PELIOTROPIC REGULATORY LOCUS 2 exhibits unequal genetic redundancy with its homolog PRL1

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In plants, signaling leading to resistance against biotrophic pathogens is complex. Perception of pathogenic microbes by resistance (R) proteins is relayed through successive activities of downstream components, in a network that is not well understood. PLEIOTROPIC REGULATORY LOCUS 1 (PRL1) and >20 other proteins are members of the MOS4-associated complex (MAC), a regulatory node in defense signaling. Of all characterized MAC members, mutations in PRL1 cause the most severe susceptibility towards both virulent and avirulent microbial pathogens. Genetic suppressors of prl1 represent new signaling elements and may aid in further unraveling of defense mechanisms. Our identification and characterization of a dominant suppressor of prl1 revealed a regulatory, gain-of-function mutation in PLEIOTROPIC REGULATORY LOCUS 2 (PRL2), a close homolog of PRL1. Loss-of-function mutants of PRL2 do not exhibit altered phenotypes; however, prl1 prl2 double mutants exhibit enhanced morphological defects consistent with unequal genetic redundancy between the homologs. Up-regulated gene expression mediated by the dominant prl2-1D allele completely suppresses disease susceptibility in the prl1 mutant background and also restores wild-type appearance, further supporting functional equivalence between the two PRL proteins.

Keywords: Genetic redundancy • MOS4-associated complex • Plant immunity • PRL1 • PRL2 • SOP.

Abbreviations: CC, coiled-coil; EMS, ethyl methanesulfonate; ETI, effector-triggered immunity; MAC, MOS4-associated complex; MOS, modifier of snc1; MS, Murashige and Skoog; NB-LRR, nucleotide-binding leucine-rich repeat; PAMP, pathogen-associated molecular pattern; PRL, PLEIOTROPIC REGULATORY LOCUS; P.s.m., Pseudomonas syringae pathovar maculicola; PTI, PAMP-triggered immunity; P.t.m., Pseudomonas syringae pathovar tomato; R gene, resistance gene; RT–PCR, reverse transcription–PCR; SA, salicylic acid; SOP, suppressor of prl1; TIR, Toll and interleukin-1 receptor; UTR, untranslated region.

Introduction

Plant resistance to microbial pathogens is conferred by several layers of immunity: pre-formed barriers to infection, a non-specific response to the presence of microbes and pathogen-specific defense mechanisms (Osborn 1996, Heath 2000, Jones and Dangl 2006). Microbes at the plant surface are detected by pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs), e.g. bacterial flagellin, and induce a general resistance response termed PAMP-triggered immunity (PTI). Some pathogens are able to evade or suppress PTI by delivering effector molecules into the host cell which interfere with defense signaling. In turn, plants recognize the activity of such effector molecules via a surveillance system comprised of receptor complexes which, upon induction, establish local and systemic resistance termed effector-triggered immunity (ETI; Chisholm et al. 2006, van der Hoorn and Kamoun 2008).

Most of the receptors in ETI are products of resistance (R) genes, containing protein regions with NB-LRRs, named after their central nucleotide-binding (NB) and C-terminal leucine-rich repeat (LRR) domains, although various exceptions exist (Caplan et al. 2008). The N-terminal domains of NB-LRR R proteins fall into two broad categories: those with homology to Drosophila Toll and mammalian interleukin-1 receptor (TIR), or those with predicted coiled-coil (CC) regions (Collier and Moffett 2009). TIR- and CC-NB-LRRs require EDS1 and NDR1, respectively, and trigger the onset of salicylic acid (SA) production, mediated by SID2 (SA INDUCTION DEFICIENT 2) and EDSS (ENHANCED DISEASE SUSCEPTIBILITY 5) (Century et al. 1995, Parker et al. 1996, Feyes et al. 2001, Wildermuth et al. 2001, Nawrath et al. 2002, Venugopal et al. 2009, Knepper et al. 2011). The regulatory protein NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1) functions downstream of SA accumulation to induce defense gene expression through interaction with TGA transcription factors (Cao et al. 1994, Zhang et al. 2003a, Durrant and Dong 2004). Only virulent pathogens can evade recognition
by R proteins, yet their presence is still perceived, causing a weaker and delayed response overlapping in parts with ETI signaling (Navarro et al. 2004, Qi et al. 2011).

In snc1 plants, a point mutation in a TIR-NB-LRR-encoding gene causes constitutive defense in the absence of pathogens (Zhang et al. 2003b). Dissection of snc1-induced signaling through MODIFIER OF SNC1 (MOS) forward genetic screens has unveiled substantial involvement of the nucleocytoplasmic transport machinery in plant resistance. MOS3 and MOS7 are nuclear pore components, whereas MOS6 and MOS11 are facilitators of protein import and mRNA export, respectively (Germain et al. 2010, Monaghan et al. 2010a). Comprehensive analysis of the recessive mos4 mutant revealed an important role in defense for the evolutionarily conserved MOS4-associated complex (MAC), which consists of approximately 20 proteins including MOS4, the putative transcription factor CELL DIVISION CYCLE 5 (AtCDC5) and WD40 repeat protein PLEIOTROPIC REGULATORY LOCUS 1 (PRL1; Palma et al. 2007, Monaghan et al. 2010b). Homologous complexes in yeast and humans function in pre-mRNA splicing, although it is as yet unknown whether these are involved in pathogen response in other kingdoms (Cheng et al. 1993, Ajuh et al. 2000).

The MAC seems to be involved in the regulation of both TIR- and CC-type R protein-mediated immunity. Single mutants of all three first described complex members display defects in basal resistance and ETI; however, mutations in PRL1 result in substantially more compromised resistance relative to mutants of MOS4 and AtCDC5 (Palma et al. 2007). PRL1 is predicted to facilitate transient or stable interactions between proteins and it is hypothesized that it may represent a structural component of the MAC (Stirmann et al. 2010). This multifunctional protein also modulates sugar, hormone and stress signaling (Nemeth et al. 1998, Salchert et al. 1998, Baruah et al. 2009, Flores-Perez et al. 2010). To better understand PRL1 function, we conducted a suppressor screen in the prl1 mutant background and obtained suppressor of prl1 (sop) mutants. The cloning of sop1 revealed functional redundancy between PRL1 and its close homolog PRL2.

**Results**

**Complete and partial suppression of morphological phenotypes in prl1 sop mutants**

prl1 exhibits a number of characteristic phenotypes including serrated leaf margins, short roots and growth arrest under high sugar conditions (Nemeth et al. 1998). After the primary and secondary screens, we obtained 22 sop mutants—among these, five completely suppressed prl1-related phenotypes (prl1 sop1, prl1 sop2, prl1 sop8, prl1 sop13 and prl1 sop15) and 17 mutants exhibited partial suppression (Supplementary Table S1, Supplementary Fig. S1). Morphological suppression levels varied significantly. A mutant was declared a complete suppressor if overall wild-type-like colour, root length and leaf morphology were exhibited. We focused the rest of this study on sop1.

**Restored ETI in sop1 prl1 plants is caused by a dominant mutation in sop1**

When prl1-2 sop1 was backcrossed with prl1-2, all 26 F1 plants were wild-type like, indicating that the sop1 mutation is dominant (Supplementary Table S1). We therefore named this allele sop1-1D.

Morphologically, prl1-2 sop1-1D resembles a wild-type plant (Fig. 1A). The attenuated root growth and slightly early flowering of prl1 were also no longer observed in the double mutant (Fig. 1B, C). When prl1-2 sop1-1D plants were infiltrated with *Pseudomonas syringae* pathovar *maculicola* (P.s.m.) ES4326, they displayed resistance similar to the wild type whereas prl1 plants supported close to 1,000-fold more bacterial growth (Fig. 1D). sop1-1D also reverted the susceptibility of prl1-2 against *Hyaloperonospora arabidopsidis* EMWA1 to wild type (Fig. 1E). Taken together, these findings show that sop1-1D is a complete dominant suppressor of prl1-2.

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

To map the sop1-1D mutation, we crossed the prl1 sop1-1D double mutant (in the Col-0 background) with Ler ecotype plants and created a segregating mapping population. In the F2 generation of the mapping cross, our analysis of 48 plants with prl1 mutant morphology identified the approximate location of sop1-1D between insertion/deletion (InDel) markers MIE1 (4.87 Mb) and MRC8 (6.21 Mb) on the top arm of chromosome 3 (Fig. 2A). Progeny of prl1 homozygous F2 plants with heterozygosity for the sop1-1D locus were used in further mapping steps. Using selected recombinants, we mapped the sop1-1D locus to a 62 kb region between 5.63 Mb (MGL6) and 5.69 Mb (MGL6-SNP5) encompassing 20 predicted genes (Fig. 2A; Huala et al. 2001). One of these genes, At3g16650, encodes PLEIOTROPIC REGULATORY LOCUS 2 (PRL2), a WD40 family protein homologous to PRL1 (Nemeth et al. 1998). Through sequencing the At3g16650 open reading frame in the prl1 sop1-1D double mutant, a C to T substitution was identified in the first exon of the PRL2 gene, 58 bp upstream of the translational start codon (Fig. 2B).

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

PRL2 has been previously reported as the homolog of PRL1 in Arabidopsis (Nemeth et al. 1998, Baruah et al. 2009). Public data from the transcriptome platform AtGenExpress identifies on average 80% lower expression of PRL2 compared with PRL1 across all tested developmental stages (http://jsp.weigelworld.org/expviz/expviz.jsp, Schmid et al. 2005). Since the encountered mutation in prl1 sop1-1D is located in the S’ untranslated region (S’UTR) of PRL2, we hypothesized a regulatory modification affecting PRL2 transcript levels. PRL2 mRNA is approximately twice as abundant in seedling tissues of prl1 sop1-1D and sop1-1D mutants compared with prl1 and wild-type controls (Fig. 2C, D). These results are consistent with a regulatory
Fig. 1 sop1-1D suppresses prl1-related phenotypes. (A) Morphology of soil-grown wild type (Col-0), prl1-2 and prl1-2 sop1-1D. (B) Root length analysis of 1-week-old seedlings of the indicated phenotypes; results represent an average of 10 seedlings each ±SD. (C) Flowering time analysis: rosette leaves of 10 plants per genotype wild type (Col-0), prl1-2 and prl1-2 sop1-1D were counted when the emerging flower measured 1 cm in height. Values represent averages ±SD. (D) Infection experiments with P.s.m. ES4326 (OD600 = 0.0001) using 5-week-old soil-grown plants of the indicated phenotypes. Bacterial titer was quantified at 0 and 3 d post-inoculation; values represent the average of six replicates per genotype ±SD. (E) Soil-grown, 2.5-week-old plants of the indicated genotypes were spray inoculated with 100,000 spores ml⁻¹ of H. arabidopsidis EMWA1 and colonization quantified after 9 d. Values represent averages ±SD of two replicates with 15 plants each. For (B–E), experiments were repeated at least three times with similar results. Statistical significance compared with Col-0 control was calculated using Student’s t-test: *P < 0.001 and **P < 0.0001 for all graphs. cfu, colony-forming units.

Genetic redundancy between PRL1 and PRL2

Fig. 2 Map-based cloning of sop1-1D. (A) Map position of sop1-1D; indicated are recombinants and sequence-anchored positions of flanking markers and bacterial artificial chromosome (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 cDNA obtained from the indicated genotypes of tissue series #1. Values represent averages of two experimental replicates. (D) Semi-quantitative RT–PCR of tissue series described in (C).
gain-of-function allele of PRL2 in sop1-1D, acting as a suppressor of prl1.

To confirm the role of PRL2 in the dominant suppression of prl1, we transformed three partially overlapping clones from the Arabidopsis JAtY library into prl1 and prl1 sop1-1D mutants. The JATY clone 69M23 covers a 67 kb region on chromosome 3 encompassing PRL2, whereas 79F11 (78 kb) and 51K01 (31 kb) are adjacent (Fig. 3A). Since experimental data derived from our expression analysis demonstrated up-regulated PRL2 transcripts in prl1 sop1-1D plants, we reasoned that addition of a second copy of PRL2 (JATY69M23) into prl1 would result in similarly increased transcript levels and mimic complementation. Consistent with this hypothesis, two of the three prl1 T1 plants transformed with JATY69M23 exhibited wild-type morphology (Fig. 3B). The phenotype of prl1 was not affected by 51K01 and we did not recover transformants when using the large 79F11 clone (Fig. 3C). Transformation of prl1 sop1-1D double mutants with any of these three clones resulted in phenotypically unaltered plants in all cases (Fig. 3C, D). In addition, elevated PRL2 expression is observed only in the two complementing transgenic lines carrying JATY69M23 (Fig. 3E; lines #1 and #2). These data support PRL2 transcript-level-dependent suppression of prl1 phenotypes in prl1 sop1-1D, as only JATY69M23-transformed prl1 plants phenocopied the dominant suppressor. The prl2 allele present in sop1-1D probably carries a regulatory gain-of-function mutation (prl2-1D), conferring a dominant suppression of prl1 mutant phenotypes.

PRL1 and PRL2 share high structural and sequence homology

PRL1 and PRL2 belong to the Transducin/WD40 repeat family, a motif that mediates protein–protein interactions (Stirnimann et al. 2010, Xu and Min 2011). Significant sequence homology between these proteins has been previously established through hybridization experiments and sequence alignment (Nemeth et al. 1998, Baruah et al. 2009). 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 (Supplementary Fig. S2). A second motif, designated DWD (DDB1 binding WD40), is located within the WD3/4 and WD4/5 repeats of PRL1 and PRL2 (Supplementary Fig. S2). Recent evidence suggests that a subset of WD40 proteins with this motif, one of them being PRL1, may interact with CUL4-based E3 ligases in the nucleus (Lee et al. 2008). The high level of sequence conservation between PRL1 and PRL2 suggests functional redundancy between the homologs and is consistent with the ability of prl2-1D to suppress prl1 phenotypes.

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 observable

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Fig. 3 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 harbors PRL2, into prl1-2. (C) Summary of JATY transformations; indicated are JATY clones, genotypes of transformed plants as well as the number and phenotypes of the obtained transgenic plants. (D) Morphology of transgenic plants obtained through transformation of JATY69M23 into prl1-2 sop1-1D. (E) Semi-quantitative RT–PCR of JATY69M23-transformed prl1-2 lines as well as controls prl1-2, prl1-2 sop1-1D and the wild type. Lines #1 and #2 are those shown in B, while line 3 is the prl1-like transgenic line in C.
morphological changes (Fig. 4A, B). To test further the redundancy between PRL1 and PRL2, we crossed their respective T-DNA insertion mutants and identified the prl1 prl2 double mutant in the F2 generation by PCR-based genotyping. Leaves of 3-week-old prl1 prl2 plants are darker and smaller than those of the prl1 single mutant (Fig. 4A, B). prl1 prl2 double mutants derived from a second T-DNA allele of prl2 (prl2-3, Salk_133878) also exhibited similar enhanced phenotypes (data not shown). An early flowering phenotype, which is usually observed in prl1, is more pronounced in the double mutant (Fig. 4C). In addition, we noted a statistically insignificant slight reduction in root length in prl1 prl2 (mean = 4.3, SD = 0.48) compared with already strongly reduced prl1 roots (mean = 4.9, SD = 0.87, Fig. 4D). These enhanced phenotypes observed in the double mutant suggest an unequal genetic redundancy between PRL1 and PRL2.

prl1 prl2 mutants are impaired in basal and effector-triggered immunity

Consistent with morphological data, our infection assays revealed that prl2 loss-of-function and wild-type plants exhibited no differences in susceptibility to virulent P. s. m. ES4326 (Fig. 4E). Substantially impaired basal signaling was observed for prl1 prl2 although bacteria did not grow more than in prl1 plants (Fig. 4E).

To test R protein-dependent ETI, we infiltrated rosette leaves with avirulent Pseudomonas syringae pathovar tomato (P. s. t.) DC3000 expressing avrRPS4, which is recognized by the TIR-NB-LRR protein RESISTANCE TO PSEUDOMONAS SYRINGAE 4 and confers resistance in wild-type and prl2 mutant plants. This resistance is not evident in prl1 and prl1 prl2, since the normally avirulent pathogen grows to an order of magnitude higher at 3 d post-inoculation (Fig. 4G). In addition, minor impaired defence to avirulent H. arabidopsis EMWA1 was detected in the prl1 prl2 double mutant (Fig. 4F). It should be noted that the prl1 prl2 plants grew extremely poorly under the conditions of H. arabidopsis infection (18°C and >90% humidity). The plants turned purple and were extremely dwarf. The outcome of the susceptibility tests may have been affected under these growth conditions.

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

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

We generated the prl2-1D single mutant to investigate whether wild-type PRL1 expression and increased levels of PRL2 transcript would result in a mutant phenotype. prl1 prl2-1D was crossed with Col-0 and single mutants were identified in the F2 using PCR-based genotyping. prl2-1D plants are phenotypically wild type, apart from a slightly late flowering phenotype which we also observed in prl1 prl2-1D double mutants. Both mutants initiate flowering with at least 20 rosette leaves present, in contrast to prl1 and wild-type plants, which flower on average at the 11- and 15-leaf stage, respectively (Fig. 5A, B).

Following infection with virulent bacterial and oomycete pathogens, we detected no difference in pathogen colonization of prl2-1D plants compared with prl1-2 prl2-1D and the Col-0 wild type (Fig. 5C, D). Similarly, colonization by two avirulent strains was limited to wild-type levels in prl2-1D when evaluated after 3 d. P. s. t. DC3000 expressing avrRPS4 and P. s. t. DC3000 expressing avrRphB are detected in the plant cytoplasm by TIR-type and CC-type R proteins, respectively (Fig. 5E, F). When challenged with avirulent H. arabidopsis EMWA1 (detected by TIR-NB-LRR immune receptor RPP4), the same trend was observed (Fig. 5G). Since both tested layers of plant immunity are unaffected in prl2-1D, it is unlikely that the function of PRL1/PRL2 in resistance signaling can be enhanced through a dosage-dependent mechanism such as overexpression of PRL2.

Discussion

The WD40 protein PRL1 is part of the MAC, a multiprotein complex with a role in plant defense signaling. Mutant prl1 plants sustain higher pathogen growth than MAC mutants mos4, Atcdc5 and mac3a mac3b, leading us to hypothesize a more substantial role for PRL1 in immunity (Palma et al. 2007, Monaghan et al. 2009). With the goal of identifying members of the PRL1- and MAC-associated signaling network, we carried out a genetic screen based on suppression of prl1 morphology, i.e. serrated, dark green leaves and short roots. Our investigation of dominant suppressor prl2-1D and loss-of-function prl2 suggests that PRL1 and its homolog PRL2 have partly redundant roles in the regulation of plant immunity, sugar-related signaling and morphogenesis.

The majority of the PRL2 and PRL1 protein structure consists of seven WD40 repeat sequences, whereas the remaining C-terminal region does not seem to comprise another motif. The encoded 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 (Nemeth et al. 1998, Palma et al. 2007, Lee et al. 2008, Baruah et al. 2009, Flores-Perez et al. 2010). The WD40 structure is approximately 90% conserved between the PRL2 and PRL1 homologs and thus is probably capable of a similar range of interactions and assumed to be responsible for re-established wild-type phenotypes in the prl1 prl2-1D mutant.
In the prl2-1D single mutant, increased PRL2 mRNA levels did not result in a detectable phenotype, which is further evidence for a lack of enzymatic activity of both homologs. This finding is consistent with its proposed function as an adaptor protein and an important component in signal relay. So far, WD40 proteins have not been shown to exhibit enzymatic activity but rather are suggested to function as adaptor components through protein–protein interactions in signal relay (Stirnimann et al. 2010). Through reversible or stable association with pathway-controlling elements, adaptors often provide essential platforms for regulatory interaction. Such a role could explain the added susceptibility displayed by prl1 and prl1 prl2 mutants compared with other MAC mutants. The presence of another predicted structural protein in the MAC core complex, MOS4, emphasizes the importance of stable interplay for functional immunity signaling.

Since phenotypes of prl1 and prl1 prl2 are not limited to defects in immunity, transient association with MAC components or independent interaction of PRL1/PRL2 with other proteins appears likely. A presumed function as a signaling-enabling adaptor could explain PRL1 involvement in diverse pathways such as sugar, light, stress, resistance and hormone signaling, reported previously (Nemeth et al. 1998, Palma et al. 2007, Lee et al. 2008, Barua et al. 2009, Flores-Perez et al. 2010). The influence of PRL1/PRL2 over additional pathways independent from the MAC pathway is further supported by cloning of sop2-1D, which specifically reverses prl1 phenotypes but does not affect mutant Atcdc5 or mos4 signaling (Weihmann et al. unpublished results).

Fig. 4 Enhanced phenotypes in prl1 prl2 double mutants compared with single mutants. (A) Gene structure of PRL1 and PRL2. Indicated are the 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

WT prl1-2 prl2-2

C

Number of rosette leaves at bolting

D

Average root length (mm)

P.s.m. ES4326

Day 0 Day 3

log cfu/cm²

H.a. EMWA1

0 2 4 6 8 10

log cfu/cm²

P.s.t. avrRps4

Day 0 Day 3

log cfu/ml

0 2 4 6 8 10

E

F

G

0 2 4 6 8 10

(P. s. m.)

0 2 4 6 8 10

Fig. 4 Continued

(B) Morphology of 4-week-old soil-grown wild type (Col-0), prl1-2, prl2-2 and prl1-2 prl2-2. (C) Flowering time analysis of wild type, prl1-2, prl2-2 and prl1-2 prl2-2. Rosette leaves of 10 plants per genotype were counted when the emerging flower measured 1 cm in height. Values represent means ± SD. Means were compared using analysis of variance [ANOVA; F(3,36) = 102.34, P < 0.0001] and a Tukey HSD post-hoc test was conducted to test for significant differences between the means (P < 0.01). (D) Root length analysis of the indicated genotypes. The results represent means of 10 plate-grown seedlings for each genotype ±SD. Means were compared using ANOVA [F(3,36) = 266.13, P < 0.0001] and a Tukey HSD post-hoc test was conducted to test for significant differences between the means (P < 0.01). (E) Bacterial infection of the indicated phenotypes with virulent P. s. m. ES4326 (OD600 = 0.0001) using 5-week-old soil-grown plants. Bacterial titer quantification at 0 and 3 d post-infection; averages represent six replicates ±SD. (F) Evaluation of disease susceptibility of the indicated genotypes towards avirulent oomycete H. arabidopsidis EMWA1 (200,000 spores ml⁻¹); values represent the averages of two replicates of 15 plants ±SD. (G) Inoculation of the indicated genotypes with avirulent P. s. t. avrRps4 (OD600 = 0.002) using 5-week-old soil-grown plants. Bacterial titer quantification at 0 and 3 d post-infection; averages represent six replicates ±SD. For (C–G), experiments were repeated at least three times with similar results. Statistical significance compared with Col-0 control was calculated using Student’s t-test: **P < 0.0001 for all graphs. cfu, colony-forming units.
In contrast to the protein-coding sequences, the PRL1 and PRL2 5’UTRs have diverged significantly. Having identified the 12th position of the PRL2 5’ non-translated region as highly relevant for mRNA levels, a regulatory function for this region can be hypothesized. The nucleotides surrounding the sop1-1D mutation site could contribute to regulating either the transcription or stability of the mRNA molecule, thus affecting PRL2 expression levels.

There have been several large-scale duplication events in Arabidopsis, since approximately 80% of 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 the E3 ubiquitin ligase subunits CUL3A/CUL3B have been documented, although diversification of function and expression patterns is predicted for many of these duplicated genes (Figueroa et al. 2005, Briggs et al. 2006). In the MAC, the 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/MAC5B-encoded proteins and non-redundancy between MAC5A/MAC5B (Monaghan et al. 2009, Monaghan et al. 2010b). The absence of a mutant phenotype in prl2 plants and an enhanced phenotype in prl1 prl2 mutants are hallmarks of unequal redundancy caused by substantially reduced gene expression of one of the duplicated genes (PRL2) compared with the levels of PRL1, which is likely to be the ancestral gene (Briggs et al. 2006).

Subfunctionalization is an evolutionary mechanism believed to be contributing to the preservation of duplicated genes found in eukaryotic genomes. In this process, both members of a gene pair resulting from duplication experience degenerative mutations that affect patterns and levels of activities (Lynch and Force 2000). We did not detect novel properties

Fig. 5 Continued

indicated genotypes. Rosette leaves of 10 plants per genotype were counted when the emerging flower measured 1 cm in height. (C) Infection assay of the indicated genotypes with 5-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 d post-inoculation (dpi). Values represent six replicates ±SD. (D) Evaluation of resistance towards virulent oomycete H. arabidopsidis NOCO2 of the indicated genotypes. Spray inoculation of 2.5-week-old plants with 50,000 spores ml$^{-1}$ followed by conidiospore count after 8 d. 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$_{600}$ = 0.0002) of P.s.t. DC3000 expressing avrRps4 or avrPphB into leaves of 5-week-old plants of the indicated genotypes showed wild-type resistance in prl2-1D. Bacterial titers were quantified at 0 and 3 dpi. Values represent six replicates ±SD. (G) Spray inoculation of 2.5-week-old plants of the indicated genotypes with 150,000 spores ml$^{-1}$ of avirulent oomycete H. arabidopsidis EMWA1 followed by conidiospore count after 8 d; values represent averages of two replicates with 15 plants. Statistical significance compared with Col-0 control was calculated using Student’s t-test: *P < 0.003 and **P < 0.0001 for all graphs.
of the PRL2 protein in the overexpressing sop2-1D mutant, which argues against significant alteration at the protein level. 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 that of PRL1. Such inverse expression patterns could be seen as evidence of subfunctionalization leading to tissue specificity. Mutations in the regulatory promoter region are probably responsible for the attenuated expression of PRL2 and may ultimately lead to full pseudogenization of the PRL1 homolog (Adams and Wendel 2005, Yang et al. 2011).

Materials and Methods

Plant material and growth

Wild-type Arabidopsis ecotypes Columbia (Col-0), Landsberg erecta (Ler) and Wassilewskija (Ws) and derived mutants were grown on soil with a 16 h light/8 h dark regime. We obtained T-DNA insertion mutants prl1-2 (Salk_008466), prl2-2 (Salk_075970) and prl2-3 (Salk_133878) from the Arabidopsis Biological Research Centre (ABRC); these were genotyped by PCR using PRL1-Salk-NF (5'-CATGAAAGTTGCCTTGAGG-3') with PRL1-NR-A (5’-ACTACCTACTACCTAGACG-3') for prl1-2 and PRL2-Salk-F (5’-TCTGAAACGCGCTTAAATGAGG-3') with PRL2-Salk-R (5’-TGAGCCCTACTTGCAGG-3') for prl2-2 and prl2-3. Unless otherwise noted in the text, prl1 refers to Salk_008466 (prl1-2) and prl2 refers to Salk_075970 (prl2-2).

EMS mutagenesis and primary screen

We treated 0.5 g (~25,000) of prl1 seeds with ethyl methanesulfonate (EMS), selfed the mutagenized M1 plants and harvested M2 progeny into 79 pools containing seeds from 20–25 plants each. Approximately 500 seeds per M1 pool were screened on soil or Murashige and Skoog (MS) plates containing 6% sucrose. prl1 mutant plants germinate late and display characteristic serrated and dark green leaf morphology, these phenotypes intensify under high sugar conditions, eventually resulting in growth arrest (Nemeth et al. 1998). Seedlings exhibiting suppression of prl1 phenotypes were transplanted and the homozygous prl1 background was confirmed by PCR. We obtained 49 putative mutants from 22 pools, some of which we crossed to Ler ecotype plants, and two were eventually cloned by positional mapping (Supplementary Table S1).

Pathogen susceptibility screen

Susceptibility of sop mutants to the obligate biotrophic oomycete H. arabidopsidis EMWA1 (Slusarenko and Schlaich 2003, Holub 2008) was utilized as a secondary screen for defects in pathogen response. This pathogen isolate is virulent on the Ws ecotype but avirulent on Col-0, where resistance is mediated by the R gene RESISTANCE TO PERONOSPORA PARASITICA 4 (RPP4). We infected 2-week old soil-grown progeny of confirmed sop mutants with a conidiospore solution of H. arabidopsidis EMWA1 (1.5 x 10⁵ spores ml⁻¹) and disease symptoms were assessed 7 days post-infection. These were scored using five categories of disease severity, with resistant Col-0 scored as '1', highly susceptible Ws as '5' and prl1 as '4'. Suppressor mutants were classified as resistant with a ranking of '1–2' and classified as susceptible when scored '3' or higher (Supplementary Table S1).

Assessment of genetic inheritance

To assess the genetic nature of sop mutations, we back-crossed various prl1 sop double mutants with homozygous prl1 single mutants. Homozygous recessive sop mutations resulted in exclusively prl1-like progeny, whereas homozygous dominant sop mutations gave only wild-type-like progeny. Phenotypically intermediate progeny indicated semi-dominant inheritance of the respective sop mutation (Supplementary Table S1).

Morphological characterization

We measured the root length of 7-day-old seedlings (10 per genotype), grown on vertical MS plates, to assay root growth. To evaluate flowering time, we counted rosette leaves present at the time of bolting (1 cm in height) for 10 plants per genotype.

Pathogen infection assays

P.s.m. ES4326 and P.s.t. DC3000 are hemi-biotrophic bacterial pathogens responsible for leaf spot and bacterial speck disease on Arabidopsis, respectively. We cultured these at 28–30 °C in media containing 50 mg ml⁻¹ streptomycin (P.s.m. ES4326) or 25 mg ml⁻¹ rifampicin and 50 mg ml⁻¹ kanamycin (P.s.t. avrPphB and avrRps4). We inoculated the abaxial leaf sides of 4- to 5-week-old Arabidopsis plants using a small needle-less plastic syringe to inject bacteria into the plant apoplast at two concentrations; a low dose (OD₆₀₀ = 0.0001) for virulent P.s.m. ES4326, and a high dose (OD₆₀₀ = 0.002) for avirulent P.s.t. expressing avrPphB or avrRps4. At 0 and 3 d after inoculation, we cut 0.32 cm² leaf discs with a standard paper hole punch, homogenized samples in 10 mM MgCl₂ and plated a series of six dilutions on growth media. Bacterial colony-forming units were calculated after 2 d of incubation at 28 °C.

For H. arabidopsidis EMWA1 infection assays, we spray-inoculated 2.5-week-old plants with either 50,000 or 100,000 spores ml⁻¹ of conidiospores to determine resistance defects in compatible and incompatible interactions, respectively. After 7–10 d, we harvested the infected plants, vortexed them for 7–10 s in 5 ml of water and quantified conidiospores using a hemocytometer.

Quantification of PRL2 mRNA levels

We collected tissue from seedlings of various genotypes grown for 10 d on 0.5 MS plates and extracted RNA (Totally RNA kit, Ambion). After reverse transcribing into cDNA (SuperScript II,
Invitrogen), relative expression of PRL2. Tubulin and Actin genes was measured by real-time PCR with gene-specific primers: PRL2-RT-F2 `5'-ATATCAAAACTGAGCCACA-3' with PRL2-RT-R `5'-AACCTCGAAATAGTT-3', Tubulin (control) `5'-ACGTATGATGCTATTTCAACG-3' with `5'-ATATCGTAA GAGCCCTATTGCC-3' and Actin (control) `5'-CGATGAA GCTCAATCCAAAGA-3' with `5'-CAGAGTCGAGGCAAAATCG CG-3', respectively.

**Single and double mutant construction**
To create the sop1-1D/prl1-1D single mutant, we crossed homozygous prl1 sop1-1D to Col-0 wild-type plants and genotyped the resulting F2 progeny with allele-specific primers P2A2-M-F2 (`5'-GTCGATTTAATCCTATTTGT-3') and P2A2-WT-R (`5'-GGCAAACGTTGATTAACT-3'). To obtain the prl1 prl2 double mutant, we crossed homozygous prl1 and prl2 plants and confirmed double mutants in the F2 using insertion-flanking primers PRL1-Salk-NF with PRL1-NR-A for prl1 and PRL2-Salk-F with PRL2-Salk-R for prl2.

**Transformation of JATY clones**
The genomic, TAC-based JATY library (http://www.genome-enterprise.com/services/dna-libraries) was created at the John Innes Centre (UK) with the pYLTAC17 vector, which confers glufosinate resistance to transgenic plants (Liu et al. 2002). Innes Centre (UK) with the pYLTAC17 vector, which confers glufosinate resistance to transgenic plants (Liu et al. 2002).

**Supplementary data**
Supplementary data are available at PCP online.

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