

**A study of mutual antagonism between  
EDS1 and transcription factor MYC2  
in Arabidopsis immunity**

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## **Publications**

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## **Abbreviations**

°C	degree Celsius
aa	amino acid
Arabidopsis	<i>Arabidopsis thaliana</i>
avr	Avirulence
bp	base pair
C-terminal	carboxy-terminal
CC	Coiled-coil
cDNA	complementary DNA
cfu	colony forming unit(s)
d	day(s)
dH <sub>2</sub> O	deionised water
ddH <sub>2</sub> O	distilled, deionized water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleosidetriphosphate
dpi	days post inoculation
EDTA	ethylenediaminetetraacetic acid
ETI	effector triggered immunity
GFP	green fluorescent protein
YFP	yellow fluorescent protein
h	hour(s)
HA	hemagglutinin
hpi	hours post inoculation
HRP	horseradish peroxidase
JA	jasmonic acid

kb	kilo base(s)
kDa	kilo Dalton
l	litre
LRR	leucine-rich repeat
M	molar (mole/l)
μ	micro
MAMP	microbe-associated molecular pattern
MAPK	mitogen activated protein kinase
mg	milligram
min	minute(s)
mM	millimolar
MPIPZ	Max-Planck-Institute for Plant Breeding Research
mRNA	messenger RNA
MW	molecular weight
NB	nucleotide-binding
ng	nanogram
NLR	nucleotide-binding leucine-rich repeat
nM	nanomolar
NOD	nucleotide-binding-oligomerization domain
N-terminal	amino-terminal
OD	optical density
p35S	35S promoter of Cauliflower Mosaic Virus
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
pH	negative decimal logarithm of the H <sup>+</sup> concentration
PR	pathogenesis related

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PRR	PAMP/ pattern recognition receptor
<i>Pst</i>	<i>Pseudomonas syringae</i> pv. <i>tomato</i>
PTI	PAMP/ pattern-triggered immunity
pv.	pathovar
qRT-PCR	quantitative real-time PCR
R	resistance
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription -PCR
SA	salicylic acid
SDS	sodium dodecyl sulphate
T3SS	type III secretion system
TBS	Tris buffered saline
T-DNA	transfer-DNA
TF	transcription factor
TIR	Toll/ interleukin-1 receptor
TNL	TIR-NLR
Tris	Tris- (hydroxymethyl-) aminomethane
V	Volt(s)
v/v	volume per volume
WT	wild type
w/v	weight/volume
Y2H	yeast two-hybrid

**Amino acids**

<b>Amino acid</b>	<b>3-letter code</b>	<b>1-letter code</b>
Alanine	Ala	A
Arginine	Arg	R
Isoleucine	Ile	I
Leucine	Leu	L
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W



## Summary

Intracellular immune signaling plays an important role in modulating plant defense responses against pathogens. Arabidopsis nucleo-cytoplasmic protein EDS1 (Enhanced Disease Susceptibility1), together with its sequence-related signaling partners, PAD4 (Phytoalexin-Deficient4) and SAG101 (Senescence-Associated Gene101), is essential for transcriptional reprogramming during intracellular signaling in basal and TNL (TIR-NB-LRR) receptor-triggered immunity. EDS1 regulates both SA (salicylic acid)-dependent and SA-independent pathways in immune responses. Interactions between TNLs and EDS1 place EDS1 as a bridge between TNLs and induced transcriptional reprogramming in cells. How EDS1 signaling is regulated and which molecular events connect EDS1 to transcriptional defense reprogramming are still unclear.

A genetic screen was used to identify suppressors of Arabidopsis *eds1-2* hypersusceptibility to *Pst* (*Pseudomonas syringae* pv. *tomato*) DC3000 *avrRps4* with the purpose to identify potential components of EDS1 signaling in immunity. Bacterial effector *avrRps4* is recognized by the paired Arabidopsis TNL receptors RRS1 (Resistance to *Ralstonia solanacearum*1)/RPS4 (Resistance to *Pseudomonas syringae*4). I identified seven mutants with restored resistance to *Pst* DC3000 *avrRps4*. Among these, four mutants contain different mutations in *COI1* (*Coronatine-Insensitive1*). Further analysis of several mutants suggests that four different signaling pathways can compensate for defects of *eds1-2* in defense responses in Arabidopsis.

Because *COI1* is essential for activating JA (jasmonic acid) signaling which antagonizes SA (salicylic acid), I hypothesized that EDS1 negatively regulates JA signaling in order to promote SA resistance. In transient expression assays, EDS1, PAD4 and SAG101 formed complexes with MYC2-family transcription factors (TFs) which regulate an important JA signaling branch. EDS1-MYC2 association was found to interfere with MYC2 transcriptional activity in transient expression assays. This is the first evidence that EDS1 regulates transcriptional reprogramming through association with TFs such as MYC2. Because EDS1 interferes with MYC2 transcriptional activity, I tested whether MYC2 reciprocally affects EDS1 protein and/or gene expression. MYC2 specifically suppressed *EDS1* promoter activity independently of MYC2-binding G-box and G-box-related motifs but, surprisingly, requiring MYC2 bHLH domain DNA-binding activity. After exogenous application of a bacterial mimic of bioactive JA, coronatine (COR), MYC2-family TFs were found to act redundantly to repress *EDS1* expression. This repressive function on *EDS1* was

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manifested in Arabidopsis protoplasts transient assays and at an early stage of *Pst* DC3000 infection and was not detectable at the late infection stage, probably due to activated EDS1 signaling.

In summary, results presented in this thesis reveal a new and important level of regulation of immunity and SA-JA pathway balance by mutual antagonism between EDS1- and MYC2-dependent processes.

## Zusammenfassung

Intrazelluläre Signalwege spielen eine wesentliche Rolle für die pflanzliche Immunantwort gegen Pathogene. Transkriptionales Umprogrammieren, welches im Zuge der intrazellulären Signaltransduktion in basaler und TNL (TIR-NB-LRR) Rezeptor-abhängiger Immunantwort in Arabidopsis auftritt, erfordert nucleo-zytoplasmatisch lokalisiertes EDS1 (Enhanced Disease Susceptibility1). EDS1 wiederum bedarf seiner sequenzähnlichen Partnerproteine PAD4 (Phytoalexin Deficient4) und SAG101 (Senescence-Associated Gene101) um voll funktional zu sein. Sowohl SA- (Salicylsäure) abhängige, als auch SA-unabhängige Signalwege werden von EDS1 während einer Immunreaktion reguliert. Die Interaktion von TNLs mit EDS1 platziert EDS1 als Verbindung zwischen TNLs und induzierter Umprogrammierung der Genexpression innerhalb der Zelle. Es ist jedoch noch immer unklar, wie EDS1 reguliert wird und welche molekularen Vorgänge EDS1 mit veränderter Transkription, welche mit der pflanzlichen Immunantwort einhergeht, verbinden.

Um potentielle Komponenten der EDS1 Signaltransduktion zu finden, welche die Hyperanfälligkeit von Arabidopsis *eds1-2* Pflanzen gegenüber *Pst* (*Pseudomonas syringae* pv. *tomato*) DC3000 *avrRps4* aufheben, wurde ein genetischer Screen durchgeführt. Das bakterielle Effektorprotein *avrRps4* wird von dem Arabidopsis TNL Rezeptorpaar RRS1 (Resistance to *Ralstonia solanacearum*1) und RPS4 (Resistance to *Pseudomonas syringae*4) erkannt. Ich konnte sieben Mutanten mit wiederhergestellter Resistenz gegenüber *Pst* DC3000 *avrRPS4* identifizieren. Von diesen enthielten vier unterschiedliche Mutationen in COI1 (*Coronatine Insensitive1*). Die tiefere Untersuchung diverser Mutanten legt nahe, dass vier eigenständige Signalwege die auftretenden Defekte in der Immunantwort von Arabidopsis *eds1-2* Pflanzen kompensieren können.

Da COI1 unabdingbar ist für die Aktivierung des JA (Jasmonsäure) Signalweges, welcher SA entgegenwirkt, stellte ich die Hypothese auf, dass EDS1 negativ auf JA wirkt, um so SA abhängige Pathogenresistenz voranzutreiben. In transienten Expressionsanalysen bildeten EDS1, PAD4 und SAG101 Proteinkomplexe mit Transkriptionsfaktoren (TFs) der MYC2 Familie. Diese TFs regulieren einen wichtigen Teil des JA Signalweges. In transienten Expressionsversuchen beeinträchtigte die Interaktion von EDS1 mit MYC2 die Aktivität von MYC2 als TF. Dies zeigt zum ersten Mal, dass EDS1 transkriptionales Umprogrammieren durch die Interaktion mit TFs

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wie MYC2 reguliert. Da EDS1 die Aktivität von MYC2 reguliert, testete ich, ob MYC2 auf reziproke Weise EDS1 Genexpression und/oder Proteinlevel beeinflusst. Hierbei inhibierte MYC2 spezifisch EDS1 Promoteraktivität. Dies war unabhängig von G-Box und G-Box-ähnlichen Motiven, an welche MYC2 binden kann, benötigte aber überraschenderweise die MYC2 bHLH Domäne, welche Bindung an DNA vermittelt. Die exogene Zugabe von Coronatine (COR), welches ein bakterieller Imitator bioaktiver JA ist, verdeutlichte, dass TFs der MYC2 Familie EDS1 Genexpression auf redundante Art und Weise inhibieren. Diese hemmende Wirkung auf EDS1 wurde in transienten Arabidopsis Protoplasten Versuchen und in frühen Stadien von *Pst* DC3000 Infektion weiter bekräftigt. In späteren Infektionsstadien konnte die inhibierende Wirkung jedoch nicht nachgewiesen werden, was wahrscheinlich auf die Aktivierung von EDS1 Signaltransduktion zurückzuführen ist.

Die Ergebnisse dieser Arbeit zeigen eine neue und wichtige Regulationsebene der Immunantwort und der Balance zwischen SA-JA Signalwegen auf, welche von dem gegenseitigen EDS1-MYC2 Antagonismus gesteuert wird.

# 1 Introduction

## 1.1 The two major layers of innate immunity in plants

Plants have evolved a multi-layered immune system to defend themselves from infection by potential pathogenic microorganisms. The first layer of defense takes place on the surface of the plant cell, where PRRs (Pattern-Recognition Receptors) reside. PRRs can specifically detect PAMPs (Pathogen-Associated Molecular Patterns), such as bacteria flagellin, lipopolysaccharides, peptidoglycans, chitin and chitin derivatives, which are conserved across microbial species (Jones and Dangl, 2006; Couto and Zipfel, 2016). PRRs activated by PAMPs initiate a signaling cascade resulting in the onset of PTI (PAMPs-Triggered Immunity) (Jones and Dangl, 2006; Boller and Felix, 2009; Spoel and Dong, 2012). The PTI signal transduction cascade promotes phosphorylation of RBOHD (Respiratory Burst Oxidase Homologue Protein D) which is responsible for ROS (Reactive Oxygen Species) production in the apoplast (Kadota et al., 2014; Li et al., 2014). Next to this, PTI responses rely on cytosolic  $Ca^{2+}$  signaling which activates CDPKs ( $Ca^{2+}$ -dependent Protein Kinases), as well as MAPKs (Mitogen-Activated Protein Kinases) signaling cascades, to induce transcriptional reprogramming (Asai et al., 2002; Couto and Zipfel, 2016). The transcriptional changes translate into diverse cellular responses at the metabolic level: phytoalexin production, callose deposition at the cell wall and hormone synthesis, which are generally effective to fend off host non-adapted pathogens (Boller and Felix, 2009).

Pathogens that have become adapted to a particular host genotype can disable PTI by secreting effector molecules into the plant apoplast or cytoplasm. Some effectors are delivered by a T3SS (Type III Secretion System) in the case of bacterial pathogens or by feeding structures (haustoria) in the case of fungal and oomycete pathogens (Buttner and He, 2009; Kemen and Jones, 2012). Effectors target host cellular processes to aid pathogen colonization, often by dampening PTI (Dangl et al., 2013). In response, host plants have evolved intracellular immune receptors known as R (Resistance) proteins which can specifically recognize the presence or monitor the activity of certain effectors (Cui et al., 2015). Upon perception of effectors, activated R proteins lead to ETI (Effector-Triggered Immunity) involving a boost in PTI-related defenses (Jones and Dangl, 2006; Cui et al., 2015). The major class of R proteins belongs to a polymorphic family of intracellular NLRs [Nucleotide oligomerization domain (NOD)-like receptors] with NB-LRR (Nucleotide-

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Binding and Leucine-Rich-Repeat) domains (Spoel and Dong, 2012). ETI responses are qualitatively similar to PTI but are generally faster and characterized by stronger and sustained transcriptional changes, which are usually coupled with a local HR (Hypersensitive Response) (Tao et al., 2003; Navarro et al., 2004; Dodds and Rathjen, 2010; Cui et al., 2015).

In this study, a low-level post-infection immune responses against virulent pathogens which are not obviously recognized by NLRs is defined as basal resistance. Plant basal resistance is likely to be “PTI plus weak ETI, minus ETS (Effector-Triggered Susceptibility)”, which allow virulent pathogen slow growth (Jones and Dangl, 2006).

### **1.2 The role of NLRs in ETI**

As in mammals, plant NLR proteins belong to a subclass of the STAND (Signal Transduction ATPases with Numerous Domains) superfamily of proteins (Danot et al., 2009). The molecular function of STAND proteins is modulated by a P-loop motif (GxxxxGKT/S) which can be switched from an ADP-bound “off” position to an ATP-bound “on” state (Leipe et al., 2004). NLR proteins usually contain variable domains at the N-terminus, an LRR domain at the C-terminus and a central conserved NB-ARC [Nucleotide-Binding, shared by Apoptotic protease activating factor1 (Apaf-1), certain R-proteins and Cell death protein 4 (CED-4)] domain (Danot et al., 2009; Takken and Govere, 2012). Based on the sequences of the N-terminal domains, plant NLR proteins are generally divided into two groups: TNLs which contain a TIR (Toll/Interleukin1 receptor)-like domain and CNLs which contain a CC (coiled-coil) domain (Maekawa et al., 2011).

#### **1.2.1 NLRs reside in different cellular compartments**

NLR proteins localize to and function in diverse cellular compartments, such as at the plasma membrane, endomembrane and/ or nucleus. Upon perception of *Pseudomonas syringae* effectors avrRpt2, avrRphB and avrRpm1, respectively, the Arabidopsis CNLs, RPS2, RPS5 (Resistance to *Pseudomonas syringae*2 and 5, respectively) and RPM1 (Resistance to *Pseudomonas syringae* pv. *maculicola*1) are activated at and function on the plasma membrane (Axtell and Staskawicz, 2003; Gao et al., 2011; Qi et al., 2012). N-termini of some NLR proteins function as membrane-attached domains. The N termini of Flax TNL L6 and M, recognizing fungal effectors AvrL567 and AvrM, respectively, function as anchor domains to attach L6 and M to endomembranes system (Takemoto et al., 2012). Potato CNL protein Rx, conferring resistance to Potato virus X, localizes in both the

nucleus and cytosol, and the movement of Rx from the nucleus to cytoplasm promotes immune signaling, which is critical for resistance to virus (Slootweg et al., 2010; Tameling et al., 2010). In barley, nuclear localization of the nucleocytoplasmic CNL MLA10 (Polymorphic barley Mildew A10) is essential for resistance to *Blumeria graminis* expressing AVR<sub>A10</sub>, while the cytosolic localized MLA10 or its CC-NB<sub>1-225</sub> domain is required for cell-death signaling (Shen et al., 2007; Chang et al., 2013). These results suggest that the subcellular localization of MLA10 is critical for its function. However, a recent study showed that Sr33 (Stem Rust resistance33), an orthology of MLA in wheat, induces disease resistance signaling from the cytosol, suggesting that cytosolic Sr33 activates both cell death and stem rust resistance signaling (Cesari et al., 2016). Thus, the signaling components and regulatory mechanism for cytosolic Sr33-mediated signaling pathways may differ from cytosolic MLA10 although they share ~80% amino acid sequence identity (Cesari et al., 2016). Nuclear accumulation of Arabidopsis TNL RPS4 (Resistance to *Pseudomonas syringae*4) is necessary for triggering defense response against *Pst* (*Pseudomonas syringae* pv. *tomato*) DC3000 expressing effector avrRps4 (Wirthmueller et al., 2007). Thus, the localization and spatial dynamics are critical for NLRs activation and function in ETI.

### **1.2.2 NLRs recognize specific pathogen effectors**

NLRs can recognize effectors directly by physical interaction or indirectly by monitoring effector-caused perturbations of host targets, many of which are PTI components (Cui et al., 2015). The direct and indirect recognition are consistent with the gene-for-gene model proposed to address the disease resistance specificity (Flor, 1971). As examples of direct recognition, effector ATR1 secreted by *Hpa* (*Hyaloperonospora arabidopsidis*) into host cells is specifically recognized by TNL protein RPP1-WsB [an allele of Recognition of *Peronospora parasitica*1 in Arabidopsis accession Ws-2 (Wassilewskija)] (Krasileva et al., 2010; Steinbrenner et al., 2015), and the effectors AVR-Pia and AVR1-CO39 from *Magnaporthe oryzae* can be recognized by resistance protein pair RGA4/RGA5 (R-gene analog4/5) via directly binding in rice (Cesari et al., 2013; Cesari et al., 2014b). NLRs can also be activated by monitoring pathogen effector-triggered modification of host factors. For example, RPM1 and RPS2 in Arabidopsis sense the phosphorylation or cleavage of RIN4 (RPM1-interacting protein4) caused by *Pseudomonas syringae* effectors AvrB, AvrRpm1 or AvrRpt2, leading to activation of immune signaling (Axtell and Staskawicz, 2003; Mackey et al., 2003; Kim et al., 2005; Chung et al., 2014). Indirect

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recognition has the potential advantage of increasing NLR perception space for rapidly evolving pathogen effectors (Cui et al., 2015).

Recent studies show that there are variations of NLR direct and indirect recognition, and therefore the distinction is not so clear between these modes of NLR action. For example, some NLRs contain domains resembling effector targets and acting as “integrated decoys” (Cesari et al., 2014a). Some of these NLRs function as pairs. Crystal structure analysis of homo- and heterodimer forming TIR domains from Arabidopsis TNL receptors RRS1 (Resistance to *Ralstonia solanacearum*1) and RPS4 suggests that RRS1 and RPS4 act as a heterodimer or oligomer and cooperatively intercept two unrelated effectors, avrRps4 from *Pseudomonas syringae* and PopP2 from *Ralstonia solanacearum* (Deslandes et al., 2003; Narusaka et al., 2009; Williams et al., 2014). In the absence of RRS1/RPS4 recognition, effector PopP2 dislodges WRKY TFs (Transcription Factors) from the DNA and reduces their transcriptional activity by acetylating a conserved lysine within WRKY domains, leading to effector-induced susceptibility (Le Roux et al., 2015; Sarris et al., 2015). A WRKY domain integrated within TNL RRS1-R is also targeted by PopP2 (Le Roux et al., 2015; Sarris et al., 2015). The PopP2-mediated acetylation dislodges RRS1-R from the DNA and thereby activates RPS4-dependent immune responses, including upregulation of defense genes required for disease resistance (Le Roux et al., 2015; Sarris et al., 2015). Therefore, RRS1 acts as an NLR sensor with an integrated effector decoy domain, while RPS4 activates as a signaling NLR, likely via forming signaling heterodimer with RRS1 (Cesari et al., 2014a; Griebel et al., 2014).

### **1.2.3 Certain NLR families function as “helpers” in resistance signaling**

The current view that some NLRs function as “helpers” to relay and amplify resistance signaling is supported by studies on NLR receptors in mammalian immunity. For example, in mouse, NAIPs (NLR family, Apoptosis Inhibitory Proteins), which provide recognition specificity for distinct bacterial pathogens, are required for NLRC4 (NLR family, CARD domain containing4) activation and formation of NLRC4 oligomerization, which in turn regulates downstream signaling (Kofoed and Vance, 2011; Zhao et al., 2011; Hu et al., 2013; Hu et al., 2015; Zhang et al., 2015c). These results suggest that a “sensor-executor” framework, in which NLRs such as NAIPs serving as “sensor” and the other partner NLRs such as NLRC4 acting as “executor” to regulate downstream signaling (Griebel et al., 2014). In plant ETI, several NLR proteins have no known effector-sensor function, referred to as “helper” NLRs, but assist other immune receptors in transducing signals

after pathogen recognition to downstream responses (Griebel et al., 2014). Recently, NRC1 (NLR protein required for HR-associated cell death1)-like proteins NRC2a/b and NRC3, without known effector recognition activity, were discovered to be critical for the cell death and resistance mediated by Pto (*Pseudomonas syringae*. pv. *tomato*), suggesting NRC2a/b and NRC3 function as helper for intracellular receptor Pto-mediated cell death in *N. benthamiana* (Wu et al., 2016). In *N. benthamiana*, the CNL NRG1 (N requirement gene1), acts together with TNL N to trigger resistance against tobacco mosaic virus (Peart et al., 2005). In a genetic screen for *lsd1* (*lesions simulating disease1*) suppressors, mutations in *ADR1-L2* (*Activated Disease Resistance1-Like2*) were isolated (Bonardi et al., 2011). ADR1 and its paralogs, ADR1-L1 and ADR1-L2, are necessary for autoimmune phenotypes of the Arabidopsis typical TNL mutants *sncl* (*suppressor of npr1-1, constitutive1*) and *chs2-1* (*chilling-sensitive2-1*) (Dong et al., 2016). In contrast to canonical NLRs, the helper function of ADR1-L2 does not require an intact P-loop in the NB-ARC domain. (Bonardi et al., 2011). Although these “helper” non-canonical NLRs operate downstream of “sensor” NLRs, it is unclear whether sensor and helper receptors reside in a complex (Griebel et al., 2014).

#### **1.2.4 Several nuclear NLRs regulate transcription changes**

Currently, some evidence indicate that nuclear-localized NLRs are able to regulate transcriptional reprogramming via association with transcription regulators. In barley, in the presence of the cognate powdery mildew effector AvrA10, the N-terminal signaling domain of the nucleocytoplasmic MLA10 interacts with TF MYB6 and stimulates its DNA binding activity by releasing TF WRKY1 suppression, which in turn activates defense-related gene expression (Shen et al., 2007; Chang et al., 2013). In rice, CNL protein Pb1 (Panicle blast1) physically interacts with TF WRKY54, a positive regulator of SA signaling, which was proposed to contribute to Pb1-dependent blast resistance (Inoue et al., 2013). In Arabidopsis, the TNL SNC1 associates with TF bHLH84 and transcription co-repressor TPR1 (Topless-Related1) to mediate immune responses (Zhu et al., 2010; Xu et al., 2014). Tobacco TNL receptor N forms a complex with the TF SPL6 [Squamosa Promoter Binding Protein (SBP)-domain] to enable the activation of transcription of defense genes (Padmanabhan et al., 2013). These findings link NLR function with transcriptional regulation in cells.

### **1.3 Modulation of hormones in plant immunity**

Downstream of PTI and ETI activation, a complex network of antagonistic and synergistic crosstalk occurs between phytohormone pathways which play an important role in fine-tuning the strength and dynamics of immune response and maintaining the balance with other stress pathways and growth (Pieterse et al., 2012; Lozano-Duran and Zipfel, 2015). Among the phytohormones, SA (salicylic acid) and JA (jasmonic acid) are two critical defense hormones and the crosstalk between them has been extensively explored in determining plant-microbe interactions (Pieterse et al., 2012). SA is generally considered to be effective against (hemi) biotrophic pathogens, such as *Pseudomonas syringae* or *Hpa*, whereas JA is important for plants to defend against necrotrophic pathogens and insect herbivores, as well as serving as a developmental hormone (Pieterse et al., 2012; Wasternack and Hause, 2013).

#### **1.3.1 SA biosynthesis, metabolism, accumulation and signaling in immunity**

SA is a phenolic compound, which is synthesized by two distinct enzymatic pathways, the isochorismate and the phenylalanine ammonia-lyase pathway (Dempsey et al., 2011). Isolation and characterization of the *ics1* (*isochorismate synthase1*) mutant have demonstrated an essential role of *ICS1* in pathogen-induced SA synthesis in the chloroplast by *ICS1* (Wildermuth et al., 2001; Dempsey et al., 2011). Stress-induced SA appears to be trapped in the chloroplast in the *Arabidopsis eds5* (*enhanced disease susceptibility5*) susceptible mutant with mutation in *EDS5* encoding a putative SA-transporter localized on the chloroplast envelope membrane, suggesting that SA export from the chloroplast to different cellular compartments is critical for immune responses (Nawrath et al., 2002; Serrano et al., 2013; Yamasaki et al., 2013). However, SA level is also tightly controlled by further metabolism and regulated by different signaling pathways in plants, since constitutive SA accumulation is often associated with autoimmunity and defects in growth (Jirage et al., 2001; Zhang et al., 2003; Chandran et al., 2014). SA can be chemically modified to other forms of SA, most of which inactivate SA and allow fine-tuning of SA accumulation, function and/or mobility (Dempsey et al., 2011). For example, SAG (SA glucoside) is produced from accumulating SA after pathogen infection (Dean and Delaney, 2008), and MeSA (methyl salicylate), which is catalyzed by SA methyl transferase such as BSMT1 (Benzoic acid/SA carboxyl Methyltransferase1), negatively interfere with SA-associated defense responses (Chen et al., 2003; Zhu and Park, 2005; Attaran et al., 2009).

Furthermore, SA biosynthesis and metabolism are finely regulated by different signaling pathways. In Arabidopsis, mutation of *SR1* (*Signal Responsive1*), also known as *CAMTA3* (*Ca<sup>2+</sup>/Calmodulin-binding Transcription Activator3*), leads to enhanced spontaneous lesions and resistance to *Pseudomonas syringae*, suggesting that SR1 acts as a negative regulator of plant immunity (Galon et al., 2008; Du et al., 2009). SR1 represses *EDS1* (*Enhanced Disease Susceptibility1*) (details in section 1.4) via directly binding to a typical CGCG- box (ACGCGT) motif in the *EDS1* promoter, resulting also in suppression of SA accumulation (Du et al., 2009). The finding that TF CBP60g (Calmodulin Binding Protein60g) activates expression of *ICS1* via binding directly to its promoter, provides a direct link between Ca<sup>2+</sup> signaling and SA-dependent defense responses (Sun et al., 2015). Thus, calcium signaling cascades likely positively and negatively regulate SA-dependent resistance signaling.

Besides calcium signaling cascades, MAPK signaling pathways also modulate SA-dependent immune responses. Pathogen infection of Arabidopsis pretreated with BTH (Benzothiadiazole), a bioactive analog of SA, enhanced MPK3 and MPK6 accumulation and their mRNA levels, which was correlated with increased defense gene expression and induced disease resistance (Beckers et al., 2009). These results suggested that MPK3/6 are important in SA-mediated priming disease resistance, in which plants are able to trigger faster and stronger activation of defense in response to biotic stress (Beckers et al., 2009; Conrath et al., 2015). However, it is unknown how MPK3 and MPK6 modulate the SA signaling pathway because they function redundantly in the same signaling cascade and an *mpk3 mpk6* double mutant is embryo-lethal (Wang et al., 2007). In contrast to MPK3 and MPK6, MPK4 were shown to negatively regulate defense responses with the findings that disruption of MPK signaling cascade by loss-of-function mutation in *MPK4* leads to activation of a CNL SUMM2 (Suppressor of *mkk1 mkk2*, 2), which in turn constitutively activates defense responses including increased SA accumulation (Petersen et al., 2000; Brodersen et al., 2006; Zhang et al., 2012). NPR1, a transcriptional co-activator acting downstream of SA accumulation, is stabilized and activated by its homologs NPR3 and NPR4 which function as SA receptors to sense a gradient of SA concentration, which modulates a large set of defense-related genes (Dong, 2004; Fu et al., 2012; Yan and Dong, 2014).

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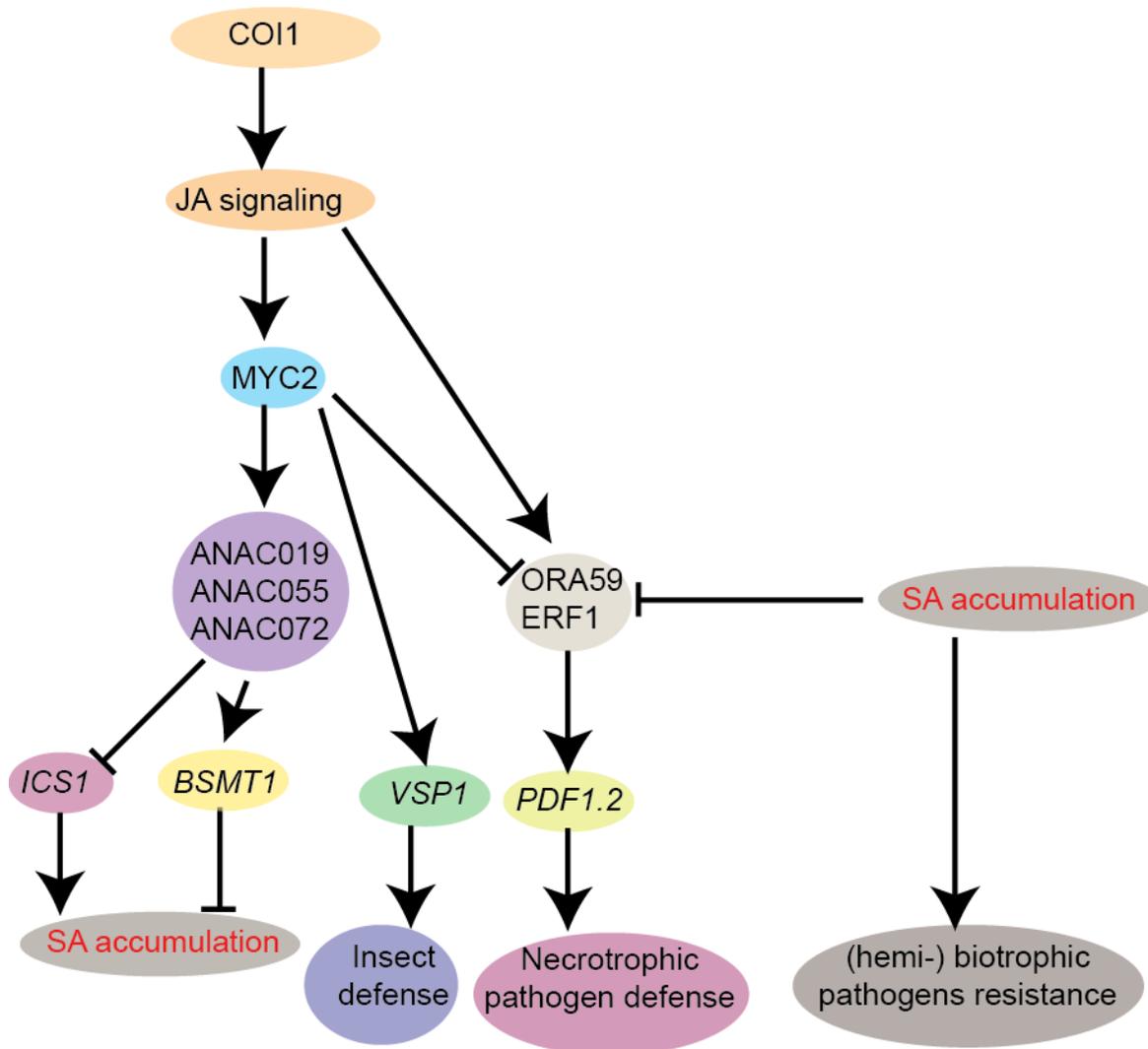
### **1.3.2 JA biosynthesis and signaling in defense responses**

JA is synthesized through a series of enzymatic reactions, and biologically active JA-Ile (jasmonoyl-isoleucine) is rapidly synthesized by JAR1 (Jasmonic acid-Amido Synthetase1) upon necrotrophic pathogens infection, pest attack or wounding (Fonseca et al., 2009; Wasternack and Hause, 2013). JA-Ile, together with an inositol phosphate cofactor, glues the F-box protein COI1 (Coronatine-insensitive1) with JAZ (Jasmonate-ZIM-domain) proteins (Xie et al., 1998; Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). In the absence of JA-Ile, JAZ proteins repress activation of JA-responsive genes expression via recruitment of chromatin modifying proteins such as HDA6 (Histone Deacetylase6) or transcriptional co-repressors such as TPL (Topless) (Pauwels et al., 2010; Zhu et al., 2011b; Shyu et al., 2012). Enhanced COI1-JAZs association in the presence of JA-Ile leads to degradation of JAZs via 26S proteasome, coupled with releasing TFs from repression of HDA6, TPL or TPR (TPL-Related) proteins, thereby leading to activation of two major branches of signaling pathways involved in regulating diverse JA responses (Chini et al., 2007; Thines et al., 2007; Wasternack and Hause, 2013) (Figure 1.1). Two TFs ERF1 (Ethylene-Responsive transcription Factor1) and ORA59 (Octadecanoid-Responsive Arabidopsis AP2/ERF59) positively regulate one branch signaling pathway upon necrotrophic pathogen attack, which upregulates the expression of JA-responsive marker gene *PDF1.2* (*Plant Defensin1.2*) (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003) (Figure 1.1). Upon the perception of JA-Ile, degradation of JAZs activate MYC2 (MYC-related protein2), a bHLH (basic helix-loop-helix) TF (Chini et al., 2007). The activated MYC2 acts together with its related TFs MYC3 and MYC4 (MYC2-family TFs) to regulate another branch of JA signaling, in which the stress marker genes such as *VSP1&2* (*Vegetative Storage Proteins1&2*), are induced in response to wounding and insect herbivores (Lorenzo et al., 2004; Fernandez-Calvo et al., 2011; Pieterse et al., 2012; Schweizer et al., 2013). Importantly, MYC2 directly binds to the promoter of *ORA59* and represses *ORA59* expression, leading to suppression of *ERF1/ORA59*-controlled genes expression (Zhai et al., 2013). This negative role of MYC2 on *ERF1/ORA59* branch signaling make it is possible to fine-tune the expression of a specific subset of JA-responsive genes in different stress responses.

### **1.3.3 MYC2 and the SA-JA crosstalk in stress responses**

MYC2 with its conserved bHLH DNA-binding domain preferentially binds to G-box and G-box-related motifs which are present in the 5' upstream regions of nearly 30% Arabidopsis genes

(Dombrecht et al., 2007). Due to the abundance of bHLH domain-containing proteins as well as different variations of the G-box, it is likely that MYC2 does not regulate all G-box-containing genes (Dombrecht et al., 2007; Fernandez-Calvo et al., 2011). A series of studies revealed that MYC2 positively and negatively regulates a large number of genes involved in variety of JA-regulated functions, such as tryptophan and flavonoid metabolism, hormone biosynthesis and signaling, insect pest resistance and senescence (Abe et al., 2003; Lorenzo et al., 2004; Nickstadt et al., 2004; Laurie-Berry et al., 2006; Chini et al., 2007; Dombrecht et al., 2007; Fernandez-Calvo et al., 2011; Schweizer et al., 2013). Recent emerged evidence has demonstrated that MYC2 serves as a master in modulating crosstalk between the JA signaling pathway and other phytohormone pathways: SA, ABA (abscisic acid), GA (gibberellin) and ethylene signaling pathways (Kazan and Manners, 2013).



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### **Figure 1.1. Current network of MYC2 functions and crosstalk between JA and SA signaling pathways**

COI1 mediates two branches of JA signaling, including MYC2-mediated branch and ORA59/ERF1-controlled branch. In response to insect, JA signaling positively regulates insect defense gene *VSP1* through MYC2. In contrast, both increased SA accumulation and activated MYC2 negatively regulate necrotrophic pathogen-responsive gene *PDF1.2* via repressing TFs ORA59 and ERF1. In response to COR secreted by *Pseudomonas syringae*, MYC2 activates TFs ANAC019, ANAC055 and ANAC072, which in turn suppress salicylic acid levels by activating the SA metabolism gene *BSMT1* and by repressing SA biosynthesis gene *ICS1*, leading to a reduction of SA accumulation. The increased SA accumulation leads to enhance resistance to biotrophic or hemi-biotrophic pathogens. Arrows indicate positive regulation while the arrows with blunt end denote negative regulation. See text for details.

Through changing cellular redox state, SA modulates modification, sequestering and even degradation of TFs involved in JA signaling, thereby antagonizing the JA pathway (Caarls et al., 2015). Downstream of COI1, accumulation of SA interferes with ORA59 stability, leading to suppression of JA-responsive genes, such as *PDF1.2* (Van der Does et al., 2013). (Figure 1.1) Recently, it was shown that insect egg extracts suppress JA signaling-controlled defense via promoting the degradation of TFs MYC2 and its related paralogs MYC3 and MYC2 in an SA-dependent manner (Schmiesing et al., 2016). Thus, in the context of biotic stress, an activated SA signaling pathway targets both the ORA59- and MYC2-mediated JA-responsive signaling pathways to repress JA-related responses.

Reciprocally, JA also antagonizes SA signaling pathways, as demonstrated predominantly through exploring the interactions between pathogens and plants (Pieterse et al., 2012). Effectors secreted by pathogens have been shown to dampen plant defense responses by suppressing SA accumulation or signaling or by activating JA signaling which then interferes with SA outputs. Two *Pseudomonas syringae* effectors HopZ1a and HopX1 promote bacterial virulence by directly targeting JAZ proteins for degradation which activates JA signaling (Jiang et al., 2013; Gimenez-Ibanez et al., 2014). The virulence factor, COR (coronatine) secreted by certain *Pseudomonas syringae* and some other bacterial strains is a potent structural and functional mimic of JA-Ile, which binds to COI1-JAZ complexes (Feys et al., 1994; Sheard et al., 2010). COR promotes bacteria entry into the apoplast by reopening stomata and subsequently increasing bacterial growth by overcoming SA-dependent resistance (Mittal and Davis, 1995; Brooks et al., 2005; Melotto et al., 2006; Sheard et al., 2010; Zheng et al., 2012). Acting downstream of COI1-JAZ release, MYC2

negatively regulates SA-dependent resistance, because *myc2/jin1* mutant displays increased SA-dependent resistance to *Pseudomonas syringae* strains (Lorenzo et al., 2004; Laurie-Berry et al., 2006). Recently, MYC2 was shown to directly upregulate ANAC [*Arabidopsis NAM (no apical meristem)*, *ATAF*, *CUC (cup-shaped cotyledon)*] TFs, such as *ANAC019*, *ANAC055* and *ANAC072* (Zheng et al., 2012). These ANAC TFs act redundantly to reduce SA accumulation via direct repressing the *ICS1*, a SA biosynthesis gene, and upregulating an SA metabolism gene *BMST1*, leading to a general reduction in SA-mediated resistance (Zheng et al., 2012) (Figure 1.1). Thus, MYC2 is a master positive regulator of JA-related responses and negative regulator of SA-related defenses.

#### **1.4 The EDS1 signaling node in immunity**

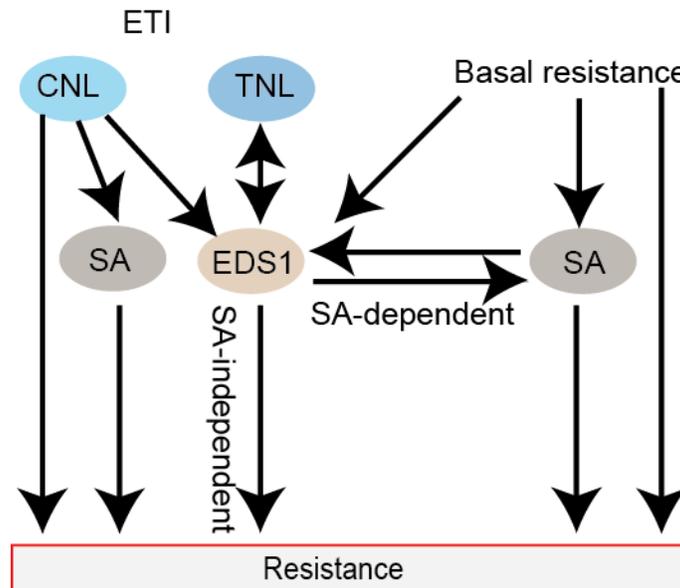
*EDS1*, encoding a protein with a lipase-like domain in its N-terminus, was originally isolated in a genetic screen in *Arabidopsis* for suppressors of *RPP5 (Peronospora parasitica5)*- and *RPP14*-mediated resistance against oomycete *Hpa* isolates (Parker et al., 1996; Falk et al., 1999). Subsequently, EDS1 was shown to be an essential components for all tested TNL ETI and certain autoimmune responses (Aarts et al., 1998; Li et al., 2001; Rusterucci et al., 2001; Hu et al., 2005; Wirthmueller et al., 2007; Heidrich et al., 2013; Xu et al., 2015).

##### **1.4.1 Nuclear EDS1 accumulation is necessary for immunity**

The *Arabidopsis* EDS1 is a nucleocytoplasmic protein (Feys et al., 2005; Garcia et al., 2010). Based on analysis of EDS1 mislocalized to the cytoplasm or the nucleus in transgenic *Arabidopsis* plants, it was concluded that a full immune response requires coordinated EDS1 nuclear-cytoplasmic activities (Wirthmueller et al., 2007; Cheng et al., 2009; Garcia et al., 2010). More specifically, nuclear EDS1 is necessary to activate transcriptional reprogramming in defense responses against biotrophic or hemi-biotrophic pathogens in TNL and basal immunity (Garcia et al., 2010). *Arabidopsis* plants expressing high levels of nuclear EDS1 display TNL autoimmunity-like phenotype and transcriptional reprogramming, which is dependent on a potentially weakly active TNL *RPP1-like<sup>Ler</sup> DM2h (Dangerous mix2)*, suggesting that the nuclear activity of EDS1 needs to be tightly controlled (Stuttman et al., 2016). This study also showed that very small amounts of nuclear pool EDS1 are sufficient for mediating basal and TNL immunity deleterious consequences for plant rosette development (Stuttman et al., 2016). Thus, an appropriate amount of EDS1 is necessary for plant defense responses.

**1.4.2 EDS1 acts as a hub in NLRs signaling and is a virulence target for effectors**

Arabidopsis EDS1 forms complexes with several TNLs: RPS4, RPS6 (Resistance to *Pseudomonas syringae* 6), SNC1 and VICTR (Variation in Compound Triggered Root growth response), inside nuclei, indicating that EDS1 may act as hub for TNL-triggered immunity and bridge TNLs with downstream resistance pathways (Bhattacharjee et al., 2011; Heidrich et al., 2011; Kim et al., 2012). Apart from its essential role in TNL signaling, EDS1 is also involved in certain CNL receptor-mediated defense responses. EDS1 functions genetically redundantly with SA, potentially compensating for SA defects in resistance mediated by three CNL receptors: RPS2, HRT (HR to TCV) and RPP8 (Recognition of *Peronospora parasitica*8), recognizing *Pst* DC3000 *avrRpt2*, TCV (Turnip Crinkle Virus) and *Hpa* Emco5, respectively (Venugopal et al., 2009) (Figure 1.2). This has been evidenced by the finding that *eds1-22 sid2-1* double mutant showed susceptibility to *Pst* DC3000 *avrRpt2*, TCV and *Hpa* Emco5, whereas *eds1-22* and *sid2-1* single mutant were resistant (Venugopal et al., 2009). Therefore, EDS1 acts as a core signaling hub involved in basal, CNL- and TNL-triggered immunity (Figure 1.2).



**Figure 1.2. Simplified illustration of EDS1 signaling in both basal, CNL- and TNL-triggered resistance.**

EDS1 is required for basal resistance, in which SA is also important. EDS1 is essential for all TNL-mediated signaling, while functions redundantly with SA in certain CNLs-mediated resistance. Downstream of *EDS1*, both SA-dependent and SA-independent responses are activated. Arrows indicate positive regulation.

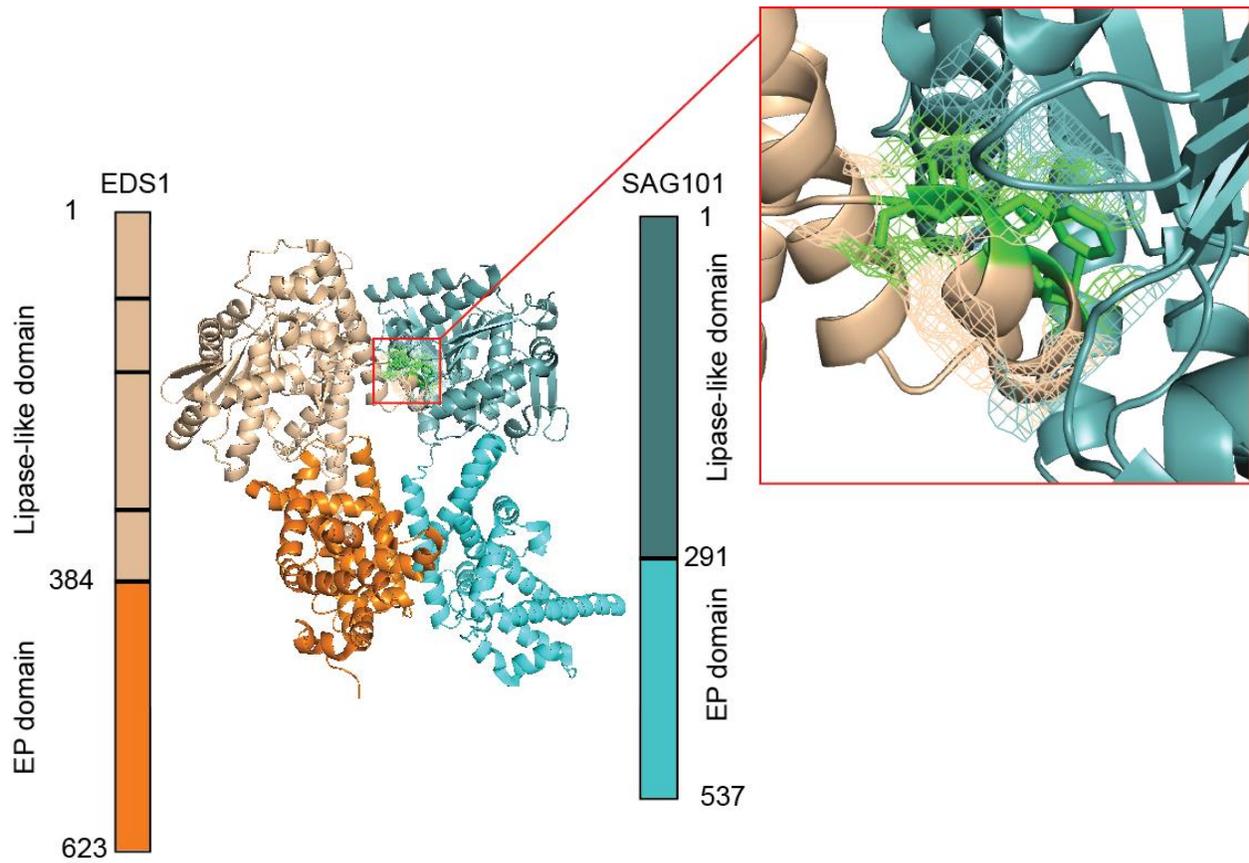
EDS1 interacts with unrelated effectors, *avrRps4* and *HopA1*, and EDS1-*avrRps4* association alters the complex of EDS1 with RPS4 and a repressor protein SRFR1 (SUPPRESSOR OF *RPS4-RLD1*) (Bhattacharjee et al., 2011; Heidrich et al., 2011). It was therefore proposed that EDS1 is a probable common virulence target for effectors to dampen the immune signaling. This is supported by the finding that bacterial pathogen effector *AvrA1* directly targets soybean GmEDS1 for virulence (Wang et al., 2014a). However, it is ambiguous whether EDS1 is a common virulence target for pathogen effectors because of the finding that *rrs1a/rrs1b* mutant, defective in recognition *Pst* DC3000 *avrRps4*, only displayed partially increased and susceptibility to *Pst* DC3000 *avrRps4* and *Pst* DC3000 compared to *eds1-2* (Saucet et al., 2015). If EDS1 is a virulence target for *avrRps4*, *rrs1a/rrs1b* mutant should show similar susceptibility as *eds1-2* to *Pst* DC3000 *avrRps4* due to the suppressive target virulent function of EDS1 (Saucet et al., 2015). Thus, more studies are required to examine whether EDS1 is a common virulence target for pathogen effectors.

#### **1.4.3 EDS1 functions with its sequence-related partners PAD4 and SAG101 in innate immunity**

Through molecular and genetic analysis in *Arabidopsis*, EDS1 has been demonstrated to function together with its sequence-related partners, PAD4 (PHYTOALEXIN DEFICIENT4) and SAG101 (SENESCENCE-ASSOCIATED GENE101), in both basal and TNL-mediated resistance (Aarts et al., 1998; Feys et al., 2001; Feys et al., 2005; Rietz et al., 2011; Wagner et al., 2013). The EDS1-family proteins (EDS1, PAD4 and SAG101) contain a lipase-like domain at their N-termini and share a conserved uncharacterized domain referred to EP (EDS1-PAD4) domain (Feys et al., 2001; Feys et al., 2005). EDS1 physically interacts with PAD4 in both nuclei and the cytoplasm, while the association of EDS1 with SAG101 occurs only in nuclei where SAG101 is restricted to (Feys et al., 2005). Together with the separate complexes among EDS1-family proteins, it was therefore proposed that dynamic interaction of EDS1 with its signaling partners in different cell compartments are important for plant immune signaling relay (Feys et al., 2005). Loss of interaction with EDS1 reduces post-transcriptional accumulation of PAD4 as well as SAG101 (Feys et al., 2005; Rietz et al., 2011). Random mutagenesis of EDS1 established that one variant EDS1<sup>L262P</sup> disrupts the interaction with PAD4 but still retains a physical association with SAG101 (Rietz et al., 2011). EDS1<sup>L262P</sup> reduced PAD4 accumulation and compromised basal resistance but did not affect TNL-triggered resistance in the *Arabidopsis* Ws-2 background (Rietz et al., 2011).

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A recently resolved crystal structure of an EDS1-SAG101 heterodimer and a derived EDS1-PAD4 heterodimer model revealed the large interface of EDS1-SAG101 or EDS1-PAD4 proteins in a heterodimers and provided further evidence that EDS1-PAD4 and EDS1-SAG101 complexes are exclusive (Wagner et al., 2013) (Figure 1.3). This is contrary to the finding that EDS1-PAD4-SAG101 formed a ternary complex (Zhu et al., 2011a). Mutations in a hydrophobic helix of EDS1, EDS1<sup>LLIF</sup>, which is a necessary contact site between the two partner proteins, losses ability to interact with both PAD4 and SAG101 (Wagner et al., 2013). Corresponding contact site mutations in PAD4 and SAG101, PAD4<sup>MLF</sup> and SAG101<sup>LLIY</sup>, dramatically reduce association with EDS1 (Wagner et al., 2013). Genetically, EDS1<sup>LLIF</sup> transgenic plants failed to complement the defect of *eds1-2* in resistance to *Hpa* Cala2 (Wagner et al., 2013). Moreover, the *pad4-1 sag101-1* double mutant showed similar hypersusceptibility as *eds1-2*, a null *EDS1* mutant, in *RPP2*-mediated resistance to *Hpa* Cala2 (Feys et al., 2005; Wagner et al., 2013). Therefore, these genetic data indicate that formation of EDS1-PAD4 and EDS1-SAG101 heterodimers are essential for EDS1 resistance signaling (Wagner et al., 2013).



**Figure 1.3. Structural features of an EDS1-SAG101 heterodimer**

Crystal structure of EDS1-SAG101 heterodimer is represented in cartoon form. EDS1 lipase-like domain (sandy brown) is juxtaposed with the lipase-like domain of SAG101 (dark cyan), while the EP domains of EDS1 (chocolate) and SAG101 (dark turquoise) interact with each other. Red box highlights the amino acids L258, L262, I254, F261A in a hydrophobic helix of EDS1 (in green), which are represented in mesh. The molecular arrangement of the heterodimer is represented by vertical bars. (Adapted from Wagner, et al., 2013)

The crystal structure analysis also indicated that the N-terminal domains contribute dominantly in forming heterodimers EDS1-PAD4 and EDS1-SAG101 (Wagner et al., 2013). Although EDS1<sup>1-384</sup>, consisting of the EDS1 lipase-like domain, is sufficient and necessary for heterodimer formation with SAG101 in *N. benthamiana*, and also stable and nucleocytoplasmic in Arabidopsis transgenic plants, it is insufficient to complement the defects of *eds1-2* in resistance (Wagner et al., 2013). Therefore, the C-terminal EDS1 consisting of amino acid residues 385-623 is required for EDS1 to modulate defense responses (Wagner et al., 2013).

**1.4.4 EDS1 modulates SA-JA crosstalk**

Genetic studies in Arabidopsis show that *EDS1* and its signaling partner gene *PAD4* positively regulate SA accumulation and are essential for SA-mediated basal resistance (Zhou et al., 1998; Feys et al., 2001). Reciprocally, *EDS1* and *PAD4* mRNAs are upregulated by exogenous SA application, consistent with *EDS1/PAD4* and SA forming a positive feedback loop which amplifies and reinforces resistance against biotrophic pathogens (Zhou et al., 1998; Feys et al., 2001). Previous observations that constitutive defense responses of *sncl* and *smg7* (*suppressor with morphogenetic effects on genitalia7*) are fully dependent on *EDS1* and *PAD4*, but only partially dependent on *ICS1* suggests that *EDS1/PAD4* signaling node also induces SA-independent pathways to regulate part of the defense responses (Li et al., 2001; Zhang et al., 2003; Gloggnitzer et al., 2014). We recently showed that the autoimmune transgenic Arabidopsis lines with co-overexpressing *EDS1* and *PAD4* trigger both SA-dependent and SA-independent signaling and *EDS1/PAD4* and SA signaling act in parallel in pathogen resistance (Cui et al., in Press). Arabidopsis microarray analyses of wild-type and signaling-defective mutants, *coil*, *pad4*, and *sid2*, in which *PAD4* not only affected SA signaling pathway but also JA/ET signaling pathway (Glazebrook et al., 2003; Bartsch et al., 2006; Wang et al., 2008). Therefore, as a positive regulator

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in SA signaling pathway, EDS1/PAD4 signaling node negatively controls JA/ET signaling pathway via an unknown mechanism.

### **1.5 Repression and de-repression in plant immunity**

To maintain the balance between immune responses and growth fitness, plants employ various strategies to adjust the strength and duration of PTI responses (Couto and Zipfel, 2016). In Arabidopsis, to completely implement BIK1 (Botrytis-Induced Kinase1)-mediated responses upon PAMP perception, BIK1 needs to be released from repression from protein phosphatase type 2C PP2C38 (Couto et al., 2016). PP2C38 regulates phosphorylation stasis of BIK1 via direct interaction, resulting in inhibition of BIK1-mediated immunity (Couto et al., 2016). Upon the flg22 perception, the induced microRNA *miR393* removes repression on SA signaling from auxin signaling, leading to certain activation of SA signaling (Navarro et al., 2006; Robert-Seilaniantz et al., 2011). Thus, upon PAMP perception, a process of de-repression is necessary to activate PTI.

ETI is quantitatively stronger than PTI, and it was proposed that this involves the removal of negative regulators upon NLR specific recognition of effector (Cui et al., 2015). In barley ETI, CNL MLA10 interacts with HvWRKY1 and removes its suppression of transcription factor HvMYB6, leading to activation of both basal and MLA-mediated resistance (Shen et al., 2007; Chang et al., 2013). In rice, CNL Pb1 associates with and protects OsWRKY45, a positive regulator of SA signaling pathway, from ubiquitin-mediated degradation, which is critical for Pb1-mediated resistance (Inoue et al., 2013). In Arabidopsis, SNC1 interacts with TPR1, a transcriptional corepressor, probably leading to downregulation of negative regulators of immunity (Xu et al., 2014). These findings suggest a de-repression model in which activated NLRs remove the suppression of negative immune regulators to trigger robust immunity.

### **1.6 Thesis aims**

Under the pressure of natural selection, plant pathogens have developed diverse strategies, including the secretion of virulence factors, to colonize hosts and circumvent innate immunity. Reciprocally, plants have evolved to activate robust ETI against pathogen infection. As an early NLR convergence point and immune signaling hub, EDS1 functions as a crucial bridge between activated NLRs and nuclear transcriptional reprogramming (Garcia et al., 2010; Bhattacharjee et al., 2011; Heidrich et al., 2011). However, the molecular mechanism by which EDS1 relays signaling information from an activated NLR to the transcriptional machinery is not yet known.

Crosstalk between phytohormone pathways is essential for plants to keep a balance between immunity, responsiveness to competing stresses, and growth (Pieterse et al., 2012; Lozano-Duran and Zipfel, 2015). EDS1 with PAD4 promotes SA accumulation to activate SA-dependent resistance but also negatively regulates JA signaling-related responses independently of SA (Falk et al., 1999; Feys et al., 2001; Glazebrook et al., 2003; Brodersen et al., 2006). The question how EDS1 modulates SA-JA crosstalk in immune response remains to be addressed. Also, although EDS1/PAD4 are central immunity components, our knowledge is very limited about how they are themselves are regulated and whether EDS1/PAD4 counteracts the action of negative components to fine-tune immune responses, consistent with a de-repression signaling model.

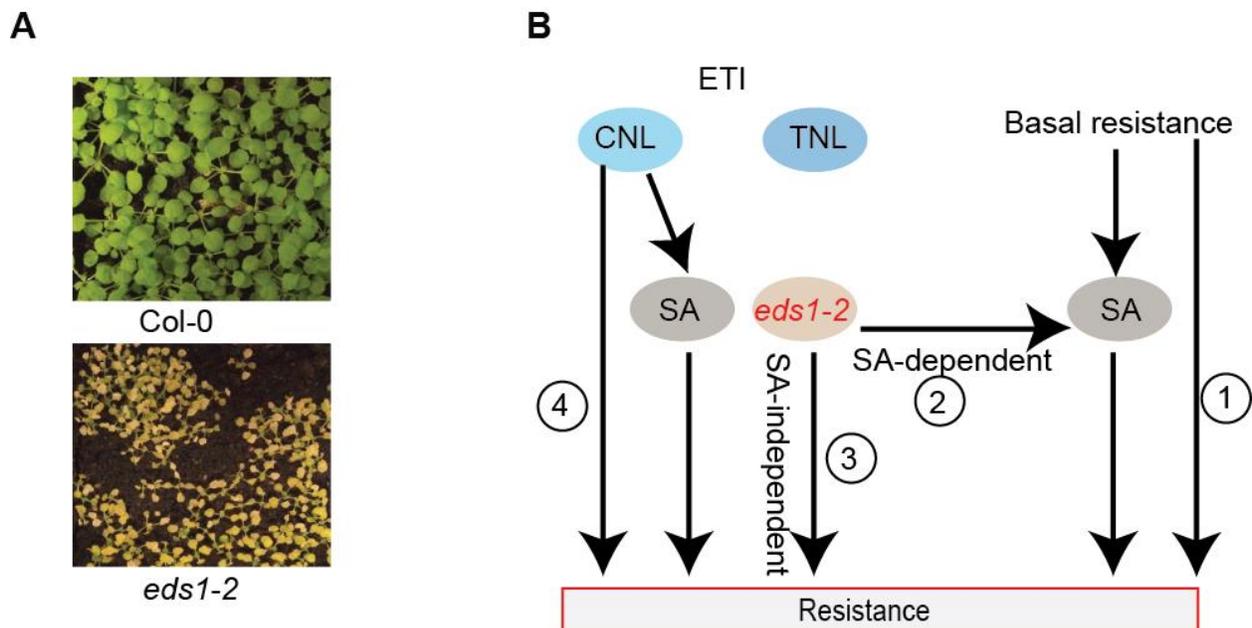
My aim in this Ph.D. thesis was to test the hypothesis that EDS1 resistance-promoting activity is negatively regulated by repressors which can themselves be overcome by activated EDS1 signaling in TNL-mediated ETI. For this, I started my Ph.D. project with a genetic screen to identify mutants that have recovered EDS1-dependent TNL (*RRS1/RPS4*) resistance to *Pst* DC3000 *avrRps4* in a hypersusceptible Col-0 *eds1-2* mutant background. Specifically, the aims of my Ph.D. thesis were to 1) identify negative regulators that are potentially antagonized by EDS1 resistance signaling, 2) explore how EDS1 activates downstream transcriptional reprogramming, 3) investigate the role of EDS1 in modulating SA-JA crosstalk in stress signaling.

## 2 Results

### 2.1 Forward genetic screening for suppressors of eds1 (*sed* mutants)

#### 2.1.1 Isolation of *Arabidopsis sed* mutants in an EMS mutagenized M<sub>2</sub> population

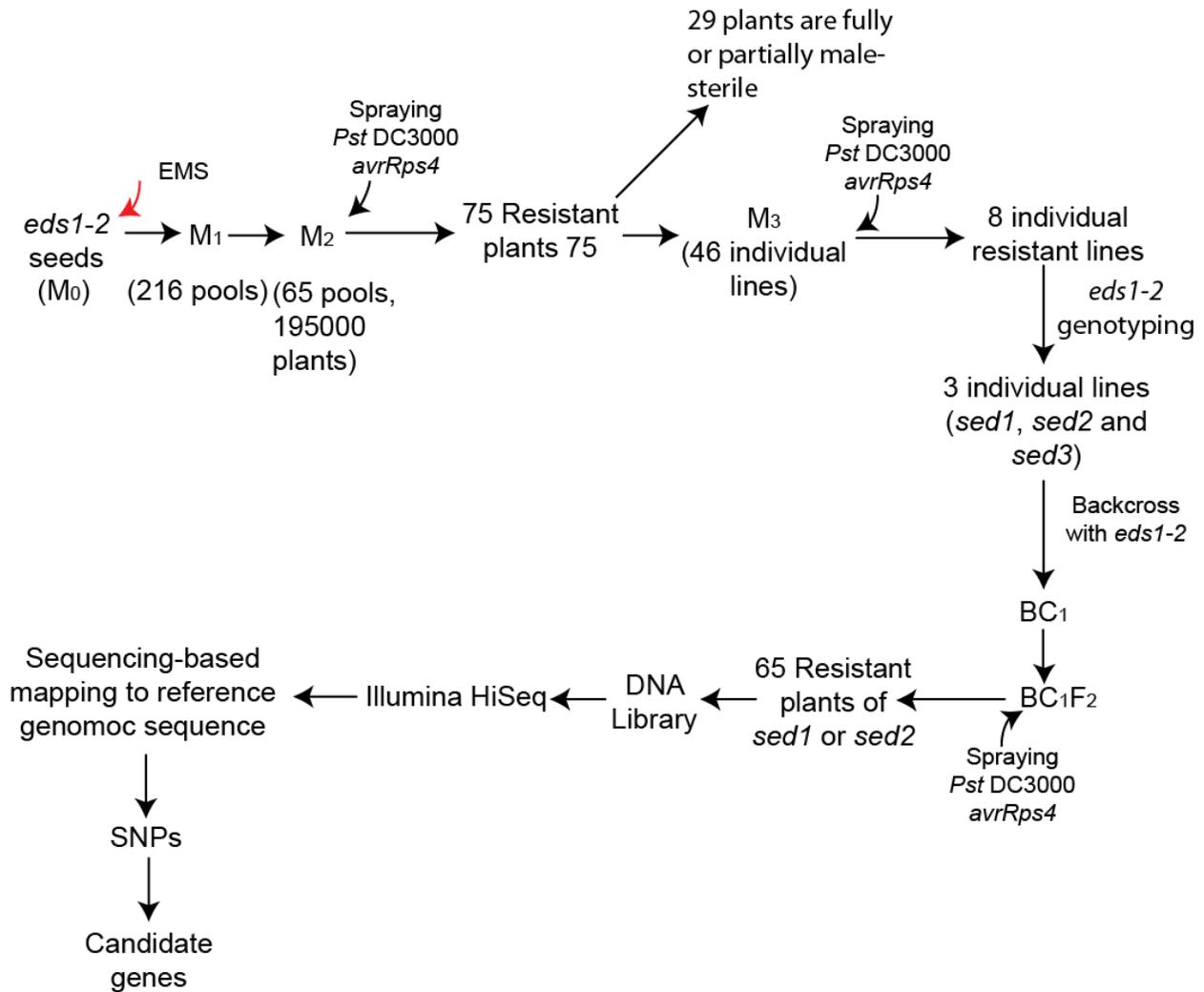
*Arabidopsis* Col-0 recognizes the effector *avrRps4* via the TNL RPS4/RRS1 receptors pair and is therefore resistant to *Pst* DC3000 *avrRps4* (Hinsch and Staskawicz, 1996; Narusaka et al., 2009; Le Roux et al., 2015; Sarris et al., 2015). The Col-0 null (fast-neutron-generated deletion) *eds1-2* mutant is hypersusceptible to *Pst* DC3000 *avrRps4* due to defective TNL signaling (Figure 2.1A). To identify suppressors of *eds1-2* (*sed*), ethyl methanesulfonate (EMS) mutagenesis was initiated by Dr. Haitao Cui with the expectation that *sed* mutants display restored resistance of *eds1-2* to *Pst* DC3000 *avrRps4*. The loss-of-function mutations in negative regulators or gain-of-function mutations in positive regulators of different resistance signaling pathways may lead to restored resistance phenotype in *eds1-2* (Figure 2.2B). These regulators may be involved in basal resistance or CNL-mediated resistance, which are *EDS1*-independent. These regulators may also regulate defense responses in *EDS1*-dependent manners, such as *EDS1*-triggered SA-dependent and SA-independent resistance signaling.



**Figure 2.1. Expected signaling defects in the recovery of *eds1-2* resistance to *Pst* DC3000 *avrRps4***

- A. Col-0 *eds1-2* is defective in TNL (*RRS1/RPS4*) resistance to *Pst* DC3000 *avrRps4*.** Col-0 *eds1-2* mutant seedlings, in contrast to the wild type Col-0, do not survive *Pst* DC3000 *avrRps4* infection. Photos were taken 7 d after spray-inoculation with bacteria ( $10^8$  CFU/mL) of two-week old seedlings.
- B. Four possible disrupted signaling pathways may lead to the recovery of *eds1-2* resistance to *Pst* DC3000 *avrRps4*.** The *eds1-2* mutant is defective in basal, TNL- and certain CNL-mediated resistance. The loss-of-function mutations in negative regulators or gain-of-function mutations in positive regulators of resistance signaling may restore *eds1-2* resistance. These regulators may be involved in *EDS1*-independent signaling pathway in basal resistance (1) or CNL-mediated resistance (4). It is also possible that these regulators are in *EDS1*-dependent resistance signaling, such as *EDS1*-triggered SA-dependent (2) and SA-independent signaling (3). Arrows indicate positive regulation.

Approximately 195000 two-week-old  $M_2$  mutant plants from a total of 65-independent  $M_1$  pools were spray-inoculated with *Pst* DC3000 *avrRps4* and 75 resistant plants were selected (Figure 2.2). From the 75 putative  $M_2$  plants, 15 plants from 2 independent pools were found to be male-sterile, while 14 plants from two other pools were partially sterile (Details in section 2.2). At the  $M_3$  generation, eight of 46 fertile and resistant lines were confirmed to rescue the *eds1-2* hypersusceptibility after spray-inoculation of two-week-old seedlings with *Pst* DC3000 *avrRps4*. To rule out the possibility of pollen or seed contamination, the confirmed 8 resistant lines were genotyped with specific *EDS1* primers which detect the deletion of parental *eds1-2*. Three EMS-mutagenized *eds1-2* mutants, which originate from different  $M_1$  pools and had restored resistance to *Pst* DC3000 *avrRps4*, are further referred to here as *sed1*, *sed2* and *sed3*.

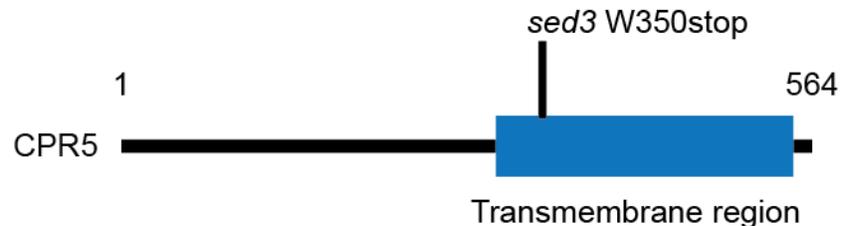


**Figure 2.2. Genetic screen for suppressors of *eds1-2* hypersusceptibility identifies 3 *sed* mutants.**

Ethyl methanesulfonate (EMS) treated *eds1-2* seeds (M<sub>0</sub>) were sown, each of 216 pools of M<sub>2</sub> seeds contained seeds from ~150 M<sub>1</sub> plants. Two-week-old M<sub>2</sub> seedlings (~195,000) from 65 pools were sprayed with *Pst* DC3000 *avrRps4* at 10<sup>8</sup> CFU/mL. At 7 d post infection, 75 resistant plants were selected, among which 29 were fully or partially male-sterile. Seeds from the remaining 46 plants were harvested as individual M<sub>3</sub> lines. In the M<sub>3</sub> generation, three of 46 individual lines were confirmed to be resistant to *Pst* DC3000 *avrRps4* and to contain the parental *eds1-2* mutation. These three lines were referred as *sed1*, *sed2* and *sed3*. The *sed1* and *sed5* were backcrossed with Col-0 *eds1-2* to generate BC<sub>1</sub> seeds. A DNA library was prepared by pooling genomic DNA from 65 BC<sub>1</sub>F<sub>2</sub> resistant plants for each *sed* mutant. DNA libraries were sequenced on an Illumina Hiseq machine. The number of potentially causative SNPs was reduced by removing SNPs shared by all *sed* mutants and found in the Arabidopsis line *pEDS1:EDS1-YFP<sup>NLS</sup> #A3/ eds1-2*, which has the same Col-0 *eds1-2* genetic background as the *sed* mutants. After this, only SNPs with G to A nucleotide transition and with an effect on the protein sequence were considered as putative causative SNPs responsible for suppression of *eds1-2* hypersusceptibility.

### 2.1.2 A mutation in *CPR5* leads to autoimmune phenotype of *sed3*

The *sed3* mutant displayed autoimmune phenotype with dwarfism, early senescence and defect in trichome development (not shown). These defective developmental phenotypes are well known in *cpr5-1* (*constitutive expressor of PR genes5-1*) mutant which was identified in a genetic screen for mutants with spontaneous programmed cell death and constitutively defense responses to biotrophic pathogens (Bowling et al., 1997). Previous findings showed that mutations in *CPR5* result in activation of CNL and TNL resistance, which is partially *EDS1*-dependent (Boch et al., 1998; Clarke et al., 2001; Wang et al., 2014b). Thus, I tested whether *sed3* contains a mutation in this negative regulator of defense. Based on sequencing of PCR products of *CPR5* genomic DNA from *sed3*, a G1049A nucleotide transition changed tryptophan (W350) to a premature stop codon in *CPR5* was found, resulting in a truncated *CPR5* lacking most of the transmembrane region (Figure 2.3). A recent report revealed that *CPR5* resides at the NPC (nuclear pore complex) and a conformational change of *CPR5* at NPC leads to activation of diverse pathways in TNL and CNL ETI (Gu et al., 2016). All these results highly suggest that *CPR5* functions upstream of *EDS1*. Thus, no further analysis is carried out on *sed3*.



**Figure 2.3** The diagram displays an amino acid change in *CPR5* identified in *sed3*. In the *CPR5* gene, the G1049A nucleotide substitution causes a premature stop codon in *CPR5*. the truncated *CPR5* lacks most of the transmembrane region in *sed1* mutant. Numbers refer to amino acid positions in *CPR5* protein. The blue box indicates the transmembrane region.

### 2.1.3 *eds1-2* pathogen hypersusceptibility is reduced in *sed1* and *sed2*

To reduce the number of background mutations introduced by EMS treatment, *Pst* DC3000 *avrRps4*-resistant *sed1* and *sed2* mutants ( $M_3$  generation) were backcrossed to Col-0 *eds1-2* ( $BC_1$ ) and the resulting  $BC_1$  lines were self-pollinated to obtain  $BC_1F_2$  generation. Two-week-old seedlings from the  $BC_1F_2$  population were sprayed with *Pst* DC3000 *avrRps4*. For both *sed1* and *sed2*-derived  $BC_2F_2$  populations, the ratio of resistant and susceptible plants was 1:3 ( $X^2$  test,  $p > 0.05$ ), suggesting that *sed1* and *sed2* mutations are inherited as a single recessive locus (Table

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2.1). In the BC<sub>1</sub>F<sub>3</sub> generation, *sed1* and *sed2* displayed normal rosette growth similar as *eds1-2* under short-day growth condition (10 h light, 14 h dark) (Figure 2.4A).

**Table 2.1. Segregation ratio of resistant and susceptible plants of *sed1* and *sed2* in the BC<sub>1</sub>F<sub>2</sub> generation**

Mutant	Resistant individuals	Susceptible individuals	X <sup>2</sup>	p
<i>sed1</i> BC <sub>1</sub> F <sub>2</sub>	24	60	0.571	0.45
<i>sed2</i> BC <sub>1</sub> F <sub>2</sub>	30	69	1.465	0.226

To assess more quantitatively the degree of resistance in *sed1* and *sed2* lines, the homozygous mutant lines (BC<sub>1</sub>F<sub>3</sub>) were infiltrated with *Pst* DC3000 *avrRps4*, and bacteria titres in leaves were estimated at 3 d post infection (dpi). Importantly, infiltration instead of spraying allowed me to exclude an effect of stomatal closure on bacteria entry into the apoplast, which could reduce host susceptibility (Melotto et al., 2006). As shown in Figure 2.4B, bacterial growth was lower in both *sed1* and *sed2* than in *eds1-2*, but more than in wild-type Col-0 (Tukey HSD test, p<0.05), suggesting that both mutants partially restore resistance of *eds1-2* against *Pst* DC3000 *avrRps4*. To test whether *sed1* and *sed2* mutations restore other TNL-dependent signaling pathways, I have inoculated the mutants with downy mildew *Hpa* (*Hyaloperonospora arabidopsidis*) EMWA1. EDS1 is essential for *RPP4*-mediated resistance to *Hpa* EMWA1 in Col-0 (Parker et al., 1996; Aarts et al., 1998). The *sed1* mutant displayed reduced sporulation of *Hpa* EMWA1 compared to *eds1-2*, while *sed2* fully recovered resistance against *Hpa* EMWA1 as evidenced by the absence of *Hpa* sporulation similar to Col-0 (Figure 2.4C, Tukey HSD test, p<0.05). Thus, both *sed1* and *sed2* mutations partially restore TNL-mediated resistance compromised by the *eds1-2* mutation.

Since *EDS1* is also essential for basal resistance (Parker et al., 1996), I tested whether *sed1* and *sed2* have elevated resistance to the virulent strain *Pst* DC3000. *sed1* as well as *sed2* mutant had susceptibility that was intermediate between *eds1-2* (hypersusceptible) and Col-0 with intact basal immunity to this pathogen (Figure 2.4D, Tukey HSD test, p<0.05). Taken together, the pathogen infection assays show *sed1* and *sed2* mutations partially counteract the defect of *eds1-2* in both basal and TNL-mediated immunity. Because *eds1-2* is a deletion mutation, the recovered basal and TNL resistance in *sed1* and *sed2* cannot be due to an intragenic suppressor, I concluded that

*sed1* and *sed2* represent loss-of-function or compromised function mutations in a component which is negatively regulated by *EDS1*, or mutations leading to increased resistance in a pathway unrelated to *EDS1*.

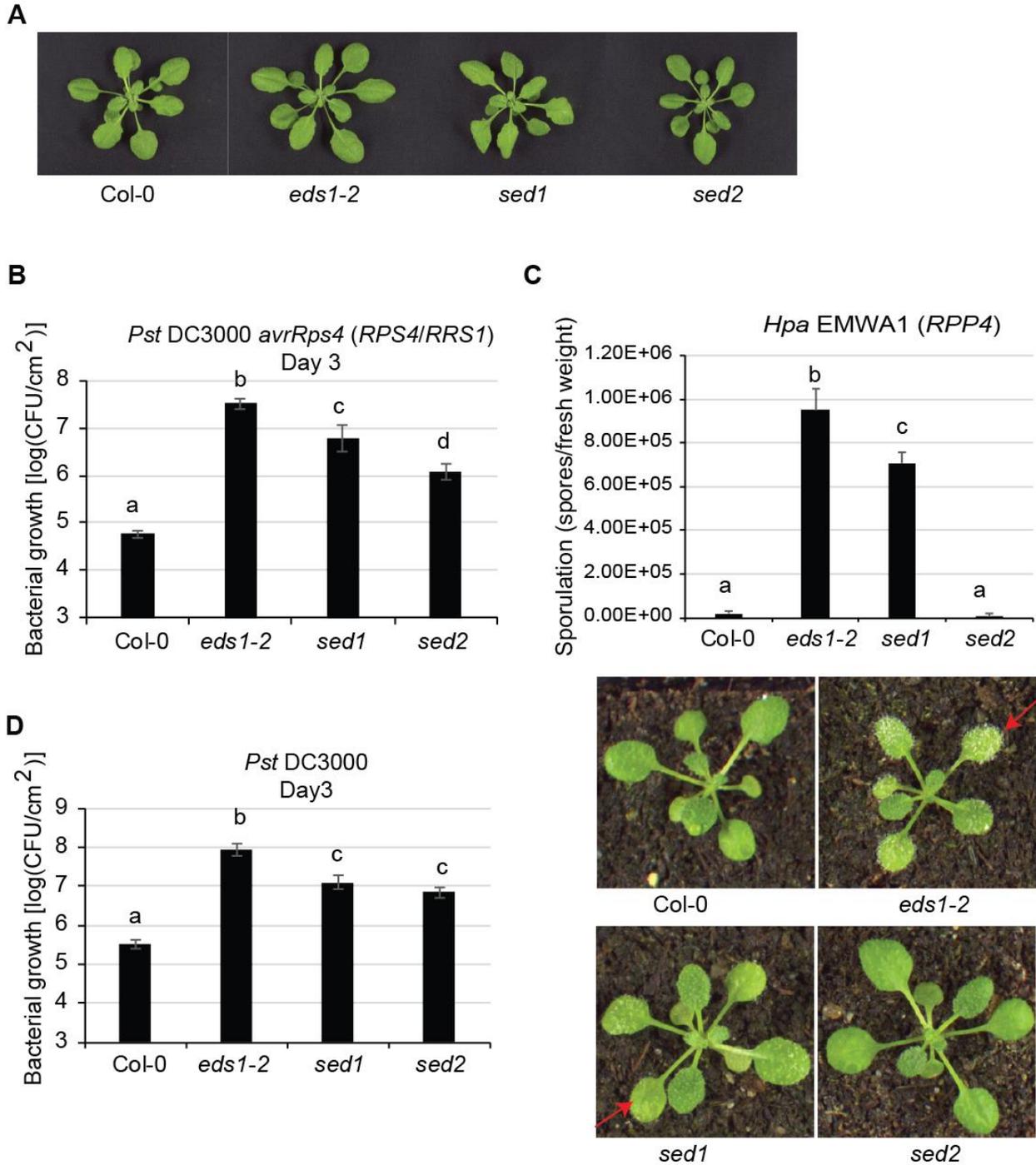


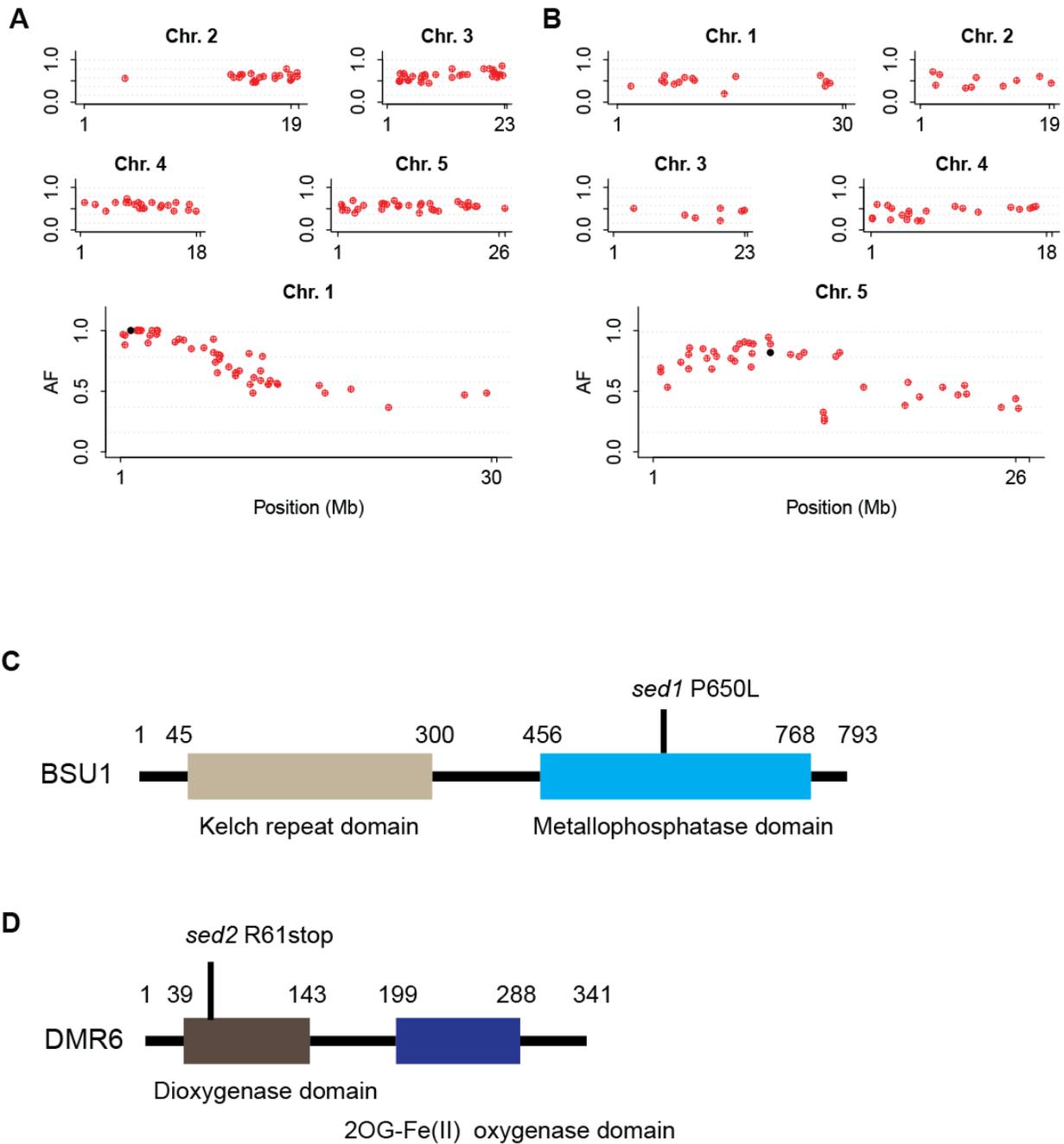
Figure 2.4. Characterization of *sed1* and *sed2* mutant

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- A. The *sed1* and *sed2* mutants do not have obvious growth abnormalities in comparison to both Col-0 and *eds1-2* plants.** Rosettes of 4-week-old Col-0, *eds1-2*, *sed1* and *sed2* plants (BC1F3 generation) grown on soil under the short-day conditions (10 h light and 14 h dark).
- B. The *sed1* and *sed2* mutants partially restore *RRS1/RPS4*-mediated resistance in the susceptible *eds1-2* mutant to *Pst* DC3000 *avrRps4*.** 4.5-week-old plants of Col-0, *eds1-2*, *sed1* and *sed2* mutants were infiltrated with *Pst* DC3000 *avrRps4* at  $10^5$  CFU/mL. Bacterial titers were determined at 3 d post infection (dpi). Error bars represent standard deviation (SD). Different characters above the error bars indicate significant differences in bacterial growth among genotypes (one-way ANOVA with Tukey-HSD test;  $p < 0.05$ ,  $n = 4$ ). The experiment was repeated three times with similar results. Results from one experiment are shown.
- C. The *sed1* and *sed2* mutants restore different levels of resistance in *eds1-2* to *Hpa* EMWA1 mediated by *RPP4*.** 2-week-old seedlings of Col-0, *eds1-2*, *sed1* and *sed2* mutants were sprayed with *Hpa* EMWA1 at  $4 \times 10^4$  spores/mL. *Hpa* spores number per gram fresh leaf was measured at 5 d post infection (dpi). The pictures were taken at 5 dpi. Red arrows indicate sporulation on leaves. Error bars represent standard deviation (SD). Different characters above the error bars indicate significant differences in the oomycete growth among genotypes (one-way ANOVA with Tukey-HSD test;  $p < 0.05$ ,  $n = 3$ ). The experiment was repeated three times with similar results. Results from one experiment are shown.
- D. The *sed1* and *sed2* mutants partially reverse hypersusceptibility of *eds1-2* to *Pst* DC3000.** 4.5-week-old plants of Col-0, *eds1-2*, *sed1* and *sed2* mutants were infiltrated with *Pst* DC3000 at  $10^5$  CFU/mL. Bacterial titers were determined at 3 d post infection (dpi). Error bars represent standard deviation (SD). Different characters above the error bars indicate significant differences in bacterial growth among genotypes (one-way ANOVA with Tukey-HSD test;  $p < 0.05$ ,  $n = 4$ ). The experiment was repeated three times with similar results. Results from one experiment are shown.

### **2.1.4 Candidate genes corresponding to *sed1* and *sed2* were isolated via sequencing-based mapping**

To identify *sed1* and *sed2* mutations responsible for partially inverting the *eds1-2* hypersusceptibility, I applied a sequencing-based mapping approach. For this, genomic DNA was extracted from 65 resistant plants from each of the *sed1*- and *sed2*-derived BC<sub>1</sub>F<sub>2</sub> populations. DNA from individual plants was quantified and mixed in equimolar amounts and sent for sequencing to the Genome Center (MPI, Cologne). The Illumina HiSeq sequence reads were mapped to a reference, genomic sequence of *pEDS1:EDS1-YFP<sup>NLS</sup>* #A3 in the Col-0 *eds1-2* background (Stuttman et al., 2016) (Figure 2.5A and B).



**Figure 2.5. Candidate genes corresponding to *sed1* and *sed2* were isolated via sequencing-based mapping**

- A. The most likely SNP on Arabidopsis chromosome 1 underlying the phenotype of *sed1*.** Bulk segregant DNA from *sed1* BC<sub>1</sub>F<sub>2</sub> were Illumina sequenced. Allele frequency (AF) estimation of EMS changes on the five Arabidopsis chromosomes in *sed1* bulk segregants after subtraction of SNPs from the parental line, EDS1-YFP<sup>NLS</sup> #A3 (Stuttman et al., 2016), are shown in red. Black dots indicate the most likely SNPs underlying the phenotype. AF indicates SNP confidence.
- B. An SNP on Arabidopsis chromosome 5 might be responsible for the phenotype of *sed2*.** Bulk segregant DNA from *sed2* BC<sub>1</sub>F<sub>2</sub> were Illumina sequenced. Allele frequency (AF) estimation of EMS changes on the five

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Arabidopsis chromosomes in *sed2* bulked segregants after subtraction of SNPs from the parental line, EDS1-YFP<sup>NLS</sup> #A3 (Stuttman et al., 2016), are shown in red. Black dots indicate the most likely SNPs underlying the phenotype. AF indicates SNP confidence.

- C. Amino acid change in BSU1 caused by putative causative SNPs in the *sed1* mutant.** In the *BSU1* gene, the G1949A nucleotide substitution causes a non-synonymous amino acid change P650L in the metallophosphatase domain in *sed1* mutant. Numbers refer to the position of amino acid in protein. Colored boxes indicate different functional domains.
- D. Amino acid change in DMR6 resulted from putative causative SNPs in the *sed2* mutants.** A premature stop in *DMR6* is caused by G181A nucleotide transition. Numbers refer to the position of amino acid in protein. Colored boxes indicate different functional domains.

To narrow down the number of single nucleotide polymorphisms (SNPs) which potentially underlie the resistance phenotype, I also compared sequencing results between *sed1* and *sed2*. The mapping results suggest that an amino acid change, P650L in BRI1 Suppressor (BSU1, AT1G03445), caused by a G1949A substitution is responsible for the resistance phenotype of *sed1* (Figure 2.5C). BSU1 contains an N-terminal Kelch-repeat domain and a C-terminal serine-threonine phosphatase domain and positively regulates brassinosteroid signaling pathway (Mora-Garcia et al., 2004). For *sed2* mutant, a nucleotide transition G181A changed arginine (R) at position 61 (R61) to a premature stop codon in the *Downy Mildew Resistance 6* (*DMR6*, AT5G24530) gene (Figure 2.5D). *DMR6* encodes a 2-oxoglutarate (2OG)-Fe (II) oxygenase and was isolated in an independent genetic suppressor screen of Ler *eds1-2* in which the *dmr6-1* mutation fully restored TNL RPP2-mediated resistance against *Hpa* Cala2 (Van Damme et al., 2005; van Damme et al., 2008).

### **2.1.5 A defect in COR perception causes *sed4* resistance against *Pst* DC3000 *avrRps4***

29 plants from four independent M<sub>2</sub> pools were resistant to *Pst* DC3000 *avrRps4* and were fully or partially male-sterile (Table 2.2). Resistance to *Pst* DC3000, coupled with male-sterility, is well known in *coil-1* mutant, which was initially identified in a forward genetic screen for mutants insensitive to the bacterial signal coronatine (COR) (Feys et al., 1994). Bacterial COR plays a critical role in promoting the virulence of *Pst* DC3000 via reopening stomata closed upon PAMP recognition and also enhancing bacterial multiplication and disease symptoms development (Brooks et al., 2005; Melotto et al., 2006; Zheng et al., 2012). COI1 is a co-receptor for bacterial

COR and the Arabidopsis resistance to *Pseudomonas syringae* is conferred by *coi1* mutations (Xie et al., 1998; Kloek et al., 2001; Sheard et al., 2010). Therefore, I tested whether any of the 29 partially or fully resistant plants arising from the *sed*-genetic screen also contain mutations in this important regulator of JA signaling. Based on sequencing of PCR products of COI1 genomic DNA from four plants per pool (M<sub>2</sub> generation), the same *coi1* mutation was found in each pool and four *coi1* mutations were identified from the different four M<sub>2</sub> pools (Table 2.2). The respective mutants were referred as *sed4*, *sed5*, *sed6* and *sed7* (Table 2.2). The partially male-sterile *sed4* and *sed5* mutant have a G330A and A384T mutation in COI1, respectively (Table 2.2 and Figure 2.6). The male-sterile *sed6* and *sed7* mutants have G/A transitions in COI1, which leads to an E359K and G369R substitutions in the COI1 protein, respectively (Table 2.2 and Figure 2.6).

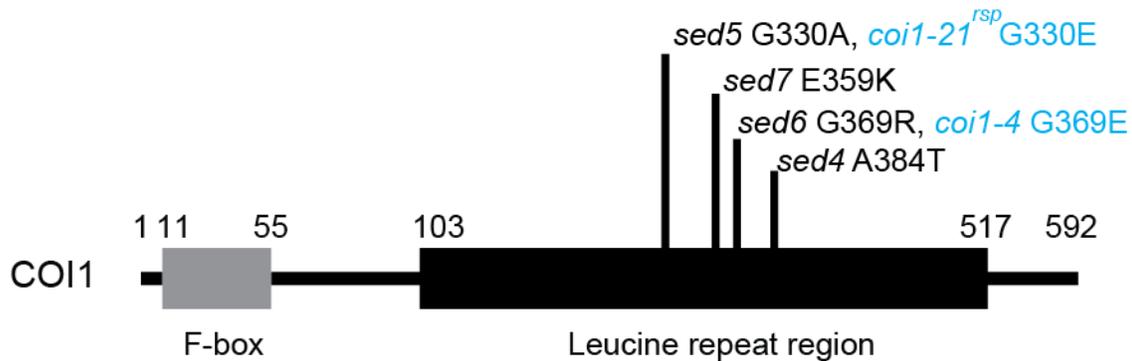
**Table 2.2. Four *coi1* mutations identified from four different M<sub>2</sub> pools**

M <sub>2</sub> pool	Number of genotyped mutants	Mutation	Amino acid change	Fertility	Mutant name
CM2-27	4	G1150A	A384T	Partially male-sterile	<i>sed4</i>
CM2-45	4	G989A	G330A	Partially male-sterile	<i>sed5</i>
CM2-94	4	G1105A	G369R	male-sterile	<i>sed6</i>
CM2-95	4	G1075A	E359K	male-sterile	<i>sed7</i>

COI1 contains a degenerate F-box motif and a LRRs (Leucine-Repeat Repeats) region (Figure 2.6), both of them are involved in the protein-protein interaction (Xie et al., 1998). The F-box is required for COI1 to form an SCF<sup>COI1</sup> complex with SKP1 (S PHASE KINASE-ASSOCIATED PROTEIN 1)-like proteins and cullin proteins, thereby COI1 functions as an E3-type ubiquitin ligase which interacts with JAZ (Jasmonate-ZIM-domain) proteins via LRRs and promotes their degradation in a ubiquitin-dependent manner in JA response (Devoto et al., 2002; Chini et al., 2007; Thines et al., 2007). The *coi1-1* mutant with a truncated COI1<sup>W467\*</sup> displayed enhanced resistance to bacterial pathogen *Pseudomonas syringae* pv *atropurpurea* (Feys et al., 1994; Xie et al., 1998). Two different layers of mechanisms have been demonstrated to be responsible for enhanced resistance phenotype in *coi1* mutants (Melotto et al., 2006). For the first layer, upon the perception of COR secreted by *Pseudomonas syringae*, COI1 facilitates bacterial entry into the plant apoplast by

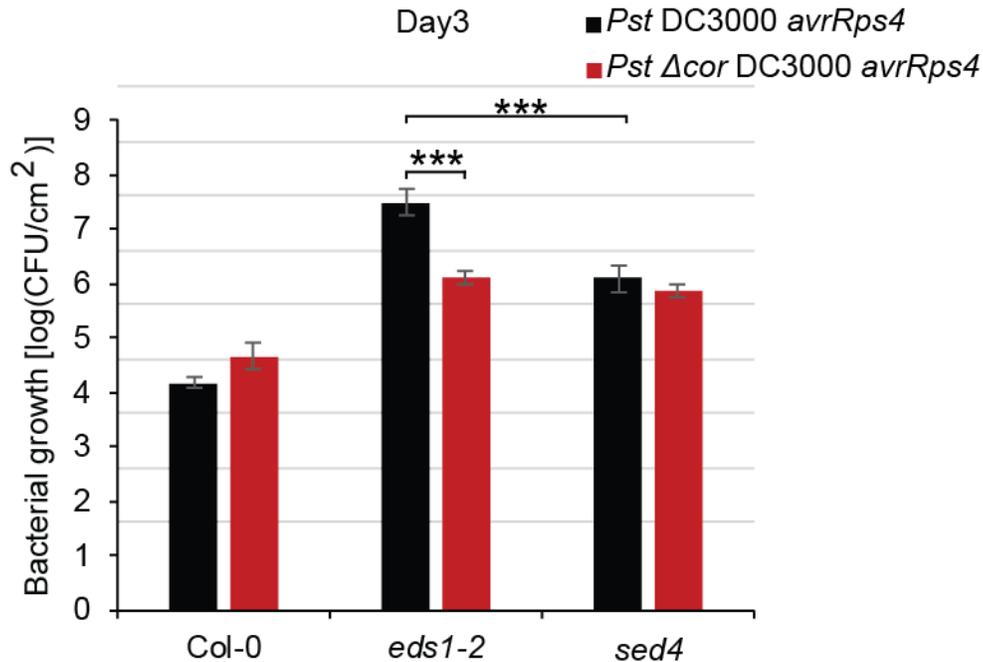
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stimulating reopening of stomata, which normally close in response to flagellin and other PAMPs (Melotto et al., 2006; Sheard et al., 2010). The second layer of the COI1 function is in the apoplast, in which COI1 represses an SA-dependent defense signaling responsible for restricting pathogen growth and impairs an SA-independent disease symptom development (Kloek et al., 2001; Melotto et al., 2006; Zheng et al., 2012).



**Figure 2.6.** The diagram displays four new amino acid substitution mutations in COI1 identified in *sed4*, *sed5*, *sed6* and *sed7*. Characters in blue refer to the amino acid substitutions in previously published *coi1* mutants (He et al., 2012; Huang et al., 2014). Grey and black box indicate F-box domain and Leucine repeat region in COI1, respectively. Numbers refer to amino acid positions in the COI1 protein.

I therefore determined whether the enhanced resistance of the *sed4* mutant is caused by reduced entry into the apoplast of *Pst* DC3000 *avrRps4* bacteria which produce virulence factor COR (Melotto et al., 2006). For this, *Pst* DC3000 *avrRps4* bacteria were infiltrated into leaves of 4.5-week-old plants to bypass stomatal resistance, rather than sprayed on the leaf surface. The *sed4* mutant still exhibited lower susceptibility compared to *eds1-2* (Figure 2.6), suggesting that the increased resistance is not due to the inability of bacteria to reopen stomata. Also, 4.5-week-old plants were infiltrated with *Pst*  $\Delta$ *cor* DC3000 *avrRps4* lacking COR secretion. At 3 dpi, no significant difference in bacterial growth was observed between *sed4* and *eds1-2* (Figure 2.7, Student's *t*-test,  $p < 0.001$ ). Furthermore, the growth of *Pst* DC3000 *avrRps4* and *Pst*  $\Delta$ *cor* DC3000 *avrRps4* was comparable in *sed4* mutant (Figure 2.7, Student's *t*-test,  $p < 0.001$ ). Together, these results suggest that a post-stomatal *coi1* defect in the perception of COR is responsible for *sed4* resistance to *Pst* DC3000 *avrRps4*. These data further show that in wild-type EDS1-mediated TNL (*RRS1/RPS4*) resistance signaling suppresses the virulent effects promoted by COI1 receptor-mediated perception of bacterial COR.



**Figure 2.7. Enhanced resistance of the *sed4* mutant to *Pst* DC3000 *avrRps4* is due to defect in COR perception.** 4.5-week-old plants of Col-0, *eds1-2* and *sed4* were infiltrated with *Pst* DC3000 *avrRps4* (black bar) or *Pst* Δ*cor* DC3000 *avrRps4* (red bar) at 10<sup>5</sup> CFU/mL, and bacterial titers were determined at 3 d post infiltration (dpi). Error bars represent standard deviation (SD). Different characters above the error bars indicate significant differences in bacterial growth among genotypes (Student's t-test;  $p < 0.001$ ,  $n = 4$ ). The experiment was repeated three times with similar results. Results from one experiment are shown.

To sum up, seven *sed* mutants were identified from the *eds1* suppressor genetic screening. I found a truncated CPR5 resulted in constitutive defense responses in *sed3*. Detailed analysis of *sed1* and *sed2* suggested that mutations in *BSU1* and *DMR6* are responsible for restored resistance of *sed1* and *sed2*, respectively. However, no further experiments and characterization of *sed1*, *sed2* and *sed3* were included in my thesis here. Four mutants were identified with mutations in COI1 that is essential for triggering JA signaling. With a COI1<sup>A384T</sup> mutation, *sed4* was insensitive to bacterial COR, whose virulence was also abolished in wild-type Col-0, suggesting that TNL (*RRS1/RPS4*)-triggered resistance suppresses COI1-activated JA signaling upon bacterial COR perception. Moreover, *RRS1/RPS4*-triggered EDS1 resistance signaling was shown to specifically suppress MYC2-mediated JA-responsive genes such as *VSP1* and *BSMT1* (H. Cui, et al., unpublished). These results underlie a negative role of EDS1 in regulating JA signaling responses and further

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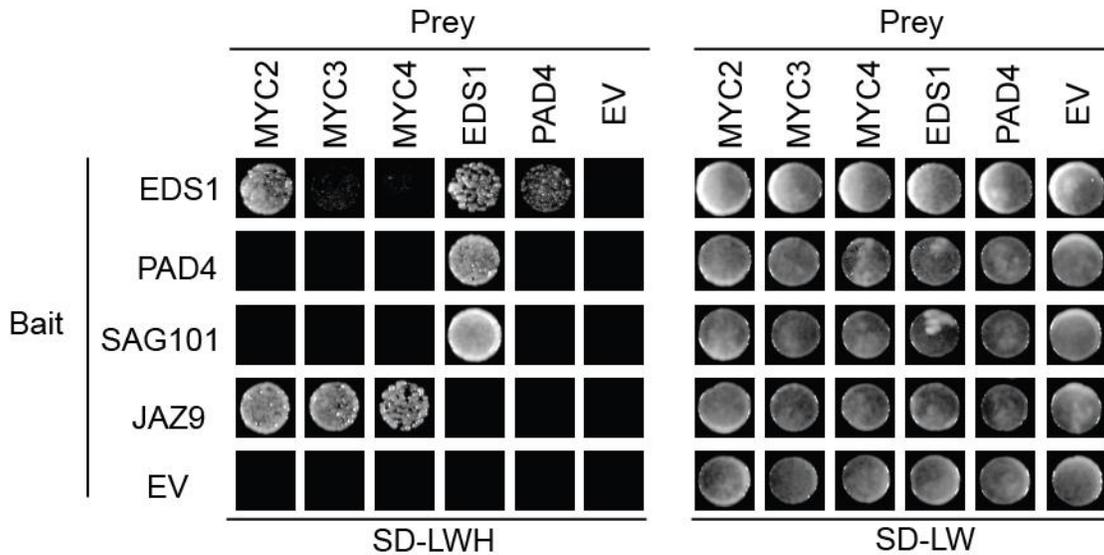
investigation on how EDS1 negatively regulates JA signaling may reveal a molecular function of EDS1 in modulating SA-JA crosstalk in plant immunity.

## **2.2 Mutual antagonism between EDS1 and MYC2 in defense responses**

### **2.2.1 EDS1, PAD4 and SAG101 interact with MYC2 family TFs in plants**

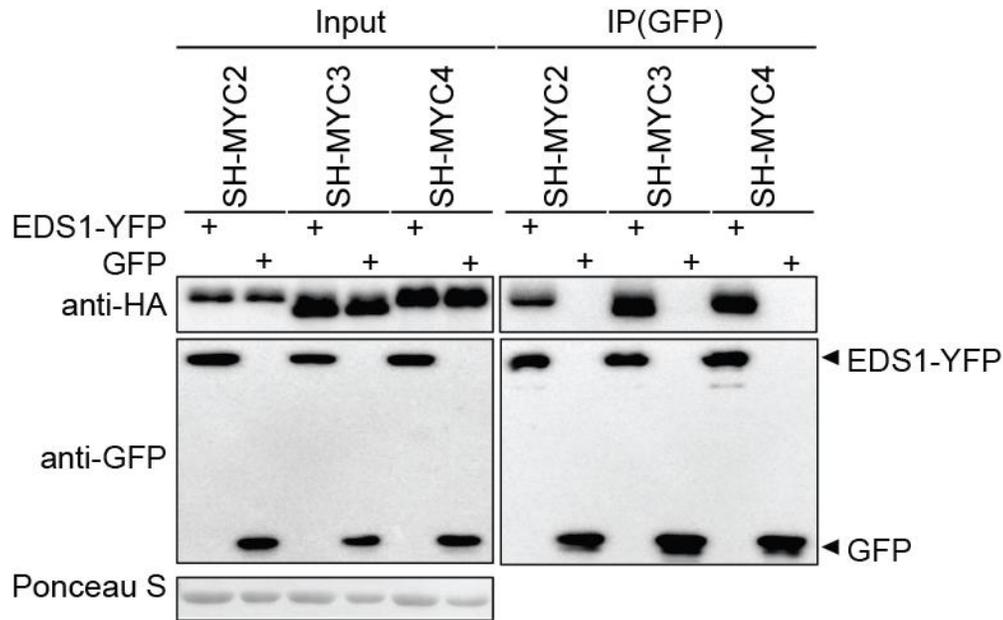
Unpublished data from our lab demonstrate that TNL (RRS1/RPS4)-mediated activation of *EDS1* resistance is associated with the suppression of known MYC2 target genes operating in COR/JA-Ile signaling (H. Cui, et al., unpublished). As a master transcription factor (TF) downstream of *COII*, MYC2 modulates transcription of a large set of genes involved in diverse aspects of JA-triggered response (Kazan and Manners, 2013). For different transcriptional activities, MYC2 associates with diverse TFs. MYC2 interacts with and attenuate the transcriptional activity of EIN3 (Ethylene-Insensitive3), resulting in repression of ethylene (ET)-induced responses, such as apical hook curvature (Song et al., 2014). Moreover, MYC2, MYC3 and MYC4 directly interact with glucosinolates (GS) biosynthesis-related MYB TFs, which play a critical role in the regulation of secondary metabolite production in defense responses (Schweizer et al., 2013). MYC2 was also found to interact functionally with general transcription regulators such as MED25 (Mediator unit25) (Chen et al., 2012).

A yeast 2-hybrid (Y2H) system was used to test the association of EDS1 with MYC2 family TFs. A positive control protein JAZ9, interacted with all MYC2, MYC3 and MYC4 TFs (Niu et al., 2011), but not with EDS1 or PAD4 (Figure 2.8). Yeast growth on SD-LWH medium, indicative of the protein interaction, was observed when EDS1 was co-expressed with MYC2, its positive control, PAD4 and itself, but not with MYC3 or MYC4 (Figure 2.8). By contrast, PAD4 and SAG101 did not show interaction with any of the MYC2-family TFs in this Y2H assay (Figure 2.8). In summary, EDS1 but not PAD4 or SAG101 interacts with MYC2 in yeast.



**Figure 2.8. EDS1 interacts with MYC2 in a yeast 2-hybrid (Y2H) assay.** EDS1, PAD4 and SAG101 fused to LexA-DB as baits were tested for interaction with Arabidopsis MYC2, MYC3 and MYC4 proteins fused to GAL4-AD as preys. Empty vector (EV) was used as a negative control. JAZ9 protein fused with LexA-DB was used as a positive control for interaction with MYC2-family TFs in yeast (Niu et al., 2011). LexA-DB-EDS1 and PAD4 fused with GAL4-AD, are known to form a heterodimer (Feys et al., 2001), and were used as a further positive control. Yeast transformed with both prey and bait plasmids were grown on SD-Leu-Trp (SD-LW, right) or selective the SD-Leu-Trp-His (SD-LWH, left) medium, and interactions between proteins were indicated by yeast growth on the selective medium (SD-LWH) at 72 h after plating. The experiment was repeated three times with similar results. Results from one experiment are shown.

To validate the interaction between EDS1 and MYC2 *in planta*, EDS1 fused to a C-terminal YFP tag (EDS1-YFP, ~95 kDa), and GFP alone (~27 kDa), used as negative control, were each transiently co-expressed with StrepII and triple hemagglutinin (HA) epitope-tagged MYC2 (SH-MYC2, ~105 kDa), MYC3 (SH-MYC3, ~97 kDa) and MYC4 (SH-MYC4, ~100 kDa) in *Nicotiana benthamiana* (*N. benthamiana*). In these plant transient over-expression assays, SH-MYC2, SH-MYC3 and SH-MYC4 all were co-immunoprecipitated (co-IPed) with EDS1-YFP but not with GFP (Figure 2.9), indicating that EDS1 forms complexes with MYC2, MYC3 and MYC4 *in planta*.

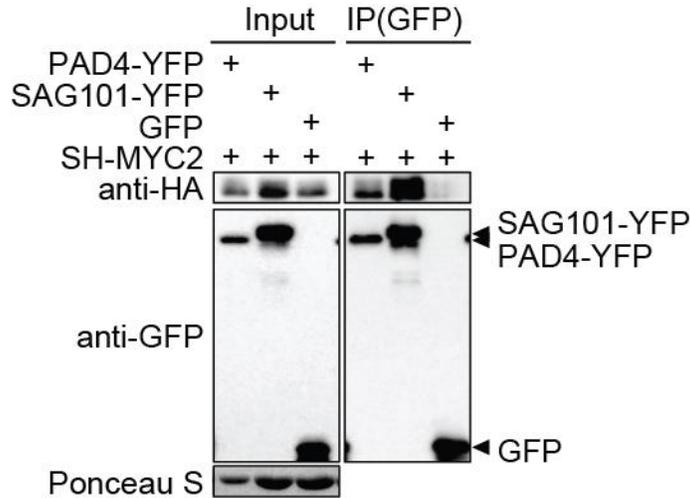


**Figure 2.9. EDS1 associates with MYC2 family TFs in *planta*.** EDS1-YFP and GFP alone under the control of cauliflower mosaic virus 35S promoter were transiently co-expressed with StrepII-3HA-tagged (SH)-MYC2, -MYC3 and -MYC4 driven by 35S in *N. benthamiana* leaves. At 2 d post infiltration, soluble protein extracts were incubated with GFP-trap beads and eluates analyzed by immunoblotting with anti-HA- and anti-GFP-specific antibodies. Equal sample loading on the gel was estimated with Ponceau S staining. The experiment was repeated three times independently with similar results.

Although no interaction was found between EDS1 sequence-related partners PAD4 or SAG101 with MYC2 family TFs in Y2H assay, I tested whether PAD4 and SAG101 could form complexes with MYC2 in plants. In transient co-expression assays in *N. benthamiana*, SH-MYC2 was co-IPed with PAD4-YFP and SAG101-YFP, but not with GFP (Figure 2.10), suggesting that PAD4 as well as SAG101 interacts with MYC2 in plant extracts. Taken together, these results suggest that all three members of EDS1-family form complexes with MYC2 and its bHLH related TFs, MYC3 and MYC4 in *planta*. N-terminal GST-tagged EDS1 (GST-EDS1) and SAG101 (GST-SAG101) expressed in *E. coli* failed to pull-down His-tagged MYC2 (His-MYC2) expressed in *E. coli* *in vitro* (Not shown), suggesting the interactions of EDS1 and SAG101 with MYC2 are indirect or the post-transcriptional modifications on EDS1, SAG101 and MYC2, which do not occur in *E. coli*, are required for direct interaction. Thus, it is unclear whether these interactions between EDS1 family proteins and MYC2 family TFs are direct or indirect. Nonetheless,

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interactions between EDS1 family proteins and MYC2 and its related TFs, MYC3 and MYC4, provide the first evidence of a potentially functional connection between EDS1 and transcriptional regulation of plant resistance responses.

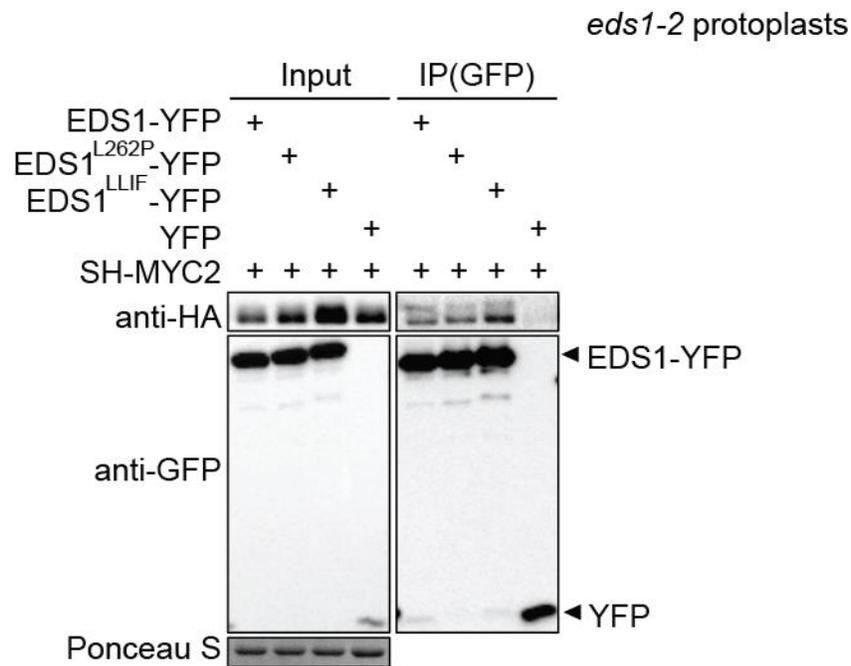


**Figure 2.10. PAD4 and SAG101 form complexes with MYC2 in planta.** PAD4-YFP, SAG101-YFP or GFP alone, were transiently co-expressed with SH-MYC2 in *N. benthamiana* leaves. Co-immunoprecipitation (co-IP) with GFP-trap beads was performed on total protein extracts at 2 d post infiltration. Immunoblotting was carried out using specific anti-bodies, anti-HA and anti-GFP. Equal sample loading on the gel was estimated with Ponceau S staining. The experiment was repeated two times independently with similar results.

### 2.2.2 EDS1, PAD4 and SAG101 each form complexes with MYC2 in Arabidopsis

A structure-guided mutational analysis revealed an essential role for EDS1 N-terminal  $\alpha$ H hydrophobic helix in EDS1 heterodimers with PAD4 and SAG101 (Wagner et al., 2013). Two EDS1 variants with mutations in the  $\alpha$ H helix were used to explore whether EDS1 forms complex with MYC2 using the same interface. One variant is EDS1<sup>L262P</sup>, which has lost its ability to associate with PAD4 but retains association with SAG101 (Rietz et al., 2011). The other variant is EDS1<sup>LLIF</sup> (L258A+L262A+I254A+F261A), which strongly reduces EDS1 association with both PAD4 and SAG101 (Wagner et al., 2013). To rule out potential interference by endogenous *N. benthamiana* EDS1 in the co-IP experiments, Arabidopsis mesophyll protoplasts were prepared from *eds1-2* mutants and used for further transient co-expression assays. EDS1-YFP, EDS1<sup>L262P</sup>-

YFP, EDS1<sup>LLIF</sup>-YFP, and a YFP control were each transiently co-expressed with SH-MYC2 in Arabidopsis *eds1-2* protoplasts. The SH-MYC2 protein co-IPed with EDS1-YFP, EDS1<sup>L262P</sup>-YFP, EDS1<sup>LLIF</sup>-YFP, but not with YFP in the IP assays (Figure 2.11), implying that an intact  $\alpha$ H helix needed for direct interaction with EDS1 partners PAD4 and SAG101 is not essential for the association of EDS1 with MYC2 in Arabidopsis. Notably, SH-MYC2 interaction with EDS1<sup>LLIF</sup>-YFP was as strong as with EDS1-YFP (Figure 2.11). This result suggests that EDS1 alone, without association with PAD4 or SAG101, forms complex with MYC2 in Arabidopsis.

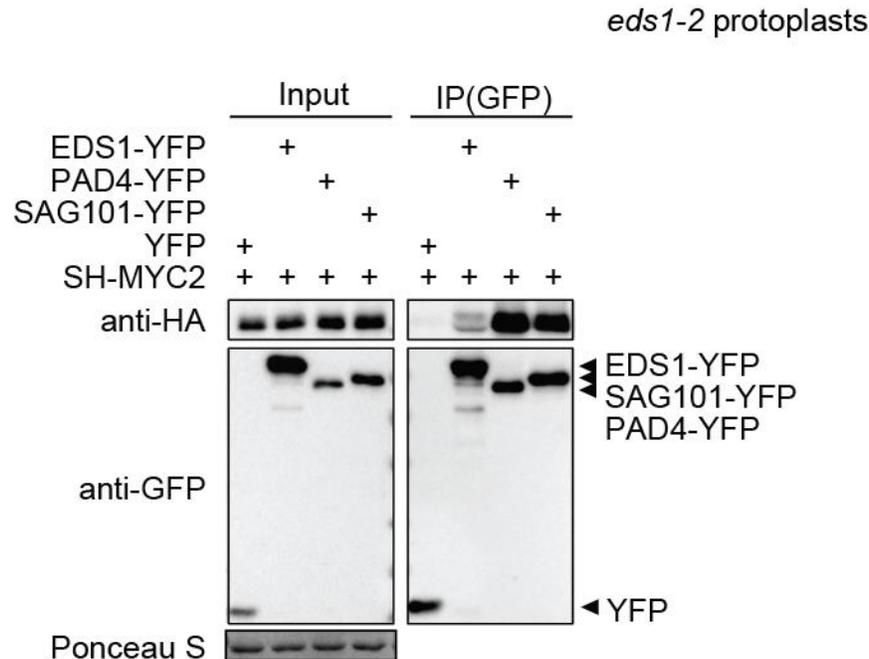


**Figure 2.11. The EDS1  $\alpha$ H helix, critical for the EDS1-PAD4 and EDS1-SAG101 heterodimer formation, is not required for EDS1 to associate with MYC2 in Arabidopsis.** The fusion proteins, EDS1-, EDS1<sup>L262P</sup>- and EDS1<sup>LLIF</sup>-YFP and YFP each was transiently co-expressed with SH-MYC2 in protoplasts prepared from the Arabidopsis *eds1-2* mutant. Protoplasts were harvested at 16 h post transfection. GFP-trap beads were added to the protein extracts, and eluates of the IP were analyzed with immunoblotting using anti-HA- and anti-GFP-specific antibodies. Equal sample loading on the gel was estimated with Ponceau S staining. The experiment was repeated two times independently with similar results.

Next, I investigated whether PAD4 and SAG101 are also able to associate with MYC2 without forming complexes with EDS1 *in planta*. I transiently co-expressed each EDS1-YFP, PAD4-YFP,

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SAG101-YFP or YFP alone with SH-MYC2 in Arabidopsis *eds1-2* protoplasts. Strikingly, more SH-MYC2 was co-purified with PAD4-YFP and SAG101-YFP compared to EDS1-YFP, although both PAD4 and SAG101 YFP fusions had lower accumulation than EDS1 in the input samples (Figure 2.12). Thus, PAD4 and SAG101, even without forming complexes with EDS1, have strong direct or indirect association with MYC2 in Arabidopsis. Taken together, these results suggest that EDS1, PAD4 and SAG101 alone can form complexes with MYC2 in Arabidopsis protoplasts.



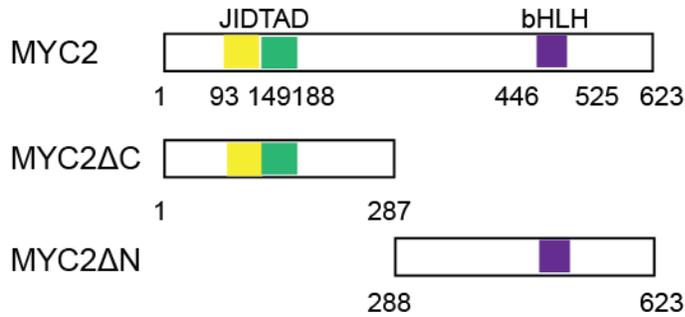
**Figure 2.12. EDS1, PAD4 and SAG101 alone associate with MYC2 in Arabidopsis.** C-terminal YFP fused EDS1-, PAD4- or SAG101-YFP, or YFP alone were each expressed in *eds1-2* protoplast with SH-MYC2. At 16 h post transfection, GFP-trap beads were added to protein extracts, and the eluates were immunoblotted with anti-HA- or anti-GFP-specific antibodies. Equal sample loading on the gel was estimated with Ponceau S staining. The experiment was repeated two times independently with similar results.

### 2.2.3 N-terminal and C-terminal parts of MYC2 associate with PAD4 and SAG101

I explored which determinants of MYC2 promote association with PAD4 or SAG101, give that these proteins interact more strongly with MYC2 in Arabidopsis protoplast assays (Figure 2.12). For this, N- and C-terminal deletion variants of MYC2 were generated (Figure 2.13A). MYC2 $\Delta$ C contains the N-terminal JID (JAZ interaction domain) and a TAD (transcriptional transactivation

domain), while MYC2 $\Delta$ N contains the C-terminal DNA-binding, basic helix-leucine-helix (bHLH) domain (Kazan and Manners, 2013).

**A**



**B**

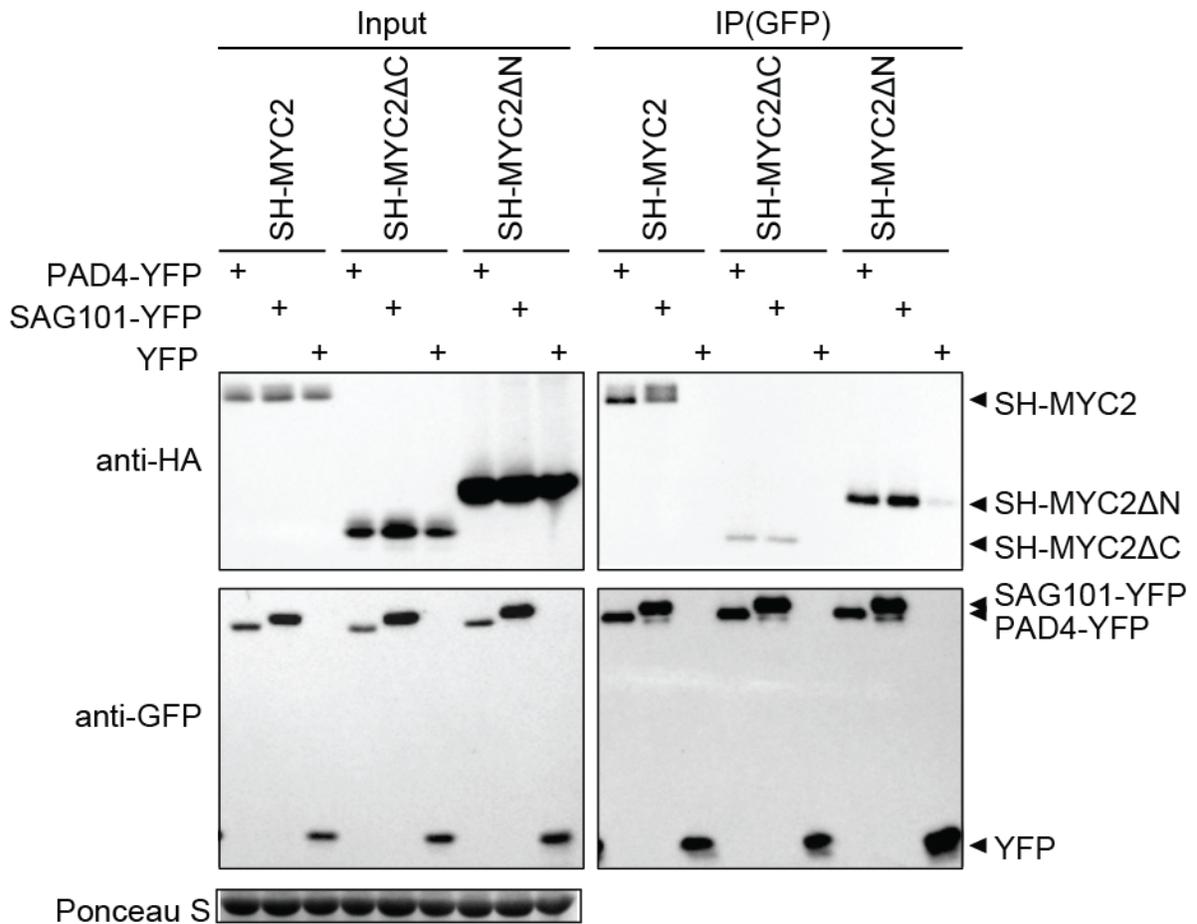


Figure 2.13. N-terminal and C-terminal parts of MYC2 associate with PAD4 and SAG101 in *N. benthamiana*

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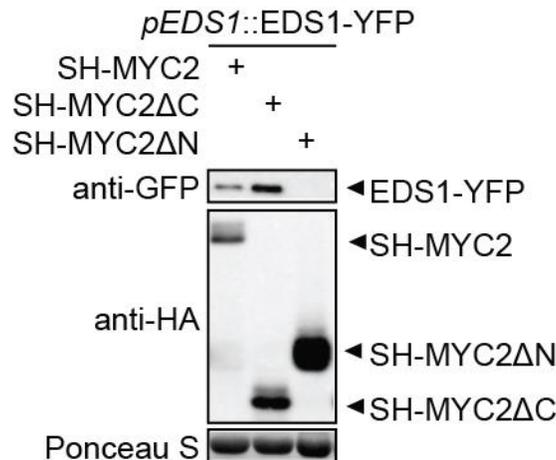
- A. Diagram of full-length MYC2 and deletion derivatives, MYC2 $\Delta$ C and MYC2 $\Delta$ N used in *N. benthamiana* transient assays.** The yellow box indicates the JAZ-interacting domain (JID), green box - transactivation domain (TAD) and purple - the basic-helix-loop-helix (bHLH) domain. Numbers refer to the amino acid position in MYC2.
- B. PAD4 and SAG101 associate with MYC2 $\Delta$ C or MYC2 $\Delta$ N in *N. benthamiana*.** PAD4-YFP, SAG101-YFP or GFP were transiently co-expressed with SH-MYC2, SH-MYC2 $\Delta$ C and SH-MYC2 $\Delta$ N in *N. benthamiana*. At 2-day post infiltration, protein extracts were co-IPed with GFP-trap beads, and the pull-down was analyzed by immunoblotting using specific anti-HA and anti-GFP anti-bodies. Equal sample loading on the blot was estimated with Ponceau S staining. The experiment was repeated three times independently with similar results.

To determine which part of MYC2 is important for association with PAD4 and SAG101, PAD4-YFP and SAG101-YFP were transiently co-expressed with SH-MYC2 $\Delta$ C and SH-MYC2 $\Delta$ N in *N. benthamiana*. Higher SH-MYC2 $\Delta$ C and SH-MYC2 $\Delta$ N accumulation than SH-MYC2 were observed in the input samples (Figure 2.13B), suggesting that both variants are more stable than the full-length MYC2 *in planta*. Both SH-MYC2 $\Delta$ C and SH-MYC2 $\Delta$ N were co-IPed with PAD4-YFP and SAG101-YFP, which indicated that MYC2 $\Delta$ C as well as MYC2 $\Delta$ N can form a complex with PAD4 and SAG101.

### **2.2.4 MYC2 $\Delta$ N suppresses *pEDS1* driven *EDS1* transcription**

The above data show that there is a possible association between EDS1 signaling pathway proteins and MYC2-family TFs. This might explain my colleague Haitao Cui's data that TNL/EDS1 resistance signaling negatively regulates COI1/MYC2 signaling, potentially to dampen the JA signaling pathway and boost SA signaling (H. Cui, et al., unpublished). In co-IP experiments with *EDS1* promoter driven *EDS1* expression, the EDS1 accumulation was reproducibly lower when MYC2 was expressed in the *N. benthamiana* tissue (not shown), which suggested that it is probable that MYC2 antagonizes EDS1 function. Therefore, I decided to further analyze if MYC2 affects EDS1 promoter activity. For this, EDS1-YFP driven by its native promoter *pEDS1* (*pEDS1::EDS1*-YFP), which contains 1426 bp nucleotides upstream of the EDS1 start codon (Garcia et al., 2010), was co-expressed with SH-MYC2, SH-MYC2 $\Delta$ C and SH-MYC2 $\Delta$ N under the 35S promoter in *N. benthamiana*. Strikingly, EDS1-YFP was almost undetectable in extracts expressing SH-MYC2 $\Delta$ N and EDS1-YFP, compared to the samples co-expressing SH-MYC2 and EDS1-YFP (Figure 2.14). By contrast, extract with co-expressed SH-MYC2 $\Delta$ C displayed higher

EDS1-YFP accumulation (Figure 2.14). These observations suggest that MYC2 $\Delta$ C either suppresses *pEDS1* activity or mediates more rapid EDS1 protein turnover.

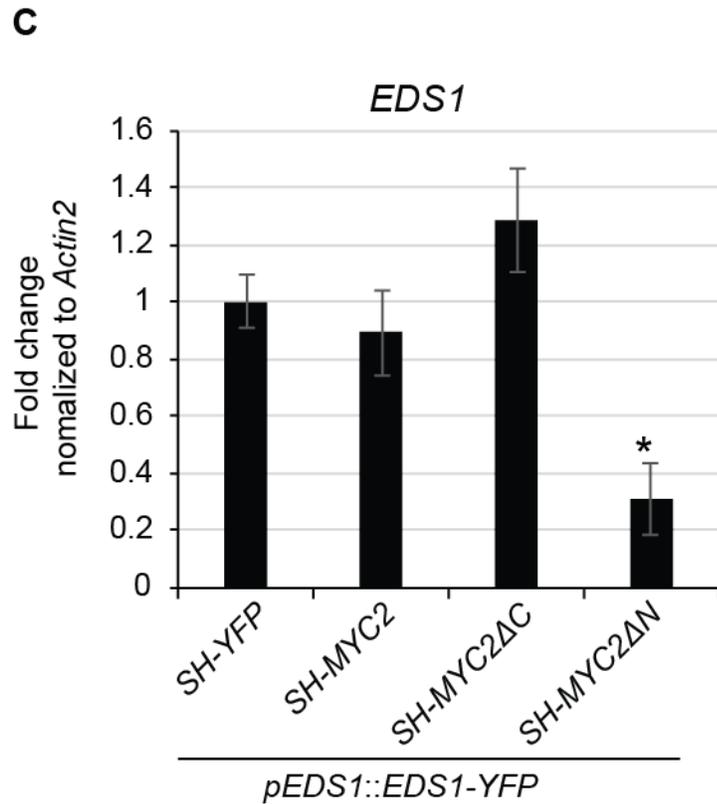
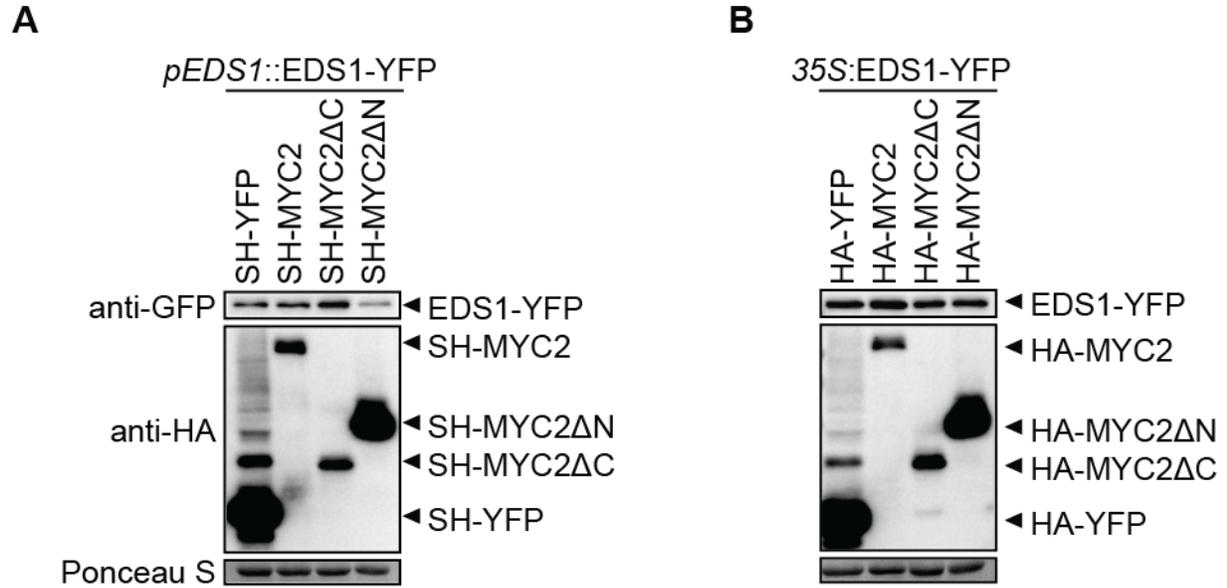


**Figure 2.14. MYC2 $\Delta$ N reduces EDS1 protein accumulation in *N. benthamiana*.** EDS-YFP, under control of the *EDS1* promoter (*pEDS1*) was transiently co-expressed with 35S-driven SH-MYC2, SH-MYC2 $\Delta$ C or SH-MYC2 $\Delta$ N in *N. benthamiana*. Protein extracts were analyzed by immunoblotting with specific anti-GFP and anti-HA antibodies. Equal sample loading on the blot was estimated with Ponceau S staining. The experiment was repeated three times independently with similar results.

Due to the potential effects of endogenous *N. benthamiana* MYC2 orthologous TFs, transient co-expression assays were also performed in the Arabidopsis protoplast system to validate results obtained in *N. benthamiana*. In Arabidopsis, MYC2, MYC3 and MYC4 function redundantly to modulate the transcription of certain target genes (Fernandez-Calvo et al., 2011; Schweizer et al., 2013). To determine whether MYC2 $\Delta$ C inhibits *pEDS1* activity in Arabidopsis, the triple mutant *myc234* was used to prepare protoplasts. In these assays, EDS1-YFP under control of *pEDS1* was co-expressed with SH-MYC2, SH-MYC2 $\Delta$ C, SH-MYC2 $\Delta$ N or SH-YFP as a control. In accordance with the results of the *N. benthamiana* assays, co-expression of SH-MYC2 $\Delta$ N with *pEDS1::EDS1-YFP* led to reduced EDS1-YFP accumulation compared to co-expression of SH-YFP, even though SH-MYC2 $\Delta$ N was expressed to a similar level as SH-YFP (Figure 2.15A). The *pEDS1::EDS1-YFP* samples with co-expression of SH-MYC2 $\Delta$ C displayed increased accumulation of EDS1-YFP compared to the samples expressing SH-YFP, while EDS1-YFP levels were

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comparable between samples expressing SH-MYC2 and SH-YFP (Figure 2.15A). These results again suggest that MYC2ΔN suppresses *pEDS1* activity or accelerates EDS1-YFP protein turnover.



**Figure 2.15. MYC2ΔN suppresses *pEDS1* activity in Arabidopsis protoplast transient assays**

- A. MYC2ΔN represses the accumulation of EDS1 in Arabidopsis transient assays.** EDS1-YFP under control of *pEDS1* was transiently co-expressed with SH-MYC2, SH-MYC2ΔC and SH-MYC2ΔN or SH-YFP in protoplasts prepared from the Arabidopsis *myc234* mutant. At 16 h post transfection, protein extracts were harvested and were immunoblotted using specific anti-GFP and anti-HA antibodies. Equal sample loading on the blot was estimated with Ponceau S staining. The experiment was repeated three times independently with similar results.
- B. MYC2ΔN does not affect EDS1 turn-over in Arabidopsis transient assays.** EDS1-YFP under control of CaMV (Cauliflower Mosaic virus) 35S promoter was transiently co-expressed with SH-MYC2, SH-MYC2ΔC and SH-MYC2ΔN or SH-YFP in Arabidopsis *myc234* protoplasts. At 16 h post transfection, protein extracts were harvested and were immunoblotted using specific anti-GFP and anti-HA antibodies. Equal sample loading on the blot was estimated with Ponceau S staining. The experiment was repeated three times independently with similar results.
- C. MYC2ΔN likely suppresses *pEDS1* activity in Arabidopsis transient assays.** *pEDS1::EDS1-YFP* was co-transformed with SH-MYC2, SH-MYC2ΔC, SH-MYC2ΔN or SH-YFP driven by the 35S promoter in Arabidopsis *myc234* protoplasts. At 8 h post transfection, samples were harvested for RNA isolation. RNA samples were first treated with RNase-free DNase to digest residual DNA then analyzed by one-step qPCR to quantify transcripts of *EDS1-YFP*, and the data were normalized to *Actin2* expression in the same samples. Error bars represent standard deviation. \* indicates a significant difference of *EDS1* expression compared to the samples co-transformed with *pEDS1::EDS1-YFP* or SH-YFP (Student's *t*-test,  $p < 0.05$ ). The experiment was repeated two times independently with similar results, results from one are shown.

To test whether different actions of MYC2 domain on EDS1 accumulation are mediated at the EDS1-YFP protein, the 35S promoter, instead of *pEDS1*, was used to drive *EDS1-YFP*, and the 35S::*EDS1-YFP* was co-expressed with SH-MYC2, SH-MYC2ΔC, SH-MYC2ΔN or SH-YFP in Arabidopsis *myc234* protoplasts as described above. The accumulation of EDS1-YFP was similar in all co-expressed samples (Figure 2.15B), suggesting that MYC2ΔN does not affect the turn-over of EDS1-YFP, and MYC2ΔN likely suppresses *pEDS1* activity. To confirm the repression of *pEDS1* by MYC2ΔN, I next measured the transcript levels of EDS1 in Arabidopsis *myc234* protoplasts co-transformed with *pEDS1::EDS1-YFP* and SH-MYC2, SH-MYC2ΔC, SH-MYC2ΔN or SH-YFP under control of 35S. Samples co-expressing HA-MYC2ΔN, but not HA-MYC2, or HA-MYC2ΔC, exhibited reduced *EDS1* transcript levels compared to the samples co-expressing with HA-YFP (Figure 2.15C, Student's *t*-test,  $p < 0.05$ ). However, *EDS1* transcript levels were not significantly different between samples with *pEDS1::EDS1-YFP* co-transformed with SH-

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*MYC2ΔC* and *SH-YFP* (Figure 2.15C, Student's *t*-test,  $p < 0.05$ ). Taken together, these results indicate that *MYC2ΔN* represses *pEDS1* activity.

### 2.2.5 MYC2 specifically represses *pEDS1* promoter activity

*MYC2ΔN* contains the DNA-binding domain bHLH which facilitates full-length MYC2 binding to G-box (CACGTG) and G-box-related hexamers (Abe et al., 1997; Boter et al., 2004; Dombrecht et al., 2007). I searched the *pEDS1* sequence for such sequences. The *EDS1* promoter region has one G-box and one G-box variant (Figure 2.16A), indicating that the suppression of *pEDS1* activity by *MYC2ΔN* could arise from direct binding to G-box. Because interaction of *EDS1* with MYC2 by protein interaction may interfere with MYC2 DNA-binding (Figure 2.15), a reporter construct for transient assays in *Arabidopsis myc234* protoplasts was generated in which *pEDS1* was fused to YFP-CFP (*pEDS1::YFP-CFP*), instead of *EDS1*-YFP in previous experiments (Figure 2.16A).

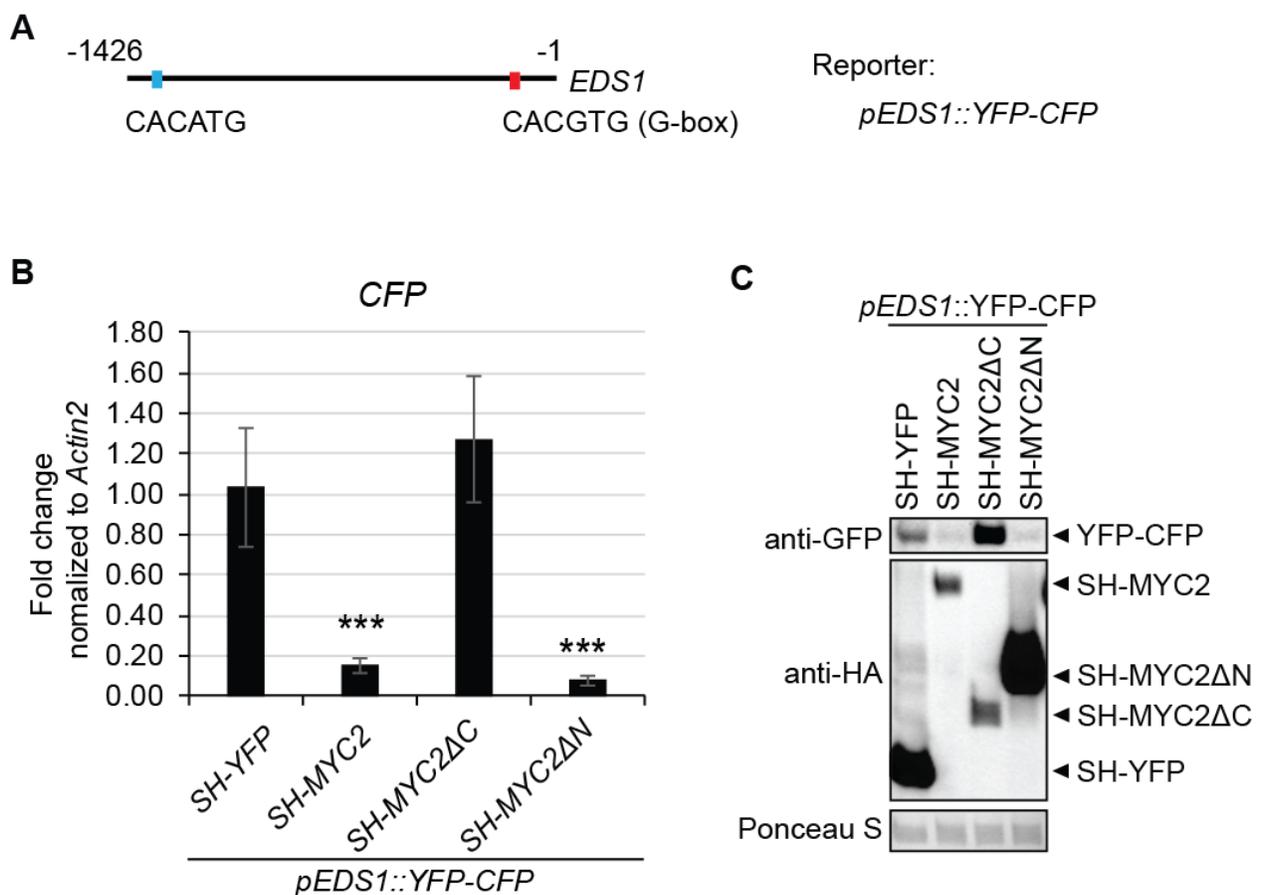


Figure 2.16. MYC2 represses *pEDS1* promoter activity in *Arabidopsis* protoplast transient assays

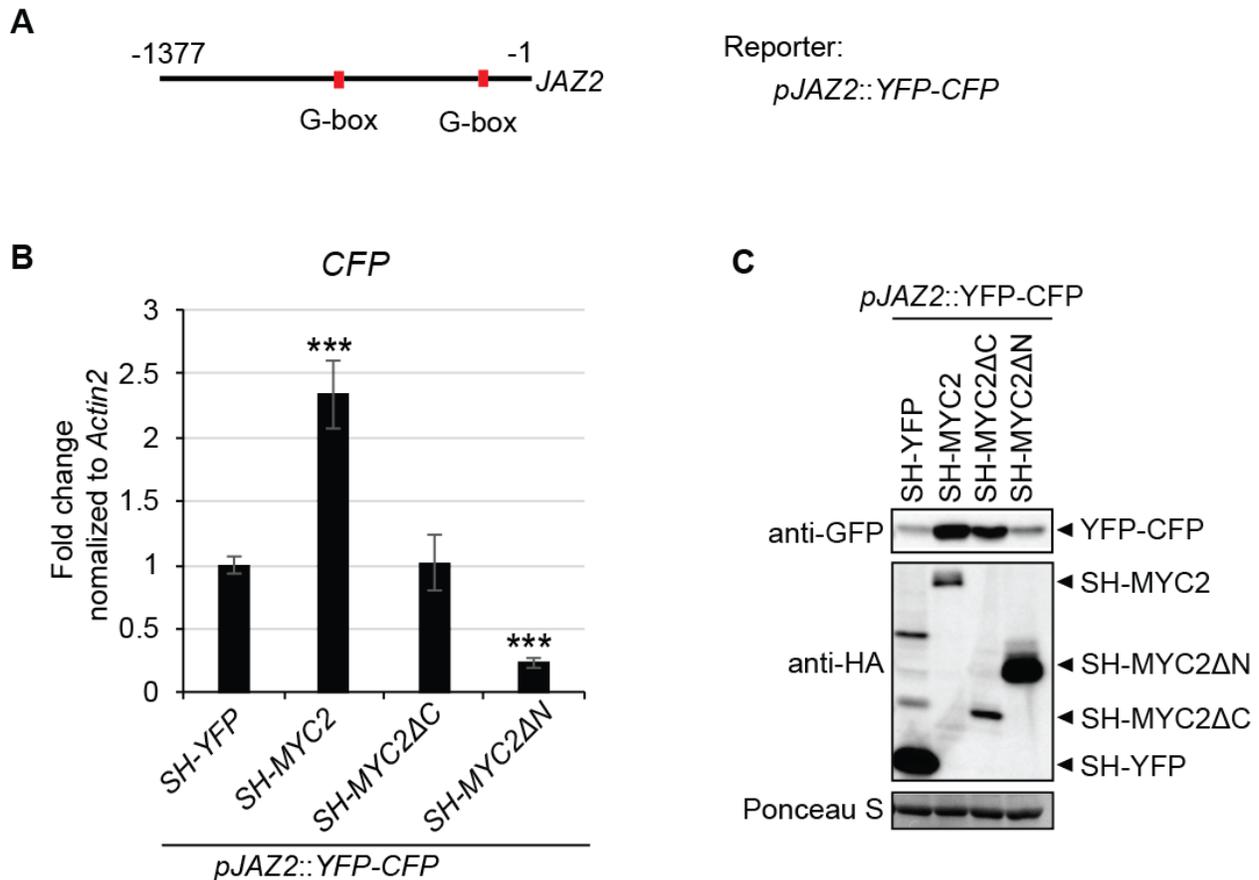
- A. Diagram of the *EDS1* promoter *pEDS1* and reporter construct *pEDS1::YFP-CFP*.** Numbers refer to the nucleotide position upstream of the start codon ATG. The red box represents G-box (CACGTG) and blue box a G-box variant (CACATG).
- B. Full-length MYC2 as well as MYC2ΔN represses *pEDS1* activity in Arabidopsis *myc234* protoplasts transient assays.** The reporter plasmid *pEDS1::YFP-CFP* was co-transformed with *SH-MYC2*, *SH-MYC2ΔC*, *SH-MYC2ΔN* or *SH-YFP* driven by the *35S* promoter into Arabidopsis *myc234* protoplasts. RNA was isolated from the samples harvested at 8 h post transfection. RNA samples were first treated with RNase-free DNase and then analyzed by one-step qPCR. *YFP-CFP* transcript levels were determined by normalizing to the expression of *Actin2* in the same samples. Error bars represent the standard deviation. \*\*\* indicates significant differences in *pEDS1::YFP-CFP* expression compared to a control sample transformed with *p35S::SH-YFP* (Student's *t*-test,  $p < 0.001$ ). The experiment was repeated two times independently, each time contains 2 biological replicates, with similar results, results from one are shown.
- C. MYC2 and MYC2ΔN reduce accumulation of YFP-CFP in Arabidopsis *myc234* protoplasts transient assays.** The reporter protein YFP-CFP, under control of *pEDS1* was transiently co-expressed with *SH-MYC2*, *SH-MYC2ΔC*, *SH-MYC2ΔN* or *SH-YFP* in Arabidopsis *myc234* protoplasts. At 16 h post transfection, protein extracts were immunoblotted with specific anti-GFP and anti-HA antibodies. Equal sample loading on the blot was estimated with Ponceau S staining. The experiment was repeated two times independently with similar results.

To test *pEDS1* activity, I measured the transcript levels of *CFP* in Arabidopsis *myc234* protoplasts after co-transformation of *pEDS1::YFP-CFP* with the *MYC2* constructs. Lower *CFP* transcript accumulation was detected in samples expressing with *SH-MYC2* and *SH-MYC2ΔN* compared to those with *SH-YFP* (Figure 2.16B, Student's *t*-test,  $p < 0.001$ ). A similar pattern was observed when YFP-CFP protein levels were analyzed (Figure 2.16C). These results suggest that both MYC2ΔN and full-length MYC2 repress *pEDS1* activity when EDS1 protein itself is not expressed (compared to Figure 2.15B). However, *SH-MYC2ΔC* expressing samples accumulated higher YFP-CFP protein levels compared to samples with *SH-YFP* (Figure 2.16C). This difference is not clear at the transcript levels (Figure 2.16B), suggesting MYC2ΔC possibly acts on the *YFP-CFP* expression at the post-transcriptional level. In summary, the above transient assays provide evidence that full-length MYC2 and MYC2ΔN, but not MYC2ΔC, can act as a repressor of *EDS1* expression at the level of the *EDS1* promoter.

In transient co-expression assays, high expression levels might interfere with the physiological function of a protein, especially a TF which is normally expressed at low levels. To rule out that suppression of *pEDS1* activity by MYC2 is a result of non-specific transcription regulation, I tested

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whether, in the same assays, MYC2 is able to exert its transcription stimulating activity. MYC2 directly and positively regulates *JAZ2* via targeting its promoter (Figuroa and Browse, 2012). The *JAZ2* promoter (*pJAZ2*), containing two G-box motifs, was cloned to generate the *pJAZ2::YFP-CFP* for transient assays (Figure 2.17A). Higher levels of *YFP-CFP* transcripts were observed in *pJAZ2::YFP-CFP* samples co-transformed with *p35S::SH-MYC2* compared to those co-transformed with *p35S::HA-YFP* (Figure 2.17B). This was also evident at YFP-CFP protein level (Figure 2.17C). Transcript levels of *YFP-CFP* under the control of *pJAZ2* were significantly lower in samples co-transformed with *p35S::SH-MYC2ΔN* than in samples with *p35S::SH-YFP* (Figure 2.17B, Student's *t*-test,  $p < 0.001$ ). However, this difference was not manifested at the YFP-CFP protein level (Figure 2.17C). Nonetheless, this experiments shows that MYC2 specifically promotes, while MYC2ΔN represses, *pJAZ2* activity in Arabidopsis. In summary, I conclude that MYC2 specifically suppresses *pEDS1* activity likely via the MYC2 C-terminal portion (MYC2ΔN) in Arabidopsis protoplasts transient assays.



**Figure 2.17. MYC2 promotes *pJAZ2* transcription activity in Arabidopsis transient assays**

- A. Diagram of *JAZ2* promoter (*pJAZ2*) and reporter construct *pJAZ2::YFP-CFP*.** Numbers refer to the position of nucleotides upstream of the start codon ATG. The red box represents a G-box (CACGTG) element.
- B. MYC2 promotes *pJAZ2* transcription activity in Arabidopsis *myc234* protoplasts transient assays.** *pJAZ2::YFP-CFP*, as reporter plasmid, was co-transformed with *SH-MYC2*, *SH-MYC2ΔC*, *SH-MYC2ΔN* or *SH-YFP* driven by *35S* promoter in *myc234* protoplasts. A one-step qPCR was used to analyze the RNA isolated from the samples harvested at 8 h post transfection. The relative *YFP-CFP* transcript levels were determined by normalization with expression of *Actin2* in the same sample. Error bars represent the standard deviation. \*\*\* indicates significant differences in *YFP-CFP* expression compared to the control samples transformed *SH-YFP* (Student's *t*-test,  $p < 0.001$ ). The experiment with two biological replicates was repeated two times independently with similar results. Results from one experiment are shown.
- C. MYC2 promotes accumulation of YFP-CFP under control of *pJAZ2* in Arabidopsis *myc234* protoplasts transient assays.** The reporter protein YFP-CFP driven by *pJAZ2* was transiently co-expressed with *SH-MYC2*, *SH-MYC2ΔC*, *SH-MYC2ΔN* or *SH-YFP*, in *myc234* protoplasts. At 16 h post transfection, protein extracts were immunoblotted with specific anti-GFP and anti-HA anti-bodies. Equal sample loading on the blot was estimated with Ponceau S staining. The experiment was repeated two times independently with similar results.

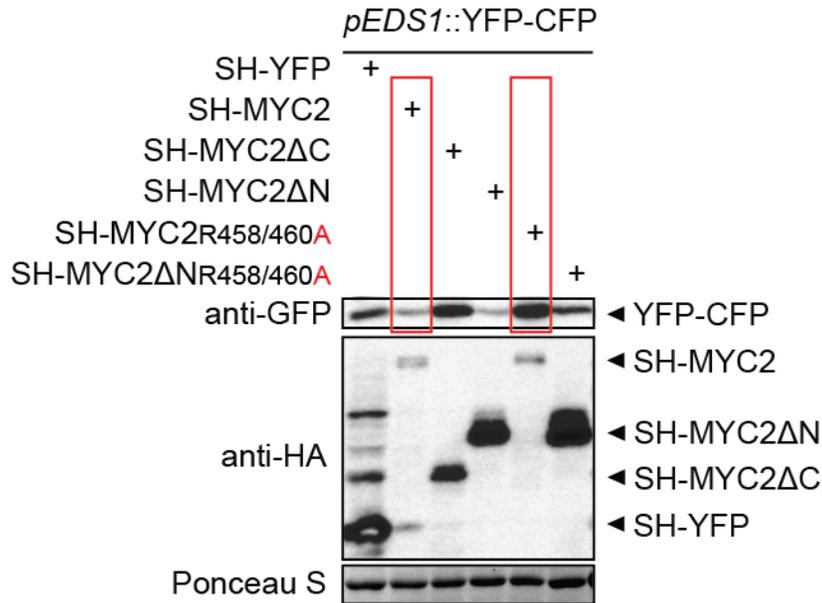
### 2.2.6 MYC2 DNA-binding is essential for MYC2 suppression of *pEDS1* activity

The above data provide strong evidence that MYC2 suppresses *EDS1* expression at the level of the *EDS1* promoter. However, this does not inform us on whether MYC2 exerts repression activity directly on *EDS1* promoter or via other proteins. I therefore checked requirements of MYC2 DNA-binding activity in *EDS1* promoter repression. The conserved bHLH domain of MYC2 is responsible for direct binding to the G-box in the promoter of *rd22*, a dehydration-responsive gene, in Arabidopsis (Abe et al., 1997). Analysis of the crystal structure of the mammalian MyoD (Myoblast determination protein1) bHLH domain indicated that two arginine (R) residues directly contact the G-box motif in the DNA (Ma et al., 1994). I aligned amino acid sequences of bHLH domains from MYC2, MYC3 and MYC4 with MyoD and found that R458 and R460 in MYC2 correspond to the conserved R residues involved in DNA binding in c-MYC (Figure 2.18A).

A



B



**Figure 2.18. DNA-binding ability is essential for MYC2 to suppress *pEDS1* activity in Arabidopsis protoplasts transient assays.**

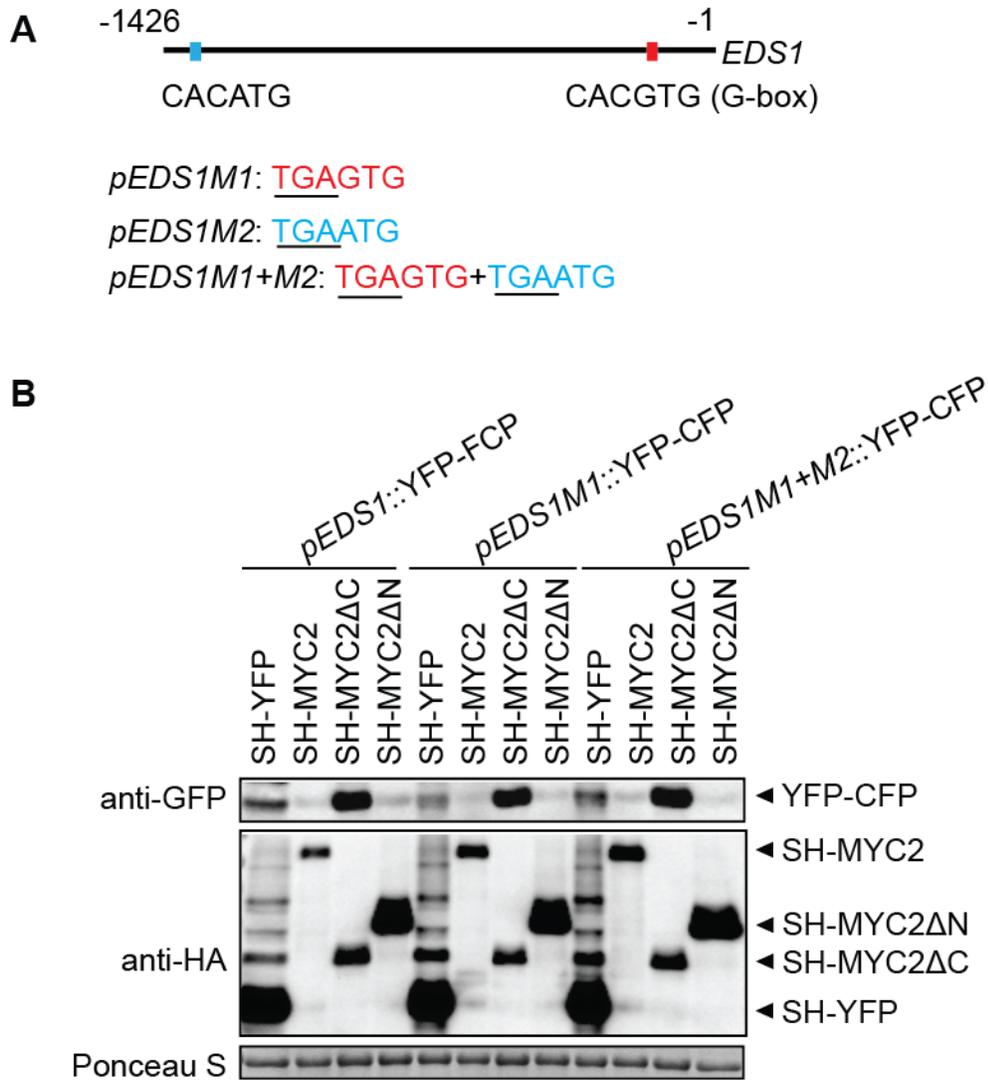
- A. Alignment of the bHLH domain from Arabidopsis MYC2, MYC3, MYC4 and human c-MYC.** The numbers refer to the amino acid position in MYC2 and the black triangles highlight two conserved arginines (R).
- B. R458A/R460A substitution of MYC2 losses repression on *pEDS1* in Arabidopsis protoplasts transient assays.** *pEDS1::YFP-CFP* was transiently co-expressed with SH-YFP, SH-MYC2, SH-MYC2ΔC, SH-MYC2ΔN, SH-MYC2<sup>R458A/R460A</sup> or SH-MYC2<sup>R458A/R460A</sup> in *myc234* protoplasts. At 16 h post transfection, immunoblotting was used to analyze protein extracts using specific anti-GFP and anti-HA antibodies. The red boxes highlight the different YFP-CFP protein accumulation between the samples with the presence of SH-MYC2 and SH-MYC2<sup>R458A/R460A</sup>. Equal sample loading on the blot was estimated with Ponceau S staining. The experiment was repeated two times independently with similar results.

These two R residues were mutated to alanines (A) (SH-MYC2<sup>R458A/R460A</sup> and SH-MYC2ΔN<sup>R458A/R460A</sup>) to assess the requirement of DNA-binding for repression of *pEDS1* by MYC2 and MYC2ΔN. SH-MYC2<sup>R458A/R460A</sup> and SH-MYC2ΔN<sup>R458A/R460A</sup> lost their suppression activity on *pEDS1* because the SH-MYC2<sup>R458A/R460A</sup> and SH-MYC2ΔN<sup>R458A/R460A</sup> expressing samples displayed increased accumulation of YFP-CFP compared to the samples with wild-type versions of the respective MYC2 constructs (Figure 2.18B). The R458A and R460A mutations did not influence MYC2 and MYC2ΔN protein accumulation (Figure 2.18B). I concluded that direct DNA-binding activity is essential for MYC2 to repress *pEDS1* activity.

### **2.2.7 Suppression of *pEDS1* activity by MYC2 is G-box-independent**

Since DNA-binding is essential for MYC2 to suppress *pEDS1* transcriptional activity in the Arabidopsis protoplast transient assays, and G-box and one G-box-related motif were found in the *EDS1* promoter, I tested whether binding to the G-box or the G-box variant is required for MYC2 suppression of *pEDS1* activity. Two reporter constructs with a mutated G-box and its variant in *pEDS1* were generated. G-box CACGTG was mutated to TGAGTG in the *pEDSIM1::YFP-CFP* construct, while *pEDSIM1+M2::YFP-CFP* contains an additional mutation in the G-box variant: from CACATG to TGAATG (Figure 2.19A). Each mutated reporter construct was co-expressed with SH-MYC2ΔC, SH-MYC2ΔN, SH-MYC2 or SH-YFP in Arabidopsis *myc234* protoplasts. Surprisingly, expression of SH-MYC2 and SH-MYC2ΔN reduced YFP-CFP protein to similar levels when the reporter gene was driven by both mutated *pEDSIM1* and *pEDSIM1+M2* promoters, or by the wild-type *pEDS1* (Figure 2.19B). However, additional experiments are required to confirm whether the protein accumulation levels correspond to the mRNA levels. The suppression activity of MYC2 on *pEDS1* is independent of G-box and G-box variant motifs because the repressive activity of MYC2 and MYC2ΔC on *EDS1* promoter was previously manifested at both *YFP-CFP* mRNA levels and its protein accumulation (Figure 2.16 B and C). I concluded that MYC2 exerts its repressive activity on *EDS1* expression either by binding at other non-canonical sites on the *EDS1* promoter, or indirectly by affecting the binding/ or repressor activities of the other TFs. However, additional mRNA quantitative experiments at the transcript levels are required to test whether the protein accumulation is positively correlated with the transcript levels.

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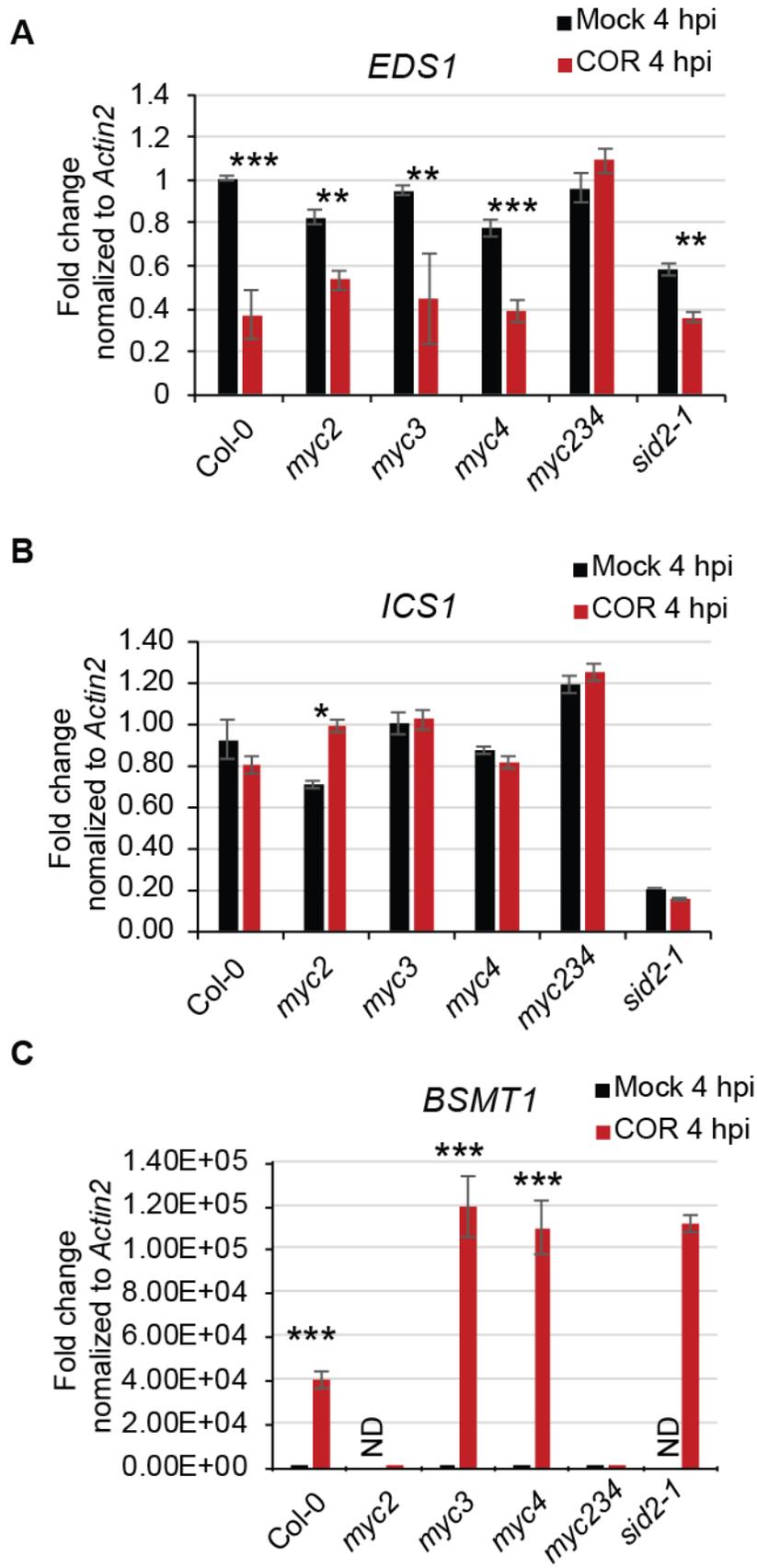
**Figure 2.19. Suppression of *pEDS1* activity by MYC2 is G-box-independent in Arabidopsis protoplasts transient assays.**

- A.** Diagram of *pEDS1* and underlines indicate the mutated sequences of G-box (red) and G-box variant (blue) in *pEDS1*. *pEDS1M1* contains a mutated G-box, *pEDS1M2* contains a mutated G-box variant and *pEDS1M1+M2* contains both mutated G-box and G-box variant.
- B.** The suppression of MYC2 on *pEDS1* activity is G-box-independent in Arabidopsis *myc234* protoplasts transient assay. The reporter YFP-CFP under control of *pEDS1*, *pEDS1M1* or *pEDS1M1+M2*, were transiently co-expressed with SH-YFP, SH-MYC2, SH-MYC2ΔC or SH-MYC2ΔN in *myc234* protoplasts. At 16 h post transfection, protein extracts were immunoblotted with specific anti-GFP and anti-HA antibodies. Equal sample loading on the blot was estimated with Ponceau S staining. The experiment was repeated two times independently with similar results.

### **2.2.8 MYC2-family TFs function redundantly to negatively regulate *EDS1* expression**

The finding that MYC2 specifically suppresses *pEDS1* activity in transient assays raises a question whether it is a physiological function of MYC2 to reduce EDS1 expression in order, possibly, to maintain a balance between stress hormone pathways. I applied COR, a structural mimic JA-Ile which is able to induce MYC2 expression and increase MYC2 steady-state levels (Zhai et al., 2013), to examine whether MYC2 represses *EDS1* expression in Arabidopsis plants growing on soil. At 4 h after COR spraying, expression of *EDS1* was reduced in the *myc2*, *myc3*, and *myc4* single mutants, and in the wild-type Col-0 compared to mock treatments (Figure 2.20A). By contrast, COR treatment did not cause a reduction in *EDS1* transcript levels in the triple mutant *myc234* (Figure 2.20A). Thus, MYC2 family TFs function redundantly to repress *EDS1* expression in Arabidopsis.

SA accumulation through *ICS1* (*Isochorismate synthase1*) upregulates *EDS1* transcription (Feys et al., 2001), and *ICS1*-dependent SA accumulation is in turn negatively regulated by COR secreted by *Psm* ES4326 (*Pseudomonas syringae* pv. *maculicola* ES4326) through TFs ANAC019, 055 and 072 (Zheng et al., 2012). Therefore, *sid2-1*, a null mutant of *ICS1* which is unable to accumulate pathogen-induced SA (Nawrath and Metraux, 1999; Wildermuth et al., 2001), was used to test whether COR-induced repression of the basal *EDS1* transcription is due to a reduced *ICS1* expression. COR treatment did not reduce *ICS1* expression in Col-0 or in *myc2*, *myc3*, *myc4*, *sid2-1* single mutants or the triple mutant *myc234* (Figure 2.20B). However, the reduction of *EDS1* mRNA levels caused by COR treatment in the same tissues was observed in the *sid2-1* mutant and in Col-0 (Figure 2.20A). Taken together, these results suggest that COR-induced suppression of *EDS1* by combined activities of the MYC2, MYC3 and MYC4 TFs is largely *ICS1*-independent and therefore is unlikely to be due to *ICS1*-mediated SA accumulation.

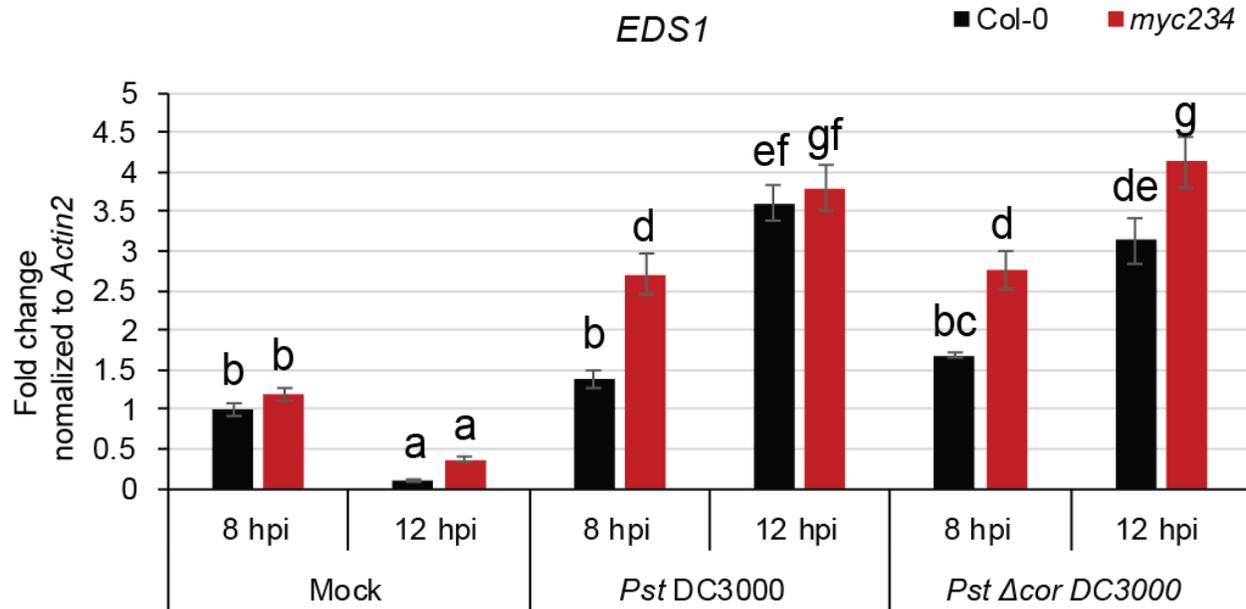


**Figure 2.20. MYC2-family transcription factors function redundantly to negatively regulate *EDS1* expression in response to COR.** Two-week-old seedlings of Col-0, the single *myc2*, *myc3*, *myc4* and *sid2-1* mutants and triple *myc234* mutant were sprayed with 2 nM COR or water as control (mock). At 4 h post treatment, RNA was isolated and analyzed by qPCR, and the relative transcript levels of *EDS1* (A), *ICS1* (B) and *BSMT1* (C) were determined by normalization with the expression of *Actin2*. ND, no detectable signal. Error bars represent standard deviation. The star \* represents significant difference of gene expression between mock and COR treatment (*t*-test; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ,  $n=3$ ). The experiment was repeated three times independently with similar results, results from one are shown.

I considered other paths by which COR via MYC2-family TFs might antagonizing *EDS1* expression. *BSMT1* (*Salicylate/benzoate carboxyl methyltransferase 1*) encodes a methyltransferase that methylates SA, converting it to an inactive and volatile form (Chen et al., 2003). The *BSMT1* expression is upregulated by *Psm* ES4326 secreted COR (Chen et al., 2003; Zheng et al., 2012). I found that *BSMT1* was also induced by COR application in Col-0, *myc3*, *myc4* and *sid2-1* single mutants but not in *myc2* single or *myc234* triple mutant plants (Figure 2.20C). Since the *myc2* mutation alone does not affect *EDS1* transcription repression after applying COR (Figure 2.20A), but does significantly reduce *BSMT1* mRNA levels, I concluded that COR-induced MYC2-family TFs-modulated suppression of *EDS1* is independent of *BSMT1*. In conclusion, MYC2, MYC3 and MYC4 function redundantly with each other but independently of SA to repress *EDS1* transcription in Arabidopsis plants with exogenous COR treatment.

### **2.2.9 MYC2-family TFs delay induction of *EDS1* in basal resistance**

The experiments described above revealing an activity of COR-induced MYC2-family TFs in suppressing *EDS1* expression at the level of the *EDS1* promoter were performed without pathogen infection. The virulent strain *Pst* DC3000 was applied to determine whether MYC2-family TFs also repress *EDS1* expression in basal resistance as well. At 8 h after *Pst* DC3000 infiltration, induction of *EDS1* expression was higher in *myc234* compared to that in Col-0 (Figure 2.21, Tukey HSD test,  $p < 0.05$ ,  $n=4$ ). However, by 12 hpi, *EDS1* expression was similar between Col-0 and the *myc234* mutant (Figure 2.21, Tukey HSD test,  $p < 0.05$ ,  $n=4$ ). These data suggest that MYC2-family TFs activities lead to delayed induction of *EDS1* expression during the basal resistance.



**Figure 2.21. MYC2-family TFs negatively regulate the expression of *EDS1* in basal immunity.** 4.5-week-old leaves of Col-0 and the *myc234* triple mutant were pressure infiltrated with H<sub>2</sub>O, *Pst* DC3000 and *Pst Δcor* DC3000. RNA was isolated from the samples harvested at 8 h and 12 h post infection. The transcript levels of *EDS1* were determined by qPCR and normalized to *Actin2* expression. Different letters above error bars indicate significant differences of *EDS1* expression in different samples (one-way ANOVA with Tukey HSD test;  $p < 0.05$ ,  $n = 4$ ). The experiment was repeated two times independently with similar results. Results from one are shown.

Since COR application downregulates *EDS1* expression in Arabidopsis, I reasoned that *Pst* DC3000 secreted COR could dampen the transcription of *EDS1*. To test this possibility, I analyzed the *EDS1* expression post leaf infiltration with *Pst Δcor* DC3000. No significant difference in *EDS1* mRNA levels was observed between *Pst* DC3000 and *Pst Δcor* DC3000 in both Col-0 and *myc234* (Figure 2.21, Tukey HSD test,  $p < 0.05$ ,  $n = 4$ ). These preliminary results imply that a MYC2-family TFs-dependent delay of *EDS1* transcription after *Pst* DC3000 infection is not due to bacterial COR. Taken together, these results point to an important action of MYC2-family TFs in negatively regulating *EDS1* transcription in basal resistance. Preliminary analysis also suggests that these MYC2-family TFs actions on *EDS1* are not affected by bacterial COR.

In summary, I found associations between *EDS1*-family proteins and MYC2-family TFs. In Arabidopsis protoplasts transient assays, *EDS1*, *PAD4* and *SAG101* each formed a complex with MYC2, and compared to *EDS1*, *PAD4* and *SAG101* displayed stronger association with MYC2.

Furthermore, the presence of EDS1 interfered with MYC2 transcriptional activity. These results provide the first evidence that EDS1 is involved in transcriptional regulation via association with and antagonism of TFs, such as MYC2. The Arabidopsis protoplasts assays also showed that the DNA-binding is essential for MYC2 to repress *pEDS1* activity, which is independent of G-box and G-box variant motif in *pEDS1*. MYC2-family TFs were shown to function redundantly in downregulating *EDS1* after exogenous COR application. Induction of *EDS1* was delayed by MYC2-family TFs at an early stage of *Pst* DC3000 and *Pst Δcor* DC3000 infection, which was counteracted, probably by active EDS1 resistance signaling, at a later infection stage. To conclude, these data suggest a mutual antagonism between EDS1- and MYC2-dependent defense responses.

### 3 Discussion

EDS1 is essential for basal, TNL-and certain TNL-mediated innate immunity. However, little is known about how EDS1 signaling is regulated and which molecular events connect EDS1 to transcriptional defense reprogramming. In this thesis, I applied genetic screen for suppressors of *eds1-2* to identify signaling components downstream of *EDS1*. Seven *sed* mutants were identified with restored *eds1-2* resistance to *Pst* DC3000 *avrRps4* (Figure 2.3, 2.4 and 2.5). Among these mutants, four of them contained four different mutations in *COI1* (*Coronatine-Insensitive1*) (Figure 2.6 and Table 2.2), which is essential for JA signaling and bacterial COR-promoted virulence (Xie et al., 1998; Brooks et al., 2005; Laurie-Berry et al., 2006; Melotto et al., 2006; Zheng et al., 2012). Downstream of *COI1*, TF *MYC2* is a master in regulating JA signaling-dependent responses, such as modulating JA-SA crosstalk in response to pathogen infection (Laurie-Berry et al., 2006; Chini et al., 2007; Dombrecht et al., 2007; Zheng et al., 2012; Zhang et al., 2015a; Schmiesing et al., 2016).

Bacterial COR promoted *Pst* DC3000 *avrRps4* virulence in *eds1-2*, which was abolished in Col-0 (Figure 2.7), suggesting EDS1 resistance signaling activated by TNL *RRS1/RPS4* suppresses *COI1*-mediated COR-triggered JA signaling pathway. The associations between EDS1-family proteins and *MYC2*-family TFs (Figures 2.8, 2.9, 2.10, 2.11, 2.12 and 2.13) provide the first evidence that EDS1 modulates transcriptional reprogramming via association with TFs. This was supported by the finding that the presence of EDS1 interfered *MYC2* transcriptional regulation in Arabidopsis protoplasts transient assays (Figures 2.15 and 2.16). Reciprocally, *MYC2* was shown to repress *EDS1* promoter activity in Arabidopsis protoplasts transient assays (Figures 2.16, 2.18 and 2.19), and *MYC2* with its sequence-related *MYC3* and *MYC4* redundantly downregulated *EDS1* transcription with exogenous COR application (Figure 2.20). This repressive function of *MYC2*-family TFs on induction of *EDS1* was also manifested at an early stage of *Pst* DC3000 infection, which appears to be counteracted by EDS1 resistance signaling at a later stage (Figure 2.21). This body of evidence here illuminates a mutual antagonism between EDS1- and *MYC2*-mediated signaling, which modulates SA-JA crosstalk in Arabidopsis immunity. The implications and shortfalls of the results achieved here will be discussed within the current knowledge of EDS1 and *MYC2*. And further required experiments will be proposed in detail below.

### **3.1 Forward genetic screening for suppressors of *eds1***

Here, I hypothesized that EDS1 resistance signaling is negatively regulated by suppressors which can be antagonized or eliminated by activated EDS1 signaling in TNL-mediated resistance responses (ETI). With this hypothesis as a basis, I found seven mutants with restored resistance to *Pst* DC3000 *avrRps4* in *eds1-2* background. A truncated CPR5 was identified to be responsible for resistance to *Pst* DC3000 *avrRps4* recognized by *RRS1/RPS4* in *sed3* that was coupled with constitutive defense responses (Figure 2.3, and J. Qiu, unpublished). Defective BR signaling pathway caused by *BSU1*<sup>P650L</sup> and truncated DMR6 probably restored paired TNL *RRS1/RPS4*-mediated resistance to *Pst* DC3000 *avrRps4* in *sed1* and *sed2*, respectively (Figures 2.4 and 2.5). Notably, four different mutations in *COI1* were identified with enhanced resistance to *Pst* DC3000 *avrRps4* in *sed4*, *sed5*, *sed6* and *sed7*, respectively (Table 2.2, Figures 2.6 and 2.7), suggesting that *COI1* mutation partially restores the defect of *eds1-2* to *Pst* DC3000 *avrRps4*. Previously, bacterial COR secreted by *Pseudomonas syringae* was shown to promote bacterial virulence by triggering *COI1*-mediated JA signaling, which in turn suppresses SA accumulation-dependent resistance signaling (Brooks et al., 2005; Zheng et al., 2012). This COR virulence was not manifested in wild-type Col-0 (Figure 2.7), suggesting that EDS1-controlled resistance signaling suppresses bacterial COR-triggered virulence. These data suggest that *COI1* is likely a negative signaling component that is antagonized by EDS1-triggered resistance signaling.

#### **3.1.1 Truncated CPR5 restores TNL (*RRS1/RPS4*) resistance to *Pst* DC3000 *avrRps4* in *sed3***

Several mutants in potential negative regulators of plant immunity have been identified as plants displaying constitutive resistance, such as Arabidopsis *edr1* (*enhanced disease resistance1*, a MAPKKK mutant) (Frye et al., 2001) and *cpr5* (*constitutive expresser of PR genes5*) (Bowling et al., 1994; Bowling et al., 1997), and also *dmr3* (*downy mildew-resistance3*) mutant (Van Damme et al., 2005). In this *sed* mutant screen, I found a mutation in Arabidopsis *CPR5* resulting in a truncated CPR5 protein in *sed3* (Figure 2.3) and two other *cpr5* alleles were also identified by Dr. Haitao Cui (H. Cui, unpublished), which highly suggests that the truncated CPR5 is responsible for enhanced constitutive defense in *sed3*. The observed enhanced immune responses in *sed3* are also consistent with a previous study that *eds1* partially reduced constitutive defenses of *cpr5-1* (Clarke et al., 2001). Moreover, CPR5 was shown to negatively regulate CNL- and TNL-triggered

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ETI and autoimmune responses of *cpr5* were dependent on CKIs (Cyclin-dependent Kinase Inhibitors) and a cell-cycle TFs E2F (Wang et al., 2014b). Loss-of-function *cki* and *e2f* mutations in a WT background also compromised ETI responses induced by TNL and CNL, consistent with these factors operating in a convergent NLR resistance pathway that is negatively regulated by CPR5 (Wang et al., 2014b). Recent work have suggested that CPR5 resides at the NPC (nuclear pore complex) and a conformational change in CPR5 releases CKIs which causes over-activation of E2F (Wang et al., 2014b; Gu et al., 2016). This change at the NPC leads to transcriptional stimulation of diverse pathways in TNL and CNL ETI activation and programmed cell death (Gu et al., 2016). Based on these findings, it is likely that EDS1 acts downstream of CPR5.

### **3.1.2 Disrupted BR signaling by BSU1<sup>P650L</sup> might restore basal and TNL resistance in *eds1-2***

The plant growth-promoting hormone BR (brassinosteroid) signaling is involved in regulating the trade-off between growth and immunity (Lozano-Duran and Zipfel, 2015). Previously, a gain-of-function mutant *BRI1<sup>sudl</sup>* showing constitutive activation of BR signaling, displayed enhanced susceptibility to virulent *Pst* DC3000 and *Hpa* Noco2 (Belkhadir et al., 2012). Moreover, the BR signaling mutant, *bak1-3* (*BRI1-associated kinase1*) showed enhanced basal resistance to *Hpa* Noco2 and also *RPP4*-mediated resistance to *Hpa* EMWA1 (Belkhadir et al., 2012). A recent report showed that overexpressing the TF gene *HBII* positively regulated BR-responsive genes and enhanced susceptibility to *Pst* DC3000, whereas the *HBII* co-suppressed plants displayed increased basal resistance to *Pst* DC3000 (Fan et al., 2014). These results suggest that BR signaling pathway negatively regulates basal resistance. Here, I found a P650L mutation in BSU1 (*BRI1-Suppressor1*) in *sed1* (Figure 2.4). Acting as a positive regulator of BR-signaling pathway, BSU1 dephosphorylates BIN2 (*BR-Insensitive2*) which consequently activates two TFs, BZR1 (*Brassinazole-Resistant1*) and BES1 (*BRI1-EMS Suppressor1*), leading to transcriptional changes of BR-responsive genes (Yin et al., 2002; Kim et al., 2011). The BSU1<sup>P650L</sup> mutation might disrupt BR signaling, thereby leading to restored basal resistance of *sed1* to *Pst* DC3000, TNL (*RRS1/RPS4*) resistance to *Pst* DC3000 *avrRps4* and TNL (*RPP4*) resistance to *Hpa* EMWA1 (Figure 2.3 B, C and D). In a microarray analysis of *Ws-0* and *eds1-1* to *Pst* DC3000 *avrRps4*, *BZR1* and *BES1* were down-regulated by 2.4 fold in an *EDS1*-dependent manner after *Pst* DC3000 *avrRps4* infection (Bartsch et al., 2006). These results suggest that BR signaling components function as repressors of *EDS1* signaling, which can be removed by activated EDS1 resistance

signaling. However, this view requires to be evidenced by further experiments, such as genetic transformation *BSU1* into *sed1*, because only one *bsu1* mutant was identified here.

### **3.1.3 DMR6 acts as negative regulator in plant immunity**

The negative role of DMR6 (Downy Mildew Resistance6) in plant immunity has been demonstrated in different studies. Arabidopsis Ler-0 *dmr6-1* fully restored resistance to *Hpa* Cala2 in an *eds1-2* mutant (Van Damme et al., 2005; van Damme et al., 2008). It was further reported that *dmr6-1* was more resistant than Ler *eds1-2* to virulent *Pst* DC3000 and DMR6 over-expression in Col-0 caused enhanced susceptibility towards *Pst* DC3000 (Zeilmaker et al., 2015). Here, *sed2* was identified as a G181A nucleotide transition in *DMR6* resulting in a truncated DMR6, which caused partially enhanced basal resistance to *Pst* DC3000 and TNL (*RRS1/RPS4*) resistance to *Pst* DC3000 *avrRps4*, while completely restored TNL (*RPP4*) resistance to *Hpa* EMWA1 (Figure 2.3 B, C and D). DMR6 is a 2-oxoglutarate (2OG)-Fe (II) oxygenase which acts redundantly with DLO1 (DMR6-Like Oxygenase1) and DLO2 to suppress plant immunity (Zeilmaker et al., 2015). DMR6 was further hypothesized to catabolize SA via a hydroxylation reaction in the same way as DLO1 which hydroxylates SA to form an inactive 2,3-DHBA (2,3-dihydroxybenzoic acid molecule) (Zeilmaker et al., 2015). The Col-0 *dmr6-3* mutant accumulated more SA than the *dlo1* mutant and, the dwarf phenotype of Col-0 *dmr6-3 dlo1* double mutant was *ICS1*-dependent (Zeilmaker et al., 2015). It is therefore likely that truncated DMR6 in *sed2* compensates for a defect of *eds1-2* in SA accumulation, thereby leading to restored resistance phenotype of *sed2*. These data support the negative role of DMR6 in plant defense responses. However, it is unclear whether the negative role of DMR6 is *EDS1*-dependent due to a feedback loop between SA accumulation and *EDS1* resistance signaling (Feys et al., 2001).

### **3.1.4 Disrupted JA signaling partially relieves *eds1-2* defects in TNL and basal immunity**

Bacterial COR promotes pathogen entry into plant apoplastic spaces by stimulating stomatal reopening and enhances bacterial multiplication after entry (Mittal and Davis, 1995; Melotto et al., 2006). As a potent JA-Ile mimic, COR antagonizes SA-dependent defenses by activating JA signaling (Zheng et al., 2012). In this antagonism, three TFs ANAC019, ANAC055 and ANAC072 down-regulate the SA biosynthetic gene *ICS1* and up-regulate an SA metabolic gene *BSMT1*, leading to lower SA accumulation and promotion of *Pseudomonas syringae* virulence (Zheng et al., 2012) (see Figure 1.1). As a co-receptor for bacterial COR, COI1 transmits COR-induced

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susceptibility, as different *coil* mutants exhibit enhanced resistance to COR-secreting *Pseudomonas syringae* strains compared to wild-type Col-0 (Xie et al., 1998; Kloek et al., 2001; Sheard et al., 2010; Huang et al., 2014; Zhang et al., 2015b). A single amino acid exchange in COI1<sup>G330E</sup> reduced COI1 protein accumulation in *coil-21<sup>rps</sup>* which exhibited slightly less resistance to *Pst* DC3000 compared to *coil-1* and *coil-16* null mutants (He et al., 2012). By contrast, COI1<sup>G369E</sup>, COI1<sup>G155E</sup>, COI1<sup>D452A</sup> and COI1<sup>L490A</sup> mutations abolished COI1 protein accumulation in *coil-4*, *coil-7*, *coil-9* and *coil-10* respectively, and disrupted all JA-related responses (Huang et al., 2014). Based on these insights, I concluded that the COI1<sup>G330A</sup> mutation in *sed5* and COI1<sup>G369E</sup> mutation in *sed6* likely interfere with COI1 protein accumulation, thereby restoring TNL resistance to *Pst* DC3000 *avrRps4* in screening.

Interestingly, a recent publication reported that COI1<sup>A384V</sup> accumulates normally but has greatly reduced COR-binding and enhanced resistance to *Pst* DC3000, suggesting that disruption of COR perception by COI1<sup>A384V</sup> leads to a loss of COR-induced susceptibility without interfering with COI1 stability (Zhang et al., 2015b). I therefore speculated that the restored resistance of *sed4* might be a consequence of reduced COI1<sup>A384T</sup> binding of the COR molecule upon *Pst* DC3000 *avrRps4* infection (Figure 2.7). This was supported by similar TNL resistance to *Pst* DC3000 *avrRps4* and *Pst cor-* DC3000 *avrRps4* in *sed4* (Figure 2.7). I also found that the *sed4* mutant is partially male-fertile, suggesting that the COI1<sup>A384T</sup> mutation only partially disrupts the perception of JA-Ile. This contrasts with a previous claim that COI1<sup>A384T</sup> loses recognition of both JA-Ile and COR because COI1<sup>A384T</sup> completely disrupted JA-Ile- and COR-dependent COI1-JAZ9 interactions in yeast (Zhang et al., 2015b). This difference might be due to the different penetrance of COI1<sup>A384T</sup> in wild-type Col-0 and *eds1-2* mutant. I also found a new amino acid substitution COI1<sup>E359K</sup> in *sed7* (Table 2.2 and Figure 2.5), which is fully male-sterile. Whether the enhanced resistance to *Pst* DC3000 *avrRps4* and male-sterility of *sed7* is due to disrupted COI1 protein accumulation or abolished perception of COR and JA-Ile is not yet known. However, COI1 protein accumulation can be tested by transiently expressing COI1 and COI1<sup>A384T</sup> in Arabidopsis protoplasts. The results presented here suggest that disrupted perception of bacterial COR in multiple independent *coil* alleles reduces *eds1-2* disease susceptibility. Because COI1 is an essential component in JA signaling (Xie et al., 1998; Fonseca et al., 2009; Sheard et al., 2010), my results further suggest that JA/COI1 signaling leading to pathogen susceptibility becomes exposed in an Arabidopsis *eds1-2* mutant background when EDS1 signaling is disrupted.

### **3.1.5 EDS1-dependent resistance signaling suppresses bacterial COR virulence**

COR-mediated bacterial virulence is abolished in wild-type Col-0 because Col-0 with intact TNL (*RRS1/RPS4*) signaling had similar resistance to *Pst* DC3000 *avrRps4* and *Pst*  $\Delta$ *cor* DC3000 *avrRps4* (Figure 2.7). This result suggests that TNL/EDS1 signaling suppresses bacterial COR-promoted virulence and thus likely represses COR-triggered COI1-mediated JA signaling. JA signaling is antagonized by SA accumulation downstream of COI1 by reducing TF ORA59 stability, thereby interfering with ORA59 transcriptional regulation, such as in induction of the JA pathway marker, *PDF1.2* (Van der Does et al., 2013) (Figure 1.1). Moreover, TNL-activated EDS1 resistance signaling promotes SA accumulation (Falk et al., 1999; Feys et al., 2001). Therefore, it is reasonable to argue that TNL/EDS1 resistance suppresses COR-triggered JA signaling by promoting SA accumulation. However, our recent results show that TNL (*RRS1/PR54*) activated EDS1 signaling represses the expression of JA marker genes such as *VSP1* and *BSMT1* in an SA-independent manner (H. Cui, et al., unpublished). This suggests that an EDS1-triggered SA-independent mechanism is responsible for repressing JA signaling activated by COI1 upon bacterial COR perception. Further investigation of how EDS1 suppresses JA signaling may illuminate the molecular role of EDS1 in modulating SA-JA crosstalk.

### 3.2 Mutual antagonism between EDS1 and MYC2 in defense responses

Here, I found and explored the association between EDS1-family proteins and MYC2-family TFs (Figures 2.8, 2.9, 2.10, 2.11, 2.12 and 2.13), and the EDS1-MYC2 association was shown to repress MYC2 transcriptional regulation activity without affecting MYC2 protein accumulation (Figures 2.15 and 2.16). These data provide the first evidence that EDS1 regulates transcriptional reprogramming through interaction with TFs. I also found that MYC2 specifically represses *pEDS1* promoter activity independently of known MYC2-binding G-box and G-box variant motifs, but requiring the MYC2 bHLH DNA-binding domain, and thus likely needed MYC2-DNA association (Figures 2.16, 2.18 and 2.19). Using exogenous COR applications, I established that MYC2 acts redundantly with MYC3 and MYC4 in the negative regulation of *EDS1* expression and in an SA-dependent manner (Figure 2.20). At an early stage of *Pst* DC3000 infection, MYC2-family TFs delay *EDS1* induction, which appears to be counteracted by EDS1 resistance signaling at a later infection stage (Figure 2.21). These data suggest that MYC2-family TFs antagonize EDS1 at the transcriptional level. Therefore, the results achieved here have revealed a mutual antagonism between EDS1- and MYC2-dependent signaling in defense responses.

#### 3.2.1 Arabidopsis EDS1, PAD4 and SAG101 each forms complexes with MYC2 in planta

Arabidopsis EDS1, an essential regulator for TNL-mediated transcriptional reprogramming, is hypothesized to link effector-activated TNL receptors to downstream transcription reprogramming (Bhattacharjee et al., 2011; Heidrich et al., 2011). However, it was not known how EDS1 mediates transcriptional regulation. The first indication that EDS1 interacts with transcriptional regulators came out of a large scale EDS1 Y2H screen using ~1500 Arabidopsis TFs in a prey library, in which EDS1 was found to interact with ~30 TFs, including several bHLH TFs (J. Qiu, unpublished). In independent *N. benthamiana* transient expression assays, EDS1, PAD4 and SAG101 each formed complexes with MYC2-family TFs (Figures 2.9 and 2.10). Only an EDS-MYC2 interaction was found in Y2H assays (Figure 2.8). It is possible that a MYC2 ortholog present in *N. benthamiana* but absent in yeast connects Arabidopsis MYC3 and MYC4 with Arabidopsis EDS1 because Arabidopsis MYC2 forms heterodimers with Arabidopsis MYC3 and MYC4 in *N. benthamiana* (Fernandez-Calvo et al., 2011). Heterodimer formation between *N. benthamiana* MYC2 and Arabidopsis MYC3 and MYC4 would need to be confirmed in transient assays or *in vitro* pull-down experiments.

Interestingly, EDS1, PAD4 and SAG101 each formed a complex with MYC2 in Arabidopsis transient expression assays using the *eds1-2* null mutant background (Figures 2.11 and 2.12). However, direct interaction between EDS1, SAG101 or EDS1-SAG101 complexes and MYC2 was not found with recombinant proteins expressed in *E.coli* (H. Cui, unpublished). I have two models for EDS1-family protein association with MYC2. First, an intermediate protein bridges the association between EDS1-family proteins and MYC2. The conserved C-terminal EP domains of EDS1-family proteins (Figure 1.3) contain repeating pairs of  $\alpha$  helices which are structurally similar to tetratricopeptide repeat (TPR) motifs mediating protein-protein interactions (Wagner et al., 2013). TPR domain-containing transcriptional co-repressors TPL (Topless) and TPR (Topless-like) proteins form complexes with MYC2 via JAZ proteins (Pauwels et al., 2010; Fernandez-Calvo et al., 2011). Because JAZ repressors physically interact with JID (JAZ-Interaction Domain) at the N-terminal of MYC2, it is likely that JAZs proteins bridge EDS1-family proteins with MYC2 via association with the N-terminus of MYC2. This idea is supported by the observation that PAD4 and SAG101 associate with N-terminal part of MYC2 (Figure 2.13). In future assays, I will investigate possible MYC2-EDS1 family interacting modules further and ask whether JAZ proteins bridge EDS1-family proteins with MYC2 by testing whether transiently co-expressed JAZ proteins also form complexes with EDS1-family proteins *in planta*. I also used EDS1<sup>1-384</sup> lacking its EP domain and sufficient to form EDS1-SAG101 heterodimers (Wagner et al., 2013) to examine whether the EDS1 EP domain is required for association with MYC2 *in planta*. However, no conclusive data were obtained because the accumulation of transiently expressed EDS1<sup>1-384</sup> was much lower compared to that of EDS1 in *N. benthamiana* (J. Qiu, unpublished).

The second possibility is that interaction of EDS1-family protein with MYC2 requires post-transcriptional modifications which may only occur *in planta*. This might explain why PAD4-MYC2 and SAG101-MYC2 interactions were found in *N. benthamiana* transient assays but not in Y2H assays (Figures 2.8 and 2.10). All EDS1, PAD4 and SAG101 orthology have conserved SUMO (Small Ubiquitin-Like Modifier) attachment motifs (Lee et al., 2007) and are therefore potential substrates for SUMO modification which might alter protein-protein interactions and function (Johnson, 2004; Lee et al., 2007). It is notable that SIZ1, a SUMO E3 ligase, is required for EDS1/PAD4-mediated TNL-triggered signaling (Lee et al., 2007). A site-directed mutagenesis of SUMO-latable amino acids in EDS1, PAD4 and SAG101 would be useful to determine whether SUMO modification of EDS1-family proteins is important for their association with MYC2 *in*

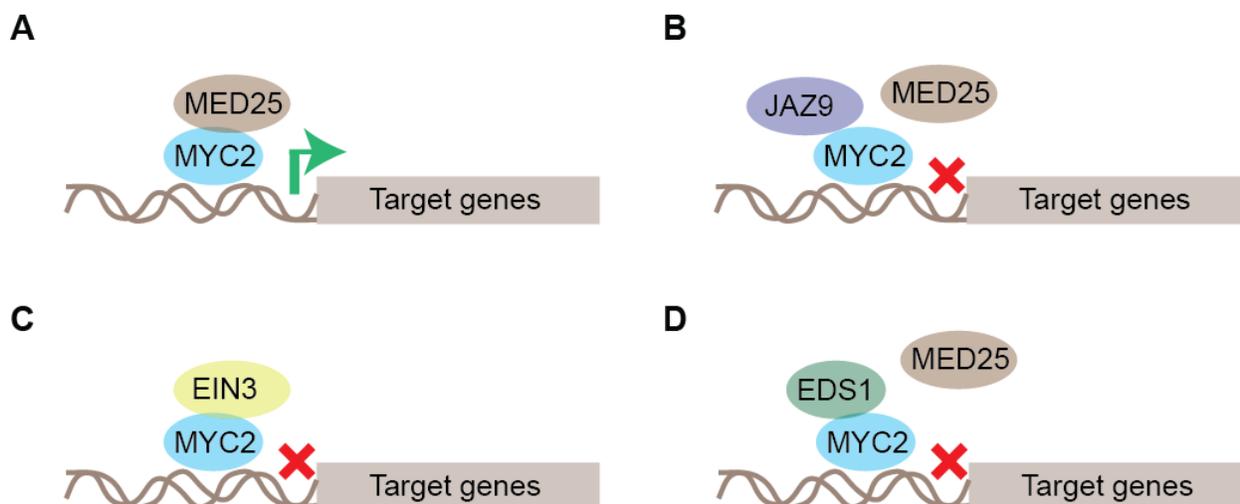
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*planta*. Also, MYC2 contains different phosphorylation sites (Zhai et al., 2013) and MYC2 can be polyubiquitinated by PUB10 (Plant U-BOX Protein10) (Jung et al., 2015). More experiments are necessary to explore the post-transcriptional mechanisms underlying EDS1-family association with and antagonism of MYC2-family TFs.

MYC2 was shown to form complex with each of EDS1-family proteins independently of EDS1-PAD4 and EDS1-SAG101 heterodimers (Figures 2.11 and 2.12). However, these heterodimers are essential for EDS1-mediated resistance signaling (Feys et al., 2001; Feys et al., 2005; Rietz et al., 2011; Wagner et al., 2013). Recently, we also found that resistance to biotrophic pathogens was greatly increased by combined enhanced accumulation of EDS1 and PAD4, but not by enhanced accumulation of them individually in stable transgenic *Arabidopsis* plants (H. Cui, et al., in press). These results suggest that formation of EDS1-PAD4 heterodimer likely triggers post-transcriptional regulatory step, which is critical for EDS1/PAD4 signaling. Therefore, we are in process of testing whether EDS1-PAD4 and EDS1-SAG101 heterodimers interfere with their associations with MYC2 via co-IP experiments using *Arabidopsis* protoplasts with a combined expression of EDS1, PAD4/ or SAG101 and MYC2, and testing whether co-expressed EDS1 and PAD4 affect MYC2 post-transcriptional modifications.

### **3.2.2 EDS1-family proteins modulate MYC2-family TF transcriptional activity**

MYC2, together with sequence-related MYC3 and MYC4 TFs, modulates the expression of a large number of genes involved in diverse signaling pathways (Dombrecht et al., 2007; Fernandez-Calvo et al., 2011). MYC2 transcriptional regulation activity was shown to require interaction with other proteins. For example, MYC2 recruits MED25 (a subunit of plant Mediator complex) via a TAD (Transcriptional Activation Domain) at the MYC2 N-terminus, which brings essential components of the PIC (Transcription Pre-initiation Complex) to enrich at MYC2 target promoters (Amoutzias et al., 2008; Kidd et al., 2009; Chen et al., 2012) (Figure 3.1A). Thus, the association of PAD4/SAG101 with the MYC2 N-terminus (Figure 2.13) may prevent recruitment of PIC by blocking MYC2-MED25 interaction, leading to suppressing MYC2 transcriptional regulation activity (Figure 3.1D). This is supported by recent finding that JAZ9, which physically interacts with JID domain in MYC3 N-terminus, inhibits the MYC3-MED25 interaction (Zhang et al., 2015a) (Figure 3.1B).



**Figure 3.1. Proposed model indicates how MYC2 transcriptional activity is influenced by protein-protein interaction.** MED25 is recruited by MYC2 and enriches at promoters targeted by MYC2, which in turn activates the transcription of MYC2 target genes (A). JAZ9 competes with MED25 to associate with MYC2, leading to suppression of MYC2 transcriptional regulation (B). TF EIN3 interacts with MYC2 and represses MYC2 transcriptional regulation activity by interfering with MYC2 DNA-binding to its target genes (C). EDS1 associates with MYC2 and interferes with MYC2 transcriptional activity, probably by dislodge MYC2 from MED25 or from DNA (D). Green arrows and red crosses indicate positive and negative regulation, respectively.

MYC2 transcriptional regulation is also inhibited by the TF EIN3 (Ethylene Insensitive3) which directly interacts with MYC2 and interferes with MYC2 DNA-binding to several target genes in ethylene responses (Song et al., 2014) (Figure 3.1C). Therefore, the association of PAD4/SAG101 with MYC2 C-terminus (Figure 2.13) also likely influences bHLH domain-mediated DNA-binding at promoters of its target genes (Figure 3.1D), thereby leading to inhibition of MYC2 transcriptional regulation activity. This is consistent with a recent finding that TNL (*RRS1/RPS4*)-activated EDS1 signaling reduces the enrichment of MYC2 at certain target gene promoters (H. Cui, unpublished). Notably, co-expression of EDS1 inhibits MYC2 transcriptional repressive function on the *EDS1* promoter (*pEDS1*) in Arabidopsis protoplast transient assays (Figure 2.15 and 2.16), further supporting the notion that association between EDS1-family proteins and MYC2 interferes with MYC2 transcriptional activity. The data presented here provide the first evidence that EDS1-family proteins modulate transcriptional reprogramming through antagonizing MYC2 transcriptional regulation.

**3.2.3 MYC2 suppresses *pEDS1* activity independently of G-box and G-box variant motifs**

In Arabidopsis, the conserved bHLH domain of MYC2 directly binds to G-boxes in the promoter of *rd22*, a dehydration-responsive gene (Abe et al., 1997). The role of MYC2 as a transcriptional activator was demonstrated by reduced induction of many *JAZ* genes in *myc2* mutants compared to wild type in response to JA treatment (Chini et al., 2007; Dombrecht et al., 2007). Also, transgenic plants over-expressing MYC2 displayed constitutive expression of certain *JAZ* genes (Chini et al., 2007). There is additional evidence that MYC2 activates transcription of certain *JAZ* genes, such as *JAZ2* and *JAZ3*, through direct binding to their promoters (Chini et al., 2007; Figueroa and Browse, 2012), which is further supported in this study by MYC2-stimulated *JAZ2* promoter activity in Arabidopsis protoplasts transient assays (Figure 2.17).

MYC2 represses TF genes *PLT1* (*PLETHORA1*) and *PLT2* (*PLETHORA2*) and TF *ORA59* by directly binding to the G-box motif in their promoters (Chen et al., 2011; Zhai et al., 2013). I found that MYC2 also represses *pEDS1* activity (Figures 2.15 and 2.16). The repression activity of MYC2 on *pEDS1* was independent of known MYC2-binding G-box and G-box variant motifs (Figure 2.19). Crystal structure analysis of the mammalian MyoD (Myoblast determination protein 1) bHLH domain with DNA has revealed two conserved R residues in the bHLH domain which directly contact the G-box motif and its flanking DNA sequence (Ma et al., 1994). The two corresponding R residues in MYC2 bHLH domain are required for repressive transcriptional regulation on *pEDS1* (Figure 2.18), consistent with previous report in mammals that MYC $\Delta$ C lacking the bHLH domain loses its transcriptional repression function (Cowling and Cole, 2007). My results suggest that MYC2 exerts its repressive activity on *pEDS1* either by binding to other non-canonical sites or indirectly by affecting the binding/repressor activities of other TFs.

The first point of view was supported by early experiments in mammals showing that suppression of c-Myc on *C/EBP $\alpha$*  (*CCAAT-Enhancer-Binding Proteina*) was mediated by initiator (Inr) elements [CTCA(+1)CTCTCT] rather than E-box (identical with G-box in plants) on *C/EBP $\alpha$*  promoter (Li et al., 1994). MYC, a transcription factor containing a transactivation domain at N-terminus and a bHLH domain at C-terminus, functions as transcription activator by forming a heterodimer with TF MAX (MYC-Associated factorX) containing only the bHLH domain (Blackwood and Eisenman, 1991; Amin et al., 1993). Moreover, physical interaction of MYC with two TFs SP1 (Protein Coding1) and MIZ1 (Myc-interacting zinc-finger protein1) typically binding

to the Inr elements, transforms these two transcription activators into transcriptional repressors (Gartel et al., 2001). Therefore, next experiments should involve in testing whether the Inr element in the *EDS1* promoter is required for MYC2 repressive function by measuring whether MYC2 shows repressive function on a mutated *EDS1* promoter.

Alternatively, for repression, MYC2 might recruit a co-repressor complexes, such as histone deacetylase (HDAC) and DNA methyl transferases because MYC2-interacting JAZ proteins, such as JAZ1, JAZ3 and JAZ9, physically interact with HDA6 in Arabidopsis (Zhu et al., 2011b). Studies in mammals found that c-MYC recruits HDAC3 to repress *Id2* (*Inhibitor of DNA binding2*) and *Gadd153* (*Growth Arrest And DNA Damage-Inducible Protein153*) (Kurland and Tansey, 2008). Further evidence illustrated that mammalian c-MYC interacts with MIZ1 to recruit DNA methyl transferase Dmmt3a (DNA 5-cytosine methyltransferase3a) to the *CDKN1A* (*Cyclin-Dependent Kinase Inhibitor1A*) promoter, leading to cytosine methylation of the DNA and silencing of gene expression (Brenner et al., 2005). Therefore, it will be interesting to test whether MYC2 associates with HDA6, whether HDA6 also enriches at the promoter of MYC2 target genes in co-IP experiments and ChIP-qPCR, and whether such associations and activities are affected by EDS1-family proteins.

#### **3.2.4 MYC2, without direct DNA-binding, promotes protein post-transcriptional accumulation**

The N-terminal TAD (Transcriptional Activation Domain) of MYC2 is responsible for physical association with MED25 at the promoter of MYC2 target genes (Chen et al., 2012). Also, association of MYC2 with MED25 is required for both MYC2 transcription activation and repression activities (Cevik et al., 2012). In Arabidopsis protoplast transient expression assays, I found that MYC2 $\Delta$ C enhances YFP-CFP accumulation, regardless of whether it is under control of *pEDS1* or *pJAZ2* (Figures 2.16C and 2.17C). These results suggest that MYC2 $\Delta$ C with the TAD domain and without an intact bHLH DNA-binding domain promotes protein translation, independently of changing bHLH domain-mediated DNA binding and altered target gene transcript levels (Figures 2.16, 2.17 and 2.18). This finding also suggests that the MYC2 bHLH domain might negatively regulate protein post-transcriptional accumulation-promoting activity of MYC2 $\Delta$ C (Figure 2.18). My findings are consistent with previous observations in mammals that protein levels of several CDKs (Cyclin and Cyclin-Dependent Kinases) increased in response to

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MYC expression without changes in their mRNA levels or requirement for the bHLH DNA-binding domain of MYC (Cowling and Cole, 2007). The possible mechanism behind this is that MYC promotes the methylation of the 5' mRNA guanine or 'cap' by promoting global phosphorylation of the RNA polymerase II C-terminal domain (Cowling and Cole, 2007). The cap stabilizes mRNAs and cap methylation is critical for recruiting translation factors, such as RNMT (RNA methyltransferase), to mRNAs for translation (Caldwell and Emerson, 1985; Schwer et al., 1998). However, more experiments such as RNA methylation profiling in *myc234* transgenic plants expressing *MYC2ΔC* or *MYC2<sup>R458A/R460A</sup>*, would be required to establish whether MYC2 regulates methylation of a mRNA cap and which target genes are cap-methylated in response to MYC2, independently of MYC2 promoter binding activity. It would be interesting to investigate how the bHLH domain suppresses MYC2 translation-promoting activity. Previous studies have shown that the bHLH domain is required for dimerization and for MAX to recognize its cognate DNA (Ferre-D'Amare et al., 1993). Therefore, it is possible that bHLH domain-mediated dimerization inhibits the translation-promoting activity of MYC2. To test this possibility, *in vitro* pull-down experiments could be performed to investigate whether *MYC2<sup>R458A/R460A</sup>* forms dimers with itself or with MYC2ΔN, and EMSA (Electrophoretic Mobility Shift Assays) to check MYC2 DNA-binding activity.

### **3.2.5 MYC2-family TFs redundantly negatively regulate *EDS1* expression**

I found that MYC2-family TFs negatively regulate *EDS1* expression in an *ICS1*- and *BSMT1*-independent manner in response to exogenous COR application (Figure 2.20), providing genetic evidence that MYC2-family TFs function redundantly and negatively regulate *EDS1* transcription. The redundant role of MYC2-family TFs is consistent with previous findings that MYC3 and MYC4 function additively to MYC2 in activation of JA responses (Fernandez-Calvo et al., 2011; Schweizer et al., 2013). However, the down-regulation of *EDS1* by MYC2-family TFs appears to be SA-independent upon exogenous COR application (Figure 2.20). However, the negative role of MYC2 in regulating defense responses was evidenced to be dependent on SA accumulation (Laurie-Berry et al., 2006; Zheng et al., 2012). These contrasting results might be down to SA accumulation which is coupled with bacterial COR-triggered JA signaling at an early stage of *Pseudomonas syringae* infection, whereas exogenously applied COR chemical does not change basal SA-related gene expression (Figure 2.20).

Because there is little repressive effect of MYC2-family TFs on *EDSI* basal expression (Figure 2.20A), it is likely that down-regulation of *EDSI* is due to increased amount of active repressive MYC2 pool upon exogenous application of COR, consistent with MeJA-triggered JA signaling, resulting in activation of MYC2 transcriptional activity and promotion of MYC2 stability (Zhai et al., 2013). Furthermore, transcriptional activity of MYC2 is tightly controlled and therefore other factors might contribute to modulation of *EDSI* expression under non-challenged condition. Notably, MYC2 transcriptional activity is fine-tuned by different pathways, such as association with other proteins such as EIN3 (Song et al., 2014), and the stability of MYC2 is controlled by post-translational modifications, such as phosphorylation and PUB10-mediated ubiquitination (Zhai et al., 2013; Jung et al., 2015). MYC2 protein accumulation is also reduced by association with TIC (Time for Coffee) in an evening-phase specific manner (Shin et al., 2012). Therefore, it is worth checking whether the *EDSI* basal expression is regulated by MYC2-family TFs in a circadian clock-dependent manner. The *EDSI* expression is also negatively regulated by the TF SR1 (Signal Responsive 1) also known as CAMTA3 (Calmodulin-binding Transcription Activator3) (Du et al., 2009). SR1 represses *EDSI* expression by binding to CGCG box (ACGCGT) in *EDSI* promoter, dependent on CAMTA3 Ca<sup>2+</sup> / CaM-binding (Du et al., 2009).

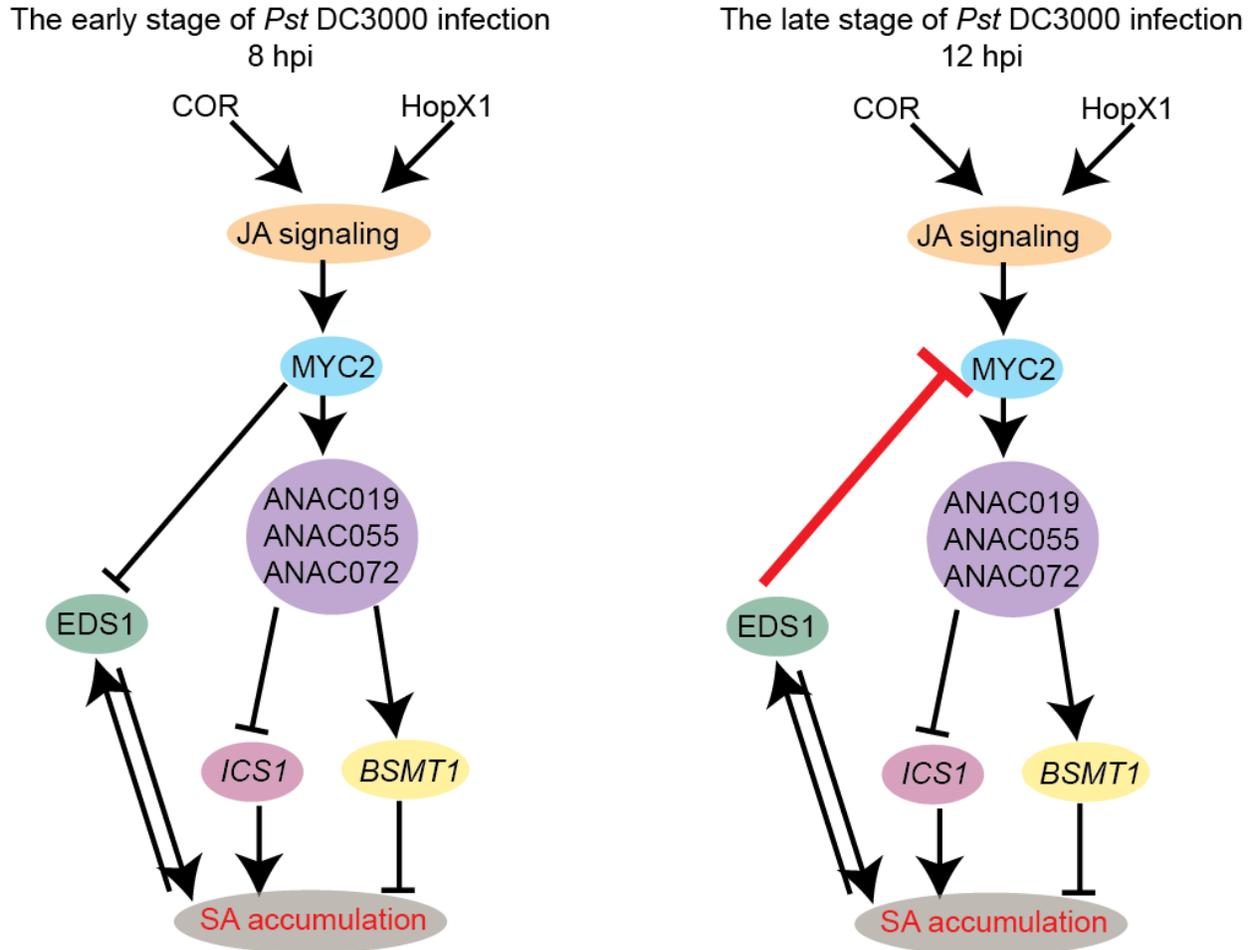
### **3.2.6 MYC2-family TFs delay induction of *EDSI* in basal resistance**

MYC2 is involved in modulating the mutual antagonism between SA and JA in response to pathogen infection (Kazan and Manners, 2013). A negative role of MYC2 in regulating plant resistance to *Pseudomonas syringae* was illustrated by *jin1/myc2* increased resistance to *Pst* DC3000 (Lorenzo et al., 2004; Nickstadt et al., 2004). Bacterial COR-activated MYC2 stimulates the expression of *ANAC019*, *ANAC055* and *ANAC072* by MYC2 direct binding to their promoters (Zheng et al., 2012) (Figure 3.2). The activated ANAC TFs down-regulate SA biosynthesis gene *ICS1* while up-regulate SA metabolism gene *BSMT1*, leading to reduced SA accumulation, thereby promoting the virulence of *Pseudomonas syringae* (Zheng et al., 2012) (Figure 3.2). Here, I found that MYC2-family TFs delayed the induction of *EDSI* in response to *Pst* DC3000 at an early stage of infection (8 h) (Figure 2.21), suggesting the MYC2-family TFs activated by bacterial COR may target *EDSI* and repress its transcription to promote *Pseudomonas syringae* virulence (Figure 3.2). This delayed *EDSI* expression was counteracted at later infection stages (12 h) (Figure 2.21), suggesting that activated *EDSI*-signaling overcomes MYC2-family TFs repressive function via forming complexes with MYC2-family TFs at the later *Pst* DC3000 infection stage (Figure 3.2).

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This is supported by finding that presence of EDS1 strongly reduced the repressive activity of MYC2 on *pEDS1* (Figure 2.15 and 2.16). This negative effect of EDS1 on MYC2 repression might be examined further by quantifying *EDS1* transcript under control of *pJAZ2* co-transformed with *35S::SH-MYC2* in Arabidopsis *eds1-2* protoplasts. Further experiments, such as gene expression assays of *EDS1* expression in *coi1* and *myc234 sid2-1* quadruple mutants upon *Pst* DC3000 infection, will be used to disentangle the MYC2-family TFs-delayed expression of *EDS1* from the negative effects of MYC2-family TFs on SA accumulation.

The MYC2-family mediated delay in *EDS1* gene induction in basal resistance could not be explained by the presence of bacterial COR (Figure 2.21). Previous studies have shown that COR and Type III effectors function cooperatively to promote bacterial virulence by hijacking JA signaling (Cohn and Martin, 2005; Navarro et al., 2006; Jiang et al., 2013; Gimenez-Ibanez et al., 2014). *Pseudomonas syringae* effector HopZ1 and its homologous HopZ2 and HopZ3 interact with and promote the degradation of JAZ proteins (Jiang et al., 2013). However, HopZ1 on its own does not contribute to the virulence of *Pst* DC3000 (Lewis et al., 2008; Jiang et al., 2013). Another effector HopX1 from *Pta* (*Pseudomonas syringae* pv. *tabaci*) 11528 (which can not produce COR) interacts with and promotes the degradation of JAZ proteins, requiring HopX1 cysteine protease activity (Gimenez-Ibanez et al., 2014). Although HopX1 only shares 72 % identity to HopX from *Pst* DC3000, they do have conserved N-terminal domains and a consensus cysteine-based catalytic triad (Gimenez-Ibanez et al., 2014). Thus, it is likely that HopX from *Pst cor* DC3000 functions contributes to virulence of *Pst cor* DC3000 (Figure 3.2).



**Figure 3.2. A signaling scheme underlies a mutual antagonism between EDS1 and MYC2 in basal resistance.**

At an early stage of *Pst* DC3000 infection (8 hpi), bacterial virulence factor COR and effector HopX1 activated MYC2 through triggering JA signaling, which delays the induction of *EDS1* and reduces SA accumulation by repressing *ICS1* and increasing *BSMT1* in an ANAC TFs (ANAC019, ANAC055 and ANAC072)-dependent manner. However, at the late infection stage, the active EDS1 associates with MYC2 and interferes with MYC2 transcriptional activity, which blocks COR and effector-triggered MYC2-mediated JA signaling, thereby leading to activation of resistance outputs. Arrows indicate positive regulation while the arrows with blunt end denote negative regulation.

### **3.3 Summary and Perspectives**

Results from the Arabidopsis *eds1-2* genetic suppressor screen point to four different pathways negatively impacting plant disease resistance, and disruption of any of these can alleviate defects of Arabidopsis *eds1-2* in basal and TNL-triggered immunity. The most interesting pathway with regard to EDS1 signaling is that controlled by COI1 for promoting COR-stimulated virulence and JA responses. Activated EDS1 basal or TNL immunity suppresses bacterial COR-triggered COI1-mediated JA signaling, thereby highlighting an EDS1 function in modulation of SA-JA crosstalk independently of ICS1-generated SA. Associations between EDS1-family proteins and MYC2-family TFs and the suppressive function of EDS1 on MYC2 transcriptional activity suggest that EDS1 regulates transcriptional reprogramming and that MYC2-family TFs are direct targets for EDS1 repression of COR/JA signaling. A body of evidence also suggests that MYC2 reciprocally negatively suppresses EDS1 activity. This MYC2 activity is dependent on the MYC2 bHLH DNA-binding domain but independent of G-box and G-box-related MYC2-binding motifs in the promoter of *EDS1*. The repressive function of MYC2 on *EDS1* was manifested in the expression of *EDS1* after exogenous COR treatment, which is also independent of SA. A picture emerges in which MYC2-family TFs act redundantly to negatively regulate *EDS1* at an early stage of *Pst* DC3000 infection, and this is counteracted by activated EDS1 resistance signaling at a later infection stage. The data presented here suggest that mutual antagonism between EDS1 and MYC2 activities is an important fine-tuning mechanism for plant immunity.

Recent reports have demonstrated that EDS1-family proteins play important role in abiotic stresses, such as freezing and drought tolerance (Chen et al., 2015; Szechynska-Hebda et al., 2016). MYC2 was also shown to positively regulate drought stress with the observation of enhanced drought stress resistance phenotype of *myc2/jin1* mutant (Abe et al., 2003). Therefore, it is interesting to test whether the mutual antagonism between EDS1- and MYC2-dependent signaling impacts drought stress tolerance.

Here, I found associations between EDS1-family proteins and MYC2-family TFs *in vivo* and that the presence of EDS1 represses MYC2 transcriptional activities in Arabidopsis protoplast transient assays. However, it is unknown what determines this antagonistic relationship. A next step is therefore to determine which amino acid residues or sub-domains/ or surfaces are important for EDS1-family proteins to associate with MYC2.

My data highlight a suppressive function of MYC2 needing bHLH DNA-binding activity but not known MYC2-binding G-box or G-box-related motifs in *EDS1* promoter. Next experiments will determine which part of the *EDS1* promoter sequence is essential for MYC2 suppressive function and whether MYC2 can directly bind to this motif. If a *pEDS1* motif and direct DNA binding can be confirmed, the Arabidopsis protoplast transient expression system will provide a platform to interrogate molecular mechanisms underlying MYC2 transcriptional repression activity. To further investigate which other components are recruited by MYC2 for repressive function, DNA immunoprecipitation assays can be performed in which a reporter construct driven by a biotin-tagged promoter such as *pEDS1* is co-transformed with MYC2 into Arabidopsis protoplasts. Potential co-acting components might be identified using mass spectrometry from chromatin-IP assays.

## **4 Materials and methods**

### **4.1 Materials**

#### **4.1.1 Plant materials**

##### **4.1.1.1 *Arabidopsis thaliana***

*Arabidopsis* wild-type and mutant lines use in this study are listed in Table 4.1 and Table 4.2, respectively.

**Table 4.1.** Wild-type *Arabidopsis* accessions used in this study

Accession	Abbreviation	Original source
Columbia	Col-0	J. Dangl <sup>a</sup>

<sup>a</sup> University of North Caroline, Chape Hill, NC, USA

**Table 4.2.** Mutant *Arabidopsis* lines used in this study

Gen	Accession	Reference / Source
<i>eds1-2</i>	Col-0/ (Ler) <sup>a</sup>	(Bartsch et al., 2006)
<i>myc2-3</i>	Col-0	(Shin et al., 2012)
<i>myc3</i>	Col-0	(Fernandez-Calvo et al., 2011)
<i>myc4</i>	Col-0	(Fernandez-Calvo et al., 2011)
<i>myc234</i>	Col-0	(Fernandez-Calvo et al., 2011)
<i>sid2-1</i>	Col-0	(Nawrath and Mettraux, 1999)

<sup>a</sup> Ler *eds1-2* allele introgressed into the Col-0 background, 8<sup>th</sup> backcrossed generation, referred to as “*eds1-2*” in this study.

##### **4.1.1.2 *Nicotiana benthamiana***

*Nicotiana benthamiana* (310A) plants expressing the *N* resistance gene were obtained from MPIPZ (Cologne) and used for transient *Agrobacterium*-mediated transformation of leaf tissues.

## **4.1.2 Pathogens**

*Arabidopsis* plants were infected with isogenic *Pseudomonas syringae* pv. *tomato* (*Pst*) strains (DC3000) expressing different *Pseudomonas* effector proteins as specified in section 4.2.4 and 4.2.5

### **4.1.2.1 *Pseudomonas syringae* strains**

*Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 harbouring either the empty vector pVSP61 or expressing the *Pseudomonas syringae* pv. *pisi* effector *avrRps4* from the same plasmid (Hinsch and Staskawicz, 1996) were obtained from R. Innes (Indiana University, Bloomington Indiana, USA) and used throughout this study. *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 lacking coronatine (COR) (*Pst*  $\Delta$ *cor* DC3000) was obtained from Renier van der Hoorn (MPIPZ). Plasmid *pVSP61-avrRps4* was transformed into *Pst*  $\Delta$ *cor* DC3000 to generate strain *Pst*  $\Delta$ *cor* DC3000 expressing *avrRps4*.

### **4.1.2.2 *Hyaloperonospora arabidopsidis***

*Hyaloperonospora arabidopsidis* (*Hpa*) isolate EMWA1 (Holub *et al.*, 1994) recognized by Col-0 (*RPP4*) was used in this study.

## **4.1.3 Bacterial strains**

### **4.1.3.1 *Escherichia coli* strains**

**Table 4.3.** All *E. coli* strains were obtained from Invitrogen™ (Karlsruhe, Germany).

<b>Strain</b>	<b>Genotype</b>
DH5 $\alpha$	F- $\Phi$ 80 $\Delta$ <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> ) U169 <i>deoR recA1 endA1 hsdR17</i> (rk-, mk+) <i>phoA supE44</i> $\lambda$ - <i>thi-1 gyrA96 relA1</i>
DH10B	F- <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\Phi$ 80 $\Delta$ <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74 deoR recA1 endA1 ara</i> $\Delta$ 139 $\Delta$ ( <i>ara, leu</i> )7697 <i>galU galK</i> $\lambda$ - <i>rpsL</i> (StrR) <i>nupG</i>
DB3.1	F- <i>gyrA462 endA</i> $\Delta$ ( <i>sr1-recA</i> ) <i>mcrB mrr hsdS20</i> (rB- mB-) <i>supE44 ara14 galK2 lacY1 proA2 rpsL20</i> (StrR) <i>xyl5</i> $\lambda$ - <i>leu mtl1</i>

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### 4.1.3.2 *Agrobacterium tumefaciens* strains

DNA constructs for transient expression in *Nicotiana benthamiana* (4.2.6) were transformed in *Agrobacterium tumefaciens* strain GV3101 carrying the helper plasmids pMP90RK with antibiotic resistance Rif, Kan and Gent.

### 4.1.4 *Saccharomyces cerevisiae* (yeast) strain

Yeast strain L40 was used in this study (Mitsuda et al., 2010).

### 4.1.5 Vectors

The vectors used or generated in this study were listed in Table 4.4.

**Table 4.4. Vectors**

Vector	Annotation
pXCSG-mYFP-GW	Binary Gateway® destination vector for expression of a fusion protein under control of 35S promoter with a C-terminal mYFP tag
pXCSG-YFP-mYFP	Binary Gateway® destination vector for expression of YFP (with stop codon) control of 35S promoter with a C-terminal mYFP tag
pXCSG-gEDS1-mYFP	Binary Gateway® destination vector for expression of genomic EDS under control of 35S promoter with a C-terminal mYFP tag
pXCSG-PAD4-mYFP	Binary Gateway® destination vector for expression of PAD4 under control of 35S promoter with a C-terminal mYFP tag
pXCSG-SAG101-mYFP	Binary Gateway® destination vector for expression of SAG101 under control of 35S promoter with a C-terminal mYFP tag
pXCSG-gEDS1 <sup>L262P</sup> -mYFP	Binary Gateway® destination vector for expression of mutated genomic EDS under control of 35S promoter with a C-terminal mYFP tag

pXCSG-gEDS1 <sup>LLIF</sup> -mYFP	Binary Gateway® destination vector for expression of mutated genomic EDS under control of 35S promoter with a C-terminal mYFP tag
pXCSG-3*Flag-GW	Binary Gateway® destination vector for expression of a fusion protein under control of 35S promoter with a C-terminal 3*Flag tag
pXCSG-gEDS1-Flag	Binary Gateway® destination vector for expression of genomic EDS1 under control of 35S promoter with a C-terminal 3*Flag tag
pXCSG-gEDS1 <sup>L262P</sup> -Flag	Binary Gateway® destination vector for expression of mutated genomic EDS1 under control of 35S promoter with a C-terminal 3*Flag tag
pXCSG-gEDS1 <sup>LLIF</sup> -Flag	Binary Gateway® destination vector for expression of mutated genomic EDS1 under control of 35S promoter with a C-terminal 3*Flag tag
pENS-StrepII-3*HA (SH)-GW	Binary Gateway® destination vector for expression of a fusion protein under control of 35S promoter with an N-terminal StrepII-3*HA tag
pENS-SH-MYC2	Binary Gateway® destination vector for expression of MYC2 under control of 35S promoter with an N-terminal StrepII-3*HA tag
pENS-SH-MYC2ΔC	Binary Gateway® destination vector for expression of MYC2ΔC under control of 35S promoter with an N-terminal StrepII-3*HA tag
pENS-SH-MYC2ΔN	Binary Gateway® destination vector for expression of MYC2ΔN under control of 35S promoter with an N-terminal StrepII-3*HA tag
pENS-SH-MYC2 <sup>R458/460A</sup>	Binary Gateway® destination vector for expression of mutated MYC2 under control of 35S promoter with an N-terminal StrepII-3*HA tag

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pENS-SH-MYC2ΔN <sup>R458/460A</sup>	Binary Gateway® destination vector for expression of mutated MYC2ΔN under control of 35S promoter with an N-terminal StrepII-3*HA tag
pENS-SH-MYC3	Binary Gateway® destination vector for expression of mutated MYC3 under control of 35S promoter with an N-terminal StrepII-3*HA tag
pENS-SH-MYC4	Binary Gateway® destination vector for expression of mutated MYC4 under control of 35S promoter with an N-terminal StrepII-3*HA tag
pENS-SH-YFP	Binary Gateway® destination vector for expression of YFP under control of 35S promoter with an N-terminal StrepII-3*HA tag
pEDS1XCG-YFP-CFP	Binary Gateway® destination vector for expression of YFP under control of EDS1 promoter with a C-terminal mCFP tag
pANAC019XCG-YFP-CFP	Binary Gateway® destination vector for expression of YFP under control of ANAC019 promoter with a C-terminal mCFP tag
pEDS1M1XCG-YFP-CFP	Binary Gateway® destination vector for expression of YFP under control of mutated EDS1 promoter with a C-terminal mCFP tag
pEDS1(M1+M2)XCG-YFP-CFP	Binary Gateway® destination vector for expression of YFP under control of mutated EDS1 promoter with a C-terminal mCFP tag
pDEST-BTM116-GW	Gateway® destination vector for expression of a fusion protein under control of ADH1 promoter with an N-terminal fused LexA DB
pDEST-BTM116-EDS1	Gateway® destination vector for expression of EDS1 under control of ADH1 promoter with an N-terminal fused LexA DB

pDEST-BTM116-PAD4	Gateway® destination vector for expression of PAD4 under control of ADH1 promoter with an N-terminal fused LexA DB
pDEST-BTM116-SAG101	Gateway® destination vector for expression of SAG101 under control of ADH1 promoter with an N-terminal fused LexA DB
pDEST-BTM116-JAZ9	Gateway® destination vector for expression of JAZ9 under control of ADH1 promoter with an N-terminal fused LexA DB
pDEST-GAD424-GW	Gateway® destination vector for expression of a fusion protein under control of ADH1 promoter with an N-terminal fused GAL4 AD
pDEST-GAD424-MYC2	Gateway® destination vector for expression of MYC2 under control of ADH1 promoter with an N-terminal fused GAL4 AD
pDEST-GAD424-MYC3	Gateway® destination vector for expression of MYC3 under control of ADH1 promoter with an N-terminal fused GAL4 AD
pDEST-GAD424-MYC4	Gateway® destination vector for expression of MYC4 under control of ADH1 promoter with an N-terminal fused GAL4 AD
pDEST-GAD424-EDS1	Gateway® destination vector for expression of EDS1 under control of ADH1 promoter with an N-terminal fused GAL4 AD
pDEST-GAD424-PAD4	Gateway® destination vector for expression of PAD4 under control of ADH1 promoter with an N-terminal fused GAL4 AD
pENTRY-gEDS1	Gateway® entry plasmid that contains genomic EDS1 without stop codon from Ler-0
pENTRY-gEDS1 <sup>L262P</sup>	Gateway® entry plasmid that contains mutated genomic <i>EDS1</i> without stop codon from Ler-0

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pENTRY-gEDS1 <sup>LLIF</sup>	Gateway® entry plasmid that contains mutated genomic <i>EDS1</i> without stop codon from Ler-0
pENTRY-EDS1	Gateway® entry plasmid that contains CDS of <i>EDS1</i> with stop codon from Ler-0
pENTRY-PAD4	Gateway® entry plasmid that contains CDS of <i>EDS1</i> with stop codon from Ler-0
pENTRY-SAG101	Gateway® entry plasmid that contains CDS of <i>PAD4</i> with stop codon from Ler-0
pENTRY-MYC2	Gateway® entry plasmid that contains CDS of <i>MYC2</i> without stop codon from Col-0
pENTRY-MYC3	Gateway® entry plasmid that contains CDS of <i>MYC3</i> without stop codon from Col-0
pENTRY-MYC4	Gateway® entry plasmid that contains CDS of <i>MYC4</i> without stop codon from Col-0
pENTRY-MYC2ΔC	Gateway® entry plasmid that contains CDS of N-terminal <i>MYC2</i> (aa 1-287) without stop codon from Col-0
pENTRY-MYC2ΔN	Gateway® entry plasmid that contains CDS of C-terminal <i>MYC2</i> (aa 288-623) without stop codon from Col-0
pENTRY-MYC2 <sup>R458/460A</sup>	Gateway® entry plasmid that contains CDS of <i>MYC2</i> (R458/460A) without stop codon from Col-0
pENTRY-MYC2ΔN <sup>R458/460A</sup>	Gateway® entry plasmid that contains CDS of C-terminal <i>MYC2</i> (aa 288-623, R458/460A) without stop codon from Col-0
pENTRY-YFP	Gateway® entry plasmid that contains CDS of <i>YFP</i> without stop codon from Col-0
pENTRY-YFP	Gateway® entry plasmid that contains CDS of <i>YFP</i> with stop codon from Col-0

pEDS1XCG-CFP	Binary Gateway® destination vector for expression of a fusion protein under control of <i>EDS1</i> promoter with a C-terminal mCFP tag
pEDS1M1XCG-CFP	Binary Gateway® destination vector for expression of a fusion protein under control of mutated <i>EDS1</i> promoter with a C-terminal mCFP tag
pEDS1(M1+M2)XCG-CFP	Binary Gateway® destination vector for expression of a fusion protein under control of mutated <i>EDS1</i> promoter with a C-terminal mCFP tag
pANAC019XCG-CFP	Binary Gateway® destination vector for expression of a fusion protein under control of <i>ANAC019</i> promoter with a C-terminal mCFP tag

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#### 4.1.6 Oligonucleotides

Primers used in this study are listed in Table 4.5. Oligonucleotides were purchased from Invitrogen (Martinsried, Germany). Start and Stop codons are highlighted in red and recognition sites for restriction endonucleases are underlined. Lyophilised primers were resuspended in ddH<sub>2</sub>O to a final concentration of 100 pmol/μL (= 100 μM). Working solutions for PCR were diluted to 10 pmol/μL (=10 μM), while qPCR working solutions were diluted to 5 pmol/ μL (=10 μM).

**Table 4.5.** Oligonucleotides used in this study

Namer	Purpose	Sequence (5' → 3')
MYC2-F	Cloning <i>MYC2</i>	CACCATGACTGATTACCGGCTAC
MYC2-R		TCCGATTTTTGAAATCAAACCTTGC
MYC2ΔC-R	MYC2ΔC deletion	tcaTGTTC CAATCGGGTCATTA A
MYC2ΔN-F	MYC2ΔN deletion	caccATGCCTGGATCTAACGAACCGGGT
MYC2R458A/R460A-F	Mutagenesis, R458A/R460A	CAGAGgcACAAGcACGCGAGAACTAAACCAA A
MYC2R458A/R460A-R	Mutagenesis, R458A/R460A	CGCGTgcTTGTgcCTCTGCTTCGACGTGGTTT
pEDS1M1-F	Mutagenesis, CAC-TGA	ACCAATGTAAAACCGAtgaGTG

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pEDS1M1-F	Mutagenesis, CAC-TGA	GAATCTTAGCTTTCCACTcatCG
pEDS1M2-F	Mutagenesis, CAC-TGA	AGCTCAAAGATTCACAtgaATG
pEDS1M2-R	Mutagenesis, CAC-TGA	GAATCAGGACATGCATtcaTGT
pJAZ2-F	Cloning <i>JAZ2</i> promoter, <i>AscI</i>	GGCGCGCCGTTGCATACTCTAAATAA
pJAZ2-R	Cloning <i>JAZ2</i> promoter, <i>XhoI</i>	CCGCTCGAGCGTTGAAACCGAAATTGA
qCFP-F	qRT-PCR	AAAGTGGTTGATGGGATGGTGAGC
qCFP-R		TGCACGCCCCAGGTCAGG
qP_ICS1_F		TTCTGGGCTCAAACACTAAAAC
qP_ICS1_R		GGCGTCTTGAAATCTCCATC
Actin2_short_F		ATGGAAGCTGCTGGAATCCAC
Actin2_short_R		TTGCTCATACGGTCAGCGATA
EDS1-qF		AGATTATTCAGGTGATCGAGCA
EDS1-qR		TTTATGGGCTTGACACTTTGG
BSMT1-qF		AGTCTTTGAGGGTCTTGTGAG
BSMT1-qR		GTCATCTTCTTCGTAGTAGTG
coil-141-f		sequencing <i>COII</i> genomic DNA
coil-1436-r	GGACAAGACAACAGACAGTTG	
coil-1117-f	CTTTGCAACTGTTATGCTTGC	
coil-2301-f	ATCTCTTGAAAGCATAGGCAC	
coil-2503-r	ACTGTCCGATGTAGCTTAAG	
coil-2987-r	ACCAAAGTAGTATAGTACAC	
CPR5-269-F	sequencing <i>CPR5</i> genomic DNA	CACTAGTCACGAAGCGACCA
CPR5-1482-R		CGACAAGAGTAGCAAAGATC
CPR5-1340-F		TGTTGATGATCTTGTTGAGG
CPR5-2471-F		GGTAAATTGTGTGTGGACAC
CPR5-2634-R		CAACGGTTCGATCTGGTCTC
CPR5-2791-R		TCAAGCATAGTCAGACCCAC

## 4.1.7 Enzymes

### 4.1.7.1 Restriction endonucleases

Restriction enzymes were purchased from New England Biolabs (Frankfurt, Germany) unless otherwise stated. Enzymes were used with supplied 10x reaction buffer for restriction digests.

### 4.1.7.2 Nucleic acid modifying enzymes

Standard PCR reactions were performed using home-made *Taq* DNA polymerase. To achieve high accuracy, *Pfu* polymerases were used when PCR products were generated for cloning. Modifying enzymes and their suppliers are listed below:

Taq DNA Polymerase	Home made
Phusion High-Fidelity DNA Polymerase	ThermoFisher, Germany
T4 DNA ligase	Roche, Germany
SuperScript™ II RNase H <sup>-</sup> Reverse Transcriptase	Invitrogen, Germany
Gateway™ LR Clonase™ Enzyme mix	Invitrogen, Germany

## 4.1.8 Chemicals

Laboratory grade chemicals and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Invitrogen™ (Karlsruhe, Germany), Serva (Heidelberg, Germany), and Gibco™ BRL® (Neu Isenburg, Germany) unless otherwise stated.

## 4.1.9 Antibiotics (stock solutions)

**Table 4.6.** Antibiotics and concentration

Antibiotics	Stock solution
Ampicillin (Amp)	100 mg/ml in ddH <sub>2</sub> O
Carbeninillin (Carb)	100 mg/ml in ddH <sub>2</sub> O
Gentamycin (Gent)	15 mg/ml in ddH <sub>2</sub> O
Kanamycin (kan)	50 mg/ml in ddH <sub>2</sub> O
Rifampicin (Rif)	100 mg/ml in ddH <sub>2</sub> O

Stock solutions (1000×) stored at -20 °C. Aqueous solutions were sterile filtrated.

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### 4.1.10 Media

Media were sterilised by autoclaving at 121°C for 20 min. For the addition of antibiotics and other heat labile compounds the solution or media were cooled to 55°C. Heat labile compounds were sterilised using filter sterilisation units prior to addition.

#### *Escherichia coli* media

##### LB (Luria-Bertani) broth

Tryptone 10.0 g/l

Yeast extract 5.0 g/l

NaCl 5.0 g/l

pH 7.0

❖ For LB agar plates 1.5 % (w/v) agar was added to the above broth.

#### *Pseudomonas syringae* media

##### NYG broth

Peptone 5.0 g/l

Yeast extract 3.0 g/l

Glycerol 20 ml/l

pH 7.0

❖ For NYG agar plates 1.5 % (w/v) agar was added to the above broth.

#### *Agrobacterium tumefaciens* media

##### YEB

Beef extract 5.0 g/l

Yeast extract 1.0 g/l

Peptone 5.0 g/l

Sucrose 5.0 g/l

1M MgSO<sub>4</sub> 2.0 ml/l

pH 7.2

❖ For YEB agar plates 1.5 % (w/v) agar was added to the above broth.

#### Yeast media

##### YPAD medium

Peptone 20 g/L

Yeast extract 10 g/L

Adenine 100 mg/L

Glucose 2 %

pH 5.8

##### SD/-LW media

SD base 26.7 g/L

Leu/-Trp DropOut 0.64 g/L

pH 5.8

##### SD/-LWH media

SD base 26.7 g/L

Leu/-Trp/-His DropOut 0.62 g/L

pH 5.8

❖ For agar plates, 1.8 % agar was added. The amino acids or amino acid DropOuts were added after autoclaving.

#### 4.1.11 Antibodies

The used primary and secondary antibodies for immunoblot detection are listed below.

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**Table 4.7.** Primary antibodies

<b>Antibody</b>	<b>Source</b>	<b>Dilution</b>	<b>Supplier</b>
$\alpha$ -GFP	Mouse polyclonal	1:5000	Roche
$\alpha$ -Flag	Mouse polyclonal	1:5000	
$\alpha$ -HA	Rat polyclonal	1:5000	Roche

**Table 4.8.** Secondary antibodies

<b>Antibody</b>	<b>Source</b>	<b>Dilution</b>	<b>Supplier</b>
Goat anti-mouse IgG-HRP	Horseradish peroxidase conjugated	1:5000	Santa Cruz (Santa Cruz, USA)
Goat anti-rat IgG-HRP			

### 4.1.12 Buffers and solutions

**Table 4.9.** Buffers and components

	<b>Buffer</b>	<b>Components</b>
DNA electrophoresis	10x running buffer	0.4 M Tris, 0.2 M acetic acid, 10 mM EDTA, pH 8.5
	6x loading buffer	40 % (w/v) sucrose, 0.5 M EDTA, 0.2 % (w/v) bromophenol blue
	DNA ladder	10 % (v/v) 6x loading buffer, 5 % (v/v) 1 Kb DNA ladder (Roth)
Protein electrophoresis	10x Tris-glycine running buffer	250 mM Tris, 1.92 M glycine, 1 % (w/v) SDS
	2x SDS sample buffer	60 mM Tris pH6.8, 4 % (w/v) SDS, 200 mM DTT, 20 % (v/v) glycerol, 0.2 % (w/v) bromophenol blue
Immunoblotting	TBS buffer	10 mM Tris, 150 mM NaCl, pH 7.5
	TBS-T buffer	10 mM Tris, 150 mM NaCl, 0.05 % (v/v) Tween 20, pH7.5

	10x transfer buffer	250 mM Tris, 1.92 M glycine, 1 % (w/v) SDS, 20 % (v/v) Methanol
	Ponceau S	Dilution of ATX Ponceau concentrates (Fluka) 1:5 in water
Protein purification	GFP co-IP extraction buffer and wash buffer	50 mM Tris (PH7.5), 150 mM NaCl, 10 % (v/v) Glycerol, 2 mM EDTA, 5 mM DTT, Protease inhibitor (Roche, 1 tablet per 50 mL) , 0.1 % Triton
Ponceau S		Ponceau S working solution was prepared by dilution of ATX Ponceau S concentrate (Fluka) 1:5 in H <sub>2</sub> O.
Yeast transformation	10×TE buffer	100 mM Tris-HCL (PH8.0), 10 mM EDTA (PH8.0)
	10×LiAc solution	1 M LiAc (Lithium Acetate)
	1×TE/LiAc/PEG	1 volume of 10×TE buffer and 1 volume of 10×LiAc solution with 8 volumes of 44% (V/V) PEG3350
Arabidopsis transient expression solution and buffer	Enzyme solution	1-1.5% cellulose R10, 0.2-0.4% macerozyme R10 (Yakult Honsha, Tokyo, Japan), 0.4 M mannitol, 20 mM KCl, 20 mM MES (PH5.7), heat the enzyme solution at 55 °C for 10 min and cool it to room temperature before adding: 10 mM CaCl <sub>2</sub> , 0.1% BSA (Sigma A-6793)
	PEG solution (40%, V/V)	4 g PEG (Fluka, #81240), 3 mL H <sub>2</sub> O, 2.5 mL 0.8 M mannitol, 1 mL 1 M CaCl <sub>2</sub>
	W5 solution	154 mM NaCl, 125 mM CaCl <sub>2</sub> , 5 mM KCl, 2 mM MES (pH 5.7)

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	MMg solution	0.4 M mannitol, 15 mM MgCl <sub>2</sub> , 4 mM MES (pH 5.7)
<i>N. benthamiana</i>	Infiltration buffer	10 mM MES, 10 mM MgCl <sub>2</sub> , PH5.6 (NaOH), before use add acetosyringone to final concentration 0.15 mM
	infiltration buffer	

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## 4.2 Methods

### 4.2.1 Maintenance and cultivation of *Arabidopsis* plants

*Arabidopsis* seeds were sowed directly onto moist compost (Stender, Schermbeck, Germany) containing 10 mg/L Confidor® WG 70 (Bayer, Germany). The soil with seeds was covered with a lid and keep it at 4 °C for 24-48 h in the dark for vernalization. Then the seeds were moved to a controlled environment growth chamber and maintained under short day conditions (10 h photoperiod, the light intensity of approximately 200  $\mu$ Einsteins m<sup>-2</sup> sec<sup>-1</sup>, 22 °C and 65 % humidity). Lids were removed around 5 d post germination. 2-week old plants were transferred to long day conditions (16 h photoperiod) and allowed to flower for propagation. Aerial tissue was enveloped with a paper bag and sealed with tape at its base until siliques shattered.

### 4.2.2 Generation of *Arabidopsis* F1 and F2 progeny

Fine tweezer was used to emasculate an individual flower. Only flowers with well-developed stigma but immature stamen, which can prevent self-pollination, were used for crossing. At least 2 independent fresh pollen was dabbed onto the stigma. Mature siliques containing F1 seed were bagged and harvested. At least five F1 seeds per cross were grown as described above and allowed to self-pollinate to produce F2 seeds.

### 4.2.3 Maintenance of *P. syringae* pv. *Tomato* cultures

*Pseudomonas syringae* pv. *tomato* strains were streaked onto selective NYG agar plates containing proper antibiotics from -80 °C glycerol stocks. Then the plates were incubated at 28 °C for 48 h before storing at 4 °C and refreshed weekly.

#### **4.2.4 *P. syringae* pv. *tomato* spraying treatment**

One day before infection, bacterial strains, streaked on NYG agar plates, were restreaked and incubated ON at 28 °C. The concentration of bacteria was adjusted to  $1 \times 10^7$  CFU/mL in 10 mM MgCl<sub>2</sub> containing 0.04 % Silwet L-77 (Lehle seeds, USA). Two hours before spray-infection, two-week old seedlings grown under short day conditions were watered and covered by a lid to allow opening of stomata. Spray-infected plants were covered by the lid for 3 h and the lid was removed to dry the plants. Then infected plants were covered with a lid and moved to a growth chamber with short day conditions. One week after infection, survived plants were selected and moved to long day growth chamber for harvesting seeds.

#### **4.2.5 *P. syringae* pv. *tomato* growth assay**

One day before infection, bacterial strains were re-streaked on NYG plates containing the appropriate antibiotics and incubated ON at 28 °C. The concentration of bacteria was adjusted to OD<sub>600</sub> 0.0002 in ddH<sub>2</sub>O. Three leaves per 4.5-week-old plant were infiltrated with a needle-less syringe. At 3 d post infiltration, 4 replicate samples each with 3 leaf discs (d=6 mm) were taken from 4 different plants and transferred to a 2 mL centrifuge tubes containing 10 mM MgCl<sub>2</sub> supplemented with 0.01% Silwet L-77. Bacterial titers were determined by shaking the samples with the speed of 650 rpm at 28 °C for 1 h with. 20 µL of the resulting bacterial suspension were plated on NYGA plates with appropriate antibiotics and incubated at 28 °C for 40 h before colony counting.

#### **4.2.6 Transient protein expression in *N. benthamiana***

*Agrobacteria* carrying specific constructs (Table 4.4) were incubated for 2 d on selective LB plates under 28 °C and inoculated to LB liquid for over-night culture. Liquid cultures were centrifuged for 5 min at 3000 rpm. The pellets were incubated for 3-4 h in infiltration buffer (10mM MES pH 5.6, 10 mM MgCl<sub>2</sub>, 0.15 mM acetosyringone) at OD<sub>600</sub>=1. Leaves from 3-4 week-old *Nicotiana benthamiana* plants were syringe-infiltrated with different v/v mixes of the prepared *Agrobacteria* strains. Leaf samples were taken around 40 hpi.

#### **4.2.7 Transient expression assays using *Arabidopsis* mesophyll protoplasts**

The method was previously developed by Sheen's Lab (Yoo et al., 2007). In shortly, leaves from 3-4 weeks old plants were cut into 0.5-1 mm strips with fresh razor blades without wounding. Put

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the leaf strips from 10-20 leaves in a flask with 10 mL cellulose/macerozyme solution, and then apply vacuum infiltration for 15 min. Continue the digestion for 2 h with gentle shaking (40 rpm on a platform shaker). Filter the enzyme solution containing protoplasts with a 35-75 mm nylon mesh. Spin at 100 g to pellet the protoplasts in a round-bottomed tube for 2min. Wash protoplasts twice in W5 solution, and resuspend protoplasts in the same solution. Keep the protoplasts on ice for 30 min in W5 solution before transfection. Spin down protoplasts and resuspend in MMg solution ( $5 \times 10^5$  protoplasts/mL) before PEG transfection. Prepare plasmids combination, add 200 uL protoplasts to microfuge tube contained plasmids and mix well, then add 220 uL PEG/Ca solution and mix well again. Incubate at room temperature for 15 min. Dilute with 1 mL W5 solution and mix well. Spin down at 100 g for 2 min, remove PEG. Resuspend protoplasts gently by adding 1 mL W5 solution and mix well. Spin down at 100 g for 2 min. Resuspend the protoplasts by adding 1 mL W5 solution and mix well. Samples for transcript assay were harvested at 8 h post transfection by centrifugation at 100 g for 2 min, while samples for immunoblotting were incubated at room temperature with light for 16 h by centrifugation at 100 g for 2 min. Discard supernatant, freeze and store samples at -80 °C until ready for analysis.

### **4.2.8 Biochemical methods**

#### **4.2.8.1 Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

Mini-PROTEAN 3 system (BioRad) and discontinuous polyacrylamide (PAA) gels were applied to run denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Resolving gels were firstly cast between two glass plates and overlaid with 100% isopropanol which was removed by when gels were polymerized which is normally 30 min. Then, the stacking gels were poured onto the top of resolving gels, a comb with a specific number of wells was inserted and the gel was polymerized for 30 min. In this study, 8% and 10% resolving gels were used, overlaid by 4% stacking gels. Gels were 1.0 mm in thickness.

If protein samples were not directly extracted in 2x SDS-PAGE sample buffer, 1 volume of 2x SDS-PAGE sample buffer was added to protein sample and followed by boiling for 5 min for denaturation. After removing the combs under running water, each gel was placed into the electrophoresis tank and submerged in 1x running buffer. A pre-stained molecular weight marker (Precision plus protein standard dual colour, BioRad) and denatured protein samples were loaded

onto the gel and run at 80 V for 20 min (stacking gel) and 120 V (resolving gel) until the marker line suggested the samples had resolved sufficiently.

#### **4.2.8.2 Immunoblot analysis**

Proteins that had been resolved on PAA gels were transferred to Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences) after gels were released from the glass plates and stacking gels were removed with a scalpel. The blotting apparatus (Mini Trans-Blot® Cell, BioRad) was assembled according to the manufacturer instructions. The transfer was carried out at 100 V for 70 min. The transfer cassette was dismantled and membranes were checked for equal loading by staining with Ponceau S for 5 min before rinsing with deionised water. Ponceau S stained membranes were imaged by ChemiDoc Imaging system (Bio-Rad) and thereafter washed for 5 min in TBS-T before membranes were blocked for 1 h at room temperature in TBS-T containing 5% (w/v) non-fat dry milk. The blocking solution was removed and membranes were washed briefly with TBS-T. Incubation with primary antibodies was carried out overnight by slowly shaking on a rotary shaker at 4°C in TBS-T supplemented with 3 % (w/v) non-fat dry milk. Next morning the primary antibody solution was removed and membranes were washed 3 x 10 min with TBS-T at room temperature on a rotary shaker. Bound primary antibodies were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies. Antibody details in 2.1.10. Membranes were incubated in the secondary antibody solution for 1 h at room temperature at slow rotation. The antibody solution was removed and membranes were washed as described above. This was followed by chemiluminescence detection using the SuperSignal® West Pico Chemiluminescent kit or a 9:1 - 1:1 mixture of the SuperSignal® West Pico Chemiluminescent- and SuperSignal® West Femto Maximum Sensitivity-kits (Pierce) according to the manufacturer instructions. ChemiDoc Imaging system (Bio-Rad) were used to detect the luminescence.

#### **4.2.8.3 Immunoprecipitation with GFP\_Trap beads (Chromtech)**

5 leaf discs (∅1 cm) were finely ground in liquid nitrogen. 1.5 mL cold extraction buffer was added and incubated on ice for 30 min with 3 times thoroughly vortexing. Centrifuge 2 times with speed of 14000 RPM under 4 °C. Transfer supernatant to 15 mL falcons and add another 3mL extraction buffer to dilute the samples. 20 µL of GFP\_Trap beads were added to samples and incubate for 2.5 h at 4 °C on a wheel roller. Afterward, samples were spun down at 2500 g at 4 °C for 2 min. The supernatant was removed by pipetting. Next, 1 mL extraction buffer was added to wash Trap

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beads on wheel roller at 4 °C for 5 min. To pellet bead, samples were centrifuged for 2 min at 2500 g at 4 °C. Washing with 1 mL extraction buffer was repeated 4 times. After the last wash, a syringe with a needle was used to completely remove the remaining liquid. To elute protein, 100 µL 2×loading buffer was added and heated at 95 °C for 5 min. To pellet beads, samples were spun down by centrifuging for 1 min at 2500 g at room temperature.

### **4.2.9 Molecular biological methods**

#### **4.2.9.1 Isolation of total RNA from Arabidopsis**

Total RNA was prepared from 3 to 6-week-old plant materials or from transfected protoplasts. Liquid nitrogen froze samples (approximately 50 mg) were homogenized 2 x 15 sec to a fine powder using a Mini-Bead-Beater-8™ (Biospec Products) and 1.2 mm stainless steel beads (Roth) in 2 ml centrifuge tubes. After the first 15 sec of homogenisation samples were transferred back to liquid nitrogen and the procedure was repeated. Thereafter, RNA was isolated using RNeasy kit from Bio-Budget according to the manufacturer's instruction. Samples were stored at -80°C.

#### **4.2.9.2 Polymerase chain reaction (PCR)**

Standard PCR reactions were performed using home-made *Taq* DNA polymerase while for cloning of PCR products *Pfu* polymerase was used (see 2.1.6.2) according to the manufacturer's instructions. All PCRs were carried out using a PTC-225 Peltier thermal cycler (MJ Research). A typical PCR reaction mix and thermal profile are shown below.

**Table 4.10.** PCR mix (20 µL total volume)

<b>Component</b>	<b>Volume</b>
Template DNA	0.2-10 ng
10x PCR buffer	2 µL
dNTP (2.5 mM each) mix	1 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
<i>Taq</i> DNA polymerase (4U/ml)	0.5 µL
Nuclease-free water	Make up to 20 µL total volume

**Table 4.11.** Thermal cycling

Stage	Temperature (°C)	Time	Cycles
Initial denaturation	94	5 min	1×
Denaturation	94	30 sec	30-35×
Annealing	55-60	30 sec	
Extension	72	1 min/kb	
Final extension	72	5 min	1×

**4.2.9.3 One-Step quantitative PCR (qPCR)**

SYBR Green One-Step qPCR Kit (Biotool) was used to analyze the RNA samples prepared from the transfected protoplasts.

**Table 4.12.** PCR mix (20 µL total volume)

Components	Volume
2x SYBR Green One-Step Mix	10 µL
SYBR Green One-Step Enzyme Mix	1 µL
RNA template	100 ng
Forward primer (5 µM)	1 µL
Reverse primer (5 µM)	1 µL
RNase-free ddH2O	Add to 20 µL

**Table 4.13.** One-Step amplification program

Stage	Temperature (°C)	Time	Cycles
Reverse Transcription	50	15 min	1×
Initial denaturation	95	5 min	1×
Denaturation	95	10 sec	40×
Annealing	60	20 sec	
Extension	72	20 sec	

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Melt Curve	65 to 95	5 sec per 0.5 °C	1×
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### 4.2.9.4 Site-directed mutagenesis

Site-directed mutagenesis was performed with minor modifications as described in the instruction manual of the QuickChange® site-directed mutagenesis kit of Stratagene®.

**Table 4.14.** PCR mix (20 µL total volume)

Component	Volume
Template plasmid (7.5 ng/µL)	1 µL
10x pfu Turbo reaction buffer	2 µL
dNTP (2.5 mM each) mix	1 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
<i>Pfu Turbo</i> DNA polymerase (2.5U/ml)	0.4 µL
Nuclease-free water	Make up to 20 µL total volume

**Table 4.15.** Thermal cycling

Stage	Temperature (°C)	Time	Cycles
Initial denaturation	94	1 min	1×
Denaturation	94	10 sec	18×
Annealing	55-60	10 sec	
Extension	72	15 sec /kb	
Final extension	72	5 min	1×

After the PCR, 1 µL DpnI (20 U/µL) were added to the reaction mix to digest methylated, parental DNA and to enable selection of mutation-containing synthesized DNA. The reaction was incubated for 1 h at 37°C. 3 µL of the reaction mixture, containing the circular, nicked vector DNA with the desired mutations were then transformed into DH10B cells and plated on LB agar containing the appropriate antibiotic.

#### **4.2.9.5 Reverse transcription-polymerase chain reaction (RT-PCR)**

RT-PCR was carried out in two steps. Thermo Scientific RevertAid RT Kit was used for first strand cDNA synthesis by combining 1 µg total RNA, 1 µL oligo dT (0.5 µg/µL), in a volume of 12 µL (made up with H<sub>2</sub>O). The sample was incubated at 65°C for 5 min to destroy secondary structures before cooling on ice. Subsequently, the reaction was filled up to a total volume of 20 µL by adding 4 µL of 5x reaction buffer, 2 µL dNTP mix (each dNTP 10 mM), 1 µL of RiboLock RNase Inhibitor (20 U/µL) and 1 µL of RevertAid RT (200 U/µL). The reaction was incubated at 42°C for 60 min before the enzyme was heat inactivated at 70°C for 5 min. For subsequent PCR, synthesised cDNA was diluted to 10 ng/µL.

#### **4.2.9.6 Plasmid DNA isolation from bacteria**

Standard alkaline cell lysis miniprep of plasmid DNA were carried out using the MACHEREY-NAGEL (MN) miniprep plasmid isolation kit according to the manufacturer's instructions. Larger amounts of plasmid DNA were isolated using Qiagen Maxi or Mega preparation kits.

#### **4.2.9.7 Restriction endonuclease digestion of DNA**

Restriction digests were carried out according to the recommended manufacturer's conditions. Typically, reactions were carried out in 0.2 ml tubes, using 1 µL of restriction enzyme per 20 µL reaction. All digests were carried out at the appropriate temperature for a minimum of 60 min.

#### **4.2.9.8 Agarose gels electrophoresis of DNA**

DNA fragments were separated by agarose gel electrophoresis in gels consisting of 1-2 % (w/v) agarose in TAE buffer. Agarose was dissolved in TAE buffer by heating in a microwave. Molten agarose was cooled to 50° C before 2.5 µL of ethidium bromide solution (10 mg/ml) was added. The agarose was poured and allowed to solidify before being placed in TAE in an electrophoresis tank. DNA samples were loaded onto an agarose gel after addition of 2 µL 6x DNA loading buffer to 10 µL PCR- or restriction reaction. Separated DNA fragments were visualized by placing the gel on a 312 nm UV trans-illuminator and photographed.

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### **4.2.9.9 Isolation of DNA fragments from agarose gels**

DNA fragments separated by agarose gel electrophoresis were excised from the gel with a clean razor blade and extracted using the MACHEREY-NAGEL (MN) NucleoSpin® Gel and PCR Clean-up kit according to the manufacturer's protocol.

### **4.2.9.10 Site-specific recombination of DNA in Gateway®-compatible vectors**

The Gateway® system was used to generate the proper constructs. The pENTR/D vector was used to clone the gene fragments. To transfer the fragment of interest into gene expression constructs, an LR reaction between the entry clone and a Gateway® destination vector was performed.

**Table 4.16.** Basic LR reaction approach:

<b>Components</b>	<b>Volume</b>
LR reaction buffer (5x)	1 µL
Entry clone (75 ng/µL)	1 µL
Destination vector (100 ng/µL)	1 µL
LR clonase enzyme mix	0.5 µL
TE buffer	Make up to 6 µL

Reactions were incubated for 1 h at room temperature before adding 0.5 µL proteinase K solution. Reactions were incubated at 37° C for 10 min. Entire reaction was transformed into *E. coli* strain DH10B.

### **4.2.9.11 DNA sequencing**

DNA sequences were determined by Sanger sequencing at the “Automatische DNA Isolierung und Sequenzierung” (ADIS) service unit at the MPIPZ, Cologne.

### **4.2.9.12 DNA sequence analysis**

Sequence data were analyzed mainly using various packages from DNASTAR and Clone Manager 6 (Scientific and Educational software, USA).

**4.2.9.13 Preparation of chemically competent *E. coli* cells**

Media and solutions required for preparation of rubidium chloride *E. coli* chemically competent cells

**Table 4.17.** Media for preparation of competent cells

<b>ΦB</b>	<b>TFB1</b>	<b>TFB2</b>
Yeast extract 0.5 %	KAc 30 mM	MOPS 10 mM
Tryptone 2 %	MnCl <sub>2</sub> 50 mM	CaCl <sub>2</sub> 75 mM
MgSO <sub>4</sub> 0.4 %	RbCl 100 mM	RbCl 10 mM
KCl 10 mM	CaCl <sub>2</sub> 10 mM	Glycerol 15 %
pH 7.6	Glycerol 15 %	
autoclave	pH 5.8 sterile-filter	sterile-filter

5 ml of an *E. coli* strain DH10B over-night culture grown in ΦB was added to 400 ml of ΦB and shaken at 37°C until the bacterial growth reached an OD<sub>600</sub> 0.4 - 0.5. Cells were cooled on ice and all following steps were carried out on ice or in a 4° C cold room. The bacteria were pelleted at 5000 g for 15 min at 4°C. The pellet was gently resuspended in 120 ml ice-cold TFB1 solution and incubated on ice for 10 min. The cells were pelleted as before and carefully resuspended in 16 ml ice-cold TFB2 solution. 1.5 ml Eppendorf reaction tubes containing 50 μL aliquots of cells were frozen in liquid nitrogen and stored at -80°C until use.

**4.2.9.14 Transformation of chemically competent *E. coli* cells**

A 50 μL aliquot of chemically competent cells was thawed on ice. 10 to 25 ng of ligated plasmid DNA (or ~ 5 μL of ligated mix from 10 μL ligation reaction) was mixed with the aliquot and incubated on ice for 20 min. The mixture was heat-shocked for 45 sec at 42°C and immediately put on ice for 2 min. 500 μL of SOC medium or LB medium was added to the microcentrifuge tube and incubated at 37° C for 40 min on a rotary shaker. The transformation mixture was centrifuged for 5 min at 1500×g, resuspended in 50 μL LB broth and plated onto selective media plates.

**4.2.9.15 Preparation of electro-competent *A. tumefaciens* cells**

The desired *Agrobacterium* strain was streaked out onto YEB agar plate containing adequate antibiotics and grown at 28 °C for two days. A single colony was picked and a 5 ml YEB culture, containing appropriate antibiotics, was grown overnight at 28 °C. The whole overnight culture was added to 200 ml YEB (without antibiotics) and grown to an OD600 of 0.6. Subsequently, the culture was chilled on ice for 15 - 30 min. From this point onwards bacteria were maintained at 4 °C. Bacteria were centrifuged at 6000 g for 15 min and 4°C and the pellet was resuspended in 200 ml of ice-cold sterile water. Bacteria were again centrifuged at 6000 g for 15 min and 4°C. Bacteria were resuspended in 100 ml of ice-cold sterile water and centrifuged as described above. The bacterial pellet was resuspended in 4 ml of ice-cold 10 % glycerol and centrifuged as described above. Bacteria were resuspended in 600 µL of ice-cold 10 % glycerol. 40 µL aliquots were frozen in liquid nitrogen and stored at -80°C.

**4.2.9.16 Transformation of electro-competent *A. tumefaciens* cells**

100 ng of plasmid DNA was mixed with 40 µL of electro-competent *A. tumefaciens* cells and transferred to an electroporation cuvette on ice (2 mm electrode distance; Eurogentec, Seraing, Belgium). The BioRad Gene Pulse™ apparatus was set to 25 µF, 2.5 kV and 400 Ω. The cells were pulsed once at the above settings for a second, the cuvette was put back on ice and immediately 1 ml of YEB medium was added to the cuvette. Cells were quickly resuspended by slowly pipetting and transferred to a 2 mL microcentrifuge tube. The tube was incubated for 3 h in an Eppendorf thermomixer at 28°C. A 5 µL fraction of the transformation mixture was plated onto selection YEB agar plates.

**4.2.9.17 Yeast transformation**

The method for yeast transformation was adjusted from the protocol developed by the Lab of Dr. Pascal Braun (Dreze et al., 2010). Streak yeast strain L40 on YPAD plates and incubate at 30°C for 48-72 h to obtain isolated colonies. Pick out 10 isolated colonies and inoculate in 20 mL YPAD liquid medium and incubate at 30 °C on a shaker for 14-18 h. Dilute cells with OD600 between 4.0 and 6.0 into 100 ml YEPD media to obtain a final OD600= 0.1 which is enough for a 96-well plate of transformations. Incubate at 30 °C on a shaker until OD600 reaches 0.6–0.8. Harvest cells by centrifugation at 800 g for 5 min and discard the supernatant and resuspend cells gently in 10

ml of sterile water. Repeat this resuspending and centrifugation once and discard the supernatant. Then resuspends cell in 10 mL TE/LiAc solution, centrifuge, and discard the supernatant. The left cells were resuspended in 2 mL TE/LiAc solution and 10 mL TE/LiAc/PEG solution supplemented with 200  $\mu$ L of pre-boiled carrier DNA were added and then mix the solution by inversion. Dispense 120  $\mu$ L of this solution into one 200  $\mu$ L PCR tube which contains 300 ng plasmid DNA and then mixes by pipetting up and down. Then incubate at 30 °C for 30 min followed by 15 min heat shock in a 42 °C water bath. Then the PCR tubes were centrifuged for 5 min at 800 g. Around 90  $\mu$ L water were carefully removed and then resuspend the left cell pellets by vortexing. Plate half of the cell resuspension onto appropriate selective plates (SD-LW). Then the plates were incubated at 30 °C for 72 h. Use a sterile toothpick to pick transformed yeast colonies into sterile glass tubes containing 1.5 mL appropriate selective media. Then incubate on a shaker at 30 °C for overnight, which were diluted with 10 times serial to 1000 times. Then drop 5  $\mu$ L of undiluted and diluted cultures onto appropriate selective media (SD-LW and SD-LHW) and incubate at 30 °C for 72 h. ChemiDoc Imaging system (Bio-Rad) was used to take picture of the plates.

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