Functional characterisation of the EDS1–MYC2 regulatory node in *Arabidopsis*

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Stay hungry. Stay foolish.

Stewart Brand

Summary

Plants rely on a multi-layered, cell autonomous immune system to combat a plethora of pathogens. While a first, broad defence response is sufficient to restrict growth of most invaders, some pathogens have evolved means to overcome this. In an evolutionary arms race plants have evolved intracellular receptors that recognise host-adapted pathogens and initiate a sustained and potent immune response call ETI (effector-triggered immunity). EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) and its signalling partners PAD4 (PHYTOALEXIN DEFICIENT 4) or SAG101 (SENESCENCE-ASSOCIATED GENE 101) form a central convergence point for those intracellular receptors and act as a decision-making node for SA (salicylic acid) dependent and SA independent transcriptional reprogramming. Despite our increasing knowledge about plant immunity the molecular function of the EDS1/PAD4 complex and how these proteins are regulated remains unclear. Recent work established an antagonistic regulation between the JA (jasmonic acid) key TF (transcription factor) MYC2 and EDS1. While MYC2 enhances bacterial virulence by repressing the *EDS1* promoter, ETI activated EDS1 represses MYC2 signalling and dampens pathogen growth. This cross-regulation represents an intersection of ETI and JA signalling and allows fine-tuning of the plant's immune response. How EDS1 controls MYC2 accumulation and activity is not known.

Here, I show regulation of MYC2 abundance and MYC2 transactivation activity by EDS1 family proteins. Further, I present evidence for the underlying molecular mechanisms of this regulation and identify new components in this pathway. Specifically, PAD4 and SAG101 but not EDS1, stabilise MYC2 protein while EDS1 but not PAD4 or SAG101, promote MYC2 transactivation activity. Thus, protein accumulation is not indicative of MYC2 transcriptional output. MYC2 activity promotion is lost in a JAZ repressor uncoupled MYC2 variant ($MYC2^{s}$) or when co-expressed with the bacterial virulence protein avrRPS4, indicating i) regulation of JAZ proteins by EDS1 and ii) immunity context dependent regulation of MYC2. Functional characterisation of this regulation shows that besides JAZ repression MYC2 is phosphorylated in an EDS1 dependent manner. In *eds1-2* plants $MYC2^{S123}$ is phosphorylated, suggesting that EDS1 either represses a protein kinase or activates a protein phosphatase. I show interaction of the protein kinase EDR1 (ENHANCED DISEASE RESISTANCE 1), a negative regulator of plant immunity, with PAD4 and with MYC2 in Arabidopsis (*Arabidopsis thaliana*) protoplasts. Whether EDS1 regulates MYC2 via EDR1 or another, so far unknown, component remains to be tested.

Results presented in this work provide insights into the regulatory relationship of EDS1 and MYC2. EDS1 promotes MYC2 transactivation activity as shown by enhanced MYC2 target gene expression. Molecularly, EDS1 regulates MYC2 via JAZ proteins and likely via MYC2 phosphorylation. More detailed analysis will be necessary to address this entirely. Ultimately, the impact of the presented data depends on the functional relevance of this regulation. For this, *in planta* experiments in pathogen challenged and unchallenged conditions will be key.

Zusammenfassung

Pflanzen sind auf ein vielschichtiges, zellautonomes Immunsystem angewiesen, um eine Vielzahl von Krankheitserregern zu bekämpfen. Während eine erste, breite Abwehrreaktion ausreicht, um das Wachstum der meisten Eindringlinge einzuschränken, haben einige Krankheitserreger Wege gefunden, um dies zu überwinden. In einem evolutionären Wettrüsten haben Pflanzen intrazelluläre Rezeptoren entwickelt, die wirtsangepasste Pathogene erkennen und eine dauerhafte und starke Immunantwort (ETI - Effektor-ausgelöste Immunität) auslösen. EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) und seine signalgebende Partnerproteine PAD4 (PHYTOALEXIN DEFICIENT 4) oder SAG101 (SENESCENCE-ASSOCIATED GENE 101) bilden einen zentralen Konvergenzpunkt für diese intrazellulären Rezeptoren und fungieren als Entscheidungsknoten für SA (Salicylsäure) abhängige und SA-unabhängige Umprogrammierung der Genexpression. Trotz unseres zunehmenden Wissens über die Immunität von Pflanzen ist die molekulare Funktion des EDS1 / PAD4-Komplexes und dessen Regulierung unklar. Jüngste Arbeiten haben eine antagonistische Regulierung zwischen dem JA (Jasmonsäure)-Kern-TF (Transkriptionsfaktor) MYC2 und EDS1 etabliert. Während MYC2 die bakterielle Virulenz durch Unterdrückung des EDS1-Promotors verstärkt, unterdrückt ETI-aktiviertes EDS1 den MYC2-Signalweg und dämmt das Wachstum von Erregern ein. Diese Kreuzregulierung stellt eine Kreuzung von ETI- und JA-Signalwegen dar und ermöglicht eine Feinabstimmung der pflanzlichen Immunantwort. Wie EDS1 die Menge und Aktivität von MYC2 steuert, ist nicht bekannt.

Hier zeige ich die Regulierung der MYC2 Menge und der MYC2 Transaktivierungsaktivität durch Proteine der EDS1-Familie. Darüber hinaus präsentiere ich die molekularen Mechanismen dieser Regulation und identifiziere neue Komponenten in diesem Signalweg. Insbesondere stabilisieren PAD4 und SAG101, aber nicht EDS1, das MYC2-Protein, während EDS1, nicht jedoch PAD4 oder SAG101 die MYC2 Transaktivierungsaktivität fördert. Daher ist die Proteinakkumulation kein Indikator für die Aktivität von MYC2 als TF. Die Erhöhung der MYC2 Aktivität geht in einer JAZ-entkoppelten MYC2-Variante ($MYC2^{s}$) oder bei gleichzeitiger Expression des bakteriellen Virulenzproteins avrRPS4 verloren, was auf i) die Regulierung von JAZ-Proteinen durch EDS1 und ii) die immun-kontextabhängige Regulation von MYC2 hinweist. Die funktionale Charakterisierung dieser Regulierung zeigt, dass MYC2, neben Regulation durch JAZ-Proteine, EDS1-abhängig phosphoryliert wird. In *eds1-2* Pflanzen ist $MYC2^{S123}$ phosphoryliert, was darauf schließen

lässt, dass EDS1 entweder eine Proteinkinase unterdrückt oder eine Proteinphosphatase aktiviert. Ich zeige die Interaktion der Proteinkinase EDR1 (ENHANCED DISEASE RESISTANCE 1), einem negativen Regulator der Pflanzenimmunität, mit PAD4 und mit MYC2 in Arabidopsis (*Arabidopsis thaliana*) Protoplasten. Ob EDS1 MYC2 über EDR1 oder eine andere bisher unbekannte Komponente reguliert, muss noch getestet werden.

Die in dieser Arbeit präsentierten Ergebnisse geben Einblick in die regulatorischen Beziehungen zwischen EDS1 und MYC2. EDS1 fördert die MYC2 Transaktivierungsaktivität, wie durch die verstärkte Expression von MYC2 abhängigen Genen gezeigt wird. Insgesamt reguliert EDS1 die MYC2 Aktivität über JAZ-Proteine und wahrscheinlich über MYC2 Phosphorylierung. Eine genauere Analyse wird jedoch erforderlich sein, um dieses Problem vollständig zu lösen. Letztendlich hängt die Wichtigkeit der dargestellten Daten von der funktionalen Relevanz dieser Regulierung ab. Um dies zu klären, werden *in planta* Experimente in Pathogen infizierten und nicht-infizierten Bedingungen ausschlaggebend sein.

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Abbreviations

°C	degree Celsius
aa	amino acid
ACN	acetonitrile
Arabidopsis	*Arabidopsis thaliana*
avr	avirulence
bp	base pair
C-terminal	carboxy-terminal
CC	coiled-coil
cDNA	complementary DNA
d	day(s)
ddH2O	distilled, deionized water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleosidetriphosphate
dpi	days post infiltration
EDTA	ethylenediaminetetraacetic acid
eps	*eds1-2/pad4-1/sag101-2*
ETI	effector-triggered immunity
FA	Formic acid
fw	forward
FWHM	Full Width at Half Maximum
g	gravity
GFP	green fluorescent protein
YFP	yellow fluorescent protein
h	hour(s)
HA	hemagglutinin
HCD	High-energy collisional dissociation
HF	High Fidelity
hpi	hours post infiltration
HRP	horseradish peroxidase
iBAQ	intensity Based Absolute Quantification

JA	jasmonic acid
kb	kilo base(s)
kDa	kilo Dalton
l	litre
LFQ	label-free quantification
LRR	leucine-rich repeat
M	molar (mol / l)
µ	micro
MAMP	microbe-associated molecular pattern
mg	milligram
min	minute(s)
mM	millimolar
MPIPZ	Max Planck Institute for Plant Breeding Research
mRNA	messenger RNA
ng	nanogram
nLC-MS/MS	nano-scale liquid chromatographic tandem mass spectrometry
NLR	nucleotide-binding leucine-rich repeat
nM	nanomolar
NOD	nucleotide-binding-oligomerisation domain
N-terminal	amino-terminal
OD	optical density
p35S	35S promoter from *Cauliflower Mosaic Virus*
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PEG	Polyethylene glycol
pH	negative decimal logarithm of the H^+ concentration
PRR	PAMP/pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato DC3000*
PTI	PAMP/ pattern-triggered immunity
pv.	pathovar
qRT-PCR	quantitative reverse-transcription PCR
R	resistance
RNA	ribonucleic acid

ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
rv	reverse
SA	salicylic acid
SDM	Site directed mutagenesis
SDS	sodium dodecyl sulphate
SH	StrepII-3xHA
T3SS	type III secretion system
TBS	Tris buffered saline
T-DNA	transfer-DNA
TF	transcription factor
TFA	Trifluoroacetic acid
TIR	Toll/ interleukin-1 receptor
TNL	TIR-NLR
V	Volt(s)
v/v	volume per volume
WT	wild-type, here: *Arabidopsis thaliana*, *Col-0*
w/v	weight/volume
Y2H	yeast-two-hybrid

1 Introduction

Given the evolutionary distance between plants and animals, their innate immune systems are rather alike. Both rely on a two-phase system that firstly recognises intruders via unspecific, commonly present clues. Secondly, a more specific and more powerful immune response is initiated. Compared to animals, plants face numerous complications in terms of pathogen and pest defence. Being sessile organisms, plants cannot physically move away from pathogen pressure. Further, plants cannot rely on mobile, highly specialised immunity cells, since the cell wall prevents cell motility. Rather, every individual cell needs to be equipped with the full defence potential. This is reflected by the fact that plants do not possess adaptive immunity like animals, but rather an innate and a systemic immune response. In the past 20 years we have gained extensive insights into the sophisticated principles of the plant immunity network (Dangl et al. 2001, Jones et al. 2006) and while we understand pathogen recognition and subsequent defence outputs rather well, our knowledge on signal integration and decision-making remains limited.

1.1 Recognition of conserved microbial patterns - an initial barrier for pathogens

The first layer of plant immunity is typically referred to as non-host, or basal immunity. It relies on perception of pathogens via cell surface receptors called PRRs (pattern recognition receptors) that recognise conserved molecular patterns common to most microbes. PRR mediated immunity is therefore called PAMP- (pathogen-associated molecular pattern) or MAMP- (microbe-associated molecular pattern) triggered immunity (PTI) (Zipfel 2009). This form of immune response is usually potent enough to restrict growth of host non-adapted microbes. The Arabidopsis (*Arabidopsis thaliana*) genome encodes more than 600 *PRR* genes of which almost half are up-regulated by biotic stresses (Lehti-Shiu et al. 2009). Of this plethora of PRRs, numerous members have been characterised and shown to differ in their phylogenetic distribution, epitope recognition, and interaction partners. The best characterised PRR is FLS2 (FLAGELLIN SENSING 2). Upon recognition of the bacterial peptide flg22 (flagellin), FLS2 and its signalling partner BAK1 (BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE 1) phosphorylate the cytoplasmic kinase BIK1 (BOTRYTIS-INDUCED KINASE 1), which in turn phosphorylates the NADPH oxidase RBOHD (RESPIRATORY BURST OXIDASE HOMOLOGUE D). This results in an

antimicrobial ROS (reactive oxygen species) burst. In parallel, activated FLS2/BAK1 induce MAPK (MITOGEN-ACTIVATED PROTEIN KINASE) signalling leading to WRKY TF phosphorylation and ultimately to transcriptional reprogramming (Couto et al. 2016, Wang et al. 2018). A hallmark of PTI is its rapid induction with kinase phosphorylation and ROS burst occurring within 5-10 min after receptor activation.

1.2 NLR resistance - the next level

Selection pressure on pathogens to overcome PTI led to the evolution of effector proteins that suppress PTI and cause ETS (effector-triggered susceptibility). These virulence factors have numerous structures and functions and many are secreted into the plant cell, usually via a T3SS (type III secretion system) to suppress PTI. In an arms race plants evolved intracellular receptors that sense bacterial effector proteins and trigger a second, more powerful immune response named ETI (effector-triggered immunity). These intracellular receptors resemble mammalian NOD-like receptors and are called nucleotide binding domain-leucine rich repeat receptors (NLRs). Dependent on their N-terminal domain NLRs can be further specified into CC-NLRs (coiled-coil domain, CNLs) and TIR-NLRs (Toll-Interleukin1-Receptor domain, TNLs) (Jones et al. 2016). The long-standing dogma of NLR signalling was that each NLR represents a *R* (resistance) gene that directly recognises a bacterial effector, thereby conferring resistance (fitting the gene-for-gene hypothesis) (Flor 1971, Ellis et al. 2000, Jones et al. 2016). In this scenario pathogens face substantial selective pressure to evolve their effector proteins in order to dodge plant immunity. Likewise, plants have to keep up with effector diversification by adapting existing or inventing new *R* genes. Nowadays a more universal notion is accepted.

Besides direct NLR-effector interaction, other recognition mechanisms have been proposed and characterised. Numerous NLRs do not directly detect an effector, but monitor effector target proteins, so called “guardees”, or “decoys”. Hence, modification of a guardee/decoy leads to NLR activation. While the function of guardees and decoys is similar, guardees usually have an additional role in immune signalling. Decoys, however, serve exclusively as baits for effectors. A variation of the decoy model is a class of NLRs with an integrated decoy domain. Here, the NLR contains an effector virulence target domain. Combination of these recognition mechanisms enables relatively few NLRs (ca. 150 in Arabidopsis) to cover a vast diversity of bacterial effectors (Jones et al. 2016).

How NLRs are activated mechanistically is still subject to debate. Generally, activation is thought to correlate with conformational changes and possibly via oligomerisation (Jones et al. 2016, Zhang et al. 2017). Our understanding of the events after NLR activation is more advanced. All TNLs, and certain CNLs, converge on the EDS1/PAD4 signalling hub. This protein complex functions as a signal relay positioned downstream of TNL activation, but upstream of transcriptional reprogramming (Wirthmueller et al. 2007, Garcia et al. 2010). Successful initiation of ETI includes various defence responses, among which pcd (programmed cell death), also known as HR (hypersensitivity response), and accumulation of SA are the most prominent ones. A simplified overview of PTI, ETI, and EDS1/PAD4 positioning within immune signalling is depicted in Figure 1.1.

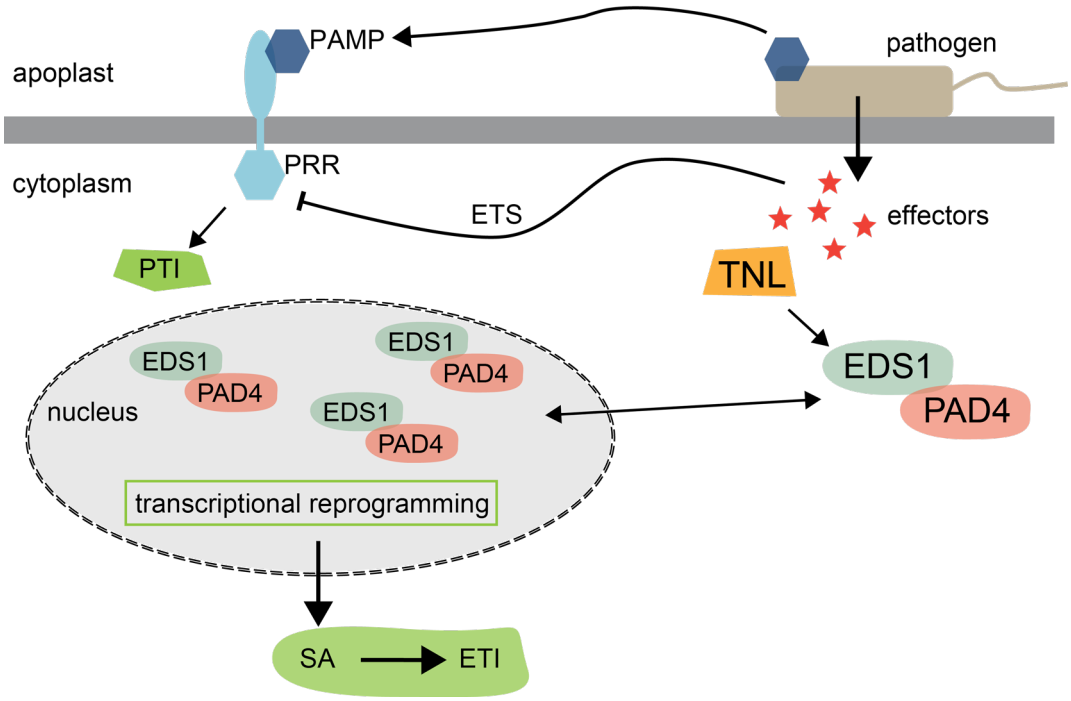


Figure 1.1 Plant immune signalling. Simplified illustration of different layers of plant immunity. Pathogens are recognised by two mechanisms. Extracellular PRRs (pattern recognition receptors) detect conserved molecular patterns called PAMPs (pathogen-associated molecular patterns) and initiate a universal first immune response, PTI (pattern-triggered immunity). In the cytoplasm, secreted bacterial effectors counteract PTI, causing ETS (effector-triggered susceptibility). Adapted hosts in turn detect bacterial effectors via TNLs that convert on the signalling hub EDS1/PAD4. Upon nuclear localisation, EDS1/PAD4 heterodimer dependent transcriptional reprogramming occurs which results in SA accumulation and a sustained, pathogen specific immune response, ETI (effector-triggered immunity).

PTI and ETI were seen as processes employing distinct recognition and output machineries. In recent years however, evidence for interlaced immunity signalling between PTI and ETI has accumulated. Rather than quality, quantity seems to be the decisive factor between PTI and ETI. For instance genes that are differentially expressed during PTI and ETI overlap strongly, but vary in their abundance and timing (Tao et al. 2003). MAPK activation also suggests shared signalling components. During PTI, MPK3/6 activation is short-lived, but rapid. In presence of effectors though, MPK3/6 activation was sustained over several hours (Tsuda et al. 2013), suggesting context-specific adjustment of common signalling sets. Interestingly, successful PTI can partially suppress ETI, thereby limiting fitness costs for the plant (Hatsugai et al. 2017). In a recent review, Alhoraibi et al. discuss the distinct, but also the common features of PTI and ETI and propose a more interconnected relationship of the two immune responses (Alhoraibi et al. 2018).

1.3 The EDS1/PAD4 signalling node

EDS1 was identified in a forward genetic suppressor screen of Arabidopsis resistance against the oomycete *Hyaloperonospora parasitica* (Parker et al. 1996). Over time, EDS1 has emerged as a major component of various aspects of plant immunity. In the following chapter I will summarise our current understanding of the EDS1 resistance node and the implications that arise from it. EDS1 is a nucleo-cytoplasmic protein with multi-facetted functions in plant immunity. It functions in basal (post-infection) and TNL-triggered immunity, but also in some CNL-triggered immune responses (Aarts et al. 1998, Rusterucci et al. 2001, Venugopal et al. 2009, Wang et al. 2009, Garcia et al. 2010, Makandar et al. 2015, Cui et al. 2018). Further, SA dependent and SA independent roles have been shown (Glazebrook et al. 2003, Zhang et al. 2003, Bartsch et al. 2006, Gloggnitzer et al. 2014, Cui et al. 2016).

1.3.1 Subcellular localisation determines EDS1 function

EDS1 localisation depends on the cell's immune status. In unchallenged tissues EDS1 localises nucleo-cytoplasmically, while infection with avirulent *Pseudomonas syringae* pv. *tomato DC3000* (hereinafter *Pst*) *avrRPS4* causes EDS1 accumulation in the nucleus. This nuclear pool of EDS1 is essential for transcriptional reprogramming and resistance to bacterial and oomycete pathogens. Simultaneously, a cytoplasmic EDS1 fraction is

maintained and necessary for complete resistance (Garcia et al. 2010). Following this observation, distinct functions of nuclear and cytoplasmic EDS1 were found. While nuclear EDS1 steers transcriptional reprogramming to dampen bacterial growth, cytoplasmic EDS1 elicits host cell death. This might allow the plant to fine-tune defence pathways for most efficient pathogen growth inhibition (Heidrich et al. 2011). Tight control of this nucleo-cytoplasmic shuttling is crucial, as high levels of nuclear EDS1 cause DM2h (DANGEROUS MIX 2) dependent auto-immunity (Stuttmann et al. 2016).

1.3.2 EDS1 and its signalling partners PAD4 and SAG101 constitute a unique plant protein family

Genetic and molecular studies identified two signalling partners of EDS1, PAD4 (PHYTOALEXIN-DEFICIENT 4) and SAG101 (SENESCENCE-ASSOCIATED GENE 101) (Falk et al. 1999, Feys et al. 2001, Feys et al. 2005). Together these three proteins form a unique plant protein family with a N-terminal LP (lipase-like) domain with homology to α/β hydrolases (Ollis et al. 1992) and an unique C-terminal "EP" (EDS1-PAD4) domain with no known homologies (Feys et al. 2001, Feys et al. 2005, Wagner et al. 2013). EDS1 and PAD4 contain a S-D-H catalytic triad common to lipase-like domains. Yet no enzymatic activity has been shown for EDS1, and catalytic triad mutants were fully functional in basal and TNL-triggered immunity, suggesting that EDS1 and PAD4 are not enzymatically active (Rietz et al. 2011, Wagner et al. 2013). SAG101 lacks the catalytic triad (Feys et al. 2005). With no catalytic activity of the LP domain and a previously unknown EP domain, mechanistic insights of the EDS1 protein family were difficult to gain.

On a protein level, EDS1 forms exclusive heterodimers with PAD4 or SAG101, resulting in stabilisation of both interaction partners (Feys et al. 2001, Feys et al. 2005). Resolving the crystal structure of the EDS1/SAG101 heterodimer and subsequent modelling of the EDS1/PAD4 heterodimer provided crucial insights into the interaction mode of the three proteins (Wagner et al. 2013) (Figure 1.2). Heterodimer formation mainly relies on an interface of a hydrophobic EDS1 helix that fits into a corresponding pocket of SAG101, or PAD4, respectively. Mutation of several residues in the EDS1 hydrophobic helix ($EDS1^{LLIF}$) abolishes interaction with SAG101, or PAD4, and renders EDS1 non-functional. Likewise, corresponding mutations in SAG101 ($SAG101^{LLIY}$), or PAD4 ($PAD4^{MLF}$) strongly, but not entirely, impair heterodimer formation (Wagner et al. 2013). Genetically, pathogen

challenged EDS1^{LLIF} expressing plants resemble an *eds1-2* null mutant, with hypersusceptibility to the oomycete *Hpa* (*Hyaloperonospora arabidopsisdis*).

These features underline the importance of the EDS1/PAD4 or EDS1/SAG101 heterodimer in Arabidopsis immunity. *SAG101* genetically partially compensates for loss of *PAD4*, suggesting similar signalling properties of the EDS1/PAD4 and the EDS1/SAG101 heterodimer. However, SAG101 localises exclusively to the nucleus, while PAD4 is present nucleo-cytoplasmically, suggesting potential functional distinction (Feys et al. 2005, Wagner et al. 2013). Recent work in our group supports distinct roles of PAD4 and SAG101 in plant immunity. While the TNL-activated EDS1/SAG101 heterodimer promotes cell death, the TNL-activated EDS1/PAD4 heterodimer limits bacterial growth (Lapin et al., submitted).

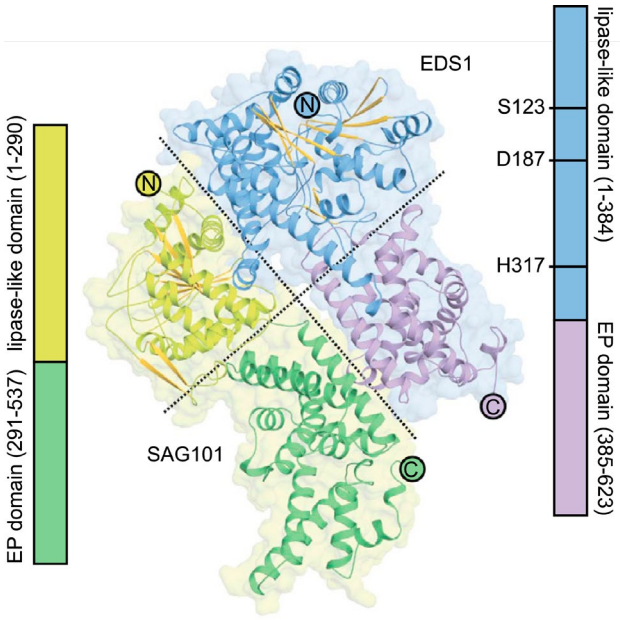


Figure 1.2 Crystal structure of the EDS1/SAG101 heterodimer. Both proteins contain a lipase-like domain (EDS1: light blue, SAG101: yellow) and the family specific EP domain (EDS1: light purple, SAG101: green). EDS1 contains a conserved S-D-H triad typical for lipases. Taken from Wagner et al. 2013

Further, Wagner et al. (2013) showed that both the LP and EP domains are necessary for full EDS1 resistance signalling and while the LP domain is stable by itself, the EP domain relies on stabilisation by the LP domain. Together with the fact that heterodimer formation depends on residues in the LP domain, and the absence of catalytic activity, this suggests a scaffolding function of the LP domain and a signalling function of the EP domain. This is in line with recent work in the group which established a specific role of the EP domain within

an EDS1/PAD4 heterodimer in timely reprogramming of the Arabidopsis immune response (Bhandari et al. 2019).

1.3.3 EDS1/PAD4 control SA dependent and SA independent pathways

The characterisation of EDS1/PAD4 proteins revealed that the EDS1/PAD4 complex signals upstream of SA in both basal and TNL-triggered immunity with EDS1/PAD4 supporting SA accumulation (Zhou et al. 1998, Jirage et al. 1999, Feys et al. 2001). On the other hand, exogenous application of SA enhances transcript and protein levels of EDS1 and PAD4, supporting the idea of an EDS1/PAD4 - SA positive feedback loop which fortifies SA dependent resistance signalling (Jirage et al. 1999, Feys et al. 2001).

Adding another layer of complexity to this signalling network, EDS1/PAD4 also employ SA independent pathways for resistance signalling (Li et al. 2001, Zhang et al. 2003, Bartsch et al. 2006). Recently, our group disentangled these SA dependent and SA independent processes. My colleagues showed that overexpressed EDS1 and PAD4 act in parallel with SA signalling and protect the SA pathway from pathogenic or genetic perturbations (Cui et al. 2016). For this, EDS1 and PAD4 engage hormone crosstalk. Specifically, TNL-activated EDS1/PAD4 shelter SA signalling from JA antagonism by interfering with the JA master TF MYC2 (Cui et al. 2018). This exemplifies a characteristic of ETI which protects crucial defence sectors like the SA node to ensure robust resistance signalling. The EDS1/PAD4 compensatory mechanism fits with the notion of network buffering where interactions within the immune signalling network constitute a robust and dynamic defence response (Cui et al. 2016, Hillmer et al. 2017).

Hormone crosstalk regulation presents a new function of the EDS1/PAD4 complex and will be addressed in more detail in this study. In summary, the EDS1 regulatory node allows the plant to respond to multi-facetted immune challenges in an effective and efficient manner by prioritising and integrating signalling pathways.

1.4 SA signalling

The role of SA in plant defence is rather well understood. SA is typically associated with resistance against microbial biotrophic pathogens (Glazebrook 2005). Chemically, SA is a phenolic acid and is predominantly synthesised via ICS1 (ISOCHORISMATE SYNTHASE 1) (Wildermuth et al. 2001). Upon pathogen attack SA levels increase significantly, with

EDS1 and PAD4 mediating TNL-triggered immune responses (Wiermer et al. 2005) and the resistance protein NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE 1) mediating CNL-triggered signalling (Aarts et al. 1998). Once SA accumulates, it initiates relocalisation of NPR1 (NON-EXPRESSOR OF PR GENES 1), which in the absence of SA forms oligomers in the cytoplasm. SA induced changes in the cell's redox state promote NPR1 monomerisation and translocation into the nucleus where it acts as a co-activator of defence genes (Tada et al. 2008). The underlying mechanism is well understood. Within the nucleus, NPR1 binds to and activates TGA TFs (members of the bZIP TF family) that sit at the promoter of SA-responsive genes like *PR1* (*PATHOGENESIS-RELATED GENE 1)* or *WRKYs* (Despres et al. 2000, Eulgem et al. 2007). To restrict SA signalling NPR1 is phosphorylated and subjected to 26S proteasomal degradation (Spoel et al. 2009).

The nature of the SA receptor has been a long-standing matter of debate in the scientific community. While NPR1 acts as the SA signal transducer (Dong 2004, Yan et al. 2014), opposing views on the SA binding capabilities of NPR1 exist. In 2012 the NPR1 paralogues NPR3/NPR4 were shown to bind SA with different affinities (Fu et al. 2012) and in the same year, NPR1 was proposed to be the SA receptor (Wu et al. 2012). More recently, both NPR1 and NPR3/NPR4 were described as SA receptors, but with opposing roles in transcriptional reprogramming during plant immunity (Ding et al. 2018). The perception of SA by multiple, non-redundant receptors is unique in the plant hormone network and explains the seemingly controversial observations in the search for a SA receptor.

1.5 The core JA pathway

Besides SA, JA is of exceptional importance for plant defence. Analogous to SA repressing biotrophic microbes, JA primarily antagonises necrotrophic pathogens (Glazebrook 2005). The starting point for JA biosynthesis are fatty acid precursors, which are processed via multiple biochemical reactions to JA. "JA" is usually used as a generic term that spans a broad spectrum of JA metabolites. These can be conjugated and modified in multiple ways, resulting in JA derivatives with diverse functions. Generally, the conjugate jasmonoyl isoleucine (JA-Ile) is the predominant biologically active compound (Wasternack 2007, Wasternack et al. 2013). Interestingly, plant pathogens like *Pst* have evolved functional mimics of JA-Ile such as COR (coronatine) (Mittal et al. 1995, Brooks et al. 2004). COR is a small molecule that competes with JA-Ile to activate the JA pathway. Initiation of JA signalling antagonises SA dependent immunity and enhances *Pst* virulence (Brooks et al.

2004, Brooks et al. 2005, Zheng et al. 2012). JA signalling relies on the repression of repressors. Molecularly, this means that key JA TFs are kept inactive by a family of repressor proteins, the so-called JAZ (JASMONATE ZIM DOMAIN) proteins (Chini et al. 2007, Thines et al. 2007). Together with the co-repressors NINJA (NOVEL INTERACTOR OF JAZ) and TPL (TOPLESS) JAZ proteins keep the JA pathway in check (Pauwels et al. 2010).

Active JA-Ile is bound by COI1 (CORONATINE-INSENSITIVE 1), an E3 ubiquitin-ligase that is part of the SKP1-Cullin-F-box complex SCF^{COI1}, which leads to ubiquitin dependent proteasomal degradation of JAZ proteins (Sheard et al. 2010). The removal of JAZ proteins sets key JA TFs free. JA signalling can be divided in two branches: the MYC (MYELOCYTOMATOSIS ONCOGENE HOMOLOG) branch (Lorenzo et al. 2004, Dombrecht et al. 2007) and the ERF (ETHYLENE RESPONSE FACTOR) branch (Lorenzo et al. 2003, Pre et al. 2008). The MYC branch is controlled by MYC class IIIe bHLH (basic helix-loop-helix) TFs, with its most prominent members being MYC2/3/4. Well-known marker genes of this branch are *VSP1/2* (*VEGETATIVE STORAGE PROTEIN 1/2*) and *LOX3* (*LIPOXYGENASE 3*) for instance. The ERF branch on the other hand is regulated by AP2/ERF (APETALA2/ETHYLENE RESPONSE FACTOR) TFs. Typical genes regulated by this branch are *PDF1.2* (*PLANT DEFENSIN 1.2*) or *ORA59* (*OCTADECANOID-RESPONSIVE ARABIDOPSIS 59*). Interestingly, ERF branch genes can be repressed by MYC2 (Zhai et al. 2013). On contrast to the MYC branch, activation of the ERF branch involves the plant hormone ethylene and is more complex and less well understood (Lorenzo et al. 2003, Pre et al. 2008). Functionally, the two pathways are thought to differ in their responsiveness to different attackers. The MYC pathway is activated by wounding and herbivorous damage (Lorenzo et al. 2004, Kazan et al. 2013), while microbial necrotrophic pathogens activate the ERF branch (Berrocal-Lobo et al. 2002, Lorenzo et al. 2003). A simplified overview of JA signalling is depicted in Figure 1.3.

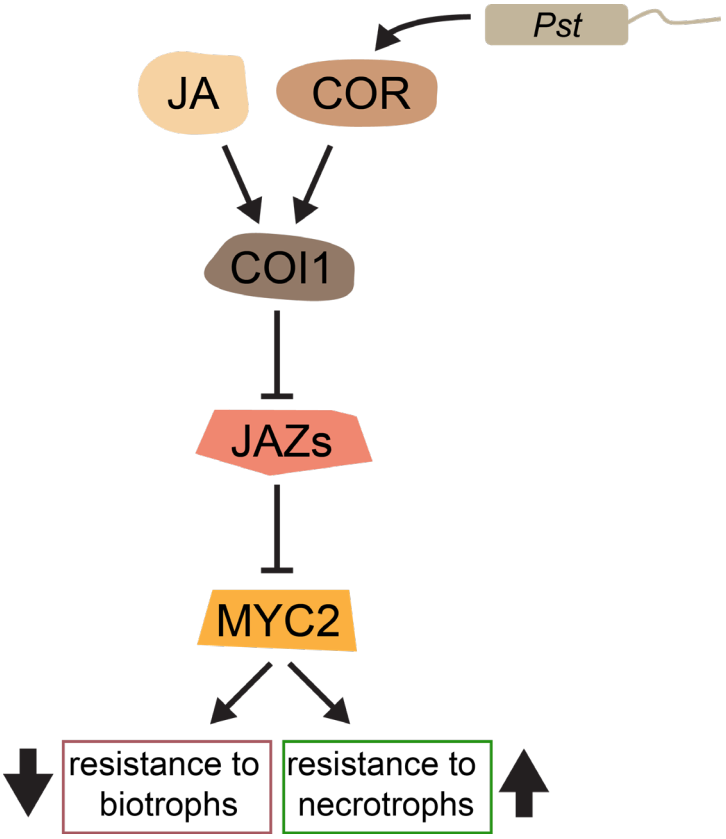


Figure 1.3 Scheme of JA signalling in context of plant immunity. In absence of JA the JA master TF MYC2 is repressed by the JAZ protein family. JA or its bacteria derived functional mimic COR lead to COI1 dependent proteasomal JAZ degradation, thereby releasing MYC2 and activating MYC2 specific gene expression. Generally, MYC2 activation correlates with decreased resistance to biotrophic pathogens and increased resistance to necrotrophic ones.

1.5.1 The JAZ protein family represses JA signalling

Since JA signalling needs to be tightly regulated, diverse (auto-)regulatory mechanisms have been observed. For instance, JA biosynthesis is promoted by JA itself, ensuring a robust JA response when necessary. Further downstream in the signalling cascade MYC2 restricts its own transcription twofold: i) by binding a G-box motif in its own promoter, and ii) by rapidly inducing JAZ transcription (Chini et al. 2007, Dombrecht et al. 2007). Besides this transcriptional regulation, an additional auto-regulatory MYC2 mechanism has been described. Once activated, MYC2 promotes the MTB (MYC2-TARGETED BHLH) proteins, a small subgroup of bHLH proteins. MTBs compete with MYC2 for binding of MED25 (MEDIATOR 25), a transcriptional co-activator of MYC2, thereby attenuating MYC2 transactivation activity (Liu et al. 2019).

The probably most dynamic and versatile tool to fine-tune JA signalling are the JAZ repressor proteins. 13 JAZs have been identified so far (Chini et al. 2016) and while the majority seems to act redundantly, there are gradually more insights gained into functional specification of single JAZ proteins. Common to all JAZs is their transcriptional regulation. In presence of JA, JAZ proteins are degraded by the 26S proteasome, thus releasing their target genes. At the same time, *JAZ* transcription is enhanced which allows a quick replenishment of JAZ proteins if necessary (Chini et al. 2007, Pauwels et al. 2011). JAZ specificity is accomplished by different means, for instance *JAZ1/2/5-10* are highly up regulated after *Pst* infection (Demianski et al. 2012). Yet only *jaz10* mutants show hypersusceptibility to *Pst* (Demianski et al. 2012). This was further supported by the finding that JAZ10 acts synergistically with JAZ5 in restricting COR cytotoxicity, as *jaz10/jaz5* double mutants showed enhanced chlorosis in *Pst* infected leaves (de Torres Zabala et al. 2016). JAZ6 takes part in circadian regulation of plant defence against *Botrytis cinerea*. Arabidopsis is more susceptible to *B. cinerea* during the night, than at dawn. However, *jaz6* mutant plants lose this daytime dependent susceptibility, indicating a specific role for JAZ6 in this context (Ingle et al. 2015). JAZ2 localises exclusively to guard cells and mediates COR induced stomatal re-opening (Gimenez-Ibanez et al. 2017) and JAZ4/7/8 have been identified as important components of JA-induced leaf senescence (Jiang et al. 2014, Yu et al. 2016). Finally, JAZ3 interacts with the bacterial effector HopBB1 leading to degradation of the JA pathway repressor TCP14 and promotion of bacterial virulence (Yang et al. 2017).

To extend the regulatory repertoire of JAZ proteins they are able to form homo- and heteromers within the JAZ family, but we are still missing a detailed understanding of the functional implementations this has (Chini et al. 2009, Chung et al. 2009). The diversity of JAZ proteins also helps to re-suppress JA signalling and avoids hyperactivation. Means to accomplish this are specific JAZ degrons and alternative splice variants. JAZ8 for instance lacks the typical COI1 recognition motif that mediates COI1-JAZ binding and is therefore less prone to COI1 dependent proteasomal degradation (Shyu et al. 2012). A similar observation has been made for JAZ13 (Thireault et al. 2015). To increase the regulatory range even further, alternative splice variants of certain JAZ proteins have been described in context of re-suppressing JA signalling. A well-known example is JAZ10, which exists in three isoforms. The shortest of these isoforms (JAZ10.4) lacks the Jas domain that is necessary for COI1 binding and subsequent degradation (Chung et al. 2009). Consistent with this, JAZ10.4 did not bind COI1, even in presence of high (200 µM) COR. Other tested

interactions including MYC2 and several JAZs were unchanged, suggesting a specific COI1 related function. Overexpressing this variant led to *coi1* like hyposensitivity to JA and associated elongated root length and impaired seed set phenotypes (Chung et al. 2009, Chung et al. 2010). This dominant-negative effect has been also observed in other JAZ proteins and supports the notion that alternatively spliced JAZ proteins that evade COI1 mediated degradation keep activated JA signalling in check (Chung et al. 2010).

1.5.2 MYC2 is the master TF in JA signalling

As mentioned, a large number of JA outputs rely on the bHLH TF MYC2 (Kazan et al. 2013). MYC2 function is largely, but not exclusively, redundant with MYC3 and MYC4. For instance, MYC3 and MYC4 act additively to MYC2 in transducing JA signalling (Fernandez-Calvo et al. 2011) and in driving gene expression for producing glucosinolates to restrict insect feeding (Schweizer et al. 2013). Recent work from our group shows that MYC2/3/4 also act additively in antagonising expression of *EDS1* (Qiu et al., in prep.). The high redundancy again raises the question of functional and regulatory specificity. Part of the answer might lie in the spatio-temporal expression of these proteins. While *MYC2* transcript is found predominantly in the root, *MYC3* and *MYC4* mRNAs are present in the aerial parts of the plant (Fernandez-Calvo et al. 2011, Gasperini et al. 2015). MYC5, a less regarded member of the MYC family, localises exclusively to the stamen where it acts together with MYC2/3/4 in stamen development (Qi et al. 2015). Temporally, MYC2 accumulation correlates with circadian clock connecting time-of-day regulation with the JA pathway (Shin et al. 2012). MYC2/3/4 homo- and heterodimerise with each other and interact with nearly all JAZ proteins, indicating that signalling specificity is not achieved via distinct protein interaction (Fernandez-Calvo et al. 2011, Chini et al. 2016).

A major step forward in understanding how JAZ proteins repress MYC was the successful crystallisation the JAZ9/MYC3 complex. When bound to JAZ9, MYC3 undergoes substantial conformational changes and, consequently, cannot interact with its transcriptional activator MED25. Thus, JAZ9/MYC3 interaction provides a molecular switch that allows changing between transcriptional repression and activation (Zhang et al. 2015).

For a detailed discussion of SA-JA crosstalk during plant immunity please refer to chapter 3.3.

1.6 Thesis aims

In an evolutionary arms race plants have evolved sophisticated defence mechanisms against a plethora of pathogens. In turn, pathogens have evolved strategies to maintain and/or enhance their virulence. In this context, the EDS1 signalling node represents a central convergence point for basal and TNL-triggered immunity which acts in signal relay between activated NLRs and the transcriptional reprogramming machinery. Despite new mechanistic insights, our understanding of how EDS1 family proteins ensure a robust and strong immune response remains limited.

Antagonistic crosstalk between SA and JA is well established and distinct roles for SA and JA have been identified in context of plant immunity (Robert-Seilaniantz et al. 2011, Pieterse et al. 2012, Thaler et al. 2012). Recent work in our group revealed a molecular basis for this antagonism that connects EDS1 to the JA master TF MYC2. MYC2 was found to interact with all EDS1 family proteins and to repress *EDS1* promoter activity, leading to enhanced COR dependent *Pst* virulence (Cui et al. 2018, Qiu et al., in prep.). Similarly, we could show that during ETI, EDS1 represses MYC2 outputs, thus attenuating COR dependent virulence and reinforcing SA dependent defence (Cui et al. 2018). These data reveal that a central immune node intervenes with hormone crosstalk.

I started this work knowing that EDS1 and MYC2 were functionally connected. However, the nature of this relationship and its molecular basis were unclear. I decided to focus on EDS1 and its regulation of MYC2 and divided my approach in two parts. First, I tested systematically if EDS1 family members altered MYC2 accumulation on protein and transcript levels and if so, how this affected MYC2 transactivation activity. Second, I aimed to identify and functionally characterise the mechanism(s) by which EDS1 regulates MYC2 activity. Specifically, I analysed MYC2 regulation by repressor proteins and MYC2 PTMs (post-translational modifications).

2 Results

This thesis is divided into two sections. Section 2.1 addresses if and how the EDS1 protein family impacts MYC2 transcript, protein, and target genes. To test this, I first characterised and then employed the Arabidopsis mesophyll protoplast system to probe MYC2 mRNA and protein levels when co-expressed with EDS1, PAD4, and/or SAG101. To test correlation of MYC2 abundance with its transactivation activity I tested MYC2 regulated genes.

The aim of section 2.2 was to find a mechanistic explanation for EDS1/PAD4 dependent MYC2 regulation. To this end I analysed MYC2 dimerisation properties, JAZ proteins, and MYC2 post-translational modifications.

2.1 Impact of EDS1 family proteins on MYC2 abundance and transactivation activity

2.1.1 Arabidopsis protoplasts are responsive to avrRPS4 and COR

Previous work in the group had shown that EDS1, PAD4, and SAG101 can interact with MYC2 *in vitro* (Cui et al. 2018). To assess whether EDS1 family proteins modify MYC2 abundance, *i.e.* transcript and/or protein accumulation I decided to use the established Arabidopsis protoplast system used in our group, which is based on the protocol published by the Sheen group (Yoo et al. 2007). The advantages of using this system are i) relatively high sample number with results in short time, ii) use of protoplasts of specific genetic background, and iii) intact Arabidopsis cell signalling in isolated cells. Figure 2.1A shows a simplified workflow of protoplast isolation. Inevitably, the isolation procedure stresses cells and might alter cell signalling in unexpected ways. Therefore, I first tested whether protoplasts are still able to induce a defence response by transforming the bacterial effector protein avrRPS4 into *eds1-2/pad4-1/sag101-2* (hereinafter called *eps*) cells. Upon infection, *Pst avrRPS4* triggers EDS1/PAD4 dependent ETI as shown by induction of marker genes such as *ICS1* and *PBS3*. Co-expression of EDS1-YFP, PAD4-YFP and avrRPS4-HA induced *ICS1* and *PBS3* significantly, while EDS1 and PAD4 alone, or together with the inactive effector variant avrRPS4^{KRVY} (Sohn et al. 2009) did not (Figure 2.1B).

Consequently, isolated cells induce ETI-like signalling upon transient gene expression making the protoplast system a potent mimic of *in planta* conditions. To ensure similar transformation efficiency I adjusted the transformed plasmid volumes if necessary with "neutral" *35S:GUS* plasmid between samples.

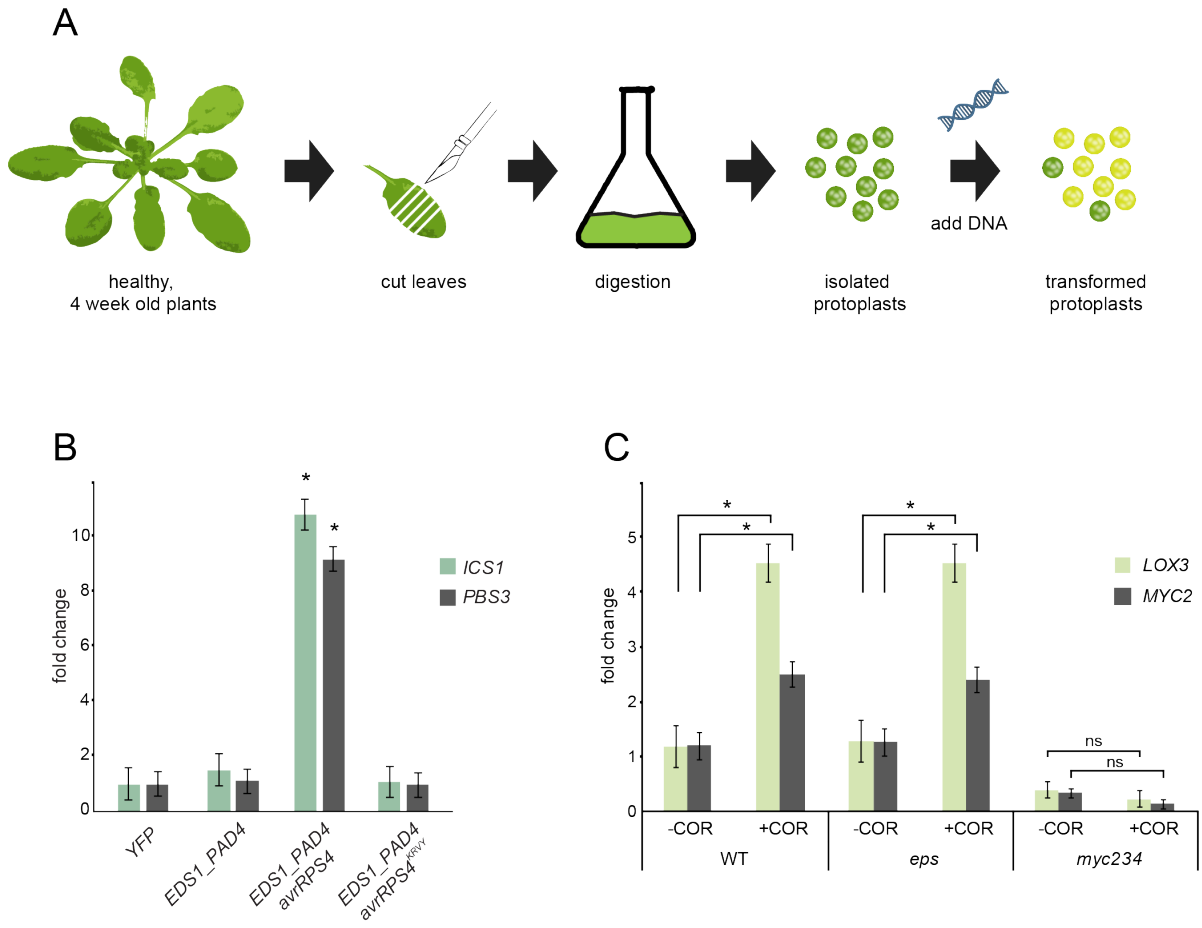


Figure 2.1 Protoplast isolation and characterisation.
(A) Schematic workflow of protoplast isolation from Arabidopsis leaves. 3 - 4 leaves from healthy, 4-week-old plants were cut with a scalpel, digested with cellulase and macerozyme, filtered, washed and transformed via PEG to obtain transiently transformed cells.
(B) *eps* protoplasts initiate ETI-like signalling if transformed with EDS1-YFP, PAD4-flag, and avrRPS4-HA, but not if transformed with EDS1-YFP and PAD4-flag alone, or with inactive avrRPS4^{KRVY}-HA. RNA was extracted 14 h after *eps* protoplast transformation. Relative transcript of *ICS1* and *PBS3* was determined by qRT-PCR and normalised to *ACTIN2*. Asterisks indicate statistical difference (mixed linear model, adjusted p-value≤0.001). Error bars represent normalised mean standard error. Data from three independent experiments.
(C) WT and *eps* protoplasts respond to 2 µM COR treatment. *myc234* protoplasts served as negative control. -COR are MOCK (DMSO) treated samples. RNA was extracted 16 h after isolation with 2 h 2 µM COR treatment preceding harvesting. Relative transcript of *LOX3* and *MYC2* was determined by qRT-PCR and normalised to *ACTIN2*. Asterisks indicate statistical difference (mixed linear model, adjusted p-value≤0.001). Error bars represent normalised mean standard error. Data from three independent experiments. ns: not significant

Since protoplast isolation exerts physical stress on cells I wanted to assess if protoplasts are still responsive to exogenous JA stimulation, *i.e.* addition of COR. To address this, I tested JA marker gene expression of *LOX3* and *MYC2* with or without COR application in WT, *eps,* and *myc234* protoplasts. 2 h treatment with 2 µM COR significantly induced *LOX3* and *MYC2* expression in WT and *eps* cells, but not in *myc234* (Figure 2.1C) demonstrating that protoplasts are responsive to COR treatment.

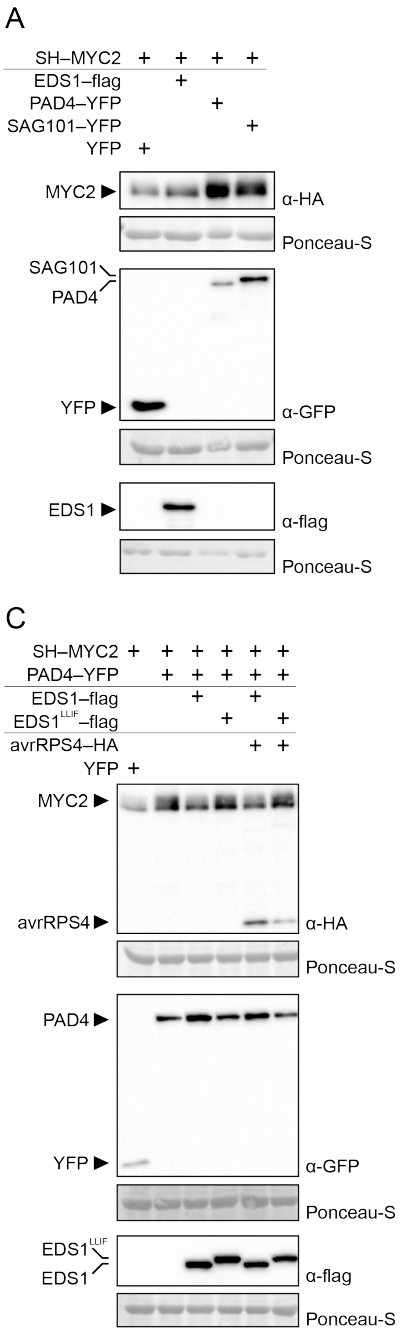
In summary, the obtained protoplasts induce ETI-like gene expression and react to COR treatment suggesting that the endogenous cell signalling network is intact and responsive.

2.1.2 PAD4 and SAG101, but not EDS1 increase MYC2 protein level

While all EDS1 family proteins interact with MYC2, the interaction of PAD4 or SAG101 with MYC2 is seemingly stronger than with EDS1 (Cui et al. 2018). To test effects of EDS1, PAD4, and SAG101 on basal MYC2 protein level I co-expressed SH-MYC2 with EDS1-flag, PAD4-YFP, or SAG101-YFP respectively and visualised protein amounts via immunoblotting. In presence of EDS1, MYC2 protein level was similar to a YFP control sample. Interestingly, in presence of PAD4 or SAG101 MYC2 was clearly more abundant (Figure 2.2A). This observation cannot be explained by transcript level, as native *MYC2* transcript was not affected by expressing YFP, EDS1-flag, PAD4-YFP, SAG101-YFP, EDS1-flag with PAD4-YFP, or EDS1^{LLIF}-flag, a heterodimerisation-deficient version with PAD4-YFP (Figure 2.2B). This suggests a post-translational effect of PAD4 and SAG101 on MYC2.

In context of ETI the formation of an EDS1/PAD4 heterodimer is necessary for intact defence signalling (Feys et al. 2001, Wagner et al. 2013). SAG101 has been shown to act largely redundantly with PAD4 (Feys et al. 2005) which is why the following experiments include PAD4, but not SAG101. So far, little is known about functions of EDS1, PAD4, (and SAG101) as monomers. This prompted me to test MYC2 protein levels in presence of the EDS1/PAD4 heterodimer in immune non-challenged (without avrRPS4) and immune challenged (with avrRPS4) cells. Co-expressing PAD4-YFP with EDS1-flag abolished the PAD4 promoting effect on MYC2 abundance while this was not the case with EDS1^{LLIF}-flag (Figure 2.2C), suggesting that heterodimer formation affects MYC2 protein accumulation. The addition of avrRPS4-HA had no effect (Figure 2.2C).

I conclude that PAD4 (and SAG101) stabilise MYC2 on protein level and that this stabilisation is impaired by EDS1/PAD4 heterodimer formation. This seems to be independent of the immune status of the cell, as this was observed in avrRPS4 expressing and non-expressing samples.



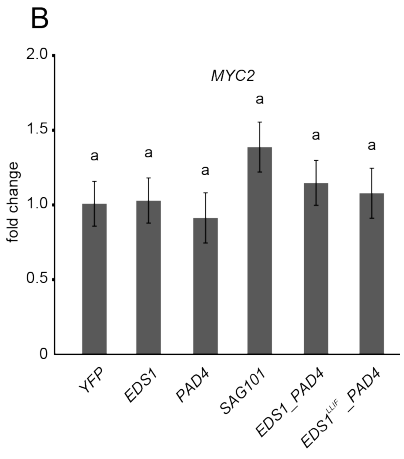


Figure 2.2 PAD4 and SAG101 stabilise MYC2 protein but not *MYC2* transcript.
(A) SH-MYC2 protein accumulation in *eps* protoplasts co-expressed with EDS1-flag, PAD4-YFP, or SAG101-YFP 14 h after transformation. YFP served as control. Note how PAD4 and SAG101, but not ESD1 stabilise MYC2.
(B) Native *MYC2* transcript is unchanged by transient expression of EDS1 family proteins. Relative transcript of *MYC2* was determined by qRT-PCR normalised to *ACTIN2*. Different letters indicate statistically significant differences (mixed linear model, adjusted p-value≤0.01). Error bars represent normalised mean standard error. Data from two independent experiments.
(C) EDS1-PAD4 heterodimer formation abolishes PAD4 stabilisation of MYC2. SH-MYC2 protein accumulation in *eps* protoplasts co-expressed with EDS1-flag and PAD4-YFP or EDS1^{LLIF}-flag and PAD4-YFP. AvrRPS4-HA did not affect this.
(A,C) Ponceau-S staining shows equal sample loading. Experiments were repeated three times with similar results. SH: StrepII-3xHA.

2.1.3 PAD4 does not stabilise MYC2 via 26S proteasome or JAZ inhibition

To find a mechanistic explanation for the observed MYC2 levels I interrogated the role of the 26S proteasome. MYC2 is under strict control of the 26S proteasome and proteasome inhibition stabilizes MYC2 protein (Shin et al. 2012, Zhai et al. 2013, Jung et al. 2015). Thus, a simple explanation for high MYC2 amounts would be PAD4 protecting MYC2 from proteasomal degradation. As before, I expressed SH-MYC2 with PAD4-YFP, with PAD4-YFP and EDS1-flag, or with PAD4-YFP and EDS1^{LLIF}-flag, but added 50 µM MG132, a potent proteasome inhibitor. MG132 increased overall MYC2 levels, yet PAD4 still enhanced MYC2 abundance and addition of EDS1 counteracted this (Figure 2.3A). Thus, the observed MYC2 amounts are unlikely to be caused by changed 26S proteasome activity.

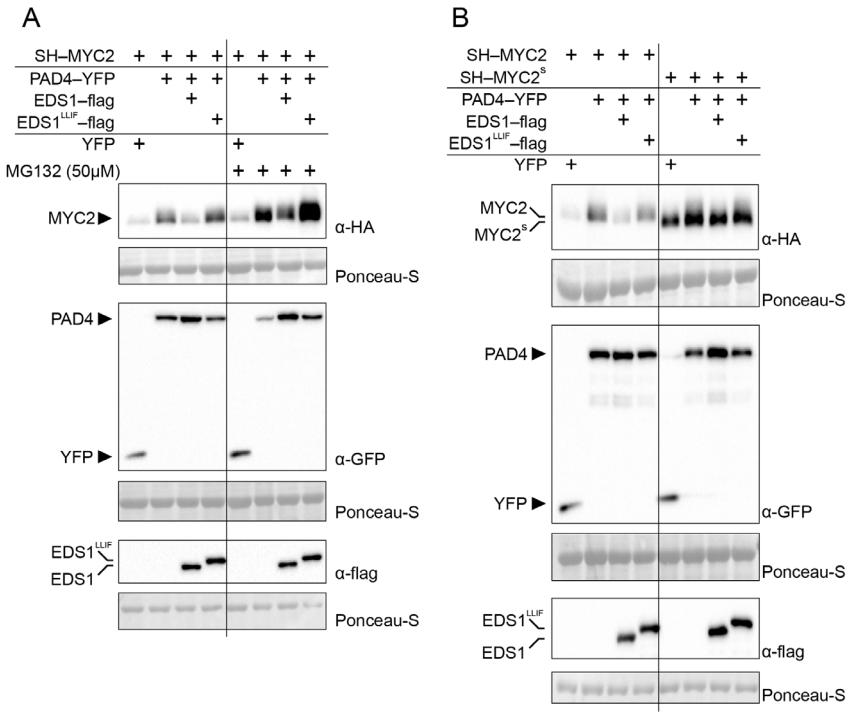


Figure 2.3 PAD4 does not stabilise MYC2 via 26S proteasome or JAZ protein regulation.
(A) SH-MYC2 protein accumulation in *eps* protoplasts co-expressed with PAD4-YFP, PAD4-YFP and EDS1-flag, or PAD4-YFP and EDS1^{LLIF}-flag, 14 h after transformation. YFP served as control. Addition of 50 µM MG132 (2 h) enhanced overall MYC2 protein levels, but did not change MYC2 stabilisation by PAD4 and EDS1 inhibition.
(B) EDS1/PAD4 dependent MYC2^{s} accumulation pattern resembles MYC2^{WT}.
(A,B) Ponceau-S staining shows equal sample loading. Experiments were repeated three times. SH: StrepII-3xHA.

Besides the 26S proteasome, JAZ proteins are well-known regulators of MYC2 (Chini et al. 2007, Kazan et al. 2013, Zhang et al. 2015). The single amino acid change D105N releases MYC2 form JAZ suppression and results in a gain-of-function MYC2 referred to as "super-MYC2", ($MYC2^{s}$) (Goossens et al. 2015). If PAD4 and/or EDS1 interfered with MYC2-JAZ regulation this JAZ-uncoupled MYC2 variant should behave different to $MYC2^{WT}$. Similar to MG132 treated samples, SH-$MYC2^{s}$ samples showed elevated MYC2 levels, yet the observed abundance pattern for $MYC2^{WT}$ was unchanged when co-expressed with various EDS1/PAD4 combinations (Figure 2.3B). The different MYC2 amounts can therefore not be explained by regulation of JAZ proteins. This means that quantity, but not quality of MYC2 accumulation depends on 26S proteasome and JAZ proteins.

2.1.4 EDS1 enhances MYC2 transactivation activity

Since MYC2 is a transcription factor mere transcript or protein abundance are of limited information when it comes to functional implications. Even very low levels of a TF can be biologically active (Cheng et al. 2007). To address this I used a transactivation assay in *eps* protoplasts that measures MYC2 activity. MYC2 activates expression of *LOX3* and *VSP1* and represses expression of *ORA59* (Lorenzo et al. 2004, Verhage et al. 2011). By adding SH-MYC2 alone or with EDS1-flag, PAD4-YFP, or EDS1-flag with PAD4-YFP I tested whether MYC2 activity was changed EDS1/PAD4 dependently. Compared to YFP, SH-MYC2 induced *LOX3* and *VSP1* levels and decreased *ORA59* levels providing functional proof for the assay. Intriguingly, co-expression of EDS1-flag and SH-MYC2 significantly increased *LOX3* and *VSP1* levels compared to YFP and SH-MYC2 suggesting a promoting effect of EDS1 on MYC2 (Figure 2.4A,B). This stimulation was not observed by co-expressing PAD4-YFP, or EDS1-flag with PAD4-YFP indicating an EDS1 specific feature, which is abolished by PAD4. EDS1 also enhanced the repressive function of MYC2 on *ORA59*, yet to similar levels as PAD4 or EDS1 with PAD4 did (Figure 2.4C). The results for *ORA59* are therefore less clear and should be interpreted with caution.

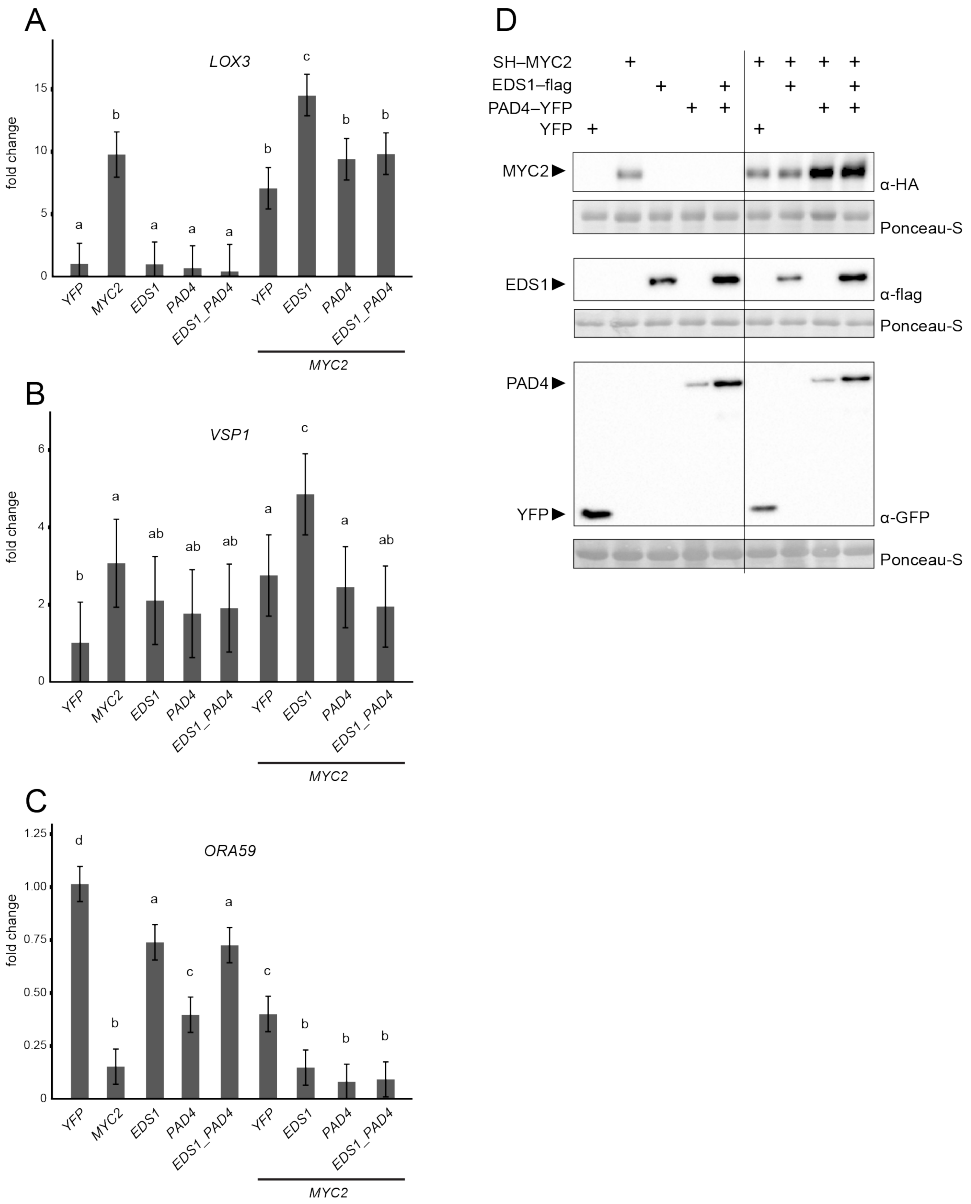


Figure 2.4 EDS1 promotes MYC2 transactivation activity.
MYC2 transactivation activity assay via qRT-PCR. SH-MYC2 was expressed separately, or together with YFP, EDS1-flag, PAD4-YFP, or EDS1-flag plus PAD4-YFP and *LOX3* (A), *VSP1* (B), and *ORA59* (C) transcript was measured 14 h after *eps* protoplast transformation. Different letters indicate statistically significant differences (mixed linear model, adjusted p-value<0.01 (A), adjusted p-value≤0.05 (B and C)). Error bars represent normalised mean standard error. Data from four independent experiments. (D) Immunoblot confirming expression of proteins in samples of (A-C). Ponceau-S staining shows equal sample loading. Note that high MYC2 transactivation activity in EDS1 co-expressed samples does not correlate with high MYC2 protein amounts. SH: StrepII-3xHA.

Testing protein accumulation confirmed proper protein expression showing that the observed changes in MYC2 transactivation activity are not due to protein amounts (Figure 2.4D).

Overall, this data demonstrates an EDS1 specific, positive function on MYC2 activity and is in contrast to the observed protein abundance described in Figure 2.2A were MYC2 was most abundant when co-expressed with PAD4. Consequently, MYC2 protein abundance and transactivation activity do not correlate, suggesting post-translational regulation.

2.1.5 EDS1 does not enhance MYC2^{s} transactivation activity

EDS1 could promote MYC2 activity by releasing it from JAZ suppression. To test this hypothesis I co-expressed YFP, EDS1-flag, PAD4-YFP, or EDS1-flag with PAD4-YFP with SH-MYC2^{WT} or with SH-MYC2^{s}. While EDS1 enhanced *LOX3* and *VSP1* levels when co-expressed with MYC2^{WT}, it failed to do so when co-expressed with MYC2^{s}. Consistent with the previous experiment, PAD4 and EDS1 with PAD4 did not affect expression (Figure 2.5A,B). *ORA59* was not tested. As EDS1 could not promote the activity of a JAZ uncoupled MYC2 this indicates that EDS1 releases MYC2 from JAZ suppression. Interestingly, *VSP1* expression was higher in all MYC2^{s} samples compared to MYC2^{WT} samples which is in line with a JAZ uncoupled MYC2. However, this was not true for *LOX3*, as expression was similar between MYC2^{s} and MYC2^{WT}. This suggests different regulatory pathways.

To rule that the absence of EDS1 promotion on MYC2^{s} was due to a lack of protein, I verified protein levels for all transformed constructs, *i.e.* YFP, EDS1-flag, PAD4-YFP, SH-MYC2^{WT} and SH-MYC2^{s}. In short, all constructs are expressed well and MYC2^{s} is more stable than MYC2^{WT} (Figure 2.5C). Again, protein amount does not correlate with transactivation activity and is therefore not indicative of functional impact.

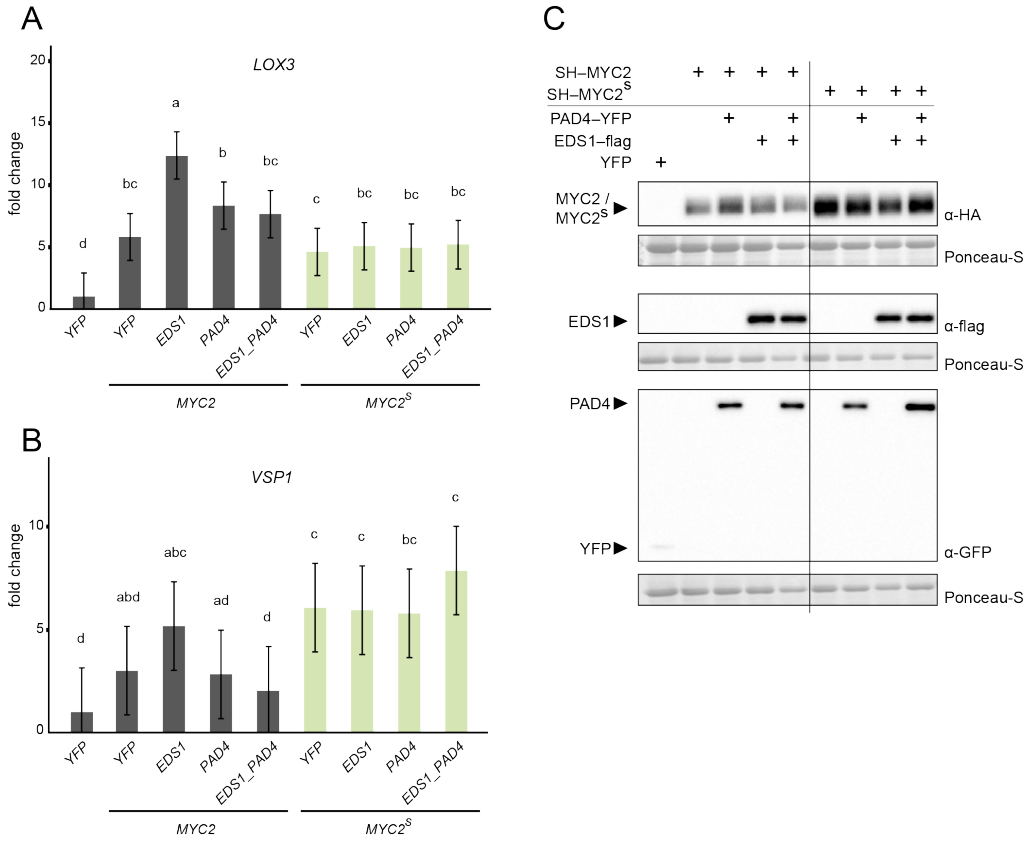


Figure 2.5 EDS1 does not promote MYC2^s transactivation activity.
MYC2 transactivation activity assay via qRT-PCR. SH-MYC2 or SH-MYC2^s was transformed with YFP, EDS1-flag, PAD4-YFP, or EDS1-flag plus PAD4-YFP and *LOX3* (A) and *VSP1* (B) transcript was measured 14 h after *eps* protoplast transformation. Note the overall enhanced *VSP1* levels in MYC2^s samples. Different letters indicate statistically significant differences (mixed linear model, adjusted p-value≤0.05). Error bars represent normalised mean standard error. Data from three independent experiments. (C) Immunoblot confirming expression of proteins in samples of (A) and (B). Ponceau-S staining shows equal sample loading. SH: StrepII-3xHA.

2.1.6 MYC2^s interacts with EDS1 family proteins

With MYC2^s not being promoted by EDS1 I hypothesised that MYC2^s might not interact with EDS1. To test this I expressed SH-MYC2^{WT} and SH-MYC2^s in *eps* protoplasts together with YFP, EDS1-YFP, PAD4-YFP, SAG101-YFP, or EDS1-flag with PAD4-YFP. As reported earlier (Cui et al. 2018), MYC2^{WT} co-immunoprecipitated (co-IPed) with all EDS1 family proteins. Interestingly, MYC2^s showed similar, yet weaker interaction (Figure 2.6).

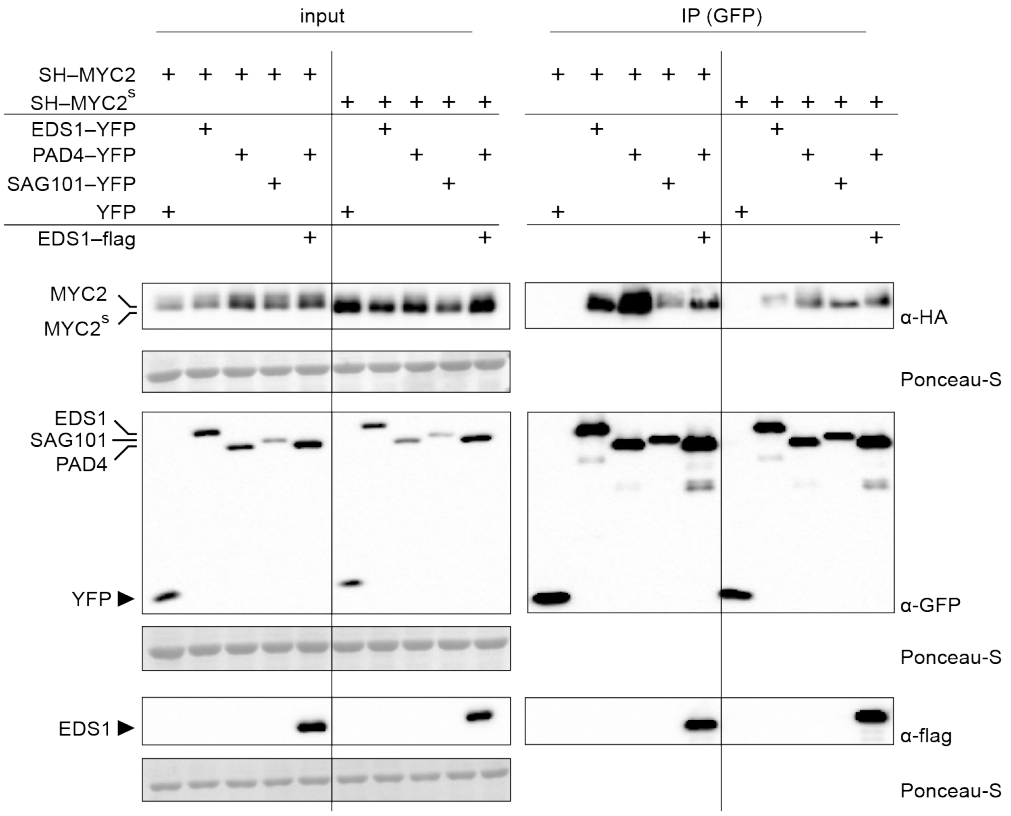


Figure 2.6 MYC2^s retains interaction with EDS1 family proteins.
Co-IP analysis of EDS1-YFP, PAD4-YFP, SAG101-YFP or PAD4-YFP plus EDS1-flag co-expressed with SH-MYC2 or SH-MYC2^s 14 h after transformation of *eps* protoplasts. GFP trap IP shows that MYC2^s retains interaction with EDS1 family proteins, but with weaker intensity than MYC2^{WT}. YFP served as control. Ponceau-S staining shows equal sample loading. SH: StrepII-3xHA. Performed thrice with similar results.

This is remarkable, as MYC2^s is more stable than MYC2^{WT} (see input), but co-IPs less, suggesting that the MYC2^{D105N} mutation affects MYC2-EDS1 interaction. Still, interaction is clear and is unlikely to be the cause for the lack of EDS1 dependent MYC2^s promotion.

2.1.7 AvrRPS4 abolishes the EDS1 promoting effect on MYC2 transactivation activity

Given the well-established SA/JA antagonism it was unexpected to find EDS1 promoting MYC2. Further, this is a rare case where EDS1 functions as a monomer and not in context of an EDS1/PAD4 heterodimer. This led me to test whether the results I obtained in pathogen unchallenged conditions were consistent in ETI-like conditions. Adding avrRPS4-HA to the previously described experiment indeed abolished EDS1 promotion of MYC2

transactivation activity (Figure 2.7A,B). As these experiments were done in *eps* protoplasts, only the last sample contains EDS1 and PAD4, *i.e*. the functional EDS1/PAD4 heterodimer, which is crucial for ETI signalling after avrRPS4 recognition. The fact that avrRPS4 changes MYC2 output in samples containing EDS1 alone indicates that avrRPS4 affects the EDS1-MYC2 relationship independent of EDS1/PAD4 dependent dimerisation and canonical ETI signalling. Again, I verified protein expression to ensure the lack of transactivation activity was not due to lack of protein (Figure 2.7C).

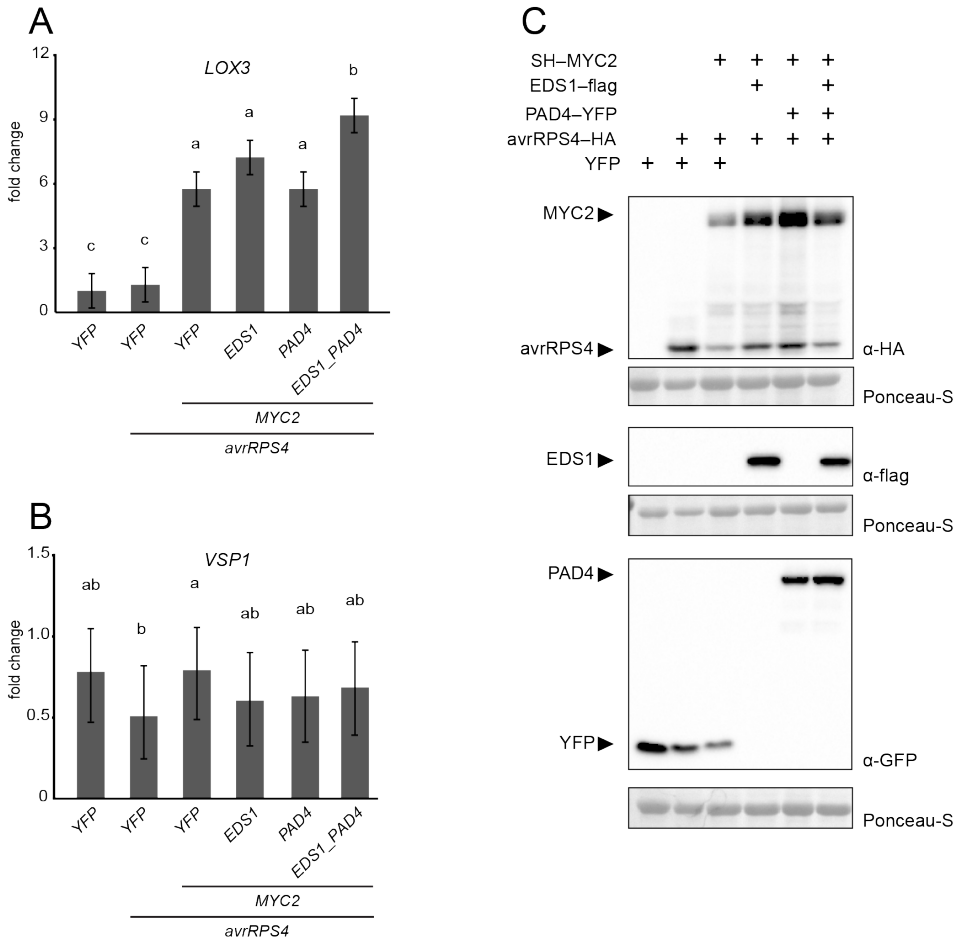


Figure 2.7 AvrRPS4 abolishes the EDS1 promoting effect on MYC2.
MYC2 transactivation activity assay via qRT-PCR. SH-MYC2 and avrRPS4-HA were co-expressed with YFP, EDS1-flag, PAD4-YFP, or EDS1-flag plus PAD4-YFP and *LOX3* (A) and *VSP1* (B) transcript was measured 14 h after *eps* protoplast transformation. YFP alone served as control. Note how in the presence of avrRPS4, EDS1 does not promote MYC2 transactivation activity. Further, MYC2 induced *LOX3* transcript, but not *VSP1* transcript. Different letters indicate statistically significant differences (mixed linear model, adjusted p-value≤0.05). Error bars represent normalised mean standard error. Data from three independent experiments. (C) Immunoblot confirming expression of proteins in samples of (A) and (B). Ponceau-S staining shows equal sample loading. Note that MYC2 transactivation activity does not correlate with high MYC2 protein amounts. SH: StrepII-3xHA.

2.1.8 EDS1 does not promote other TF classes

With EDS1 positively regulating MYC2 I wondered if this is specific to MYC2. Therefore I tested the effect of EDS1 on PIF4 (PHYTOCHROME INTERACTING FACTOR 4), like MYC2 a bHLH TF, which has not been described in EDS1 dependent immune signalling. *IAA19* is positively regulated by PIF4 and was used as a readout in this assay (Sun et al. 2013). Surprisingly, EDS1 also promoted PIF4 activity resulting in increased *IAA19* levels (Figure 2.8A).

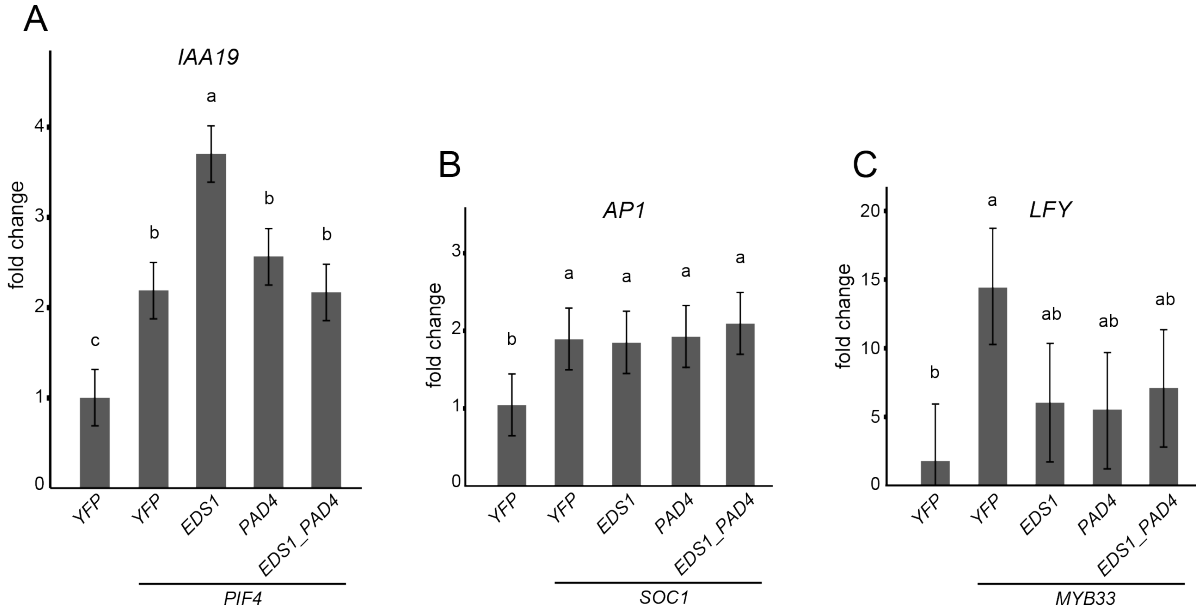


Figure 2.8 EDS1 promotes PIF4, but not SOC1 or MYB33 transcription activities.
(A) qRT-PCR analysis of SH-PIF4 co-expressed with YFP, EDS1-flag, PAD4-YFP, or EDS1-flag plus PAD4-YFP. *IAA19* transcript was measured 14 h after *eps* protoplast transformation.
(B) qRT-PCR analysis of SH-SOC1 co-expressed with YFP, EDS1-flag, PAD4-YFP, or EDS1-flag plus PAD4-YFP. *AP1* transcript was measured 14 h after *eps* protoplast transformation.
(C) qRT-PCR analysis of SH-MYB33 co-expressed with YFP, EDS1-flag, PAD4-YFP, or EDS1-flag plus PAD4-YFP. *LFY* transcript was measured 14 h after *eps* protoplast transformation.
(A-C) Different letters indicate statistically significant differences (mixed linear model, adjusted p-value≤0.05). Error bars represent normalised mean standard error. Data from three independent experiments.

Since PIF4 belongs to the same TF class as MYC2 the EDS1 promotion effect might apply to bHLH TFs in general. Therefore I decided to test two other classes of TFs, MADS-box TFs and MYB TFs. SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1) is a MADS-box TF, which leads to *AP1* expression, while MYB33 (MYB DOMAIN PROTEIN 33) increases *LFY* expression. Both SOC1 and MYB33 were not promoted by EDS1 indicating

specificity of EDS1 promoting activity to certain TFs or to bHLH TFs in general (Figure 2.8B,C).

2.1.9 MYC2 protein is elevated in *eds1-2* plants

Besides protoplast assays I wanted to quantify EDS1 dependent MYC2 protein levels *in planta*. MYC2 is regulated by the E3-Ubiquitin ligase PUB10 and was shown to accumulate after *Pst avrRPS4* infection (Jung et al. 2015, Cui et al. 2016). I used transgenic *myc2-3* or *myc2-3/eds1-2* Arabidopsis lines expressing C-terminally 3xflag tagged MYC2 under its native promoter (*pMYC2:MYC2-flag*). 4-week-old plants were either MOCK treated or infected with *Pst* (basal immunity), *Pst avrRPS4* (ETI), or *Pst avrRPS4* Δ*cor* (ETI, no COR). To bypass COR dependent effects on stomata aperture I infiltrated bacteria into the abaxial leaf side with a needleless syringe. Samples were taken at 24 hpi and protein was visualised by immunoblot. In *pMYC2:MYC2-flag, myc2-3* background MOCK samples accumulated low levels of MYC2, which increased after bacterial treatment, even in the *Pst avrRPS4* Δ*cor* sample (Figure 2.9A). In *pMYC2:MYC2-flag, myc2-3/eds1-2* background MYC2 generally accumulated to higher levels even in MOCK samples with highest levels in *Pst* and *Pst avrRPS4* samples. *Pst avrRPS4* Δ*cor* did not induce MYC2 (Figure 2.9A). From this data the following can be concluded: i) in *pMYC2:MYC2-flag, myc2-3*, *Pst* infection increases MYC2 protein which seems to be largely COR independent as *Pst avrRPS4* Δ*cor* induced MYC2 levels, too, ii) by removing EDS1 basal (MOCK treated) MYC2 increases, iii) in *pMYC2:MYC2-flag, myc2/eds1-2* MYC2 levels increase after infection mainly because of bacterial COR, as *Pst* and *Pst avrRPS4* strongly induced MYC2, while *Pst avrRPS4* Δ*cor* did not.

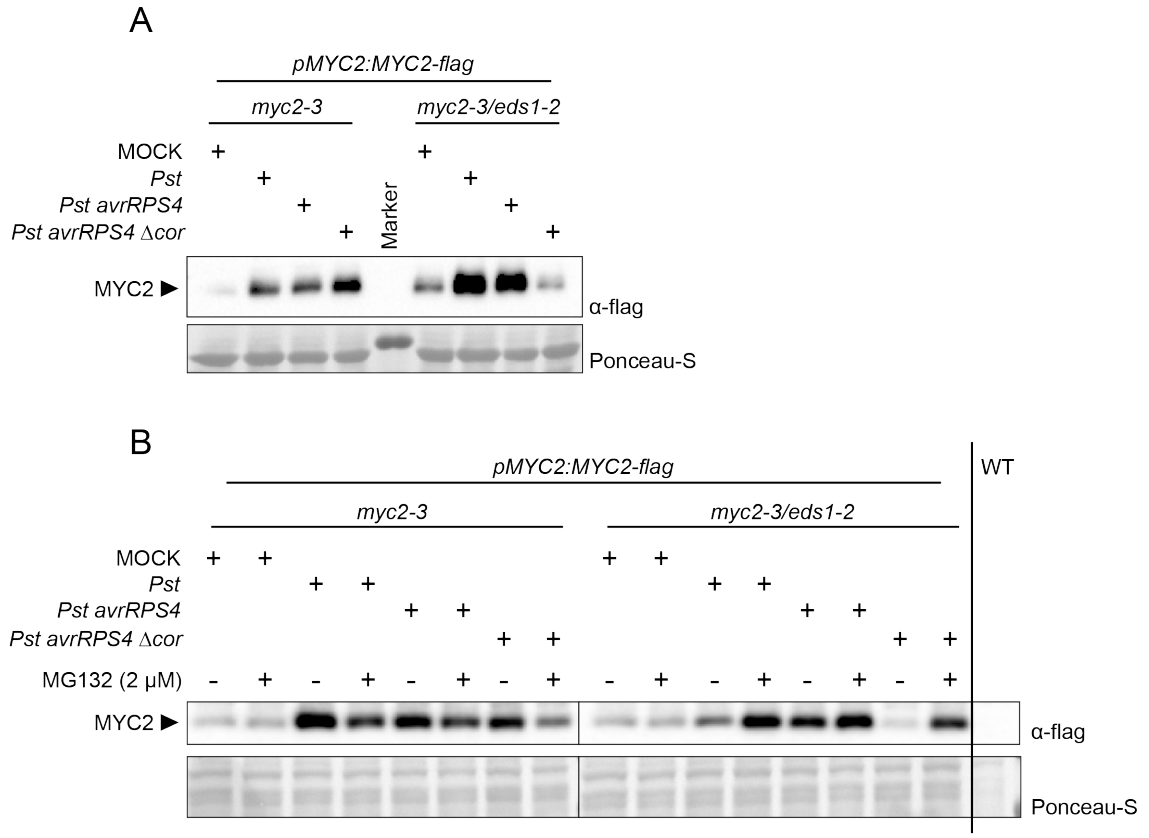


Figure 2.9 MYC2 levels depend on pathogen infection and presence of EDS1.
(A) MYC2-flag protein accumulation in transgenic *pMYC2:MYC2-flag myc2-3* or *pMYC2:MYC2-flag myc2-3/eds1-2* plants at 24 hpi with MOCK, *Pst*, *Pst avrRPS4*, or *Pst avrRPS4* Δ*cor*. 4-week-old plants were infiltrated with bacterial solutions (OD_{600}=0.002) and total protein was extracted for immunoblot analysis. Note i) overall increased MYC2 protein levels in *myc2-3/eds1-2* plants except after *Pst avrRPS4* Δ*cor* treatment ii) induced MYC2 after pathogen treatment except in *Pst avrRPS4* Δ*cor myc2-3/eds1-2*. Ponceau-S staining shows equal sample loading. Repeated four times with similar results.
(B) To the experimental setup from (A) DMSO or 2 µM MG132 was added to the infiltrated solution. MYC2-flag protein was visualized via immunoblot with similar results as in (A). Note how MG132 treatment decreased MYC2 stability in *myc2-3* plants, but increased MYC2 stability in *myc2-3/eds1-2* plants. Regardless of genetic background MOCK treated MYC2 levels were not changed by MG132. Repeated four times with varying results. One representative replicate is shown.

After establishing that MYC2 levels *in planta* at least partially depend on EDS1 I hypothesised that EDS1 might steer 26S proteasome activity to modulate MYC2. MYC2 is subject to proteasomal turnover and stabilised by MG132 (Shin et al. 2012). To test this I performed the same experiment, this time treating each sample with MOCK or 2 µM MG132. A total of 4 experiments showed high variation. Figure 2.9B is exemplary for the obtained results. Again, in *myc2-3* samples *Pst* infection increased MYC2 levels, but surprisingly MG132 lowered MYC2 levels. In *myc2-3/eds1-2* plants MG132 treatment led

to MYC2 stabilisation as previously reported. Therefore I conclude that in this setup EDS1 does influence proteasomal regulation of MYC2 although precise conclusions are difficult.

2.1.10 Summary section 2.1

Data presented in this section establishes a general regulation of MYC2 protein accumulation and MYC2 transactivation activity by EDS1/PAD4. Characterisation of the used Arabidopsis protoplast system showed that *eps* protoplasts respond to the bacterial effector avrRPS4 when co-expressed with EDS1 and PAD4, but not by mere overexpression of EDS1 with PAD4, or by replacing avrRPS4 with the inactive variant avrRPS4^{KRVY} (Figure 2.1B). Further, *eps* cells initiated JA signalling after COR treatment, indicating intact signal mechanisms and JA receptive cells (Figure 2.1C). By measuring protein accumulation of transiently expressed MYC2 I could show that PAD4 and SAG101, but not EDS1 stabilise MYC2 in protoplasts (Figure 2.2A). Native *MYC2* transcript was not affected by expression of EDS1 family proteins (Figure 2.2B). The fact that EDS1, but not the heterodimer deficient version EDS1^{LLIF} dampens the positive PAD4 effect on MYC2 (Figure 2.2C) indicates that this is a PAD4 specific function that is counteracted by EDS1. As MG132 treatment and use of MYC2^{s} showed no qualitative difference in MYC2 accumulation (Figure 2.3) it is unlikely that the observed differences are linked to 26S proteasome or JAZ activity.

Transactivation assays showed that relatively low levels of MYC2 in EDS1 samples were more active, suggesting that the presence of EDS1 is beneficial for MYC2 activity (Figure 2.4). This is supported by loss of activation of MYC2^{s} (Figure 2.5A,B). This lost promotion is not caused by lost interaction between EDS1 and MYC2^{s} although interaction was weaker than with MYC2^{WT} (Figure 2.6). Further, the immune status of the cell seems to influence EDS1 in its ability to modify MYC2 activity since addition of avrRPS4 abolished MYC2 promotion (Figure 2.7A,B). The function of avrRPS4 in this context is EDS1/PAD4 heterodimer independent, because *eps* protoplasts transformed with EDS1 or PAD4 alone showed similar results as protoplasts transformed with EDS1 and PAD4.

I further show that EDS1 can promote another bHLH TF, PIF4, suggesting that EDS1 might act TF class specifically (Figure 2.8A). This is supported by the fact that SOC1 and MYB33, a MADS-box and a MYB TF, respectively, were not promoted by EDS1 (Figure 2.8B,C).

In planta infection assays using transgenic *myc2-3* or *myc2-3/eds1-2* lines expressing *pMYC2:MYC2-flag* showed elevated MYC2 accumulation in MOCK treated *myc2-3/eds1-2* plants (Figure 2.9A). This seems contradictory to the promotion on transactivation activity, yet as I have shown previously, protein amount and protein activity do not always correlate (Figure 2.2, 2.4, 2.5, 2.7). Infection with *Pst* or *Pst avrRPS4* induced MYC2 which was stronger in *myc2-3/eds1-2* background (Figure 2.9A). Surprisingly, *Pst avrRPS4* Δ*cor* in *myc2-3* induced MYC2 even stronger, while in *myc2-3/eds1-2* the lack of COR was mirrored by low MYC2 levels. Adding the proteasome inhibitor MG132 generally had opposing effects. It reduced MYC2 accumulation in *myc2-3* background, but stabilised MYC2 in *myc2-3/eds1-2* plants (Figure 2.9B).

By unravelling a general role of EDS1 family members in MYC2 regulation the question arises how this takes place mechanistically and what the biological significance is.

2.2 Exploring mechanistic regulation of MYC2 by EDS1

Section 2.1 established a general regulatory link between EDS1/PAD4 and MYC2. The nature of this regulation depends likely on the immune status of the cell (unchallenged, basal, ETI), developmental stage, and time. While it is challenging to find the "right" setting to observe the specific regulation in the respective context, some basic regulatory features can be identified and tested. In this section I address three major ways of MYC2 regulation and test whether EDS1/PAD4 alter them. These include MYC2 homodimerisation, JAZ repression, and MYC2 post-translational modifications.

2.2.1 EDS1 does not change MYC2 dimerisation properties

For correct function MYC2 needs to dimerise and bind DNA. Given the positive effect EDS1 had on MYC2 transactivation activity (see Figure 2.4) I hypothesised that EDS1 might influence MYC2 dimerisation. I tested this by co-expressing SH-MYC2 with MYC2-flag in Arabidopsis *eps* protoplasts with YFP, EDS1-YFP, or EDS1-YFP, PAD4-YFP with avrRPS4-HA. The latter sample tells whether simulated ETI would affect MYC2 dimerisation. MYC2-flag co-IPed SH-MYC2 in all samples showing clear homodimerisation regardless of presence or absence of EDS1 or ETI signalling (Figure 2.10). Therefore I conclude that MYC2 dimerisation properties are not affected by EDS1 and are irrelevant for the observed regulation of MYC2 protein and its activity.

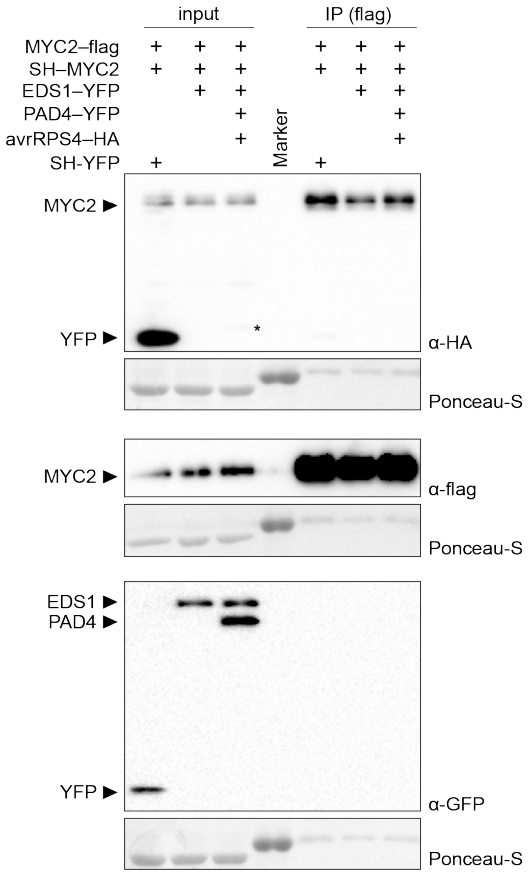


Figure 2.10 EDS1 does not interfere with MYC2 homodimerisation.
Co-IP analysis of MYC2-flag with SH-MYC2, with or without co-expressed EDS1-YFP, PAD4-YFP, avrRPS4-HA or SH-YFP. IP with flag beads 14 h after transformation of *eps* protoplasts. SH-YFP served as control. Asterisk indicates low avrRPS4 expression in input sample. SH: StrepII-3xHA. Ponceau-S staining shows equal sample loading. Performed twice with similar results.

2.2.2 EDS1 does not interact with JAZ9 or JAZ10

MYC2 activity is restricted by JAZ repressor proteins. Unpublished data from our collaborator Alain Goossens (VIB, Ghent, BE) indicated interaction between EDS1 and the majority of JAZ proteins, including JAZ9 and JAZ10, in Y2H (yeast-two-hybrid) assays (personal communication). To answer whether EDS1 interacts with JAZ proteins in Arabidopsis I co-expressed EDS1-flag with JAZ9-YFP or JAZ10-YFP. EDS1 did not IP JAZ9 or JAZ10, but strongly co-IPed PAD4 serving as positive control and giving context of interaction strength (Figure 2.11). Changing tags, the position of tags (N-terminal vs. C-terminal), or direction of IP had no effect on this (data not shown). Since I test only two JAZ members here, I cannot rule out that EDS1 interacts with other JAZ proteins.

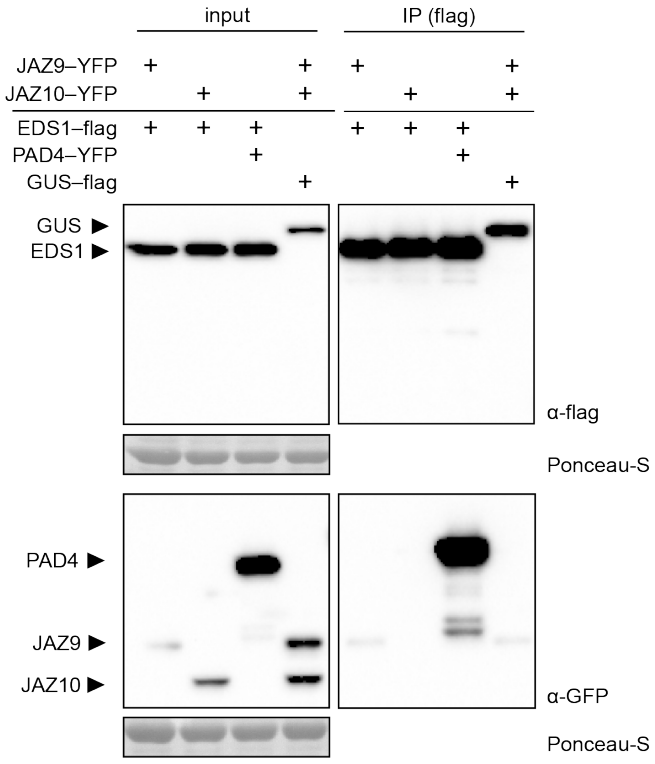


Figure 2.11 EDS1 does not interact with JAZ9 or JAZ10.
Co-IP analysis of EDS1-flag co-expressed with JAZ9-YFP, JAZ10-YFP, or PAD4-YFP. IP with flag-beads 14 h after transformation of *eps* protoplasts. GUS-flag served as control. EDS1 does not co-IP JAZ9 or JAZ10, but strongly binds to PAD4. Ponceau-S staining shows equal sample loading. SH: StrepII-3xHA. Performed thrice with similar results.

2.2.3 EDS1 does not change MYC2 ubiquitination

MYC2 is a master TF in JA signalling and has been studied extensively (Kazan et al. 2013). This is why numerous post-translational modifications have been discovered. Most well understood is the ubiquitin dependent proteasomal turnover by the 26S proteasome. Phosphorylation of MYC2^{T328} is necessary for maintaining MYC2 turnover and transactivation activity (Zhai et al. 2013) while the E3 ligase PUB10 polyubiquitinates MYC2 (Jung et al. 2015). The observed EDS1 promotion effect on MYC2 might be caused by high protein turnover ensuring a pool of active MYC2. To test whether altered MYC2 ubiquitination levels supported this I co-expressed MYC2-flag with YFP or EDS1-YFP in Arabidopsis *eps* cells. Samples with 10 µM MG132 or with GUS-flag instead of MYC2-flag served as controls. I performed flag IP to enrich protein and visualised ubiquitination in input and IP samples with a commercially available primary anti-ubiquitin antibody (see section 4.1.5 in Materials).

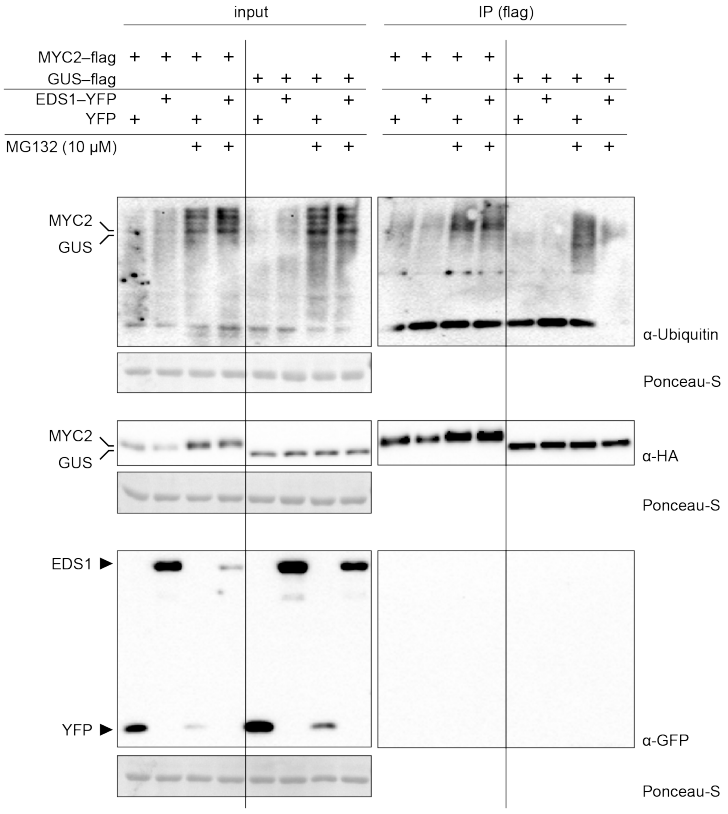


Figure 2.12 EDS1 does not affect MYC2 ubiquitination.
Immunoblot analysis of MYC2-flag expressed in *eps* protoplasts with or without EDS1-YFP and 10 µM MG132. YFP and GUS-flag served as controls. Protein was extracted 15 h after transformation and detected by anti-ubiquitin antibody. Note how MG132 treatment increases overall ubiquitination, but EDS1 does not change the MYC2 specific ubiquitination pattern. Ponceau-S staining shows equal sample loading. Performed thrice with similar results.

MG132 stabilised ubiquitination on MYC2, but I did not observe EDS1 dependent changes in the ubiquitination pattern (Figure 2.12).
While it is still possible that EDS1 affects ubiquitination of certain MYC2 residues only, I conclude that EDS1 does not change the overall MYC2 ubiquitination profile.

2.2.4 MYC2 is phosphorylated in an EDS1 dependent manner

Analysing my immunoblots of MYC2 I observed a weak second band at higher molecular weight (see Figure 2.2C, 2.3, 2.6). Given published information (Durek et al. 2010, Zhai et al. 2013, Sethi et al. 2014) I hypothesised this to be phosphorylated MYC2. To confirm this I co-expressed SH-MYC2 with YFP or PAD4-YFP in *eps* protoplasts and treated the extracted protein with lambda protein phosphatase (λ-PP) or phosphatase inhibitors (P-stop). I chose to test MYC2 in presence of PAD4 because PAD4 stabilises MYC2 protein simplifying protein detection. Separating the protein via regular SDS-PAGE was sufficient to resolve the slight migration changes caused by phosphorylation. Addition of λ-PP reduced the second band and caused the overall protein to run at lower molecular weight, indicating lost phosphorylation (Figure 2.13A). Addition of P-stop had the opposite effect (Figure 2.13A). This shows that MYC2 is phosphorylated in Arabidopsis *eps* protoplasts and that separation via regular SDS-PAGE is sufficient for resolving phosphorylation dependent migration patterns.

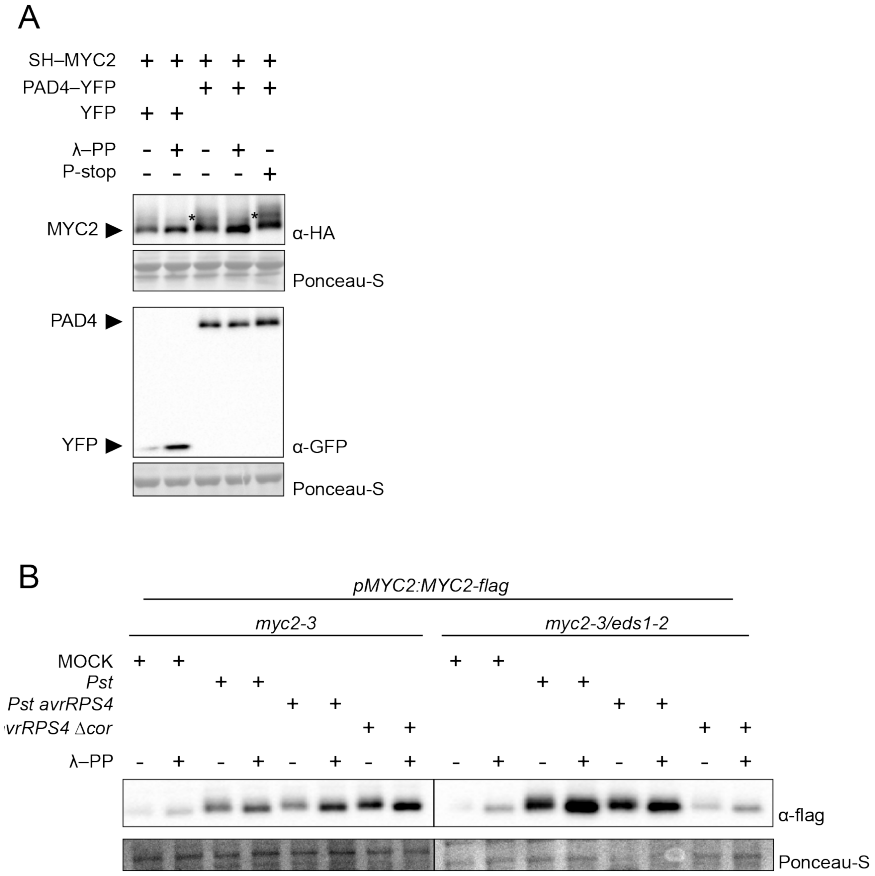


Figure 2.13 MYC2 is phosphorylated in *eps* protoplasts and transgenic plants.
(A) MYC2 phosphorylation assay of SH-MYC2 expressed in *eps* protoplasts for 14 h. Protein was separated and visualised by standard immunoblot analysis. PAD4-YFP was co-expressed to increase MYC2 accumulation. Note how λ-PP treatment decreases molecular weight. Asterisks indicate additional band of phosphorylated MYC2. λ-PP: lambda protein phosphatase; P-stop: phosphatase stop.
(B) MYC2 protein accumulation in transgenic *pMYC2:MYC2-flag myc2-3* or *pMYC2:MYC2-flag myc2-3/eds1-2* plants at 24 hpi with MOCK, *Pst*, *Pst avrRPS4*, or *Pst avrRPS4* Δ*cor*. 4-week-old plants were infiltrated with bacterial solutions (OD_{600}=0.002) and total protein was extracted, treated with or without λ-PP and used for immunoblot analysis. Neither infection, nor genotype (*myc2-3* vs. *myc2-3/eds1-2*) affected the observed MYC2 phosphorylation.
(A,B) Ponceau-S staining shows equal sample loading. Performed thrice with similar results.

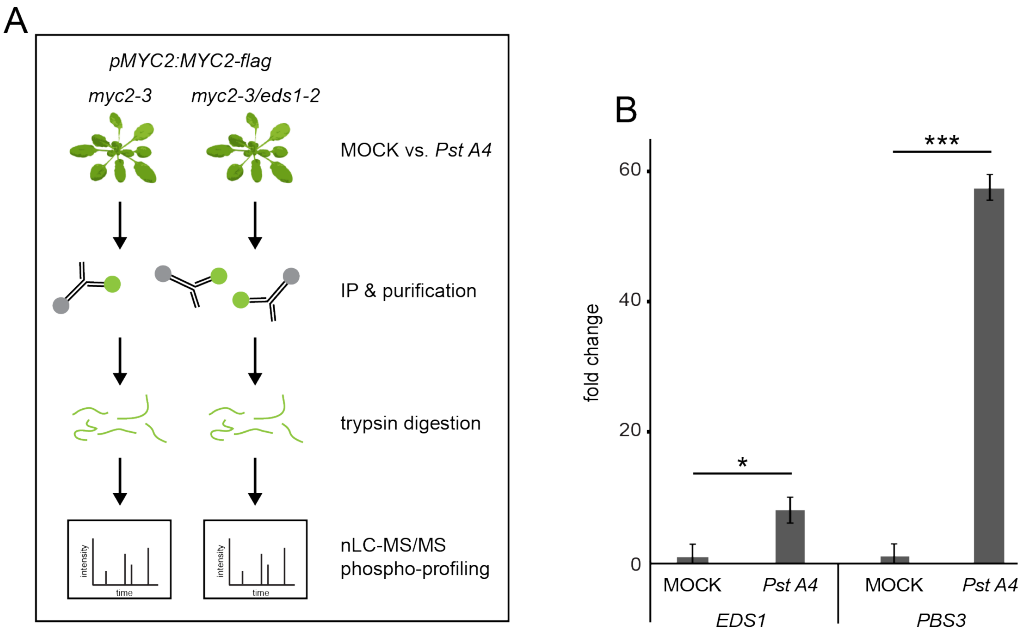
To connect MYC2 phosphorylation to EDS1 function I extracted MYC2 protein from *pMYC2:MYC2-flag myc2-3* or *myc2-3/eds1-2* plants infiltrated with MOCK, *Pst*, *Pst avrRPS4*, or *Pst avrRPS4* Δ*cor* (OD_{600}=0.002, 24 hpi). Protein was then split in a λ-PP treated and a non-treated fraction. I hypothesised that MYC2 phosphorylation would either depend on immune status of the plant, or presence/absence of EDS1. Again, λ-PP treatment caused the overall protein to run lower, but this was observed in all treatments (MOCK and

infection) and therefore not immunity related (Figure 2.13B). Further this seems to be independent of EDS1, as phosphorylation patterns were identical between *myc2-3* and *myc2-3/eds1* plants (Figure 2.13B). I therefore conclude that overall MYC2 phosphorylation is independent of pathogen infection and/or EDS1.

2.2.5 Mass spectrometry reveals EDS1 dependent phosphorylation of MYC2^{S123}

Testing global protein phosphorylation via SDS-PAGE is rather crude and modifications on single residues are easily missed or cannot be mapped. To investigate the role of EDS1 on MYC2 phosphorylation in more detail I designed an IP-nLC-MS/MS experiment. My goal was to create an EDS1 dependent *in planta* phosphorylation profile of MYC2 in pathogen challenged and unchallenged conditions. The experimental setup can be seen in Figure 2.14A. *pMYC2:MYC2-flag* plants with (*myc2-3*) or without EDS1 (*myc2-3/eds1-2*) were either MOCK or *Pst avrRPS4* (OD_{600}=0.1, 6 hpi) treated followed by protein extraction, MYC2-flag IP, purification, digestion, and nLC-MS/MS phospho-analysis. I chose a high bacterial load to ensure rapid and strong initiation of ETI and harvested samples 6 hpi in the hope to catch early, transient changes of MYC2 status. For feasibility, I focused on comparing MOCK and *Pst avrRPS4,* omitting *Pst* and *Pst avrRPS4* Δ*cor.* ETI was induced in harvested samples as shown by *EDS1* and *PBS3* induction in *pMYC2:MYC2-flag myc2-3* leaves (Figure 2.14B). Protein digestion, handling of mass spectrometer, and data analysis was performed by our MPIPZ core facility. Analysis showed a MYC2 sequence coverage of 47.2 % consisting of 22 unique peptides. Within the identified peptides, one amino acid was phosphorylated in an EDS1 dependent, but infection independent manner. MYC2^{S123} (hereinafter S123) was phosphorylated in both MOCK and *Pst avrRPS4* treated samples lacking EDS1 (for analytical details see section 4.2.3.6 in Methods) but was not phosphorylated in presence of EDS1. This suggests that EDS1 either removes, or prevents S123 phosphorylation. Upstream of S123 are two more serines, S121 and S122, that were also identified to be phosphorylated, although with a lower probability (Figure 2.14C). Interestingly, S123 lies in the JID (JAZ interaction domain) of MYC2 and is therefore an interesting candidate for EDS1 regulation on MYC2 via JAZ proteins (Figure 2.14D). If S123 had a crucial role in mediating the MYC2-JAZ regulation it should be phylogenetically conserved. To address this I aligned MYC2 protein sequences ranging from monocot species (*e.g. Zea mays*), to solanaceous species (*e.g. Solanum lycopersicum*), to *Brassicaceae* (*e.g.*

Arabidopsis thaliana). S123 was conserved in 5 out of 9 tested species, suggesting an important role across many, but not all tested plant species (Figure 2.14E).



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Position	Modified Peptide Sequence	Sample	Phosphorylation Probability
S121	YKGEEDKANPRRR(pS)SSPPFSTPADQEYRKKV	*myc2/eds1*	0.067
S122	YKGEEDKANPRRRS(pS)SPPFSTPADQEYRKKV	*myc2/eds1*	0.067
S123	YKGEEDKANPRRRSS(pS)PPFSTPADQEYRKKV	*myc2/eds1*	0.086

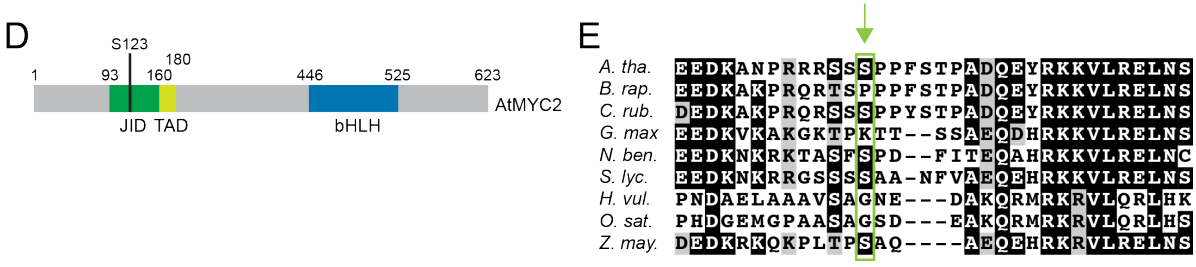

Figure 2.14 MYC2^{S123} is phosphorylated EDS1 dependently.
(A) Scheme of IP-nLC-MS/MS experimental setup. 4-week-old transgenic *pMYC2:MYC2-flag myc2-3* or *pMYC2:MYC2-flag myc2-3/eds1-2* plants were infiltrated with MOCK or *Pst avrRPS4* (OD_{600} = 0.1) and samples were taken 6 hpi. After flag IP and washing, protein extracts were trypsin digested and analysed for MYC2 phosphorylation by nLC-MS/MS. Three replicates from the same experiment were analysed, showing similar results.
(B) qRT-PCR analysis of *EDS1* and *PBS3* showing activation of immune signalling in sampled tissue. Statistical analysis with mixed linear model, * = adjusted p-value≤0.01, *** = adjusted p-value≤0.001. Error bars represent normalised mean standard error.

(C) Identified MYC2 peptide with EDS1 dependent phosphorylation pattern. S123 was phosphorylated only in absence of EDS1. *Pst avrRPS4* treatment did not have an effect. S121 and S122 were identified to be phosphorylated, too, but with a lower probability (for detailed cut-offs, FDR, and data analysis please see section 4.2.3.6 in Methods). Three replicates from the same experiment were analysed, showing similar results.
(D) Scheme of MYC2 protein domains. S123 lies in the JID. JID: JAZ interaction domain, TAD: transactivation domain, bHLH: DNA binding domain. Numbers indicate amino acid position.
(E) Phylogenetic conversation of AtMYC2^{S123} (highlighted box) in various species including *Poaceae* (monocots), *Solanaceae* and *Brassicaceae* (both dicots).

2.2.6 MYC2$^{S123A/D}$ retain interaction with EDS1 family proteins and JAZ9/10

With S123 lying in the JID and being partly conserved I decided to mutate this serine to phospho-mimic aspartate (D) or phospho-dead alanine (A) (Thorsness et al. 1987, Chen et al. 2015) and test putative functional implications.

I generated SH-MYC2^{S123A} and SH-MYC2^{S123D} variants. An approach to mutate S121, S122, and S123 altogether did not succeed (for discussion of this see section 3.2.3). First, I tested whether MYC2$^{S123A/D}$ retained interaction with EDS1 family proteins in *eps* protoplasts. Both SH-MYC2^{S123A} and SH-MYC2^{S123D} showed similar interaction like SH-MYC2^{WT} with strong interaction with PAD4-YFP and weak interaction with EDS1-YFP, SAG101-YFP or EDS1-flag/PAD4-YFP heterodimer (Figure 2.15, compare to (Cui et al. 2018)). Thus, changing S123 to alanine or aspartate does not affect MYC2 interaction with EDS1 family proteins.

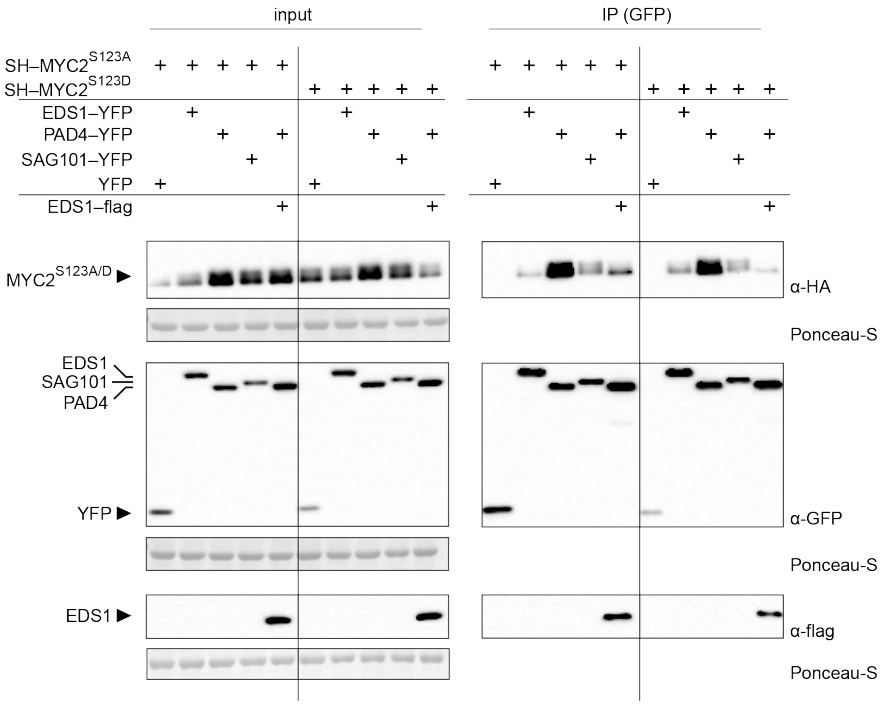

Figure 2.15 MYC2^{S123A} and MYC2^{S123D} retain interaction with EDS1 family proteins.
Co-IP analysis of SH-MYC2^{S123A} or SH-MYC2^{S123D} with EDS1-YFP, PAD4-YFP, SAG101-YFP, or PAD4-YFP plus EDS1-flag. Proteins were transiently expressed in *eps* protoplasts, harvested 14 h after transformation, and IPed using GFP-trap beads. YFP served as control. Interaction resembles MYC2^{WT} (Cui et al. 2018). SH: StrepII-3xHA. Ponceau-S staining shows equal sample loading. Performed twice with similar results.

Given that S123 lies in the JID I decided to test MYC2$^{S123A/D}$ interaction with JAZ proteins. As before I tested two JAZ members for MYC2 interaction, JAZ9 and JAZ10. Like SH-MYC2^{WT}, SH-MYC2^{S123A} and SH-MYC2^{S123D} were co-IPed by JAZ9-YFP and JAZ10-YFP, respectively (Figure 2.16). Therefore the S123A/D mutation does not affect MYC2 binding to JAZ9 or JAZ10, but could interfere with interaction of other JAZ proteins.

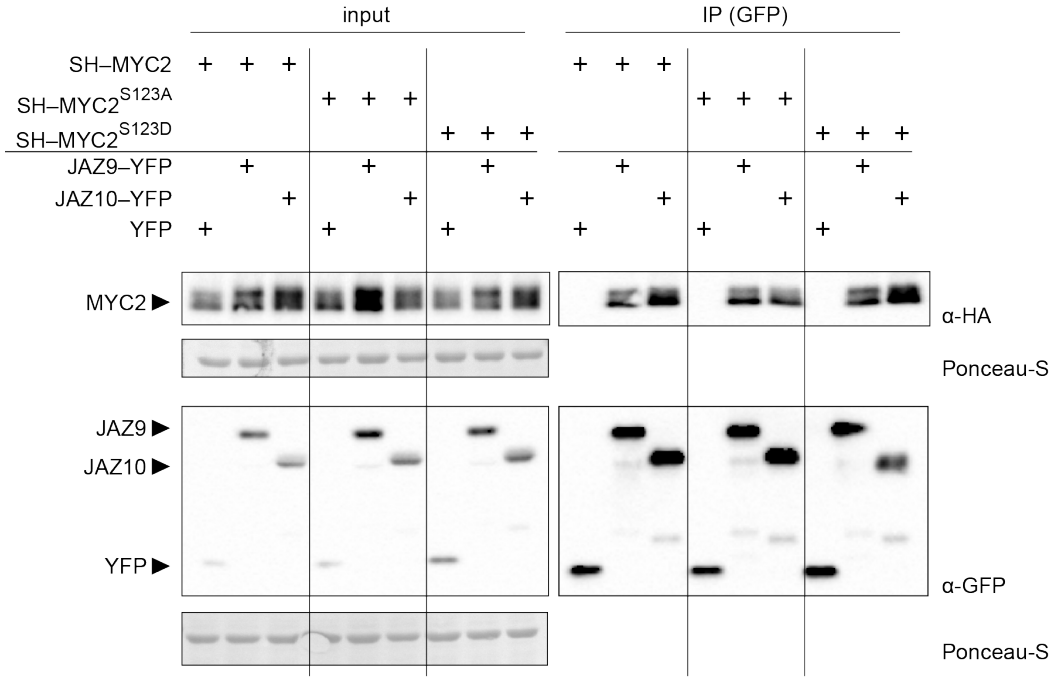

Figure 2.16 MYC2^{S123A} and MYC2^{S123D} retain interaction with JAZ9 and JAZ10.
Co-IP analysis of SH-MYC2, SH-MYC2^{S123A}, or SH-MYC2^{S123D} with JAZ9-YFP or JAZ10-YFP. Proteins were transiently expressed in *eps* protoplasts, harvested 14 h after transformation, and IPed using GFP-trap beads. YFP served as control. Like MYC2^{WT}, MYC2^{S123A} and MYC2^{S123D} interact with JAZ9 and JAZ10. SH: StrepII-3xHA. Ponceau-S staining shows equal sample loading. Performed twice with similar results.

2.2.7 MYC2$^{S123A/D}$ transactivation activity is changed

With protein-protein interactions unchanged I investigated whether MYC2$^{S123A/D}$ transactivation activity was still enhanced by EDS1. I transformed *eps* protoplasts with SH-MYC2^{WT}, SH-MYC2^{S123A}, or SH-MYC2^{S123D} and checked *LOX3* and *VSP1* transcript. MYC2^{WT} replicated the earlier observation of EDS1 promoting MYC2 outputs (Figure 2.17A,B). The same was true for MYC2^{S123D} (Figure 2.17A,B). Interestingly, EDS1 did not promote MYC2^{S123A} activity in the *VSP1* branch, but showed MYC2^{WT} like expression in the *LOX3* branch (Figure 2.17A,B). *LOX3* expression levels in YFP plus MYC2^{WT}, MYC2^{S123A}, and MYC2^{S123D} were similar, but for *VSP1* YFP plus MYC2^{S123A} samples showed elevated *VSP1* levels even in YFP co-expressed samples (Figure 2.17A,B). This suggests a higher basal activity of MYC2^{S123A} and might explain why EDS1 does not promote its activity further. This data indicates that *LOX3* and *VSP1* are differentially affected by S123A/D mutation, possibly because of different MYC2 signalling branches.

Again, I verified correct protein expression to exclude that the observed effects are caused by lack of protein (Figure 2.17C).

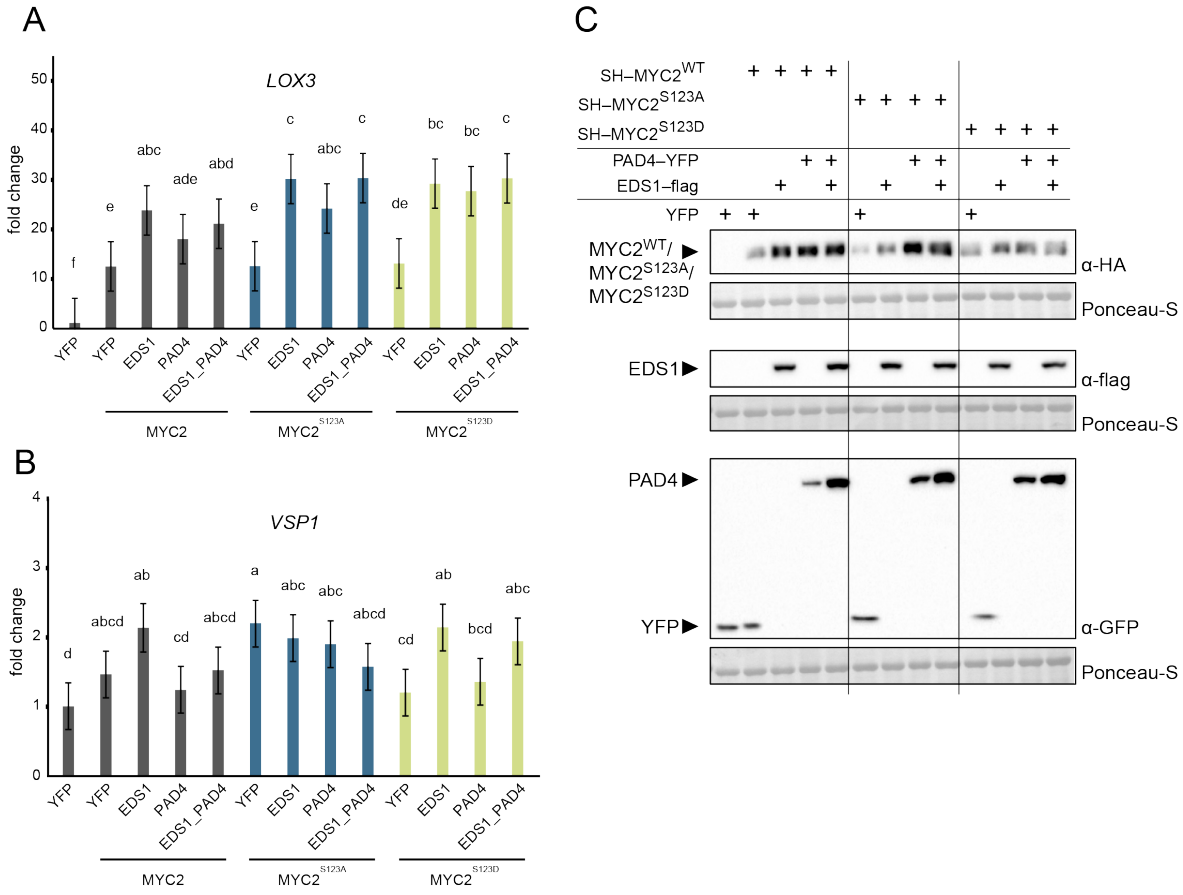

Figure 2.17 EDS1 does not promote the VSP1 branch of MYC2^{S123A}.
MYC2 transactivation activity assay via qRT-PCR. SH-MYC2^{WT} (grey bars), SH-MYC2^{S123A} (blue bars), or SH-MYC2^{S123D} (green bars) was expressed with YFP, EDS1-flag, PAD4-YFP, or EDS1-flag plus PAD4-YFP and *LOX3* (A), or *VSP1* (B) transcript was measured 14 h after *eps* protoplast transformation. Different letters indicate statistically significant differences (mixed linear model, adjusted p-value≤0.05). Error bars represent normalised mean standard error. (C) Immunoblot confirming expression of proteins in samples of (A,B). Ponceau-S staining shows equal sample loading. SH: StrepII-3xHA. Data from three independent experiments.

2.2.8 The protein kinase EDR1 interacts with MYC2

The fact that EDS1 prevents S123 phosphorylation suggests the involvement of protein kinases or phosphatases. Y2H data from Roger Innes' group indicated interaction of the protein kinase EDR1 (ENHANCED DISEASE RESISTANCE 1) with EDS1 and PAD4 as well as with MYC2 (personal communication). I decided to verify these observations in our Arabidopsis protoplast system. I used *eps* protoplasts to co-express YFP-EDR1 and SH-

MYC2 with or without EDS1-flag/PAD4-flag and with or without avrRPS4-HA. These combinations cover basal and immune activated cell states and address whether MYC2-EDR1 interaction relies on activated immune signalling or not. Regardless of presence of EDS1-flag/PAD4-flag or avrRPS4-HA, YFP-EDR1 co-IPed SH-MYC2 (Figure 2.18), confirming previous results from the Innes group. Therefore, the MYC2-EDR1 interaction is independent of EDS1/PAD4 presence and EDS1/PAD4 heterodimer activity.

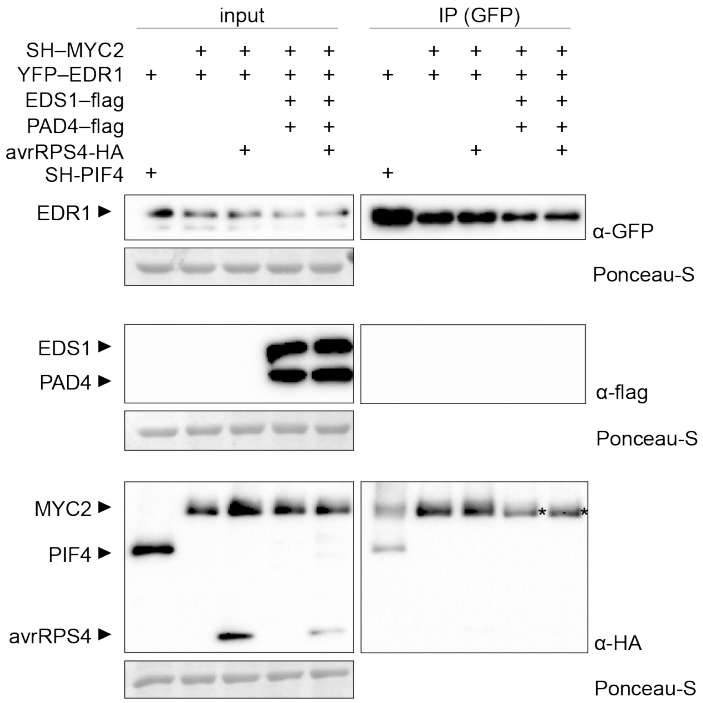

Figure 2.18 MYC2 interacts with EDR1.
Co-IP analysis of SH-MYC2 with YFP-EDR1 with or without EDS1-flag, PAD4-flag, and avrRPS4-HA. SH-PIF4 served as control. Proteins were transiently expressed in *eps* protoplasts, harvested 14 h after transformation and IPed using GFP-trap beads. Asterisks indicate MYC2 bands with lower molecular weight. Note: the PIF4 negative control seems contaminated here. In two additional, independent experiments this was not the case. However, in those replicates I did not observe the shifted MYC2 band when co-expressed with EDS1/PAD4. In total this experiment was performed thrice and showed consistently binding of EDR1 to MYC2. SH: StrepII-3xHA. Ponceau-S staining shows equal sample loading.

2.2.9 EDR1 interacts with PAD4, but not EDS1

To probe the relationship between MYC2, EDR1, and EDS1/PAD4 further I decided to test interaction of YFP-EDR1 with EDS1-YFP and with PAD4-YFP, respectively. Again, I co-expressed avrRPS4-HA to address putative differences in basal vs. ETI conditions. GFP IP

shows that SH-EDR1 is co-IPed by PAD4-YFP in presence or absence of avrRPS4-HA, but not by EDS1-YFP (Figure 2.19A). The same is true for reciprocal GFP IP, where YFP-EDR1 co-IPed PAD4-flag, but not EDS1-flag (Figure 2.19B). However, none of the samples contains EDS1 plus PAD4 meaning that avrRPS4 would not trigger EDS1/PAD4 heterodimer dependent ETI.

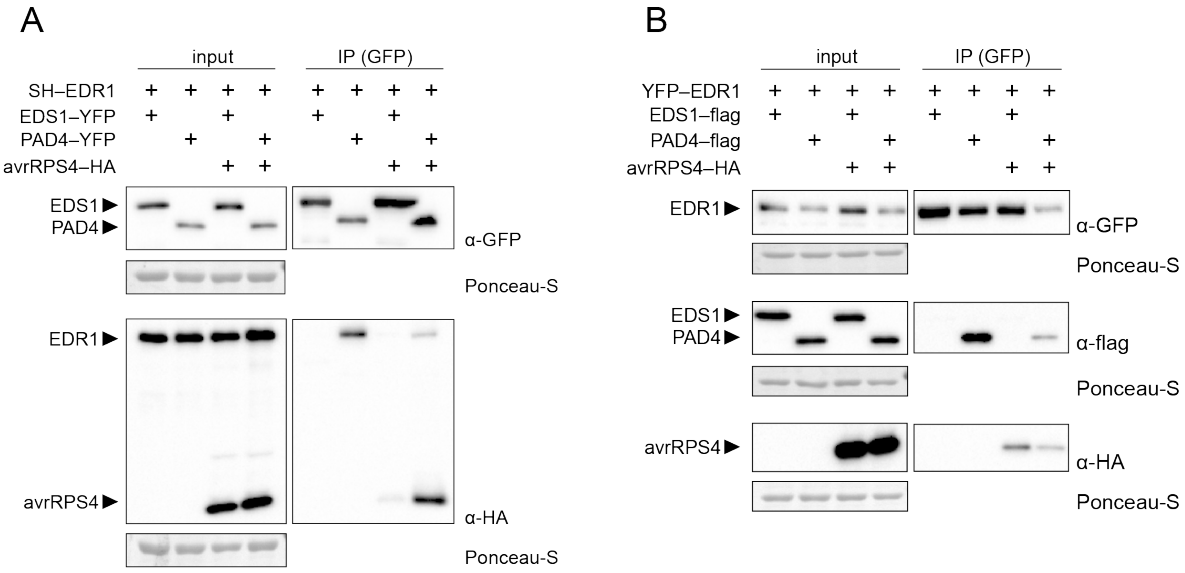

Figure 2.19 EDR1 interacts with PAD4, but not with EDS1.
(A) Co-IP analysis of SH-EDR1 with EDS1-YFP or PAD4-YFP, with or without avrRPS4-HA. Proteins were transiently expressed in *eps* protoplasts, harvested 14 h after transformation, and IPed using GFP-trap beads. SH: StrepII-3xHA. Ponceau-S staining shows equal sample loading. Performed three times with similar results.
(B) Reciprocal GFP-IP with EDR1-YFP, EDS1-flag, PAD4-flag, and avrRPS4-HA.

2.2.10 Overall MYC2 phosphorylation is independent of EDS1 or PAD4 and EDR1

With EDS1 not interacting with EDR1 a functional connection seems unlikely. However, PAD4 might act as a scaffolding protein that brings EDS1 and EDR1 in close proximity that could allow functional interaction. Next, I wanted to test the effect of EDS1/PAD4 on MYC2 phosphorylation in more detail. For this, I co-expressed SH-MYC2 with YFP-EDR1 alone, with EDS1-flag, with PAD4-flag, with EDS1-flag/PAD4-flag, with EDS1^{LLIF}-flag, or with EDS1^{LLIF}-flag/PAD4-flag. SH-SOC1 served as control. After GFP IP, samples were split in two fractions, treated with λ-PP or MOCK, and separated via SDS-PAGE. MYC2 co-IPed in all samples with EDR1. λ-PP treatment decreased MYC2 molecular weight as seen previously (Figure 2.20A), but this was independent of any EDS1-PAD4 combination.

In conclusion, EDS1 and PAD4 do not alter overall MYC2 phosphorylation in an EDR1 dependent manner in Arabidopsis protoplasts. Remarkably, here EDR1 did not interact with EDS1 or PAD4, which suggests MYC2 out-competing EDS1 and PAD4 for EDR1 binding.

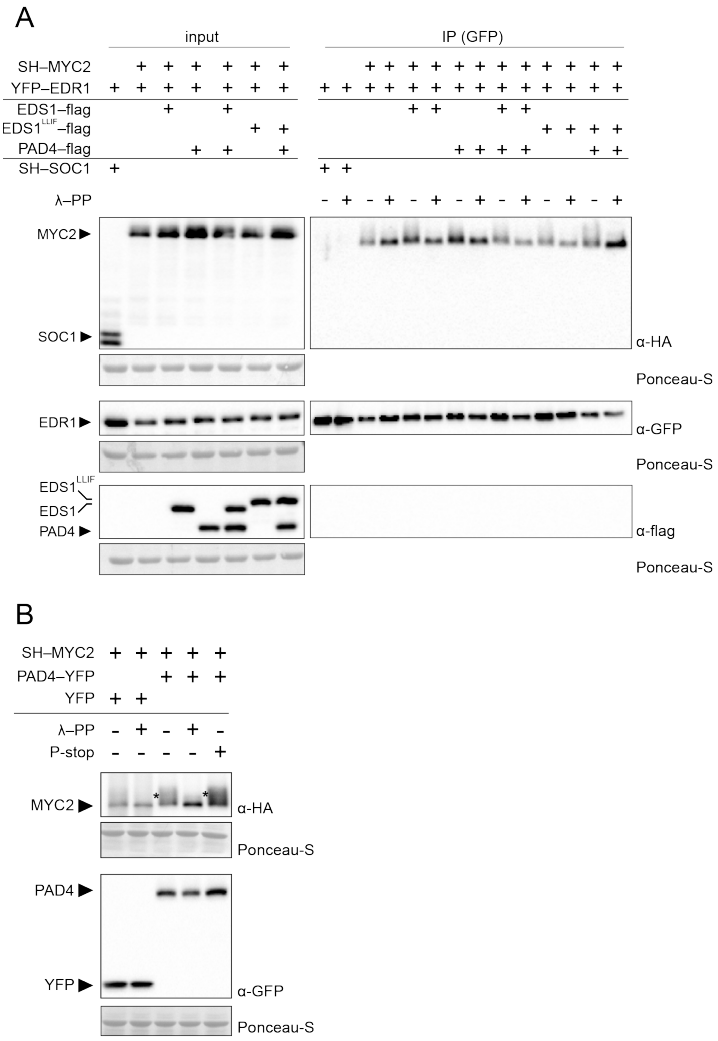

Figure 2.20 Overall MYC2 phosphorylation does not depend on EDR1 or EDS1 family proteins.
(A) Co-IP analysis of SH-MYC2 and YFP-EDR1 with or without EDS1-flag, PAD4-flag, or EDS1^{LLIF}-flag. SH-SOC1 served as control. Proteins were transiently expressed in *eps* protoplasts, harvested 14 h after transformation and IPed using GFP-trap beads. Beads were treated with λ-PP which resulted in lower MYC2 molecular weight. Co-expressing EDS1-family proteins did not affect this. SH: StrepII-3xHA. Ponceau-S staining shows equal sample loading. Performed three times with similar results.
(B) Identical experiment as 2.13A, but in *edr1* protoplasts. MYC2 phosphorylation in *edr1* and *eps* protoplasts is identical. Performed twice with similar results.

Figure 2.13A shows clear phosphorylation of MYC2 when expressed in *eps* protoplasts. If this phosphorylation was EDR1 dependent it should be lost in *edr1* protoplasts. Therefore I

repeated the experiment shown in Figure 2.13A using *edr1* protoplasts and tested SH-MYC2 phosphorylation in presence of YFP or PAD4-YFP and treated with λ-PP or P-stop. MYC2 phosphorylation was not affected by the *edr1* mutation indicating that the overall changes of MYC2 phosphorylation seen on SDS gels are not EDR1 dependent (Figure 2.20B).

Consequently, it is unlikely that EDS1 (and PAD4) regulate MYC2 phosphorylation via EDR1. While this holds true for overall MYC2 phosphorylation, it is still possible that EDR1 acts on specific MYC2 residues only and that this is regulated by EDS1.

2.2.11 Phospho-regulatory proteins are mis-expressed in *eds1-2* plants

EDR1 was an interesting candidate to test as it is a protein kinase reported to be a negative regulator of immunity that interacts with MYC2 and PAD4. However, EDS1 might target any other kinase, phosphatase or immunity-related gene that would lead to altered MYC2 phosphorylation. For further insights I made use of an available RNA-seq (RNA-sequencing) dataset described by our group earlier (Bhandari et al. 2019). I analysed RNA-seq data from Arabidopsis WT and *eds1-2* plants focussing on genes linked to phosphorylation or immunity. For statistical analysis a cut-off of |log2 FC| ≥1, and a FDR≤0.05 was used. Transcript of genes of interest like *MYC2*, *EDR1*, *FER* (*FERONIA*, see section 3.2.6 in discussion), *MKK6*, *MPK3/6*, *BIK1*, *CNI1* was unchanged. Genes repressed by EDS1 (transcript up in *eds1-2*) include *MAPKKK13, 15, 17*, *GSO1*, *JAZ9,* and *JAZ10* although the latter two were not statistically significantly changed. Genes promoted by EDS1 (transcript down in *eds1-2*) include *MKK4*, *MPK11*, *SERK4*, *WAK3*, *CRK7*, *CRK36*, *PBS3* and *PYL6* (Figure 2.21).

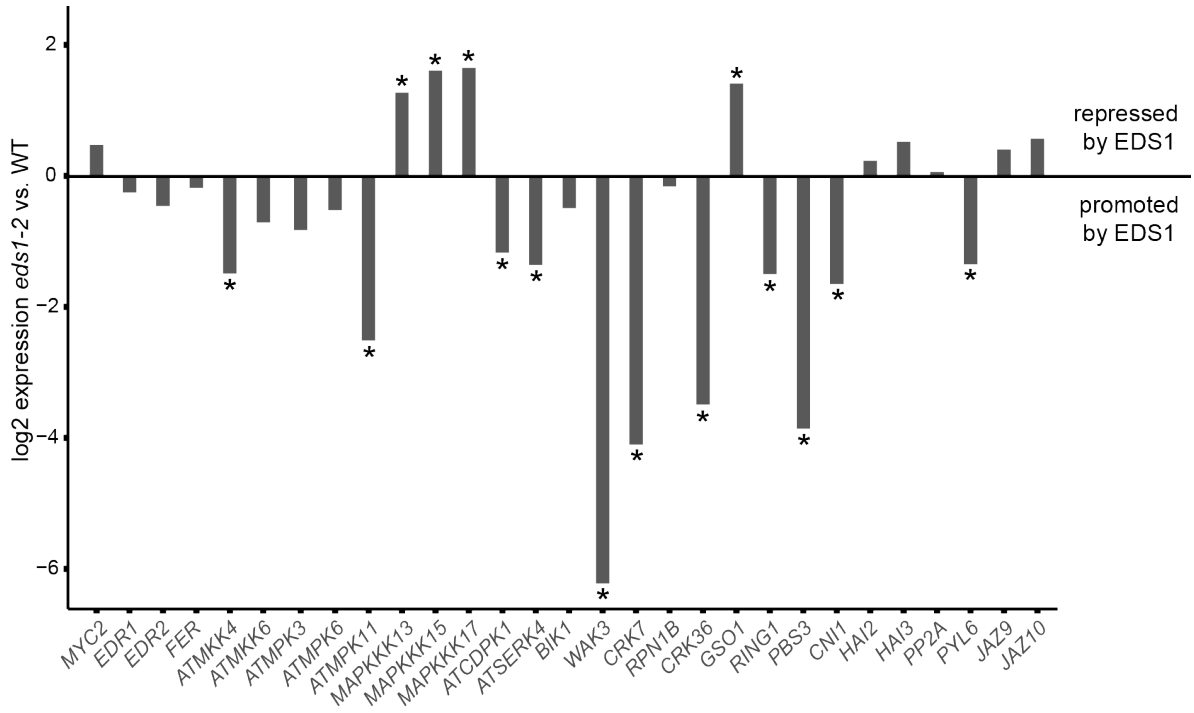

Figure 2.21 Numerous phosphorylation-related proteins are mis-regulated in *eds1-2* plants. RNA-seq data analysis of WT (Col-0) and *eds1-2* plants showing genes related to immunity and/or protein phosphorylation. Asterisks indicate genes passing filtering with a |log2 FC| ≥1, FDR≤0.05 cut-off.

While this list by no means is exhaustive, it does show that the transcript of numerous genes that are linked to protein phosphorylation or immunity is regulated by EDS1 and provides potential new candidates for further studies.

2.2.12 Summary section 2.2

The EDS1 dependent regulation of MYC2 transactivation activity shown in section 2.1 could be explained by various mechanisms. In this section I have demonstrated that EDS1 regulates MYC2 most likely via MYC2 phosphorylation, but not via MYC2 dimerisation (Figure 2.10) or MYC2 ubiquitination (Figure 2.12). To what extent JAZ proteins and 26S proteasome are involved needs further investigation.

MYC2 is known to be phosphorylated *in planta* (Heazlewood et al. 2008, Zhai et al. 2013, Sethi et al. 2014) and I observed phosphorylation of transiently expressed MYC2 in Arabidopsis protoplasts (Figure 2.13A, 2.20) as well as in plants treated with MOCK or *Pst* (Figure 2.13B). Mass spectrometry identified MYC2^{S123} to be phosphorylated in an EDS1 dependent manner, indicating direct or indirect regulation of S123 phosphorylation status by

EDS1 (Figure 2.14). This is of interest since S123 is positioned in the JID of MYC2 and is partly conserved (Figure 2.14). Mutation of S123 to phospho-mimic aspartate (D) or phospho-dead alanine (A) did not affect MYC2 interaction with EDS1 or PAD4 (Figure 2.15), or with JAZ9 or JAZ10 (Figure 2.16).

Testing the ability of EDS1 to promote $MYC2^{S123A/D}$ transactivation activity, I found that $MYC2^{S123A/D}$ was behaving like $MYC2^{WT}$ when testing *LOX3* levels (Figure 2.17A). Regarding *VSP1*, EDS1 enhanced $MYC2^{S123D}$ transactivation activity, but not $MYC2^{S123A}$ transactivation activity (Figure 2.17B). This suggests phospho-dependent, EDS1-dependent MYC2 regulation via S123 that might differ between distinct MYC2 signalling branches.

S123 being phosphorylated in the absence of EDS1 could be explained by EDS1 inhibiting a kinase or promoting a phosphatase. I demonstrate that PAD4, but not EDS1 binds the protein kinase EDR1 (Figure 2.19) and that EDR1 does bind MYC2 (Figure 2.18). These interactions are independent of avrRPS4, *i.e.* independent of the cell's immune status. However, I could not connect overall MYC2 phosphorylation to be regulated by EDR1. Also, my data do not suggest EDS1 (or PAD4) regulating EDR1 kinase activity (Figure 2.20A). The fact that overall MYC2 phosphorylation is unchanged in *eps* and *edr1* protoplasts (compare Figure2.13A and 2.20B) suggests that EDR1 does not affect the MYC2 phosphorylation pattern, but it is still feasible that EDS1 regulates EDR1 in order to control single residues like S123.

Comparing WT and *eds1-2* RNA-seq data allows to screen for genes of interest like kinases, phosphatases, or genes related to the 26S proteasome machinery or in general plant immunity in order to provide new potential candidates to test. Interesting candidates include FER, MKK4/11, MPK3/6, MAPKKK13/15/17, CDPK1, SERK4, WAK3, PYL6 and others (Figure 2.21).

3 Discussion

EDS1 and its interaction partners PAD4 and SAG101 are crucial proteins in plant immunity. Roles for EDS1 have been described in basal and TNL triggered immunity (Falk et al. 1999, Feys et al. 2001, Wiermer et al. 2005, Rietz et al. 2011). In context of TNL signalling EDS1 functions as a decision-making node, triggering SA dependent and independent pathways (Wiermer et al. 2005, Venugopal et al. 2009, Cui et al. 2016). While *eds1* or *pad4/sag101* plants are unable to transduce TNL signalling, many (but not all) CNLs act EDS1 independently (Wiermer et al. 2005, Xiao et al. 2005, Day et al. 2006, Bhandari et al. 2019). Why this is and if EDS1/PAD4 phylogenetic conservation across seed plants is important remains elusive. EDS1 interacts with various TNLs such as RPS4, RPS6, and SNC1 (Bhattacharjee et al. 2011), VICTR (Kim et al. 2012) and the effector protein avrRPS4 (Bhattacharjee et al. 2011, Huh et al. 2017) suggesting that EDS1 serves as a junction for further immune signalling. With its central role the EDS1/PAD4 node represents a worthwhile target for effectors in order to overcome plant immunity. Despite its importance, our understanding of EDS1 molecular function remains scarce.

Recently, our group employed a forward genetic screen in Arabidopsis to find components that are repressed by TNL-activated EDS1. To this end, EMS (ethane-methyl sulfonate)-mutagenised *eds1-2* plants were infected with *Pst avrRPS4* and screened for restored resistance. From ~650000 M2 plants, 12 independent mutations in *COI1* were found. *COI1* encodes a F-box protein that binds active JA-Ile or COR and initiates JA signalling (Sheard et al. 2010). Further analysis showed that *COI1* is required for COR dependent virulence of *Pst avrRPS4* and that TNL-activated EDS1 represses JA signalling. Bacterial growth was similar in *eds1-2/myc2-3*, and *eds1-2/coi1-41* plants, being intermediate between WT and *eds1-2*. This indicates that EDS1 represses COR induced bacterial virulence mainly by repressing the MYC2 branch of JA signalling. These experiments reveal a new intersection between ETI and JA signalling with ETI-activated EDS1 repressing MYC2 activity (Cui et al. 2018).

With MYC2 being a promising candidate to gain insights about the molecular function of EDS1, I decided to interrogate the relationship between EDS1 and MYC2 further. My aim for this thesis was to i) probe the effect of EDS1 family proteins on MYC2 accumulation and activity in non-triggered conditions, and to ii) find a mechanistic basis for EDS1

regulation of MYC2 activity. In the following section I will summarise my results, discuss their functional implications and their shortcomings, and put them in context of current literature. In the last paragraph I will discuss future experiments.

3.1 Impact of EDS1 family proteins on MYC2 abundance and transactivation activity

EDS1 represses the MYC2 branch of JA signalling during *Pst avrRPS4* infection, but not in non-infected conditions (Cui et al. 2018). However, all EDS1 family members interact with MYC2 in basal, *i.e.* non-infected conditions (Cui et al. 2018). While it is reasonable that an external trigger like avrRPS4 initiates EDS1 repression on MYC2, two observations are striking. First, the aforementioned interaction of EDS1, PAD4, and SAG101 with MYC2 in basal conditions and second, the difference in protein accumulation and the seemingly different interaction strength with strong interaction between MYC2 and PAD4 or SAG101 and weak interaction with EDS1 (Cui et al. 2018). If EDS1-MYC2 interaction is necessary and sufficient for EDS1 repression of MYC2 signalling remains unknown.

3.1.1 PAD4 and SAG101 stabilise MYC2 protein levels

To get a basic understanding of the EDS1-MYC2 relationship I co-expressed MYC2 with each one of the EDS1 family proteins in Arabidopsis *eps* protoplasts. Interestingly, MYC2 protein was stabilised by PAD4 and SAG101, but not by EDS1 (Figure 2.2A). This cannot be explained by elevated *MYC2* transcript levels, as native *MYC2* transcript was not affected by expression of EDS1 family proteins (Figure 2.2B). Further tests showed that formation of the EDS1/PAD4 heterodimer abolishes the PAD4 stabilising effect on MYC2 (Figure 2.2C). This data indicates that PAD4 (and SAG101) regulate MYC2 post-translationally. This was not changed by addition of avrRPS4, which induces ETI-like signalling when co-expressed with EDS1 and PAD4 (Figure 2.1B), and is therefore independent of the cells immune status in this transient assay (Figure 2.2C). Higher MYC2 accumulation in presence of PAD4 would explain the apparent stronger interaction observed by Cui et al. (2018). Noteworthy, PAD4 or SAG101 did not bind MYC2 in Y2H assays, indicating indirect interaction of these proteins (Cui et al. 2018). An interesting aspect is that EDS1, PAD4, and SAG101 interact with MYC2 in their monomeric, *i.e.* non-EDS1/PAD4 or EDS1-SAG101 heterodimer form (Cui et al. 2018) and here I show monomer specific effects on MYC2 protein accumulation (Figure 2.2A) and MYC2 transactivation activity (Figure 2.4, 2.5). So far, we know very

little about the function of EDS1 family proteins as monomers. Overexpression of either EDS1 or PAD4 does not lead to autoimmunity or defence priming, while constitutive expression of EDS1 with PAD4 does cause autoimmunity (Cui et al. 2016). For PAD4, numerous studies reported an EDS1 independent function in resistance signalling against green peach aphid (GPA). Detailed analysis showed that PAD4 is necessary and sufficient for proper resistance to GPA (Pegadaraju et al. 2005, Pegadaraju et al. 2007, Louis et al. 2012). Recent work in our group further showed that PAD4^{LLD}, an EDS1 non-interacting mutant, is fully functional in GPA resistance signalling, while being defective in ETI signalling (Dongus, J., personal communication). Thus, PAD4 acts distinctly different in resistance to GPA compared to ETI. EDS1 and PAD4 are best characterised in the context of ETI, where they act as a heterodimer and heterodimerisation-deficient mutants like *EDS1*^{LLIF} are non-functional during ETI (Feys et al. 2001, Wagner et al. 2013). Further, PAD4 did not stabilise MYC2 in presence of EDS1 (Figure 2.2C), indicating different functions of PAD4 and EDS1/PAD4 in context of MYC2 protein accumulation.

3.1.2 PAD4 specific MYC2 stabilisation is independent of proteasomal activity and JAZ repression

PAD4 could stabilise MYC2 by different means. Among other things, it could protect MYC2 from protein turnover by the 26S proteasome, or by releasing it from JAZ suppression. Proteasomal turnover of MYC2 had been observed before (Shin et al. 2012, Zhai et al. 2013) and had been confirmed by Jung et al. (2015) showing PUB10 dependent polyubiquitination and subsequent degradation of MYC2. Since neither MG132 treatment, nor use of the JAZ repressor uncoupled MYC2 variant MYC2^{s} (Goossens et al. 2015) showed qualitative difference in MYC2 stabilisation by PAD4 (Figure 2.3), I concluded that the observed differences in MYC2 accumulation have another cause.

3.1.3 EDS1 promotes MYC2 transactivation activity

While protein accumulation can be informative about a protein's function, protein amount and protein activity are not necessarily correlated (Lipford et al. 2005, Collins et al. 2006, Spoel et al. 2009). To understand the functional relevance of stabilised MYC2 by PAD4, I employed a qRT-PCR based transactivation activity assay. To my surprise, this assay showed that MYC2 activity, measured by relative expression of MYC2 target genes *LOX3*, *VSP1*, and *ORA59*, was promoted by EDS1, but not by PAD4, or by EDS1 with PAD4

(Figure 2.4A-C). This is in clear contrast to the observed MYC2 protein accumulation pattern in which PAD4, but not EDS1 promoted MYC2 accumulation (Figure 2.2A,C, 2.4D). Notably, MYC2 repression of *ORA59* was promoted by EDS1, PAD4, and EDS1 with PAD4 making the *ORA59* results more difficult to interpret. Nevertheless, the observed trend holds true for promotion of MYC2 transcriptional activation and repression functions. MYC2 is a TF and has been shown to rely on an "activation by destruction" mechanism (Zhai et al. 2013). In their work the authors hypothesise that constant destruction and subsequent *de novo* synthesis of MYC2 ensures a primed, "ready-to-act" protein pool. This could explain the discrepancy between high MYC2 protein accumulation and low MYC2 activity in presence of PAD4 and low MYC2 protein abundance, but high MYC2 activity in presence of EDS1. In this scenario, the accumulated MYC2 in PAD4 samples represents an inactive pool of MYC2, which either cannot be activated or has been activated earlier. The "activation by destruction" relies on phosphorylation of $MYC2^{T328}$ (Zhai et al. 2013) and I will discuss MYC2 phosphorylation more detailed in section 3.2.3.

3.1.4 $MYC2^{s}$ is not promoted by EDS1 but retains interaction with EDS1 family proteins

To find the mechanism behind MYC2 regulation by EDS1 I made use of the JAZ uncoupled $MYC2^{s}$ ($MYC2^{D105N}$, Goossens et al. 2015). My reasoning was that EDS1 might promote MYC2 activity by releasing it from JAZ suppression. If true, $MYC2^{s}$ should not be promoted by EDS1. Indeed, *LOX3* and *VSP1* levels were unchanged in $MYC2^{s}$ samples, but promoted by EDS1 in $MYC2^{WT}$ samples (Figure 2.5A,B). This was not caused by low protein expression (Figure 2.5C), or lost interaction of EDS1 with $MYC2^{s}$ (Figure 2.6). However, interaction with $MYC2^{s}$ was considerably weaker than with $MYC2^{WT}$, suggesting that the JID, and specifically $MYC2^{D105}$ is important for EDS1 protein family interaction with MYC2. It is conceivable that EDS1 and JAZ proteins share a common structural motif via which they competitively bind MYC2. Preliminary tests by my colleague Deepak Bhandari do not support this, but more detailed analysis will be needed to answer this definitely (Bhandari, D., personal communication).

Taken together, EDS1 is likely to steer MYC2 activity by interfering with MYC2-JAZ regulation. Another experiment to address this in more detail *in planta* relies on *myc2-3* or *eds1-2/myc2-3* transgenic lines expressing *35S:SH-*$MYC2^{WT}$ or *35S:SH-*$MYC2^{s}$. These lines

are being selected and results will hopefully be obtained in summer 2019. Please refer to Perspectives (section 3.4) for further notes on this.

When co-expressed with PAD4, EDS1 lost its promoting activity, resembling my previous observations that these results are specific to the monomeric protein and not to EDS1/PAD4 heterodimers.

3.1.5 Expression of avrRPS4 abolishes EDS1 promotion of MYC2

Since Cui et al. (2018) showed EDS1 repressing MYC2 in TNL-triggered immunity conditions my opposing observation of EDS1 promoting MYC2 transactivation activity (Figure 2.4) was puzzling. However, a major difference of the two analyses is the immune context of the experiment. EDS1 repressed MYC2 only in *Pst avrRPS4* infected tissues. In my assays cells are non-infected. Therefore I examined whether promotion of MYC2 by EDS1 would be affected by addition of the bacterial virulence factor avrRPS4. Co-expression of avrRPS4 with MYC2 and EDS1 indeed abolished EDS1 dependent MYC2 promotion (Figure 2.7). This shows that EDS1 regulates MYC2 differently depending on the immunity context and subsequently on the status of EDS1. An alternative explanation is that in presence of avrRPS4, EDS1 focuses on its heterodimer function with PAD4 and is therefore not available to promote MYC2. However, in this assay I use *eps* protoplasts, which means that transiently expressed EDS1 cannot heterodimerise with native PAD4 or SAG101. The observed differences in avrRPS4 expressing samples can therefore not be due to EDS1/PAD4 dependent ETI signalling, but are likely to account for an EDS1/PAD4 independent activity of avrRPS4. In terms of EDS1 independent functions little to nothing is known about avrRPS4. Full virulence function relies on proteolytic processing of the effector within the plant cell where both N- and C-terminal peptides contribute to virulence. Further, the N-terminal KRVY motif is essential (Sohn et al. 2009, Halane et al. 2018). AvrRPS4 interacts with WRKY TFs and likely targets them to promote bacterial virulence (Sarris et al. 2015), yet no biochemical activity has been found so far.

Notably, *LOX3* and *VSP1* showed different expression patterns in transactivation assays. While the EDS1 promoting effect holds true for both genes, *VSP1* was elevated in MYC2^{s} samples, consistent with a JAZ uncoupled MYC2, while *LOX3* was not (Figure 2.5A,B). Similarly, in presence of avrRPS4 *VSP1* was hardly induced by MYC2, but *LOX3* was strongly increased, although not promoted by EDS1 (Figure 2.7A,B). This suggests that

MYC2 outputs vary between different targets and provides a possibility for EDS1 specific regulation of certain, but not all MYC2 outputs.

Overall, these results suggest an EDS1 specific promotion of MYC2 transactivation activity that is related to JAZ proteins and affected by avrRPS4.

3.1.6 EDS1 specifically promotes bHLH TFs

EDS1 promoting MYC2 transactivation activity might be a generic feature of EDS1 and not limited to MYC2. To address EDS1 promotion specificity I tested if EDS1 affects PIF4 transactivation activity. PIF4 has been found to balance temperature sensitive plant growth and defence by repressing the plant immune system (Gangappa et al. 2016). Still, it was unexpected to find EDS1 promoting PIF4 (Figure 2.8A) as no direct functional connection between these two proteins had been reported. However, PIF4 and MYC2 are both class IIIe bHLH TFs suggesting a more general function of EDS1. This could mean that i) EDS1 is not promoting MYC2 by releasing it from JAZ suppression, ii) that EDS1 has different ways of promoting different bHLH TFs, or iii) that MYC2 and PIF4 are regulated by the same components. MYC2 and PIF4 belong to the type IIIe class of DNA binding bHLH TFs and dimerise in order to bind to DNA and initiate transcriptional reprogramming (Toledo-Ortiz et al. 2003, Kazan et al. 2013, Goossens et al. 2016). Our group could show via FRET-FLIM experiments that EDS1 associates with DNA, likely on chromatin level (Lapin, D., Bhandari, D., personal communication). Therefore it is feasible that EDS1 might regulate DNA associated TFs when encountering them at the DNA, or in general, making the chromatin more accessible for DNA-binding TFs. This is in line with data showing that EDS1 does not promote SOC1 and MYB33, both non-bHLH TFs (Figure 2.8B,C). In this context it would be interesting to test EDS1 dependent DNA methylation as DNA methylation and TF binding can be linked (Zhu et al. 2016). Another, maybe more likely explanation of EDS1 promotion of PIF4 is JAZ related. There is no evidence for direct regulation of PIF4 by JAZ proteins. However, analogous to MYC2 repression by JAZs, PIF4 is repressed by DELLA proteins, the key repressors of GA signalling (Sun 2011). Hou et al. (2010) have shown that DELLA proteins interact with JAZ1. This interaction sequesters JAZ1 away from MYC2, thereby activating MYC2 target genes like *LOX2*. With this interesting observation of JA pathway regulation by GA signalling components it seems possible that this regulation works *vice versa*, too. Hypothetically, by interacting with DELLA proteins, JAZ proteins could repress DELLAs and promote GA signalling. In *della*

quadruple or *pif4* mutants, JA inhibition of hypocotyl growth was significantly attenuated, suggesting that JA steers photomorphogenesis via DELLAs and/or PIF4 (de Lucas et al. 2008, Hou et al. 2010). It is tempting to speculate that JAZ proteins indeed release PIF4 from DELLA suppression. Assuming that EDS1 binds JAZs this would explain how EDS1 could promote both MYC2 and PIF4 transactivation activities. Further, this is in line with the notion that MYC2^{s} cannot be promoted by EDS1 because it is already uncoupled from JAZ suppression (Figure 2.5). A caveat of this model is that I could not detect interaction of EDS1 with two tested JAZ proteins, JAZ9 and JAZ10 (Figure 2.11). With these promising results it will be worth testing the remaining 11 JAZs (JAZ1-8, JAZ11-13) for interaction with EDS1 to identify the relevant ones.

My results further show that the mere over-expression of EDS1 with a TF does not lead to enhanced TF activity. The functional relevance of this observation is still to be tested. If EDS1 indeed promotes MYC2 activity in a biological meaningful manner this could be a means to fine-tune SA-JA crosstalk in basal and ETI conditions. My colleague Jingde Qiu showed that MYC2 inhibits *EDS1* expression by binding to the *EDS1* promoter. With this mutual regulation enhanced MYC2 activity might be a self-regulatory loop by which *EDS1* regulates itself (Qiu et al., in prep.).

3.1.7 MYC2 accumulates in *eds1-2* plants

While transient expression in Arabidopsis protoplasts has its benefits, *in planta* experiments are necessary to address functional implications of the observed results. Comparing MYC2 protein accumulation in a stable transgenic lines expressing *pMYC2:MYC2-flag* in *myc2-3* or *myc2-3/eds1-2* background revealed elevated MYC2 levels in i) EDS1 lacking lines (*myc2-3/eds1-2*), ii) all infected *myc2-3* lines, and iii) *Pst* and *Pst avrRPS4* infected *myc2-3/eds1-2* lines (Figure 2.9A). Elevated MYC2 levels in basal, *i.e.* MOCK treated *myc2-3/eds1-2* lines indicate a negative role of EDS1 on MYC2 accumulation even in basal conditions. This effect is consistent in *Pst* and *Pst avrRPS4* infected *myc2-3/eds1-2* lines, but not in *Pst avrRPS4* Δ*cor* infected tissue. One explanation for the high MYC2 levels in *Pst* and *Pst avrRPS4* and the low MYC2 levels in *Pst avrRPS4* Δ*cor* infected *myc2-3/eds1-2* lines is pathogen growth. In *eds1-2* plants, growth of avirulent bacteria is ~100-1000 fold higher than in WT plants (Feys et al. 2005). Numbers of bacteria producing COR is therefore higher, leading to higher MYC2 accumulation. Consequently, COR lacking *Pst avrRPS4* Δ*cor* does not induce MYC2 accumulation. The limitation of this model is that i) MYC2

was also enriched in MOCK treated samples, and ii) MYC2 is enriched in *Pst avrRPS4* Δ*cor* treated *myc2-3* background. Similar to data shown in Figure 2.2A and 2.4, high protein in MOCK treated *myc2-3/eds1-2* plants could be inactive, while low protein in *myc2-3* could represent an active protein pool. Therefore I propose a model in which MYC2 accumulation depends on EDS1 (basal and TNL-activated), bacterial growth, and COR. Which of these components is relevant for MYC2 functional outputs in specific conditions remains to be tested in more detail.

MYC2 accumulation at least partially depends on the 26S proteasome as MG132 treatment stabilised MYC2 levels in all *myc2-3/eds1-2* lines infected with *Pst*, but not in MOCK treated *myc2-3* lines (Figure 2.9B). This is consistent with published results where MYC2 protein level was elevated in *eds1-2* plants (Shin et al. 2012). Strikingly, I observed the opposite for *myc2-3* lines, suggesting EDS1 dependent regulation of MYC2 by the 26S proteasome. Possibly, EDS1-MYC2 regulation on protein level is in a fine-tuned balance, which is revealed in absence of COR, but overshadowed by COR during bacterial infection. Further complexity is added by the fact that MYC2 is regulated by the circadian clock (Shin et al. 2012). To avoid daytime dependent effects on my experiments, all samples were taken at 10 a.m.

The new dimension in my presented data is that monomeric EDS1 and PAD4 influence MYC2 in basal, *i.e*. non-challenged conditions as seen by elevated MYC2 protein levels in presence of PAD4, increased transactivation activity in presence of EDS1, and higher basal protein accumulation in MOCK treated *pMYC2:MYC2-flag myc2-3/eds1-2* plants (Figure 2.2, 2.4, 2.9).

The impact of these results ultimately relies on whether one can show function implications for EDS1/PAD4 regulation of MYC2 protein accumulation and transactivation activity. *In planta* experiments are necessary to address this in full scope and to elucidate the physiological relevance in context of basal and pathogen challenged conditions.

3.2 Exploring mechanistic regulation of MYC2 by EDS1

Together with data from Cui et al. (2018) the previously discussed results establish a functional connection between the ETI signalling node EDS1 and the JA master TF MYC2. In section 2.2 I addressed possible mechanisms by which EDS1 might regulate MYC2. Specifically, I tested MYC2 dimerisation properties, regulation by JAZ proteins, and the post-translational modification phosphorylation.

3.2.1 MYC2 dimerisation is not affected by EDS1

As discussed section 3.1.6, MYC2 is a bHLH TF that needs to dimerise and to bind to DNA for proper function. (Activated) EDS1 did not affect MYC2 dimerisation in transient assays (Figure 2.10), making it unlikely that EDS1 would steer MYC2 activity by regulating MYC2 DNA binding. Yet this experiment is rather crude and two alternatives remain. First, EDS1 could impact MYC2 dimerisation in a more subtle manner which cannot be resolved by immunoblot analysis. This could be addressed by using microscale thermophoresis (MST), a method that allows high-resolution quantification of protein-protein, or protein-nucleic acid interaction. It would be interesting to test if and how MYC2 homodimerisation and/or DNA binding changes in presence or absence of EDS1. Second, EDS1 might change MYC2 localisation, thereby interfering with its nuclear TF activity. However, we have not observed this (multiple group members, unpublished). EDS1 shuttles between nucleus and cytoplasm with distinct, compartment specific immunity outputs (Garcia et al. 2010, Heidrich et al. 2011). It is therefore possible that localisation of EDS1 determines its impact on MYC2. TNL-activated, nuclear EDS1 might repress MYC2 signalling (Cui et al. 2018), while this might differ for non-activated nuclear or cytoplasmic EDS1. However, I have not tested this.

3.2.2 EDS1 does not interact with JAZ9 or JAZ10

Unpublished Y2H data from Alain Goossens' group suggested interaction between EDS1 and the majority of JAZ proteins (personal communication). This would be in line with the idea of EDS1 competing with JAZ proteins for MYC2 binding, thereby releasing MYC2 from JAZ suppression. However, I could not show interaction of EDS1 with JAZ9 or JAZ10 (Figure 2.11), suggesting that either other JAZ proteins are of importance or that EDS1 indirectly affects JAZ function. JAZ9 and JAZ10 were promising candidates, as JAZ9 participates in COI1 dependent JA signalling during stomatal plant immunity (Lee et al.

2018) and JAZ10 confers enhanced resistance to *Pst* infection (de Torres Zabala et al. 2016). Further, both *JAZ9* and *JAZ10* were induced upon *Pst* infection in a COR dependent, but MYC2 independent manner (Demianski et al. 2012).

With lost promotion of MYC2^{s} by EDS1 in transient assays it is clear that EDS1 does regulate MYC2 via JAZ proteins, but the precise mechanism remains unknown. It is unlikely that EDS1 needs to be activated or relies on additional components to interact with JAZs, because EDS1 did interact with JAZ proteins in Y2H assays (A. Goossens, personal communication). Alternatively, EDS1 might interact with COI1 and guide it to close proximity with MYC2. This would represent a more upstream control of JA signalling by EDS1 and could be tested easily (see Figure 3.1).

Even if EDS1-JAZ interaction were observed it would be difficult to show functional relevance. Therefore I designed an experiment to answer to what extent JAZ proteins are relevant for MYC2-EDS1 regulation. Transgenic Arabidopsis lines are being generated and first results are expected in June 2019. In this setup C-terminally 3xflag tagged MYC2 driven by its own promoter (*pMYC2:MYC2-flag*) is expressed in a *myc2-3* or a *myc2-3/eds1-2* genetic background and compared to the JAZ uncoupled MYC2^{s} in the same genetic backgrounds (*pMYC2:MYC2^{s}-flag*). Testing transcript, protein, and target gene expression before and after *Pst avrRPS4* infection will tell whether EDS1 is affecting the MYC2-JAZ regulation and if this has a role in EDS1 immune signalling.

3.2.3 MYC2^{S123} is phosphorylated in an EDS1 dependent manner

Another possibility for MYC2 regulation is via post-translational modifications (PTMs). Even though MYC2 has been characterised extensively, information on PTMs is scarce. The most well understood MYC2 PTM is phosphorylation. The PhosPhAt database counts approximately 10 MYC2 phosphorylation sites (Heazlewood et al. 2008) and functional relevance for certain residues has been shown. For instance, MPK6 phosphorylates MYC2^{S123} in blue light conditions during seedling development (Sethi et al. 2014). Zhai et al. (2013) connected phosphorylation coupled proteasomal turnover to MYC2^{T328} in promoting JA responses. In their proposed model, MYC2 proteolysis is part of an "activation by destruction" regulatory loop that ensures a pool of "ready-to-act" protein.

Phosphorylation often precedes ubiquitination and subsequent activation of the 26S proteasome (Geng et al. 2012). Consequently, two studies show regulation of MYC2 via

ubiquitination by the U-box protein PUB10 (Jung et al. 2015), and deubiquitination by the ubiquitin proteases UBP12 and UBP13 (Jeong et al. 2017). MYC2 was destabilised by overexpression of *PUB10* and stabilised in *pub10*, or $PUB10^{C249A}$ mutants, indicating ubiquitin dependent proteolysis of MYC2. Deubiquitination of MYC2 by UBP12 and UBP13 prolongs MYC2 half-life and acts antagonistically to PUB10. Direct evidence for phosphorylation dependent MYC2 ubiquitination is still elusive, though. When testing MYC2 ubiquitination in a transient assay, I did not observe EDS1 dependent changes in the MYC2 ubiquitination pattern (Figure 2.12).

Regarding phosphorylation, I was able to resolve MYC2 phosphorylation via SDS-PAGE and show that transiently overexpressed MYC2 is phosphorylated and that MYC2 from stable transgenic plants is phosphorylated independently of EDS1 or infection (Figure 2.13).

An IP-nLC-MS/MS experiment to identify residue specific MYC2 phosphorylation showed EDS1 dependent, but infection independent phosphorylation of $MYC2^{S123}$ (Figure 2.14). Since published literature (Heazlewood et al. 2008, Zhai et al. 2013, Sethi et al. 2014) and previous test runs had identified multiple phosphorylation sites of MYC2 it is surprising that here I identified one phosphorylated residue only. This residue was phosphorylated in 0/6 replicates (*pMYC2:MYC2-flag*, *myc2-3*), and in 5/6 replicates (*pMYC2:MYC2-flag*, *myc2-3/eds1-2*). S123 is the last in a row of three serines and one could claim that if S123 is mutated, S121 or S122 might compensate for a non-functional S123. Two instances argue against this. First, Sethi et al. demonstrated that MPK6 phosphorylates MYC2 specifically at S123 (Sethi et al. 2014) and second, S123, but not S121 or S122 was identified in a large Arabidopsis phospho-proteasome profiling study (PhosPhAt database, version 4.0) (Heazlewood et al. 2008), making it unlikely that S121 or S122 would act redundantly with S123.

Since S123 is situated in the JID of MYC2 phosphorylation of S123 might prevent MYC2-JAZ binding consistent with the idea that absence of EDS1 leads to phosphorylation and consequently to lost JAZ binding. To test this further I created phospho-dead $MYC2^{S123A}$ and phospho-mimic $MYC2^{S123D}$ variants which both retained interaction with JAZ9 and JAZ10 similar to $MYC2^{WT}$ level (Figure 2.16). Again, it is possible that other JAZ proteins are involved. Also, $MYC2^{S123A/D}$ interacted with EDS1 family members resembling $MYC2^{WT}$, indicating that phosphorylation status of S123 is not important for these protein-protein interactions (Figure 2.15).

With nLC-MS/MS one can also test for other PTMs such as ubiquitination or acetylation. However, this increases the experimental and analytical efforts drastically. The advantage of a high-resolution methods like nLC-MS/MS is the capture of more transient, single residue changes. While we know very little about the functional implications of TF acetylation (Pireyre et al. 2015), ubiquitination is rather well understood (Sharma et al. 2016, Adams et al. 2018). Commonly, polyubiquitinated proteins are subject to 26S proteasomal degradation. Monoubiquitination on the other hand can be a means to regulate protein activity independent of 26S proteasomal degradation enhancing both, positive and negative outputs (Geng et al. 2012). The existence of deubiquitinating enzymes like UBP12 and UBP13 supports a transient, proteasome independent role of (mono)ubiquitination, also with relevance for MYC2 regulation (Jeong et al. 2017).

EDS1 family members are highly conserved among flowering plants (Wagner et al. 2013) and so is JA signalling (Thaler et al. 2012). If S123 were of functional importance one would expect it to be phylogenetically conserved. As Figure 2.14E shows S123 was partially conserved across tested species, supporting an important role in many, though not all species. How the respective MPK-target sites are conserved across these species still needs to be addressed.

3.2.4 EDS1 does not promote MYC2^{S123A} transactivation activity on *VSP1*

Next, I tested whether EDS1 could still promote MYC2$^{S123A/D}$ transactivation activity. While MYC2$^{S123A/D}$ resembled MYC2^{WT} activity in promoting *LOX3* expression, *VSP1* was not promoted by EDS1 phospho-dead MYC2^{S123A} (Figure 2.17B). Two conclusions can be made: first, *LOX3* and *VSP1* differ in their expression profiles, supporting the notion that distinct MYC2 signalling branches are regulated differently by EDS1. Second, MYC2^{S123D} was still promoted by EDS1, suggesting that phosphorylation of S123 is necessary for the EDS1 effect. For detailed analysis of MYC2$^{S123A/D}$ function I have transformed *myc2-3* and *myc2-3/eds1-2* Arabidopsis plants with *35S:SH-MYC2^{S123A}* and *35S:SH-MYC2^{S123D}*, respectively. Plants are being selected and will help to gain insights in MYC2$^{S123A/D}$ function in the context of EDS1 (*i.e.* in *myc2-3* vs. *myc2-3/eds1-2*) and *Pst avrRPS4* induced ETI.

3.2.5 The protein kinase EDR1 associates with MYC2 and PAD4 but is unlikely to regulate MYC2 phosphorylation

Searching for an EDS1 associated kinase, I found that EDR1 interacts with MYC2 and with PAD4, making it an interesting candidate for functional studies (Figure 2.18 and 2.19). Interaction was independent of avrRPS4, supporting the notion that EDS1 effects MYC2 independent of ETI induction. *EDR1* was identified in a forward genetic screen for gain-of-resistance mutants against *Pst* (Frye et al. 1998). Interestingly, *edr1* hyper-resistant plants are not autoimmune and do not show constitutive expression of defence genes like *PR1* (Frye et al. 1998). Therefore, loss of *EDR1* either primes plants for defence, or EDR1 is activated upon pathogen infection only. Further work showed that EDR1 antagonises SA dependent defence against the powdery mildew pathogen *E. cichoracearum* (Frye et al. 2001) and more recently, the molecular mechanism underlying EDR1 repression was described (Zhao, et al. 2014). EDR1 suppresses the MKK4/MKK5-MPK3/MPK6 kinase cascade by binding to MKK4/MKK5, providing functional insights into how EDR1 works (Zhao, et al. 2014).

In spite of EDR1 interaction with PAD4 and MYC2 in co-IP assays, I did not detect EDR1 dependent changes in MYC2 phosphorylation on protein SDS-gels (Figure 2.20). Higher resolution methods like mass spectrometry will provide details about single residue modifications that cannot be observed on SDS gels. Furthermore, *in vitro* kinase activity assays using recombinant proteins expressed in *E. coli* could answer if EDS1 and/or PAD4 regulate EDR1 kinase activity or other kinases in general.

3.2.6 Finding genes of interest - how does EDS1 regulate phosphorylation?

RNA-seq data analysis of genes involved in protein (de)phosphorylation, proteolysis, or ETI showed that some but not all tested genes were differentially regulated in an EDS1 dependent manner. The receptor-like kinase *FER* (*FERONIA*) for instance is unchanged between WT and *eds1-2* plants, but might be still worth testing as an EDS1 regulated kinase. FER phosphorylates MYC2 at more than a dozen residues, including S123 and MYC2 phosphorylation leads to its destabilisation (Guo et al. 2018). Further, FER inhibits MYC2 dependent JA pathway gene expression and restricts MYC2 promoted *Pst* virulence (Guo et al. 2018). With FER modulating MYC2 phosphorylation in bacteria challenged and

unchallenged conditions it is tempting to speculate that FER is being utilized by EDS1 to control MYC2 phosphorylation. S123 was phosphorylated in *myc2-3/eds1-2* background only (Figure 2.14), which could be explained by EDS1 preventing FER activity. This could be tested by comparing MYC2 phosphorylation in WT plants with *eds1*, *fer*, and *eds1/fer* mutants.

Alternatively, *MPK6* is an interesting candidate gene. MPK6 has been reported function largely redundantly with MPK3 in promoting plant immunity both in PTI and ETI (Meng et al. 2013). Like FER, MPK6 was shown to phosphorylate $MYC2^{S123}$ (Sethi et al. 2014) and earlier work demonstrated that MPK6 inhibits MYC2 dependent JA signalling (Takahashi et al. 2007). This is in line with the observation that COR represses MPK3/6 activity by inducing protein phosphatases that dephosphorylate MPK6 and render it less active (Mine et al. 2017). Our group addressed whether MPK3/6 kinase activity is EDS1 dependent. Preliminary results suggest that MPK3/6 are more active (*i.e.* phosphorylated) in *eds1-2* plants compared to WT in MOCK treatment, indicating repression of MPK3/6 by EDS1 (Bhandari, D., personal communication). If EDS1 suppressed MPK3/6 mediated phosphorylation of S123, this would explain how EDS1 modulates MYC2 activity. Intriguingly, MPK3/6 are downstream targets of EDR1 (Zhao et al. 2014), providing new support for EDR1 involvement in this regulation.

Ultimately, a functional connection between EDS1 and FER or MPK3/6 in phosphorylating MYC2 and its relevance to MYC2 transcriptional activity needs to be demonstrated. For instance it will be interesting to test whether EDS1 has general potential to change protein kinase activity. In context of interesting candidates, activity of FER and MPK3/6 with and without EDS1 could be tested in a recombinant system using protein expressed in *E. coli*. Further, genetic evidence by crossing kinase mutants to *eds1-2* plants would be informative. Assuming that EDS1 antagonises phosphorylation of MYC2, phosphorylation will be enhanced in *eds1-2* mutants and lost in double mutants of *eds1-2* with the functional kinase. Because *mpk3/6* double mutants are lethal conditional loss-of-function mutants exist and could be used (Wang et al. 2007, Wang et al. 2008).

A final remark regarding the shown RNA-seq data is that naturally, genes with high expression changes between WT and *eds1-2* plants seem most interesting. However, as I show in this work and others have shown before, gene expression often does not correlate

with protein activity making it possible that even transcriptionally unchanged genes represent useful candidates to test.

The regulatory network shown in Figure 3.1 connects data presented in this and other studies in order to explain the observed EDS1-MYC2 regulation. Here, I assume EDS1 to be "non-activated", *i.e.* in non-triggered conditions. As EDS1 promoted $MYC2^{WT}$, but not JAZ uncoupled $MYC2^{s}$ in transient transactivation assays (Figure 2.4, 2.5), I propose that EDS1 releases MYC2, directly or indirectly, from JAZ suppression. If this occurs by competitive binding, or by other means, and which JAZ proteins are involved remains to be tested. Further, I could show that $MYC2^{S123}$ is phosphorylated in absence of EDS1 (Figure 2.14). MPK6 phosphorylates MYC2 at S123 (Sethi et al. 2014) and inhibits its transactivation activity (Takahashi et al. 2007). If S123 is the only phosphorylation site and thus the decisive one for MPK6 dependent regulation is unclear. Nevertheless, this raises the possibility that EDS1 enhances MYC2 transactivation activity by dampening MPK6 activity, possibly via EDR1. This is supported by low levels of phosphorylated MPK6 in MOCK treated WT plants, compared to *eds1-2* plants (Bhandari, D., personal communication, preliminary). This aspect needs further testing, as $MYC2^{S123A/D}$ *LOX3* transcription was still promoted by EDS1 (Figure 2.17A), but EDS1 did not enhance $MYC2^{S123A}$ dependent *VSP1* expression (Figure 2.17B). A possible explanation for this would be different regulatory mechanisms for *LOX3* and *VSP1* signalling by MYC2. Alternatively, EDS1 could act via another, unknown component (Figure 3.1, "FACTOR X"). Figure 2.21 illustrates EDS1 dependent candidate genes that are involved in protein phosphorylation and/or plant immunity and might be worth testing.

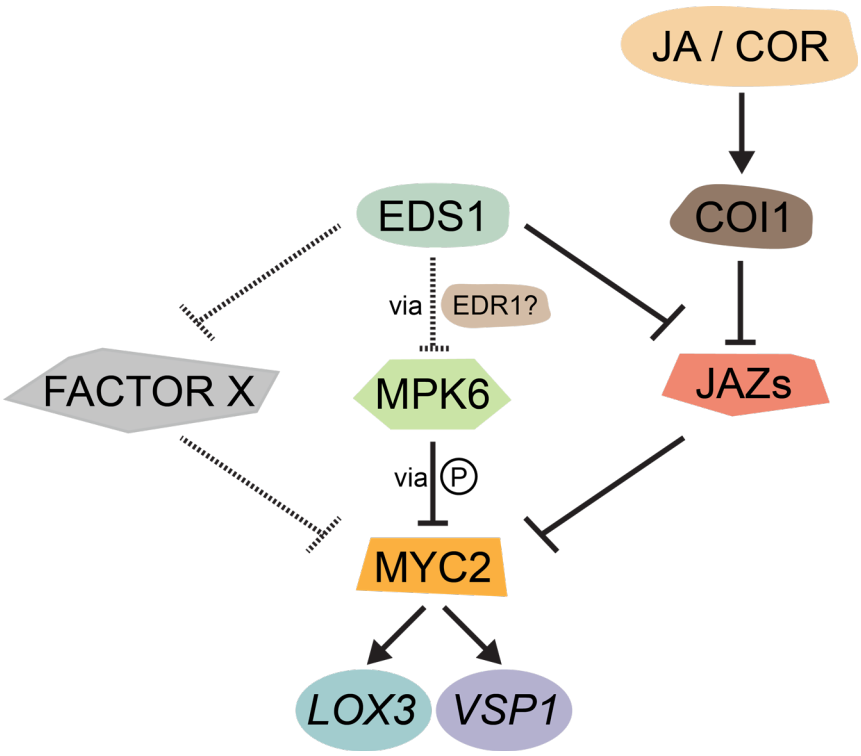

Figure 3.1 Scheme illustrating EDS1 regulation of MYC2 transactivation activity in pathogen unchallenged conditions. MYC2 is repressed via MPK6 dependent phosphorylation and JAZ protein binding. The observed promotion of MYC2 transactivation activity as measured by *LOX3* and *VSP1* transcript can be explained by EDS1 inhibiting MPK6 or JAZ activity. If EDR1 is connected to the EDS1 regulation of MYC2, directly or indirectly, is unclear. Alternatively, a so far unknown "FACTOR X" could be involved. Dashed lines represent hypothesised or preliminary connections. P: phosphorylation

One mode of MYC2 regulation that I haven't addressed in my work is the relationship of MYC2 and MED25. MED25 is a subunit of the MEDIATOR complex that links DNA-associated TFs to RNA-polymerase II, thereby steering gene transcription (An et al. 2017, Kazan 2017). MYC2 relies on MED25 binding for its transactivation activity. Interestingly, JAZ proteins bind MED25, too. This leads to competitive binding to MED25 and allows JAZ proteins to repress MYC2 via MED25 (Kazan 2017). My colleague Dacheng Wang investigated whether EDS1 family proteins interfere with MYC2-MED25 binding and if so, if this has functional implications. He showed that EDS1 or PAD4, but not SAG101 interact with MED25 in Arabidopsis protoplasts, but this interaction did not affect MYC2 binding to MED25, suggesting distinct interaction motifs (Wang, D., personal communication). I concluded that EDS1 most likely does not regulate MYC2 by directly interfering with MYC2-MED25 interaction. Rather, EDS1 might release MED25 and promote MYC2 indirectly by sequestering JAZ proteins.

3.3 SA-JA crosstalk in plant immunity

This study needs to be considered in the context of hormonal crosstalk during plant immunity. By the end of the last century it became increasingly clear that crosstalk between certain phytohormones provides a means for the plant to fine-tune and prioritise defence pathways (Reymond et al. 1998). Now, the concept of hormonal crosstalk is well-established and all known phytohormones take part in fine-tuning the plant's immune response (Robert-Seilaniantz et al. 2011, Pieterse et al. 2012). Traditionally, the crosstalk between SA and JA pathways has received the most attention in context of plant-microbe/insect interactions although it is still unclear whether the evolution of SA-JA crosstalk goes along with adaptive advantages for the plant (Thaler et al. 2012). The common notion is that SA mainly prevents growth of microbial (hemi-) biotrophic pathogens. By contrast, JA signalling primarily counteracts necrotrophic pathogens and herbivores (Glazebrook 2005). The molecular basis for SA and JA pathway signalling is well understood and described in Introduction (section 1.4 and 1.5). Despite, or maybe because of our sound knowledge of SA and JA signalling, a major conceptual question remains: how do these two pathways coexist and mutually regulate each other in context of plant immunity, while maintaining highly specific, yet functionally diverse outputs?

First, I will focus on the SA portion of SA-JA crosstalk. Spoel et al. were among the first to identify a molecular player of the SA-JA crosstalk in context of *Pst* infection. Upon infection, SA levels rise and induce *PR1*. This is accompanied by decreased JA levels and reduced *LOX2* and *VSP1* transcript. The novel finding was that NPR1, the key SA signal transducer, was required for this crosstalk (Spoel et al. 2003). Later, it was found that infection with (hemi-) biotrophic *Pst* renders plants more susceptible to the necrotrophic *Alternaria brassicicola*, providing further functional proof of NPR1 dependent SA-JA crosstalk (Spoel et al. 2007). Another component in SA-JA crosstalk regulation is MPK4. *Mpk4* mutants were autoimmune, and with high SA accumulation and *PR1* expression, failed to induce the JA marker *PDF1.2* (Petersen et al. 2000). Multiple other components have been implicated in SA-JA crosstalk, too, including WRKY TFs, redox regulators like GRX480 (GLUTAREDOXIN 480), TGA (TGACG MOTIF-BINDING PROTEIN) TFs, the fatty acid desaturase SSI2 (SUPPRESSOR OF SA INSENSITIVE 2), and the catalase CAT2 (CATALASE 2) (Pieterse et al. 2012, Yuan et al. 2017). In ETI specifically, our group showed that EDS1/PAD4 protect and boost the SA pathway in part by repressing MYC2 (Cui et al. 2018). The described components that establish the SA-JA crosstalk indicate that

this mutual regulation occurs downstream of hormone accumulation. This is further supported by work showing that even though SA targets JA biosynthesis genes, SA represses *PDF1.2* and *VSP2* in JA biosynthesis deficient *aos/dde2* plants (Leon-Reyes et al. 2010). Consistent with this, the JA responsive TF ORA59 was identified as a target for SA to repress JA pathway genes (Van der Does et al. 2013). Overall, the molecular mechanisms by which SA pathways dampen JA signalling are manifold and mirror the high complexity of hormone signalling networks in Arabidopsis.

Interestingly, JAZ proteins have not been found to be directly involved in SA-JA crosstalk, but present an integrative node for JA crosstalk with other plant hormones like ABA and GA (Chini et al. 2016).

How the JA pathway represses SA outputs is less well understood. Our group could show that on the molecular level MYC2 attenuates *EDS1* expression. This effect depends on MYC2 binding to the *EDS1* promoter, suggesting transcriptional rather than translational regulation (Qiu et al., in prep.).

Additionally, numerous examples show that the SA-JA antagonism can be exploited by (hemi-) biotrophic pathogens such as *Pst*. By producing COR, a functional mimic of bioactive JA-Ile, *Pst* inhibits the SA pathway, resulting in enhanced bacterial virulence (Brooks et al. 2004, Brooks et al. 2005, Zheng et al. 2012). Besides small molecules like COR, *Pst* also produces effector proteins, which act as virulence factors. One such effector is HopX1 which was shown to initiate JA signalling by degrading JAZ repressors, providing an alternative means for *Pst* to dampen the plant's SA response (Gimenez-Ibanez et al. 2014). Another example of effector mediated virulence is HopBB1. Once secreted into the plant cell HopBB1 interacts with JAZ3 and the JA repressor TCP14. This causes TCP14 degradation by the SCF^{COI1} degradation complex, de-repression of JA pathway signalling, and ultimately enhanced pathogen virulence (Yang et al. 2017). An overview of bacterial effectors and their respective impact on hormone signalling was provided by Kazan and Lyons (Kazan et al. 2014).

While not the focus of this discussion, it is worth mentioning that beneficial microbes evolved distinct means to manipulate plant defence, including SA-JA crosstalk (Pieterse et al. 2014).

Besides mutually negative regulation, neutral and positive correlations in SA-JA crosstalk have been observed as well (Schenk et al. 2000, van Wees et al. 2000, Mur et al. 2006, Liu

et al. 2016). Recently, Mine et al. (2017) identified an incoherent feed forward loop that allows JA to promote or attenuate SA accumulation dependent on the immune status of the plant. The authors showed that JA induces expression of *EDS5*, a crucial gene for SA accumulation, via MYC2, while simultaneously dampening expression of *PAD4*, a positive regulator of *EDS5* expression. With this mechanism the plant is able to limit SA dependent growth repression in absence of pathogens and to initiate robust SA accumulation during PTI (Mine et al. 2017).

The seemingly contradictory observations that JA accumulation coincides with SA accumulation could be explained by segmenting SA-JA occurrence into spatially distinct domains. Indeed, a recent study showed spatio-temporal dynamics of the SA marker gene *PR1* and the JA marker gene *VSP1* during ETI in Arabidopsis (Betsuyaku et al. 2017). The concentric pattern of SA accumulation in the centre and JA accumulation at the periphery of the infection site separates contributions of the SA and JA pathways during ETI and allows spatially restricted, domain specific immune signalling (Betsuyaku et al. 2017).

Given this multifaceted and complex relationship between the two pathways, general statements like “SA and JA antagonise each other” are often oversimplifying. The nature of SA-JA crosstalk usually depends on i) time and duration of elicitation, ii) sequence of triggers, and iii) strength of stimulus and is therefore highly dynamic and context specific (Koornneef et al. 2008, Leon-Reyes et al. 2010). This multi-layered, finely tuneable regulation makes sense since plants usually face a plethora of harmful organisms. Proper timing and prioritisation of responses determine the best use of limited resources and the most promising strategy of defence. Taken together, this explains how two distinct, yet connected pathways can coexists and mutually regulate each other over the plant’s lifespan.

As mentioned, we know relatively little about the molecular mechanisms underlying the SA-JA crosstalk. This study, together with the latest work by colleagues in the group, illuminates the molecular basis by connecting the SA pathway component EDS1 with the JA master TF MYC2. EDS1 (and PAD4) antagonise MYC2 in ETI, thereby ensuring a strong SA response during pathogen infection (Cui et al. 2018). Conversely, MYC2 dampens EDS1 expression by binding the *EDS1* promoter, making the plant more susceptible to *Pst* infection (Qiu et al., in prep.). Here, I interrogated how EDS1 modulates MYC2 activity mechanistically. Collectively, my data argue for EDS1 control of MYC2 by directly or indirectly releasing MYC2 from JAZ inhibition. Further, I provide evidence of EDS1

dependent MYC2 phosphorylation. As these are partially contradictory observations, it will be crucial to test the biological relevance of the observed molecular relationships. With the previously described highly diverse regulatory network of SA-JA crosstalk, multiple, synergistic and antagonistic connections of EDS1 and MYC2 seem plausible.

3.4 Perspectives

Our group recently established an antagonistic relationship between EDS1/PAD4 complexes and the JA key TF MYC2. By antagonizing bacterial COR-stimulated MYC2 transcriptional induction of JA pathway genes EDS1/PAD4 restrict bacterial virulence during TNL-ETI (Cui et al. 2018).

In this study I identify a positive connection between MYC2 and EDS1 with EDS1 promoting MYC2 transactivation activity. Functionally, this involves EDS1 dependent regulation of JAZ proteins (indirect evidence) and protein phosphorylation. For a more detailed understanding and especially for biological relevance however additional experiments are necessary.

Assuming that EDS1 releases MYC2 from JAZ suppression it would be interesting to see whether EDS1 shares any surface structure with (certain) JAZ proteins, which might explain competition with JAZs for MYC2 binding. Preliminary analysis by my colleague Deepak Bhandari suggests that this is not the case, but more detailed analysis will be needed to answer this definitely (Bhandari, D., personal communication). Also, it is worth testing EDS1 interaction with further JAZ proteins. To address functional relevance of promoted MYC2 activity by EDS1 stable transgenic Arabidopsis lines are needed. For this I am generating transgenic lines expressing C-terminally 3xflag tagged MYC2 or JAZ uncoupled MYC2^{s}, driven by its own promoter (*pMYC2:MYC2-flag or pMYC2:MYC2^{s}-flag*) in *myc2-3* or *myc2-3/eds1-2* background. By testing transcript, protein, and target gene expression before and after *Pst avrRPS4* infection this will tell whether EDS1 is affecting the MYC2-JAZ regulation and to what extent pathogen challenge influences this.

The nLC-MS/MS experiment described in Figure 2.14 focused on phosphorylation as a putatively changed PTM by EDS1. With more analytical power and time one could rather easily test for SUMOylation, ubiquitination, or acetylation and resolve even single residue modifications that might be missed by other, more crude methods.

With MYC2^{S123} phosphorylated in an EDS1 dependent manner, it will be interesting to test whether S123 phosphorylation is important for MYC2 function in context of EDS1 signalling. As for MYC2 and MYC2^{s}, I am generating transgenic lines expressing *pMYC2:MYC2^{S123A}-flag* or *pMYC2:MYC2^{S123D}-flag* in *myc2-3* or *myc2-3/eds1-2* background. This will allow to interrogate MYC2 activity *in planta* in pathogen challenged and unchallenged conditions.

Further, it will be interesting to test whether EDS1 is capable of directly modulating kinase activities of potential strong candidate protein kinases such as EDR1, FER, and MPK6, in a recombinant system. If so, this will also tell whether EDS1 requires additional components for this regulation or not. Also, predicting the likelihood of MYC2^{S123} being a MPK target site will be useful for further characterisation.

The complexity of the EDS1 and the MYC2 node respectively complicates identification of biologically relevant regulation. Providing evidence for functionally relevant promotion of MYC2 transactivation signalling by EDS1, whether through JAZ interference or protein phosphorylation control is therefore of highest priority.

4 Materials and Methods

Materials and methods are separated into two parts. Section 4.1 lists materials used in this work including plant lines, pathogen and bacterial strains, antibodies, chemicals, enzymes, media, etc. Experimental procedures and peculiarities are described in the second part, 4.2.

4.1 Materials

4.1.1 Plant Materials

The *Arabidopsis thaliana* lines used in this study are listed in table 1.

Table 1 - *Arabidopsis thaliana* lines used in this work

Genotype	Accession	Reference
WT	Col-0	Dangl lab, University of North Carolina, NC, USA
edr1	Col-0	(Frye et al. 1998)
eds1-2	Col-0/(Ler)*	(Bartsch et al. 2006)
eds1-2/pad4-1/sag101-2	Col-0	Parker lab, MPIPZ, Cologne
myc2-3	Col-0	(Shin et al. 2012)
myc2-3/eds1-2	Col-0	Parker lab, MPIPZ, Cologne
myc234	Col-0	(Fernandez-Calvo et al. 2011)
pMYC2:MYC2-flag	Col-0	(Hou et al. 2010)
pMYC2:MYC2-flag/eds1-2	Co-0	Parker lab, MPIPZ, Cologne

* *Ler eds1-2* allele introgressed into Col-0 background, 8th backcrossed generation, referred to as "*eds1-2*" in this work

For *Agrobacterium tumefaciens* dependent transient expression assays I used *Nicotiana benthamiana 310A* plants from the seed stock of MPIPZ, Cologne, GER.

4.1.2 Pathogen Strains

Throughout this work *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) was used as a hemi-biotrophic pathogen strain. The strain was either transformed with the empty vector *pVSP61*

or with *pVSP61* carrying *avrRPS4*, an effector gene from *Pseudomonas syringae* pv. *pisi* (Hinsch et al. 1996).

The coronatine lacking strain *Pst* DC3000 Δcor was obtained from Renier van der Hoorn (MPIPZ). To generate *Pst avrRPS4* Δ*cor*, the *pVSP61-avrRPS4* plasmid was transformed into *Pst* Δ*cor*.

4.1.3 Bacterial Strains

An overview of bacteria used in this study is given in table 2.

Table 2 - Bacterial strains used in this work

Species	Strain	Genotype
E.coli	DB3.1	F- *gyr*A462 *end*A Δ(*sr*1-*rec*A) *mcr*B *mrr hsd*S20 (rB-mB-) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20 (StrR) *xyl*5 λ- *leu mtl*1
E.coli	DH5α	F- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk -, mk+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1
E.coli	DH10b	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*Δ139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L (StrR) *nup*G
A. tumefaciens	GV3101	*pMP90RK* (Deak et al. 1986)

4.1.4 Antibiotics

Table 3 shows antibiotics used in this study. Aqueous stocks were sterile filtered before use.

Table 3 - Antibiotic stock solutions

Name	**stock concentration (mg/ml)**	**working concentration (µg/ml)**	**solved in**
Ampicillin	100	100	ddH_2O
Carbenicillin	100	50	ddH_2O
Kanamycin	50	25	ddH_2O
Gentamycin	25	25 (*Pst*)/15 (*Agrobac.*)	ddH_2O
Rifampicin	40	100	DMSO
Spectinomycin	100	100	ddH_2O

4.1.5 Antibodies

Antibodies used in this study are listed in table 4.

Table 4 - Antibodies

Antibody	Source	Dilution	Supplier	Group
α-flag	rabbit polyclonal	1:5000	Sigma-Aldrich	primary
α-GFP	rabbit monoclonal	1:5000	Cell Signaling Technology	primary
α-HA	rabbit monoclonal	1:5000	Cell Signaling Technology	primary
α-myc	rabbit monoclonal	1:5000	Cell Signaling Technology	primary
α-Ubiquitin	rabbit polyclonal	1:1500	Cell Signaling Technology	primary
α-rabbit IgG-HRP	goat polyclonal	1:5000	Sigma-Aldrich	secondary

4.1.6 Chemicals

All chemicals met laboratory use purity and were obtained by various laboratory suppliers including Merck (Darmstadt, GER), Roth (Karlsruhe, GER), SERVA (Heidelberg, GER), Sigma-Aldrich (Hamburg, GER), ThermoFisher (MA, USA), and VWR (Langenfeld, GER).

4.1.7 Enzymes

4.1.7.1 Restriction Enzymes

For DNA digestion restriction enzymes from either New England Biolabs (NEB, Frankfurt, GER) or ThermoFisher Scientific (MA, USA) were used to the manufacturer's recommendations.

4.1.7.2 DNA Polymerases

Different DNA Polymerases were used according to cloning purpose and complexity. An overview is given in table 5.

Table 5 - DNA Polymerases used in this work

Name	Purpose	Supplier
Phire II	standard PCR	ThermoFisher
Phusion HF	HF, proofreading for cloning	ThermoFisher
Takara PrimeStar	HF, proofreading, for GC-rich templates	CloneTech

4.1.7.3 Other enzymes

Other enzymes used include T4 DNA ligase (ThermoFisher), cDNA Synthesis SuperMix (Bimake, Munich, GER), Gateway® pENTR™/D-TOPO™ Kit (ThermoFisher), Gateway® LR Clonase® II Enzyme Mix (ThermoFisher), PhosSTOP™ Phosphatase Inhibitor (Merck, Darmstadt, GER), Lambda Protein Phosphatase (NEB), Cellulase Onozuka R-10, and Macerozyme R-10 (both SERVA, Heidelberg, GER).

4.1.8 Oligonucleotides

Primers are given in table 6. For regular oligo design primer3 (http://bioinfo.ut.ee/primer3/) was used while SDM primer were created by the primerX (http://www.bioinformatics.org/primerx). Oligonucleotides were ordered at Sigma-Aldrich (Hamburg, GER). Lyophilised primer were resuspended in ddH2O to 100 µM stock concentration and diluted 1:10 to reach a working concentration of 10 µM.

Table 6 - Oligonucleotides

Name	Orientation	Sequence 5' - 3'	purpose
ACTIN	fw	ATGGAAGCTGCTGGAATCCAC	qRT-PCR
ACTIN	rv	TTGCTCATACGGTCAGCGATA	qRT-PCR
AP1	fw	CTCTCTCATCAGCCATCTC	qRT-PCR
AP1	rv	AACGGGTTCAAGAGTCAGTTC	qRT-PCR
EDS1	fw	AGATTATTCAGGTGATCGAGCA	qRT-PCR
EDS1	rv	TTTATGGGCTTGACACTTTGG	qRT-PCR
IAA19	fw	GGTGTGGCCTTGAAAGATGG	qRT-PCR
IAA19	rv	TCTCAACCTCTTGCATGACTCT	qRT-PCR
ICS1	fw	TTCTGGGCTCAAACACTAAAAC	qRT-PCR

ICS1	rv	GGCGTCTTGAAATCTCCATC	qRT-PCR
LFY	fw	TCAGGTACGCGAAGAAATCA	qRT-PCR
LFY	rv	GAAGCTTCTTCGTCTAGGCA	qRT-PCR
LOX3	fw	CGCGAGTTTCATCTCCCATC	qRT-PCR
LOX3	rv	CGCATCTCCTGTCCAAATCG	qRT-PCR
MYC2	fw	GAACGACCCGTCTATGTG	qRT-PCR
MYC2	rv	TTCGGTTATTGTGCTTGA	qRT-PCR
ORA59	fw	GCCGAGATAAGAGACTCAACG	qRT-PCR
ORA59	rv	AGATTCTTCAACGACATCCGC	qRT-PCR
PBS3	fw	ACACCAGCCCTGATGAAGTC	qRT-PCR
PBS3	rv	CCCAAGTCTGTGACCCAGTT	qRT-PCR
VSP1	fw	TCATACTCAAGCCAAACGG	qRT-PCR
VSP1	rv	ATCCTCAACCAAATCAGC	qRT-PCR
MYC2_S123A	fw	GGAGATCGAGTGCTCCGCCGTTTTC	SDM
MYC2_S123A	rv	GAAAACGGCGGAGCACTCGATCTCC	SDM
MYC2_S123D	fw	GGAGATCGAGTGATCCGCCGTTTTC	SDM
MYC2_S123D	rv	GAAAACGGCGGATCACTCGATCTCC	SDM

4.1.9 Vectors

Vectors used or generated in this work are listed in table 7.

Table 7 - Vectors

Vector	Notes
pENTR-EDR1	Gateway® entry plasmid that contains CDS of EDR1 with stop codon from Col-0
pENTR-gEDS1	Gateway® entry plasmid that contains genomic EDS1 without stop codon from Ler-0
pENTR-gEDS1^{LLIF}	Gateway® entry plasmid that contains mutated genomic EDS1 without stop codon from Ler-0, described in (Wagner et al. 2013)
pENTR-JAZ9	Gateway® entry plasmid that contains CDS of JAZ9 with stop codon from Col-0

pENTR-JAZ9	Gateway® entry plasmid that contains CDS of JAZ9 without stop codon from Col-0
pENTR-JAZ10	Gateway® entry plasmid that contains CDS of JAZ10 with stop codon from Col-0
pENTR-JAZ10	Gateway® entry plasmid that contains CDS of JAZ10 without stop codon from Col-0
pENTR-MYB33	Gateway® entry plasmid that contains CDS of MYB33 with stop codon from Col-0
pENTR-MYC2^{D105N} named super-MYC2 (MYC2^{s})	Gateway® entry plasmid that contains CDS of mutated MYC2 with stop codon from Col-0, described in (Goossens et al. 2015)
pENTR-MYC2^{S123A}	Gateway® entry plasmid that contains CDS of mutated MYC2 with stop codon from Col-0
pENTR-MYC2^{S123D}	Gateway® entry plasmid that contains CDS of mutated MYC2 with stop codon from Col-0
pENTR-PAD4	Gateway® entry plasmid that contains CDS of PAD4 with stop codon from Ler-0
pENTR-PIF4	Gateway® entry plasmid that contains CDS of PIF4 without stop codon from Col-0
pENTR-SAG101	Gateway® entry plasmid that contains CDS of SAG101 with stop codon from Ler-0
pENTR-SOC1	Gateway® entry plasmid that contains CDS of SOC1 without stop codon from Col-0
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-gEDS1-mYFP	Binary Gateway® destination vector for expression of genomic EDS under control of 35S promoter with a C-terminal mYFP tag
pXCSG-gEDS1^{LLIF}-mYFP	Binary Gateway® destination vector for expression of mutated genomic EDS under control of 35S promoter with a C-terminal mYFP tag
pXCSG-JAZ9-mYFP	Binary Gateway® destination vector for expression of

	JAZ9 under control of 35S promoter with a C-terminal mYFP tag
pXCSG-JAZ10-mYFP	Binary Gateway® destination vector for expression of JAZ10 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-PAD4$^{1-300}$-mYFP	Binary Gateway® destination vector for expression of truncated 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-YFP-mYFP	Binary Gateway® destination vector for expression of YFP (with stop codon) control of 35S promoter with a C-terminal mYFP tag
pENSG-mYFP-GW	Binary Gateway® destination vector for expression of a fusion protein under control of 35S promoter with a N-terminal mYFP tag
pENSG-mYFP-EDR1	Binary Gateway® destination vector for expression of EDR1 under control of 35S promoter with a N-terminal mYFP tag
pENSG-mYFP-JAZ9	Binary Gateway® destination vector for expression of JAZ9 under control of 35S promoter with a N-terminal mYFP tag
pENSG-mYFP-JAZ10	Binary Gateway® destination vector for expression of JAZ10 under control of 35S promoter with a N-terminal mYFP tag
pXCSG-3xflag-GW	Binary Gateway® destination vector for expression of a fusion protein under control of 35S promoter with a C-terminal 3xflag tag

pXCSG-gEDS1-flag	Binary Gateway® destination vector for expression of genomic EDS1 under control of 35S promoter with a C-terminal 3xflag tag
pXCSG-gEDS1^{LLIF}-flag	Binary Gateway® destination vector for expression of mutated genomic EDS1 under control of 35S promoter with a C-terminal 3xflag tag
pXCSG-JAZ9-flag	Binary Gateway® destination vector for expression of JAZ9 under control of 35S promoter with a C-terminal 3xflag tag
pXCSG-JAZ10-flag	Binary Gateway® destination vector for expression of JAZ10 under control of 35S promoter with a C-terminal 3xflag tag
pXCSG-PAD4-flag	Binary Gateway® destination vector for expression of PAD4 under control of 35S promoter with a C-terminal 3xflag tag
pXCSG-MYC2-flag	Binary Gateway® destination vector for expression of MYC2 under control of 35S promoter with a C-terminal 3xflag tag
pXCSG-GUS-flag	Binary Gateway® destination vector for expression of GUS under control of 35S promoter with a C-terminal 3xflag tag
pENS-StrepII-3xHA (SH)-GW	Binary Gateway® destination vector for expression of a fusion protein under control of 35S promoter with an N-terminal StrepII-3xHA tag
pENS-SH-EDR1	Binary Gateway® destination vector for expression of EDR1 under control of 35S promoter with an N-terminal StrepII-3xHA tag
pENS-SH-MYB33	Binary Gateway® destination vector for expression of MYB33 under control of 35S promoter with an N-terminal StrepII-3xHA tag
pENS-SH-MYC2	Binary Gateway® destination vector for expression of MYC2 under control of 35S promoter with an N-terminal StrepII-3xHA tag

pENS-SH-MYC2^{D105N} named super-MYC2 (MYC2^{s})	Binary Gateway® destination vector for expression of mutated MYC2 under control of 35S promoter with an N-terminal StrepII-3xHA tag
pENS-SH-MYC2^{S123A}	Binary Gateway® destination vector for expression of mutated MYC2 under control of 35S promoter with an N-terminal StrepII-3xHA tag
pENS-SH-MYC2^{S123D}	Binary Gateway® destination vector for expression of mutated MYC2 under control of 35S promoter with an N-terminal StrepII-3xHA tag
pENS-SH-PIF4	Binary Gateway® destination vector for expression of PIF4 under control of 35S promoter with an N-terminal StrepII-3xHA tag
pENS-SH-SOC1	Binary Gateway® destination vector for expression of SOC1 under control of 35S promoter with an N-terminal StrepII-3xHA tag
pENS-SH-YFP	Binary Gateway® destination vector for expression of YFP under control of 35S promoter with an N-terminal StrepII-3xHA tag
pXCSG-3xHA-GW	Binary Gateway® destination vector for expression of a fusion protein under control of 35S promoter with an C-terminal 3xHA tag
pXCSG-avrRPS4-3xHA	Binary Gateway® destination vector for expression of avrRPS4 under control of 35S promoter with an C-terminal 3xHA tag

4.1.10 Media

All media were sterilised by autoclaving at 121 °C for 20 min. Heat sensitive additives like antibiotics were added once the media cooled to approximately 50 °C.

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 MgSO4 2.0 ml/l
pH 7.2
For YEB agar plates 1.5 % (w/v) agar was added.

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.

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 (NYGA) 1.5 % (w/v) agar was added.

4.1.11 Buffers and Solutions

Buffers and their components used in this work are summarised in table 8.

Table 8 - Buffers and Components

Application	Buffer	Components
DNA extraction (quick and dirty)	DNA extraction buffer	200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 7.5, 0.5 % SDS
DNA extraction (sucrose prep)	sucrose solution	50 mM Tris pH 7.5, 300 mM NaCl, 300 mM sucrose
DNA electrophoresis	10x TAE running buffer	0.4 M Tris, 0.2 M acetic acid, 10 mM EDTA, pH 8.5
	6x DNA loading buffer	40 % (w/v) sucrose, 0.5 M EDTA, 0.2 %(w/v) bromophenol blue
	DNA ladder	10 %(v/v) 6× loading buffer, 5 %(v/v) 1 kb DNA ladder (ThermoFisher)
SDS-PAGE	10x Tris-glycine running buffer	250 mM Tris, 1.92 M glycine, 1 %(w/v) SDS
	2x SDS sample buffer (Lämmli buffer)	60 mM Tris pH6.8, 4 %(w/v) SDS, 200 mM DTT, 20 %(v/v) glycerol, 0.2 %(w/v) bromophenol blue
Immunoblotting	TBST buffer	10 mM Tris, 150 mM NaCl, 0.05 %(v/v) Tween 20, pH 7.5
	10x transfer buffer	250 mM Tris, 1.92 M glycine, 1 %(w/v) SDS, 20 %(v/v) Methanol
	Ponceau-S	Dilution of ATX Ponceau concentrate (Fluka) 1:5 in ddH2O
Protein Extraction	Extraction- and wash	50 mM Tris (PH7.5), 150 mM NaCl, 10 % (v/v) Glycerol, 2 mM

	buffer	EDTA, 5 mM DTT, Protease inhibitor (Roche, 1 tablet per 50 mL), 0.1 % Triton
Arabidopsis protoplast preparation	Digestion enzyme solution	1.5% cellulose R10, 0.4% macerozyme R10 (SERVA), 0.4 M mannitol, 20 mM KCl, 20 mM MES (PH5.7), 55 °C for 10 min followed by cooling to room temperature before adding 10 mM $CaCl_2$, 0.1% BSA (Sigma A-6793)
	PEG 40 % (w/v) transformation solution	4 g PEG 4000 (Sigma-Aldrich, #81240), 0.2 M mannitol, 0.1 M $CaCl_2$
	W5 wash buffer	154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES (pH 5.7)
	MMg resuspension solution	0.4 M mannitol, 15 mM MgCl2, 4 mM MES (pH 5.7)
N. benthamiana transient expression	infiltration solution	10 mM MES, 10 mM $MgCl_2$, pH5.6, 0.15 mM acetosyringone
Pst infection	infiltration solution	10 mM $MgCl_2$

4.2 Methods

4.2.1 Plant methods

4.2.1.1 Maintenance and cultivation of *Arabidopsis thaliana* plants

Arabidopsis seeds were sown on moist soil supplemented with 10 mg/l Confidor® WG 70 (Bayer, GER) and vernalised in the dark for 48 h at 4 °C. After this, seeds for experiments were covered with a propagator lid and placed in growth chambers with the following conditions: 10 h light, 14 h dark, ~ 150 µmol m^{-2} s^{-1}, 22 °C, 65 % humidity. Seeds that were sown for plant propagation or crossings were grown in "fast breeding" settings: 22 h light, 2 h dark, ~ 150 µmol m^{-2} s^{-1}, 22 °C, 65 % humidity. Propagator lids were removed 5 - 7 days after germination. For seed collection mature plants were wrapped in a breathable plastic bag and harvested once plants had dried completely.

4.2.1.2 *Agrobacterium tumefaciens* mediated transformation of Arabidopsis ("floral dip")

To obtain stably transformed Arabidopsis plants I used the well-established "floral dip" method (Clough et al. 1998). Stems of Arabidopsis plants grown under the aforementioned fast breeding conditions were cut once after inflorescences set to promote the number of shoots. Ca. 5-week-old plants were then dipped in 5 % sucrose, 0.01 % Silwet L-77 solution containing the *Agrobacteria* (OD_{600} = 1.5). Inflorescences were submerged for 45 sec with gentle agitation, then dapped on tissue to remove excessive solution and covered in plastic bags for 24 h without direct exposure to light. After this the plastic bags were removed and plants were grown in the greenhouse.

4.2.1.3 Crossing of Arabidopsis plants

To cross genetically defined backgrounds, Arabidopsis plants were grown until inflorescence emergence. Flowers with immature pollen, but fully developed stigma were emasculated and received donor pollen via dabbing donor stamen onto each stigma. Cross-pollinated stigmata were sealed in paper bags and left to set seeds. Progeny was then analysed for segregation and desired gene combinations.

4.2.1.4 Isolation of Arabidopsis mesophyll protoplasts

For preparation of Arabidopsis mesophyll protoplasts I used an adapted version of the protocol published by the Sheen group (Yoo et al. 2007). Arabidopsis plants of defined genetic backgrounds were grown and leaves were harvested 4.5 weeks after sowing. Ca. 20 leaves were stacked and cut on tissue with a razor blade into 1 mm thin stripes. Cut leaves were placed in enzyme solution (see section 4.1.11) immediately and vacuum infiltrated twice for 5 min each. The digestion mix was then incubated at RT for 3 h with gentle shaking (30 rpm). To isolate the protoplasts, the solution was filtered through a nylon mesh (100 µm), collected in a flacon tube and washed with the same volume W5 buffer. Cells were centrifuged at 100 g, RT, 1 min. The wash was repeated twice followed by incubating the cells on ice for 30 min. After this, protoplasts were spun down and resuspended in MMg solution to the desired volume ($5x10^5$ protoplasts/ml). 200 µl protoplasts were added to the prepared plasmid combinations, mixed, and transformed by adding 220 µl PEG 4000 solution. Tubes were inverted 3 times and incubated at RT for 12 min before the transformation was stopped by adding 800 µl W5 buffer. Cells were centrifuged (100 g, RT, 2 min), washed with 1 ml W5 buffer, and finally resuspended in 1 ml fresh W5 buffer. Samples were taken 14 h after transformation by either adding RNA extraction buffer, Lämmli buffer, or IP extraction buffer and processed accordingly. For Immunoprecipitation assays all volumes were scaled up 10 times.

4.2.2 Bacterial methods

4.2.2.1 *E. coli*

In general, *E. coli* laboratory strains were grown at 37 °C in LB medium supplemented with the respective antibiotic to ensure plasmid maintenance.

Transformation of chemically competent *E. coli* cells was performed as follows: 50 µl competent cells were thawed on ice and incubated with 2 - 10 ng plasmid DNA for 10 min on ice. The mixture was heat shocked using a water bath at 42 °C for 30 sec and was immediately put to ice for 2 min. After adding 700 µl LB medium, cells were incubated at 37 °C and 200 rpm for 1 h to allow expression of the resistance cassette. Cells were then centrifuged (6000 g, 1 min) and resuspended in 200 µl of which 100 µl were plated on selective LB media plates.

4.2.2.2 *A. tumefaciens*

Agrobacteria were grown in liquid or solid YEB medium with respective antibiotic resistance at 28 °C for 1 - 2 days.

Agrobacteria were transformed via electroporation. Electro competent cells were incubated with 20 ng plasmid DNA for 10 min on ice before being transferred to a precooled electroporation cuvette (1 mm, Eurogentec, BE). The BioRad Gene Pulser Xcell™ with the following settings was used for electroporation. 25 µF, 2.5 kV, 5 ms, and 400 Ω. After pulsing, cells were supplied with 700 µl YEB medium immediately and incubated at 28 °C, 200 rpm for 2 h to allow resistance cassette expression. To obtain single colonies, 50 µl of cells were plated on selective LB media plates.

For *Nicotiana benthamiana* infiltration *Agrobacteria* were grown on plate overnight and resuspended in infiltration medium with an adjusted OD_{600} = 0.2. A needleless syringae was used to infiltrate the abaxial side of mature leaves.

4.2.2.3 *P. syringae*

Pseudomonas syringae strains were grown on selective NYGA plates at 28 °C for 2 days. Stock plates were kept for 2 weeks at 4 °C before being re-streaked.

For infection experiments, bacteria were streaked on fresh plates and grown overnight before being resuspended in infiltration medium and infiltrated in the abaxial leaf side using a needleless syringae. General infection assays were performed by infiltrating *Pst* with an OD_{600} = 0.002. Samples were taken either 6 hpi or 24 hpi.

For infection of plants for nLC-MS/MS analysis bacterial OD_{600} was adjusted to 0.1 and samples were taken at 6 hpi.

After infiltration, plants were covered with a propagator lid for 6 h to ensure high humidity. In general, plants were always infiltrated at 10 a.m. to avoid influence by the circadian rhythm.

4.2.3 Biochemical methods

4.2.3.1 Total protein extraction for immunoblot analysis

Plant tissue was collected from 4-week-old Arabidopsis plants and snap frozen in liquid nitrogen followed by homogenisation with the Qiagen TissueLyser II (Qiagen, Hilden, GER). 50 - 100 µl Lämmli Buffer were added to the powder, vortexed, boiled at 95 °C for 10 min, and centrifuged at 14000 rpm, 4 °C, 5 min. Supernatant was transferred to a new tube and used for immunoblot analysis or stored at -20 °C.

4.2.3.2 Immunoprecipitation of transiently expressed protein

For immunoprecipitation of proteins expressed in Arabidopsis protoplasts, cells were harvested by centrifugation (100 g, 1 min) followed by adding 600 µl extraction buffer directly to the cells. Samples were incubated on ice for 5 min with interspersed vortexing before being centrifuged for 2 min, 4 °C, 14000 rpm. 50 µl supernatant were taken as input sample. For immunoprecipitation, 12 µl of GFP-Trap or myc-Trap (Chromotek, Martinsried, GER) or ANTI-FLAG® M1 Gel (Sigma-Aldrich) were added to the supernatant and incubated on a rotating mixer for 2.5 h at 4 °C. After incubation, beads were spun down at 2500 g at 4 °C for 2 min and washed 4 times with 1 ml extraction buffer. To elute the protein, 100 µl of Lämmli buffer were added to the beads and heated to 95 °C, 10 min with 3 vortex steps. Finally, the eluted beads were collected at the bottom by centrifugation (14000 rpm, 4 °C, 1 min) and supernatant was transferred to a fresh tube and used for immunoblot analysis or stored at -20 °C.

4.2.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

To separate proteins based on their size, the Mini-PROTEAN 3 SDS-PAGE system (BioRad) was used. Samples were extracted in Lämmli buffer and boiled at 95 °C for 10 min. Samples were loaded on discontinuous, self-cast polyacrylamide gels (8 - 10 % for running gel, see table 9, and 6 % for stacking gel, see table 10) with 1.5 mm width. Electrophoresis took place in 1x Running buffer at 80 V for 15 min, followed by 120 V for 70 - 90 min. As protein size marker 3 µl of the PageRuler™ Prestained Protein Ladder (ThermoFisher) were loaded.

Table 9 - Composition SDS PAGE running gels (for 4 1.5 mm gels)

Component	8 % running gel	10 % running gel
ddH_2O	18.5 ml	15.7 ml
1.5 M Tris-HCl pH 8.8	10 ml	10 ml
10 % SDS	400 µl	400 µl
30 % Acrylamide/Bis solution 29:1	10.7 ml	13.3 ml
10 % APS	400 µl	400 µl
TEMED	25 µl	25 µl

Table 10 - Composition SDS-PAGE stacking gel (for 4 1.5 mm gels)

Component	6 % stacking gel
ddH_2O	9 ml
0.5 M Tris-HCl pH 6.8	4 ml
10 % SDS	160 µl
30 % Acrylamide/Bis solution 29:1	2.6 ml
10 % APS	160 µl
TEMED	25 µl

4.2.3.4 Immunoblot analysis

After successful SDS-PAGE proteins were transferred to a Hybond™-ECL™ nitrocellulose membrane (GE Healthcare, Freiburg, GER). For this the BioRad Mini Trans-Blot® cell system was employed. Gels were submerged for 10 min in ice-cold 1x transfer buffer and transfer cassettes were assembled to the manufacturer's instructions. Transfer was performed at 100 V for 70 min at 4 °C. To avoid high background signal after immunoblotting, membranes were blocked with 5 % (w/v) low-fat milk TBST solution for 60 min at RT on a shaker (50 rpm). Primary antibodies were diluted in 2 % (w/v) milk TBST solution (see table 4) and blocked membranes were incubated with the primary antibody over night at 4 °C on a shaker (50 rpm). In the morning, membranes were washed 3 times with TBST (5 min each) and then incubated with secondary antibody diluted in 2 % (w/v) milk TBST at RT for 60 min at 50 rpm. Primary antibodies bound by protein of interest were detected with a horseradish peroxidase (HRP) conjugated secondary antibody. After 4 washes with TBST (5 min each) membranes were supplied with the BioRad Clarity™ or Clarity Max™

Western ECL Substrate. For highly abundant proteins Clarity substrate was used. For low abundance proteins a 1:1 ratio of Clarity™:Clarity Max™ or pure Clarity Max™ was used. Chemiluminescence was detected with the BioRad ChemiDoc™ XRS+ system.

4.2.3.5 Immunoprecipitation for nLC-MS/MS

Immunoprecipitation for nLC-MS/MS analysis was performed with tissue from stable transgenic Arabidopsis plants. For infiltration process please see chapter 4.2.2.3. For each sample 1 g of leaf tissue was collected and snap frozen. Tissue was then homogenised by using a pestle and mortar and afterwards by using the Precellys® Evolution Homogeniser at 7500 rpm, 10 sec, 3 repeats (VWR, Darmstadt, GER). The powder was then resuspended in 8 ml immunoprecipitation extraction buffer supplemented with PhosSTOP phosphatase inhibitor to prevent protein dephosphorylation. After 4 centrifugation steps (14000 rpm, 4 °C, 10 min) I took 50 µl as input control and added 30 µl ANTI-FLAG® M1 Gel to each sample. Immunoprecipitation was performed for 3 h at 4 °C on a rotating mixer. Beads were collected by centrifugation at 2500 rpm, 4 °C, 2 min and washed with extraction buffer 4 times. Protein was eluted from beads by adding 100 µl 0.1 % TFA (Trifluoroacetic acid), incubated 10 min at RT and neutralised by adding 100 µl 8 M urea. Samples were stored at -20 °C until further processing.

4.2.3.6 nLC-MS/MS analysis

Immunoprecipitated proteins in 4 M urea 50 mM Tris-HCl pH 8.5 were reduced with dithiothreitol, alkylated with chloroacetamide, and digested with trypsin (1:100) over night. Samples were desalted using stage tips with C18 Empore disk membranes (3 M, Neuss, GER) (Rappsilber et al. 2003). For data acquisition, dried peptides were re-dissolved in 10 µL 2 % acetonitrile (ACN), 0.1% Trifluoroacetic acid (TFA). Samples were analysed using an EASY-nLC™ 1200 coupled to a Q Exactive™ Plus mass spectrometer (both ThermoFisher). Peptides were separated on 16 cm frit-less silica emitters (New Objective, 0.75 µm inner diameter), packed in-house with reversed-phase ReproSil-Pur C18 AQ 1.9 µm resin (Dr. Maisch). 5 µl of peptides were loaded on the column and eluted for 115 min using a segmented linear gradient of 5% to 95% solvent B (0 min: 5 % B; 0-5 min -> 5 % B; 5-65 min -> 20 % B; 65-90 min -> 35 % B; 90-100 min -> 55 %; 100-105 min -> 95 %, 105-115 min -> 95 %) (solvent A: 0 % ACN, 0.1 % formic acid (FA); solvent B: 80 % ACN, 0.1 % FA) at a flow rate of 300 nl/min. Mass spectra were acquired in data-dependent

acquisition mode with a TOP15 method by using an Orbitrap analyser with a mass range of 300–1750 m/z at a resolution of 70,000 FWHM (Full Width at Half Maximum) and a target value of 3×10^6 ions. Precursors were selected with an isolation window of 1.3 m/z. HCD (High-energy collisional dissociation) fragmentation was performed at a normalised collision energy of 25. MS/MS spectra were acquired with a target value of 10^5 ions at a resolution of 17,500 FWHM, a maximum injection time (max.) of 120 ms and a fixed first mass of m/z 100. Peptides with a charge of +1, greater than 6, or with unassigned charge state were excluded from fragmentation for MS/MS, dynamic exclusion for 30 s prevented repeated selection of precursors.

For data analysis obtained data was processed using MaxQuant software (version 1.5.7.4, http://www.maxquant.org/) (Cox et al. 2008) with label-free quantification (LFQ) and iBAQ enabled (Tyanova et al. 2016). MS/MS spectra were searched by the Andromeda search engine against a combined database containing the sequences from Arabidopsis (TAIR10_pep_20101214), the sequence of MYC2-flag and sequences of 248 common contaminant proteins and decoy sequences. Further, trypsin specificity was required and a maximum of two missed cleavages allowed. Minimal peptide length was set to seven amino acids. Carbamidomethylation of cysteine residues was set as fixed, phosphorylation of serine, threonine and tyrosine, oxidation of methionine and protein N-terminal acetylation were set as variable modifications. Peptide-spectrum-matches and proteins had to pass a false discovery rate of 1 %.

Alternatively, raw data was processed with Proteome Discoverer software (version 2.2.0.388, ThermoFisher). MS/MS spectra were searched using the Sequest HT function implemented in Proteome Discoverer software; using a database containing the sequences from Arabidopsis (TAIR10_pep_20101214), the sequence of MYC2-flag and sequences of 248 common contaminant proteins and decoy sequences. Mass tolerances were set to 10 ppm for precursor and 0.02 Da for fragment ion masses, respectively. Trypsin was set as protease with two allowed missed cleavages and the minimum peptide length was set to 6 amino acids. Carbamidomethylation of cysteine residues was set as fixed, phosphorylation of serine, threonine and tyrosine, oxidation of methionine and protein N-terminal acetylation were set as variable modifications. Peptide spectrum match validation was carried out using the Percolator function implemented in Proteome Discoverer software. Only high confident peptide identifications with false discovery rates ≤ 1 % were considered in the analysis.

4.2.4 Molecular biological methods

4.2.4.1 Isolation of genomic DNA (quick and dirty prep)

Fresh leaf material (ca. 0.5 cm^2) was collected in a test tube and crushed with a pestle. 400 µl DNA extraction buffer were added to the tube and vortexed. The mixture was cleared by centrifugation at 13000 rpm, RT, 3 min. 300 µl supernatant were transferred to a new tube and DNA was precipitated by adding 300 µl isopropanol. Tubes were centrifuged at 13000 rpm, RT, 5 min, washed in 600 µl 70 % EtOH and dried at 40 °C for 5 - 10 min. Dried DNA was resuspended in 50 µl ddH_2O and samples were kept at 4 °C.

4.2.4.2 Isolation of genomic DNA (sucrose prep)

For large numbers of DNA extraction (*e.g.* for genotyping) I used a method based on sucrose that allows DNA extraction in a 96 well plate format (Berendzen et al. 2005). Few mg of leaf material were collected in collection tubes (Qiagen) containing one metal bead. 200 µl sucrose solution were added to each tube and samples were homogenised with TissueLyser II (Qiagen). Tubes were then centrifuged at 1000 g, RT for 1 min and then placed in a water bath for 15 min at 97 °C. After this, samples were placed on ice for 30 min, before 1 µl of solution was used for PCR analysis. DNA extracted by this method should not be frozen and can be used for up to 7 days.

4.2.4.3 Isolation of total RNA

Total RNA was extracted from 4-week-old Arabidopsis plants or Arabidopsis protoplasts by using the Plant RNA Kit (Bio-Budget, Krefeld, GER) according to the provided instructions. Briefly, samples were homogenised in extraction buffer, loaded on DNA binding columns to remove gDNA, precipitated, loaded on RNA binding columns, washed, dried and eluted into collection tubes. RNA was processed immediately or stored at -80 °C.

4.2.4.4 Polymerase Chain Reaction (PCR)

For standard PCRs I used non-proofreading Phire II DNA polymerase (ThermoFisher), while for cloning purposes I used proofreading Phusion HF polymerase (ThermoFisher) or Takara PrimeStar (CloneTech). An overview of used polymerases can be found in table 5. PCR mix was identical for all polymerases and is shown in table 11. The thermal cycling program was adjusted to each polymerase and is shown in table 12.

Table 11 - PCR reaction mix

Component	Volume
10x PCR buffer	2 µl
dNTP mix (2.5 mM)	1.6 µl
forward primer (10 µM)	1 µl
reverse primer (10 µM)	1 µl
template DNA	0.2 - 10 ng
polymerase	0.2 - 0.5 µl
ddH_2O	to 20 µl

Table 12 - Thermo-cycling programs

Stage	Temperature (°C)	Time (Phire II)	Time (Phusion, Takara)	Cycles
Initiation	95	30 sec	5 min	1x
Denaturation	95	10 sec	10 sec	
Annealing	55 - 60	15 sec	30 sec	30 - 35x
Elongation	72	15 sec/kb	30 sec/kb	
Final extension	72	5	5	1x

4.2.4.5 Site-directed Mutagenesis

To mutate specific nucleotides in a sequence of interest site-directed mutagenesis was performed with minor alterations according to the instructions of the QuickChange Site-Directed Mutagenesis Kit (Agilent, Waldbronn, GER). PCR mix can be seen in table 11. Thermo-cycling program differed to the one shown in table 12 in the sense that the PCR was stopped after 18 cycles in order to avoid PCR induced sequence mistakes.

To remove template plasmid from the reaction the methylation sensitive restriction enzyme DpnI (NEB) was used. 1 µl DpnI was added to 20 µl PCR mix and incubated for 1 h at 37 °C. As template DNA is methylated it will be digested, while the mutagenesis carrying non-methylated DNA will not be affected. 5 µl of digested PCR mix were transformed into DH10b and plated on selective LB plates for colony isolation and further cloning.

4.2.4.6 cDNA Synthesis

Total RNA was isolated as described in section 4.2.4.3 and 250 - 1000 ng were used for cDNA synthesis. I used cDNA Synthesis SuperMix (Bimake) following manufacturer's instructions and diluted cDNA 1:1 when using 250 ng total RNA and 1:5 when using 1000 ng total RNA. cDNA was stored at -20 °C.

4.2.4.7 quantitative reverse-transcription PCR (qRT-PCR)

qRT-PCR was performed with a BioRad CFX96 Touch™ Real-Time PCR Detection System. For details see table 13 and table 14. Primer sequences can be found in table 6. For data analysis CFX Maestro™ Software (BioRad) was used.

Table 13 - qRT-PCR reaction mix

Component	Volume
SYBR® Green Supermix	5 µl
forward primer (10 µM)	0.5 µl
reverse primer (10 µM)	0.5 µl
template cDNA	1 µl
ddH_2O	to 10 µl

Table 14 - qRT-PCR thermo-cycling program

Stage	Temperature (°C)	Time	Cycles
Initiation	95	30 sec	1x
Denaturation	95	10 sec	
Annealing	55	15 sec	40x
Elongation	72	10 sec	
Melt Curve	60 - 95	5 sec per 0.5 °C	1x

4.2.4.8 Plasmid DNA isolation from bacteria

For standard plasmid prep the NucleoSpin® Plasmid Kit (Macherey-Nagel, Düren, GER) was used to the manufacturer's instructions. For prepping *Agrobacteria* the low copy protocol was used.

Protoplast transformation requires large amounts of high quality DNA. For this purpose, the NucleoBond® Xtra Maxi Kit (Macherey-Nagel) was used. Typically, 250 ml of bacterial

culture were grown and DNA was purified using NucleoBond® Finalizers yielding ~1 mg DNA.

4.2.4.9 Restriction endonuclease digestion of DNA

DNA digestion was performed to the respective enzyme manufacturer's instructions. Typically, 5 - 15 µl DNA were mixed with reaction buffer, 0.3 µl enzyme and brought to a final volume of 20 µl. The reaction was incubated at the correct temperature for 1 - 2 h and stored at 4 °C.

4.2.4.10 Agarose gel electrophoresis of DNA

If not pre-mixed, DNA was mixed with DNA loading dye and loaded on a 0.8 - 2 % (w/v) agarose gel in TAE buffer. Typically, gels were run at 120 V for 30 - 45 min. Agarose gels were supplemented with 0.2 mg/l ethidium bromide and visualised on a 312 nm UV trans-illuminator.

4.2.4.11 DNA purification from agarose gels

Separated DNA fragments were illuminated on an UV trans-illuminator and cut out with a clean razor blade. For further processing the PCR clean-up and gel extraction kit (Macherey-Nagel) was used to the manufacturer's instructions.

4.2.4.12 Gateway® DNA cloning

In this work I mainly used the Gateway® system for DNA cloning (ThermoFisher). To create entry clones the sequence of a gene of interest was amplified by PCR adding a 5' "CACC" 3' overhang upstream of the start codon. Purified PCR product was then ligated into the pENTR™/TOPO-D™ vector (ThermoFisher) following the manufacturer's instructions and 2 µl were transformed into *E. coli*. To create expression clones a LR reaction was performed using Gateway LR Clonase II mix (ThermoFisher). Typically, reactions were incubated for 1 h at RT and 2 µl were transformed into *E. coli* (DH10b). For reaction mix see table 15.

Table 15 - LR reaction mix

Component	Volume / Amount
ENTR clone	25 - 75 ng
destination clone	75 ng
ddH_2O	to 4 µl
LR Clonase II mix	1 µl

4.2.4.13 Golden Gate DNA cloning

Golden Gate cloning was performed as published (Engler et al. 2008, Weber et al. 2011, Engler et al. 2014, Patron et al. 2015). Concretely, for level 0 constructs, DNA was amplified using primers with overhangs containing BpiI restriction sites, and a 4 nucleotide attachment overhang (5' "TACA" 3'). A typical Golden Gate reaction and the thermo-cycling conditions are shown in table 16 and table 17, respectively. 2 µl of reaction mix were transformed into chemically competent *E. coli* (DH10b) and plated on selective LB plates.

Table 16 - Golden Gate reaction mix

Component	Volume
10x FastDigest buffer	2 µl
10 mM ATP	2 µl
plasmid (insert)	50 ng
plasmid (backbone	15 ng
ddH_2O	to 20 µl
HF restriction enzyme (BpiI or BsaI)	0.5 µl
T4 DNA ligase*	0.5 µl

* For level 0 construction T4 DNA ligase, 1 U/µl was used. More complex assemblies like level 1 construction were performed with T4 DNA ligase, HC 30 U/µl.

Table 17 - Thermo-cycling Golden Gate reaction

Stage	Temperature (°C)	Time	Cycles*
digestion	37	5 min	8 - 50x
ligation	16	5 min	
inactivation 1	55	5 min	1x
inactivation 2	85	5 min	1x

* For level 0 construction 8 cycles were sufficient. More complex assemblies like level 1 construction were performed with 30 - 50 cycles overnight.

4.2.4.14 DNA sequencing

DNA sequencing was carried out by Eurofins Genomics (Ebersberg, GER) using their Mix2Seq kit. Instructions were followed precisely.

4.2.4.15 *In silico* sequence analysis

All sequence data was modified and analysed using CLC Main Workbench software (Qiagen).

For phylogenetic protein alignment, protein sequences were obtained via the Phytozome database (http://phytozome.jgi.doe.gov), aligned via MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle), and coloured via BoxShade (https://embnet.vital-it.ch/software/BOX_form).

5 References

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Köln, 10. Mai 2019

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Patrick von Born