Exploring the Roles of GCN2 and the m^{6}A RNA Modification in Plant Immunity

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

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Abstract

Plants have evolved a robust immune system to fend off pathogens. This response must be tightly regulated, as aberrant activation can have detrimental effects. Much work has been done to understand the transcriptional responses in plant immunity, but less is known about post-transcriptional mechanisms. Here I examine the roles of the general control nonderepressible 2 (GCN2) kinase and the N^6-methyladenosine (m^6A) RNA modification in regulating plant immunity at the post-transcriptional level.

A previous study revealed that the master immune regulator TBF1 (a transcription factor) is essential for mediating a defense response. TBF1 is under the control of 2 upstream open reading frames (uORFs) which inhibit translation of TBF1's major open reading frame (mORF). These uORFs' sequences are enriched with codons for aromatic amino acids, especially phenylalanine. Pathogen treatment resulted in the induction of uncharged $tRNA^{phe}$ and eIF2α phosphorylation. It was proposed that GCN2 would mediate eIF2α phosphorylation and allow readthrough of TBF1's mORF, analogous to GCN2's role in promoting translation of GCN4 upon amino acid starvation. To test this, eIF2α phosphorylation was examined in *gcn2* mutant plants upon pathogen infection using a phoshpo-specific eIF2α antibody. eIF2α phosphorylation was not observed in *gcn2* plants upon pathogen treatment and

therefore GCN2 is likely to be the kinase responsible for this induced eIF2α phosphorylation. To test GCN2's role in positively regulating TBF1, a bacterial infection assay was undertaken with *gcn2* plants. Compared to wild-type plants, *gcn2* plants did not have any significant difference in bacterial growth and therefore GCN2 is unlikely to play a role in regulating TBF1 or plant immunity.

The m^{6}A modification is the most abundant internal modification present in mRNAs and has been found to regulate several aspects of mRNA metabolism and biological processes. There is less known about m^{6}A in plants, though it has been found to regulate stability of transcripts and is important for development and the salt stress response. It is unknown whether m^{6}A plays a role in plant immunity. To address this, m^{6}A deficient plant lines were used and bacterial infection assays undertaken. These lines displayed significantly higher levels of bacterial growth (susceptibility) and thus m^{6}A plays a positive role in plant immunity. Further assays found that m^{6}A was essential for fully mediating pattern-triggered immunity (PTI) and salicylic acid (SA)-mediated immune responses. m^{6}A-seq was used to map the dynamics of m^{6}A across the transcriptome in response to the immune inducers SA and elf18 (a microbe-associated molecular pattern). Hundreds of pathogen-induced methylation sites were uncovered and gene ontology (GO) analysis revealed a predominance of defense/immune-related transcripts.

In summary, this dissertation work found that although GCN2 is required for pathogen induced eIF2α phosphorylation, it is dispensable for defense against a bacterial pathogen, and thus is unlikely to be a regulator of TBF1 or the immune response. On the other hand, m^{6}A was established to be broadly essential and dynamic upon immune induction. These findings open the door for future studies to elucidate how m^{6}A machinery is interacting with the defense response and establish a new area for post-transcriptional control of plant immunity.

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Abbreviations

BCSC breast cancer stem cell

CBB comassie brillant blue

CDKC cyclin-dependent kinase complex

CDPK calcium-dependent protein kinase

DEX dexamethasone

EDS enhanced disease susceptibility

EFR EF-TU RECEPTOR

elf18 18 amino acids peptide of the elongation factor thermo unstable

ER endoplasmic reticulum

ETI effector-triggered immunity

ETS effector-triggered susceptibility

FTO Fat mass and obesity-associated protein

Hpa *Hyaloperonospora arabidopsidis*

HR hypersensitive response

m^{6}A N^6-methyladenosine

MAMP microbe-associated molecular pattern

MAPK mitogen activated protein kinase

mORF major open reading frame

NLR Nucleotide-binding leucine-rich repeat

NPR1	NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS 1
PR	pathogenesis related
PRR	pattern recognition receptor
Psm	*Pseudomonas syringae* pathovar *maculicola* strain ES4326
Pst	*Pseudomonas syringae* pathovar *tomato* strain DC3000
PTI	pattern-triggered immunity
PTM	post-translational modification
RLCK	receptor like cytoplasmic kinase
SA	salicylic acid
SAR	systemic acquired resistance
TBF1	TL1-binding transcription factor 1
uORF	upstream open reading frame
WT	wild-type
YTH	YT521-B homology

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1. Introduction to the plant immune system, GCN2, and m^{6}A

There is a wide body of research in the areas of plant immunity, GCN2, and *N6*-methyladenosine (m^{6}A) modification of RNA. This introductory chapter is not meant to be a complete review of all the literature in these areas, but rather aims to highlight findings relevant to the dissertation work.

1.1 Introduction to the plant immune system

Pathogens represent a significant threat to crops, causing 13%-16% of crop yield loss worldwide (Pagan and Garcia-Arenal 2018). Microbial pathogens, such as bacteria and fungi, are significant players. For example, the oomcyte (a fungus-like organism) *Phytophthora infestans* caused the devastating Irish potato famine in the 1800s (Yoshida et al. 2013). Understanding the regulation and signaling events of plant immunity is essential to creating pathogen resistant crops and thus improving crop yields. This dissertation seeks to uncover the role of post-transcriptional regulation mediated by GCN2 and the m^{6}A RNA modification in plant immunity.

Plants have evolved a robust immune system to fend off pathogens, although unlike animals they do not have specialized immune cells (Jones and Dangl 2006). Instead, each cell of the plant can recognize a pathogen infection and mount a defense response. The plant immune response consists of multiple layers- from the initial cell surface perception of conserved microbe associated molecular patterns (MAMPs), to

recognition of secreted intracellular pathogen effectors, to the long term and systemic resistance mediated by the plant hormone salicylic acid (Spoel and Dong 2012).

This dissertation work used the model plant *Arabidopsis thaliana* (hereafter called *Arabidopsis*) and the model plant pathogens *Pseudomonas syringae* pathovar *maculicola* strain ES4326 (*Psm*, a bacterial pathogen), *Pseudomonas syringae* pathovar *tomato* strain DC3000 (*Pst*, a bacterial pathogen), and *Hyaloperonospora arabidopsidis* (*Hpa,* an oomycote). These systems have been used extensively to elucidate immune mechanisms in plants, an overview of which is discussed below.

1.1.1 Pattern-Triggered Immunity (PTI)

The first layer of defense that the plant utilizes is recognition of conserved microbial associated patterns (MAMPs) by pattern recognition receptors (PRRs) residing on the plasma membrane (Zipfel 2014). All PRRs contain an ectodomain involved in ligand binding, a transmembrane domain, and an intracellular kinase domain (Zipfel 2014). The most studied PRRs are the leucine-rich repeat (LRR) family members EF-TU RECEPTOR (EFR; binds bacterial EF-TU peptide elf18) and FLAGELLIN SENSING 2 (FLS2; binds the bacterial flagellin peptide flg22) (Zipfel et al. 2006, Chinchilla et al. 2006). Other PRRs include LYK5 (binds chitin), LYM1/LYM3 (bind bacterial peptidoglycan), and LORE (binds bacterial lipopolysaccharides), along with receptors whose ligands have yet to be discovered (Couto and Zipfel 2016).

Upon MAMP binding, PRRs heterodimerize with co-receptors to activate downstream signaling, with the major co-receptors being BAK1 for LRRs and CERK1 for LysM-receptor proteins (e.g. the chitin receptor) (Couto and Zipfel 2016). These co-receptors appear to be specific to the type of PRR, as BAK1 is not required for chitin-triggered responses and CERK1 does not participate in FLS2 signaling (Wan et al. 2008, Gimenez-Ibanez et al. 2009).

Receptor-like cytoplasmic kinases (RLCKs) are the direct targets of PRR complexes and further mediate downstream PTI signaling (Macho and Zipfel 2014). There are hundreds of RLCKs in *Arabidopsis* and several have been identified as being important for the PTI response (Lehti-Shiu et al. 2009). Some RLCKs mediate a response to several different MAMPs, and thus represent a convergent point in PTI signaling, while others appear to have specificity (Couto and Zipfel 2016). For example, BOTRYTIS-INDUCED KINASE 1 (BIK1) and PBS1-LIKE KINASEs (PBLs) are required for flg22, elf18, and chitin mediated responses (Couto and Zipfel 2016). On the other hand, PBL27 is specific to the chitin response and BSK1 is required for flg22 but not elf18-mediated immunity (Shi et al. 2013, Shinya et al. 2014) .

Further PTI responses include an ROS burst which is preceded by a calcium burst (Couto and Zipfel 2016). ROS is an important signaling molecule which also possess anti-microbial properties (Saijo, Loo, and Yasuda 2018). Both ROS and calcium bursts appear to be dependent on the RLCKs (Couto and Zipfel 2016). RESPIRATORY

BURST OXIDASE HOMOLOGUE PROTEIN D (RBOHD) is the enzyme responsible for PRR-triggered ROS bursts which associates with the PRR complex and is phosphorylated (and thus activated) by the RLCKs BIK1 and PBLs upon MAMP elicitation (Li, Li, et al. 2014, Kadota et al. 2014).

To produce the calcium burst the RLCKs BIK1 and PBL1 are required, though the identity of the calcium channels that produce this burst remain unknown (Couto and Zipfel 2016). Cytoplasmic calcium activates calcium-dependent protein kinases (CDPKs) which are important for transcriptional reprograming during PTI (Boudsocq et al. 2010, Dubiella et al. 2013). Interestingly, calcium helps activate RBOHD by binding to its EF-hand motifs and facilitates CDPK-mediated phosphorylation of RBOHD (Ogasawara et al. 2008, Dubiella et al. 2013). Thus, there is a synergistic effect on RBOHD by both calcium (through direct binding and CDPKs) and phosphorylation by BIK1, and this requirement for two types of activators may help to maintain signal specificity (Kadota, Shirasu, and Zipfel 2015).

The MAPKs (mitogen activated protein kinases) cascade is another signaling event that leads to transcriptional reprogramming during PTI. MAPK activation appears to be a distinct event after MAMP binding to PRR, as the aforementioned RLCKs or CDPKs are generally not found to be required (Couto and Zipfel 2016). There are two discrete MAPK cascades: the first starts with an unknown MAPKK-kinase, targeting MKK4/MKK5 (MAPK kinases), which then activate MPK3/MPK6 (MAPKs); the second

starts with the MAPKK-kinase MAPKKK8 (MEKK1), targeting MKK1/MKK2 (MAPKKs), which then target MPK4/MPK11 (MAPKs) (Meng and Zhang 2013). Substrates of these MAPKs include cyclin-dependent kinase Cs (CDKCs) as well as transcription factors (Meng and Zhang 2013). MAPKs are generally found to be activators of the PTI response, though they may induce negative regulators as part of a negative feedback loop to inactivate PTI (Couto and Zipfel 2016).

Transcriptional reprogramming is a major downstream PTI output with ~1000 genes induced upon MAMP treatment, which are largely shared among different MAMP elicitors (Li et al. 2016). Many PRRs and signaling components are among those transcriptionally upregulated (i.e. FLS2, BIK1, and MAPKs) suggesting a positive feedback loop (Li et al. 2016). Both general transcriptional machinery and specific transcription factors are regulated by PTI signaling. The carboxyl-terminal domain (CTD) of RNA polymerase II is phosphorylated by CDKCs upon MAMP treatment, and this phosphorylation is required for activating immune genes and mediating pathogen resistance (Li, Cheng, et al. 2014). Specific transcription factors involved in PTI include WRKY33 (targeted by MAPKs) and CBP60g (activated by calcium) (Li et al. 2016).

Negative regulation of immunity is important to plant health as well, as too much or aberrant activation can have adverse effects. Negative regulators exist at many of the major signaling components discussed above, from PRR complexes to the transcriptional output (Couto and Zipfel 2016). PRRs are negatively regulated by

psuedokinases, such as BIR2, which inhibits PRR complex formation (Halter et al. 2014). As phosphorylation is important for activating many PTI signaling components, phosphatases are integral negative regulators (Couto and Zipfel 2016). For example, PRRs and MAPKs are negatively regulated by protein phosphatases type 2C (PP2Cs) (Holton et al. 2015, Schweighofer et al. 2007). At the transcriptional level WRKY TFs (activators of PTI) can be sequestered by VQ motif-containing proteins (VQPs, i.e. MKS1) and are released from this inhibitory complex upon MAMP elicitation (Qiu et al. 2008). The transcriptional repressor ASR3 is responsible for downregulating expression of PTI genes (Li et al. 2015). Interestingly ASR3 binding activity is increased by MAMP induced MPK4 phosphorylation, suggesting a negative feedback mechanism for PTI signaling (Li et al. 2015).

1.1.2 Effector-Triggered Susceptibility (ETS) and Effector-Triggered Immunity (ETI)

To overcome the plant PTI defense response, pathogens have evolved effector molecules to suppress PTI and promote virulence, and this is known as effector-triggered susceptibility (ETS) (De Vleesschauwer, Höfte, and Xu 2014). Effectors have been found to target and inhibit several integral PTI signaling components, such as PRRs and MAPKs (Macho and Zipfel 2015). Pathogens that successfully mount this program are considered “virulent”.

To counteract these effectors, plants have evolved nucleotide-binding domain leucine-rich repeats (NLRs) proteins that can either directly or indirectly detect the

presence of the effectors (Jones and Dangl 2006). Indirect detection seems to be the predominant action of NLRs and has given rise to the "guard hypothesis", where NLRs recognize ("guard") integral host components ("gaurdees") which are targeted by pathogen effectors (Couto and Zipfel 2016). Upon successful recognition, NLRs trigger an immune signaling response known as effector-triggered immunity (ETI) (Jones and Dangl 2006). Pathogens that produce effectors that are recognized by the plant host are known as "avirulent" as they trigger robust ETI in the plant host that severely limits their virulence.

ETI shares many components with PTI, including ROS burst, calcium spikes, and MAPK activation (Tsuda and Katagiri 2010). Furthermore, the transcriptional output between ETI and PTI largely overlaps (Tao et al. 2003, Navarro et al. 2004). It is thought that the difference lies in the amplitude of the responses- ETI is stronger/longer lasting than PTI (Cui, Tsuda, and Parker 2015). Another major difference in ETI is the induction of cell death at the site of infection, known as the hypersensitive response (HR), which is thought to limit the spread of the pathogen as well as send systemic signals to induce systemic immunity (Spoel and Dong 2012).

1.1.3 Systemic Acquired Resistance (SAR)

Systemic acquired resistance (SAR) is an immune response that is induced by a local infection and provides broad-spectrum and long-lasting resistance to distal

uninfected tissues (Durrant and Dong 2004). SAR can also prime defense responses across generations (Luna et al. 2012).

SAR is mediated by the defense hormone salicylic acid (from which aspirin was derived from) and treatment of plants with exogenous SA can induce SAR (Durrant and Dong 2004, Vlot, Dempsey, and Klessig 2009). SAR induces transcriptional reprogramming, with upregulation of pathogenesis-related (PR) genes along with ER associated genes that help with PR secretion (Fu and Dong 2013). Most of these transcriptional changes are mediated by the master regulator non-expresser of PR1 (NPR1), which is a transcriptional co-activator (Wang, Amornsiripanitch, and Dong 2006, Zhang et al. 1999, Cao et al. 1997). Additionally, the *TL1*-binding transcription factor 1 (TBF1) was found to induce the expression of ER associated genes, while repressing genes with chloroplast or growth-related activities (Pajerowska-Mukhtar et al. 2012).

Salicylic acid is synthesized by isochorismate synthase 1 (ICS1) in response to pathogens (Wildermuth et al. 2001). Transcription of ICS1 is positively regulated by the transcription factors CBP60g and SARD1, while CBP60a acts a negative regulator (Seyfferth and Tsuda 2014). Members of the WRKY family of transcription factors, such as WRKY28 and WRKY46, are also positive regulators of ICS1 expression (van Verk, Bol, and Linthorst 2011). SA is perceived by the receptors NPR3/NPR4, which regulate NPR1 stability to induce SAR target genes (Fu et al. 2012). NPR1 is thought to activate gene

expression by association with TGA transcription factors, which bind to the as-1 element in target gene promoters, such as in *PR1* (Gatz 2013). Interestingly NPR1 suppressor screens identified DNA repair proteins, such as BRCA2A (BREAST CANCER 2A) and RAD51D as being required for SA-mediated transcription (Durrant, Wang, and Dong 2007, Wang et al. 2010, Song et al. 2011).

1.1.4 Epigenetic regulators of plant immunity

Epigenetics is defined as a change in gene expression without change in DNA sequence, which can potentially be heritable (Goldberg, Allis, and Bernstein 2007). Major players in epigenetic regulation include DNA methylation and histone post-translational modifications, both of which have been found to regulate plant immunity (Chen et al. 2017). DNA methylation was found to be dynamic upon SA or pathogen challenge, and mutants in the DNA methylation pathway resulted in increased bacterial resistance in *Arabidopsis* (Dowen et al. 2012). Both histone acetyltransferases (HATs) and deacytlases (HDACs) have been found to regulate plant defenses (Ramirez-Prado, Abulfaraj, et al. 2018). The HDAC, HD2B, is directly phosphorylated by MAPK3 and is a positive regulator of plant immunity (Latrasse et al. 2017). Further underscoring the importance of epigenetic regulators, many pathogens target these components in the plant host to promote virulence (Ramirez-Prado, Piquerez, et al. 2018).

1.1.5 Post-transcriptional regulation of plant immunity

Aside from transcriptional outputs, post-transcriptional control of plant immunity has emerged as an important process (Staiger et al. 2013). Nonsense-mediated decay components, such as *UPF1* and *UPF3*, play a negative role in plant immunity, as mutants show enhanced resistance to *Pst* DC3000 along with elevated levels of SA and defense gene expression (Jeong et al. 2011). Plants with mutations in components required for mRNA export display susceptibility to microbial pathogens (Wiermer et al. 2012, Pan, Liu, and Tang 2012). Alternative splicing and splicing factors are also important for plant immunity (Zhang and Gassmann 2003, 2007, Xu et al. 2012).

Along with the post-transcriptional processes described above, the importance of translational control has been uncovered. Overall the correlation between mRNA levels and protein levels across the transcriptome is modest, thus highlighting the importance of translation in regulating gene expression (Roy and Arnim 2013). Studies using ribosome profiling, a technique used to measure translational output, found translational reprogramming of hundreds of genes upon both PTI and ETI inductions (Xu et al. 2017, Meteignier et al. 2017).

A master regulator of plant immunity, TBF1, was found to be under translational control via the presence of upstream open reading frames (uORFs) and an R-motiff in its 5′ UTR (Xu et al. 2017, Pajerowska-Mukhtar et al. 2012). The uORFs appeared to have an inhibitory effect on translation of the mORF of TBF1 and analysis of the uORFs′

sequence composition revealed an enrichment of codons for aromatic amino acids (especially Phe) (Pajerowska-Mukhtar et al. 2012). Thus, it was proposed that Phe starvation could occur upon pathogen challenge which would allow translation initiation of TBF1 downstream of the uORFs. Then the GCN2-eIF2α pathway would be activated and help to mediate readthrough and translation of TBF1's mORF.

1.2 Introduction to GCN2

GCN2 (general control nonderepressible 2) is a protein kinase that phosphorylates eIF2α (creating eIF2α-P), which in turn causes global translational arrest with translational readthrough of mORFs in certain transcripts, such as GCN4 (Roussou, Thireos, and Hauge 1988). GCN2 is conserved in almost all eukaryotes, from yeast to humans, as well as in plants (Castilho et al. 2014). GCN2 has been found to be important for a variety of biological processes in diverse organisms, such as oxidative stress, immunity, development, and metabolism (Chaveroux et al. 2011, Liu, Huang, et al. 2014, Malzer et al. 2013, Guo and Cavener 2007).

As mentioned in the previous section, GCN2 was proposed to regulate the plant immune response through TBF1. TBF1 was found to be under the control of uORFs, which were found to be derepressed upon pathogen challenge to allow translation of the mORF (Pajerowska-Mukhtar et al. 2012). Analysis of the uORF sequence revealed an enrichment of aromatic amino acids such as phenylalanine (Phe) (Pajerowska-Mukhtar et al. 2012). Thus, it was proposed that pathogen challenge would induce starvation of

aromatic amino acids, which was observed indirectly by an accumulation of uncharged $tRNA^{Phe}$ (Pajerowska-Mukhtar et al. 2012). The accumulation of uncharged tRNAs would then trigger phosphorylation of eIF2α, which was also observed upon pathogen challenge (Pajerowska-Mukhtar et al. 2012). Ultimately, this would allow readthrough of uORFs and translation of TBF1 mORF. GCN2 was proposed to be the kinase that mediates phosphorylation of eIF2α during the defense response, analogous to the regulation of GCN4 translation upon amino acid starvation in yeast (Pajerowska-Mukhtar et al. 2012). Consistent with this hypothesis, the GCN2-eIF2α pathway was found to be important for mediating plant stress responses, such as herbicide treatment and amino acid starvation (Lageix et al. 2008). However, whether this pathway is involved in plant defense against pathogen challenge has not been tested before my study.

1.3 Introduction to the m^6A RNA modification

There are over 100 chemical modifications that occur on RNA (Zhao, Roundtree, and He 2016). N^6-methyladenosine (m^6A) is the most abundant internal mRNA modification in eukaryotes (Knuckles and Bühler 2018). m^6A was first discovered in the 1970s, but it has recently gained renewed interest, due to the development of sequencing technologies (m^6A-seq) that can map the m^6A modification and the discovery of the demethylase FTO (Jia et al. 2011, Dominissini et al. 2013) The m^6A modification occurs on a consensus motif, RRACH, which is conserved across eukaryotic species (Meyer and

Jaffrey 2014). m^{6}A is deposited on transcripts by "writers" (methyltransferase complex components), removed by "erasers" (demethylases), and recognized by "readers" (proteins that bind m^{6}A). The m^{6}A modification has been implicated in many facets of RNA metabolism and diverse biological processes.

1.3.1: m^{6}A writers, readers, and erasers and effects on RNA metabolism and biological processes

The m^{6}A modification is deposited co-transcriptionally on RNAs in the nucleus by a multi-component methyltransferase complex (Knuckles and Bühler 2018). The catalytic component of the complex is METTL3 (MTA in *Arabidopsis*) which forms a dimer with METTL14 (MTB in *Arabidopsis*) (Bokar et al. 1997, Liu, Yue, et al. 2014, Zhong et al. 2008, Růžička et al. 2017). METTL14 does not have catalytic activity, but helps the writer complex bind to RNA (Śledź and Jinek 2016). WTAP (FIP37 in *Arabidopsis*) serves as a scaffold for the methyltransferase complex and WTAP knock-down results in significant depletion of m^{6}A globally (Ping et al. 2014, Růžička et al. 2017). Other members of the complex include VIRMA, RBM15, Hakai, and Zc3h13 (Knuckles and Bühler 2018).

Writers that have been well characterized in *Arabidopsis* include MTA and FIP37. Studies of writers in plants have revealed functions of m^{6}A in development and stress response (salt stress), and in regulating the stability of transcripts (Shen et al. 2016, Anderson et al. 2018). Complete null mutants of plant writers are embryonic lethal (Bhat et al. 2018). Partial knockouts of *FIP37* in *Arabidopsis* revealed a role for this m^{6}A writer

in mediating development of the shoot apical meristem (SAM) with increased stability of key SAM development gene transcripts in the *fip37* mutant. Transcripts of these genes were m^{6}A modified in wild type plants, and their m^{6}A levels were diminished in the *fip37* writer mutant (Shen et al. 2016). Thus, m^{6}A may cause destabilization of certain transcripts. A more global analysis of m^{6}A modified transcripts, however, found that m^{6}A generally causes increased stability of transcripts (Anderson et al. 2018). Upon salt stress m^{6}A was deposited on stress-specific transcripts, which resulted in their stabilization (Anderson et al. 2018).

Two studies have found that m^{6}A methyltransferase components contain post-translation modifications (PTMs) that could regulate their function. The first study examined phosphorylation of human METTL3 and METTL14 using mass spectrometry (Scholler et al. 2018). METTL3 was found to have a total of eight phosphorylation sites with varying levels of conservation. Despite fairly high conservation in most of these residues, mutating them to glutamate (E, phospho-mimic) or alanine (A, phosho-dead) did not affect METTL3's activity, localization, or interaction with METTL14/WTAP. METTL14 was also found to be phosphorylated at S399, which is conserved among vertebrates as well as *D. melanogaster*. Like METTL3, mutating S399 in METTL14 to E/A did not affect METTL14 interactions or activity of the METTL3/METTL14 heterodimer. Although these results suggest that these phosphorylation sites may be non-functional,

the authors note that they could be important for specific physiological conditions not tested in the study.

Along with phosphorylation, the SUMOylation status of human METTL3 has also been studied (Du et al. 2018). SUMOylation is a post-translational modification process that attaches the small-ubiquitin-like modifier protein (SUMO) to proteins at specific lysine residues (Hay 2005). METTL3 is SUMOylated at four major sites: K^{177}, K^{211}, K^{212}, and K^{215}, which are highly conserved among species (Du et al. 2018). SUMOylation of METTL3 inhibited its methyltransferase activity, but did not affect its stability, localization, or interaction with its known partners (METTL14, WTAP, CBP80, eIF4E, eIF3B) (Du et al. 2018). METTL14 was not found to be SUMOylated, and other methyltransferase complex components were not examined (Du et al. 2018).

Currently, it remains to be seen whether other methyltransferase complex components have PTMs, and what their functions may be under different physiological or stress conditions. Additionally, studies of writer PTMs in non-human species (including plants) have not been undertaken.

There are several m^6A binding proteins or m^6A "readers" that can influence the fate of m^6A containing mRNAs. These reader proteins have been found to be localized in different cellular compartments and cause different fates for mRNAs: translation, degradation, splicing, and nuclear export. The major m^6A reader proteins are YTH (YT521-B homology) domain containing proteins, which have a conserved YTH domain

of aromatic amino acids responsible for binding m^{6}A (Patil, Pickering, and Jaffrey 2018). Primarily m^{6}A readers have been studied in mammalian systems although reader proteins (ECT family) have been uncovered in plants.

Cytoplasmic readers that have been found to increase translation include YTHDF1, YTHDF3, EIF3, and METTL3. YTHDF1 was found to have an effect of increasing translation of m^{6}A target transcripts by associating with translational initiation factors and ribosomal subunits (Wang et al. 2015). m^{6}A was also found to promote cap-independent translation by directly binding to the initiation factor EIF3 when present in the 5′ UTR of transcripts (Meyer et al. 2015). Stresses such as heat shock have been found to increase distribution of m^{6}A to the 5′UTR and thus promote cap-independent translation of transcripts (Zhou et al. 2015, Meyer et al. 2015). The related reader YTHDF3 synergizes with YTHDF1 by potentiating YTHDF1 binding to target RNAs and thus promoting their translation (Shi et al. 2017). Interestingly, although METTL3 was initially found to be a nuclear localized writer, it has also been found in the cytoplasm where it can promote translation of m^{6}A target transcripts independent of its catalytic activity (Lin et al. 2016). METTL3 achieves enhanced translation of targets through mRNA circularization and association with translational initiation factors (Choe et al. 2018, Lin et al. 2016).

Cytoplasmic readers that increase decay of targets include YTHDF2, YTHDF3, and YTHDC2. RNA half-life profiling of YTHDF2-knockdown cells revealed increased

stabilization of RNAs compared to the control, and the majority of these RNAs were direct targets of YTHDF2 (Wang et al. 2013). Furthermore, YTHDF2 co-localizes with markers of P-bodies (DCP1a, GW182, and DDX6) where degradation of RNAs occurs in the cytoplasm (Wang et al. 2013). A subsequent study found that YTHDF2 recruits the CCR4-NOT deadenylase to m^{6}A containing transcripts to promote their deadenylation and degradation (Du et al. 2016). Similar to the synergistic effect on YTHDF1, YTHDF3 has been found to promote YTHDF2's effect on decay of target RNAs (Shi et al. 2017). YTHDC2 is an RNA helicase found to be essential for meiosis in mice (Knuckles and Bühler 2018). YTHDC2 likely promotes RNA degradation, as *Ythdc2* knockout testes had upregulated m^{6}A-containing transcripts and a top binding partner of YTHDC2 is the 5'-3' exoribonuclease XRN1 (Wojtas et al. 2017).

YTHDC1 is the nuclear localized m^{6}A reader protein, and has been found to have functions in splicing and nuclear export of m^{6}A containing mRNAs (Knuckles and Buhler 2018). YTHDC1 promotes exon inclusion of target mRNAs through recruitment of splicing factor 3 (SRSF3) (Xiao et al. 2016). YTHDC1 also mediates nuclear export of mRNAs by facilitating their binding to the export factors SRSF3 and NXF1 (Roundtree et al. 2017).

Thirteen proteins with YTH domains have been found in *Arabidopsis* (Li, Zhang, et al. 2014). Among these members, ECT2 was initially selected for further study as it is more highly and ubiquitously expressed than the other candidate readers (Wei et al.

2018). ECT2 binding to m^{6}A transcripts was confirmed with RNA immunoprecipitation (RIP) methods, and binding was dependent on a functional YTH domain (Wei et al. 2018). The *ect2* plants were found to have abnormal trichomes, which could be complemented by wild-type ECT2, but not ECT2 containing mutations in the m^{6}A binding site (Wei et al. 2018). A novel protein RNA-binding assay, FA-CLIP (formaldehyde cross-linking and immunoprecipitation), revealed ECT2 targets as mostly mRNAs with an m^{6}A enrichment in the 3'UTR (Wei et al. 2018). ECT2 has a role in m^{6}A-mediated stability, as ECT2 was found to bind to trichome morphogenesis related transcripts with m^{6}A modifications and *ect2* plants have accelerated degradation of these transcripts (Wei et al. 2018).

Along with ECT2, ECT3 was also found to be an m^{6}A reader with functions in *Arabidopsis* leaf development and trichome morphology (Arribas-Hernández et al. 2018). The developmental phenotype of *ect2*/*ect3* is exacerbated by mutation of the *ECT4* reader gene in the *ect2*/*ect3*/*ect4* triple mutant which has a morphology similar to that of m^{6}A-writer depleted plants (Arribas-Hernández et al. 2018). Thus, redundancy exists among these reader members. These ECT members may also be involved in mediating stress responses, as ECT2 and ECT4 were found to localize to cytoplasmic foci upon osmotic stress in the root (Arribas-Hernández et al. 2018).

Along with the writers and readers, there are "eraser" proteins (demethylases). There are two demethylases identified in mammals, FTO and ALKBH5, both of which

belong to the Fe(II)/α-KG-dependent dioxygenase superfamily (Liu and Pan 2016). GWAS studies uncovered FTO as the first body-mass index-associated gene (Scuteri et al. 2007, Frayling et al. 2007). FTO-deficient mice have decreased adipose tissue and are protected from obesity, while overexpression of FTO in mice results in increased obesity (Fischer et al. 2009, Church et al. 2010). FTO was found to demethylate m^6A to unmodified A, and this occurs in the nucleus, as FTO was found to be localized to nuclear speckles (Jia et al. 2011). A subsequent study found that FTO may preferentially demethylate m^6Am over m^6A, as it has a greater catalytic efficiency towards m^6Am (Mauer et al. 2016).

ALKBH5 is the second mammalian demethylase discovered, and like FTO, it is localized in nuclear speckles (Zheng et al. 2013). *Alkbh5* is strongly expressed in the testes and ALKBH5 deficient mice have defects in spermatogenesis (Zheng et al. 2013). ALKBH5 knockdown in human cells results in accelerated nuclear export of mRNA (its major substrate) and an increased rate of nascent RNA synthesis (Zheng et al. 2013). ALKBH5 was also found to play a role promoting the hypoxia induced breast cancer stem cell (BCSC) phenotype (Zhang et al. 2016). Exposure of breast cells to hypoxia results in ALKBH5-mediated m^6A demethylation and stabilization of NANOG mRNA (a pluripotency factor) which induces the BCSC phenotype (Zhang et al. 2016).

The *Arabidopsis* genome contains 13 AlkB family members (atALKBH1-10B) (Mielecki et al. 2012). Two have been functionally characterized as bona-fide

demethylases: ALKBH9B and ALKBH10B. ALKBH9B, localized in the cytoplasm, has been found to positively regulate alfalfa mosaic virus (AMV) infection of *Arabidopsis* plants (Martinez-Perez et al. 2017). ALKBH9B interacts with AMV genomic RNA, and there are increased levels of m^{6}A in AMV genomic RNA from *alkbh9b* plants, which have decreased levels of AMV infection compared to wild-type plants (Martinez-Perez et al. 2017).

ALKBH10B plays a role in regulating flowering time by targeting FT, SPL3, and SPL9 which are positive regulators of flowering (Duan et al. 2017). The *alkbh10b* plants have a delayed flowering phenotype, and this is associated with increased m^{6}A and decreased stability of these regulators (Duan et al. 2017). Aside from floral transition and AMV viral infection, it remains to be seen what other biological processes plant demethylases might be involved with or if other AlkB family members are m^{6}A demethylases.

1.3.2 m^{6}A-seq method overview:

The m^{6}A-seq method is a significant improvement over previous m^{6}A detection technologies which involved laborious biochemical techniques at low throughput (Dominissini et al. 2013). The m^{6}A-seq method is capable of providing a global, transcriptome wide overview of m^{6}A sites within transcripts at relatively high resolution. It utilizes immunoprecipitation with an m^{6}A-specific antibody and is analogous to the ChIP-seq method. An mRNA pool is first fragmented (~200bp) and

then separated into two aliquots, an input and an IP portion. The IP aliquot is subjected to immunoprecipitation with the anti-m^{6}A antibody while the input remains unchanged. These two pools are then subjected to high-throughput RNA-seq and input vs IP reads are compared to identify regions of enrichment which correspond to m^{6}A sites. Although m^{6}A-seq is a significant improvement over older techniques, it still has some limitations. Due to the reliance of fragmentation, it is does not reveal m^{6}A sites at single base pair resolution and the stoichiometry of a particular m^{6}A site within a specific transcript is lost (Dominissini et al. 2013). Overall, m^{6}A-seq is an established method to map m^{6}A throughout the transcriptome and has been used in a variety of species and physiological conditions.

1.4 Dissertation outline:

Plant immunity helps protect plants from microbial pathogens, but it must be tightly regulated as it is a costly process that can have detrimental effects. Multiple layers of regulation of the plant immune system have been explored, but less is known about post-transcriptional regulation.

Chapter 2 explores the potential role of GCN2 in regulating the plant immune response. It was previously proposed that the GCN2-eIF2α pathway could regulate the plant immune system through post-transcriptional (translational) control of the master immune regulator TBF1. To explore this, *gcn2* mutant plants were utilized and eIF2α

phosphorylation assays undertaken upon pathogen challenge. Furthermore, a bacterial infection assay was performed to explore the role of GCN2 in plant immunity.

Chapter 3 explores the role of the post-transcriptional RNA modification m^6A in regulating plant immunity. Other layers of gene expression important for mediating plant defense are regulated by chemical modifications, such as DNA methylation and post-translational modifications of proteins. Therefore, it makes sense that RNA modifications (m^6A here) could also play an important regulatory role. As a first step, the requirement of m^6A in mediating the defense response was explored using m^6A deficient plant lines and bacterial infection assays. Next, pathogen induced dynamics of the m^6A modification across the transcriptome was uncovered using the m^6A-seq technique. Overall this chapter sought to establish an integral and dynamic role for m^6A in plant immunity.

Chapter 4 concludes and offers future research directions based on the results described in Chapters 2 and 3.

2. GCN2 mediates pathogen induced eIF2α phosphorylation but is not required for plant immunity against *Psm*

Parts of this chapter (Figure 1b) were modified from: Xu, G., et al. (2017). "Global translational reprogramming is a fundamental layer of immune regulation in plants." Nature 545(7655): 487-490. Figure 1 was produced in collaboration with Dr. Guoyong Xu.

2.1 Introduction

As discussed in Chapter 1, it is proposed that upon pathogen challenge, GCN2 would phosphorylate eIF2α to produce eIF2α-P. This would in turn inhibit global translation and allow ribosomal readthrough of *TBF1*'s uORFs to *TBF1*'s mORF, allowing for *TBF1* translation. *TBF1* expression would thus mediate the growth to defense transition. In this chapter, I tested this hypothesis by looking at whether eIF2α is phosphorylated in *gcn2* knockout mutant plants upon pathogen challenge. I also tested the bacterial growth phenotype of *gcn2* plants upon *Psm* infection. If GCN2 mediates the defense response through TBF1, it would be expected that *gcn2* plants would have a susceptibility phenotype, as do *tbf1* plants.

2.2 Results

2.2.1 eIF2α-P phosphorylation is induced by *Psm avrRpt2* and elf18 in a GCN2-dependent manner

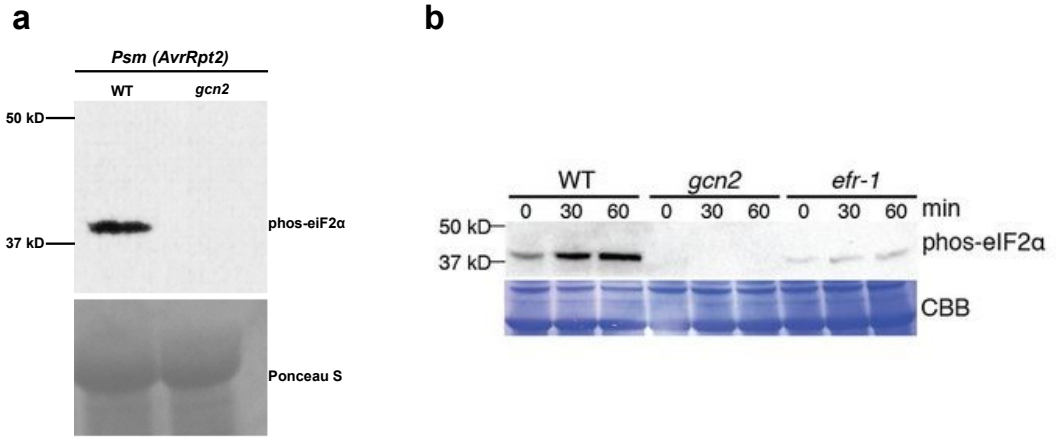

Figure 1: GCN2 is required for pathogen induced eIF2α phosphorylation (Done in collaboartion with Dr. Guoyong Xu, b is modified from "Gu et al 2017") a, Phosphorylated eIF2α was detected using a phospho-specific antibody in protein extracts from WT and *gcn2* plants collected after a 2 hour infiltration with *Psm avrRpt2* (OD_{600nm} = 0.02). Ponceau S was used to confirm equal loading of protein. **b,** Phosphorylated eIF2α was detected using a phospho-specific antibody in protein extracts from WT and *gcn2* seedlings after treatment with 1uM elf18 at the indicated timepoints. CBB was used to confirm equal loading. Experiments were repeated at least twice with similar results.

A previous study found that phosphorylated eIF2α (eIF2α-P) was induced by *Psm avrRpt2* (Pajerowska-Mukhtar et al. 2012). To test whether GCN2 is the kinase responsible for this eIF2α phosphorylation, an eIF2α-P western blot was done on *Psm avrRpt2*-treated WT and *gcn2* plant samples. I found that eIF2α-P was induced in WT as previously shown, but completely abolished in the *gcn2* mutant (Figure 1a). Like *Psm avrRpt2*, the MAMP signal, elf18, was shown to induce translation of TBF1, which then mediates the PTI response (Xu et al. 2017). Therefore, elf18 treatment was also tested for

eIF2α-P in both WT and *gcn2* plants. I observed eIF2α-P in WT, but not *gcn2* plants, indicating that GCN2 mediates eIF2α-P in response to both *Psm avrRpt2*-induced ETI and elf18-induced PTI (Figure 1b).

Interestingly, the elf18 receptor mutant, *efr-1*, still has residual phosphorylation, suggesting that elf18 induced eIF2α-P is not completely dependent on the receptor (figure 1b). This phosphorylation is likely due to the flooding stress the seedlings undergo during this assay.

These results suggest that GCN2 is the kinase that phosphorylates eIF2α in response to pathogen challenge, which could then mediate the translation of TBF1 and promote the defense response.

2.2.2 GCN2 is not required for defense against *Psm*

To test if GCN2 is required for the defense response, a bacterial infection assay was done with *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*) on WT and *gcn2* plants. The *tbf1* mutant was previously shown to be susceptible with a higher level of bacterial growth as compared to WT plants. Thus, *gcn2* plants would be expected to have the same susceptibility phenotype if GCN2 promotes expression of TBF1. However, *gcn2* plants were found to have similar levels of bacterial growth compared to WT while the master immune regulator mutant, *npr1*, showed significantly enhanced disease susceptibility (Figure 2). This suggests that *gcn2* does not mediate TBF1

translation/expression nor is involved in the defense response even though it completely blocks eIF2α-P.

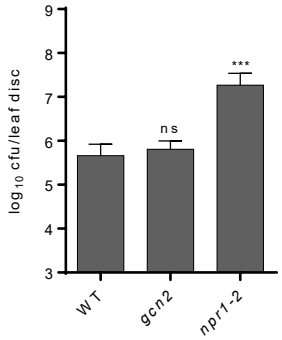

Figure 2: *Psm* bacterial growth assay of *gcn2* plants Leaves of 3.5-week-old WT, *gcn2*, and *npr1-2* plants were infiltrated with *Psm* (OD600nm = 0.0001). After 3 days bacterial growth was quantified. Error bars represent 95% confidence intervals (n=8). Statistical analysis was performed using Bonferroni post hoc test, ***$p < 0.0005$. Experiement repeated three times with similar results.

2.3 Discussion

The work in this chapter uncovered GCN2 as the kinase required for pathogen (*Psm avrRpt2* and the MAMP elf18) induced eIF2α-P. Despite this finding, GCN2 is not likely to regulate TBF1, as GCN2 is dispensable for bacterial disease resistance. GCN2 was also tested for the elf18-induced PTI response in another study (Xu et al. 2017) that found it not to be essential. The Xu et al. study also found that GCN2 is likely not required for mediating TBF1 translation, because a TBF1 translational reporter in *Arabidopsis* was still induced by elf18 (MAMP) treatment when crossed into the *gcn2* background (Xu et al. 2017). This translational reporter consists of a 35s promoter driving expression of TBF1's exon 1 fused to the luciferase gene. Thus, expression of

luciferase is controlled at the translational level by the 5'UTR elements within the first exon of TBF1. Collectively these results suggest that a GCN2-eIF2α-P pathway is not involved in regulating TBF1 or plant immunity.

Novel translational regulators of TBF1 may be revealed by an EMS mutant screen performed in Dr. Dong's lab using the aforementioned plant line containing the TBF1 translational reporter. Here upon immune induction, mutants that have altered translation of the reporter (that is altered luciferase signal in comparison to controls) would be followed up as candidate genes that regulate TBF1 translational expression. This is currently being undertaken by other members of the Dong lab.

2.4: Materials and Methods

2.4.1: *Arabidopsis* growth conditions

Plants were grown on soil (Metro Mix 360) under 12/12-h light/dark cycles at 22 °C with 55% relative humidity. Wildtype (WT) plants are the Columbia accession and the *gcn2* mutant is GABI_862B02 (also in Columbia accession, from the *Arabidopsis* Biological Resource Center).

2.4.2: Bacterial infection assays

Leaves from 3.5-week-old soil grown plants were infiltrated with *Psm* ($OD_{600\ nm}$ = 0.0001). Three days later, bacterial growth was scored.

2.4.3: Detection of eIF2α-P through western blotting

Leaves of 3.5-week-old soil grown plants were infiltrated with *Psm avrRpt2* ($OD_{600\ nm}$ = 0.02) for 2 hours. Leaf tissue was ground in extraction buffer (10 mM HEPES pH 7.7, 2 mM EDTA, 150 mM NaCl, 10 mM $MgCl_2$, 0.3% Triton X-100, 0.1 mM MG115) supplemented with Protease Inhibitor Cocktail (SIGMA-ALDRICH, P9599) and 1X PhosSTOP (Roche). The primary antibody was anti-phospho-eIF2α antibody (pS51; Epitomics, 1:1000 ON at 4°C) and the secondary antibody was goat anti-rabbit IgG (BioRad, #170-6515, 1:4000 1 hr at room temperature). For elf18 treatment, 12-day-old seedlings were grown on MS agar plates and flooded with 1 μM elf18 solution for the indicated timepoints. The tissue was then processed as described for *Psm avrRpt2* treated plants.

3. The m^{6}A RNA modification is required for plant immunity and is dynamic in mRNAs upon immune induction

Results from this chapter were done in collaboration with Dr. Guoyong Xu, Dr. Tianyuan Chen, Scott Zhang, Dr. Sargis Karapetyan, and George Greene.

3.1: Introduction

As discussed in chapter 1, m^{6}A methylation is an important post-transcriptional regulator of gene expression in a variety of biological processes and stress responses. Here instead of focusing on post-transcriptional (translational) regulation of a single master immune regulator TBF1 (Chapter 2), I sought uncover the global impact of m^{6}A throughout the transcriptome in different plant immune responses. To achieve this, I utilized m^{6}A deficient plant lines and found a broadly integral requirement for this modification in plant defense. The m^{6}A-seq method was used on purified mRNA from salicylic acid (SA)- and elf18-treated plants to uncover transcriptome-wide methylation dynamics upon immune inductions.

3.2: Results

3.2.2: *Arabidopsis* lines deficient in m^{6}A are defective in plant immunity

To elucidate the role of the m^{6}A methylation machinery in promoting the defense response, I used a dexamethasone (DEX) inducible siRNA silencing line against *MTA*, which was found to induce a greater than 50% reduction in the target transcript levels

(generated in collaboration with Dr. Guoyong Xu). Two independent lines were generated. In addition to the inducible siRNA silencing MTA line, the methyltransferase scaffold gene *FIP37* mutant, *fip37-4,* was also used. This mutant is a T-DNA insertion line that is a viable hypomorphic mutant of *FIP37*, in which the *FIP37* transcript levels are reduced to ~10% and methylation in mRNA is reduced to ~11% of wild-type levels (Růžička et al. 2017). The *fip37* mutants also have developmental defects including over proliferation of the SAM (Shen et al. 2016). Transformation of the *fip37-4* line with the wild-type genomic FIP37 locus fused with GFP (*fip37-4* gFIP37-GFP) rescued these defects (Shen et al. 2016). I hypothesized that as m^6A is important for meditating stress responses in a variety of organisms, it would be integral for plant defense against pathogens.

To uncover the role of m^6A in plant immunity, I utilized the bacterial infection assay to examine the pathogen growth phenotype of these writer deficient lines with the virulent bacterial plant pathogen *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*). This assay revealed highly significant levels of increased bacterial growth (susceptibility) in both *fip37-4* and MTA silencing lines (siMTA1,2) as compared to wild-type or silencing control (Ctrl is a DEX inducible GFP line) (Figure 3a,b). As a positive control, the null mutant of the master immune regulator NPR1 showed high levels of bacterial growth as expected (Figure 3a,b). Importantly, the susceptibility observed in the m^6A-deficient lines was abolished in the complemented *fip37-4* gFIP37-GFP line (Figure 3a) and in the

absence of DEX treatment for siMTA1,2 lines (Figure 3b). This suggests that m^{6}A is a positive regulator of basal immunity, which includes PTI and SA-mediated pathways.

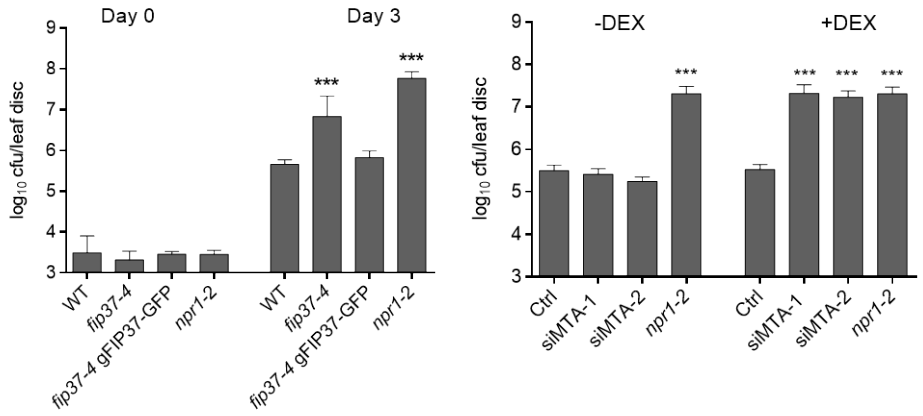

Figure 3: *Psm* bacterial growth assay of m^{6}A deficient plant lines a, Leaves of 3.5-week-old WT, *fip37-4*, *fip37-4* gFIP37-GFP, and *npr1-2* plants were infiltrated with *Psm* (OD_{600nm} = 0.0001). Baterial growth was quantified at day 0 and day 3. Error bars represent 95% confidence intervals (n=4 for day 0, n=8 for day 3). Statistical analysis was performed using Bonferroni post hoc test, ***p < 0.0005. **b,** 3.5-week-old plants of each of the indicated genotypes were pretreated with mock (-DEX) or 50uM DEX (+DEX). 1 day later, plants were infected with *Psm* (OD_{600nm} = 0.0001) and after 3 days bacterial growth was quantified. Error bars represent 95% confidence intervals (n=8) Statistical analysis was performed using Bonferroni post hoc test, ***p < 0.0005. Ctrl is the GVG vector expressing GFP. Experiements were repeated three times with similar results.

To further delineate the level within plant immunity that m^{6}A is involved with, a PTI assay was undertaken. This involves an alteration of the above mentioned *Psm* infection assay. Prior to infection with *Psm*, plant leaf tissue is pre-treated with a MAMP (elf18 here) thus activating PTI signaling. These pretreated plants are consequently resistant to infection and have reduced levels of bacterial growth in comparison to mock-treated plants. If a plant line has deficient PTI signaling, then the protection

offered by the MAMP pre-treatment will be reduced in comparison to WT control plants.

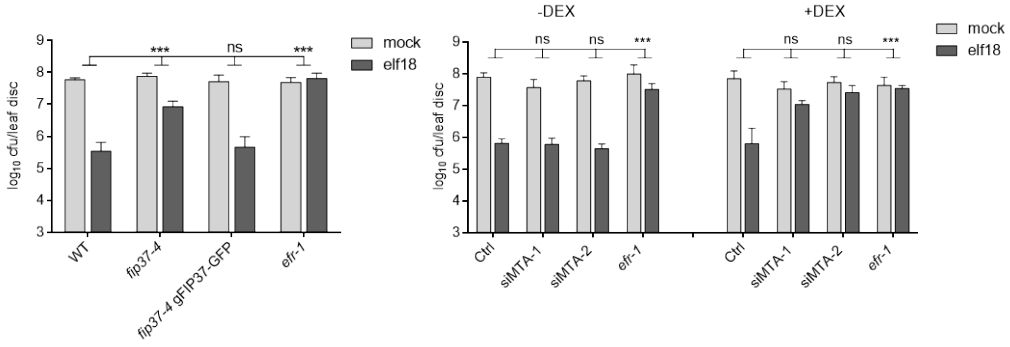

Figure 4: PTI bacterial growth assay of m^{6}A deficient plant lines a, Leaves of 3.5-week-old WT, *fip37-4*, *fip37-4* gFIP37-GFP, and *efr-1* plants were infiltrated with mock (dH_2O) or 1uM elf18. 1 day later, the same leaves were infiltrated with *Psm* (OD_{600nm} = 0.001). After 2 days, bacterial growth was quantified. Error bars represent 95% confidence intervals (n=8). Statistical analysis was performed using Bonferroni post hoc test, ***p < 0.0005. **b,** 3.5-week-old plants of each of the indicated genotypes were pretreated with -DEX (mock) or +DEX (50uM DEX). 1 day later leaves were infiltrated with mock (dH_2O) or 1uM elf18. 1 additional day later, the same leaves were infiltrated with *Psm* (OD_{600nm} = 0.001). After 2 days bacterial growth was quantified. Error bars represent 95% confidence intervals (n=8). Statistical analysis was performed using Bonferroni post hoc test, ***p < 0.0005. Ctrl is the GVG vector expressing the GFP cassette. Experiments repeated three times with similar results.

This PTI assay was utilized to test the m^{6}A-deficent plant lines for their impact on PTI. el18 was used as the MAMP, as it was previously found to globally affect transcripts at the post-transcriptional (translational) level (Xu et al. 2017). I found that the methylation deficient lines all showed significantly reduced protection in comparison to WT or the silencing controls (Ctrl and -DEX) (Figure 4a,b). The *efr-1* mutant containing a defective elf18 receptor was insensitive to induction as expected (Figure 4a,b). Again, the *fip37-4* gFIP37-GFP complemented line rescued this defective

phenotype from *fip37-4* (Figure 4a). These results suggest that elf18-mediated PTI signaling is defective in these m^{6}A-deficient lines, and thus m^{6}A plays a positive role in PTI.

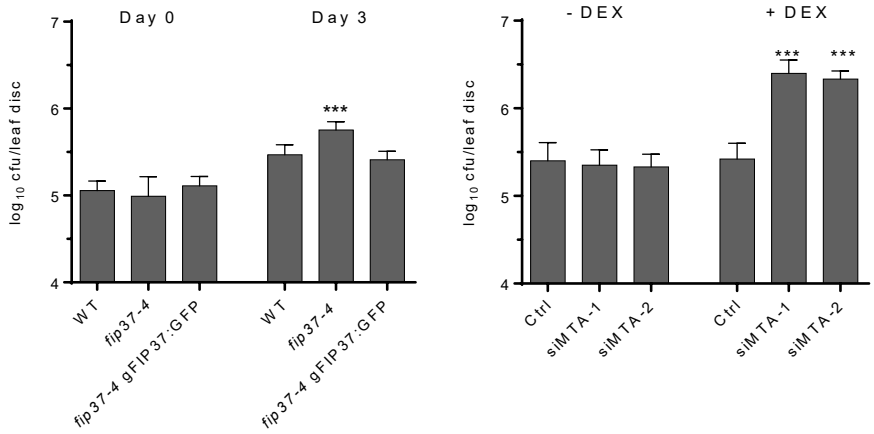

Figure 5: *Pst hrcC*$^-$ bacterial growth assay of m^{6}A deficient plant lines a, Leaves of 3.5-week-old WT, *fip37-4*, *fip37-4* gFIP37-GFP, plants were infiltrated with *Psm* (OD_{600nm} = 0.01). Bacterial growth was quantified at day 0 and 3 days post inoculation. Error bars represent 95% confidence intervals (n=4 at day 0, n=8 at day 3). Statistical analysis was performed using Bonferroni post hoc test, ***p < 0.0005. **b,** 3.5-week-old plants of each of the indicated genotypes were pretreated with mock (-DEX) or 50uM DEX (+DEX). 1 day later plants were infected with *Pst hrcC*$^-$ (OD_{600nm} = 0.01) and after 3 days, bacterial growth was quantified. Error bars represent 95% confidence intervals (n=8). Statistical analysis was performed using Bonferroni post hoc test, ***p < 0.0005. Ctrl is the GVG vector expressing the GFP cassette. Experiments were repeated three times with similar results.

To further confirm the role of m^{6}A in PTI, the *Pst hrcC*$^-$ strain was used. This is a *Pseudomonas* strain that is defective in the type III secretion system and thus the contribution to the host response is solely by MAMPs. My bacterial infiltration assays with *Pst hrcC*$^-$ revealed significantly increased susceptibility in the m^{6}A deficient lines as compared with the WT and complemented *fip37-4* gFIP37-GFP lines as well as the

silencing controls (Ctrl, -DEX) (Figure 5a,b). This indicates that m^{6}A deficient lines have a broad defect in PTI, which is not restricted to that induced by elf18, and further supports a positive role for m^{6}A modification in this immune response.

To understand the stage of PTI signaling that was affected in m^{6}A deficient plants, MAPK phosphorylation of MAPK3/6 was examined upon elf18 treatment in the *fip37-4* mutant. MAPK3/6 phosphorylation is an early PTI signaling event, just downstream of PRR elicitation (Couto and Zipfel 2016). Compared to WT, *fip37-4* had similar activation of MAPK3/6 phosphorylation, though this appeared to persist more strongly at the later 60-minute timepoint (Figure 6). This indicates that the PTI defect caused by m^{6}A deficiency lies downstream of MAPK3/6 phosphorylation.

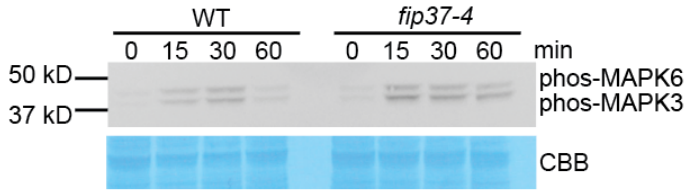

Figure 6: MAPK phosphorylation in *fip37-4* upon elf18 treatment WT, *fip37-4*, and *efr-1* seedlings were treated with 1uM elf18 and collected at the indicated timepoints. MAPK activation (phosphorylation) was detected using the MAPK phospho-specific antibody. Staining with CBB was done to confirm equal loading of proteins. This experiment was repeated twice with similar results.

Next, the salicylic acid (SA) mediated immune response was examined in *fip37-4*. A modified version of the bacterial infection assay was used, with pre-treatment of plants with SA followed by *Psm* inoculation. Here SA-pretreated plants have activation of the SA signaling pathway and thus have increased resistance to subsequent *Psm*

infection vs the mock pre-treated plants. Plants with defective SA signaling (such as *npr1*) will have significantly more bacterial growth in the SA treated plants. In this experiment, I observed that *fip37*-4 plants showed significantly reduced protection relative to WT or the complemented *fip37-4* gFIP37-GFP line and thus SA-mediating signaling is abrogated (Figure 7).

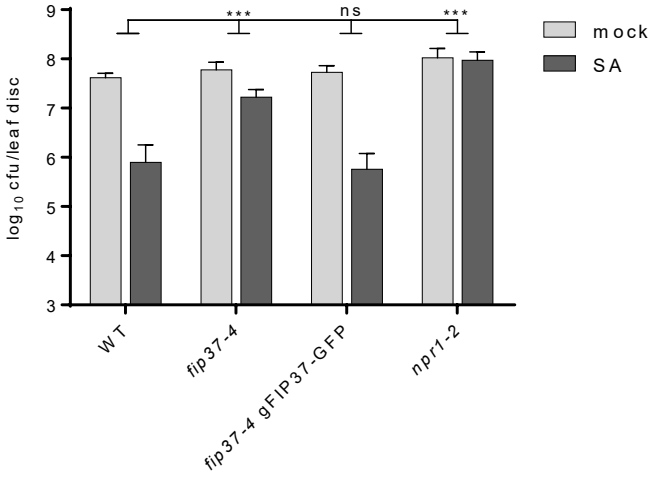

Figure 7: SA-mediated bacterial growth assay of *fip37-4* Leaves of 3.5-week-old WT, *fip37-4*, *fip37-4* gFIP37-GFP, and *npr1-2* plants were sprayed with mock (dH_2O) or SA (1mM). 1 day later the leaves were infiltrated with *Psm* (OD_{600nm} = 0.001). After 2 days, bacterial growth was quantified. Error bars represent 95% confidence intervals (n=8). Statistical analysis was performed using Bonferroni post hoc test, ***$p < 0.0005$. This experiment was repeated three times with similar results.

This SA-mediated resistance assay should also be repeated with the siMTA lines to further confirm the role of m^6A in mediating this response. As SA is the major mediator of systemic acquired resistance (SAR), it would be interesting to see whether biological SAR (that is pre-treatment with a pathogen, followed by systemic treatment

with *Psm*) is affected. Also, whether m^{6}A is important for cross-generational SAR, such a seen with DNA methylation and histone acetylation, is another open question (Luna et al. 2012).

To understand whether the requirement of m^{6}A in defense is specific to *Psm* or could be more broadly required, infection with *Hyaloperonospora arabidopsidis* (*Hpa*) Noco2 was undertaken. *Hpa* is a natural biotrophic pathogen of *Arabidopsis*, and is an oomycte, a fungus-like organism (Coates and Beynon 2010). In comparison to WT, *fip37-4* plants had significantly more conidiospores (the offspring of *Hpa* produced upon a successful infection) (Figure 8). Thus, m^{6}A appears to be broadly required for defense against multiple pathogens, aside from *Psm*.

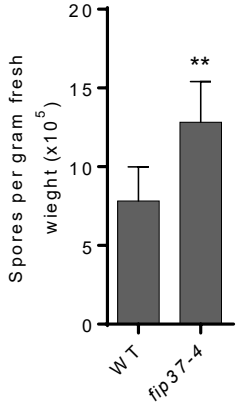

Figure 8: *fip37-4* infection wth *Hpa* Noco2 (Done in collaboration with Dr. Sargis Karapetyan) Condiospore count at 7 dpi by *Hpa* Noco2 (n=12). Error bars 95% confidence interval. The mann-whitney test was used for stastical analysis. This experiement was repeated three times with similar results.

3.2.3: *Arabidopsis* m^{6}A reader and eraser proteins do not appear to be involved in plant immunity

As methyltransferase components appear to be essential for a robust immune response, it stands to reason that other m^{6}A related proteins, namely the readers and erasers, may also play a role. Reader mutants would be expected to have a susceptibility phenotype like the writer mutants, as readers are mediators of the m^{6}A mRNA modification. Since m^{6}A deficient plants have a susceptibility phenotype, I hypothesized that eraser mutant plants (m^{6}A hypermethylated) would have the opposite, resistant phenotype.

To elucidate the role of readers, mutant T-DNA lines of ECTs were tested with the *Psm* bacterial growth assay and PTI bacterial growth assay. As mentioned in Chapter 1 there are 13 YTH domain containing proteins in *Arabidopsis*, and 3- ECT2, ECT3, and ECT4 were found to be m^{6}A reader proteins (Arribas-Hernández et al. 2018). T-DNA insertion lines for ECT2, ECT3, and ECT4 along with other selected ECT family members were subject to both the bacterial growth assay and PTI protection assay with elf18 (Figure 9). None of the reader t-DNA lines showed a susceptibility phenotype. This is not too unexpected as single mutants of the three readers (*ect2*, *ect3*, *ect4*) showed more minor phenotypes (developmental) as compared with higher order double or triple mutants (i.e. *ect2/3/4*) (Arribas-Hernández et al. 2018). The other ECT mutants tested may not have shown a susceptibility phenotype as they may not be bona-fide m^{6}A readers. Alternatively, these ECT members may be bona-fide readers but do not bind

m^{6}A containing transcripts related to defense, and so mutants of these readers would not show a susceptibility phenotype.

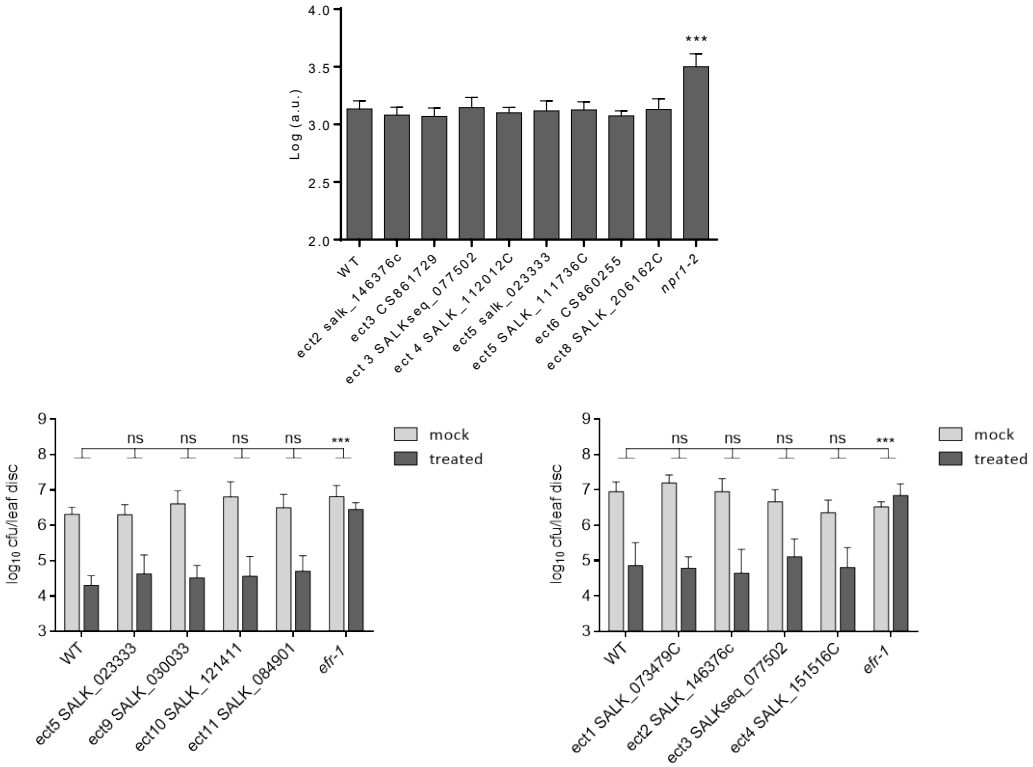

Figure 9: *Psm* bacterial growth and PTI assay of ECT family member T-DNA lines, (b and c done in collaboration with Dr. Tianyuan Chen) a, bacterial growth assay with *Psm lux* (OD_{600nm}=0.0001). Bacterial growth measured by luminesce after 3 days. n = 6, Error bars represent 95% confidence intervals. Statistical analysis was performed using Bonferroni post hoc test, ***p < 0.0005. **b,** Leaves of 3.5-week-old plants were infiltrated with mock (dH_2O) or 1uM elf18. 1 day later the same leaves were infiltrated with *Psm* (OD_{600nm} = 0.001). After 2 days bacterial growth was quantified. Error bars represent 95% confidence intervals (n=8). Statistical analysis was performed using Bonferroni post hoc test, ***p < 0.0005. Experiments were repeated twice with similar results.

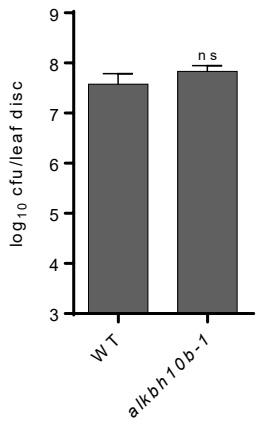

Figure 10: Bacterial growth assay of *albh10b-1* plants Leaves of 3.5-week-old plants were infiltrated with *Psm* (OD_{600nm} = 0.001). Bacterial growth quantified 3 days post-infiltration. Error bars represent 95% confidence intervals (n=8). Statistical analysis was performed using Bonferroni post hoc test, ***$p < 0.0005$. Experiment was repeated twice with similar results.

ALKBH10B is an eraser protein that was found to demethylate mRNA and has a role in floral development (Duan et al. 2017). Therefore, I tested a mutant T-DNA line, Salk_004215c (*alkbh10b-1*) for its bacterial resistance phenotype. The results showed no significant difference in bacterial growth between WT and *alkbh10b-1*, meaning that resistance to *Psm* is not increased in this eraser mutant (Figure 10). Other infection assays were not undertaken, so it is possible that ALKBH10B could play a role in other layers of immunity such as PTI or SAR, which was seen with the writer deficient plant lines. Other mutant "AlkB" family members were not tested and these may play a role in mediating bacterial resistance. Alternatively, it may be that m^6A demethylation is not involved in the immune response.

3.2.4: m^{6}A transcriptome dynamics of *Arabidopsis* plants upon elf18 and SA treatments

To determine m^{6}A dynamics in mRNAs upon immune induction by elf18 and SA, m^{6}A-seq was undertaken. Both mock and treated samples were prepared for this analysis for both sets of inductions. m^{6}A-seq requires a large amount of input total RNA (100-200 mg), therefore infection assays were scaled up and ~1.5 g of mock and treated tissue was collected for both elf18 and SA samples. Once total RNA was isolated, and quality confirmed with the bioanalyzer, ~1 ug mRNA was obtained using Oligo-dt magnetic beads. m^{6}A-seq was then undertaken in collaboration with Scott Zhang from the group of Dr. Chaun He at the University of Chicago.

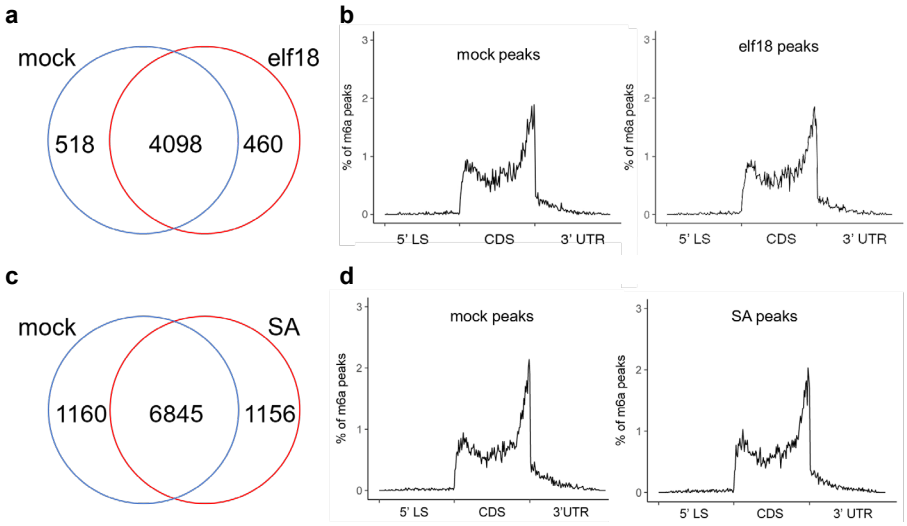

Figure 11: m^{6}A-seq upon immune inductions (Done in collaboration with Scott Zhang and George Greene) a, venn diagram of m^{6}A transcripts specific to mock and elf18 conditons. **b**, metagene profile of m^{6}A in the gene body of mock and elf18 specific transcripts.**c,d,** same as **a,b** except with SA-treated samples.

The m^{6}A-seq results show dramatic changes in m^{6}A throughout the transcriptome, with hundreds of transcripts having m^{6}A peaks in both mock and treatment specific conditions (Figure 11). This m^{6}A-seq analysis was done in collaboration with George Greene. Metagene profiles of all mock m^{6}A peak containing transcripts found the expected enrichment of peaks in the start of the CDS and stop codon, as previously described for m^{6}A in *Arabidopsis* (Figure 11b,d, left panel). Interestingly, this metagene profile did not change upon elf18/SA treatments (Figure 11b,d, right panel). This differs from what was observed in stress treatments in other systems, such as zika virus infection or heat shock, where an enrichment of m^{6}A peaks was found in the 5'UTR (Lichinchi et al. 2016, Zhou et al. 2015).

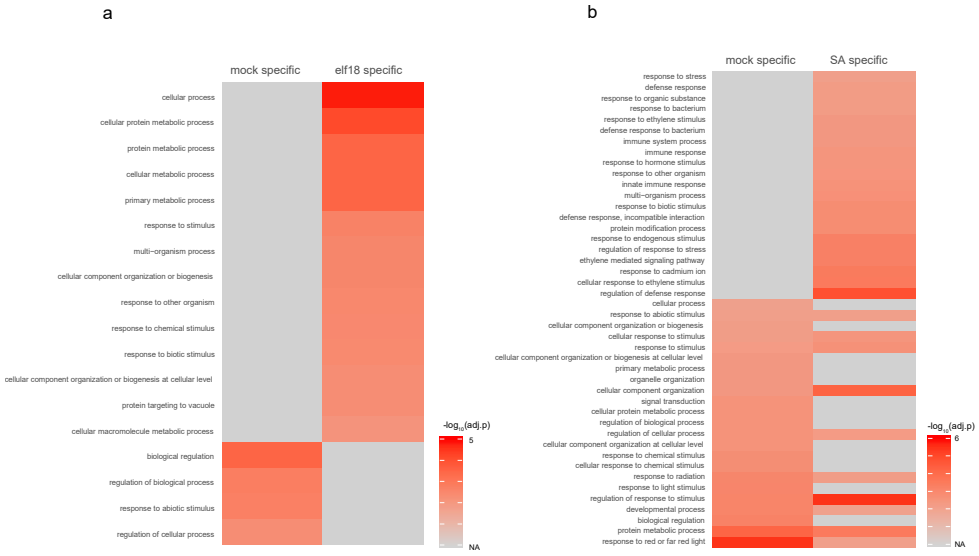

Figure 12: GO analysis of m^{6}A mock-specific and treatment-specific transcripts (Done in collaboration with George Greene) a, elf18 GO, red is higher P-value **b,** SA GO, red is higher p-value. GO generated using the Virtual Plant Program (Katari et al. 2010). Fischers exact test used to calculate p-value with a cutoff of 0.01. p-values are -log$_{10}$ transformed.

GO analysis revealed enrichment of biotic stress or immune-related genes in the both the elf18 and SA specific sets, with a greater amount of these categories found in the SA treatment (Figure 12a,b). In contrast, mock-specific peaks did not show great enrichment in stress-related categories, but instead showed more predominance in general metabolism/biological regulation (Figure 12a,b). This suggests that immune/stress-related transcripts are reprogrammed with m^{6}A, which could promote their expression, possibly through increased translation/stability, to further promote a defense response.

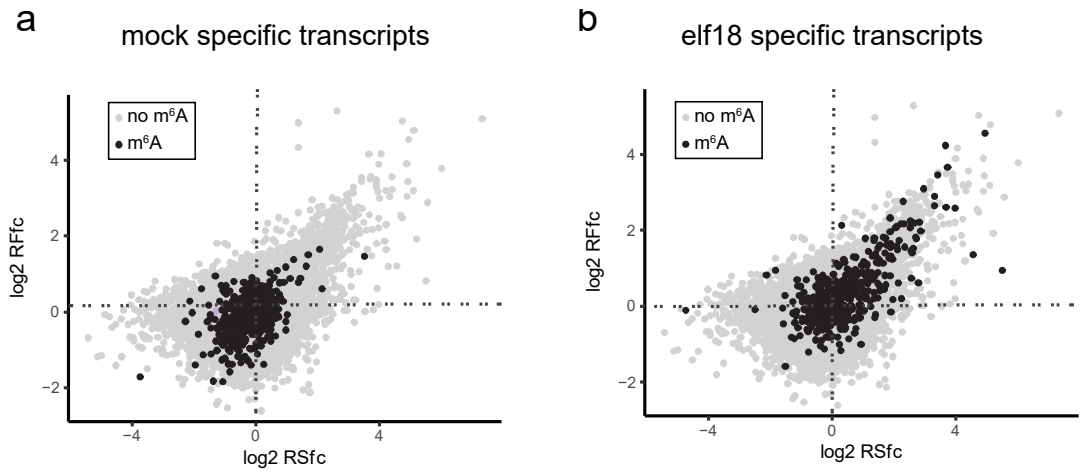

Figure 13: Relationship between m^{6}A containing transcripts and transcription/translation upon elf18 treatment (Done in collaboration with George Greene) a, RFfc vs RSfc of mock specific m^{6}A transcripts. Grey dots are all transcripts with RFfc and RSfc, black dots are m^{6}A containing transcripts. **b,** RFfc vs RSfc of elf18 treatment specific m^{6}A transcripts. Grey dots are all transcripts with RFfc and RSfc, black dots are m^{6}A containing transcripts. Both RSfc and RFfc are log_2 transformed.

Treatment of *Arabidopsis* plants with elf18 was previously shown to increase both the transcription and translation of a variety of transcripts, including those in immune-

related GO categories (Zipfel et al. 2006, Xu et al. 2017). To uncover the relationship between m^{6}A containing transcripts and their transcription/translation, all transcripts with RNA-seq fold changes (RSfc) and Ribo-seq fold changes (RFfc) found in a previous study (Xu et al. 2017) were plotted with mock-specific and elf18-specific m^{6}A transcripts highlighted (Figure 13a,b). This revealed that mock-specific transcripts (which can also be thought of as transcripts with m^{6}A lost upon elf18 treatment) had a low amount of expression, with low levels of transcription and translation (Figure 13a). The elf18-specific transcripts (which can also be thought of as transcripts with m^{6}A gained upon elf18 treatment) had increased expression, with both transcription and translation up (Figure 13b). This suggests that m^{6}A reprograms genes to be more expressed upon elf18 induction to support immune reprogramming. Translational profiling of these induced transcripts in the methylation deficient mutant could help to confirm the role of m^{6}A in promoting translation in these transcripts. The translational status of transcripts upon SA treatment is not known, though it may play a similar role as elf18 in promoting their translation. Aside from translation, increased stability could aid in the expression of defense-related transcripts with m^{6}A gained in either SA/elf18 treatment. mRNA stability assays comparing m^{6}A-induced transcripts in both WT and m^{6}A-deficient plant lines upon elf18/SA treatments could help to uncover this.

3.3: Discussion

In summary, this chapter revealed a role for m^{6}A in regulating the plant immune response at multiple levels as revealed through bacterial growth assays of writer deficient plant lines. Dynamic m^{6}A changes of mRNAs were observed in both elf18 and SA treatments, with a predominance of GO terms in immune/biotic stress related categories induced by both treatments. SA showed a wider variety of GO terms induced, suggesting some differences in the PTI- and SA-mediated immune responses. Differential expression analysis of genes between SA and elf18 treatments was noted in a previous study, implying that these two layers of immunity are distinct (Pajerowska-Mukhtar et al. 2012). Given the apparent requirement for m^{6}A and the immune induced dynamics, it seems that m^{6}A mediates reprogramming towards a defense response. How m^{6}A mediates gene expression during immune response is an open question and could be answered through translational/stability assays comparing m^{6}A containing transcripts in WT and m^{6}A-depleted plant lines.

Future directions include profiling m^{6}A modifications in other RNAs besides mRNAs. Non-coding RNAs have also been found to be m^{6}A modified and could also regulate the immune response, though they are less characterized in plants. How m^{6}A mediates immunity in different plant organs, such as the root is another interesting question. Aside from pathogenic microbial interactions, plants have a native microbiome. Could m^{6}A regulate plant-microbiome interactions as seen with immune

components (Castrillo et al. 2017)? Analysis of the microbiome in m^{6}A-deficient plant lines could help answer this question. Overall, m^{6}A represents a new avenue of regulation of plant immunity with many questions yet to be answered.

3.4: Materials and Methods

3.4.1: *Arabidopsis* plant lines and growth conditions

Plants were grown on soil (Metro Mix 360) under 12/12-h light/dark cycles at 22 °C with 55% relative humidity. Wildtype (WT) plants are the Columbia accession and all plant lines used are within this background. ECT T-DNA lines and SALK_018636 (*fip37-4)* are from the *Arabidopsis* Biological Resource Center. The *fip37-4* gFIP37-GFP transgenic line was provided by Hao Yu from the National University of Singapore (Shen et al. 2016).

2.4.2: Bacterial and *Hpa* infection assays

For bacterial infection assays leaves from 3.5-week-old plants were infiltrated with *Psm* ES4326 (OD_{600nm} = 0.0001). 3 days later bacterial growth was scored. *Psm lux* treatment was done in the same manner, except an imaging chamber was used to quantify luciferase signal as a proxy for bacterial growth. For elf18 PTI bacterial growth assays leaves from 3.5-week-old plants were infiltrated with 1uM elf18 in dH_2O or mock (dH_2O). After 1 day, the same leaves were then infiltrated with *Psm* ES4326 (OD_{600nm} = 0.001) and bacterial growth was scored 2 days later. For the SA protection assay 3.5-week-old plants were sprayed with 1mM SA in dH_2O or mock treated (dH_2O). After 1

day, leaves were infiltrated with *Psm* ES4326 (OD_{600nm} = 0.001) and bacterial growth scored 2 days later.

For *Hpa* Noco2 infection, 12-day-old plants were grown. The plants were sprayed with a suspension of 3-5×10^4 spores/mL in water and covered with a dome to achieve 100% saturated humidity for 1 day. The plants were then exposed to ambient humidity for 3 days before being covered again. Spores were collected 7 days the after the initial infection by suspending infected plants in 1 mL of water and were counted in a hemocytometer under microscopy.

2.4.3: elf18-induced MAPK activation

12-day-old seedlings were grown on MS agar plates and flooded with 1 uM elf18. 25 seedlings were collected at the indicated timepoints and protein extracted with the following extraction buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 0.2% (v/v) Nonidet P-40, protease inhibitor cocktail (Roche), phos-stop phosphatase inhibitor cocktail (Roche). The primary antibody was the Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (Cell Signaling Cat: #9101, 1:1000 at 4°C overnight) and the secondary antibody was goat-anti rabbit-HRP antibody (Bio-Rad Cat: 1706515, 1:10000 for 1 hour at room temperature).

2.4.3: Total RNA isolation of elf18-treated and SA-treated plants for m^6A-seq

3.5-week-old WT plants were infiltrated with 10 uM elf18 or mock (dH_2O) for 1 hr. For the SA samples, WT plants were sprayed with 1 mM SA or mock (dH_2O) for 24 hours. Leaf tissue (~1.5g) was then collected and flash frozen in liquid nitrogen. Total RNA was extracted using TRIZOL (Ambion) according to the manufacturer's instructions.

2.4.4: m^6A-seq

mRNA was isolated from total RNA using Dynabeads mRNA DIRECT purification kit (Thermo Fisher, Cat 61011). mRNA was adjusted to 15 ng/ul in 100ul and fragmented using Bioruptor ultrasonicator (Diagenode) with 30s on/off for 30 cycles. m^6A-immunoprecipitation (m^6A-IP) and library preparation were performed according to the published protocol with minor changes (Dominissini et al. 2013). Specifically, the input mRNA was scaled down to 1 ug and the anti-m^6A antibody used is scaled down to 2.5 μg. Input and m^6A-IP eluted RNA were constructed into libraries using TruSeq Stranded mRNA kit (Illumina) following the standard protocol. Sequencing was carried out on Illumina HiSeq 4000 according to the manufacturer's instructions.

2.4.5: Data processing for m^6A-seq

Three replicates of m^6A input and IP libraries were aligned to the *Arabidopsis* genome (TAIR10.27) using Bowtie2.0. Reads were assigned to genes by alignment to the exons of the longest isoform of each gene. Differentially expressed genes were called through analysis with DESeq2.

Peak calling: For each library, a sliding window of 100 nt in length with a step of 50 nt was used to determine read coverage of the longest isoform of each gene. Read coverage in each window was pseudo-count transformed and normalized by the median window read coverage. In m^6A IP libraries, windows with a greater than 3 peak-over-median (POM) score were condensed. These regions were normalized by the read coverage in the corresponding input library to determine a peak-over-input (POI) score. A region of greater than 3 POI in at least two of the three replicates were determined to be putative m^6A methylation sites. Putative peaks are considered specific to a condition if none of the windows under the peak are found with a significant POI score in the other condition.

4. Conclusions and future perspectives

In summary, this dissertation work found that GCN2 is not likely to be involved in regulating TBF1 translation or plant immunity, while uncovering m^{6}A transcriptome dynamics and a positive role for m^{6}A in regulating plant immunity. There are several avenues of future directions for these areas, particularly for m^{6}A, which is still a relatively new field in plant biology.

GCN2 may be important for mediating other plant stress responses outside of pathogen infection. Indeed, GCN2 was found to be important for stresses such as herbicide treatment or amino acid deprivation (Lageix et al. 2008).

If GCN2 and eIF2α-P are not involved in TBF1 regulation, how is TBF1 translation induced? To answer this question, a forward genetic screen is being done using a TBF1 translational reporter to uncover upstream regulatory factors that control the translational induction of TBF1. Downstream factors involved in mediating TBF1's transcriptional activity are also not known. Co-IP/Mass spec analysis of TBF1 could help to reveal co-factors or perhaps enzymes that add PTMs to TBF1 and regulate its expression.

There are many future directions that can be followed up in the context of the m^{6}A modification in plant immunity. Major questions include how pathogen-induced m^{6}A mRNA dynamics occur and how is an immune signal translated to the nuclear writer complex? It has previously been shown that a transcription factor can direct m^{6}A

writers to specific genomic locations to add m^{6}A to certain transcripts (Aguilo et al. 2015). There are many transcription factors known in plant immunity to target defense related genes, such as WRKYs or TGAs, and these could help guide the writer complex to target genes. Aside from transcription factors, DNA damage components were found to specifically associate with defense gene promoters and promote their transcription (Song et al. 2011, Yan et al. 2013). These could also be promising candidates as DNA damage has been found to be associated with m^{6}A in other systems (Xiang et al. 2017). Plant writer components may be regulated by PTMs, such as phosphorylation, upon immune induction. Phosphorylation of nuclear components has been reported for both specific transcription factors and general transcriptional machinery (e.g. RNA poll II) during the defense response in plants (Couto and Zipfel 2016). A MAPK or CDPK could phosphorylate writer components, leading to their activation or association with other activating factors. Reverse genetic screens or interaction analysis with these known components could help to reveal these factors. Co-IP/mass spec of writers upon immune induction would also help to identify interacting regulators. As *fip37-4* produces a strong immunity phenotype, a suppressor screen could also be undertaken which may identify novel regulators.

Aside from m^{6}A, do other RNA modifications play a role in regulating the immune response? RNA modifications besides m^{6}A have not been well studied in plants, but *N*^{1}-methyladenosine (m^{1}A), pseudouridine (Ψ), and N4-acetylcytidine (ac4C),

have been found to regulate RNAs in other systems (Zhao, Roundtree, and He 2016). There may be a dynamic combinatorial effect of these modifications in fine tuning gene expression during the defense response. This would be analogous to the "histone code" where multiple histone PTMs work in concert to regulate gene expression (Rothbart and Strahl 2014).

Besides the immune responses studied here, can m^6A regulate other stress responses in plants? Effector-triggered immunity (ETI) was not studied, but this may also be regulated by m^6A, as pattern-triggered-immunity (PTI) shares many components with ETI. Other abiotic stresses such as drought may also be regulated through m^6A.

Along with chemical modifications on DNA and proteins regulating gene expression in plant immunity, this dissertation work uncovered a role for chemical modifications (m^6A) at the RNA level. A thorough understanding of regulation at all levels of plant immunity is essential for creating disease resistant plants and m^6A meditated regulation presents a new avenue for this important goal.

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