due to attenuation of proteasomal processivity (Figure 4.5 and 4.6), we next tested whether constitutive activation of ethylene signalling in this mutant induced ethylene-dependent phenotypes. The commonly used method to analyse phenotypes in response to ethylene is to treat etiolated seedlings with ethylene or ACC, which causes specific morphological changes that are known as the triple response, including shortening and thickening of the

hypocotyl, formation of a pronounced apical hook, and inhibition of root growth (Guzman and Ecker, 1990). Indeed, ACC treatment largely prevented hypocotyl and root elongation in etiolated WT seedlings (Figure 4.10). As previously reported (Guo and Ecker, 2003, Potuschak et al., 2003), ethylene responses were dramatically attenuated when *EIN3* was mutated, whereas the *ebf1 ebf2* double mutant that highly accumulates EIN3, displayed constitutive ethylene response phenotypes (Figure 4.10). Similar to the *ebf1 ebf2* mutant, untreated *upl3 upl4* seedlings exhibited constitutive ethylene-responsive phenotypes, including markedly shorter hypocotyl and root lengths compared to WT (Figure 4.10). Moreover, the constitutive triple response phenotypes of *upl3 upl4* were enhanced further when seeds were treated with ACC (Figure 4.10). The *upl3* and *upl4* single mutants also showed significantly shorter hypocotyls compared to WT in response to ACC, but their phenotypes were less severe than that of the double mutant (Figure 4.10).

Because UPL3/4-regulated ethylene-responsive genes were also dependent on EIN3 (Figure 4.8), we next investigated whether the constitutive triple response phenotypes of the *upl3 upl4* mutant could also be recovered by loss-of-function of *EIN3*. As shown in Figure 4.11, the constitutive ethylene response phenotype was partially lost in the *upl3 upl4 ein3-1* triple mutant with effects on hypocotyl elongation being larger than those on root elongation. Residual ethylene signalling was likely due to a notable number of ACC-responsive genes that are independent of EIN3 but regulated by UPL3/4 (Figure 4.8), which may indicate that UPL3/4 also target EIL activators. Finally, mutation of *EIN3* completely abolished enhanced responsiveness of *upl3 upl4* plants to ACC (Figure 4.11). Taken together with the fact that phenotypes in the *upl3 upl4* mutant

resembled those of the *ebf1 ebf2* mutant, these data indicate that ubiquitination of EIN3 by relays of EBF1/2 and UPL3/4 is pivotal for developmental ethylene signalling.

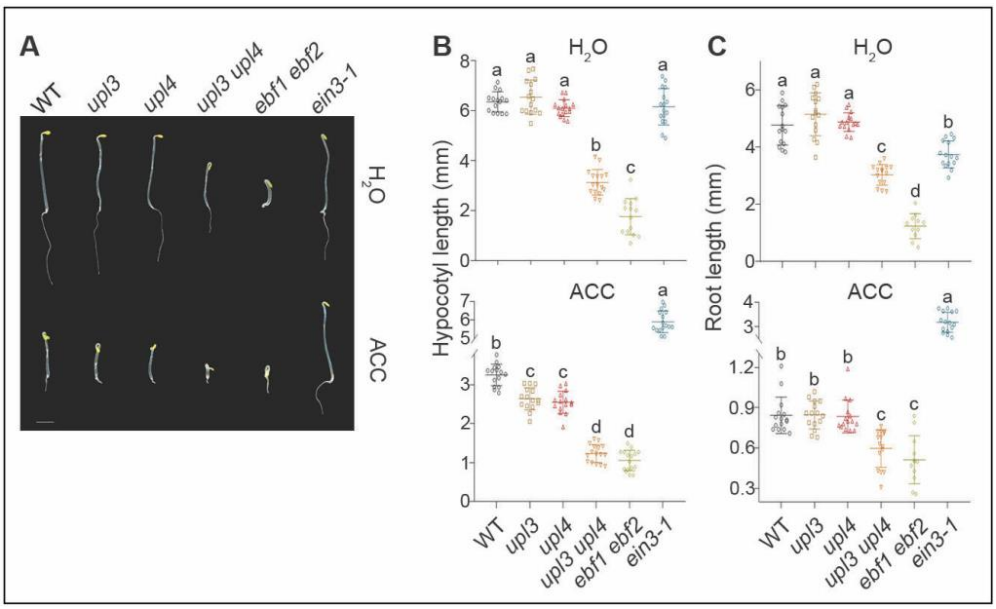

Figure 4. 10 Mutant *upl3 upl4* plants display constitutive ethylene response phenotypes.

Etiolated Seedlings were grown on MS medium supplemented with or without 10 μM ACC. Hypocotyl (B) and root (C) length of 4-day-old seedlings was measured. Data represent the mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 15).

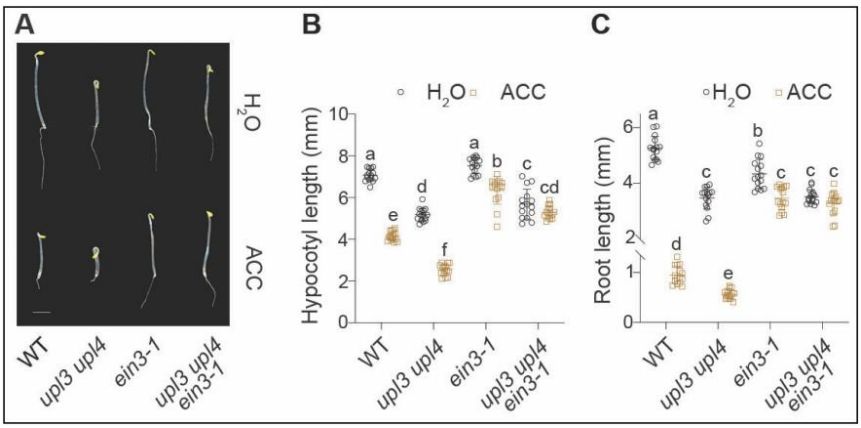

Figure 4. 11 Constitutive ethylene responses of *upl3 upl4* mutants are partially dependent on *EIN3*.

ET-responsive phenotypes of 4-day-old seedlings of the indicated genotypes were analysed. Hypocotyl (B) and root (C) length of 4-day-old seedlings was measured. Data represent the mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 15).

4.2.5 UPL3/4-mediated ubiquitination is required for clearing chromatin-bound EIN3

In response to ethylene, EIN3 directly binds to ethylene-responsive promoters to trigger a myriad of transcriptional events (Chang et al., 2013). Because we detected elevated expression of EIN3 target genes in the *upl3 upl4* mutant, we then assessed if this was due to enhanced binding of EIN3 to ethylene-responsive promoters. To this end, we performed chromatin immunoprecipitation (ChIP) experiments using an antibody that recognizes endogenous EIN3. In WT plants, ACC treatment induced enrichment of EIN3 at its *ERF1* target promoter, whereas binding of EIN3 was not detectable in the *ein3-1* mutant (Figure 4.12A). In agreement with its constitutive ethylene responsive phenotype, much more EIN3 was bound to the *ERF1* promoter of *upl3 upl4* mutants compared to the

WT, even under steady-state conditions (Figure 4.12A). This indicates that UPL3/4 are required for constitutive clearing of EIN3 from the chromatin.

Ubiquitinated substrates are normally recruited to the proteasome via ubiquitin receptors that directly bind to the substrate-attached ubiquitin chains (Verma et al., 2004, Elsasser et al., 2004). Although loss of *UPL3* and *UPL4* function inhibits proteasome processivity, $SCF^{EBF1/2}$-ubiquitinated EIN3 can still be recognised by the proteasome (Figure 4.4). Moreover, enhanced occupancy of EIN3 on the *ERF1* promoter of *upl3 upl4* mutants raises the possibility that UPL3/4 and the proteasome might be recruited to the chromatin to capture and processively degrade EIN3. To test this, we first performed ChIP experiments using an antibody against the proteasomal subunit RPN10. Compared to the no antibody control sample, no obvious enrichment of the proteasome at the *ERF1* promoter was detected in WT plants (Figure 4.12B). By contrast, the *ERF1* promoter was substantially enriched with proteasomes in the *upl3 upl4* mutant (Figure 4.12B). Importantly, as a proteasome-associated protein, YFP-UPL3 was also bound to the *ERF1* promoter (Figure 4.12C), suggesting that UPL3/4 mediates ubiquitination of EIN3 at the chromatin. Taken together with our finding that UPL3 interacts with EIN3 in an $SCF^{EBF1/2}$-dependent manner, these data demonstrate that EIN3 is relayed from the $SCF^{EBF1/2}$ ligase to proteasome-associated UPL3/4, which further remodels the ubiquitin chains on EIN3 at the chromatin to promote proteasomal degradation.

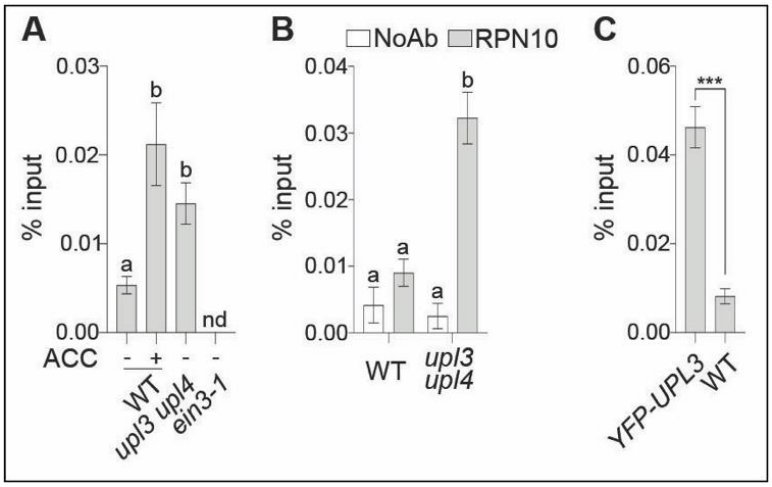

Figure 4. 12 EIN3 and UPL3-containing proteasome accumulate at ET-responsive promoters of *upl3 upl4* mutants.

(A) Seedlings were treated with 50 μM ACC or H_2O for 3 h before assessing EIN3 binding to the *ERF1* promoter by ChIP. The *ein3-1* mutant was used as a negative control. Data represent mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; $\alpha = 0.05$, $n = 3$); nd, not determined.

(B) ChIP was performed on seedlings using antibody against RPN10, no antibody immunoprecipitates (NoAb) served as a negative control. Data were analysed as in (A).

(C) YFP-UPL3 was immunoprecipitated using a GFP antibody, WT plants were used as a negative control. Data represent mean ± SD, asterisk (two-tailed t test, ***$p \leq 0.001$, $n = 3$) indicate statistically significant between samples.

4.3 Discussion

Developmental signals in eukaryotic cells are controlled and integrated by numerous TAs. Maintaining homeostasis of these master regulators to ensure precise gene expression often relies on the ubiquitin-proteasome pathway (Kodadek et al., 2006). The central node of the ubiquitin-proteasome pathway is selective recognition of unstable proteins by

E3 ligases, followed by catalysing formation of ubiquitin chains on the substrate. After being recognised by the proteasome, substrates are further processed for degradation, which includes ubiquitin chain remodelling. However, there is still a missing link between substrate ubiquitination by E3 ligases and substrate processing at the proteasome. Our study on the plant developmental regulator, EIN3, indicates that substrates may be physically handed over from E3 ligases to proteasome-associated HECT-type ligases, and that sequential ubiquitination by both is pivotal for processive proteasome-mediated clearance of transcription activators from the chromatin (Figure 4.13).

To continuously clear EIN3 in resting cells, the $SCF^{EBF1/2}$ complex recognises and initiates ubiquitination of EIN3 (Guo and Ecker, 2003, Potuschak et al., 2003). The fact that the interaction between UPL3 and EIN3 depends on the $SCF^{EBF1/2}$ complex suggests that the $SCF^{EBF1/2}$ complex may carry ubiquitinated EIN3 and deliver it to UPL3 (Figure 4.1). Interestingly, EIN3 stalled on proteasomes of *upl3 upl4* double mutants (Figure 4.4), indicating that proteasome-associated UPL3/4 are not required for recognition of ubiquitinated EIN3 by the proteasome. Rather, the SCF complex might contribute to unloading the substrate at the proteasome as has been previously suggested (Farras et al., 2001). Therefore, recognition and modification of EIN3 by the proteasome might be a two-step process that starts with capturing EIN3-attached ubiquitin chains by proteasome-associated ubiquitin receptors (Verma et al., 2004, Elsasser et al., 2004). After recognition by the proteasome, the $SCF^{EBF1/2}$ complex then engages with UPL3/4, which in turn directly interact with EIN3 to further remodel its attached ubiquitin chains. This substrate hand-over mechanism between the SCF complex and the proteasome-associated ubiquitin ligase might be a universal phenomenon, as several F-box proteins were found to associate with the proteasome (Peng et al., 2003, Verma et al., 2000).

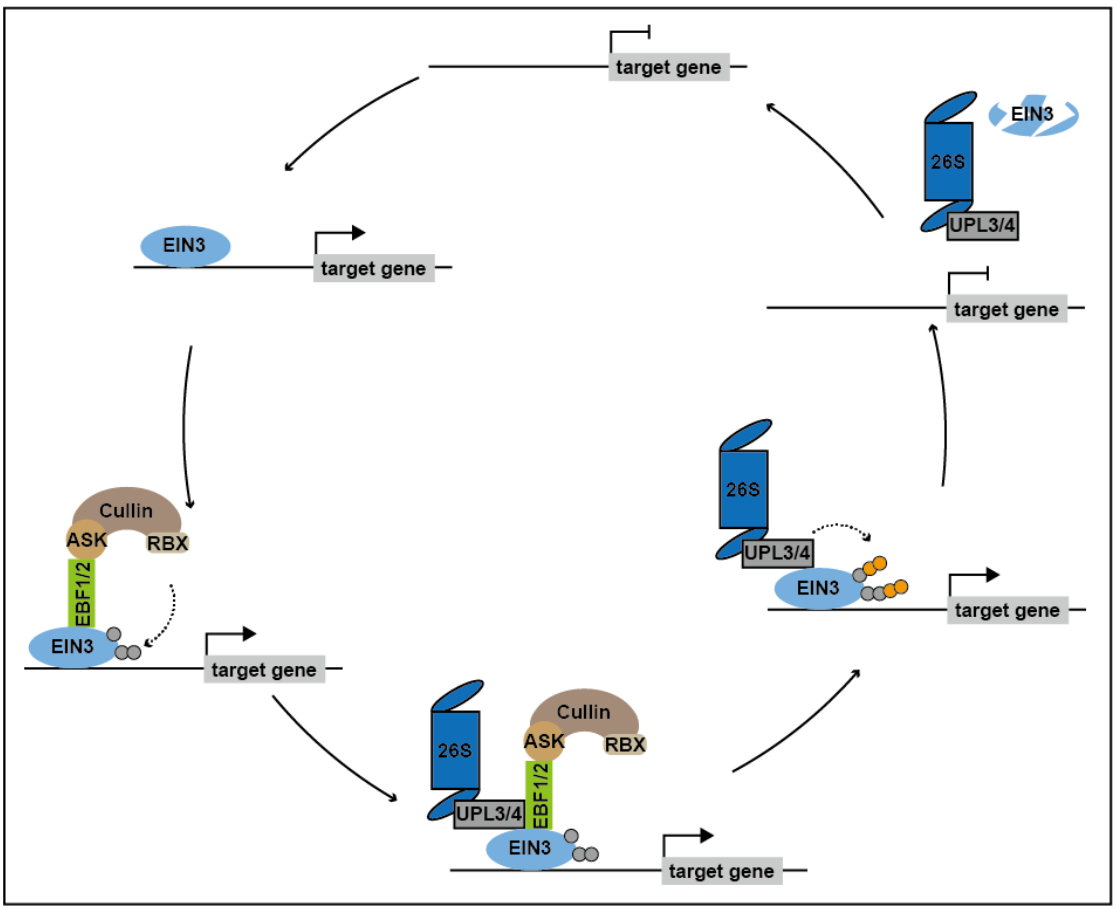

Figure 4. 13 Working model for how the relay of ubiquitin ligases is involved in proteasomal degradation of EIN3.

Occupancy of EIN3 to ethylene-responsive promoters triggers expression of target genes. To fine-tune transcriptional events, the $SCF^{EBF1/2}$ complex mediates formation of ubiquitin chains (grey circles) on the promoter-bound EIN3. EIN3 is then relayed from the $SCF^{EBF1/2}$ complex to the proteasome-associated UPL3/4, which further remodels EIN3 attached ubiquitin chains (orange circles), thereby promoting its processive degradation.

Taken together with our finding that UPL3/4 mediate polyubiquitination of EIN3, these data suggest that degradation of substrates requires remodelling of ubiquitin chains attached to proteasome-bound substrates. Importantly, this E3/E4-like ligases-mediated sequential ubiquitination of EIN3 likely occurs at the chromatin, as suggested by our evidence indicating that the proteasome and UPL3 localized to the *ERF1* promoter region (Figure 4.12). An impaired UPS enhanced accumulation of EIN3 at ethylene-responsive promoters and led to dysregulation of EIN3-dependent genes (Figure 4.8). GO analysis of these genes showed an enrichment for transcription factor activity (Figure 4.9), indicating that UPL3/4 are essential for EIN3-dependent transcriptional reprogramming and downstream transcription factor cascades. Our transcriptome analysis showed that in response to ethylene, UPL3/4 also regulate EIN3-independent pathways (Figure 4.8A), indicating that besides ubiquitinating EIN3, UPL3/4 might also target other EILs, which are functionally redundant with EIN3 (Chao et al., 1997). Another possibility is that UPL3/4 may regulate other signalling pathways that contribute to the ethylene response. Indeed, UPL3 has been identified as an essential regulator of global cellular ubiquitination (Figure 3.1) (Furniss et al., 2018), suggesting that it targets a large repertoire of substrates. Additionally, the functional significance of UPL3/4 in various aspects of plant biology, including leaf senescence, seed size, and trichome development, has been widely reported (Miao and Zentgraf, 2010, Miller et al., 2019, Downes et al., 2003). Therefore, besides targeting the EIN3 family transcription factors, UPL3/4 might also indirectly affect the ethylene signalling pathway through other cellular and biological processes.

The significance of remodelling substrate-anchored ubiquitin chains at the proteasome is also supported by studies on the yeast ubiquitin ligase, HUL5. Similar to

the role of UPL3, recruitment of HUL5 endows the proteasome with ubiquitin ligase activity (Crosas et al., 2006, Furniss et al., 2018), and depletion of *HUL5* resulted in incomplete degradation of an artificial substrate (Aviram and Kornitzer, 2010). These findings suggest that progressive ubiquitination by proteasome-associated ligases may be necessary for complete translocation of the substrate into the 20S proteolytic barrel. Moreover, another HECT-type protein, UFD4, was previously identified as a proteasome-associated ubiquitin ligase (Xie and Varshavsky, 2000). The mutated $UFD4^{\Delta N}$ that lacked the N-terminal proteasome-binding region can still bind and catalyse ubiquitination of substrates, however, it was impaired in proteasomal substrate proteolysis (Xie and Varshavsky, 2002, Ju et al., 2007). These data suggest that ubiquitination of the substrate at the proteasome is an indispensable process that possibly enhances the substrate's affinity for the proteasome and/or activates the ability of the proteasome to unfold and translocate the substrate (Finley, 2009, Reichard et al., 2016). Overall, proteasome-associated ubiquitin remodelling is a fundamental step to ensure processive degradation of substrates. Here we showed that this process is harnessed directly by $SCF^{EBF1/2}$ ligase to ensure its substrate EIN3 is appropriately cleared from ethylene-responsive target gene promoters.

Chapter 5:

UPL3 and UPL4 modulate plant immunity by targeting the U-box-type E3 ligase PUB22 for degradation

5.1 Introduction

To effectively fend off pathogenic microbes, plants have developed a sophisticated immune system to recognise and stop invaders. This starts with cell surface-localized pattern recognition receptors (PRRs) that detect conserved microbial molecules known as pathogen-associated molecular patterns (PAMPs), including bacterial flagellin and Elongation Factor-Tu (EF-Tu) as well as fungal chitin (Zhang and Zhou, 2010). Recognition of PAMPs triggers a response known as pattern-triggered immunity (PTI). Activation of PTI results in kinase signalling, a burst of reactive oxygen species (ROS) and calcium influxes, which together activate a series of cellular events, including expression of defence genes (Segonzac and Zipfel, 2011). At the physiological level, PTI leads to deposition of callose to reinforce the cell wall, the closing of stomata, and accumulation of chitinases, glucanases and other anti-microbial proteins (Yu et al., 2017). Consequently, prioritization of PTI responses usually slows the plant's growth (Huot et al., 2014). To achieve a defence-growth balance and avoid hypersensitivity to pathogens, plants tightly govern the activation of the immune system and also utilize a feedback mechanism to limit immune responses.

The ubiquitin-proteasome system not only plays a role in activating plant immune responses, but also in limiting their extend by modulating the homeostasis and activity of immune signalling proteins. In Arabidopsis, the U-box-type E3 ligase, PUB22, was reported to negatively regulate PTI and it is functionally redundant with its closely related homologs PUB23 and PUB24 (Trujillo et al., 2008). PUB22 regulates immune responses by directly ubiquitinating and promoting degradation of Exo70B2, a subunit of the exocyst complex that regulates homeostasis of transmembrane receptors (Wang et al., 2020). Accordingly, loss of *PUB22*, *PUB23* and *PUB24* functions results in increased cellular

immune responses, including an enhanced oxidative burst and up-regulation of PTI-responsive genes (Trujillo et al., 2008). Accumulation of PUB22 is induced by pathogen infection, and this allows plants to inhibit excessive and prolonged activation of the immune system (Furlan et al., 2017).

As a negative regulator of PTI, accumulation and activity of PUB22 are tightly regulated by diverse post-translational modifications, which provide a self-regulatory mechanism to manipulate the cellular immune response. Upon pathogen infection, association of Mitogen-Activated Protein Kinase 3 (MPK3) with PUB22 is rapidly induced, resulting in phosphorylation of PUB22 residues Thr-62 and Thr-88 that are located in and adjacent to the U-box domain, respectively (Furlan et al., 2017). Phosphorylation of PUB22 promotes its dimer-to-monomer switch and inhibits self-ubiquitination, thereby stabilizing PUB22 and inducing its accumulation. By contrast, under steady-state conditions, PUB22 forms dimers through the U-box domain, which mediates its self-ubiquitination and eventual targeting it to the proteasome for proteolysis (Furlan et al., 2017).

Previously, a yeast two-hybrid screen revealed that the N-terminal region of UPL3 interacts with PUB23 ligase (Furniss et al., 2018). This suggests that UPL3 may play a role in regulating the activities or stabilities of the closely related PUB22, PUB23 and PUB24. In the previous two chapters I reported that UPL3 acts at the proteasome to remodel ubiquitin chains of substrates involved in immune and developmental responses. In case of the developmental hormone ethylene, UPL3 directly interacted with an SCF ubiquitin ligase to receive substrates via a physical relay for further ubiquitination, which promoted proteasome processivity. Here, we demonstrate that UPL3 physically interacts with PUB22, but rather than functioning as a ubiquitin ligase relay, this interaction leads

to polyubiquitination and degradation of PUB22. We show that the *upl3 upl4* double mutant is impaired in perception of the PAMP, flagellin, and is highly susceptible to pathogens. Importantly, these phenotypes were largely due to dysregulation of PUB22 protein levels. Our findings imply that UPL3/4 not only regulate the degradation of substrates relayed by other ligases, but also target E3 ligases themselves. Thus, proteasome-associated UPL3/4 are a final checkpoint that may allow the proteasome to regulate the stability of E3 ligases in plant immunity.

5.2 Results

5.2.1 UPL3 interacts with PUB22

Our previous yeast two-hybrid screening showed that the N-terminal region of UPL3 interacts with the U-box-type E3 ligase PUB23 (Furniss et al., 2018). Given that PUB23 is closely related to major immune regulator PUB22, we tested if UPL3 also interacts with this protein. YFP-UPL3 protein was immunoprecipitated from *35S:YFP-UPL3* (in *upl3*) plants and incubated with PUB22-FLAG, which was produced using a cell-free protein synthesis system (Nomoto and Tada, 2018). As shown in Figure 5.1, YFP-UPL3 was found to specifically interact with PUB22-FLAG. Previous results have shown that MPK3-mediated phosphorylation and stabilisation of PUB22 occurs at the plasma membrane, whereas PUB22 interacts with its target substrate Exo70B2 in the cytoplasm (Furlan et al., 2017, Stegmann et al., 2012). Thus, the different stages of PUB22 activation correlate with its cellular localization. Therefore, we next performed a bimolecular fluorescence complementation (BiFC) assay to test the *in vivo* subcellular localization of the UPL3-PUB22 interaction. Specific fluorescent signals could only be detected when UPL3 and

PUB22 were co-expressed, indicating the two proteins also interact *in planta* (Figure 5.2).

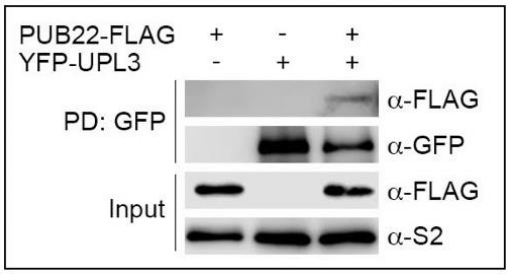

Figure 5. 1. UPL3 physically interacts with PUB22.

YFP-UPL3 was purified with GFP-Trap from *35S:YFP-UPL3/upl3* plants and incubated with *in vitro* synthesised PUB22-FLAG. Immunoprecipitated proteins were analysed by western blotting with antibodies against GFP and FLAG. Protein level of S2 was used as a loading control.

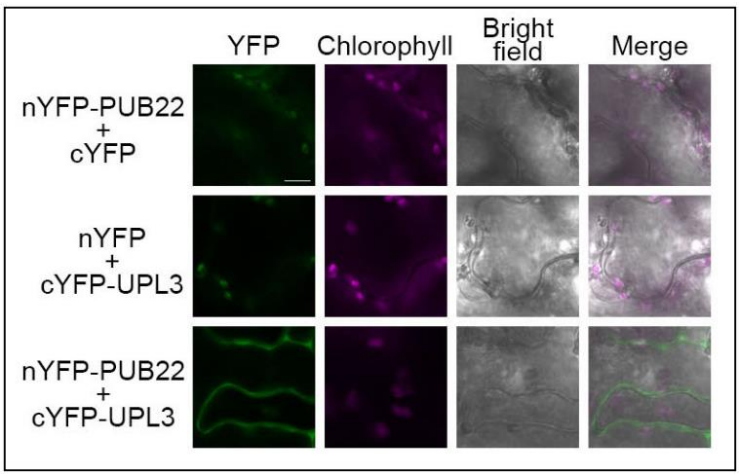

Figure 5. 2. Interaction between UPL3 and PUB22 detected by BiFC assay in *N. benthamiana* cells.

N. benthamiana leaves were infiltrated with *Agrobacterium* suspensions harbouring expression constructs with UPL3 and PUB22 fused to the N-terminal half (nYFP) or C-terminal half (cYFP) of YFP. Reconstituted YFP fluorescence and chloroplast autofluorescence (Chlorophyll) were evaluated 3 days after infiltration. Bar = 10 µm.

5.2.2 Ubiquitination and turnover of PUB22 is regulated by UPL3/4

In Chapter 3 and 4, I showed that UPL3 and UPL4 regulate proteasomal turnover of multiple unstable proteins by remodelling substrate-attached ubiquitin chains. Since UPL3 physically interacts with PUB22, we examined whether UPL3 could directly catalyse ubiquitination of PUB22. To this end, FLAG-UPL3 and YFP-PUB22 were transiently co-expressed in *N. benthamiana* leaves. While YFP-PUB22 was expressed at both the mRNA and protein levels, polyubiquitinated YFP-PUB22 was only detected when it was co-expressed with FLAG-UPL3 (Figure 5.3A and B). These results indicate that UPL3 catalyses ubiquitination of PUB22.

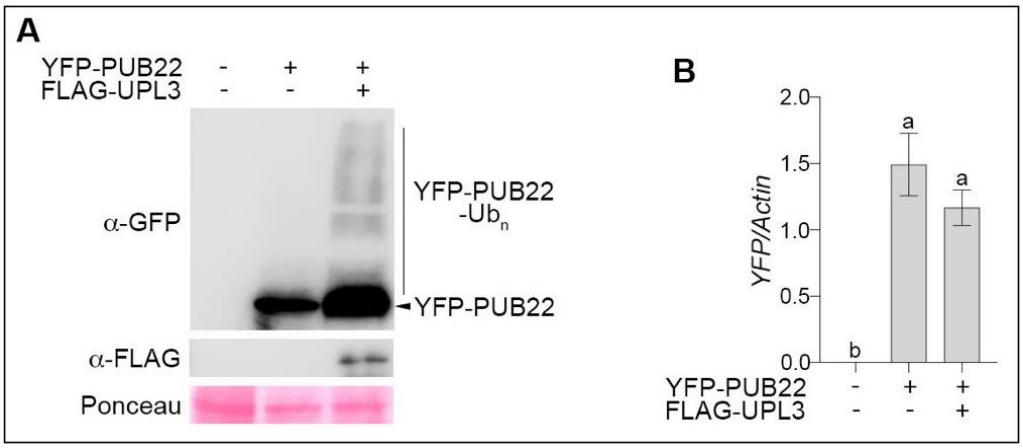

Figure 5. 3 UPL3 ubiquitinates PUB22 *in vivo*.

(A) *N. benthamiana* leaves were infiltrated with *Agrobacterium* suspensions harbouring indicated constructs. YFP-PUB22 and FLAG-UPL3 were detected by western blotting using GFP and FLAG antibodies, respectively. Ponceau S staining is shown as a loading control. Unmodified (arrowhead) and ubiquitinated (Ub_n) forms of YFP-PUB22 are indicated.

(B) Gene expression of *YFP-PUB22* in the indicated samples was examined. *NbActin* was used as a reference gene. Data represent mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 3).

Next, we tested whether UPL3/4 regulate stability of PUB22. We performed a cell-free degradation assay by incubating recombinant YFP-PUB22 with protein extracts from WT or *upl3 upl4* mutant plants. YFP-PUB22 protein was degraded rapidly when incubated with protein extracts from WT plants (Figure 5.4). However, high level of YFP-PUB22 remained in protein extracts from the *upl3 upl4* mutant even at 60 min after incubation (Figure 5.4A and B). Because PUB22 is degraded by the proteasome under steady-state condition (Furlan et al., 2017), we then questioned if accumulation of PUB22 is affected by mutation of *UPL3* and *UPL4*. To test this, we generated transgenic plants that constitutively expressed *GFP-PUB22* in either the WT or *upl3 upl4* mutant

background. Although *GFP-PUB22* transcript levels were comparable, *upl3 upl4* mutants accumulated much higher levels of GFP-PUB22 protein than WT plants in which it was barely detectable (Figure 5.5A and B). Moreover, treatment with flg22 enhanced this accumulation of GFP-PUB22 even further (Figure 5.5A). Taken together, our findings show that UPL3/4 directly catalyse ubiquitination of PUB22, thereby targeting it for proteasome-mediated turnover under stead-state conditions.

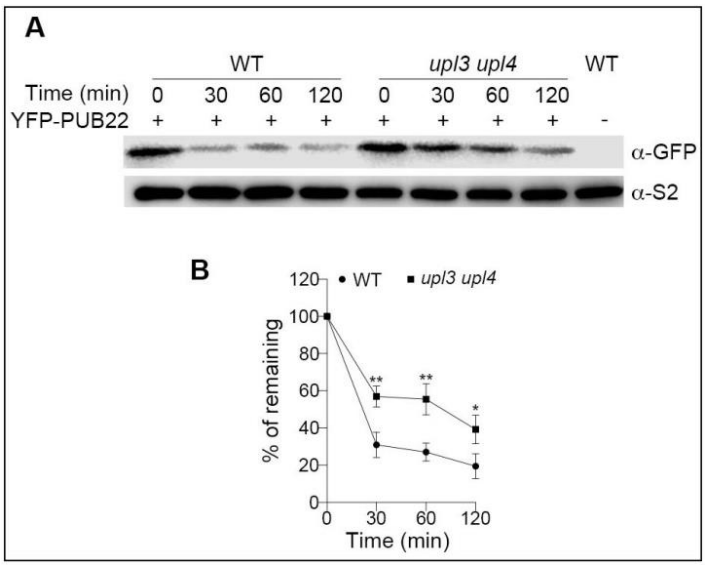

Figure 5. 4 YFP-PUB22 is stabilized in the *upl3 upl4* mutant.

Recombinant YFP-PUB22 was incubated with protein extracts from WT or the *upl3 upl4* mutant, and samples collected at the indicated time points. YFP-PUB22 was detected by using a GFP antibody and protein levels of S2 were used as a loading control (A). The percentage of remaining proteins was calculated relative to the quantification at 0 min (B). Data represent the mean ± SD, statistical significance compared to WT plants is indicated by asterisks (Student's *t*-test, **p* < 0.05, ***p* < 0.01, n=3).

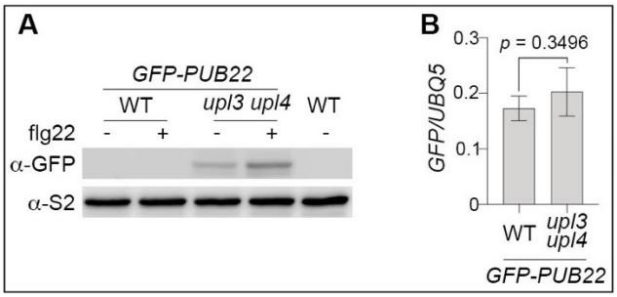

Figure 5. 5 Loss of *UPL3* and *UPL4* function enhances accumulation of PUB22.

(A) Seedlings expressing *pUBQ10:GFP-PUB22* in the indicated backgrounds were treated with 1 μM flg22 for 1 h. GFP-PUB22 protein levels were monitored by immunoblotting using a GFP antibody, S2 level was used as a loading control.

(B) *GFP-PUB22* transgene expression was normalized to constitutively expressed *UBQ5*. Data represent the mean ± SD with Student's *t*-test p value indicated (n=3).

5.2.3 UPL3/4 regulate PUB22-mediated PTI signalling

PUB22 and its paralogues, PUB23 and PUB24, regulate immunity by limiting PTI signalling. A major hallmark of PTI is the rapid production of ROS upon detection of pathogen-derived PAMPs at the early stage of pathogen infection (Torres et al., 2006). Because UPL3 and UPL4 regulate PUB22 stability, we examined if PAMP-induced ROS production is affected in the *upl3 upl4* mutant. Compared to WT plants, the *upl3 upl4* mutant exhibited reduced amplitude and duration of ROS production upon treatment with the bacterial PAMP, flg22 (Figure 5.6). By contrast, ROS production was dramatically elevated in the *pub22 pub23 pub24* mutant (Figure 5.6). To examine if dampened ROS accumulation in the *upl3 upl4* mutant was due to elevated PUB22 and possibly PUB23/24 levels, we generated a *upl3 upl4 pub22 pub23 pub24* quintuple mutant. Indeed, compared to *upl3 upl4* mutants, the flg22-induced ROS burst was recovered in the

quintuple mutant (Figure 5.6), indicating that UPL3/4 may positively modulate PAMP-triggered oxidative burst by regulating PUB ligases. Although not significant, the flg22-induced ROS burst was slightly higher in the quintuple mutant compared to the *pub22 pub23 pub24* triple mutant, suggesting UPL3/4 might also control additional factors (Figure 5.6).

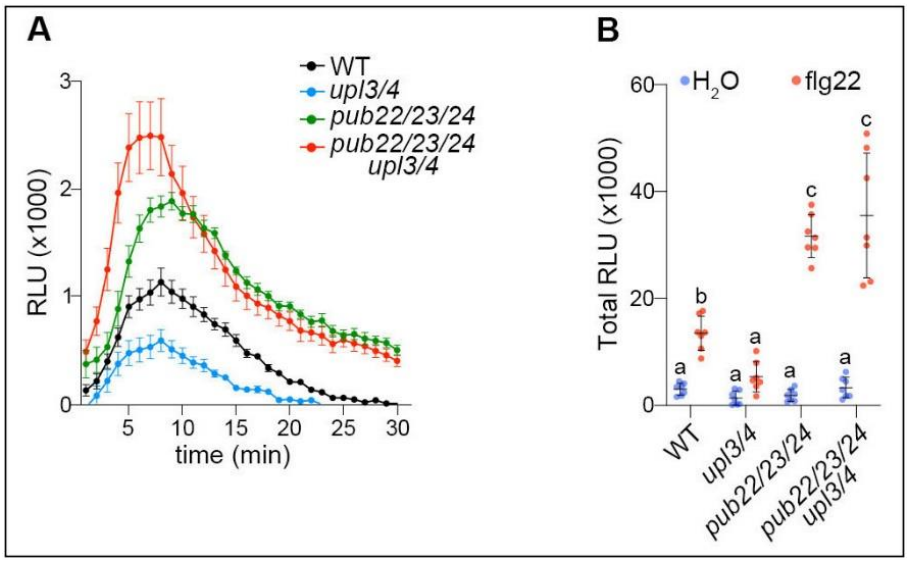

Figure 5. 6 UPL3/4 positively regulate flg22-induced oxidative burst

(A) Leaf discs from 4-week-old plants were treated with 1 µM flg22, and ROS production is represented as relative light units. Data represent the mean ± SE (n = 7).

(B) Total ROS production over 30 min was analysed, data represent the mean ± SD, lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 7).

Due to energetic defence-growth trade-offs, sustained activation of PTI is associated with attenuation of plant growth. To further examine if UPL3 and UPL4 are required for sustained PAMP-induced PTI responses, we examined root growth in in different genotypes in response to the elicitor flg22. As expected, treatment with flg22 strongly inhibited root growth of WT plants (Figure 5.7A and B). By contrast, flg22 inhibited root growth significantly less in *upl3* and *upl4* single mutants (Figure 5.7 A and B). The *upl3 upl4* double mutant exhibited shorter roots even in absence of flg22 (Figure 5.7A), which is likely due to enhanced ethylene signalling as described in Chapter 4. Nonetheless, compared to the single mutants, the deficiency of flg22-induced root inhibition was further accentuated in the *upl3 upl4* double mutant (Figure 5.7 A and B), indicating that *upl3 upl4* mutants are even less sensitive to flg22. These data show that UPL3 and UPL4 regulate sustained PTI responses.

Next, we assessed if reduced sensitivity of *upl3 upl4* mutants to flg22 was due to a lack of PUB22/23/24 degradation. Because PUB22/23/24 suppress PTI, the corresponding *pub22 pub23 pub24* triple mutant displays elevated immune responses and increased flg22-induced root inhibition (Trujillo et al., 2008)(Figure 5.7C and D). More importantly, when we mutated all three PUBs in the *upl3 upl4* mutant background, this resulted in complete recovery of the *pub22 pub23 pub24* triple mutant root growth phenotype (Figure 5.7C and D). These data demonstrate that UPL3/4 control sustained PTI signalling by modulating the levels of PUB ligases.

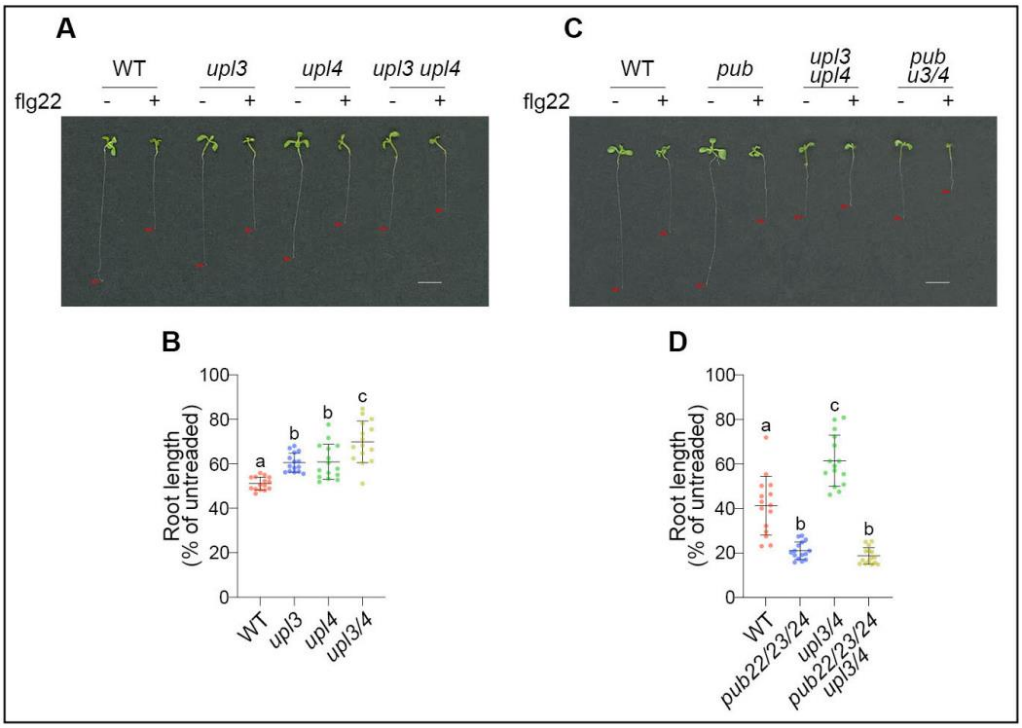

Figure 5. 7 UPL3/4 are required for sustained PUB22-mediated PTI responses.

(A) and (C) 4-day-old seedlings were transferred onto MS agar medium supplemented with or without 1 µM flg22. Morphological phenotypes were analysed 6 days after transfer. Arrowheads point to the root tips. Bar = 50 mm.

(B) and (D) Length of the main root after flg22 treatment is shown as a percentage of the control root length. Data present the mean ± SD; lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 15).

5.2.4 UPL3/4-mediated regulation of PUB22 stability is required for disease resistance

Because UPL3/4 directly target PUB22/23/24 for ubiquitination and proteasomal degradation, this regulatory mechanism might be required for appropriate activation of immune responses. To test this hypothesis, WT, *upl3*, *upl4*, and *upl3 upl4* plants were spray-inoculated with *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Compared to WT, the *upl3* mutant showed enhanced susceptibility to this pathogen, whereas the *upl4* mutant displayed similar levels of resistance as the WT plants (Figure 5.8A and B). Strikingly, inoculation of the *upl3 upl4* double mutant resulted in severely enhanced growth of *Pst* DC3000 to levels beyond those observed in the *upl3* single mutant (Figure 5.8A and B), indicating that *UPL3* and *UPL4* share functional redundancy in controlling disease resistance.

Next, we tested whether the defective immune response of *upl3 upl4* mutants was due to dysregulation of PUB22 and its closest homologues. Consistent with previous finding, the *pub22 pub23 pub24* mutant was more resistant to *Pst* DC3000 infection than the WT, whereas the *upl3 upl4* mutant was more susceptible (Figure 5.8C and D) (Trujillo et al., 2008). However, mutation of *PUB22/23/24* significantly supressed the disease susceptible phenotype of the *upl3 upl4* mutant to levels similar to those seen in WT plants (Figure 5.8C and D). These results suggest that the disease susceptible phenotype of *upl3 upl4* mutants is due to elevated levels of PUB ligases. In summary, our findings have uncovered that UPL3/4 modulate PTI signalling and its effectiveness against pathogens by mediating ubiquitination and promoting proteasomal degradation of PUB22.

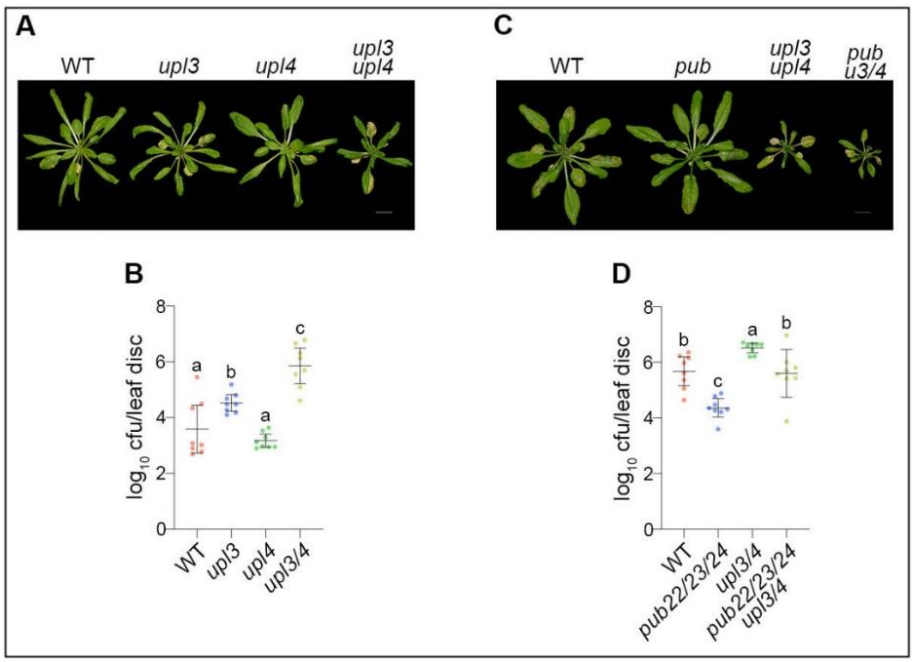

Figure 5. 8 UPL3/4-mediated regulation of PUB22/23/24 is required for disease resistance.

(A) and (C) Adult plants were spray inoculated with bacterial suspension of 5 x 10^8 cfu/mL. Morphological phenotypes were analysed at 4 days after inoculation. Bar = 1 cm.

(B) and (D) Bacterial growth was assessed, data represent the mean ± 95% confidence limits. Lowercase letters indicate statistically significant differences between samples (Tukey HSD ANOVA test; α = 0.05, n = 8).

5.3 Discussion

One of the major mechanisms that manipulates biological events in eukaryotic cells is the modification of proteins with ubiquitin and subsequent targeting for proteasomal degradation. This process tightly modulates homeostasis of various unstable proteins, and serves an important role in fine-tuning multiple signalling pathways. In addition to this, components of the ubiquitin system, particularly E3 ligases that selectively recognise target proteins, are also strictly regulated. In many cases E3 ligases auto- or self-ubiquitinate, which can alter their activities or promotes their proteasomal turnover, thereby fine-tuning the timing and rate of substrate ubiquitination (de Bie and Ciechanover, 2011).

Self-regulation of E3 ligases has been implicated in the plant defence response (Trujillo and Shirasu, 2010). In Arabidopsis, the U-box-type E3 ligase, PUB22, is known as a negative regulator of PTI. Under steady-state conditions, PUB22 forms oligomers and the U-box catalyses self-ubiquitination that promotes proteasomal degradation (Furlan et al., 2017). In this study, we show that the proteasome-associated ubiquitin ligases, UPL3/4, also mediate polyubiquitination of PUB22 and that this process is required for its turnover. In accordance, degradation of PUB22 was impaired in the *upl3 upl4* mutant, which led to elevated levels of PUB22 and compromised PTI signalling (Figure 5.4 and 5.5). Upon flg22 treatment, the *upl3 upl4* mutant exhibited attenuated ROS burst and reduced root growth inhibition (Figure 5.6 and 5.7), indicating that UPL3/4 are essential for both early and late immune responses. These defects were rescued by mutation of the *PUB22*, *PUB23*, and *PUB24* genes (Figure 5.6 and 5.7), indicating that UPL3/4 control immune responses at least in part via PUB22 and its closest paralogues. The fact that the *upl3 upl4 pub22 pub23 pub24* quintuple mutant behaved like WT rather

than attaining elevated disease resistance similar to the *pub22 pub23 pub24* triple mutant may suggest that other immune pathways are also regulated by UPL3/4. Indeed, UPL3 is required for establishing the SA-dependent immune responses, as the *upl3* mutant displayed attenuated SA-induced transcriptional reprogramming (Chapter 3)(Furniss et al., 2018). SA plays key roles in activating systemic acquired resistance (SAR) and effector-triggered immunity (An and Mou, 2011, Fu et al., 2012, Spoel and Dong, 2012). In this respect, defects in different SA-dependent responses might also contribute to the compromised immune system of the *upl3 upl4* mutant. Taken together, UPL3/4 are essential for limiting pathogen invasion via multi-layered regulation of the immune response.

We discovered that UPL3 interacts with PUB22 at the plasma membrane (Figure 5.2), where the PUB22 ubiquitination is thought to take place (Figure 5.3). Likewise, the interaction between MPK3 and PUB22 was also detected at the plasma membrane (Furlan et al., 2017). MPK3-mediated phosphorylation promotes the dimer-to-monomer switch of PUB22, thereby preventing its self-ubiquitination and stabilizing PUB22 (Furlan et al., 2017). Previous results showed that PUB22 exerts its effect on the immune response by targeting Exo70B2 for degradation (Stegmann et al., 2012). Interestingly, Exo70B2 physically interacts with PUB22 in the cytoplasm, where the ubiquitination of Exo70B2 is catalysed (Stegmann et al., 2012). These results suggest that under steady-state conditions, PUB22 is ubiquitinated and degraded at the plasma membrane to maintain a low protein level, and this action requires UPL3/4-mediated ubiquitin chain assembly or remodelling at the proteasome. In pathogen infected cells, monomerization of PUB22 is triggered by phosphorylation, and the PUB22 monomer translocates from the plasma membrane to the cytoplasm, where it recognises and ubiquitinates substrates

to moderate the immune responses.

Besides PUB22/23/24, the function of several other PUBs has been implicated in plant defence signalling (Trujillo, 2018). Perception of bacterial flagellin by FLS2 was the first characterised PAMP-receptor pair *in planta* (Gomez-Gomez and Boller, 2000, Chinchilla et al., 2007). Plasma membrane localized FLS2 recognises a conserved 22 amino acid peptide fragment of flagellin, known as flg22 (Sun et al., 2013, Chinchilla et al., 2006), and this process rapidly triggers association of PUB12/13 with FLS2 (Lu et al., 2011). The closely related PUB12 and PUB13 directly catalyse ubiquitination of FLS2, which induces its turnover and suppresses plant innate immunity (Lu et al., 2011). Moreover, perception of flg22 causes internalization of FLS2, and this process is indispensable for the flg22-triggered responses (Beck et al., 2012, Robatzek et al., 2006). Recent findings found trafficking of FLS2 is partially regulated by Exo70B1 and Exo70B2, which promote accumulation of FLS2 at the plasma membrane (Wang et al., 2020). Thus, PUB ligases are involved in controlling homeostasis of immune receptors at multiple levels. Our study now predicts that UPL3/4 may also be involved in regulating immune receptors by modulating the stability of PUB ligases.

Chapter 6:

General discussion

Targeting of proteins for degradation by the eukaryotic proteasome plays a critical role in shaping the intracellular signalling network. The central step of this mechanism is to label substrates with ubiquitin molecules, which involves E3 and E4 ligases that catalyse formation and elongation of ubiquitin chains. However, how ubiquitinated substrates are processed upon arrival at the proteasome, and influenced by proteasome-associated accessory proteins remains largely unknown. In this study, we revealed that proteasome-associated HECT-type ubiquitin ligases ensure substrate-attached ubiquitin chains are further remodelled at the proteasome and that this process is indispensable for processive degradation of substrates. Importantly, we found that HECT-type ubiquitin ligase activities were imperative for proteasome-mediated regulation of plant developmental and stress responses.

6.1 Ubiquitin chain remodelling at the proteasome

The proteasome is a multi-subunit protease that is ubiquitous in eukaryotic organisms. In addition to the highly conserved subunits, proteasome function is also influenced by various proteasome-associated accessory proteins, such as ubiquitin ligases. In Chapter 3 we found that the N-terminal armadillo repeat-containing region of UPL1 associated with the proteasome (Figure 3.2A). Although the cellular level of ubiquitin conjugates was reduced in *upl1* mutants (Figure 3.1B), *in vitro* proteasome-associated ubiquitin ligase activity was not affected (Figure 3.2B). Since UPL1 and UPL2 exhibited extremely high sequence similarity (Bates and Vierstra, 1999), one explanation is that UPL1 might be functionally redundant with UPL2. In absence of UPL1, association of UPL2 with the proteasome might be sufficient for forming ubiquitin conjugates *in vitro*, particularly because our *in vitro* assays utilised the promiscuous yeast E2 enzyme UbcH5c, which

may be more active than the unknown Arabidopsis E2 enzymes that UPL1 and UPL2 utilise *in vivo*. However, redundancy between UPL1 and UPL2 cannot be examined as the *upl1 upl2* double mutant was unobtainable, likely because these two genes are linked on the same chromosome or because of homogenous lethality. Functional redundancies between different UPLs were also apparent from our SA-induced transcriptome analysis of *upl1* and *upl5* mutants in which the lists of dysregulated genes partially overlap (Figure 3.4C).

Our findings show that UPLs are recruited by the proteasome and UPL3 in particular, offers the proteasome with ubiquitin ligase activity. Association of UPL3 with the proteasome enables ubiquitinated substrates to be further remodelled, which is required for substrate turnover (Figure 3.8, 4.3, 5.3, and 5.4). At present it remains unclear if UPL3 remodels existing substrate-attached ubiquitin chains or if it generates new chains to ensure the substrate maintains high affinity for the proteasome during translocation into the proteolytic barrel. Regardless, the enhanced stability of multiple substrates in the *upl3* or *upl3 upl4* mutants suggests that remodelling or generation of substrate-attached ubiquitin chains at the proteasome might be a universal process (Figure 3.8, 4.3, and 5.4). We showed that this process is particularly important for appropriate regulation of hormone-mediated developmental and stress-induced transcriptional responses. Loss of *UPL3* and *UPL4* function led to accumulation of TAs at the promoter region (Figure 3.10 and 4.12), indicating that UPL3/4 are essential for clearing TAs from the chromatin. Nevertheless, lack of UPL3/4-mediated ubiquitination has distinct effects on different TAs. In Chapter 3, we found that absence of UPL3/4 activity led to accumulation of inactive NPR1 at a SA-responsive promoter, which prevented expression of target genes (Figure 3.6C and 3.10A). As a well-known

transcriptional cofactor, NPR1 associates with WRKY70 to silence immune genes under steady-state conditions (Saleh et al., 2015). However, accumulation of SA leads to dissociation of NPR1 from WRKY70, and promotes interaction between NPR1 and TGA transcription factors to form a transcriptional trans-activation complex (Despres et al., 2000, Boyle et al., 2009). Therefore, one possible explanation of why inactive NPR1 accumulates in the *upl3* mutant is that ubiquitination of NPR1 might manipulate its interaction with different transcription factors or components of the transcription preinitiation complex, thereby fine-tuning expression of immune-responsive genes.

By contrast, active EIN3 was highly accumulated in the *upl3 upl4* mutant, and this led to induction of downstream ethylene-responsive genes and constitutive ethylene-dependent phenotypes (Figure 4.8 and 4.10). The occupancy of EIN3 at the *ERF1* promoter region was highly enhanced in the *upl3 upl4* mutant (Figure 4.12A). Strikingly, the proteasome also occupied this ethylene-responsive promoter and it could only be detected in the *upl3 upl4* mutant but not in WT plants (Figure 4.12B). Since proteasomal degradation is a transient process, this suggests the proteasome is released from the promoter region after destroying TAs, and this association is hence difficult to detect in WT plants. Nonetheless, strong binding of YFP-UPL3 at a *ERF1* promoter region was detected under steady-state condition (Figure 4.12C). This suggests that either the association of UPL3 with the proteasome is not constitutive but rather hormone-induced, or that expression of *YFP-UPL3* did not completely recover the *upl3* mutant phenotype, thereby trapping some proteasomes at the *ERF1* promoter.

Association of the proteasome with chromatin has been described in yeast and animals, and the non-proteolytic activity of the proteasome is also important for activating gene expression (Muratani and Tansey, 2003, Morris et al., 2003, Gonzalez et al., 2002).

For example, components of both yeast 19S and 20S particles were found to bind to the promoter of *CDC20*, which is a substrate adaptor of the APC/C ubiquitin ligase complex. Expression of *CDC20* was not induced in a mutant of the proteasome ATPase subunit Rpt6 (Morris et al., 2003), illustrating the proteasome is required for transcriptional responses. Interestingly, blocking proteolytic activity of the proteasome had limited effects on *CDC20* expression, implying that activation of *CDC20* requires non-proteolytic activity of the proteasome (Morris et al., 2003). We cannot completely rule out that proteasome non-proteolytic activity might contribute to the enhanced expression of ethylene-responsive genes in the *upl3 upl4* mutant. However, the evidence that EIN3 stalled at proteasomes of *upl3 upl4* mutants suggests that chromatin-bound proteasomes are more likely responsible for mediating turnover of EIN3.

6.2 UPLs integrate plant hormone signalling

Plants have developed a wide range of strategies to adapt to various environmental conditions. Among these mechanisms, plant hormones play a vital role not only in perceiving and fine-tuning stress signals, but also in ensuring optimal growth. Plant hormones exert their effects via different transcriptional regulators that orchestrate transcriptional reprogramming. Hormonal signalling networks are highly sophisticated with points of crosstalk amongst different hormones, which is thought to maximize plant performance. For example, prioritisation of immune responses over plant growth and development is a well-recognised phenomena that is orchestrated by various plant hormones (Denance et al., 2013). Moreover, to avoid the hijacking of hormone pathways by pathogens, plants have evolved specific antagonistic and synergistic interactions between different defence hormones (Spoel and Dong, 2008). SA and ethylene are

essential plant hormones that manipulate many developmental processes and immune responses (Leon-Reyes et al., 2009, Robert-Seilaniantz et al., 2011). To provide optimal responses to different environmental cues, plants need to maintain a balance between the SA- and ethylene-dependent signalling pathways. This requires strict regulation of protein levels of the key hormone-specific TAs in the cell.

In this thesis, we found that UPL3 and UPL4 mediate ubiquitination of both NPR1 and EIN3, and are required for proteasomal turnover of these TAs. Recent studies show that SA-induced NPR1 interferes with binding of EIN3 to the target gene promoter, thereby repressing ethylene-dependent apical hook development (Huang et al., 2020). Conversely, EIN3 and EIL1, negatively regulate SA accumulation by directly repressing the SA biosynthesis gene, *SID2* (Chen et al., 2009). Therefore, to mediate turnover of NPR1 and EIN3, UPL3/4 might play an important role in fine-tuning the SA/ethylene-directed growth-defence trade-off. Besides SA and ethylene signalling pathways, UPL3 also modulates gibberellin (GA) responses, as mutation of *UPL3* caused a GA-hypersensitivity phenotype (Downes et al., 2003). Interestingly, our transcriptome analysis showed numerous JA- and auxin-responsive genes are differentially expressed in the *upl3 upl4* mutant (data not shown), suggesting potential involvement of UPL3/4 in JA and auxin signalling. In addition, it was previously reported that UPL3 controls multiple aspects of plant development by mediating proteasomal degradation of several transcriptional regulators, including LEC2, GL3 and EGL3, and WRKY53, which regulate seed size, trichome development, and leaf senescence, respectively (Miller et al., 2019, Patra et al., 2013, Miao and Zentgraf, 2010). Taken together, these findings suggest that UPL3 and UPL4 regulate various developmental signals by targeting different regulatory proteins and TAs, and therefore could potentially play a key role in hormonal crosstalk.

6.3 Multi-layered regulation of immune response by UPLs

Pathogens enter plant cells via wounds or penetrate through the epidermis, and acquire nutrients in the apoplast to proliferate. Plants, in turn, utilize a two-branched immune system to surveillance and withstand pathogen attack. The first branch, known as PTI, recognizes conserved pathogen molecules and then establishes a series of immune responses. In Chapter 5 it was demonstrated that upon pathogen recognition, UPL3/4 are involved in activation of PTI by interacting with PUB ligases. The *upl3 upl4* mutant was more susceptible to pathogen infection than the WT, while this defective immune phenotype was suppressed by mutation of *PUB22*, *PUB23*, and *PUB24* (Figure 5.8). Indeed, loss of *UPL3* and *UPL4* function led to overaccumulation of PUB22, which negatively regulates plant defences. Nevertheless, the fact that the *upl3 upl4 pub22 pub23 pub24* mutant was less resistant to pathogens than the *pub22 pub23 pub24* mutant supports the hypothesis that UPL3/4 might also regulate other pathways to prevent bacterial entry or propagation.

The functions of PUB22 and PUB23 have been previously linked to water stress responses (Cho et al., 2008), whereas a patent filed by KEYGENE implicates UPL3 in drought resistance (Deslattes Mays et al., 2013). It is well known that stomatal aperture is dramatically affected by water balance and the primary reaction to drought stress is stomatal closure, which prevents water loss (Daszkowska-Golec and Szarejko, 2013). In addition, stomata also serve as ports for microbial entry into plant tissues. Consequently, plant immune responses induce stomatal closure to restrict pathogen ingress (Melotto et al., 2006). In Chapter 5, adult plants were spray inoculated with pathogens rather than pressure infiltrated, so that stomatal immune responses were not bypassed. Because the *pub22 pub23 pub24* mutant was more resistant, whereas the *upl3 upl4* mutant was more

susceptible to pathogen infection (Figure 5.8), one possibility is that PUB22, PUB23, and PUB24 might regulate stomatal activity or patterning in response to abiotic and biotic stresses. Hence, the *pub22 pub23 pub24* mutant may have displayed enhanced stomatal restriction of bacterial entry, while the *upl3 upl4* mutant failed to close its stomata due to elevated levels of PUB22/23/24. Additionally, compromised pathogen-induced SA accumulation and attenuated SA-dependent transcriptional reprogramming in the *upl3 upl4* mutant might also affect stomatal closure (Melotto et al., 2006).

To counteract host PTI responses, pathogens deliver effectors that suppress host immunity. Interestingly, some pathogen effectors such as HopM1, HopAO1, HopA1, and HopG1 associate with the proteasome and act as putative proteasome inhibitors (Ustun et al., 2016). Moreover, an effector from the potato cyst nematode, Globodera pallida, interacts with the potato functional homologue of UPL3. Interestingly, nematode-induced transcriptional responses were dysregulated in the *upl3* mutant (Diaz-Granados et al., 2020). As pathogen and parasite effectors are considered to target well connected signalling hubs, these reports and our findings reported here suggest that UPL3 is an essential player in orchestrating multiple cellular signalling networks.

In response to pathogen effectors, plants have developed intracellular receptors that recognise their presence in the plant cell and trigger a second layer of plant immune responses known as ETI. The major hallmark of ETI is the hypersensitive response (HR), which results in host programmed cell death (PCD) to stop pathogen growth (Cui et al., 2015). Studies have shown that SA signalling underpins ETI. Notably, the SA-deficient mutants, *sid2* and *eds2*, displayed attenuated ETI response (Nawrath and Metraux, 1999). It was described in Chapter 3 that pathogen-induced accumulation of SA was reduced in the *upl* mutants, (Figure 3.5C), suggesting that UPLs might also manipulate ETI by

regulating biosynthesis of SA.

Taken together, this study uncovers a multi-layered regulation of the plant immune system by UPLs. In the nucleus, UPLs catalyse ubiquitination of transcriptional regulators to govern transcriptional reprogramming. Turnover of cytoplasm-localized immune-related proteins, such as PUBs, also requires UPLs, and this is critical for perceiving pathogens and stimulating the immune responses. UPL-mediated regulation of diverse layers of the defence system is indispensable for PTI and possibly ETI, and is also crucial for establishing systemic immunity.

6.4 Conclusions and impact

The work in this thesis uncovers vital roles of HECT-type ubiquitin ligases in mediating substrate ubiquitination at the proteasome. The data presented has shown that substrates are relayed from E3/E4 ligases to proteasome-associated UPL ligases for further ubiquitin chain remodelling. By testing different target proteins, we can conclude this process is vital for proteasomal turnover of unstable proteins *in planta*. Functionally, UPLs are involved in multiple signalling pathways, and thus are essential for coordinating plant responses to environmental and developmental cues. Exploring the roles of HECT-type ligases in immune signalling and developmental responses in Arabidopsis can provide important clues for development of agricultural improvement strategies that mitigate against crop disease and improve crop yield.

HECT-type ubiquitin ligases are implicated in a wide range of biological functions, including tumorigenesis, cell proliferation, and cancer development. Moreover, association of HECT ubiquitin ligases with the proteasome has been shown in several organisms, including yeast and humans. Accordingly, rather than being specific only to

plants, proteasome-associated ubiquitin chain remodelling by HECT-type ligases is likely a universal mechanism for proteasomal substrate degradation in eukaryotic cells. As many regulators and transcription factors implicated in human disease are unstable, our mechanistic findings that substrates are relayed between E3/E4 ligases and proteasome-associated HECT ubiquitin ligases could therefore be used as a basis for future research in mammalian cells and the biomedical field.

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