Towards comprehensive understanding of *PLEIOTROPIC REGULATORY LOCUS 1* associated resistance signalling

by

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Abstract

Plants employ a multi-layered protection system to recognize pathogen presence and act upon intrusion. The conserved MOS4-associated complex (MAC) participates in the triggered signal transduction relay and contributes to the build-up of sound resistance. PLEIOTROPIC REGULATORY LOCUS 1 (PRL1), a MAC component with predicted structural function, is needed for a healthy immune response. Loss of this WD40 protein results in substantially higher pathogen colonization in *Arabidopsis* mutants. To dissect signalling steps downstream of the MAC, a mutant allele of *PRL1* was chosen as the basis for a genetic suppressor screen. From this screen, both dominant and recessive mutants with defects in candidate genes were isolated, and two suppressors were cloned using map-based cloning techniques.

Characterization of the first dominant mutant revealed a gain-of-function mutation in *PRL2*, the homolog of *PRL1*. Although similar in sequence, the expression of *PRL2* is greatly reduced in wild-type plants and functional analysis had not been attempted. Using the dominant *prl2-1d* allele and complementary mutants, full functional equivalence between the related proteins was established by means of defence –testing assays and evaluation of morphological criteria. This investigation revealed unequal genetic redundancy between the homologs; *PRL2* has retained residual but relevant expression levels compared to the higher expressed *PRL1. PRL2* also displays modified expression patterns, potentially indicative of developing tissue specificity.

The haplo-insufficient *SUPPRESSOR OF prl1, 2* (*SOP2*) gene is an intriguing discovery in *PRL1* signal relay. Devoid of known sequence motifs, *SOP2* encodes a novel nuclear protein with homologs limited to the plant kingdom. Several lines of evidence support a dosage-dependent mechanism, mediated by SOP2, which is prone to interference by a spoiler protein. Both the obtained dominant-negative *sop2-1D* allele and a recessive *sop2* mutation fully suppress *prl1*-related phenotypes, however neither one causes impaired resistance in single mutant analysis. Although specifics of *SOP2* functionality in the context of plant resistance signalling remain to be fully resolved, clues from epistasis analysis point towards a *PRL1* centered relationship and do not support SOP2 as a target of the MAC.

Preface

Some of the work presented in this thesis has been a collaborative effort. Contributions made by the candidate and fellow scientists are outlined below.

Chapter 1: The candidate wrote the chapter. The figure used in this chapter has been designed by the candidate and has been published in the chapter "Plant Innate Immunity" of *Plant – Environment Interactions, Signalling and Communication in Plants,* Springer, 2009. Editorial support was provided by Dr. Li, Dr. Kronstad and Dr. Samuels.

Chapter 2: The candidate wrote the chapter. A version of this chapter will be included in a manuscript tentatively titled " A gain-of-function mutation in *PLEIOTROPIC REGULATORY LOCUS 2* reveals unequal genetic redundancy and functional equivalence with *PRL1*", which is in preparation for publication. Anticipated authors list: Weihmann, T., Palma, K. and Li, X.

Experiments were co-designed by the candidate and Dr. Li and carried out by the candidate. Dr. Palma (BC Cancer Research, Vancouver) carried out the first stages of the *prl1* suppressor screen until screening of M2 mutants. Dr. Yuelin Zhang (NIBS, P.R. China) provided all three JAtY clones and Shuxin Li, from Dr. Zhang's laboratory, obtained ratios of segregating F2 progeny used in Table 1. Angela Chiang (Bohlmann Lab, UBC) assisted in real-time PCR-analysis and James Robertson, MSc. provided *PAC* polymerase used in *prl2-1D* cloning approaches. Dr. Li supervised the work and manuscript preparation and provided editorial support. Dr. Kronstad and Dr. Samuals also provided editorial support.

Chapter 3: The candidate wrote the chapter. A version of this chapter tentatively titled " A gain-of-function mutation in *PLEIOTROPIC REGULATORY LOCUS 2* reveals unequal genetic

redundancy and functional equivalence with *PRL1*", is in preparation for publication. Anticipated authors list: Weihmann, T., Palma, K. and Li, X.

Experiments were co-designed by the candidate and Dr. Li and carried out by the candidate. Dr. Palma (BC Cancer Research, Vancouver) isolated the *prl1-2 prl2-2* double mutant. Shuxin Li (Zhang laboratory, NIBS, P.R. China) assisted in mapping *prl2-1D*. Dr. Li supervised the work and manuscript preparation and provided editorial support. Dr. Kronstad and Dr. Samuels also provided editorial support.

Chapter 4: The candidate wrote the chapter. A version of this chapter tentatively titled " The plant specific SOP2 functions in signal relay mediated by the evolutionary conserved WD40 protein PRL1" is in preparation for publication. Anticipated authors list: Weihmann, T., Xia, S., Lee, E., Sack, F. and Li, X.

Experiments were co-designed by the candidate and Dr. Li and carried out by the candidate. Dr. EunKyoung Lee (Sack laboratory, UBC) performed fluorescence microscopy for localization of SOP2 shown in Figure 15. Shuxin Li (Zhang laboratory, NIBS, P.R. China) assisted in mapping *sop2-1D*. Jaclyn Dee, MSc. (Berbee laboratory, UBC) assisted in preparing phylogenetic relationships shown in Figure 12. Dr. Li supervised the work and manuscript preparation and provided editorial support. Dr. Kronstad and Dr. Samuels also provided editorial support.

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AtCDC5: *Arabidopsis thaliana* CELL DIVISION CYCLE 5

Avr: Avirulence

BAK1: BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1

BIK1: BOTRYTIS-INDUCED KINASE 1

CC: coiled-coil

cfu: colony forming units

Col: *Arabidopsis thaliana* ecotype Columbia

DCX: DDB1/CULLEN4/protein X

DWD: DDB1 binding WD40

EDS1: ENHANCED DISEASE SUSCEPTIBILITY 1

EFR: EF-TU RECEPTOR

EMS: ethyl methanesulfonate

ETI: effector-triggered immunity

FLS2: FLAGELLIN SENSING 2

F1: first filial generation

F2: second filial generation

GFP: GREEN FLUORESCENT PROTEIN

H.a.: *Hyaloperonospora arabidopsidis*

InDel: Insertion/Deletion

kD: kilodalton

LB: luria bertani

Ler: *Arabidopsis thaliana* ecotype Landsberg

MAC: MOS4-associated complex

MAPK: MITOGEN ACTIVATED PROTEIN KINASE

MOS: MODIFIER OF SNC1

MS: murashige-skoog

NB-LRR: mucleotide binding – leucine rich repeat

NCBI: National Centre for Biotechnology Information

NLS: nuclear localisation signal

NPR1: NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1

OD: optical density

PAD4: PHYTOALEXIN DEFICIENT 4

PAMP: pathogen associated molecular pattern

PCR: polymerase chain reaction

PR: PATHOGENESIS RELATED

PRL1: PLEIOTROPIC REGULATORY LOCUS 1

PRL2: PLEIOTROPIC REGULATORY LOCUS 2

P.s.m.: *Pseudomonas syringae* pv *maculicola*

P.s.t.: *Pseudomonas syringae* pv *tomato*

PTI: PAMP-triggered immunity

RPP4:

R protein: Resistance protein

SA: salicylic acid

snc1: suppressor of npr1, constitutive 1

SNP: single nucleotide polymorphism

SOP: SUPPRESSOR OF PRL1

SOP2h: SOP2 homolog

T-DNA: transfer DNA

T1: first transgenic generation

T2: second transgenic generation

TIR: Toll/Interleukin-1

TTSS: type three secretion system

WD40: Trp-Asp (W-D)

WRKY: Trp-Arg-Lys-Tyr (W-R-K-Y)

Ws: *Arabidopsis thaliana* ecotype Wssilewskija

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who are unwavering in their support for my endeavours.

1   Introduction

1.1   Inducible plant defence systems

Plants are constantly exposed to a wealth of microbes which seek access to nutrients.

Fortunately, inducible layers of defence are capable of conveying sound resistance towards

many microbes thus rendering disease an exception. Following detection of conserved

pathogen-associated molecular patterns (PAMPs) by immunity receptors located on the cell

surface, broad range protection is activated and only specialized pathogens can overcome

these defences. Innovative pathogens have found ways to evade or suppress such early

resistance mechanisms through the use of virulence factors which are delivered into a host cell.

Intracellular receptors, denoted resistance proteins (R proteins), detect the presence or activity

of these pathogen-delivered molecules and induce strong immunity responses often leading to

localized cell death and halting of pathogen invasion. PAMP and effector triggered immunity

signalling is complex. Both proteins and small molecules with diverse functions are utilised and

also involves substantial redistribution of cellular resources and efforts. Whereas infections by

avirulent pathogens are halted by the combined defence layers, virulent pathogens defy these

efforts and disease ensues.

1.2   Pathogen perception and signalling induced by cell surface receptors

Plants are equipped with a receptor-based surveillance system oriented towards the

extracellular environment which recognizes components central to microbial life. Fungal chitin,

bacterial flagellin, lipopolysaccharides or the cellulose binding elicitor lectin (CBEL) from

Phytophthora parasitica, a filamentous oomycete, are PAMPs that are recognized by

specialized pattern receptors (Fig. 1) (Gust *et al.*, 2007; Newman *et al.*, 2007; Gaulin *et al.*, 2006).

Closure of stomata contributes to the plants immune response and is in part mediated by the detection of microbial flagellin and lipopolysaccharides on the plant surface, in an effort to restrict bacterial invasion (Zhang *et al.*, 2008; Melotto *et al.*, 2006).To counteract this, the bacterial strain *Pseudomonas syringae* pv *tomato (P.s.t)* DC3000 releases the phytotoxin coronatine which induces reopening of stomata and is critical for overall virulence (Zeng and He, 2010; Melotto *et al.*, 2006).

Recognition of fungal microbes through chitin detection is mediated by CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1), a LysM domain receptor kinase (Iizasa *et al.*, 2010; Petutschnig *et al.*, 2010; Miya *et al.*, 2007). CERK1, and two other LysM domain proteins LYM1 (LysM DOMAIN PROTEIN 1) and LYM3, also participate in peptidoglycan sensing and thus immunity to bacterial pathogens (Willmann *et al.*, 2011). The perception of bacterial flagellin is probably the best understood mechanism involving a PAMP molecule and its respective receptor: in this interaction, the leucine-rich repeat receptor kinases (LRR-RK) FLAGELLIN SENSING2 (FLS2) recognizes the flg22 epitope of flagellin and subsequently interacts with several other LRR-RK's, among them BAK1/SERK3 (BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1/ SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE3), SERK1 and BKK1/SERK4 (BAK1-LIKE1) (Roux *et al.*, 2011; Chinchilla *et al.*, 2007; Heese *et al.*, 2007).

The cytoplasmic kinase BIK1 (BOTRYTIS-INDUCED KINASE 1), originally discovered in the immunity response against necrotrophic fungal pathogens, also associates with FLS2-BAK1 and may be the first to be phosphorylated in a sequential series of reciprocal trans phosphorylation steps between kinases (Lu *et al.*, 2010b; Wang *et al.*, 2008). BAK1 might also differentially phosphorylate other complex members, leading to varying signal outputs

(Schwessinger *et al.*, 2011). PAMP signalling might be further propagated through release of BIK1 from the receptor complex (Lu *et al.*, 2010b).

To fine tune responses, activated FLS2 receptors are degraded following ligand-induced endocytosis (Robatzek *et al.*, 2006). Ubiquitination of FLS2 by two BAK1 associated U-box E3 ligases, PUB12 and PUB13, suggests degradation via the plants proteasome machinery (Lu *et al.*, 2011). Recently, a role of the gaseous phytohormone ethylene in regulation of FLS2 has been demonstrated. Presence of ETHYLENE-INSENSITIVE 2 (EIN2) and endogenous levels of ethylene result in binding of the transcription factor EIN2 to the *FLS2* promoter sequence, possibly replenishing receptor numbers in a positive feedback loop (Boutrot *et al.*, 2010).

There is growing evidence that BAK1 forms similar ligand-induced complexes with other receptors including PEPR1(PEP RECEPTOR1) and PEPR2, which recognize the PAMP and wound-induced endogenous molecule Pep1, and EFR (EF-TU RECEPTOR), the receptor recognizing the bacterial elongation factor Tu (Roux *et al.*, 2011; Krol *et al.*, 2010; Postel *et al.*, 2010; Zipfel *et al.*, 2006). Mutants of pattern recognition receptors usually show heightened susceptibility towards pathogens underlining the importance of PAMP-triggered immunity (PTI) in the overall defence response (Nicaise *et al.*, 2009).

Following PAMP perception, a cascade of mitogen activated protein kinases (MAPK) is induced, likely including sequential phosphorylation of at least a MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and a MAPK. Using a protoplast system to identify MAPK's in flg22-activated PTI outputs, signal routing through MEKK1, MKK4/5 and MPK 3/6 was initially suggested (Asai *et al.*, 2002). Subsequent work, however, suggests that MEKK1 instead functions in a cascade with MKK1/2 and MPK4, which negatively regulates resistance (Gao *et al.*, 2008; Suarez-Rodriguez *et al.*, 2007; Ichimura *et al.*, 2006). Interaction of MEKK1 with MKK1/2 mainly occurs at the plasma membrane, whereas MPK4 and MKK2 have been shown to associate both on the

membrane surface and in the nucleus, suggesting that signal relay might function through relocation of MKK1 and MKK2 (Gao *et al.*, 2008). It thus appears that FLS2 activates an unknown MAPKKK followed by phosphorylation of MKK4/5 and MPK3/4 which positively regulate PTI but also triggers a MAPK cascade with inhibitory influence on immunity (Fig. 1) (Rodriguez *et al.*, 2010) . A negative defence response modulated by an MPK4 homolog was also monitored in soybean (Liu *et al.*, 2011).

Both antagonistic pathways are expected to affect defence gene expression through WRKY-type transcription factors such as WRKY22 and WRKY29, which probably function downstream of MPK3/6 (Asai *et al.*, 2002). The MPK4 substrate MKS1, which is required for resistance in *mpk4* mutants, has been recently shown to interact with both WRKY33 and MPK4 using the N-terminal domain (Petersen *et al.*, 2010; Andreasson *et al.*, 2005). A complex consisting of MPK4, WRKY33 and MKS1 appears to be activated through defence signalling, leading to phosphorylation of MKS1 and release of the transcription factor. WRKY33 is later recruited to the promoter of *PHYTOALEXIN DEFICIENT 3*, a gene required for synthesis of phytoalexin camalexin (Qiu *et al.*, 2008). Phosphatases also contribute to the coordination of defence responses through de-phosphorylation of kinases. MAPK phosphatase 2 interacts with both MPK3 and MPK6, likely influencing signalling capacities (Lumbreras *et al.*, 2010).

1.3   Virulence factors of pathogenic microbes

Due in large parts to broad range PAMP-signalling, plants fight off pathogenic take-over attempts in most cases. The mechanisms resulting in PTI are consequently the main targets of pathogen-delivered virulence factors, called effectors. An essential factor in bacterial virulence, the type three secretion system (TTSS), is encoded by the *hrp/hrc (hypersensitivity response and pathogenicity/hrp conserved)* gene cluster which assembles into a multiprotein structure capable of translocating molecules into the host cytoplasm (Fig.1) (Tampakaki *et al.*, 2010).

The inventory of effectors thought to be injected by *Pseudomonas syringae* pv *phaseolicola* 1448a amounts to 27 molecules and which is similar in scale to the predicted 28 effector repertoire of *P.s.t.*DC3000 (Zumaquero *et al.*, 2010; Cunnac *et al.*, 2009). Between 30-40 molecules are translocated by plant pathogenic *Xanthomonas* bacteria (Buttner and Bonas, 2010).

The virulence promoting function of many effectors remains elusive although progress has been made, e.g. early targets of effectors are pattern recognition receptors. Binding of *P.s.* effector AvrPto to FLS2 and EFR in *Arabidopsis* and to LeFLS2 in tomato has been previously shown to correlate with hinderance of PTI, possibly through inhibiting of kinase signalling ability (Xiang *et al.*, 2008). Phosphorylation of BIK1, the proposed first target of FLS2 is blocked in the presence of AvrPto (Xiang *et al.*, 2011).

The sequence-distinct bacterial effector avrPtoB mimics the structure and function of an eukaryotic E3 ligase to mediate ubiquitination and subsequent degradation of host PTI kinases in susceptible plants (Ntoukakis *et al.*, 2009; Rosebrock *et al.*, 2007; Janjusevic *et al.*, 2006). Targets of AvrPtoB, which is widely found among *Erwinia, Xanthomonas* and *Pseudomonas* strains, include the tomato kinase Fen as well as CERK1, the receptor kinase responding to both chitin and peptidoglycan patterns (Willmann *et al.*, 2011; Gimenez-Ibanez *et al.*, 2009). In vitro kinase activity of Bti9, the most sequence similar LysM receptor-like kinase to CERK1 in tomato, is also halted in the presence of AvrPtoB (Zeng *et al.*, 2011). AvrPto and AvrPtoB may also bind the regulatory protein BAK1, potentially interfering with signal relay mediated by multiple receptors (Lu *et al.*, 2010a; Shan *et al.*, 2008).

Another example of an effector hijacking host mechanisms is the TAL family of transcription activator-like molecules used by *Xanthomonas* spp. and *Ralstonia solanacearum*. TAL effectors imitate eukaryotic transcription factors and specifically initiate gene expression upon binding to

the UPT (UPREGULATED BY TAL EFFECTORS) box of host target genes (Boch *et al.*, 2009; Moscou and Bogdanove, 2009).

Delivery and virulence functions of oomycete and fungal effectors are less well understood. An uptake signal motif with the consensus sequence RXLR (arginine, "any amino acid", leucine, arginine), is shared by many oomycete effectors and is important for translocation into the host plant (Dou *et al.*, 2008; Whisson *et al.*, 2007). One possible mechanism involves binding of the RXLR motif to phosphatidyl inositol phosphatases (PIP) on the outer surface of the plant plasma membrane, followed by lipid raft-mediated endocytosis of the complex (Kale *et al.*, 2010). Requirement of the RXLR-PIP interaction for translocation is currently debated (Yaeno *et al.*, 2011).

Avr3b, an RXLR effector from *Phytophthora sojae*, is an ADP-ribose/NADH pyrophosphorylase (Dong *et al.*, 2011). In plants, some proteins with a similar Nudix motif have been shown to act as negative regulators of resistance, i.e. AtNUDT7 (*ARABIDOPSIS THALIANA* NUDIX HYDROLASE HOMOLOG 7) (Bartsch *et al.*, 2006b). Defects in *AtNUDT7* result in altered cellular redox levels, suggesting a role for this protein and similarly, Avr3b, in early defence modulation (Ge *et al.*, 2007).

Phytophthora infestans encodes two forms of the RXLR effector Avr3a which suppress PAMP-like elicitin INF1 induced programmed cell death (Bos *et al.*, 2009). INF1 interacts with CMPG1, a host E3 ubiquitin ligase which in turn is stabilised by Avr3a (Gonzalez-Lamothe *et al.*, 2006). Although targets of CMPG1 activity are unknown, maintaining processes is beneficial to the pathogen. Silencing of the Avr3a genes results in significantly reduced virulence, suggesting these factors are required for full pathogenicity (Bos *et al.*, 2010).

Functional characterization of individual effectors has proven difficult since mutating one virulence factor rarely reduces virulence, likely due to redundancy between pathogen-delivered molecules. Creation and comprehensive analysis of pathogenic bacterial strains with double and triple effector mutations may overcome some of the problems (Zumaquero *et al.*, 2010). A TTSS based system in *P.s.t.* DC3000-LUX was recently used to investigate oomycete effectors and may also facilitate accelerated screening procedures for this type of pathogen (Fabro *et al.*, 2011)

1.4   Intracellular receptors initiate effector-triggered defence

Despite the amount and functional diversity of virulence factors, the plant immune system often succeeds in limiting pathogen proliferation. A second layer of inducible defences is mediated by the products of *resistance* (*R*) genes which detect the presence or activities of effectors, also called Avirulence (Avr) proteins. In many cases, such activity is an attempt by the pathogen to interfere with PTI responses. *R* genes encode intracellular receptors that initiate effector-triggered immunity (ETI) after perception of a Avr-associated danger cues (Fig. 1) (Jones and Dangl, 2006).

Plant immunity receptors are encoded by at least five different classes of resistance-associated genes (Glowacki *et al.*, 2011; Dangl and Jones, 2001). PAMP receptors usually reside in plasma membranes whereas the largest class of ETI receptors localize to the cytosol. Recent genome-wide predictions suggest a large number of proteins, between 159 – 174, as members of the nucleotide binding – leucine rich repeat (NB-LRR) family of R proteins (Guo *et al.*, 2011; Chen *et al.*, 2010). NB-LRR's are further subdivided into two categories depending on either homology of their N-terminal domain with *Drosophila* Toll and human interleukin-1 proteins (TIR) or according to an N-terminal coiled coil motif (CC). RPM1 (RESISTANCE TO PSEUDOMONAS SYRINGAE PV MACULICOLA 1) is a CC-NB-LRR class protein whereas

RPS4 (RESISTANCE TO PSEUDOMONAS SYRINGAE 4) and RPP1 (RESISTANCE TO PERONOSPORA PARASITICA 1) are examples of TIR-type R proteins (Takken and Tameling, 2009b).

In *Arabidopsis*, *P.s.* strains expressing the effector molecule AvrPphB elicit local cell death known as the hypersensitive response (HR), which halts the infection process. This process is dependent on the CC-NB-LRR R-protein RPS5, which is associated with the immediate target of the effector, the host kinase PBS1. Proteolytic cleavage of PBS1 by the effector protease AvrPphB presumably aids in bacterial colonization, however leads to ETI signalling and resistance in plants with the *RPS5* gene (Shao *et al.*, 2003). Cleavage of the host protein causes a modified phosphorylation state of the R protein and induces immunity responses (Ade *et al.*, 2007).

The ETI response triggered by AvrPphB relies on AtMIN7 (*ARABIDOPSIS THALIANA* HOPM INTERACTOR 7), a guanine nucleotide-exchange factor targeted by the HopM1 effector (Nomura *et al.*, 2011; Nomura *et al.*, 2006). HopM1 destabilizes AtMIN7 levels in the plant via a 26S proteasome-dependent mechanism thus resulting in susceptibility towards *P.s.*pv.*tomato.* DC3000 (Nomura *et al.*, 2006). In contrast, presence of avirulence proteins AvrPphB, AvrRpt2 or HopA1 blocks degradation. This suggests a mechanism in which earlier ETI responses triggered by avirulence proteins work to protect downstream targets of the signal transduction (Nomura *et al.*, 2011). AtMIN7 and HopM1 are localized in the trans-Golgi/early endosome system, which supports increasing evidence towards a critical role of vesicle traffic in defence (Nomura *et al.*, 2011; Frei dit Frey and Robatzek, 2009; Hoefle and Huckelhoven, 2008).

Activity associated with the previously introduced TAL effectors of *Xanthomonas campestris* pv. *vesicatoria* is recognized via an intriguing mechanism in pepper plants. Target genes of the TAL effector AvrBs3 include host genes with assumed beneficial function to the pathogen. But the

transcription factor- like effector will also bind a UPT box in the promoter region of the pepper resistance gene *Bs3*. Presence of *Bs3* confers ETI signalling likely through induced synthesis of and subsequent signalling by Bs3, leading to resistance in plants (Romer *et al.*, 2007). A similar mechanism might also mediate resistance of rice towards *X. oryzae* pv. *oryzae*. Effective defence in resistant plants is activated by the TAL effector AvrXa27, and is characterized through increased transcription of the rice R gene *Xa27,* which also carries an UPT box (Romer *et al.*, 2009).

ETI receptors may also be directly interacting with Avr proteins, e.g. the TIR-NB-LRR RPP1 proteins present in *Arabidopsis thaliana* ecotypes co-purify with ATR1 (ARABIDOSIS THALIANA RECOGNIZED 1), an oomycete effector from several *Hyaloperonospora arabidopsidis* (*H.a.*) subspecies including EMOY, CALA and NOCO (Krasileva *et al.*, 2010; Rehmany *et al.*, 2005). Depending on recognition surface variants between RPP1-encoding ecotypes, interaction specifics may differ (Chou *et al.*, 2011). Evidence further suggests that RPP1 variants associate with the effector in an inactive state. This would support the current hyptesis of an R protein activation switch, which possibly includes conformational changes (Krasileva *et al.*, 2010; Lukasik and Takken, 2009).

Crystallization of both the CC domain of barley MLA10 and the TIR domain of flax L6 as protein dimers are consistent with activation models of R-proteins requiring conformational changes potentially through oligomerization. Site-directed mutagenesis of dimerization relevant residues resulted in the loss of signalling ability *in planta,* further supporting a role for multimeric receptor complexes at the beginning of ETI (Bernoux *et al.*, 2011; Maekawa *et al.*, 2011; Takken and Tameling, 2009a). Examples of proteins that aid in NB-LRR complex stabilization include RAR1, HSP90 and SGT1 (REQUIRED FOR MLA12 RESISTANCE, HEAT SHOCK PROTEIN 90, SUPPRESSOR OF THE G2 ALLELE OF SKP1) (Shirasu, 2009). Negative regulation of R-

protein signalling may be provided by the tetratrico peptide repeat protein SRFR1 (SUPPRESSOR OF *rps4-RLD)*, which interacts with SGT1 *in vivo* (Li *et al.*, 2010).

Indirect or direct association, as well as a general capability to recognize effector-induced modification of host proteins, are mechanisms predicted by the guard model of plant resistance (Dangl and Jones, 2001). However, in recent times, examples of effector recognition have been described that do not fit the hypothesized guarding mechanism of PTI-involved subjects. The AvrPto-Pto relationship is such an example. As described earlier, AvrPto interacts and inhibits PAMP receptors FLS2 and EFR; however, it also binds the tomato resistance protein Pto which closely resembles the structure of these receptor kinases and competes with FLS2 for interaction. An AvrPto-FLS2 complex promotes virulence, whereas interaction of AvrPto with Pto triggers strong defences and may suggest that the R-proteins' main function is that of a decoy for AvrPto (Xiang *et al.*, 2008). The two models differ solely in the proposed recognition mechanism of pathogenic activity. ETI is either triggered by an R protein after a guarded host protein has been compromised or through an alternative relationship between a decoy R gene and a virulence factor. The current understanding allows for both models (Block and Alfano, 2011; van der Hoorn and Kamoun, 2008).

1.5   Biotrophic pathogens induce salicylic acid-dependent signalling

PTI and ETI receptors both recognize ligands to activate defence and the induced signalling cascades use in some cases molecules common to both pathways. A major branch in plant immunity signalling is the salicylic acid (SA) signal route which is used by both PTI signal relay and ETI networks in response to (hemi) biotrophic pathogens such as *H.a.* ssp. and *P.s.* strains. In our current understanding, the lipase like protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) is a primary component upstream of SA production and is required for activation of both TIR and CC-type R-protein signalling (Venugopal *et al.*, 2009; Parker *et al.*, 1996). EDS1 and

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related protein PHYTOALEXIN DEFICIENT 4 (PAD4) localize to the cytosol as well as to the nucleus and are able to interact with each other (Wiermer *et al.*, 2005; Feys *et al.*, 2001). A third, similar protein, SENESCENCE ASSOCIATED GENE 101 (SAG101) is preferentially found in the nucleus where it forms a ternary complex which EDS1 and PAD4 (Zhu *et al.*, 2011; Feys *et al.*, 2005). Localisation of the components and also of the complex appear to be dependent on relative levels of binding partners; both SAG101 and PAD4 affect EDS1 distribution, however with antagonistic effects (Zhu *et al.*, 2011). Such redistribution could be a mechanism to adjust defence outputs, as suggested by the need for balanced levels of EDS1 for fully functional resistance (Garcia *et al.*, 2010).

The NDR1 (NONRACE-SPECIFIC DISEASE RESISTANCE) protein functions early in multiple CC-type R-protein signalling pathways and shows homology to integrins, a class of surface receptors that bind extracellular proteins and relate signals into the cell through connection to the cytoskeleton (Knepper *et al.*, 2011; Gee *et al.*, 2008; Century *et al.*, 1995). Consequently, *ndr1* mutants are compromised in the adhesion between the plasma membrane and the cell wall, and also show altered pathogen-induced electrolyte leakage, outlining the importance of maintaining cell integrity in plant resistance (Knepper *et al.*, 2011)

Several genes important for SA production and accumulation have been previously identified. In response towards pathogen infection, SA responses are controlled by EDS1, PAD4 and Isochorismate synthase SID2/ICS1 (SA INDUCTION DEFICIENT2/ISOCHORISMATE SYNTHASE 1) which synthesises SA from the precursor chorismate (Wildermuth *et al.*, 2001). ENHANCED DISEASE SUSCEPTIBILITY5 (EDS5) has homology to MATE transport family proteins, and may be involved in the transport of SA related molecules (Nawrath *et al.*, 2002) The ALD1 protein (AGD2-LIKE DEFENCE RESPONSE PROTEIN 1) also contributes to the accumulation of SA and acts additively with PAD4 (Song *et al.*, 2004).

In PTI responses, the association of calmodulin (CaM) with CBP60g, a member of the CaM-binding CBP60 family, positively affects SA accumulation either independent or potentially upstream of PAD4 (Wang *et al.*, 2009). CaM binding is not required for the function of SARD1 (SAR DEFICIENT 1), a closely related and functionally redundant member of the CBP60 family of plant specific DNA binding proteins. In the presence of pathogens, both SARD1 and CBP60g are recruited to the promoter of *SID2/ICS1* potentially affecting transcription of the SA-producing enzyme (Wang *et al.*, 2011; Zhang *et al.*, 2010).

PBS3 (AvrPphB SUSCEPTIBLE*),* a member of the GH3-like family of acyl-adenyl/thioester-forming enzymes, plays a critical yet unresolved role in SA metabolism (Nobuta *et al.*, 2007; Warren *et al.*, 1999). Interestingly, JAR1 (JASMONIC ACID RESPONSE LOCUS 1) of the same family, catalyzes formation of the hormone-amino acid conjugate JA-Ile, the active form of jasmonic acid (JA). JA-Ile binds to the E3 ubiquitin ligase F-box protein CORONATINE INSENSITIVE 1 (COI1) which targets a family of jasmonate ZIM domain (JAZ) transcriptional repressors for degradation via the 26S proteasome, thus resulting in activation of jasmonate responsive genes. (Chini *et al.*, 2007; Thines *et al.*, 2007; Staswick *et al.*, 2002).

During SA-mediated immunity against (hemi)biotrophic pathogens, redox changes in the cytoplasm lead to monomerization of usually inactive complexed multimers of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) (Tada *et al.*, 2008; Mou *et al.*, 2003). Monomeric NPR1 relocates into the nucleus where it facilitates gene expression through interaction with TGA and possible recruitment of WRKY transcription factors (Wang *et al.*, 2006; Zhang *et al.*, 2003). TGA transcription factors bind to motifs in SA-inducible *PATHOGENESIS-RELATED (PR)* genes and other genes that are either dependent or independent of the regulator NPR1, consistent with the influence of multiple signalling routes over pathogen-induced genetic reprogramming (Fode *et al.*, 2008; Thibaud-Nissen *et al.*, 2006; Zhang *et al.*, 1999).

Regulation of *PR* genes, which encode vacuole-targeted or secreted proteins with antimicrobial activities, is aided negatively by SNI1 and positively by RAD51, BRC2A and SSN2 (SUPPRESSOR OF NPR1, INDUCIBLE1; RAS ASSOCIATED WITH DIABETES 51D; BREAST CANCER 2A; SUPPRESSOR OF SNI1,2) (Song *et al.*, 2011; Wang *et al.*, 2010; Durrant *et al.*, 2007; Mosher *et al.*, 2006; Li *et al.*, 1999; Van Loon and Van Strien, 1999). SNI1, a protein exhibiting structural similarity to Armadillo repeat proteins, functions as an inhibitor of gene expression. It is likely relieved through SA-induced activities of RAD51, SSN2 and TGA7 in the presence of NPR1, acting collectively as coactivators (Moore *et al.*, 2011; Song *et al.*, 2011; Pape *et al.*, 2010).

Some components of the SA pathway are prone to modification by AtSIZ1 (A. THALIANA SAP and MIZ), the small ubiquitin-like modifier (SUMO) E3 ligase. Loss-of-function mutants of *SIZ1* exhibit high SA levels and increased resistance to pathogens pointing towards a negative role for sumoylation in this branch of immunity (Lee *et al.*, 2007). Post-translational addition of SUMO to proteins is reversible and may change abilities of modified proteins in molecular interactions (van den Burg *et al.*, 2010).

NPR1-dependent expression of *PR* genes is essential for establishing systemic acquired resistance (SAR), a state of broad range immunity towards pathogens effective in both local and distal tissues. SAR is induced after a local infection and results in local and systemic resistance towards a broad range of pathogens. Recent evidence suggests that DIR1 (DEFECTIVE IN INDUCED RESISTANCE 1), in complex with a derivative of glycerol-3-phosphate, is the long-distance defence signal responsible for SAR outside of the local infection realm (Chanda *et al.*, 2011; Maldonado *et al.*, 2002). Levels of NPR1 in the nucleus are controlled through a CULLEN3-based E3 ligase complex which tags the protein for degradation. Dynamic amounts of NPR1 are important for proper induction of genes as well as inhibition of this activity in the absence of pathogens (Spoel *et al.*, 2009).

1.6 Modifier of snc1 genes and the MOS4-associated complex

Genetic screens are a widely used approach to identify components in pathways of interest. In a standard screen, wild-type plants are mutagenized and desired mutants identified using selection criteria. Suppressor screens are a subcategory, in which the plants to be mutated are already mutants that exhibit phenotypes. Desired double mutants display suppression of mutant phenotypes and restored wild-type traits. Suppressor genes often function in the same pathway than the original mutated gene and can be identified using map-based cloning approaches (Zhang *et al.*, 2007).

Functional involvement of a group of genes in plant immunity was discovered through a genetic suppressor screen based on the gain-of-function mutant *suppressor of npr1, constitutive 1* (*snc1*) (Li *et al.*, 2001). This mutant produces an altered form of a predicted TIR-NB-LRR immunity receptor which constitutively activates otherwise tightly regulated defence mechanisms. Redistribution of resources is likely responsible for the typical dwarf phenotype displayed by *snc1* plants. However, the mutant is fertile and produces many siliques. *snc1* also does not exhibit localized cell death which is a typical defence phenotype induced after detection of pathogen invasion and aimed at containing pathogen spread (Zhang *et al.*, 2003; Li *et al.*, 2001). Several types of mutagens were used to create second site mutations in the *snc1* and *snc1 npr1* mutant background, producing a collection of suppressor double mutants with largely restored wild-type phenotypes caused by mutations in *modifier of snc1* (*mos*)genes (Monaghan *et al.*, 2010).

Several *MOS* genes encode components of nucleo-cytoplasmic transport which was established as a major mechanism in defence responses. MOS3 and MOS7 share homology with nucleoporins, the main building blocks of the nuclear pore complex and which are required for mRNA export and for protein retention/export from the nucleus, respectively (Cheng *et al.*,

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2009; Zhang and Li, 2005). MOS6/IMPORTIN ALPHA 3 is an import receptor binding to the nuclear localisation signal of a nucleus-bound protein and aiding in crossing the nuclear envelope (Palma *et al.*, 2005). MOS11 also contributes to mRNA export, potentially in the same pathway although upstream of MOS3 (Germain *et al.*, 2010; Cheng *et al.*, 2009; Zhang and Li, 2005). The importance of RNA pathways in defence is further supported through cloning of *MOS2*, which encodes a protein containing a G-patch and two KOW repeats, domains linked to m-RNA binding properties (Woloshen *et al.*, 2011; Zhang *et al.*, 2005).

A subset of *snc1* suppressors are proteins aiding in post-translational modification processes such as farnesylation, which allows anchoring of a protein to the nuclear envelope. The β-subunit of farnesyltransferase, an enzyme that attaches the hydrophobic farnesyl group to proteins, is encoded by *ERA1/MOS8* (Goritschnig *et al.*, 2008). Plant defence also includes ubiquitination mechanism. *MOS5* is allelic to *UBA1*, one of only two E1 ubiquitin activating enzymes in *Arabidopsis*. E1 enzymes initiate an ubiquitin conjugation cascade which often leads to a tagged proteins' degradation via the 26S proteasome (Goritschnig *et al.*, 2007). Participating in this pathway are also E3 ligases which are responsible for identification of proteins that should be ubiquitinated. The F-box protein CPR1 (CONSTITUTIVE EXPRESSER OF PR GENES 1) interacts with SNC1 *in vivo* and a related SKP1-CULLIN1-F-box (SCF) E3 ligase likely regulates relative levels of this and other R proteins via the ubiquitin proteasome pathway (Cheng *et al.*, 2011; Hua and Vierstra, 2010)

The exact function of MOS4 is still unclear; however, it plays an important role as a core protein in the MOS4-associated complex (MAC). MOS4 co-purifies with 24 other proteins, most of which have homologs in human and yeast and are organized in similar aggregates (Johnson *et al.*, 2011). Other MAC core proteins are AtCDC5/MAC1 (CELL DIVISION CYCLE 5), an R2-R3 Myb transcription factor, PRL1 (PLEIOTROPIC REGULATORY LOCUS 1), a WD40 protein likely providing structural support, and the redundant U-box (PUB) proteins MAC3A/MAC3B,

belonging to a class of single polypeptide E3 ligases (Monaghan *et al.*, 2009; Yee and Goring, 2009; Palma *et al.*, 2007). The human and yeast analogues of the MAC are associated with the spliceosome in their respective systems, a similar role is conceivable for the MAC. For example, the unequally redundant MAC5A/MAC5B proteins are putative RNA binding proteins (Monaghan *et al.*, 2010). Importantly, MAC5B suppresses *snc1* signalling and all MAC core proteins also display increased susceptibility towards a range of pathogens in single mutant analysis (Monaghan *et al.*, 2010; Monaghan *et al.*, 2009; Palma *et al.*, 2007).

The *snc1*-induced defence array is built up through pathways utilizing key regulatory molecules NPR1 and SA, but signals are seemingly also routed through additional branches (Li *et al.*, 2001). Double mutants of *MOS4* and *NPR1* exhibit increased vulnerability to pathogens compared to either of the single mutants, suggesting two independent affected pathways. Furthermore, expression of *PATHOGENESIS RELATED 2*, a hallmark of *NPR1* regulation, is abolished in the *snc1 mos4* mutant, further supporting *MOS4* functionality in *NPR1*-independent signalling. Since SA levels are unaffected in *atcdc5*, *mos4* and *prl1* mutants, MAC signalling might be also routed through pathways independent to that of SA (Palma *et al.*, 2007).

Characterization of several MAC components has revealed a complex picture of potential MAC functions; however immediate targets of the complex remain unknown

1.7   *Pleiotropic regulatory locus 1 (PRL1)*

PRL1 is one of the core components of the MAC and homologous to PRLG1, Prp46p and Prp5p/Cwf1p in *H. sapiens*, *S cerevisae* and *S. pombe*, respectively (Johnson *et al.*, 2011). Large parts of the PRL1 amino acid sequence are arranged in WD40 repeats and folding into a seven-blade β-propeller, first described in the G_β subunit of heterotrimeric G proteins (Xu and

Min, 2011). Through this interface, WD40 proteins participate in protein-protein, protein-peptide and protein-DNA interaction (Stirnimann *et al.*, 2010).

Our current hypothesis of MAC organization assigns an important structural role to the PRL1 protein. Plants with mutations in *PRL1* are severely susceptible to pathogens in addition to other pleiotropic effects (Palma *et al.*, 2007). First identified as a mutant exhibiting growth arrest in media containing 6% sucrose, regulatory influence of PRL1 over a growing number of sugar, light, hormone, isoprenoid and stress-responsive pathways has been revealed (Flores-Perez *et al.*, 2010; Baruah *et al.*, 2009; Li *et al.*, 2007; Abraham *et al.*, 2003; Nemeth *et al.*, 1998; Salchert *et al.*, 1998). Morphologically, *prl1* mutants exhibit phenotypes such as compromised root development, altered leaf morphology with serrated leaf margins, and an overall darker green colour, due to accumulation of isoprenoids such as chlorophyll (Flores-Perez *et al.*, 2010; Nemeth *et al.*, 1998).

PRL1 has been shown to directly bind AKIN10 and AKIN11 (*ARABIDOPSIS* SNF1 KINASE HOMOLOG 10/11) which are conserved protein kinases central to stress-, sugar- and developmental signalling (Baena-Gonzalez *et al.*, 2007; Bhalerao *et al.*, 1999). PRL1 inhibits AKIN kinase activities *in vitro*, potentially through a ubiquitination-based mechanism (Farras *et al.*, 2001; Bhalerao *et al.*, 1999). AKINs were co-purified with the α4/PAD1 (20S PROTEASOME ALPHA SUBUNIT PAD1) unit of the 26S proteasome, and both AKINs bind PRL1 with the same domain than used for interacting with the SKP1/ASK1 (*ARABIDOPSIS* SKP-LIKE 1) subunit of an SCF E3 ubiquitin ligase (Farras *et al.*, 2001). A potential link between PRL1 and the plant ubiquitin machinery was further strengthened through co-precipitation of PRL1 with CULLEN4 (CUL4), a major scaffolding subunit of DCX-type E3 ligases, *in vivo*. Mutants of *PRL1* and *CUL4* exhibit similar phenotypes (Lee *et al.*, 2008).

DCX-type E3 ligases are composed of several subunits. The CUL4 backbone interacts with the RING-finger protein RBX1 which in turn can bind an E2 protein; CUL4 also binds DDB1 (DAMAGED DNA BINDING 1) which recruits substrate receptors, bound to substrate, for ubiquitination (Biedermann and Hellmann, 2011; Vierstra, 2009). DDB1 is capable of interacting with a variety of substrate receptors through the 16aa DWD (DDB1 BINDING WD40) motif which is found in 78 proteins of rice and in 85 *Arabidopsi*s proteins, including PRL1 (Lee *et al.*, 2008). PRL1 features two DWD repeats imbedded into WD40 sequences which are needed to bind DDB1 in vivo, suggesting that PRL1 may act as a substrate receptor for DCX-type ubiquitination (Lee *et al.*, 2008)

1.8   *Research objectives*

Much progress has been made in understanding the multitude of resistance mechanisms displayed by plants. Through progressive dissection of signalling pathways, it has also become clear that defence mechanisms are complex and new questions arise just after others have been answered. The MOS4-associated complex plays a role in effector-triggered immunity as well as in PAMP-triggered defences and, although advances in the characterization of MAC members have been made, the targets of the complex and its components remain elusive. A prominent member of the MAC is the WD40 protein PRL1. Comprised largely comprised of domains associated with protein-protein interactions, PRL1 is a good candidate for interactions with other defence molecules. PRL1 function is important to plant defence since mutant *prl1* plants display severe susceptibility towards pathogens.

The research presented in this thesis describes a genetic suppressor screen aimed at identifying genes that function in *PRL1*-dependent resistance signalling. It also describes the cloning and subsequent characterization of two such genes, *PRL2* and *SOP2*. Based on

previous experience with screens carried out in our laboratory, the following objectives were established:

1) Compilation and preliminary analysis of *suppressor of prl1* (*sop*) mutants

2) Analysis of the relationships between *PRL1* and homologous gene, *PRL2*

3) Characterization of *SOP2*, a member of a novel gene family with unknown function

To address the objectives, a combination of approaches including screening methodologies, positional cloning, infection assays and PCR-based protocols were used.

1.8.1 Objective 1: Compilation and preliminary analysis of suppressor of prl1 (sop) mutants

We previously identified a number of defence signalling components by means of a similar suppressor screen based on the plant resistance gene *snc1 (Monaghan et al., 2010)*. The research described in Chapter 2*, The prl1 suppressor screen results in twenty-two mutants with defects in candidate genes*, aimed to identify *prl1* suppressor mutants. These suppressors were expected to no longer exhibit *prl1*-associated traits but morphologically resemble a wild-type plant. Through backcrosses with the original *prl1* mutant, the recessive, dominant or semi-dominant nature of the respective suppressing mutation was evaluated. To test whether the mutations affect defence, obtained morphological suppressors were subsequently subjected to a second screening step in which defence responses towards oomycete pathogen *Hyaloperonospora arabidopsidis* EMWA1 were tested.

1.8.2 Objective 2: Analysis of the relationships between PRL1 and homologous gene, PRL2

Using map-based cloning methods and sequencing, we identified a regulatory gain-of-function allele of *PLEIOTROPIC REGULATORY LOCUS 2 (PRL2)* as dominant suppressor of *prl1* phenotypes. Based on high sequence similarity, this gene had been previously documented as a homolog of *PRL1*, however, due to very low expression levels, has not been studied yet (Nemeth *et al.*, 1998). Due to the regulatory nature of the discovered mutation in the *prl2-1D* mutant, we were in a unique position to analyse the relationship between the two related proteins and test whether they carry out similar functions. In a comprehensive approach, we examined defence abilities directed against both virulent and avirulent pathogens as well as developmental characteristics such as root length and flowering time. This work is described in Chapter 3, A gain-of-function mutation in *PLEIOTROPIC REGULATORY LOCUS 2* reveals unequal redundancy and functional equivalence between the WD40 protein and close homolog *PRL1*.

1.8.3 Objective 3: Characterization of SOP2, member of a novel gene family with unknown function

sop2-1D is the second dominant mutant derived from our screen, exhibiting fully restored wild-type appearance and resistance. To identify the responsible mutation, we followed a positional cloning approach with subsequent sequencing of candidate genes. Intriguingly, we determined that an unknown gene functions in *PRL1* signalling. This finding led us to ask a number of questions: What causes the dominant nature of the mutation? Do homologs of *SOP2* exist in *Arabidopsis*, in other plants, in other kingdoms? What is the subcellular localization of the encoded protein? Is SOP2 required for plant resistance and what can we learn about its function? And also, is SOP2 a target of the MAC? In Chapter 4, *A dominant mutation in an*

uncharacterized gene identifies a component of PRL1 signalling specific to the plant kingdom,
the cloning and characterization of *SOP2* is described. In this process, we used methods
including PCR-based techniques, infection assays with pathogens and a number of publicly
available computer algorithms.
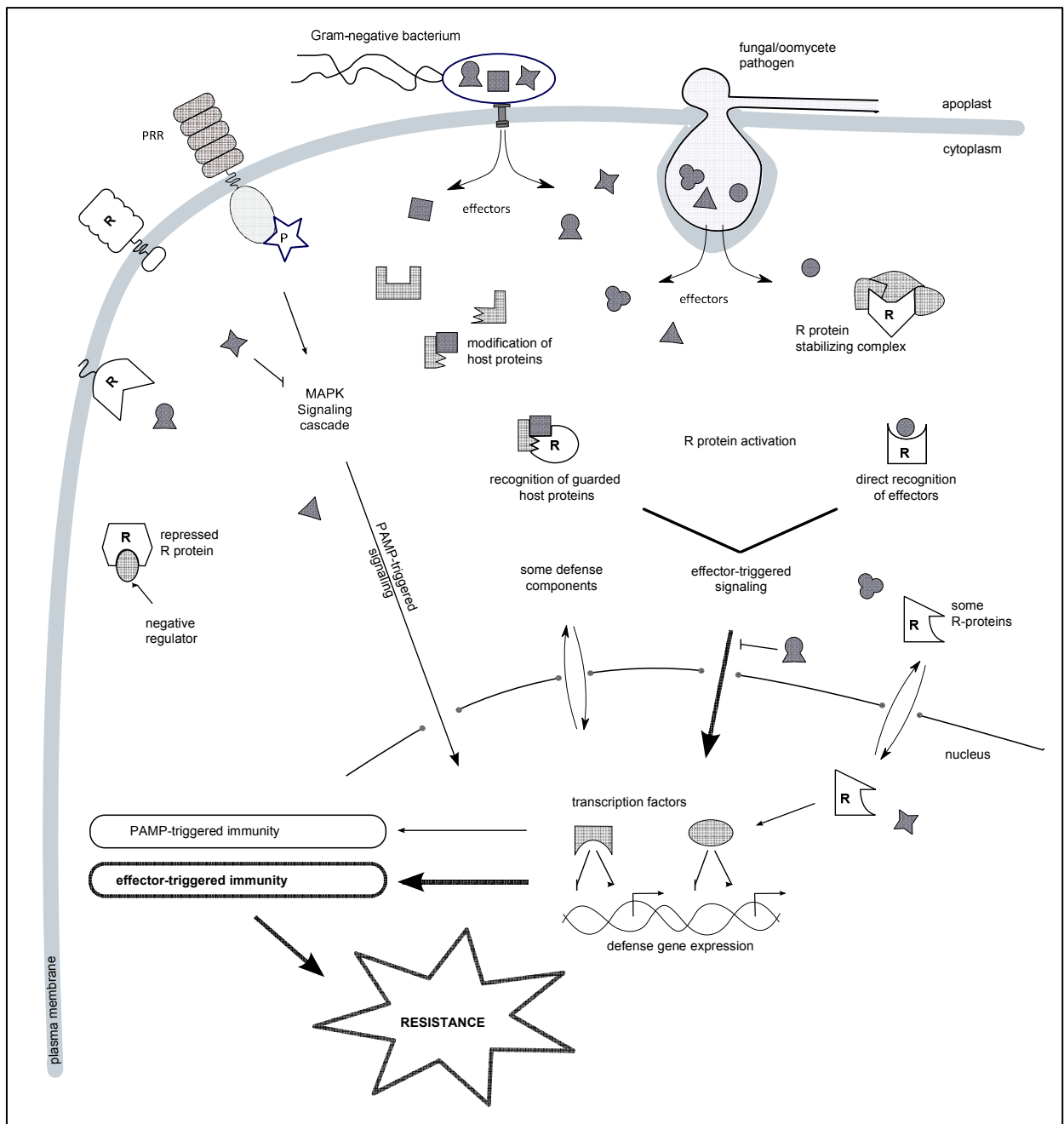
***Figure 1*. Signalling events involved in plant innate immunity**
Plants have evolved the ability to perceive highly conserved pathogen-associated molecular patterns (PAMPs) via transmembrane pattern recognition receptors (PRRs). PRR activation triggers mitogen-activated protein kinase (MAPK) signaling cascades that induce defense gene expression and hinder the growth of some microbial populations. During infection, pathogenic microbes deliver effector proteins into host cells, where they function to suppress or interfere with PAMP-triggered immunity and other defense responses. In resistant plants, cytoplasmic and membrane-associated resistance (R) proteins recognize effectors either directly or indirectly through the surveillance of guarded plant proteins and trigger effector-triggered immunity. Activated R proteins result in genetic reprogramming and pronounced physiological changes in the infected plant cell that ultimately result in resistance. Adapted from Monaghan *et al*. 2009.

2 A prl1 suppressor screen yielded twenty-two mutants with defects in candidate genes

2.1 Introduction

Plant resistance is conferred by preformed barriers and components, broad range detection of microbes and pathogen-specific defence mechanisms (Jones and Dangl, 2006; Heath, 2000; Osbourn, 1996). If pathogens overcome these layers of protection, ensuing disease symptoms will affect crop yield and horticultural success. To reduce or prevent economic losses, a comprehensive understanding of natural plant defence mechanisms is needed. However, efforts are impeded by the complex genomes of important cultivars such as broccoli, rape seed and cauliflower, (members of the *Brassicaceae* family). Research focussing on the related, but more simply organized model plant *Arabidopsis thaliana* has significantly advanced our knowledge of plant resistance mechanisms, not least due to the feasibility of mutant screens in this plant (Malinovsky *et al.*, 2010; Zhang and Li, 2005; Glazebrook *et al.*, 1996; Glazebrook and Ausubel, 1994).

In forward genetic screens, radiation or chemicals such as ethyl methanesulfonate (EMS) are often used as mutagens and resulting mutants with defects in defence responses can be identified. In *Arabidopsis* and other plants, insertional mutagenesis methods mediated by *Agrobacterium tumefaciens* are also an option. In a suppressor screen, mutants are mutagenized a second time and screened for desired double mutants exhibiting reverted wild-type attributes, often due to an additional mutation in the same signalling pathway. In our laboratory, a number of previously unknown resistance components have been identified as suppressors of s*nc1*, a mutant allele of a predicted resistance gene (Germain *et al.*, 2010; Monaghan *et al.*, 2010; Zhang *et al.*, 2003). An in depth analysis of *MOS4 (MODIFIER OR*

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SNC1,4) revealed the existence of a MOS4-associated complex (MAC), playing a role in plant defence (Johnson *et al.*, 2011; Monaghan *et al.*, 2009; Palma *et al.*, 2007) The MAC displays attributes of a nodal point, channeling signals initiated by both major types of R proteins and routing through pathways both dependent and independent of the marker molecules salicylic acid (SA) and NPR1 (NONEXPRESSOR OF PATHOGENESIS RELATED PROTEINS1) (Palma *et al.*, 2007). Signalling steps controlled by the MAC will be best understood through the identification of targets, which remain elusive to this point.

Counting as one of the five core members of the MAC, the WD40 repeat protein PRL1 (PLEIOTROPIC REGULATORY LOCUS 1) belongs to a family with prominent protein-protein interactions domains (Stirnimann *et al.*, 2010; Nemeth *et al.*, 1998). Importantly, mutations in *PRL1* result in substantially compromised plant resistance (Palma *et al.*, 2007). Both characteristics make *PRL1* a promising candidate for a suppressor screen with the goal of finding downstream components of MAC-related defence signalling. Using EMS as a mutagen, we induced random second site point mutations in T-DNA mutants of *prl1* (*prl1-2*) and screened progeny for morphological as well as defence-related phenotypes. The goal was to identify novel elements in *PRL1* mediated signal relay by cloning of obtained *suppressors of prl1 (sop)* mutations.

2.2   *Material and methods*

2.2.1 *Plant material and growth*

Wild-type *Arabidopsis* ecotypes Columbia (Col-0), Landsberg *erecta* (Ler) and derived mutants were usually grown on soil in a 16h light / 8h dark regime. The T-DNA mutants *prl1-2* (Salk_008466) and *prl1-3* (Salk_039427) were obtained from the *Arabidopsis* Biological Research Centre and genotyped by PCR using insertion flanking oligonucleotides PRL1-Salk-

NF (5'-GATGAAAGTTGCGTTTGGAG-'3) and PRL1-NR-A (5'-ACTACCTACACTACCTAGAGC-'3).

2.2.2 Agrobacterium tumefaciens mediated mutagenesis

Agrobacterium tumefaciens mediated T-DNA mutagenesis was carried out on approximately 128,000 *prl1-2* and *prl1-3* mutant plants. M1 and M2 progeny from transformed plants was treated with the herbicide BASTA® to select for successful genomic integration of the T-DNA, encoding the relevant resistance gene.

2.2.3 EMS mutagenesis and primary morphological screen

Approximately 25,000 *prl1-2* seeds (0.5g) were subject to EMS treatment (The *Arabidopsis* Information Resource (TAIR),
[http://www.arabidopsis.org/comguide/chap_1_plants/6_EMS_mutagenesis.html]). Resulting M1 plants were allowed to self and M2 seeds harvested into 79 pools each containing seeds of 20-25 plants. Approximately 500 seeds per M2 pool were either screened on soil or alternatively on MS plates containing 6% sucrose. *prl1* mutant plants germinate late and display characteristic serrated and dark green leaf morphology.Tthese symptoms intensify in high sugar conditions finally resulting in growth arrest (Nemeth *et al.*, 1998) .

During the primary screen based on suppression of described phenotypes, between one and eleven candidates from 29 M2 seed pools, totalling 86 candidate plants, were transplanted. Using the primers PRL1-Salk-NF and PRL1-NR-A, homozygous *prl1-2* background was confirmed and contaminants discarded. Morphological verification of M3 and M4 plants excluded some lines with low levels of suppression. Forty-nine putative mutants from 22 pools were obtained and a representative for each pool chosen. The selected mutants were named

according to their respective pools P1 through P71, followed by the nomination "A" for plate grown, "B" for soil grown and a number.

2.2.4 Secondary resistance screen

Hyaloperonospora arabidopsidis EMWA1 (formerly *Peronospora parasitica* and *Hyaloperonospora parasitica*) is a biotrophic oomycete pathogen which causes downy mildew on *Arabidopsis* (Holub, 2008; Slusarenko and Schlaich, 2003). It is an obligate pathogen which is propagated on the susceptible Ws ecotype usually completing a life cycle in one week at 16°C and high humidity (Li *et al.*, 2001). For the secondary screen based on disease resistance traits, two-week old soil-grown progeny of confirmed *sop* mutants were infected with a conidiospore solution of *H.a.* EMWA1 (1 x 10^6 spores/ml) and disease ratings (n=3) were assessed 7 days post-infection (DPI). Using a five category ranking system, resistant Col-0 is scored as "1", highly susceptible Ws as "5" and *prl1-2* as "4". Suppressor mutants were classified as resistant with a ranking of "1-2" and classified as susceptible when scored "3" or higher.

2.2.5 Assessment of genetic inheritance

To assess the genetic nature of *sop* mutations, backcrosses between the *sop prl1-2* and homozygous *prl1-2* single mutants were carried. In such a backcross, the *prl1-2* mutation is fixed and only the suppressing locus is segregating. Homozygous recessive *sop* mutations resulted in exclusive *prl1* like progeny, whereas homozygous dominant *sops* gave only wild-type like backcross progeny. Phenotypically intermediate progeny indicated semi-dominant (incomplete dominant) inheritance of the respective *sop* mutation.

2.3 Results

2.3.1 Complete and partial suppression of prl1 phenotypes by sop mutations

The recessive T-DNA mutant *prl1-2* displays a number of visible phenotypes including serrated leaf margins, short roots and growth arrest under high sugar conditions (Nemeth *et al.*, 1998). We screened for *suppressor or prl1* (*sop*) mutants with restored wild-type morphology among M1 and M2 progeny of the T-DNA screen and in the M2 generation of the EMS screen. We identified 22 *sops* from EMS M2 pools and confirmed suppression through examination of progeny in following generations whereas our T-DNA approach was not successful. Among the mutants that were obtained, five showed complete suppression of the assayed *prl1*-associated phenotypes (*prl1-2 sop1*, *prl1-2 sop2*, *prl1-2 sop8*, *prl1-2 sop13* and *prl1-2 sop15*); seventeen additional mutants exhibited partial suppression (Tab. 1 and Fig. 2).

2.3.2 Restored R protein mediated resistance in most prl1-2 sop mutants

Resistance protein mediated signalling is impaired in single mutants of *prl1-2*, resulting in successful growth of the avirulent oomycete *Hyaloperonospora arabidopsidis* (*H.a.*) EMWA1 compared to resistant Col-0 wild-type plants (Palma *et al.*, 2007). When *prl1-2 sop* suppressor mutants were challenged with a high dose (150,000 spores/ml) of *H.a.* EMWA1, 19 mutants displayed restored wild-type resistance. The remaining three mutants (*prl1-2 sop9*, *prl1-2 sop16* and *prl1-2 sop17*) allowed pathogen colonization at a level that was similar to *prl1-2* single mutants (Tab. 1).

2.3.3 Suppressor phenotypes are caused by dominant, semi-dominant and recessive mutations

Backcrossing *prl1-2 sop* mutants to the *prl1-2* parent according to standard Mendelian tests allowed us to determine the inheritance pattern of the *sop* alleles. Exclusively *prl1*-like progeny in the F1 generation of such a cross would provide evidence for a recessive *sop* allele that is present for both copies of the gene (homozygous). Recessive patterns were obtained for nine suppressors (Table 1). In contrast, progeny consisting of nine *prl1*- like and twelve wild-type plants were obtained after back-crossing a phenotypically wild-type *prl1-2 sop2* individual. This ratio suggests dominant inheritance of *sop2* and heterozygous gene configuration at the *sop2* locus in the tested specimen (expected 1:1, $\chi^2 = 0.42$, P = 0.51). Dominant inheritance was also established for *sop1* since only wild-type progeny (26 plants) resulted from the backcross. *sop1* and *sop2* are different genetic loci and located on chromosome three and two, respectively, as was determined in preliminary mapping analysis. For suppressors *sop15, sop 17* and *sop10,* a semi-dominant (incomplete dominant) relationship is most likely because the backcross progeny exhibited phenotypes intermediate to both parents (Table 1). Several *sop* lines were investigated further in our collaborating laboratory at the National Institute for Biological Sciences (NIBS, P.R.China), where approximate numbers of *prl1*-like progeny in the F2 of mapping crosses were determined. The observed F2 segregation ratios support inheritance patterns obtained through backcrossing in most cases; i.e., for recessive mutants approximately 1/16 of F2 progeny should be *prl1*-like. Contradicting results were obtained for *sop11* and *sop20,* which have been listed as non-classified mutants (Tab. 1).

2.4 Discussion

Using EMS as mutagen, we succeeded in producing more than twenty *prl1* suppressors harbouring mutations in candidate genes relevant to the *PRL1* signalling branch. Analysis of

prl1-typical attributes such as pointy, serrated leaves and dark green colour allowed us to efficiently process M2 pools since morphological suppressors were clearly visible due to fully or partially restored wild-type phenotypes. The isolated mutants were designated *suppressor of prl1* (*sop*).

As previously noted, mutations in *PRL1* impact transformation efficiencies (Nemeth *et al.*, 1998). Mutagenesis mediated by *A. tumefaciens* is based on a transformation protocol and thus our lack of success in producing suppressors with insertional mutations can at least be partially explained through low transformation efficiency. Odds of a successful insertion were likely further reduced through additional biotic stress caused by greenhouse pathogens. The chemical agent EMS, which is used on *Arabidopsis* seeds thus represents the better choice in mutagen when dealing with stress and disease-sensitive mutants. Observations made during the T-DNA screen resulted in a modified transformation protocol, which was successfully employed for cloning of *sop2* (see Chapter 4 and Appendix 1).

After exposure to an established *Arabidopsis* pathogen, most *sop* mutants displayed restored R protein mediated resistance, independent of the level of morphological suppression. Whereas limits in quantification of resistance cannot be excluded, these results might suggest that *PRL1*-related morphology and resistance are not fully linked. In a reverse example, *sop17*, which is highly susceptible to pathogen *H.a. EMWA1*, shows largely restored wild-type morphology. Next to an important role in plant defence, PRL1 has been reported to function as a regulator of sugar, hormone, light and O_2 responsive genes (Flores-Perez *et al.*, 2010; Baruah *et al.*, 2009; Bhalerao *et al.*, 1999; Nemeth *et al.*, 1998; Salchert *et al.*, 1998). It is tempting to hypothesize that *sop17* and partial suppressors such as *sop10* or *sop12* might signal in parallel or through shared components relative to defence signalling.

The *prl1* suppressor screen resulted in 13 recessive mutants, more than 70% of all classified suppressors. Screens usually result in a majority of recessive mutants since most genes in *Arabidopsis* are haplosufficient, able to sustain wild-type function even if one allele is defective. Since EMS induces point mutations, functionality of a protein can be affected on a quantitative scale which manifests as a partial or fully suppressed phenotype in a homozygous suppressor mutant plant. If a member of a partially redundant gene family is affected, the effect could be similar since the remaining family genes still provide most of the functionality needed. A different type of dosage effect is often responsible for semi-dominant mutations, which make up 16% of obtained mutants. A portion of *Arabidopsis* genes are haplo-insufficient, referring to the need for product produced by both gene alleles to confer normal function. If impairment of one allele reduces product levels below a critical threshold, a mutant phenotype is visible which is intensified if both copies are affected. In essence, these are dominant loss-of-function mutations.

The smallest number of *sop* mutants fall into the dominant class. Working with non-recessive mutants is challenging since genetic analysis, complementation tests and pathway classification through epistasis are more time consuming and often difficult. However, the study of dominant mutations can lead to unique insights into biological proceedings and both *sop1* and *sop2* have been studied in detail in Chapters 3 and 4.

All suppressors were isolated from separate screening pools and since both dominant mutations have already been mapped to different areas of the genome, other independent and potentially novel genetic loci may be found among the yet uncharacterized *prl1* suppressors. Similar looking *sop* mutants such as *sop5* and *sop19* or *sop4* and *sop10* might harbour mutations in the same respective gene, however, comprehensive complementation tests are required to determine the actual number of affected loci among the suppressor mutants.

Table 1. Analysis of *sop* mutants

Screening name[a]	22 *sop*s	Morphological suppression[b]	Backross[c] (N⁰ of plants)	Nº in F2 of mapping cross (*prl1*-like)[d]	*H.a.* EMWA1 resistant[e]	Chr.
			Dominant mutants obtained			
P2-A2	*sop1*	complete	WT (26)	n.d.	Yes	3
P20-A4	*sop2*	complete	prl1 (9), WT (12)	n.d.	Yes	2
			Semi-dominant mutants obtained			
P33-A1	*sop15*	complete	neither (3)	1/16	Yes	
P38-A1	*sop17*	complete	neither (49)	n.d.	No	
P48-A1	*sop19*	partial	neither (10)	n.d.	Yes	
			Recessive mutants obtained			
P6-A1	*sop3*	partial	neither (24)	<10/400	Yes	
P12-B2	*sop5*	partial	n.d.	~20/500	Yes	
P13-B2	*sop6*	partial	n.d.	~40/400	Yes	
P16-A1	*sop7*	partial	prl1 (26)	<10/400	Yes	
P18-A7	*sop8*	complete	n.d.	~10/200	Yes	
P21-A1	*sop9*	partial	prl1 (21)	n.d.	No	
P23-A1	*sop10*	partial	prl1 (2)	n.d.	Yes	
P27-B1	*sop12*	partial	prl1 (32)	n.d.	Yes	
P28-B1	*sop13*	partial	n.d.	~10/300	Yes	
P31-B2	*sop14*	partial	prl1 (17)	~40/400	Yes	
P34-B1	*sop16*	partial	prl1 (45)	~40/400	No	
P67-A1	*sop21*	partial	prl1 (6)	n.d.	Yes	
P71-A1	*sop22*	partial	prl1 (6)	~10/400	Yes	
			Non-classified mutants			
P7-B1	*sop4*	partial	n.d.	n.d.	Yes	
P25-A1	*sop11*	partial	prl1(26)	25%	Yes	
P43-A1	*sop18*	partial	n.d.	n.d.	Yes	
P61-A1	*sop20*	partial	neither (27)	~10/400	Yes	
prl1-2					No	
WT					Yes	

[a] Identification name in original screen
[b] Morphological suppression levels varied significantly. A mutant was declared a complete suppressor if WT-like colour, root length and leaf morphology was exhibited.
[c] Number of progeny and their phenotypes obtained through backcrossing of respective *prl1-2 sop* mutants with *prl1-2* single mutant
[d] Approximate number of *prl1*-like progeny obtained in F2 of mapping population
[e] Suppression of *prl1*- related disease susceptibility was quantified using avirulent pathogen *H.a.* EMWA1, see Materials and Methods

WT, wild-type   n.d., not determined
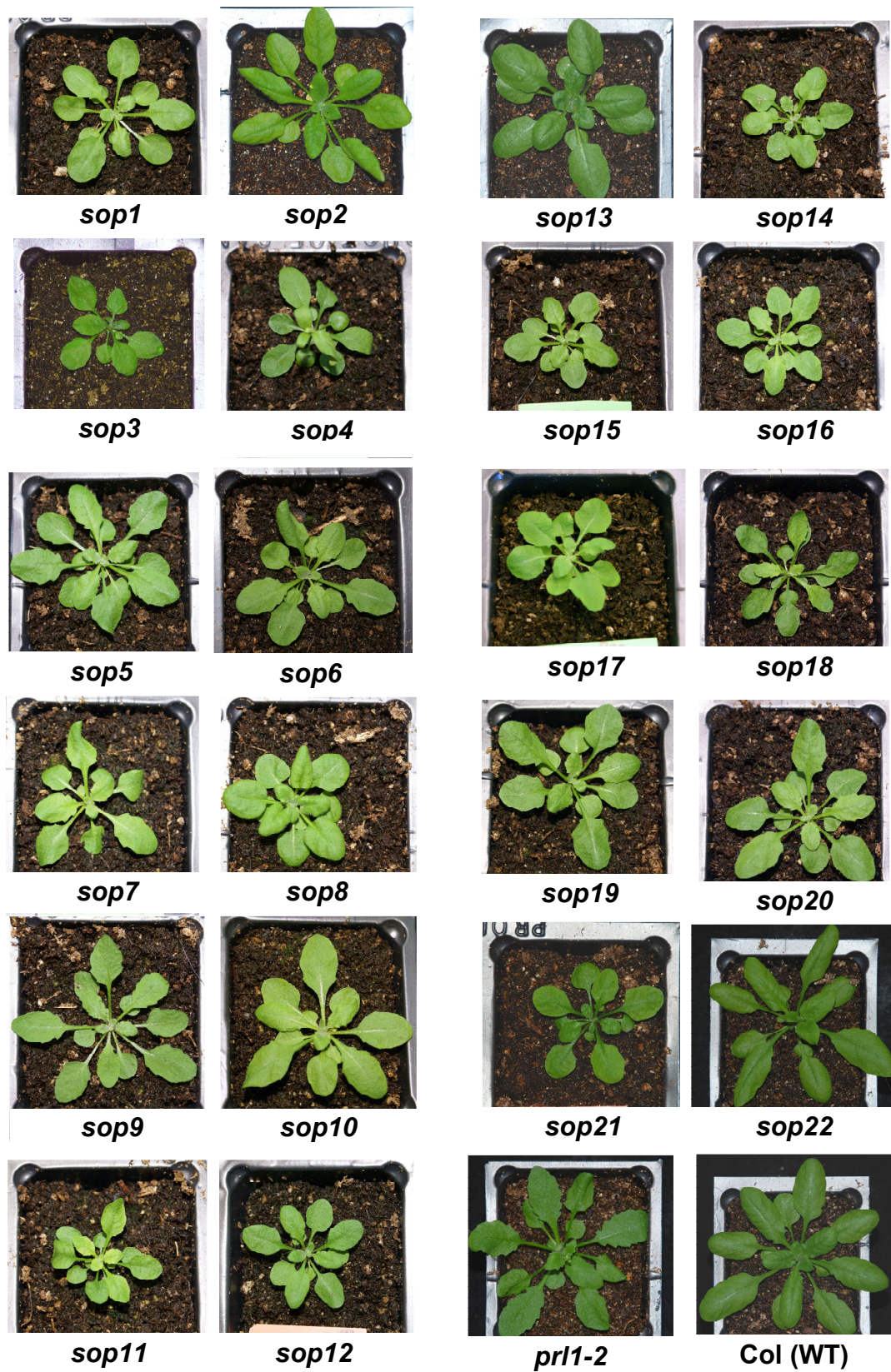
Table 2. *sop* mutants exhibit complete and partial suppression of *prl1* phenotypes

Most images were taken when plants were four weeks old. Images of *sop2, sop3, sop13, sop21, sop22,* Col-0 and *prl1-2* show five-week-old plants.

3   A gain-of-function mutation in *PLEIOTROPIC REGULATORY LOCUS 2* reveals unequal redundancy and functional equivalence between the WD40 protein and the close homolog PRL1

3.1   Introduction

To colonize a plant host, pathogens need to overcome an array of defence mechanisms (Jones and Dangl, 2006). The presence of microbes at the plant surface is detected by pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMP) and a general resistance reponse is induced. Pathogenic microbes are able to evade or suppress PAMP-triggered immunity (PTI) by delivering effector molecules into the host cell which interfere with PTI signalling. Activity of such pathogen-derived effector molecules is detected by the second layer of plant defence, a surveillance system comprised of resistance (R) protein complexes which, upon induction, signal to establish local and systemic resistance (ETI) (van der Hoorn and Kamoun, 2008).

Most R-proteins use NDR1 (NONRACE-SPECIFIC DISEASE RESISTANCE) (Knepper *et al.*, 2011; Century *et al.*, 1995) or the EDS1/PAD4 node (ENHANCED DISEASE SUSCEPTIBILITY1/PHYTOALEXIN DEFICIENT4) (Wiermer *et al.*, 2005; Feys *et al.*, 2001) early in signalling and before the onset of salicylic acid (SA) production which is mediated by SID2 and EDS5 (SA INDUCTION DEFICIENT2, ENHANCED DISEASE SUSCEPTIBILITY5)(Nawrath *et al.*, 2002; Wildermuth *et al.*, 2001). One pathway downstream of SA accumulation requires the regulatory protein NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1) to induce defence gene expression through interaction with TGA transcription factors (Zhang *et al.*, 2003; Cao *et al.*, 1994). At least two other

pathways are used by R proteins which either do not require NPR1 (Bowling *et al.*, 1997) or bypass both SA production and the later involvement of NPR1 (Shah *et al.*, 2001). Virulent pathogens evade specific detection and can colonize the plant, yet their presence is still perceived causing a weaker and delayed (basal) response overlapping in parts with R protein mediated signalling (Qi *et al.*, 2011; Zhang and Li, 2005; Navarro *et al.*, 2004)

Substantial involvement of the nucleo-cytoplasmic transport machinery in plant resistance was unveiled through characterization of *mos* (*modifier of snc1*) mutants which had been obtained through a suppressor screen based on *snc1*, a constitutively signalling predicted resistance gene, (Zhang *et al.*, 2003). MOS3 and MOS7 are nuclear pore components, MOS6 and MOS11 are facilitators of protein import and mRNA export respectively (reviewed in (Germain *et al.*, 2010; Monaghan *et al.*, 2010)). Comprehensive analysis of the recessive *mos4* mutation revealed an important role in plant signalling for the evolutionary conserved MOS4-associated complex (MAC) which consists of approximately 20 proteins (Johnson *et al.*, 2011; Monaghan *et al.*, 2009; Palma *et al.*, 2007). Homologous complexes in yeast and humans function in pre-mRNA splicing (Ajuh *et al.*, 2000; Cheng *et al.*, 1993; Tarn *et al.*, 1993).

The MAC functions in signal relay mediated by both major classes of resistanc proteins. Interestingly, the less understood SA - and NPR1 independent signal routes are also used in the relay of defence information downstream of the complex. In single mutant analysis, all three firstly described MAC components display impaired PTI and ETI however defects in *PLEIOTROPIC REGULATORY LOCUS 1 (PRL1/MAC2)* reduce plant resistance levels subtantially more than do mutations in *MOS4* or *CELL DIVISION CYCLE 5 (AtCDC5/MAC1)* (Palma *et al.*, 2007; Nemeth *et al.*, 1998)*.* PRL1 has been implicated in the modulation of sugar-mediated, hormone and stress regulated responses (Nemeth *et al.*, 1998; Salchert *et al.*, 1998). In the MAC, this WD40 protein is hypthesized to provide structural support consistant with abilities of this domain family to facilitate transient or stable interactions between proteins

(Stirnimann *et al.*, 2010). To better understand PRL1 function in plant biology, we conducted a suppressor screen in the *prl1* mutant background. Characterization of the dominant *sop1* (*suppressor of prl1, 1*) mutant revealed functional redundancy between the versatile PRL1 and its homolog *PRL2.*

3.2   Materials and methods

3.2.1 Plant material and growth

Wild-type *Arabidopsis* ecotypes Columbia (Col-0), Landsberg erecta (Ler) , Wassilewskija (Ws) and derived mutants were grown on soil in a 16h light / 8h dark regime. T-DNA mutants *prl1-2* (Salk_008466), *prl2-2* (Salk_075970), *prl2-3* (Salk_133878) were obtained from the *Arabidopsis* Biological Research Centre and genotyped by PCR using insertion flanking oligonucleotides PRL1-Salk-NF (5'-GATGAAAGTTGCGTTTGGAG-'3) and PRL1-NR-A (5'-ACTACCTACACTACCTAGAGC-'3) for *prl1-2* and PRL2-Salk-F (5'-TCTGAACCGACGCTTAATGAG-'3) and PRL2-Salk-R (5'-TGTAGGCTTACTTGCAGGTTC-'3) for *prl2-2* and *prl2-3*.

3.2.2 Morphological characterization

For root assays, seeds were vernalized for three days at 4ºC and grown for seven days on Murashige and Skoog (MS) medium. Square plates were kept upright in a growth rack and exposed to 16h light and 8h dark per day. Root lengths of 10 individual plants per genotype were scored using a small ruler. To evaluate flowering time, seeds were vernalized for at least three days and plants grown under long day conditions (16h dark/ 8h light). Plants were observed daily and onset of flowering was established when an emerging flower measured 1cm in height. Rosette leaves present at the time of bolting were counted for 10 plants per genotype.

3.2.3 Infection assays

Pseudomonas syringae pathovar *maculicola (P.s.m.)* ES4326 and pathovar *tomato (P.s.t.)* DC3000 are hemi-biotrophic bacterial pathogens responsible for leaf spot and bacterial speck disease on *Arabidopsis*, respectively. The bacteria were grown at 28-30ºC in liquid LB or LB plates containing 50 mg/ml Streptomycin (*P.s.m.* ES4326) or 25 mg/ml Rifampicin and 50 mg/ml Kanamycin (*P.s.t.* avrPphB and avrRps4). *Arabidopsis* plants are inoculated using a small needless plastic syringe with the opening lightly pressed against an *Arabidopsis* leave underside allowing injection of bacteria into the plant apoplast. A low dose (OD_{600}= 0.0001), referred to as enhanced disease susceptibility (EDS) dose, was used in virulent (*P.s.m.* 4326) infections. A higher dose (OD_{600}= 0.002) was employed with avirulent *P.s.t. avrPphB* and *avrRps4*. On the first day and three days after inoculation, leaf discs of $0.32cm^2$ were cut with a standard paper hole-punch, samples were homogenized in 10 mM $MgCl_2$ and a series of six dilutions was plated. Bacterial colony forming units (cfu) were calculated after two days of incubation at 28ºC.

For *H.a.* EMWA1 infection assays, 2.5 week old plants were spray-inoculated with a low dose (50,000 spores/ml) of conidiospores to determine resistance defects in a compatible interaction, a high dose (100,000 spores/ml) was used to evaluate incompatible interactions. After 7-10 days, plants were harvested in a 50 ml Falcon tube containing up to 5 ml water, conidiospores were released using a vortex for 10 seconds and quantified using a hemocytometer.

3.2.4 Positional cloning

Molecular markers for map-based cloning were PCR based and detected either length (InDel) or single nucleotide polymorphisms (SNP) between Col-0 and Ler ecotypes (Monsanto *Arabidopsis* Ler sequence available at TAIR:

[http://www.arabidopsis.org/browse/Cereon/index.jsp]). When employing InDel markers, one set of primers is used for both ecotypes and amplified fragments are visualized on 1-2% agarose gels. For SNP markers, two forward primers differing in the last two nucleotides were designed allowing ecotype specific binding as well as pairing with a common reverse primer (Bui and Liu, 2009). The two primer combinations are used in alternating reactions on genotypes and fragments are analysed on 1% agarose gels. For primer sequences, see Appendix 2.

3.2.5 *Quantification of PRL2 mRNA levels*

Seeds of the genotypes *prl1-2*, *prl1-2 prl2-1d*, *prl2-1d*, *prl2-2* and Col-0 wild-type were vernalized for 7 days and plated on 0.5 MS containing 100 mg/ml Ampicillin. The plates were incubated in a growth chamber for ten days using a 16h light, 8h dark regime. Tissue of 10-day-old seedlings was collected in a 2ml reaction tube containing two glass beads and frozen immediately. RNA was extracted using the Totally RNA kit (Ambion) and Reverse Transcriptase (SuperScript II, Invitrogen) was used to produce c-DNA copies of the transcriptome Relative amounts of *PRL2* cDNA (PRL2-RT-F: 5'-CGTAATGGTCACTGGAGGTG-'3, PRL2-RT-R: 5'-TTTTTCTGGCTTCGAGTTTGA-'3) and *Tubulin* (control) c-DNA (5′-ACGTATCGATGTCTATTTCAACG-3′ and 5′-ATATCGTAGAGAGCCTCATTGTCC-3′) present in the collected tissues were quantified using real-time PCR.

3.2.6 *Single and double mutant construction*

For creation of the *sop1-1d/prl2-1d* single mutant, homozygous *prl1-2 sop1-1d* mutants were crossed to Col-0 wild-type plants and the single was identified in the F2 generation using allele specific primers P2A2-M-F2 (5'-GTCGGATAAAATCCTATTTGT-3') and P2A2-WT-R (5'-GCGAAACTGTTGATTAACCT-3'). To obtain the *prl1-2 prl2-2* double mutant, homozygous *prl1-1* and *prl2-2* plants were crossed and double mutants were confirmed in the F2 using insertion

flanking primers PRL1-Salk-NF and PRL1-NR-A for *prl1-1* and PRL2-Salk-F and PRL2-Salk-R

for *prl2-2*. The *prl1-2 prl2-2* mutant was previously isolated by Dr. Palma in our laboratory.

3.2.7 Transformation of JAtY clones

The genomic JAtY library was created at the John Innes Centre (UK) and is based on the

pYLTAC17 vector allowing for the selection of transformed plants through BASTA selection.

Populations of flowering *prl1-2* and *prl1-12 sop1-1d* plants were subject to *Agrobacterium*

tumefaciens-mediated transformation and seeds collected after six weeks. T1 progeny was

sprayed 3-5 times with BASTA® in the first two weeks after germination and transformants

identified after three weeks.

3.3   Results

3.3.1 sop1-1D fully suppresses prl1-associated phenotypes

Morphological traits associated with the T-DNA mutant of *PRL1* (*prl1-2*) such as serrated leaf

margins, pointy shaped first leaves and darker leaf colour, are no longer exhibited by the *prl1-2*

sop1-1D suppressor mutant which instead resembles a wild-type plant (Fig. 2A). When

comparing average root lengths of Col-0 (WT), *prl1-2* and *prl1-2 sop1-1D* plants, double

mutants grow roots of wild-type length instead of short roots typical for *prl1* mutants (Fig. 2B)

(Nemeth *et al.*, 1998). *sop1-1D* also suppresses early flowering in the double mutant since *prl1-*

2 sop1-1D mutants initiate flowering with twice as many rosette leaves present than *prl1-2*

single mutants (Fig. 2C). Dominant suppression of *prl1* phenotypes by *sop1-1D* was

demonstrated by backcrossing of a pure breeding *prl1-2 sop1-1D* line to the *prl1-2* parent which

resulted in 26 F1 plants with wild-type morphology (Tab.1)*.*

For evaluation of defence responses in *Arabidopsis* plants, we employ both virulent and avirulent pathogens in our laboratory, with the former successfully colonizing the plant and raising only insufficient defence reactions whereas the latter is recognized by specialized immunity receptors and prevented from causing further harm. Using virulent pathogen *Pseudomonas syringae* pathovar *maculicola* (*P.s.m*) ES4326 and avirulent *Hyaloperonospora arabidopsidis* (*H.a.*) EMWA1, substantial differences both in basal defence and R protein mediated resistance between the *prl1-2 sop1-1D* double mutant and the original *prl1-2* single mutant were observed. The characteristic enhanced disease susceptibility (EDS) in basal defence, usually displayed by *prl1-2* plants when exposed to the virulent bacterial pathogen, cannot be detected in the double mutant (Fig. 2D) (Palma *et al.*, 2007). Similarly, effective resistance signalling mediated by the R protein RESISTANCE TO PERONOSPORA PARASITICA 4 (RPP4) ensures resistance towards oomycete *H.a.* EMWA1 in both Col-0 and *prl1-2 sop1-1D* plants, thereby overcoming the compromised response of the *prl1* background mutation (Fig. 2E). Morphological assessments and pathogen infection assays thus identify *sop1-1D* as a full suppressor of *prl1-* related phenotypes.

3.3.2 Map-based cloning of sop1-1D identifies a molecular lesion in PLEIOTROPIC REGULATORY LOCUS 2 (PRL2)

The *sop1-1D prl1-2* double mutant (Col-0 background) was crossed with the L*er* ecotype to create segregating progeny, a requirement for map-based cloning techniques. In the F2 generation of the mapping cross, analysis of 48 plants with *prl1* mutant morphology identified the approximate location of *sop1-1D* between Insertion/Deletion (InDel) markers MIE1 (4.87Mb) and MRC8 (6.21Mb) on the top arm of chromosome 3 (Fig. 3A). Progeny of *prl1-2* homozygous F2 plants with heterozygosity for the *sop1-1D* locus was used in further mapping steps. In total, 1008 F3 plants were examined and 61 recombinants for above markers of either *prl1-2* or wild-type morphology identified. For recombinants, allele configuration at the *sop1-1d* locus was

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confirmed using morphological segregation patterns in the following (F4) generation. Using the selected recombinants, the *sop1-1D* locus was further mapped to a region flanked by the InDel markers MDC8 (5.58Mb) and K14A17 (5.84Mb) and was finally included in a 62kb section on Chromosome 3 between InDel marker MGL6 and single nucleotide polymorphism (SNP) marker MGL6-SNP5, with two recombinants for each marker remaining. The genomic area between 5.63Mb (MGL6) and 5.69Mb (MGL6-SNP5) on the top arm of Chromosome 3 harbours 20 genes (Fig. 3A (Huala *et al.*, 2001)), among them *At3g16650* encoded PLEIOTROPIC REGULATORY LOCUS 2 (PRL2) which is homologous to described WD40 family protein PRL1 (Nemeth *et al.*, 1998). Through sequencing of the *At3g16650* open reading frame in the *prl1-2 sop1-1D* mutant, a C to T substitution was identified in the first exon of the *PRL2* gene, 58bp upstream of the translational start codon (Fig. 3B).

During mapping of *sop1-1D*, we encountered marker patterns indicative of an area with low recombination frequency on chromosome I, co-seggregating with *prl1* mutant phenotypes and correlating with Col-0 morphology. Initially, this led us to hypothesize an ecotype specific suppressor gene in Ler plants. Such specificity is not unknown; for example, the *Arabidopsis* ecotype Ws is insensitive to flagellin presence since it does not encode a functional *FLS2* receptor (Gomez-Gomez *et al.*, 1999). Comprehensive analysis of this region however led us to conclude the presence of a chromosome mutation. During the initial creation of *prl1-2* using T-DNA mutagenesis, a fragment of chromosome IV, encompassing the mutated *PRL1* locus, appears to have been relocated to chromosome I. We did not determine the exact scope or location of the assumed translocation; however, molecular marker-based flanking suggests insertion between 4.15Mb and 4.99Mb on chromosome I (data not shown). A genomic rearrangement can cause improper pairing of chromosomes during meiosis, resulting in the loss of gametes and which would manifest in observed reduced numbers of recombinants. Interchromosomal rearrangements, deletions or insertions have been previously associated with

T-DNA mutagenesis and are an unfortunate shortcoming of this technique (Tax and Vernon, 2001).

3.3.3 sop1-1D is a gain-of-function allele of PRL2

PRL2 has been previously reported as the homolog of *PRL1* in *Arabidopsis* (Baruah *et al.*, 2009; Nemeth *et al.*, 1998). Public data from the transcriptome platform AtGenExpress (http://jsp.weigelworld.org/expviz/expviz.jsp (Schmid *et al.*, 2005)) identifies on average 80% lower expression of the *PRL2* gene compared to *PRL1* across tested developmental stages in the database (Appendix 3). Similar expression patterns are also detected in response to abiotic stress, hormones and pathogens (Appendix 3). The encountered mutation in *prl1-2 sop1-1D* is located in the 5' untranslated region (5'UTR) of *PRL2,* thus excluding changes to the protein structure. Consequently, we hypothesized a regulatory effect, potentially impacting *PRL2* transcript levels. We examined seedling tissues of *prl1-2 sop1-1D* and *sop1-1D* mutants and detected *PRL2* cDNA levels approximately twice as high in *prl1-2 sop1-1D* compared to the *prl1-2* single mutant and wild-type controls as determined by quantitative reverse transcription (RT)-PCR analysis (Fig. 3C,D) and as validated by semi-quantitative PCR-analysis using primers PRL2-RT-F2/R2 (Fig. 3E). Both results suggest the presence of an upregulated allele of *PRL2* in *sop1-1D*, acting as suppressor *of prl1-2*.

PCR-based amplification of the *PRL2* allele present in *prl1-2 sop1-1D* using primers PRL2-CL-F1 and PRL2-CL-R1 did not yield an amplification product whereas no problems were encountered when genomic wild-type DNA was used in the reaction. Small amounts of PCR product were obtained using a non-commercial Paq and primers PRL2-CL-F1 and PRL2-SQ-R1 for amplification of a 2.7kb 5' fragment and PRL2-SQ-F2 and PRL2-CL-R1 for amplification of a partially overlapping 2.2kb 3' fragment, however amino acid codon changing mutations were detected in each of four sequenced sections. An attempt to produce a collection of full-length

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products from twenty independently cloned and restriction nuclease treated 5' and 3' fragments failed repeatedly and we changed strategies to achieve complementation.

In an alternative approach, three clones of the *Arabidopsis* JAtY library were used to transform *prl1-2* and *prl1-2 sop1-1D* mutants (John Innes Centre, http://orders2.genome-enterprise.com/libraries/arabidopsis/jaty). JAtY clone 69M23 covers a 67kb region on Chromosome 3 encompassing *PRL2*, whereas 79F11 (78kb) and 51K01 (31kb) are adjacent and partially overlapping clones to the left and right, respectively (Fig. 4A). Since experimental data derived from our expression analysis demonstrated upregulated *PRL2* transcripts in *prl1-2 sop1-1D* plants, we proposed that introduction of a second *PRL2* copy (JAtY69M23) into the original *prl1-2* mutant would result in similar transcript levels and mimic complementation. Consistent with our hypothesis, two of the three JAtY69M23-derived *prl1-2* T1 transgenics no longer exhibited mutant phenotypes but wild-type morphology instead (Fig. 4B). The third line displayed a different mutant phenotype (data not shown).Transformation of *prl1-2* with JAtY51K01 yielded transgenics with unchanged mutant phenotypes, no transformants were recovered using the largest clone, JAtY79F11 (Fig. 4C). Transformation of wild-type looking *prl1-2 sop1-1D* plants with any of the three clones resulted in phenotypically unaltered transgenics in all cases (Fig. 4D). The results of our transformation series strongly support *PRL2* transcript-level dependent suppression of *prl1-2* phenotypes in *prl1-2 sop1-1D,* as only JAtY69M23-transformed *prl1-2* plants exhibited a change in phenotype. In conclusion, the *PRL2* allele present in *sop1-1D* most likely carries a regulatory gain-of-function mutation (*prl2-1D)*, conferring dominant suppression of *prl1* mutant phenotypes.

3.3.4 PRL2 and PRL1 exhibit high structural and sequence homology

Significant sequence homology between PRL1 and PRL2 proteins has been previously established through hybridization experiments and sequence alignments (Baruah *et al*., 2009;

Nemeth *et al.*, 1998). Both proteins belong to the Transducin/WD40 repeat family, a motif which has been shown to mediate protein-protein interactions (Xu and Min, 2011; Stirnimann *et al.*, 2010). The seven highly conserved WD40 repeats starting at amino acid position 142 of PRL1 and position 135 of PRL2, respectively, make up the majority of the protein structure. In contrast to 89% amino acid identity across this C-terminal region, only 59% identity is shared among the N-terminal amino acids leading up to the previous positions (Fig. 5). A second motif, designated DWD (DDB1 binding WD40) is located within the WD3/4 and WD4/5 repeats of PRL1 and PRL2 (Fig. 5). Recent evidence suggests that a subset of WD40 proteins carrying this motif, among them PRL1, may interact with CUL4-based E3 ligases (Lee *et al.*, 2008). PRL1 localizes to the nucleus using either nuclear localisation signal (NLS) based translocation or potentially through interaction with IMPORTIN ALPHA 3/MOS6 (Kosugi *et al.*, 2009). The high level of sequence conservation between PRL1 and PRL2 proteins accounts as further evidence for functional redundancy between the homologs and is consistent with the ability of *prl2-1D* to suppress *prl1-2* phenotypes.

3.3.5 Unequal genetic redundancy between PRL1 and PRL2

In contrast to *prl1* mutant plants, recessive loss-of-function of *PRL2* (*prl2-2*, Salk_075970) does not lead to morphological changes. To test for redundancy between *PRL2* and *PRL1*, we crossed exonic T-DNA insertion alleles *prl1-2* and *prl2-2* and identified the *prl1-2 prl2-2* double in the F2 generation by PCR-based genotyping. The double mutant can be distinguished from the *prl1-2* single mutant in the third week of development since leaves of *prl1-2 prl2-2* plants are darker, smaller and rounder (Fig. 6A, B). When a second T-DNA allele, *prl2-3* (Salk_133878) was used to generate the *prl1-1 prl2-3* double mutant, it also displayed the enhanced phenotype (data not shown). An early flowering phenotype, which is usually observed in *prl1-2* single mutants, manifests itself even more pronounced in the double mutant. *prl1-2 prl2-2* plants initiate bolting with 7.4 ± 0.5 leaves present, compared to 12.8 ± 1.8 leaves for *prl1-1* single

mutants and 18.7 ± 1.9 leaves in wild-type plants (Fig. 6C). This trend is less obvious when considering average root lengths, however a slight reduction in lengths was recorded compared to already strongly reduced *prl1-2* roots (Fig. 6D). The enhanced phenotypes observed in the double mutant suggest unequal genetic redundancy between *PRL1* and *PRL2*.

3.3.6 *prl1-2 prl2-2* mutants are impaired in basal and R-protein mediated resistance

Consistent with the obtained morphological data, infection assays reveal no differences in resistance between *prl2* loss-of-function and wild-type plants when using a low dose of virulent *P.s.m.* 4326. Substantially impaired basal defence signalling however was observed for *prl1-1 prl2-1* although not surpassing bacteria titer of *prl1-2* plants (Fig. 6E). The infection series with virulent oomycete *H.a.* NOCO2 also indicates less functional low-level defence in the double mutant (Fig. 6F).

For testing R protein mediated resistance, rosette leaves were infiltrated with avirulent *Pseudomonas syringae pathovar tomatoe* (*P.s.t.*) DC3000 expressing avrRPS4. This effector is recognized by the TIR-NB-LRR protein RESISTANCE TO PSEUDOMONAS SYRINGAE 4 which triggers effective defence in wild-type (Col-0) and *prl2-2* mutant plants. Modification of the responsible signal relay however allows for ten times more growth of the pathogen in *prl1-2* and *prl1-2 prl2-2* mutants, when titers were calculated at three days past exposure (Fig. 6G). Using oomycete *H.a.* EMWA1 conidiospores in spray-inoculation experiments, some impaired defence was detected in *prl1-2 prl2-2* but *prl1-2* plants still sustained more growth of the oomycete (Fig. 6H).

3.3.7 prl2-1D single mutants do not show enhanced resistance to pathogens

At the genomic level, *PRL2 is* larger than *PRL1* (4.3kb and 3.9kb, respectively) due to increased intron size and longer non-translated regions. The *prl2-1D* mutation affects the 12th position in the 69bp long 5' untranslated region of *PRL2* thus identifying an important segment for transcript level regulation. We attempted to compare 5'UTR sequences between the homologs but the regions are too different to allow an alignment. The 12th position of the shorter, 47bp measuring 5'UTR of *PRL1* is also occupied by a cytosine, both adjacent nucleic acids are however different (data not shown).

We generated the *prl2-1D* single mutant to investigate whether increased levels of *PRL2* transcript combined with wild-type *PRL1* expression rates would result in an observable mutant phenotype. The single mutant was created through crossing of *prl1-2 prl2-1D* with Col-0 and identified in the F2 generation using PCR-based genotyping. Morphologically, *prl2-1D* mutants resemble a wild-type plant aside from a late flowering phenotype which we have also noted in *prl1-2 prl2-1D* double mutants. Both mutant types initiate flowering with at least 20 rosette leaves present in contrast to *prl1-2* and wild-type plants which flower on average at the 11- and 15-leave stage, respectively (Fig. 7A,B).

Inoculation with virulent bacterial and oomycete pathogens detected no change in basal resistance levels in *prl2-1D* compared to pathogen growth sustained by *prl1-2 prl2-1D* plants and by the Col-0 control (Fig. 7C,D). Similarly, colonization by avirulent strains was limited to wild-type levels in *prl2-1D* when evaluated after three days. *P.s.t.* DC3000 expressing avrRPS4 and *P.s.t.* DC3000 expressing avrPphB are detected in a plants cytoplasm by TIR-type and CC-type R proteins, respectively (Fig. 7E, F). When challenged with avirulent *H.a.* EMWA1 (detected by TIR-NB-LRR immune receptor RPP4), we also did not detect changes in

resistance (Fig. 7G). These results suggest that both basal and ETI immunity are unaffected in *prl2-1D* plants which express *PRL2* at significantly increased levels. It is thus unlikely, that the function of PRL1/PRL2 in resistance signalling can be enhanced through a dosage-dependent mechanism such as overexpression of *PRL2*.

3.4   Discussion

PRL1 is part of the MAC, a multi-protein complex with a role in plant defence signalling. It is suggested, that the WD40 protein contributes substantially to a plants defence output since loss of PRL1 results in higher pathogen colonization than observed in other MAC mutants i.e. *mos4*, *Atcdc5* and *mac3a mac3b* (Monaghan *et al.*, 2009; Palma *et al.*, 2007). With the goal of identifying new defence components functioning in PRL1 - and MAC associated signal relay, we have carried out a genetic suppressor screen in the *prl1* mutant background. Investigation of the dominant suppressor *prl2-1D* suggests that not only well-known PRL1 but also the PRL2 homolog are involved in the regulation of plant immunity, sugar sensitivity and morphogenesis.

Important aspects of cellular functions such as transcription and ubiquitination are mediated with the help of specialized protein sequences such as the zinc-finger motif or the Ring-finger domain, respectively. Notably, these domains are among the most abundant in eukaryotic proteomes whereas modules that have been implicated in signalling events, such as the interactor domains SH2 (*src* homology 2), SH3 and PDZ (postsynaptic density 95/ discs large/zonula occludens-1) are not as common and less conserved in importance across kingdoms (Stirnimann *et al.*, 2010). For example, the interactor domain SH2 connects an extensive network of phosphorylated tyrosine-containing proteins in animals whereas only two *Arabidopsis* proteins feature a predicted homologous motif (Pawson, 2007; Williams and Zvelebil, 2004). More recently, the importance of the WD40 family is emerging which is the most utilized interaction module in baker's yeast and also makes up approximately 1% and 0.8% of

proteins in a limited human and *Arabidopsis* proteome analysis, respectively (Stirnimann *et al.*, 2010)

The majority of the PRL2 and PRL1 protein structure, about 70%, is taken up by seven WD40 repeat sequences whereas the remaining C-terminal region does not seem to comprise another motif. The encoded 7-fold WD40 propeller is a highly symmetrical structure allowing interaction to occur on all sides of the surface including the top, bottom and circumference (Xu and Min, 2011). This binding flexibility is certain to play a role in modulating the multitude of regulatory activities established for PRL1 (Flores-Perez *et al.*, 2010; Baruah *et al.*, 2009; Lee *et al.*, 2008; Palma *et al.*, 2007; Nemeth *et al.*, 1998). The WD40 structure is approximately 90% conserved between the PRL2 and PRL1 homologs thus likely capable of a similar range of interactions and assumed to be responsible for re-established wild-type phenotypes in the *prl1-2 prl2-1D* mutant.

So far, WD40 proteins have not been shown to exhibit enzymatic activity but rather are suggested to function as adapter components in signal relay (Stirnimann *et al.*, 2010). Increased *PRL2* mRNA levels in the *prl2-1D* single mutant did not result in a detectable mutant phenotype, in line with a presumed structural role of PRL2 and PRL1. Through reversible or stable association with pathway controlling elements, adapter proteins often provide essential platforms for regulatory interaction. Such a role could explain the enhanced susceptibility displayed by *prl1-2* and *prl1-2 prl2-1* mutants compared to other MAC mutants. The presence of yet another predicted structural protein in the MAC core complex, MOS4, emphasizes the importance of stable interplay for functional immunity signalling.

Phenotypes of *prl1-2* and *prl1-2 prl2-2* mutants are not limited to defects in immunity. It is conceivable, that PRL1 and PRL2 might be only transiently associated with MAC components for defence purposes while also mediating interactions in other pathways. A presumed function as a signalling-enabling adapter could explain involvement of PRL1 in diverse signalling

branches such as sugar, light, stress, resistance and hormone responses (Flores-Perez *et al.*, 2010; Baruah *et al.*, 2009; Lee *et al.*, 2008; Palma *et al.*, 2007; Nemeth *et al.*, 1998). Influence of PRL1/PRL2 over additional pathways independent from the MAC is also supported by cloning *of suppressor of prl1, 2Dominant*, (*sop2-1D*), which specifically reverses *prl1*-phenotypes however does not affect mutant *atcdc5* or *mos4* signalling (Weihmann et al., unpublished results).

In contrast to the highly conserved protein-coding segments, regulatory sequences of *PRL2* and *PRL1* have diversified significantly. *PRL2* transcript levels are far lower than those of *PRL1* in wild-type *Arabidopsis* plants potentially as a result of *PRL2* down regulation. Despite the differences, a single polymorphism at the 12th position of the *PRL2* 5' untranslated region (5'UTR) substantially increases mRNA levels suggesting that an important regulatory motif is present in this region. Single nucleotide polymorphisms (SNP) in 5'UTRs have been studied in some detail in human genes since such changes often correlate with disease phenotypes (Chatterjee and Pal, 2009). For example, six different SNPs were found in alleles of *ANKRD26* (*ankirin repeat domain 26*) and are associated with thrombocytepemia, a condition of abnormally low amounts of platelets. Reporter gene fusion constructs consisting of mutated 5'UTRs and the luciferase ORF demonstrated significant overexpression for all tested constructs (Pippucci *et al.*, 2011). Similarly, a single base substitution in the 5' UTR of *TGFβ3* (*transforming growth factor-beta3*) causes more than two-fold increased expression in a *TGFβ3* 5'UTR-luciferase assay. This mutant allele of *TGFβ3* contributes to the development of a specific type of myocardial disease in young adults (Beffagna *et al.*, 2005). The upstream untranslated region of *TGFβ3* contains 11 ATG sequences which potentially initiate several upstream open reading frames (uORFs) (Beffagna *et al.*, 2005). uORFs can affect translation of the main open reading frame, a regulatory mechanism that is also been present in yeast and plants (Calvo *et al.*, 2009; Tran *et al.*, 2008; Zhang and Dietrich, 2005). Neither *PRL1* nor *PRL2* UTRs however contain any additional ATG sequences upstream of the inferred translational

start codon and there are no strong indications of alternative transcripts (J. Robertson, personal communication). The rate of translation may also depend on the length of a given UTRs and potential secondary structures among other mechanisms. Finally, we cannot rule out that the polymorphism in the *PRL2* gene affects the rate of transcription rather than translation and stability of produced RNA molecules.

An often cited aspect for the decision of positioning *Arabidopsis thaliana* en route to the successful model it is today has been the compact genome size and low amount of repetitive sequences compared to the complex genomes of related crop plants. ollowing the *Arabidopsis* genome release and extensive *in silico* analysis, it became clear that there had also been several large-scale duplication events in the adopted model plant, since approximately 80% of the identified genes are present in two or more copies (Blanc and Wolfe, 2004). Examples of largely maintained redundancy such as for the two loci encoding E3 ubiquitin ligases subunits ATCUL3A/3A have been documented, however predicted outcomes for at least one copy of a duplicated gene also include diversification of function, expression patterns or even a fate as non-functional pseudogene (Briggs *et al.*, 2006; Figueroa *et al.*, 2005). In the MAC, U-BOX proteins MAC3A and MAC3B function redundantly in plant immunity whereas the relationships between the three MAC5 loci are more complex, displaying unequal genetic redundancy between *MAC5A* and *MAC5B*-encoded proteins and non-reduncancy between MAC5C and MAC5A/MAC5B (Monaghan *et al.*, 2010; Monaghan *et al.*, 2009). Absence of a mutant phenotype in *prl2-2* plants and an enhanced phenotype displayed by *prl1-2 prl2-2* mutants are hallmarks of unequal genetic redundancy caused by substantially reduced expression of one of the duplicates (Briggs *et al.*, 2006). However, *PRL2* appears still as functional as *PRL1*.

The fate of both the ancestral and a daughter gene is not pre-assigned following a duplication event. Duplicated genes can be the source of novel gene functions (neofunctionalization) or may result in an inactive pseudogene state for one of the copies (nonfunctionalization) (Ohta,

49

2000). Subfunctionalization on the other hand, is an evolutionary mechanism believed to be contributing to the preservation of duplicated genes. In this process, both members of a gene pair experience degenerative mutations that affect expression patterns and level of activities (Lynch and Force, 2000). We did not detect novel properties of the PRL2 protein in the overexpressing *sop2-1D* mutant which argues against neofunctionalization. According to the AtGenExpress platform, *PRL2* expression ranks consistently five times lower than *PRL1* throughout developmental stages, with the exception of pollen tissue. In this floral organ, *PRL1* expression drops whereas *PRL2* expression reaches levels five times higher than *PRL1.* Such inverted expression patterns could be seen as evidence towards subfunctionalization, i.e. developing tissue specificity. Mutations in the regulatory promoter region likely are responsible for the already strongly attenuated expression of *PRL2* and might ultimately lead to full pseudogenization of the homolog (Yang *et al.*, 2011; Adams and Wendel, 2005).
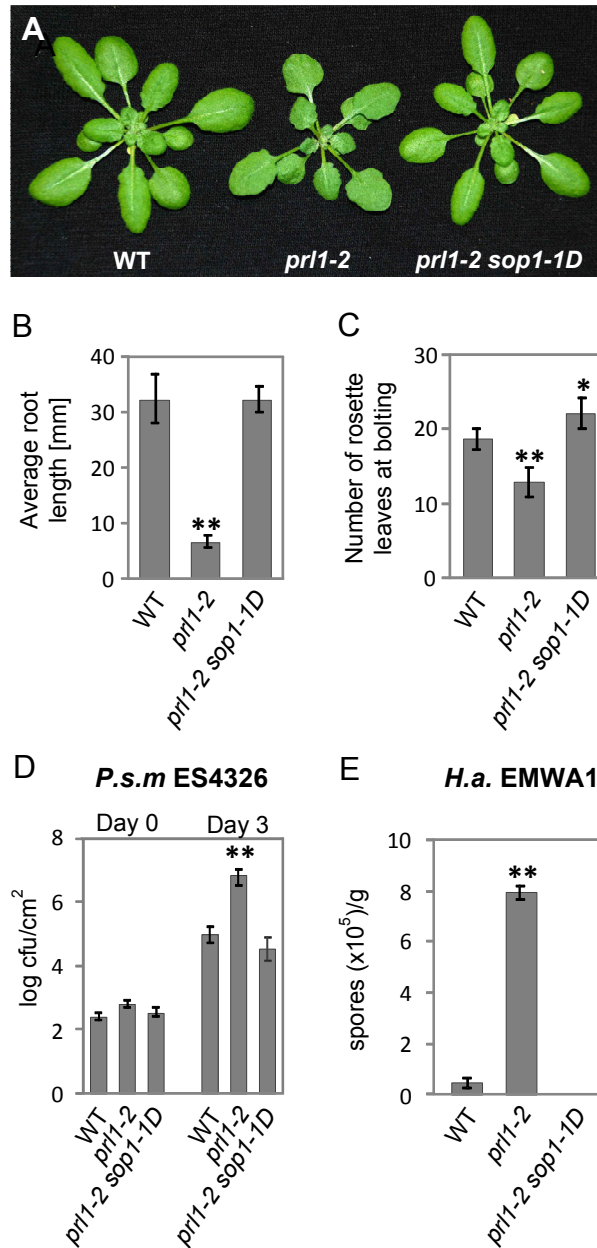
Figure 2. sop1-1D suppresses prl1 related phenotypes
(A) Morphology of soil-grown WT (Col-0), *prl1-2* and *prl1-2 sop1-1D*. (B) Root length analysis of one week old seedlings of indicated phenotypes, results represent an average of ten seedlings each ±SD. (C) Flowering time analysis: rosette leaves of ten plants per genotype WT (Col-0), *prl1-2* and *prl1-2 sop1-1D* were counted when emerging flower measured 1cm in height. Values represent averages ±SD. (D) Infection experiments with *P.s.m*. ES4326 (OD_{600}= 0.0001) using five-week-old soil-grown plants of indicated phenotypes. Bacterial titer was quantified at 0 and 3 days post inoculation, values represent average of six replicates per genotype ±SD. (E) Soil-grown, 2.5 week old plants of indicated genotypes were spray inoculated with 100,000 spores/ml of *H.a*. EMWA1 and colonisation quantified after nine days. Values represent averages ±SD of two replicates with 15 plants each. For (B) through (E), experiments were repeated at least three times with similar results. Statistical significance compared to Col-0 control was calculated using Student's *t*-test: *$P < 0.001$ and **$P < 0.0001$ for all graphs. cfu, colony forming units.

A

marker → MIE1   MRC8   Chromosome 3
4   8 (Mb)

MDC8   K14A17
4.87   5.58   5.84   6.21 (Mb)

BAC clones

MDC8   K20I9   K14A17
MGL6   MUH15

MDC8   MGL6   MGL6   MGL6   K14A17
            -SNP1   -SNP5
3   2   0   2   # of recombinants   7

5.64   5.66   5.68 (Mb)
Genes

*
At3g16650

B

(prl2-1D/)   C → T transition
sop1-1D   AATCCTATTTC**T**CTCTCTTCTCTTCCGTCTT
PRL2   AATCCTATTTC**C**CTCTCTTCTCTTCCGTCTT

sop1-1D   TCTCGTTCTGTTTCTGATTGTTCGTGTAAAA
PRL2   TCTCGTTCTGTTTCTGATTGTTCGTGTAAAA
            5' non-translated region (5'UTR)

sop1-1D   CCCTAGAATGACGATGATAGCTTTGAACAGG
PRL2   CCCTAGAATGACGATGATAGCTTTGAACAGG
                    M T M I A L N R

C

D

Figure 3. **Map-based cloning of *sop1-1D***
(A) Map position of *sop1-1D;* indicated are recombinants and sequence-anchored positions of flanking markers and BAC clones. A mutation (*) was identified in *At3g16650/PRL2*. (B) Sequence analysis reveals a point mutation in the *PRL2* 5'UTR of *prl1-2 sop1-1D*. (C) Quantification of real-time RT-PCR data using exon-specific *PRL2* primer on c-DNA obtained from indicated genotypes of tissue series #1. Values represent averages of two experimental replicates. (D) Semi-quantitative RT-PCR of tissue series described in C).

A

Chromosome 3

5.6   5.7   (Mb)

JAtY79F11   JAtY51K01

library clones

JAtY69M23

At3g16650/
PRL2

C

	79F11	69M23	51K01
prl1-2 *(mutant phenotype)*	n.d.	2 *WT* / 3 total	6 *mutant* / 6 total
prl1-2 sop1-1D *(WT phenotype)*	4 *WT* / 4 total	5 *WT* / 5 total	5 *WT* / 5 total

B

prl1-2
sop1-1D   *prl1-2*

prl1-2 with   *prl1-2* with
JAtY69M23,   JAtY69M23,
T1, line #1   T1, line #2

D

prl1-2 sop1-1D with JAtY69M23, T1
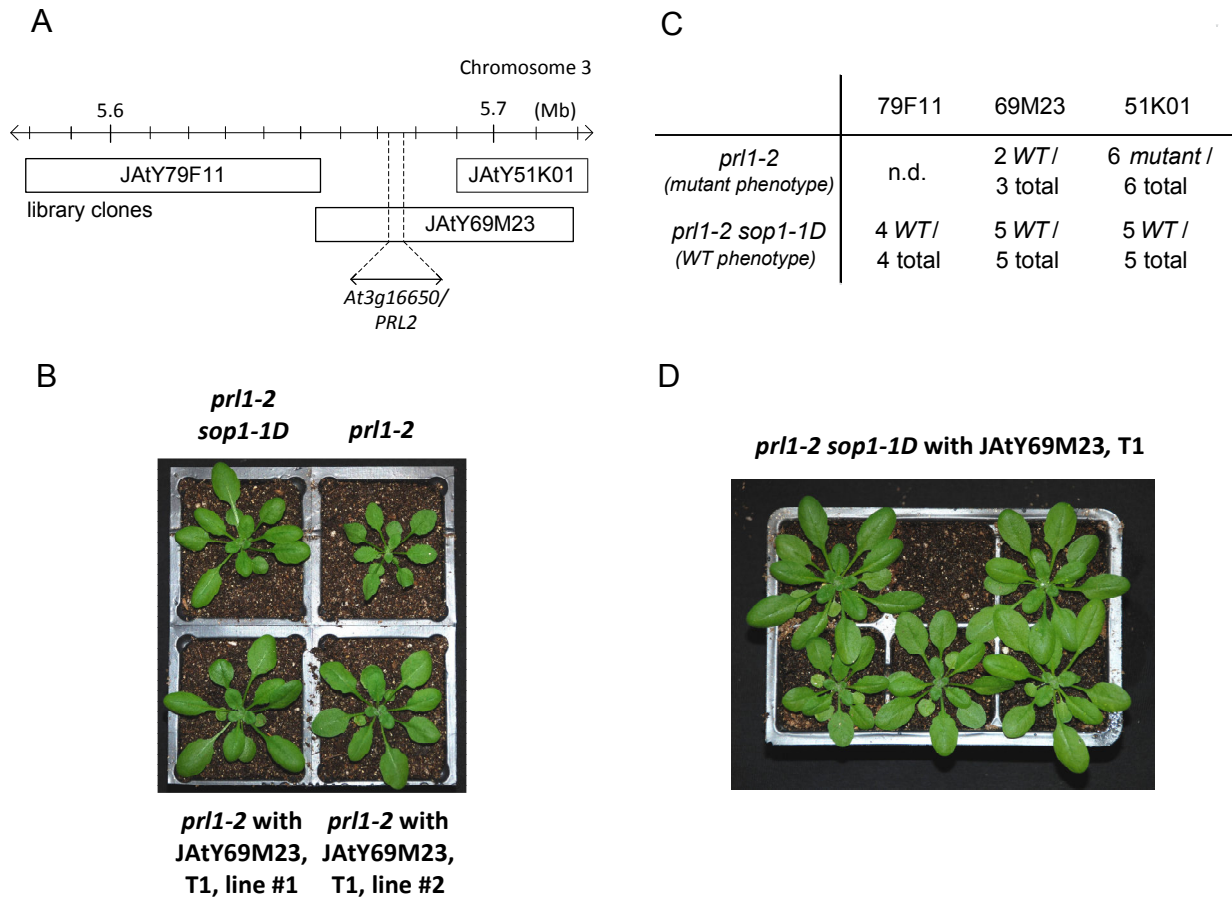
Figure 4. **Overexpression of *PRL2* is able to complement *prl1* mutant defects**
(A) Arrangement and sequence-anchored positions of JAtY clones 79F11, 69M23 and 51K01. The *PRL2* gene is only covered by JAtY69M23. (B) Morphology of *prl1-2 sop1-1D, prl1-2* and two transgenic lines obtained through introduction of JAtY69M23, which harbours *PRL2, into prl1-2.* (C) Summary of JAtY transformations; Indicated are JAtY clones, genotypes of transformed plants as well as number and phenotypes of obtained transgenic plants. (D) Morphology of transgenic plants obtained through transformation of JAtY69M23 into *prl1-2 sop1-1D.*

A

bipartite NLS

```
PRL1    1  MPAPTTEIEPIEAQSLKKLSLKSLKRSLEIFSPVHGQFPPPDPEAKQIRLSHKMKVAFGG
PRL2    1  MTMIALNRE-VETQSLKKLSLKSVRRAREIFSPVHGQFPQPDPESKRIRLCHKIQVAFGG
```

monopartite NLS

```
PRL1   61  VEPVVSQPPRQPDRINEQPGPSNALSLAAPEGSKSTQKGATESAIVVGPTLLRPILPKGL
PRL2   60  VEP-ASKPTRIADHNSEKTAPLKALALPGPKGSKELRKSATEKALVVGPTLP----PRDL
```

WD1

```
PRL1  121  NYTGSSGKSTTIIPANVSSYQRNLSTAALMERIPSRWPRPEWHAPWKNYRVIQGHLGWVR
PRL2  115  NNTGNPGKSTAILPAPGSFSERNLSTAALMERMPSRWPRPEWHAPWKNYRVLQGHLGWVR
```

WD2

```
PRL1  181  SVAFDPSNEWFCTGSADRTIKIWDVATGVLKLTLTGHIEQVRGLAVSNRHTYMFSAGDDK
PRL2  175  SVAFDPSNEWFCTGSADRTIKIWDVATGVLKLTLTGHIGQVRGLAVSNRHTYMFSAGDDK
```

WD3   DWD box 1

```
PRL1  241  QVKCWDLEQNKVIRSYHGHLSGVYCLALHPTLDVLLTGGRDSVCRVWDIRTKMQIFALSG
PRL2  235  QVKCWDLEQNKVIRSYHGHLHGVYCLALHPTLDVVLTGGRDSVCRVWDIRTKMQIFVLP-
```

WD4   DWD box 2   WD5

```
PRL1  301  HDNTVCSVFTRPTDPQVVTGSHDTTIKFWDLRYGKTMSTLTHHKKSVRAMTLHPKENAFA
PRL2  294  HDSDVFSVLARPTDPQVITGSHDSTIKFWDLRYGKSMATITNHKKTVRAMALHPKENDFV
```

WD6

```
PRL1  361  SASADNTKKFSLPKGEFCHNMLSQQKTIINAMAVNEDGVMVTGGDNGSIWFWDWKSGHSF
PRL2  354  SASADNIKKFSLPKGEFCHNMLSLQRDIINAVAVNEDGVMVTGGDKGGLWFWDWKSGHNF
```

WD7

```
PRL1  421  QQSETIVQPGSLESEAGIYAACYDNTGSRLVTCEADKTIKMWKEDENATPETHPINFKPP
PRL2  414  QRAETIVQPGSLESEAGIYAACYDQTGSRLVTCEGDKTIKMWKEDEDATPETHPLNFKPP
```

```
PRL1  481  KEIRRF
PRL2  474  KEIRRF
```

Figure 5. **Alignment of PRL1 and PRL2 protein sequences**
Dark shaded areas denote identical amino acids, light grey areas indicate similar amino acids. Predicted NLS according to cNLS mapper shown for PRL1 above and for PRL2 below the alignment (Kosugi *et al*., 2009). WD40 repeats 1-7 (adapted from Nemeth *et al*., 1998 and Baruah *et al*., 2009) and DWD boxes 1 and 2 (Lee *et al*., 2008) are indicated for both sequences. NLS, nuclear localization signal. DWD, DDB1 binding WD40.
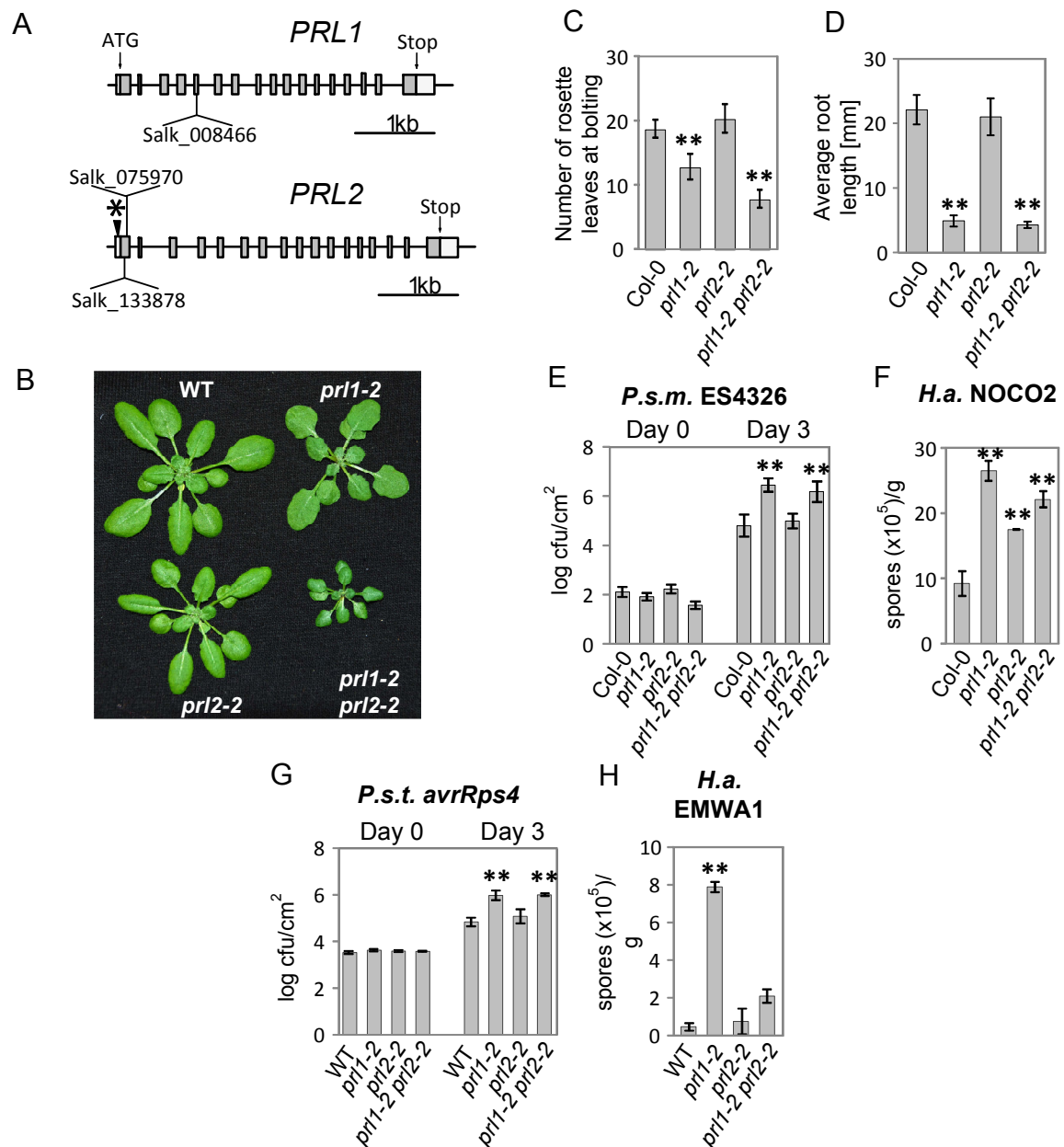
Figure 6*. **Enhanced phenotypes in *prl1 prl2* double mutants compared to single mutants***
(A) Gene structure of *PRL1* and *PRL2*. Indicated are positions of Salk lines S_008466 (*prl1-2*), S_075970 (*prl2-2*) and S_13378 (*prl2-3*). The *prl2-1D* mutation (*) is located in the first exon. (B) Morphology of soil-grown WT (Col-0), *prl1-2*, *prl2-2 and prl1-2 prl2-2.* (C) Flowering time analysis of WT, *prl1-2, prl2-2* and *prl1-2 prl2-2*. Rosette leaves of ten plants per genotype where counted when emerging flower measured 1cm in height. Values represent averages ±SD. (D) Root length analysis of indicated genotypes. Results represent an average of ten plate-grown seedlings for each genotype ±SD. (E) Bacterial infection of indicated phenotypes with virulent *P.s.m.* ES4326 (OD_{600}= 0.0001) using five week old soil-grown plants. Bacterial titer quantification at 0 and 3 dpi, averages represent six replicates ±SD. (F) Evaluation of disease susceptibility of indicated genotypes towards virulent oomycete *H.a.* NOCO2 (50,000 spores/ml), values represent averages of two replicates of 15 plants ±SD. (G) Inoculation of indicated genotypes with avirulent *P.s.t. avrRPS4* (OD_{600}= 0.002) using five week old soil-grown plants. Bacterial titer quantification at 0 and 3 dpi, averages represent six replicates ±SD. (H) Evaluation of disease susceptibility of indicated genotypes towards avirulent oomycete *H.a.* EMWA1 (200,000 spores/ml), values represent averages of two replicates of 15 plants ±SD. For (C) through (H), experiments were repeated at least three times with similar results. Statistical significance compared to Col-0 control was calculated using Student's *t*-test: **P < 0.0001 for all graphs. cfu, colony forming units.
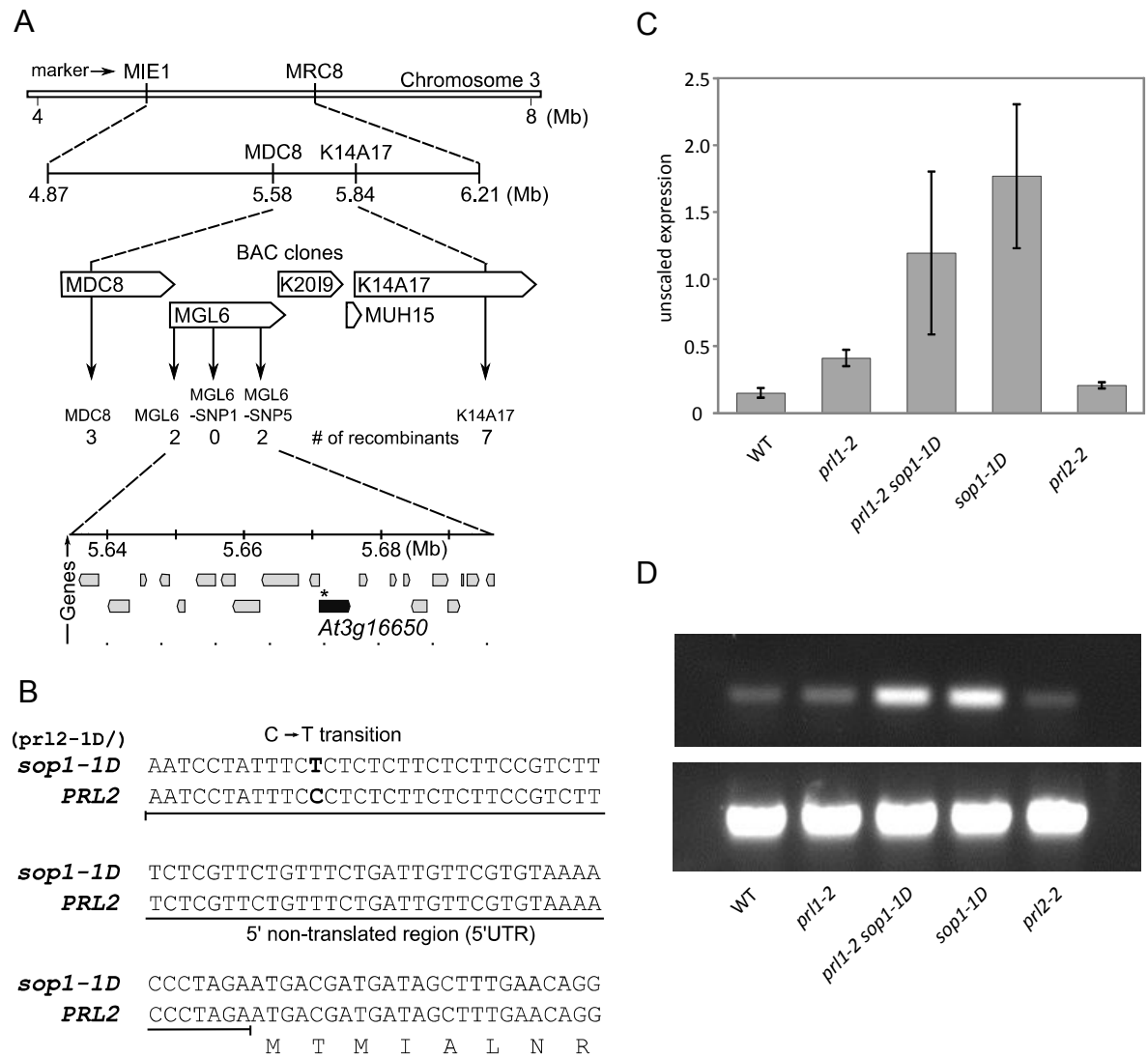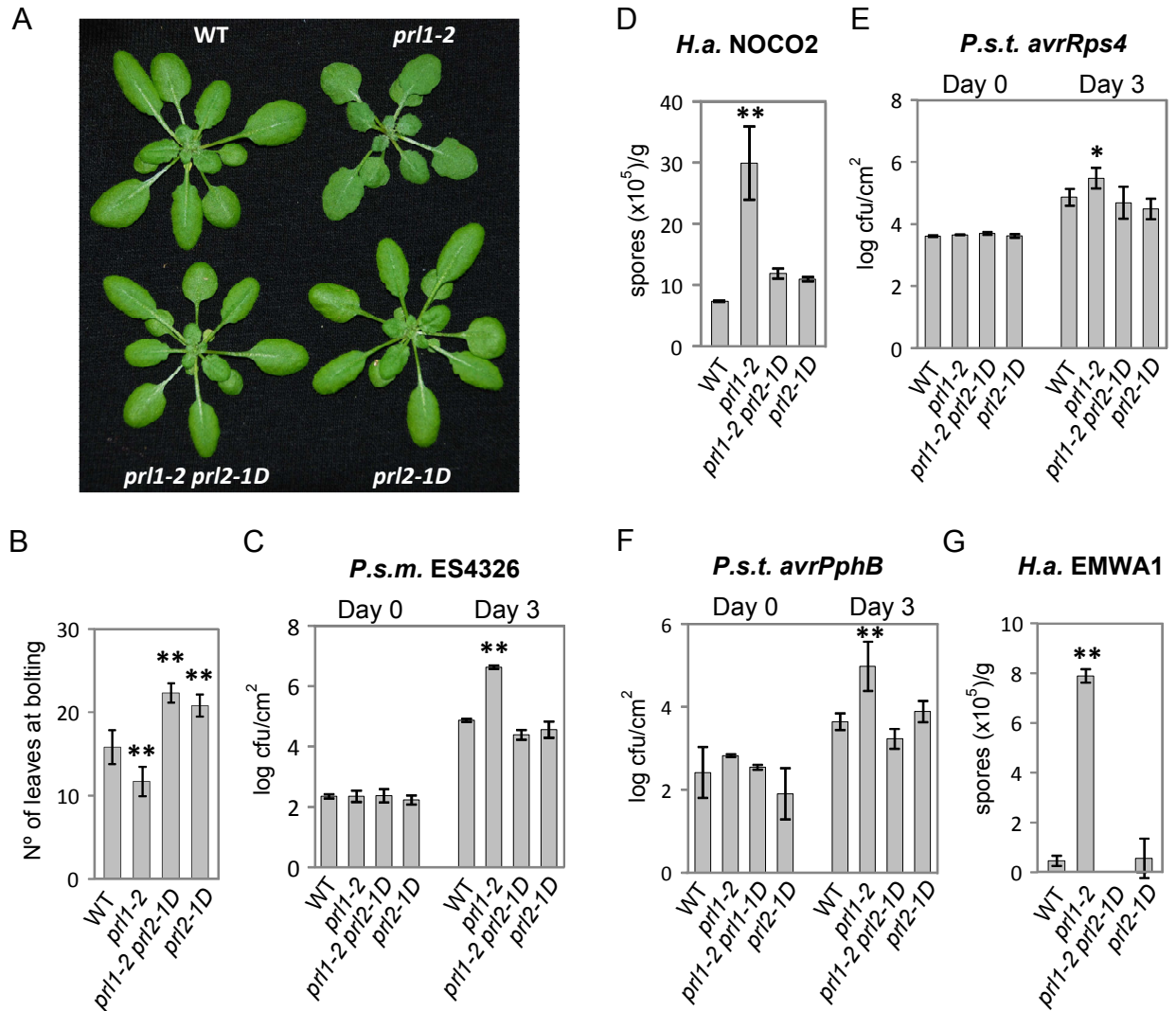
Figure 7*. Basal and R protein mediated resistance is unaffected in *prl2-1D

(A) Morphology of five-week-old soil-grown Col-0 (WT), *prl1-2*, *pr1-2 prl2-1D* and *prl2-1D*. (B) Flowering time analysis of indicated genotypes. Rosette leaves of ten plants per genotype where counted when emerging flower measured 1cm in height. (C) Infection assay of indicated genotypes with five-week-old plants using a low dose (OD_{600}= 0.0001) of *P.s.m*. ES4326 and quantification of bacterial titer at 0 and 3 dpi. Values represent six replicates ±SD. (D) Evaluation of resistance towards virulent oomycete *H.a.* NOCO2 of indicated genotypes. Spray-inoculation of 2.5-week-old plants with 50.000 spores/ml followed by conidiospore count after 8 days. Values represent two replicates of 15 plants ±SD. (E) and (F) Evaluation of disease resistance towards avirulent bacterial pathogens. Injection of a high dose (OD_{600}= 0.002) of *P.s.t.* DC3000 expressing *avrRPS4* or *avrPphB* into leaves of five-week-old plants of indicated phenotypes showed wild-type resistance in *prl2-1D*. Bacterial titer ere quantified at 0 and 3 dpi. Values represent six replicates ±SD. (G) Spray-inoculation of 2.5-week-old plants of indicated genotypes with 150.000 spores/ml of avirulent oomycete *H.a.* EMWA1 followed by conidiospore count after 8 days, values represent averages of two replicates with 15 plants. Statistical significance compared to Col-0 control was calculated using Student's *t*-test: *P < 0.003 and **P < 0.0001 for all graphs. dpi, days post inoculation.

4   A dominant mutation in an uncharacterized gene identifies a component of PRL1 signalling specific to the plant kingdom

4.1   Introduction

In plants, perception of pathogens is facilitated by receptors, which monitor the extracellular environment and activities in the cytosol. The first line in active defence is provoked once conserved epitopes of bacterial and fungal pathogens such as flagellin, lipopolysaccharides, chitin or other pathogen associated molecular patterns (PAMPs) have been detected by pattern recognition receptors at the cell surface (Willmann *et al.*, 2011; Newman *et al.*, 2007; Gomez-Gomez and Boller, 2000). PAMP-triggered immunity (PTI) is effective against a broad range of pathogens; early activities include ion fluxes across the plasma membrane as well as mobilisation of reactive oxygen species and signalling via the mitogen-activated protein kinase (MAPK) network which helps regulate the PTI defence output through activation of WRKY-type transcription factors (Petersen *et al.*, 2010; Nicaise *et al.*, 2009; Qiu *et al.*, 2008; Asai *et al.*, 2002). Successful pathogens are nonetheless able to deliver effector molecules into the host cell, which often function to diminish or suppress PTI, thus allowing virulent pathogen growth. In incompatible interactions however, the presence or activity of effectors is detected by resistance (R) proteins which are intracellular immunity receptors. Activation of R protein complexes initiates effector-triggered immunity (ETI), an amplified form of PTI which confers resistance against specialized pathogens.

R protein induced ETI signalling in response to biotrophic pathogens is usually first routed through lipase-like proteins ENHANCED SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) which control salicylic acid –dependent responses (Venugopal *et al.*,

2009; Wiermer *et al.*, 2005; Parker *et al.*, 1996). Some CC-type R proteins also signal though NONRACE-SPECIFIC DISEASE RESISTANCE (NDR1), a transmembrane protein with homology to integrins (Knepper *et al.*, 2011; Aarts *et al.*, 1998). Pathogen-induced SA production and accumulation are facilitated by the isochorismate synthase SID2 (SA INDUCTION DEFICIENT2) and MATE transport family protein ENHANCED DISEASE SUSCEPTIBILITY5 (EDS5) and supported by the ALD1 protein (AGD2-LIKE DEFENCE RESPONSE) (Song *et al.*, 2004; Nawrath *et al.*, 2002; Wildermuth *et al.*, 2001) SENESCENCE ASSOCIATED GENE 101 (SAG 101), a third lipase –like protein forms a ternary complex with EDS1 and PAD4 in the nucleus with a role in SA mediated defence (Zhu *et al.*, 2011). Redox changes in the cytoplasm lead to monomerization and relocation of usually inactive complexed multimers of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) (Tada *et al.*, 2008; Mou *et al.*, 2003). In the nucleus, NPR1 binds TGA family transcription factors which orchestrate defence-related transcription , likely supported by WRKY proteins (Kesarwani *et al.*, 2007; Eulgem, 2006; Wang *et al.*, 2006; Zhang *et al.*, 2003). Mutants deficient in SA production or acculmulation, or NPR1 function, have demonstrated abilities to build up partial defences thus identifying signalling routes independent of both regulators (Lu, 2009; Lee *et al.*, 2007; Palma *et al.*, 2007; Bartsch *et al.*, 2006a; Bowling *et al.*, 1997)

Resistance pathways have been further examined through genetic dissection of *snc1* (*suppressor of npr1-1, constitutive 1*) signalling, a dwarf mutant encoding a constitutively activated predicted R protein (Zhang *et al.*, 2003). New defence elements were identified through cloning of *mos* (*modifier of snc1*) mutations which partially or fully restored wild-type phenotypes in *snc1 mos* suppressor mutants (Monaghan *et al.*, 2010). For example, the nuclear transport machinery was found to be required for plant resistance since *MOS3, MOS6* , *MOS7* and *MOS11*-encoded proteins function in messenger RNA export as well as both import and export of nucleus-targeted proteins (Germain *et al.*, 2010; Monaghan *et al.*, 2010). Post-translational farnesylation and ubiquitination mechanisms do also play a role in defence

(MOS5/UBA1 and MOS8/ERA1) (Cheng *et al.*, 2011; Goritschnig *et al.*, 2008; Goritschnig *et al.*, 2007).

Characterization of MOS4 revealed association with ~20 proteins, either stable or transiently organized in a complex termed MAC (for MOS4-associated complex) (Monaghan *et al.*, 2009; Palma *et al.*, 2007). Loss-of-function mutations in predicted MAC structural elements *MOS4*, *PRL1* (*PLEIOTROPIC REGULATORY LOCUS 1*) or putative transcription factor *CELL DIVISION CYCLE 5 (AtCDC5),* result in increased colonization by both virulent and avirulent pathogens (Palma *et al.*, 2007). Similar susceptibility was also demonstrated in *mac3a mac3b* mutants which lack two MAC U-box proteins with homology to the yeast and human E3 ubiquitin ligase Prp19 (Monaghan *et al.*, 2009). Defence defects have been established for all tested MAC proteins although plants with mutations in *PRL1* tested most impaired. With the goal of identifying potential targets of this specific MAC member and aiming to clarify tasks of the complex in plant defence, we based a suppressor screen on a mutant allele of *PRL1*. Our screen resulted in a range of suppressors, the first of which was described recently (Weihmann et al., submitted). Here we describe cloning and characterization of *sop2-1D,* a dominant mutant obtained from the *prl1* suppressor screen.

4.2   *Material and methods*

4.2.1 *Plant material and growth*

Wild-type *Arabidopsis* ecotypes Columbia (Col-0), Landsberg erecta (Ler), Wassilewskija (Ws) and derived mutants were grown on soil in a 16h light / 8h dark regime. T-DNA mutants *prl1-2* (Salk_008466), *sop2-2* (Salk_058710) and *sop2h-1* (Salk_121713c) were obtained from the *Arabidopsis* Biological Research Centre*, atcdc5-2* (GABI_278B09) was obtained from the Max-Planck-Institute for Plant Breeding Research. All lines were genotyped by PCR, using insertion

flanking oligonucleotides PRL1-Salk-NF (5'-GATGAAAGTTGCGTTTGGAG-'3) and PRL1-NR-A

(5'-ACTACCTACACTACCTAGAGC-'3) to identify *prl1-2* mutants, Salk_058710-F (5'-

AGCCACATCTCTTGCTGTTG-'3) and Salk_058710-R (5'-TGAAGCGAGAAGCGGTAACT-'3)

for identification of *sop2-2* mutants, Salk_121713c-F (5'-CTAGGACTCCCATCTGCACTA-'3)

and Salk_121713c-R (5'-AATGGCATGTCTCAAGTTGG-'3) for identification *of sop2h-1*

mutants.

4.2.2 *Plant pathogens and infection assays*

Bacterial pathogens *Pseudomonas syringae* pathovar *maculicola (P.s.m.)* ES4326 and pathovar

tomato (P.s.t.) are hemi-biotrophic bacterial pathogens responsible for leaf spot and bacterial

speck disease on *Arabidopsis,* respectively. For propagation, bacteria were cultivated on LB

plates containing 50 mg/ml Streptomycin (*P.s.m.* ES4326) or 25 mg/ml Rifampicin and 50 mg/ml

Kanamycin (*P.s.t.* avrPphB and avrRps4). For inoculation, bacteria were grown at 28-30ºC in

liquid LB to high densities, cross-inoculated to fresh media and allowed to reach OD_{600} = 0.2.

Using a needless small plastic syringe lightly pressed against an *Arabidopsis* leave underside,

bacterial solution is injected into the apoplast. A low dose (OD_{600}= 0.0001), referred to as

enhanced disease susceptibility (EDS) dose, was used in virulent (*P.s.m.* 4326) infections. A

high dose (OD_{600}= 0.002) was employed with avirulent *P.s.t. avrPphB* and *P.s.t. avrRps4*. On

the first day and three days after inoculation, leave discs of $0.32cm^2$ were cut with a standard

paper hole-punch, samples were homogenized in 10mM $MgCl_2$ and a series of six dilutions was

plated. The bacterial titer was measured after two days of incubation at 28ºC.

Hyaloperonospora arabidopsidis (formerly *Peronospora parasitica* and *Hyaloperonospora*

parasitica) (Holub, 2008; Slusarenko and Schlaich, 2003) is a biotrophic oomycete pathogen

which causes downy mildew on *Arabidopsis*. It is an obligate pathogen which has to be

propagated on susceptible *Arabidopsis* ecotypes usually completing a life cycle in one week at

16ºC and > 50% humidity (McDowell *et al.*, 2011). The susceptible *Arabidopsis* ecotype Col-0 was used to propagate *H.a.* NOCO2, *H.a*. EMWA1 was grown on susceptible Ws plants. For infection assays, 2.5-week-old plants were spray-inoculated using either a low dose (50,000 spores/ml) to determine resistance defects in a compatible interaction, or a high dose (100,000 spores/ml) for evaluation of incompatible interactions. After 7-10 days, plants were harvested in a 50 ml Falcon tube containing up to 5 ml water, conidiospores were released using a vortex for 10 seconds and quantified using a hemocytometer.

4.2.3 Positional cloning

Molecular markers used for map-based cloning are PCR based and detect either length (InDel) or single nucleotide polymorphisms (SNP) between Col-0 and Ler (Monsanto *Arabidopsis* Ler sequence available at TAIR: [http://www.arabidopsis.org/browse/Cereon/index.jsp]).ecotypes. When employing InDel markers, one set of primers is used for both ecotypes and amplified fragments are visualized on 1-2% agarose gels. For SNP markers, two ecotype specific forward primers and one common reverse primer were designed. The forward primers differ in the last two positions with the ultimate nucleotide complementary to the respective ecotype polymorphism, and the penultimate chosen to stabilize specific binding (Bui and Liu, 2009). The primer combinations are used in alternating reactions on genotypes and fragments are analysed on 1% agarose gels. For primer sequences, see Appendix 2.

4.2.4 Single and double mutant construction

For creation of the *sop2-1D* single mutant, homozygous *prl1-2 sop2-1D* mutants were crossed with Col-0 wild-type plants and the single was identified in the F2 generation using allele specific primers P20A4-M-F (5'-AATGGTTTTCTATCAGGCAT-'3) and P20A4-WT-R2 (5'-GAGCAGACCTGTTTCTTAGTCC-'3). Double mutants *prl1-2 sop2-2*, *prl1-2 sop2h-1*, *sop2-2*

mos4-1 and *sop2-2 atcdc5*-2 were generated through crossing of respective single mutants. In all cases, double mutants were identified in the F2 by PCR-based genotyping.

4.2.5 Phylogenetic analysis

Protein sequences were obtained from NCBI and PLAZA (Sayers *et al.*, 2011; Proost *et al.*, 2009). For full gene ID's, see Appendix 4. The 31 plant protein sequences were aligned with *MAFFT* v.6 using default settings (Katoh *et al.*, 2002). Sequences were edited in *SeaView (Gouy et al., 2010)*. We then used the *ProtTest* server (http://darwin.uvigo.es/software/prottest_server.html) to determine the best model of protein evolution for our sequences (Abascal *et al.*, 2005).The model chosen according to the Akaike Information Criterion was the JTT model (Jones *et al.*, 1992). Thus, we inferred a maximum likelihood tree under this model using *RAxML* (Stamatakis *et al.*, 2005). Using RAxML's rapid bootstrapping method, we performed 500 bootstrap replicates to establish significance for interior branch points (Stamatakis *et al.*, 2008).

4.2.6 Localisation of SOP2-GFP

Using primers At2g40638-CL-F (5'-CGGGGTACCTGTTTTATCGCGGGTTTATGTG-3') and At2g40630-CL-GFP-R (5'-CGCGGATCCATGAAGCCGCCGCCTACCG-3') we amplified a 3.5kb genomic fragment encompassing the *SOP2* open reading frame and introduced *KpnI* and *BamHI* restriction sites on the 5' and 3' ends, respectively, The digested fragment was cloned into a modified pGreen vector just upstream of a GFP open reading frame. Through sequencing of a *SOP2-GFP* fusion clone we confirmed in- frame insertion and absence of mutations. The construct was transformed into *prl1-2 sop2-1D* mutants and transgenic plants identified through BASTA® selection. Fluorescence from propidium iodide-stained tissue of BASTA® resistant T2 progeny complementing the *sop2* phenotypes was analysed using a Nikon Eclipse 80i confocal

laser-scanning microscope (Lucas *et al.*, 2006). Images of GFP expression used the Zeiss (LSM5 Pascal, Germany) confocal microscope as well as the Nikon Eclipse 80i and s using 40x and 60x objective and laser lines, 488 nm and 543nm.

4.2.7 Quantification of SOP2 mRNA levels

Seeds of the genotypes *prl1-2*, *atcdc5-2, mos4-1, mac3a mac3b, mac5a* and Col-0 wild-type were vernalized for 7 days and plated on 0.5 MS containing 100 mg/ml Ampicillin. The plates were incubated in a growth chamber for ten days using a 16h light, 8h dark regime. Tissue of 10-day-old seedlings was collected in a 2ml tube containing two glass beads and frozen immediately. RNA was extracted using the Totally RNA kit (Ambion) and Reverse Transcriptase (SuperScript II, Invitrogen) was used to produce c-DNA copies of the transcriptome. Relative amounts of *SOP2* cDNA (At2g40630_40-F2: 5'-ATAAGCGGAGAGGTGGTGAG-'3, At2g40630-40-R2: 5'-TGATTACCGTCTTTCCCAAA-'3) and *Tubulin* (control) c-DNA (5′-ACGTATCGATGTCTATTTCAACG-3′ and 5′-ATATCGTAGAGAGCCTCATTGTCC-3′) present in the collected tissues were quantified using real-time PCR.

4.3   Results

4.3.1 Dominant suppression of prl1 loss-of-function phenotypes by sop2-1D

To facilitate a more complete picture of *PRL1*-related contributions to plant defence, we have carried out a genetic screen based on suppression of mutant *prl1-* associated disease symptoms and morphological criteria (Palma *et al.*, 2007; Nemeth *et al.*, 1998). One of the obtained mutants harbours a dominant suppressor allele, which we named *suppressor of prl1,2 Dominant* (*sop2-1D*). The dominant phenotype was detected after back crossing of a *prl1*

homozygous wild type-like progeny plant of a selfing *sop2* line with the parental *prl1-2* line. The cross resulted in nine *prl1*-like and twelve wild-type progeny in the first filial generation, indicative of a segregating dominant mutant allele in the tested *sop2* plant (expected 1:1, $\chi^2 = 0.42$, P = 0.51). *sop2-1D* is a complete suppressor of mutant leaf colour and shape in the *prl1-2 sop2-1D* double mutant and also suppresses characteristically short root length, usually associated with the *prl1-2* mutant background (Fig. 8A,B).

To investigate the impact of *sop2-1D* on immunity signalling in the *prl1* loss-of-function background, we employed virulent bacterial pathogen *Pseudomonas syringae* pathovar *maculicola* (*P.s.m*) ES4326. In a compatible interaction between a plant and a virulent pathogen, plant defence responses are outperformed by pathogenic proliferation leading to disease symptoms. In the MAC mutant *prl1-2*, defence-related signalling is compromised leading to even higher colonization compared to a susceptible wild-type plant. In line with our morphological data, *prl1*-associated enhanced susceptibility is no longer observed in *prl1-2 sop2-1D* leaves and instead pathogen growth is restricted to wild-type levels (Fig. 8C). *sop2-1D* suppression thus also extends to defence phenotypes.

4.3.2 A leucine-for-proline substitution in an uncharacterized protein causes differential interference of prl1-related signalling

For positional cloning of *sop2-1D*, we crossed the *prl1-2 sop2-1D* double mutant (in the Col-0 background) with *Arabidopsis* ecotype Landsberg *erecta* (Ler). From the second filial generation (F2) of the mapping cross, we used 48 *prl1*-like plants to locate *sop2-1D* on the bottom arm of chromosome 2. Seeds of F2 plants that were *prl1-2* homozygous but *sop2-1D* heterozygous were collected and marker patterns of 1192 progeny plants used to flank the *sop2-1D* locus using Insertion/Deletion (InDel) markers T2N18 (15.57Mb) and F16B22 (18.40Mb). Dominant suppression of the recessive *prl1* mutation allowed us to use 127

recombinants of both mutant and wild-type phenotypes in these mapping steps. Allele configuration at the suppressor locus in wild-type plants was extrapolated from segregation ratios in progeny populations. Next, the candidate region was further narrowed down by means of InDel marker T3G21 (16.86MB) and T3K9 (17.10Mb), for which thirteen recombinants remained. Final flanking of *sop2-1D* was achieved with three and two recombinants for InDel marker T2P4(2) and single nucleotide polymorphism (SNP) marker T7D17-SNP2, respectively (Fig. 9A). Through sequencing of candidate open reading frames, we identified a base pair change in the third exon of *At2g40630* (Fig. 9B). The genomic modification results in substitution of proline with leucine in the encoded predicted protein (Fig. 9C)

At2g40630 is an uncharacterized gene of approximately 2.9Kb, with eight exons encoding a 535 amino acid protein (Arabidopsis Genome Initiative, 2000). To confirm that *At2g40630* is *SOP2*, we transformed *prl1-2 sop2-1D* plants with a 4kb genomic clone encompassing the wild-type *At2g40630* open reading frame and 2.3kb of 5' and 3' regulatory and non-translated sequences. Two transgenic plants were recovered, both exhibiting *prl1*-like features although growing slightly larger and exhibiting less serrated leaves (Fig. 9D). When the homozygous T2 plants were challenged with virulent *P.s.m.* ES4326, they exhibited *prl1*-like susceptibility indicating that *At2g40630* can complement the mutation in *prl1-2 sop2-1D* (Fig. 9E).

Since *sop2-1D* is dominant, we also tested the effect of the *sop2-1D* allele on *prl1-2* plants. When *prl1-2* single mutants were transformed with an analogue 4kb clone derived from a *sop2-1D* homozygous plant and consisting of the mutant *sop2* sequence and 2.3kb of adjacent 5' and 3' regions, the resulting six T1 plants showed varying levels of *prl1* suppression, one line exhibiting a near wild-type phenotype (Fig. 9F).

Since introduction of the mutant *sop2-1D* allele into *prl1-2* resulted in partial suppression and introduction of genomic *SOP2* into *prl1-2 sop2-1D* lead to partial complementation, a dominant-

negative mechanism can be hypothesized. In the presence of both a wild-type SOP2 protein

and an altered *sop2-1D* gene product (such as in all transgenics), the latter may act

antagonistically. Considering the range of intermediate traits in the transgenic lines, this

interference could be dosage-dependent.

4.3.3 SOP2 and SOP2h are plant-specific proteins

In silico BLAST analysis identified 32% amino acid identity of SOP2 with *At5g05240*-encoded

SOP2h, a homolog likely arisen from a genome duplication event (Tang *et al.*, 2008; Altschul *et al.*, 1997). Sequence homology between the proteins is scored throughout the alignment,

however little evidence towards a functional domain could be found. A putative twenty-two

amino acid coiled-coil domain starting at position 452 (SOP2) and 443 (SOP2h) respectively, is

predicted by MIPS (Fig. 10 (Rattei *et al.*, 2010)). When we searched public data bases, we

identified further homologous sequences using the NCBI and PLAZA platforms, the latter being

a resource for plant genomes (Proost *et al.*, 2009). Similarity with hypothetical proteins present

in plant species ranging from established crop plants to newly sequenced cacao and strawberry

were established and relationships evaluated using maximum likelihood analysis (Fig. 11).

Whereas SOP2 homologs are present in a variety of plant species, none were found in species

outside the plant kingdom. Despite PRL1 conservation in eukaryotes, it appears that SOP2 and

SOP2h may have a plant specific function.

4.3.4 SOP2 and SOP2h are not essential for plant defence

Arabidopsis transcriptome analysis using the AtGenExpress data set revealed no impact of

pathogen exposure, abiotic stress or developmental state on the expression of *SOP2* (Appendix

5). To examine potential involvement of *SOP2* and *SOP2h* in plant immunity signalling, we

investigated whether plants with mutations in these genes would exhibit compromised

resistance. We ordered insertional loss-of-function mutants *sop2-2* (Salk_058710) and *sop2h-1* (Salk_121713c) and obtained the *sop2-1D* single mutant through crossing of the *prl1-2 sop2-1D* double mutant with a Col-0 wild-type plant and subsequent PCR-based genotyping in the F2 generation. Morphologically, neither of the two recessive T-DNA alleles nor the dominant *sop2-1D* allele caused any obvious morphological defects in singe mutant analysis (Fig. 12A,B). Similarly, when challenged with bacterial pathogen *P.s.m.* ES4326, all three single mutants sustained normal levels of bacteria three days after inoculation (Fig. 12C).

Genetic redundancy between homologous proteins is considered responsible for the absence of phenotypes in the majority of *Arabidopsis* single mutants (Briggs *et al.*, 2006).. To investigate, whether the loss of both homologs would produce a mutant phenotype, we crossed s*op2-2* and *sop2h-1* single mutants and identified the *sop2-2 sop2h*-double mutant in the F2 by PCR-based genotyping. Transition to flowering was induced slightly earlier in *sop2-2 sop2h-1* plants (data not shown) which is a mild phenotype we observed. The approximately wild-type like double mutant however did not show deficiencies in defence mechanisms after inoculation with *P.s.m.* ES4326. Bacterial colonization levels were similar to those reached in *sop2-2*, *sop2h-1* and in wild-type plants thereby ruling out an essential involvement of SOP2 and SOP2h in plant defence (Fig. 12B,C).

4.3.5 Dosage-dependent suppression of prl1-2 by a loss-of-function mutation in SOP2 supports dominant-negative activity of sop2-1D

Since the dominant *sop2-1d* allele suppresses *prl1* phenotypes, we were interested in finding out whether use of the loss-of-function allele (*sop2-2)* would restore wild-type phenotypes as well. Phenotypic changes were already observed in the F1 generation of the cross, among the plants with homozygous *prl1-2* - and heterozygous sop2-2/SOP2 configuration. A representative plants produced segregation ratios of 45:19:22 (intermediate:WT-like:*prl1*-like), suggesting

semi-dominance of the *sop2-2* allele (expected 2:1:1, χ^2 = 0.4, P = 0.82). PCR-based genotyping identified homozygous *prl1-2 sop2-2* mutants among the plants with wild-type traits and intermediate specimen as plants with heterozygous *sop2-2/SOP2* configuration (Fig. 13A). The observed genetic ratios and phenotypes indicate dosage-dependent suppression of *prl1-2* by loss-of-function of *SOP2*.

To test whether a homozygous *sop2-2* genotype is functionally equivalent to *sop2-1D* activity, we compared resistance levels displayed by *prl1-2 sop2-1D* and *prl1-2 sop2-2* double mutants three days after inoculation with the established virulent inoculum. As shown in Fig. 13B, loss-of-function of *SOP2* restored wild-type levels of resistance in *prl1-2 sop2-2* double mutants, comparative to the defence output displayed by *prl1-2 sop2-1D* plants. This trend is consistent with morphological data showing similar root lengths for wild-type, *prl1-2 sop2-1D* and *prl1-2 sop2-2* double mutants plants (Fig. 13C). In contrast, mutating the SOP2 homolog did not affect morphology nor defence and this could indicate functional diversification of SOP2h-1 (Fig. 13B,D).

Taken together, our findings are consistent with semi-dominant suppression of *prl1* mutant phenotypes by *sop2-2*, which further strengthens our hypothesis of a dosage dependent dominant-negative mechanism for *sop2-1D.*

4.3.6 SOP2 gene expression is only slightly altered in MAC mutants

Public databases rank *SOP2* expression at 25% and *SOP2h* at 15% of average deposited gene profiles. In contrast, MAC genes *PRL1, MOS4, AtCDC5, MAC3a/MAC3b* and *MAC5a* are highly expressed, at levels ranging from 1.4 to 2.8 times the average gene expression (Ace View: www.ncbi.nlm.nih.gov/IEB/Research/Acembly). To address a potential role of PRL1 or the MAC in regulating *SOP2* gene expression we examined *SOP2* mRNA levels in *prl1-2* as well as in

mos4-1, *atcdc5-2*, *mac3a mac3b* and *mac5a* plants. Quantitative RT-PCR revealed unchanged levels of transcript in *mos4-1* and *mac5a* mutants whereas slight elevated expression was detected in *atcdc5-2* and *mac3a mac3b* mutants. In contrast, *SOP2* expression in *prl1-2* mutants is reduced, however not below 35% of wild-type levels (Fig. 13E). Suppression data thus do not support substantial regulatory control of MAC member over *SOP2* gene expression although a minor role of PRL1 and potentially AtCDC5 might be possible.

4.3.7 SOP2 is a nuclear protein

Since PRL1 and other members of the MAC localize to the nuclear compartment, a corresponding localization for SOP2 is a reasonable hypothesis (Palma *et al.*, 2007; Nemeth *et al.*, 1998). Recently, SOP2 was found in a large-scale analysis of chloroplast preparations, however current prediction programmes do not detect a reliable sorting signal for this compartment (Zybailov *et al.*, 2008). Using an algorithm which specializes on importin α-dependent nuclear import however, a putative nuclear localisation signal (NLS) was calculated for positions 50 to 79 of the SOP2 amino acid sequence, supporting co-localisation with MAC members (Fig. 10) (Kosugi *et al.*, 2009). To visualize the intracellular distribution of SOP2, we created a C-terminal SOP2-GFP fusion construct encompassing 1kb of 5' regulatory sequence and the *SOP2* open reading frame. Transformation of the construct into *prl1-2 sop2-1D* resulted in six T1 transgenics, four of which exhibited *prl1*-like or intermediate phenotypes (Fig. 14A). Complementing, BASTA® resistant T2 progeny of *prl1*-like GFP-lines exhibited susceptibility similar to *prl1* single mutants in infection experiments demonstrating, that the SOP2-GFP fusion protein functions properly and should be localized to its normal subcellular compartment (Fig. 14B). Although observed SOP2-GFP fluorescence was very weak, the fusion protein was detected in the nucleus using confocal microscopy (Fig. 14C). Low intensity of the GFP signal is probably due to low expression levels mediated by the genomic *SOP2* promoter.

4.3.8 sop2-2 does not suppress MAC mutations atcdc5-2 or mos4-1

To test whether mutations in *SOP2* affect signalling mediated by other MAC components, we examined double mutants of *sop2* crossed with loss-of-function alleles of transcription factor-encoding *CELL DIVISION CYCLE 5* (*AtCDC5*) and predicted structural member *MOS4* (Palma *et al.*, 2007). Since both *sop2-1D* and *sop2-2* were able to suppress *prl1* phenotypes, we decided to use the insertion allele *sop2-2* which allowed efficient PCR-based genotyping of *atcdc5-2 sop2-2* and *mos4-1 sop2-2* double mutants. If SOP2 functions in a MAC dependent pathway, the double mutants should resemble a wild-type plant analogue to the suppression phenotypes observed in *prl1-2 sop2-2* plants. However, *atcdc5-2 sop2-2* double mutants resemble *atcdc5-2* single mutants, indicating that ATCDC5 and SOP2 act independently (Fig. 15A). Similarly, *mos4-1* associated phenotypes such as broad leaves and a late flowering phenotype are still evident in the *mos4-1 sop2-2* double mutant (Fig. 15B). Considering that a mutation in *SOP*2 solely suppresses *prl1* phenotypes and does not seem to impact signalling in *atcdc5-2 sop2-2* and *mos4-1 sop2-2* plants, a function of SOP2 specific to PRL1 appears likely.

4.4 Discussion

We identified the dominant *sop2-1D* mutant from a *prl1* suppressor screen aimed to identify signalling components downstream of the MAC, a multi-protein complex with a role in plant immunity. We found that *SOP2* functions in *PRL1*-dependent signal relay but independent of two other MAC genes, *AtCDC5* and *MOS4*. This suggests that SOP2 is not a target of the MAC but rather specific to PRL1. The PRL1 protein may operate only temporarily as part of the MAC, a hypothesis that is consistent with research demonstrating PRL1 activity in sugar, hormonal and abiotic stress related pathways (Flores-Perez *et al.*, 2010; Baruah *et al.*, 2009; Abraham *et al.*, 2003; Nemeth *et al.*, 1998). If PRL1 functions as a flexible facilitator of multiple protein interaction, SOP2 may in fact be part of yet another complex.

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Mutations in *PRL1* result in compromised resistance, a phenotype that is no longer observed in plants that also carry defects in the *SOP2* gene. In mechanistic terms, PRL1 acts as a positive regulator and is required for sound resistance; SOP2 on the other hand appears to contribute negatively to resistance. Increased resistance towards fungal pathogen *Golovinomyces cichoracearum* (powdery mildew) and upregulation of defence related genes has been established for *edr1* (*enhanced disease resistance 1,)* a negative regulator of resistance in *Arabidopsis.* The gene encodes a protein kinase possibly targeting transcription factors to the proteasome (Christiansen *et al.*, 2011; Frye *et al.*, 2001). Transcription factors themselves also may act as negative regulators; loss of WRKY11 and WRKY17 increases resistance to virulent and avirulent *P.s.t.* strains (Journot-Catalino *et al.*, 2006). However, neither dominant *sop2-1D* nor recessive *sop2-2* mutants displayed any signs of compromised resistance (increased or decreased) in our infection assays, which leaves the nature of *SOP2* contribution to resistance currently unsolved.

A possible explanation for a lack of phenotypes might be functional redundancy between *SOP*2 and other homologs. We included a mutant allele of the only homolog *SOP2h-1* and the *sop2-2 sop2h-1* double mutant in our analysis to address redundancy but again recorded defence outputs similar to wild-type plants. *mac5a*, a putative RNA binding protein and component of the MAC does not exhibit susceptibility in pathogen infections but partially suppresses *snc1*-associated phenotypes suggesting a contributing function in plant immunity (Monaghan *et al.*, 2010). A *snc1 sop2-2* double is currently generated in our laboratory and should address involvement of SOP2 in the constitutive defence response triggered in this mutant background.

Expression levels of *SOP2* in a wild-type plant are approximately 80% lower than those of *PRL1*. Further reduction of functional protein amounts revealed a dosage-dependent relationship between *SOP*2 and *PRL1* in heterozygous mutant plants and identified the *sop2-2* loss-of-function allele as semi-dominant. We have thus identified a haplo-insufficient gene

acting in *PRL1* signalling, for which the product of both alleles is needed to confer normal operations (Mao *et al.*, 2011; Wang *et al.*, 2008; Pillitteri *et al.*, 2007). Intriguingly, the phenotype is only visible in the *prl1* mutant background. Semi-dominant as well as dominant mutations have often been found in resistance genes, i.e. NB-LRR type R-proteins SSI4, SNC1, SLH1 and CHS3 in *Arabidopsis* and NLS1 in rice (Tang *et al.*, 2011; Yang *et al.*, 2010; Noutoshi *et al.*, 2005; Zhang *et al.*, 2003; Shirano *et al.*, 2002). In these cases, sequence modifications usually led to upregulation of signalling responses and increased resistance, none of which were observed in *sop2-2*. Additionally, as a genetic suppressor of *prl1*, the *SOP2* gene likely functions further downstream in a signal cascade.

Since the insertional T-DNA allele *sop2-2* suppressed *prl1* phenotypes, we hypothesized that *sop2-1D* was in fact also a loss-of-function mutation, however dominant. We observed a range of intermediate phenotypes among transgenic *pr1-2 sop2-1D* expressing wild type *SOP2* and in *prl1-2* mutants expressing *sop2-1D.* These phenotypes suggest a *sop2-1D*-encoded protein causing a dosage-dependent dominant negative effect. Association of SOP2 with other proteins in form of an oligomer or complex is one hypothesis that could explain the displayed intermediate morphology in transgenics as well as dosage dependency of *prl1* suppression. Full functionality of a putative multi-subunit conglomerate might rely on sufficient amounts of SOP2 and such an oligomer could be sensitive to a spoiler version of SOP2. Increased amounts of one complex component would not be expected to result in a mutant phenotype due to stoichiometric interactions and is consistent with an observed absence of phenotypes in *SOP2* overexpressing lines. The observation, that *SOP2* is expressed at very low levels in the plant yet able to suppress *prl1* signalling completely when defect, may further support this hypothesis.

Nineteen proteins of the MAC share homology with proteins of the yeast NINETEEN COMPLEX (NTC) and human complex CELL DIVISION CYCLE 5-LIKE-SENESCENCE EVASION FACTOR (CDC5L) (Monaghan *et al.*, 2009). In the latter two systems, complex members are

either components of or are associated with the spliceosome, a connection that likely extends to the plant complex (Johnson *et al.*, 2011). We hypothesized that targets of the MAC would be found among similarly conserved proteins; however homologs of SOP2 appear to be plant line exclusive. Using the PLAZA platform we identified homologous sequences in crop plants such as rice, soybean, apple, corn and cacao but also in poplar and in the model legume plants *Medicago truncatula*, *Lotus japonicas* and the grass *Brachypodium distachyon* (Proost *et al.*, 2009). Although PRL1 is conserved among eukaryotes, its binding partners need not necessarily fall into the same category. WD40 proteins are versatile in their interactions with other proteins (Stirnimann *et al.*, 2010). The majority of amino acid sequence of both PRL1 and close homolog PRL2 are taken up by seven WD40 repeats which assemble into a flexible binding interface (Xu and Min, 2011). Both PRL1 and close homolog PRL2 likely interact with a range of proteins and seem to be a more likely signalling partner of SOP2 by themselves than the conserved complex as a whole (Weihmann et al. submitted).

We detected SOP2-GFP fluorescence in the nucleus in line with a proposed NLS signal situated among the first 51 N-terminal amino acids. However, with a molecular weight of 58kD, SOP2 falls within the diffusion limit of the nuclear pore complex and does not necessarily need an NLS (Nardozzi *et al.*, 2010; Wang and Brattain, 2007). Localising to the same subcellular compartment, SOP2 and PRL1 could potentially interact directly. Although we did not confidently identify a known domain among the SOP2 and SOP2h protein sequences, a novel plant specific motif might have not been recognized.

PRL1 belongs to a subset of WD40 proteins that also contain DWD (DDB1 binding WD40) motifs. Named after the UV-DAMAGED DNA BINDING PROTEIN1, this domain is credited to facilitate binding between DDB1 and diverse receptor proteins, including PRL1, as part of the *Arabidopsis* CUL4 E3 ubiquitin ligase (DCX - type)(Zhang *et al.*, 2008; He *et al.*, 2006). It is tempting to speculate a role for SOP2 in the ubiquitin proteasome pathway. We did not find a

strong correlation between PRL1 protein and *SOP2* transcription levels however association

between SOP2 and PRL1 remains to be explored.
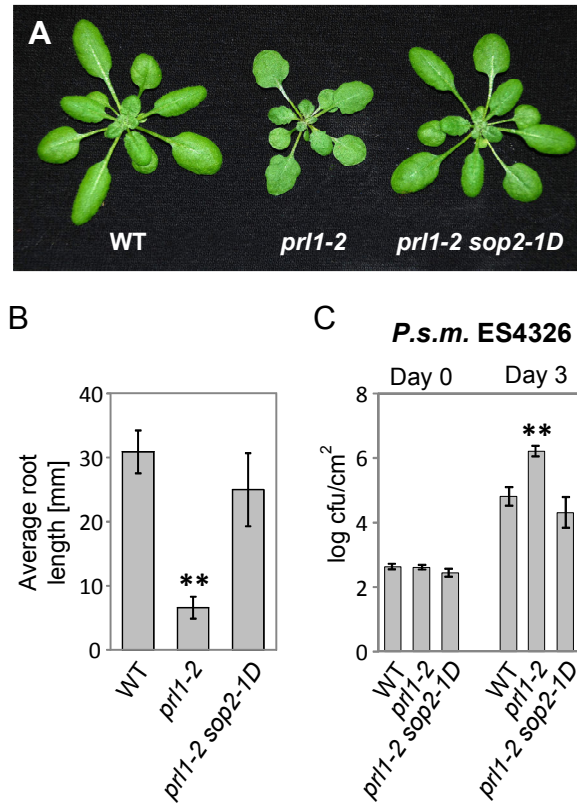
Figure 8. *prl1* **phenotypes are suppressed in** *prl1-2 sop2-1D*
(A) Morphology of 5-week-old soil grown plants of WT (Col-0), *prl1-2* and *prl1-2 sop2-1D*. (B) Root length analysis of 1-week-old seedlings of indicated phenotypes. The results represent an average of 10 seedlings each ±SD. (C)Infection of 5-week-old plants (OD_{600}= 0.0001) of indicated phenotypes and quantification of titer at 0 and 3 days post inoculation. Values represent averages of six replicates ±SD. Statistical significance compared to Col-0 control was calculated using Student's *t*-test: **P < 0.0001 for both graphs.
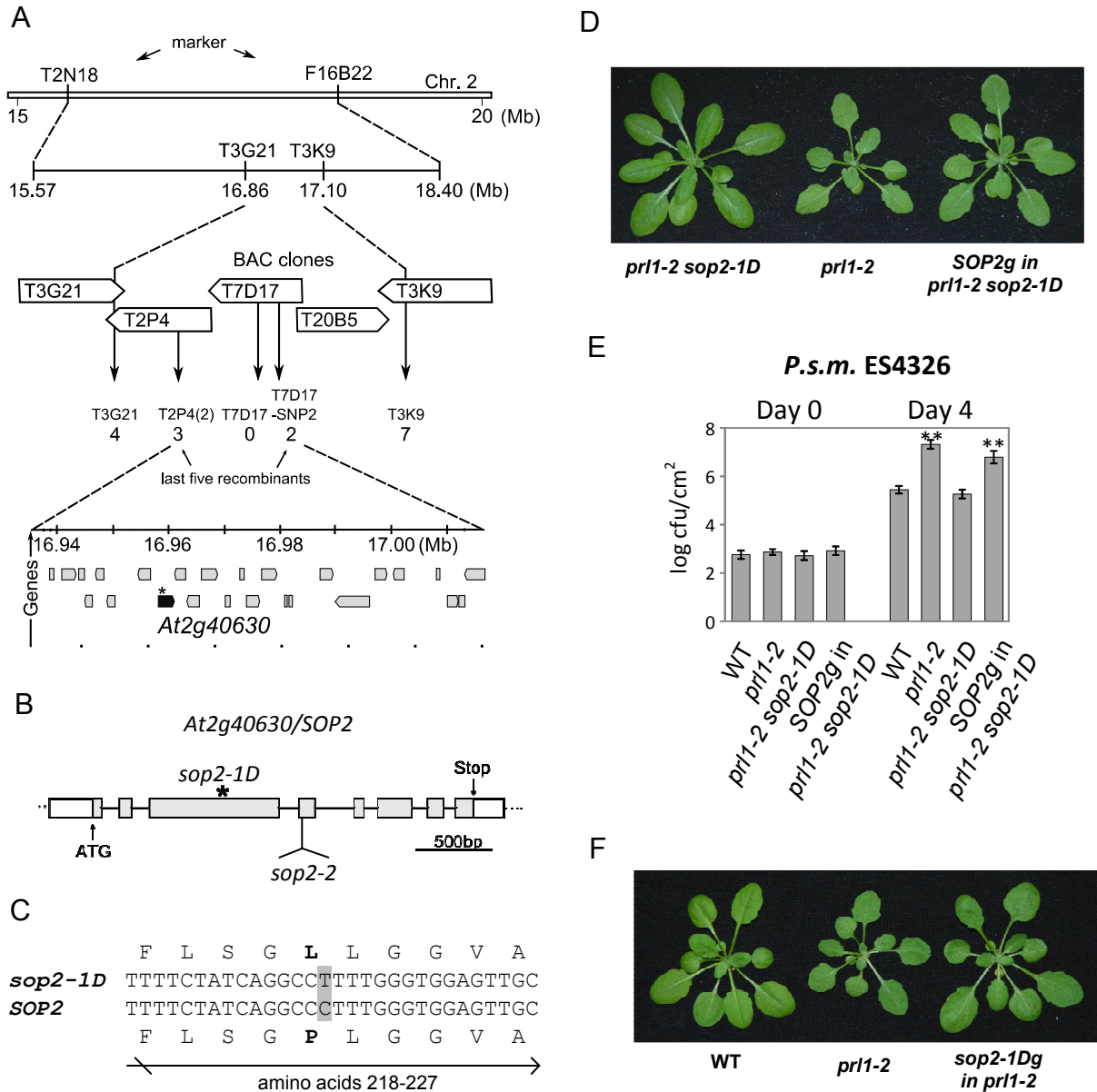
Figure 9. Map-based cloning of *sop2-1D*
(A) Positional mapping locates *sop2-1D* on the bottom arm of chromosome 2. Indicated are recombinants, markers and BAC clones as well as their respective sequence-anchored positions. A mutation (*) was identified in *At2g40630*. (B) *At2g40630* was named *SUPPRESSOR OF PRL1, 2* (*SOP2*), the *sop2-1D* mutation is located in the third exon. The T-DNA insertion site in sop2-2 (Salk_058710(C) Sequence analysis reveals a C to T transition in *prl1-2 sop2-1D* resulting in an amino acid change from proline to leucine. (D) Morphology of *prl1-2 sop2-1D, prl1-2* and transgenic *prl1-2 sop2-1D* mutants expressing the genomic wild-type *SOP2* gene. (E) Bacterial infection of the indicated phenotypes with *P.s.m*. ES4326. Plants were inoculated with a low dose (OD_{600}= 0.0001) and titer quantified at 0 and 4 days past inoculation. Values represent averages of six replicates ±SD, Infections were repeated four times with similar results. Statistical significance relative to the wild-type was calculated with a Students *t*-test: **$P < 0.0001$ (F) Morphology of Col-0*, prl1-2* and transgenic *prl1-2* mutants expressing the genomic *sop2-1D* mutant allele (T1).

```
SOP2     1  MEEQRGISSGVVSEPASNSVISGEKRNGNGLDEKDELGSKRVKVPDLASDAKTSSLQSHG
SOP2h    1  MEEHDAVSGGCSPIPRSDLKIGG-----NELDERQELSKIEPVTPTLNSGNTVHGSKDVE
                              bipartite NLS

SOP2    61  NSNSVQQPNLSSEKLSKVSKVLVAPDAEGIRRVVRENDVLSKDIKPSSTVETRTYLPKAK
SOP2h   56  KANGMSQVGKVSESFVTE--------------------GKNIKTELLVGQEVKSSSSK


SOP2   121  SISTDDNRRVVNSGKQALLENHTVKTDDSKCRVVKNISLLKPRETTESVVSQRGAAEPSV
SOP2h   94  LVLNTD----VDCDKRALLKRCDNADAVSSCLNDDLTSVCSSRISQKTSS----------


SOP2   181  SVPVGDKVSPFQMCSSADGSLGESDSMRRWREMKRNGFLSGPLGGVAAPTSTVVTTP---
SOP2h  140  -------MDVYSECGSSNGSVAKRDPMKVWTEMKQNGYLSNPNGGISTTSSSCLISSSHG


SOP2   238  -VEVPAPKQQKNKRRGGESLKKKNDVPKKEQQLVDRFANVTAPSGLLTELNPGIINHVRT
SOP2h  193  GIPAPKKRGRKTKINNDAAVAKKRKIERKEE--VDRFARLAAPSGLLNELNPGIINHVRN
                            bipartite NLS

SOP2   297  KKQVCSIIEALIRS--------------SNDDATTRERHGDFN----------------
SOP2h  251  KKQVLSIIENIVKSERDAGNYHSTLRHSNSADGSPRKNLGDACRSEFYQVFQYALPKDMY


SOP2   326  ----------------VRDAIREDRALAFKLPSTGVSDNAISITNPEQATSLAVEAATVA
SOP2h  311  SMRYYAEKCADDEFSEENNTVRSRFQVAGKFSENDSSLSSEDASDLNSASVLTVNAATVA


SOP2   370  SQWLEFLQQDLSGRLSAVQDSRNRVQNILTTELPLLASSRESSSNQANSLEMVTTNTSGD
SOP2h  371  SQWLELLHQDIKGRVSALRRSRKRVRAVVTIELPHLIR-KEFPADQENDPTLLG-----
                                     coiled-coil domain

SOP2   430  ASSDKAATETHQKRWTAKEDQINKALYDEQRDLERSLNQVKEMQSRCNHGLRQMEEYSPF
SOP2h  425  GASKASTVDIHKSRWMTLFKQLEHKLSEEESQLESWLNQVRYMQSHCDEGLQHLSLSSGQ
                                     coiled-coil domain

SOP2   490  SSQSS----DSSFGKDGNQETSMAVQAAAASIFSTCSFLLSMMKPPPTGS
SOP2h  485  NFLQLGMPLDSRAANALISDKDLVIKAAAASIYSTCSFLEENITCS----
```

Figure 10. **SOP2 and SOP2h are homologous proteins**
Sequence alignment with identical amino acids shaded in black and similar amino acids indicated in light grey. Predicted nuclear localization signals (NLS) and coiled-coil domains are shown for SOP2 above and for SOP2h below the alignment (Kosugi *et al*., 2009; Rattei *et al*., 2010).
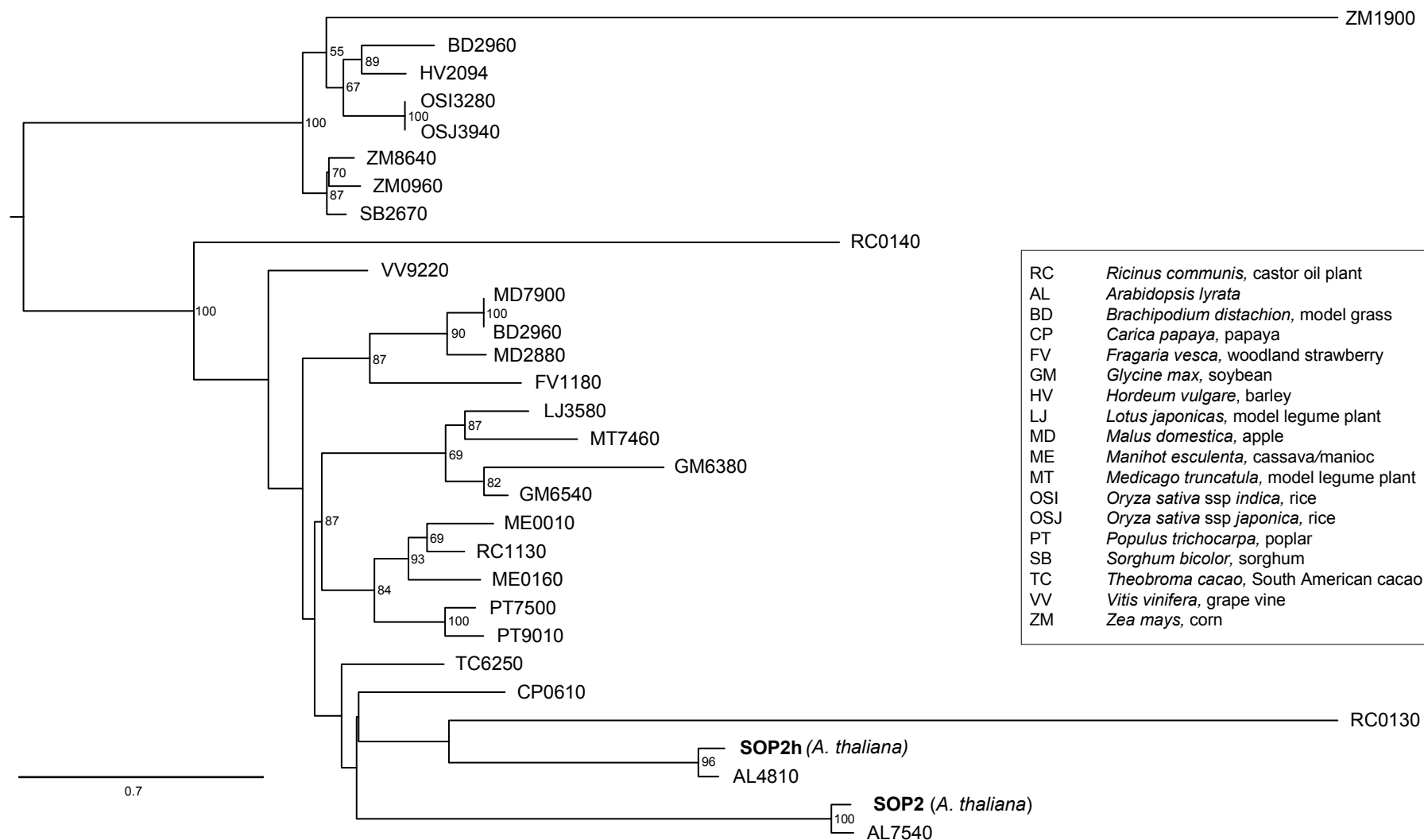
Figure 11. **Phylogenetic analysis of SOP2 homologs in plants**

A phylogenetic tree based on maximum likelihood analysis was constructed from 31 plant protein sequences. Numbers indicate bootstrap support values expressed as a percentage for 500 bootstrap replicates; bootstrap supports below 50% are not shown. The scale represents the rate of amino acid substitutions. The tree was rooted at midpoint. For details, see Materials and Methods

A

WT   *sop2-2*   *sop2-1D*

B

WT   *prl1-2*   *sop2-2*

sop2h-1   *sop2-2 sop2h-1*

C

***P.s.m* ES4326**
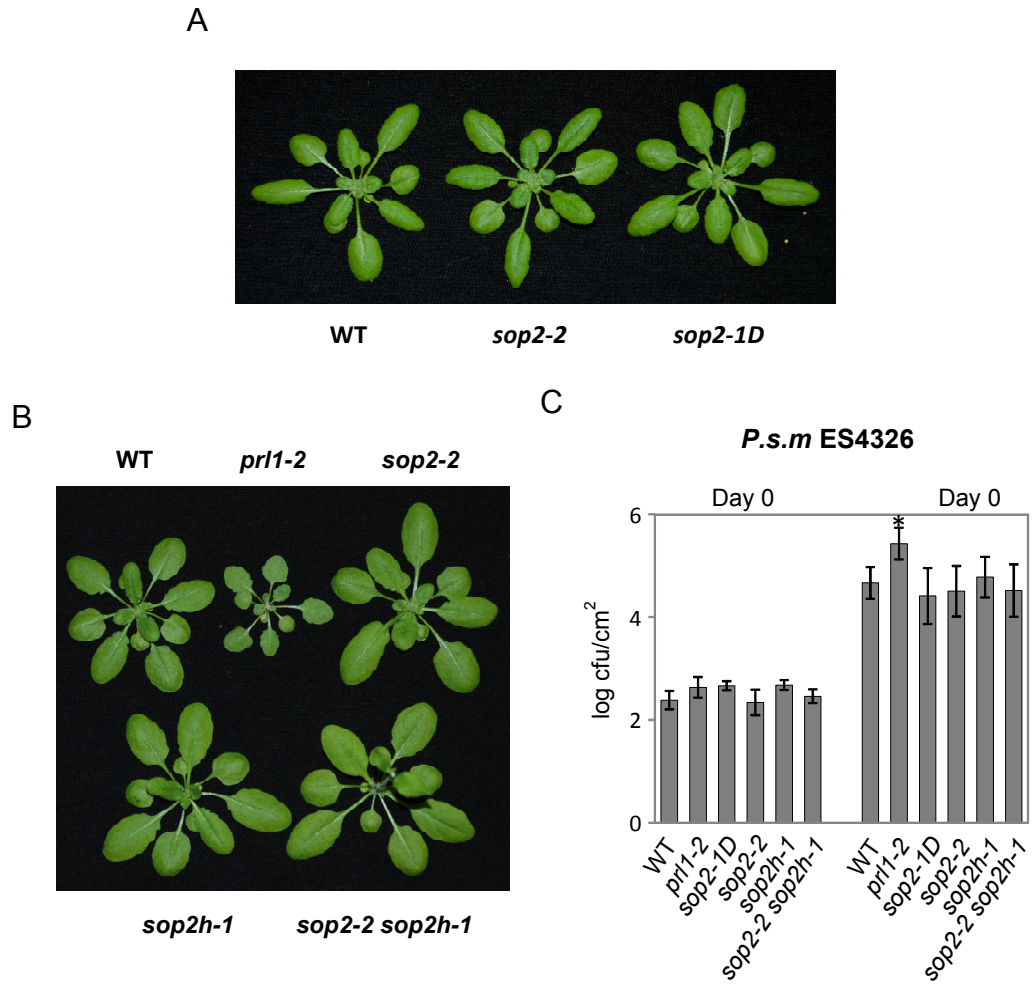
Figure 12. **Mutations in *SOP2* and *SOP2h* do not cause morphological or disease-related phenotypes**

(A) and (B) Morphology of 5-week-old soil-grown plants of indicated genotypes. (C) Infection of Col-0, *prl1-2*, *sop2-1D, sop2-2, sop2h-1* and *sop2-2 sop2h-1* mutants with virulent *P.s.m.* ES4326. Leaves were inoculated with a low dose (OD_{600}= 0.0001) and bacterial titer quantified at 0 and 3 days post inoculation. Values represent averages of six replicates, statistical significance relative to the wild-type plant was calculated using Students *t*-test: *P < 0.002. Experiments were repeated at least three times with similar results.
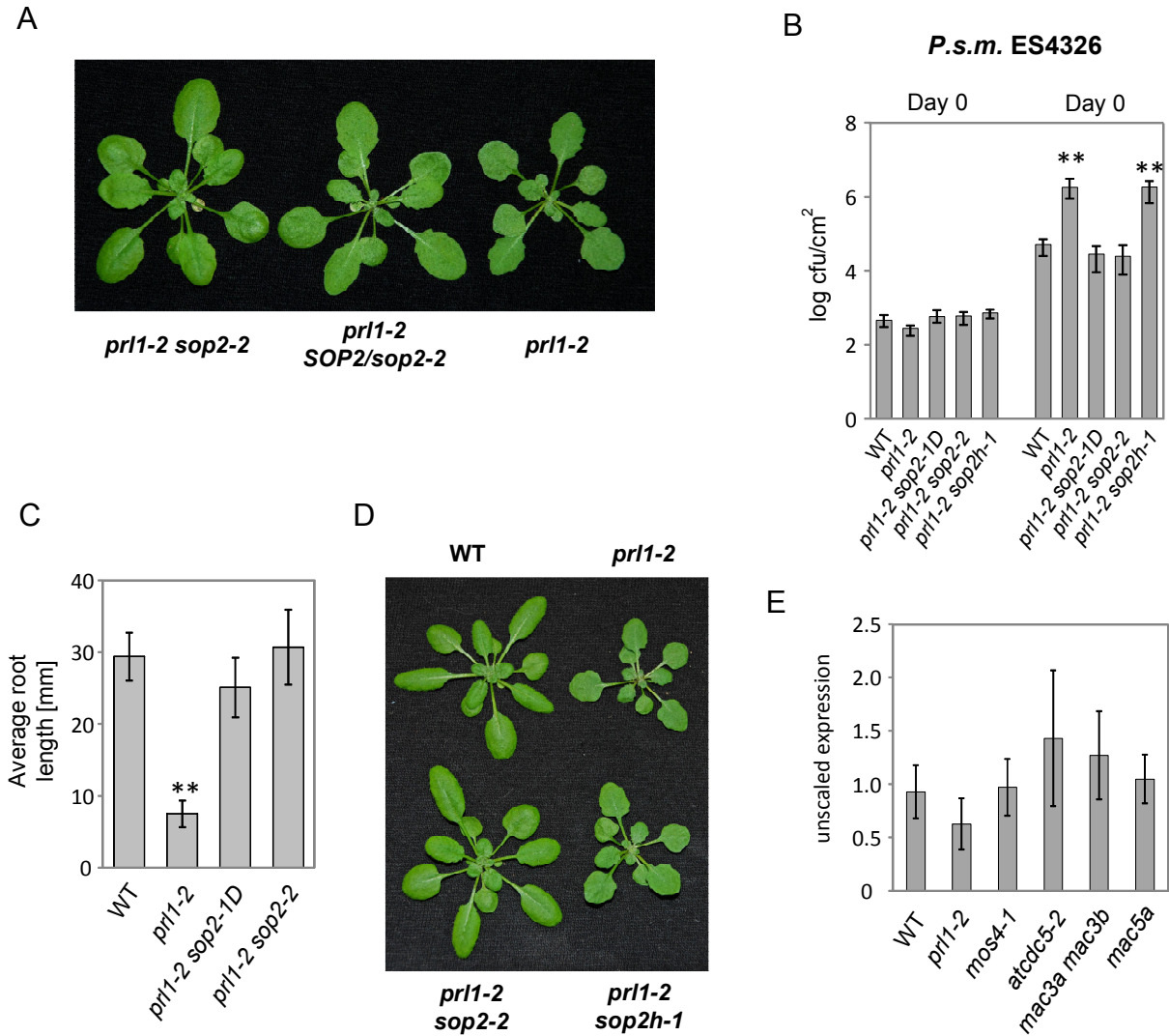
Figure 13. **sop2-2 is a semi-dominant suppressor of *prl1*-phenotypes**
(A) Morphology of five-week-old soil-grown plants of *prl1-2 sop2-2, prl1-2 SOP2/sop2-2* and *prl1-2.* (B) A low dose (OD_{600}= 0.0001) of *P.s.m.* ES4326 was infiltrated into rosette leaves of indicated phenotypes and bacterial titer quantified after 0 and 3 days past inoculation. Values represent an average of six replicates ±SD. Analysis was repeated at least three times. (C) Root length analysis of 1-week-old seedlings of indicated phenotypes. The results represent an average of 10 seedlings each ±SD. Experiment was repeated twice. (D) Morphology of 5-week-old soil-grown plants of indicated phenotypes. *prl1-2 sop2-2* is phenotypically wild-type whereas the *prl1-2 sop2h-1* mutant resembles *prl1-2.* (E) Quantitative RT-PCR analysis of *SOP2* expression in MAC mutants. Values are normalized to tubulin expression. Experiment was repeated twice with similar results. Statistical significance for (B) and (C) was calculated using a Students t-test: **P < 0.0001 for both graphs.

A

prl1-2 sop2-1D   *SOP2-GFP in prl1-2 sop2-1D*

B

P.s.m. ES4326

C

PI   GFP   Merged
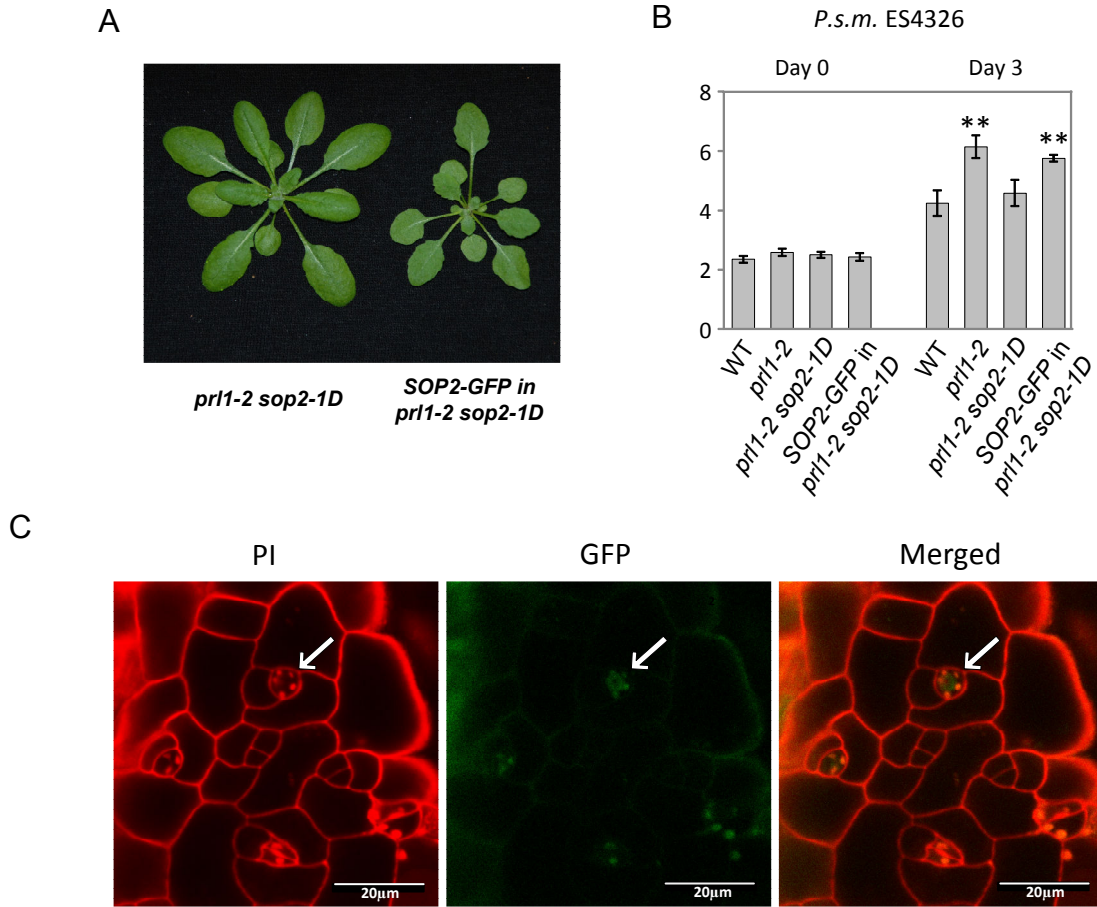
Figure 14**. **SOP2 localizes to the nucleus
(A) Morphology of *prl1-2 prl2-1D* mutant and transgenic *prl1-2 prl2-1D* plants expressing a genomic SOP2-GFP fusion protein (B) Bacterial infection of the indicated phenotypes with virulent *P.s.m.* ES4326. Statistical significance was calculated using a Students t-test: **P < 0.0004 (C) Fluorescence in guard cells of transgenic plants was observed using confocal microscopy, cell walls were stained using propidium iodine (PI).
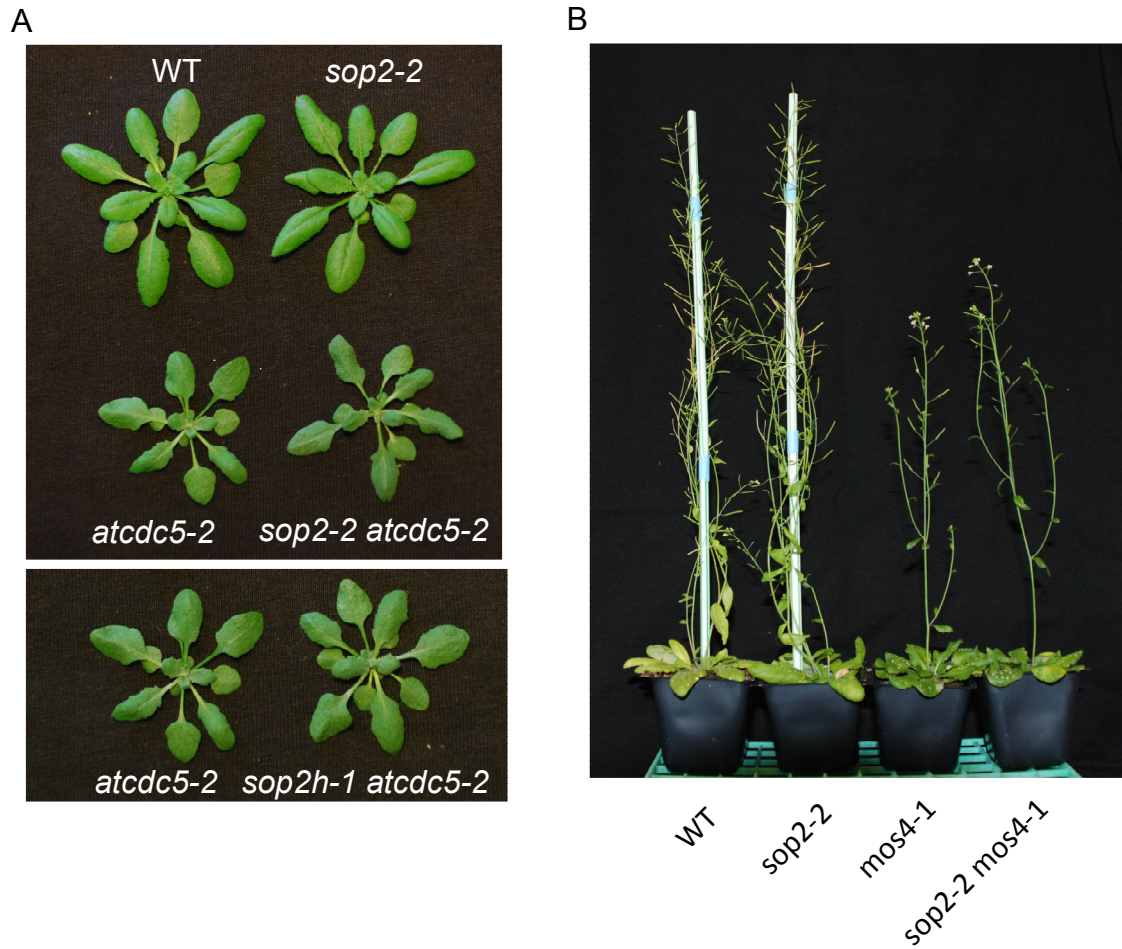
Figure 15. *sop2-2* **does not affect mutant phenotypes of** *atcdc5-2* **or** *mos4-1*
(A) Morphology of five-week-old soil- grown plants of indicated phenotypes. (B) Morphology of fully grown Col-0, *sop2-2, mos4-1* and *sop2-2 mos4-1* plants.

5   *Concluding discussion and outlook*

The main goal of this thesis is to deepen our understanding of signalling steps leading to plant resistance. In particular, signalling associated with PRL1, a member of the MAC, has been at the centre of the presented research.

5.1   *Suppressors of prl1 signalling*

We set out to genetically dissect *PRL1* signal relay since we hypothesized a considerable contributing role of this protein to plant defence. To find new components of the desired pathway, we carried out two suppressor screens with the goal of introducing mutations in genes downstream of *PRL1* and potential targets of the MAC. Chronologically, the EMS screen was executed first. We succeeded in producing both complete and a range of partial morphological suppressors, most of which displayed restored R protein mediated resistance when exposed to an established *Arabidopsis* pathogen thus demonstrating strong correlation between morphological and disease-related phenotypes.

During preliminary examination of the complete suppressors, we established dominant inheritance for two of these mutants. This finding prompted us to execute a second screen based on *Agrobacterium* mediated insertional T-DNA mutagenesis. The rationale for this decision lies in the accelerated cloning possibilities for dominant mutations caused by a loss-of-function mechanism. As introduced in Chapter 2, a portion of *Arabidopsis* genes fall into the haplo-insufficient category thus causing a dominant or semi-dominant phenotype with functional loss of already one allele copy (Mao *et al.*, 2011; Wang *et al.*, 2008; Pillitteri *et al.*, 2007).

Haplo-insufficient genes in a pathway can also be identified through insertional mutagenesis followed by PCR-based protocols, referred to as T-DNA tagging. This cloning approach can

identify a mutated haplo-insufficient gene faster than conventional map-based cloning techniques. It is unfortunate that we were not able to obtain mutants through this screen. Closer examination of *SOP2* revealed this gene to be indeed haplo-insufficient and thus it could have been identified through T-DNA tagging.

Optimized environmental conditions are vital to the success of a screen, and they were not ideal in our studies because soil-borne an air-borne pathogens infested our growth chambers. These circumstances visibly increased stress levels in *prl1* plants, which are both stress sensitive and highly susceptible. Considering these observations, it becomes clear that the choice of mutagen is important when dealing with mutants that are less robust than a wild-type plant. Transformation efficiencies are affected by the health of the plant and thus chemical (EMS) mutagenesis, carried out during the seed stage, is recommended for future screens using sensitive mutants. Additionally, single nucleotide polymorphisms often produce a range of mutation, i.e., we created not only a dominant negative but also a dominant gain-of-function mutant and a collection of recessive mutants.

The scope of this thesis does not include comprehensive complementation analysis which leaves the number of loci among the remaining *sop* mutants undetermined. The *SOP2* gene was sequenced in the three semi-dominant mutants *sop15*, *sop17* and *sop19* that we obtained, with the hope of finding additional alleles. However, the phenotypes in these mutants appear to be caused by mutations at different site(s). Further adding to the potential number of *sop* loci are the recessive mutants. Crosses among them should be undertaken to establish overall complementation group numbers for future mapping projects.

Although designed similar to the *snc1* screen, less affected loci are expected among the obtained *prl1* suppressors. The deregulated, predicted resistance protein in *snc1 is* positioned at the beginning of a defence network, constantly emitting danger cues (Zhu *et al.*, 2010; Li *et*

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al., 2001). PRL1, on the other hand, is a structural protein presumably functioning further downstream in the pathway and for which we used available recessive alleles in the screen. Since we are narrowing in on signalling steps further downstream in a signal cascade, the scope of defence components that are potentially involved also becomes understandably more limited.

5.2   *Analysis of PRL2*

The suppression caused by *sop1-1D/prl2-1D* is due to a dominant gain-of-function mutation in the homolog of *PRL1*. The mutation however is not located in the protein-coding open reading frame – as is the case for *snc1* - but rather just upstream of the translational start codon. Consequentially, the encoded PRL2 protein is unaffected. A gain-of-function mutation may alter a gene's function or expression patterns, in both cases diverging from the natural profile and resulting in a dominant phenotype. *sop1-1D/prl2-1D* is an example of a regulatory mutation, dramatically altering gene expression.

PRL2 had been previously identified as a close sequence homolog to *PRL1* and therefore functional overlap of the encoded proteins is a reasonable assumption (Nemeth *et al.*, 1998). However, in contrast to the pleiotropic effects displayed by *prl1* mutants, the *prl2* mutants that have been tested are indistinguishable from wild-type plants and thus have not allowed functional characterization of the homolog. Our attempts to prove that an allele of *PRL2* is the suppressor in the *prl1-2 sop1-1D* double mutant have been laborious. For unknown reasons we were unable to create a *PRL2* specific primer pair which would result in sufficient amounts of PCR product. By attempting to amplify only half of the *PRL2* gene, we determined that the genomic region upstream of the open reading frame - and in which left primers were binding - was problematic. It would be interesting to determine the cause of his problem. Is the chromatin

in this region organized tighter or differently in *prl1-2 sop1-1D* than in *prl1-2* or a wild-type plant? Could there be steric hindrance of the utilized polymerases?

These questions tie in with the yet unknown mechanism by which gene expression in *sop1-1D/prl2-1D* is enhanced. Potentially, a *cis*-acting (promoter) motif influencing transcription rate has been altered. Such an element could be involved in recruiting transcription factors or other related proteins to the *PRL2* gene. Enhanced binding of a factor or alternatively, reduced binding affinity for an inhibitor of transcription could be envisioned. Mutating a controlling motif may have substantial impacts on overall expression patterns.

Transcript levels could also be affected post-transcriptionally. If stability of a given mRNA molecule is increased, more protein product can be synthesized through repeated translation events. Fluorescence in situ hybridization or radioactive labelling of transcripts could be attempted to determine whether increased transcription or stability-enhanced RNA molecules are present in the *prl2-1D* mutant.

Using JAtY clone 69M23 and a transformation protocol that had been substantially modified during the T-DNA screen, we succeeded in creating transgenics that carried two *PRL2* alleles in the *prl1* homozygous background. Although only three transgenics were identified, the phenotypes supported our hypothesis and we proceeded with the comprehensive analysis of *PRL2*.

The two WD40 proteins are functionally equivalent, suggesting that the less conserved C-terminal regions are not essential for protein interaction. As previously noted, sequences adjacent to the *PRL2* open reading frame have however diversified significantly and are likely responsible for the observed unequal genetic redundancy. After a gene duplication event, expression patterns of one or both copies may be altered to compensate for undesired dose effects (Yang *et al.*, 2011; Schuster-Bockler *et al.*, 2010; Zou *et al.*, 2009). We did record

enhanced phenotypes in *prl1-2 prl2-2* double loss-of-function mutants, confirming at least residual activity of *PRL2* in a wild-type plant. Consequently, *PRL1* expression might be lower now than before the assumed duplication event, to accommodate for remaining *PRL2* activity. A dynamic relationship would also leave room for the development of specialized patterns, such as a suggested tissue specificity for *PRL2*.

Most likely due to low abundance, PRL2 has not been detected in the recent immunoprecipitation of the MAC (Monaghan *et al.*, 2009). Nonetheless, PRL2 is probably regularly incorporated into a small number of MAC complexes and acting interchangeable with PRL1 in resistance. Other phenotypes typical for *prl1* mutants, i.e. sugar sensitivity and stunted roots are also suppressed by *prl2-1D* which serves as evidence towards equivalence beyond defence signalling. PRL2 likely acts as an equally capable regulator of sugar, hormone and stress influenced genes, functions which have been repeatedly associated with PRL1 (Flores-Perez *et al.*, 2010; Baruah *et al.*, 2009; Li *et al.*, 2007; Bhalerao *et al.*, 1999; Nemeth *et al.*, 1998; Salchert *et al.*, 1998).

5.3   *Discovery of SOP2*

sop2-1D was pinpointed using a positional cloning approach and followed by candidate sequencing analogue to mapping of *prl2-1D.* A large number of conserved genes with predicted homologs across kingdoms were found among the twenty-five candidates present in the flanked region. We chose to sequence these genes first, in line with the evolutionary conservation of the MAC, however we did not find a sequence polymorphism. Thereafter we investigated genes with predicted functions conceivable as targets of PRL1 or the MAC. Eventually, the last two genes which were positioned closest to one of the flanking makers were analysed, and the *sop2-1D* mutation was found in a plant specific gene with unknown function. This finding was

unexpected and underlines the need for constant reassessment of hypotheses in our efforts to unravel pathways.

SOP2 does not fit our profile of a MAC target. Epistasis analysis later revealed that *SOP2* functions in *PRL1* signalling but is not part of *AtCDC5* or *MOS4* signal transduction, two other core MAC components. In this context, *SOP2* as a target of *PRL1* only is a more likely hypothesis considering the plant specific nature of the suppresser gene and the high level of conservation for the MAC as a complex. A versatile adaptor protein may facilitate interaction and interact with a range of partners, independent of their level of overall conservation. PRL1 is largely comprised of WD40 repeats, a prominent motif in plants (Stirnimann *et al.*, 2010).

All investigated members of the MAC are expressed at levels well above average gene data in the AceView database, PRL1 ranks at 140% of average levels. In contrast, *SOP2* and even more so, *SOP2h*, are expressed at very low levels. This added a complication to efforts of localizing a SOP2-GFP fusion protein and left us with the need to explain how mutations in *SOP2* are able to suppress *prl1* signalling so completely. Could a rate limiting step be involved in which a small number of SOP2 molecules control subsequent signalling? Are SOP2 and PRL1 associated as an oligomer? Or is this dependency not based on mutual protein activity? We tested transcription levels of *SOP2* in all core MAC mutants but did not find strong evidence of a regulatory relationship existing on the transcription level. In a next step, we have initiated a cross between transgenic *prl1-2 sop2-1D* plants expressing genomic SOP2-GFP and a double mutant of *MAC3a* and *MAC3b*, both MAC core components with predicted E3 ubiquitin ligases activity. If SOP2 is post-translationally modified by MAC3a/3b, absence of ubiquitination could lead to higher observable GFP fluorescence.

Despite our efforts, SOP2 function remains elusive. We ordered T-DNA mutants to examine whether loss-of-function would result in a mutant phenotype however single and double mutants

of *SOP2* and homolog *SOP2*h are largely wild-type in appearance. Additionally, none of the mutants exhibited increased resistance or related defects when exposed to pathogens. These findings argue against a possible role of the homologs as negative regulators. It should be established next, whether SOP2 functions directly in immunity signalling. All investigated members of the MAC, except for the untested relationship with *PRL1*, suppress *snc1* signalling fully or partially. S*NC1* and *PRL1* are located in close proximity on chromosome 4 and because of this genetic linkage, a double mutant could not be created for use in such an analysis (Palma *et al.*, 2007). Thus, examination of a *snc1 sop2-2* double mutant would address several questions: a) if wild-type morphology is at least partially restored, *SOP2* would be considered a component of *snc1* resistance signalling with a potential role in overall immunity and b) if this is the case, a so far theorized ability of *prl1* to suppress the constitutive resistance signalling in *snc1* would be assured.

Endorsement of SOP2 as a defence molecule should lead to further investigation, i.e. crossing the mutant with SA-deficient mutants and *npr1*, a major regulator in resistance. To clarify the role of SOP2 relative to SA - dependent signalling, measurement of SA levels in *sop2* mutants could be a first step. Also, crossing of *sop2* with *sid2* or *eds5* mutants could be attempted and resistance levels of double mutants analysed. To evaluate the relationship between SOP2 and NPR1, *sop2 npr1* or *sop2 npr1 snc1* mutants could be generated and levels of marker genes observed, i.e. *PR2/BGL2* expression is a hallmark of the NPR1-independent pathway.

Two lines of evidence support a dominant negative mutation in *sop2-1D*. Classical examples for this type of mutation have been factors that oligomerize or associate as homodimers and in which the mutant form acts as a spoiler protein. Disruption of structural polymers is another example (Veitia, 2007). *SOP2* however is also a haplo-insufficient gene, a phenotype that has often been found in enzyme-encoding genes and transcription factors (Qian and Zhang, 2008; Seidman and Seidman, 2002). In an effort to learn more about the *SOP2*-encoded protein, a

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yeast-two-hybrid or co-immunoprecipitation approach could be employed to identify potential binding partners. If SOP2 associates with other proteins, identities of such partners could serve as valuable clues towards SOP2 function.

PRL1 has been suggested to function as a substrate receptor, a part of the ubiquitin proteasome pathway in plants (Lee *et al.*, 2008; Farras *et al.*, 2001). Two DWD boxes, which are found embedded in the WD40 repeats, may be the motifs mediating interaction between PRL1 and DDB1 (DAMAGED DNA BINDING 1), a substrate adaptor for CULLEN 4-type (DCX) E3 ubiquitin ligases (Biedermann and Hellmann, 2011; Lee *et al.*, 2008; Lee and Zhou, 2007). E3 ligases are multi protein complexes and potentially susceptible to a spoiler subunit, i.e. a dominant negative form of SOP2. However, defects in genes of DCX complex members, *CULLEN 4*, *DDB1* and *RBX1* lead to mutant phenotypes which we did not observe in *sop2-1D* or *sop2-2* or in double mutants of *SOP2* and homolog *SOP2h (Lee et al., 2008; Lechner et al., 2002; Schroeder et al., 2002)*. Aside from being theoretically possible, participation of SOP2 in this important pathway is thus not supported from our data.

In conclusion, further investigation of *SOP2* will deepen our understanding of *PRL1* associated signalling in defence, dependent or independent of the MAC. Using the remaining *sop* mutants as a resource, a more complete picture of interactions facilitated by this WD40 protein can be revealed and might also lead to discoveries in signalling branches other than immunity.

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Appendices

Appendix 1: Arabidopsis transformation protocol

<u>*Arabidopsis* transformation (Spray method)</u>

(Adapted from Diana Hall)

*Working with the sensitive prl1 mutant, we observed high levels of seed abortion and general senescence in floral parts. This is probably due to the weight of the spray emulsion and exposure to high light and heat conditions in our growth chambers after spraying. To reduce these phenotypes and increase transformation efficiency, several modification were introduced (highlighted with * and **in bold**).*

- Grow 4 ml of O/N Agro culture 28-30ºC
- Innoculate 300 mls of LB (+ appropriate antibiotics) with 50µl of O/N Agro culture, let grow to OD_{600} 0.8-1.0 at 28-30ºC

1. Spin down 300 mls of culture at approximately 5,000 rpm in the Sorvall RC5C plus centrifuge (Rotor SLA-1500) for 15 minutes at RT

2. Resuspend in 300 mls of 5% sucrose and 0.01% wetting agent (Silwet)

3. Spray plants ***only briefly to minimize weight on aerial parts**

4. ***Place plants in a darker area for 2-3 days, without direct light from the top. Do not cover plants with plastic bag.**

5. ***Transfer back to growth chamber but leave rack lights off for 5-8 days** (Indirect light from neighbouring racks will trigger slower growth but less aborted seeds with desired mutations)

The sucrose is made up fresh just before use (not sterile).

Appendix 2: Molecular marker

InDel and SNP marker sequences used in the mapping of *sop1* and *sop2*

Marker	Chr.	Location	Polymorphism:	Sequence
K14A17-F	3	5.84Mb	Col: 397bp / Ler: 367bp 29bp InDel	5'-CCAAGCCTCTGCGTCTCTAC-'3
K14A17-R				5'-CTTTGTTTGAAGTCGCATCG-'3
MGL6-F	3	5.63Mb	Col: 330bp / Ler: 384bp 53bp InDel	5'-CTTCAGCGGCTTGCATCTAT-'3
MGL6-R				5'-CCCTTCATAACTAATTCCACACA-'3
MGL6-SNP1-Ler	3	5.66Mb	Col/Ler: T/C SNP (PERL0458534) 92bp fragment for both	5'-CTGGTACATTCACTTCCTTC-'3
MGL6-SNP1-Col				5'-CTGGTACATTCACTTCCTTT-'3
MGL6-SNP1-R				5'-GAATTCAATGCCTCCGGTTA-'3
MGL6-SNP5-Ler	3	5.69Mb	Col/Ler: G/A SNP (PERL0458860) 173bp fragment for both	5'-AGAATGATGGTGGAGCTGAT-'3
MGL6-SNP5-Col				5'-AGAATGATGGTGGAGCTGTC-'3
MGL6-SNP5-R				5'-AACCTAATGCGGTCTACTGA-'3
MDC8-F	3	5.58Mb	Col: 557bp/Ler: 461bp 96bp InDel	5'-TGGCAAACTTGTTGGGTTCC-'3
MDC8-R				5'-CATATGTGCCTTCAACTGCAG-'3
MIE1-A	3	4.87Mb	Col: 450bp/Ler: 318bp 132bp InDel	5'-CTAAGTTCTTCCACCATCTG-3'
MIE1-B				5'-CAAGGAGCATCTAGCCAGAG-'3
MRC8-F	3	6.21Mb	Col: 232bp/Ler: 246bp 14bp InDel	5'-GATGTCGGAATTGTCGATCG-'3
MRC8-R				5'-TCGCAGAAACCACACTAAACC-3'
T2N18-F	2	15.57Mb	Col: 299bp/Ler: 227bp 72bp InDel	5'-TTGGTCACTAGTAAGATCTTG-3'
T2N18-R				5'-GTCGTCTAGTGTACTTGTAGC-3'
T2P4(2)-F	2	16.94Mb	Col: 95bp / Ler: 110bp 14bp InDel	5'-CGTACGTGAGAGATATGCAA-'3
T2P4(2)-R				5'-ATCACCAGATGGAAGTCTTG-'3
T3G21-F	2	16.86Mb	Col: 469bp / Ler: 538bp 68bp InDel	5'-TTTCTTGGAAATTCGGGTTG-'3
T3G21-R				5'-AGTTTGAAGCCAAGCAAACG-3'
T3K9-F	2	17.10Mb	Col: 443bp / Ler: 355bp 87bp InDel	5'-TGGTGTTGACGAACTTCCAA-'3
T3K9-R				5'-TCGGAAGGAGCATTATGGAC-'3
T7D17-F	2	17.01Mb	Col: 249bp / Ler: 231bp 17bp InDel	5'-GGCGTTTTTAATGGCAGTTC-'3
T7D17-R				5'-GCATTTACGGAAGCAGAAGG-'3
T7D17-SNP2-Col	2	17.03Mb	Col/Ler: C/G SNP (PERL0398101) 270bp fragment for both	5'-CGTTCTTTGTCTCTCTCTTAC-'3
T7D17-SNP2-Ler				5'-CGTTCTTTGTCTCTCTCTTAG-'3
T7D17-SNP2-R				5'-AATGTGACCAAGACAACTTCC-'3

# Appendix 3: Gene expression profiles of PRL1 and PRL2
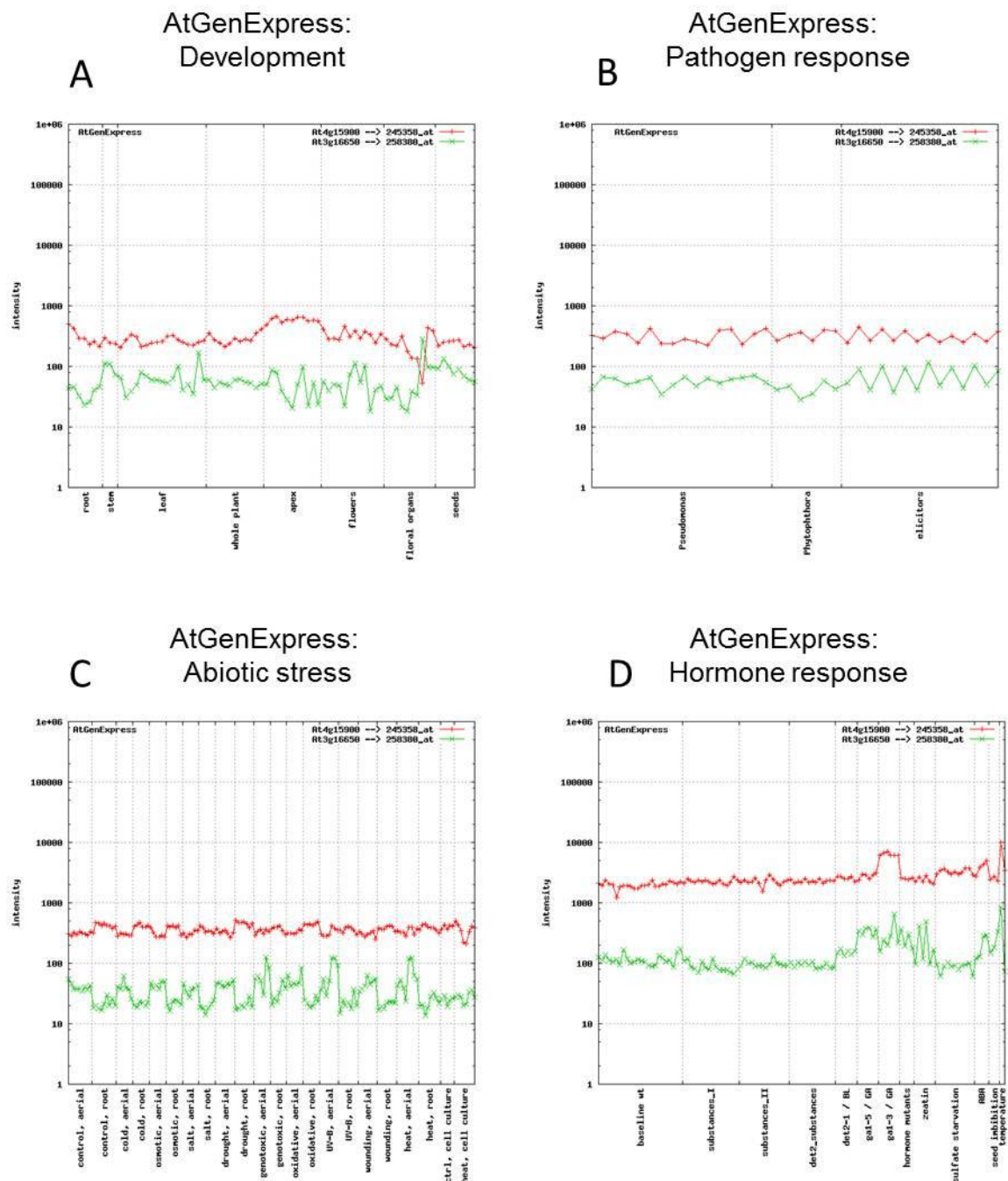
Comparison of *PRL1* (*At4g15900*) and *PRL2* (*At3g16650*) expression patterns using the AtGenExpress visualisation tool. Data are derived from Affymetrics (Santa Clara, CA, USA) gene chip oligonucleotide arrays and are expression estimates by gcRMA.

Appendix 4: Gene ID's of SOP2 homologs

Abbreviated ID	Full gene ID	Species
AL4810	AL6G04810	*Arabidopsis lyrata*
AL7540	AL4G27540	*Arabidopsis lyrata*
AT0630	AT2G40630	*Arabidopsis thaliana*
AT5240	AT5G05240	*Arabidopsis thaliana*
BD2960	BD3G02960	*Brachypodium distachyon*
CP0610	CP00019G00610	*Carica papaya*
FV1180	FV7G31180	*Fragaria vesca*
GM6380	GM14G26380	*Glycine max*
GM6540	GM17G26540	*Glycine max*
HV2094	HV326532094	*Hordeum vulgare*
LJ3580	LJ1G003580	*Lotus japonicas*
MD2880	MD04G002880	*Malus domestica*
MD3850	MD00G333850	*Malus domestica*
MD7900	MD00G137900	*Malus domestica*
ME0010	ME10895G00010	*Manihot esculenta*
ME0160	ME08315G00160	*Manihot esculenta*
MT7460	MT1G007460	*Medicago truncatula*
OSI3280	OSINDICA_02G03280	*Oryza sative spp. indica*
OSJ3940	OS02G03940	*Oryza sative spp. japonica*
PT7500	PT19G07500	*Populus trichocarpa*
PT9010	PT13G09010	*Populus trichocarpa*
RC0130	RC29976G00130	*Ricinus communis*
RC0140	RC29976G00140	*Ricinus communis*
RC1130	RC29729G01130	*Ricinus communis*
SB2670	SB04G002670	*Sorghum bicolor*
TC6250	TC10G016250	*Theobroma cacao*
VV9220	VV13G09220	*Vitis vinifera*
ZM0960	ZM04G40960	*Zea mays*
ZM1900	ZM05G21900	*zea mays*
ZM8640	ZM05G18640	*Zea mays*
ZM9510	ZM05G29510	*Zea mays*

Appendix 5: Gene expression profile of SOP2
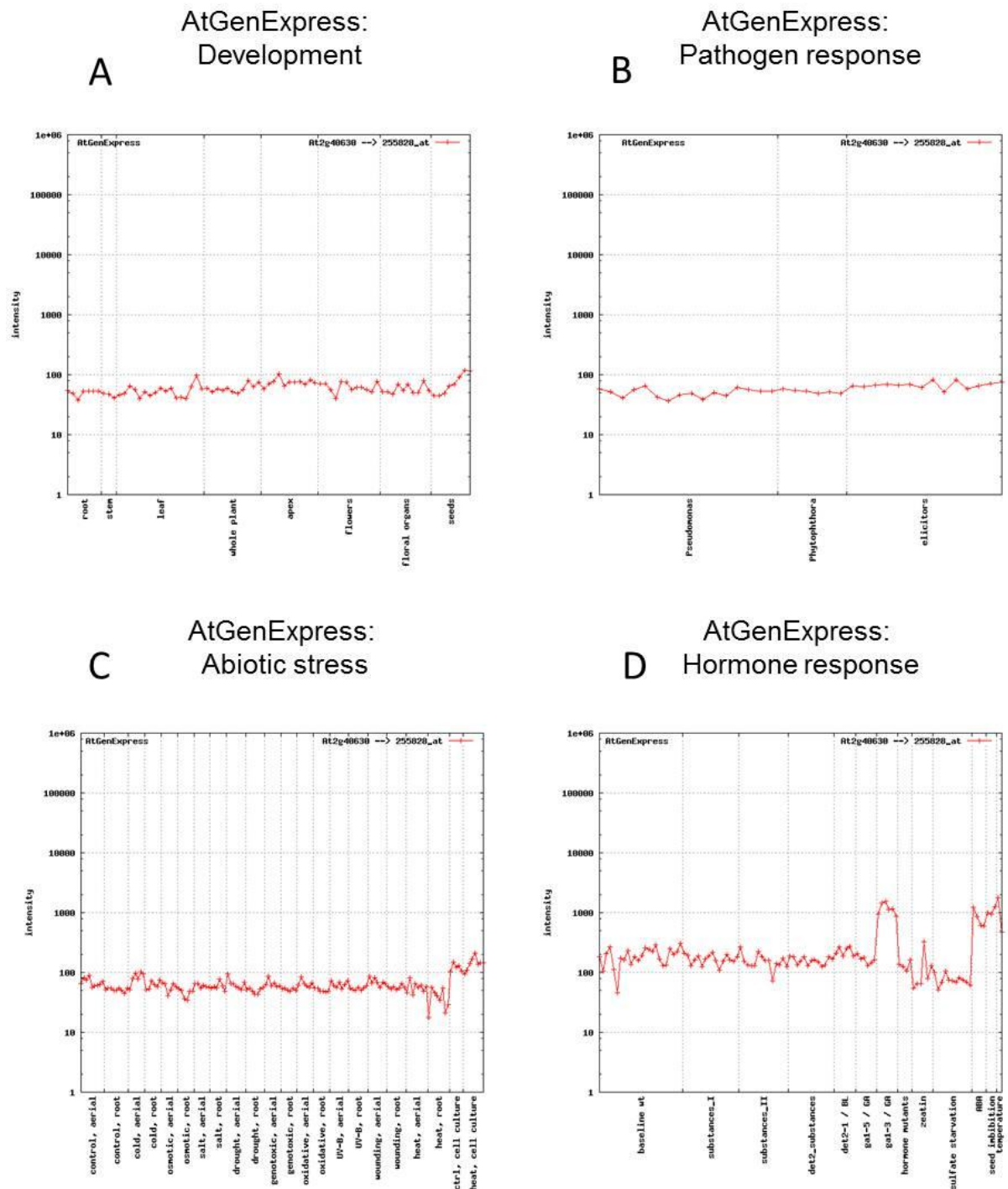
Visualisation of *SOP2* (*At2g40630*) expression patterns as available from AtGenExpress. Data are derived from Affymetrics (Santa Clara, CA, USA) gene chip oligonucleotide arrays and are expression estimates by gcRMA. *SOP2h (At5g05240)* is not present on the arrays.

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