A novel class of MYB factors controls sperm cell formation in plants

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Running head: Control of sperm cell formation in plants

Summary

In contrast to animals, the plant male germ line is established after meiosis in distinctive haploid structures, termed pollen grains. Germ line arises by a distinct asymmetric division of the meiotic products [1, 2, 3]. The fate of the resulting vegetative and generative cells are distinct. In contrast to the larger vegetative cell arrested in the G1 phase of the cell cycle, the smaller generative cell divides once to produce the two male gametes or sperm cells. Sperm cells are delivered to the female gametes by the pollen tube, which develops from the vegetative cell. In spite of recent efforts to understand pollen development [4 – 8] the molecular pathway controlling sperm cell ontogenesis is unknown. Here we present the isolation of *DUO1*, a novel *R2R3 MYB* gene of *Arabidopsis*, as the first gene shown to control male gamete formation in plants. *DUO1* is specifically expressed in the male germ line and DUO1 protein accumulates in sperm cell nuclei. Mutations in *DUO1* produce a single larger diploid sperm cell unable to perform fertilisation. *DUO1* appears to be evolutionarily conserved in several plant species and defines a new subfamily of pollen-specific *MYB* genes.

Results and Discussion

Isolation and genetic characterization of mutants with altered sperm cell development

The *duo pollen* (*duo*) mutant lines, *duo1-1* and *duo1-2*, were isolated in two separate genetic screens on the basis of morphological [9] or semi-sterility defects [10]. Genetic transmission of *duo1-2* was investigated by analysis of seed set obtained after reciprocal crosses between wild type (WT) and mutant parents (Supplemental Table 1). In contrast to pollination of *duo1-2* ovules with WT pollen, pollination of wild type ovules with pollen from *duo1-2/DUO1* plants leads to failure of fertilisation in 50% of ovules leading to ovule abortion (chi square test: P>0.2). Accordingly *duo1-2* is not transmitted through pollen, whereas *duo1-2* ovules transmit the mutation as WT.

Similar results were obtained for the *duo1-1* allele (not shown). Therefore *duo1* mutations exhibit fully penetrant, strict male gametophytic control.

We could not detect anomalies during microspore development *in duo1/+* flower buds until mitotic division of the generative cell (Figure 1A; n>400). However, when the WT generative cell has divided producing two sperm cells, 50% of pollen from *duo1/+* plants showed a single nucleus associated with the vegetative cell nucleus (Figure 1B; n>500). The absence of division of the generative cell in *duo1* pollen may result either from a specific defect in cell cycle regulation, or from a more general deregulation of cell fate, between vegetative cell fate with one division, as opposed to generative cell fate with two divisions. Therefore, we introduced different marker lines into the *duo1/+* background to monitor cell identity. Pollen from WT plants expressing the *promACTIN11-H2B::mRFP1* marker only showed monomeric Red Fluorescent Protein (mRFP1, [11]) in the vegetative nucleus (Figures 1C and 1D; n>500). Mutant plants *duo1-2/+* homozygous for *promACTIN11-H2B::mRFP1* produce mature pollen grains that all show a single labelled nucleus corresponding to the vegetative nucleus (Figures 1C and 1D; n>200). Thus, vegetative cell fate is correctly specified in mature *duo1* pollen. Accordingly, the *duo1* generative-like cell does not express the *promACTIN11-H2B::mRFP1* reporter but expresses the cell identity reporter for *ANTIKEVORKIAN* (*AKV*) that is expressed in sperm cells in mature pollen grains (Figure 1E and Supplemental Figure 1; n>200). As *AKV* is expressed in microspores and subsequently only in WT generative and sperm cells, the undivided generative-like cell in mature *duo1* pollen may be either arrested with a generative cell identity or may have acquired or retained some patterns of gene expression associated with sperm cell identity. Both such hypotheses are supported by the high condensation of chromatin typical of WT generative and sperm cells (Figure 1B) and by the tight association of the unique *duo1* sperm-like cell with the vegetative nucleus as in the WT male germ unit (Figures 1B and 1D; [2]).

Upon failure of the *duo1* generative cell to divide, the expectation might be that further DNA replication would not occur, especially in light of the resulting sterility that we observed in *duo1-2*. As expected the mean DNA content of generative cell nuclei in wild type and *duo1-2*/+ pollen increased significantly between early bicellular and late bicellular stages to reach approximately 2C

(Table 1; Supplemental Table 2). However, after division nuclear DNA content increased significantly between mid and late tricellular stages to more than 2C, nearly matching the relative increase observed in sperm nuclei (Table 1 and Supplemental Table 2). Apparently, *duo1* and the failure of the generative cell to undergo mitosis do not interfere with its subsequent entry into S phase. The undivided cell has therefore acquired at least one characteristic typical of *Arabidopsis* sperm cells, which is the onset of S phase in the anther during pollen maturation [12]. This result supports the proposal that mature *duo1* pollen contains a unique homodiploid germ line cell in S phase at the time of anthesis. In conclusion, *duo1* mutations identify a specific control of male germ line mitosis that leads to sperm cell formation, however this operates independently of some features of sperm cell identity.

DUO1 encodes a novel R2R3 MYB transcription factor that is specific to the male germ line

We identified the *DUO1* gene via map based cloning (see Experimental procedures). Sequencing of the At3g60460 predicted ORF revealed mutations in both *duo1-1* and *duo1-2*. At3g60460 is predicted to consist of 3 exons and to encode a protein of 298 amino acids (aa) (Figure 2A). In *duo1-2*, an insertion of 14 bp in the third predicted exon gives rise to a frame shift and a predicted truncated protein of 186 aa (Figures 2A and 2B). A transgene carrying the genomic sequence of At3g60460 with putative 5' and 3' control elements restored a WT phenotype to pollen of *duo1-2* plants demonstrating complementation (see Experimental procedures). In *duo1-1*, a C to T substitution within the third exon creates a stop codon and the predicted mutant protein consists of 214 aa (Figures 2A and 2B). Therefore, we identify At3g60460 as *DUO1* and *duo1-1* and *duo1-2* as mutant alleles.

DUO1 expression is not detected in vegetative tissues but only in inflorescences (Figure 3A). We compared *DUO1* expression between mutants impaired for flower development. *pistilata-1* (*pi-1*) flowers contain only sepals and carpels, *pistilata-5* (*pi-5*) flowers contain sepals, carpels and stamens, in which pollen develops and *agamous* (*ag*) forms flowers that contain only sepals and petals. RT-PCR analyses show that *DUO1 expression* is dramatically enhanced in *pi-5* compared with *pi-1* and *ag* (Figure 3B) suggesting that *DUO1* is predominantly active in stamens. *DUO1* is

specifically expressed in pollen as shown by monitoring *in planta* the expression of the HISTONE2B::mRFP1 fusion protein under the control of the *DUO1* promoter (Supplemental Figure 2). The *DUO1* promoter is specifically active in the generative cell and in sperm cells (Figures 3C-H). In conclusion *DUO1* is expressed specifically in the male germ line in agreement with the strict male gametophytic control associated with *duo1-1* and *duo1-2*.

DUO1 belongs to the large family of *R2R3 MYB* transcription factors of *A. thaliana*, but was not included in previous studies [13, 14]. *In silico* analysis indicates that *DUO1* represents a unique gene within the R2R3 family of *A. thaliana* (see Experimental procedures). A Tobacco protein, as well as a Rice putative protein and three Maize predicted proteins are more closely related to DUO1 than to any other R2R3 MYB protein in *A. thaliana* (Figure 2C). Interestingly putative *DUO1* orthologues in Tobacco and in Maize seem to be expressed in stamens or in pollen (see Experimental procedures). We refer to these putative orthologues as the *DUO1* family. The MYB domain of the *DUO1* family shows a supplementary lysine residue at position 58 (Figure 2C), which is never observed in the other plant MYB sequences we analysed. This could represent a signature of the *DUO1* family among plant MYB proteins. We suggest that distinct members of the *MYB* gene family have been recruited during evolution as specific regulators of gametophytic development and that the *DUO1* family consists of pollen-specific *MYB* factors. In order to identify the subcellular localisation of the DUO1 protein we expressed a fusion of the entire coding sequence of *DUO1* with the *mRFP1* reporter gene under the control of the putative *DUO1* promoter. This construct complemented the *duo1-2* mutation, strongly suggesting that the fusion protein DUO1::mRFP1 is functional (see Experimental procedures). DUO1::mRFP1 co-localises with DAPI staining during interphase, which shows that it is located in the nucleus of the generative cell and of the sperm cells (Figures 3I-N). Interestingly the DUO1::mRFP1 fusion protein may be co-localised with chromosomes during mitosis of the generative cell (Figures 3K and 3L).

Conclusion

DUO1 is specifically expressed in the male germ line in *Arabidopsis* and defective function of *DUO1*, as defined by the analysis of two mutant alleles, prevents entry of the generative cell into

mitosis. DUO1 may promote generative cell division by activating specific targets such as cyclin genes. *GL1*, an *Arabidopsis* R2R3 MYB gene controls the transition from cell proliferation to endoreduplication during trichome development [15], which supports the view that cell cycle regulation and specific developmental processes are tightly coupled and are regulated by *R2R3* MYB genes in plants. Alternatively, DUO1 may control competence to respond to activities controlling the G2/M checkpoint of the cell cycle.

In some flowering plant species generative cell division takes place during pollen maturation and produces tricellular pollen. Other species produce mature bicellular pollen and the generative cell divides in the pollen tube formed by the vegetative cell [12, 16]. If the essential function of DUO1 in G2/M transition is conserved for putative orthologues, DUO1, perhaps in combination with other factors not yet characterised, may have controlled the timing of generative cell division during the evolution of bicellular and tricellular species.

Supplemental data

Supplemental data including Experimental procedures, Tables and Figures are available at http://www.current-biology.com/cgi/content/full/XX/XX/XXXX/XXX/

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References

1. Eady, C., Lindsey, K. and Twell, D. (1995). The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. Plant Cell *7,* 65-74.
2. Lalanne, E. and Twell D. (2002). Genetic control of male germ unit organization in *Arabidopsis*. Plant Physiol. *129,* 865-875.
3. McCormick, S. (2004). Control of male gametophyte development. Plant Cell *16,* S142-S153.
4. Zhang, Z., Xu, H., Singh, M. B. and Russell, S. D. (1998). Isolation and collection of two populations of viable sperm cells from the pollen of *Plumbago zeylanica*. Zygote *6,* 295-298.
5. Xu, H., Swoboda, I., Bhalla, P. L. and Singh, M. B. (1999). Male gametic cell-specific gene expression in flowering plants. Proc. Natl. Acad. Sci. U. S. A. *96,* 2554-2558.
6. Engel, M. L., Chaboud, A., Dumas, C. and McCormick, S. (2003). Sperm cells of *Zea mays* have a complex complement of mRNAs. Plant J. *34,* 697-707.

7. Becker J. D., Boavida, L. C., Carneiro, J., Haury, M. and Feijo, J. A. (2003). Transcriptional profiling of *Arabidopsis* tissues reveals the unique characteristics of the pollen transcriptome. Plant Physiol. *133,* 713-725.

8. Honys, D. and Twell, D. (2003). Comparative analysis of the *Arabidopsis* pollen transcriptome. Plant Physiol. *132,* 640-652.

9. Park, S. K., Howden, R. and Twell, D. (1998). The *Arabidopsis thaliana* gametophytic mutation *gemini pollen1* disrupts microspore polarity, division asymmetry and pollen cell fate. Development *125,* 3789-3799.

10. Rotman, N., Rozier, F., Boavida, L., Dumas, C., Berger, F. and Faure, J. E. (2003). Female control of male gamete delivery during fertilization in *Arabidopsis thaliana*. Curr. Biol. *13,* 432-436.

11. Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A. and Tsien, R. Y. (2002). A monomeric red fluorescent protein. Proc Natl. Acad. Sci. USA *99,* 7877-7882.

12. Friedman, W. E. (1999). Expression of the cell cycle in sperm of *Arabidopsis*: implications for understanding patterns of gametogenesis and fertilization in plants and other eukaryotes. Development *126,* 1065-1075.

13. Martin, C. and Paz-Ares J. (1997). MYB transcription factors in plants. Trends Genet. *13,* 67-73.

14. Stracke, R., Werber, M. and Weisshaar, B. (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr. Opin. Plant Biol. *4,* 447-456.

15. Szymanski, D. B. and Marks M. D. (1998). GLABROUS1 overexpression and TRIPTYCHON alter the cell cycle and trichome cell fate in *Arabidopsis*. Plant Cell *10,* 2047-2062.

16. Brewbaker, J. L. (1967). The distribution and phylogenetic significance of binucleate and trinucleate pollen grains in the Angiosperms. Am. J. Bot. *54,* 1069-1083.

17. Faure, J-E., Rotman, N., Fortuné, P. and Dumas, C. (2002). Fertilization in *Arabidopsis thaliana* wild type: developmental stages and time course. Plant J. *30,* 481-488.

18. Galtier, N., Gouy, M. and Gautier, C. (1996). SEAVIEW and PHYLOWIN: two graphic tools for sequence alignment and molecular phylogeny. Comput. Applied Biosci. *12,* 543-548.

19. Rabinowicz, P. D. (2003). Constructing gene-enriched plant genomic libraries using methylation filtration technology. Methods Mol. Biol. *236,* 21-36.

20. Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J. and Berger, F. (2001). Dynamic analyses of the expression of the HISTONE::YFP fusion protein in *Arabidopsis* show that syncytial endosperm is divided in mitotic domains. Plant Cell *13,* 495-509.

Figure legends

Figure 1. Pollen phenotype of *duo1-2*. (A) *duo1-2* pollen at the bicellular stage (epifluorescence microscopy after Hoechst staining for DNA). Arrowheads indicate the vegetative nucleus. Arrows indicate the generative-like nucleus. Bar is 10 µm. (B) Pollen from *duo1-2/DUO1* plants, observed by fluorescence microscopy after Hoechst staining. Arrowheads indicate the vegetative nucleus. Arrows indicate the single generative-like cell nucleus in *duo1-2* pollen and sperm cell nuclei in WT pollen. Bar is 25 µm. (C) Mature pollen from *duo1-2/DUO1* plants homozygous for the *promACTIN11-H2B::mRFP1* transgene, observed by epifluorescence microscopy with mRFP1 detection settings. White arrowheads indicate the vegetative nucleus. (D) Same pollen as in C*,* but observed by epifluorescence microscopy for DNA staining by Hoechst. Arrowheads indicate the vegetative nucleus. Arrows indicate the generative-like nucleus of *duo1* pollen and sperm cell nuclei of WT pollen. (E) Mature pollen from *duo1-2/DUO1* plants homozygous for the prom*AKV-H2B::YFP* reporter, observed by epifluorescence microscopy with GFP detection settings. Arrows indicate the generative-like nucleus of *duo1* pollen and sperm cell nuclei of WT pollen. Bar is 25 µm.

Figure 2. The unigene *DUO1* encodes an atypical R2R3 MYB protein. (A) *DUO1* predicted Open Reading Frame structure. Exons are shown as boxes and introns with a broken line. The *duo1-1* mutation consists of a C to T substitution in position 812. The *duo1-2* mutation consists of a 14 bp insertion at position 672. Black boxes represent DNA regions encoding the *R2R3 MYB* domain [14]. (B) DUO1 predicted protein and the two predicted mutant proteins corresponding to *duo1-1* and *duo1-2* mutations. Black boxes correspond to the R2R3 MYB domain. The grey box corresponds to 18 amino acids that are different from those of DUO1 at the same position. (C) Alignment of the R2R3 MYB domain of the DUO1 putative orthologues with the *A. thaliana* consensus. The upper alignment concerns the R2 repeat, and the lower one the R3 repeat. The tryptophan (W) and phenylalanine (F) residues that are known to be important in maintaining the hydrophobic core of the DNA-binding domain are highlighted in yellow. The supernumerary lysine residue from each DUO1 putative orthologue is highlighted in red. The shading of the boxes for the

other amino acid residues (black, gray or white) depends on their chemical properties. Consensus 1 consists of the most frequent amino acid residue for each position in all *R2R3 MYB* related genes of *A. thaliana* [14]. Consensus 2 consists of the second most frequent amino-acid (14). Black stars (*) indicate putative base-contacting amino acid residues, and red stars (*), those that are conserved among plant MYB proteins [13].

Figure 3. Expression of *DUO1*. (A) RT-PCR analysis of *DUO1* expression in wild type C24. R: roots, R.L: rosette leaves, L: developed leaves, S: shoots, B: unopened flower buds, Fl: open flowers, Sil: green siliques, C: DNA control (C24). *DUO1* expression is only detected in inflorescences. (B) RT-PCR analysis of *DUO1* expression in floral mutants. *pi-1: pistilata 1, pi-5: pistilata 5, ag: agamous*, C: DNA control (Ler). *DUO1* is dramatically more expressed in *pi-5* flowers, which contain stamens. The low level of expression found in *pi-1* and *ag* backgrounds could reveal a native basal expression in every floral organs or leaky expression due to the mutant background. (C-H) Expression of *promDUO1-H2B::mRFP1* in WT pollen is detected in the nucleus of the generative cell (C), during generative cell mitosis (E) and in the nucleus of each sperm cell (G). D, F and H show epifluorescence of DAPI stained nuclei of pollen corresponding to C, E and G respectively. (I-N) Subcellular localisation of the DUO1::mRFP1 protein fusion during pollen development expressed under the control of the *DUO1* promoter in WT. mRFP1 fluorescence of the reporter is confined to the nucleus of the generative cell (L) and of sperm cells (M). The fusion protein appears to be associated with chromosomes during generative cell division (K with inset showing a confocal section of a metaphase plate). J, L and N show DAPI stained nuclei of pollen corresponding to I, K and M respectively. Arrowheads indicate the vegetative nucleus. Arrows indicate the single generative-like cell nucleus in *duo1-2* pollen and sperm cell nuclei in WT pollen. Bar is 25 µm.