Investigating the initial signalling mechanisms underpinning gene-for-gene mediated Systemic Acquired Resistance in *Arabidopsis thaliana*

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Abstract

Plants deploy two key active defensive strategies to combat microbial pathogens; (i) Recognition of Pathogen-Associated Molecular Patterns (PAMPs) by extracellular surface receptors leading to the activation of PAMP-Triggered Immunity (PTI); (ii) Recognition of pathogen effector activity, usually intracellularly, by host Resistance (R) proteins leading to Effector-Triggered Immunity (ETI). ETI is characterised by a rapid localised Hypersensitive Response (HR). HR induces Systemic Acquired Resistance (SAR) through the production of an inducible immune signal(s), leading to broad spectrum systemic resistance. I investigated the earliest events associated with SAR signalling using plant electrophysiology, SAR mutants and a unique promoter-luciferase fusion that captures early systemic transcriptional events associated with ETI. We describe the transcriptional dynamics of *A70* (At5g56980), a gene of unknown function (Truman *et al.* 2007), in local and systemic tissue following challenge with different elicitors and virulent or avirulent pathogen challenges. We provide evidence that *A70* responds to a jasmonate (JA) related signal that is rapidly generated following ETI recognition. We further evaluate *A70::LUC* reporter activity in response to JA stimulus and correlate activity with histological expression of a JA repressor reporter (*JAZ10::GUS*) and *A70::GFP* reporter in systemically responding leaves following avirulent pathogen challenges. Finally, we examine changes in electrophysiological signals following ETI in local and systemic leaves. Focussing on events underpinning initiation, propagation and perception of SAR-inducing signals within the first 6-8 h of pathogen challenge we provide new insight into the integrated signalling mechanisms, dynamics and connectivity underpinning systemic immune responses. We conclude that there are multicomponent signals that link ETI induced transcriptional and electrical signals, with

a COI1 receptor dependent propagative transcriptional wave the leads to rapid temporal spatial activation of jasmonate responsive genes in systemic responding leaves.

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Abbreviations

%	percent
°C	degree celsius
µg	microgram
µL	microliter
µM	micromolar
c.f.u.	colony forming units
cm	centimetre
dpi	days post inoculation
hpi	hours post inoculation
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	gram
h(s)	hour(s)
HYG	hygromycin
Kb	kilobase
KOH	potassium hydroxide
L	litre
LF	left flank
M	molar
mg	milligram
min(s)	minute(s)
mL	millilitre

mm	millimetre
mM	millimolar
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
ORF	open reading frame
PCR	polymerase chain reaction
pH	power of hydrogen' measure of acidity/basicity
r.p.m.	rotations per minute
RF	right flank
bp	base pair
WT	wild type
ROS	reactive oxygen species
NADPH	nicotinamide adenine dinucleotide phosphate
BIT	1,2-benzisothiazol-3(2*H*)-one1,1-dioxide
BTH	benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester

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Dedicated to my *late* grandmother Indubai (Atti) 1928-2017

1 Chapter 1 General Introduction

1.1 Plant defense responses

Plants have evolved complex defense mechanisms against microbial pathogens, (Jones & Dangl, 2006) simplistically categorized into two categories (**Figure 1**). A first line of defense is based on the recognition of Pathogen-Associated Molecular Patterns (PAMPs, or alternatively referred to as MAMPs for Microbial-Associated Molecular Patterns) by extracellular surface receptors that leads to the activation of PAMP (MAMP)-Triggered Immunity (PTI or MTI). The second line of defense acts largely inside the cell, using the polymorphic cytosolic localized plant disease resistance gene products. These comprise two main classes. Both contain a central nucleotide binding (NB) and carboxyl terminal Leucine-Rich Repeat (LRR domain), but encode either a coiled-coil (CC) or TIR (Toll/Interlukin 1) domain at their amino terminus. These TIR-NB-LRR or CC-NB-LRR proteins, can recognize specific effector molecules delivered by the pathogen, known as avirulence proteins providing resistance to a broad range of pathogens plant pathogens (Staskawicz *et al.*, 1995). Recognition of a single pathogen effector by a single host R protein (called a gene-for-gene interaction) leads to Effector-Triggered Immunity (ETI), which is characterized by rapid Programmed Cell Death (PCD) and is commonly known as the Hypersensitive Response (HR) (**Figure 1**). The HR is a localized cell death at the site of infection which contains the biotrophic pathogen and prevents it from spreading within the host tissue.

The *Arabidopsis thaliana* disease resistance genes *RPS2* and *RPM1* belong to a class of plant disease resistance genes that encode proteins that contain an N-terminal tripartite nucleotide binding site (NBS) and a C-terminal tandem array of leucine-rich repeats. *Arabidopsis thaliana* (Col-0) is resistant to *Pseudomonas*

syringae pv. *tomato* DC3000 carrying either the *Avr* gene *avrRpm1* (DC3000*avrRpm1*) due to *RPM1*-mediated resistance (Mackey *et al.*, 2002) or the *avrRpt2* which is recognised by RPS2 (Leister *et al.*, 1996, Mudgett & Staskawicz, 1999). This molecular recognition of *P. syringae* effectors by RPS2 or RPM1 occurs inside of plant cells and is classically referred to as gene-for-gene (Flor, 1971) resistance (Leister *et al.*, 1996).

Besides the macroscopic HR, ETI is accompanied by other defense responses such as increased extracellular pH, one or more oxidative bursts, nitric oxide (NO) generation, secondary metabolite synthesis, cell wall strengthening, and the expression of pathogenesis-related (PR) proteins (Silipo *et al.*, 2010). PTI and ETI are effective against biotrophic and hemi-biotrophic pathogens, but not against necrotrophic pathogens (Glazebrook, 2005). Although PTI and ETI use different immune receptors, they seem to deploy similar signalling network components (Tsuda *et al.*, 2009), activating a largely overlapping set of genes (Navarro *et al.*, 2004, Zipfel *et al.*, 2006). In addition to these local responses such as PTI and ETI, many systemic signals are translocated and decoded in the distal tissue of the plant leading to a broad-spectrum and long-lasting resistance to pathogen infection known as Systemic Acquired Resistance (SAR) (Glazebrook, 2005, Durrant & Dong, 2004, Shah, 2009).

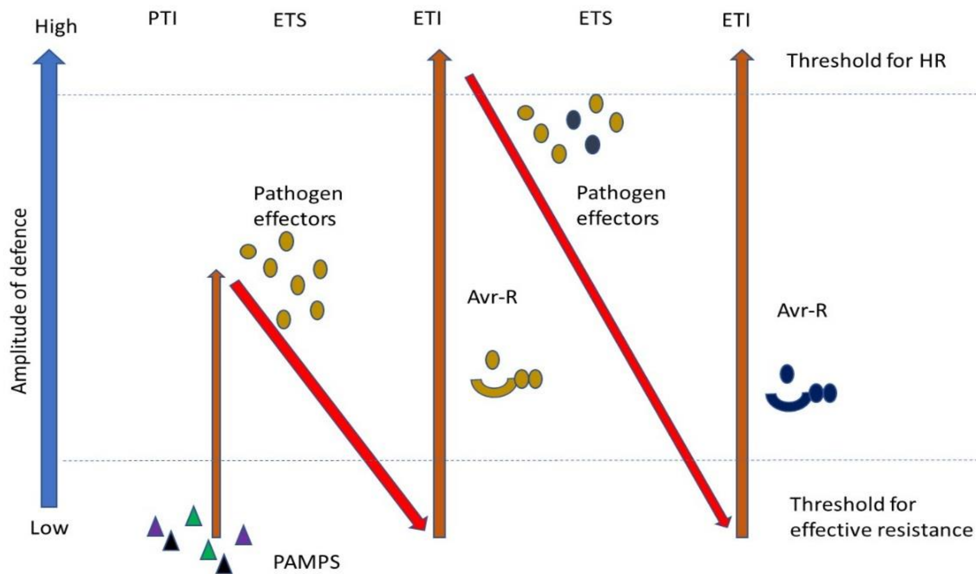


Figure 1. Zig zag model of plant defense*:*

The zig zag model describes the four phases of the plant-pathogen interaction. Firstly, PAMPs are recognized by their cognate PRRs to induce PTI. Secondly, the pathogen can develop effectors that disable PAMP recognition result in to effector-triggered susceptibility (ETS). Thirdly, plants evolve cognate R-proteins recognizing these effectors leading to effector-triggered immunity, the magnitude of this ETI response generally exceeding a threshold for induction HR. Finally, the pathogen develops new effectors to overcome to ETI leading to ETS. This evolutionary "Tug of war" can continue, creating a zig zag between ETI and ETS. Modified from Jones and Dangl (2006).

1.2 The *Pseudomonas syringae* - *Arabidopsis* pathosystem; an ideal model to study "gene-for-gene" interactions and systemic immunity

The *Arabidopsis*-*Pseudomonas syringae* interaction is a model host-pathogen system to study gene-for-gene resistance and functional characterization of innate immunity (Jones and Takemoto (2004). *Pseudomonas syringae* is a Gram-negative rod shaped bacterium and many strains of *Pseudomonas syringae* are known to interact with plants in a host-specific manner (Hirano & Upper, 2000). In nature *Pseudomonas syringae* typically exhibits an epiphytic growth habit, i.e., surviving

and/or multiplying on the plant surface. Here, the limited nutrients on the surface of the plant restricts the growth and development of the *Pseudomonas syringae*, thus these hemi-biotrophic pathogens have developed strategies to enter the plant through wounds or natural openings like stomata or hydathodes (Katagiri *et al.*, 2002). Once *Pseudomonas syringae* enter the apoplastic space, a highly evolved process involving host triggered activation of a *hypersensitive response* and *pathogenicity* (*hrp*) signalling cascade that leads to the establishment of a TTSS (Type III secretion system). The TTSS acts as a molecular syringe to deliver small molecule and proteinaceous effectors directly into the plant cell. Collectively, these collaborate to suppress plant immunity (Jones & Dangl, 2006) and reconfigure host metabolism leading to rapid multiplication and subsequent disease symptom development (Hirano & Upper, 2000, Cunnac *et al.*, 2011).

Pseudomonas mutant strains which are unable to deliver effectors into plant cells, such as *Pst* DC3000*hrpA*$^-$ (*hrpA*$^-$) which has a mutation in the hrpA protein, a major structural component of the T3SS pilus (Wei *et al.*, 2000), cannot establish an infection but can still activate basal, or innate immunity. In this case, basal resistance mechanisms of the host are sufficient to restrict pathogen growth (Roine *et al.*, 1997). By contrast, the 28 effectors delivered by the virulent strain *Pst* DC3000 (Cunnac *et al.*, 2011) are capable of suppressing host immune responses and causing disease (Kaffarnik *et al.*, 2009).

1.3 Role of peptides in defense mechanism

One of the first lines of defense is mediated by the recognition of highly conserved PAMPs such as flagellin and EF-Tu (Elongation Factor Thermo unstable). PTI is primarily mediated by transmembrane pattern recognition receptor (PRR)

proteins, and is presumed to be sufficient to protect plants from incoming pathogens (Macho & Zipfel, 2014). In Arabidopsis, the best studied PAMPs are the bacterial flagellin (specifically its 22 aa epitope flg22) and bacterial EF-Tu (specifically its 18 aa epitope elf18) which are recognized by their cognate LRR-receptor-like kinases FLS2 (flagellin-sensing 2) (Felix *et al.*, 1999) and EFR (EF-Tu receptor), respectively (Lu *et al.*, 2009, Kunze *et al.*, 2004a). In addition, lipopolysaccharides (LPS), chitins and glucans serve as PAMPs (Nurnberger *et al.*, 2004, Ron & Avni, 2004, Zipfel, 2008). Additional PRRs are central to another surveillance system which recognizes plant-derived molecules or DAMPs (Damage-Associated Molecular Patterns), inducing similar defense responses (Boller & Felix, 2009). DAMPs are endogenous plant derived molecules that appear in the intercellular space in response to the damage caused by a pathogen infection, e.g. cell wall damage or effectors derived from cytoplasmic proteins (Ryan *et al.*, 2007). A 23-amino-acid peptide from *Arabidopsis*, called *At*Pep1, represents the archetypal endogenous peptide elicitor activating defense genes associated with the innate immune response (Huffaker *et al.*, 2006).

Induction of SAR was originally believed to be activated specifically by HR-inducing pathogens but has recently also been shown to occur following local leaf challenge with bacterial PAMPs, such as flagellin or lipopolysaccharides (Mishina & Zeier, 2007). Irrespective of the stimulant, the initial molecular events in inoculated leaves that lead to SAR in distant leaves are only partially understood.

Avirulent (Vincent *et al.*, 2017), ETI/HR-inducing pathogens are well known to induce SAR although an HR is not an obligatory signal for SAR induction. Some pathogens cause necrotic disease symptoms instead of HR and trigger SAR (Cameron *et al.*, 1994, Mishina & Zeier, 2006) and recently it has been reported that

insect eggs can enhance resistance against bacterial infection in systemic leaves results in SAR. (Hilfiker *et al.*, 2014).

1.4 Secrets of Systemic Acquired Resistance

Downstream from PTI or ETI, various plant hormones act as central players in triggering the plant immune signalling network (Bari & Jones, 2009, Pieterse *et al.*, 2009, Katagiri & Tsuda, 2010, Grant *et al.*, 2013, Robert-Seilaniantz *et al.*, 2011). As initially articulated by Ross 1966, SAR is an inducible defense response that leads broad-spectrum resistance against pathogenic fungi, oomycetes, viruses, and bacteria. Moreover, SAR-conferred as immune "memory" in plants can last for weeks to months, or possibly even for the whole growing season (KuĆ, 1987). Here, the long-distance signal(s) are predicted to be propagated from the site of primary infection to the remote parts of the plant to induce *PR* gene expression (Conrath *et al.*, 2006, Durrant & Dong, 2004, Dempsey & Klessig, 2012, Yun *et al.*, 2012). Classically, it was believed that after local (HR inducing) infection, the challenged leaf started to accumulate the endogenous signalling molecule SA, which mediates activation of a large set of *PR* genes (Durrant & Dong, 2004) which propagated a signalling cascade throughout the plant via SA and activates *PR* genes expression in the distal tissues. This generated a "primed" situation in the uninfected leaves and when these SAR induced plants are inoculated with a pathogen, defences are induced faster, conferring broad spectrum resistance. It has recently been reported that this primed state can be passed onto the progeny resulting in higher level of disease resistance than progeny of the same parent that had not had a SAR inducing challenge; this phenomenon is termed 'transgenerational SAR' (Luna *et al.*, 2012). The memory linked with the inheritance of SAR is probably of epigenetic nature (Luna & Ton, 2012, Luna *et al.*, 2012).

1.4.1 Signalling molecules involved in SAR

1.4.1.1 Salicylic acid: a signalling molecule classically associated with systemic plant defence

Plant hormones were initially recognized as PGR (Plant Growth Regulators) (Santner *et al.*, 2009). However, subsequent research has shown that hormones are intimately involved in plant defence and are manipulated by pathogens to promote disease (Robert-Seilaniantz *et al.*, 2011, Grant & Jones, 2009). **SA** (Salicylic acid), **JA** (Jasmonic acid) and **ET** (Ethylene) play key roles in plant defence to pathogens with different lifestyles. Initial studies in the early 1990s with transgenic tobacco and *Arabidopsis* plants that constitutively express a bacterial salicylic acid (SA) hydroxylase, involved in catabolism of SA, clearly demonstrated requirement of SA in the distal tissue for SAR to be expressed (Delaney *et al.*, 1994, Gaffney *et al.*, 1993, Vernooij *et al.*, 1994). In response to pathogens, plants synthesise a variety of defense-related small metabolites, including the defense hormones SA and JA, which can activate distinct plant defence pathways. JA induces resistance against several necrotrophic pathogens, whereas SA-mediated defense responses are effective against biotrophic pathogens (Glazebrook, 2005). Classically, SA and JA signalling are antagonistic (Koornneef *et al.*, 2008).

The source of pathogen induced SA is synthesized is not fully understood; it can be synthesized by two different pathways. One is via phenylalanine (PAL pathway) which is then converted to SA, either through free benzoic acid, benzoyl glucose or through *o*-coumaric acid as a precursor, depending on the plant species (Garcion *et al.*, 2008). Alternatively, the isochorismate (IC) pathway first identified in bacteria can be used to synthesise SA (Leistner, 1999). It is most likely that the IC pathway appears to be the major route to synthesize SA in plants following pathogen infection

(Wildermuth *et al.*, 2001, Garcion *et al.*, 2008). Supporting this conclusion, the SA-deficient *Arabidopsis* mutant *sid2*, impaired in ICS, is also impaired in SAR (Wildermuth *et al.*, 2001).

During the course of SAR, SA accumulates in inoculated leaves and was then proposed to translocate in systemic leaves via phloem (Malamy *et al.*, 1990, Smirnov *et al.*, 1997). However, grafting studies in tobacco provided compelling evidence that SA is not transported from inoculated to distal leaves but it is the accumulation of SA in distal leaves that is essential for SAR (Vernooij *et al.*, 1994). Then, it was clarified by (Park *et al.*, 2007) that methyl-SA (MeSA) produced from SA in pathogen-inoculated leaves and then translocated systemically to the distal leaves, could be hydrolyzed by a MeSA esterase to release SA and thus contribute to the activation of SAR in tobacco. MeSA has been reported to be involved as a light dependent mobile SAR signal in tobacco, *Arabidopsis*, and potato (Park *et al.*, 2007, Vlot *et al.*, 2008, Manosalva *et al.*, 2010, Liu *et al.*, 2011a).

Systemic SA accumulation proceeds via upregulation of *ISOCHORISMATE SYNTHASE 1* (*ICS1)* and *de novo* SA biosynthesis (Attaran *et al.*, 2009) which is likely triggered by perception of mobile long-distance signals in the cells of distal leaves (Shah, 2009). SA biosynthesis via ICS1, its positive regulation by PHYTOALEXIN-DEFICIENT4 (PAD4), and SA downstream signalling via NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) is essential for PTI, ETI, and SAR (Durrant & Dong, 2004).

In *Arabidopsis*, the transcription cofactor NONEXPRESSOR OF PR1 (NPR1) plays a vital role in SAR, the degradation of which acts as a molecular switch for inducing *PR* gene expression. Recently, it was reported that NPR3 and NPR4,

paralogues of NPR1, were SA receptors that bind SA with different affinities (Zhang *et al.*, 2006, Fu *et al.*, 2012). NPR3 and NPR4 function as adaptors of the Cullin 3 ubiquitin E3 ligase which in turn mediates NPR1 degradation in an SA dependent manner (Pintard *et al.*, 2004). A double *npr3/4* mutant contains elevated levels of NPR1, as well as it was highly resistant in basal and induced SAR expression and compromised in ETI-triggered HR cell death (Fu *et al.*, 2012). By contrast, the *npr1/3/4* triple mutant lack this phenotype and the plant is more susceptible to *Psm* ES4326/*avrRpt2*.

1.4.1.2 A role for jasmonates in systemic signalling?

Another proposed signal is possible derived from jasmonate. These are lipid-derived molecules originating from α-linolenic acid from the plasma membrane (Schaller & Stintzi, 2009) and may be generated by either enzymatic or non-enzymatic mechanisms. Among all JAs found in nature, (+)-7-iso-JA–L-Ile (JA-Ile) is the molecularly active form of the hormone (Fonseca *et al.*, 2009) as this is perceived through a co-receptor complex formed by the jasmonate receptor – an F-box protein CORONATINE- INSENSITIVE 1 (COI1) and JAZ (jasmonate ZIM domain) proteins, a family that comprise 12 members in *Arabidopsis* (Chini *et al.*, 2007, Chini *et al.*, 2009b, Thines *et al.*, 2007, Sheard *et al.*, 2010). SAR is abolished in JA mutants, but the link between SAR and JA seems to be unclear since SAR is not altered in all JA signalling mutants (Cui *et al.*, 2005, Mishina & Zeier, 2007). Based upon reporter genes studies, transcriptomics and metabolite profiling, Truman and colleagues (Truman *et al.*, 2007) proposed that a JA-based signal may contribute to systemic immunity, before systemic SA accumulation. Indeed, the JA perception, biosynthetic and signalling mutants, *sgt1b* (for Suppressor of G2 allele of *skp1)*, *opr3* (12-oxophytodienoate reductase), and *jin1* (Jasmonate-insensitive 1, subsequently identified as a MYC2 mutant) all

attenuate SAR (Truman *et al.*, 2007). Analysis of JA pathway mutants suggested that pathogen-induced MeSA production is fully dependent on JA signalling (Attaran *et al.*, 2009).

Many pathogens have elaborated several mechanisms to suppress plant defense responses and promote virulence (Spoel & Dong, 2008). In addition, to the 28 Type III Secreted effectors characterized in *Pseudomonas syringae* (Cunnac *et al.*, 2011), several bacterial toxins are also quite potent. One of the best characterized, coronatine (COR), a non-host specific polyketide phytotoxin, is a known mimic of JA-Ile. JA-Ile promotes direct binding of (Jasmonate ZIM domain) JAZ repressors to the F-box protein COI1 with very high affinity (Katsir *et al.*, 2008, Fonseca *et al.*, 2009). COR is also known to inhibit callose deposition in leaf mesophyll cells and root cells (Clay *et al.*, 2009, Millet *et al.*, 2010). Notably, coronatine is only produced by some *Pst* strains such as *Pseudomonas syringae* pv. *atropurpurea*, *P. syringae* pv. glycinea, *P. syringae* pv. *maculicola* and *P. syringae* pv. *Morsprunorum* (Mitchell, 1982).

Coronatine is predicted to reopen stomatal during a virulent *Pst* interaction. It is therefore worth considering ion channels regulation forms an integral part of the light mediated stomatal opening [reviewed by (Shimazaki *et al.*, 2007)] and bacterial flagellin is known to inhibit light-induced stomata opening in an FLS2-dependent manner by inhibiting the K^+ channels of guard cells that mediate K^+ uptake during stomatal opening (Zhang *et al.*, 2008). DC3000 reverses this inhibitory effect indicating an interplay between plant and pathogen in ion channel regulation associated with stomatal immunity (Zhang *et al.*, 2008). The inhibitory effect of coronatine on *A. thaliana* stomata depends on the presence of the JA receptor COI1 and the (bHLH) domain–containing JA responsive transcription factor MYC2, and three NAC petunia **N**AM and Arabidopsis **A**TAF1, ATAF2, and **C**UC2) transcription factors ANAC019,

ANAC055, and ANAC072, whose expression is induced by COR directly through MYC2 (Zheng *et al.*, 2012, Melotto *et al.*, 2006). Thus, coronatine appears to use the same signalling pathway as JA to inhibit stomatal closure. MYC2 acts as both activator and repressor of distinct JA-responsive gene expression in *Arabidopsis* (Lorenzo *et al.*, 2004). The three NACs (ANAC019; At1G52890, ANAC055; At3G15500, and ANAC072; At4G27410) are implicated in ethylene and JA signalling, and are inducible by both bacterial infection and treatment with hormones. They are integral parts of the ABA and JA signalling pathways, necessary for regulating plant developmental processes. These NACs are also involved in inhibition of host defence by repressing the expression of the SA-biosynthesis gene *ICS1*, while activating the expression of the SA metabolic genes *SAGT1* and *BSMT1* through direct binding to their promoters. Consequently, they promote bacterial virulence by interfering with the SA level through COR production (Zheng *et al.*, 2012).

It should be noted however that both MYC2 and ANAC072 (also known as RD26, RESPONSIVE TO DESICCATION 26) are both implicated in ABA signalling (Fujita *et al.*, 2004, Abe *et al.*, 2003).

1.5 Other defense factors

Over the past few years, several other signalling molecules have emerged as possible candidates for the endogenous long-distance signal for SAR (Vlot *et al.*, 2008). Most likely all are involved depending upon the pathosystem, growth conditions and time of inoculation as well as the inducing challenge, yet the specific individual contributions to SAR remain somewhat controversial.

In addition to JA, SA and MeSA, different SAR signals associated with lipids or lipid metabolism have been described (Alvarez *et al.*, 1998, Maldonado *et al.*, 2002,

Chanda *et al.*, 2011, Chaturvedi *et al.*, 2012, Dempsey & Klessig, 2012). the dicarboxylic acid azelaic acid (AzA) (Jung *et al.*, 2009) the diterpenoid dehydroabietinal (DA) (Chaturvedi *et al.*, 2012), pipecolic acid (PiP) (Navarova *et al.*, 2012a), reactive oxygen species (ROS) (Alvarez *et al.*, 1998) and glycerol-3-phosphate (G3P) (Mandal *et al.*, 2011, Chanda *et al.*, 2011) have all been reported to play a role in SAR (**Figure 2**).

The Arabidopsis *DIR1* (*Defective in Induced Resistance1*) gene, which encodes a non-specific lipid transfer protein, is required for SAR (Maldonado *et al.*, 2002). The *dir1* mutant abolishes both local and systemic SA accumulation after virulent and avirulent *Pst* infection as well as systemic *PR* gene expression compared to wild type plants (Maldonado *et al.*, 2002). Thus, it was suggested that DIR1 is required for the systemic movement of a SAR inducing factor. A DIR1-ortholog is also required for the activation of SAR in tobacco (Liu *et al.*, 2011b). Furthermore, petiole exudates collected from *dir1* lack the SAR-inducing activity, but the *dir1* mutant can induce SAR when challenged with petiole exudates collected from induced wild-type plants. This suggests that DIR1, which encodes a putative lipid-transfer protein, is probably involved in the synthesis or transport of a lipid molecule, which is itself, or a derivative thereof, a mobile signal for SAR (Maldonado *et al.*, 2002). Consistent with this possibility, DIR1 contains a signal peptide at its N-terminus that targets it for secretion to the cell surface (Champigny *et al.*, 2011).

Azelaic acid (AzA; also, called nonanoic acid), a nine carbon di carboxylic acid was identified as a potential SAR signal in pathogen-induced resistance in *Arabidopsis (*Jung *et al., 2009).* AzA treated plants shows increased SA accumulation and *PR1* expression. So, AzA may prime plant cells for SA production upon infection (Jung *et al.*, 2009). The *AZI1* (*AZELAIC ACID INDUCED 1*) gene, which encodes a putative

lipid-transfer protein, was transiently expressed at elevated levels in AzA-treated plants. Experiments with the *azi1* mutant confirmed that *AZI1* is required for both AzA synthesis and biologically induced SAR (Jung *et al.*, 2009). Interestingly 9-hydroperoxy octadecadienoic acid (9- HPOD) and 9-oxo nonanoic acid (ONA) are the precursors of AzA (Wittek *et al.*, 2014). In Arabidopsis, ONA is converted into AzA immediately after exogenous application, and is then converted into the C7 dicarboxylic acid, pimelic acid (Zoeller *et al.*, 2012).

Interestingly, (Navarova *et al.*, 2012b) proved that SAR can occur without the accumulation of AzA in phloem exudates collected from virulent pathogen treated plants and (Zoeller *et al.*, 2012) reported that AzA content in virulent pathogen-inoculated leaves was only slightly higher than in mock-inoculated leaves.

After considering these recent two studies by (Zoeller *et al.*, 2012) and (Navarova *et al.*, 2012b) one could conclude that systemic translocation of AzA might not be essential for the establishment of SAR, but when it is translocated, AzA can strengthen systemic immunity during SAR. Alternatively, AzA is important in gene-for-gene induced SAR and alternative SAR inducing molecules are engaged during SAR induced by MAMPs/virulent challenges.

In Arabidopsis, G3P levels were reported to be elevated in the pathogen-inoculated and the distal pathogen-free leaves, as well as with petiole exudates from leaves infected with avirulent bacteria (Chanda *et al.*, 2011), thus implicating a possible role for G3P in long-distance signalling associated with SAR. G3P can be produced by the activity of the G3P dehydrogenase GLY1. GLY1 reduces dihydroxyacetone to G3P and is required for SAR (Chanda *et al.*, 2011, Mandal *et al.*, 2011) In addition, G3P accumulation is enhanced by the physical interaction of DIR1

(Chanda *et al.*, 2011, Maldonado *et al.*, 2002) and AZI1. G3P accumulation also stabilizes the transcript accumulation of *DIR1* and *AZI1* (Yu *et al.*, 2013). It has recently been shown that there is a feedback regulatory loop among AZI1, G3P and DIR1 which regulates SAR and that AzA functions upstream of G3P (Yu *et al.*, 2013).

A newly identified putative SAR signal is Pip (Pipecolic acid). Genetic analyses suggest a crucial role for Pip in systemic plant immunity because it accumulates in systemic leaves and is also found in petiole exudates of pathogen-inoculated leaves (Navarova *et al.*, 2012b). Elevated levels of Pip were found in *Psm (Pseudomonas syringae* pv. *maculicola*) inoculated leaves, as well as systemic leaves. *ald1* (*AGD2-like defence response protein1*) mutant plants lack production of Pip and were defective in SAR as well as PTI and ETI. Exogenously applied Pip complements these resistance defects of the *ald1* mutant and increases pathogen resistance in WT plants (Navarova *et al.*, 2012b).

Finally, the local application of dehydroabietinal (DA), an abietane diterpenoid, induced SA accumulation and *PR1* expression in the systemic untreated leaves (Chaturvedi *et al.*, 2012). DA is a SAR-activating compound identified in avirulent petiole exudates of Arabidopsis which is systemically translocated through the plant and is one of the most potent inducers of SAR (Chaturvedi *et al.*, 2012). DA is a SAR inducer that is active when applied at picomolar concentrations to leaves of *Arabidopsis*, tobacco, and tomato (Chaturvedi *et al.*, 2012). DA-induced SAR required *NPR1*, *FMO1* (*flavin-dependent monooxygenase 1*), and *DIR1* genes (Chaturvedi *et al.*, 2012).

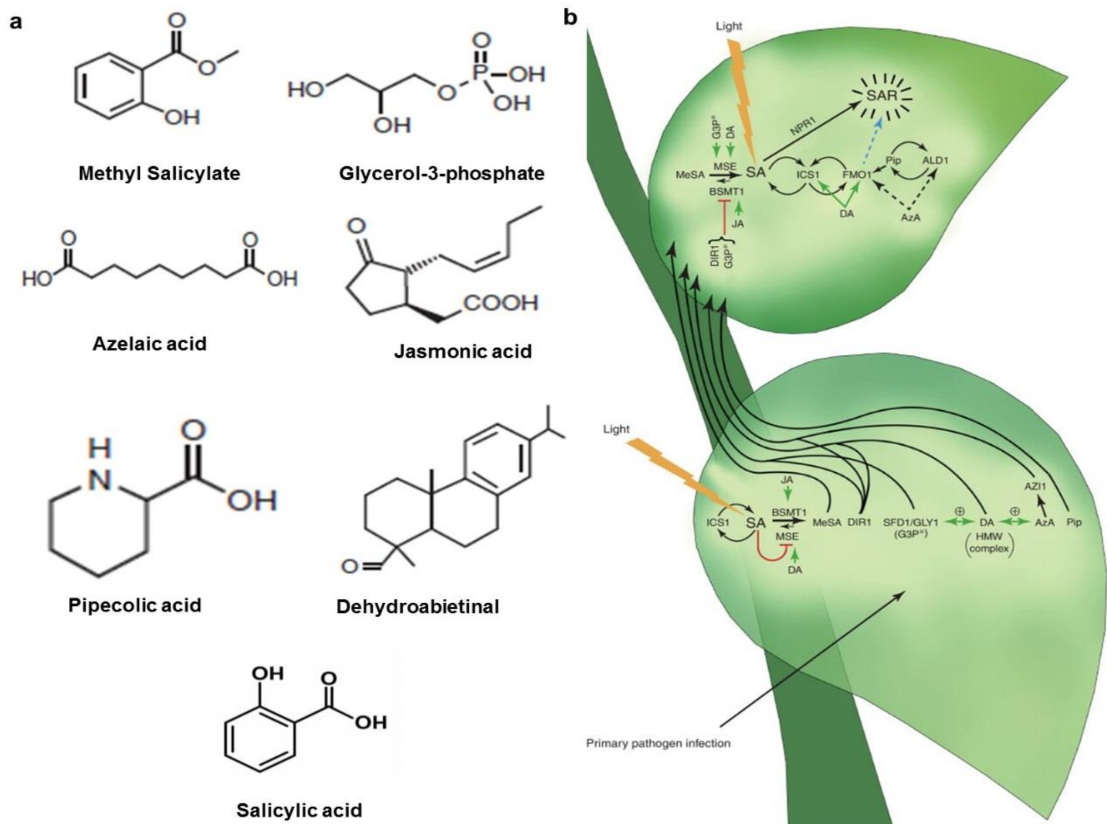


Figure 2. SAR network of signalling molecules:

a. Structure of metabolites putatively involved in long-distance signalling associated with SAR (Dempsey and Klessig 2012). **b.** Multiple signalling molecules participate in SAR. **Events in the pathogen-infected (local) leaf:** The metabolites SA, MeSA, JA, G3P, AzA, DA and PiP accumulate in the local leaf as well as DIR1, a putative non-specific lipid-transfer protein. They all appear essential for the generation and transport of a nonautonomus signal (G3P, DA, and AzA) required for SAR. **Events in the distal (systemic) leaf:** The contributions of SA, JA, G3P, AzA, DA, PiP and DIR1 to SAR establishment. Symbols: synergistic interaction: double-headed green arrow ⊕, red mark: enhancement, SAR signal-induced gene expression: green arrows, amplification loop: black circle, bold and longer arrows: primary direction of MeSA/SA, requirement for induced resistance: dashed lines, alternative pathway: dashed blue line, light: yellow arrows. For further explanations see introductory text. Illustration taken from (Dempsey & Klessig, 2012)

1.6 Surface potential change is associated with long-distance signalling

Plants respond to local injury/wounding with changes in gene expression and the accumulation of defense proteins (Green & Ryan, 1972, Graham *et al.*, 1986). Wounding mechanisms provoke plasma membrane depolarization throughout the whole wounded leaves (Maffei *et al.*, 2004). Plasma membrane depolarization is a common mechanism associated with signalling in plants (Fromm & Lautner, 2007). For instance, exposure of cells to damage-associated molecular patterns DAMPs leads to plasma membrane depolarization, predicted to be associated with generation of peptide danger signals (Krol *et al.*, 2010a). Many studies on herbivory-initiated signalling focused on chemical signals such as JA (Farmer & Ryan, 1990) ethylene (O'Donnell *et al.*, 1996), abscisic acid (ABA) (Pena-Cortes *et al.*, 1989, Herde *et al.*, 1996), oligosaccharides (Walker-Simmons & Ryan, 1984), and systemin polypeptides (Pearce *et al.*, 1991). ABA induces the depolarization of the plasma membrane by activation of anion channels (Thiel *et al.*, 1992) and inhibition of H^+-ATPases (Brault *et al.*, 2004). Whereas electrophysiological changes are largely overlooked as potential signalling components, certainly systemin and oligouronides have been reported to evoke electrical responses at the plasma membrane (Thain *et al.*, 1995, Moyen & Johannes, 1996). However, controversy exists as to whether the long-distance transport of systemin or the propagation of an electrical signal is responsible for triggering the systemic wound response (Wildon *et al.*, 1992, Malone, 1996). Chewing insects induce a wound like response and in 1992, Wildon *et al.* showed a correlation between electrical signalling and activation of proteinase inhibitor gene expression in the distal leaves of tomato seedlings that were subject to herbivory.

1.7 Different type of electrical signals associated with stimuli

Three different electrical signals can propagate over long distances in higher plants: action potential (AP), variation potential (VP) and system potential (SP) (**Figure 3**). These signals travel possibly via the phloem or the xylem, inducing the accumulation of JA and ABA in systemic leaves (Pena-Cortes *et al.*, 1995, Hlavackova *et al.*, 2006, Mousavi *et al.*, 2013b, Huber & Bauerle, 2016). AP and VP are typical depolarisation events of a plasma membrane resulting in changes in voltage pattern, ionic mechanism and velocity (Stahlberg & Cosgrove, 1996, Stahlberg & Cosgrove, 1997, Vian & Davies, 2006, Zimmermann & Felle, 2009). The APs are rapidly propagated electrical signals and can be generated by touching the leaf. In contrast, SPs are systemically transmitted hyperpolarisation events of a plasma membrane (Zimmermann & Felle, 2009, Zimmermann *et al.*, 2009). The SPs are recognized as signals with reverse polarity, and are not caused by a hydraulic pressure surge (Zimmermann & Felle, 2009). The VPs are slow wave potentials induced by wounding, and the signal varies with the intensity of the stimulus and has a longer delayed repolarization (Fromm & Lautner, 2007). It has been reported that VPs are evoked by hydraulic waves that affect ion channels in xylem (Mancuso, 1999).

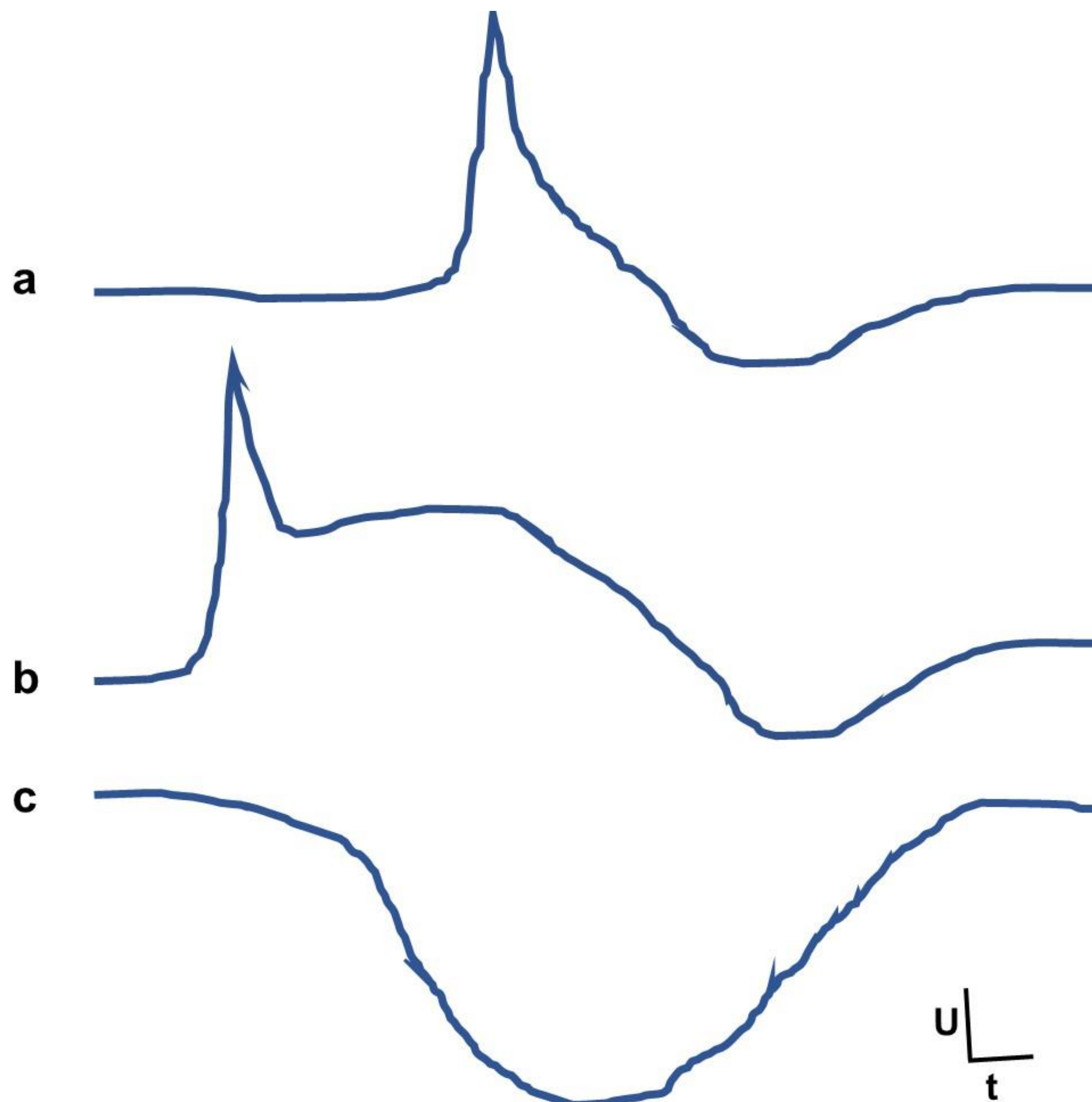


Figure 3. Illustration of three type of potential:

a. action potential: the typical rapid and steep depolarization, **b.** variable potential: this is mixture of AP and VP, and the voltage pattern is mostly variable **c.** System potential: SPs represent self-propagating hyperpolarization potentials. U= voltage in mV and t= time in min, modified from (Zimmermann & Mithöfer, 2013).

1.8 Relation of electrical signals with jasmonate signalling

Plants contain many jasmonate derivatives and synthesise precursors of JA, with different in biological activities (Wasternack & Strnad, 2016). Jasmonates are essential regulators in plant growth and development and responses to biotic and abiotic stresses (Koo *et al.*, 2009, Wasternack & Hause, 2013), but are also important for the initiation of the defence response via electrical signals (Mousavi *et al.*, 2013b). The isoleucine conjugate of JA, JA-Ile, is the only jasmonate for which the molecular basis of its gene-regulatory activity has been clarified (Fonseca *et al.*, 2009). Detailed knowledge of molecular mechanisms initiated by other jasmonate molecules are unknown (Stintzi *et al.*, 2001, Taki *et al.*, 2005, Nakamura *et al.*, 2011, Bosch *et al.*, 2014). The experimental studies show that the binding of JA-Ile to COI1 receptor mediates the ubiquitin-dependent degradation of JAZ proteins, resulting in the activation of JA-dependent gene expression (Thines *et al.*, 2007, Fonseca *et al.*, 2009, Sheard *et al.*, 2010). In 2013, Mousavi *et al.* reported that GLUTAMATE RECEPTOR-LIKE genes, can control the distal wound-stimulated expression of several key jasmonate-inducible regulators of jasmonate signalling (*JAZ* genes) in adult plants. Plant glutamate receptor-like (GLR) homologs are intimately associated with Ca2+ influx through plasma membrane and participate in various physiological processes (Manzoor *et al.*, 2013). AtGLR3.3 plays a vital role in innate immunity and GSH (γ-glutamate [Glu]-cysteine [Cys]-glycine)-mediated defense responses in Arabidopsis leaves (Li *et al.*, 2013). *GLR* genes encode putative cation channels and GLR3.3 functions in plasma membrane depolarization (Qi *et al.*, 2006, Stephens *et al.*, 2008). GLR3.3, and several other GLRs expressed in pollen can control cytosolic Ca^{2+} influxes (Michard *et al.*, 2011), and GLRs have been involved in mediating calcium influxes in response to the perception of MAMPs (Kwaaitaal *et al.*, 2011). In addition,

GLRs are also involved in plant immunity through regulation of elicitor/pathogen mediated plant defence signalling pathways in *Arabidopsis* (Manzoor *et al.*, 2013). In PAMP-mediated resistance, Ca^{2+} fluxes are necessary for activating downstream signalling events related to plant defense (Manzoor *et al.*, 2013). The fungal PAMP cryptogein can induce in glutamate and $[Ca^{2+}]_{cyt}$ extracellularly through exocytosis (Vatsa *et al.*, 2011), recently it has been shown that GLRs involve in ER Ca^{2+} release from the cell is downstream of PAMP perception (Weiland, 2016). Recently, it has been demonstrated that the role of GLRs in local Ca^{2+} signalling and also identified that GLRs as a mechanism leading to of $[Ca^{2+}]_{cyt}$ elevations during biotic interactions (Vincent *et al.*, 2017).

1.9 Introduction to current study

The overall aim of this Project was to study the early events underpinning systemic signal generation and translocation, focusing on the “classical” systemic signalling processes following ETI. This involves dissecting the genetic, temporal and electrophysiological components that contribute to initiating and propagating the signalling events that eventually induce SAR systemically.

There are four main objectives of this study:

1. To study the temporal spatial development of SAR using a luciferase reporter assay
2. To understand the early events in initiating and propagating the SAR signal with the help of plant electrophysiology in combination with the luciferase reporter line.
3. To characterize the establishment of SAR in systemic tissue with a range of published SA, JA and glutamate receptor signalling mutants.
4. Establish whether SAR is associated with specific surface electrical potential signatures and address what contribution, if any, these have to SAR.

2 Chapter 2 Materials and methods

2.1 Plant material

The model plant utilized throughout this study was *Arabidopsis thaliana*, ecotype Columbia (Col-0) or the Col-5 glabrous mutant, which responds to bacterial challenges in an identical manner to Col-0. T-DNA insertion mutants in the Col-0 background for the following genes were obtained from NASC (Nottingham Arabidopsis Stock Centre, Loughborough, UK) or collaborators **(Table 1)**.

Table 1. T-DNA insertion mutant from NASC

npr1	SALK_204100
npr3	GABI_684H02
npr4	SALK_098460.35.75
sid2	SALK_133146.39.30
A70 homolog1.1	SALK_057032.21.05.x
A70 homolog1.2	SALK_067538.49.95.x
A70	SALK_002838.49.30
***coi1*-16**	SALK_045434

Some lines e.g. *jaz10::GUS*, *glr3.3a*, *glr3.6a* and *glr3.3a glr3.6a* were a generous gift from Ted Farmer. Additionally, *npr3* (deleted) and *npr-4* (deleted), *npr3/4, npr1/3/4, nac019/055* and *nac019/055/072* mutants were a gift from the Xinian Dong lab and correspond to *anac019* (SALK_096295), *anac055* (SALK_014331), and *anac072* (SALK_083756) (Zheng *et al.*, 2012). The *coi1*-16 (SALK_045434) with the associated *pen2* mutation removed, were kindly provided by John Turner.

2.2 Bacterial strains

2.2.1 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens (EHA105) was used during this work for transforming plants with constructs of interest (Hood *et al.*, 1993), and was grown at 28°C in Luria Bertani (LB) medium containing 1% bacto-tryptone, 0.5% bacterial yeast extract and 1% sodium chloride supplemented with rifampicin (Rif 50µg/ml).

2.2.2 *Escherichia coli*

E. coli DH5α strain (Hanahan, 1983) was used for transformation and propagation of plasmids of interest and was cultured at 37°C in LB medium.

2.2.3 Vectors for cloning

pCAMBIA1305 (Roberts, 1998) was used for generating the T-DNA transformable *A70* promoter- amino terminal GFP and YFP fusion constructs. All primers used in this study are listed in **(Table 2)**. For the translational fusion, a 1633 nucleotide promoter region of *A70* plus the N-terminal 262 nucleotides, predicted with SMART (http://smart.embl-heidelberg.de) to encode a transit sequence (signal peptide), of *A70* were isolated by PCR using primers incorporating EcoRI and NcoI restriction sites, enabling cloning into the pCAMBIA 1305 containing GFP or YFP as a NcoI translational fusion (**Figure 4**).

Table 2. Primers used for isolation of *A70* pro::*A70* SP

Name	Primer (5'>>>3')
***A70* pro FP**	TCAAAGAATTCCGTAAAAGGTCGGTGTAGC
***A70* RP**	GTGCCATGGAGAAAAGCTCAGTTTCTGGATG

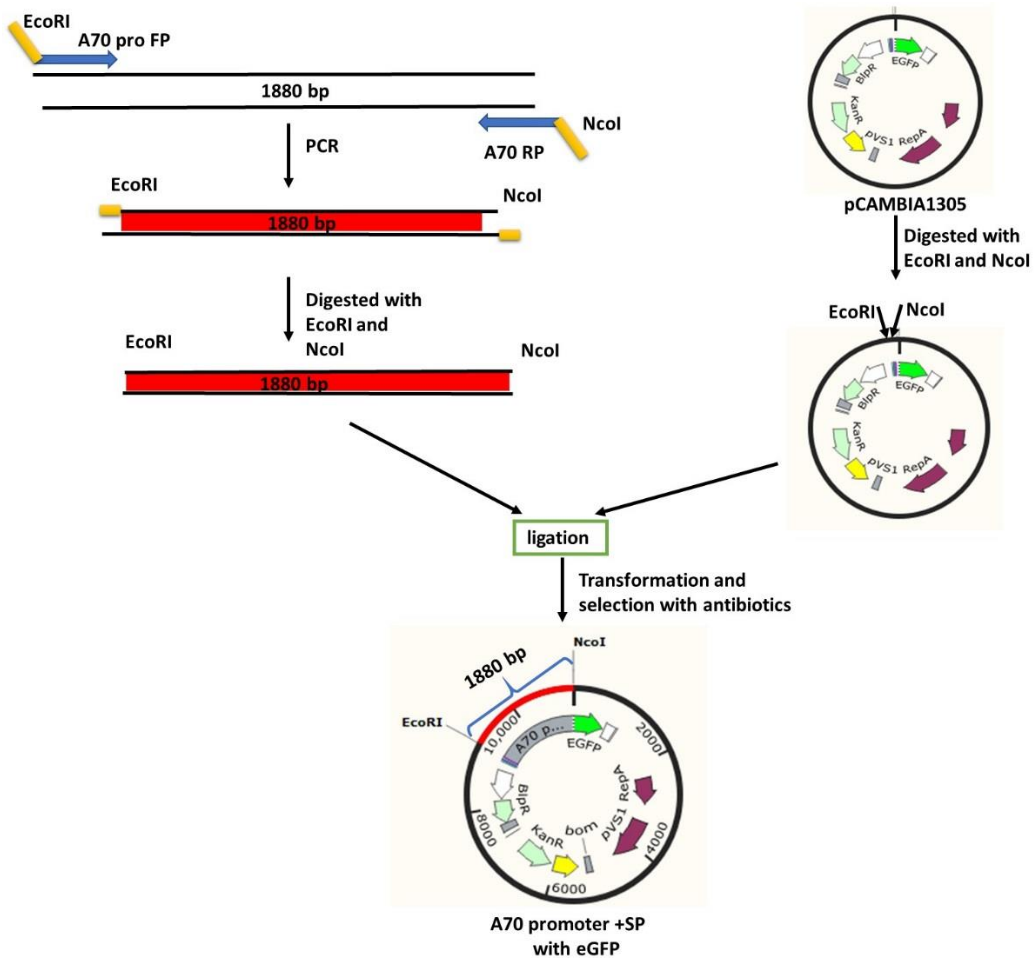


Figure 4. Cloning a *A70*pro::*A70*SP::eGFP into the pCAMBIA 1305 vector:

PCR primers are designed to incorporate EcoRI and NcoI sites onto the gene-coding region. After amplification, the PCR product is purified to remove the DNA polymerase and primers and digested with EcoRI and NcoI, then gel purified. This digested PCR product was ligated into an acceptor pCAMBIA1305 Vector that has been digested with EcoRI and NcoI. Following transformation, the cells are selected with the appropriate antibiotic for the pCAMBIA1305 Vector used.

Heat shock transformation of competent *E. coli* DH5α cells was carried out as described (Sambrook, 1989). The transformed cells were plated on LB plates with appropriate antibiotics and incubated overnight at 37°C.

The transformation of competent *A. tumefaciens* (EHA105) was carried out by adding 500 ng of the plasmid DNA of interest into aliquots of 100 µl of competent cells. Then the aliquot was placed on ice for 15-30 min, and then in to liquid nitrogen for 5 min. Competent cells were heat shocked using heating block at 37 °C for 5 min and placed on ice for 5 min. 1 ml of LB liquid was added and cells incubated at 28°C for 3-4 h on a rotating shaker. A 100 µl aliquot was plated on LB plates with antibiotic selection and incubated at 28°C for 48 h.

2.2.4 Bacterial phytopathogens

The bacterial strains used for expression analysis were; *Pseudomonas syringae* pv. *tomato* strain DC3000 containing either the empty broad host range vector plasmid pVSP61 (Innes *et al.*, 1993), or with either *avrRpm1, avrRpt2* or *avrRps4* avirulence genes cloned into pVSP61 (Innes *et al.*, 1993, Debener *et al.*, 1991); *Pseudomonas syringae pv. tomato* DC3000*hrpA* contain a mutation in the HrpA structural component of the T3SS pilus (Wei *et al.*, 2000)*; Pseudomonas syringae pv. tomato* DC3000 strain DB4G3, a coronatine-deficient (*cor*$^{-1}$/*cor*$^{-2}$) DC3000 mutant (Brooks *et al.*, 2004) or *Pseudomonas syringae* pv. *maculicola* (Schreiber *et al.*, 2012). DC3000*avrRpm1*/*cor*$^{-1}$/*cor*$^{-2}$, was derived in the lab using standard conjugation methods (Holmes & Jobling, 1996)

All *Pseudomonas syringae* strains were cultured on King's B (KB) medium (King *et al.*, 1954) (PH- 7.2) containing 10g Peptone meat, 10g N-Z casein, 1.5g $MgSO_4 \cdot 7H_2O$, 1.5g K_2HPO_4, 10ml Glycerol and 15g Agar/liter. The medium was

supplemented with antibiotics corresponding to each strain; 50 µg ml^{-1} rifampicin and 25 µg ml^{-1} kanamycin or 20 µg ml^{-1} Spectinomycin was for selection of *Pseudomonas syringae* pv. *maculicola.*

2.3 Plant Growth Conditions

A. thaliana seeds were sown in a 3:1 mixture of Levington F2 compost and vermiculite and stratified for 2 days at 4°C. Plants were grown under short day conditions in a controlled environment chamber with 10h light, 14 h dark cycle (100-120 µEinsteins - provided by a combination of Osram White L100/23 and Warm White L100/30 38 (2400mm) fluorescent tubes with a rating of 8600 lumen) at 22°C day and 20°C night. After germination, the seedlings were transferred to trays of 24 (6 X 4 matrix) individual pots from (H. Smith Plastics Ltd) (52x 50 x 48 mm) and grown, for 3-5 weeks before use.

2.4 Bacterial Infiltration

The DC3000 strains were grown on KB medium plates containing appropriate antibiotics. Single colonies were picked to inoculate 10 ml of liquid KB also containing selective antibiotics and incubated at 28 °C and 270 rpm for 16 h in an INNOVA orbital shaker. Cultures were spun down at 1600 g for 7 min at 22 °C, and the pellets were re-suspended in 10 mM $MgCl_2$ and the bacteria re-pelleted as above. These washed bacteria were then re-suspended to give a final OD_{600} of 0.2 and serial diluted to 0.002 or 0.0002 for the measurement of bacterial growth in plants.

2.4.1 SAR assay

The bacterial cultures were grown overnight in KB medium containing 50 µg ml^{-1} rifampicin and 25 µg ml^{-1} kanamycin. For analysis of SAR, one leaf of 3-4-week-old plants were in-filtrated with a needless syringe on the abaxial surface with either 10 mM $MgCl_2$ or DC3000*avrRpm1* diluted to OD_{600} of 0.002 (~2 x 10^6 cfu ml^{-1}). After 2-3

days, three systemic leaves were infected with M4 (*Pseudomonas syringae* pv. *maculicola*) bacteria at OD_{600} 0.002. Three leaf discs were taken from systemic leaves at 2 d.p.i. (days post inoculation) and macerated in 10 mM $MgCl_2$. Appropriate dilutions were made in 10mM $MgCl_2$ and plated on KB agar containing 50 µg ml^{-1} rifampicin and 20 µg ml^{-1} spectinomycin.

To study SAR assay, two different bacterial strains has been used one is DC3000*avrRpm1* and other is M4 (*Pseudomonas syringae* pv. *Maculicola).* This SAR experiment run for 6 days, therefore bacterial inoculum is lower (~2 x 10^6 cfu ml^{-1}) compared to other experiment such as luciferase or electrophysilogical studies.

2.5 Selection of *A70::LUC* plants showing strong systemic signals.

2.5.1 A systemic signal reporter, the *A70::LUC* construct

A70 (At5g56980) was first identified as an early systemically induced gene following DC3000*avrRpm1* challenge, being detected 4 hpi in naïve leaves. Interestingly, this gene, whose function is unknown, was also induced locally in a PAMP associated manner (Truman *et al.*, 2007).

An *A70* (1.6kb promoter-luciferase fusion) (**Figure 5**) line in *A. thaliana* Col-5 was constructed (by Marta de Torres Zabala) to allow expression of *A70* to be visualized in systemic responding tissue in real time. The At5g56980 promoter enables temporal and spatial visualization of SAR activities induced by classical gene-for-gene reactions. Localization of photon emission generated by the luciferase activity (synthetic firefly luciferase 2P, Promega) on the luciferin substrate was captured by a cooled charge coupled device (CCD) camera (Hammamatsu ORCAII ER, Hamamatsu City, Japan), providing information on the location of induced expression of At5g56980

and the magnitude of the response. All *A70::LUC* transgenic lines were selected on hygromycin (50 µg ml^{-1}) and homozygous lines identified based upon *A70*::*LUC* PCR and rapid induction of the reporter in systemic leaves following DC3000*avrRpm1* challenge, consistent with transcript activity documented previously (Truman *et al.*, 2007).

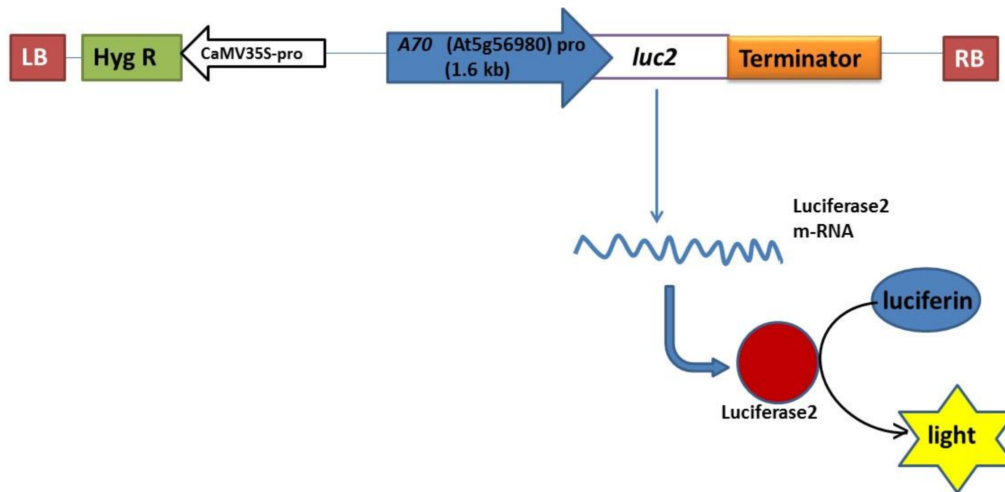


Figure 5. Promoter-Luciferase fusions of At5g56980:

Binary construct used for *Agrobacterium*-mediated transformation. The *A70* promoter was fused to a luciferase reporter gene (Promega Luc-2P) and cloned into a modified version of the pCAMBIA 1302 binary vector. Hyg^R, hygromycin resistance gene; LB, left border sequence; RB, right border sequence; luc2, synthetic firefly luciferase 2P (Promega); terminator, NOS terminator.

2.5.2 Preparation of luciferin

10 µl of the surfactant Silwet L77 (Loveland industries, LTD) was thoroughly mixed in 25 ml of sterile MQ water. 4.8 ml of this solution was added to a spray bottle with 200 µl of (25 mM) luciferin (Promega) (final conc. of sprayed luciferin 1 mM) and mixed well before spraying on plants. Plants were kept under the dark for 30 min after spraying the luciferin, prior to bacterial infiltration and real time imaging.

2.5.3 Real-Time Imaging

A70::LUC plants expressing firefly luciferase under the control of the *A70* promoter were sprayed with luciferin 30 min before bacterial inoculation. Challenged plants were placed inside a dark box, and digital Monochrome images were captured on an ORCAII ER CCD camera with a 35 mm f2.8 micro Nikkor lens after photon counting for 10 min at 2 × 2 binning mode and acquisition using Wasabi imaging software (Hamamatsu). Later in the thesis a Retiga R6 Scientific CCD camera (Qimaging/Photometric) was used under the same settings and images acquired using ImageJ (Micro-Manager 1.4).

2.6 Investigating PAMP/DAMP-induced defence signalling

2.6.1 Elicitors

Peptides of (i) flg22 (QRLSTGSRINSAKDDAAGLQIA), the inactive *A. tumfaciens* tum.flg22 (ARVSSGLRVGDASDNAAYWSIA), (ii) elf18 (SKEKFERTKPHVNVGTIG), an inactive mutant derivative, mut.elf18 (SAEKAERTKPHVNVGTIG) or *At*Pep1 (ATKVKAKQRGKEKVSSGRPGQHN), were synthesized by GENECUST EUROPE-LABBX. Stock solutions (1mM) were prepared and stored at -80°c.

2.6.2 PAMP/DAMP in activation of *A70::LUC* expression

A70::LUC plants were syringe-infiltrated with 2.5 µM of elf 18, flg 22, *At*Pep1 or water as a mock challenge. Plants were immediately imaged under the CCD camera.

2.7 Role of plant hormones in signalling

2.7.1 JA, SA and ABA stock

JA, SA and ABA (Sigma, Dorset, UK) were solubilized in ethanol (final concentration of 1%) and working stocks, JA (250µM), SA (1 mM) or ABA (1 mM) were infiltrated with a needleless syringe in *Arabidopsis* plants. Control plants were treated identically with a solution of 1 % ethanol

2.7.1 Role of JA, SA and ABA in activation of *A70::LUC* expression

Individual hormones were diluted from stock solutions and JA, SA and ABA treatments were carried out by infiltrating *A70::LUC* plants with concentrations ranging from 1 mM to 10 µM. typically, one leaf was infiltrated per plant. Care was taken to remove any residue hormonal solution from un-inoculated leaves. Plants were then transferred to the cooled CCD camera to observe the signals.

Initially, serial dilutions of different concentrations of JA, SA and ABA were tested and the final concentrations selected for further work were determined to be 1 mM for SA and ABA and 250 µM for JA.

2.8 Pathogen Infection and Collection of Phloem Exudates

Plant leaves were infiltrated with *P. syringae* DC3000, DC3000*avrRpm1* DC3000*hrpA* or 10mM $MgCl_2$ (mock control) as described previously. Leaves were immediately excised after infection and petiole exudates were collected immediately in water using an EDTA facilitated method (Tetyuk *et al.*, 2013).

2.8.1 Collection of phloem exudates

Rosette leaves were harvested from four to five-week-old plants by cutting them at the base of the petiole, close to the centre of the rosette. Leaves were then

immediately laid around the edges of a plastic petri dish with petioles submerged in 20 mM Na_2-EDTA at PH- 7. After completing harvesting, petioles were aligned with each other and re-cut at the base of the petioles (~1 mm) then immediately transferred into microfuge (1.5 ml) tubes - four leaves per tube - containing 20 mM Na_2-EDTA solution.

All detached leaves were positioned in microfuge tubes to ensure all leaves were exposed equally to light. Exudates were collected in a clear plastic container covered with cling film and lined with wet paper towels to maintain a humid environment. Collection conditions were identical to plant growth conditions. After 1 h, leaves were removed from the microfuge tubes and washed thoroughly with distilled water to remove all EDTA. Leaves were immediately transferred into newly prepared microfuge tubes containing sterile distilled water, and returned to the humidified container for exudate collection. After the intended collection time (e.g. 8 h), leaves were transferred to another tube to collect overnight phloem exudates, e.g. from 8h-24h, and the phloem exudates were flash frozen in liquid N_2 and stored at -80 °C.

2.9 Isolation of homozygous T-DNA lines compromised in SAR

The following T-DNA insertion lines implicated in SAR were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK); SALK_204100 (*npr1*), GABI_684H02 (*npr3*), SALK_098460.35 (*npr4*) and SALK_133146.39 (*sid2*). To identify homozygous T-DNA insertion lines by PCR, the method described by Alonso *et al.* (2003) was applied, using the T-DNA-specific primers detailed in **(Table 3).**

Table 3. Oligonucleotides used for Isolation of homozygous T-DNA lines during PCR

Mutant	Gene	t- DNA	Primers Sequence (5' to 3')	KO. Product size
npr1	AT1G64280.1	SALK_204100	LP - GAGCAGCGTCATCTTCAATTC RP - TTGTCAGCGAGAAGCTCTTTC	523-823
npr3	AT5G45110.1	GABI_684H02	LP - CGTTGGAAAGAACAACTGAGC RP - GCTGTTGCTCATGAAGCTTTC	471-771
npr4	AT4G19660.1	SALK_098460.35.75	LP - GCATTTCTGCATTTCTTGAGC RP - CTGCTGGGAAGAACAACTGAG	584-884
sid2	AT1G74710	SALK_133146.39.30	LP - TCTGATGGATCTCCAATCGTC RP - GAGATTTCAAGACGCCACTTG	577-877
nac 055	AT3G15500	SALK_014331.54	LP - TAAACGATGAGCGATAGCGAG RP - AAAGGAACCAAAACCAATTGG	467-767
nac 019	AT1G52890	SALK_096295.49.30	LP - TCAATGAACTCAAGGGATTGC RP - ATGCGGTTTGGGTTAGAAAAC	459-759
nac 072	AT4G27410.2	SALK_083756.50.50	LP - GACTGGTCTTTTATCTCCGGG RP - ACAACACATCGATAAGGTCGG	527-827
A70 homolog1-1	AT2G26110	SALK_057032.21.05.x	LP- CATAAAGCTCGCAATCCACTC RP- CTTCGGAGGTCTAATTCCAGC	569-869
A70 homolog1-2	AT2G26110	SALK_067538.49.95.x	LP-TAGGCGCTTTTTCCATAGATG RP-TTTCACCAACCAGCTTCAATC	601-901
A70	AT5G56980	SALK_002838.49.30	LP-ATGTTTACCCGGATCCAAATC RP-GCCACACATACTTCGCTAAGC	552-852
glr3.3a	AT1G42540	SALK-099757	LP-GATGCTGCATATGGTTGTGTG RP-GTTGAACGATAAGCTTGCGAG	700
glr3.6a	AT3G51480	SALK_091801	LP-TTCGTTCAAAGGTGGCATAAC RP-CGACTATGAGGAAAGACGCAG	550
npr-3* **(deleted)**	AT5G45110	SALK_043055	LP1-TGATTGTTGTCGACCTGCCA RP1-AGATCTGACCTCGCCACTCT LP2- TTGGTTCTTTTGCCTTCTCTTTGA RP2-GGCATCCCTATCACCATCTGT	307 209
npr-4* **(deleted)**	AT4G19650	SALK_098460	LP1-TTGGCGATGAAGCTAAGGGG RP1-CTGGCAGAGAGCATGAACCA LP2- TACGCTACTGCTGTTCCAGA RP2-CTTGCACGTGTGCTTTTTGG	526 341
npr1*	AT1G64280	EMS mutagenized	LP-CTCGAATGTACATAAGGCAC RP-GTGCGGTTCTACCTTCC	296

* These lines were derived from the *npr1/3/4* triple mutant obtained from the lab of Xinian Dong.

2.10 Genetic analysis of all crosses

To determine how mutants in these SAR signalling components impact transcriptional dynamics of the *A70::LUC* reporter, crosses were made between all SAR mutants and *A70::LUC*. Artificial pollination was done by opening the flowers at the bud stage and removing all the other floral parts of one parent plant and carefully exposing the stigma without damaging it. Pollen grains from the other parent under study were dusted on the open stigma of the recipient plant.

2.10.1 Crosses between mutant lines and *A70::LUC*

To determine how mutations in these SAR signalling components impact dynamics of the *A70::LUC* reporter, crosses were made between all SAR mutants and *A70::LUC* **(Table 4)**

Table 4. *A70::LUC* lines generated in different immune compromised lines:

1. *A70::LUC/npr1*	2. *A70::LUC/coi1*-16
3. *A70::LUC/sid2*	4. *A70::LUC/nac19/55*/72
5. *A70::LUC/npr3*	6. *A70::LUC/npr3/4*
7. *A70::LUC/glr3.3a*	8. *A70::LUC/glr3.3a*
9. *A70::LUC/glr3.3a3.6a*	10. *A70::LUC/nac19/55*

2.11 Insertion of the *A70::LUC* in the genome

To facilitate such large scale crossing, an adapter ligation based PCR method (O'Malley *et al.*, 2007) was used to identify the genomic localization of *A70:: LUC* gene (**Figure 6**).

2.11.1 Preparation of 10x stocks of Hind III and EcoRI adapters for ligation mediated PCR

10 µl of 10 µM long strand adapter 1, and 10 µl of 10µM short strand adapter (HindIII or EcoRI) was diluted to 1,230 µl in 1 mM Tris, pH 8.3, in a 1.5 ml microfuge tube. The final concentration of each adapter primer was 80 nM. The tube was vortexed and placed on a heat block at 96 ^{o}C for 2 min. The heat was then turned off and the sample left to cool to room temperature (20–24 ^{o}C), facilitating annealing of the long and short adapters.

2.11.2 Digestion of gDNA and ligation of adapters

A master mix, final volume depending upon the number of samples for analysis, comprising the following components listed in **(Table 5)** was prepared and added to each PCR tube.

Table 5. Components for ligation PCR reaction:

Component	amount per reaction (µl)	final conc.
Sterile MQ H_2O	3.25	-
10x ligase buffer +10 mM ATP	1	1x
Hind III adapter	0.25	2 nM
EcoR I adapter	0.25	2 nM
Hind III	0.10	0.2 U
EcoR1	0.10	0.2 U
T4 DNA ligase	0.05	0.10 U

5 µl (~30 ng) of genomic DNA (gDNA) from different *A70* lines was aliquoted into a 200 µl thin-walled PCR tube then 5 µl of the master mix added and the samples mixed by pipetting. Samples were incubated overnight at room temperature. The digestion of the gDNA was checked by running the digested sample on a 1 % (wt/vol) TAE agarose gel and visualized by ethidium bromide staining. A digested sample should appear as a lower molecular weight smear averaging about 4,000 bp. The adapter-ligated DNA was stored at -20 ^{o}C.

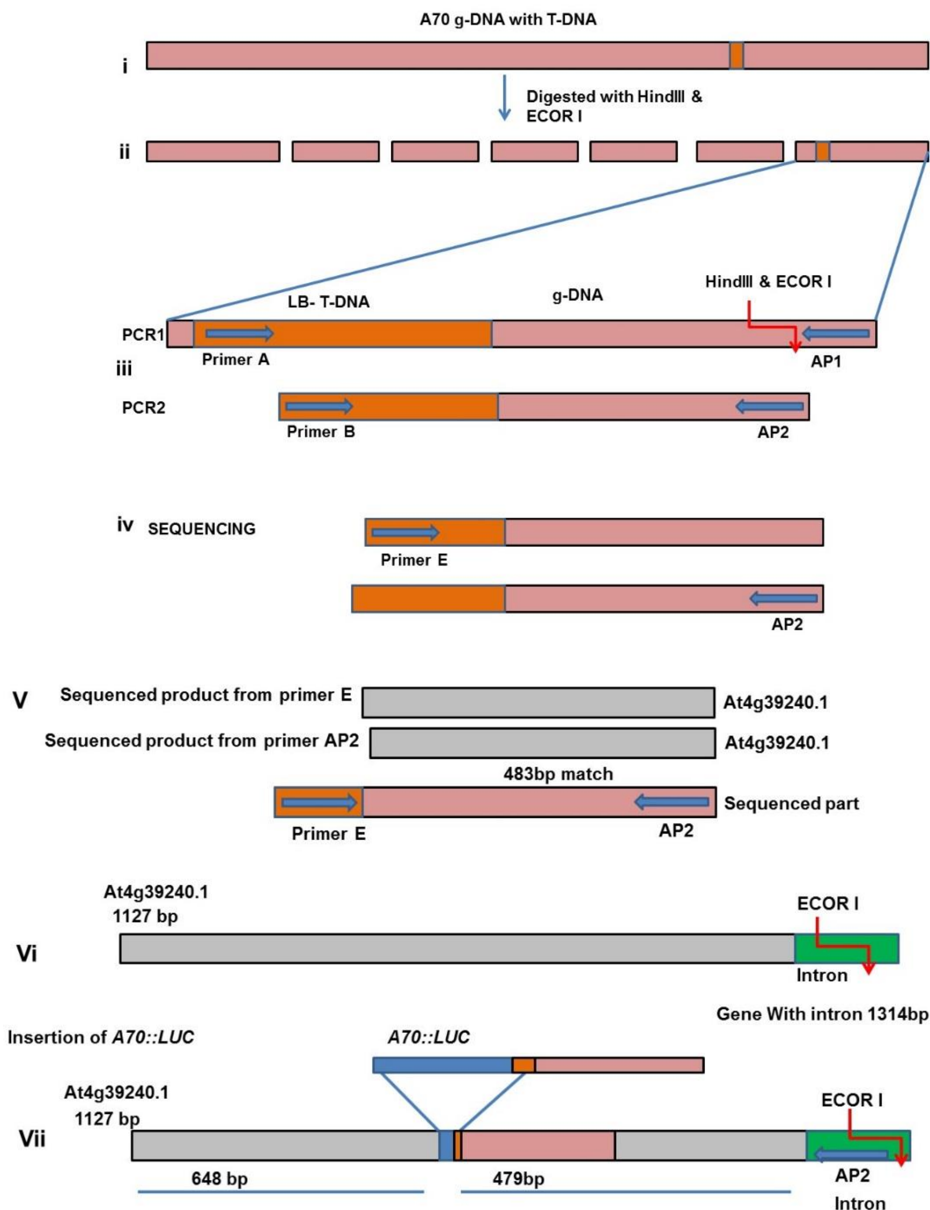


Figure 6. Locating the *A70::LUC* insertion with adapter ligation-mediated PCR:

(i) *A70::LUC* g DNA is isolated from *A70::LUC* plant. **(ii)** *A70::LUC* g DNA digested with either EcoR1 or HindIII. Adapters are ligated to the restriction sites creating adapter-flanked templates. **(iii)** The first PCR is conducted using T-DNA LB primer (primer A) and AP1 (adaptor primer 1), followed by nested PCR with T-DNA LB primer (primer B) and AP2 (adaptor primer 2). **(iv)** The end product of the 2nd PCR fragment was sent for sequencing. **(v)** Sequencing results aligned with the 20% of T-DNA border after the primer B and 80% of additional sequence was At2g39240.1. **(vi)** At2g39240.1 gene with intron. One Primer was designed 479bp left side of intron and other primer designed on T-DNA LB. **(vii)** After PCR we identified that the position of insertion of the T-DNA was in the At2g39240.1 gene. Modified from (O'Malley *et al.*, 2007).

2.11.3 PCR with the T-DNA and adapter primer pairs

1 µl of adapter-ligated (HindIII/EcoR1) gDNA was transferred to a PCR tube and first PCR carried out to amplify the desired T-DNA/gDNA junction using the standard T-DNA primer (LBa1) (Primer A) and adapter primer (AP1) **(Table 8)**. 19 µl of the following master mix **(Table 6)** was added to each tube containing the adapter-ligated gDNA. Specific PCR programmed for amplification of first interested fragment **(Table 7)**.

Table 6. Components for first PCR reaction:

Component	**amount per reaction (µl)**	**final conc.**
sterile MQ H2O	11.6	-
5x phusion HF buffer	4	1x
2 mM dNTP	2	0.2mM
LBa1 primer A (10 µM)	1	250nM
AP1 primer (10 µM)	1	250nM
DNA polymerase (Phusion)	0.2	0.25U

Table 7. Program used for first ligation PCR:

cycle number	**Denature**	**Anneal and extend**
1 to 10	96ºC for 20 s	72ºC for 2:20
11 to 25	96ºC for 20 s	67 ºC for 2:20

6.5 µl of PCR product was separated on a 1.5% (wt/vol) TAE agarose gel and products were visualized by ethidium bromide staining.

Table 8. Oligonucleotides used for ligation based PCR:

Primer code	**Oligonucleotide name**	**Sequence (5' to 3')**
A	primer 281-304	CGATCGACAAGCTCGAGTTTCTC
B	primer 249-270	GTGAGTAGTTCCCAGATAAGG
C	primer 230- 252	AGGGAATTAGGGTTCCTATAGG
D	primer 110-132	CCAGTACTAAAATCCAGATCCC
E	primer 95-117	GATCCCCCGAATTAATTCGGCG
	Long strand of adapter 1	GTAATACGACTCACTTAGGGCACGCGTGGTCGACGGCCCGGGCTGC
	Long strand of adapter 2	GTAATACGACTCACTTAGGGCACGCGTGGTCGACGGCCCGGGCTGTGC
	Adapter primer 1 (AP1)	GTAATACGACTCACTTAGGGC
	Adapter primer 2 (AP2)	TGGTCGACGGCCCGGGCTGC
F	Primer 15-35	GTGGTGTAAACAAATTGACGC
G	Primer 117- 97	CGCCGAATTAATTCGGGGGATC

0.2 µl product of the first PCR and 19.8 µl of the following master mix **(Table 9)** Was added to each tube for the second PCR, which amplifies the T-DNA/gDNA junction using the standard T-DNA primer (LBa1) (Primer B) and adapter primer (AP2) **(Table 8)**.

Table 9. Components for second PCR reaction:

Component	**amount per reaction (µl)**	**final conc.**
sterile MQ H2O	11.6	-
5x Phusion HF buffer	4	1x
2 mM dNTP	2	0.2mM
LBa1 primer B (10 µM)	1	250nM
AP2 primer (10 µM)	1	250nM
DNA polymerase (Phusion)	0.2	0.25U

PCR was carried under the conditions specified in (**Table 10**)

Table 10. Program used for second PCR:

cycle number	**Denature**	**Anneal**	**extend**
1 to 5	96ºC for 0:30	67ºC for 20 s	72ºC for 2:20
6 to 28	96ºC for 0:20	67ºC for 20 s	72 ºC for 2:10

2.5 µl of the PCR product was separated on a 1.5% (wt/vol) TAE agarose gel and products were visualized by ethidium bromide staining. Visualized bands were extracted using a QIAquick gel extraction kit and sequenced.

2.12 Genotyping of *A70::LUC* plant

Based on the sequencing results, the *A70::LUC* T-DNA insertion was located in At4g39240.1 and confirmed by a diagnostic PCR of At4g39240.1. Subsequently, the following protocol used to genotype *A70::LUC* plants. 2 µl of gDNA of *A70::LUC* lines and 28 µl of master mix **(Table 11)** was added to each tube to continue PCR.

Table 11. Components for *A70::LUC* PCR reaction:

Component	amount per reaction (µl)	final conc.
sterile MQ H2O	18.2	-
Taq buffer 10X	3	1x
2.5 mM dNTP's	2.4	200 µM
Primer 1 (10 µM)	1.5	0.5 µM
Primer 2 (10 µM)	1.5	0.5 µM
Taq polymerase	0.5	0.5 U

PCR was preformed according to the conditions in tubes Covered with rubber mat and immediately loaded onto the PCR machine, paused at the initial 95^0C 1st step. Run the PCR on a thermocycler as listed in the **Table 13**. The PCR amplicon generated with primer1 (E) and primer2 (R2) **(Table 12)** gives final product 478bp if *A70::LUC* is present, while the primer F1 and R2 combination gives 585bp which is WT band.

Table 12: Oligonucleotides used for A70::LUC PCR:

Oligonucleotide name	Sequence (5' to 3')
F1	GTCCTTGGTGGATGCATTGAT
R2	CTCCGTGCAACAGATTTTGGTT
E	GATCCCCCGAATTAATTCGGCG

Table 13. PCR program used for *A70::LUC*:

Step 1	95 ^{o}C for 2min
Step 2	94 ^{o}C for 30 sec
Step 3	54 ^{o}C for 30 sec
Step 4	72 ^{o}C for 50 sec
Step 5	step 2 to 4 ; 34 cycles
Step 6	72 ^{o}C for 10min
Step 7	15 ^{o}C forever

2.13 Transformation of Arabidopsis with *A70*

The *A70*::GFP and *A70*::YFP constructs in *Agrobacterium* were transformed into Col-5 plants by floral dipping (Clough & Bent, 1998). Only *A70*::GFP line is used to localisation experiment.

2.13.1 Floral dipping

Twenty four Arabidopsis seedlings were pricked into 2 pots and grown under short days. These short day grown plants were transferred to long days and primary inflorescence bolts were clipped to encourage proliferation of secondary bolts. At the time of transformation (~ 10 days after clipping) plants had many immature floral buds with few open flowers. To transform, inflorescence stems were submerged into a 5%

sucrose solution containing the *Agrobacterium* construct (OD_{600} = ~1.0) and Silwet L-77 (0.02%), agitated for 1 min, then dipped plants were laid on their side on trays inside a plastic cover for 16-24 h to maintain high humidity. The next day plants were placed upright and grown through to flowering and seed set under 16 h day length.

2.13.2 Selection of transgenic plants

The F1 transformants were selected by sowing the seeds in a soil medium containing BASTA herbicide (trade name Kaspar, Certis, UK) Ammonium glyfosinate (150g/L active chemical solution) at a 1/1000 ratio. The transformants were pricked into pots after ~15 days and grown until seed set. F2 seeds were selected for BASTA resistance and the segregation of the transgene was observed for each transformant. Transformants showing 3:1 segregation of the transgene were predicted to contain a single copy of the transgene and were selected for seed collection. The resultant F3 seeds were checked for homozygosity and GFP and YFP reporter activity following DC3000*avRpm1* infiltration using confocal laser scanning microscopy.

2.14 Monitoring *JAZ10::GUS* expression in systemic leaves.

In the histochemical GUS assay, mature leaves were infiltrated with different virulent or avirulent bacteria. GUS activity in systemic leaves was assessed at 4 h, 8 h and 24 h by staining in GUS staining solution (1 mM X-Gluc, 100 mM $NaPO_4$ buffer pH 7.0, 10 mM EDTA, and 0.1% [v/v] Triton X-100). Tubes were incubated at 37 ^{o}C, and leaves were de-stained by repeated washes with 70% ethanol. The GUS expression technique is based on β-glucuronidase. Were, β-glucuronidase converts the colourless X-Gluc into a blue precipitate. This blue precipitate specific to the tissue in which *JAZ10* expressed.

2.15 Plant electrophysiology

To determine whether, like during wounding, any electrophysiological signatures were associated with SAR, electrical potentials were measured following avirulent bacterial challenge of *Arabidopsis* using non-invasive surface electrodes.

2.15.1 Experimental set up

To conduct the electrophysiological experiment, a four-channel circuit was constructed, as shown in **(Figure 7c)**, with the help of Dr. David Horsell. The circuit design was based on that described in (https://www.picotech.com/library/experiment/Four-Channel-pH-Data-Logger). The circuit provided separate reference and working electrodes for each channel. Because of the Common reference electrode input, we were able to use multiple working electrodes against a single reference electrode. Most importantly, the reference electrode input was connected to the ground to provide a stable potential recording.

To conduct the Surface potential recordings, the output voltages from the 4-channel module were read by a Pico Technology PicoLog ADC-20 data logger **(Figure 7d)** via PicoLog software running on a PC **(Figure 7e)**. Silver electrodes 0.5mm in diameter were chloridized with HCl (0.5 M), stored at room temperature and rechloridized after several uses. The contact at the electrode–leaf interface was a drop of 10mM KCl in 0.5% (w/v) agar placed so that the silver electrode did not damage to the cuticle, and the reference electrode was placed in the soil by creating an agar-bridge **(Figure 7a)**.

2.15.2 Bacterial-activated electrical signals

To investigate whether an electrical activity is associated with gene-for-gene responses a fully developed leaf of 5-week-old Col-5 rosettes were challenged with DC3000*avrRpm1* and the working electrode (W2) placed on the petiole of an infected leaf. The other electrodes W1, W3 and W4 were placed on the petiole of systemic leaves, while the reference electrode (R) was placed in the soil **(Figure 7a)**. Surface potential change was measured from the reference electrode to the working electrode. Control recordings over extended time, predominately showed consistent surface potential. Two different parameters were implemented to characterize these signals, amplitude (change in wavelength over period of time from infection) and duration (time from amplitude midpoint to arrival at original basal value) **(Figure 7b)**.

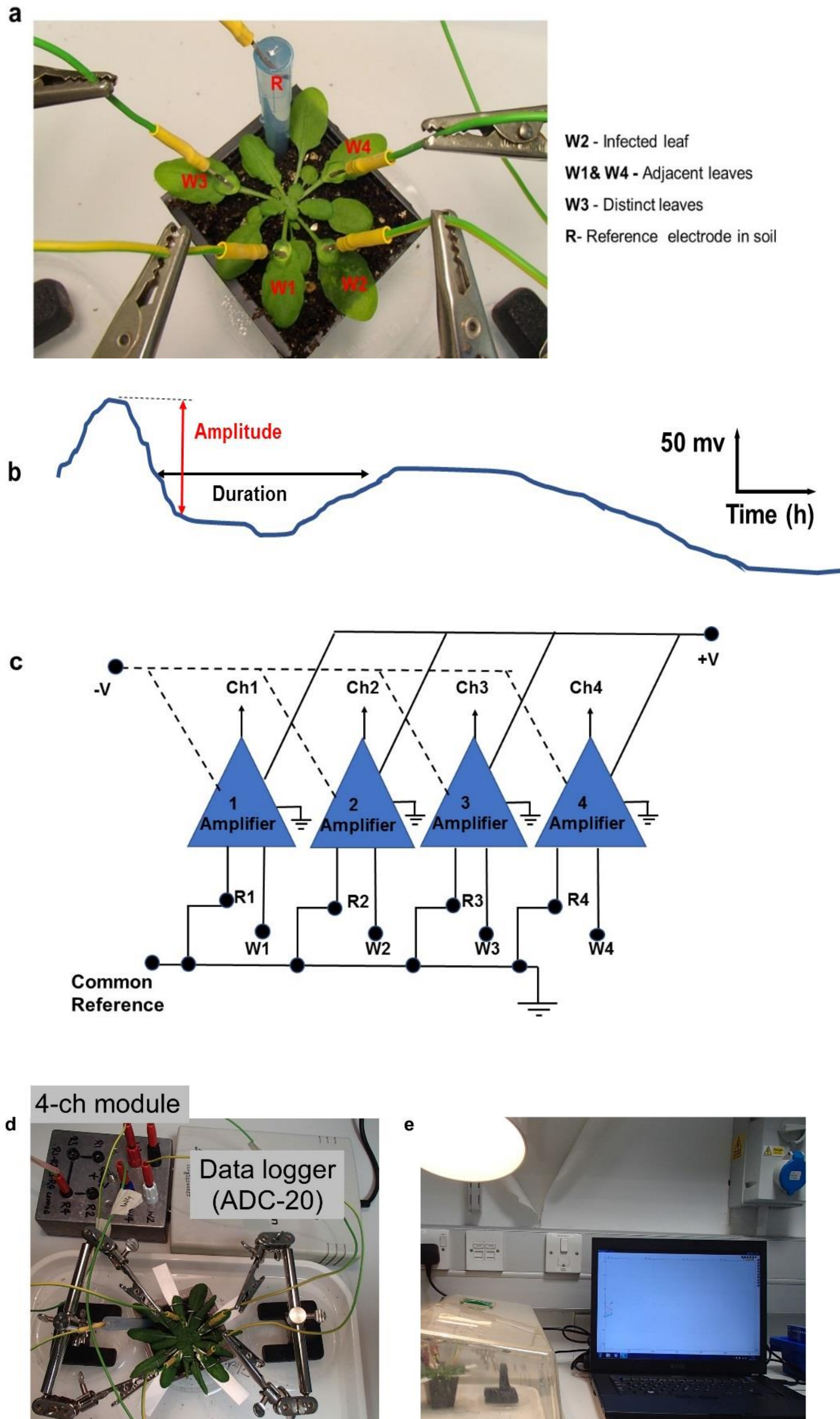


Figure 7. Schematic image for plant electrophysiology set up:

a. Set up of plant electrophysiology. W2 - Infected leaf, W1& W4 - Adjacent leaves, W3 - Distinct leaves, R- Reference electrode in soil. **b.** Two distinct variables, duration and amplitude of surface potential were recorded. **c**. Instrumentation amplifier circuit diagram of a four channel module. **d.** Connected the 4-ch module to the ADC-20. **e.** Recorded data by connecting Voltage input data logger (ADC-20) to a PC.

2.16 Extraction and analysis of nucleic acids

2.16.1 Extraction of plasmid DNA

For extracting high quality plasmid DNA in the range of 20 µg from a 5-10 ml culture, a commercially available QIAPrep Spin Miniprep kit was used in which DNA binds to a silica based spin column. For culture volumes of 100 ml or more, a QIAGEN Plasmid Kit was used. The Plasmid DNA extracted was quantified using a spectrophotometer, where 1 OD at λ_{260nm} equates to 50 mg/ml of DNA

2.16.2 Extraction of plant DNA

Plant genomic DNA was extracted by a phenol-chloroform extraction method described in (Stephen L. Dellaporta, 1983), using “Shorty buffer” (0.2 M Tris-HCL, pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS). Excised leaf tissue (1-2 leaves) from each plant was put in a 1.5ml microfuge tube and this was crushed with pestle by adding 500µl of Shorty buffer and the sample extracted with 500µl Phenol/chloroform by vortexing (~ 30 s). This emulsion was spun for 5 min at RT in micro-centrifuge at max speed. Next, 400µl of the aqueous (upper phase) was transferred into a clean microfuge tube and 400 µl of isopropanol (RT) added. The solution was mixed by inversion and spun down for 10min at RT at max speed in a microfuge. The pellet was washed with ethanol 70%, briefly vortexed, spun down as before and the supernatant decanted onto a blue towel. The sample was quickly spun and any excess liquid removed using a pipette tip and the pellet re-suspended in 100µl sterile water and the re-suspended genomic DNA stored at -20° freezer.

2.16.3 Amplification of DNA fragment

Polymerase Chain Reaction (PCR) was used to amplify DNA fragments of interest (Saiki *et al.*, 1985). The relevant primers were purchased from MWG (Eurofin Genomics, United Kingdom).

2.16.4 Electrophoresis of DNA samples

DNA fragments obtained by PCR and restriction digestion were visualized by agarose gel electrophoresis using ethidium bromide in 0.5X TAE buffer (45 mM Tris Acetate, 1 mM EDTA, pH 8), the agarose concentration reflecting the amplicon sizes to be visualized. The separated DNA fragments were then eluted using QIAquick Gel Extraction kit according to the manufacturer’s instructions (Qiagen).

2.16.5 Extraction of RNA from plant

Total RNA from plants was extracted using guanidinium hydrochloride as described (Logemann *et al.*, 1987). In brief, the plant tissue (2-3 leaves) was harvested into liquid nitrogen. Frozen leaves were ground into a fine powder in a mortar and pestle pre-cooled with liquid nitrogen. Special care was taken to ensure the powdered tissue did not thaw. Next, 600µl of Z6 solution (8M guanidinium hydrochloride, 20mM MES PH-7.0, 20mM EDTA) was added to the powdered tissue and mixed thoroughly with a pestle (should freeze and must be mixed as it thaws).The mixture was transferred to a microfuge tube on ice and 1 vol. of Phenol/chloroform was added and the tube vortexed vigorously then centrifuged for 5min at 4°C at max speed in a microfuge. The aqueous phase was transferred to a new Eppendorf tube. 1/20 vol. of acetic acid 1M and 0.7 vol. ethanol 100% were added and samples mixed by vortexing. These RNA samples were kept on ice for 30 min then spun down at for 20 min at max speed in a microfuge at 4°C. The pellet was washed with 70% ethanol and re-suspended in 100µl of ddH_2O. Total RNA was quantified using a Nano drop.

2.16.6 Electrophoresis of RNA samples

Gel electrophoresis of RNA was performed using denaturing formaldehyde agarose gels as described by (Sambrook, 1989). In brief, 2µg of RNA + water (38%) was denatured at 65°C for 15 min in formamide (40%), formaldehyde (12%), 10X MEN and ethidium bromide (10%) and resolved in 1.5% agarose gels containing 15% formaldehyde and MEN buffer at PH- 7.0 (200mM Morpholino Propane Sulfonic acid (MOPS), 50mM sodium acetate and 20mM EDTA). Gels were run gel at 100 V until marker dye has migrated 2/3 down the gel in MEN buffer under a fume hood. Confocal microscopy

2.17 Confocal Laser Scanning Microscopy

2.17.1 Sample preparation

Pro*A70*::*A70*SP::GFP (T2) plants were challenged with DC3000*avrRpm1*. Systemic leaf of infected plant were cut around ~4 hpi and then dissected the leaf petiole in small section (4-5mm) and mounted on glass slide with the drop of water or PP2 solution and then covered the sample with cover slip. Monitored the samples under microscope.

2.17.2 Parameters for confocal microscopy

Freshly excised leaf samples were mounted in water and imaged on a Zeiss LSM 880 confocal microscope with a 63× oil immersion objective lens. GFP was excited at 488. GFP was detected in the 494 to 513 nm range. Propodeum iodide (PI) was excited using the laser according to the fluorescent protein observed (488 nm for GFP) and the signal was detected in the range from 596 to 710 nm. Images were acquired using a defined region of interest with an average of four, with 1,024 × 1,024 pixels of image size and 8-bit image depth, taking care that every part of each image remained fully within the dynamic range of pixel intensity.

3 Chapter 3 Use of the *A70::LUC* reporter to study SAR signalling mechanisms

3.1 Introduction

Luciferase reporter systems have been widely used to visualize in real time, changes in gene expression (Velten *et al.*, 2008, Greer & Szalay, 2002). Transgenic *Arabidopsis* lines expressing pathogen responsive promoter-luciferase fusions, including *A70::LUC*, were first generated in our lab by Dr Marta de Torres Zabala. This thesis will specifically focus on systemic signals following DC3000*avrRpm1* infection, and specifically, one of these lines; the *A70* (At5g56980) promoter fused with a *LUC* reporter (**Figure 5**). *A70* (At5g56980), a gene of unknown function, was identified by AFLP as an early (4 hpi) systemically induced gene following DC3000*avrRpm1* inoculation. Interestingly, it is also induced locally in a PAMP associated manner (Truman *et al.*, 2007).

This chapter is primarily focusses on several underpinning activities such as screening for strong systemic expressing *A70::LUC* plants in response to elicitation and establishing the dynamics of gene-for-gene induced *A70::LUC* expression. This chapter answered several questions such as; Is there any specific pattern of activation?; What hormonal compound acts as an inducer molecule for activation of *A70::LUC*?; Are PAMPs involved in the *A70::LUC* expression?; How does *A70::LUC* activation correlate to SAR and if it does, can we use this *A70::LUC* reporter model to study and understand the defence mechanism in plant against bio-trophic pathogen?

With the aim of identifying new components of the plant immune system, the molecular mechanism of *A70* activation in the defense response triggered by AvrRpm1 in *Arabidopsis thaliana* was investigated. Based upon the results, *A70* gene we

conclude that *A70* expression is rapidly transiently induced by avirulent strains of *P. syringae* pv. *tomato* via an JA-mediated pathway in a complex manner that involves multicomponent inputs.

3.2 Results

3.2.1 Selection of *A70::LUC* plants with strong reporter expression

Total 8 Independent *A70::LUC* transgenic lines were initially screened for luciferase expression in laboratory before I start my project. I started to work on only one independent line which is selected based on luciferase expression up on avrRpm1 infection. I screened *A70::LUC* plant for resistance to hygromycin (50 µg ml^{-1}). For a secondary screen, *A70::LUC* transgenic plants were infiltrated with DC3000*avrRpm1* and selected for strong luciferase activity. *A70::LUC* plants infiltrated with DC3000*avrRpm1* (OD_{600} = 0.2) were placed immediately into a dark cabinet, were photon emission was recorded with a CCD camera for 24 h, capturing images every 10 min. Out of all inoculated *A70::LUC* plants from T2 generation, 12 individual plants were selected for a strong luciferase signal. Luciferase activity could first be detected in DC3000*avrRpm1* challenged leaf petioles at around 2.5-3 hpi, the signal then moved towards the centre of the rosette (over a period of 30-40 minutes) and subsequently propagated into the petioles of systemic leaves (**Figure 8**). Thus a mobile signal is initiated around 2.5 hpi following DC3000*avrRpm1* challenge and this propagates to the rest of the plant causing transcriptional activation of *A70*. Subsequently, the luciferase signal intensity declined and no signal could be detected with our imaging system 10 hpi in the dark.

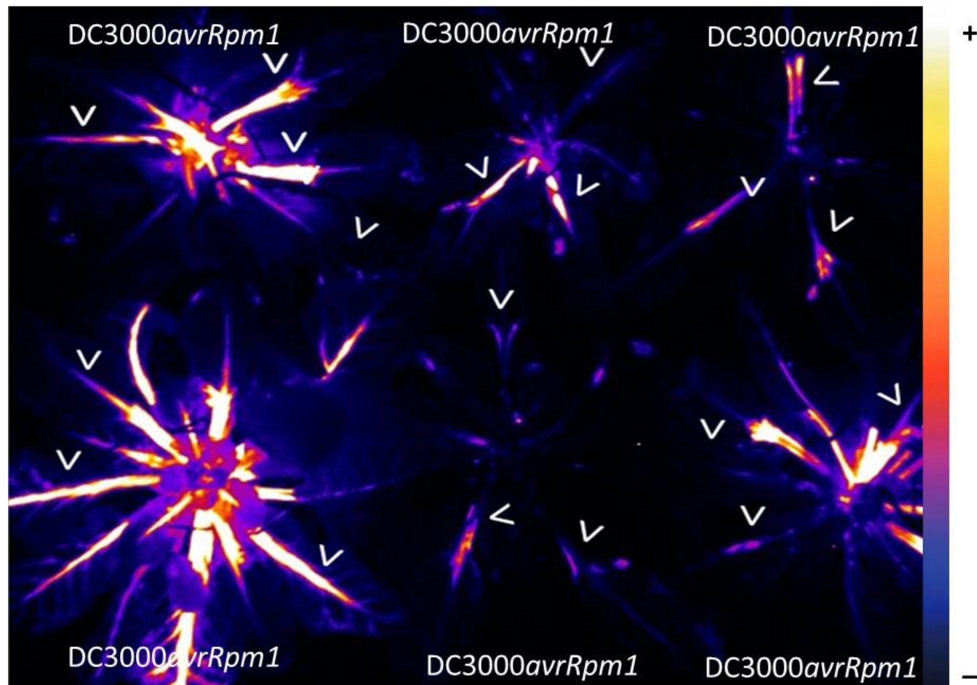


Figure 8. Selection of *A70::LUC* T2 plant based on luciferase activity*:*

Image of luciferase activity of the *A70::LUC* expressing plants challenged with DC3000*avrRpm1* (~2 x 10^8 cfu ml^{-1}). Arrowheads indicate challenged leaves. Plants were screened for strong A*70::LUC* expression. Images were captured 3 h after transfer to a dark box. Acquisition time 10 min. Picture was false coloured. The color scale indicates signal intensity from 0 (blue) to saturation (white).

Based on the preliminary experiments conducted above, plants showing the strongest *A70::LUC* expression were selected and seeds were bulked. Progenies of the selected plants were then tested for consistently strong luciferase activation by inoculation with DC3000*avrRpm1* compared to $MgCl_2$ (mock) (**Figure 9**).

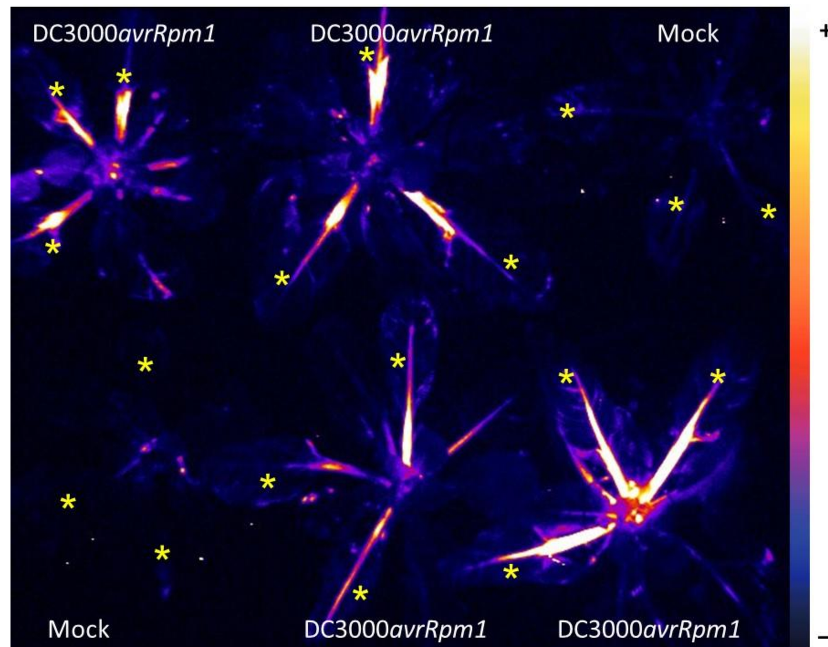


Figure 9. Validation of *A70::LUC* expression:

A70::LUC expressing plants (T3 generation) challenged with DC3000*avrRpm1* (~2 x 10^8 cfu ml^{-1}) asterisks indicate the challenged leaves. All infiltrated plants show strong *A70::LUC* expression. No luciferase signals were evident after $MgCl_2$ challenge (mock). Images were captured 3:40 hpi. Acquisition time 10 min. Picture was false coloured. The colour scale indicates signal intensity from 0 (blue) to saturation (white).

3.2.2 Investigating dynamics of gene for gene resistance-induced *A70::LUC* expression

If the host plant possesses a disease resistance (R) protein competent to directly or indirectly recognize T3Es (avirulence gene products), it can induce different signalling pathways resulting in a localized HR and restricted pathogen growth (Belkhadir *et al.*, 2004, Dangl & Jones, 2001). As *A70* is induced systemically remarkably rapidly and specifically following a gene-for-gene interaction, we hypothesized that this was part of the very early systemic signalling network.

To verify this, *A70::LUC* plants were infiltrated with virulent *Pst* DC3000, a type III secretion-defective mutant (DC3000*hrpA*), and avirulent DC3000*avrRpm1* at a concentration of 2 × 10^8 cfu ml^{-1} to investigate the role of *A70* in response to virulent,

T3Es-deficient, and avirulent interactions. After initial infection, all infected plants were placed immediately into a dark box. **Figure 10** shows by real-time integration of photons that LUC activity was induced in the inoculated leaf as well as in systemic leaves of DC3000*avrRpm1* but not the other challenges (full video is provided in appendix 3 CD). This shows that *A70::LUC* expression is specific to gene-for-gene recognition. DC3000*avrRpm1* induced rapid and sustained *A70::LUC* expression in challenged leaves, beginning around 3 h post inoculation (hpi) which almost immediately propagated down the petiole of the challenged leaf into systemic leaves where *A70::LUC* expression lasted for approximately 5 to 5:30 h, with maximal intensity recorded between 4 to 5 hpi. No *A70::LUC* expression was detected in mock-challenged ($MgCl_2$) leaves, indicating that *A70::LUC* expression was not generated through perturbation of the apoplastic osmotic pressure or wounding associated with the mock inoculation process.

3.2.3 Spatial specificity of *A70::LUC* expression

The spatial distribution of *A70::LUC* expression during an incompatible interaction was monitored by examining sequential images of photon counts integrated for 10 min. **Figure 10b** traces the spatial-temporal patterns of *A70::LUC* expression in a single leaf inoculated with DC3000(*avrRpm1*) and in systemic leaves. *A70::LUC* expression was first generated within the leaf midrib beginning 3 hpi. As the interaction develops, a wave of *A70::LUC* expression then moves rapidly down the petiole and then propagates through the midrib of the systemic leaves (**Figure 10b).**

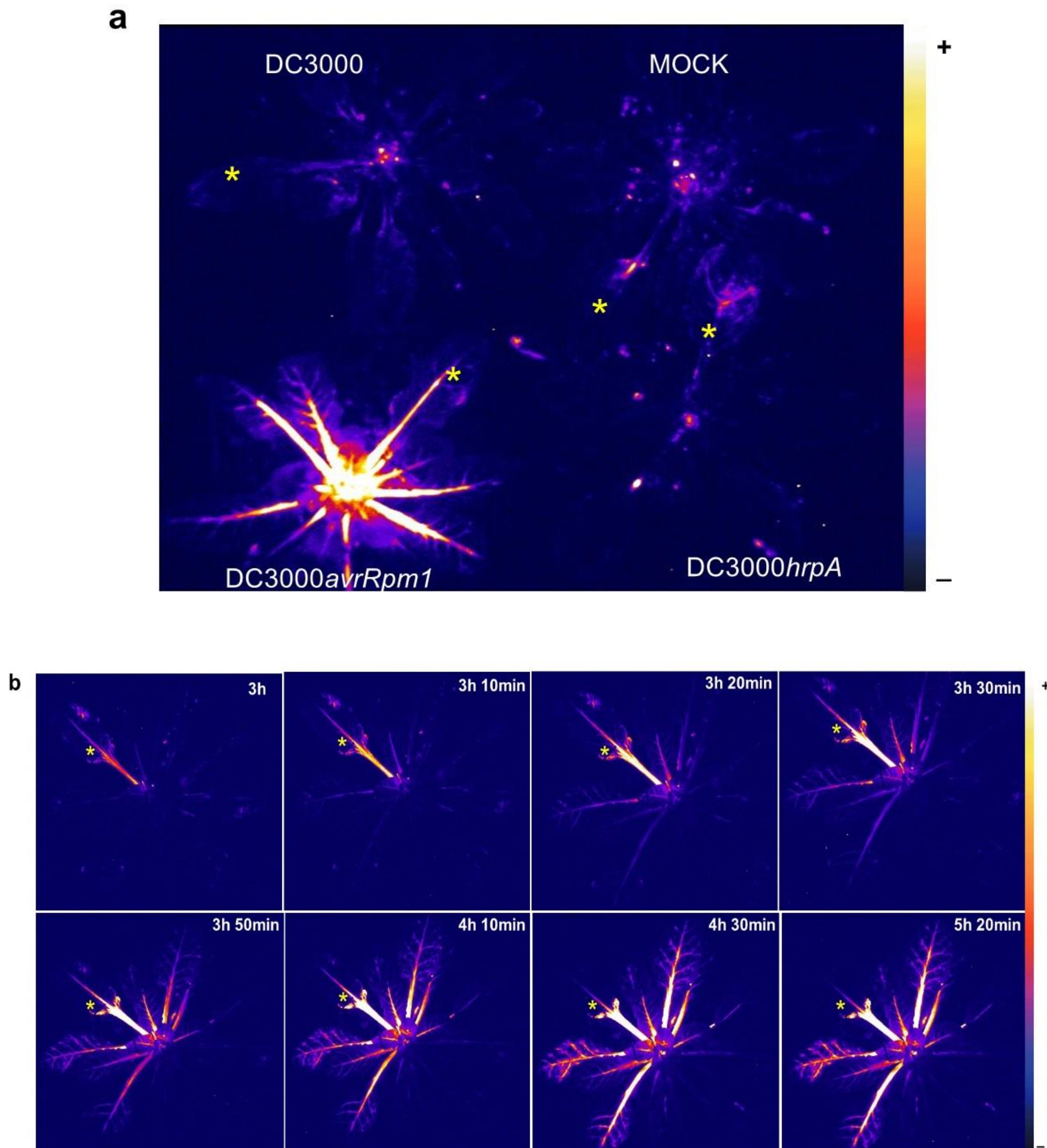


Figure 10. *A70::LUC* expression by *P. syringae* strains:

a. To assess gene for gene induced *A70::LUC* expression in primarily treated leaves, *A70::LUC* plants were infiltrated with 10 mM $MgCl_2$, *Pst* DC3000*avrRpm1*, *Pst* DC3000 or *Pst* DC3000*hrpA* (~2 x 10^8 cfu ml^{-1}). Only *A70::LUC* plants inoculated with *Pst* DC3000*avrRpm1* showed strong luciferase activity at 4hpi. **b.** In order to examine spatial specificity of *A70::LUC* expression dynamics, an *A70::LUC* plant was infiltrated with *Pst* DC3000*avrRpm1* (~2 x 10^8 cfu ml^{-1}), and LUC activity was assessed as indicated in sequential images. Pictures are false colored. The color scale indicates signal intensity from 0 (blue) to saturation (white). The figure **a** and **b** representative of six separate experiments.

3.2.4 ETI based *A70::LUC* expression

As *A70* is induced systemically rapidly, and specifically, following a gene-for-gene interaction, therefore, to address whether the luciferase expression of *A70* in local and systemic tissue is effector based? We infiltrated *A70::LUC* plants with different elicitors DC3000*avrRpt2* or DC3000*avrRps4* and DC3000*avrRpm1* as a positive control at a concentration of 2 × 10^8 cfu ml^{-1} to understand the gene for gene mediated defense mechanism, and after infection all infected plants were placed immediately into a dark box. () shows the real-time integration of light arising from LUC activity that was induced in the inoculated leaf as well as in systemic leaves by DC3000*avrRpm1* followed by DC3000*avrRps4* and then DC3000*avrRpt2* (**Figure 11**) (Full video is provided in appendix 3 CD). This shows that *A70::LUC* expression is specific to gene for gene recognition. The avirulent DC3000*avrRpm1* bacteria induce a rapid and sustained *A70::LUC* expression in challenged leaves, beginning around 3.50 hpi (**Figure 11b**) followed by DC3000*avrRps4* *A70::LUC* expression starts at 14.30 hpi (**Figure 11c**) after that finally DC3000*avrRpt2* shows *A70::LUC* expression at 18.20 hpi (**Figure 11d**). Therefore, these results confirm that Recognition of specific effectors leads to ETI and this ETI induces *A70::LUC* expression. Activation of *A70::LUC* expression requires effector delivery and timing of induction is specific to particular *R-Avr* combinations (**Figure 11**).

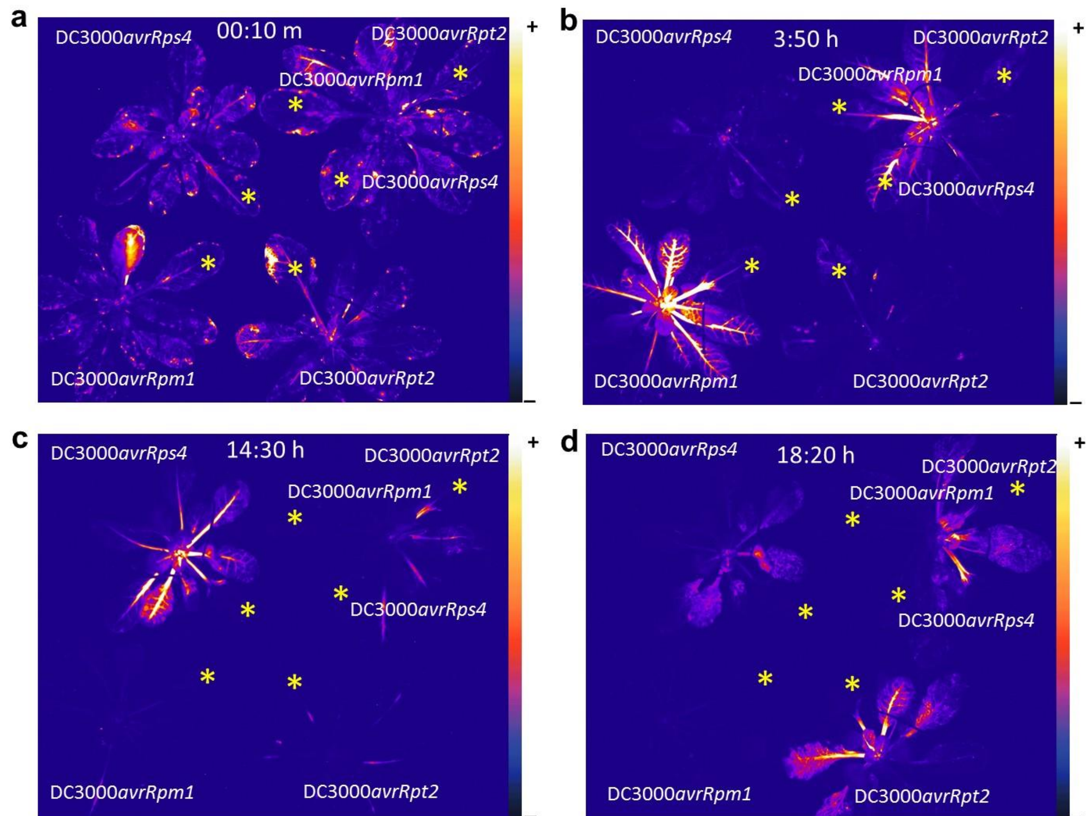


Figure 11. ETI activates *A70::LUC* expression:

A70::LUC driven LUC activity in local or systemic tissues after infecting single individual leaf with bacterial suspensions of avirulent DC3000*avrRpm1*, DC3000*avrRps4*, DC3000*avrRpt2*, at an OD_{600} 0.2. Images taken at indicated time point (**a. b. c. d.)**. Out of four plants, three plants infected with particular bacterial as labeled. Forth plant which in in top right corner in the image infected with all three bacteria as per mentioned in label. LUC activity that was induced in the inoculated leaf as well as in systemic leaves by DC3000*avrRpm1* followed by DC3000*avrRps4* and then DC3000*avrRpt2*.

3.2.5 Investigating PAMP-induced defense signalling in *A70::LUC* plants.

Recently emerging evidence shows that PTI triggers SAR (Mishina & Zeier, 2007). General immune elicitors were tested to check potential contribution to *A70::LUC* induction. To determine whether flagellin, a well-characterized PAMP involved in basal and non-host resistance, can trigger *A70::LUC* expression, *A70::LUC* plants were infiltrated with 5 µM of flg22, a peptide corresponding to the elicitor-active epitope of flagellin (Gomez-Gomez *et al.*, 1999). Likewise, other well-known elicitors such as the bacterial elongation factor Tu (EF-Tu epitopes) elf18, or the *At*Pep1 DAMP, a 23-amino acid endogenous peptide elicitor, were infiltrated into one leaf of *A70::LUC* plants and LUC activity was compared with the positive control, *Pst* DC3000*avrRpm1* infiltrated at a concentration of 2×10^{8} cfu ml^{-1}. There was no significant difference in *A70::LUC* expression at 2 hpi in elf18, flg22 or *At*Pep1 and *Pst* DC3000*avrRpm1* treated plants (**Figure 12**) (full video is provided in appendix 3 CD). This was expected because elicitors would show a rapid local response in *A70::LUC* as *A70* was originally identified as PAMP responsive (Truman *et al.*, 2007). However, no systemic luciferase expression was induced by these peptides whereas *Pst* DC3000*avrRpm1* infiltration induced a strong *A70::LUC* expression in systemic leaves at 4:10 h after infection. So, *A70* is involves in the SAR pathway.

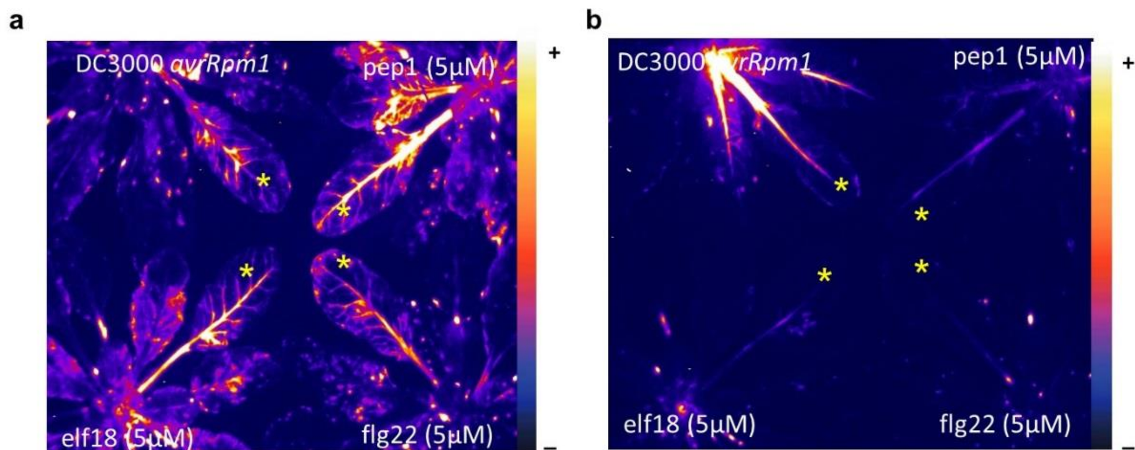


Figure 12. ***A70::LUC* expression after infiltration of flg22, elf18 and *At*Pep1:**

A70::LUC plants were infiltrated with 5µM flg22, elf18, or *At*Pep1 peptide solutions, or *Pst* DC3000*avrRpm1* at OD_{600} = 0.2. **a.** The image was taken at 2 hpi and **b.** at 4:10 hpi. Asterisks indicate challenged leaves. *A70::LUC* challenged leaves do not show a systemic *A70::LUC* expression after treatment with 5µM flg22, elf18 or *At*Pep1. Pictures are representative of three independent experiments. Pictures are false colored. The color scale indicates signal intensity from 0 (blue) to saturation (white).

3.2.6 PTI Inhibits the RPM1-Dependent HR and *A70::LUC* expression.

Arabidopsis induces PTI in response to PAMP treatments (Felix *et al.*, 1999), but PTI has been reported to inhibit gene-for-gene induced HR responses (Newman *et al.*, 2000, Crabill *et al.*, 2010a), To further expand these studies into our pathosystem pre-treatment flg22, elf18 and *At*Pep1 prior to challenge with *Pst* DC3000*avrRpm1* was tested.

PTI-inducing peptides (5 µM) or water as a mock (pre-treatment) were infiltrated into an *Arabidopsis* leaf and 16 h later leaves challenged with *Pst* DC3000*avrRpm1* (OD_{600} 0.2). Presence or absence of HR was scored in the same leaves 8 h after the bacteria infiltration. Pre-treatment with 5 µM flg22, elf18 and *At*Pep1 can restrict the RPM1-dependent HR, as DC3000*avrRpm1* was unable to cause an HR in PTI-induced leaves (**Figure 13**).

Next, the effect of pre-treatment with flg22, elf18 and *At*Pep1 on local and systemic *A70::LUC* expression was examined. Leaves were pre-treated with 5 μM of flg22, elf18 and *At*Pep1 and 16 h later, the same leaves were infiltrated with DC3000*avrRpm1* (OD_{600} 0.2). Interestingly, the DC3000*avrRpm1*-induced *A70::LUC* signal could only be detected in mock pre-challenged leaves, but not leaves pre-treated with flg22, elf18 and *At*Pep1 (**Figure 13**) (full video is provided in appendix 3 CD).

In summary, *avrRpm1*-triggered HR and its suppression by flg22, elf18 & *At*Pep1 were observed in wild-type col-0 and RPM1-mediated luciferase activation was abolished in *A70::LUC* plants after pre-treatment of PTI-inducing peptides, indicating that the *A70::LUC* inducing signal is most likely HR dependent.

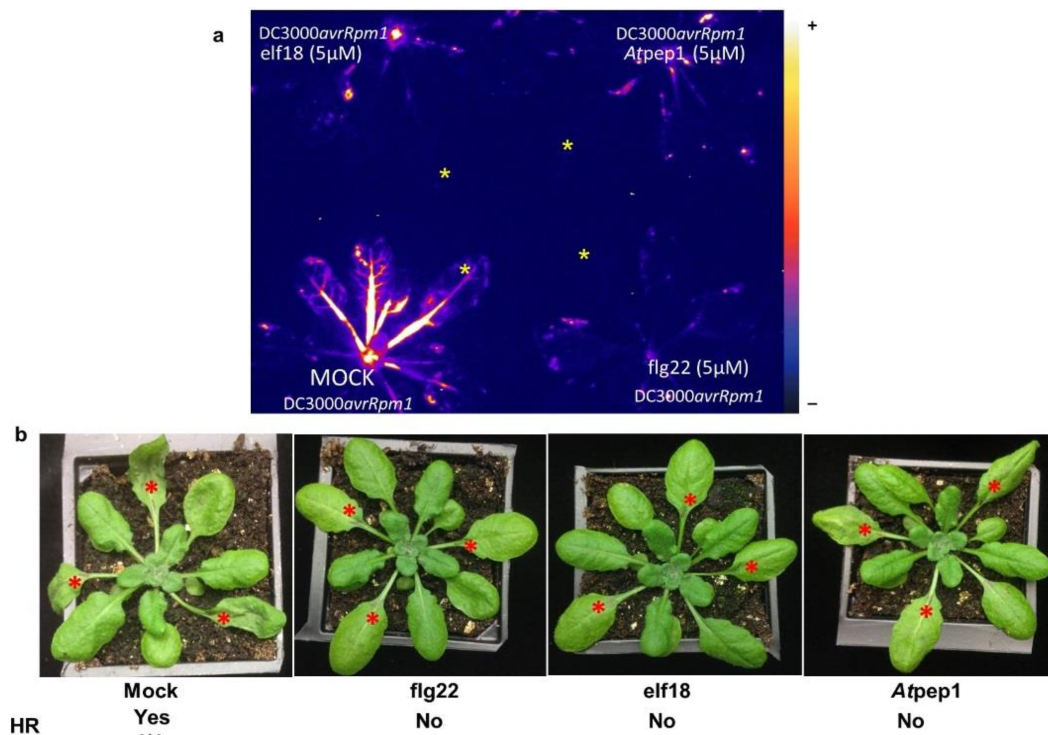


Figure 13. RPM1-dependent luciferase activity in *A70::LUC* plant pre-treated with flg22, elf18 or *At*Pep1:

a. *A70::LUC* plants were pre-infiltrated with 5 µM flg22, elf18, *At*Pep1 or mock (water). After 16 h, the same leaves were infected with *Pst* DC3000*avrRpm1* (OD_{600} 0.2). The image was taken 4:40 h after infiltration (acquisition time 10 min). There was a strong RPM1-based signal in *A70::LUC* plants pre-infiltrated with water (mock), which was absent in leaves pre-challenged with PTI-inducing peptides. Representative of three independent experiments.

b. PTI-induction inhibits avrRpm1-induced HR in *A. thaliana.* Leaves of Col-0 plants was pre-infiltrated with 5 µM flg22, elf1, *At*Pep1, or mock (water). 16 hpi DC3000*avrRpm1* (OD_{600} 0.2) was infiltrated into the same leaves. Leaves were assessed for the presence or absence of an HR 8 hpi. The fraction below each plant indicates the number of times that the HR was inhibited over the total number of times the assay was performed. Photos were taken 7 h after DC3000*avrRpm1* infiltration.

3.2.7 The role of the plant hormones, JA, SA, and ABA in *A70::LUC* expression

To determine whether plant hormones played a role in activating *A70* during gene-for-gene responses we explored the impact of the plant hormones SA, JA and ABA on *A70::LUC* expression. The rationale was as follows: SA accumulation in non-inoculated leaves is essential for SAR development (Vernooij *et al.*, 1994, de Torres-Zabala *et al.*, 2007). ABA induces or suppresses the immune response depending upon the type of plant pathogen interactions (Yazawa *et al.*, 2012). A JA signalling system modulates plant immune responses and confers resistance or susceptibility against pathogens mainly because of antagonistic crosstalk between the JA/Et and SA signalling pathways. (Moffat *et al.*, 2012). As part of the hypothesis that MeSA functions as a SAR signal (Park *et al.*, 2007), JA was suggested to strengthen the MeSA component of SAR signalling (Vlot *et al.*, 2008). However, the significance of JA *per se* for SAR signalling has been questioned as Long distance signal (Chaturvedi *et al.*, 2008). Chaturvedi shown that SAR inducing activity in petiole exudates of Arabidopsis is independent of JA Therefore the role of JA signalling in activation of *A70* in *A70::LUC* plants was explored, while simultaneously determining the impact of ABA and SA challenges.

JA, SA and ABA were infiltrated into *A70::LUC* plants at concentrations of 1mM SA and ABA and 250 μM of JA (**Figure 14a**). Luciferase activity was only recorded in JA treated leaves and importantly, this signal does not move to systemic leaves, therefore JA can induce *A70::LUC*, but it is unclear whether JA is involved in systemic *A70::LUC* induction or not. No *A70::LUC* activation in local and distant leaves was seen in ABA and SA challenged leaves (**Figure 14a**) (full video is provided in appendix 3 CD), indicating that local *A70::LUC* expression is JA but not SA or ABA inducible.

In addition, other forms of jasmonates were tested to see if they can activate *A70::LUC* expression. *A70::LUC* plants were infiltrated with 2.5 µM solutions of coronatine, MeJA the JA-Ile's; JA-S.ILe and JA-L.Ile or 1% ethanol as a mock treatment. It has been reported that bacterial pathogens use coronatine as a molecular mimic of JA-Ile, (Katsir *et al.*, 2008) to cause hormone imbalance in plants and thereby suppress plant defence responses to bacterial colonization. (+)-7-Iso-JA-Ile, a conjugate between one of the four JA stereoisomers, (3R,7S)-JA, and L-isoleucine (Fonseca *et al.*, 2009), is the most active naturally occurring jasmonate synthesized by plants**.** Results (**Figure 14b** (full video is provided in appendix 3 CD)) indicated that the coronatine infected leaf shows by far the strongest *A70::LUC* activation compared to any other treatment.

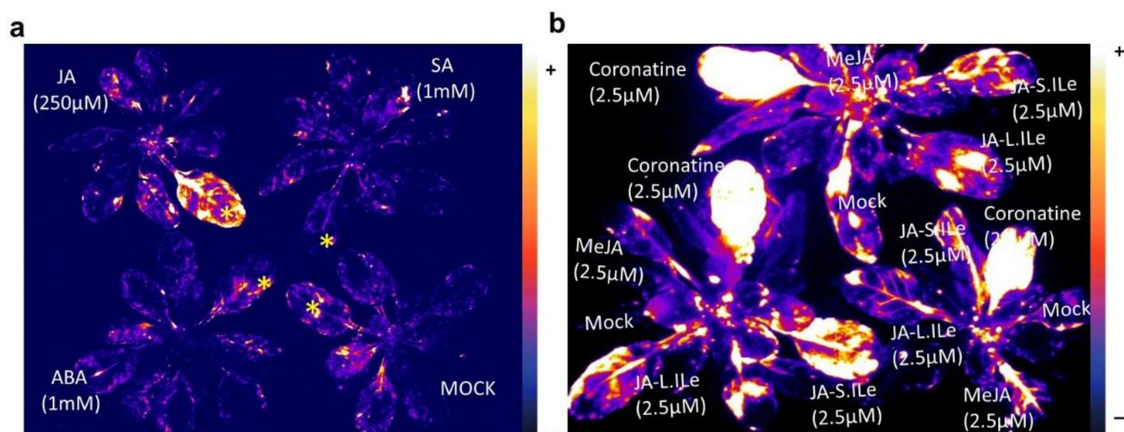


Figure 14. Luciferase activity in *A70::LUC* plants treated with JA, ABA or SA:

a. *A70::LUC* plants infiltrated with 250 µM JA, 1 mM ABA, 1 mM SA or mock (1% ethanol). The image was taken 1:30 h after infiltration (acquisition time 10 min). A strong signal in *A70::LUC* plants was only detected in the JA infiltrated leaf. No *A70::LUC* signal was observed in leaves treated with 1 mM SA, ABA or mock challenges. **b.** *A70::LUC* plants were infiltrated with the 2.5µM coronatine, MeJA, JA-S.Ile, JA-L.Ile or 1 % ethanol as a mock. The luciferase image was taken 1:30 h after treatment (Acquisition time 10 min). *A70::LUC* plants show strong *A70::LUC* expression to coronatine and weak response to JA-S Ile, JA-L Ile and MeJA. The picture is representative of three independent experiments. Both picture were false colored. The color scale indicates signal intensity from 0 (blue) to saturation (white).

To investigate, whether pre-treatment of ABA, JA, or SA affect local and systemic RPM1-based *A70::LUC* induction in Arabidopsis leaves, their role in long-distance transport was examined. Leaves were infiltrated with 1 mM ABA, 1 mM SA or 250 μM JA and 16 h after treatment, the same leaves were infiltrated with *Pst* DC3000*avrRpm1.* Infected plants were monitored with a CCD camera for luciferase activity during 24 h. interestingly and unexpectedly, the DC3000*avrRpm1*-induced *A70::LUC* signal was abolished in ABA pre-treated leaves (**Figure 15**). In contrast, the induction of the DC3000*avrRpm1* signal in *A70::LUC* plants was neither enhanced nor suppressed by JA and SA pre-treatment (**Figure 15** (full video is provided in appendix 3 CD)).

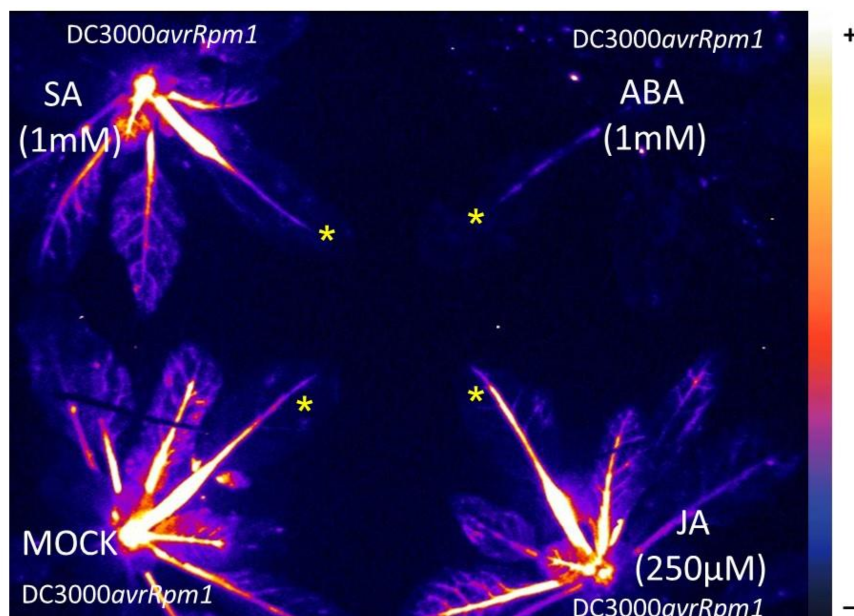


Figure 15. RPM1-dependent luciferase activity in *A70::LUC* plants pre-treated with JA, ABA or SA:

A70::LUC plants were pre-infiltrated with the 250 μM JA, 1 mM ABA, 1 mM SA, or mock (1% ethanol), and then after 16 h, the same leaves were inoculated with *Pst* DC3000*avrRpm1* (OD_{600} 0.2). The image was taken 4:30 h after bacteria infiltration (acquisition time 10 min). There was a strong RPM1-based signal in *A70::LUC* plants in JA, SA and mock pre-infiltrated leaves, but no RPM1-based *A70::LUC* signal observed in leaves pre-treated with 1 mM ABA.

3.2.8 RPM1-dependent *A70::LUC* systemic expression is independent of bacterial coronatine.

The plant immune signal SA plays an essential role in plant defence against biotrophs and hemi-biotrophs such as *P. syringae*. However, JA can counteract the SA-mediated defense to adjust the immune response through signalling crosstalk (Kunkel & Brooks, 2002, Spoel & Dong, 2008). Since *Pst* derived coronatine (COR) can activate the JA signalling pathway by mimicking to structurally similar JA-isoleucine (JA-Ile) (Browse, 2009), and suppress SA accumulation in *Arabidopsis* (Brooks *et al.*, 2005), we used coronatine-deficient (cor^-) *Pst* mutants (Brooks *et al.*, 2004) to test whether *RPM1*-mediated *A70::LUC* expression was coronatine dependent. *A70::LUC* leaves were infected with *Pst* DC3000, *Pst* DC3000*avrRpm1*, or their COR-deficient mutants *Pst* DC3000cor^{-1}/cor^{-2} or *Pst* DC3000*avrRpm1/*cor^{-1}/cor^{-2}. The results shows that both DC3000*avrRpm1* and DC3000*avrRpm1/*cor^{-1}/cor^{-2} can induce *A70::LUC* expression to the same extent based on strength of signal. Ruling out coronatine production in *Pst* DC3000*avrRpm1* as the driver of the RPM1-mediated *A70::LUC* systemic signature (**Figure 16**) (full video is provided in appendix 3 CD).

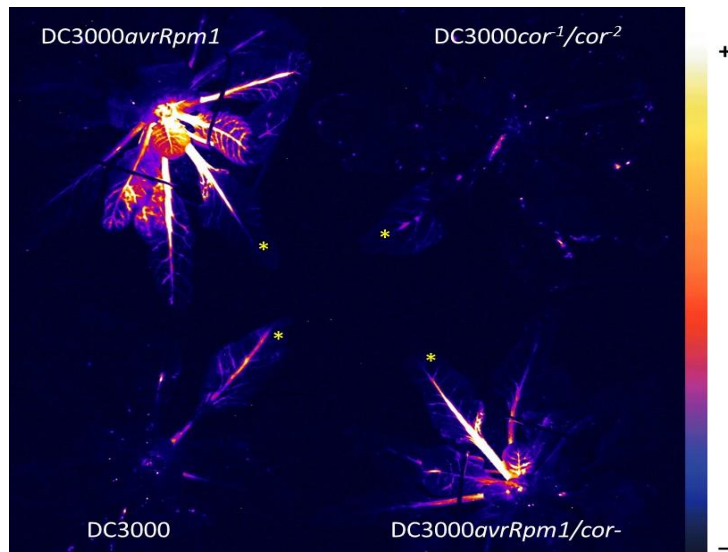


Figure 16. Coronatine production is not required for the RPM1 dependent systemic luciferase activity in *A70::LUC* plants:

A70::LUC plant infiltrated with *Pst* DC3000, DC3000*avrRpm1,* DC3000*cor^{-1}/cor^{2}*, DC3000*avrRpm1/cor $^{-1}$/cor^{2}* (OD_{600} 0.2). The image was taken 4:40 h after infiltration (acquisition time 10 min). Picture was false coloured. The colour scale indicates signal intensity from 0 (blue) to saturation (white).

3.2.9 Role of phloem exudates in activating *A70::LUC* expression

Immune signals are transferred from infected to non-infected leaves through the phloem to generate SAR (Tuzun S, 1985). Therefore, exudates were collected from Col-0 plant leaves that had been infected with *pst* DC3000, DC3000*avrRpm1,* DC3000*hrpA*, or treated with 10 mM $MgCl_2$ or non-treated (mock). Phloem exudates were collected from the same leaves in two sets, firstly 2-7 hpi after treatment, corresponding to the time frame for DC3000*avrRpm1*-induced systemic *A70::LUC* expression), and secondly overnight between 7-24 hpi. These phloem exudates were infiltrated into leaves to test whether they could elicit *A70::LUC* expression. All phloem exudates collected in the first set, 2-7 hpi post infection, were able to induce *A70::LUC* expression in the locally challenged leaf (**Figure 17**) Full video is provided in appendix 3 CD).

Figure 17. Role of phloem exudates (collected 2-7 hpi) in activation of *A70::LUC* expression: *A70::LUC* plants infiltrated with the phloem exudates collected from Col-0 leaves infected with *Pst* DC3000, DC3000*avrRpm1*, DC3000*hrpA*, or treated with 10 mM $MgCl_2$ or non-treated. Luciferase image taken 1 h after treatment. Acquisition time 10 min. *A70::LUC* leaves show strong *LUC* expression with all phloem exudates.

Unexpectedly, only phloem exudates from DC3000 infiltrated plants collected overnight, i.e. 7-24 h after treatment, induced *A70::LUC* expression (**Figure 18**) (Full video is provided in appendix 3 CD). This might be because of the accumulation of coronatine, which can be observed after 8 hpi in DC3000 infected leaves (de Torres Zabala *et al.*, 2009). To confirm this signal is because of coronatine, it would be exciting to see effect of Ph. Exudate collected in 7-24 h after treatment of cor- bacteria.

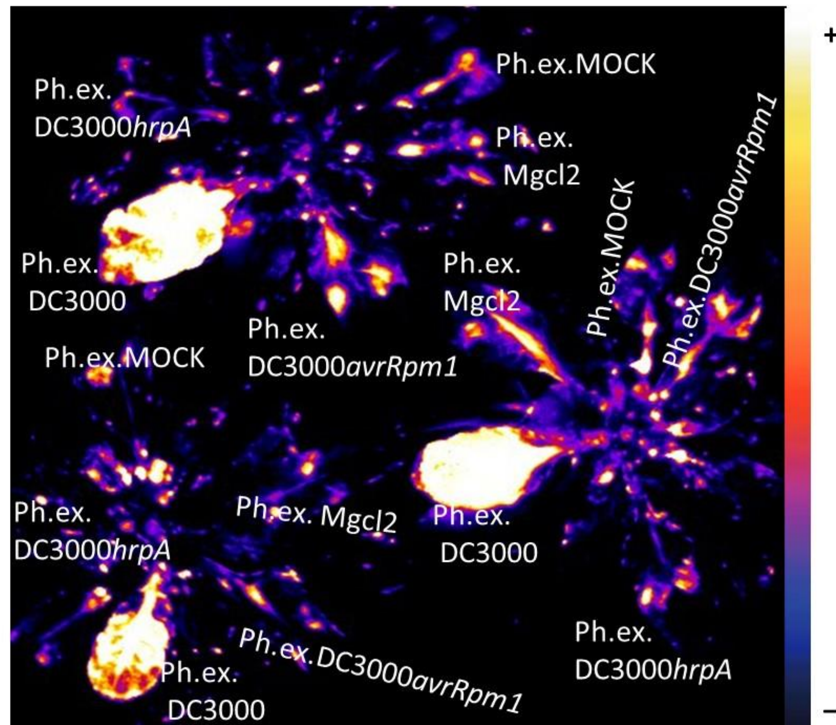


Figure 18. Role of phloem exudates (collected 7-24 hpi) in activation of *A70::LUC* expression:

A70::LUC plants were infiltrated with phloem exudates collected 7-24 hpi from Col-0 leaves infected with DC3000, DC3000*avrRpm1*, DC3000*hrpA*, or treated with 10 mM $MgCl_2$ or non-treated. The luciferase image was taken 1 h after treatment, with acquisition over 10 min. *A70::LUC* plants show strong LUC expression after treatment with phloem exudates from DC3000 infected plants. Picture is false coloured. The color scale indicates signal intensity from 0 (blue) to saturation (white). The picture is a representative of 6 independent experiments.

3.3 Utility of *A70::LUC* expression to report activation of plant defence responses

There are various parameters associated with this defense response include rapid changes in ion fluxes, followed by NO (Nitric Oxide) generation (Delledonne *et al.*, 1998), an oxidative burst (Grant *et al.*, 2000) and induction of a specific set of genes (de Torres *et al.*, 2003), which results in restriction of pathogen growth by leaf

collapse around five hours after infection. Here I investigated the role of Oxidative burst and NO generation in *A70::LUC* expression.

3.3.1 RPM1-dependent *A70::LUC* expression is independent of an oxidative burst.

Since increases in intracellular calcium and generation of ROS are integral to active defense responses (Grant & Loake, 2000, Grant *et al.*, 2000), the impact of selected pharmacological inhibitors of the oxidative burst on RPM1-dependent *A70::LUC* expression were tested. Plants were co-infiltrated with the avirulent pathogen DC3000*avrRpm1* at OD_{600} 0.2 and the NADPH oxidase inhibitor diphenyl iodonium (DPI, 9 µM). Results show that, DPI was not capable of preventing RPM1-dependent *A70::LUC* expression (**Figure 19**) (Full video is provided in appendix 3 CD), suggesting increases in NADPH oxidase are not necessary for *A70::LUC* expression.

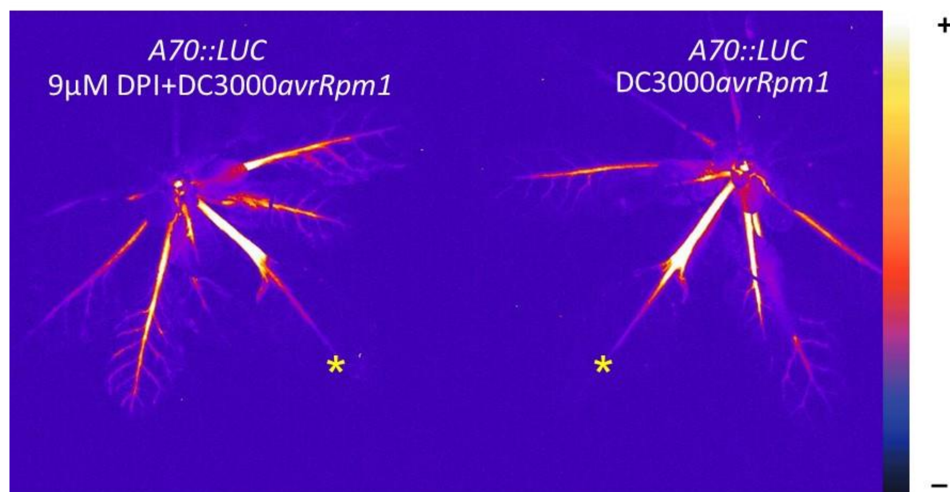


Figure 19. RPM1-dependent luciferase activity in *A70::LUC* plants treated with the NADPH oxidase inhibitor DPI:

A70::LUC plants were infiltrated with DC3000*avrRpm1* (OD_{600} 0.2), DC3000*avrRpm1* together with 9 µM DPI. The image was taken 4 h 50 min after infiltration (Acquisition time 10 min). Picture is false coloured. The color scale indicates signal intensity from 0 (blue) to saturation (white).

3.3.2 RPM1-dependent *A70::LUC* expression is abolished by inhibitors of NO synthase.

It has been demonstrated that NO plays a crucial role in gene-for-gene resistance, but its role is totally dependent on the type of pathogen. *S*,*S*9-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea (PBITU), prevents RPM1-specified HR (Delledonne *et al.*, 1998). Plants were co-infiltrated with the avirulent pathogen DC3000*avrRpm1* and 3 mM of PBITU + DC3000*avrRpm1* and RPM1-dependent *A70::LUC* expression monitored. **Figure 20** shows that PBITU also attenuated RPM1-dependent *A70::LUC* expression but required co-infiltration at a much higher concentration (3 mM) (Full video is provided in appendix 3 CD). In summary, these data suggest that NO generation is necessary for RPM1-dependent *A70::LUC* expression. But then bacterial growth assay shows that 3 mM of PBITU can affect bacterial growth. It means higher conc. Of PBITU affect bacterial growth as well as *A70::LUC* expression. In conclusion, to Induce *A70::LUC* expression significant bacterial growth is essential.

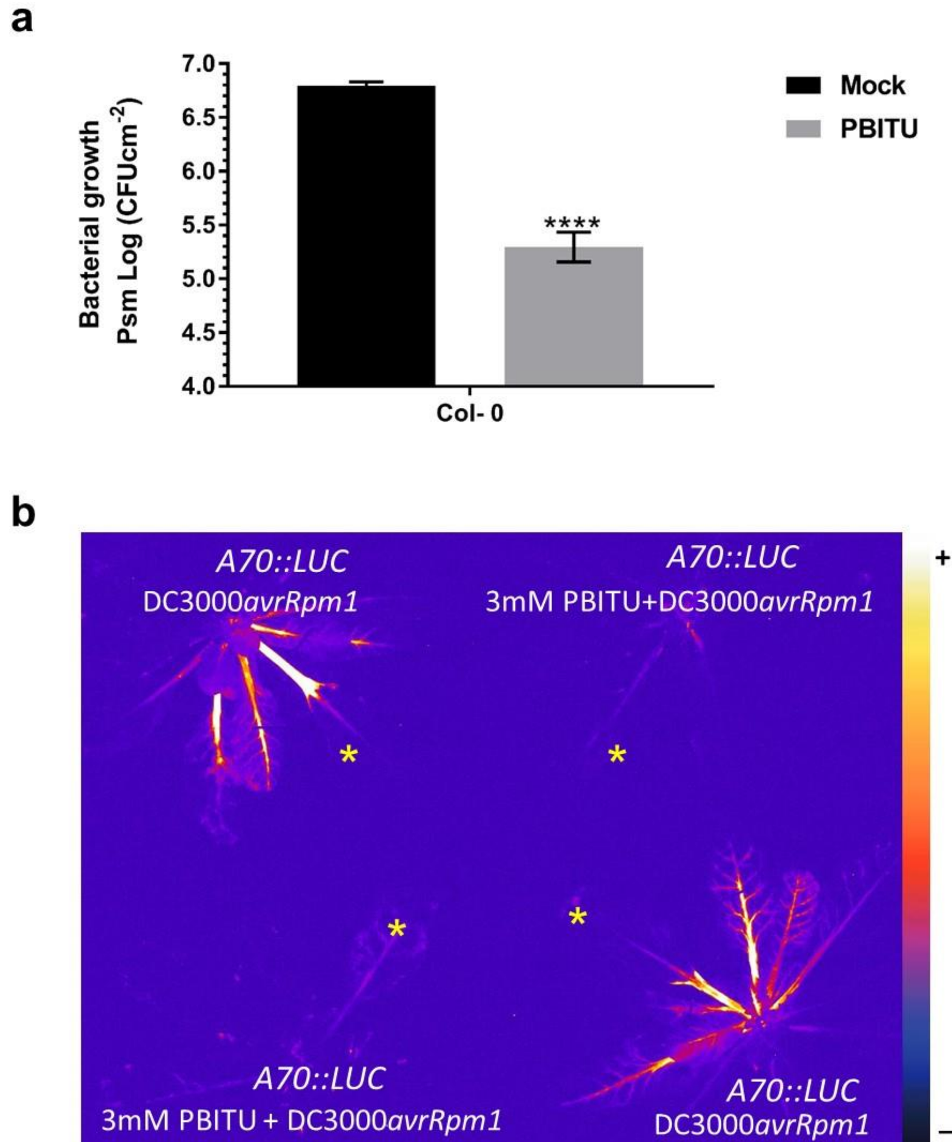


Figure 20. Bacterial growth assay and RPM1-dependent luciferase activity in *A70::LUC* plants after co-infiltration with PBITU:

a. Bacterial growth assay: bar chart shows Col-0 plant co-infiltrated with DC3000*avrRpm1* (OD_{600} 0.2), or DC3000*avrRpm1* + 3 mM PBITU or DC3000*avrRpm1* in 1% ethanol as control. And after 12hr of infection measured for the bacterial growth. Values are means and standard errors obtained in six replicates. Asterisks denotes a significant difference (**** at $P \leq 0.0001$ paired t-test) when compared with the corresponding $MgCl_2$ treated controls. **b.** *A70::LUC* plants were co-infiltrated with either DC3000*avrRpm1* (OD_{600} 0.2), or DC3000*avrRpm1* + 3 mM PBITU or DC3000*avrRpm1* in 1% ethanol as control. The image was taken 4 h 50min after infiltration (Acquisition time 10 min). Picture is false coloured. The color scale indicates signal intensity from 0 (blue) to saturation (white).

3.4 SAR Assay

Arabidopsis leaves infiltrated with virulent hemi-biotrophs, *P. syringae* pv. tomato (*Pst* DC3000) and *P. syringae* pv. maculicola (*Psm*) M4 can multiply rapidly in the plant apoplast causing water-soaked lesions and eventually a chlorotic/necrotic appearance concurrent with a shift from biotrophic to necrotrophic phases (Dong *et al.*, 1991). These avirulent HR-inducing strains are well known to induce SAR in Arabidopsis (Cameron *et al.*, 1994, Mishina & Zeier, 2006).

To quantify the efficiency of SAR induction by avirulent bacterial strains, only one primary lower leaf of a Col-0 plant was first inoculated with $MgCl_2$ (mock) or avirulent *Pst* DC3000*avrRpm1* (~2 x 10^6 cfu ml^{-1}) and 2 days later, a secondary infection with virulent *Psm* M4 (~2 x 10^6 cfu ml^{-1}) was performed on three systemic leaves (upper leaves). Determination of bacterial growth in M4 infected leaves was performed 2 days later **(Figure 21**).

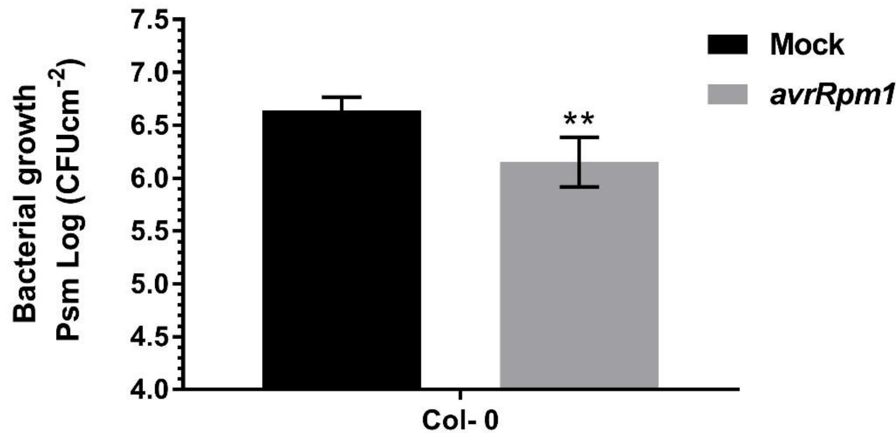


Figure 21. Gene-for-gene mediated systemic disease resistance

Growth of virulent bacteria *Psm* M4 on WT Col-0 plants. Lower leaves (1°) were infiltrated with either 10 mM $MgCl_2$ or *Pst* DC3000*avrRpm1* (OD_{600} 0.002) and 2 d later, three upper leaves (2°) were challenge infected with *Psm* M4 (OD_{600} 0.002). Bacterial growth in upper leaves was assessed 2 d after the 2° leaf inoculation. Values are means and standard errors obtained in six replicates. Experiment repeated three times. An asterisk (**) denotes a significant difference ($P < 0.002$; paired t-test) when compared with the corresponding $MgCl_2$ treated controls.

As can be seen in **(Figure 21)** growth of M4 in systemic leaves 2 dpi was reduced about 5 fold in plants first inoculated with DC3000*avrRpm1* compared with the $MgCl_2$ control treatment. This assay demonstrates that recognition of the avirulent bacteria DC3000*avrRpm1* by functional RPM1 can efficiently initiate SAR under our conditions. The mobile SAR signal(s) is produced, and translocated to the rest of the plant to trigger an enhanced immune state.

3.4.1 SAR assays in *A70* T-DNA KO mutant and *A70 homologs*

To study role of *A70* in SAR signalling, I obtained an *A70* T-DNA knockout mutant from NASC (Nottingham Arabidopsis Stock Centre (Loughborough, UK)). To quantify the efficiency of SAR induction in *A70 KO* by avirulent bacterial challenge, one primary leaf was first inoculated with $MgCl_2$ (mock) or *Pst* DC3000*avrRpm1* (OD_{600} 0.002) and 2 days later, a secondary infection with virulent *Psm* M4 (OD_{600} 0.002) was

performed on three systemic leaves/plant and bacterial growth enumerated 2 days later. Results showed that *A70 KO* doesn't affect SAR signalling (**Figure 22a**). Therefore, I decided to look for *A70 homologs* to rule out possible redundancy in SAR signalling. I found *A70 homolog* (At2g26110) with 93% similarity with A70 protein using BLAST (https://www.ncbi.nlm.nih.gov/blast/) and SMART data base (smart.embl-heidelberg.de/) searches. I studied SAR in the two different allels of the *A70 homolog* (At2g26110); one is *A70 homolog1-1* and other is *A70 homolog 1-2*. While our SAR assay shows that *A70 homolog 1-1* and *A70 homolog1-2* fully affect SAR (**Figure 22b**). It means *A70 homologs* play crucial role in SAR signalling.

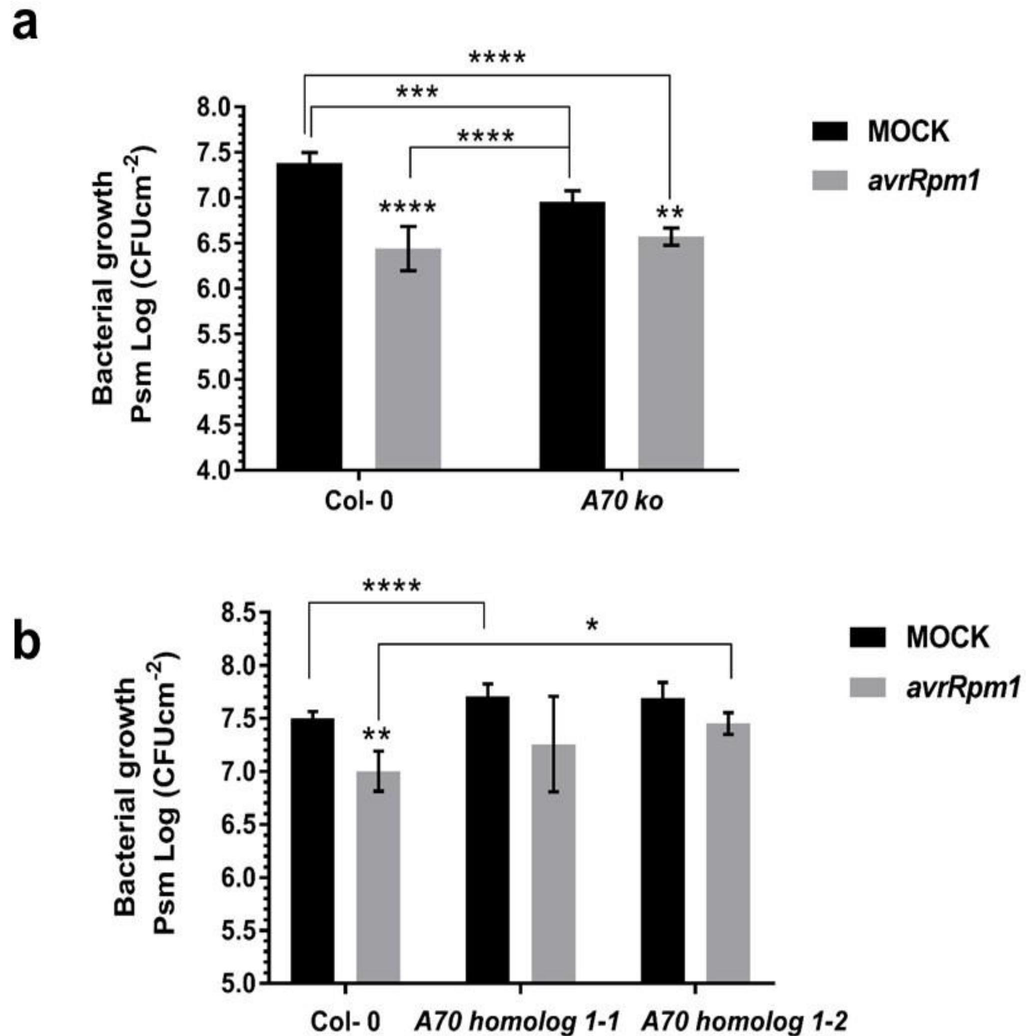


Figure 22. SAR assay in *A70* t-DNA *KO* mutant and *A70 homologs*:

a. SAR assay in Col-0, and *A70 KO* plants. **b**. SAR assay in Col-0 and *A70 homolog1-1*, *A70 homolog1-2*. Lower leaves (1°) were infiltrated with either 10 mM $MgCl_2$ or *Pst* DC3000*avrRpm1* (OD_{600} 0.002), and 2 d later, three upper leaves (2°) were challenged with *Psm* (OD_{600} 0.002). Bacterial growth in upper leaves was assessed 2 d after 2° leaf inoculation. Values are means and standard errors representative of six replicates. This experiment was repeated three times. An asterisk denotes a significant difference (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$; two way ANOVA).

3.5 Discussion

The expression of *A70::LUC* is strongly induced in systemic leaves of *Arabidopsis* plants inoculated with avirulent DC3000*avrRpm1*, reaching its peak 3.5 hpi, (**Figure 10**). Inoculation of plant leaves with virulent DC3000, or with the non-pathogenic DC3000*hrpA* that triggers PTI, did not induce *A70::LUC* expression (**Figure 10**), suggesting a potential role of this gene in the systemic ETI response triggered by avirulent strains of *Pseudomonas* DC3000*avrRpm1.*

Involvement of *A70::LUC* in the ETI response was reinforced by lack of expression of the *A70::LUC* gene in the T3SS deficient mutant. Both restriction of bacterial growth and cell death are consequences of the ETI response triggered by *AvrRpm1* (Pieterse *et al.*, 2009, Alvarez, 2000) implicating *A70::LUC* expression is linked to ETI responses. Although it was previously thought that cell death accompanying HR is responsible for pathogen growth inhibition, a study analysing the phenotype of mutant plants in the metacaspase 1 gene (*atmc1*) indicates that inhibition of pathogen growth and cell death can be uncoupled processes (Coll *et al.*, 2011). Whether *A70* expression is dependent one or other process needs further research. We have determined that *A70* appears to play an early role in ETI mediated systemic immunity signalling, though the KO has no phenotype.

We next examined the role of peptides (PAMPs and DAMPs) in *A70::LUC* expression was then examined. *At*Pep1 is an endogenous peptide elicitor activating defence genes associated with the innate immune response. However, pre-treatment of *Arabidopsis thaliana* with the elicitor-active flagellin peptide flg22, elf18 or *At*pep1 16 h prior to challenge with DC3000*avrRpm1* restricted both HR development and *A70::LUC* expression (**Figure 13**). This suppression of HR by prior PTI induction may be due to restriction of the T3SS's ability to inject type III effectors (Crabill *et al.*, 2010a)

and *A70::LUC* expression is based on effectors because *A70* is activated only by effectors i.e *avrRpm1*, *avrRpt2* and *avrRps4*. If effectors don't enter the plant cell then neither HR induction and nor *A70::LUC* expression would be activated. These results support studies by Kunze and colleagues who reported that pre-treatment with crude bacterial extracts or with flg22 induces resistance to subsequent infection with the bacterial pathogen *Pseudomonas syringae* pv *tomato (Kunze et al., 2004b).* In conclusion, PTI triggering peptides are not directly involved in systemic *A70::LUC* expression but they indirectly play a role in abolishing ETI based *A70::LUC* expression in local as well as systemic leaves.

This Chapter also focused on role of plant hormones in *A70::LUC* activation and results shows that only JA, but not ABA nor SA, could induce *A70::LUC* expression. However, induction was only in infiltrated leaves and the signal did not move systemically. Collectively, the *A70::LUC* gene is moderately and transiently induced locally by JA treatment (**Figure 14a**). Furthermore, to understand the context of *A70::LUC* induction with respect to hormonal interactions, *A70::LUC* expression was examined in plants were pre-treated with JA, ABA and SA. Surprisingly, pre-treatment of ABA abolished the RPM1-induced *A70::LUC* expression but did not restrict HR (data not shown) (**Figure 15**). How ABA modifies the transcriptional activation of *A70::LUC*, e.g. is it a direct modification of the transcriptional complex or perturbation of the upstream signalling pathways, remains to be determined. Recently, it has been shown that ABA suppresses SAR by inhibiting SA biosynthesis (Kusajima *et al.*, 2017)

Furthermore, Results also shows that *A70::LUC* can be activated locally but not systemically by the jasmonates; MeJA, JA-L-Ile or the JA-Ile mimic, coronatine (**Figure 14b**). Interestingly, these results shows that coronatine induced stronger *A70::LUC* expression compared to other components.

Since the *Pst* derived coronatine (COR) can activate the JA signalling by mimicking the structurally similar JA-isoleucine (JA-Ile) (Browse, 2009), the impact of COR on RPM1-induced *A70::LUC* expression was examined. Using a COR deficient DC3000 strain it was demonstrated that coronatine production doesn't affect RPM1-induced *A70::LUC* expression (**Figure 16**) which means that bacterial coronatine production does not contribute to the RPM1-dependent *A70::LUC* systemic signature. It has been previously reported that SAR is established independent to coronatine in the *Arabidopsis–Pseudomonas* interaction (Attaran *et al.*, 2009), however this study did not explore gene-for-gene interactions.

This thesis focuses on SAR triggered by an incompatible bacterial infection. As expected, virulent bacteria in systemic leaves of Col-0 plants multiplied to higher levels in mock treated plants compared to bacterial growth in the Col-0 plants previously immunized with DC3000*avrRpm1* (from ~5 fold to ~9 fold **Figure 21**), indicative of an RPM1 mediated SAR response. As previous work had shown the RPM1-AvrRpm1 interaction generated biophotons ~3h after challenge with *Pst* DC3000*avrRpm1* (Bennett *et al.,* 2005) here, Bennet represents the bio-photon as a ultra-weak photon emission which is associated with hypersensitive cell death. The induction and propagation of the *A70::LUC* signal down the petiole occurs remarkably rapidly, almost instaneously after biophoton generation. Notably, biophoton generation appears to be localized to the infected leaf, hence it is proposed that some inducing

signal is generated concomitant with biophoton generation and this is responsible for the propagative *A70::LUC* expression down the petiole and into systemic leaves.

The final part of this chapter briefly explored the function of *A70* and its homolog by examining whether T-DNA insertion mutations affected SAR. While requiring more detailed study, preliminary results suggest that two independent alleles of the *A70* homolog showed consistent loss of SAR inducing activity. Further experimentation is required to understand the interaction between these two genes in establishment of SAR. Generation of the double mutant lines is current underway.

In summary, these data implicate that *A70::LUC* is specifically induced systemically within 4 h of DC3000*avrRpm1* challenge and the specific induction signal requires an HR response classically associated with ETI. Local induction of *A70* is also induced by external JA application but is independent of bacterial coronatine production. It is noted that it has been reported that significant early production of JA occurs in Arabidopsis leaves following recognition of avirulent *P. syringae* (Mishina, 2008). These data suggest that *A70::LUC* plants challenged with DC3000*avrRpm1* most likely respond to some, as yet, unidentified jasmonate signal produced within the first 3 h after infection initiates *A70::LUC* expression in local leaves, and drives rapid propagation of a signal through systemic leaves.

4 Chapter 4 *A70::LUC* expression in defence mutant backgrounds

4.1 Introduction

Localised bacterial infections often trigger the synthesis of endogenous signalling molecules, which act as secondary signals for induction of SAR. Depending on specific host–pathogen interactions, one or more of these secondary signals may play dominant roles in systemic resistance. For example, the phenolic compound, SA is important in both local and systemic resistance to biotrophic pathogen infection. In many plant species, an increase in SA levels usually precedes the expression of *PR* genes and is associated with the development of SAR. Building on the results in Chapter 3 tools were developed to study *A70::LUC* expression in different SA dependent SAR mutant backgrounds. *A70::LUC* lines were crossed into the following mutant lines of *Arabidopsis thaliana, salicylic acid induction-deficient 2* (*sid2)*, *npr1* (*n*on-expresser of *PR* genes 1 also known as *nim1*, or *no inducible immunity 1*)*, npr3, npr4,* and triple mutant *npr1/3/4, petunia NAM* and *Arabidopsis ATAF1, ATAF2, and CUC2* (*nac*) transcription factors *nac19/55, nac19/55/72, coronatine insensitive 1* (*coi1-16*), and *clade 3 glutamate receptor-like* (*glr3*) genes *glr3.3a, glr3.6a* and *glr3.3/glr3.6a.* Homozygous *A70::LUC/mutant* lines were generated and *A70::LUC* expression induced by DC3000*avrRpm1* measured.

A key component in the induction of *PR* genes in *Arabidopsis* by SA is NPR1. *npr1* is a SAR regulatory mutant, that does not express *PR* genes after exposure to SA or ETI-inducing pathogens (Cao *et al.*, 1994). *npr1* is SA receptor in the SAR signalling pathway and as a result is blocked in the SAR response (Cao *et al.*, 1994). Recently, additional members of the NPR family, NPR3, and NPR4, were shown to

negatively regulate SAR (Liu *et al.*, 2005, Zhang *et al.*, 2006, Fu *et al.*, 2012). NPR3 and NPR4, are SA receptors and negative regulators of immunity (Kuai *et al.*, 2015).

Recent studies on the *clade 3 glutamate receptor-like* mutants link the wound response and disease susceptibility (Mousavi *et al.*, 2013a). Moreover, there is evidence that some members of this clade of *GLR* genes encode important components of the plant's defence response and plant innate immune response (Forde & Roberts, 2014).

MYC2, MYC3 and MYC4 are required for full COR-dependent induction of *ANAC19, ANAC55* and *ANAC72* to modulate stomata aperture during bacterial invasion (Gimenez-Ibanez *et al.*, 2017). (**Table 14**) Listed the homozygous lines containing *A70::LUC* generated from the above mutants that were used for further experimentation.

Table 14. Immune compromised mutant gene used for crossing with *A70::LUC*

Gene involves in SAR	Function
npr-1	SA receptor; transcriptional coactivator
npr-3	SA receptor involved in proteasomal turnover of NPR1
sid -2	Isochorismate synthase required for stress-induced SA biosynthesis
nac 019/055/072	COR suppresses SA accumulation directly through MYC2 – these NACs act downstream of MYC2
npr1/3/4	Compromised in SA signalling
***coi1*-16**	Loss of the JA response

The family of JASMONATE ZIM-domain containing (JAZ) proteins were identified as repressors of JA signalling (Chini *et al.*, 2007). *jaz10* loss-of-function mutants (e.g. *jaz10-1*) are hypersensitive to JA, and *JAZ10* expression is rapidly induced by JA (Yan *et al.*, 2007, Demianski *et al.*, 2012). While, the "JA receptor" COI1 was identified in *Arabidopsis thaliana* in 1998, and the corresponding mutant *coi1-1* is the most prominent JA signalling mutant (Xie *et al.*, 1998), identification of *jaz* mutants provided the first mechanistic explanations of JA perception, including identification of (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) as the ligand of COI1 (Fonseca *et al.*, 2009). The identification of these key components in JA perception and signalling allowed identification of downstream targets, notably the transcription factors like MYC2, acting specifically in numerous JA-dependent processes.

The positive feedback loop in JA biosynthesis can be explained now by the SCFCOI1–JAZ regulatory module. For JA perception and signalling, COI1 acts as an F-box protein (Xie *et al.*, 1998). JAZ proteins are targets of the SCF/COI1 complex. The formation of JA/JA-Ile triggers degradation of JAZ negative regulators by the 26S proteasome in the presence of JA/JA-Ile. Since JAZs are transcriptional repressors, their degradation leads to de-repression of JA signalling, most notably the activation of MYC2 and homologues MYC3 and MYC4. As part of the transcriptional activation of JA-responsive promoters and biosynthetic pathways, both JAZ and MYC genes are induced, elegantly activating a negative feedback loop to attenuate JA signalling (Moreno *et al.*, 2013, Wasternack & Hause, 2013, Goossens *et al.*, 2016, Dombrecht *et al.*, 2007, Chini *et al.*, 2007, Chung *et al.*, 2008).

This chapter mainly concentrated on the role of *A70::LUC* in different mutant backgrounds to find out which signalling molecules are directly or indirectly involved in RPM1-mediated *A70::LUC* expression.

4.2 Results

4.2.1 *A70::LUC* expression in SA based SAR-deficient *Arabidopsis* lines

SAR is fully compromised in the Arabidopsis SA biosynthesis mutant *sid2* and in mutants of *non-expresser of PR genes 1* (*npr1*), which encodes a regulatory protein acting downstream of SA biosynthesis (Cao *et al.*, 1994, Delaney *et al.*, 1995, Lawton *et al.*, 1995, Nawrath & Metraux, 1999). It was reported that NPR3 and NPR4, paralogues of NPR1, were SA receptors that bind SA with different affinities (Zhang *et al.*, 2006, Fu *et al.*, 2012). NPR3 and NPR4 function as adaptors of the Cullin 3 ubiquitin E3 ligase which in turn mediates NPR1 degradation in an SA dependent manner (Zhang *et al.*, 2006, Fu *et al.*, 2012). A double *npr3/4* mutant contains elevated levels of NPR1, as well as it was highly upregulated in basal immunity compared to WT and induced SAR expression. The role of SA signalling in *A70::LUC* expression and its relationship to SAR induction were investigated in this mutant. Here, *sid2/A70::LUC, npr3/npr4/A70::LUC* and *npr3/A70::LUC* plants generated through crossing *A70::LUC* into the respective mutant backgrounds were infiltrated with DC3000*avrRpm1* (OD_{600} 0.2). RPM1-mediated *A70::LUC* expression in *npr1*, *sid2, npr3* and *npr3/npr4* mutant backgrounds was unaffected (**Figure 23 & Figure 24).** This indicated that *A70::LUC* signalling is not dependent on SA biosynthesis nor SA signalling events. Consistent with the literature, *sid2, npr3, npr3/4* and *npr1* were all compromised in induction of RPM1-mediated SAR (**Figure 23 and Figure 24**) (Full video is provided in appendix 3 CD) as distal accumulation of SA is a prerequisite for effective SAR.

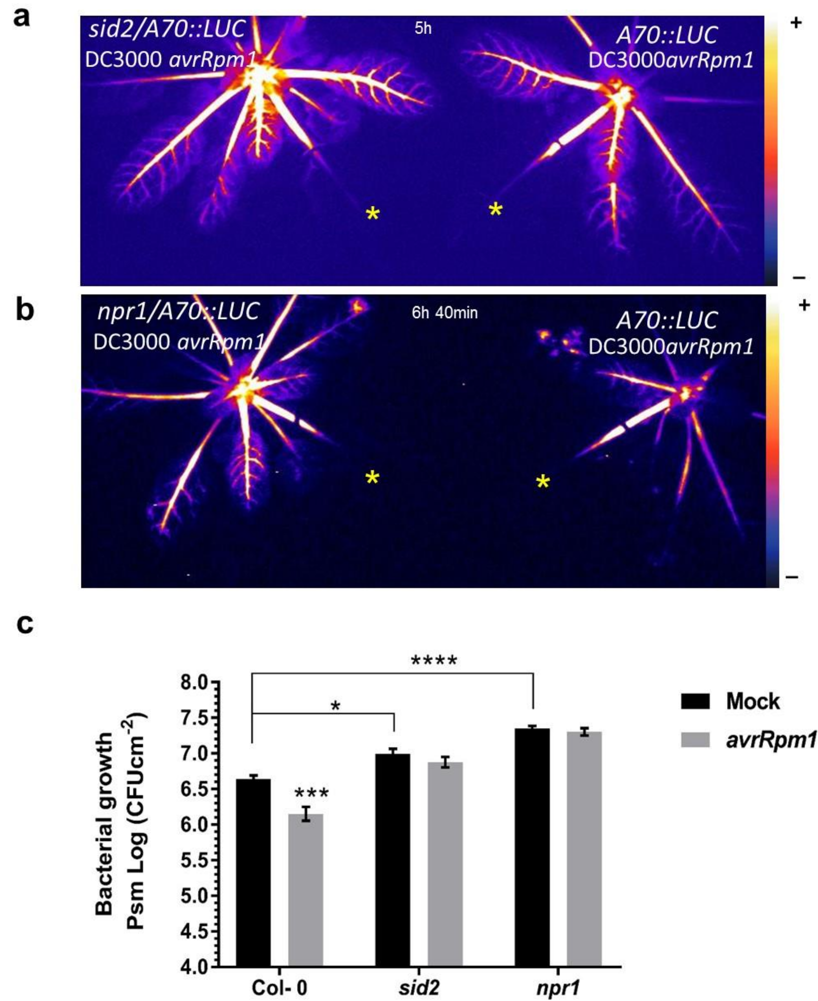


Figure 23. ***A70::LUC*** **expression and SAR assay in Col-0,** ***sid2*** **and** ***npr1*** **plants:**

a. *sid2/A70::LUC* and **b**. *npr1/A70::LUC* plants were challenged with DC3000*avrRpm1* (OD_{600} 0.2). Infiltrated *A70::LUC*, *sid2/A70::LUC* and *npr1/A70::LUC* plants showed strong LUC activity around 5-6 h in response to DC3000*avrRpm1.* Asterisks (yellow) indicates challenged leaves.

b. Bar chart shows SAR assay with *sid2* and *npr1*. Lower leaves (1°) were infiltrated with either 10 mM $MgCl_2$ or DC3000*avrRpm1* (OD_{600} 0.002), and 2 d later, three upper leaves (2°) were challenge infected with *Psm* (OD_{600} 0.002). Bacterial growth in upper leaves was assessed 2 d after 2° leaf inoculation. Values are means and standard errors obtained within the 6 replicates. This experiment was repeated three times. An asterisk denotes a significant difference (* $P \leq 0.05$, *** $P \leq 0.001$ and **** $P \leq 0.0001$; two way ANOVA).

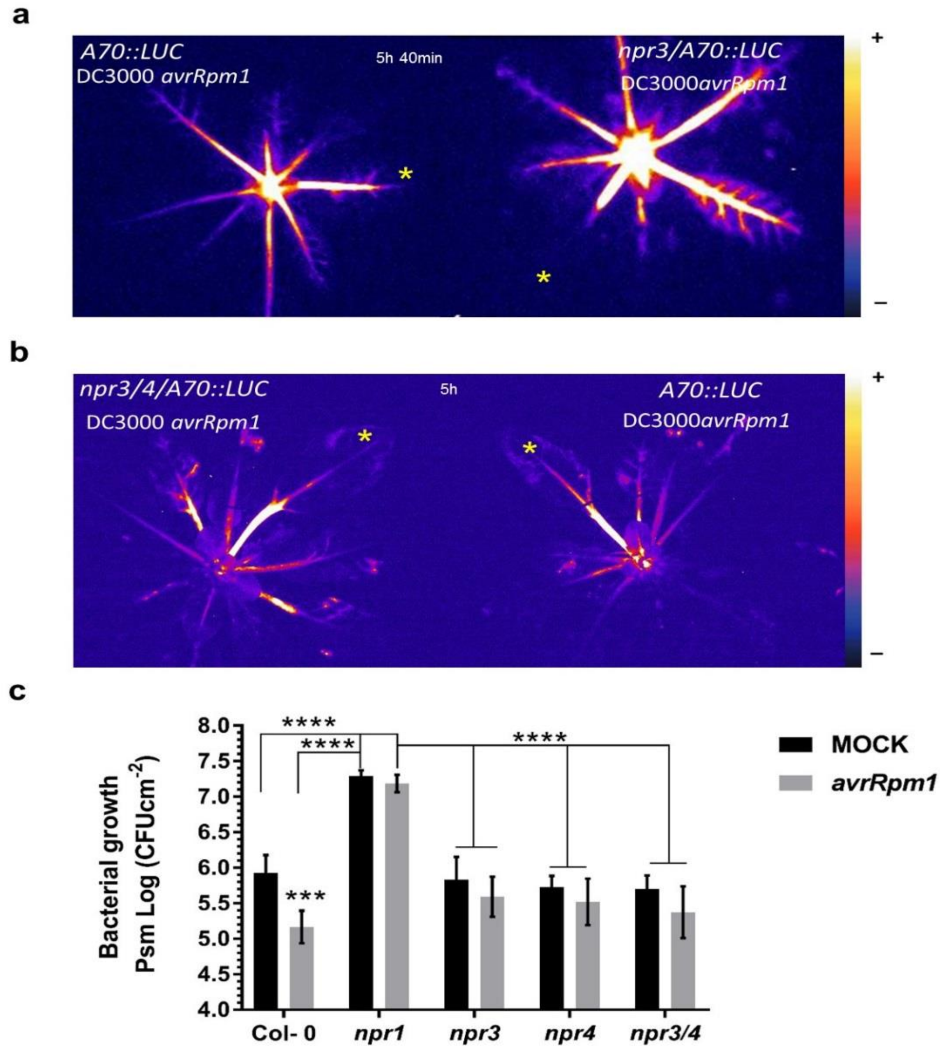


Figure 24 *A70::LUC* expression and SAR assay in Col-0, *npr3* and *npr3/4* plants

a. To study luciferase assay *npr3/A70::LUC* and **b.** *npr3/4/A70::LUC* plants were infected with DC3000*avrRpm1* (OD_{600} 0.2). Infiltrated *A70::LUC*, *npr3/4/A70::LUC* and *npr3/4/A70::LUC* plants showed strong LUC activity around 5-6 h in response to DC3000*avrRpm1.* Challenged leaves are indicated by yellow asterisks.

b. To test SAR : Col-0 , *npr1, npr3, npr4* and *npr3/4* lower leaves (1°) were infiltrated with either 10 mM $MgCl_2$ or DC3000*avrRpm1* (OD_{600} 0.002), and 2 d later, three upper leaves (2°) were challenge infected with *Psm* (OD_{600} 0.002). Bacterial growth in upper leaves was assessed 2 d after 2° leaf inoculation. Values are means and standard errors obtained from the 6 replicates. This experiment was repeated three times. An asterisk denotes a significant difference (*** $P \leq 0.001$ and **** $P \leq 0.0001$; two way ANOVA).

Further support for no direct role for SA in initiation of *A70::LUC* was derived from challenge of a homozygous triple *npr1/3/4* expressing *A70::LUC* with DC3000*avrRpm1* (OD_{600} 0.2). The RPM1-mediated *A70::LUC* signalling in this *npr1/3/4* background was unaltered (**Figure 25**) (Full video is provided in appendix 3 CD). Interestingly, *npr1/3/4* mutants were more susceptible to virulent *Psm* compared to wild type Col-0 plants and SAR was affected (**Figure 25**), suggesting that NPR1 is dominant over NPR3/4 function and that *A70::LUC* signal generation and propagation is independent to SA mediated SAR signalling.

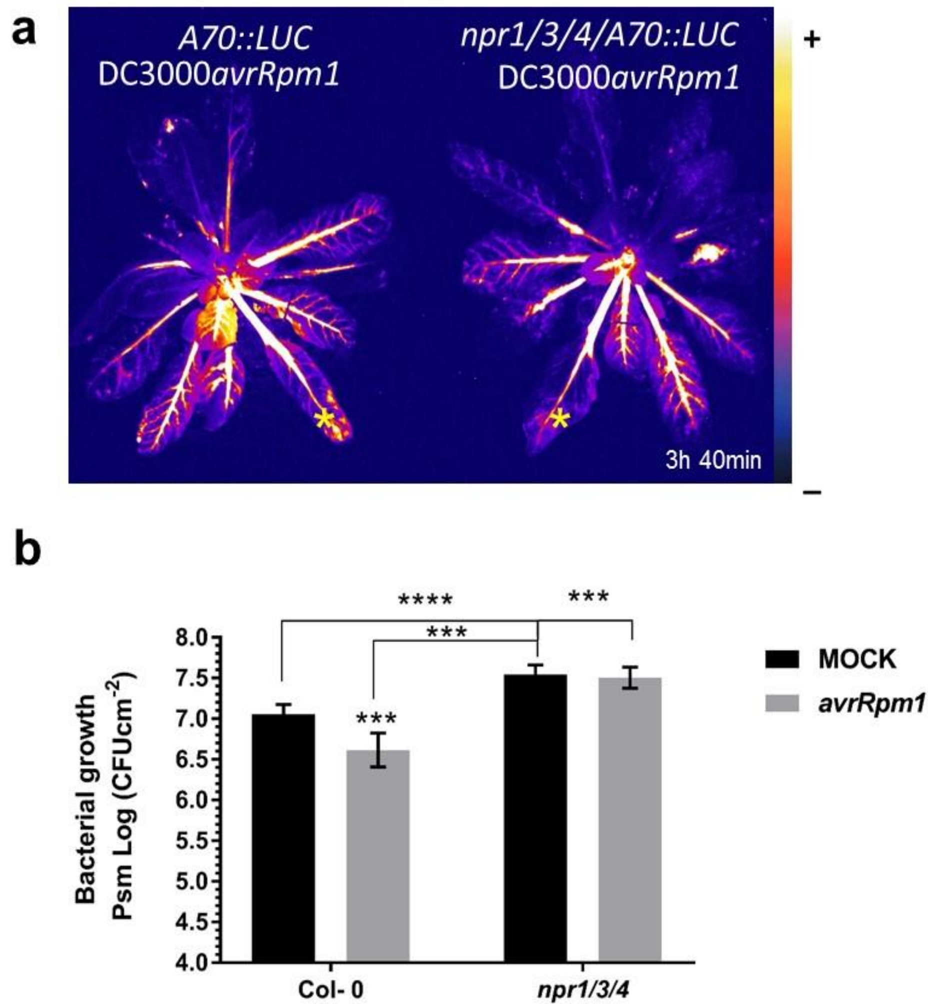


Figure 25. Analysis of SAR and *A70::LUC* expression in an *npr1/3/4* mutant background:

a. *A70::LUC* expression induced by avirulent strain of DC3000*avrRpm1* (OD_{600} 0.2) in *npr1/3/4/A70::LUC* and in *A70::LUC*. Both plants showed strong LUC activity at 3:40 h. Asterisks (yellow) indicate challenged leaves.

b. SAR growth assay in Col-0 and *npr1/3/4* plants. One lower leaf (1°) was infiltrated with either 10 mM $MgCl_2$ or DC3000*avrRpm1* (OD_{600} 0.002), and 2 d later, three upper leaves (2°) were challenge with *Psm* (OD_{600} 0.002). Bacterial growth in upper leaves was assessed 2 d after *Psm* inoculation. Values are means and standard errors obtained in six replicates. This experiment was repeated three times. An asterisk denotes a significant difference (*** $P \leq 0.001$ and **** $P \leq 0.0001$; two way ANOVA).

4.2.2 *A70::LUC* expression in the *nac19/55/72* mutant background.

Next the role of NAC homologs in *A70::LUC* expression was investigated since *Arabidopsis nac19/55/72* triple mutants show enhanced resistance against *Psm* ES4326 (Zheng *et al*., 2012). A homozygous triple *nac19/55/72* expressing *A70::LUC* was generated and challenged with DC3000*avrRpm1* (OD_{600} 0.2). RPM1-mediated *A70* signalling was unchanged in the *nac19/55/72* mutant background (**Figure 26a**) (Full video is provided in appendix 3 CD). This infers that *A70* signalling is independent to MYC2. In addition, testing the role of *nac19/55/72* in SAR revealed that there was no significant difference in bacterial growth for basal resistance *nac19/55/72* **(Figure 26a)** nor *nac19/55* **(Figure 26b)** compared to wild type. However, SAR was affected, as there was no significant difference in *Psm* infection after DC3000*avrRpm1* inoculation when compared to mock treatment in *nac19/55/72* (**Figure 26c**). These results suggest that NAC mutant doesn't affect RPM1-mediated *A70::LUC* expression and that *A70* may not play a role in SAR.

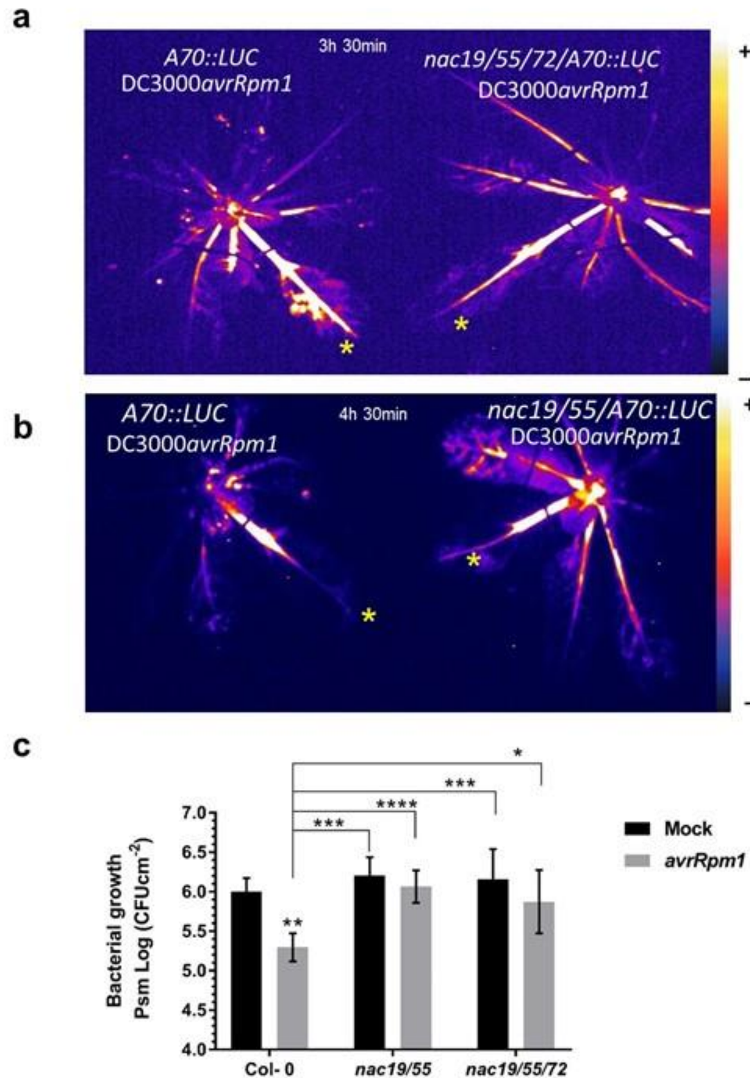


Figure 26. Induction of *A70::LUC* expression and SAR in a *nac19/55* and *nac19/55/72* mutant background:

a. Luciferase assay in *nac19/55/72/A70* **b.** *nac19/55/A70.* Both plants were infected with DC3000*avrRpm1* (OD_{600} 0.2). Strong LUC activity observed at 3:30 h after DC3000*avrRpm1* challenge in both *A70::LUC* and *nac19/55/72/A70* plants. Asterisks (yellow) indicate challenged leaves.

c. SAR assay in Col-0, *nac19/55* and *nac19/55/72* plants. Lower one leaf (1°) was infiltrated with either 10 mM $MgCl_2$ or DC3000*avrRpm1* (OD_{600} 0.002), and 2 d later, three upper leaves (2°) were infected with Psm (OD_{600} 0.002). Bacterial growth in upper leaves was assessed 2 d after *Psm* inoculation. Values are means and standard errors obtained in six replicates. (Values are means and standard errors obtained in three replicates). This experiment was repeated three times. An asterisk denotes a significant difference (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$; two way ANOVA).

4.2.3 *A70::LUC* expression is independent to glutamate receptors

Plant GLR proteins GLR3.3a and GLR3.6a are closely associated with the plasma membrane. AtGLR3.3 are also involved in defence signalling and resistance to *Hyaloperonospora arabidopsidis* through its role in elicitor-/pathogen-mediated plant defence signalling in *Arabidopsis* (Manzoor *et al.*, 2013). Jasmonate-response gene expression in leaves distal to wounds was reduced in a *glr3.3 glr3.6* double mutant and *JAZ10* expression was reduced in the single mutant *glr3.3a* (Mousavi *et al.*, 2013b). To study role of jasmonate signalling mechanisms associated with initiation and propagation of the SAR signal(s) *glr3.3a/A70::LUC, glr3.6a/A70::LUC* and *glr3.3a/glr3.6a/A70::LUC* plants were generated by crossing and homozygous lines infiltrated with DC3000*avrRpm1* (OD_{600} 0.2) and luciferase activity recorded. (**Figure 27 a-c)** clearly shows that RPM1-based *A70::LUC* signalling in the *glr* 3 mutant backgrounds remained unchanged, indicating that *A70* signalling is not dependent on the GLRs (Full video is provided in appendix 3 CD). However, SAR assays measuring restriction of bacterial growth showed that *glr3.3a* and *grl3.3agrl3.6a*, but not *grl3.6a* affect SAR signalling (**Figure 27 d-e**). Thus although not affecting propagation and transduction of *A70::LUC* signalling, the *glr3.3a* mutant, which affects jasmonate wound responses, also affects SAR signalling, underlining a broad role for jasmonate in both production and perception of SAR and wound signals.

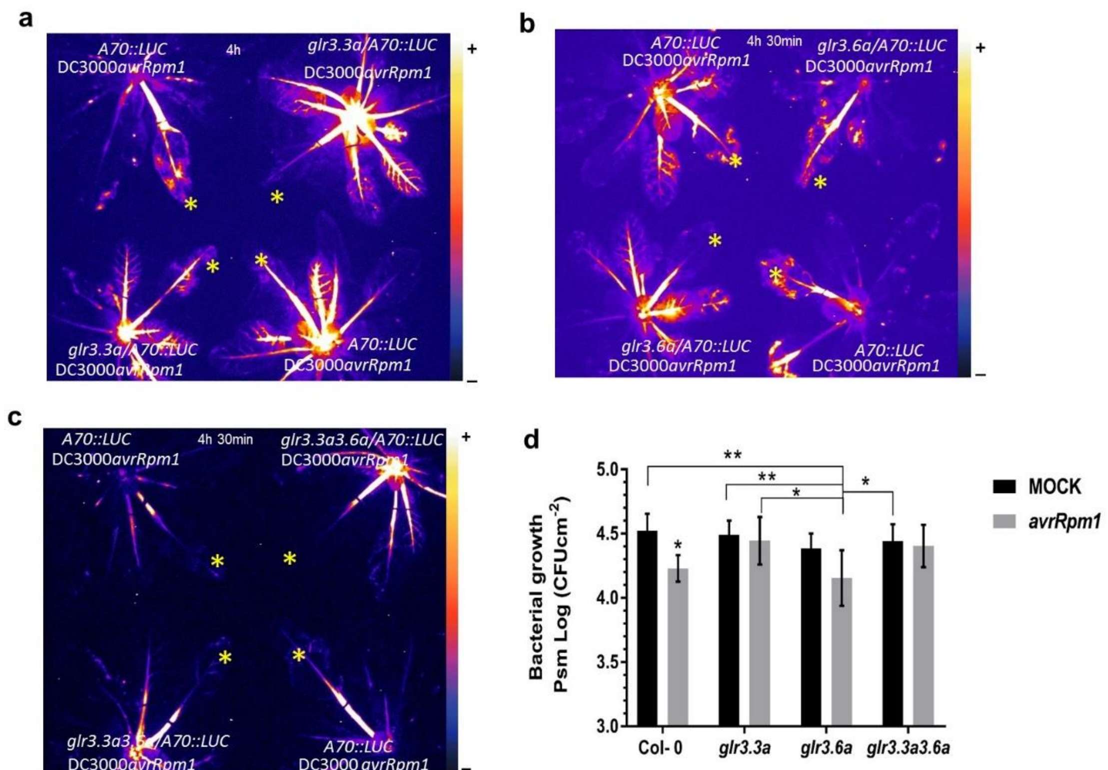


Figure 27. Expression of *A70::LUC* and SAR in *glr* mutant backgrounds:

Luciferase expression in *glr3* mutants. **a.** *glr3.3a/A70::LUC*, **b.** *glr3.6a/A70::LUC* and **c.** *glr3.3a/glr3.6a/A70::LUC* plants inoculated with DC3000*avrRpm1* (OD_{600} 0.2). Infected *A70::LUC* plants showed strong LUC activity at 4 h, *glr3.3a/A70::LUC* at 4 h, (*glr3.3a/glr3.6a/A70*) and (*glr3.6a/A70::LUC*) at 4:30 h in response to DC3000*avrRpm1*. Asterisks indicate challenged leaves.

d. Bar chart showing SAR responses in Col-0 and *glr3.3a, glr3.6a* and *glr3.3a/glr3.6a* mutant plants. Lower leaves (1°) were infiltrated with either 10 mM $MgCl_2$ or DC3000*avrRpm1* (OD_{600} 0.002), and 2 d later, three upper leaves (2°) were challenge infected with *Psm* (OD_{600} 0.002). *Psm* growth in upper leaves was assessed 2 dpi. Values are means and standard errors obtained in six replicates. Experiments were repeated at least three times. An asterisk denotes a significant difference (* $P \leq 0.05$, ** $P \leq 0.01$; two way ANOVA).

Activation of jasmonate synthesis by signals depends on clade 3 *GLR* genes and these GLRs control the distal wound-stimulated expression of several key jasmonate-inducible regulators of jasmonate signalling (*JAZ* genes) in the adult-phase plant (Mousavi *et al.*, 2013b). To determine whether JA and coronatine affect the *A70* signal in *glr3* mutant backgrounds, *glr3.3a/A70::LUC, glr3.6a/A70::LUC, glr3.3a/glr3.6a/A70::LUC* and *A70::LUC* plants were infiltrated with 250 μM JA and 500 nM coronatine (**Figure 28 a, b, c**) (Full video is provided in appendix 3 CD). Challenged *A70::LUC* plants showed strong LUC activity after 50 min in response to JA and coronatine but *glr3.3a/A70::LUC* and *glr3.6a/A70::LUC* shows weak signal to JA and glr3.3a/*glr3.6a/A70::LUC* almost completely abolished the expression of *A70::LUC* in both JA and coronatine challenged leaves.

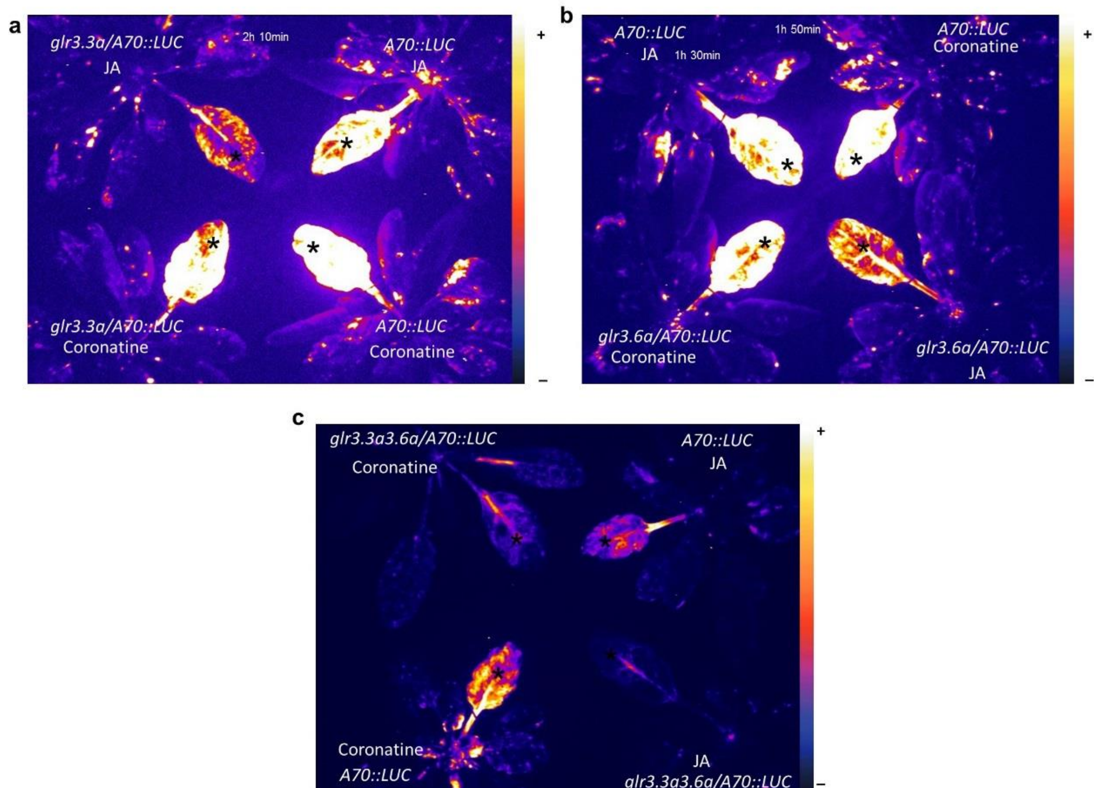


Figure 28. Role of JA and coronatine in *A70::LUC* expression in *glr* mutant backgrounds:

Comparing luciferase expression in *A70::LUC* (positive control) with, **a.** *glr3.3a/A70::LUC*, **b.** *glr3.6a/A70::LUC* or **c.** *glr3.3a/glr3.6a/A70::LUC* lines. All indicated leaves were infiltrated with either 250 µM JA or 500nM coronatine as indicated. Leaves of *A70::LUC* plants showed strong LUC activity after 50 min in response to JA and coronatine but both single mutant lines had reduced signal, this being more pronounced in *glr3.3a/A70::LUC*, whereas in the *glr3.3a/glr3.6a/A70::LUC* line JA and coronatine signals were abolished. *(black) indicates challenged leaves. This experiment was repeated six times.

4.2.4 A70*::LUC* expression is dependent on *coi1*-16

In order to study whether jasmonates might be signals that propagate SAR in *A70::LUC* signalling, the role of the COI1 JA receptor was investigated. *coi1*-16*/A70::LUC* plants were generated and challenged with DC3000*avrRpm1* (OD_{600} 0.2) and luciferase activity measured. **Figure 29a** shows that the RPM1-based *A70::LUC* signalling was almost completely abolished in the *coi1*-16 mutant background indicative of a critical role for jasmonate signaling in *A70* induction (Full

video is provided in appendix 3 CD). Consistent with this, *coi1*-16 plants do not undergo an RPM1-mediated SAR to *Psm* (**Figure 29b**). However, like the *npr3/4* double mutant, *coi1*-16 is already become resistant, presumably because of its elevated SA levels. Because of high level of SA, it has been reported that a phenotype of *coi1*-16 is associated with elevated SA levels and higher resistance to both virulent pathogens *Psm* ES4326 and *Pst* DC3000 (Kloek *et al.*, 2001). Thus, it may be challenging to mount an additive SAR defence response. However, (de Torres Zabala *et al.*, 2016a) found that *coi1*-16 only shows significantly higher SA 18 hpi with DC3000 compared to WT, and showed that COR-dependent suppression of SA during DC3000 infection is not fully COI1 dependent. We show that *A70::LUC* plants still retain its RPM1-based luciferase signature following SA pre-treatment **(**above **Figure 15)**, implying that *A70* plays an important role in systemic signalling and this is absolutely dependent on the *COI1* signalling pathway.

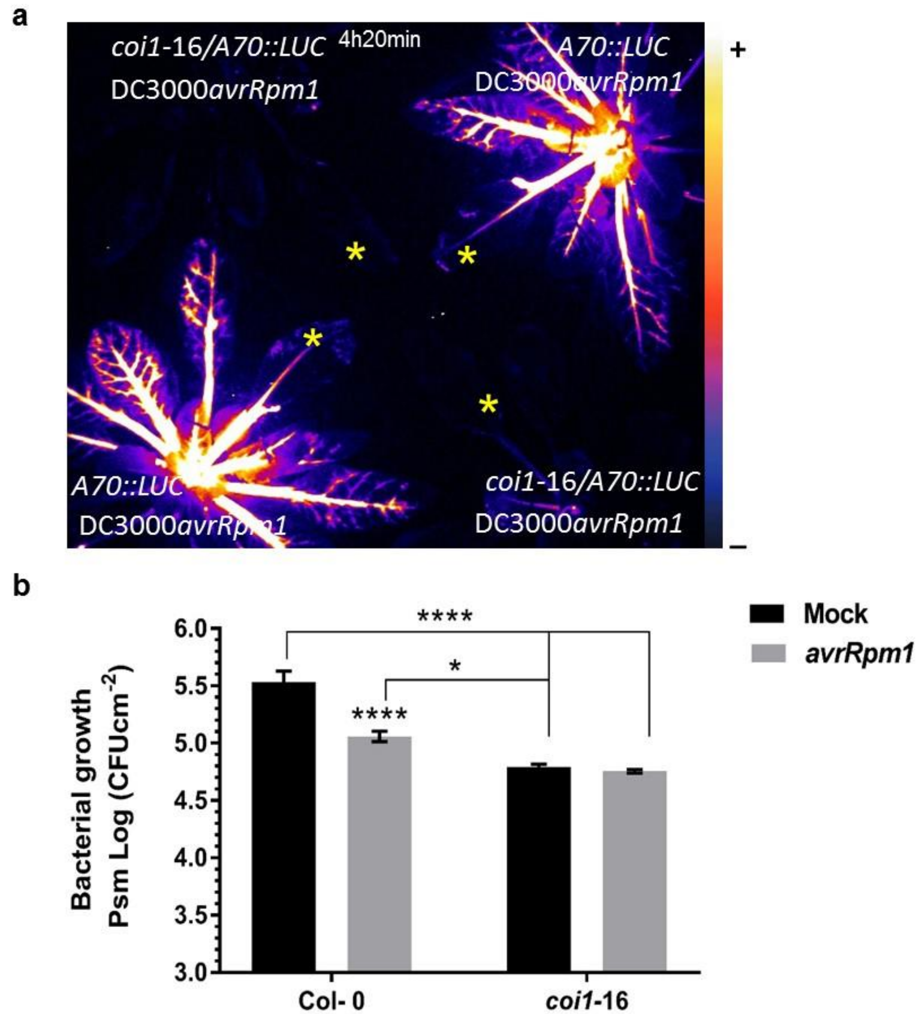


Figure 29. Analysis of SAR and *A70::LUC* expression in the *coi1*-16 mutant background:

a. To study luciferase expression under *coi1*-16 mutant background, *A70::LUC* and *coi1*-16*/A70::LUC* plants were infected with DC3000*avrRpm1* (OD_{600} 0.2). *A70::LUC* plants showed strong LUC activity at 4:20 h in response to *Pst* DC3000*avrRpm1* while signal was virtually absent in *coi1*-16*/A70::LUC* plants. Asterisks indicate challenged leaves. Image is false coloured. The colour scale indicates signal intensity from 0 (blue) to saturation (white).

b. SAR assay in Col-0 and *coi1*-16 plants. The lower leaf (1°) was infiltrated with either 10 mM $MgCl_2$ or DC3000*avrRpm1* (OD_{600} 0.002), and 2 d later, three upper (2°) leaves were challenged with *Psm* (OD_{600} 0.002). *Psm* growth in upper leaves was assessed 2 d after inoculation. Values are means and standard errors obtained in six replicates. This experiment was repeated three times. An asterisk denotes a significant difference (* $P \leq 0.05$ and **** $P \leq 0.0001$; two way ANOVA).

Previous studies show that the COI1-mediated degradation of JAZ proteins can activate COR-dependent gene expression (Thines *et al.*, 2007, Fonseca *et al.*, 2009, Sheard *et al.*, 2010). This means that JA and coronatine signalling depends on COI1. To test, whether JA or coronatine can induce the *A70* signal in the *coi1*-16 mutant background, *coi1*-16*/A70::LUC* and *A70::LUC* were challenged with 250 μM JA or 500 nM coronatine. **Figure 30** shows that JA and coronatine induced LUC activity in *A70::LUC* plants but not in *coi1*-16*/A70::LUC* (Full video is provided in appendix 3 CD). This means *coi1*-16 controls JA and coronatine-related signal activation of *A70* during the RPM1-mediated HR.

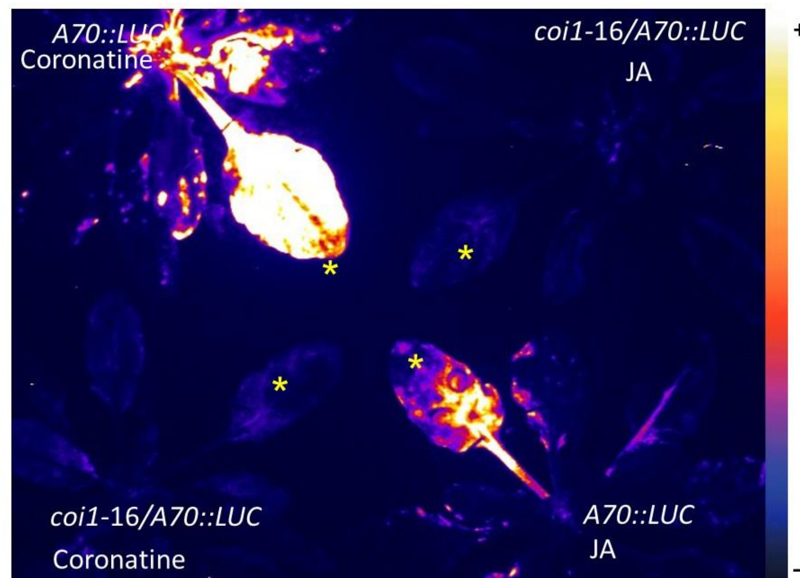


Figure 30. Role of JA and coronatine in *A70::LUC* expression in a *coi1*-16 mutant background:

Luciferase images shows, *coi1*-16*/A70::LUC* and *A70::LUC* plants were infiltrated with 250 μM JA or 500 nM coronatine. *A70::LUC* plants showed strong LUC activity within 50 min in response to JA or coronatine but *coi1*-16*/A70::LUC* plants did not. Asterisks indicate infiltrated leaves.

4.2.5 The *JAZ10::GUS* expression patterns are similar to *A70::LUC* expression

In, 2013 Mousavi *et al.*, detected *JAZ10* transcript induction in systemic leaves 1 h after wounding. Interestingly GUS staining showed that the staining was restricted by the plant orthostichies. Following wounding of leaf 8 (**Figure 31a**), *JAZ10* transcript increased ≥100-fold in adjacent leaves 5, 11, 13 and 16 compared to distant leaves 7, 9, 10, 12 and 14. To investigate the possibility commonality of systemic wound responses with establishment of SAR, a single leaf of *JAZ10::GUS* plants were infected with DC3000*avrRpm1*, DC3000, or DC3000*hrpA* (OD_{600} 0.2) or mock challenged (10 mM $MgCl_2$). Systemic leaves (adjacent and distant (**Figure 31b**)) were then harvested at 4, 8 or 24 hpi and GUS activity determined by GUS staining. Visualization of GUS activity in petioles of unchallenged leaves adjacent to the DC3000*avrRpm1* challenged leaf only at 8hpi (**Figure 31b**) showed a similar pattern to that of *A70* expression was seen at 4h in the *A70::LUC* expressing lines (**Figure 10**). GUS activity were not strong at 24 hpi (**Figure 31c**) The systemic induction of transcripts of *JAZ10* implicates modulation of JA signalling in systemic responding leaves, which is consistent with a previous study that reported JA transcripts being upregulated in systemic leaves in response to DC3000*avrRpm1* (Truman *et al.*, 2007).

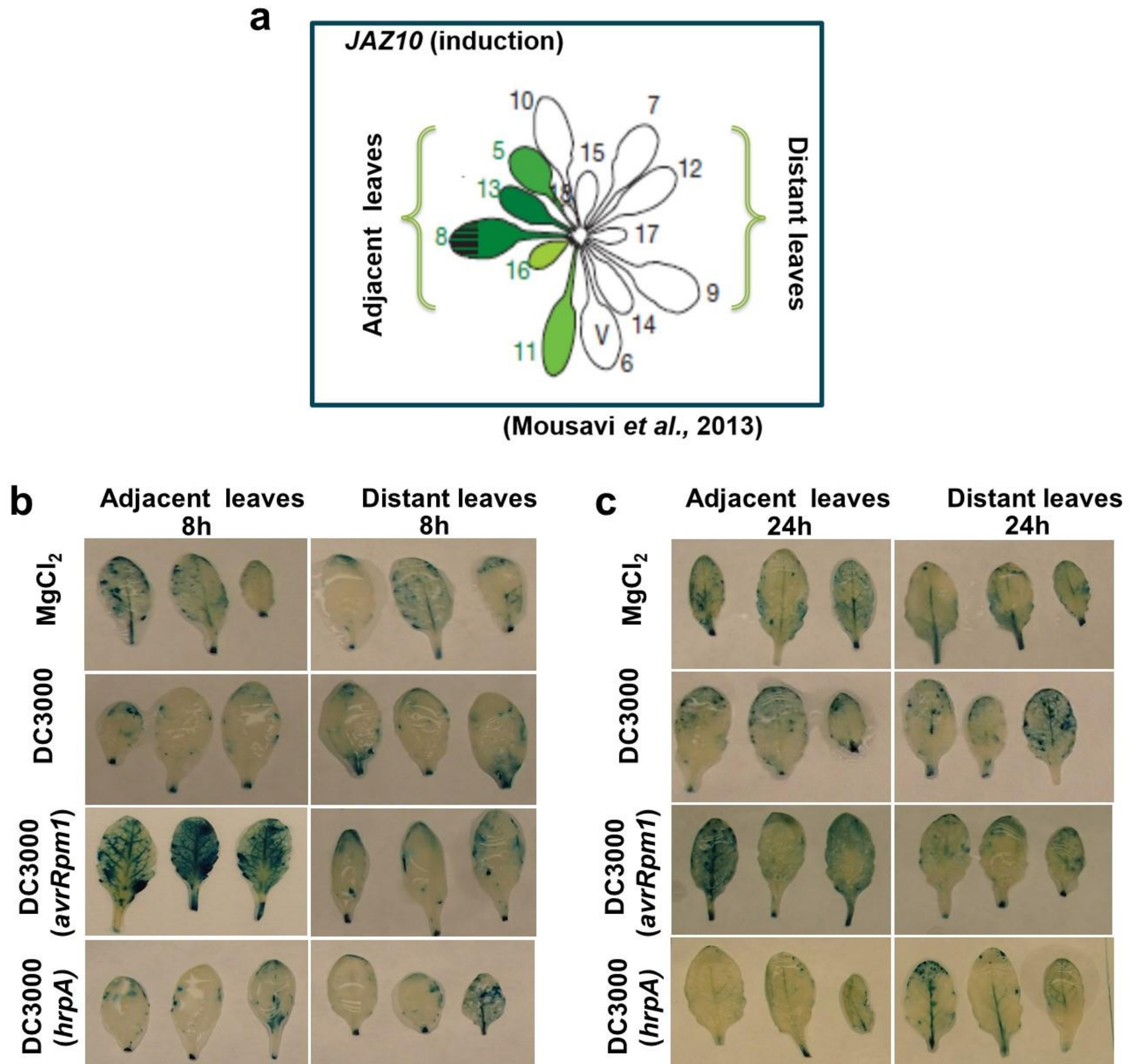


Figure 31. GUS expression in leaves of *JAZ10::GUS* plants:

a. Heat maps of *JAZ10* transcript induction 1 h after wounding, (image taken from (Mousavi *et al.*, 2013a) **b.** *JAZ10::GUS*-expressing lines were infiltrated with DC3000*avrRpm1*, DC3000 and DC3000*hrpA* (OD $_{600}$ 0.2) and 10 mM $Mgcl_2$ (mock) in single lower (primary) leaves, and adjacent and distal systemic leaves were collected at the indicated time intervals and stained for GUS activity. Only DC3000*avrRpm1* treated plant showed clear GUS activity at 8 hpi and no GUS activity was observed after infiltration of DC3000 and DC3000*hrpA* or 10 mM $MgCl_2$. **c**. GUS activity at 24 hpi Note, GUS staining at the petiole excision, consistent with a local wound response.

4.2.6 *JAZ10*::GUS expression in the *coi1*-16 background.

JAZ10 induction in response to wounding requires the production of JA, specifically the biologically active form, JA-Ile (Fonseca *et al.*, 2009). The interaction of JA-Ile with the receptor COI1, not only leads to defence gene expression (Browse, 2009, Fonseca *et al.*, 2009) , but also to the induction of JA synthesis (Wasternack & Hause, 2013).

The currently accepted hypothesis is that JA is a bioactive signal mediates the degradation of JAZ proteins, repressors of JA signlling, via SKP1/Cullin/COI1 degradation.

To study *JAZ10* expression in a *coi1*-16 mutant background the *JAZ10::GUS* transgenic plant was crossed into the *coi1*-16 mutant background. *JAZ10::GUS/coi1*-16 plants were selected for homozygous *coi1*-16 by plating on 0.5x MS + 25 µM MeJA and looked for long root length (**Figure 32a & b**) . Leaves of a homozygous *JAZ::GUS/coi1*-16 expressing line were infiltrated with DC3000*avrRpm1* or DC3000 *hrpA* at OD_{600} 0.2 or with 10 mM $MgCl_2$ (mock). After this, systemic responding leaves were harvested at 8h and GUS activity determined in both leaves adjacent to the immunizing challenge and distal leaves. As expected, the *JAZ10::GUS/coi1*-16 reporter leaves were devoid of detectable GUS activity in systemic leaves at 8 hpi, which strongly supports a role of activation of JA signalling very early in the systemic responding leaves during ETI mediated SAR responses (**Figure 32c**).

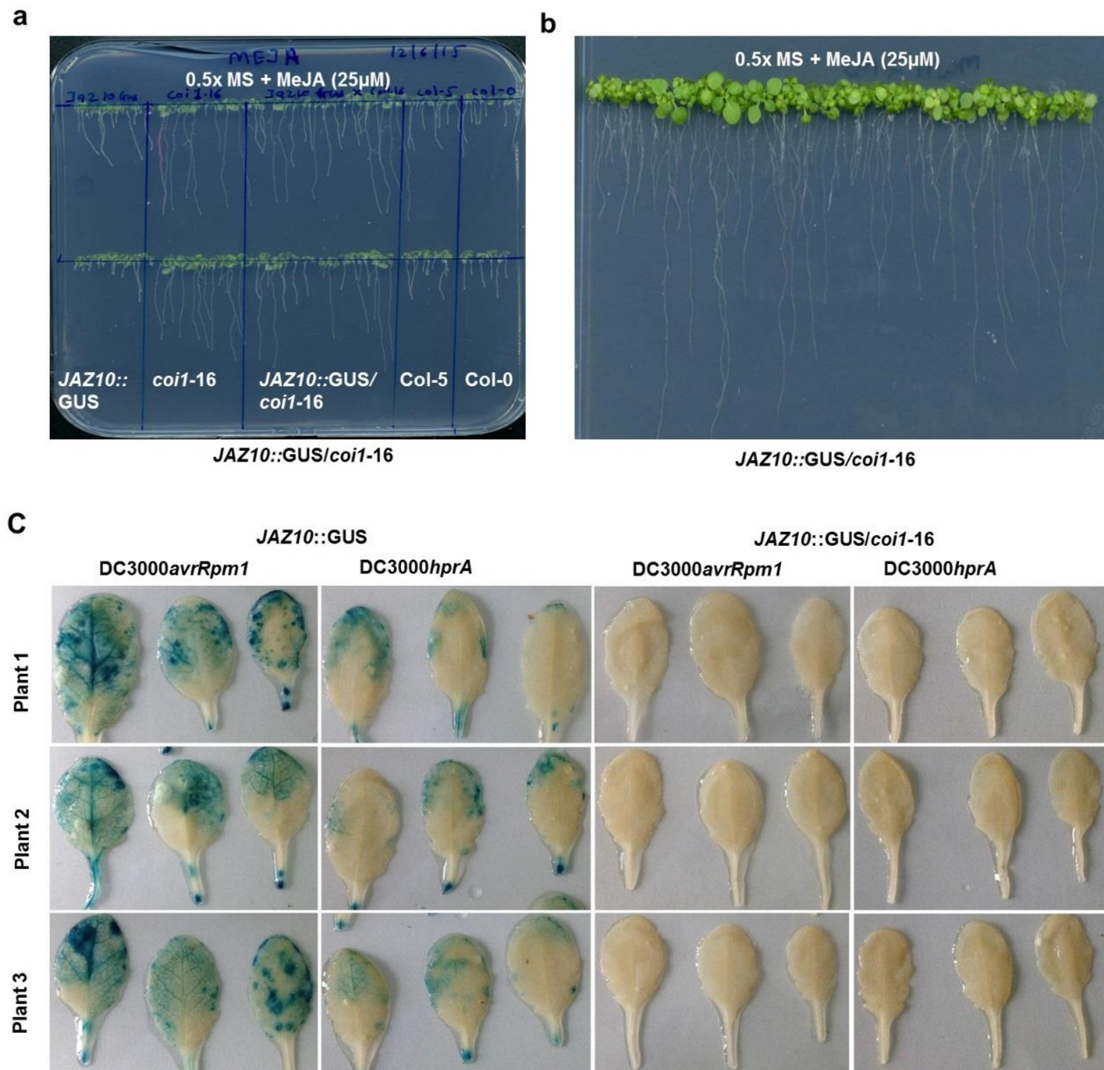


Figure 32. Selection of *JAZ10::GUS/coi1*-16 plants and systemic GUS expression in *JAZ10::GUS/coi1*-16 plants:

a and **b.** selection of F2 generation of *JAZ10::GUS/coi1*-16 based on long root length on 0.5x MS + 25 µM MeJA. **c.** *JAZ10::GUS and JAZ10::GUS/coi1*-16-expressing lines were infiltrated with DC3000*avrRpm1*, DC3000*hrpA* (mock) into single lower leaves, and adjacent systemic leaves (only) were collected at the indicated time intervals and stained for GUS activity. No GUS activity was observed at 8h in systemic leaves of *JAZ10::GUS/coi1*-16-expressing lines after infiltration with DC3000*avrRpm1* or DC3000*hrpA.*

4.3 Discussion

In this Chapter the results indicate that *A70::LUC* expression is independent of currently known SA-mediated defence processes but appears to be controlled by jasmonate responses in a complex manner. SAR is fully compromised in the Arabidopsis SA biosynthesis mutant *ics1* (*sid2*) and in mutants of NON-EXPRESSOR OF PR1 (NPR1), which encodes a regulatory protein acting downstream of SA (Delaney *et al.*, 1995) (Cao *et al.*, 1994) (Nawrath & Metraux, 1999). *A70::LUC* expression after DC3000*avrRpm1* inoculation in the SAR-defective line *sid-2*/*A70::LUC* was indistinguishable from col-0 control lines (**Figure 23**), underlining that *de novo* SA biosynthesis is not necessary for RPM1-mediated *A70* induction. To rule out NPR1, which plays an important role in SA signalling in the nucleus and SA-JA cross-talk in the cytoplasm (Spoel, 2003, Felton & Korth, 2000, Feys & Parker, 2000, Spoel *et al.*, 2007, Beckers & Spoel, 2006, Bostock, 2005, Pieterse, 2001), *npr1/A70::LUC* plants were tested. *A70::LUC* expression was not affected in *npr1/A70::LUC* plants*,* suggesting that NPR1 doesn't impact RPM1-induced *A70::LUC* expression. In SAR assays, the *npr1* mutant is compromised in basal resistance and is more susceptible. Similarly, both *sid-2* and *npr-1* are compromised in SAR signalling (**Figure 23**).

The link between NPR1 activation and SA accumulation has long remained elusive. Recently, progress in understanding NPR1-mediated signalling and transcriptional reprogramming has been made via the identification of multiple SA receptors (Fu *et al.*, 2012) (Wu *et al.*, 2012). Recombinant NPR3 and NPR4, paralogs of NPR1, strongly bind SA (Fu *et al.*, 2012). So, we extended this study to investigate RPM1-induced *A70::LUC* expression and SAR establishment in *npr3, npr3/4* and *npr1/3/4* triple mutant backgrounds. Our results show strong induction of RPM1-

induced *A70::LUC* expression in single double and triple mutant *npr3/A70::LUC, npr3/4/A70::LUC* and *npr1/3/4/A70::LUC* respectively **(Figure 24 & Figure 25**), indicating that neither *npr3* nor *npr3/4* affect *A70::LUC* expression. Interestingly, in contrast to the *npr1* mutant which is compromised in basal resistance, the *npr3/4* mutant exhibits enhanced basal disease resistance to virulent M4 bacteria but not *npr1/3/4* **(Figure 24 & Figure 25)**. However, the *npr3/npr4* mutant is not able to elicit SAR in response to inoculation with the avirulent DC3000*avrRpm1* and is also partially compromised in ETI (Bonardi & Dangl, 2012). This suggests that NPR1, NPR3 and NPR 4 are required for SA-defence pathways but not for RPM1-mediated *A70::LUC* expression.

To similarly explore whether "classical" JA mediated signalling networks impact *A70::LUC* expression a detailed assessment of the three homologous NAC family TF genes, ANAC019, ANAC055 and ANAC072, which are induced by *Pst* DC3000 infection and methyl-JA treatment (Ooka *et al.*, 2003) as well as implicated as a core JA signalling module downstream of MYC2, a key regulator of JA responses (Chini *et al.*, 2009a, Yan & Xie, 2015, Kazan & Lyons, 2014). To study their roles and contributions to *A70::LUC* expression, we generated *nac19/55* double and *nac19/55/72* triple mutant lines. RPM1-mediated *A70::LUC* expression in both *nac19/55/A70::LUC* and *nac19/55/72/ A70::LUC* plant was indistinguishable from controls indicating that they had no direct role in regulation of *A70* induction nor propagation systemically (**Figure 26**). These data suggest that NACs are not required for the RPM1-mediated *A70::LUC* expression.

Finally, we tested clade 3 GLR mutants for RPM1-mediated *A70::LUC* expression, because *GLUTAMATE RECEPTOR-LIKE* genes (GLRs), regulate the distal wound-stimulated expression of several key jasmonate-inducible regulators of

jasmonate signalling (*JAZ* genes) in the adult plants (Mousavi *et al.*, 2013a). Here, all our results showed no significant change in RPM1-mediated *A70::LUC* expression in *glr3.3a/A70::LUC, glr3.6a/A70::LUC* or *glr3.3a/glr3.6a/A70::LUC* lines (**Figure 27**). Thus *A70::LUC* expression is independent to GLRs, yet GLR mutants compromised SAR (**Figure 27**) indicating that establishment of SAR necessitates a role for GLR transporters, possibly through a jasmonate based signal. Notably however, JA and coronatine challenged leaves of *grl3.3a/A70::LUC* and *grl3.6a/A70::LUC* plants showed strongly reduced *A70::LUC* expression. Interestingly, the double mutant *glr3.3a/glr3.6a/A70::LUC* line was strongly compromised in both JA as well as coronatine signal (**Figure 28**). This suggests that GLRs controls jasmonate-inducible regulators as reported by (Mousavi *et al.*, 2013a) but not the predicted JA signal that is generated in a local ETI interaction and activates *A70::LUC* expression.

The *Arabidopsis thaliana* coronatine insensitive mutant *coi1* is the only definitive jasmonate receptor described to date and binds the bioactive jasmonate JA-Ile to regulate a wide range of jasmonate based responses (Yan *et al.*, 2016). *coi1*-16 is a conditional fertile *coi1* allele (Ellis & Turner, 2002) and we used this to address the role of jasmonate signalling in RPM1-mediated *A70::LUC* expression. Strikingly, induction of *A70::LUC* expression is completely abolished in *coi1*-16*/A70::LUC* plants (**Figure 29**), strongly supporting the idea that *A70::LUC* expression induced by DC3000*avrRpm1* bacteria is controlled via a yet to be identified JA-mediated pathway. As previously described, *coi1-16* plant shows enhanced basal resistance, probably due to the higher SA levels observed in the *coi1* mutant (Kloek *et al.*, 2001), so it was not possible to assess the contribution of COI1 to SAR, other than to observe that there was no additional enhancement or suppression in systemically responding *coi1* leaves (**Figure 29**).

Histochemical GUS assays enabled us to study the link between *JAZ10* related wound signalling and RPM1-induced systemic signalling. To compare *JAZ10::GUS*-expressing lines with *A70::LUC* results DC3000*avrRpm1* challenged *JAZ10::GUS* leaves were harvested at 4, 8 and 24 hpi and GUS activity was assessed in local and distal leaves (4h time point data is not shown). Histochemical staining of leaves 8 and 24 hpi demonstrate that the expression pattern of GUS activity in systemic leaves (**Figure 31**) closely resembled LUC activity visualized in systemic petioles and leaves in close proximity to the DC3000*avrRpm1* challenged leaf (**Figure 10**). Like LUC activity, GUS staining was dependent upon functional COI1. The similarity of the expression/staining patterns strongly supports an important role for jasmonates in early SAR responses. Collectively these results support a scenario whereby after infecting with DC3000*avrRpm1*, local and systemic leaves produce a jasmonate like compound(s) that can promote COI1-JAZ interactions that de-repress the expression of JA responsive genes. The identity of this compound, and whether this is the same inducing compound in local and systemic leaves remains to be determined. A previous study indicated that JA is upregulated in petiole exudates in response to DC3000*avrRpm1* challenge (Truman *et al.*, 2007) but genetic studies with petiole exudates ruled out a direct role for JA as the systemic signal (Chaturvedi *et al.*, 2008). The jasmonate nature of the inducing signal is re-informed by the quite specific activation of expression of JA-responsive genes within 4 hpi in systemic responding leaves (Truman *et al.*, 2007). Moreover, re-examination of that data following the discovery of *JAZ* genes revealed that at least 7 of the 12 *JAZ* genes are up-regulated in systemic responding leaves within 4h of DC3000*avrRpm1* challenge (M. Grant per com.).

The dependence of RPM1-induced *JAZ* expression on COI1 (**Figure 32**) indicates that a bioactive JA signal(s) produced in DC3000*avrRpm1* infected leaves triggers $SCF^{COI1/26S}$ proteasome-mediated destruction of JAZ repressors and subsequent transcription of primary response genes. These data support a jasmonate based signal – currently whether this is an enzymatically or non-enzymatically derived signal is unknown. Several studies show that *coi1* plants are deficient in the accumulation of OPDA- and dinor-OPDA-containing galactolipids that may function as precursors for JA synthesis (Buseman *et al.*, 2006, Kourtchenko *et al.*, 2007). Our data demonstrating that RPM1-induced accumulation of *JAZ* transcripts is attenuated in *coi1*-16 plants is consistent with the idea that JAZ proteins are destabilized by SCF/COI1-mediated ubiquitination (Chini *et al.*, 2007, Thines *et al.*, 2007).

To summarize, this study provided evidence that RPM1-mediated *A70::LUC* expression is dependent upon a jasmonate based signal that requires a functional COI1 receptor (Katsir *et al.*, 2008, Li *et al.*, 2004, Feys *et al.*, 1994) and independent of previously characterized NAC, GLRs and SA-mediated defence signalling mutants. Moreover, *A70::LUC* expression in systemic leaves mimics the jasmonate dependent *JAZ10*::GUS expression seen in the wound response (Mousavi *et al.*, 2013a). Together we argue this provides strong evidence to prove that RPM1-mediated *A70::LUC* expression is JA dependent signal.

5 Chapter 5 Plant electrophysiology

5.1 Introduction

Small wounds inflicted by insect herbivores can induce electrical responses due to change in ion efflux/influx in plants (Bricchi *et al.*, 2013, Bricchi *et al.*, 2012). The genes involved in the propagation of electrical activity, leading to defense gene expression such as *GLUTAMATE RECEPTOR-LIKE* genes (GLRs), control the distal wound-stimulated expression of several key jasmonate-inducible regulators of jasmonate signalling (*JAZ* genes) in the adult-phase plant (Mousavi *et al.*, 2013a). Analogous to the *JAZ10*::GUS results from the same group, this study, stimulated us to investigate whether, based upon our evidence for jasmonate based signalling in early SAR responses, specific electrical signatures, similar to those propagated in the wound response, may also be involved in SAR signalling.

There are several studies revealing that electrical signals are a component of wound signalling (Zimmermann *et al.*, 2009, Maffei *et al.*, 2004, Salvador-Recatala *et al.*, 2014, Felle & Zimmermann, 2007, Zimmermann *et al.*, 2016), but information on long distance signalling in plant microbe interactions is, to my knowledge, absent. For wound induced electrical signals, it was reported that both negative and positive extracellular voltage changes can be induced by wounding and these can propagate into adjacent leaves of *Arabidopsis thaliana* during feeding of *Spodoptera littoralis* larvae (Mousavi *et al.*, 2013a). These voltage changes, were designated as wound-activated surface potentials (WASPs). This study reported negative WASPs in the local leaf as well as adjacent leaves whereas the same stimulus simultaneously triggered positive WASPs in other distant leaves of the same plant. The negative voltage changes were correlated with the jasmonate pathway due to an increase (up

to approximately 130-fold) of *JAZ10* transcript levels. Furthermore, they also reported herbivore-induced (*Pieris brassicae*) electrophysiological reactions in *Arabidopsis* sieve elements of intact neighboring leaves by direct current electrical penetration graphs using a living aphid as bio-electrode (Zimmermann *et al.*, 2009, Maffei *et al.*, 2004, Salvador-Recatala *et al.*, 2014, Felle & Zimmermann, 2007, Zimmermann *et al.*, 2016).

This chapter characterizes the electrical surface potential changes generated during DC3000*avrRpm1* challenge, in both bacterial infected (local) and non-infected (systemic) leaves. Furthermore, we asked how these signals might act as long-distance electrical signals to induce plant defense mechanism in systemic leaves. In addition, we investigated whether there was any specificity in the electrical signal and whether electrical signals could be induced in immune compromised mutants by DC3000*avrRpm1.*

5.2 Results

5.2.1 RPM1- mediated electrical signals

DC3000*avrRpm1* (~2 x 10^8 cfu ml^{-1}) was infiltrated in a single Col-5 leaf on a rosette on which voltage recording electrodes were assembled as detailed and illustrated in Chapter 2. Surface potential change was measured from the reference electrode to the working electrode. Representative images of all electrophysiological experiments shown in Apendix-2.

In the presence of a drop of 10 mM KCl in 0.08% agar, the surface potential of a non-treated plant leaf was consistently between ±20 mV. Upon infiltration with DC3000, mock (10mM $MgCl_2$) or T3SS deficient DC3000*hrpA* as a control, surface potential changes after lag phase of ~50min and return to its constant phase as in non-

treated plants **(Figure 33)**. Interestingly, infection with DC3000*avrRpm1* resulted in a change in surface potential of the infected leaf (electrode W2). After an initial lag phase of 1:40 h post challenge, the signal starts to drop to negative and by 3-4 hpi it's about (-100mV), returning to the pre-stimulus level within 5 h. Measuring electric signals required very sensitive apparatus and only one plant can be analysed at a time. While there was some noise in the system after many replicates we can confidently say that following DC3000*avrRpm1* we could reproducibly see, in systemic leaves (corresponding to electrodes W1, W3 and W4), similar changes in surface potential from ~ 5hpi until ~ 8 hpi. These were, as one might anticipated given the local leaf is undergoing a major physiological perturbation, of significantly reduced duration and of smaller amplitude compared to the infected leaf **(Figure 33)**.

The fact that DC3000 expressing *avrRpm1* could induce changes in surface potential in local and systemic tissue indicated a possible role for electrical signal propagation during HR and subsequent establishment of SAR. To further understand the propagation of electrical signal during pathogenesis, two additional DC3000 strains expressing avirulent effector proteins were tested for their ability to induce changes in surface potential, DC3000*avrRpt2* and DC3000*avrRps4.* Both ETI producing challenges produced electrical signals, notably the systemic electrical signal after DC3000*avrRpt2* infection was stronger than that induced by DC3000*avrRps4* challenge **(Figure 34)**, consistent with *A70::LUC* expression patterns observed by others in the laboratory (data not shown).

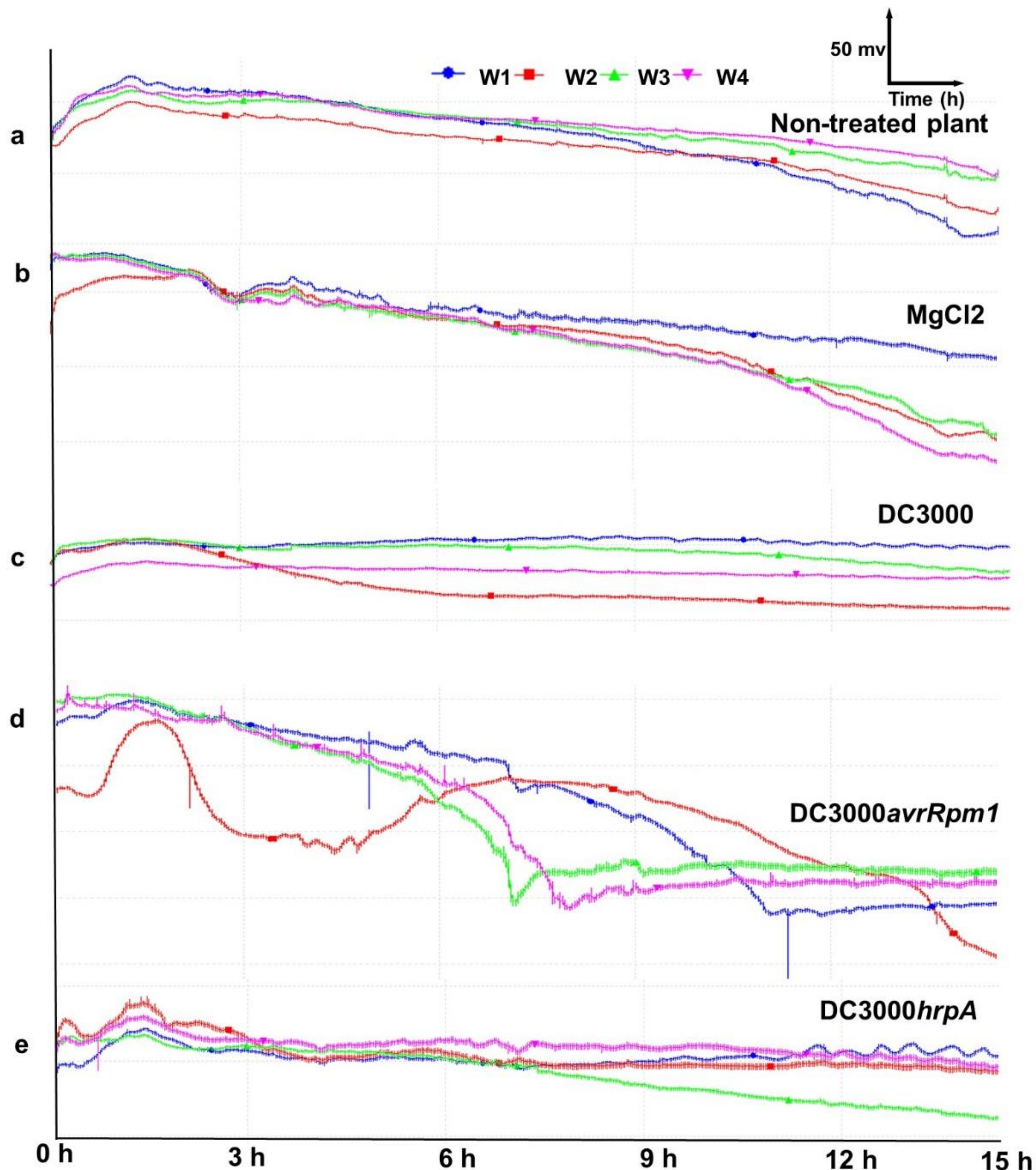


Figure 33. Surface potential changes following challenge with virulent (DC3000) or T3SS deficient bacteria:

a. An unchallenged Col-5 plant, in comparison to changes in surface potential measured by the working electrode after infiltration with **b.** 10mM $MgCl_2$, **C**. DC3000, **d.** DC3000*avrRpm1* or, **e.** DC3000*hrpA* at (~2 x 10^8 cfu ml^{-1}). Figure captures typical surface potential changes recorded between the four electrodes, W1, W2, W3 and W4 after treatment. The electrical potential difference on the leaf surface was registered continuously after infecting with DC3000*avrRpm1* as indicated in the figure. Surface potential changes measured over time were in the voltage 50mv/h. Individual experiment repeated 12 times.

In a leaf infected with DC3000*avRpt2* a signal with a duration of 1:30 h and a peak amplitude of 40±10 mV was observed. In systemic leaves (electrodes W3 and W1) the change is surface potential was of same direction, beginning after 8 h, but of a shorter duration and smaller amplitude than the infected leaf, with the surface potential returning to normal 12 hpi. While the locally challenged DC3000*avRps4* leaf showed a peak amplitude of 80±10 mV, signals in systemic leaves were not reproducible and while trending, no significant change in surface potential could be reliably measured **(Figure 34).**

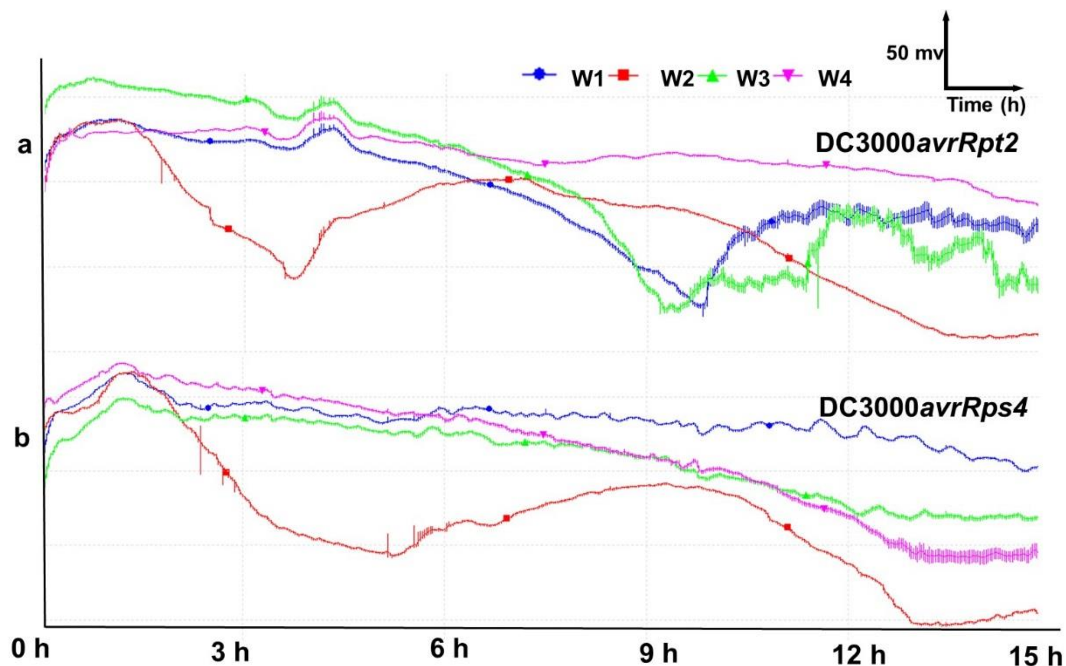


Figure 34. Surface potential changes with various effectors:

Change in surface potential recorded on working electrode after infiltration of ***a.*** *DC3000*avrRpt2 *and* ***b.*** *DC3000*avrRps4 *at (~2 x 10^8 cfu ml^{-1}).* Surface potential changes measured over time were in the voltage 50mv/h. Individual experiment repeated 3 times.

5.2.2 Pre-treatment of peptides affects DC3000*avrRpm1* mediated electrical signalling

PTI can inhibit the HR in *Arabidopsis* (Newman *et al.*, 2000, Crabill *et al.*, 2010a) and our earlier results shows RPM1-mediated *A70::LUC* expression as well as HR were abolished after pre-treatment of PTI eliciting peptides. To study the effect of pre-activation of PTI on RPM1-based electrical signalling, leaves were first pre-treated with 5 µM flg22, elf18 or *At*Pep. 16 h later, the same leaves were infiltrated with *DC3000avrRpm1* and plants monitored for DC3000*avrRpm1* mediated electrical signal. Interestingly, results show that the *DC3000avrRpm1*-induced electrical signal was abolished in all pre-treated peptide leaves as well as systemic leaves **(Figure 35)**. This clearly indicated that PTI plays an important role in induction of effector based electrical signal and raised the question whether pre-treatment with PTI inducers can abolish the ETI induced systemic electrical signal?

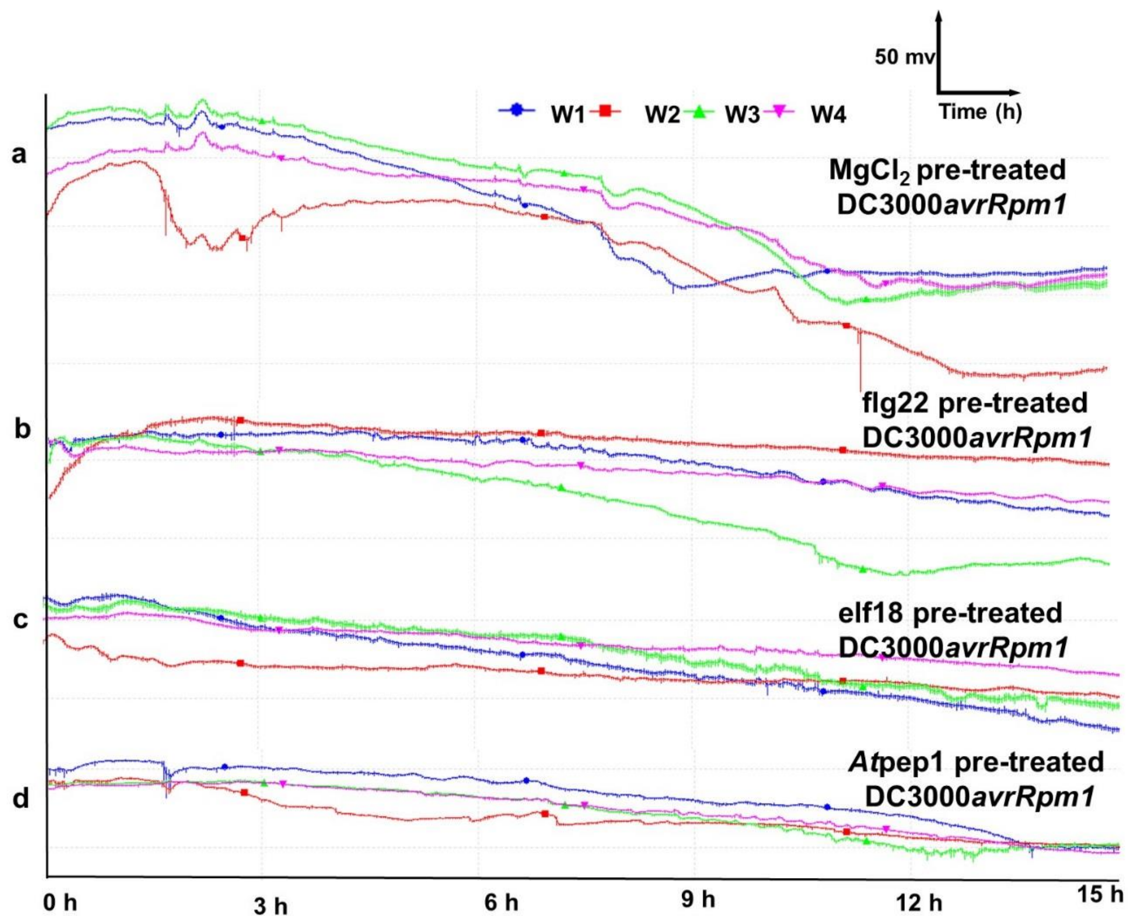


Figure 35. Role of PAMP pretreatment in DC3000*avrRpm1* mediated electrical signal generation
Col-5 plants were pre-infiltrated with 5 µM **a.** mock ($MgCl_2$) **b.**flg22, **c.** elf18, **d.** *At*Pep1. After 16 h, the same leaves were challenged with DC3000*avrRpm1* (OD_{600} 0.2) and changes in Surface potential changes measured over time were in the voltage 50mv/h. Individual experiment repeated 3 times.

5.2.3 Role of PAMPs in systemic signalling:

Surprisingly, up to now, little has been known with respect to the changes at the plasma membrane underlying PAMP/MAMP based signalling. Early and robust responses of cells to PAMPs are linked to alkalinisation of the apoplast or ROS generation (Felix *et al.*, 1999, Kunze *et al.*, 2004b, Bauer *et al.*, 2001). It has been reported that electrical signalling in response to peptides (flg22 or elf 18) fully depends on the activity of the FLS2-associated receptor-like kinase BAK1, it also suggesting

that calcium-associated plasma membrane anion channel opening as an initial step in the pathogen defence pathway (Jeworutzki *et al.*, 2010). PAMPs significantly contribute to SAR initiation in *Arabidopsis* (Mishina & Zeier, 2007), though as alluded to throughout this thesis, evidence to date suggests that PAMP induced SAR is almost certainly induced through different signalling pathways than gene-for-gene mediated SAR.

To understand the role of PTI in induction of this systemic electrical signature a transgenic line carrying the bacterial *avrRpm1* protein under the control of an inducible promoter dexamethasone (DEX::*avrRpm1* plants) was used to look at the generation of an HR in the absence of associated PAMP triggering molecules. This addresses whether AvrRpm1 alone can induce a systemic electrical signal. Painting DEX (10μM) onto a DEX::*avrRpm1* leaf led to an RPM1-mediated electrical signal. **(Figure 36)**.

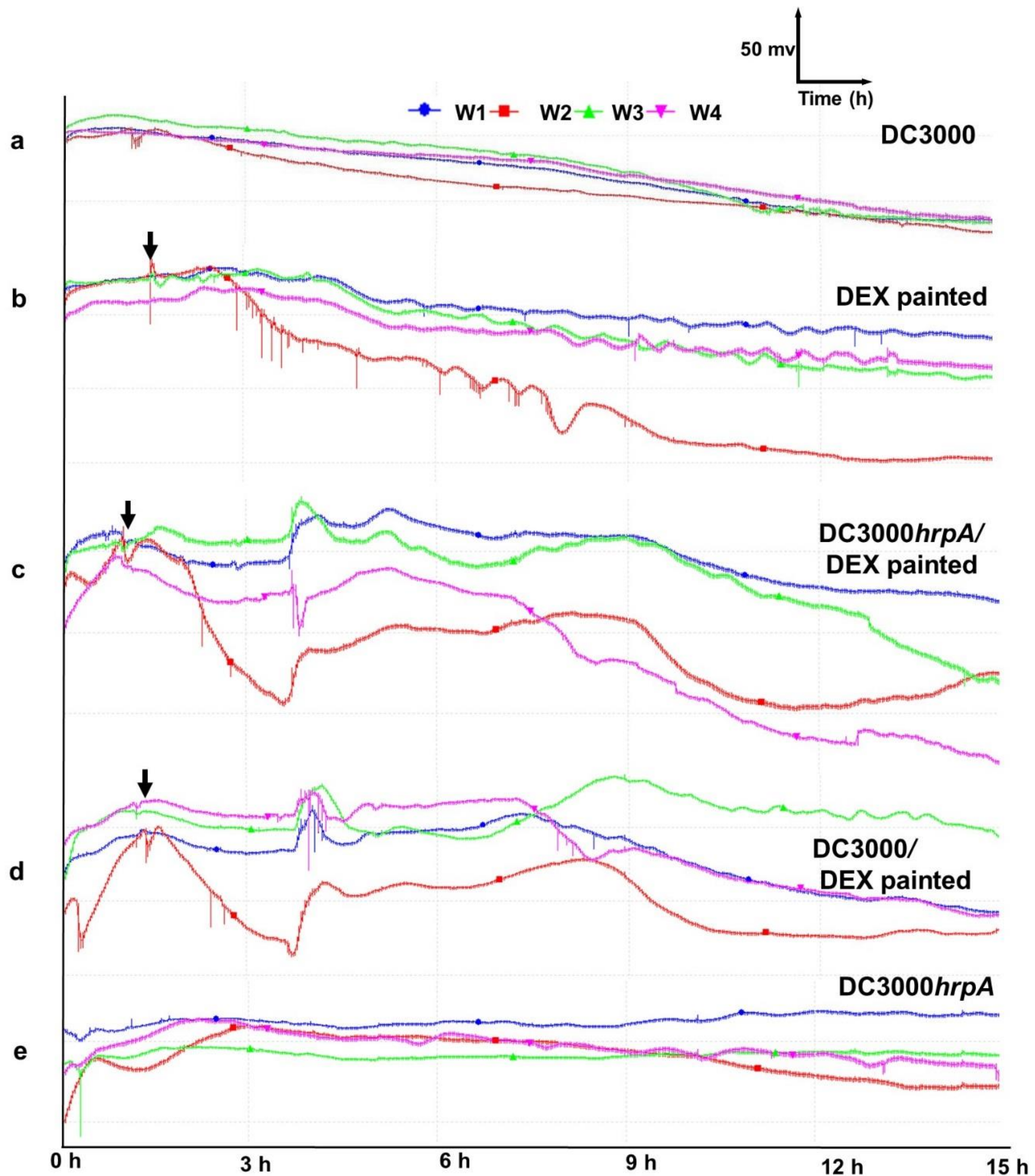


Figure 36. Role of PAMPs in change in Surface potential in systemic leaves:

Change in surface potential recorded on working electrode on leaf of DEX::*avrRpm1* plant after infiltration of **a.** DC3000 (Mock) at (~2 x 10^8 cfu ml^{-1}) **b.** 10μM Dex **c**. DC3000*hrpA* at (~2 x 10^8 cfu ml^{-1}) followed by DEX painting and **d.** DC3000 followed by DEX painting. **e.** DC3000*hrpA* (Mock) All arrows indicate time point at which DEX painted on leaf. Surface potential changes measured over time were in the voltage 50mv/h. Individual experiment repeated 4 times.

Surprisingly, while there was gradual reduction of change in surface potential after painting DEX on leaf of DEX::*avrRpm1* plant but no systemic electrical signal recorded in on induced leaves of DEX::*avrRpm1* plants.

To investigate the impact of PAMPs in the context of a normal electrical AvrRPM1 specified ETI response, a DEX::*avrRpm1* leaf was infected with DC3000 or DC3000*hrpA* then after 1h that leaf was painted with DEX and surface potential changes recorded (electrode W2). Electrical signals dropped to ~ -80mV and returned to the pre-stimulus level within ~6 h in both DC3000/DEX and DC3000*hrpA*/DEX treated leaves. Strikingly, compared to DEX application alone, systemic leaves (electrode W3 and W4) with either DC3000 or DC3000*hrpA*, like challenge with DC3000*avrRpm1*, showed a change in surface potential beginning ~6:30 h, though the magnitude of change was less than the infected leaf **(Figure 36)**. As infiltration of DC3000 or DC3000*hrpA* alone do not lead to significant changes in electrical signal (either in local leaves or systemic leaves), these data clearly indicate that PAMPs are an essential component of an AvrRPM1 mediated systemic electrical signal generation.

5.2.4 DC3000*avrRpm1* induced electrical signalling in SAR compromised mutants

To further explore the role of electrical signals generation in systemic responding leaves DC3000*avrRpm1* (~2 x 10^8 cfu ml^{-1}) induced systemic electrical signals were monitored in the SAR mutants used to investigate *A70::LUC* signalling in Chapter 4; i.e. *npr-1*, *npr3/4*, *npr1/3/4* and *sid2*. **Figure 37** shows that all SAR mutants generated surface potential change similar to wild type upon DC3000*avrRpm1* in the infected leaf (W2 electrode). The systemic signal was persistent in the adjacent systemic leaves (W1 and W4 electrodes) in *npr1, npr1/3/4, npr3/4* and *sid2*. As,

npr1/3/4 and *sid2* plants show strong and significant changes in surface potential in systemic leaves compared to other mutant plants, it is likely that the propagation of the electric signal is independent of SA signalling. All parameters recorded in various SAR mutant plants in the DC3000*avrRpm1* infected leaf are shown in (**Table 15**).

Table 15. Electrical Parameters recorded in SAR mutant plants

Mutant plants	**Amplitude (mV)**	**Duration (h)**
npr1	80±5	3h 10min
npr3/4	70±5	3h 50min
npr1/3/4	90±5	3h 10min
sid2	80±5	2h 20min
WT	100±5	3h 2min

Out of all SAR mutant's tested, the highest amplitude of 90±5 mV was recorded in the infected leaf of *npr1/3/4* plant and the longest duration of 3:50 h was recorded for *npr3/4*.

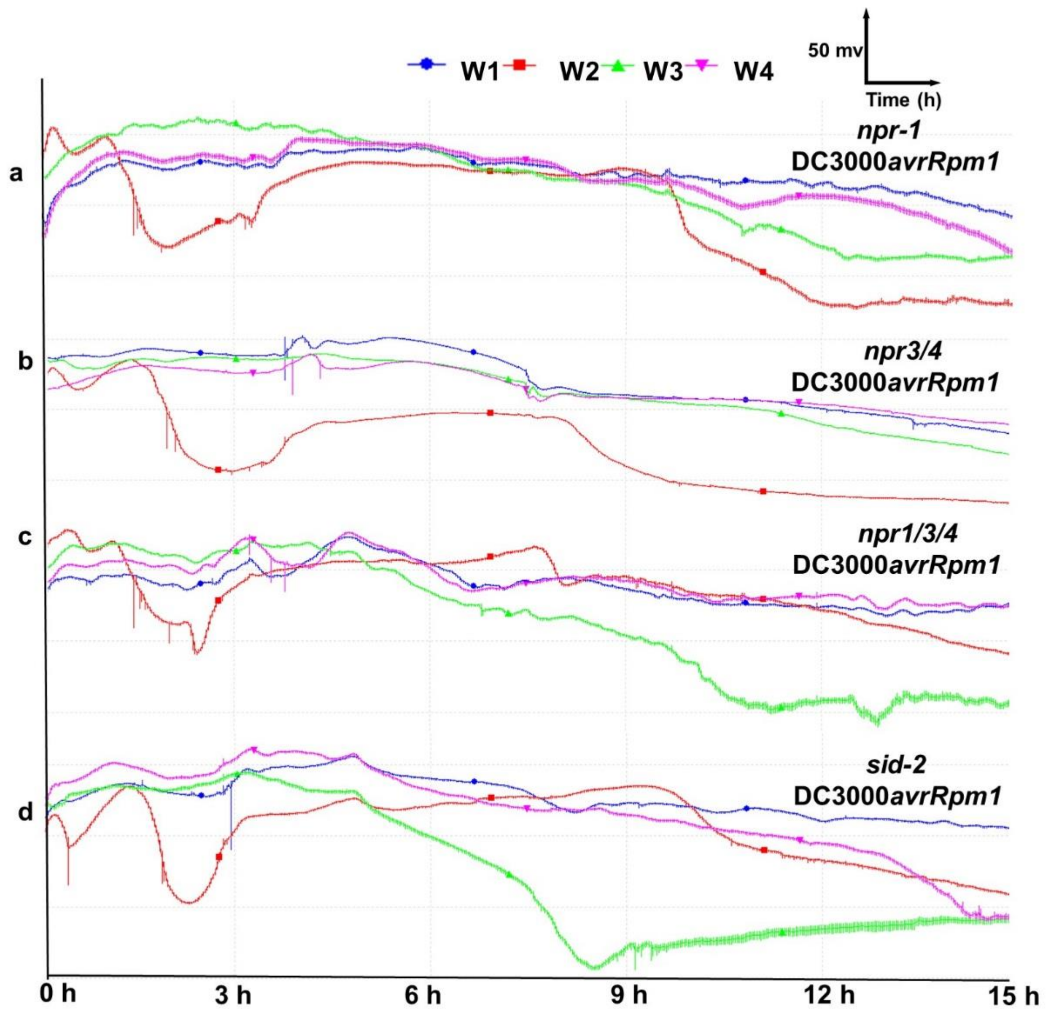


Figure 37. Surface potential changes in SAR mutant plants:

Change in surface potential recorded on working electrode after infiltration of DC3000*avrRpm1* at (~2 x 10^8 cfu ml^{-1}) in **a.** *npr-1,* **b.** *npr3/4,* **C**. *npr1/3/4* and **d.** *sid-2*. Surface potential changes measured over time were in the voltage 50mv/h. Individual experiment repeated 4 times.

5.2.5 The role of GLR's in RPM1-mediated electrical signalling

Plant glutamate receptor-like (GLR) homologs *glr3.3a* and *glr3.6a* are membrane associated channels predicted to transport glutamate, but most likely other compounds as well. Metabolic fluxes are important in plant pathogen interactions and Grant et al. (2000) showed that in addition to the well characterised Ca^{2+} transient associated with PAMP recognition, AvrRpm1-RPM1 interactions induced a slow increase in cytosolic calcium. Given results from Chapter 4 and findings from jasmonate mediated wound signalling (Mousavi *et al.*, 2013a), we also explored what role GRLs may play in generating the systemic surface potentials we observed during ETI.

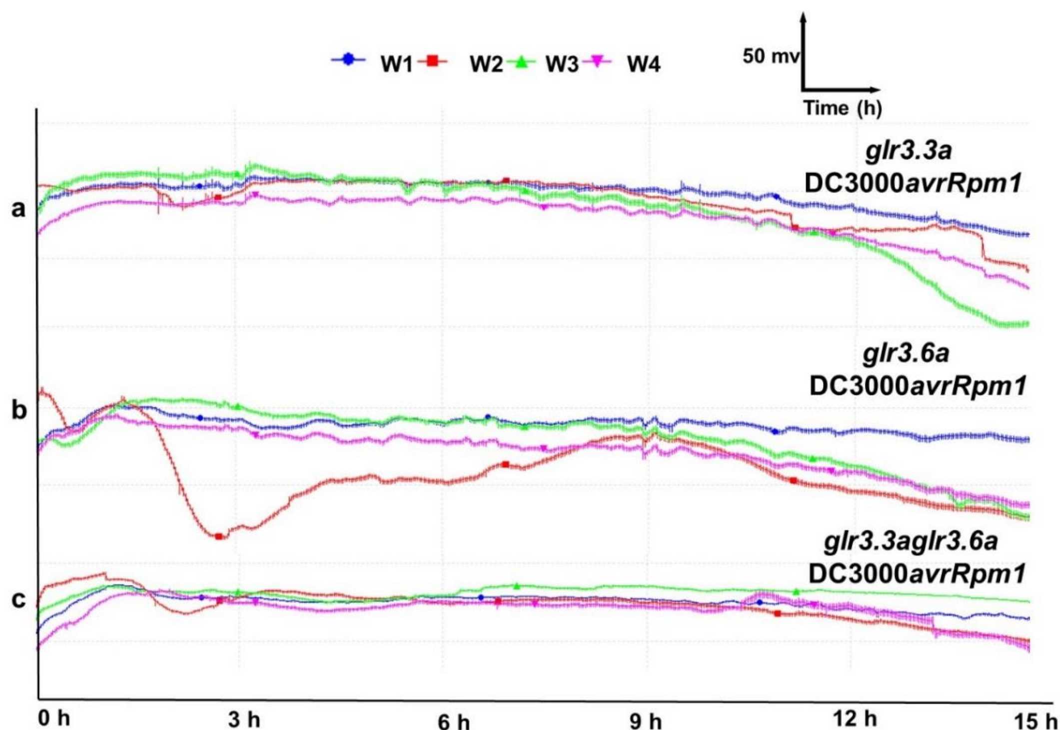


Figure 38. Surface potential changes in *glr's* mutant plants:

Change in surface potential recorded on working electrode W2 after infiltration with DC3000*avrRpm1* at (~2 x 10^8 cfu ml^{-1}); **a.** *glr3.3a*, **b.** *glr3.6a*, **c.** *glr3.3a3.6a*. Surface potential changes measured over time were in the voltage 50mv/h. Individual experiment repeated 6 times.

glr3.3a, glr3.6a and *glr3.3a3.6a* were infected with DC3000*avrRpm1* (~2 x 10^8 cfu ml^{-1}) and changes in surface potential were recorded in systemic and local leaves **(Figure 38)**. Interestingly, after infection of DC3000*avrRpm1* changes in surface potential were observed only in *glr3.6a* (electrode W2). DC3000*avRpm1* infected *glr3.6a* leaves shows negative peak amplitude of 80±10 mV which was lasted for 6h. On other hand, no change in surface potential was observed in *glr3.3a* nor *glr3.3a3.6a* plants, either in the local or systemic tissue following DC3000*avrRpm1* challenge, indicating that the GLR3.3a regulated a pathway that appears to play a role in transduction of electrical signals generated during ETI.

5.2.6 DC3000*avrRpm1* induced electrical signalling in *coi1*-16, *nac19/55/72* and *jaz5/10* backgrounds

As the earlier results indicate that the JA signalling mutant, the *coi1*-16 mutant abolished the SAR response and both *A70::LUC* and *JAZ10::GUS* expression in DC3000*avrRpm1* challenged plants (Chapter 4), we examined whether DC3000*avrRpm1* induced an electrical signal in *coi1*-16. Changes in the surface potential of *coi1*-16 plants infected with DC3000*avrRpm1* (~2 x 10^8 cfu ml^{-1}) were recorded. DC3000*avrRpm1* challenge of *coi1*-16 induced reproducible surface potential changes only in the infected leaf but not in systemic leaves. In the infected leaf changes in surface potential lasted for ~2:20 h with a peak amplitude of -80±10 mV. No changes in surface potential were observed in systemic leaves after DC3000*avrRpm1* infection. Thus *coi1*-16 also plays a vital role in facilitating the systemic electrical potentials during ETI **(Figure 39)**.

The two phylogenetically distinct JAZs, JAZ5 and JAZ10 (Oh *et al.*, 2013), collectively function to mitigate COR virulence functions and contribute to innate

immunity. Recently, Marta de Torres Zabala reported JA dynamics during DC3000 infection of *Arabidopsis* and how JAZ5 and JAZ10 together restricts COR phytotoxicity. Moreover, mRNA-seq predicts compromised SA signalling in a *jaz5/10* mutant and rapid suppression of JA-related components on bacterial infection. (de Torres Zabala *et al.*, 2016b). Therefore, *jaz5/10* (de Torres Zabala *et al.*, 2016a) lines were also studied for RPM1-based electrical signal.

Both *nac19/55/72* and *jaz5/10* (de Torres Zabala *et al.*, 2016a) lines were also studied for RPM1-based electrical signal. *nac19/55/72* affected systemic electrical potentials but *jaz5/10* lines were similar to WT challenged with DC3000*avrRpm1* **(Figure 39)**. Thus, *jaz5/10*, despite being hypersensitive to COR, doesn't play any kind to role in systemic electrical signalling, however, *nac19/55/72* appears to be involved in signal perception required for elaborating the systemic electrical signal.

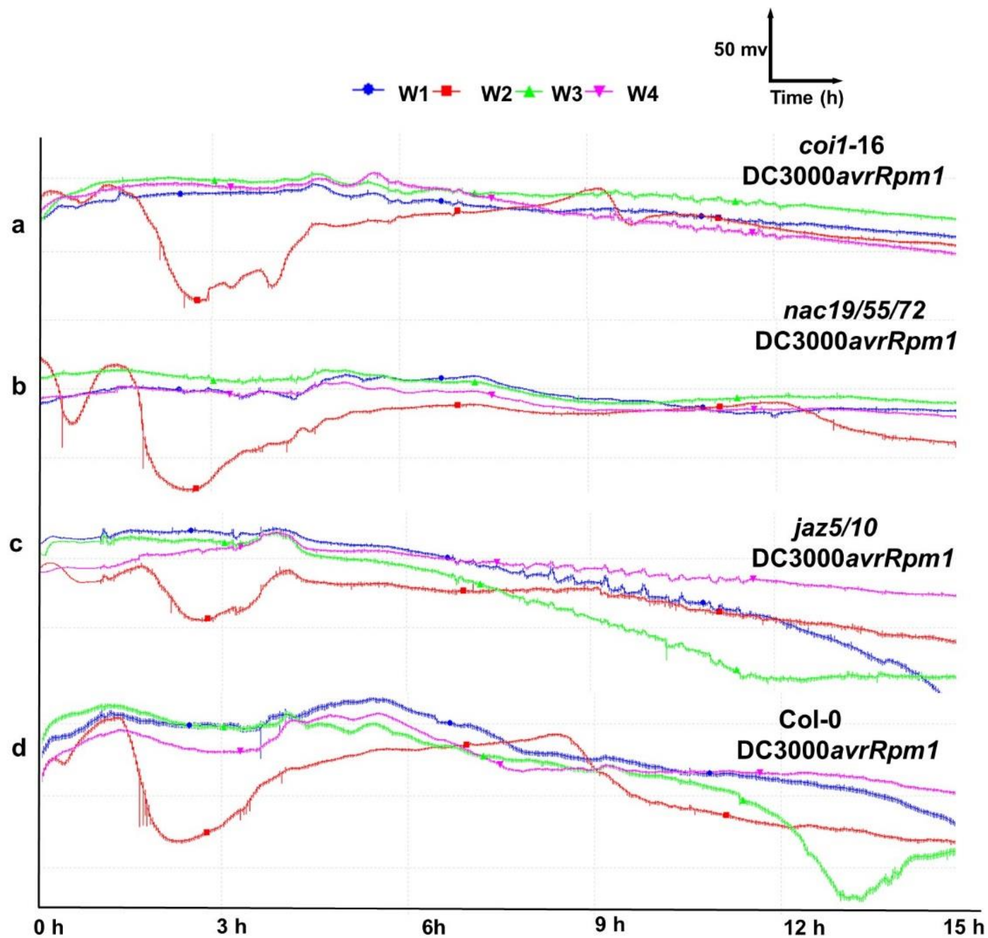


Figure 39. Surface potential changes in *coi1*-16, *nac19/55/72* and *jaz5/10* mutant plants: Change in surface potential were recorded in mutants (**a**) *coi1*-16*,* (**b**) *nac19/55/72,* (**c**) *jaz5/10* and (**d**) Col-0 after challenge with DC3000*avrRpm1* (OD_{600} 0.2). Surface potential changes measured over time were in the voltage 50mv/h. Individual experiment repeated 3 times.

5.2.7 DC3000*avrRpm1* induced electrical signalling in *A70* homologs and *A70* mutant

Because of potential redundancy (supported by the lack of a SAR phenotype in individual A70 *homolog* lines), electrophysiological changes in mutants of the actual SAR marker, *A70,* and its homologue were examined. DC3000*avrRpm1* challenged *A70 homolog1-1 (A70* like 1-1*), A70 homolog1-2 (A70* like *1-2)* and *A70KO* leaves showed typical surface potential changes (i.e. W2 electrode) Strikingly, whereas no

change in surface potential was recorded across electrodes W1, W3 and W4, reporting the systemic leaf electrical responses **(Figure 40)**. These results show that both functional *A70 homolog* and *A70* are essential to induce systemic electrical signalling.

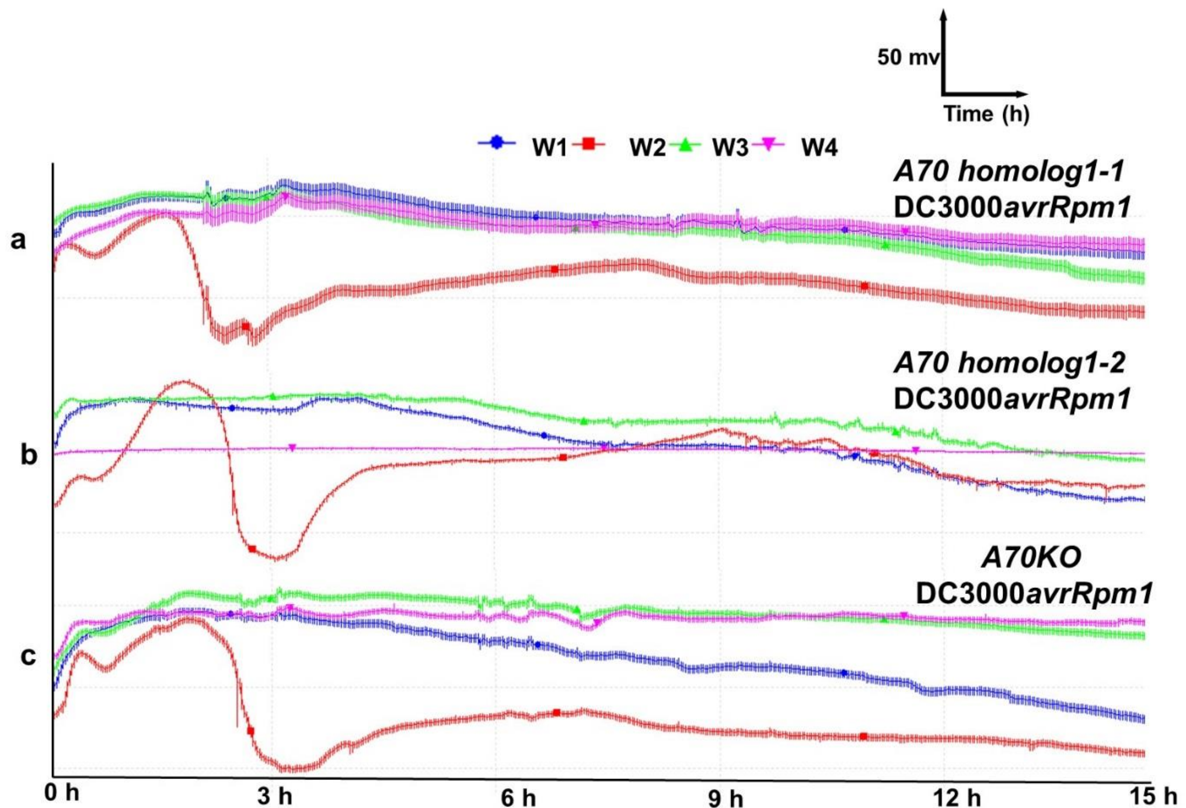


Figure 40. RPM1 mediated surface potential changes in *A70 homolog's* and the *A70 knock out* mutant:

Change in surface potential recorded on working electrode after infiltration of DC3000*avrRpm1* at (~2 x 10^8 cfu ml^{-1}) in **a.** *A70 homolog1-1*, **b.** *A70 homolog1-2*, or **c**. *A70KO*. Surface potential changes measured over time were in the voltage 50mv/h. Individual experiment repeated 3 times.

5.3 Discussion

Understanding the molecular mechanisms of systemic signalling in plants has been a major scientific challenge. In reality, systemic signalling, induced either by herbivore attack or pathogen infection, is likely to be complex and involve chemical and electrical signals. Indeed, several reports support a role for electrical and chemical systemic signalling upon wounding, either through the vasculature, or possibly as volatile signals (Davies, 1987, Rhodes JD, 1996, Heil & Silva Bueno, 2007a, Heil & Ton, 2008, Howe, 2008, Mithöfer A, 2009).

This chapter is focussed on changes in leaf electrical potential generated upon DC3000*avrRpm1* challenge and addressed the question of the contribution of PTI, in the context of specific gene-for-gene responses, to SAR signalling. It looked at the components involved in the evolution and propagation of the signal using a variety of mutants.

Electrical signals in plants are established as a rapidly propagated signal in response to both biotic and abiotic stimuli (Maffei & Bossi, 2006), and are defined as an ion imbalance across the plasma membrane leading to a voltage transient (Trebacz *et al.*, 2006). Here we demonstrated, changes in leaf surface potentials in both the infected (local) and non-infected (systemic) leaves after DC3000*avrRpm1* challenge. The whole depolarization and repolarization process takes place between 2.5 h to 5h in the local leaf and between 5h to 8h in the adjacent systemically responding leaves. No change in surface potential was observed either in local leaves or in systemic leaves after infection of DC3000, DC3000*hrpA* or mock ($MgCl_2$) (**Figure 33**), strongly supporting the hypothesis that in addition to a local HR, DC3000*avrRpm1* infection also orchestrates complex electrical signalling events.

Our work also identified an unexpected role for PAMP activation in the generation of systemic signals. The RPM1-induced electrical response was abolished after pre-treatment with the PAMPs flg22 or elf18, or the DAMP *At*Pep1 (**Figure 35**). Our results lend support to the Krol studies proposing that the overall response pattern of the plants to *At*Pep(s) is similar to the response to MAMPs such as flg22 or elf18, despite differing in amplitude (Krol *et al.*, 2010b). Notably, several studies have reported that DAMPs and PAMPs, after being recognized through their receptors, lead to the activation of anion and calcium channels. (Schulze *et al.*, 2010, Postel *et al.*, 2010). Thus MAMP- and DAMP-induced signalling pathways appear to converge at very early stages, sharing common signalling components, including ion channels (Krol *et al.*, 2010a), NADPH oxidases, MAPK cascades and several defence gene combinations (Navarro *et al.*, 2004, Zipfel *et al.*, 2006, Denoux *et al.*, 2008). Our results with DEX::*avrRpm1* inducible lines unexpectedly revealed that PAMP perception appears an essential component of the generation and propagation of a systemic signal because DEX doesn't induce systemic electrical signals by itself but after infecting plant with peptides followed by DEX painting able to induce Systemic electrical signal. (**Figure 36**).

Interestingly the electrical potential signatures of the SA signalling and biosynthetic mutant's *sid-2, npr-1, npr3/4* and *npr1/3/4* were identical to wild type Col-0 responses based on amplitude and duration of signal (**Figure 37**). By contrast, of the *Arabidopsis* GLRs mutant lines tested, *glr3.3a* and *glr3.3a/3.6a* don't show any change in systemic surface potentials, whereas glr3.6a displayed changes in electrical surface potential only in infected leaves but not in systemic leaves (**Figure 38**). These results support a role for GLRs in systemic electrical signalling. This is consistent with our earlier results (Chapter 4) showing that bacterial growth in systemic leaves of

GLRs mutants was not restricted. *glr3* mutants do not affect RPM1-mediated *A70::LUC* expression (Chapter 4), but *glr3.3a/3.6a* affect *A70::LUC* expression induced by JA and coronatine.

We previously showed that the *coi1*-16 mutant abolished *A70::LUC* expression and was compromised in SAR (Chapter 4). Furthermore, Mousavi *et al.* reported that the genes involved in the propagation of electrical activity, leading to defense gene expression, can also control jasmonate-inducible regulators of jasmonate signalling (*JAZ* genes) (Mousavi *et al.*, 2013a). Consistent with an important role for electrical signals in ETI mediated systemic signalling, *coi1*-16 abolished systemic RPM1-mediated electrical signalling (**Figure 39**).

We also studied two additional mutant lines, *jaz5/10* and *nac19/55/72*. The logic was that COI1, JAZs and MYC protein plays important role in JA signalling defence and COI1 controls the turnover of the JAZ co-receptors which directly interact with MYC2 to control JA-regulated genes (Thines *et al.*, 2007, Sheard *et al.*, 2010, Chini *et al.*, 2007, Pauwels & Goossens, 2011, Fernandez-Calvo *et al.*, 2011). Notably, the NAC19/55/72 signalling module has been predicted to function downstream of MYC2 in jasmonate based defence responses (Zheng *et al.*, 2012). Chapter-4 demonstrated that *nac19/55/72* abolished DC3000*avrRpm1* induced SAR. Surprisingly, *nac19/55/72* also could not induce RPM1-based electrical signal in systemic leaves although changes in surface electrical potential in the local challenge leaf was similar to wild type (**Figure 39**). Thus, both *nac19/55/72* and *coi1*-16 both induce electrical signal in local leaves upon DC3000*avrRpm1* but fail to elicit changes in systemic leaves. These data suggest the electrical surface changes in systemic leaves are dependent upon COI1 and propagated through the NAC19/55/72 signalling module.

Additionally, the *jaz5/10* was tested for systemic RPM1 based electrical activity. Collectively, *JAZ5* and *JAZ10* play an important role in restricting coronatine cytotoxicity and pathogen growth through a complex transcriptional reprogramming during infection of *Arabidopsis* with virulent *P. syringae* infection. Moreover, upon bacterial infection the *jaz5/10* mutant is compromised in SA- signalling and shows a rapid suppression of JA-related components based on the mRNA-seq data (de Torres Zabala *et al*., 2016b). However, despite this impact on virulent infections, the electrophysiological responses were similar to WT after DC3000*avrRpm1* challenge (**Figure 39**). These data suggest the JA signalling components required for ETI initiation and propagation of COI1 dependent jasmonates signals don't require JAZ5/10 function. Given the role for *coi1* and *nac19/55/72* demonstrate above, we cannot discount that any JAZ requirement for SAR is compensated for by either other specific JAZ's or the extensive redundancy in the JAZ family.

Finally, the contributions of *A70* and its homologue were also analyzed for electrophysiological activity after DC3000*avrRpm1* infection. Strikingly, both *A70 homologs* and *A70KO* abolished RPM1-mediated systemic electrical signal but not local (**Figure 40**) providing further evidence that *A70* (and its homologue) plays important role in systemic electrical signaling.

In summary, this chapter presents novel results demonstrating gene-for-gene interactions generate specific electrical signatures in both local challenged and systemic leaves. The RPM1-induced electrical signature is not just dependent upon AvrRpm1 delivery but strikingly, PAMPs play a crucial role in orchestrating RPM1-mediated systemic electrical signal. Somewhat counterintuitively, prior pre-treatment of with PAMPs can abolish the RPM1-based changes in surface potential. These data strongly correlate with *A70::LUC* expression and onset of macroscopic HR.

DC3000*avrRpm1* mediated electrical signatures (both local and systemic) are shown in SAR mutant lines, *npr1, npr1/3/4, sid2* and *jaz5/10*. Interestingly, *npr3/4, coi1*-16*, glr3.6a, A70KO* and *A70 homologs* showed local electrical signal but abolished systemic RPM1-mediated electrical signals, providing evidence that these components are required for signal propagation and/or perception in systemic leaves. Strikingly, *glr3.3*a and *glr3.3a/3.6a* completely abolished in RPM1-mediated electrical signals suggesting a key role for GLR3.3a in initial generation of the electrical surface potential, and possibly also in its perception in systemic responding tissue.

6 Chapter 6 Localization of *A70*

6.1 Introduction

The TargetP (http://www.cbs.dtu.dk/services/TargetP) and Tair database predicts that the A70 (At5g56980) and *A70 homolog* (At2g26110) protein are localised in the chloroplast. The first ~ 300 nucleotides of *A70* are predicted to encode a signal peptide and a conserved sequence of unknown function. Main rationale for Pro*A70::A70SP* design is to visualisation the dynamics of *A70* expression and localisation at the subcellular level. Therefore a transgenic *Arabidopsis* line carrying a Pro*A70*::*A70*SP::eGFP was generated (SP corresponding to the ~100 N-terminal amino acids) and determined its localisation after *DC3000avrRpm1* challenge by confocal microscopy. The *A70*-green fluorescent protein (eGFP) fusion was found to follow similar dynamics of induction to the *A70::LUC* fusion and was localised to the vasculature and endoplasmic reticulum of systemically responding leaves. Cell-to-cell movement is predicted to be via plasmodesmata.

6.2 Results

6.2.1 Cloning of Pro*A70*::*A70*SP::eGFP fusion protein

To gain further insight into how *A70* contributes in defence and better understand the dynamics of *A70* systemic signalling, the subcellular localization of the A70 protein was examined by constructing a GFP fusion with the first ~100 aa of A70. **Figure 41** illustrates the construct comprising the same 1633 nucleotide *A70* promoter region used in the *A70::*LUC line plus the N-terminal 262 nucleotides of the putative transit sequence of *A70* isolated by PCR with primers designed to generate EcoRI and NcoI ends, thus enabling cloning of purified, digested fragments into pCambia1305.

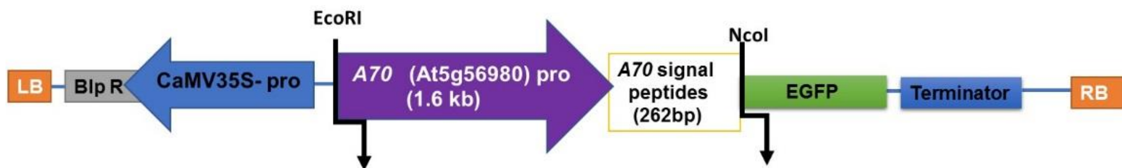


Figure 41. Schematic diagram of Pro*A70*::*A70*SP::eGFP fusion protein:

The 1633 nucleotides of the full length *A70* promoter plus the first N-terminal 262 nucleotides of the putative transit sequence and domain of unknown function of *A70* was cloned in front of eGFP in pCAMBIA1305 and transformed into *E.coli*. The validated T-DNA plasmid was subsequently transformed into *Arabidopsis* via *Agrobacterium* transformation. T1 plants were selected on BASTA.

6.2.2 Pathogen induced expression of *A70* in the leaf petiole and the epidermal layer of systemically responding leaves

Pro*A70*::*A70*SP::eGFP (T1-generation) plants were selected on BASTA as described in chapter 2. Then selected plants infected with DC3000*avrRpm1* (~2 x 10^8 cfu ml^{-1}) and analysed for GFP signal in local and systemic leaves under stereomicroscope. Plant confirmed for GFP signal in systemic leaves used for further studies.

Pro*A70*::*A70*SP::eGFP (T2) plants were challenged with DC3000*avrRpm1* and samples from the epidermal layer of the systemic leaf and its petiole were analysed for GFP fluorescence by confocal microscope ~4 hpi Pro*A70*::*A70*SP::eGFP was strongly activated in the systemic leaf, particularly in the epidermal layer and in the petiole region.

Examination of the petiole in this systemically responding tissue revealed eGFP accumulation in the vasculature. Cross sections of the leaf petiole were imaged, and consistent with the predicted rapid localisation of *A70* expression in petioles, eGFP accumulation was recorded in the vasculature tissue of the systemic responding petiole **(Figure 42 a & b)** as well as, unexpectedly, in the epidermal cells. To distinguish better the components vascular tissue, the xylem, which is composed of dead cells was stained with propidium iodide to increases visibility of the cell walls in the leaf petiole. In contrast to strong propidium iodide staining shown in the xylem, the adjacent phloem cells appeared to show strong accumulation of eGFP **(Figure 42 c & d)**. These data support a conclusion that the propagation of a systemic signal activates both *A70* expression and accumulation of the protein in the petiole.

Furthermore, the systemic responding leaves expressing Pro*A70*::*A70*SP::eGFP showed strong eGFP localisation in the endoplasmic reticulum of systemic tissue. (**Figure 43 a & b)** as well as around plasmodesmata of the systemic responding cell within 4 hpi (**Figure 43 c & d)**.

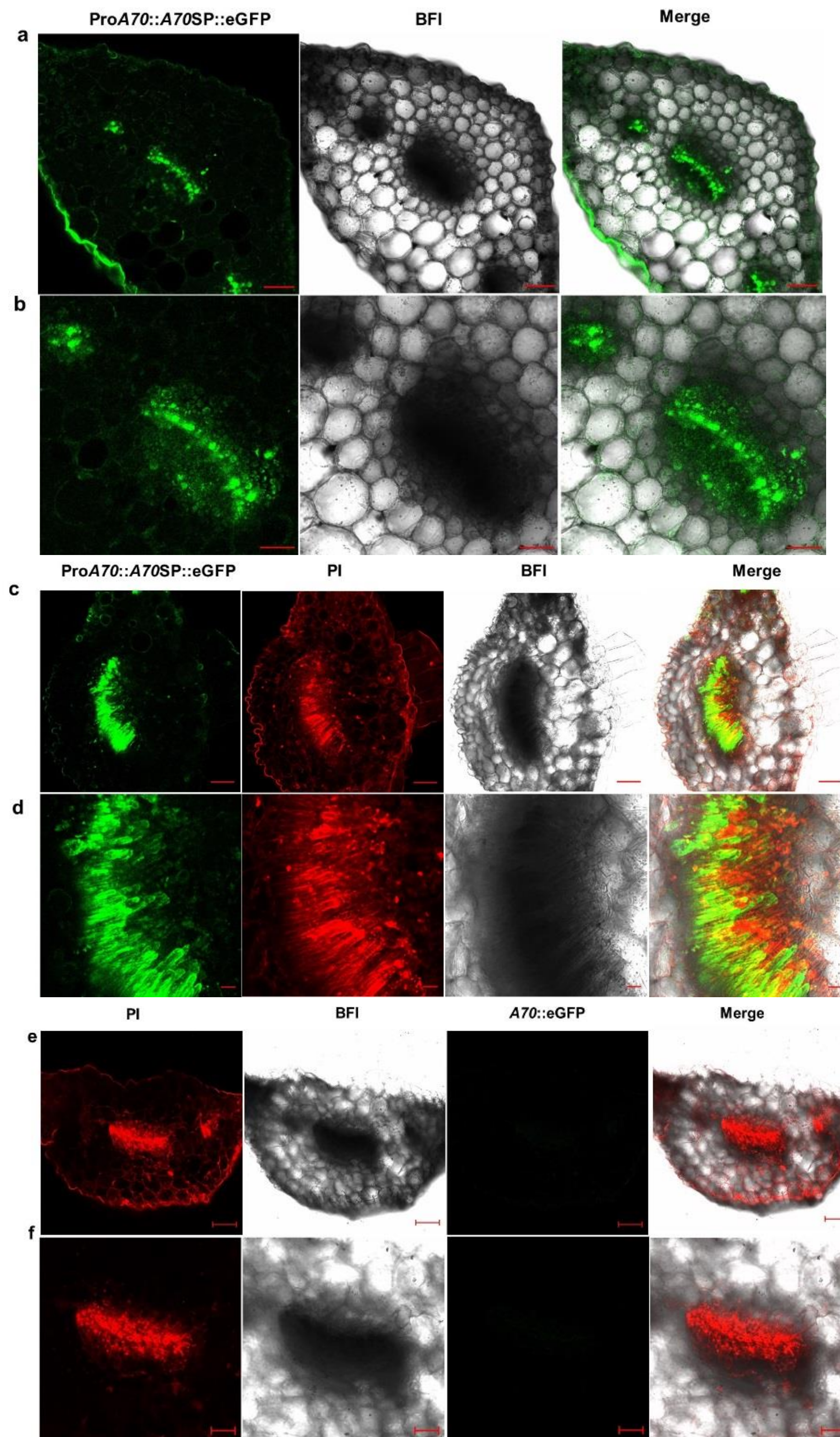


Figure 42. Systemic translocation of GFP through the phloem in Arabidopsis Pro*A70*::*A70*SP::eGFP leaf petioles: Confocal micrographs of eGFP accumulation by DC3000*avrRpm1* ~4 hpi in Pro*A70*::*A70*SP::eGFP transgenic Arabidopsis systemic leaf petiole showing the *A70* N-terminal GFP fusion protein is localized (and possibly translocating) through the phloem to systemic tissue. (**a & b)** petiole of systemic leaves were directly analyzed by confocal microscopy. Left panel, GFP (green channel); middle panel, BFI (Bright Filed Image) (white); right panel, merged images. **a**. Transverse cross section of petiole. Scale bars, 100μm. **b**. Close-up view of leaf petiole. Scale bars, 50μm. (**c**, d, e & f) For cell wall staining, WT (e & f) and Pro*A70*::*A70*SP::eGFP (c & d) systemic leaves were submerged in propidium iodide (PI) at 50μg/ml and leaf petiole was directly analyzed by confocal microscopy. Left panel, Pro*A70*::*A70*SP::eGFP (green); middle 2nd panel, PI (red); middle 3rd panel, BFI (white); right panel, merged images. Scale bars, 100μm. **d**. Close-up view of leaf petiole. Scale bars, 20μm.

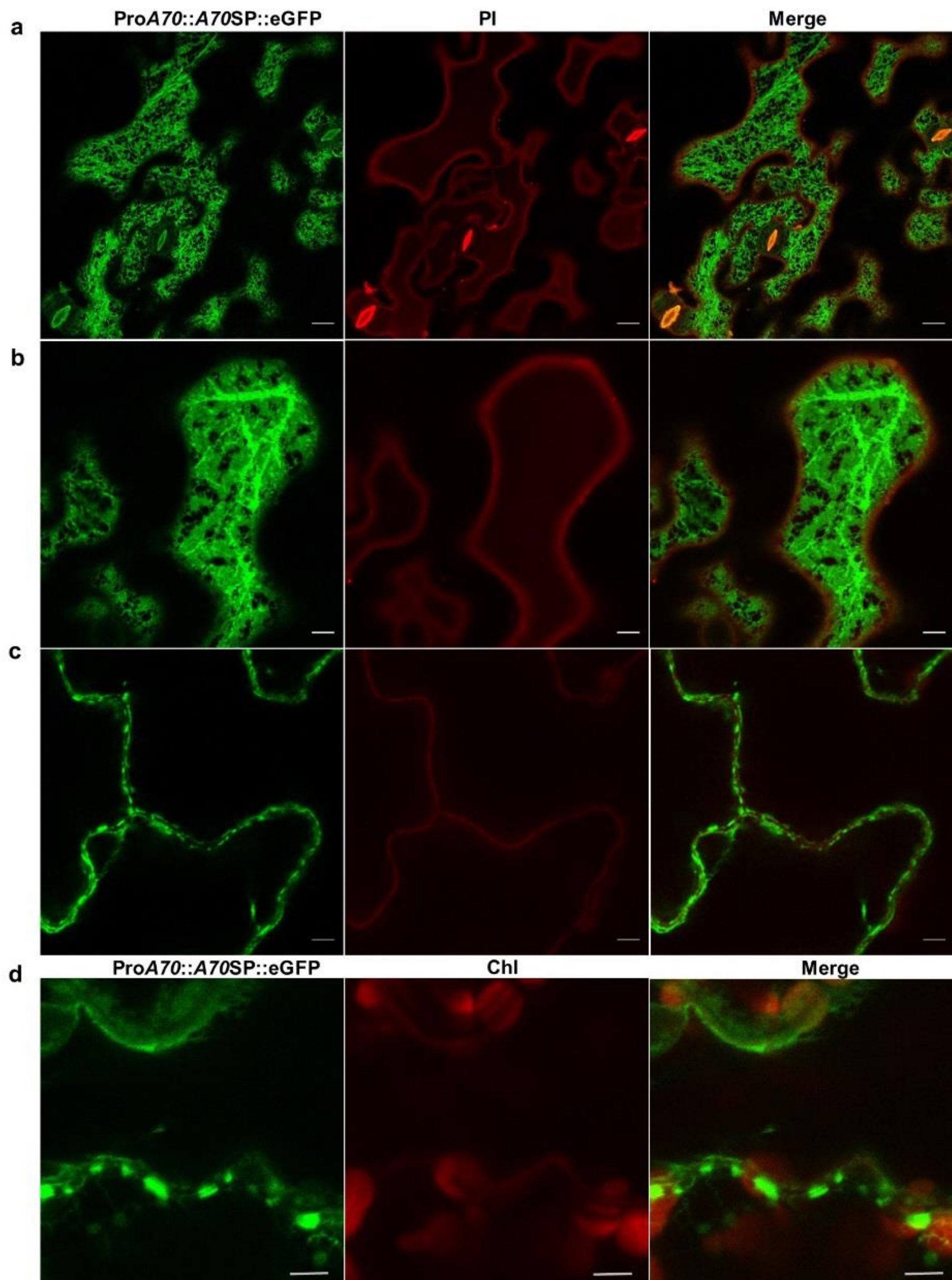


Figure 43. Subcellular localization of GFP in Pro*A70*::*A70*SP::eGFP *Arabidopsis*:

Laser scanning confocal microscopy micrographs of epidermal cells of systemic leaf of Pro*A70*::*A70*SP::eGFP transgenic *Arabidopsis* lines after AvrRpm1 challenge. Pro*A70*::*A70*SP::eGFP localized in the ER (**a & b**) and around plasmodesmata (**c & d**). For cell wall staining, Pro*A70*::*A70*SP::eGFP systemic leaves were submerged in propidium iodide (PI) at 50µg/ml and leaf epidermal cells were directly analyzed by confocal microscopy. Left panel, GFP channel; middle panel, PI (red); right panel, merged images. For image (**d**) the middle panel is chlorophyll fluorescence (chl), (red). The scale bar represents **a.** 10 µm. **b.** 5 µm **c.** 20 µm **d.** 5 µm.

A damaged plant leaf can induce *JAZ10::GUS* expression not only in the wounded leaf but also in some distal leaves that share vascular connections (orthostichy) with the wounded leaf (Farmer *et al.*, 2014). Moreover, transport of chemical elicitors of the wound response has been reported in the xylem (Malone & Alarcon, 1995, Rhodes *et al.*, 2006) or in the phloem (Stenzel *et al.*, 2003). It has also been reported that jasmonate signalling was associated with the phloem of *Solanaceae* family plants (Jacinto T, 1997). Here, following DC3000*avrRpm1* challenge of Pro*A70*::*A70*SP::eGFP lines, GFP signal was detected in the petiole of distal leaves that share vascular connection with the infected leaf. It still needs to be confirmed whether this signal is traveling though xylem or phloem-associated cells, or both cell types.

6.3 Discussion

This chapter focused on the localization of A70 using a Pro*A70*::*A70*SP::eGFP transgenic line. Our results reveals rapid and strong accumulation GFP signal in the endoplasmic reticulum as well as around plasmodesmata in systemic responding leaves. The spatial accumulation of GFP and the timing thereof strongly correlate with *A70::LUC* expression (Chapter 3). The data support systemic expression and subsequent accumulation of the GFP fusion in leaves comprising the same orthosticy as the inoculated leaf, consistent with an inducing signal propagating from the challenged leaf and translocating up the petioles of the systemic responding leaves. These data are remarkably similar to the wound signal responses reported by the Farmer Lab showing that mechanically damaged leaves can induce *JAZ10::GUS* expression in wounded leaf and also in distal leaves that share vascular connections with the wounded leaf (Farmer *et al.*, 2014). *A70::LUC* expression is COI1 dependent which means RPM1-mediated luciferase *A70* signal is most likely jasmonate derived. The GFP signal indicates both an epidermal and phloem localization. It has previously been reported that the vascular bundle is a site of activation of AOC (allene oxide cyclase) and generation of JA (Stenzel *et al.*, 2003, Hause et al., 2003) and the phloem is also associated with jasmonate signaling, being suggested as the site of systemin production and jasmonate signalling in *Solanaceous* spp (Jacinto T, 1997).

In summary, our fusion of GFP expressed from the Pro*A70*::*A70*SP::eGFP *Arabidopsis* line localizes in the ER and plasmodesmata of systemic leaves. Which is completely different from predicated native *A70* compartments i.e. chloroplast. Patterns of GFP accumulation were observed in systemic responding leaf petioles as well as the epidermal cell layer of the leaf. Further work is required to validate this localization by cloning with the full genome sequence.

7 Chapter 7 General Discussion and Future Perspectives

The aim of this thesis was investigate the earliest signalling mechanisms underpinning SAR induced by gene-for-gene responses, thus addressing the "classical" systemic signalling processes following ETI. By specifically focussing on the AvrRpm1-RPM1 interaction we found a very complex, synergistic signalling system that appeared to be driven both locally and in systemic responding leaves by one or more unideintified jasmonate based signals. Reverse genetics, real time whole plant and cellular imaging and electrophysiology were used to tease out the components and pathways engaged in SAR. The study sought to address three principal questions:

1. Can we use a unique SAR reporter promoter (*A70*) in a luciferase reporter assay to study the temporal spatial dynamics underpinning development of SAR? If so, what is the nature of this signal generation and can we dissect this using classical SAR mutants?
2. Do changes in plant electrophysiology contribute to the early events in initiating and propagating the SAR signal?
3. What is the chemical nature of the signal(s) and how is it translocated to systemic tissues?

7.1 Use of the *A70::LUC* reporter to study SAR signalling mechanism

Using an *A70::LUC* reporter construct it was shown that whole plants displayed a strong reporter gene activation in response to DC3000*avrRpm1* infection both locally and systemically after 4 hpi (**Figure 10**) whereas no signal was observed for the mock treated local or systemic leaves. In addition, the compatible DC3000 strain and the T3S deficient strain DC3000*hrpA* were unable to trigger a luciferase based signal

(**Figure 10**). Thus, although *A70* function is unknown it appears to be an important component and marker of the systemic ETI response triggered by avirulent strains of *Pseudomonas* DC3000*avrRpm1.*

Notably, the *A70::LUC* signal is initiated almost straight away following bio-photon generation. These bio-photons are specific to ETI responses and emission which appears to be the result of lipid peroxidation events intimately associated with development of the HR (Bennett et al. 2005). Importantly, bacteria-induced bio-photons emission only occurred in local leaves and not in systemic leaves. Thus the *A70* signal is intimately linked to the local HR. The gene-for-gene interaction (Flor, 1971) leads to transcriptional reprogramming within and around the infection sites and also a localized programmed cell death, which is termed the hypersensitive response (HR) (Nimchuk *et al.*, 2003). A detailed understanding of the systemic transcriptional response, particularly any information on temporal dynamics is limited, but clearly *A70* induction is one of the earliest responses, its transcriptional activation mimicking the propagation of an inducing signal, rather than being the consequence of a downstream transcriptional process in a systemic responding leaf.

Here we studied the classical SAR response induced by recognition of AvrRpm1 delivery by the recognition by RPM1 (Grant *et al.*, 1995) and then used virulent *P. syringae* pv *maculicola* (M4) to quantify SAR (**Figure 21**). However, it has been reported that the virulent strain M4 as well as the avirulent DC3000*avrRpm1* triggers a robust SAR response in Col-0 plants based on gene for gene interaction (Mishina & Zeier, 2006, Mishina & Zeier, 2007) suggesting multiple routes to activation of a SAR signal. We also studied the ability of *A70* and *A70* homologues mutants to mount SAR. While requiring more detailed study, SAR induction by DC3000*avrRpm1* was totally compromised in *A70 homolog1-1*, *A70 homolog1-2* mutants whereas the

A70KO showed wild type expression of SAR (**Figure 22**). Further experimentation is required to understand the how these two alleles of *A70* homologue able to compromise SAR expression. Generation of the double mutant lines is currently underway. These will help to understand role of *A70* in SAR signalling.

In summary, these data imply that *A70::LUC* is specifically induced after 4 h of DC3000*avrRpm1* challenge and the specific induction signal is generated through activation of an HR response classically associated with ETI. More importantly, these results suggest that the *A70* gene most likely plays an important role in orchestrating systemic immunity.

7.1.1 Role of PAMPs and DAMP in *A70::LUC* expression

PAMPs are molecular microbial components (e.g. proteins, small peptides, oligosaccharides and glycolipids) with a conserved feature and an important role in the microbial life style. Here we showed that *A70::LUC* expression was induced after either elf18, flg22 or *At*Pep1 inoculation (**Figure 12**) consistent with the initial findings of Truman *et al.* (2006). However, no systemic luciferase expression was induced by these peptides whereas *Pst* DC3000*avrRpm1* infiltration induced a strong *A70::LUC* expression in systemic leaves at 4:10 h after infection (**Figure 12**). These data indicate that these peptides do not induce *A70::LUC* systemically, but rather a local gene-for-gene event is required to initiate *A70* expression.

In *Arabidopsis,* pre-treatment with the PAMP (flg22) induces resistance to the necrotrophic fungus *Botrytis cinerea* as well as the phytopathogenic bacterium DC3000 (Zipfel, 2009). Therefore, we investigated RPM1-mediated *A70::LUC* expression and HR after pretreatment with flg22, elf18 and *At*pep1. Results indicated that pre-treatment with PAMPs and DAMP inhibited RPM1-mediated HR as well as

completely abolished RPM1-mediated *A70::LUC* expression (**Figure 13**). This result is consistent with previous studies demonstrating that PTI could inhibit the ability of the plant to mount an HR in response to an ETI-inducing bacterial strain (Oh & Collmer, 2005) (Newman *et al.*, 2000, Klement *et al.*, 2003). The most likely explanation is that PAMP or DAMP pretreatment blocks effector delivery thus preventing the HR and consequently *A70::LUC* expression and SAR (**Figure 13**). Indeed, it has been proposed that the mechanisms for inhibition of the HR caused by PTI may involve impairment of delivery of T3Es, modification of the events downstream of T3E recognition, or a shutdown of programmed cell death (Newman *et al.*, 2000). In 2010, Crabill proved that PTI-induced HR inhibition is due to direct or indirect restriction of T3E injection and that T3Es can relieve this restriction by suppressing PTI (Crabill *et al.*, 2010b).

In summary, PTI triggering peptides locally but not systemically induce *A70::LUC* expression but pre-treatment abolishes both the HR and ETI based *A70::LUC* expression.

7.1.2 The potential roles of JA, SA, and ABA in *A70::LUC* expression

Lipid-derived molecules including JA have been shown to be important signals in systemic resistance. Most importantly, JA plays a leading role in induced systemic resistance (ISR) (Pieterse *et al.*, 1998), but its practical contribution to SAR remains controversial. In 2007 Truman *et al.,* reported that some JA biosynthetic and JA-insensitive mutants exhibit an attenuation of SAR but in 2009 Attaran reported that JA biosynthesis or downstream signalling are not required for SAR (Attaran *et al.*, 2009). Thus, it remains to be determined if, and how JA signalling contributes to SAR? Does JA function as a long-distance mobile signal in SAR?

This research work provided surprising results on the role of jasmonates in *A70::LUC* induction. JA can moderately induce *A70::LUC* expression but the response remains local and is not propagated systemically (**Figure 14**). More research is clearly required to establish the actual role of JA or derivatives thereof in SAR. Recently, it has been demonstrated that JA synthesis and signalling are positive regulators of RPS2-mediated ETI (Liu *et al.*, 2016).

Till now, various approaches have been used to investigate the impact of ABA and SA in plant immunity. These include the use of mutants with altered ABA biosynthesis or signalling pathway, as well as exogenous application of ABA (de Torres Zabala *et al.*, 2009, de Torres-Zabala *et al.*, 2007). The results of these studies largely support a negative role of ABA in plant immunity to many biotrophic pathogens. Here we investigated the role of ABA and SA during local and systemic induction of *A70::LUC* expression and its' role in long-distance transport. We first established that neither ABA nor SA treatment induced luciferase activity in the infected leaf or in systemic leaves. (**Figure 14**). We then pre-treated plants with JA, ABA or SA and analysed RPM1-induced *A70::LUC* expression. Surprisingly, pre-treatment of ABA abolished RPM1-induced *A70::LUC* expression but JA and SA didn't show any effect (**Figure 15**). While, Pre-treatment of ABA does not affect HR induction (data not shown). It has been shown that exogenous ABA application enhanced susceptibility to DC3000 in *Arabidopsis* (de Torres-Zabala *et al.*, 2007), but ABA suppression of RPM1-induced *A70::LUC* expression was unexpected and requires further examination.

Since the *Pst* derived coronatine (COR) can activate JA signalling by mimicking the structurally similar JA-isoleucine (JA-Ile) (Browse, 2009), the impact of COR on RPM1-induced *A70::LUC* expression was examined. Exogenous application of

coronatine strongly induced *A70::LUC* expression locally at very low concentrations (500nM) relative to JA (**Figure 14**) however expression was restricted to the area of application. Using a COR deficient DC3000 strain it was demonstrated that coronatine production doesn't affect RPM1-induced *A70::LUC* expression (**Figure 16**) which means that bacterial coronatine production does not contribute to the RPM1-dependent *A70::LUC* systemic signature, but some other jasmonate derivative is the inducing signal. This is consistent with the finding that non gene-for-gene SAR is established independently of coronatine in the *Arabidopsis*–*Pseudomonas* interaction (Attaran *et al.*, 2009).

In summary, *A70::LUC* is transiently induced locally by JA and coronatine treatment, with coronatine being highly inductive but COR is not the mobile signal. This suggests an HR mediated jasmonate derivative is the inducing signal. Consistent with the importance of the HR, pre-treatment with ABA abolished *A70::LUC* expression but not RPM1-mediated HR. In fact, ABA pre-treated leaves are more susceptible for further infection (such as M4). It has been reported that, ABA affects JA biosynthesis during the activation of defences against oomycete (Adie *et al.*, 2007). Thus a capacity of ABA to interfere with RPM1-induced signalling was studied. Results represent that pre-treatment of ABA alters the SAR expression. Several report suggest that, ABA supresses SAR by inhibiting SA biosynthesis (Kusajima *et al.*, 2017). Abscisic acid suppresses chemically (BIT and BTH) induced systemic acquired resistance. However, SAR and ABA signaling are mutually antagonistic (Hofmann, 2008).

7.2 *A70::LUC* expression in SAR compromised backgrounds

Several signalling molecules are able to trigger SAR in *Arabidopsis*. Although SA plays an important role, other metabolites involved in SAR have been reported but this

list of SAR signalling molecules is probably still not exhaustive (see Dempsey & Klessig 2012 for a review).

To investigate the impact of SAR signalling mutants on *A70::LUC* expression the reporter line was crossed into different *Arabidopsis* SAR mutant lines, including *npr*, *nac* and *glr3* lines. *A70::LUC* expression was induced by DC3000*avrRpm1* in the following mutant lines of *Arabidopsis thaliana: sid2, npr1*, *npr3, npr4,* and the triple mutant *npr1/3/4* (**Figure 23**, **Figure 24**, **Figure 25**)*, nac19/55, nac19/55/72* (**Figure 26**) and *glr3.3a, glr3.6a* and *glr3.3/glr3.6a* (**Figure 27**). The only mutant background in which, *A70::LUC* induction by DC3000*avrRpm1* was abolished was in the jasmonate receptor *coi1-16* background (**Figure 29**). All these results indicate that *A70::LUC* expression is SA, GLRs and NAC independent but dependent on *COI1*. As *coi1-16* shows already more resistant to *M4* bacteria than wildtype in mock treated plants and bacteria growth is not reduced further during SAR (**Figure 29**) it is difficult to quantify the direct contribution of COI1 to SAR. However, these results support previously published data (Thines *et al.*, 2007) (Kloek *et al.*, 2001) (Melotto *et al.*, 2008) and confirm that COI1 improves basal defence, likely via JA-SA antagonism in which JA deficient plants leads to higher in SA level, this leads to increases the basal defence response.

Notably, *A70::LUC* expression is independent of GLRs, yet GLR mutants compromised SAR (**Figure 27**) indicating that establishment of SAR necessitates a role for GLR transporters, possibly through a jasmonate based signal. Notably however, JA and coronatine challenged leaves of *glr3.3a/A70::LUC* and *grl3.6a/A70::LUC* plants showed strongly reduced *A70::LUC* expression, as did the double mutant (**Figure 28**). This suggests that GLRs constrain jasmonate-inducible

regulators as reported by (Mousavi *et al.*, 2013a) but not the predicted JA signal that is generated in a local ETI interaction and activates *A70::LUC* expression.

Through this study, we discovered that, RPM1-mediated *A70::LUC* signalling is independent of previously characterized NAC, GLRs and SA-mediated defence signalling mutants but completely dependent on COI1-mediated defence. These results provide strong evidences that RPM1-mediated *A70::LUC* signalling is a jasmonate based signal.

7.3 Using a *JAZ10::GUS* reporter to understand the spatial context of SAR signaling

Several molecular, biochemical, and genetic studies show that the best-characterized JA-dependent signalling cascade in *A. thaliana* is mediated by the COI1, JAZ, and MYC genes (Thines *et al.*, 2007, Chini *et al.*, 2007, Lorenzo *et al.*, 2004). A direct role for *JAZ10* as a negative feedback control of JA signalling is supported by the JA-hypersensitive phenotype of *jaz10* null mutants and the ability of specific JAZ10 splice variants to complement the hypersensitive phenotype of *jaz10* mutants (Cerrudo *et al.*, 2012, Demianski *et al.*, 2012, Moreno *et al.*, 2013, Yan *et al.*, 2007). Here, *JAZ10::GUS* reporter lines were used to explore our hypothesis that a jasmonate based signal is perceived systemically following RPM1-induced SAR, similar to that recently described for systemic wounding responses. After 8 hpi *JAZ10::GUS* plants challenged with DC3000*avrRpm1* showed strong *JAZ10::GUS* expression systemic leaves adjacent to the DC3000*avrRpm1* infected leaf (**Figure 31**). Among the 12 *JAZ* genes in *Arabidopsis*, most are rapidly and strongly expressed in response to exogenous JA or stress-induced accumulation of endogenous JA (Yan *et al.*, 2007, Chini *et al.*, 2007, Chung *et al.*, 2008, Thines *et al.*, 2007). Furthermore, a previous

study indicated that JA is increased in petiole exudates in response to DC3000*avrRpm1* challenge (Truman *et al.*, 2007) but genetic studies with petiole exudates ruled out a direct role for JA as the systemic signal (Chaturvedi *et al.*, 2008). Then we analysed the role of COI1 in RPM1-induced *JAZ10::GUS* expression since *COI1* encodes a main component of the JA-Ile receptor complex with JAZ proteins as co-receptors (Thines *et al.*, 2007, Katsir *et al.*, 2008, Melotto *et al.*, 2008, Sheard *et al.*, 2010). Our experiments showed that RPM1-based *JAZ10::GUS* expression in systemic leaves is *COI1* dependent (**Figure 32**). This suggests that RPM1-induced accumulation of *JAZ* transcripts is attenuated in *coi1*-16 seedlings. JAZ proteins are destabilized by SCF/COI1-mediated ubiquitination (Chini *et al.*, 2007, Thines *et al.*, 2007) so in the absence of COI1 one might anticipate accumulation of JAZ10::GUS. However, if, as we predict, an RPM1 induced systemic jasmonate signal is needed to induce JAZ via COI1 function then this would explain lack of GUS staining. It is noteworthy that JAZs are often induced to re-impose negative regulation on JA signalling via a positive feedback loop. As RPM1-mediated *JAZ10::GUS* signatures at 8 hpi are correlated with RPM1-mediated *A70::LUC* expression at 4-5 hpi is dependent upon a jasmonate based signal that requires a functional COI1 receptor (Katsir *et al.*, 2008, Li *et al.*, 2004, Feys *et al.*, 1994), it would be interesting to ascertain the respective timings of *A70* and *JAZ10* accumulation in systemic responding leaves.

7.4 Plant electrophysiology

Several reports shows that jasmonate signals to plasma membrane depolarization, but these are quite a common phenomenon in all plants (Fromm & Lautner, 2007). For example, Egyptian cotton leaf worm (*Spodoptera littoralis*) provokes plasma membrane depolarizations while feeding, which can spread through entire wounded leaves of beans (Maffei *et al.*, 2004) and *S. littoralis* herbivory of *A. thaliana*, stimulates jasmonate-regulated transcription in close proximity to wounded tissues (Reymond *et al.*, 2004). Given the systemic induction of JAZ10::GUS following either wounding or gene-for-gene recognition (see above) it was decided to investigate whether the changes in electrical surface potential seen in systemic leaves following wounding (Mousavi *et al.*, 2013b, Salvador-Recatala *et al.*, 2014) were also replicated in early SAR responses. Col-5 plants infected with DC3000*avrRpm1* showed significant changes in the surface potential within 2 to 3 hpi of both the infected and later, the systemic leaves. The whole depolarization and repolarization process takes place between 2.5 h to 5h in the local leaf and between 5h to 8h in the adjacent systemically responding leaves. No change in surface potential was observed either in local leaves or in systemic leaves after infection of DC3000, DC3000*hrpA* or mock ($MgCl_2$) (**Figure 33**). These results indicated that the change in surface potential recorded after DC3000*avrRpm1* infection is an RPM1 induced electrical signal, which combined with JAZ10::GUS expression (Chapter 4) is remarkably similar to the WASP (Wound Activated Surface potential) and systemic induction of JAZ10::GUS seen in the jasmonate mediated wound response. A wounded leaf generates a signal that moves first towards the centre of the rosette and then disperses into a restricted number of distal leaves within 1h, inducing distal JA accumulation and signalling

(Mousavi *et al.*, 2013a), and presumably a specific transcriptional signature (Heil & Ton, 2008, Mithöfer A, 2009) (Howe & Jander, 2008) (Heil & Silva Bueno, 2007b).

We have seen previously (Chapter 3) that pre-treatment with PAMPs and DAMP inhibited RPM1-mediated HR as well as *A70::LUC* expression. Interestingly, we showed that RPM1-induced electrical responses were also abolished after pre-stimulation with PAMPs, flg22 or elf18 and DAMPs *At*Pep1 (**Figure 35**). It has been reported that DAMPs and PAMPs, after being recognized through their PRRs, lead to the activation of anion and calcium channels. (Schulze *et al.*, 2010, Postel *et al.*, 2010). Our results lend support to the Krol studies proposing that the overall response pattern of the plants to *At*Pep(s) is similar to the response to MAMPs such as flg22 or elf18, despite differing in amplitude (Krol *et al.*, 2010b). Our results with DEX::*avrRPM1* inducible lines unexpectedly revealed that PAMP perception appears to be an essential for the generation and propagation of a systemic signal (**Figure 36**).

The changes in surface potential in the SA signalling and biosynthetic mutants *sid-2, npr-1, npr3/4* and *npr1/3/4* were almost similar to wild type Col-0 responses (**Figure 37**). By contrast, in *glr3.6a* changes in electrical surface potential were only registered in the DC3000*avrRpm1* challenged leaf, but not in systemic leaves. Moreover, *glr3.3a* and *glr3.3a/3.6a* don't show any change in surface potential (**Figure 38**). This is consistent with our earlier results (Chapter 4) showing that bacterial growth in systemic leaves of GLR3 mutants was not restricted. While, *glr3* mutants do not affect RPM1-mediated *A70::LUC* expression (Chapter 4) *glr3.3a/3.6a* affects *A70::LUC* expression induced by JA and coronatine. These results support a role for GLRs in systemic electrical signalling. Mousavi (2013) reported wound-induced electrical signals are propagated by glutamate-like receptors to activate JA biosynthesis in systemic tissue. Our results differ in that only the double mutant line,

but not with single mutant line regulate SAR specific electrical signals. The mechanism by which these propagating signals are perceived and subsequently connected to jasmonate biosynthesis/signalling is still unknown. However, there is evidence to suggest that calcium ions may be involved in propagating and/or interpreting the signal in the responding target cells (Felle and Zimmermann 2007; Maffei et al. 2006; Qi et al. 2006)

A wounded leaf activates JA-responsive genes and induced resistance, plus it also triggers rapid systemic responses, JA-Ile accumulation and degradation of JAZ proteins (Acosta et al 2013; Green and Ryan 1972; Koo et al. 2009; Mousavi et al. 2013; Zhang and Turner 2008). We have demonstrated that the *coi1*-16 mutant abolished systemic *A70::LUC* expression, JAZ10::GUS accumulation, was compromised in SAR and abolished systemic RPM1-mediated electrical signalling. This gives strong evidence for an important role for electrical signals in ETI-mediated systemic signalling, (**Figure 39**) and that COI1 plays an important role in systemic electrical signalling. COI1 controls the turnover of the JAZ co-receptors by directly interacting with MYC2 that control JA-regulated genes (Thines *et al.*, 2007, Sheard *et al.*, 2010, Chini *et al.*, 2007, Pauwels & Goossens, 2011, Fernandez-Calvo *et al.*, 2011). Our results lend support to Mousavi group's report that, the genes involved in the propagation of electrical activity, leading to defense gene expression can also control jasmonate-inducible regulators of jasmonate signalling (*JAZ* genes) (Mousavi *et al.*, 2013a). *JAZ5* and *JAZ10* play an important role in restricting coronatine cytotoxicity and pathogen growth through a complex transcriptional reprogramming during infection of *Arabidopsis* with virulent *P. syringae* infection. Moreover, upon bacterial infection the *jaz5/10* mutant is compromised in SA- signalling and shows a rapid suppression of JA-related components based on the mRNA-seq predict data (de

Torres Zabala *et al.*, 2016b). In contrast we show that, *jaz5/10* mutant changes in surface electrical potential in the local challenged leaf was almost similar to wild type upon DC3000*avrRpm1* infection (**Figure 39**). These data suggest the JA signalling components required for ETI initiation and propagation of COI1 dependent jasmonate signals don't require JAZ5/10 function. It would be interesting to study the SAR (assay) signalling mechanism and *A70::LUC* expression in a *JAZ5/10* mutant background.

The NAC19/55/72 signalling module has been predicted to function downstream of MYC2 in jasmonate based defence responses (Zheng *et al.*, 2012). We observed that this NAC triple mutant could not induce RPM1-based electrical signals in systemic leaves although changes in surface electrical potential in the local challenge leaf was similar to wild type (**Figure 39**), thus mirroring the *coi1* surface electrical potential signature. Moreover, in Chapter-4 it was demonstrated that *nac19/55/72* abolished DC3000*avrRpm1* induced SAR. It means *nac19/55/72* and *coi1*-16 both induce electrical signal in local leaves upon RPM1-recongnization but but fail to elicit changes in systemic leaves. These data suggest the electrical surface changes in systemic leaves are dependent upon COI1 and propagated through the NAC19/55/72 signalling module. We note that this result doesn't correlate with *A70::LUC* expression. It means systemic *A70::LUC* expression is independent to NAC19/55/72 signalling module.

In addition, our main aim to characterize the *A70* gene in defense mechanism. *A70 homologs* and *A70KO* were tested for electrophysiological activity after DC3000*avrRpm1* infection. Both *A70 homologs* and *A70KO* abolished the RPM1-mediated systemic electrical signal (**Figure 40**). These results provided further evidence that *A70* (and its homologs) plays important role in systemic electrical signaling.

In summary, this chapter presents novel results demonstrating a specific RPM1-induced electrical signature in local and systemic leaves of *Arabidopsis thaliana.* Unexpectedly, PAMPs play a crucial role in orchestrating RPM1-mediated systemic electrical signal, yet prior pre-treatment of with PAMPs can abolish RPM1-based changes in surface potential. These data also strongly correlate with *A70::LUC* expression and onset of macroscopic HR. DC3000*avrRpm1* mediated electrical signatures are independent of the SAR mutant lines, *npr1, npr1/3/4, sid2* and *jaz5/10*. Interesting, *npr3/4, coi1*-16*, glr3.6a, A70KO* and *A70 homologs* showed local electrical signal but abolished systemic RPM1-mediated electrical signals, providing evidence on components required for signal propagation and/or perception in systemic leaves. Strikingly, *glr3.3*a and *glr3.3a/3.6a* completely abolished in RPM1-mediated electrical signals consistent with a key role in signal generation.

7.5 Localization of A70

GFP expressed from the Pro*A70*::*A70*SP::eGFP *Arabidopsis* line localizes to the ER and plasmodesmata of systemic leaves (**Figure 43**). Patterns of Pro*A70*::*A70*SP::*GFP* signal observed in systemic leaf petiole as well as epidermal cell layer. Propidium iodide (PI) is a fluorescent intercalating agent that can be used to stain cells. Propidium Iodide cannot cross the membrane of live cells, making it useful to differentiate necrotic, apoptotic and healthy cells (Lecoeur, 2002). Propidium iodide staining helped differentiate a phloem based Pro*A70*::*A70*SP*::*eGFP signal but further work is need to validate this localization by A70 full genome cloning (**Figure 42**). Here it was possible to co-relate *A70::LUC* expression with localization of the Pro*A70*::*A70*SP::eGFP signal. As *A70::LUC* expression shows that RPM1-based luciferase signal travel though leaf petiole to systemic leaves, it means infected leaf and non-infected systemic adjacent leaves are attached with each other by vascular

connection (i.e. phloem and xylem). Similar to the Farmer group who showed that mechanically damaged leaves can induce *JAZ10::GUS* expression in distal leaves that share vascular connections with the wounded leaf (Farmer *et al.*, 2014) we show that *A70::LUC* expression shows that RPM1-based luciferase signal travel though leaf petiole to systemic leaves and this transcriptional wave appears to be propagated through the phloem and is localised to the ER. Preliminary observations suggest that the ER is reorganised into a more planar sheet structure which is indicative of enhanced protein production, and thus I may be observing a very early sub-cellular structural change associated with the so-called “priming” phenomena (Conrath *et al.*, 2002).

7.6 Conclusion and Future perspective

In conclusion, this study provides a fascinating insight into early temporal-spatial systemic signaling dynamics facilitated by *A70* reporter construct, electrophysiology and well characterized SA and JA signaling mutants **(Figure 44)**.

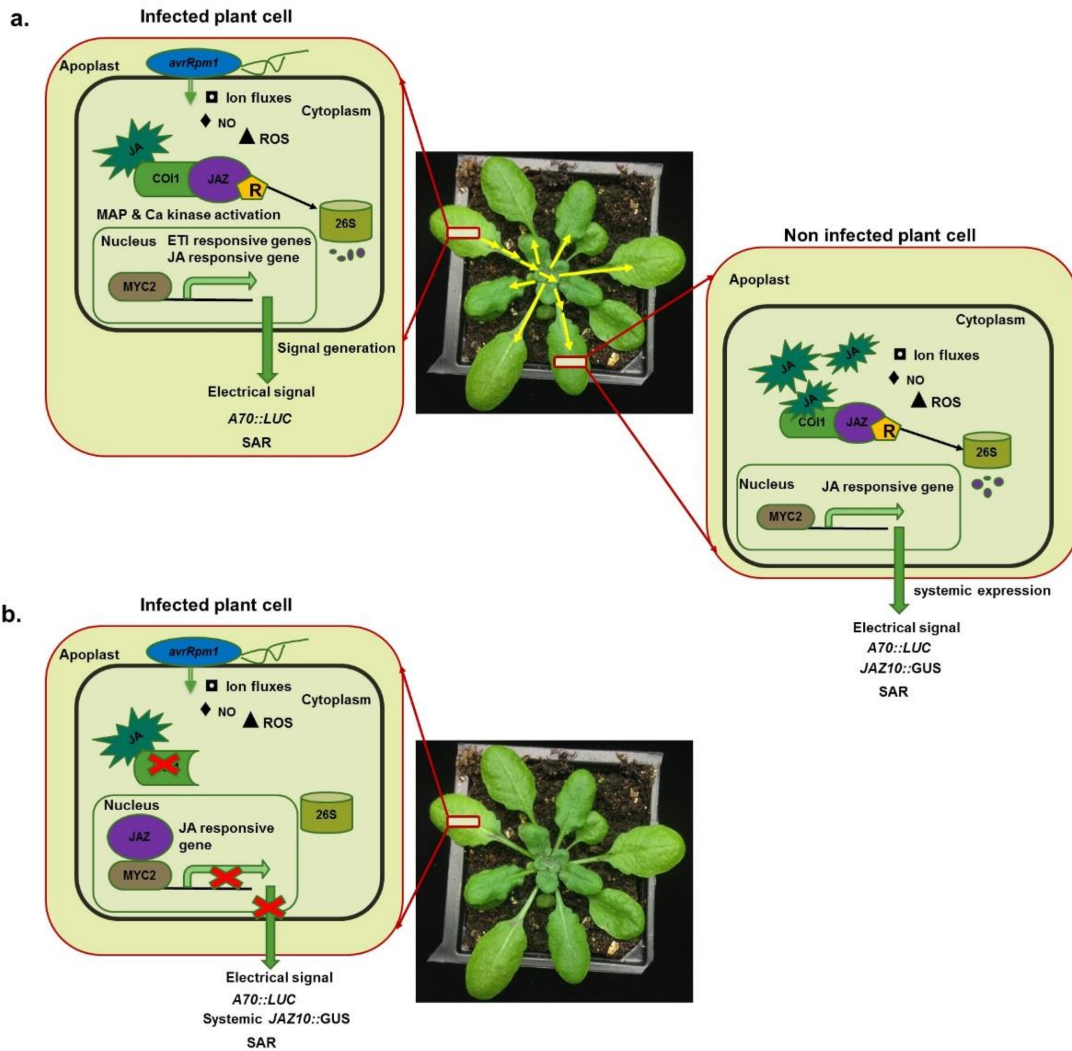


Figure 44: Working model shows JA as a long-distance SAR signal in Arabidopsis:

Under normal growth conditions JA hormone levels are low and JA-mediated responses are kept in a repressed state by JAZ proteins. **a**. In the presence of COI1: **Infected leaf:** DC3000*avrRpm1* infection induces ROS (reactive oxygen species), NO (nitiric oxide), ion influxes and increase the level of an unknown jasmonate derived molecule, facilitating an interaction between JAZ repressors and COI1. This interaction targets JAZs for ubiquitination and degradation via the 26S proteasome pathway, de-repressing transcription factors which induce a COI1 dependent SAR transcriptional network(s) including *A70::LUC* expression and concomitantly activating a local electrical potential. **Signal induction:** Rapid propagation of SAR specific signals, which includes systemic movement of *A70*, collaborate to activate a priming response in distal naïve leaves. **Non infected leaf:** Systemic induction of jasmonate signalling, as evidenced by activation of JAZ10::GUS expression, in conjunction with establishment of an electrical potential represent the earliest known events in systemic responding leaves. These responses are quantitatively stronger in the leaves physically closer to the challenged leaves. **b**. In the absence of COI1: JAZ repressors maintain SAR propagating transcription factors in an inactive state. A coi1 mutant fails to activate *A70::LUC* expression and although exhibiting a local electrical potential, fails to systemically induce *A70::LUC*, JAZ10::GUS, propagate an electrical signals nor activate SAR.

The evidence that a jasmonate based signal is responsible for the *A70* and electrical surface potential changes in systemic responding leaves is overwhelming. Key findings during this project are as follows:

1. RPM1-mediated *A70::LUC* expression is a unique signature signal based on gene-for-gene induction, this involves signal generation, propagation and perception.
2. *A70::LUC* expression is transiently induced locally by JA and coronatine treatment, with coronatine being highly inductive but COR is not the mobile signal.
3. RPM1-mediated *A70::LUC* signalling is independent of previously characterized NAC and GLRs signalling mutants, which means they had no direct role in regulation of *A70* induction nor propagation systemically. Yet GLR and NAC mutants were compromised in SAR, indicating that establishment of SAR necessitates a role for NAC and GLR transporters, possibly through a jasmonate based signal.
4. RPM1-mediated *A70::LUC* signalling is completely dependent on COI1-mediated defence, these results cleared that *A70::LUC* expression is dependent upon a jasmonate based signal that requires a functional COI1 receptor. It was not possible to assess the contribution of COI1 to SAR due to the higher SA levels observed in the *coi1* mutant.
5. Interestingly, *npr3/4, coi1*-16*, glr3.6a, NAC, A70KO* and *A70 homologs* showed local electrical signal but abolished systemic RPM1-mediated electrical signals, providing evidence that these components are required for signal propagation and/or perception in systemic leaves.

6. Strikingly, *glr3.3*a and *glr3.3a/3.6a* completely abolished in RPM1-mediated electrical signals suggesting a key role for GLR3.3a in initial generation of the electrical surface potential, and possibly also in its perception or transduction in systemic responding tissue.
7. *glr3.3a/3.6a* is sufficient to understand the link between SAR and electrophysiological signal(s).
8. Results with DEX::*avrRPM1* inducible lines unexpectedly revealed that PAMP perception appears an essential component of the generation and propagation of a systemic electrical signal.
9. Unexpected findings with PAMPs: pre-treatment of PAMPs abolished RPM-induced HR, *A70::LUC* expression and electrical signal. To understand the mechanism behind it further study needed.

Finally, this project provides strong evidence that there are strong links between ETI induced electrical signals and the spatial pattern in systemic leaves of *A70::LUC* and *JAZ10::GUS* expression following DC3000*avrRpm1* challenge. Systemic RPM1-induced *A70::LUC* expression, *JAZ10::GUS* expression and electrical signal, all are COI1 dependent. Collectively these data implicates rapid generation and propagation of an early jasmonate based signal(s) in establishment of systemic immunity.

There are number of specific questions regarding the *A70::LUC* expression that remain to be addressed:

1. Is *A70::LUC* expressed following challenge with a necrotrophic pathogen?
2. What are the *A70::LUC* signatures under a DEX::*avrRpm1* activated background?

3. What is the chemical structure that drives *A70::LUC* expression given COR is the most potent tested but not the propagative signal generated by the HR.
4. What are the *A70::LUC* expression and SAR signatures in ABA compromised background?
5. What is the localization pattern of full length *A70*::GFP?
6. How to design experiments to enhance our knowledge of the molecular mechanisms underpinning RPM1-induced *A70::LUC* signalling?
7. What is the impact of expressing Pro*A70*::*A70*SP::eGFP under a DEX inducible line?

To continue this study in future, I have also generated several resources to understand more about the role of *A70* in plant immunity and characterize *A70* gene in different signalling networks. e.g. To study role of PAMPs in *A70::LUC* expression, I have generated a DEX::*avrRpm1*/*A70::LUC* line. Moreover, I have crossed DEX::*avrRpm1* with Pro*A70*::*A70*SP::eGFP lines. These cross will help to analyse bacterial PAMP is essential to induce ETI based *A70::LUC* expression as well as *A70*::GFP activation.

To clarify the role of *A70 homologs in A70::LUC* expression, I generated *A70::LUC/A70 homolog1-1* and *A70::LUC/A70 homolog1-2.* These mutants will give more information about how *A70* is involved in defence mechanism. As well as to study *A70* signaling mechanism in *JAZ5/10* background and (*aao3*) ABA biosynthetic mutant background. I crossed between *JAZ5/10* and *aao3* with *A70::LUC* to explore the unexpected roles for ABA and possibly JAZs in the *A70* signaling mechanism.

8 APPENDIX-1

8.1 Genotyping of all gene used in study and crosses

All PCR products run on 1.5 % agarose gel staining with ethidium bromide and documented gel based on Band size for wild type and Knock out product.

All homozygous mutant line involve in SAR signalling are used for study are following: *nac19/55/72, nac19/55* (**Figure 45**), *npr3, npr4, sid2* **(Figure 46)**, *glr3.3a/A70::LUC, glr3.6a/A70::LUC, glr3.3a/3.6*a/*A70::LUC* **(Figure 47)**, npr3/*A70::LUC* **(Figure 48)**, sid2/*A70::LUC* **(Figure 49)**, *nac*19/55/*A70::LUC* **(Figure 50)**, *nac*19/55/72/*A70::LUC* **(Figure 51)**, *npr1/3/4/A70::LUC* **(Figure 52*)***, *coi1*-16*/A70::LUC* **(Figure 53),** *coi1-16/Jaz::GUS* **(Figure 54),** A70KO (**Figure 55**), A70 homolog1-1 and A70 homolog1-2 (**Figure 55**)**.**

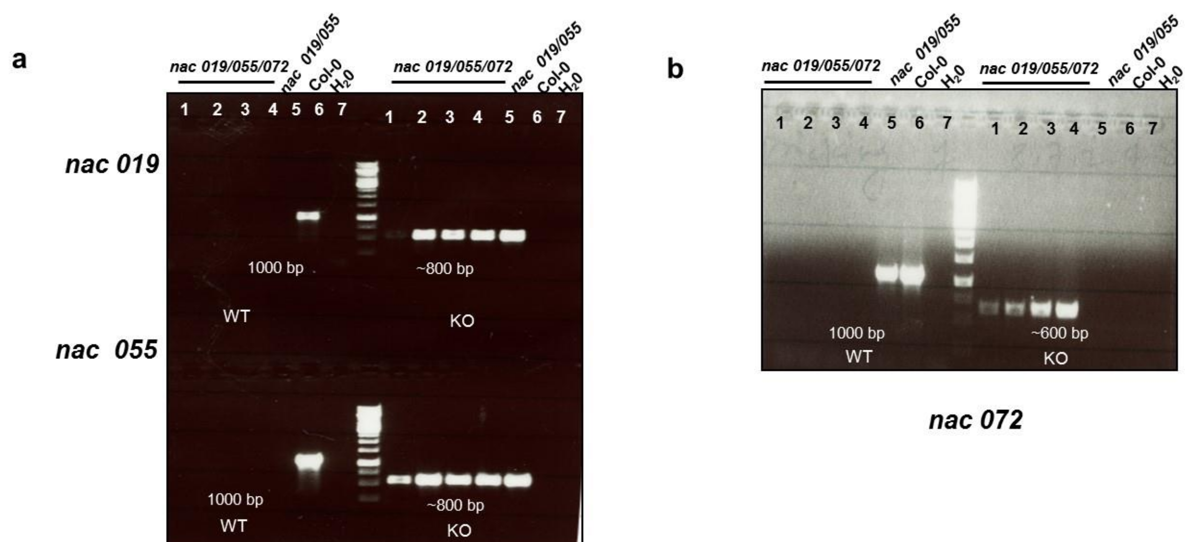


Figure 45. PCR gel documentation photo showing amplification of homozygous T-DNA knockout of NAC lines:

a. *nac19* and *nac55* **b.** *nac72* PCR product ran on an 0.8% agarose gel and staining with ethidium bromide shows that plant 1, 2, 3 and 4 are homozygous for *nac19/55/72* and plant 5 is homozygous for nac19/55.

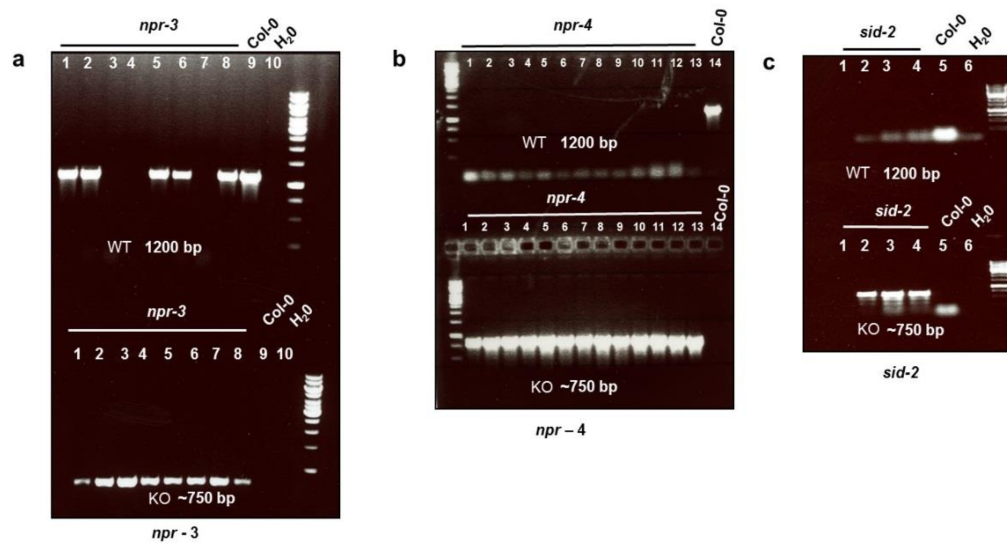


Figure 46. Characterization of homozygous T-DNA knockout lines for SA signalling:

a. gel electrophoresis of *npr-3* PCR product on an 0.8% agarose gel and staining with ethidium bromide shows *npr*-3 only sample 3, 4 and 7 indicate homozygous lines and others are heterozygous. **b.** *npr-4* all lines are homozygous*.* **c.** *sid-2* samples show all plants are homozygous.

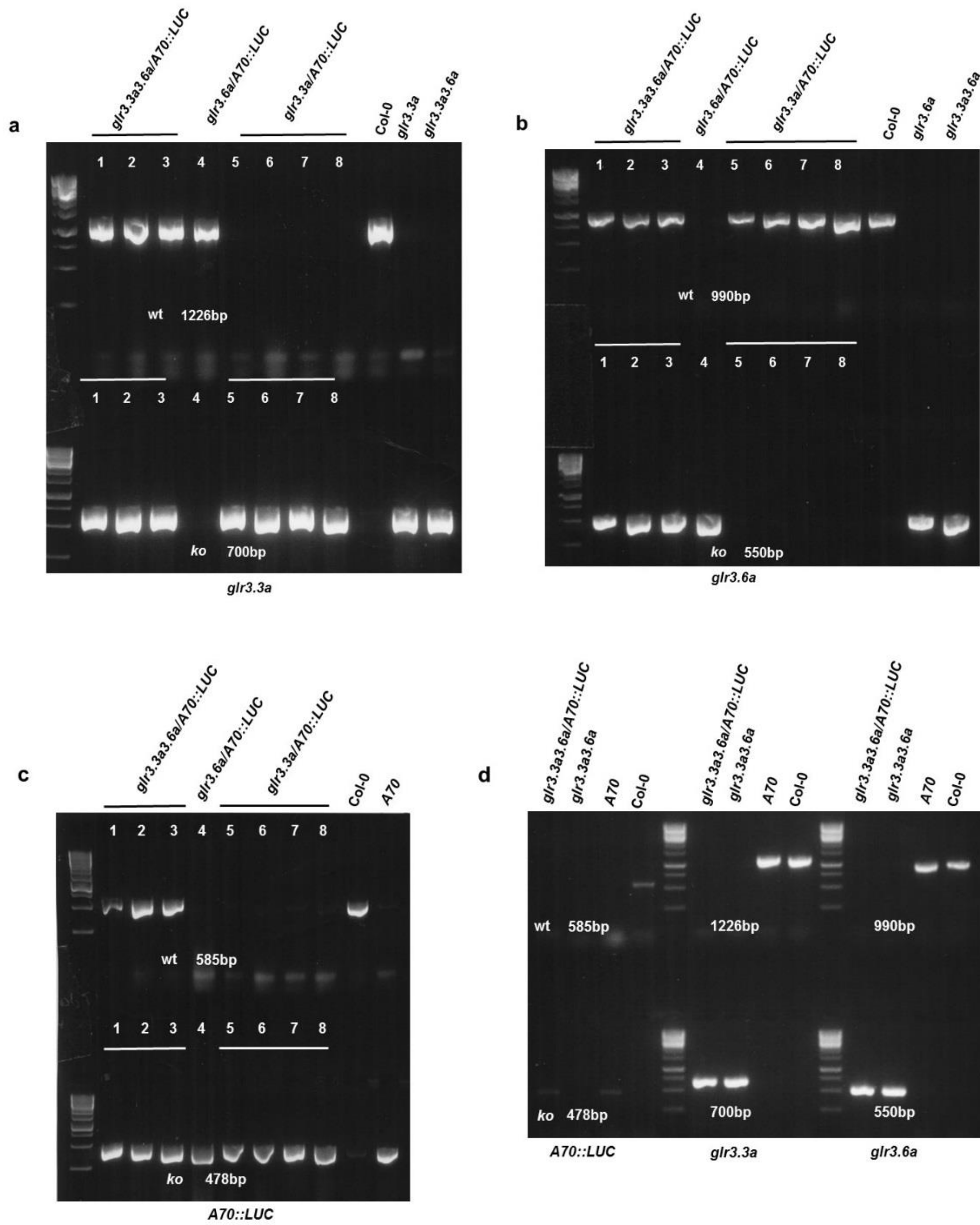


Figure 47. Genotyping of *glr3.3a3.6a/A70::LUC* for homozygous crossed lines:

PCR amplification of the three target genes was carried out **a.** *glr3.3a* **b.** *glr3.6a* **c.** *A70 ::LUC* PCR gel documentation shows that plant 5, 6, 7 and 8 are homozygous for *glr3.3a/A70::LUC* and plant 4 is homozygous for *glr3.6a/A70::LUC* **d.** after several selection only one plant is homozygous for all three targeted genes *glr3.3a3.6a/A70*.

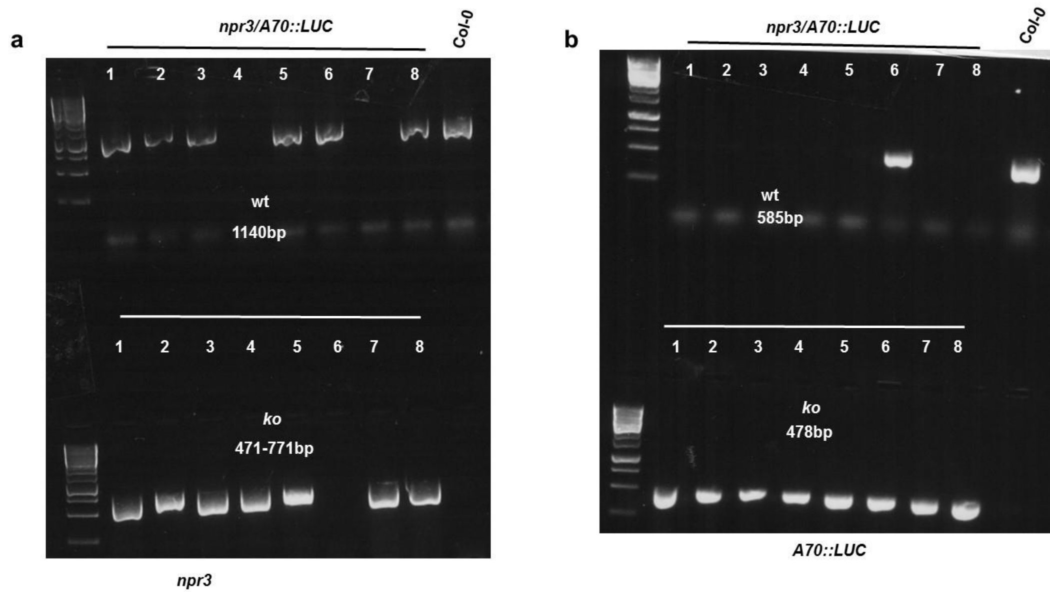


Figure 48. Photograph of agarose gel showing the PCR products for *A70::LUC/npr3* cross:

a. out of 8 plants only no 4 and 7 plants is homozygous for *npr-3* **b.** shows plant 4 and 7 is homozygous for *A70::LUC*. Last lane of Col-0 is negative control.

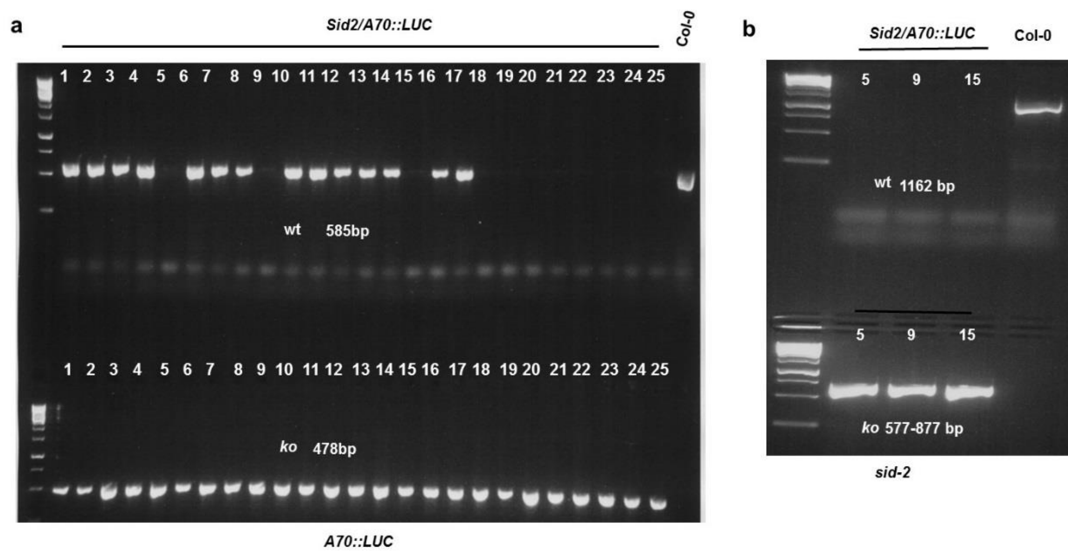


Figure 49. Representative agarose gel documentation of PCR amplification of homozygous cross of *A70::LUC/sid-2* :

Gel documentation shows that 5, 9 and 15 plants is homozygous for **a.** *A70::LUC* and **b.** *sid-2*.

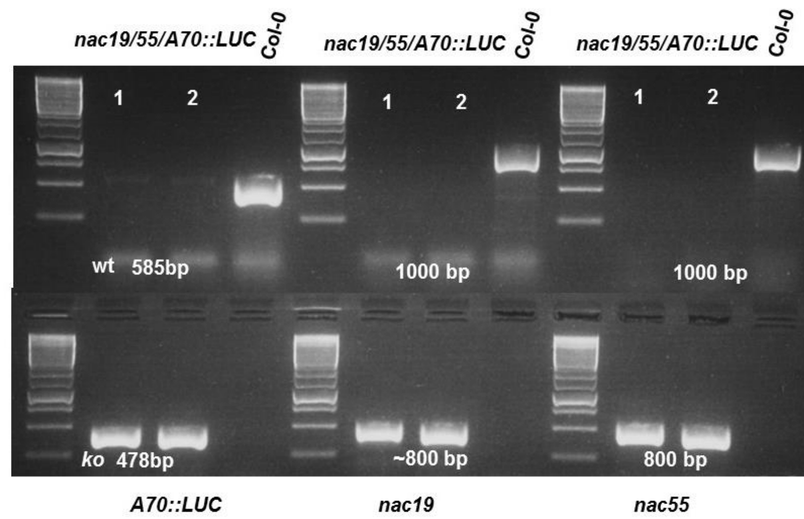


Figure 50. Gel Documentation picture showing PCR of *A70::LUC/nac19/55* :

Gel electrophoresis of *A70, nac19* and *nac55* PCR product on an 0.8% agarose gel and staining with ethidium bromide shows that 1,2 and 3 plants are homozygous for *A70::LUC*, *nac19 and nac55.*

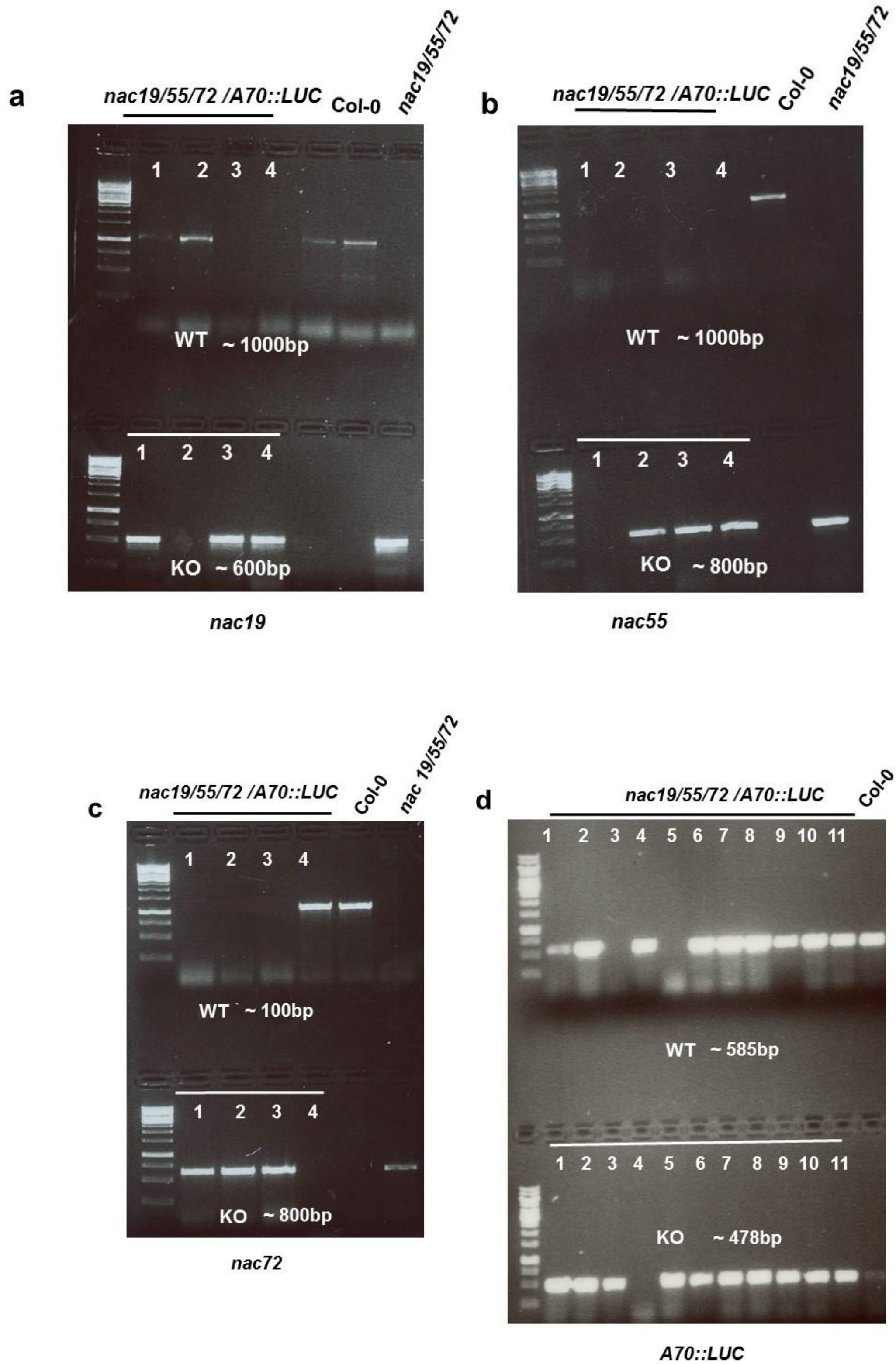


Figure 51. Agarose gel electrophoresis of homozygous cross of *A70::LUC/nac19/55/72* :

*G*el electrophoresis of **a.** *nac19,* **b.** *nac55,* **c.** *nac72* and **d.** *A70::LUC* PCR shows that only plant no. 3 is homozygous for all targeted gene.

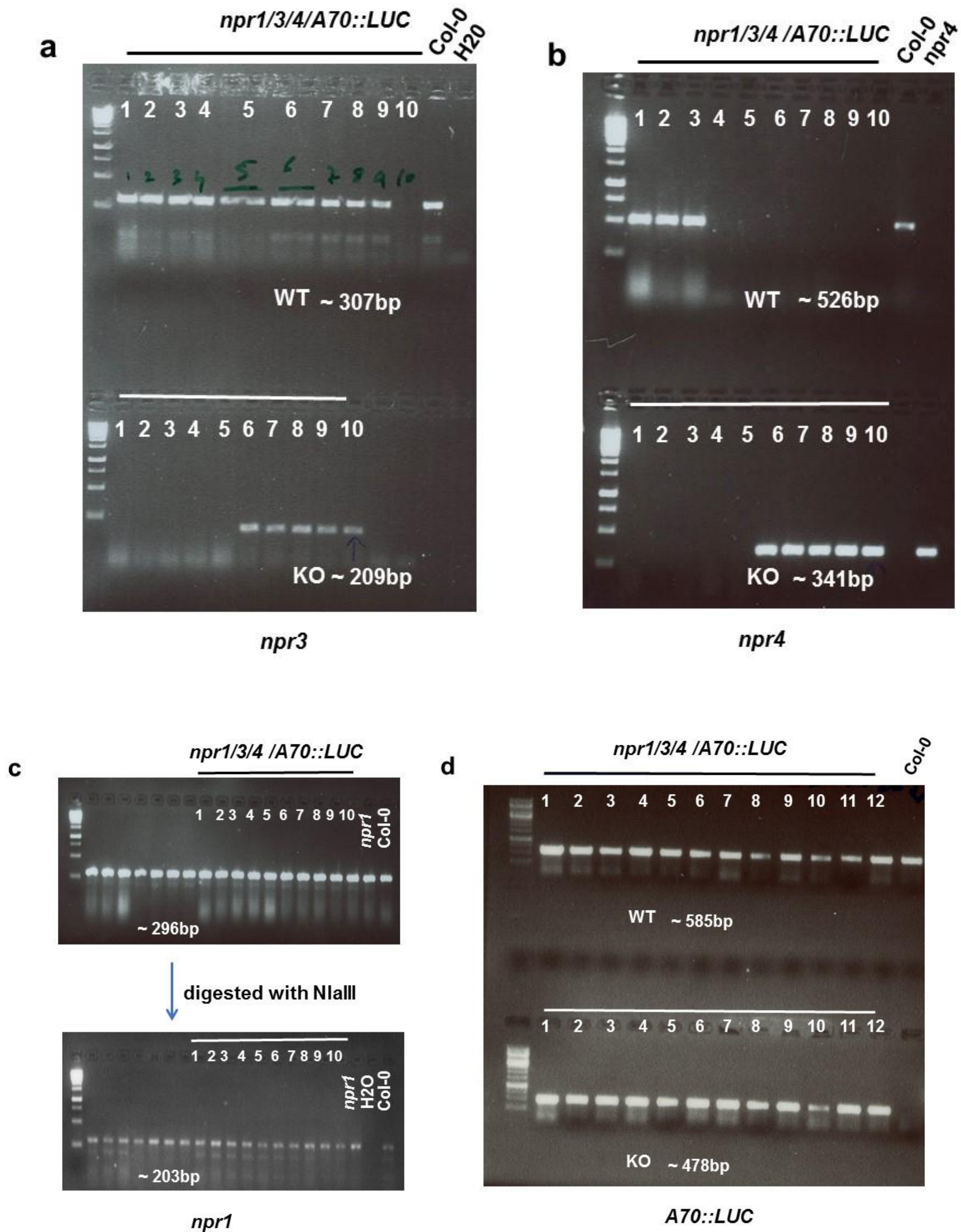


Figure 52. Characterization of homozygous cross of *A70::LUC/npr1/3/4*:

Gel electrophoresis of **a.** *npr3* and **b.** *npr4* PCR product on an 0.8% agarose gel shows that 10 plants is homozygous for *npr3* and *npr4* deleted. **C.** npr1 PCR product of plant no 10 is not digested by Nla **III** i.e no 10 plant is homozygous for *npr1* **d.** *A70* PCR shows that plant 10 is heterozygous for *A70*.

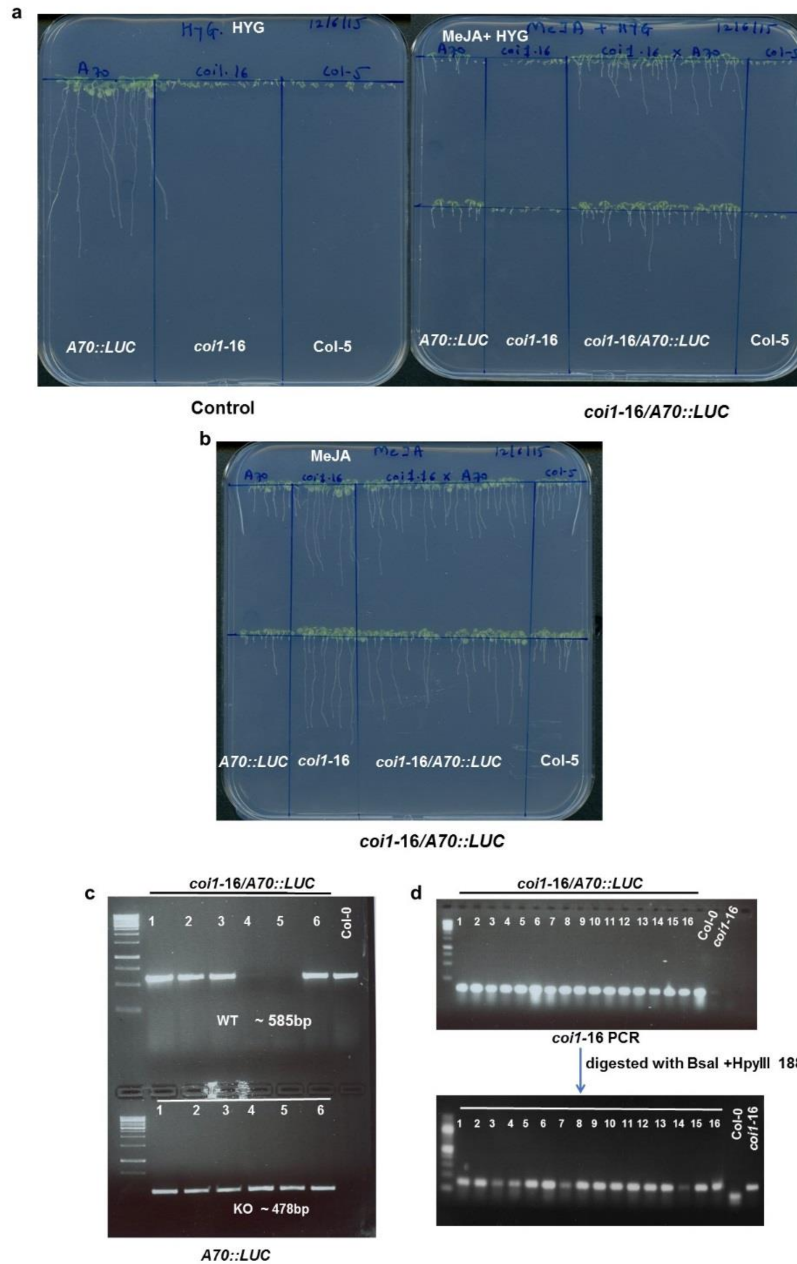


Figure 53. PCR gel documentation photo showing amplification of *coi1*-16*/A70::LUC*:

a. selected long root *coi1*-16*/A70::LUC* plants on 0.5x MS + hyg + MeJA (25µM)vvvv and **b.** selected long root *coi1*-16*/A70::LUC* plants on 0.5x MS + MeJA (25µM). **c.** *A70::LUC* PCR shows plant no 4 and 5 are homozygous and **d.** *coi1*-16 PCR product on an 0.8% agarose gel and staining with ethidium bromide shows that all plant are homozygous for *coi1*-16 since PCR product doesn't digested by BsaI+HpyIII

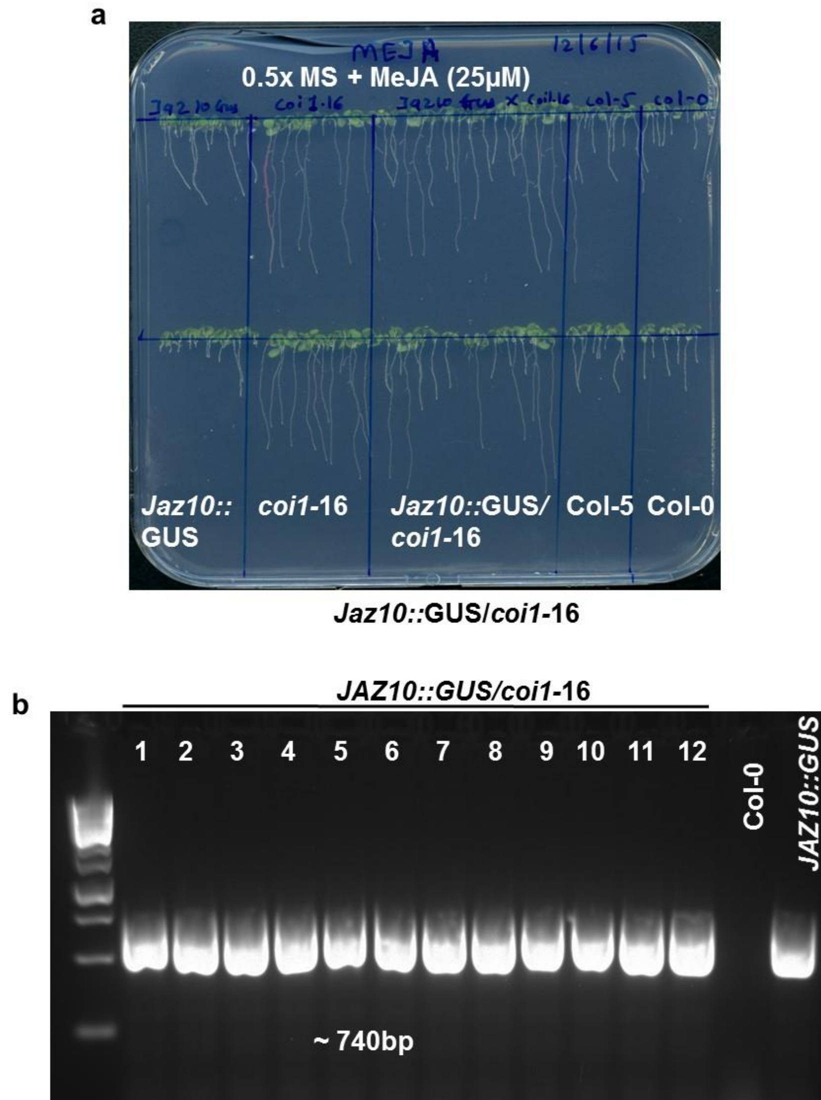


Figure 54. PCR gel documentation showing amplification of *coi1*-16*/JAZ::GUS*:

a. Plants with long roots selected *coi1*-16*/Jaz10::GUS* plants on 0.5x MS + MeJA (25µM). **b.** PCR for *JAZ10::GUS* reporter, all plant shows presence to Jaz10 GUS reporter in the *coi1*-16*/Jaz::GUS* cross.

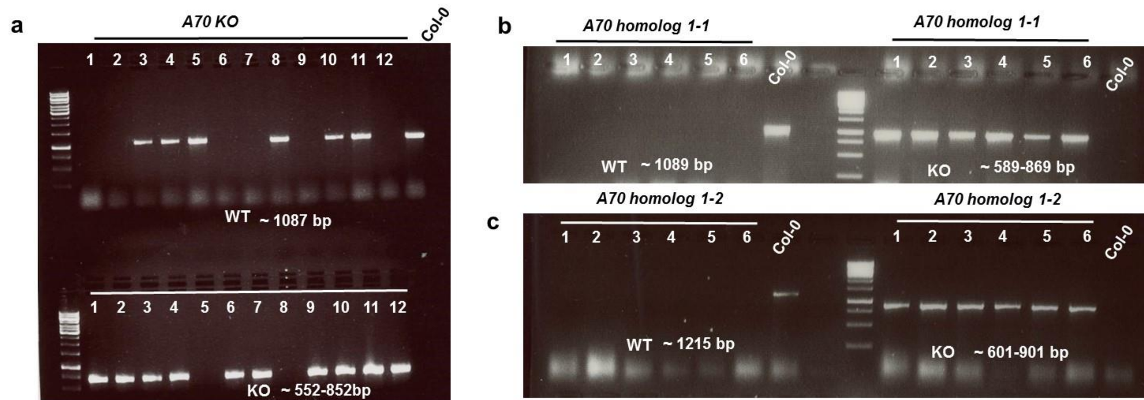
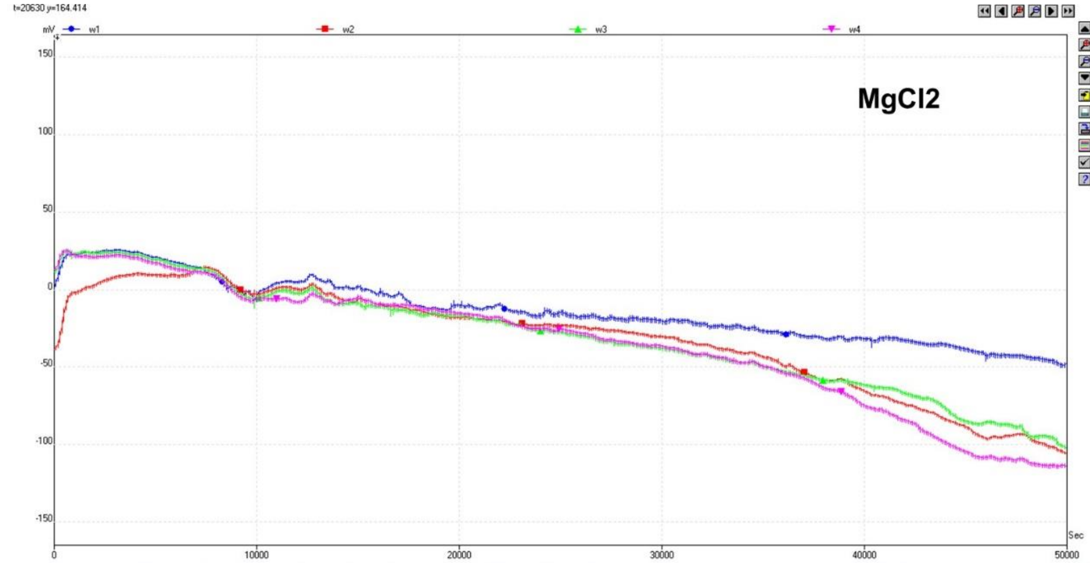


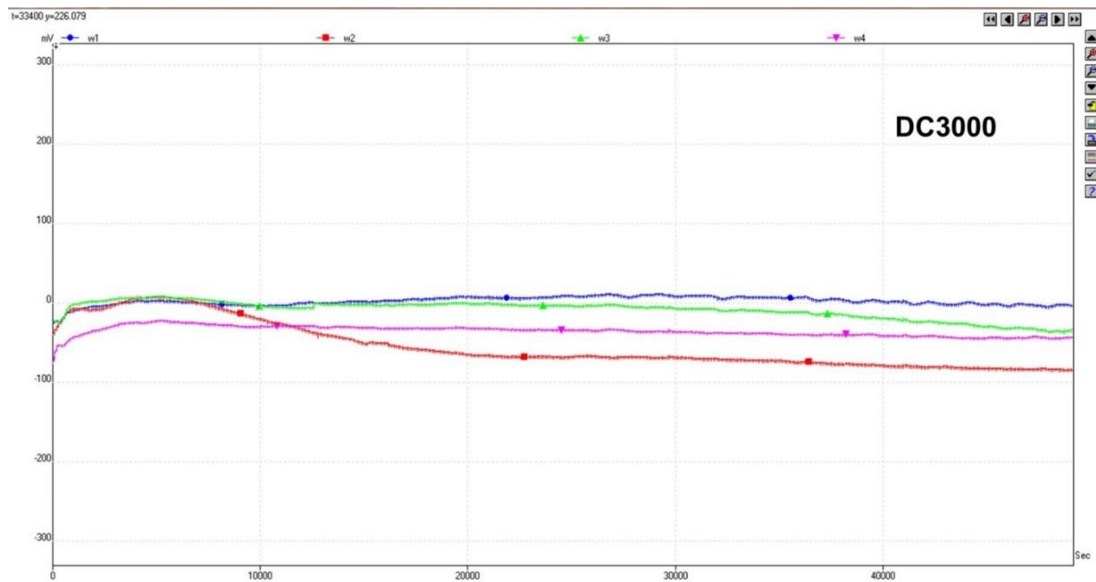
Figure 55. PCR gel documentation photo showing amplification of homozygous T-DNA knockout of *A70* and *A70 homologs* lines:

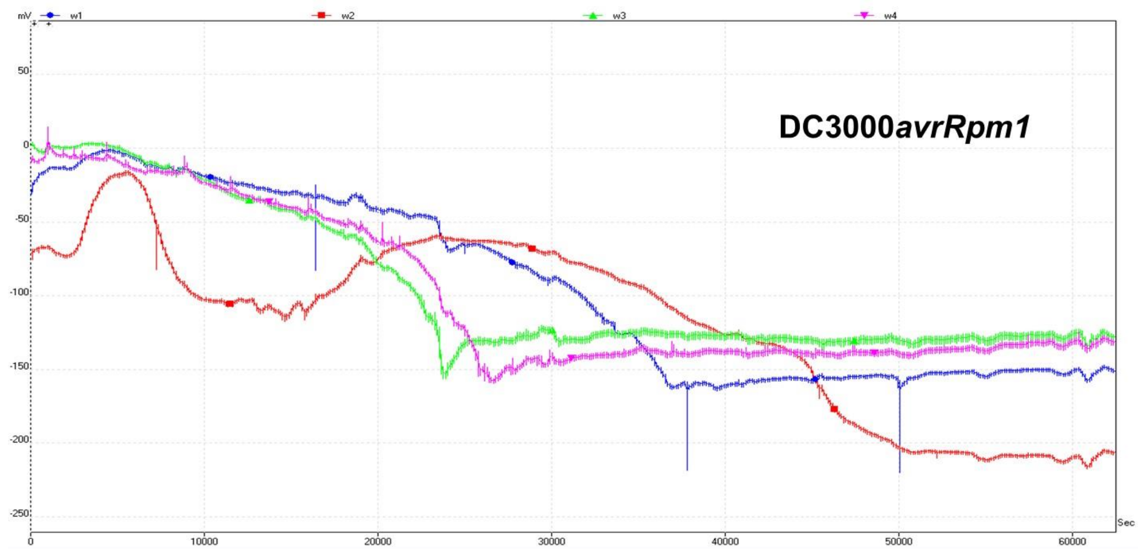
PCR amplification of the three target genes was carried out **a.** *A70KO* **b.** *A70 homolog 1-1* **c.** *A70 homolog 1-2.* PCR gel documentation of (**a)** shows that plant 1, 2, 6, 7, 9 and 12 are homozygous for *A70KO* and (**b & c)** all plants are homozygous for targeted genes *A70 homologs1-1* & *A70 homolog1-2*.

9 APPENDIX-2

Representative images of all electrophysiological experiments.



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w3
w4
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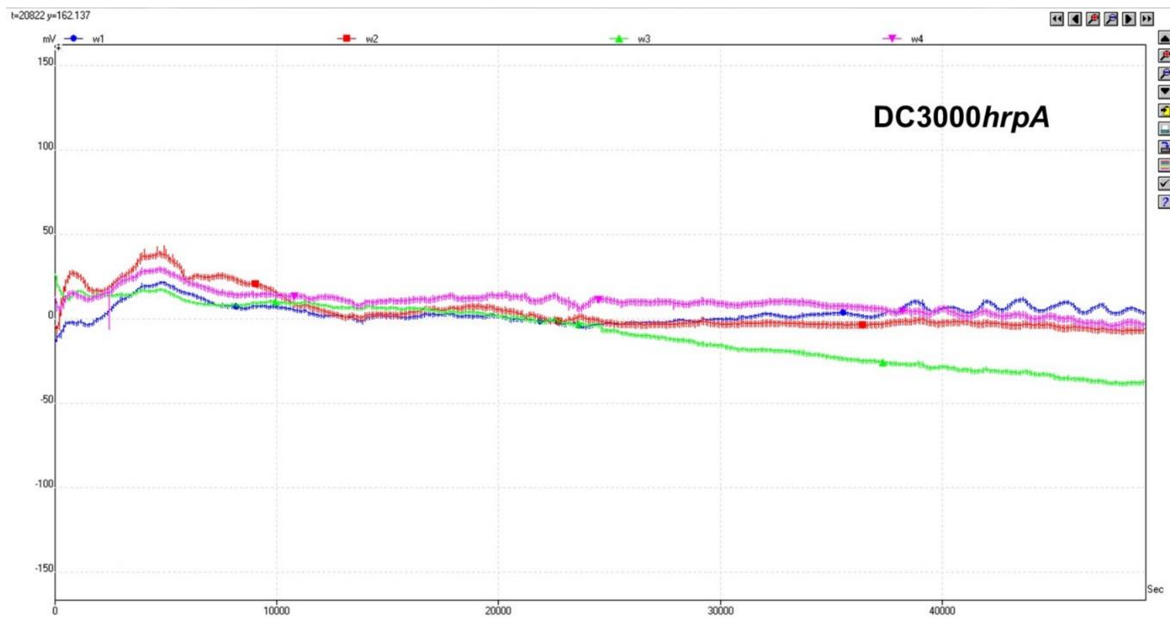
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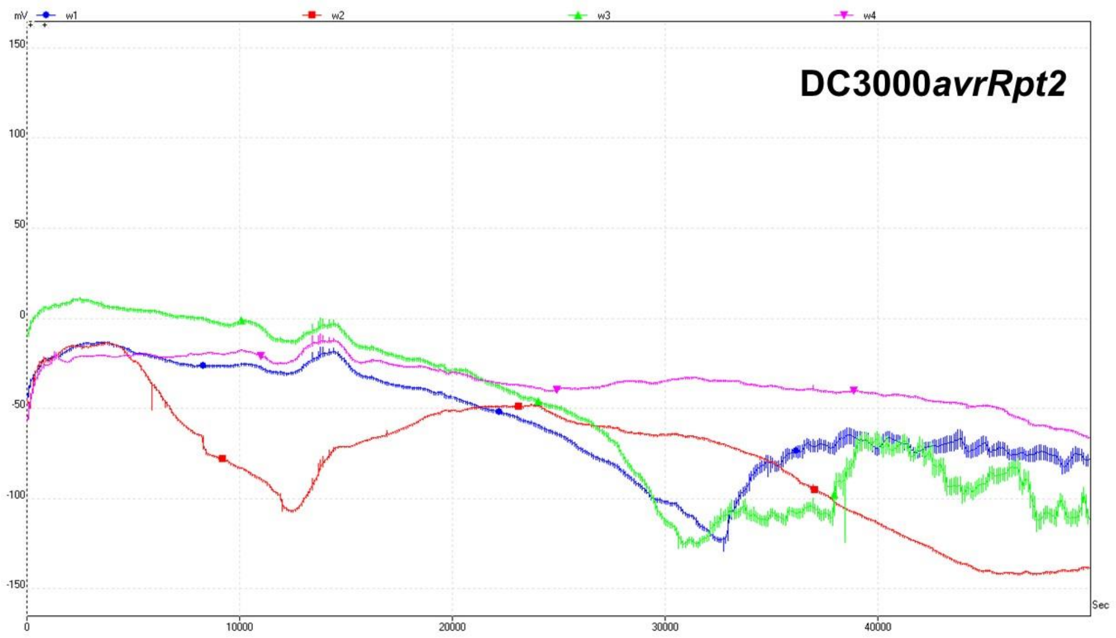
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40000
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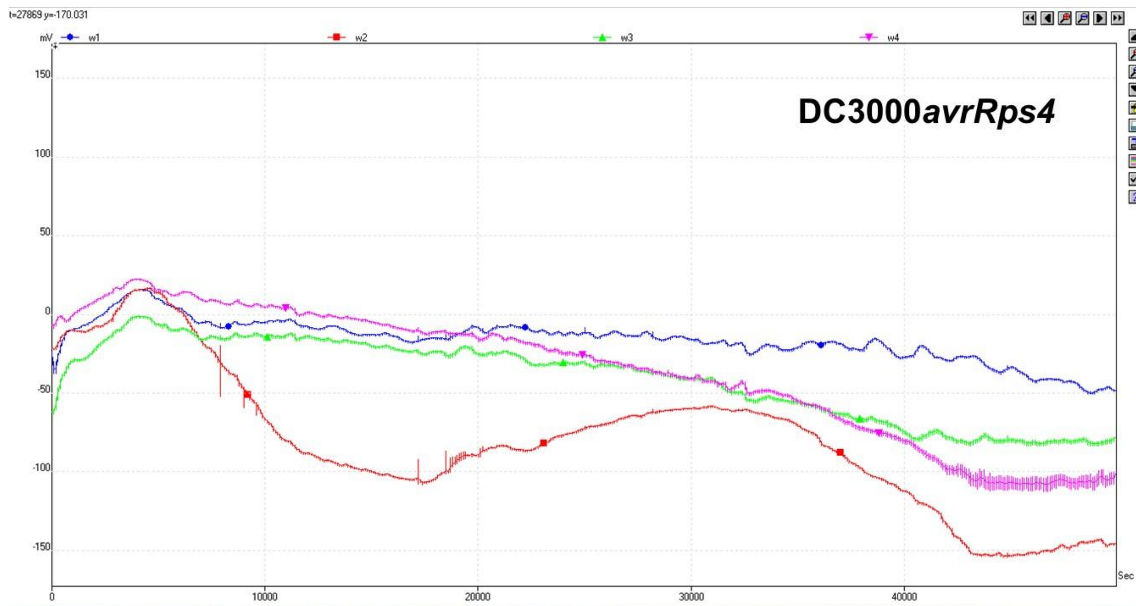
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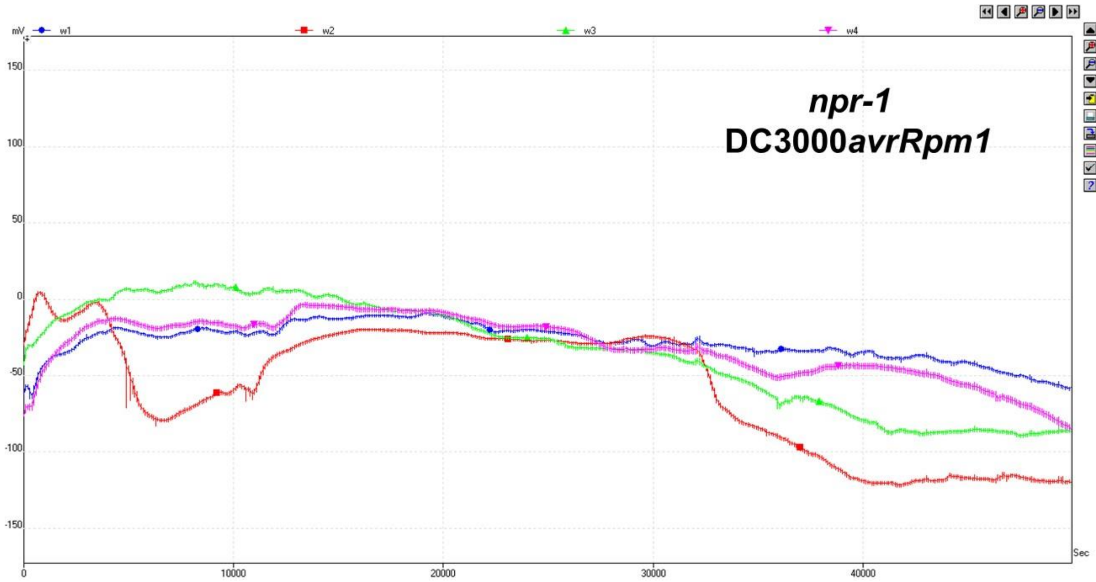
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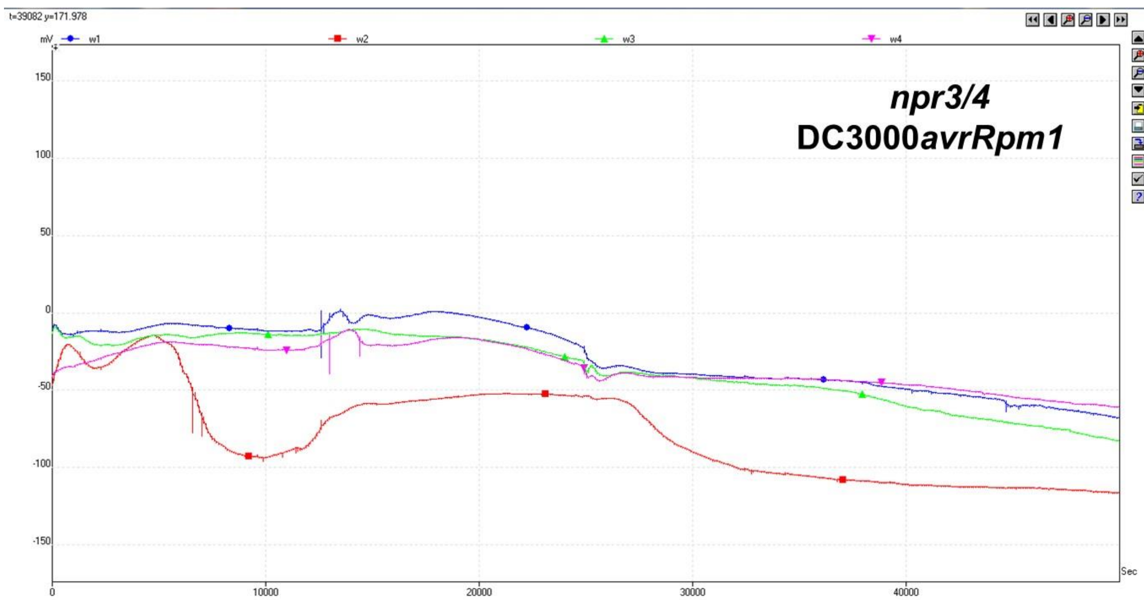
Sec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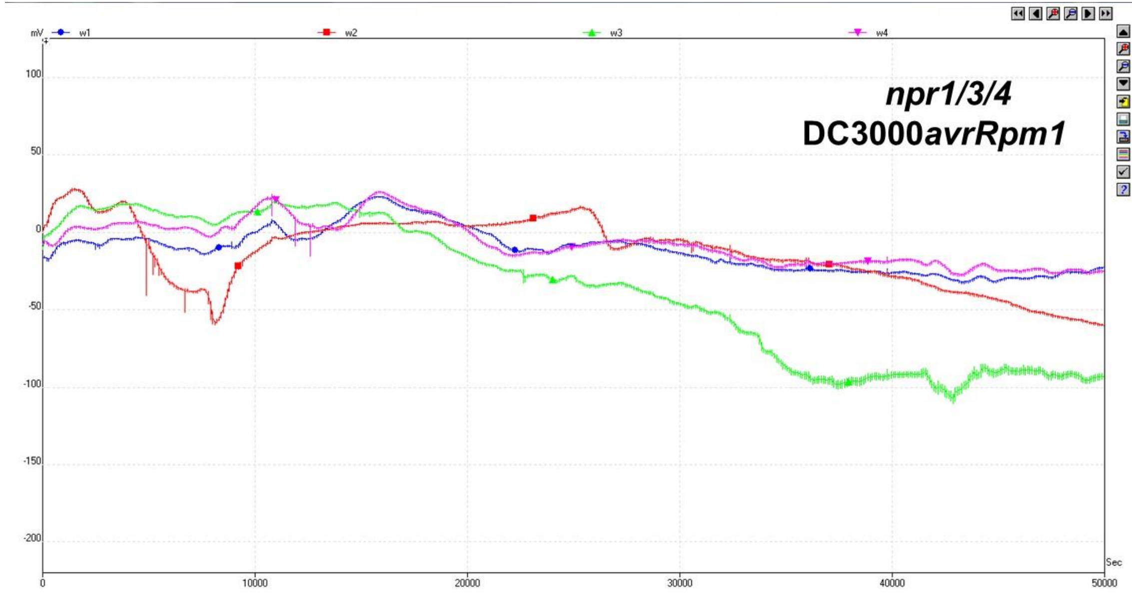
40000
Sec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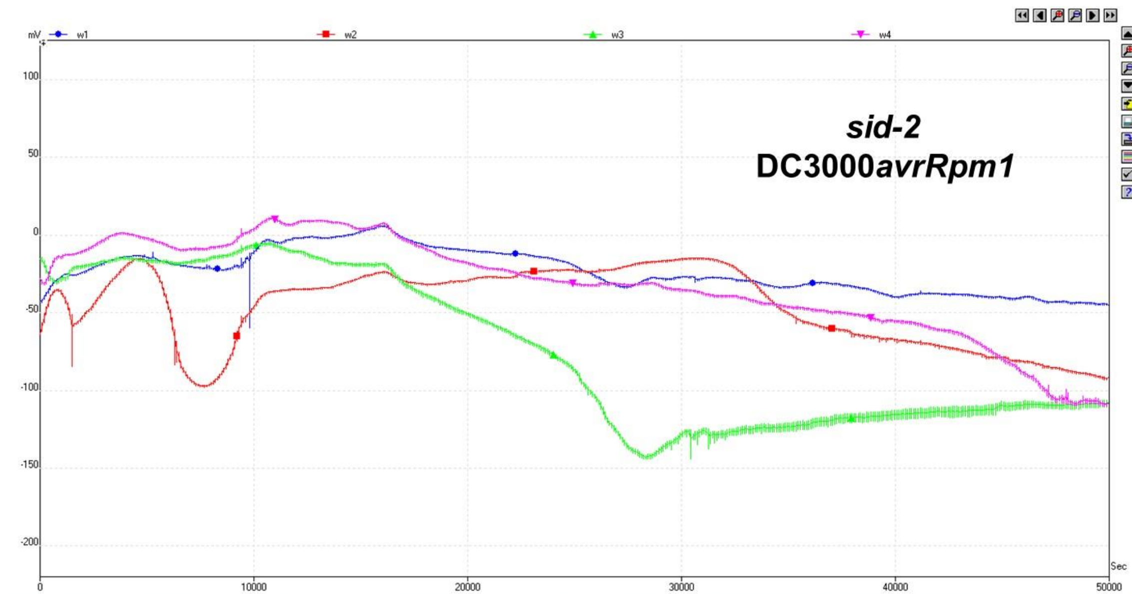
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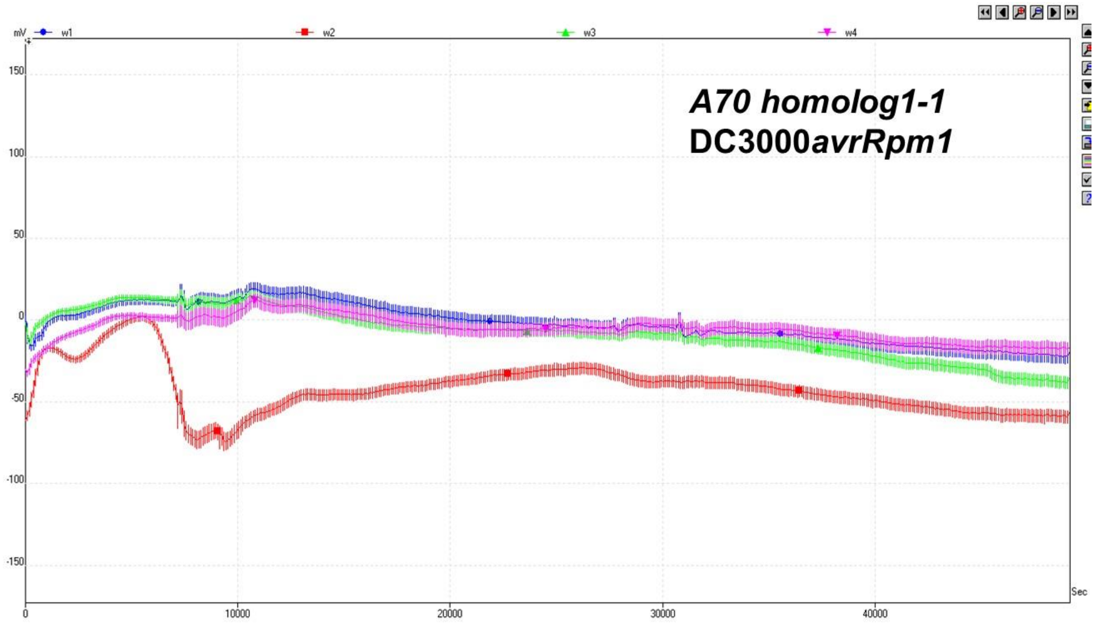
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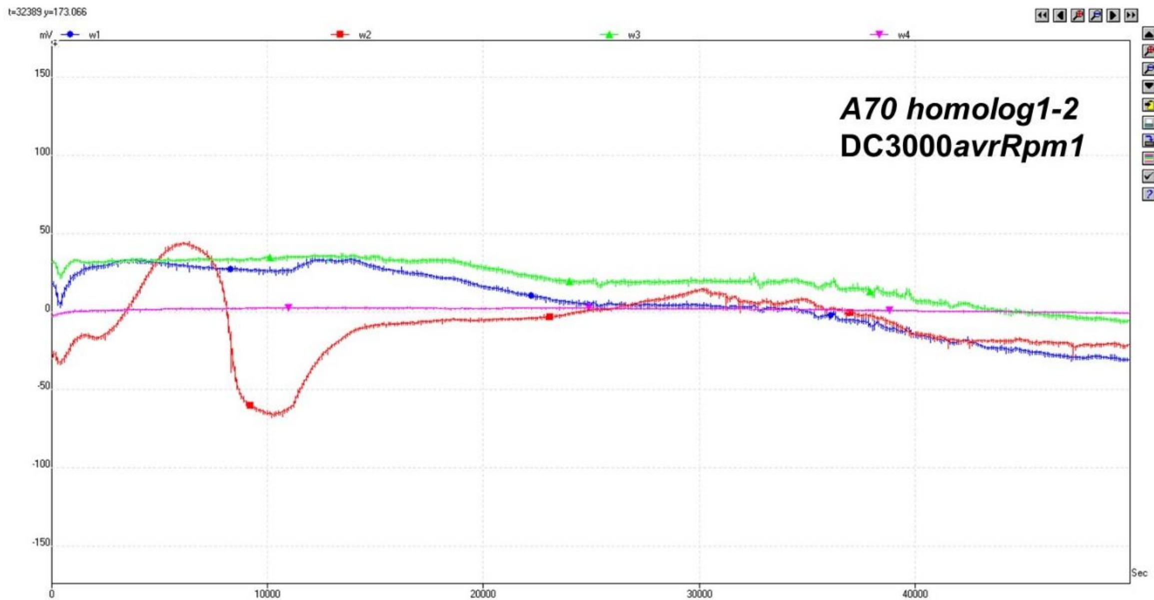
DC3000avrRpm1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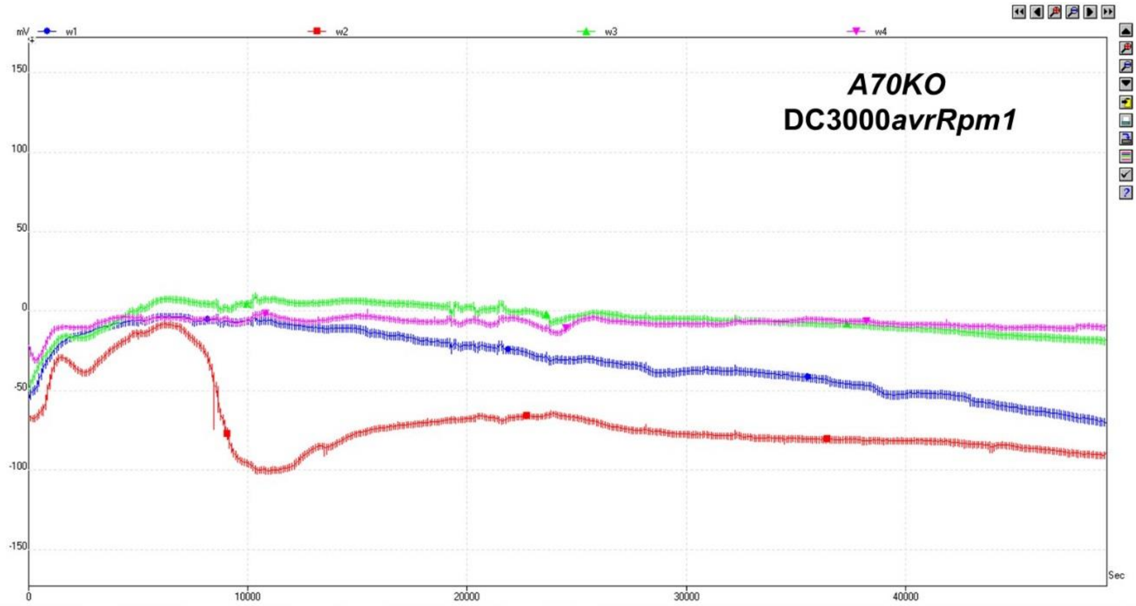
A70 homolog1-2
DC3000avrRpm1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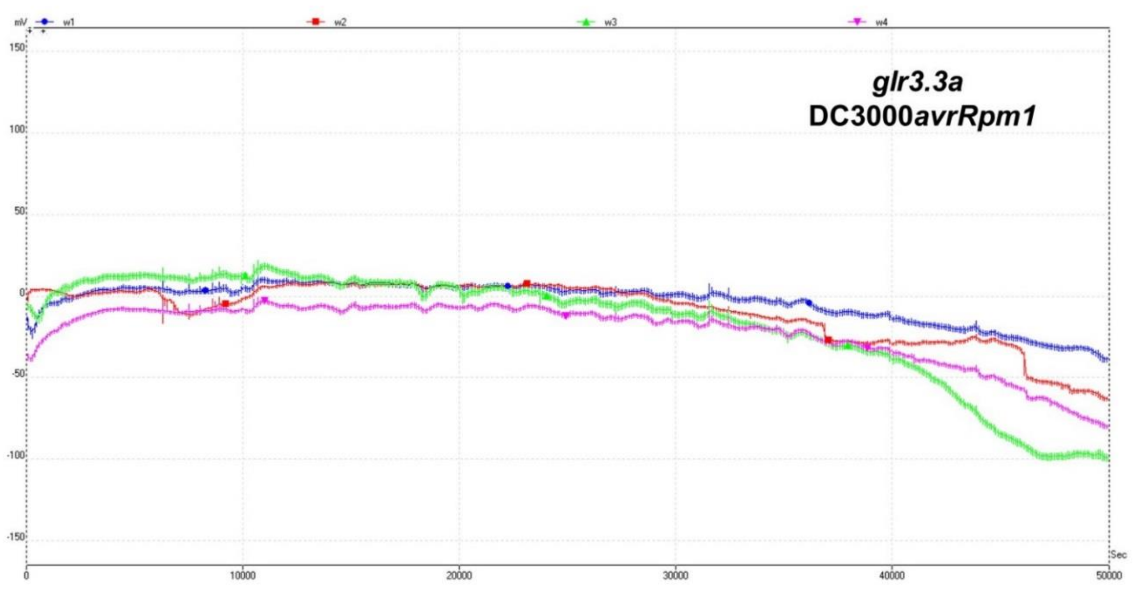
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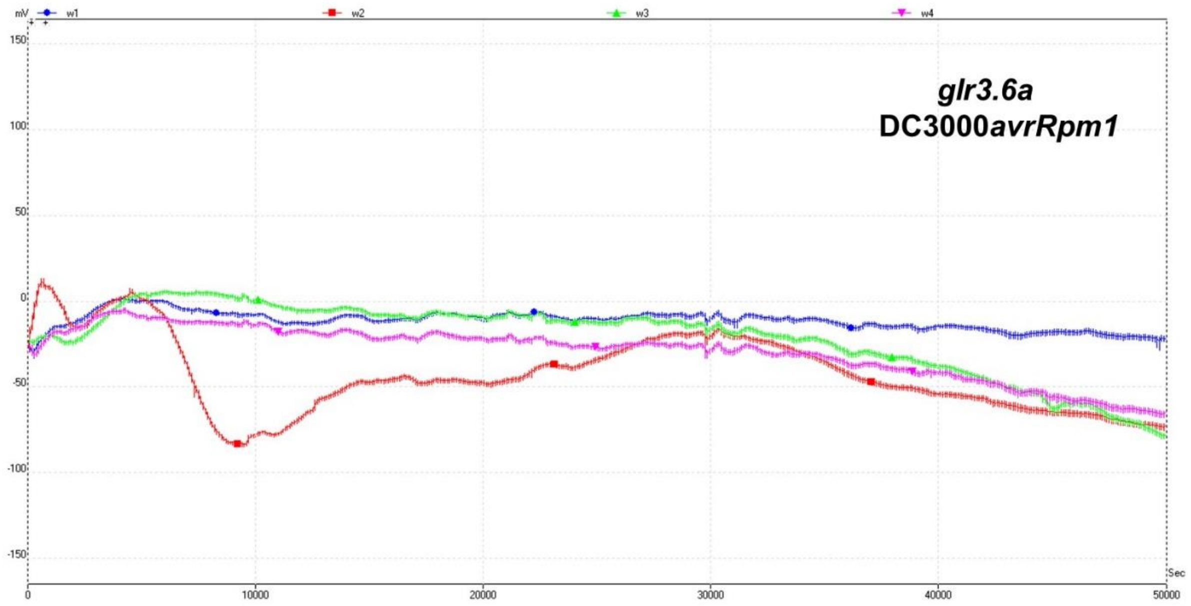
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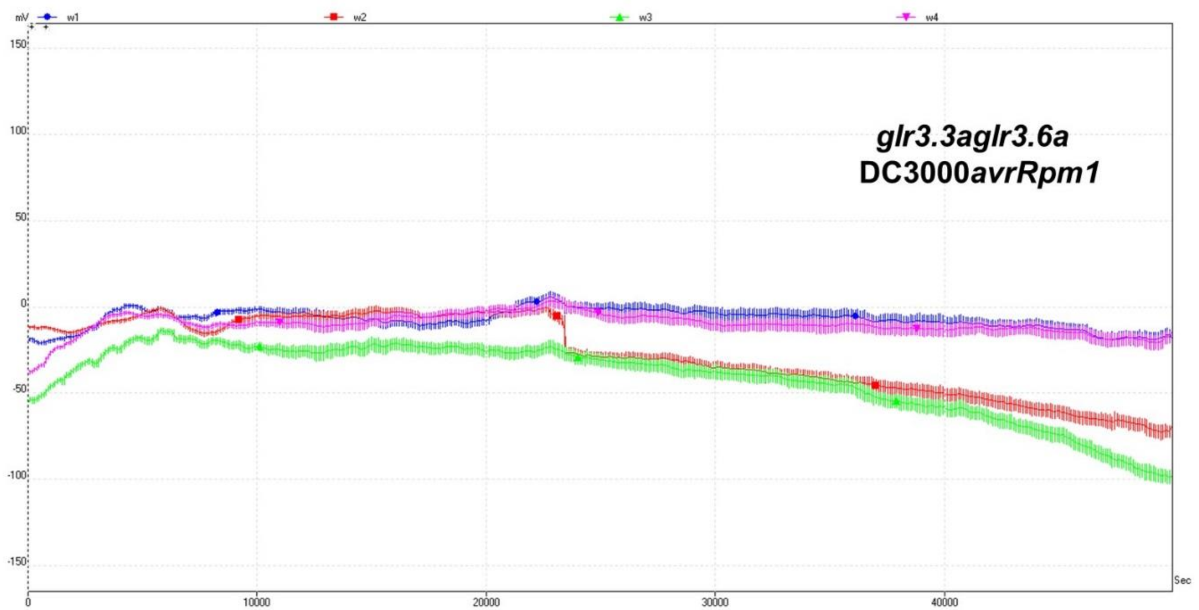
50000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w3
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-150
Sec
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30000
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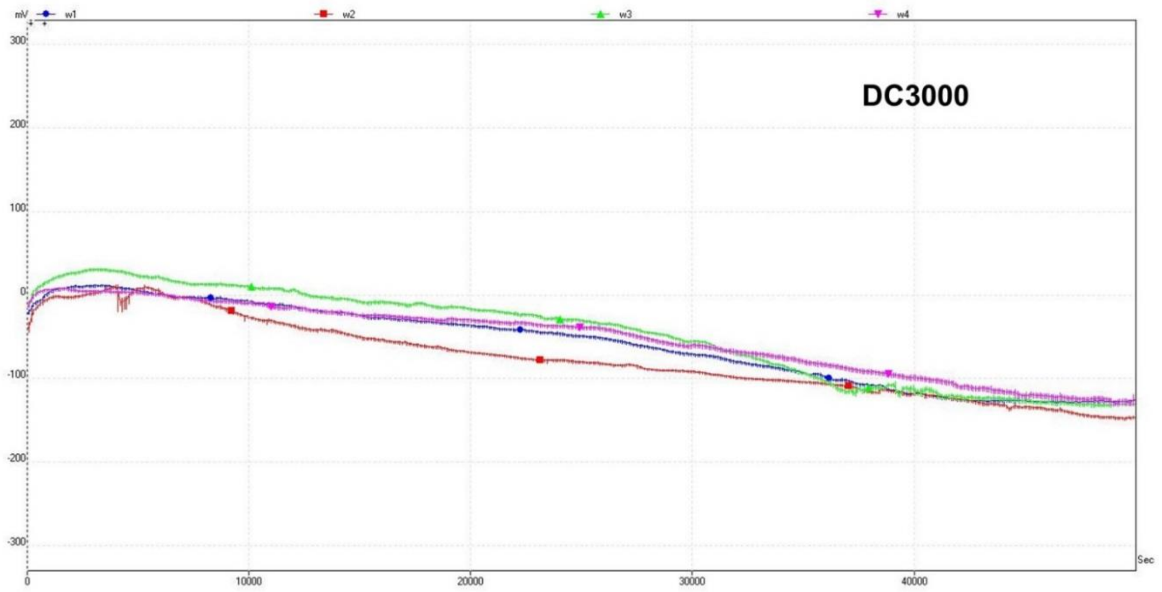
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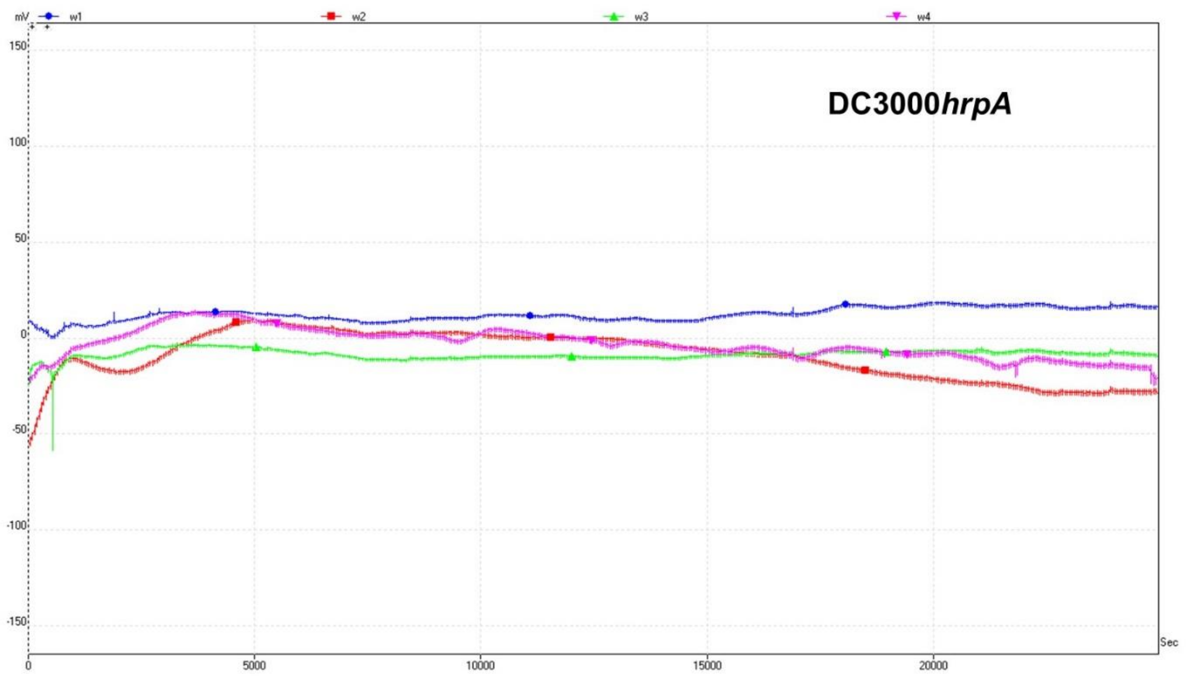
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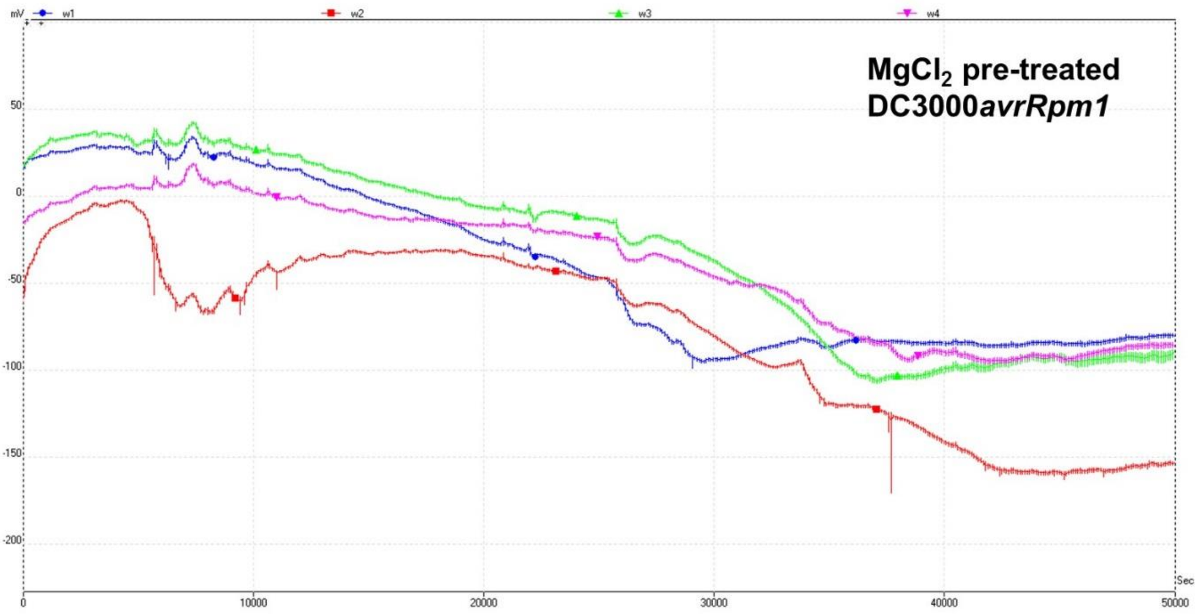
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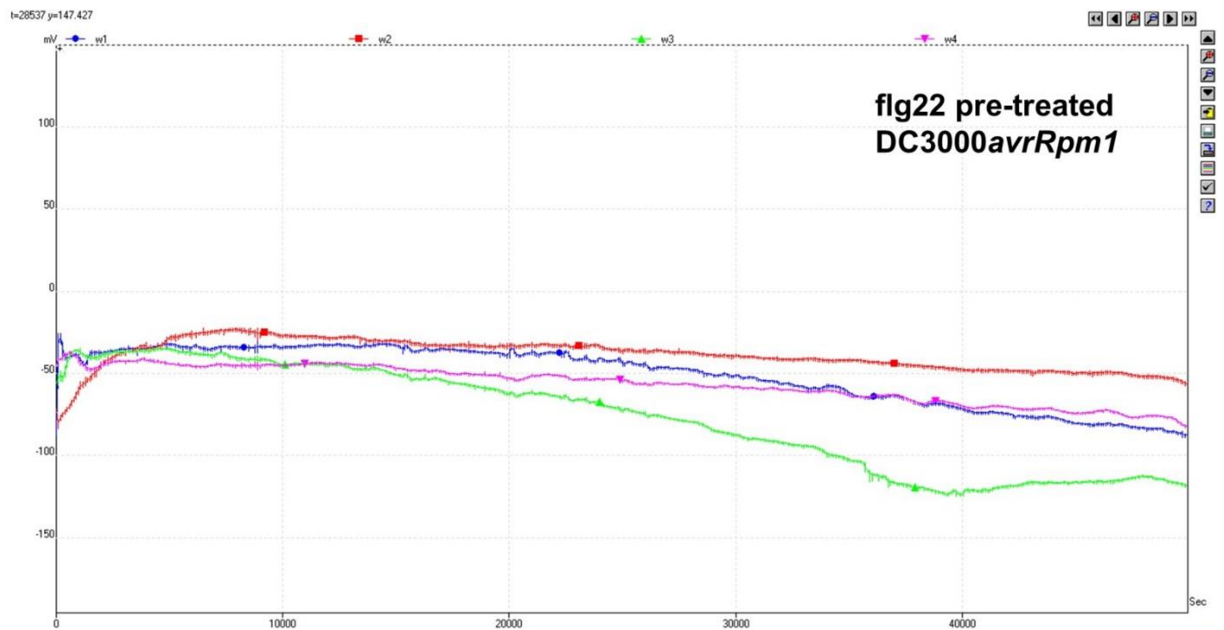
50000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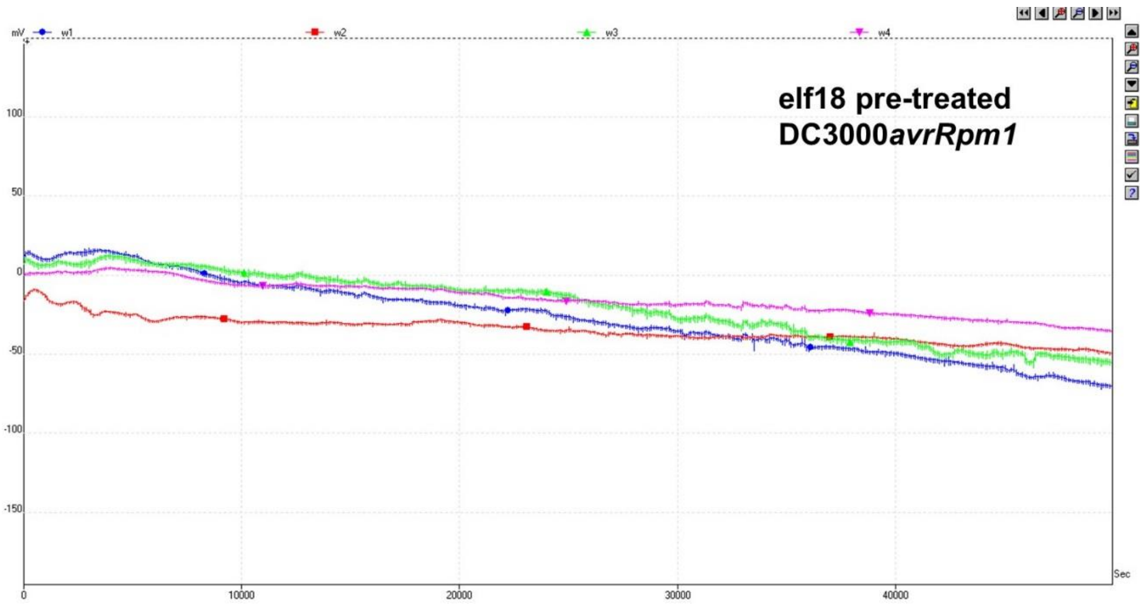
30000
40000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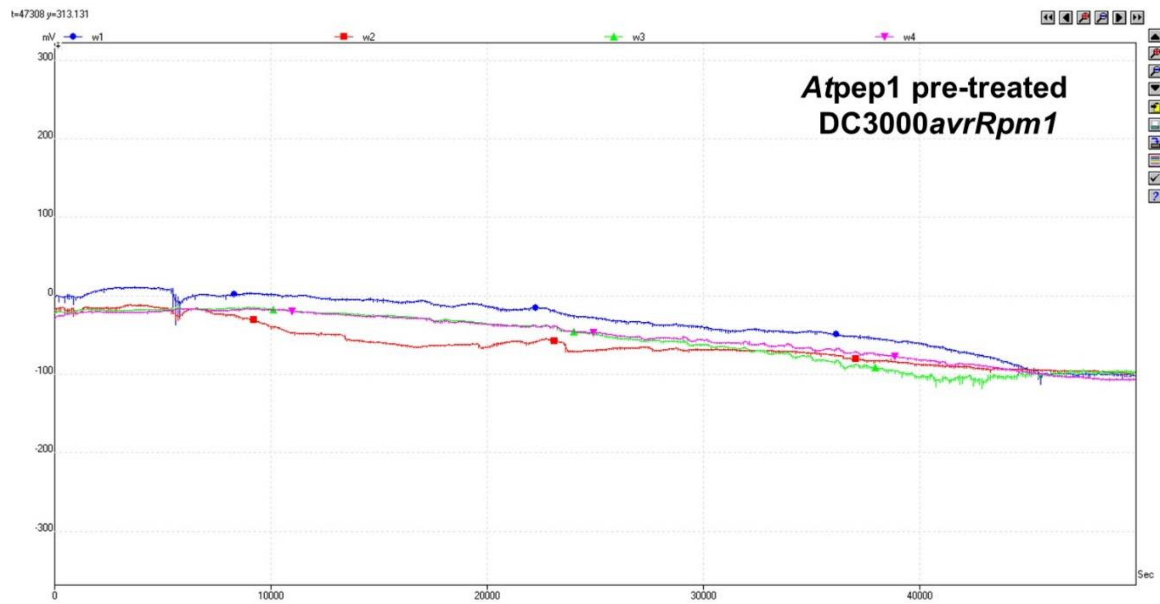
20000
30000
40000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-300
Sec
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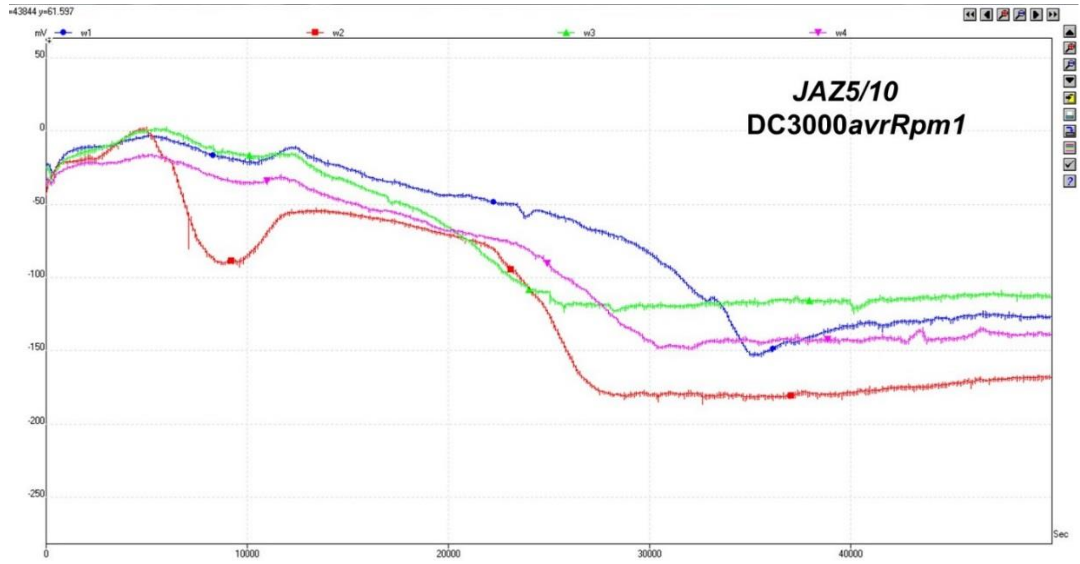
40000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20000
30000
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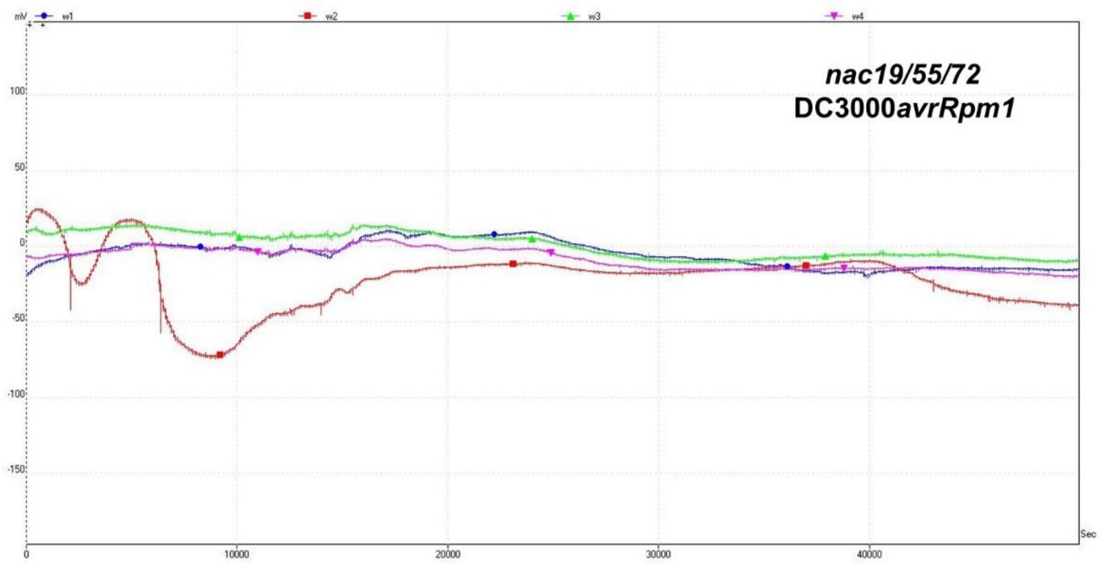
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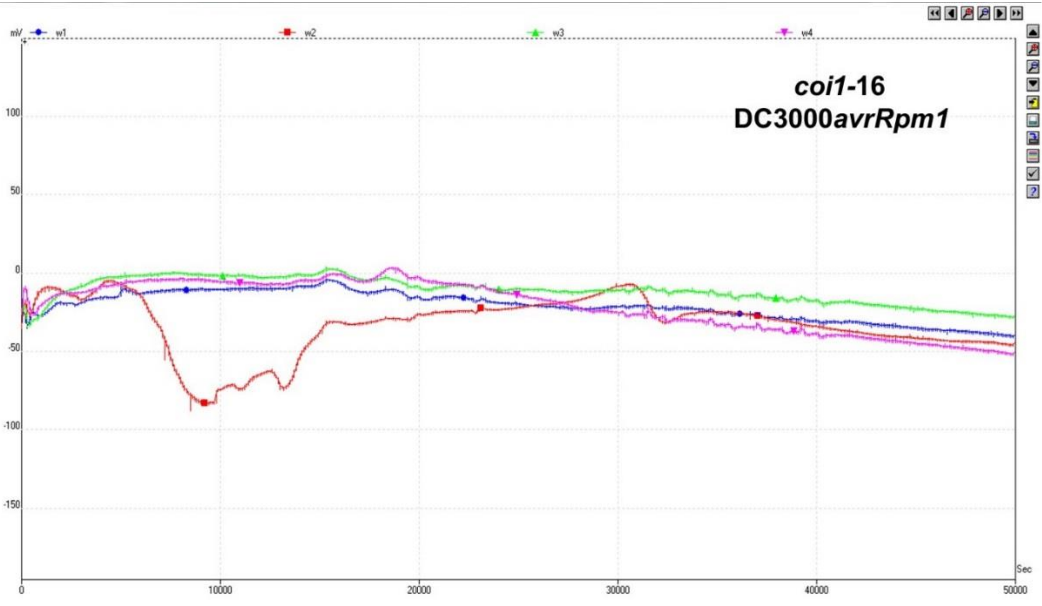
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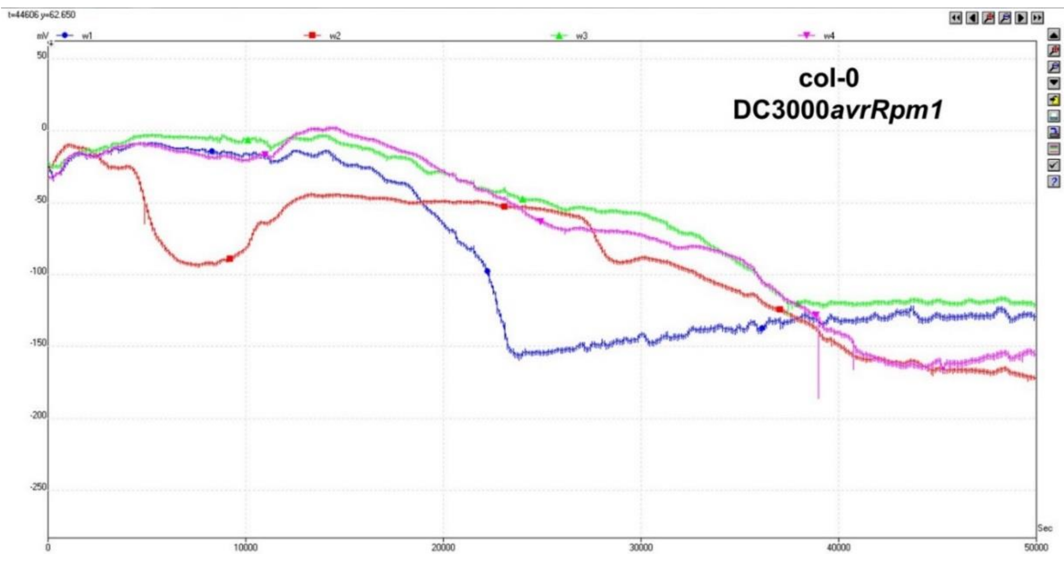
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0
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-100
-150
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-250
0
10000
20000
30000
40000
50000
Sec

10 APPENDIX-3

CD is attached with the thesis for all luciferase experiments.

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