

Leveraging Autoimmunity to Explore Regulatory Mechanisms of *Arabidopsis* Helper NLRs

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Summary

The plant immune system is based on an arsenal of specialized immune receptors that activate immune responses after the perception of pathogen-derived signals. These receptors can be located at the cell surface or inside the cell. The RNLs form a subclade of intracellular immune receptors (NLRs), consisting of two families: ADR1 and NRG1. RNLs act as 'Helper NLRs' that do not perceive the pathogen-derived signals directly but instead aid other receptors to mount a full immune response. The outcome of RNL activation appears to vary in amplitude and duration depending on the receptor that initiated the immune response, suggesting that RNL activity is finely tuned. In this PhD thesis, I address the question how *Arabidopsis* RNL activity is regulated to achieve the desired immune response.

In the first chapter, I used the autoimmunity observed in a combinatorial RNL mutant (*adr1-l1-1 adr1-l2-4 nrg1.1-2*) as a proxy to study ADR1-dependent immune responses. We hypothesized that ADR1 is auto-active in the absence of ADR1-L1, ADR1-L2, and NRG1.1. However, we uncovered that the autoimmunity was not directly caused by the absence of other RNLs. Instead, the autoimmunity was caused by an unknown background mutation that we called '*sith*'. Continued investigation should reveal what exactly the *sith* mutation is and expand our understanding of ADR1-dependent (auto)immune responses.

In the second chapter, I continued with the initial hypothesis from the previous chapter that ADR1 is redundantly regulated by ADR1-L1, ADR1-L2, and NRG1.1. Using transient expression in *Nicotiana benthamiana*, we found that expression of *NRG1.1* could suppress cell death induced by ADR1. During this investigation, we discovered a putative variant of *NRG1.1* generated by alternative splicing. However, we ultimately identified that the suppression of cell death did not correlate with the co-expression of *NRG1.1*. Nevertheless, the function of the alternatively spliced NRG1.1 isoform requires further investigation, providing grounds for new research directions into RNL function.

In the final chapter, I focussed on the role of phosphorylation in regulating the activity of *Arabidopsis* ADR1-L2. Based on our findings, we hypothesize that ADR1-L2 is dynamically regulated by the phosphorylation status of multiple residues. This 'phosphorylation code' would allow for the integration of multiple signals into ADR1-L2 to finetune the immune response. Identifying which kinases/phosphatases are involved and how phosphorylation modifies the activity of ADR1-L2 is essential to further improve our understanding of RNL function and their central role in plant immunity.

Improving our understanding of RNL function and regulation is essential to unlock the full potential of RNLs to enhance crop resistances against pathogens. This work revealed novel insights on how *Arabidopsis* RNL activity could be shaped by various regulatory mechanisms.

Zusammenfassung

Das Immunsystem der Pflanzen basiert auf einem Arsenal an Immunrezeptoren, die nach der Wahrnehmung von Erregern Immunreaktionen aktivieren. Diese Rezeptoren befinden sich sowohl an der Zelloberfläche als auch im Zellinneren. RNLs sind eine Untergruppe der intrazelluläre Immunrezeptoren (NLRs), bestehend aus zwei Genfamilien: ADR1 und NRG1. RNLs fungieren als 'Helper NLRs', die nicht selbst Erreger wahrnehmen, sondern andere NLRs dabei unterstützen, um eine vollständige Immunreaktion zu aktivieren. Das Ergebnis der RNL-Aktivierung variiert je nach Rezeptor, der die Immunität ausgelöst hat, weshalb der RNL-Aktivität fein abgestimmt werden muss. In dieser Doktorarbeit gehe ich auf die Frage ein, wie die Aktivität von *Arabidopsis* RNLs reguliert wird, um die gewünschten Immunreaktion zu erreichen.

Im ersten Kapitel benutzte ich der Autoimmunität eine RNL-Mutante (*adr1-l1-1 adr1-l2-4 nrg1.1-2*) um ADR1-abhängige Immunreaktionen zu studieren. Wir gingen zunächst davon aus, dass ADR1 in Abwesenheit von ADR1-L1, ADR1-L2, und NRG1.1 autoaktiv ist. Jedoch entdeckten wir, dass die Autoaktivität nicht direkt durch die Abwesenheit von andere RNLs verursacht wurde. Stattdessen würde die Autoaktivität durch eine bisher unbekannte Hintergrundmutation verursacht, die wir '*sith*' nannten. Weitere Untersuchungen sind notwendig, um herauszufinden was genau die *sith* Mutation ist und um unser Verständnis von ADR1-abhängigen Immunreaktionen zu verbessern.

Im zweiten Kapitel setzte ich die Untersuchung der Regulation von ADR1 durch ADR1-L1, ADR1-L2, und NRG1.1 fort. Mithilfe transienter Expression in *Nicotiana benthamiana* stellten wir zunächst fest, dass die Expression von *NRG1.1* den durch ADR1 verursachten Zelltod unterdrücken kann. Innerhalb dieser Analysen konnten wir jedoch eine neue Isoform von NRG1.1 identifizieren. Allerdings letztlich haben wir festgestellt, dass die Unterdrückung des Zelltods nicht mit der Expression von *NRG1.1* korreliert. Dennoch sollte die Funktion dieser neuen NRG1.1 Variante weiter untersucht werden, da dies eine neue Richtung innerhalb der Forschung zu RNL-Funktionen eröffnet.

Im letzten Kapitel untersuchte ich den Einfluss von Phosphorylierung auf die Aktivität von ADR1-L2. Basierend auf unseren Erkenntnissen, haben wir die Hypothese aufgestellt, dass ADR1-L2 durch die Phosphorylierung mehrerer Aminosäurereste dynamisch reguliert wird. Dieser 'Phosphorylierungscode' ermöglicht die Integration mehrerer Signale und dadurch die Feinabstimmung der Aktivität von ADR1-L2. Die Identifizierung der Kinasen/Phosphatasen welche ADR1-L2 regulieren könnten ist unerlässlich, um die zentrale Rolle der RNLs im pflanzlichen Immunsystem besser zu verstehen.

Zusammenfassend liefert diese Doktorarbeit neuen Erkenntnissen darüber, wie die Aktivität von *Arabidopsis* RNLs durch verschiedene Mechanismen reguliert wird. Unser Verständnis von RNL-Funktion zu verbessern ist essenziell, um das volle Potenzial von RNLs zur Verbesserung der Pflanzenresistenz zu verwenden.

Abbreviations

Abbreviation	Definition
ADP	Adenosine diphosphate
ADR1	Activated disease resistance 1
ARC	APAF-1, R proteins, and CED4 domain
ATP	Adenosine triphosphate
BAK1	Brassinosteroid insensitive 1-associated receptor kinase 1
BIK1	<i>Botrytis</i> -induced kinase 1
BKK1	BAK1-like 1
CC	Coiled coil
CFU	Colony forming units
CNL	Coiled coil NLR
CRK	Cysteine-rich receptor-like kinase
DAMP	Damage-associated molecular pattern
EDS1	Enhanced disease susceptibility 1
ETI	Effector-triggered immunity
FLS2	Flagellin sensitive 2
GRDP2	Glycine-rich domain protein 2
(co-)IP	(Co-)Immunoprecipitation
LRR	Leucine-rich repeat
LSD1	Lesion stimulating disease 1
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NB	Nucleotide binding domain
NLR	Nucleotide-binding leucine-rich repeat receptor
NOS	Nopaline synthase
NRG1	N requirement gene 1
OD	Optical density
PAD4	Phytoalexin deficient 4
(RT-)PCR	(Reverse transcriptase-) Polymerase chain reaction
PBL	AvrPphB susceptible 1-like
PDB	Protein data bank
PML	Potentially membrane localized NLR

Abbreviation	Definition
PR1	Pathogenesis-related protein 1
PRIMER	Primary immune responder
PRR	Pattern recognition receptor
<i>Pst</i>	<i>Pseudomonas syringae</i>
PTI	Pattern-triggered immunity
RAR1	Required for Mla12 resistance 1
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNL	RPW8-like coiled-coil NLR
Roq1	Recognition of XopQ 1
RPM1	Resistance to <i>Pseudomonas syringae</i> pv. <i>Maculicola</i> 1
RPS2	Resistance to <i>Pseudomonas syringae</i> 2
RPS4	Resistance to <i>Pseudomonas syringae</i> 4
RPW8	Resistance to powdery mildew 8
RRS1	Resistance to <i>Ralstonia solanacearum</i> 1
SA	Salicylic acid
SAG101	Senescence-associated gene 101
SDM	Site-directed mutagenesis
SERK	Somatic embryogenesis receptor kinase
SID2	SA induction-deficient 2
<i>sith</i>	Stunting induced by triggered helper NLR
SOBIR1	Suppressor of <i>bir1-1</i>
SNC1	Suppressor of <i>npr1-1</i> , constitutive 1
TIR	Toll/Interleukin-1 receptor
TNL	Toll/Interleukin-1 receptor NLR
ZAR1	HopZ-activated resistance 1

General introduction

In this general introduction, I will broadly introduce the plant immune system and discuss several concepts that are relevant to all chapters. At the start of each individual chapter of this PhD thesis, I will introduce additional concepts that apply to that chapter specifically.

The importance of plant immunity

Throughout their lifetime, plants are constantly sensing and adapting to their environment, dealing with biotic and abiotic stresses. Biotic stress is generated by micro-organisms (bacteria/fungi/oomycetes/viruses¹) living on or inside various plant tissues. Interactions between a plant and microbes can provide a benefit to the plant, but can also be detrimental (Delaux & Schornack, 2021). Detrimental microbes, referred to as pathogens, cause disease which can have major effects on plant growth leading to reduced crop yield in agriculture (Jones et al., 2024). To combat these pathogenic microbes, plants evolved an intricate innate immune system that consists of two interconnected layers: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). Fundamental understanding of the plant immune system is essential for generating plants that are more resilient plants against invading pathogens (Snoeck et al., 2025; Zdrzałek et al., 2023).

Pattern-triggered immunity

PTI forms the first layer of molecular defence against pathogens (Figure 1). Initiation of PTI occurs at the cell surface after perception of pathogens by pattern recognition receptors (PRR) (X. Yu et al., 2017). The diverse arsenal of PRRs in plants can perceive a range of microbe- or damage-associated molecular patterns (MAMP or DAMP, respectively) (Ngou et al., 2024; X. Yu et al., 2017). PRRs can be classified into receptor-like kinases (RLK) and receptor-like proteins (RLP), depending on the presence or absence of a cytosolic kinase domain (Jamieson et al., 2018; D. Tang et al., 2017). The type of extracellular domain determines the type of ligand bound by the receptor (Ngou et al., 2024; J. Wang & Chai, 2020).

Perception of MAMPs is mediated by the extracellular domain and leads to the recruitment of a co-receptor (Roux et al., 2011). For immune signalling, the co-receptors ‘brassinosteroid insensitive 1-associated kinase 1’ (BAK1/SERK3) or ‘BAK1-like 1’ (BKK1/SERK4) are recruited by PRRs to activate downstream signalling (Roux et al., 2011). RLPs, lacking an intracellular kinase domain, additionally require ‘suppressor of *bir1-1*’ (SOBIR1) to form a functional signalling unit (Bi et al., 2014; Gust & Felix, 2014; Jehle et al., 2013). Together with their co-receptors, PRRs initiate a cascade of immune signalling through phosphorylation of downstream components leading to a rapid initial immune response.

¹ Viruses are not classified as living organisms, but are relevant in the context of plant immunity

PTI signalling is further dependent on receptor-like cytoplasmic kinases (RLCK), specifically those of the RLCK subfamily VII (Rao et al., 2018). The most well-characterized RLCK from this family, '*Botrytis*-induced kinase 1' (BIK1), associates with various PRR complexes and is required for proper initiation of immune responses (Z. Liu et al., 2013; Lu et al., 2010; J. Zhang et al., 2010). Upon ligand perception, PRR complexes phosphorylate BIK1 (and potentially other RLCKs). Activation of the RLCKs then mediates phosphorylation of other immune components to propagate immune signalling (L. Li et al., 2014).

Initiation of the PTI response is characterized by several molecular events, such as a reactive oxygen species (ROS) burst, activation of mitogen-activated protein kinase (MAPK) cascades, callose deposition, and transcriptional reprogramming (Tsuda & Katagiri, 2010).

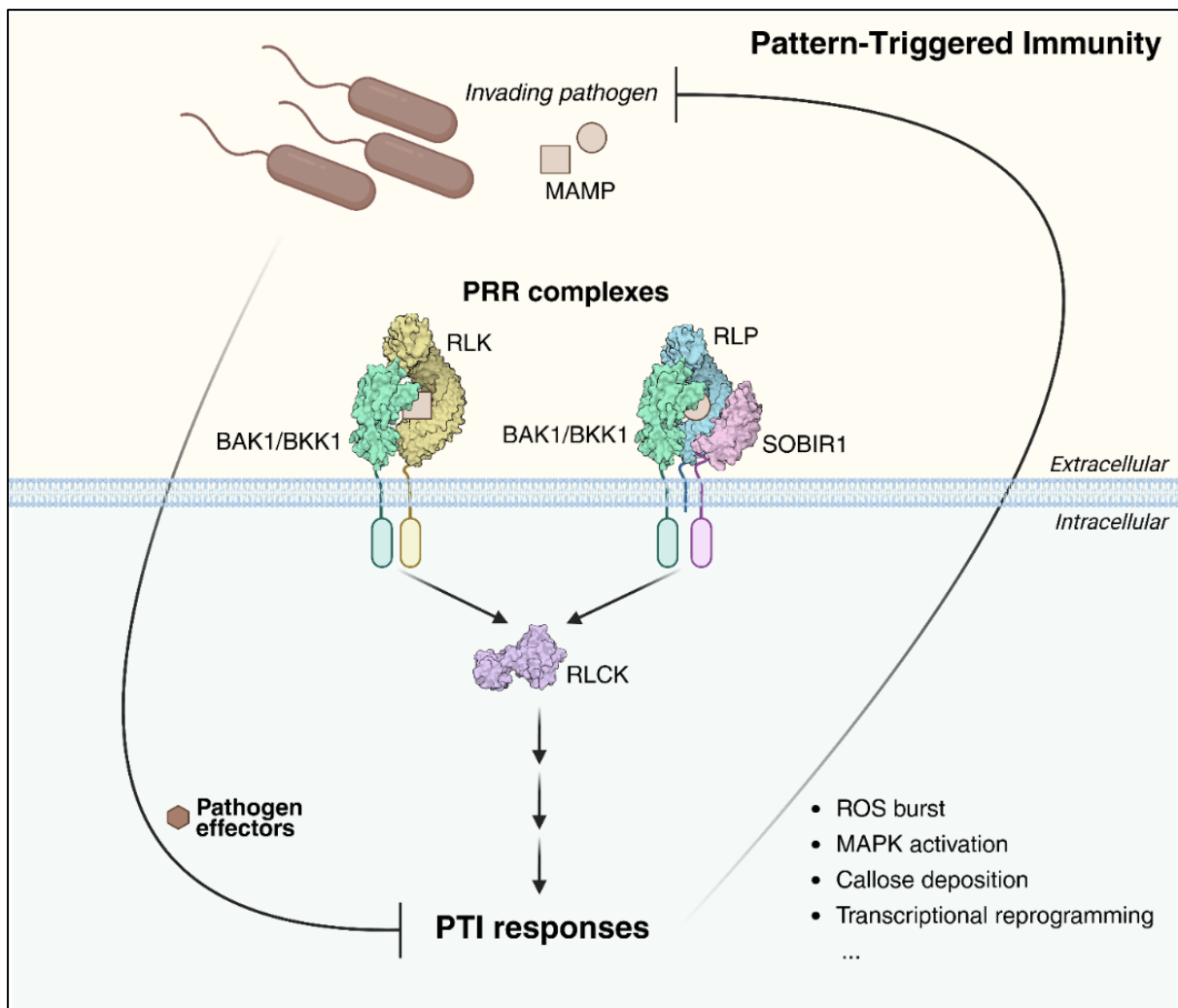


Figure 1: Overview of the pattern-triggered immunity in plants. Conserved molecular patterns (MAMPs) from invading pathogens are perceived by RLKs or RLPs with their respective co-receptors. Activation of these PRR complexes leads to large scale changes in the phosphorylation landscape within the cell. Together with RLCKs, these PRR complexes initiate molecular events like the reactive oxygen species burst, MAPK signalling cascades, and transcriptional reprogramming. Adapted pathogens secrete effectors into the plant cell to suppress these PTI responses. Proteins are represented by various crystal structures acquired from the RCSB Protein Data Bank (PDB). The PDB accession codes and references for protein structure models used in this figure are available in Table S1. This figure was created using BioRender (www.biorender.com).

The immune response from PTI is enough to provide resistance against non-adapted microbes. However, adapted pathogens evolved specialized proteins known as effectors that suppress/manipulate PTI and promote effective colonization of plant tissues (Figure 1) (S. Zhang et al., 2022). To combat against pathogens that can overcome PTI responses, plants evolved ETI as a second layer of immunity (Jones & Dangl, 2006).

Effector-triggered immunity

ETI is activated upon the perception of effectors or their activity inside the cell and initiates a stronger immune response than PTI alone. Often, ETI culminates in a hypersensitive response (HR) to the invading pathogen, characterized by local cell death to prevent pathogen spread (Balint-Kurti, 2019). Effector recognition and the initiation of ETI is mediated by intracellular receptors known as nucleotide-binding leucine-rich repeat receptors (NLR) (Iakovidis et al., 2023; Jones & Dangl, 2006). NLR-like proteins are conserved across the tree of life, exhibiting various combinations of conserved protein domains (K. Chia & Carella, 2023). In plants, NLRs consists of a variable N-terminal domain, a nucleotide-binding domain shared by APAF-1, R proteins and CED-4 (NB-ARC), and a leucine-rich repeat (LRR) domain (Figure 2a) (K.-S. Chia et al., 2024; Van Der Biezen & Jones, 1998; Van Ooijen et al., 2008).

During activation, NLRs undergo conformational changes to relieve autoinhibition by the NB-ARC and LRR domain and to initiate signalling (Z. Hu et al., 2013; Van Ooijen et al., 2008). Activation of the NLR is achieved by switching from an ADP-bound off-state to an ATP-bound on-state (Figure 2a) (Leipe et al., 2004). Various motifs in the NB-ARC domain are crucial for this 'molecular switch'. The Walker A motif or phosphate-binding loop (P-loop) is responsible for nucleotide binding and the downstream Walker B motif mediates nucleotide hydrolysis (Tameling et al., 2006). The MHD motif forms another highly conserved motif that plays a crucial role in the inhibition/activation of NLRs, and can be leveraged to generate auto-activated versions (Roberts et al., 2013; Van Ooijen et al., 2008).

NLR activation occurs after the recognition of a pathogen effector inside the cell (Figure 2b). Recognition can occur through direct binding of the effector (Chen et al., 2022; Krasileva et al., 2010; Salcedo et al., 2017; Saur et al., 2019). Other NLRs monitor important effector targets for indication of effector activity, such as modifications or degradation (Axtell & Staskawicz, 2003; Mackey et al., 2002, 2003; Schultink et al., 2017; M.-Y. Wang et al., 2023). Often effector recognition is attributed to the LRR domain (Dodds et al., 2006; Krasileva et al., 2010), but some NLRs evolved integrated domains that resemble effector targets to capture effectors and activate immune signalling (Cesari et al., 2014; Kroj et al., 2016; Williams et al., 2014).

Some NLRs are multifunctional singleton NLRs that require no other NLRs to achieve resistance (Figure 2c) (Boyes et al., 1998; J. Wang, Wang, et al., 2019). Other NLRs form signalling pairs or networks consisting of ‘sensor’ and ‘helper’ NLRs.

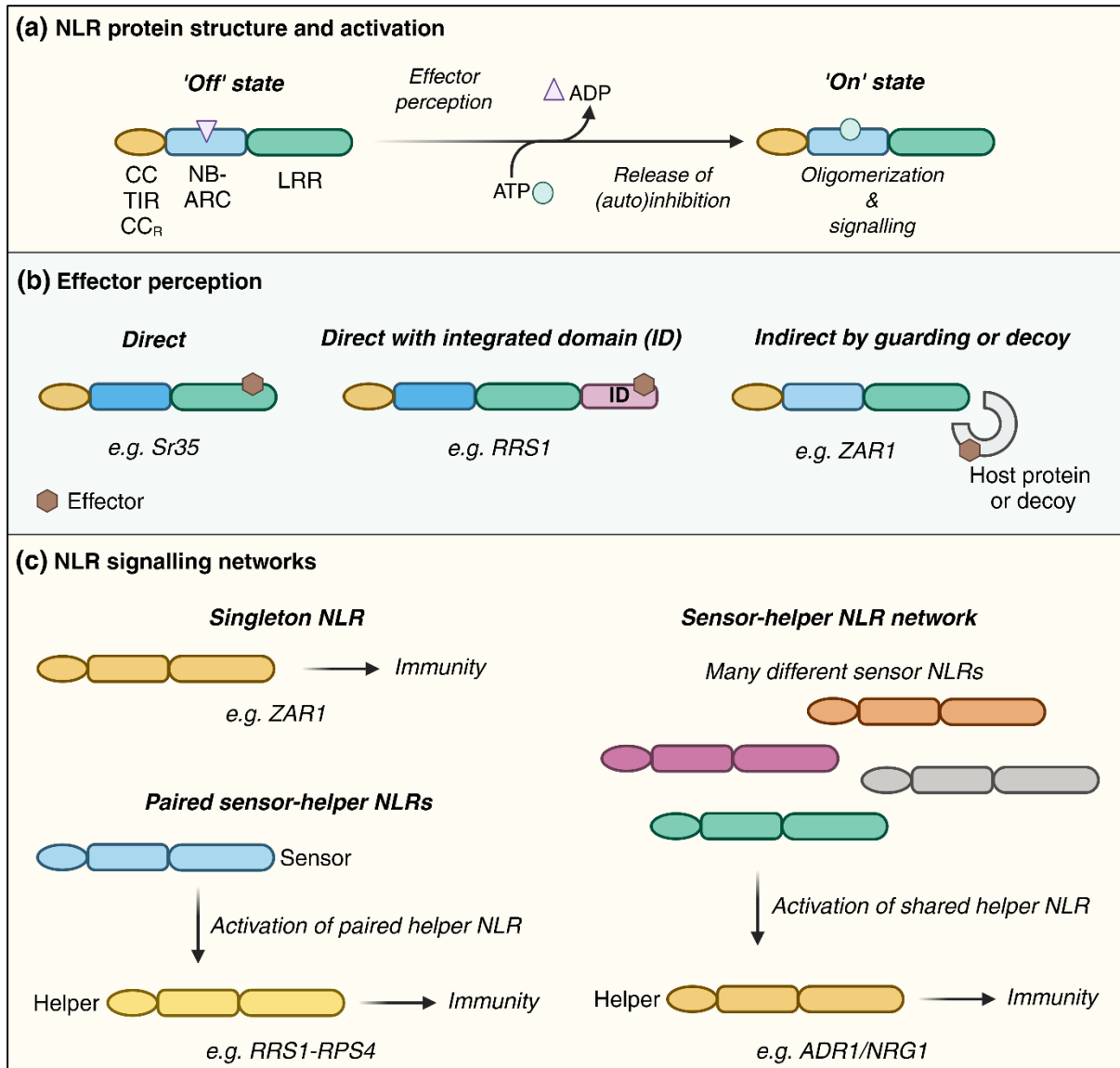


Figure 2: Schematic overview of NLR structure, effector perception, and signalling. (a) NLR proteins consist of a variable N-terminal domain, a central NB-ARC, and C-terminal LRR domain. The N-terminal domain determines the mode of action of the NLR. The NB-ARC mediates activation by switching from an ADP-bound ‘off’ state to an ATP-bound ‘on’ state. The LRR plays a role in autoinhibition and perception of effectors. Activation of NLRs leads to the initiation of immune signalling, often by oligomerization of the NLR. (b) Effector perception by NLRs can be direct or indirect. In direct recognition, the NLR itself binds the effector, commonly through the LRR domain or an integrated domain. Indirect effector perception is commonly called ‘guarding’. The NLR monitors a host protein (or a decoy) for signs of effector activity, such as modifications or degradation. (c) NLR signalling can be mediated by single NLRs, pairs of NLRs or extensive NLR networks. Singleton NLRs require no additional NLRs to achieve immunity. Paired NLRs are comprised of a genetically linked sensor- and helper-NLR that function together. The sensor NLR mediates effector recognition and activates the helper NLR to initiate immune responses. Sensor-helper NLR networks function like paired NLRs but comprise a multitude of sensor NLRs that converge on a small number of helper NLRs to achieve immunity. This figure was created using BioRender (www.biorender.com).

In these networks, effector recognition by the sensor NLR activates the helper NLR to mediate downstream signalling. Paired NLRs are genetically linked NLR genes that encode for a sensor- and helper NLR which act as a functional unit to induce resistance (Cesari et al., 2013; Narusaka et al., 2009). Other helper NLRs have evolved to mediate signalling for a large number of sensor NLRs, forming extensive sensor-helper NLR networks (Figure 2c) (Contreras, Lüdke, et al., 2023; Goh et al., 2024; Gong et al., 2023; Saile & El Kasmi, 2023).

After activation, most NLRs oligomerize to mediate downstream signalling. The resulting protein complex, referred to as the ‘resistosome’, may vary between NLRs in its stoichiometry (Contreras, Pai, et al., 2023; G. Guo et al., 2026; Martin et al., 2020; J. Wang, Hu, et al., 2019; Zhao et al., 2022). The mode of action of the resistosome is determined by the N-terminal domain of the NLR (Figure 3). Based on this N-terminal domain, we can classify NLRs into three distinct clades: coiled-coil NLRs (CNL), Toll/Interleukin-1 NLRs (TNL), and ‘resistance to powdery mildew 8’ (RPW8)-like coiled coil NLRs (RNL) (Shao et al., 2016).

Coiled-coil NLR (CNL)

Coiled-coil (CC) NLRs are found abundant and widespread in all land plants, but show high levels of divergence (H. Lee, Mang, et al., 2021; Seo et al., 2016; J.-Y. Wu et al., 2021). CNLs can be divided into subclasses based on conserved but distinct motifs in their CC domain: CC_{EDVID}, CC_{G10}, and CC_{RPW8} (A. R. Bentham et al., 2018; G. Guo et al., 2026; Sulkowski et al., 2025; Sunil et al., 2024). The RNLs (CC_{RPW8}) are functionally distinct from the other CNLs, acting solely as helper NLRs in extensive sensor-helper networks and will be discussed separately. The precise difference between CC_{EDVID}- and CC_{G10}-type CNLs is not known, but are proposed to underpin variations in CNL localization and oligomerization (Sulkowski et al., 2025; Sunil et al., 2024).

The CC domain of CNLs forms the basis for oligomerization and signalling. The CNL ‘HopZ activated resistance 1’ (ZAR1) was the first plant NLR to have its resistosome structure solved (J. Wang, Hu, et al., 2019). ZAR1 forms a pentameric structure, but other CNLs have since been shown to form hexamers and octamers (G. Guo et al., 2026; Selvaraj et al., 2024; Zhao et al., 2022). These CNL resistosomes function as a Ca²⁺-permeable channel in the plasma membrane (Figure 3) (Bi et al., 2021). The rapid influx of calcium ions induced by CNLs is believed to initiate downstream response and contribute to cell death (Köster et al., 2022).

Toll/Interleukin-1 NLR (TNL)

Toll/Interleukin-1 receptor (TIR) domains are conserved signalling components in immune responses of prokaryotes, mammals, and plants (Essuman et al., 2022). In plants, the TNL clade of NLRs integrated TIR domains as their N-terminal domain (Meyers et al., 2003). Upon effector recognition, TNLs indirectly induce potent cell death (Horsefield et al., 2019; Swiderski

et al., 2009; Wan et al., 2019). This response is initiated by oligomerization of TNLs to form tetrameric complexes (Figure 3) (Ma et al., 2020; Martin et al., 2020). Together, the TIR domains in the TNL resistosome form a holoenzyme with NAD cleaving activity (Horsefield et al., 2019; Wan et al., 2019). The TIR enzymatic activity leads to the accumulation of pRib-AMP/pRib-ADP and ADPR-ATP/di-ADPR (S. Huang et al., 2022; Jia et al., 2022).

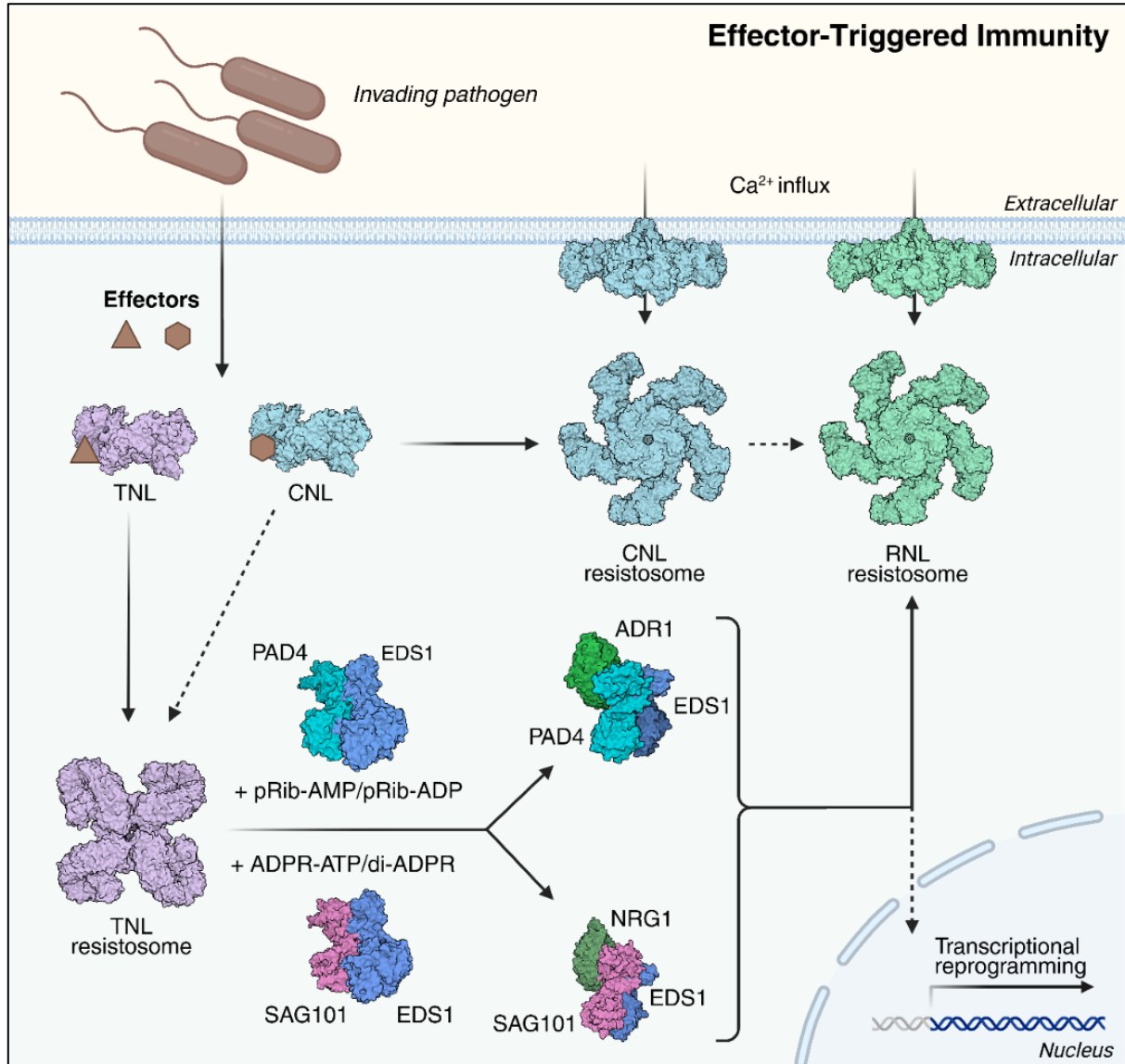


Figure 3: Overview of NLR function in *Arabidopsis* effector-triggered immunity. Effectors from invading pathogens are perceived (directly or indirectly) by TNLs or CNLs (sensor NLRs). Upon activation, CNLs oligomerize to form calcium-permeable channels in membranes, leading to rapid influx of Ca^{2+} . Activated TNLs also form resistosomes that are NADase holoenzymes. The enzymatic activity leads to the production of pRib-AMP/pRib-ADP and ADPR-ATP/di-ADPR which are bound by EDS1-PAD4 or EDS1-SAG101, respectively. Binding of these small molecules recruits and activates the RNL families, ADR1 (with EDS1-PAD4) or NRG1 (with EDS1-SAG101). Activation of RNLs mediates resistance through transcriptional reprogramming and the formation of RNL resistosomes. Like CNLs, RNL resistosomes act as calcium-permeable ion channels in membrane compartments like the plasma membrane. Proteins are represented by various crystal structures acquired from the RCSB Protein Data Bank (PDB). The PDB accession codes and references for protein structure models used in this figure are available in Table S1. This figure was created using BioRender (www.biorender.com).

These small signalling molecules are essential for downstream signalling through lipase-like proteins EDS1, PAD4, and SAG101 (Gantner et al., 2019; Rietz et al., 2011; Wagner et al., 2013). EDS1 forms mutually exclusive hetero-dimers with either PAD4 or SAG101 (Dongus et al., 2022; Wagner et al., 2013). The EDS1-PAD4 dimer binds pRib-AMP/pRib-ADP, whereas EDS1-SAG101 binds ADPR-ATP/di-ADPR (S. Huang et al., 2022; Jia et al., 2022). The binding of these small molecules by the EDS1 hetero-dimers introduces similar conformational changes into their structure (S. Huang et al., 2025; Xiao et al., 2025; H. Yu et al., 2024). This conformational change leads to the recruitment of RNLs by the EDS1-complexes and is thought to lead to the activation of the RNLs to mediate further immune signalling (Figure 3) (T. Qi et al., 2018; Z. Wu et al., 2021).

RPW8-like coiled coil NLR (RNL)

RNLs are a subclade of CNLs, containing a N-terminal CC-domain that resembles the CC domain of the resistance gene 'resistance to powdery mildew 8' (RPW8) (Shao et al., 2016). RNLs are not known to directly perceive effectors. Instead, RNLs act as helper NLRs that mediate signalling from sensor NLRs to form the basis for an extensive NLR signalling network (Gong et al., 2023; Saile & El Kasmi, 2023). This functional distinction between sensor- and helper NLR allows for extensive sensor-helper networks to form, where multiple sensor NLRs can signal through a few helper NLRs (Figure 2c) (Gong et al., 2023). The RNLs function downstream of the aforementioned TNLs through EDS1-complexes (Figure 3), but some CNLs also rely on RNLs for full immunity (Ge et al., 2026; Saile et al., 2020).

The RNL clade is made up by the 'activated disease resistance 1' (ADR1) and 'N requirement gene 1' (NRG1) families (Grant et al., 2003; Peart et al., 2005; Shao et al., 2016). The number of RNL members in either family varies between plant species (Collier et al., 2011). In *Arabidopsis thaliana*, the ADR1 family consists of three full-length members (ADR1, ADR1-L1, and ADR1-L2) and the truncated (pseudogene) ADR1-L3 (Bonardi et al., 2011; Gravot et al., 2024). The NRG1 family consists of two full-length members (NRG1.1 and NRG1.2) and the N-terminally truncated NRG1.3 (Castel et al., 2019; Z. Wu et al., 2022). These two gene families act unequally redundant in various *Arabidopsis* immune responses (Pruitt, Locci, et al., 2021; Saile et al., 2020; H. Wang, Tan, et al., 2024). *ADR1* family genes are commonly associated with salicylic acid (SA) dependent responses, transcriptional changes, and pathogen restriction (Cui et al., 2017; Jacob et al., 2023; Lapin et al., 2019). The *NRG1* family genes are more commonly associated with the (hypersensitive) cell death observed at the infection site (Lapin et al., 2019; X. Sun et al., 2021).

Binding of small molecules generated from TIR enzymatic activity by EDS1-complexes is required for the activation of RNLs (Figure 3) (S. Huang et al., 2022; Jia et al., 2022). The ADR1 family specifically interacts with EDS1-PAD4 dimers whereas the NRG1 family interacts with

EDS1-SAG101 dimers (X. Sun et al., 2021; Wagner et al., 2013; Z. Wu et al., 2021). These interactions are proposed to introduce conformational changes in the RNLs, possibly promoting the binding of ATP to switch into an active state (S. Huang et al., 2025; Xiao et al., 2025; H. Yu et al., 2024). After activation, RNLs can form resistosomes resembling those of CNLs (Feehan et al., 2023; Jacob et al., 2021). The precise stoichiometry of RNL resistosome is not yet known as no experimentally determined structures of ADR1 or NRG1 resistosomes have been resolved at this time. The proposed mode of action of RNLs is consistent with CNLs, forming calcium-ion permeable channels in membranes (Jacob et al., 2021). ADR1 members localize primarily at the plasma membrane of the cell through phospholipid interactions (Saile et al., 2021). NRG1 members are proposed to also function at the plasma membrane but may additionally function at other compartments (Ibrahim et al., 2026; Jacob et al., 2021). However, more and more evidence is accumulating that RNLs are additionally translocated into the nucleus and fulfil a function there that is required to mediate full resistance (Bernoux et al., 2023; García et al., 2010; Jianyu Li et al., 2025; X. Sun et al., 2021).

The interconnections between PTI and ETI

The two branches of plant immunity, PTI and ETI, were long considered and studied as distinct and separate immune responses (Jones & Dangl, 2006). However, scientific advances allowed for studying ETI in absence of PTI and revealed that PTI signalling is required for full ETI responses (Yuan et al., 2021). Changes in the transcriptional landscape induced by PTI include the increased expression of various NLR genes, suggesting that PTI prepares the plant cell for a potential ETI response (Feehan et al., 2023; Tian et al., 2021). Various complexes consisting of combinations of ‘canonical’ PTI and ETI components have also been identified, suggesting that plant immunity is a collaborative effort by the two branches of the plant immune system (Pruitt, Locci, et al., 2021; Schulze et al., 2022; Z. Wang et al., 2026). The interconnectivity of PTI and ETI is further reinforced by the requirement of TNL activity and EDS1-signalling for PTI signalling (Pruitt, Locci, et al., 2021; Tian et al., 2021). Specifically, EDS1-PAD4-ADR1 is required in *Arabidopsis* for cell surface signalling by some RLPs, but not for two tested RLKs (Pruitt, Locci, et al., 2021). These findings conceptually fit with natural ETI-eliciting infections, where the physical presence of the pathogen should also trigger (suppressed) PTI. However, the presence of a MAMP should not immediately initiate a full immune response, since plant defence is an energetically costly process. So, plants must carefully regulate and finetune their immune system, balancing growth and defence (He et al., 2022). Understanding how plants prioritize between growth and defence is crucial for furthering the engineering of plant immunity without affecting crop yield. However, there is still much to be learned about how the plant immune system is tightly regulated, and how the activation RNLs leads to an appropriate immune response.

Objectives

To address this knowledge gap on the regulation of *Arabidopsis* RNLs, I address the following overarching research question in this PhD thesis:

How are *Arabidopsis* RNLs regulated to achieve the desired immune response?

This question was investigated and discussed from various angles across three chapters. In Chapter 1, I investigated the autoimmune phenotype of a combinatorial *Arabidopsis* RNL mutant (*adr1-l1-1 adr1-l2-4 nrg1.1-2*). Autoimmunity in plants is often caused by mis-regulation of immune components (Van Wersch et al., 2016). Here, the objective was to identify which branch of the plant immune system is mis-regulated in this RNL mutant and how the absence of three RNLs leads to autoimmunity.

Then in Chapter 2, I continued the investigation from the previous chapter from a mechanistic point-of-view. Helper NLR networks can be regulated by other family members, but regulation of the ADR1 family is largely unknown (Adachi et al., 2023; Z. Wu et al., 2022). Using biochemical approaches, I studied the initial hypothesis proposed in Chapter 1 about regulation of ADR1 by other RNLs. The objective in this chapter was to identify how other RNLs regulate the activity of ADR1.

Finally, in Chapter 3, I studied how phosphorylation regulates the activity of *Arabidopsis* ADR1-L2. Phosphorylation is essential for plant immune signalling, and various NLRs have been reported to undergo phosphorylation to regulate the immune response (Y. Yan et al., 2025). The objective here was to use a wide range of (de)phosphorylation mimicking mutations in ADR1-L2 to uncover how phosphorylation affects the activity of ADR1-L2.

The three chapters are then connected in the General discussion where I discuss the multifaceted roles of *Arabidopsis* RNLs in plant immunity and how the activity of RNLs is carefully regulated. I conclude with the main findings from this work, highlighting new directions for future research into the function and regulation of *Arabidopsis* RNLs.

Chapter 1

Defining the Basis of Autoimmunity in an *Arabidopsis* Helper NLR Mutant

Introduction

Autoimmunity in mammals is defined “an immune response leading to reaction with self-antigen, i.e. any molecule that is a normal body constituent of the animal mounting the response” (Delves, 1998). In plants, autoimmunity commonly refers to the constitutive activation of the plant immune system due to mis-regulation of immune components (Freh et al., 2022; Van Wersch et al., 2016). This distinction arises from plant NLRs naturally monitoring important components for plant physiology and immunity (Van Der Hoorn & Kamoun, 2008). Modification or absence of these crucial components due to effector activity then leads to the activation of an immune response. Thus, autoimmunity in plants is often not due to the recognition of self-antigens, but rather by the disruption of critical components monitored by NLRs leading to immune activation.

The autoactivation of the plant immune system is costly and leads to a trade-off in growth and development (He et al., 2022). In *Arabidopsis*, autoimmunity is characterized by stunted growth, curled leaf morphology, and often spontaneous cell death (Van Wersch et al., 2016). The striking phenotype associated with autoimmunity is ideal for genetic screens. Genetic screens using autoimmunity have resulted in many new insights in immune signalling pathways in plants (Bonardi et al., 2011; Rate et al., 1999; Schulze et al., 2022; Y. Wu et al., 2020; Z. Wu et al., 2020; Zhu et al., 2010). Mutagenesis of wildtype plants can induce autoimmunity through autoactivating mutations or disrupting regulation of immune components (Dong et al., 2016; Grant et al., 2003; X. Li et al., 2001; Roberts et al., 2013; Y. Zhang et al., 2003). Genetic screens can also be used with plants portraying autoimmunity to look for enhanced or rescued phenotypes. Targeted approaches using loss-of-function alleles of common immune components are also informative to identify the responsible gene or signalling pathway behind an autoimmune phenotype (Roberts et al., 2013; Y. Zhang et al., 2003). A relevant example of these approaches is *ADR1*, which was identified by random activation-tagging in a ‘*pathogenesis related 1*’ (PR1) reporter-line as indicator of autoimmunity (Grant et al., 2003). This initial discovery of *ADR1* was crucial for uncovering the helper NLR branch of the plant immune system. Further studies then revealed the expanded *ADR1* family and their central role in plant immune responses (Bonardi et al., 2011; Dong et al., 2016; M. Hu & Zhou, 2022; Pruitt, Locci, et al., 2021).

As indicated by the many discoveries that relied on autoimmune phenotypes, autoimmunity forms a potent tool to probe the plant immune system (Van Wersch et al., 2016). In this chapter, we identified an autoimmune phenotype specific to the *adr1-l1-1 adr1-l2-4 nrg1.1-2* triple mutant. Using targeted genetic approaches, we believed the autoimmunity to rely on *ADR1* and associated signalling pathways. Based on our genetic analysis, we could unlink the autoimmunity from the absence of *ADR1-L1*, *ADR1-L2*, and *NRG1.1*. Despite these

efforts, the direct cause of the autoimmunity remains unknown, but we generated a list of candidate mutations that may be causal. We investigated the most promising candidate but could show that the autoimmune phenotype established independent of this mutation.

Results

The *adr1-l1 adr1-l2 nrg1.1* mutant portrays ADR1-dependent autoimmunity

While crossing various T-DNA loss-of-function alleles of *Arabidopsis thaliana Col-0* RNLs (Table S2), we observed that the *adr1-l1-1 adr1-l2-4 nrg1.1-2* triple mutant was stunted in growth and curled leaf morphology (Figure 1.1a, b). For the remainder of this chapter, I will refer to the *adr1-l1-1 adr1-l2-4 nrg1.1-2* triple mutant as *adr1-l1 adr1-l2 nrg1.1* unless the specific alleles are relevant. We quantified the stunted growth by fresh weight measurements, and indeed the *adr1-l1 adr1-l2 nrg1.1* was significantly reduced in fresh weight compared to *Col-0* (One-way ANOVA with post-hoc TukeyHSD, $F_{2,87} = 76.37$, $n = 20$, $p = 3.09 \times 10^{-10}$). Loss of immune components can lead to autoimmunity (Van Wersch et al., 2016), so we hypothesized that the stunted phenotype was caused by an auto-active immune system. Using ‘pathogenesis-related protein 1’ (PR1) protein accumulation as a measure, we showed that *adr1-l1 adr1-l2 nrg1.1* indeed portrayed autoimmunity (Figure 1.1c).

In crossing all these RNL loss-of-function T-DNA lines, we also observed that the *adr1-1 adr1-l1 adr1-l2 nrg1.1* quadruple mutant was not stunted (Figure 1.1a, b). This suggested to us that the loss of *ADR1* rescued the stunted phenotype. Loss of individual *ADR1*-family genes was previously shown to affect the expression of remaining *ADR1* genes in a different autoimmune context (Dong et al., 2016), so we wondered whether overexpression of *ADR1* could be causing the observed autoimmunity. Semi-quantitative RT-PCR revealed that *ADR1* was expressed at comparable levels in *Col-0* and in the *adr1-l1 adr1-l2 nrg1.1* mutant (Figure 1.1d). Thus, overexpression of *ADR1* was unlikely to be the reason behind this autoimmune phenotype. Instead, we hypothesized that *ADR1* is autoactivated in absence of *ADR1-L1*, *ADR1-L2* and *NRG1.1* and that under normal circumstances these three RNLs redundantly suppress *ADR1* activity (Figure 1.1e). In this chapter, we further investigated the cause of the autoimmunity in the *adr1-l1 adr1-l2 nrg1.1* triple mutant. Simultaneously, we set out to understand the molecular mechanism by which *ADR1-L1*, *ADR1-L2* and *NRG1.1* may be suppressing *ADR1* activity (Chapter 2).

Canonical signalling partners of ADR1 are required for autoimmunity

For all known immune functions, *ADR1* requires lipase-like proteins *EDS1* and *PAD4* to initiate immune signalling (Cui et al., 2017; Lapin et al., 2019). We hypothesized that *ADR1*-dependent autoimmunity would also require *EDS1* and *PAD4*, so we generated the *adr1-l1 adr1-l2 nrg1.1 eds1-12* and *adr1-l1 adr1-l2 nrg1.1 pad4-2* quadruple mutants. As expected, removing either *EDS1* or *PAD4* rescued the autoimmune phenotype of *adr1-l1 adr1-l2 nrg1.1* (Figure 1.2). *SAG101* on the other hand acts in a mutually-exclusive way to *PAD4*, signalling with *EDS1* and *NRG1* family proteins (Lapin et al., 2019; X. Sun et al., 2021). Thus, we hypothesized that *SAG101* would not be required for *ADR1*-dependent autoimmunity, unlike

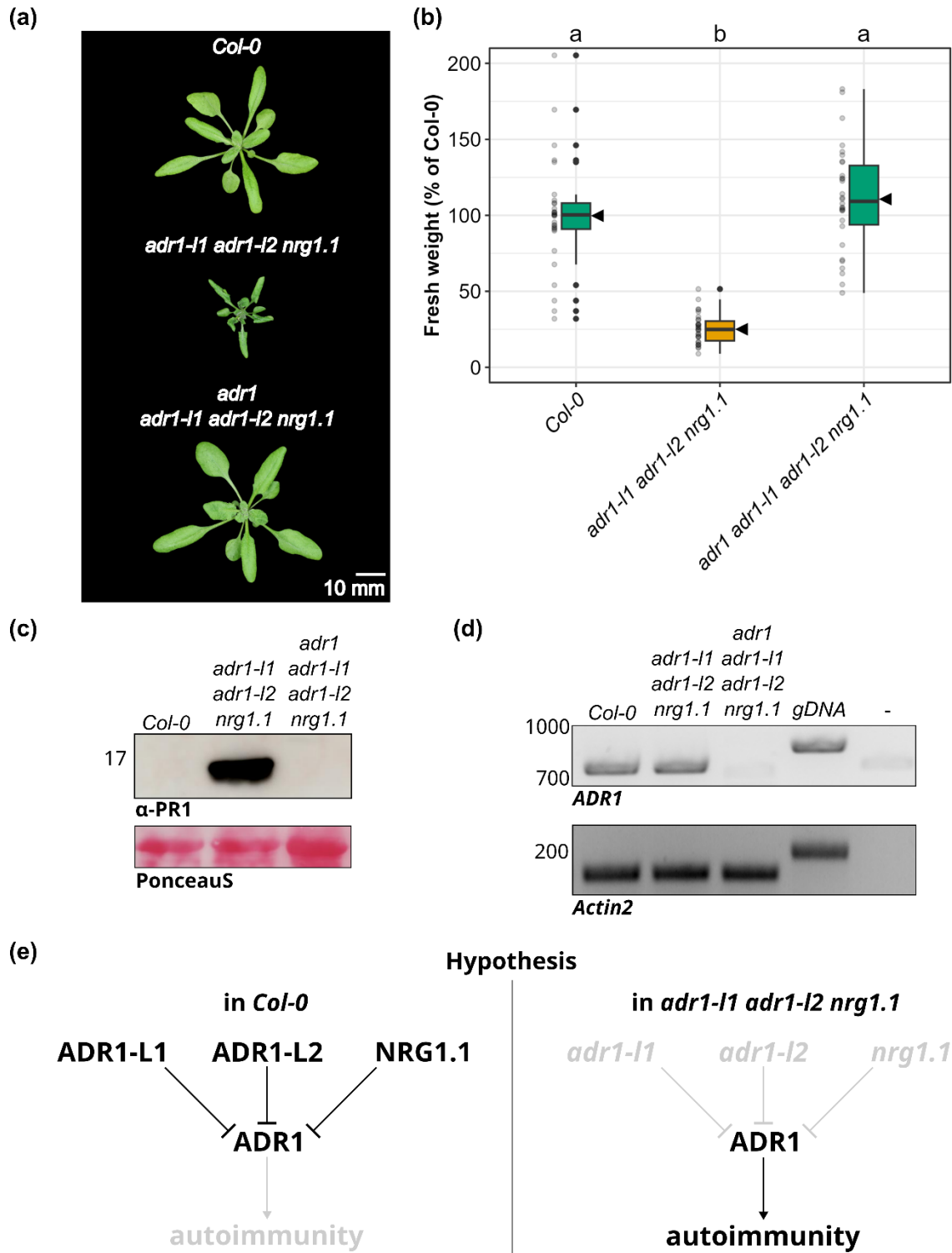


Figure 1.1: The *adr1-l1 adr1-l2 nrg1.1* triple mutant portrayed ADR1-dependent autoimmunity. (a) *adr1-l1 adr1-l2 nrg1.1* plants displayed stunted growth and curled leaves, indicative of autoimmunity. Loss of ADR1 restored the stunted growth phenotype. Pictures were taken of 4-week-old *Arabidopsis* plants grown under short-day conditions. All loss-of-function alleles used in this work are listed in Table S2. (b) Quantification of fresh weight for the genotypes depicted in (a) relative to *Col-0* (100%). The arrow (◄) indicates the mean fresh weight of each genotype. The letters depict the statistical result of a One-Way ANOVA with post-hoc TukeyHSD ($F_{2,87} = 76.37$, $n = 20$). Genotypes sharing the same letter are *not* statistically different in fresh weight. Genotypes that were significantly reduced in fresh weight compared to *Col-0* are depicted in orange (c) PR1 was upregulated in *adr1-l1 adr1-l2 nrg1.1* plants, but not in *Col-0* or *adr1 adr1-l1 adr1-l2 nrg1.1* plants. (continued on the next page)

(continued) Western blot was performed with anti-PR1. The number on the left indicates the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. **(d)** *ADR1* transcription is not upregulated in *adr1-l1 adr1-l2 nrg1.1*. Expression was assessed using semi-quantitative RT-PCR detecting *ADR1* transcript (40 cycles). *Actin2* is shown as loading control (30 cycles). Primers are listed in Table S3. **(e)** Schematic representation of our working hypothesis on redundant regulation of ADR1 activity by ADR1-L1, ADR1-L2, and NRG1.1. We hypothesized that in absence of these three RNLs, an ADR1-dependent immune response is initiated, leading to autoimmunity

PAD4. Indeed, crossing the *sag101-2* allele into the *adr1-l1 adr1-l2 nrg1.1* background did not rescue the autoimmune phenotype (Figure 1.2). Taken together, these findings reinforce the idea that ADR1 is auto-actively signalling in the *adr1-l1 ar1-l2 nrg1.1* mutant in an EDS1/PAD4-dependent manner.

The ADR1-EDS1-PAD4 node is intrinsically linked to salicylic acid (SA)-mediated defences (Bonardi et al., 2011; Cui et al., 2017). So, we hypothesized that ADR1-dependent autoimmunity might involve SA signalling. To test this hypothesis, we crossed the *sid2-1* allele into the *adr1-l1 adr1-l2 nrg1.1* background to disrupt SA biosynthesis (Nawrath & Métraux, 1999). The *adr1-l1 adr1-l2 nrg1.1 sid2-1* quadruple mutant was not stunted in growth, suggesting that SA biosynthesis is required to establish or maintain the ADR1-dependent autoimmunity (Figure 1.2).

Chaperone proteins, like ‘required for Mla12 resistance 1’ (RAR1), have been proposed to be required for various NLRs to ensure proper protein folding and accumulation (Azevedo et al., 2002; Belkhadir et al., 2004; Bieri et al., 2004; Holt et al., 2005; Tornero et al., 2002). We crossed the *rar1-21* loss-of-function allele into the *adr1-l1 adr1-l2 nrg1.1* triple mutant to assess whether ADR1 autoimmunity was dependent on RAR1. Autoimmunity was not abolished in absence of RAR1, suggesting that ADR1 auto-activity does not require RAR1 (Figure 1.2). This observation aligned with previous findings that ADR1-L2 protein accumulation did not require RAR1 either (Roberts et al., 2013).

The *adr1-l1-1* allele was previously shown to produce a truncated version of ADR1-L1 that attenuates the TNL ‘suppressor of npr1-1, constitutive’ (SNC1) from signalling in an ADR1-dependent manner (M.-Y. Wang et al., 2023). To ensure that the autoimmune phenotype we study is not caused by the same SNC1-dependent signalling cascade, we generated a CRISPR-Cas9 knock-out allele for SNC1, *snc1-c* (Figure S1.1a). Introducing *snc1-c* into the *adr1-l1 adr1-l2 nrg1.1* triple mutant did not rescue the autoimmune phenotype (Figure 1.2). This suggested that SNC1 is attenuated by the *adr1-l1-1* allele in this triple mutant and SNC1 is not causal for the observed autoimmunity. These findings reinforce the hypothesis that the autoimmunity in *adr1-l1 adr1-l2 nrg1.1* is caused by ADR1 (auto-)activity.

Taken together, these findings suggested to us that ADR1 is signalling through its canonical partners EDS1 and PAD4 in a SA-dependent manner only when *ADR1-L1*, *ADR1-L2* and *NRG1.1* are absent.

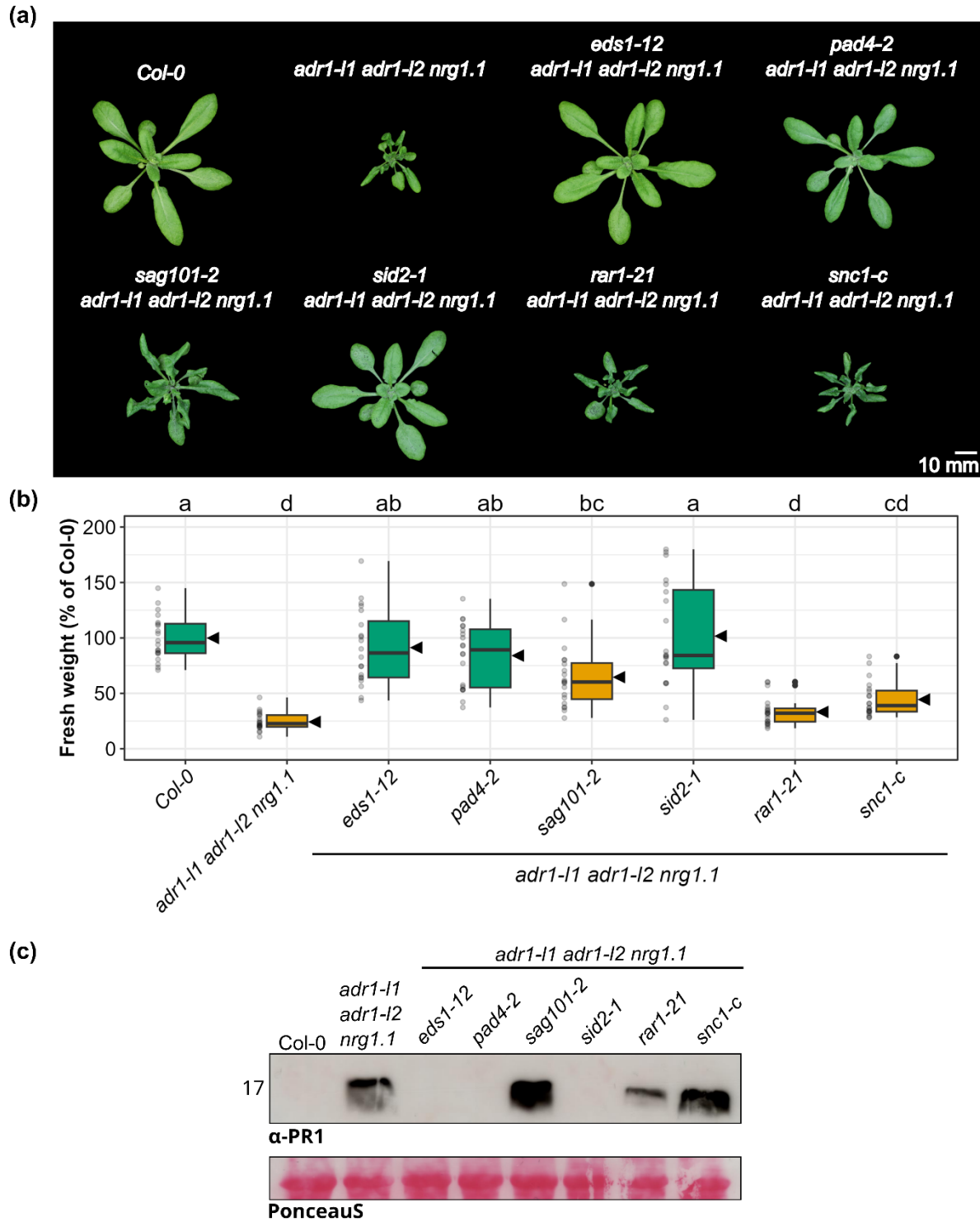


Figure 1.2: Autoimmunity in *adr1-11-1 adr1-12-4 nrg1.1-2* relies on the ADR1-EDS1-PAD4 branch and salicylic acid. (a) Requirements of common RNL immune signalling components for the autoimmunity in *adr1-11 adr1-12 nrg1.1*. Pictures were taken of 4-week-old *Arabidopsis* plants grown under short-day conditions. All loss-of-function alleles used in this work are listed in Table S2. (b) Quantification of fresh weight for genotypes depicted in (a) relative to *Col-0* (100%). The arrow ($\blacktriangleleft$) indicates the mean fresh weight of each genotype. The letters depict the statistical result of a One-Way ANOVA with post-hoc TukeyHSD ($F_{7,152} = 25.16$, $n = 20$). Genotypes sharing the same letter are *not* statistically different in fresh weight. Genotypes that were significantly reduced in fresh weight compared to *Col-0* are depicted in orange. (continued on the next page)

(continued) (c) PR1 accumulation correlated with the autoimmune phenotypes observed in (a). Western blot was performed with anti-PR1. The number on the left indicates the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control.

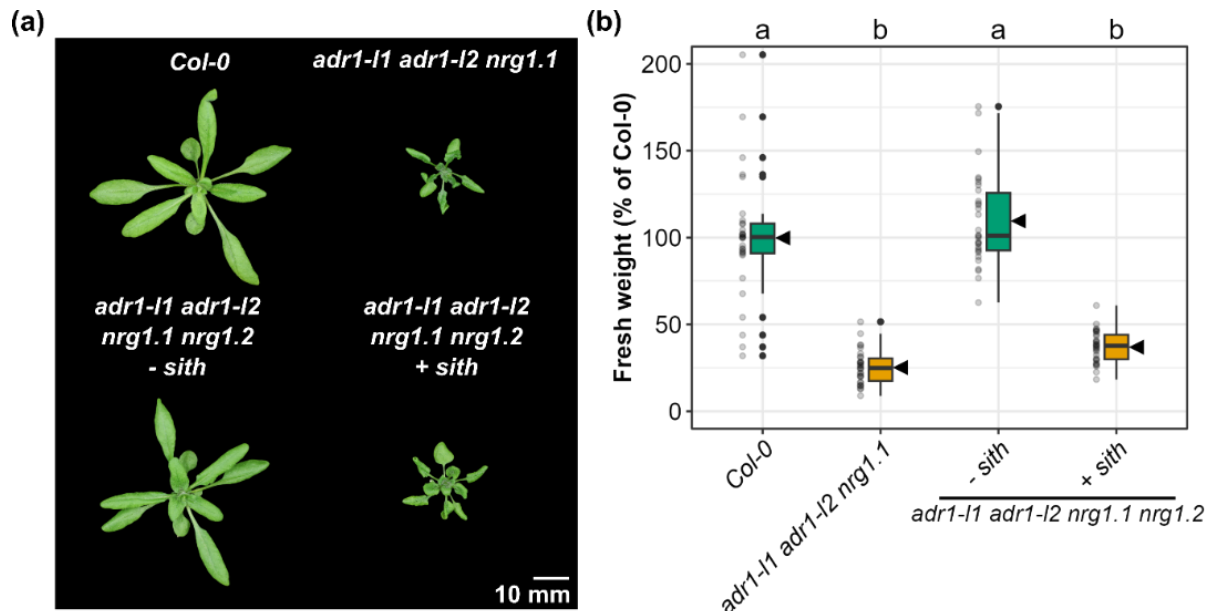
ADR1 auto-activity is not caused by the absence of other RNLs

Unlike the other RNLs, *NRG1.2* appeared to have no clear role in this autoimmune phenotype. Nevertheless, we set out to investigate whether *NRG1.2* contributes in any way to ADR1 auto-activity or the suppression thereof. *NRG1.1* (*At5g66900*) and *NRG1.2* (*At5g66910*) are neighbouring genes on chromosome 5 of *Arabidopsis*, and as a result these genes are tightly genetically linked. Therefore, generating a *nrg1.1-2 nrg1.2-2* double mutant by crossing the respective T-DNA lines is statistically improbable. Instead, we used a previously generated CRISPR-Cas9 allele (*nrg1.1 nrg1.2*) to generate the remaining combinatorial RNL mutants, including the *adr1-l1 adr1-l2 nrg1.1 nrg1.2 quadruple* mutant (Castel et al., 2019). To our surprise, we initially found that this genotype portrayed no autoimmunity, despite lacking the hypothesized negative regulators of ADR1 (Figure 1.3a, b). We speculated that ADR1 may rely on *NRG1.2* to establish autoimmunity. Alternatively, the CRISPR-Cas9 *nrg1.1* allele behaves differently from the T-DNA *nrg1.1-2* allele in the context of ADR1-dependent autoimmunity.

To untangle this complex genetic structure, we crossed the *adr1-l1-1 adr1-l2-4 nrg1.1-2* mutant to the ‘Helperless’ mutant (*adr1-1 adr1-l1-1 adr1-l2-4 nrg1.1 nrg1.2*) to study co-inheritance of *ADR1* and *NRG1.2* with the autoimmune phenotype (Saile et al., 2020). The F2 from this cross segregated for the *ADR1* and *NRG1.2* loci, along with the two different *nrg1.1* loss-of-function alleles. In 80 F2 plants, we found that five plants (6.25% or 1/16) portrayed stunted growth resembling the *adr1-l1 adr1-l2 nrg1.1* parent. The remaining 75 F2 plants (93.75%) portrayed the wildtype-like morphology of the ‘Helperless’ parent. Genotyping the five stunted plants revealed that all five carried homozygous wildtype *ADR1* alleles, further reinforcing the role of ADR1 in this autoimmune phenotype. However, we identified all possible combinations of alleles in the *NRG1* locus in these five stunted plants. This included a second identification of the *adr1-l1 adr1-l2 nrg1.1 nrg1.2 quadruple* mutant which now portrayed autoimmunity (Figure 1.3a, b). This finding pointed us towards the likely possibility that an unknown background mutation was directly causal for the observed autoimmune phenotype, rather than the absence of *ADR1-L1*, *ADR1-L2* and *NRG1.1*. Additionally, *NRG1.2* is not required for the auto-immune phenotype. We named this putative background mutating ‘Stunting Induced by Triggered Helper NLR’ (*sith*).

To identify *sith*, we performed Whole Genome Sequencing on *adr1-l1 adr1-l2 nrg1.1-2*, and the *adr1-l1 adr1-l2 nrg1.1 nrg1.2 quadruple* mutants depicting opposing phenotypes (‘-*sith*’ and ‘+ *sith*’) (Figure 1.3a). We could confirm that our PCR-based genotyping results of the RNL loss-of-function alleles in these genotypes was correct. We identified a total of 30361

variants across these three genotypes compared to the TAIR10 reference genome (Figure 1.3c) (Lamesch et al., 2012).



(c) Whole Genome Sequencing + Variant Calling to identify candidate mutations

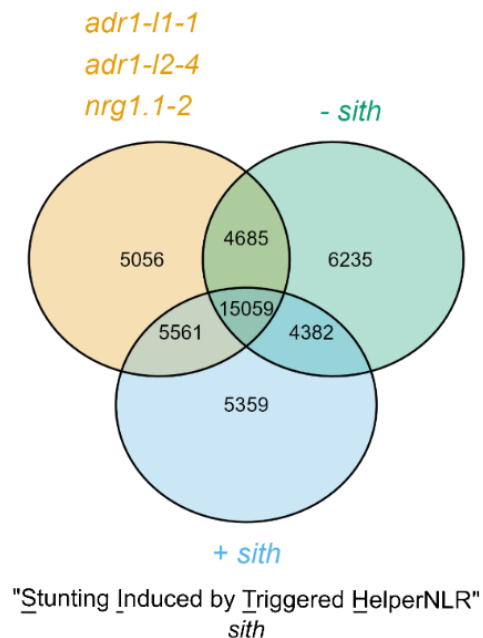


Figure 1.3: Two plants with identical RNL genotypes portrayed opposing phenotypes. (a) The *adr1-l1 adr1-l2 nrg1.1 nrg1.2* genotype was identified with and without autoimmune phenotype. Pictures were taken of 4-week-old *Arabidopsis* plants grown under short-day conditions. **(b)** Quantification of fresh weight for plants depicted in (a) relative to *Col-0* (100%). The arrow (◄) indicates the mean fresh weight of each genotype. The letters depict the statistical result of a One-Way ANOVA with post-hoc TukeyHSD ($F_{3,116} = 102.5$, $n = 20$). Genotypes sharing the same letter are *not* statistically different in fresh weight. Genotypes that were significantly reduced in fresh weight compared to *Col-0* are depicted in orange. **(c)** The Venn diagram depicts shared and non-shared variants between the *adr1-l1 adr1-l2 nrg1.1*, *adr1-l1 adr1-l2 nrg1.1 nrg1.2 +sith*, and *adr1-l1 adr1-l2 nrg1.1 nrg1.2 -sith* plants. Variant calling was performed using TAIR10 as reference genome (Lamesch et al., 2012).

From our previous findings derived from crosses, we could narrow down candidates among these variants drastically by applying the following knowledge:

1. The causal mutation is present in the autoimmune plants (*adr1-l1 adr1-l2 nrg1.1* and '+ *sith*') but absent in the wildtype-like plant ('- *sith*').
2. None of these plants segregated for the auto-immune phenotype, therefore candidate loci must be homozygous for the respective alleles.
3. The *sith* mutation segregated independently of *ADR1* (*At1g33560*).

Applying these conditions on our variant calling results allowed us to generate a list of 94 candidate *sith* mutations. However, 45 candidate variants were omitted due to insufficient sequencing coverage for one (or more) of the genotypes, leaving us with 49 candidate *sith* mutations (Table S1.1). Manual assessment of these candidates focussed our attention to a single mutation: a 22 bp deletion in *At4g23210* only found in the autoimmune plants. *At4g23210* encodes for 'cysteine-rich receptor-like kinase 13' (*CRK13*), which was previously implicated in immunity (Acharya et al., 2007). The 22 bp deletion is located towards the 5' end of the *CRK13* gene (between nucleotides 393 and 415), causing a frameshift followed by an early stop codon which likely leads to a full loss-of-function allele (Figure 1.4a, Figure S1.2). So, we followed up on this 22 bp deletion in *CRK13* as the most promising candidate to be *sith*.

The deletion in *CRK13* is also not causal for the observed autoimmunity

We wanted to investigate whether the deletion in *CRK13* was causal for the autoimmune phenotype in *adr1-l1 adr1-l2 nrg1.1*. So, we generated a F2 population that segregated for the causal mutation by crossing the plants depicted in Figure 1.3 ('- *sith*' x '+ *sith*'). If the *crk13* allele is directly causing the autoimmune phenotype, all offspring that are autoimmune should be homozygous for the deletion in *CRK13*. In 78 F2 plants, 11 plants (14.1%) portrayed stunted growth similar to the '+ *sith*' parent (Figure 1.4b). We designed genotyping primers to amplify the region carrying the 22 bp deletion (Table S3). Genotyping of these stunted plants revealed that all but one carried homozygous deletions in *CRK13* (Figure 1.4c). We carried the non-homozygous F2 plant (#3) into the F3 generation. We found that the offspring were all stunted in growth yet carried all variations of the *CRK13* alleles (Figure 1.4d). Notably, we now identified that the deletion in *CRK13* was heterozygous in the *adr1-l1 adr1-l2 nrg1.1* control plant for genotyping. This suggested that the individual plant sampled for Whole Genome Sequencing was homozygous for the 22 bp deletion in *CRK13* (Figure 1.3c), but that this deletion was segregating in the *adr1-l1 adr1-l2 nrg1.1* population. Although the deletion in *CRK13* was initially seen as a promising *sith* candidate, these finding proved that the autoimmunity is established independently of this deletion. The identity of the *sith* mutation remains unknown, with now 48 candidates remaining.

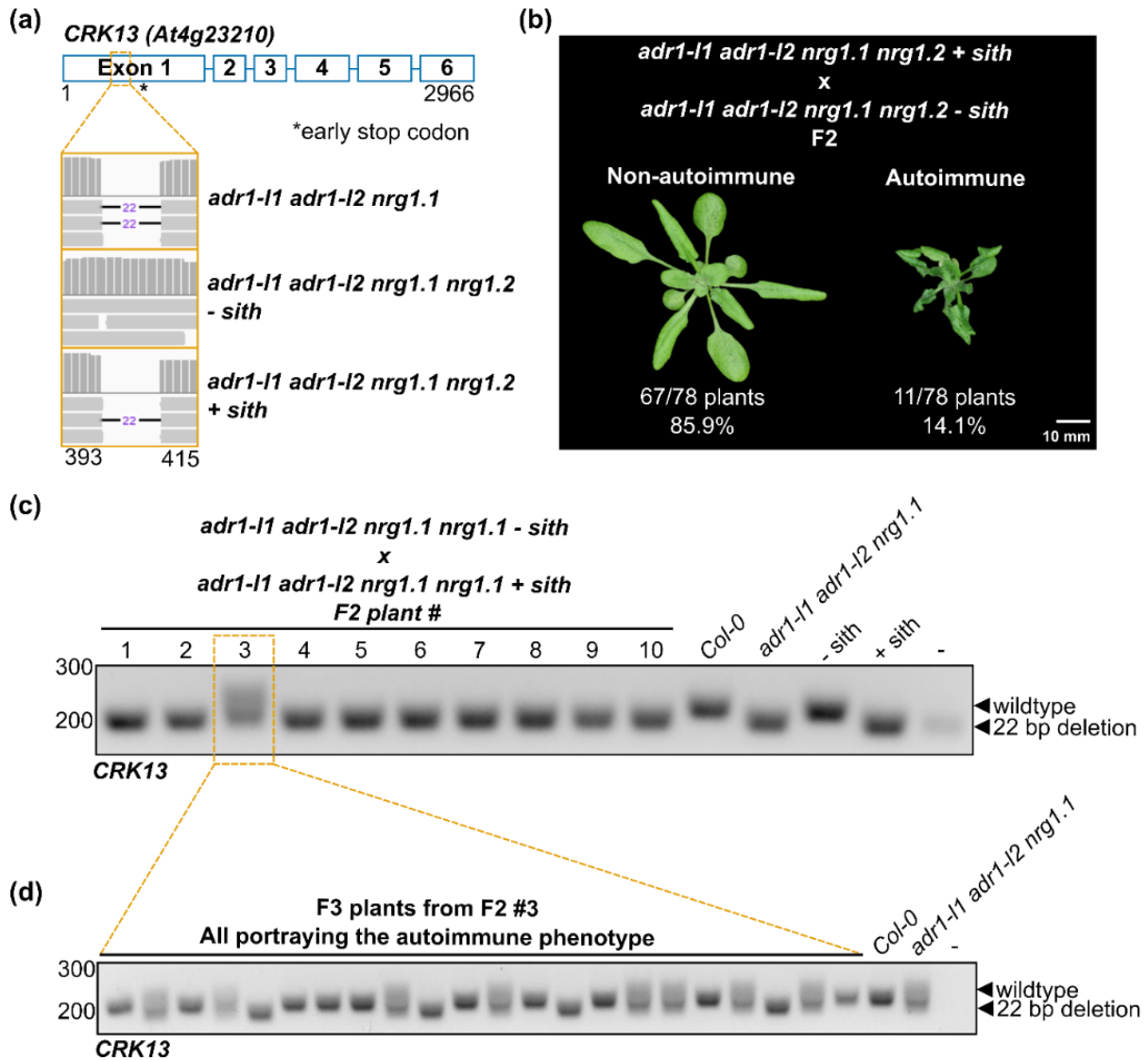


Figure 1.4: The 22 bp deletion in *CRK13* (At4g23210) is not causal for the autoimmunity observed in *adr1-11 adr1-12 nrg1.1*. (a) Identification and location of the 22 bp deletion in the At4g23210 gene locus, encoding for *CRK13*. The 22 bp deletion leads to an early stop codon (*) in exon 1. Partial screenshots from Integrated Genome Viewer depict alignment of reads to this region inside the locus. Full screenshots are available in Figure S1.2 (b) F2 phenotypes from the cross between *adr1-11 adr1-12 nrg1.1 nrg1.2* '-sith' x *adr1-11 adr1-12 nrg1.1 nrg1.2* '+ sith' (Figure 1.3a). Two plants with representative phenotypes (non-autoimmune or autoimmune morphology) are shown. Numbers and percentages indicate the occurrences of the observed phenotypes. Pictures were taken of 4-week-old *Arabidopsis* plants grown under short-day conditions. (c) Out of 10 tested autoimmune F2 plants, 9 were homozygous for the deletion in *CRK13*, but #3 was heterozygous. Primers for genotyping the deletion in *CRK13* are listed in Table S3. *Col-0*, *adr1-11 adr1-12 nrg1.1*, and the parental lines were included as controls. (d) Genotyping of the F3 plants from F2 plant #3 identified in (c) showed segregation of the 22 bp deletion in *CRK13*. All these F3 plants portrayed autoimmune phenotypes. *Col-0* and *adr1-11 adr1-12 nrg1.1* were included as genotyping controls.

Discussion

The finding of autoimmunity in the *adr1-l1-1 adr1-l2-4 nrg1.1-2* triple mutant made for a compelling hypothesis of intra- and inter-familial regulation of *Arabidopsis* RNLs (Figure 1.1). We identified ADR1 as a crucial component of this autoimmunity, which was further supported by the requirement for EDS1, PAD4, and SA biosynthesis (Figure 1.2). However, our genetic evidence and Whole Genome Sequencing suggested that the autoimmunity is not due to absence of *ADR1-L1*, *ADR1-L2*, and *NRG1.1* (Figure 1.3). Instead, we hypothesize a background mutation (*sith*) in the *adr1-l1 adr1-l2 nrg1.1* triple mutant causes the ADR1-dependent autoimmunity. A 22 bp deletion in *CRK13* was seen as the most promising candidate, but we could not show that the autoimmunity was not caused by this deletion (Figure 1.4).

Functional interactions between RNLs warrants further investigations

The concept of regulation among RNLs is not novel. *NRG1.3* was previously reported to regulate its subfamily members *NRG1.1* and *NRG1.2* by competing for EDS1-SAG101 interaction (S. Huang et al., 2025; Z. Wu et al., 2022; Xiao et al., 2025). The truncated ADR1 family member, ADR1-L3, does not inhibit the function of ADR1, ADR1-L1 or ADR1-L2 in *Col-0* and is believed to be a pseudogene (Z. Wu et al., 2022). However, in the ecotype *Bur-0* ADR1-L3 functions together with its genomic neighbour *At5g47260* in resistance to clubroot disease caused by *Plasmodiophora brassicae*, although the mechanism is unclear (Gravot et al., 2024). We initially provided a novel hypothesis ADR1 is redundantly regulated by members from both the ADR1 and NRG1 family (Figure 1.1e). Although we disproved this hypothesis on RNL regulation (Figure 1.3), much remains unknown about the functional interactions between different RNLs. *Arabidopsis* ADR1 family proteins were reported to hetero-associate in *Nicotiana benthamiana*, but the functional relevance of these protein-protein interactions is unknown (Saile et al., 2021). The runaway cell death observed in the *Arabidopsis* ‘lesion simulating disease 1’ (*lsd1*) mutant requires a combination of ADR1-L1 and ADR1-L2, suggesting that certain responses may indeed rely on different ADR1 family members for non-redundant functions (Roberts et al., 2013). Whether immunity mediated by the ADR1 family requires a combination of ADR1 family members is only partially investigated and much remains unclear about the redundancies between these genes (Bonardi et al., 2011).

Genetic studies should rely on multiple alleles and complementation

Our observation of the autoimmune phenotype of *adr1-l1-1 adr1-l2-4 nrg1.1-2* was based solely on these T-DNA loss-of-function alleles of the RNL genes (Table S2). In hindsight, more emphasis should have been placed on acquiring secondary loss-of-function alleles and complementation of the autoimmune phenotype to validate our findings. We generated new loss-of-function alleles for these RNLs with CRISPR-Cas9 (Figure S1.1b, c, d), but the *adr1-*

l1-c1 adr1-l2-c nrg1.1-c triple mutant was not yet obtained. However, full loss of *ADR1-L1* was shown to cause SNC1-dependent autoimmunity (M.-Y. Wang et al., 2023). We could show that the autoimmunity of *adr1-l1 adr1-l2 nrg1.1* is independent of SNC1 (Figure 1.2). So, these CRISPR-Cas9 alleles would have falsely supported our initial hypothesis unless we followed up on the role of SNC1. These findings emphasize the importance of using multiple alleles in combination with transgenic complementation to support a hypothesis based on combinatorial mutants.

The various T-DNA lines for *ADR1* family genes used in this chapter (Table S1.1) have been widely used in previous studies to investigate RNL function (Bonardi et al., 2011; Dong et al., 2016; Pruitt, Locci, et al., 2021; Saile et al., 2020). Now, our findings raise some concern about these T-DNA alleles as a potential source for introducing background mutations, and in our case: autoimmunity (Figure 1.1). However, we have not yet been able to trace back the origin of *sith* to any particular T-DNA line. The *adr1-l1-1* and *adr1-l2-4* alleles were previously investigated as single mutants and did not depict autoimmunity (Roberts et al., 2013; M.-Y. Wang et al., 2023). All *adr1* family T-DNA lines were additionally investigated in the context of *snc1-1* autoimmunity (Dong et al., 2016). The *adr1-l1-1* was shown to produce truncated version of *adr1-l1* mRNA and is hypothesized to produce N-terminally truncated ADR1-L1 which attenuates SNC1 (M.-Y. Wang et al., 2023). To our knowledge, the *nrg1.1-2*, and *nrg1.2-2* alleles were never used in other studies. Instead, research on the *NRG1* genes has relied on the CRISPR-Cas9 *nrg1 double* allele (Castel et al., 2019; Saile et al., 2020). Although caution is required, published findings regarding the function of RNLs in immunity gained from these RNL T-DNA lines are unlikely to be compromised. Many studies on RNL function included loss-of-function alleles for *EDS1*, *PAD4*, or *SAG101* as controls, which portrayed a similar susceptibility as the loss of the *Arabidopsis* RNL families (Castel et al., 2019; Saile et al., 2020; X. Sun et al., 2021; H. Wang, Song, et al., 2024). Nevertheless, full gene locus deletions of RNL family genes were generated during this work and are now available to generate additional versions of the *adr1 triple*, *nrg1 double*, and *Helperless* to validate previous findings (Figure S1.1b-d, g).

The identity of *sith* remains unknown after ruling out *CRK13*

The 22 bp deletion in *CRK13* was the most promising *sith* candidate out of the 49 (Table S1.1). *CRK13* was previously published to promote immunity against *Pseudomonas syringae* (Acharya et al., 2007). Loss of cell surface co-receptors from the SERK family can lead to NLR-dependent (auto)immunity as a result of NLRs guarding PTI components (Schulze et al., 2022; Y. Wu et al., 2020). We hypothesized that the 22 bp deletion in *CRK13* leads to a full loss-of-function by introducing an early stop codon and that the absence of *CRK13* triggers a NLR that requires *ADR1*. However, we disproved that the deletion in *CRK13* is causal for the

autoimmune phenotype and the identity of *sith* remains unknown (Figure 1.4d). Nevertheless, the 22 bp in *CRK13* we identified may be a potent loss-of-function allele that could be used in parallel to the published T-DNA line *crk13-1* (Acharya et al., 2007). This is especially notable since generating a CRISPR-Cas9 allele for *CRK13* alone may be challenging due to its high sequence homology to the closely related *CRK11* and *CRK22* (Martin-Ramirez et al., 2025). Moreover, a link between other CRKs like *CRK13* and RNLs may still warrant further investigation despite our findings. Recently, the connection between two CRKs (*CRK7* and *CRK43*) and the *ADR1-EDS1-PAD4* node was shown in the perception of MAMPs from Gram-positive bacteria (Pierdzig et al., 2025). More and more evidence is accumulating that CRKs play important roles in various biotic and abiotic stress responses, but much remains unknown about the molecular mechanisms underlying CRK function (Acharya et al., 2007; Bourdais et al., 2015; Hunter et al., 2019).

After ruling out *CRK13* as a candidate, we are left with 48 remaining *sith* candidates. Fifteen of these candidates are located within annotated gene loci, but only four introduce non-synonymous mutations (Table S1.1). None of these candidates immediately stand out based on gene annotations. However, intergenic mutations can also affect immune phenotypes, as was recently reported for a mutation located downstream of a TNL gene, *MMF1* (Van Rensburg et al., 2025). So, we cannot confidently rule out any of the remaining candidates but may be able to narrow it down further. Our Whole Genome Sequencing was performed on individual plants of each genotype, which may capture variants as homozygous in the individual that are segregating in the population. We observed this problem for the 22 bp deletion in *CRK13*, which we later found to be segregating in the *adr1-l1 adr1-l2 nrg1.1* population (Figure 1.4d). As a result, other *sith* candidates could also be false positively identified. A second round of Whole Genome Sequencing on DNA isolated from multiple seedlings per genotype should reduce false positive discovery rates. Alternatively, crossings for positional cloning could be performed with the pre-existing knowledge that *sith* is likely linked to *ADR1-L1* on chromosome 4 (Figure 1.4c) (Lukowitz et al., 2000). Therefore, the non-synonymous mutation (Cysteine → Arginine) identified in ‘glycine-rich domain protein 2’ (*GRDP2*, *At4g37900*) is the next best candidate, given its location on chromosome 4 (Table S1.1). The function of *GRDP2* was not previously linked to immunity, unlike *CRK13*. Instead, *GRDP2* may be a modulator of plant growth and stress responses (Castro-Bustos et al., 2022; Ortega-Amaro et al., 2015). The non-synonymous mutation in *GRDP2* forms a promising new candidate to focus on but now requires further investigation to determine whether this mutation directly causes autoimmunity. Uncovering the identity of *sith* may still yield valuable information in how *sith* causes autoimmunity and shed new light on the plant immune system.

Autoimmunity caused by *sith* is likely dependent on ADR1

The current hypothesis is that *sith* induces ADR1-dependent autoimmunity through unknown means (Figure 1.1). However, at this time we cannot say with certainty that the *sith* mutation was present in the *adr1-1 adr1-l1 adr1-l2 nrg1.1* quadruple mutant, which showed wildtype-like morphology (Figure 1.1). The same holds true for other crosses that yielded wildtype-like quadruple mutants, like the crosses with *eds1-12*, *pad4-2*, and *sid2-1* (Figure 1.2). To circumvent this problem, we set out to introduce the *adr1-c* allele (Figure S1.1b) into the *adr1-l1 adr1-l2 nrg1.1* background. However, currently we have only identified the *adr1-c* allele in the *Col-0* background. Unfortunately, we cannot cross *adr1-c* with *adr1-l1 adr1-l2 nrg1.1* to obtain this quadruple mutant, as we would risk losing the *sith* mutation in the process. Nevertheless, we hypothesize that ADR1 is indeed required for the autoimmunity in *adr1-l1 adr1-l2 nrg1.1*. The observed segregation patterns in the F2 from the cross between *adr1-l1 adr1-l2 nrg1.1* x *Helperless* supported ADR1 as required for this autoimmunity (Figure 1.3). Five plants portrayed autoimmunity out of the 80 total F2 plants, as expected for Mendelian segregation (1/16) of two independently segregating loci. These five plants were all homozygous for the wildtype ADR1 allele. Considering independent Mendelian segregation, this observation would have approximately 0.06% chance of occurring². Therefore, we still reason that ADR1 is likely required for the autoimmunity in the *adr1-l1-1 adr1-l2-4 nrg1.1-2* triple mutant.

² based on Hypergeometric Distribution Probabilities with the following parameters:

Population size =	80	(total number of F2 plants)
Number of successes =	20	(predicted homozygous wildtype ADR1 in the population)
Sample size =	5	(number of plants portraying autoimmunity)
Successes in sample =	5	(homozygous wildtype ADR1 alleles in autoimmune plants)

Conclusion

Taken together, we could show that the source of autoimmunity in *adr1-l1-1 adr1-l2-4 nrg1.1-2* is not the absence of *ADR1-L1*, *ADR1-L2*, and *NRG1.1*, but instead is caused by a background mutation that we called *sith*. The main takeaway from this work is to rely on multiple alleles of the genes-of-interest and to generate transgenic complementation lines before continuing too far ahead. The experimental framework described here also provides a pivotal example to critically assess, consider, and reflect on unexpected observations from experiments. Unexpected observations might not support a current hypothesis and provide a platform to adjust the hypothesis to best fit all experimental observations. Unfortunately, we were unable to determine the identity of *sith* at this time. Uncovering the causal mutation forms a crucial next step for this project and we proposed a next best candidate to assess. Notably, we reason that the autoimmunity caused by *sith* is dependent on *ADR1* and continued investigation may further improve our understanding of *ADR1*-dependent (auto)immunity.

Contributions

This chapter includes work from the internship students Anna Gurtmann, Johann Moore, Manon Corcia, and Isabell Baier, all of whom I co-supervised during my PhD thesis. The project on *adr1-l1-1 adr1-l2-4 nrg1.1-2* autoimmunity was initially worked on by Dr. Lance Jubic in the lab of Prof. Jeffery L. Dangl (University of North Carolina at Chapel Hill). Whole genome sequencing and subsequent variant calling was done in collaboration with Dr. Miriam Lucke and Dr. Rebecca Schwab in the lab of Prof. Detlef Weigel (Max Planck Institute for Biology Tübingen).

Supplementary material

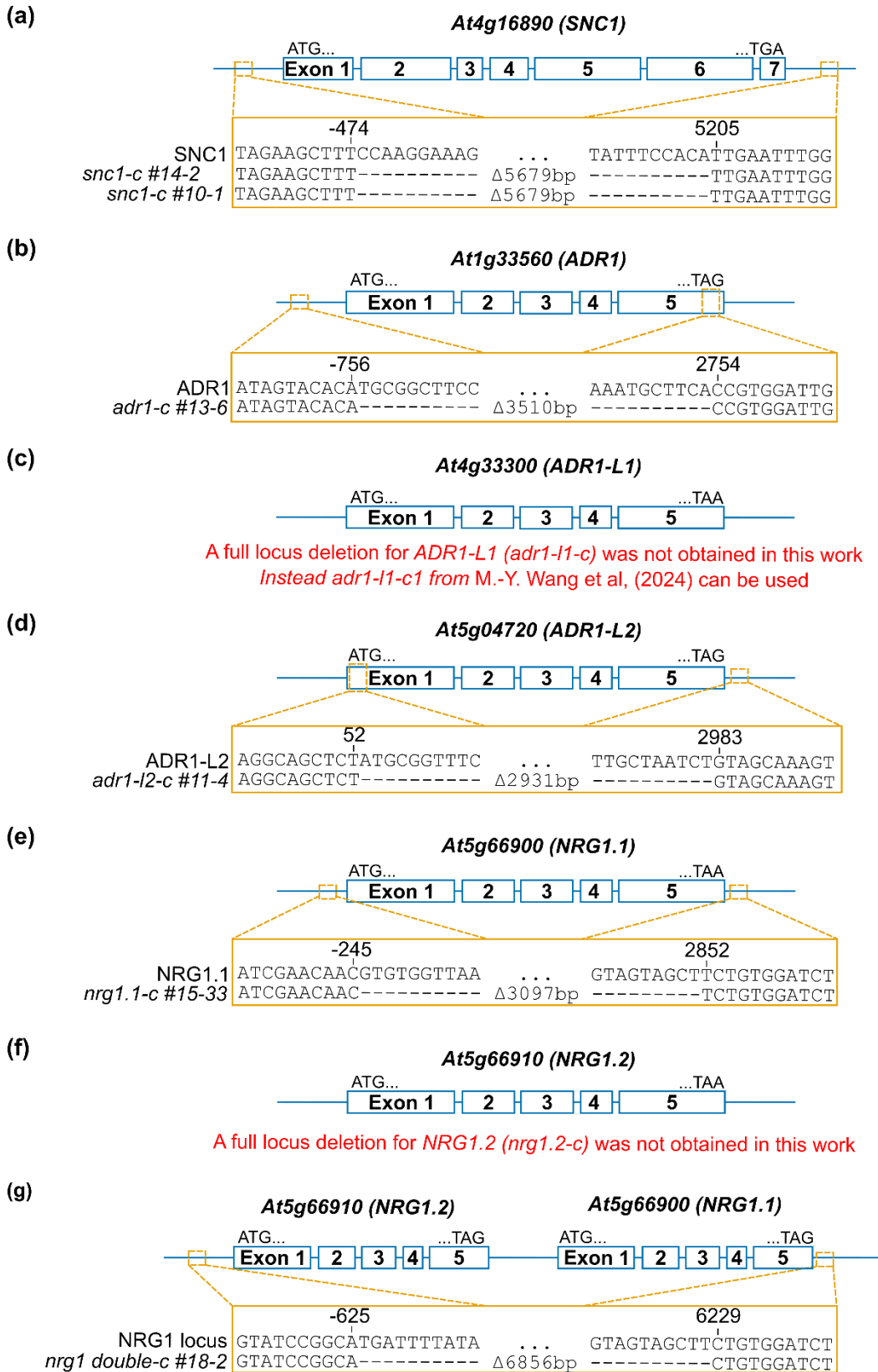


Figure S1.1: New full-locus deletion alleles generated with CRISPR-Cas9 for (a) *adr1*, (b) *adr1-l1*, (c) *adr1-l2*, (d) *nrg1.1*, (e) *nrg1.2*, (f) *nrg1 double*, (g) *snc1*. Oligonucleotides used for cloning sgRNA cassettes are available in Supplementary Table S3.

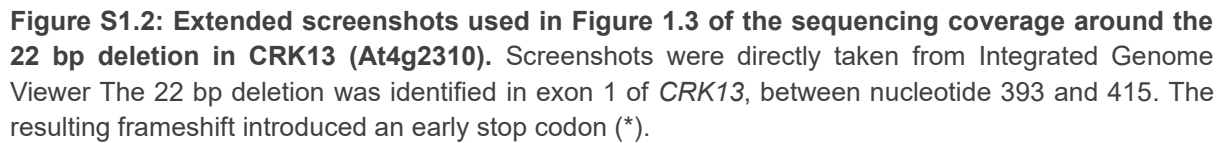


Table S1.1: Overview of all *sith* candidate mutations. For each candidate, the chromosome number and position are listed. The reference allele (Ref.) is the sequence found in that position in the TAIR10 genome assembly. The alternative allele (Alt.) is the sequence variant identified in that position in our whole genome sequencing data. The location specifies whether the variant is found inside a gene locus (5' UTR, exon, intron, 3' UTR) or in between genes (nothing listed). For candidates inside gene loci, the gene number and description according to TAIR is provided. For mutations inside exons, non-synonymous changes in the gene product are indicated.

Chr.	Position	Ref.	Alt.	Location	Gene	Gene description	Non-synonymous
1	1339379	AAT	A				
1	4244761	G	GT	Intron	<i>At1g12450</i>	SNARE associated Golgi protein family	
1	9388597	CA	C	Intron	<i>At1G27040</i>	Major facilitator superfamily protein	
1	9636104	T	TTA				
1	10727813	CT	C	5' UTR	<i>At1g30400</i>	Multidrug resistance-associated protein 1	
1	14151676	C	T				
1	18491966	C	CATGT				
1	21449318	GT	G				
1	27440776	CA	C				
2	2707628	C	T				
2	11088429	AAT	A				
2	11413835	GTA	G	Intron	<i>At2g26780</i>	ARM repeat superfamily protein	
2	11720959	C	T	3' UTR	<i>At2g27389</i>	Hypothetical protein	
2	13978309	C	T	Exon	<i>At2g32950</i>	Transducing/WD40 repeat-like superfamily protein	Leu (CTC) → Phe (TTC)
2	14515818	A	T	3' UTR	<i>At2g34390</i>	NOD26-like Intrinsic protein 2	
2	16339572	AATA TATA TATA TATA TATA TATA TATA	A				

Chr.	Position	Ref.	Alt.	Location	Gene	Gene description	Non-synonymous
		TATA TATA TATA TATA TAT					
2	18061497	C	CTA				
2	19365847	GA	G				
2	19618011	G	GAT	3' UTR	<i>At2g47940</i>	DEGP protease 2	
3	6471113	A	T		<i>At3g18777</i>	Pseudogene of Ring/U-box superfamily protein	
3	8492486	G	A				
3	10538659	T	C	5' UTR	<i>At1g30400</i>	Glutathione S-conjugate Transporting ATPase	
3	10878065	CAT	C				
3	16555898	C	CA				
3	20428272	T	TA				
3	22661754	T	G				
4	3984007	C	T				
4	11208219	C	CAT				
4	11214721	G	A				
4	12085166	AAT	A				
4	12151003	GGCT CAAT CTCC CAAA AAGC TGC	G	Exon	<i>At4g23210</i>	Cysteine-rich receptor-like kinase 13	Deletion with frameshift → early stop codon
4	12315894	A	G	Intron	<i>At4g23620</i>	Ribosomal protein L25/Gln-tRNA synthetase	
4	13318737	C	CA				
4	17823901	A	G	Exon	<i>At4g37900</i>	Glycine Rich Domain Protein 2	Cys (TCG) → Arg (CGC)
4	18521820	GT	G				

Chr.	Position	Ref.	Alt.	Location	Gene	Gene description	Non-synonymous
5	862573	A	T	5' UTR	<i>At5g03455</i>	Rhodanese/ Cell cycle control phosphatase family protein	
5	8179785	A	G				
5	8390131	CT	C				
5	11887248	A	C				
5	11887261	C	A				
5	11887263	G	C				
5	11887278	A	G				
5	11887284	G	A				
5	17011107	G	A	Exon	<i>At5g42540</i>	Exoribonucle ase 2	Arg (CGA) → Gln (CAA)
5	17274013	C	CA				
5	18214981	C	CAT				
5	18657309	TTA	T				
5	20959256	G	GTT				
5	24103016	C	CA	5' UTR	<i>At5g59820</i>	C2H2-type zinc finger family protein	

Chapter 2

Investigating Interfamilial Regulation of *Arabidopsis* Helper NLRs by NRG1.1

Introduction

Unwarranted activation of the immune system comes at a high cost, and so the plant immune system must be tightly regulated to balance between growth and defence (He et al., 2022). Mis-regulation of NLRs often leads to autoimmunity (introduced in Chapter 1). To avoid auto-activity, NLRs are regulated at both the transcriptional and translational level (Kourelis & Adachi, 2022).

At the transcriptional level, NLRs are generally regulated by keeping a low basal level of expression and only upregulating NLR genes during the initial transcriptional reprogramming of the plant immune response (Aerts et al., 2022; Lai & Eulgem, 2018; Tian et al., 2021). The accumulation of NLR transcripts is then further regulated by small interfering RNAs which were identified to broadly target NLR transcripts (F. Li et al., 2012; Seo et al., 2018).

Regulation on NLRs also occurs at the protein level through various mechanisms. Post-translational modification of NLR proteins can alter their function, localization, or longevity (Zhong et al., 2026). Post-translational modifications include processes such as ubiquitination, lipidation, or phosphorylation. Ubiquitination primarily regulates the degradation of NLRs, but may also positively influence NLR function (Dong et al., 2018; Z. Wang et al., 2026; Z. Wu et al., 2020). The degradation of NLRs forms an important regulatory pathway to maintain proper homeostasis. Disruption of NLR degradation can lead to (auto)immunity (C. Wang et al., 2024). The addition of lipids to NLRs is commonly associated with altered localization to affect their function (Hemsley, 2015; D. Qi et al., 2012). Phosphorylation events are a crucial signalling mechanism of the plant immune system, and are involved in many regulatory processes (Withers & Dong, 2017). The role of phosphorylation regulating NLRs will be further explored in Chapter 3.

Alternative splicing of immunity-related transcripts forms an additional layer of regulation on the immune system that is gaining more attention in recent years (Kufel et al., 2022). Alternative splicing allows multiple mRNA transcripts to be produced by a single gene through variations of the intron-exon inclusions in the final transcript (Tognacca et al., 2023). The translation of these alternatively spliced transcripts can then lead to the production of protein isoforms that can have similar or distinct functions from the constitutively spliced variant. This regulatory mechanism extends to some NLRs which have been reported to undergo alternative splicing. These NLR isoforms can exist in unchallenged plants to balance the accumulation of the constitutively spliced NLR (B. Sun et al., 2024). In other cases, the products of the alternatively spliced NLR transcripts are required for the full immune response (Xu et al., 2012; X.-C. Zhang & Gassmann, 2003).

The activation requirement of NLRs forms an additional layer of regulation. Under normal circumstances, NLRs are kept in an inactive state (Figure 2a). This is achieved through

autoinhibition (self-regulation) by the NB-ARC and LRR domains or by association with other proteins (Figure 2b) (Z. Hu et al., 2013; Van Ooijen et al., 2008). Perception of an effector then leads to conformational changes to relieve autoinhibition and activate the NLR. NLRs that guard other plant protein are kept inactivated until modification or degradation of their guardee (Van Der Hoorn & Kamoun, 2008). This suggests that guardees can regulate the activity of NLRs to ensure activation only occurs after effectors were perceived.

For RNLs specifically, the activation is thought to be mediated by association with EDS1-PAD4 or EDS1-SAG101 (Figure 3). Small molecules from TIR (TNL) enzymatic activity are bound by EDS1 dimers to induce conformational changes, which leads to the recruitment of RNLs and promotes their activation (S. Huang et al., 2022; Jia et al., 2022; X. Sun et al., 2021; H. Yu et al., 2024). Thus, the activation of RNLs is thereby gated behind TNL activity. In *Arabidopsis*, the NRG1 family is further regulated by the N-terminally truncated NRG1.3, which competes for EDS1-SAG101 association (S. Huang et al., 2025; Z. Wu et al., 2022; Xiao et al., 2025). Nevertheless, much remains unknown about the regulation of RNLs, especially the ADR1 family.

As central components for both PTI and ETI signalling, RNLs must be tightly regulated to avoid unwarranted execution of immunity and cell death (Pruitt, Gust, et al., 2021). Here, we further investigate regulation of ADR1 by other RNLs based on our initial findings presented in Chapter 1 (Figure 1.1). We initially found NRG1.1 to be a promising candidate regulator of ADR1 cell death. Continued investigation led to the identification of a putative splice junction in *NRG1.1*, leading to the hypothesis that alternative splicing is required to suppress ADR1. We additionally found that the suppressive effect extended beyond ADR1 to other RNLs. However, we ultimately uncovered that the inhibited activity of *Arabidopsis* RNLs in *N. benthamiana* did not correlate with the co-expression of *NRG1.1*.

Results

In *Chapter 1*, we formed the initial hypothesis that the activity of ADR1 is redundantly suppressed by ADR1-L1, ADR1-L2, and NRG1.1 (Figure 1.1e). We turned to transient overexpression in *Nicotiana benthamiana* to gain a clearer understanding of the mechanisms driving this regulation of ADR1 function. Although the genetic evidence from *Arabidopsis* no longer supports this initial hypothesis (Figure 1.3), this investigation into the mechanism still led us towards new discoveries that are presented in this chapter.

ADR1-induced cell death can be suppressed by NRG1.1

To further investigate the initial hypothesis proposed in Chapter 1, we used transient expression of *Arabidopsis* RNLs in *N. benthamiana* *adr1 nrg1* (T. Qi et al., 2018; H. Wang, Song, et al., 2024). Overexpression of wildtype ADR1 in *N. benthamiana* was previously shown to induce potent cell death (Collier et al., 2011; Saile et al., 2021). Indeed, we could observe cell death by ADR1, unlike the remaining *Arabidopsis* RNLs (Figure 2.1a). We then wondered whether co-expression of ADR1 with other RNLs would suppress the ADR1-induced cell death. We found that ADR1-L1, ADR1-L2, and NRG1.2 were unable to suppress ADR1 activity. However, co-expression of NRG1.1^{genomic} with ADR1 prevented cell death from occurring.

We previously showed that the autoimmune phenotype of *adr1-l1-1 adr1-l2-4 nrg1.1-2* was dependent on EDS1 and PAD4 (Figure 1.2). Previously, cell death from overexpressing ADR1 was shown to be independent of the native *N. benthamiana* EDS1 (Jacob et al., 2021). To confirm this finding under our conditions, we expressed *Arabidopsis* ADR1 in *N. benthamiana* *eds1 pad4 sag101a sag101b* quadruple mutant (Lapin et al., 2019). We still observed cell death after overexpression of *Arabidopsis* ADR1, suggesting that EDS1 and PAD4 from *N. benthamiana* are indeed not required (Figure S2.1). Moreover, within the same experiment we showed that the suppression of ADR1 by NRG1.1^{genomic} was also independent of *N. benthamiana* EDS1. We continued to leverage transient expression of RNLs in *N. benthamiana* for further mechanistic investigations, despite the contrasting findings of the EDS1-dependency of *Arabidopsis* ADR1 auto-activity between *Arabidopsis* and *N. benthamiana*.

So far, all our findings in *N. benthamiana* were based on NRG1.1^{genomic}, because cloning the coding sequence of NRG1.1 from *Arabidopsis* cDNA was unsuccessful for a long time. Since the other RNL constructs we used were all derived from coding sequences, we tested coding sequence NRG1.1 (NRG1.1^{cds}) after we successfully obtained the clone. Surprisingly, co-expression of NRG1.1^{cds} did not suppress ADR1-induced cell death (Figure 2.1a). This suggested a functional difference between NRG1.1^{genomic} and NRG1.1^{cds}. We observed that overexpression of NRG1.1^{genomic} led to two visible protein bands, whereas NRG1.1^{cds} only

produced a single protein band (Figure 2.1b). This suggested that the secondary band is produced at the transcript level, rather than the protein level. So, we continued our investigation by assessing whether alternative splicing of *NRG1.1^{genomic}* is involved in the suppression of ADR1.

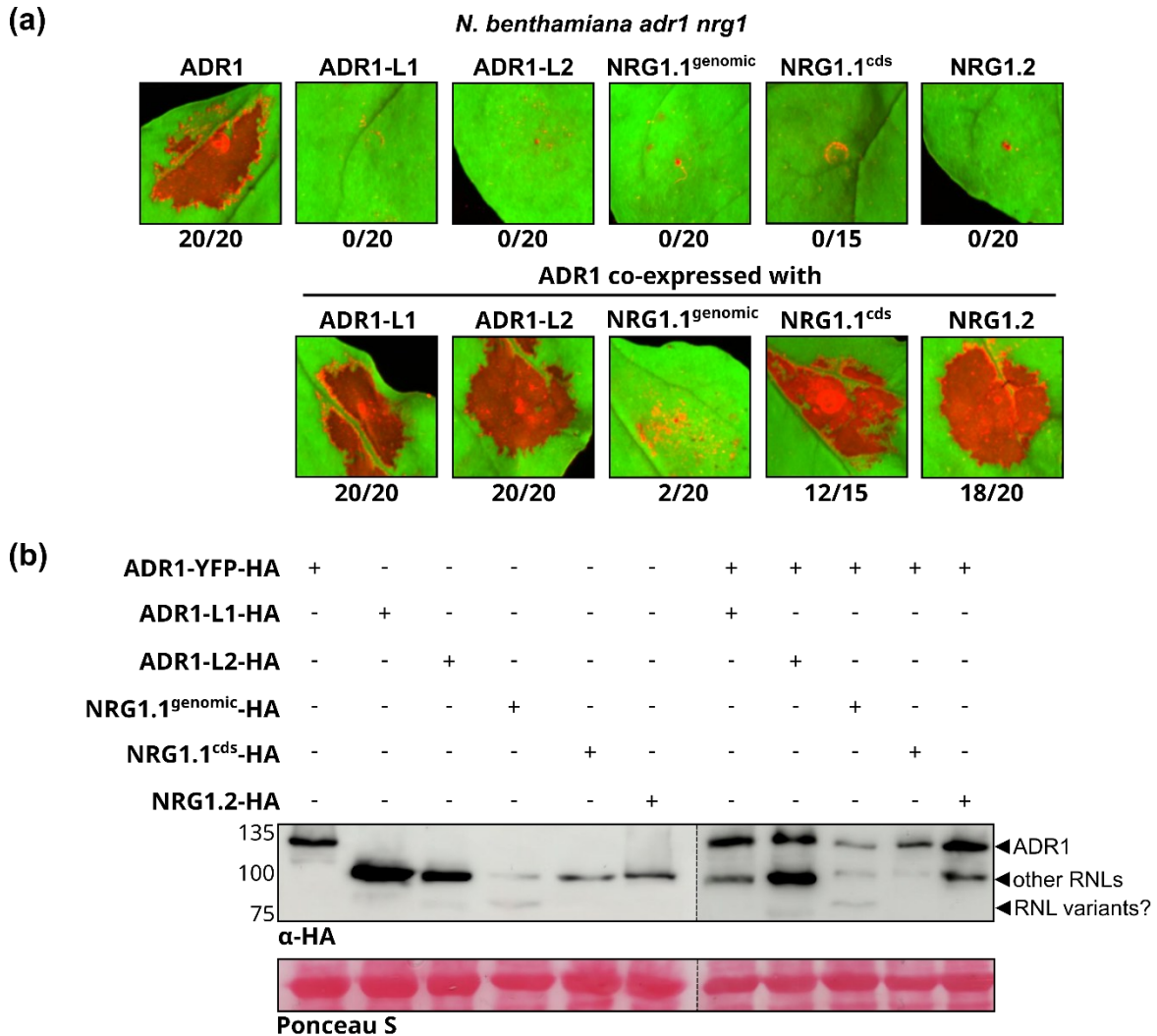


Figure 2.1: Cell death caused by overexpression of *Arabidopsis* ADR1 in *N. benthamiana* is only suppressed by co-expression of *NRG1.1^{genomic}*. (a) Co-expression of *NRG1.1^{genomic}* but not ADR1-L1, ADR1-L2, *NRG1.1^{cds}*, or *NRG1.2* could suppress ADR1 induced cell death in *N. benthamiana*. *Agrobacterium* infiltrations were done in *N. benthamiana adr1 nrg1*. ADR1 was expressed under an estradiol-inducible 35S promoter. All other RNLs were expressed under a 35S promoter. Images were taken 24 hours after inducing expression of ADR1. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) Protein accumulation of RNLs in (a) was confirmed by western blot. For ADR1-L2 and *NRG1.1^{genomic}*, a secondary protein band was observed. *NRG1.1^{cds}* lacked this secondary band. Proteins were detected using anti-HA. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The dotted line indicates where digital stitching of membranes occurred in the presented western blots.

Alternative splicing of *NRG1.1* is required for suppression of *ADR1*

To further investigate the role of splicing in the suppression of *ADR1*, we set out to identify splicing events in *NRG1.1*. We used publicly available databases (Plant Intron-Splicing Efficiency database, PastDB) of RNA sequencing datasets to assess the feasibility of spliced isoforms existing, but failed to find conclusive evidence (Martín et al., 2021; H. Zhang et al., 2023). So, we generated primers to analyse whether we could detect putative splice variants of *NRG1.1*. Using primers located in exon 3 and exon 5 of *NRG1.1* (Table S3), we were able to amplify a region that yielded amplicons of two distinct sizes from *Col-0* cDNA (Figure 2.2a). An amplicon of similar size was detected in *N. benthamiana* transiently expressing *NRG1.1^{genomic}*. We sequenced both the observed amplicons from *Col-0* cDNA and found that the larger amplicon corresponded to the constitutively spliced sequence of *NRG1.1* (Figure 2.2b). In the smaller amplicon, we identified a putative splice site located in exon 3 of *NRG1.1*. We found that the sequence after this splice site continued into exon 5, rather than exon 4. Despite this splicing amplicon lacking parts of exon 3 and all of exon 4, the frame was perfectly maintained between exon 3 and exon 5. So, we hypothesized that this putative splicing event would generate a *NRG1.1* transcript that encodes for a version of *NRG1.1* with a deletion in the NB-ARC domain, lacking half of exon 3 and all of exon 4.

We continued our investigation by assessing whether this splicing even is relevant for the suppression of *ADR1*-induced cell death. We generated a version of our *NRG1.1^{genomic}* construct that had the splice junction silently mutated without affecting the amino acid sequence of constitutively spliced *NRG1.1* (*NRG1.1^{genomic} G391G**). Co-expression of *ADR1* with *NRG1.1^{genomic} G391G** led to cell death, indicating that the splice junction is indeed crucial to suppress *ADR1*-induced cell death (Figure 2.2c). Notably, the secondary protein band observed for *NRG1.1^{genomic}* was no longer visible on western blots (Figure 2.2d). This suggested to us that this secondary isoform of *NRG1.1* we consistently detected is indeed generated by alternative splicing.

Next, we synthesized the hypothesized spliced amplicon of *NRG1.1* (*NRG1.1^{synth}*) and cloned it into the pGWB614 expression vector (Table S4). When we co-expressed *ADR1* with *NRG1.1^{synth}*, we still observed cell death (Figure 2.2c, d). Co-expression of *NRG1.1^{cds}* together with *NRG1.1^{synth}* also did not suppress *ADR1* cell death. We noted that *NRG1.1^{synth}* appeared to consistently run as a slightly larger protein on western blots than the secondary protein band of *NRG1.1^{genomic}* (Figure 2.2d). Taken together, the *NRG1.1^{synth}* sequence likely did not match the sequence of *NRG1.1^{spliced}*, the latter of which we hypothesized is required to suppress *ADR1* induced cell death. At this point, the identity of the suppressive unit within *NRG1.1^{genomic}* remains unknown. We continue our investigation of *NRG1.1* as a negative regulator of immunity, utilizing *NRG1.1^{genomic}* as a genetic ‘toolbox’ to express the hypothesized suppressor of *ADR1* induced cell death.

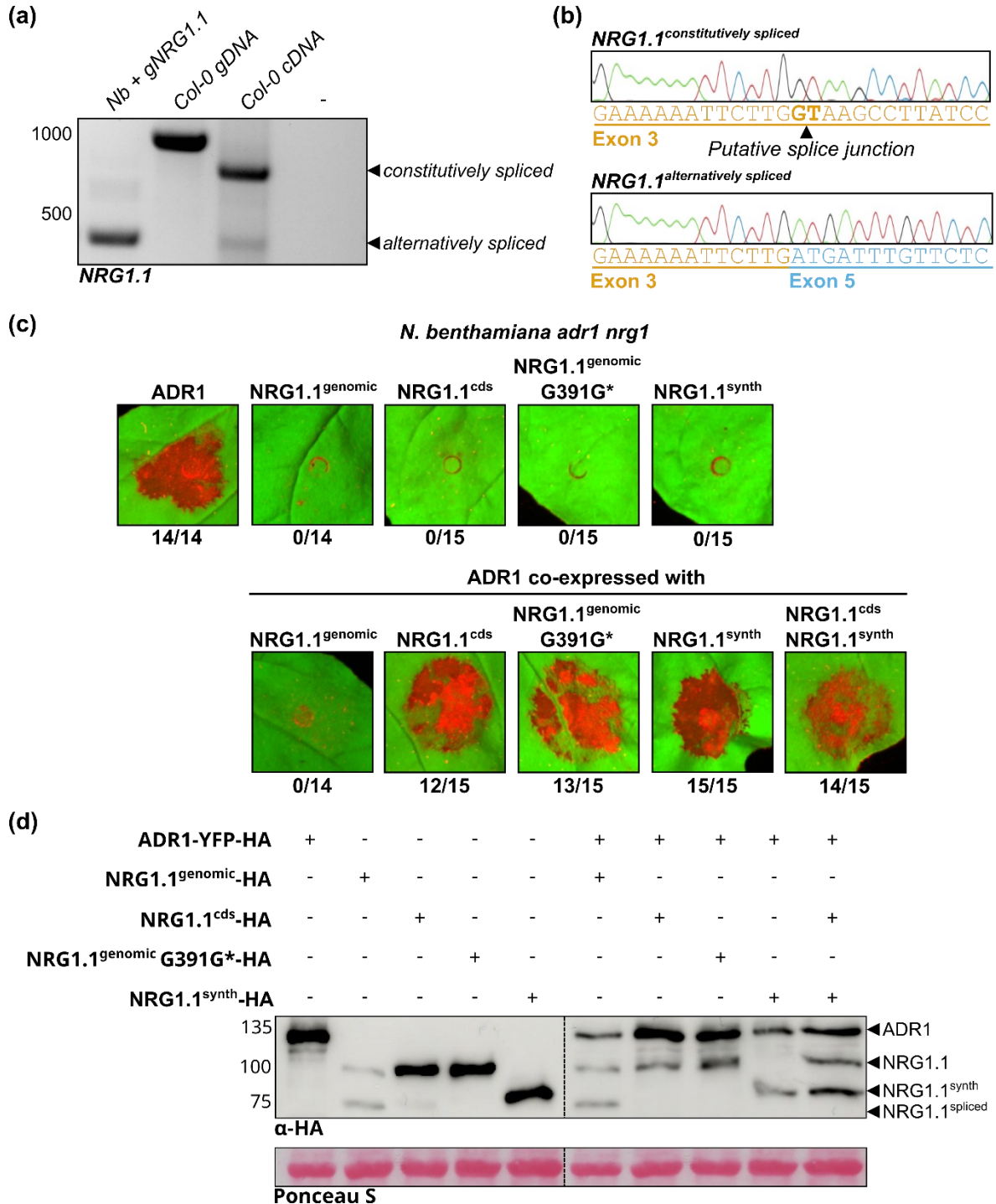


Figure 2.2: An alternatively spliced variant of *NRG1.1* is required to suppress ADR1 cell death in *N. benthamiana*. (a) Amplification of the region between exon 3 and 5 of *NRG1.1* produced two amplicons. Amplicons were generated by RT-PCR using primers specific to exon 3 and exon 5 from *NRG1.1*. Primers are listed in Table S3. (b) Sanger sequencing of the amplicons from *Col-0* in (a) revealed a putative splice junction in exon 3 of *NRG1.1*. Sequencing traces from Sanger sequencing are shown with their respective base-calling. The putative splice junction is indicated in bold in the full-length sequence. (c) The putative splice junction in *NRG1.1^{genomic}* is required to suppress ADR1 cell death in *N. benthamiana*. *Agrobacterium* infiltrations were done in *N. benthamiana* *adr1 nrg1*. ADR1 was expressed under an estradiol-inducible 35S promoter. All *NRG1.1* variants were expressed under a 35S promoter. (continued on the next page)

(continued) Images were taken 24 hours after inducing expression of *ADR1*. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (d) The secondary protein band of *NRG1.1^{genomic}* was lost by mutating the putative splice junction (G391G*). Protein accumulation of RNLs in (c) was confirmed by western blot. Proteins were detected using anti-HA. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The dotted line indicates where digital stitching of membranes occurred in the presented western blots.

The expression pattern of *Arabidopsis NRG1.1* contrasts other RNLs

In current literature, *NRG1.1* is primarily associated with mediating hypersensitive cell death after effector recognition by various TNLs (Castel et al., 2019; Saile et al., 2020). We questioned how a positive regulator of immunity (and cell death) could have dual functionality as a negative regulator. Alternative splicing forms an elegant hypothesis on how *NRG1.1* could have two opposing functions. The *NRG1.1* variant generated by alternative splicing could fulfil a distinct role (e.g. regulatory) from the constitutively spliced variant.

To further investigate *NRG1.1* as a potential negative regulator of *ADR1* (auto-)activity, we analysed the expression patterns of all five full-length *Arabidopsis* RNLs using a previously published RNA sequencing dataset (Saile et al., 2020). This dataset was generated from infections with the *Pseudomonas fluorescence Pf0-1* expressing various effectors to trigger specific types of immunity across three timepoints (30 minutes, 4 hours, and 8 hours post infection). Four types of immune responses are investigated: (1) PTI only (empty vector), (2) PTI + RNL-independent ETI (*AvrRpm1*), (3) PTI + partially RNL-dependent ETI (*AvrRpt2*), and (4) PTI + fully RNL-dependent ETI (*AvrRps4*). Focussing on the expression pattern of *NRG1.1*, we observed that *NRG1.1* is upregulated during early immune responses (after 30 minutes) and then steadily decreased to a basal level after 4-8 hours post-infection (Figure 2.3). This pattern of expression level is unique to *NRG1.1* and inverted compared to the other RNLs which are gradually upregulated over the course of the infection. To confirm this observation, we analysed the expression pattern of *NRG1.1* in another RNA sequencing dataset using the epitope of bacterial flagellin (flg22) as MAMP to elicit PTI responses (Winkelmüller et al., 2021). We indeed again observed that *NRG1.1* is highly upregulated early (1 hour after flg22 treatment) and gradually downregulated over the next 24 hours (Figure S2.2a).

We compared this expression pattern of the *NRG1.1* to the N-terminally truncated *NRG1.3*, which has a reported negative regulatory function (Z. Wu et al., 2022). In infections with *Pf0-1 empty vector* (PTI only), *NRG1.3* is slightly upregulated after 30 minutes similar to *NRG1.1* (Figure S2.2b). In ETI-eliciting infections, *NRG1.3* is steadily upregulated over the course of the immune response, contrasting the early peak of *NRG1.1* expression. Nevertheless, we reason that the observed expression pattern of *NRG1.1* may indeed support the concept of a negative regulatory function for *NRG1.1* in plant immunity.

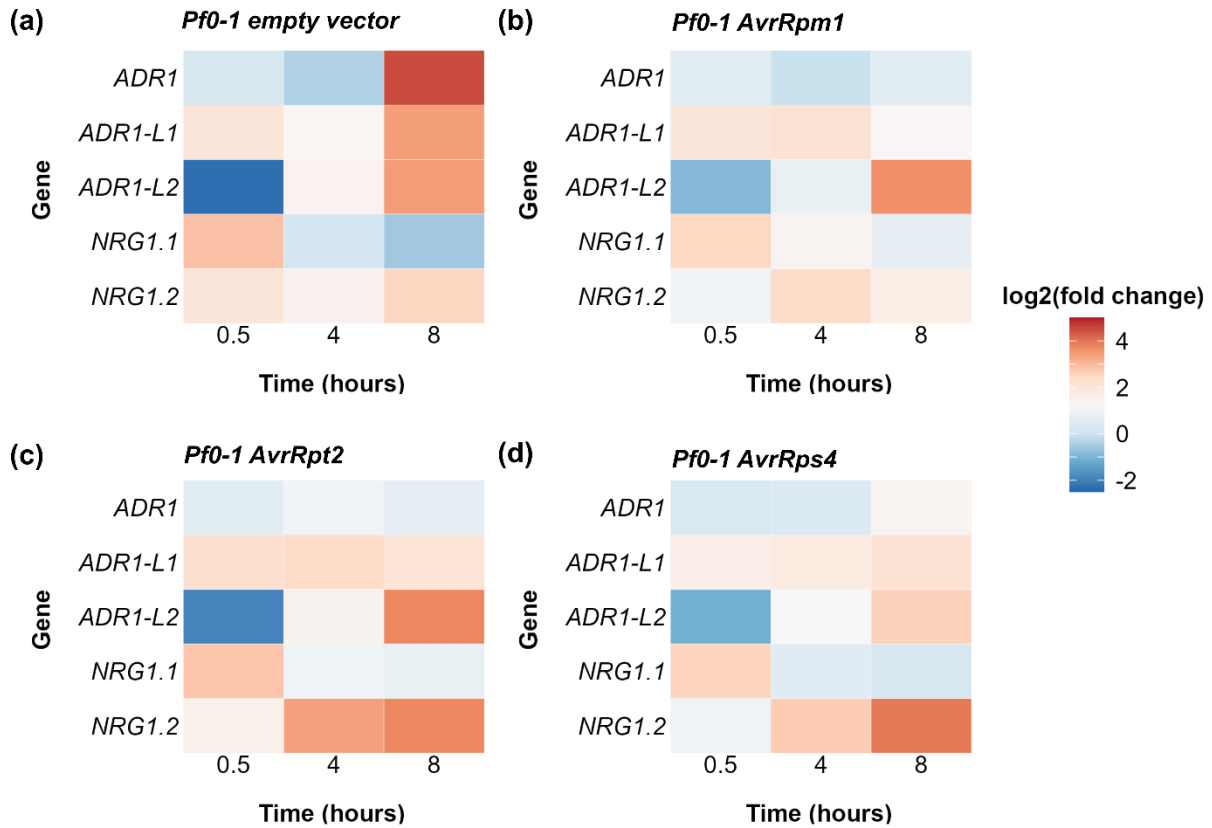


Figure 2.3: The expression pattern of *NRG1.1* is unique compared to other RNLs. RNL expression levels were analysed at three timepoints (30 min, 4 hours, and 8 hours) after infection with (a) *Pf0-1* empty vector, (b) *Pf0-1* *AvrRpm1*, (c) *Pf0-1* *AvrRpt2*, and (d) *Pf0-1* *AvrRps4*. The RNA sequencing dataset was previously generated by Saile et al. (2020). Expression levels were normalized against untreated *Col-0* plants and depicted as $\log_2(\text{fold change})$. The same legend applies to all heatmaps in this figure. *NRG1.3* was omitted here due to its comparatively high expression level and instead is included in Figure S2.2b.

We additionally noted that *ADR1* was strongly upregulated after 8 hours during PTI (Figure 2.3a), but not during infections with ETI-eliciting bacteria (Figure 2.3b-d). Despite the lack of *ADR1* upregulation by ETI-eliciting bacteria, the expression pattern of *NRG1.1* was consistent across all treatments (Figure 2.3). Based on this difference in transcriptional response between *ADR1*, *NRG1.1*, and the other RNLs, we wondered whether the suppressive effect of *NRG1.1* extends beyond *ADR1* to the remaining RNLs.

NRG1.1 can suppress cell death from other RNLs

To investigate the suppression of other RNLs by *NRG1.1*, we leveraged auto-activated variants of these RNLs by introducing a mutation in the MHD motif (Roberts et al., 2013). Overexpression of *ADR1* D461V and *ADR1-L1* D489V caused strong cell death in *N. benthamiana* (Figure 2.4a). Despite proper protein accumulation, *ADR1-L2* D484V only caused a weak and inconsistent cell death response in *N. benthamiana*, consistent with previous reports (Saile et al., 2021). Co-expression of *NRG1.1^{genomic}* suppressed the cell death induced by *ADR1* D461V and *ADR1-L1* D489V. We also did not observe cell death for the co-

expression of *ADR1-L2 D484V* with *NRG1.1* but cannot conclude that this is due to the presence of *NRG1.1*. Nevertheless, these findings suggested to us that the cell death induced by *Arabidopsis* ADR1-family proteins in *N. benthamiana* is suppressed by the co-expression of *NRG1.1^{genomic}*.

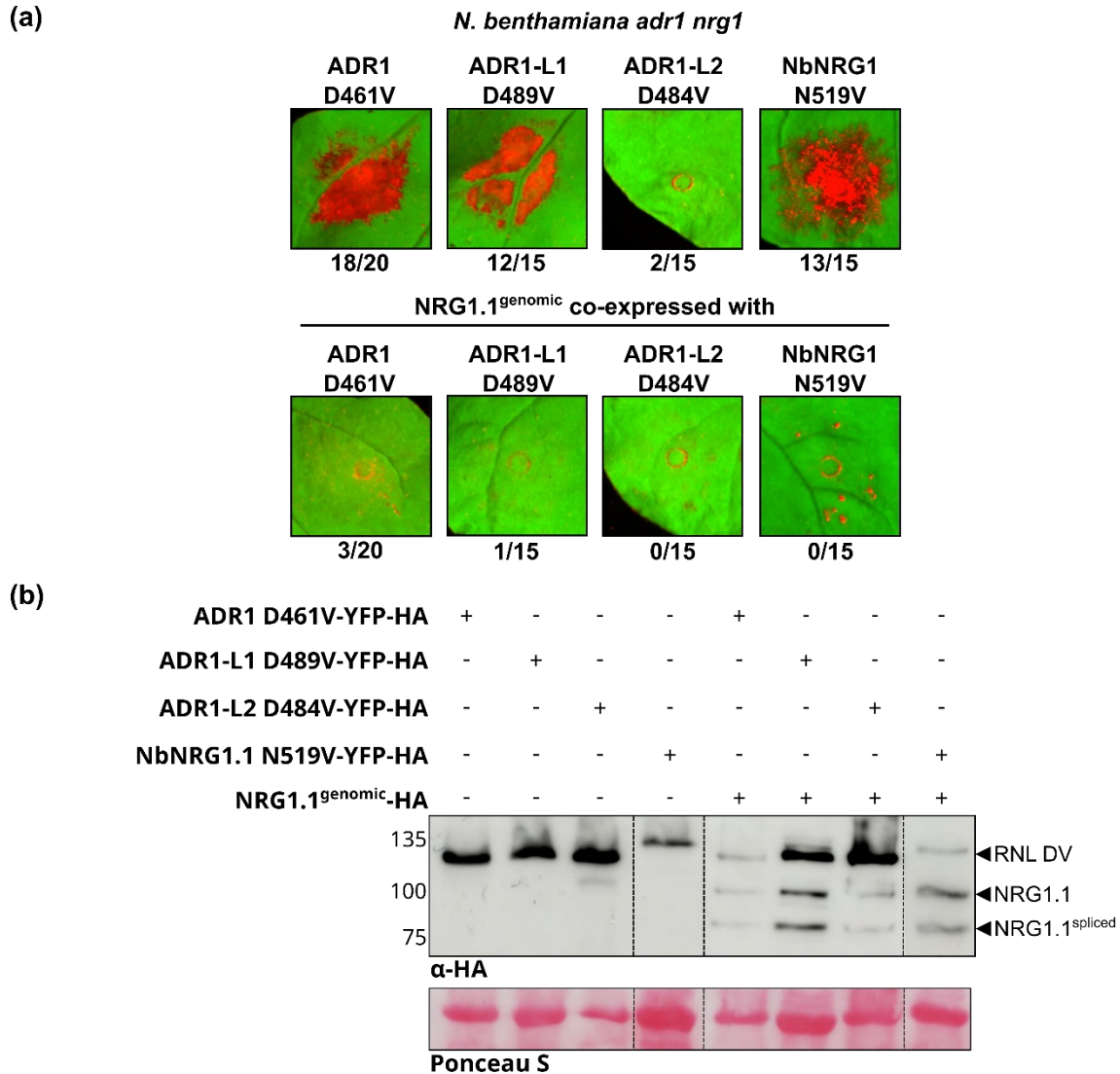


Figure 2.4: Co-expression of *NRG1.1^{genomic}* can suppress cell death from other tested RNLs.

(a) Auto-activated variants of ADR1, ADR1-L1 and NbNRG1 caused potent cell death in *N. benthamiana* that was suppressed by co-expression of *NRG1.1^{genomic}*. *Agrobacterium* infiltrations were done in *N. benthamiana adr1 nrg1*. Auto-activated RNLs was expressed under an estradiol-inducible 35S promoter. *NRG1.1^{genomic}* was expressed under a 35S promoter. Images were taken 24 hours after inducing expression of auto-active ADR1, ADR1-L1, or ADR1-L2 and after 72 hours after inducing expression of auto-active NbNRG1. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) Protein accumulation of RNLs in (a) was confirmed by western blot. Proteins were detected using anti-HA. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The dotted lines indicate where digital stitching of membranes occurred in the presented western blots.

For the auto-activated NRG1-family proteins, we observed strong cell death for NRG1.1 D485V, but not for NRG1.2 N490V despite proper protein accumulation (Figure S2.3). Upon co-expression with *NRG1.1^{genomic}*, we did not observe cell death for NRG1.1 D485V or NRG1.2 N490V. However, we could never detect NRG1.1 D485V or NRG1.2 N490V proteins when co-expressed with *NRG1.1^{genomic}*. Therefore, we cannot make direct conclusions on the effect of *NRG1.1^{genomic}* on cell death induced by NRG1.1 D485V or NRG1.2 N490V. To circumvent this limitation, we leveraged auto-activated *N. benthamiana* NRG1 (NbNRG1 N519V). Expression of *NbNRG1 N519V* caused cell death 72 hours post induction under our conditions (Figure 2.4a). Cell death from NbNRG1 N519V was suppressed by the presence of *NRG1.1^{genomic}*. Unlike *Arabidopsis* NRG1 proteins, we could detect protein accumulation of NbNRG1 N519V when co-expressed with *NRG1.1^{genomic}* (Figure 2.4b). Despite the more distant evolutionary relationship between *Arabidopsis* and *N. benthamiana*, *NRG1.1* and *NRG1.2* are more closely related to *NbNRG1* than to *Arabidopsis ADR1*-family genes (Collier et al., 2011). Based on these evolutionary relationships, we hypothesize that *NRG1.1^{genomic}* may also suppress cell death from *Arabidopsis* NRG1-family proteins even though our assay failed to conclusively prove this.

Cell death from CNLs is not suppress by NRG1.1

Next, we investigated whether the suppressive effect of *NRG1.1^{genomic}* extended beyond RNLs to *Arabidopsis* CNLs. RNLs are a subclade of CNLs and thus relatively closely related to RNLs (Jubic et al., 2019). CNLs and RNLs are thought to initiate cell death through conserved mechanisms (Figure 3) (J. Wang, Hu, et al., 2019; Z. Wang et al., 2023). To study the effect of *NRG1.1^{genomic}* expression on CNL cell death, we selected four CNLs from *Arabidopsis*: (1) ‘resistance to *Pseudomonas syringae* pv. *Maculicola* 1’ (RPM1) (Boyces et al., 1998; Gao et al., 2011), (2) ‘resistance to *Pseudomonas syringae* 2’ (RPS2) (Kunkel et al., 1993), (3) ‘potentially membrane localized NLR 5’ (PML5) and (4) PML11 (Sunil et al., 2024). We once more leveraged auto-activating mutations in the MHD motif to generate auto-active versions of these CNLs. All four auto-activated CNLs caused potent cell death when overexpressed in *N. benthamiana* (Figure 2.5a). Unlike the RNLs, induction of cell death was unaffected by co-expression of *NRG1.1^{genomic}*. Detecting the accumulation of these CNLs by western blot proved difficult (Figure 2.5b). We failed to detect RPM1 D505V and RPS2 N473V throughout our experiments, and PML5 could not be detected when co-expressed with *NRG1.1^{genomic}*. However, the occurrence of cell death indicated the presence of these CNLs and *NRG1.1^{genomic}* was always observed when co-expressed with the various CNLs. Thus, the cell death-suppressing effect of NRG1.1 is unique to RNLs and does not extend to CNLs.

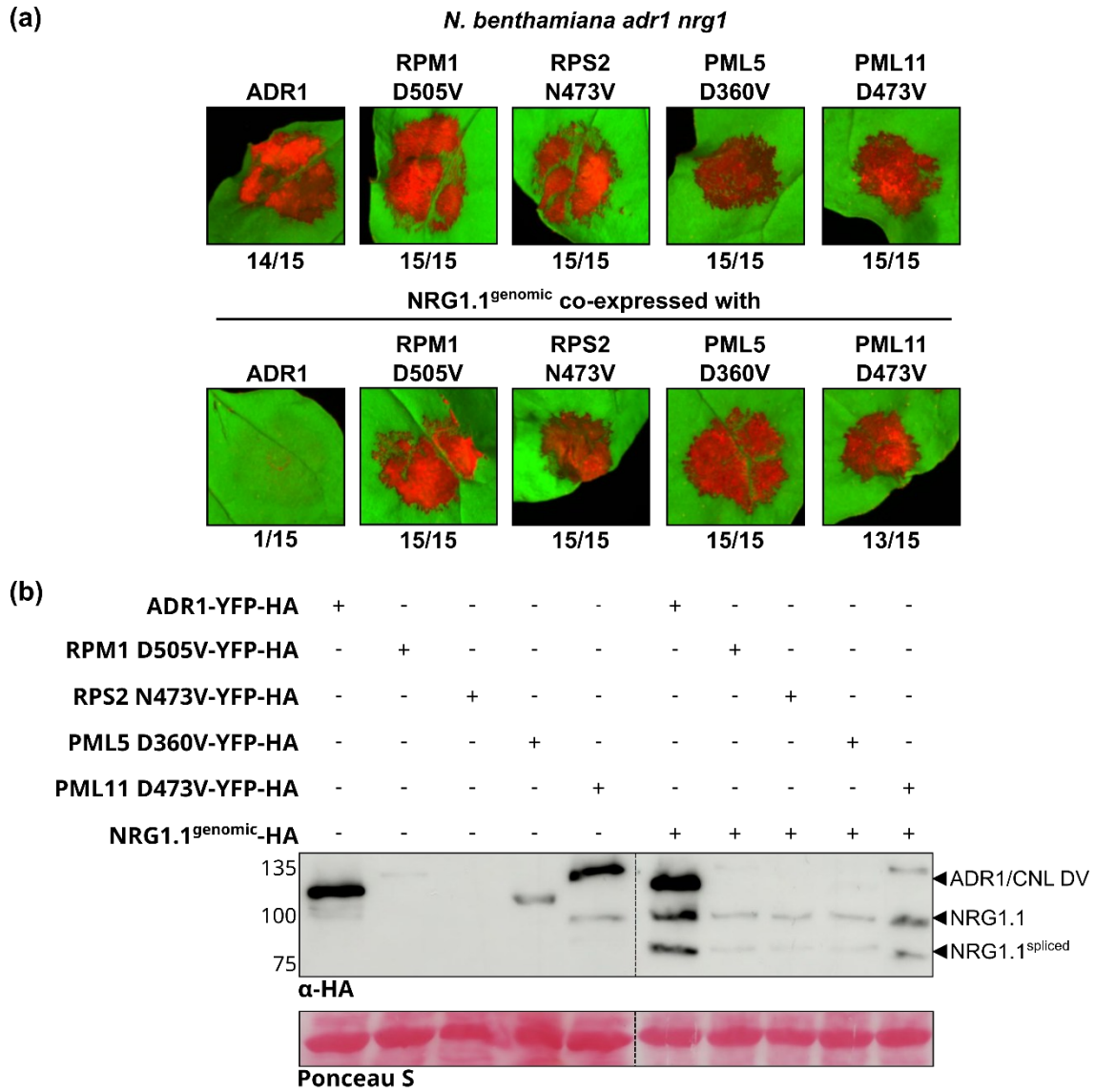


Figure 2.5: Cell death induced by CNLs is not suppressed by co-expression of *NRG1.1*^{genomic} in *N. benthamiana*. (a) Autoactivated versions of RPM1, RPS2, PML5, and PML11 cause potent cell death that is unaffected by co-expression of *NRG1.1*^{genomic}. *Agrobacterium* infiltrations were done in *N. benthamiana* *adr1 nrg1*. Auto-activated ADR1 and CNLs were expressed under an estradiol-inducible 35S promoter. *NRG1.1*^{genomic} was expressed under a 35S promoter. Images were taken 24 hours after inducing expression of the auto-activated NLRs. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) Protein accumulation of NLRs in (a) was assessed by western blot. Proteins were detected using anti-HA. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The dotted line indicates where digital stitching of membranes occurred in the presented western blots.

NRG1.1 requires nucleotide-binding and oligomerization to suppress ADR1

To better understand how NRG1.1 can suppress other RNLs, we wondered what functional and structural requirements NRG1.1^{genomic} may have to suppress the other RNLs. The structure of NLRs is well studied and many critical domains for NLR function have been identified (Förderer & Kourelis, 2023). The P-loop is a crucial motif in the NB-ARC domain of NLRs that mediates nucleotide binding, allowing the switch from the ADP-bound 'off' state to

the ATP-bound 'on' state (A. Bentham et al., 2016; Bonardi et al., 2012; Leipe et al., 2004). We mutated the P-loop of *NRG1.1^{genomic}* (*NRG1.1^{genomic} P-loop*) and found that co-expression of *NRG1.1^{genomic} P-loop* with *ADR1* could no longer suppress the cell death induced by *ADR1* (Figure 2.6).

Additionally, *NRG1.1* was shown to form higher order oligomers when (auto-)activated, presumed to be the *NRG1.1* resistosome (Jacob et al., 2021). Based on structural findings, the leucine in position 134 (L134) was shown to be crucial for oligomerization since mutation of this leucine to glutamic acid (L134E) abolished the oligomerization of *NRG1.1* D485V. We wondered whether this residue is also crucial for the suppression of *ADR1*. By introducing the

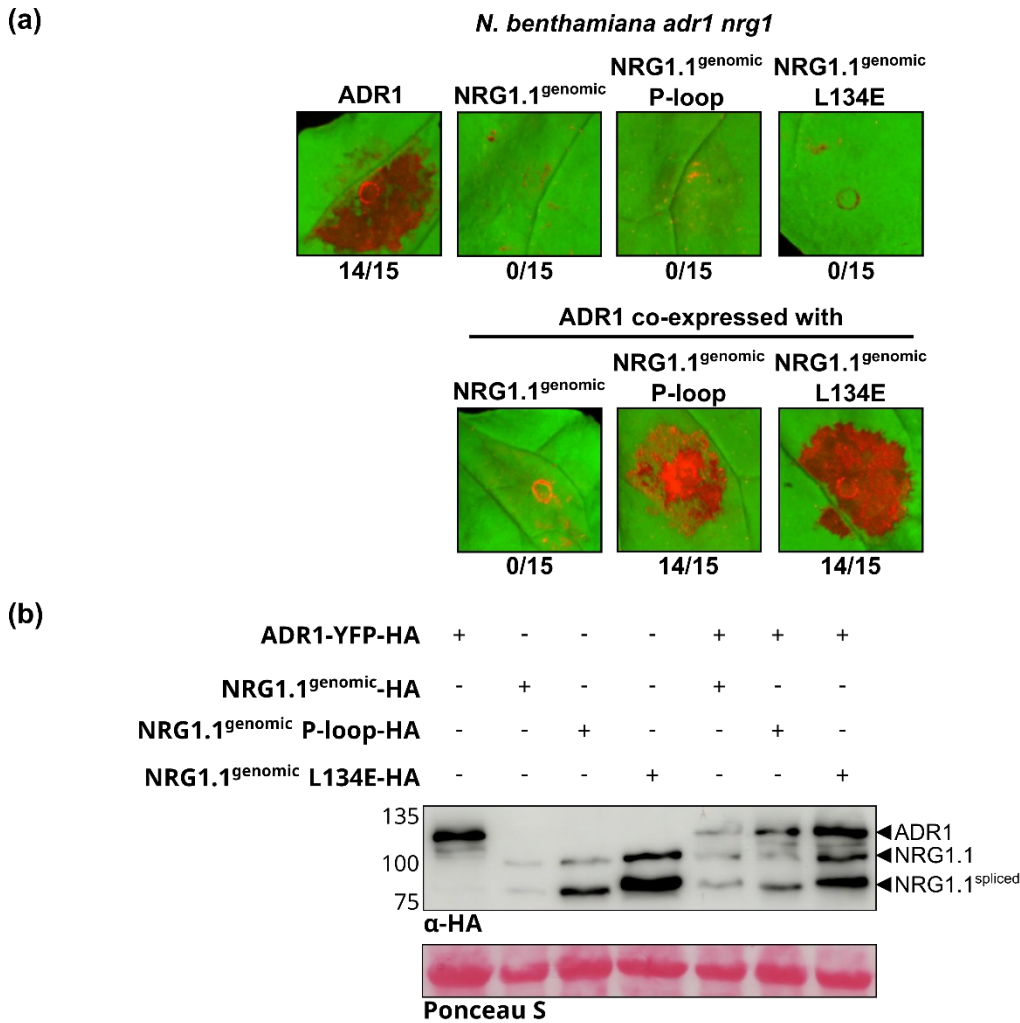


Figure 2.6: Nucleotide binding and oligomerization are required features for *NRG1.1* to suppress *ADR1* cell death. (a) Variants of *NRG1.1^{genomic}* mutated in the P-loop motif or L134E lost the ability to suppress *ADR1* cell death. *Agrobacterium* infiltrations were done in *N. benthamiana adr1 nrg1*. *ADR1* was expressed under an estradiol-inducible 35S promoter. All *NRG1.1^{genomic}* variants were expressed under a 35S promoter. Images were taken 24 hours after inducing expression of *ADR1*. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) Protein accumulation of RNLs in (a) was confirmed by western blot. Proteins were detected using anti-HA. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control.

L134E mutation in *NRG1.1^{genomic}* we found that expression of *NRG1.1^{genomic}* L134E did not suppress cell death induced by ADR1 (Figure 2.6). Taken together, these findings suggested that the suppressive unit produced by *NRG1.1^{genomic}* required nucleotide binding and oligomerization for to suppress other RNLs.

The regulatory effect of *NRG1.1* on ADR1 cannot be properly replicated

At this point, identifying the sequence of the *NRG1.1* splice variant was crucial to continue this work. In our efforts to elucidate its sequence, we started to encounter inconsistencies and discrepancies that needed to be explained. Our findings that *NRG1.1^{genomic}* required an intact P-loop and the Leucine at position 134 suggested that these regions must be included in the mRNA sequence of the *NRG1.1* splice variant. However, in the process of identifying the sequence of *NRG1.1^{spliced}*, we had trouble amplifying the part of exon 1 containing the P-loop with a splicing-specific reverse primer (Figure S2.4a, Table S3). This finding, while not conclusive, suggested to us that additional splicing events may be occurring in exon 1.

We also observed that after transient expression of *NRG1.1^{genomic}* in *N. benthamiana* we could amplify two distinct amplicons from *NRG1.1* using primers annealing directly at the start- and stop-codon (Figure S2.4b, Table S3). In *Arabidopsis*, this combination of primers led to only a single amplicon, which corresponded to the size of the constitutively spliced *NRG1.1* sequence. This may suggest differential splicing after transient expression of *NRG1.1^{genomic}* in *N. benthamiana* compared to *Arabidopsis*.

Because of the trouble identifying the *NRG1.1* splice variant, we critically reflected on all findings presented in this chapter. We realized that each instance of site-directed mutagenesis of the *NRG1.1^{genomic}* plasmid led to loss of ADR1 suppression (Figure 2.2 and Figure 2.6). This was certainly plausible because the selection of mutations was informed by NLR literature. But taken together with the inconsistencies regarding alternative splicing of *NRG1.1*, we decided to double-check our findings. We used the template pDONR207 *NRG1.1^{genomic}* plasmid utilized for these site-directed mutagenesis reactions to clone a new *NRG1.1^{genomic}*-HA plasmid (*NRG1.1^{genomic}*-HA^{recreated}). We transiently co-expressed ADR1 and *NRG1.1^{genomic}* from this newly generated plasmid in *N. benthamiana* and compared the result to the original plasmid (*NRG1.1^{genomic}*-HA^{original}). We found that expression of *NRG1.1^{genomic}*-HA^{recreated} was unable to suppress cell death induced by ADR1 (Figure 2.7). Comparative Whole Plasmid Sequencing did not reveal any causative mutations in the *NRG1.1^{genomic}* sequence of either plasmid. With two identical constructs portraying opposing results, we decided to generate another functionally identical construct using GoldenGate cloning (*NRG1.1^{genomic}*-HA^{GoldenGate}) (Binder et al., 2014). Expression of *NRG1.1^{genomic}*-HA^{GoldenGate} was also unable to suppress cell death induced by ADR1 (Figure 2.7). Taken together these findings suggested to us that the observed suppression of ADR1-induced cell death was unique to *NRG1.1^{genomic}*-HA^{original} and

could be traced back to a specific glycerol stock of *Agrobacterium tumefaciens* GV3101 used for all the previous experiments.

We wondered whether this glycerol stock may have been heterogeneous, carrying two (or more) distinct expression vectors. To investigate this, we streaked out the glycerol stock to single colonies and inoculated twenty of these for further analysis. We mixed *Agrobacterium* carrying our *ADR1* overexpression plasmid together with the isolated *Agrobacterium* colonies. We found that 2 out of 20 colonies (10%) could not suppress cell death from *ADR1* (Figure S2.5a). However, both colonies (nr. 5 and 13) resulted in proper expression of *NRG1.1^{genomic}* (Figure S2.5b). Other colonies (nr. 3, 9, 11, and 15) did not lead to proper expression

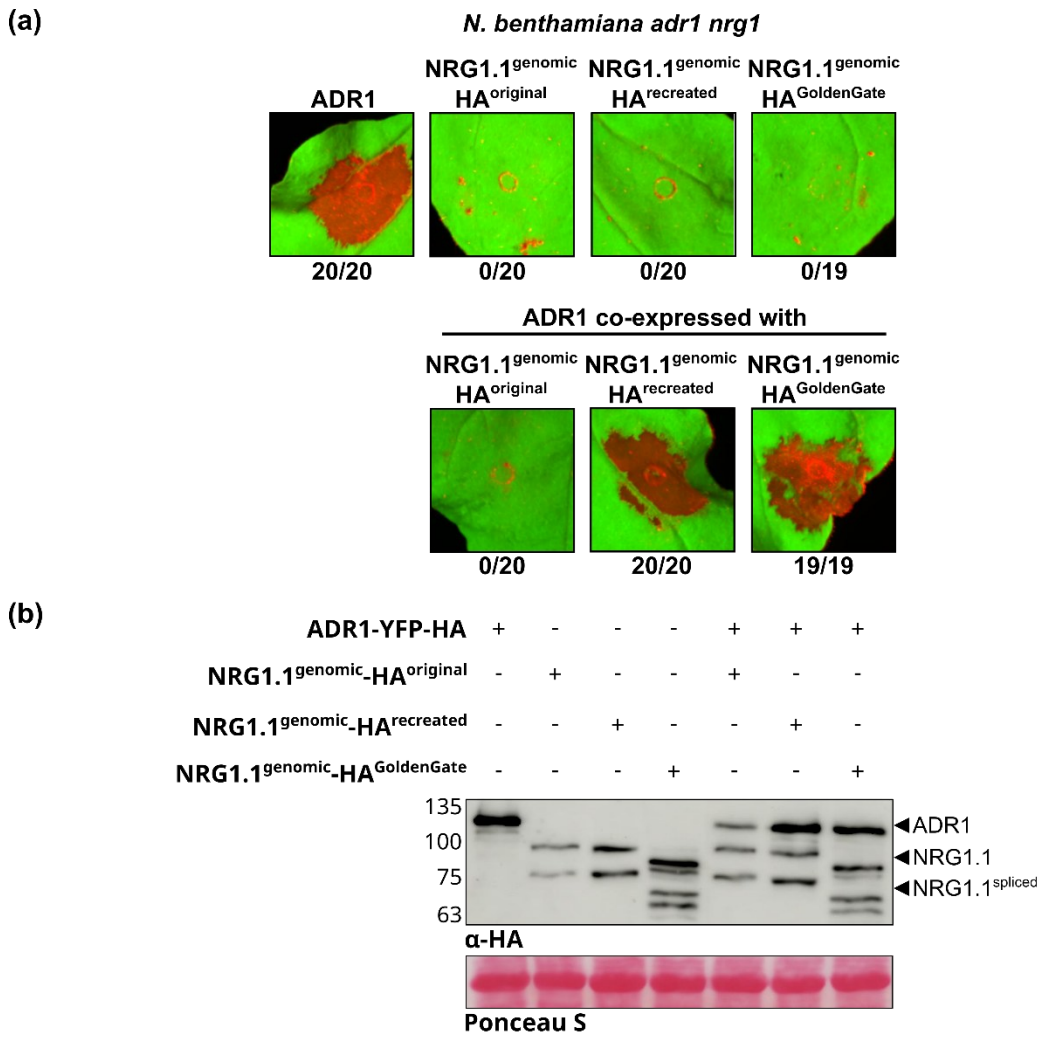


Figure 2.7: Only the original glycerol stock of *Agrobacterium* carrying the *NRG1.1^{genomic}* construct led to the suppression of *ADR1* cell death. (a) Comparison of three *NRG1.1^{genomic}*-HA constructs in their ability to suppress *ADR1*. *Agrobacterium* infiltrations were done in *N. benthamiana* *adr1 nrg1*. *ADR1* was expressed under an estradiol-inducible 35S promoter. All *NRG1.1^{genomic}* variants were expressed under a 35S promoter. Images were taken 24 hours after inducing expression of *ADR1*. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. **(b)** Protein accumulation of RNLs in (a) was confirmed by western blot. All proteins were detected using anti-HA. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control.

NRG1.1^{genomic} but could still suppress cell death induced by ADR1. Based on these findings, we cannot correlate the suppression of ADR1 cell death to the expression of *NRG1.1^{genomic}*. Instead, we hypothesize that the *Agrobacterium* glycerol stock carrying the *NRG1.1^{genomic}-HA^{original}* plasmid may be heterogeneous, with a subset of these *Agrobacteria* leading to the introduction an unknown RNL-suppressing unit.

We did not yet perform a full genome comparison between suppressing and non-suppressing *Agrobacterium* colonies from this glycerol stock. However, we had one candidate gene in mind that could explain the RNL-specific suppression we had observed. *NRG1.3* is a N-terminally truncated *NRG1* gene that negatively regulates *NRG1.1* and *NRG1.2* by competing for EDS1-SAG101 complex formation (S. Huang et al., 2025; Z. Wu et al., 2022; Xiao et al., 2025). *NRG1.3* was previously reported to also suppress *NRG1.1* D485V cell death in *N. benthamiana* (Z. Wu et al., 2022). We tested whether *NRG1.3* could similarly suppress cell death by ADR1. We found that *NRG1.3* was unable to suppress ADR1 cell death (Figure S2.6). Thus, accidental introduction of *NRG1.3* into the *NRG1.1^{genomic}-HA^{original}* *Agrobacterium* glycerol stock was not causing the ADR1-suppressing effect we studied. Since this project had now strayed so far from the original hypothesis and lacked biological relevance, we decided to halt further investigations.

Discussion

In this chapter, we attempted to discover the mechanism behind the regulation of ADR1 activity regulation by other *Arabidopsis* RNLs, as hypothesized in the previous chapter (Figure 1.1e). Using transient expression in *N. benthamiana*, we initially found that only *NRG1.1^{genomic}* could suppress cell death from ADR1, unlike other RNLs and *NRG1.1^{cds}* (Figure 2.1). This observation led to the discovery of a putative splice junction in *NRG1.1* also present in *Col-0* plants (Figure 2.2). Based on these results, we hypothesized that the resulting *NRG1.1* isoform could be responsible for the suppression of cell death induced by ADR1. However, during our continued investigation, we found discrepancies between different experiments that were contradictory (Figure 2.6 and Figure S2.4). Ultimately, we discovered that the suppression of ADR1 did not correlate with the co-expression of *NRG1.1^{genomic}* (Figure 2.7 and Figure S2.5). Despite this course-changing discovery, we did uncover novelties about *NRG1.1* that could pave the way for new research directions on *Arabidopsis* RNL function and regulation.

An unknown factor suppresses cell death from ADR1 and other RNLs

At this time, we do not know why *NRG1.1^{genomic}-HA^{original}* suppressed ADR1, but *NRG1.1^{genomic}-HA^{recreated}* did not. We could determine that the expression of *NRG1.1* did not correlate with the suppression of ADR1 (Figure 2.7 and Figure S2.5). So, we hypothesize that the glycerol stock containing *Agrobacterium* of *NRG1.1^{genomic}-HA^{original}* contained and introduced an unknown factor that can suppress cell death from overexpression of RNLs. We currently do not yet know what exactly this suppressive factor is. Based on literature and considering the observed RNL specificity (Figure 2.4 and Figure 2.5), we speculated that *Agrobacterium* carrying *NRG1.3* expression vectors could have been accidentally mixed into the *NRG1.1^{genomic}-HA^{original}* glycerol stock. The *NRG1.3* gene encodes for a N-terminally truncated *NRG1* protein that is highly upregulated during immunity (Figure S2.2) (Z. Wu et al., 2022). The N-terminal truncation of *NRG1.3* is distinct from the deletion in our putative alternatively spliced *NRG1.1* isoform, which would lack a region in the NB-ARC domain. Functionally, *NRG1.3* was shown to compete with other *NRG1* proteins for EDS1-SAG101, thereby negatively regulating their activation (S. Huang et al., 2025; Xiao et al., 2025). Expression of *NRG1.3* was additionally reported to suppress cell death of *NRG1.1* D485V in *N. benthamiana* (Z. Wu et al., 2022). However, this observation contrasts the proposed model for *NRG1.3* function, because we and others have shown that that cell death from (auto-)activated *Arabidopsis* RNLs is EDS1-independent in *N. benthamiana* (Figure S2.1 and Figure S2.6) (Jacob et al., 2021). Indeed, we were unable to corroborate the previous report that *NRG1.1* D485V cell death in *N. benthamiana* is suppressed by co-expression of *NRG1.3* under our conditions. Neither did we observe suppression of ADR1 cell death by co-expression of

NRG1.3. These findings rule out the hypothesis that *NRG1.3* was causal for the suppression of ADR1 we observed during our experiments with *NRG1.1^{genomic}-HA^{original}*.

After ruling out *NRG1.3* as a candidate, the identity of the suppressive factor inside our *NRG1.1^{genomic}-HA^{original}* *Agrobacterium* glycerol stock remains unknown. We speculate that the source is RNL-derived due to the specificity of cell death suppression to RNLs (Figure 2.4 and Figure 2.5). Comparative Whole Genome Sequencing of suppressing and non-suppressing *Agrobacterium* colonies could lead to the identification of plasmid variants carried by these bacteria (Figure S2.5). An alternative hypothesis is that the suppressing *Agrobacteria* carry the suppressive factor on their own genome. However, due to the RNL-specificity of the suppressive factor, we favour the hypothesis of a secondary expression plasmid.

We also did not uncover the mechanism behind the suppression of ADR1 cell death by this unknown suppressive factor. The specificity to suppress cell death from RNLs but not CNLs hinted towards directly affecting the RNL function, rather than a general effect on NLR-mediated cell death in *N. benthamiana*. Speculating on the potential mechanism behind cell death suppression may inform future experimental design. Since RNLs are reported to have functions at membrane compartments, one hypothesis is that the localization of RNLs is affected by the suppressive factor (Ibrahim et al., 2026; Jacob et al., 2021; Saile et al., 2021). Alternatively, we hypothesize that the suppressive unit targets the function of the RNL resistosome to suppress cell death. This could be achieved by preventing RNL oligomerization or altering the structure of the resistosome to inhibit its role as calcium-permeable channel (Jacob et al., 2021). Uncovering the identity and function of the suppressive factor could provide a useful tool to avoid auto-activity when engineering RNL regulatory networks in plants.

Alternative splicing of *NRG1.1* warrants further investigation

While the suppression of ADR1 by *NRG1.1^{genomic}* was disproven in this work, we did identify an isoform of *NRG1.1* generated by alternative splicing (*NRG1.1^{spliced}*) (Figure 2.2 and Figure 2.6). *NRG1.1^{spliced}* is generated through a splice junction within exon 3 of *NRG1.1*. Notably, based on our Sanger sequencing the resulting transcript after utilizing this splice junction would not lead to a frameshift. The full sequence of the spliced transcript will first need to be discovered, for instance by Rapid Amplification of cDNA Ends (5' and 3' RACE). Our preliminary findings point towards the possibility of additional splicing events occurring on the same transcript (Figure S2.4), but the evidence is far from conclusive. Notably, *NRG1.1^{spliced}* was not previously been annotated in gene annotations such as Araport11 (C. Cheng et al., 2017) or in alternative splicing databases (Plant Intron-Splicing Efficiency database, PastDB). Combined with a 40-cycle RT-PCR underlying the identification of this putative splice variant, we assume that the spliced transcript accumulation is low (Figure 2.2a). This could perhaps

be attributed to the expression pattern of *NRG1.1* (Figure 2.3). RNLs are generally low expressed genes in unchallenged plants and upregulated during infection (Lai & Eulgem, 2018; Tian et al., 2021). For *NRG1.1*, we observed an early peak in expression upon infection with various immunity triggers. A high-resolution Isoform Sequencing study designed around changes in the transcriptomic landscape of early infection may have the highest odds of capturing this *NRG1.1* variant.

Alternative splicing of immunity-related transcripts has previously been identified as an important feature of the plant immune system (Bui et al., 2025; Kufel et al., 2022). The importance of alternative splicing is reflected by pathogen effectors also targeting splicing machinery to disrupt plant immune responses and promote infection (J. Huang et al., 2020; Mejias et al., 2021). Many NLRs have also been reported to undergo splicing (Lai & Eulgem, 2018). For example, the paired NLRs ‘resistance to *Ralstonia solanacearum*’ (RRS1) and ‘resistance to *Pseudomonas syringae* 4’ (RPS4) were both reported to undergo alternative splicing, and all isoforms of *RPS4* are essential for its function (C.-Y. Huang et al., 2021; X.-C. Zhang & Gassmann, 2003). Similarly, the function of the TNL *SNC1* is also modulated by alternative splicing machinery (Xu et al., 2012). Recently, differential splicing of immunity-related transcripts was reported to function in a cell type specific manner (Barff et al., 2025). This adds yet another layer of complexity and finetuning of the plant immune system. Alternative splicing of a helper NLR (*NRG1.1*) central to an extensive sensor-helper NLR network (Figure 2c) could be a mechanism to regulate the entire signalling network.

The protein level evidence that the splice junction mutant *NRG1.1*^{genomic} G391G* lost the secondary band from *NRG1.1*^{genomic} is compelling and should be assessed in *Arabidopsis* (Figure 2.2d). Performing IP-MS on the secondary protein band from *NRG1.1*^{genomic} after expression in *N. benthamiana* could be an alternative approach to identifying *NRG1.1*^{spliced}. Along with the identification of the *NRG1.1*^{spliced} sequence, further investigation into the biological and functional importance of this *NRG1.1* isoform is warranted. The splice junction mutant (*NRG1.1* G391G*) could already be used to further study the function of *NRG1.1*^{spliced} until the full sequence of *NRG1.1*^{spliced} is known. Transgenic *Arabidopsis* plants expression *NRG1.1* G391G* (and eventually *NRG1.1*^{spliced}) could be used to compare common immune outputs to wildtype *NRG1.1*.

The early peak in expression of *NRG1.1* raises more questions

Despite finding that *NRG1.1* was not regulating the cell death activity of other RNLs in *N. benthamiana*, the early peak in expression of *NRG1.1* in *Arabidopsis* raised questions on the biological function of this expression pattern (Figure 2.3). *NRG1.1* is commonly associated with the induction of cell death (Lapin et al., 2019). Indeed, complementation with *NRG1.1* can restore cell death in the *nrg1 double* mutant (Castel et al., 2019). Early upregulation of a cell

death inducing gene seems counterintuitive since cell death is not observed as an early immune response, but rather during later stages of immunity (Balint-Kurti, 2019; Coll et al., 2011). To prevent unwarranted early induction of cell death, we reason that *NRG1.1* must be tightly regulated, for instance by *NRG1.3* (Z. Wu et al., 2022). A secondary function unrelated to cell death induction (e.g. transcriptional reprogramming) could explain the early upregulation of *NRG1.1*. Another theory is that changes in the alternative splicing landscape during immune responses could also regulate *NRG1.1* activity, by regulating the accumulation of the (cell death capable) constitutively spliced *NRG1.1*. However, further investigation into the alternative splicing event in *NRG1.1* and the function of the *NRG1.1^{spliced}* isoform is required (Figure 2.2). Notably, our findings were based on a bulk RNAseq dataset, rather than single cell RNAseq (Figure 2.3) (Saile et al., 2020). Thus, the observed expression pattern for *NRG1.1* could for instance also be unique to cells directly in contact with the pathogen. Taken together, our findings on the expression patterns of RNLs using a published RNAseq dataset raise additional questions about RNL expression dynamics and function (see General discussion).

Conclusion

Taken together, we initially observed that co-expression of *NRG1.1^{genomic}* could suppress cell death induced by ADR1. However, we showed that the suppression of cell death from RNLs in *N. benthamiana* did not correlate with the co-expression of *NRG1.1*. The presented results in this chapter form a warning to others that experimental evidence may support your current hypothesis, but considering alternative hypotheses that can explain the same observations is equally important. Nevertheless, our investigation led to the identification of a putative splice junction in *NRG1.1* that was directly responsible for a detectable protein isoform. The function of this novel NRG1.1 isoform is currently unknown. Together with the unique expression pattern we uncovered for *NRG1.1*, our findings pave the way for continued research on alternative functions of *Arabidopsis* RNLs.

Contributions

This chapter contains work from the internship students Anna Gurtmann, Johann Moore and Florian Mangler, all of whom I co-supervised during my PhD thesis. Dr. Lance Jubic in the lab of Prof. Jeff Dangl (University of North Carolina at Chapel Hill) made the initial finding that co-expression of *NRG1.1^{genomic}* suppressed cell death induced by overexpression of *Arabidopsis* ADR1 in *N. benthamiana*.

Supplementary material

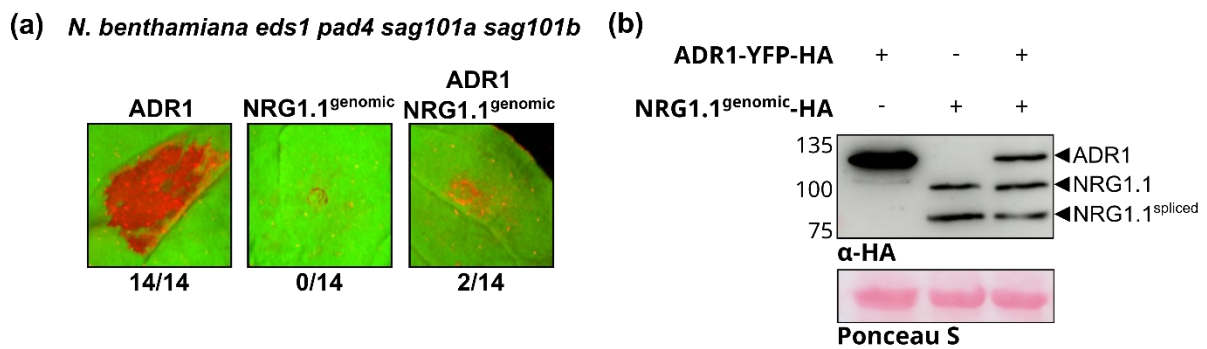
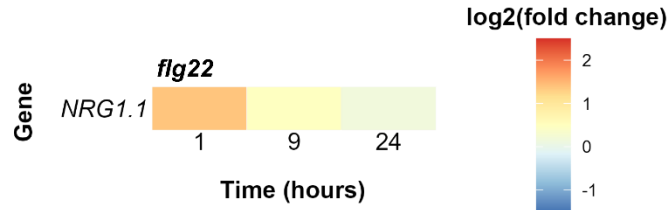


Figure S2.1: ADR1 induced cell death and suppression by NRG1.1 are independent of EDS1, PAD4, and SAG101a/b in *N. benthamiana*. (a) Co-expression of *NRG1.1^{genomic}* with *ADR1* could still suppress cell death in the *N. benthamiana eds1 pad4 sag101a sag101b* quadruple mutant (compared to *N. benthamiana adr1 nrg1*, Figure 2.1). *ADR1* was expressed under an estradiol-inducible 35S promoter. *NRG1.1^{genomic}* was expressed under a 35S promoter. Images were taken 24 hours after inducing expression of *ADR1*. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) Protein accumulation of RNLs in (a) was confirmed by western blot. Proteins were detected using anti-HA. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control.

(a)



(b)

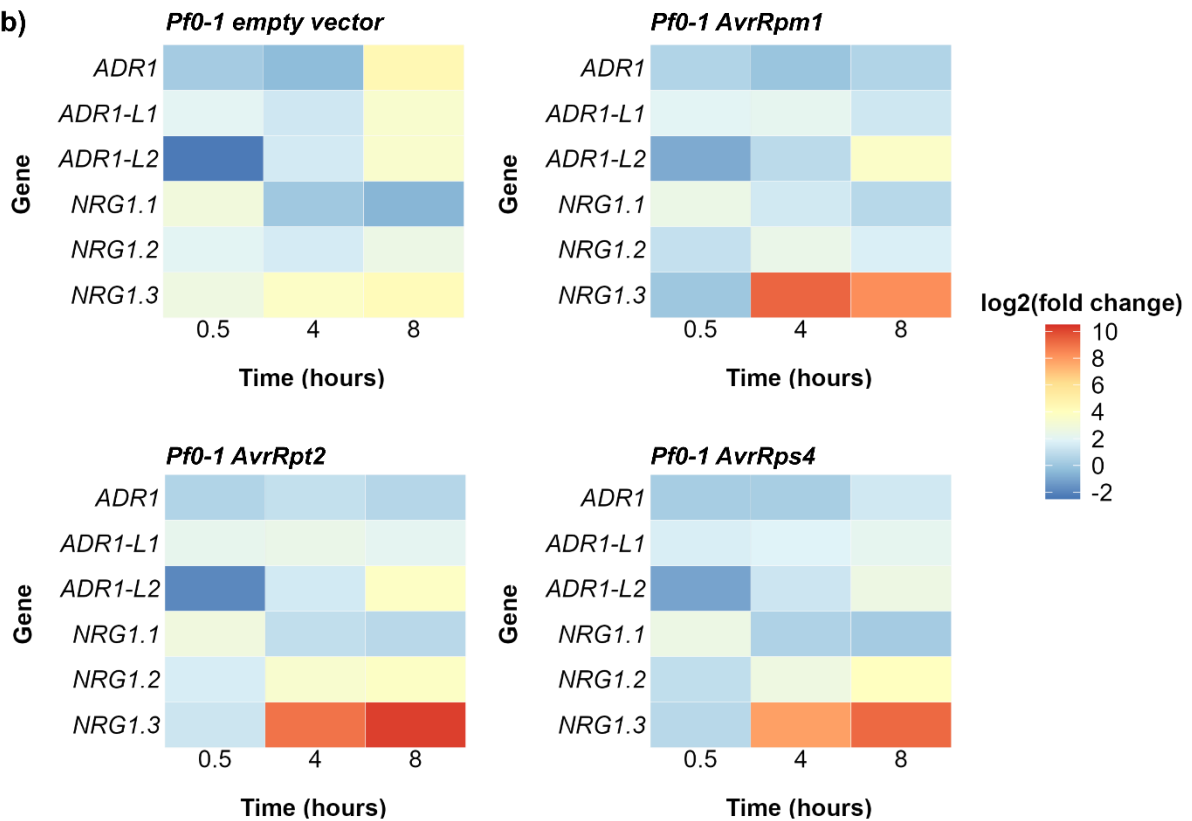
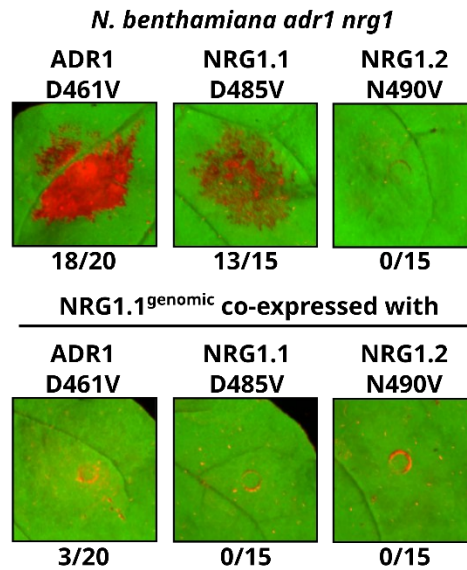


Figure S2.2: The early peak in expression of *NRG1.1* is distinct from all other *Arabidopsis* RNLs.

(a) The expression pattern of *NRG1.1* was confirmed with a second published RNA sequencing dataset (Winkelmüller et al., 2021). In this work, PTI responses were elicited using a 22 amino acid epitope of bacterial flagellin (flg22). Expression levels are depicted as log2(fold change) compared to mock-treated plants. (b) *NRG1.3* (*At5g66890*) expression levels are gradually upregulated over time, unlike *NRG1.1*. The same data is depicted as seen in Figure 2.3 but now accommodating for *NRG1.3*. The same legend applies to all heatmaps depicted in (b).

(a)



(b)

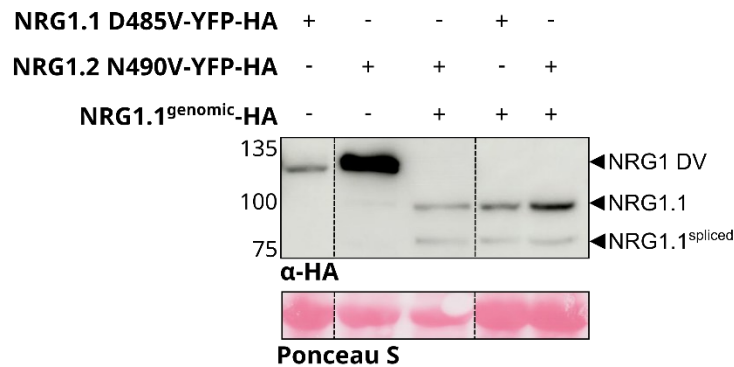


Figure S2.3: NRG1.1 D485V and NRG1.2 N490V protein could not be detected upon co-expression with *NRG1.1*^{genomic}. (a) Overexpression of *NRG1.1 D485V* caused cell death in *N. benthamiana* whereas *NRG1.2 N490V* did not. In co-expression with *NRG1.1*^{genomic}, we did not observe cell death induced by *NRG1.1 D485V* and *NRG1.2 N490V*. *Agrobacterium* infiltrations were done in *N. benthamiana* *adr1 nrg1*. *ADR1 D461V*, *NRG1.1 D485V*, and *NRG1.2 N490V* were expressed under an estradiol-inducible 35S promoter. *NRG1.1*^{genomic} was expressed under a 35S promoter. Images were taken 24 hours after inducing expression of the auto-active RNLs. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) *NRG1.1 D485V* and *NRG1.2 N490V* could be detected in standalone infiltrations, but not upon co-expression with *NRG1.1*^{genomic}. Protein accumulation of RNLs in (a) was assessed by western blot. Proteins were detected using anti-HA. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The dotted line indicates where digital stitching of membranes occurred in the presented western blot.

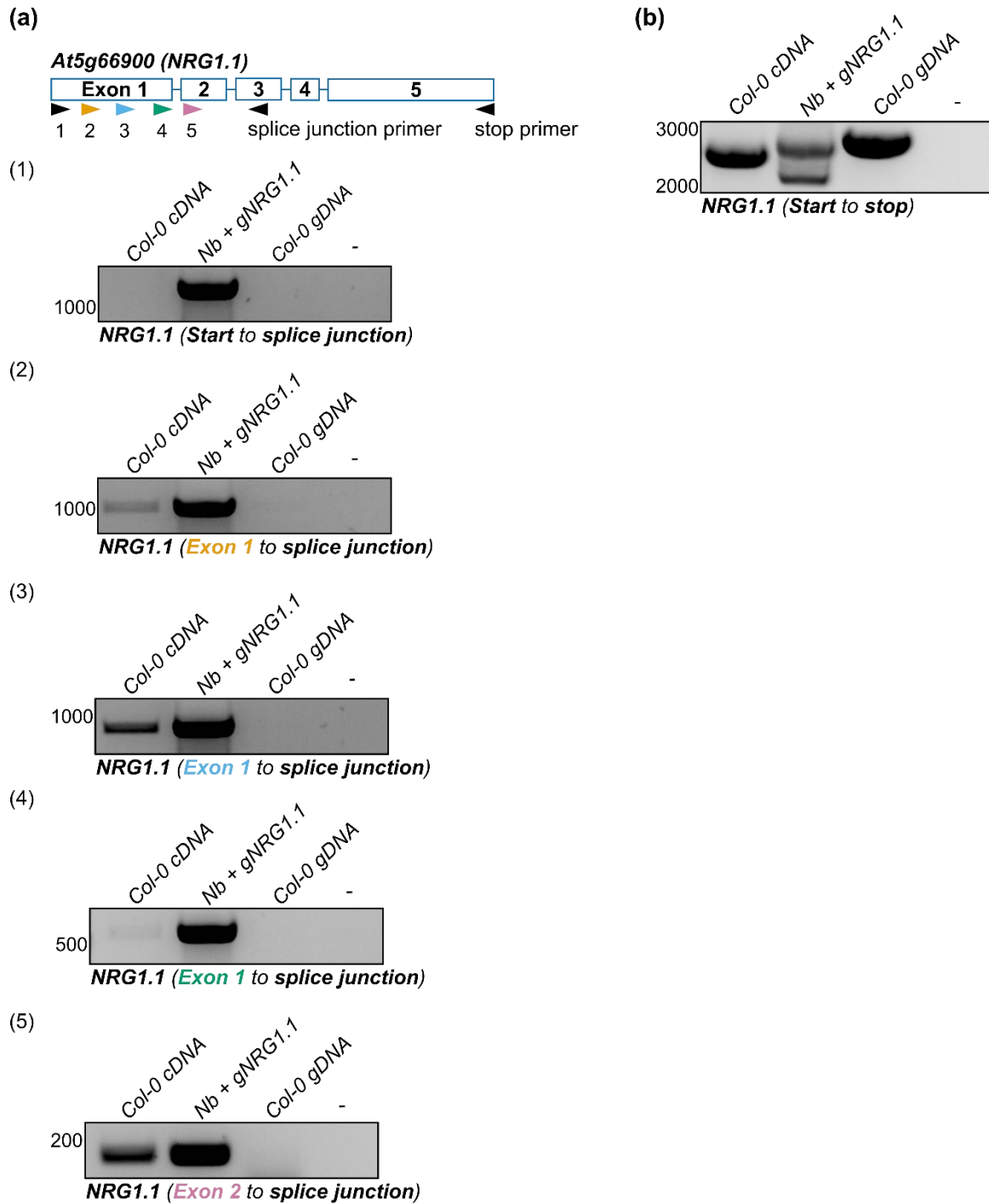


Figure S2.4: Findings on *NRG1.1^{spliced}* were inconsistent between protein- and transcript level evidence. (a) Determination of exon inclusion in *NRG1.1^{spliced}* by RT-PCR using a splice-variant specific primer. Various forward primers (1-5) were combined with a splice junction specific primer to assess sequence inclusion based on the primer binding site. Primers are listed in Table S3. Copy DNA from *N. benthamiana* overexpressing *Arabidopsis NRG1.1^{genomic}* was included as a positive control. *Col-0* gDNA was included as a negative control to assess the specificity of the splice junction-specific primer. **(b)** Amplification of the *NRG1.1* sequence from the start codon to the stop codon revealed contrasting splicing behaviour from *Arabidopsis* and *N. benthamiana*. Primers annealed directly at the start- and stop-codon of *NRG1.1*. Genomic DNA from *Col-0* was included as a positive control. The negative control contained no DNA

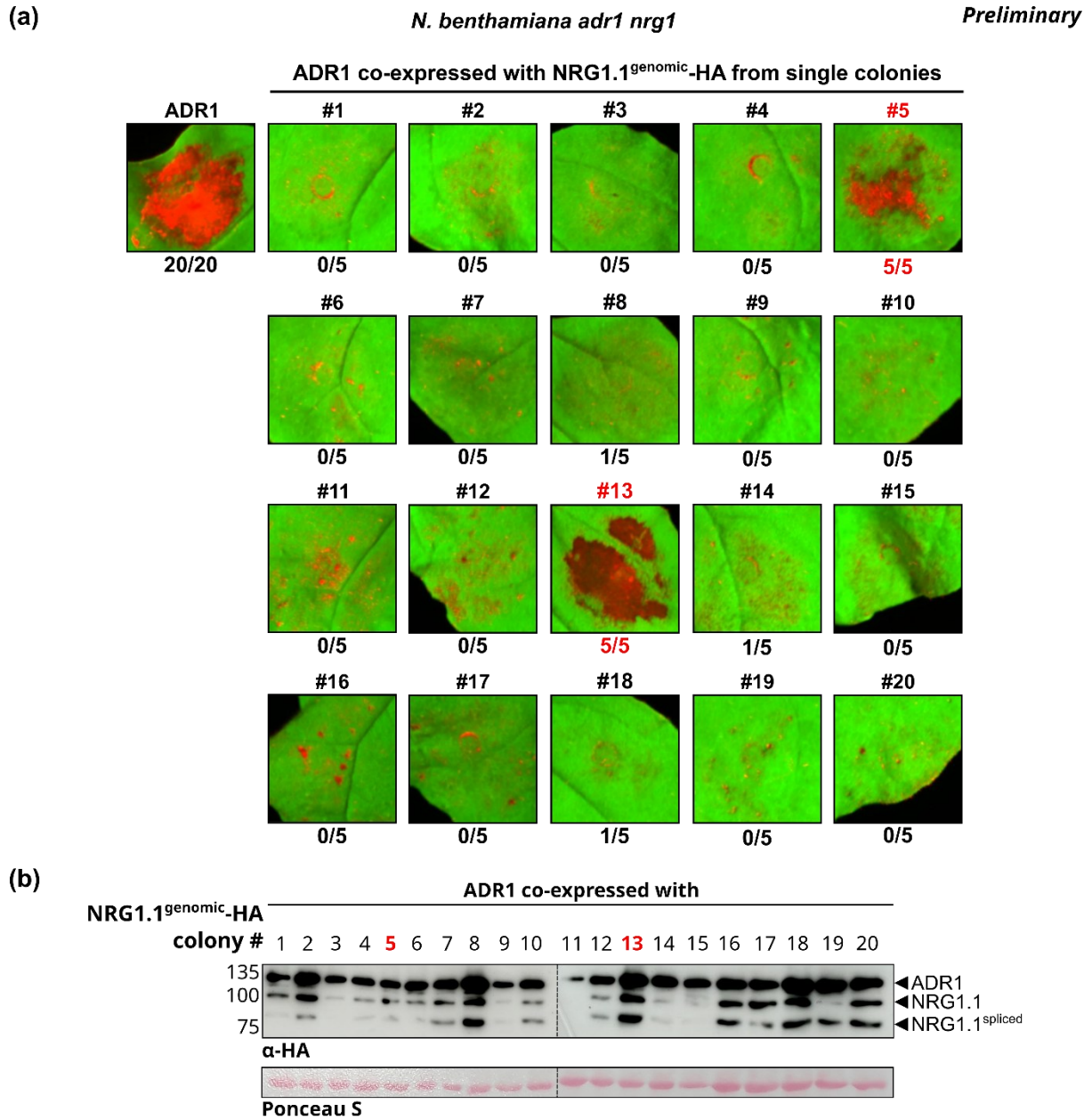


Figure S2.5: (Preliminary) Individual colonies from the same *Agrobacterium* glycerol stock carrying NRG1.1^{genomic}-HA^{original} had varying effects ADR1 induced cell death. (a) NRG1.1^{genomic}-HA *Agrobacterium* colony #5 and #13 were unable to suppress ADR1 cell death. *Agrobacterium* infiltrations were done in *N. benthamiana adr1 nrg1*. ADR1 was expressed under an estradiol-inducible 35S promoter. NRG1.1^{genomic} was expressed under a 35S promoter. Images were taken 24 hours after inducing expression of the ADR1. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. This experiment was only performed once. (b) NRG1.1^{genomic}-HA *Agrobacterium* colony #3 and #13 both expressed NRG1.1^{genomic}. Protein accumulation of RNLs in (a) was assessed by western blot. Proteins were detected using anti-HA. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The dotted line indicates where digital stitching of membranes occurred in the presented western blot.

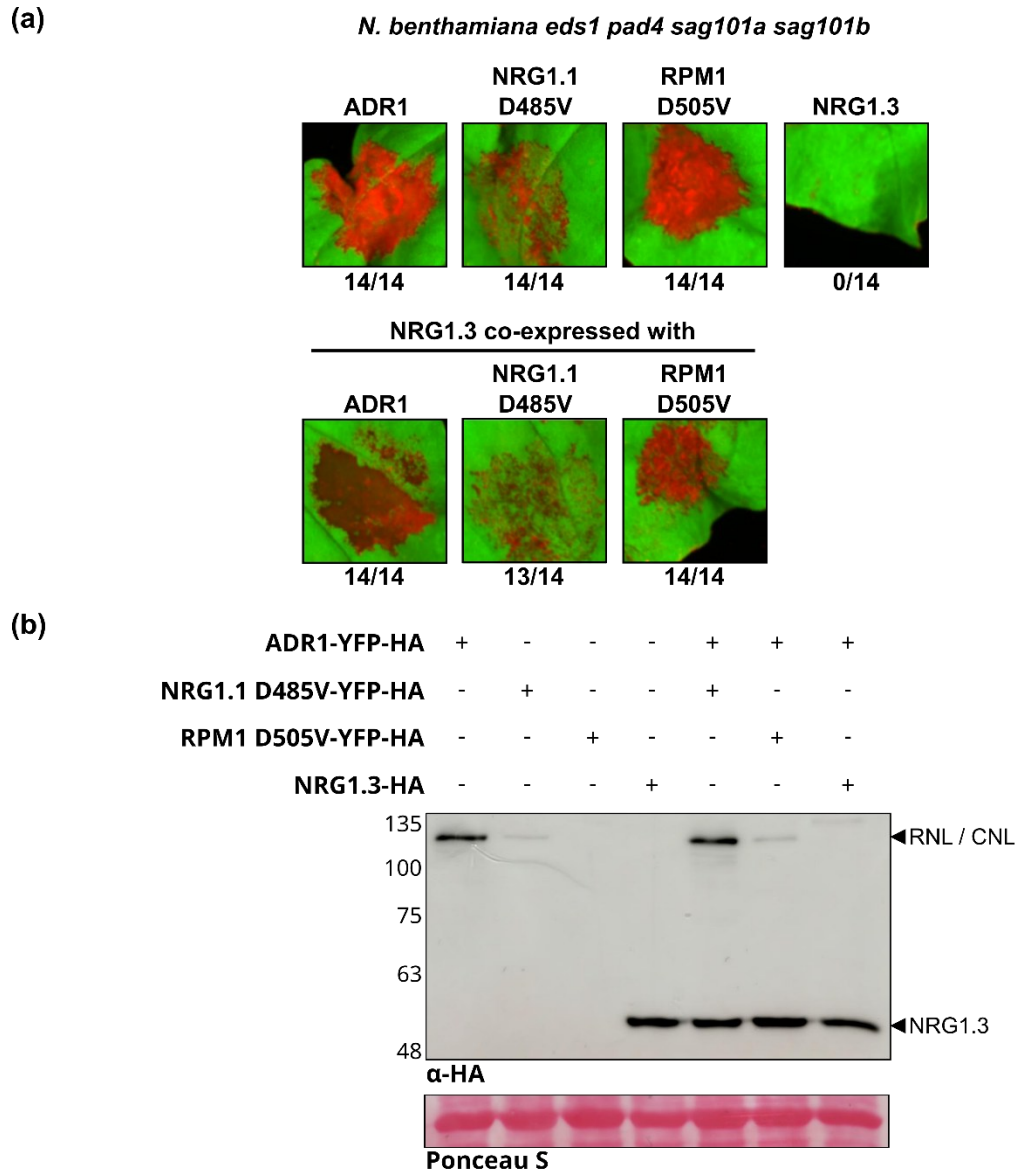


Figure S2.6: Co-expression of *NRG1.3* did not suppress cell death induced by overexpression of *Arabidopsis* RNLs in *N. benthamiana*. (a) Overexpression of *ADR1*, *NRG1.1 D485V*, or *RPM1 D505V* caused potent cell death which was unaffected by co-expression of *NRG1.3*. *Agrobacterium* infiltrations were done in *N. benthamiana eds1 pad4 sag101a sag101b*. Images were taken 24 hours after inducing expression of the *ADR1*. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) Protein accumulation of NLRs in (a) was assessed by western blot. Proteins were detected using anti-HA. *RPM1 D505V* could not be detected properly, but we could still observe cell death. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control.

Chapter 3

Phosphorylation Modulates the Activity of *Arabidopsis* Helper NLR ADR1-L2

Introduction

Phosphorylation events are an essential part of cellular signalling. Phosphorylation as post-translational modification modulates properties of a protein, by altering the activity, stability, or localization (W. J. Zhang et al., 2023). The phosphorylation status of Serine (S), Threonine (T), and Tyrosine (Y) residues is regulated by the interplay between kinases and phosphatases. This 'molecular switch' allows for the rapid adaptation to external cues and altering the physiology of the plant where necessary. The effect phosphorylation has on a protein-of-interest is determined on a case-by-case basis. Research on phosphorylation frequently rely on phosphomimetic substitutions of potentially phosphorylated residues. The negative charges introduced by phosphorylation can be partially mimicked by exchanging the phosphorylated residue with aspartic acid (D) or glutamic acid (E). Inversely, substitution to alanine (A) renders the residue unable to be phosphorylated, thereby mimicking a dephosphorylated state.

Phosphorylation events are common in plant immune signalling for the transduction of externally perceived signals and regulation of immune components (Y. Yan et al., 2025). The early recognition of MAMPs by RLKs or RLPs initiates the formation of PRR complexes and activates their kinase domains to initiate phosphorylation of downstream signalling components (Figure 1) (D. Tang et al., 2017). These downstream immune components also involve many cytoplasmic kinases, most notably RLCKs and MAPKs (Rao et al., 2018; M. Zhang & Zhang, 2022). Upon activation, these kinases mediate signalling cascades that activate different branches of the plant immune system (Bi et al., 2018; Frei Dit Frey et al., 2014; Lal et al., 2018).

The importance of phosphorylation in plant immunity is reflected by the evolution of pathogen effectors targeting these signalling cascades. Many effectors interfere with kinases involved in plant immunity to disrupt their function, suppress immune responses, and promote the virulence of the pathogen. As a result, kinases are often monitored by NLRs (Breit-McNally et al., 2022; DeYoung et al., 2012; Schulze et al., 2022; Y. Wu et al., 2020; Z. Zhang et al., 2012). In other cases, the phosphorylation status of effector targets can be monitored by NLRs (e.g. RPM1) to initiate immunity when specific residues are phosphorylated (Chung et al., 2011; J. Liu et al., 2011). Phosphorylation events are essential for full immune activation, but phosphorylation can also negatively regulate the components of the plant immune system. This extends to NLRs where certain phosphorylation events were reported to inhibit their function, for instance by promoting autoinhibition or preventing resistosome formation (H. Guo et al., 2020; Toth et al., 2026; Zhong et al., 2025). These examples indicate the crucial role of phosphorylation in regulating NLR activity, both positively and negatively.

The ADR1 family of RNLs form a central signalling hub that connects parts of the two branches of plant immunity, PTI and ETI (Pruitt, Locci, et al., 2021). Immune responses induced by ADR1 activation come at high costs, and mis-regulation often leads to autoimmunity (Chapter 1) (Grant et al., 2003; Roberts et al., 2013; M.-Y. Wang et al., 2023). Thus, the activity of ADR1 family members must be carefully fine-tuned to activate the appropriate response, depending on the elicitor of immunity (MAMP or effector). Phosphorylation forms an elegant hypothesis as potential molecular regulator of the ADR1 family to achieve the desired immune output. Here, we investigate the role of phosphorylation on *Arabidopsis* ADR1-L2. We identified several Serine and Threonine residues that are phosphorylated *in planta*. Using (de)phosphorylation mimicking mutations we showed that the phosphorylation status of these residues regulates the (auto-)activity of ADR1-L2. We then set out to mechanistically investigate how phosphorylation affects immune responses mediated by ADR1-L2.

Results

Arabidopsis ADR1-L2 is phosphorylated on residues in *N. benthamiana*

We set out to study the effect of phosphorylation on RNL function using *Arabidopsis* ADR1-L2 as our protein-of-interest. During trial experiments to optimize the immunoprecipitation with subsequent mass spectrometry (IP-MS) of ADR1-L2³, we already identified a list of candidate residues in ADR1-L2 that may be phosphorylated in *Nicotiana benthamiana* with and without MAMP treatment (Table S3.1). Two residues (Threonine-56 and Serine-148) stood out due to their higher MS/MS count in our preliminary data. We decided to continue with these two residues as candidates to study the effect of phosphorylation on ADR1-L2 activity (Figure 3.1a). Additionally, we included Serine-141 as an additional candidate residue since this residue was identified as phosphorylated in a previously published dataset (Umezawa et al., 2013). Finally, Serine-342 was predicted to be a phosphorylation target of the *Arabidopsis* RLCK BIK1 and also included as a residue of interest (Toth et al., 2026).

We next used AlphaFold3 structural prediction to assess where our candidate residues are located on the ADR1-L2 protein structure (Abramson et al., 2024). The residues T56, S141, and S148 are located on the CC domain of ADR1-L2 (Figure 3.1b). Based on our AlphaFold3 predictions, T56 is found in between the second and third α -helix of the ADR1-L2 CC_{RPW8} domain (Figure 3.1b, d). S141 and S148 are located further downstream of the α -helices, in the region where the CC_{RPW8} domain and NB domain connect. Notably, S141 and S148 are in a region of the CC domain where the prediction confidence is relatively low (Figure S3.1). The predicted BIK1 phosphorylation site S342 is in the NB-ARC domain.

We additionally predicted the structure of an ADR1-L2 pentamer, resembling the ZAR1 resistosome (J. Wang, Hu, et al., 2019). Within this predicted structure, we can observe a channel formed by the CC domains of ADR1-L2 (Figure 3.1c, d). The residues S141 and S148 are located at the end of this channel, but do not face towards the channel interior. However, the function of phosphorylation on these residues cannot be determined from structural location based on predictions alone. So, we continued with our four candidate residues (T56, S141, S148, and S342) and set out to study the effect of phosphorylation on *Arabidopsis* ADR1-L2 activity.

³ Data courtesy of Dr. Marie Le Naour-Vernet and Prof. Dr. Cyril Zipfel at the University of Zürich

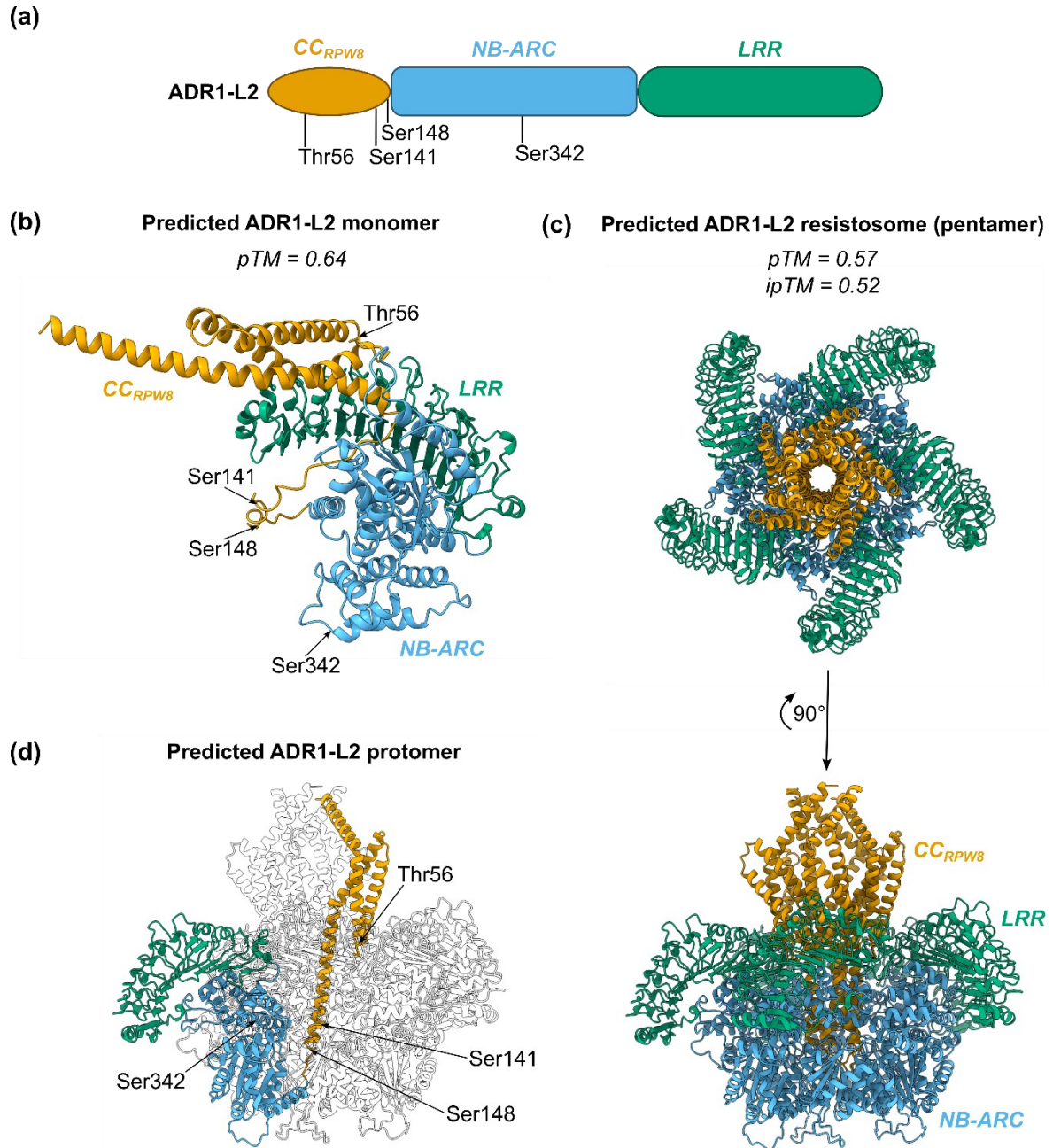


Figure 3.1: Arabidopsis ADR1-L2 is phosphorylated in *N. benthamiana*. (a) Schematic representation of the ADR1-L2 protein with candidate phosphorylation sites indicated. Phosphorylated residues were identified by IP-MS of ADR1-L2-FLAG after overexpression in *N. benthamiana*. The full overview of identified phosphorylation sites in ADR1-L2 are available in Table S3.1. (b, c, d) AlphaFold3 predictions of ADR1-L2 as a monomer (b), pentamer (c) and protomer in the pentamer (d) (Abramson et al., 2024). The position of residues T56, S141, S148, and S342 indicated. The CC domain is indicated in orange, the NB-ARC domain in blue, and the LRR domain in green. The predicted Template Modelling score (pTM) and predicted interface Modelling Score (ipTM) are included to indicate the predicted accuracy of the structure. Prediction confidence scores and expected position error are available in Figure S3.1.

Phosphomimetics modulate ADR1-L2 D484V autoimmunity in *Arabidopsis*

To investigate the effect of these phosphorylated residues in ADR1-L2, we generated transgenic lines expressing ADR1-L2 D484V in *cis* with various combinations of phosphorylation-mimic or dephosphorylation-mimicking mutations in the *adr1-l2-4* background⁴. We found that the introduction of the T56D S141D S148D or S141D S148D S342D mutations into ADR1-L2 D484V restored a wildtype-like morphology (Figure 3.2). However, in the current generation we could no longer identify accumulation of ADR1-L2 D484V T56D S141D S148D protein, requiring additional validation that phosphorylation-mimicking mutations impair ADR1-L2 D484V autoimmunity (Figure 3.2b).

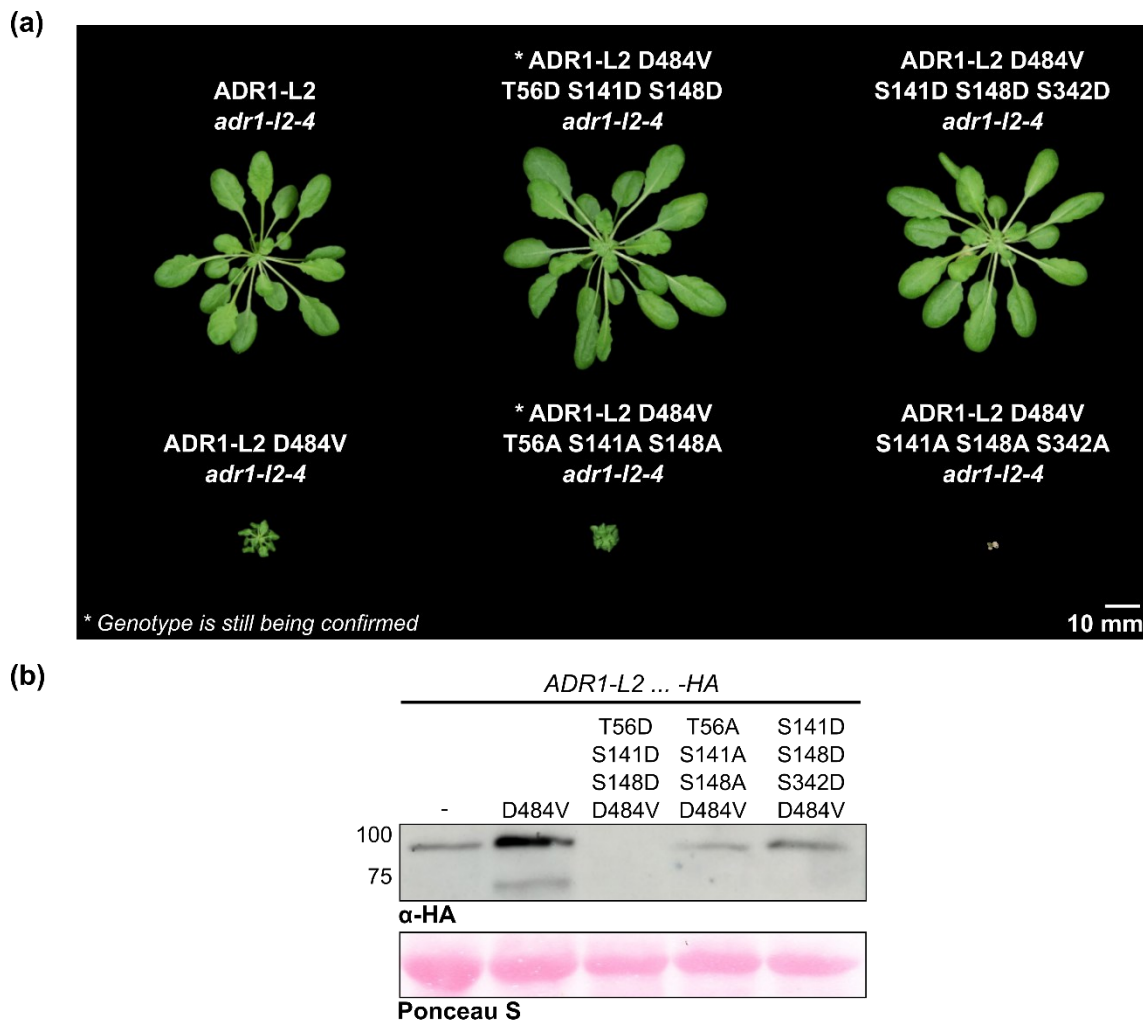


Figure 3.2: The phosphorylation status of our candidate residues in ADR1-L2 D484V affected the autoimmune phenotype in *Arabidopsis*. (a) Autoimmunity caused by the expression of ADR1-L2 D484V under its native promoter was rescued by the introduction of T56D S141D S148D or S141D S148D S342D mutations. Dephosphorylation-mimicking mutations of the same residues did not visibly alter the autoimmune phenotype. Pictures were taken of 4-week-old *Arabidopsis* plants grown under long-day conditions. (continued on the next page)

⁴ Generation, characterization, and imaging of all transgenic *Arabidopsis* lines was done by Frank D. Vogt (ZMBP, University of Tübingen)

(continued) **(b)** Accumulation of the ADR1-L2 protein variants in plants depicted in (a) was confirmed by western blot. Proteins were detected using anti-HA. For ADR1-L2 D484V S141A S148A S342A, the enhanced autoimmune phenotype prevented the collection of sufficient protein samples. The number on the left indicates the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The generation and characterization of these transgenic lines, as well as the images and western blot are courtesy of Frank D. Vogt (Vogt, 2026).

We also generated the equivalent dephosphorylation-mimicking variants (T56A S141A S148A and S141A S148A S342A) in *cis* with ADR1-L2 D484V and observed maintained (T56A S141A S148A) or enhanced (S141A S148A S342A) autoimmunity (Figure 3.2a). Taken together, these observations suggested to us that phosphorylation of one or more of these residues hinders ADR1-L2 auto-activity.

ADR1-L2 alone cannot mediate resistance to *Pseudomonas* in *Arabidopsis*

To investigate the biological function of ADR1-L2 phosphorylation, we wanted to utilize various triggers for PTI and ETI using *Pseudomonas syringae* (*Pst*). The *ADR1* family genes were previously reported to play a major role in basal resistance to *Pst* DC3000 (Bonardi et al., 2011; Saile et al., 2020). ADR1-L2 specifically was previously reported to play a partial role in TNL-mediated (RRS1-RPS4) resistance (Jacob et al., 2023). We used all combinations of *adr1* family mutants (Table S2) to analyse individual roles of *ADR1* genes in mediating resistance against *Pst* DC3000 (basal resistance) and *Pst* DC3000 *AvrRps4* (ETI).

For basal resistance (Figure 3.3a), we found that bacterial growth of *Pst* DC3000 varied significantly among the loss-of-function *adr1* family alleles (Two-Way ANOVA, $F_{7,111} = 22.880$, $p < 2 \times 10^{-16}$). Comparing the colony-forming units (CFU) of *Pst* *AvrRps4* in *adr1-l1-1* (mean $\pm$ standard deviation: $7.448 \pm 0.351 \text{ Log}_{10} \text{ CFU/cm}^2$) to *Col-0* ($6.446 \pm 0.595 \text{ Log}_{10} \text{ CFU/cm}^2$) revealed a significant contribution to basal resistance by *ADR1-L1* (post hoc TukeyHSD, $p = 0.0000121$). For *adr1-l2-4*, a weaker, non-significant trend was observed ($6.889 \pm 0.546 \text{ Log}_{10} \text{ CFU/cm}^2$, $p = 0.264$). Notably, all three double *adr1* family mutants (*adr1-1 adr1-l1-1*, *adr1-1 adr1-l2-4*, and *adr1-l1-1 adr1-l2-4*) were all significantly more susceptible than *Col-0*, resembling the *adr1* triple. Taken together, these findings suggested that basal resistance is primarily mediated by *ADR1-L1* with a minor contribution from *ADR1-L2*, but neither *ADR1-L1* or *ADR1-L2* can mediate full resistance alone.

Bacterial growth of *Pst* *AvrRps4* varied significantly among the *adr1* family loss-of-function alleles (Two-Way ANOVA, $F_{7,111} = 5.391$, $p = 0.0000243$). Consistent with previous reports, the *adr1* triple mutant was more susceptible to *Pst* *AvrRps4* than *Col-0* (Figure 3.3b, post-hoc Tukey HSD, $p = 0.00249$) (Saile et al., 2020). We found that single *adr1* family mutants (*adr1-1*, *adr1-l1-1*, or *adr1-l2-4*) were comparable to *Col-0* in resistance against *Pst* *AvrRps4*. The double *adr1* family mutants showed a weak intermediate resistance phenotype between *Col-0* and the *adr1* triple, but this trend was not statistically significant. We focussed on the role of ADR1-L2 using the *adr1-1 adr1-l1-1* double mutant. This mutant portrayed slightly reduced

bacterial growth compared to the *adr1 triple* mutant (5.679 ± 0.287 and $6.014 \pm 0.477 \text{ Log}_{10} \text{ CFU/cm}^2$, respectively), but this trend was not significant (post-hoc TukeyHSD, $p = 0.247$). Taken together, these findings suggested that under our conditions any combination of two *ADR1* family genes can confer resistance to *Pst AvrRps4*. However, a single *ADR1* family gene appeared insufficient to fully mediate signalling downstream of RRS1/RPS4.

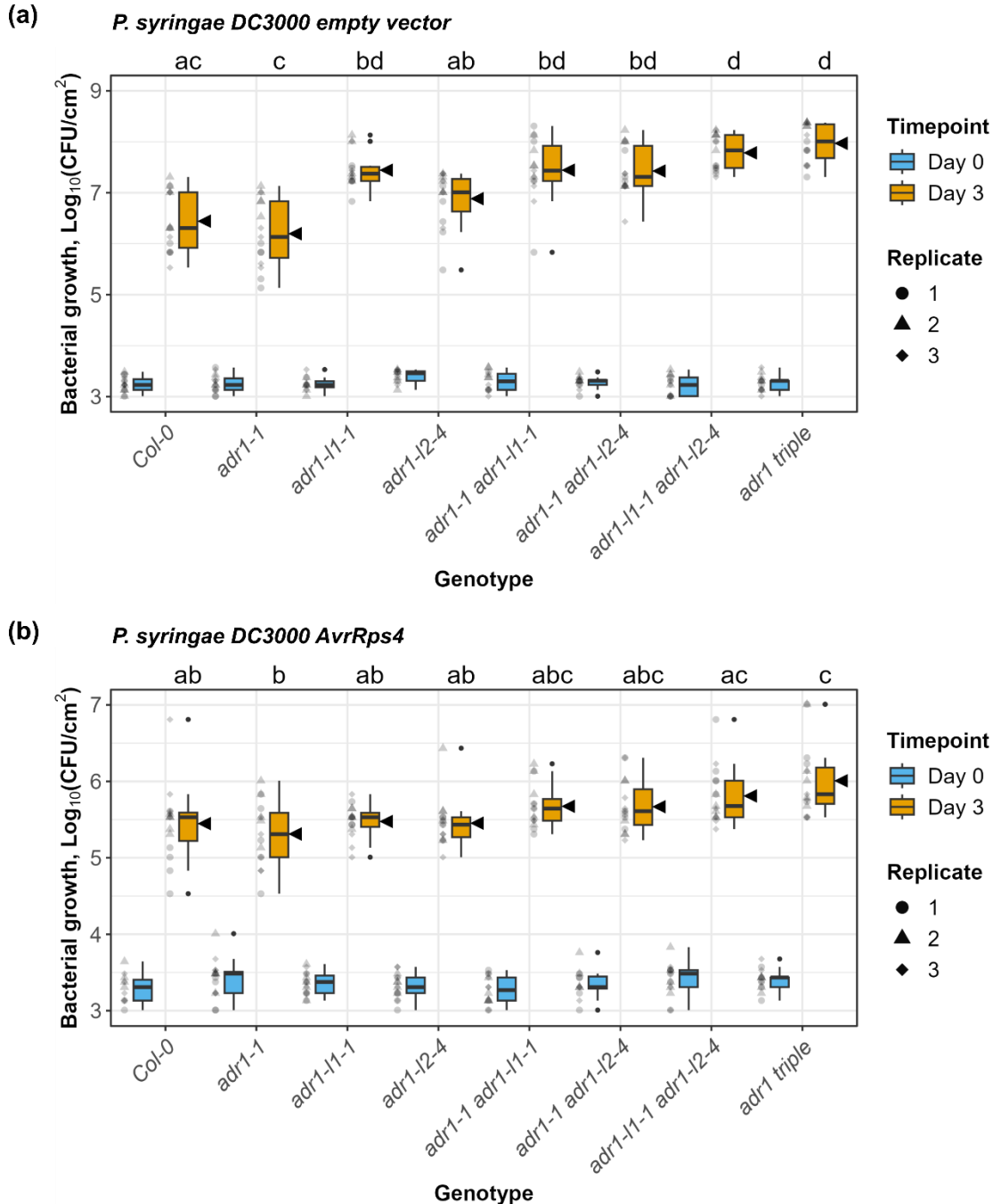


Figure 3.3: ADR1-L2 cannot mediate resistance to *Pst* DC3000 empty vector or *Pst* DC3000 *AvrRps4* alone. All ADR1-family loss-of-function mutants were investigated for resistance to *Pst* DC3000 empty vector (a) or *Pst* DC3000 *AvrRps4* (b). Colony forming unit (CFU) counts per cm² were determined on Day 0 and on Day 3 for all combinations of *adr1* family mutants (Table S2). The arrow (◄) indicates the mean bacterial growth for each genotype. The letters depict the result of statistical testing (Two-Way ANOVA with post-hoc TukeyHSD, $n = 15$). Genotypes sharing the same letter are *not* statistically different in bacterial counts on Day 3.

These observations complicated our efforts to study the effect of phosphorylation on ADR1-L2 in *Arabidopsis*. All transgenic lines we generated were expressing ADR1-L2 phosphorylation mutants in the *adr1 triple* background to study ADR1-L2 in absence of ADR1 and ADR1-L1 (Vogt, 2026). However, now we questioned whether *ADR1-L2* (or any *ADR1* family gene) is sufficient to mediate any commonly studied immune read-outs. To circumvent this issue, we turned towards heterologous transient expression in *N. benthamiana* to study the effect of phosphorylation on ADR1-L2.

Phosphorylation regulates cell death by ADR1-L2 D484V in *N. benthamiana*

To validate the use of *N. benthamiana* transient expression for studying the effect of phosphorylation on ADR1-L2, we focused our efforts on ADR1-L2 phosphorylation mutants that showed a visible phenotype in *Arabidopsis* (Figure 3.2) (Vogt, 2026). ADR1-L2 D484V causes very weak and inconsistent cell death in *N. benthamiana* (Saile et al., 2021). We hypothesized that ADR1-L2 D484V might be regulated by phosphorylation in *N. benthamiana* on the candidate residues that we identified (Figure 3.1a). To test this hypothesis, we transiently expressed ADR1-L2 variants from the same constructs used to create the transgenic *Arabidopsis* lines (Figure 3.2).

We found that both ADR1-L2 D484V T56A S141A S148A (Figure 3.4) and ADR1-L2 D484V S141A S148A S342A (Figure S3.2) depicted a gain-of-function, now causing strong cell death in *N. benthamiana*. The equivalent phosphorylation-mimic triple mutants did not cause cell death. These observations were consistent with our findings on autoimmunity in *Arabidopsis* (Figure 3.2). To further dissect these findings, we generated various combinations of phosphorylation-mimic and dephosphorylation-mimic versions of ADR1-L2 D484V and investigated which of these versions are sufficient to induce a cell death response in *N. benthamiana*. We additionally quantified the cell death intensity in an attempt to capture smaller quantitative effects of phosphorylation on cell death. We identified that ADR1-L2 D484V S148A caused potent cell death (Figure 3.4 and Figure S3.2). Notably, this cell death was lost by the addition of the T56A or S342A *in cis*. This observation suggested to us that the phosphorylation status of T56 or S342 may further regulate the cell death-promoting effect of the S148A mutation. However, we had already identified that the introduction of T56A S141A S148A or S148A S342A into ADR1-L2 D484V caused strong cell death. In both cases, we observed that the addition of S141A appeared to overcome the cell death suppressing effect of T56A or S342A. Notably, ADR1-L2 D484V S141A did not cause cell death, suggesting that dephosphorylation of S141 specifically promoted cell death from ADR1-L2 D484V S148A.

To better understand the functional relationship between (de)phosphorylation of T56 and S148, we generated ADR1-L2 D484V T56D S148A and ADR1-L2 D484V T56A S148D. Based on our previous findings, we expected that ADR1-L2 D484V T56D S148A should induce strong

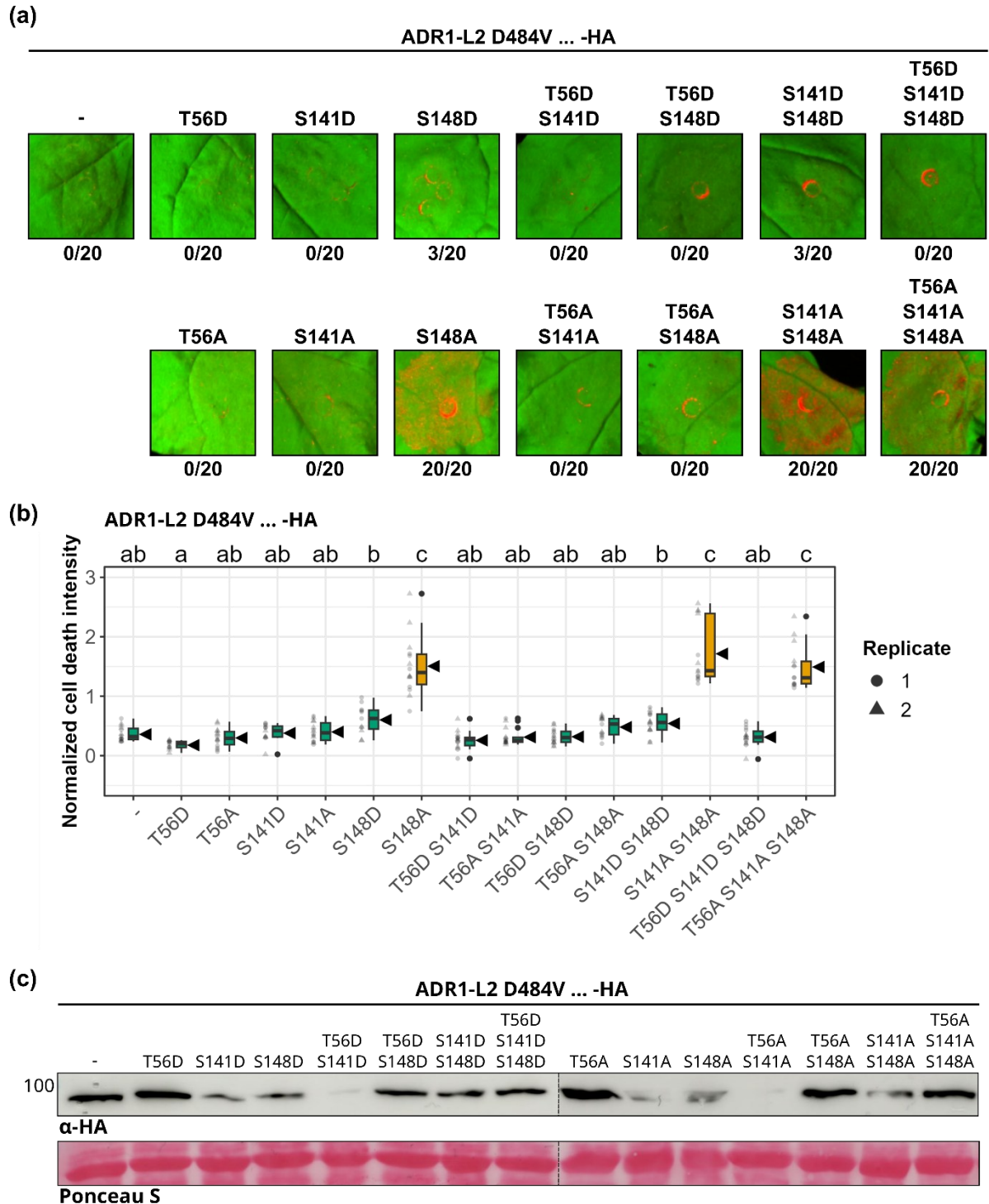


Figure 3.4: The dephosphorylation-mimic ADR1-L2 D484V S148A caused cell death in *N. benthamiana* and was differentially affected by the addition of the T56A or S141A mutation *in cis*. (a) Cell death was investigated for each combination of phosphorylation- or dephosphorylation-mimic of the residues T56, S141, and S148 in ADR1-L2 D484V. *Agrobacterium* infiltrations were done in *N. benthamiana adr1 nrg1*. ADR1-L2 variants were expressed using the *Arabidopsis* ADR1-L2 promoter. Images were taken five days after infiltration. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) Quantification of cell death by pixel intensity for the ADR1-L2 (de)phosphorylation mimicking mutants in (a). Mean pixel intensity was measured and normalized against the background signal. The arrow (◄) indicates the mean cell death intensity for each ADR1-L2 variant. (continued on the next page)

(continued) The letters depict the result of statistical testing (Two-Way ANOVA with post-hoc TukeyHSD, $n = 20$). Genotypes sharing the same letter are *not* statistically different in cell death intensity. The **orange** colour indicates a significant difference from ADR1-L2 D484V. **(c)** Protein accumulation of all ADR1-L2 variants in (a) was assessed by western blot. Proteins were detected using anti-HA. The number on the left indicates the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The dotted line indicates where digital stitching of membranes occurred in the presented western blots.

cell death, since the T56A mutation negatively affected the gain-of-function from the S148A mutation *in cis* (Figure 3.4). In contrast to our expectation, we observed weak cell death for both ADR1-L2 D484V variants (Figure S3.3). Quantification of cell death using pixel intensity and comparing the result to previous experiments confirmed that the strength of the cell death was weaker than ADR1-L2 D484V S148A. In this particular analysis, we observed that ADR1-L2 D484V S148D had a slight but statistically significant increase in cell death intensity compared to ADR1-L2 D484V (Figure S3.3b). The strength of cell death observed for ADR1-L2 D484V T56D S148A and T56A S148D rather resembled that of ADR1-L2 D484V S148D alone.

Altogether, our results indicated that cell death by auto-activated ADR1-L2 is indeed regulated by phosphorylation. We could show that the candidate residues we identified are directly involved in the regulation of ADR1-L2 D484V. Moreover, we uncovered complex functional relationships between the phosphorylation status of these residues.

ADR1-L2 cannot mediate XopQ-triggered cell death in *N. benthamiana*

The auto-activating D484V mutation is a rather artificial way to study ADR1-L2 function post-activation. To complement the previous findings with auto-active ADR1-L2, we continued to study the effect of phosphorylation in a more biologically relevant setting using effector-triggered cell death in *N. benthamiana*. The effector XopQ from *Xanthomonas* is recognized by the TNL ‘recognition of XopQ 1’ (Roq1) in *N. benthamiana* and relies on the NRG1-EDS1-SAG101b node for signalling and cell death (Lapin et al., 2019; Prautsch et al., 2023; T. Qi et al., 2018; Schultink et al., 2017). However, the *N. benthamiana* *adr1 nrg1* or *eds1 pad4 sag101a/b* mutants can be complemented by expressing *Arabidopsis* ADR1-L1, EDS1, and PAD4 to restore the XopQ-triggered cell death (H. Yu et al., 2024). Unlike ADR1-L1, ADR1-L2 lacks the ability to cause cell death after NbRoq1 activation by XopQ (Lapin et al., 2019).

We asked whether phosphorylation mutants that could rescue the cell death from ADR1-L2 D484V, could also rescue the cell death after XopQ perception by Roq1. We focused on the most promising candidate residues based on our findings from ADR1-L2 D484V variants (Figure 3.4). We then complemented *N. benthamiana* *eds1 pad4 sag101a sag101b* with variants of *Arabidopsis* ADR1-L2 along with EDS1/PAD4, and co-expressed XopQ (Lapin et al., 2019). ADR1-L1 was included as a positive control for XopQ-triggered cell death as previously reported (H. Yu et al., 2024). Indeed, we could confirm that ADR1-L1 induced potent

cell death upon co-expression with XopQ, but ADR1-L2 was unable to mediate XopQ-triggered cell death (Figure 3.5). The lack of cell death from ADR1-L2 was not rescued by any of the (de)phosphorylation-mimicking mutants in response to XopQ, contrary to the findings on autoactivated ADR1-L2 (Figure 3.4). These findings suggested that the inability of ADR1-L2 to signal downstream of Roq1 activation is not due to inhibition by phosphorylation of the tested residues.

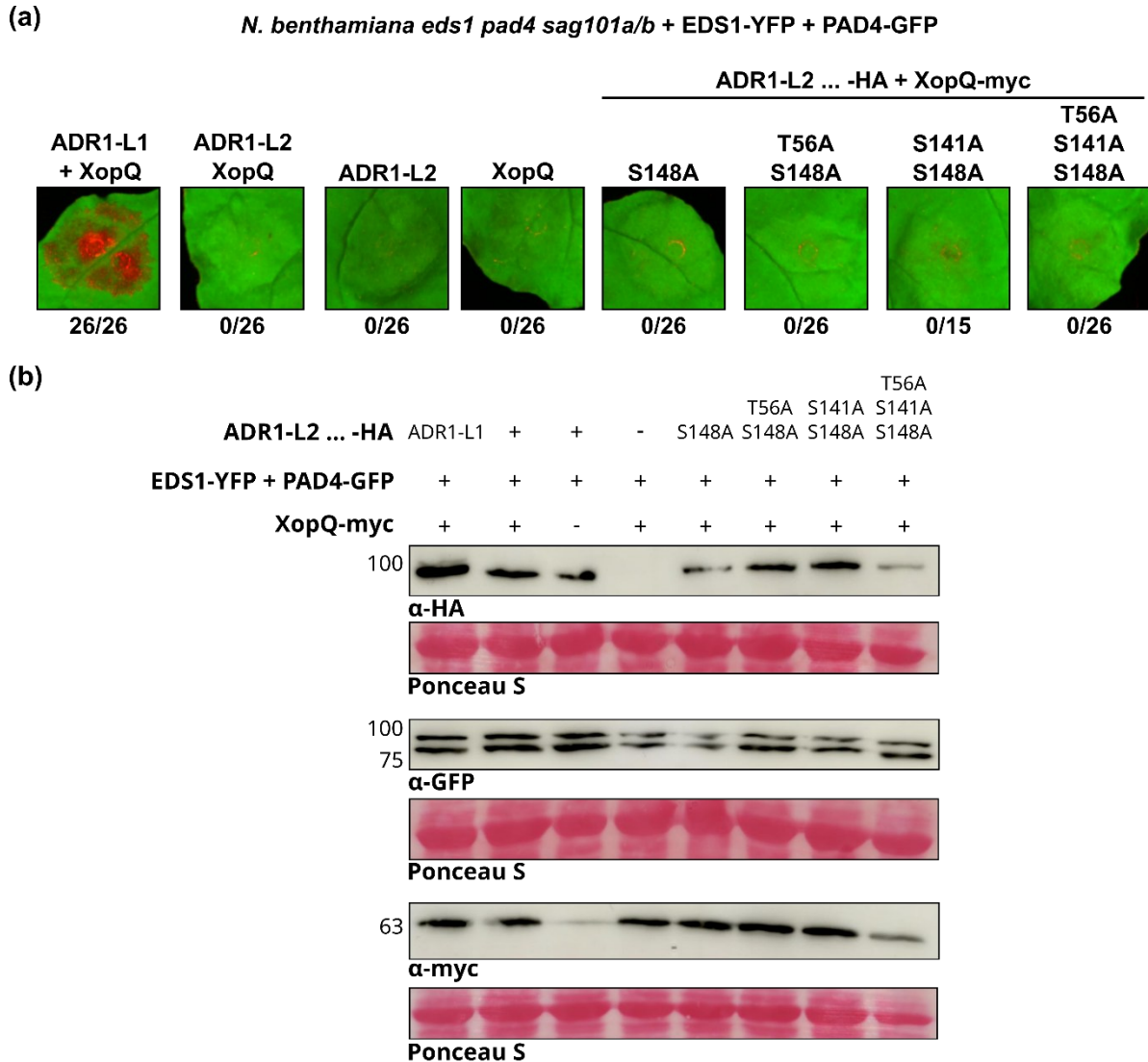


Figure 3.5: ADR1-L2 is unable to mediate XopQ-triggered cell death in *N. benthamiana*. (a) XopQ recognition by *N. benthamiana* Roq1 did not induce cell death through *Arabidopsis* ADR1-L2. *N. benthamiana eds1 pad4 sag101a sag101b* was complemented with *Arabidopsis* EDS1, PAD4 and ADR1-L2 variants. Cell death was induced by expression of XopQ. *Arabidopsis* ADR1-L1 was included as a positive control. ADR1-L1 and ADR1-L2 variants were expressed under their native *Arabidopsis* promoter. EDS1, PAD4 and XopQ were expressed under a 35S promoter. Images were taken five days after infiltration. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) Protein accumulation of combinations depicted in (a) was confirmed by western blot. RNLs were detected with anti-HA, EDS1 and PAD4 with anti-GFP, and XopQ with anti-myc. The numbers on the left indicated the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control.

ADR1-L2 S148 is conserved in ADR1 and ADR1-L1 with unknown function

So far, our investigation of the function of RNL phosphorylation was limited by the lack of biological phenotypes mediated by ADR1-L2 alone (Figure 3.3, Figure 3.5). To circumvent these issues, we assessed whether our findings could be translated over to the closely related ADR1 and ADR1-L1 from *Arabidopsis*. We had already shown that ADR1-L1, unlike ADR1-L2, is functional in XopQ-triggered cell death in *N. benthamiana* (Figure 3.5), consistent with previous reports (Lapin et al., 2019; H. Yu et al., 2024). So, we assessed whether we could utilize ADR1 or ADR1-L1 to study the effects of phosphorylation on RNL function using orthologous residues. Protein sequence alignment revealed that the ADR1-L2 S148 residue is conserved in ADR1 (S147) and ADR1-L1 (S150) (Figure S3.4a). In AlphaFold3 predicted structures, the position of these residues appeared less conserved due to different orientations of the CC domains (Figure S3.4b and Figure S3.5a, b). We attempted to resolve this issue by modelling only the CC domains (amino acid 1-160) of each ADR1 family member. While the alpha helix bundles of the CC domains are highly structurally conserved, the orientation of the C-terminal end of the CC domain remained different between the proteins (Figure S3.4c and Figure S3.5c-e). Nevertheless, this serine was always positioned towards the end of the CC domain, near the start of the NB domain. Additionally, the IP-MS dataset that supported the phosphorylation of ADR1-L2 S141 (Figure 3.1) also identified phosphorylation of ADR1-L1 S131 (Umezawa et al., 2013), so we decided to also include this residue in our investigation.

Based on our findings from ADR1-L2 D484V (Figure 3.4 and Figure S3.2), we hypothesized that phosphorylation on ADR1 S147 and ADR1-L1 S150 should negatively impact cell death activity. For ADR1-L1 S131 we had no prior evidence that hinted towards its potential role in ADR1-L1 function. For each of these residues in ADR1 and ADR1-L1, we generated phosphorylation-mimic and dephosphorylation-mimic versions and assessed the effect on XopQ-triggered cell death. For ADR1, we observed strong cell death upon co-expression with *EDS1*, *PAD4*, and *XopQ* in *N. benthamiana* (Figure 3.6a). Despite reduced accumulation of *EDS1* and *PAD4* (Figure S3.6), both ADR1 S147D and ADR1 S147A were not impaired in XopQ-triggered cell death. ADR1-L1 also caused potent cell death after XopQ recognition by *N. benthamiana* Roq1, consistent with previous reports (H. Yu et al., 2024). Neither ADR-L1 S150D or ADR1-L1 S150A were impaired in XopQ-triggered cell death (Figure 3.6b). ADR1-L1 S131D and ADR1-L1 S131A also showed comparable cell death to wildtype ADR1-L1. We additionally generated combinatorial phosphorylation mutants for ADR1-L1 S131 and S150, like we did for ADR1-L2 T56 and S148 (Figure S3.3). However, induction of cell death was unaffected by any combination of S131D/A and S150D/A. All in all, we observed a sequence-level conservation of ADR1-L2 S148 in ADR1 and ADR1-L1 but could not identify a clear effect on cell death by (de)phosphorylation on ADR1 S147 or ADR1-L1 S150.

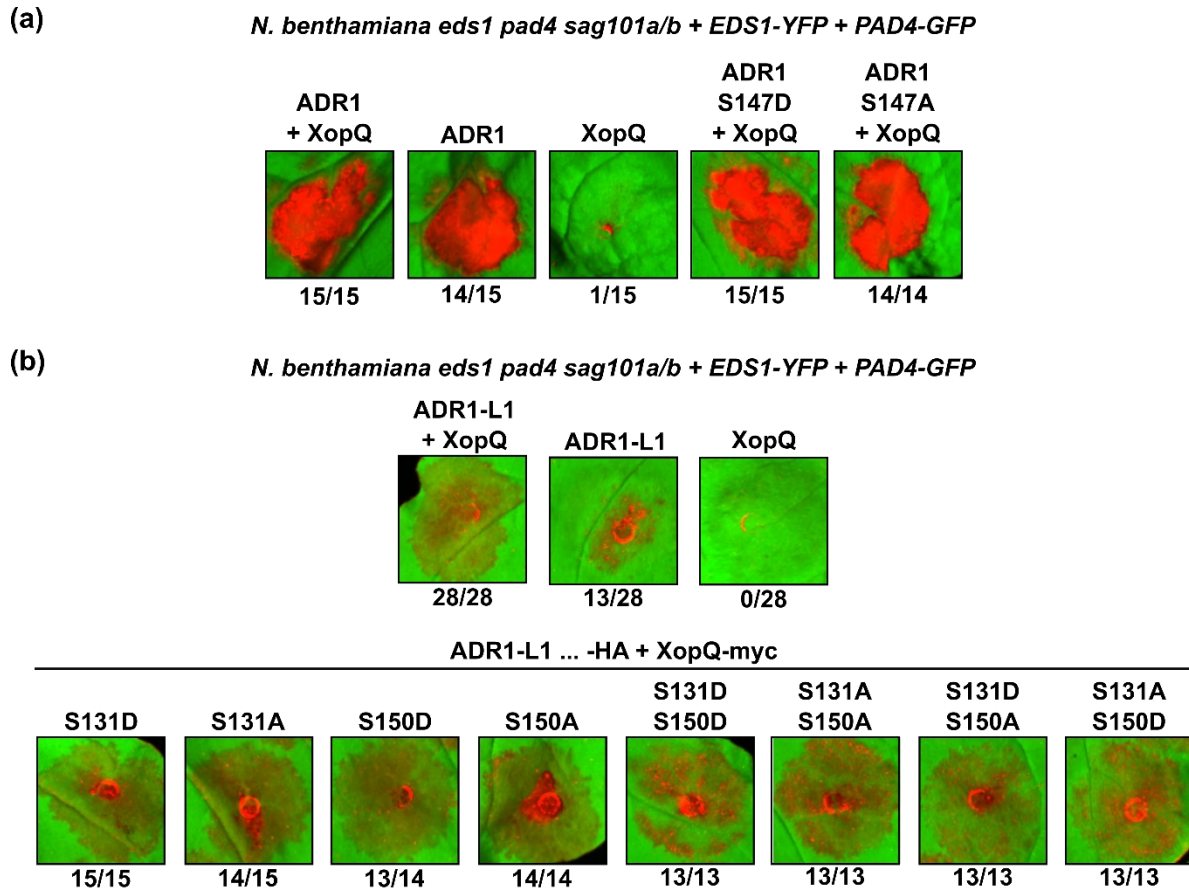


Figure 3.6: (De)phosphorylation mimicking mutations in ADR1 and ADR1-L1 had no visible effect on XopQ-triggered cell death. XopQ-triggered cell death induced by ADR1 (a) and ADR1-L1 (b) variants co-expressed with *Arabidopsis* EDS1 and PAD4 in *N. benthamiana eds1 pad4 sag101a/b*. ADR1 and ADR1-L1 variants were expressed under their native *Arabidopsis* promoters. EDS1, PAD4 and XopQ were expressed under a 35S promoter. Images were taken five days after infiltration. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. Expression of all ADR1 and ADR1-L1 variants was confirmed by western blot (Figure S3.6).

HopQ1 resistance may be regulated by phosphorylation of ADR1-L2

Cell death is commonly used as a read-out for NLR function in *N. benthamiana*, but perhaps for ADR1-L2 this is not the correct measure. In the literature, ADR1-family genes from *Arabidopsis* are frequently suggested to induce resistance to pathogens as their primary function, and cell death as a secondary function (Dongus & Parker, 2021; Lapin et al., 2019). The auto-active cell death observed after expression of ADR1 or ADR1-L1 when co-expressed with EDS1/PAD4 would be problematic for studying resistance against pathogens (Figure 3.6). So, we decided to omit ADR1 and ADR1-L1 from further investigation and continued only with ADR1-L2. The model bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 carries the effector HopQ1, which is also recognized by the TNL NbRoq1 (Schultink et al., 2017). To assess whether ADR1-L2 can initiate resistance against pathogens that trigger a TNL, we designed an 'Agromonas' assay (Figure 3.7a) (Buscaill et al., 2021). We reconstituted the EDS1-PAD4 node together with ADR1-L2 (de)phosphorylation mutants transiently in *N.*

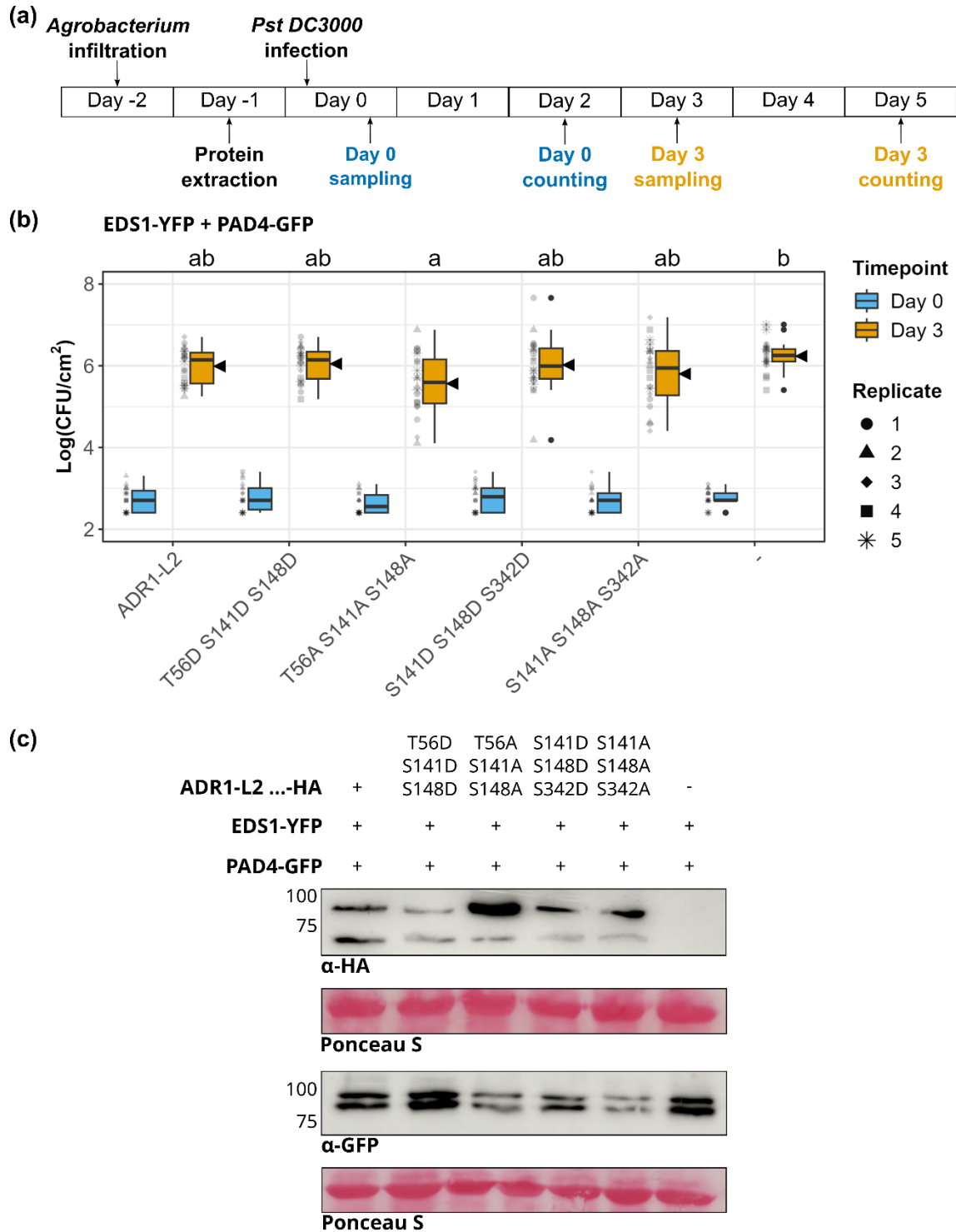


Figure 3.7: Resistance to *Pst* DC3000 was weakly enhanced by the dephosphorylation mimicking ADR1-L2 T56A S141A S148A variant. (a) Experimental design for the ‘*Agromonas*’ assay adapted from Buscaill et al. (2021). Transient expression of ADR1-L2 variants together with EDS1 and PAD4 in *N. benthamiana eds1 pad4 sag101a/b* was initiated 48 hours prior to *Pst* DC3000 infection (OD = 0.0001). (b) De-phosphorylation mimicking mutations in ADR1-L2 were slightly increased resistance to *Pst* DC3000 in *Agromonas* assays. ADR1-L2 variants were expressed using the native ADR1-L2 promoter. EDS1, PAD4 and XopQ were expressed under a 35S promoter. Colony forming unit (CFU) counts per cm² were determined on Day 0 and on Day 3 for all combinations. The arrow (◀) indicates the mean bacterial growth for each genotype. The letters depict the result of statistical testing (Two-Way ANOVA with post-hoc TukeyHSD, n = 20). (continued on the next page)

(continued) Variants sharing the same letter are not statistically different in bacterial growth on **Day 3**. (c) Protein accumulation of ADR1-L2 variants, EDS1, and PAD4 during the *Agromonas* assay was confirmed by western blot. ADR1-L2 variants were detected with anti-HA, EDS1 and PAD4 with anti-GFP. The numbers on the left indicated the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control.

benthamiana eds1 pad4 sag101a/b adr1 nrg1 mutants (J. Wang, Sun, et al., 2025). Based on CFU counting, we identified a significant contribution of the infiltrated combination to resistance (Two-way ANOVA, $F_{5, 112} = 2.818$, $p = 0.0195$). However, we found that there was no statistical difference between the ADR1-L2 variants (Figure 3.7b). We did observe that specifically ADR1-L2 T56A S141A S148A (mean $\pm$ standard deviation: $5.569 \pm 0.758 \text{ Log}_{10} \text{ CFU/cm}^2$) was significantly elevated in resistance to *Pst DC3000* compared to the negative control of EDS1/PAD4 alone ($6.240 \pm 0.381 \text{ Log}_{10} \text{ CFU/cm}^2$, post-hoc TukeyHSD, $p = 0.011$). ADR1-L2 S141A S148A S342A portrayed a similar trend but was not statistically significant ($5.811 \pm 0.790 \text{ Log}_{10} \text{ CFU/cm}^2$, post-hoc TukeyHSD, $p = 0.251$). Although not definitive, this observed trend matched our observations regarding ADR1-L2 cell death (Figure 3.4). Taken together, these findings point towards the phosphorylation status of T56, S141, and S148 as a key regulatory mechanism of ADR1-L2 function.

Plasma membrane localization is unaltered by phosphorylation of ADR1-L2

Next, we wondered how phosphorylation was directly affecting the protein function of ADR1-L2. A common feature of *Arabidopsis* RNLs is their localization and function at the plasma-membrane (Saile et al., 2021; Z. Wang et al., 2023). ADR1-L2 was previously shown to localize at the plasma membrane when transiently expressed in *N. benthamiana*. (Saile et al., 2021). This localization is mediated by interactions between specific negatively charged phospholipids and positively charged amino acid residues in the CC domain of ADR1-L2 (Saile et al., 2021; Z. Wang et al., 2023). We set out to investigate whether phosphorylation of the candidate residues affected the steady-state localization of ADR1-L2, and thereby impacting its function. We generated YFP-tagged versions of the ADR1-L2 variants from our initial findings in *Arabidopsis* (Figure 3.2). We opted not to introduce the autoactivating D484V mutation to prevent any complications from cell death during confocal microscopy imaging.

Expression of ADR1-L2 driven by its native promoter was low and required high laser intensity to capture fluorescent signal. Additionally, these results are preliminary as we have not yet performed additional replicates of these findings. Nevertheless, we could confirm that ADR1-L2-YFP indeed co-localized with the plasma-membrane marker Lti6b-RFP (Figure 3.8a) (Saile et al., 2021). This localization was not affected by phosphorylation or dephosphorylation mimicking mutations. Despite the low expression level, we made an additional observation that stomatal guard cells often portrayed strong accumulation of

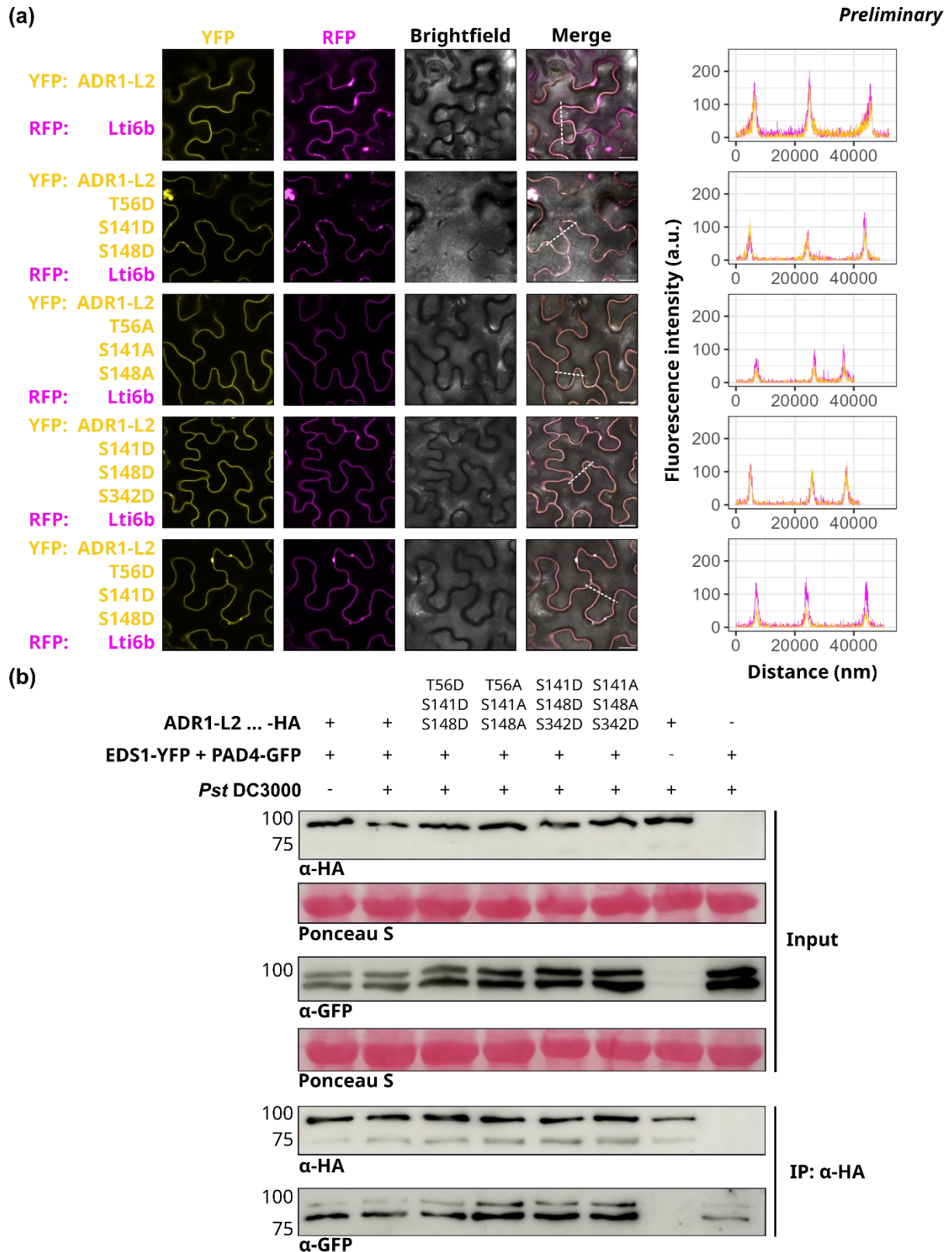


Figure 3.8: (Preliminary) The phosphorylation status of ADR1-L2 did not affect plasma membrane localization or association with EDS1-PAD4. (a) Plasma membrane localization of ADR1-L2 was unaffected by (de)phosphorylation mimicking mutations of T56, S141, S148, and S342. ADR1-L2 variants (**YFP**) co-localized with the plasma membrane marker Lti6b (**RFP**) in *N. benthamiana*. Fluorescence intensities (in arbitrary units) were measured across the dotted line in the merged images and depicted as a line graph to show co-localization. (continued on the next page)

(continued) Scale bars in the merged images indicate 20 μm . **(b)** All tested ADR1-L2 (de)phosphorylation mimics still associated with EDS1 and PAD4 in co-immunoprecipitation. *Pst DC3000* was used as an elicitor for the association of ADR1-L2 with EDS1/PAD4, as reported by H. Wang, Tan, et al. (2024). The negative control (no ADR1-L2-HA expression) showed weak but visible immunoprecipitation of EDS1/PAD4, suggesting that the HA agarose beads showed unspecific binding under our conditions. ADR1-L2 variants were detected with anti-HA, EDS1 and PAD4 with anti-GFP. The numbers on the left indicate the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control.

ADR1-L2-YFP signal (Figure S3.7). Taken together, we found that phosphorylation of ADR1-L2 did not impact or alter the steady-state localization of ADR1-L2 in *N. benthamiana*.

Phosphorylation of ADR1-L2 did not affect the association with EDS1-PAD4

RNLs require the lipase-like proteins EDS1 and PAD4/SAG101 to trigger full immunity (T. Qi et al., 2018; Z. Wu et al., 2019, 2021). The ADR1 family, including ADR1-L2, specifically associates with EDS1-PAD4 after binding pRib-AMP/pRib-ADP (S. Huang et al., 2022, p. 2022; Jia et al., 2022; Wagner et al., 2013; H. Yu et al., 2024). Given this requirement for the function of ADR1 family proteins, we wondered whether phosphorylation may affect the association with EDS1-PAD4. In *N. benthamiana*, ADR1-L2 was reported to associate with EDS1-PAD4 after bacterial infection (H. Wang, Tan, et al., 2024). So, we performed co-immunoprecipitation (co-IP) of EDS1 and PAD4 with ADR1-L2, using *Pst DC3000* as an elicitor of immunity.

Contrary to previous reports, we found that ADR1-L2 associated with EDS1 and PAD4 prior to bacterial infection in *N. benthamiana* (Figure 3.8b). We also did not observe an increase in association after bacterial elicitation. Moreover, all (de)phosphorylation mimicking variants of ADR1-L2 could still associate with EDS1-PAD4, similar to wildtype ADR1-L2. Notably, we observed weaker but visible immunoprecipitation of EDS1-YFP/PAD4-GFP in the negative control (without ADR1-L2-HA expression) across two replicates. Although the detection of EDS1-PAD4 after co-IP with ADR1-L2 was much stronger, the findings presented here may be false-positive under our current conditions. At the time of writing, no additional replications have been performed. Taken together, we cautiously conclude that the association of ADR1-L2 with EDS1-PAD4 is unaffected by phosphorylation of our candidate residues.

Discussion

In this chapter, we identified potential phosphorylated residues in *Arabidopsis* ADR1-L2 and started to characterize the effect of (de)phosphorylation on T56, S141, S148, and S342. We could show that phosphorylation mimicking mutations of these residues could attenuate the autoimmunity caused by expression of *ADR1-L2 D484V* (Figure 3.2). The importance of these residues was corroborated by transient expression in *N. benthamiana*. Introducing the T56A S141A S148A (Figure 3.4) or S141A S148A S342A (Figure S3.2) mutations into ADR1-L2 D484V caused potent cell death. We identified S148 as the main contributor to cell death but simultaneously uncovered a complex regulatory system between our candidate residues. However, none of our ADR1-L2 variants were able to mediate XopQ-triggered cell death (Figure 3.5). Despite the lack of cell death, resistance to *Pst DC3000* was weakly enhanced by dephosphorylation mimicking mutations (Figure 3.7). How phosphorylation is mechanistically affecting ADR1-L2 function is currently not known, as (de)phosphorylation mutants of ADR1-L2 were unaffected in their steady-state localization and association to EDS1-PAD4 (Figure 3.8). We additionally found that S148 is relatively conserved in *Arabidopsis* ADR1 (S147) and ADR1-L1 (S150) but mutating these orthologous residues did not affect the cell death activity of ADR1 or ADR1-L1 (Figure 3.6 and Figure S3.4).

Limitations and challenges of studying *Arabidopsis* ADR1-L2

Although we study *Arabidopsis* RNLs, most of our research described here was performed in *N. benthamiana*. Transient expression in *N. benthamiana* had large benefits in terms of speed but also circumvented additional issues we identified in *Arabidopsis*. Under our conditions, we found that ADR1-L2 alone is insufficient to mediate resistance to commonly used infection assays with *Pst DC3000* or *Pst AvrRps4* (Figure 3.3). This contradicts published results where a partial contribution of ADR1-L2 in RPS4-mediated immunity was observed (Jacob et al., 2023). Growth conditions for infection assays may vary between labs, and some may slightly favour bacterial growth more so than others. More statistical power (higher number of replicates) may aid in the reproducibility between labs. Nevertheless, these findings under our growth conditions complicated the use of transgenic lines expressing (de)phosphorylation mimicking variants of ADR1-L2 in the *adr1 triple* background, as ADR1-L2 alone would not rescue the resistance phenotypes. In this work, we circumvented this problem by studying the potential effect(s) of (de)phosphorylated residues in ADR1-L2 using transient expression in *N. benthamiana*. However, the role of phosphorylation on ADR1-L2 activity may still be further investigated using transgenic *Arabidopsis* plants expressing (de)phosphorylation mimicking variants of ADR1-L2.

The *ADR1* family was reported to function in stomatal immunity (H. Wang, Song, et al., 2024). In our *Pst* infections (Figure 3.3), we performed syringe infiltrations which largely

circumvent stomatal immunity by directly introducing the bacterial inoculum into the apoplast. Instead, spray or dipping infections with *Pst* could be performed. Indeed, a minor role of ADR1-L2 was previously observed in dipping infections with *Pseudomonas* and could be further investigated using ADR1-L2 phosphorylation mutants (Bonardi et al., 2011). In the same study, ADR1-L2 was also shown to contribute partially to resistance against the oomycete pathogen *Hyaloperonospora arabidopsidis*. Alternatively, the role of ADR1-L2 in (RLP-dependent) priming of the immune system could be assessed (Pruitt, Locci, et al., 2021). Preliminary results suggest that ADR1-L2 might be partially capable of mediating defence priming alone (Y. Liu, 2026). Cell death by RNLs is primarily attributed to the *NRG1* family whereas disease resistance is more dependent on the *ADR1* family (Lapin et al., 2019). Nevertheless, the *ADR1* family is capable of mediating cell death in certain circumstances (Pierdzig et al., 2025; Roberts et al., 2013; Z. Wu et al., 2019). Our (de)phosphorylation mutants of ADR1-L2 could be further investigated in *Arabidopsis* for known ADR1-dependent cell death phenotypes.

Dynamic regulation of ADR1-L2 by phosphorylation on multiple residues

In *N. benthamiana*, ADR1 and ADR1-L1 cause potent cell death upon transient overexpression of auto-active variants (Figure 2.4) or upon XopQ-triggered immunity (Figure 3.6) (H. Yu et al., 2024). ADR1-L2 appeared to be the outlier, not causing reliable cell death after introduction of the D484V mutation (Figure 3.4 and Figure S3.2) or when co-expressed with XopQ (Figure 3.5) (Lapin et al., 2019). We could show that cell death from ADR1-L2 D484V can be restored by introducing dephosphorylation mimicking mutations on S148 (Figure 3.4 and Figure S3.2). However, XopQ-triggered cell death could not be gained through the introduction of the cell death enhancing S148A mutation (Figure 3.5). This suggested that S148A could promote auto-activated cell death but not promote the activation by TNL-triggered immunity (Roq1). Phosphorylation was previously reported as a regulator of NLR autoinhibition (H. Guo et al., 2020). Perhaps phosphorylation of a different residue in ADR1-L2 or a secondary regulatory mechanism is modulating autoinhibition and thereby preventing activation through TNL signalling. The D-to-V mutation circumvents the requirements of 'canonical' activation by preferentially binding ATP, thereby becoming auto-activated (Tameling et al., 2006; Williams et al., 2011).

Further investigation of our candidate phosphorylation sites in ADR1-L2 revealed dynamic regulation of cell death activity by the phosphorylation status of T56 and S148 (Figure 3.4 and Figure S3.3). Based on our initial finding that the cell death from ADR1-L2 D484V S148A is suppressed by addition of the T56A mutation, we hypothesized that ADR1-L2 D484V T56D S148A should cause stronger cell death. Inversely, we reasoned that T56A S148D should not cause cell death. Instead, we found that both T56D S148A and T56A S148D caused mild cell death in *N. benthamiana*. One hypothesis is that the phosphorylation-mimicking mutations are

not fully resembling a phosphorylated Serine/Threonine, as previously shown in other contexts (Kozeleková et al., 2022; Paleologou et al., 2008). And indeed, we observed a marginal (sometimes statistically significant) increase in cell death intensity for ADR1-L2 D484V S148D as well (Figure 3.4, Figure S3.3, Figure S3.2). An alternative hypothesis is that the phosphorylation status of ADR1-L2 T56 and S148 may act like a logic XOR gate. We observed that phosphorylation or de-phosphorylation of *both* residues led to impaired cell death (Figure 3.4) and so phosphorylation of either T56 or S148 would cause (mild) cell death (Figure S3.3). Our findings from single residue mutations do not fully support this hypothesis, since the (de)phosphorylation mimicking mutations of T56 in ADR1-L2 D484V did cause cell death (Figure 3.4 and Figure S3.2). However, the unknown phosphorylation status of ADR1-L2 S148 (and other residues) introduced ambiguity, preventing us from drawing concrete conclusions from these observations. Nevertheless, our findings supported the idea that the phosphorylation status of multiple amino acid residues modulates the activity of *Arabidopsis* ADR1-L2. The resulting 'phosphorylation code' would enable a single kinase/phosphatase to alter the activity of ADR1-L2 while also allowing for the integration of various signals into a single protein (H. Lee, Ganguly, et al., 2021; Y. Sun et al., 2025; W. J. Zhang et al., 2023). How the phosphorylation status of these various residues can be integrated into the function of ADR1-L2 is not yet known. First, the effect of T56 and S148 (de)phosphorylation mimics on ADR1-L2 (auto-)activity should be further investigated in *Arabidopsis*.

To add another layer of complexity, we identified ADR1-L2 S141 as an additional modulator of cell death in the context of phosphorylation. Neither ADR1-L2 D484V T56A S148A nor ADR1-L2 D484V S148A S342A caused cell death in *N. benthamiana* (Figure 3.4 and Figure S3.2). However, addition of the S141A mutation into these ADR1-L2 D484V variants restored the cell death gained from the S148A mutation. S141A alone did not induce cell death, suggesting that this effect required the cell death initiating effect from S148A. We did not yet study the full role of S141 in the context of the proposed T56 S148 XOR gate. The combination of T56 and S141 (de)phosphorylation mimicking mutations also requires additional investigation, as we observed poor protein accumulation ADR1-L2 D484V T56D S141D and ADR1-L2 D484V T56A S141A (Figure 3.4).

The role of S342, which was recently identified as a candidate for phosphorylation by BIK1, also requires additional investigation (Toth et al., 2026). The generation of the ADR1-L2 D484V T56A S141A S148A S342A quadruple mutant could be relevant to investigate whether the outcome of cell death is simply an addition of positive and negative regulatory signals. Taken together, our results pointed towards a complex regulatory system of ADR1-L2 that can potentially be dynamically regulated by multiple kinases and phosphatases. Extending this hypothesis over a multitude of potential phosphorylation sites (Table S3.1), we can envision how signals from multiple sources can activate a fine-tuned outcome in terms of resistance

and cell death. Throughout all transient expression assays in *N. benthamiana* presented in this chapter, the phosphorylation status of non-mutated residues in ADR1-L2 is uncertain. Phosphorylation by kinases in *N. benthamiana* might have affected our results in unforeseen ways (Table S3.1). Continued investigation into the phosphorylation on ADR1-L2 should consider comparative approaches between unchallenged and challenged plants to identify *when* specific residues are phosphorylated in *N. benthamiana* and in *Arabidopsis*.

Regulation by phosphorylation may have diverged in the ADR1 family

Due to the complicated nature of working with ADR1-L2, we assessed whether our findings on ADR1-L2 S148 translated over to orthologous residues in ADR1 (S147) and ADR1-L1 (S150) (Figure S3.4). Both ADR1 and ADR1-L1 could mediate XopQ-triggered cell death in *N. benthamiana* upon co-expression of *Arabidopsis* *EDS1* and *PAD4* (Figure 3.6). We found that ADR1 S147 and ADR1-L1 S150 (de)phosphorylation variants could still cause cell death comparable to wildtype versions. Notably, only auto-active cell death by ADR1-L2 D484V could be restored by dephosphorylation mimicking mutations (Figure 3.4 and Figure S3.2), but XopQ-triggered cell death by ADR1-L2 could not be gained by those same mutations (Figure 3.5). The conditions used for the XopQ cell death assay were optimized for ADR1-L2 and not altered/optimized for ADR1 and ADR1-L1. Cell death from ADR1 and ADR1-L1 in response to XopQ was generally stronger (and potentially faster) than cell death from ADR1-L2 D484V variants. Thus, five days after infiltration, smaller quantitative effects of a (de)phosphorylation mimicking mutation may not be detectable. Instead, conductivity measurements or calcium reporters may be more suitable to study cell death in a quantitative manner, rather than the qualitative method used here (DeFalco et al., 2017).

So far, we identified up to 18 potential phosphorylation sites on ADR1-L2 in *N. benthamiana* that could all affect its function (Table S3.1). Besides ADR1-L2 S148, we found several additional residues (T36, T44, S51) that are conserved in the CC domain of all ADR1 genes. These residues could be of additional interest as potentially conserved regulatory mechanism across the ADR1 family and may warrant further investigation. Other residues do not appear entirely conserved among the ADR1s and could underpin divergence in the function of *ADR1* family genes. ADR1-L2 T56 was of particular interest to us and only appeared sequentially conserved in ADR1 (S59) but not in ADR1-L1 (Figure S3.4). Notably, ADR1-L2 S342 was predicted to be phosphorylated by BIK1, but ADR1 and ADR1-L1 were not identified as candidate substrates (Toth et al., 2026). In other NLRs, phosphorylation by BIK1 was shown to inhibit oligomerization, thereby modulating their function. The role of phosphorylation on ADR1-L2 S342 is not yet known. Here, we identified that the introduction of the S342A mutation into ADR1-L2 D484V S148A did not cause cell death, unlike ADR1-L2 D484V S148A (Figure S3.2). This suggested that phosphorylation on S342 might promote cell

death, but we did not observe cell death for ADR1-L2 D484V S342D. Potentially, S342D functions similar to S141A and enhances cell death of ADR1-L2. To investigate this, we could introduce the S342D mutation *in cis* with S148A or T56A S148A and assess the strength of the cell death.

Phosphorylation affects ADR1-L2 activity through unknown mechanisms

Phosphorylation of NLRs can inhibit or promote NLR activation and immune signalling function through various mechanisms (H. Guo et al., 2020; Toth et al., 2026; Y. Yan et al., 2025; Zhong et al., 2025). We were not yet able to determine how the phosphorylation status of our candidate residues alters the function of *Arabidopsis* ADR1-L2. In this work, we started to investigate how the activity of ADR1-L2 were disrupted in (de)phosphorylation mutants of our candidate residues. Plasma membrane localization relies on several positive charges in the CC domain that interact with negatively charged lipids in the plasma membrane (Saile et al., 2021; Z. Wang et al., 2023). Phosphorylation introduces additional negative charges to the amino acid residue, which we hypothesized may affect membrane association. However, we did not observe a difference in pre-activation localization between the (de)phosphorylation mimicking variants of ADR1-L2 (Figure 3.8a). However, this data is preliminary (repeated once), and we ran into some additional problems with the expression level of ADR1-L2-YFP driven by its native promoter for confocal microscopy. High laser intensity was required to visualize the fluorescence from ADR1-L2-YFP after transient expression in *N. benthamiana*. In *Arabidopsis*, *ADR1-L2* expression is upregulated during various immune responses (Figure 2.3). Perhaps introducing a bacterial elicitor prior to confocal microscopy can boost the expression driven by the native *ADR1-L2* promoter to increase fluorescent signal and would enable us to investigate changes in localization post-activation. Notably, we consistently observed increased accumulation of ADR1-L2-YFP in the stomatal guard cells (Figure S3.7). This observation may support the idea that ADR1-L2 functions in stomatal immunity, under the assumption that promoter elements driving expression in the stomatal lineage is conserved between *Arabidopsis* and *N. benthamiana*.

Phosphorylation was previously reported to modulate NLR activation either directly by phosphorylation of the NLR itself or indirectly by phosphorylation of guardees (H. Guo et al., 2020; D. Lee et al., 2015). For RNLs, association with EDS1-PAD4 or EDS1-SAG101 is crucial for activation and subsequent oligomerization (Feehan et al., 2023; Lapin et al., 2019; T. Qi et al., 2018; X. Sun et al., 2021). Recently, the protein structure of N-terminally truncated RNLs in complex with EDS1-PAD4 or EDS1-SAG101 was resolved (S. Huang et al., 2025; Y. Wu et al., 2024; Xiao et al., 2025; H. Yu et al., 2024). This structure revealed that the association between RNLs and their respective lipase-like signalling partners primarily relies on two interfaces located within the LRR of the RNLs. Our candidate phosphorylated amino acid

residues in ADR1-L2 primarily reside in the CC domain (T56, S141, S148) and the NB-ARC (S342), decreasing the likelihood that EDS1-PAD4 association would be affected (Figure 3.1). Indeed, we found that the (de)phosphorylation variants of our candidate residues were unaffected in the association to EDS1-PAD4 (Figure 3.8b). However, our current findings should be considered with caution, as we could observe weaker but visible immunoprecipitation of EDS1/PAD4 without ADR1-L2 present (false positive). Further replications or alternative protein-protein interaction studies (e.g. bimolecular fluorescence complementation) are necessary to draw a clear conclusion. NRG1.1 and SAG101 could be used as ideal negative controls, as these proteins should not interact with PAD4 and ADR1-L2 respectively. Nevertheless, based on our preliminary findings and the known structure of the ADR1-EDS1-PAD4 trimer, we reason that ADR1-L2 association with EDS1/PAD4 is unaffected by phosphorylation of our candidate residues.

Hypotheses on how phosphorylation might regulate ADR1-L2 activity

Our findings suggested that phosphorylation can alter ADR1-L2 (cell death) activity, without affecting pre-activation localization or EDS1-PAD4 association (Figure 3.8). So, phosphorylation must act on other function(s) of ADR1-L2 to modulate its activity. We hypothesize that phosphorylation may affect the oligomerization of ADR1-L2. Phosphorylation was recently shown to negatively affect NLR oligomerization (Toth et al., 2026; Zhong et al., 2025). For ADR1-L2, this can be studied next using the auto-activating D484V mutation or bacteria-induced activation (resulting in oligomerization) as previously reported (H. Wang, Tan, et al., 2024). In *N. benthamiana*, ADR1-L2 was shown to weakly self-associate but only forms a higher order oligomer after TNL activation (Saile et al., 2021; H. Wang, Tan, et al., 2024). Perhaps *Arabidopsis* ADR1-L2 forms a dimeric resting state that maintains autoinhibition, resembling NRC2 from *N. benthamiana* (Selvaraj et al., 2024). Phosphorylation could be a mechanism to maintain or disrupt such a resting state, acting as a molecular switch (W. J. Zhang et al., 2023). However, alongside phosphorylation, activation of ADR1-L2 may be regulated by additional mechanisms, since mutation of our candidate phosphorylated residues could only introduce auto-activated cell death but not TNL-triggered cell death (Figure 3.4, Figure 3.5, and Figure S3.2).

An alternative hypothesis is that phosphorylation of ADR1-L2 does not alter the resistosome formation, but rather the function of the resistosome. For TNLs, phosphorylation of key residues was shown to negatively affect the NADase activity of the TNL resistosome (J. Li et al., 2025). Perhaps a similar effect may exist for CNLs and RNLs, modulating their function as a calcium-permeable channel (Bi et al., 2021; Jacob et al., 2021). Based on our AlphaFold3 predictions, the candidate residues in the ADR1-L2 CC domain (T56, S141, S148) are not located in the channel interior but could perhaps alter the overall structure of the

channel (Figure 3.1c, d). However, there is currently no experimentally resolved protein structure for RNL resistosomes to confirm the position of these residues. If oligomerization of ADR1-L2 is unaffected by phosphorylation at our candidate residues, then the channel function should be studied next using conductivity assays or calcium-reporters (DeFalco et al., 2017).

Identifying new players in regulating ADR1-L2 activity

Another major question remaining in this project is which kinases and phosphatases are responsible for (de)phosphorylation of ADR1-L2 in *Arabidopsis*. Given the large role phosphorylation has in plant immune signalling, many kinases involved in immunity have been identified (Jamieson et al., 2018; D. Tang et al., 2017). Phosphatases are functionally opposite to kinases, and can also fulfil similar regulatory roles in plant immunity (Ayatollahi et al., 2022; Segonzac et al., 2014). Candidate kinases for the phosphorylation of ADR1-L2 can be studied with *in-vitro* kinase assays or protein-protein interaction studies. Dephosphorylation by candidate phosphatases is more difficult to study without known kinases to phosphorylate ADR1-L2, but protein-protein interaction studies could still hint towards a functional relationship with ADR1-L2. Promising candidates could be further investigated in *Arabidopsis* by crossing loss-of-function alleles of these candidates to ADR1-L2 D484V plants.

To identify candidate kinases and phosphatases that may regulate ADR1-L2, both targeted and untargeted approaches can be considered. For untargeted identification of kinases/phosphatases, the strong autoimmune phenotype of ADR1-L2 D484V could be leveraged for a forward genetic screen. This phenotype was previously successfully used to identify a crucial TNL for ADR1-L2 function (Jacob et al., 2023). Similarly, disruption of kinases or phosphatases that positively regulated the activity of ADR1-L2 could lead to a restoration of the wildtype-like phenotype. However, genetic redundancy might hinder this approach. Just the RLK family in *Arabidopsis* represents almost 2.5% of protein coding genes, with over 600 members (Shiu & Bleecker, 2001). The total number of kinases in *Arabidopsis* is predicted to be around 4% of the protein coding genes (Champion et al., 2004; Zulawski et al., 2014). Proximity labelling, such as TurboID, could help narrow down candidate kinases and phosphatases by identifying those that are in close proximity to ADR1-L2 (Branon et al., 2018). The experimental design could include challenged and unchallenged plants to assess proteins that are differentially associating with ADR1-L2 during an immune response.

Alternatively, targeted approaches can be used to study candidates for ADR1-L2 (de)phosphorylation. Using published literature, we can identify kinases or phosphatases that are involved in plant immunity and could be screened for their capability to phosphorylate ADR1-L2. A high priority candidate is the RLCK BIK1, which was predicted to phosphorylate ADR1-L2 S342 (Toth et al., 2026). Other RLCKs may also be promising candidates, such as

those from the 'AvrPphB susceptible 1-like' (PBL) family. PBL30/31 were previously shown to be involved in RLP23 signalling, which requires ADR1-EDS1-PAD4 (Pruitt, Locci, et al., 2021). Similarly, SOBIR1 is crucial for RLP-dependent immune responses and was shown to associate with ADR1 family proteins (Pruitt, Locci, et al., 2021). Preliminary evidence from our lab now also suggested that loss of the RLCK-VII-4 family in *Arabidopsis* can rescue the ADR1-L2 D484V phenotype, suggesting one or more members of this kinase family may positively regulate ADR1-L2 (Rao et al., 2018; Saile, 2021; Vogt, 2026).

Conclusion

In this chapter, we showed that the phosphorylation status of multiple amino acid residues could regulate the (auto-)activity of *Arabidopsis* ADR1-L2. Examining the biological effect of phosphorylation on ADR1-L2 was difficult since we found that *ADR1* genes may function in a collaborative fashion. Preliminarily, we can rule out that phosphorylation of ADR1-L2 affected pre-activation localization or association with EDS1-PAD4. Our findings pointed towards a ‘phosphorylation code’ on ADR1-L2 that can integrate signals from multiple kinases/phosphatases to dynamically fine-tune the immune response. The next steps would be the validation of our findings in *Arabidopsis* and identifying kinases/phosphatases that are involved in the regulation of ADR1-L2. Ultimately, we aim to crack the ADR1-L2 phosphorylation code by uncovering under which conditions each residue is phosphorylated and how the phosphorylation of these residues alters the activity of ADR1-L2.

Contributions

This chapter contains contributions from my internship student Finola Knöll, whom I co-supervised during my PhD thesis. The IP-MS data (Table S3.1) was generated by Dr. Marie Le Naour-Vernet in the lab of Prof. Cyril Zipfel (UZH, Zürich) in collaboration with Dr. Kyle Bender (UZH, Zürich) and Dr. Frank Menke (TSL, Norwich). The work on *Arabidopsis* presented in this chapter (Figure 3.2) was performed by my colleague Frank Vogt as part of his PhD thesis (Vogt, 2026).

Supplementary material

Table S3.1: (Preliminary) Phosphorylated residues identified by IP-MS in ADR1-L2-FLAG after expression in *N. benthamiana* with flg22 or mock treatment. The peptide count of each residue is listed per replicate performed so far. Percentage coverage indicates how much of the ADR1-L2-FLAG sequence could be reconstructed using the obtained peptide sequences. The large difference in peptide counts between experiments is attributed to on-going optimization of the IP-MS experiments. All IP-MS experiments and analysis were performed by Dr. Marie Le Naour-Vernet (UZH, Zürich) in the lab of Prof. Dr. Cyril Zipfel (UZH, Zürich & TSL, Norwich) in collaboration with Dr. Kyle Bender (UZH, Zürich) and Dr. Frank Menke (TSL, Norwich). The phosphorylated residues in ADR1-L2 that were functionally investigated in this chapter are indicated in **green**.

Experiment		Experiment 1				Experiment 2			
Treatment		Mock		flg22		Mock			
Replicate		#1	#2	#1	#2	#1	#2	#3	#4
ADR1-L2 residue	S21	1			1	1	1	1	1
	T24			1					1
	T36						1		2
	T44					1			
	Y50	1		1	1		2	1	3
	S51					1		1	
	T56	1	1	1	2	4	7	3	5
	S141				1	1	1		1
	S148	3		2	3	9	6	8	6
	S154				1	1		1	1
	S156						1		
	S211					1			
	S281								
	S342								1
	Y472					1	1		1
	T535					2		1	
	T539					1		5	3
	S803							1	
% Coverage		85	84	86	87	90	90	87	89

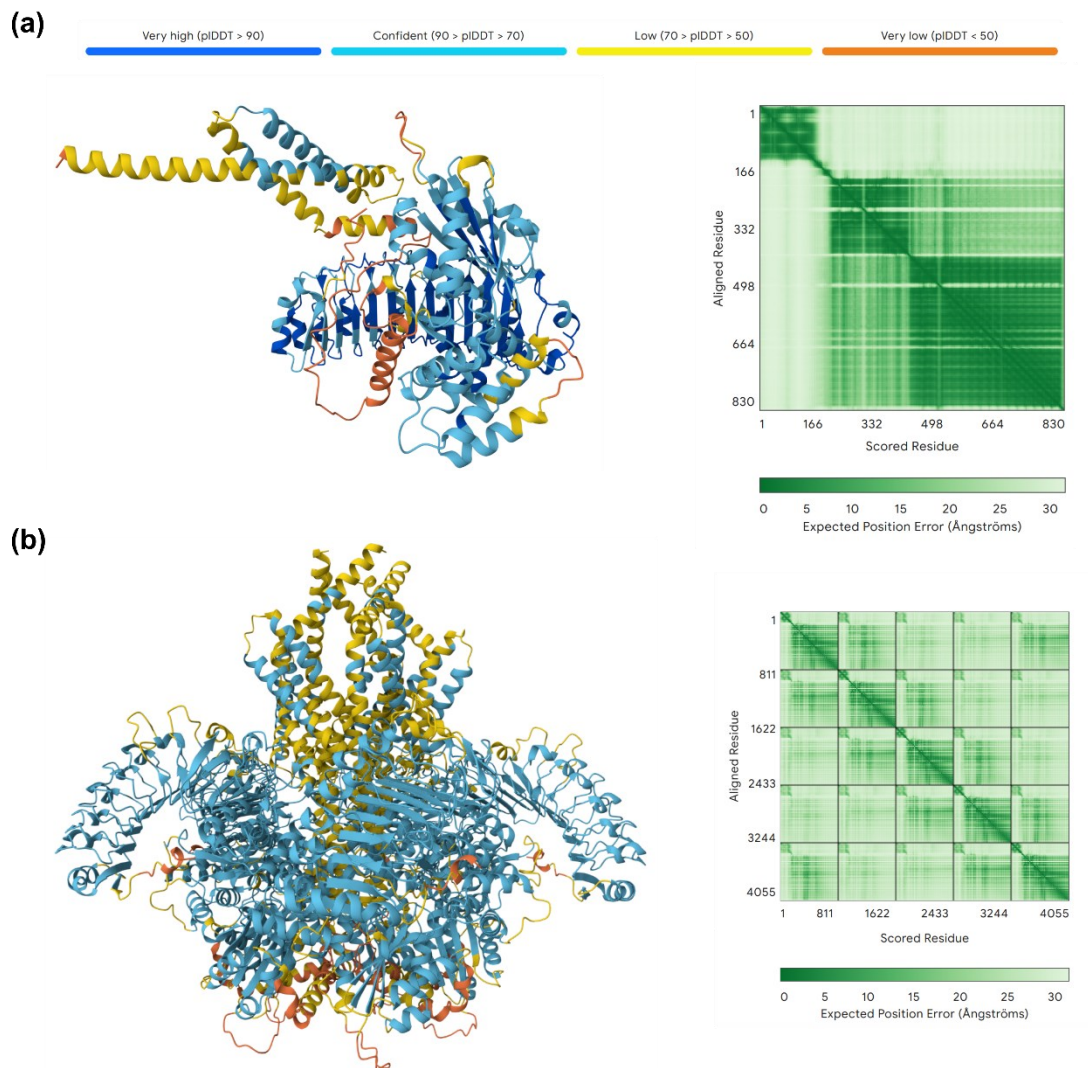


Figure S3.1: Confidence levels of AlphaFold models depicted in Figure 3.1. Model with confidence score (pLDDT = predicted Local Distance Difference Test) and expected position error for the predicted ADR1-L2 monomer **(a)** and pentamer **(b)** predictions. Predictions and the confidence scores were directly taken from the AlphaFold3 server (Abramson et al., 2024).

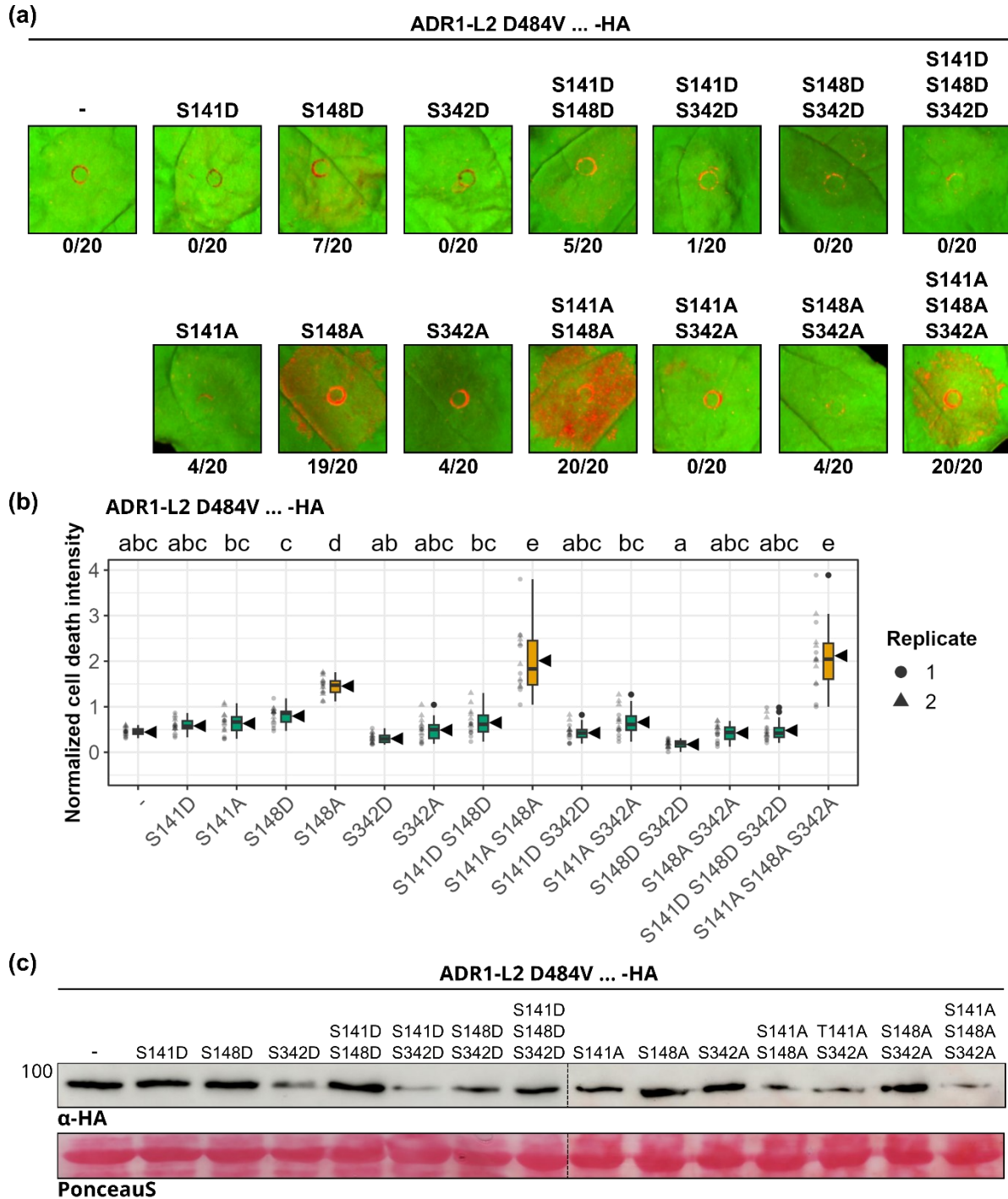


Figure S3.2: The dephosphorylation-mimic ADR1-L2 D484V S342A also negatively regulated the gain-of-function from ADR1-L2 S148A (a) Cell death was investigated for each combination of phosphorylation- or dephosphorylation-mimic of the residues S141, S148, and S342 in ADR1-L2 D484V. *Agrobacterium* infiltrations were done in *N. benthamiana adr1 nrg1*. ADR1-L2 variants were expressed under the *Arabidopsis* ADR1-L2 promoter. Images were taken five days after infiltration. Numbers below the images indicate the occurrence of cell death out of the total number of replicates. False colours indicate healthy (green) and dead (red) tissue. (b) Quantification of cell death by pixel intensity for the ADR1-L2 (de)phosphorylation mimicking mutants in (a). Mean pixel intensity was measured and normalized against the background signal. The arrow (◄) indicates the mean cell death intensity for each ADR1-L2 variant. The letters depict the result of statistical testing (Two-Way ANOVA with post-hoc TukeyHSD, $n=20$). Genotypes sharing the same letter are *not* statistically different in cell death intensity ($n=20$). (continued on the next page)

(continued) The **orange** colour indicates a significant difference from ADR1-L2 D484V. **(c)** Protein accumulation of all ADR1-L2 variants in (a) was confirmed by western blot. The number on the left indicates the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The dotted line indicates where digital stitching of membranes occurred in the presented western blots.

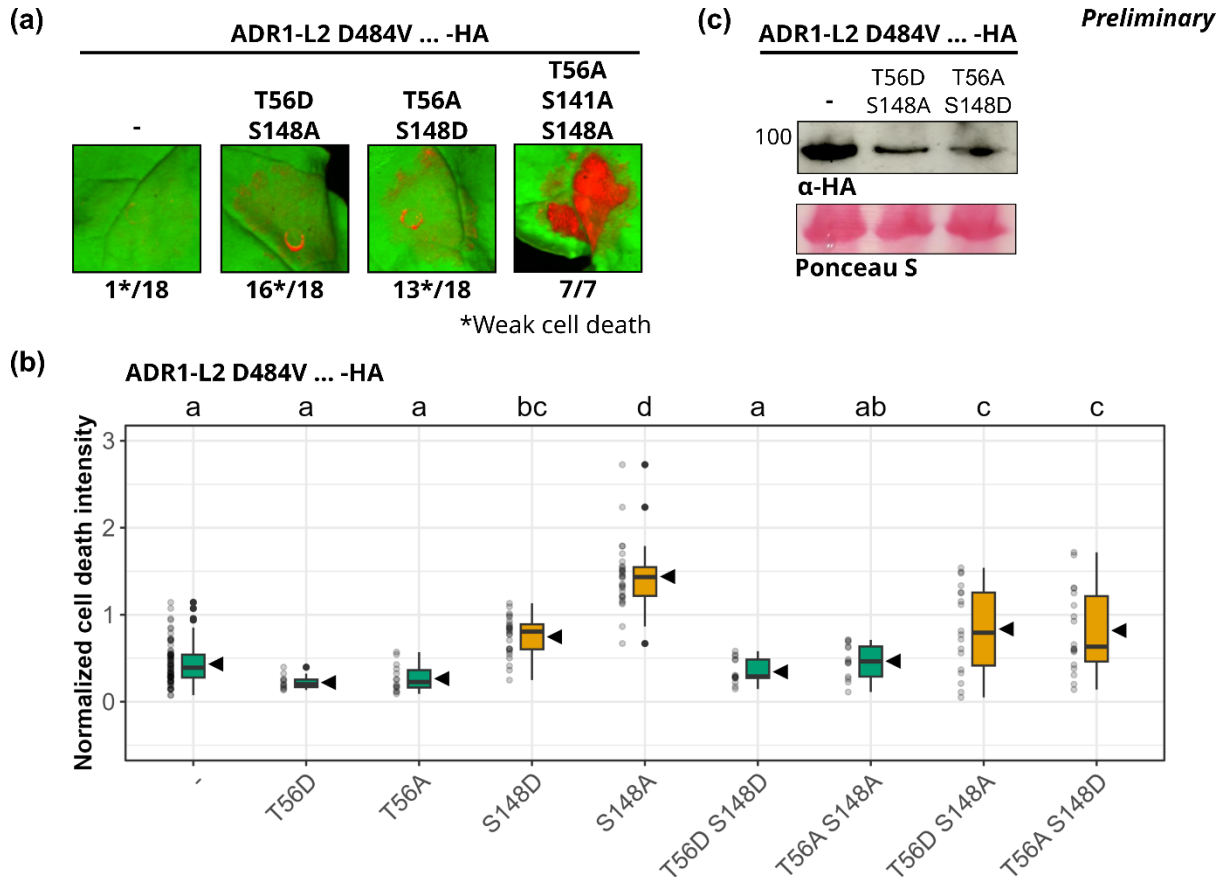


Figure S3.3: (Preliminary) Dynamic regulation of ADR1-L2 D484V cell death by the phosphorylation status of the residues T56 and S148. **(a)** Cell death from ADR1-L2 D484V is partly restored by T56D S148A and T56A S148D. *Agrobacterium* infiltrations were done in *N. benthamiana* *adr1 nrg1* leaves. Images were taken 5 days after infiltration. Numbers below the images indicate the occurrence of cell death. The asterisk (*) indicates a consistently weaker onset of cell death compared to S148A. False colours indicate healthy (**green**) and dead (**red**) tissue. **(b)** Quantification of cell death by pixel intensity for the ADR1-L2 phosphorylation mutants in (a). Mean pixel intensity was measured and normalized against the background signal. Data was used from multiple experiments (including Figure 3.4 and Figure S3.3). The arrow (◄) indicates the mean cell death intensity for each ADR1-L2 variant. The letters depict the result of statistical testing (Two-Way ANOVA with post-hoc TukeyHSD). Variants sharing the same letter are *not* statistically different in cell death intensity. The **orange** colour indicates a significant difference from ADR1-L2 D484V. **(c)** Protein accumulation of combinations depicted in (a) was confirmed by western blot. Proteins were detected with anti-HA. The numbers on the left indicated the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control.

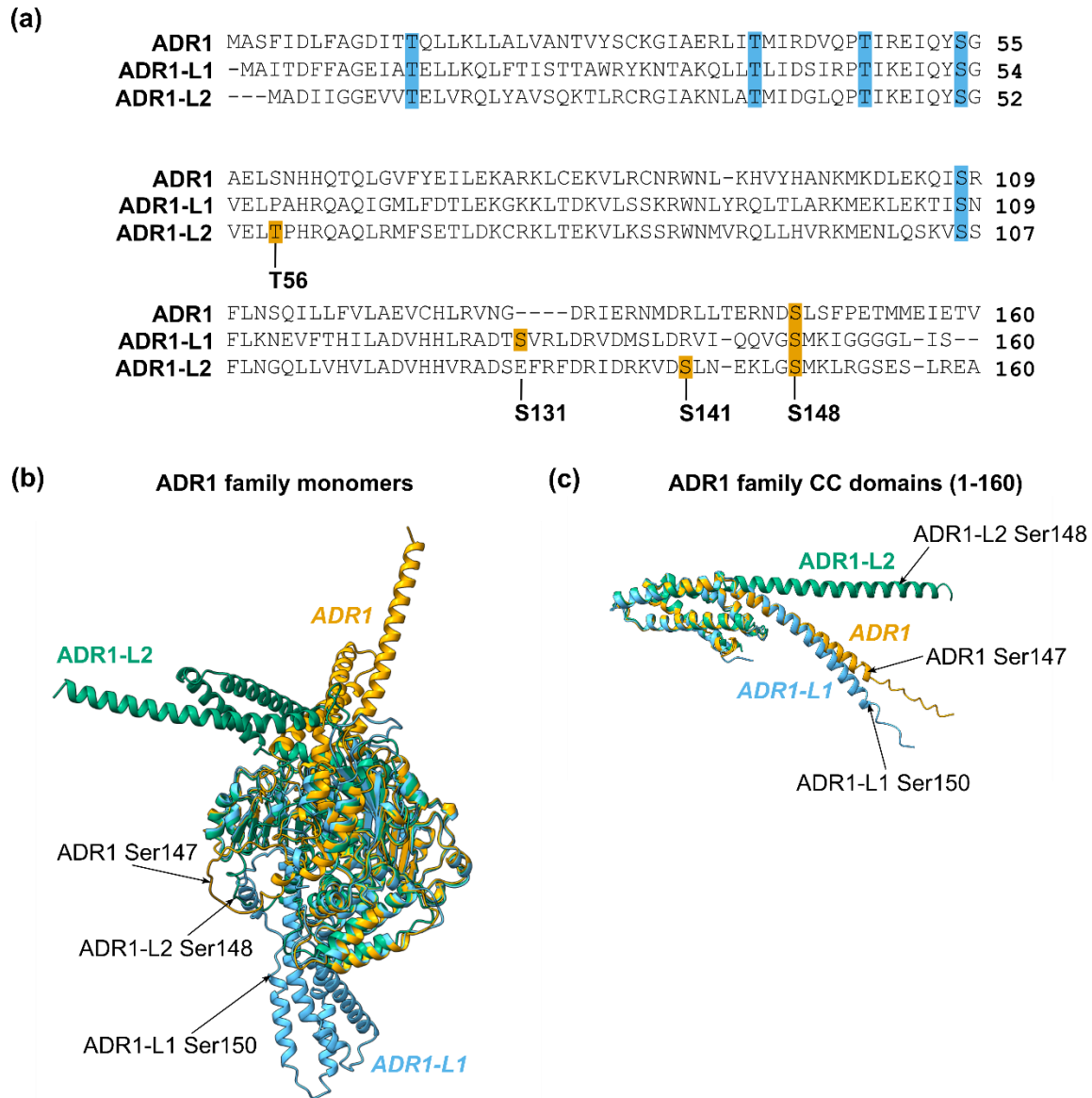


Figure S3.4: Homologous residues to ADR1-L2 S148 are found in ADR1 (S147) and ADR1-L1 (S150), but structural conservation is limited. (a) Protein alignment of the ADR1, ADR1-L1, and ADR1-L2 CC-domains (amino acid 1-160). Residues studied in this work are indicated with labels and highlighted in orange. Serine or Threonine residues that are conserved in all three genes are highlighted in blue. **(b)** Structural alignment of AlphaFold3 predicted monomers **(b)** and CC-domains **(c)** for ADR1, ADR1-L1, and ADR1-L2 (Abramson et al., 2024). The ADR1-L2 S148 homologous residues are indicated. ADR1 is depicted in orange, ADR1-L1 in blue, and ADR1-L2 in green. The confidence of the predicted structures is shown in Supplementary Figure S3.1 and Supplementary Figure S3.5.

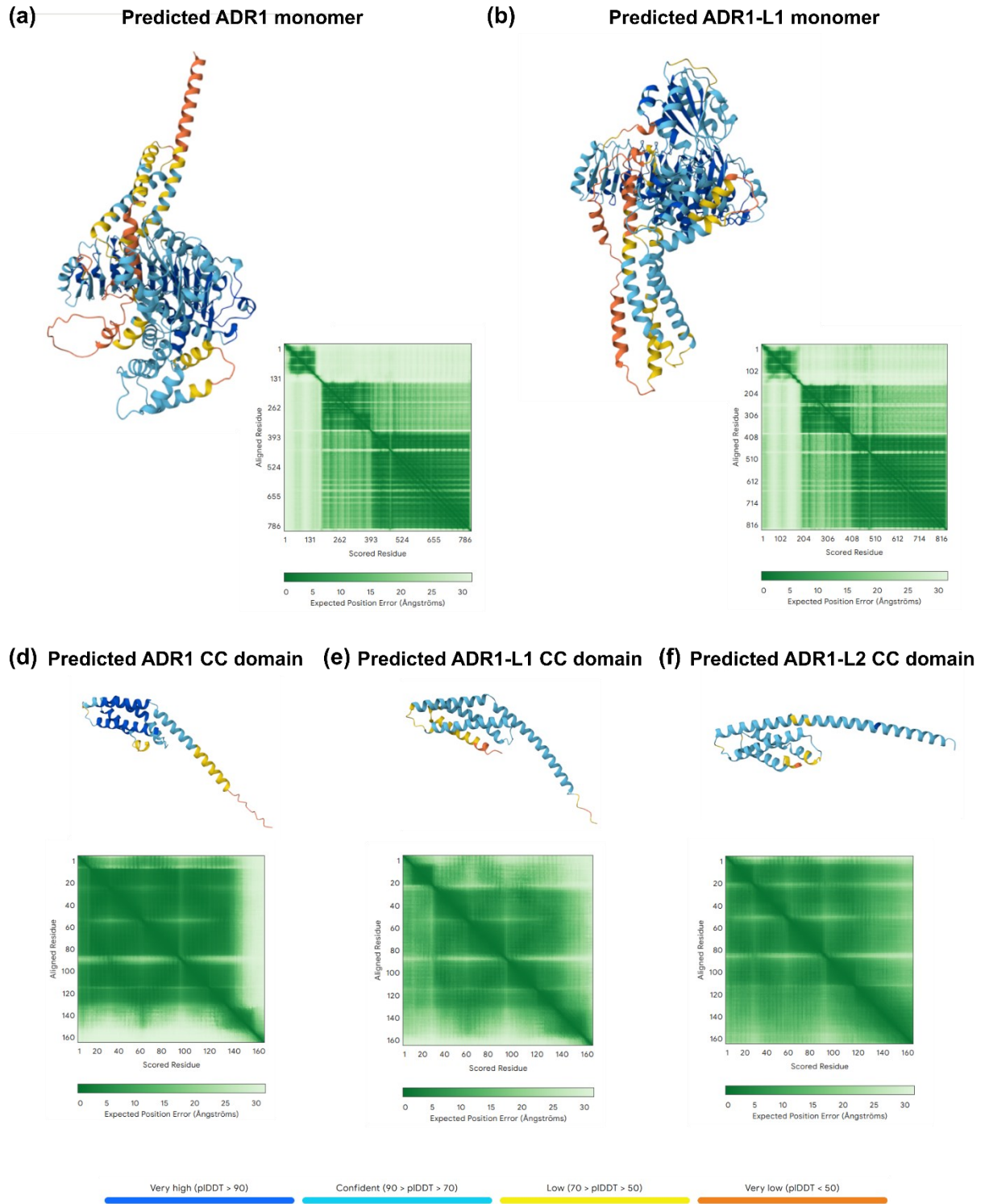
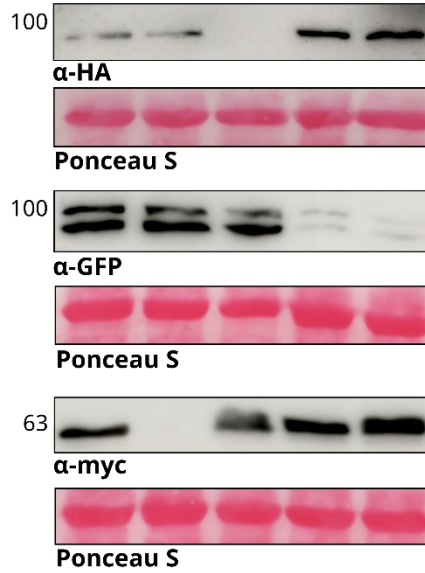


Figure S3.5: Confidence levels of AlphaFold models depicted in Supplementary Figure S3.4. Model with confidence score (pLDDT = predicted Local Distance Difference Test) and expected position error for the predicted monomers of ADR1 (a) and ADR1-L1 (b) and CC-domains (amino acids 1-160) of ADR1 (c), ADR1-L1 (d), and ADR1-L2 (e). Predictions and the confidence scores were directly taken from the AlphaFold3 server (Abramson et al., 2024).

(a)

ADR1 ...-HA	+	+	-	S147D	S147A
EDS1-YFP + PAD4-GFP	+	+	+	+	+
XopQ-myc	+	-	+	+	+



(b)

ADR1-L1 ...-HA	+	+	-	S131D	S131A	S150D	S150A	S131D S150D	S131A S150A	S131D S150A	S131A S150D
EDS1-YFP + PAD4-GFP	+	+	+	+	+	+	+	+	+	+	+
XopQ-myc	+	-	+	+	+	+	+	+	+	+	+

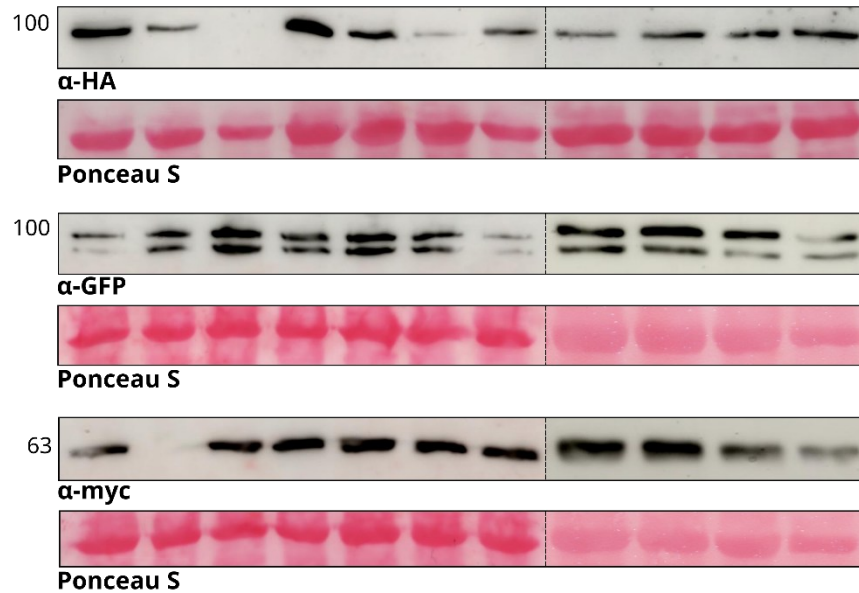


Figure S3.6: Protein accumulation for the transient expression depicted in Figure 3.6. ADR1 variants (a) and ADR1-L1 variants (b) were detected using anti-HA, EDS1 and PAD4 were detected by anti-GFP, and XopQ by anti-myc. The numbers on the left indicated the approximated protein weight in kDa as indicated by the molecular protein marker. Ponceau S staining is shown as loading control. The dotted lines indicate where digital stitching of membranes occurred in the presented western blots.

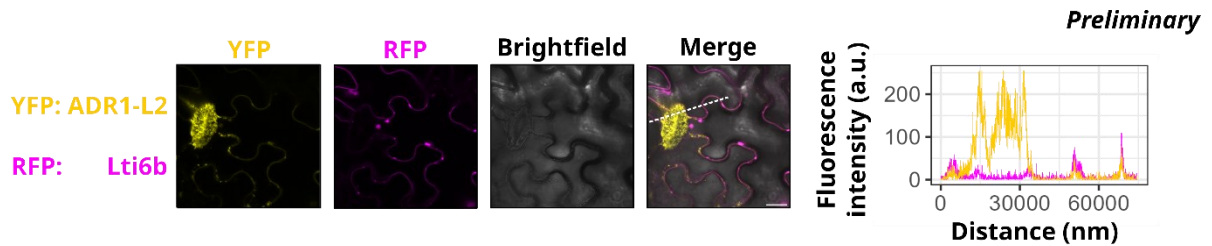


Figure S3.7: (Preliminary) Fluorescent signal from *ADR1-L2*-YFP was stronger in stomatal guard cells in *N. benthamiana*. *ADR1-L2* (**YFP**) was driven under its native *Arabidopsis* promoter. The plasma membrane marker *Lti6b* (**RFP**) driven by a 35S promoter did not accumulate in these cells. Fluorescence intensities (in arbitrary units) were measured across the dotted line in the merged image and depicted as a line graph to show co-localization. Scale bars in the merged images indicate 20 μm .

General discussion

Here, I place the findings presented in this PhD thesis in the context of current literature. I will primarily focus on current ideas and concepts within the field of plant NLRs, and how my work presented in this thesis relates to and builds upon those ideas.

Autoimmunity remains a valuable tool to study the plant immune system

Autoimmunity provides a potent tool to probe, poke, and prod at the plant immune system (Van Wersch et al., 2016). Uncovering the source of autoimmunity can lead to identification of novel immune components that would normally negatively regulate immunity, or activation mechanisms of positive immune regulators. Autoimmunity can also be used to analyse signalling partners of the auto-activated component, as the loss of function of these signalling partners should (partially) turn off the immune system. Fundamental understanding of the plant immune system and autoimmunity is crucial for developing new methods to protect plants from pathogens. A recent study illustrated this well by leveraging auto-activated NLRs, including NRG1.1, with a N-terminal cleavage peptide that would be processed by viral proteases (J. Wang, Chen, et al., 2025). Upon cleaving this N-terminal sequence, the auto-activated NLR could fulfil channel function and provided full immunity against several viruses. This highlights the importance of studies that improve our fundamental molecular understanding of plant-microbe interactions, from both the pathogen (virus) and plant (NLR) perspective. Combining (auto-activated) NLRs with appropriate regulatory systems may provide a new platform for resistance engineering in crops.

In this PhD thesis, I leveraged autoimmunity in *Arabidopsis thaliana* and auto-activity in *Nicotiana benthamiana* to dissect regulatory mechanisms of *Arabidopsis* RNLs. In *Arabidopsis*, the autoimmunity of the *adr1-1-1 adr1-1-2 nrg1.1-2* triple mutant was not caused by the absence of other RNLs (Figure 1.3). However, the *sith* autoimmune phenotype is likely *ADR1*-dependent, which we are currently confirming with CRISPR-Cas9 deletion of the *ADR1* locus in the *adr1-1-1 adr1-1-2 nrg1.1-2* mutant (Figure S1.1b). Uncovering the identity of the *sith* mutation may still lead to important discoveries on regulation of *ADR1* signalling in the plant immunity. However, our findings also act as a warning to others regarding the importance of using secondary alleles or complementation lines in genetic studies (Chapter 1). Use of only a single loss-of-function allele per gene is vulnerable to background mutations underlying the observed phenotype (B. Song et al., 2026).

In *N. benthamiana*, we commonly leveraged cell death as a readout for NLR activity to study regulatory mechanisms (Chapter 2, Chapter 3). We identified that cell death by autoactivated *ADR1-L2* is regulated by phosphorylation (Figure 3.4) but were unable to observe the same effect in effector-triggered context (Figure 3.5). This observation illustrated that translating conclusions drawn from autoimmunity back into biological context (e.g. effector

recognition or pathogen infection) can be complicated. However, this can also spark new insights or questions due to differences between autoimmunity and natural infections. A recent study showed that drastic autoimmune phenotypes in laboratory settings do not always translate to growth penalties under field-grown conditions (Lundberg et al., 2024). This observation may for instance point towards a large role in plant microbiomes in regulating (auto)immunity that are not observed under standard lab conditions (Y. T. Cheng et al., 2024). Similarly, from the observations in this thesis we can now raise the question *why* ADR1-L2 is unable to signal in Roq1-triggered immunity, unlike ADR1-L1 (H. Yu et al., 2024). Perhaps TNLs like Roq1 can specifically activate certain RNLs. Alternatively, there could be additional regulation in place that impairs ADR1-L2 but not ADR1-L1. These examples highlight how investigating autoimmunity can simultaneously further our understanding of the plant immune system and provide grounds for new research directions.

Benefits and pitfalls of using *N. benthamiana* to study *Arabidopsis* NLRs

The model system *N. benthamiana* has been invaluable in furthering our understanding of the plant immune system. Transient expression of *Arabidopsis* NLRs has broadly been used to study their activity. Cell death by overexpression in *N. benthamiana* provides a rapid screening method for functional studies, as seen in this work (Chapter 2, Chapter 3). However, all findings from heterologous systems should be considered with caution and always be validated in the native system whenever possible.

In Chapter 2, we used transient overexpression of *Arabidopsis* RNLs in *N. benthamiana* to test our initial hypothesis from Chapter 1 (Figure 1.1e). Our findings directed us towards the possibility that alternative splicing was involved in the suppression of ADR1 by NRG1.1^{genomic}. Although we did not identify the full sequence of the splice variant, we could identify a putative splice junction in *NRG1.1* (Figure 2.2a, b). Our initial evidence showed that the *Arabidopsis* *NRG1.1* splice variant was also generated in *N. benthamiana* (Figure 2.2d). However, based on amplification from start-to-stop of *NRG1.1* variants, we found that the splicing of transcripts may vary between *Arabidopsis* and *N. benthamiana* (Figure S2.1). For many studies, this finding may not be relevant since coding sequence variants are frequently used. However, evidence is accumulating that alternative splicing plays a crucial role in plant immunity (Kufel et al., 2022). Future studies on alternative splicing of *Arabidopsis* NLRs should confirm that splice variants that were identified through transient expression in *N. benthamiana* also exist in *Arabidopsis*.

Interpretation of cell death in *N. benthamiana* is often done visually, either in a qualitative manner (Chapter 2) or semi-quantitative using a metric to score the strength of cell death (Chapter 3). Both approaches have drawbacks in being too subjective or lacking in consistency. In Chapter 3, we measured pixel intensity of recorded images to quantify cell

death (see Material and methods). However, visually the strength of cell death could vary between plants. This raises the question how useful quantification of cell death from overexpression in *N. benthamiana* is, or whether qualitative approaches are sufficient. Many studies on NLR function (including this work) would benefit from quantification of cell death by measuring conductivity in *N. benthamiana*. Conductivity measurements provide an additional benefit of recording temporal dynamics, which may aid capturing differences in cell death on an additional axis.

Observations regarding cell death may also vary between different labs. NRG1.1 D485V was previously reported to cause cell death in *N. benthamiana* which could be inhibited by co-expression of *NRG1.3* (Z. Wu et al., 2022). Under our conditions, we could not corroborate this observation (Figure S2.6). While the model on NRG1.1/NRG1.2 regulation by NRG1.3 remains unaffected, our findings raise concerns about the reproducibility of certain results using *N. benthamiana*. Variation in quantitative measurements using *Agrobacterium* infiltrated *N. benthamiana* plants gained traction recently, providing better guidelines to reduce variability (Bashandy et al., 2015; S. N. Tang et al., 2026). However, tackling a qualitative difference between labs as we observed for NRG1.1 D485V/NRG1.3 is difficult. The source for this variation between labs is not known but may be due to differences in growth conditions like temperature, light settings, humidity, or microbiome availability.

Not all *Arabidopsis* RNLs caused cell death in *N. benthamiana*, as seen for ADR1-L2 D484V and NRG1.2 N490V under our conditions (Figure 2.4, Figure 3.4, and Figure S3.2). This can be due to incompatibility with required signalling components in *N. benthamiana*, like EDS1 (Lapin et al., 2019). However, overexpression of some RNLs may circumvent EDS1 requirements (Figure S2.1). EDS1 is required for the upregulation of many immune components in *Arabidopsis* (Bhandari et al., 2019). Previous work proposed the idea that plant NLRs are in an equilibrium between ATP-bound ('on') and ADP-bound ('off') state in unchallenged plants (Bernoux et al., 2016; Richard et al., 2018). Activation of immunity would then shift this equilibrium towards the ATP-bound on-state to mediate immune signalling. Overexpression of *Arabidopsis* RNLs may represent an immune state post-EDS1-dependent upregulation and thereby inducing auto-active cell death by sheer number of ATP-bound RNLs. This may complicate inferring requirements for signalling or cell death from overexpression in *N. benthamiana*. Contrary, some NLRs (like ADR1-L2) may be inhibited by regulatory systems native to *N. benthamiana*, such as phosphorylation (Chapter 3). Unforeseen regulation of non-native NLRs may also occur in crop species and could alter the function of NLRs in unexpected manners. Other plant species may also lack the proper (co-evolved) regulation of introduced NLRs, thereby rendering them auto-active and leading to autoimmunity. Therefore, understanding the regulatory mechanisms of NLR activity is crucial for the development of more resistant crops.

Regulation of the RNLs balances resistance and cell death

In current RNL literature, there is a central dogma that the *ADR1* family primarily contributes to resistance and the *NRG1* family mediates cell death (Lapin et al., 2019; Saile et al., 2020). However, more exceptions are becoming known that contradict this generalization of RNL function. For example, the PRRs RLP42 and CRK7/43 were shown to initiate ADR1-dependent cell death from the cell surface (Pierdzig et al., 2025; L. Zhang et al., 2021). Specific TNLs also appear to initiate cell death via the ADR1 branch, rather than the NRG1 (Dong et al., 2016; Z. Wu et al., 2019). These findings challenge the idea that *ADR1* genes are less involved in cell death, instead proving that ADR1-dependent cell death occurs under certain conditions. Cell death from the *ADR1* branch after activation of TNL signalling was also observed in *Arabidopsis* plants that were deficient in SA synthesis (X. Sun et al., 2021). This finding suggested that SA may negatively regulate cell death by *ADR1* genes. SA is a crucial phytohormone in many interactions between plants and microbes (Tian et al., 2025). As a result, (hemi-)biotrophic pathogens evolved various methods to disrupt SA-dependent responses in plants to promote virulence (G. Qi et al., 2018). Previously, the EDS1-PAD4 node was proposed to protect the SA pathway (Cui et al., 2017). Regulation of cell death from the *ADR1* family in a SA-dependent manner conceptually fits as disruption of SA accumulation by pathogens would then lead to ADR1-dependent cell death. Under normal SA accumulation, *LSD1* is a likely candidate to be involved in the negative regulation of cell death from the *ADR1* branch, as is evident from the SA-triggered runaway cell death in loss-of-function *lsd1* mutants (Roberts et al., 2013). However, the mechanism behind the negative regulation of *ADR1* cell death is not yet known. Involvement of multiple kinases/phosphatases to create a phosphorylation code (Chapter 3) forms an elegant hypothesis in how cell death from *ADR1* family members could be fine-tuned during specific immune responses (Figure 4).

Sensor NLRs may also shape the activation and function of RNLs, balancing resistance and cell death to achieve the desired response. This is best illustrated by comparing infections where the pathogen is identical except for one effector. For resistance to *Pst DC3000 AvrRpt2*, the *ADR1* family is required for timely onset of cell death, directly correlating with their role in resistance (Bonardi et al., 2011; Saile et al., 2020). Contrary, resistance to *Pst DC3000 AvrRps4* is primarily mediated by the *ADR1* family (Figure 3.3), but cell death requires the *NRG1* family thereby unlinking resistance and cell death (Saile et al., 2020). These observations suggest that sensor NLRs can differentially require the RNL families while also influencing the activity of RNLs. The mechanism underlying RNL specificity (*ADR1* or *NRG1*) by sensor NLRs is poorly understood and forms an exciting new research direction. Regulation of RNLs by, for example, phosphorylation (Chapter 3) could also contribute to the differential activation of the RNL families in *Arabidopsis*.

In plant immunity, cell death is often seen as a crucial part of resistance by inhibiting the growth of (biotrophic) pathogens (Balint-Kurti, 2019). Necrotrophic pathogens, however, may benefit from cell death induced by the plant immune system, unlinking cell death from resistance (Faris et al., 2010; J. Lorang et al., 2012; J. M. Lorang et al., 2007; Pitsili et al., 2020). Local cell death is also not (always) necessary to achieve systemic acquired resistance (P.-P. Liu et al., 2010). Instead, primed immune responses mediated resistance while negatively regulating cell death, likely to prevent unnecessary fitness costs associated with cell death (D. Wang et al., 2023). Perhaps cell death is initiated locally by ‘primary immune responder’ (PRIMER) cells and activates the immune signalling in neighbouring cells through mobile damage-associated signals to avoid further spreading of the pathogen (Jacob et al., 2023; Nobori et al., 2025).

These examples support the idea that plants sense the ‘danger level’ of an invading pathogen based on the receptor that was triggered (Thomma et al., 2011). Receptor evolution favoured receptor variants and signalling pathways that induced an appropriate response that mediated effective resistance at a minimal cost to growth and development. Cell surface receptors that induce cell death are likely to perceive signals associated to a higher level of danger, thereby immediately activating an amplified immune response. However, generally signals from the cell surface require more attenuated or transient responses to avoid fitness costs associated with immunity after perceiving any MAMP in the extracellular space. Effector recognition inside the cell is naturally associated with a higher danger level as an indicator of active invasion of the cell by a pathogen. This concept still maintains the consensus in literature that immunity from NLRs (ETI) is more amplified and prolonged compared to cell surface responses (PTI) but also accommodates for exceptions where PRR signalling causes cell death (Tsuda & Katagiri, 2010). Integration of multiple concurrent signals could then also additively indicate a higher danger level and activate a stronger immune response.

All in all, we may not be able to generalize the initiated immune response based on the type of receptor alone. Instead, receptors may have evolved to induce an appropriate immune response for the perceived danger signal, which can vary between different pathogens. Differential regulation of the *Arabidopsis* RNL families can be a mechanism underlying the fine-tuning of the immune response to balance resistance and cell death (Figure 4).

Expansion of RNL families may drive functional diversity

The two RNL families in *Arabidopsis* are known to be unequally redundant, fulfilling partially overlapping but also distinct functions (Saile et al., 2020). However, redundancy between members of the same RNL family is not as clear (Y. Liu, 2026). The NRG1 family members can redundantly induce cell death in response to AvrRps4 (Castel et al., 2019). Redundancy within the ADR1 family is largely unknown.

The ADR1 family functions in immune responses initiated by various types of immune receptors, like RLPs, TNLs, and CNLs (Dong et al., 2016; Ge et al., 2026; Pruitt, Locci, et al., 2021; Saile et al., 2020). Whether all ADR1 family members can act redundantly in mediating these immune responses is not known. In this work, we could show that ADR1-L1 is the main contributor to basal resistance (Figure 3.3a). *ADR1-L1* was also consistently upregulated across all tested immune responses (Figure 2.3), perhaps contributing to the role of ADR1-L1 in basal immunity. However, ADR1-L1 required at least one other ADR1 family member to achieve full resistance. This could suggest that RNL dependent immune responses require a threshold of ADR1 family members that a single gene locus is unable to achieve. Introduction of a loss-of-function allele for one *ADR1* family gene led to an upregulation of the remaining family members genes during autoimmunity, suggesting that the total accumulation of ADR1 family members may be carefully maintained (Dong et al., 2016). Nevertheless, loss of *ADR1-L1* (*adr1-l1-1*) still significantly impacted basal resistance (Figure 3.3a), despite the presumed upregulation of *ADR1* and *ADR1-L2*.

An alternative hypothesis is that ADR1 family members have distinct functions that are individually required to achieve full immunity. Indeed, the redundancy among ADR1 family members appeared to vary between different types of infections (Bonardi et al., 2011). This concept was also observed in the runaway cell death phenotype from the loss-of-function *Isd1* mutant which requires ADR1-L1 and ADR1-L2 (Roberts et al., 2013). Additionally, ADR1 was shown to have diverged from ADR1-L1 and ADR1-L2, lacking a ubiquitination site that is targeted by the bacterial effector *AvrPtoB* to promote degradation of ADR1 family proteins (M.-Y. Wang et al., 2023). Notably, the expansion of RNL families occurred in Rosids, and many other angiosperms (including *N. benthamiana*) instead carry only single copies of RNLs (Shao et al., 2016). Nevertheless, these examples suggest that expansion of the *ADR1* gene family may have allowed for functional diversification. Our findings now provide additional evidence for collaborative functions between ADR1 family members to mount a full immune response. Previous work showed that members of the ADR1 family can hetero-associate (Saile et al., 2021). The function and stoichiometry of these *ADR1* complexes are currently not known but could provide a potential mechanism for their collaborative function (Figure 4).

In Chapter 2, we investigated expression patterns of *Arabidopsis* RNLs during various immune responses (Figure 2.3). Here we identified that *NRG1.1* portrayed a unique expression pattern compared to other RNLs, characterized by an early peak in expression level upon infections with virulent and avirulent bacteria. Although the functional relevancy of this observation is up for debate, the contrasting transcriptional regulation between *NRG1.1* and *NRG1.2* could hint towards undiscovered diverging functions in the *NRG1* family. We also found that *ADR1* is strongly upregulated during PTI (log2 fold change of ~4.5 compared to unchallenged plants), but not during ETI-eliciting infections (Figure 2.3). The *ADR1* family was

shown to be required for PTI responses initiated by RLPs at the cell surface, so perhaps *ADR1* specifically plays an important role in those responses (Pruitt, Locci, et al., 2021). However, the upregulation of *ADR1* was observed at a relatively late stage of infection (8 hpi, Figure 2.3a), suggesting that a role in early PTI responses is unlikely. Instead, the timing of *ADR1* upregulation might coincide with the *de novo* synthesis of pRib-AMP/pRib-ADP by TNLs to mediate SA-dependent responses and resistance during RLP-mediated immunity (Fliegmann et al., 2025).

Notably, our findings on RNL expression are based on bulk RNA sequencing and lack any information on tissue or cell type specificity. Perhaps some RNLs are specifically upregulated in PRIMER cells (Nobori et al., 2025). Meanwhile, bystander cells surrounding the infection site mount an immune response to limit spreading of the pathogen, which was shown to depend primarily on the *ADR1* family (Jacob et al., 2023). Moreover, *ADR1* members were recently shown to be involved in stomatal immunity in plants (H. Wang, Song, et al., 2024). We observed that the fluorescent signal from *Arabidopsis* *ADR1-L2-YFP* was especially potent in *N. benthamiana* stomatal guard cells. This could indicate that the native *Arabidopsis* *ADR1-L2* promoter drives potent expression of *ADR1-L2* in guard cells and may hint towards a potential function for *ADR1-L2* in stomatal immunity (Figure S3.7). Another recent publication reported that *Arabidopsis* guard cells do not activate SA-dependent immunity upon infection with (hemi-)biotrophic pathogens, but do display rapid calcium influx and hypersensitive cell death (J. Song et al., 2026). These findings raise the question whether hypersensitive cell death in guard cells is *ADR1*-dependent, thereby linking the observed cell death to the role of the *ADR1* family in stomatal immunity and the proposed negative regulation of *ADR1*-dependent cell death by SA signalling (X. Sun et al., 2021; H. Wang, Song, et al., 2024). Generating reporter lines for RNL promoters will be extremely valuable to gain spatial context on RNL expression during infection and further investigate functional divergence in different cell types.

Taken together, these observations support the idea that the *ADR1* family in *Arabidopsis* may be functionally diverging. This functional diversification of RNLs may not only exist on the protein level but also at the transcriptional level and paves the way for future research directions (Figure 4).

Variants of *Arabidopsis* RNLs provide a basis for a new research direction

In this work, we uncovered the existence of a putative spliced isoform of *NRG1.1* (Chapter 2). We identified the responsible alternative splicing event that was directly responsible for the observed *NRG1.1* isoform observed on western blots (Figure 2.2). However, the full sequence of the spliced transcript and the function of the *NRG1.1* isoform remain unknown. The role of alternative splicing of NLR transcripts varies between NLRs. The homeostasis of some NLRs

is regulated by alternative splicing (B. Sun et al., 2024). Other NLRs require isoforms generated by alternative splicing to achieve full immunity (Xu et al., 2012; X.-C. Zhang & Gassmann, 2003). Our increasing understanding of the role alternative splicing plays in plant immunity warrants continued investigation into the function of the putative *NRG1.1* isoform described in Chapter 2 (Kufel et al., 2022).

We also frequently observed a secondary protein band for ADR1-L2 (Figure 2.1, Figure 2.4, and Chapter 3), consistent with previous reports (Saile et al., 2021). Notably, our ADR1-L2 expression constructs are coding sequences (Table S4), suggesting that the secondary band is less likely to be the product of alternative splicing. Instead, I hypothesize that this variant of ADR1-L2 is likely generated by proteolytic activity. Throughout this work, all NLRs were always tagged at their C-terminus to maintain their functionality. So, presumably the N-terminal part of ADR1-L2 is cleaved off to generate this variant. IP-MS analysis of the secondary bands could be used to identify the amino acid sequence of this putative ADR1-L2 variant. The N-terminal CC domain of RNLs is the minimal unit required for cell death, forming the basis for the calcium-permeable channel (Collier et al., 2011; Jacob et al., 2021). So, N-terminal truncations of RNLs could provide additional ways to regulate RNL activity. In the *NRG1* family, *NRG1.3* is also N-terminally truncated and negatively regulates the activity of *NRG1.1* and *NRG1.2* by competing for EDS1-SAG101 interaction (S. Huang et al., 2025; Z. Wu et al., 2022; Xiao et al., 2025). However, the truncation in *NRG1.3* is much larger compared to the putative ADR1-L2 truncation (Figure S2.6). Nevertheless, the consistent generation of this ADR1-L2 variant and its presumed truncation warrant further investigation.

Together, these findings support the existence of RNL variants that may play a role in immune responses. So far, we only identified variants for *NRG1.1* and ADR1-L2, but other RNLs may also generate additional variants that are undiscovered at this time. I believe that exploring variants of RNLs could form an entirely novel research direction that could uncover new functions of RNLs within plant immunity (Figure 4).

The importance of solving the structure of RNL resistosomes

Since the structure of the first NLR resistosome was resolved, focus of NLR research has shifted largely towards solving and understanding resistosome function (J. Wang, Hu, et al., 2019). Since then, resistosomes for various TNLs and CNLs have been resolved and revealed variation in the stoichiometry of these structures (G. Guo et al., 2026; Madhuprakash et al., 2024; Martin et al., 2020; J. Wang, Hu, et al., 2019; Zhao et al., 2022). Higher order oligomers (presumed resistosomes) of *Arabidopsis* RNLs were observed in heterologous systems (Jacob et al., 2021; H. Wang, Tan, et al., 2024, 2024; Z. Wang et al., 2023). These oligomeric complexes were also seen in *Arabidopsis* for *NRG1.2* (Feehan et al., 2023). Crucially, no resistosome structures have been solved for RNLs at this time. In Chapter 3, we depicted the

predicted ADR1-L2 resistosome as a pentameric complex, resembling the ZAR1 resistosome (Figure 3.1c, and Figure S3.1) (J. Wang, Hu, et al., 2019). The actual stoichiometry of RNL resistosomes may differ from our predicted structure. Obtaining experimental RNL resistosome structures will be essential for furthering RNL research, allowing targeted mutagenesis approaches to disrupt resistosome formation or function (Jacob et al., 2021).

In recent literature, many studies focus primarily on NLR resistosome formation and function, neglecting any potential functions outside of resistosome formation. The widespread role of RNLs in various plant immune responses raises the question whether resistosomes are required for all RNL functions. In *Arabidopsis*, the ADR1 family functions downstream of RLP signalling from the cell surface (Pruitt, Locci, et al., 2021). Indeed, PRR signalling was reported to enhance the association between ADR1-L2 and EDS1/PAD4 (H. Wang, Tan, et al., 2024). In our preliminary co-IP results, we could not corroborate this finding, although this could be attributed to unspecific immunoprecipitation of EDS1/PAD4-GFP by HA-beads in absence of ADR1-L2-HA (Figure 3.7b). Despite the association to EDS1/PAD4, PRR signalling alone was insufficient to induce oligomerization of ADR1-L2 in *N. benthamiana* (H. Wang, Tan, et al., 2024). This could indicate that ADR1-EDS1-PAD4 complexes may have additional functions outside of promoting the formation of RNL resistosomes (H. Yu et al., 2024). However, this should be further investigated in *Arabidopsis*, since native PRR responses in *N. benthamiana* were reported to not require EDS1-complexes (Zönnchen et al., 2022). Nevertheless, I believe an important next question to address is whether ADR1 resistosomes form after MAMP perception in *Arabidopsis*. Here, the distinction could be made between cell surface responses that are ADR1-independent (e.g. FLS2), ADR1-dependent without cell death (e.g. RLP23), and ADR1-dependent with cell death (e.g. RLP42, CRK7) (Pierdzig et al., 2025; L. Zhang et al., 2021, 2021)

The ADR1 family was also shown to be essential for establishing the SA-dependent immune response in bystander cells bordering the infection site (Jacob et al., 2023). Here, I would raise the question whether the formation of ADR1 resistosomes occurs within these bystander cells, or only in the PRIMER cells (Nobori et al., 2025). RNLs and lipase-like proteins were previously identified in IP-MS analysis of nuclear extracts, suggesting that these proteins may also function in the nucleus (X. Sun et al., 2021). Indeed, nuclear-localization of EDS1 and RNLs is sufficient to mediate resistance, but not cell death in TNL-activated immunity (Bernoux et al., 2023; García et al., 2010; Jianyu Li et al., 2025). Perhaps transcriptional responses leading to resistance are primarily mediated by nuclear-localized RNL/EDS1-complexes, and cell death is mediated by plasma-membrane localized RNLs through resistosome function (Jacob et al., 2021; Z. Wang et al., 2023).

Many genetic tools are now available to dissect the specific function of RNLs in various immune responses. Specific residues have been identified in *Arabidopsis* RNLs than can

disrupt their association with EDS1-PAD4 or EDS1-SAG101 (S. Huang et al., 2025; Xiao et al., 2025; H. Yu et al., 2024), plasma membrane localization (Z. Wang et al., 2023), resistosome formation, or channel function (Jacob et al., 2021). Solving the structure of RNL resistosomes will guide future mutagenesis-based approaches and help improve our understanding of all aspects of *Arabidopsis* RNL function.

Future challenges for engineering RNL-dependent immunity

In the context of climate change, plants face increasing pressure from biotic and abiotic stressors. Elevated temperatures drastically affect the plant immune system due to thermosensitivity of many immune components (C. Cheng et al., 2013; Demont et al., 2025; Kim et al., 2022; Shields et al., 2025). Engineering NLRs is a potent strategy to expand effector recognition capabilities and improve the durability of important crops to major pathogenic threats (Zdrzałek et al., 2023). Future NLR engineering efforts may shift towards maintaining NLR functionality at elevated temperatures. Many full-length TNLs are not functional at elevated temperatures, despite the thermostability of the TIR domain activity, suggesting that ligand (effector) binding or oligomerization is affected by elevated temperatures (Demont et al., 2025). Indeed, the latter was recently confirmed, connecting thermosensitivity to impaired resistosome formation for several NLRs (Grech-Baran et al., 2025). Structural comparison between thermostable and thermosensitive NLRs revealed that intramolecular interactions between the NB-ARC and LRR domain enhance the stability of NLRs. For the RNL families, NRG1 proteins remained functional at elevated temperatures, whereas ADR1 proteins lost functionality (Demont et al., 2025). Perhaps this difference in thermostability between the RNL families can guide the identification and exchange of crucial residues underlying RNL thermostability to enhance ADR1 function under heat stress. The broad involvement of TIR signalling and the ADR1 family in various branches of plant immunity emphasize the importance of understanding the thermosensitivity of these proteins (Pruitt, Gust, et al., 2021). Fundamental understanding of thermosensitivity can guide NLR engineering approaches to introduce thermostability in RNL-mediated immune responses.

Recently, a sensor-helper NLR pair (General Introduction, Figure 2c) was shown to be transferrable as a function signalling unit between plant species (Du et al., 2025). Transfer of a TNL between closely related species also mediated functional resistance (Xun et al., 2019). This may allow for engineering broad sensor-RNL networks in crops to establish resistance against a multitude of pathogens. However, EDS1 complexes from one plant species may be incompatible with RNLs from another, as seen for *Arabidopsis* and *N. benthamiana* (Lapin et al., 2019). To overcome this limitation, we can reconstitute TNL immunity in *N. benthamiana* by introduction of the full *Arabidopsis* ADR1-EDS1-PAD4 (Figure 3.5) or NRG1-EDS1-SAG101 (Lapin et al., 2019; H. Yu et al., 2024). This could potentially open the possibility of

co-transferring RNLs and their respective lipase-like proteins into new crop species (Zuijdgeest & El Kasmi, 2025). An alternative approach would be to study the functional incompatibility between RNLs and EDS1 complexes from different species. Recent studies have solved the structure of ADR1-EDS1-PAD4 and NRG1-EDS1-SAG101 trimers using N-terminally truncated RNLs and identified the primary interaction interfaces (S. Huang et al., 2025; Y. Wu et al., 2024; Xiao et al., 2025; H. Yu et al., 2024). Comparing RNL sequences for diverging amino acid residues within these interfaces could guide mutagenesis-based approaches to resurrect EDS1-compatibility between RNLs from distantly related species. Improving thermostability and enabling the transfer of RNL-based immunity across plant species would open a rich arsenal of TNLs to be utilized for crop protection in a warming climate. *Arabidopsis* alone carries hundreds of genes encoding for TIR-domain containing proteins that could then be employed (Bayless et al., 2025). The durability of resistance mediated by introduction of RNL pathways requires further investigation, as pathogens have been reported to interfere with helper NLR function (Derevnina et al., 2021; M.-Y. Wang et al., 2023). Taken together, many challenges still lie ahead for engineering RNL-based immunity across important crop species. To overcome these challenges, we require a solid fundamental understanding of the function and regulation of RNLs in the plant immune system.

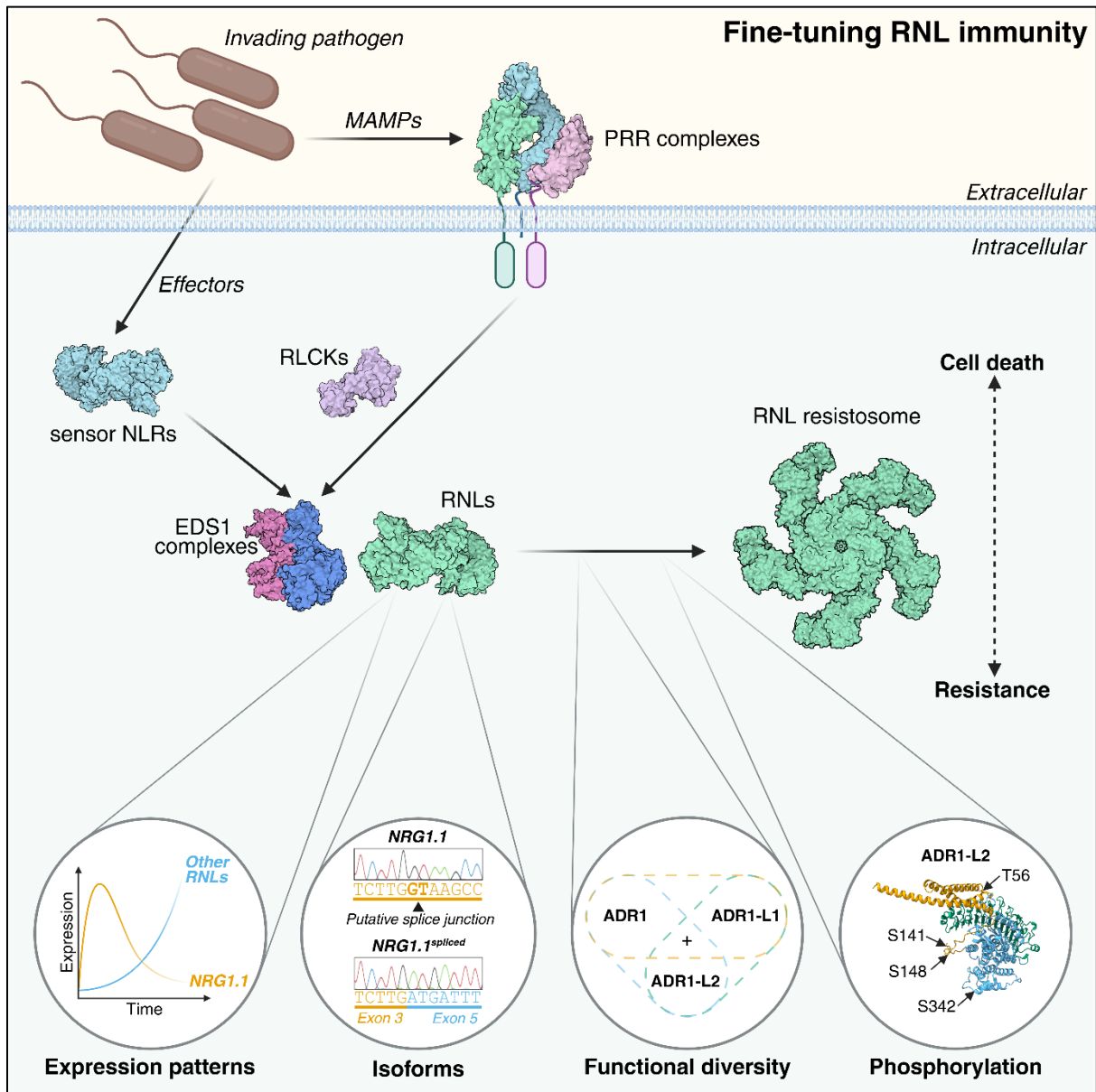


Figure 4: Schematic overview of RNL-dependent immunity in *Arabidopsis* and several mechanisms that may regulate and fine-tune RNL immune responses. Together with EDS1-complexes, RNLs function downstream of both PRR complexes and sensor NLRs. Tight regulation of RNL activity is essential to prevent unwarranted activation and achieve the desired immune response, balancing between resistance and cell death. Based on findings presented in this work, I propose various potential mechanisms that could contribute to the regulation of RNL immunity. For *NRG1.1*, we identified a unique expression pattern compared to other RNLs and a putative splice variant with unknown function. For the ADR1 family, we observed that at least two ADR1 family members are required for full immune responses, potentially also hinting towards functional diversity. For ADR1-L2 specifically, we found that phosphorylation of various serine and threonine residues can regulate the (auto-)activity of ADR1-L2. These observed properties of RNLs and their function provide grounds for new research directions and warrant further investigation to improve our understanding of RNL-dependent immune responses. Proteins are represented by various crystal structures acquired from the RCSB Protein Data Bank (PDB). The PDB accession codes and references for protein structure models used in this figure are available in Table S1. This figure was created using BioRender (www.biorender.com).

General conclusion

Helper NLRs are a central component of the plant immune system and their activity shapes the plant immune response. The work presented in this PhD thesis contributes to better understanding the function and regulation of helper NLRs by addressing the main research question:

How are *Arabidopsis* RNLs regulated to achieve the desired immune response?

In this work, I uncovered and proposed various means by which the *Arabidopsis* RNLs may be regulated to fine-tune their activity (Figure 4). Despite several unexpected challenges faced during this work, the results presented here led to new ideas on the regulation of *Arabidopsis* RNL activity, the functional divergence of RNLs, and the potential roles for RNL isoforms. These ideas provide a foundation for exciting new research directions regarding RNL function. The importance of continued research into RNLs is highlighted by their crucial role in signalling from a variety of receptor types. Understanding how RNL activity is carefully regulated to achieve the desired immune responses may ultimately make RNLs intriguing targets for engineering fine-tuned immunity against plant pathogens of all lifestyles. However, the full potential of RNLs in engineering efforts is still locked behind several challenges. Fundamental research on RNL function and regulation, like presented in this thesis, is essential to overcome these challenges.

Material and methods

Here, I describe all the methods that were used throughout this thesis. The General Supplementary Material contains additional information about all the plant genotypes (Table S2), primers (Table S3), and expression constructs (Table S4) are listed that were used and/or generated in this thesis.

Plant material and growth conditions

Arabidopsis thaliana plants were grown under either short-day conditions (8 hours light at 22°C, 16 hours dark at 20°C; 40-60% relative humidity) or long-day conditions (16 hours light at 22°C, 8 hours dark at 20°C; 40-50% relative humidity). All *Nicotiana benthamiana* plants were grown under 12 hours light at 24°C, 12 hours dark at 22°C, and 60-70% relative humidity.

Genotyping *Arabidopsis* plants

For genomic DNA extractions, plant material from *Arabidopsis* rosette leaves was snap frozen in liquid nitrogen and grinded at 30 Hz using the Mill Retsch MM400 (Retsch GmbH). Grinded tissue was resuspended in SENT buffer (200 mM Tris pH 7.5, 250 mM NaCl, 75 mM EDTA, 0.5% (w/v) SDS). Samples were centrifuged at 16000 g for 10 minutes at 4°C. The supernatant was mixed 1:1 with isopropanol and briefly incubated at -20°C. Samples were centrifuged at 16000 g for 20 minutes at 4°C. The pellet was washed with 70% ethanol and centrifuged for 8 minutes at 16000 g and 4°C. After air-drying overnight, the pellet was resuspended in 50 µl water.

Genotyping was performed by PCR using standard PCR protocols. In case of (d)CAPS-based genotyping, the required restriction reactions (documented in Table S3) were performed according to manufacturer's protocol. Genotyping results were visualized on agarose gel using Midori Green Advance (Nippon Genetics) as nucleic acid stain and documented by UV exposure using the E-Box CX5 TS Gel Documentation system (Vilber).

Phenotyping of *Arabidopsis* plants

Photographs were of 4-week-old *Arabidopsis* plants grown under long-day conditions using a Canon EOS 80D with a Canon EW-88C lens. Scalebars were added using GIMP (version 2.10.34). Fresh weight was measured of 4-week-old *Arabidopsis* rosettes and normalized against *Col-0* from the respective batch of plants. Accumulation of the immunity marker 'pathogenesis related 1' (PR1) was determined by western blot.

Semi-quantitative RT-PCR

RNA was extracted using 100 mg plant material from *Arabidopsis* or *N. benthamiana* leaves. Plant material was snap frozen in liquid nitrogen and grinded using a micro-pestle. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) with on-column DNase digestion following manufacturer's specification.

Copy DNA (cDNA) was generated from 500 ng RNA using SuperScript IV Reverse Transcriptase (Invitrogen) according to manufacturer's specifications. 2 µl of cDNA was used as template for standard PCR protocols. Amplicons were compared on agarose gel using Midori Green Advance (Nippon Genetics) as nucleic acid stain and documented by UV exposure using the E-Box CX5 TS Gel Documentation system (Vilber).

Cloning of expression constructs

Expression constructs for *Agrobacterium tumefaciens* transient assays were constructed using standard Gateway (Invitrogen) cloning procedures or by GoldenGate assembly (Binder et al., 2014).

For Gateway cloning, pDONR207 was used as entry vector and pMDC7 (Curtis & Grossniklaus, 2003) and pGWB614 (Nakagawa et al., 2007) were used as destination vectors. BP-flanked genes-of-interest were generated by PCR. Subsequent BP and LR reactions were performed according to manufacturer's specifications. Inducible expression constructs were generated using the estradiol-inducible receptor-based trans-activator XVE system (Zuo et al., 2000).

For GoldenGate cloning, promoter regions and genes-of-interest flanked with BsaI sites were generated by PCR. Level I constructs were generated by blunt-end cloning of these PCR fragments into the pGEM T-Easy vector (Promega). Level II constructs were created by BsaI cut-ligation using various level I modules and the BB10 vector as backbone (Binder et al., 2014).

Correctly assembled constructs were identified using a combination of colony PCRs, restriction-digest, and Sanger sequencing (Genewiz). The expression constructs were transformed into chemically competent *Agrobacterium tumefaciens* GV3101 by heatshock at 37°C and subsequent recovery at 28°C.

Generation of stable transgenic *Arabidopsis* lines

For transgenic *Arabidopsis* lines, genes-of-interest were cloned using GoldenGate methods under their native promoters with either HA or YFP C-terminal tags, a Nopaline Synthase (NOS) gene terminator, and a FastRed selection marker (Table S4). RNL genes were cloned as coding sequence, unless specified otherwise. *Arabidopsis* plants were transformed by floral dipping in *Agrobacterium tumefaciens* carrying the generated constructs (Bent, 2006). Transformed seeds were selected by red fluorescence from the encoded FastRed selection marker (Shimada et al., 2010). Protein samples were harvested from transgenic plants to investigate the expression of the transgene by western blot. Segregation analysis using the FastRed selection markers was done to identify homozygous stable transgenic lines carrying only a single transgene insertion.

CRISPR-Cas9 knock-out of *Arabidopsis* genes

Single guide RNAs targeting the *Arabidopsis* *RNL* and *SNC1* gene loci were designed using ChopChop v3 (Labun et al., 2019). 10 μ M oligonucleotides (**Table S3**) containing the single guide RNA sequences were hybridized by a denaturing step at 98°C and subsequent cooling down to room temperature. A 1:200 dilution of the hybridized oligos was used for cloning. The CRISPR-Cas9 constructs were generated with the pDGE vector system using GoldenGate cloning procedures (Stuttman et al., 2021). Constructs were transformed into *Arabidopsis Col-0* by floral dipping in *Agrobacterium* cultures. Transformed seeds were selected using the encoded FastRed selection marker. Successful gene locus deletions were identified by PCR and confirmed by Sanger sequencing (Genewiz). An overview of all the generated deletions is provided in Figure S1.1.

***Pseudomonas syringae* infection of *Arabidopsis* plants**

Infections were performed with *Pseudomonas syringae* pv. *DC3000* with an empty vector or a vector carrying *AvrRps4* from *P. syringae* pv. *pisi* (Sohn et al., 2009). *P. syringae* strains were grown on LB agar (Luria/Miller, Roth) with 25 μ g/ml Rifampicin and 50 μ g/ml Kanamycin. Bacteria were harvested from the plates by washing with 10 mM $MgCl_2$. The OD₆₀₀ (optical density) for all strains was set to 0.001 and hand-infiltrated using a 1 ml syringe. After 4 hours (day 0) and 72 hours (day 3), bacteria were extracted from 3 leaf disks ($\varnothing$ 0.5 cm) for counting colony forming units (CFU). Bacterial extracts were plated onto LB agar with 25 μ g/ml Rifampicin, 50 μ g/ml Kanamycin, and 50 μ g/ml Cyclohexamide and incubated for 40+ hours at 28°C before counting the CFU.

Agrobacterium* transient transformation assays in *N. benthamiana

Agrobacterium tumefaciens GV3101 overnight cultures were centrifuged at 8000 g for 2 minutes and resuspended in induction buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, 150 μ M acetosyringone). The OD₆₀₀ was adjusted to 0.3 for all expression constructs, except for 35S::P19 which was set to 0.05 (Win & Kamoun, 2004). *Agrobacterium* was infiltrated into leaves from 4-5-week-old *N. benthamiana* plants. Induction of estradiol-inducible promoters was done 48 hours after infection by spraying 20 μ M estradiol (Sigma-Aldrich) with 0.001% (v/v) Silwet L-77. Cell death was captured after 24 hours after estradiol treatment (Chapter 2) or 5 days after *Agrobacterium* transformation for native RNL promoters (Chapter 3)

Agromonas* infection assays in *N. benthamiana

The workflow for the *Agromonas* infection assays in *N. benthamiana* was adapted from a previous report (Buscaill et al., 2021). Leaves from *N. benthamiana* *adr1 nrg1 eds1 pad4 sag101a sag101b* plants were infiltrated with *Agrobacterium* 48 hours before infecting with *Pseudomonas* (J. Wang, Sun, et al., 2025). Samples for protein extraction were taken 24 hours after *Agrobacterium* infiltration and accumulation of proteins was determined by western blot.

P. syringae pv. *tomato* DC3000 empty vector was grown overnight on LB with 25 µg/ml Rifampicin and 50 µg/ml Kanamycin. Bacteria were collected using 10 mM MgCl₂ and the OD₆₀₀ was adjusted to 0.0001. The transiently transformed *N. benthamiana* leaves were infiltrated with *P. syringae* using a needleless syringe. After 3 hours (day 0) and after 72 hours (day 3), bacteria were extracted from four leaf disks (ø 0.5 cm) for CFU counting. Bacterial extracts were plated onto LB agar with C-F-C supplement (Oxoid) to selectively grow *Pseudomonas* and incubated for 40+ hours at 28°C before counting the CFU.

Protein extraction

Plant material was snap frozen in liquid nitrogen and lysed at 30 Hz using a Mill Retsch MM400 (Retsch GmbH). Lysed tissue was resuspended in grinding buffer (20 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 5 mM DTT, 1x Halt™ Proteinase Inhibitor Cocktail (Thermo Fischer Scientific), vortexed vigorously and then incubated on ice for 5 minutes. Samples were centrifuged at 16000 g for 15 min at 4°C. 5x SDS loading buffer (250 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 500 mM DTT, 10% (w/v) SDS, 0.005% (w/v) bromophenol blue) was added to the supernatant. Proteins were denatured at 95°C for 5 minutes and then used for western blotting.

Co-immunoprecipitation

Plants were infiltrated with *Agrobacterium* GV3101 carrying expression constructs as previously described. After 48 hours, infiltrated leaves were infected with *P. syringae* pv. *DC3000* empty vector (OD₆₀₀ = 0.2). Leaf tissue was harvested 8 hours after the *Pseudomonas* infection. Leaf tissue was snap-frozen with liquid nitrogen and then ground using a pestle and mortar before adding extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% Triton X-100 (Sigma-Aldrich), 10% glycerol, 10 mM DTT and 1× plant protease inhibitor cocktail). Cell debris was precipitated and removed by subsequent centrifugation steps at >14000 g for 5 minutes and then 15 minutes. Input samples were collected from the supernatant and 5x SDS loading buffer was added. The remainder of the supernatant was added to 20 µl HA-Trap Agarose beads (Chromotek) and incubated on a rotor for 2 hours at 10°C. The beads were then washed four times with extraction buffer (without DTT). 2x SDS loading buffer (100 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 200 mM DTT, 2% (w/v) SDS, 0.0002% (w/v) bromophenol blue) was added before boiling the samples at 95°C for 5 minutes. Input and (co-)IP samples were analysed by western blot.

Western blot analysis

Western blotting and immunodetection followed standard procedures. SDS-PAGE gels were loaded with 60-80 µg of protein and run at 140V in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell chamber (BioRad). The proteins were transferred onto a nitrocellulose membrane using a Trans-Blot Turbo Transfer system (BioRad) at 25V for 30 minutes. Ponceau

S staining (1% (w/v) Ponceau S, 2% (w/v) acetic acid) was used as loading control. Primary antibodies used throughout this thesis were α -HA (1:2000, from Rat, Roche), α -GFP (1:1500, from Mouse, Roche), α -Myc (1:5000, from Rat, Chromotek), and α -PR1 (1:2500, from Rabbit, Agrisera). Appropriate secondary antibodies used were α -rat (1:5000, Invitrogen), α -mouse (1:10000, Sigma-Aldrich), and α -rabbit (1:10000, Sigma-Aldrich). Chemiluminescence was detected using ECLTM Prime Western Blotting Detection Reagent (Cytiva) and the Amersham ImageQuant 800 (GE Healthcare). TriColor Protein Ladder (10-180 kDa, Biotechrabbit) was used to assess protein sizes on the western blot.

Cell death documentation and quantification

Cell death in *N. benthamiana* was recorded using the Amersham ImageQuant 800 (GE Healthcare). Images were taken using the Cy3 (4.70 sec), Cy5 (0.10 sec) and visible light channels. False colours were attributed to healthy tissue (green) and dead tissue (red). Cell death quantification was done by measuring pixel intensity from the Cy3 channel using FIJI (Schindelin et al., 2012). Pixel intensity was measured across three circular sites ($\varnothing$ 50 pixels) per infiltration spot. The mean pixel intensity was then calculated for each infiltration spot and adjusted for the background signal from the respective *N. benthamiana* leaf. The normalized pixel intensity mean was used as a measure for cell death intensity.

Confocal microscopy imaging and analysis

Co-localization of ADR1-L2 variants (YFP) and the plasma membrane marker Lti6b (RFP) was performed with an inverse confocal laser scanning microscope LSM88 (Zeiss) using a 63x water immersion objective. YFP fluorophores were excited at 514 nm and emission was detected at 520-555 nm. RFP fluorophores were excited at 561 nm and emission was detected at 580-660 nm. Image analysis was performed using FIJI (Schindelin et al., 2012). Co-localization was analysed in Zeiss Zen 3.8 by capturing the fluorescence intensity profile of each channel across the indicated segment.

Protein alignment and structural predictions

Protein sequences were aligned with the MUSCLE Multiple Sequence Alignment tool at EMBL-EBI (Madeira et al., 2024). AlphaFold3 was used to predict protein structures based on their amino acid sequences (Abramson et al., 2024). Molecular graphics and analyses of AlphaFold3 structures were performed with UCSF ChimeraX 1.11.1 (Meng et al., 2023).

Data visualization and statistical analysis

Figures in the result sections were created in Inkscape 1.2. Data visualization was done in RStudio R4.3.1 (R Core Team, 2021) using the 'ggplot2' package (Wickham, 2016). Colourblind-friendly colours were selected from the colour palette Okabe & Ito (Ichihara et al., 2008). Statistical analysis was performed in R (version 4.3.1). The significant level (α) was always set to 0.05 in all analyses. The performed statistical tests are listed in the captions of

respective figures. Normality of data and homogeneity of variance was assessed prior to testing. Statistical analyses were integrated into graphs using a compact letter display generated with the packages 'emmeans', 'multcomp', and 'multcompView' in R.

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General supplementary material

Table S1: Structures used in Figures 1-4 in this PhD thesis and their Protein Data Bank (PDB) identifiers. Each object is represented by a protein structure available at the Protein Data Bank using the identifier listed in this table. The reference to the source publication of each structure is also provided.

Object	Represented by	Identifier	Reference
BAK1/BKK1	BAK1 ectodomain	3UIM	(L. Yan et al., 2012)
CNL monomer	NAIP5	8FML	(Paidimuddala et al., 2023)
CNL resistosome	ZAR1 resistosome	6J5T	(J. Wang, Hu, et al., 2019)
EDS1-PAD4	EDS1-PAD4-ADR1-L1	8ZW9	(H. Yu et al., 2024)
EDS1-PAD4-ADR1	EDS1-PAD4-ADR1-L1	8ZW9	(H. Yu et al., 2024)
EDS1-SAG101	EDS1-SAG101-NRG1.1	8YL7	(Xiao et al., 2025)
EDS1-SAG101-NRG1	EDS1-SAG101-NRG1.1	8YL7	(Xiao et al., 2025)
RLCK	BIK1	5TOS	(Lal et al., 2018)
RLK	FLS ectodomain	4MNA	(Y. Sun et al., 2013)
RLP	FLS ectodomain	4MNA	(Y. Sun et al., 2013)
RNL monomer	NAIP5	8FML	(Paidimuddala et al., 2023)
RNL resistosome	ZAR1 resistosome	6J5T	(J. Wang, Hu, et al., 2019)
SOBIR1	SOBIR1 ectodomain	6R1H	(Hohmann & Hothorn, 2019)
TNL monomer	NAIP5	8FML	(Paidimuddala et al., 2023)
TNL resistosome	RPP1 resistosome	7CRC	(Ma et al., 2020)

Table S2: Plant genotypes used in this work and their origin. The source publication is listed for each genotype. T-DNA insert lines were obtained from the Syngenta *Arabidopsis* Insertion Library (SAIL) or from the Salk Institute Genomic Analysis Laboratory (SALK).

Plant	Genotype	Reference
<i>A. thaliana</i>	<i>Col-0</i> (wildtype)	
<i>A. thaliana</i>	<i>adr1-1</i>	SAIL_842_B05
<i>A. thaliana</i>	<i>adr1-l1-1</i>	SAIL_302_C06
<i>A. thaliana</i>	<i>adr1-l2-4</i>	SALK_126422
<i>A. thaliana</i>	<i>nrg1.1-2</i>	SALK_005656
<i>A. thaliana</i>	<i>nrg1.2-2</i>	SALK_020974
<i>A. thaliana</i>	<i>nrg1 double</i>	(Castel et al., 2019)
<i>A. thaliana</i>	<i>eds1-12</i>	(Ordon et al., 2017)
<i>A. thaliana</i>	<i>pad4-1</i>	(Jirage et al., 1999)
<i>A. thaliana</i>	<i>sag101-2</i>	(Feys et al., 2005)
<i>A. thaliana</i>	<i>sid2-1</i>	(Nawrath & Métraux, 1999)
<i>A. thaliana</i>	<i>rar1-21</i>	(Tornerio et al., 2002)
<i>A. thaliana</i>	<i>pADR1-L2::ADR1-L2 D484V adr1-l2-4</i>	(Roberts et al., 2013)
<i>A. thaliana</i>	<i>adr1-c</i>	This work (Figure S1.1)
<i>A. thaliana</i>	<i>adr1-l1-c1</i>	(M.-Y. Wang et al., 2023)
<i>A. thaliana</i>	<i>adr1-l2-c</i>	This work (Figure S1.1)
<i>A. thaliana</i>	<i>nrg1.1-c</i>	This work (Figure S1.1)
<i>A. thaliana</i>	<i>nrg1 double-c</i>	This work (Figure S1.1)
<i>A. thaliana</i>	<i>snc1-c</i>	This work (Figure S1.1)
<i>N. benthamiana</i>	Wildtype	
<i>N. benthamiana</i>	<i>adr1 nrg1</i>	(T. Qi et al., 2018) (H. Wang, Song, et al., 2024)
<i>N. benthamiana</i>	<i>eds1 pad4 sag101a sag101b</i>	(Lapin et al., 2019)
<i>N. benthamiana</i>	<i>adr1 nrg1 eds1 pad4 sag101a sag101b</i>	(J. Wang, Sun, et al., 2025)

Table S3: Primers used in this work. All primers are listed with their name, sequence and a description of the purpose. The primer list is separated into primers for genotyping, RT-PCRs, CRISPR-Cas9 single guide RNAs, cloning, and site-directed mutagenesis.

Name	Sequence	Description
Genotyping		
<i>ADR1_fw</i>	CAAAGGACGATGATGTTTCGAG	Genotyping <i>adr1-1</i>
<i>ADR1_rv</i>	CGGATTGTTCACTATAGTAAGG	
<i>adr1-1_fw</i>	TTTCATAACCAATCTCGATACAC	
<i>ADR1-L1_fw</i>	ATGGCCATCACCATTTC	Genotyping <i>adr1-l1-1</i>
<i>ADR1-L1_rv</i>	GTCAGGAACAGGATTTCCAG	
<i>adr1-l1-1_fw</i>	TAGCATCTGAATTTTCATAACCAATCTCG	
<i>ADR1-L2_fw</i>	TGGGAGATTGTGACACAGTC	Genotyping <i>adr1-l2-4</i>
<i>ADR1-L2_rv</i>	ATGGCAGATATAATCGGCGG	
<i>adr1-l2-4_fw</i>	CAACATCTCCTTCACCTTCC	
<i>NRG1.1_fw</i>	GATTCGATGCAAAAAGAGCTTGA	Genotyping <i>nrg1.1-2</i>
<i>NRG1.1_rv</i>	GGTTTCAAACGATAATTGGAATCA	
<i>nrg1.1-2_rv</i>	TGGTTCACGTAGTGGGCCATCG	
<i>NRG1.2_fw</i>	AGAACTCCTTGACAATTCCG	Genotyping <i>nrg1.2-2</i>
<i>NRG1.2_rv</i>	CCGAGATGAACAAGTTTAAGC	
<i>nrg1.2-2_rv</i>	GCGTGGACCGCTTGCTGCAACT	
<i>NRG1double_fw</i>	GCATCTCCACCTCTTCA	Genotyping <i>nrg1 double</i> Avall digest required
<i>NRG1double_rv</i>	CTGAAGAAATGAACCCATGT	
<i>Actin2_fw</i>	AATCACAGCACTTGACCAAGCAGC	Actin2 RT-PCR control
<i>Actin2_rv</i>	CATCATACTCGGCCTTGAGATC	
<i>EDS1_fw</i>	AACTAGCATAAGAGGGGCA	Genotyping <i>eds1-12</i> Three-primer PCR
<i>EDS1_rv</i>	GCTGAGAGAAATCGAACCGG	
<i>eds1-12_rv</i>	CTGGTTTCCACTTCACGATGA	
<i>PAD4_fw</i>	ATGAGTCGCATAAGACTAGCCAAG	Genotyping <i>pad4-1</i> PflmI digest required
<i>PAD4_rv</i>	CCATTTCTTTCTAAATGAAAATCA	
<i>SAG101_fw</i>	GATCTTGAGATACATAACCC	Genotyping <i>sag101-2</i>
<i>SAG101_rv</i>	CATCTTCAGGGATCAATCTTGAATCCG	
<i>sag101-2_rv</i>	GGTGCAGCAAAACCCACACTTTTACTTC	
<i>SID2_fw</i>	AAGCTTGCAAGAGTGCAACA	Genotyping <i>sid2-1</i> MseI digest required
<i>SID2_rv</i>	AAACAGCTGGAGTTGGATGC	
<i>RAR1_fw</i>	CACGACGGAATGAAAGAGTGGAGCTGCTGCTAG	Genotyping <i>rar1-21</i> NheI digest required
<i>RAR1_rv</i>	CCGCAACAGGATGTTTTGGAACCGATTGGCC	
<i>CRK13_fw</i>	GATTGTTCTAACTGTATCAAGGGCG	Genotyping <i>crk13 deletion</i> <i>AcuI</i> digest optional
<i>CRK13_rv</i>	GTCTTCCCATATTGTCTTGAACCTCTG	
<i>ADR1_fw_2</i>	CAGCATAACCAACTGTCC	Genotyping <i>adr1-c</i>
<i>ADR1_rv_2</i>	CTGCTGATCAATGATTAATACAAGGG	

Name	Sequence	Description
<i>adr1-c_fw</i>	GGTACTAACC GGTTGACTATTCC	
<i>ADR1-L1_fw_2</i>	TGTCCCACATGCTAGTTTC	Genotyping <i>adr1-l1-c</i>
<i>ADR1-L1_rv</i>	GTCAGGAACAGGATTTCCAG	
<i>adr1-l1-c_rv</i>	GTCGAGGAATTTGGAACAG	
<i>ADR1-L1_fw_3</i>	GACGATACTAGGATTGTTGTTTGG	
<i>ADR1-L1_rv_2</i>	CCTAGACTGGCTTGACGAATAA	Genotyping <i>adr1-l1-c1</i>
<i>adr1-l1-c1_rv</i>	TTCTTGCAAATCACCTAAATTC	
<i>ADR1-L2_fw_2</i>	CAAAAGGTGACTGCAACGAAG	Genotyping <i>adr1-l2-c</i>
<i>ADR1-L2_rv</i>	TGGGAGATTGTGACACAGTC	
<i>adr1-l2-c_rv</i>	TTTGCACTGACTCTTGACTC	
<i>NRG1.1_fw_2</i>	TTACTAACCACGCTGTATC	Genotyping <i>nrg1.1-c</i>
<i>NRG1.1_rv_2</i>	ACGTCAACTGATTCCCTATGCTGAAGAAGC	
<i>nrg1.1-c_rv</i>	GGCATGACATTTTCTCTGTAC	
<i>NRG1.2_fw_2</i>	ACGTTGCAATGGATTCC	Genotyping <i>nrg1.2-c</i>
<i>NRG1.2_rv_2</i>	TTCTTTGCATGATTGGTTCC	
<i>nrg1.2-c_fw</i>	TGGAGTATTATGCTTTTGGG	
<i>SNC1_fw</i>	TCTGTGGAGAGGAAAAGTAGC	Genotyping <i>snc1-c</i>
<i>SNC1_rv</i>	CCACGAAAGCTTGGGAAAAC	
<i>snc1-c_rv</i>	CAGCGGATATGCAAATGTTG	
Semi-quantitative RT-PCRs		
<i>ADR1_fw</i>	CAAAGGACGATGATGTTTCGAG	RT-PCR for <i>ADR1</i>
<i>ADR1_rv</i>	CGGATTGTTCACTATAGTAAGG	
<i>NRG1.1_spliced_fw</i>	AGTAGTGGGTGTTTCACTC	<i>NRG1.1^{spliced}</i> identification
<i>NRG1.1_spliced_rv</i>	CCGTTTCAGGTTTGGTAATGAGC	
<i>NRG1.1_start_fw</i>	ATGAACGATTGGGCTAGTTTGGG	<i>NRG1.1</i> start-to-stop
<i>NRG1.1_stop_rv</i>	AAACATTTGAAGCAAGTTCAGGTTATGC	
<i>NRG1.1_exon1_1_fw</i>	GATTCGATGCAAAAAGAGCTTGACT	<i>NRG1.1</i> exon 1 primer
<i>NRG1.1_exon1_2_fw</i>	GCTGATGTAGCTGTTTCGGAAGTTTCCAAGAGTC	<i>NRG1.1</i> exon 1 primer
<i>NRG1.1_exon1_3_fw</i>	GACGATCCAGATATCAAAG	<i>NRG1.1</i> exon 1 primer
<i>NRG1.1_exon2_fw</i>	CACGTCTCCTGATGAGTA	<i>NRG1.1</i> exon 2 primer
<i>NRG1.1_splice-junction_rv</i>	GAGAACAAATCATCAAGAATTTTTTC	Putative <i>NRG1.1</i> splice junction primer
CRISPR-Cas9 single guide RNA oligonucleotides		
<i>sgRNA_adr1-c_5'_fw</i>	attgCCACCGTATAGTACACATG	sgRNA cassette for CRISPR-Cas9 deletion of <i>ADR1</i>
<i>sgRNA_adr1-c_5'_rv</i>	aaacCATGTGTACTATACGGTGGC	
<i>sgRNA_adr1-c_3'_fw</i>	attgCGCCAAAAAATGCTTCACCG	
<i>sgRNA_adr1-c_3'_rv</i>	aaacCGGTGAAGCATTTTTTGGCG	
<i>sgRNA_adr1-l1-c_5'_fw</i>	attgATTGCTAGGAAATTTTCAAG	sgRNA cassette for CRISPR-Cas9 deletion of <i>ADR1-L1</i>
<i>sgRNA_adr1-l1-c_5'_rv</i>	aaacCTTGAAAATTTCTAGCAAT	
<i>sgRNA_adr1-l1-c_3'_fw</i>	attgTTCCCACATAAATGCAACGT	

Name	Sequence	Description
<i>sgRNA_adr1-l1-c_3'_rv</i>	aaacACGTTGCATTTATGTGGGAA	sgRNA cassette for CRISPR-Cas9 deletion of <i>ADR1-L2</i>
<i>sgRNA_adr1-l2-c_5'_fw</i>	attgCTCGTGAGGCAGCTCTATG	
<i>sgRNA_adr1-l2-c_5'_rv</i>	aaacCATAGAGCTGCCTCACGAGC	
<i>sgRNA_adr1-l2-c_3'_fw</i>	attgACATGCTTCTAAGGGCTCGG	
<i>sgRNA_adr1-l2-c_3'_rv</i>	aaacCCGAGCCCTTAGAAGCATGT	
<i>sgRNA_nrg1.1-c_5'_fw</i>	attgTCCGTCTATCGAACAACGTG	sgRNA cassette for CRISPR-Cas9 deletion of <i>NRG1.1</i>
<i>sgRNA_nrg1.1-c_5'_rv</i>	aaacCACGTTGTTTCGATAGACGGA	
<i>sgRNA_nrg1.1-c_3'_fw</i>	attgCGTTATCGTAGTAGCTTCTG	
<i>sgRNA_nrg1.1-c_3'_rv</i>	aaacCAGAAGCTACTACGATAACG	
<i>sgRNA_nrg1.2-c_5'_fw</i>	attgTCTAATATATAAAATCATGC	sgRNA cassette for CRISPR-Cas9 deletion of <i>NRG1.2</i>
<i>sgRNA_nrg1.2-c_5'_rv</i>	aaacGCATGATTTTATATATTAGA	
<i>sgRNA_nrg1.2-c_3'_fw</i>	attgTCACTCAACATAATATTCTG	
<i>sgRNA_nrg1.2-c_3'_rv</i>	aaacCAGAATATTATGTTGAGTGA	
<i>sgRNA_snc1-c_5'_fw</i>	attgTATAGAGTAGAAGCTTTCCA	sgRNA cassette for CRISPR-Cas9 deletion of <i>SNC1</i>
<i>sgRNA_snc1-c_5'_rv</i>	aaacTGGAAAGCTTCTACTCTATA	
<i>sgRNA_snc1-c_3'_fw</i>	attgGTACGCTCCCAAATTCAATG	
<i>sgRNA_snc1-c_3'_rv</i>	aaacCATTGAATTTGGGAGCGTAC	
Cloning		
<i>ADR1_attB1_fw</i>	GGGGACAAGTTTGTACAAAAAAGCAG GCTtaATGGCTTCGTTTCATAGATCT	GateWay cloning for <i>ADR1</i>
<i>ADR1_attB2_rv</i>	GGGGACCACTTTGTACAAGAAAGCTG GGTgATCGTCAAGCCAATCCACGG	
<i>ADR1-L1_attB1_fw</i>	GGGGACAAGTTTGTACAAAAAAGCAG GCTtaATGGCCATCACCATTGTTTTT	GateWay cloning of <i>ADR1-L1</i>
<i>ADR1-L1_attB2_rv</i>	GGGGACCACTTTGTACAAGAAAGCTG GGTgTTCGTCAAGCCAGTCTAGGC	
<i>ADR1-L2_attB1_fw</i>	GGGGACAAGTTTGTACAAAAAAGCAG GCTtaATGGCAGATATAATCGGCGG	GateWay cloning of <i>ADR1-L2</i>
<i>ADR1-L2_attB2_rv</i>	GGGGACCACTTTGTACAAGAAAGCTG GGTgATCGTCGAGCCAATCCCTGC	
<i>NRG1.1_attB1_fw</i>	GGGGACAAGTTTGTACAAAAAAGCAG GCTtaATGAACGATTGGGCTAGTTTGG	GateWay cloning of <i>NRG1.1</i>
<i>NRG1.1_attB2_rv</i>	GGGGACCACTTTGTACAAGAAAGCTG GGTtAAACATTTGAAGCAAGTTCAGGTTATGC	
<i>NRG1.2_attB1_fw</i>	GGGGACAAGTTTGTACAAAAAAGCAG GCTtaATGGTCGTGGTCGATTGGCTTGG	GateWay cloning of <i>NRG1.2</i>
<i>NRG1.2_attB2_rv</i>	GGGGACCACTTTGTACAAGAAAGCTG GGTtAAACGTTAGAAGCAACTTCAGG	
<i>NRG1.1_BsaI_fw</i>	gtGGTCTCaTCTGATGAACGATTGGGCTAGTTTGGGA	GoldenGate cloning of <i>NRG1.1</i>
<i>NRG1.1_BsaI_rv</i>	atGGTCTCaCCTTAAACATTTGAAGCAAGTTCAGGTTATG	

Name	Sequence	Description
<i>NRG1.3_Bpil_Bsal_fw</i>	ggGAAGACTaTACGGGTCTCaCACCA TGAATTCGAATTCGATTCAAAG	GoldenGate cloning of <i>NRG1.3</i>
<i>NRG1.3_Bpil_Bsal_rv</i>	ggGAAGACTaCAGAGGTCTCaCCTTA AACAGTTGAAGCAAGTTGAG	
<i>pADR1-L2_Bsal_fw</i>	gtGGTCTCaGCGGTTTGTCTTACT GTGTGTCCCCAG	GoldenGate cloning of <i>ADR1-L2</i> promoter
<i>pADR1-L2_Bsal_rv</i>	atGGTCTCaCAGAGGCAAAGGCCGAT GAGAAAAAGAGAG	
<i>ADR1-L2_Bsal_fw</i>	gtGGTCTCaTCTGATGGCAGATATAA TCGGCGGCCGAAG	GoldenGate cloning of <i>ADR1-L2</i>
<i>ADR1-L2_Bsal_rv</i>	atGGTCTCaCCTTATCGTCGAGCCAA TCCCTGCTGAAAG	
<i>pADR1_Bsal_fw</i>	gtGGTCTCaGCGGGCTAACTTATGTT GACTTCACGA	GoldenGate cloning of <i>ADR1</i> promoter
<i>pADR1_Bsal_rv</i>	atGGTCTCaCAGACGAGACCGATCTT GGAGTGTAAG	
<i>ADR1_Bsal_fw</i>	gtGGTCTCaTCTGATGGCTTCGTTCA TAGATCTTTTCG	GoldenGate cloning of <i>ADR1</i>
<i>ADR1_Bsal_rv</i>	atGGTCTCaCCTTATCGTCAAGCCAA TCCACGGTGAAGC	
<i>pADR1-L1_Bsal_fw</i>	gtGGTCTCaGCGGTCTATTGTCCAAT TTATTCTTTAC	GoldenGate cloning of <i>ADR1-L1</i> promoter
<i>pADR1-L1_Bsal_rv</i>	atGGTCTCaCAGATGGATACCACCAA GTCAAGTCAATGG	
<i>ADR1-L1_Bsal_fw</i>	gtGGTCTCaTCTGATGGCCATCACCG ATTTTTTCGCCG	GoldenGate cloning of <i>ADR1-L1</i>
<i>ADR1-L1_Bsal_rv</i>	atGGTCTCaCCTTTTCGTCAAGCCAG TCTAGGCTGAAGC	
Site-Directed Mutagenesis		
<i>SDM_NRG1.1_G391G*_fw</i>	GAAAAAATTCTTGGgAAGCCTTATCC AAC	SDM of <i>NRG1.1 G391G*</i>
<i>SDM_NRG1.1_G391G*_rv</i>	GTTGGATAAGGCTTcCCAAGAATTTT TTC	
<i>SDM_ADR1_D461V_fw</i>	GACGCAACACGtTGTTTTGAGAGACC TAG	SDM of <i>ADR1 D461V</i>
<i>SDM_ADR1_D461V_rv</i>	CTAGGTCTCTCAAAACAaCGTGTTGC GTC	
<i>SDM_ADR1-L1_D489V_fw</i>	GTGACACAGCATGtTGTTCTGCGAGA C	SDM of <i>ADR1-L1 D489V</i>
<i>SDM_ADR1-L1_D489V_rv</i>	GTCTCGCAGAACAaCATGCTGTGTCA C	
<i>SDM_ADR1-L2_D484V_fw</i>	GTCACGCAGCATGtTGTTCTAAGAGA TG	SDM of <i>ADR1-L2 D484V</i>
<i>SDM_ADR1-L2_D484V_rv</i>	CATCTCTTAGAACAaCATGCTGCGTG AC	
<i>SDM_NRG1.1_D485V_fw</i>	GTGACTCAACATGtTATCCTTAGGGA G	SDM of <i>NRG1.1 D485V</i>
<i>SDM_NRG1.1_D485V_rv</i>	CTCCCTAAGGATAaCATGTTGAGTCA C	
<i>SDM_NRG1.2_N490V_fw</i>	GTCACTCAACATgtTATTCTGAGGGA G	SDM of <i>NRG1.2 N490V</i>

Name	Sequence	Description
<i>SDM_NRG1.2_N490V_rv</i>	CTCCCTCAGAATAacATGTTGAGTGA C	
<i>SDM_NbNRG1_D519V_fw</i>	CATACAGCAGCATGtTCTGCTGAGAG AACTG	SDM of <i>NbNRG1 D519V</i>
<i>SDM_NbNRG1_D519V_rv</i>	CAGTTCTCTCAGCAGAAcATGCTGCT GTATG	
<i>SDM_RPM1_D505V_fw</i>	CAAGGCGTTCAAGATGCATGtCGTGA TATGGGAG	SDM of <i>RPM1 D505V</i>
<i>SDM_RPM1_D505V_rv</i>	CTCCCATATCACGaCATGCATCTTGA ACGCCTTG	
<i>SDM_RPS2_N473V_fw</i>	CAGGTGAAGATGCATgtTGTGGTCAG AAGCTTTG	SDM of <i>RPS2 N473V</i>
<i>SDM_RPS2_N473V_rv</i>	CAAAGCTTCTGACCACAacATGCATC TTCACCTG	
<i>SDM_PML5_D360V_fw</i>	GTTGTGATGCATGtCGTGGTTCGTG	SDM of <i>PML5 D360V</i>
<i>SDM_PML5_D360V_rv</i>	CACGAACCACGaCATGCATCACAAC	
<i>SDM_PML11_D473V_fw</i>	ATTGTGTGATGCATGtCGTGGTTCGT GAAAT	SDM of <i>PML11 D473V</i>
<i>SDM_PML11_D473V_rv</i>	ATTTACGAACCACGaCATGCATCAC ACAAT	
<i>SDM_NRG1.1_P-loop_fw</i>	CCTCCTGGTTGCGcGgcAgCCACGCT GGTTAGT	SDM of <i>NRG1.1 P-loop</i>
<i>SDM_NRG1.1_P-loop_rv</i>	ACTAACCAGCGTGGcTgcCgCGCAAC CAGGAGG	
<i>SDM_NRG1.1_L134E_fw</i>	GACGGGAAACgagGTGAATTCGGTTG	SDM of <i>NRG1.1 L134E</i>
<i>SDM_NRG1.1_L134E_rv</i>	CAACCGAATTCACctcGTTTCCCGTC	
<i>SDM_ADR1-L2_T56D_fw</i>	GCGTCGAGCTCgacCCTCATCGC	SDM of <i>ADR1-L2 T56D</i>
<i>SDM_ADR1-L2_T56D_rv</i>	GCGATGAGGgtcGAGCTCGACGC	
<i>SDM_ADR1-L2_T56A_fw</i>	GCGGCGTCGAGCTCgCACCTCATCGC	SDM of <i>ADR1-L2 T56A</i>
<i>SDM_ADR1-L2_T56A_rv</i>	GCGATGAGGTGcGAGCTCGACGCCGC	
<i>SDM_ADR1-L2_S141D_fw</i>	GGAAGGTTGATgaTTTGAATGAGAAG C	SDM of <i>ADR1-L2 S141D</i>
<i>SDM_ADR1-L2_S141D_rv</i>	GCTTCTCATTCAAAtcATCAACCTTC C	
<i>SDM_ADR1-L2_S141A_fw</i>	GGAAGGTTGATgcTTTGAATGAGAAG C	SDM of <i>ADR1-L2 S141A</i>
<i>SDM_ADR1-L2_S141A_rv</i>	GCTTCTCATTCAAAGcATCAACCTTC C	
<i>SDM_ADR1-L2_S148D_fw</i>	GAAGCTTGGTgaTATGAAACTCAG	SDM of <i>ADR1-L2 S148D</i>
<i>SDM_ADR1-L2_S148D_rv</i>	CTGAGTTTCATAtcACCAAGCTTC	
<i>SDM_ADR1-L2_S148A_fw</i>	GAGAAGCTTGGTgCTATGAAACTC	SDM of <i>ADR1-L2 S148A</i>
<i>SDM_ADR1-L2_S148A_rv</i>	GAGTTTCATAGcACCAAGCTTCTC	
<i>SDM_ADR1-L2_S342D_fw</i>	GTGCCTTCAGGGTTCgcCCAAAGTTT GGTCAAG	SDM of <i>ADR1-L2 S342D</i>
<i>SDM_ADR1-L2_S342D_rv</i>	CTTGACCAAACCTTGGgcGAACCCTG AAGGCAC	
<i>SDM_ADR1-L2_S342A_fw</i>	GTGCCTTCAGGGTTCgaCCAAAGTTT GGTCAAG	SDM of <i>ADR1-L2 S342A</i>
<i>SDM_ADR1-L2_S342A_rv</i>	CTTGACCAAACCTTGGtcGAACCCTG AAGGCAC	
<i>SDM_ADR1_S147D_fw</i>	GCGGAATGATgaTTTGTGCTTTTC	SDM of <i>ADR1 S147D</i>
<i>SDM_ADR1_S147D_rv</i>	GAAACGACAAAtcATCATTCCGC	

Name	Sequence	Description
<i>SDM_ADR1_S147A_fw</i>	GCGGAATGATgcTTTGTCGTTTC	SDM of <i>ADR1 S147A</i>
<i>SDM_ADR1_S147A_rv</i>	GAAACGACAAAgcATCATTCCGC	
<i>SDM_ADR1-L1_S131D_fw</i>	GCCGATACCgaTGTGCGTTTG	SDM of <i>ADR1-L1 S131D</i>
<i>SDM_ADR1-L1_S131D_rv</i>	CAAACGCACAtcGGTATCGGC	
<i>SDM_ADR1-L1_S131A_fw</i>	GCCGATACCgcTGTGCGTTTG	SDM of <i>ADR1-L1 S131A</i>
<i>SDM_ADR1-L1_S131A_rv</i>	CAAACGCACAgcGGTATCGGC	
<i>SDM_ADR1-L1_S150D_fw</i>	GCAAGTGGGTgaCATGAAAATTG	SDM of <i>ADR1-L1 S150D</i>
<i>SDM_ADR1-L1_S150D_rv</i>	CAATTTTCATGtcACCCACTTGC	
<i>SDM_ADR1-L1_S150A_fw</i>	GCAAGTGGGTgcCATGAAAATTG	SDM of <i>ADR1-L1 S150A</i>
<i>SDM_ADR1-L1_S150A_rv</i>	CAATTTTCATGgcACCCACTTGC	

Table S4: Expression constructs used in this work. Each expression construct is listed as the promoter, gene, and tag. The terminator and selection marker are specified if these were cloned separately into the backbone vector. The backbone is listed separately. For each construct, the checkmark under “This work” indicated whether I personally created the expression construct during this work. The cloning method is listed under ‘Cloning method(s)’ for these constructs.

Expression construct	Backbone	This work	Cloning method(s)
<i>XVE(35S)::ADR1-YFP-HA</i>	pMDC7		
<i>35S::ADR1-L1-HA</i>	pGWB614		
<i>35S::ADR1-L2-HA</i>	pGWB614		
<i>35S::NRG1.1^{genomic}-HA</i>	pGWB614		
<i>35S::NRG1.1^{cds}-HA</i>	pGWB614	✓	GateWay
<i>35S::NRG1.2-HA</i>	pGWB614		
<i>35S::NRG1.1^{genomic} G391G*-HA</i>	pGWB614	✓	SDM + GateWay
<i>35S::NRG1.1^{spliced}-HA</i>	pGWB614	✓	GateWay
<i>XVE(35S)::ADR1 D461V-YFP-HA</i>	pMDC7		
<i>XVE(35S)::ADR1-L1 D489V-YFP-HA</i>	pMDC7		
<i>XVE(35S)::ADR1-L2 D484V-YFP-HA</i>	pMDC7		
<i>XVE(35S)::NbNRG1 N419V-YFP-HA</i>	pMDC7	✓	GateWay
<i>XVE(35S)::NRG1.1 D485V-YFP-HA</i>	pMDC7		
<i>XVE(35S)::NRG1.2 N490V-YFP-HA</i>	pMDC7	✓	GateWay
<i>XVE(35S)::RPM1 D505V-YFP-HA</i>	pMDC7		
<i>XVE(35S)::RPS2 N473V-YFP-HA</i>	pMDC7		
<i>XVE(35S)::PML5 D360V-YFP-HA</i>	pMDC7		
<i>XVE(35S)::PML11 D473V-YFP-HA</i>	pMDC7		
<i>35S::NRG1.1^{genomic} P-loop-HA</i>	pGWB614	✓	SDM + GateWay
<i>35S::NRG1.1^{genomic} L134E-HA</i>	pGWB614	✓	SDM + GateWay
<i>35S::NRG1.1^{genomic}-HA^{recreated}</i>	pGWB614	✓	GateWay
<i>35S::NRG1.1^{genomic}-HA tNOS FastRed</i>	BB10	✓	GoldenGate
<i>35S::NRG1.3-HA tNOS FastRed</i>	BB10	✓	GoldenGate
<i>pADR1-L2::ADR1-L2 D484V-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V T56D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V T56A-HA tNOS FastRed</i>	BB10		

Expression construct	Backbone	This work	Cloning method(s)
<i>pADR1-L2::ADR1-L2 D484V S141D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V S141A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V S148D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V S148A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V T56D S141D-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 D484V T56D S141A-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 D484V T56D S148D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V T56A S148A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V S141D S148D-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 D484V S141A S148A-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 D484V S342D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V S342A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V S141D S342D-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 D484V S141A S342A-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 D484V S148D S342D-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 D484V S148A S342A-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 D484V T56D S141D S148D-HA tNOS FastRed</i>	BB10		

Expression construct	Backbone	This work	Cloning method(s)
<i>pADR1-L2::ADR1-L2 D484V T56A S141A S148A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V S141D S148D S342D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V S141A S148A S342A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 D484V T56D S148A-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 D484V T56A S148D-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 T56D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 T56A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 S141D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 S141A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 S148D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 S148A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 T56D S141D-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 T56D S141A-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 T56D S148D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 T56A S148A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 S141D S148D-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L2::ADR1-L2 S141A S148A-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate

Expression construct	Backbone	This work	Cloning method(s)
<i>pADR1-L2::ADR1-L2 T56D S141D S148D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 T56A S141A S148A-HA tNOS FastRed</i>	BB10		
<i>35S::EDS1-YFP</i>	pGWB641		
<i>35S::PAD4-GFP</i>	pGWB5		
<i>35S::SAG101-GFP</i>	pGWB5		
<i>XopQ-myc</i>	pJOG272		
<i>pADR1-L2::ADR1-L2 S141D S148D S342D-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 S141A S148A S342A-HA tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2-YFP tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 T56D S141D S148D-YFP tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 T56A S141A S148A-YFP tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 S141D S148D S342D-YFP tNOS FastRed</i>	BB10		
<i>pADR1-L2::ADR1-L2 S141A S148A S342A-YFP tNOS FastRed</i>	BB10		
<i>35S::Lti6b-RFP</i>	pB7RWG2		
<i>pADR1::ADR1-HA tNOS FastRed</i>	BB10		
<i>pADR1::ADR1 S147D-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1::ADR1 S147A-HA tNOS FastRed</i>	BB10	✓	SDM + GoldenGate
<i>pADR1-L1::ADR1-L1-HA tNOS FastRed</i>	BB10		
<i>pADR1-L1::ADR1-L1 S131D-HA tNOS FastRed</i>	BB10	✓	GoldenGate
<i>pADR1-L1::ADR1-L1 S131A-HA tNOS FastRed</i>	BB10	✓	GoldenGate
<i>pADR1-L1::ADR1-L1 S150D-HA tNOS FastRed</i>	BB10	✓	GoldenGate

Expression construct	Backbone	This work	Cloning method(s)
<i>pADR1-L1::ADR1-L1 S150A-HA tNOS FastRed</i>	BB10	✓	GoldenGate
<i>pADR1-L1::ADR1-L1 S131D S150D-HA tNOS FastRed</i>	BB10	✓	GoldenGate
<i>pADR1-L1::ADR1-L1 S131A S150A-HA tNOS FastRed</i>	BB10	✓	GoldenGate
<i>pADR1-L1::ADR1-L1S131D S150A-HA tNOS FastRed</i>	BB10	✓	GoldenGate, Bsal assembly
<i>pADR1-L1::ADR1-L1 S131A S150D-HA tNOS FastRed</i>	BB10	✓	GoldenGate, Bsal assembly