Development 138, 3409-3420 (2011) doi:10.1242/dev.060384

Female gametophytic cell specification and seed development require the function of the putative *Arabidopsis* INCENP ortholog *WYRD*

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SUMMARY

In plants, gametes, along with accessory cells, are formed by the haploid gametophytes through a series of mitotic divisions, cell specification and differentiation events. How the cells in the female gametophyte of flowering plants differentiate into gametes (the egg and central cell) and accessory cells remains largely unknown. In a screen for mutations that affect egg cell differentiation in *Arabidopsis*, we identified the *wyrd* (*wyr*) mutant, which produces additional egg cells at the expense of the accessory synergids. *WYR* not only restricts gametic fate in the egg apparatus, but is also necessary for central cell differentiation. In addition, *wyr* mutants impair mitotic divisions in the male gametophyte and endosperm, and have a parental effect on embryo cytokinesis, consistent with a function of *WYR* in cell cycle regulation. *WYR* is upregulated in gametic cells and encodes a putative plant ortholog of the inner centromere protein (INCENP), which is implicated in the control of chromosome segregation and cytokinesis in yeast and animals. Our data reveal a novel developmental function of the conserved cell cycle-associated INCENP protein in plant reproduction, in particular in the regulation of egg and central cell fate and differentiation.

KEY WORDS: Cell specification, Differentiation, Gametophytes, INCENP, Maternal effect, Seed development, Sexual reproduction, Cell cycle regulation, *WYRD*

INTRODUCTION

The life cycle of plants alternates between a diploid sporophytic and a haploid gametophytic generation. In flowering plants (angiosperms) gametes are formed by sexually dimorphic gametophytes, which are derived from the meiotic products of sporophytic cells, the megaspore mother cells (MMCs) and pollen mother cells (PMCs), respectively. Angiosperm gametophytes comprise gametic and accessory cells, the latter aiding the delivery of gametes and/or double fertilization (for reviews, see Boavida et al., 2005; Brukhin et al., 2005). The *Arabidopsis* male gametophyte (pollen) develops from the meiotic products (microspores) by an asymmetric division (pollen mitosis I, PMI) producing a large vegetative and a small generative cell that divides again (PMII) to form two sperm cells (Borg et al., 2009). After pollen germination, the growing pollen tube delivers the sperm cells to the female gametophyte (embryo sac), which develops within the ovule from one of the meiotic products, the functional megaspore, through three syncytial divisions. Subsequent cellularization forms four cell types: the two female gametes, the haploid egg and the homo-diploid central cell, and the accessory synergids and antipodals (for reviews, see Brukhin et al., 2005; Pagnussat et al., 2009). After the pollen tube penetrates one of the synergids, ruptures and discharges the sperm (pollen tube reception), one sperm each fertilizes the egg and central cell to form the diploid zygote (and resulting embryo) and the triploid endosperm, respectively.

Although genetic and molecular approaches have identified many factors involved in male and female gametophyte development (for reviews, see Borg et al., 2009; Brukhin et al., 2005; Dresselhaus and Marton, 2009; Kägi and Gross-Hardt, 2007; Liu and Qu, 2008; Sundaresan and Alandete-Saez, 2010; Yadegari and Drews, 2004; Yang et al., 2010) and maternal effects on seed formation (for reviews, see Berger and Chaudhury, 2009; Grossniklaus, 2005; Huh et al., 2008; North et al., 2010), the molecular processes underlying cell specification and differentiation are still poorly understood. Only a small number of genes involved in the cellular differentiation of gametophytic cell types has been described. Because manipulation of auxin response factors or auxin biosynthesis in the embryo sac affects cell identity, it was suggested that an auxin gradient might provide the spatial information for cell specification (Pagnussat et al., 2009). How the auxin gradient is interpreted to determine cell fate is, however, unknown. In *Arabidopsis*, core components of the splicing machinery are required for the maintenance of gametic versus accessory cell fate (Gross-Hardt et al., 2007; Moll et al., 2008). In addition, central cell fate depends on type I MADS-domain transcription factors (Bemer et al., 2008; Portereiko et al., 2006; Steffen et al., 2008), whereas a MADS target gene is necessary for the differentiation of accessory cells (Matias-Hernandez et al., 2010) and a MYB transcription factor for synergid differentiation (Kasahara et al., 2005). In maize, a LOB-domain transcription

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Accepted 31 May 2011

factor (Evans, 2007; Guo et al., 2004) and a diSUMO-like protein (Srilunchang et al., 2010) were shown to be involved in embryo sac differentiation.

A loss-of-function analysis in *Arabidopsis* showed that RETINOBLASTOMA RELATED (RBR), a conserved cell cycle regulator controlling S-phase entry (Inze and De Veylder, 2006), is crucial for differentiation of all cell types, not only in female (Johnston et al., 2010; Johnston et al., 2008) but also in male gametophytes (Chen et al., 2009; Johnston et al., 2008). In addition to *rbr*, several mutations in *Arabidopsis* impairing cell cycling affect cell fate and mitotic progression in the male gametophyte. Most of the mutants arrest at PMII (Brownfield et al., 2009; Chen et al., 2008; Durbarry et al., 2005; Iwakawa et al., 2006; Nowack et al., 2006; Rotman et al., 2005) and affect the identity of the generative cell. By contrast, the role of cell cycle regulators in the female gametophyte is less well understood. The *prolifera* (*prl*) mutant disrupting an S-phase-specific DNA replication licensing factor affects mitotic divisions late during female gametogenesis and produces maternal effects on embryogenesis (Springer et al., 2000). Maternal effects play an important role in embryonic development in most animal species (for reviews, see Glover, 2005; Li et al., 2010; Lindeman and Pelegri, 2010) but have only recently been investigated in higher plants (for reviews, see Grossniklaus, 2005; Grossniklaus and Schneitz, 1998; Rodrigues et al., 2010). Like *prl*, about half of the gametophytic mutants in *Arabidopsis* display gametophytic maternal effects early in seed development (Moore et al., 2002; Pagnussat et al., 2005), including the well-studied *FERTILIZATION-INDEPENDENT SEED* (*FIS*) class genes (Chaudhury et al., 1997; Grossniklaus et al., 1998; Guitton et al., 2004; Köhler et al., 2003; Luo et al., 1999; Ohad et al., 1996; Ohad et al., 1999) and several cell cycle genes (Andreuzza et al., 2010; Pignocchi et al., 2009). How cell cycle regulators contribute to cellular differentiation is unclear because it is difficult to separate effects on proliferation and differentiation; but some, such as *RBR*, affect differentiation independently of their role in cell cycle regulation in both animals and plants (Inze and De Veylder, 2006; Johnston et al., 2010; Korenjak and Brehm, 2006).

Here, we report the characterization of *WYRD* (*WYR*), identified by its role in cell fate specification and thus named after one of the three Norns, goddesses of fate in Norse mythology (Brodeur, 1916). *WYR* encodes a putative *Arabidopsis* ortholog of the inner centromere protein (INCENP) and plays a role during both gametophytic differentiation and postfertilization development. Disruption of *WYR* affects cell fate establishment in the female gametophyte and progression through PMI during pollen development. Consistent with the developmental phenotypes of *wyr*, *WYR* transcripts are detected primarily within the developing male and female gametophytes. This is the first report elucidating the developmental function of a plant INCENP, which, in yeast and animals, has been implicated in chromosome segregation and cytokinesis via a functional complex with aurora kinases and other chromosome passenger complex (CPC) proteins. This suggests that these proteins not only play a role in cell cycle regulation, but also in cell fate determination, possibly through equal but non-random segregation of chromosomes.

MATERIALS AND METHODS

Plant material and growth conditions

The wild-type plants used were *Arabidopsis thaliana* (L.) Heynh. var. Landsberg (*erecta* mutant: L*er*) or var. Columbia-0 (Col-0). Plant growth conditions were as previously described (Boisson-Dernier et al., 2009; Groß-Hardt et al., 2007). The *wyr-1* allele was isolated in a mutagenesis screen as described (Groß-Hardt et al., 2007); the *wyr-2* (*GK-065B09*; Col-0) and *wyr-3* (*ET12763*; L*er*) alleles were obtained from GABI-Kat (http://www.gabi-kat.de) and Cold Spring Harbor Laboratory (http://genetrap.cshl.org). Marker lines were as previously described (Chen et al., 2007; Groß-Hardt et al., 2007; Köszegi et al., 2011; Luo et al., 2000; Twell, 1992). Tetraploid *wyr-2* plants were generated by repeated backcrossing to a tetraploid wild-type plant (Col-0) (Johnston et al., 2010) as previously described (Grossniklaus et al., 1998; Huck et al., 2003). Expected phenotypic ratios for recessive and dominant models were based on maximal double reduction (Burnham, 1964) and reduced transmission of the *wyr-2* allele through both gametophytes.

Morphological and histological analyses

Ovule and seed clearing, whole-mount histochemical β-glucuronidase (GUS) assays, and Alexander staining for pollen viability were performed as previously described (Groß-Hardt et al., 2007; Johnston et al., 2008; Vielle-Calzada et al., 2000). Microscopic analyses were performed on a Leica HC microscope with differential interference contrast (DIC) optics or a Leica DM6000 fluorescence microscope (Leica Microsystems, Mannheim, Germany).

Positional cloning of *wyrd-1*

Genetic mapping was performed on a Col-0×*wyr-1*(L*er*) F_2 population (n>1000) using accession-specific polymorphisms (Cereon database, www.arabidopsis.org) for simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequences (CAPS) marker design. Wild-type and semisterile mutant plants were separated into two subgroups for independent mapping of the *WYR* and *wyr-1* alleles, respectively. Because the mapping interval of 117 kb contained no Col-0/L*er* polymorphisms, the Surveyor nuclease assay (Transgenomic, Omaha, NE, USA) was used according to the manufacturer's recommendations for *wyr-1* fine mapping. Primers were designed to obtain overlapping fragments spanning 2- to 2.5-kb regions of predicted coding sequences (see Table S1 in the supplementary material) and PCR products were digested using the Cel-A nuclease to identify nucleotide mismatches specific to *wyr-1*. The PCR fragment with a specific Surveyor restriction pattern in the mutant was directly sequenced to identify the lesion in *wyr-1* (see Fig. S1 in the supplementary material). Despite repeated attempts using various methods, we were unable to clone the full-length *WYR* cDNA in different bacterial strains, suggesting that it is toxic or unstable in bacteria. The cDNA sequence submitted to NCBI (accession number JF817219) is therefore derived from the direct sequencing of the RT-PCR product of the full-length cDNA (Fig. 7A).

RNA extraction, RT-PCR and RACE

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RT-PCR was performed as published (Johnston et al., 2007) using SuperScript III (Invitrogen). RACE was carried out according to the manufacturer's instructions (GeneRacer Kit, Invitrogen) with gene-specific primers designed on the basis of sequence of RT-PCR fragments (see Table S1 in the supplementary material).

In situ hybridization

In situ hybridization was performed as described previously (Johnston et al., 2007; Vielle-Calzada et al., 1999) using an antisense probe synthesized from a pDrive vector (Qiagen, Hilden, Germany) with a *WYR* cDNA fragment spanning the IN-box domain (see Table S1 in the supplementary material).

RESULTS

The gametophytic mutation *wyrd* affects reproduction

We identified *wyr-1* in a screen for mutants affecting egg cell identity, from deviations in the expression of the egg cell marker *ET1119* (Groß-Hardt et al., 2007). In *wyr-1* mutant embryo sacs, *ET1119* expression was extended, including the egg cell and the domain of the synergids (see below). Because we could not recover

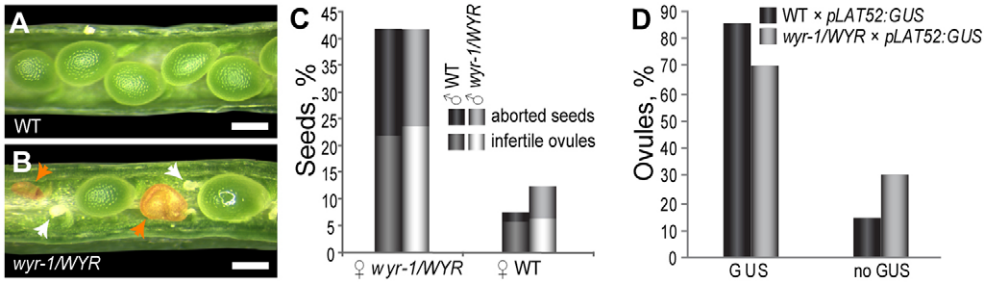

Fig. 1. The *wyr-1* mutant has reduced seed set and exhibits gametophytic phenotypes. (**A-C**) Seed set reduction in *wyr-1/WYR* plants. (A) Dissected wild-type silique with viable seeds at the late walking stick embryo stage. (B) *wyr-1/WYR* silique at a comparable stage; note infertile ovules (white arrows) and aborted seeds (orange arrows). (C) Percentage seed set in reciprocal crosses. Pollinating *wyr-1/WYR* mothers with wild-type pollen did not rescue seed abortion, in contrast to pollination with pollen from *wyr-1/WYR* plants (*n*=416 and 364, respectively). Crosses of the wild type with pollen from *wyr-1/WYR* plants slightly increased seed abortion (*n*=2468 and 1562, respectively). (**D**) Successful pollen tube reception is reduced in *wyr-1/WYR* female plants as evidenced by the percentage of paternal *pLAT52:GUS* marker expression in ovules upon fertilization. Scale bars: 300 μm in A,B.

any homozygous *wyr-1* plants, only heterozygous *wyr-1/WYR* mutants were analyzed. The *wyr-1/WYR* plants appeared normal (not shown), suggesting that the mutation is recessive in the sporophyte but that the gene is essential for gametogenesis and/or seed development. In contrast to siliques of wild-type segregants, in which 95% of the seeds develop normally, siliques of *wyr-1/WYR* mutants showed a reduced seed set of about 58% (Fig. 1A,B), demonstrating that *wyr-1* affects seed formation. We noticed equal proportions of infertile ovules and seeds aborted at distinct stages after fertilization (Fig. 1B,C, white and orange arrows, respectively). In order to examine the genetic nature of *wyr-1*, we assessed seed set in reciprocal crosses. Pollination of *wyr-1/WYR* plants with wild-type pollen neither restored fertility nor changed the ratio between infertile ovules and aborted seeds (Fig. 1C). This indicates that seed abortion is under gametophytic maternal control and that zygotic embryo lethality plays no or only a minor role in reducing fertility. Paternally inherited *wyr-1* had only a minor effect on seed set, which was reduced by 5%, in contrast to a reduction of 42% if *wyr-1* was maternally inherited (Fig. 1C).

To investigate fertilization success in *wyr-1/WYR* plants we crossed them with a *pLAT52::GUS* marker line, where GUS activity is observed in the receptive synergid after the pollen tube penetrates the synergid and ruptures. In heterozygous mutants, pollen tube reception was reduced by 15% (Fig. 1D), roughly correlating with the proportion of infertile ovules in *wyr-1/WYR* siliques (Fig. 1C). This finding demonstrates that *WYR* plays a role in the female gametophyte in addition to its gametophytic maternal requirement for seed development.

Concomitant with the reduction in seed set, the proportion of mutants in the progeny of self-fertilized *wyr-1/WYR* plants (Table 1) was much lower (0.38:1) than the expected Mendelian segregation ratio for diploid sporophytic (3:1) or embryo lethal (2:1) mutants. This suggests an effect of *wyr-1* on both male and female gametophytes, because a female- or male-specific gametophytic defect does not exceed a 1:1 segregation ratio distortion in the progeny of a self-fertilized heterozygote (Howden et al., 1998; Moore et al., 1997). Transmission efficiency (TE=mutant/wild-type offspring) of *wyr-1* determined from reciprocal crosses with the wild type was reduced through both gametophytes, confirming gametophytic lethality (Table 1). The TE of *wyr-1* through female and male gametophytes was almost equal [TE_{female}(*wyr-1*)=0.18 and TE_{male}(*wyr-1*)=0.21], although only maternal *wyr-1* caused a strong decrease in seed set, suggesting an effect of *wyr-1* on pollen development. Moreover, 8% of the viable seeds carried a maternal *wyr-1* allele (difference between 50% of *wyr-1* female gametophytes in *wyr-1/WYR* plants and 42% reduction in seed set), correlating with TE_{female}=18%, (0.08/0.50=0.16≈0.18). These data indicate that *wyr-1* affects both female and male gametophytes in addition to exerting a gametophytic maternal – and a minor paternal – effect on seed formation.

wyrd affects pollen mitosis I

Because the TE_{male} of *wyr-1* was only 21% (Table 1) but paternally inherited *wyr-1* had very little effect on seed set (Fig. 1C), we hypothesized that *wyr-1* affects male gametophyte development before pollen tube reception and fertilization. We examined the viability of mature pollen at anthesis when a significant fraction of male gametophytes from heterozygous *wyr-1/WYR* plants had aborted and were of variable size (Fig. 2A,B). A detailed analysis by DAPI staining showed that, although mature wild-type pollen was at the trinucleate stage (Fig. 2C,F), only 63% of pollen from *wyr-1/WYR* plants had reached this stage, approximately 24% and 12% had aborted and appeared larger, containing only one nucleus, respectively (Fig. 2C-F). Thus, in the absence of *WYR* activity a subset of microspores aborts, and a smaller fraction continues to grow but fails to undergo two pollen mitoses.

Approximately 13% of *wyr-1* mutant male gametophytes (the difference between 50% of wild-type *WYR* pollen produced by *wyr-1/WYR* plant and 63% of trinucleate pollen observed)

Table 1. Transmission of the *wyr-1* mutant allele to the progeny

Transmission	Cross	Segregation ratio (mutant:wild-type)	Expected Mendelian ratio	*n*
	wyr-1/WYR selfed	0.38:1	3:1	423
Male	*wyr-1/WYR* × WT	0.18:1	1:1	162
Female	WT × *wyr-1/WYR*	0.21:1	1:1	442

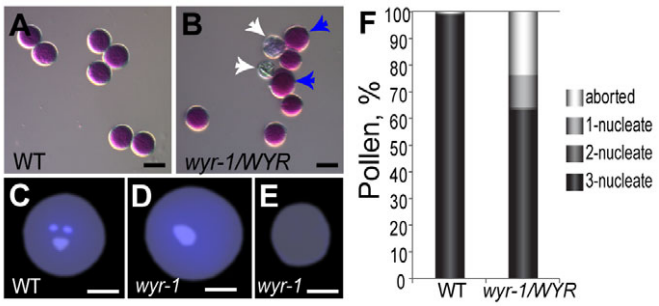

Fig. 2. Disruption of *WYR* impairs cell divisions in the male gametophyte. (**A,B**) Alexander staining for pollen viability reveals abortion of *wyr-1* male gametophytes. (A) Viable wild-type pollen grains at anthesis (purple). (B) A fraction of *wyr-1* pollen is shrunken and aborted (greenish color, white arrows); note that some viable pollen grains appear larger (blue arrows). (**C-F**) The microspore nucleus fails to divide in *wyr-1* mutants. Micrographs of DAPI-stained pollen at anthesis. (C) Mature wild-type trinucleate pollen grain with a large vegetative and two small sperm nuclei. (D) A *wyr-1* uninucleate pollen grain. (E) A *wyr-1* aborted pollen grain. (F) Male gametophyte classes at anthesis in wild-type and *wyr-1/WYR* plants (*n*=810 and 2755, respectively). Scale bars: 20 μm in A,B; 5 μm in C-E.

developed normally to the trinucleate stage (Fig. 2C,F), indicating incomplete penetrance of *wyr-1* in the male gametophyte. These data are in agreement with the observed TE_{male} of 21% (Table 1), which, considering the minor paternal effect of *wyr-1* on seed set (Fig. 1C), indicates that most trinucleate *wyr-1* pollen grains are functional. Therefore, the *wyr-1* mutation primarily affects early stages of male gametophyte development by impairing PMI.

Mutant *wyrd* female gametophytes have aberrant cellular differentiation

To investigate female gametophytic defects in *wyr-1* mutants, we performed cytological analyses and investigated the expression of cell type-specific markers. Although over 97% of wild-type central cells contained a fused nucleus (Fig. 3A,B, black arrows; histogram 3H, *n*=395), approximately 60% of *wyr-1* central cells (30% of all embryo sacs from *wyr-1/WYR* mutants) contained unfused polar nuclei, which were of different size in about half of the cases (Fig. 3C-E, gray arrows; histogram 3H, *n*=437). In addition, we found *wyr-1* embryo sacs that had an abnormal egg apparatus. In the wild type, egg and synergid nuclei are positioned at the chalazal and micropylar pole of the cell, respectively, and thus have opposite cell polarity (Fig. 3A,B, red and green arrows, respectively). Some *wyr-1* embryo sacs with atypical egg apparatuses had two cells with a nuclear orientation typical of the egg, but had only one with a micropylarly positioned nucleus typical of synergids (Fig. 3D,E, red and green arrows, respectively). In addition, a few *wyr-1* embryo sacs were arrested at the uninucleate stage and some collapsed during development (Fig. 3G-H). Apart from these rare aberrant embryo sacs, the *wyr-1* phenotypes become visible only after cellularization (see Fig. S2 in the supplementary material). Thus, *wyr-1* female gametophytes can contain two egg cells at the expense of a synergid, and polar nuclei that failed to undergo karyogamy were often a different size; these phenotypes were observed either alone or together in the same embryo sac. Given that nuclear size is indicative of the cell type (Groß-Hardt et al., 2007) (J. M. Moore, PhD thesis, State University of New York, 2002), these data suggest that *WYR* is involved in cell specification.

To determine how the *wyr-1* phenotypes correlate with the identity and differentiation status of cells in the embryo sac, we analyzed expression patterns of molecular markers specific to

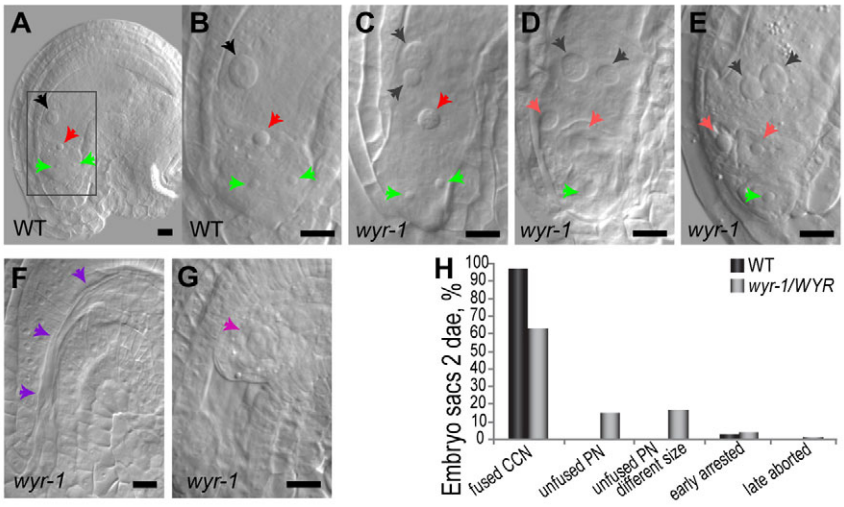

Fig. 3. Mutant *wyr-1* female gametophytes contain morphologically aberrant central cells and egg apparatuses. (**A-G**) Cleared mature embryo sacs 2 days after emasculation (2 dae; no fertilization). (A) Ovule bearing a mature four-celled wild-type embryo sac with a fused homo-diploid central cell nucleus (CCN, black arrow), an egg cell nucleus (red arrow) and two synergid nuclei (green arrows; the nuclear structures seen are the nucleoli). The box indicates the region of the female gametophyte (FG) with the egg apparatus and most of the central cell, which is shown in all other panels. (B) Wild-type FG. (C-E) Mutant *wyr-1* FGs. In all of FGs shown here, unfused polar nuclei (PN) of different size are visible (dark-gray arrows, compare to black arrow in B), whereas the FG in C has a morphologically normal egg apparatus consisting of an egg cell and two synergid nuclei (red and green arrows, respectively), the FGs in D,E have two egg-like cells (bright red arrows) but only one synergid (green arrow) as inferred from their cell polarity (i.e. the position of the nucleus). (F) A *wyr-1* FG aborted at a later stage of development (violet arrows indicate a collapsed FG). (G) A *wyr-1* FG arrested at the uninucleate stage (pink arrow). (**H**) FG phenotypic classes at 2 dae in wild-type (*n*=395) and *wyr-1/WYR* (*n*=437) ovules. Using a Fisher's exact test, normal and abnormal (all phenotypic classes combined) embryo sacs in wild-type versus mutant ovules are significantly different ($P<0.0001$). Scale bars: 30 μm in A,F,G; 10 μm in B-E.

synergids, egg and central cell. The central cell marker *FIS2::GUS* was expressed in all *wyr-1* central cell nuclei, including unfused polar nuclei of different sizes (Fig. 4A-D). By contrast, about 18% of *wyr-1* central cells showed no expression of the central cell marker *ET956* (in *wyr-1/WYR; ET956/–* plants 33% and 5% have normal or weak GUS expression, respectively, and 3% showed misexpression, which leaves 9% without expression in comparison with the wild type, translating to 18% of the *wyr-1* embryo sacs; Fig. 4E,F,H). Another 3% of ovules from *wyr-1/WYR; ET956/–* plants (corresponding to 6% of *wyr-1;ET956* and thus extrapolated to 12% of *wyr-1* embryo sacs) showed ectopic patterns such as expression in the synergids (Fig. 4G,H, green arrows). Expression of the synergid marker *ET2634* was reduced by 8% in *wyr-1/WYR* plants compared with the wild type (Fig. 4I,J,L). Interestingly, in

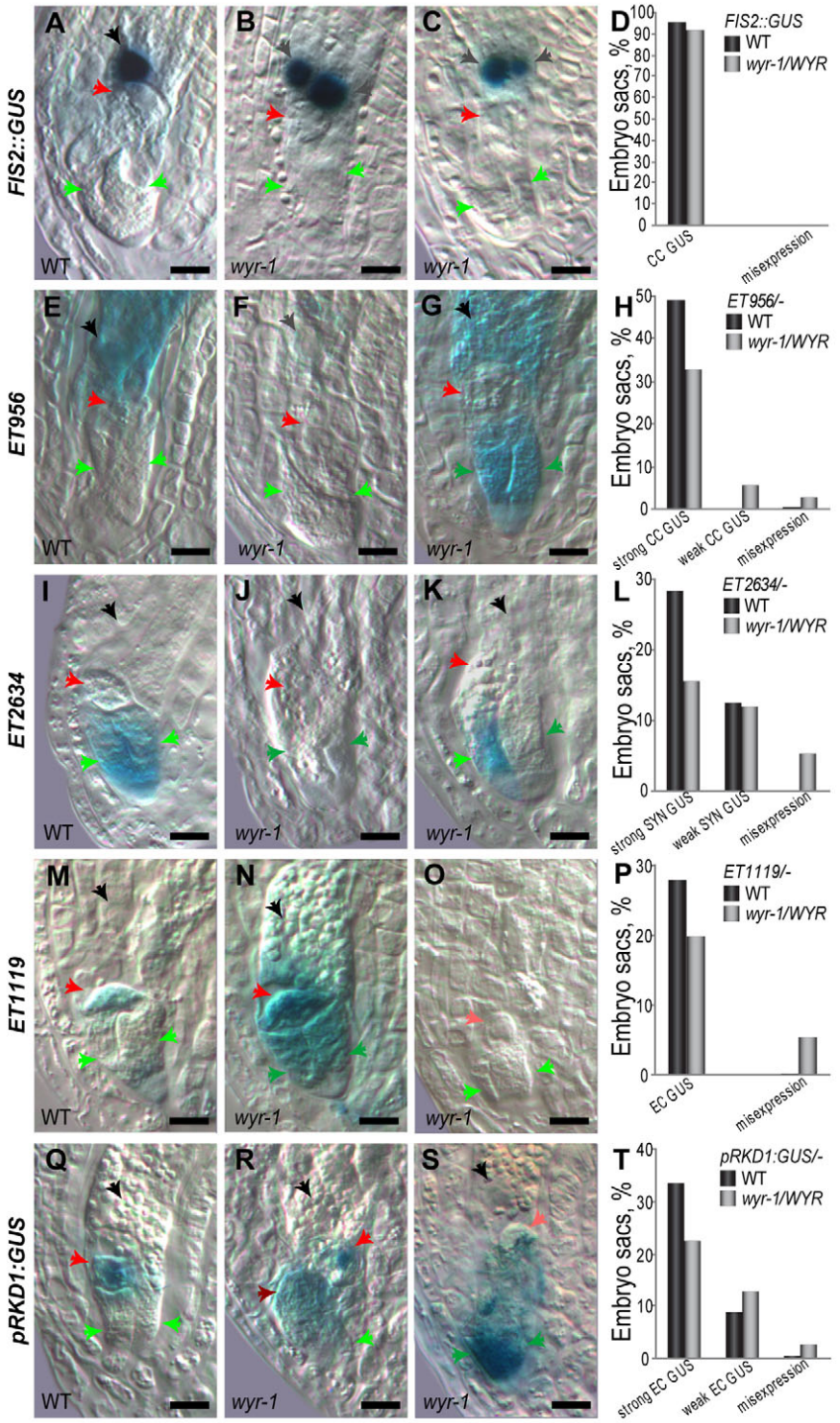

Fig. 4. Improper differentiation of cell types in *wyr-1* female gametophytes. Histochemical GUS assays with cell type-specific markers in micropylar halves of the female gametophytes (FGs) including the egg apparatus [consisting of egg cell (red arrow) and two synergids (green arrows)] and a part of the central cell (black arrow); note that all markers except *FIS2::GUS* were analyzed in the hemizygous condition. (**A-D**) Central cell-specific marker *FIS2::GUS*, which is expressed in (A) the homo-diploid nucleus of a wild-type central cell (black arrow) and (B,C) in *wyr-1* unfused polar nuclei of different size (dark gray arrows). (D) Classes of *FIS2::GUS* expression patterns in *wyr-1* mutants in comparison with the corresponding wild-type segregants (*n*=509 and 443, respectively). CC, central cell. (**E-H**) Central cell-specific marker *ET956*, which is expressed in (E) the wild-type central cell (red arrow) and (F) weakly in a *wyr-1* central cell (dark-gray arrow). (G) A misexpression example of *ET956* in *wyr-1* synergids (dark green arrows), where the egg cell remained unstained (red arrow). (H) Classes of *ET956* expression patterns in *wyr-1* mutants in comparison with the corresponding wild-type segregants (*n*=438 and 270, respectively). (**I-L**) Synergid cell-specific marker *ET2634*, which is expressed in (I) wild-type synergids (green arrows) but (J) sometimes absent in *wyr-1* embryo sacs (dark green arrows), based on the overall reduction in embryo sacs expressing GUS. The faint background staining indicates that this embryo sac carried the marker transgene. (K) Misexpression of *ET2634* in *wyr-1* embryo sacs: partial loss of expression. In many cases, only one *wyr-1* synergids lost GUS expression (dark green arrow), while the other was properly stained (green arrow). (L) Classes of *ET2634* expression patterns in *wyr-1* mutants in comparison with the corresponding wild-type segregants (*n*=211 and 194, respectively); note that the *wyr-1/WYR* misexpression class consisted mainly of FGs with only one stained synergid (SYN; K). (**M-P**) Egg cell-specific marker *ET1119*, which is expressed in (M) the wild-type egg cell (red arrow) and (N) misexpressed in *wyr-1* synergids (dark green arrows). (O) Loss of *ET1119* expression in a fraction of *wyr-1* egg apparatuses (dark green arrows), based on the overall reduction in embryo sacs expressing GUS. This specific embryo sac could not unambiguously be identified as carrying the maker transgene. (P) Classes of *ET1119* expression patterns in *wyr-1* mutants in comparison with the corresponding wild-type segregants (*n*=628 and 239, respectively); note that the misexpression class consisted only of *wyr-1/WYR* FGs with stained synergids (N). EC, egg cell. (**Q-T**) Egg cell-specific marker *pRKD1:GUS*, which is (Q) expressed in the wild-type egg cell (red arrow) and (R) misexpressed in two egg-like cells of a *wyr-1* egg apparatus (red and dark red arrows) with one remaining synergid (green arrow). (S) A misexpression example of *pRKD1:GUS* in *wyr-1* synergids (dark green arrows), while the cell positioned in the egg-cell domain shows no GUS expression (light red arrow). (T) Classes of *pRKD1:GUS* expression patterns in *wyr-1* mutants in comparison with the corresponding wild-type segregants (*n*=698 and 403, respectively). Scale bars: 10 μm.

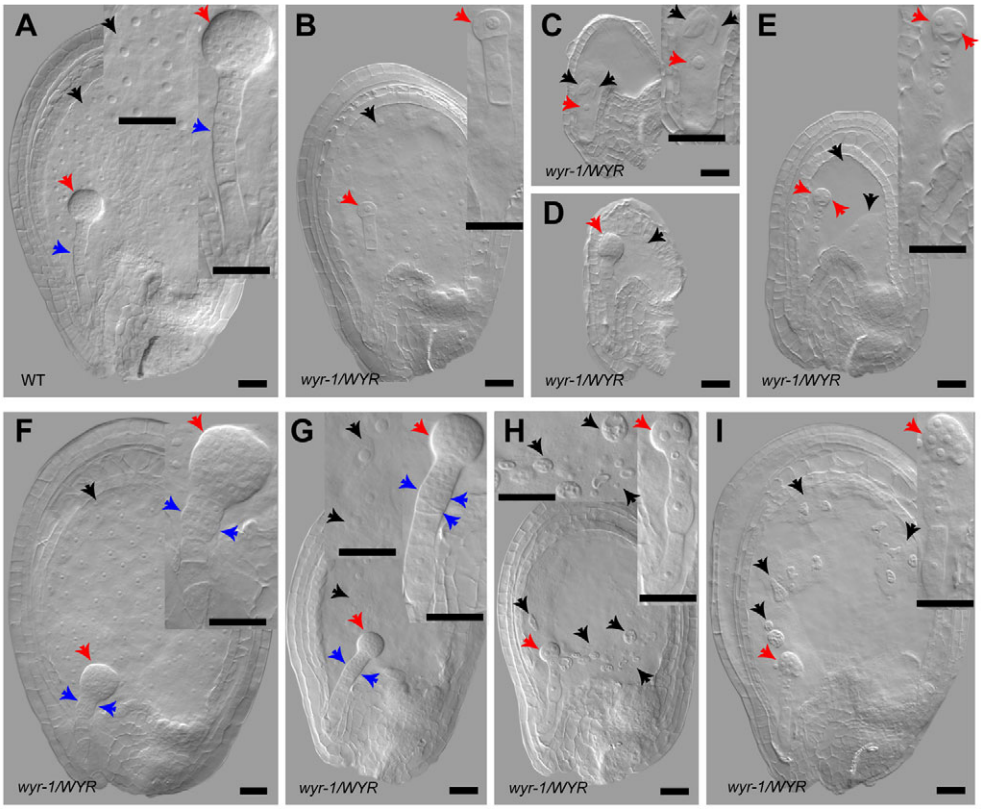

Fig. 5. Development of both endosperm and embryo is impaired in *wyr-1* mutants. (A-I) Cleared seeds from self-fertilized wild-type (WT) and *wyr-1/WYR* siliques at the late globular embryo stage; the genotype of the mother plant is indicated for each panel. (**A**) A properly developed wild-type seed with an embryo at the late globular stage (red arrow) with suspensor (blue arrow) and free-nuclear endosperm (black arrow).(**B**) A delayed *wyr-1/WYR* seed with one-cell embryo (red arrow). (**C**) A fertilized *wyr-1/WYR* egg cell formed a zygote (red arrow) with a centrally positioned nucleus; the two unfused polar nuclei (black arrows) apparently failed to be fertilized. (**D**) A *wyr-1/WYR* seed with normal globular embryo (red arrow) despite absence of endosperm proliferation (black arrow). (**E**) Delayed endosperm formation (black arrows) is accompanied by asynchronous cell divisions in the embryo (red arrows): one nucleus of the two-cell embryo is at interphase (left cell), while the other is dividing (right cell). (**F**) A *wyr-1/WYR* seed with embryo at the late globular stage (red arrow) showing cytokinetic defects in the suspensor (two cell files, blue arrows); endosperm development seems normal (black arrow). (**G**) In this *wyr-1/WYR* seed, the suspensor has division plane defects (blue arrows) similar to those in F; note that the endosperm contains nuclei of irregular size (black arrows, inset). (**H**,**I**) *wyr-1* seeds with endosperm breakdown; note unevenly distributed huge endosperm nucleoli of irregular size and shape (black arrows). Seed in H has a normal looking two-cell embryo (red arrow); the early globular embryo (red arrow in I) has a 'raspberry-like' shape probably as a result of irregular cell divisions. The aberrant endosperm phenotype (G-I) is only seen when homozygous (*wyr-1/wyr-1/wyr-1*) and is caused by a recessive postfertilization effect of the mutation. Scale bars: 30 μm.

5.2% of all embryo sacs (corresponding to 20.8% of *wyr-1* female gametophytes) only one synergid expressed the marker (Fig. 4K,L, light green versus dark green arrow). Correspondingly, the egg cell maker *ET1119* was expressed in the synergid domain in 5% of the ovules from *wyr-1/WYR* plants (Fig. 4M,N,P, dark green arrows). The egg cell marker *pRKD1:GUS* showed a variable pattern in 3% of the female gametophytes from *wyr-1/WYR* plants (Fig. 4Q-T) in which it was expressed in either two cells of the egg apparatus (Fig. 4R, red and dark red arrows), only in the synergid domain (Fig. 4S, green and red arrows, respectively) or not at all (Fig. 4T, see also Fig. 4O,P). Therefore, we infer that between 12% (based on *pRKD1:GUS*) and 21% (based on *ET1119* and *ET2634*) of all *wyr-1* embryo sacs differentiated egg cells at the expense of synergids, consistent with a 16% decrease in the number of pollen tubes entering *wyr-1/WYR* ovules (Fig. 1D). Thus, the differentiation of an egg cell-like fate by the synergid cells might be responsible for blocking fertilization and seed initiation, as indicated by 22% (n=416) infertile ovules in *wyr-1/WYR* plants.

wyrd exerts gametophytic effects on embryo development and recessive postfertilization effects on endosperm nuclear divisions

Seed set reduction in *wyr-1/WYR* plants comprises not only infertile ovules but also seeds arrested at different stages after fertilization (Fig. 1A-C), probably as a consequence of variable expressivity of the mutation in the female gametophyte. Seeds from self-fertilized *wyr-1/WYR* plants showed various phenotypes in the embryo and endosperm. By the time wild-type embryos had reached the late globular stage (Fig. 5A), some seeds from self-fertilized *wyr-1/WYR* plants were delayed (Fig. 5B,C,E) and/or showed asynchronous developmental progression of embryo and endosperm (Fig. 5C-E). In others, polar nuclei appeared unfertilized, preventing endosperm formation (Fig. 5C,D). Mutant embryos exhibited defects in cytokinesis ranging from asynchronous cell divisions and disorganized cell layers (Fig. 5E,I) to irregular cytokinetic planes in the suspensor, forming two cell files (Fig. 5F,G). Some

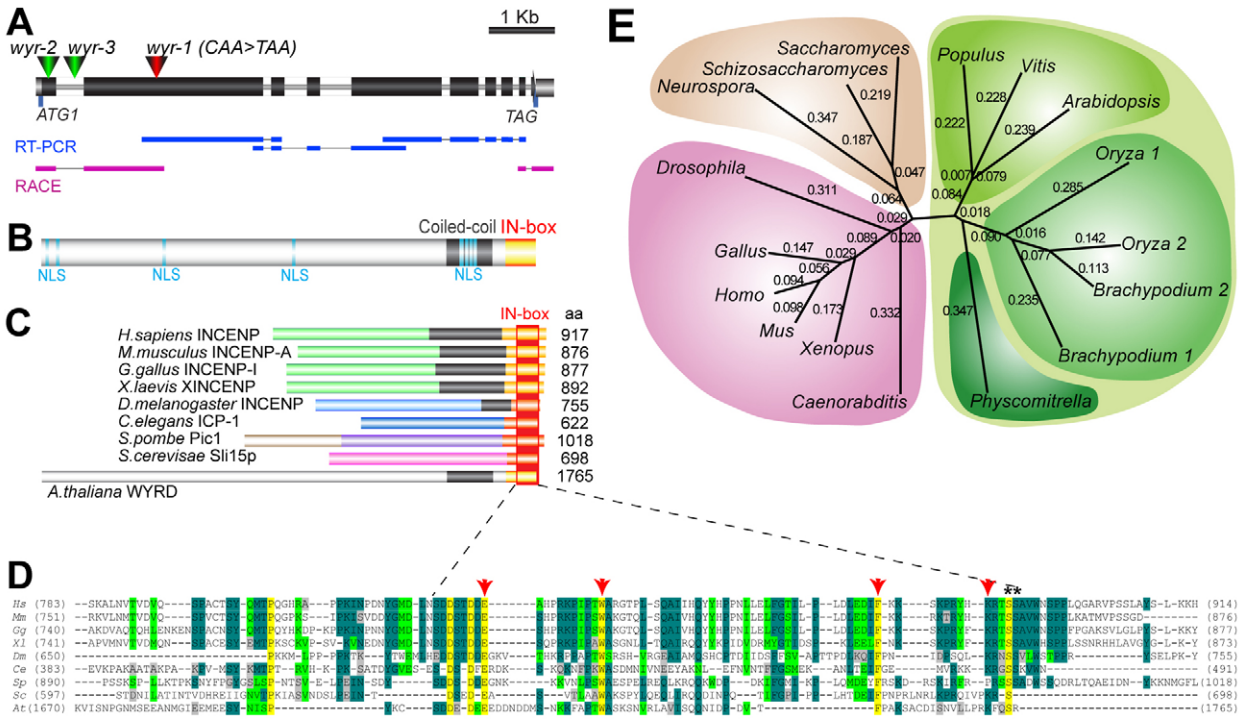

Fig. 6. *WYRD* encodes the putative *Arabidopsis* INCENP ortholog. (**A**) Identified gene structure of *WYR* (upper panel), examples of RT-PCR products (blue) used for design of gene-specific primers, and RACE-determined 5′- and 3′-ends (pink) of *At5g55820*. Positions of *wyr-1*, *wyr-2* and *wyr-3* mutations, and predicted start and stop codons are shown. (**B**) Predicted WYRD protein with IN-box (yellow-red), coiled-coil region (gray) and putative nuclear localization signals (NLS; turquoise). (**C**) INCENPs in animals, yeasts and *Arabidopsis*: comparison of the predicted domain structure. The coiled-coil domain (gray shadowed) and IN-box (aurora B binding) domain (red box) at the C-terminus are indicated. aa, amino acids. (**D**) Multiple sequence alignment of IN-box domains. Identical, conservative, similar and weakly similar amino acids are highlighted in yellow, teal, green and gray, respectively (Vector NTI, Invitrogen, with manual adjustment). Arrows point to conserved IN-box amino acids (Xu et al., 2009); asterisks indicate conserved Ser residues phosphorylated by the aurora B kinase (Bishop and Schumacher, 2002). (**E**) Phylogenetics relationships of INCENP IN-box domains in animal, yeast, fungi and plants (NJ method). Species used for D and/or E were: *Caenorhabditis elegans* (*Ce*), *Drosophila melanogaster* (*Dm*), *Gallus gallus* (*Gg*), *Homo sapiens* (*Hs*), *Mus musculus* (*Mm*), *Xenopus laevis* (*Xl*), *Neurospora crassa*, *Saccharomyces cerevisae* (*Sc*), *Schizosaccharomyces pombe* (*Sp*), *Arabidopsis thaliana* (*At*), *Brachipodium distachyon, Oryza sativa*, *Populus trichocarpa*, *Vitis vinifera*, *Physcomitrella patens*; note that INCENP is duplicated in grass genomes (*B. distachyon*, *O. sativa*).

seeds contained endosperm with fewer and severely deformed nuclei of irregular size, often clustered in patches (Fig. 5H,I; *n*=23).

To determine which of these developmental aberrations resulted from the gametophytic maternal effect of *wyr-1*, we analyzed *wyr-1/WYR* siliques after crossing with wild-type pollen. At approximately the heart stage of wild-type embryogenesis, free-nuclear endosperm initiates cellularization (see Fig. S3A in the supplementary material). As in self-fertilized *wyr-1/WYR* plants, 20% of the ovules arrested before or immediately after fertilization (early aborted class, see Fig. S3O in the supplementary material). In many seeds development was delayed (see Fig. S2E,H,L,O in the supplementary material) and/or asynchronous (see Fig. S3F,G,K in the supplementary material), including seeds that formed heart stage embryos despite an arrested endosperm (see Fig. S3B,D in the supplementary material). About 12% of the embryos (see Fig. S3C,D,H-K in the supplementary material) and/or suspensors were abnormal (see Fig. S3H,K-N in the supplementary material), often with cytokinesis defects. Albeit only half as frequent, similar abnormalities were seen if *wyr-1* was introduced paternally (see Fig. S4 in the supplementary material), suggesting this aspect of the phenotype to be caused by haplo-insufficiency. Intriguingly, the breakdown of endosperm nuclear proliferation we observed after self-fertilization (Fig. 5H,I) was not seen in seeds derived from crosses with the wild type. This suggests that *wyr-1* causes a failure of endosperm proliferation only when inherited from both parents and that one paternal *WYR* allele is sufficient to rescue endosperm proliferation upon fertilization of a *wyr-1* mutant central cell. In summary, the *wyr-1* mutation shows a complex genetic behavior with defects in cell specification and fertilization because of a requirement for *WYR* in the female gametophyte, early seed abortion due to a gametophytic maternal effect, cytokinesis defects in embryo and suspensor that are, at least partly, due to haplo-insufficiency, and irregular proliferation of the endosperm caused by a recessive postfertilization effect of *wyr-1*.

WYRD is an essential gene encoding a putative INCENP ortholog

In a positional cloning approach, we mapped the *wyr-1* mutation to the predicted coding sequence of *At5g55820*, where it caused a C to T nucleotide change (see Fig. S1 in the supplementary material; Fig. 6A). To confirm the predicted gene model of *At5g55820*, we amplified fragments of the corresponding cDNA by RT-PCR. Recurrent failure to amplify 5′- and 3′-ends of the *At5g55820* cDNA suggested an incorrect prediction of the gene model (http://www.arabidopsis.org). Therefore, we amplified corresponding cDNA ends by RACE and sequenced a full-length cDNA to identify the gene structure with ten exons and nine introns (Fig. 6A), where

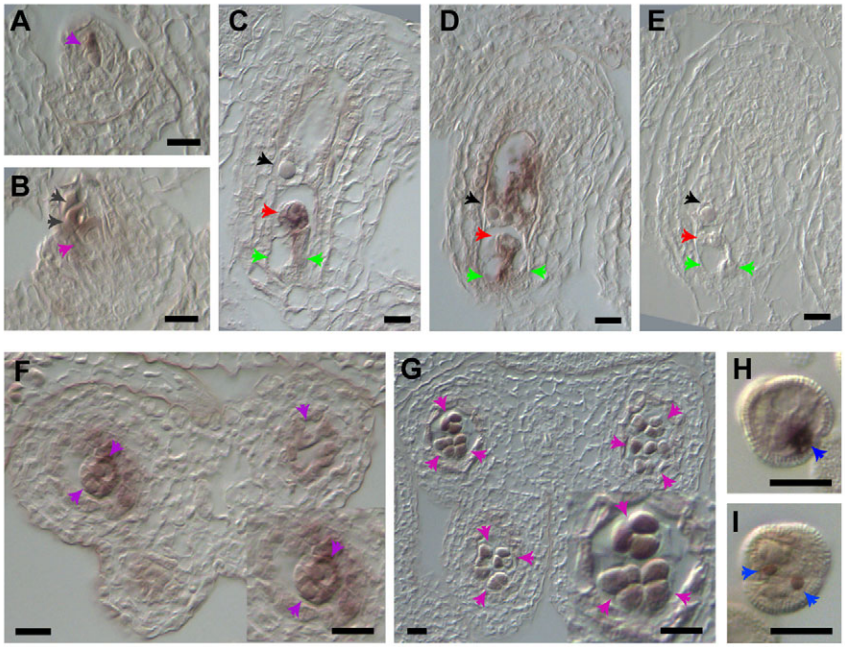

Fig. 7. *WYRD* is expressed at high levels in gametophytic cells. (**A-E**) *WYR* mRNA was detected by in situ hybridization before the onset of (A,B), and at the very end of (C-E) female gametogenesis, 2 dae. (A) A developing ovule with an MMC shows a strong *WYR* signal (arrow). (B) Tetrad of megaspores after meiosis. *WYR* is detected in the whole tetrad, especially in the three upper megaspores showing signs of degeneration (gray arrows); the functional megaspore is indicated by a pink arrow. (C) At low *WYR* riboprobe concentration, the mature egg shows a strong *WYR* signal, whereas the signal in the central cell appears much weaker (red and black arrows, respectively). (D) An increased riboprobe concentration reveals *WYR* expression in both the egg and central cell (red and black arrows, respectively), but not in the synergids (green arrows). (E) The sense probe (negative control) gives no signal in the mature embryo sac. (**F-I**) In situ hybridization at stages of postmeiotic male gametophyte development. (F) *WYR* is expressed during male meiosis (arrows indicate dyads). (G) Tetrads of microspores after meiosis (arrows) exhibit strong *WYR* signals. (H) Pollen grain at the binucleate stage: *WYR* is detected only in the generative cell (arrow). (I) Pollen grain at the trinucleate stage; note *WYR* signal in the two sperm cells (arrows). Scale bars: 10 μm.

wyr-1 created an in-frame stop codon in the second exon (at position Q^{420} of the corresponding protein). To confirm that *At5g55820* corresponds to the *WYR* locus, we identified two additional alleles, *wyr-2* and *wyr-3*, which carried a T-DNA and a *Ds*-element insertion in the first exon and intron of *At5g55820*, respectively (Fig. 6A). Both insertion alleles faithfully reproduced the phenotypes of the *wyr-1* point mutation (see Table S2 and Figs S5, S6, S7 in the supplementary material). The analysis of additional alleles confirmed that disruption of the *AT5g55820* coding sequence indeed caused the reproductive phenotypes observed in *wyr* mutants.

WYR is a unique gene in the *Arabidopsis* genome and codes for a putative plant ortholog of INCENP (Ruchaud et al., 2007; Vagnarelli and Earnshaw, 2004) with characteristic C-terminal domains, a coiled-coil domain and an IN-box (aurora B binding domain; Fig. 6B,C). The WYR IN-box contains four amino acid residues (Fig. 6D, arrows) that are conserved from yeasts to mammals (Xu et al., 2009). The putative plant INCENP ortholog is almost twice as long as its non-plant counterparts (Fig. 6C). Predicted plant INCENPs exhibit similarity at their C-termini, consisting of the coiled-coil and IN-box domains, and of an additional region at their N-termini (see Fig. S8 in the supplementary material). Phylogenetic analysis of conserved IN-box domains revealed that plant, animal and yeast INCENPs form distinct clusters, with plants subdivided into subclusters for dicots, monocots and mosses (Fig. 6D,E).

In order to ascertain the possible function of an INCENP protein in female gametophyte development, we determined whether the gametophytic effect of the *wyr* mutation is due to loss of *WYR* function or caused by a dominant effect of a truncated *WYR* product. Because dominance-recessiveness relationships cannot be investigated in haploid gametophytes that carry only a single allele, we analyzed whether *wyr-2* is recessive or dominant in diploid gametophytes of tetraploid plants (Grossniklaus et al., 1998; Huck et al., 2003; Johnston et al., 2010). Analysis of seed set in parental tetraploid plants in combination with segregation data of their progeny (see Table S3 in the supplementary material) showed that *wyr-2* is a recessive, loss-of-function allele.

WYRD is expressed in a cell cycle-dependent manner and upregulated in gametes

Before investigating of the spatiotemporal expression pattern of *WYR* during reproduction, we examined *WYR* expression by RT-PCR and detected its mRNA in leaves, inflorescences and siliques (data not shown), consistent with the ubiquitous expression of *WYR* as listed in tissue- and organ-specific expression databases (Hruz et al., 2008), which show elevated expression in mitotically active apex tissues, pollen, carpels and seeds (see Fig. S8A in the supplementary material). In synchronized *Arabidopsis* cell culture, *WYR* expression peaks at the onset of mitosis (see Fig. S8B in the supplementary material) (Menges et al., 2003), suggesting that *WYR* expression is regulated during plant development in a cell cycle-dependent manner.

To investigate *WYR* expression at the cellular level, we performed RNA in situ hybridization on reproductive tissues. We detected a very strong *WYR* signal in the MMC at the onset of meiosis, and in the resulting tetrad of megaspores (Fig. 7A,B).

However, during female gametogenesis no signal was detected (not shown) until maturity of the female gametophyte, when *WYR* expression strongly increased in the female gametes: the egg cell and, at a slightly lower level, in the central cell (Fig. 7C,D). In anthers, we detected a strong *WYR* signal in the dyads and tetrads of microspores (Fig. 7E,F) and later in the generative cell and the sperm cells of mature pollen (Fig. 7G,H). In summary, both M-phase-dependent regulation and prominent expression of *WYR* in the gametophytes are consistent with the mitotic and developmental phenotypes we observed in *wyr* mutants.

DISCUSSION

WYRD is a putative plant INCENP ortholog

INCENP was the first subunit of the CPC identified in animals, and subsequently the INCENP orthologs Pic1 and Sli15p were described in yeasts (Ruchaud et al., 2007; Vagnarelli and Earnshaw, 2004). INCENP functions in a complex with aurora kinases, survivin and borealin to ensure proper chromosome condensation, regulation of the spindle assembly checkpoint, chromosomal segregation and cytokinesis. A functional role for INCENP-like proteins has not previously been reported in plants. We provide insight into the function of WYR – the putative *Arabidopsis* ortholog of INCENP – during development and differentiation of the gametophytes and the developing seed. Although a plant-specific CPC has not been purified, WYR is the second conserved CPC subunit identified in plants after the aurora kinases, the central CPC players in fungi, animals and plants. In *Arabidopsis*, the three aurora kinases AUR1, AUR2 and AUR3 are thought to play a conserved function during cell division (Demidov et al., 2005; Kawabe et al., 2005). A CPC function in cell differentiation and reproductive development, however, has not been described. It is conceivable that the IN-box domain of WYR acts as a docking station for *Arabidopsis* aurora kinases, as in yeast and animal systems (Ruchaud et al., 2007). The *Arabidopsis* WYR protein comprises all essential domains of INCENP that are crucial for cell division in other systems, but the sequence divergence of *WYR* across kingdoms may indicate that *WYR* has plant-specific developmental functions, such as those uncovered in *wyr* mutants.

Arabidopsis WYR is an essential gene similar to the studied metazoan CPC members (Ruchaud et al., 2007). We observed occasional triploid offspring from *wyr-1/WYR* plants (0.3%, data not shown). Because *wyr* is gametophytic recessive, triploid progeny may result from a failure in CPC-dependent chromosomal segregation during male and/or female gametophytic mitoses, as observed in other systems if CPC members are deregulated in somatic tissues (Nguyen and Ravid, 2006). Support for a role of *WYR* in cell cycle control as reported for the metazoan INCENP orthologs comes from the finding that *WYR* expression is regulated in a cell cycle-dependent manner, increasing at the onset of mitosis together with all three aurora kinase genes (Menges et al., 2003) (see Fig. S8B in the supplementary material). Thus, WYR may participate in a putative plant CPC complex with aurora kinases during cell division and development.

WYRD is crucial for postmeiotic progression of male mitosis

Some microspores lacking *WYR* fail to proceed through PMI and either abort or survive with a single enlarged nucleus (Fig. 2). This phenotype is consistent with the proposed function of *WYR* in cell division. Except for *rbr* mutants, which affect both PMI and PMII, causing overproliferation (Chen et al., 2009; Gusti et al., 2009; Johnston et al., 2008; Kim et al., 2008), most known male gametophytic mutants in cell cycle genes arrest at PMII producing bicellular pollen (Durbarry et al., 2005; Rotman et al., 2005). By contrast, *wyr*/*WYR* plants do not produce a significant fraction of bicellular pollen (Fig. 2; see Fig. S3 in the supplementary material). Therefore, if the amount of residual *WYR* product inherited by the meiotically derived microspores is sufficient to complete PMI, it might also ensure progression through PMII. Alternatively, *WYR* might specifically control chromosome segregation in the asymmetric PMI, although this seems less probable, as other data indicate a general INCENP-like role of WYR in cell division. Accordingly, we found *WYR* transcripts in all mitotically active cells of the male gametophyte, indicating that *WYR* function is crucial for progression through both pollen mitoses, either because of a direct function in chromosome segregation and cytokinesis and/or a putative role in the establishment of cell polarity (see below).

WYRD is required postfertilization for endosperm development and embryogenesis

Between 36% and 40% of *wyr* embryo sacs initiate seed formation but abort later during development (Fig. 1; see Fig. S2 in the supplementary material). Although maternal inheritance of *wyr* results in very early arrest in about 20% of the seeds, other phenotypes such as cytokinetic defects in the embryo and suspensor are observed upon both maternal and paternal inheritance, albeit at different frequencies (see Figs S3, S4 in the supplementary material). The reduced number of misshapen nuclei in the endosperm and the cytokinetic defects are reminiscent of cell cycle defects in embryo-lethal mutants such as *orc2*, a mutant in one of the origin recognition complex subunits (Collinge et al., 2004), and members of the *pilz* and *titan* (*ttn*) class mutants affecting genes encoding cohesins, condensins and tubulin-folding cofactors (Liu et al., 2002; Liu and Meinke, 1998; Steinborn et al., 2002). The *wyr* phenotypes observed in the embryo and endosperm support a general function of *WYR* in regulating mitotic divisions. The endosperm proliferation defect is due to a recessive postfertilization effect of *wyr* similar to those of *orc2*, *ttn* and *pilz* mutants, whereas the cytokinetic anomalies during early embryogenesis are under gametophytic parental control, probably caused by haplo-insufficiency.

Interestingly, the postfertilization defects observed in *wyr* mutants resemble loss-of-function phenotypes of CPC components in animals: mutant embryos of mice, *Drosophila* and *C. elegans* contain giant nuclei of irregular shape (Chang et al., 2006; Cutts et al., 1999; Kaitna et al., 2000; Uren et al., 2000), similar to those in endosperm lacking *WYR* activity. Likewise, the effect of *wyr* on postzygotic cytokinesis in the embryo is reminiscent of the maternal effects of the *Drosophila Incenp* and zebrafish *cellular island* (*cei*) mutants affecting the Aurora kinase B gene, respectively (Resnick et al., 2009; Yabe et al., 2009), which cause mitotic arrest and impair cytokinesis during embryogenesis. Taken together, the conserved consequences of CPC deregulation across several model systems supports a role of *WYR* in cell cycle regulation.

Cell-cycle-independent role of *WYRD* in cell fate establishment and differentiation

Although *wyr* embryo sacs properly complete syncytial mitoses and cellularize (Fig. 3; see Fig. S2 in the supplementary material), cell fate determination and differentiation are compromised. Firstly, central cell fate is not established correctly in the absence of *WYR*,

resulting in the failure of polar nuclei to fuse, and sometimes blocking central cell fertilization. Although expression of the central cell marker *ET956* is affected in *wyr*, expression of *FIS2* is not, suggesting that some aspects of central cell differentiation are normal. Relatively few genes have been shown to be required for both central cell identity and fusion of polar nuclei, e.g. the transcription factors DIANA/AGAMOUS LIKE 61 (DIA/AGL61) and AGL80 (Bemer et al., 2008; Portereiko et al., 2006; Steffen et al., 2008). In addition to other phenotypes, loss of *RBR* activity can also prevent fusion of polar nuclei and central cell differentiation (Johnston et al., 2010; Johnston et al., 2008). Like *RBR*, *WYR* thus appears to function in both cell cycle regulation and cellular differentiation.

In *wyr* embryo sacs, additional eggs are formed at the expense of synergid cells, a phenotype also observed in the *Arabidopsis eostre*, *lachesis* (*lis*), *clotho* (*clo*/*gfa1*), *atropos* (*ato*) and, rarely, *rbr* mutants (Groß-Hardt et al., 2007; Johnston et al., 2010; Moll et al., 2008; Pagnussat et al., 2007). *LIS*, *CLO/GFA1* and *ATO* encode basic spliceosome factors, whereas *eostre* leads to ectopic expression of a BEL1-like homeobox gene usually not expressed in the embryo sac, indicating that the effect of these mutants on cell specification may be rather indirect. Multiple eggs were also observed in the maize mutant *indeterminate gametophyte1* (*ig1*), which forms supernumerary nuclei and cells in the embryo sac (Evans, 2007; Guo et al., 2004). Unlike in *eostre*, *rbr* and *ig1* mutants, where both eggs can be fertilized (Guo et al., 2004; Ingouff et al., 2009; Pagnussat et al., 2007), we never observed twin embryos in *wyr*, possibly because the ectopic egg cells are not fully functional.

In animals, a functional link between maintenance of cellular identity and regulation of CPC proteins has been proposed. For instance, genes encoding CPC subunits such as INCENP are often deregulated in tumors that have lost differentiation characteristics (Ke et al., 2003; Nguyen and Ravid, 2006). Given that *WYR* is strongly expressed in female gametes, we propose that it plays a role in the establishment and/or maintenance of egg and central cell identity, as some *wyr* gametes do not express markers specific to these cell types (Fig. 4F,O). In addition, *WYR* is involved in preventing synergids from acquiring egg cell fate, perhaps via a yet to be identified non-cell-autonomous pathway as previously suggested (Groß-Hardt et al., 2007; Moll et al., 2008).

A cell cycle-independent developmental role of CPC members was reported for *Drosophila* Incenp, which is necessary for the asymmetric allocation of the Prospero protein during unequal divisions in the developing nervous system (Chang et al., 2006). Analogously, *WYR* may be involved in establishing cellular polarity, as *wyr* mutants can produce both non-polarized microspores (Fig. 2D) and zygotes (Fig. 5C). The cell specification defects in *wyr* embryo sacs might arise from the distorted distribution of a yet to be identified morphogen such as Prospero in *Drosophila*. Alternatively, *WYR* may be involved in the selective segregation of sister centromeres to daughter nuclei as recently proposed for various developmental decisions (Armakolas et al., 2010). Indeed, proteins of four different centromere-bound kinetochore complexes were found to be asymmetrically distributed upon division in yeast, thus establishing a specific cell fate (Thorpe et al., 2009). Because one of these complexes is regulated by the CPC (Widlund et al., 2006) it is tempting to speculate that cell fate decisions in *Arabidopsis* embryo sacs may involve the non-random segregation of chromatids. The finding that the plant INCENP ortholog is essential for gamete differentiation uncouples cell cycle and differentiation functions of *WYR*, indicating a novel role for the CPC in gametogenesis.

WYRD has sex-specific functions in gametophytic development

The timing of mitotic divisions and cellular differentiation are different in female and male gametophytes. Although nuclei in the female gametophyte undergo three syncytial, synchronous divisions followed by differentiation of sister cells, in male gametogenesis cell specification begins immediately during/after PMI (Borg et al., 2009; Brukhin et al., 2005). Genetic requirements are expected to differ in the two gametophytes, as evident from sex-specific, phenotypic differences in some gametophytic mutants, for instance *rbr* (Chen et al., 2009; Johnston et al., 2010; Johnston et al., 2008). Loss of *WYR* also causes sex-specific defects, such as abolishing PMI in the male, and affecting cell specification and differentiation in the female gametophyte. High levels of *WYR* transcripts were detected only in PMCs and MMCs, meiotic products, and the generative cell of bicellular pollen. Elevated *WYR* expression may be necessary both for PMI and the differentiation of the generative cell, but the female gametophyte does not seem to require high *WYR* expression to progress through the mitoses, as *WYR* mRNAs were undetectable during nuclear divisions and were highly expressed only in the female gametes.

Likewise, impairing INCENP has no effect on oocyte development in *Xenopus*, zebrafish and *Drosophila* (Yamamoto et al., 2008; Yabe et al., 2009; Resnick et al., 2009; Resnick et al., 2006), but causes a maternal effect on embryogenesis as observed for *wyr*. Although homozygous *cei* male zebrafish are fertile (Yabe et al., 2009), *Drosophila* males with reduced INCENP activity are impaired in spermatogenesis (Chang et al., 2006; Resnick et al., 2006). These gender-dependent requirements and distinct parent-of-origin effects support the view that CPC proteins, including WYR, have distinct, sex-specific roles in animals and plants.

Conclusion

Gametophyte development in angiosperms requires a tightly orchestrated developmental program, coordinating cell division and cellular differentiation. The putative plant INCENP ortholog *WYR* plays a role in both processes. Although its role in cell specification appears independent of cell cycle regulation, it controls mitotic divisions during pollen development and after fertilization in both embryo and endosperm. Intriguingly, INCENP and other proteins of the CPC have been shown to play a role in cell specification and cell cycle progression in both animals and plants. This suggests that conserved CPC functions existing in the common unicellular ancestor have independently been co-opted during evolution to regulate developmental decisions in distinct multicellular contexts. It is possible that these two functions are linked through the selective segregation of chromosomes, possibly with associated cell fate determinants, to daughter nuclei. Future analyses of the biochemical function(s) of WYR will shed light on the role(s) of CPC proteins in plant development and cell cycle control.

Acknowledgements

We thank Célia Baroux and Sharon Kessler for valuable discussions and suggestions; Abed Chaudhury and David Twell for providing seeds; Christof Eichenberger for help with phylogenetic analyses; Arturo Bolaños for ploidy measurements; and Peter Kopf, Valeria Gagliardini and Annett Busching for technical support. A.J.J. acknowledges Wilhelm Gruissem for support and partial funding via an SNF grant (31-109475). This project was supported by the University of Zürich and grants of the Swiss National Science Foundation (31-64061 and 31-112489) to U.G., by IPK-Gatersleben core funding to H.B., and by an EMBO Long-Term Fellowship to R.G.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.060384/-/DC1

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