Planta (2011) 233:423–431
DOI 10.1007/s00425-010-1324-5

RAPID COMMUNICATION

Effect of sporophytic *PIRL9* genotype on post-meiotic expression of the Arabidopsis *pirl1*;*pirl9* mutant pollen phenotype

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Received: 12 September 2010 / Accepted: 12 November 2010 / Published online: 8 December 2010

Abstract Plant intracellular ras-group-related leucine-rich repeat proteins (PIRLs) are a novel class of plant leucine-rich repeat (LRR) proteins structurally related to animal ras-group LRRs involved in cell signaling and gene regulation. Gene knockout analysis has shown that two members of the *Arabidopsis thaliana PIRL* gene family, *PIRL*1 and *PIRL*9, are redundant and essential for pollen development and viability: *pirl1*;*pirl9* microspores produced by *pirl1*/*PIRL1*;*pirl9* plants consistently abort just before pollen mitosis I. *qrt*1 tetrad analysis demonstrated that the genes become essential after meiosis, during anther stage 10. In this study, we characterized the phenotype of *pirl1*;*pirl9* pollen produced by plants heterozygous for *pirl9* (*pirl1*;*pirl9*/*PIRL9*). Alexander's staining, scanning electron microscopy, and fluorescence microscopy indicated that *pirl1*;*pirl9* double mutants produced by *pirl*9 heterozygotes have a less severe phenotype and more variable morphology than *pirl1*;*pirl9* pollen from *pirl1*/*PIRL1*;*pirl*9 plants. Mutant pollen underwent developmental arrest with variable timing, often progressing beyond pollen mitosis I and arresting at the binucleate stage. Thus, although the *pirl*1 and *pirl*9 mutations act post-meiosis, the timing and expressivity of the *pirl1*;*pirl9* pollen phenotype depends on the *pirl*9 genotype of the parent plant. These results suggest a continued requirement for *PIRL1* and *PIRL9* beyond the initiation of pollen mitosis. Furthermore, they reveal a modest but novel sporophytic effect in which parent plant genotype influences a mutant phenotype expressed in the haploid generation.

Keywords *Arabidopsis thaliana* · Gametophyte · Gene knockout · Genetic redundancy · Paternal effect · Pollen development

Abbreviations

DAPI	4′,6-Diamidino-2-phenylindole
LRR	Leucine-rich repeat
PIRL	Plant intracellular ras-group-related LRR protein
SEM	Scanning electron microscopy

Introduction

Leucine-rich repeat proteins (LRRs) constitute a diverse and widespread eukaryotic superfamily defined by the presence of distinct domains consisting of tandem repeats of short leu-rich motifs. In plants, various LRR families function in important developmental and physiological regulatory pathways (reviewed by Vernon and Forsthoefel 2002). Prominent examples include LRR-receptor kinases (Morris and Walker 2003; Dievart and Clark 2004; Morillo and Tax 2006; De Smet et al. 2009), F-box LRR proteins that mediate signal-responsive protein degradation (Hellmann and Estelle 2002; Somers and Fujiwara 2009), and two different classes of disease resistance proteins, NBS-LRRs and *cf*-type receptors, which feature LRR domains involved in pathogen recognition or response

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(Fluhr 2001; Belkhadir et al. 2004; McHale et al. 2006; Eitas and Dangl 2010).

Plant intracellular Ras-group LRRs (PIRLs) are a novel class of plant LRRs first identified in the *Arabidopsis thaliana* (L.) Heynh genome (Forsthoefel et al. 2005). Based on their repeated leu-rich motif sequence, PIRLs are most closely related to animal and fungal ras-group LRRs (Claudianos and Campbell 1995; Buchanan and Gay 1996), which take part in developmental cell signaling and gene regulation by serving as transcriptional coactivators (Lee et al. 2004; Jeong et al. 2009) or as components of ras signaling complexes (Cutler et al. 1992; Sieburth et al. 1998; Sternberg and Alberola-Ila 1998; Li et al. 2000; Dai et al. 2006; Dougherty et al. 2008). While the PIRL LRR consensus sequence reflects a relationship to animal and fungal ras-group LRRs, divergent sequences outside the LRR domain define PIRLs as a novel protein class unique to plants (Forsthoefel et al. 2005).

The Arabidopsis genome encodes nine *PIRL*s, with *PIRL1* (At5g05850) and *PIRL9* (At3g11330) constituting the most closely related pair, encoding products sharing 77% amino acid identity. Analysis of T-DNA knockout mutants has shown that *PIRL1* and *PIRL9* are genetically redundant and essential for pollen development (Forsthoefel et al. 2010). In *pirl1*;*pirl9* microspores produced by *pirl1*/*PIRL1*;*pirl9* parent plants, microspore growth, exine development, and vacuolization at first appear normal. However, after that initial period of normal development, *pirl1*;*pirl9* microspores produced by this genotype consistently arrest and are unable to complete the first pollen mitosis. Consistent with the late-microspore onset of the mutant phenotype, analysis of *pirl1*;*pirl9* phenotype segregation in *qrt*1 tetrads confirmed that abnormalities originate after meiosis, thus indicating that these *PIRL* genes have essential function in the developing haploid microspores (Forsthoefel et al. 2010).

In this study, we describe development of *pirl1*;*pirl9* pollen produced by *pirl1*;*pirl9*/*PIRL9* plants. We find that double mutant pollen produced by this parental genotype have a more variable and often less severe terminal phenotype than do double mutants produced by *pirl9* homozygotes. 4′,6-diamidino-2-phenylindole (DAPI) staining indicates this variability results from inconsistent timing of developmental arrest, with mutants containing from one to three nuclei at anther maturation. This suggests that the developmental requirement for *PIRL1* and *PIRL9* function persists into the pollen mitotic stages. The *pirl1*;*pirl9* phenotype variability indicates that the presence of a functional *PIRL9* allele in the parent sporophyte can partially and temporarily rescue the expression of the *pirl1*;*pirl9* phenotype and delay pollen lethality. Because the *pirl1*;*pirl9* phenotype is expressed post-meiosis, this reflects a developmental "sporophytic effect" mechanism, manifested in the observed genetic relationship between the sporophyte and microgametophyte generations.

Materials and methods

Plant materials

Arabidopsis [*Arabidopsis thaliana* (L.) Heynh] plants were grown on soil from seed under 16 h light/8 h dark conditions as described by Cushing et al. (2005). *Pirl* T-DNA knockout alleles used in this study have been described previously and shown to be knockout alleles based on reverse transcription-PCR analysis of disrupted loci (Forsthoefel et al. 2010). *Pirl1-1* and *pirl9-1* are in the Wassilewskija (Ws) ecotype, and *pirl1-2* and *pirl9-2* are in Columbia. Lines used for tetrad analyses were generated by introduction of *pirl*1-1 and *pirl*9-1 into *qrt*1 (Preuss et al. 1994), followed by identification of *qrt*1 homozygotes in the subsequent F2 by bright field microscopy of pollen, and PCR genotyping (see below) to identify individuals of indicated *pirl*;*qrt*1 genotypes.

Plant genotypes for pollen transmission and phenotype analyses were determined by PCR using gene-specific forward and reverse primer combinations to detect wild-type alleles and T-DNA/*PIRL* primer combinations to detect mutant alleles, as described in Tax and Vernon (2001), using the primer combinations described in Forsthoefel et al. (2010). Mature pollen for viability staining or scanning electron microscopy (SEM) was collected from open flowers from wild type, *pirl*1 or *pirl*9 homozygotes, or *pirl1*/*PIRL1*;*pirl9* or *pirl*;*pirl9*/*PIRL9* plants, which produce pollen segregating 50% for the double mutant genotypes. Pollen for fluorescence microscopy was viewed in anther squashes as described below.

Pollen genetic transmission and microscopy

Analysis of *pirl*1 and *pirl*9 mutant allele transmission was done by PCR genotyping of F1 seedling populations produced by reciprocal crosses between wild type and either *pirl1*/*PIRL1*;*pirl9* or *pirl*;*pirl9*/*PIRL9* plants, which both produce pollen segregating 50% for the double mutant phenotypes. Viability staining and fluorescence microscopy were carried out as previously described (Forsthoefel et al. 2010). For SEM, mature flowers from PCR-genotyped *pirl1-1*/*PIRL1*;*pirl9-1* or *pirl1-1*;*pirl9-1*/*PIRL9* plants or from wild-type controls were excised and dried for at least 48 h. Pollen was transferred to double-sided carbon tape on a stub, and samples were viewed and digitally imaged using an FEI Quanta200 scanning electron microscope with variable vacuum and Peltier cold stage capabilities (FEI, Hillsboro, OR, USA).

Results

Transmission failure and inviability of *pirl1*;*pirl9* pollen

Two independent null T-DNA insertion alleles for both *pirl1* (*pirl1-1* and *pirl1-2*) and *pirl9* (*pirl9-1* and *pirl9-2*) have been identified and described previously (Forsthoefel et al. 2010). To assess *pirl1*;*pirl9* allele transmission from plants heterozygous for *pirl9*, reciprocal crosses to wild type were carried out with *pirl*1;*pirl*9/PIRL9 plants, which produce 50% *pirl1*;*pirl9* double-mutant gametophytes. F1 progeny were genotyped by PCR using gene-specific and T-DNA/gene-specific primer combinations. Results are shown in Table 1, along with data from similar crosses with *pirl9* homozygotes (*pirl1*/*PIRL1*;*pirl9*). When mutant plants were used as pollen donors, only a very low frequency of *pirl1*;*pirl9* transmission was observed: none from *pirl1*/*PIRL1*;*pirl9* plants ($n = 117$) and 4% from *pirl1*;*pirl9*/*PIRL9* plants ($n = 183$). Transmission failure was observed with both the *pirl1-1*;*pirl9-1* and the *pirl1-2*;*pirl9-2* allele combinations, verifying that it was due to *PIRL* gene disruption. As observed previously (Forsthoefel et al. 2010), the double mutant allele combination was transmitted normally through the female parent, resulting in approximately 50% of F1 progeny containing mutant alleles for both *pirl*1 and *pirl*9 when wild-type plants were used as pollen donors (Table 1, bottom).

To determine if *pirl1*;*pirl9* pollen transmission failure was due to defective early pollen development, we assessed viability of mature dehisced *pirl1*;*pirl9* pollen using Alexander's viability stain (Alexander 1969; Johnson-Brousseau and McCormick 2004). First, *pirl1-1* and *pirl9-1* were introduced into a *qrt1* background (Preuss et al. 1994) to allow for analysis of phenotype segregation. *qrt1*;*pirl1*/*PIRL1*;*pirl9* and *qrt1*;*pirl1*;*pirl9*/*PIRL9* plants were identified by PCR, and tetrads produced by these genotypes were subjected to Alexander's staining. Results are shown in Fig. 1. As previously reported (Forsthoefel et al. 2010), *qrt1*;*pirl1*/*PIRL1*;*pirl9* plants produced approximately 50% inviable (fully aborted) pollen, consistent with the 50% segregation of the *pirl1*;*pirl9* genotype in pollen from such plants. However, *qrt1*;*pirl1*;*pirl9*/*PIRL9* plants, which also produce 50% *pirl1*;*pirl9* pollen, yielded only approximately 30% fully aborted pollen, indicating that some of the *pirl1*;*pirl9* mutant pollen produced by these parents stain positive for cytoplasmic content.

Importantly, the distribution of aborted pollen within tetrads indicated that pollen defects segregated in parallel with the *pirl1*;*pirl9* genotype. Tetrads from *pirl1*/*PIRL1*;*pirl9* plants consistently contained two inviable grains, reflecting post-meiotic expression of the *pirl1*;*pirl9* phenotype (Fig. 1c). Less than 10% of tetrads contained a third aborted grain, consistent with the occasional pollen abortion observed in *pirl9* single mutants (Fig. 1a), which also were segregating within these tetrads. Similarly, tetrads from *pirl1*;*pirl9*/*PIRL9* plants exhibited meiotic segregation of the pollen-lethal phenotype (Fig. 1c). Because the pollen abortion frequency is less than 50% for this genotype (Fig. 1a), not all tetrads contained two aborted grains; many contained just one. However, the distribution of aborted pollen in these tetrads was

Table 1 Reduced transmission of *pirl1*;*pirl9* mutant pollen in crosses to wild type

Parent genotype[a]	Expected heterozygotes in F1[b]	Observed heterozygotes in F1[c] (%)	χ^2 (P value)[d]
WT × *pirl*1-1 +/−, *pirl*9-1 −/−	34.5/69 (50%) *pirl*1 heterozygotes	0/69 (0)	69 (≪0.005)
WT × *pirl*1-1 −/−, *pirl*9-1 +/−	67.5/135 (50%) *pirl*9 heterozygotes	6/135 (4)	112.1 (≪0.005)
WT × *pirl*1-2 +/−, *pirl*9-2 −/−	24/48 (50%) *pirl*1 heterozygotes	0/48 (0)	48 (≪0.005)
WT × *pirl*1-2 −/−, *pirl*9-2 +/−	24/48 (50%) *pirl*9 heterozygotes	2/48 (4)	40.3 (≪0.005)
*pirl*1-1 +/−, *pirl*9 −/− × WT	24/48 (50%) *pirl1-1* heterozygotes	22/48 (46)	NS
*pirl*1-1 −/−, *pirl*9-1 +/− × WT	41/82 (50%) *pirl9-1* heterozygotes	43/82 (52)	NS
*pirl*1-2 +/−, *pirl*9-2 −/− × WT	23/46 (50%) *pirl1-2* heterozygotes	23/46 (50)	NS
*pirl*1-2 −/−, *pirl*9-2 +/− × WT	24/48 (50%) *pirl9-2* heterozygotes	23/48 (48)	NS

Significant P values (<0.05) indicate aberrant transmission

NS χ^2 value not significant

[a] Crosses with wild type (WT), using mutants with indicated homozygous/heterozygous *pirl* allele combinations. Parents used as pollen donors are listed second in each cross. Reciprocal crosses with wild type used as pollen donor are shown in the bottom tier (Forsthoefel et al. 2010)

[b] Predictions for expected values focused on the *pirl* gene for which the pollen donor plant was heterozygous: with normal transmission of mutant alleles, approximately 50% of F1 progeny should receive the *pirl* knockout allele from the parent heterozygous for that mutation, and 50% should contain only the wild-type allele of that *PIRL*

[c] F1 seedlings were genotyped by PCR using gene- and gene/T-DNA-specific primer combinations as described in "Materials and methods"

[d] χ^2 was calculated based on an expected 1:1 ratio of heterozygotes:homozygous wild types for the *PIRL* gene for which the parent was heterozygous

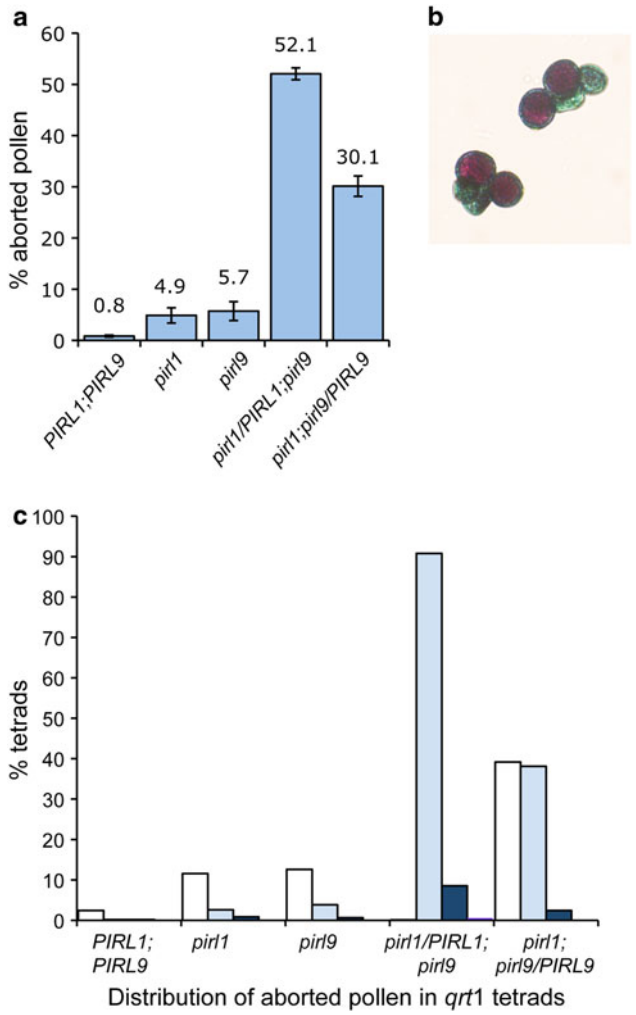

Fig. 1 Lethality of the *pirl*1;*pirl*9 double mutant genotype. Pollen produced by plants of the indicated genotypes in a *qrt*1 background was stained with Alexander's viability stain and viewed by bright field microscopy. Single mutant homozygotes produce 100% single-mutant pollen; genotypes with both knockout mutations produce 50% double-mutant pollen. **a** Frequency of fully aborted pollen produced by plants of different *pirl* genotypes. Mean values (±SE) derived from three to ten plants of each genotype are plotted. *N* values: 2,468, 1,384; 1,872; 3,560; 2,308, respectively. **b** Pollen produced by a *qrt1*;*pirl1-1*;*pirl9-1*/*PIRL9* plant, illustrating the segregation of the *pirl1*;*pirl9* pollen-lethal phenotype within tetrads. **c** Post-meiotic distribution of aborted pollen in *qrt*1 tetrads segregating for 50% *pirl1*;*pirl9* mutant pollen. *White* percentage of tetrads with 1 dead; *light blue* tetrads with 2 dead; *dark blue* tetrads with 3 dead pollen. Given the 30.1% frequency of the aborted pollen phenotype in *pirl1*;*pirl9*/*PIRL9* anthers (**a**), the distribution in tetrads for this genotype is non-random ($\chi^2 = 52.27$; $P \ll 0.001$; $n = 577$ tetrads), reflecting post-meiotic segregation of the aborted pollen phenotype

non-random and skewed toward two aborted grains per tetrad ($\chi^2 = 52.27$, $P \ll 0.001$, $n = 577$). This distribution indicated that *pirl1*;*pirl9* pollen defects were due to loss of *pirl1* and *pirl9* function in the haploid generation, not to pre-meiotic defects in the parental anther. This was consistent with prior studies of *pirl1*;*pirl9* phenotype segregation (Forsthoefel et al. 2010) and was subsequently further supported by fluorescence microscopy (see below).

Variable phenotype in double mutant pollen produced by *pirl1*/*PIRL1*;*pirl9* plants

Closer inspection of pollen following viability staining revealed further differences between pollen produced by *pirl1*/*PIRL1*;*pirl9* versus *pirl1*;*pirl9*/*PIRL9* plants. As previously reported, the homozygous *pirl9* genotype produced approximately 50% phenotypically normal and 50% small, clearly aborted *pirl1*;*pirl9* grains (Fig. 2), a result of consistent developmental arrest prior to pollen mitosis I (Forsthoefel et al. 2010). *pirl1*;*pirl9*/*PIRL9* plants, however, produced pollen with a range of sizes and staining characteristics, including small and intermediate-size pollen that stained positive with Alexander's stain, as well as clearly aborted grains (Fig. 2c). This phenotype range was observed with both *pirl1-1*;*pirl9-1* and *pirl1-2*;*pirl9-2* allele combinations. The total number of abnormal pollen produced by these plants, including both aborted and small positive-staining pollen, was between 40 and 50%, which approached the expected segregation of *pirl1*;*pirl9* genotype (Fig. 2d). Observation of terminal morphology by SEM revealed severely deformed, clearly inviable grains as well as others with abnormal morphology (Fig. 3). These varied in size and degree of deformation. This observation was consistent with the phenotypic variability seen in the hydrated Alexander-stained samples from this genotype.

Variable timing of developmental arrest in *pirl1*;*pirl9* mutant pollen

The range of pollen phenotypes observed with Alexander's staining and SEM suggested that developmental arrest may have been occurring with variable timing. To further investigate, we used DAPI staining to determine the terminal nuclear content of double mutants produced by *qrt1*;*pirl1*;*pirl9*/*PIRL9* plants, which produce tetrads segregating for two *pirl1*;*pirl9* pollen. Representative results are shown in Fig. 4. As predicted, tetrads each contained two phenotypically normal pollen with properly organized trinucleate male germ units. Tetrads also featured abnormal grains, ranging from aborted microspores to binucleate pollen. 94.4% of tetrads observed contained at least one aborted microspore and/or abnormal pollen. 81.5% of tetrads contained two abnormal grains, consistent with the post-meiotic expression of the *pirl1*;*pirl9* phenotype. In total, 88% of segregating *pirl1*;*pirl9* mutant pollen were aborted or had abnormal nuclear content ($n = 498$). Of those, 50.5% arrested as binucleate pollen and 49.5% arrested as microspores, which appeared either denucleated

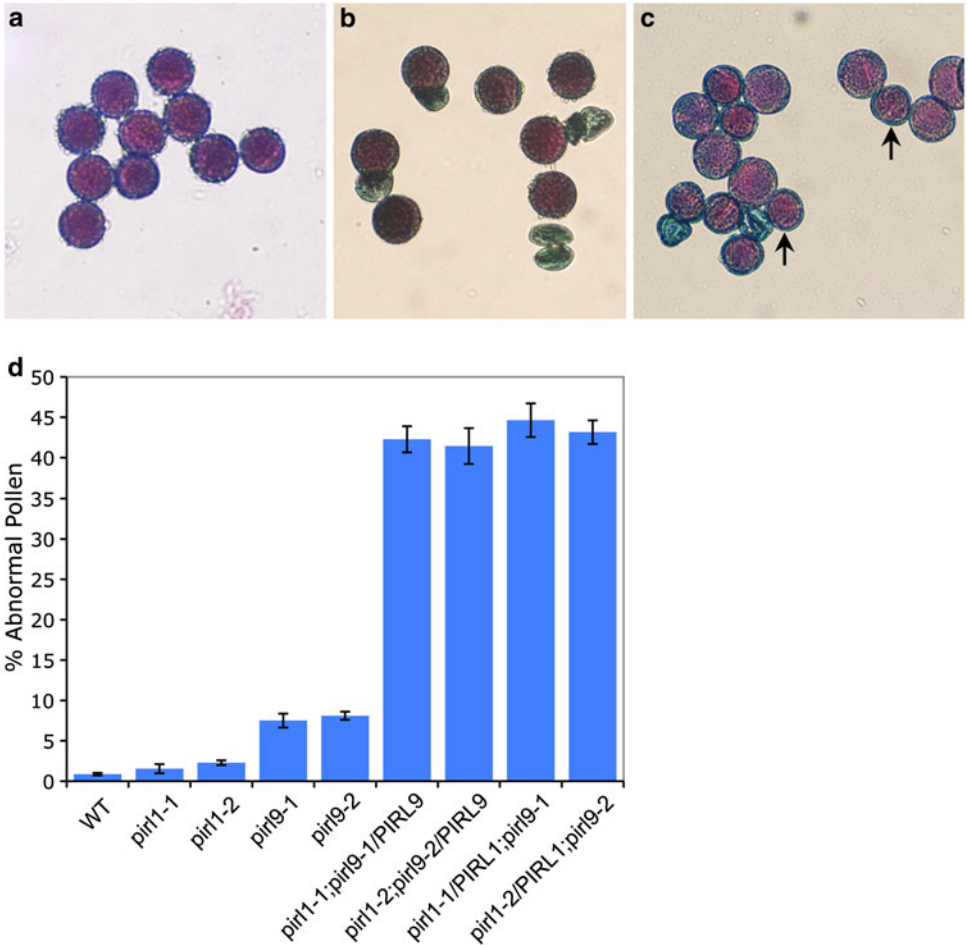

Fig. 2 Variable expressivity of the *pirl1*;*pirl9* pollen phenotype in pollen produced by *pirl1*;*pirl9*/*PIRL9* parents. Dehisced pollen from wild type or from plants producing double mutant pollen was treated with Alexander's stain and visualized by bright field microscopy. **a** wild-type pollen, uniform in size and staining intensity; **b** Pollen produced by a *pirl1*/*PIRL1*;*pirl9* plant, which produces 50% *pirl1*;*pirl9* double mutant pollen; **c** Pollen from a *pirl1*;*pirl9*/*PIRL9* plant, which also produces 50% *pirl1*;*pirl9* double mutant pollen. *Arrows* indicate representative examples of abnormally small pollen that stain positive with Alexander's viability reagent. **d** Comparison of the percentages of total abnormal pollen (small/*purple* plus aborted/*green*) from parents with indicated genotypes. Mean values (±SE) derived from three to ten plants of each genotype are plotted. *N* values: 1,608, 1,402, 1,078, 1,734, 1,851, 1,507, 687, 584, and 1,103, respectively

or with a residual single nucleus. In contrast, tetrads from *pirl1*/*PIRL1*;*pirl9* plants consistently featured two clearly aborted, denucleated grains (Fig. 4b) as expected from the consistent microspore arrest reported previously for that genotype (Forsthoefel et al. 2010). Thus, *pirl1*;*pirl9* pollen produced by *pirl9* heterozygotes have more variable nuclear content and terminal morphology than their *pirl1*;*pirl9* counterparts from *pirl9* homozygotes.

Discussion

We have previously shown that *PIRL1* and *PIRL9* have redundant functions essential for pollen development: *pirl1*;*pirl9* double mutants produced by *pirl9* homozygotes are severely shrunken and distorted, due to consistent developmental arrest of microspores just prior to pollen mitosis I (Forsthoefel et al. 2010). Here, we show that *pirl1*;*pirl9* pollen produced by *pirl9* heterozygotes have a more variable, often less severe, terminal phenotype. This appears to be a result of variable time of developmental arrest: *pirl1*;*pirl9* pollen from *pirl9* heterozygotes can arrest as microspores or later, as binucleate stage pollen.

Double mutant pollen from *pirl9* heterozygotes exhibited extremely poor transmission in reciprocal crosses (Table 1) while initial viability staining suggested an incomplete penetrance of pollen defects (based on 30.1% pollen abortion in anthers segregating 50% for *pirl1*;*pirl9* pollen; Fig. 1.) Alexander's stain has the potential to overestimate actual pollen viability because it simply detects cytoplasmic content (Dafini et al. 2005; Baez et al. 2002). Providing a more accurate view of pollen abnormalities, DAPI staining demonstrated that the vast majority (88%) of segregating *pirl1*;*pirl9* pollen from *pirl9* heterozygotes were abnormal in terms nuclear content (Fig. 4). This indicates that development of a normal trinucleate germ unit was not completed prior to anther maturation, and it can explain the poor genetic transmission observed for the *pirl1*;*pirl9* allele combination.

pirl1;*pirl9* phenotype variability suggests a requirement for PIRLs after pollen mitosis I

The variable developmental arrest time observed in this study suggests a requirement for *PIRL* gene functions beyond the microspore stage. Prior characterization of *pirl1*;*pirl9* pollen derived from *pirl9* homozygotes indicated

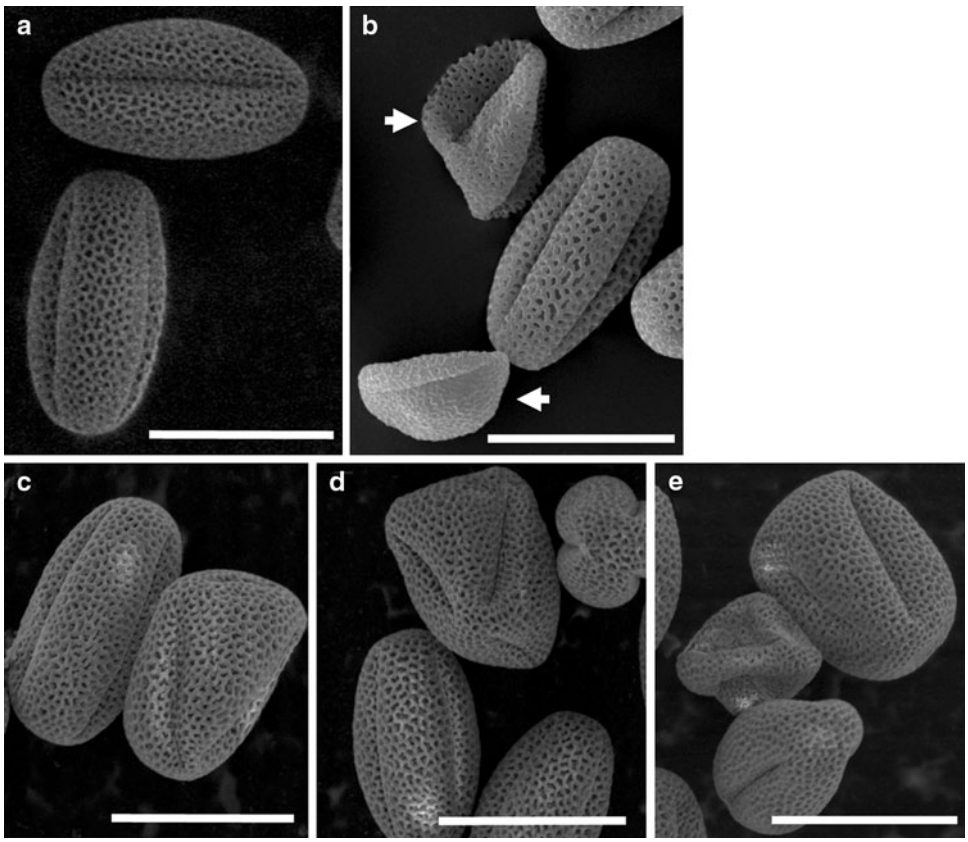

Fig. 3 Variable morphology of double-mutant pollen produced by *pirl1*;*pirl9*/*PIRL9* plants. Dehisced pollen produced by plants with different genotypes was viewed by scanning electron microscopy. **a** Wild-type pollen; **b** pollen from a *pirl1*/*PIRL1*;*pirl9* plant; *arrows* indicate segregating *pirl1*;*pirl9* grains, which appear severely shrunken and deformed; **c**–**e** pollen from *pirl1*;*pirl9*/*PIRL9* anthers segregating for *pirl1*;*pirl9* double mutants, illustrating a range of observed pollen morphologies. *Bars* 20 μm

a clear requirement for *PIRL* genes in the late microspore, before the onset of pollen mitosis (Forsthoefel et al. 2010). If *PIRL1* and *PIRL9* were only transiently required at that point in the late microspore, then mutant pollen that progressed beyond the microspore arrest point would be predicted to successfully complete development to form functional pollen. However, this was not observed; *pirl1*;*pirl9* pollen that managed to progress beyond pollen mitosis I in this study still failed to complete germ unit development and efficiently transmit in reciprocal crosses. Therefore, the lack of both *pirl1* and *pirl9* continues to hinder pollen development after the onset of mitosis. This finding is consistent with transcript expression profiles from microarray data, which suggest that both *PIRL1* and *PIRL9* are expressed in developing pollen through the mitotic stages (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al. 2007). Thus, while *PIRL1* and *PIRL9* are clearly essential in the microspore, the variable phenotype reported here suggests that they must also normally function at least into the mitotic stages of pollen development.

PIRL9 in the sporophyte influences *pirl1*;*pirl9* phenotype expression in the haploid generation

The meiotic segregation of the pollen defects in *qrt1* tetrads and the fact that developmental defects do not appear in *pirl1*;*pirl9* microspores until anther stage 10 (Forsthoefel et al. 2010) indicate clearly that pollen defects are due to the compromised activity of these genes in the haploid generation. Given that *pirl1* and *pirl9* first become essential after meiosis, reduction in phenotype severity in *pirl1*;*pirl9* pollen from *PIRL9* heterozygotes must occur by the following mechanism: sporophytic *PIRL9* activity partially compensates for the lack of *PIRL9* function in the haploid phase, thus delaying expression of the *pirl1*;*pirl9* pollen-lethal phenotype.

This genetic mechanism accounts for observed phenotypic variability, and it raises a new set of questions about the molecular mechanism of the sporophytic effect. We propose two further mechanistic models. The most straightforward model is one in which some residual *PIRL9* mRNA or protein from the pollen mother cell persists in microspores after meiotic divisions, occasionally allowing for some continued development of *pirl1*;*pirl9* pollen beyond pollen mitosis I (the point at which *pirl1*;*pirl9* pollen from *pirl9* homozygotes arrests). Gradual loss of this residual *PIRL9* product could explain the variable arrest observed in mutant pollen in this study (Fig. 4) and the ultimately poor *pirl1*;*pirl9* transmission observed in genetic experiments (Table 1). In the second model, *PIRL9* expression in surrounding anther tissue could indirectly provide an environment that better supports development

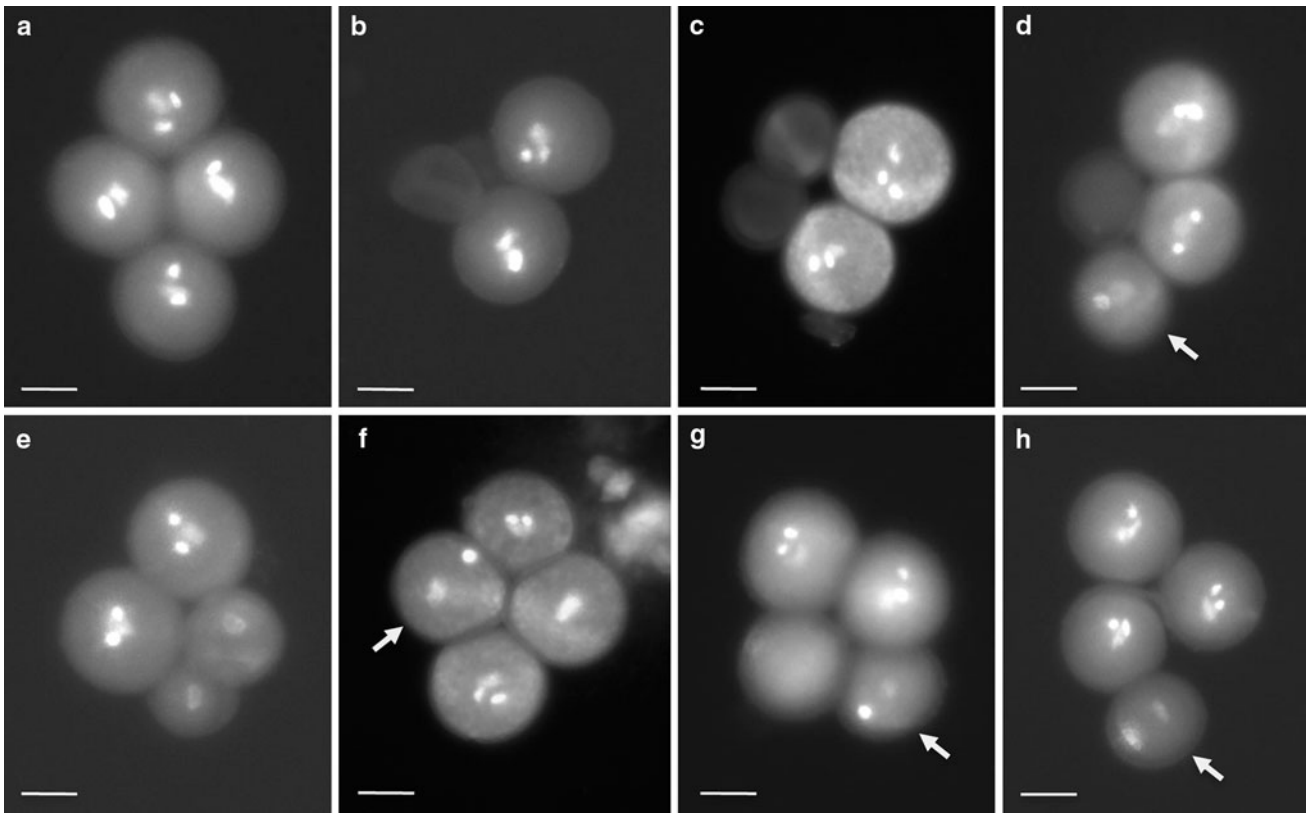

Fig. 4 Variable timing of developmental arrest in *pirl*1;*pirl*9 mutant pollen from *qrt1*;*pirl1*;*pirl9*/*PIRL9* plants. *qrt*1 control tetrads and tetrads produced by *qrt1*;*pirl1*/*PIRL1*;*pirl9* and *qrt1*;*pirl1*;*pirl9*/*PIRL9* plants, which segregate 50% for *pirl1*;*pirl9* double mutant pollen, were obtained from anthers near maturity, stained with DAPI, and observed by fluorescence microscopy. **a** Tetrad from a *qrt1*;*PIRL1*;-*PIRL9* plant, illustrating normal trinucleate germ unit organization. **b** A representative tetrad from a *qrt1*;*pirl1*/*PIRL1*;*pirl9* plant, with two aborted denucleate pollen, illustrating post-meiotic expression and full penetrance of early lethality in *pirl1*;*pirl9* pollen produced by plants homozygous for *pirl*9. **c**–**h** Representative tetrads produced by *qrt1*;*pirl1*;*pirl9*/*PIRL9* plants, illustrating variable developmental arrest. **c** Tetrad with double mutants arrested as microspores; both have apparently undergone loss of DNA. **d** Tetrad with one binucleate mutant arrested after the first pollen mitosis (*arrow*) and one apparently denucleate grain; **e** tetrad with both double mutants arrested as mononucleate microspores; **f**, **g** tetrads each with a *pirl1*;*pirl9* mutant arrested at the binucleate stage (*arrows*); in each, the generative nucleus remains near the pollen wall; **h** tetrad in which one mutant is arrested at the binucleate stage (*arrow*), and the other is indistinguishable from normal trinucleate siblings. 88% of *pirl1*;*pirl9* pollen segregating in *qrt1*;*pirl1*;*pirl9*/*PIRL9* tetrads appeared aborted or had abnormal nuclear content ($n = 498$), with 50.5% of those arrested at the binucleate stage and 49.5% arrested as either uninucleate or aborted, denucleated microspores. *Scale bar* 10 μm

of *pirl1*;*pirl9* pollen, such that the severity of the post-meiotic phenotype is temporarily and partially reduced. This model is not meant to imply that *PIRL9* functions only in surrounding anther tissue: *PIRL* function must still be required after meiosis in microspores and pollen to account for the observed meiotic segregation of the mutant phenotype and the late-microspore timing of developmental defects. This model only implies that *pirl1*;*pirl9* microspores could be sensitive to the *pirl*9 status of surrounding tissue. The exact nature of this "supportive environment" cannot be deduced from our genetic experiments; we offer it only as an alternative model to explain the observed variability in *pirl1*;*pirl9* pollen phenotype.

Both of these models would predict *PIRL9* expression in diploid tissues in the anther as well as microspores and mitotic pollen. The first model, for example, predicts *PIRL9* expression in pollen mother cells, while the second model predicts expression in surrounding anther tissue while pollen develops. These scenarios are consistent with previous *PIRL9* expression data showing transcript expression in developing flowers (Forsthoefel et al. 2005) and stamens (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al. 2007; Zimmermann et al. 2004). Future detailed investigation of spatio-temporal patterns of *PIRL9* expression in developing anthers should help distinguish between these two models, as would reverse genetic investigation of *PIRL* functions in the sporophyte. Such experiments may be complicated by low abundance of the *PIRL9* transcript and by the pollen lethality of the *pirl1*;*pirl9* genotype which prevents recovery of double-mutant sporophytes. Regardless of the precise molecular mechanism, the genetic results reported here imply some

form of *PIRL9* sporophytic effect on the expression of the *pirl1*;*pirl9* phenotype in developing pollen.

A novel example of unequal redundancy

Genetic redundancy is a prominent feature of plant genomes, in which gene duplication by various mechanisms has played an important role in genome evolution (The Arabidopsis Genome Initiative 2000; Bouche and Bouchez 2001; Briggs et al. 2006 and references therein). Quite often, one gene of a redundant gene pair has a stronger or more widespread effect on phenotype, a phenomenon that has been termed unequal redundancy (Briggs et al. 2006). In the case of *pirl*1 and *pirl*9 in developing pollen, redundancy appears to be almost complete: pollen inviability in single mutants for either *pirl*1 or *pirl*9 is only slightly higher than that of wild type, suggesting that pollen function is usually unaffected as long as one of the genes is intact (Forsthoefel et al. 2010; Fig. 2). However, we did observe a slightly higher percentage of abnormal pollen produced by *pirl9* homozygotes than *pirl1* homozygotes (approximately 7–8 vs. 1–2% in *pirl1* homozygotes; Fig. 2). In that respect, there is a straightforward minor inequality in the redundancy between these genes, with *pirl9* having a somewhat higher impact as a single mutant. A more unusual example of unequal redundancy is offered by the differential effects on *pirl1*;*pirl9* pollen by *PIRL1* versus *PIRL9* wild-type alleles in the sporophyte. The presence of *PIRL1* in the sporophyte allows for consistent expression of the lethal mutant phenotype in *pirl1*;*pirl9* microspores, while sporophytic *PIRL9* temporarily complements the mutant pollen phenotype, delaying the onset of *pirl1*;*pirl9* pollen defects and resulting in more phenotypic variability.

Acknowledgments This work was supported by NSF award 0616166 to D.M.V. Whitman College's SEM facility was provided by NSF grant 0922978. We thank Thuy Dao for assistance with PCR and phenotype analyses, and Michelle Shafer and Caroline Reinhart for assistance with SEM. Caroline Reinhart was supported in part by a Voyles summer research scholarship, provided by a gift to Whitman College.

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