Transcriptional regulation of effector-triggered immunity (ETI) in plants: from tissue to cells

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Chapter 5
General Discussion

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5.1 A general overview of challenges in improving plant disease resistance

Plant innate immunity relies on a multilayered defense system involving both cell-surface pattern recognition receptors (PRRs) and intracellular nucleotide-binding leucine-rich repeat receptors (NLRs)[1]. While significant advances have been made in identifying the components and downstream signaling modules associated with both pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), many critical questions remain unanswered[2]. In particular**,** how different NLR signaling branches (*e.g.*, ADR1- versus NRG1-mediated) coordinate distinct immune outputs, such as transcriptional reprogramming versus hypersensitive cell death[3–5], remains incompletely understood. Moreover, the spatio-temporal regulation of ETI across different cell types, and how such regulation contributes to immune specificity, strength, or trade-offs with growth, are emerging as key areas of investigation. While recent studies have begun to characterize individual modules, there is still limited clarity on how these modules function independently, redundantly, or synergistically under various immunogenic contexts. This study provides a multi-dimensional dissection of ETI in plants, integrating functional, spatial, and regulatory insights into how plants orchestrate immune responses with precision and flexibility. Building on a foundation of genetic, transcriptional, and cell-type-resolved approaches, we reveal a modular organization of the ETI network that is contextually specialized across signaling nodes, cell types, and transcriptional regulators.

5.2 Dissecting EDS1 module function under isolated ETI activation

At the core of Toll/interleukin-1 receptor (TIR)-NLR-mediated ETI signaling lies the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) node, which forms mutually exclusive heteromeric complexes with either PHYTOALEXIN DEFICIENT 4 (PAD4) or SENESCENCE-ASSOCIATED

GENE 101 (SAG101) to propagate downstream immune signaling via helper NLRs ACTIVATED DISEASE RESISTANCE 1 (ADR1s) and N REQUIREMENT GENE 1 (NRG1s)[4,6], respectively. Our analyses support a model of unequal redundancy[3,5,7], in which the EDS1-PAD4-ADR1 module predominantly contributes to disease resistance and transcriptional reprogramming, while the EDS1-SAG101-NRG1 module is more tightly linked to cell death (HR). We have employed the synthetic estradiol inducible ETI system i.e. Super ETI (SETI)[8] to isolate ETI specific responses of these modules. The functional divergence becomes especially evident under isolated ETI conditions, where SETI_*pad4* mutants show partial relief of growth inhibition and retain priming capacity **(Chapter 2)**, whereas SETI_*sag101* mutants exhibit more severe defects in HR and immune suppression[9]. These findings not only reinforce the idea of functional bifurcation within ETI signaling nodes but also highlight their synergistic roles in mediating immune robustness (**Chapter 2**). Curiously, the retention of ETI activated priming ability by immune compromised plants such as SETI_*pad4* open new venues for harnessing enhanced resistance in plants through prior learning, even when the immune system is not fully functional (**Chapter 2**).

5.3 Interplay between PTI and ETI

Importantly, our transcriptome data during different immune conditions (PTI, ETI, PTI+ETI) underscore the dominant influence of PTI in shaping early immune landscapes, where co-activation with PTI (PTI+ETI) can mask ETI-specific gene expression due to pathway convergence and cellular response saturation (**Chapter 2**). This suggests that while ETI contributes additional layers of defense**,** its distinct transcriptional signature is best revealed in isolation**.** Notably, the requirement of PTI for NRG1 resistosome formation[10], further illustrates the functional

interdependence between surface-localized and intracellular immune pathways.

5.4 Spatial regulation of immune responses

We further build on our understanding of modular ETI regulation by exploring its spatial dynamics at the single-cell level. While earlier sections of our study demonstrate that PAD4-ADR1 and SAG101-NRG1 modules contribute differentially to transcriptional reprogramming and cell death, respectively, it remained unclear how these immune outputs are orchestrated across the complex cellular architecture of a leaf. Our ETI single-cell transcriptomic atlas paints a compelling picture of broad transcriptional competence but spatially refined execution of immunity. Genes encoding core signaling nodes such as *EDS1*, *PAD4*, *SAG101*, and enzymes in the salicylic acid (SA) biosynthesis pathway are widely expressed across diverse cell types (**Chapter 3**), suggesting a uniform capacity for immune perception and signal propagation.

However, the downstream immune responses especially those involving metabolic branches such as N-hydroxypipecolic acid (NHP) and jasmonic acid (JA) pathways display marked cell-type-specific expression patterns, indicating that tissue identity imposes critical regulatory constraints. This tissue-intrinsic logic of immunity is further shaped by differential transcription factor activity, such as specific induction of WRKY8 in pavement cells and the upregulation of trihelix DNA-binding factors in hydathodes. Together, these findings challenge the previously assumed notion of homogeneous immune activation and instead support a model wherein cell-type-specific chromatin accessibility, transcription factor availability, and structural vulnerabilities collectively dictate the amplitude and nature of immune responses.

Importantly, the functional relevance of this spatial heterogeneity becomes particularly evident in the context of nonhost resistance (NHR). Although CBP60g and SARD1 are pathogen-responsive transcription factors and established master regulators of immune gene expression[11,12], our data show that their absence in specific outer tissue layers such as the epidermis or hydathodes allows haustorial entry by non-adapted pathogens (**Chapter 3**), underscoring that immune competence alone is not sufficient because precise spatial execution of transcriptional programs is essential for effective resistance**.** This provides direct evidence that cell-type-specific transcriptional regulation underlies a layered immune architecture**,** one that ensures maximal immune responsiveness at the most vulnerable tissue interfaces.

5.5 CBP60G and SARD1 as toolkit to uncouple disease resistance and cell death

This spatial dependence also complements the broader role of CBP60g and SARD1 in maintaining immune balance across tissues. In addition to their roles in activating SA biosynthesis, CBP60g and SARD1 also serve as key modulators of immune homeostasis (**Chapter 4**). The *cbp60g sard1* (*gh*) mutant exhibits an uncoupling of HR from disease resistance without any growth defects, a phenomenon rarely observed. Elevated HR in *gh* mutants, despite reduced SA levels and compromised ETI, points to transcriptional misregulation of genes encoding negative immune regulators (*e.g.*, NUDIX HYDROLASE 7 (*NUDT7*), *BON1 ASSOCIATED PROTEIN 1* (*BAP1*), *LESION SIMULATING DISEASE 1* (*LSD1*))[13–15], suggesting an imbalance in immune suppression **(Chapter 4**). These findings not only extend the functional repertoire of CBP60g/SARD1 beyond SA control but also provide a model for studying immune activated programmed cell death in plants.

Together, our findings converge on a modular model of ETI, wherein uniformly initiated immune signaling is interpreted through layers of spatial, functional, and transcriptional logic**.** The PAD4-ADR1 and SAG101-NRG1 branches act as parallel and partially redundant modules tuned to distinct immune outputs; cell-type-specific TFs provide regulatory precision; and PTI-ETI cross-talk modulates overall signal amplitude. This layered architecture ensures not only effective defense but also minimizes collateral damage and preserves growth under stress. The exaggerated HR observed in *gh* mutants where heightened cell death occurs in the absence of robust disease resistance serves as a striking example of what happens when transcriptional regulation within this modular framework is disrupted. This phenotype illustrates how loss of immune homeostasis can lead to the uncoupling of defense outputs, further emphasizing the need for tight regulatory coordination across all layers of the immune network.

5.6 Future perspectives

While these studies have illuminated modular aspects of ETI signaling and spatial immune execution, a comprehensive understanding of how transcriptional and signaling networks are integrated at the whole-organism and single-cell level remains incomplete. The robustness and specificity of plant immunity are contingent upon finely tuned gene regulatory mechanisms that coordinate responses across diverse cellular and environmental contexts. Yet the integration of these regulatory layers how they interface, under what circumstances they are engaged, and how they collectively shape immune outputs is still poorly understood.

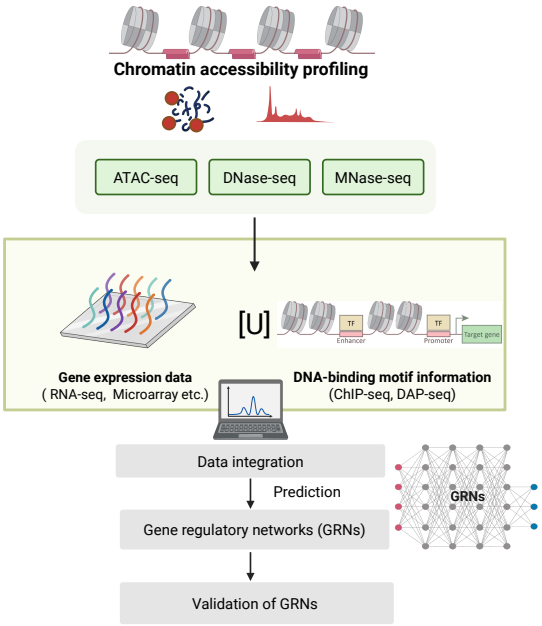

Figure 1. Next-generation toolkit for elucidating immune-responsive GRNs. Integration of data on TF DNA-binding, chromatin accessibility, and gene expression can be employed as a powerful tool to elucidate the highly interconnected gene regulatory networks (GRNs) that determine the plant immune transcriptome, even at single cell resolution. For instance, information related to TF-binding sites can be obtained from chromatin-immunoprecipitation followed by sequencing (ChIP-seq), and DNA affinity purification sequencing (DAP-seq). Information about chromatin status can be derived from methods such as Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), micrococcal nuclease digestion with deep-sequencing (MNase-seq), or DNase-I hypersensitive sites sequencing (DNase-seq). Different variants of RNA (e.g. mRNA, miRNA, lncRNA) can be measured by RNA-seq. These data can be integrated to reveal GRNs that shape the plant immune transcriptome. The functionality of these GRNs can be tested and validated by mutant analysis under different conditions or in different tissues or cell types.

To unravel these complexities, future research must embrace a systems biology framework, leveraging the convergence of multi-omics technologies, advanced data integration, and predictive modeling[16,17]. This includes systematic comparisons of diverse immune-stimulatory treatments (e.g., PTI, ETI, and their combinations), coupled with genome-wide assays such as chromatin accessibility profiling (*e.g.*, ATAC-seq), epigenomic mapping (DNA/histone modifications), and transcriptomic analyses encompassing mRNAs, miRNAs, and lncRNAs. By integrating these datasets through computational modeling and machine learning approaches, we can begin to reconstruct the gene regulatory networks (GRNs) that govern immune signaling with both spatial and temporal resolution (**Figure 1**).

In parallel, single-cell technologies will be essential for dissecting GRNs at the resolution of individual cell types. Such approaches can identify cell-autonomous versus non-cell-autonomous responses, reveal transcriptional heterogeneity within tissues, and elucidate how immune signaling propagates across spatial domains. Addressing questions such as which immune responses are inherently cell-type-specific, and how factors like developmental stage, circadian rhythms, abiotic stress, or distance from the infection site influence immunity, will require high-throughput methods tailored for spatial and temporal precision. Tools like laser-capture microdissection, spatial transcriptomics, and dual host-pathogen single-cell profiling represent promising avenues to interrogate the intimate host-pathogen interface *in planta*.

Collectively, such integrative, high-resolution analyses will pave the way for a network-level understanding of plant immunity one that accounts for both global regulatory dynamics and local cellular context. These insights will not only clarify how plants maintain immune robustness under variable

conditions but will also provide actionable frameworks for rational engineering of crops with enhanced and durable resistance across diverse environmental and pathogen challenges.

Future work should aim to define the temporal dynamics of ETI execution, integrate chromatin accessibility data (*e.g.*, ATAC-seq, ChIP-seq), and expand cross-pathogen functional validation to better understand how these modules adapt to diverse pathogens. By dissecting the immune circuitry at such high resolution, we lay the groundwork for engineering durable and spatially targeted immune responses in crops a promising step toward achieving sustainable agriculture.

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Summary

Plants possess a sophisticated innate immune system composed of two major defense layers: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). Rather acting as independent pathways, PTI and ETI are highly interconnected and mutually reinforcing, together generating robust and durable immune responses. Over the past decades, extensive research has elucidated key components of plant immune architecture and signalling. However, the complexity and interconnectedness of immune signalling mean that key gaps remain in our understanding of the organisation and execution of ETI The research described in this thesis employs genetics and transcriptomics to define how ETI signalling is partitioned across distinct pathways and leaf cell types in *Arabidopsis thaliana* (Arabidopsis).

Chapter 1 provides an overview of the organizational principles underlying plant immunity, from pathogen perception at the cell surface and intracellular immune receptors to downstream signalling cascades and transcriptional reprogramming. Particular emphasis is placed on the regulatory layers that shape immune output, including calcium signaling and the CALMODULIN-BINDING PROTEIN 60 (CBP60) family of transcription factors, CBP60g and SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1)**,** which are central regulators of salicylic acid (SA)-dependent immunity. Although recent studies have demonstrated that PTI and ETI synergize to amplify immune responses, this interdependence has made it difficult to disentangle ETI-specific signalling. Because natural pathogen infection invariably activates PTI prior to ETI, dissecting ETI in isolation has remained a major experimental challenge. To overcome this limitation, this thesis employs a synthetic, estradiol-inducible ETI (SETI)

system, enabling precise activation and analysis of ETI signaling in absence of confounding PTI inputs.

Chapter 2 uses this inducible ETI system to dissect the functional contributions of two partially redundant signaling branches in Arabidopsis TIR-NLR–mediated immunity. We demonstrate that the two ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) associated modules, EDS1-PHYTOALEXIN DEFICIENT 4 (PAD4)-ACTIVATED DISEASE RESISTANCE 1 (ADR1) and EDS1-SENESCENCE-ASSOCIATED GENE 101 (SAG101)-N REQUIREMENT GENE 1 (NRG1), play distinct roles during ETI execution. The PAD4-ADR1 is involved in disease resistance while NRG1-SAG101 branch is primarily responsible for triggering the hypersensitive response (HR), a localized form of programmed cell death. These results establish that disease resistance and HR represent separable outputs of ETI, rather than obligatorily coupled consequences of the same signalling process.

To uncover the transcriptional basis of this functional separation, we performed global transcriptome profiling in SETI_*pad4*, SETI_*sag101*, and SETI_*pad4 sag101* backgrounds following PTI, ETI, and combined PTI+ETI activation. These analyses revealed that PAD4 is the dominant driver of immune-induced transcriptional reprogramming, whereas the contribution of SAG101 becomes evident mainly in the SETI_*pad4 sag101* double mutant, highlighting a context-dependent redundancy between the two signalling branches. Furthermore, we identified node-specific gene regulatory signatures, with distinct sets of defense-related genes preferentially controlled by the PAD4–ADR1 or SAG101–NRG1 modules. Importantly, this chapter also demonstrates that immune priming can be retained even if immune signaling is partially compromised, indicating that plants can maintain immune memory without fully engaging

all ETI branches. This finding has practical implications for crop improvement, as it suggests that durable disease resistance may be achieved while minimizing the fitness costs associated with excessive immune activation.

Chapter 3 extends the concept of ETI modularity from signalling pathways to cellular and spatial organization. Using single-cell RNA sequencing (scRNA-seq), we resolved immune ETI-induced transcriptional responses at the level of individual leaf cell types. Although ETI activation is initiated broadly across the tissue, its transcriptional execution varies substantially between cell identities. A conserved core defense program is activated in most cell types, representing a shared ETI backbone, while additional immune modules are selectively deployed in specific cell types, reflecting differences in developmental state, chromatin accessibility, and transcription factor availability.

Comparison of single-cell ETI-responsive genes with bulk RNA-seq data confirms that these transcriptional programs represent authentic ETI outputs rather than artifacts of cell isolation. We further classify ETI-responsive genes into “generalists”, expressed across multiple cell types, and “specialists”, restricted to one or a few cell types. This distinction reveals a hierarchical immune organization in which broadly distributed defenses coexist with highly localized, cell-type-specific immune functions.

A key finding of this chapter is that transcription factors CBP60g and SARD1 are preferentially induced in epidermal pavement cells, the plant’s first physical barrier against pathogen invasion. Functional analyses show that loss of these regulators compromises the epidermis ability to restrict pathogen entry, even when inner tissues retain immune competence. These results demonstrate that effective ETI requires not only robust

signaling but also precise spatial deployment of immune regulators across tissue layers.

Chapter 4 focuses on CBP60g and SARD1 as central transcriptional regulators that enable plants to balance immune activation with cellular viability during ETI. Through genetic analysis, inducible ETI activation, and transcriptome profiling, we show that these transcription factors function not merely as positive regulators of SA-mediated immunity, but as master coordinators that integrate immune-promoting and immune-restraining programs.

The *cbp60g sard1* double mutant provides a striking genetic uncoupling of ETI outputs: ETI induction triggers exaggerated HR, yet pathogen resistance is markedly reduced. This demonstrates that HR is neither sufficient nor required for effective pathogen restriction, challenging the long-standing assumption that stronger cell death necessarily correlates with stronger immunity. Notably, the absence of major developmental defects in this mutant allows immune-specific phenotypes to be interpreted without confounding pleiotropic effects. Transcriptome analyses reveal that CBP60g and SARD1 simultaneously activate defense genes while maintaining expression of negative immune regulators, including Nudix hydrolases, thereby preventing uncontrolled or self-destructive immune activation. Their regulatory role can thus be understood as establishing a dynamic equilibrium that permits strong pathogen control while safeguarding cellular integrity.

In the concluding **Chapter 5**, these findings are integrated into a broader conceptual framework of plant immunity, and future research directions are discussed. Collectively, this thesis demonstrates that ETI is not a uniform or binary response, but a modular, tunable, and spatially coordinated immune system. By elucidating how core immune signaling

nodes interface with central transcriptional regulators across distinct cellular contexts, this work provides a foundation for developing strategies to engineer disease-resistant crops that maintain growth and yield, an essential goal for sustainable agriculture and plant biotechnology.